NOTTINGHAM TRENT UNIVERSITY

THE EFFECTS OF FLAVONOIDS IN REDUCING TROPHOBLAST OXIDATIVE STRESS AND APOPTOSIS

A Thesis Submitted by

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Vernon Justice Ebegboni

Communications resulting from this study

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- Balahmar, R., **Ebegboni, V. J.,** Ray, S., & Sivasubramaniam, S. (2016). Understanding the behaviour of "stem like cells" derived from transformed first trimester trophoblast cell lines. Placenta, 45, 126. https://doi.org/10.1016/j.placenta.2016.06.225.
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Acknowledgements

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Abbreviations

BME	Base membrane extract
BSA	Bovine serum albumin
CTBs	Cytotrophoblasts
DMSO	Dimethyl sulfoxide
egEVTs	Endoglandular extravillious trophoblasts
EGF	Epidermal growth factor
enEVTs	Endovascular extravillious trophoblasts
ERK	Extracellular signal-regulated kinase
EVTs	Extravillious trophoblasts
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GrH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin hormone
HGF	Hepatocyte growth factor
hPL	Human placental lactogen
HR	Hypoxia reoxygenation
iEVTs	Interstitial extravillious trophoblasts
IgG	Immunoglobulin G
IUGR	Intrauterine growth restriction
JNK	Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
NADPH	Nicotinamide adenine dinucleotide phosphate
PE	Pre-eclampsia
PKB	protein kinase B

Q3G	Quercetin-3-O-β-glucuronide
ROS	Reactive oxygen species
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulphate
STBs	Syncytiotrophoblasts
TBS	Tris buffered saline
TGF	Transforming growth factor
TNF	Tumour necrotic factor
VEGF	Vascular endothelial growth factor

Abstract

Pregnancy is a complex state characterised by changes in maternal physiology to favour the development and growth of the developing foetus. This is mainly achieved by the placenta, a pregnancy specific temporary organ. In human's, the placenta is a highly specialised organ that acts as an interface for the exchange of nutrients, gaseous products, wastes and hormones between the mother and the foetus. Most importantly, the fate of human pregnancy relies on the successful invasion of early placental precursor cells called extravillous trophoblasts. These highly invasive cells, form a "plug" into material spiral arteries. This transforms the placental circulation from high-resistant low flow to low-resistant high flow state. Inadequate remodelling of the maternal spiral arteries can cause pregnancy complications such as pre-eclampsia, miscarriage and foetal/maternal death. The main cause for the incomplete transformation of spiral arteries is believed to be placental oxidative stress during early gestation. Therefore, measures to reduced early trophoblast oxidative stress have taken precedence in placental research. It has been hypothesised that the introduction of non-enzymatic dietary antioxidants such as flavonoids, metal chelates and vitamins may prevent this oxidative stress, especially during early pregnancy.

Flavonoids are phenolic compounds derived from fruits and vegetable and possess antioxidant properties. In fact, normal pregnant mothers usually "crave" for certain plant products (such as fruits) which apparently have high flavonoids concentrations. Although, their antioxidant properties have been well documented in myocardial and neuronal cells, their role in the prevention of trophoblast oxidative stress is unknown. Thus, exploring the antioxidative oxidative effects of selected flavonoids, their metabolites alone or in combination is deemed essential.

This study aims to understand the antioxidative properties of selected flavonoids (and their metabolite combinations) on a transformed early extravillous trophoblast cell line HTR-8/SVneo. It also explores the cytoprotective effects of these flavonoids during *in vitro* trophoblast invasion. To achieve this aim, optimisation of the non-toxic concentrations of flavonoids (quercetin, morin, naringin and hesperidin)/metabolites (Q3G, naringenin and hesperetin) on HTR-8/SVneo cells was carried out using MTT and CellTox™ green cytotoxicity assays. Afterwards, oxidative stress model for HTR-8/SVneo cells was established by hypoxia reoxygenation (HR). The effects of 24 h pre-treatment with flavonoids prior to HR-induced oxidative stress on HTR-8/SVneo cell viability was assessed using MTT and CellTiter-Glo® assays. Further investigation on the antioxidative effects of

flavonoids was carried out by assessing their effects on glutathione levels, nicotinamide adenine dinucleotide phosphate system, H_2O_2 scavenging and apoptosis. Flavonoid involvement in the modulation of protein kinases (ERK1/2, PKB, JNK/SAPK and p38 MAPK), trophoblast invasion and migration as well as spheroid formation/invasion was also investigated.

Flavonoids/metabolites below 10 µM were well tolerated by HTR-8/SVneo cells and pre-treatment with 3 µM flavonoid or 1 µM metabolite shown significant protection against H/R insult. Further investigation revealed that flavonoid cytoprotective effects against H/R insult were associated with their ability to interact with other antioxidant systems such as increasing GSH and decreasing GSSG levels, redox balancing (restoring NADP/NADPH ratio), inhibition of apoptosis by decreasing caspase 3/7 activity, and scavenging of H_2O_2. The data from this study also indicates that flavonoid pre-treatment significantly inhibited HR-induced protein kinase activation/phosphorylation ($p<0.0001$), enhanced HTR-8/SVneo invasive capacity ($p<0.0001$) and increased spheroid formation and invasion ($p<0.001$).

In summary, the study has shown for the first time that 24 h pre-treatment with flavonoids, their metabolites or in combination on HTR-8/SVneo cells significantly protected against HR-induced oxidative stress. Most importantly these combinations have enhanced trophoblast cell line invasion by reducing oxidative stress. These findings suggest that the ingestion of flavonoid rich foods during pregnancy may benefit placental development and health.

Chapter 1
Introduction

1.0 Introduction

Understanding the relationship between placental/or foetal development, increased oxidative stress and the cytoprotective effects of natural and/or synthetic antioxidants during early gestation can lead to the advancement of new concepts on preventing pregnancy complications such as pre-eclampsia and intrauterine growth restriction. Placental oxidative stress is a result of reduced antioxidant activities or inability of antioxidants to scavenge free radicals leading to shallow trophoblast invasion and slow development of the placenta, which then results in pregnancy complications (Wu *et al.*, 2016; Burton and Jauniaux, 2011). However, the exploitation of edible plants and vegetables that are rich in antioxidants such as flavonoids may help improved placental health during early gestation. This study aims to explore the cytoprotective effects of various classes and subclasses of flavonoids on a trimester trophoblast cell line, subjected to oxidative stress.

1.1 Human Placenta

The word placenta is derived from both the Latin ("a flat cake") and Greek ("*plakoenta –* meaning flat) (**www[1]**). The placenta is a highly specialised multifunctional transient organ that forms during pregnancy to support and maintain the development and growth of the foetus, by acting as a medium of exchange between the mother and developing foetus (Pereira *et al.*, 2015; Yoshizawa, 2013; Ji *et al.*, 2013). In fact, the health of the mother and foetus during pregnancy is paramount to proper development and function of the human placenta (Pereira et al., 2015). The placenta forms the maternal-foetal interface and regulates the *in utero* environment by the dense network of blood vessels that are responsible for the exchange of respiratory gases, nutrients and waste products between the maternal and foetal tissues throughout pregnancy. Furthermore, the vasculature of the human placenta evolves throughout the pregnancy to accommodate the increase demand of the developing foetus. Therefore both intrinsic and extrinsic stress to the placenta can also impact its development, structure and function (Pereira *et al.*, 2015). Due to the central role of the placenta throughout gestation, the placenta is often referred to as the "diary" of pregnancy (Yoshizawa, 2013). The cross section of a term placenta is shown in figure 1.1 (Pereira *et al.*, 2015; Ji *et al.*, 2013).
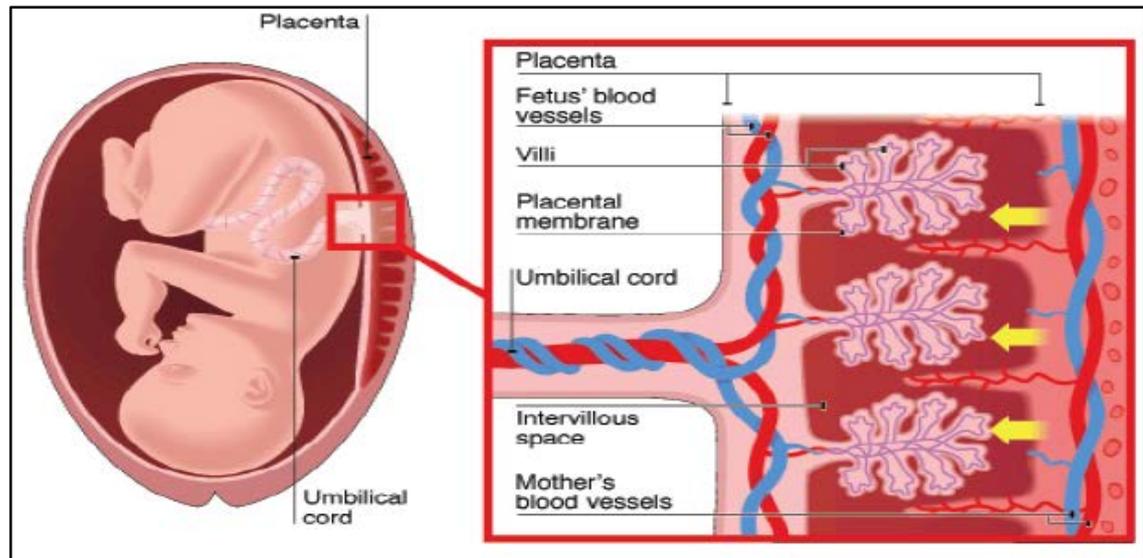
Figure 1.1: The materno-foetal interface of the human placenta

This image reveals the localisation of the placenta in the *in utero* environment and the cross-section showing the blood circulation. *Figure adapted from www[2]*

1.1.1 Functions of the human placenta

The placenta acts as a medium of exchange between the mother and the developing foetus throughout gestation. The multifaceted nature of the human placenta has proved to be important for the health and survival of the foetus and mother (Ji *et al.*, 2013). The placenta is involved in the following functions;

o **Exchange of gases**

As the foetal lungs do not take part in respiration while *in utero*, the placenta is wholly responsible for respiratory gas exchange (oxygen and carbon dioxide) to and from the developing foetus via diffusion (Donnelly and Campling, 2016; Vause and Saroya, 2005; Griffiths and Campbell, 2015). The rate and quantity of gaseous exchange is dependent on the concentration of both maternal and foetal side of the placenta, and dissociation curve in either sides of the blood circulation (Vause and Saroya, 2005). Oxygen transfer to the developing foetus is enhanced by the Bohr effect; the haemoglobin of the foetus has higher affinity for oxygen than that of maternal haemoglobin (Griffiths and Campbell, 2015). Carbon dioxide (CO_2) exchange across the placenta is via passive diffusion, which is dependent on mainly the partial pressure gradient for CO_2 between foetal blood at the umbilical arteries and maternal blood at the intervillous space; this exchange is facilitated by Haldane effects (Donnelly and Campling, 2016).

o **Metabolic transfer**

Metabolic transfer across the human placenta includes the transfer of glucose, amino acids, fatty acids, electrolytes, vitamins and water (Donnelly and Campling, 2016). Since the developing foetus has very little or no capacity for gluconeogenesis, maternal glucose is transferred to the foetus via the placenta (Vause and Saroya, 2005; Donnelly and Campling, 2016).

o **Endocrine and immunological functions**

The human placenta is an endocrine organ which secretes very important peptides and hormones as well as performing some immunological functions (Donnelly and Campling, 2016). Human chorionic gonadotropin hormone (hCG) and human placental lactogen (hPL) are the two major peptide hormones secreted by the placenta (Donnelly and Campling, 2016). hCG is produced in early gestation by the syncytiotrophoblast, and is responsible for extending the lifespan of the corpus luteum and secretion of progesterone, which prevents shedding of the endometrium, hence sustaining the viability of pregnancy (Donnelly and Campling, 2016; Griffiths and Campbell, 2015). hPL also secreted by the synctiotrophoblast, is responsible for the growth of breast tissue in preparation for lactation (Vause and Saroya, 2005). Maternal antibodies such as immunoglobulin G (IgG) cross the placenta by pinocytosis to provide immunity for the foetus. Other endocrine functions of the placenta include the transfer/secretion of human placental lactogen, human growth hormone variants, oestrogens and progesterone (Donnelly and Campling, 2016; Vause and Saroya, 2005; Griffiths and Campbell, 2015).

o **Placental drug transfer**

The transplacental passage of drugs can have some beneficial or teratogenic effects. Drug transfer from the maternal to the foetal circulation must be carried into the intervillous space and pass via the syncytiotrophoblast, endothelium of the foetal capillaries and foetal connective tissues. There are three types of transplacental passage of drug that have been identified, which are; (a) complete transfer (such as type 1 drugs; thiopental drugs), (b) exceeding transfer (such as type 2 drugs; ketamine) and (c) incomplete transfer (type 3 drugs; succinylcholine) (Griffiths and Campbell, 2015). Physical factors such as placental surface area, metabolism, thickness, and pharmacological factors such as molecular weights of drugs, protein binding, liquid solubility, can affect drug transfer across the placenta (Donnelly and Campling, 2016; Griffiths and Campbell, 2015)

In addition, there are four major mechanisms of drugs transfer across the placenta, namely: simple diffusion, facilitated diffusion, active transport and pinocytosis (Griffiths and Campbell, 2015)

1.1.2 Structure of full term human placenta

The architecture of the full term human placenta can be described as a disc-shaped organ with a diameter of 15 – 22 cm, a thickness of 2 – 3 cm, a surface area of 10 – 15 m^2 and a weight of between 470 – 500 g (Huppertz, 2008; Griffiths and Campbell, 2015). These parameters differ from one placenta to another, with the mode of delivery being a major factor in these variations. However, the weight of the placenta is directly related to the size of the foetus (Huppertz, 2008). The main structural unit of the human placenta is the chorionic villus, which are the vascular representation of the foetal tissue surrounded by chorions. These chorions consist of two layers, namely; the outer syncytiotrophoblast and inner cytotrophoblast layers (Griffiths and Campbell, 2015). The placental membrane/barrier separates the maternal circulation in the intervillous space as well as the foetal circulation in the maternal-foetal environment (Gude *et al.*, 2004). At full term, the placental membrane consist of two surfaces; (a) foetal surface/membrane and (b) maternal surface/membrane as shown in figure 1.2 (Gude *et al.*, 2004; Huppertz, 2008).
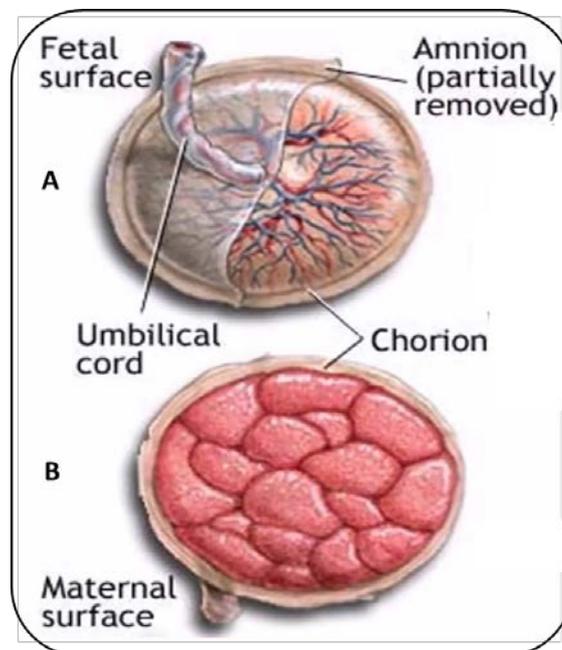
Figure 1.2: Graphical representation of placental surfaces.
 (A) represents the foetal surface; (B) represents the maternal surface. *Figure was slightly modified from **www³**.*

▪ **Foetal surface**

The foetal surface also known as the chorionic plate is covered by the amnions as seen in figure 1.2, A. These amnions consist of a single layer of epithelium and amniotic mesenchyme (connective tissue) (Huppertz, 2008). Attached to the chorionic plate in an eccentric position is the umbilical cord with continuous chorionic vessels arising from the chronic mesenchyme. The chorionic artery is derived from the two umbilical arteries, which supplies the villous trees (Gude *et al.*, 2004; Huppertz, 2008). In addition, the chorionic veins arises from the veins of the villous trees which give rise to the single umbilical vein (Huppertz, 2008).

▪ **Maternal surface**

The maternal surface is an artificial surface arising from the separation of the placenta from the uterine wall during delivery. This surface consists of a mixture of foetal extravillious trophoblasts and maternal cells such as macrophages, natural killer cells and stroma cells (Gude *et al.*, 2004; Huppertz, 2008). In addition, large amounts of extracellular matrix, fibroid and blood clots can be found on the maternal surface (Huppertz, 2008). The maternal surface is subdivided by a system of flat grooves or deeper clefts into 10 – 40 slightly heightened regions called lobes as seen in figure 1.2, B (Huppertz, 2008).

1.1.3   Development of the human placenta

Placentation and other early pregnancy processes such as pre-implantation and implantation are the most crucial determinants of pregnancy outcome (Anin *et al.*, 2004). The formation and development of the placenta first requires successful fertilization of the ovum by a sperm followed by tightly regulated molecular and cellular processes /or factors such as uterus, blastocyst into maternal decidua, and invasion of the trophoblast cells (Anin *et al.*, 2004; Pereira *et al.*, 2015). Human implantation can be defined as interstitial implantation, a process where the embryo is completely embedded with the endometrium without being destroyed (Su and Fazleabas, 2015). The next section aims to discuss the intricate sequence of events leading to the formation and development of the human placenta.

1.1.3.1   Pre-implantation events

Fertilization of the haploid oocyte by the spermatozoa occurs between 24 to 48 h after conception in the fallopian tube which results in the formation of a diploid zygote. This stage is usually referred to as day 0 of placental development and pregnancy (Imakawa *et al.*, 2004). The diploid zygote moves along the fallopian tube into the uterus during which it undergoes several stages including cleavage and differentiation (Norwitz *et al.*, 2001). The diploid zygote undergoes the first cleavage on day 1 leading to mitotic division of two cells and four cell stages on day 2. These divisions continue to day 4 where the 8-cell uncompacted morula becomes compacted (Norwitz *et al.*, 2001; Imakawa *et al.*, 2004).

On day 5, the 32- cell (8-cell compacted) morula enters the uterine cavity, the appearance of a blastocoel (fluid-filled inner cavity) marks the transition of morula to early blastocyst that is accompanied by cellular differentiation such as trophoblast (embryonic structure; placenta), and the inner mass cells (which results to the embryo) (Norwitz *et al.*, 2001). Within 24 to 48 h (day 6-7) of the blastocyst entering the uterus, it hatches from the surrounding zona pellucida (late stage blastocyst) by constant contraction and expansion, thereby exposing the outer surface of the multinucleated (syncytial) trophoblasts which leads to the implantation of the blastocyst in the uterine wall (Red-Horse *et al.*, 2004; Norwitz *et al.*, 2001). Figure 1.3 illustrates the series of events and their position in the female reproductive tracts during pre-implantation.
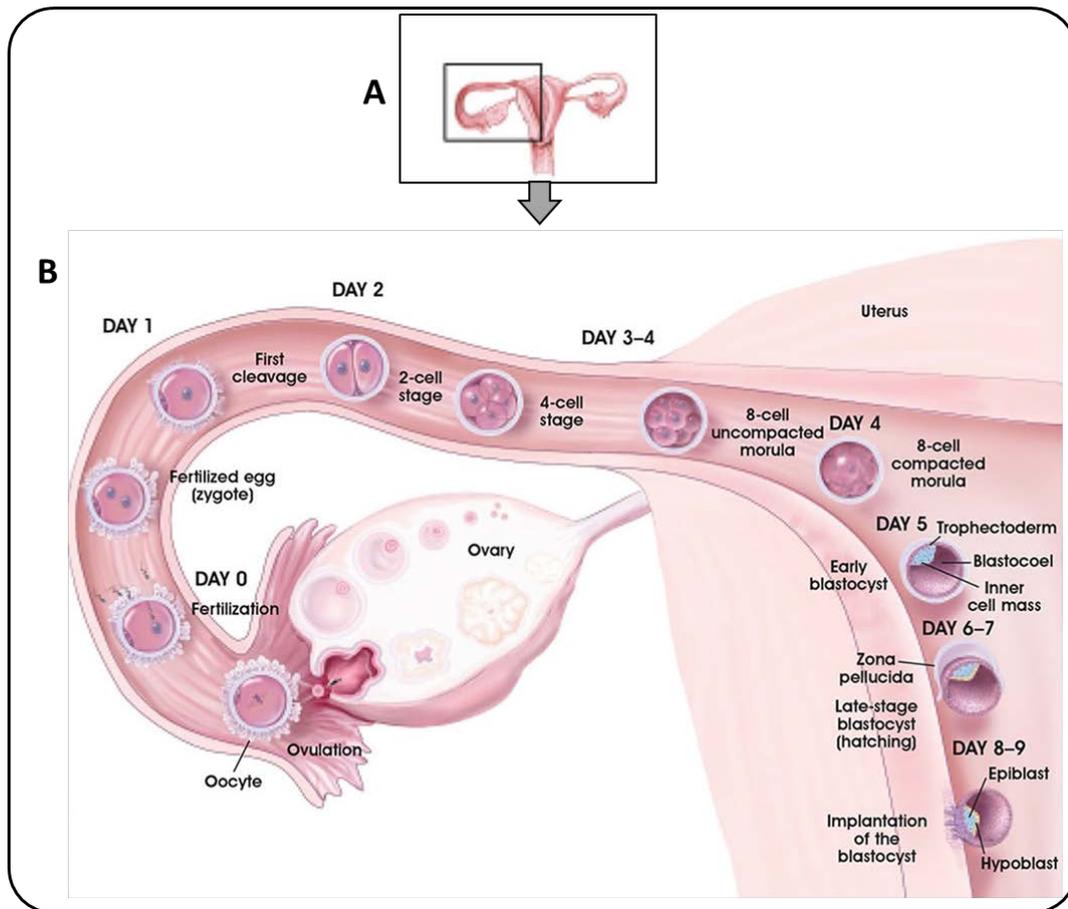
Figure 1.3: Diagrammatic illustration of the events leading to placentation.
(A) Image represents the entire female reproductive tract; (B) represents a zoomed section and series of events from day 0 – 9. *Figure was slightly modified from www[4].*

1.1.3.2   Implantation

In humans, the implantation process is one of the most important biological events as it is considered as the first biological interaction between the developing foetus (blastocyst) and uterus. Implantation of the blastocyst usually commences from about day 6 when the blastocyst differentiate into early embryonic structures (such as inner {embryo} and outer {trophoblasts} cell masses) (Gude *et al.*, 2004; Norwitz *et al.*, 2001; Su and Fazleabas, 2015). The period in which implantation occurs is relatively short and is often referred to as the window of receptivity/implantation (Su and Fazleabas, 2015). During this window period, the endometrium, primed by oestrogen and progesterone, undergoes extensive changes (intensive morphological, biochemical and molecular) (Su and Fazleabas, 2015). The process of implantation in human consist of three stages, namely; (i) apposition, (ii) adhesion and (iii) invasion (Staun-Ram and Shalev, 2005). See figure 1.4 for diagrammatic representations

- **Apposition stage:** This stage is the first connection between the blastocyst and the maternal uterine wall, during which the blastocyst finds a location to implant, guided by the maternal endometrium (Su and Fazleabas, 2015). At this stage the blastocyst dislodges from the uterine surfaces by flushing the uterus without bringing damage to itself. Herein, the pinopodes, which are the micro protrusions from the apical surface of the maternal uterine epithelium engage with the microvilli on the apical surface of the syncytiotrophoblast of the blastocyst (Norwitz *et al.*, 2001; Staun-Ram and Shalev, 2005). The apposition stage usually takes place in the fundal (upper posterior wall) of the uterus. See figure 1.4A for illustration.

- **Adhesion stage:** The next step is referred to as the stable adhesion stage. This stage shows stronger physical connection between blastocyst and the maternal uterine epithelium (Staun-Ram and Shalev, 2005). During this stage, there is direct interaction between the endometrial epithelium and the trophoblast without dislodging the blastocyst, as seen in figure 1.4A (Su and Fazleabas, 2015).

- **Invasion stage:** The invasion stage takes place shortly after the stable adhesion stage, which is estimated to take place on day 10 after fertilisation (Staun-Ram and Shalev, 2005). This stage begins with the trophoblasts (extravillious cytotrophoblast) penetrating and infiltrating the entire endometrium as well as the inner third of the myometrium and uterine vasculature to reach the maternal blood vessels as revealed in figure 1.4B (Staun-Ram and Shalev, 2005). The invasion process that occurs at this stage is called the interstitial and endovascular invasion (Norwitz *et al.*, 2001). This stage allows the trophoblast cells to come into direct contact with maternal blood, thereby establishing uteroplacental circulation. See section 1.1.3.4.2 for the details of interstitial and endovascular trophoblast cells.
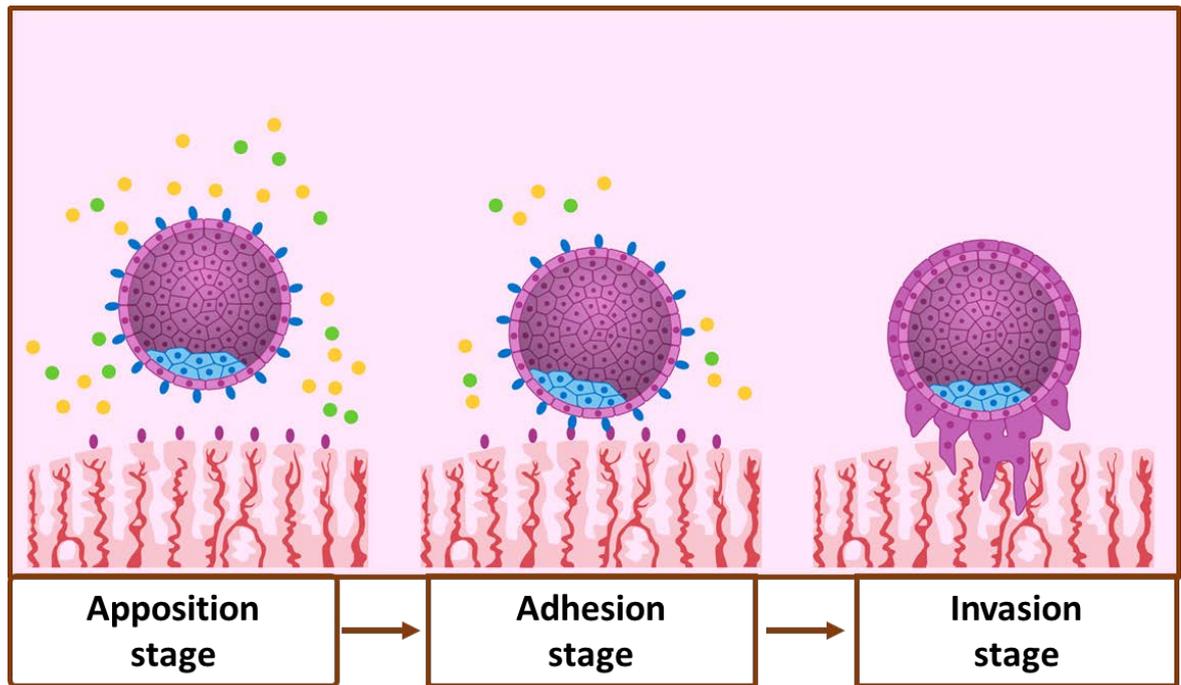
Figure 1.4: Images showing detailed illustration of blastocyst implantation stages.
Image showing the series of events that occurs during apposition, adhesion and invasion stage. *Figure was adapted from **www**[5].*

1.1.3.3   Trophoblast differentiation

As previously described in section 1.1.3.2, the trophectoderm (also known as the trophoblast) of the blastocyst is the first cell lineage that exhibits an extensive proliferation and differentiation function during gestation (Ji *et al.*, 2013). During implantation, trophoblast invasion and migration is tightly regulated by components of the maternal microenvironment and the trophoblast itself, via cellular and molecular interaction (Ji *et al.*, 2013). These interactions are the major determinants for a successful pregnancy (Staun-Ram and Shalev, 2005). Shortly after implantation, the trophectoderm gives rise to diverse trophoblast cells by undergoing differentiation that results in the formation of the multinucleated syncytiotrophoblasts (STBs). These STBs are mainly involved in the exchange of nutrients, waste products, and hormone production between the developing foetus and mother (Ji *et al.*, 2013; Huppertz, 2008). The remaining mononucleated trophoblast cells are called the cytotrophoblasts (CTBs), which are usually seen in the second layer and are never in contact with the maternal tissues (Huppertz, 2008). The CTB forms the anchoring villi that attach to the maternal uterine walls and also act as stem cells, which are responsible for the continuous expansion of the STBs by rapidly dividing and fusing with STBs (Huppertz, 2008). The differentiation of trophoblast cells during placental

development is tightly controlled by various growth factors, hormones and environmental factors such as oxygen concentration within the maternal-foetal interface (Ji *et al.*, 2013). Fluid-filled spaces appear within the STB on the 8th day after fertilization which are important in the development of the villous trees of the human placenta and the identification of the three fundamental zones (chorionic plate, villous trees and basal plate) of the placenta (Huppertz, 2008).

1.1.3.4 Trophoblast cell differentiation pathways

The human trophoblast progenitor cells differentiate via two pathways; (1) non-invasive villous trophoblasts and (2) the invasive extravillous trophoblasts (Ji *et al.*, 2013)

1.1.3.4.1 Villous trophoblast differentiation pathway

In this pathway, the mononucleated CTBs integrate with the multinucleated STBs to form the syncytial layer that house the placental villous tree, therefore consisting of both CTBs and STBs (Ji *et al.*, 2013). The villous trophoblasts are involved in gaseous, waste and nutrient exchange within the maternal-foetal interface (Gude *et al.*, 2004). They also play an important role in the maintenance of pregnancy by producing the necessary hormones such hCG and hPL (Gude *et al.*, 2004; Ji *et al.*, 2013). In addition, the STB are usually in contact with the maternal circulation and exhibit some degree of immune tolerance. Since STBs are non-proliferative, they rely on the fusion with CTB for continuous replenishment and syncytial knots (shedding of the aged portion) throughout gestation (Ji *et al.*, 2013). See figure 1.5 and figure 1.6 for events leading trophoblast differentiation and function.
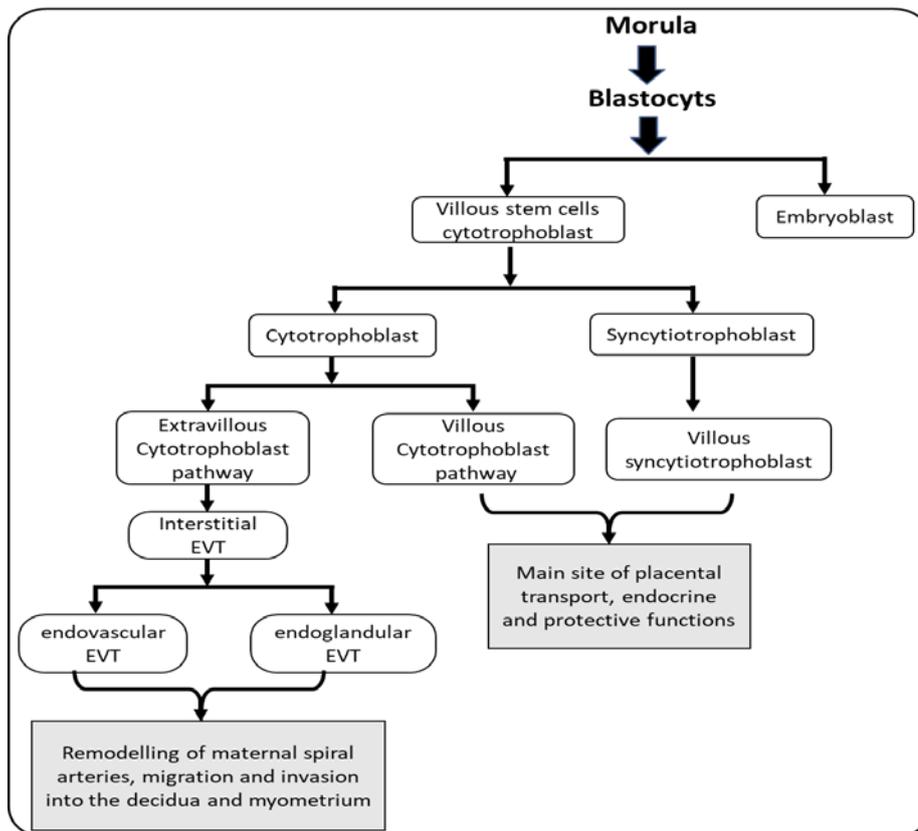
Figure 1.5: Trophoblast differentiation pathways and functions.
The blastocyst on day 5 gives rise to the trophectoderm (trophoblast) and inner cell mass (embryoblast). The cytotrophoblast differentiates into two distinct pathways with autonomous functions. *Figure was reconstructed from* (Gude *et al.*, 2004; Huppertz, 2011)

1.1.3.4.2   Extravillous trophoblast differentiation pathway

Extravillous trophoblasts (EVTs) are the highly invasive, proliferative and migrative trophoblast population. They are responsible for the development of the floating villi and anchoring villi that invade into the maternal uterine wall for the purpose of remodelling the maternal spiral arteries, which is important for the sustenance of the developing foetus (Gude *et al.*, 2004; Ji *et al.*, 2013). The EVTs are mainly mononucleated cells that can be found in the smooth chorion, chorionic plate and basal plate.

For the successful invasion into the maternal uterine wall, EVT performs several functions. These includes remodelling of the maternal spiral arteries, proliferation, differentiation, programmed cell death (apoptosis), tolerance to hypoxia, adherence and digestion of the extracellular matrix, as well as migration and maternal immune evasion (Anin *et al.*, 2004). A tightly regulated invasion of the EVTs is a main step in placentation and it is therefore necessary for a successful pregnancy (Gude *et al.*, 2004). EVTs within the maternal

compartment consist of several different populations of trophoblast cells with different phenotypes with regards to functions and morphology. These populations of EVTs are distinguished based on their location within the maternal uterine tissues (Hammer, 2011). The three sub-populations of EVT are; (i) interstitial EVT (iEVT), (ii) endovascular EVT (enEVT) and (iii) endoglandular EVT (egEVT) (Huppertz, 2011).

i. Interstitial extravillous trophoblast (iEVT)

iEVT is the main source of all other subtypes of the EVTs and they invade as deep as the inner third of the maternal myometrium, migrate towards the spiral arteries as well as differentiate into giant multinuclear cells (Anin *et al.*, 2004; Huppertz, 2011). iEVT consist of three structurally and functionally distinct subtypes, namely; large polygonal, small spindle-shaped and multinucleated giant cells iEVT.

- **Large polygonal iEVTs** also referred to as X cells and are suggested to stay around the transition phase of placental-decidua (Huppertz, 2007). They are also involved in securing appropriate placentation of the uterus during the entire gestational period by secreting "trophoblast glue" composed of matrix-type fibrinoid (Ji *et al.*, 2013). This subpopulation are rarely seen in early gestation, but increase significantly from 45% (in weeks 9-12) to 69% (in weeks 16-24) and eventually reach about 90% (in weeks 31-39) (Huppertz, 2007). These iEVTs are evenly distributed across the basal plate and invade as far as the superficial third of the maternal myometrium (less invasive but more differentiative) and it is the more abundant phenotype seen in term placenta (Huppertz, 2007).

- **Small spindle-shaped iEVTs** are highly invasive and have been proposed to invade deeper into the decidua, up until the inner third of the myometrium (Ji *et al.*, 2013). This subtype is similar to that of large polygonal iEVT based on their spatial distribution patterns (Huppertz, 2011). They can be described morphologically as small ovoid nuclei, elongated and partly filiform bodies (mostly observed on the uterine wall). This subpopulation reduces significantly towards term time from 55% (first trimester) to 31% (second trimester) and eventually 11% (term time) (Huppertz, 2007). Small spindle-shaped iEVTs mostly form loose arranged arrays of cells in the mist of extracellular matrix and are separated from each other by decidua, myometrial cells and large polygonal iEVTs (Huppertz, 2007).

- **Multinucleated giant cells** also called placental bed giant cells are derived from invading iEVTs differentiation and they play a role in the maintenance of pregnancy by secreting both hPL and hCG hormones (Ji *et al.*, 2013).

  By secreting protease inhibitors, they are also involved in regulating trophoblast invasion (by ensuring that the invasion do not exceed the myometrium) (Huppertz, 2007). The major difference between these subtypes and others (large polygonal and small spindle-shaped iEVTs), is that the multinucleated giant cells consist of over ten irregular shaped nuclei of various sizes (Huppertz, 2007). See figure 1.5 and figure 1.6 for events leading to trophoblast differentiation and function flowchart.
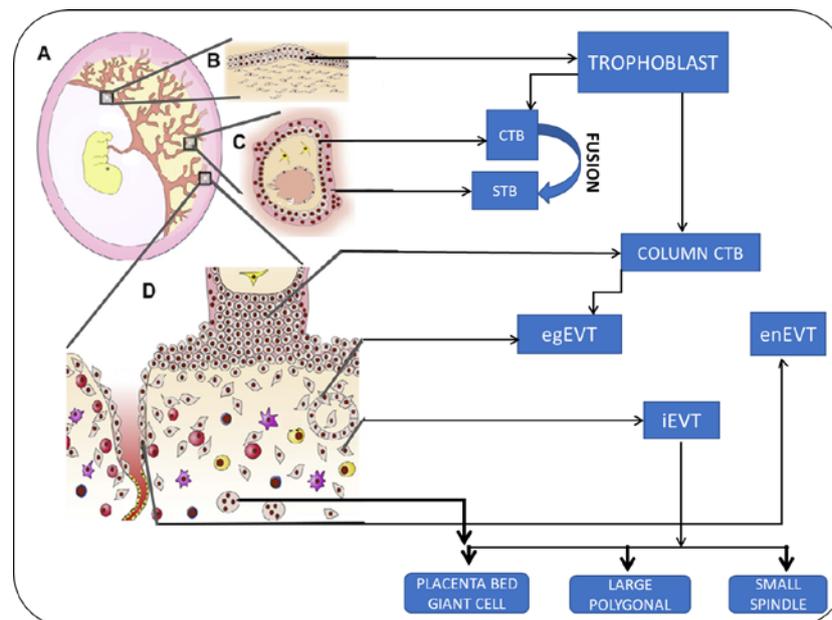
Figure 1.6: Events involved in extravillous trophoblast differentiation.
(A) Foetus anchored to the uterine walls via the formation of the placenta. (B) Magnification of the chorionic membrane that contains the trophoblast and further differentiation of the trophoblast. (C) Magnification of the floating villus with the mononucleated CTBs covered by multinucleated STBs. (D) Magnification of the anchoring villus. At anchoring villus, the active proliferating CTBs form column CTBs which detach from the villus at the distal portion of the column and migrate into the maternal decidua. **CTB**- cytotrophoblast cell, **STB**- syntiotrophoblast cell, **egEVT**- endoglandular extravillous trophoblast, **enEVT**- endovascular extravilous trophoblast, **iEVT**- interstitial extravillous trophoblast. *Figure was modified from* (Ji *et al.*, 2013)*.*

ii. Endovascular extravillous trophoblast (enEVT)

This category of EVTs make up the loose plugs seen in the apical portion of the maternal spiral arteries. They are situated either in the wall of the spiral arteries or substituting the endothelial surface of the spiral arteries through a process known as pseudo-

vasculogenesis/vascular mimicry (Huppertz, 2011; Khankin *et al.*, 2010). enEVTs are believed to play a major role in the remodelling of the maternal spiral arteries starting from week 8 to approximately week 22 (Ji *et al.*, 2013). They play a vital role in transforming the maternal spiral arteries from "high-resistance-low flow", to "low-resistance-high-flow" sac-like vessels. This is accomplished by the destruction of the arterial layer and enEVTs replacing the endothelium vessels (Huppertz, 2007). This transformation is necessary for the normal growth and development for both the foetus and placenta (Ji *et al.*, 2013). In fact, maternal uterine spiral artery remodelling occurs in five stages, namely:

- ❖ Decidua-associated early vascular remodelling
- ❖ iEVTs-associated vascular remodelling
- ❖ migration of enEVTs
- ❖ plugin of enEVTs
- ❖ maternal vascular repairs (Pijnenborg *et al.*, 2006). See figure 1.7 for illustrations. The spiral artery remodelling is explained further in section 1.1.4.
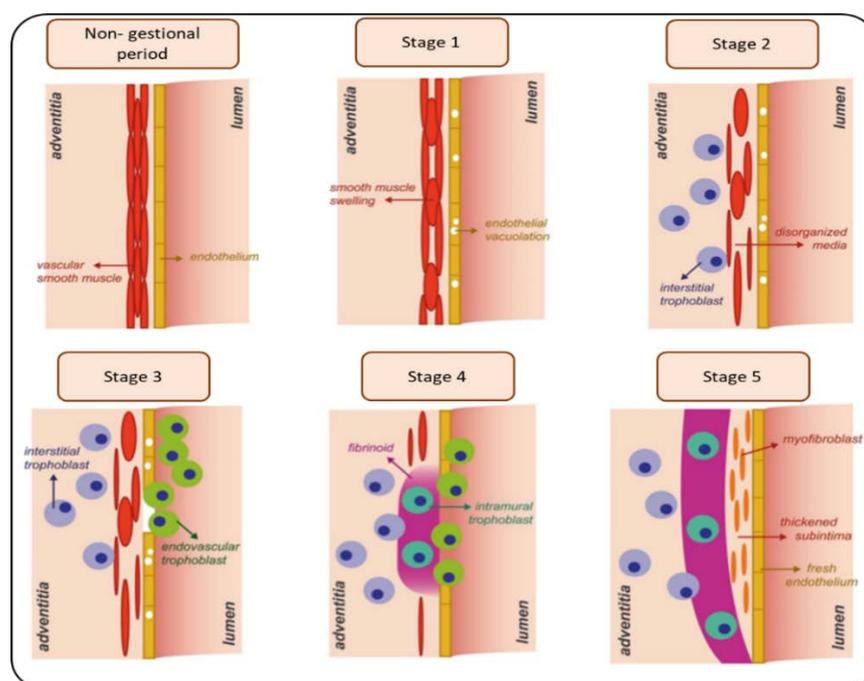
Figure 1.7: The various stages involved in maternal spiral artery remodelling.
Figure is slightly modified from (Pijnenborg *et al.*, 2006)**.**

iii.   Endoglandular extravillous trophoblast (egEVT)

Recent evidence suggests the existence of a third subpopulation of EVT, which are the endoglandular EVTs (Ji *et al.*, 2013). egEVT are derived from iEVT and they mainly differentiate and migrate towards the maternal uterine glands via penetration of their basement membrane (Ji *et al.*, 2013; Huppertz, 2011). It is believed that egEVT mediate histiotrophic nutrition of the embryo prior to the completion of the maternal spiral artery remodelling as well as opening the maternal uterine glands to the intervillous space (Huppertz, 2011; Ji *et al.*, 2013).

1.1.4   Trophoblast cell regulation

The differentiation of the highly proliferative EVTs into migratory and invasive trophoblasts is tightly regulated by numbers of factors acting such as environmental factors (oxygen), hormones, cytokines, proteases (matrix metalloproteinase), and growth factors (Ji *et al.*, 2013). These factors are most likely to be derived from uterine stromal and glandular cells, trophoblast cells, myometrial cells, villous mesenchymal cells, endothelial cells and several immune cells within the maternal-foetal interface (Ji *et al.*, 2013).

Oxygen tension is highly considered to play a major role in the balancing of trophoblast cell proliferation and differentiation using hypoxia-inducible factors as oxygen sensors (Ji *et al.*, 2013). During gestation, the partial pressure of oxygen in the intervillous space constantly varies until the completion of maternal spiral artery remodelling. Oxygen pressure at 8-10 weeks of pregnancy is estimated to be as low as 17.9 – 39.6 mmHg (Ji *et al.*, 2013). This values increases to about 80 -100 mmHg after spiral artery remodelling by the endovascular EVTs (Ji *et al.*, 2013; Pollheimer and Knöfler, 2012). Initial implantation, placentation and trophoblast invasions takes place in a physiological hypoxic environment estimated to be around 1 – 2% oxygen (Anin *et al.*, 2004; James *et al.*, 2006). Other factors involve in the regulation of trophoblast differentiation and invasion as well as their functions are summarised in table 1.1.

Table 1.1: **Summary of important factors involved in trophoblast differentiation and invasion.**

Factors	Function
Vascular Endothelial Growth Factor	Vascular development, stimulates cell proliferation, induces endothelial division, migration and survival, and activation MAP kinases.
Epidermal Growth Factor	Endocrine functions such as stimulating the secretion of hCG, induces trophoblast differentiation, proliferation, invasion and implantation.
Transforming Growth Factor β	Inhibits trophoblast proliferation and invasion, induces syncytialization.
Hepatocyte Growth Factor	Increases proliferation, migration, invasion by targeting HIF-1α.
Notch signalling	Promoting vascular invasion and proliferation of trophoblast cells.
Wnt signalling	Controls invasion of trophoblast in paracrine manner.
Gonadotropin-releasing hormone	Enhances EVT invasion
Human Chorionic Gonadotropin	Maintenance of corpus luteum, syncytialization process.
Integrins	Embryo implantation via adhesive molecules such as E-cadherin.
Mucin 1	Provide barrier to trophoblast invasion, acts as anti-adhesive molecules hindering blastocyst attachment until the 3rd day.
Leukaemia inhibitory factor	Involve in implantation and immune tolerance.

*Table adapted from (Staun-Ram and Shalev, 2005; Ji et al., 2013).

1.1.5 Complications during placentation

Failure in controlled implantation and placentation processes can lead to placental insufficiency resulting in reduced nutrient and waste exchange between the foetal and maternal circulations (Ji *et al.*, 2013). One key process that can result in placenta insufficiency is altered uteroplacental haemodynamic (impairment of the maternal spiral arteries) or shallow invasion of the trophoblast cells which can result in spontaneous miscarriages, pre-eclampsia, intrauterine growth restriction, stillbirth, and preterm delivery (Malhotra *et al.*, 2016; Pereira *et al.*, 2015). In addition, chromosomal anomalies and insufficient maternal arteriole plugging have also been associated with early pregnancy complications such as miscarriage (Malhotra *et al.*, 2016). However, the cause of impaired spiral arteries remodelling or shallow trophoblast invasion is not fully understood, placental oxidative stress has been proposed to a major key factor in the pathophysiology of pregnancy complications (Malhotra *et al.*, 2016; Pereira *et al.*, 2015; Aouache *et al.*, 2018).

1.1.5.1 Oxidative stress

Oxidative stress can be described as an imbalance in the production of oxidants (reactive oxygen species) and the ability of antioxidants to scavenge them, resulting in a disruption of redox signalling, control and/or molecular damage as demonstrated in figure 1.9 (Myatt and Cui, 2004; Aouache *et al.*, 2018). Reactive oxygen species (ROS) refers to molecules derived from the oxygen that have gained an extra electron and have the ability to oxidize other molecules (Sullivan and Chandel, 2014). ROS is one of the main types of free radical species and they are known to be very unstable and reactive (Agarwal and Gupta, 2005). A schematic representation of the interplay between ROS and anti-oxidants in normal cells is shown in figure 1.8
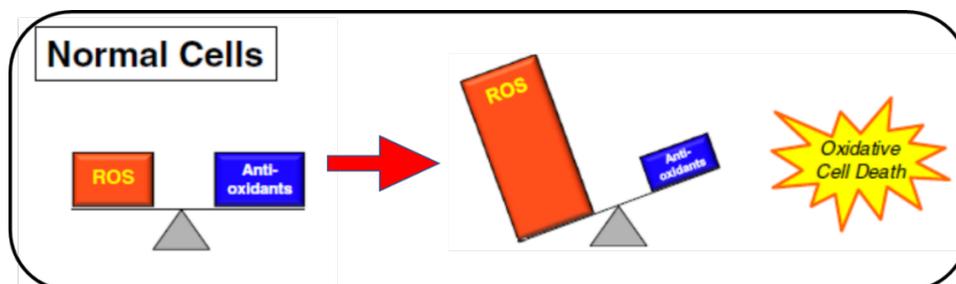
Figure 1.8: **Overview of oxidative stress**.
In healthy/normal cells, ROS and antioxidants remain in balance whereas, during oxidative stress there is an imbalance because of increased ROS production or depletion of antioxidant defence system resulting in cellular damage and death. *Figure was slightly modified from* (Sullivan and Chandel, 2014)**.**

There are three major types of ROS, namely; superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl ($OH^\bullet$) which are generated in cells (Ashok Agarwal, Sajal Gupta, 2005). The most common being superoxide ($O_2^{\bullet-}$), which is formed as the first step in the leakage of electrons from the electron transport chain, particularly from complex I and III or from endoplasmic reticulum (one electron reduction from oxygen). The reactivity is created as the result of the unpaired electron in $O_2^{\bullet-}$ which leads to the formation of H_2O_2 and OH• (Myatt and Cui, 2004; Ashok Agarwal, Sajal Gupta, 2005). Hydrogen peroxide is formed from the dismutation of superoxide i.e. two molecules of $O_2^{\bullet-}$ being converted to H_2O_2 and water (H_2O) by superoxide dismutases (SODs) as shown in figure 1.10. The hydroxyl radical is formed when H_2O_2 accepts another electron from ferrous ion (Fe^{2+}) mediated by the Fenton reaction. Hydroxy radical is very reactive and can result in DNA damage by causing strand breaks (Ashok Agarwal, Sajal Gupta, 2005). These major types of ROS have varied reactivity and can result in different physiological and cellular effects. See figure 1.10 for the summary of ROS subtypes formation.
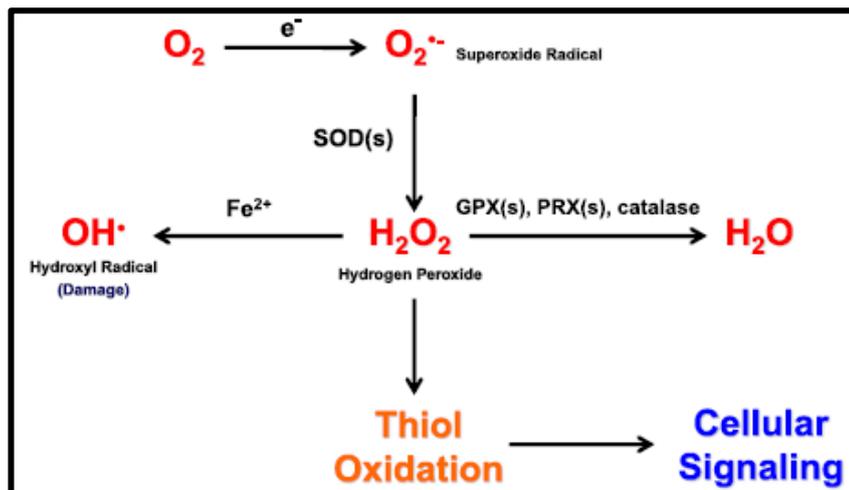
Figure 1.9: **Formation and interconversion of ROS**.
Figure adapted from (Sullivan and Chandel, 2014).

The primary site for ROS production is the mitochondria, where the majority of oxygen consumption and ATP generation takes place. The major source of intracellular ROS is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Sullivan and Chandel, 2014). NADPH oxidases catalyses the formation of superoxide from oxygen and NADPH and are capable of generating increase amount of ROS as they are present in several tissues in the human body (Sullivan and Chandel, 2014).

Oxidative stress has been implicated in over 100 diseases including several types of cancers, diabetes, cardiovascular diseases and pregnancy disorders (Aouache *et al.*, 2018; Sullivan and Chandel, 2014; Daubney *et al.*, 2015). Bodies of evidence from both human and animal studies have shown the presence of ROS in female reproductive tracts such as fallopian tubes, ovaries, placenta and embryos (Ashok Agarwal, Sajal Gupta, 2005; Aouache *et al.*, 2018). When ROS is maintained at a reduced or moderate amount, they can play beneficial roles in cellular and molecular processes. For instance, ROS is required for the synthesis of cellular structures (phagocytes) used to combat pathogen by the host immune system (Pizzino *et al.*, 2017). Other beneficial functions of ROS at low or moderate amount include, activation of mitogen-activated protein kinase (ERK1/2, SAPK-JNK, p38MAPK), and redox-sensitive transcription factors (p53, NF-κB) (Burton and Jauniaux, 2011).

1.1.6.1 Implication of ROS in pregnancy

Pregnancy is considered to be a state of oxidative stress as a result of increased placental mitochondrial activity and formation of ROS, especially superoxide anion (Myatt and Cui, 2004). ROS produced by the placenta are important for trophoblast proliferation, differentiation and vasculogenesis (Myatt and Cui, 2004). The excessive production of ROS, which overwhelms the antioxidant capacity (oxidative stress), results in several pregnancy complications as summarised in figure 1.11.
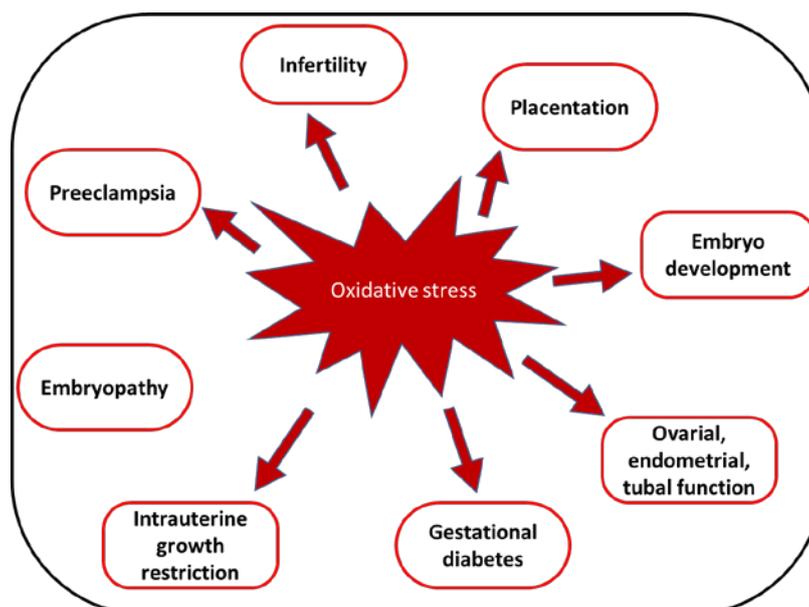
Figure 1.10: **Contribution of oxidative stress during pregnancy**. *Figure was adapted from* (Lázár, 2012).

1.1.6.1.2 Pre-eclampsia

Pre-eclampsia (PE) is major disease of human pregnancy characterised by hypertension (160/110 mmHg) and proteinuria (>300 mg/24 h) developing after 20 weeks of gestation (Aouache *et al.*, 2018). PE is estimated to affect about 3 – 14 % of pregnant women worldwide and the incidence varies according to time of the year, geographical location, nutrition, and race/ethnicity (Aouache *et al.*, 2018). It is the leading cause of perinatal and maternal mortality and morbidity worldwide (Fayyad and Harrington, 2005). Low birth weight, prematurity and death are potential outcomes of foetal complications whilst haemolysis, thrombocytopenia, elevated liver enzymes/liver failure, seizures and death potential outcomes of maternal complications (Mutter and Karumanchi, 2008).

The exact etiology of PE is still unknown. However, PE has been classified into two subtypes by some researchers, namely; (a) early-onset PE and (b) late-onset PE, developing after 34 weeks (Huppertz, 2011; Poon and Nicolaides, 2014).

