Lactogenic activity of *Gunnera perpensa* L. (*Gunneraceae*) from South Africa

Mthokozisi B.C Simelane

20054768

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Supervisor : Prof A.R Opoku
Co-supervisor: Prof T.G Djarova

2010

Dedication

To

My family

DECLARATION

This is to certify that the work reported in the dissertation entitled “Lactogenic activity of *Gunnera perpensa* L *(Gunneraceae)* from South Africa” is an original work of Mr. Mthokozisi B.C Simelane, carried out under our supervision and directions. The dissertation has been submitted to fulfill the requirements for the degree Master of Science (Msc) with the approval of the undersigned.

I, M.B.C Simelane, declare that the dissertation has not previously been submitted by me for a degree at this or any other University, that this is my own work in design and in execution, and that all the material contained therein has been duly acknowledged.

M.B.C. Simelane	Prof A.R. Opoku	Prof T.G. Djarova
(Student)	(Supervisor)	(Co-Supervisor)
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ABSTRACT

Gunnera perpensa L. (Gunneraceae) is a medicinal plant used by Zulu traditional healers to induce labor, expel the placenta after birth, to relieve menstrual pains, and to stimulate milk production. Phytochemical screening of the rhizomes revealed the presence of alkaloids, flavonoids, steroids, saponins, tannins and glycosides. Methanol extracts of *G*. *perpensa* exhibited strong scavenging of 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) and 3-ethylbenzothiazoline-6-sulfonate (ABTS), but showed poor radical scavenging of nitric oxide, superoxide and hydroxyl radicals. At a concentration of 5 mg/100 ml, the extract was able to inhibit lipid peroxidation of the whole rat brain homogenate (71%) and lipoxygenase (30%) activity. The plant extract also contained reduced form of nicotinamide adenine dinucleotide (NADH, 3.8 ρm/g), and total phenol (248.45 mg/g). The total antioxidant capacity was 36% relative to ascorbic acid (AA) and 64% relative to butylated hydroxyl toluene (BHT).

The effect of an aqueous extract of the rhizome of the plant on milk production in rats was also investigated. Female lactating rats that received oral doses of aqueous extract of *G perpensa* produced more milk than controls (P<0·05). Pup weight gain was also significantly higher than that in the control group (P<0.05). In addition, the mammary glands of rats treated with the extract showed lobuloalveolar development. The extract of *G perpensa* was found to stimulate the contraction of the uterus; the highest amplitude was 5.06±08mm. *G perpensa* extract inhibited (23%) fish-brain acetylcholinesterase activity. The plant extract did not significantly influence prolactin,

growth hormone, progesterone, cortisol, ALT, AST, and albumin levels. It is inferred that the plant extract exerts its activity on milk production and secretion by stimulating lobuloalveolar cell development and the contraction of myoepithelial cells in the alveoli.

The cytotoxicity of the extract (LC_{50}) to brine shrimp larvae was 137.62 mg/ml and to two human cell lines (HEK293 and HEPG2) it was 279.43µg/ml and 222.33µg/ml respectively.

It is apparent that the antioxidant and lactogenic activity of G. *perpensa* contributes to its effectiveness in folk medicine.

Key words: *Gunnera perpensa, gunneraceae*, antioxidant activity, cytotoxicity, lactation, muscle contractility

ABBREVIATIONS

ABTS *	3-ethylbenzothiazoline-6-sulfonate
DPPH	2,2-Diphenyl-1-picryl-hydrazyl
$^{\cdot}OH$	Hydroxyl radical
$O^{2\cdot-}$	Superoxide anion radical
$NO^{\cdot}$	Nitric oxide radical
DMSO	Dimethylsulfoxide
MDA	Malondialdehyde
TBA	Thiobarbituric acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
ASChi	s-acetyl- thiocholine iodide
EDTA	Ethylenediaminetetra-acetic acid
BHA	Butylated hydroxyl-anisole
BHT	Butylated hydroxyl toluene
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ALP	Alkaline phosphatase
WHO	World Health Organisation
UZ	University of Zululand
KZN	KwaZulu Natal
AA	Ascorbic acid
CA	Citric acid

CONTRIBUTION TO KNOWLEDGE

See Appendix D

A. Publication

1. Simelane, M.B.C., Lawal, O.A., Djarova, T.G., **Opoku**, **A.R.** (2010). "In vitro antioxidant and cytotoxic activity of *Gunnera perpensa* L. (Gunneraceae) from South Africa". *Journal of Medicinal Plant Research* Vol.**4** (21) pp. 2181-2188.

