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Screening for potential embryotoxicity of phytochemicals and gestational diabetes mellitus using the chick cardiomyocyte micromass system and stem cell differentiation: Prediction by molecular targets

By

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This thesis is submitted to the University of Nottingham for the degree of doctor of philosophy

March 2017

Abstract

Congenital heart defects are a leading cause of postnatal loss; they could genetically or environmentally induced. Herbal remedies are often used during the early stages of pregnancy, being considered 'harmless' and 'natural'. To alleviate pregnancy-induced symptoms, women frequently use herbal medicines such as ginger to relieve nausea and vomiting - 'morning sickness', gingko biloba and ginseng as dietary supplements or tonics to boost body energy and blood circulation, particularly to the brain. Also, chamomile and holy basil are recommended to promote calmness and reduce stress, often related to planned or unplanned pregnancy. These easily available and accessible medicinal herbs could be possible causes of congenital malformations. Additionally, diabetes mellitus in gestation is a considerable medical challenge, since it is related to augmented dangerous morbidity and mortality for both the fetus and the pregnant woman. Two *in vitro* methods were utilised; chick embryonic heart micromass (MM) and mouse embryonic D3 stem cells (ESD3).

The potential effects of the tested herbal components in both *in vitro* systems were evaluated by monitoring the alteration in several endpoints. These include contractile activity (morphological scoring system), cell activity, total protein content, ROS production, DNA damage, transmembrane proteins expression (connexin43, integrin β1) and stem cell migration.

In MM, 6-gingerol decreased contractility, cell activity and protein content. It caused an increase in ROS production and DNA damage and a decrease in transmembrane protein expression (connexin43, integrin β1) at high

concentrations. In ESD3, 6-gingerol severely affected differentiation into cardiomyocytes cell activity and protein content at both low and high concentrations. With regards to ginkgolide A and ginkgolide B, there were alterations for few endpoints in both systems at moderate to high concentrations.

G-Rg1, from ginseng, decreased contractile activity, cell activity, protein content and elevated ROS production in both systems only at high concentrations. α-bisabolol (chamomile) showed no immediate effects on all end points at low concentrations, but several disturbances occurred at high concentrations. Eugenol (holy basil) at moderate to high concentrations, significantly decreased contractility, cell activity and protein content. The diabetic formula used showed an increase in DNA damage and a decline in cell migration in mouse embryonic stem cells.

Molecular endpoints indicate a role for reactive oxygen species and changes in cell membrane proteins.

To summarise, these data indicate that some herbal remedies used in the first trimester of pregnancy might not be safe for fetal development. Also care needs to be taken to ensure good glycaemic control in diabetic pregnancy.

Publications

Mohammed OJ, Pratten MK **(2014)** Evaluation of embryotoxicity for herb extracts using chick embryonic heart micromass system. Reproductive Toxicology **48**:33-34. doi: 10.1016/j.reprotox.2014.07.054. *(Poster presented at Annual Meeting of ETS, Hamburg, Germany, September 2014).*

Mohammed OJ, McAlpine R, Chiewhatpong P, Pratten MK **(2015)** Assessment of developmental cardiotoxic effects in chick embryonic cardiomyocyte micromass culture and the mouse embryonic stem cell differentiation model of novel phytochemicals. Reproductive Toxicology **56**:21 doi: 10.10. 6/j.reprotox.2015.07.047. *(Poster presented at Annual Meeting of ETS, Amsterdam, The Netherlands, September 2015).*

Mohammed OJ, Latif ML, Pratten MK **(2015)** Evaluation of embryotoxicity for major components of herbal extracts using the chick embryonic heart micromass and mouse D3 embryonic stem cell systems. Reproductive Toxicology **59**:117-127. doi:10.1016/j.reprotox.2015.12.003.

Mohammed OJ, McAlpine R, Chiewhatpong P, Latif ML, Pratten MK **(2016)** Assessment of developmental cardiotoxic effects of some commonly used phytochemicals in mouse embryonic D3 stem cell differentiation and chick embryonic cardiomyocyte micromass culture models. Reproductive Toxicology **64**:86-97. doi:10.1016/j.reprotox.2016.04.011.

Mohammed OJ, Latif ML, Pratten MK **(2017)** Diabetes-induced effects on cardiomyocytes in chick embryonic heart micromass and mouse embryonic D3 differentiated stem cells. Reproductive Toxicology **69**:242-253. doi:10.1016/j.reprotox.2017.03.006.

Acknowledgment

All praise and gratitude are to ALLAH Almighty for all His blessings that He bestowed on me to fulfil this hard work. First and foremost I must admit there would be no words enough to describe my supervisor, Dr. Margaret Pratten. I would like to sincerely thank you, Margy, for offering this project to study in the field of molecular developmental toxicology, for the guidance and patience in all the time of the research program but also for being supportive, kind and compassionate in all tough times that I encountered during my work. I also owe sincere thanks to my co-supervisor Dr. Siobhan Loughna for her suggestions and support which left a significant impact in my research but also in my life. Without the supervisory team, I would not have finished this thesis.

I am immensely grateful to my sponsor the Higher Committee of Education in Iraq (HCED) for offering me this opportunity and funding. Big thanks should also go to the University of Mosul for their continuous support.

I owe sincere and earnest thanks to Mr. Liaque Latif (my Taekwondo teacher) for all the support, help and motivation in the lab and also in Taekwondo sessions. I would miss your sessions, Sir!

I am also very appreciative to Mrs. Monica Owen, Dr. David Onion and all AMU unit team for their help and technical support. Big thanks also for School of life Sciences administrative team particularly, Dr. Ian Kerr, Leanne Mitchell and Susan Stelmak for being so helpful and supportive during my study.

Many thanks to our project students Roseanna McAlpine and Phasawee Chiewhatpong who worked on bisabolol and eugenol and published a paper

with us, well done! Moreover, also for Jevana Jeevahan and Priyan Magan who introduce anethole and curcumin for embryotoxicity testing.

A great big thank you to the amazing friends and colleagues Ayob, Entidar, Ghayth, Valerie, Matt, Karlai, Jen, Mosul College of Pharmacy collegues and all school mates who loved and helped me one by one, YOU were my family away from home and made my time at Nottingham so enjoyable and memorable. You're all brilliant.

Finally, thanks a million for my mother and father the secret of my success who guide, advice and pray for me through tough times, may ALLAH reward you the paradise for breeding me in such excellent way. A bow of respect and unlimited thanks should go to my lover wife, Parween, who offered me her love, time, care and support. To my sons Hayder, Ammar and Muhammed and for our new little princess Alsultana Mariyyah, I say thanks guys for making my life very interesting and exciting, you are very special gifts, May ALLAH bless you all.

Abbreviations

2’, 7’ dichlorofluorescein diacetate (DCFH-DA)
5-flourouracil (5-FU)
6-gingerol (6G)
Actin-Related Proteins (Arp2/3 complex)
activator protein 1 (AP-1)
adenosine triphosphate (ATP)
antiepileptic drugs (AEDs)
ascorbic acid (AA)
atrioventricular (AV)
Australian Drug Evaluation Committee (ADEC)
bovine serum albumin (BSA)
brain monoaminergic system (BMS)
cardiac neural crest cells (CNCCs)
casein kinase 1 (CK1)
collective cell migration (CCM)
Confidential Enquiry into Maternal and Child Health (CEMACH)
congenital heart defects (CHD)
connexins (Cxs)
death-inducing signaling complex (DISC)
deoxyribonucleic acid (DNA)
diabetes mellitus (DM)
dichlorofluorescein (DCF)
dimethyl sulfoxide (DMSO)
distilled water (DW)
DNA double-strand breaks (DSBs)
dulbecco’s modified eagles culture medium (DMEM)
embryoid bodies (EBs)
integrin-linked kinase (ILK)
Environmental Protection Agency (EPA)
ethanol (ETH)
European Centre for the Validation of Alternative Methods (ECVAM)
extracellular milieu (ECM)
Fas-Associated protein with Death Domain (FADD)
fetal bovine serum (FBS)
fetal alcohol spectrum disorders (FASDs)
fibroblast growth factors (FGF)
focal adhesion kinase (FAK)
folic acid (FA)
Food and Drug Administration (FDA)
gap junctions (GJs)
gestational diabetes mellitus (GDM)
gestational DM (GDM)
ginkgolide A (GA)
ginkgolide B (GB)
ginsenoside Rg1 (G-Rg1)
glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
Hamburger and Hamilton (HH)
hanging drop (HD)
hanks balanced salt solution (HBSS)
high-density lipoprotein (HDL)
horse serum (HS)
human embryonic stem cell test (hEST)
induced pluripotent stem cells test (iPSC)

International Committee on Harmonization (ICH)
janus-associated tyrosine kinases (JAK)
jun N-terminal kinases or (JNKs)
kenacid blue (KB)
kisspeptin 1 receptor (KISS1R)
leukemia inhibitory factor (LIF)
low-density lipoprotein (LDL)
micromass (MM)
mitogen-activated protein kinase (MAPK)
mouse embryonic D3 stem cell-derived cardiomyocyte (ESD3)
mouse embryonic stem cell test (mEST)
myocyte enhancer factor-2 (MEF2)
National Institutes of Health (NIH)
National Toxicology Program (NTP)
non-essential amino acids (NEAA)
paraformaldehyde (PFA)
permeability transition pore (PTP)
phosphate buffered saline (PBS)
Phosphatidylinositol (3,4,5) trisphosphate (PIP3)
phosphatidylinositol kinase 3 (PI3K-like)
phosphoinositide 3-kinase (PI3K)
prenatal alcohol exposure (PAE)
protein kinase A (PKA)
protein kinase C (PKC)
quantitative structure–activity relationship (QSAR)
Receptor type 1-associated DEATH domain protein (TRADD)
retinoic acid (RA)
retinoic acid receptors (RAR)
retinoid X receptor (RXR)
room temperature (RT)
Signal transducer and activator of transcription 3 (STAT3)
standard protective molecules (SPM)
stem cells-derived systems (SCD)
superoxide dismutase (SOD)
Swedish Catalogue of Approved Drugs (FASS)
tumor necrosis factor (TNF)
valproic acid (VPA)
Western blot (WB)
whole embryo culture (WEC)
wingless-related integration (Wnt)
Wiskott-Aldrich syndrome family proteins (WASP)
World Health Organisation (WHO)
Ras–extracellular signal-regulated kinase (ERK)
reactive oxygen species (ROS)

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CHAPTER 1

1 Chapter one – General introduction

1.1 Developmental toxicity and teratogenesis

Abnormal development of the conceptus, which may result from exposure to exogenous agents or conditions, could lead to one of these outcomes: structural malformation, growth retardation, functional impairment, and/or death of the organism. The science studying the cause of these adverse effects is a relatively new research branch which is called "developmental toxicology", whereas teratology, the study of human structural birth defects, appeared before written language [1]. The phrase 'developmental malformation' describes permanent fetal abnormalities which occur during the prenatal period, when many fundamental cellular processes could be affected, such as cell communication, cellular differentiation, proliferation and migration [2]. Whilst birth defects are considered as multifactorial conditions, the cause of 40-60% of these congenital anomalies is unknown. Also, it has been recorded that 15% of the cases are caused by genetic aspects such as gene mutation or chromosomal abnormalities. However, environmental factors per se could cause 10% of the birth defects, but they might be combined with genetic factors in approximately 20-25% of cases [3]. In general, teratogenic exposure during pregnancy could be classified into four categories: drug and chemical agents (including pesticides and herbal remedies), infectious disease (e.g., rubella, syphilis and mumps) physical agents (e.g., ionizing radiation, mechanical factors and temperature), and metabolic disorder (e.g., diabetes and phenylketonuria). There is very limited quality data regarding the safety of herbal medicines during pregnancy. The emphasis of this thesis is the

investigation of embryotoxic effects of some herbal components, mainly on development of cardiac cells. The selected phytochemicals might be taken by pregnant women during the first stages of pregnancy, which mostly includes organogenesis, to treat or ease certain conditions which might be triggered due to the pregnancy itself. The project is an attempt to negate the public view that herbal medicines are intrinsically 'safe' because they are 'natural', but also to rationalise the non-essential use of herbal remedies during pregnancy.

1.1.1 Principles of teratogenesis

James Wilson, a well-known teratologist, proposed five general principles of teratogenesis in 1959 which were revised in 1977 after the thalidomide disaster [4,5]. These teratogenesis principles are still acceptable at present and have continued to guide scientific research in teratology [6]; these are:

'1. Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which this interacts with adverse environmental factors.

2. Susceptibility to teratogenesis varies with the developmental stage at the time of exposure to an adverse influence.

3. Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate sequences of abnormal developmental events (pathogenesis).

4. The access of adverse influences to developing tissues depends on the nature of the influence (agent).

5. The four manifestations of deviant development are death, malformation, growth retardation, and impaired function.

6. Manifestations of deviant development increase in frequency and degree as dosage increases from no-effect to the totally lethal level.'

Wilson encouraged the scientific community to revise his principles and formulate new ones for a better understanding of teratogenesis, but in fact, since that time, no new principles have been originated or recognised [7]. However, the first revisiting of these principles was attempted by an invited lecturer at the second meeting of the International Federation of Teratology Societies held on July 14–16, 1988 in Kyoto, Japan. The author stated that individual development and teratogenesis are not single cell-dependent processes, but are part of a morphogenetic system, which is defined as a set of cell populations which communicate to create and execute the developmental process for a definitive part of any organism [7]. Molecular pharmacological mechanisms have been found to be implicated in embryotoxicity processes for various compounds, for instance, valproic acid, and many chemotherapeutic drugs [8]. However, reproductive toxicants could achieve their action through cellular disturbances at the genetic or molecular levels, such as mutation, chromosomal aberrations, cell division, energy supply for embryonic development, cellular enzyme systems, membrane characteristics, genetic transcription, RNA splicing, translation, and post-translational modification [9].

1.1.2 Cardiac teratology

Congenital heart defects (CHD) are defects in the structure of the heart or great vessels at birth. These faults often result from the diversion of several molecular pathways that are involved in embryonic heart development [10]. CHD are considered a leading cause of postnatal death, almost one out of 100

newborns are estimated to be affected directly, but the exact causes of these defects are yet to be specified [11,12]. The etiologies of 90% of CHD cases are considered complex and multifactorial; 8% are chromosomal and genetic in nature but only 2% caused by known environmental teratogens [10]. However, it is challenging to assess the incidence of non-genetic cardiac developmental defects due to a lack of information on environmental risk factors, such as radiation and air pollution and toxic chemicals [12]. The difficulty and complexity of collecting human paternal or maternal exposure data has made animal models for cardiac developmental defects become an important tool for understanding, detecting, and isolating different cardiac teratogens [13]. Understanding normal cardiac development is a fundamental element for applying the animal model data (chick, mouse, frog, and zebrafish) to human congenital heart disorders and could make the understanding of the mechanism for cardiac teratogens more reliable [14]. Although the cardiac developmental process is quite conserved among vertebrates, there is variation in genetics, anatomy, pharmacokinetic processes and bioavailability between human and animals, so, all these aspects should be considered in selecting an animal model and in the analysis of results [13]. It has been agreed that drugs, chemicals, extracts and herbs and nanoparticles may be considered as the causes of environmentally-induced CHD, as they interfere with the normal embryonic cardiac developmental processes [15].

1.2 Approaches to teratogenicity assessment

A substance that reaches the blood stream and causes developmental abnormalities or fetal death is called a teratogen. This substance is assumed

to have embryonic or placental penetrating capacity in sufficient concentrations to be toxic at a specific time in gestation [16]. Drugs and chemicals are supposed to cause 1-3% of all birth defects, but the figure is a rough approximation [17]. Such substances need to be assessed by different methods. The well-known protocols that have been developed to investigate the teratogenicity of substances are discussed below.

1.2.1 Analytical methods for structure detection

The identification of the structure of a substance is one of the methods that can give predictions about possible teratogenicity. Notably alkaloid-related elements have been shown to have developmental toxicity in different published literature, especially indolizidine [18], pyrrolizidine [19], piperidine and triterpene [16]. The structural alerts could be detected by various methods that include gas chromatography–mass spectrometry, liquid chromatography–mass spectrometry, spectrophotometry and thin layer chromatography [20].

1.2.2 *In vitro* approaches for teratogenicity assessments and the three Rs

It has been estimated that several millions of experimental animals would be sacrificed per year to perform *in vivo* developmental toxicity tests for existing and manufactured chemicals. Therefore, there is a need to find appropriate alternatives to cease or at least reduce the use of animal models [21]. The growing efforts in this area have been mainly led by the European Centre for the Validation of Alternative Methods (EVCAM), since the 1990s, when it became responsible for identifying, evaluating and validating alternative methods for developmental toxicity [22]. In February 2008, three US agencies (the Environmental Protection Agency (EPA), the National Institutes of Health

(NIH), and the consortium of the National Toxicology Program (NTP)) had met and signed a memorandum which contained an agreement to cease the use of animals in chemical safety assessments by 2020 [23]. All the efforts made in the past 40 years, to find alternative tests, have stemmed from the three R's principles which were proposed by Russell and Burch in 1959 in a paper entitled "The Principles of Humane Experimental Technique" [24]. The three Rs concept stated that 'if animals were to be used in experiments, every effort should be made to **R**eplace them with non-sentient alternatives, to **R**educe to a minimum the number of animals used, and to **R**efine experiments which used animals so that they caused the minimum pain and distress' [25]. In the United Kingdom, these principles have become formally incorporated into the Animal (Scientific) Procedures Act (1986) and they have progressively influenced new legislation intended to control the use of laboratory animals elsewhere around the world [25]. In general, *in vitro* tests for embryotoxicity can be classified into three subcategories: cell cultures (e.g. embryonic stem cell test), organ cultures (micromass models) and embryo cultures (e.g. whole embryo culture).

1.2.2.1 Approach based on stem cell-derived systems (SCD)

Three stem cell-derived systems are in common use, namely: the mouse embryonic stem cell test (mEST), human embryonic stem cell test (hEST) and induced pluripotent stem cells test (iPSC). However, the most widely used test among the developmental toxicity assays is the mES cells [26]. The potential of mEST to detect embryotoxicity is approximately 73, 69, and 100% to identify whether tested chemicals are non, weak or strong teratogens, respectively [27]. ECVAM has approved the use of non-human embryonic stem

cells in culture studies as an alternative model [28]. The stem cells' pluripotency, which theoretically enables them to differentiate into all types of mammalian cells, offers a valuable tool to assess adverse developmental effects on the differentiation process of any substance [17]. The *in vitro* differentiation of EST into cardiomyocytes has been widely used in developmental toxicity studies because it is easily monitored by clearly identified beating cells in the cultures [29]. Nonetheless, there are some limitations to this approach, which are the long culture duration and subjectivity of endpoint scoring [30]. However, Karen *et al.* (2010) suggested three goals to refine this test; these includes: finding and validating a molecular end point of differentiation instead of the current phenotype based assessment, shortening the duration and increasing the throughput of the assay [31]. Attempts at 'humanisation' of mEST have been undertaken to overcome the interspecies variation. Similar to mES cells, the ability of hES cells to produce embryoid bodies (EBs) with three germ layers (ectoderm, mesoderm, and endoderm) maintained by all required transcriptional genes has been recently verified [32]. However, the biggest obstacle encountered in this system before its validation could be accomplished is the ethical issue of human embryo usage in the research field. The regulations for the use of hES cells in the European member states, which were last updated in September 2003, have been very controversial. Only Belgium and the United Kingdom have clearly stated the legal position for the production of human embryos for ES cell research; the other countries have made no regulations, or restrictive regulations or prohibited derivation of ES cells from human embryos [21]. However, an alternative to hES cells was introduced recently, iPSC, in which

differentiated adult cells are reprogrammed to regain pluripotency and can then differentiate into any cell type [33,34]. The main advantage of this model is the possibility of selection of a compatible donor, which may allow for more precise data collection, but also it circumvents the ethical issues of using human embryonic stem cells as a test model [35]. Several experimental hurdles were identified when using reprogrammed stem cells. It has been demonstrated that reprogrammed stem cells were associated with nucleotide mutations in 22 cell lines [36]. In addition, many studies have shown that mRNA and gene expression of iPSC hugely differ from ES cells, which adds another challenge that should be addressed before mES or hES cells might be replaced by iPSC [37-40].

1.2.2.2 Approach based on mammalian embryonic primary cells - micromass assay (MM assay)

Many *in vitro* culture methods have been developed including whole embryo culture or even single cell culture. However, the micromass culture could be placed in the middle, between the two culture approaches [41]. The method for micromass was developed in the 1980s using rat midbrain and limb bud embryonic cells grown in special media to allow the cells to differentiate, launching a new *in vitro* test supporting other tests to evaluate compounds for their embryotoxicity potential [42]. Later, this system was employed in mouse [43] and recently in chick [44]. This test is based upon using primary early embryonic cells from any animal species; these have to be disaggregated before seeding them *in vitro* at a high density when they reassemble, forming fully differentiated cultures. The teratogenic potential of chemicals could be investigated using this method, especially studying cell-cell communication,

cellular differentiation, and cell-matrix reactions [45]. Rat limb bud micromass is the only ECVAM registered and validated (using 20 well known teratogenic compounds) micromass test for developmental toxicity [22]. Primary cells from several organs have been employed in the micromass assay including limb buds, digits, palatal shelves, lung, intestine, midbrain and sex organs [46]. Pros and cons of the micromass system as a cell-based model have been apparent. Firstly, the lack of a 3D culture environment, which means the system is functionally restricted and naturally disconnected from interacting tissues, secondly, the difficulty of quantifying physiological parameters, but also thirdly the absence of the effect of maternal toxicity and indistinct extrapolation of the test duration and dose into human data [47]. However, the main advantages of this model are cost effectiveness, fewer animals are required, and it overcomes some interfering factors from maternal toxicity such as placental metabolism [48]. Except for rat limb bud micromass, all other organ micromass models are still in the pre-validation stage, and many efforts and studies are in progress toward their validation.

1.2.2.3 Approach based on whole embryo culture test (WEC tests)

The rodent post implantation WEC system is one of three currently validated developmental toxicity models. The initial purpose of this system was for studying the regulation of embryogenesis [49], but since then it has been widely utilised in teratogenicity screening of drugs and chemicals [50-52]. The system has been considered quite accurate regarding detecting the teratogens, and its accuracy approaches 80% [53]. In the WEC test, whole embryos from different rodents and lagomorphs are maintained intact in their visceral yolk

sac, supported by growth media for a period, until the organogenesis process is completed [54]. Monitoring allows the main phases of organogenesis, such as neural tube formation, cardiac development, early development of eyes and ears and limb buds to be investigated; malformation for any organ could happen as a result of either proliferation or differentiation disturbances [54,55]. The incorporation of drug-metabolising enzymes is another advantage of this test system; also the drug or metabolite concentration in embryonic tissues could be easily monitored [56]. One suitable measure of embryonic development in this method is the total embryonic protein because it increases logarithmically during early development [57,58]. However, some researchers argue that the lack of maternal-embryo interactions can be considered a drawback for this system, where maternal metabolites might have an effect on embryonic development and eventually result in birth defects [26]. The system was also criticised for the period where the embryotoxicity might happen. For instance, any chemical showing an effect on implantation or the early stages of gastrulation might be missed, as the system deals with the post-implantation period; further, as the embryo can persist for only short period of time in culture (technically about 72 hours), this means embryotoxicity in the advanced organogenesis period would be ignored [26].

1.2.2.4 Approach based on lower order classes test

The use of lower order species (frog and fish embryos) in the field of embryonic toxicity has increased greatly because it supports the current interest in applying the 3 Rs principle (refinement, reduction, replacement) in decreasing the number of animals required for teratogenicity assessment [59]. Different vertebrates (amphibians, zebrafish) have gradually started to take their

position as an alternative for mammals; the similarity of their embryogenesis to those of high-order species at both morphological and molecular levels make them a reasonable alternative [60]. These systems offer many advantages for teratogenicity evaluation, including low cost, good embryo fecundity, acceptable productivity and reproducibility [61,62].

1.2.2.5 Approach based on invertebrates test

Invertebrates could be a very powerful alternative technique for toxicity tests. Hydra, a simple metazoan animal found in clean slow-moving fresh waters, has been suggested to replace or reduce animal usage in teratogenicity screening tests [63]. However, because the invertebrates have less advanced organ systems, and they lack the adaptive immune system, these factors were considered drawbacks of the system [64]. Further, Hydra shows limitations in studying water-insoluble chemicals [48]. However, invertebrate models possess several advantages such as their small size and simple anatomy, overcoming the social and scientific ethical debate of using laboratory animals in experiments and they are inexpensive compared with the cost of housing of higher animals [65]. This model is yet to be validated, and many more studies are required to show its validation might be achieved.

1.2.3 *In vivo* approaches for teratogenicity assessments

In vivo embryonic toxicity tests basically use rats, rabbits, mice and monkeys. The embryos are exposed to test compounds in their mother's uterus, and the mother is periodically weighed and checked for any toxicity signs along the gestational period [66,67]. Wilson in 1975 advised that metabolism, distribution and transferring test chemicals cross the placenta can be

accomplished using animal models in teratogenicity screening tests [68]. Three years later, Wilson highlighted the importance of having mother-placenta-embryo interaction in any developmental toxicity model used [69]. Schardein and Keller have evaluated some animal models, namely, rat, mouse, hamster, several species of primate and rabbit. Although no one animal species has been identified to be a perfect animal model in developmental toxicity screening tests, their data showed that these models were reliable for identification of human developmental toxicants, after being tested against some 50 already known teratogens [70]. In order to increase the potential detecting accuracy for teratogens and to improve the opportunity to distinguish human teratogens, testing in more than one species is very necessary; rat and rabbits are typically recommended for historical and physiological reasons (where thalidomide showed teratogenicity in some strains of rabbits but not in rats) [71]. Furthermore, one of the big challenges that is encountered in animal models is the appropriate extrapolation of data from animal species to humans; factors such as the difference in sensitivity between animal species and human toward tested compounds, doses and exposure periods need further understanding and standardisation [72]. Using animals as testing tools is increasingly facing public protests worldwide and many governments are now under pressure to support alternative methods and programs to cease using animals in experiments. This is backed up by the view of the EU REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) program, which is aimed at completing a minimal toxicity database for thousands of existing chemicals during the coming decade [73], hence, a call for alternative embryotoxicity models is urgently required.

1.2.4 Computational (In silico) toxicology model

In silico toxicology methods are also known as computational toxicology, predictive quantitative structure–activity relationship (QSAR) modeling of toxicity, e-tox, i-drug discovery or predictive ADMET (absorption, distribution, metabolism, excretion, and toxicity). They can be defined as a computer-based informative tool which shows a chemical structure utility for producing compound characteristic information to help in compound risk assessment at the design stage of the pharmaceutical industry [74]. Another definition of computational toxicology (i.e., *in silico* toxicology) has been proposed by the U.S. Environmental Protection Agency (EPA), which is "integration of modern computing and information technology with molecular biology to improve agency prioritization of data requirements and risk assessment of chemicals" [75]. The system has been suggested to be a very powerful alternative toxicity method but primarily it has to successfully address some essential challenges that hamper it from being a fully reliable model. However, being considered as a high throughput approach, several regulators have recommended using the QSAR model as a preliminary screen to evaluate the degree of further testing that is needed [76]. Attention should be drawn to some limitations in the current computational system; as it relies totally on input data, which in turns relies on human credibility, thereby, assurance and transparency of high-quality experimental data (e.g. molecular structure animal toxicity data) used to build the QSAR model is necessitated [77]. The applicability domain of this model (the limit of the setup information of the model) has been established as a major limitation in the use of *in silico* QSARs in toxicology and pharmacology, simply because the prediction is invalid if an

in silico screened pharmaceutical is outside its applicability domain [78,79]. A long-term goal to develop this system has been proposed to incorporate more complex data (such as receptors and signal pathways) into the computer to provide simulations of multicellular behaviour and dynamic interactions. It is aimed to help in better understanding and interpretation of the emerging data, which may assist in reducing uncertainty in risk assessment for embryonic teratogenicity [80].

1.2.5 Approach of alternative *in vitro* test battery

In the past few years, some attempts have been made to combine several alternatives *in vitro* tests to assess the augmented value of a test battery over an individual assay. It is the complexity of the reproductive cycle, the necessity to reduce the number of experimental animals and presence of thousands of compounds in the market which need to be tested for their embryotoxicity, that encouraged embryotoxicologists and teratologists to employ more complex *in vitro* procedures for investigating chemicals that might carry reproductive hazards [81]. Recently, 10 compounds have been studied in a battery of 14 tests by The EU Framework 7 Integrated Project ReProTect [82]. The study demonstrated the concept that a combination of *in vitro* alternative tests was able to identify all teratogens whose modes of action were represented in at least one of the tests within the battery. According to recent studies, the commonly used battery includes these tests: zebrafish embryo test to monitor any effect on egg development from fertilisation until hatching; embryonic stem cell test to assess effects on cardiomyocyte differentiation; ReProGlo assay which includes the wingless-related integration (Wnt) pathway monitoring; and metabolic assays for evaluating CYP17 and CYP19 which are

essential in hormone reproductive homeostasis [81,83]. In the absence of any *in vivo* or pertinent structural information of tested chemicals, the battery test serves as an optimising filter which prevents further additional animal studies for detecting teratogens or at least prioritises them for further limited animal studies [84,85]. However, although this battery assay seems to be promising, more studies should be performed before it is applied as a validated assay to replace the *in vivo* tests.

1.2.6 Omics approaches for teratogenicity assessments

Transcriptomic, proteomic, genomic and metabonomic techniques are starting to occupy an interesting place in supporting the substantial efforts of using alternative methods for teratogenicity testing. However, their use is still in the early stages, although their initiation has given a strong push to the reduction of use of animals *in vivo* toxicity tests [86,87].

1.3 Use-in-pregnancy ratings categories

The United States Food and Drug Administration (FDA) employs "Use-in-Pregnancy Ratings" to categorise the maternal advantage to fetal hazard with respect to drug exposures; the categories are as follows [88]:

Category A: Controlled studies in women failed to demonstrate a risk to the fetus in the first trimester, and the possibility of fetal harm appeared remote.

Category B: Either animal reproductive studies have not demonstrated fetal risks, but no controlled studies in pregnant women have been reported, or animal reproductive studies have shown an adverse effect that was not confirmed in controlled studies in women in the first trimester.

Category C: Either studies in animals have revealed adverse effects on the fetus but no controlled studies have been reported, or studies in women and animals are not available. Drugs should be given only if the potential benefit justifies the potential risk to the fetus.

Category D: Positive evidence of human fetal risk exists but the benefits for use in pregnant women may be acceptable despite the risk.

Category X: Contraindicated in pregnancies: Studies in animals or human beings have demonstrated fetal anomalies or evidence exists of fetal risks based on human experience or both, and the fetal risk clearly outweighs any possible benefit.

The Teratology Society has recommended that the drug labelling of the FDA Use-in-Pregnancy Ratings be removed and substituted by descriptive statements that summarise and explain available data regarding hazards of developmental toxicity and present some estimations of teratogenic risk [89]. The using of 'letters' in this system might suggest the utilisation of a risk gradation system, while the original classification does not necessarily follow such gradation. For example, category C is the most misleading category in the system as it implies some degree of risk rather than just lack of safety information regarding particular agent [89]. Addis *et al.* (2000) analysed 645, 446 and 527 drugs included in three risk classification systems, the US FDA, the Australian Drug Evaluation Committee (ADEC) and the Swedish Catalogue of Approved Drugs (FASS), respectively, and he found that only quarter of analysed drugs were assigned to a similar risk class [90]. It was concluded that differences in the same drug allocation would result in confusion for practitioners and may limit the practicality and reliability of the current risk

classification systems. Medical staff should be informed by a better risk classification method about the teratogenic potential of drugs and perhaps a comprehensive revision of the current systems should be considered soon.

1.4 Herbal Remedies for Early Pregnancy-Induced Conditions

1.4.1 Historical view

It has been recorded that the first use of herbs as medicines effectively began at the start of history itself [91]. However, this ancient inclination to treat by herbs has been extended to become a modern trend in different parts of the world [92,93]. It has been shown, by several studies, that almost half of pregnant women have chosen herbal medicines for various purposes during their gestation time [94].

1.4.2 Common early pregnancy conditions and their eminent herbal treatment

1.4.2.1 Pregnancy-induced nausea and vomiting

Nausea and vomiting during gestation, also known as morning sickness, are considered as an annoying and offensive symptom which is encountered by almost two-thirds of pregnant women during the first trimester [95]. Nevertheless, some pregnant women continue suffering this condition until just before delivery [96]. Sometimes the sickness develops to be severe vomiting (hyperemesis gravidarum) that it leads to dehydration, where hospitalization becomes necessary for management [97]. The underlying cause of this condition is not entirely understood, but it is realized that the central nervous system and chemoreceptor trigger zone play an essential role here. Activation

by cholinergic or serotonergic mediators of this centre leads to a slowing of gastrointestinal motility accompanied by a reduction in stomach secretion and weakness in tonicity of the oesophageal sphincter. This effect leads to expulsion of the stomach contents [98]. Other theories suggested that this is either triggered by a by-product of "mother-embryo genetic conflict" or by hormones associated with pregnancy [99]. It could be a protective measure for the fetus, by making mother expel some potentially harmful substances [100]. The three most popular herbs that can counter the above mentioned morning sickness mechanisms and are used by pregnant women to treat this condition, often without advising their doctors, are ginger, peppermint and cannabis [101-103].

1.4.2.2 Pregnancy-induced stress/depression

It has been estimated that about half of all pregnancies are unplanned, which adds further stress which might turn into depression in the early stages of gestation [104]. Untreated depression in gestation has been correlated with increased danger of miscarriages, premature birth and low birth weight [105]. The antidepressant herbal medicines are: St. John's Wort via its two main constituents, hypericin and hyperforin [106-109]; recently discovered antidepressant activity of ginseng, Rhodiola Rosea (roseroot) and Crocus sativus (saffron) [110]. Several mechanisms were suggested through monoamine (serotonin, dopamine and noradrenaline) reuptake inhibition, improved binding and sensitisation of serotonin receptors, inhibition of monoamine oxidase, and neuroendocrine modulation [111,112]. Chamomile and holy basil have also shown anti-stress and anti-anxiety activities which are

the main reasons to encourage pregnant women to use these herbs during their gestation period [113,114].

1.4.2.3 Common cold during pregnancy

Echinacea, also called purple coneflower, has reached a high selling point as an herbal remedy for flu and common cold conditions in Europe and the USA [115,116]. The three top species of this plant are Echinacea Purpurea, Echinacea Angustifolia and Echinacea Pallidum [117]. The reason behind using this plant is the fact that echinacea can activate macrophages, polymorphonuclear leukocytes and natural killer cells, which enables it to modulate the immune system and improve defence mechanisms, which in turn made it popular as a "cure" or prophylactic herb for flu and the common cold [118]. Regarding safety during pregnancy, the concern for the teratogenic potential of echinacea is raised due to its effects on embryonic angiogenesis in mice. Therefore, there is an opportunity for the echinacea ingredients to influence fetal development in the human as well [119]. Further, chamomile has also been recorded to be commonly used in the Middle East during pregnancy to ease flu and cold symptoms [120].

1.4.2.4 Pregnancy-induced weakness and anaemia

The relationship between nutrition during development, birth weight at delivery and appearance of diseases through later life stages has been well documented [121,122]. The alteration of nourishment levels in pregnancy could cause different defects in the offspring's biological systems [123-126]. The herbs that have been used as tonics during the gestation period are ginkgo biloba, which is one of the most commonly used herbal supplements [127],

and ginseng, which is according to Chinese traditional medicine. The 'tonic' features and powerful accumulative actions of ginseng appear particularly when the herb is taken regularly [110].

1.4.2.5 Unexplained infertility in women

Infertility itself is another reason that might expose the early fetus to herbal medicines. Many herbal supplements contain phytoestrogens and have been examined and marketed purporting positive fertility effects [128]. Shahin *et al*. (2009) demonstrated that 120mg per day of black cohosh (Cimicifuga racemosa), a herb native in the USA, shows an agonist effect with clomiphene by increasing luteinizing hormones, progesterone and oestradiol in addition to increasing the endometrial thickness with significant improvement in clinical pregnancy [129,130]. So far, no safety information is available regarding this herb in terms of its effect on fetal development.

1.4.2.6 Diabetes mellitus during pregnancy

Diabetes mellitus (DM) is the most frequently encountered metabolic disorder during gestation which has a high potential risk to harm both the mother and her fetus [131]. Diabetes mellitus could either develop during the gestation (GDM), but it could also represent pregnant women who already have type I or II diabetes. A new program was initiated in the UK (2002-2003) by the Confidential Enquiry into Maternal and Child Health (CEMACH) committee to compare the events of perinatal morbidity and mortality of type I and type II diabetic pregnancy deliveries to non-diabetic pregnancies. This showed in 2005 that 1 adverse event in 364 babies was born from type I and 1 adverse event in 955 babies were born from type II diabetic mothers. However, the study

showed that major defects of diabetic mothers was 41.8 per 1000 births compared with 21 per 1000 births for births of mothers in general [132]. The most common risk factor for the development of GDM is obesity. It has been found that approximately 1 in 5 obese pregnant women can develop GDM [133]. Whilst fetal morbidity should always be considered as one of the GDM consequences, McCance (2015) has demonstrated that the main concern regarding the mortality is the long-term metabolic and cardiovascular implications for both mother and child [134]. One of the herbal components of holy basil, which is called eugenol, showed some hypoglycaemic characteristics through inhibition some diabetes related enzymes (pancreatic α-amylase, lipase, and angiotensin converting enzyme activity) in diabetic rats, but also brings about a decrease in cholesterol levels where low-density lipoprotein (LDL) cholesterol decreases whilst high-density lipoprotein (HDL) increases [135]. Several further herbal alternatives which might be offered by traditional Chinese medicine to treat DM such as herbs with a bitter flavour based on San Huang Tang, Kushen (Radix Sophorae Flavescentis), Longdancao (Radix et Rhizoma Gentianae), Kuding (Herba Corydalis Bungeanae), and Shanzhizi (Fructus Gardeniae) also could be considered. Herbs of sour flavour are represented by Fructus Mume formula, Shanzhuyu (Fructus Corni), Suanzaoren (Semen Ziziphi Spinosae), and Shiliupi (Pericarpium Granati) should also be considered [136]. Collectively speaking, much more needs to be done in terms of embryotoxic investigations for these herbs before they might be advocated for pregnant women.

1.5 Current herbal medicines regulations

The Chinese pharmacopeia contains over 2000 Chinese drugs and Chinese crude drugs, fats and extracts, vegetable oil, patented Chinese traditional medicines, and single components of Chinese crude drugs [137]. Reports showed that there are over 300 herbal medicines which have been used during pregnancy on a daily basis, all being considered safe and effective [138]. However, among hundreds of herbal medicines, only 30 of them were classified as contraindicated, not recommended or used with caution in pregnancy [138]. Marcus and Snodgrass in 2005 have suggested that there is not sufficient scientific data to indicate that herbal remedies are safe and gentle in pregnancy. Their proposed commentary stated 'The active ingredients of plant extracts are chemicals that are similar to those in purified medications, and they have the same potential to cause serious adverse effects', which criticised the quality control and very limited toxicity studies of herbal medicines [139]. The Teratology Society has also indicated that the safety of herbal medicinal products for the embryo or fetus should not be assumed by the public, or even by some health care providers and obstetricians, who should advise women that fetal exposure to herbal medicines is not a risk-free measure [140]. Hence, there is a current necessity to apply pharmaceutical style safety for herbs to ensure that they meet governmental regulations and the demands of pregnant women. The International Committee on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has grouped the US FDA and European Medicines Agency guidelines for pharmaceutical testing and their registration regulations together. This also suggested that all pharmaceuticals of human use must undergo embryotoxic

evaluations to identify any risks for fetus or mother [141]. However, the main strategy of the World Health Organisation (WHO) is to focus more on the development of traditional medicine as a field rather than only concentrate on the traditional medicine products [142]. While many might consider traditional knowledge, skills, and practices are far away from the principle of scientific methods, the European framework for traditional herbal medicinal products has recognised the position of traditional medicine based upon long-standing use [142]. The decision was based partially on the definition of traditional medicine by WHO, which states 'Traditional medicine has a long history. It is the total of the knowledge, skill, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness' [143]. An herbal medicine product may be registered in the EU only if it has been used for at least thirty years within the EU. However, medicinal use outside the EU can be considered only if the product has been used for at least 15 years in the EU [142]. On the other hand, US FDA defines dietary supplements as 'ingredients as vitamins, minerals, *herbs*, amino acids, and enzymes'. Dietary supplements are marketed in forms such as tablets, capsules, softgels, gelcaps, powders, and liquids [144]. What essentially comes out is that an herb being part of a diet supplement does not have to meet the same guidelines as drugs and over the counter medications for proof of strict safety, effectiveness, and good manufacturing practices. With these facts, one can conclude that at the moment, drugs are considered unsafe until proven safe, whereas dietary supplements are considered safe until proven unsafe.

1.6 Aims and objectives

The main aims and objectives of this thesis are

- To evaluate two *in vitro* techniques to be used for developmental toxicity screening purposes.

- To investigate and compare the teratogenic potential for some commonly used herbal components by using chick embryonic cardiomyocytes and mouse embryonic D3 stem cell-derived cardiomyocytes models employing different end points.

- To detect possible protective effects of the selected herbal components against standard teratogens in both utilised systems.

- To determine the potential teratogenic mechanisms at the molecular level of the effects on embryonic heart cell development induced by herbal components.

- To study the embryonic cardiotoxic effects of experimental diabetic conditions utilizing *in vitro* micromass and stem cell systems.

CHAPTER 2

2 Chapter two - Optimising and evaluation of two *in vitro* alternative models; the chick embryonic heart micromass and mouse embryonic D3 stem cell system

2.1 Introduction

Experimental design and optimization are essential elements of scientific research. It is a necessity to plan the experiments in such a way that valid data will be obtained [145]. However, the main purpose in any optimisation is to determine the experimental conditions that generate reproducible results. It is essential that the influence of one variable at a time be monitored [146]. In this study, two *in vitro* models were employed, namely, the chick embryonic cardiomyocyte micromass (MM) system and the (ECVAM-validated) mouse embryonic D3 stem cell-derived cardiomyocyte (ESD3) system. Both systems were optimised and evaluated by using different cell seeding densities, by identification of cardiomyocytes within the cultures and by treating the cells with known standard teratogens (ethanol, retinoic acid and valproic acid) and standard non-teratogens (folic acid, ascorbic acid and superoxide dismutase). Three endpoints were monitored: contractile activity, cell activity (resazurin reduction assay) and total protein content (kenacid blue assay).

2.1.1 Major chick heart developmental stages

Among various available vertebrate models, such as zebrafish, mouse or xenopus, chick heart is a longstanding model used by cardiovascular scientists and embryologists. This is perhaps because it is easy to obtain but also because it provides ready observation of a living embryo even at early stages of embryogenesis [147]. The main cardiac development stages are extremely well-maintained processes in all vertebrate organisms; in particular, the stages from the linear heart tube phases into the early stages of looping and chamber formation, which are nearly indistinguishable [148], especially when it comes to comparison of chick heart development stages with those of the human [149,150]. The critical period of embryonic exposure to a cardiac teratogen in human is the very first weeks of gestation (week 2 to 7), where the outcome is most likely to be cardiac malformation [12]. The early milestones of chick cardiogenesis will be described and correlated to the same stages in the human according to the staging of Hamburger and Hamilton (HH, 1951).

2.1.1.1 Cardiac Tube Formation

The early human embryo is composed of a flat disk of cells called a blastula, which is formed after implantation into the endometrium; subsequently, the blastula converts to a trilaminar structure (ectoderm, mesoderm, and endoderm) by a process called gastrulation, which includes massive cell migration and differentiation processes [151]. A structure called the 'primitive streak' appears as a thickened layer of cells in the caudal or tail end of the embryo. The formation of the primitive streak begins the gastrulation process (Figure 2-1A and B) [152]. Indeed, gastrulation is a very sensitive period for teratogen exposure because it involves cellular migration, differentiation and

proliferation, which result in establishing the basic morphogenic fields of the embryo. In the chick, it starts at HH3 (12-13 hours of incubation) [153,151]. However, organogenesis starts at HH4 (18 hours of incubation) and the first organ to be formed is the heart; this period is correlated to 15-16 days of the human embryo [13,153]. At HH5 (22 hours of incubation), endoderm initiates a signal to the anterior lateral plate mesoderm in order to induce progenitor cells to perpetrate a cardiogenic destiny and form two crescent-like cardiogenic plates [154,155]. The primitive heart tube is formed after fusion of these two plates at the midline [156]. Furthermore, the tubular heart starts beating at HH10 (33 hours of incubation) correlating to day 23 in human embryo; at this stage, the looping process for the ventricular region of the tubular heart begins and any disturbances of this process leads to heart malformations later (Figure 2-1A) [155,153].

2.1.1.2 Cardiac Looping

At this stage, HH17 (52 hours of incubation), structures called cushions are formed due to thickening and swellings of the extracellular matrix at valve forming regions of the heart tube, outflow tract, and atrioventricular (AV) junction. These structures end up differentiating into the AV and semilunar valves [155] (Figure 2-1C). The AV cushions contribute to the interventricular and interatrial septa [157,153]. However, many extensive repositioning-remodelling processes for these inner structures of the looped heart tube must be completed to make the cardiac chambers, aortic and pulmonary arteries possess their final structure [158]. The heart finishes the looping period by HH18 (69 hours of incubation) by completing the S-shaped heart loop; this period compares to about day 30 in the human embryo [159,153].

2.1.1.3 Cardiac septation and chamber formation

The atria and ventricles do not acquire their proper final shape until stages HH 21-23 (day 3.5-4 after incubation) [13,153]. During this period of development, the fusion of the ostium primum and ostium secundum is typically completed by the development of the primary and secondary inter-atrial and AV septum (Figure 2-1D) [13,160]. However, an insufficient fusion of these septa results in different heart septal defects such as the AV septum defect, primary atrial septum defect, and atrial secundum septum defect [161]. In general, communication and physical location of the cardiac chambers are recognised by almost HH32 (day 7.5 after incubation) and cardiac morphogenesis is completed at approximately HH36 (day 10 after incubation) [13,153].

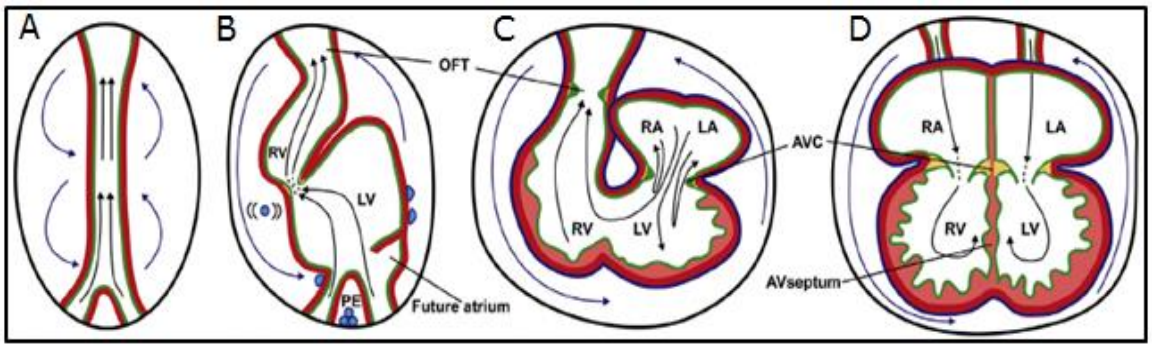


Figure 2-1: Illustration of stages of vertebrate heart development
(A) primary heart tube starting of myocardial contraction, (B) initiation of looping and chamber and PE formation, (C) ventricular trabeculation and valve and epicardium formation, (D) Chamber septation. Arrows illustrate turbulent flow. The endocardium is marked green, myocardium red, epicardium blue, and the developing valves yellow. Blue circles represent proepicardial cells. A, atrium; AVC, atrioventricular canal; LA, left atrium; LV, left ventricle; OFT, outflow tract; PE, proepicardium; RA, right atrium; RV, right ventricle; V, ventricle.Taken from[160].

2.1.2 Principal of stem cells

Stem cells are characterised from other cell types by two important features. First, they are unspecialised cells which can undergo unlimited self-renewing through cell division, sometimes after long periods of dormancy. Second, 'pluripotency', which means that under certain physiologic or experimental conditions, they can be ultimately differentiated to become any tissue- or organ-specific cell with special functions [162]. However, plasticity can also be considered an attribute of stem cells where they can differentiate into cell types beyond the tissue where they originally reside. Stem cells can be categorised into three types, totipotent, pluripotent and multipotent [163] and two origins, embryo and adult stem cells [164]. Bongso and Richards (2004) have clearly defined these terminologies [165], see Figure 2-2:

- Totipotency is the ability of stem cells to differentiate into all cell types of the conceptus, including the entire fetus and placenta. Early mammalian embryos are clusters of totipotent cells.
- Pluripotency is the ability to form several cell types (almost 200) from the three germ layers (ectoderm, mesoderm and endoderm) but it lacks the ability to generate the whole organism.

- Multipotency is the ability of stem cells to give rise to a limited range of cells and tissues appropriate to their location, e.g. skin stem cells give rise to the different types of skin cells, whereas blood stem cells give rise to red blood cells, white blood cells and platelets.

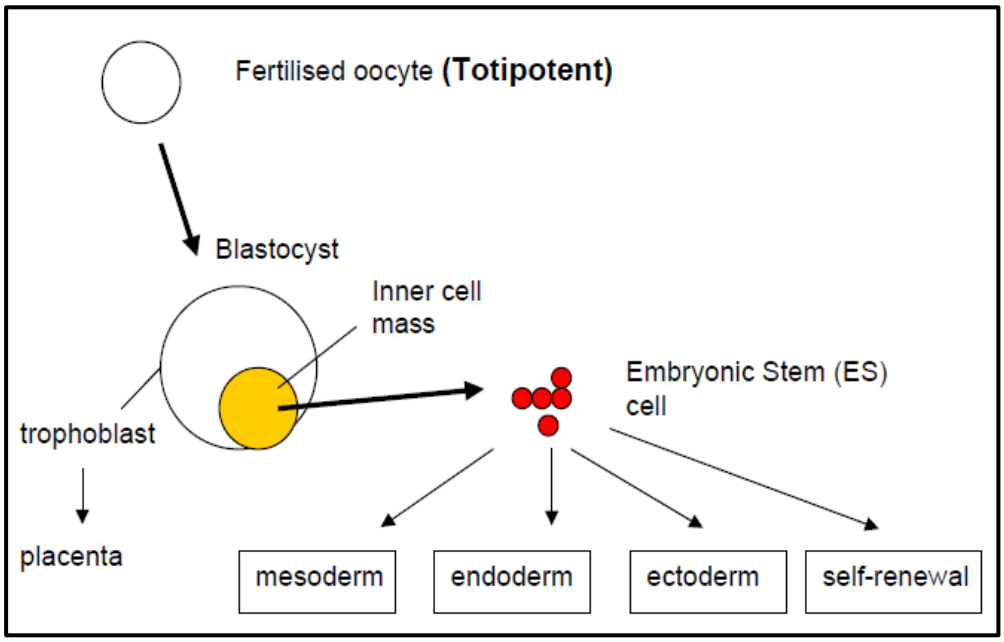


Figure 2-2: Illustration of stem cell origin
The diagram demonstrates that the fertilised oocyte (totipotent) can give rise to all tissue types and can produce an embryo. Blastocyst (inner cell mass) is derived from a fertilised oocyte. It also shows that embryonic stem cells are derived from the inner cell mass of a preimplantation embryo. These cells are termed (pluripotent) as they can give rise to most, however, not all, tissue types within the body. Adopted from [162].

2.1.2.1 Embryonic stem cell self-renewal signalling networks

Embryonic stem cell self-renewal depends on the presence of leukemia inhibitory factor (LIF) - an interleukin (IL-6) cytokine family member - or other cytokines that activate intracellular signal pathways from cell surface receptors [166,167]. Human ESC differ from mouse ESC in their response to LIF, perhaps due to fewer receptors being available in the human than in the mouse ESC [168,169]. The LIF receptor comprises of the LIF-specific receptor subunit (LIFRβ) and the common signal transducer (gp130), and the activation of this

receptor can be achieved by cytokines other than LIF [170]. For a long time, scientists thought that the renewal process was completely governed by the 'JAK-STAT' activation pathway. It was proposed that LIF stabilises the association of LIF receptor and gp130 transducer. Resultant activation of receptor-associated Janus-associated tyrosine kinases (JAK) causes the recruitment, phosphorylation, and dimerisation of signal transducer and activator of transcription 3 (STAT3). Next, the STAT3 dimers translocate to the nucleus, where they bind to a specific DNA region that controls the transcription of genes adjusting the self-renewal process (Figure 2-3) [171]. One of the transcription factors that plays a major role in this pathway is octamer-binding transcription factor 4 (Oct4). It is considered a key element in maintaining pluripotency of the ICM, but it also relies on LIF availability for functioning [172,173]. On the contrary, Chambers *et al*. and Mitsui *et al*., in 2003, have discovered what they called Nanog, the gene that has an essential role in the stem cell renewal pathway. It has been found that overexpression of the Nanog gene resulted in stem cells renewal even when STAT, JAK and LIF effects were terminated [174,175]. However, Nanog overexpressed cells showed enhanced proliferation when LIF was introduced, which suggests Nanog and LIF may work in synergy [162]. The other important gene transcription in the stem cell renewal pathway and maintenance of their pluripotency is sex determining region Y-box 2 (SOX2) [176]. This gene transcription co-factor cooperatively functions with Oct4 in such a way to either suppress or activate other stem cell renewal related genes [177].

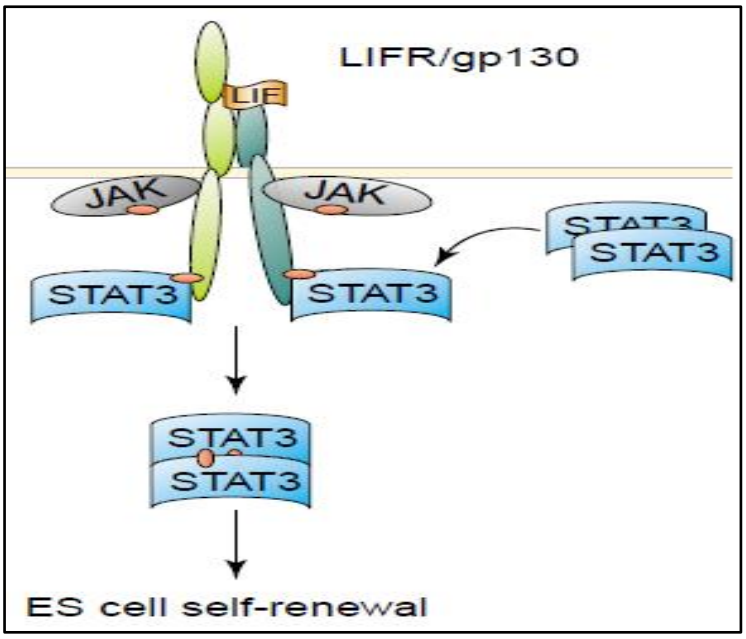


Figure 2-3: A schematic illustration for LIF activated the process

The process is believed to undergo via JAK-STAT pathway to keep stem cell pluripotency characteristic operational taken from [168].

2.1.2.2 Embryonic stem cell differentiation into cardiac lineage

The formation of three-dimensional aggregates termed embryoid bodies (EBs), has been widely used and been considered the main step in the differentiation of ES cells. The first pluripotent cell line isolation of mESC had been achieved almost 40 years ago, which was derived from the inner cell mass of a preimplantation blastocyst [178,179]. The withdrawal of LIF or mouse embryonic fibroblast (MEF) feeder layers enhances spontaneous differentiation of ES cells to form EBs. These spheres contain three germ layers (ectodermal, mesodermal, and endodermal) and can show multicellular dynamic interactions, recapitulating many aspects of cell differentiation during early mammalian embryogenesis [180,181]. The key technique of EB formation is to culture ES cells in suspension without differentiation hindering factors such as LIF and feeder layer cells. The three basic methods which are mainly utilised for EB formation are culture in methylcellulose semisolid media, liquid

suspension culture in bacterial-grade dishes, and culture in hanging drops [182,183]. However, EB-independent strategies for differentiation of hESC have also been developed but less frequently utilised [168]. Although two types of EBs were described, simple and cystic EBs according to the stage of their development, researchers tend to use the term 'EBs' regardless of the methodology or the features [184]. Several transcription factors and signalling molecules have been shown to play a vital role in the differentiation of stem cells into the cardiac lineage, but their combinatorial interactions need further elucidation [185]. These are from several families: NK-2 homeodomain protein, GATA, and myocyte enhancer factor-2 (MEF2) [186]. NK-2 homeobox genes also termed 'Nkx genes', are expressed in mammals in early overlapping patterns. However, Nkx2-5 (also called Csx) seems to perform a unique function in cardiac development. Among all other cardiac-specific genes which are involved in initial vertebrate heart development, Nkx2-5 has been proven to be the earliest known cardiogenic marker [187]. Jamali *et al*. (2001) revealed that Nkx2-5 knockdown in mice led to the failure of looping morphogenesis and abnormal cardiac muscle growth. The mutant embryos were able to develop a primitive heart tube and express some cardiac-specific genes, but eventually they died midway through gestation [188]. Similarly, the role of GATA transcription factors has been confirmed in cardiomyogenesis. There are six GATA transcription factors, but according to their expression patterns, the GATA factors have been allocated into two subfamilies, GATA-1, -2, and -3 and GATA-4, -5, and -6 [189]. The latter family has been implicated as important regulators of gene expression in some tissues such as heart, liver, gonads, gut epithelium, and lungs [190]. Also, Molkentin *et al.* (2000) have

demonstrated that mouse GATA factors directly regulate the Nkx2.5 gene in the developing heart and reveal a jointly reinforcing regulatory circuit of Nkx2.5 and GATA transcription factors during cardiogenesis [191]. GATA-4 and -5 null mice show a similar pattern of expression and die between embryonic day 8 and 9 because of defects in cardiogenesis and ventral closure of the foregut. However, mice null of GATA-6 die during day 5.5-7.5 of embryonic development because of defects in visceral endoderm function [189]. Collectively speaking, GATA transcriptional factors are important regulators of both structural and regulatory genes in the heart. The other important transcription regulator in heart development is myocyte enhancer factor-2 (MEF2). Indeed, MEF2 is a subfamily of the MADS-box family of transcriptional regulators. MEF2 has been shown to play an essential role in morphogenesis and myogenesis of muscle cells (skeletal, cardiac, and smooth). In vertebrates, four MEF2 genes have been discovered; these are referred as MEF2A, -B, -C, and -D, and are located on different chromosomes [192]. Some other molecules have been discovered to play a role in early heart development, such as cardiotrophin-1 (CT-1) (a member of the IL-6 cytokine family), which was believed to play a fundamental role in the normal development of the heart, particularly in ensuring that the myocardium grows to a normal thickness [193]. Additionally, heart and neural crest derivatives-expressed proteins, dHAND and eHAND, were found to be transcription factors which are mainly expressed in chamber specific patterns and, perhaps, they are responsible for the specification of chamber identity [194]. Finally, the extracellular environment is said to be involved in directing stem cell differentiation toward cardiomyocytes by providing signalling molecules or

growth factors that act in this manner, including bone morphogenetic proteins (BMP), sonic hedgehog (SHH), fibroblast growth factors (FGF), Wnt and Notch proteins, where each one of them down or up-regulates specific embryonic cardiogenic genes [195].

2.1.3 Well-known cytoprotective molecules

2.1.3.1 Folic acid

The Latin word 'folium', which means leaf, is the origin of the word 'folate' which was first isolated from spinach leaves. Folic acid is a synthetically produced water-soluble vitamin B9. This vitamin cannot be synthesised in the body *de novo*. Therefore, naturally-occurring foods and fortified dietary supplements are essential sources to meet the daily requirements of humans [196,197]. Several essential folate-dependent reactions are present in the human body where folate acts as a co-enzyme or co-substrate in the metabolism of amino acids and synthesis or the repair of nucleic acids (DNA and RNA) [198]. It is particularly important in childbearing age or pregnant women for continuous cell division and embryonic development. Folate is mainly stored in the liver but also in blood and other tissues. The approximate total body content of folate is about 10 to 30 mg. In serum, its total level is about 5 to 15 ng/ml, while the normal levels in erythrocytes vary from 175 to 316 ng/ml. Folate deficiency is associated with serum levels below 5 ng/ml, but megaloblastic anemia (a hallmark of folate deficiency) is viewed at a serum level less than 2 ng/ml [198,199]. It has been advised that the daily recommended allowance of folic acid is 400 µg for teenagers and adults, 500 µg for breastfeeding women and 600 µg in pregnancy [196]. An adequate supply of dietary folate in the early stages pregnancy has been confirmed to

be pivotal for normal embryo development, but also to lower the incidence of congenital defects, including congenital heart defects (CHD) and neural tube defects (NTDs) [200,201]. Folic acid has antioxidant characteristics, and this has been proven by using an *in vitro* cellular model where an induced oxidative stress environment was counteracted [202]. While some scientists are yet to support the periconceptional folic acid fortification policy, Atta *et al*. (2016) have meta-analysed global data of folic acid supplementation and congenital defects, and they strongly recommended mandatory folic acid fortification policies to be legislated immediately [203].

2.1.3.2 Ascorbic acid (Vitamin C)

Ascorbic acid or vitamin C is a water-soluble micronutrient. It is essential in the biosynthesis of connective tissue as it is involved in the synthesis of collagen, and in antioxidant defence mechanisms. Humans lack the ability to synthesise ascorbic acid due to the absence of required enzymes, hence why an adequate dietary intake of ascorbic acid is necessary in order to replenish body stores. However, it is available in many fruits and vegetables, such as blackcurrants, guava, strawberries, citrus fruits, capsicum, tomatoes, potatoes and broccoli [204]. The ascorbic acid daily recommended intake during pregnancy has been suggested to be 60mg, which is higher than the non-pregnant women because ascorbic acid can cross the placenta reducing the total ascorbic acid maternal levels [205]. Ascorbic acid deficiency results in a disease known as 'scurvy' which is mainly characterised by damage to connective tissue and haemorrhage of capillaries leading to bleeding in the gums and deeper tissues, delayed wound healing, skin rashes, fatigue and megaloblastic anaemia or iron deficiency anaemia [206]. Ascorbic acid protects

the biological system from the harmful effect of free radicals, which eventually result in several oxidative stress related diseases, by quenching the free radicals from the body [204]. Dietary supplementation with a therapeutic dose of ascorbic acid (1 g/day) has not been shown to cause adverse effects in humans, neither to the mother nor the fetus [207]. This fact was confirmed by Chappell *et al*. (1999) who used 1g ascorbic acid per day, but the results showed no adverse effect on fetus [208]. Therefore, it has been suggested that appropriate doses of ascorbic acid are safe in pregnancy; however, chronic intake of high doses might cause renal stones in the mother [209].

2.1.3.3 Superoxide dismutase (SOD)

SODs are antioxidant metalloproteins that dismutate the superoxide radical ($O_2^{-\cdot}$) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) [210]. Thereafter, two enzymes, namely, catalase and glutathione peroxidases, convert H_2O_2 into H_2O [211]. However, it must be remembered that the risk of ($O_2^{-\cdot}$) starts when it reacts with nitric oxide (NO) to produce a non-free radical strong oxidant peroxynitrite ($ONOO^-$) [212]. Generally speaking, there are three types of SOD: copper/zinc (CuZnSOD) which is found mainly in the cytosol of eukaryotes; manganese (MnSOD) which locates in the mitochondria of eukaryotes and prokaryotes and iron (FeSOD) in prokaryotes [213]. Lequarré *et al*. (2001) investigated the expression of SOD in the bovine embryo at transcriptional and protein levels. The results confirmed that MnSOD is transcribed and that CuZn and MnSOD proteins are expressed in bovine preimplantation embryos [214]. Similarly, it has been evidenced that extracellular SOD is expressed spatiotemporally in developing embryos and

extraembryonic tissues during mouse organogenesis, suggesting its role in antioxidant defence mechanisms against oxidative stresses at early stages of embryonic development [215]. Nevertheless, Zaken *et al*. (2000) measured the SOD activity in rat embryos and yolk-sac and found a gradual increase with progress in embryonic age, thus indicating a weak embryonic antioxidant defense, particularly in the early stages of organogenesis [216].

2.1.4 Well-known teratogenic molecules

2.1.4.1 Alcohol (Ethanol)

Alcohol or ethanol has been recognised as a common teratogen. Fetal alcohol spectrum disorders (FASDs) are a common cause of cognitive dysfunction and maldevelopment in vital organs including the embryonic heart, and it is an outcome of prenatal alcohol exposure (PAE) [217]. The latter condition has been associated with higher incidence of spontaneous abortion [218], a sixfold increase in the rate of stillbirth [219], prematurity and fetal growth impairment [220]. The inefficient ethanol metabolic elimination by the fetus adds insult to injury. Further, ethanol excreted by the fetus to the amniotic fluid is re-ingested by the fetus, prolonging the exposure of the fetus to ethanol via ethanol recycling [217]. It is agreed that more efforts should be undertaken to detect early and treat PAE. Neonatologists and paediatricians have encountered clinical difficulties to determine the maximum safe levels of alcohol in pregnancy. However, the current UK Department of Health guidelines recommends that 'women who are planning a pregnancy or during her gestation should not exceed drinking 1 or 2 units of alcohol (1 UK unit contains 10ml or 8 g of alcohol) once or twice a week and should avoid episodes of intoxication' [221]. In Western communities, it has been documented, by

The National Institute of Drug Abuse Survey, that moderate drinking during gestation is common. Perhaps most of the cases occurred unknowingly particularly in unplanned pregnancy where mothers were unaware of being pregnant [222]. The UK Chief Medical Officer in a published guidance (2016) has recommended a 'precautionary' approach to alcohol during pregnancy. Nonetheless, avoidance of drinking in pregnancy was regarded the safest approach for the fetus and expectant mother [223].

2.1.4.2 Retinoic acid (RA)

RA - an analogue of vitamin A - is available in all human cells. Two RA receptors (retinoid X receptor (RXR) and retinoic acid receptors (RAR)) are located on DNA and play essential roles in cell differentiation and proliferation via expression of several transcriptional factors binding to RA [224]. As a classic morphogen, RA coordinates embryogenesis signalling pathways by forming a precise concentration gradient to guide the development of fetal organs including the heart [225]. RA-induced congenital heart malformations have been recently highlighted by Sakabe *et al*. (2012) who found that high maternal RA levels lead to defects in the formation of the outflow tract in mouse hearts [226]. Since the 1980s, RA embryopathy has been identified after treatment given to pregnant women, which has led to heart defects and craniofacial malformations that seemed similar to those recorded in experimental animals [227]. However, it is well documented now that either lack or excess of RA results in embryonic malformations which indicate the importance and precision of RA signalling pathways in embryonic development [228].

2.1.4.3 Valproic acid (VPA)

Valproic acid (VPA), has been marketed since 1974 and is well documented to be used in the treatment of several CNS disorders, for example, epilepsy, bipolar disorder and in the prophylaxis of a migraine [229]. Clinical studies have shown VPA exhibits a potential to cause teratogenicity in the form of 'valproate syndrome', i.e. birth defects, developmental delay [230], and reduced intellectual functions [231,232]. Unfortunately, the epileptic expectant mother is unable to abandon their antiepileptic drugs (AEDs), as this will endanger both the mother and her fetus due to harmful consequences of seizures during pregnancy [233]. Whilst it has been discovered that the teratogenic potential of AEDs varies, VPA has been classified as a certain teratogenic drug [230]. The American Academy of Neurology has recommended avoiding VPA during pregnancy if possible [234]. Alternatively, when VPA treatment is mandatory, the lowest therapeutic dose three times a day should be administered to reach the steady state serum level, thus reducing the risk to the fetus by being exposed to VPA serum level fluctuations arising from a single high dose [230].

2.1.5 Chapter objective

This Chapter will describe the effect of cell density on cultured cells and also will examine the effects of well-known chemicals on embryonic cardiomyocytes in both systems to test the reliability and reproducibility of the utilised models.

2.2 Materials and methods

2.2.1 Chick primary embryonic cardiomyocytes MM cultures:

The method for MM culture has been described by Pratten *et al. (2012)* [235]. Fertilised White Leghorn chickens eggs (Henry Stewart & Co., Louth, UK) were stored at 11°C for no more than two weeks before use. When required the eggs (30 per session) were transferred to an automatic egg turner and placed at 37°C, 90% humidity in an incubator (Sanyo, Japan). Under aseptic conditions and after 5 days incubation, 6 eggs at a time were placed into a cleaned laminar flow hood. The embryo was revealed using forceps and transferred to a 90mm petri-dish containing enough Hanks Balanced Salt Solution (HBSS) (Sigma–Aldrich, UK) to cover the bottom of the dish, at RT (Figure 2-4A). Under a dissecting microscope (Nikon, Japan), the heart was extracted and transferred to a 7ml bijou tube containing horse serum (HS) (Life Technologies, UK) and HBSS, in 50:50 (v/v) ratio (Figure 2-4B). Once all the hearts had been collected, the HS:HBSS solution was removed, and the hearts were rinsed three times in 5ml HBSS to remove remaining HS. Following rinsing, the collected hearts were dissociated using 2ml 2.5x trypsin/EDTA – the hearts were incubated at 37°C, 95% air: 5% CO_2 for 20min with brief agitation every 5min (Figure 2-4B). The cell suspension produced was neutralized with MM medium (500 ml DMEM supplemented with 10% (v/v) heat-inactivated FBS, 50units/ml penicillin, 50µg/ml streptomycin, 2mM L-glutamine). The suspension was centrifuged at 1500rpm for 5min at RT. Cells were disassociated, counted using a TC10™ Automated Cell Counter (Bio-Rad, USA) and the trypan blue assay, see Appendix II. The cells, then, were plated as 20µl drops at different densities (Figure 2-4C, D). For cell optimisation

purpose, five different cell densities (4, 3, 2, 1 and 0.5 x10^6 /ml) were tested. The cells were incubated for at least 2h at 37°C, 95% air: 5% CO_2 before being flooded with 500µl MM medium (Figure 2-4E). The seeding of the cells was said to occur on Day 0 (0h). On day 1 (24h), the cells were observed and rated morphologically (Section 2.2.3.1). An additional 500µl of MM medium was added to the control and blank wells, and 500µl of medium containing test chemicals was added to the test wells as per each individual experimental method. The cells were further assessed morphologically on day 2 (48h) and on the final day 6 (144h) after culturing. A resazurin assay (Section 2.2.3.2) to detect cellular activity and kenacid blue assay (Section 2.2.3.4) to detect cellular protein content were also performed as end tests on the same plates. Each experiment had at least three individual runs (n=3) performed on separate days, with a minimum of 4 wells designated to each environment per experiment. For chemical MM tests, a cell density of 3x10^6/ml was utilised.

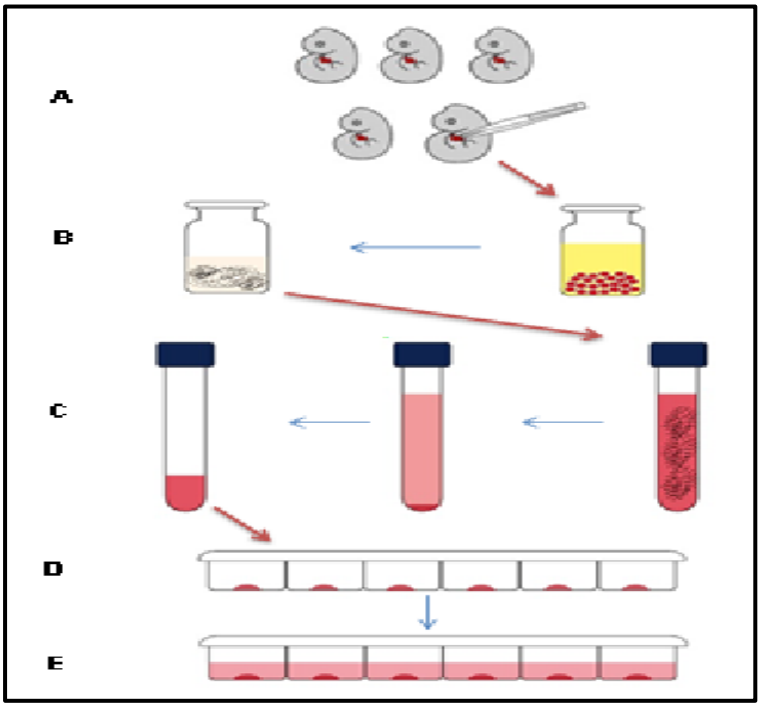


Figure 2-4: Chick embryonic heart micromass system protocol

(A) surgical removing of embryonic heart by special forceps under sterile conditions, (B) collecting the hearts in 50%:50% HBSS:HS, then washing and trypsinising the cells, (C) Neutralising trypsin action by MM medium then centrifugation and cell counting, (D) plating 20µl drop of the cell suspension for 2h in the incubator and (E) flooding the drops with 500µl MM medium.

2.2.2 Mouse embryonic D3 stem cells (ESD3) culture

The technique has been described previously [236], ESD3 were obtained from American Type Culture Collection (ATCC) organisation, Rockville, USA. The undifferentiated cells were grown in a T-25 culture flask (Thermo-Scientific, UK) using ESD3 medium, 500ml DMEM supplemented with (0.1mM β-mercaptoethanol, 20% (v/v) heat-inactivated fetal bovine serum (FBS), 50units/ml penicillin, 50µg/ml streptomycin, 2mM L-glutamine, 1000U/ml leukemia inhibitory factor (LIF) and 1% (v/v) non-essential amino acids (NEAA) (all reagents Sigma-Aldrich, UK) (Figure 2-5A) . After the ESD3 were 80-90 % confluent in the T-25 flask, the cells were either passaged or harvested prior the cells become 16 passage number for all experiments. For experimental purpose, the cells were trypsinised by 2ml 1x trypsin/EDTA (Sigma–Aldrich, UK) and centrifuged at 900rpm for 5min at room temperature (RT); then the cell pellet was resuspended in 5ml ESD3 medium without LIF to allow differentiation. A cell suspension of $4x10^4$ cell/ml was prepared (with or without test chemicals), and 20µl drops were seeded on the internal side of the lid of a 90mm Petri dish (Sterilin, UK) (Figure 2-5B). The drops were cultured as hanging drops after reverting the lids back to the petri dishes containing 5ml phosphate buffered saline (PBS) (Sigma–Aldrich, UK) to prevent the drops being dried in the 37°C/ 95% air: 5% CO_2 incubator (Figure 2-5C). However, in cell optimisation experiment, four cell density concentrations (16, 8, 4 and 2 $x10^4$ cell/ml) were used. The first day of drop plating was recorded as day 0. After 72h (day 3), the EBs formed within the hanging drops were moved to other petri dishes with 10ml ESD3 medium without LIF, maintaining the same environment (Figure 2-5D). The

EBs were then transferred after two days into 24 well plates as 1 EB/ml ESD3 differentiation medium in each well (Figure 2-5E), maintaining the same environment up to day 12 when the EBs attach the plastic and spread out (Figure 2-5F). The starting time for differentiation in the well was recorded as day 5 and the differentiation scoring started on day 10 and continued for two more days, 11 and 12.

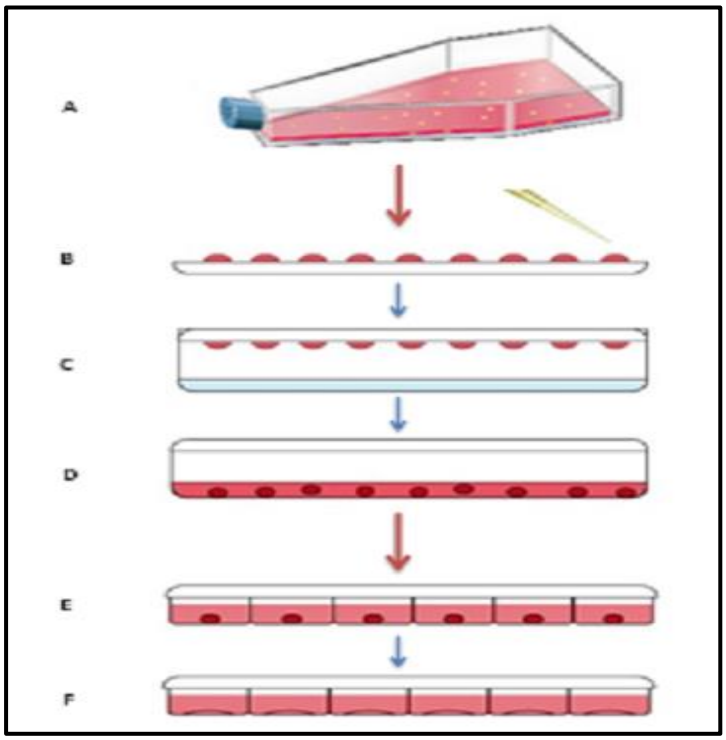


Figure 2-5: Mouse embryonic D3 stem cell (ESD3) culture hanging drop protocol
(A) confluent stem cells in T-25 flask, (B) plating 20µl cell suspension drop on the inner surface of petri dish lid, (C) inverting the lid to the dish and incubating the hanging drops for three days, (D) transferring the formed EBs into another petri dish with the same experimental environments for two days in an incubator (E) plating EBs into 24 well plate one per well maintaining the same environments for further seven days, and (F) differentiating cultures.

2.2.3 Endpoint measurments

2.2.3.1 Morphological scoring system

Cellular contractility was rated against a numerical morphological score with a maximum available score of 3. This was designed to evaluate the cellular differentiation in the cultures of both systems. Scoring in MM system was performed at days 1, 2 and 6 while in ESD3 system it was performed at days

Beating Foci in MM culture	Score	Localised beating area in ESD3 culture	Score
Whole well beating	3	5 and >	3
Numerous foci beating	2	3-4	2
Few foci beating	1	1-2	1
No beating	0	No beating	0

Table 2-1: Morphological scoring system for beating cardiomyocytes

10, 11 and 12. The system relied on a subjective scale of observation under an ordinary microscope as detailed in Table 2-1.

2.2.3.2 Resazurin Reduction Assay

The resazurin assay aims to quantify cell culture viability/activity [3]. The principle of the assay is based on the reduction of non-fluorescent resazurin into a pink fluorescent molecule, resorufin, by the action of living cells which indicates their activity (Figure 2-6) [237]. The reduction occurs due to cell metabolism where the colour intensity is directly proportional to the cell activity. Resazurin acts as an intermediate electron acceptor in the electron transport chain where the electron is transported from the cytochrome c oxidase to reduce resazurin to fluorescent resorufin, instead of molecular oxygen; therefore, it acts as oxidation-reduction indicator for cellular metabolism [238]. This test is sensitive, rapid, reproducible, cheap and does not harm the cells. Therefore, the cells are still available for further tests [239].

However, with longer incubation time or at a high cell density, further resorufin reduction occurs resulting in a colourless product (hydroxyresorufin) (Figure 2-6) and achieves a plateau [240].

Resazurin stock solution was prepared by dissolving 5mg resazurin (Sigma–Aldrich, UK) in 5ml HBSS to achieve 1000 µg/ml. The solution was filtered into a 50ml universal container using Minisart® filter (Sigma–Aldrich, UK) with pore size 0.45µm before it was diluted to 50ml with HBSS to create 100µg/ml. This stock resazurin solution was aliquoted, wrapped with aluminium foil and kept at (-20^{0}C) to avoid light reduction. The working solution was prepared by adding 45 ml of HBSS to 5ml of stock solution (1:10 dilution) to create a working solution of 10µg/ml. Resazurin solution was pre-warmed to 37^{0}C prior to introducing to the cells. To carry out the resazurin assay, on day 6 of MM and day12 of ESD3 cultures, the cells were incubated with 500µl of resazurin (10µg/ml) in HBSS at 37°C, 95% air: 5% CO_2 for 1h before being read on the Star plate reader. The optical density was determined using an emission filter wavelength of 590 ± 12.5nm and excitation filter wavelength of 530 ± 12.5nm, with the sensitivity gain set at 60. The raw fluorescence data was converted to resorufin concentration, by plotting against the resorufin standard curve (Section 2.2.3.3), which in turn determined the cell activity. The resazurin working solution was aspirated and replaced with a 300µl kenacid blue fixative solution (500ml ethanol: 490ml distilled water (DW): 10ml acetic acid) and the plates refrigerated at 4°C overnight.