Early-onset PE can be defined as PE that develops before the 34 weeks of gestation. Early-onset PE accounts for a small proportion of about 5 – 20% of all cases and it is associated with changes in blood circulation within the maternal uterine arteries (Poon and Nicolaides, 2014). This alteration can be linked to incomplete conversion of the maternal spiral arteries by impaired trophoblast invasion as seen in figure 1.12. Early-onset PE is the most severe clinical variant and it is associated with neonatal morbidity and mortality (Gomathy *et al.*, 2018). On the other hand, the late-onset of PE develops after 34 weeks of gestation and accounts for about 80% of PE cases (Raymond and Peterson, 2011). This case is associated with maternal morbidity (such as impaired glucose tolerance, obesity, chronic hypertension, metabolic syndrome), normal placental weight and volume, and normal birth weight (Huppertz, 2011; Gomathy *et al.*, 2018). Although the clinical features of both early and late-onset PE overlap, there are differences in prognosis, perinatal and maternal outcome (Raymond and Peterson, 2011).
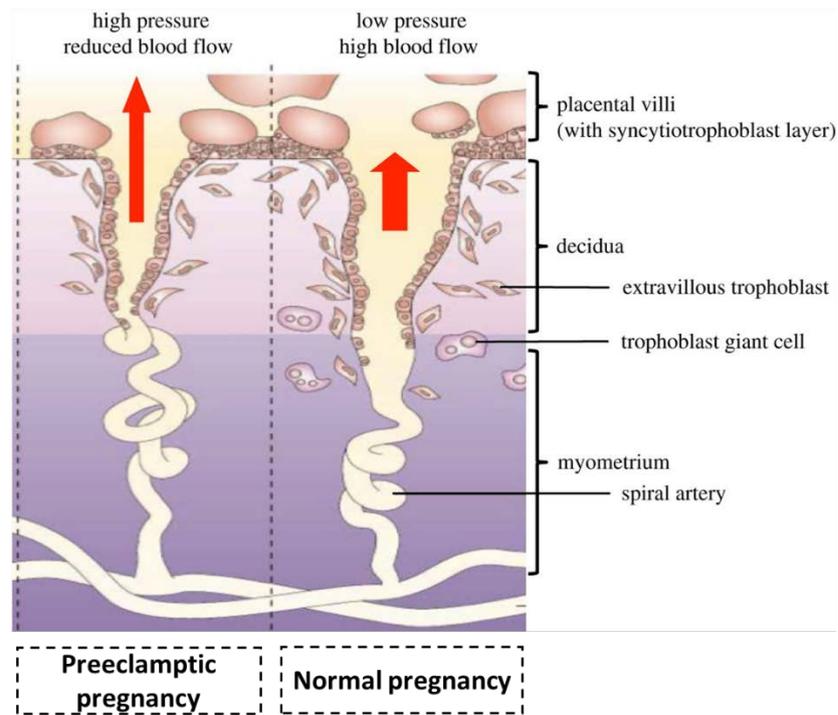
Figure 1.11: **Preeclampsia associated with spiral artery remodelling.**
Inadequate remodelling of the maternal spiral arteries by the trophoblasts cells will result in high blood pressure and reduced blood flow, which eventually leads to preeclampsia. *Figure adapted from* (Moffett *et al.*, 2015)**.**

1.1.6.1.3   Pathophysiology of pre-eclampsia

The exact aetiology of PE still remains a mystery, however it is almost certain that the pathology is caused by the placenta (Irminger-Finger *et al.*, 2008). It has been proposed that the physiopathological mechanism originates from the inadequate invasion of the trophoblast cells into spiral arteries as a result of severe hypoxia triggering hypoxia/ reoxygenation-induced oxidative stress (Irminger-Finger *et al.*, 2008; Ji *et al.*, 2013). Placenta has been reported to be solely involved in placenta PE rather than the foetus, as studies has shown that removing the foetus alone was not sufficient to stop the symptoms of PE until the placenta was delivered (Petla *et al.*, 2013). Incomplete or reduced spiral arteries remodelling as shown in figure 1.11, will lead to placental insufficiency, increased placental apoptosis and necrosis, endothelial dysfunction and abnormal increase in inflammatory responses (Petla *et al.*, 2013). Endothelium is responsible for the regulation of vessel permeability and platelet adhesion, hence this dysfunctionality will lead to an imbalance in vasoconstriction, intravascular coagulation and thrombocytopenia (Irminger-Finger *et al.*, 2008). See figure 1.12 for summary of the pathogenesis of PE.
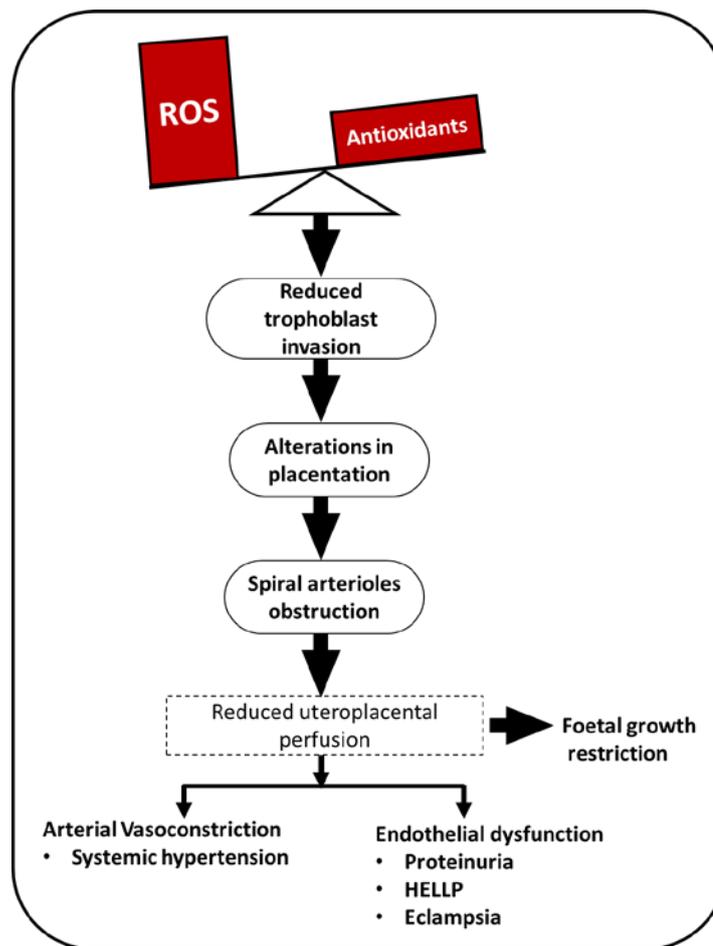
Figure 1.12: Pathogenesis of preeclampsia.
Oxidative stress resulting for severe hypoxic or hypoxia reoxygenation within maternal foetal interface leads to reduced trophoblast invasion which results in incomplete remodelling of the maternal spiral arteries. *Figure modified from* (Irminger-Finger *et al.*, 2008)**.**

1.1.6.1.4   Pre-eclampsia and hypertension

Although the exact cause of hypertension is not fully understood, it is clear the pathological changes within placenta are the main contributors. According to latest theory, PE is believed to result from shallow/reduced trophoblast invasion into the maternal uterine wall leading to placental ischemia, which initiates the release of antiangiogenic factors such as soluble fms-like tryrosine kinase-1 (sFlt-1) and soluble endoglin (sEng) (Enkhmaa *et al.*, 2016). sFlt-1 binds and halt angiogenic factors including VEGF and placental growth factors (PIGF) while sEng binds and inhibits TGF-β leading to an imbalance in angiogenic factors depriving the maternal vascular endothelium and resulting in systemic endothelial dysfunction,

hypertension, proteinuria(Enkhmaa *et al.*, 2016; Maynard *et al.*, 2003). Elevated levels of sFlt-1 and sEng have been seen in preeclamptic patients (Maynard *et al.*, 2003).

1.2 Antioxidants

Antioxidants are scavenging molecules that are capable of preventing and slowing the oxidation of other molecules by converting ROS to H_2O, thereby preventing ROS overproduction (Ashok Agarwal, Sajal Gupta, 2005; Siddiqui *et al.*, 2010). This process is accomplished by terminating the ROS cascade reaction and elimination of free radical intermediates followed by the inhibition of other oxidation reactions (Siddiqui *et al.*, 2010). This shows that antioxidants are central in the protection of cells from oxidative stress (Khera *et al.*, 2013).

There are two types of antioxidants namely; enzymatic and non-enzymatic (Aouache *et al.*, 2018). Enzymatic antioxidants also referred to as natural antioxidants include superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, thioredoxin, and hemoxygenase (Aouache *et al.*, 2018; Ashok Agarwal, Sajal Gupta, 2005). Non-enzymatic antioxidants are mainly dietary supplements or synthetic antioxidants such as vitamins (A, C, E), minerals (zinc, selenium), glutathione, nicotinamide adenine dinucleotide system and flavonoids (Birben *et al.*, 2012; Aouache *et al.*, 2018; Pizzino *et al.*, 2017).

1.2.1 Pre-eclampsia and antioxidant therapy

To date, the only known solution to PE is preterm delivery or termination of pregnancy to safeguard mother's life. Therefore, prevention and treatment are of major clinical importance and research focus. Since PE has been linked to oxidative stress, research to date, has focused on reducing and/ preventing placental oxidative stress using antioxidant (non-enzymatic) therapy (Aouache *et al.*, 2018; Kiondo *et al.*, 2014). Several researchers have focussed on the use of vitamin C and E alone or in combinations (Basaran *et al.*, 2010; Rossi and Mullin, 2011; Fu *et al.*, 2018).

In a clinical trial conducted by Kiondo *et al.* 2014 on the effect of vitamin C supplements on PE in Kampala, Uganda has shown that supplementation with vitamin C did not prevent or reduce the incidence of PE nor reduce the adverse maternal neonatal outcome. This clinical trial consisted of 932 pregnant women with half of them randomised to vitamin C and the other half on placebo.

Clinical trials by Stratta *et al.* 1994 and Bastani *et al.* 2011, concluded that vitamin E supplement did not appear to decrease PE outcome or occurrence and failed to improve foetal outcome.In addition, clinical trial by Basaran *et al.* 2010 investigating the combination of vitamin C and E supplementation for the prevention of PE also conducted that these combination does not decrease the onset/risk of PE and should not be offered to pregnant women for the prevention of PE or other pregnancy combination. This conclusion was drawn with data from 9 independent studies consisting of about 20,000 pregnant women.

Interestingly, Chen *et al*, 2012 carried out extensive clinical study to investigate the effects of pregnant women consuming pomegranate juice (rich in polyphenolic antioxidant) to attenuate oxidative stress and trophoblast apoptosis. This study involved tissue samples from 12 patients (35weeks of gestation till birth) with the daily consumption (8 oz/day) of pomegranate juice. They concluded that pomegranate juice reduces placental oxidative stress *in vivo* and *in vitro*.

1.2.2   Flavonoids

Flavonoids are large group of naturally occurring phenolic compounds with significant antioxidant and chelating properties ubiquitously distributed in plants (Erlund, 2004; Panche *et al.*, 2016). To date, there are more than 8000 identified flavonoids, although only a small number are relevant from a dietary point of view (Erlund, 2004). They are low molecular weight and widely distributed in barks, seeds, leaves and flowers of plants and vegetables (Heim *et al.*, 2002). Flavonoids have been associated with a large spectrum of health promoting effects such as anti-cancer, cardio-protection, neuroprotection, anti-inflammatory and antioxidant properties (Heim *et al.*, 2002; Panche *et al.*, 2016). The dietary intake of flavonoids varies by geographical location and cultural practices such as tea or wine drinking (Heim *et al.*, 2002). The average intake of flavonoids in developed countries (e.g. Netherland, USA, and Italy) is estimated to be between 23 – 38 mg/day (Erlund, 2004).

1.2.3   Chemical structure and classification of flavonoid

The chemical structure of flavonoids is based on their degree of polymerization, structural class, degree of hydroxylation, other substitutions and conjugations (Yao *et al.*, 2004). Although flavonoids vary in structure they all consist of a two phenyl chromone parent compound (A and C) connected to a phenolic ring (B) as shown in figure 1.14 (Yao *et al.*, 2004; Erlund, 2004).
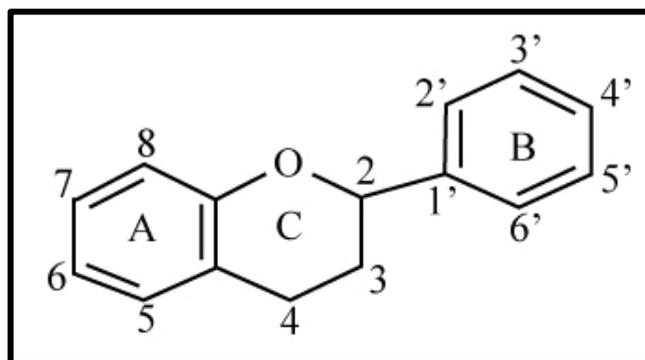
Figure 1.13: The basic structure of flavonoid.
Figure adapted from (Heim *et al.*, 2002).

These phenolic and pyrane rings all exhibit several levels of hydroxylation and methoxylation (Yao *et al.*, 2004). The biochemical properties and functions of flavonoids/their metabolites relies on their chemical structure and the position of various moieties in the structure (Erlund, 2004; Yao *et al.*, 2004). Flavonoids can be classified as either 3-hydroxyflavonoids (flavonoids containing hydroxyl group in position C-3 of the C ring) or 3-desoxyflavonoids (flavonoids with the absence of an hydroxyl group in position C-3 of the C ring) (Erlund, 2004). In addition, classifications are also based on the presence and position of additional hydroxyl or methyl groups (Erlund, 2004). Flavonols, anthocyanidins, leucoanthocyanidins and flavan-3-ol are subclasses of 3-hydroxyflavonoids while flavonones and flavones are subclasses of 3-desoxyflavonoids (Erlund, 2004). Isoflavonoids are different from other groups due to the fact that their B ring is attached to position C-3 instead of C-2 (Erlund, 2004). This study will be emphasising on flavonols and flavanones. See table 1.2 for details of other subclasses of flavonoids.

- **Flavonols**: Flavonols are the most abundant of flavonoids and quercetin is the most common flavonol. It is present in numerous fruits and vegetables with onion having the highest concentrations (Erlund, 2004). In addition, the source of quercetin varies according to the country, for example, the main source of quercetin in Japan and Netherland is tea, while in Italy, quercetin is predominantly found in wine. In the USA, Finland and Greece, the major source of quercetin is apple and onion (Erlund, 2004; Panche *et al.*, 2016). Kaemferol and myricetin are the two other common flavonols which are mostly found in broccoli and berries respectively (Yao *et al.*, 2004). The chemical structure of quercetin can be found in table 1.2.

- **Flavanones**: These are another important subclass that are predominantly found in citrus fruits (with the highest concentrations seen in solid tissues) and considerable amounts have also been found in juices as well (Panche *et al.*, 2016). Hesperidin is the major flavonoids in this subclass and it is mainly found in oranges and mandarins. Naringin is another example of a flavanone and it is mainly found in grapefruits. Others examples of flavanones includes hesperetin and naringenin (tomatoes) (Erlund, 2004). See table 1.2 for chemical structures.

Table 1.2: subclasses of flavonoids, food sources and chemical structures

Flavonoid subclasses	Example compound	Dietary source	Structures
Flavonols	Quercetin, kaempferol, myricetin, isorhamnetin	Onions, berries, broccoli, tea, wine, apples, kale, lectuce	**Quercetin**: R1=OH, R2=OH, R4=OH. **Myricetin**: R1=OH, R2=OH, R3 =OH, R4=OH. **Kaempferol**: R2=OH, R4=OH.
Flavanones	Hesperidin, naringin, hesperitin, naringenin	Oranges, grapefruits, tomatoes	**Hesperidin**: R1=6-O-α-L-rhammosyl-D-glucose, R2=OH, R3=OCH$_3$. **Naringin**: R1=2-O-α-L-rhammosyl-D-glucose, R3=OH.
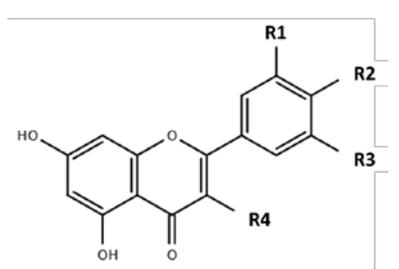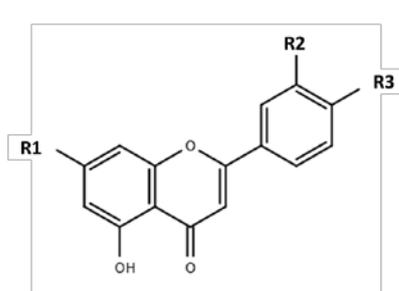
			Hesperetin: R1=OH, R2=OH, R3= OCH$_3$. **Naringenin**: R1=OH, R3=OH.
Flavan-3-ols	Catechin, epicatechin	Apples, pear, grapes, peaches, cocoa or dark chocolate, red wine, green tea.	
Flavones	Apigenin, luteolin, tangeretin	Red pepper, celery, chamomile tea, herbs (parsley).	 **Apigenin**: R2=OH **Luteolin**: R1=OH, R2=OH
Anthocyanidins	Pelargonidin, cyanidin, delphinidin, malvidin	Plum, apples, eggplant, berries	 **Pelargonidin**: R2=OH. **Cyanidin**: R1=OH, R2=OH. **Malvidin**: R1=OCH$_3$, R2=OH, R3=OCH$_3$.
Isoflavonoids	Genistein, daidzein,	Soybean, soy products (tofu, tempeh, miso)	
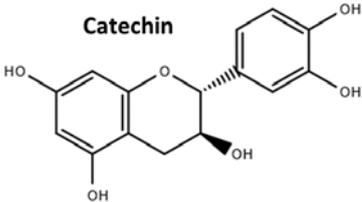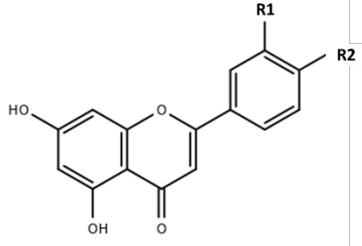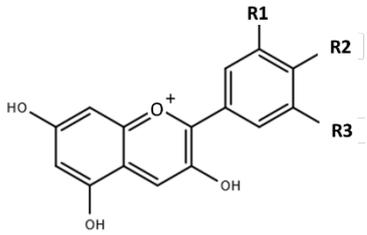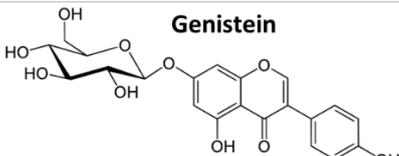
Ideas were adapted from (Hui *et al.*, 2013; Erlund *et al.*, 2001; Panche *et al.*, 2016) and structures were sketched using ChemSpider, UK.

1.2.4 Absorption and metabolism of flavonoids

Ingestion of dietary flavonoids from fruits and vegetables is mainly by oral route, and their absorption is dependent on their physico-chemical properties like molecular weight, lipophilicity, structural configuration, solubility and acid dissociation constant (pKa) (Kumar and Pandey, 2013). The main site for flavonoid absorption is the gastrointestinal tract (small intestine and colon), and therefore is influenced by the form of flavonoids whether they are glycoside or aglycone. Aglycones are readily absorbed by the small intestine, whereas glycosides are converted into aglycones before they can be absorbed (Kumar and Pandey, 2013). In order for flavonoids to be absorbed, conjugated, metabolised and/or excreted, they have to be degraded into phenyl acids (mainly by the colonic bacteria) by splitting them into heterocyclic rings (Yao *et al.*, 2004). Flavonoids in the form of glycosides are rapidly deglycosylated. The rate and extent of this deglycosylation is dependent on the position or nature of sugar attached to the flavonoid as well as the structure of the flavonoid (Yao *et al.*, 2004). The mechanisms and events leading to flavonoid absorption are poorly understood and are a matters of debate. It was previously thought that flavonoids are absorbed in the large intestine only (Erlund, 2004). Recent evidence has shown that flavonoids are metabolised in the liver, small intestine and colon by *O*-methylation, glucuronidation and sulphation (Bentz, 2009; Thilakarathna and Vasantha Rupasinghe, 2013). Flavonoid conjugation takes place in the small intestine followed by the liver for further metabolism. Liver metabolism produces sulphates and glucuronide derivatives, and excretion occurs via urine or bile. Flavonoids that are not processed in the intestine will reach the colon, where they will undergo structural modifications by colonic microflora. Some flavonoid glucuronides re-enter the enterohepatic circulation via bile excretion where they are hydrolysed to aglycones by the microbiota. Aglycones are further metabolised to low molecular weight compounds for easy absorption. Plasma and urine measurement of flavonoids after ingestion revealed that absorption of flavonoids is rapid and flavonoids are present in systemic circulation shortly after ingestion (Yao *et al.*, 2004). See figure 1.15 for summary of flavonoids absorption and metabolism.
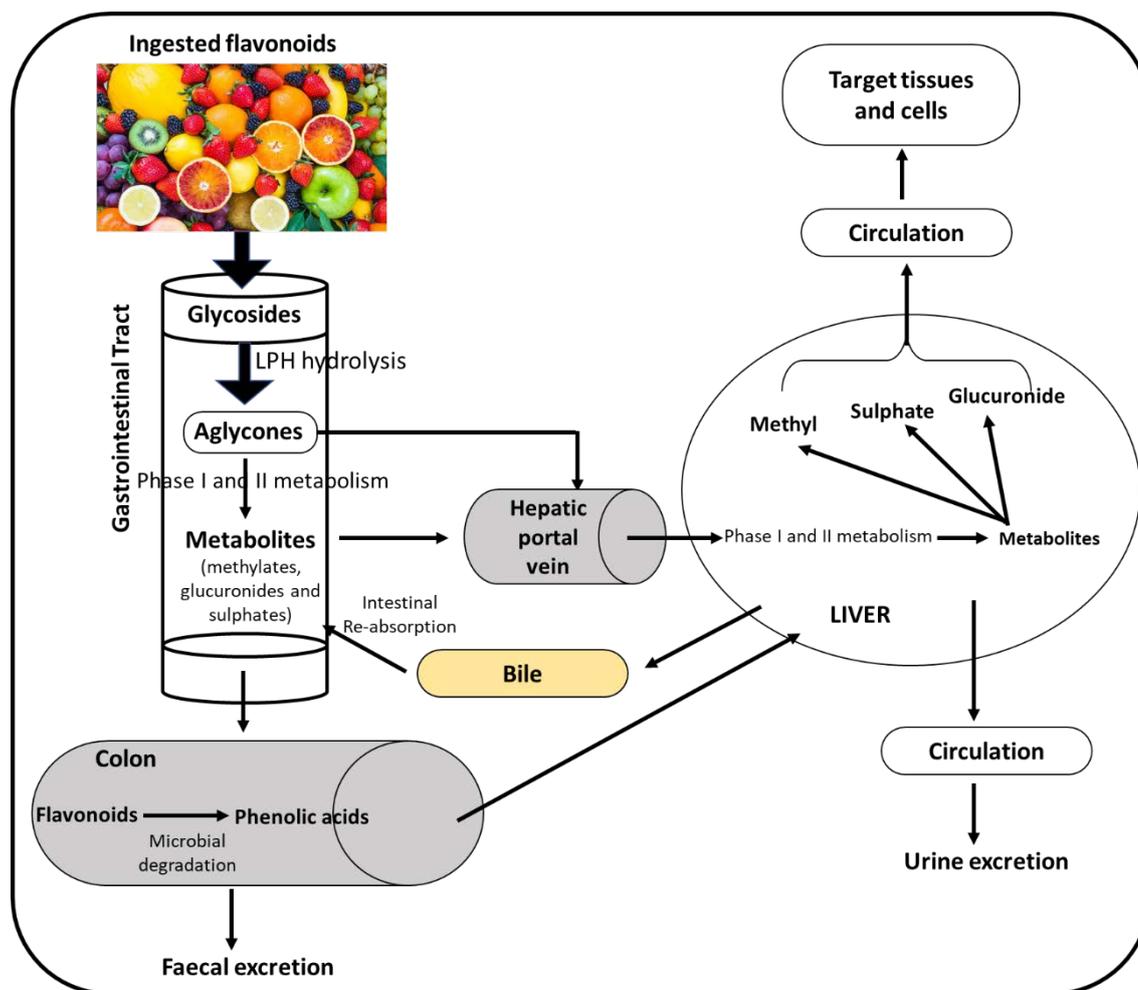
Figure 1.14: **Schematic representation of flavonoids metabolism.**
Ingestion flavonoids undergoes intestinal biotransformation producing metabolites. These metabolites are transported to the liver through the hepatic portal vein. Metabolites in the liver are then transferred to target tissues and cells or excreted to bile for enterohepatic re-circulation or eliminated through faeces or urine. Flavonoids aglycones or metabolites that get to the colon undergo degradation by microbial population and then reabsorbed. **LPH:** lactase-phlorizin hydrolase. *Figure adapted from* (Thilakarathna and Vasantha Rupasinghe, 2013) *with modifications*.

1.2.5   Bioavailability of flavonoids

Bioavailability can be defined as "the rate and extent to which an active moiety or active ingredient is absorbed from a drug product and becomes available at the site of action" (U.S Food and drug Administration). This same principle can be applicable to flavonoids as the rate of absorption and presence at the site of action is crucial for them to be effective in the biological system. Flavonoid bioavailability is low and varies among different flavonoid subclasses and individual flavonoid compounds (Panche *et al.*, 2016).

For instance the absorption of quercetin from onion is fourfold greater than that from apples or tea (Yao *et al.*, 2004). According to Crozier, Del Rio and Clifford (2010). Flavonoids with complex structures and higher molecular weights have lower bioavailability. In a clinical study, quercetin from onion powder consumption showed faster absorption, increase plasma concentrations and greater bioavailability when compared to apple peel powder (Banjarnahor and Artanti, 2014). Study from Erlund *et al*, (2001) has also shown that bioavailability and pharmacokinetics of flavanones (hesperidin and narigin) varies in different studies. For example, hesperetin recovery was 3% after 500 mg of hesperidin in one study and in another 24% was recovered in 5 subjects ingesting 1250 ml of oranges. Similar observations was seen in naringenin recovery, 5% in one subject and 15% in two subjects after a single ingestion of naringin (214-700 mg) (Bentz, 2009; Crozier *et al.*, 2010).

1.2.6 Flavonoids as prooxidants

As explained above (section1.2.5) flavonoids can be beneficiary, however they also can produce deleterious effects on the dells by exhibiting prooxidant properties (Eghbaliferiz and Iranshahi, 2016). The prooxidant properties of flavonoids has been proposed to be directly proportional to the total number of hydroxyl groups in the structure of flavonoid as multiple hydroxyl groups in the B ring significantly elevates the production of the hydroxyl radical in Fenton reaction (Procházková *et al.*, 2011). Evidence has also revealed that the presence of 2,3-double bond and 4-oxo structural arrangement of flavones may increase the chances of ROS production stimulated by divalent copper and oxygen (Eghbaliferiz and Iranshahi, 2016). They are also known for their metal chelating ability which can be deleterious when flavonoids mediate the reduction of Cu(II) to Cu(I) resulting to the development of ROS and other organic compound that can harm lipids (Eghbaliferiz and Iranshahi, 2016). Flavonoid prooxidant properties is also concentration dependent; the prooxidant properties of four flavonoids (quercetin, morin naringenin and hesperetin) was investigated by Yen *et al*, (2003) in human lymphocyte system. In this study, when flavanones (namely naringenin and hesperetin) were added in a concentration range from 0 – 200 μM, intracellular H_2O_2 production could not be detected whereas addition of quercetin and morin in concentration range 25 – 200 μM and 125 – 200 μM respectively increased the concentration of H_2O_2. Another mechanism for prooxidant effects of flavonoids involves several peroxidases that catalyse the oxidation of polyphenols such as inducing lipid peroxidation and co-oxidising of GSH to thiol radical formation (Procházková *et al.*, 2011).

Prooxidant properties of flavonoids are also considered to be one of many mechanisms in which flavonoids promote apoptosis, bactericidal activity and inhibit mitochondrial breathing (Eghbaliferiz and Iranshahi, 2016; Procházková *et al.*, 2011).

1.2.7 Flavonoids as antioxidants

Flavonoids have shown both antioxidant and prooxidant properties in animal and *in vitro* models. Their antioxidant property has been labelled as high level natural antioxidant due to following reasons (Yao *et al.*, 2004; Procházková *et al.*, 2011);

❖ **Direct involvement in scavenging ROS**: Flavonoids are capable of scavenging free radicals due to their ability to donate hydrogen atom due to their high reactivity of the hydroxyl group, hence resulting less-reactive and stable free radicals (Panche *et al.*, 2016). Some flavonoids have been found to directly scavenge superoxide while others scavenge peroxynitrite, a highly reactive oxygen derived radical (Panche *et al.*, 2016). *In vitro* studies of flavonoids have shown that their f antioxidant properties depend on the structural arrangement of functional groups as well as both the total number of hydroxyl groups and their configuration. The most significant determinant of ROS scavenging by flavonoids is the configuration of the B ring hydroxyl group (Bentz, 2009). However, inserting the hydroxy group in ring A and C has little or no impact on superoxide scavenging effect. Other structural features of flavonoids in combating free radicals include the following; the presence of catechol (ortho-dihydroxy) structure in the B ring which is responsible for delocalization of electron, presence of hydroxyl group at positive 5 (A ring) and 3 (C ring) which is responsible for providing hydrogen bond to the oxo group and the presence of 2,3-double bond in combination with a 4-oxo function the C ring which is responsible for the delocalization of election from the B ring (Procházková *et al.*, 2011; Robert J. Williams *et al.*, 2004).

❖ **Activation of antioxidant enzymes**: Flavonoids can indirectly scavenge free radicals by interacting various antioxidant enzymes such as glutathione and nicotinamide adenine dinucleotide phosphate system (Panche *et al.*, 2016). This can be achieved by their ability to induce phase II detoxifying enzymes like NAD(P)H-quinone oxidoreductase, and glutathione S-transferase, which are the main defence enzymes against oxidative stress and electrophilic toxicants (Procházková *et al.*, 2011).

❖ Flavonoids with the hydroxy functional group at position C-3 (e.g. quercetin) are more effective in the activation of other antioxidant mechanisms and effective inducer of electrophile-responsive element (Brunetti *et al.*, 2013).

❖ **Metal chelating potentials**: Flavonoids have shown the ability to chelate iron and copper, thereby deleting a causal factor for the formation of free radicals (Procházková *et al.*, 2011). Quercetin was found to prevent oxidative cellular injury by a number of oxidising agents (such as acrolein) that influenced the release of iron in its redox form in erythrocyte membrane (Procházková *et al.*, 2011). According to Pietta (2000), the binding site for trace metal in the flavonoid structure are the 3-hydroxyl and 4-oxo group in the C ring, catechol moiety in the B ring, and 4-oxo and 5-hydroxyl groups in the A and C ring. The main contributor of the metal chelation is the catechol moiety in the B ring due to its ability to chelate cupric cation (Brunetti *et al.*, 2013).

❖ **Inhibition of oxidases**: Flavonoids have the ability to inhibit enzymes such as xanthine oxidases and protein kinase C that are responsible for the generation of superoxide (Panche *et al.*, 2016). Flavonoids have also shown to be potent inhibitors of cyclooxygenase, microsomal succinoxidase, lipoxygenase and NADH oxidase (Banjarnahor and Artanti, 2014). Based on structural function of flavonoids, luteolin a member of the subclass flavone was found to be the most potent inhibitor of xanthine oxidase which eventually led to the decreased oxidative cellular injury (Procházková *et al.*, 2011).

Other antioxidant properties of flavonoids include; increasing uric acid levels, mitigating oxidative stress as a of nitric oxide, reduction of α-tocopheryl radicals, and modification of prooxidant properties of low molecular antioxidants (Panche *et al.*, 2016). As can be seen above (state sections 1.1.6.1.2) oxidative stress is found to be main contributor to several diseases which include pregnancy related disorders. It is also known that the flavonoids in edible plants and fruits do have protective effects against oxidative stress mainly by preventing the formation of intracellular ROS. Interestingly, scientific research is now concentrating on the use of natural food supplementations to prevent many of these disorders. In fact, several studies have been carried out on the antioxidant effects of flavonoids in cancer, cardiovascular diseases, neurodegeneration, anti-inflammatory, diabetes, and anti-bacterial (Panche *et al.*, 2016; Eghbaliferiz and Iranshahi, 2016; Hwang and Yen, 2011; Bournival *et al.*, 2009).

However, no study has explored the antioxidant effects of flavonoids during early gestational disorders (such as pre-eclampsia), which result from placental oxidative stress. Perhaps, the main reason for these are due to the ethical constraints of performing clinical trials on pregnant women. Although it is possible to study the overall effects of ingesting edible plants, and/or fruits to prevent early pregnancy disorders, it is virtually impossible to extract a particular ingredient (such as flavonoids) from other bio-protective substances (such as vitamins) without affecting their biological activities. The best alternative to this is to use *in vitro* studies using cell lines originated from early trophoblast.

1.2.8   The role of flavonoids in vasculo-endothelial functions

Endothelial dysfunction is a significant event in the pathogenesis of pre-eclampsia, cardiovascular disease (atherosclerosis) and cancer (Aouache *et al.*, 2018; Bondonno *et al.*, 2012; Franses *et al.*, 2013). Healthy endothelial cells promote vascular repairs and enhances trophoblast invasion by maintaining vascular homeostasis and regulation of vascular tone (Aouache *et al.*, 2018; Loke *et al.*, 2008). This maintenance is achieved by balancing the production of vasodilators such as nitric oxide. Dietary flavonoids increase the nitric oxide level which enhances endothelial function by increasing the production endogenous nitric oxide and reducing their breakdown (Bondonno *et al.*, 2012). Human clinical trials have shown that flavonoids improve endothelial functions in acute and short-term intervention (Hodgson and Croft, 2006; Chen *et al.*, 2012). The improvement of endothelial – dependent and nitric oxide – dependent vasodilation have been proposed to be one of the possible mechanism of flavonoid involvement in endothelial and vascular function (Loke *et al.*, 2008). The improvement in endothelial function can also contribute to lower blood pressure.

1.2.9   The role of flavonoids in glucose and lipid metabolism

Lipids and glucose are important macromolecules required for the maintaining various physiologic, homeostatic and cellular processes in the human body. Dysfunction in lipid, lipoprotein and glucose metabolism have been linked in the pathogenesis of several diseases including obesity, cardiovascular diseases, diabetes and inflammation(Assini *et al.*, 2013; Jung *et al.*, 2006). Flavonoids from citrus origin (especially naringin) have been found to exhibit lipid-lowering properties in humans by significantly reducing plasma triglyceride and apolipoprotein B levels in hypertriglyceridemic patients (Assini *et al.*, 2013). Citrus flavonoid (naringin) was found to significantly lower plasma low density lipoprotein (LDL) in a clinical trial involving hypercholesterolemic patients (Jung *et al.*, 2003). The lipid lowering mechanism of citrus flavonoids have been fully established in human subjects. However, other studies exhibited inhibitory effect on hepatic HMG-CoA (3-hydroxy-3-

methyl-glutaryl-coenzyme A) reductase and acyl CoA (Jung *et al.*, 2003). In fact flavonoids' cholesterol lowering properties have been attributed to their direct binding to/interaction with oestrogen receptors (Bok *et al.*, 2000).

Flavonoids have also been identified as an antidiabetic agent, the mechanisms in which they exert their hypoglycaemic and hypolipidemic effects in type-2 diabetes have not been well documented (Jung *et al.*, 2006). Although, animal studies shows that hesperidin and naringin significantly increases glucokinase mRNA, as well as markedly reducing the mRNA expression of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase in the liver of C57BL/KsJ-db/db mice (Jung *et al.*, 2006).

1.3   Trophoblast models

Lack of suitable animal model has made the investigation on the pathogenesis of PE more difficult. There were several attempts to create animal models using rats, rabbits, dogs, and guinea pigs and nonhuman primates (Lu *et al.*, 2007). These attempts failed due to the nature of surgical and methodological interventions that altered the vascular functions (Lu *et al.*, 2007). It should be noted PE is a human specific disease, therefore establishing animal models needs to be carried out either by surgical interventions (such as renal artery ligation) or dietary modifications (high salt diet) Since the 1890s, there have been increasing interest in the isolation of trophoblasts from human placenta for the purpose of primary culture. However, isolated primary trophoblast culture has its setback of being very difficult to maintain in culture due to their short lifespan (maximum of 7 days) and inability to subculture (Rothbauer *et al.*, 2017; Barrak *et al.*, 2016). These disadvantage inspired the generation trophoblast cell lines that able to differentiate, invade and migrate (Abou-Kheir *et al.*, 2017). Different cell lines were developed to study the function of the placenta including BeWo, JEG-3 and JAR from choriocarcinomas, HTR-8/SVneo, TEV-1, SW71 from first trimester EVTs (Rothbauer *et al.*, 2017; Abou-Kheir *et al.*, 2017; Barrak *et al.*, 2016)

HTR-8/SVneo was the main cell line used for this study, as it is a well-established extravillious cytotrophoblast (EVTs) cell line. It was generated from the isolation of evCTB from the first trimester placenta tissue and transfected with a plasmid (simian virus 40 large T antigen) by Graham et al. (Abou-Kheir *et al.*, 2017; see also Graham *et al.*, 1993). Also, it has been validated as trophoblast cell line with extended lifespan and shares phenotypic features with primary cytotrophoblast cell cultures such as E-cadherin, vimentin, cytokeratin 7, hCG production and the expression of type IV collagenase and cytokeratin (Abou-Kheir

et al., 2017; Kilburn, 2000). The study also initially considered using TEV-1 and Swan 71 (SW71) evCTB first trimester trophoblast cell lines. However, HTR-8/SVneo cell line was found to generate consistent and reproducible results. In fact HTR-8/SVneo cells are the most commonly used *in vitro* model to study trophoblast/placental physiology and development such as proliferation, invasion, migration and regulation (Abou-Kheir *et al.*, 2017).

1.4   Aims

The aims of this *in vitro* study using transformed first trimester early extra-villous trophoblast cell line (HTR-8/Svneo) are to;

- Optimise a range of non-toxic concentrations of flavonoids, their metabolites alone or combination that is tolerated by these cells.

- Establish an oxidative stress model in this cell line

- Investigate the cytoprotective effects of flavonoids against oxidative stress

- Investigate the intracellular mechanisms associated with flavonoid-mediated cytoprotection.

- Study the effects of flavonoids on intra-cellular protein kinase(s) activation and apoptosis pathways with/without oxidative stress

- Study the effects of flavonoids on trophoblast cell invasions using 2D and 3D models as well as stem cell generation.

Chapter 2
Materials and Methods

2.1   Materials

All the chemicals and compounds used in this study are analytical grade. Thiazolyl Blue Tetrazolium bromide (MTT) and all flavonoids/metabolites used in this study were purchased from Sigma-Aldrich®, UK. Dimethyl sulfoxide (DMSO) and bovine serum albumin (fractive V, heat shock treated) were purchased from Fisher Scientific, UK. CellTox™ green cytotoxicity, CellTiter-Glo® luminescent cell viability, NADP/NADPH-Glo™, ROS-Glo™, and GSH/GSSG-Glo™ assays were purchased from Promega, UK. RPMI-1640, trypsin (0.05% w/v) - EDTA (0.02% w/v) solution, penicillin (100 U/ml) and streptomycin (100 µg/ml) were purchased from Lonza, UK. Foetal bovine serum (FBS) was purchased from Gibco®, UK. Invasion and migration 96-well plates were purchased from Corning®, UK. Cultrex® Basement Membrane Matrix (3D spheroid invasion kit) was purchased from AMS Biotechnology, UK. Tissue culture flasks (T75 and T25) were purchased from Sarstedt AG and Co. KG, Germany. Modular incubator chamber was purchased from Billups-Rothenberg Inc., USA. Gas mixture of 0.5% O_2, 5% CO_2 and 94.8% N_2 was bought from BOC limited, UK and certified by HiQ®, UK. Absorbance, fluorescence and luminescence were measured using FLUOstar Omega plate reader (BMG LABTECH, UK).

2.1.1   Buffers and Solutions

Buffers and solutions used for western blotting were prepared using sterile glassware and Millipore water (where necessary). They were stored at the recommended temperature (usually 4ºC or room temperature) when not used while some of them needed to be prepared fresh. Table 2.1 summarise the list of buffers and solutions.

Table 2.1: **Buffers and Solutions**

Reagent	Recipe
TBS 10X	24.9 g Tris HCl, 5.6 g Tris base, 88 g NaCl, 1 L millipore H_2O
TBS-Tween 20 (0.1% v/v)	100 ml TBS (10X), 900 ml millipore H_2O, 1 ml Tween 20, pH 7.6
Electrophoresis Buffer 10X Electrophoresis Buffer 1X	30.3 g Tris base, 143 g Glycine, 10 g SDS, 1 L millipore H_2O 100 ml 10X, 900 ml Millipore H_2O, pH 8.3
Transfer buffer 10X Transfer buffer 1X	30.3 g Tris base, 143 g Glycine, 1 L millipore H_2O 100 ml 10X, 200 ml methanol, 700 ml Millipore H_2O, pH 8.3

SDS – Sodium dodecyl sulphate, TBS – Tris-buffered saline, NaCl – Sodium Chloride, H_2O - Water

2.1.2 Antibodies

All antibodies were prepared and stored in accordance with manufacturer's instructions and was used before their expiration date. All antibodies were prepared in either 3% (w/v) BSA or 5% (w/v) non-fat milk dissolved in TBS-Tween 20 (0.1% v/v plus 0.02% (w/v) sodium azide) Abcam, UK and their dilutions are summarised in table 2.2. Prism ultra protein ladder (10-245 kDa), Abcam, UK was also used to identify protein positions and ensure proper separation of proteins.

Table 2.2: **Details of antibodies with their dilutions**

Primary antibodies	Description	Supplier	Antibody dilution
Rabbit Anti-AKT1/2/3	Monoclonal	Abcam	WB, 1:10000
Rabbit Phospho-AKT (Ser473)	Monoclonal	CST	WB, 1:2000
Rabbit Anti-ERK1/2	Polyclonal	Abcam	WB, 1:1000
Rabbit Phospho-ERK1 (T202/Y205) + ERK2 (T185/Y187)	Monoclonal	Abcam	WB, 1:5000
Rabbit Anti-JNK1/2/3	Monoclonal	Abcam	WB, 1:1000
Rabbit Phospho-SAPK/JNK (Thr183/Tyr185)	Monoclonal	CST	WB, 1:1000
Rabbit p38 MAPK	Monoclonal	CST	WB, 1:1000
Rabbit Phospho-p38 MAPK	Monoclonal	CST	WB, 1:1000
Rabbit Anti-Beta Actin	Polyclonal	Abcam	1:5000
Rabbit GAPDH	Monoclonal	CST	1:1000
Rabbit Beta Tubulin	Monoclonal	CST	1:1000
Secondary antibody	**Description**	**Supplier**	**Antibody dilution**
Goat Anti-Rabbit IgH H&L (HRP)	Polyclonal	Abcam	WB, 1:3000

CST: Cell Signalling Technology®, WB: Western Blot

2.2   Methods

2.2.1   *In vitro* cell culture - Cell revival and maintenance

The transformed first trimester human trophoblast cell line (HTR-8/SVneo) was kindly provided by Dr Charles Graham, University of Kingston, Canada. Cells were cultured in growth medium; RPMI-1640 with L-glutamine (Lonza UK) supplemented with 10% (v/v) Foetal Bovine Serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml) and maintained in a humidified incubator (95% air/5% CO_2 at 37ºC). The cells were retrieved from the liquid nitrogen and thawed. The cells were thawed by adding 10 ml of growth medium to eliminate the cytotoxic effects of freeze medium and centrifuged in a Harrier 15/80 centrifuge (Sanyo) at 1000 x g for 3 min. Supernatant was discarded and pellet (containing cells) was re-suspended in 1ml growth medium before being transferred to Sarstedt T25 flasks. Daily checks were carried out and growth medium (RPMI-1640) was changed every 2 days (or as appropriate).

Sub-culturing was carried out when cells reached 70-95% confluency, by discarding the growth medium and washing the cells three times with 1X PBS (Lonza, UK). Cells were detached from the flask by adding 1X trypsin (Sigma-Aldrich, UK) and placed at 37˚C for 2-3 min, checking for cell detachment every 30 secs using an inverted light microscope (AE2000, Scientific Laboratory Supplies Ltd). Afterwards, RPMI-1640 growth medium was added to the flask to inhibit the effect of trypsin before collecting the cells in a Sterilin® tube (Sarstedt, UK) and centrifuged in a Harrier 15/80 centrifuge (Sanyo) at 1000 x g for 5 min.

The supernatant was discarded, and the cell pellet was resuspended in 1ml RPMI-1640 growth medium to achieve a single cell suspension. Total number of cells and viability were determined using trypan blue exclusion (0.4% v/v trypan blue, Sigma-Aldrich, UK), 100µl of cell suspension was added to 300 µl of trypan blue before vortexing briefly. The cell (10µl) was transferred into a haemocytometer for counting. The total number of cells were estimated using the formula below.

$$\textbf{Total cells/ml} = \text{Total cells counted} \times \frac{\text{dilution factor}}{\text{\# of squares}} \times 10,000 \text{ cells/ml}$$

These cells were either bulked up into T75 flask (Sarstedt, UK), used for further experiments or frozen as described below. The same protocol was followed in all subsequent cell culture experiments unless stated otherwise.

2.2.1.1 Cryopreservation of HTR-8/SVneo cells

HTR-8/SVneo cells were cryopreserved in liquid nitrogen. Following centrifugation (as described in section 2.2.1) the cell pellet was resuspended in freeze medium (FBS, 92%; DMSO, 8%) and then aliquoted into cryogenic storage vials. Cryogenic storage vials were placed in -20ºC for 2 h before transferring to -80ºC for another 22 h. After a total of 24 h, cryogenic storage vials were transferred to liquid nitrogen for long term storage.

2.2.2 Flavonoids and Metabolites preparation

Stock solution for both flavonoids (quercetin, morin, naringin and hesperidin) and their respective metabolites (Quercetin-3-O-β-glucuronide [Q3G], naringenin and hesperetin) were prepared to the concentration of 10 mM dissolved in dimethyl sulfoxide (DMSO) and stored in the -20ºC freezer. Further dilution (working concentrations) to 1, 3, 10, 20 and 40 μM in RPMI-1640 growth medium to a final DMSO concentration of 0.1% (v/v) was carried out before treating the cells. HTR-8/SVneo cells were treated with indicated working concentrations of flavonoids for 24 and 48 h to achieve an optimal non-toxic concentration. Control cells were supplemented with growth medium. MTT and CellTox™ green cytotoxicity assays were used to determine the non-toxic concentrations for further studies.

2.2.3 Establishing oxidative stress model

Stage 1 (Hypoxic conditions): To induce oxidative stress, HTR-8/SVneo cells were seeded at the density of 5×10^4 in a 24 well plate (Sarstedt, UK) and allowed to attain 70-80% confluency before exposure to hypoxia (either 0.5% or 2% O_2). Hypoxia was achieved by placing cultured cells (24-well plate) in a humidified modular incubator chamber (Billups-Rothenberg, USA) that was flushed with a gas mixture of 0.5% O_2, 5% CO_2 and 94.5% N_2 (BOC/HiQ®, UK). An electronic oxygen analyser (Teledyne Analytical Instruments, UK) was used to confirm O_2 concentrations before sealing the chamber and subsequent incubation at 37ºC for the desired duration of experiment/exposure.

 Stage 2 (conditions for hypoxia-reoxygenation): Herein, the same procedure from stage 1 was adopted, but hypoxia was established for 2 h or 4 h followed by re-oxygenation for 6 h in a humidified incubator (95% air/5% CO_2 at 37ºC). Both hypoxic and re-oxygenation conditions were carried out in glucose and serum free Gibco® RPMI-1640 medium (ThermoFisher Scientific, UK).

2.2.4 Cell viability assays

Cell viability was performed using 3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide (MTT), CellTox™ green cytotoxicity and CellTiter-Glo® luminescent cell viability assays.

2.2.4.1   3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide (MTT)

The viability of HTR-8/SVneo cells in culture was assessed by MTT assay (Sigma-Aldrich, UK). This assay is based on the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide to violet formazan crystals by both mitochondrial and extra-mitochondrial dehydrogenases. In the MTT assay, formazan formation is the direct representation of viable cells.

HTR-8/SVneo cells were seeded at a density of 5×10^4 cells/well in a 24-well flat-bottomed plate and incubated overnight in fully supplemented RPMI-1640 growth medium. Following treatments HTR-8/SVneo cells were incubated for 1 h in 5 mg/ml of MTT solution (dissolved in 1X PBS) at 37ºC. The culture medium in each well was aspirated without disturbing the blue, insoluble formazan crystals. Dissolving the formazan crystal was achieved by adding 500 µl of DMSO (Fisher Scientific, UK), and was agitated in a plate shaker to dissolve the crystals wrapped in aluminium foil. 200 µl of the resulting solution was transferred into a 96-well plate (Sarstedt, UK) and then absorbance was read at 570 nm using a FLUOstar Omega (BMG LABTECH) plate reader. The absorbance of the DMSO blank was subtracted from each reading assay absorbance.

2.2.4.2   CellTox™ reagent preparations and storage

CellTox™ green cytotoxicity assay components (CellTox™ green dye, 1000X; assay buffer and lysis solution) were thawed in a 37ºC water bath. Afterwards, each component was vortexed briefly to ensure homogeneity and briefly centrifuged. To create CellTox™ green reagent, 20 µl of the CellTox™ green dye was transferred into 10 ml assay buffer (2 X solution). Unused CellTox™ green dye (2X) was stored at 4ºC for up to 7 days and stock reagent was stored at -20ºC.

2.2.4.2.1   CellTox™ Green Cytotoxicity Assay

CellTox™ green cytotoxicity (Promega, UK) assesses the changes in membrane integrity that occur as a result of cell death. This assay uses a propriety asymmetric cyanine dye that stains dead cells' DNA but is excluded from viable cells.

HTR-8/SVneo cells were cultured in a 96-well black with clear bottom plate (Falcon®) at a density of 2×10^4 cells/well (100 µl) and was incubated overnight to allow cells to adhere before treating with the indicated flavonoids or metabolites for further 24 h or 48 h. The cytotoxic effects of different concentrations of flavonoids or hypoxia/re-oxygenation was assessed by CellTox™ green cytotoxicity assay. CellTox™ green reagent (2X) was prepared according to the manufacturer's instructions (see section 2.2.4.2) to achieve a 1:1 mixture with treatment medium. The addition of 100 µl CellTox™ green dye (2X) to experimental wells was carried out and the plate was placed in an orbital shaker briefly before allowing to incubate at room temperature for 15 min covered in aluminium foil. Afterwards, fluorescence was read at 485 nm$_{EX}$ / 520 nm$_{EM}$ using FLUOstar Omega (BMG LABTECH) plate reader (see figure 2.1 for assay flowchart).
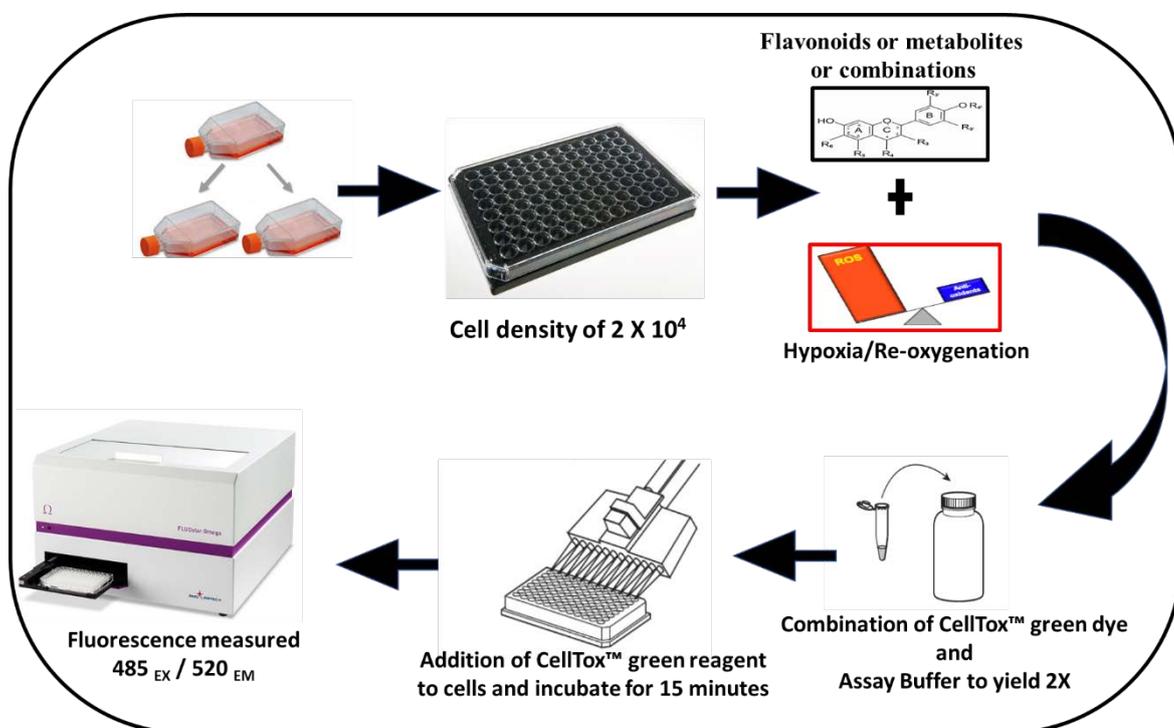
Figure 2.1: Overview of CellTox™ green cytotoxicity assay protocol
Primary control (toxicity control) for necrosis was performed by the addition of lysis solution which represented the maximum dead-cell signal from non-proliferating cells. The untreated cells represented the minimum signal obtainable from proliferating cells. Blank wells (medium containing treatment and CellTox™ green dye 2X) represent background signals which was subtracted from experimental readings.

2.2.4.3   CellTiter-Glo® Luminescent Cell Viability Assay

This assay is a homogeneous method to determine the number of viable cells based on quantitation of the Adenosine Triphosphate (ATP) present. The presence of ATP signals represent metabolically active cells. CellTiter-Glo® assay is a luciferase-based assay and relies on cell lysis and generation of luminescent signal proportional to the amount of ATP present (the amount of ATP is directly proportional the number of viable cells). Before carrying out this experiment, generation of an ATP standard curve and determination of the cell seeding density was carried out.

2.2.4.3.1   Generation of ATP standard curve

ATP disodium stock solution 1 µM was prepared in growth medium. Serial dilutions of the stock solution ranging from 1 µM – 10pM were prepared. These were transferred (100 µl) into a 96-well black with clear bottom plate (Falcon®) and equal volume of CellTiter-Glo® reagent was added to serial dilutions before being placed on an orbital shaker for 2 min. Plates were incubated at room temperature for 10 min to stabilize the luminescent signal and was read using FLUOstar Omega (BMG LABTECH) plate reader. Data were analysed using GraphPad Prism (see figure 2.2).
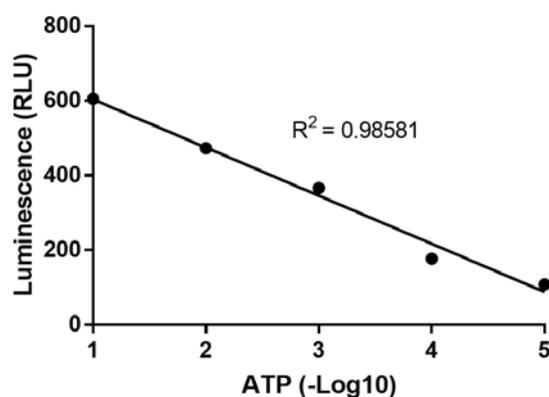
Figure 2.2: **Standard curve for ATP quantitation**

2.2.4.3.2   CellTiter-Glo® Assay

Appropriate cell seeding density was determined (see appendix A.1) to ensure the number of cells was within the linear range of the CellTiter-Glo® assay. HTR-8/SVneo cells were cultured in a 96-well black with clear bottom plate (Falcon®, UK) using 5×10^4 cells/well (100 µl). Cells were incubated overnight before treating with flavonoids, their metabolites alone or in combination for a further 24 h prior to HR-induced oxidative stress exposure. Afterwards, an equal volume of CellTiter-Glo® reagent (100 µl) was added to each experimental well.

Plates were mixed on an orbital shaker for 2 min (to induce cell lysis) and kept at room temperature for 10 min to stabilize the luminescent signal. Luminescence was recorded using FLUOstar Omega (BMG LABTECH) plate reader. Blank wells (culture medium containing treatment and CellTiter-Glo® reagent) represent background signals which were subtracted from experimental reading.

2.2.5   Determination of flavonoids' redox potential

The effect of HR-induced oxidative stress, flavonoids, their metabolites alone or in combination on redox balancing was measured using GSH/GSSG-Glo™ and NADP/NADPH-Glo™ assays.

2.2.5.1   Reagents preparation

Total glutathione and oxidized glutathione lysis reagent were prepared according to the manufacturer's instruction. Preparation of GSH and GSSG lysis reagents is summarised in table 2.3.

Table 2.3: GSH and GSSG lysis reagent for adherent cells

Total Glutathione lysis reagent		Oxidized Glutathione lysis reagent	
Components	Volume/reaction for 96 well plate (µl)	Components	Volume/reaction for 96 well plate (µl)
Millipore water	39.0	Millipore water	38.5
Passive lysis buffer (5X)	10.0	Passive lysis buffer (5X)	10
Luciferin-NT	1.0	Luciferin-NT	1.0
		NEM (25 mM)	0.5

2.2.6   Determination of reduced and oxidized glutathione

The effect of HR-induced oxidative stress, flavonoids, metabolites or in combinations on HTR-8/SVneo cells was assessed using GSH/GSSG-Glo™ assay (Promega, UK). This assay is a luminescence-based assay used in the detection and quantification of total glutathione (GSH + GSSG), oxidized glutathione (GSSG) and GSH/GSSG ratio in cultured cells. HTR-8/SVneo cells were cultured in two 96-well black with clear bottom plate (Falcon®) using a cell density of 2×10^4 cells/well (100 µl). One plate was used to determine total glutathione and the other for oxidized glutathione. Both plates were incubated for 24 h before treating

with flavonoids, their metabolites alone or in combinations for further a 24 h prior to HR-induced oxidative stress exposure.

Culture medium was aspirated from both plates and replaced with 50 µl total glutathione lysis reagent (for the first plate) or 50 µl oxidized glutathione lysis reagent (the second plate) (see section 2.2.5.1 for reagent preparation). Both plates were kept on a plate shaker for 5 min at room temperature. Afterwards, 50 µl of luciferin generation reagent was added to both plates before incubating at room temperature for another 30 min. Then 100 µl of luciferin detection reagent was added to both plates and luminescence was recorded after 15 min using a FLUOstar Omega plate reader (BMG LABTECH, UK). No-cell control reflects the assay background signal. Calculating the GSH/GSSG ratio was carried out using manufacturer's formula (see below).

$$\text{GSH/GSSG ratio} = \frac{(\text{Net total glutathione RLU} - \text{Net oxidized glutathione RLU})}{[\text{Net oxidized glutathione RLU/2}]}$$

2.2.6.1 NADP/NADPH- Glo™ Assay

NADP/NADPH-Glo™ Assay (Promega, UK) is a bioluminescent based assay for the detection of reduced and oxidized nicotinamide adenine dinucleotide phosphates ($NADP^+$ and NADPH) as well as determining their ratio in biological samples. Both NADP and NADPH play an important role in the maintenance of reduction and oxidation (redox) reaction (Miller, et al. 2018).