B. Paper presented at scientific conference

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CHAPTER 1

INTRODUCTION

Milk is a food that is of great biological significance for people from the moment they are born; it is accredited by the quantity and quality of nutrients it contains (high biological value proteins, and significant quantities of inorganic salts—phosphorus and calcium) which are structural materials for the skeleton. Milk is produced by the breast through endocrine and autocrine systems. Alveoli (hollow cavities, a few millimetres large) are the primary components of a mature mammary gland lined with milk-secreting cuboidal cells and surrounded by myoepithelial cells. These alveoli join together to form groups known as lobules, and each lobule has a lactiferous duct that drains into openings in the nipple. The myoepithelial cells can contract under the stimulation of oxytocin thereby excreting milk secreted from alveolar units into the lobule lumen toward the nipple, where it collects in sinuses of the ducts. As the infant begins to suck, the hormonally (oxytocin) mediated "let down reflex" ensues and the mother's milk is secreted (Watson and Khaled, 2008).

The key hormone that initiates milk biosynthesis is prolactin (Vonderhaar, 1987), which exerts a direct effect on the mammary gland through prolactin receptors.

When a mother has low prolactin level, milk supply may be affected. The drugs used for increasing milk supply work by blocking dopamine—a major inhibitor of prolactin. These drugs do not work in all women and would not be likely to increase milk production in a woman who already has normal or high prolactin levels (Brown, *et al*, 2000). Drugs like metoclopramide (Reglan) cause severe depression in mothers (Gabay, 2002). The toxicity of the currently available drugs has stimulated the search for alternative medicine.

Traditional healing has been the main form of medicine to cure diseases and providing primary healthcare to rural people. This is because modern health care to the rural people is still a far-reaching goal, due to economic limitations (Grover *et al*., 2002). Traditional healers have used various herbal preparations in addressing various illnesses. Ethnobotanical survey of traditional healers around Kwa-Zulu Natal recommend *Gunnera perpensa* L (*ugobho*-- Zulu name) to have lactogenic activity, Decoctions prepared from it are used to remove excess fluid from the body and to remove placenta after birth (Ngwenya *et al.* 2003; Van Wyk and Gericke 2000; Von Ahlenfeldt *et al.* 2003). Considering the extensive utilization of *G. perpensa* in Zulu traditional medicine, the present study was undertaken to investigate the effect of the rhizomes of *G perpensa* on milk production in lactating rats.

CHAPTER 2

LITERATURE REVIEW

Milk is an important natural food for infants from the minute they are born; it is the food for a new born for the period of first six months of his or her life. The importance of milk is due to the different nutrients it contains (high biological value proteins, essential amino acids and significant quantities of inorganic salts which are essential for the building up the skeleton) (Hale, 2004). Milk is the primary diet of mammalian infants, because it contains immunoglobulins (Birgens *et al*, 1983), antimicrobial peptides e.g. lactoferrin and lactoperoxidase (Tomita *et al*, 1991), and growth factors (Nichols *et al,* 1987).

2.1 MILK PRODUCTION AND SECRETION

Milk secretion is a very complicated process that undergoes so many changes; most of the factors influencing milk secretion are unacknowledged (Neville *et al,* 2002). Fully grown breasts are comparable to a flowering tree in spring time**,** with lobular alveolar complexes, which are referred to as terminal duct lobular units (TDLU) by pathologists, developing frequently from the main ducts. At times the breast reaches a stage of dormancy which is revealed by some waxing and waning of the TDLU controlled by the hormonal changes of the menstrual cycle (Neville and Morton, 2001). Thus, induction of the terminal differentiation of mammary alveolar cells before birth and after birth requires the presence of hormones like prolactin (Topper and Freeman, 1980). See figure 2.1.

Milk lactose biosynthesis is extremely dependent on the action of α-lactalbumin (LA). LA confers the binding of glucose to the complex and consequently facilitates the biosynthesis of lactose (Pike, *et al*, 1996). Glucose is required to sustain milk production during lactation

and its production can be obtained by increasing the process of gluconeogenesis. Glucagon is a key hormone that stimulates gluconeogenesis and increase glucose production from liver (Williams *et al*, 2006). Thyroid hormones have a major responsibility in the production of α-lactalbumin (Vonderhaar and Greco, 1979; Bhattacharjee and Vonderhaar, 1984).

