IN VITRO ANTIOXIDATIVE EVALUATION OF *Plukenetia volubilis* AQUEOUS EXTRACT IN OVARIAN AGING PROCESS

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by

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

Muhammad Alzam Nafiz Bin Rusidi

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
20x	20 magnifications
2D	Two dimensions
3D	Three dimensions
40x	40 magnifications
ABTS	2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
A_C	Absorbance of control
ALA	Alpha-linolenic acid
AMH	Anti-Müllerian hormone
A_S	Absorbance of sample
ATP	Adenosine triphosphate
BSC Class II	Biosafety cabinet class 2
°C	Degree Celsius
Cells/mL	Cells per millilitre
COV434	Ovarian granulosa cell line
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-dyphenyl-1-picrylhydrazyl
EC_{50}	Half-maximal effective concentration
FBS	Foetal Bovine Serum
F-C	Follin-Ciocalteu

FRAP	Ferric reducing antioxidant power
FSH	Follicle-stimulating hormone
H_2O_2	Hydrogen peroxide
HRT	Hormone replacement therapy
IC_{50}	Half-maximal inhibitory concentration
iPSCs	Induced pluripotent stem cells
LH	Luteinising hormone
M	Molar
mg GAE/g	Milligram gallic acid equivalent per gram
mg/mL	Milligram per millilitre
min	minutes
mM	Millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
Na_2CO_3	Sodium carbonate
nm	Nanometre
OD	Optical density
OHS	Ovarian hyperstimulation syndrome
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate Buffer Saline
PCOS	Polycystic ovary syndrome
POF	Premature ovarian failure
POI	Premature ovarian insufficiency
RNA	Ribonucleic acid
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
RSA	Radical scavenging activity
RSS	Reactive sulphur species
SOD	Superoxide dismutase
T25	25ml culture flask
TPC	Total phenolic content
v/v	Volume per volume
w/v	Weight per volume
xg	Centrifugal Force
µL	microlitre

PENILAIAN KESAN ANTIOKSIDATIF SECARA IN VITRO BAGI EKSTRAK AKUEUS *Plukenetia volubilis* TERHADAP PROSES PENUAAN OVARI

ABSTRAK

Penuaan ovari merujuk kepada proses penuaan yang berkaitan dengan penurunan fungsi sel ovari, yang sebahagian besarnya disebabkan oleh tekanan oksidatif. Keadaan ini menjejaskan penghasilan hormon yang menyebabkan penurunan kesuburan dan penghasilan hormon dalam kalangan wanita. Walaupun terdapat banyak ubat-ubatan yang telah dibangunkan untuk meningkatkan kesuburan dan hormon reproduktif, seperti clomiphene citrate dan gonadotropins, ubat-ubatan ini boleh menyebabkan kesan sampingan jangka pendek dan jangka panjang kepada wanita. *Plukenetia volubilis* (Sacha Inchi) ialah tumbuhan yang berasal dari barat laut Brazil dan Peru, yang telah terbukti mengandungi kandungan antioksidan yang tinggi seperti tokoferol dan sebatian fenolik. Oleh itu, kajian kami bertujuan untuk menilai potensi antioksidan ekstrak akueus *P. volubilis* dalam menangani tekanan oksidatif semasa proses penuaan ovari menggunakan model secara *in vitro.* Kajian ini menilai jumlah kandungan fenolik (TPC) ekstrak akueus *P. volubilis* melalui ujian TPC, keupayaan penurunan radikalnya melalui ujian DPPH, dan kesan pelindungnya terhadap sel granulosa (COV434) yang terdedah kepada tekanan oksidatif melalui ujian MTT. Asid askorbik digunakan sebagai antioksidan piawai untuk membandingkan keputusan. Hasilnya, ujian TPC mengesahkan kehadiran jumlah kandungan fenolik yang signifikan dalam ekstrak, di mana kandungan fenolik meningkat seiring dengan peningkatan kepekatan dari 0.2 mg/ml hingga 15 mg/ml. Seterusnya, ujian DPPH menunjukkan peningkatan aktiviti penurunan radikal DPPH dengan nilai IC_{50} sebanyak 2.27 mg/ml. Ekstrak akueus *P. volubilis* juga menunjukkan aktiviti penurunan radikal yang signifikan berbanding asid askorbik ($p = 0.009$). Tambahan pula, ujian MTT mendedahkan kesan pelindungan yang signifikan oleh ekstrak terhadap tekanan oksidatif yang disebabkan oleh hidrogen peroksida pada sel COV434 berbanding asid askorbik ($p = 0.089$) dengan nilai EC_{50} sebanyak 23.82 mg/ml. Ini dibuktikan dengan peningkatan daya hidup sel apabila dirawat dengan ekstrak akueus *P. volubilis*. Secara keseluruhan, penemuan ini berjaya menunjukkan potensi *P. volubilis* sebagai antioksidan semula jadi untuk mengurangkan penuaan ovari yang berkaitan dengan tekanan oksidatif.

IN VITRO ANTIOXIDATIVE EVALUATION OF *Plukenetia volubilis* AQUEOUS EXTRACT IN OVARIAN AGING PROCESS