HTR-8/SV-neo cells were seeded at 2×10^4 cells/well in a 96- well black with clear bottom plate (Falcon®) for overnight before treating with flavonoids, their metabolites alone or in combinations for further 24 h prior to exposure to HR-induced oxidative stress. NADP/NADPH-Glo™ detection reagent was prepared according to the manufacturer's guidelines. Equal volumes of NADP/NADPH™ detection reagent was added to samples (1:1), followed by gentle and brief shaking. The plate was kept at room temperature for 45 mins. Luminescence was recorded using a FLUOstar Omega plate reader (BMG LABTECH, UK).

2.2.7 Measurement of Intracellular ROS generation

The effect of HR-induced oxidative stress, flavonoids, their metabolites alone or in combinations on intracellular hydrogen peroxide generation was measure using ROS-Glo™ H_2O_2 assay.

2.2.7.1 ROS-Glo™ H₂O₂ Assay

Generation of hydrogen peroxide (H_2O_2) was measured in HTR-8/SVneo cells treated with flavonoids, their metabolites alone or in combination prior to HR-induced oxidative stress using ROS-Glo™ assay (Promega, UK). Cells were cultured at a density of 2×10^4 cells/well in a 96-well with black with clear bottom plate (Falcon®) as previously described in section 2.2.6.1 ROS-Glo™ H_2O_2 detection solution was prepared according to the manufacturer's guidelines. The addition of H_2O_2 substrate solution in all experiments was carried out in the last 6 h of the experiment. ROS-Glo™ H_2O_2 detection substrate was added to all experimental wells at a 1:1 with culture medium and kept at room temperature for 20 min before luminescence was measured using a FLUOstar Omega plate reader (BMG LABTECH).

2.2.8 Caspase-Glo® 3/7 Assay

Caspase-Glo® 3/7 assay (Promega, UK) is a homogenous, luminescent assay that measures caspase-3 and -7 activities in cultured cells. HTR-8/SVneo cells were cultured at a density of 2×10^4 cells/well in a 96-well black with clear bottom plate (Falcon®) as previously described in section 2.2.6.1 Caspase-Glo® 3/7 reagent was prepared according to manufacturer's instruction and was added to experimental wells (1:1). Plate was gently mixed on a plate shaker for 30 secs and was kept at room temperature for 45 min. Luminescence was recorded using a FLUOstar Omega plate reader (BMG LABTECH). Blank reaction (Caspase-Glo® 3/7 and culture medium without cells) was used to measure background luminescence signal and was subtracted from all readings.

2.2.9 Immunoblotting

2.2.9.1 Cell lysate preparation for protein analysis

Cells were cultured in T75 to about 70% confluency before treating with flavonoids, their metabolites alone or in combinations prior to exposure to HR-induced oxidative stress. Cells were harvested using 1X trypsin as described in section 2.2.1. Cell pellets were directly lysed with Pierce® RIPA buffer (ThermoScientific, UK) supplemented with Roche cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, UK) and phosphate inhibitor cocktail (Roche PhosSTOP™, Sigma-Aldrich, UK) according to manufacturer's guidelines and allowed to incubate on ice for 1 h whilst maintaining agitation every 15 min. Afterwards, the samples were centrifuged at 27000 x g for 15 min at 4ºC (Harrier 15/80 refrigerated centrifuge, Sanyo). Following this, supernatants were carefully transferred to 1.5 ml Eppendorf™ tubes and then stored at -80ºC and pellets were discarded.

2.2.9.2 Determination of protein concentration by Bicinchoninic Acid (BCA) Assay

Estimating the protein concentration in all samples from section 2.2.9.1 was carried using BCA (Sigma-Aldrich, UK). BCA assay mixture was prepared according to manufacturer's guidelines by mixing 50 parts of reagent A (BCA) and 1 part of reagent of B (4% w/v Copper (II) sulphate solution). Bovine serum albumin (BSA) (Fisher Scientific, UK) in the concentration of 0.2, 0.4, 0.6, 0.8 and 1 mg/ml was used as protein standard to generate a standard curve (see figure 2.3). Cell lysates, BSA standards, and blank (RIPA buffer) were added to 96 well plate before adding the BCA mixture. The plate was incubated at 37⁰C for 30 min. Absorbance was read at 570 nm using a FLUOstar Omega plate reader (BMG LABTECH) and the protein concentrations were calculated based on the standard curve.
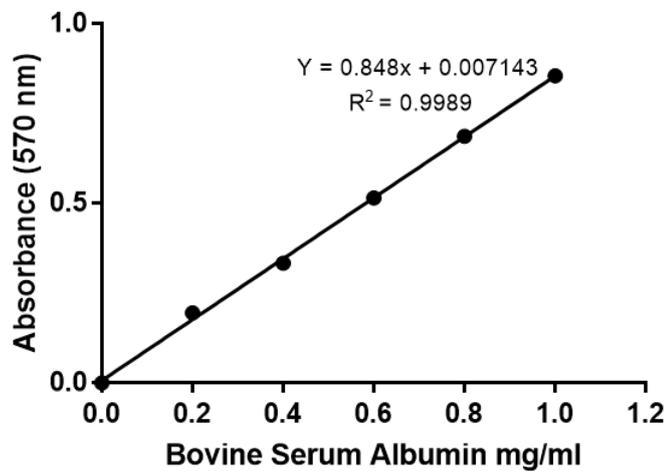
Figure 2.3: **Protein standard curved produced with BCA method and known BSA concentrations.**

2.2.9.3 Protein separation by SDS-PAGE

Since antibodies typically recognise a small portion of their target protein (epitope), denaturing (protein unfolding) the samples will enable the antibodies access to this portion. To denature the protein, 2X Laemmli buffer (4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol, 0.004 % bromphenol blue and 0.125 M Tris HCl, pH approx. 6.8) (Sigma-Aldrich, UK) was added to cell lysates and the mixture was boiled at 95⁰C for 5 min on a heating block. Gel preparation was performed using ProtoGel® 30% w/v acrylamide, ProtoGel® 4X resolving (Tris-base 18 % w/v and SDS 0.5 % w/v) and ProtoGel® stacking (Tris-base 6 % w/v and SDS 0.5 % w/v) buffers in accordance with manufacturer's guidelines (National Diagnostics, UK). Equal amount of denatured proteins (30 µg) were loaded into 12.5 % w/v SDS-PAGE gel along with molecular weight maker; prism ultra-protein ladder, 10-245 kDa (Abcam, UK).

Electrophoresis was performed at 50 volts for 15 min followed by 100 volts for 90 min in electrophoresis buffer. Coomassie Brilliant Blue (Bio-Rad Laboratories, Ltd, UK) was used to visualise proteins following SDS-PAGE to ensure proteins were electrophoresed uniformly and evenly (during voltage and duration optimization process).

2.2.9.4 Transfer, antibody incubation, imaging and data analysis

The separated proteins from the SDS-PAGE as described in section 2.2.9.3 were transferred onto 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories, Ltd, UK) by wet-transfer (see table 2.1 for transfer buffer composition) method overnight in a cold room. Afterwards, the nitrocellulose membrane was stained with ponceau red (Sigma-Aldrich, UK) to visualise successful transfer before incubating the membrane in a blocking solution (3% w/v BSA or 5% w/v non-fat milk in TBS-Tween 20) for 1 h at room temperature with gentle agitation (this process prevents non-specific background binding). After incubation with blocking solution, the nitrocellulose membrane was washed three times for five min with TBS-Tween20 before incubation with the indicated primary antibody (see dilution in table 2.2) overnight on a shaker at 4ºC. Following incubation, primary antibody was removed, and the membrane was washed with TBS-Tween20 three times for five min followed by further incubation with horseradish peroxidase (HRP) conjugated secondary antibody (see dilution in table 2.2) for 1 h at room temperature. After which, the membrane was washed with TBS-Tween20 three times for five min to remove excess secondary antibody.

Nitrocellulose membrane was developed using EZ-Chemiluminescence Detection Kit for HRP (Geneflow Ltd, UK). Imaging was performed using FujiFilm LAS 4000 and quantified using Li-Cor image studio™ lite (Li-Cor®, UK). GAPDH, β actin or β tubulin were used to check that all lanes in the gel have been loaded evenly (loading control) and were also used to normalise the target proteins during quantification.

2.2.10 Invasion assay

Invasion assay was used to assess the protective effect of flavonoids, their metabolites or in combinations in enabling HTR-8/SVneo cell invasion after HR-induced oxidative stress using Corning® BioCoat™ Tumour Invasion system. HTR-8/SVneo cells were treated with flavonoids, their metabolites alone or in combinations for 24 h prior to HR-induced oxidative stress. Cells were harvested as described in section 2.2.1 and the following protocol was carried out:

❖ **Rehydration:** Corning® BioCoat™ Tumour Invasion system was removed from -20°C and allowed to equilibrate at room temperature for 30 min. Then the apical chambers were filled with 75 µl of warm RPMI-1640 serum free medium and allowed to rehydrate for 2 h at 37°C in a 5% CO_2 environment. After 2 h of rehydration, 50 µl of the RPMI-1640 serum free medium was removed from all apical chamber without disturbing the layer of Matrigel matrix on the membrane. In contrast, the uncoated Corning® FluoroBlok™ 96 multiwell insert system (migration control) does not require rehydrating.

❖ **Pre-labelling of cells:** Harvested cells were incubated with 5 µM CellTrace™ CFSE dye (Molecular Probes®, UK) for 45 min at 37°C and shielded from light. After which, RPMI-1640 growth medium was added (5 times the volume of CellTrace™ CFSE dye) to the cells and allowed incubate for at room temperature for 5 min to eliminate excess dye. After the incubation, cells were centrifuged and re-suspended in fresh warm RPMI-1640 serum free medium.

❖ **Assay set-up:** The addition of 25 µl of cell suspension (1.25×10^4 cell density) in RPMI-1640 serum free medium to the apical chambers of the coated 96-multiwell plate (invasion plate) was carried out. Similarly, addition of 25 µl of cell suspension (1.25×10^4 cell density) in RPMI-1640 serum free medium to the apical chambers of the uncoated 96-multiwell plate (migration plate). The basal chamber for both invasion and migration plates were filled with 200 µl of RPMI-1640 growth medium containing 5% v/v FBS serving as chemoattractant. Then both plates were allowed to incubate at 37°C in CO_2 environment for 20-22 h (according to manufacturer's guidelines).

❖ **Imaging:** After 22 h incubation, membranes for both plates were carefully detached using tweezers and mounted on slides. Imaging of invaded/migrated cells were taken using EVOS FL microscope (ThermoFisher Scientific, UK).

❖ **Image analysis:** The number of cells invaded/migrated were analysed using WimCounting (Wimasis Image Analysis, Germany). Data were presented as either number of cells invaded or invasion percentage using manufacturer's formula (see below). See figure 2.4 for image summary of this assay.

$$Invasion\ \% = \frac{Number\ of\ cells\ invaded}{Number\ of\ cells\ migrated} \times 100$$
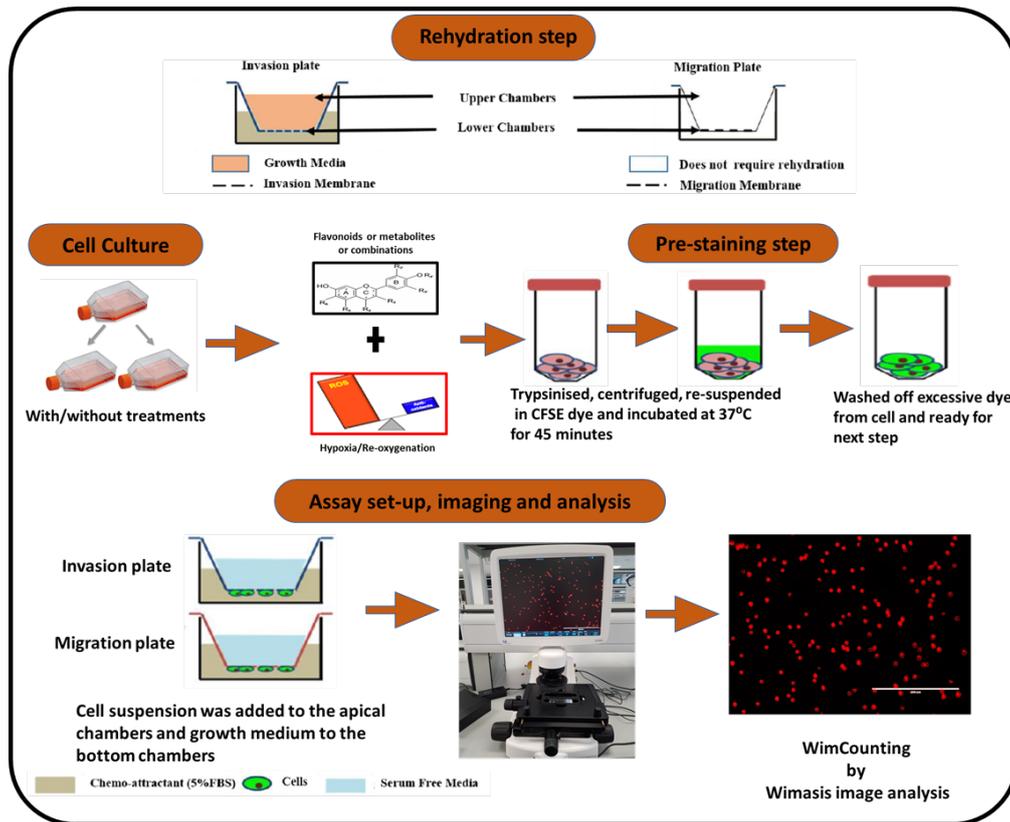
Figure 2.4: Summary of the invasion assay protocol.

2.2.11 Generation of spheroidal stem-like cells

Spheroidal stem-like cells were generated to assess the effect of HR-induced oxidative stress on formation of spheroids and the effects of 24 h pre-treatment of cells with flavonoids, their metabolites alone or in combinations prior to HR-induced oxidative stress on the generation of spheroid. The process for this assay are highlighted in stages below;

➢ **Stage 1 (Bulking up of HTR-8/SVneo cells)**

HTR-8/SVneo cells were grown in a T75 flask usually at a density of 5 x 10^6 cells/ml for up to 72 h. Once a confluency of 70-80% was achieved, cells were treated with or without the indicated flavonoids, their metabolites alone or in combinations for 24 h prior to exposure to HR-induced oxidative stress. Afterwards, cells were harvested and ready for stage 2.

➢ **Stage 2 (Spheroid formation)**

Harvested HTR-8/SVneo cells from stage 1 were sub-cultured in non-adherent ultra-low attachment T75 flask (Sigma-Aldrich, UK) in 20 ml spheroid formation medium that is made up with the following; RPMI-1640 serum free medium supplemented with 1% w/v L-glutamine, 1% v/v penicillin/streptomycin, 5 µg/ml bFGF (Sigma-Aldrich, UK), 5 µg/ml EGF (Sigma-Aldrich, UK), 5 µg/ml insulin (Sigma-Aldrich, UK) and 0.4% (w/v) BSA

(Sigma-Aldrich, UK). Cells were incubated at 37ºC in 5% CO_2 and 95% air for 3-5 days monitoring the formation of spheroid under the microscope every 24 h.

➢ **Stage 3 (Cell sorting by gravity separation)**

Single cells were separated from spheroids by gravity separation. Cell mixtures consisting of both single and spheroid cells were transferred into Sterlin® tubes (Sarstedt, UK) and left at room temperature for 10 min allowing the separation of spheroids from single cells. This technique allows the spheroid cells to settle at the bottom of the tube while the single cells keep floating in the upper layer with the medium. Then the medium is discarded without disturbing the pellet of spheroids. Afterwards, the spheroids were centrifuged at 1000 x g for 5 min.

2.2.12 Soft-Agar Colony Formation Assay

To quantify the effects of flavonoids, their metabolites alone or in combinations on the spheroid growth area, soft-agar colony formation assay was carried out. This assay is semi-quantitative and measures the morphological transformation of *in vitro* cellular colonies. HTR-8/SVneo cells were cultured in T75 flask to about 80% confluency before being treated with or without flavonoids, their metabolites alone or in combinations prior to HR-induced oxidative stress (as described previously). Cells were harvested and counted before adjusting the cell density to 2.5 x 10^3 cell/well in a 6 well plate (Sarstedt, UK). Preparations of soft-agar are as follows:

❖ **Preparation of Base Aga**r: Difco Agar noble (France) at 1% (w/v) was dissolved in Millipore water by microwave heating, then allowed to cool to 40ºC in water bath. RPMI-1640 2X was prepared by adjusting the growth medium from 10% FBS (as previously described in section 2.2.1) to 20% FBS and was also kept in the water bath (40ºC). After 45 min of equilibration of both base agar and 2X RPMI-1640 culture medium, equal volume (1:1) was mixed in a fresh Sterlin® 50 ml tube and 1 ml was transferred into a 6-well plate to form the base layer, carefully shaking the plate to ensure even distribution of the base agar. Plate was keep at room temperature for 5 min to allow solidification for the base agar.

❖ **Preparation of Top Agarose**: Agarose (Sigma-Aldrich, UK) at 0.7% (w/v) was melted in Millipore water using microwave and allowed to cool in a 37ºC water bath. Cells concentration as described earlier in this section was made up in 2X RPMI medium. Equal volume of cell suspension (2.5 x 10^3 cell/well) and agarose (1:1) was mixed in a fresh Sterlin® 20ml tube and 1 ml was transferred in the same 6-well plate containing

the base layer. The plate was kept at humidified 37ºC in CO_2 environment for up to 20 days. Cells were fed twice a week with RMPI-1640 culture medium and the spheroid colony formation was monitored every 5 days using a microscope.

2.2.13 Cultrex® 96 well 3D spheroid Base Membrane Extract (BME) cell invasion assay

This assay offers a standardized, three-dimensional cell structure for quantitating the degree to which invasive cells penetrate a barrier consisting of components resembling basement membrane in response to chemoattractant. The procedure for this experiment was preformed according to manufacturer's guidelines, see below for highlights;

➢ **Step 1/Day 0:** HTR-8/SVneo cells were cultured in T75 flask to about 80% confluency before being treated with or without flavonoids, their metabolites alone prior to HR-induced oxidative stress (as described previously). Cells were harvested and counted before adjusting the cell density to 3×10^3 cell/well (single cell suspension). The single cell suspension (50 µl) was prepared in 1X formation extracellular matrix per well and then transferred to the 3D 96-well plate. The plate was then centrifuged at 200 x g for 5 min at room temperature and incubated in a humidified 37ºC, 5% CO_2 and 95% air environment for 72 h to promote the formation of spheroids.

➢ **Step 2/Day 3:** After 72 h, the 3D plate was taken out from the incubator and placed on ice for 5 min to cool. Working on ice, invasion matrix (50 µl) was added to all wells and the plate was centrifuged at 300 x g at 4ºC for 5 min to eliminate bubbles and position spheroids towards the middle of the well. The 3D 96-well plate was transferred to the incubator for 1 h to promote gel formation. After which, 100 µl of warm growth medium was added to each well and images were taken using a confocal microscope before returning the plate to the incubator for additional 72 h.

➢ **Step 3/Day 6:** After 72 h, plate was taken out and images of spheroids were taken using a confocal microscope and analysed using Image-J software. Image data were analysed using GraphPad prism software.

2.2.15 Statistical analysis

Statistical analysis was performed using GraphPad prism 7.0 software. One-way ANOVA was used to compare three or more sets of data applying with Turkey's/Dunnett's multiple comparisons to further demonstrate the significant difference. Data are presented as mean $\pm$ SEM (standard error of mean) and statistical significance was deemed to be represented as $P < 0.05$.

Chapter 3
Optimisation of non-toxic concentrations of flavonoids and trophoblast oxidative stress model

Chapter 3: Optimisation of non-toxic concentrations of flavonoids

3.1 Introduction

As previously described in chapter 1, oxidative stress can be defined as the imbalance in the generation and accumulation of reactive oxygen species (ROS) in cells/tissues, and the ability of antioxidants to detoxify them. This can occur as the result of increased production of ROS and/or decrease in effectiveness of antioxidant defence system (Myatt and Cui, 2004; Pizzino *et al.*, 2017). Considerable evidence has linked oxidative stress to the pathophysiology of several diseases including cardiovascular diseases, neurological diseases, cancers, respiratory disease, rheumatoid arthritis as well as pregnancy related disorders (Lázár, 2012; Pizzino *et al.*, 2017). In fact, pregnancy can be considered as a state of oxidative stress due to the increased placental mitochondrial activity and production of ROS as a low/moderate level of oxidative stress is essential for the normal development of foetal growth (Khera *et al.*, 2013). Over-production of ROS during early gestation period has been associated with shallow trophoblast/placental invasion that leads to pregnancy complications such as intrauterine growth restriction (IUGR), prematurity, placenta percreta, placenta accreta, foetal/maternal death, miscarriage and preeclampsia (PE) (Khera *et al.*, 2013; Jauniaux *et al.*, 2007). PE is a pregnancy-specific condition characterised by hypertension ($\geq$160/100 mmHg) and proteinuria (>300 mg/day) usually after 20 weeks of gestation (early onset) and occasionally after 34 weeks (late onset). It is the world leading cause of maternal and foetal morbidity and mortality and complicates about 5 – 8% of all pregnancies (Bolnick *et al.*, 2015). Additional consequences of PE include retardation of the foetal growth leading to seizure and coma (Cross *et al.*, 2015; Wu *et al.*, 2015; Petla *et al.*, 2013). Incidentally, it was first reported in the nineteenth century and its prevention has been a major issue for clinicians and researchers as the cause remains unknown (Fayyad and Harrington, 2005). Also, the only cure is to deliver the placenta and the foetus. In recent years, several antioxidant therapies such as vitamin C and E, polyphenols, aspirin, calcium, magnesium and zinc supplements, have been used early in gestation period to reduced oxidative stress and they have all proved to be controversial and mostly ineffective (Carty *et al.*, 2008; Fayyad and Harrington, 2005).

However, very little is known about the effects of dietary flavonoids as antioxidants during gestation. Flavonoids are a large group of polyphenolic compounds and phytochemicals that are present in fruit and vegetable rich diets (e.g. apples, red grapes, oranges and grapefruit juice, soybeans, green leafy spices). In the last two decades, extensive body of evidences from *in vitro* research have revealed that flavonoids may be beneficial to health *in vivo*, due to their antioxidant properties (Day *et al.*, 2000; R.J. Williams *et al.*, 2004; Procházková *et*

al., 2011). However, the exact mechanism by which flavonoids carry out their beneficial effects remains a mystery. Their biological effects are attributed to be highly dependent upon their structural configurations, especially the availability of their hydroxyl group (Day *et al.*, 2000). As mentioned in chapter 1, the presence or absence of a hydroxyl functional group plays a crucial role in the classification of flavonoids (Erlund, 2004; Bentz, 2009). Interestingly, flavonoids can act as pro-oxidants under circumstances such as high concentrations, presence of O_2 molecules, alkali pH and the presence of too many hydroxyl groups in the B-ring (Procházková *et al.*, 2011; Eghbaliferiz and Iranshahi, 2016). Therefore, optimising the non-toxic concentrations was important for this study and selection of flavonoids belonging to different classes and subclasses.

Table 3.1: **Summaries of flavonoids/metabolites used in this study and their sources**

Flavonoids	Metabolites	Sources
Quercetin	Quercetin 3-O-β-glucuronide (Q3G)	Apples, Onions, Tea, and Red wines
Morin	Q3G (No commercial available metabolites)	Guavas, Osage oranges, Old fustics, Coffee, Seaweeds, Onions, and Apples.
Naringin	Naringenin	Citrus fruits, Chinese herbs, and Tomatoes
Hesperidin	Hesperetin	Citrus fruits, and Tomatoes

Ideas adapted from (Gonzalez *et al.*, 2015; Panche *et al.*, 2016; Erlund *et al.*, 2001).

3.2   Aims

➤ Optimisations of non-toxic concentrations of

    I.   Flavonoids

    II.   Their metabolites alone

    III.   Flavonoid-metabolite combinations

    IV.   Flavonoid combinations

➤ Effects of different concentrations of O_2 on HTR-8/SVneo cell viability

    I.   Effects of 2% O_2

    II.   Effects of 0.5% O_2

    III.   Effects of hypoxia/reoxygenation

3.3   Results

Four different flavonoids (quercetin, morin, naringin and hesperidin) were selected for this study based on the on the classification and sub-classification of flavonoids. Metabolites (Q3G, naringenin and hesperetin) for flavonoids were chosen based on availability except for morin which is not commercially available. In this section, the effects of different concentrations of flavonoids (1, 3, 10, 20 and 40 µM) or their metabolite alone or in combinations was assessed.

3.3.1   Optimization of non-toxic concentration of flavonoids

MTT and CellTox™ green cytotoxicity assays were used to assess the non-toxic concentrations for flavonoids and their metabolites after 24 and 48 h treatment on cell viability in HTR-8/SVneo cells. As shown in figure 3.1 (A-D), MTT assay showed that 24 h treatment of HTR-8/SVneo cells with 1 and 3 µM concentrations of quercetin, morin, naringin and hesperidin were not toxic. However, unlike quercetin and naringin, morin and hesperidin did not show any indication of toxicity at 10 µM after 24 h treatment. However, concentrations of 20 and 40 µM of all flavonoids triggered significant cell death on HTR-8/SVneo cells after 24 h. Further validation was carried using CellTox™ green cytotoxicity assay to measure the changes in membrane integrity as a result of cell death (figure 3.1, E-H). Data from CellTox™ green cytotoxicity assay was in agreement with MTT assays indicating that concentration 10, 20 and 40 µM of flavonoids were indeed toxic to HTR-8/SVneo cells after 24 h treatment.
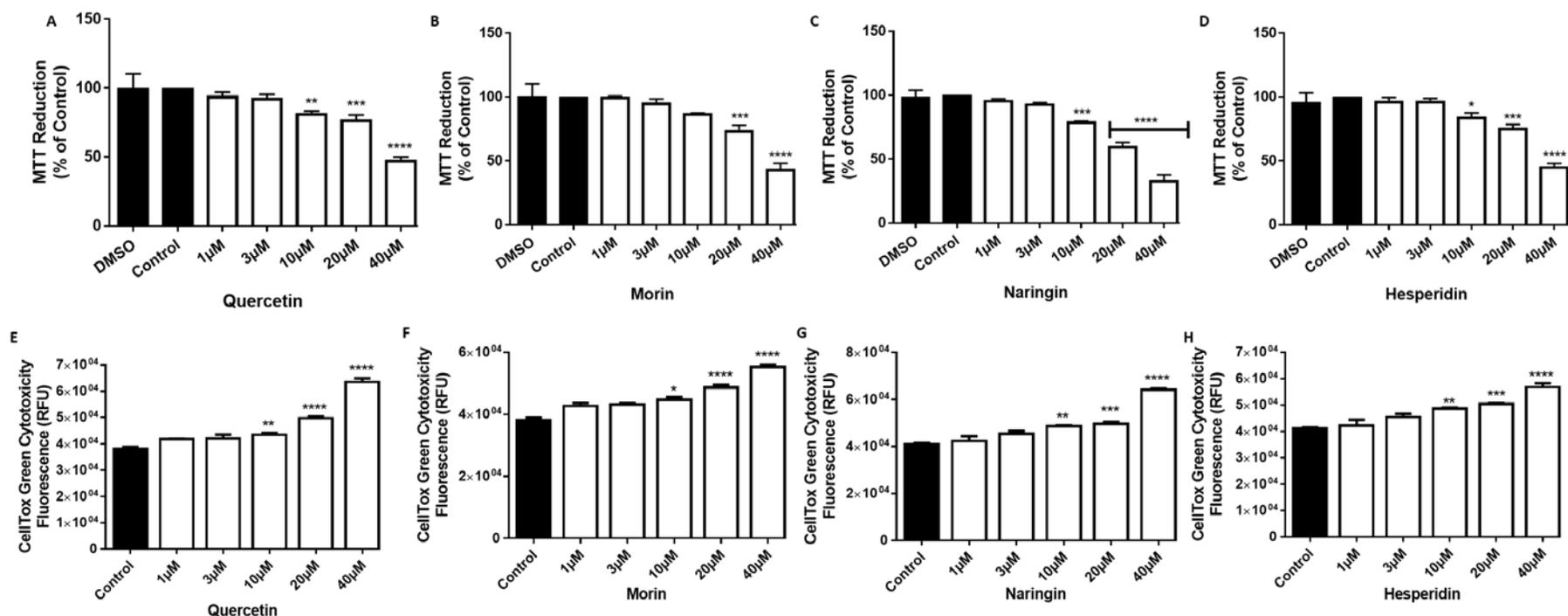
Figure 3.1: **Effects of 24 h flavonoids treatment on HTR-8/SVneo cell viability.**

HTR-8/SVneo cells were treated with the indicated concentrations (1-40 µM) of quercetin, morin, naringin and hesperidin for 24 h. Following flavonoid treatment, cell viability was assessed by MTT reduction assay (A-D) and membrane integrity (cell death) via CellTox™ green cytotoxicity assay (E-H). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against control). Data are expressed as the percentage of control cells and represent the mean ± SEM of nine (A-D) and three (E-H) independent experiments performed in quadruplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$ versus control.

Further experiment was carried out using MTT assay alone to determine the effects of the indicated flavonoids and concentrations on HTR-8/SVneo after 48 h treatment. The MTT data as shown in figure 3.2 (A-D) showed that again 1 and 3 µM of all flavonoids were not toxic to HTR-8/SVneo cells. Again, concentrations of 10, 20 and 40 µM of all flavonoids showed more toxicity after 48 h treatment.
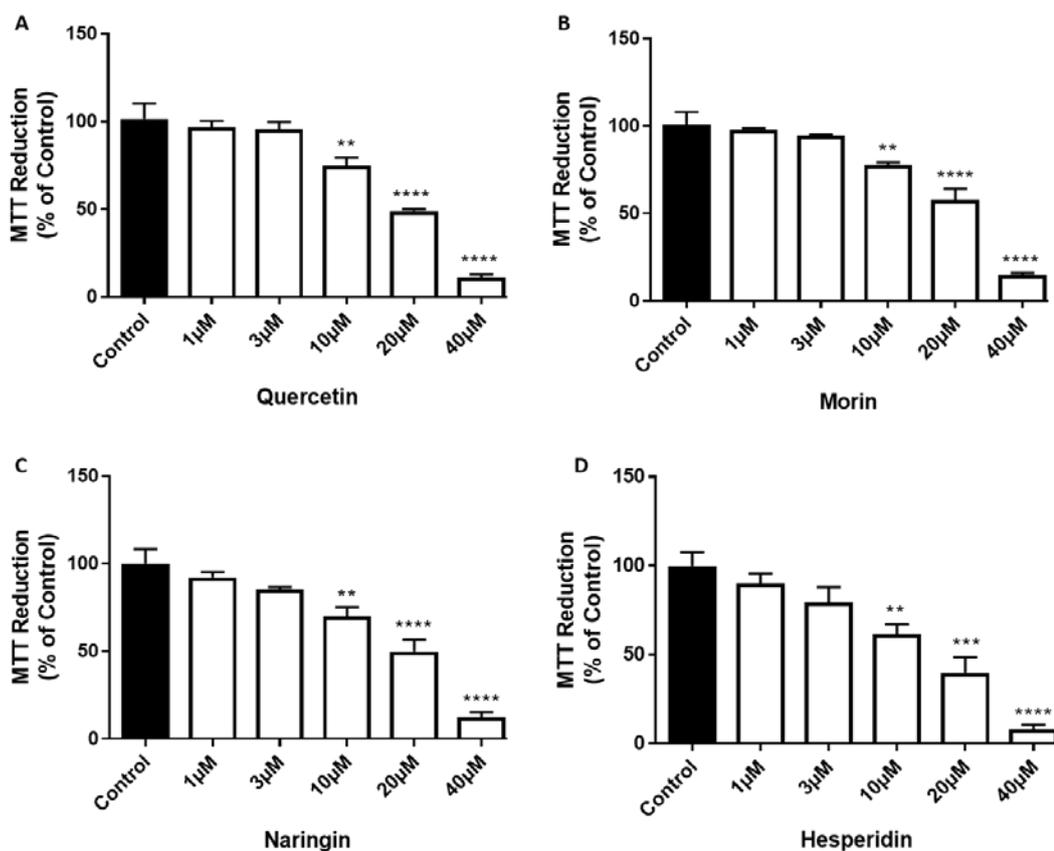
Figure 3.2: **Effects of 48 h flavonoids treatment on HTR-8/SVneo cell**.
HTR-8/SVneo cells were treated with the indicated concentrations (1-40 µM) of quercetin, morin, naringin and hesperidin for 48 h. Following flavonoid exposure, cell viability was measured by MTT reduction assay (A-D). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against control). Data are expressed as the percentage of control cells and represent the mean ± SEM of six independent experiments performed in quadruplicate. **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$ versus control.

3.3.2 Optimization of non-toxic concentration of metabolites

After optimising the non-toxic concentration for quercetin, morin, naringin and hesperidin for both 24 h and 48 h, it was essential to determine the working concentration for their metabolites (Q3G, naringenin and hesperetin) for both 24 h and 48 h treatment as the form in which they exist in plant and vegetable differs from their in vivo form. Flavonoids undergo extensive biotransformation in the liver and small intestine to produce their metabolite. Therefore, cell viability following 24 h and 48 h treatment of HTR-8/SVneo cells with Q3G, naringenin and hesperetin was determined using MTT and CellTox™ green cytotoxicity assay.

Herein, 1 and 3 µM of these metabolites did not trigger any cell death after 24 h as seen in figure 3.3 (A-C) MTT assay and figure 3.3 (D-F) CellTox™ green. HTR-8/SVneo cells tolerated 10 µM of naringenin (figure 3.3B), although CellTox™ green assay showed traces of cell death (figure 3.3E). As seen figure 3.3 (A-C and D-F) both MTT and CellTox™ assays agreed that concentrations 10 µM and above were toxic after 24 h treatment.
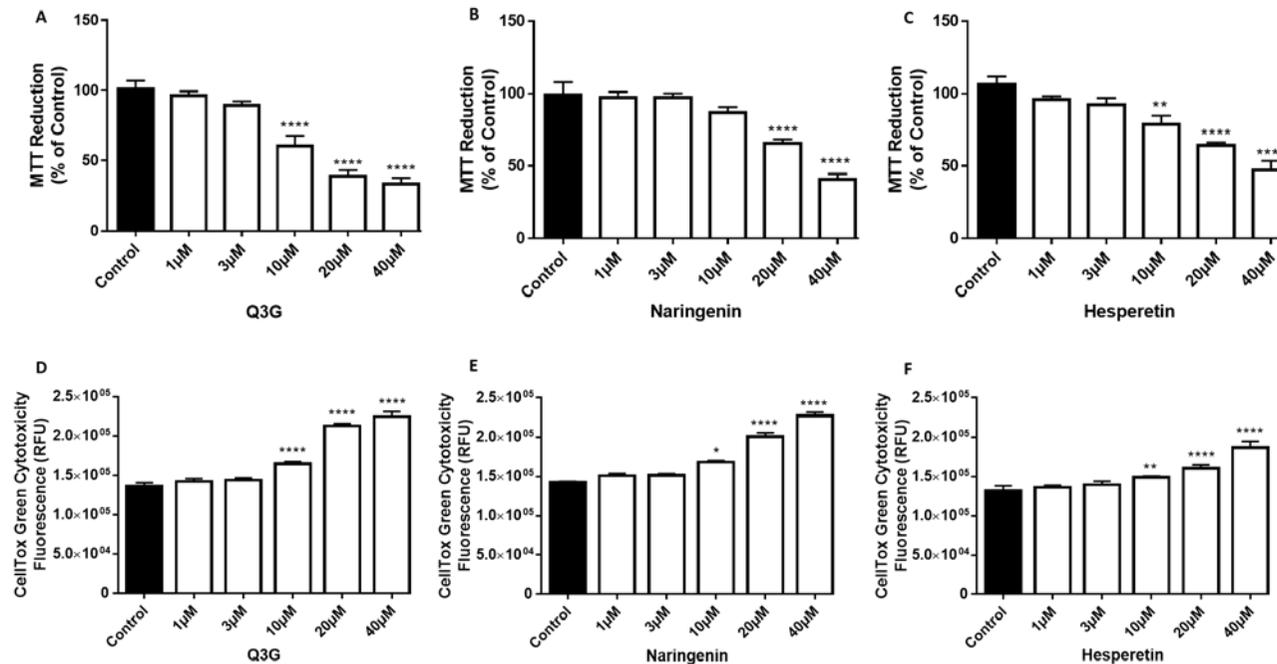
Figure 3.3: **Effects of 24 h metabolites treatment on HTR-8/SVneo cell viability.**

HTR-8/SVneo cells were treated with the indicated concentrations (1-40 µM) of Q3G, naringenin and hesperetin for 24 h. Following flavonoid treatment, cell viability was assessed by MTT reduction assay (A-C) and membrane integrity (cell death) via CellTox™ green cytotoxicity assay (D-F). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against control). Data are expressed as the percentage of control cells and represent the mean ± SEM of nine (A-C) and three (D-F) independent experiments performed in quadruplicate. **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$ versus control.

Determination of prolonged exposure (48 h) of HTR-8/SVneo to the indicated concentrations of Q3G, naringenin and hesperetin was carried out using MTT assay alone. Unsurprisingly, similar results as that of 24 h exposure was seen following 48 h exposure (figure 3.4, A-C) 1 and 3 µM of all metabolites was tolerated by HTR-8/SVneo. In contrast, concentrations of 10, 20 and 40 µM induced toxic effects as seen by the MTT reduction.
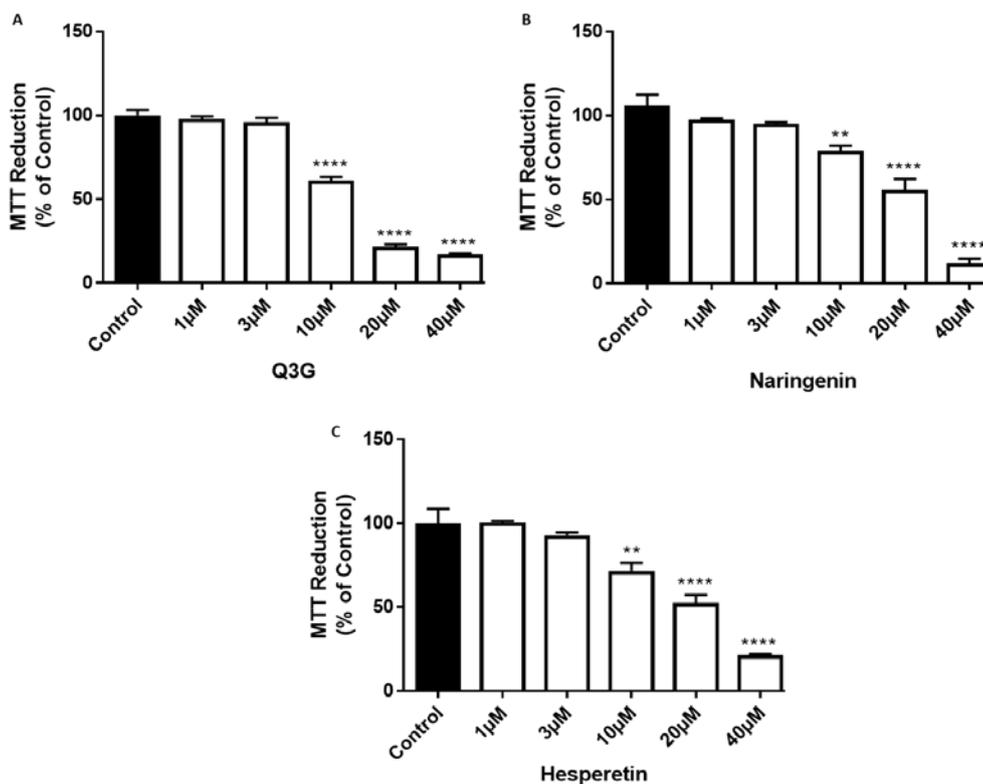
Figure 3.4: Effects of 48 h metabolites treatment on HTR-8/SVneo cell viability.
HTR-8/SVneo cells were treated with the indicated concentrations (1-40 µM) of Q3G, naringenin and hesperetin for 48 h. Following flavonoid treatment, cell viability was assessed by MTT reduction assay (A-C). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against control). Data are expressed as the percentage of control cells and represent the mean ± SEM of six independent experiments performed in quadruplicate. **P<0.01, ***P<0.001 and ****P<0.0001 versus control.

3.3.4 Determination of working concentration for flavonoid-metabolite combinations.

Flavonoids are proposed to exist in glyosidic or aglycone form (parent compound), upon metabolism they exist as glucuronide and sulphate conjugates (metabolites) in the bloodstream. Most researches on flavonoids focus on studying either their aglycone form or glucuronide/sulphate form but not together. Therefore, it was important to assess the effect

of the combinations of flavonoid-metabolites to mimic the *in vivo* environment. Therefore, flavonoids at 3 µM was combined with their metabolites at 1 µM (quercetin/Q3G; morin/Q3G; naringin/naringenin and hesperidin/hesperetin). Cells viability on 24 h treatment of flavonoid-metabolite combinations on HTR-8/SVneo cells was assessed using MTT and CellTiter-Glo® luminescent cell viability assays. As shown in fig 3.5 (A), the MTT assay data showed no indication of toxicity and this data was further validated using CellTiter-Glo® viability assay, which monitors intracellular ATP levels (figure 3.5, B).
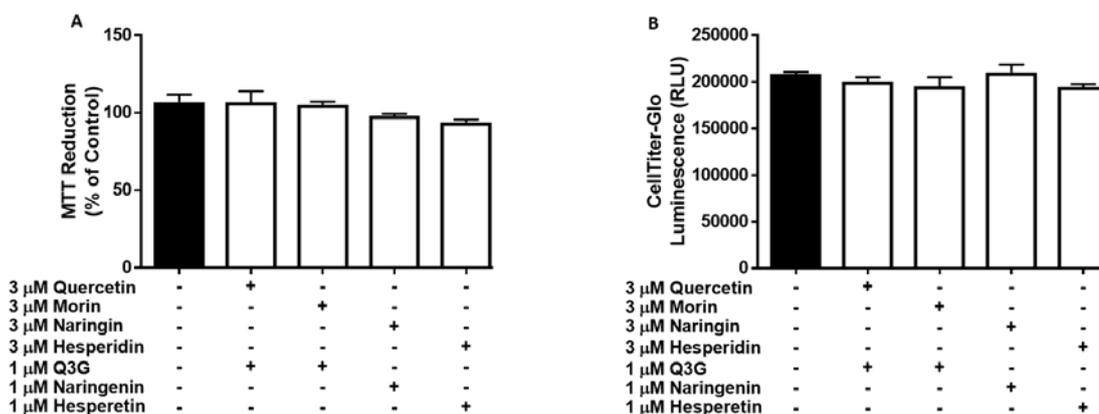
Figure 3.5: Effects of 24 h treatment with flavonoid-metabolite combinations on HTR-8/SVneo cell viability.

HTR-8/SVneo cells were treated with the indicated flavonoids (3 µM) and their respective metabolites (1 µM) for 24 h. Following the combinations of flavonoid-metabolite treatment, cell viability was measured by MTT reduction assay (A) and Cell proliferation via CellTiter-Glo® Luminescent assay (B). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against control). Data are expressed as the percentage of control cells and represent the mean ± SEM of six (A) and three (B) independent experiments performed in quadruplicate.

The effect of prolonged (48 h) exposure of flavonoid-metabolite was carried out to check for toxicity using MTT and CellTiter-Glo® luminescent cell viability assays. Again, the data from figure 3.6 (A and B) showed that there was no sign of toxicity.
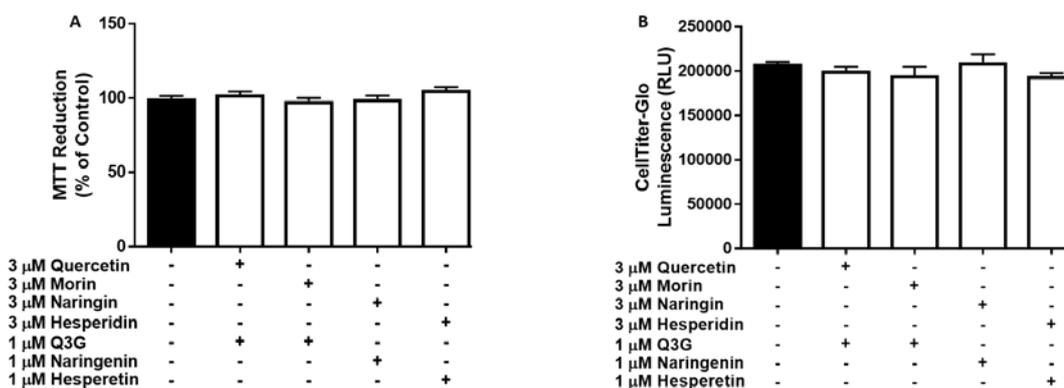
Figure 3.6: Effects of 48 h treatment with flavonoid-metabolite combinations on HTR-8/SVneo cell viability.

HTR-8/SVneo cells were treated with the indicated flavonoids (3 µM) and their respective metabolites (1 µM) for 48 h. Following the combinations of flavonoid-metabolite treatment, cell viability was assessed by MTT reduction assay (A) and Cell proliferation via CellTiter-Glo® Luminescent assay (B). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against control). Data are expressed as the percentage of control cells and represent the mean ± SEM of six (A) and three (B) independent experiments performed in quadruplicate.

3.3.5 Determination of working concentration for flavonoids combination

Another important aspect of this project was to examine the effect of combining two different flavonoids. This is because flavonoids in fruits and other natural sources are mostly distributed as combined forms. Since quercetin is the most abundant flavonoid in fruits (and the fact that it is the most studied), it was used as a reference for other flavonoids. Combination of flavonoids (quercetin + morin; quercetin + naringin and quercetin + hesperdin) at 3 µM was carried to check for synergistic, addictive or antagonistic effects. The effects of flavonoids combination on HTR-8/SVneo cells after 24 and 48 h was examined using MTT and CellTiter-Glo® luminescent cell viability assays. The data shown in figure 3.7 (A) showed that there was no cytotoxic effect after 24 h treatment. MTT assay data was validated using CellTiter-Glo® luminescent cell viability assay (figure 3.7, B), which also showed that there were no cytotoxic effects. Furthermore, the effect of flavonoids combinations on HTR-8/SVneo cells after 48 h showed that there was no indication of toxicity as shown in figure 3.8A (MTT assay) and figure 3.8B (CellTiter-Glo® assay).
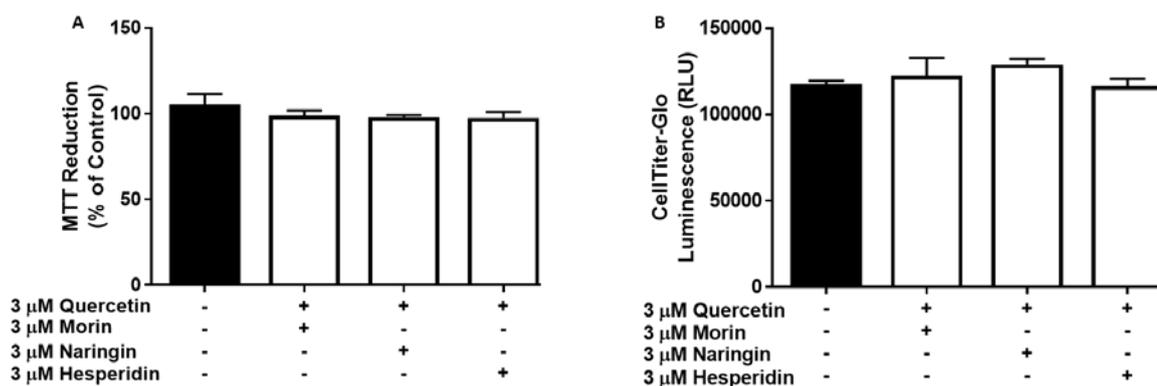
Figure 3.7: Effects of 24 h treatment with flavonoids combination on HTR-8/SVneo cell viability.

HTR-8/SVneo cells were treated with the indicated flavonoids at 3 µM (individually) for 24 h. The combinations are indicated as + for presence (and – for absence). Following the combination of flavonoids treatment, cell viability was measured by MTT reduction assay (A) and Cell proliferation via CellTiter-Glo® Luminescent assay (B). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against control). Data are expressed as the percentage of control cells and represent the mean ± SEM of six (A) and three (B) independent experiments performed in quadruplicate.
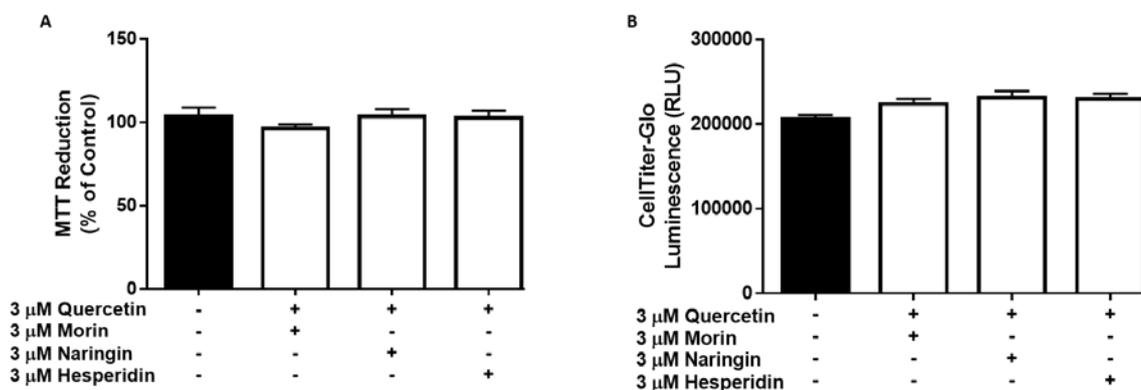
Figure 3.8: Effects of 48 h treatment with flavonoid-metabolite combinations on HTR-8/SVneo cell viability.

HTR-8/SVneo cells were treated with the indicated flavonoids at 3 µM (individually) for 48 h. The combinations are indicated as + for presence (and – for absence). Following the combination of flavonoids treatment, cell viability was assessed by MTT reduction assay (A) and Cell proliferation via CellTiter-Glo® Luminescent assay (B). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against control). Data are expressed as the percentage of control cells and represent the mean ± SEM of six (A) and three (B) independent experiments performed in quadruplicate.

3.3.6 Establishing an oxidative stress model

To induce oxidative stress, cells were exposed to either 2 or 0.5% oxygen (O_2) in glucose and serum free medium for up to 24 h. The effect of reduced oxygen (hypoxia) was assessed using the MTT assay. Firstly, to determine the optimal duration of hypoxia to cause cell death or induce oxidative stress, HTR-8/SVneo cells were exposed to 2% O_2 for up to 24 h. The data from the MTT assay showed that HTR-8/SVneo cells were proliferating under hypoxic, glucose free and serum free condition for up to 24 h (see figure 3.9, A). Since 2% O_2 was ineffective in inducing oxidative stress as shown by MTT assay, reducing the O_2 concentration to 0.5% (under glucose- and serum- free medium condition) was considered and again cell viability was measured using MTT assay. As shown in figure 3.9 (B) 0.5% O_2 did not induced oxidative stress or cause cell death in HTR-8/SVneo cells. Therefore, hypoxia alone (2 or 0.5% O_2) coupled with glucose- and serum- free medium was not sufficient to cause cell death or induced oxidative stress in the human first trimester trophoblast cell line HTR-8/SVneo.
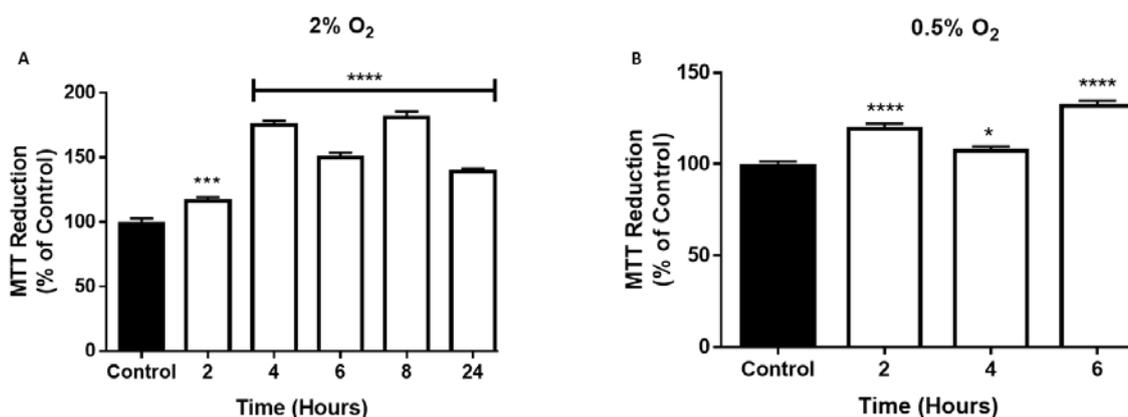
Figure 3.9: The effects of hypoxia alone on survival of HTR-8/SVneo cells.
HTR-8/SVneo cells were exposed to different concentrationss of oxygen (O_2) for up to 24 h in glucose and serum free medium. HTR-8/SVneo cell viability was assessed using MTT reduction assay. (A) the effect of 2% O_2 on HTR-8/SVneo survival and (B) the effects of 0.5% O_2 on HTR-8/SVneo survival. Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against control). Data are expressed as the percentage of normoxic control cells and data represent the mean ± SEM of at least six independent experiments performed in quadruplicate. *$P<0.05$, ***$P<0.001$ and ****$P<0.0001$.

Oxidative stress model was achieved by the exposure of HTR-8/SVneo cells to 2 h hypoxia (0.5% O_2) followed by 6 h re-oxygenation in a humidified incubator (37ºC, 5% CO_2, 95% air). Both hypoxic and re-oxygenation conditions were carried out using glucose- and serum-

free medium. Cell viability was determined using MTT and CellTox™ green cytotoxicity assays.

As seen in figure 3.10 (A), the MTT assay showed that upon re-oxygenation (4 and 6 h), there was in a significant reduction in cell viability (figure 3.10A). However, re-oxygenation after 2 h did not show significant reduction from the normoxic control but when compared to hypoxia alone, there was a significant reduction (figure 3.10, A). The data from CellTox™ green confirmed that cell death started from 2 h of re-oxygenation (figure 3.10, B). This assay also confirmed that hypoxia alone did not induced cell death as seen figure 3.10 (B or D). Summary of MTT and CellTox™ green assays can be found in figure 3.10 (C and D respectively). Therefore, oxidative stress model will be represented as hypoxia/re-oxygenation (HR)-induced oxidative stress for the rest of the research.
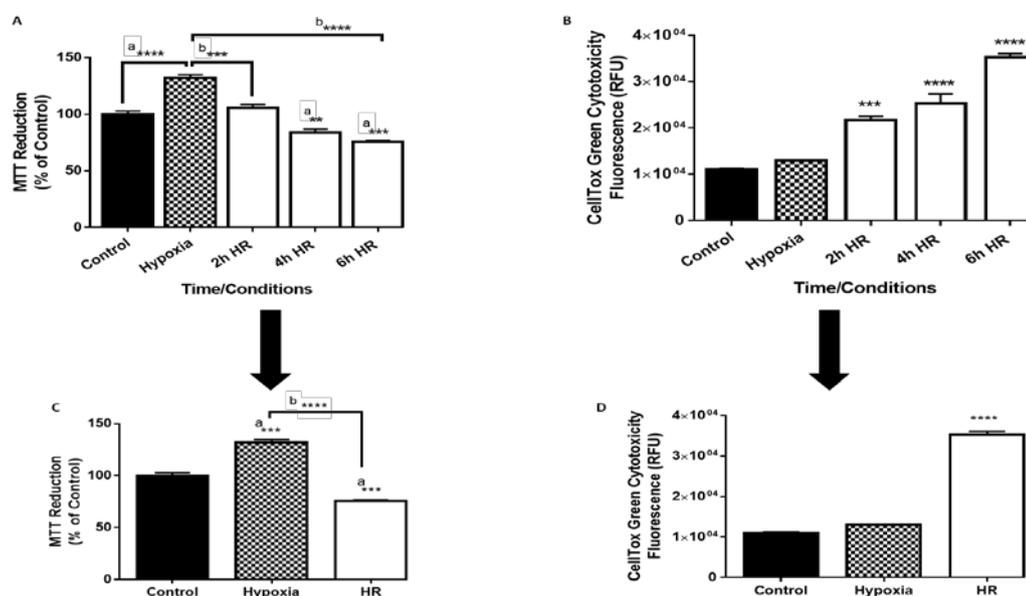
Figure 3.10: The effects of hypoxia alone or hypoxia/re-oxygenation on HTR-8/SVneo cell survival.

HTR-8/SVneo cells were exposed to hypoxia (0.5% O_2) for 2 h followed by re-oxygenation for up to 6 h in glucose- and serum- free medium. HTR-8/SVneo cell viability was assessed by MTT reduction assay (A and C) and membrane integrity (cell death) via celltox™ green cytotoxicity assay (B and D). Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as the percentage of normoxic control cells and data represent the mean ± SEM of six (A, C) and three (B,D) independent experiments performed in quadruplicate .**$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus normoxia control and (b) versus hypoxia alone.