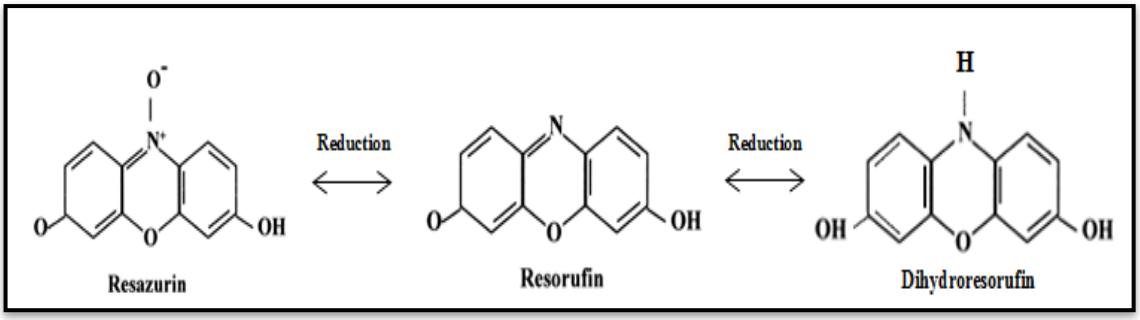


Figure 2-6: Schematic depiction of resazurin reduction pathway. Taken from [237]

2.2.3.3 Resazurin standard curve

The optical densities of resazurin values that were obtained from the plate reader were converted to actual resorufin amounts, which presumably had been produced by the cells, by plotting the resorufin standard curve. Five different concentrations of resorufin solutions were prepared (125, 250, 500, 1000, 2000 ng/ml in HBSS). 500µl aliquot per concentration was added into four wells of the 24-well plate, while HBSS alone represents blank. The optical density was determined with an emission filter wavelength of 590± 12.5nm and excitation filter wavelength of 530±12.5nm, and a gain of 60. The results were plotted, and the best-fit straight line was calculated (Figure 2-7).

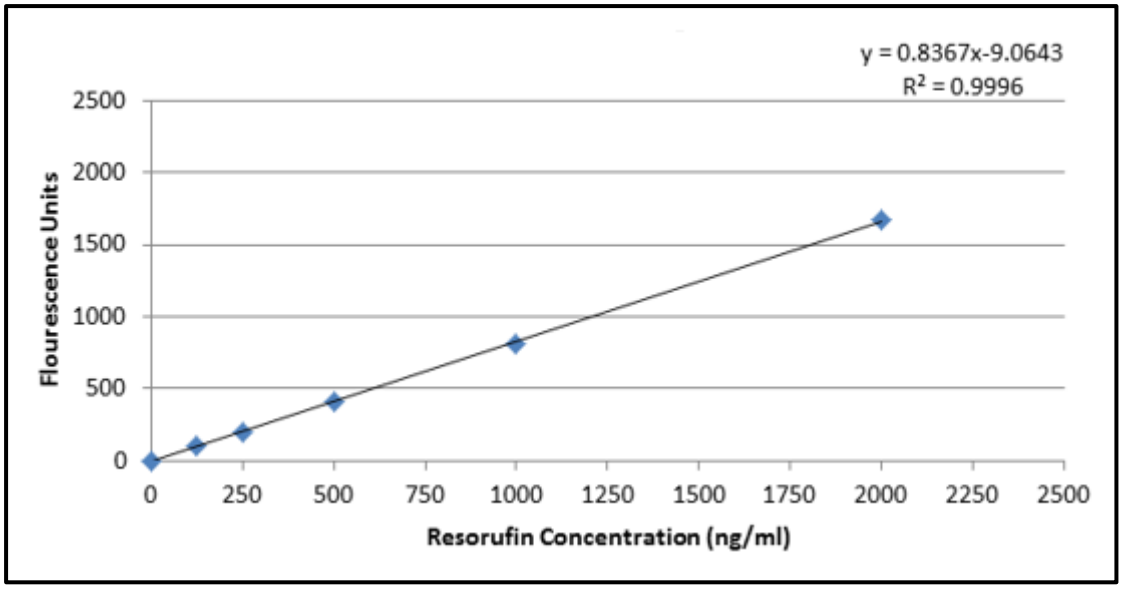


Figure 2-7: Representative resorufin standard curve
The equation is x=(y+9.0643)/0.8367 where y= emission filter wavelength of 590± 12.5nm and x= resorufin concentration (ng/ml).

2.2.3.4 Kenacid Blue Assay

The total protein present in the cell culture was quantified using the kenacid blue (KB) assay by measuring the quantity of kenacid blue dye taken up by the cells. Cellular protein is an important factor as it indicates the rate of growth, proliferation or death of the cells cultured in a system [4]. The principle of this test is based on dye binding with the amino (-NH_3) terminal end of an amino acid of a protein forming a dye-protein complex regardless whether the protein is active or not. The total dye-protein binding expresses the content of cellular proteins [241]. For measurement purposes, the 300µl kenacid blue fixative solution was removed and replaced with 500µl kenacid working solution (6ml glacial acetic acid + 44ml kenacid stock solution [0.4g kenacid blue powder dissolved in 630ml DW + 250ml ethanol]). The plate was placed for 2h on a plate shaker at a low speed. Each well was individually washed with 400µl kenacid washing solution (100ml ethanol:50ml acetic acid:850ml DW)

twice – the first 400µl was quickly removed, the second 400µl was left in the wells for 20min on the shaker. The washing solution was then aspirated away and replaced with 400µl kenacid desorb solution (700ml ethanol: 98.15g potassium acetate : 300ml DW) and the plate shaken for 1hr at a higher speed. The resulting solution was transferred in triplicate to a 96 well plate (Nunc, UK), and the optical density was read on a Multiskan Ascent plate reader, reference filter 405nm and reading filter 570nm. Using bovine serum protein standard curve (Section 2.2.3.5), the raw colorimetric data was converted to actual protein content. (All reagents Sigma-Aldrich, UK except potassium acetate, Fisher Scientific, UK).

2.2.3.5 Kenacid blue standard curve

A kenacid blue standard curve was plotted in order to measure the protein content intracellularly. The procedure starts by dissolving 2.4mg bovine serum albumin (BSA) in 70% (v/v) ethanol solution. 3ml of albumin solution was dissolved in 6ml of 70% (v/v) ethanol to create 80µg/ml albumin concentration. From this concentration, 70, 50, 40, 20 and 10µg/ml albumin concentrations were prepared. The plate was kept at 4^0C overnight to permit ethanol to evaporate and albumin to attach to the surface. Then, the wells were fixed with 300µl kenacid blue fixative overnight at 4^0C. The kenacid blue protocol was carried out as described in Section 2.2.3.4. The results were plotted on a scatter graph, and the best fit line was drawn to recognize the actual protein values (Figure 2-8).

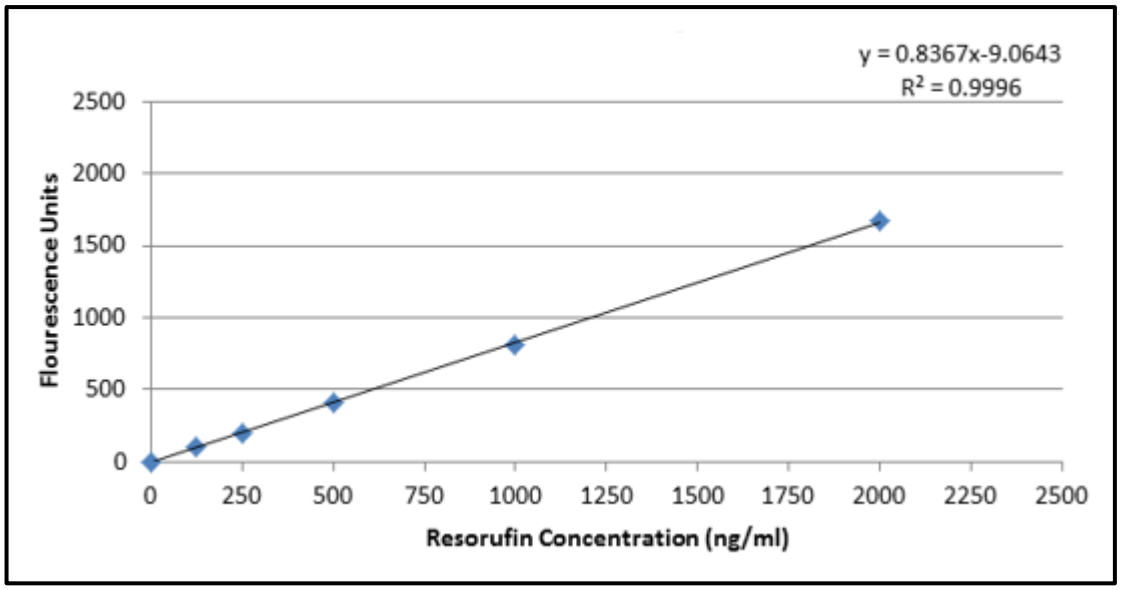


Figure 2-8: Representative Kenacid blue standard curve

2.2.4 Immunocytochemistry

2.2.4.1 Immunocytochemistry staining of MM and ESD3 cell culture

In order to verify the presence of cardiomyocytes in MM and ESD3 cultures, immunocytochemical examination was performed using a cardiomyocyte-specific marker, mouse anti-α-actinin (Sigma–Aldrich, UK). Untreated contractile chick MM cardiomyocytes or ESD3 (cultured on autoclaved coverslips, Fisher Scientific, UK) were washed with PBS then 1% (w/v) BSA (Sigma–Aldrich, UK) in PBS for 2min. The cells were fixed in 4% (w/v) paraformaldehyde for 15min at RT, then the cells were permeabilised by 0.3% (v/v) Triton X-100 for 20min. For blocking nonspecific binding, 5% (v/v) normal goat serum (Vector Laboratories Ltd, USA) was added to the cells for 30min at RT. 1% (w/v) BSA in PBS was used for all washing steps. The primary antibody dilution (α-actinin mouse antisarcomeric) was diluted in the

blocking solution (1:100) and it was incubated with the cells at 4°C overnight. On the next day, after 15min x 3 washing, FITC Goat anti-mouse secondary antibody (Sigma–Aldrich, UK) 1:100 was incubated with the cells at RT for 1h 15min x 3 washing was followed, and then cell nuclei were labelled with 1:2000 (v/v) DAPI. Finally, the cells were examined with an OLYMPUS fluorescence microscopy (Nikon, Japan).

2.2.4.2 Immunohistochemistry staining of 9-day chick embryonic heart

9 day chick embryonic heart was used as a positive control to optimise the cardiomyocyte marker (α-actinin). The embryonic heart was removed and post-fixed in 4% w/v paraformaldehyde (PFA) overnight. The heart then was cryoprotected in 30% (v/v) sucrose solution for 3h at 4^{o}C then submerged in OCT-compound (VWR International Ltd, UK). The OCT mould was stored at -80 ^{o}C until being sectioned in coronal plane using a Leica CM 100 cryostat (Leica Microsystem, UK) with a thickness of 20µm at -20 ^{o}C. The sections were thaw mounted onto slides and stored at -80 ^{o}C until being used for immunohistochemistry. On the day of the experiment, a selected section was washed with PBS three times and then immediately permeabilised with 0.3% (v/v) Triton X-100 for 1h at room temperature. The section was given three washes with PBS before it was blocked for 1h with 5% (w/v) milk in 1% (w/v) BSA in PBS at RT. For the rest of the experiment, the same steps were followed as in Section 2.2.4.1 except that the sections were incubated with (TRITC)-labelled goat anti-mouse IgG (Life Technologies, UK) in a dilution 1:1000 being diluted in the blocking solution.

2.2.4.3 Immunocytochemistry staining of undifferentiated stem cells

In the presence of LIF (a cytokine that inhibits stem cell differentiation), untreated stem cells were cultured on autoclaved coverslips with the same supplemented ESD3 medium (Section 2.2.2). To confirm the stem cells were kept in an undifferentiated state, SOX2 (Abcam, UK), a marker of the undifferentiated state, was stained within the culture. The cells were washed quickly with PBS twice at RT. Then the cells were fixed with ice-cold methanol (Sigma–Aldrich, UK) and kept at -20^{0}C for 5min followed by 30min wash with PBS at RT. 500µl Triton X-100 (0.3% (v/v) in PBS) was added for 30 min to permeabilise the cells. This was followed by 30min rinse with PBS at RT. The cells were blocked for 1h with 5% (w/v) milk in 1% (w/v) BSA in PBS (MBP) at RT. Cells were incubated with SOX2 antibody diluted (1:250) in MBP overnight at 4°C. The cultures were rinsed three times with 1% (w/v) BSA in PBS solution for 5min per wash before they were incubated with (TRITC)-labelled goat anti-mouse IgG (Life Technologies, UK) in a dilution 1:1000 with MBP for 1h at RT in the dark. This was followed by 45 minutes washing with 1% (w/v) BSA in PBS solution, and then DAPI staining was performed by incubating 1:2000 in PBS for 5 min at RT. Then, 2 times 5 min rinses were given to the cells before they were mounted on pre-labelled slides in mounting medium.

2.2.5 Preparation of drug solutions

Depending on their solubility, five chemicals stock solutions were prepared by dissolving directly in the culture medium (folic acid (13.47mM), ascorbic acid (5.67mM), SOD (338.4 µM), ethanol (100%) and valproic acid (6mM)). Only retinoic acid was firstly dissolved in ethanol (20mM) then diluted with culture

medium. The appropriate concentrations of drugs required for the experiment were prepared by diluting the stock solutions. The final concentration of solvent in controls, when applicable, and blanks were kept constant. All these chemicals were purchased from (Sigma–Aldrich, UK).

2.2.6 Statistical analysis

Raw data was collated in Microsoft Excel (2008, Version 12.2.0). Three independent repeats were performed for each experiment with at least four technical repeats per experiment. The data was imported into GraphPad Prism software (Version 6.0, August 5, 2014) for statistical analysis. For non-parametric data – cellular scoring morphology – a Kruskall-Wallis and post-hoc Dunn's test were used and mean ± SEM were computed. For parametric data – resazurin and kenacid blue assays – the data was analysed using one-way ANOVA test, with a post-hoc Dunnett's test and mean ± SEM were computed. $P<0.05$ was considered statistically significant.

2.3 Results

2.3.1 Determination of optimal cellular seeding condition in MM and ESD3 systems

2.3.1.1 Evaluation of cell density in MM

For synchronised contractile units to form in the MM system, there needs an appropriate cardiomyocyte density seeded. Cardiomyocytes are affected by the distance from surrounding cells, which governs the likelihood of signals being transported among them, which are essential for establishing the foci. An initial chemical free experiment was performed to ensure that $3x10^6$cell/ml was indeed a suitable density to establish most favourable cell culture

conditions, but also to make certain that the experimental protocols of heart extraction, cell culture and end points were being performed successfully. The biochemical results with microscopic observations confirmed that the best cell density for this system is $3x10^6$cell/ml. This was consistent with previous studies where the most widely accepted cell density ($3x10^6$cell/ml) was already employed [242,45]. The results showed that resorufin production in the resazurin test and protein content in the kenacid blue test increased in direct proportion to cell densities (Figure 2-9A, B). When low cell density was utilised, the cell to cell communications made become less, resulting in lower cell contractile scores (Figure 2-9C). The cell to cell distances was noticed clearly after seeding. While there were no spaces or gaps at both 3 and $4x10^6$cell/ml, these became evident with lesser concentrations and increased as the cardiomyocyte density decreased (Figure 2-10A-E). After 24h of seeding, the cell density of $2x10^6$ cell/ml and above managed to form contractile clump-like units (Figure 2-11C-E). However, at 0.5 and $1x10^6$cell/ml, only a few cells communicated and gathered together to form small weakly contractile bead-like units within the cultures (Figure 2-11A, B). On the next day (48h after seeding) a complete cellular sheet was observed only at 3 and $4x10^6$cell/ml (Figure 2-12D, E), with a very condensed cellular sheet at $4x10^6$cell/ml. No completed cellular sheets were seen at lower seeded concentrations of cardiomyocytes (0.5, 1 and $2x10^6$cell/ml) (Figure 2-12A-C). The images at 144h showed that $0.5x10^6$cell/ml failed to initiate beating foci but only few scattered clusters of beating cardiomyocytes (Figure 2-13A). Some contractile clumps were evident at $1x10^6$cell/ml which also failed to form foci within a cellular sheet (Figure 2-13B). The cardiomyocytes cultured at $2x10^6$cell/ml

density were partially successful in forming foci within cellular sheets but also some spaces and gaps were available which makes the cardiomyocytes beat asynchronously (Figure 2-13C). Ideal foci and sheets were seen at $3x10^6$cell/ml cardiomyocyte density, the cells formed full sheets with distinguishable synchronously beating foci (Figure 2-13D). Similarly, the cellular sheets and foci were beating in harmony at $4x10^6$cell/ml cardiomyocyte density, but it was difficult for the foci to be recognised perhaps because of crowded of cultured cardiomyocytes due to high cell density seeding (Figure 2-13E). Therefore, 3 $x10^6$ cell/ml was selected for chemical MM tests.

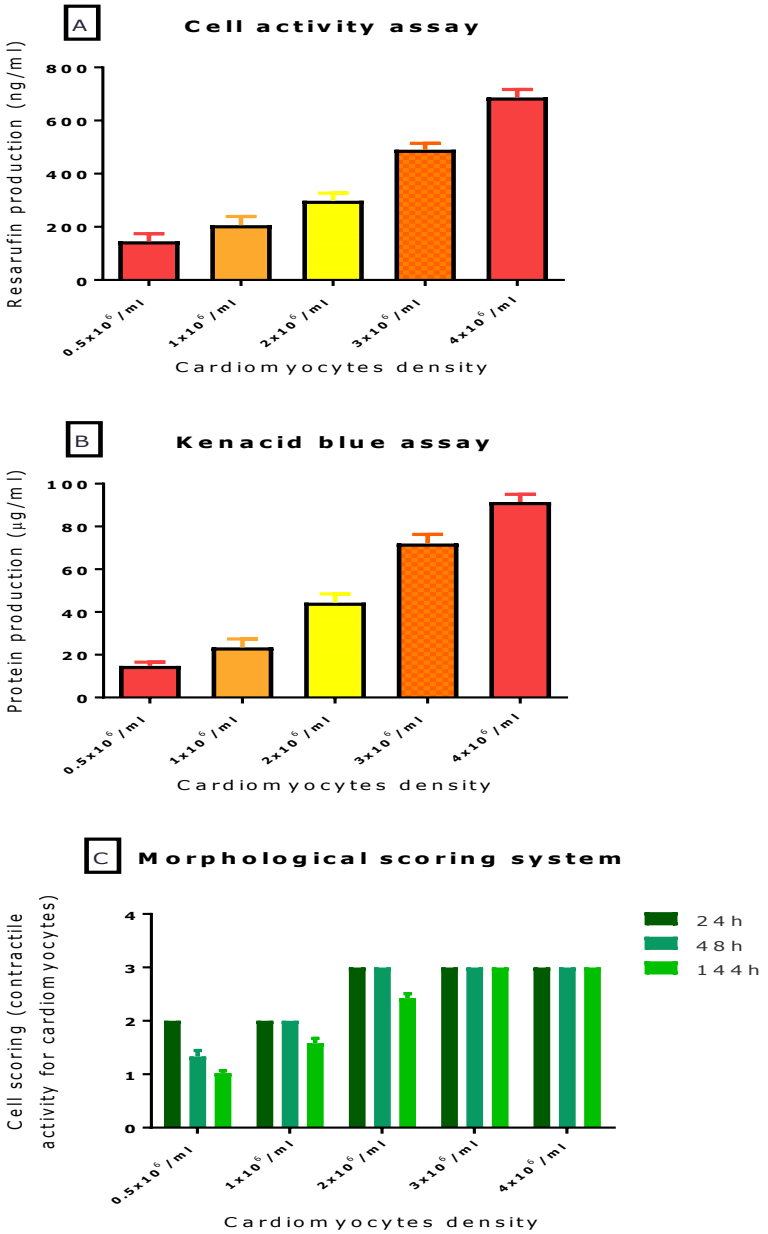


Figure 2-9: Effects of reducing cardiomyocyte seeding density in MM system

(A) Resazurin reduction assay for measuring cell viability 144h post seeding for the same MM cells grown in DMEM supplemented medium, (B) Kenacid blue assay for total protein content 144h post seeding for the same MM cells grown in DMEM supplemented medium, (C) Morphological scoring system for assessing cell redifferentiation on three time points -24h, 48h and 144h after seeding, for MM cells grown in DMEM supplemented medium. Three independent repeats were performed, (n=3).

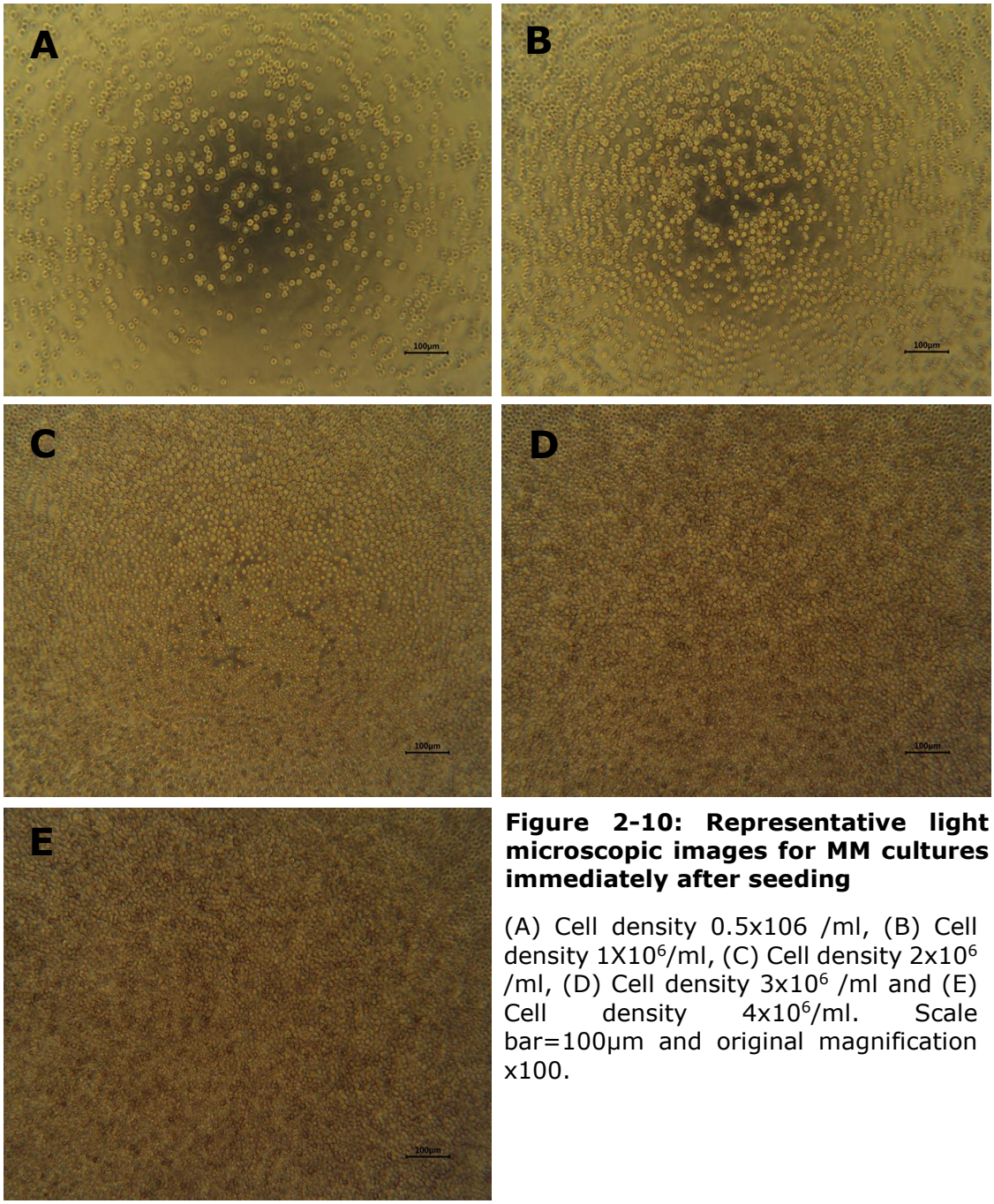


Figure 2-10: Representative light microscopic images for MM cultures immediately after seeding

(A) Cell density 0.5x106 /ml, (B) Cell density $1X10^6$/ml, (C) Cell density $2x10^6$ /ml, (D) Cell density $3x10^6$ /ml and (E) Cell density $4x10^6$/ml. Scale bar=100µm and original magnification x100.

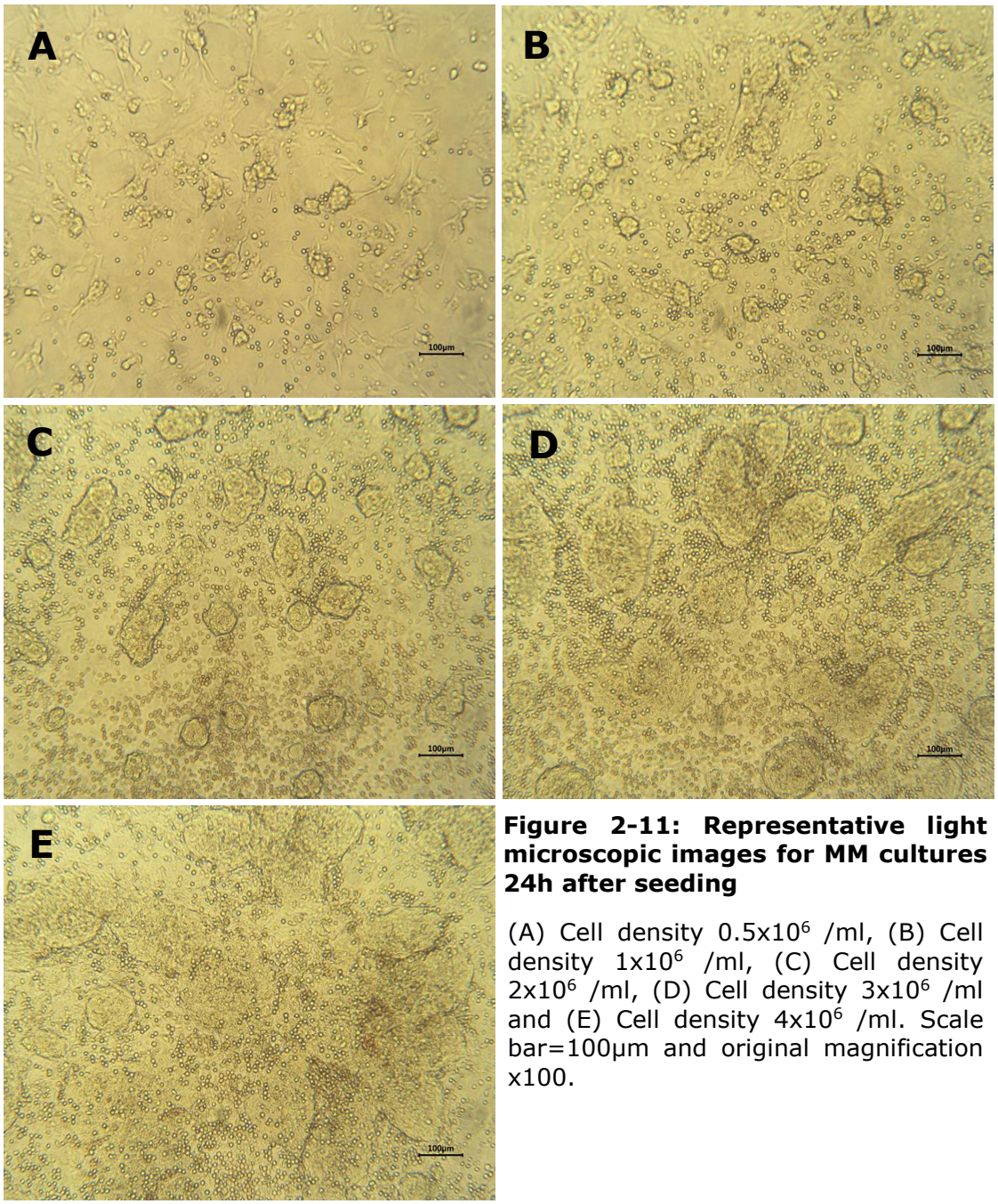


Figure 2-11: Representative light microscopic images for MM cultures 24h after seeding

(A) Cell density $0.5x10^6$ /ml, (B) Cell density $1x10^6$ /ml, (C) Cell density $2x10^6$ /ml, (D) Cell density $3x10^6$ /ml and (E) Cell density $4x10^6$ /ml. Scale bar=100µm and original magnification x100.

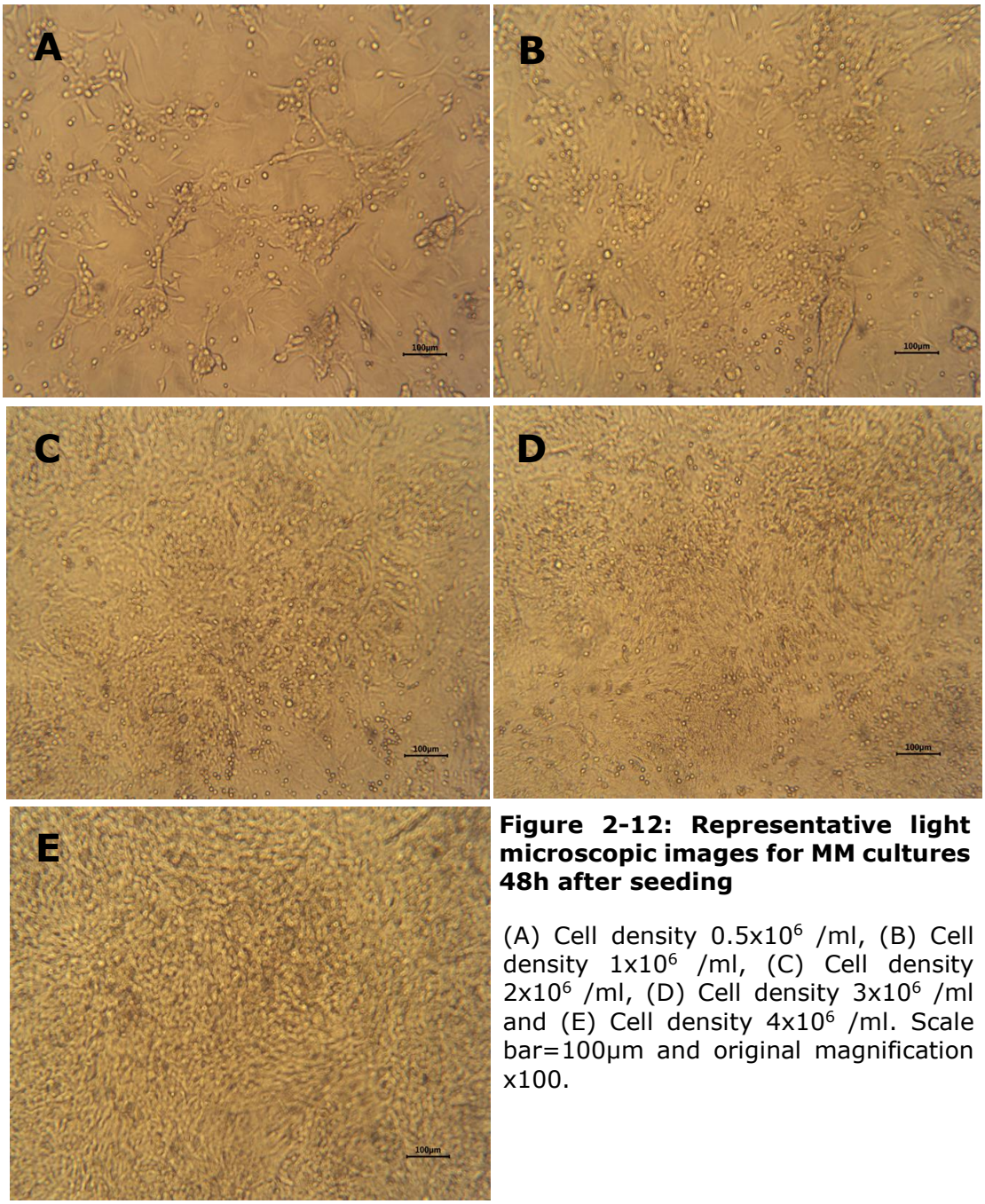


Figure 2-12: Representative light microscopic images for MM cultures 48h after seeding

(A) Cell density $0.5x10^6$ /ml, (B) Cell density $1x10^6$ /ml, (C) Cell density $2x10^6$ /ml, (D) Cell density $3x10^6$ /ml and (E) Cell density $4x10^6$ /ml. Scale bar=100µm and original magnification x100.

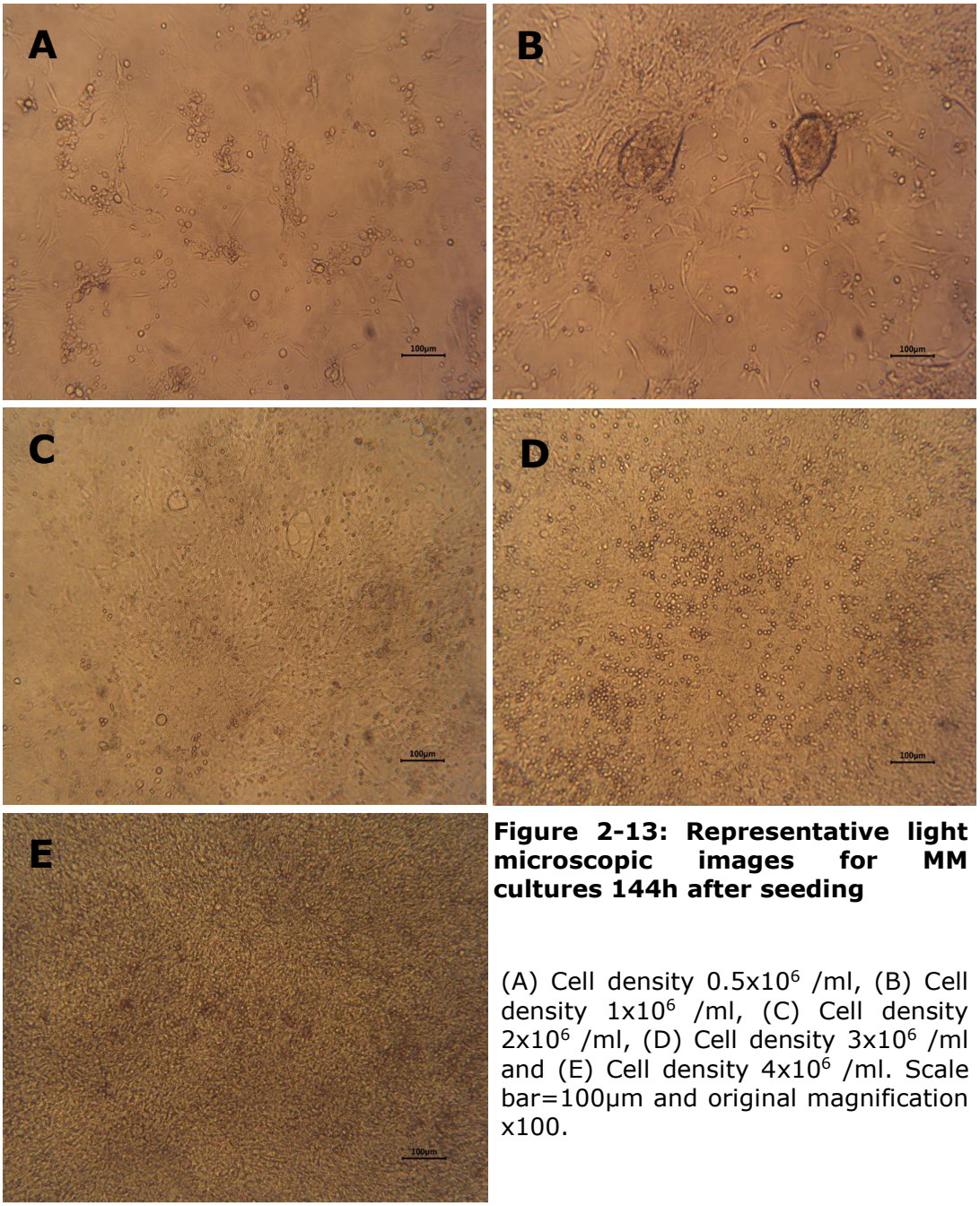


Figure 2-13: Representative light microscopic images for MM cultures 144h after seeding

(A) Cell density $0.5x10^6$ /ml, (B) Cell density $1x10^6$ /ml, (C) Cell density $2x10^6$ /ml, (D) Cell density $3x10^6$ /ml and (E) Cell density $4x10^6$ /ml. Scale bar=100µm and original magnification x100.

2.3.1.2 Evaluation of cell density in ESD3

At all selected densities (2, 4, 8 and 16 x 10^4 cells/ml), resorufin production increased in direct proportion to stem cell densities but also cellular protein measurement increased proportionally with increasing density (Figure 2-14A, B). At both the highest (16x10^4 cells/ml) and lowest (2x10^4 cells/ml) seeding densities less ESD3 differentiated into cardiomyocytes were detected. This was shown by low morphological scoring in Figure 2-14C. However, at seeding densities (8x10^4 cells/ml and 4x10^4 cells/ml) most of the cultures showed 5 beating areas, giving a high score (Figure 2-14C). Further, EB formation was also visually assessed. It was noticed that EBs enlarge their size by increasing densities in a period between day 3 and day 5. Some erratic EBs started to be observed more commonly with the increase in stem cell concentrations. However, the seeded EBs have shown different behaviour with the different stem cell concentrations in terms of the extent of cellular proliferation, migration and differentiation for each culture. The least cell concentration (2x10^4 cells/ml) showed less differentiating cells being spread out per well. All other densities showed comparable covered areas per well (Figure 2-15). Therefore, 4 x10^4 cell/ml was selected for chemical ESD3 tests.

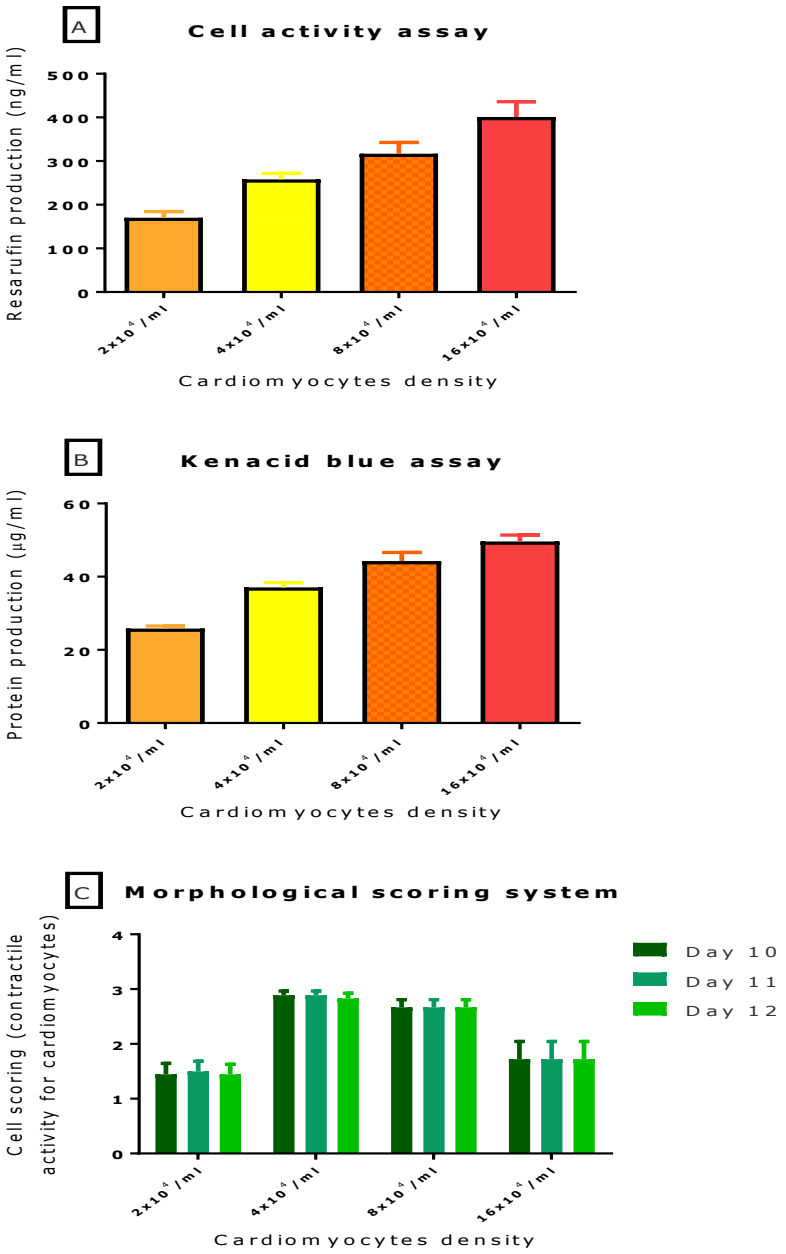


Figure 2-14: Effects of reducing cardiomyocyte seeding density in the ESD3 system

(A) Resazurin reduction assay for measuring cell viability at day 12 of ESD3 procedure, (B) Kenacid blue assay for total protein content at day 12 of ESD3 procedure, (C) Morphological scoring system for assessing stem cell differentiation on three time points at day 10, 11 and 12 of ESD3 procedure. Four different cell densities were used (16, 8, 4 and $2x10^4$ cell per ml). Three independent repeats were performed, (n=3).

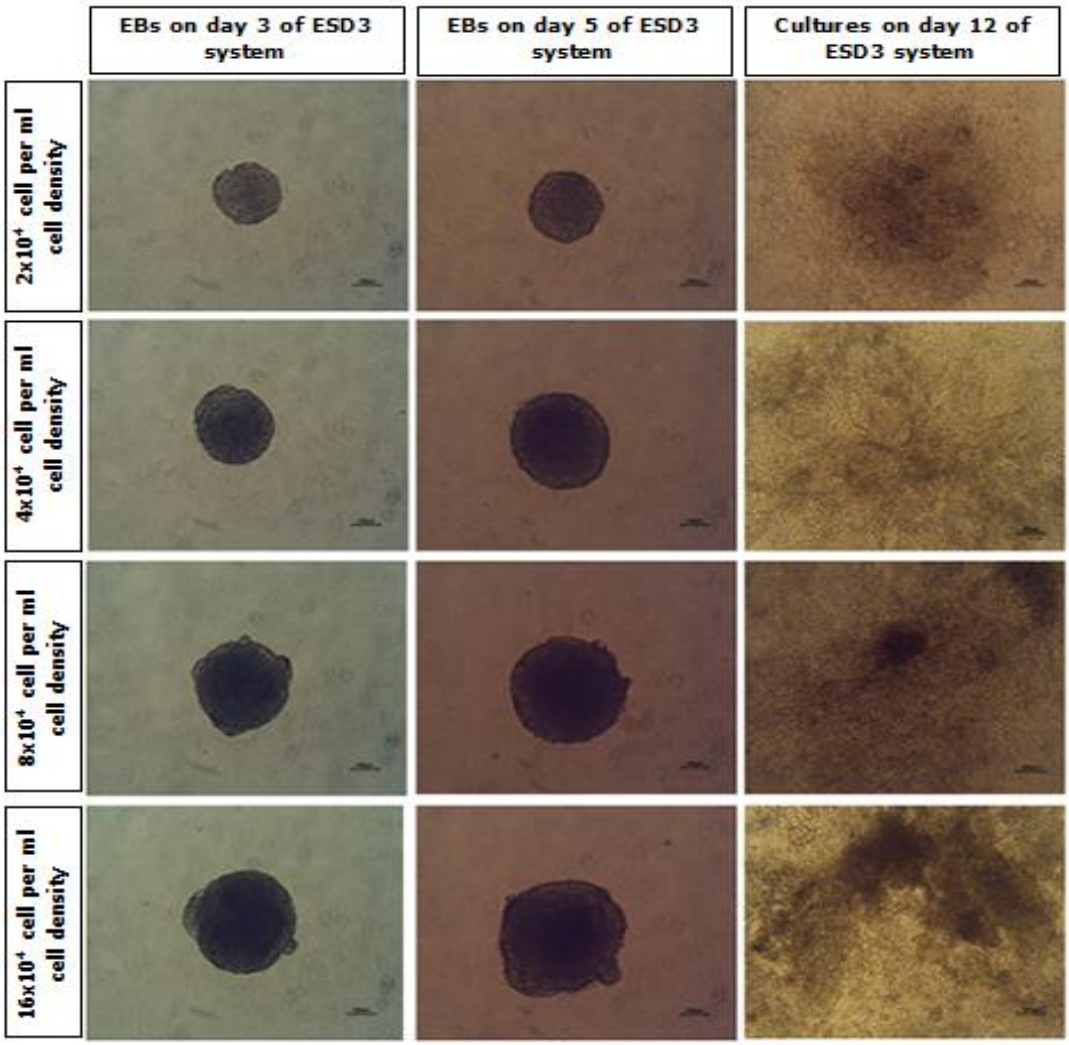


Figure 2-15: Visual comparison of the effect for cell density of EBs produced by the hanging drop method
Four different cell concentrations (2, 4, 8 and $16x10^4$) were selected to be monitored at day 3 and 5 for EBs size and at day 12 for culture behaving and contractile areas available at each concentration. Scale bar=100μm and origional magnification x100.

2.3.2 Cardiomyocyte verification in both MM and ESD3 cultures:

Although cardiomyocytes, whether primary or stem cell-derived, show relaxation and contraction phases as an intrinsic property and whilst these phases can be easily observed under a microscope, there always remains a need to confirm the cell type present by labelling cardiomyocyte-specific

proteins. This is more appropriate when it comes to the MM method as there would be some uncertainty that surrounding tissues might be removed alongside the heart such as fibroblast and endothelial cells. 9-day chick embryonic heart sections were utilised as a positive control to optimise the α-actinin marker (Figure 2-16). The cells of untreated control MM cultures were then stained with the cardiomyocyte-specific marker, α-actinin. This is localized in z-discs, providing the anchor position for actin microfilaments, and the staining pattern showed that cardiomyocytes predominated in the culture. Two different areas of the culture were imaged, at the periphery (Figure 2-17A2) and in the middle of the culture (Figure 2-17B2). This was also supported by video recording of whole culture contractility of the same culture which provided evidence that cardiomyocytes are predominant in the culture. Also to make certain that ESD3 cultures had been differentiated to cardiomyocytes, the α-actinin protein was stained within the cultures (Figure 2-18A2). Short video clips of stem cell-derived cardiomyocyte culture and beating embryoid bodies were recorded to provide further proof of ESD3 being differentiated into contracting cardiomyocytes. The images in both systems show z-lines localised in cardiac cell sarcomeres, where α-actinin in positive structures appeared as short lines or random striations, indicating that cardiomyocytes were present in that particular area of the cultures. The pluripotent characteristic of undifferentiated ESD3 was also verified by staining with the undifferentiated mouse embryonic stem cell marker SOX2 (Figure 2-19D).

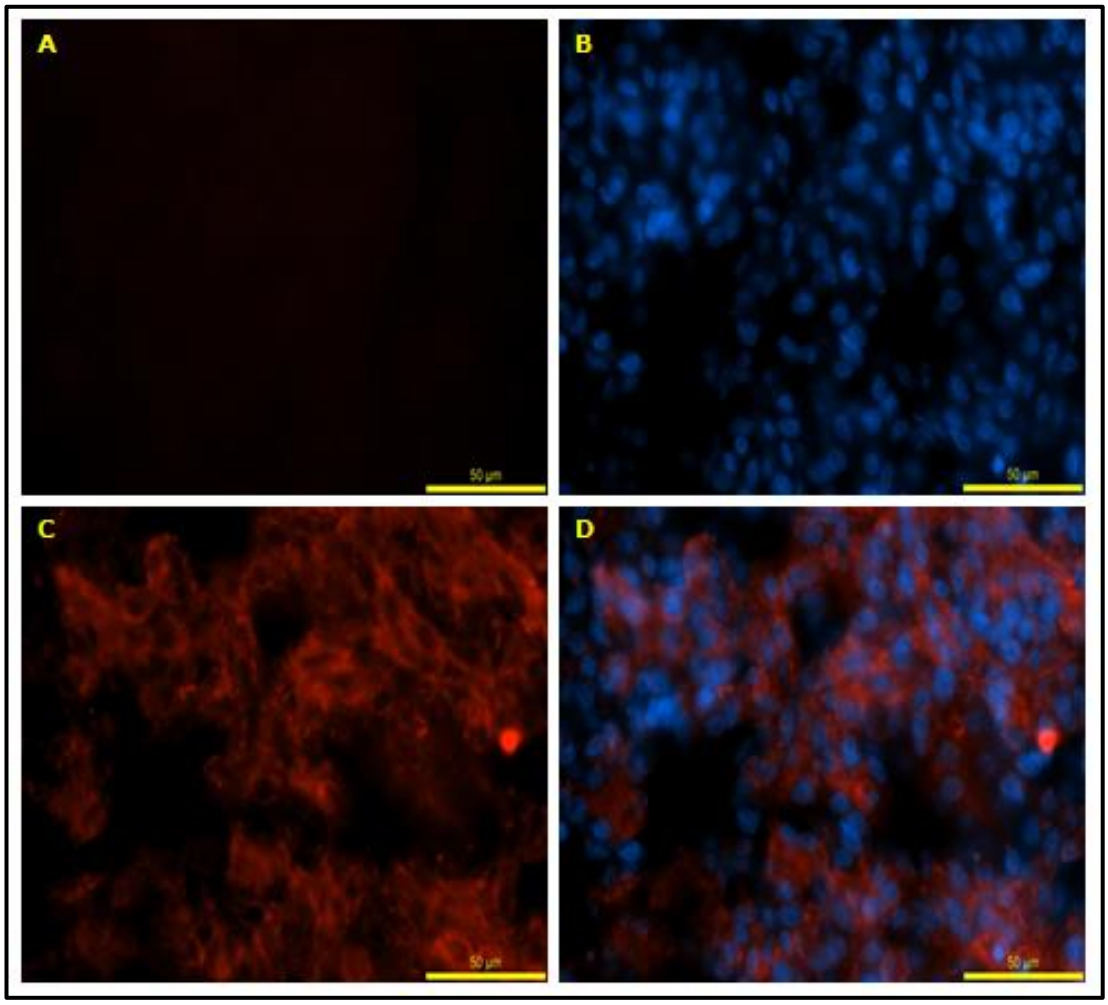


Figure 2-16: Immunohistochemistry staining of α-actinin marker of 9 day chick embryonic cardiomyocytes

(A) Section of only secondary antibody staining cardiomyocyte, (B) 9-day chick embryonic cardiomyocytes nuclei stained with DAPI, (C) 9-day chick embryonic cardiomyocytes stained with α-actinin and (D) overlay of (B and C). Scale bar=50μm.

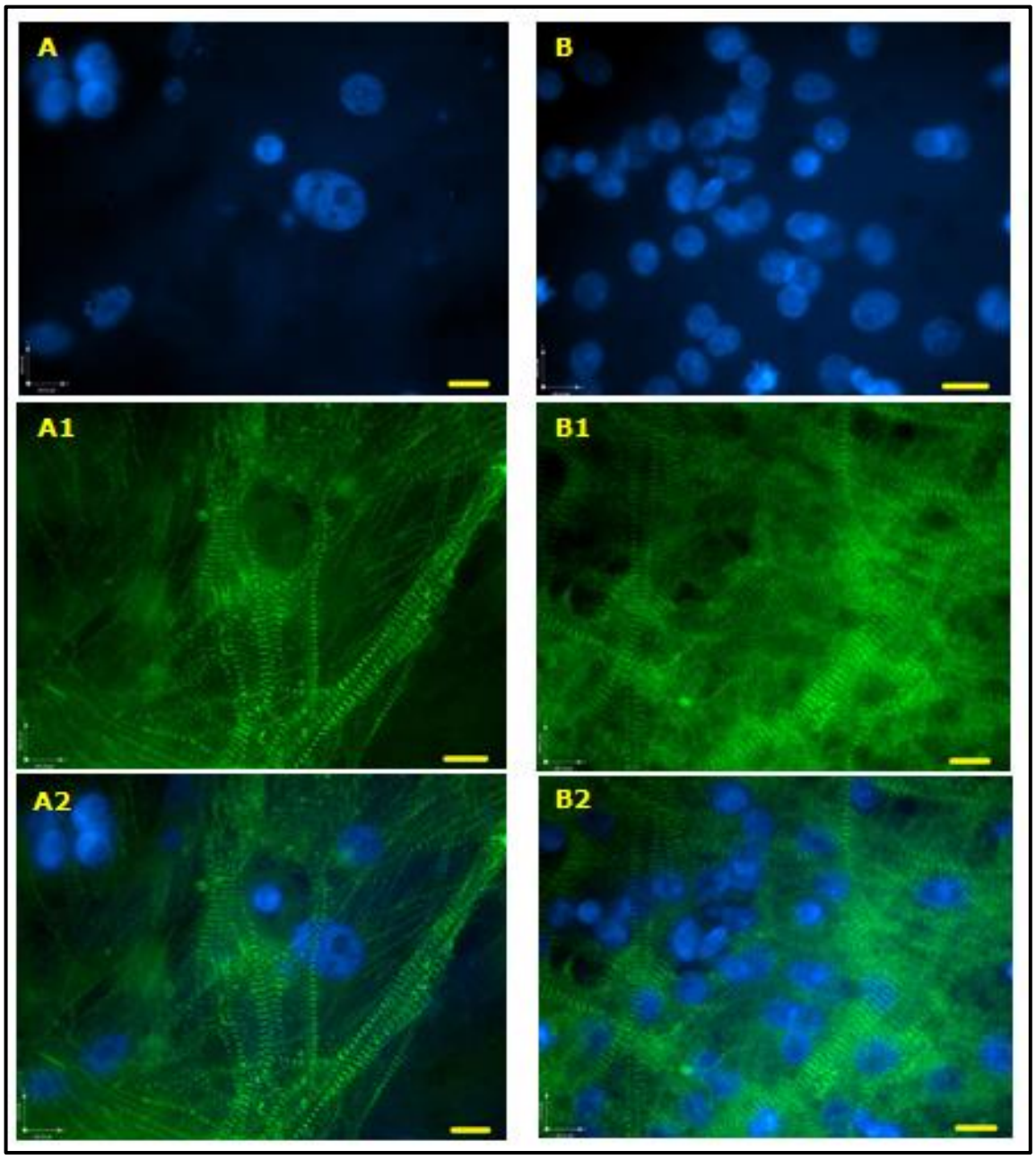


Figure 2-17: Immunostaining of α-actinin marker of untreated MM culture at 144h

(A) Image shows nuclei stained with DAPI from the peripheral side of culture, (A1) represents cardiomyocyte specific marker which shows z-lines of α-actinin sarcomeric structures appearing as small lines, image (A2) is the overlay of both images (A) and (A1); (B) image shows nuclei stained with DAPI from middle of the culture, (B1) represents cardiomyocyte specific marker α-actinin, and image (B2) is the overlay of both images (B) and (B1). Scale bar 12μm.

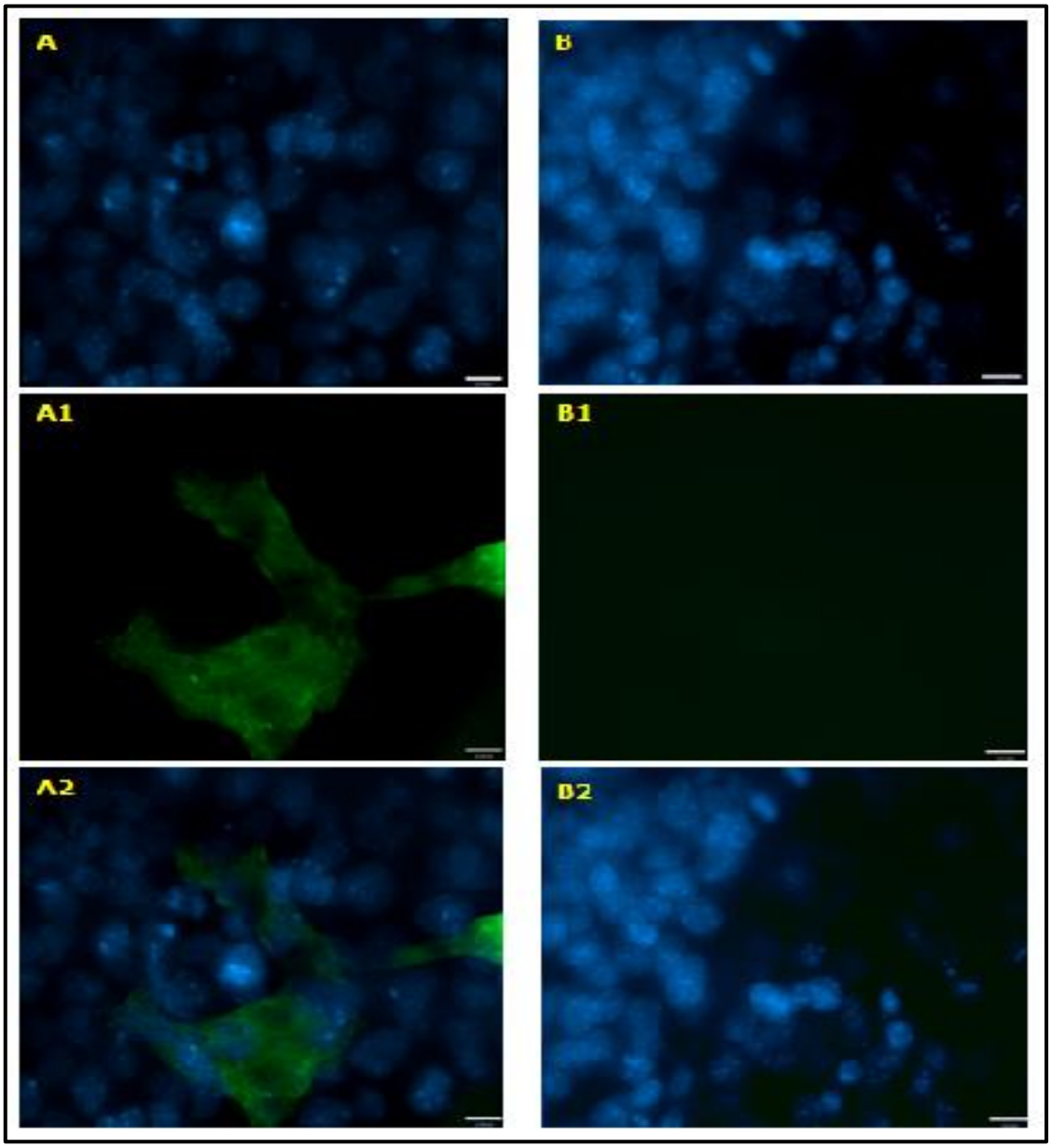


Figure 2-18: Immunofluorescent images of untreated ESD3 culture twelve days after plating (post EB culturing)
Image (A) and (B) are nuclei stained with DAPI, image (A1) is stained with α-actinin which is characteristic of cardiomyocytes, which shows z-lines of α-actinin sarcomeric structures appearing as small lines, all nuclei which are unstained with α-actinin represent non-cardiomyocytes cells in the culture, (B1) is stained with secondary antibody alone, image (A2) and (B2) are overlays for images (A and A1) and (B and B1), respectively. Scale bar 12μm.

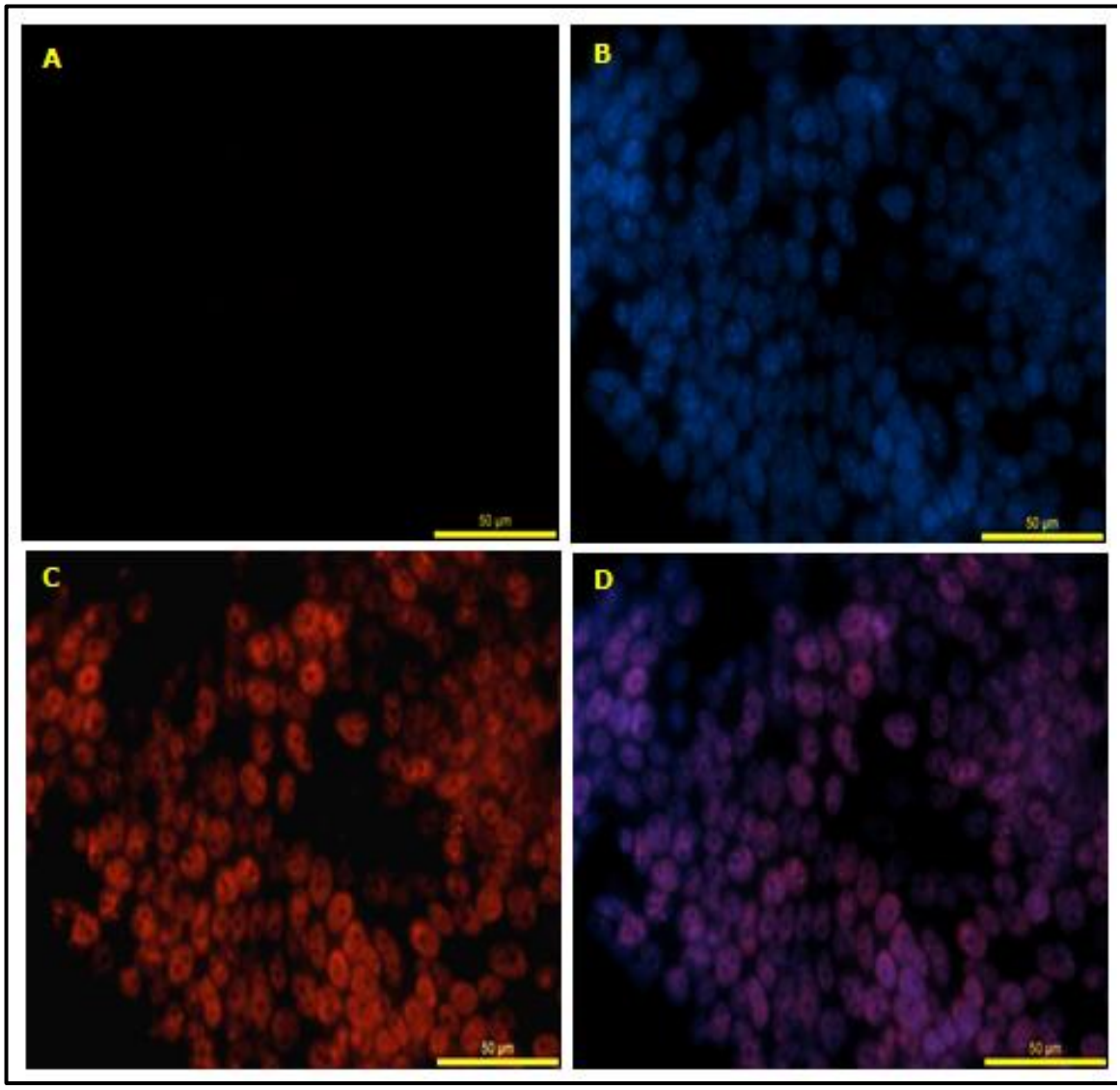


Figure 2-19: Immunofluorescent characterization of untreated ESD3 culture in the presence of LIF

Image (A) image is negative control stained with secondary antibody alone (B) is stained with DAPI and appears as a cluster of cells, image (C) is stained with SOX2 which is characteristic of undifferentiated ESD3, and image (D) is overlay for images (B) and (C). Scale bar 50 μm.

2.3.3 The effects of some protective molecules (FA, AA and SOD) on cardiomyocytes in MM and ESD3

2.3.3.1 The effects of folic acid

Exposure of embryonic cardiomyocytes to different concentrations (50, 175, 375 and 750μM) of folic acid (FA) in both systems did not show any significant alteration ($p>0.05$) in contractile activity (Figure 2-20A, D), cell activity (Figure 2-20B, E), or total protein content (Figure 2-20C, F).

2.3.3.2 The effects of ascorbic acid

Four ascorbic acid (AA) concentrations (5, 20, 50 and 100μM) were used to treat the chich MM and mouse ESD3 cultures. These concentrations did not show any specific sign of embryotoxicity or teratogenicity in both systems. The statistical analysis showed no siginificant changes ($p>0.05$) in contractile activity (Figure 2-21A, D), cell activity (Figure 2-21B, E) or total protein content (Figure 2-21C, F).

2.3.3.3 The effects of superoxide dismutase

MM and ESD3 cultures exposed to superoxide dismutase (SOD) concentrations (0.25, 0.5, 1 and 2μM) did not show any significant ($p>0.05$) alterations in the contractile activity (Figure 2-22A, D), cell activity (Figure 2-22B, E) or total cell protein content (Figure 2-22C, F).

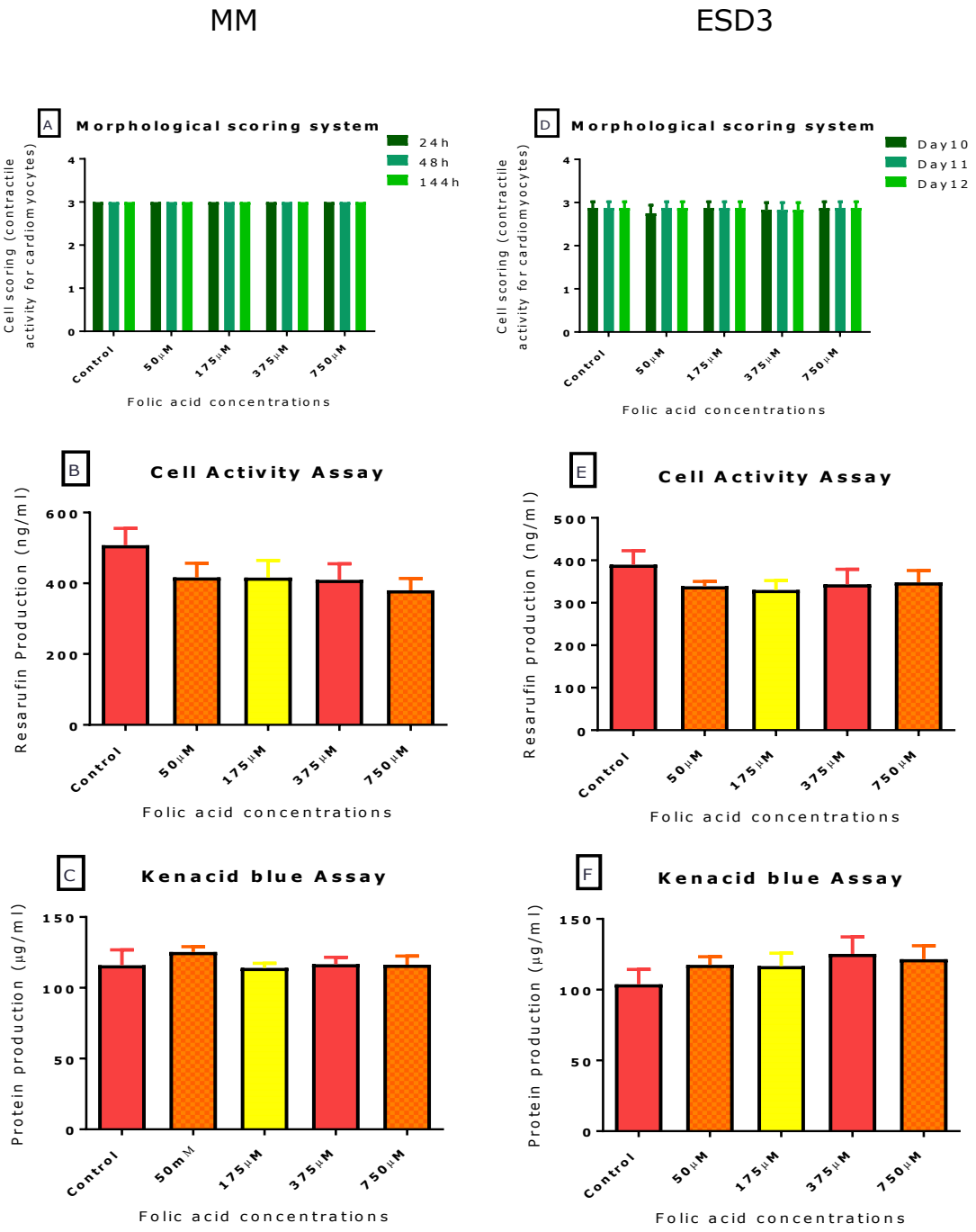


Figure 2-20: The effects of folic acid on MM and ESD3 systems
(A) Effects on contractile activity in MM system, (B) effects on cell activity in MM system, (C) effects on total protein content in MM system, (D) effects on contractile activity in ESD3, (E) effects on cell activity in ESD3 and (F) effects on total protein content in ESD3. Three independent repeats were performed, (n=3).

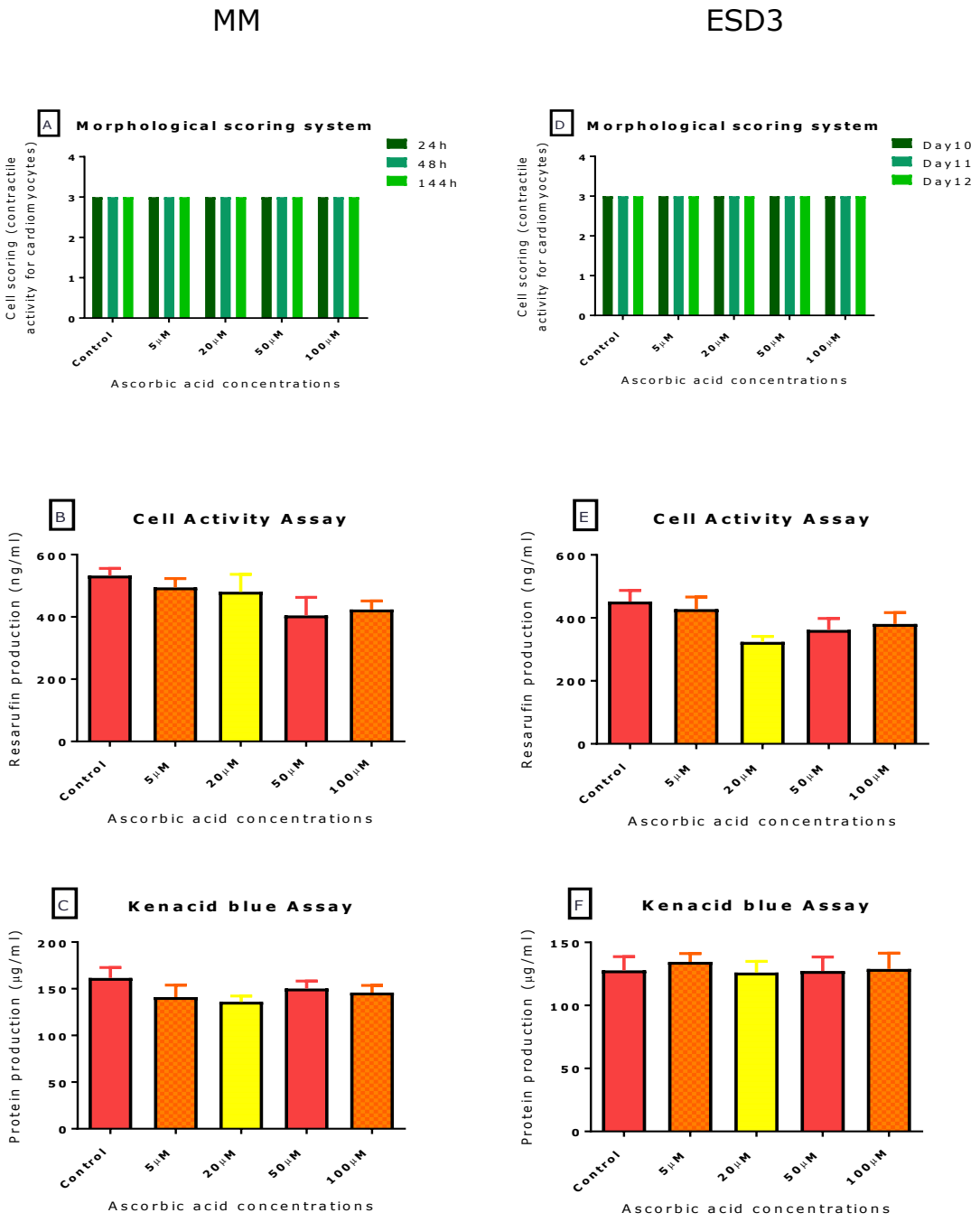


Figure 2-21: The effects of ascorbic acid on MM and ESD3 systems

(A) Effects on contractile activity in MM system, (B) effects on cell activity in MM system, (C) effects on total protein content in MM system, (D) effects on contractile activity in ESD3, (E) effects on cell activity in ESD3 and (F) effects on total protein content in ESD3. Three independent repeats were performed, (n=3).

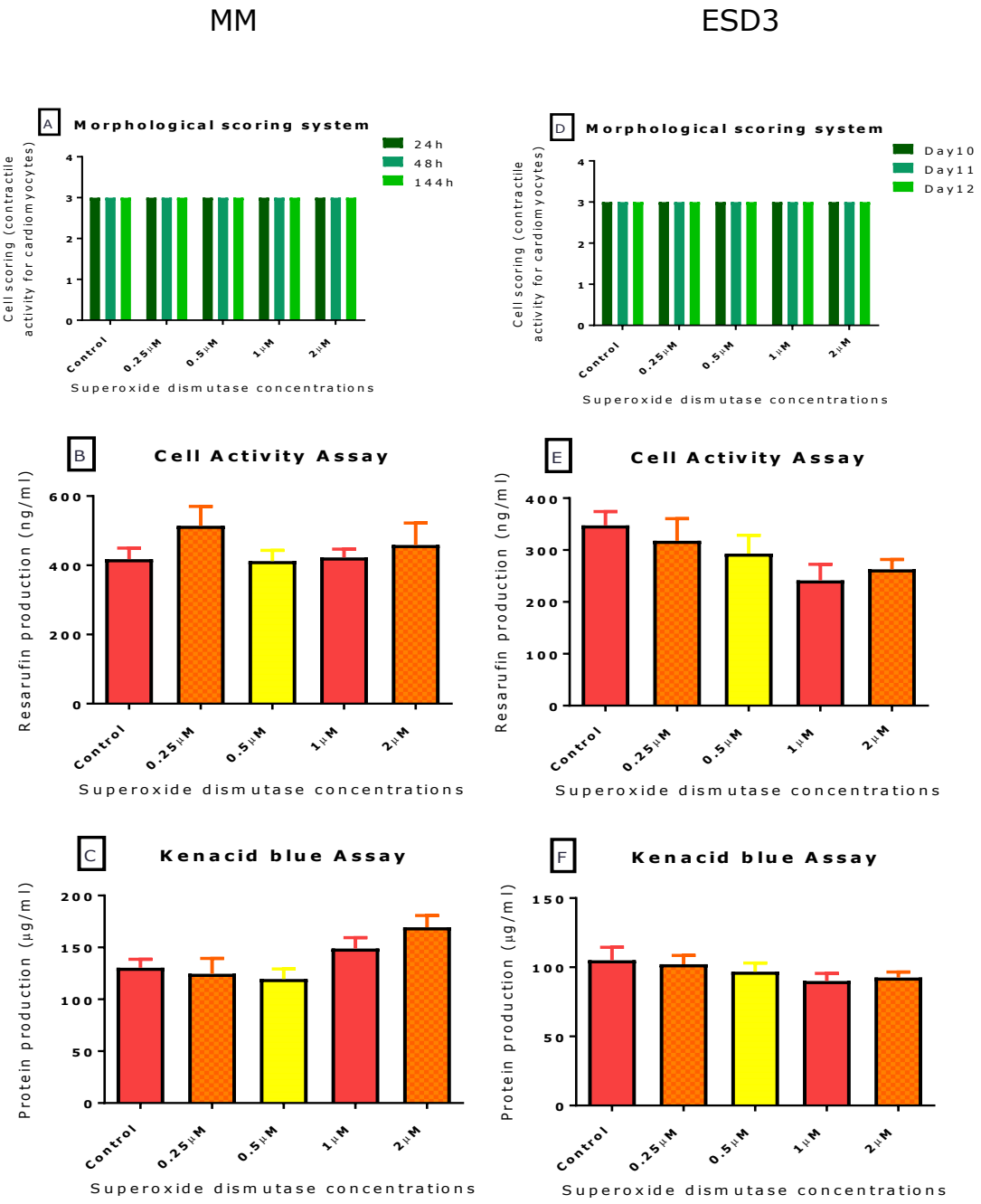


Figure 2-22: The effects of superoxide dismutase on MM and ESD3 systems

(A) Effects on contractile activity in MM system, (B) effects on cell activity in MM system, (C) effects on total protein content in MM system, (D) effects on contractile activity in ESD3, (E) effects on cell activity in ESD3 and (F) effects on total protein content in ESD3. Three independent repeats were performed, (n=3).

2.3.4 The effects of some teratogens (ETH, RA and VPA) on cardiomyocytes in MM and ESD3

2.3.4.1 The effects of ethanol

Exposure of the embryonic chick cardiomyocyte MM cultures to ethanol (ETH) at different concentrations (12.5, 25, 75 and 100µl/ml) caused a disruption in cell cultures at all end points measured. For the cell morphological scoring, the embryonic chick cardiomyocyte MM cultures were observed for contractile activity immediately before ETH addition (at 24h post-seeding) and further observations were noted at 48h post-seeding/24h post-exposure and 144h post-seeding/ 120h post-exposure. Statistical analysis showed no significant difference in contractile activity before the addition of ETH. MM cultures exposed to 25µl/ml ETH and above were found to have significantly reduced contractile activity at 48 and 144h (Figure 2-23A). At 48h there was a complete cessation of beating at 75 and 100µl/ml in ETH treated cultures. At 144h there was no contractile activity for 100µl/ml ETH-exposed cultures with no sign of recovery. However, some recovery has been viewed with the 75µl/ml treated group (Figure 2-23A). The cell activity was found to be significantly decreased for the 25µl/ml group and above when compared to controls (Figure 2-23B). The total protein content was also significantly altered at all ETH concentrations used (Figure 2-23C).

With regarding the effects of ETH on ESD3, the same concentrations used in MM were applied in this system. Severe consequences have been detected after ETH exposure at all endpoints utilised. ETH significantly inhibited contractile activity at 12.5µl/ml and above (Figure 2-23D). ETH totally prevented stem cells from being differentiated to cardiomyocytes at 25µl/ml

and above on all days observed (Figure 2-23D). Statistical analyses showed that ETH significantly inhibited cell activity at all doses used (Figure 2-23E). Graphs also revealed that total protein content of ETH treated cultures was significantly reduced compared to controls (Figure 2-23F). A reduction in EBs size was observed in ETH exposed cultures in a dose-dependent manner which might explain the recorded decrease in cellular protein. However, ETH did not cause ESD3 cells to be completely eradicated as some cells are still active and express some proteins but it caused the differentiation process to be completely terminated.