ABSTRACT

Ovarian aging is the aging process related to the decline in ovarian cellular function primarily driven by oxidative stress which affects hormonal production, resulting in reduced fertility and hormonal dysfunction in women. Although there are many medications have been developed to increase fertility and reproductive hormones such as clomiphene citrate and gonadotropins, they can cause short- and long-term effects on women. *Plukenetia volubilis* (Sacha Inchi) is a plant that originated from Northwestern Brazil and Peru and has proven to contain high antioxidant contents such as tocopherols and phenolic compounds. Therefore, our study aims to evaluate the antioxidative potential of *P. volubilis* aqueous extract in combating oxidative stress during the ovarian aging process using in vitro models. This study assesses the total phenolic content of *P. volubilis* aqueous extract via total phenolic content (TPC) assay, its radical scavenging ability via DPPH assay, and its protective effects on granulosa cells (COV434) subjected to oxidative stress via MTT assay. Ascorbic acid is used as a standard antioxidant to compare the results. As a result, the TPC assay confirms the presence of significant phenolic content in the extract where the phenolic contents increase as the concentration increases from 0.2 mg/ml to 15 mg/ml. Next, The DPPH assay shows the increase of DPPH radical scavenging with the IC_{50} value of 2.27 mg/ml. *P. volubilis* aqueous extract also shows a significant radical scavenging activity when compared to ascorbic acid ($p = 0.009$). Additionally, the MTT assay reveals the significant protective effects of the extract against hydrogen peroxide-induced oxidative stress in COV434 cells when compared to ascorbic acid ($p = 0.089$) with the EC_{50} value of 23.82 mg/ml. This is proven by increasing cell viability when treated with *P. volubilis* aqueous extract. Overall, the findings successfully underscore the promise of *P. volubilis* as a natural antioxidant to mitigate oxidative stress-related ovarian aging.

CHAPTER 1

INTRODUCTION

1.1 Background of The Study

Healthy ovaries are the indicator of the health and longevity of a woman's body. It produces hormones that are not only essential in reproductive function but also help in protecting the body and enhancing the immune system of women. However, these organs cannot retain their function forever. It cannot escape the aging process as the woman age. As the ovary ages, it loses its ability to reproduce due to decreased production of sex hormones as well as unable to protect the body from infections, illnesses and disorders.

Oxidative stress is the condition where there is an imbalance between pro-oxidants and antioxidants in the body. Increased pro-oxidants and reduced antioxidants can cause the accumulation of free radicals in the body such as reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulphur species (RSS) (Jomova *et al*., 2023). These free radicals can cause damage in the body at cellular and molecular levels disrupting the cellular membrane and interfering with genetic components like DNA and RNA (Chaudhary *et al*., 2023). These mechanisms are associated with several diseases such as cardiovascular disease, neurological disease, cancer and aging. In ovarian aging, oxidative stress is involved in impairing ovary function. The imbalance of pro-oxidants and antioxidants in ovaries causes damage to cells in the ovaries (Liang *et al*., 2023). Prolonged exposure to oxidative stress in the ovary can reduce the longevity of women's reproductive system and health (Wang *et al.*, 2021).

Ovarian aging is becoming a concern in female reproductive health since it is related to reduced fertility and increased age-related diseases such as osteoporosis, arthritis and cardiovascular disease. These problems are closely linked to the effect of oxidative stress in the ovary due to the ongoing aging process which can lead to cellular and hormonal dysfunction (Wang *et al*., 2021). In recent years, researchers have been focussing on the effect of oxidative stress towards granulosa cells due to its function in producing oestrogen. Oestrogen is the primary female sexual hormone which is crucial for the development and maturation of the female reproductive system. On the other hand, its function is also vital in maintaining ovarian health and enhancing the immune system. The destruction of granulosa cells by oxidative stress could lead to the decline of oestrogen secretion (Sun *et al*., 2023). It can directly affect the quality and quantity of the oocytes produced by the ovary (Secomandi *et al*., 2022). Subsequently, it can cause infertility in women and limit pregnancy.

Plukenetia volubilis, also known as Sacha Inchi, originates from the Amazon region and has been traditionally used to treat various health conditions due to its rich nutritional and medicinal properties. The seeds of *P. volubilis* are highly prized for their high content of essential fatty acids, particularly omega-3, omega-6, and omega-9, which contribute to their anti-inflammatory and heart-protective effects (Morales & Garcia, 2023). Apart from its lipid composition, Sacha Inchi is renowned for its powerful antioxidant properties, which are attributed to its bioactive compounds such as tocopherols, polyphenols, and flavonoids. Recent research has explored its potential for addressing metabolic disorders, enhancing skin health, and reducing oxidative stress, positioning it as a promising natural remedy for aging and chronic diseases (Samrit *et al*., 2024). As a result, *P. volubilis* continues to garner attention in both traditional and modern medicine for its diverse therapeutic uses.

1.2 Problem Statement

Ovarian aging is believed to be associated with increased oxidative stress and its action in damaging granulosa cells in the ovary. The increased damage of granulosa cells causes a decrease in oestrogen levels in the body which directly affects the fertility and chance of pregnancy in a woman (Secomandi *et al.*, 2022). As the woman ages, the endogenous antioxidants in the body begin to decrease, allowing the increase of prooxidant molecules which contribute to oxidative stress.

In this era, late marriage has become a common practice in society. Marriage at a later age has a significant impact on pregnancy in women due to the aging process. The chances of pregnancy begin to decrease as follicular development and hormone production decrease. It is mainly due to the cellular changes within the cell of the ovary such as theca cell and granulosa cell caused by increased oxidative stress, affecting the production of reproductive hormones (Wang *et al.*, 2021). The consumption of commercial antioxidants such as vitamin C and vitamin E can significantly help in slowing down the aging process, allowing the production of healthy oocytes (Fusco *et al.*, 2007). However, it must be taken with caution to prevent overdosage and adverse effects such as kidney problems, iron overload and gastrointestinal problems.

On the other hand, medications used to increase reproductive hormones in women, such as gonadotropins and clomiphene citrate, are commonly prescribed to treat infertility and hormonal imbalances. These drugs stimulate the ovaries to produce and release eggs, thereby improving the chances of conception. Hormone replacement therapy (HRT) with oestrogen and progesterone is also used to regulate menstrual cycles and alleviate symptoms of hormonal deficiencies, such as those seen during menopause. However, these medications come with potential disadvantages. The side effects may include mood swings, headaches, nausea, bloating, and weight gain which have more severe risks including ovarian hyperstimulation syndrome (OHSS), and can cause abdominal pain and fluid retention, as well as an increased chance of multiple pregnancies. Long-term use or misuse of hormonal therapies may also elevate the risk of certain cancers, such as breast or ovarian cancer (Feh *et al*., 2024). Therefore, these medications should be used under close medical supervision to minimize risks.