Although 2 h hypoxia followed by 6 h re-oxygenation was an established model for oxidative stress, further experiment was carried out to assess the effect of 4 h hypoxia followed by up to 6 h re-oxygenation. Cell viability assay was measured using MTT assay.

Data shown in figure 3.11 indicate that 4 h hypoxia followed by up to 6 h re-oxygenation did not cause any significant cell death to HTR-8/SVneo cells. Therefore 4 h hypoxia and 6 h re-oxygenation was not suitable for this study.
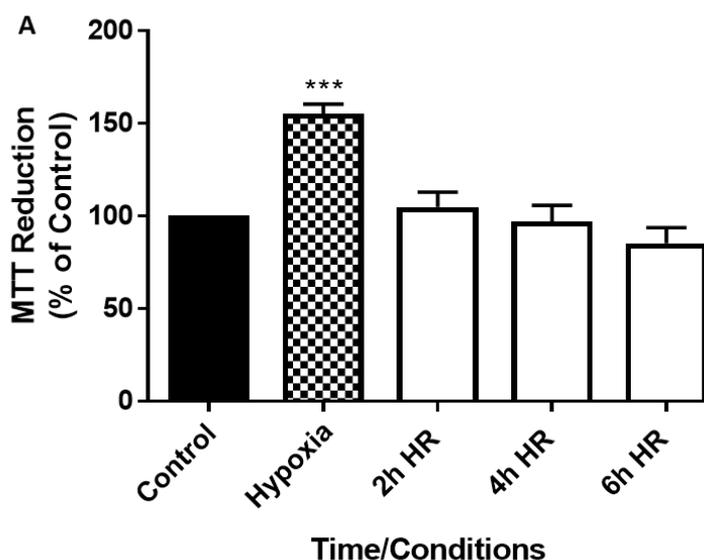
Figure 3.11: The effects of 4 h hypoxia followed by up to 6 h reoxygenation on survival of HTR-8/SVneo cells.
HTR-8/SVneo cells were exposed to hypoxia (0.5% O_2) for 4 h followed by re-oxygenation for 6 h in glucose and serum free medium. HTR-8/SVneo cell viability was assessed by MTT reduction assay (A). Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as the percentage of normoxic control cells and data represent the mean ± SEM of six independent experiments performed in quadruplicate. ***$P<0.001$ versus normoxia control.

3.3.7 Morphological alteration of HTR-8/SVneo during hypoxia/re-oxygenation

Light microscopy was used to track morphology alteration in HTR-8/SVneo cell during HR-induced oxidative stress. As observed in figure 3.12 (A-C), there were no changes in cell morphology between normoxic control and hypoxia alone (2 h). Comparing normoxic control (A) or hypoxia alone (B) to hypoxia/re-oxygenation (C), there were changes in cell morphology. Hypoxia/re-oxygenation cells had so many cells with altered morphological structure, clusters of cells and confluency was reduced.
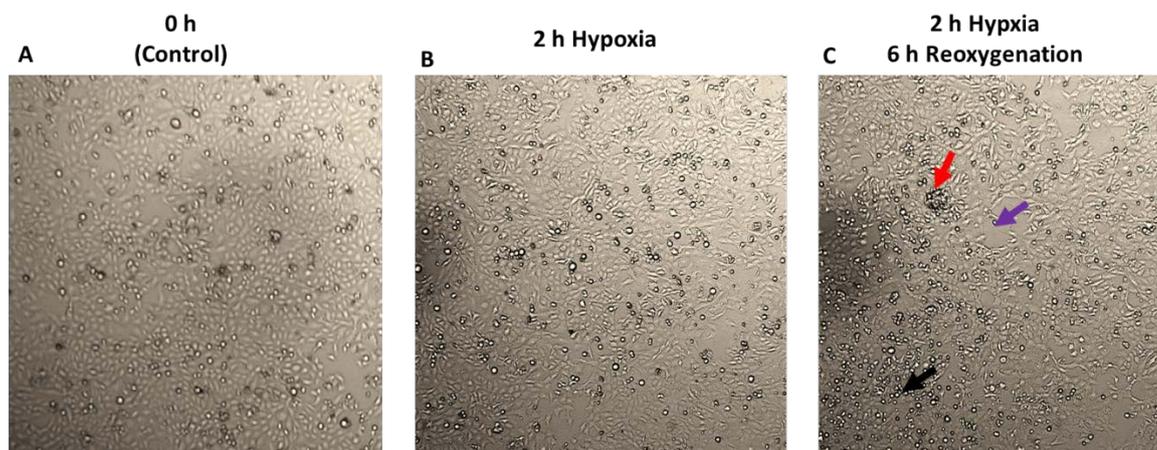
Figure 3.12: Morphological alteration of HTR-8/SVneo cells in response hypoxia/re-oxygenation.

HTR-8/SVneo cells were exposed to hypoxia (0.5% O_2) for 2 h followed by 6 h re-oxygenation in glucose and serum free medium. Images were taking using Motic AE2000 inverted microscope and GT vision GXCAM-5 camera with objective magnification 10X (Scale bar = 100 µm). (A) shows the morphology of HTR-8/SVneo cells before exposure to hypoxia or hypoxia/re-oxygenation, (B) shows the morphology of HTR-8/SVneo cells after 2 h of hypoxia alone and (C) shows the morphology of HTR-8/SVneo cells after 2 h hypoxia followed by 6 h re-oxygenation. Red bold arrow indicates the formation of cell clusters, which was only seen in hypoxia/re-oxygenated cells and occasionally in cell exposed to hypoxia. Purple bold arrow shows that there are more spacing between cells and black bold arrow shows the roundish single cells and elongated structures.

3.4   Result Summary

It was demonstrated that flavonoids or their metabolites can have cytotoxic effects when cells are exposed to higher concentrations (10, 20 and 40 µM) at 24 h and 48 h. However, at low concentrations (1 or 3 µM), flavonoids, metabolites or their combinations used herein, did not trigger cell death after 24 h or 48 h treatment. Hypoxia at 2 or 0.5% O_2 was not adequate to induce oxidative stress or cause cell death. Hypoxia (2 h) followed by re-oxygenation for up to 6 h induced significant cell death. In contrast, hypoxia (4 h) followed by re-oxygenation for up to 6 h did not cause significant cell death. Light microscopy confirmed changes in HTR-8/SVneo cells during 2 h hypoxia followed by 6 h re-oxygenation.

Chapter 3: Optimisation of non-toxic concentrations of flavonoids

3.5 Discussion

The aim of this chapter was to optimise the non-toxic concentrations of flavonoids, (their metabolites alone or in combinations) on HTR-8/SVneo cell viability as well establishing an oxidative stress model for further research (see Chapters 4 to 6).

3.5.1 Selection and optimisation of flavonoids/concentrations

This study began with the determination of non-toxic concentrations of flavonoids, their metabolites alone or in combination (flavonoid-metabolite or flavonoids). The study started with the selection of flavonoids, which was based on the structural configurations; presence and absence of a hydroxyl group in C-3 position of the C-ring (3-hydroxy and 3-desoxyflavonoids respectively). Quercetin belong to the 3-hydroxyflavonoid class and flavonol subclass, it is the most common and studied flavonoid (over 3000 citations on PubMed). In this study, quercetin served as a reference as it is the most abundant in fruits and vegetables as well as the most studied. Since morin is an isomer of quercetin, it was essential to investigate if both flavonoids have similar antioxidant properties and if the structural arrangement would be a factor. Naringin and hesperidin are classified as the 3-desoxyflavonoid class and flavanones subclass, they have not gained much interest like that of flavonols. Herein, it was aimed to determine if the presence of hydroxyl group could influence the antioxidant or pro-oxidant properties of flavonoids.

After the selection of flavonoids, the next step was to optimise the non-toxic concentration for further studies. Since there were no previous studies conducted on this subject using a human first trimester trophoblast cell line (HTR-8/SVneo), a range of concentrations (1 – 40 µM) were selected based on other studies carried out using non-trophoblast cells (PeiMing *et al.*, 2012; Yamamoto *et al.*, 2013; Daubney *et al.*, 2015). This present study has shown flavonoids (quercetin, morin, naringin and hesperidin) at high concentrations (10, 20 and 40 µM) induced cytotoxic effects in HTR-8/SVneo cells as shown from the MTT and CellTox™ assays. In a study conducted by Boesch-Saadatmandi *et al.* (2011), concentration of flavonoid (quercetin) above 25 µM decreased cell viability in a mouse macrophage (RAW264.7) cell line. In addition, quercetin at 10 µM and above was found to induce cytotoxicity in a leukaemia cell line (Nalm6) after 48 h treatment (Srivastava *et al.*, 2013). Since flavonoids exist in plants as mainly glycosidic forms and occasionally as aglycones, most research has focused on studying the antioxidant properties of flavonoids in the glycosidic forms (Erlund, 2004). However, the compounds found in the plasma and urine after ingesting flavonoids are different and these compounds are known as metabolites.

Flavonoids are absorbed by the gastrointestinal tracts of humans and are intensively metabolised (first past metabolism, both intestinal and hepatic) resulting in methylated derivatives and conjugates (glucuronides and sulphates). Interestingly, many flavonoid glycosidic forms have not been detected in plasma even if the retention time is similar to their glucuronides (Erlund, 2004; Bentz, 2009; Boesch-Saadatmandi *et al.*, 2011). Therefore, studying the effect on their metabolites on HTR-8/SVneo cells was important. Until recently, flavonoid metabolites were rarely used because data about them were scare and their commercial availability were limited due to chemical standards. Herein, flavonoid metabolites (Q3G, naringenin and hesperetin), triggered cell death from concentrations 10 µM and above in HTR-8/SVneo cells after 24 and 48 h as seen in the MTT and CellTox™ assays.

To mimic the *in vivo* environment, combination of flavonoids (3 µM) and their metabolites (1 µM) (quercetin + Q3G, morin + Q3G, naringin + naringenin and hesperidin + hesperetin) were carried out as well as combinations of flavonoids at 3 µM (quercetin + morin, quercetin + naringin and quercetin + hesperidin). As mentioned earlier in this section, daily intake of flavonoids cannot be estimated as they are widely distributed in several fruits and vegetables, and since the craving of fruits cannot be controlled during gestation, there is a tendency that two or more flavonoids will be consumed. Also, these combinations were carried out using non-toxic concentrations from individual flavonoids or metabolites. There was no indication that these combinations were toxic to the cells as shown in the MTT and CellTiter-Glo® assays.

3.5.1   Oxidative stress model

Oxygen (O_2) is often referred to as the Janus gas because it is necessary for healthy living and potentially damaging to the cells (as well toxic) at high or very low concentrations/partial pressure (or radicalised). In this study, the toxic effect of O_2 on HTR-8/SVneo cells was investigated by exposing the cells to a hypoxic environment. Initially, HTR-8/SVneo cells were subjected to 2% and 0.5% O_2 respectively for up to 24 h and cell viability was monitored by MTT assay. The data from this experiment showed that the human first trimester trophoblast cell line tolerated and adapted to the hypoxic environment. In fact, there are bodies of evidence that shows that trophoblast cells are acclimatised to a low O_2 environment (Sagrillo-Fagundes *et al.*, 2018; Hung and Burton, 2006; Armant *et al.*, 2006). In fact, initial trophoblast cells development occurs in a low oxygen environment estimated to be around an ambient pO_2 of ~18 mmHg (or 2%) (Armant, et al. 2006).

This low oxygen environment is beneficiary to trophoblast cells in promoting proliferation, invasion and angiogenesis in the placenta. This adaptation to changes in O_2 concentrations by the placenta is brought about by the modulation of hypoxia inducible factor 1α and by increasing antioxidant defences system (Burton and Jauniaux 2011, Tuuli, Longtine and Nelson 2011). This led to the conclusion that hypoxia alone was not enough to induce oxidative cell death in HTR-8/SVneo cells. This conclusion was in agreement with studies by Kilburn et al. (2000) and Armant et al (2006), where they found that HTR-8/SVneo cells increased in proliferation and were resistant to hypoxia-induced apoptosis upon exposure to 2% O_2 for up to 24 h. Therefore, the oxidative stress model used in this study was established by 2 h hypoxia (either 2 or 0.5% O_2) followed by 6 h re-oxygenation, which was a minor modification from the protocol employed by Armant et al (2006) and Leach et al (2008). Cell viability was assessed using MTT and CellTox™ green cytotoxicity assays. Increasing hypoxia to 4 h and reoxygenation for up to 6 h did not reveal any significant MTT reduction. Therefore, 2 h hypoxia followed by 6 h reoxygenation was a suitable model for oxidative stress in HTR-8/SVneo cells.

3.6   Conclusions

The collective data from MTT, CellTox™ green cytotoxicity and CellTiter-Glo® assays suggest that 1 and 3 µM of flavonoids (quercetin, morin, naringin and hesperidin) and their metabolites (Q3G, naringenin and hesperetin) are not toxic to HTR-8/SVneo cells. These data also suggest that concentrations above 10 µM of flavonoids or metabolites trigger cytotoxic effects which could suggest pro-oxidant properties of flavonoids and their metabolites. Combinations of flavonoids and their metabolites, or flavonoids at low concentrations were tolerated by HTR-8/SVneo cells. It can also be concluded that hypoxia alone either at 2% or 0.5% was not enough to induce oxidative cell death and that H/R is a better model of oxidative stress for this study.

Chapter 4
Antioxidative mechanisms of flavonoids

4.0   Introduction

As described in chapter 1, during oxidative stress there is increased production of reactive oxygen species (ROS). This deleterious effect can negatively affect numerous cellular processes. ROS are free radicals generated from biotransformation of molecular oxygen, which relies on either enzymatic (such as respiratory chain, phagocytosis and cytochrome P450 system) or non-enzymatic (O_2 reacting with organic compound) reactions (Banjarnahor and Artanti, 2014; Pizzino *et al.*, 2017). Free radicals can be defined as species containing one more unpaired electrons and are considered to be very unstable and extremely reactive and their stability is associated with their acquisition of extra election from nucleic acids, lipids, carbohydrates or proteins (Banjarnahor and Artanti, 2014). Superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl (OH•) are the three major types of ROS that are of physiological significance. Superoxide anion radical is produced from the leakage of electrons from the electron transport chain (Aouache *et al.*, 2018). The mitochondria are the main site for the production of superoxide anion and its mediated by nicotine adenine dinucleotide phosphate [NAD(P)H] oxidase, or xanthine oxidase (Birben *et al.*, 2012). Detoxification of superoxide by superoxide dismutase leads to the production of hydrogen peroxide (Burton and Jauniaux, 2011). Hydrogen peroxide is less reactive than superoxide and it is not a free radical. Hydroxyl ion is the most reactive of all three major ROS and it is generated by the reaction of superoxide with hydrogen peroxide in the presence of reaction catalyst such as ferrous (Fe^{2+}) or cuprous (Cu^+) ions (Lázár, 2012; Pizzino *et al.*, 2017).

ROS have been linked to the pathophysiology of over 100 diseases and involved several organ systems such as human reproductive tract; ovaries, fallopian tube, placenta or even embryos (Agarwal and Gupta, 2005). In placenta, hypoxia followed by re-oxygenation is a very common source of ROS during gestational period resulting in oxidative stress and eventually incomplete remodelling of the maternal spiral arteries during placentation (Leach *et al.*, 2008; Wu *et al.*, 2016). Placental oxidative stress can be adversely destructive such as shallow invasion of the trophoblast cells (see chapter 6), DNA damage, apoptosis (see chapter 5), endothelial dysfunction and immune disturbance (Wu *et al.*, 2016). These adverse effects lead to spontaneous abortion (30-50% of conceptions), pre-eclampsia (8-10%) and intrauterine growth restriction (3-10%) (Wu *et al.*, 2016). One major indicator of oxidative stress is the ratio between reduced and oxidised glutathione (GSH/GSSG).

This ratio is often used to explain reduction and oxidation (redox) regulation and other cellular/biological processes (Flohé, 2013). Reduced glutathione (GSH) is an essential intracellular, small-molecular (tripeptide) antioxidant and detoxification agent.

GSH is the main intracellular redox buffer that protects the cells/tissue against ROS and its generated by-products. It acts as an independent antioxidant or combination with other enzymes such as glutathione peroxidase. GSH is converted to a disulphide/oxidised form represented as oxidised glutathione (GSSG) (Massarsky *et al.*, 2017). Within cells, GSH constitute > 98% of total glutathione while GSSG usually constitute < 2%. This ratio is tightly regulated, and it is an essential mechanism for cell survival (proliferation, differentiation and apoptosis). During severe oxidative stress, there is a drastic shift in the GSH/GSSG ratio that results in the reduction of GSH and increment of GSSG levels resulting in GSH/GSSH redox imbalance (Giustarini *et al.*, 2015; Li *et al.*, 2016). Altered GSH/GSSG ratio plays a major role in the development of human diseases such as diabetes mellitus, cancer, cardiovascular diseases and pre-eclampsia (Giustarini *et al.*, 2015). GSSG reduction to GSH is achieved by glutathione disulphide reductase in a reaction using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. NADP/NADPH ratio is a redox couple of one of nicotinamide adenine dinucleotide system in cells that is responsible for several biological processes including maintenance of redox balance (Miller *et al.*, 2018). This ratio determines the intracellular redox state that influences the thermodynamic effects of several reactions *in vivo* (Miller *et al.*, 2018). NADP (oxidised form) is reduced to NADPH which is ten-fold more in abundance. In addition, NADPH is predominantly utilised by the cells for reduction reactions such as anabolic or antioxidant (converting GSSG to GSH) activities whereas NADP synthesis is dependent on NAD availability and it functions as a co-enzyme in cellular electron transport reactions (Agledal *et al.*, 2010). NADP/NADPH ratio is maintained at a very low level and disruption in ratio eventually results in increased ROS leading to oxidative stress (Miller *et al.*, 2018).

Interestingly, there are numerous enzymatic and non-enzymatic antioxidant defence systems against ROS and oxidative cell damage, which includes glutathione, Vitamin C & E and flavonoids. However, the antioxidative effects of flavonoids are yet to be investigated during early pregnancy. Therefore, investigating the non-toxic concentrations of flavonoids, their metabolites alone or combinations (from Chapter 3) will be used to determine their cytoprotective effects and potential intracellular mechanisms in this chapter.

4.1 Aims

- To assess the effects of pre-treatment of HTR-8/SVneo with the indicated flavonoids, their metabolites alone or in combinations prior to HR-induced oxidative stress on;

    i. Cell Viability

    ii. GSH and GSSG levels

    iii. NADP/NADPH ratio

    iv. Hydrogen peroxide (H_2O_2) levels

4.2   Results

4.2.1   Cytoprotective effects of flavonoids on HR-induced oxidative stress

The cytoprotective effects of flavonoids, their metabolites alone or in combinations on HTR-8/SVneo cells exposed to HR-induced oxidative stress was assessed using MTT and CellTiter-Glo® assays. As seen in chapter 3, HR-induced oxidative stress significantly increased cell death and increased MTT reduction as demonstrated by CellTox™ green and MTT assays respectively. Therefore, pre-treating HTR-8/SVneo cells with the non-toxic concentrations (1 and 3 µM) of the indicated flavonoids for 24 h prior to exposure to H/R insult was carried out. The data from MTT assay as shown in figure 4.1 (A-D) suggested that cells pre-treated with flavonoids (quercetin, morin, naringin and hesperidin) were associated with significant protection against HR-induced inhibition of MTT reduction.
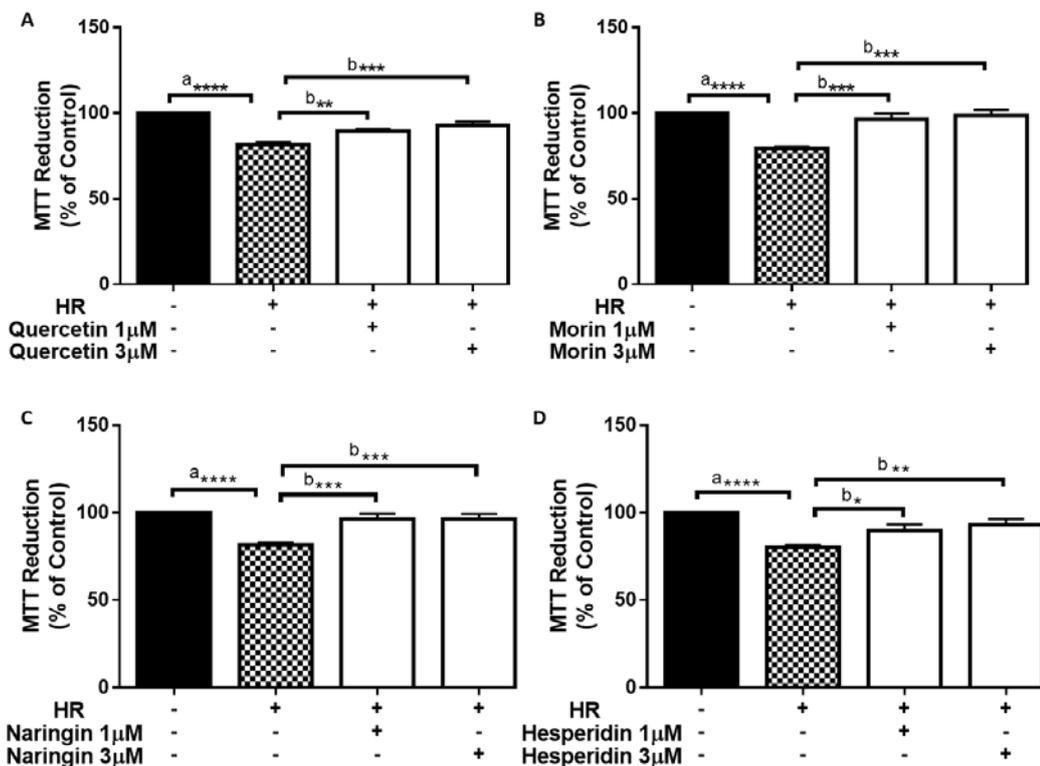
Figure 4.1: Effect of 24 h flavonoid treatment on the viability of HTR-8/SVneo cells subjected to HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with either 1 or 3 µM of quercetin, morin, naringin and hesperidin for 24 h prior to HR-induced oxidative stress exposure. Cell viability was measured by MTT reduction assay (A-D). Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as the percentage of control cells and represent the mean ± SEM of six independent experiments performed in quadruplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus control, (b) versus H/R alone.

Comparing to untreated cells (H/R), pre-treating with 1 and 3 µM flavonoids has a significant protective effect against oxidative stress. However, there was no significant difference between flavonoid treated cells and control cells.

Comparable results were achieved by monitoring cell viability based on ATP quantitation during this flavonoid-mediated cytoprotection using CellTiter-Glo® assay (figure 4.2, A-D). The data showed that 24 h pre-treated cells with 3 µM showed significantly increased ATP levels compared to H/R cells indicating flavonoid mediated proliferation. Again, comparison between 24 h pre-treated cells and control showed no significant changes in ATP level.
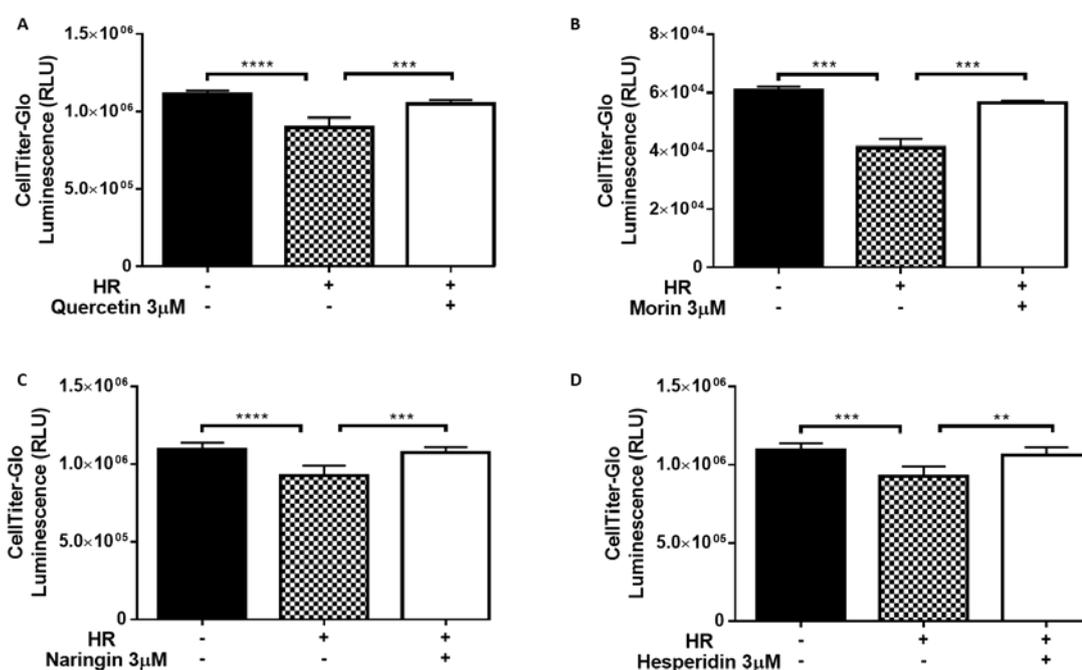
Figure 4.2: Effect of 24 h treatment with flavonoids on HTR-8/SVneo cells viability based on ATP detection in response to HR-induced oxidative stress.
HTR-8/SVneo cells were pre-treated with 3 µM of quercetin, morin, naringin and hesperidin for 24 h prior to HR-induced oxidative stress exposure. Cell viability was measured by CellTiter-Glo® Luminescent assay (A-D). Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as relative luminescence unit and represent the mean ± SEM of three independent experiments performed in quadruplicate. **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, versus control.

Since 24 h pre-treated HTR-8/SVneo cells showed significant protection against HR-induced oxidative stress, the effect of 48 h pre-treatment was assessed by MTT assay alone. Although, the usual maximum plasma peak recovery for flavonoids never exceed 24 h,

flavonoids have been recovered from the bloodstream after 48 h in some studies. The data from figure 4.3 (A-D) showed that the indicated flavonoids at both 1 and 3 µM showed significant protection against HR-induced MTT reduction. The MTT assay data from figure 4.1 (A-D) and figure 4.3 (A-D) suggest that flavonoids' cytoprotective is not only restricted to 24 h pre-treatment but also 48 h.
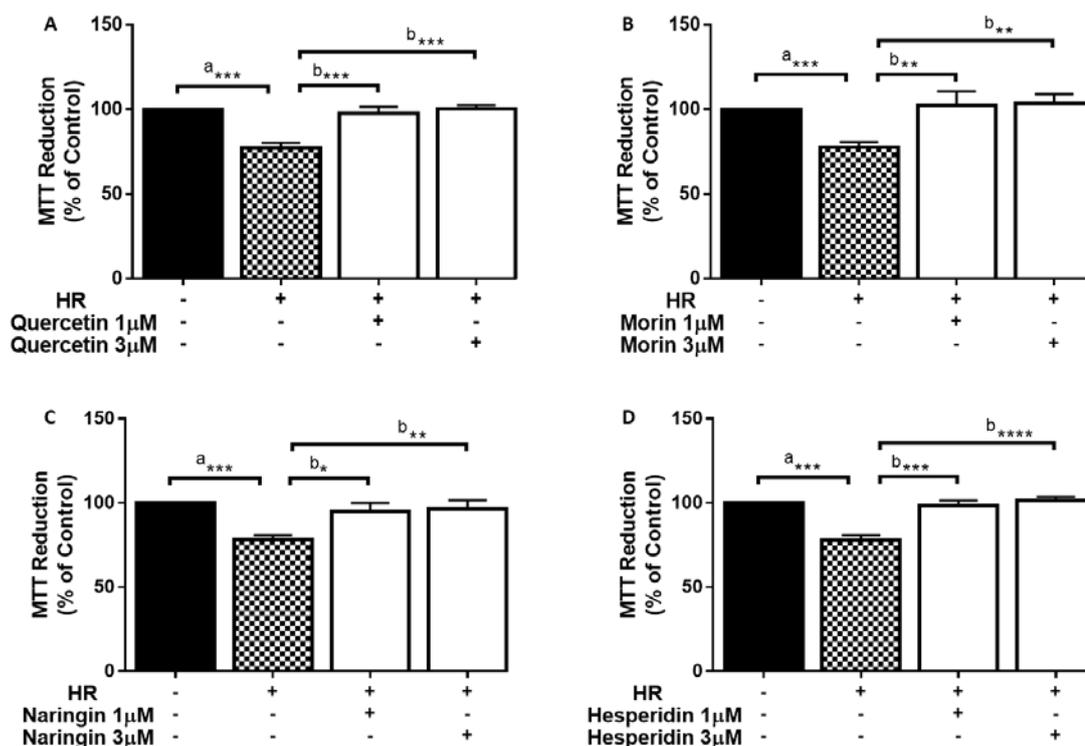
Figure 4.3: Effect of 48 h treatment with flavonoids on the viability of HTR-8/SVneo cells subjected to HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with either 1 or 3 µM of quercetin, morin, naringin and hesperidin for 48 h prior to HR-induced oxidative stress exposure. Cell viability was measured by MTT reduction assay (A-D). Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as the percentage of control cells and represent the mean ± SEM of six independent experiments performed in quadruplicate. .*$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus control, (b) versus H/R alone.

As previously described in chapter 1, after the absorption of flavonoids, their metabolites were found in bloodstream, urine and faeces. Therefore, assessing the effects of these metabolites is necessary. Similar to parent compounds, 24 h pre-treatment with 1 and 3 µM of respective metabolites (namely Q3G, naringenin and hesperetin) exhibited significant

protection against MTT reduction as shown in figure 4.4 (A-C). Further validation was carried out using CellTiter- Glo® assay. The results as seen in figure 4.5 (A-C) were in agreement with the MTT assay data, indicting that their metabolites were significantly protective against HR-induced oxidative stress.
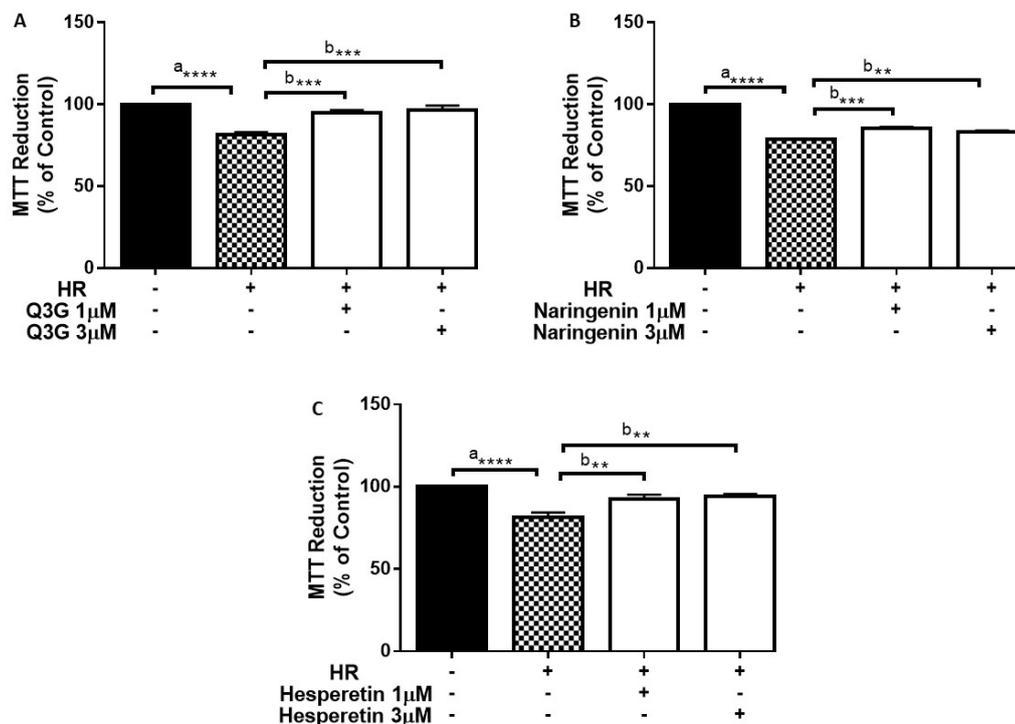
Figure 4.4: Effect of 24 h treatment with metabolites on the viability of HTR-8/SVneo cells subjected to HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with either 1 or 3 µM of Q3G, naringenin and hesperetin for 24 h prior to HR-induced oxidative stress exposure. Cell viability was measured by MTT reduction assay (A-C). Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as percentage of control cells and represent the mean ± SEM of six independent experiments performed in quadruplicate. $**P<0.01$, $***P<0.001$ and $****P<0.0001$, (a) versus control, (b) versus H/R alone.
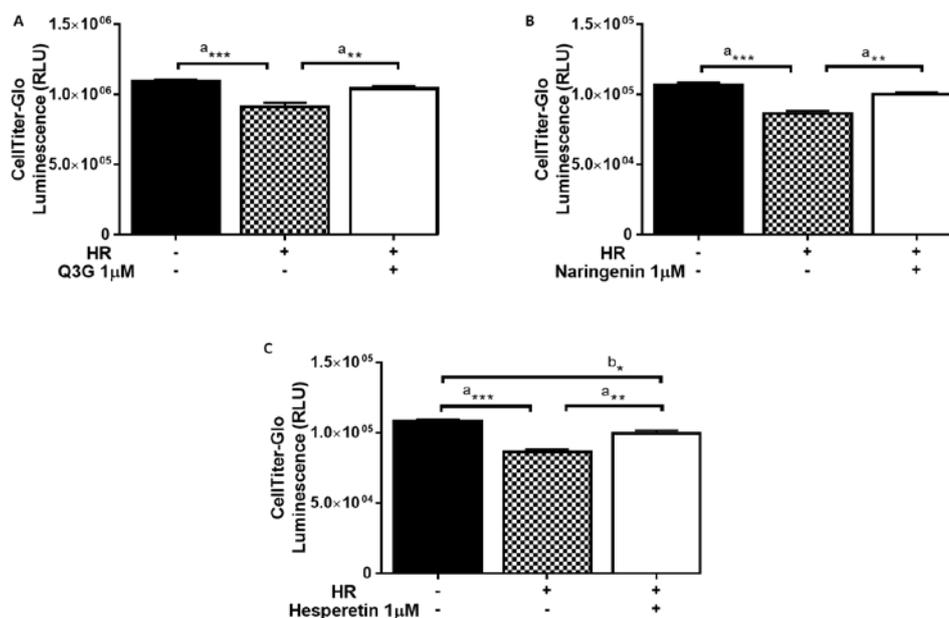
Figure 4.5: Effect of 24 h treatment with flavonoid metabolites on HTR-8/SVneo cells viability based on ATP detection subjected to HR-induced oxidative stress.
HTR-8/SVneo cells were pre-treated with 1 µM of Q3G, naringenin and hesperetin for 24 h prior to exposure to HR-induced oxidative stress. Cell viability was measured by CellTiter-Glo® Luminescent assay (A-C). Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as relative luminescence units and represent the mean ± SEM of three independent experiments performed in quadruplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus H/R alone, (b) versus control.

As explained earlier, it was important to mimic the *in vivo* environment by combining the parent compounds (flavonoids) and their respective metabolites. The effect on flavonoid-metabolite combinations as explained in section 3.2.4 (quercetin/Q3G, morin/Q3G, naringin/naringenin and hesperidin/hesperetin) with 24 h pre-treatment prior to H/R insult was investigated. The MTT assay data as seen in figure 4.6 (A-D) shows that flavonoid-metabolite combinations exhibited significant protection against HR-induced MTT reduction.
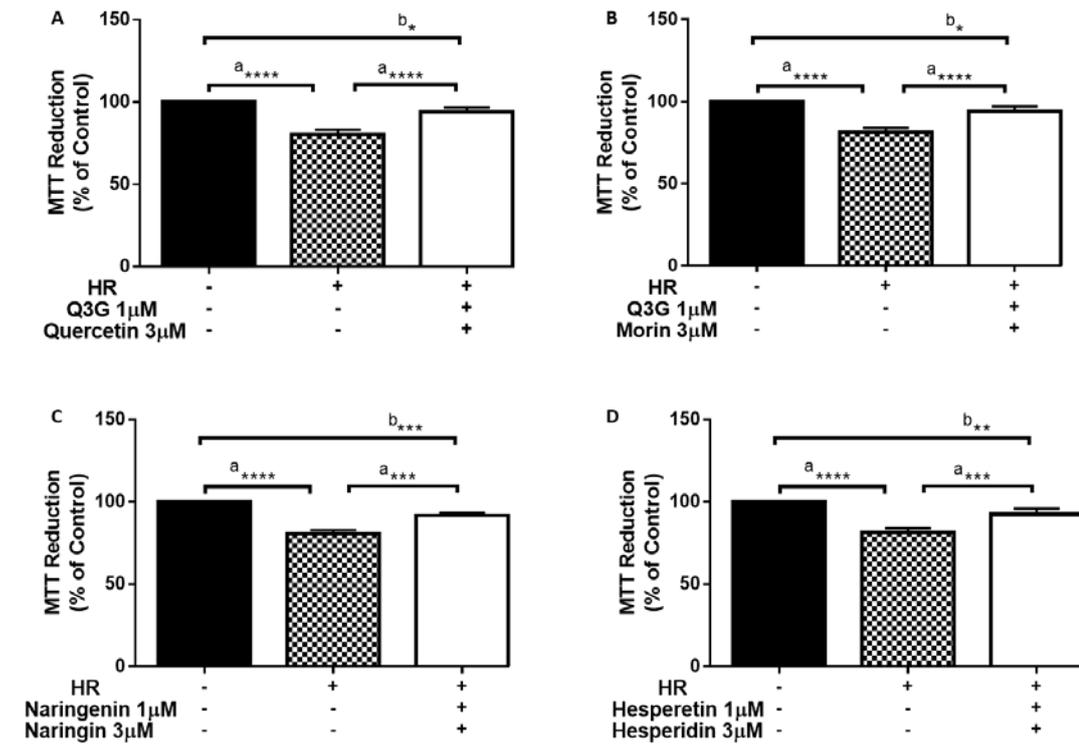
Figure 4.6: Effect of 24 h treatment with flavonoid-metabolite combinations on the viability of HTR-8/SVneo cells subjected to HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with 3 µM of flavonoid and 1 µM metabolite for 24 h prior to HR-induced oxidative stress exposure. Cell viability was measured by MTT reduction assay (A-D). Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as percentage of control cells and represent the mean ± SEM of six independent experiments performed in quadruplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus H/R alone, (b) versus control.

Similar results were obtained from the CellTiter-Glo® assay (figure 4.7, A-D). Again, the protective effects of flavonoid-metabolites combinations were seen, but unlike the MTT assay there was no significance difference between morin/Q3G, naringin/naringenin and hesperidin/hesperetin pre-treated cell compared to control.
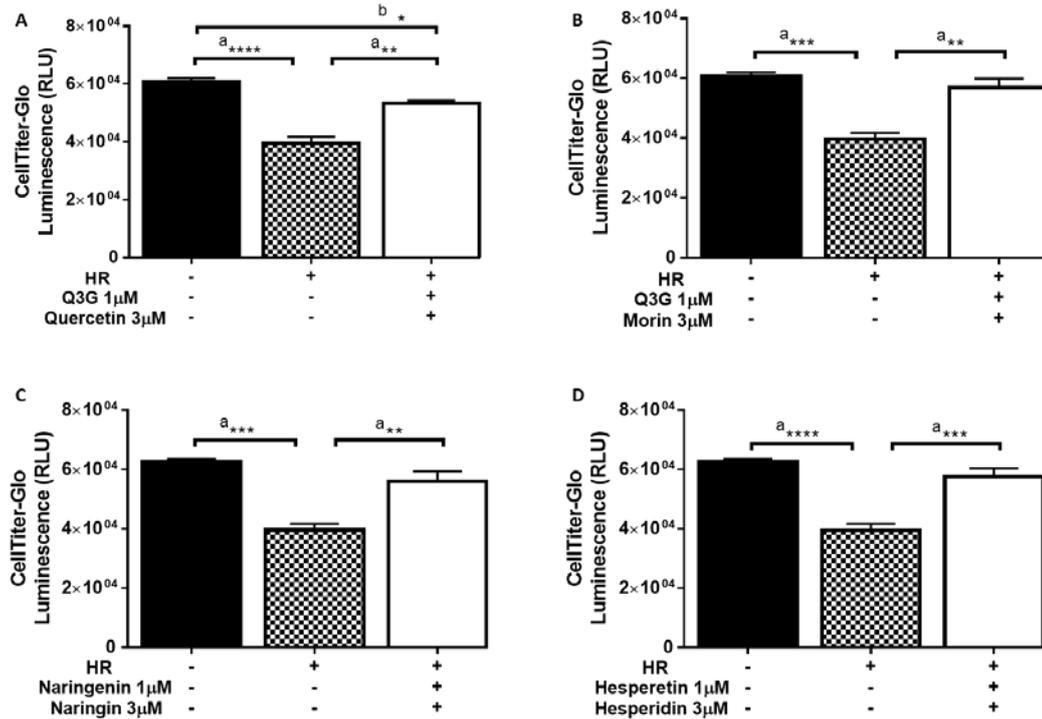
Figure 4.7: Effect of 24 h treatment with flavonoid-metabolite combinations on HTR-8/SVneo cells viability based on ATP detection subjected to HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with 3 µM of flavonoid and 1 µM metabolite for 24 h prior to HR-induced oxidative stress exposure. Cell viability was measured by CellTiter-Glo® Luminescent assay (A-D). Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as relative luminescence units and represent the mean ± SEM of three independent experiments performed in quadruplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus H/R alone, (b) versus control.

Since the daily intake of flavonoids cannot be precise and the craving of flavonoid rich fruits and vegetables cannot be controlled during early gestation, it was important to investigate the synergistic, addictive or antagonistic effect of combining two or more flavonoids. Therefore, the effects of 24 h pre-treatment with flavonoids combination as described in section 3.2.4 (quercetin/morin, quercetin/naringin and quercetin/hesepredin) was investigated by MTT and CellTire-Glo® assays. As shown in figure 4.8 (A-C), the MTT assay data showed that combination of flavonoids exhibited significant protection against MTT reduction induced by HR-induced oxidative.
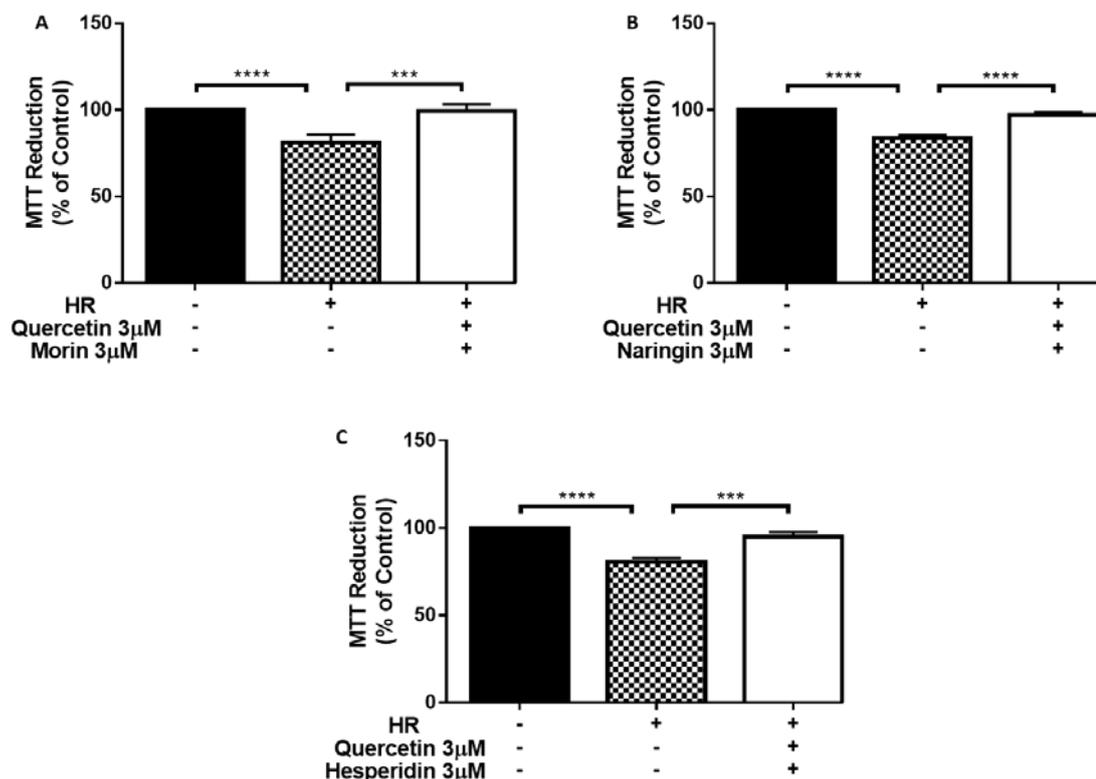
Figure 4.8: Effect of 24 h treatment with flavonoids combination on the viability of HTR-8/SVneo cells subjected to HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with the combination of flavonoids at 3 µM (individually) for 24 h prior to HR-induced oxidative stress exposure. Cell viability was measured by MTT reduction assay. Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as the percentage of control cells and represent the mean ± SEM of six independent experiments performed in quadruplicate. ***$P<0.001$ and ****$P<0.0001$, versus H/R alone.

As before, to validate the data from the MTT assay (figure 4.8), CellTiter-Glo® assay was performed. Comparable results were achieved as seen in figure 4.9 (A-C). Apart from the protective effects of combined flavonoids, the viability assay also indicated that 24 h pre-treated cells had significant reduction in viability when compared to controls unlike that of MTT assay. Both assays agreed upon comparison between pre-treated cells and untreated H/R cells.
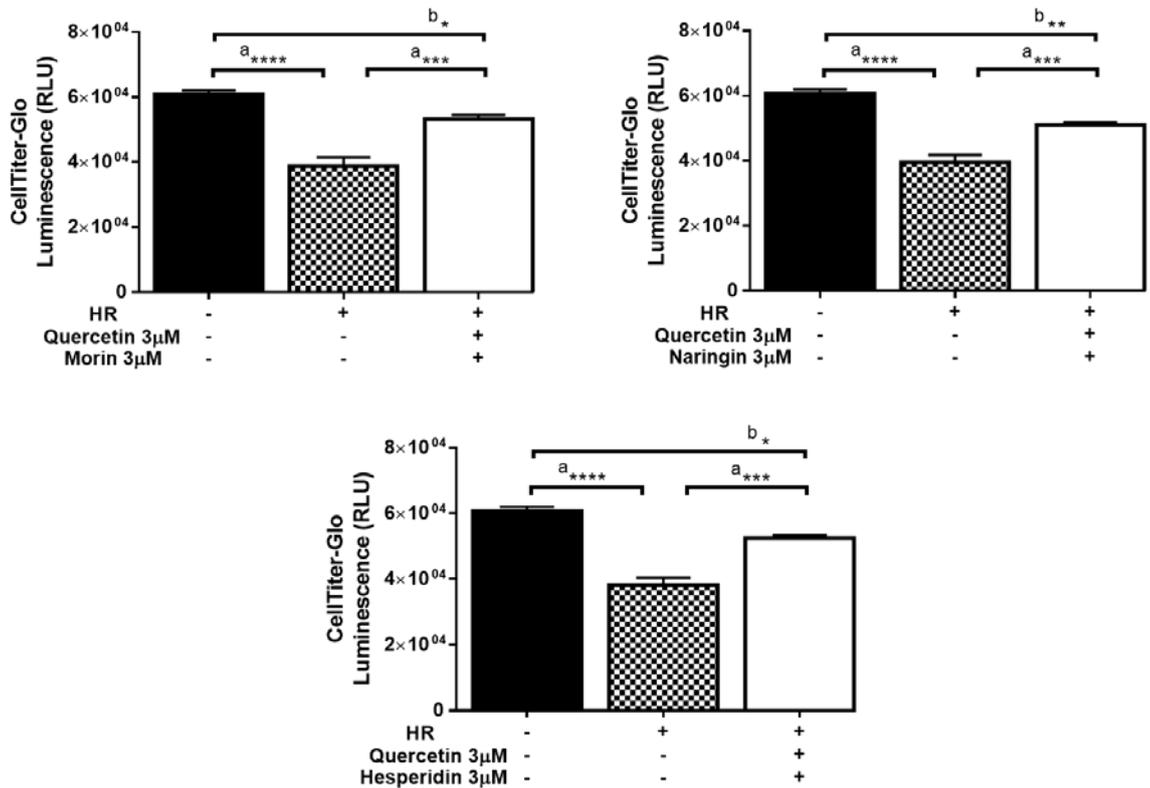
Figure 4.9: Effect of 24 h treatment with flavonoids combination on HTR-8/SVneo cells viability based on ATP detection subjected to HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with the combination of flavonoids at 3 µM (individually) for 24 h prior to HR-induced oxidative stress exposure. Cell viability was measured by CellTiter-Glo® Luminescent assay. Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as the relative luminescence unit and represent the mean ± SEM of three independent experiments performed in quadruplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus H/R alone, (b) versus control.

4.2.1.1 Effects of lower concentrations of flavonoids/metabolites on HR-induced oxidative stress

Since 1 and 3 µM of flavonoids/metabolites were protective against HR-induced oxidative stress in HTR-8/SVneo cell line, further investigation was carried out to assess the effects of flavonoids/metabolites below 1 µM. As seen in figure 4.10 (A-D), 24 h pre-treatment with the selected flavonoids (quercetin, morin, naringin or hesperidin) did not protect HTR-8/SVneo against HR-induced oxidative at either 0.3 or 0.5 µM. Similarly, 24 h pre-treatment with their respective metabolites (Q3G, naringenin and hesperetin) failed to protect HTR-8/SVneo from HR-induced oxidative stress as shown in figure 4.11 (A-C).
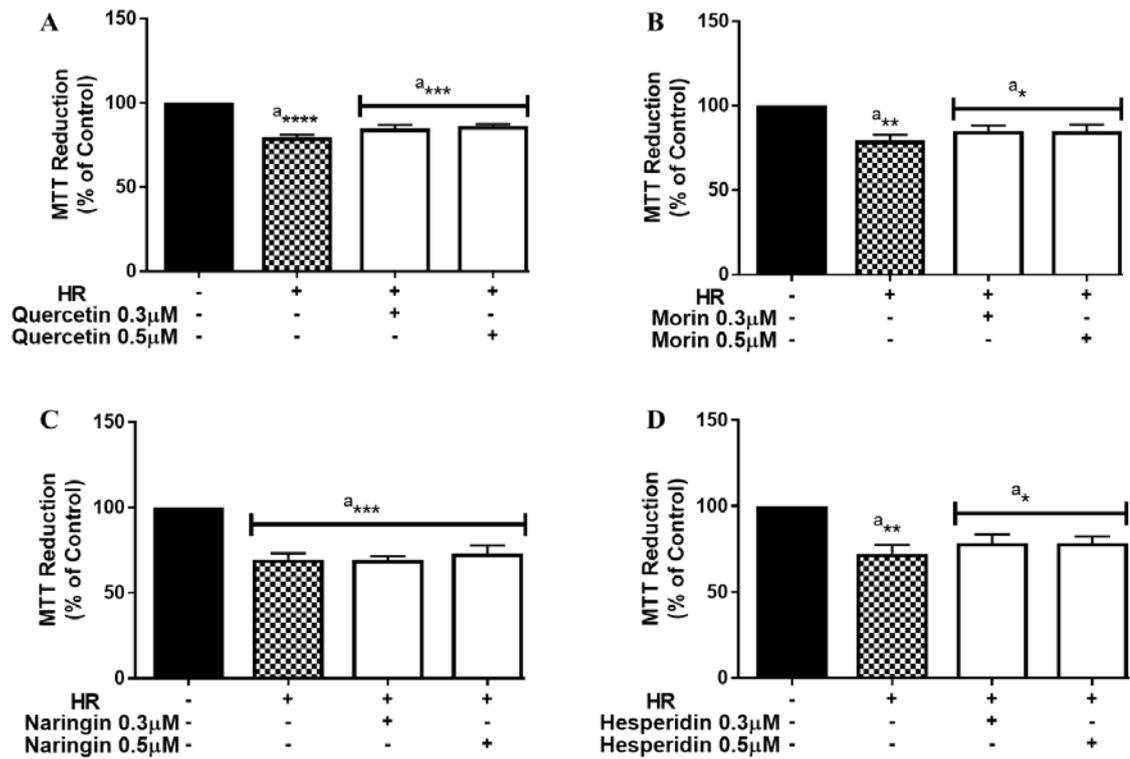
Figure 4.10: Effect of 24 h treatment with concentrations below 1 µM of flavonoids on the viability of HTR-8/SVneo cells subjected to HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with either 0.3 or 0.5 µM of quercetin, morin, naringin and hesperidin for 24 h prior to HR-induced oxidative stress exposure. Cell viability was measured by MTT reduction assay (A-D). Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as the percentage of control cells and represent the mean ± SEM of three independent experiments performed in quadruplicate. .*$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus control, (b) versus H/R alone.
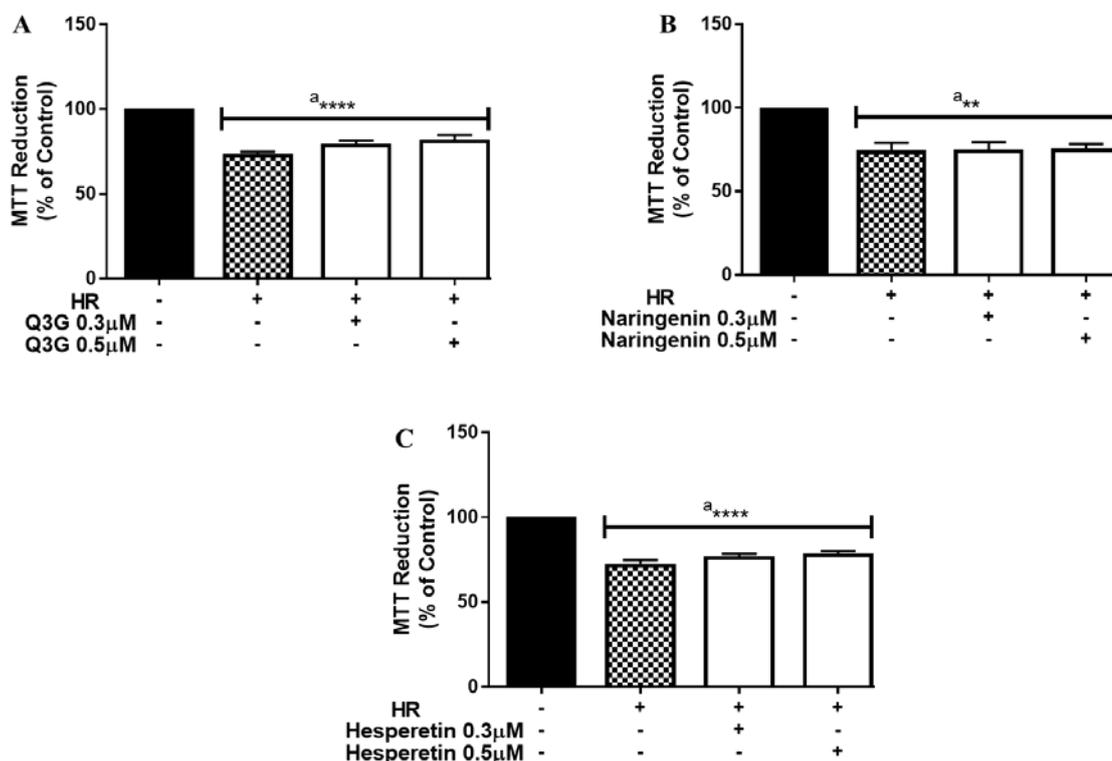
Figure 4.11: Effect of 24 h treatment with concentrations below 1 µM of metabolites on the viability of HTR-8/SVneo cells subjected to HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with either 0.3 or 0.5 µM of Q3G, naringenin and hesperetin for 24 h prior to HR-induced oxidative stress exposure. Cell viability was measured by MTT reduction assay (A-C). Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as the percentage of control cells and represent the mean ± SEM of six independent experiments performed in quadruplicate. **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus control, (b) versus H/R alone.

4.2.2 Effect of flavonoids on redox state

The effects of 24 h pre-treatment with flavonoids, their metabolites alone or in combination prior to HR-induced oxidative stress on redox state was assessed using GSH/GSSG-Glo™ and NADP/NADPH-Glo™ assays.

4.2.2.1 Effect of flavonoids on glutathione (GSH/GSSG) levels

This sub-section aimed to assess the effect of 24 h pre-treatment of HTR-8/SVneo cells with flavonoids on total (GSH+GSSG), reduced (GSH) and oxidised (GSSG) glutathione in response to HR-induced oxidative stress. As seen in figure 4.10 (A-D), 24 h pre-treatment with flavonoids (quercetin, morin, naringin or hesperidin) or H/R insult did not affect the

total glutathione level. However, the data from GSH (figure 4.10, E-H) indicated that levels of GSH were significantly depleted in H/R untreated cells while cells pre-treated with the indicated flavonoids significantly increased GSH levels when compared to both control and H/R cells. Again, there was a significant increase in the level of GSSG during HR-induced oxidative stress, yet cells pre-treated with indicated flavonoids significantly reduce the generation of GSSG. These data indicate that flavonoids were associated with the elevation of GSH and depletion of GSSG levels, restoring the imbalance produced by HR-induced oxidative stress. Comparable results were obtained from their respective metabolites (Q3G, naringenin or hesperetin; figure 4.11A-C). There was no change in the total glutathione; pre-treatment with the indicated metabolites significantly elevated GSH (figure 4.11, D-F) and significantly decreased GSSG levels (figure 4.11, G-I). Not surprisingly, pre-treatment with combinations of flavonoids and their respective metabolites (quercetin/Q3G, morin/Q3G, naringin/naringenin or hesperidin/hesperetin) did not significantly increase the total glutathione (figure 4.12A-D). However, these combinations significantly increased the GSH level (see figure 4.12, E-H) and significantly depleted GSSG level as shown in figure 4.12 (I-L). Surprisingly, the combination of flavonoids (quercetin/morin, quercetin/naringin or quercetin/hesperidin) significantly increased the total glutathione level as seen in figure 4.13 (A, B) with the exception of the quercetin/hesperidin combination as shown in figure 4.13 (C).