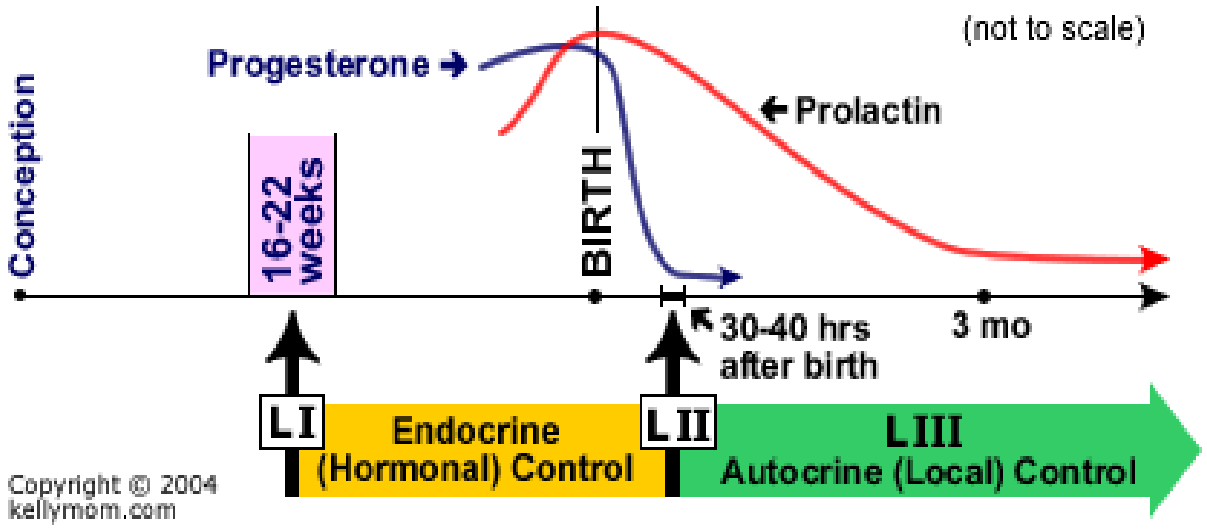


Fig 2.1. Hormonal changes during conception and lactation (L) at different time intervals (www.kellymom.com/bf/supply/milkproduction.html).

Milk secretion is suppressed by high circulating levels of progesterone (Figure 2.1). This is referred to as lactogenesis I. Lactogenesis II occurs after birth, normally 30–40 hours after the delivery of a full-term infant. Once the placenta is detached, progesterone levels decrease sharply and prolactin levels increase to initiate milk secretion.

Prolactin (PRL) is the key hormone that initiates lactation (Vonderhaar, 1987). However, growth hormone (GH) is required to sustain milk production when PRL is decreased (Flint *et al*, 1992). Moreover, without the fall of progesterone level after birth milk production is suppressed even though prolactin is produced in high amounts. Progesterone prevents

lactose and lipid production in mammary glands (Kuhn, 1969) and there are reports indicating that the increase in PRL synthesis and release may increase vulnerability to carcinoma (Venugopal *et al*. 1999). Gene targeting approaches have discovered the exact roles of the progesterone and estrogen receptors, plus other coactivators and transcription factors that mediate signaling processes in the developing mammary gland. The function of progesterone is to bind to specific nuclear receptors, which trigger defined genes in a ligand-dependent manner (Bocchinfuso *et al*., 2000).

The mammary glands become bigger during pregnancy. The functions of prolactin on the mammary gland embrace growth and development of the mammary gland (mammogenesis), synthesis of milk (lactogenesis), and regulation of milk secretion (galactopoiesis). Prolactin is mainly produced from the anterior lobe of pituitary gland, but it is also produced from central nervous system, the immune system, the uterus and its associated tissues of conception, and even the mammary gland itself (Freeman *et al*, 2000). Prolactin belongs to the prolactin/growth hormone/placental lactogen family [group I of the helix bundle protein hormones] based on its genetic, structural, binding and functional properties, (Boulay and Paul, 1992; Horseman *et al*, 1994). Genes encoding prolactin, growth hormone, and placental lactogen evolved from a common ancestral gene by gene duplication (Niall *et al*, 1971). Proliferation and differentiation of secretory mammary epithelium is dependent on the presence of the prolactin receptor (PrlR) (Ormandy *et al*., 1997) and the downstream Jak2-Stat5 pathway (Liu *et al*., 1997). See figure 2.2.