Many researchers have been looking for natural alternatives to commercial antioxidants, aiming to ensure that it is effective and safe for consumption as well as prevention of adverse effects which can cause discomfort and problems to women. One such natural product, *P. volubilis*, commonly known as Sacha Inchi, has gained widespread attention for its numerous health benefits, including its strong antioxidant properties. However, its potential effects on ovarian aging have not been fully explored yet. Thus, this study will evaluate how *P. volubilis* extract can help combat oxidative stress during the ovarian aging process.

1.3 Objectives

1.3.1 General Objective

To study the antioxidative effect of *P. volubilis* aqueous extract on the ovarian aging process

1.3.2 Specific Objectives

1. To determine the total phenolic content in *P. volubilis* aqueous extract by using Total Phenolic Content (TPC) assay
2. To evaluate the antioxidative ability of *P. volubilis* aqueous extract in scavenging DPPH radical by using DPPH radical scavenging assay

3. To assess the protective effect of *P. volubilis* aqueous extract against oxidative stress on ovarian granulosa cells (COV434) by using MTT assay

1.4 Hypothesis

1. *P. volubilis* aqueous extract has significant amount of total phenolic content expressed as gallic acid equivalents (GAE)
2. *P. volubilis* aqueous extract demonstrates a significant high DPPH scavenging activity compared to ascorbic acid
3. *P. volubilis* aqueous extract shows significant protective effect on ovarian granulosa cell line (COV434) compared to ascorbic acid

1.5 Significance of The Study

The effort to slow down the ovarian aging process is essential towards individuals, society and country. This study emphasises the use of *P. volubilis* in scavenging oxidative stress which is the main factor of ovarian aging in women. This study focusses on the effectiveness of *P. volubilis* extract in ensuring the longevity of ovarian health. It can benefit all women around the world especially those of late marriage to conceive. In addition, this study can also help to reduce the risk of diseases and disorders due to insufficiency of reproductive hormones such as osteoporosis, autoimmune and cardiovascular disease. The effort of sustaining ovarian function can help in maintaining woman's health in general.

CHAPTER 2

LITERATURE REVIEW

2.1 Anatomy and Physiological Function of Ovary

The ovary is a vital organ in the female reproductive system, serving dual roles as an endocrine gland and a reproductive organ. Its primary functions include the production of female sex hormones and the release of oocytes necessary for reproduction. The paired, almond-shaped ovaries are located within the pelvic cavity, on either side of the uterus and it integral to the regulation of the menstrual cycle, fertility, and the maintenance of secondary sexual characteristics. Each ovary measures approximately 3 cm in length, 1.5 cm in width, and 1 cm in thickness. However, these dimensions can vary based on age and physiological conditions such as puberty, pregnancy, and menopause. The ovaries are connected to the uterus via the ovarian ligament and to the lateral pelvic wall by the suspensory ligament, which also contains the ovarian vessels and nerves. These structural attachments ensure the stability of the ovaries while facilitating vascular and neural connections (Soloyan *et al*., 2019).

Figure 2.1 shows the anatomical structure of the ovary which includes the internal architecture, vascular supply and follicular stages. The internal architecture of the ovary is divided into two primary regions known as the cortex and the medulla. The cortex forms the outer portion of the ovary and is the functional region containing ovarian follicles at various stages of development. These follicles progress through well-defined stages: primordial, primary, secondary, and tertiary (Graafian) follicles. The primordial follicles, which are present at birth, represent the finite reservoir of oocytes available throughout a female's reproductive lifespan. Upon hormonal stimulation, the follicles mature, with one of the ovaries typically becoming dominant to release a mature oocyte during ovulation. After ovulation, the remnants of the follicle form the corpus luteum, a temporary endocrine structure that secretes progesterone to support early pregnancy. In the absence of fertilization, the corpus luteum degenerates into the corpus albicans, a fibrous scar-like structure. Next, the medulla occupies the central portion of the ovary and comprises a loose connective tissue matrix interspersed with blood vessels, lymphatic vessels, and nerves. This region provides the necessary vascular supply and neural control to support follicular development and endocrine function (Avellar & Barreto, 2022).

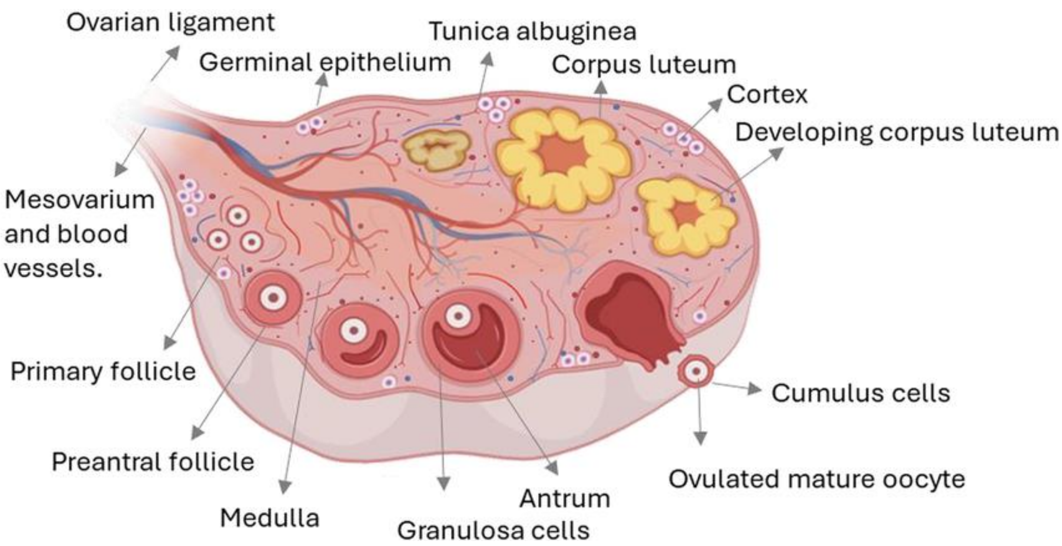

Figure 2.1: The anatomical structure of the ovary (Adapted from Nair *et. al.,* 2024)