Furthermore, flavonoid combination pre-treatment significantly elevated GSH as indicated in figure 4.13 (D-F) and significantly reduced the GSSG level (figure 4.13, G-I). The data obtained in this section indicates that cells pre-treated with either flavonoids, their metabolites alone or in combinations significantly restore GSH/GSSG ratio.
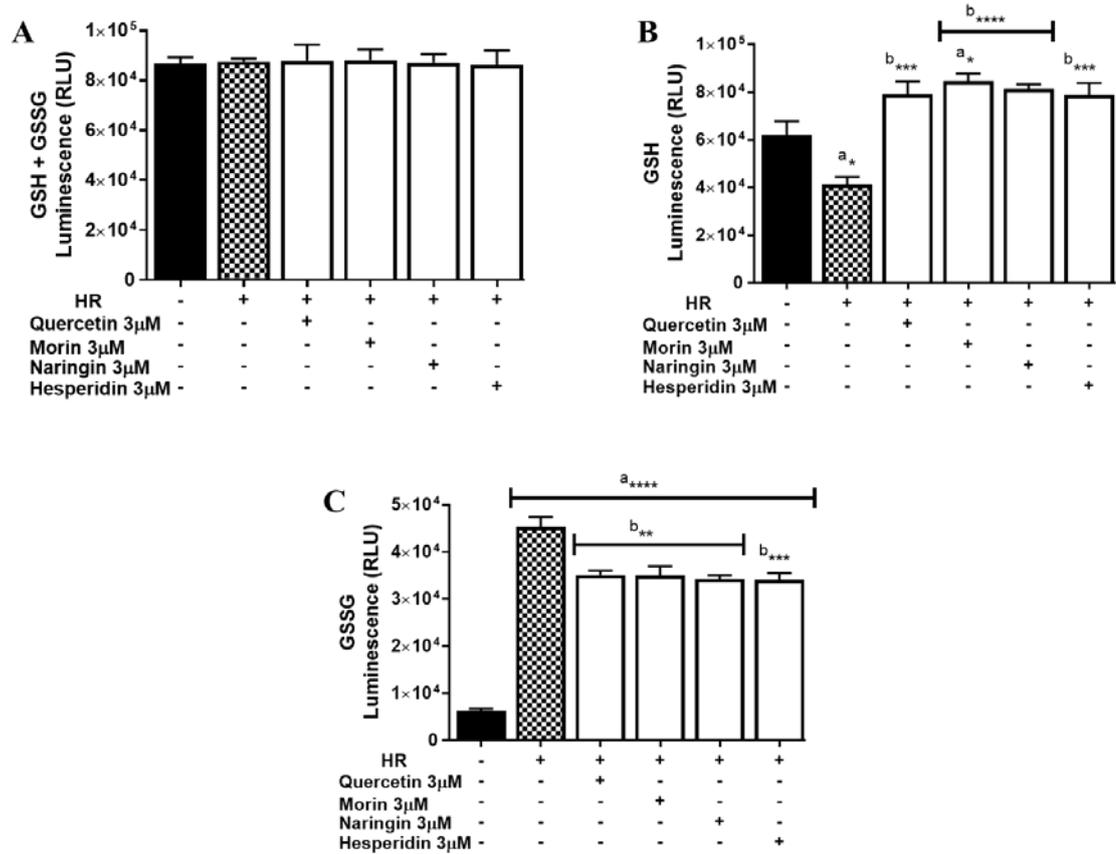
Figure 4.12: Effects of flavonoids on glutathione in response to HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with the indicated flavonoids at 3 µM for 24 h prior to exposure to HR-induced oxidative stress. Total, reduced and oxidised glutathione was measured using GSH/GSSG-Glo™ assay. (A) represent the effect of flavonoids on total glutathione, (B) represent the effects of flavonoids on reduced glutathione, and (C) represent the effect of flavonoids on oxidised glutathione. Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as the relative luminescence units (RFU) and represent the mean ± SEM of at least three independent experiments performed in quadruplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus control and (b) versus HR.
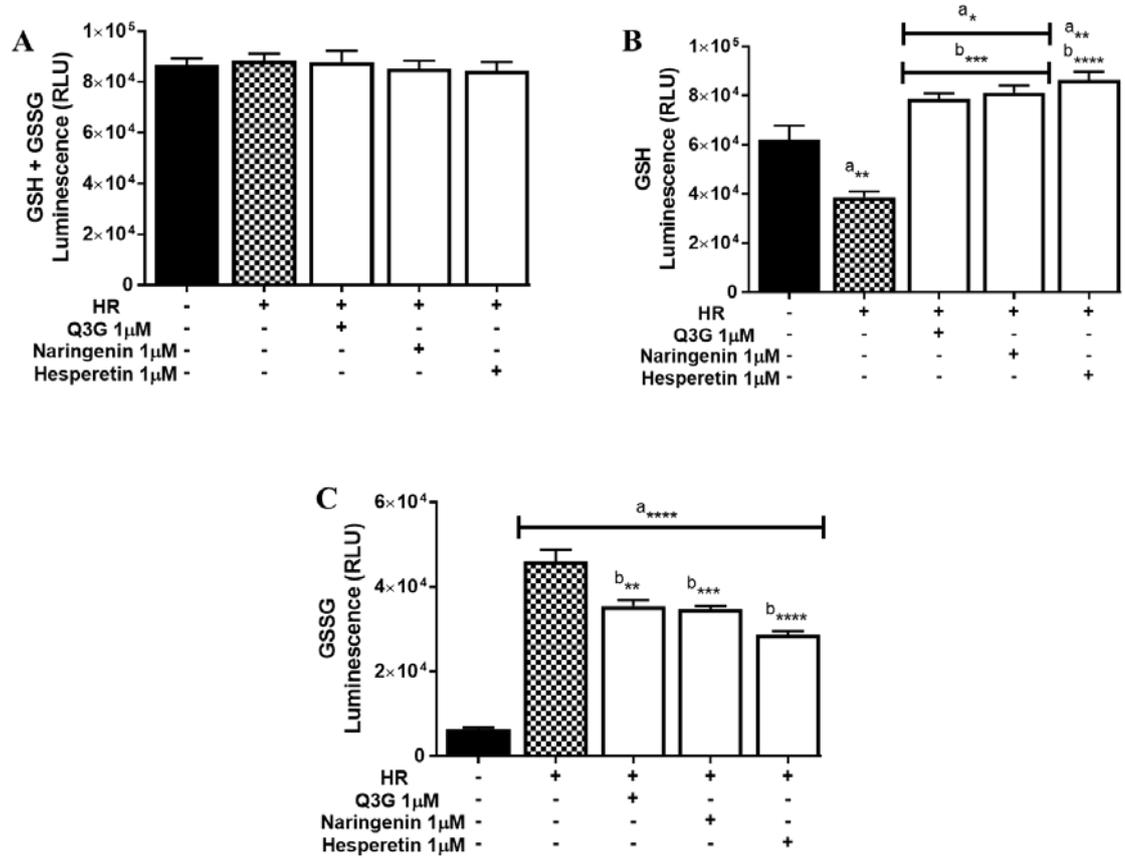
Figure 4.13: Effects of flavonoid metabolites on glutathione after HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with the indicated flavonoid metabolites at 1 µM for 24 h prior to exposure to HR-induced oxidative stress. Total, reduced and oxidised glutathione was assessed using GSH/GSSG-Glo™ assay. (A) represent the effect of metabolites on total glutathione, (B) represent the effects of metabolites on reduced glutathione, and (C) represent the effect of metabolites on oxidised glutathione. Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as the relative luminescence units (RFU) and represent the mean ± SEM of at least three independent experiments performed in quadruplicate. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001, (a) versus control and (b) versus HR.
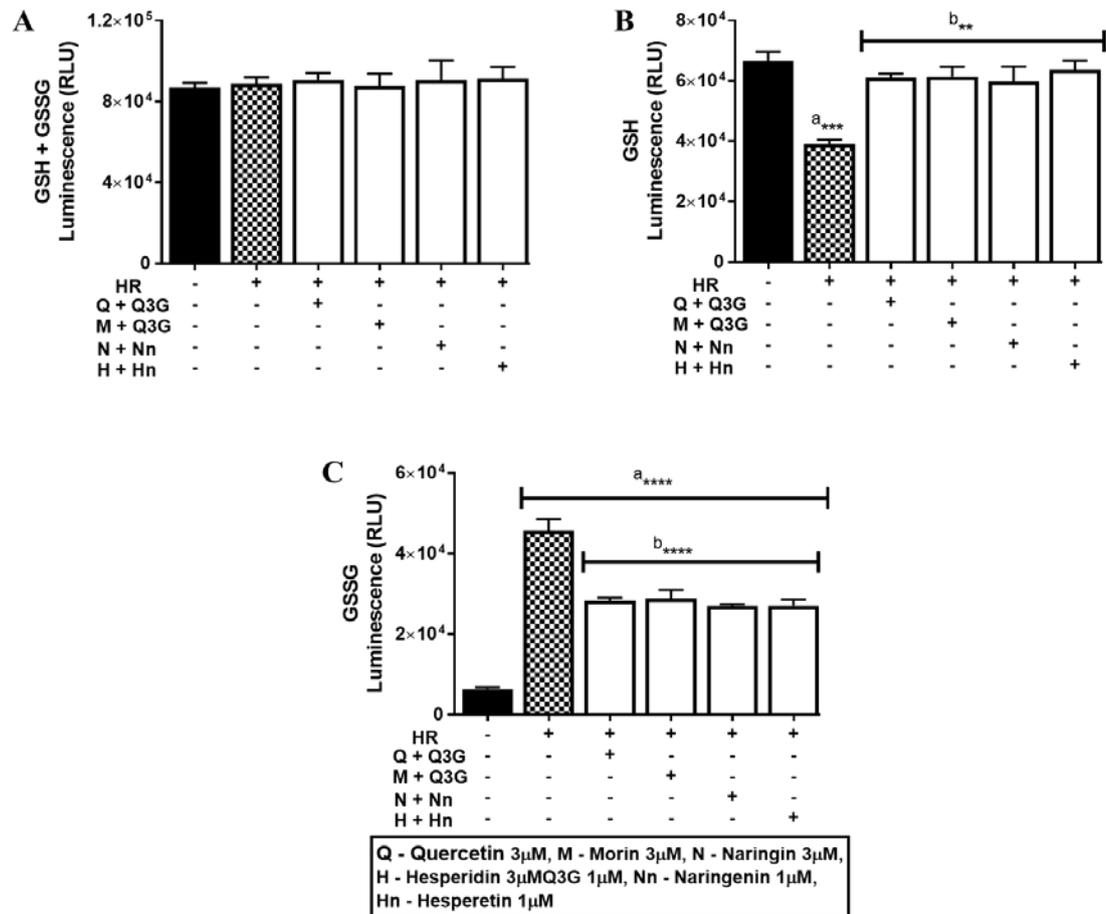
Figure 4.14: Effects of flavonoid-metabolite combinations on glutathione after HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with the indicated flavonoids (3 µM) and their metabolites (1 µM) combinations for 24 h prior to exposure to HR-induced oxidative stress. Total, reduced and oxidised glutathione was determined using GSH/GSSG-Glo™ assay. (A) represent the effect of flavonoid-metabolite combinations on total glutathione, (B) represent the effects of flavonoid-metabolite combinations on reduced glutathione, and (C) represent the effect of flavonoid-metabolite combinations on oxidised glutathione. Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as relative luminescence units (RFU) and represent the mean ± SEM of at least three independent experiments performed in quadruplicate. ***P<0.01* and *****P<0.0001*, (a) versus control and (b) versus HR.
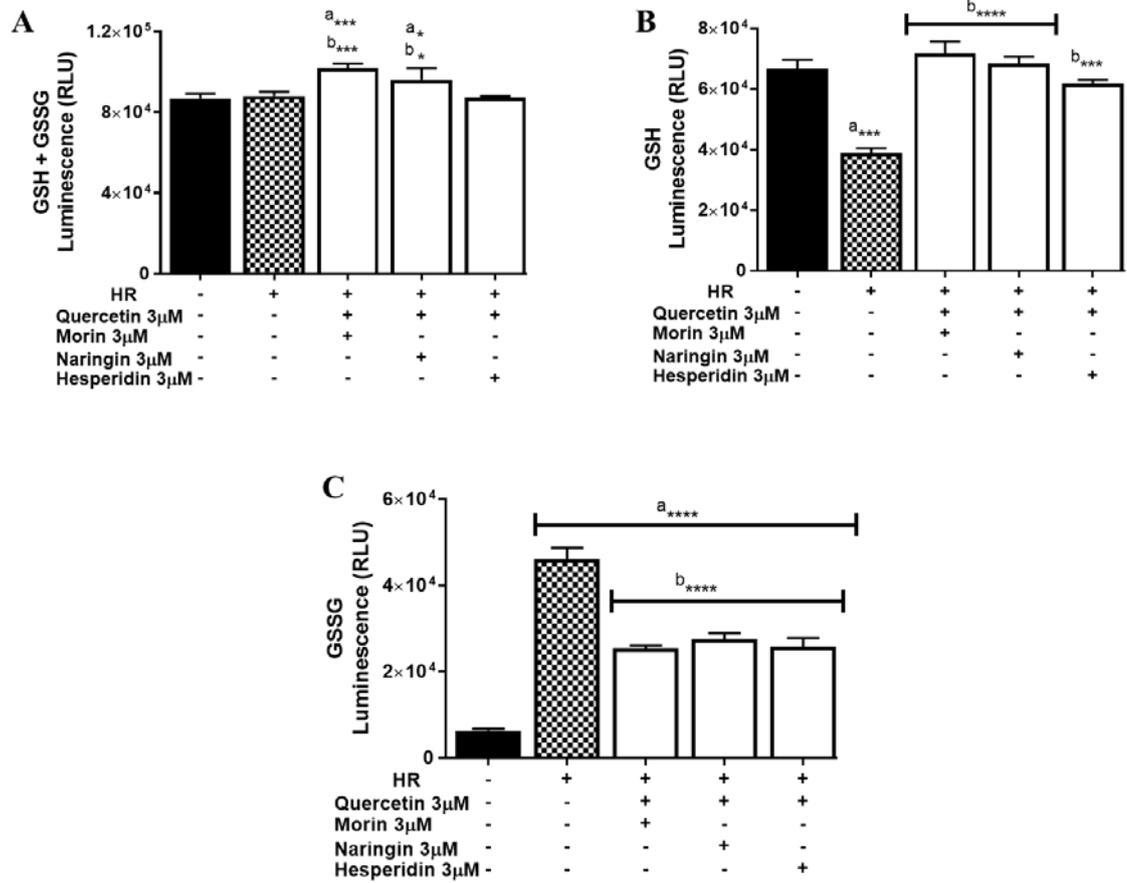
Figure 4.15: Effects of flavonoid combinations on glutathione after HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with the indicated combinations of flavonoids (3 μM each) for 24 h prior to exposure to HR-induced oxidative stress. Total, reduced and oxidised glutathione was assessed using GSH/GSSG-Glo™ assay. (A) represent the effect of combined flavonoids on total glutathione, (B) represent the effects of combined flavonoids on reduced glutathione, and (C) represent the effect of combined flavonoids on oxidised glutathione. Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as relative luminescence units (RFU) and represent the mean ± SEM of at least three independent experiments performed in quadruplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus control and (b) versus HR.

4.2.2.2 Effect of flavonoids on the nicotinamide adenine dinucleotide system

The effects of 24 h flavonoid pre-treatment prior to H/R insult on the nicotinamide adenine dinucleotide system was investigated by measuring NADP/NADPH ratio. As explained previously, the intracellular concentrations of NADP and NADPH are kept low levels as they are very important molecules responsible for major biological processes including the maintenance of redox balance. Most importantly, the mechanisms for redox balancing and subcellular ratio of NADP/NADPH has been proposed to be crucial in influencing the physiology of cells and their survival. The NADP/NADPH ratio data seen in figure 4.14 (A-D) indicated H/R control cells show high levels of the NADP/NADPH ratio which is an indication of oxidative stress. 24 h pre-treatment with flavonoids (quercetin, morin, naringin or hesperidin) significantly restored the balance in NADP and NADPH.

Figure 4.16: Effects of flavonoids on the redox state of HTR-8/SVneo cells after HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with the indicated flavonoids at 3 µM for 24 h before exposure to HR-induced oxidative stress. Redox state was assessed using NADP/NADPH-Glo™ assay (A-D). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against HR). Data are expressed as the relative luminescence units and represent the mean $\pm$ SEM of three independent experiments performed in quadruplicate. ***$P<0.001$ and ****$P<0.0001$.

Comparable data were obtained from their respective metabolites alone (Q3G, naringenin or hesperetin), as shown in figure 4.15 (A-C) there was significant reduction in the NADP/NADPH ratio when compared to H/R cells.
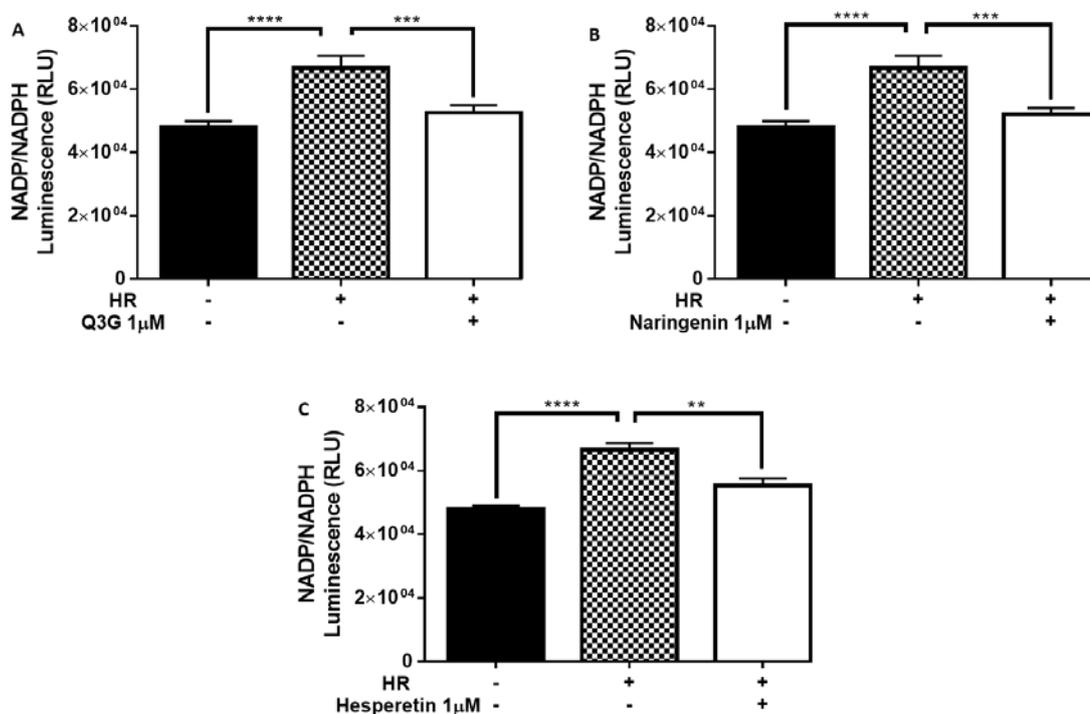
Figure 4.17: Effects of flavonoid metabolites on the redox state of HTR-8/SVneo cells after HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with the indicated flavonoid metabolites at 1 µM for 24 h before exposure to HR-induced oxidative stress. Redox state was assessed using NADP/NADPH-Glo™ assay (A-C). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against HR). Data are expressed as relative luminescence units and represent the mean ± SEM of three independent experiments performed in quadruplicate. **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$.

In addition, the effects of 24 h pre-treatment with flavonoid/metabolite combinations (quercetin/Q3G, morin/Q3G, naringin/naringenin or hesperidin/hesperetin) on NADP/NADPH was investigated. The data from figure 4.16 (A-D) shows that these combinations significantly restored the ratio balance of the nicotinamide adenine dinucleotide system. Similar results were also achieved in the investigation of 24 h pre-treatment with flavonoid combinations (quercetin/morin, quecetin/naringin or quercetin/hesperidin) as seen in figure 4.17 (A-C).
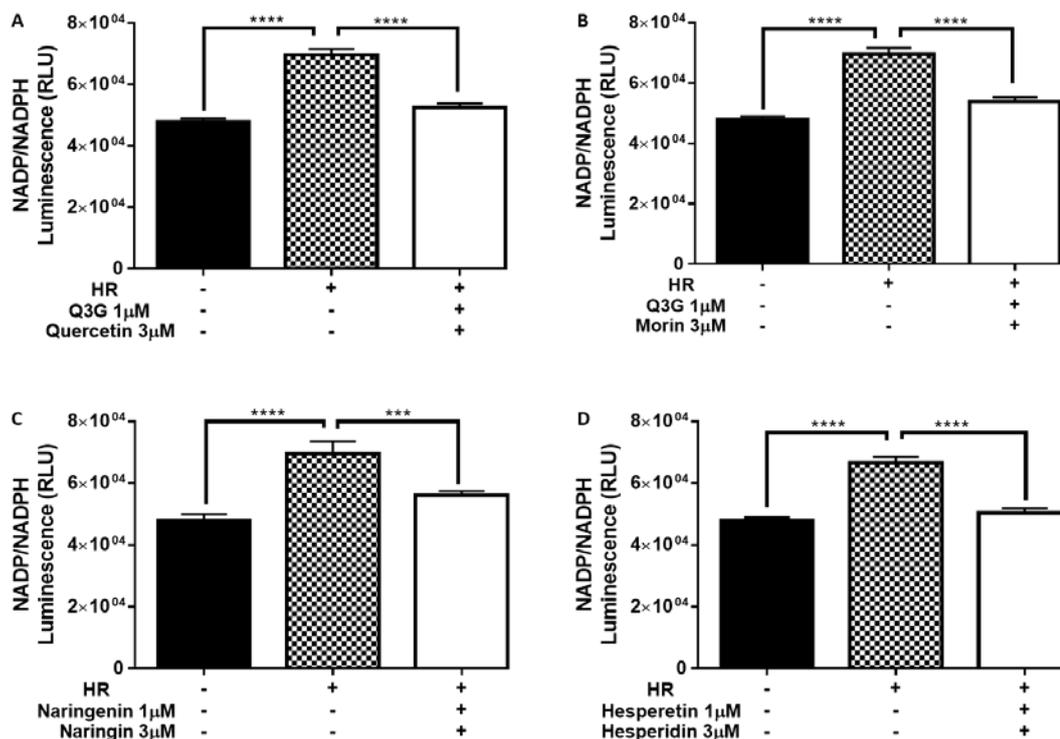
Figure 4.18: Effects of flavonoid-metabolite combinations on the redox state of HTR-8/SVneo cells after HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with the indicated combinations flavonoid (3 µM) and flavonoid metabolite (1 µM) for 24 h before exposure to HR-induced oxidative stress. Redox state was assessed using NADP/NADPH-Glo™ assay (A-D). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against HR). Data are expressed as relative luminescence units and represent the mean ± SEM of three independent experiments performed in quadruplicate. ***$P<0.001$ and ****$P<0.0001$.
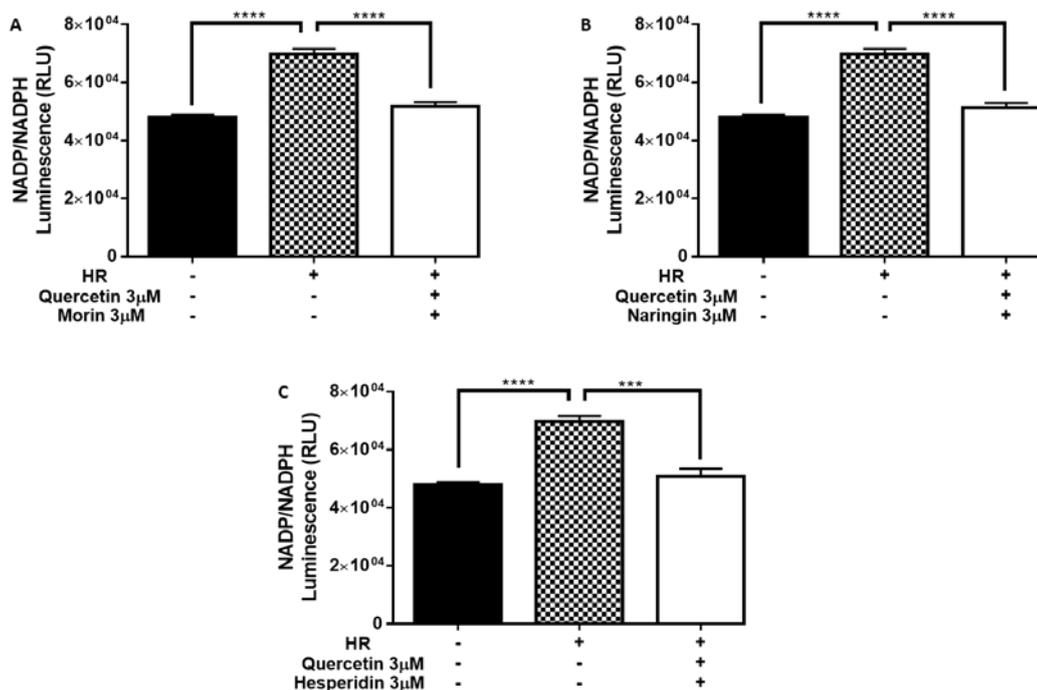
Figure 4.19: Effects of flavonoid combinations on the redox state of HTR-8/SVneo cells after HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with the indicated combinations of flavonoids at 3 µM for 24 h before exposure to HR-induced oxidative stress. Redox state was assessed using NADP/NADPH-Glo™ assay (A-C). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against HR). Data are expressed as relative luminescence units and represent the mean ± SEM of three independent experiments performed in quadruplicate. ***$P<0.001$ and ****$P<0.0001$.

4.2.3 Scavenging effect of flavonoids on ROS

In this section the role of flavonoids, their metabolites alone or in combinations in scavenging of ROS (hydrogen peroxide) was investigated. The effects of 24 h pre-treatment was assessed using ROS-Glo™ H_2O_2. As expected, exposure to H/R insult significantly increased the generation of hydrogen peroxide as seen in figures 4.18-21 (H/R untreated cells). The data from figure 4.18 (A-D) indicated that flavonoids (quercetin, morin, naringin or hesperidin) exhibited significant scavenging effects on hydrogen peroxide (generated by HR-induced oxidative stress) was significantly reduced.
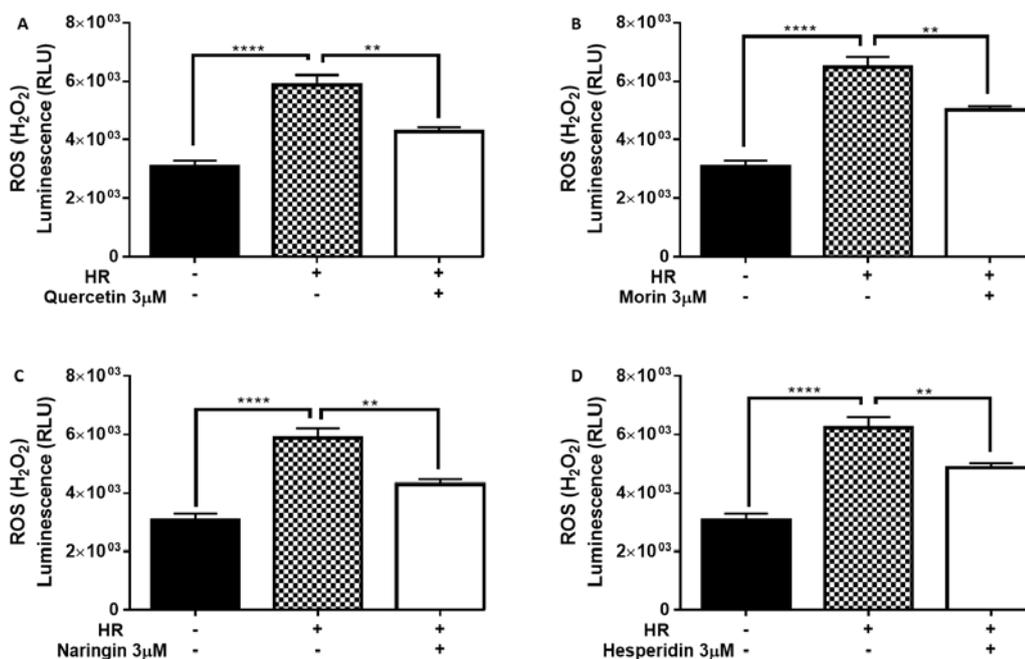
Figure 4.20: Scavenging effects of flavonoids on intracellular ROS during HR-induced oxidative stress in HTR-8/SVneo cells.

HTR-8/SVneo cells were pre-treated with the indicated flavonoids (3 µM) for 24 h before exposure to HR-induced oxidative stress. Intracellular ROS was assessed using ROS-Glo H_2O_2 assay (A-D). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against HR). Data are expressed as relative luminescence units and represent the mean ± SEM of three independent experiments performed in quadruplicate. **$P<0.01$, and ****$P<0.0001$.

Investigating the effects of 24 h pre-treatment with their metabolites alone, again there was significant reduction in HR-induced hydrogen peroxide generation as shown in figure 4.19 (A-C). Comparable results were obtained from the combination of flavonoid-metabolite/flavonoids as seen in figure 4.20 (A-D) and figure 4.21 (A-C) respectively.
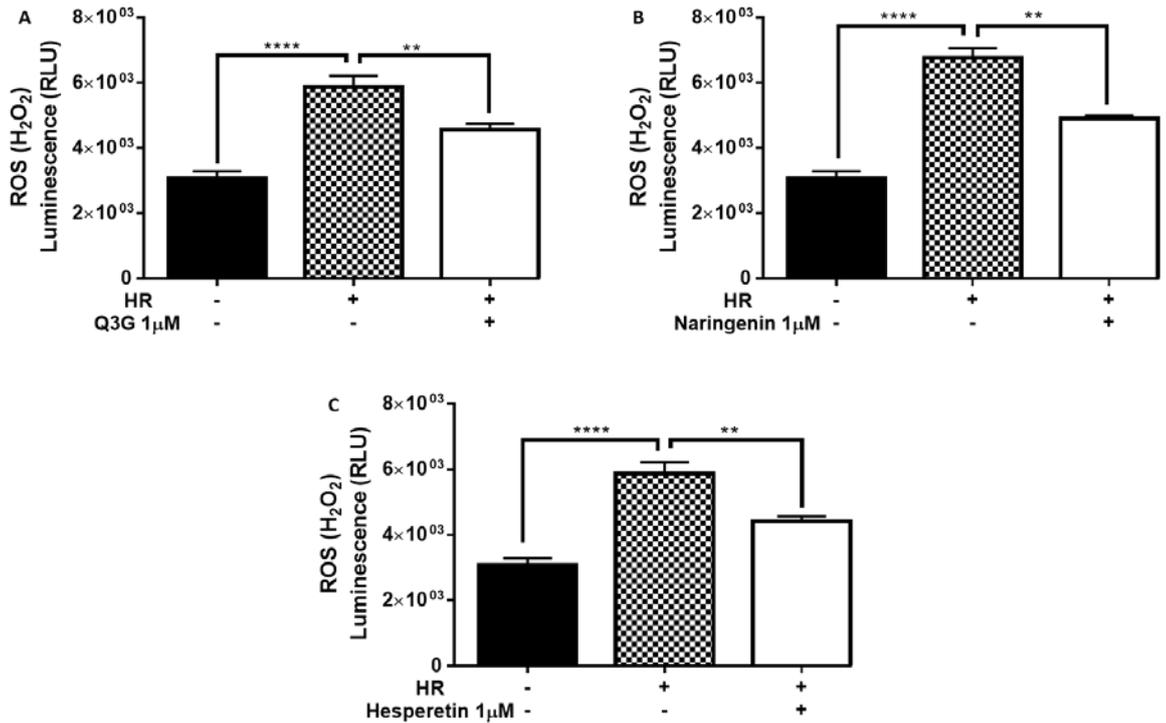
Figure 4.21: Scavenging effects of metabolites on intracellular ROS during HR-induced oxidative stress in HTR-8/SVneo cells.

HTR-8/SVneo cells were pre-treated with the indicated flavonoid metabolites at 1 µM for 24 h before exposure to HR-induced oxidative stress. Intracellular ROS was assessed using ROS-Glo H_2O_2 assay (A-C). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against HR). Data are expressed as relative luminescence units and represent the mean ± SEM of three independent experiments performed in quadruplicate. **$P<0.01$ and ****$P<0.0001$.
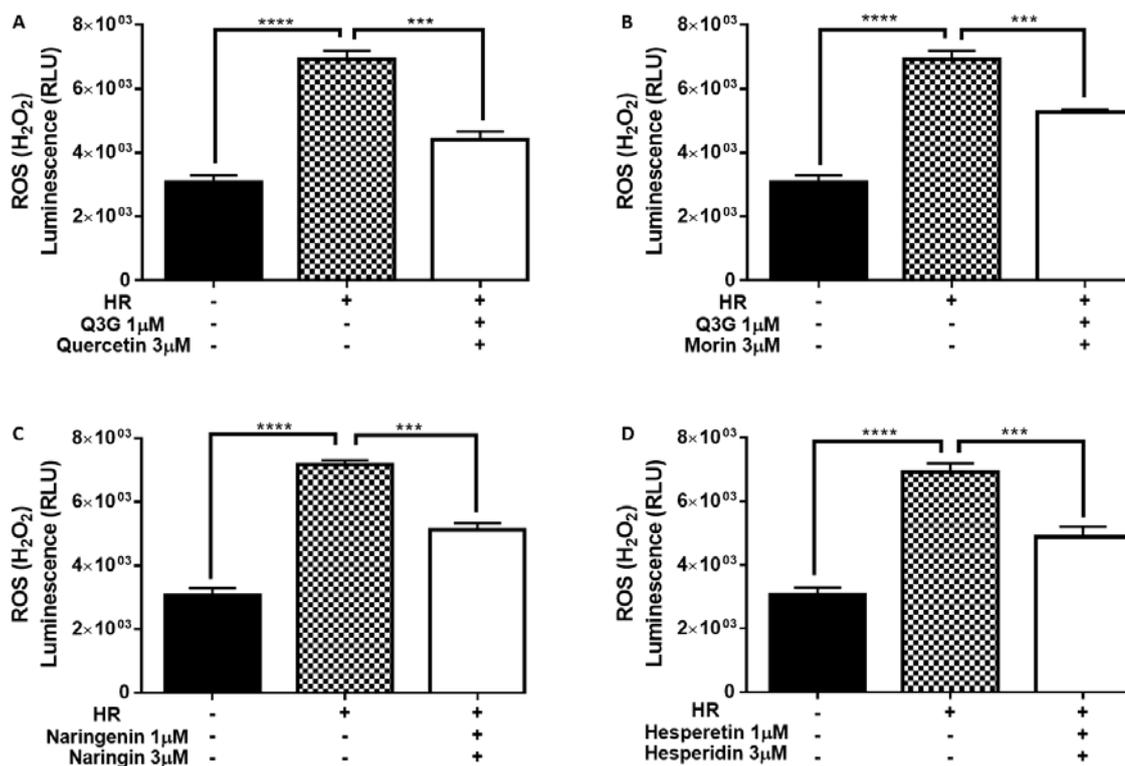
Figure 4.22: Scavenging effects of flavonoid-metabolite combinations on intracellular ROS during HR-induced oxidative stress in HTR-8/SVneo cells.

HTR-8/SVneo cells were pre-treated with the indicated combinations of flavonoid (3 µM) and flavonoid metabolite (1µM) for 24 h before exposure to HR-induced oxidative stress. Intracellular ROS was assessed using ROS-Glo H_2O_2 assay (A-D). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against HR). Data are expressed as relative luminescence units and represent the mean ± SEM of three independent experiments performed in quadruplicate. ***$P<0.001$ and ****$P<0.0001$.
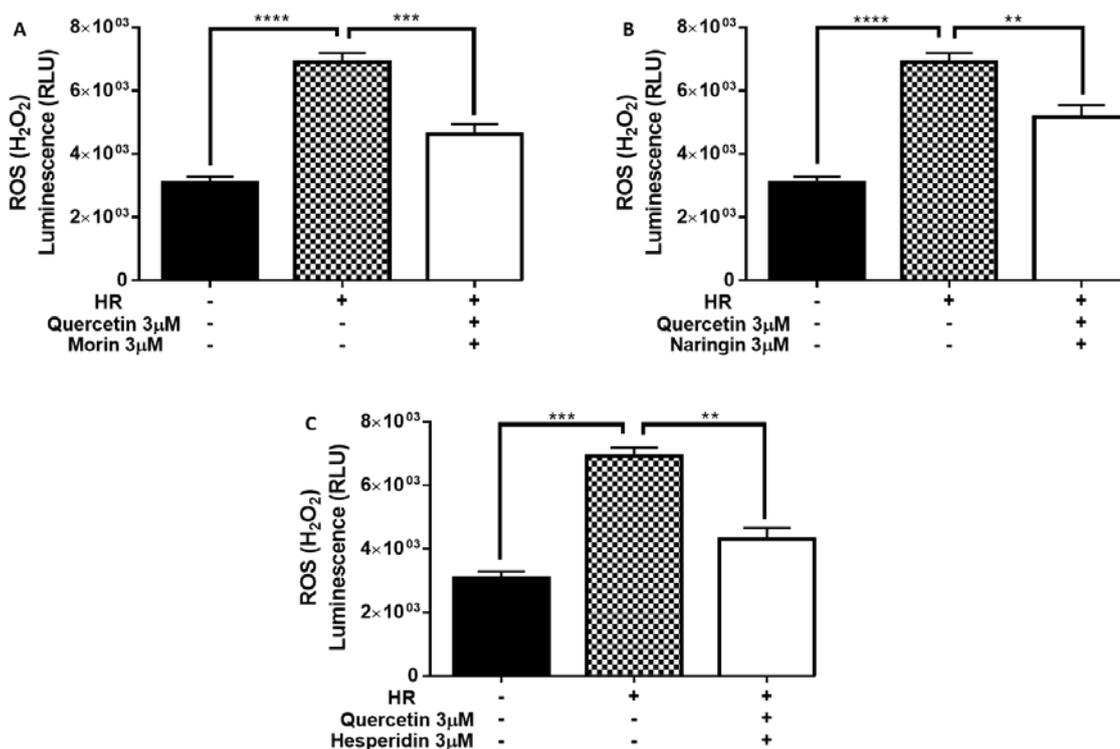
Figure 4.23: Scavenging effects of flavonoid combinations on intracellular ROS during HR-induced oxidative stress in HTR-8/SVneo cells.

HTR-8/SVneo cells were pre-treated with the indicated combinations of flavonoid (3 µM) for 24 h before exposure to HR-induced oxidative stress. Intracellular ROS was assessed using ROS-Glo H_2O_2 assay (A-D). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against HR). Data are expressed as relative luminescence units and represent the mean ± SEM of three independent experiments performed in quadruplicate. **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$.

4.3 Result Summary

Flavonoid (quercetin, morin, naringin or hesperidin) pre-treatment for 24 and 48 h exhibited cytoprotective effects against HR-induced oxidative stress in HTR-8/SVneo cells. This cytoprotective effect was also observed with their metabolites alone (Q3G, naringenin or hesperetin) and their combinations (flavonoid/metabolites or flavonoid/flavonoid). Initial data from the MTT reduction assay confirmed that both 1 and 3 µM of the indicated flavonoids or metabolites, alone showed significant cytoprotection against MTT reduction from HR-induced oxidative cell death. On the contrary, concentrations 0.3 and 0.5 µM of flavonoids/metabolites alone failed to show cytoprotective effects against HR-induced oxidative stress in HTR-8/SVneo cells. Investigating the intracellular mechanism of the cytoprotective effects of these flavonoids, pre-treated cells (24 h) showed restorative effects

in redox balancing during HR-induce oxidative stress by significantly elevating the levels of GSH, depleting GSSG levels and maintaining the ratio balance in the nicotinamide adenine dinucleotide system (NADP/NADPH). Unexpectedly, combination of flavonoids such as quercetin/morin and quercetin/ naringin significantly increased the total glutathione (GSH+GSSG) level during HR-induced oxidative stress despite the finding that there was no change in total glutathione level between control and H/R cells. Finally, there was significant ROS (H_2O_2) scavenging effects exhibited by 24 h pre-treated cells with flavonoids/metabolites.

4.4   Discussion

The aim of this section was to assess the cytoprotective effects of the indicated flavonoids, their metabolites alone or in combinations (flavonoids-metabolites or flavonoids) as well as their intracellular pathways such as redox balance (GSH/GSSG, NADP/NADPH) and scavenging of ROS (H_2O_2) on HR-induced oxidative stress in first trimester trophoblast cell line (HTR-8/SVneo).

4.4.1   Cytoprotective effects of flavonoids against H/R

To the author's knowledge, this is the first study to investigate the cytoprotective effects of flavonoids on HTR-8/SVneo cells during HR-induced oxidative stress. The data obtained from MTT assays showed that HTR-8/SVneo cells pre-treated with flavonoids for 24 h prior to H/R insult showed significant cytoprotection against MTT reduction. Both 1 and 3 µM of flavonoids and metabolites alone, induced cytoprotective effects but more consistent protection was observed in 3 µM for flavonoids and 1 µM for metabolites (Q3G, naringenin or hesperetin) pre-treated cells as seen from the MTT assay. These protective effects seen from both flavonoids and their metabolites alone was further validated with using CellTiter-Glo® viability assay, which assesses cellular proliferation/viability based on ATP quantitation and was in agreement. Furthermore, cells pre-treated with flavonoids alone for 48 h also showed protective effects against HR-induced oxidative stress as seen by the MTT reduction. However, 24 h pre-treatment was used for this study as the absorption, metabolism and excretion of flavonoids as well as the peak plasma levels are achieved in the first 24 h (Lotito and Frei, 2006; Thilakarathna and Vasantha Rupasinghe, 2013). Assessing the effects of 24 h pre-treatment with flavonoid-metabolite/flavonoid combinations, there was also a significant protection against H/R insult as seen from the MTT reduction and CellTiter-Glo® assays. This protective effect of flavonoids, their metabolites or in combination against H/R can be associated with their anti-oxidative properties. Since this is the first study on the effects of flavonoids on a human first trimester trophoblast cell line,

comparable data or studies are not available. However, there are numerous studies of flavonoids on non-trophoblast origin, for instance, quercetin and it's metabolite (Q3G) were associated with protection against H_2O_2-induced oxidative stress in mitotic and differentiated H9c2 cardiomyoblasts as well as neuronal PC12 cells (Daubney *et al.*, 2015; Ho and Chang, 2004a). In another study conducted by Lee *et al.* (2017) morin exhibited cytoprotective effects in lung fibroblast cells (V79-4) following H_2O_2-induced oxidative DNA damage and cell death. In addition, naringin attenuated the effect of H_2O_2-induced cytotoxicity in mouse leukemic (P388) and lung fibroblast (V79) cells (Bacanli *et al.*, 2015; Kanno *et al.*, 2003). Furthermore, in a study conducted by Chen *et al.* (2010), hesperidin was found to be cytoprotective against *tert*-butyl hydroperoxide in human hepatocyte L02 cells. The data from this current study is in agreement with the above studies that flavonoids are protective against oxidative stress. Another unique feature of this current study is that fact that cytoprotection was achieved using low concentrations that are within physiological range and within the daily recommended intake. In contrast to other studies (stated above), higher concentrations (20-500 µM) of flavonoids are used that cannot be achieved physiologically by the daily intake of fruits and vegetables. Also, the daily average intake of flavonoids cannot be estimated due to their extensive distribution in plants and varieties. Considering these factors, the dietary daily intake of flavonoids has been proposed to range from 100 to 1000 mg/day (Yao *et al.*, 2004; Mullie *et al.*, 2007).

Investigating the effects of flavonoid-metabolite combinations (quercetin/Q3G, morin/Q3G, naringin/naringenin or hesperidin/hesperetin), the data from MTT and CellTiter-Glo® assays showed that, there is significant protection against inhibition of MTT reduction and significant increase in ATP levels in pre-treated cells when compared with H/R cells. Although synergistic or addictive effects could not be confirmed, the data clearly indicated that these combinations were not antagonist. Again, this is the first study to report the combination of flavonoid-metabolite combination in HTR-8/SVneo cells prior to H/R insult. Therefore, comparable results were not available both from trophoblast or non-trophoblast origins due to limited studies. Furthermore, the combinations of flavonoids (quercetin/morin, quercetin/naringin or quercetin/hesperidin) also showed significant protective against HR-induced oxidative stress as demonstrated by MTT and CellTiter-Glo® assays. Combinations of flavonoids were found to have more reducing power when compared to single flavonoid during oxidative stress using the pancreatic ductal ademonocarcinoma cell line (BxPc-3) and PaCaDD-183, and primary cells (Appari *et al.*, 2014).

4.4.2 Regulation of redox balance

To investigate the effects of 24 h pre-treatment with flavonoids, their metabolites alone or in combinations on the redox state of HTR-8/SVneo cells during HR-induced oxidative stress, GSH/GSSG-Glo™ and NADP/NADPH-Glo® assays were carried out.

GSH/GSSG-Glo™ assay was used to assess the intracellular levels of total (GSH+GSSG), reduced (GSH) and oxidised (GSSG) glutathione in HTR-8/SVneo cells during H/R insult. As glutathione plays a vital role in the cellular defence system, maintaining the cellular GSH/GSSG ratio is important as depletion of GSH or elevation of GSSG levels increases cellular vulnerability to oxidative stress-induced cell death (Cavia-Saiz *et al.*, 2010). Data obtained from this report indicated that 24 h pre-treated cells (except for quercetin/morin and quercetin/naringin), did not significantly increase or decrease the level of total glutathione. Comparisons between the control and H/R cells did not show any significant difference in total glutathione. This may suggest that the total glutathione is not a factor during the oxidative stress and more emphasis should be focused on GSH and GSSG, alone as well as their ratio. Investigating GSH, the data from GSH/GSSG-Glo™ assay showed that H/R significantly depleted the level of GSH when compared to control cells. Interestingly, cells pre-treated with flavonoid (24 h) prior to H/R showed elevated levels of GSH. Comparing pre-treated to control cells, flavonoids (morin and hesperidin) and metabolites (Q3G, naringenin and hesperetin) showed significant elevation of GSH levels. Although other flavonoids or combinations did not significantly increase GSH levels when compared to control but was significantly increased when compared to H/R cells. This data may suggest that flavonoid-mediated cytoprotection can be linked to their ability to elevate GSH levels. In a study carried out by Myhrstad *et al.* (2002), flavonoids significantly increased cellular GSH by approximately 50% in COS-1 fibroblast-like cells. In addition, quercetin was found to significantly elevate GSH in the human laryngeal carcinoma cell line HEp2 cells (Myers *et al.*, 2013). Comparable results were also obtained from *in vivo* studies concluding that flavonoids significantly increase the level of GSH as a protective mechanism against oxidative stress (Subash and Subramanian, 2009; Golechha *et al.*, 2011; Pari *et al.*, 2015; Chandra Jagetia, 2015).

Elevated levels of GSSG is an indicator of oxidative stress as it only constitutes less than 1% of total glutathione as previously described in section 4.0. The data from GSH/GSSG-Glo™ assay showed that there was a significant increase in GSSG level in H/R untreated cells when compared to controls. Pre-treated cells before exposure to HR-induced oxidative stress significantly decreased the intracellular GSSG levels. This indicates that these flavonoids,

their metabolite alone or their combinations restore the redox state by increasing GSH and decreasing GSSG level. GSSG has been proposed to significantly increase in cells when exposed to oxidative stress or electrophilic chemicals. This elevation leads to an imbalance in the GSH/GSSG ratio, loss of GSH as well as severe nuclear protein modifications such as insolubilization, oxidation and cross-linking (Ballatori *et al.*, 2009). Although comparable data from both trophoblast and non-trophoblast *in vitro* studies are limited, these flavonoids were found to significantly increase GSH and deplete GSSG levels in mouse macrophage RAW264.7 cell line following oxidative stress caused by PCV2 (viral) infection (Chen *et al.*, 2017). In addition, comparable data was also obtained from an *in vivo* study conducted by Wu *et al.* (2016), in their study, they discovered that PCV2 infection in mice induced oxidative stress by significantly reducing GSH and remarkably elevating GSSG level, and the introduction of flavonoids (post treatment) reversed the changes seen in GSH and GSSH level as well as restoring the ratio.

The nicotinamide adenine dinucleotide system (NADP/NADPH) is crucial to cellular oxidative stress defence system and reductive synthesis (Pollak *et al.*, 2007). Physiological redox balance is important for the development of the foetus and embryogenesis; overproduction of free radicals has detrimental effects on placentation and embryogenesis leading to disturbed metabolism and impaired intracellular milieu (Agarwal and Gupta, 2005). The data from this section showed that H/R insult significantly increased the NADP/NADPH ratio when compared to control cells. However, 24 h pre-treatment either flavonoids, their metabolite alone or in combination revealed that there was significant decrease in NADP/NADPH ratio when compared to H/R untreated cells. Cells pre-treated for 24 h prior to HR-induced oxidative stress reduced the NADP/NADPH ratio to almost baseline level (control cells). This decrease once again shows that flavonoids are capable of maintaining redox via mechanisms other than GSH/GSSG balancing in HTR-8/SVneo cells As explained before, there are limited studies about the effects of flavonoids on NADP/NADPH ratio during oxidative stress in both trophoblast and non-trophoblast cells; however, there are considerable evidence linking NADP/NADPH ratio to increases in oxidative stress (Itsumi *et al.*, 2015; Blacker and Duchen, 2016; Corpas and Barroso, 2014).

This current study has shown that flavonoids regulate oxidative stress caused by H/R insult by elevating GSH and decreasing GSSG as well as restoring the balance in NADP/NADPH ratio in HTR-8/SVneo cells, thereby maintaining the redox state.

4.4.3 ROS scavenging activity of flavonoids

To further investigate the antioxidant properties of flavonoids, their metabolites alone or in combinations prior to HR-induced oxidative stress on HTR-8/SVneo cells, their ability to scavenge free radical such as H_2O_2 was assessed using the ROS-Glo™ H_2O_2 assay. The data obtained from this assay indicated that flavonoids, their metabolites or combinations significantly scavenged H_2O_2 activity generated by HR-induced oxidative stress. H_2O_2 is generated from the dismutation of superoxide and it is an indicator of oxidative stress (Agarwal and Gupta, 2005). Increase in free radicals (especially H_2O_2) can have detrimental effects on the development of the placenta, remodelling of the maternal spiral arteries, the foetus, as well at the mother and it is known to mediate cell death via apoptosis. Thereby leading to health implications such as pre-eclampsia as a result of shallow invasion during early gestation period (Dennery, 2010). The anti-oxidative properties of flavonoids have been linked to their ability to scavenge free radicals which was shown in this present study. The mechanisms behind flavonoid anti-oxidant properties remain unclear although studies have speculated their ability to donate hydrogen molecules is not the only mechanism (Williams *et al.*, 2004). Another study suggested that the B ring hydroxyl group configuration (at position 3) plays an important role in the ability of flavonoids to scavenge ROS (Procházková *et al.*, 2011). Other mechanisms that have been suggested include their ability to interact with various antioxidant enzymes such as inducing phase II detoxifying enzymes (glutathione S-transferase) and regulation of protective gene expression (Procházková *et al.*, 2011). Herein, the data from this section, revealed that flavonoids can interact with other antioxidants as seen in the GSH and GSSG levels. In addition, flavonoids have been suggested to inhibit oxidase enzymes (e.g. xanthine oxidase) responsible for the production of superoxide leading to reduction in oxidative injures or cell death. (Procházková *et al.*, 2011). In a study conducted by Chuenkitiyanon, Pengsuparp and Jianmongkol (2010), they concluded that quercetin was protective against H_2O_2-induced tight junction disruption and hyperpermeability in the umbilical vein endothelial cell line (ECV304). In addition, morin was found to attenuate H_2O_2-induced apoptosis in the hamster lung fibroblast cell line V79-4 (Lee *et al.*, 2017). In an *in vivo* study, naringin and its metabolite, naringenin were found to significantly scavenge free radicals (superoxide and hydroxyl) in male Wistar rats livers induced with xanthine oxidase (enzymatic) and nitro blue tetrazolium (non-enzymatic) oxidative stress (Cavia-Saiz *et al.*, 2010). Flavonoids are known not only to influence the propagation reaction of free radicals but also their generation

by halting the enzymes involved in the production or by metal chelating activity (Procházková *et al.*, 2011; Cavia-Saiz *et al.*, 2010).

4.5   Conclusions

The data from this chapter showed that flavonoids, their metabolites alone or in combination exhibited cytoprotective effects against HR-induced oxidative stress by increasing cell viability and proliferation. Further investigations on their cytoprotective intracellular mechanisms revealed that their protective effects are brought about by maintaining balance in the redox state; such as elevating GSH and depleting GSSG level thereby restoring the GSH/GSSG ratio as well as their ability to balance the nicotinamide adenine dinucleotide system (NADP/NADPH ratio). The data also suggest that flavonoids, their metabolites alone or in combinations are potent scavengers of free radicals such H_2O_2 which is one of the major free radicals associated with increased ROS and oxidative stress.

Chapter 5
Effect of flavonoid-mediated cytoprotection on apoptosis
and
protein kinases activation

5.0   Introduction

As discussed in chapter 4, cells pre-treated for 24 h with flavonoids, their metabolites or in combinations prior to HR insult showed that they are associated with cytoprotection by increasing viability and proliferation, elevating GSH and depleting GSSG levels, scavenging of ROS (H_2O_2) and restoring the balance in NADP/NADPH ratio. Herein, the focus of this chapter is to determine their effects on apoptosis and their involvement in the activation or inhibition of protein kinases such as protein kinase B (PKB), extracellular signal-regulated kinases (ERK1/2), p38 mitogen-activated protein kinase (p38 MAPK) and stress-activated protein kinase/c-Jun N-terminal kinases (SAPK/JNK).

Overproduction of free radicals leading to oxidative stress has been linked to cell death by apoptosis. Apoptosis (or programmed cell death) is a natural occurrence of cell death, which is important for the normal development, functioning and maintenance of homeostasis for all multicellular organisms (Kannan and Jain, 2000; Sinha *et al.*, 2013). Changes in the redox state in favour of oxidation leads to the activation of cysteine proteases and caspases. Depending on sequences of activation during apoptosis, caspases can be classified into two groups, which are the initiator caspases (-2, -8, -9 and -10) and the effector/executioner caspases (-3, -6 and -7) (Sinha *et al.*, 2013; Tan *et al.*, 2016). There are three main mechanisms that result in the activation of caspases; first, receptor-ligand binding also known as the extrinsic pathway (via activation of caspase -8); second, intracellular stresses leading to a mitochondria-dependent mechanism also known as the intrinsic pathway (via activation of caspase-9) and finally, the involvement of the endoplasmic reticulum and activation of caspase-12 (Sinha *et al.*, 2013). Only the first and second mechanisms lead to the activation of the effector/executioner caspases (Sinha *et al.*, 2013). Summarising the first two mechanisms, receptor-ligand binding mechanism involves cell death receptors that are members of the tumour necrosis factor receptor super family and also involves transmitting of death signals from the surface to the inside of the cells (Sinha *et al.*, 2013). This pathway utilises ligands such as FasL/R, TNF-α/TNFR$_1$, which activate caspase-8 at the death-inducing signal complex resulting in two different mechanisms depending on the extent of activation. Normal activation of caspase-8 activates caspase-3 directly and low caspase-8 activation induces the activation of caspase-3 indirectly by involving the mitochondria (Barnhart *et al.*, 2003). The intrinsic /mitochondria-mediated pathway involves and plays a crucial role in the integration and circulation of death signals initiated from inside the cells such as oxidative stress and DNA damage. Mitochondria is the origin of excessive ROS production; and, in pathological states, become the target of oxidative stress assault resulting

in mitochondrial swelling, superoxide formation and cytochrome c leakage (Wu *et al.*, 2016). The main regulatory mechanism in this pathway is the release of cytochrome c into the cytoplasm, which triggers a rapid apoptotic response by activating cysteine proteases, caspase -9 and -3 (Sinha *et al.*, 2013; Kralova *et al.*, 2008).