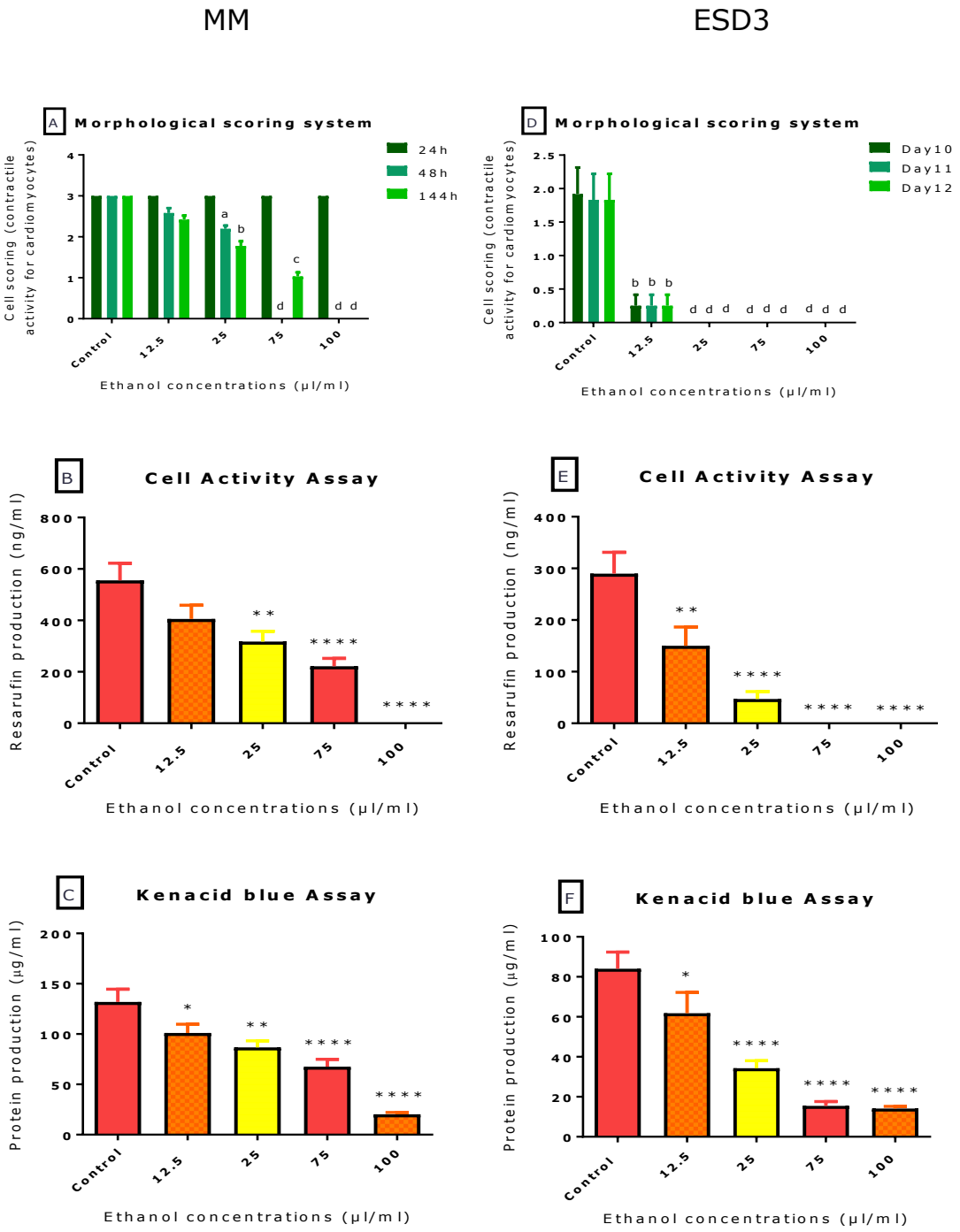


Figure 2-23: The effects of ethanol on MM and ESD3 systems

(A) Effects on contractile activity in MM system, (B) effects on cell activity in MM system, (C) effects on total protein content in MM system, (D) effects on contractile activity in ESD3, (E) effects on cell activity in ESD3 and (F) effects on total protein content in ESD3. * or (a) $p<0.05$, ** or (b) $p<0.01$, *** or (c) $p<0.001$ and **** or (d) $p<0.0001$ vs. control. Three independent repeats were performed, (n=3).

2.3.4.2 The effects of retinoic acid

The addition of retinoic acid (RA) at different concentrations (5, 10, 20 and 40μM) to chick embryonic heart cell in MM system results in dramatic impacts on all endpoints employed. The cultures were monitored for the morphological scoring system at three-time points, 24h, 48h and 144h post-seeding by recording the contractile activity. No changes of contractile activity at 24h post-seeding were recorded, but this was expected because cultures were scored before treatment (Figure 2-24A). At 48h post-seeding, the whole range of RA concentrations tested had a non-significant reduction in contractile activity (Figure 2-24A). The scores decreased significantly at the 144h post-seeding period in a dose-dependent trend compared to controls (Figure 2-24A). The graphs of cell activity and protein content showed a dramatic decline in all tested RA concentrations. Significant effects on cell activity were found after resazurin reduction assay data were analysed at 144h post-seeding period for all RA concentrations tested (Figure 2-24B). In the same way, at 144h post-seeding kenacid blue data analyses showed that RA at tested concentrations significantly decreased protein content either by killing cells or prevent proliferation (Figure 2-24C).

The ESD3 derived cardiomyocytes were observed for contractile activity on day 10, 11 and 12 following retinoic acid exposure. There was a significant difference observed for contractile activity after the addition of retinoic acid (Figure 2-24D). This is caused by a complete cessation of stem cell differentiation to cardiomyocytes at all RA concentrations tested. The graph of resazurin reduction assay showed that the available cells exposed to RA 5μM or above produced significantly less resorufin than control (Figure 2-24E). Total

protein content analysed data of the RA exposed cultures showed a significant reduction at all tested concentrations compared to controls (Figure 2-24E). It was observed that treated EBs size was subtle in comparison to control and only slightly expanded after culturing which was the reason behind the significant reduction in cell activity and protein content.

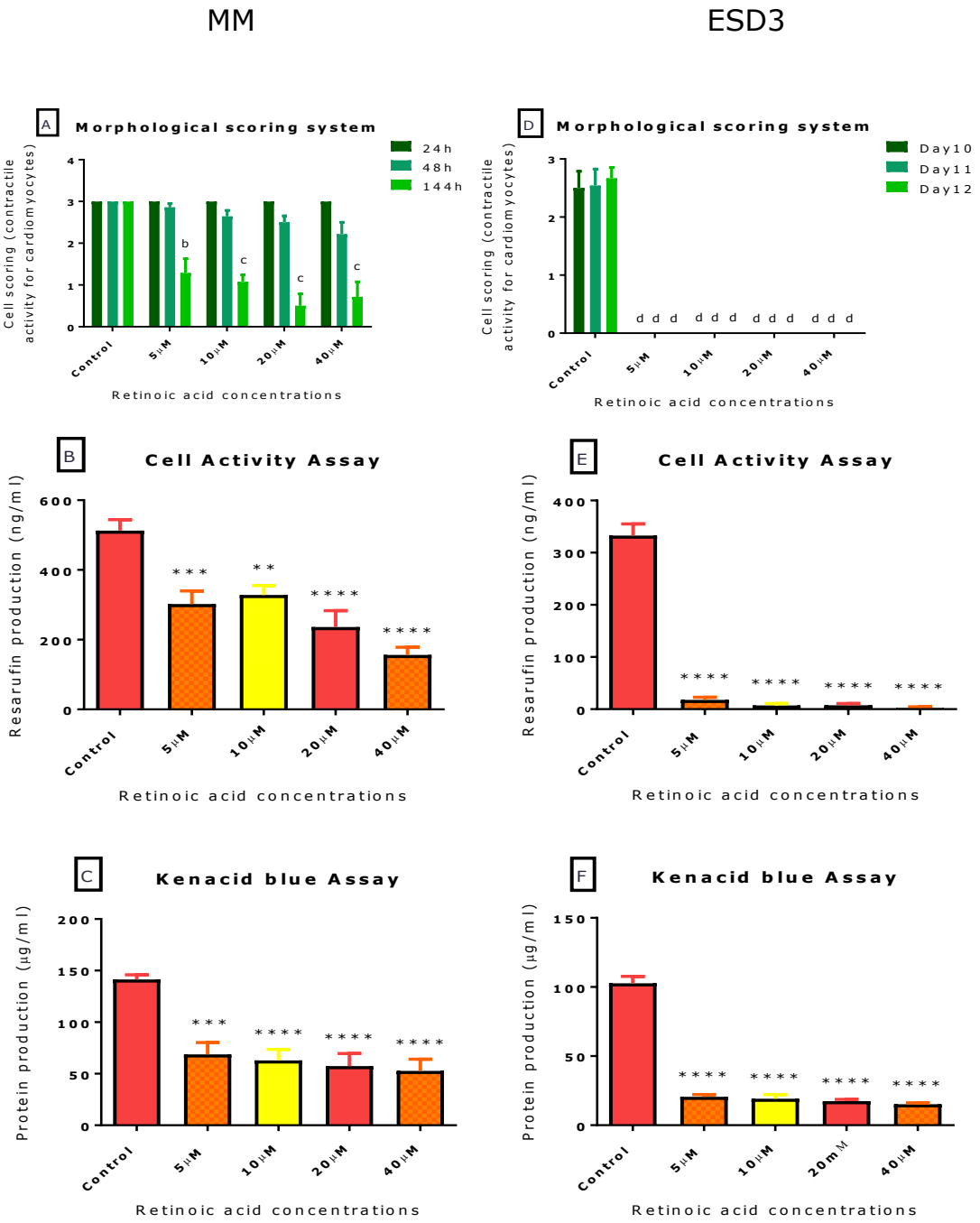


Figure 2-24: The effects of retinoic acid on MM and ESD3 systems

(A) Effects on contractile activity in MM system, (B) effects on cell activity in MM system, (C) effects on total protein content in MM system, (D) effects on contractile activity in ESD3, (E) effects on cell activity in ESD3 and (F) effects on total protein content in ESD3. * or (a) $p<0.05$, ** or (b) $p<0.01$, *** or (c) $p<0.001$ and **** or (d) $p<0.0001$ vs. control. Three independent repeats were performed, (n=3).

2.3.4.3 The effects of sodium valproate

Four sodium valproate (VPA) concentrations (100, 300, 750 and 1500 μM) were tested in MM system. The cultured beating chick cardiomyocytes were treated after 24h of seeding. The cultures were recorded for their contractile activity at 24h, 48h and 144h post-seeding. It was noticed that scoring was decreasing in a dose dependent manner until it becomes significant at 750 and 1500μM VPA on both 48h and 144h (Figure 2-25A). The addition of VPA to MM cultures caused chick embryonic cardiomyocytes to convert a significantly less amount of resazurin into resorufin at all tested concentrations due to cells being less active compared to controls (Figure 2-25B). In contrast, chick embryonic cardiomyocytes exposed to VPA did not have a significant decrease in total protein content after 120h VPA exposure (Figure 2-25C). However, the graph of the kenacid blue test shows that there was an apparent non-significant increase in protein content in VPA dose-dependent trend (Figure 2-25C).

The same VPA doses (100, 300, 750 and 1500μM) were tested in differentiating cardiomyocytes in the ESD3 system. The toxic effect on contractile activity, i.e. differentiation process, was evident and significant at all doses tested except at 100μM where the contractile activity was reduced insignificantly in comparison to controls (Figure 2-25D). The declining trend was evident for resazurin cell activity with increased dose until it becomes significant at the highest VPA dose (1500μM) (Figure 2-25E). The highest two tested concentrations (750 and 1500μM) showed a significant decline in total protein content of exposed ESD3 cultures compared to controls (Figure 2-25F). This was backed up by observation of the cultures where little EBs were obtained

and cultured after (750 and 1500μM) VPA treatment, and very slightly expansion on the plate was evident.

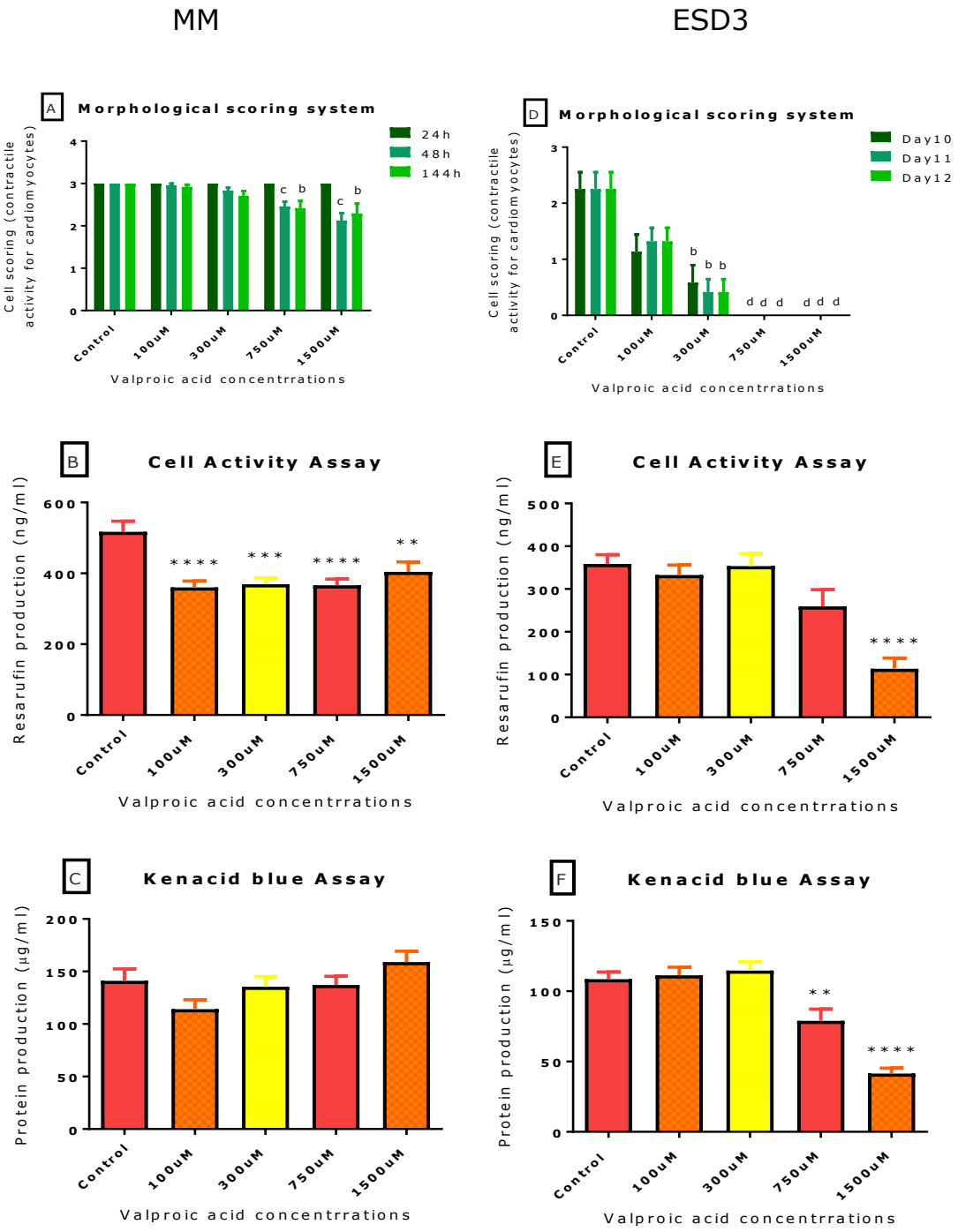


Figure 2-25: The effects of VPA on MM and ESD3 systems

(A) Effects on contractile activity in MM system, (B) effects on cell activity in MM system, (C) effects on total protein content in MM system, (D) effects on contractile activity in ESD3, (E) effects on cell activity in ESD3 and (F) effects on total protein content in ESD3. * or (a) $p<0.05$, ** or (b) $p<0.01$, *** or (c) $p<0.001$ and **** or (d) $p<0.0001$ vs. control. Three independent repeats were performed, (n=3).

2.4 Discussion

The evaluation of MM and ESD3 systems was essential to make certain they are reliable and consistent or reproducible. In this Chapter, the MM and ESD3 protocols of cardiomyocyte differentiation were evaluated with chemical free cell density experiments and exposure experiments for some known protective agents and teratogens. This was necessary before commencing the herbal components study (Chapter 3). The cultures in each system were verified to contain cardiac myocytes by staining them with cardiomyocyte marker (α-actinin).

The recently developed chick embryonic cardiomyocytes MM system has some advantages in the field of developmental toxicology. The cardiotoxic effects of test chemical are investigated without maternal drug metabolic interference or placental chemical bioactivation or inactivation while pre-determined chemical concentrations can be directly delivered to the culture at any stage [243]. The cardiomyocyte density optimisation experiment was performed in the MM system to observe cell communication and redifferentiation, in terms of the re-establishment of contractile units at different cell densities. Decreasing the cell density from $4x10^6$ cell/ml to $0.5x10^6$ cell/ml was accompanied by a decrease in contractile activity scoring but also there was a declining trend in cell activity and total protein content. However, the optimum cell density was found to be $3x10^6$ cell/ml, where the cells appeared to be in harmony and beating foci are distinguishable with the absence of crowded sheets which appeared at $4x10^6$ cell/ml or clumps which appeared $2x10^6$ cell/ml or less cell.

Mouse ESD3 derived cardiomyocytes have been confirmed to express the same signal molecules, transcription factors and differentiation regulated genes as those discovered in *in vivo* early cardiogenesis [244]. Therefore, the system has been considered very reliable to study heart embryotoxicity. Cardiomyocytes derived from ESD3 are efficient resources used to study cardiac development and teratogens. However, this had to be optimised in the laboratory to assure the reliability and consistency of the experiments. The hanging drop technique was employed in order to produce 3D aggregates which are termed EBs. This EBs-producing technique has been widely utilised to differentiate ESD3 to cardiomyocytes [245]. The starting number of embryonic stem cells in the EBs has been ranked as a first factor which has effects on the cardiomyocytes differentiation [244]. Additionally, other factors have been mentioned such as media, FBS, growth factors, additives, ES cell lines, and the time of EB plating. The results of cell density optimisation exhibited an expected declining pattern in cell activity and protein content with the cell number reduction. This may be because the size of EBs reduced with the reduction of cell density. Interestingly, this did not match with the contractile activity (differentiation potential) pattern observed where the both extreme densities (16 and $2x10^4$ cell/ml) showed fewer differentiation events than the mid densities. The results were comparable to Chen *et al*. findings in 2011 where three cell densities of mouse embryonic stem cell have been studied, 400, 1000 and 4000 cell per 30µl (which are approximately $1.35x10^4$, $3.3x10^4$ and 13.5 cell/ml). The Chen study investigated EBs beating at several time points and found the number of beating EBs on day 14 (two days more compared to this study-day12) showed no significant difference between the

400 group (lowest) and the 4000 group (highest). The 1000 group (the nearest to $4x10^4$ cell/ml in this study) had a higher percentage of contractile foci compared with the other groups [245]. The cell density of $8x10^4$ cell/ml showed some 'erratic' EBs formation with high differentiation ability. In conclusion, the optimal cell densities that have been chosen for MM and ESD3 systems for testing the chemicals in this thesis were $3x10^6$ cell/ml and $4x10^4$ cell/ml, respectively.

Treatment with folic acid, ascorbic acid or superoxide dismutase in chick embryonic and mouse embryonic D3 stem cell-derived cardiomyocytes did not show any alterations in contractile activity, cell activity and total protein content at all concentrations used.

- **Folic acid**

The association between maternal folate supplementation and CHDs has been studied clinically, *in vivo* and *in vitro*. Feng *et al*. in 2015 showed by a meta-analysis that maternal folate supplementation is associated with a significantly decreased risk of CHDs [246]. The effect of periconceptional folic acid-containing multivitamin supplementation on CHDs was studied in a Hungarian population-based case control which included 22,843 cases of different congenital abnormalities. The study concluded that pregnant women who were supplemented with folic acid have shown significant reduction in the risk of development of CHD [247]. Mao *et al*. (2012) studied the *in utero* effect of 5mg FA per day on fetal cardiac, hepatic, and renal functions, during the third trimester of gestation in ewes. They concluded that FA did not trigger biochemical changes related to cardiac, hepatic, and renal functions in both maternal and fetal sheep [248]. A murine study showed that maternal FA

deficiency increased the incidence of heart birth defects in embryos [249]. However, one study has investigated the effect of high FA dose in mice, 40mg/kg that is 20 fold more than normal dose. The study suggested that FA deficiency may lead to growth retardation and birth defects particularly thinner ventricular walls in embryonic hearts [250].

- **Ascorbic acid**

Ascorbic acid as an important antioxidant molecule in extracellular fluids was also tested in the MM and ESD3 systems. Due to the fact that chick embryonic hearts were explanted on day 5 of incubation, it is unlikely for AA to be synthesised at this stage as its synthesis starts on day 8 onwards of development in the yolk sac membrane [251]. Therefore, the addition of AA to embryonic cardiomyocyte is not expected to be harmful, if not advantageous, due to its role in the embryonic tissue development. The results were in accordance with several studies in that AA shows no deleterious effects on fetal development. Hossein *et al*. (2007) demonstrated that adding AA to *in vitro* fertilised gilt follicles has improved blastocyst formation and elevated the number of cells in the blastocyst depending on exposure time and concentration [252]. AA was also found to act as a potent supplement that enhances embryonic cardiomyoblast survival or induces induced pluripotent stem cells to improve cardiomyocyte differentiation *in vitro* [253,254]. Further, AA has rescued fibroblast growth factor receptor 1 gene knockout murine embryonic stem cells, a condition that leads to impair cardiomyocyte differentiation, and enables contractile cell differentiation [255]. Rumbold *et al*. in 2015 have published a paper which was entitled '*Vitamin C supplementation in pregnancy'* endeavouring to evaluate AA efficacy and

safety in pregnancy [256]. Cochrane Pregnancy and Childbirth Group's Trials Register in Australia was employed to collect and analyse the data. Whilst the data revealed that AA supplementation showed no substantial evidence of any damaging or beneficial effects, further studies to evaluate its role in pregnancy have been suggested [256].

- **Superoxide dismutase**

Besides AA, several other antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, also act as antioxidants throughout embryonic development [257]. SOD is slightly expressed at various levels in different embryonic tissues including the heart which has comparable SOD activity to other tissues [257,258]. Despite the fact that there is no study which has investigated SOD expression for the whole chick embryonic development period, it is improbable that SOD addition to 5-day old chick heart cells would affect differentiation and development because it has been confirmed to be expressed at least after this stage. In accordance with our study, Shaikh Qureshi and co-authors (2014) have shown that addition of 2μM of SOD to chick embryonic heart MM and ESD3 cells alter no end points measured [259]. On the contrary, another study has demonstrated that MnSOD but not CuZnSOD plays a role in stabilizing the turnover of pluripotent proteins (Oct4 and Nanog) at the post-translational level by modulating (inhibiting) proteasomal activity via an STAT3 mediated pathway; this inhibition resulted in the self-renewal of mouse ESD3 and fewer stem cells being differentiated [260]. However, the study did not specify the effect of addition SOD to differentiating stem cells or to which extent extrinsic SOD can prevent the ESD3 from being differentiating to cardiomyocytes.

With regards to the teratogens (ETH, RA and VPA), their teratogenic effects were revealed on exposed cultures in the MM and ESD3 systems. The results showed a dose-dependent declining trend for the end points utilised (contractile activity, cell activity and protein content) in both systems.

- **Ethanol**

For ETH, the association of intrauterine alcohol exposure and CHDs has been reported in several studies. Pan *et al*. (2014) investigated the effect of alcohol consumption during gestation in mice. They found that a single dose of 56% (v/v) alcohol (10 µl/g/d) by gavage every morning from embryonic day 7.5 to 15.5 had led to alteration of the expression of some heart development-related genes, such as Gata4, during cardiogenesis which could be linked to CHD [261]. In agreement with the findings reported here in ESD3, Worley *et al.* during 2015 showed that as low as 0.3% (v/v) ETH (equivalent to 3µl/ml) for 11 days significantly reduced cardiac differentiation. They proposed that this happened by decreasing the fraction of mouse ESD3 cultures containing beating foci (contractile activity), and this was accompanied by a significant decline in cell numbers [262]. ETH exposure was also found to delay the normal decline of Oct4 expression (undifferentiation gene) for mouse ESD3 throughout differentiating stages at applied concentration of 0.25% (v/v) (2.5µl/ml) for 7 days which in turn had led to an interruption in the cardiomyocyte differentiation process [263]. Whether relatively low doses of ETH can affect cardiac development, Nguyen *et al*. during 2014 reported that low doses of intrauterine exposure of ETH did not alter cardiomyocyte growth-related genes in rat offspring in their first few weeks after delivery. However, this has been harmful and caused several cardiac defects such as left

ventricular hypertrophy, cardiac fibrosis and reduced in aortic flow velocity with eight-month-old offspring [264]. As such, ETH has detrimental effects on both differentiating and growing cardiomyocytes and its toxic mechanisms would be further discussed in the next Chapters.

- **Retinoic acid**

Likewise, RA at the tested concentrations (5-40μM) has been damaging to the growing or differentiating cardiomyocytes in MM and ESD3, respectively. It has been confirmed that RA is essential for the patterning and development of the heart. Further, embryos with RA signalling attenuated showed several cardiac phenotypes suggesting multiple roles for RA in cardiogenesis [265]. However, Drysdale *et al*. (1997) studied the effect of addition 1μM RA to Xenopus laevis embryos and their explanted cardiomyocytes. The study has shown that RA effects on heart looping and gene expression and led to blocking of cardiac differentiation and developing a contractile tissue [266]. Interestingly, it has been confirmed that 0.01 and 0.001μM RA resulted in an increase the level of cardiac myosin heavy and light chain mRNA in embryoid bodies in early developmental stages. This had led to conclude that RA at very low doses accelerates cardiac differentiation particularly towards ventricular-like cardiomyocytes [267].

- **Valproic acid**

VPA has been employed as a positive control in several previous studies where the same *in vitro* systems have been utilised [268,44]. VPA effects at the concentration range 100-1500μM in chick embryonic heart cells and stem cell-derived cardiomyocytes were slightly different compared with ETH and RA. In

the MM system, VPA mainly reduced the contractile and cell activities of the culture but did not alter the protein content which renders VPA to be classified as mild to moderately embryotoxic instead of a cytotoxic drug with relation to its effect on growing cardiomyocytes. The decrease in cell activity observed in MM could be because VPA disturbed mitochondrial redox status which decreased the enzymes required to reduce resazurin to resorufin (cell activity indicator). However, dose-dependent declining pattern for all endpoints employed particularly the inhibition of cardiomyocyte differentiation even at therapeutic levels (350–1162μM) [269] was observed in the ESD3 system which means VPA should be prescribed with caution for women planning for pregnancy. Also, reduced protein content in the ESD3 system at the highest tested concentration (1500μM) was observed due to reducing the size of exposed EBs compared to untreated group. These findings are to some extent consistent with Ahir *et al.* results published in 2014. In their study, VPA was used at a range of 100 to 2000μM using the same *in vitro* systems and observed reduced cell beating in MM and declined cell differentiation in ESD3 with no effect on total protein content or cell activity [269]. Additionally, Na and co-workers (2003) also reported that VPA (100-1000μM) retarded the cardiac cell formation and differentiation of stem cell EBs dose-dependently without cytotoxicity being detected [270]. With regards to *in vivo* studies, it has been shown that 600 and 800mg/kg (15x and 20x human dose) caused cardiac anomalies which consisted of great vessel malformations which might be accompanied by ventricular septal defects in treated rat embryos [271].

Finally, it is important to remember that the cultures in both systems inevitably contain some other cell types in addition to the majority of cardiomyocytes;

the possibility cannot be fully ignored that teratogens used are damaging myocardial development by altering another tissue. Herein it was shown that the systems are highly reliable in terms of their sensitivity to developmental toxicants. Consequently, these two *in vitro* systems are now applicable to investigate the embryonic cardiotoxic potential of some new phytochemicals using the same biochemical end points in addition to some molecular assays.

CHAPTER 3

3 Chapter three - Evaluation of embryotoxicity for commonly used herbal components using the chick embryonic heart micromass and mouse D3 embryonic stem cell systems

3.1 Introduction

Congenital heart defects are considered a leading cause of postnatal loss; almost one out of 100 newborns are estimated to be affected directly, but at present the exact causes of these defects are yet to be specified [11,12]. The difficulty and complexity of collecting human paternal or maternal exposure data have made animal models for cardiac developmental defects become an important tool for understanding, detecting and isolating different cardiac teratogens [13]. The embryotoxicity of thousands of chemicals marketed each year must be tested for their developmental toxicity according to European legislation [43,272]. The use of herbs as medicines effectively began at the start of history itself [91] but has now been extended to become an 'optimal choice' to treat various kinds of illnesses in different parts of the world [92,93]. It has been shown, by several studies, that frequently pregnant women have chosen to consume various herbal preparations for different purposes during their pregnancy [94]. Despite their increasing popularity, few herbs have been tested for their effects on the development of the embryo [273]. Moreover, a literature review into the safety of 4 herbs commonly

consumed in pregnancy (ginger, chamomile, peppermint and lemongrass) found that 34% of sources analysed deemed these herbs to be unsafe. More confusingly the information on the safety of each herb varied from paper to paper [274]. Further research, by Da Silva Costa *et al.* in 2012, has also performed a database search and shown the wide range of adverse effects from a greater spread of medicinal herbs [275]. For this reason, the investigation of herbal supplements is imperative, not only for evidence of beneficial effects but primarily to ensure their safety for the developing embryo.

3.2 Herbal extracts

3.2.1 Ginger and [6]-gingerol

Ginger (Zingiber officinale Roscoe) has acquired an excellent reputation as an herbal remedy for treating many conditions including morning sickness, which affects about 80-90% of pregnant women [276-278], and it is widely used in the Asian kitchen as a cooking additive as well [276,277]. Unfortunately, the safety of using this herb during pregnancy has not been proved, compared with much evidence about its clinical effectiveness [277]. At the commencement of the 21st century, it was declared that there were not enough studies that investigated the risk of ginger to fetal development [279]. The safety of ginger regarding the development of the fetus still needs more work, as there is a lack of agreement about this issue among different countries. For example, in the USA, ginger has been considered as "Generally Regarded as Safe", whereas in other countries like Germany and Denmark, it was recommended to avoid ginger during pregnancy [277,280].

Ginger contains many phenolic compounds collectively known as gingerols [281] (Figure 3-1A). The pungent bioactive constituent of ginger has been shown to be 6-gingerol (6G) [282], which plays a role in counteracting vomiting conditions [283-286]. 6G has been shown pharmacokinetically to distribute to nearly all human tissues, particularly the gastrointestinal tract, and to have a terminal half-life up to 1.7 hours in rats after an oral dose of 120mg/kg [287,288]. In contrast, Ding *et al*. (1991) have found that terminal half-life of 6G after an intravenous dose of 3mg/kg in rats was 7.23 minutes with 92.4% protein binding [289]. 6G exerts several pharmacological actions, including anti-inflammatory, antioxidant and anticancer effects, by interfering with a variety of cellular pathways, such as the cell cycle and programmed cell death, and it also has cytotoxic properties [281]. Nevertheless, a few recent studies mentioned gingerol as having fetus-protective effects against well-known teratogenic compounds and/or conditions. Using the whole embryo culture technique, the results indicated that gingerol might protect the fetus from anomalies by its antioxidant effect via increasing superoxide dismutase (SOD) activity [290,291]. The concern about the teratogenic potential of 6G was raised as a result of its effect on some essential embryonic developmental processes, such as angiogenesis. This was confirmed by its ability to inhibit the proliferation and tube formation of primary cultured human endothelial cells by downregulation of cyclin D and also to inhibit tumour growth in mice through its anti-angiogenic activity [292]. Furthermore, it can induce apoptosis and/or DNA mutation in different tumour cell lines [293-295] and decrease cell migration and motility in a dose-dependent manner in a human breast cancer cell line [296]. It can also arrest the cell cycle and halt the proliferation of

cancer cells [297]. Hence, congenital malformation becomes a concern during the organogenesis period of the first trimester, where ginger might be taken to relieve morning sickness. However, the safety profile for ginger is still indistinct, and evidence-based data on the safety of use during pregnancy is limited [298]. Indeed, until recently, only one study was performed to assess specifically the safety of ginger use during human pregnancy [299].

3.2.2 Ginkgo biloba and ginkgolides

Ginkgo biloba is considered as one of the oldest herbal species in the world [127]. Ginkgolides are diterpene trilactones of the maiden hair tree, Ginkgo biloba. The two main active components of ginkgo biloba extract are ginkgolide A (GA) and ginkgolide B (GB) [300] (Figure 3-1B). Ginkgo leaves have been used for a long time in traditional Chinese medicine. It has been reported that its extract exhibits antibacterial, antitumor, antiviral, antiparasitic and antifungal activities [301]. Nowadays usage utilises extracts of the leaves for the treatment of cerebrovascular and peripheral vascular disorders, symptoms associated with cognitive decline and neurosensory impairments that may be linked to dementia, aging, and senility [302]. Ginkgolides can augment cellular energy and enhance tissue perfusion by the capillaries [303]. Moreover, Ginkgo biloba extracts have been shown to inhibit β-amyloid accumulation-induced apoptosis by inhibition of caspase 3 activation [304]. On the contrary, other reports concerning oral cavity tumor cells have shown pro-apoptotic characteristics of Ginkgo biloba extracts through activation of the caspase 3 enzyme system [305]. In spite of their extensive clinical uses, there is a lack in their safety profile, including their effects on the developing conceptus [306]. Different medical organisations recommend the avoidance of Ginkgo

biloba during gestation, as more studies are required to investigate its reproductive and developmental toxicity [307,308].

3.2.3 Ginseng and ginsenoside Rg1

Ginseng is said to be one of the most valuable herbs that improve general well-being, and it is extensively used in traditional Chinese medicine as discussed by Leung (2013) [309]. There are different ways in which Ginseng can be ingested, such as in the diet or as medicines, as well as nutritional and/or health supplements [310] and, thus, different forms could be registered for safety investigations. Ginseng is widely used among pregnant women, whether the Asian (Panax japonicus), American (Panax quinquefolius), Siberian or Ashwagandha (Indian Ginseng) type of ginseng, but the possible embryonic cardiotoxicity effects are of concern because Ginseng has cardiotonic and Ca^{+2} elevation characteristics [311]. Ginsenosides are dammarane triterpene saponins and are categorized by structure into two groups, the protopanaxatriol-type (ppt G-Rg1) (Figure 3-1C), and the protopanaxadiol-type (ppd G-Rb1) [312]. Ginsenoside Rg1 (G-Rg1) is believed to be one of the most active ingredients in ginseng, and it has multiple beneficial bioactivities. These particularly are neuroprotective, hypolipidaemic, anti-oxidant and anti-inflammatory activity, with little toxicity reported in both human and animal species both *in vivo* and *in vitro* [313-315], although there are limited data available about its impact on fetal growth and its embryotoxic effects as discussed by Liu *et al*. (2006) [316].

3.2.4 Chamomile and α-bisabolol

Chamomile (Matricaria recutica) has been commonly used in the Western world for many centuries. The multiple ways to administer chamomile have led it to be used as an anti-inflammatory, antiseptic, antispasmodic, anxiolytic and sedative but also to soothe external rashes such as diaper rash or chicken pox [114]. It is chamomile's effect as an anxiolytic [317] and anti-emetic that promotes its use in pregnancy [318]. α-Bisabolol (bisabolol) is the most abundant molecule in the essential oil (Figure 3-1D). Chamomile as a whole herb is attributed with anti-inflammatory properties, the potential ability to inhibit tumour growth and to reduce the risk of death from coronary heart conditions in certain subjects, as well as the capacity to relieve colic/diarrhea especially in infants [318]. Taken as a single ingredient tea, it has been used as an anxiolytic and sedative [114], with a randomized placebo-controlled pilot trial concluding it to have mixed and modest effects on both mild to moderate insomnia and mild to moderate generalized anxiety disorder (GAD). However, toxicity of bisabolol has been reported at high levels. The dose that kills 50% of treated animals (LD50) was determined to exceed 5g/kg in rats dependent on sex [319]. The pro-apoptotic effects of bisabolol also suggest them to be protective molecules against tumour cells. However, cascade pathways induced by apoptogenic stimuli may result in either the survival or death of the targeted cell with the potential to cause embryonic maldevelopment [320].

3.2.5 Holy basil and eugenol

Holy basil (Ocimum sanctum, OS), whilst lesser known in the Northern hemisphere, is an herb used in traditional medicine in the eastern world. It is credited with a whole range of pharmacological characteristics such as antimicrobial activity, hypoglycemic and hypolipidaemic activity, anti-stress activity, immunomodulation, psycho-pharmacological activity, antioxidant, anti-inflammatory, anticancer activity and radio protective activity [321]. Eugenol is one of the main active ingredients in OS (see the chemical structure Figure 3-1E). It has been pharmacologically shown in animals that eugenol is quickly distributed throughout the body and has a lethal dose (LD50) of 1930mg/kg if orally consumed in rats [322]. It plays a major role in the anti-stress and antioxidant activity of holy basil [113] but is found in multiple plants including those readily added as flavourings to be ingested – cloves, cinnamon, nutmeg, basil (sweet, African and Holy), star anise, dill, vanilla, bay laurel and celery. One study, investigating the anti-stress activity of eugenol, has concluded that the mechanism of reducing restrained stress in rats is by its modulatory effect on the hypothalamus (H), pituitary gland (P), and adrenal gland (A) association communication, or the HPA axis, which is involved in regulating hormones such as cortisol, and the brain monoaminergic system (BMS). The modulatory effect results in a decrease in corticosterone and 5-HT levels in the brain [323]. It has been reported that stress hormones provoke symptoms such as a headache, fatigue, muscle pains, nausea and vomiting in pregnancy [324]. Therefore, if eugenol can suppress these hormones, theoretically eugenol can be effective in reducing some common pregnancy symptoms.

This set of experiments was conducted to investigate the possible embryotoxic potential of six active phytochemicals (6G, GA, and GB and G-Rg1, bisabolol and eugenol), which might be taken by women in the early stages of pregnancy for various conditions. This was performed by monitoring cardiomyocyte differentiation, cell viability, and total protein content (total cell number) for chick embryonic cardiomyocytes and ESD3 cultured *in vitro*. The former system was used to investigate the test phytochemicals with respect to growing differentiated chick embryonic cardiomyocytes, and the latter was utilised to test their role in preventing or changing stem cell differentiation to cardiomyocytes. Accordingly, it is possible to see effects on one system, but not the other. According to their effects on the end points, the tested herbal components may be classified as safe, teratogenic or cytotoxic. An ingredient was considered cytotoxic when the total protein content in the treated cultures was significantly lower than the control since this would be indicative of a non-specific toxic effect. Moreover, the ability of chemicals to decrease the cellular activity and especially contractile activity could indicate more specific potential harmful teratogenic effects. However, contractile activity is indicative of cardiomyocyte differentiation, which could be altered even when a teratogen will not modify other endpoints.

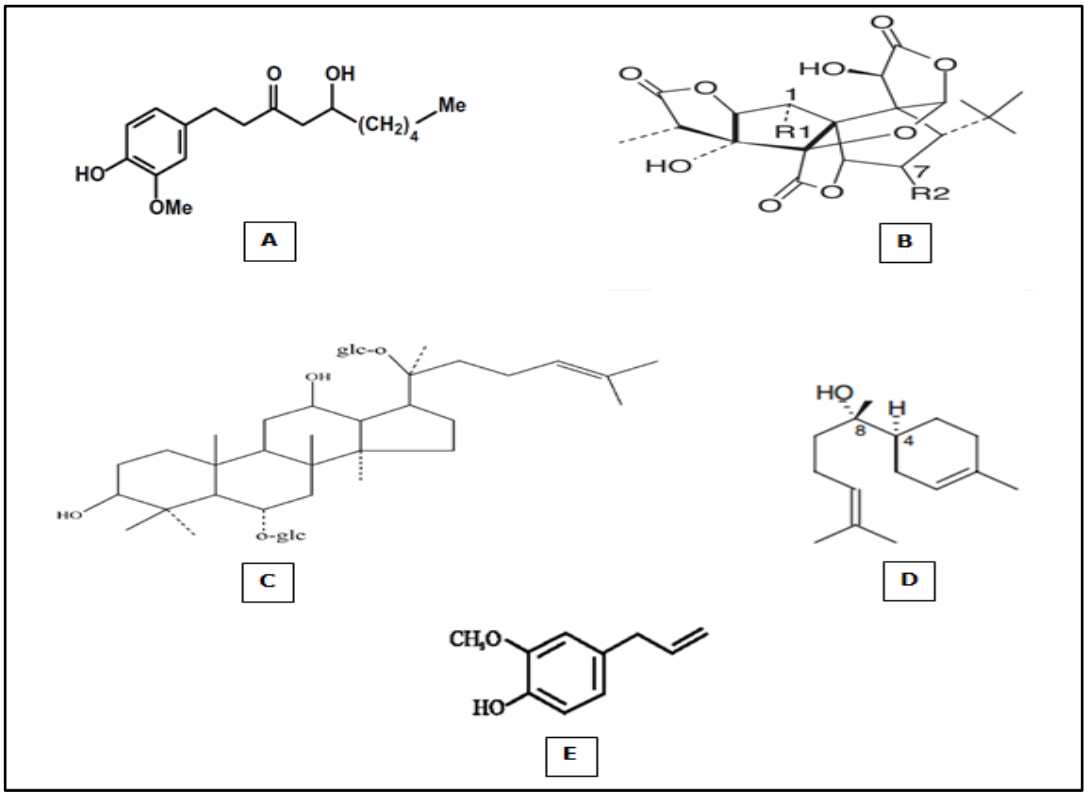


Figure 3-1: Chemical structures for tested herbal components
(A) [6]-gingerol, (B) ginkgolide A –R1 and R2=H, ginkgolide B-R1=OH and R2=H, (C) ginsenoside Rg1, (D) α-bisabolol and (E) eugenol.

3.3 Materials and methods

3.3.1 Herbal ingredients

The herbal ingredients were dissolved in dimethyl sulfoxide (DMSO) (6G, GA, GB, G-Rg1- Sigma–Aldrich, UK) or ethanol (α-bisabolol and eugenol-Sigma–Aldrich, UK). These were then serially diluted from their stock concentrations to the working concentrations in MM Dulbecco's Modified Eagles culture medium (DMEM) or ESD3 differentiation DMEM: 6G (0.75-100μM); GA (25-2000nM); GB (12.5-1000nM) and G-Rg1 (0.0625-10μM); α-bisabolol (0.01-80μM) and eugenol (0.01-80μM). The herbal ingredient concentrations corresponded to plasma levels in pharmacokinetic studies, see Table 3-1 [325-328] and Sections 3.3.1.1 and 3.3.1.2 for bisabolol and eugenol concentrations calculations.

Active components	Dose delivered	Mode of administration	Study type	Plasma Cmax	Commonly available forms on the market
6-Gingerol [327] MW 294.39	Ginger extract capsules contain 100mg-2g	Single oral dose	Human PKs	0.85μg/ml (= 2.88 μM)	Tablet, capsule, bread, spices, tea and lozenges.
Ginkgolide A [326] MW 408.40	Ginkgo biloba extract tablets contain 298-1571 μg GA	Single oral dose	Human PKs	3.62 ng/ml (= 88.6 nM)	Tablet, capsule, tea, injection, and syrup.
Ginkgolide B [326] MW 424.4	Ginkgo biloba extract tablets contain 147-836 μg GB	Single oral dose	Human PKs	1.38 ng/ml (= 32.51 nM)	Tablet, capsule, tea, injection and syrup.
Ginsenoside Rg1 [325,328] MW 801.01	Pure Ginsenoside Rg1 administered at 150mg/kg	Single oral dose	Rat PKs	1134.4ng/ml (= 14.00 μM)	Capsules, tea, gel, shampoo and injection.
	Pure Ginsenoside Rg1 administered at 150mg/kg			0.9 μg/ml (= 1.1236 μM)	

Table 3-1: Pharmacokinetic studies as references for 6G, GA, GB and G-Rg1 concentrations

The data base does not include any pharmacokinetic study for bisabolol and eugenol. These were calculated with some assumptions based upon the available data.

3.3.1.1 Bisabolol – concentration calculations

Whilst there is little research into the pharmacokinetics and dosage of bisabolol, its state as a volatile compound leads to the conclusion that it is readily absorbed when consumed orally. A randomized placebo-controlled pilot study into the use of chamomile as a treatment for insomnia used a daily regimen of 6x90mg of chamomile extract taken in capsular form. The University of Michigan Investigational Drug Service concluded that per 90mg of chamomile extract there is 1.8mg of bisabolol [7], although how this relates to chamomile brewed as a tea, or capsules containing higher purity doses is unknown. With regards to chamomile brewed as a tea, the advised method for creating the hot drink is to steep 2 teaspoons of dried flowers in hot water for 5 minutes, after which 10-15% of the essential oil would have entered the consumable liquid [8]. In order to relate this to a plasma level, some assumptions need to be made so this can be estimated. The range of concentrations was calculated assuming 1000mg chamomile (20mg bisabolol) was completely dissolved in 5 liters blood of a 70 kg pregnant woman. Therefore, the theoretically predicted blood level of bisabolol that has 222.37 Dalton molecular weight might reach up to 18μM. Due to the lack of precise data as to the possible peak blood level after ingestion, a wide range of concentrations of bisabolol was tested. The initial investigations used a wide range of concentrations of bisabolol (0.01-80μM), after being dissolved in ethanol. The maximum safe concentration, 1μM, was selected alongside the highest concentration, 80μM, to monitor the EBs size and culture morphology. The ethanol concentration was kept around (0.08 % - 15 times less than the

minimum tested concentration of ETH 12.5µl/ml in Chapter 2) for treated and untreated cultures in bisabolol experiments.

3.3.1.2 Eugenol – concentration calculations

As there are no clinical pharmacokinetic studies to relate the plasma levels of eugenol directly, concentrations derived from *in vivo* studies were utilized. The suggested dose for holy basil is 800-1000mg daily. From studies, holy basil contains 18% essential oils of which 70% is eugenol [329]. Thus, from a dose of 1000mg holy basil daily, a consumer will be taking 126mg of eugenol daily. A pharmacokinetic study on rats with an oral dose of 40mg/kg eugenol has shown a maximum concentration in the blood of 0.27µg/ml or 1.64µM eugenol [330]. By using human equivalent dosage (HED) conversion [331], dividing the rat dose factor (6.2) [331] to a rat dose (40mg/kg), hence, a rat oral dose of 40mg/kg is equivalent to a human oral dose of 6.4mg/kg eugenol – 448mg eugenol in an average 70kg woman, which is much higher than 126mg eugenol daily. This suggests that the maximum blood concentration of an average consumer taking 1000mg holy basil daily will not exceed 1.64µM, and the predicted blood concentration of 126mg eugenol is almost 500nM, overlooking the differences between rat and human physiology. Therefore this study investigated a broad range of concentrations of eugenol surrounding 1.64µM, from 0.01µM up to 80µM, assuming free crossing of the placental barrier, as well as taking into consideration the pharmacokinetic differences between species. Eugenol was dissolved in ethanol then diluted into either MM or ESD3 medium in the concentrations of 0.01-80µM to be delivered to chick embryonic cardiomyocytes and mouse stem cell-derived cardiomyocytes, respectively. For EBs/culture microscopic imaging, only two concentrations were tested,

0.01µM (which was the eugenol concentration where no effect appears at all endpoints in both systems) and 80µM the highest test concentration. The final ethanol concentration was kept as low as (0.03% - 40 times less than the minimum tested concentration of ETH 12.5µl/ml in Chapter 2) for treated and untreated cultures in eugenol experiments.

3.3.2 Primary embryonic micromass preparation

3.3.2.1 Preparation of MM growth media

For the MM embryonic cardiomyocyte culture medium, 10% (v/v) heat inactivated FBS, 50 U/ml penicillin, 50µg/ml streptomycin were added to 500ml DMEM. After preparation, the medium was kept in a refrigerator (4^0C) and utilised for no more than two weeks. Fresh 2mM L-glutamine was added to each 50ml of medium prior to use.

3.3.2.2 Micromass chick heart embryo protocol

MM culture was described in more details in Chapter 2, Section 2.2.1. The material being tested was prepared in double the required concentration in 500µl culture medium and added after 24h (making the total volume of culture medium 1000µl). The plate was scored again after 24h of treatment (48h of culture), and after 120h of treatment (144h of culture). The same solvent proportion was maintained in all wells, including the control, for all repeats.

3.3.3 Mouse D3 embryonic stem cells hanging drop preparation

3.3.3.1 Preparation of ESD3 growth media

For mouse embryonic D3 stem cell medium, 0.1mM β-mercaptoethanol, 20% (v/v) FBS, 50units/ml penicillin, 50µg/ml streptomycin, 2mM L-glutamine, and 1% (v/v) NEAA were added to 500ml DMEM. After preparation, the medium

was kept in a refrigerator (4^0C) and utilised for no more than two weeks. 2mM L-glutamine was added freshly on an experimental day for each 50ml medium. 1000U/ml LIF was added to passaged cells to maintain the pluripotency, but it was removed since day 0 of ESD3 procedure to allow cell differentiation.

3.3.3.2 Mouse D3 embryonic stem cell differentiation protocol

The procedure was described earlier, and more details are available in Chapter 2, Section 2.2.2. The same chemical concentration and environment were maintained from day 0 to day 12.

3.3.4 Endpoint measurements

The same endpoints in Chapter 2, Section 2.2.3 were performed in this study to determine the effect of phytochemicals on the embryonic cardiomyocytes. Additionally, the effect on MM cultures and EBs formation before and after cultivation was imaged in this Chapter.

3.3.4.1 Cardiomyocyte differentiation assessment

The beating foci were recorded manually in both systems with the aid of an inverted light microscope. The score was progressively decreased when the number of beating foci dropped in a range between 3 and 0 for no contractility, Table 2-1. For each experiment, both untreated and treated cells were seeded on the same plate. For more details see (Chapter 2, Section 2.2.3.1). The morphology of EBs (formation and size) at day 5 of the ESD3 system was monitored but also at day 12 after their cultivation to examine their speed of proliferation.

3.3.4.2 Resazurin-Cell activity assay

Resazurin (Alamar Blue dye) is a non-toxic non-fluorescent substrate, which is used to measure the activity of living cells by yielding a strongly fluorescent pink reagent (resorufin) after its reduction by viable cells [332]. The procedure was described in details in Chapter 2, Section 2.2.3.2. The obtained values were plotted on the standard curve to obtain the actual resorufin values, Section 2.2.3.3.

3.3.4.3 Kenacid Blue (protein content) Assay

This assays total cellular protein by quantifying the uptake of the dye kenacid blue [333]. The protein content could be considered as a useful endpoint to establish whether toxicity results from modifying differentiation, proliferation or causing a decrease in cellular protein expression [242]. The detailed procedure and solutions components are mentioned in Chapter 2, Section 2.2.3.4. The raw values were converted to actual protein content by employing the kenacid protein standard curve, Section 2.2.3.5.

3.3.5 Statistical analysis

Raw data was collated in Microsoft Excel (2008, Version 12.2.0). Three independent repeats (n=3) were performed for each experiment with at least four technical repeats per each. The data was imported into GraphPad Prism software (Version 6.0, August 5, 2014) for statistical analysis. For non-parametric data – cellular scoring morphology – a Kruskall-Wallis and post-hoc Dunn's test were used and mean ± SEM were computed. For parametric data – resazurin and kenacid blue assays – the data was analysed using one-way ANOVA test, with a post-hoc Dunnett's test. For resazurin and kenacid blue

assays, the percentage of means was presented with considering the control as 100%. P<0.05 was considered statistically significant.

3.4 Results

3.4.1 Effect of [6]-gingerol

At low to moderate concentrations (0.75μM-6μM), 6G-treated primary embryonic chick cardiomyocytes showed no significant changes in contractile activity, cellular activity or changes in total protein content in comparison to the control (P>0.05) (Figure 3-2A, B and C). At 6G concentrations of 12.5-100μM, inhibition of contractile activity was significant (P<0.05) at 48h -except at 100μM- and 144h in comparison to control (Figure 3-2A). All high 6G concentrations, 12.5-100μM, tested in MM, significantly altered (p<0.05) both the cellular activity and protein content in a dose-dependent manner (Figure 3-2B, C). At 144h, the cultures of control, 3μM and 100μM have been imaged to observe the changes within the cultures. Although 3μM did not show microscopic changes to the culture, 100μM 6G prevented cardiomyocytes from maintaining a confluent status by causing a cell-absent zone to appear in the middle of the culture (Figure 3-3A, B and C).

The same concentrations (0.75uM-100μM) of 6G were used in the ESD3 system, which showed a significant (p<0.05) decrease in cardiomyocyte differentiation for all tested concentrations except 0.75μM (Figure 3-2D). Furthermore, the cellular activity and protein content of stem cell-derived cardiomyocytes followed the same pattern of significant decrease with increased 6G concentration exposure (Figure 3-2E and F). There was a slight anomaly with 6μM concentration, where the morphological score showed

almost no differentiation on day 10 and 11, although there was a small amount of differentiation evident on day 12 (Figure 3-2D). This was paralleled by somewhat greater effects on the other endpoints. The 5-day-old EBs size was monitored at both 0.75μM and 100μM 6G treatments to compare them with the control. The EBs were followed up even after they were being plated up until day 12 of the protocol where images have been taken at that point. At 0.75μM 6G, there was very mild decrease of EB size but its culture at day 12 showed normal cell proliferation (Figure 3-3E and H) in comparison to the control (Figure 3-3D and G). However, EBs treated with 100μM 6G exhibited a very clear reduction in EBs size pre- and post-plating (Figure 3-3F and I).

MM ESD3

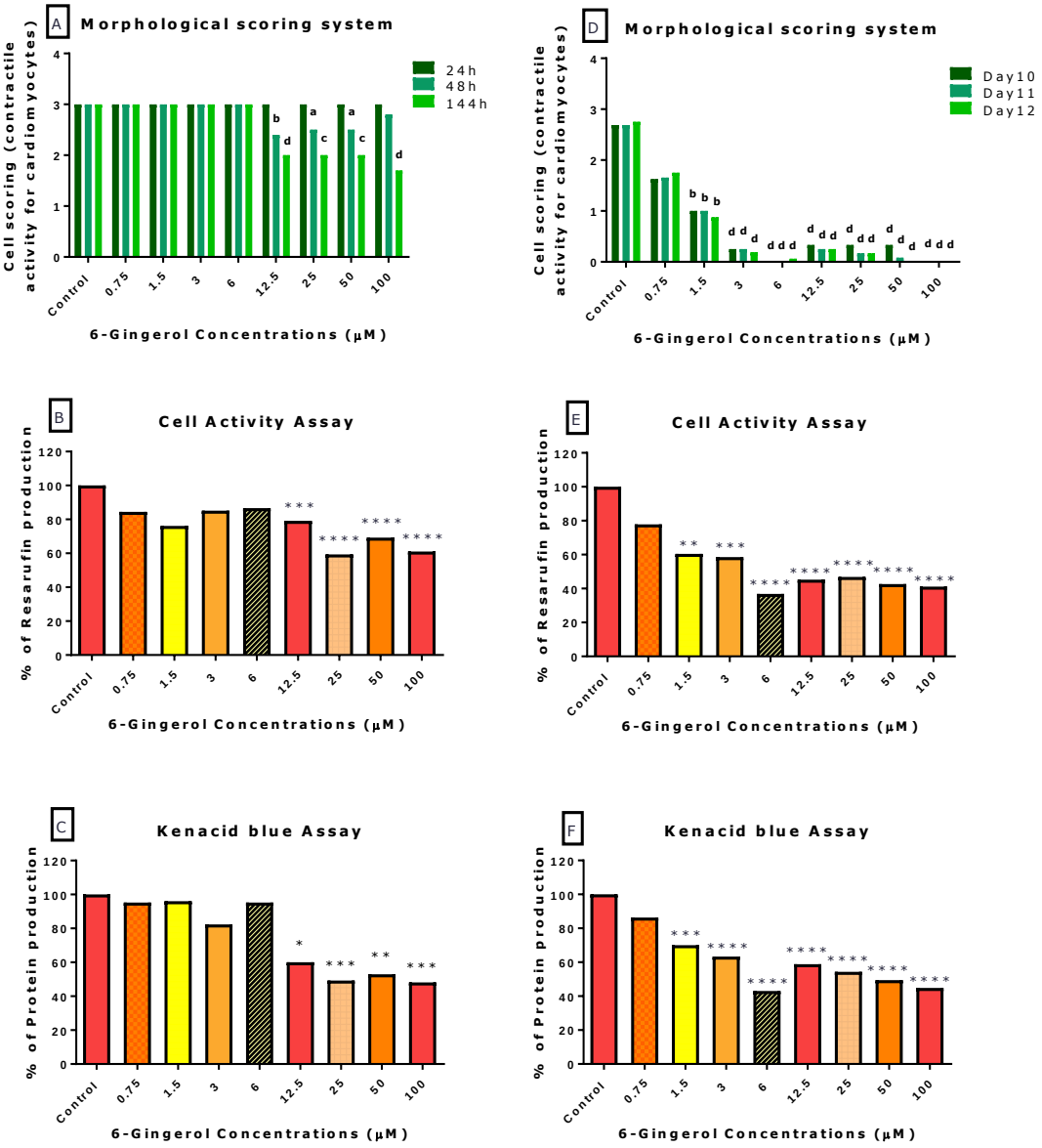


Figure 3-2: Effects of 6-gingerol on embryonic cardiomyocyte in MM and ESD3 systems

In MM, (A) effect of 6-gingerol on contractile activity, (B) effect of 6-gingerol on cell activity (resazurin assay) at 144h, and (C) effect of 6-gingerol on total protein content (kenacid blue assay) at 144h. In stem cell-derived cardiomyocytes, (D) effect of 6-gingerol on stem cell differentiation to cardiomyocytes, (E) effect of 6-gingerol on cell activity (resazurin assay) on day 12, and (F) effect of 6-gingerol on total protein content (kenacid blue assay) on day 12. Three independent repeats (n=3) of each experiment were performed. The data are presented as mean for morphological scoring and % of the mean control for resazurin and protein content. * or (a) $p<0.05$, ** or (b) $p<0.01$, *** or (c) $p<0.001$ and **** or (d) $p<0.0001$ vs. control.

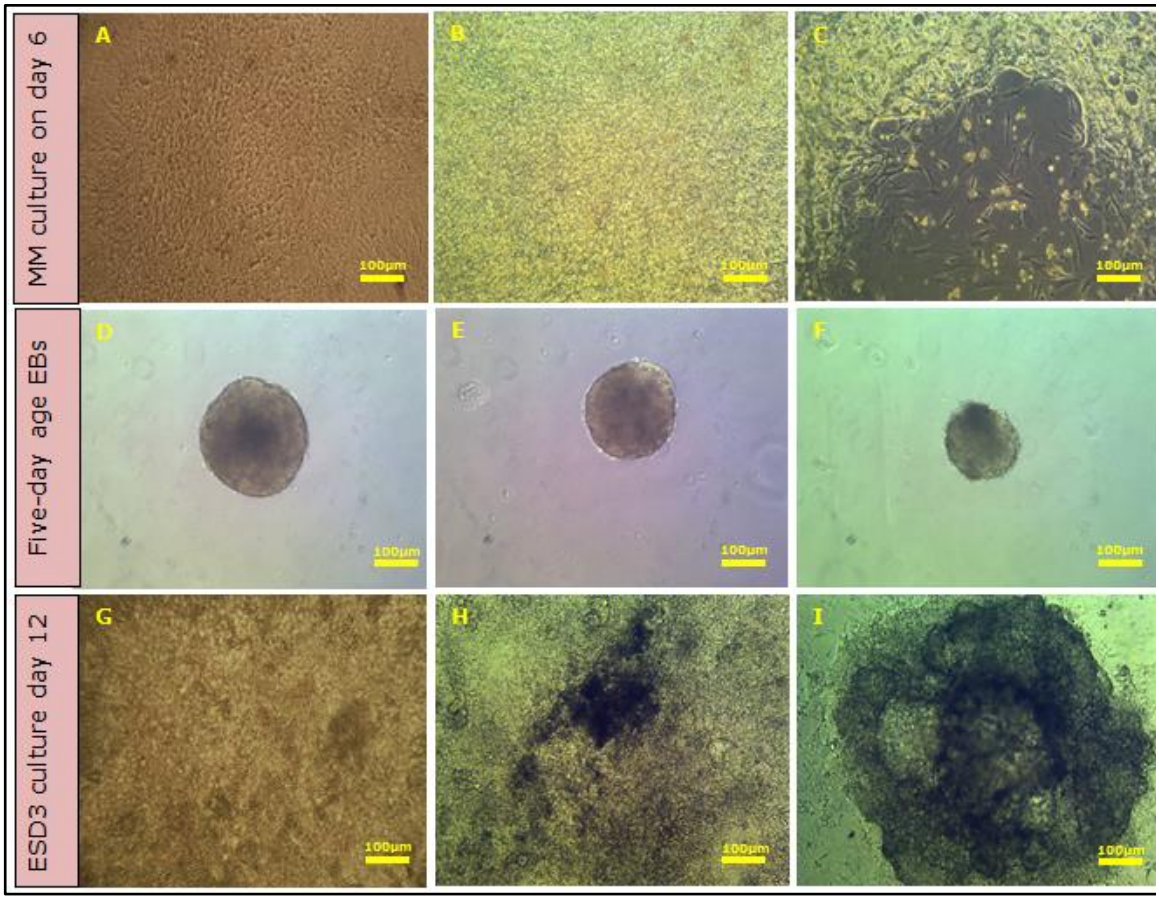


Figure 3-3: Representative light microscopy photographs of 6-gingerol effects on MM and ESD3 cultures

(A) untreated (vehicle control) cardiomyocytes at 144h after MM culturing, (B) 3µM 6-gingerol-treated cardiomyocytes at 144h after MM culturing, (C) 100µM 6-gingerol-treated cardiomyocytes at 144h after MM culturing, (D) untreated (vehicle control) 5-day-age EB in ESD3 system, (E) 0.75µM 6-gingerol-treated 5-day-age EB in ESD3 system, (F) 100µM 6-gingerol-treated 5-day-age EB in ESD3 system, (G) untreated (vehicle control) EB post-culturing on day 12 in ESD3, (H) 0.75µM 6-gingerol-treated EB post-culturing on day 12 in ESD3, (I) 100µM 6-gingerol-treated EB post-culturing on day 12 in ESD3. (Scale bar 100µm) with original magnification x100 for all images.

3.4.2 Effect of ginkgolides

The effects of GA at concentrations (25-2000nM) were similar in MM and ESD3 systems. GA (25-100nM) did not alter the cardiomyocyte differentiation (morphological scoring system), either in the MM system (Figure 3-4A) or in the embryonic stem cell system (Figure 3-4D). The higher concentrations of GA (250-2000nM) showed disruption in the redifferentiation process of the MM cultures (Figure 3-4A) and also disrupted the cardiomyocyte differentiation process of ESD3 (Figure 3-4D). This was represented by a dramatic decrease in the morphological score in both systems ($p<0.05$). The conversion of resazurin to resorufin, an indicator of cellular activity, does not vary significantly ($P>0.05$) in GA-treated groups compared to control at the evaluated concentrations (25-2000nM) in either system (Figure 3-4B and E). An exception was for 500nM which did show a significant ($p<0.05$) cellular activity reduction in MM culture (Figure 3-4B). Thus, these GA concentrations did not show any specific trend of cytotoxicity. The protein content, which indicates total cell number and cellular proliferation, also does not significantly alter after treatment compared to control in either method (Figure 3-4C and F), except for the 50nM concentration with the embryonic stem cells (Figure 3-4F). The microscopic images of 6-day age MM cultures (Figure 3-5A, B and C), 5-day-old EBs (Figure 3-5D, E and F) and post-cultured EBs in ESD3 system (Figure 3-5G, H and I), at 100nM and 2000nM GA exhibited no culture alterations and they appeared very comparable to the corresponding controls.

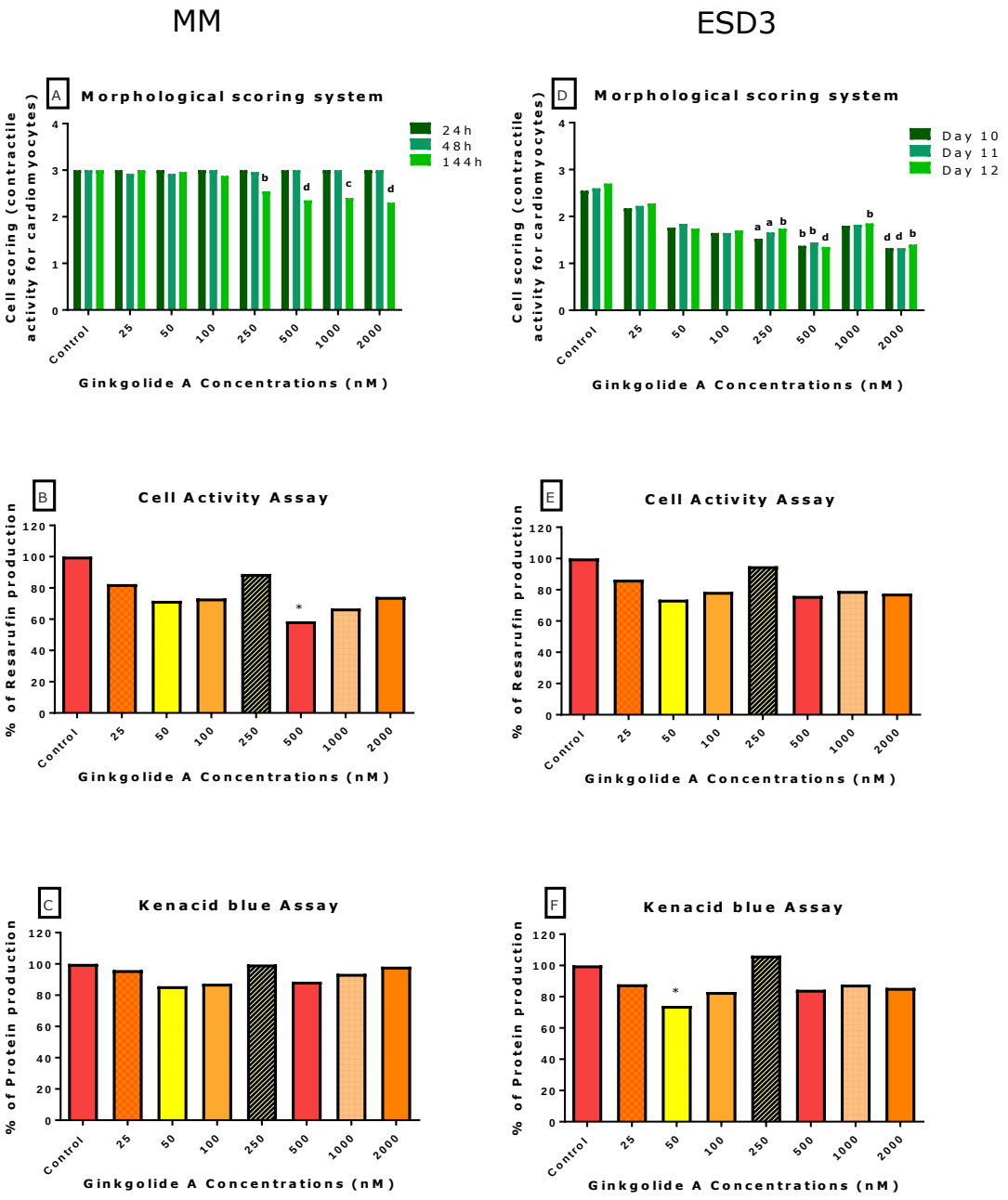


Figure 3-4: Effects of ginkgolide A on embryonic cardiomyocyte in MM and ESD3 systems

In MM, (A) effect of ginkgolide A on contractile activity, (B) effect of ginkgolide A on cell activity (resazurin assay) at 144h, and (C) effect of ginkgolide A on total protein content (kenacid blue assay) at 144h. In stem cell-derived cardiomyocytes, (D) effect of ginkgolide A on stem cell differentiation to cardiomyocytes, (E) effect of ginkgolide A on cell activity (resazurin assay) on day 12, and (F) effect of ginkgolide A on total protein content (kenacid blue assay) on day 12. Three independent repeats (n=3) of each experiment were performed. The data are presented as mean for morphological scoring and % of the mean control for resazurin and protein content. * or (a) $p<0.05$, ** or (b) $p<0.01$, *** or (c) $p<0.001$ and **** or (d) $p<0.0001$ vs. control.

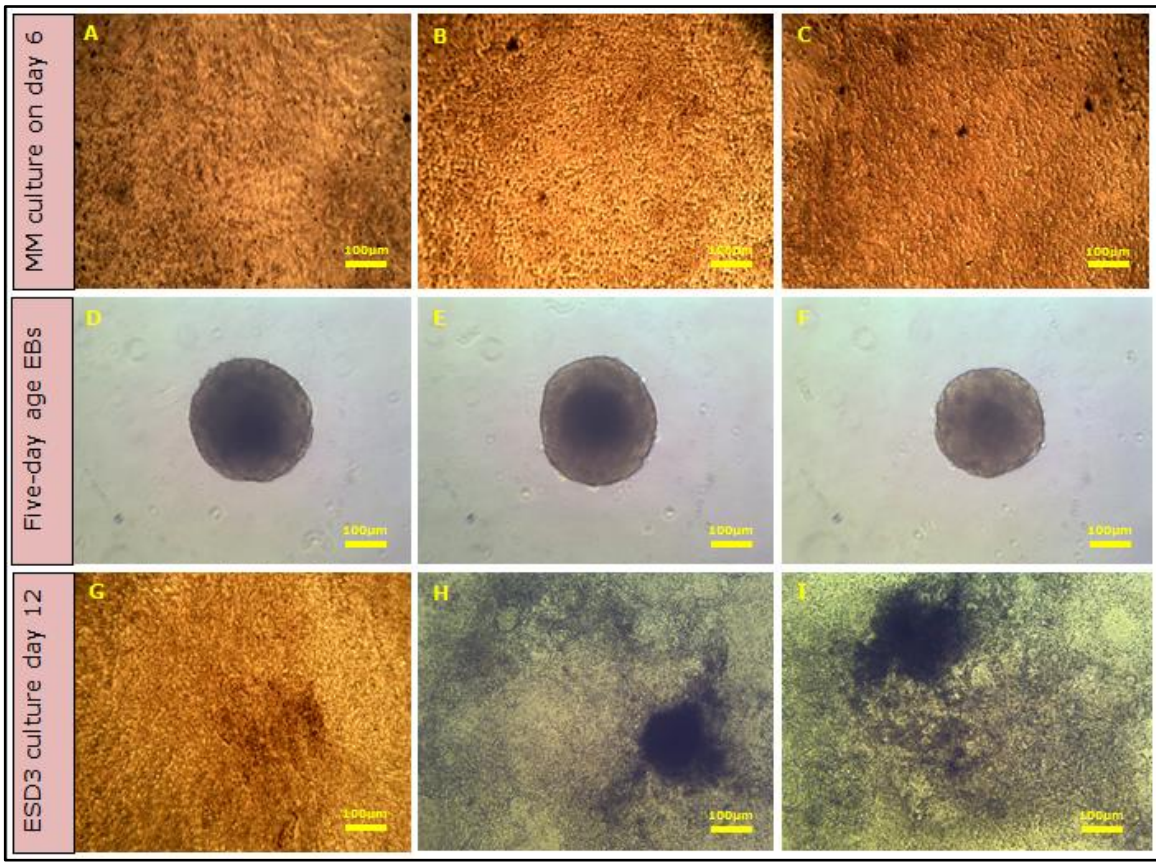


Figure 3-5: Representative light microscopy photographs of ginkgolide A effects on MM and ESD3 cultures

(A) untreated (vehicle control) cardiomyocytes at 144h after MM culturing, (B) 100nM ginkgolide A-treated cardiomyocytes at 144h after MM culturing, (C) 2000nM ginkgolide A-treated cardiomyocytes at 144h after MM culturing, (D) untreated (vehicle control) 5-day-age EB in ESD3 system, (E) 100nM ginkgolide A-treated 5-day-age EB in ESD3 system, (F) 2000nM ginkgolide A-treated 5-day-age EB in ESD3 system, (G) untreated (vehicle control) EB post-culturing on day 12 in ESD3, (H) 100nM ginkgolide A-treated EB post-culturing on day 12 in ESD3, (I) 2000nM ginkgolide A-treated EB post-culturing on day 12 in ESD3. (Scale bar 100µm) with original magnification x100 for all images.

The data obtained from concentrations (12.5-50nM) of GB in the MM method, showed that these concentrations showed no dramatic alteration for the morphological score ($p>0.05$) (Figure 3-6A). In contrast, the results of moderate to high concentrations (125-1000nM) showed a significant reduction of the morphological score in MM (Figure 3-6A). However, when GB was applied to ESD3 (12.5-1000nM), the cardiomyocyte differentiation showed no specific declining trend with an increase in GB concentration (Figure 3-6D). However, the cellular activity in MM culture was significantly affected after GB (250-1000nM) treatment (Figure 3-6B). In contrast, only 12.5nM showed a significant ($P < 0.05$) reduction of cell activity in the ESD3 system (Figure 3-6E). However, GB 12.5nM gave a normal score in the morphological scoring system (Figure 3-6D). GB does not seem to have the ability to reduce the cell number and hence reduce protein content (Figure 3-6C and F) in a significant manner in either system at any tested concentration. Only GB 12.5nM in ESD3 system induced a slight significant ($P<0.05$) decline in total protein content (Figure 3-6F).

The phase contrast images which were taken at day 6 in MM, day 5 EBs and day 12 cultures in ESD3 clearly showed that there was no disturbance caused at any selected tested GB concentrations (50nM and 1000nM) in comparison to their corresponding controls (Figure 3-7A-I).

MM ESD3

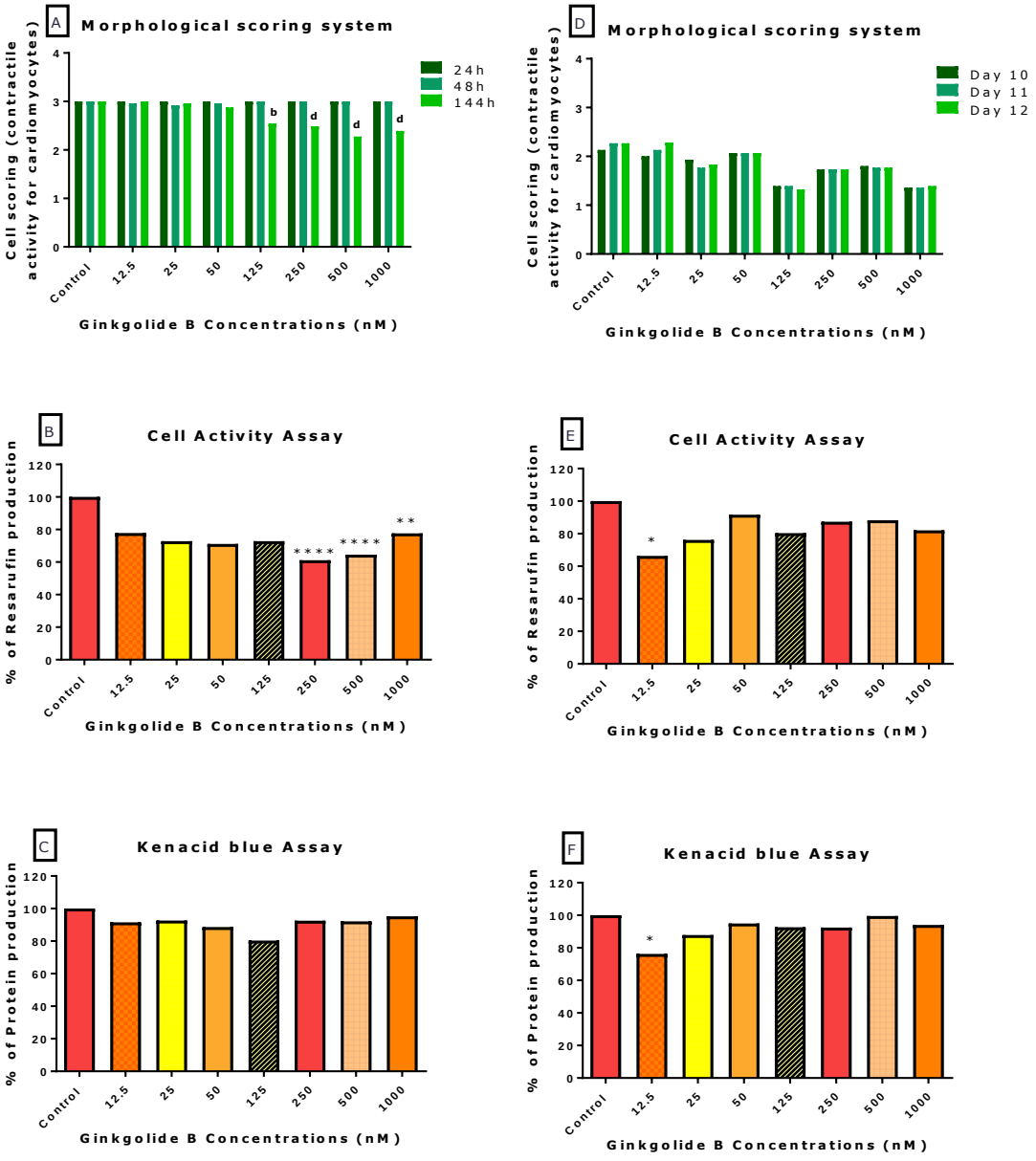


Figure 3-6: Effects of ginkgolide B on embryonic cardiomyocyte in MM and ESD3 systems

In MM, (A) effect of ginkgolide B on contractile activity, (B) effect of ginkgolide B on cell activity (resazurin assay) at 144h, and (C) effect of ginkgolide B on total protein content (kenacid blue assay) at 144h. In stem cell-derived cardiomyocytes, (D) effect of ginkgolide B on stem cell differentiation to cardiomyocytes, (E) effect of ginkgolide B on cell activity (resazurin assay) on day 12, and (F) effect of ginkgolide B on total protein content (kenacid blue assay) on day 12. Three independent repeats (n=3) of each experiment were performed. The data are presented as mean for morphological scoring and % of the mean control for resazurin and protein content. * or (a) $p<0.05$, ** or (b) $p<0.01$, *** or (c) $p<0.001$ and **** or (d) $p<0.0001$ vs. control.

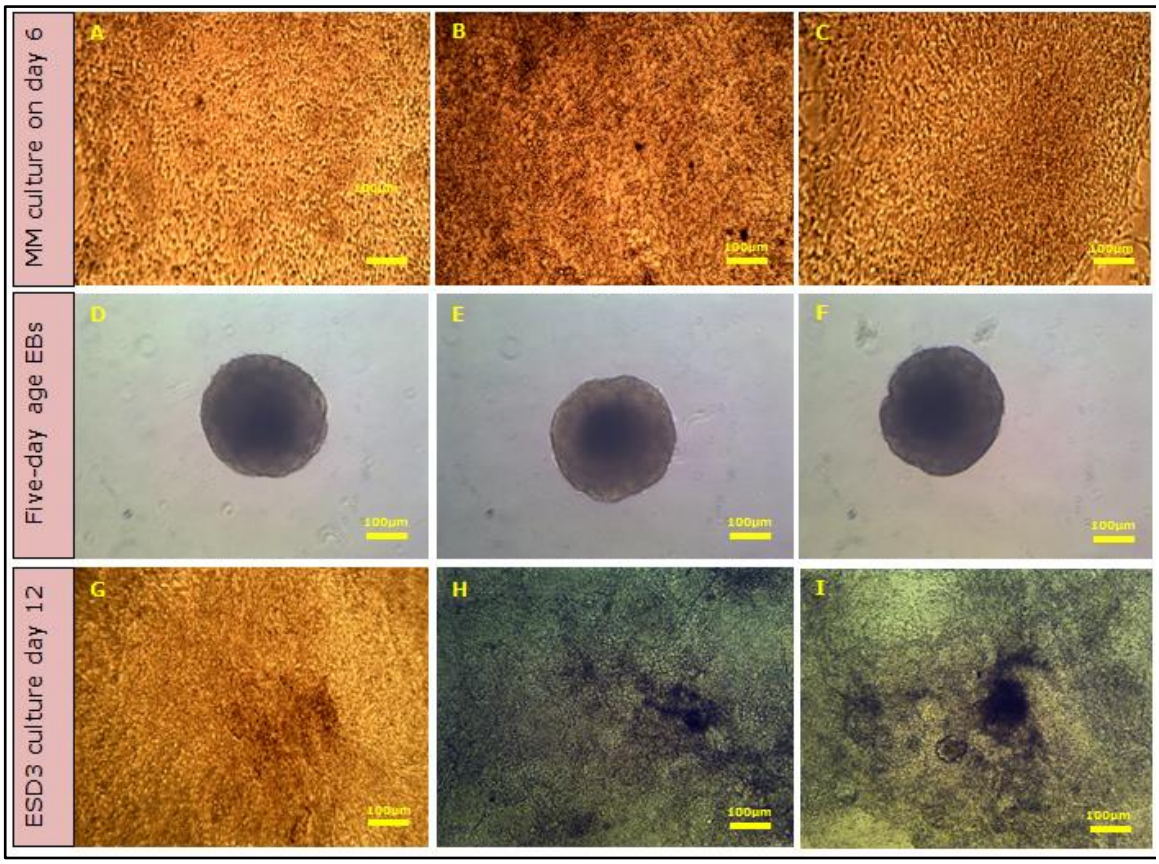


Figure 3-7: Representative light microscopy photographs of ginkgolide B effects on MM and ESD3 cultures

(A) untreated (vehicle control) cardiomyocytes at 144h after MM culturing, (B) 50nM ginkgolide B-treated cardiomyocytes at 144h after MM culturing, (C) 1000nM ginkgolide B-treated cardiomyocytes at 144h after MM culturing, (D) untreated (vehicle control) 5-day-age EB in ESD3 system, (E) 50nM ginkgolide B-treated 5-day-age EB in ESD3 system, (F) 1000nM ginkgolide B-treated 5-day-age EB in ESD3 system, (G) untreated (vehicle control) EB post-culturing on day 12 in ESD3, (H) 50nM ginkgolide B-treated EB post-culturing on day 12 in ESD3, (I) 1000nM ginkgolide B treated-EB post-culturing on day 12 in ESD3. (Scale bar 100µm) with original magnification x100 for all images.