The ovary's endocrine function involves the synthesis and secretion of hormones, including oestrogen, progesterone, inhibin, and activin. These hormones are critical in regulating the menstrual cycle, promoting the development of secondary sexual characteristics, and preparing the reproductive tract for potential pregnancy. Concurrently, the ovary's reproductive function entails the cyclic release of oocytes through ovulation, a process tightly regulated by hormonal feedback mechanisms involving the hypothalamus, pituitary gland, and ovary. Within the ovarian cortex, a variety of specialized cells are involved in the synthesis and secretion of hormones such as granulosa cells, theca cells, luteal cells, stromal cells and oocytes, each playing distinct roles in endocrine regulation. The focus would be on granulosa cells and theca cells as they are the first-line cells involved in hormonal regulation during follicular development in the ovaries (Avellar & Barreto, 2022).

Figure 2.2 shows the cellular structure of follicles including granulosa cells, theca cells and oocytes. Granulosa cell is the main type of cells involved in the regulation of hormones and the first line cell that determines the chance of pregnancy in women. These cells are located within the follicle, surrounding the developing oocyte. These cells are primarily responsible for producing oestrogen, particularly during the follicular phase of the menstrual cycle. Oestrogen synthesis involves the conversion of androgens that are produced by the theca cells into oestradiol, a process mediated by the enzyme aromatase. Granulosa cells also secrete inhibin, a hormone that negatively regulates follicle-stimulating hormone (FSH) production from the anterior pituitary, ensuring the proper selection of the dominant follicle. Post-ovulation, granulosa cells, along with theca cells, transform into luteinised granulosa cells, which are critical for progesterone production in the corpus luteum (Alam & Miyano, 2020).

Next, theca cells are also important in hormonal regulation in the ovary. Theca cells form the outer layer of the follicle and are subdivided into two layers which are theca interna and theca externa. The theca interna cells are responsible for synthesising androgens, such as androstenedione, under the influence of luteinising hormone (LH) from the anterior pituitary. These androgens serve as precursors for oestrogen production in granulosa cells. On the other hand, theca externa cells provide structural support to the follicle but do not play a direct role in hormone synthesis (Liu *et. al.*, 2020). Theca cells alongside granulosa cells work synergistically in the ovary to ensure the fertility of a woman is preserved. Any declined of these cells can lead to various consequences such as ovarian aging, menopause and infertility.

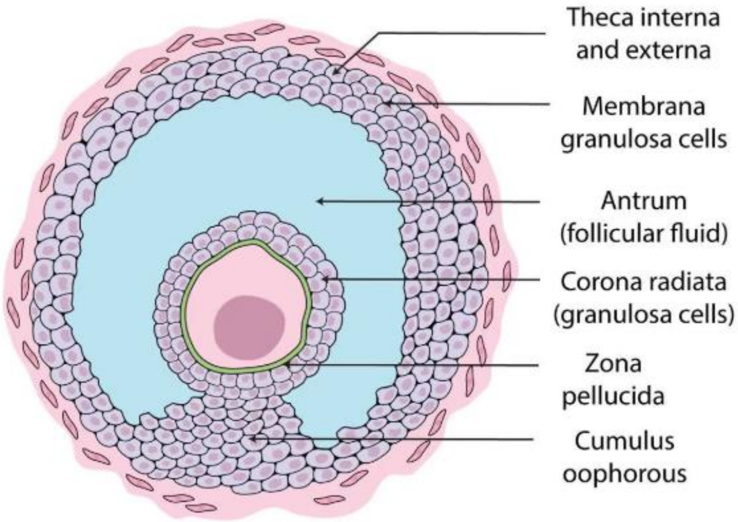

Figure 2.2: Cellular structure of follicle (Adapted from MyEndoConsult.com)

2.2 Ovarian Aging Process

Ovarian aging is a natural process characterised by a gradual decrease in both the quantity and quality of oocytes, affecting female fertility and ultimately leading to menopause (Wang *et al*., 2023). The ovarian reserve, which refers to the number of primordial follicles in the ovaries, begins to decline early as women approach the average age of menopause (over 40 years old) (Park *et al*., 2021). Oxidative stress is a major factor contributing to this decline as the accumulation of reactive oxygen species (ROS) causes cellular damage in the ovaries which leads to decreased oocyte quality (Ra *et al*., 2023).

Mitochondrial dysfunction is closely linked to ovarian aging as mitochondria play a crucial role in energy production within oocytes. As women age, mitochondrial function will deteriorate, resulting in reduced ATP production, increased oxidative damage and eventually contribute to decreased fertility (May-Panloup *et al*., 2016). Additionally, the shortening of telomeres, which protect chromosome ends, is another characteristic of ovarian aging. This can lead to cellular senescence and a decrease in reproductive potential (Secomandi *et al*., 2022).

As the ovarian reserve diminishes, hormonal changes occur, with an increase in follicle-stimulating hormone (FSH) levels due to the reduced number of responsive follicles, and a decline in anti-Müllerian hormone (AMH) levels (Broer *et al*., 2014). These hormonal shifts are linked to a decrease in oocyte quality, particularly an increased occurrence of aneuploidy, a significant cause of infertility and miscarriage in older women (Herbert *et al.*, 2015).

2.3 Role of Oxidative Stress in Ovarian Aging

Oxidative stress is increasingly recognised as a key factor in the progression of ovarian aging, which results from an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defence mechanisms. Within the ovaries, ROS are naturally formed during metabolic processes, particularly in the mitochondria. However, as individuals age, the accumulation of ROS exceeds the capacity of antioxidant defences, leading to cellular damage that accelerates ovarian decline (Yan *et al.*, 2022).