Oxidative stress activates multiple signalling pathways that decide the fate of the cell i.e. either to die or survive. Among the signalling pathways are the mitogen-activated protein kinase (MAPK) cascades that transmit extracellular signals regulating survival, proliferation, cell cycle arrest, death, differentiation, migration, invasion, etc (Kralova *et al.*, 2008; Wada and Penninger, 2004; Sui *et al.*, 2014). To date, 14 members of the MAPK family have been established, including ERK (1/2), JNK (1/2/3) and p38 MAPK (Yao *et al.*, 2017). ERK activation is important for survival (anti-apoptotic), whereas p38 MAPK and JNK/SAPK activation are responsible and involved in apoptosis (pro-apoptotic). Different external stimuli such as oxidative stress and overproduction of free radicals can activate one or more MAPK pathways, subsequently activating a series of signal transductions and regulation to induce different physiological responses (Wada and Penninger, 2004; Yao *et al.*, 2017). Upon phosphorylation at the serine/threonine sites, MAPKs can further mediate the activation of downstream kinases in the cytoplasm or translocated into the nucleus through the nuclear pore to enhance transcription of genes that can either be a positive or negative outcome to the entire signalling cascades (Yao *et al.*, 2017; Wada and Penninger, 2004).

Another signalling pathway is that is crucial to this study is the protein kinase B or AKT (PKB/AKT) pathway. This pathway is a serine/threonine protein kinase that is regulated via the phosphoinositide 3-kinase (PI3k) pathway and it comprises of three isoforms (AKT1/2/3) that play unique or vague roles (Fayard *et al.*, 2010; Nicholson and Anderson, 2002). Like other signalling pathways, PKB is activated by stress stimuli but its activation mechanism is not fully understood. However, PKB/AKT phosphorylates and mediates the function of several cellular proteins responsible for glucose metabolism and transport, apoptosis, migration and proliferation (Fayard *et al.*, 2010; Burghardt *et al.*, 2018). Dysfunction in this pathway has been linked to numerous diseases including cancer, diabetes mellitus as well as pregnancy complications like preeclampsia (Burghardt *et al.*, 2018; Ferreira *et al.*, 2011)

The regulation of apoptosis by either PKB or MAPKs is very complex and still under debate. In this chapter, investigating the effects of flavonoids, their metabolites alone or combination

prior to H/R insult on caspase-3 and -7 activation, MAPKs and protein kinase B activation was important to further understand flavonoid-mediated cytoprotection.

5.1   Results

In this chapter, the pro-apoptotic effects of H/R-induced oxidative stress and anti-apoptotic effects of 24 h pre-treatment with the indicated flavonoids, their metabolites alone or in combinations was investigated using Caspase-Glo® 3/7 assay and MAPK activation status via western blot.

5.1.1   The modulation of flavonoids on caspase 3/7

The data from the Caspase-Glo® assay as shown in figure 5.1 (A-D) indicates that H/R insult activates caspase -3 and -7. Furthermore 24 h pre-treatment with flavonoids (quercetin, morin, naringin or hesperidin) prior to H/R insult significantly reduced the H/R-induced activity of caspase -3/-7 in HTR-8/SVneo cells suggesting that flavonoids pre-treatment attenuates the activation of caspases.
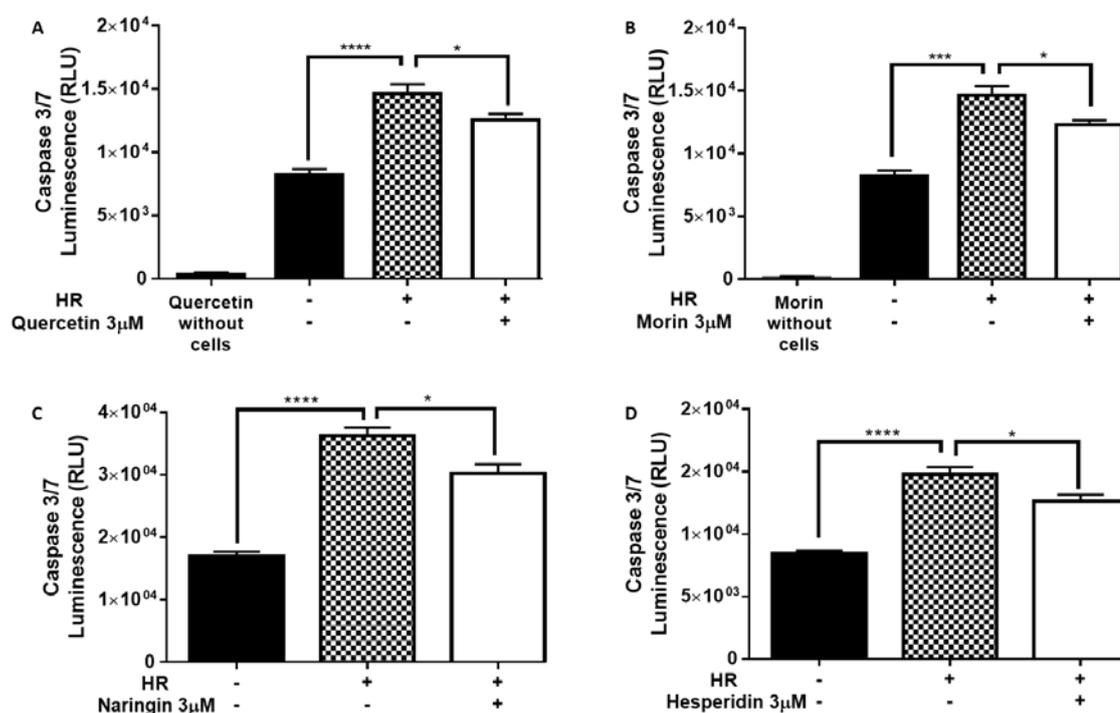
Figure 5.1: Modulation by flavonoids of caspase 3/7 activation in response to HR-induced oxidative stress.

HTR-8/SVneo cells were treated with the indicated flavonoids at 3 µM for 24 h prior to HR-induced oxidative stress. Caspase 3/7 activation was measured using Caspase-Glo® Assay (A-D). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against HR). Data are expressed as relative luminescence units and represent the mean ± SEM of at least three independent experiments. *P<0.05, ***P<0.001 and ****P<0.0001 versus HR.

Comparable data were achieved from their respective metabolites (Q3G, naringenin or hesperetin). As seen in figure 5.2 (A-C), there was a significant reduction in the caspase -3/-7 activity from cells pre-treated with Q3G, naringenin or hesperetin (24 h) prior to H/R insult.
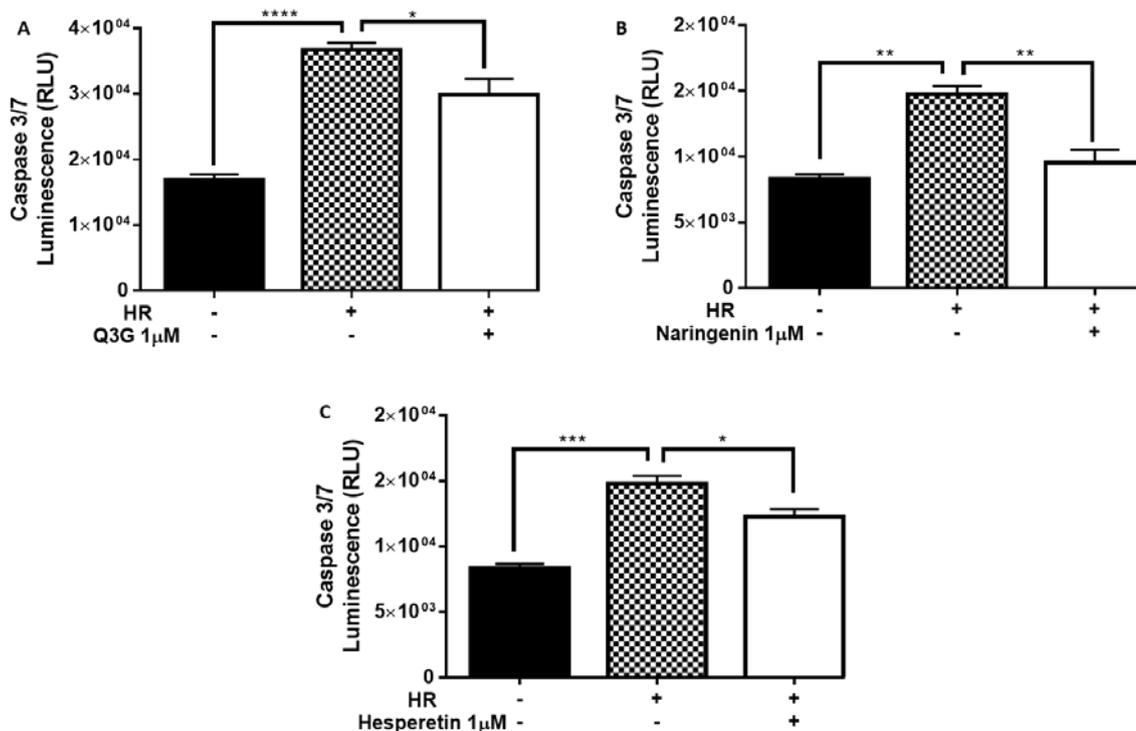
Figure 5.2: Effect of flavonoid metabolites on caspase 3/7 activation in response HR-induced oxidative stress.

HTR-8/SVneo cells were treated with the indicated metabolites at 1 µM for 24 h prior to HR-induced oxidative stress. Caspase 3/7 activation was measured using Caspase-Glo® Assay (A-C). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against HR). Data are expressed as relative luminescence units and represent the mean ± SEM of at least three independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$ versus HR.

Further investigation was carried out to study the effects of flavonoid-metabolite and flavonoid combinations on apoptosis (caspase 3/7). As seen in figure 5.3 (A-D) and 5.4 (A-C), there was a significant reduction in H/R-induced caspase (3/7) activity in cells pre-treated with either flavonoid-metabolite (quercetin/Q3G, morin/Q3G, naringin/naringenin or hesperidin/hesperetin) or flavonoid combinations (quercetin/morin, quercetin/naringin or quercetin/hesperidin).
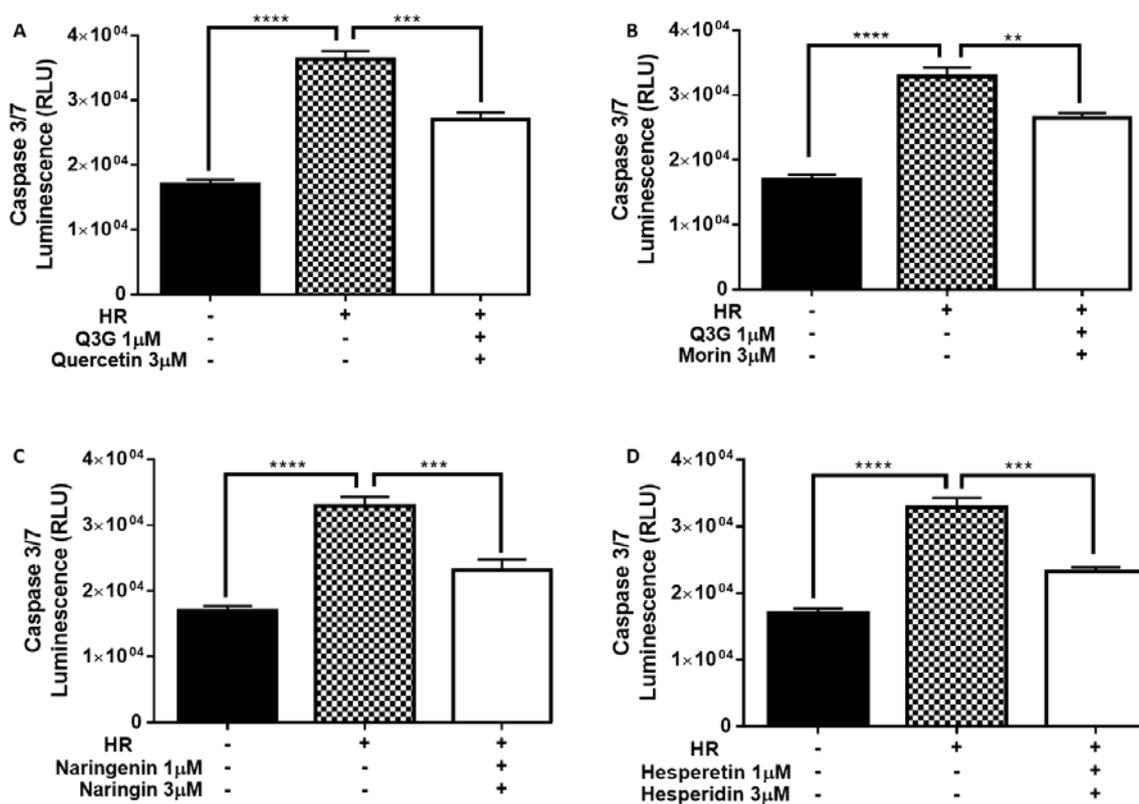
Figure 5.3: Effect of flavonoid-metabolite combinations on caspase 3/7 activation in response HR-induced oxidative stress.

HTR-8/SVneo cells were treated with the indicated flavonoids at 3 µM and metabolites at 1 µM for 24 h prior to HR-induced oxidative stress. Caspase 3/7 activation was measured using Caspase-Glo® Assay (A-D). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against HR). Data are expressed as relative luminescence units and represent the mean ± SEM of at least three independent experiments. **P<0.01, ***P<0.001 and ****P<0.0001 versus HR.
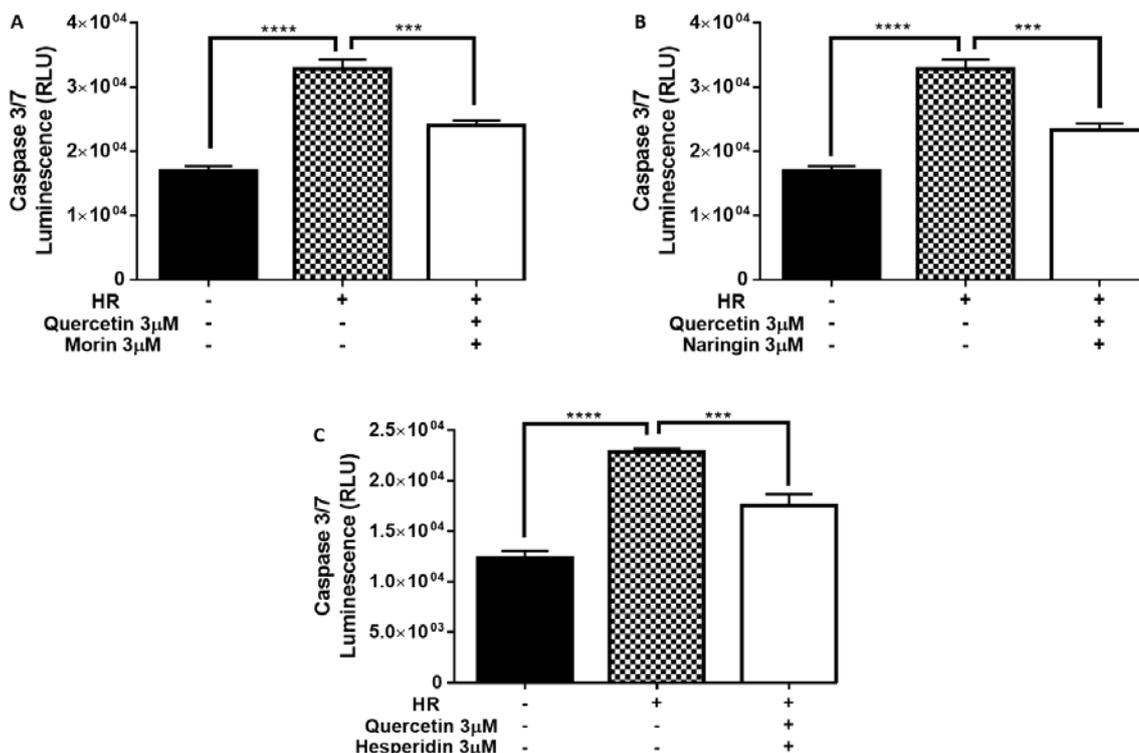
Figure 5.4: Effect of flavonoid combinations on caspase 3/7 activation in response HR-induced oxidative stress.

HTR-8/SVneo cells were treated with the indicated combination of flavonoids at 3 µM (individually) for 24 h prior to HR-induced oxidative stress. Caspase 3/7 activation was measured using Caspase-Glo® Assay (A-C). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against HR). Data are expressed as relative luminescence units and represent the mean ± SEM of at least three independent experiments. ***P<0.001 and ****P<0.0001 versus HR.

5.1.2 Involvement of protein kinases in flavonoid-mediated cytoprotection

As previously observed in chapter 4, 24 h pre-treatment with 3 µM of flavonoids and 1 µM of metabolites or in combinations was able to induce significant cytoprotection in HTR-8/SVneo against HR-induced oxidative stress. Here the intracellular mechanism of the cytoprotective effect was investigated using western blot analysis to assess involvement of total and phosphorylated protein kinases associated with cell survival and death; PKB/AKT, ERK1/2, SAPK/JNK and p38 MAPK were investigated. As seen in figures 5.4-8, H/R insult consistently induced significant activation of PKB, ERK1/2, SAPK/JNK and p38 MAPK in HTR-8/SVneo cells. Furthermore, the data from figure 5.5-5.8 (A-D) showed that 24 h pre-treatment with quercetin, morin, naringin and hesperidin inhibited HR-induced activation of PKB, ERK/1/2, p38 MAPK and JNK.
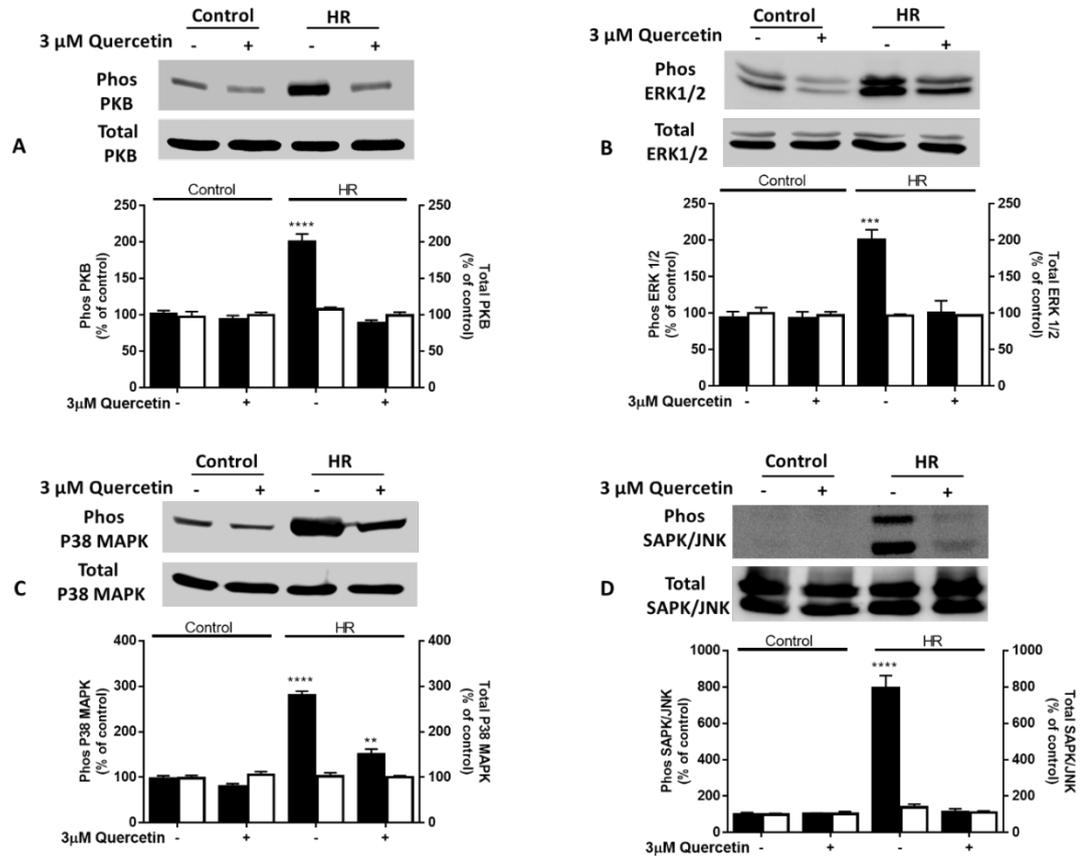
Figure 5.5: Effect of quercetin on HR-induced protein kinase activation
HTR-8/SVneo cells were treated with the 3 μM quercetin for 24 h prior to HR-induced oxidative stress. Cells were then lysed and western blotting was performed using specific antibodies (phosphorylated and total). (A) effects of quercetin on HR-induced activation of PKB, (B) effects of quercetin on HR-induced activation of ERK1/2, (C) effects of quercetin on HR-induced activation of P38 MAPK and (D) effects of quercetin on HR-induced activation of SAPK/JNK. Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test). Data are expressed as percentage of control and represent the mean ± SEM of at least three independent experiments. **P<0.01 and ****P<0.0001 versus control untreated.
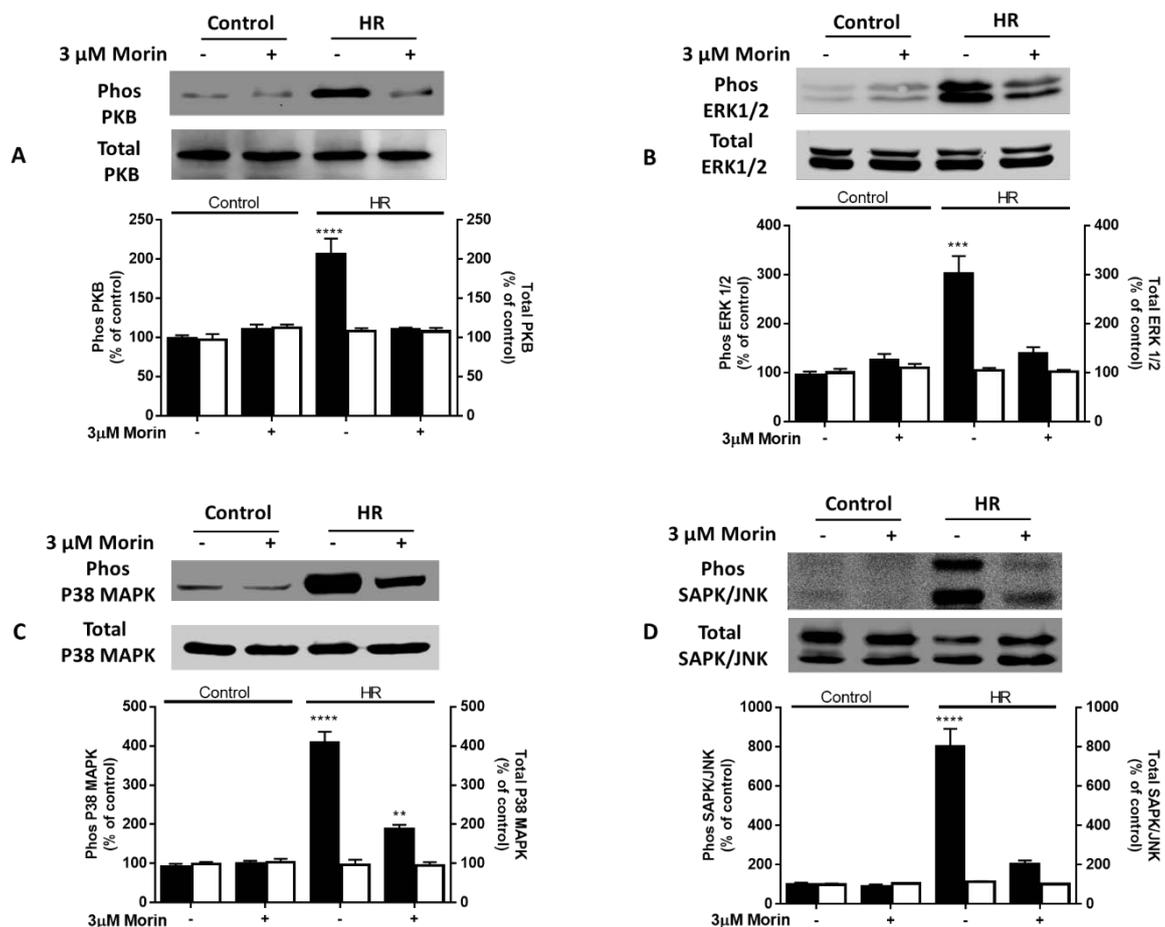
Figure 5.6: **Effect of morin on HR-induced protein kinase activation.**
HTR-8/SVneo cells were treated with or without 3 µM of morin for 24 h prior to HR-induced oxidative stress exposure. Cells were then lysed and western blotting was performed using specific antibodies (phosphorylated and total). (A) effects of morin on HR-induced activation of PKB, (B) effects of morin on HR-induced activation of ERK1/2, (C) effects of morin on HR-induced activation of P38 MAPK and (D) effects of morin on HR-induced activation of SAPK/JNK. Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test). Data are expressed as percentage of control and represent the mean ± SEM of at least three independent experiments. ***P<0.001 and ****P<0.0001 versus control untreated.

Figure 5.7: Effect of naringin on HR-induced protein kinase activation.

HTR-8/SVneo cells were treated with or without 3 µM of naringin for 24 h prior to HR-induced oxidative stress exposure. Cells were then lysed and western blotting was performed using specific antibodies (phosphorylated and total). (A) effects of naringin on HR-induced activation of PKB, (B) effects of naringin on HR-induced activation of ERK1/2, (C) effects of naringin on HR-induced activation of P38 MAPK and (D) effects of naringin on HR-induced activation of SAPK/JNK. Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test). Data are expressed as percentage of control and represent the mean ± SEM of at least three independent experiments. *P<0.05, ***P<0.001 and ****P<0.0001 versus control untreated.

Figure 5.8: Effect of hesperidin on HR-induced protein kinase activation.
HTR-8/SVneo cells were treated with or without 3 µM of hesperidin for 24 h prior to HR-induced oxidative stress exposure. Cells were then lysed and western blotting was performed using specific antibodies (phosphorylated and total). (A) effects of naringin on HR-induced activation of PKB, (B) effects of naringin on HR-induced activation of ERK1/2, (C) effects of naringin on HR-induced activation of p38 MAPK and (D) effects of naringin on HR-induced activation of SAPK/JNK. Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test). Data are expressed as percentage of control and represent the mean ± SEM of at least three independent experiments. ***P<0.001 and ****P<0.0001 versus control untreated.

Similar sets of data were obtained from their respective metabolites, as seen in figures 5.9 - 5.11 (A-D respectively). Cells pre-treated with Q3G, naringenin and hesperetin fully inhibited HR-induced PKB, ERK1/2, p38 MAPK and JNK activation. Comparing the pre-treated (Q3G, naringenin and hesperetin) with untreated control showed that there was not significant difference in protein kinase activation as shown in figure 5.9 – 11.
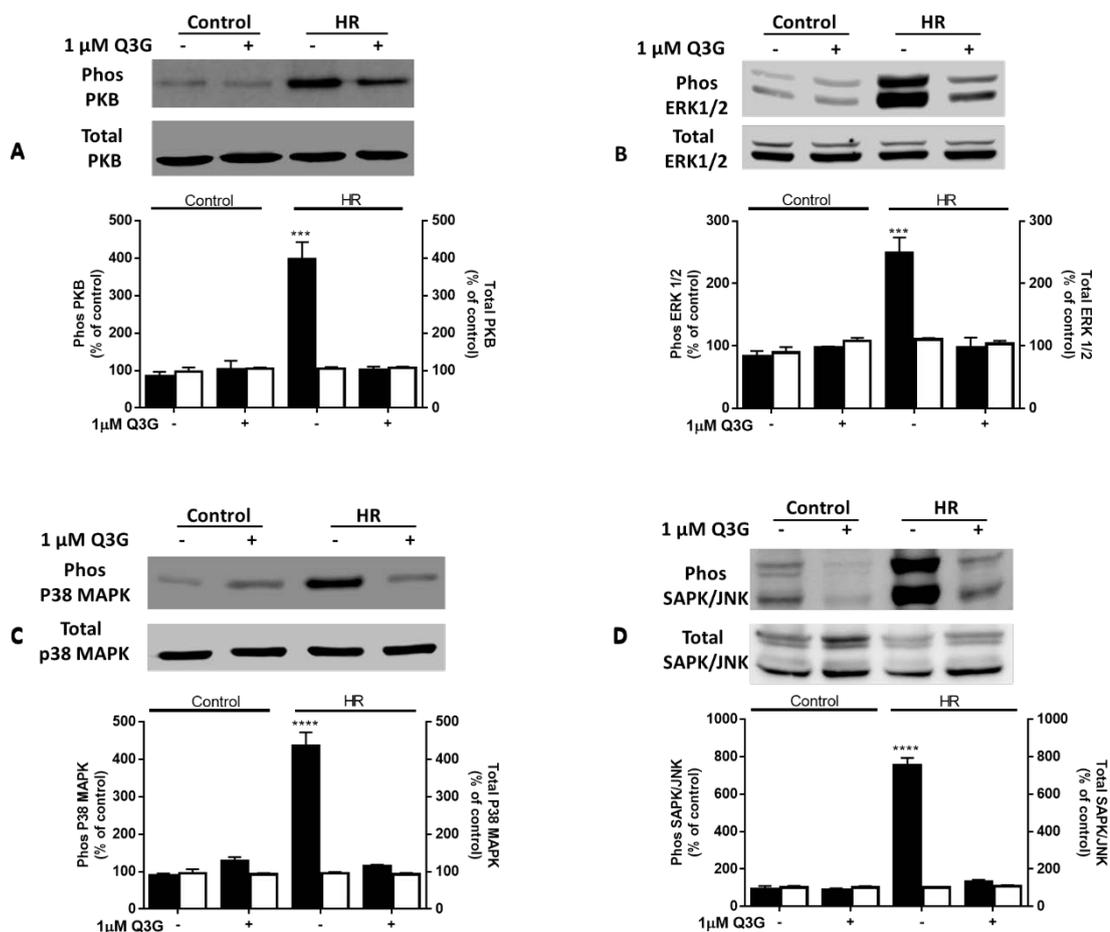
Figure 5.9: Effect of Q3G on HR-induced protein kinase activation.

HTR-8/SVneo cells were treated with or without 1 µM of Q3G for 24 h prior to HR-induced oxidative stress exposure. Cells were then lysed and western blotting was performed using specific antibodies (phosphorylated and total). (A) effects of Q3G on HR-induced activation of PKB, (B) effects of Q3G on HR-induced activation of ERK1/2, (C) effects of Q3G on HR-induced activation of p38 MAPK and (D) effects of Q3G on HR-induced activation of SAPK/JNK. Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test). Data are expressed as percentage of control and represent the mean $\pm$ SEM of at least three independent experiments. ***$P<0.001$ and ****$P<0.0001$ versus control untreated.
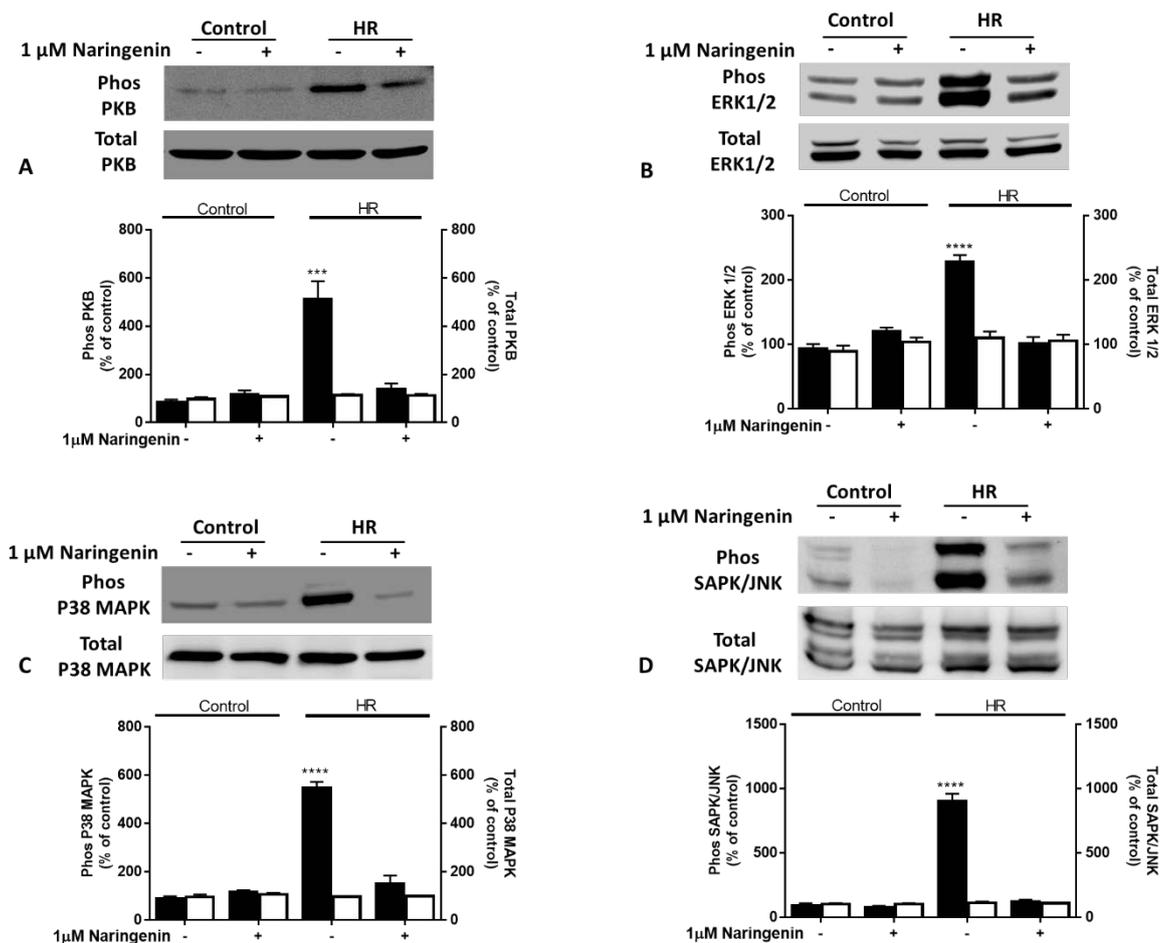
Figure 5.10: Effect of naringenin on HR-induced protein kinase activation.
HTR-8/SVneo cells were treated with or without 1 µM of naringenin for 24 h prior to HR-induced oxidative stress exposure. Cells were then lysed and western blotting was performed using specific antibodies (phosphorylated and total). (A) effects of naringenin on HR-induced activation of PKB, (B) effects of naringenin on HR-induced activation of ERK1/2, (C) effects of naringenin on HR-induced activation of p38 MAPK and (D) effects of naringenin on HR-induced activation of SAPK/JNK. Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test). Data are expressed as percentage of control and represent the mean ± SEM of at least three independent experiments. ***$P<0.001$ and ****$P<0.0001$ versus control untreated.

Figure 5.11: Effect of hesperetin on HR-induced protein kinase activation.
HTR-8/SVneo cells were treated with or without 1 µM of hesperetin for 24 h prior to HR-induced oxidative stress exposure. Cells were then lysed and western blotting was performed using specific antibodies (phosphorylated and total). (A) effects of hesperetin on HR-induced activation of PKB, (B) effects of hesperetin on HR-induced activation of ERK1/2, (C) effects of hesperetin on HR-induced activation of p38 MAPK and (D) effects of hesperetin on HR-induced activation of SAPK/JNK. Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test). Data are expressed as percentage of control and represent the mean ± SEM of at least three independent experiments. ***$P<0.001$ and ****$P<0.0001$ versus control untreated.

Further investigation was carried out to monitor the effect of pre-treatment with either flavonoid combinations or flavonoid-metabolite combinations on HR-induced protein kinase activation. As seen from the data in figure 5.12 – 5.18 (A-D respectively), flavonoid-metabolite combinations (quercetin/Q3G, morin/Q3G, naringin/naringenin or hesperidin/hesperetin) or flavonoid combinations (quercetin/morin, quercetin/naringin or quercetin/hesperidin) significantly inhibited HR-induced protein kinase activation.
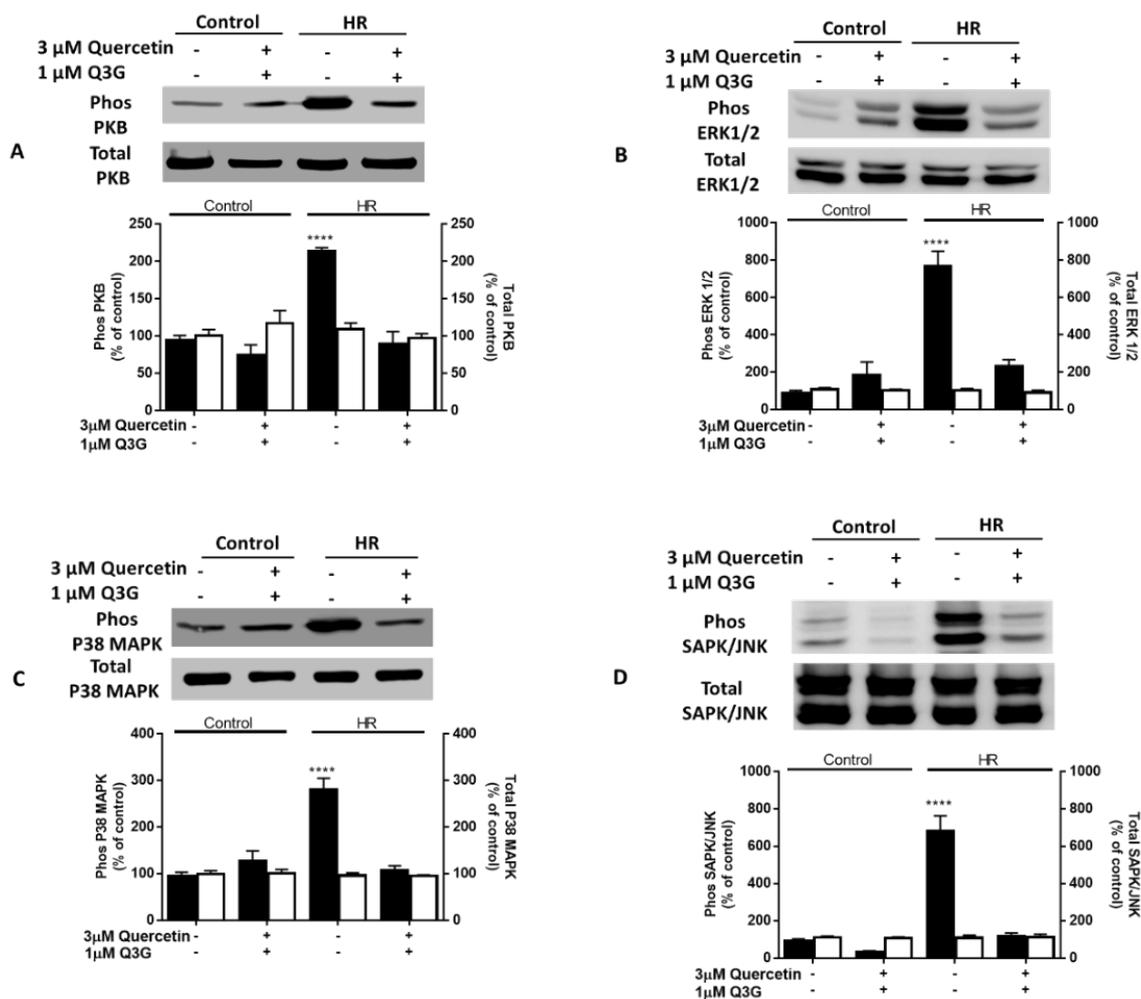
Figure 5.12: Effects of quercetin and Q3G combination on HR-induced protein kinase activation.

HTR-8/SVneo cells were treated with or without 3 µM quercetin and 1 µM Q3G combination for 24 h prior to HR-induced oxidative stress exposure. Cells were then lysed and western blotting was performed using specific antibodies (phosphorylated and total). (A) effects of quercetin and Q3G combination on HR-induced activation of PKB, (B) effects of quercetin and Q3G combination on HR-induced activation of ERK1/2, (C) effects of quercetin and Q3G combination on HR-induced activation of p38 MAPK and (D) effects of quercetin and Q3G combination on HR-induced activation of SAPK/JNK. Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test). Data are expressed as percentage of control and represent the mean ± SEM of at least three independent experiments. ****$P<0.0001$ versus control untreated.

Figure 5.13: Effects of morin and Q3G combination on HR-induced protein kinase activation.

HTR-8/SVneo cells were treated with or without 3 μM morin and 1 μM Q3G combination for 24 h prior to HR-induced oxidative stress exposure. Cells were then lysed and western blotting was performed using specific antibodies (phosphorylated and total). (A) effects of morin and Q3G combination on HR-induced activation of PKB, (B) effects of morin and Q3G combination on HR-induced activation of ERK1/2, (C) effects of morin and Q3G combination on HR-induced activation of p38 MAPK and (D) effects of morin and Q3G combination on HR-induced activation of SAPK/JNK. Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test). Data are expressed as percentage of control and represent the mean ± SEM of at least three independent experiments. ****$P<0.0001$ versus control untreated.

Figure 5.14: Effects of naringin and naringenin combination on HR-induced protein kinase activation.

HTR-8/SVneo cells were treated with or without 3 µM naringin and 1 µM naringenin combination for 24 h prior to HR-induced oxidative stress exposure. Cells were then lysed and western blotting was performed using specific antibodies (phosphorylated and total). (A) effects of naringin and naringenin combination on HR-induced activation of PKB, (B) effects of naringin and naringenin combination on HR-induced activation of ERK1/2, (C) effects of naringin and naringenin combination on HR-induced activation of p38 MAPK and (D) effects of naringin and naringenin combination on HR-induced activation of SAPK/JNK. Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test). Data are expressed as percentage of control and represent the mean ± SEM of at least three independent experiments. ***$P<0.001$ and ****$P<0.0001$ versus control untreated.

Figure 5.15: Effects of hesperidin and hesperetin combination on HR-induced protein kinase activation.

HTR-8/SVneo cells were treated with or without 3 µM hesperidin and 1 µM hesperetin combination for 24 h prior to HR-induced oxidative stress exposure. Cells were then lysed and western blotting was performed using specific antibodies (phosphorylated and total). (A) effects of hesperidin and hesperetin combination on HR-induced activation of PKB, (B) effects of hesperidin and hesperetin combination on HR-induced activation of ERK1/2, (C) effects of hesperidin and hesperetin combination on HR-induced activation of p38 MAPK and (D) effects of hesperidin and hesperetin combination on HR-induced activation of SAPK/JNK. Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test). Data are expressed as percentage of control and represent the mean ± SEM of at least three independent experiments. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 versus control untreated.

Figure 5.16: Effect of quercetin and morin combination on HR-induced protein kinase activation.

HTR-8/SVneo cells were treated with or without 3 µM of quercetin and 3 µM of morin combination for 24 h prior to HR-induced oxidative stress exposure. Cells were then lysed and western blotting was performed using specific antibodies (phosphorylated and total). (A) effects of quercetin and morin combination on HR-induced activation of PKB, (B) effects of quercetin and morin combination on HR-induced activation of ERK1/2, (C) effects of quercetin and morin combination on HR-induced activation of p38 MAPK and (D) effects of quercetin and morin combination on HR-induced activation of SAPK/JNK. Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test). Data are expressed as percentage of control and represent the mean ± SEM of at least three independent experiments. ****$P<0.0001$ versus control untreated.

Figure 5.17: Effect of quercetin and naringin combination on HR-induced protein kinase activation.

HTR-8/SVneo cells were treated with or without 3 µM of quercetin and 3 µM of naringin combination for 24 h prior to HR-induced oxidative stress exposure. Cells were then lysed and western blotting was performed using specific antibodies (phosphorylated and total). (A) effects of quercetin and naringin combination on HR-induced activation of PKB, (B) effects of quercetin and naringin combination on HR-induced activation of ERK1/2, (C) effects of quercetin and naringin combination on HR-induced activation of p38 MAPK and (D) effects of quercetin and naringin combination on HR-induced activation of SAPK/JNK. Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test). Data are expressed as percentage of control and represent the mean ± SEM of at least three independent experiments. ***P<0.001 and ****P<0.0001 versus control untreated.

Figure 5.18: Effect of quercetin and hesperidin combination on HR-induced protein kinase activation.

HTR-8/SVneo cells were treated with or without 3 μM of quercetin and 3 μM of hesperidin combined for 24 h prior to HR-induced oxidative stress exposure. Cells were then lysed and western blotting was performed using specific antibodies (phosphorylated and total). (A) effects of quercetin and hesperidin combination on HR-induced activation of PKB, (B) effects of quercetin and hesperidin combination on HR-induced activation of ERK1/2, (C) effects of quercetin and hesperidin combination on HR-induced activation of P38 MAPK and (D) effects of quercetin and hesperidin combination on HR-induced activation of SAPK/JNK. Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test). Data are expressed as percentage of control and represent the mean ± SEM of at least three independent experiments. *P<0.05, ***P<0.001 and ****P<0.0001 versus control untreated.

5.2   Results Summary

The flavonoids quercetin, morin, naringin and hesperidin significantly reduced caspase -3/-7 activity during HR-induced oxidative stress. The data also revealed that H/R insult induces apoptosis as seen from the caspase 3/7 assay. Similarly, their respective metabolites (Q3G, naringenin, hesperetin), and their combinations (flavonoid-metabolite/flavonoids) significantly reduced the activity of caspase -3/-7. In addition, flavonoid-metabolite/flavonoids combinations showed very significant reductions in caspase -3/-7 activity compared to single flavonoid treatment. The cytoprotective effects of pre-treatment with flavonoids, their metabolites or combinations is likely to be associated with an inhibitory effect on HR-induced protein kinase activation as shown in figure 5.5 – 5.18 (A-D), which may suggest that this is a potential cytoprotective mechanism.

5.3   Discussion

The aim of this chapter was to investigate the effect of 24 h pre-treated cells on HR-induced oxidative stress apoptosis (caspase 3/7) and total/phosphorylated protein kinases (PKT/AKT, ERK1/2, SAPK/JNK and p38 MAPK) in HTR-8/SVneo as a potential mechanism for flavonoid-mediated cytoprotection.

5.3.1   Flavonoid modulation of apoptosis

The data from Caspase-Glo® 3/7 assays revealed that HR-induced oxidative significantly increased the activity of executioner caspases (3/7) in HTR-8/SVneo cells. These data are in agreement with several studies that showed that oxidative stress induces apoptosis; for example, in non-trophoblast cell origin such as H_2O_2-induced caspase activation in human epithelial RPE cell *in vitro*, human melanoma M14 cell line (Kook *et al.*, 2008; Clément *et al.*, 1998). With Respect to trophoblast cell origin, H/R- and H_2O_2-induced oxidative stress significantly activated caspases in HTR-8/SVneo, BEWO, JEG-3 cells (the latter two are originated from choriocarcinoma) (Kilburn, 2000; Hung *et al.*, 2002; Leach *et al.*, 2008; Aouache *et al.*, 2018). Pre-treatment (24 h) with either flavonoids, their metabolites or combinations significantly reduced HR-induced caspase 3/7 activation in HTR-8/SVneo cells. Although the effects of flavonoids or their metabolites on trophoblast cells have not been explored before, studies have revealed that they are potent inhibitors of caspase during oxidative stress in non-trophoblast cells. For example; quercetin significantly reduced the activation of caspase -3 in RPE cells exposed to H_2O_2-induced oxidative stress (Kook *et al.*, 2008). Morin was found to attenuate H_2O_2-induced apoptosis in hamster lung V79-4 fibroblasts cells and significantly reduce caspase -3 activity in rat primary cells exposed to high glucose-induced oxidative stress which mimics diabetes mellitus (Lee *et al.*, 2017;

Kapoor and Kakkar, 2012). Furthermore, naringin was found to significantly attenuate apoptosis in mouse leukaemia P388 cells from H_2O_2-induced cell death as well as significantly reducing caspase -3 activity in human neuroblastoma SH-SY5Y cells following rotenone-induced cell death (Kim *et al.*, 2009; Kanno *et al.*, 2003). Hesperidin significantly downregulated the expression of caspase -3 in human keratinocyte (HaCaT) cell line during ultraviolet B-induced cell apoptosis (Hewage *et al.*, 2016). Hesperidin was also found to effectively ameliorate RPE (ARPE-19) cell line from high glucose-induced damages by reducing the activity of caspase 3/9 (Liu *et al.*, 2018). In addition, their respective metabolites have also been found to reduce apoptosis during oxidative stress by downregulating the expression of executioner caspases such as -3, -7 and -9 (Kapoor *et al.*, 2013; Han *et al.*, 2008; Bai *et al.*, 2017). Apoptosis is important in the maintenance of tissue homeostasis by equilibrating the balance between cell survival and death, and therefore must be tightly regulated. During gestation, unregulated apoptosis resulting from increased oxidative stress leads to trophoblast death. Trophoblast death leads to poor placentation and shallow trophoblast invasion, resulting in incomplete maternal spiral arteries remodelling, which eventually results in pregnancy complications such as spontaneous pregnancy loss, intrauterine growth restriction (IUGR), gestational diabetes mellitus, and preeclampsia (Aouache *et al.*, 2018).

5.3.2 Effect of flavonoids on HR-induced phosphorylation of protein kinases

Protein kinase activation is a key decider of cell fate by either mediating cell survival or death. Herein, the data obtained indicated that HR-induced oxidative stress activated all four phosphorylated protein kinases. These findings agreed with others that reported oxidative stress-induced phosphorylation of protein kinase activation *in vitro*. It has also been reported that H_2O_2 induced phosphorylation of protein kinase (PKB, ERK1/2, SAPK/JNK and p38 MAPK) in cardiomyocyte (H9c2) cells and epithelial tight junction (ECV304) cells (Daubney *et al.*, 2015; Chuenkitiyanon *et al.*, 2010). In this present study, the introduction (24 h pre-treatment) of flavonoids, their metabolites alone or in combinations significantly inhibited HR-induced protein kinase activation (PKB, ERK1/2, SAPK/JNK and p38 MAPK). Pre-treatment of cells (24 h) with flavonoids/metabolites alone or combination had no effects on the levels of phosphorylated forms of PKB, ERK1/2, SAPK/JNK and p38 MAPK in the absence of H/R. However, pre-treatment (24 h) prior to HR-induced oxidative stress significantly inhibited the HR-induced phosphorylation of PKB, ERK1/2, SAPK/JNK and p38 MAPK. Herein, the data indicated that pre-treated cells showed significant anti-apoptotic (SAPK/JNK and p38 MAPK inhibition) effect against HR-induce oxidative stress.

In other studies, naringin was concluded to significantly inhibit SAPK/JNK and p38 MAPK activation against rotenone-induce apoptosis in human SH-SY5Y cells (Kim *et al.*, 2009). Morin was found to significantly inhibit the phosphorylation of p38 MAPK against *t*-BHP induced ROS generation in YPEN-1 cells (Kim *et al.*, 2010). In addition, quercetin successfully inhibited the activation of phosphorylated p38 MAPK in ECV304 cells against H_2O_2-induce protein kinase activation whilst hesperidin significantly inhibited the activation of phosphorylated p38 MAPK and JNK against high glucose-induced protein kinase activation in retinal ganglion (RGC-5) cells (Chuenkitiyanon *et al.*, 2010; Chen *et al.*, 2010). It is known that the anti-oxidative properties of flavonoids are capable of preventing MAPK activation under oxidative state as seen in these previous studies. This present study supports the protective role of flavonoids, their metabolites alone or combinations in HTR-8/SVneo cells exposed to H/R insult via the inhibition of phosphorylation of p38 MAPK and SAPK/JNK activation.

Exploring the role of pro-survival/anti-apoptotic protein kinases (ERK1/2 and PKB), flavonoids, their metabolites or combinations also inhibited their activation against HR-induced activation. ERK1/2 and PKB activation is generally considered to be pro-survival but under certain circumstances, they can have pro-apoptotic functions (Lu and Xu, 2006; Song *et al.*, 2008). Traditionally, the ERK1/2 signalling cascade consists of Ras-Raf family members followed by MEK1/2 activation. PKB activation is mediated via PI3K as described earlier (Lu and Xu, 2006; Lee *et al.*, 2003; Martindale and Holbrook, 2002). The activation of ERK1/2 have been suggested to inhibit apoptosis in response to several stimuli, however, their activation in response to cellular DNA damage fosters apoptosis by increasing the activity of some pro-apoptotic signalling molecules such as BAX, cytochrome *c* release, caspase 3 activation, and p53 transcriptional factors (Lu and Xu, 2006).

Activation of PKB in an important mediator of cell survival in response to growth factor withdrawal, disturbance in cell cycle and extracellular matrix detachment but there have been controversies over the role of their activation under other conditions of stress (Martindale and Holbrook, 2002). Other studies have shown oxidative stress-mediated activation of ERK1/2 and PKB whilst the introduction of flavonoids or their metabolites significantly inhibited this activation to promote cell survival (Kim *et al.*, 2010). This inhibition during oxidative stress is likely to be induced through a pathway independent of MEK1 and PI3K inhibition. However, how these protein kinases promote apoptosis is still unclear. The intricate balance between cell survival and cell death pathways is an important determinant of cell fate, 24 h pre-treatment with flavonoids, their metabolites or in

combination have shown to balance HTR-8/SVneo cell fate during HR-induced oxidative stress.

5.4   Conclusions

To conclude this chapter, 24 h pre-treatment with either flavonoids, their metabolites alone or in combination significantly reduced HR-induced apoptosis in HTR-8/SVneo cells by reducing the activation of caspases (3/7) resulting in trophoblast cell survival and potentially preventing poor placentation. This cytoprotective effect of 24 h pre-treatment of cells with flavonoids were also associated with their ability to significantly inhibit the HR-induced protein kinase phosphorylation (PKB, ERK, JNK and p38 MAPK), thereby deciding cell fate (pro-survival).

Chapter 6
The effects of flavonoids on trophoblast invasion under oxidative stress

6.0   Introduction

As previously described in chapter 1, immediately after implantation, the trophoblast cell layers of the blastocysts proliferate into the two pathways, namely; extravillous (highly invasive/less migratory) and villous (non-invasive/migratory) trophoblast cells (Gude *et al.*, 2004). It is the former extra-villous trophoblast cells that invade the maternal spiral arteries and are responsible for the physiological conversion of these arteries into a high flow system. This is the key to a successful human pregnancy (Burton *et al.*, 2009; Burton *et al.*, 2010). Proper implantation and placental development/function are crucial to the well-being of the mother and foetus during gestation. However, defects in these processes (maternal-foetal interface) especially the poor invasion leading to insufficient spiral artery remodelling can lead to foetal growth restriction, preeclampsia, foetal/maternal death, maternal/foetal immune disturbance, and miscarriage (Pereira *et al.*, 2015; Yu qi Li *et al.*, 2017). Thus, insufficient invasion and incomplete spiral artery remodelling have been associated with placental oxidative stress that results from overproduction of free radicals (ROS) as a result of a prolonged hypoxic environment, hypoxia/reoxygenation. This prolonged hypoxic insult is usually aggravated in conditions such as maternal obesity and smoking (Whitley and Cartwright, 2010; Yu qi Li *et al.*, 2017; Pereira *et al.*, 2015). Therefore, investigating the anti-oxidative effects of dietary flavonoids, their respective metabolites or combinations during trophoblast cell (HTR-8/SVneo cells as an *in vitro* model of extravillous trophoblast) invasion under H/R insult is crucial to this study.

Another unique feature of the placenta is that it comprises of cytotrophoblast stem cells, which are attached to the basement membrane of the trophoblast cells. These cytotrophoblast stem cell populations are responsible for the promotion of self-renewal processes, which are proposed to play a vital role in the generation of intraembryonic lineage (Ferretti *et al.*, 2007; Red-Horse *et al.*, 2004). Apart from their self-renewal function, cytotrophoblast stem cells also play an important role during trophoblast invasion, differentiation as well as maturation of the maternal-foetal interface (Red-Horse *et al.*, 2004). Although there are a limited number of reports assessing the generation and function of cytotrophoblast stem cells during oxidative stress. High levels of free radicals from ROS generation impair stem cell function by mediating the activation of signalling pathways that inhibit self-renewal, exhaustion of stem-cell population, pre-mature aging as well as defective DNA repair machinery (Schieber and Chandel, 2014; Cieślar-Pobuda *et al.*, 2017).

6.1 Aims

The aims of this chapter are to assess the effect of 24 h pre-treatment of HTR-8/SVneo cells with flavonoids, their respective metabolites alone or in combinations prior to HR-induced oxidative stress on the ability of transformed first trimester trophoblast cell line HTR-8/SVneo to:

a) invade through Basement Membrane Extract (BME) (in vitro 2D invasion) and

b) generate spheroidal stem-like cells under normoxic and hypoxic conditions.

Experiments also compared the ability of these self-assembling spheroidal stem-like cells to invade a 3-D invasion under normoxia and hypoxia conditions and to identify whether the flavonoids are capable of assisting invasion under severe hypoxia. Finally, using proteomic analysis the potential intracellular proteins/molecules that were involved in flavonoid-mediated cytoprotection during invasion were identified.

6.3 Results

In vitro invasion assay was carried out first using 2D Matrigel, followed by the optimisation and generation of spheroids from either flavonoid-treated or untreated HTR-8/SVneo cells after H/R insult. The invasive capacity of the spheroids was generated subsequently assessed using 3D spheroid BME.

6.3.1 Effects of flavonoids on 2D *in vitro* cell invasion

2-D invasion assay was carried out using Corning® BioCoat™ Tumour Invasion system as previously described in section 2.10. This investigation was performed to monitor the effects of HR-induced oxidative stress on HTR-8/SVneo cells with or without 24 h pre-treatment with the indicated flavonoids (quercetin, morin, naringin or hesperidin), their respective metabolites alone (Q3G, naringenin or hesperetin) or their combinations (flavonoid/flavonoid and metabolite/flavonoid). The number of cells invaded and migrated were analysed by using WimCounting (Wimasis®) and represented as either percentage invasion (number of cells invaded/number of cells migrated x 100) or number of cells invaded using GraphPad Prism statistical software.