3.4.3 Effect of ginsenoside Rg1

The effects of G-Rg1 were less pronounced in both *in vitro* systems for all endpoints compared to the other ingredients. With the selected concentrations (0.0625-10μM), only the highest two concentrations of G-Rg1 showed effects on some evaluated endpoints. In the MM system, all the G-Rg1 concentrations at 48h (24h post drug addition) do not affect the morphological score significantly ($P>0.05$) except at 10μM. After 144h of cell culture, G-Rg1 at 5 and 10μM reduced the cardiomyocyte contractile activity dramatically ($P<0.05$) (Figure 3-8A). In the ESD3 system, while 5μM G-Rg1 inhibited the morphological score significantly at day 12, 10μM reduced the morphological score significantly at day 10, 11 and 12 (Figure 3-8D). Apart from the highest tested concentration (10μM), neither the cell activity nor total protein content were affected by G-Rg1 with both primary chick embryonic cardiomyocytes Figure 3-8B and C) and ESD3 differentiated cardiomyocytes (Figure 3-8E and F). No differences were noted on cultures or EBs size (Figure 3-9E) at 0.5μM G-Rg1 whether in MM (Figure 3-9B) or ESD3 system (Figure 3-9H) when compared to their controls (Figure 3-9A, D and G). However, at 10μM G-Rg1, some MM cultures started to form folded edges in the middle of the culture with some detached cells floating around (Figure 3-9C). At 10μM G-Rg1 in ESD3 system, this caused an unusual and/or poor EBs formation on day 5 of the hang drop procedure (Figure 3-9F), when the EB was seeded for further 7 days, less spreading cells and smaller diameter culture were observed (Fig 3-9I), when compared to controls.

MM ESD3

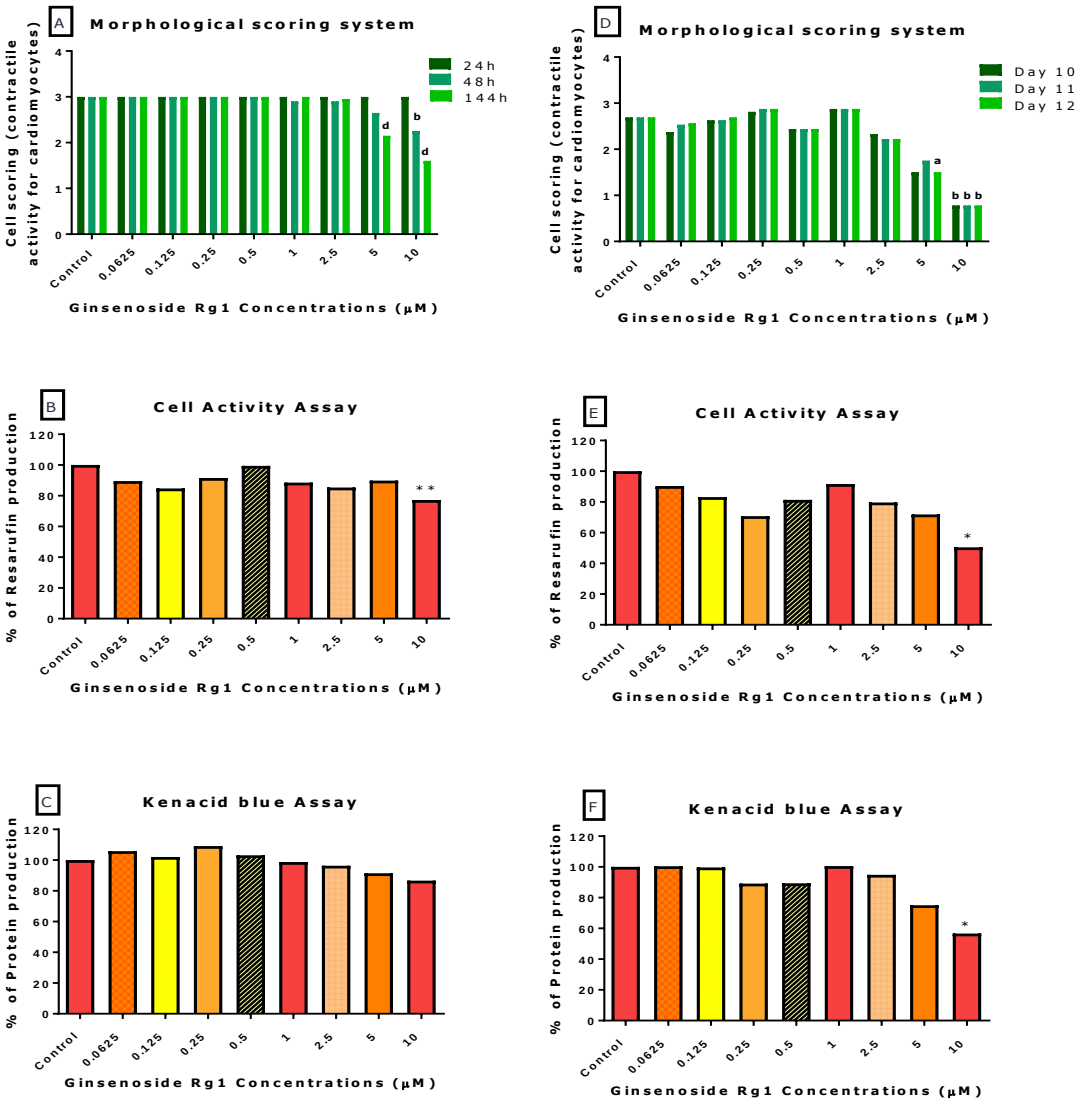


Figure 3-8: Effects of ginsenoside Rg1 on embryonic cardiomyocyte in MM and ESD3 systems

In MM, (A) effect of ginsenoside Rg1 on contractile activity, (B) effect of ginsenoside Rg1 on cell activity (resazurin assay) at 144h, and (C) effect of ginsenoside Rg1 on total protein content (kenacid blue assay) at 144h. In stem cell-derived cardiomyocytes, (D) effect of ginsenoside Rg1 on stem cell differentiation to cardiomyocytes, (E) effect of ginsenoside Rg1 on cell activity (resazurin assay) on day 12, and (F) effect of ginsenoside Rg1 on total protein content (kenacid blue assay) on day 12. Three independent repeats (n=3) of each experiment were performed. The data are presented as mean for morphological scoring and % of the mean control for resazurin and protein content. * or (a) $p<0.05$, ** or (b) $p<0.01$, *** or (c) $p<0.001$ and **** or (d) $p<0.0001$ vs. control.

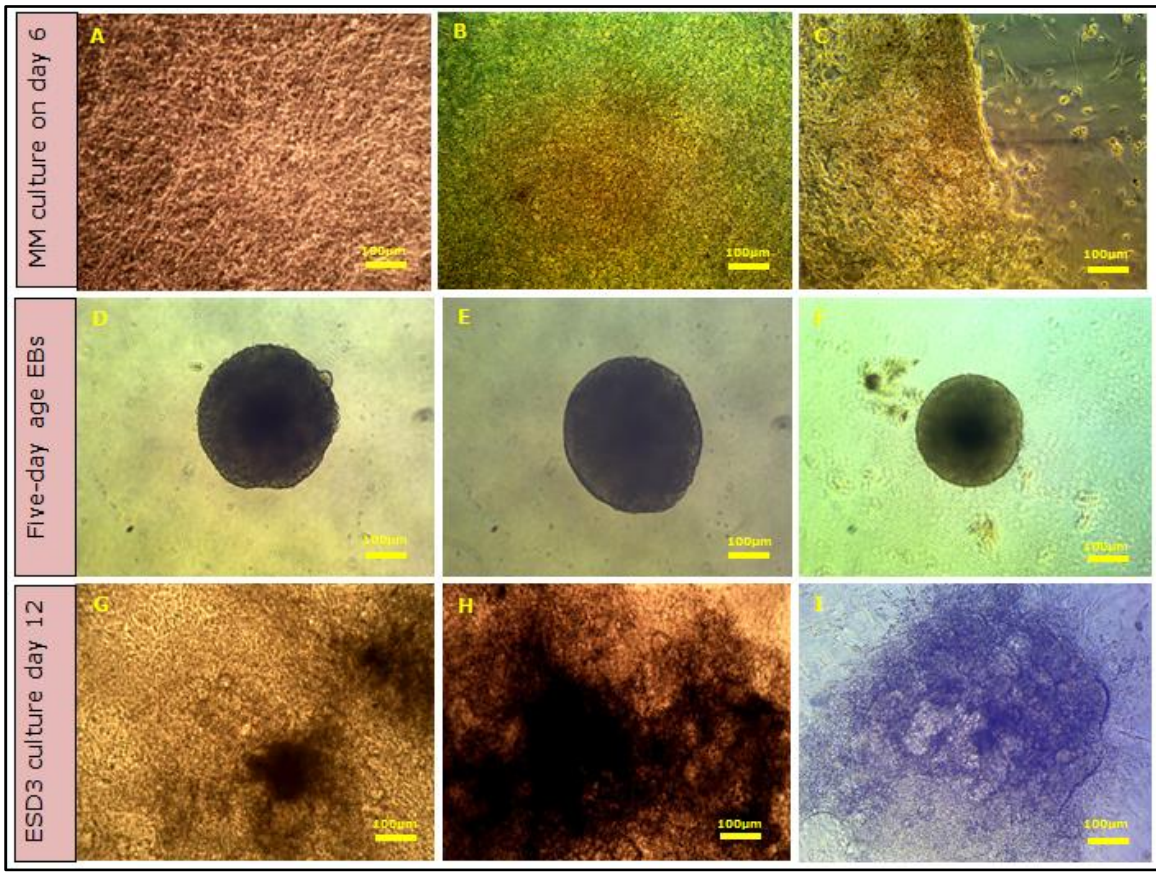


Figure 3-9: Representative light microscopy photographs of ginsenoside Rg1 effects on MM and ESD3 cultures

(A) untreated (vehicle control) cardiomyocytes at 144h after MM culturing, (B) 0.5µM ginsenoside Rg1-treated cardiomyocytes at 144h after MM culturing, (C) 10µM ginsenoside Rg-treated cardiomyocytes at 144h after MM culturing, (D) untreated (vehicle control) 5-day-age EB in ESD3 system, (E) 0.5µM ginsenoside Rg1-treated 5-day-age EB in ESD3 system, (F) 10µM ginsenoside Rg1-treated 5-day-age EB in ESD3 system, (G) untreated (vehicle control) EB post-culturing on day 12 in ESD3, (H) 0.5µM ginsenoside Rg1-treated EB post-culturing on day 12 in ESD3, (I) 10µM ginsenoside Rg-treated EB post-culturing on day 12 in ESD3. (Scale bar 100µm) with original magnification x100 for all images.

3.4.4 Effect of bisabolol

As far as the MM system is concerned, whilst there were significant ($p<0.05$) dips of contractile activity at 48h and 144h at 40 and 80μM bisabolol, there was no significant difference at 0.01-20μM bisabolol (Figure 3-10A). Bisabolol, at 0.01-20μM, exhibited no effect on the cellular activity in MM system, but it significantly affected the cell activity at 40 and 80μM (Figure 3-10B). Figure 3-10C shows that there was no significant difference in the protein content amongst primary embryonic cardiomyocytes despite the rising concentrations of bisabolol. Light microscopic images of the MM cultures showed no apparent alteration in the cell adherence, proliferation and cellular sheet forming at 1μM (Figure 3-11B) and 80μM bisabolol (Figure 3-11C) comparing to control (Figure 3-11A).

With bisabolol, when used at concentration range (0.01-80μM) in the ESD3 system, only 40 and 80μM concentrations exhibited a significant decline in contractile activity (morphological scoring) in comparison to the control (Figure 3-10D). The results of stem cell-derived cardiomyocyte activity (resazurin assay) showed a dose-dependent decline, but this was not statistically significant except at 80μM (Figure 3-10E). The analysis of total protein content data showed no significant effect in whole tested range except at 40 and 80μM bisabolol (Figure 3-10F). With regard to the effects on EBs formation, bisabolol at 80μM reduced the 5-day-old EBs size in a noticeable manner when compared to control (Figure 3-11D and F), but such effect was not observed with 1μM bisabolol-treated EBs (Figure 3-11D and E). When the same EBs were seeded in 24 well plate and checked after 7 days, 80μM bisabolol (Figure 3-11I) prevented cells being expanded to cover the same

plate area as it appeared in both 1μM bisabolol (Figure 3-11H) and control (Figure 3-11G).

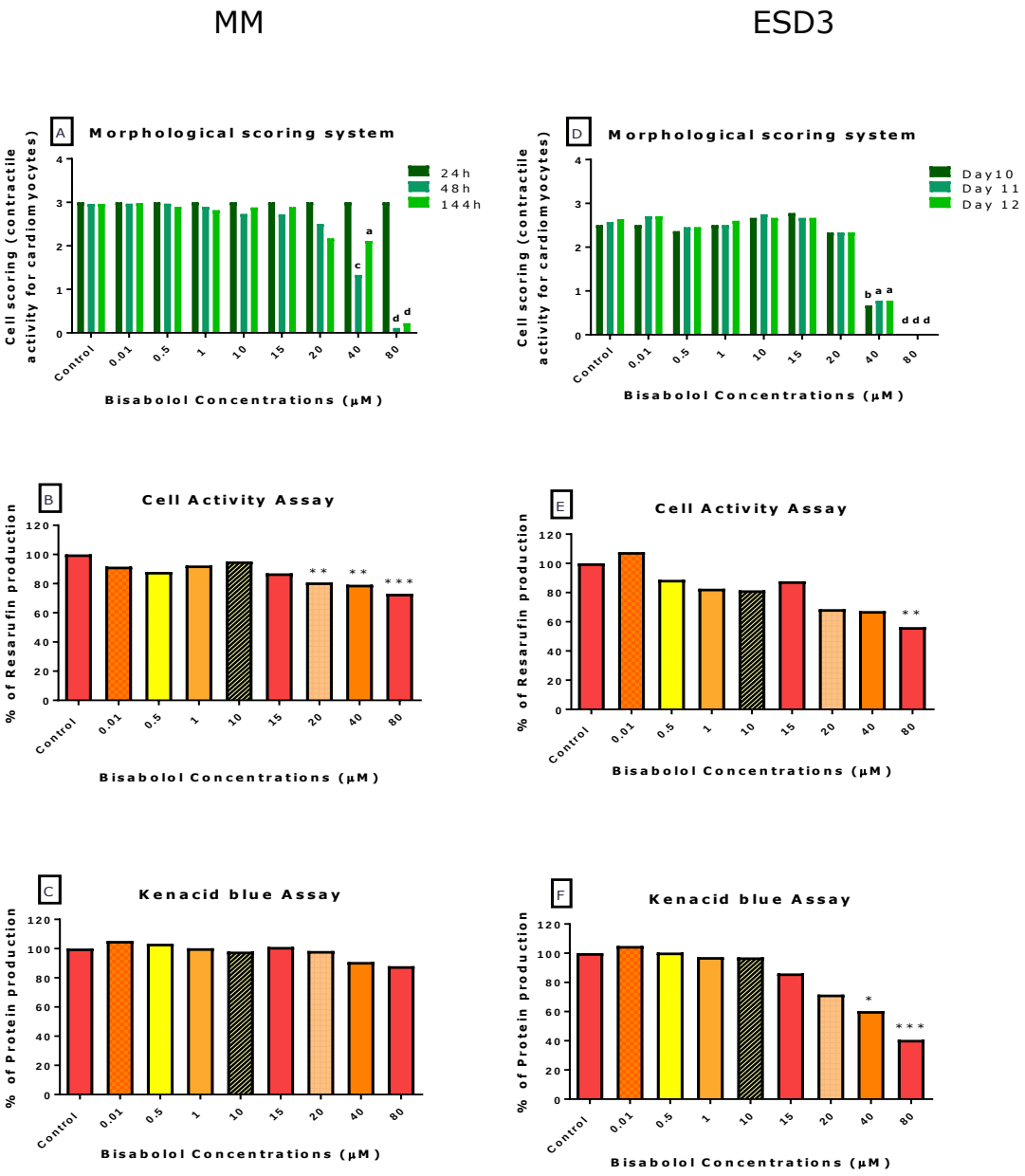


Figure 3-10: Effects of α-bisabolol on embryonic cardiomyocyte in MM and ESD3 systems

In MM, (A) effect of α-bisabolol on contractile activity, (B) effect of α-bisabolol on cell activity (resazurin assay) at 144h, and (C) effect of α-bisabolol on total protein content (kenacid blue assay) at 144h. In stem cell-derived cardiomyocytes, (D) effect of α-bisabolol on stem cell differentiation to cardiomyocytes, (E) effect of α-bisabolol on cell activity (resazurin assay) on day 12, and (F) effect of α-bisabolol on total protein content (kenacid blue assay) on day 12. Three independent repeats (n=3) of each experiment were performed. The data are presented as mean for morphological scoring and % of the mean control for resazurin and protein content. * or (a) $p<0.05$, ** or (b) $p<0.01$, *** or (c) $p<0.001$ and **** or (d) $p<0.0001$ vs. control.

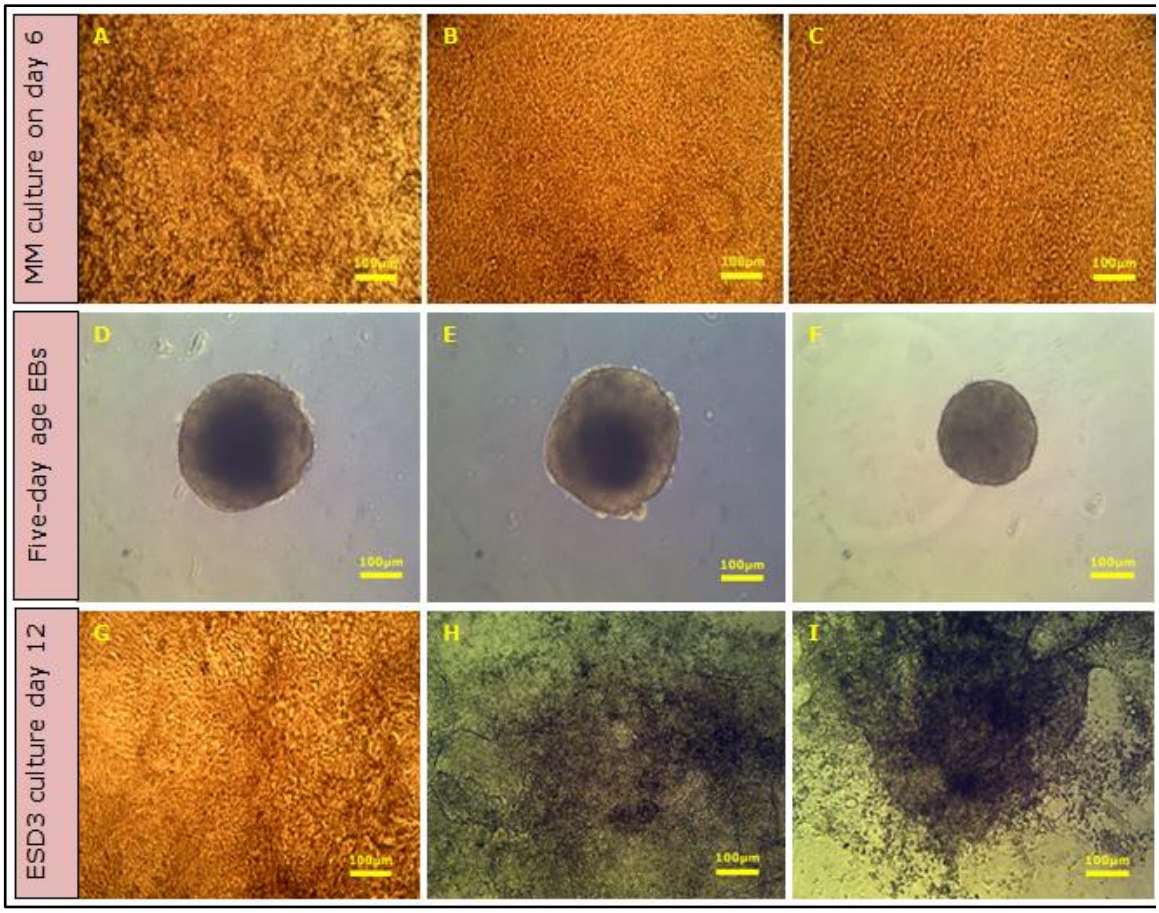


Figure 3-11: Representative light microscopy photographs of α-bisabolol effects on MM and ESD3 cultures

(A) untreated (vehicle control) cardiomyocytes at 144h after MM culturing, (B) 1µM α-bisabolol-treated cardiomyocytes at 144h after MM culturing, (C) 80µM α-bisabolol-treated cardiomyocytes at 144h after MM culturing, (D) untreated (vehicle control) 5-day-age EB in ESD3 system, (E) 1µM α-bisabolol-treated 5-day-age EB in ESD3 system, (F) 80µM α-bisabolol-treated 5-day-age EB in ESD3 system, (G) untreated (vehicle control) EB post-culturing on day 12 in ESD3, (H) 1µM α-bisabolol-treated EB post-culturing on day 12 in ESD3, (I) 80µM α-bisabolol-treated EB post-culturing on day 12 in ESD3. (Scale bar 100µm) with original magnification x100 for all images.

3.4.5 Effect of eugenol

When MM cultures were treated with eugenol, the morphological scoring showed a significant ($p<0.05$) difference at most of the high concentrations in a dose-dependent declining trend (0.5μM and 20-80μM) at 144h (Figure 3-12A). Eugenol at 40 and 80μM also showed a significant decrease at 48h as shown in Figure 3-12A. On the other hand Figure 3-12B depicts the cellular activity measurements where the cellular activity dramatically deteriorated with 15μM to 80μM eugenol. Eugenol exhibited a dose-dependent declining trend on the protein content which became significant ($p<0.05$) at 40 and 80μM (Figure 3-12C). Light microscopy images of the untreated MM culture and treated with 0.01μM eugenol at 144h were similar to normal healthy cellular sheet features (Figure 3-13A and B). The cellular sheets were severely disturbed with 80μM eugenol, forming a folded sheet and leaving a big groove in the middle of the culture (Figure 3-13C).

Eugenol at concentrations 0.01μM-20μM, when evaluated in ESD3 culture, showed no effect on the differentiation (the contractile activity) of the stem cell-derived cardiomyocytes as shown in Figure 3-12D. However, there was a significant ($p<0.05$) change at concentrations of 40 and 80μM eugenol on days 10, 11 and 12. The effects on cellular activity in the ESD3 system was more obvious in a dose-dependent manner where the concentrations (1-80μM) result in a significant ($p<0.05$) reduction in the cellular activity of the cultures as shown in Figure 3-12E. The results of protein content assay showed statistically significant ($p<0.05$) decrease only with the highest concentration (80μM) but no effect with the lower concentrations (Figure 3-12F). The size of 0.01μM eugenol treated 5-day-old EBs was slightly smaller than control;

however, with 80μM eugenol, the EBs formation was clearly affected and reduced in size (Figure 3-13D, E and F). Maintaining the same exposure environment, the same EBs were seeded for further 7 days and observed under a light microscope to monitor the adherence and proliferation. The cultured EBs of control and 0.01μM eugenol concentration have attached and spread out to form normal sheets (Figure 3-13G and H). Comparing to control, 80μM eugenol-treated EBs showed less proliferating rate after culturing and covered less area on the plate as in Figure 3-13I.

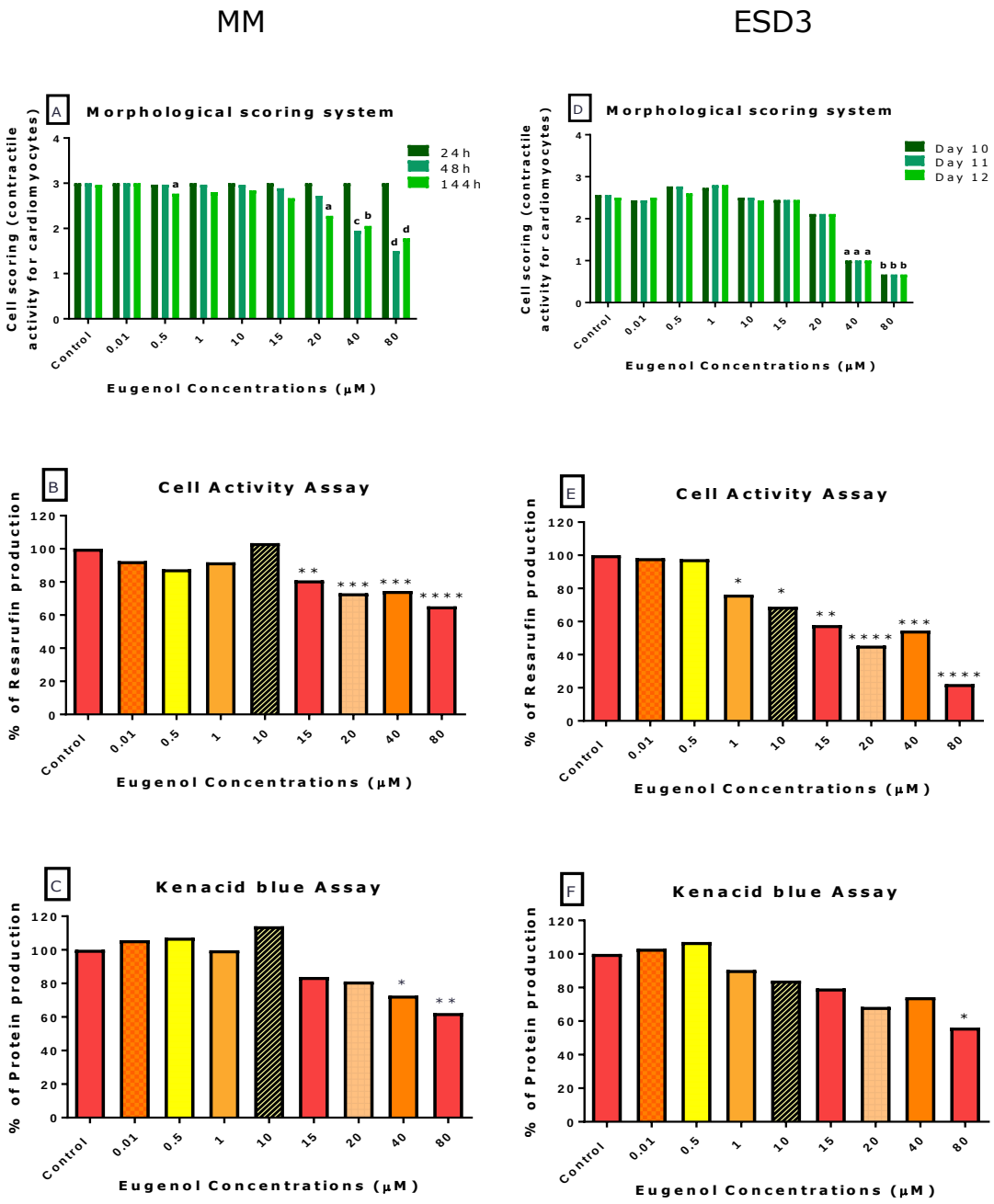


Figure 3-12: Effects of eugenol on embryonic cardiomyocyte in MM and ESD3 systems

In MM, (A) effect of eugenol on contractile activity, (B) effect of eugenol on cell activity (resazurin assay) at 144h, and (C) effect of eugenol on total protein content (kenacid blue assay) at 144h. In stem cell-derived cardiomyocytes, (D) effect of eugenol on stem cell differentiation to cardiomyocytes, (E) effect of eugenol on cell activity (resazurin assay) on day 12, and (F) effect of eugenol on total protein content (kenacid blue assay) on day 12. Three independent repeats (n=3) of each experiment were performed. The data are presented as mean for morphological scoring and % of the mean control for resazurin and protein content. * or (a) $p<0.05$, ** or (b) $p<0.01$, *** or (c) $p<0.001$ and **** or (d) $p<0.0001$ vs. control.

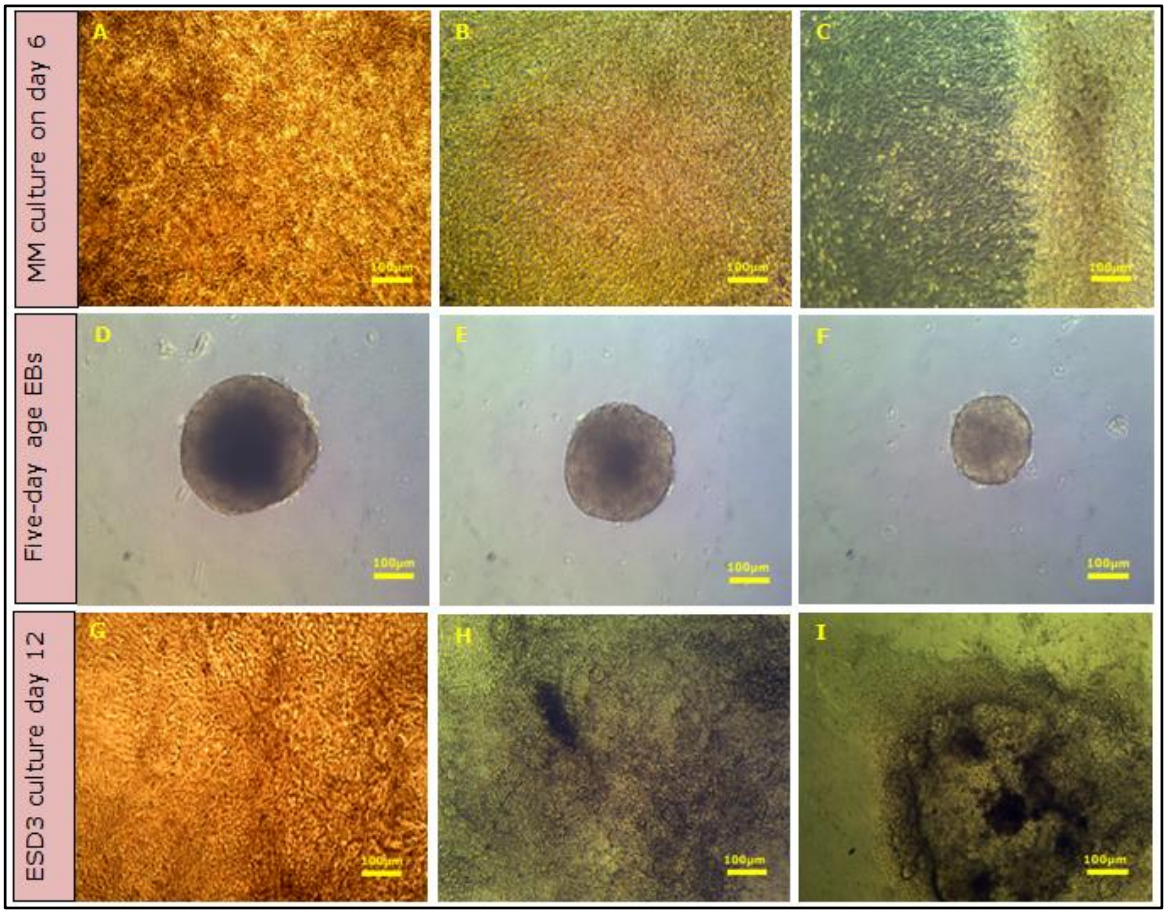


Figure 3-13: Representative light microscopy photographs of eugenol effects on MM and ESD3 cultures

(A) untreated (vehicle control) cardiomyocytes at 144h after MM culturing, (B) 0.01µM eugenol-treated cardiomyocytes at 144h after MM culturing, (C) 80µM eugenol-treated cardiomyocytes at 144h after MM culturing, (D) untreated (vehicle control) 5-day-age EB in ESD3 system, (E) 0.01µM eugenol-treated 5-day-age EB in ESD3 system, (F) 80µM eugenol-treated 5-day-age EB in ESD3 system, (G) untreated (vehicle control) EB post-culturing on day 12 in ESD3, (H) 100nM eugenol-treated EB post-culturing on day 12 in ESD3, (I) 80µM eugenol-treated EB post-culturing on day 12 in ESD3. (Scale bar 100µm) with original magnification x100 for all images.

3.5 Discussion

Herbal remedies have been credited with medicinal properties and their use advocated in pregnancy because of their apparent safety, although there is in fact little evidence to support this. However, herbal products from a legal standpoint are categorized as dietary supplements; therefore, they are exempt from legislation regarding safety testing. As a result, data regarding the safety profiles of many herbal products are very limited [334]. These two *in vitro* systems, MM and ESD3, represent different growing phases of cardiac development. The cardiomyocytes in MM culture were already differentiated at the time of heart explantation, whereas in ESD3, the stem cells undergo spontaneous differentiation into contracting cardiomyocytes. The results from the two systems are complementary as each system represents a particular lineage of cardiomyocytes. Based on that, one test chemical might show an effect on one system but not on the other, or may show effects on both.

Most of the discovered human teratogens were identified retrospectively through human epidemiologic studies after tragedies have already happened [335]. However, it is essential to predict the teratogenicity potential of new manufactured or extracted chemicals before they are introduced to the market. The two *in vitro* models that have been used herein, allowed the measurement of the effect of tested herbal components on contractile activity (representing the cardiac cell differentiation), cell activity and total protein content (representing cell number and cell proliferation). In addition, EBs size/formation was also monitored in order to enhance the versatility of ESD3 system to identify various aspects of developmental effect for the tested

phytochemicals. The importance of studying the EBs morphogenesis has been published by Warkus *et al*. in 2016 [336]. 16 contraindicated drugs (FDA category X) and 4 safe drugs (FDA category A) have been employed in that exploratory study using P19C5 mouse embryonal carcinoma stem cells. After 4 days of exposed hanging drops, the size and shape of formed EBs were measured morphometrically. It was concluded that such models could be potentially effective as an alternative method in the field of embryotoxicology [336].

3.5.1 [6]-Gingerol in MM and ESD3

Through various studies that have been performed to investigate the action of gingerol, it has been shown that 6G has various pharmacological properties, such as analgesic, anti-inflammatory, antitumor, antiemetic, antipyretic, and anti-diabetic effects [337,338]. The antiemetic action of 6G has promoted ginger as a medication for morning sickness. The results here with primary chick embryonic cardiomyocytes and stem cell-derived cardiomyocytes showed that 6G at a concentration range (12.5-100μM) exhibits teratogenicity and cytotoxicity potential. In recent studies, it was found that 6G has an ability to invoke apoptotic mediators such as caspase 3, within the mitochondria, and can disturb the membrane potential and the electron transport chain, creating a high amount of ROS that eventually has effects on DNA in different tumour cell lines [294,295,339]. These could be the possible embryotoxicity mechanisms for gingerol in this study. In contrast, a recent report has shown protective features of 6G through inhibiting reactive oxygen species [340]. However, the concentrations of 6G might be the factor that governs its safety status. The results of 6G at lower concentrations on the growing

cardiomyocytes in the MM culture showed no abnormal effects. On the other hand, 6G prevented the differentiation of embryoid bodies into cardiomyocytes. It has also been reported that 6G can prevent myofibroblast differentiation [341], which might explain the results in the ESD3 system since it is also a differentiating cell population. The study reported herein also shows that 6G could be considered a cytotoxic teratogen for growing fetal cardiomyocytes particularly at high levels and for stem cell differentiation at any level.

Weidner and Sigwart (2000) have investigated the embryotoxicity of patented ginger extract (EV.EXT 33) on rats. The extract was administered at concentrations of 100, 333 and 1000 mg/kg orally. It was concluded that the ginger preparation was well tolerated [342]. In contrast to the previous study, ginger tea (15 g/l, 20 g/l or 50 g/l) was given in the drinking bottles of pregnant Sprague–Dawley rats. Although no gross morphologic malformations in the treated fetuses and no maternal toxicity were observed, embryonic loss in the treatment groups was twofold as that of the controls. The researchers of this study suggested that *in utero* exposure to ginger tea results in increased early embryo loss [343].

This would indicate that 6G might not be safe for the embryo during the early stages of pregnancy, mainly in the first trimester during the period of organogenesis. Further studies are required to determine the teratogenic potential of ginger (or one or more of its constituents) and to establish what, if any, adverse effects are observed, see Section 3.6.

3.5.2 Ginkgolides in MM and ESD3

Ginkgo biloba is one of the most commonly used herbal supplements. The standardized extract of the leaves (commonly known as EGb 761) is known to play a role in a free radical scavenging and antioxidant processes [344]. The protective effect of GA and GB was demonstrated by their ability to block apoptosis pathways in different studies which show their antioxidant activity [345,346]. However, the effect of GB on mouse oocyte maturation and subsequent pre and post implantation development, both *in vitro* and *in vivo*, has been investigated. At the concentration range 1, 3 and 6μM, the results showed that consumption of 3-6μM GB with drinking water has negative impacts upon mouse oocyte maturation and fertilization, as well as *in vitro* embryonic development, showing inhibition of the development of fertilised oocytes to blastocysts [303]. Interestingly, two concentrations, 5 and 10μM, of both GA and GB were tested on the blastocyst stage of mouse embryos and on subsequent early post-implantation embryonic development *in vitro*. Both GA and GB brought about increased apoptosis and a reduction in the average number of total cells in the blastocyst in treatment groups versus control. Further, exposed blastocysts died before reaching the final stages of development in culture dishes [300]. The apoptosis mechanism of GB was studied, and it is thought to induce caspase 3 by activation of protein kinase JNK in mouse embryonic stem cells [347,348]. In addition, the effect of Ginkgo biloba extract was examined by Pinto and colleagues (2007) in pregnant Wistar rats. It was concluded that ginkgo biloba extract resulted in fetal intrauterine growth retardation as it reduced the total fetal body weight, although it was nontoxic for the mothers [349]. The results reported herein show that GA

mainly interfered with the contractile activity of cardiomyocytes whether growing embryonic cells or stem cell-derived cardiomyocytes, particularly at high concentrations. In contrast, GB tended to disrupt the growing cardiomyocytes rather than inhibiting the stem cells differentiation pathways. High levels of these two ingredients could have interfered with some mitochondrial processes, disturbing embryonic cardiomyocyte communication. There were some differential effects of both GA and GB between the two experimental systems, but the fact that the stem cell system contains different cell types gives a possible explanation for this phenomenon. It is possible that GB at very low concentration hinders stem cells from being differentiated to other cell types and as a result, the culture will contain fewer cell numbers with a majority of cardiomyocytes, this being reflected as low cellular activity and low protein content compared to the control. To conclude, GA primarily suppresses stem cell differentiation and growing embryonic heart cells at high concentrations. GB has no detrimental effect on the stem cell differentiation process at any concentration, but at high concentrations, GB interrupts the developing chick cardiomyocytes. Study the effects of GA and GB combination can enrich the safety profile of these phytochemicals. However, our study only concentrated on the effects of each phytochemical separately where some pharmaceutical companies marketed them as isolated products such as Ginkgo B® 60mg capsules.

3.5.3 Ginsenoside Rg1 in MM and ESD3

According to Chinese traditional medicine, the Panax ginseng C.A. Meyer (Araliaceae) is a general 'tonic' herb and a cumulative effect is believed to occur if the herb is taken regularly. It is also prescribed for depression for its

neuroprotective actions [350-352,110]. Ginseng is widely used among pregnant women, but there is little awareness of possible embryotoxicity [311]. Despite its varied usage, there have been very few investigations carried out to study its effects on embryonic development [316,353]. The results reported here for G-Rg1, the main active constituent of Ginseng, show a high safety profile in both methods. G-Rg1 was only teratogenic at the highest tested concentrations for embryonic cardiomyocytes and stem cell-derived cardiomyocytes. G-Rg1 has been investigated using the whole embryo culture technique for both mice and rats and the study exhibited embryotoxic effects for different organs in mice and rats (including rat heart) [316]. However, the concentrations in that study (50µg/ml = 62.4 mM to mice and 30µg/ml = 37.4mM to rats) are much higher than the levels used in this study (0.0625-10µM). On the other hand, another study revealed that when Korean red ginseng extract was given to mice before and during their gestation, normal offspring were obtained even when they were exposed to the extract during the organogenesis period [353]. It might be assumed that G-Rg1 has a high potential safety with respect to induction of embryonic teratogenicity as long as low to moderate levels are maintained.

3.5.4 α-Bisabolol in MM and ESD3

Chamomile possesses anti-stress characteristics that potentially may be of value for pregnant women, especially in unplanned pregnancy. Little is known regarding its embryotoxicity profile. However, emerging studies have shown some toxicity features for its active components, such as bisabolol-induced proapoptotic characteristics [354,355]. Therefore, it is important to study possible harmful effects of these herbs on embryonic development.

Experiments on bisabolol, especially during pregnancy, are very limited. Nevertheless, several reports mentioned bisabolol – which forms up to 50% of the essential oil, which in turn forms up to 2% of the whole herb [356] – has displayed pro-apoptotic effects in glioma cells [357], human acute leukaemia cells [355,358] and breast cancers [359].

In the MM system with increasing concentration, bisabolol affected the cardiomyocyte ability to reestablish the beating system. The cells proliferate and their ability to form normal flat sheeted-cultures like the control was maintained, and this was evident by normal cellular protein content over the entire tested range (0.01-80μM). However, beating foci (morphological scoring system) was suppressed at high bisabolol concentrations (40 and 80μM) in the MM culture system. This might be associated with activity cell disturbances showed at high bisabolol concentrations (20-80μM). Regarding our bisabolol observations in the ESD3 system, (0.01-20μM) concentrations did not reveal any effects on all endpoints. Bisabolol high concentration (80μM) dramatically decreased cell activity which probably results in cell death being reflected by the small EBs size, and by decreased total cellular protein. Consequently, 80μM bisabolol completely stopped the stem cells being differentiated to cardiomyocytes with limited ability for spreading out over the plate. In this study, both the ESD3 and MM systems have shown bisabolol as non-teratogenic at low to moderate concentrations (0.01-20μM), but teratogenicity and cytotoxicity with extreme suppression of cellular differentiation occur at high concentrations (40 and 80μM).

Two similarly designed studies were performed on pregnant rats and New Zealand rabbits. Animals received bisabolol (98% pure) daily by stomach tube

at doses of 0.25, 0.50, 1.0, and 3.0 ml/kg body weight, on days 6–15 of gestation. In order to link up these doses (0.25-3.0 ml/kg) with our study concentrations (0.01-80μM) and human plasma levels, we extrapolate them as follows: the 98% of highest dose (3.0 ml/kg) is equivalent to 2.94mg/kg, so the human dose corresponding to this is 0.47mg/kg (using 6.2 rat factor) and 0.94mg/kg (using 3.1 rabbit factor). This was done using the dose conversion table [331]. Performing the same calculations on Sections 3.3.1.1 and 3.3.1.2, therefore, the rat doses in the study might correspond to 29.5 μM in human plasma and the rabbit doses could reach up to 59.18 μM when we extrapolate these doses to 70kg pregnant woman. In the studies, fetuses were removed and examined on day 20 and 30 for rats and rabbits respectively. Similar findings were revealed from both studies, where no effect on embryonic development was observed at doses up to 1.0 ml/kg. A significant reduction in fetal number was observed in both studies at the 3.0 ml/kg group. No deformities were found for any dose in both studies [360]. With these observations, the researchers concluded the lowest toxic oral dose is between 1.0 and 3.0 ml/kg body weight. However, studies on bisabolol have been controversial. Some reports have shown bisabolol is toxic at 53-73μM by inducing apoptosis through targeting several factors within tumor cells (glioma cells) such as plasma membrane integrity and the mitochondrial permeability transition pore, and via disruption of mitochondrial potential, inhibition of oxygen consumption and an increase of intracellular reactive oxygen species [358,357]. Others have shown 100-200mg/kg bisabolol to be a protective molecule against ethanol-induced gastric lesions in male Swiss mice [361]. The results indicated that protection characteristics of bisabolol were via decreasing

oxidative stress and inflammatory events associated with the lesions induced by ethanol but also by showing the ability to raise superoxide dismutase (SOD) levels [361]. The study reported here is probably the first to evaluate the direct effect of bisabolol on embryonic cardiomyocyte differentiation and development.

To sum up, the overall result encourages a careful approach to the use of chamomile, with low doses advisable for pregnant women who feel treatment with chamomile may be beneficial for their pregnancy related symptoms. Further research on chamomile and its bioactive components are essential as without such evidence, see Section 3.6; it remains uncertain whether these untested and undocumented herbal components are truly safe for fetus/embryo despite the fact that currently chamomile is listed on the FDA's GRAS (generally recognized as safe) list [318].

3.5.5 Eugenol in MM and ESD3

Eugenol is a chemical that has not been widely studied, especially the direct effect of oral intake of eugenol during pregnancy on embryonic development. However, there is growing evidence that eugenol has demonstrated anti-cancer activity via triggering apoptosis and/or by showing an anti-angiogenic effect, specifically in breast and colon cancers [362-364].

When tested in the MM system, eugenol exhibited less teratogenic features on the differentiated/developed growing cardiomyocytes. At the low concentrations (0.01-10μM), eugenol showed no effects on contractile activity, cell activity and protein content. However, at the higher concentrations (20-80μM), eugenol interfered with re-differentiation process of cardiomyocytes by preventing the cultured cardiomyocytes from producing flat sheets as in

controls; instead, folded and compromised sheets were observed. This significantly lowered the morphological score (contractile activity) via reducing the total foci number in the culture. The same cells showed significant changes when they were tested, for cell activity at 15-80μM and total protein content at 40-80μM. Nevertheless, the overall data from ESD3 and MM chick embryonic cardiomyocytes hints at eugenol being potentially teratogenic at moderate to high concentrations by affecting various end points. In contrast to these findings, Amini *et al*. (2003) tested 10mg/kg bodyweight (equivalent to 56.9 mg for 70 kg pregnant woman –mouse converting factor is 12.3 [331]- which is equivalent to 225nM blood level) oral intake of eugenol in pregnant mice to observe effects on the embryo. The results showed eugenol as non-teratogenic and have shown significant protective effects towards retinoic acid teratogenicity in pregnant mice [365]. On the other hand, a study has tested the effect of essential oils from the herb, containing high levels of eugenol, on the growth and development of preimplantation mouse embryos *in vivo*. The results here showed that essential oils containing eugenol relate to the higher incidence of cell death [366]. Another study on Syrian hamster embryo cells indicated eugenol has cytotoxicity potential by inducing chromosome aberration at a concentration of 195μM [354]. Al-Sharif *et al*. (2013) have shown eugenol can also trigger a proapoptotic pathway, in human breast tumor cells. This has been demonstrated to be mediated mainly by induction of the internal apoptotic pathway and down-regulation of E2F1 and its antiapoptosis target survivin, both *in vivo* and *in vitro* using 2μM eugenol [367]. To the best of our knowledge, our study is the first report to investigate the effect of eugenol directly on embryonic cardiomyocytes; therefore, there is no available

comparable data. Furthermore, in this study, eugenol showed several effects upon ESD3 in all endpoints, with increasing concentrations of eugenol, the stem cells' ability to differentiate into cardiomyocytes was compromised, and this was significant at 40 and 80μM. Eugenol also showed cell activity decreases, which represents cell oxidoreductase disturbances; these were accompanied by cytotoxic consequences (total protein content) only at the highest concentration (80μM). At a low concentration (0.01μM), eugenol showed EBs slightly reduced in size, but this was not reflected in the differentiation process. When EBs were cultured at 0.01μM, a normal expansion (proliferation) was observed with an intact differentiation process, probably because the damage intensity produced by this eugenol concentration has been counteracted by cell defense mechanisms. Conversely, reductions in cell activity and protein content, at the highest eugenol test concentrations (80μM), were sufficient to be suggested explanations behind the severe reduction in EBs size/formation. This decrease was further noticed by a limited expansion on the plate after culture and a serious deterioration in foci formation (contractile activity).

3.6 Conclusion

These data could give a new insight concerning the safety of the major components of certain herbal extracts. Whilst they may be 'beneficial' for expectant mothers, they should also be 'safe' for fetuses. Contrary to the mother's perceptions and beliefs regarding herbal medicines, consuming certain herbal remedies, particularly at high doses, to treat or ease some pregnancy-induced conditions through the first trimester of pregnancy might

not be safe for fetal differentiation, development and growth. This study is possibly a step forward to negate the general view of most pregnant women and health advisors that 'herbs should be "safe" because they are "natural"'. With more experimentation and knowledge about how the action of active components of herbs is mediated, it is possible to educate and inform potential mothers on the usefulness of herbal remedies taken alongside modern medical interventions. Should modern doctors understand more about herbal remedies and usage, it could improve the doctor-patient relationship, since the patient can feel at ease to discuss what they are taking at home, which can only improve and enhance health services overall. Therefore, in the next Chapters, the role of some important molecular targets (ROS production, DNA damage, transmembrane proteins and cell migration) in teratogenicity-induced or embryotoxicity-induced phytochemical concentrations, would be evaluated.

However, the results obtained from this Chapter could be more interesting if the actual concentrations of the tested phytochemicals in the culture media have been measured. This would also imply whether or not the tested chemicals are actually stable for certain interval. Unfortunately, this was not feasible in our project for limited resources and time. More experiments could be performed in the future by adding the tested phytochemicals later on to the cultures to study the effects at advanced stages of growing. It would also have been interesting to know whether these effects, showed in this Chapter, are permanent or temporary by treating the cultures briefly for short period of time followed by chemical-free cell growing. All these ideas could have been enhanced the current data obtained from this Chapter.

CHAPTER 4

4 Chapter four - Evaluation of cytoprotective features of herbal extracts

4.1 Introduction

For eras, different parts of herbs such as seeds, roots, stems and leaves have been used in different parts of the world for the treatment of certain ailments, to maintain healthy body balance and to improve lifestyle [368]. Traditional Chinese medicines, 2500 years ago, relied on the 'Yin-Yang theory' which expresses two contradictory qualities of matter. Rationally, anti-oxidation processes pertain to yin and pro-oxidation processes belong to yang [369]. The main protective property of many phytochemicals was attributed to antioxidant activity to protect cells against free radicals-induced oxidative damage. Quercetin is a well-known example of a phytochemical with strong antioxidant activity which belongs to a group of polyphenolic substances termed as flavonoids [370]. In a recent study, 13 herbal extracts alleged to have anti-ageing properties were investigated against hydrogen peroxide for their antioxidant characteristic as a main modulator of ageing using human astrocytes. The phenol content of each extract was strongly correlated with its antioxidant property. However, the free radical scavenger capacity and phenol group content were only moderately correlated with the cytoprotection, indicating the contribution of multiple protection mechanisms of the tested herbs [371]. In addition, five more herbs (Mangifera indica, Camellia sinensis, Phyllanthus emblica, Punica granatum and Acacia catechu), shown to have prevalent pharmacological applications against a variety of diseases such as

diabetes, cancer, hepatic disorders, and oxidative stress, were tested against *tert*-butyl hydroperoxide (*t*-BH) induced hepatotoxicity. The results demonstrated radical scavenging potential for these herbal extracts and protection of hepatocytes from *t*-BH harmful effects [372].

4.1.1 Cytoprotective properties of tested herbal components

4.1.1.1 Cytoprotective properties of ginger

Several scientific reports have convincingly demonstrated that ginger provides various therapeutic benefits such as anticancer, antiemetic, anti-infectious diseases, hepatoprotective, hypoglycemic, antihypertensive, gastroprotective, cardioprotective, chemopreventive and immune- modulatory activities [373-375,282]. Two studies on rats [376] and mice [377] have shown that administration ginger extract for a few weeks improved antioxidant capacity (SOD, Glutathione peroxidase and Glutathione) and reduced ethanol-induced hepatotoxicity. Abdel-Azeem *et al.* (2013) also showed that ginger (100mg/ml) was beneficial in reducing acetaminophen-induced hepatotoxicity in rats particularly the necrosis and vacuolisation of exposed cells [378]. It has been established that gingerols and shogaols are responsible for suppression of free radical-induced oxidative stress and various protective characteristics and pharmacological actions of ginger have been attributed to them [282].

4.1.1.2 Cytoprotective properties of ginkgo biloba

Extract of ginkgo biloba leaves has been utilised remedially for centuries in traditional Chinese medicine to treat bronchitis and asthma, but also in Western societies to improve blood circulation and as a cardiovascular protective agent [379]. Several reports *in vivo* or *in vitro* have established the free radical scavenging potential for ginkgo biloba extract [380,381]. As an

example of the cytoprotective capacity of this extract, a study showed that acetic acid-induced duodenal ulcer in rats was dramatically counteracted by administration of ginkgo biloba extract [382]. It was concluded that the antioxidant (by increasing the activity of SOD) and cytoprotective capacity of the extract prevented ulcer formation [382]. The protective activity of ginkgo biloba extract was mainly attributed to its active constituents, namely, terpenoids (ginkgolides and bilobalides) and flavonoids (ginkgoflavone glycosides) [383].

4.1.1.3 Cytoprotective properties of ginseng

As an herbal medicinal product, ginseng has gained great popularity around the world for thousands of years as a remedy to treat some common health problems. In the last two decades, extensive literature regarding its functional ingredients, ginsenosides, and their pharmacological actions have been generated but more studies are required to understand their complex activities. With respect to protective effects of ginseng, a study showed that rats pre-treated with American ginseng demonstrated less changes in the histological and biochemical gastric mucosa induced by ethanol in comparison to the controls [384]. This protection was associated with elevation in the expression of anti-apoptotic proteins Bcl-xL, Bcl-2 and p-Bad and reduction in the levels of pro-apoptotic proteins cytochrome C, tBid and both caspases-9 and-3, in addition to pro-inflammatory mediators, interleukin 1β and cyclooxygenase 2 [384]. Yeo *et al.* (2008) found that the protective effect of ginseng against ethanol-induced ulceration was attributed to the induction of cytoprotective heat-shock proteins HSP27 and HSP70 [385].

4.1.1.4 Cytoprotective properties of Chamomile

Chamomile is one of the oldest and best-documented folk herbs which has been recommended for a variety of healing applications because of its multiple biological and pharmacological activities [318]. It has been established that terpenoids and flavonoids are behind the diversity of activities of chamomile. One of the main active constituents is the terpenoid α-bisabolol [318]. In *in vitro* studies, chamomile has been shown to modify the levels of many pro-inflammatory mediators such as TNF, interleukins, superoxide anion and prostaglandins as a consequence of targeting cyclooxygenase-2 [386-388]. In a recent study, Bhaskaran *et al.* (2012) reported that chamomile can protect murine macrophages from the cytotoxic effect of H_2O_2 in a dose-dependent manner by increasing the expression of antioxidant enzymes heme peroxiredoxin-1, oxygenase-1 and thioredoxin-1 [389]. The authors found that nuclear translocation of phosphorylated Nrf2 protein might occur, stimulating the antioxidant pathway elements in the nucleus [389].

4.1.1.5 Cytoprotective properties of holy basil

Holy basil, Ocimum sanctum, has versatile medicinal properties and has been used by several ancient societies [321]. The medicinal actions of the herb, such as anti-stress, hypoglycaemic and anti-lipidemic, were mainly credited to eugenol as an active constituent, alongside flavonoids and polysaccharides [390-392]. In a recent report, holy basil demonstrated protective capacity against paracetamol-induced liver damage in rats. It also showed synergism with silymarin for hepatoprotective activity [393]. Another study on rabbits found that holy basil significantly increased levels of some antioxidant enzymes such as catalase and SOD in an herb-exposed group compared to control

[394]. The gastroprotective properties of this herb were also established against cold stress, ethanol and pyloric ligation- induced gastric ulcers with optimal effective dose was 100mg/kg [113]. The inhibition of lipid peroxidation and increased life span of mucosal cells were among the protective mechanisms against the gastric ulcers [113].

4.1.2 Chapter objective

Herein, two models were used MM and ESD3 to investigate the possible cytoprotective capacity of each phytochemical against some well-documented teratogens (ETH, RA, VPA). The potential effects were investigated by monitoring the contractile activity, cell activity and protein content in both systems.

4.2 Materials and methods

All materials used in this Chapter are already described in the previous Chapters. The chemicals were dissolved freshly in culture media from their stocks to prepare the selected concentrations. Only one non-teratogenic concentration per phytochemical was selected depending on the results in Chapter 3 in both systems. The selection of teratogen concentration was based on the results in Chapter 2, see Table 4-1 for more details. Each experiment contained blank, untreated control, tested phytochemical, tested teratogen and tested phytochemical plus tested teratogen.

4.2.1 Primary chick heart micromass (MM) preparation

Primary chick embryonic cardiomyocytes were extracted, cultured and treated with the selected chemicals. The details of the procedure are available in Section 2.2.1.

4.2.2 Mouse embryonic D3 stem cells - derived cardiomyocytes

Mouse embryonic stem cells were differentiated to cardiomyocytes using hanging drop technique and treated with the selected chemicals. The details of the procedure are available in Section 2.2.2.

4.2.3 Cardiomyocyte contractile activity scoring system

The contractile activity was monitored using manual scoring system in MM and ESD3. See Section 2.2.3.1 and Table 2-1. The morphological scores recorded from the experiments in each group were pooled and the mean of each group was converted into (%) in relation to the mean of the control while the mean of the control considered as 100%.

4.2.4 Resazurin reduction assay (cell activity assay)

The cell activity in both systems were examined by measuring the ability of mitochondrial oxidoreductase enzymes to convert resazurin to resorufin. See Section 2.2.3.2.

4.2.5 Kenacid blue assay (total protein assay)

The total protein expression was measured using kenacid staining test. See Section 2.2.3.4.

Micromass		Mouse embryonic D3 stem cells	
Herbal component	**Teratogens**	**Herbal component**	**Teratogens**
[6]-Gingerol (3μM)	ETH (75μl/ml)	[6]-Gingerol (0.75μM)	ETH (12.5μl/ml)
	RA (20μM)		RA (5μM)
	VPA (1500μM)		VPA (750μM)
Ginkgolide A (100nM)	ETH (75μl/ml)	Ginkgolide A (100nM)	ETH (12.5μl/ml)
	RA (20μM)		RA (5μM)
	VPA (1500μM)		VPA (750μM)
Ginkgolide B (50nM)	ETH (75μl/ml)	Ginkgolide B (50nM)	ETH (12.5μl/ml)
	RA (20μM)		RA (5μM)
	VPA (1500μM)		VPA (750μM)
Ginsenoside Rg1 (0.5μM)	ETH (75μl/ml)	Ginsenoside Rg1 (0.5μM)	ETH (12.5μl/ml)
	RA (20μM)		RA (5μM)
	VPA (1500μM)		VPA (750μM)
α-Bisabolol (1μM)	ETH (75μl/ml)	α-Bisabolol (1μM)	ETH (12.5μl/ml)
	RA (20μM)		RA (5μM)
	VPA (1500μM)		VPA (750μM)
Eugenol (0.01μM)	ETH (75μl/ml)	Eugenol (0.01μM)	ETH (12.5μl/ml)
	RA (20μM)		RA (5μM)
	VPA (1500μM)		VPA (750μM)
Folic acid (375μM)	ETH (75μl/ml)	Folic acid (375μM)	ETH (12.5μl/ml)
	RA (20μM)		RA (5μM)
	VPA (1500μM)		VPA (750μM)
Ascorbic acid (100μM)	ETH (75μl/ml)	Ascorbic acid (100μM)	ETH (12.5μl/ml)
	RA (20μM)		RA (5μM)
	VPA (1500μM)		VPA (750μM)
Superoxide dismutase (1μM)	ETH (75μl/ml)	Superoxide dismutase (1μM)	ETH (12.5μl/ml)
	RA (20μM)		RA (5μM)
	VPA (1500μM)		VPA (750μM)

Table 4-1: Concentrations of herbal components, standard protective substances and standard teratogens to study potential cytoprotective capacity

4.3 Results

4.3.1 Protective effect of herbal components and standard protective molecules against ethanol

❖ In MM system

Ethanol at concentration 75µl/ml caused cessation in beating of cultured chick embryonic cardiomyocytes after 24h of treatment. However, some beating recovery has been detected in the ETH alone-treated group after 144h of treatment (Figure 4-1A). The cell activity and protein content was significantly reduced compared to untreated control. Although there were some improvements in the contractile activity for the **s**tandard **p**rotective **m**olecules (SPM) -FA, AA and SOD- and all tested phytochemicals, except for GB and bisabolol, when they were mixed with ETH, the difference was not significant in comparison with the ETH alone-treated group (Figure 4-1A). All SPM and phytochemicals, except GB and bisabolol, significantly increased the cell activity of the ETH-treated group (Figure 4-1B). However, the protein content of the ETH-treated group was significantly increased after co-treatment with all SPM, 6G, GA and eugenol (Figure 4-1C). There was no significant difference for protective molecules alone in comparison to the control for all endpoints, (results not shown).

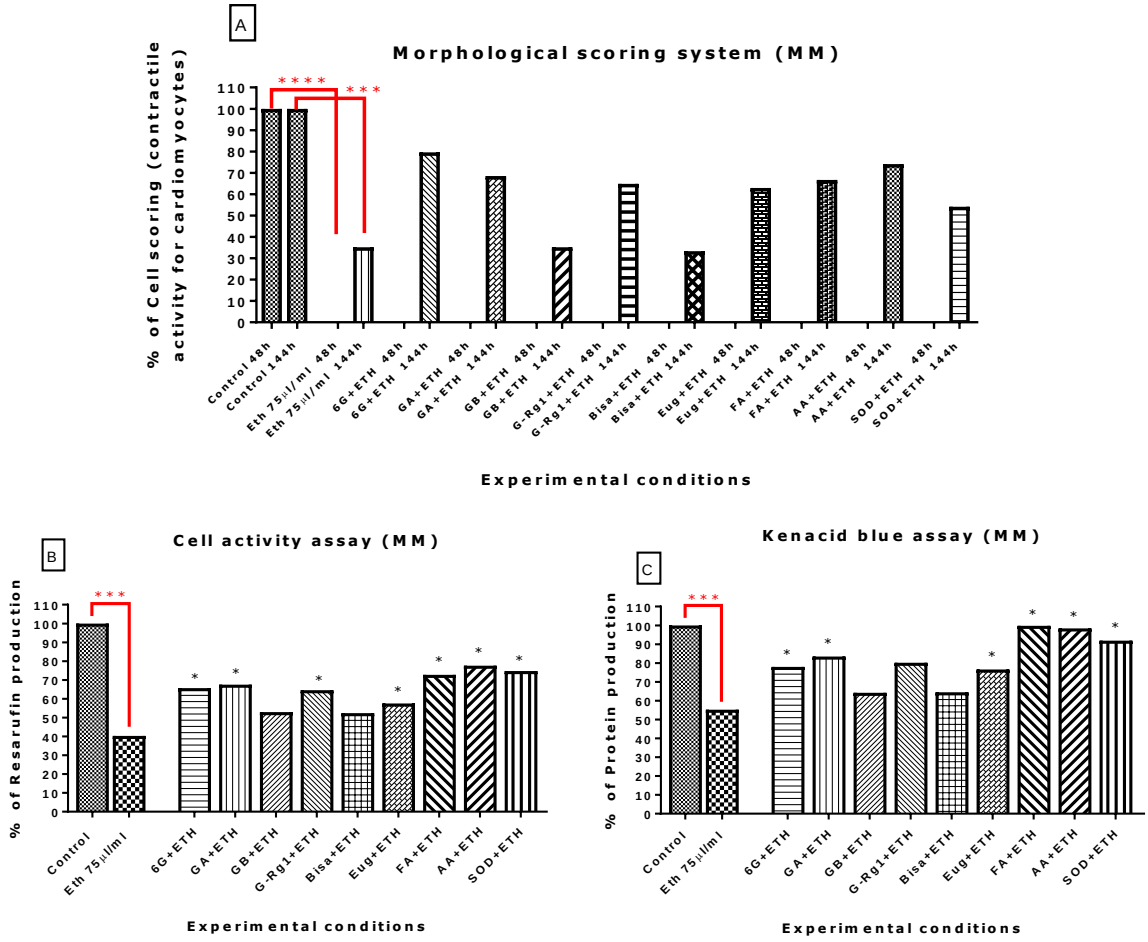


Figure 4-1: Protective effect of phytochemicals and standard molecules against ethanol in MM

(A) Morphological scoring system (contractile activity), (B) resazurin assay (cell activity), (C) kenacid blue assay (protein content). The data in each graph represent three independent experiments (n=3) with 4 intra-repeats per experiment per each environment. The 24 well plate in each experiment contained blank, control, tested phytochemical, 75µl/ml ETH and (tested phytochemical plus 75µl/ml ETH). All groups that contain (ETH + chemicals) were statistically compared with (ETH alone) group. The untreated group was statistically compared with (ETH alone) group. The data are presented in (%) but the statistical analysis was performed on raw data. *p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.

❖ In ESD3 system

Introduction of 12.5µl/ml ETH into ESD3 cultures prevented the stem cells from being differentiated into cardiomyocytes but it did not affect the cell activity and protein content. Co-treatment of ETH with SPM improved the differentiation and contractility, except of AA, but difference was not significant when compared with ETH alone-treated group (Figure 4-2A). Among the tested phytochemicals, only 0.75µM 6G and 0.5µM G-Rg1 have partially recovered the stem cells to be differentiated and contracted but also this was not significant compared to the ETH alone-treated group (Figure 4-2A). The ETH and all other molecules co-treatment groups displayed no significant difference in cell activity and protein content when compared with ETH-treated control (Figure 4-2B and C).

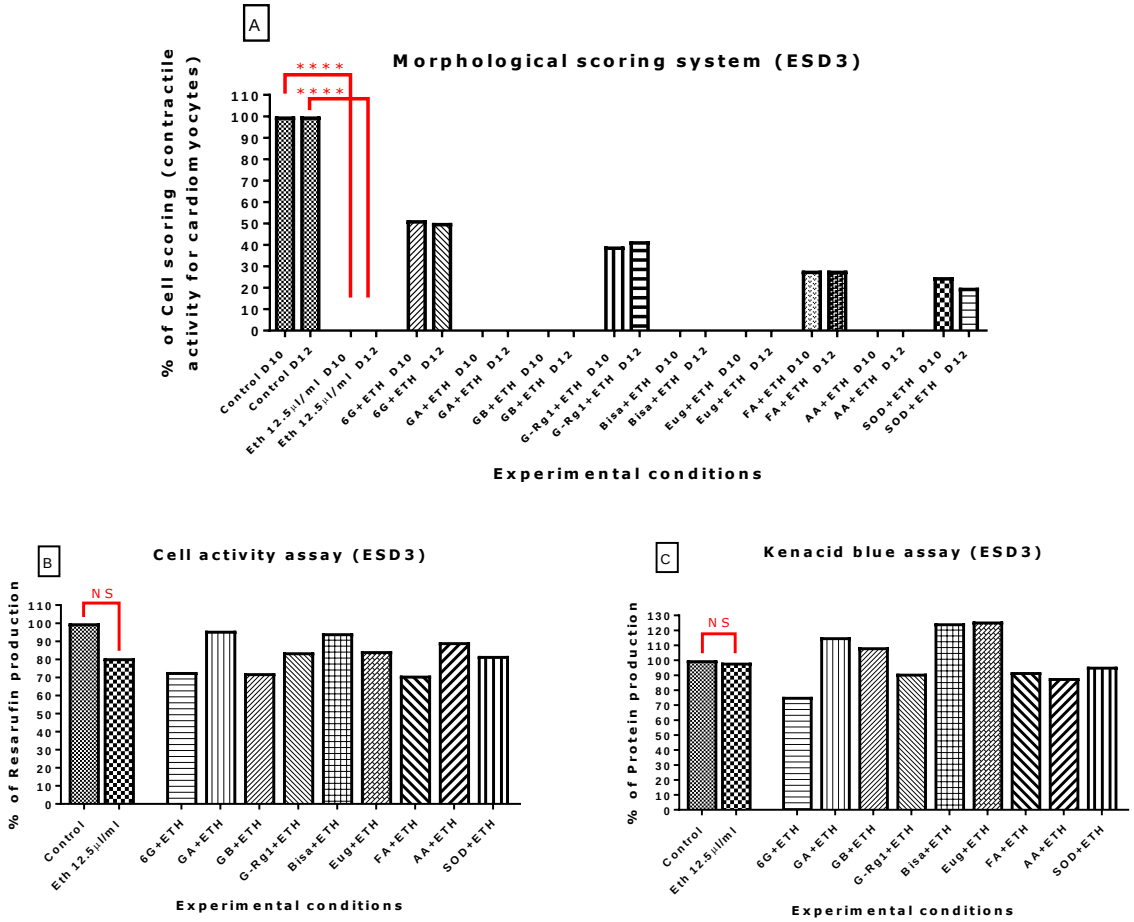


Figure 4-2: Protective effect of phytochemicals and standard molecules against ethanol in ESD3

(A) Morphological scoring system (contractile activity), (B) resazurin assay (cell activity), (C) kenacid blue assay (protein content). The data in each graph represent three independent experiments (n=3) with 4 intra-repeats per experiment per environment. The 24 well plate in each experiment contained blank, control, tested phytochemical, 12.5µl/ml ETH and (tested phytochemical plus 12.5µl/ml ETH). All groups that contain (ETH + chemicals) were statistically compared with (ETH alone) group. The untreated group was statistically compared with (ETH alone) group. The data are presented in (%) but the statistical analysis was performed on raw data. NS; non-significant, *p<0.05, **p<0.01, *** p<0.001 and **** p<0.0001.

4.3.2 Protective effect of herbal components and standard protective molecules against Retinoic acid

❖ In MM system

RA at 20μM treatment resulted in a statistically significant decline in morphological score at 48h and 144h in chick embryonic cardiomyocytes (Figure 4-3A), as well as a significant decrease in both cell activity and protein content by almost 75% and 70% respectively, in comparison to the untreated control (Figure 4-3B and C). After 120h of exposure to the tested phytochemicals or SPM, the contractile activity showed no significant recovery when compared to that of the RA-alone group, although some improvements were noted with GA, eugenol and SOD (Figure 4-3A). None of the tested molecules significantly rectified the cell activity or rescued the protein content after 120h of treatment. Only SOD treatment displayed some elevation in cell activity but it significantly increased the protein content when co-treated with RA (Figure 4-3B and C).

❖ In ESD3 system

ESD3 cells were treated with 5μM RA for 12 days and the same endpoints were monitored. At this concentration, RA prevented the stem cells from differentiating into cardiomyocytes but also reduced the EBs size substantially, where it was reflected by both a significant reduction of cell activity and protein content (Figure 4-4A, B and C). None of phytochemicals or SPM alone had a significant effect on the scoring, cell activity or protein content when compared with the control, (data not shown). The RA co-exposure with the selected concentrations of phytochemicals or SPM showed no amendments at any of the measured endpoints at all co-exposure groups (Figure 4-4A, B and C).

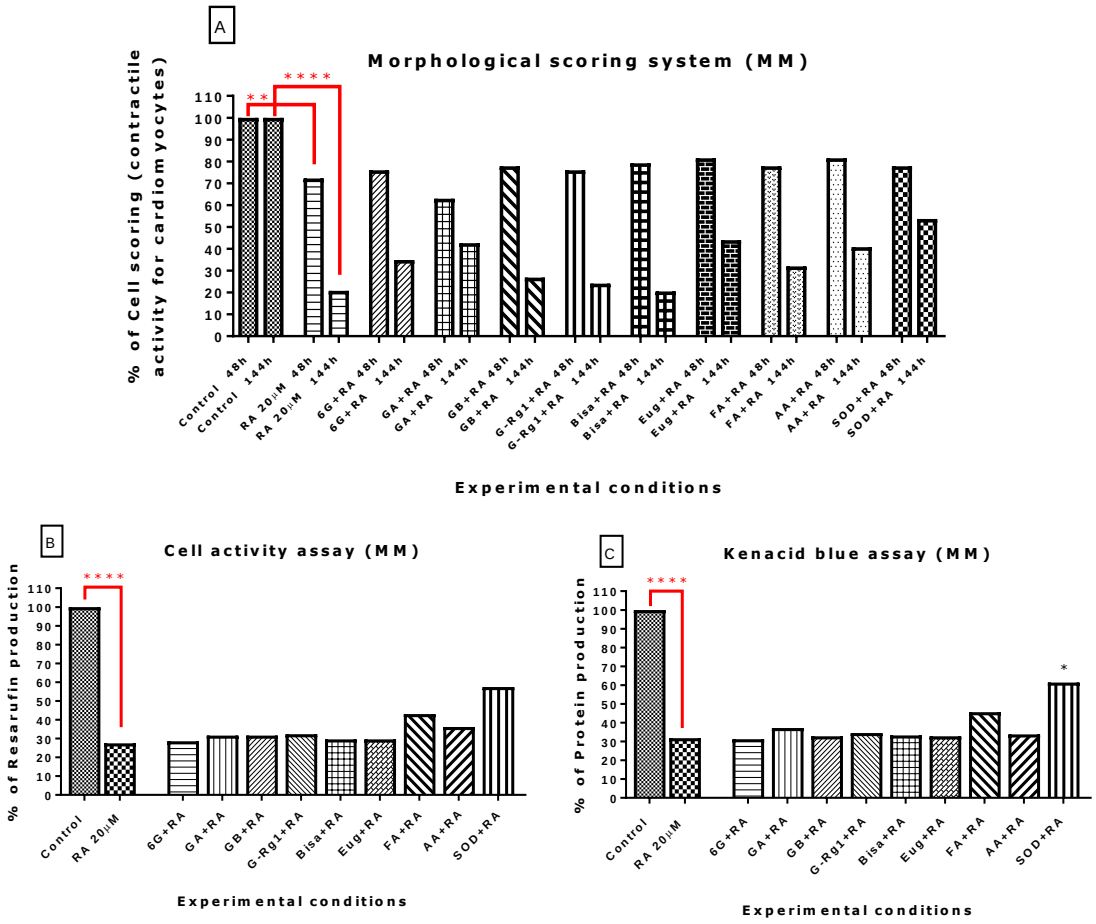


Figure 4-3: Protective effect of phytochemicals and standard molecules against Retinoic acid in MM

(A) Morphological scoring system (contractile activity), (B) resazurin assay (cell activity), (C) kenacid blue assay (protein content). The data in each graph represent three independent experiments (n=3) with 4 intra-repeats per experiment per environment. The 24 well plate in each experiment contained blank, control, tested phytochemical, 20μM RA and (tested phytochemical plus 20μM RA). All groups that contain (RA + chemicals) were statistically compared with (RA alone) group. The untreated group was statistically compared with (RA alone) group. The data are presented in (%) but the statistical analysis was performed on raw data. *p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.

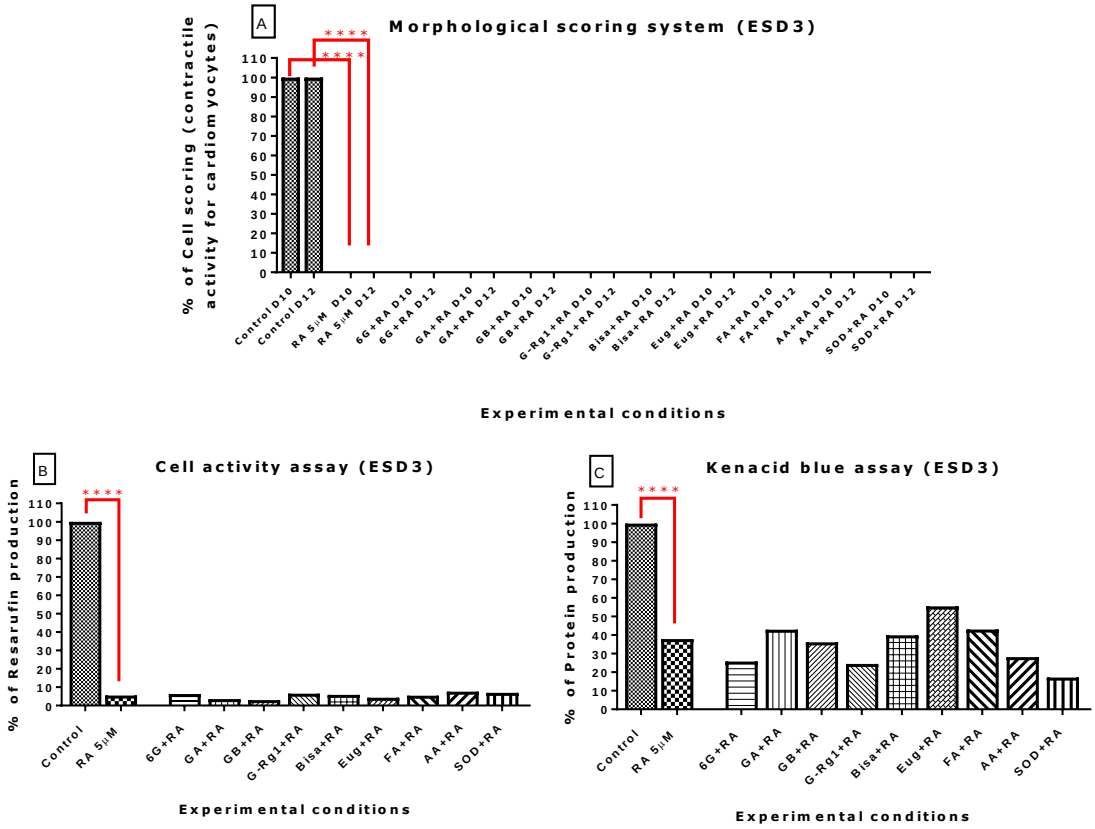


Figure 4-4: Protective effect of phytochemicals and standard molecules against Retinoic acid in ESD3

(A) Morphological scoring system (contractile activity), (B) resazurin assay (cell activity), (C) kenacid blue assay (protein content). The data in each graph represent three independent experiments (n=3) with 4 intra-repeats per experiment per environment. The 24 well plate in each experiment contained blank, control, tested phytochemical, 5µM RA and (tested phytochemical plus 5µM RA). All groups that contain (RA + chemicals) were statistically compared with (RA alone) group. The untreated group was statistically compared with (RA alone) group. The data are presented in (%) but the statistical analysis was performed on raw data. *p<0.05, **p<0.01, *** p<0.001 and **** p<0.0001.