The impacts of oxidative stress on the ovaries manifest in the reduction of the ovarian follicle reserve, DNA damage in oocytes, and impaired mitochondrial function. Oocytes are especially susceptible to oxidative stress due to their high metabolic activity and large surface area. Research has indicated that oxidative stress causes DNA fragmentation and telomere shortening in oocytes, which are significant indicators of aging and contribute to reduced fertility (Kordowitzki, 2021). This process can be observed in Figure 2.3 which emphasises the effect of oxidative stress towards telomere in oocytes which can lead to ovarian aging. Furthermore, oxidative stress disrupts mitochondrial function in oocytes and granulosa cells, reducing ATP production and hindering the energy supply necessary for oocyte maturation and follicular development (Gao *et al.*, 2023).

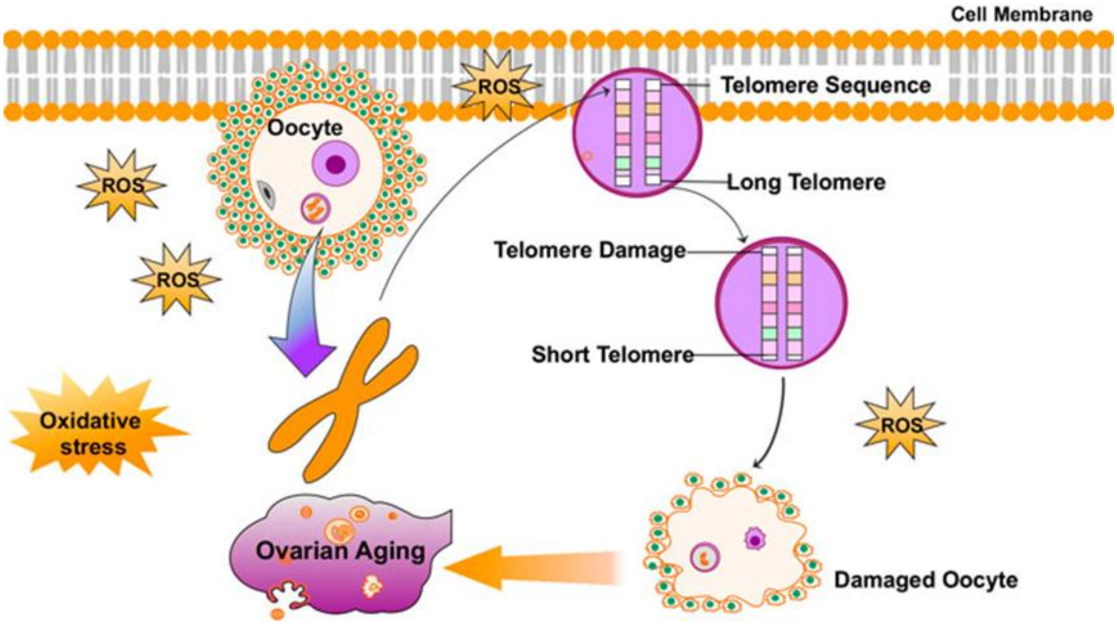

Figure 2.3: Ovarian aging process due to oxidative stress (Adapted from Yang *et al.*, 2021)

Increased oxidative stress is also associated with several ovarian disorders such as polycystic ovary syndrome (PCOS) and premature ovarian insufficiency (POI), both of these disorders can further accelerate the ovarian aging process. Studies have demonstrated that women with these conditions exhibit higher levels of oxidative markers and lower antioxidant enzyme activity, indicating that oxidative stress plays a central role in both normal and pathological ovarian aging (Ra *et al.*, 2023). Hence, reducing oxidative stress through antioxidant therapies or lifestyle interventions is being investigated as a potential strategy to delay ovarian aging and preserve reproductive function.

2.4 Antioxidants and Their Role in Slowing Ovarian Aging

Antioxidants have become a potential treatment method for counteracting oxidative stress which aims to slow down the aging of ovaries. Both exogenous and endogenous antioxidants are essential in neutralising ROS and safeguarding ovarian tissues from oxidative damage, making them a promising target for interventions to decelerate ovarian aging. Various research studies have emphasised the potential of external antioxidants like vitamin C, vitamin E, and coenzyme Q10 in reducing oxidative stress in the ovaries. For example, vitamin E, a lipid-soluble antioxidant, shields cell membranes from lipid peroxidation, a process that leads to cellular aging and malfunction. Studies have demonstrated that supplementing with vitamin E can diminish oxidative damage in ovarian granulosa cells, enhancing oocyte quality and prolonging ovarian function (Rodríguez-Varela & Labarta, 2020). Similarly, coenzyme Q10, which is crucial for mitochondrial function, has been found to improve mitochondrial efficiency and decrease ROS production in aging ovaries, thereby enhancing ovarian reserve and delaying ovarian aging (Ben-Meir *et al*., 2015).

Endogenous antioxidants such as superoxide dismutase (SOD), catalase, and glutathione also play vital roles in shielding the ovaries from oxidative stress. As individuals age, the activity of these antioxidant enzymes decreases, making ovarian cells more susceptible to oxidative damage. Increasing the activity or levels of these enzymes through antioxidant treatments has shown the potential to slow down ovarian aging. A previous study found that increasing SOD activity in ovarian tissues can prolong reproductive lifespan by reducing oxidative stress and preserving follicle viability (Liang *et al.*, 2023). This indicates that antioxidant treatments are crucial in enhancing the activity of internal antioxidants in neutralizing oxidative stress and decelerating the ovarian aging process. Both dietary antioxidants and interventions aimed at enhancing internal antioxidant systems hold promise as strategies to postpone ovarian aging and extend reproductive health.