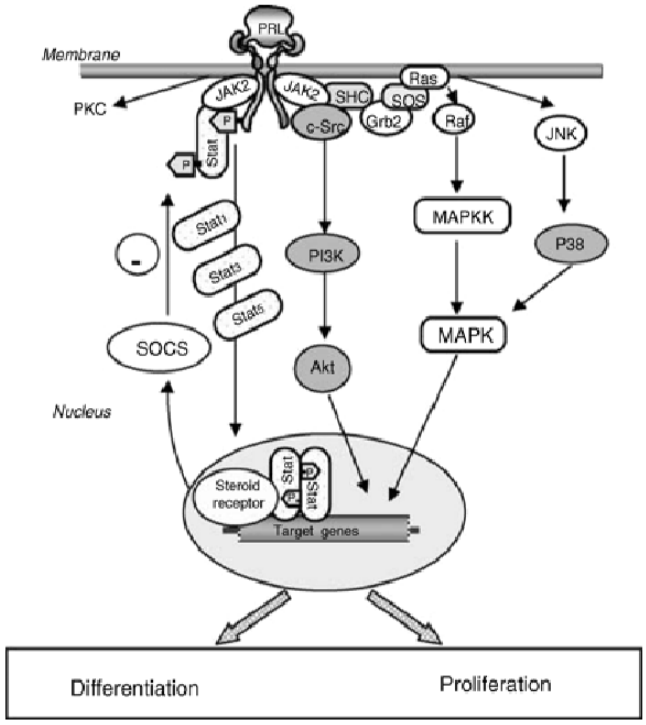


Fig 2.2 Binding of prolactin to the receptors (Bole-Feysot *et al*, 1998)

According to Schindler *et al* (1995) prolactin increase milk production by binding to the receptors which are located on the epithelial cell and induces dimerization of receptors and activate Janus kinase 2 which in turn**,** phosphorylates transcription factors that belong to the family of signal transducers and activators of transcription (STAT). This confers DNA-binding activity to STAT proteins and results in the transcriptional activation of genes containing 7-interferon activation sites (GAS) (TTCNNNGAA) (Liu, *et al,* 1997). STAT have a DNA-binding domain, an SH3-like domain, an SH2-like domain, and an NH_2 and a COOH-terminal transactivating domain (Finidori, *et al*, 1995). A number of promoters contain the GAS consensus motif, and numerous cytokines were found to activate these promoters in vitro (Ferrag *et al,* 1996). Prolactin has been shown to signal through STAT1,

STAT3 and STAT5 in cell culture (DaSilva *et al*. 1996) and to trigger promoters containing the β-casein gene GAS site (Liu, *et al*, 1995). STAT5a is an obligatory for adult mammary gland development and lactogenesis, the deficiency of STAT5a hinders milk production since it is responsible for activating the genes for milk biosynthesis (Liu, *et al,* 1997). In the process of lactogenesis, prolactin stimulates uptake of some amino acids, the synthesis of the milk proteins casein and α-lactalbumin, uptake of glucose, and synthesis of the milk sugar lactose as well as milk fats (Barber, *et al,* 1992).

Dopamine inhibits prolactin release from pituitary lactotrophs both *in vivo* and *in vitro* (MacLeod, 1969). It has, however, been reported that the amount of dopamine in stalk blood is enough to account for only about two thirds of the prolactin inhibition normally observed (De Greef and Neill*,* 1979).

According to Sadler (1984) lactation is the main active part of maternal investment in mammals. Milk removal is the most important control mechanism for supply. Milk removal is believed to be determined by a baby's appetite; hormonal levels participate in a much lesser role in already established lactation. Oxytocin is responsible for milk removal from the breast and is accomplished by the contraction of myoepithelial cells, whose processes form a basket-like network around the alveoli, where milk is stored and is increased by infant suckling (Hurst, 2007). Normally, the breasts will carry on producing milk as long as milk removal continues (Neville and Morton, 2001; Neill, 1974). Milk transfer to the suckling infants starts at a volume of 100 ml/d on day 1 postpartum, and starts to rise at 36 h postpartum and decreases at an average of 500 ml at day 4. Milk composition changes noticeably during this period, with a decline in the sodium and chloride concentrations and

raised lactose concentration that starts instantly after birth and are mostly complete by 72 h postpartum (Neville *et al*, 1991).