4.3.3 Protective effect of herbal components and standard protective molecules against Valproic acid

❖ In MM system

The chick embryonic cardiomyocytes were treated with 1500μM VPA to induce a mild to moderate adverse effect on the contractile activity without disturbing the cell activity or reducing the protein content. VPA, at this concentration, decreased the contractile activity significantly at 48h and 144h (Figure 4-5A). The contractile activity (morphological scoring) did not display any significant enhancement when co-treated with the tested phytochemicals or SPM in comparison to VPA alone group. However, some enhance were noticed with 3μM 6G and 0.5μM G-Rg1 at 144h when compared to untreated control (Figure 4-5A). The statistical analysis of co-exposure groups show no significant alterations of cell activity and protein content when compared to VPA alone group (Figure 4-5B and C). The only exception was 6G+VPA group, where the cell activity becomes significantly different from VPA-alone group after 120h of co-exposure (Figure 4-5B).

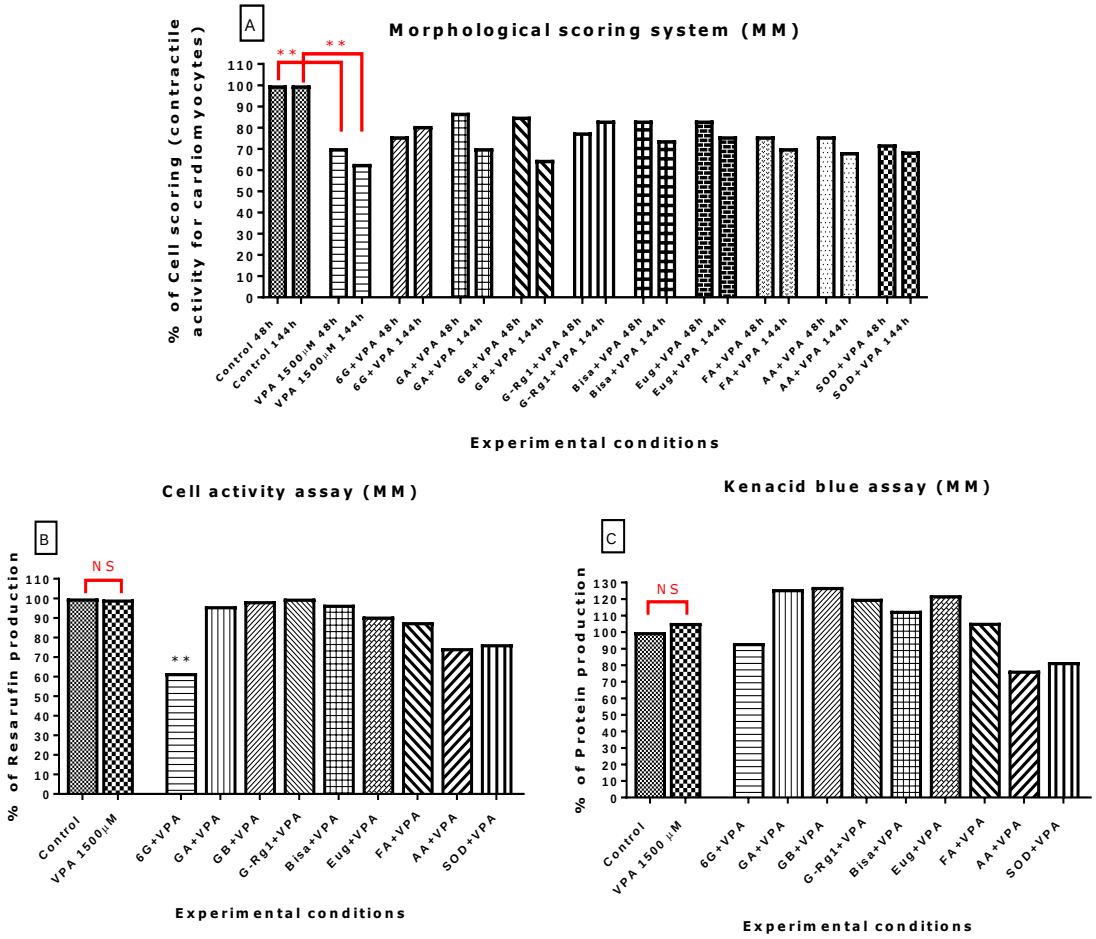


Figure 4-5: Protective effect of phytochemicals and standard molecules against Valproic acid in MM

(A) Morphological scoring system (contractile activity), (B) resazurin assay (cell activity), (C) kenacid blue assay (protein content). The data in each graph represent three independent experiments (n=3) with 4 intra-repeats per experiment per environment. The 24 well plate in each experiment contained blank, control, tested phytochemical, 1500µM VPA and (tested phytochemical plus 1500µM VPA). All groups that contain (VPA + chemicals) were statistically compared with (VPA alone) group. The untreated group was statistically compared with (VPA alone) group. The data are presented in (%) but the statistical analysis was performed on raw data. NS; non-significant, *p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.

❖ In ESD3 system

VPA at 750μM had completely prevented the stem cell differentiation into cardiomyocytes as the cultures did not show any contractile activity (Figure 4-6A). In addition, the cell activity and the protein content at 750μM VPA became significantly altered when compared with the untreated group (Figure 4-6B and C). When the phytochemicals and SPM were co-treated with VPA, none of the measured endpoints significantly changed when compared with VPA-alone group (Figure 4-6A, B and C). However, 0.75μM 6G and 0.5μM G-Rg1 had triggered a few beating foci within the cultures which means they facilitated the stem cells to differentiate to cardiomyocytes even in presence of VPA (Figure 4-6A).

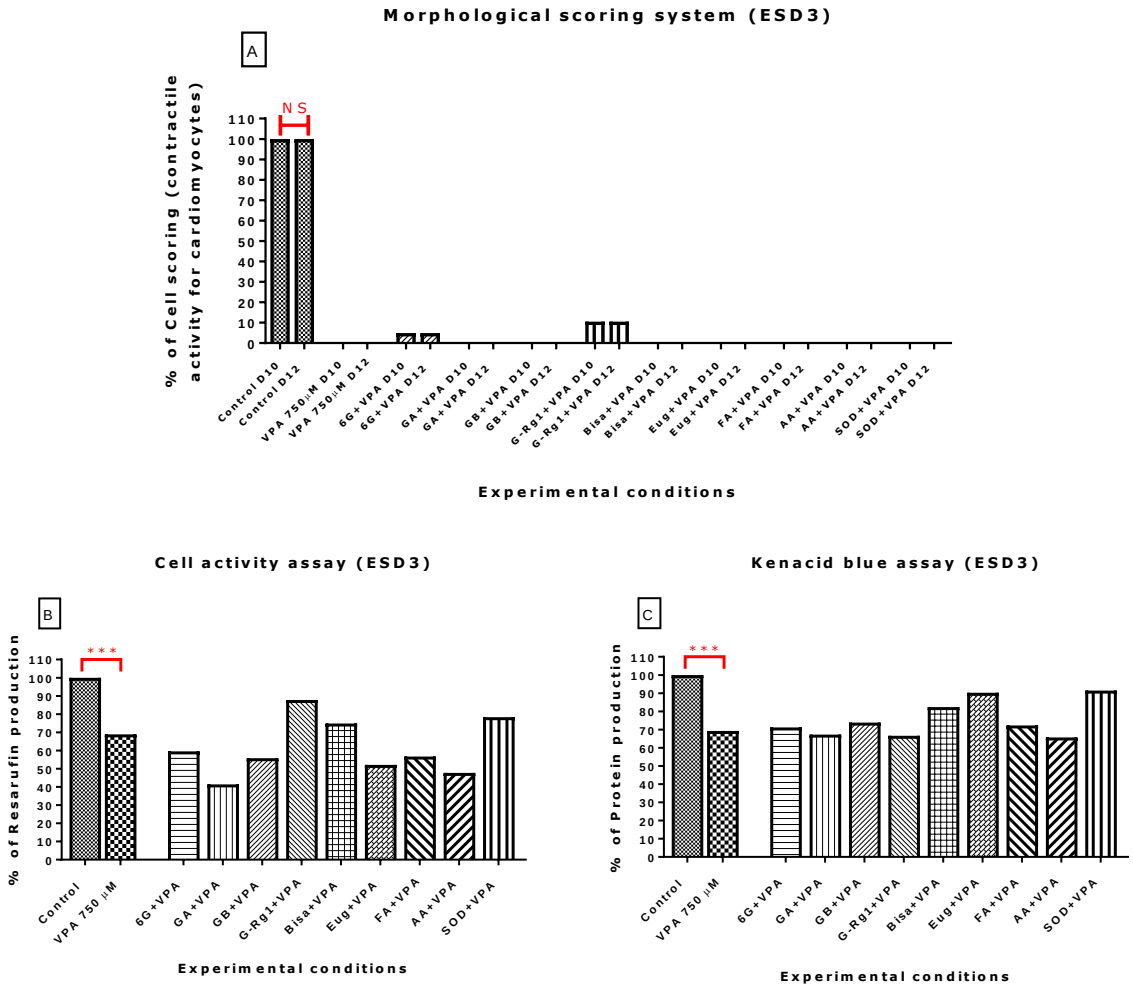


Figure 4-6: Protective effect of phytochemicals and standard molecules against Valproic acid in ESD3

(A) Morphological scoring system (contractile activity), (B) resazurin assay (cell activity), (C) kenacid blue assay (protein content). The data in each graph represent three independent experiments (n=3) with 4 intra-repeats per experiment per environment. The 24 well plate in each experiment contained blank, control, tested phytochemical, 750µM VPA and (tested phytochemical plus 750µM VPA). All groups that contain (VPA + chemicals) were statistically compared with (VPA alone) group. The untreated group was statistically compared with (VPA alone) group. The data are presented in (%) but the statistical analysis was performed on raw data. NS; non-significant, *p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.

4.4 Discussion

It was shown in the previous Chapter that the herbal components could induced teratogenicity or embryotoxicity above certain concentrations. The experiments that have been reported in this Chapter were constructed to investigate whether the selected 'non-embryotoxic non-teratogenic' herbal components concentrations have intrinsic protective characteristics or they are inert. The experiments involved three well-known protective molecules (FA, AA and SOD) alongside to make certain that both utilized systems, MM and ESD3, are reliable for such a study. The cellular insults were performed by treating the chick and mouse cell cultures in both systems with established teratogenic molecules (ETH, RA and VPA). These teratogens demonstrate different mechanisms of embryotoxicty in various severities to give opportunities to explore any possible protective mechanism of the tested herbal components. Our results may represent the protective capacity of these herbal components in embryonic cells, but not in any adult cells. Therefore, any lack of cytoprotective eveidence here should not be generalized to all other cells.

It was observed in these experiments that the teratogenic effects of the standard teratogens on the differentiating mouse stem cells cultured in ESD3 system were more dramatic on the measured endpoints, contractile activity, cell activity and protein content than their teratogenic effects on growing chick cardiomyocytes in MM system. Saretzki *et al.* (2008) found that the telomerase activity of hESD3 was maintained at high levels resulting in protection from telomere shortening and erosion. However, during differentiation, the telomerase activity has significantly downregulated [395]. In similar reports,

ROS and DNA damage levels increased in hESD3-derived differentiated cells, indicating that they are more vulnerable to insult and less competent in protecting their genome compared to the hESD3 themselves [395]. Although the defence mechanisms during chick embryonic development, including those of the heart, are immature, the antioxidant enzymes SOD, glutathione peroxidase and catalase are still marginally expressed during different periods of development [257]. These facts might partially explain the differences in the results for each endpoint in both systems.

ETH is a reactive and easily diffusible molecule, therefore it can react with several targets either within the cell membrane, such as receptors and ion channels, or within the intracellular compartments [396]. Several molecular toxicity mechanisms for ETH-induced cellular damage have been suggested. ETH induces ROS and oxidative stress in mouse cardiomyocytes which might lead to DNA damage [397]. ETH-induced damage could also be triggered by inflammatory cytokines such as TNF-α and IL-6 which exert their action by impaired protein synthesis and increased protein catabolism through the NFκB pathway [398]. In heart sections of rats put on high alcohol doses for 4 months, ETH was also found to be able to induce apoptosis in the cardiomyocytes via caspase 3, which is a mitochondrial apoptotic mediator, [399]. Our results showed the ETH in MM cultures caused cessation of the contractility after 24h of exposure followed by a partial recovery after a few days. The reason for this observation could be ETH-induced electrophysiological disturbances [400], or it could result from impaired proteins which are important in the contractile process such as actin, myosin or actinin [398]. There were some improvements in the contractile activity for

6G, GA, G-Rg1 and eugenol in addition to SPMs but no molecules brought the contractility back to being comparable to the untreated control. The partial contractile recovery with these molecules was accompanied by a significant elevation in both cell activity and protein content which might hint that 6G, GA, G-Rg1 and eugenol could protect the embryonic cardiomyocytes from the teratogenic effects of ETH by increasing protein synthesis or cell proliferation but the antioxidant effects could not be excluded. This is because of the improvements that have also been recorded with FA, AA and SOD. Furthermore, ETH prevented the stem cells being differentiated into cardiomyocytes. ETH has been shown to delay differentiation of cells in culture, such as skeletal muscle [401]. Several studies suggested different membrane associated-signalling pathways for ETH to influence transcriptional pathways that play a part in the differentiation process, such as adenylate cyclase, protein kinase C activation, receptor-kinase phosphorylation and prostaglandin production [263]. However, only two herbal components 6G and G-Rg1 have partially stimulated cardiomyocytes differentiation in the ESD3 system. ROS could play a role in hindering differentiation, as our results also showed some marginal stimulation for differentiation with FA and SOD. Hence the antioxidant activity of 6G and G-Rg1 might play a role in enhancing stem cell differentiation to cardiomyocytes. It is possible to argue that these two phytochemicals may have a capacity to downregulate the expression of stemness-maintaining genes such as Oct-4; therefore, they facilitate the differentiation. However, further experiments are necessary to better understand the protection mechanisms of 6G and G-Rg1 in both systems. It was noticed that GB and bisabolol did not show any protection against ETH teratogenic effects for all

endpoints measured in both systems. This could be because they lack protective ability against ETH, perhaps the concentrations used were not sufficient or the duration of exposure was too brief to show any protective effects. Further work is required to elucidate their protection mechanisms against ETH.

RA at 20μM has dramatically decreased the contractile activity of growing chick embryonic cardiomyocytes which was reflected by a decrease in the morphological score (Figure 4-3A). The cell activity and protein content were also significantly decreased (Figure 4-3B and C). Unfortunately, none of phytochemicals tested has shown any protection against RA. However, there was a noticeable enhancement in all measured endpoints at 1μM SOD. This might hint that ROS play an essential role in RA-induced embryonic cardiotoxicity.

It is very well established that RA induces teratogenicity in mammalian embryonic hearts [402]. Wang *et al.* (2014) established that long-term 1μM RA treatment initiates ROS-induced apoptosis in granulocyte-differentiated cells [403]. Additionally, Miyoshi *et al.* (2010) found that RA treatment of leukemia NB4 cells led to production of toxic ROS after being stimulated by phorbol 12-myristate 13-acetate [404]. On the other hand, a study demonstrated that 0-5μM RA can dose dependently prevent the angiotensin II- and mechanical stretch-induced ROS production in primary cultures of neonatal cardiomyocytes of rat pups [405]. Although RA is essential in the developmental process, at inappropriate levels it acts conversely at the genetic level where it disturbs the transcriptional regulation of specific homeobox genes, which have essential roles in patterning [406]. It could be assumed

that none of the herbal components tested has a capacity to rectify or prevent the RA-induced defects at the genetic level. However, the RA effect on the endpoints was much more apparent in the ESD3 cultures. 5μM RA was enough to reduce the EBs size and prevent the differentiation and the proliferation on the plate which was depicted by the morphological scoring system, cell activity and protein content results in comparison to untreated control (Figure 4-4). This result is consistent with another study which demonstrated that RA alters cardiac differentiation of mESD3 in a concentration and time-dependent manner. RA at 0.001 and 0.1μM induced EB differentiation toward the atrial phenotype. In contrast, 1 and 10μM RA showed a damaging effect on EB development by causing intense apoptosis. Protection against RA-induced detrimental effects in our study was completely absent for all phytochemicals and SPM.

VPA in these experiments was added to growing chick embryonic cardiomyocytes at 1500μM in the MM system for 5 days and to differentiating mouse embryonic stem cells at 750μM in ESD3 system for 12 days. VPA exerted mild teratogenic effects in the MM system and only the contractile activity significantly declined at 48h and 144h, but the cell activity and protein content were not influenced (Figure 4-5A, B and C). However, its effect on stem cell cultures was harsher as it hindered cardiac cell differentiation and decreased the EBs size and hence the protein content and cell activity. VPA is a small fat-soluble molecule, which permits it to diffuse easily into the cytoplasm [407]. Additionally, several reports have associated human embryonic heart malformations to VPA exposure in uterus during the gestational period [408-410]. The possible mechanisms of VPA embryotoxicity

have been arguable. ROS-induced oxidative stress, through disturbing the mitochondrial electron transport chain, may play a role in VPA embryonic cardiotoxicity but to an uncertain extent [411,412]. The ability of VPA to inhibit the histone deacetylase enzyme could also modify gene expression for several transcription factors leading to defects in differentiation or proliferation [413]. In fact, all the herbal components tested have failed to fully recover the contractile activity. Interestingly, the cell activity of MM cultures co-treated with 6G and VPA was reduced significantly. It could be suggested that the combination of 6G with VPA might form a new chemical product or initiate a chemical reaction that affected the cell activity. With regard the ESD3 system, only 6G and G-Rg1 managed to trigger cardiac differentiation in VPA co-treated cultures by 5% and 10% respectively compared to the untreated control (non-significant). The addition of any of the tested herbs did not make any significant difference to cell activity and protein content in comparison to the VPA-alone group. In consistent with our findings, Na *et al.* (2003) demonstrated that VPA treatment of ESD3-derived EBs at 100-1000μM did not exert cytotoxicity but suppressed the outgrowth of EBs by inhibition the cell cycle dose dependently. Na's study also showed that ROS could play an essential role in preventing cardiac differentiation [270]. However, the possible reason behind the failure of any of the tested herbal components to significantly restore the endpoints values is that VPA could also inhibit histone deacetylase and modify the expression of many proteins that are critical in the differentiation and proliferation process.

To sum up, these experiments were performed to study the potential protective characteristics of the herbal components. They have displayed a very weak intrinsic protective ability of these phytochemicals against RA and VPA. On the other hand, 6G, GA, G-Rg1 and eugenol exerted considerable protective capacity against embryotoxicity of ETH but not ETH teratogenicity effect. The only two phytochemicals that lack any protection capacity against all standard teratogens used were GB and bisabolol. Nevertheless, these phytochemicals could only protect against teratogens where ROS played the main role in the toxicity. They probably lack any capacity to protect against teratogens which act by modification at the genetic level such as VPA and RA.

CHAPTER 5

5 Chapter five - Evaluation the role of ROS production and DNA damage induction in the embryotoxicity mechanism of selected herbal components

5.1 Introduction

Reactive oxygen species (ROS) consist of oxygen-derived radical species such as superoxide ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), peroxyl ($RO_2^{\bullet}$) and alkoxyl ($RO^{\bullet}$), and non-radical oxygen species such as the superoxide anion (O_2^{-}) and hydrogen peroxide (H_2O_2), formed by the partial reduction of oxygen. Two methods have been described for ROS generation. Cellular ROS may be generated endogenously as a byproduct due to intrinsic metabolic pathways that are localised in different cellular compartments, such as the chloroplast, mitochondria and peroxisomes, or by an exogenous pathway in which ROS may arise by cell receptor binding with xenobiotic compounds [414]. O_2 has two unpaired electrons that have the same spin momentum. This spin enables O_2 to accept more electrons one at a time, leading to the generation of ROS, which can show harmful effects on cells. ROS have a role in cell signalling pathways, as a regulator to several cellular events such as proliferation, differentiation and physiological apoptosis. In addition oxidative stress may induce apoptosis and DNA damage [415].

5.1.1 Mitochondrial respiratory chain reaction

The mitochondrial process that leads to the production of adenosine triphosphate (ATP) is termed the respiratory chain reaction (RCR), which happens by oxidative phosphorylation [416]. The main operators for RCR are the enzyme complex I (NADH-coenzyme Q reductase or NADH dehydrogenase), complex II (succinate-coenzyme Q reductase or succinate dehydrogenase), complex III (coenzyme Q reductase), complex IV (cytochrome c reductase) and complex V (ATP synthase). These are all embedded in the inner mitochondrial membrane in an organised pattern [417]. Krebs' cycle in the matrix is the main source of electrons which are moved through the complex chain until they reach oxygen, the final destination, to form water molecules. While electrons move in a donor-acceptor fashion between complexes, an electrochemical gradient builds up outside the inner membrane through the coupled transfer of electrons to oxygen and the transport of protons from the matrix across the inner membrane into the intermembrane space. The terminal complex in the chain, ATP synthase, utilises this gradient to rotate itself in the membrane, rather like a turbine machine, catalysing the synthesis of most cellular ATP [418].

5.1.2 The physiological role of ROS

More and more evidence suggests that ROS play a vital role in the regulation of several cellular physiological processes which are involved in autophagy, hormone level control and enzyme activation or inhibition, as regulatory mediators in signalling processes [419,420]. It has been evidenced that ROS concentration is the main factor that determines their role, the higher the concentration, the more hazardous for cells and their major constituents [420].

At normal levels, ROS can act as a modulator for protein post-translational modifications to adjust signalling pathways. Particularly, H_2O_2 can oxidise thiol groups (—SH) on cysteine residues to produce sulphenic acid (—SOH), which in turn reacts with GSH to convert to glutathionylated (—SSG), which together with thiols can form a disulfide bond (—SS—), or with amides to form a sulfenyl amide (—SN—) [421]. Such modification can alter the function of target proteins within the molecular signal pathways. Phosphatases were found to be the most susceptible enzymes to ROS-induced protein modification, which inhibits their dephosphorylation activity [422]. ROS are also responsible for cellular responses to stimuli such as stress, free radicals or pathogens through inducing the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcriptional factor responsible for regulation of DNA gene expression of several proteins [423]. Furthermore, an important biological role of ROS has been shown to be their ability to change the intracellular redox milieu, which has a direct impact on several physiological activities such as gating properties of voltage-sensitive ion channels and associated downstream molecular transport pathways [424]. It has been suggested that ROS involvement in signal transduction could also come from activation of endogenous Ca^{+2} and subsequent Ca^{+2} dependent processes/enzymes [424]. The two critical signalling pathways which play a fundamental role in cell proliferation and survival in response to growth factor, cytokine and hormone stimulation are the phosphoinositide 3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) cascades. However, it has been well documented that ROS can regulate both PI3K and MAPK cascades by activation or inhibition of some proteins along their pathways toward DNA

transcriptional factors. Therefore, ROS at normal levels are important signalling molecules for cell proliferation [425]. Whilst ROS exert their regulatory effects in several ways intracellularly, it is essential they remain within the normal concentrations.

5.1.3 ROS involvement in apoptosis

Apoptosis is a unique and important form of programmed cell death (i.e. genetically determined elimination of cells), which performs a vital role in the development and homeostasis of multicellular organisms. Whilst some studies confirmed a clear contribution of ROS in apoptosis induction, there is growing evidence that pro-oxidative conditions are not the only triggers for apoptotic cell death [420]. However, two ATP-dependent pathway types of apoptosis have been described. Namely, the extrinsic pathway, also known as the death receptor pathway, and the intrinsic pathway, also known as the mitochondrial pathway. Some scientists have described Perforin or the granzyme pathway as another type of apoptosis pathway.

5.1.3.1 ROS and mitochondrial permeability transition pore

It has been confirmed that the mitochondrial permeability transition pore (PTP) complex is the known target of ROS to trigger the intrinsic apoptotic cascade via interactions with its integral proteins such as VDAC, ANT and cypD, whereby oxidising PTP proteins will dramatically alter mitochondrial anion fluxes [426]. Further, H_2O_2 causes a transient elevation in mitochondrial membrane hyperpolarization and initiates breakdown of the mitochondrial membrane potential ($^{\Delta}\Psi m$), mitochondrial translocation of pro-apoptotic proteins Bax and Bad, and cytochrome *c* release to the cytosol [427,428]. The

more cytochrome *c* to be released the more disturbances to the electron transport chain, which means more ROS to be generated.

5.1.3.2 ROS and Jun N-terminal kinases

Mitochondrial intrinsic apoptotic, as well as death receptor-initiated extrinsic pathways, can be mediated by Jun N-terminal kinases or (JNKs). JNKs induce apoptotic signalling by the upregulation of pro-apoptotic genes via the transactivation of specific transcription factors or by directly modulating the activities of mitochondrial pro- and anti-apoptotic proteins through distinct phosphorylation events [429]. However, ROS appear to activate JNKs through a chain of proteins. During TNF-α induced apoptotic signalling, it seems that ROS generate and activate what is called TNF-receptor-associated factor 2 (TRAF-2) which results in subsequent activation of signal-regulating kinase 1 (ASK1). Then this enzyme phosphorylates MAPK leading to the eventual stimulation of the JNK-module and apoptosis [430,431]. So the mechanism can be abbrieviated as TNF-α-ROS-TRAF-2-ASK1-MAPK-JNK mediated apoptotic signalling. Additionally, the phosphorylated JNKs translocate to the nucleus where they phosphorylate and activate c-Jun which in turn leads to the formation of a transcription factor termed Activator Protein 1 (AP-1). Ap-1 is involved in the regulation of many protein transcriptions, including an increase in the expression of pro-apoptotic proteins and/or decrease in the expression of pro-survival proteins [432,433].

5.1.4 DNA damage mechanisms

Deoxyribonucleic acid (DNA) damage is a relatively common event in the life of all cells, prokaryotic and eukaryotic, and may lead to mutation, cancer and apoptosis [434]. The damage can occur either in mitochondrial or nuclear DNA. Several factors can induce DNA damage, but as far as ROS is concerned, the following are considered the main mechanisms.

5.1.4.1 Indirect ROS-mediated DNA damage

This pathway mainly occurs in the cytoplasm and nucleus. Ataxia telangiectasia mutated (ATM) is a phosphatidylinositol kinase 3 (PI3K-like) serine/threonine protein kinase and acts as a double-strand break sensor to detect and repair any DNA double-strand damage. Thus, it cooperates with other kinase enzymes to activate various proteins which are involved in DNA repair, apoptosis or cell cycle checkpoints/proliferation [425]. Accumulating evidence has shown that ATM can also function as an 'ROS sensor' where an ROS harmful signal is mediated to DNA via ATM. A study found that cells in which ATM is inactivated or removed were more susceptible to oxidative stress-induced DNA damaging agents [435]. This is because ATM is assumed to initiate a controlled apoptosis process instead of causing collateral damage which happened in its absence. Gue *et al.* (2010) confirmed ROS-activated ATM downstream steps in the absence of DNA double-strand damage. However, ATM being activated by ROS was found to activate AMP Kinase (the enzyme that plays a role in the energy homeostasis) and caused DNA transcriptional disturbances which led to a decrease in protein synthesis but also an increase in autophagy [436].

5.1.4.2 Direct ROS-mediated DNA damage

This type mainly happens in mitochondrial DNA (mDNA) damage. There are several reasons make mDNA vulnerable to the direct effect of ROS oxidative damage, these are namely; its location is very close to the electron transport chain which is the main source of ROS and being intron-deficient makes it very prone to any damage [437]. The jeopardy of mDNA damage comes from the fact that mDNA encodes 13 polypeptides of the respiratory chain; therefore, the damage would not only compromise mitochondrial ATP production but also elevate ROS generation as a result of disturbing the respiratory chain. However, any mDNA damage would initiate a cycle of decreased ATP production-enhanced ROS generation-mDNA damage that ultimately triggers apoptosis [438,439]. Among several chemical reactions that are encountered by DNA nucleotides, such as methylation, phosphorylation or reduction, it has been confirmed that oxidative damage of nucleobases is an important source of mutations in DNA. A particular form of this damage is the oxidation of guanine to 7,8-dihydro-8-oxoguanine (oxoG). Whilst the DNA backbone might not be affected, the oxidised form of guanine is dangerous due to its ability to mis-pair with adenine instead of cytosine in the process of replication, leading to what are called transversion mutations [440].

5.1.5 Chapter objective

This Chapter was conducted to study the effects of the herbal componets on ROS production in MM and ESD3 systems but also to investigate the DNA damage in ESD3 system.

5.2 Materials and methods

5.2.1 ROS measurement assay

To quantify the ROS in the treated and untreated cultures of both systems, the 2', 7' dichlorofluorescein diacetate (DCFH-DA) assay was employed. The assay used several characteristics of DCFH-DA which include in cell permeability, as it is non-ionic and non-polar, but also its fluorogenic ability intracellularly. After being introduced in the cytoplasm, DCFH-DA is cleaved by cellular esterase to remove a diacetate group and convert the substance into fluorogenic DCFH. The DCFH is trapped in cells and is oxidised by ROS available in the cell to produce highly fluorescent dichlorofluorescein (DCF) [441]. Therefore, the ROS levels within the cell are related to the fluorescence measured (DCF). Schematic steps for DCFH-DA for this assay are depicted in Figure 5-1. The effects of any free radical generation compounds on DCFH-DA can be measured and quantified against the fluorescence of the DCF using the standard curve as in Figure 5-2.

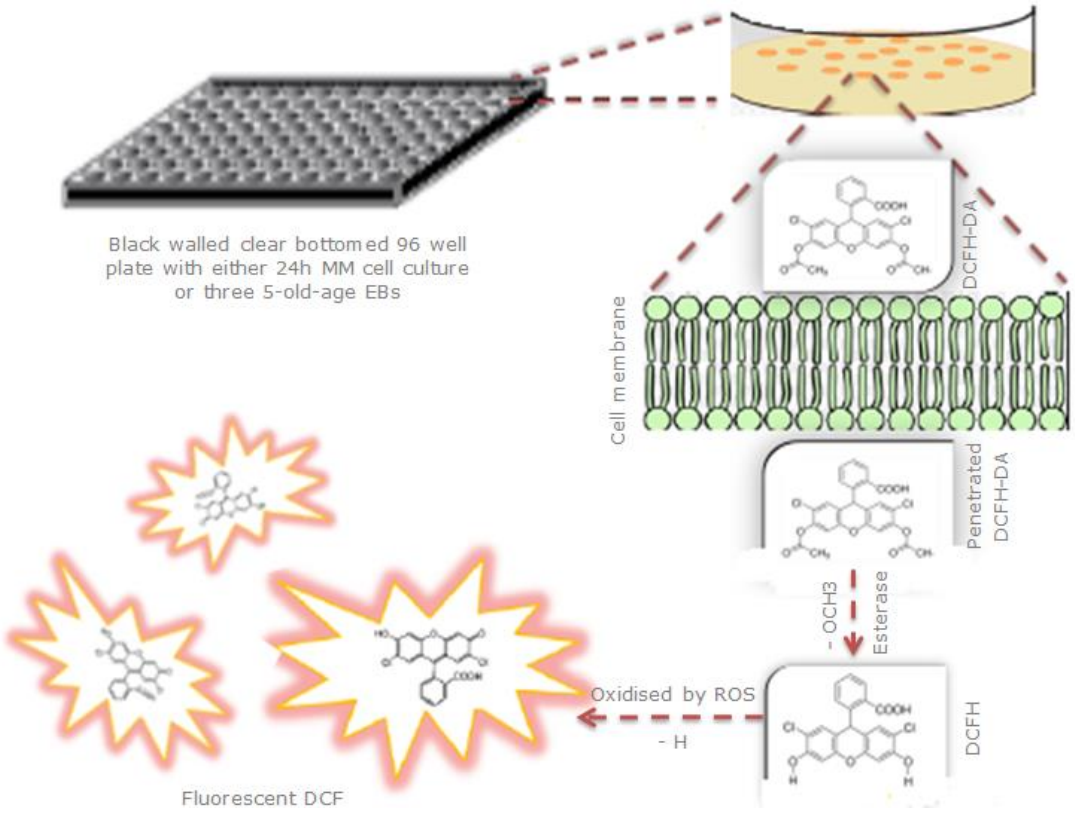


Figure 5-1: Schematic representation of cellular steps of conversion non-fluorescent DCFH-DA into fluorescent DCF in presence of ROS

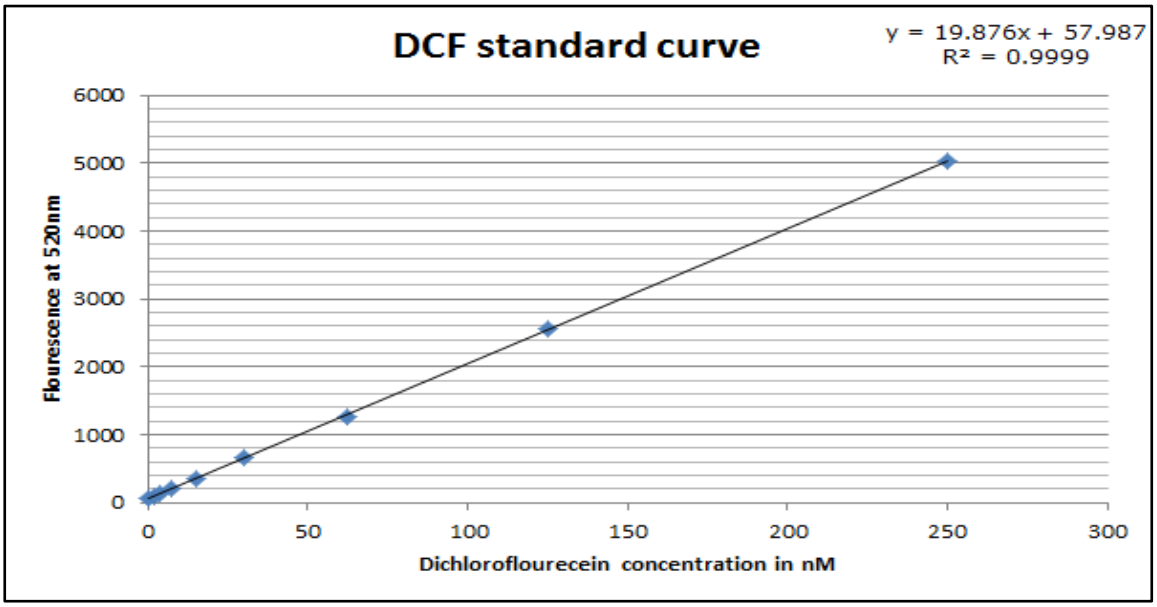


Figure 5-2: Representative DCF standard curve which shows dose-dependent elevation in fluorescence signal

5.2.1.1 Detection protocol for cellular reactive oxygen species

In this assay, the cell suspension obtained for the MM system (Section 2.2.1) was cultured in black walled, clear bottomed, 96 well plates (Corning, UK) for 24h. However, for ESD3, five day old EBs were employed (Section 2.2.2). Three EBs were plated per well of black-walled, clear bottomed, 96 well plate at the time of the experiment. The measurements were read at six time points (0, 1, 2, 3, 4, and 24h) after culture exposure. A short period point scale was used in this assay because of the short half-life of ROS [442]. Moreover, the H_2O_2, which was used as a positive control in the assay [443], could have severely damaged the cells if a longer period had been applied. Different concentrations of tested herbal components were assessed using the DCFH-DA assay. The procedure of this assay has been described by Garle *et al.* (2000) [442]. In this study, the required amounts of test chemicals were dissolved in HBSS containing 20μM (DCFH-DA). The fluorescence was detected at time periods of 0, 1, 2, 3, 4, and 24h using an emission filter of 520 nm and excitation filter of 485 nm (FLUORstar Galaxy, UK). During interval periods, the plate was wrapped with aluminium foil and kept at 37^{o}C in the 95% air: 5% CO_2 incubator to avoid light interfering.

5.2.1.1.1 Negative controls of ROS assay (AA and SOD)

Ascorbic acid and superoxide dismutase were employed as well known negative controls in regard to their ability to evoke ROS in the cells. The concentrations were selected based on the results of AA shown in Figure 2-21 and SOD as in Figure 2-22 which indicated that these two chemicals did not cause any form of embryotoxicity, teratogenicity or cytotoxicity. For AA, 50

and 100μM concentrations were selected to be assayed for their ROS induction capacity; for SOD, 0.5 and 1μM concentrations were selected to test their ROS induction ability in both MM and ESD3 systems. The analysed data showed the expected results for AA and SOD in both systems. None of the concentrations tested increased ROS in either system. This was performed to ensure the reliability of the assay and to avoid any false positive results might arisen due to a possible redox chemical reaction triggered by adding a test substance to DCFH-DA solution. However, the results confirmed such reactions are not predicted and ROS elevation should come from the effect of chemicals on the cell metabolism. The positive control of 10μM H_2O_2 elevated the ROS as expected in both systems.

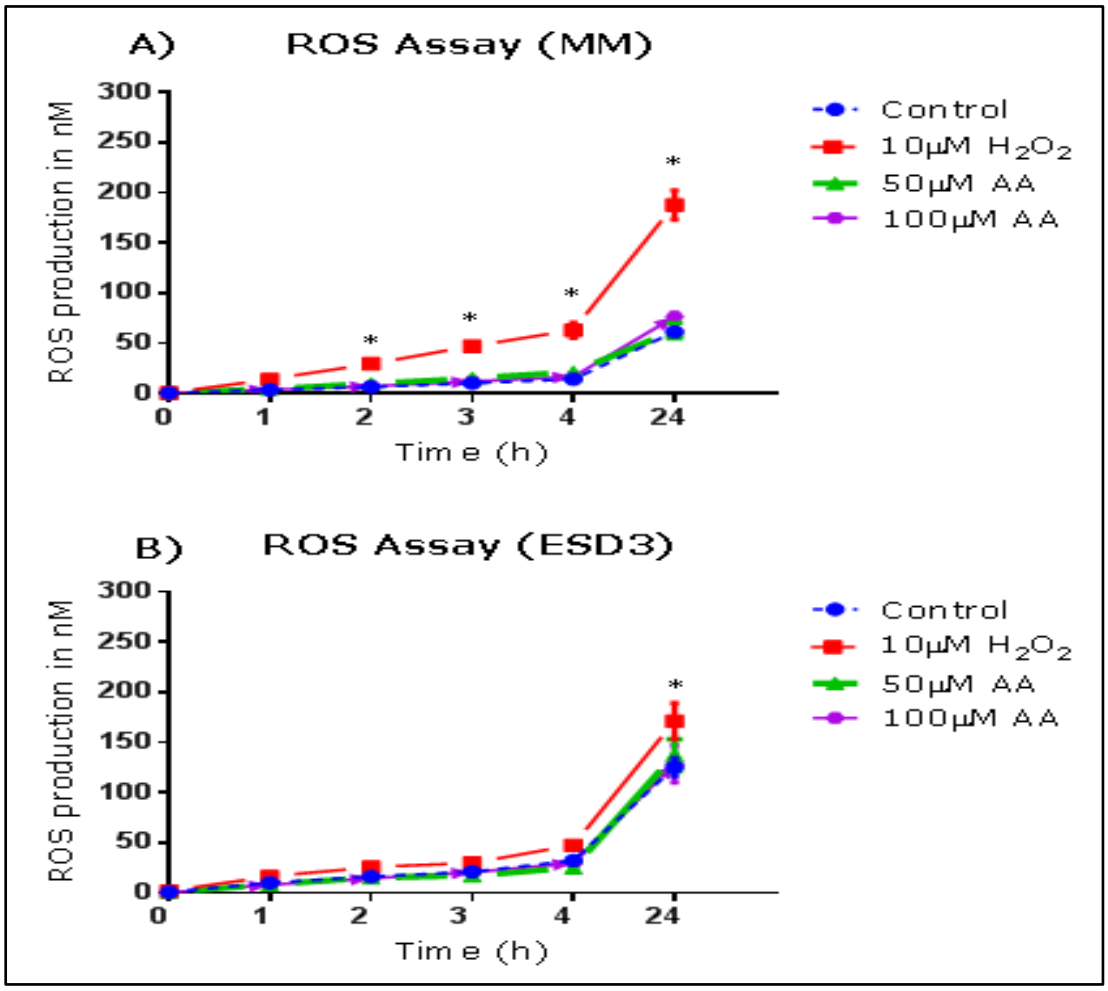


Figure 5-3: Effects of ascorbic acid (AA) on ROS production in MM and ESD3 systems

The curves, in sections (A) which represent effect of AA on MM and (B) which represents the effect of AA on ESD3, show that both tested concentrations in both systems have failed to increase the ROS production to significant levels. The effect of 10µM H_2O_2 increased ROS significantly in both systems after 24h. H_2O_2 was used as a positive control in each repeat. Three independent repeats of each experiment were performed. The data are presented as mean ± SEM and analysed statistically using two-way ANOVA with Bonferroni post-hoc test. * $p<0.05$ significant difference from control.

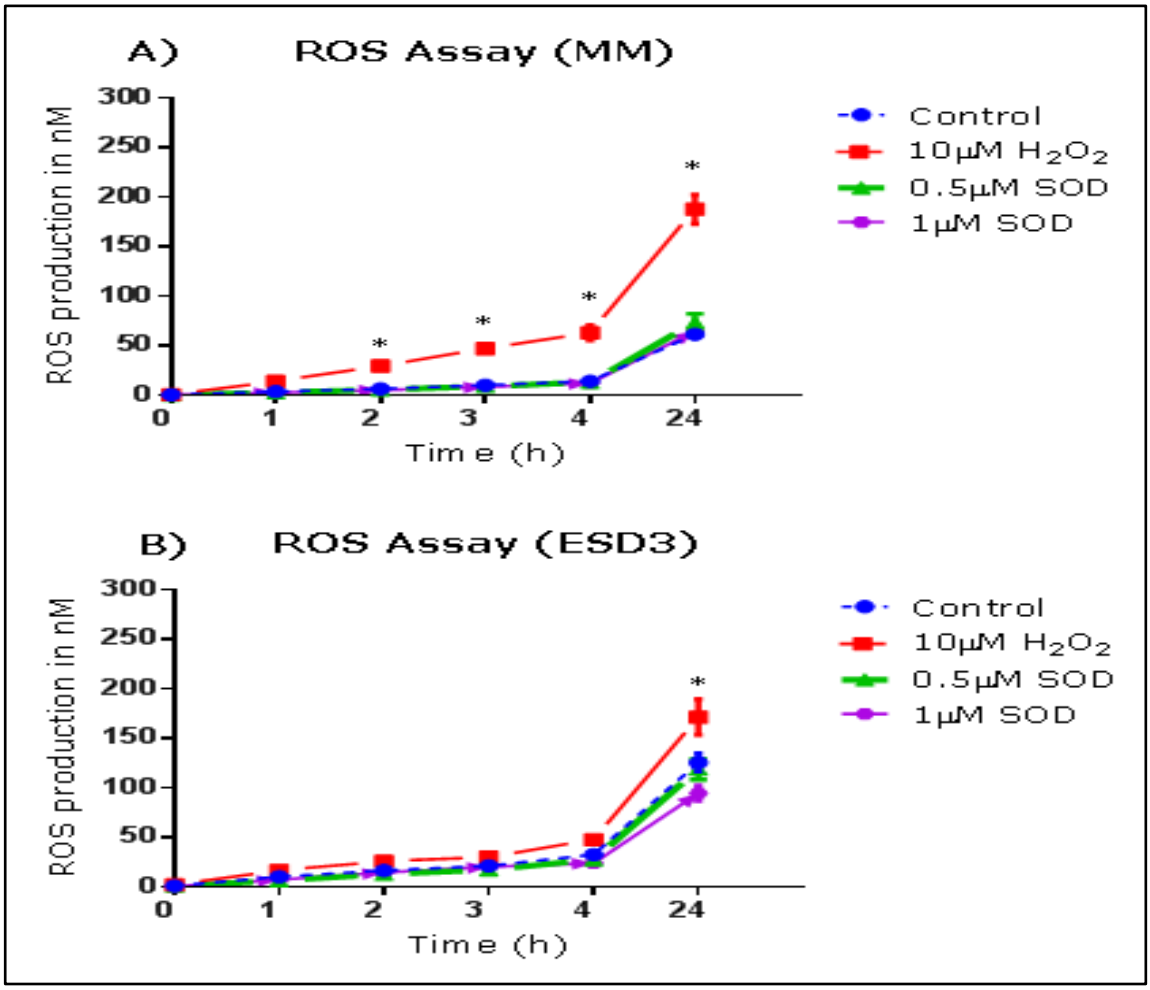


Figure 5-4: Effects of superoxide dismutase (SOD) on ROS production in MM and ESD3 systems

The curves, in sections (A) which represent effect of SOD on MM and (B) which represents the effect of SOD on ESD3, show that both tested concentrations in both systems have failed to increase the ROS production to significant levels. The effect of 10µM H_2O_2 increased ROS significantly in both systems after 24h. H_2O_2 was used as a positive control in each repeat. Three independent repeats of each experiment were performed. The data are presented as mean ± SEM and analysed statistically using two-way ANOVA with Bonferroni post-hoc test. * $p<0.05$ significant difference from control.

5.2.2 DNA double-strand breaks assay (pϒ-H2AX) (ESD3)

DNA double-strand breaks (DSBs) can occur directly or indirectly during normal cell programmed activities such as meiosis or by exposure of cells to some xenobiotics, such as ionizing radiation or some classes of

chemotherapeutic drugs like 5-FU. DSBs are one of the most serious forms of DNA damage, and their repair is vital for cell survival and the preservation of proper genomic codes [444]. When DNA damage occurs in "germ cells" and it exceeds the cellular competence to repair it, several genetic errors would be accumulated, and even transmitted to future cell generations, which may result in cell death or incorporation of genome mutations which then might be translated into embryopathy [445]. The further possible consequences of these breaks which can have serious embryonic cellular consequences include chromosomal aberrations, increased genetic instability, carcinogenesis and cytotoxicity [446]. DSBs generation results in the rapid phosphorylation of H2A variant H2AX. The phosphorylation occurs on Ser139 of H2AX (γ-H2AX), and it is described as abundant, fast and very well correlated with each DSB. Therefore, the test was considered as a most sensitive assay for DNA damage detection [447]. The phospho (p) γ-H2AX assay was implemented to investigate the possible DNA damage that might be induced by some selected herbal component concentrations. ESD3 were cultured in 1% (w/v) gelatine coated six well plates (Corning, USA) at a density of $2.5x10^5$/ml. The cells were incubated at 37^oC in the 95% air: 5% CO_2 incubator to allow the cells adhere to the plates. When the cells were 60% confluent, various test herbal component concentrations were freshly prepared (in the same ESD3 medium) and were used to replace the culture medium in each well, and these were incubated for a further 24h. At the end of 24h, floating and adherent cells were collected by trypsinization, neutralised with ESD3 medium (for each 2ml trypsin, 8ml ESD3 medium was added) and transferred to the corresponding 15ml centrifuge tubes (Sterlin, Stone, UK). The tubes were centrifuged at

900rpm for 5 minutes (but all further centrifugations were set on 2000 rpm speed for 5 minutes), the cell pellet was resuspended in 1ml PBS for cell washing. The cells were spun again, and the pellet was resuspended and fixed with 500µl 2% (w/v) formalin in PBS for 5 min at RT, with gentle agitation every 1 minute. Fixed cells were permeabilised with 500µl 0.4% (v/v) Triton X-100 in PBS for 1 minute at RT, Triton X-100 was mixed with formalin in the same tube, before the subsequent centrifugation was performed. The supernatant was aspirated, then nonspecific binding was blocked with 1ml 1% (v/v) heat inactivated FBS for each tube. The resuspended cells were left 30 minutes at RT for blocking. The blocked cells were spun again, and the cell pellet was resuspended with 200µl (1:3000 in 1% (v/v) FBS in PBS) mouse anti pϒ-H2AX antibody (Merck Millipore, Germany) and incubated at RT for 90 minutes. After this incubation, 1ml 1% (v/v) FBS was added to each tube to wash the cells, before another centrifugation was performed. Then the cells were stained with 200µl (1:1500 in 1% FBS in PBS) goat anti-mouse Alexa-fluor 488 secondary antibody (Life Technologies, UK). After 1h incubation in the dark at RT, 1ml 1% (v/v) FBS was added to each tube and final centrifugation was performed. Once the supernatant was removed, the cell pellet was resuspended with 400µl of RNase-containing propodium iodide (PI) solution (Sigma–Aldrich, UK) and kept for 10 minutes at RT. The samples were transferred into 5ml fluorescence-activated cell sorting (FACS) rounded-bottom tubes (Falcon, Mexico) to be ready for measurement. Three concentrations of chemotherapeutic drug 5-fluorouracil (5-FU) (5, 10 and 15µm) for a 24h exposure period were utilised as a 'DNA damage positive control' to identify the test reliability [448]. The 5-FU results clearly indicated

the reliability of this assay by showing highly significant DNA damage, Figure 5-5. The samples were measured using a FC500 flow cytometer (Beckman Coulter), FL-1 channel was used for colour intensity detection and in each sample, 10^4 events were recorded. At least three independent repeats of each experiment were performed using six well plates (2 wells of each group in every repeat for DNA damage flow cytometry assay). The data was analysed using Weasel software, and raw data were statistically calculated in using GraphPad Prism6

Statistics software by one-way ANOVA, but they were presented as a percentage of the pϒ-H2AX increment. The graph of positive control drug 5-FU shows a significant increase in % pϒ-H2AX increment in comparison to control, Figure 5-5 and Figure 5-6.

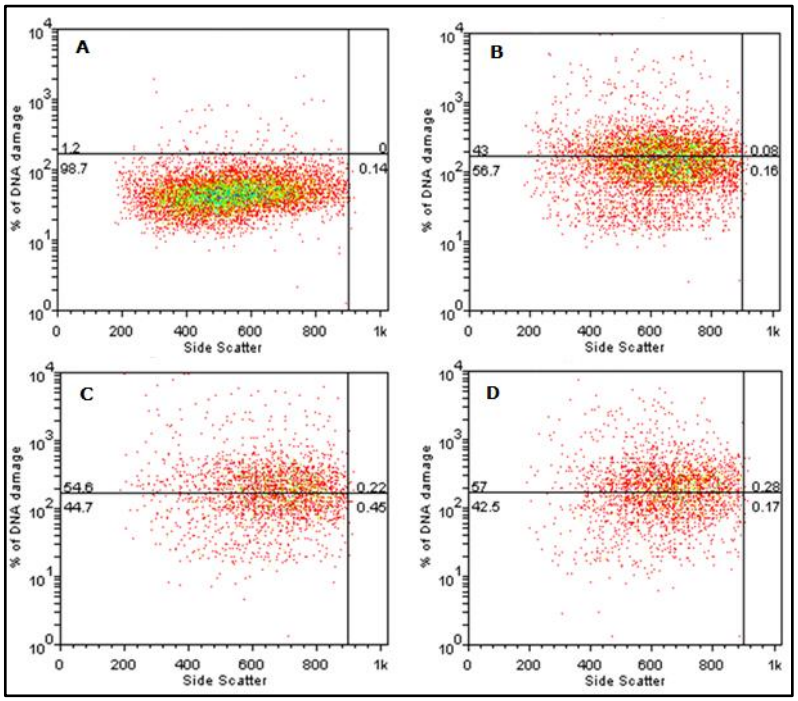


Figure 5-5: Effects of 5-FU concentrations on DNA double strand break induction in ESD3 cultures

5-FU was selected as a positive control in DNA damage assay. The figure shows (A) dot plots of the control and with the three tested 5-FU concentrations (B) 5μM, (C) 10μM and (D) 15μM. The y-axis represents DNA damage and the x-axis side scatter, which represents the cell size. There was concentration-dependent trend of increase in the H2AX signal at the three concentrations tested. Three independent repeats (n=3) were performed.

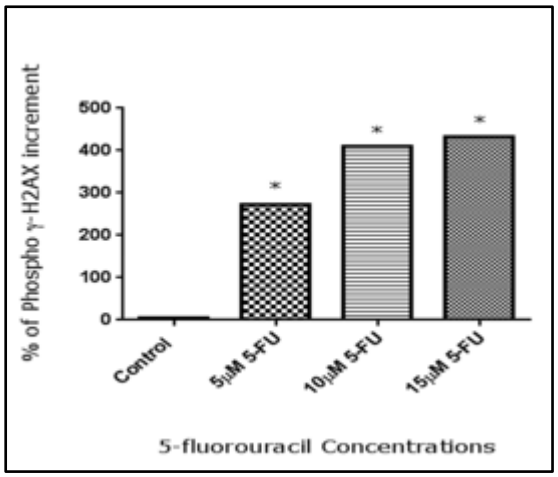


Figure 5-6: Effect of 5-FU on DNA damage in ESD3 cultures

5-FU (5, 10 and 15μM) shows significant DNA damage in comparison to control. At least three independent experiments (n=3) were performed. Data is presented in percentages but the analysis was performed on raw data using one-way ANOVA. * $P<0.05$ vs. control.

5.2.3 Immunocytochemistry of Phospho ϒ-H2AX for DNA double-strand breaks (ESD3)

To confirm the data of DNA damage obtained from flow cytometry, immunocytochemistry to stain pϒ-H2AX was employed. ESD3 were seeded onto 1% (w/v) gelatin-coated coverslips in a density of 10^5/ml in Nunc 24 well plates. The cultures were treated next day with different selected concentrations of the tested herbal components but untreated controls and secondary antibody only stained cultures were also set up. The coverslips (while they were still on the plate) were washed with PBS briefly, fixed in 4% (w/v) paraformaldehyde for 5 minutes and washed thrice with PBS again. The cells were permeabilised with 0.3% (v/v) Triton X-100 in PBS for 1h at RT. After three washes 5 minutes each, Non-specific binding sites were blocked with 1% (w/v) BSA in PBS plus 1% (w/v) low-fat milk in PBS for 1h at RT. The coverslips were removed and placed onto corresponding pre-labelled slides and were circled with a Peroxidase-Antiperoxidase (PAP) pen (Vector Laboratories). 1:500 dilution of mouse anti-pϒ-H2AX primary antibody in the blocking solution was added onto coverslips (except for those with the secondary antibody only control) and incubated overnight in a humidified chamber at 4^oC. The following day, cells were washed. Anti-mouse Alexa Fluor 488 secondary antibody was diluted in blocking solution (1:1000) and applied for 1h in the dark at RT. Coverslips were again washed thrice in PBS, and they were counterstained with DAPI (1:2000), which was added and left for 5 minutes then washed with distilled water. The cells were imaged with an OLYMPUS fluorescence microscope (Nikon, Japan).

5.3 Results

5.3.1 The effects of herbal components on ROS and DNA damage induction

5.3.1.1 The effects of herbal components on ROS production in MM and ESD3 cultures

In light of the results of the herbal components effects on MM and ESD3 cultures in Section 3.4, two concentrations of each component were selected in each system for further investigations concerning their ability to induce ROS and DNA damage. For the MM system, only 6G at 100μM (Figure 5-7A) significantly induced ROS production, but all other tested concentrations for the remaining components showed non significant changes in ROS productions. For ESD3 system, the selected 6G concentrations (0.75 and 100μM) (Figure 5-7) induced the ROD production significantly. GA failed to increase the ROS level at 100nM but it elevated ROS production significantly at 2000nM (Figure 5-10B). GB at both concentrations (50nM and 1000nM) did not alter the ROS production significantly. G-Rg1 at 10μM (Figure 5-16B) but not at 0.5μM showed significant increase in ROS production. Bisabolol (80μM) (Figure 5-19B) caused significant ROS elevation. However, both eugenol concentrations (0.01μM and 80μM) (Figure 5-22) significantly elevated the ROS levels when ESD3-derived 5-day-old differentiating EBs were treated for 24h. In both systems and in all experiments, a significant ROS increase at 10μM H_2O_2 (a positive control), has been shown. A summary is presented in Table 5-1.

5.3.1.2 The effects of herbal components on DNA damage in ESD3 cultures

The cultured differentiating mouse embryonic stem cells were treated with the same herbal component concentrations that were tested in the ROS assay to investigate the possible DNA damage occurred after 24h treatment. The results revealed that significant amounts of double strand breaks of DNA were only detected at 100μM 6G (Figure 5-8) and 80μM eugenol (Figure 5-23), when compared to untreated controls. All remaining tested concentrations failed to approach significant levels. The results of flow cytometry were confirmed by immunocytochemistry (Figure 5-9 and Figure 5-24), using the same tested concentrations and the same exposure time that have been used in flow cytometry. To exclude the autofluorescent signal, an untreated group was incubated with only secondary (without primary) antibody, and there was no signal detected. All the results of DNA damage in ESD3 for tested herbal component concentrations are summarised in Table 5-1.

5.3.2 The effects of 6G on ROS and DNA damage induction

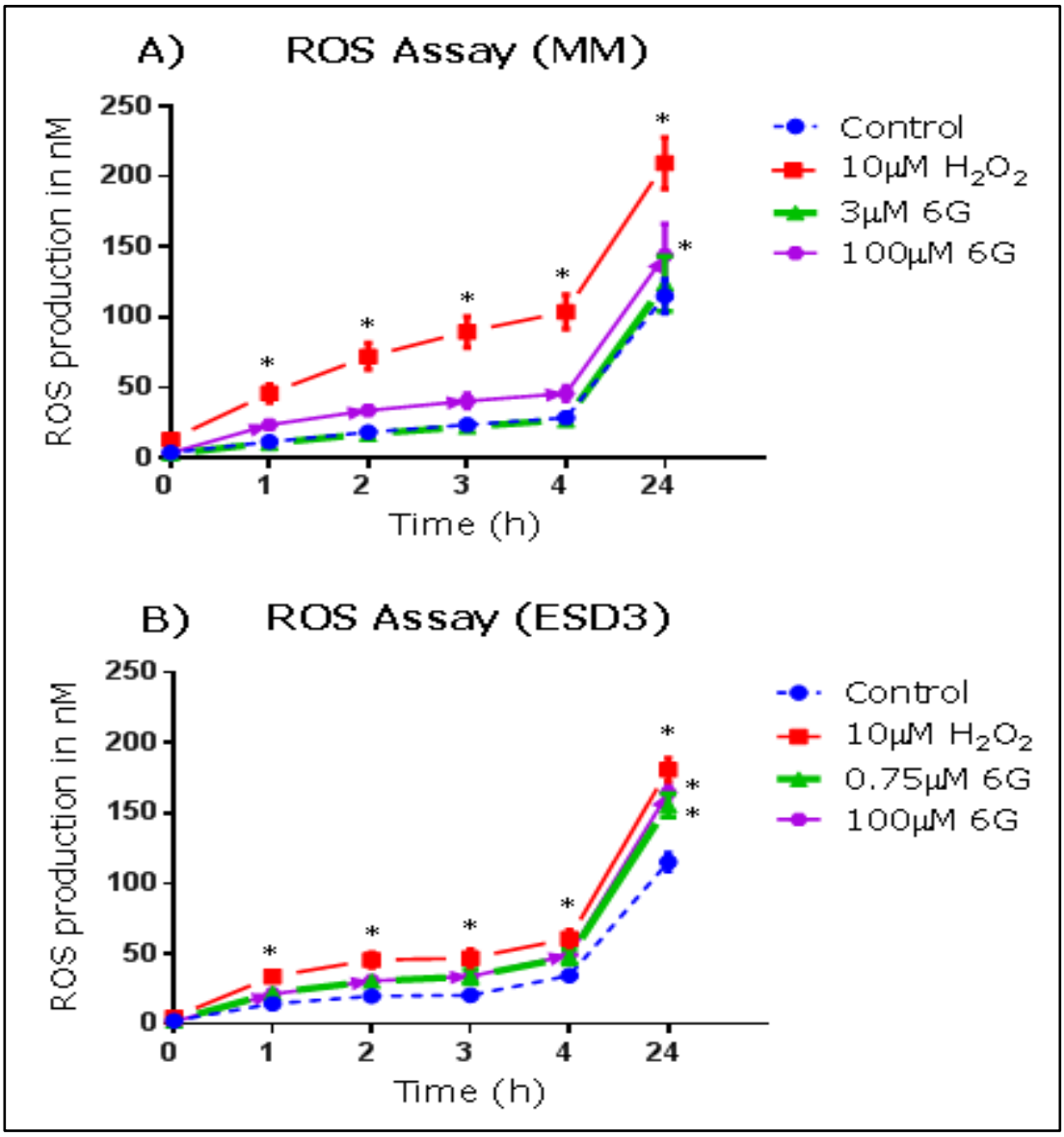


Figure 5-7 Effects of selected 6G concentrations on ROS production in MM and ESD3 systems

(A) Effects of 6G on ROS production in the MM system and (B) effects of 6G on ROS production in the ESD3 system. The curves show that 100μM 6G increased the ROS production to significant levels in both systems after 24h. At 3μM, 6G failed to elevate ROS to significant levels in MM but at 0.75μM, 6G increased ROS in ESD3 system after 24h. 10μM H_2O_2 was used as a positive control in each repeat. Three independent repeats (n=3) of each experiment were performed. The data are presented as mean ± SEM and analysed statistically using two-way ANOVA with Bonferroni post-hoc test. * $p<0.05$ significant difference from control.

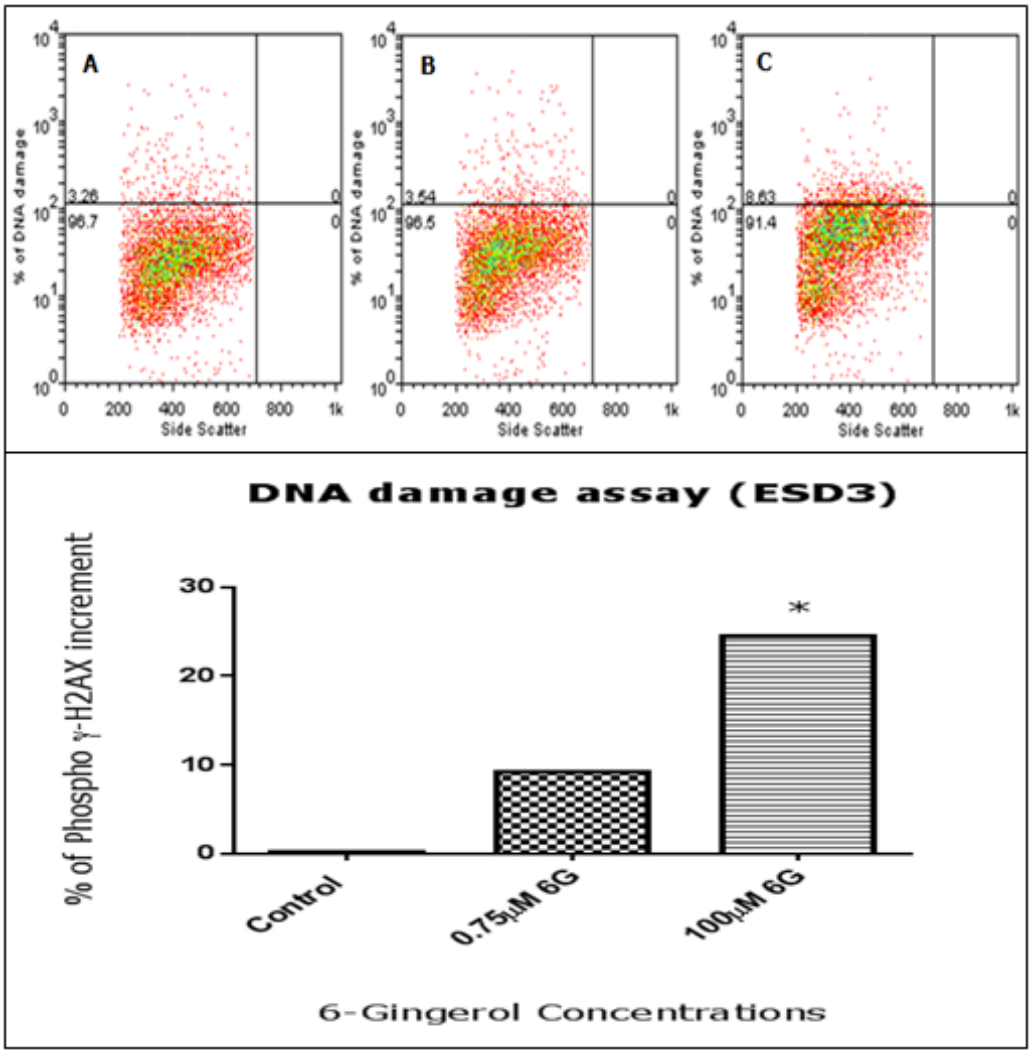


Figure 5-8: Effects of 6G on DNA double strand breaks induction
(A) DNA damage dot plots of the untreated cultures, (B) DNA damage dot plots of 0.75μM 6G and (C) DNA damage dot plots of 100μM 6G. The y-axis in the dot plot represents DNA damage and the x-axis side scatter, which represent the cell size. There was dose dependent trend of increase in the pγ-H2AX, DNA damage signal, until it becomes significant at 100μM concentration. The data from flow cytometry dot plot was converted into the % of increments as shown in bar graph. At least three independent experiments (n=3) were performed. Data is presented in percentages but the analysis was performed on raw data using one-way ANOVA. * P<0.05 vs. control.

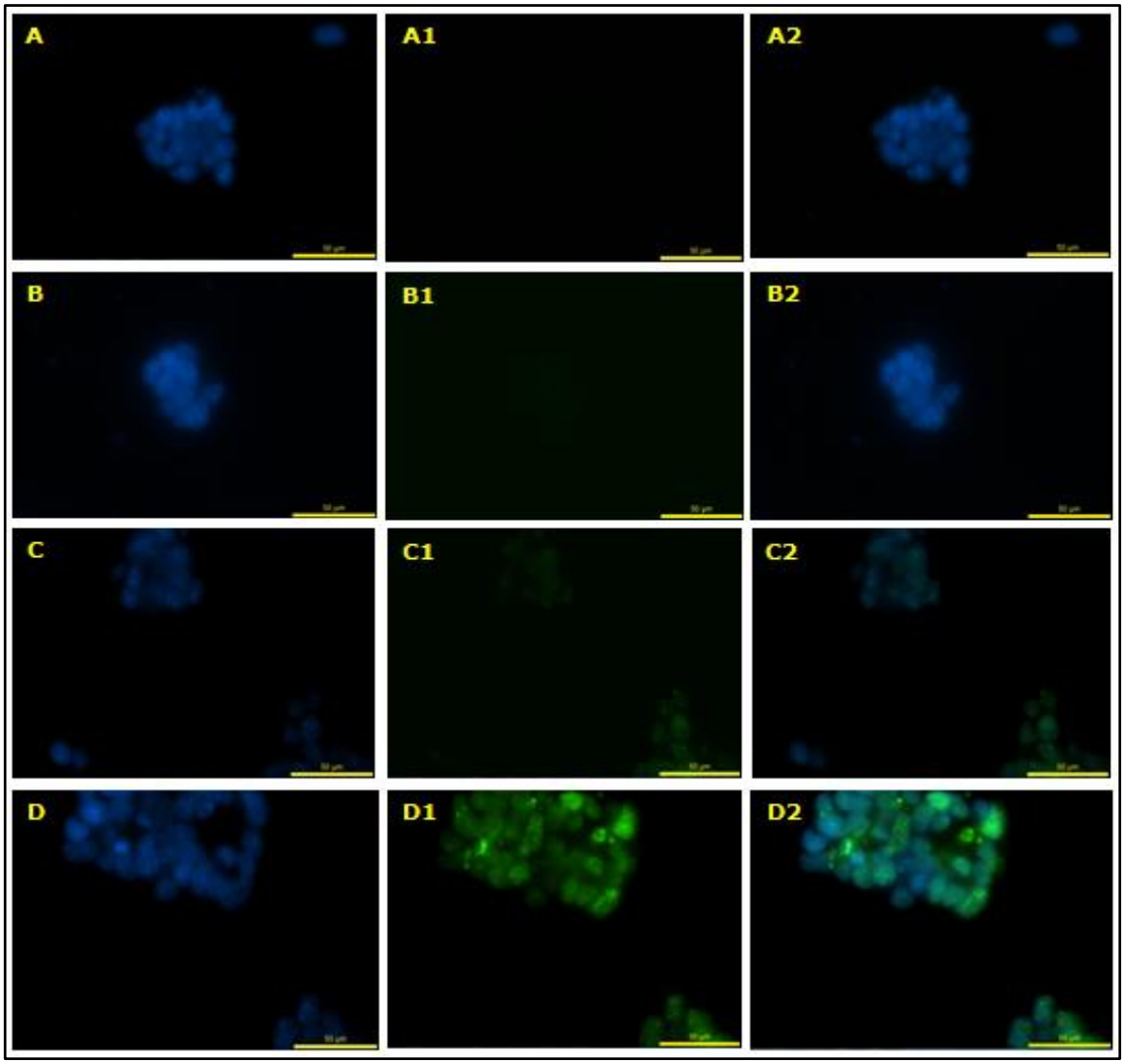


Figure 5-9: Immunocytochemistry of the effects of 6G on induction of DNA damage in differentiating mouse embryonic stem cells

(A-A2) untreated control, (A) represents DAPI nuclear staining (blue), (A1) represents pϒ-H2AX (green), and (A2) represents overlay image, (B-B2) untreated control secondary antibody only, (B) represents DAPI nuclear staining (blue), (B1) represents secondary antibody only, and (B2) represents overlay image, (C-C2) 0.75μM 6G treated stem cells, (C) represents DAPI nuclear staining (blue), (C1) represents pϒ-H2AX (green), and (C2) represents overlay image, and (D-D2) 100μM 6G treated stem cells, (D) represents DAPI nuclear staining (blue), (D1) represents pϒ-H2AX (green) and (D2) represents overlay image. The green staining indicates double-strand breaks signal within the nucleus in stem cells cultures. The green signal increased in a concentration-dependent manner. (Scale bar 50μm).

5.3.3 The effects of GA on ROS and DNA damage induction

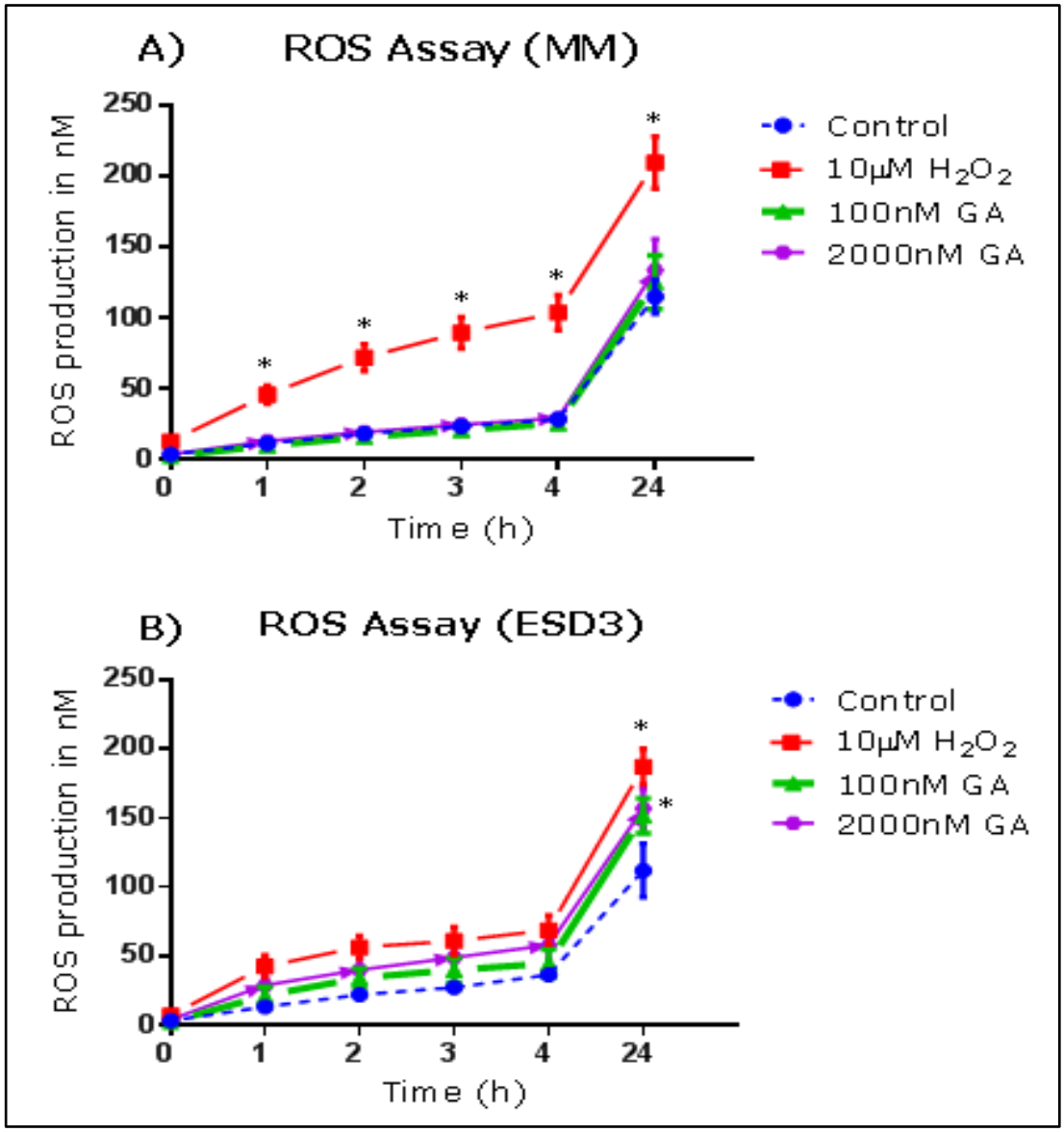


Figure 5-10: Effects of selected GA concentrations on ROS production in MM and ESD3 systems

(A) Effects of GA on ROS production in the MM system and (B) effects of GA on ROS production in the ESD3 system. The curves show that 100nM and 2000nM GA failed to increase the ROS production to significant levels in MM system after 24h. At 100nM, GA failed to elevate ROS to significant levels in ESD3 but at 2000nM, GA increased ROS in ESD3 system after 24h. 10μM H_2O_2 was used as a positive control in each repeat. Three independent repeats (n=3) of each experiment were performed. The data are presented as mean ± SEM and analysed statistically using two-way ANOVA with Bonferroni post-hoc test. * $p<0.05$ significant difference from control.

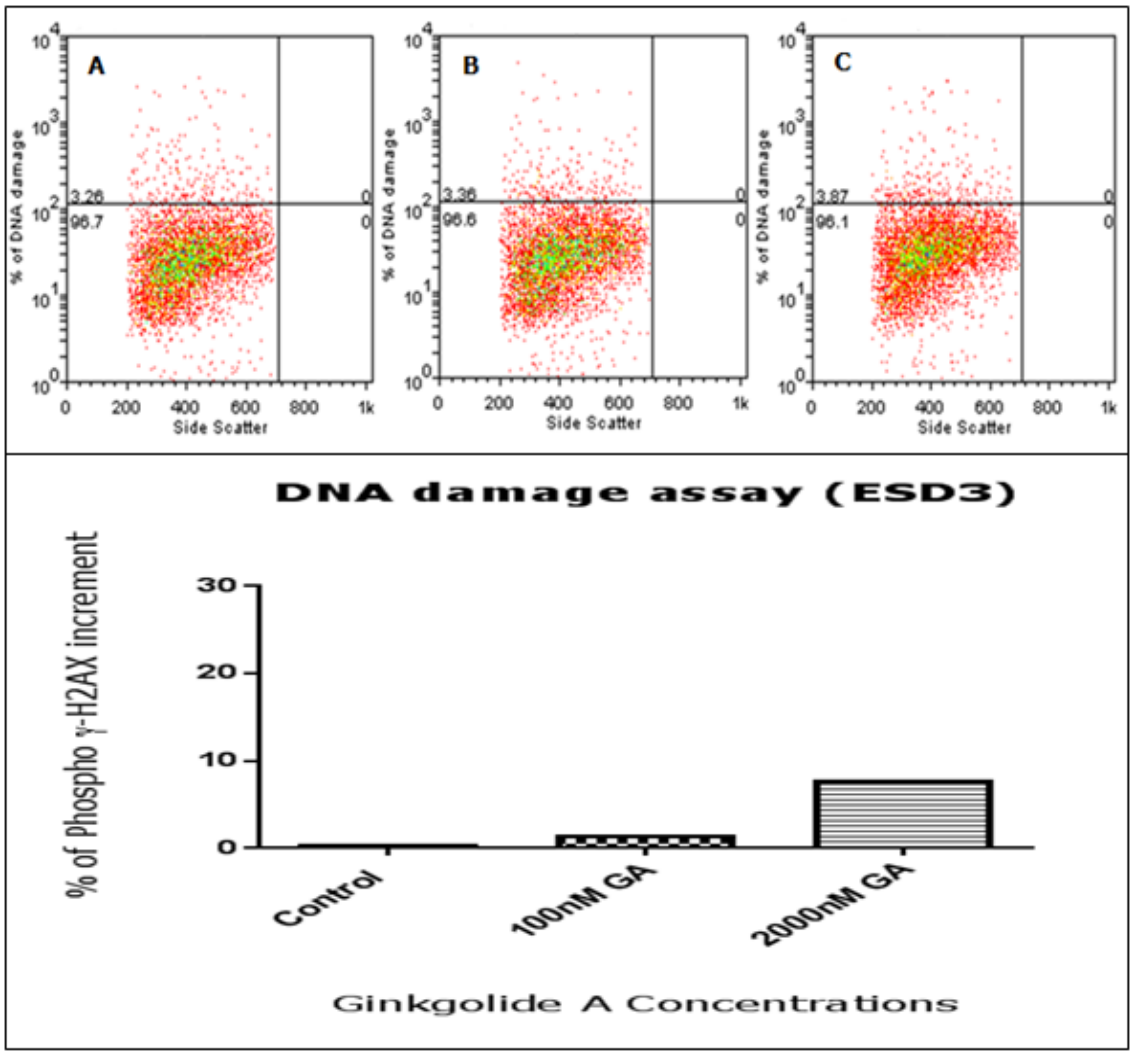


Figure 5-11: Effects of GA on DNA double strand breaks induction
(A) DNA damage dot plots of the untreated cultures, (B) DNA damage dot plots of 100nM GA and (C) DNA damage dot plots of 2000nM GA. The y-axis in the dot plot represents DNA damage, and the x-axis side scatter, which represent the cell size. There was a dose-dependent trend of increase in the pγ-H2AX, DNA damage signal, but it failed to be significant at any concentration tested. The data from flow cytometry dot plot was converted into the % of increments as shown in a bar graph. At least three independent experiments (n=3) were performed. Data is presented in percentages, but the analysis was performed on raw data using one-way ANOVA. * $P<0.05$ vs. control.