2.5 In Vitro Models of Ovarian Aging

The use of in vitro models has become crucial for examining the molecular mechanisms involved in ovarian aging due to their ability to facilitate controlled experiments and ensure high reproducibility. These models encompass a spectrum from 2D cell cultures to more advanced 3D organoid systems, which better replicate the ovarian microenvironment. Granulosa cell cultures have been extensively employed as a representation of ovarian aging as these cells play a pivotal role in follicular development and hormone production (Wang *et al.*, 2021). Research has indicated that aging granulosa cells demonstrate increased oxidative stress, DNA damage, and diminished mitochondrial function, all of which are significant indicators of ovarian aging (Lin *et al*., 2022). These cells can be extracted from animal or human ovaries or purchased from a company like the ovarian granulosa tumour cell line (COV434) and grown in culture to explore the effects of oxidative stress or other aging-related factors in vitro.

Ovarian organoids have recently garnered attention as a more physiologically relevant model for ovarian aging. Organoids are 3D structures derived from stem cells or primary ovarian tissue, and they mimic numerous aspects of ovarian follicles, including their structure and functionality. These models enable long-term investigations into follicle dynamics, hormonal responses, and age-related alterations (Heremans *et al*., 2021). Organoids also provide a promising platform for screening drugs and studying the impacts of antioxidants or anti-aging therapies on ovarian tissues (Liu *et al.*, 2020). Another approach involves using induced pluripotent stem cells (iPSCs) to generate ovarian cells in vitro. This model allows for the examination of ovarian aging in a patient-specific context, thus permitting the exploration of genetic factors contributing to age-related ovarian dysfunction (Ali *et al.*, 2022). iPSCs can differentiate into granulosa cells or other ovarian cell types, providing a versatile model to investigate the molecular pathways involved in ovarian aging and potential interventions (Smela *et al.*, 2023).

2.6 *Plukenetia volubilis* (Sacha Inchi): Bioactive Compounds and Antioxidant Properties

P. volubilis, commonly known as Sacha Inchi or the Inca peanut, is a perennial plant native to the Amazon rainforest. It is a climbing shrub with a vine-like growth habit, capable of reaching up to 2 meters in height when supported. The plant is characterized by its heart-shaped, green leaves, and small, unisexual flowers that are pollinated by insects. Its fruit is a star-shaped capsule with 4 to 7 lobes, each containing a single seed. The seeds, which resemble flattened nuts, are rich in nutrients, including essential fatty acids (omega-3, omega-6, and omega-9), proteins, and antioxidants. They have a hard outer shell and are pale brown to dark brown in colour when mature. These seeds are widely recognized for their high nutritional value and are often used in health supplements, oils, and snacks. The plant thrives in tropical climates, requiring warm temperatures, moderate rainfall, and well-drained soils for optimal growth (Lourith *et al.*, 2024). Figure 2.4 shows the tree, the parts of fruits and the oil extract of *P. volubilis* that are widely used to evaluate its health benefits to humans.

Figure 2.4: The Image of *P. volubilis* Tree (A), Dried Fruits (B), Seeds with Shell (C), Husk (D), Seeds (E) and Oil (F) *(Adapted from Rodzi & Lee, 2022)*

P. volubilis is renowned for its high concentration of bioactive compounds, such as essential fatty acids, phenolic compounds, and tocopherols, all of which contribute to its powerful antioxidant properties. The seeds of Sacha Inchi are particularly abundant in omega-3 fatty acids, particularly alpha-linolenic acid (ALA), as well as omega-6 and omega-9, which are essential for reducing inflammation and oxidative stress in the body (Cárdenas *et al*., 2021). The presence of these unsaturated fatty acids has been associated with cardiovascular health benefits and the prevention of lipid peroxidation (Wang *et al.*, 2018)

Aside from fatty acids, Sacha Inchi is rich in polyphenolic compounds, including flavonoids and tannins, which contribute to its strong antioxidant capacity. Research has shown that the phenolic content of Sacha Inchi seeds exhibits significant radical-scavenging activity, helping to counteract free radicals and safeguard cells from oxidative damage (Cárdenas *et al.*, 2021). Additionally, tocopherols (derivatives of vitamin E) in Sacha Inchi oil have been discovered to enhance the plant's antioxidant effects by preventing lipid peroxidation, which is crucial for maintaining cellular integrity (Rodríguez *et al.*, 2021).

The antioxidant properties of Sacha Inchi have been further supported by in vitro studies, demonstrating its ability to inhibit oxidative stress and enhance the activities of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (Rahman *et al.*, 2023). These findings underscore its potential as a natural source of antioxidants, which could have promising implications for promoting health and preventing disease, particularly in lowering the risk of chronic diseases associated with oxidative damage (Rahman *et al.*, 2023).

2.7 Potential Benefits of *Plukenetia volubilis* Extract in Ovarian Health

P. volubilis (Sacha Inchi) contains valuable bioactive elements such as omega-3 fatty acids, tocopherols, and polyphenols, known for their potent antioxidant and anti-inflammatory properties (Rodríguez *et al.*, 2021). These elements play a crucial role in managing oxidative stress, a significant factor in ovarian aging and dysfunction, as evidenced by research (Ra *et al.*, 2023). As women grow older, oxidative stress escalates in ovarian tissue, leading to reduced fertility and various ovarian conditions like polycystic ovary syndrome (PCOS) and premature ovarian failure (POF) (Ra *et al.*, 2023).

Research has shown that the antioxidant properties of Sacha Inchi extract can mitigate oxidative damage in ovarian cells. Notably, the abundance of omega-3 fatty acids in the extract, particularly alpha-linolenic acid, has been proven to enhance ovarian function by reducing inflammation and supporting hormonal balance (Wang *et al.*, 2020). Furthermore, the presence of tocopherols in the extract serves as robust antioxidants, safeguarding ovarian cells from lipid peroxidation, essential for maintaining the integrity of ovarian cell membranes (Cárdenas *et al.*, 2021).