The data from figure 6.1 to 6.4 showed that H/R insult significantly reduced the invasive capacity of HTR-8/SVneo. Interestingly, it did not affect the migratory capacity of HTR-8/SVneo cells (data given in appendix A.2 – 5). However, cells pre-treated (24 h) with either quercetin, morin, naringin or hesperidin prior to HR-induced oxidative stress significantly increased the invasiveness of HTR-8/SVneo cells when compared to H/R as shown in figure 6.1 (A-C). Comparing the migration control plate, there was no significant difference

between H/R and pre-treated (flavonoids) cells (see appendix A.2). This data indicates that HR-induced oxidative stress does not have any significant effect on migration but significantly decreases the invasion of HTR-8/SVneo cells. Furthermore, flavonoids do not enhance migration but significantly promote invasion. Similar results were achieved with or without migration control (B and C).

In the case of their respective metabolites (Q3G, naringenin and hesperetin), comparable results were achieved from those of flavonoids. As shown in figures 6.2, pre-treatment with the indicated metabolites alone prior to H/R insult significantly increased the invasion of HTR-8/SVneo cells. Interestingly there was a significant decrease in the migration of cells pre-treated with naringenin and hesperetin but not Q3G when compared to H/R untreated cells (see appendix A.3). Further comparison of cell migration of metabolites pre-treated cell with control did not indicate any significant decrease in migration (see appendix A.3). Again, similar trends were observed in both number of cells invaded and percentage invasion with or without migration control (Figure 6.2B and C).

Further investigation of the effects of the combinations of flavonoids and their respective metabolites (quercetin/Q3G, morin/Q3G, naringin/naringenin and hesperidin/hesperetin) prior to H/R insult revealed a significant increase in HTR-8/SVneo cell invasion as seen in figures 6.3 (A-C). Again, there was a significant decrease in the migration of cells pre-treated cells when compared with untreated H/R cells but not with control cells (refer to appendix A.4). Due to the reduced migration exhibited by the flavonoid-metabolite combination pre-treated cells, the percentage invasion was significantly increased when compared to control and H/R cells (see figures 6.3C).

In addition, 24 h pre-treatment with the combination of flavonoids (quercetin/morin, quercetin/naringin and quercetin/hesperidin) was also investigated. From the data shown in figures 6.4 (A-C), 24 h pre-treatment with these combinations significantly increased HTR-8/SVneo cell invasion and significantly reduced cell migration. Again, the data seen in figures 6.4C shows a significant increase in percentage invasion when compared to H/R untreated and control cells as a result of reduced migration from pre-treated cells (see appendix A.5 for migration data).
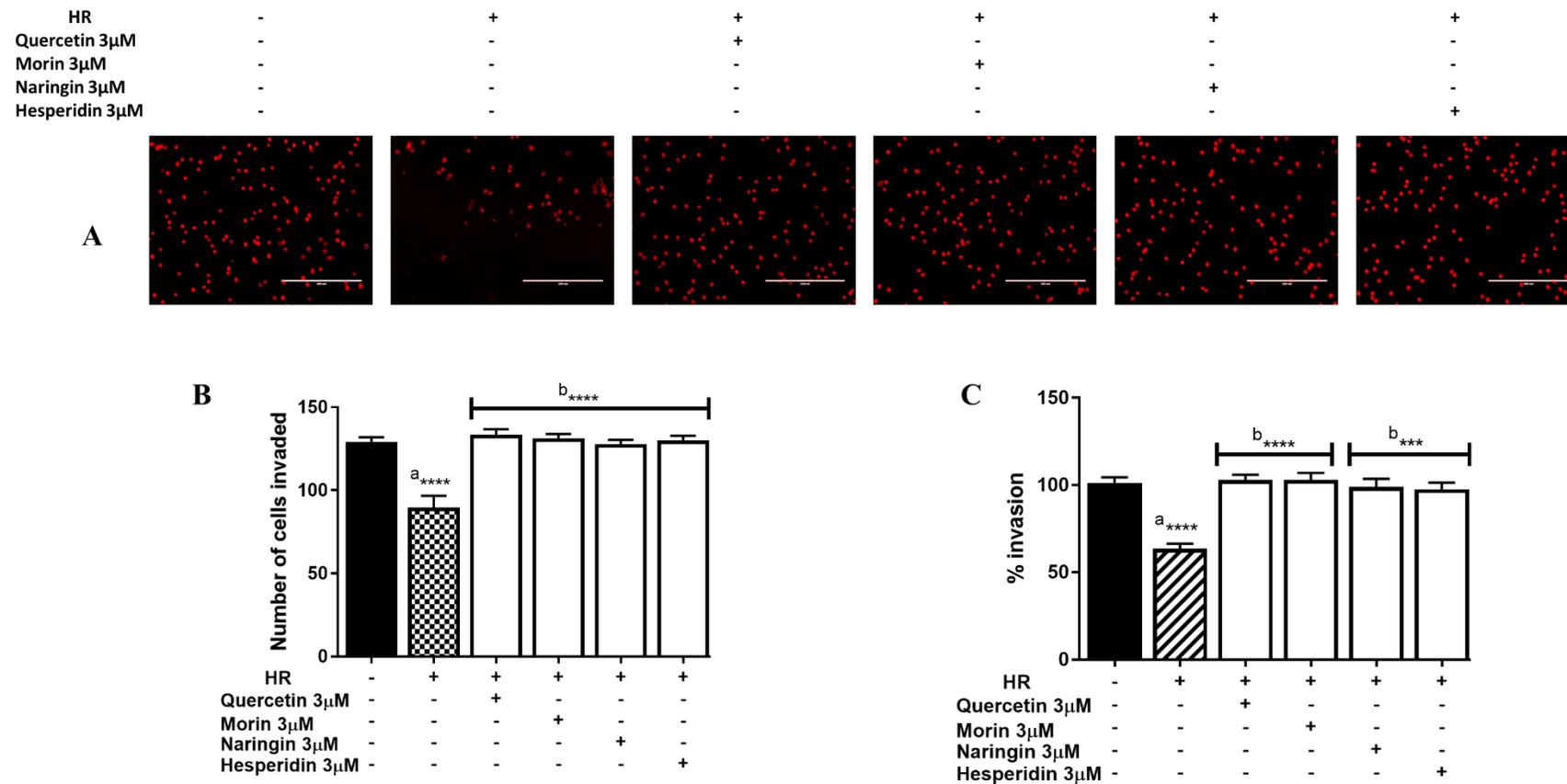
Figure 6.1: Effect of flavonoids on HTR-8/SVneo cell invasion after HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with 3 µM quercetin, Morin, naringin or hesperidin for 24 h prior to HR-induced oxidative stress. Cell invasion was assessed using Corning® BioCoat™ tumour invasion system. (A) Representative images of cell invasion after 24 h. Images were taken from the bottom coated membrane using EVOS FL with objective magnification 20X (Scale bar = 200 µm. (B) Quantitative analysis of the number of cells invaded using WimCounting and GraphPad Prism software. (C) The percentage of invasion (number of cells invaded/number of cells migrated x 100) using WimCounting and GraphPad Prism. Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as number of cells invaded (B) and percentage of control (C) and represent the mean ± SEM of at least three independent experiments. ***$P<0.001$ and ****$P<0.0001$ (a) versus control and (b) versus HR.
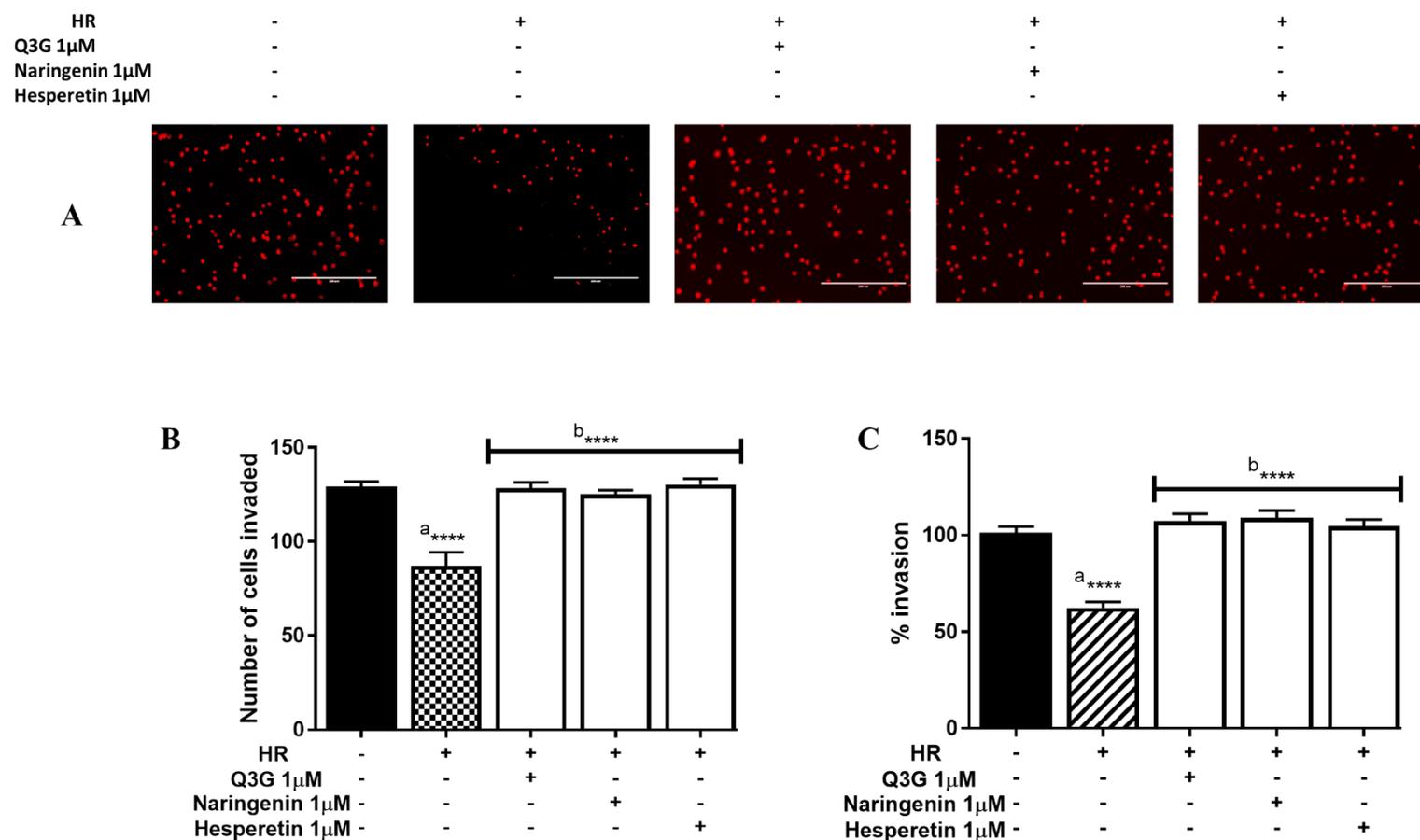
Figure 6.2: Effect of metabolites on HTR-8/SVneo cell invasion after HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with 1 µM Q3G, naringenin or hesperetin for 24 h before exposure to HR-induced oxidative stress. Cell invasion was assessed using Corning® BioCoat™ tumour invasion system. (A) Representative image of cell invasion after 24 h. Image were taken from the bottom coated membrane using EVOS FL with objective magnification 20X (Scale bar = 200 µM). (B) Quantitative analysis of the number of cells invaded using WimCounting and GraphPad Prism software. (C) The percentage of invasion (number of cells invaded/number of cells migrated x 100) using WimCounting and GraphPad Prism. Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as number of cells invaded (B) and percentage of control (C) and represent the mean ± SEM of at least three independent experiments. ****$P<0.0001$ (a) versus control and (b) versus HR.
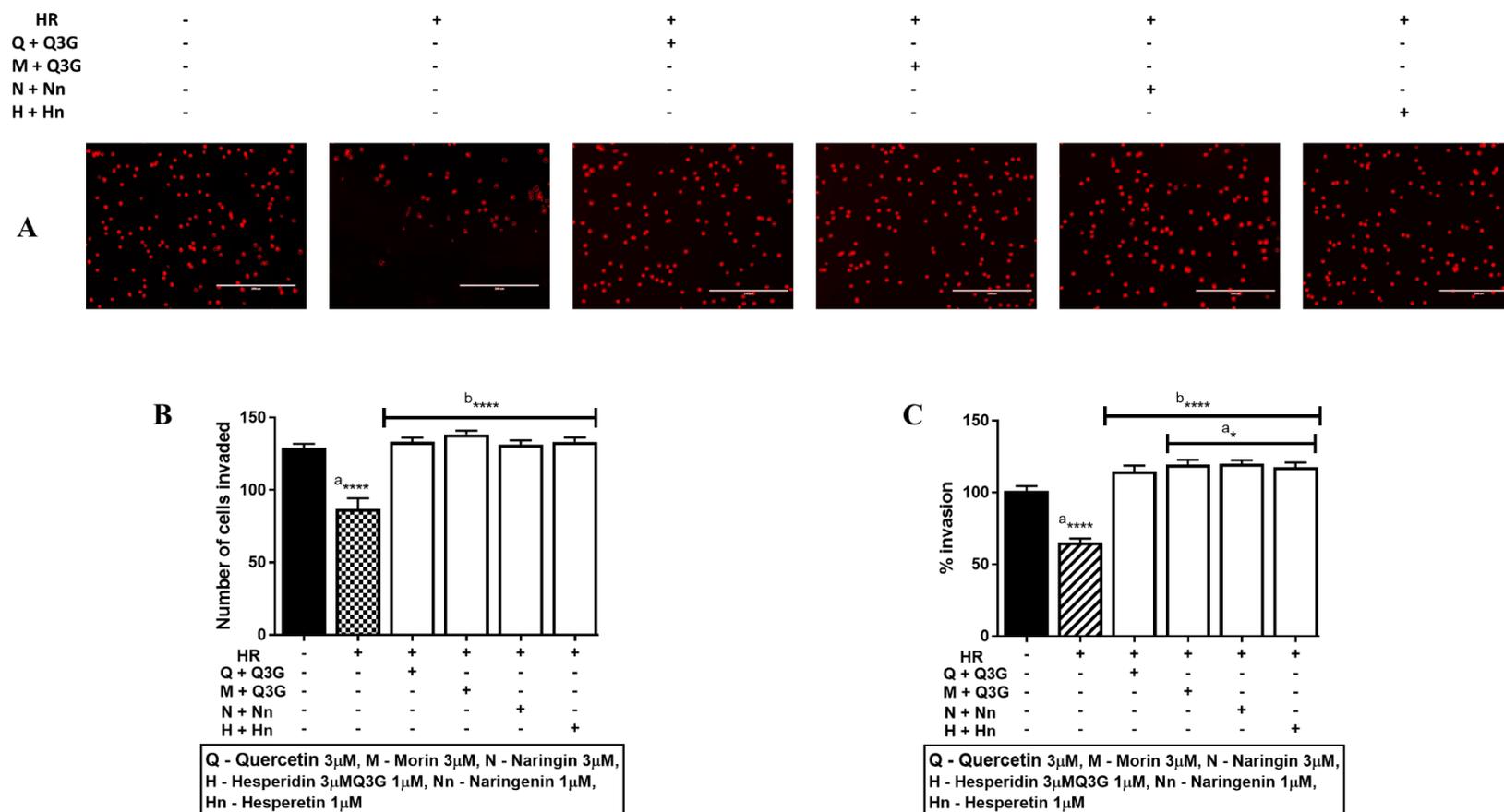
Figure 6.3: Combined effect of flavonoids and their respective metabolites on HTR-8/SVneo cell invasion after HR-induced oxidative stress.
HTR-8/SVneo cells were pre-treated with flavonoid at 3 µM and 1 µM of metabolite combination for 24 h before exposure to HR-induced oxidative stress. Cell invasion was assessed using Corning® BioCoat™ tumour invasion system. (A) Representative image of cell invasion after 24 h. Image were taken from the bottom coated membrane using EVOS FL with objective magnification 20X (Scale bar = 200 µM). (B) Quantitative analysis of the number of cells invaded using WimCounting and GraphPad Prism software. (C) The percentage of invasion (number of cells invaded/number of cells migrated x 100) using WimCounting and Graphpad. Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as number of cells invaded (B) and percentage of control (C) and represent the mean ± SEM of at least three independent experiments. *P<0.05 and ****P<0.0001, (a) versus control and (b) versus HR.
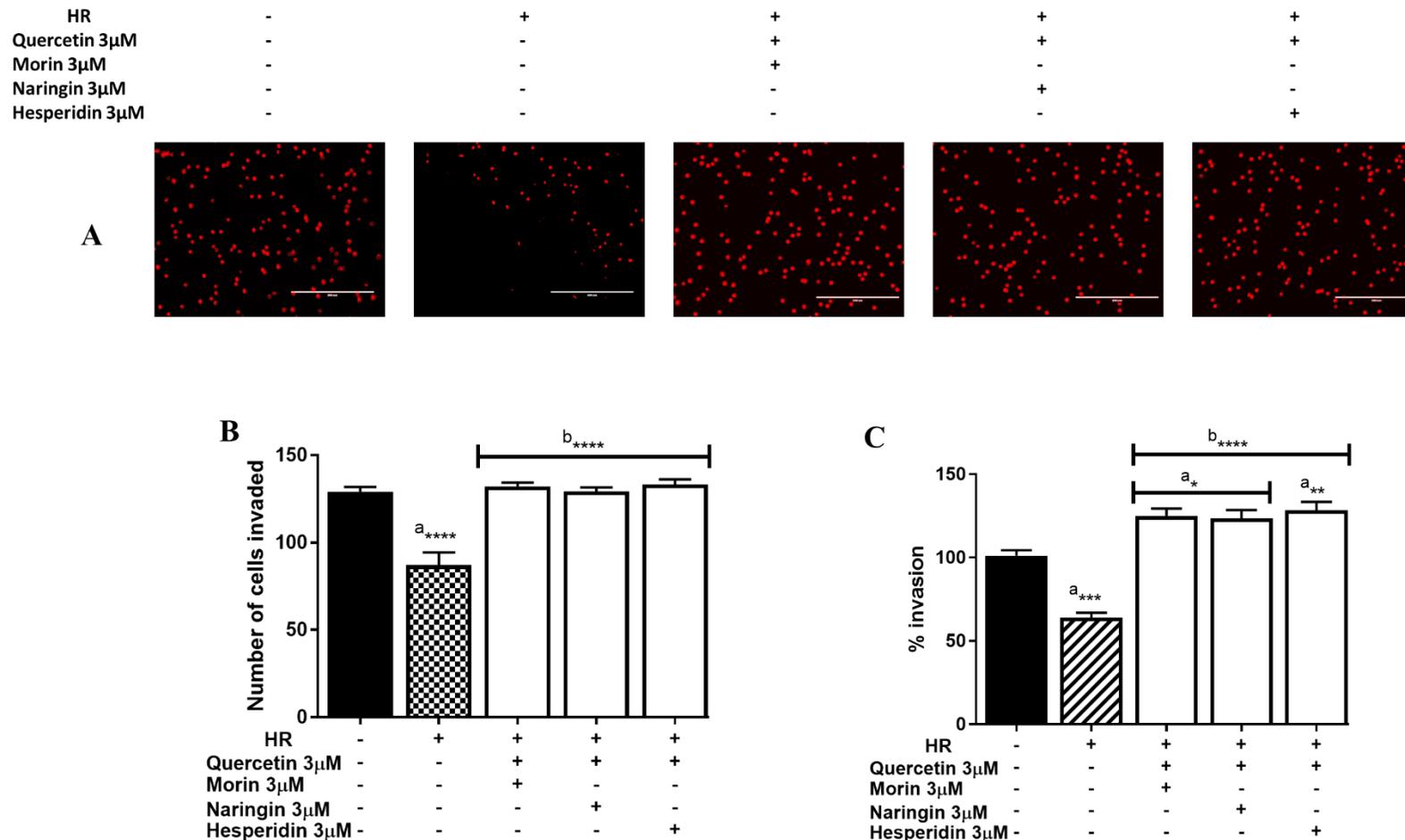
Figure 6.4: Effect of flavonoid combinations on HTR-8/SVneo cell invasion after HR-induced oxidative stress.

HTR-8/SVneo cells were pre-treated with flavonoids (3 µM) for 24 h prior to HR-induced oxidative stress exposure. Cell invasion was assessed using Corning® BioCoat™ tumour invasion system. (A) Representative image of cell invasion after 24 h. Image were taken from the bottom coated membrane using EVOS FL with objective magnification 20X (Scale bar = 200 µM). (B) Quantitative analysis of the number of cells invaded using WimCounting and GraphPad Prism software. (C) The percentage of invasion (number of cells invaded/number of cells migrated x 100) using WimCounting and Graphpad. Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as number of cells invaded (B) and percentage of control (C), and represent the mean ± SEM of at least three independent experiments. **P<0.01 and ****P<0.0001, (a) versus control and (b) versus HR.

6.3.2 Effects of flavonoids on spheroid formation

As previously described in the introduction of this chapter, high levels of ROS impair stem cell function. It has also been established that HTR-8/SVneo cells have the ability to generate spheroidal stem cells under normal conditions (Balahmar *et al.*, 2018). Herein, the effects of H/R insult on the ability of HTR-8/SVneo cells to generate spheroidal stem cells with or without the treatment of flavonoids, their metabolites or in combinations was investigated. The data from figure 6.15 (A, B) showed that 24 h pre-treatment with quercetin prior to HR-induced oxidative stress enhances the generation of spheroidal stem cells. Also, H/R on untreated cells showed impairment of spheroid formation. Similar results were obtained after 24 h pre-treatment with Q3G, quercetin/Q3G and quercetin/morin (see appendix A.6 – 8).
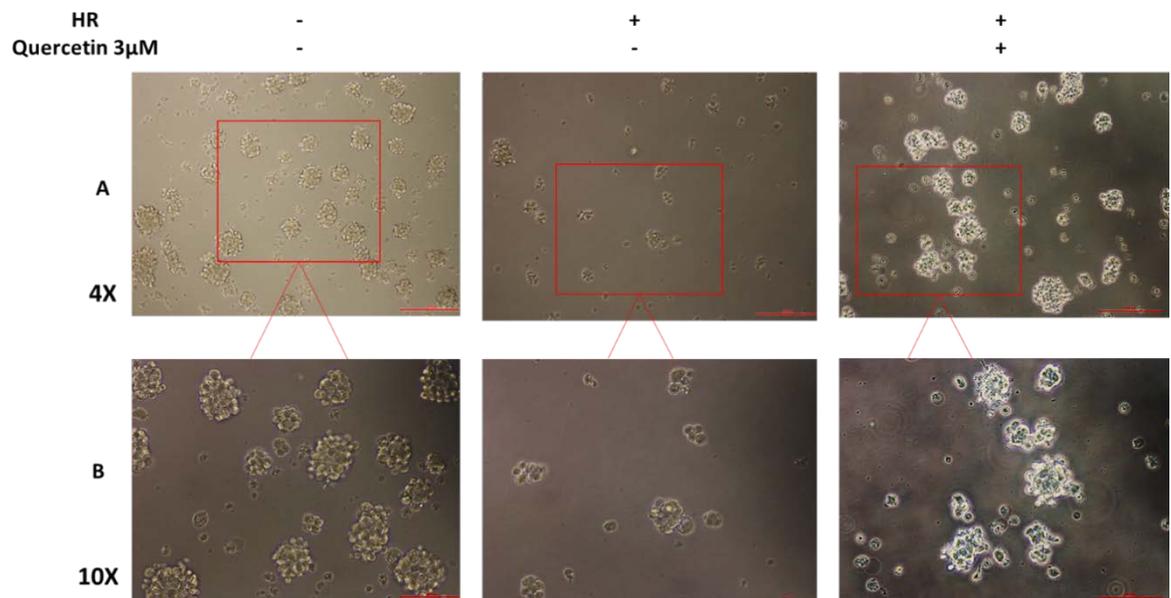
Figure 6.5: The effect of quercetin on HR-induced oxidative stress on the generation of spheroidal stem cells in HTR-8/SVneo cells.

HTR-8/SVneo cells were treated with or without quercetin (3 µM) prior to HR-induced oxidative stress exposure. Images were taken using Nikon Eclipse TS100 microscopy attached with Nikon DS-Fi2 camera. Objective magnification (A) 4X and (B) 10X.

This experiment was carried out in a T75 ultra low attachment flask (Corning®, UK) and quantification was not possible. Therefore, soft-agar colony formation assay (3-D invasion) was carried out to quantify the size and growth area of the spheroids. Again, quercetin, Q3G, quercetin/Q3G and quercetin/morin were used as representative treatments for this study.

The data shown in figures 6.6 (A, B) indicates that HTR-8/SVneo cells pre-treated with flavonoid prior to H/R insult were able to successfully generate spheroidal stem cells when compared to H/R untreated cells. Pre-treated cells produced almost the same size and growth area of spheroids as seen in the control and there was not a significant difference between pre-treated cells and contreol. However, there was significant decrease in the size and growth area of H/R untreated spheroid cells when compared to both control and pre-treated cells. These data suggest that flavonoids help to protect and restore trophoblast stem cell integrity. Investigating their invasive capacity is very important to study as shallow invasion can lead to pregnancy complications as described previously.
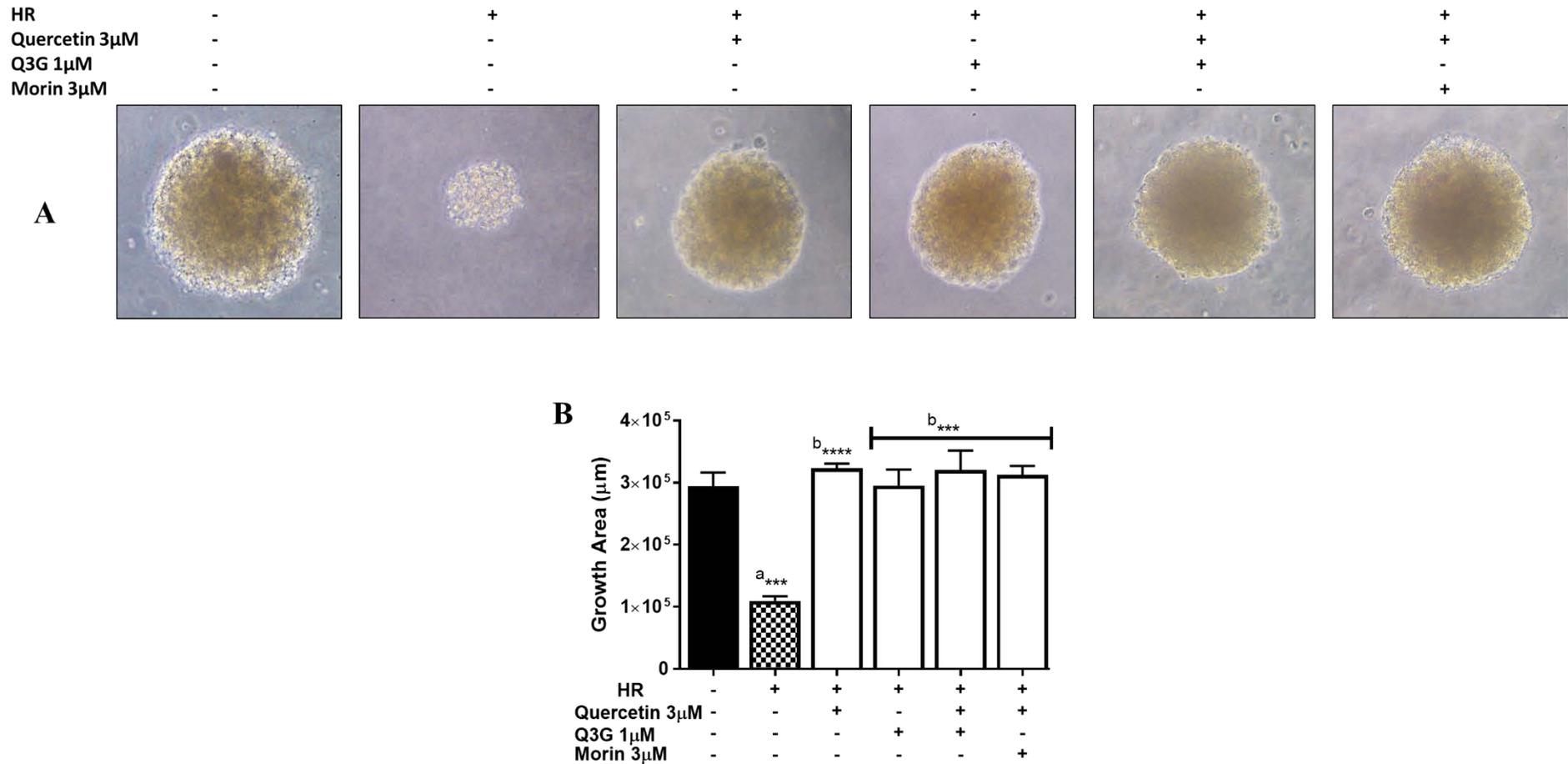
Figure 6.6: The effect of selected flavonoids on the formation of spheroidal stem cells after HR-induced oxidative stress.

HTR-8/SVeno cells were pre-treated with or without flavonoids for 24 h prior to HR-induced oxidative stress exposure. Cells were cultured in cultured in T75 to about 80-90% confluency, cells were harvested by trypsinization, counted and a single cell suspension of 5 x 10^3 cells/well (6 well plate) was seeded in the 1% agar and 0.7% agarose mixture as described in section 2.12. (A) Representative image taken after 15 days using Nikon Eclipse TS100 microscopy attached with Nikon DS-Fi2 camera with objective magnification 20X. (B) Quantitative analysis of growth area of the spheroids using WimColony and GraphPad Prism software. Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as area of cell growth (µm) and represent the mean ± SEM of at least three independent experiments. ***P<0.001 and ****P<0.0001, (a) versus control and (b) versus HR.

6.3.3   Effects of flavonoids on spheroidal stem cell invasion (3D invasion)

In this section, the invasiveness of spheroids generated from HTR-8/SVneo cells with or without pre-treatment with flavonoids/metabolites prior to HR-induced oxidative stress was investigated. The duration of this experiment was increased to 72 h due to some limitations encountered and discussed in Chapter 7. It was observed that not only does H/R insult affect the formation of spheroids but also significantly inhibited their invasiveness as shown in figures 6.7. As expected, cells pre-treated with flavonoids (quercetin, morin, naringin and hesperidin) prior to H/R insult significantly enhanced the invasive capacity of HTR-8/SVneo cells (figures 6.7). Comparing pre-treated cells to control there was no significant difference in the invasiveness of spheroidal stem cells. Comparable results were observed with their respective metabolites (Q3G, naringenin and hesperetin). The data shown in figures 6.8 indicate that pre-treatment with metabolites alone significantly increased the invasion of HTR-8/SVneo cells when compared to H/R untreated. Again, there was no significant difference in metabolite pre-treated cells and control cells.
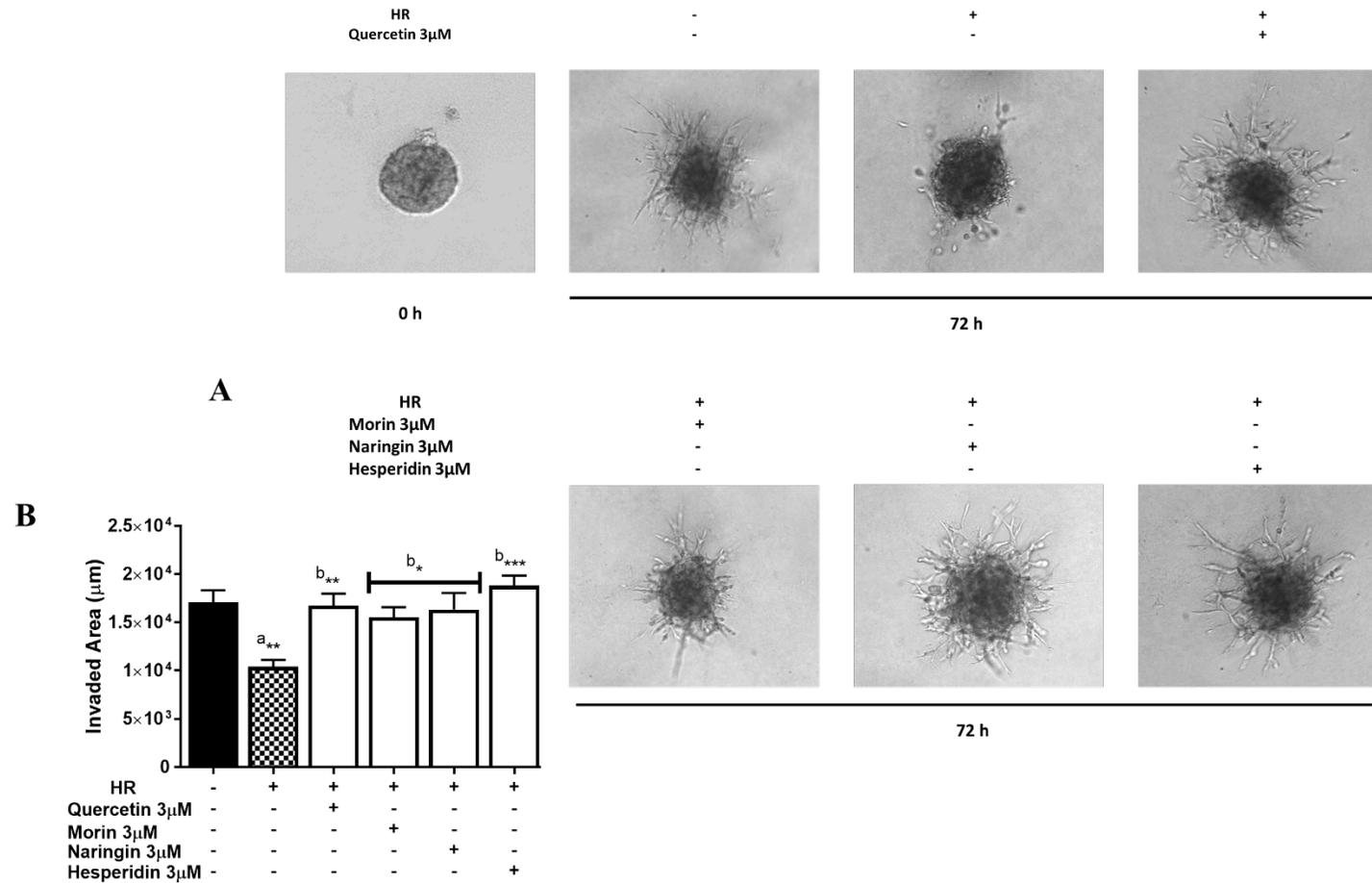
Figure 6.7: Flavonoids enhances spheroid invasion after HR-induced oxidative stress in HTR-8/SVneo cells.

HTR-8/SVneo cells were pre-treated with or without the quercetin, morin, naringin or hesperidin (3 µM) for 24 h before exposure to HR-induced oxidative stress. Single cell suspension was seeded at 3×10^3 cells/well in a 96 well 3D spheroid BME invasion plate (A) representative image taken at 0 h, just after adding the spheroid invasion matrix. Image was taken using confocal microscopy with objective magnification 10X. (B) Quantitative analysis of invasion area of the spheroids using ImageJ and GraphPad Prism software. Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as area of cell invasion (µM) and represent the mean ± SEM of at least three independent experiments. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ (a) versus control and (b) versus HR.
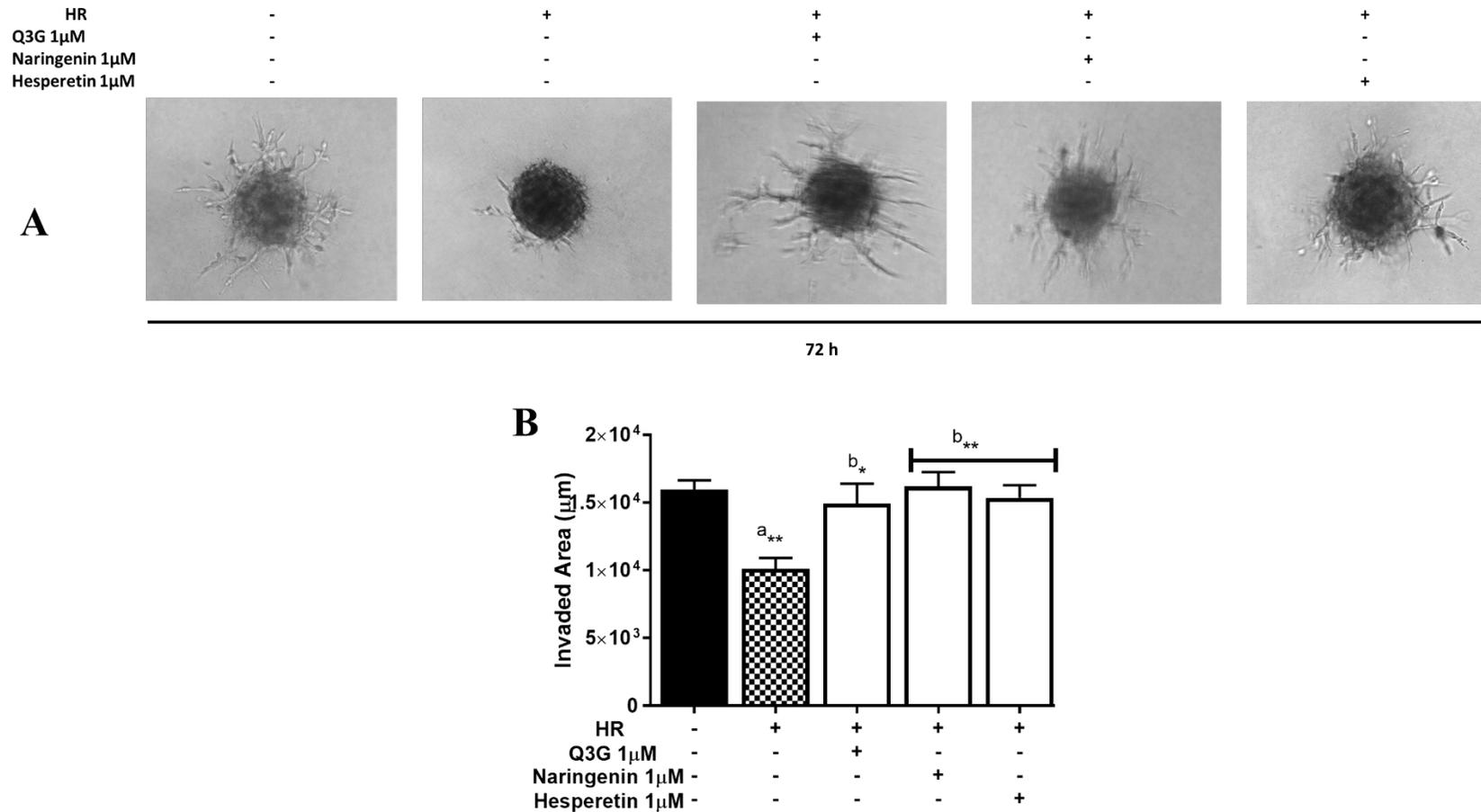
Figure 6.8: Metabolites enhances spheroid invasion after HR-induced oxidative stress in HTR-8/SVneo cells.

HTR-8/SVneo cells were pre-treated with or without the Q3G, naringenin or hesperetin (1 μM) for 24 h before exposure to HR-induced oxidative stress. Single cell suspension was seeded at 3 x 10^3 cells/well in a 96 well 3D spheroid BME invasion plate (A) representative image taken at 0 h, just after adding the spheroid invasion matrix. Image was taken using confocal microscopy with objective magnification 10X. (B) Quantitative analysis of invasion area of the spheroids using ImageJ and GraphPad Prism software. Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as area of cell invasion (μM) and represent the mean ± SEM of at least three independent experiments. *P<0.05, and **P<0.01, (a) versus control and (b) versus HR.

6.4   Result Summary

HR-induced oxidative stress significantly reduces the invasiveness of HTR-8/SVneo cells as seen in the 2D invasion assay. However, pre-treatment with flavonoids, their metabolites or combinations 24 h prior to H/R insult significantly increased the invasive capability of this cell line. Further investigations indicated that H/R has the potential to significantly inhibit the formation of spheroidal stem-like cells from HTR-8SVneo parent cells. Cells pre-treated with flavonoids prior to H/R insult showed statistically significant increase in formation size and growth area when compared to H/R untreated cells. The invasive potential of these spheroids was investigated using 3D invasion techniques. Finally, these studies revealed that not only does H/R insult significantly inhibit the formation of spheroids, it also significantly reduces their invasiveness. In contrast, flavonoid pre-treatment significantly increased the invasiveness of these spheroids.

6.5   Discussion

The aim of this chapter was to investigate the effects of 24 h pre-treatment with selected flavonoids, their metabolites alone or in combinations prior to HR-induced oxidative stress on HTR-8/SVneo invasion (2D), spheroid formation and invasiveness (3D) as well as identifying potential regulatory proteins via mass spectrometry.

6.5.1   Effect of flavonoids on single cell invasion (2D)

The effects of H/R insult and 24 h flavonoid pre-treated cells were compared using the Corning® BioCoat™ tumour invasion system. This system consists of fluoroBlok™ 96-well plates with 8 µm pores that are either coated (for invasion) and uncoated (for migration control) with matrigel matrix. The migration control was necessary as it is possible for trophoblast cells to be highly migratory but less invasive (Kilburn, 2000). The data seen in this chapter from the 2D invasion experiments showed that HR-induced oxidative stress significantly reduced the invasiveness of HTR-8/SVneo cells but did not affect their migratory capacity. This result was in agreement with other studies using H/R insult to assess the invasiveness of HTR-8/SVneo cells (Kilburn, 2000; Leach *et al.*, 2008; Yang *et al.*, 2014; Balahmar *et al.*, 2018). This present study is the first report to show that pre-treatment with flavonoids, their metabolites alone or in combinations prior to HR-induced oxidative stress can significantly increase the number of cells invaded as well as the percentage invasion of HTR-8/SVneo cells. The mechanism for the increased invasion can be attributed to flavonoids ability to increase proliferation and viability, increase GSH and reduced GSSG, restoring NADP/NADPH ratio, scavenging of H_2O_2, inhibiting caspases (3/7) and the inhibition of oxidative stress-induced activation of protein kinases during H/R insult as seen in previous chapters (3, 4 and 5). Interestingly, there was no significant difference in the migration of control and H/R untreated cells. Further investigations revealed that flavonoids (quercetin, morin, naringin or hesperidin) or flavonoid metabolite (Q3G) alone did not significantly change the migratory potentials of this cell line. Surprisingly, naringenin and hesperetin (metabolites) and

combinations of flavonoid-metabolites/flavonoids significantly reduced the migration of HTR-8/SVneo cells during H/R insult. This could indicate that the indicated metabolites and combination of flavonoid/flavonoid-metabolite increase HTR-8/SVneo cell differentiation to the invasive phenotype as the contrary is observed during hypoxia or H/R insult (Kilburn, 2000).

6.5.2 Effects of flavonoids on spheroid formation and invasion (3D)

Since the continual proliferation, migration and invasion of trophoblast stem cells is critical during pre- or post-implantation for the maintenance of pregnancy, it was important to investigate how HR-induced oxidative stress and flavonoid pre-treatment affect trophoblast stem cell function. To achieve these aims, first the effect of H/R insult and flavonoid pre-treatment on the formation of spheroids was assessed. Secondly, further investigation into the invasiveness of these spheroids was performed.

Spheroid formation, size and growth area was assessed using soft-agar colonies and results indicated that H/R insult significantly reduced the spheroidal growth area when compared to control. However, pre-treatment (24 h) with flavonoids prior to HR-induced oxidative stress significantly increased the spheroidal growth area when compared to H/R untreated spheroids and there was no significant difference when compared to control. These data were in agreement with other studies confirming that oxidative stress significantly impairs the functions of stem cells by inhibiting their capacity for self-renewal and their ability to differentiate into multiple cell types (Tower, 2012; Cieślar-Pobuda *et al.*, 2017). According to Tower (2012), prevention of oxidative stress in stem cells is important as unregulated ROS can play a lead role in the pathogenesis of disease. In another study conducted by Guo *et al*. (2010), H_2O_2-induced oxidative stress in mouse embryonic stem cells significantly reduced cell viability and proliferation, increased apoptosis, reduced number of spheroidal colonies, senescence and inhibited self-renewal. Hyperosmolar stress on trophoblast stem cells has been found to be implicated in the impairment of implantation and slow development of the placenta leading to reduced cell accumulation, increased apoptosis and cell cycle arrest (Liu *et al.*, 2009). Since there are no studies investigating the effects of flavonoids on trophoblast stem cells, therefore comparisons were made with non-trophoblast stem cell origins.

A flavonoid compound was proposed to promote mouse neuronal embryonic stem cells via the modulation of mitochondrial energy metabolism by peroxisome proliferator-activated receptors (Mei *et al.*, 2016). In another study by Srivastava, Bankar and Roy (2013), quercetin was shown to increase proliferation and differentiation of mesenchymal osteoblast stem cells. Other studies have shown that antioxidants not only increase the proliferation and mitochondrial integrity of human mesenchymal stems but they also enhance their therapeutic potentials (Sara Shaban *et al.*, 2017; Chia Jung Li *et al.*, 2017).

Dysfunctional trophoblast stem cell differentiation as a result of trophoblast stem cell lineage imbalance has been associated with gestational complications such as embryonic failure during implantation (Xie *et al.*,

2014). This leads to the investigation of spheroid invasion with or without flavonoid pre-treatment prior to HR-induced oxidative stress. This was achieved using a 3D spheroid BME cell invasion system that provides an easy, quick and quantitative process for the comparison of spheroid morphological patterns and invasion speed. The data from this chapter revealed that not only does HR-induced oxidative stress significantly reduce the formation of spheroids, but they also inhibit their invasiveness. Furthermore, the data explained herein have shown that 24 h pre-treatment with flavonoids or their metabolites alone significantly increased spheroid invasion. As described previously, the differentiation of cytotrophoblasts to invasive extravillous trophoblasts is essential for successful remodelling of the maternal uterine spiral arteries (Harun *et al.*, 2006). Again, this present study is the first study to investigate the effects of flavonoids or their metabolites on HTR-8/SVneo cell spheroid formation and invasion during HR-induced oxidative stress. It was established within this laboratory, by Balahmar *et al.* (2018) that HTR-8/SVneo cells have the ability to produce spheroids and these spheroids also possess stem cells markers such as OCT4, SOX2, NANOG1, CDX2 and NOTCH1. Limited studies have made it difficult to find comparable data.

6.6   Conclusions

According to the data from this chapter, it is clear that HR-induced oxidative stress reduces HTR-8/SVneo cell invasion and 24 h pre-treatment with the indicated flavonoids, their metabolites alone or in combinations significantly enhances trophoblast cells invasion although the mechanism behind flavonoid-enhanced trophoblast invasion is not fully understood. These data also revealed 24 h pre-treated cells increase the formation and growth area of spheroids and their invasiveness.

Chapter 7
General Discussion

7.0   Discussion

The main aim of this study was to investigate the antioxidant effects of flavonoids on HR-induced oxidative stress on human first trimester extravillous trophoblast cell line HTR-8/SVneo. This aim was achieved by (a) optimising the non-toxic concentrations of selected flavonoids (and their respective metabolites), (b) establishing a suitable oxidative stress model for HTR-8/SVneo cells to study the effects of flavonoids/metabolites and in combinations on the viability HTR-8/SVneo cells, and (c) investigating the potential mechanisms associated with flavonoid-mediated cytoprotection (role of cell survival and pro-apoptotic protein kinases and anti-apoptotic pathways) , and (d) establishing the link of flavonoid-induced cytoprotection specifically on early trophoblast invasion and spheroid formation with or without HR induced oxidative stress.

7.1   Optimising the non-toxic concentration of flavonoids

The present study started with the selection of first trimester trophoblast cell lines and flavonoids. Selection of flavonoids for this study was based on their chemical configurations. Flavonoids such as quercetin and its isomer, morin belong to the subclass of flavonol whilst naringin and hesperidin belong to the subclass of flavanones (Panche *et al.*, 2016). The beneficiary effects of flavonoids has been linked to their structural arrangements such as the presence or absence of some functional groups (hydroxyl or methyl) in certain positions (Panche *et al.*, 2016; Erlund, 2004; Yao *et al.*, 2004). It was also important to select flavonoids of different subclasses to validate their combined effects. Especially the fact that craving of fruits and vegetables during early gestational period are not specific to a particular form or source of flavonoids (Belzer *et al.*, 2010).

In addition, recent evidence revealed that the bioactive active forms of flavonoids observed *in vivo* (methyl, sulphate or glucuronide) differs from those found in fruits and vegetable (aglycones or glycosides) (Panche *et al.*, 2016; Robert J. Williams *et al.*, 2004). This confirms that flavonoids undergoes biotransformation after ingestion to produces secondary compounds known as metabolites (Erlund, 2004). Therefore, it was important for this study to investigative the effects of their respective metabolites (namely Q3G, naringenin and hesperetin). Furthermore, the effects of combination of flavonoids and their metabolites for the purpose of mimicking *in vivo* environment and assessing synergistic, addictive or antagonistic effects were studied. In addition, the combination of flavonoids was investigated as the daily intake of flavonoids, in developed countries, estimated is between 23 – 38 mg (Erlund, 2004; Heim *et al.*, 2002). However, during gestation, the estimated daily intake is expected to double as food cravings increases. In survey conducted by

Wijewardene, Fonseka and Goonaratne (1994), 40% of pregnant women craved for un-ripened fruits while 30% craved for ripened fruits. In another study by Bayley *et al.* (2002), fruits and fruits juices were among the cravings of 50% of pregnant women. However, the relationship between food craving during gestation and dietary intake remains unresolved (Belzer *et al.*, 2010). Hence, investigating the effects of combined flavonoids is crucial for identifying their combined effects on placental and foetal health.

Studies have linked the beneficial effects flavonoids/metabolites to their antioxidant properties, which resulted in cardioprotection, anti-cancer, anti-inflammatory, and neuroprotection (Daubney *et al.*, 2015; Panche *et al.*, 2016; Mei *et al.*, 2016). However, flavonoids can also have deleterious (prooxidant) effects under certain circumstances such as high concentrations, presence of O_2 molecules and involvement of free transition metal ion in oxidation processes resulting in the promotion of apoptosis, formation of H_2O_2 via bactericidal activities (Eghbaliferiz and Iranshahi, 2016; Procházková and Wilhelmová, 2011). It was imperative to establish an optimal concentration range suitable for trophoblast cells as this is the first study using trophoblast cell line. To achieve this goal, a concentration range of 1- 40 µM was selected from previous data from non-trophoblast cell lines (Daubney *et al.*, 2015; Yamamoto *et al.*, 2013; PeiMing *et al.*, 2012). Thus, optimising the non-toxic concentration of flavonoids and their metabolites was carried out using MTT and CellTox™ green cytotoxicity assays. The results suggested concentrations from 10 µM of the indicated flavonoids and their metabolites alone, triggered a robust MTT reduction and increase cell death in both HTR-8/SVneo (and TEV-1 in preliminary studies; refer to Appendix A.9 and A.10) cell lines. However, concentrations 1 and 3 µM were well tolerated by both cell lines (as there was no inhibition of MTT reduction or cell death after 24 and 48 h treatment). Hence, concentration 3 µM for flavonoids and 1 µM for metabolites were used for further experiments.

7.2 Establishing oxidative stress model

Placentation/implantation process in human and its complications (such as PE) are unique, hence no other *in vivo* animal models can provide a true representation of early gestational period. Several experimental studies have been carried out using primary cultures and cell lines (Staun-Ram and Shalev, 2005). To investigate the cytoprotective effects of the non-toxic concentrations of flavonoids on trophoblast cell line, an oxidative stress model was established. During placentation, the trophoblast cells are able to survive in low oxygen (Tuuli *et al.*, 2011). In fact until the maternal spiral arteries conversions are complete (10 – 12 weeks of gestation), the oxygen tension in the maternal-foetal environment is estimated

to be < 20 mm Hg or < 2% (Tuuli *et al.*, 2011). In this current study, the viability HTR-8/SVneo cells (together with TEV-1 and Sw71) increased at 0.5% and 2% oxygen for up to 24 h as demonstrated by the MTT assay. This data can be supported by the fact that trophoblast development and implantation occur in a hypoxic environment. Since these cells are originated from first trimester extra villous early trophoblast cells, they are physiologically tolerant to low oxygen concentrations and therefore may be resistant to hypoxia-induced cytotoxicity/apoptosis (Armant *et al.*, 2006). Rapid trophoblast growth during early gestation has been attributed to their response to low oxygen in the foeto-maternal interface (Armant *et al.*, 2006). Autocrine signalling, altered trophoblast growth factors, transcription factors, integrins, glycolytic enzymes and stress-related protein expression have all be linked as a possible mechanisms for trophoblast survival and evasion of apoptosis during low oxygen tension in the first trimester (Armant *et al.*, 2006). Successful remodelling of the maternal spiral arteries is one of the key events leading to a successful pregnancy, incomplete remodelling can results variation of maternal circulation to the intervillous space causing an inconsistency in placental perfusion (Hung *et al.*, 2001) (See chapter 1). This inconsistency and variation in maternal blood flow to the intervillous space provides the basis for hypoxia reoxygenation (ischemic-reperfusion type of injury) (Hung *et al.*, 2001). Hypoxia reoxygenation (H/R) insult in early pregnancy leads to the generation of cytotoxic ROS (and thereby oxidative stress) and increased apoptosis resulting in further reduction/shallow trophoblast invasion (Hung and Burton, 2006; Hung *et al.*, 2002; Hung *et al.*, 2001; Leach *et al.*, 2008). Hence in this current study, oxidative stress was achieved by hypoxia followed by reoxygenation which triggered a statistical significant inhibition of MTT reduction and increased cell death as observed in HTR-8/SVneo cells, (also in TEV-1 and SW71 cell lines – as shown in preliminary studies (see appendix A.11). There are evidence proposing that H/R occurrence and the events associated with it, are responsible for pregnancy complications such as preeclampsia, IUGR and miscarriages (Hung and Burton, 2006; Leach *et al.*, 2008; Aouache *et al.*, 2018).

7.3 Antioxidative effects of flavonoids

As previously mentioned in section 7.2, the occurrence of H/R insult during first trimester leads to the overproduction of ROS and decrease trophoblast cell viability leading to increased apoptosis and insufficient EVT invasion (Aouache *et al.*, 2018). These chains of events arising from increased oxidative stress have been linked with pregnancy complications such as preeclampsia. Currently, there is no known therapeutic intervention to reverse, stabilize or prevent preeclampsia apart from termination or delivery (Bolnick *et*

al., 2015). Although, antioxidant therapy such as vitamin C and E alone or in combination has been used to combat the overproduction of ROS observed in early pregnancy, hoping to reverse or prevent preeclampsia (Kiondo *et al.*, 2014; Fu *et al.*, 2018; Rumbold and Crowther, 2005; Basaran *et al.*, 2010). These clinical trials concluded that supplementation with vitamin C and E alone or in combination did not reduce the risk or onset of preeclampsia and thus are not be used to treat PE. However, the antioxidant power of flavonoids have been proposed to be higher than that of vitamin C and E (Lotito and Frei, 2006). In a study conducted by Ho and Chang (2004) on the protective effects of quercetin and vitamin C on neuronal cells against H_2O_2-induced neurodegeneration, they observed that the vitamin C had lower protection when compared to quercetin (flavonoid). Another study by Eberhardt, Lee and Liu (2000), also concluded that quercetin have a higher antioxidative activity than that of vitamin C. In this present study, it was observed that pre-treated HTR-8/SVneo cells were protected from HR-induced oxidative stress based on cell viability as demonstrated by MTT and CellTiter®-Glo assays. Similar data were observed in the TEV-1 cell line (see appendix A.12), suggesting that cytoprotective effects of flavonoids is not cell line specific. Due to time constraints, it was not possible to obtain data using alternative cell lines such as TEV-1 and SW71 cells. It would be appropriate to repeat this investigation using these cell lines to corroborate these data (See chapter 4,5 and 6).

The cytoprotective effects of flavonoids in non-trophoblast cells have been well documented (Daubney *et al.*, 2015; Shokoohinia *et al.*, 2015; Liu *et al.*, 2018). However, these previous studies used concentrations (20-500 µM) that cannot be physiologically achieved by daily intake of fruits and vegetables. In fact, the plasma peak concentration of quercetin after ingesting quercetin rich fruits/vegetables (e.g. onions) was found to be 2.1 µg/mL^{-1} (which roughly equals to 4.53 µM) suggesting that the concentrations used in this present study can be physiologically achieved from daily consumption of fruits and vegetables (Graefe *et al.*, 2001).

The possible mechanisms behind flavonoid antioxidant properties has been linked to their ability to interact/activate other antioxidant enzymes, inhibit oxidases and scavenge free radicals (Procházková and Wilhelmová, 2011). In this present study, it was observed that flavonoids, their metabolites alone (or in combinations) significantly restored GSG/GSSG (elevated GSG and depleted GSSG) and NADP/NADPH ratio, hence restoring the redox balance. The activation/interaction with other antioxidant enzymes is bought about by inducing phase II detoxifying enzymes such as glutathione S-transferase, and NAD(P)H-quinone (Panche *et al.*, 2016; Procházková *et al.*, 2011; Banjarnahor and Artanti, 2014).