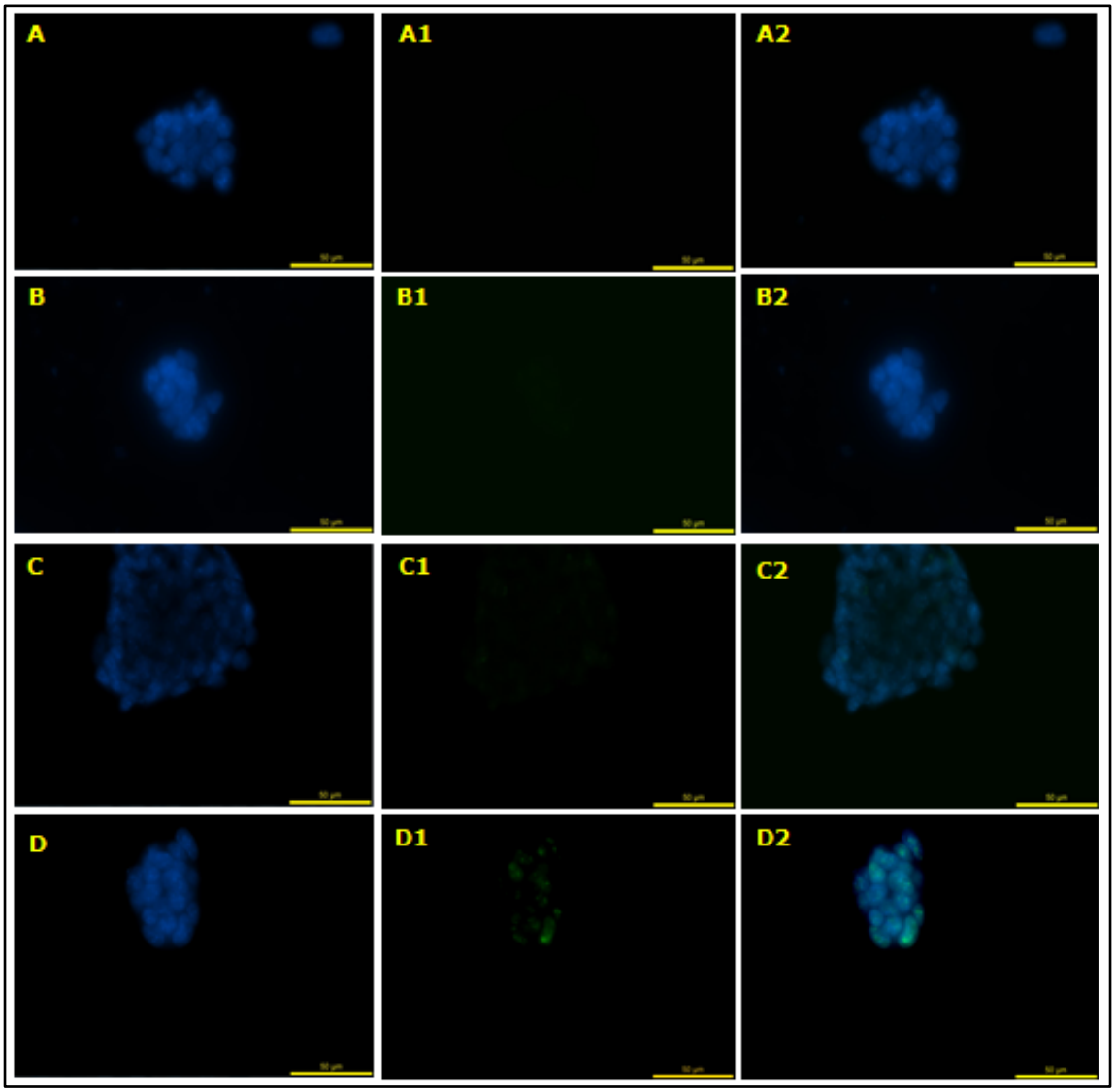


Figure 5-12: Immunocytochemistry of the effects of GA on induction of DNA damage in differentiating mouse embryonic stem cells

(A-A2) untreated control, (A) represents DAPI nuclear staining (blue), (A1) represents pϒ-H2AX (green), and (A2) represents overlay image, (B-B2) untreated control secondary antibody only, (B) represents DAPI nuclear staining (blue), (B1) represents secondary antibody only, and (B2) represents overlay image, (C-C2) 100nM GA treated stem cells, (C) represents DAPI nuclear staining (blue), (C1) represents pϒ-H2AX (green), and (C2) represents overlay image, and (D-D2) 2000nM GA treated stem cells, (D) represents DAPI nuclear staining (blue), (D1) represents pϒ-H2AX (green) and (D2) represents overlay image. The green staining indicates double-strand breaks signal within the nucleus in stem cells cultures. The green signal did not increase at both concentrations tested. (Scale bar 50µm).

5.3.4 The effects of GB on ROS and DNA damage induction

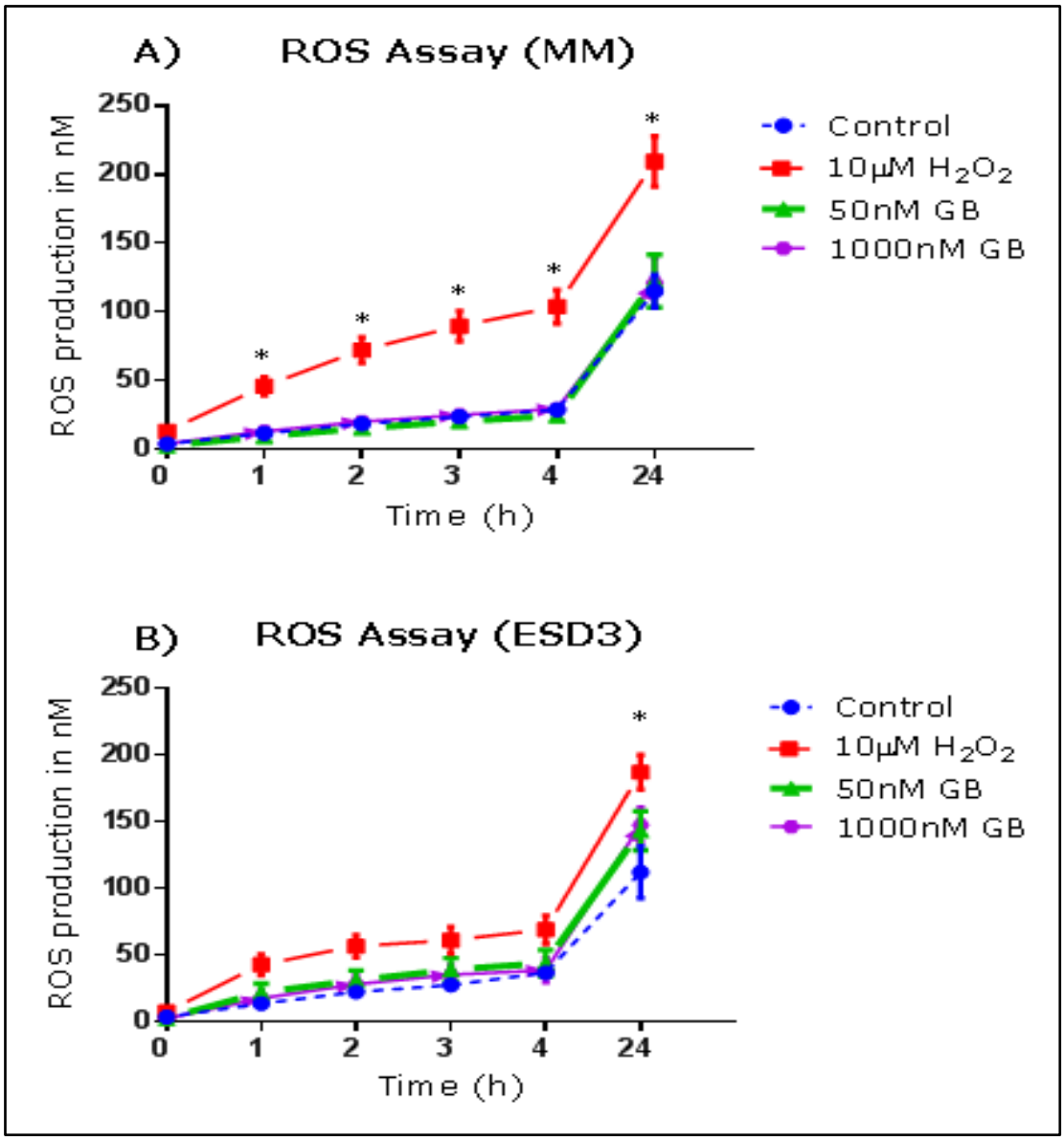


Figure 5-13: Effects of selected GB concentrations on ROS production in MM and ESD3 systems

(A) Effects of GB on ROS production in the MM system and (B) effects of GB on ROS production in the ESD3 system. The curves show that 50nM and 1000nM GB failed to increase the ROS production to significant levels in MM and ESD3 systems after 24h exposure. 10μM H_2O_2 was used as a positive control in each repeat and it shows significant ROS level elevation after 24h exposure. Three independent repeats (n=3) of each experiment were performed. The data are presented as mean ± SEM and analysed statistically using two-way ANOVA with Bonferroni post-hoc test. * $p<0.05$ significant difference from control.

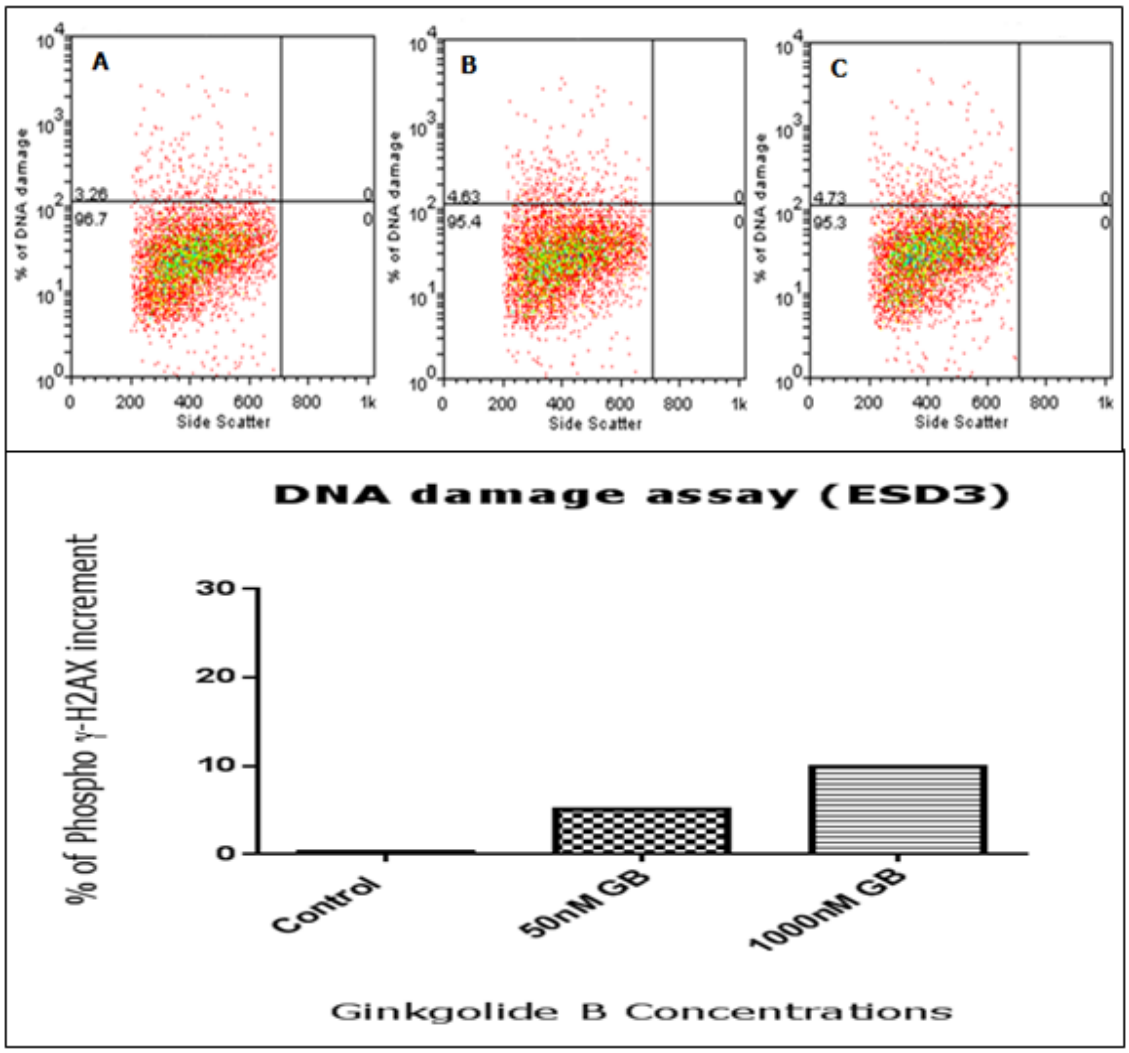


Figure 5-14: Effects of GB on DNA double strand breaks induction
(A) DNA damage dot plots of the untreated cultures, (B) DNA damage dot plots of 50nM GB and (C) DNA damage dot plots of 1000nM GB. The y-axis in the dot plot represents DNA damage and the x-axis side scatter, which represent the cell size. There was dose dependent trend of increase in the pγ-H2AX, DNA damage signal, but it failed to be significant at any concentration tested. The data from flow cytometry dot plot was converted into the % of increments as shown in bar graph. At least three independent experiments (n=3) were performed. Data is presented in percentages but the analysis was performed on raw data using one-way ANOVA. * $P<0.05$ vs. control.

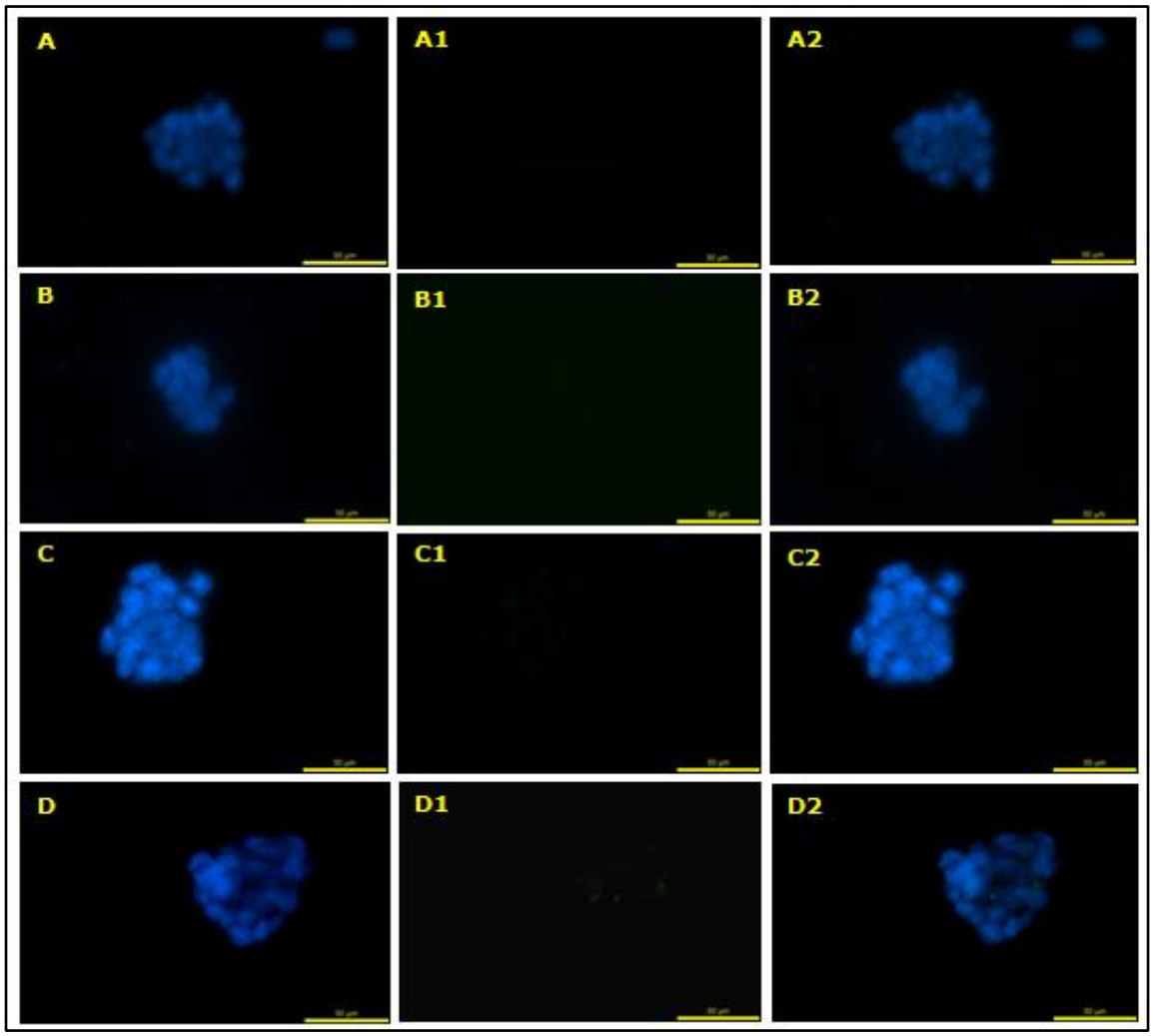


Figure 5-15: Immunocytochemistry of the effects of GB on induction of DNA damage in differentiating mouse embryonic stem cells

(A-A2) untreated control, (A) represents DAPI nuclear staining (blue), (A1) represents pϒ-H2AX (green), and (A2) represents overlay image, (B-B2) untreated control secondary antibody only, (B) represents DAPI nuclear staining (blue), (B1) represents secondary antibody only, and (B2) represents overlay image, (C-C2) 50nM GB treated stem cells, (C) represents DAPI nuclear staining (blue), (C1) represents pϒ-H2AX (green), and (C2) represents overlay image, and (D-D2) 1000nM GB treated stem cells, (D) represents DAPI nuclear staining (blue), (D1) represents pϒ-H2AX (green) and (D2) represents overlay image. The green staining indicates double-strand breaks signal within the nucleus in stem cells cultures. The green signal did not enhance at both concentrations tested. (Scale bar 50μm).

5.3.5 The effects of G-Rg1 on ROS and DNA damage induction

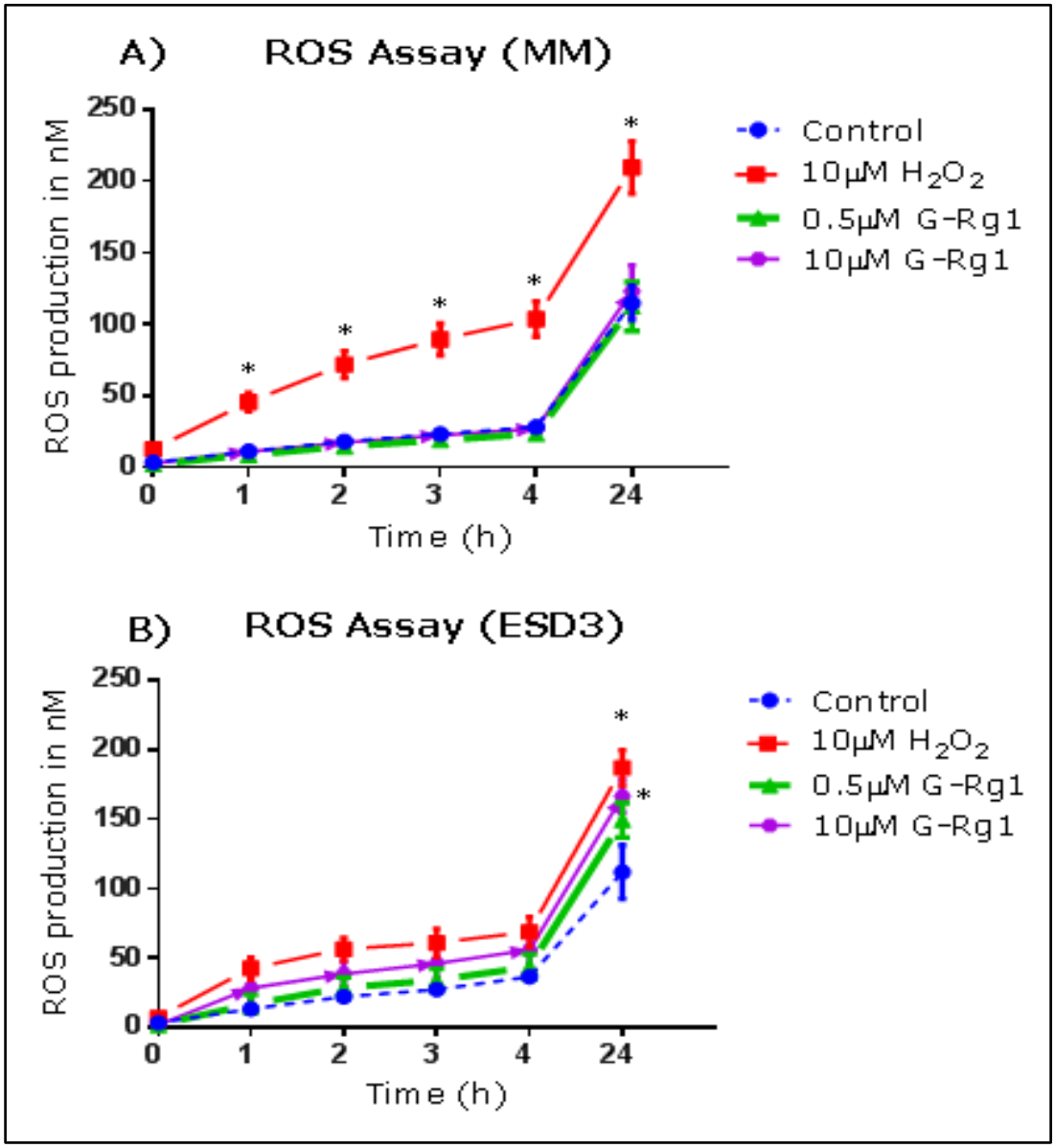


Figure 5-16: Effects of selected G-Rg1 concentrations on ROS production in MM and ESD3 systems
(A) Effects of G-Rg1 on ROS production in the MM system and (B) effects of G-Rg1 on ROS production in the ESD3 system. The curves show that 0.5μM and 10μM G-Rg1 failed to increase the ROS production to significant levels in MM system after 24h. At 0.5μM, G-Rg1 failed to elevate ROS to significant levels in ESD3 but at 10μM, G-Rg1 increased ROS in ESD3 system after 24h. 10μM H_2O_2 was used as a positive control in each repeat. Three independent repeats (n=3) of each experiment were performed. The data are presented as mean ± SEM and analysed statistically using two-way ANOVA with Bonferroni post-hoc test. * $p<0.05$ significant difference from control.

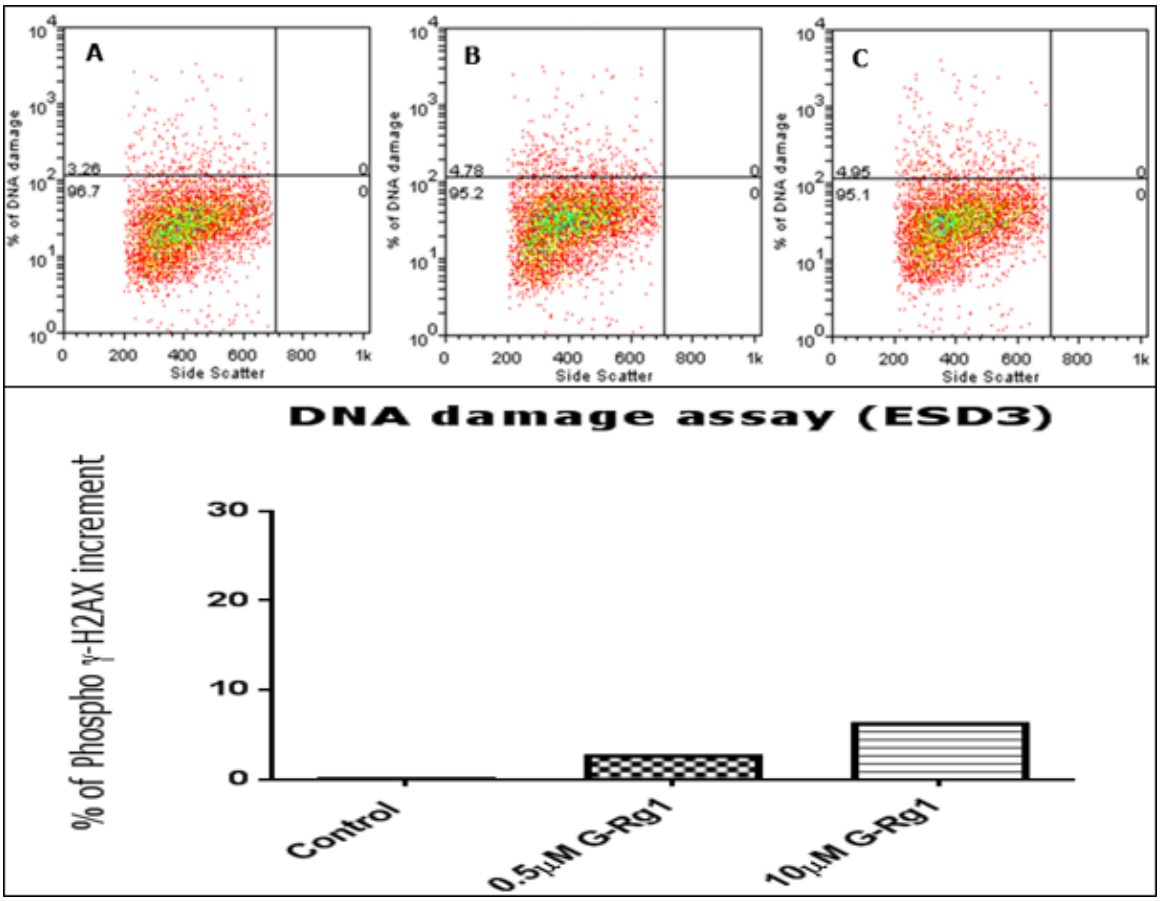


Figure 5-17: Effects of G-Rg1 on DNA double strand breaks induction
(A) DNA damage dot plots of the untreated cultures, (B) DNA damage dot plots of 0.5μM G-Rg1 and (C) DNA damage dot plots of 10μM G-Rg1. The y-axis in the dot plot represents DNA damage and the x-axis side scatter, which represent the cell size. There was dose dependent trend of increase in the pγ-H2AX, DNA damage signal, but it failed to be significant at any concentration tested. The data from flow cytometry dot plot was converted into the % of increments as shown in bar graph. At least three independent experiments (n=3) were performed. Data is presented in percentages but the analysis was performed on raw data using one-way ANOVA. * P<0.05 vs. control.

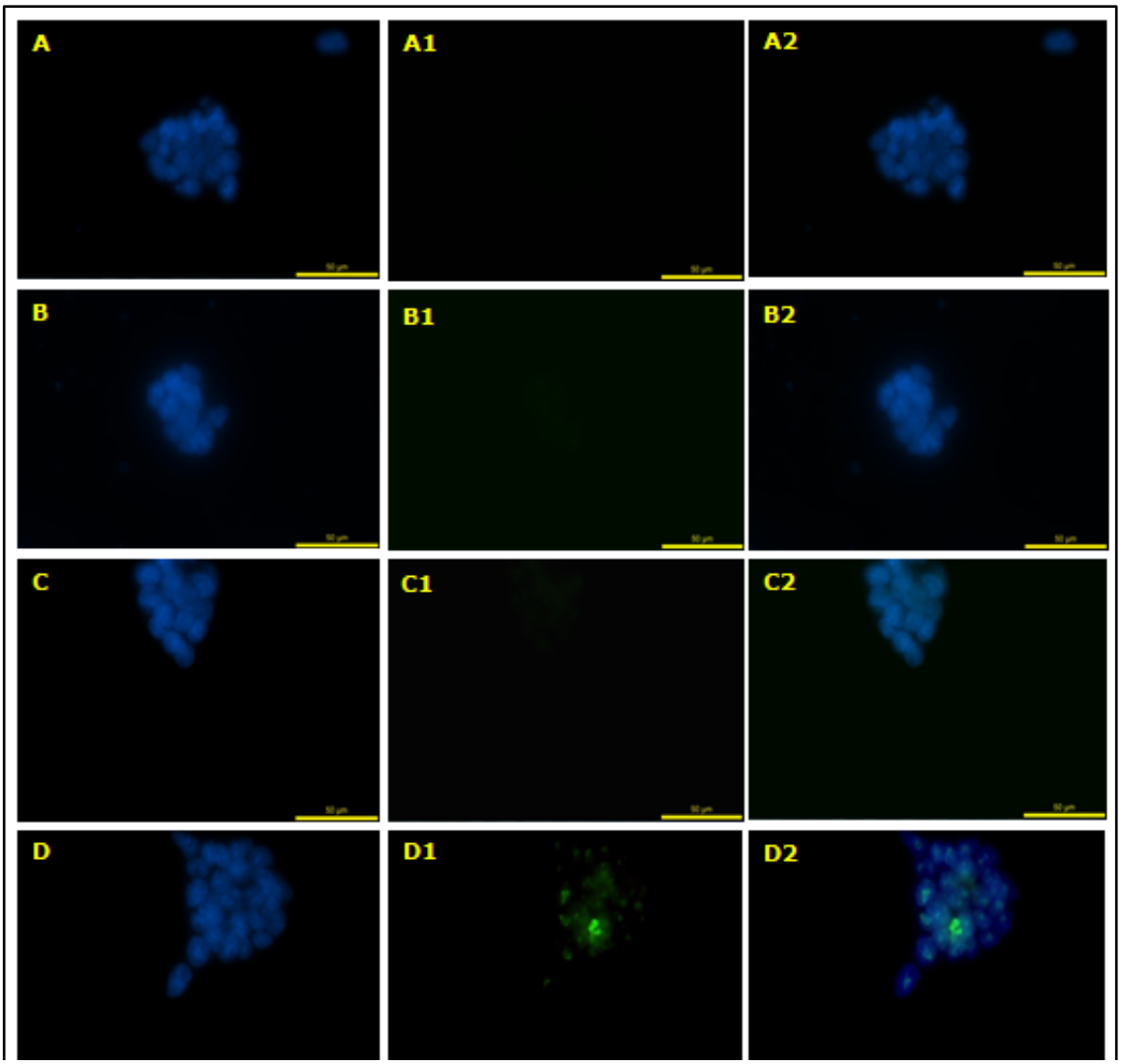


Figure 5-18: Immunocytochemistry of the effects of G-Rg1 on induction of DNA damage in differentiating mouse embryonic stem cells

(A-A2) untreated control, (A) represents DAPI nuclear staining (blue), (A1) represents pϒ-H2AX (green), and (A2) represents overlay image, (B-B2) untreated control secondary antibody only, (B) represents DAPI nuclear staining (blue), (B1) represents secondary antibody only, and (B2) represents overlay image, (C-C2) 0.5μM G-Rg1 treated stem cells, (C) represents DAPI nuclear staining (blue), (C1) represents pϒ-H2AX (green), and (C2) represents overlay image, and (D-D2) 10μM G-Rg1 treated stem cells, (D) represents DAPI nuclear staining (blue), (D1) represents pϒ-H2AX (green) and (D2) represents overlay image. The green staining indicates double-strand breaks signal within the nucleus in stem cells cultures. The green signal increased marginally at 10μM concentration tested. (Scale bar 50μm).

5.3.6 The effects of α-bisabolol on ROS and DNA damage induction

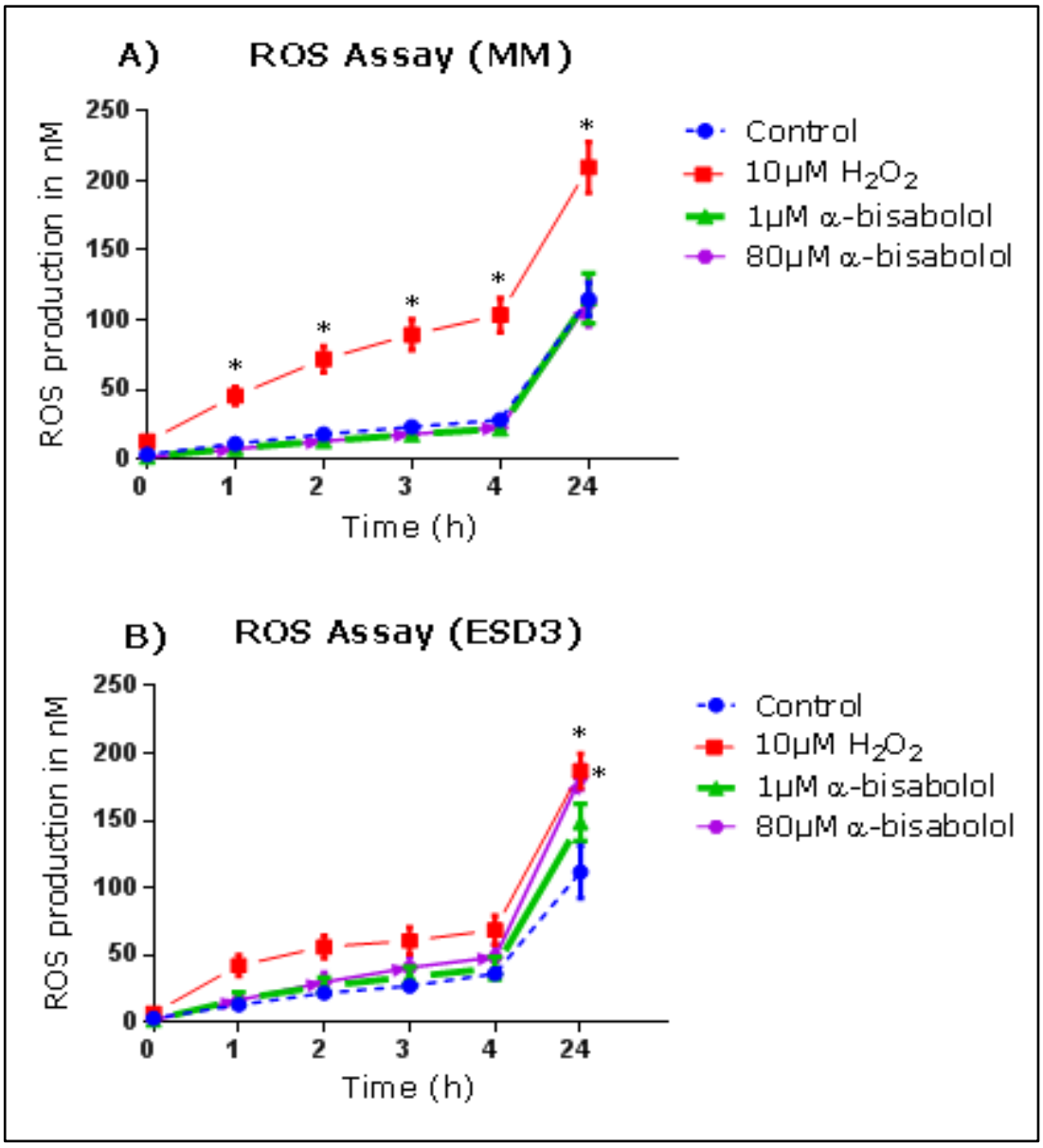


Figure 5-19: Effects of selected α-bisabolol concentrations on ROS production in MM and ESD3 systems
(A) Effects of α-bisabolol on ROS production in the MM system and (B) effects of α-bisabolol on ROS production in the ESD3 system. The curves show that 1μM and 80μM α-bisabolol failed to increase the ROS production to significant levels in MM system after 24h. At 1μM, α-bisabolol failed to elevate ROS to significant levels in ESD3 but at 80μM, α-bisabolol increased ROS in ESD3 system after 24h. 10μM H_2O_2 was used as a positive control in each repeat. Three independent repeats (n=3) of each experiment were performed. The data are presented as mean ± SEM and analysed statistically using two-way ANOVA with Bonferroni post-hoc test. * $p<0.05$ significant difference from control.

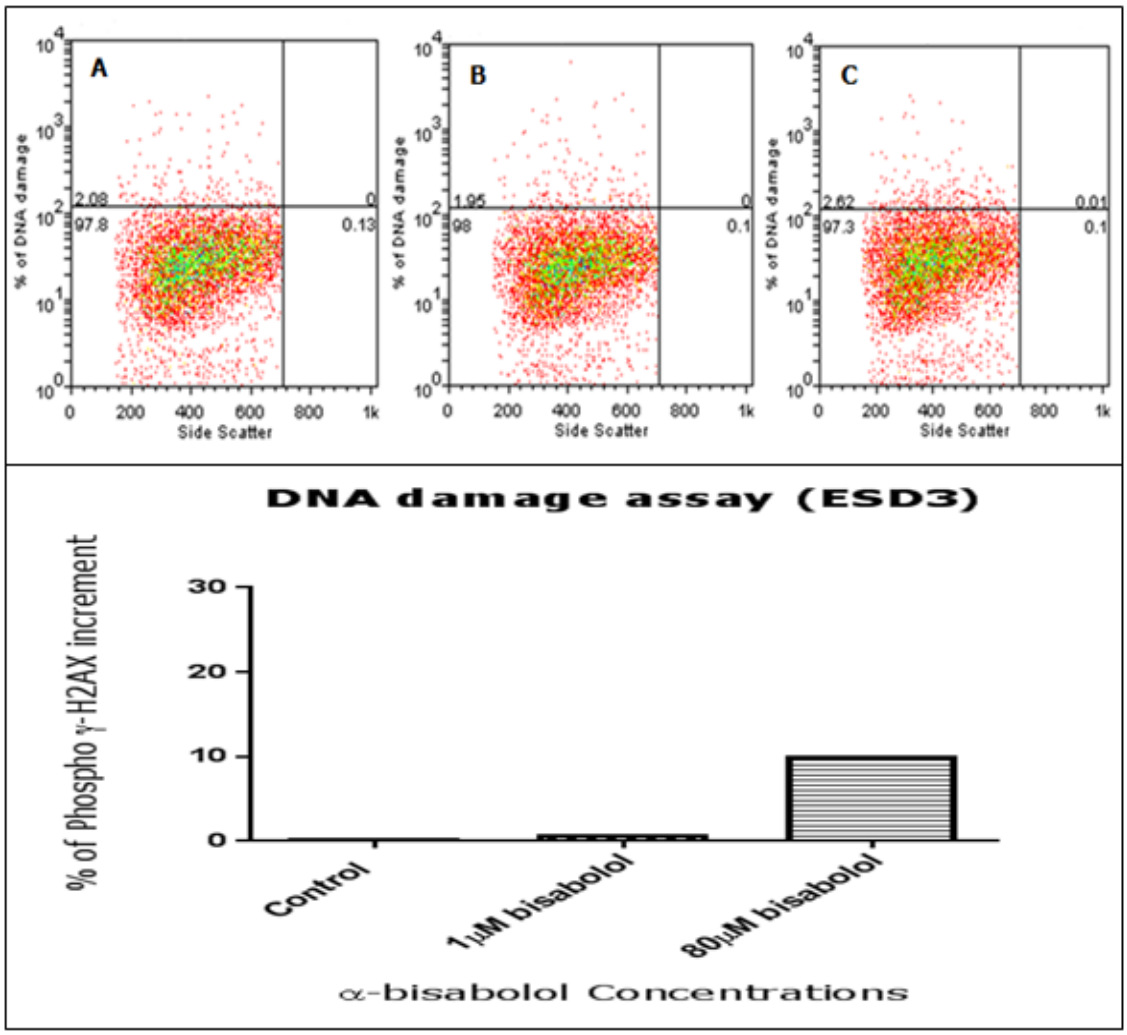


Figure 5-20: Effects of α-bisabolol on DNA double strand breaks induction
(A) DNA damage dot plots of the untreated cultures, (B) DNA damage dot plots of 1μM α-bisabolol and (C) DNA damage dot plots of 80μM α-bisabolol. The y-axis in the dot plot represents DNA damage and the x-axis side scatter, which represents the cell size. There was noticeable but non-significant increase in the pϒ-H2AX, DNA damage signal, at 80μM α-bisabolol concentration. The data from flow cytometry dot plot was converted into the % of increments as shown in bar graph. At least three independent experiments (n=3) were performed. Data is presented in percentages but the analysis was performed on raw data using one-way ANOVA. * $P<0.05$ vs. control.

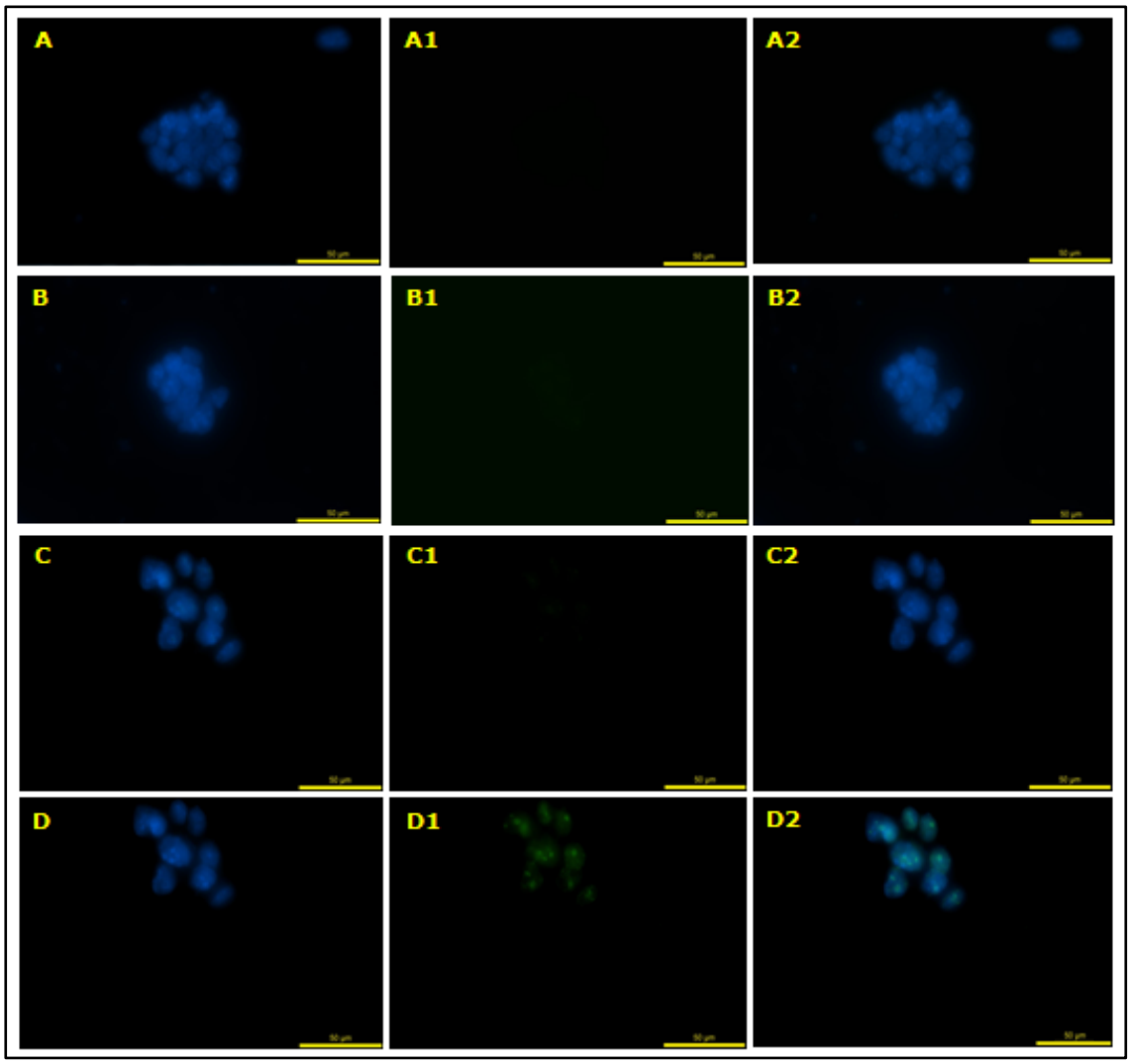


Figure 5-21: Immunocytochemistry of the effects of α-bisabolol on induction of DNA damage in differentiating mouse embryonic stem cells

(A-A2) untreated control, (A) represents DAPI nuclear staining (blue), (A1) represents pϒ-H2AX (green), and (A2) represents overlay image, (B-B2) untreated control secondary antibody only, (B) represents DAPI nuclear staining (blue), (B1) represents secondary antibody only, and (B2) represents overlay image, (C-C2) 1μM α-bisabolol treated stem cells, (C) represents DAPI nuclear staining (blue), (C1) represents pϒ-H2AX (green), and (C2) represents overlay image, and (D-D2) 80μM α-bisabolol treated stem cells, (D) represents DAPI nuclear staining (blue), (D1) represents pϒ-H2AX (green) and (D2) represents overlay image. The green staining indicates double-strand breaks signal within the nucleus in stem cells cultures. The green signal increased marginally at 80μM concentration tested. (Scale bar 50μm).

5.3.7 The effects of eugenol on ROS and DNA damage induction

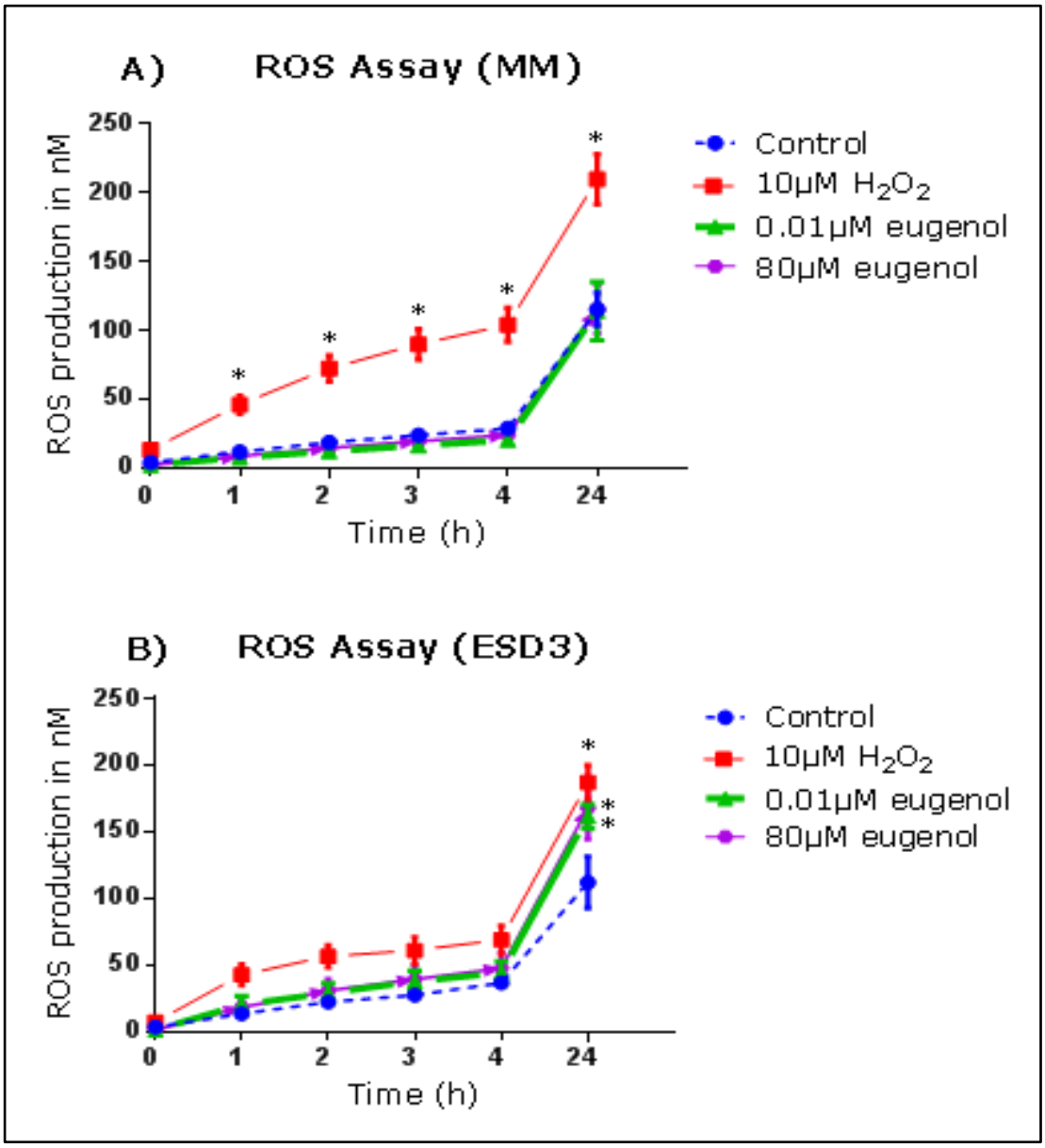


Figure 5-22: Effects of selected eugenol concentrations on ROS production in MM and ESD3 systems

(A) Effects of eugenol on ROS production in the MM system and (B) effects of eugenol on ROS production in the ESD3 system. The curves show that 0.01μM and 80μM eugenol failed to increase the ROS production to significant levels in MM system after 24h. However, both tested concentrations of eugenol significantly increased ROS in ESD3 system after 24h. 10μM H_2O_2 was used as a positive control in each repeat. Three independent repeats (n=3) of each experiment were performed. The data are presented as mean ± SEM and analysed statistically using two-way ANOVA with Bonferroni post-hoc test. * $p<0.05$ significant difference from control.

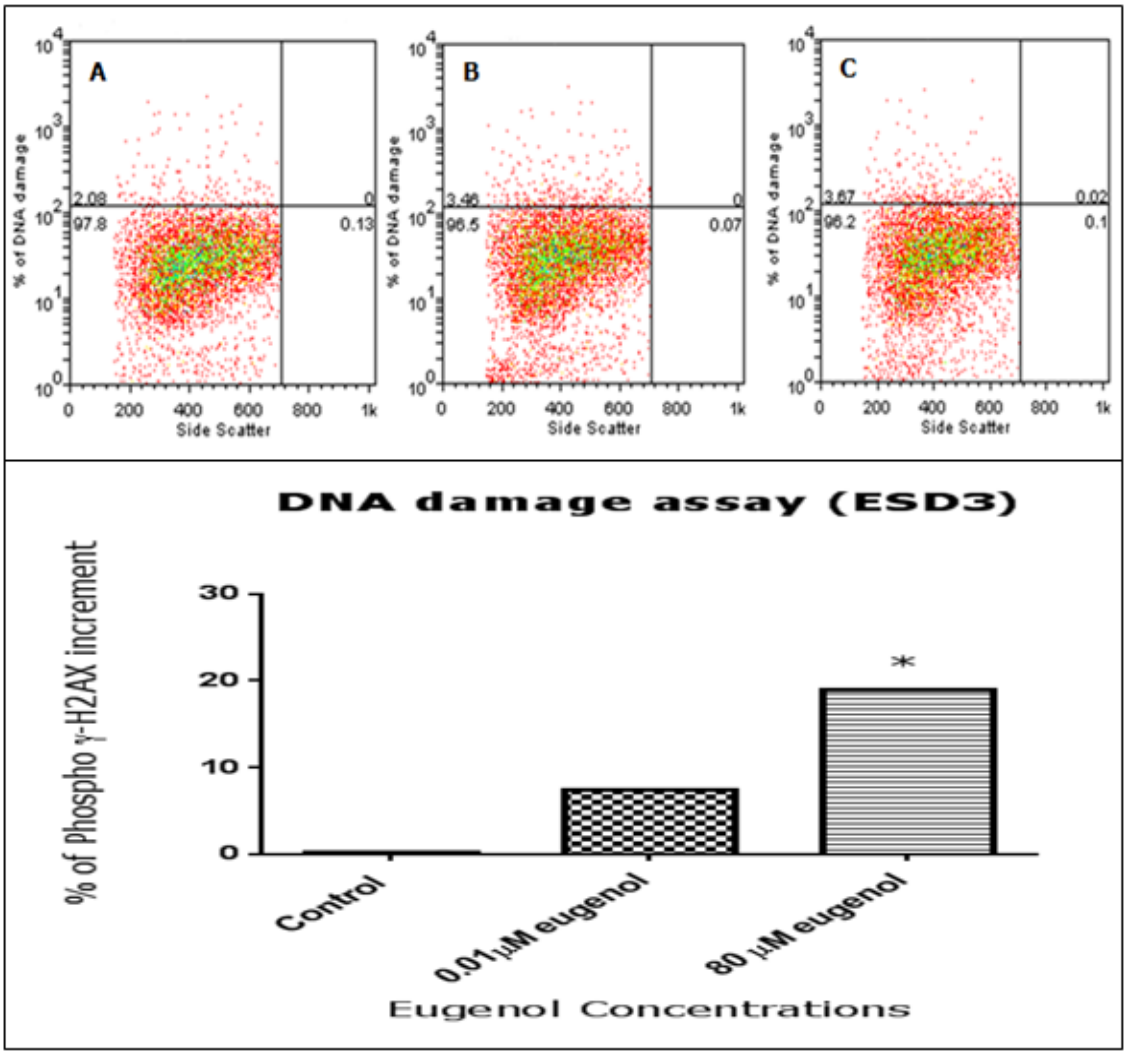


Figure 5-23: Effects of eugenol on DNA double strand breaks induction
(A) DNA damage dot plots of the untreated cultures, (B) DNA damage dot plots of 0.01μM eugenol and (C) DNA damage dot plots of 80μM eugenol. The y-axis in the dot plot represents DNA damage and the x-axis side scatter, which represents the cell size. There was noticeable but non-significant increase in the pY-H2AX, DNA damage signal, at 0.01μM eugenol concentration. However, 80μM eugenol showed significant increase in DNA damage. The data from flow cytometry dot plot was converted into the % of increments as shown in bar graph. At least three independent experiments (n=3) were performed. Data is presented in percentages but the analysis was performed on raw data using one-way ANOVA. * $P<0.05$ vs. control.

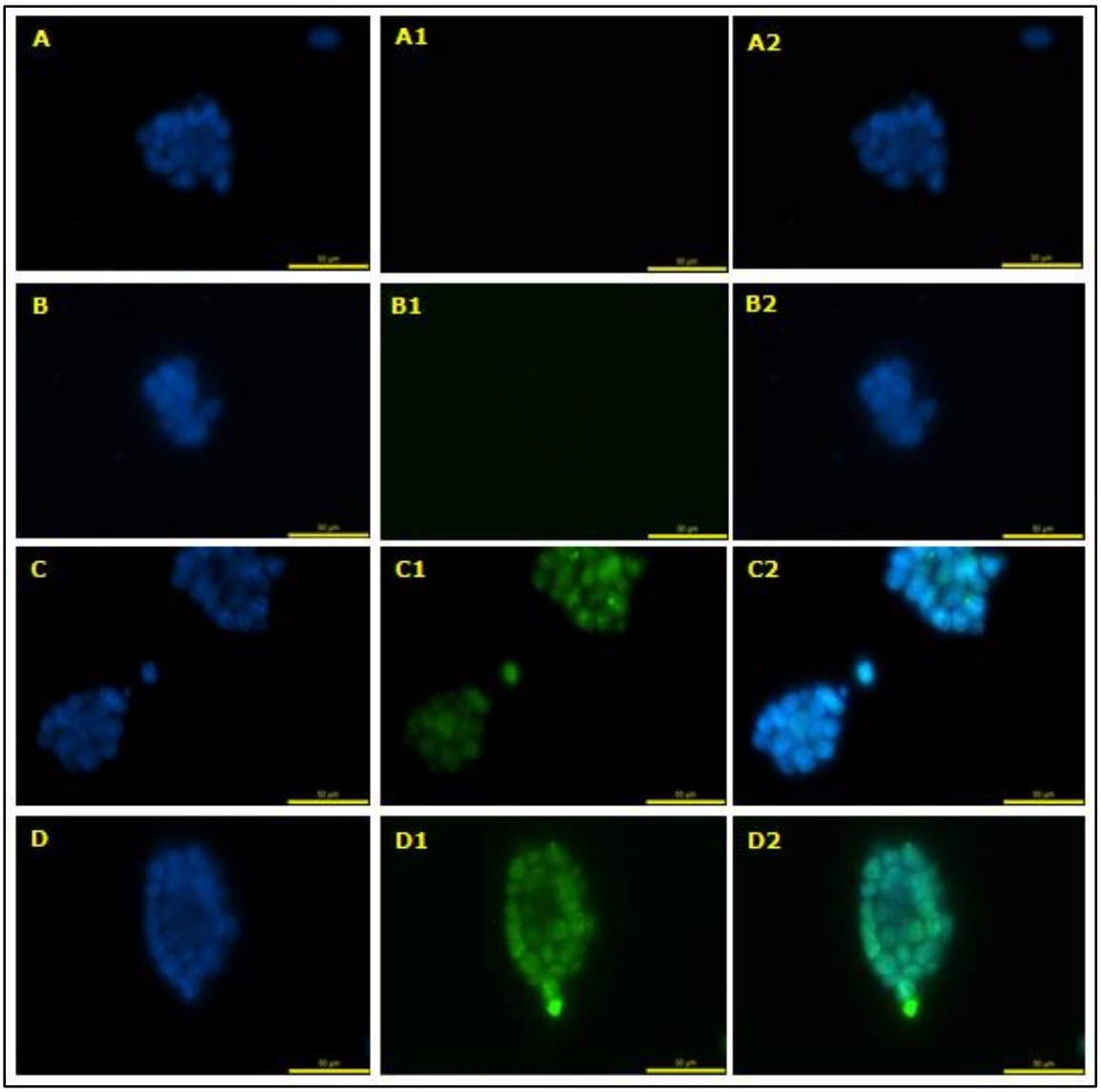


Figure 5-24: Immunocytochemistry of the effects of eugenol on induction of DNA damage in differentiating mouse embryonic stem cells
(A-A2) untreated control, (A) represents DAPI nuclear staining (blue), (A1) represents pϒ-H2AX (green), and (A2) represents overlay image, (B-B2) untreated control secondary antibody only, (B) represents DAPI nuclear staining (blue), (B1) represents secondary antibody only, and (B2) represents overlay image, (C-C2) 0.01μM eugenol treated stem cells, (C) represents DAPI nuclear staining (blue), (C1) represents pϒ-H2AX (green), and (C2) represents overlay image, and (D-D2) 80μM eugenol treated stem cells, (D) represents DAPI nuclear staining (blue), (D1) represents pϒ-H2AX (green) and (D2) represents overlay image. The green staining indicates double-strand breaks signal within the nucleus in stem cells cultures. The green signal increased marginally at 0.01μM but massively at 80μM eugenol concentration tested. (Scale bar 50μm).

Herbal components	ROS (MM)	ROS (ESD3)	DNA damage (ESD3)
Low **6G** concentration (3 µM)	×	**N/A**	**N/A**
Low **6G** concentration (0.75 µM)	**N/A**	✓	×
High **6G** concentration (100µM)	✓	✓	✓
Low **GA** concentration (100nM)	×	×	×
High **GA** concentration (2000nM)	×	✓	×
Low **GB** concentration (50nM)	×	×	×
High **GB** concentration (1000nM)	×	×	×
Low **G-Rg1** concentration (0.5µM)	×	×	×
High **G-Rg1** concentration (10µM)	×	✓	×
Low **bisabolol** concentration (1µM)	×	×	×
High **bisabolol** concentration (80µM)	×	✓	×
Low **eugenol** concentration (0.01µM)	×	✓	×
High **eugenol** concentration (80µM)	×	✓	✓

Table 5-1: The summary of the results of ROS production and DNA damage for tested herbal components in both MM and ESD3 systems
(N/A): not applicable concentration, (x): the concentration does not show significant alteration and (✓): the concentration shows significant alteration.

5.4 Discussion

The molecular mechanisms that might be involved in the phytochemical-induced defects are still obscure. In the present Chapter, ROS and DNA damage tests were performed for the tested herbal components (6G, GA, GB, G-Rg1, α-bisabolol and eugenol) in addition to AA and SOD, to investigate their influence on the development of embryonic cardiomyocytes.

In general, it was noted that ESD3–derived differentiating EBs were more likely to produce ROS than chick embryonic differentiated cardiomyocytes. This could be because the stem cells have less defensive mechanisms during differentiation than already differentiated embryonic cells. In their study (2008), Saretzki *et al.* (2008) found that two human stem cell lines developed more mitochondrial superoxide and cellular levels of ROS production during differentiation. Also, the expression of main antioxidant genes such as DNA repair genes was downregulated despite this increased oxidative stress [395].

5.4.1 6 Gingerol-induced ROS and DNA damage

Regarding 6G, the results showed that ROS levels were elevated at 100μM in MM but at both tested concentrations (0.75 and 100μM) in ESD3 system. Several studies have reported the ability of 6G to induce ROS and apoptosis in various tumor cell lines. In accordance with our findings, Nidhi *et al.* (2009) used human epidermoid carcinoma A431 cells to investigate anti-apoptotic features of 6G. Treatment the cells with 250μM-350μM 6G exhibited significant cytotoxicity as indicated by inhibited proliferation of A431 cells which was mediated via generation of ROS. Increase in ROS led to decrease in mitochondrial membrane potential followed by induction of apoptosis [339].

Further, another study used HeLa cells with 6G concentrations range from 85μM to 600μM. It has been concluded that 6G has the capability to combine with DNA and induce cell death by autophagy and caspase 3 mediated apoptosis [294]. In contrast, another study has shown a protective potential of 6G through inhibiting ROS in lipopolysaccharide-stimulated macrophages at concentrations 20μM up to 80μM in a time and dose-dependent manner [340]. The results herein showed that 6G induced DNA damage in ESD3 in a concentration-dependent manner, but it became significant at high concentration, 100μM. Conclusively, ROS generation and DNA damage could play a role in embryonic cardiac developmental disturbances, particularly at high doses. This study is unable to distinguish whether DNA damage or ROS is a cause or a result of each other. Therefore, further studies for better understanding of 6G toxicity pathways are recommended.

5.4.2 Ginkgolides induced ROS and DNA damage

The two tested herbal components, GA and GB showed less ROS production and DNA damage potential in both systems. At all tested concentrations, 100nM and 2000nM for GA and 50nM and 1000nM for GB, only high GA concentration induced ROS in ESD3 system but all other tested concentrations for both components failed to modify ROS levels or DNA damage in both systems. Accumulating evidence reveal that the increase in free radicals and associated lipid peroxidation reactions are inhibited in heart and CNS upon treatment with extracts *of* Ginkgo biloba leaves [449,450]. However, a new study was published in 2015 revealing that Ginkgo biloba leaves extract at concentration 0.2 – 1.2 mg/ml induced genotoxicity and DNA damage in human hepatic HepG2 cells [451]. Further, as has been previously mentioned

in Chapter 3, GA and GB showed apoptosis induction potential and reduction in viability in blastocysts [300]. Collectively speaking, the role of ROS and DNA damage cannot be ruled out as possible mechanisms for GA and GB-induced embryonic cardiotoxicity, particularly at high concentrations.

5.4.3 G-Rg1 induced ROS and DNA damage

The results of G-Rg1 showed at two tested concentrations, 0.5 and 10μM, only 10μM induced ROS in ESD3 system but all other endpoints failed to modify ROS levels or DNA damage in both systems. In a recent study, *in vitro* porcine embryos were exposed to G-Rg1 and has been found that 20μg/ml did not induce ROS, DNA damage or apoptosis and may still be safe for the development of preimplantation porcine embryo [452]. Yu *et al.* (2015) have found that G-Rg1 has a protective potential against diabetes-induced myocardial apoptosis in a rat model perhaps through inhibition of ROS and enhanced antioxidant enzymes [453]. Our report herein may be the first that shows G-Rg1 at 10μM can induce ROS production in the differentiation of EBs to cardiomyocytes.

5.4.4 α-Bisabolol induced ROS and DNA damage

Exposure of the chick and mouse embryonic cells in both systems to 1μM bisabolol did not cause ROS elevation or DNA damage but at 80μM, only ROS significantly differed but this was not accompanied by DNA damage. Bisabolol has been confirmed by several studies to promote apoptosis and reduce viability in various tumor cell lines. In one study, glioma (tumor cell) and normal glial cells were treated with 3.5μM bisabolol, and it was found that bisabolol reduced viability in glioma but not glial cells by induction of apoptosis [357]. Darra *et al.* (2008) rationalised the selective potential of bisabolol

toward carcinomas rather than normal cells by suggesting the ability of the molecule to bind and release mitochondrial proapoptotic proteins such as Bid [454]. However, the ROS elevation in the ESD3 system could happen through disturbing mitochondrial activities. This has been reported by Cavalieri *et al.* (2009), where they found that ROS production observed in the 5μM bisabolol treated T67 cell line (astrocytoma) was not the direct cause of apoptosis, but could be as a consequence of the loss of mitochondrial integrity [455].

5.4.5 Eugenol induced ROS and DNA damage

Treatment of growing chick embryonic cardiomyocytes with 0.01 or 80μM did not increase ROS production. In contrast, when mouse embryonic stem cells derived EBs were exposed to eugenol at 0.01 or 80μM, ROS production increased at both tested concentrations. This elevation was accompanied by DNA damage with only 80μM. Similar to our findings, Yoo *et al.* (2009) have found that ROS plays a vital role in eugenol-induced apoptosis in HL-60 (cancer cells) which also evoked DNA fragmentations and apoptotic cell death at 40μM for 1h [456]. However, Atsumi *et al.* (2005) have reported that eugenol-treated human submandibular cells showed biphasic ROS production, enhanced at 5-10μM and decreased at higher concentrations 500μM. In his report, Atsumi and his colleagues argued that eugenol may have pro-oxidant and antioxidant properties depending on the dose levels, but it was concluded that eugenol has an ROS-independent pathway [457]. The difference from this study could be that ESD3 are more vulnerable than the human submandibular cell line.

5.4.6 Summary

For tested herbal components, only 6G in MM at high concentration led to increasing in ROS production. In the ESD3 system, only 6G and eugenol showed an increase in ROS production at both low and high levels tested where this was associated with DNA damage of differentiating ESD3 only at high concertation tested. Only GB showed no sign of ROS level elevation or DNA damage in both systems at any concentration tested. GA, G-Rg1 and bisabolol showed ROS levels increasing only at high tested concentrations but without causing DNA damage.

CHAPTER 6

6 Chapter six - The effect of herbal components on the expression of Connexin43 and Integrin β1 in the MM system and α-actinin in ESD3 system

6.1 Introduction

Cell-to-cell cross talk is deemed a required process during many complex events underlying embryonic development, to direct the cells in specific multi-oriented patterns for each tissue subsystem [458]. Intercellular communications are controlled by various factors such as cell - extracellular environment interactions, chemical signals (cytokines, growth factors and hormones), mechanical signals (contractions, stretch and pressure) and electrical signals which are mainly controlled by ion channels [459]. However, the key location that governs cell communications is the cell surface which provides receptor-mediated signal exchange via messenger molecules or via direct cell-cell exchange of small molecules through gap junctional plaques [459].

6.2 Gap junctions

Gap junctions (GJs) are integral transmembrane proteins that provide intercellular connections to allow direct cytoplasmic diffusion of ions molecules and low molecular weight metabolites between adjacent cells. These connections are essential in many physiological processes such as proliferation,

differentiation, migration, electrical activation of the heart and of smooth muscles or neuronal signalling where the transmission of the electrical signal and transfer of second messenger signals are required [460,461]. The term "gap" stems from the 2-3 nm space between the plasma membrane of the two adjacent cells linked by such channels [462]. Previously, GJ channels were regarded as fixed passive conduits, but it has been discovered that these channels are continuously regulated by several factors. The level of intercellular communications varies, but it is believed to be regulated by factors such as changes in junctional protein expression, regulation of their trafficking, turnover and modulation of channel properties, variations in the level of intracellular Ca^{2+}, pH, phosphorylation status and in response to the stimulation of membrane receptors [463].

6.2.1 Molecular structure of GJs

At each GJ, a hemichannel is formed by assembling hexameric oligomers, called connexons, from one cell which couple noncovalently across a tight extracellular gap with the adjacent cell [464]. The connexon hexagonal unit is composed of six assembled subunits of connexins (Cxs) (Figure 6-1A), or innexins, in vertebrates or invertebrates, respectively [465]. The multiplicity of Cxs is clear as there are more than 20 Cx isoforms which have been identified to date in several species including humans [465]. Homomeric connexons consist of the same isoforms of Cx proteins, whereas heteromeric connexons contain different isoforms of Cx proteins. Hence, oligomerisation of two identical connexons can construct a homotypic hemichannel whereas docking two different connexons can generate a heterotypic hemichannel [466]. It has been shown that each Cx protein consists of nine domains,

namely, four transmembrane domains, two extracellular loops, one cytoplasmic loop, the C-terminus and the N-terminus [467] (Figure 6-1B). While the transmembrane domains are quite conserved, the C-terminus amino acid sequence was found to be related to the molecular mass for each Cx isoform [468]. Head to head docking of two connexons to form a gap junction is enhanced by three conserved cysteine residues in extracellular loops [468,469].

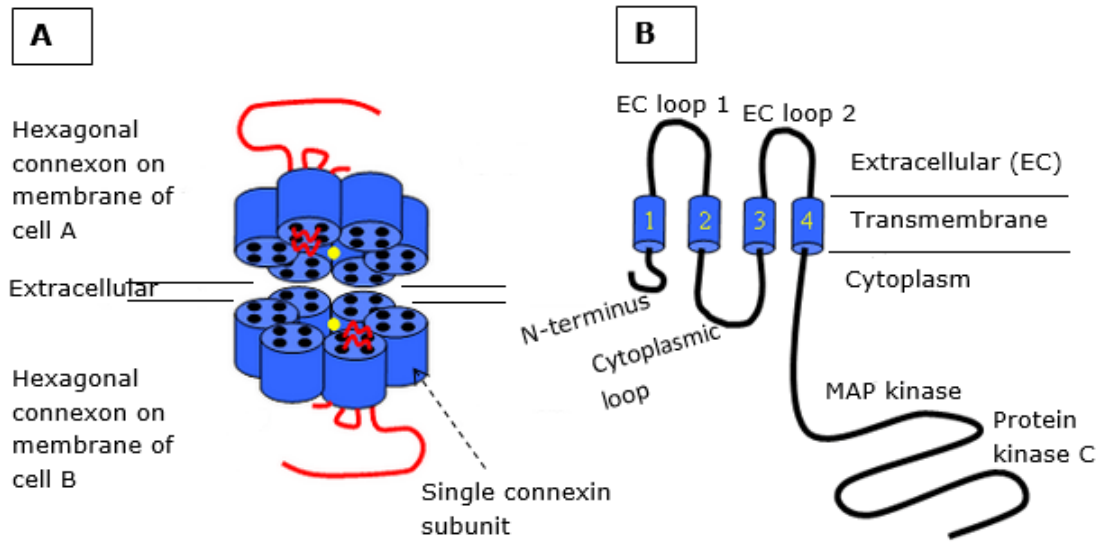


Figure 6-1: Gap junction structure
(A) Connexon hexagonal (six connexin subunits) channel formed by two cells. (B) Singly connexin unit. The figure was adapted from this website: http://www.unmc.edu/biochemistry/faculty/sorgen.html

6.2.2 Connexin phosphorylation and regulation

It has been shown that the connexin protein is subjected to several post-translational changes such as phosphorylation, hydroxylation, acetylation, disulfide bonding, and it interacts with other cellular proteins as regulatory modulators of its function [470]. However, the most investigated type of modification is connexin phosphorylation at various residues. It was confirmed that connexin export to the plasma membrane, assembly into gap junctions, gating and biotransformation all appear to be regulated via phosphorylation [470]. Among the several types of Cx, it was found that Cx43 is the most predominantly expressed connexin in tissues, cell types or *in vitro* cell lines so more is known about phosphorylation of Cx43 than any other connexin [471]. While the cytoplasmic loop does not contain serine residues, the C-terminal domain seems to be the main region that becomes phosphorylated in Cx43. The Cx43 phosphorylation can be achieved by activation of several kinases including protein kinase A (PKA), protein kinase C (PKC), casein kinase 1 (CK1), MAPK [471]. The effect of Cx43 phosphorylation on GJ channel gating is very specific and selective in that the action of the kinase may result in inhibition or enhancement of GJs depending on which residues are phosphorylated [472]. For instance, PKC activation was found to increase the phosphorylation of Cx but decrease the activity of GJ intercellular communications through down-regulation of intercellular coupling [473]. In contrast, the activation of the PKA pathway was found to increase serine 364 (S364) phosphorylation of Cx43 which in turn led to an increase in GJ intercellular communications [474].

6.2.3 Role of Cx43 in embryogenesis and embryonic cardiogenesis

Many studies have been published in the past century confirming the role of GJs in most embryonic developmental stages. Granot and Dekel in 1998 have shown that GJs enhance transport of nutrients and cAMP from follicle cells to oocytes during gametogenesis to support their growth and development [475]. It has also been confirmed that GJs are expressed during the early blastula stage, but also continue existence onward during the embryogenesis stages [476,477]. Furthermore, Hardy *et al.* (1996) have found that Cx43 is widely expressed in a preimplantation stage of the human embryo [478]. As far as the embryonic heart is concerned, there have been several Cx isoforms (Cx40, Cx45, Cx37 and Cx50) discovered which are believed to control cardiovascular development, but Cx43 was the first Cx isoform identified in the heart [479,480]. Ahir in his review (2014) has stated that Cx43 is expressed in atrial and ventricular myocytes at all stages of embryonic development, myocardial conduction tissues and coronary and aortic smooth muscle cells and is principally responsible for electrical synchrony in the heart [481]. As a consequence, Cx43 disruption or knock down has been associated with congenital heart defects in different species. Wei *et al.* (2004) have stated that Cx43 plays a vital role in the development of the coronary arteries and outflow tract morphogenesis [462]. Furthermore, Vinken *et al.* (2006) have reported that disruption of Cx43 in mice leads to right ventricular outflow tract obstruction, causing cyanosis and death at birth [467]. However, it was noticed that even in the presence of this abnormality, the hearts of these Cx43 null animals still contract rather rhythmically, though not synchronously throughout the tissue, indicating that gap junction proteins other than Cx43

may partially compensate for the loss of intercellular connections [482]. Moreover, Giepmans in 2004 has shown that Cx43 knockout in mice led to dysregulation of cardiac neural crest cell migration during embryonic development which eventually resulted in heart malformation [483]. Also, Kirchhoff *et al.* (2000) developed Cx43 and Cx40 knockout mouse models, and it has been concluded that these two Cx genes are crucial for the development of the endocardial cushion, outflow tract morphogenesis and development of coronary arteries respectively [484]. Interestingly, it has been previously demonstrated in a mouse model that not only Cx43 deficiency, but also overexpression, exhibited cardiac developmental defects including pulmonary outflow tract obstruction and conotruncal heart defects [485,486]. Despite that fact some considerable differences in GJs distribution have been identified between human and mouse heart [487], these transgenic and Cx knockout mouse models have established that GJ proteins, including Cx43, play a significant role in embryonic heart morphogenesis including cardiac conduction systems.

6.3 Transmembrane proteins (receptors)

Cells in every higher organism are surrounded by membranes, which in turn provide hydrophobic and highly dynamic barriers for maintaining equilibrium. Communications on the outside and inside of the membrane govern cellular localization and integrity. However, nutrients, signals and products have to be able to traverse the membrane in a specific fashion, often against a gradient in order to sustain cellular requirements and enable the cells to grow, migrate or differentiate [488]. Membrane proteins are incorporated and span the

entirety of 'lipid bilayer' cell membrane. Integral membrane proteins can be classified into two basic architectures, the α-helix bundle and the β-barrel. As their names infer, helix bundle proteins are built from long transmembrane helices that pack together into twisted bundles, whereas β-barrel proteins are antiparallel sheets revolved into a barrel closed by the first and last strands in the sheet [489]. However, other proteins such as cyclooxygenase 1 and 2 are only attached to one leaflet of the lipid bilayer of the membrane without penetrating it; these are termed monotopic membrane proteins [490]. It has been reported that approximately half of all proteins encoded by eukaryotic genomes are membrane proteins and almost 50% of biological processes take place on membranes. As a consequence, membrane proteins were viewed as key players in cellular physiology [491]. Integrin β1, as a transmembrane protein, is discussed in more detail in the next Sections.

6.3.1 Integrins

Extracellular milieu (ECM) materials provide cells with a structural and biological substrate that is vital for normal development and responses to pathophysiological signals. The main links between extracellular matrix ligands and intracellular structures are glycoprotein transmembrane receptors which are termed integrins. These proteins act as "integrators" of the ECM and cytoplasm, the feature from which integrin name is derived. They serve through the cell membrane bidirectionally; inside-out signaling where the extracellular interaction activity of integrins is controlled from the inside of the cell, and the outside-in signaling in which the interaction of the ECM stimulates signals that are conveyed into the cell [492,493]. Integrins organise multiple processes in the organism including organogenesis, regulation of gene

expression, cell proliferation, differentiation, migration, apoptosis and death. Most cell types can express integrins. A variety of integrin receptors can be expressed in a single cell to allow interaction with many ECM ligands [494]. It has been previously shown that integrins are expressed in cells of the vasculature, blood, neurons, cardiac myocytes and non-myocytes. Reports have stated that integrins are required for heart development and function, in addition to their role in the development of cardiac diseases [494].

6.3.2 Integrin structure

Integrins are heterodimeric transmembrane proteins compose of α and β subunits being held together by non-covalent interactions. Integrin subunits range from 90 to 180 kDa and both subunits comprise of a large extracellular domain with 700–1100 residues, a single transmembrane span and short cytoplasmic tails, ranging from 20 to 60 residues [495]. Vertebrates contain a multiple integrin family made up of 18 α and 8 β integrin subunits which heterodimerise to form 24 different receptors [492]. What is termed a globular head presents in about half of α subunits at the N-terminus, which includes seven repeating homologous sequences each folded in a β propeller. The remaining α subunits contain what is called the I-domain, which is inserted into the β propeller fold. The ligand-binding site in the α subunit consists of the I-domain and the metal ion-dependent adhesion site or ‘‘MIDAS’’ motif [494]. A similar structure to the I-domain of the α chain has also been located in β subunits and is considered as the ligand binding site within β chains [496]. The short cytoplasmic domain of integrins is thought to be the signal transducer and the part interacting with the cytoskeleton [494]. A structure model is presented in Figure 6-2.

6.3.3 Integrin-activated signal pathways

It has been established that integrins lack any intrinsic enzymatic activity, but they associate with cytoskeletal proteins of the actin contractile system. The main sequence after integrin activation starts with integrin receptor clustering on the cell membrane, which is followed by the triggering of a cytoskeleton signal through the cytoplasmic domain of the integrin receptor, which permits actin polymerisation [493]. The ensuing actin assembly results in the formation of small patches/aggregates of EC ligand-integrin-cytoskeleton proteins where this contact point known as "focal adhesion" which is also termed a "costamere" in the cardiomyocytes where it provides a firm connection between ECM and sarcomere. Other cytoskeletal proteins such as talin, α-actinin, vinculin, tensin and paxillin also accumulated at these contact points in order to bridge integrins to the actin filaments [497], but also to transmit signals to regulate cell movement, survival, proliferation, migration, differentiation and cytoskeleton reorganization [498]. Finally, an actin-myosin stress fibre is formed to provide firmness to the cell and a mechanosensitive link between the extra- and intracellular environments [499]. It has been shown that several protein tyrosine kinases are activated by integrins such as focal adhesion kinase (FAK), Src family kinases, a serine-threonine kinase and integrin-linked kinase (ILK) [500-502]. However, FAK is one of the few integrin-dependent pathways that have been studied extensively. Although the activation of FAK is not well understood, it is found to be combined with the assembly of focal adhesions. The FAK recruited to focal adhesions interacts with the cytoplasmic tail of integrin β subunits, either directly or through talin [503], α-actinin [504] and paxillin [505] proteins to be activated. Once FAK is

activated, it autophosphorylates at Tyr^{397} and creates a binding site for several adapter and cytoskeleton proteins which connect FAK to signalling pathways that eventually activate MAPK cascades to modify transcription factors accordingly [493]. Thus, it is likely that FAK, alone or by liaising with other pathways, contributes to the activation of the Ras–extracellular signal-regulated kinase (ERK) as well [493]. Figure 6-2 summarises the main intracellular events for integrin receptor activation.