The use of physiologic and pharmacologic methods can aid an adoptive mother bring in a milk supply but the quantity may not be enough to totally meet an infant's nutritional needs. There are arguments around the use of domperidone and metoclopramide to induce lactation (Bryant, 2006). Metoclopramide antagonizes the release of dopamine, which in turn inhibits the effect of prolactin inhibiting factors on the pituitary, and consequently prolactin production increases. Metoclopramide crosses the blood-brain barrier; therefore it is associated with central nervous system (CNS). Thus sedation and depressions are common side effects (Gabay, 2002). Sulpiride is a selective dopamine-2 antagonist with antipsychotic and antidepressant activity. Sedation and extra pyramidal side effects are quite common. Weight gain is also a matter of concern to mothers (Gabay, 2002). Domperidone is a safe and helpful medication in the short term (Da Silva *et al*, 2001). Its side effects are few and embrace dry mouth, skin rash or itching, headache, and gastrointestinal disturbances. Unlike metoclopramide, domperidone does not cross the blood-brain barrier but exerts its effect peripherally and is associated with little central nervous system (CNS) side effects (Barone, 1999).

Chlorpromazine is also an antipsychotic that appears to work by blocking dopamine receptors; its side effects commonly include dystonic reactions and anticholinergic effects (Gabay, 2002).

Synthetic drugs that are currently used to stimulate milk production have side effects, and are expensive for low income mothers who are staying in rural areas. Therefore in trying to

improve the health status of poor women there should be alternative drugs that are naturally derived.

Plants naturally produce a number of carbon compounds (phytochemicals) for physiological functions for use as chemical weapons against diseases, insects and predators (Fatope, 1995). A lot of these phytochemicals contain important antioxidant capacities that may possibly be associated with lower incidence and lower mortality rates of cancer in several human populations (Velioglu *et al*., 1998).Tannins have been reported to increase milk production (Bhatta *et al*, 2000).

Developed countries utilize plants derived drugs because about 25 % of the prescription drugs contain active compounds that are extracted from higher plants (Kazmi, 1991).

2.2 TRADITIONAL MEDICINE

Traditional healers are people that use traditional or indigenous plants as well as animals to cure diseases. Most of the information that they have were obtained either as a calling or inherited from their elders. A lot of traditional medicine has been used for generations and has been passed on intergenerational. Hence, traditional medicine is not a static body of knowledge and it continues to evolve with the practices of the individuals or communities that hold and use it (Correa, 2000).

About 80 % of the rural population relies mainly on the traditional practitioners and medicinal plants to meet their primary health care needs (Gumede, 1990). Although modern medicine is available, natural derived medicine (phytomedicines) have often maintained popularity for historical and cultural reasons. In the cast of characters, Zulu medicinal plants

are promising to be effective against pathological conditions, therefore, in an effort to improve the efficacy of the practice and possibly to integrate it into modern medicinal practices, researchers are increasingly turning their interest to evaluating the therapeutic potential of Zulu plant materials (McGaw *et al*., 1997; Lin *et al*, 2002).

2.2.1 SOME PLANTS THAT STIMULATE MILK PRODUCTION

Many herbs are used to stimulate milk production**;** most of them have long histories of traditional use, mainly in stimulating milk production in animals. *Hibiscus sabdariffa l.* has a lactogenic activity with favorable enhancement ability in increasing serum prolactin level. Mechanism of action of *Hibiscus sabdariffa l.* has been reported to be through dopamine receptors as dopamine antagonist (Okasha *et al*, 2008). *Acacia nilotica* ssp *adansonii* also stimulate milk production in lactating woman by stimulating the synthesis and release of prolactin and enhancing lobuloalveolar development (Lompo-Ouedraogo, *et al.* 2004). *Foeniculum vulgare* (fennel) has been shown to increase milk production in goats. This herb has been used for a very long time as a galactagogue in humans (Wynn and Fougere, 2007). *Galega officinalis* (goat's Rue) is also used to increase milk yield in cows (Bailey and Day, 2004). Raspberry leaf (Rubus idaeus L. [Rosaceae]) is believed to stimulate lactation and enrich breast milk by restoring the body's vitamins and minerals (Gladstar, 1993; Weed, 1986). Conversely, owing to its astringent qualities, it has the potential to shrink mammary glands and thereby reduce milk flow (Weed, 1986). There is no clinical evidence that raspberry leaf is a galactagogue, although the herb can be a good source of vitamins A, B complex, C, and E, as well as calcium, iron, phosphorus, and potassium (Weed, 1986).