Additionally, it is believed that the polyphenols and flavonoids in Sacha Inchi extract can regulate antioxidant enzymes like superoxide dismutase (SOD) and catalase, which play protective roles in ovarian tissue (Sławińska & Olas, 2023). This activity may promote ovarian longevity and help counteract the age-related decline in ovarian function. While research specifically focusing on the direct impact of Sacha Inchi extract on human ovarian health is still limited, its bioactive elements show potential as a natural therapeutic approach for supporting reproductive health, particularly in reducing the risks associated with ovarian aging and oxidative stress.

2.8 Antioxidant Assays in Examining the Antioxidative Ability of Natural Products

Natural products have gained significant attention for their potential antioxidant properties, which are crucial in neutralizing oxidative stress and preventing damage caused by reactive oxygen species (ROS). Various in vitro antioxidant tests have been developed to evaluate the ability of natural compounds to scavenge free radicals, inhibit oxidation, and protect cells from oxidative damage. These tests provide valuable insights into the potential therapeutic applications of natural products in preventing oxidative stress-related diseases.

One of the most widely used methods for assessing antioxidant activity is the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. This method evaluates the ability of natural compounds to donate hydrogen atoms or electrons to neutralize the stable DPPH free radical, which changes colour from purple to yellow upon reduction (Gulcin & Alwasel, 2023). The degree of discolouration is measured spectrophotometrically, and the results provide a quantitative measure of the antioxidant capacity of the sample. The DPPH assay is simple, rapid, and highly sensitive, making it one of the most popular methods for screening natural products for their antioxidative properties.

Another commonly employed test is the ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) assay, which measures the ability of antioxidants to quench the ABTS radical cation, resulting in a decrease in absorbance (Ilyasov et al., 2020). This method is particularly useful for evaluating both hydrophilic and lipophilic antioxidants and is considered more versatile than the DPPH assay. The ABTS assay is often used in conjunction with the DPPH method to provide a more comprehensive analysis of the antioxidant potential of natural products (Wołosiak *et al.*, 2022).

The FRAP (Ferric Reducing Antioxidant Power) assay is another important method that assesses the reducing ability of antioxidants. In this assay, antioxidants reduce the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}), which forms a blue-coloured complex that can be quantified spectrophotometrically (Benzie & Strain, 1996). The intensity of the colour is proportional to the antioxidant power of the sample. The FRAP assay is valued for its simplicity and ability to evaluate the electron-donating capacity of natural compounds (Silvestrini *et al*., 2023).

Other assays, such as the ORAC (Oxygen Radical Absorbance Capacity) assay, focus on measuring the ability of antioxidants to inhibit the oxidation of specific substrates in the presence of free radicals. The ORAC assay is particularly effective in evaluating the capacity of antioxidants to protect biological systems from oxidative stress (Silvestrini *et al.*, 2023). This test is widely used to measure the antioxidant potential of natural products, particularly in the context of food and nutraceutical research.

CHAPTER 3

METHODOLOGY

3.1 Materials and Apparatus

3.1.1 List of Materials

Table 3.1: The list of materials used in the study

No.	Materials	Manufacturer
1.	0.25% Trypsin	Nacalai Tesque, Japan
2.	2,2-diphenyl-1-picrylhydrazyl (DPPH) Powder	Sigma Aldrich, United States
3.	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Powder	Sigma Aldrich, United States
4.	99.5% Ethanol	HmbG Chemicals, Malaysia
5.	Ascorbic Acid (Vitamin C)	Nacalai Tesque, Japan
6.	Dimethyl Sulfoxide (DMSO)	Sigma Aldrich, United States
7.	Distilled Water	Pusat Pengajian Sains Kesihatan, Universiti Sains Malaysia, Malaysia
8.	Foetal Bovine Serum (FBS)	Nacalai Tesque, Japan
9.	Follin-Ciocalteu Reagent	Sigma Aldrich, United States
10.	Gallic Acid	Nacalai Tesque, Japan
11.	High Glucose Dulbecco's Modified Eagle Medium (DMEM)	Nacalai Tesque, Japan
12.	Hydrogen Peroxide, H_2O_2	Sigma Aldrich, United States
13.	Penicillin-Streptomycin	Nacalai Tesque, Japan
14.	Phosphate Buffer Saline (PBS)	Sigma Aldrich, United States
15.	Sodium Carbonate, Na_2CO_3	Nacalai Tesque, Japan
16	Trypan Blue	Sigma Aldrich, United States

3.1.2 List of Apparatus

Table 3.2: The list of apparatus used in the study

No.	Apparatus	Manufacturer
1.	96-well Cell Culture Plate	Biologix, China
2.	Centrifuge Machine	Hettich, Germany
3.	Centrifuge Tube	Biologix, China
4.	Cryopreservation Vial	Biologix, China
5.	Culture Flask (T25)	Biologix, China
6.	Microcentrifuge Tube	Biologix, China
7.	Micropipette	Eppendorf, Germany
8.	Microplate reader	Tecan, Switzerland
9.	Pipette tip	Biologix, China
10.	Tube rack	Biologix, China

3.1.3 Preparation of Reagents and Sample

3.1.3 (a) *Plukenetia volubilis* Aqueous Extract

P. volubilis aqueous extract was obtained from an industrial collaborator. For the antioxidant test, a 10 ml stock sample of 30 mg/ml was prepared by adding 20 µl of the sample in 9980 µl distilled water to produce 15 mg/ml of *P. volubilis* aqueous extract. For the MTT assay, 3 series of 10-fold dilutions were made by dissolving 100 µl of sample in 900 µl plain DMEM media to get 15 mg/ml which was used as the first concentration.

3.1.3 (b) Ascorbic acid

30 mg of ascorbic acid powder was weighed by using a weighing balance and dissolved in 2 ml distilled water to produce 15 mg/ml of ascorbic acid stock solution. For the MTT assay, 30 mg of ascorbic acid powder was dissolved in 2 ml plain DMEM to produce 15 mg/ml of ascorbic acid.