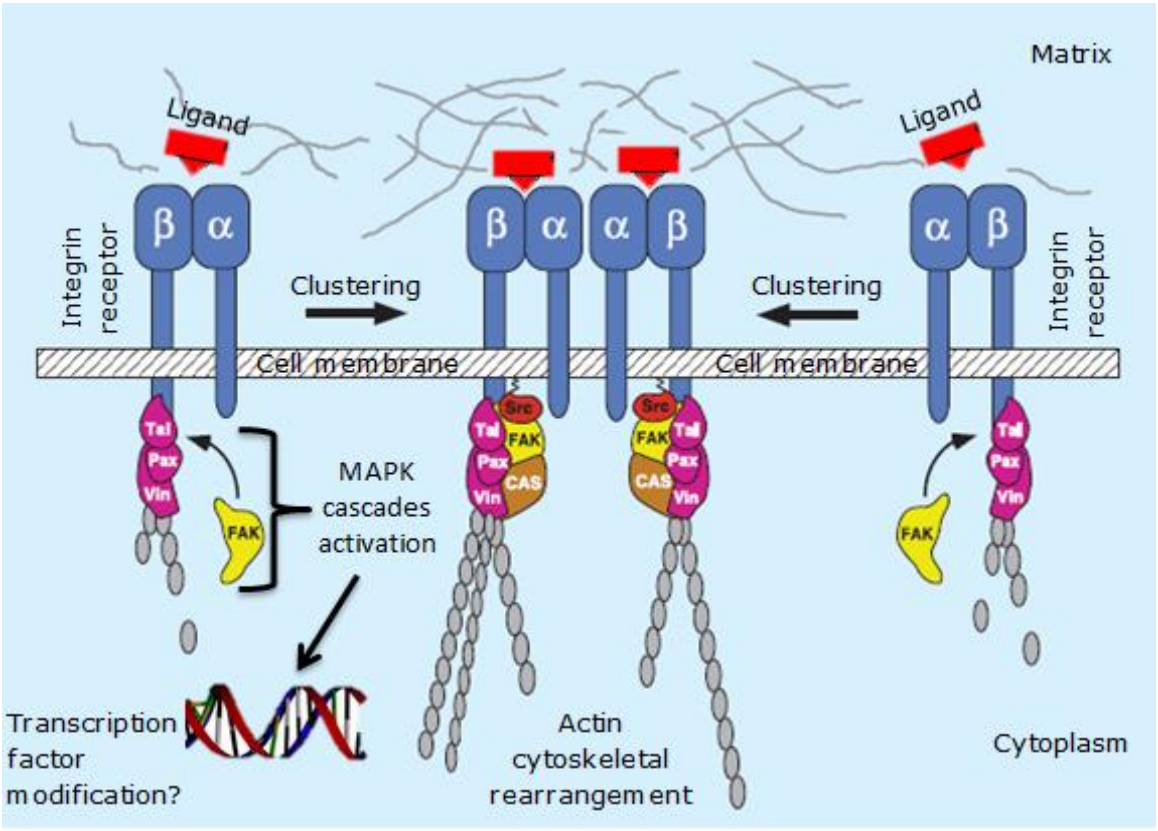


Figure 6-2: Schematic model of integrin receptor structure and signalling
Ligand binding enhances integrin clustering and linking with the cytoskeleton. This in turn provides positive feedback mechanism in terms of promoting further integrin clustering and matrix organization in a system. Tal, talin; Pax, paxillin; Vin, vinculin; CAS, p^{130}CAS. The figure was adapted from [463].

6.3.4 The role of integrin β1 in embryogenesis and heart development

It has been reported that integrin β1 is expressed ubiquitously [506]. Stephens *et al.* (1995) studied the role of integrin β1 in embryonic development, using integrin β1 null mouse embryos, and have shown that that integrin β1 is essential for normal morphogenesis of the inner cell mass and hence for implantation [507]. Integrins were also found to be required for the differentiation of visceral endoderm in mESD3 derived EBs where Liu *et al.* has reported in 2009 that β1 subunit ablation resulted in endoderm detachment [508]. Furthermore, integrin β1 function is confirmed to be vital during early development and is day 5 embryonic lethal in null mice. Further studies are required to establish which α-integrins partner with β1 to have a principal role in development, particularly in neural stem cells [509,506]. Other studies have focused on the role of the β1 subunit in cardiogenesis and have shown that β1 isoforms are the dominant myocyte β1 integrin subunit. Two major isoform splice variants of integrin β1 were found to be predominant in embryonic myocytes, β1A and β1D [510]. The Keller group (2001) has demonstrated that negative disruption of integrin β1 in transgenic mice resulted in a disturbance in heart contraction and normal functions [511]. Additionally, Fässler *et al.* (1996) have used integrin β1-deficient embryonic stem cells to study the importance of β1 in stem cell differentiation to cardiomyocytes. They found that the differentiation was retarded; the sarcomeric architecture was incomplete and disorganised, and atrial and ventricular-like cell expression was impaired, meaning that integrin β1 is necessary for cardiac cell differentiation [512].

6.3.5 Chapter objective

The aim of this Chapter was to evaluate the possible role of phytochemicals in alteration the overall expression of connexin43 and integrin β1 on growing chick embryonic cardiomyocytes but also the effect of phytochemicals on α-actinin expression in mouse embryonic stem cell-derived cardiomyocytes. Western blot was employed using the same phytochemical concentrations that have been utilised to detect ROS production in Chapter 5.

6.4 Materials and methods

6.4.1 Cell treatment

The effect of herbal components on Cx43 and integrin β1 expression in chick embryonic cardiomyocytes (MM) and on α-actinin expression in ESD3 derived cardiomyocytes was evaluated using Western blot (WB) analysis. The cells grown in 24 well plates were exposed to non-teratogenic (low) and teratogenic (high) concentrations of each phytochemical tested for 6 days in MM and 12 days in ESD3. The phytochemical concentrations tested in WB are the same as those used in the ROS assay, Chapter 5. The procedure of MM culture and cell treatment were described in more details in Chapter 2, Section 2.2.1 and Chapter 3, Section 3.3.2.2. However, for ESD3, see Sections 2.2.2 and 3.3.3.2.

6.4.2 Cell lysate preparation

After 6 days of exposure in the MM system and 12 days in the ESD3 system, the cells were placed on ice for cell lysis and protein collection. The cells were washed with ice-cold PBS (Sigma–Aldrich, UK) twice. Cells with 100µl PBS per well were scraped off and transferred into a separate labelled 1.5ml Eppendorf tube. For each tested concentration, the cultures of 5 wells (in the MM system)

and 10 wells (in the ESD3 system) were pooled together in one Eppendorf tube. The cells then were cold centrifuged (4^{o}C) at 1000rpm for 1 minute. The supernatant was aspirated off, and the pellet was resuspended with 100µl lysis buffer plus a protease inhibitor, see Appendix I for composition. The tubes were kept on a rotating wheel in the cold room for 30 min to facilitate lysis of cells, then they were homogenised using a pestle and then were centrifuged for 5 minutes at 6000rpm at 4^{o}C. The supernatant was collected into fresh 500µl Eppendorf tubes. Labelled Eppendorf tubes containing 20µl of the cells lysate were kept frozen at -80^{o}C for protein quantification (Lowry test). To the remainder of the supernatant 6X solubilisation buffer (see Appendix I for composition) was added in 1:5 ratio and stored at -20^{o}C.

6.4.3 Protein quantification using Lowry test

This method was developed by Lowry *et al.* (1951) [513]. BSA (Sigma–Aldrich, UK) standards were prepared in 1mg/ml concentrations in distilled water to generate a standard curve with a range of (0 - 0.5mg/ml) concentrations. 200µl of standard solutions were made up per Eppendorf tube. The tested samples were diluted in 1:20 ratio with distilled water to make up 200µl per Eppendorf tube. Lowry B solution was prepared by mixing 100µl of 2% (w/v) sodium-potassium tartrate -$NaKC_4H_4O_6$- with 100µl 1% (w/v) copper sulphate - $CuSO_4$-. Lowry B solution was added to 20mls of Lowry A solution (see Appendix I for composition) to make up Lowry AB solution. Then 1ml of Lowry AB was added to the 200µl of both samples and standards before they were incubated for 10 min at RT. After that, 100µl of Folin reagent in water 1:1 was added and incubated at RT for 45 min. 200µl of each sample and BSA standards were pipetted in triplicate in 96 well plates, and the plate was read at 750nm

using Spectra MAX 340pc plate reader. The protein content of each sample was determined by plotting it against BSA standard curve.

6.4.4 Western blotting

- *Protein denaturing*: the proteins were denatured by heating the samples to 95^{o}C for 5 minutes in a heating block. The samples were then vortexed and centrifuged at 13000rmp for 1 minute (Figure 6-3).
- *Gel running*: 15-20µl of each sample (containing an equivalent amount of protein) was loaded onto a Bio-Rad mini-PROTEAN TGX precast gel, UK, along with 1µl of molecular weight marker (Bio-Rad, UK). The gel was run in 1X electrode buffer (see Appendix I for composition) for approximately 60 min at 120V for Cx43 and 40 min at 175V for integrin β1 and α-actinin. When the gel had finished running, it was removed from the electrophoresis unit (Figure 6-3).
- *Proteins transferring*: the resolved proteins on the gel were then transferred to a nitrocellulose membrane by placing the gel onto a nitrocellulose membrane and holding it in a sandwich-like set up consisting of two sponge pads, two filter papers and then the gel and nitrocellulose membrane in the middle, (see the cassette model in Figure 6-3). The cassette was placed into a tank (Bio-Rad, UK) containing an ice-pack and transfer buffer. The whole tank then was put on ice. The transfer was performed in the cold transfer buffer (see Appendix I for composition) for 120 min at 70V for Cx43 and 60 min at 100V for integrin and α-actinin. The transfer was confirmed by adding a few ml of Ponceau Red solution (Sigma, UK) on the blot. Once proper transfer of protein was confirmed, the blot was washed with distilled water and Tris-buffered Saline Tween 20

(TBST) and was cut with a stainless steel scalpel into appropriate sections according to molecular weights of proteins of interest.

- *Blocking*: non-specific proteins on the blots were blocked with 1% (w/v) BSA dissolved in 5% (w/v) low-fat milk in TBST (see Appendix I for composition). The blots were placed on the shaker for 1h at RT with shaking speed of 30revs/min.
- *Primary antibody incubation*: the desired dilution of primary antibody (Table 6-1) was prepared using the same blocking solution. Then, the blots were incubated overnight at 4^{o}C on the shaker at a speed of 50revs/min. The specificity of the primary antibodies used was checked using positive control tissues as mentioned below in Sections 6.4.4.2, 6.4.4.3 and 6.4.4.4.
- *Washing*: after overnight incubation, unbound antibody was washed off using 3x1min quick wash with TBST. This was followed by 3x5min washing by TBST with agitation on the shaker. The last washing pattern of 3x15min was performed with agitation on the shaker.
- *Secondary antibody incubation*: the same blocking solution was used to dilute required concentrations of the secondary antibodies, see (Table 6-2). The blots then were incubated with the suitable antibodies in the dark at 37^{o}C for 1h.
- *Washing, scanning and analysis*: the same washing pattern of the primary antibodies was repeated to wash the secondary antibodies with final wash performed in distilled water prior to scanning. The blot scanning was performed using an Odyssey® infrared fluorescent imaging scanner (LI-COR), and the image analysis was performed using Odyssey® image studio

version 3.1. The raw data were transferred into Microsoft Excel but were analysed in Prism 6 software using one-way ANOVA with the post-hoc Dunnett's test to test the significance. For MM (Cx43 and integrin β1) and ESD3 (α-actinin), three independent repeats were performed for each experiment. In all cases p-value < 0.05 was considered significant (Figure 6-3).

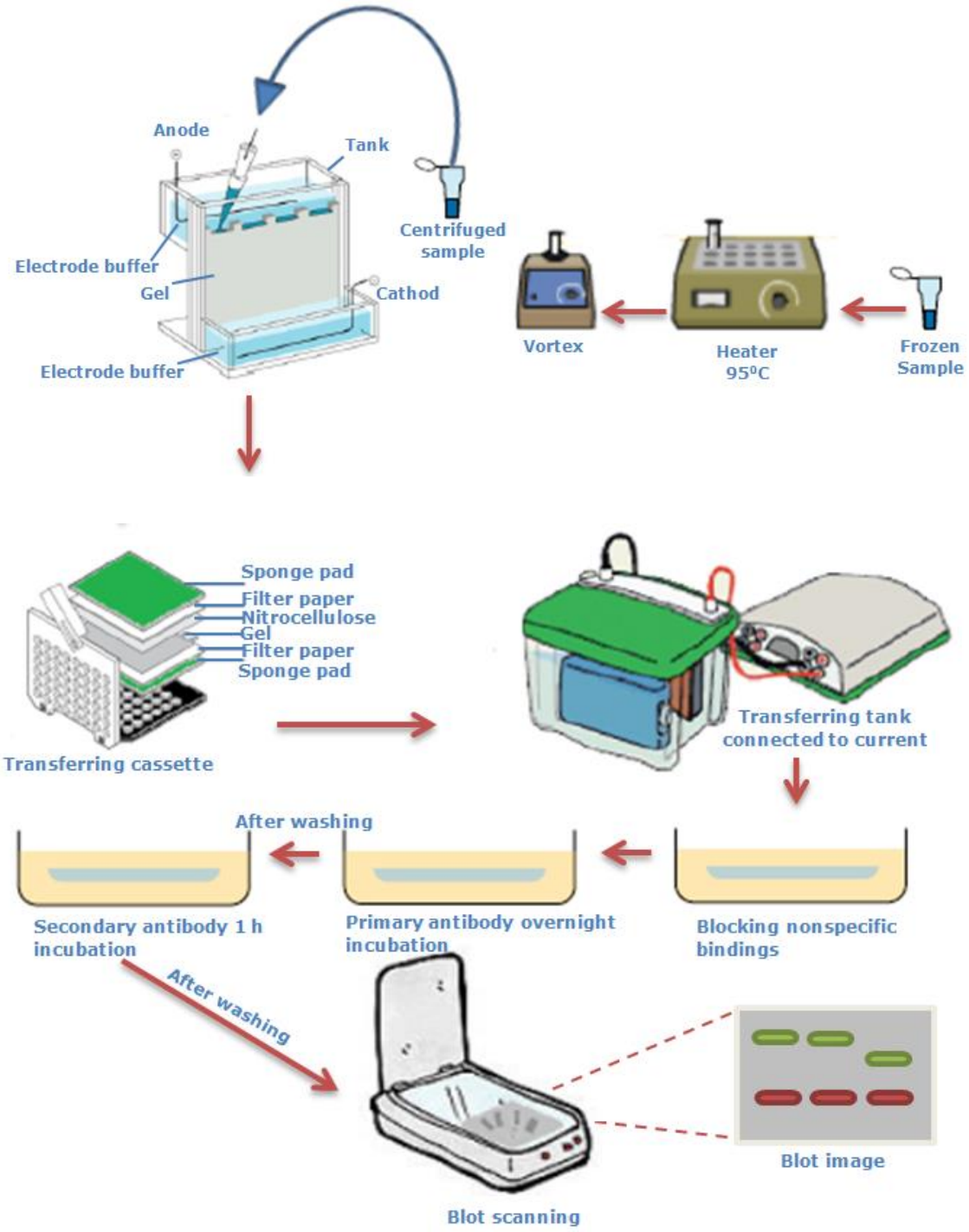


Figure 6-3: Schematic diagram represents the main steps of the Western blot technique.

6.4.4.1 Loading control for Western blot

Variations in the total amount of loaded proteins in each lane of the immunoblot have been routinely encountered in Western blot. Therefore, a "loading control" is usually used as an internal standard to normalise the loaded samples. For instance, the use of antibodies targeted to "housekeeping" genes, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is critical for the glycolytic pathway, activation of transcription and regulation of apoptosis, and actin, which is essential for cell structure and motility, has been widely considered for this purpose [514]. GAPDH has been reported to be a reliable housekeeping gene and can be used universally in prokaryotic and eukaryotic organisms because it is widely endogenously expressed [515]. Similarly, actin was found to be stable at various confluence levels in cell culture and present in almost all cells which made it useful for the ESD3 system in this study [516]. Whilst it has been reported that housekeeping gene expression remains unaltered under most experimental conditions, the saturation levels of these proteins, particularly at high load, and the effect of pathological conditions on their expression are factors which might lead to variability [517].

6.4.4.2 Positive control of Cx43

To assess the Cx43 primary antibody, see Table 6-1, against a positive control tissue and to evaluate the WB procedure utilised, day 9 chick heart was employed for detection Cx43 protein expression in Western blot analysis. Cx43 expression in chick heart on day 9 was previously confirmed by Wiens *et al.* (1995) [518]. Multiple non-specific bands between the molecular weight 50-37 were observed on the blot above and below the actual Cx43 band at 43KDa whether in heart tissue or the MM cell sample (Figure 6-4). These may represent some fluorescent proteins that are labelled by the primary or secondary antibodies used but could also be Cx isoforms that were stained with the same Cx43 antibody. The latter possibility makes more sense as the secondary antibody only stained blot showed no bands (Figure 6-4).

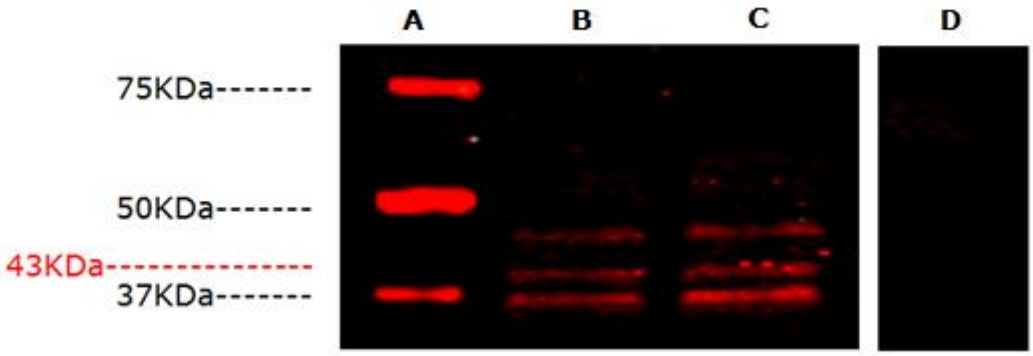


Figure 6-4: Western immunoblot of Cx43 positive control
The blot exhibits Cx43 in the expected position with possibly some non-specific Cx43 isoforms (A) Molecular weight marker, (B) chick embryonic cardiomyocytes from untreated 6 day MM culture with Cx43 band, (C) 9 day chick heart sample (positive control) with Cx43 band and (D) the same sample of (B) but incubated with secondary antibody only and shows no bands. No housekeeping protein was used in this experiment as it is only performed to determine the Cx43 band without protein quantification.

6.4.4.3 Positive control of integrin β1

To assess the integrin β1 primary antibody, see Table 6-1, against a positive control tissue and to evaluate the WB procedure utilised, day 9 chick liver (a tissue known to contain high quantities of integrin β1) was employed for detection integrin β1 protein expression by WB analysis. Integrin β1 was reported to be widely expressed in several organs including the liver. Integrin β1 expression in the liver has been reported to be one of the most dominant subunit among several integrin receptors [519]. The band size was observed in the expected position between molecular weight 100-130KDa (110KDa) (Figure 6-5). No non-specific bands were observed.

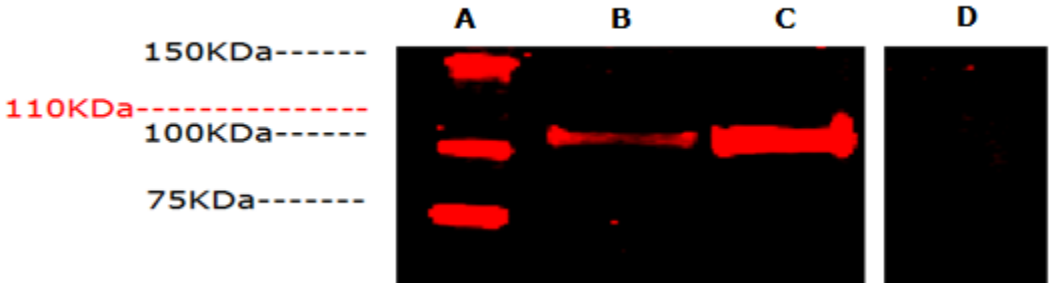


Figure 6-5: Western immunoblot of integrin β1 positive control
The blot exhibits integrin β1 band in the expected position (A) Molecular weight marker, (B) chick embryonic cardiomyocytes from untreated 6 day MM culture with integrin β1 band, (C) 9 day chick liver sample (positive control) with integrin β1 band and (D) the same sample of (B) but incubated with secondary antibody only and shows no bands. No housekeeping protein was used in this experiment as it was only performed to determine the integrin β1 band without protein quantification.

6.4.4.4 Positive control of α-actinin

To assess the α-actinin primary antibody, see (Table 6-1), against a positive control tissue and to evaluate the WB procedure utilised, day 9 chick heart was employed for detection α-actinin protein expression by Western blot analysis. α-actinin has been considered as a cardiomyocyte marker [520]. The protein was observed in its expected position on the molecular weight ladder (100KDa) (Figure 6-6). In Chapter 2, the same antibody was utilised in the immunocytochemistry to verify the α-actinin expression in day 9 chick hearts, and it positively stained the proteins (Figure 2-16).

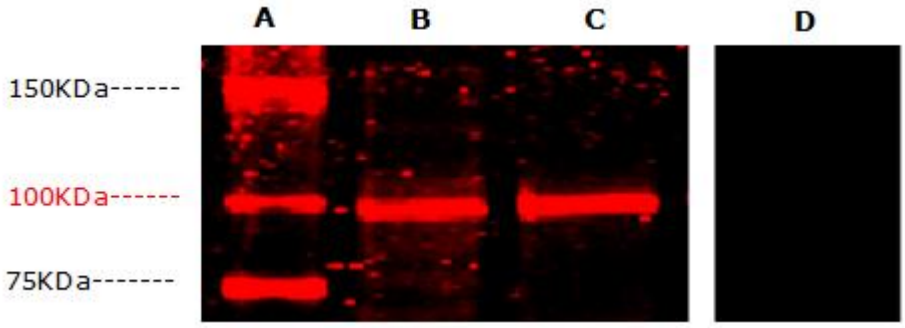


Figure 6-6: Western immunoblot of α-actinin positive control
The blot exhibits α-actinin in the expected position (A) Molecular weight marker, (B) chick embryonic cardiomyocytes from untreated 6 day MM culture with α-actinin band, (C) 9 day chick heart sample (positive control) with α-actinin band and (D) the same sample of (B) but incubated with secondary antibody only and shows no bands. No housekeeping protein was used in this experiment as it is only performed to determine the α-actinin band without protein quantification.

Antibody type (product code)	Host species	Reactivity	Dilution	Supplier
Monoclonal connexin43 (MAB3068)	Mouse	Canine,**Chicken** Human,Mouse,Rat	1:125	Merck Millipore, USA
Polyclonal integrin-β1 (251162)	Rabbit	Human,Mouse, Rat,**Chicken**, Horse, Porcine, Rabbit, Sheep, zebrafish	1:500	Abbiotec™, USA
Monoclonal α-actinin (A7732)	Mouse	Rabbit, Human, **Mouse**, Pig, Bovine, Rat, Snake, Cat, Canine, Frog, Fish, Goat, Sheep, Chicken, Hamster, Lizard	1:500	Sigma-Aldrich, UK
Housekeeping gene Polyclonal GAPDH (ab9485)	Rabbit	Mouse, Rat, **Chicken**, Dog, Human, Saccharomyces Cerevisiae, Xenopus Laevis, African Green Monkey	1:1000	Abcam, UK
Housekeeping gene Monoclonal actin (A4700)	Mouse	Goat, Viper, *Xenopus*, Bovine, Guinea Pig, Snail, Canine, Hamster, Rat, Chicken, **Mouse**, Human, Sheep, Pig, Rabbit, Carp	1:1000	Sigma-Aldrich, UK

Table 6-1: Primary antibodies used in Western blot

Antigen specificity/Source	Host species	Reactivity	Protein	Dilution
IRDye®800CW/LICOR,UK	Goat	Rabbit **IgG**	GAPDH	1:10000
IRDye®800CW/LICOR,UK	Goat	Mouse **IgG**	Actin	1:10000
IRDye®680RD/LICOR,UK	Goat	Mouse **IgG**	α-actinin, Cx43	1:10000
IRDye®680RD/LICOR,UK	Goat	Rabbit **IgG**	Integrin-β1	1:10000

Table 6-2: Secondary antibodies used in Western blot

6.5 Results

6.5.1 Effect of herbal components on total Cx43 expression in MM cultures

The effect of exposure to different concentrations of tested herbal components on total Cx43 expression was evaluated in chick embryonic cardiomyocytes (MM). Data obtained from Cx43 scanned blots were normalised against total protein expression in GAPDH scanned blots for the corresponding samples and the data were presented in the graphs as "ratio of Cx43 to GAPDH". Following treatment with 3μM 6G, 1μM bisabolol or 0.01μM eugenol, no significant alteration in the protein expression were observed in comparison with untreated control (Figure 6-7A, A1). However, when the chick embryonic cardiomyocytes were exposed to the higher concentrations of the same phytochemicals, 100μM 6G, 80μM bisabolol or 80μM eugenol, significant ($p<0.05$) downregulation of Cx43 expression was recorded (Figure 6-7B, B1). Protein expression was not affected by GA, GB or G-Rg1 under the conditions of the study (Figure 6-7).

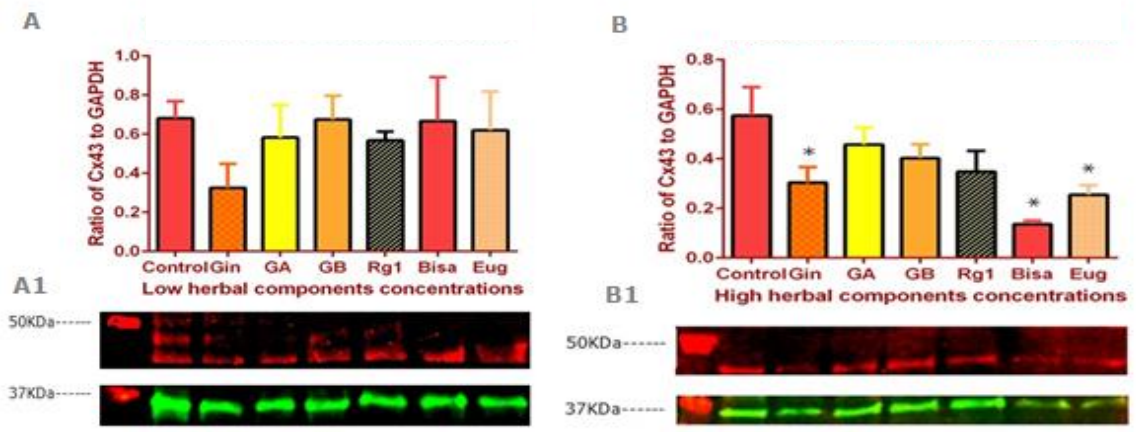


Figure 6-7: Effect of herbal components on Cx43 expression in chick embryonic cardiomyocytes (MM)

(A) Densitometric quantification of Cx43 expression for Western blots obtained from three independent experiments (n=3) of 6 day MM cultures treated with low phytochemical concentrations (Gin) 6-gingerol (3μM), (GA) ginkgolide A (100nM), (GB) ginkgolide B (50nM), (Rg1) ginsenoside Rg1 (0.5μM), (Bisa) α-bisabolol (1μM) and (Eug) eugenol (0.01μM). **(A1)** representative Western immunoblot of MM cells treated with the low phytochemical concentrations. Blot represents scan from one experiment where Cx43 (43KDa) expression shows in red and housekeeping gene GAPDH (36KDa) shows in green. **(B)** Densitometric quantification of Cx43 expression for Western blots obtained from three independent experiments of MM cultures treated with high phytochemical concentrations (Gin) 6-gingerol (100μM), (GA) ginkgolide A (2000nM), (GB) ginkgolide B (1000nM), (Rg1) ginsenoside Rg1 (10μM), (Bisa) α-bisabolol (80μM) and (Eug) eugenol (80μM). **(B1)** representative Western immunoblot of MM cells treated with the high phytochemical concentrations. Blot represents scan from one experiment where Cx43 (43KDa) expression shows in red and housekeeping gene GAPDH (36KDa) shows in green. Values are mean±SEM for from cells obtained from 5 wells of three independent experiments. The values were analysed by one way ANOVA with post-hoc test Dunnett's and (*) p value < 0.05 was considered significant in comparison to control.

6.5.2 Effect of herbal components on integrin β1 expression (MM)

The effect of exposure to different concentrations of tested herbal components on total integrin β1 expression was investigated in chick embryonic cardiomyocytes (MM). The values obtained from integrin β1 scanned blots were normalised against total protein expression in GAPDH scanned blots for the corresponding samples, and the data were presented in the graphs as "ratio of integrinβ1 to GAPDH". Observations obtained from the cultures exposed to 3μM 6G, 1μM bisabolol or 0.01μM eugenol did not exhibit a significant change in the total expression of integrin β1 when compared with untreated control (Figure 6-8A,A1). However, when the chick embryonic cardiomyocytes were exposed to the high concentrations of the same phytochemicals, 100μM 6G, 80μM bisabolol or 80μM eugenol, a significant ($p<0.05$) decline of total integrin β1 expression was noted (Figure 6-8B,B1). No effects of GA, GB or G-Rg1 were observed on integrin β1 expression whether at high or low concentrations (Figure 6-8).

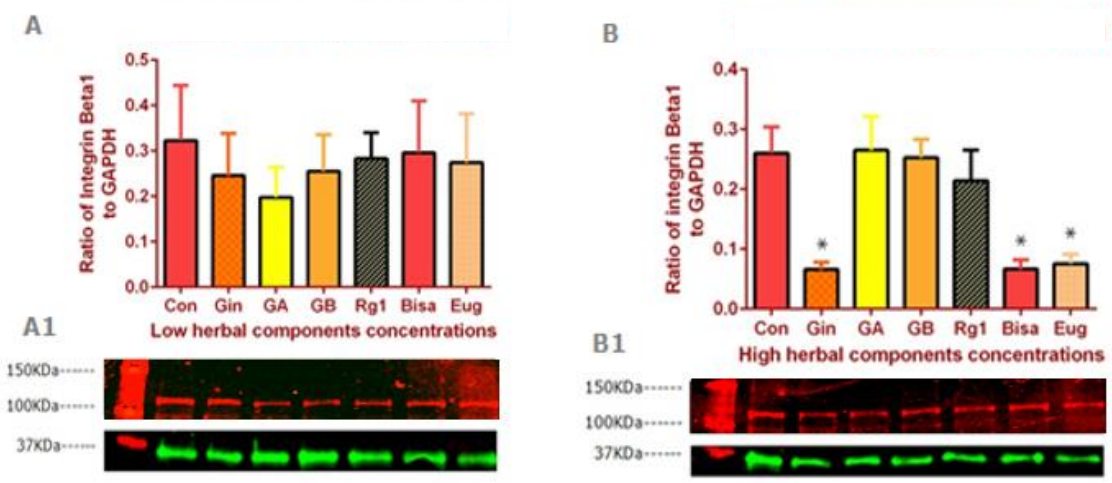


Figure 6-8: Effect of herbal components on integrin β1 expression in chick embryonic cardiomyocytes (MM).
(A) Densitometric quantification of integrin β1 expression for Western blots obtained from three independent experiments (n=3) of 6 day MM cultures treated with low phytochemical concentrations (Gin) 6-gingerol (3μM), (GA) ginkgolide A (100nM), (GB) ginkgolide B (50nM), (Rg1) ginsenoside Rg1 (0.5μM), (Bisa) α-bisabolol (1μM) and (Eug) eugenol (0.01μM). **(A1)** representative Western immunoblot of MM cells treated with the low phytochemical concentrations. Blot represents scan from one experiment where integrin β1 (110KDa) expression shows in red and housekeeping gene GAPDH (36KDa) shows in green. **(B)** Densitometric quantification of integrin β1 expression for Western blots obtained from three independent experiments of MM cultures treated with high phytochemical concentrations (Gin) 6-gingerol (100μM), (GA) ginkgolide A (2000nM), (GB) ginkgolide B (1000nM), (Rg1) ginsenoside Rg1 (10μM), (Bisa) α-bisabolol (80μM) and (Eug) eugenol (80μM). **(B1)** representative Western immunoblot of MM cells treated with the high phytochemical concentrations. Blot represents scan from one experiment where integrin β1 (110KDa) expression shows in red and housekeeping gene GAPDH (36KDa) shows in green. Values are mean±SEM for from cells obtained from 5 wells of three independent experiments. The values were analysed by one way ANOVA with post-hoc test Dunnett's and (*) p value < 0.05 was considered significant in comparison to control.

6.5.3 Effect of low and high herbal components concentrations on α-actinin expression (ESD3)

The Western blot analysis of α-actinin showed the positive presence of protein at its specific molecular weight of 100KDa. In the ESD3 system, herbal components treated cells results were compared with the untreated control. The results showed no significant changes in the overall α-actinin protein expression in ESD3 under the conditions of the study (Figure 6-9).

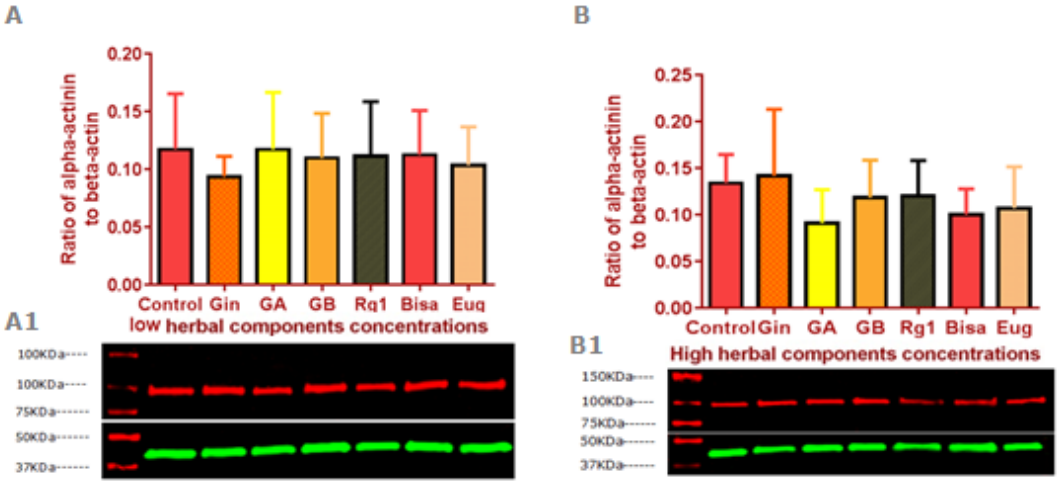


Figure 6-9: Effect of herbal components on α-actinin expression in mouse embryonic D3cardiomyocytes (ESD3)
(A) Densitometric quantification of α-actinin expression for Western blots obtained from three independent experiments (n=3) of 12 day ESD3 cultures treated with low phytochemical concentrations (Gin) 6-gingerol (0.75μM), (GA) ginkgolide A (100nM), (GB) ginkgolide B (50nM), (Rg1) ginsenoside Rg1 (0.5μM), (Bisa) α-bisabolol (1μM) and (Eug) eugenol (0.01μM). **(A1)** representative Western immunoblot of MM cells treated with the low phytochemical concentrations. Blot represents scan from one experiment where α-actinin (100KDa) expression shows in red and housekeeping gene actin (43KDa) shows in green. **(B)** Densitometric quantification of integrin β1 expression for Western blots obtained from three independent experiments of MM cultures treated with high phytochemical concentrations (Gin) 6-gingerol (100μM), (GA) ginkgolide A (2000nM), (GB) ginkgolide B (1000nM), (Rg1) ginsenoside Rg1 (10μM), (Bisa) α-bisabolol (80μM) and (Eug) eugenol (80μM). **(B1)** representative Western immunoblot of MM cells treated with the low phytochemical concentrations. Blot represents scan from one experiment where α-actinin (100KDa) expression shows in red and housekeeping gene actin (43KDa) shows in green. Values are mean±SEM for from cells obtained from 10 wells of three independent experiments. The values were analysed by one way ANOVA with post-hoc test Dunnett's and (*) p value < 0.05 was considered significant in comparison to control.

6.6 Discussion

Connexins being a part of GJs have been viewed as mediators of flow of information between adjacent cells including cardiomyocytes in a way that makes any developmental alteration in their function or expression contribute to cardiac dysfunctions, see Section 6.2.3. The results in this Chapter showed that the overall Cx43 expression of the cultured growing embryonic cardiomyocytes had downregulated significantly only when treated with 100, 80 and 80μM of 6G, bisabolol and eugenol, respectively. The effects of other chemicals on connexin expression have been reported recently. It has been shown that caffeine [521] and carbamazepine [259] disturbed contractile activity via alteration of the expression of Cx isoforms in the growing chick embryonic cardiomyocytes. However, other factors have also been found to modulate connexin expression such as tumor necrosis factor (TNF) [522] in addition to dysregulation of cardiac specific transcription factors such as Nkx2-5 and the GATA family [523]. To our knowledge, there is no report to compare our results with directly. We hypothesised that phytochemical-induced Cx43 downregulation could arise from a direct inhibition of some Cx43 transcription factors, induction of some cytokines such TNF, an increase in the Cx43 degradation process or as a result of toxic effects on cardiomyocytes but this study cannot elucidate the exact mechanism. For the rest of phytochemical concentrations tested no decrease in Cx43 expression was noted, whether non-teratogenic (low) or teratogenic (high). More studies are recommended to investigate their effects on Cx43 function which is regulated by complex mechanisms involving several players such as calcium, pH, voltage, phosphorylation and dephosphorylation [524].

As explained in Section 6.3.4, integrin β1 plays an essential role in heart development via linking matrix materials to the cytoskeleton and also initiating sophisticated bidirectional signal pathways. Its knockout leads to severe cardiac development consequences. Herein we showed that the amount of integrin β1 in chick embryonic cardiomyocytes treated with 100μM 6G, 80μM bisabolol or 80μM eugenol was significantly less than the untreated control. Integrin β subunits were recorded to be downregulated in some pathological conditions such as cytomegalovirus infection [525] and only transiently in kidney carcinoma cells [526]. In terms of chemicals, 4 mM butyrate has been associated with downregulation of expression and function of α2/β1 integrin in colorectal cancer cell line [527]. Our study might be the first that investigates the effect of herbal components on integrin expression in chick embryonic cardiomyocytes. The integrin downregulation that is presented here could result from disturbance of EC matrix composition by the tested phytochemicals. Altering some integrin β1 transcriptional factors might be another possibility for downregulation. However, the exact mechanism remains to be understood. Interestingly, we noticed that the same phytochemicals (100μM 6G, 80μM bisabolol and 80μM eugenol) showed a simultaneous dramatic decrease in the quantity of integrin β1 and Cx43. Cross-talk between these two cell surface proteins might not be excluded. Xu *et al*. (2006), in their study on cardiac neural crest cell (CNCs) migratory behaviour, have observed altered integrin β1 signalling, expression and distribution in Cx43 knockout cells [528]. In contrast, integrin β1 deficient ESD3 derived cardiomyocytes have demonstrated an upregulation of the expression of gap junctional proteins Cx40 and Cx43 [529]. Taken together, this implies that integrin

function affects connexin expression in cardiomyocytes and vice versa. However, it might be possible to suggest that Cx43 expression has been affected first by tested phytochemicals which then led to decrease in integrin β1 expression. However, growing chick embryonic cardiomyocytes might have a different behaviour in terms of expression these proteins from those of CNCs and ESD3. More information could have been obtained by studying the effects of the selected phytochemicals on the expression of some other connexin isoforms such as (Cx44, Cx45, Cx46 and Cx47) or integrin dimers such as (integrin β1α2 or integrin β1α4). Also our study exhibits no evidence about the intracellular and appositional localization of the tested proteins. Further work utilising *in situ* experiments could provide such data.

The expression of α-actinin in ESD3 derived cardiomyocytes has been examined in this Chapter. This was to determine whether phytochemicals that suppress cardiomyocytes differentiation have downregulated α-actinin expression or induced its dysfunction. Surprisingly, the expression of α-actinin did not alter at any tested phytochemical concentration. It may be, however, that phytochemicals prevent α-actinin from connecting actin filaments to adjacent sarcomeres resulting in impaired transmission of the force generated by the actin and myosin complex and therefore the formation of contractile foci in the exposed cells is inhibited. One possibility remains to be investigated, which is whether the α-actinin expression has upregulated back to normal levels after an early downregulation response. A recent report has demonstrated upregulation of α-actinin protein in the heart failure of genetically modified mouse models with impaired diastolic function [530].

To sum up, it has been shown that some phytochemicals have downregulated Cx43 and integrin β1 in growing chick embryonic cardiomyocytes which might suggest a possible cross-talk among these cell surface proteins. Furthermore, it was shown that phytochemicals do not alter α-actinin expression at least in later stages of the ESD3 model, but some of them might have impaired the protein functionality. Further molecular studies are required to be undertaken for a better understanding of the embryotoxicity mechanisms of these herbal components.

CHAPTER 7

7 Chapter seven – Evaluation the effect of herbal components on the migration of mouse embryonic D3 stem cells

7.1 Introduction

Stem cells play important roles during development through establishing fetal tissues but also their functions extend to adulthood where they maintain homeostasis through the continued replacement of defective cells and regeneration of injured or diseased tissues. However, morphogenesis during development, or the homeostasis process, require accurately organised migration of stem cells [531]. The significance of individual cell migration and collective cell migration (CCM) in organizing complex morphogenetic events during fetal development has been verified. CCM is characterised by the capacity of gathered cells to travel as clusters through coordinated permanent or transient cell-cell contacts which convey signals and affect the activities of one another [532,533]. After conception and during gastrulation, cells are first identified in a certain area of the embryo and then migrate extensively to relocate themselves and reach their target location [534]. Within the blastocyst, clusters of cells migrate collectively as sheets to establish the three germ layers from which an embryo would be developed. During embryonic development, cells within these layers eventually travel to differentiate in target locations and form various tissues and organs [535]. However, understanding the essential processes underlying cell migration is important to prevent any disturbance to migrating cells, including any caused by chemicals.

7.1.1 The migration cycle

Usually, cell migration can be conveniently conceptualised as a cyclical process, whether cell sheets or single-cell migration, Figure 7-1. For any cell to start moving, asymmetric morphology is required with distinct front and back edges [536]. The initial reaction of a cell to a migration stimulator is to polarise then develop protrusions (projections) toward the migration area. Depending on cell type, various forms of these protrusions can be produced; large and wide or spike-like protrusions, but they all adhere to ECM or neighbouring cells by integrin-mediated attachment and are directed by cytoskeletal actin. To permit a cell to move forward, there must be detachment at distinct regions of the cell to release adhesions and repeat the sequence which allows cell motility [537,536].

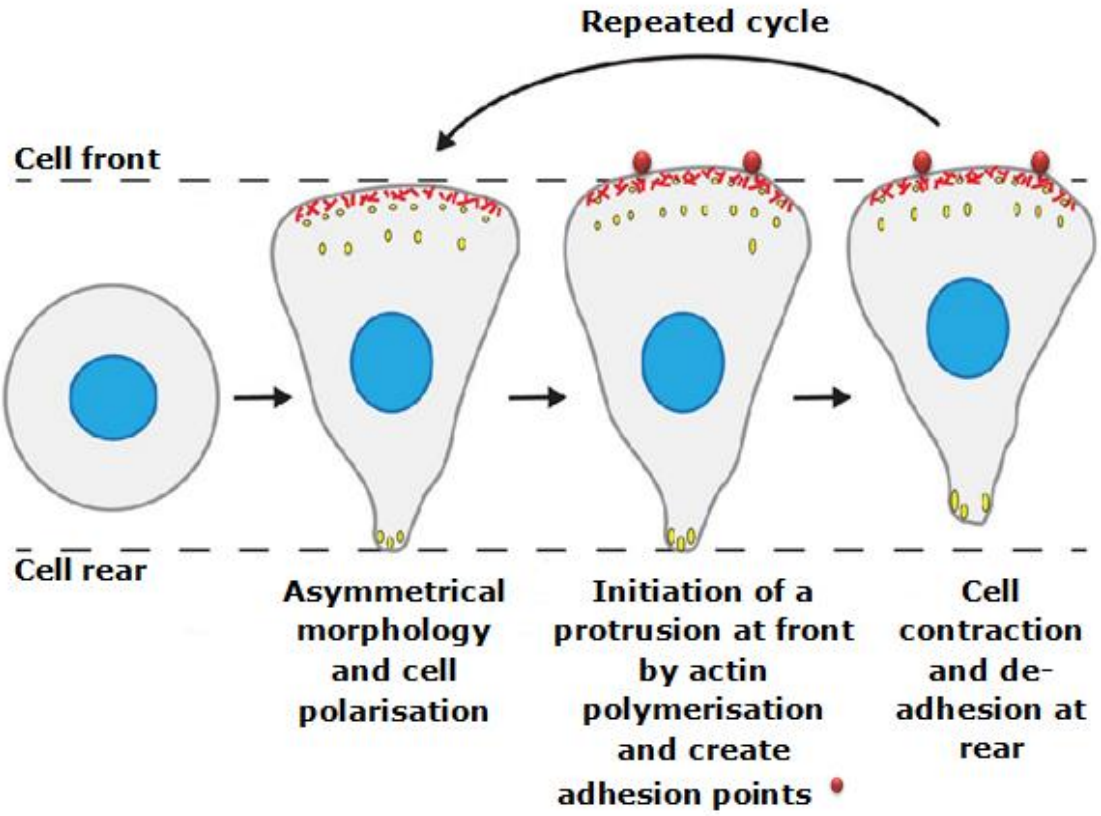


Figure 7-1: Cell migration, a simplified model
The figure was adapted from this website
http://www.biochemsoctrans.org/content/43/1/79

7.1.1.1 Cell polarisation

The cornerstone in cell migration is a polarised cell. Several external molecules (chemotactic agents) can trigger polarisation intracellularly. Whilst the chemotactic receptors do not seem to be polarised in response to a gradient, they convey the extracellular signal to the cell cytoplasm. The signal then would be converted intracellularly into polarising response [538]. Phosphatidylinositol (3,4,5) trisphosphate (PIP3), which acts as a molecule (e.g. Akt/PKB) recruiter to the cell membrane, has been found to be one of the first molecules which is polarised by chemotactic agents. As a consequence of the localised activation of PI 3-kinase and lipid phosphatases at the cell

anterior, a fast gathering of PIP3 at the leading edge is produced [535]. Therefore, an asymmetric accumulation of PIP3 would develop and lead to activation of Rho family GTPases, including Rac and/or Cdc42 [539], in addition to polymerisation of actin at the cell leading edge.

7.1.1.2 Protrusive machinery

Once actin filaments are intrinsically polarised, they rearrange by creating fast moving barbed ends (front leader) and slow moving pointed ends (rear follower), where this inherent polarity is employed to guide membrane protrusions [540]. These polarised actin filaments form a branching "dendritic" web in lamellipodia, which is mediated by the Arp2/3 complex (Actin-Related Proteins) that serves as an actin nucleation to help form projections, whereas the organisation of filaments in filopodia tends to be organised in long parallel "bundles" [540,541]. However, Arp2/3 complex is activated by WASP (Wiskott-Aldrich syndrome family proteins), which are already activated at the cell membrane [540]. Moving the cell forward in lamellipodia is believed to occur by what is called the "elastic Brownian ratchet" mechanism, in which thermal energy is utilised to bend the emerging short filaments and store elastic energy. The driving force for protrusion is derived from unbending of an elongated filament against the leading edge [541]. On the other hand, a filament treadmilling mechanism is thought to drive filopodial protrusion, where actin filaments form a firm bundle with the aid of fascin protein and elongate by building up more actin monomers at their barbed ends (where Ena/VASP proteins prevent branching), whilst simultaneously releasing more actin monomers from their pointed ends [540]. However, several proteins were found to regulate the migration process throughout various mechanisms. For

instance, profilin associates with actin monomers and prevents self-nucleation with selectively target monomers of barbed ends. Also by capping proteins which bind to the filament and prevent its elongation i.e. hampering actin polymerisation to new filaments approaching the plasma membrane. ADF/cofilin family proteins act to disassemble the existent filaments in order to generate more monomers which are required for actin polymerisation at the front end and cortactin to stabilise short actin projections, whereas filamin A and α-actinin serve as filaments cross-linkers to support the whole dendritic network [540].

7.1.1.3 Migration directionality

One of the important regulations of the directionality of cell migration is the topography of the ECM. This provides a mobility environment with physical cues from which cells can obtain mechanical information or signals, which eventually translate into constraint adhesion sites that guide the direction of migration [542]. Furthermore, the polarity signalling machinery can also effect directional cell migration. This polarisation influences the leading and trailing cell edge formation. The Rho GTPase family member Cdc42 is a principal regulator of cell polarisation that impacts directional migration. ECM components can stimulate integrin receptors, resulting in local activation of Cdc42 at sub-locations of the plasma membrane [543,544]. Additionally, any factor which interferes with actin polarisation or depolarisation can also influence the cell motility orientation including calcium, kinases and all the migration regulatory proteins mentioned previously [542].

7.1.1.4 Adhesion disassembly

For the cells to move forward, adhesion detachments at the front and rear should occur. Adhesion disassembly is observed at the leading edge, alongside the formation of new protrusions with their adhesion sites, and at the cell rear, where it endorses tail retraction [537]. However, one of the factors that has been implicated as a promoter for adhesion disassembly is the targeting of microtubules [545]. In some cases, the disassembly might fail to be accomplished allowing adhesions to persist and mature into larger, more constant assemblies. Some studies showed the importance role of kinases and phosphatases in the regulation of adhesion turnover whether at the cell front or rear. For instance, it has been reported that cells lacking tyrosine kinase FAK and Src migrate less and create more adhesion points [546,547]. Two more protein families were also discovered to have a role in regulation of disassembly turnover and migration; Rac-associated proteins [548] and mitogen-activated protein kinase ERK [549]. Several lines of evidence have indicated that myosin II and Ca^{+2} have a specific role at the rear cell to cleave the adhesions. However, Ca^{+2} targets both Ca^{+2}–regulated phosphatase calcineurin but also Ca^{+2}–activated protease calpain, which has the potential to detach and dysregulate several focal adhesion proteins such as integrins, talin, vinculin and FAK [550-552].

7.1.2 Chapter objective

In this Chapter, the selected herbal components would be investigated for their impact on the migration process of differentiating ESD3. Cell exclusion zone assay would be utilised to measure the cell migration.

7.2 Material and Methods

7.2.1 Cell exclusion zone migration assay (ESD3)

As has been explained, cell motility is a key step in many physiological and pathological processes such as embryonic organogenesis, angiogenesis, cancer metastasis and wound healing [553]. Two concentrations of each herbal component were selected; non-embryotoxic and embryotoxic, based on their biological data obtained in Chapter 3, Section 3.4. Vehicle controls were included for the untreated group to maintain the same amount of solvent among the groups. The procedure was as follows: a 96-well plate was used to evaluate cell migration into an exclusion region of fixed diameter in the centre of the well. The 96 well plates were pre-coated with 1% (w/v) gelatine and an area of cell exclusion formed using a device which is currently being investigated for patent protection (Figure 7-2A). ESD3 cells were seeded using ESD3 culture medium containing 20% (v/v) FBS with LIF, into the wells of the 96 well plate, at a cell density of $5x10^5$ cell per ml in order to approach 100% confluency on the next day. 200µl cell suspension at the required cell density was injected into each well using 1ml syringe (Figure 7-2B). After 24h and before the 96 well plate was removed from the cell exclusion zone (CEZ) device, the ESD3 culture medium was aspirated and replaced with serum-free medium without LIF (to reduce proliferation rates and emphasise the role of differentiating cell migration) with or without test phytochemicals. The plate was incubated at 37^{o}C in the 95% air: 5% CO_2 incubator 2h before the device was eventually removed to allow cell migration into the exclusion region (Figure 7-2C). The same two concentrations that were investigated for ROS production (low non-teratogenic and high teratogenic) were tested in this

assay unless the concentration caused <100% confluent cultures and so created gaps within the culture, as this would allow the cells to move in multiple directions, rather than only toward the central exclusion region. The cultures were monitored by imaging at zero time (time of removal cell exclusion mechanism) and after 24h (Figure 7-2D). Cytochalasin D was utilised as a positive control in this assay, see the details below. The size of the gap areas were determined using the ImageFocusAlpha software freehand selections and the area travelled by migrated cells was measured. The difference between areas at the two time intervals provided a quantitative index of cell migration capacity.

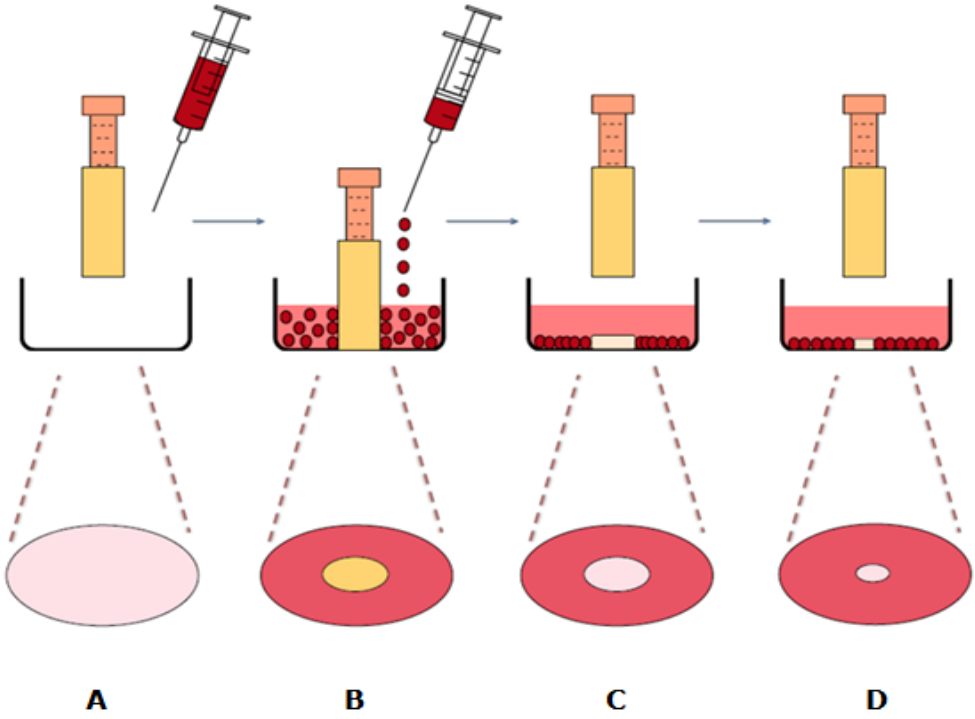


Figure 7-2: Schematic illusion of the cell exclusive zone mechanism for migration assay
(A) Represents a pre-coated well with required cell density ready for cell culture just before set up of the device, pink circle refers to empty well/area, (B) set up the device to allow pins to create cell exclusive zone before the cells being injected, red area in the circle represents the culture and orange area represent the pin, (C) after 24h, replace the medium with serum free one with or without phytochemicals and removal of the device to let cells start migrate toward the exclusive zone which appears as pink circle, this was regarded as zero time, (D) after another 24h, cells assume to reduce the gap as appears in smaller pink circle in comparison with C.

7.2.2 Procedure optimisation and positive control

To optimise the migration assay procedure, the effect of serum-free medium on stem cell pluripotency has been investigated. ESD3 were cultured in the presence of LIF but without serum for 24h. Immunostaining confirmed that the cells maintained an undifferentiated status when they were grown in serum-free medium, for protocol, see Section 2.2.4.3, (Figure 7-3 A-C). In accordance with our findings, Chin et al. (2010) had found that hESD3 maintained the expression of the undifferentiated marker when they were cultured in serum-free media and these cells successfully formed three germ layers and spontaneously differentiated into EBs [554].

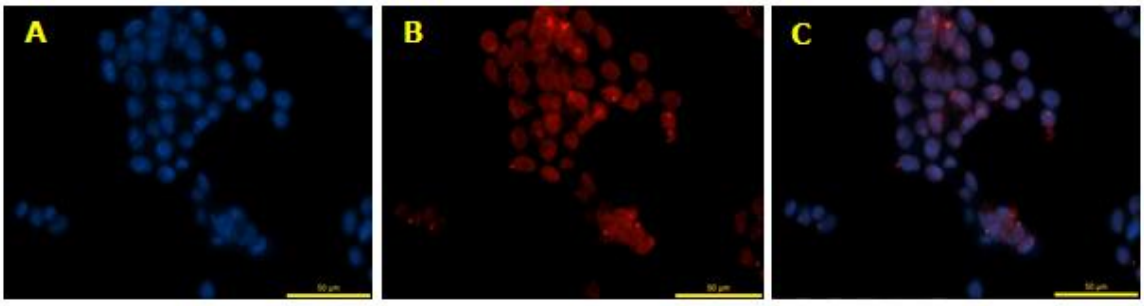


Figure 7-3: SOX2 immunofluorescent of untreated ESD3 culture with serum free medium in the presence of LIF
(A) Culture stained with DAPI, image (B) is stained for SOX2 which is characteristic of undifferentiated ESD3, and image (C) is overlay for images (A) and (B). The culture was serum starved for 12h in presence of LIF. SOX2 was positively stained in the culture which means the absence of serum has no impact on the pluripotent characterisation of stem cells. Compare this image with Figure 2-19. Scale bar is 50 µm.

Furthermore, to ensure that the system can detect any disturbance in cell migration, cytochalasin D (Fluka, Germany) has been tested as a positive control. Cytochalasin D is mycotoxin which is produced by moulds, and it has been confirmed that it inhibits cell locomotion by suppression of actin polymerisation, a process that is essential in cell migration [555,556]. Three

concentrations were tested (3.25, 7.5 and 15μM). The result shows that the migration of treated cultures was significantly inhibited at all concentrations tested (Figure 7-4). F-actin filaments were labelled by phalloidin staining (Vector Laboratories Ltd, USA) to confirm the effect of cytochalasin D. A similar immunocytochemistry procedure as for the SOX2 antibody, see Section 2.2.4.3, was followed in this staining, with use of mounting medium with TRITC phalloidin dye instead of antibodies. Under the fluorescent microscope, the exposed cells were found to express disassembled F-actin filaments (Figure 7-5) while untreated cells showed intact filaments with no gaps within them (Figure 7-5B).

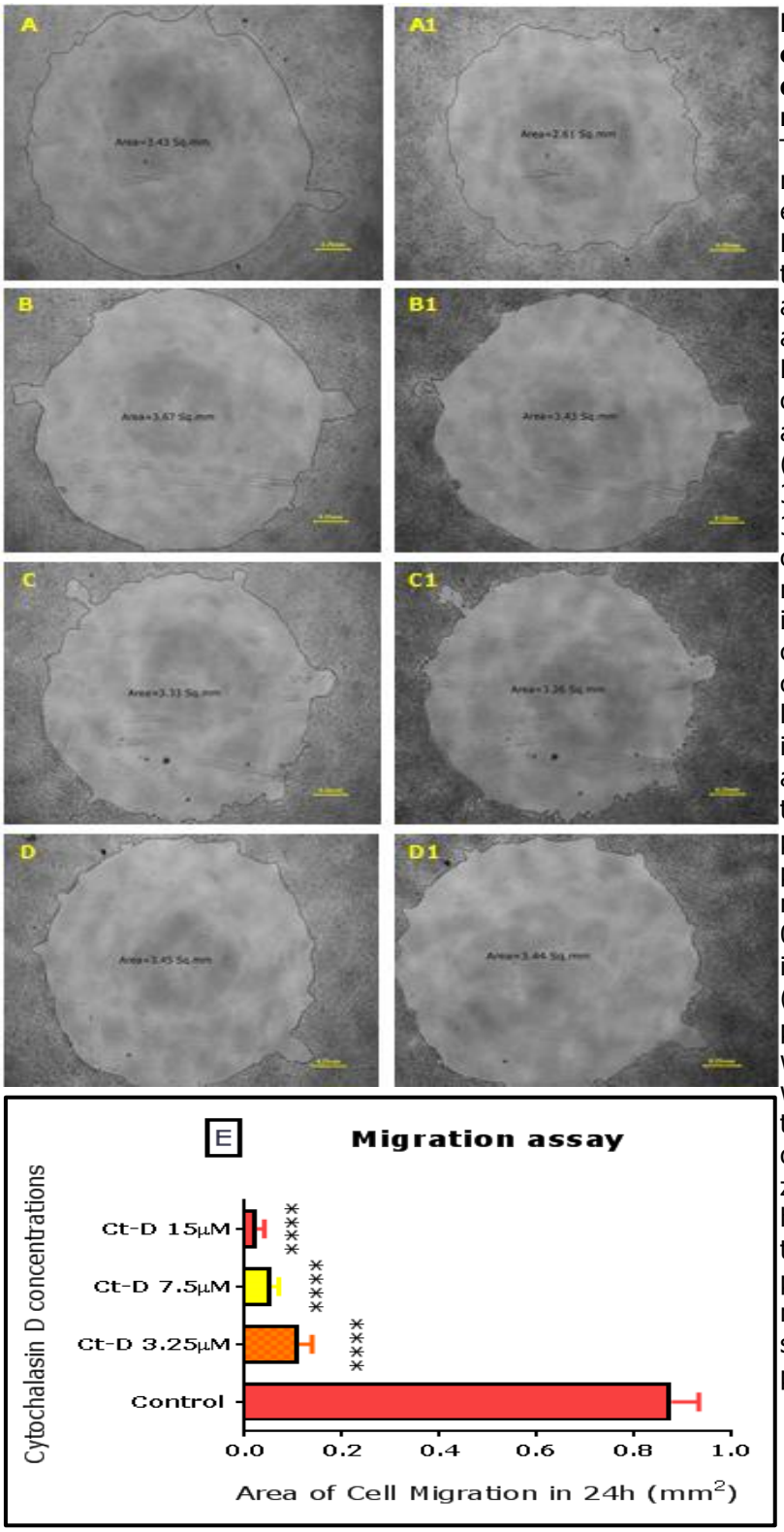


Figure 7-4: Effect of cytochalasin D on differentiating ESD3 migration

The images (A-D1) are representatives for one experiment. A, B, C and D are cultures at zero time with exclusive zone areas (3.43, 3.67, 3.33 and 3.45 mm^2) and A1, B1, C1 and D1 are corresponding images after 24h with areas (2.61, 3.43, 3.26 and 3.44 mm^2) for untreated, 3.25, 7.5 and 15µM cytochalasin D, respectively. The areas imply that cells migrated considerably in untreated culture but cell locomotion was hindered in exposed cultures and at all concentrations tested. The cells were photographed under a phase contrast microscope. Scale bar is 0.25mm. (E) Three independent repeats (n=3) with quadruplicate per each concentration were performed and one way ANOVA was utilised to analyse the data. The difference of areas at zero time and after 24h has been calculated and the results were presented as mean±SEM. **** significance indicates $p < 0.0001$ vs. control.

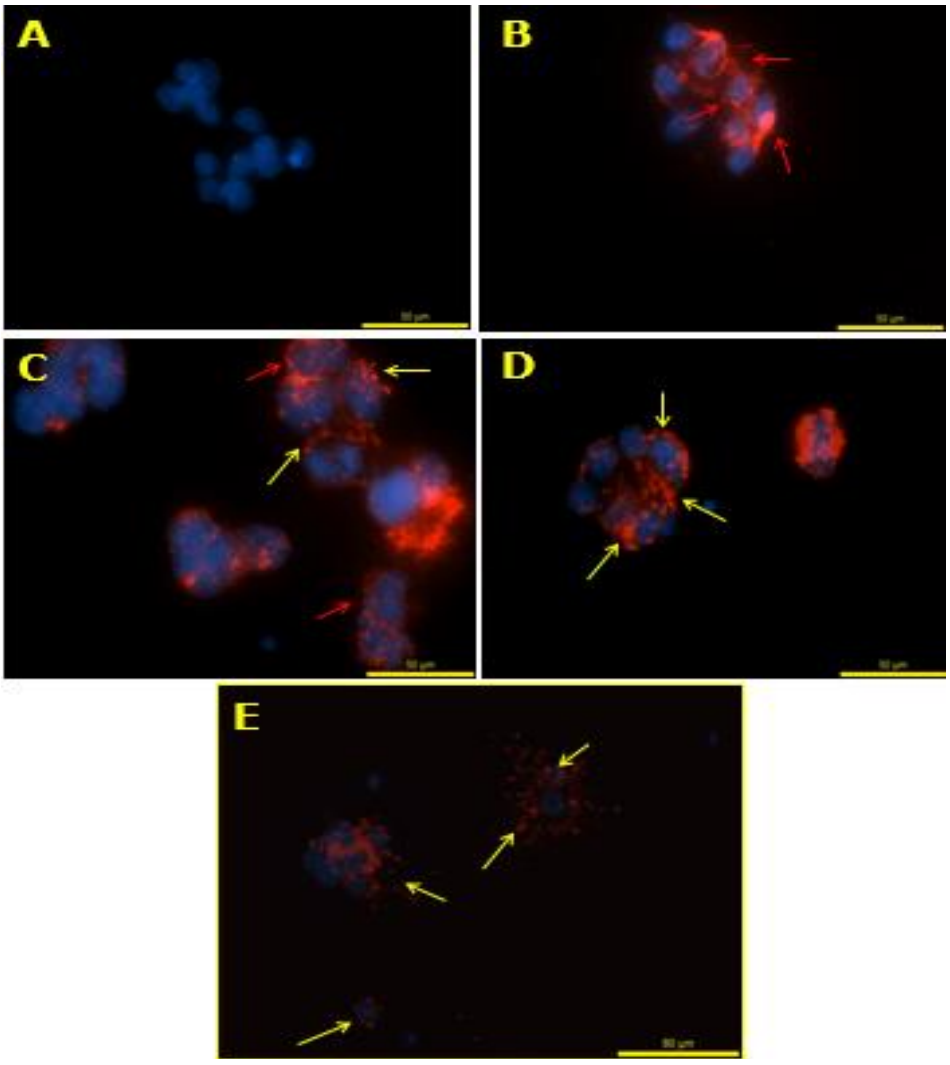


Figure 7-5: Immunostaining images labelled F-actin with phalloidin dye in cytochalasin D treated ESD3
(Red arrows in images indicate intact actin filaments but yellow arrows indicate disassembled ones), (A) DAPI staining to show image without phalloidin, (B) vehicle untreated control ESD3 showed intact filaments, (C) 3.25μM cytochalasin D treated ESD3 showed some intact filaments (red arrows) alongside the damaged ones (yellow arrows), (D) 7.5μM cytochalasin D treated ESD3 with a majority of detached filaments and (E) 15μM cytochalasin D treated ESD3 showing almost all monomer-appearance filaments. The effect on actin was believed to be the main reason preventing cytochalasin D treated ESD3 from moving toward the central gap. Scale bar is 50μm for A, B, C and D but 80μm for E.

7.3 Results

7.3.1 Effect of 6-gingerol on cell migration (ESD3)

In comparison to untreated control, the 24h 6G-exposed cells demonstrated a slight effect on the migratory behavior. Two 6G concentrations were tested, (0.75μM as a non-embryotoxic concentration and 100μM as embryotoxic concentration). At low concentration (Figure 7-6B and B1), the cells migrated to approximately the same area as the control cells (Figure 7-6A and A1) with no significant difference between the two groups over 24h exposure (Figure 7-6D). However, not significant decreasing trend in comparison with the control group was observed when the cells were treated with 100μM 6G for 24h (Figure 7-6C, C1 and D).

7.3.2 Effect of ginkgolide A on cell migration (ESD3)

As presented in Figure 7-7, the areas covered by untreated and GA-treated cells over 24h were comparable. The overall migration area of 100nM GA-treated cells (Figure 7-7B and B1) was not significantly ($p>0.05$) less than the control (Figure 7-7A, A1 and D). Likewise, treating the cells with 2000nM GA for 24h (Figure 7-7C, C1 and D) did not significantly ($p>0.05$) alter the net migration area in comparison to control.

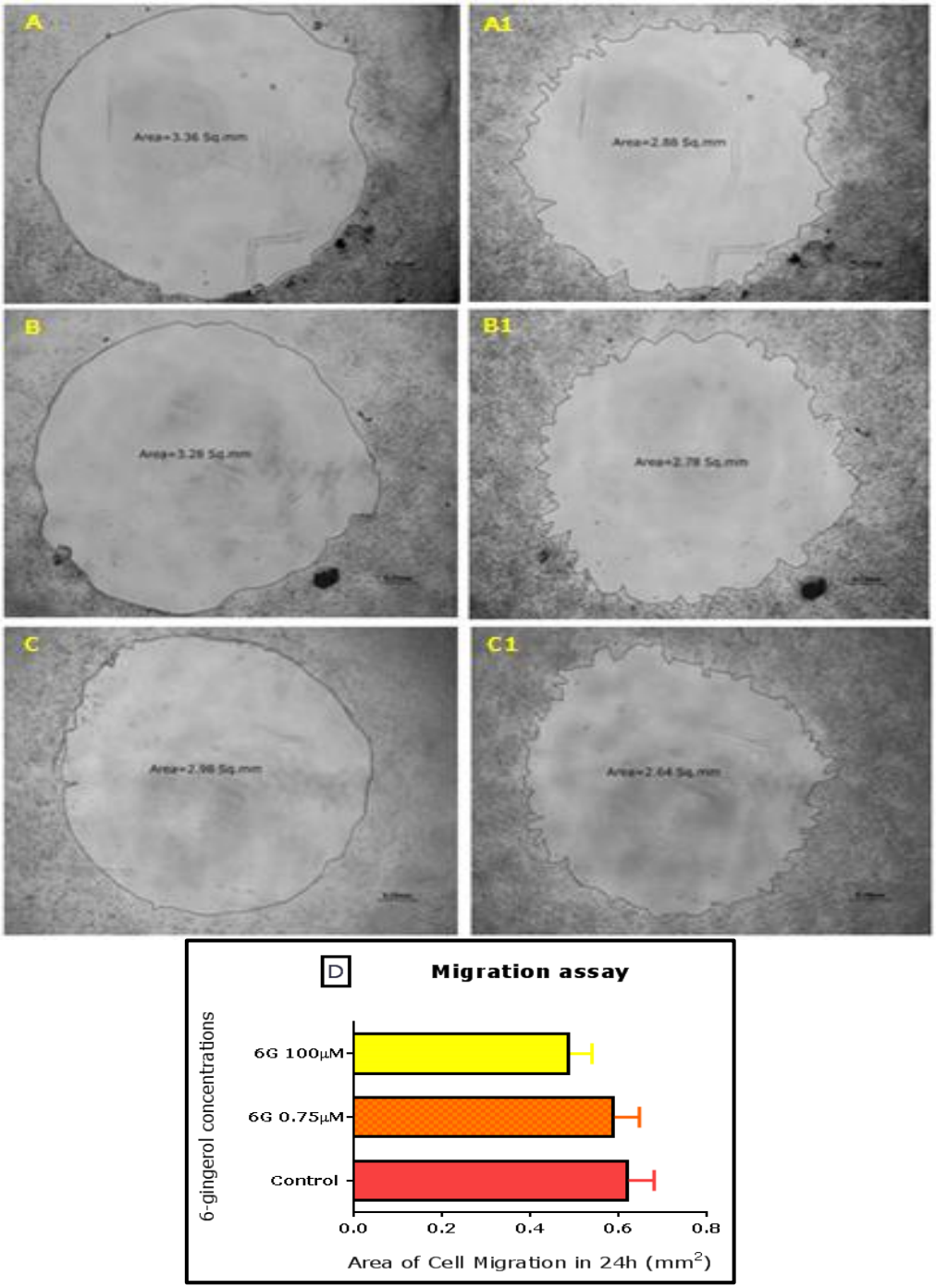


Figure 7-6: Effect of [6]-gingerol on differentiating ESD3 migration
The images (A-C1) are representatives for only one experiment, (A, B, and C) are cultures at zero time with exclusion zone areas (3.36, 3.28, and 2.98 mm^2) and (A1, B1, and C1) are corresponding images after 24h with areas (2.88, 2.78, and 2.64 mm^2) for untreated, 0.75 and 100µM 6G, respectively. The areas imply that cells migrated considerably in untreated culture but cell locomotion was decreased insignificantly in a dose dependent manner in exposed cultures particularly at 100µM. (D) Three independent repeats (n=3) with quadruplicate per each concentration were performed and one way ANOVA was utilised to analyse the data. The difference of areas at zero time and after 24h has been calculated and the results were presented as mean±SEM. * significance indicates $p < 0.05$ vs. control. The cells were photographed under a phase contrast microscope. Scale bar is 0.25mm.

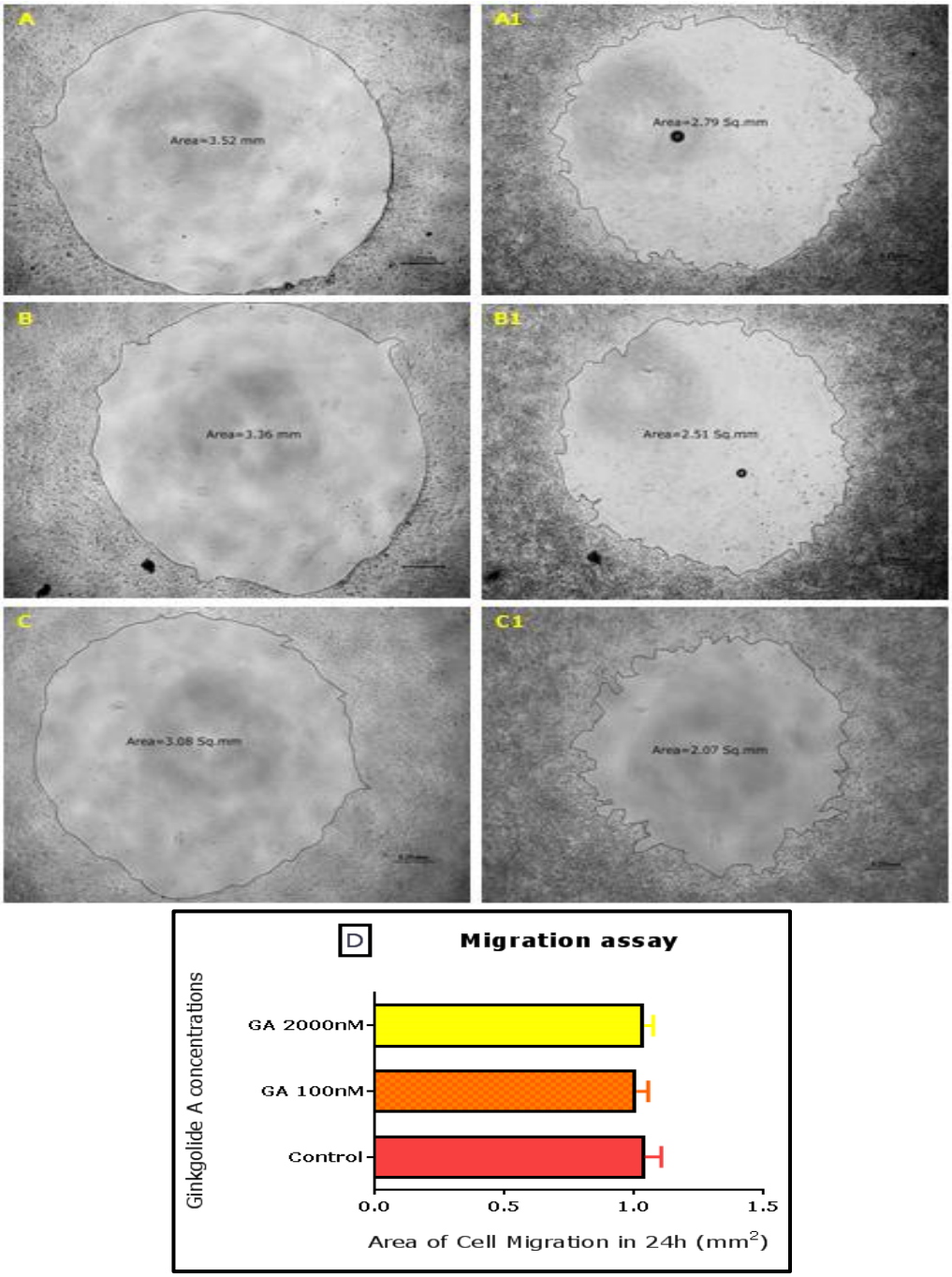


Figure 7-7: Effect of ginkgolide A on differentiating ESD3 migration
The images (A-C1) are representatives for only one experiment, (A, B, and C) are cultures at zero time with exclusive zone areas (3.52, 3.36, and 3.08 mm^2) and (A1, B1, and C1) are corresponding images after 24h with areas (2.79, 2.51, and 2.07 mm^2) for untreated, 100 and 2000nM GA, respectively. The areas imply that cells migrated considerably in untreated and exposed cultures with no difference among the groups. (D) Three independent repeats (n=3) with quadruplicate per each concentration were performed and one way ANOVA was utilised to analyse the data. The difference of areas at zero time and after 24h has been calculated and the results were presented as mean±SEM. * significance indicates $p < 0.05$ vs. control. The cells were photographed under a phase contrast microscope. Scale bar is 0.25mm.

7.3.3 Effect of ginkgolide B on cell migration (ESD3)

The effect of GB on the ESD3 migration process, following 24h exposure is presented in Figure 7-8. Without GB intervention, the cells moved toward the cell exclusion zone to cover approximately 1 mm^2 over a period of 24h (Figure 7-8A, A1 and D). However, cell migration was gradually inhibited in a dose-dependent pattern when cultures treated with 50 and 1000nM GB. The graph shows that there was significant ($p<0.05$) decrease of cell migration when treated with 50nM for 24h (Figure 7-8B, B1 and D) when compared with the untreated cells. In the same way, the migratory area was significantly ($p<0.05$) less at 1000nM GB (Figure 7-8C, C1 and D) than the untreated control.

7.3.4 Effect of ginsenoside Rg1 on cell migration (ESD3)

Interestingly, cells treated with G-Rg1 showed a slight increase in migration capacity. Although the statistical analyses did not reveal any significant differences between the tested groups, a distinct trend toward enhancing cell migration was noticeable (Figure 7-9D). Two G-Rg1 concentrations were tested (0.5μM to represent safe concentrations, Figure 7-9B, B1 and D and 10μM for cytotoxic concentrations, Figure 7-9C, C1 and D). However, over 24h exposure, none of these concentrations has shown a significant ($p>0.05$) increase in the overall migration area covered by moving cells when compared with untreated cells (Figure 7-9A, A1 and D).

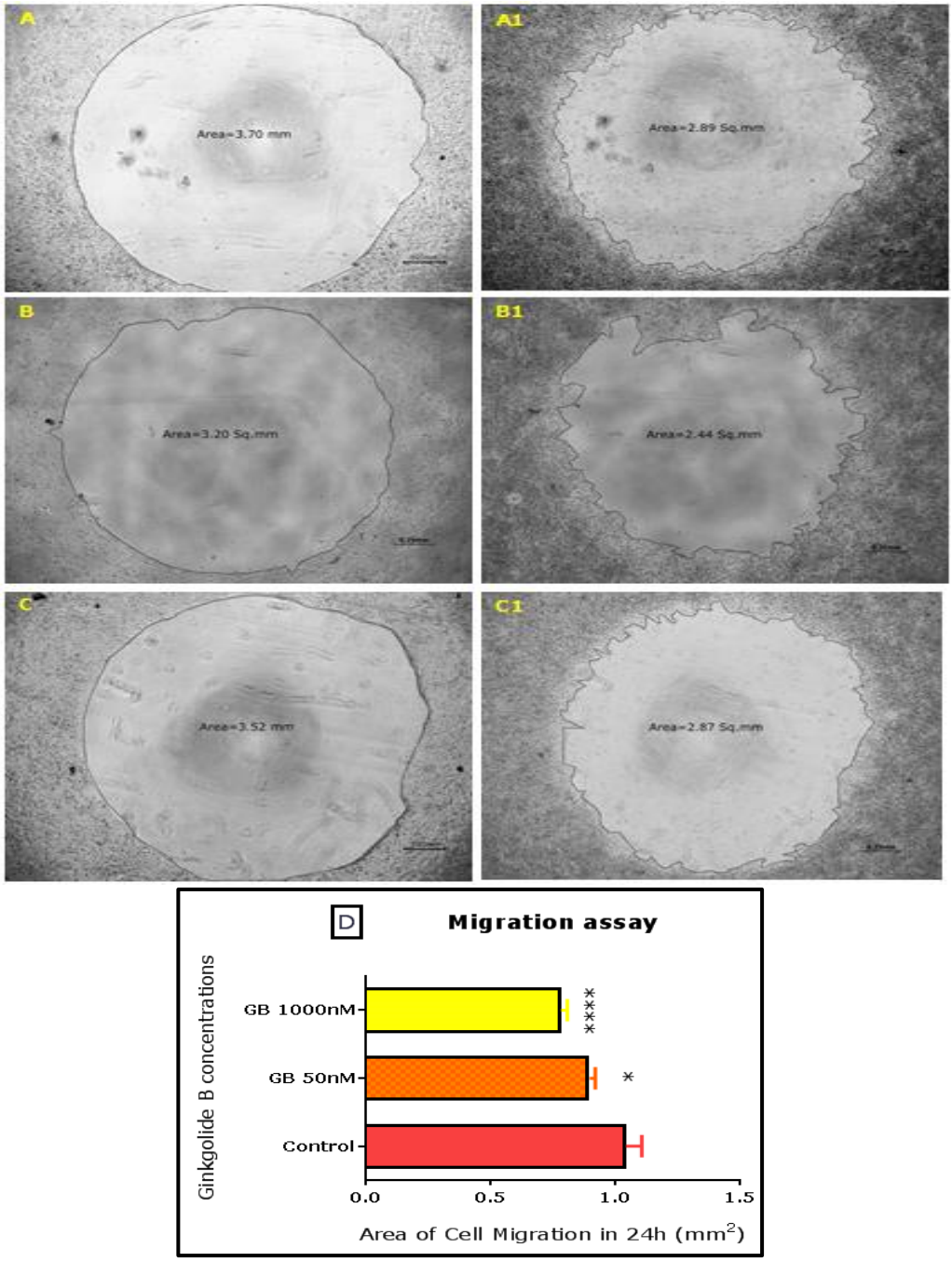


Figure 7-8: Effect of ginkgolide B on differentiating ESD3 migration
The images (A-C1) are representatives for only one experiment, (A, B, and C) are cultures at zero time with exclusive zone areas (3.70, 3.20, and 3.52 mm^2) and (A1, B1, and C1) are corresponding images after 24h with areas (2.89, 2.44, and 2.87 mm^2) for untreated, 50 and 1000nM GB, respectively. The areas imply that cells migrated considerably in untreated culture but cells migration was significantly decreased in a dose dependent manner in exposed cultures. (D) Three independent repeats (n=3) with quadruplicate per each concentration were performed and one way ANOVA was utilised to analyse the data. The difference of areas at zero time and after 24h has been calculated and the results were presented as mean±SEM. * significance indicates $p < 0.05$, **** is $p < 0.0001$ vs. control. The cells were photographed under a phase contrast microscope. Scale bar is 0.25mm.