Again, flavonoids' structural configurations such as flavonoids having a hydroxyl function group at C-3 have been found to be the most effective inducer of phase II detoxifying enzymes (Banjarnahor and Artanti, 2014; Procházková *et al.*, 2011). This current study showed that H/R insult significantly decreased GSH, thereby preventing its major function, which is to deactivate ROS and detoxify cytotoxicity during placentation and implantation (Knapen *et al.*, 1999; Knapen *et al.*, 1998). Decreased levels of GSH have been observed in preeclamptic cases (Krishna *et al.*, 2017; Vaughan and Walsh, 2002). In a study conducted by Jin *et al.* (2017) on the proteomics analysis of pre-eclamptic placentae, it was observed that glutathione metabolism disorder (decreased GSH and increase GSSG) in placenta tissues contributes to the pathogenesis of preeclampsia. The ability of flavonoids to inhibit oxidases such NADPH oxidase, which has been demonstrated by their ability to restore NADP/NADPH ratio was observed in this present study (see chapter 4). Quercetin and other flavonoids have been found to be protective due to their ability to regulate protective gene electrophile responsive element (EpRE). During oxidative stress, they activate EpRE-mediated responses, which have been correlated with their redox restoration properties (Procházková *et al.*, 2011). The data from chapter 4 may suggest that consumption of flavonoid rich fruit and vegetables during early trophoblast development can be beneficial against trophoblast oxidative stress.

It has also been well document that flavonoid-mediated cytoprotective is attributed to their free radical scavenging ability. Flavonoids' involvement in ROS scavenging is brought about by the arrangement and total number of function groups such as the B ring hydroxyl groups, which are the most major determinant to ROS scavenging (Procházková *et al.*, 2011; Banjarnahor and Artanti, 2014). Flavonoids ability to directly scavenge H_2O_2 can also be achieved with their ability to freely donate hydrogen atom described in chapter 1. Overproduction of ROS is one of the hallmarks of H/R occurrence during early placentation as previously described in section 7.2. In this current study, it was demonstrated that cells pre-treated with flavonoids, metabolites alone or in combinations significantly reduced the generation of ROS (H_2O_2) in HTR-8/SVneo cells during H/R insult, confirming that flavonoids are potent scavengers of free radicals and may be beneficiary to trophoblast survival during early pregnancy.

7.4   Effects of flavonoids on HR-induce apoptosis and protein kinase(s) activation

Apoptosis is one of the major consequence of oxidative stress. As shown by previous studies, caspase-3 and caspase-7 are the executioner caspases during apoptosis (Tan *et al.*, 2016). This current study confirmed that HR-induced oxidative stress is a mediator of apoptotic cell

death in HTR-8/SVneo cells via the activation of caspase 3/7 pathways as seen in chapter 5. Pre-treatment (24 h) with selected flavonoids, their metabolites alone or in combinations significantly inhibited HR-induced caspase 3/7 elevation (as seen in chapter 5). This current study was in agreement with previous studies from non-trophoblast origin, indicating that pre-treatment with flavonoids/metabolites prior to exposure to oxidative stress significantly inhibited apoptosis by halting the activation/elevation of caspase 3/7 (Tan *et al.*, 2016; Nadia Z Shaban *et al.*, 2017; Bournival *et al.*, 2009). The ability of flavonoids to inhibit HR-induced caspase 3/7 elevation in HTR-8/SVneo cells may be attributed to their ability to scavenge H_2O_2, elevate GSH and deplete GSSG levels, restore NADP/NADPH ratio which if left unregulated would activate the stimuli that leads to caspase(s) activation. Another possible mechanism in which flavonoids inhibit caspase activities during oxidative stress is by the restoration of mitochondrial membrane depolarisation (caused by oxidative stress), hence preventing the release of cytochrome c resulting in the downstream activation caspases (Hewage *et al.*, 2016; Liu *et al.*, 2018). In addition, flavonoids were found significantly inhibit apoptosis by increase the expression of Bcl-2 both at the mRNA and protein level, thereby inhibiting the activation of caspase-3 (Han *et al.*, 2008). During trophoblast development, increased apoptosis can result in slow development of the placenta, and reduced trophoblast invasion resulting in pregnancy complications (Aouache *et al.*, 2018). The data obtained from chapter 5, suggests that the intake of flavonoids rich food during early stages of pregnancy may help improve trophoblast survival during oxidative stress.

This current study further investigated the signalling pathways (protein kinases) activated by oxidative stress. Among the pathways investigated are the members of the MAP kinase family (ERK1/2, JNK and p38 MAPK) and protein kinase B/AKT. These kinases involved in cell survival (ERK1/2 and PKB) and cell death (p38 MAPK and JNK) (Liu *et al.*, 2018). Previous studies indicated that oxidative stress significantly activated protein kinases in various cell lines and the introduction of flavonoids significantly inhibited the activation of protein kinases (Kim *et al.*, 2009; Daubney *et al.*, 2015; Liu *et al.*, 2018). In this currently study, HR-induced oxidative significantly activated ERK1/2, SAPK/JNK, p38 MAPK and PKB phosphorylation, however, pre-treatment of HTR-8/SVneo cells significantly inhibited HR-mediated activation of these kinases. Flavonoids have been suggested to exert modulatory effects in cells via the stimulatory or inhibitory actions on protein kinases by changing the phosphorylation state of target molecules (R.J. Williams *et al.*, 2004). In this present study flavonoids/metabolites or combinations was involved in the inhibition of pro-

apoptotic SAPK/JNK and p38 MAPK phosphorylation pathways as seen in chapter 5. The inhibition of phosphorylated SAPK/JNK and p38 MAPK pathways, particularly to ROS-mediated activation, is important for protecting HTR-8/SVneo cells from cellular death. During trophoblast development, ROS-mediated activation of phosphorylated SAPK/JNK and p38 MAPK results in premature differentiation of trophoblast cells, thereby affecting trophoblast maintenance, invasion, migration, proliferation and incomplete remodelling of maternal spiral arteries (Pfeffer and Pearton, 2012). The inhibition of HR-mediated activation of phosphorylated ERK1/2 by flavonoids/metabolites (or in combination) was also seen (see chapter 5). ERK1/2 activation is generally considered to be anti-apoptotic and plays a very important role in cell survival; but under certain conditions like severe hypoxia and hypoxia reoxygenation, ERK1/2 activation can have pro-apoptotic effects (Lu and Xu, 2006). The mechanism in which flavonoids inhibit ROS-mediated ERK1/2 phosphorylation is not fully understood but has been suggested to be through a pathway independent of MEK1 inhibition (Kim *et al.*, 2010).

Further, the effects of flavonoids/metabolites (or their combinations) on HR-mediated PKB phosphorylation was also investigated. As demonstrated in chapter 5, pre-treated HTR-8/SVneo cells significantly inhibited H/R-induced phosphorylation of PKB, although the exact mechanism is not fully understood, flavonoids are believed to inhibit HR-mediated PKB phosphorylation via a pathway independent of PI3K inhibition. PKB phosphorylation during oxidative has be hypothesize to participate in either pathogenic or protective mechanisms (Uranga *et al.*, 2013). In this present study, PKB phosphorylation during HR-induced oxidative triggered apoptosis and hence was not protective to HTR-8/SVneo cell.

ROS-mediated activation of protein kinase phosphorylation has been proposed to induce apoptosis, inhibit proliferation, migration, differentiation and invasion of trophoblast cells, hence resulting the gestational complications (Aouache *et al.*, 2018). Inhibition of ROS-mediated protein kinase phosphorylation by the indicated flavonoids, their metabolites alone or in combinations suggest another mechanism in flavonoids protects HTR-8/SVneo cells.

7.5   Effects of flavonoids on HTR-8/SVneo invasion and spheroid formation

A critical component of the healthy placental development involves events like cell invasion, vasculogenesis and angiogenesis, which is crucial for the development of the foeto-placental communications and the survival of the foetus (Yu qi Li *et al.*, 2017). Insufficient trophoblast invasion as a result of H/R insult may lead to angiogenic abnormalities and poor vascularization in placenta resulting in IUGR, PE, foetal/maternal death and miscarriage (Yu

qi Li *et al.*, 2017; Pereira *et al.*, 2015). In this current study, the effects of flavonoids on trophoblast invasion prior to HR-induced oxidative stress using HTR-8/SVneo cells was investigated. The invasive capacity of HTR-8/SVneo was assessed using Corning® FluorBlok™ 96-multiwell insert plate. This system provides cultured cells with an environment that allows the assessment of their invasive potential *in vitro*. The data from this present study was in agreement with previous studies confirming that HR-induced oxidative stress significantly reduced the invasive capability of HTR-8/SVneo cell line (Kilburn, 2000; Hung *et al.*, 2002; Hung and Burton, 2006; Leach *et al.*, 2008). However, pre-treatment with the flavonoids prior to HR-induced oxidative stress significantly enhanced HTR-8/SVneo invasive capacity. In fact flavonoid treatment resulted in comparable invasive patterns of normal (without HR) controls. This suggests that flavonoids may enhance the mechanisms involved in implantation, hence ensuring successful uterine spiral remodelling. Since this is the first study on flavonoid-enhanced invasion in trophoblast cell line, the exact mechanism(s) that is/are influenced by flavonoids is/are not yet known. However, flavonoids may interact with key trophoblast regulators such as Notch signalling, WNT signalling, hypoxia inducible factor (HIF) family, vascular endothelial growth factor (VEGF) family, transforming growth factor beta (TGF-β) superfamily, and HCF/c-Met pathways to ensure proper invasion and maternal spiral artery remodelling (Ji *et al.*, 2013) (see section 7.7 for details). In addition to these key regulators, flavonoid-enhanced invasion can also be attributed to their antioxidative effects observed in this present study.

To investigate the effects of flavonoids on stem-like cells after oxidative stress, 3-D spheroidal invasion model was used. Previous work carried out within this laboratory showed that HTR-8/SVneo cells have the ability to form spheroids under normoxic conditions and these spheroids have stem cells properties (Balahmar *et al.*, 2018). As previously described in chapter 1, during early trophoblast development, the cytotrophoblast stem cells differentiate into the synctiotrophoblasts, and the latter giving rise to the invasive EVTs that are responsible for uterine spiral artery remodelling. To establish the effects flavonoid on spheroid formation using HTR-8/SVneo cells during HR-induced oxidative stress, spheroid formation/invasion assays were carried out. As observed in chapter 6, HR-induced oxidative stress significantly reduced the area of invasion by the HTR-8/SVneo spheroid growth area. However, pre-treatment with selected flavonoids, (their metabolites alone or in combinations) significantly increased the spheroid area of invasion. These data may suggest that flavonoids play a significant role in stem cell protection against oxidative stress. Oxidative stress can significantly affect stem cell self-renewal function, and their

differentiation into multiple cells types (Tower, 2012; Denu and Hematti, 2016). This present study has showed that HR-induced oxidative stress can affect this spheroidal stem cell formation which may suggest that cytotrophoblast stem cell ability to self-renew and ability to differentiate into other trophoblast linages can be impaired, hence leading to poor placental development and pregnancy complications. Flavonoids' ability to significantly improve spheroid formation in HTR-8/SVneo cells subjected to HR insult can be attributed to their antioxidant properties as antioxidant treatments have been demonstrated to increase proliferation, enhanced mitochondrial integrity and suppress H_2O_2-induced oxidative stress in human mesenchymal stem cells (Chia Jung Li *et al.*, 2017). It was also revealed that HR-induced oxidative stress not only influenced spheroid formation, but also reduced spheroid invasion (see chapter 6). Again, pre-treatment of HTR-8/SVneo cells with flavonoids/metabolites significantly enhanced spheroid invasion. The role of cytotrophoblast stem cells during placentation involves continuous cell division from day 2 of fertilization until after 10-12 week, when the spiral arteries are remodelled. There are no previous studies on the effects of flavonoids or their metabolites prior to HR-induced oxidative stress on spheroid invasion. The mechanism(s) behind their ability to increase invasion/growth of spheroids originated from HTR-8/SVneo cells is not fully understood.

However, it is known that overproduction of ROS leading to oxidative stress affects stem cell function by causing stress-induced premature senescence via telomere erosion, DNA damage and promotion of apoptosis (Tower, 2012; Dayem *et al.*, 2010). The role of flavonoids in spheroidal growth and invasion during H/R insult can also be attributed to their antioxidative properties (observed in this present study). There could also be the possibility that flavonoids mediate stem cell oxidative stress protection/response pathways such as FOXO transcription factors, WNT pathway, Nrf2/Keap1 pathway, OCT-4 transcription, lamin A signalling, NF κB transcription factor, HIF and Hippo pathways (see section 7.7 for detail) (Dayem *et al.*, 2010). These key stem cells stress response pathways plays a crucial role in the maintenance and differentiation of stem cells (Denu and Hematti, 2016). Therefore, more studies need to be carried out to exploit the mechanism(s) involved in flavonoids-enhanced invasion and spheroid growth.

7.6 Conclusion

This present study has provided novel data for possible preventive measures for pregnancy complications associated with oxidative stress, specifically by the ingestion of fruits of vegetable rich in flavonoids. For the first time, investigation into the effects of dietary

flavonoids, their metabolites alone or in combination on trophoblast oxidative stress was carried out using HTR-8/SVneo cells as a model for *in vivo* trophoblast impairment.

The data from this study suggest that concentrations 1 and 3 µM of the indicated flavonoids/metabolites were non-toxic to HTR-8/SVneo cells, and concentrations above 10 µM triggered cytotoxicity. The finding also showed that pre-treatment (24 h) with selected flavonoids, their metabolites or combinations prior to HR-induced oxidative stress significantly enhance HTR-8/SVneo cell viability, suggesting that flavonoids may enhance the activities of intracellular molecules that protect against oxidative stress. Exploring the possible mechanism(s) for flavonoid-mediated cytoprotection revealed that flavonoids are active scavengers of ROS (H_2O_2), regulate the redox state by restoring GSH/GSSG and NADP/NADPH ratios. Further investigations showed that flavonoids inhibit apoptosis by reducing HR-induced caspase 3/7 activation and inhibition of HR-mediated protein kinase phosphorylation. Overall these findings suggest that regular ingestions of flavonoid rich fruits/vegetables during the entire gestational period (especially early first trimester) may be beneficial to placental/foetal development and health.

7.7   Future works

This study needs to be extended using other first trimester trophoblast cell lines such as TEV-1 and Swan 71, and trophoblast primary cells to fully validate the data of the present study and to show that flavonoid-mediated cytoprotection/antioxidative effects are not cell line specific. This can be achieved using the methodologies already established in the current study using HTR-8/SVneo cells. It will be important to try lower concentration (below 1 µM) of flavonoids/metabolites or combinations to check for protective effects against H/R insult as concentration 0.3 or 0.5 µM did not indicate any protection in HTR-8/SVneo cells.

In addition, it is essential to explore other potential mechanisms involved in flavonoid-mediated cytoprotection and enhanced invasion such as exploiting their involvement in key regulators of trophoblast development and implantation (Notch signalling, wnt signalling, HIF family, VEG family, TGF-β superfamily, and HCF/c-Met pathway) and stem cell oxidative stress pathways (FOXO transcription factors, WNT pathway, Nrf2/Keap1 pathway, OCT-4 transcription, lamin A signalling, NF κB transcription factor, HIF and Hippo pathways). The status of these pathways can be investigated using western blot (protein expression), immunofluorescence (protein localization) and RT-PCR (gene expression analysis) for the potential pathways mentioned above. Protein expression by

western blot or immunofluorescence should be used to assess the regulation of these key regulators.

Interestingly, spheroid invasion after 72 h, showed that flavonoids can enhance spheroid invasion. However, a time course and real time image capture using confocal microscopy or IncuCtye® S3 live-cell analysis system to capture images every 12 h to monitor the exact time the effects of flavonoids kick in will be of optimum importance.

Most importantly, it will be essential to explore changes in protein expression by carrying out mass spectrometry analysis of normal cells and spheroids. This might shed more light into other unknown possible mechanisms and then validate the data with western blotting.

A parallel, balanced randomised placebo controlled clinical study/trial will be conducted using approximately 500 women for the initial study in the Niger Delta region of Nigeria. Participants for this study will include pregnant women aged 18 – 42 years with gestational ages between 12 – 22 weeks. Factors such as first-time pregnancy, single or multiple foetus, body mass index, previous preeclampsia, chronic hypertension, gestational diabetes and interval between pregnancies will all be considered. The pregnant women will be randomised to take oranges/orange juices or apple that are rich in quercetin or hesperidin or placebo daily until they deliver. Dose-finding will be carried out as described by Ursino *et al.*, 2017, to evaluate the safety of the selected flavonoids and their maximum tolerated dose (MTD). Participants, caregiver and those assessing the outcomes will be blinded to the study allocation. Primary outcome, which is the main aim of this trial (using flavonoids compounds to reduce the incidence of PE) and secondary outcomes such as no effect, clinical worsening or maternal/foetal complications will be recorded.

Appendix

A.1
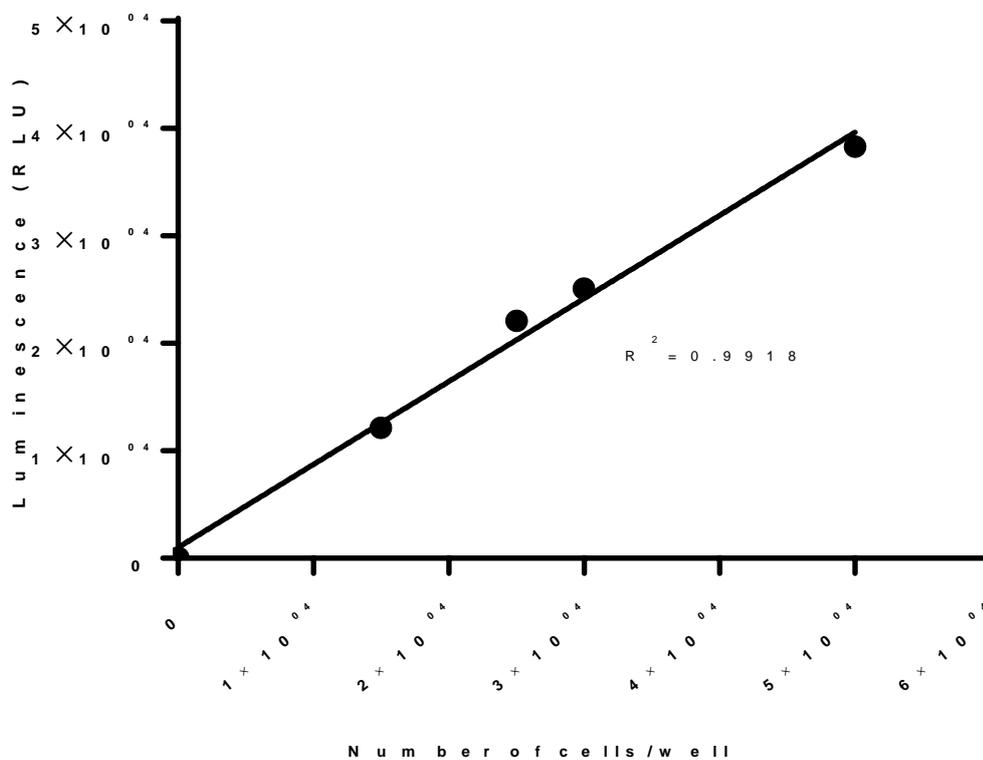
Figure A.1: Determination of HTR-8/SVneo seeding density for CellTiter-Glo® Assay.
HTR-8/SVneo was seeded at ascending density to determine the linear sensitivity of
CellTiter-Glo® assay

A.2
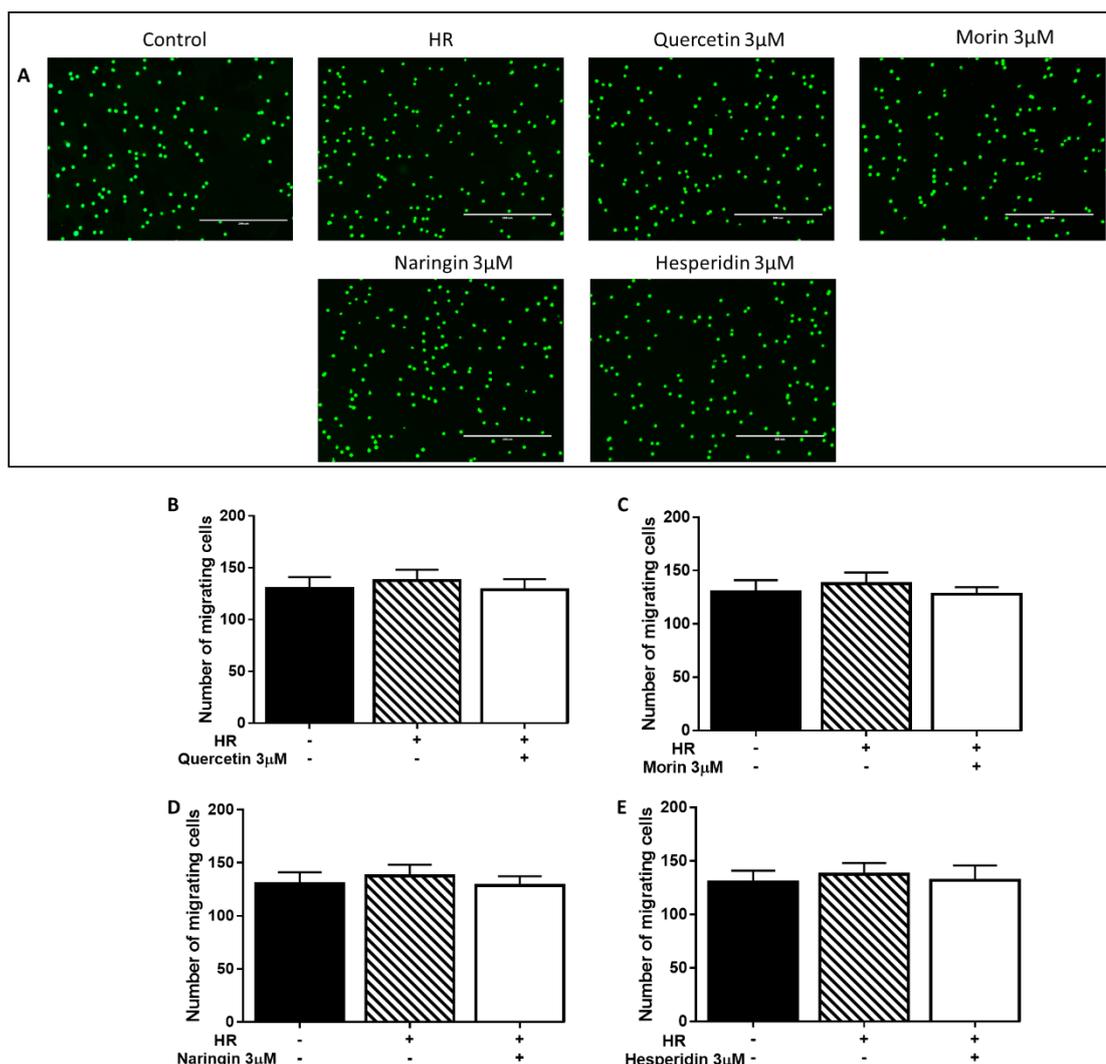
Figure A.2: Effect of flavonoids on HTR-8/SVneo cell migration after HR-induced oxidation stress.

HTR-8/SVneo cells were pre-treated with 3 µM of the selected flavonoids in for 24 h before exposure to HR-induced oxidative stress. Cell migration was assessed using Corning® BioCoat™ tumour invasion system. (A) Representative image of cells invasion after 24 h. Image were taken from the bottom coated membrane using EVOS FL with objective magnification 20X (Scale bar = 200 µM). (B) Quantitative analysing of number of cells migrated using WimCounting and GraphPad prism software. Data are expressed as number of cells invaded (B) and percentage of control (C) and represent the mean ± SEM of at least three independent experiments.

A.3
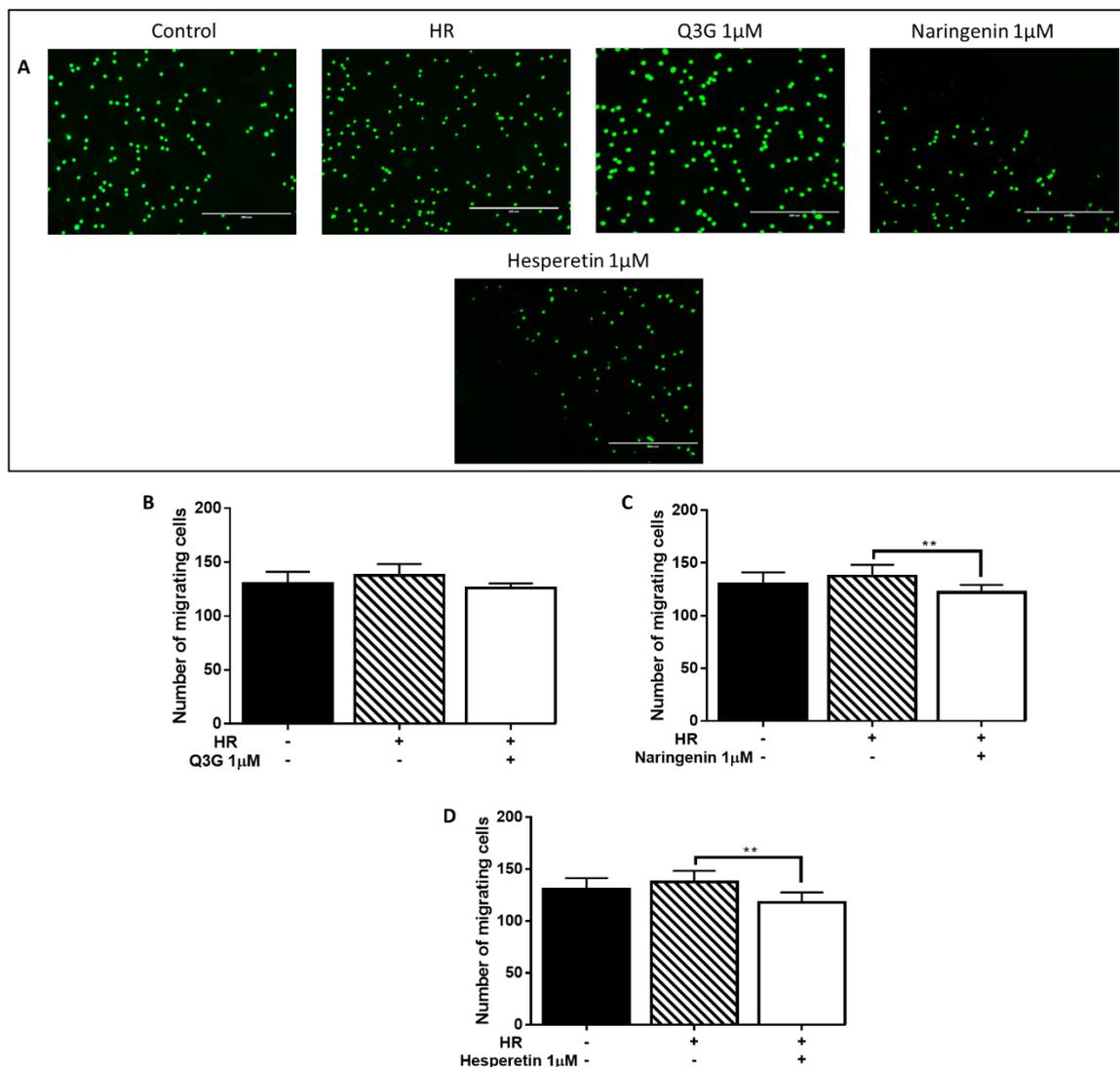
Figure A.3: Effect of metabolites on HTR-8/SVneo cell migration after HR-induced oxidation stress.

HTR-8/SVneo cells were pre-treated with 1 µM of the metabolites in for 24 h before exposure to HR-induced oxidative stress. Cell migration was assessed using Corning® BioCoat™ tumour invasion system. (A) Representative image of cells invasion after 24 h. Image were taken from the bottom coated membrane using EVOS FL with objective magnification 20X (Scale bar = 200 µM). (B) Quantitative analysing of number of cells migrated using WimCounting and GraphPad prism software. Data are expressed as number of cells invaded (B) and percentage of control (C) and represent the mean ± SEM of at least three independent experiments. **$P<0.05$ versus HR

A.4
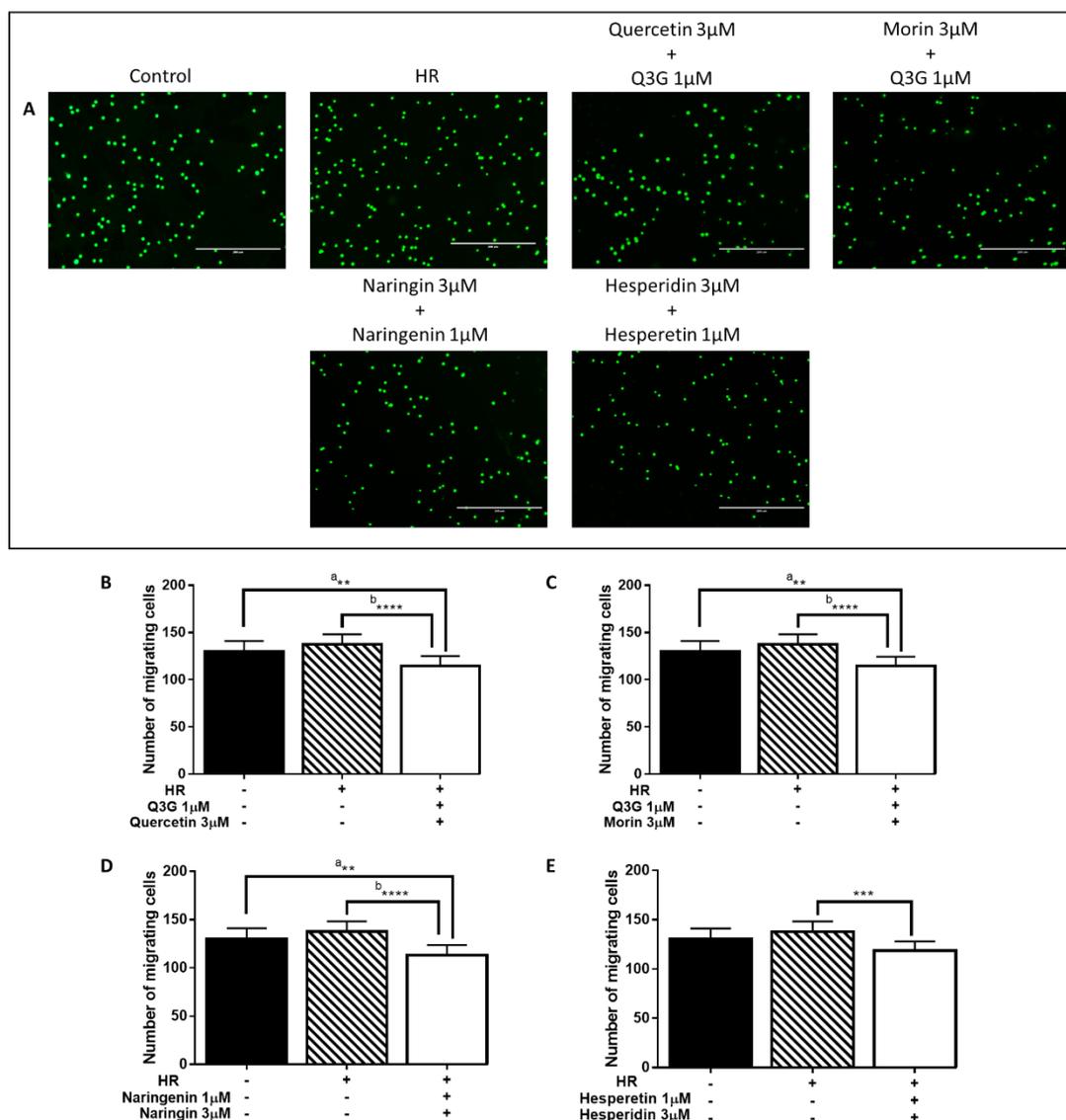
Figure A.4: Effect of flavonoids-metabolites combinations on HTR-8/SVneo cell migration after HR-induced oxidation stress.

HTR-8/SVneo cells were pre-treated with combination of 3 µM of flavonoid and 1 µM of the metabolite in for 24 h before exposure to HR-induced oxidative stress. Cell migration was assessed using Corning® BioCoat™ tumour invasion system. (A) Representative image of cells invasion after 24 h. Image were taken from the bottom coated membrane using EVOS FL with objective magnification 20X (Scale bar = 200 µM). (B) Quantitative analysing of number of cells migrated using WimCounting and GraphPad prism software. Data are expressed as number of cells invaded (B) and percentage of control (C) and represent the mean ± SEM of at least three independent experiments. **P<0.05, ***P<0.001 and ****P<0.0001, (a) versus control and (b) versus HR.

A.5
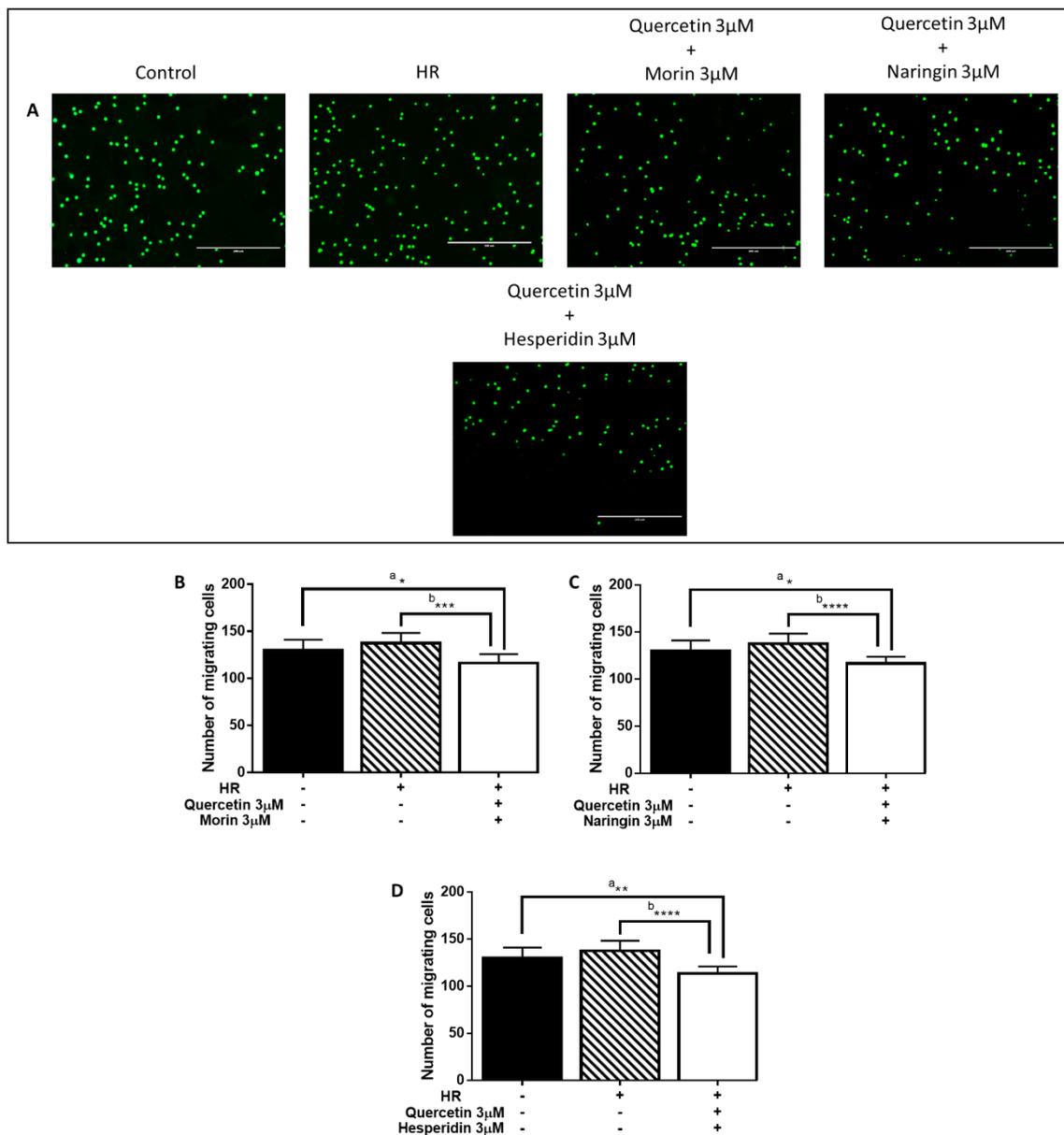
Figure A.5: Effect of flavonoid combinations on HTR-8/SVneo cell migration after HR-induced oxidation stress.

HTR-8/SVneo cells were pre-treated with combinations of flavonoid at 3 µM for 24 h before exposure to HR-induced oxidative stress. Cell migration was assessed using Corning® BioCoat™ tumour invasion system. (A) Representative image of cells invasion after 24 h. Image were taken from the bottom coated membrane using EVOS FL with objective magnification 20X (Scale bar = 200 µM). (B) Quantitative analysing of number of cells migrated using WimCounting and GraphPad prism software. Data are expressed as number of cells invaded (B) and percentage of control (C) and represent the mean ± SEM of at least three independent experiments. **P<0.05, ***P<0.001 and ****P<0.0001, (a) versus control and (b) versus HR.

A.6

| HR | - | + | + |
| Q3G 1µM | - | - | + |
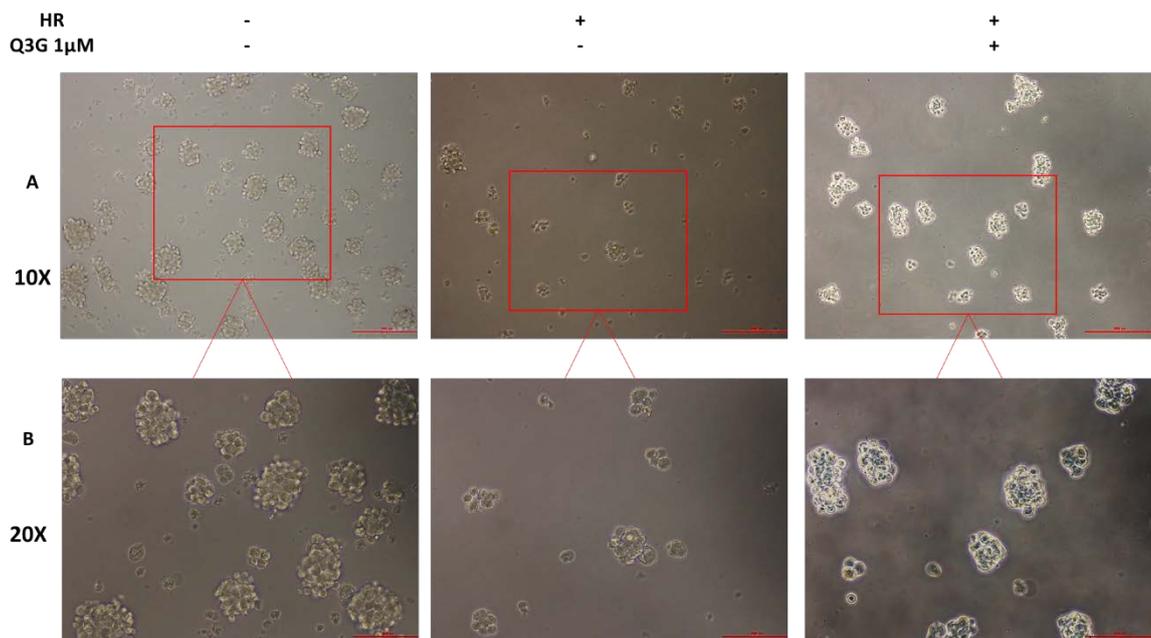
Figure A.6: The effect of Q3G on HR-induce oxidative stress on the generation of spheroidal stem cells in HTR-8/SVneo cells.

HTR-8/SVneo cells were treated with or without Q3G (1 µM) prior to HR-induced oxidative stress exposure. Images were taken using Nikon Eclipse TS100 microscopy attached with Nikon DS-Fi2 camera. Objective magnification (A) 4X and (B) 10X (Scale bar = 200 µM).

A.7
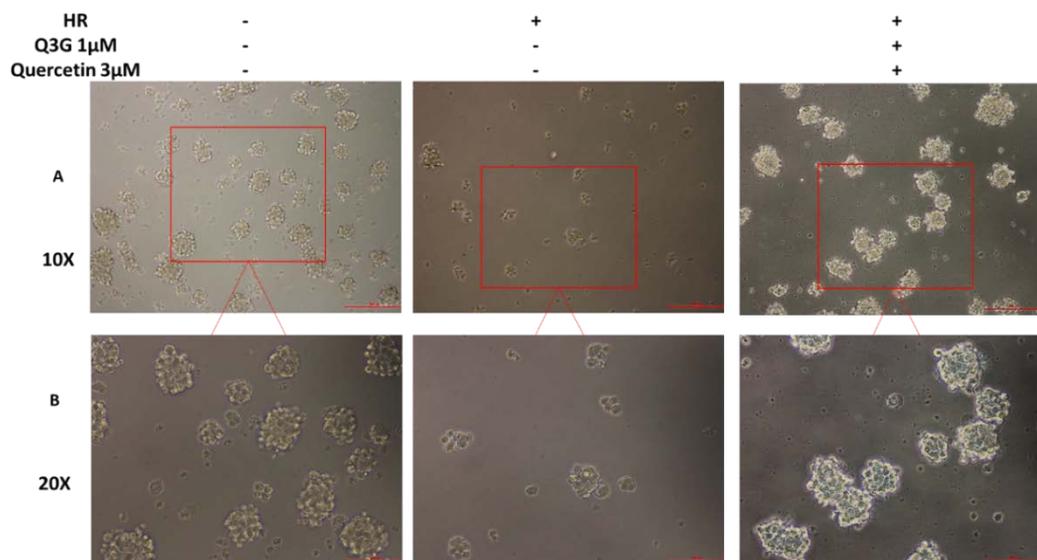
Figure A.7: The effect of Q3G/quercetin combination on HR-induce oxidative stress on the generation of spheroidal stem cells in HTR-8/SVneo cells.

HTR-8/SVneo cells were treated with or without the combination of Q3G (1 µM) and quercetin (3 µM) prior to HR-induced oxidative stress exposure. Images were taken using Nikon Eclipse TS100 microscopy attached with Nikon DS-Fi2 camera. Objective magnification (A) 4X and (B) 10X (Scale bar = 200 µM).

A.8
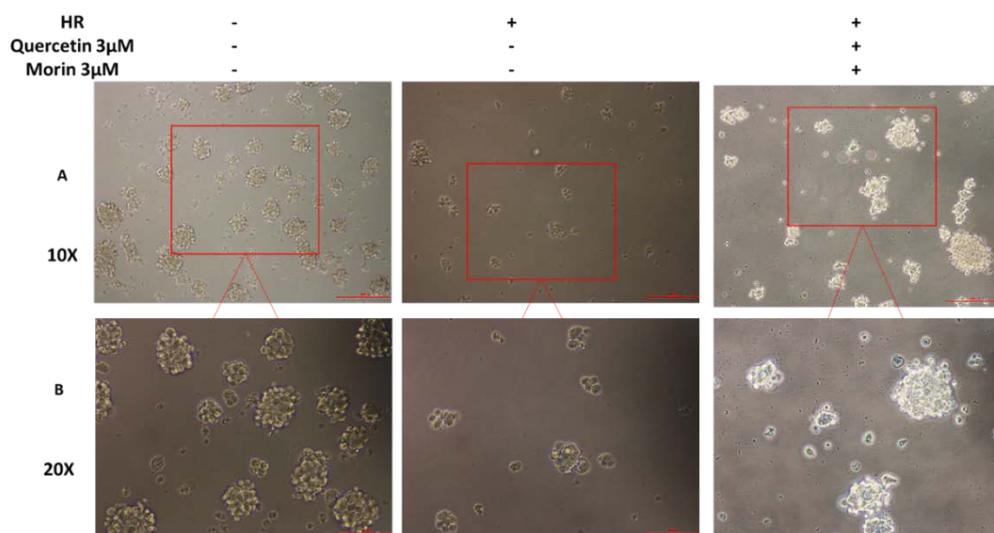
Figure A.8: The effect of quercetin/morin combination on HR-induce oxidative stress on the generation of spheroidal stem cells in HTR-8/SVneo cells

HTR-8/SVneo cells were treated with or without the combination of quercetin and morin (3 µM) and prior to HR-induced oxidative stress exposure. Images were taken using Nikon Eclipse TS100 microscopy attached with Nikon DS-Fi2 camera. Objective magnification (A) 4X and (B) 10X (Scale bar = 200 µM).

A.9
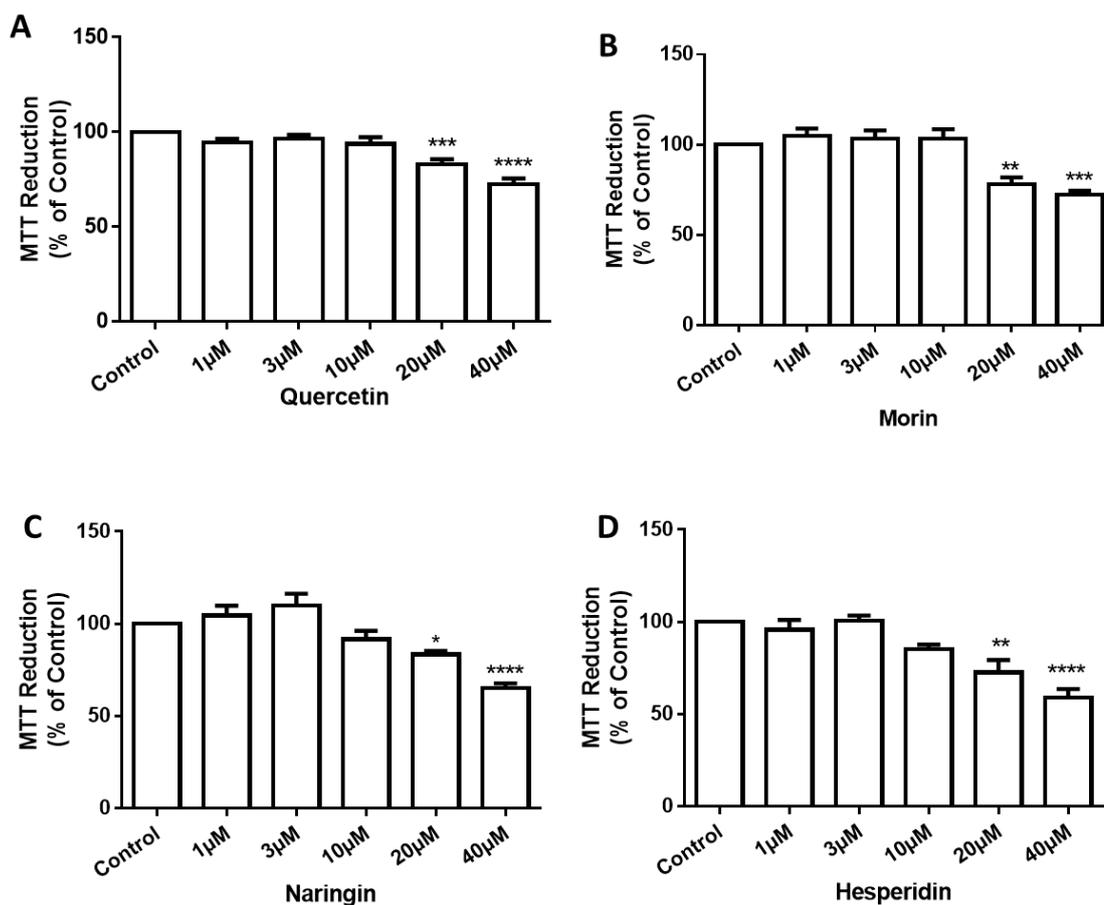
Figure A.9: Effects of 24 h flavonoids treatment on TEV-1 cells' viability.
TEV-1 cells were treated with the indicated concentrations (1-40 µM) of quercetin, morin, naringin and hesperidin for 24 h. Following flavonoid exposure, cell viability was measured by MTT reduction assay (A-D). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against control). Data are expressed as the percentage of control cells and represent the mean ± SEM of six independent experiments performed in quadruplicate. $**P<0.01$, $***P<0.001$ and $****P<0.0001$ versus control.

A.10
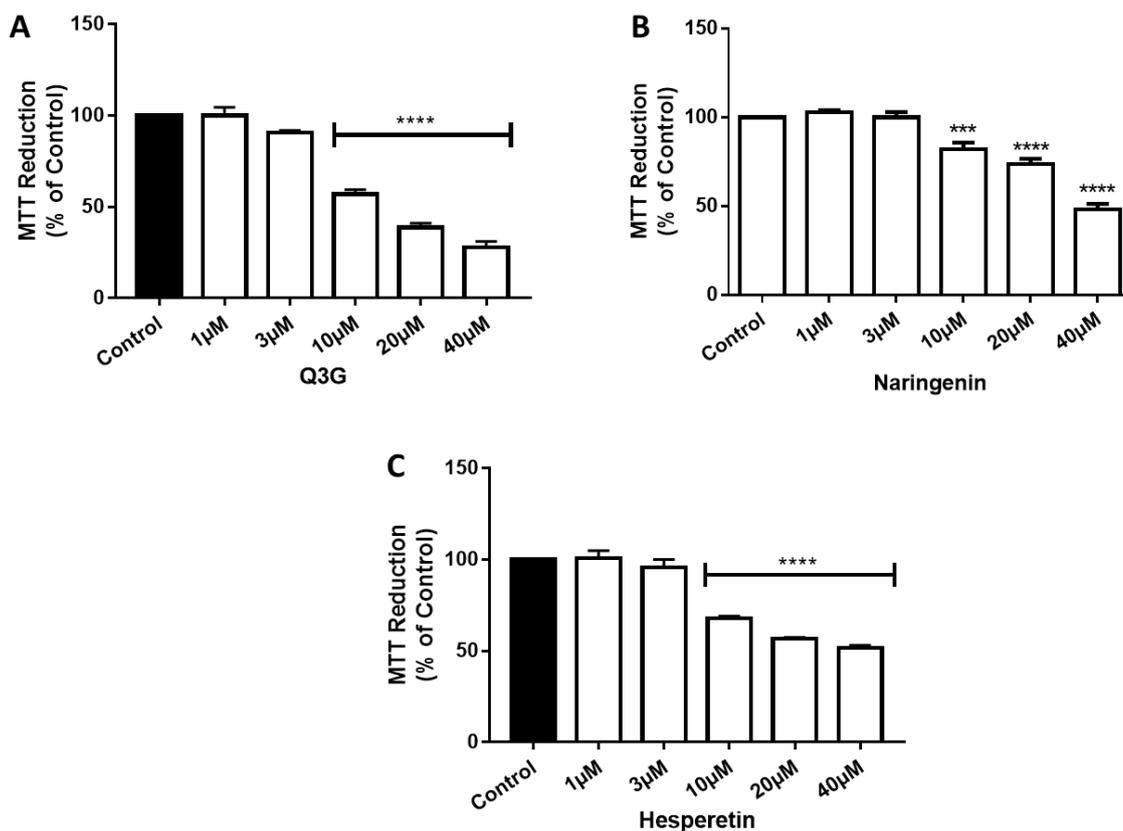
Figure A.10: Effects of 24 h metabolites treatment on TEV-1 cells' viability.

TEV-1 cells were treated with the indicated concentrations (1-40 µM) of Q3G, naringenin and hesperetin for 24 h. Following metabolite exposure, cell viability was measured by MTT reduction assay (A-D). Statistical analysis was carried out using one-way ANOVA (Dunnett's multiple comparisons test against control). Data are expressed as the percentage of control cells and represent the mean ± SEM of six independent experiments performed in quadruplicate. **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$ versus control.

A.11
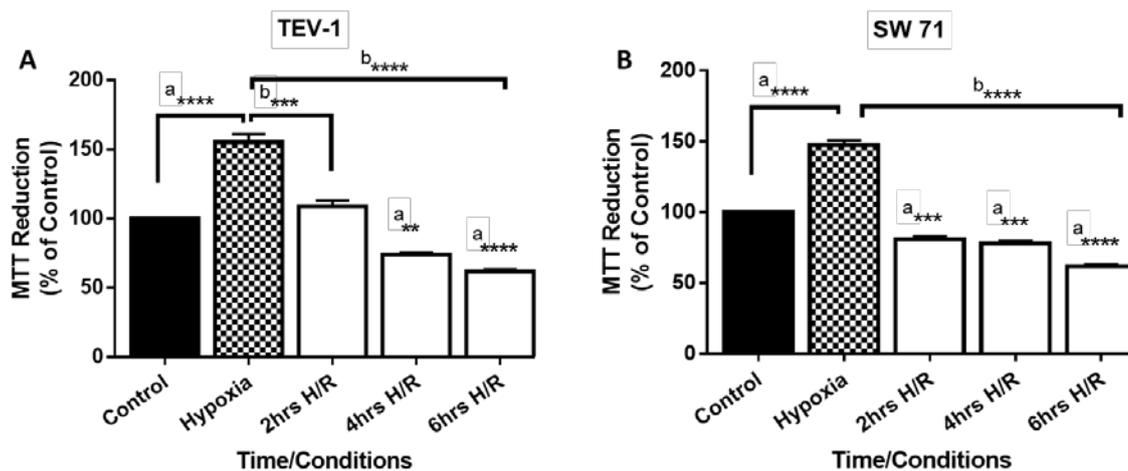
Figure A.11: The effects of hypoxia alone or hypoxia/re-oxygenation on trophoblast cell lines survival

Trophoblast cells were exposed to hypoxia (0.5% O_2) for 2 h followed by re-oxygenation for up to 6 h in glucose- and serum- free medium. Cell viability was assessed by MTT reduction assay. (A) TEV-1 cell line and (B) SW 71 cell line. Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as the percentage of normoxic control cells and data represent the mean ± SEM of six independent experiments performed in quadruplicate. **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus normoxia control and (b) versus hypoxia alone.

A.12
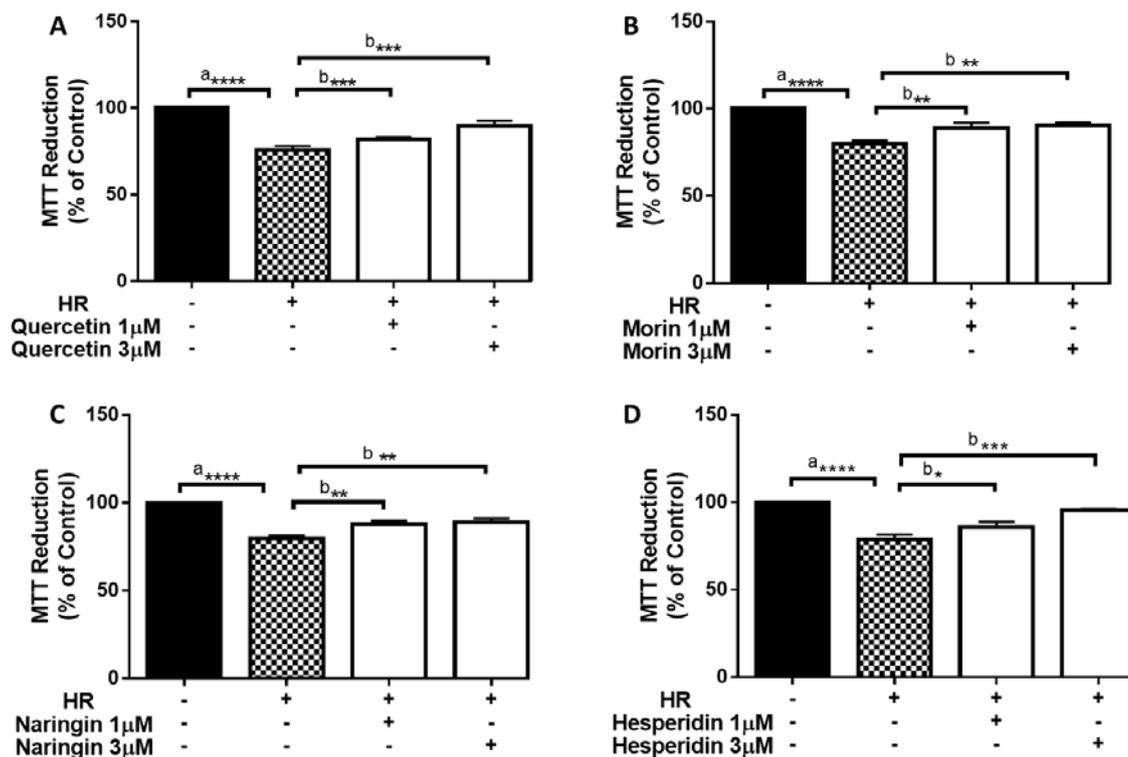
Figure A.12: Effect of 24 h flavonoid treatment on the viability of TVE-1 cells subjected to HR-induced oxidative stress.

TEV-1 cells were pre-treated with either 1 or 3 µM of quercetin, morin, naringin and hesperidin for 24 h prior to HR-induced oxidative stress exposure. Cell viability was measured by MTT reduction assay (A-D). Statistical analysis was carried out using one-way ANOVA (Tukey's multiple comparisons test). Data are expressed as the percentage of control cells and represent the mean $\pm$ SEM of six independent experiments performed in quadruplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$, (a) versus control, (b) versus H/R alone.

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