3.1.3 (c) 10% (v/v) Follin-Ciocalteu (F-C) Reagent

10% F-C reagent was prepared by diluting 2 ml of 2N F-C reagent in 18 ml of distilled water. 10% F-C reagent was used for the total phenolic content (TPC) assay to estimate the phenolic content in the sample.

3.1.3 (d) 7.5 % Sodium Carbonate, Na_2CO_3

3 mg of sodium carbonate powder was dissolved in 40 ml of distilled water.

3.1.3 (e) 0.2 mg/ml Gallic Acid

1 mg of gallic acid was dissolved in 5 ml of DMSO to make 0.2 mg/ml of gallic acid. 2-fold serial dilution was then prepared as the standard solution for TPC assay.

3.1.3 (f) Complete Dulbecco's Modified Eagle Media (DMEM)

Complete DMEM was prepared by adding 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin. Complete DMEM was used for culturing COV434 cells.

3.1.3 (g) 0.2mM DPPH reagent

0.789 mg of DPPH powder was dissolved in 10 ml absolute ethanol.

3.1.3 (h) 0.001M Hydrogen Peroxide, H_2O_2

1M hydrogen peroxide was prepared from 9.79M hydrogen peroxide stock by diluting 102 µl of stock in 898 µl of sterile distilled water. Next, a series of 10-fold serial dilutions were made to obtain 0.001M hydrogen peroxide by diluting 100 µl of hydrogen peroxide in 900 µl sterile distilled water.

3.1.3 (i) 5 mg/ml MTT reagent

A 5 mg/ml MTT reagent was prepared by dissolving 30 mg of MTT powder in 6 ml of Phosphate Buffer Saline (PBS). The mass of MTT powder was calculated based on the number of treated wells used in the MTT assay.

3.2 Ovarian Granulosa Cell (COV434) Culture

Human Ovarian Granulosa Cell, COV434 cell is an immortalised cell line which retains many characteristics of primary granulosa cells. This cell line is an invaluable model for studying ovarian function for both normal and pathological conditions. The cells were cultured and maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and 1% penicillin-streptomycin (Berg-Bakker *et al.*, 1993 & Zhang *et al.*, 2000). This cell line was used between passage 2 to 3.

3.2.1 Reviving cells

The cryopreserved COV434 cell in 1ml cryovial from liquid nitrogen was taken out for revival of cells. Complete DMEM media was prewarmed at 37°C in the water bath and the cryopreserved cells were thawed by rubbing the cryovial. The reviving process must be conducted in the Biosafety Cabinet (BSC) Class II. After the cells were thawed and the media was warmth, the cells were pipetted into a 15 ml falcon tube and mixed with 1ml of media. The falcon tube was centrifuged at 260 xg for 5 minutes to get the pellet.

The pellet was then resuspended with 1ml of media and mixed. The cell suspension was cultured into a T25 flask. 4 ml of media was then added to the T25 flask which was labelled with the name of cells, passage number, date of reviving and name of person-in-charge. The flasks were sprayed with 70% ethanol to sanitise the flask and then kept in the incubator (37°C, 5% Carbon Dioxide and high humidity).

3.2.2 Changing Culture Medium

The flasks were taken out from the incubator after 24 hours and the physical characteristics of the media such as turbidity and colour were observed. If the media was turbid, it might be a sign of contamination or unattached cells and further observation under the microscope is needed to confirm the hypothesis. The cells were then observed under the microscope at 5x magnification to confirm the attachment of COV434 cells and 40x magnification to observe any contamination such as bacteria and yeast. The flask was discarded if contamination was observed.

If there is no sign of contamination, the media was changed to new complete media. The media in the flask was pipetted out and discarded into the waste container in the BSC Class II. The cells were washed 3 times with 2 ml of PBS. After washing, 5 ml of new DMEM was added to the flask. The flask was then kept in the cell culture incubator.

3.2.3 Cell Passaging

The cells were observed to ensure they achieved 70% confluency and above before passaging. The confluence cells were washed 3 times with 2 ml of PBS. 1 ml of 0.25% trypsin was added to the cells to initiate the detachment process. The cells were incubated with trypsin for 5 minutes to allow the detachment of the cells. After incubation, the cells were observed under a microscope at 4x magnification to confirm the cells were fully detached. If the cells were fully detached, 1ml of DMEM was added to stop the action of trypsin. The cell suspension was then transferred into a 15 ml falcon tube and centrifuged at 260 xg for 5 minutes. After the centrifugation process, the supernatant was discarded, and the cell pellet was resuspended with 1 ml of DMEM. Then, the cell suspension was added into a new T25 flask with 4 ml of DMEM before incubation.

3.2.4 Cell Counting

10 µl of cell suspension was taken from the resuspended cells in the passaging steps (Section 3.2.3). 10 µl of Trypan Blue was added to the suspension and mixed completely. 10 µl of the mixture was then added to the haemocytometer and observed under the microscope at 10X magnification to count the cells. The cells need to be counted within 4 fields of the haemocytometer grid and the cell density was calculated by using the following formula.

$$Cell\ density\ (cells/ml)\ = (\frac{total\ cell\ counted\ on\ 4\ field}{4}) \times dilution\ factor \times 10^4$$

Where dilution factor depends on how many dilutions were made before adding the cells to the haemocytometer. Normally, the dilution factor was 2 due to the addition of 10 µl Trypan Blue into 10 µl of cell suspension.

3.2.5 Cryopreservation of Cells

The confluence cells were washed 3 times with 2 ml of PBS, treated with 1 ml of 0.25% trypsin and incubated to allow the detachment process to occur. After the cells fully detached, 1 ml of DMEM was added to stop the action of trypsin. The cell suspension was then transferred to a 15 ml falcon tube and centrifuged at 260 xg for 5