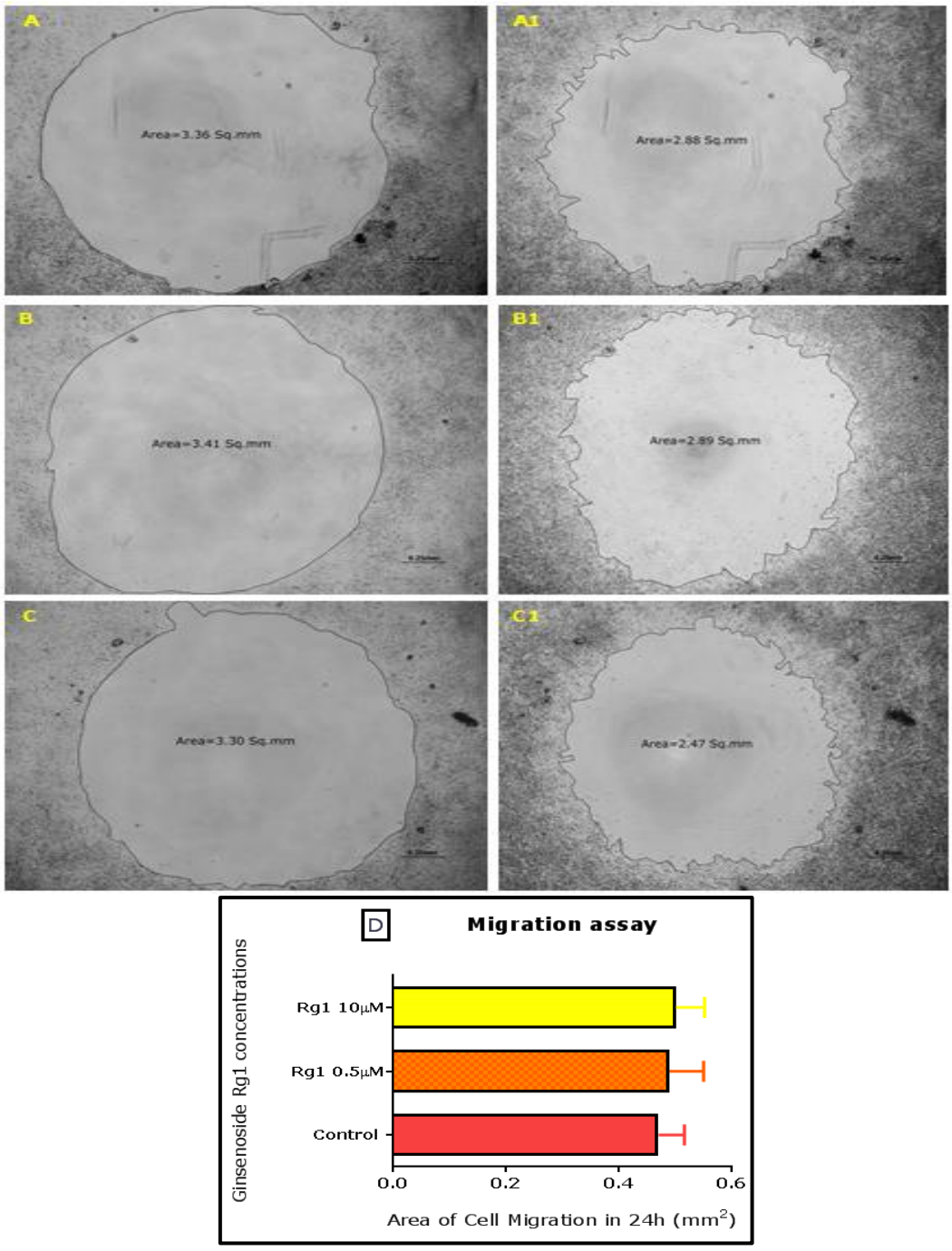


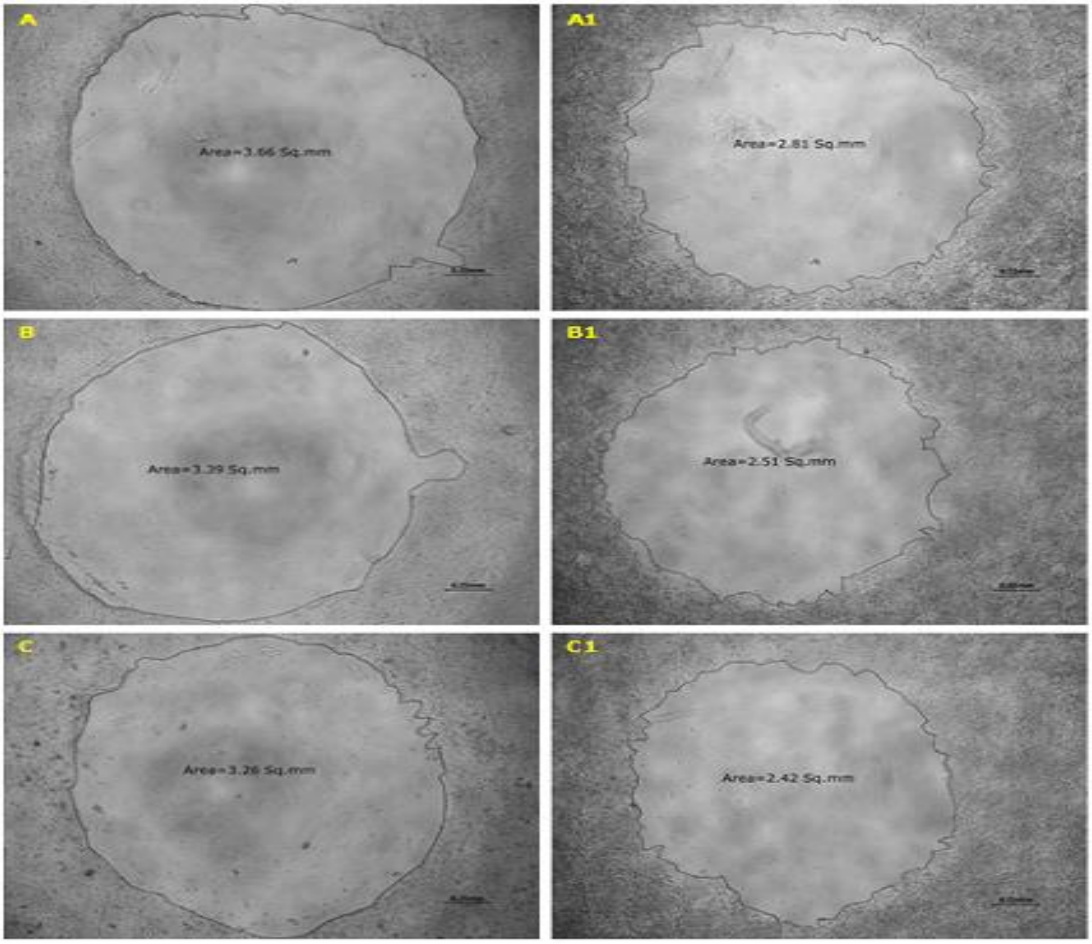
Figure 7-9: Effect of Ginsenoside Rg1 on differentiating ESD3 migration
The images (A-C1) are representatives for only one experiment, (A, B, and C) are cultures at zero time with exclusive zone areas (3.36, 3.41, and 3.30 mm^2) and (A1, B1, and C1) are corresponding images after 24h with areas (2.88, 2.89, and 2.47 mm^2) for untreated, 0.5 and 10μM G-Rg1, respectively. The areas imply that cells migrated considerably in untreated but even marginally more in exposed cultures with no significant difference among the groups. (D) Three independent repeats (n=3) with quadruplicate per each concentration were performed and one way ANOVA was utilised to analyse the data. The difference of areas at zero time and after 24h has been calculated and the results were presented as mean±SEM. * significance indicates $p < 0.05$ vs. control. The cells were photographed under a phase contrast microscope. Scale bar is 0.25mm.

7.3.5 Effect of α-bisabolol on cell migration (ESD3)

For this herbal component, also two concentrations, 1 and 80μM, were investigated representing non-embryotoxic and embryotoxic concentration, respectively. The results have shown that there was a non-significant slight enhancement in the locomotion ability of the cells exposed to 1μM bisabolol over 24h in comparison with untreated cells (Figure 7-10A-B1 and D). However, the net area of migration for the cells exposed to 80μM bisabolol for 24h was almost as comparable to the area in the unexposed cells with no significant changes between the groups (Figure 7-10C, C1 and D).

7.3.6 Effect of eugenol on cell migration (ESD3)

The impact of eugenol on the migration capacity of differentiating ESD3 was investigated employing two concentrations, non- embryotoxic 0.01μM and embryotoxic 80μM. A possible dose-dependent decreasing trend in the cell migration competence was observed after analysis. The cells when exposed to 0.01μM eugenol for 24h, they migrate slightly and non-significantly less than the untreated cells for the same period (Figure 7-11A-B1 and D). When higher eugenol concentration, 80μM, was added to the cells, the migration capacity has reduced even more when numerically compared to both the control and 0.01μM groups but this difference failed to approach a significant level (Figure 7-11C-C1).



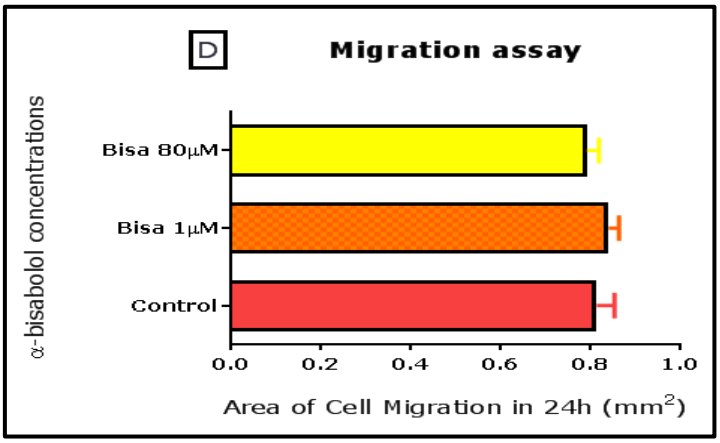


Figure 7-10: Effect of α-bisabolol on differentiating ESD3 migration

The images (A-C1) are representatives for only one experiment, (A, B, and C) are cultures at zero time with exclusive zone areas (3.66, 3.39, and 3.26 mm^2) and (A1, B1, and C1) are corresponding images after 24h with areas (2.81, 2.51, and 2.41 mm^2) for untreated, 1 and 80μM bisabolol, respectively. The areas imply that cells migrated considerably in untreated and exposed cultures with no difference among the groups. (D) Three independent repeats (n=3) with quadruplicate per each concentration were performed and one way ANOVA was utilised to analyse the data. The difference of areas at zero time and after 24h has been calculated and the results were presented as mean±SEM. * significance indicates $p < 0.05$ vs. control. The cells were photographed under a phase contrast microscope. Scale bar is 0.25mm.

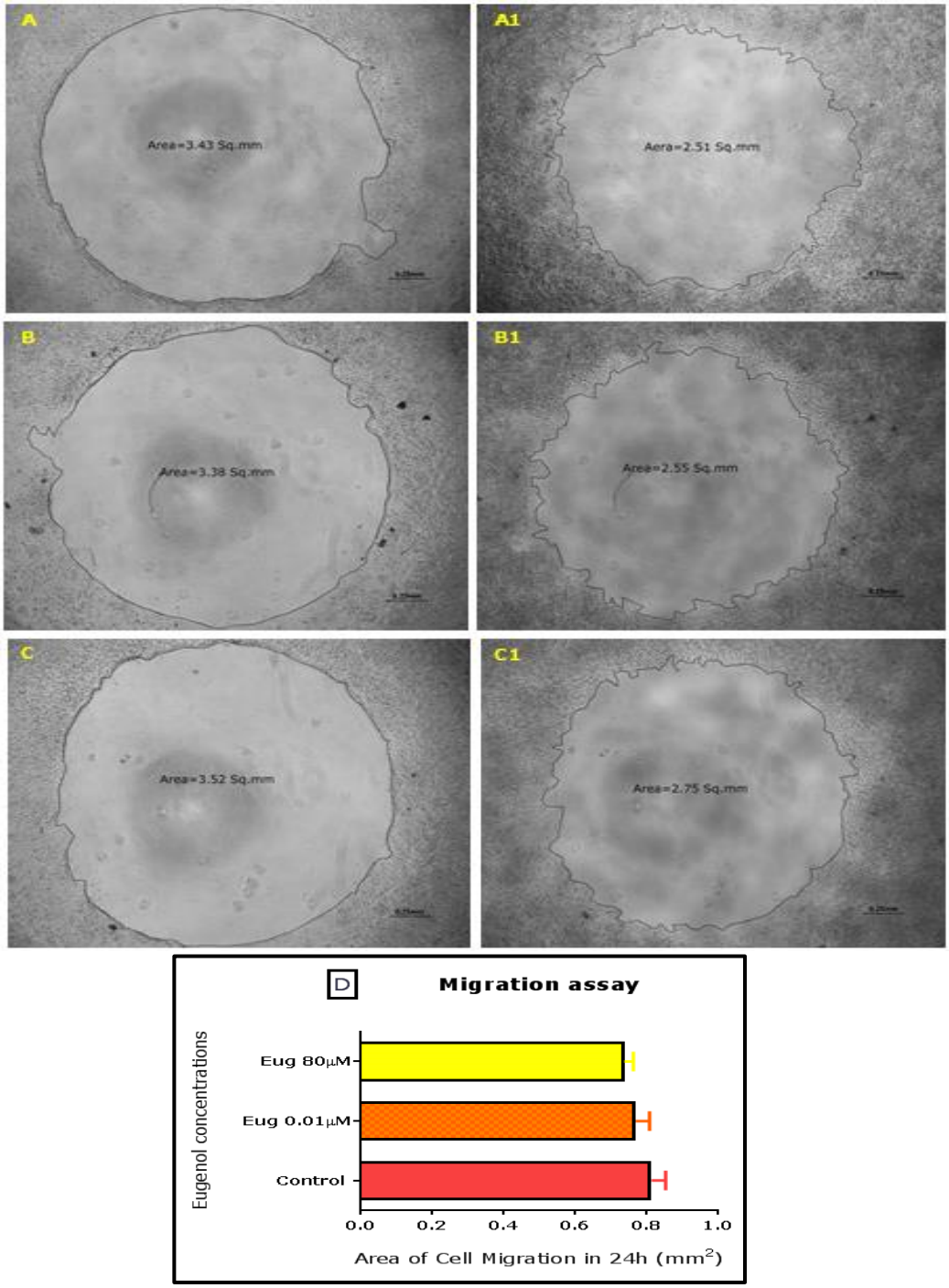


Figure 7-11: Effect of eugenol on differentiating ESD3 migration
The images (A-C1) are representatives for only one experiment, (A, B, and C) are cultures at zero time with exclusive zone areas (3.43, 3.38, and 3.52 mm^2) and (A1, B1, and C1) are corresponding images after 24h with areas (2.51, 2.55, and 2.75 mm^2) for untreated, 0.01 and 80µM eugenol, respectively. The areas imply that cells migrated considerably in untreated but slightly decrease in exposed cultures with no significant difference among the groups. (D)Three independent repeats with quadrate per each concentration were performed and one way ANOVA was utilised to analyse the data. The difference of areas at zero time and after 24h has been calculated and the results were presented as mean±SEM. * significance indicates $p < 0.05$. The cells were photographed under a phase contrast microscope. Scale bar is 0.25mm.

7.4 Discussion

In this investigation, it was attempted to address the role of some well- known herbal components in a possible disturbance of embryonic stem cell migration, which is an essential process in embryonic development and several birth defects, including the heart, could be developed as a result of its disruption. Although only GB has shown significant inhibition of cell migration, herein we showed that some herbal components might have intrinsic ability to interfere with stem cell migration by increasing their motility such as I the case with G-Rg1 or by decreasing it such as 6G and GB.

6G in this study has demonstrated a nonsignificant concentration-dependent decreasing trend in cell motility. To date, there is no relevant evidence to show that 6G exhibits embryonic stem cells motility interruption whether *in vivo* or *in vitro*. Nevertheless, some reports have suggested a role of 6G in inhibition of migration of some tumor cell lines [296],[557]. It is true that gene and protein expression differs in cancer cells from stem cells, but it could be that 6G targets some common proteins, in both cell types, that are primarily required for the migration process such as Rac, Cdc42, Arp2/3 or WASP and several more see Sections 7.1.1.1,7.1.1.2 and 7.1.1.3.

With regards to GA, the results show that there was no effect on the ESD3 migration. However, Weakley *et al*. (2011) found that GA at 50μM (alone or in conjugation with gold nanoparticles) can reduce the migration of mouse vascular smooth muscle cells [558]. However, different cells have various responses to the same chemical, but also GA being conjugated with gold

nanoparticles might modify its physicochemical properties to be able to have an effect on the migration process in the relevant study.

In contrast, GB was the only phytochemical that showed a significant inhibition of ESD3 migration. The present data agree with Liu *et al.* in 2015 where it was demonstrated that GB at 0.6 mg/ml (equivalent to 1.4mM), suppressed monocyte migration by inhibition of the expression of Cx43, cadherin and Akt phosphorylation in oxidised low density lipoprotein-treated human endothelial cells [559]. Therefore, GB might inhibit ESD3 Akt phosphorylation which is important in the asymmetry and polarisation processes of migrated cells. Interestingly, in another study GB was found to enhance the migration of human endothelial progenitor cells [560]. It might be that the 50 ng/ml vascular endothelial growth factor, which was placed in the media to direct the progenitor cells in the migration assay used, has modified the effect of GB on the cells tested. To sum up, evidence from our study and other studies have suggested that GB can modify stem cells migration.

The results of this study also showed that a nonsignificant concentration-dependent increase was observed in ESD3 migration over 24h exposure to 0.5 and 10μM G-Rg1. In accordance with our results, a study has found G-Rg1 accelerates endothelial progenitor cells at 0.5, 1 and 5μM after 24h exposure, which was considered a beneficial effect to enhance revascularisation of blood vessels [561]. Yu *et al.*, however, had contradicted this finding when they treated transforming growth factor-β1 (TGF-β1)-induced HepG2 liver cancer cells with 50-200μM G-Rg1 for 48h and found that cell migration was inhibited dose-dependently. It is obvious that the G-Rg1 concentrations used were much higher than ours; hence, G-Rg1 might behave differently toward cell motility

according to the exposure time and concentration used. Therefore, that G-Rg1 has the potential to interfere with ESD3 migratory capacity cannot be excluded but needs further verification and elucidation particularly *in vivo*.

Our results revealed no disturbances detected to ESD3 migratory capacity after treating the cells with 1 and 80μM α-bisabolol for 24h. On examining the effect of α-bisabolol on the motility of pancreatic cancer cells, Uno *et al.* in 2016 demonstrated that α-bisabolol can inhibit cell motility via induction of the Kisspeptin 1 receptor (KISS1R) [562]. However, it was concluded that other molecular mechanisms of inhibition of migration were still possible [562]. Similarly, another study using a mouse model showed that the migration of neutrophils to the gastric mucosa in ethanol-induced gastric lesions decreased after administration of α-bisabolol at doses of 100 and 200 mg/kg orally compared to animals not receiving this treatment [361]. Although the ESD3 migration was not affected at the time point tested, further migration tests should be performed with longer time intervals.

Exposure to eugenol at 0.01 and 80μM for 24h demonstrated a concentration-dependent decline in ESD3 migration, but it failed to reach significant levels. Eugenol has displayed a capacity to suppress the migration of human breast cancer cells at 1.5μg/ml (~10μM) for 24h, assayed using scratch-healing test. In the same study, eugenol failed to suppress the migration at 0.25μg/ml (~1.5μM) for 24h exposure. The authors suggested that the migration inhibition at high dose might primarily be mediated through inhibition the gene expression of matrix metalloproteinase-2, the enzyme that is also important in embryonic development, and paxillin, which is important to adhere the cells with ECM [563]. Therefore, this emphasises the need for further investigations

regarding the role of eugenol in inhibition the ESD3 migration as a mechanism for embryonic maldevelopment.

However, the data in this Chapter could have been more precise if the monitoring of cell exclusion zone migration was quantified by using the time-lapse microscopy system rather than taking snapshot pictures with a regular inverted microscope at two time intervals. Furthermore, following the assay, cells could have been fixed and various proteins involved with cytoskeletal structure and dynamics may be observed and evaluated using immunocytochemistry. This analysis may lead to further detailed biochemical variations previously unknown. The migration technique used in this Chapter needs to be more mimic to the process of extracellular matrix invasion and extravasation probably by including a layer of extracellular matrix or a layer of endothelial cells to the plates.

Collectively speaking, here we demonstrated the effect of some herbal components on ESD3 migration where the presented data, with other reports, suggest a capacity to interfere with ESD3 migration cannot be excluded, particularly at high doses.

CHAPTER 8

8 Chapter eight – Diabetes-induced cardiomyocyte defects in chick embryonic heart micromass and mouse embryonic D3 differentiated stem cells

8.1 Introduction

Diabetes mellitus (DM) is an endocrinological and metabolic disorder. It is characterised by hyperglycemia as a consequence of either inadequate pancreatic insulin secretion or poorly responding target cells or both. In addition, poorly controlled DM can result not only in hyperglycemia but also elevated levels of ketone bodies [564]. During the gestational period, three types of diabetes are of concern: Type I diabetes and Type II diabetes both present prior to pregnancy and in addition there is a type of diabetes which becomes evident for the first time during pregnancy. The former two types are called pregestational (clinical) DM, the last one is known as gestational DM (GDM) [565]. Type 1 (DM) is a result of autoimmune pancreatic β-cells destruction; type 2 exhibits insulin resistant cells and/or impaired insulin release; and gestational DM (GDM) which is defined as any glucose intolerance diagnosed during pregnancy [565].

DM in gestation is an important medical challenge since it is related to morbidity and mortality concerns for both the fetus and the pregnant woman [566]. Records show that the etiology of diabetic embryopathy is complicated as many teratological factors might be involved in the mechanism of DM-

induced congenital malformation. The absence of specific malformation patterns also supports the multifactorial theory [567,568]. Insufficient control of glycemic levels at the very first stages of conception and embryogenesis (organogenesis) is associated with an increased incidence of embryopathy [569,570]. However, it has been found that congenital heart defects and neural tube defects are the most common forms of birth malformations elicited by either of the forms of DM [571], probably because these organs develop during the early stages of embryogenesis. Alongside this, cardiomyocytes were found to be even more vulnerable to hyperglycemic-induced defects than neurons in mouse models [572]. The most frequently observed cardiac abnormalities in GDM include persistent truncus arteriosus (PTA), endocardial cushion (EC) defects and ventricular septal defects (VSD) [573]. The possible metabolic and signalling pathways that influence these heart defects included arachidonic acid metabolism, folic acid diminution, inositol depletion, sorbitol accumulation, alterations in protein kinase C (PKC) activity and elevated reactive oxygen species (ROS) levels [574]. The protective effects of several antioxidant substances such as AA, FA, SOD and vitamin E against maldevelopment induced *in vitro* [575] and *in vivo* [576,577] by diabetes-like conditions is well documented. This suggests a partial role for ROS in hyperglycemia-induced congenital defects. Also, the hyperketonemia state (beta-hydroxybutyrate elevation) was found to play a role in the regulation of cardiac glucose uptake in adult rats, but it also induced ROS production under metabolic stress conditions [578]. Diabetes-induced oxidative stress could have resulted from extreme glucose metabolism in the exposed embryo-fetal cells [575]. As a consequence, other free radical species could be generated,

modifying cellular proteins, lipids and DNA. DNA damage could be triggered as a result of alterations in expression of key genes and possibly lead to the induction of apoptosis. [579]. Thus, the hyperglycemic state results in DNA damage, oxidised lipids and proteins as a result of increased levels of ROS, possibly contributing to congenital heart defects.

The other important aspects of diabetes-induced CHD is the role of embryonic cardiac progenitors – cardiac mesoderm, epicardial and cardiac neural crest cells (CNCCs) – and biological processes which include proliferation, differentiation and migration, in heart development [580]. Although changes in these fundamental processes have not been assigned to have a direct causative role for CHD, the presence of impaired transcriptional programs of cardiac progenitors in CHD patients strongly suggest compromised cardiac progenitor biology at the origin of at least some human CHD [580]. CNCCs have shown a hyperglycemic vulnerability in an in ovo study [581]. 30mM glucose was sufficient to produce heart defects in 82% of exposed chick embryos. However, the fact that addition of 2mM N-acetylcysteine reduced the teratogenicity of glucose by 27% implies that the teratogenicity might be mediated by oxidative stress on CNCCs [581]. The inhibitory impact of glucose elevation on cell migration has been shown in an *in vitro* study on rat cranial neural crest cells. The cells from embryos of diabetic rats showed diminished migratory area expansion after growing in culture medium indicating that maternal hyperglycemia influences cranial neural crest cells migration [582].

In this Chapter, the effects of different diabetes-like formulas (β-hydroxybuterate: glucose) were investigated on primary chick embryonic cardiomyocytes and the mouse embryonic D3 stem cells (ESD3) differentiated

to cardiomyocytes. Functional parameters that were examined include contractile activity, cell activity, cytotoxicity, and any possible role of ROS, DNA damage and cell migration.

8.2 Materials and methods

All materials used in this Chapter are already described in the previous Chapters. Glucose and β-hydroxybutyrate were purchased from Sigma–Aldrich, UK. Diabetes-like formulas were constucted (β-hydroxybutyrate: glucose) with concentrations 10:20, 20:40 and 40:80mM. The chemicals were dissolved directly in culture media. These glucose concentrations were comparable to the one used in ovo by Memon and Pratten [575] and are at the random glucose levels for diabetes (11mM or more). The ketone levels were similar to those used by Sadler's group to study teratogenesis in mouse embryos [583,584]. The high concentrations that have been used in this study could be found in the most serious acute complications of diabetes, namely, diabetic ketoacidosis and hyperosmolar hyperglycemic.

8.2.1 Primary chick heart micromass (MM) preparation

Primary chick embryonic cardiomyocytes were extracted, cultured and treated with the selected chemicals. The details of the procedure are available in Section 2.2.1.

8.2.2 Mouse embryonic D3 stem cells - derived cardiomyocytes

Mouse embryonic stem cells were differentiated to cardiomyocytes using hanging drop technique and treated with the selected chemicals. The details of the procedure are available in Section 2.2.2.

8.2.3 Cardiomyocyte contractile activity scoring system - (MM and ESD3)

The contractile activity was monitored using manual scoring system in MM and ESD3. See Section 2.2.3.1.

8.2.4 Resazurin reduction assay (cell activity assay) - (MM and ESD3)

The cell activity in both systems were examined by measuring the ability of mitochondrial oxidoreductase enzymes to convert resazurin to resorufin. See Section 2.2.3.2.

8.2.5 Kenacid blue assay (total protein assay) - (MM and ESD3)

The total protein expression was measured using kenacid staining test. See Section 2.2.3.4.

8.2.6 Cellular reactive oxygen species detection assay - (MM and ESD3)

In this assay, the cell suspension obtained for the MM system was cultured in black walled, clear bottomed 96 well plates (Corning, UK) for 24h. However, for ESD3, three 5-day-old EBs were plated per well of black walled clear bottomed 96 well plate at the time of the experiment. The required amounts of glucose and β-hydroxybutyrate were dissolved in HBSS containing 20μm (DCFH-DA). The fluorescence was detected at a time periods of 0, 1, 2, 3, 4, and 24h after culture exposure. For more details see Section 5.2.1.1.

8.2.7 DNA double-strand breaks assay (pϒ-H2AX) (ESD3)

To detect pϒ-H2AX in ESD3-treated cultures, the cells were washed, fixed, permeabilised, blocked and incubated with the suitable primary and secondary antibodies. For FC see Section 5.2.2 and for ICC see Section 5.2.3.

8.2.8 Cell exclusion zone migration assay (ESD3)

The cell migration of differentiating ESD3 was measured by culturing the cells in 96 well plate with cell-free zone in the centre of each well. The migration capacity was monitored at two points, 0 and 24h after exposure. For more details see Section 7.2.1.

Statistical analysis

At least three independent repeats (n=3) of each experiment were performed using 24 well plates (four wells of each group in every repeat for resazurin and kenacid blue assays), black wall clear bottom 96 well plates (4 wells of each group in every repeat for ROS estimation assay), 6 well plates (2 wells of each group in every repeat for the DNA damage flow cytometry assay), 96 well plates (8 wells of each group in every repeat for the migration assay). The data are presented as mean ± SEM (except for DNA damage data presented in % increments) and analysed using Graph PadPrism6 statistics software. For contractile activity morphological scoring (non-parametric data), a Kruskal–Wallis test with Dunn's post-hoc test was used to determine significant differences. For parametric data (resazurin, kenacid blue, DNA damage and migration assays), one-way ANOVA followed by Dunnett's post-hoc test was employed. With ROS estimation, two-way ANOVA with Bonferroni post-hoc was used to indicate the significant difference compared to the control. $P<0.05$ was considered as significant alteration from control and specified with *.

8.3 Results

8.3.1 Effects of diabetes-like formulas on the contractile activity, cell activity and total protein content in the MM system

When the diabetes-like formulas (β-hydroxybutyrate: glucose), were used at different concentrations (10:20, 20:40 and 40:80mM) in the MM system, the inhibitory effects on contractile activity were evident (Figure 8-1A). Although the contractile activity remained unaffected in the lowest concentration formula tested, at higher concentrations (20:40 and 40:80mM) treated cardiomyocytes showed a statistically significant decrease in contractile activity at both 48h and 144h of incubation (Figure 8-1A). The cell activity measurement and the total protein content estimation followed the same inhibitory pattern with increased formula concentrations. The lowest diabetes-like formula tested (10:20mM) did not significantly affectcell activity at 24h and 144h (Figure 8-1B), nor was the total protein content altered (Figure 8-1BC). At higher concentration diabetes-like formulas (20:40 and 40:80mM) (Figure 8-1B), cell activity was significantly decreased in a dose-dependent manner. Nevertheless, significant cytotoxic effects only appeared at the highest concentration (40:80mM) (Figure 8-1C). Phase-contrast images were captured at 144h (day 6) of MM incubation, which showed a difference between normal (control) and affected (40:80mM) cultures (Figure 8-2). Increased floating cells and fewer foci number were observed, with cell-free zones within the culture, at 40:80mM, but not with the other concentrations.

8.3.2 Effects of diabetes-like formulas on the contractile activity, cell activity and total protein content in the ESD3 system

The contractile activity (differentiation) of ESD3 derived cardiomyocytes started to decrease at the lowest formula concentration (10:20mM) at day 10, 11 and 12; however, the alteration became significant compared to control at a higher concentration (20:40mM) on all scoring days recorded. The highest concentration (40:80mM) caused cardiomyocyte differentiation to cease completely (Figure 8-1D). ESD3 exposed to either (10:20mM) or (20:40mM) did not have significantly altered cell activity or cell number (total protein content) at any time point (Figure 8-1E,F), but both endpoints demonstrated a significant reduction compared to the control at the highest concentration (40:80mM) (Figure 8-1E,F). 5-day-old EBs were observed using the phase contrast microscope to monitor the size alteration. The observations made showed EBs reduced in size with increasing diabetes-like formula concentrations with the difference being very obvious at the highest concentration (DF 40:80mM) (Figure 8-2). The same EBs were further followed (at day 12 of the procedure) after seeding them in plates to observe the cultures size. The smaller the EBs, the less the culture expanded (Figure 8-2).

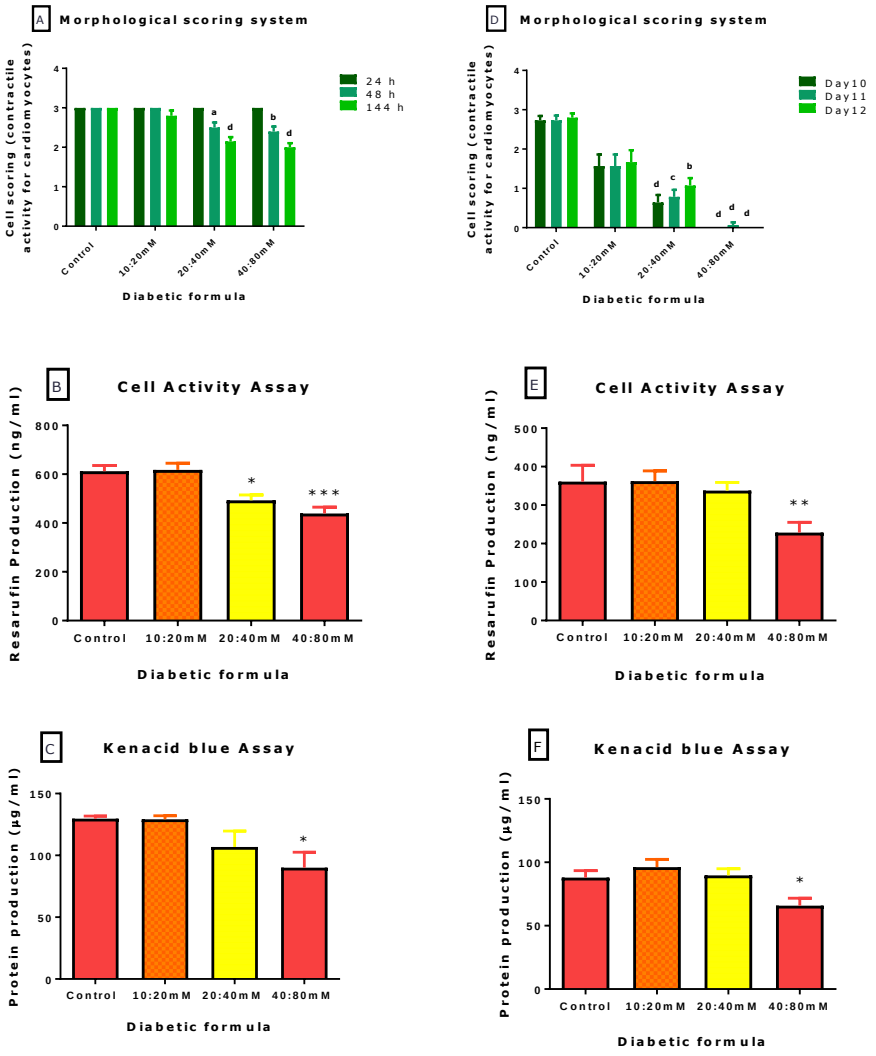


Figure 8-1: Effects of diabetic-like formulas on cardiomyocytes in the MM system (A-C) and on cardiomyocytes in the ESD3 system (D-F)
(A,D) effects of diabetic-like formulas on contractile activity, (B,E) effects of diabetic-like formulas on cell activity (resazurin assay), and (C,F) effects of diabetic-like formulas on total protein content (kenacid blue assay). Three independent repeats (n=3) of each experiment were performed. The data are presented as mean ± SEM. For contractile activity statistical comparison with control was made using the Kruskal–Wallis test with Dunn's post-hoc test. For the resazurin and kenacid blue assay, one-way ANOVA with Dunnett's post-hoc test was used. * or (a) $p<0.05$, ** or (b) $p<0.01$, *** or (c) $p<0.001$ and **** or (d) $p<0.0001$ vs. control.

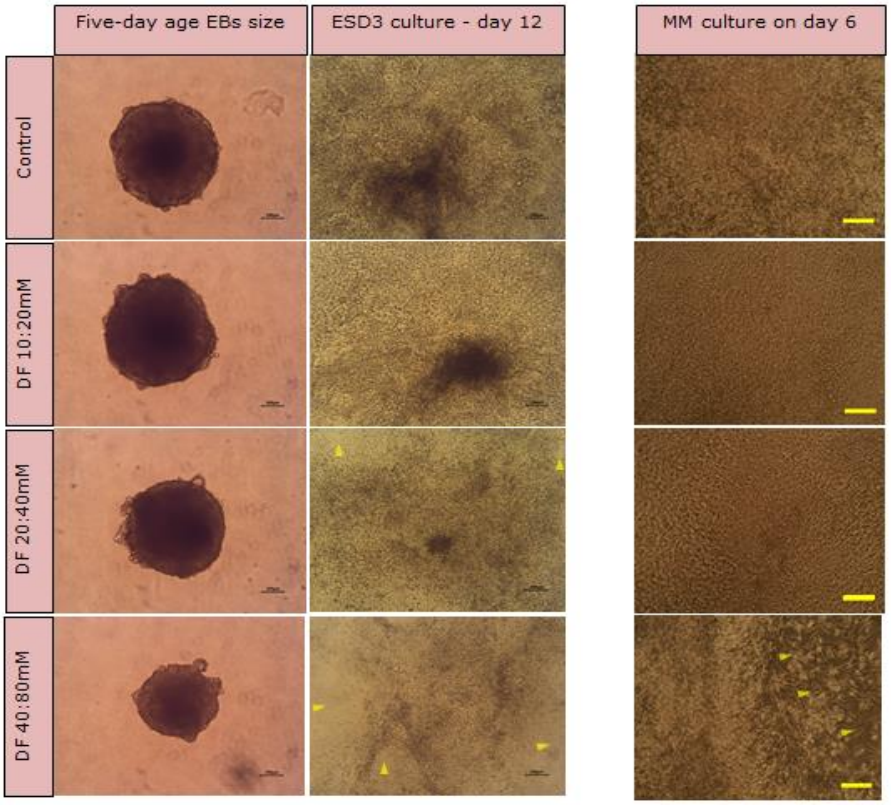


Figure 8-2: Light microscopy photographs of the effects of diabetes-like formulas in MM and ESD3
the effects of diabetes-like formulas on the MM system cardiomyocytes at 144h of culture (right panel), on 5-day-age EBs size in ESD3 system (left panel), and EBs post-culturing expansion potential (middle panel). The pictures show that with an increase in diabetes-like formula concentrations the cultured cardiomyocytes in the MM system were disturbed compared to control, the EBs reduced in size and after culturing they tend to expand less with the highest concentration. The small yellow arrows indicate free cell areas within the cultures. (Scale bar 100µm), for all images

8.3.3 Effects of diabetes-like formulas on ROS production in both MM and ESD3 systems

ROS production was assessed by using the DCF-DA method in both systems. Regarding the mild formula (10:20mM), it failed to exhibit any dramatic alteration in the amount of ROS in either system in comparison to the control group (Figure 8-3A and B). The moderate formula (20:40mM) showed a significant increase of ROS production only in the ESD3 system at 24h (Figure 8-3A and B). However; a significant increase in ROS production was detected at the highest concentration diabetes-like formula (40:80mM) in both systems after 24h exposure (Figure 8-3A and B). Only the highest concentration showed significant alteration in ROS production after 4h in ESD3 system (Figure 8-3B). The positive control used in this test (10μM H_2O_2), showed a dramatic increase in ROS production at 2h and onwards in MM system and at 4h and onwards in ESD3 system (Figure 8-3A and B).

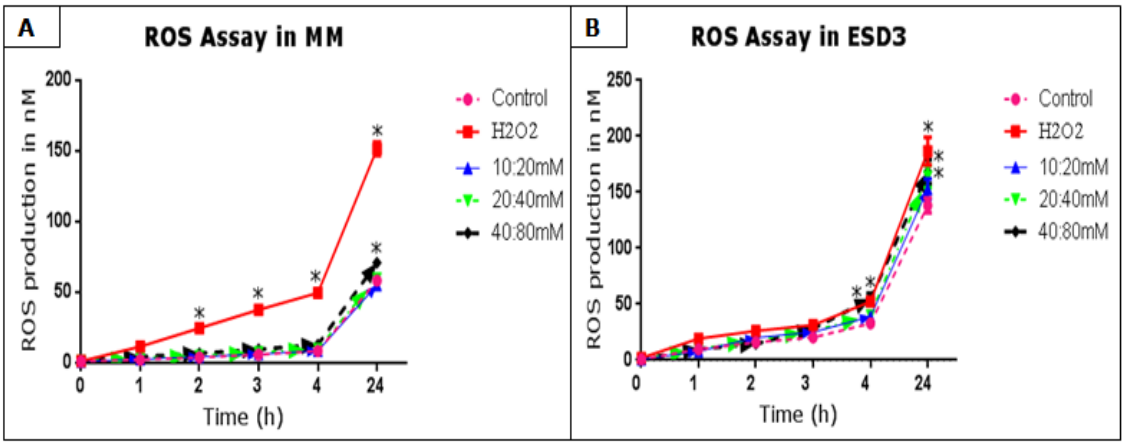


Figure 8-3: Effects of diabetes-like formulas on ROS production in MM and ESD3 systems The curves show that the (10:20mM) formula failed to increase the ROS production to significant levels in either system. The highest formula (40:80mM) increased ROS significantly in both systems. (20:40mM) increased ROS production significantly only in ESD3 after 24h. 10µM H_2O_2 was used as a positive control in each repeat. (A) effects of diabetes-like formulas on ROS production in the MM system and (B) effects of diabetes-like formulas on ROS production in the ESD3 system. Three independent repeats (n=3) of each experiment were performed. The data are presented as mean ± SEM and analysed statistically using two-way ANOVA. (*) significant difference from control and absent asterisks means none significant.

8.3.4 Assessment of diabetes-like formula induced histone γH2AX phosphorylation (DNA damage) in the ESD3 system

The ESD3 cells, when treated with different diabetes-like formulas, showed a dose-dependent increasing trend in DNA damage, until it became significantly altered from the control at the highest formula concentration (40:80mM). The dot plots in Figure 8-4 illustrate the percentage of DNA damage is increasing within the gated cell population; the control dot plot shows only 0.84% of the gated cell population demonstrated DNA damage. Further, with low and intermediate concentration formulas (10:20mM and 20:40mM), the stained H2AX (0.99 and 1.09%) respectively, showed no significant changes when compared to the control. However, at the highest formula concentration (40:80mM), the DNA damage within the gated population was 4.22%, which was statistically significant in comparison to the control (dot plots Figure 8-4).

The histogram further confirms DNA damaged ESD3 population shifting from the control and the mean of the highest formula concentration was significantly greater than the control (histogram Figure 8-4). To compare the changes in PYH2AX-labelled ESD3, between untreated (control) and treated cells, the mean of PYH2AX (integral values of gated cell population) was calculated. The % of increments among the groups was presented as it appears in the graph (Figure 8-4). Three independent repeats were performed for all experiments. These flow cytometry results were further confirmed with immunocytochemistry by staining the control and treated ESD3 cultures with PYH2AX antibody. The untreated cells showed a very faint DNA damage signal (indicated by the small yellow arrow) (Figure 8-5A1), as the diabetes-like formula concentrations increased, the number of cells developing DNA damage (small yellow arrows) became larger, and the signal under fluorescent microscope became brighter, until it became evident at the highest concentration (40:80mM) (Figure 8-5 B1, C1 and D1). The image of secondary antibody only treated culture showed no autofluorescence appeared (the image not shown).

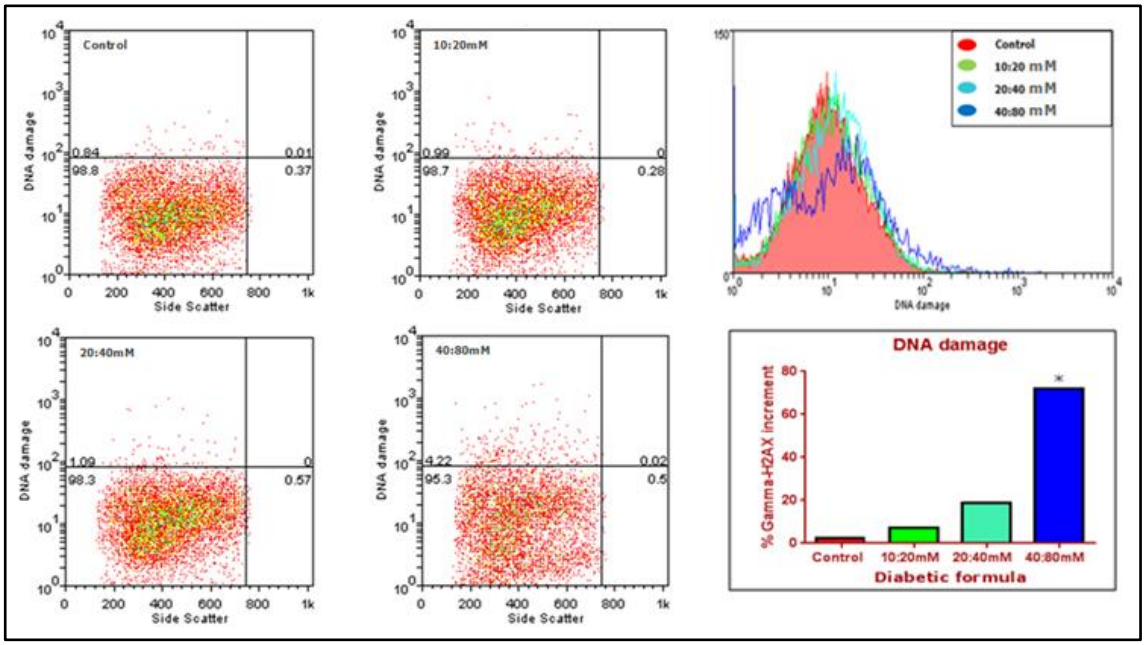


Figure 8-4: Effects of diabetes-like formulas on DNA double strand break induction

The figure shows dot plots of the control and with the three tested formulas. The y-axis represents DNA damage and the x-axis side scatter, which represent the cell size. There was dose dependent trend of increase in the H2AX signal until it becomes significant at the highest formula concentration tested. The histogram represents the H2AX flow cytometry signal within the gated cell population. The % of increments was presented in the bar graph. Control (red), 10:20mM (green), 20:40mM (turquoise) and 40:80mM (blue). At least three independent experiments (n=3) were performed. Data is presented in percentages but the analysis was performed on raw data using one-way ANOVA. (*) $P<0.05$ vs. control.

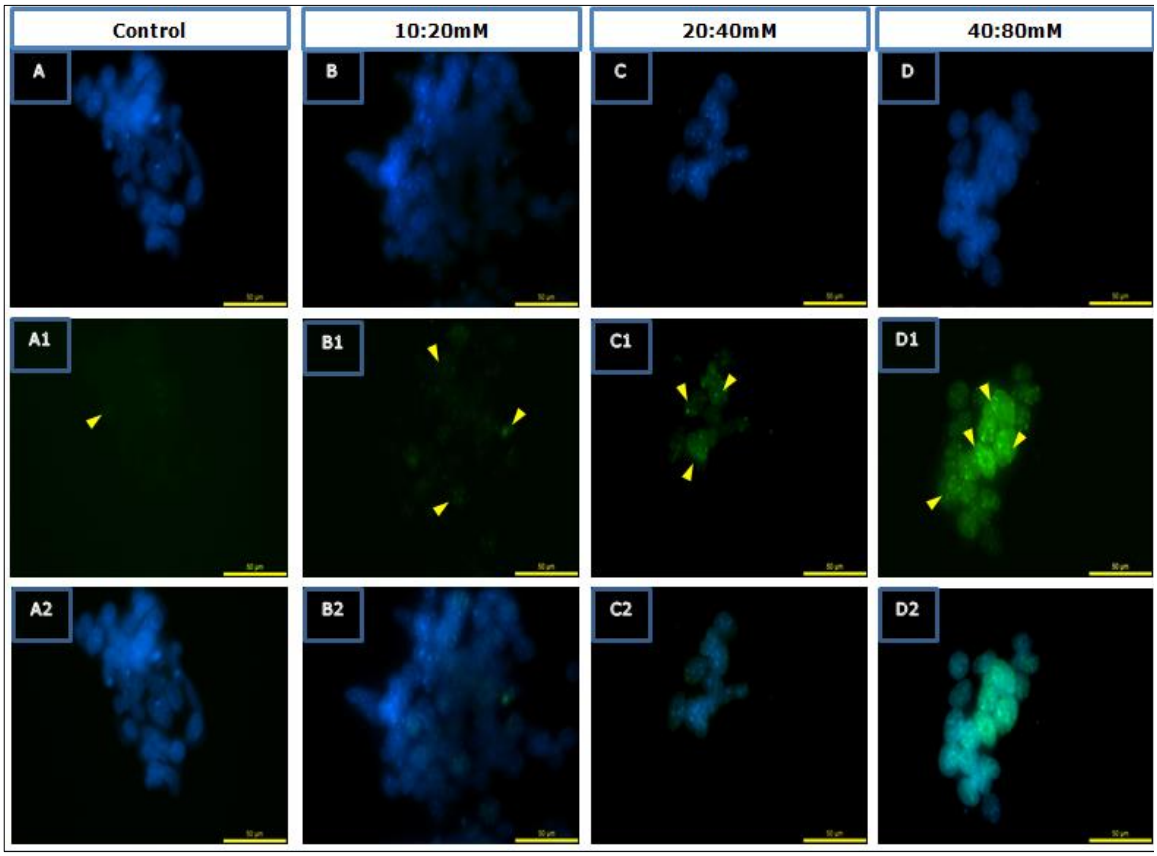


Figure 8-5: Effects of diabetes-like formulas on induction of DNA damage in differentiating mouse embryonic stem cells

The yellow arrow indicates double-strand break signals within the nucleus in treated and untreated stem cells. The green signal increased in a dose-dependent manner. (A-A2) control stem cells, (B-B2) 10:20mM formula treated stem cells, (C-C2) 20:40mM formula treated stem cells and (D-D2) 40:80mM formula treated stem cells. The top row is DAPI nuclear staining (blue), the middle row represents H2AX (green) and the bottom row represents overlay of corresponding images, (scale bar 50μm).

8.3.5 Effect of diabetes-like formulas on ESD3 migration

The effect of different diabetes-like formulas on ESD3 migration, while undergoing cardiomyocyte differentiation, has been investigated. When the cells were treated with low diabetes-like formula (10:20mM) for 24h, the pooled data showed that the cells migrated for (0.4394 mm^2) which is less than the area in the control group (0.5767 mm^2), but the difference was not significant (Figure 8-6A, B, C, D and I). In the selected representative images, the difference in areas at 0h (3.54mm^2) and 24h (2.99mm^2), which represents migration area, was (0.55mm^2) for control, and the difference in areas at 0h (3.57mm^2) and 24h (3.04mm^2) was (0.53mm^2) for (10:20mM) treated cells, which mean the untreated and treated cells had similar migration potential (Figure 8-6A, B, C and D). However, for 20:40mM formula, the pooled data showed that cells migrated area was (0.302 mm^2) which is significantly less than the control (Figure 8-6I). In the selected representative images, the difference was 3.42mm^2 - 3.19mm^2=0.23mm^2, therefore, this formula affected ESD3 migration significantly when it was compared with the migrated area (0.55mm^2) of the control (Figure 8-6E, F). At 15μM cytochalasin D, standard or reference control, the cell motility almost ceased after 24h. Three independent experiments were performed with at least five intra-experimental repeats.

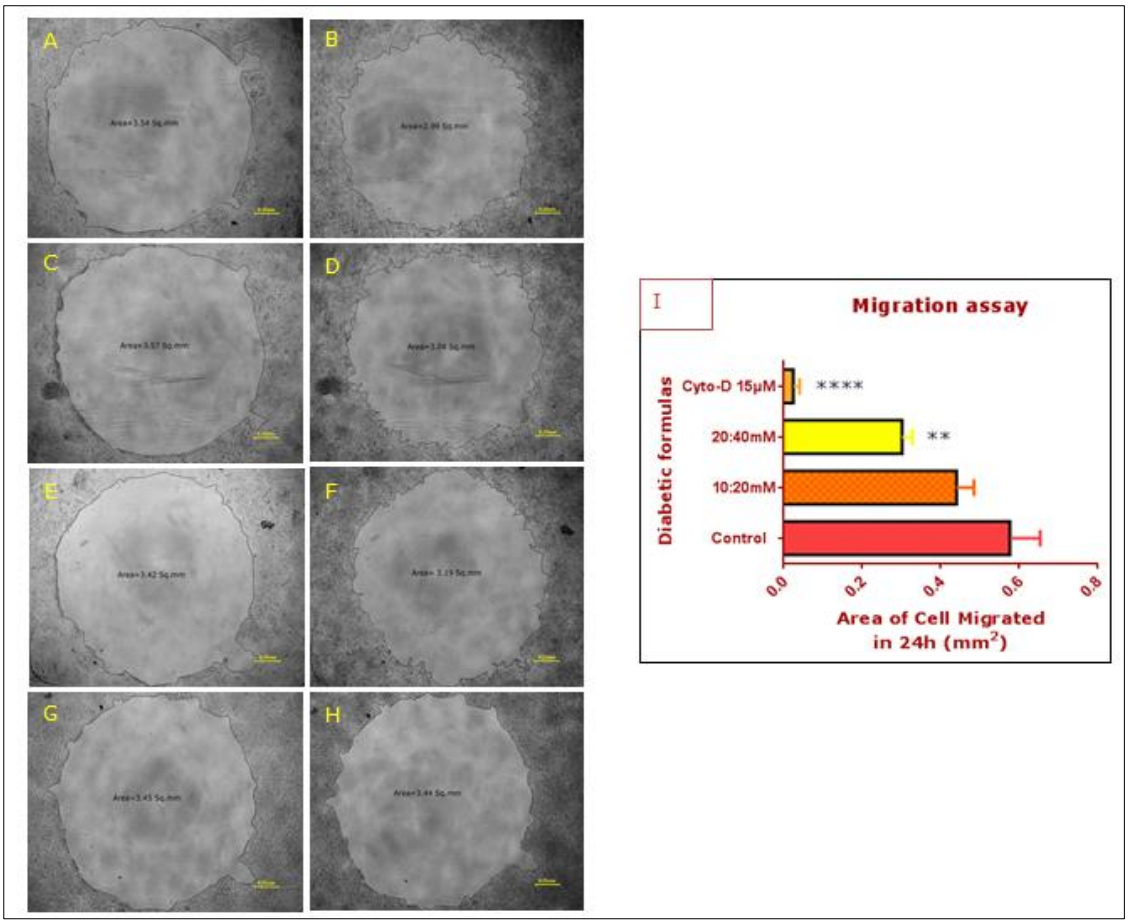


Figure 8-6: Effects of diabetes-like formulas on migration of differentiating mouse embryonic stem cells

Two formulas were tested in this assay 10:20 and 20:40mM. The results indicate the inhibitory effect of hyperglycemia on the stem cell migration process. (A) the area of untreated stem cell at 0h (3.54mm^2), (B) the area of untreated stem cell at 24h (2.99mm^2), (C) the area of cells treated with 10:20mM formula at 0h (3.57mm^2), (D) the area of cells treated with 10:20mM formula at 24h (3.04 mm^2), (E) the area of cells treated with 20:40mM formula at 0h (3.42mm^2) (F) the area of cells treated with 20:40mM formula at 24h (3.19mm^2), (G) the area of cells treated with cytochalasin D 15µm at 0h (3.45mm^2), (H) the area of cells treated with cytochalasin D 15µm at 24h (3.44mm^2) and (I) graph representing the data of three independent repeats (n=3) with 8 intra experimental repeats for each tested formula. The data is represented as mean ± SEM. (**) P<0.01, (****) p<0.0001 vs. control. (Scale bar 100µm).

8.4 Discussion

Uncontrolled DM in pregnancy is often associated with a high frequency of morbidity and mortality in the offspring and mothers [585,566]. Ignorance of hyperglycemia during gestation, particularly during the organogenesis period, leads to severe maldevelopment of the main organ systems including the cardiovascular system [575]. It has been reported that Type 1 DM causes more medically reported incidences of congenital anomalies compared to GDM or Type 2 DM [586,587].

This study was carried out to investigate the cardiotoxic effects of a diabetes like environment on primary embryonic chick heart and mouse embryonic D3 stem cell-derived cardiomyocytes and also to explore some possible mechanisms (ROS, DNA damage and cell migration), which might have roles in any of the recorded outcomes. The results from MM and ESD3 show that the designed diabetes formulas used exhibit a concentration-dependent pattern of effect on the differentiation process (determined by contractile activity), cell activity and cell protein content, whether on growing or differentiating cardiomyocytes. Memon and Pratten, (2013), have shown the effect of 20mM glucose+15mM β-hydroxybutyrate on the growing heart in the chick, which was exposed to these conditions for 6 days (day 3 up to day 9) using an in-ovo assay; the results showed abnormal heart development (particularly the ventricular chamber) of treated embryos [575]. It has been known that the major outcome of gestational diabetes is morbidity rather than mortality. This study confirms that only at high concentration (40:80mM) was there a sign of cytotoxicity, by showing a decrease in protein content (also by imaging the

cultures) in both systems, which might eventually be considered as a mortality effect and abortion promoter. This was further confirmed by EBs size reduction with higher concentration diabetes formula, which was mostly accompanied by a decline in cell activity. In support of these findings, a study in rats of the embryos of diabetic animals also demonstrated intra-cardiac anomalies in the offspring [588]. The results from the low concentration of diabetes-like formula (10:20mM) show no toxic effects. This could be because the glucose itself is a source of cell energy and the cells rely on it as nourishment.

Despite the confirmation of GDM associated congenital birth defects [585] which include congenital heart defects [589], the molecular mechanisms that might be involved in the defects are still obscure. Here were investigated three possible processes, ROS production, DNA damage and stem cell migration, which could influence the development of embryonic cardiomyocytes. The results of this study show that ROS steadily increased with the increase of diabetes formula concentrations in both systems. This was associated with the apparent increase in DNA damage in ESD3 for each corresponding formula. The role of free radicals and oxidative stress in diabetes-related embryopathy has been suggested in several studies, either directly by measuring free radical mediators or by observing the protective effects of antioxidant substances [590]. The ROS-mediated oxidative stress of hyperglycemia was evident in several experimental and clinical studies and was accompanied by a decrease in endogenous antioxidants (NADPH and GSH) with disturbed oxidation-reduction (Redox) homeostasis [591-593].

One of the possible mechanisms by which excess glucose evokes ROS production is by the increased influx of glucose through the polyol pathway,

which converts glucose to the polyalcohol sorbitol, which in turn induces intracellular O_2^- [594]. It has also been suggested that hyperglycemia could excessively drive the mitochondrial electron-transport chain in a way that would make free electrons prematurely. These could react with oxygen molecules instead of driving the necessary potential to produce ATP, leading to an increased generation of oxygen superoxide radicals [595]. ROS production could be the cause of diabetes-induced teratology since ROS could eventually react with cellular proteins, lipid and DNA resulting in alteration of gene expression, leading to mutations which might translate into underdevelopment of organs. However, the Zhao study (2010) on diabetic mice showed that there was no difference in apoptosis between diabetic and control groups in embryonic hearts, although histological examination revealed cardiac abnormalities in diabetic groups [572]. Furthermore, the main finding in the Zhao study was the role of transforming growth factor-β (TGF-β) in inducing heart abnormalities in diabetic groups. Moreover, protein kinase C (PKC) enzymes have been found to play a major role in diabetes-induced embryopathy probably because of their role in several cellular activities such as proliferation, migration, apoptosis and differentiation [596]. Prolonged activation of PKC was already linked to the hyperglycemic state of vascular smooth muscle cells [597]. The present results showed the level of diabetes formula used is related to the severity of DNA damage. This was in accordance with another *in vivo* study on rats where DNA damage was shown in the leukocytes and the fetuses of diabetic rats under highly diabetic conditions [598].

Cell migration has been found to be essential for normal heart development, particularly that of CNCCs [599]. Morgan *et al.* (2008) have discovered a role for the Pax2 gene to mediate the effect of hyperglycemia-induced oxidative stress on CNC migration in mice [600]. Cell motility in differentiating embryonic mouse stem cells was significantly affected by (20:40mM) diabetes formula in the study reported here. This might also be associated with ROS-induced oxidative stress, which could eventually affect certain genes that are essential in the migration process. Consequently, this might be the reason behind the significant decrease in the differentiation process of cardiomyocytes as a whole.

One may argue that the effects observed in this study due to variotion in osmolarity of the solution rather than high concentrations of β-hydroxybuterate: glucose per se. Some researchers include mannitol of equal osmolar concentration in their experiments to firmly state that observed effect is not due to high concentration of β-hydroxybuterate: glucose [601], but some other researchers do not [575]. However, diabetic ketoacidosis and hyperosmolar hyperglycemic state are two of the most serious acute complications of diabetes [602]. Therefore the effect of osmolarity of glucose and/or ketone bodies might not be omitted or neutralised because even if it exists, it will mimic possible clinical conditions.

To conclude, this study mainly targeted the possible effects of pre-gestational DM (type 1 or 2). It indicates that it is essential for diabetic women to be evaluated thoroughly before pregnancy since uncontrolled DM could have a major impact on both the pregnant woman and her offspring. With the current results, it is highly recommended for treatment to be oriented toward

improving the general metabolic condition by tight glucose control as indicated by normal glycosylated haemoglobin (HbA1c) levels.

CHAPTER 9

9 Chapter nine – General discussion

9.1 General discussion, conclusion and future view

After the thalidomide birth defects tragedy in the past century, research into reproductive and developmental toxicology has focussed on the prevention of chemical-induced malformations. Since that time, the study of embryonic development of different species became one of the most essential and intriguing fields of science, as it involves sophisticated transcription, signalling, differentiation, regulation and assembly. Therefore, a persistent flow of information between cells and tissue systems is required to fine tune this complex web of events [458]. Any disruptions of the fluency of information for these pathways by external or internal factors could result in devastating developmental outcomes [458].

The heart was selected in this study because it is the first organ that starts to function, therefore, any defect in heart development leads to an insufficient supply of oxygen and nutrients to other differentiating and migrating cells during the developmental process. The lack of oxygen and nutrients means more developmental defects occur in the embryo, resulting in either mortality or morbidity. Since the effect of chemicals on cardiomyocytes could happen at any embryonic developmental stage, two cardiac lineages have been chosen for the experiments. The first one is growing chick embryonic cardiomyocytes cultured in the MM system, which represents early embryonic heart cells which are anatomically underdeveloped but have obtained the cardiac lineage [268].

The second one is mouse embryonic stem cells derived cardiomyocytes cultured in the ESD3 system which represents the stage of cardiac differentiation from stem cells. In both systems, the chemicals of interest were applied to the cultures to investigate cellular and molecular effects. Several endpoints have been studied such as contractile activity, cell activity, total protein content, culture outgrowth and EBs size, ROS, DNA damage, transmembrane proteins expression and stem cell migration. The morphological scoring system was designated to mark the cell differentiation and as an indicator for cell membrane integrity. The resazurin reduction assay was utilised to measure cell activity because it allows the same cells to be submitted for protein content measurement by the kenacid blue assay. EBs size in ESD3 was monitored as a marker for stem cell proliferation. ROS production, the DNA damage test, cell migration and Cx43, Integrin β1 and α-actinin expression were explored for their important role in normal fetal development. Whilst *in vivo* embryotoxicity testing for chemicals using laboratory animals is still ongoing, our study is in support of the 3'R principle to reduce the number of animals in research by utilising *in vitro* models in addition to the fact that there are ethical barriers arising from studies on human embryos.

Many ancient civilizations have relied on herbs and herb-derived medicines in health and disease management for centuries, and recorded evidence was discovered for the prehistoric use of herbs in the treatment of different ailments [603]. The global demand for plant-derived medicines has grown significantly in recent years [604]. By 2003 and in the USA alone, more than 1500 herb-derived products marketed were exempted (because they are

regarded as food supplements) from extensive preclinical efficacy and toxicity testing by the U.S. FDA [605]. These statistics have led to increased concerns about potential detrimental effects of these products, which have eventually helped to foster toxicity testing approaches that can be applied to phytochemicals to investigate their toxicological characteristics and to explore the acute high-dose and chronic low-dose exposure effects [605]. Moreover, the lack of scientific evidence about the mechanism of action of herbal components is still an issue related to modern health care. It is therefore crucial for scientific research to investigate the effects of herbal intake. One of the global challenges with the herbal products is that phytochemical ingredients are marketed as dietary supplements and are not subject to the premarket safety assessments which would be required of new food ingredients, medications or new uses of existing food ingredients [606]. Although the U.S. FDA has the right to prohibit or withdraw harmful dietary supplements from the market, the burden of proof is on the FDA. However, the opposite is true when it comes to medications, where the manufacturer must verify effectiveness and safety. This is also means that there would be no legal obligatory system for reporting the harmful effects of dietary supplements [606]. These facts could endanger pregnant women who seek 'natural' treatments and view herbs as natural and ideally safe. Many women prefer to use herbal medicine in the early stages of pregnancy for relieving morning sickness, boosting energy and brain circulation, and as a tonic or for depression etc. Supporting our concerns, several independent organisations and groups reported that many of these herbal products failed to meet several claims stated in their leaflets which are related to the product constituents,

standardisation markers, or other elements. These reports precisely indicated the wide variation in the quality of herbal remedies [607]. It is hoped to raise awareness of the potential harm of herbal medicines to the growing embryo, and negate the view that such substances are necessarily harmless because they are natural.

Our experiments showed that all herbal components tested displayed concentration-dependent teratogenic effects on the parameters examined. Further, they all exhibited more harmful effects on the differentiating stem cells in ESD3 cultures than on growing cardiomyocytes in MM cultures. After the toxicological assessment of adverse effects of these tested herbal components was performed, a relative determination to the safe exposure levels was also identified. Depending on the number of endpoints that have been significantly modified by one tested herbal component, it could be assumed that GB, GA and G-Rg1 are relatively safer than 6G, eugenol and bisabolol. Further, our results indicate that high doses of these herbal products at earlier stages of the pregnancy would be more detrimental on embryonic heart development. Therefore it is crucial to advise pregnant mothers to abide by the minimum doses and to avoid taking extra amount of these products, by taking several forms of the same products or repeat the dose of the same product, until further studies have confirmed their minimum safe doses. Also, it is useful to recommend avoiding these products if possible during the first trimester where organogenesis, including development of the heart, is taking place.

After safe levels of the herbal components were established, it was furthermore informative to find out if these herbal components have protective potential

against standard teratogens, such as ETH, RA and VPA, or indeed if they potentiate their teratogenic effects. The experiments to study the protective potential of safe levels were undertaken in both *in vitro* systems. Based on the overall results, it can be proposed that the herbal components tested have a weak protective potential particularly against the chemicals that induce cellular modifications at the genetic level. Most of them showed antioxidant capacity which was comparable to standard protective molecules such as FA, AA and SOD, particularly in ETH-treated cells where ROS play a major role in toxicity induction. However, it was noted that GB and bisabolol did not show any capacity to rescue the injured cells in either system. The failure to show a profile of protective capacity could be because of the low concentrations of herbal components which prevent them from neutralizing the damaging effects induced by teratogens. The other possible reason is the lack of necessary defence mechanisms in embryonic cells, which might boost the protective capacity of the herbal components. The latter theory is supported by the fact shown by studies that the same herbal component behaves differently in normal and cancer cells. For example it may induce oxidative stress in cancer cells but inhibit it in normal cells. It is important to mention that our results do not deny or contradict the established reports that show a high protective capacity for these chemicals against different teratogens in *in vivo* studies [608] or in non-embryonic cells such as neurons [609], hepatocytes [610] or even in tumor cells [294]. Our experiments were only performed on chick embryonic cardiomyocytes and mouse embryonic stem cell-derived cardiomyocytes.

As aforementioned, many reports have examined the effect of diabetes during gestation that may lead to fetal morbidity and mortality [611]. Herein, different diabetes-like formulas were tested (β-hydroxybutyrate: glucose, 10:20, 20:40 and 40:80mM). The glucose concentrations were similar to those used in ovo by Memon and Pratten [575] and the lowest concentration is at the high end of the normal range for humans. The ketone levels were comparable to those used by Sadler's group to study teratogenesis in mouse embryos [583,584]. In agreement to other studies, these formulas have shown a concentration-dependent teratogenicity via decreased cardiomyocytes contractility, activity, protein content, ROS, DNA damage and differentiating stem cells migrating capacity depicting its harmful effect towards the fetus in pregnant mothers. Further explanations from other studies stated that ketones have been exhibited to be growth retardant chemicals that inhibit growth in rat embryos. As for the high glucose concentration, it is prone to cause excess free oxygen species due to its breakdown, leading to an increase in oxidative stress. During early development, embryonic cardiomyocytes are prone to oxidative stress because they lack the ability to produce sufficient antioxidants to defend themselves from ROS. Thus, it leads to a higher rate of cardiovascular and other abnormalities in babies born to a diabetic mother [612].

As foreseen in the results Chapters, these herbal components have a capacity to modify cellular, molecular and perhaps even genetic targets and cause embryonic heart maldevelopment. Although it is hard to suggest a precise mechanism of cardiac embryotoxicity from the available data, to some extent, it is possible to suggest that the measured parameters may interplay among

each other. However, for a better understanding of the mechanisms, more experiments should be carried out and more parameters have to be studied.

9.2 Future work

- Public awareness: we would strongly suggest approaching women of childbearing age to make them aware of potential adverse effects of herbal supplements on growing embryos. It is important to inform and educate more mothers on what and how to take herbal remedies along with modern medical services. This could be done through informational leaflets or, more directly, seminars, symposiums, workshops, social media or even by delivering talks in events such as TED. The alert proposal should also include the modern medical staff to give more understanding about herbal remedies and usage.
- Improve herb-marketing legislations: it is possible to review the global guidelines regarding the marketing of herbs. The first aim is to put a burden on the manufacturers to prove the herbal product's safety instead of the current situation where the FDA has to prove they are not safe. The second purpose should target the current easy access to herbal products and harden the law from this perspective.
- Effects of herbs during late pregnancy: as we studied the effects of some herbal components on early stages of embryonic cardiomyocytes, further studies may be needed to expand on the effect of these chemicals during late gestation and also apply it to other types of cells such as neurons or limb buds for generalizability. Also, many other phytochemicals or other

ingredients of the same herbs could also be examined for their teratogenicity.

- Gathering human-related information: this might be achieved by carrying out longitudinal cohort studies, where GP clinics could offer good support to initiate such ideas. These studies would include all women of childbearing age who have attempted herbal remedies for any reason with the possibility to follow them up may be for years.
- Additional herbal products knowledge: to further help their safety assessment, pharmacokinetic studies of herbal active ingredients are important to be performed. Also, ex-vivo experiments using human placenta are possible and could be utilized to provide information about placental transport.
- Connexins and integrins: further experiments to study the expression of these two protein families during different stages of embryonic development could usefully be undertaken to find out their roles in cell migration, which in turn plays an essential role in development of many parts of the heart. Heart sections from the chick embryo could be employed.
- Experimental improvement: the manual, subjective morphological scoring system used in this study could be replaced by designing a computerized electronic model to monitor and record the beating rate and magnitude of the cultured cardiomyocytes and study the immediate, short and long term effects of the chemicals.
- The effect of phytochemicals on other developmentally important mediators: the work can be further extended to study the effects of herbal

components on some ion-channels that play a role in controlling the beating activity in cardiomyocytes such as K^+ and Ca^{+2} using patch clamp techniques. Studying the effects of herbal components on caspases as apoptosis mediators using IHC and WB could be rewarding. And most importantly, application of PCR techniques would be useful to study the impact of the herbal components on the Wnt singling genes, which have a critical role in the embryonic development including the heart.

Appendices

10 Appendices

10.1 Appendix I

Western blotting solutions

Solubilisation buffer: to make up 6X, 2.4g sodium dodecyl sulphate, 3mls of 30% glycerol, 3mls of β-mercaptoethanol, 240µl bromophenol blue and 2.5µl of 1.5M Tris HCl in a 50ml tube was made up to 10ml with distilled water. The solution was warmed up at 37°C to facilitate its dissolution and the final solution was aliquoted and stored at -20°C.

Lysis buffer: this buffer was made up of 12.1g Tris (20mM), 1.9g EGTA (1mM), 51.7g sucrose (320mM), 0.1% (v/v) triton X-100 (500µl), 0.021g sodium fluoride (1mM) and 1.08g β-glycerophosphate (10mM) in distilled water. Final solution was buffered at pH 7.6 by 1.5M HCl and made up to 500ml with distilled water. The final solution was stored at 4°C.

10X electrophoresis buffer: this needs 30.3g Tris, 144g glycerine and 10g sodium dodecyl sulphate (SDS) to be dissolved in 1 liter of distilled water. Final solution was stored at room temperature.

Transfer buffer: this requires 30.3g Tris and 144g glycine to be dissolved in 8 liters of distilled water plus 2 liters of methanol and the final solution was kept at 4°C.

Tris Buffered Saline Tween20 (TBST): this contains 30.3g Tris and 73.12g NaCl in 10 liter distilled water after being adjusted to pH 7.6. to the final solution, 10ml Tween 20 was added to obtain final concentration of 0.1%. This buffer was stored at room temperature.

10.2 Appendix II

Cell density determination

Trypan blue assay was utilised to calculate the cells number. 10µl of cell suspension were put in an Eppendorf tube and mixed with 10µl trypan blue. The mixture was left for a few seconds at room temperature then a cell counting machine slide was loaded with 10µl of the mixture and carefully inserted into the TC10™ Automated Cell Counter (Bio-Rad, USA) to obtain the reading (number of cells per ml). The dead cells would be stained with trypan blue as they lost their ability to expel it.

10.3 Appendix III

Publications

Reproductive Toxicology 59 (2016) 117–127

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Evaluation of embryotoxicity for major components of herbal extracts using the chick embryonic heart micromass and mouse D3 embryonic stem cell systems

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ARTICLE INFO

Article history:
Received 11 June 2015
Received in revised form
27 November 2015
Accepted 14 December 2015
Available online 18 December 2015

Keywords:
Chick embryonic cardiomyocytes
Micromass
Mouse D3 embryonic stem cells
Herbal medicines
6-gingerol
Ginkgolide A
Ginkgolide B
Ginsenoside Rg1

ABSTRACT

Herbal remedies are often used during the early stages of pregnancy, being considered 'harmless' and 'natural'. There are insufficient data regarding their potential embryotoxicity. The main components of selected herbs, including 6-gingerol from ginger, Ginkgolide A and Ginkgolide B from gingko biloba and Ginsenoside Rg1 from ginseng, have been investigated using chick embryonic heart micromass and Mouse D3 embryonic stem cells. The potential effects were evaluated via alteration in contractility, cell viability, and cell protein content. The myocytes in both systems were also demonstrated by immunocytochemistry using a specific cardiomyocyte marker (α-actinin). For 6-gingerol, Ginkgolide A, Ginkgolide B and Ginsenoside Rg1 in both methods, at moderate to high concentrations, there were alterations in the values for the endpoints. These data indicate that herbal remedies used in the first trimester of pregnancy might not be safe for fetal development.

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1. Introduction

1.1. Overview

it may prove to be a valuable method to investigate teratogenic potential using endpoints such as contractile activity, cell viability, protein expression, apoptosis etc [6]. In addition, hanging drop

Reproductive Toxicology 64 (2016) 85–97

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Assessment of developmental cardiotoxic effects of some commonly used phytochemicals in mouse embryonic D3 stem cell differentiation and chick embryonic cardiomyocyte micromass culture models

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ARTICLE INFO

Article history:
Received 28 January 2016
Received in revised form 14 April 2016
Accepted 14 April 2016
Available online 20 April 2016

Keywords:
Mouse D3 embryonic stem cells
Chick embryonic cardiomyocytes
Eugenol
α-Bisabolol
Embryotoxicity

ABSTRACT

Pregnant women often use herbal medicines to alleviate symptoms of pregnancy. The active phytochemicals eugenol (from holy basil) and α-bisabolol (from chamomile), are recommended to promote calmness and reduce stress. There is evidence that both eugenol and α-bisabolol possess pro-apoptotic and anti-proliferative effects and induce reactive oxygen species. The potential effect was examined by monitoring cardiomyocyte contractile activity (differentiation), cell activity, protein content and ROS production for mouse D3 embryonic stem cell and chick embryonic micromass culture. The results showed that eugenol (0.01–80 μM) demonstrated effects on cell activity (both systems) and ROS production (stem cell system only), as well as decreasing the contractile activity and protein content at high concentrations in both systems. Additionally, α-bisabolol (0.01–80 μM) at high concentrations decreased the contractile activity and cell activity and in the stem cell system induced ROS production and decreased protein content. The results suggest only low concentrations should be ingested in pregnancy

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1. Introduction

1.1. Overview

Women experience a variety of pregnancy symptoms due to normal physical and biomedical changes, but the range of symptoms and their degree of severity are specific to each individual. health issues triggered by pregnancy, the pregnant woman needs close medical attention and treatment. Whilst the millennia-long history of medicinal use of certain herbs supports the idea that they are safe for a developing fetus the research to support this is very limited, and so physicians tend to not advise their use in pregnancy due to lack of evidence [6]. However there is also a preconception that 'natural' is synonymous with 'safe' [7], resulting in many

Reproductive Toxicology 69 (2017) 242–253

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Diabetes-induced effects on cardiomyocytes in chick embryonic heart micromass and mouse embryonic D3 differentiated stem cells

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ARTICLE INFO

Article history:
Received 14 October 2016
Received in revised form 3 March 2017
Accepted 7 March 2017
Available online 9 March 2017

Keywords:
Chick embryonic cardiomyocytes
Mouse embryonic D3 stem cell
Embryotoxicity
Diabetes
Micromass

ABSTRACT

Diabetes mellitus during pregnancy is a considerable medical challenge, since it is related to augmented morbidity and mortality concerns for both the fetus and the pregnant woman. Records show that the etiology of diabetic embryopathy is complicated, as many teratological factors might be involved in the mechanisms of diabetes mellitus-induced congenital malformation. In this study, the potential cardiotoxic effect of hyperglycemia with hyperketonemia was investigated by using two in vitro models; primary chick embryonic cardiomyocytes and stem cell derived cardiomyocytes, where adverse effects were recorded in both systems. The cells were evaluated by changes in beating activity, cell activity, protein content, ROS production, DNA damage and differentiating stem cell migration. The diabetic formulae used produced an increase in DNA damage and a decline in cell migration in mouse embryonic stem cells. These results provide an additional insight into adverse effects during gestational diabetes mellitus and a recommendation for expectant mothers and maternity staff to monitor glycaemic levels months ahead of conception. This study also supports the recommendation of using antioxidants during pregnancy to prevent DNA damage by the production of ROS, which might result in heart defects as well as other developmental anomalies.

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1. Introduction

Diabetes mellitus (DM) is an endocrine and metabolic disorder. It is characterised by hyperglycaemia as a consequence of either mechanism of DM-induced congenital malformation. The absence of specific malformation patterns also supports the multifactorial theory [4,5]. Insufficient control of glycaemic levels during the very first stages of conception and embryogenesis (organogenesis) is

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