As the result of an ethnobotanical survey (see section 2.3 below), it became apparent that traditional healers around Kwa-Zulu Natal recommend Gunnera *perpensa* (*ugobho,* its Zulu name) to their patients for its lactogenic activity.

2.2.1.1 GUNNERA *PERPENSA*

Gunner perpensa L. (Gunneraceae) is a Zulu medicinal plant that is traditionally used to expel the placenta after birth or to relieve menstrual pains (Ngwenya *et al.* 2003; Van Wyk and Gericke 2000; Von Ahlenfeldt *et al.* 2003). It is a perennial, robust, erect herb up to 1 m tall that always grows near water (figure 2.3). The roots are up to 300 mm thick, creeping in black, muddy soil. The inside tissues are yellow-brown (Mendes, 1978). The genus *Gunnera* L. is the only member of the family (Gunneraceae) with about 45-50 species, which occurs naturally in Central and Southern Africa, Madagascar, the Philippines, Hawaii, Mexico, Central and South America. In Africa, however, it is represented by *Gunnera perpensa* L. (Bergman *et al*, 1992).

Figure 2.3 Pictures of *Gunnera perpensa* (live plant and rhizomes)

Biological and pharmacology studies of various extracts and isolated compounds from the plant confirmed antibacterial activities (Drewes *et al*, 2005;Buwa and van Staden, 2006;

Ndhlala *et al*, 2009), antifungal activity (Buwa and van Staden, 2006; Ndhlala *et al*, 2009), antimicrobial activity (Nkomo and Kambizi, 2009), Antinociceptive and anti-inflammatory (Nkomo *et al*, 2010) antioxidant activity (Simelane *et al*, 2010) and uterine contractile activity (Khan *et al*, 2004). Considering the extensive utilization of *G. perpensa* in Zulu traditional medicine, the present study was undertaken to investigate the rhizomes of *G perpensa* for its effect on milk production in lactating rats.

2.3 ETHNOBOTANICAL SURVEY

Empangeni is a town located in KwaZulu-Natal Province, South Africa. It is about 160 kilometers north of Durban. It is located in mountainous side of the uThungulu District, KwaZulu-Natal, South Africa. It overlooks a flat coastal plain and the major harbor town of Richards Bay only 15 kilometers away. The climate is sub-tropical with an average temperature of 28.4 °C in summer and 14.5 °C in winter. Empangeni's population is 16,500 (www.shortopedia.com/K/W/KwaZulu-Natal-Province).

Ethnobotanical survey was conducted in the year 2009 (See Appendix E for copy of questionnaire). The ethnobotanical information was collected from 10 interviewees (Traditional healers) in Pongola and Empangeni—villages in Kwa-Zulu Natal province, South Africa. Most of the traditional healers that were interviewed were between the ages of 40-70 old and all of them were males, belonging to the families that believe on traditional medicine. Most of the traditional healers interviewed collect herbs from the wild and some cultivate them (home-garden) on their own and they also sell the herbs to their patients in a dry state. Most of the traditional health practitioners see their trade as a calling and that their knowledge was inherited from their elders (ancestors, grandfathers and grandmothers).

2.4 FREE RADICALS AND ANTIOXIDANTS

Free radicals, also known simply as radicals, are organic molecules responsible for aging, tissue damage, and possibly some diseases. These molecules are very unstable, therefore, they look to bond with other molecules, destroying their vigor and perpetuating the detrimental process. Reactive oxygen species (ROS) and oxygen free radicals (OFR) can change several intracellular processes; as a result they induce mutagenesis or impairment of biological systems that, in turn, can affect cell survival. The most familiar OFR are the singlet oxygen, the hydroxyl radical, and the superoxide anion, all of which are usually present in the cell because of physiologic metabolic oxidative processes which can increase in some conditions (Meffert *et al*, 1976). Among these oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to the adjacent biomolecules (Gutteridge, 1984). DNA is a major target of free radical induced damage. Under physiological conditions, the constant and endogenous rate of production of free radicals may lead to a minimal damage in DNA. However, if this production increases, oxygen radicals may attack DNA at either the sugar (deoxyribose) or the base, giving rise to a large number of products. Free radicals are known to initiate lipid peroxidation that results in potential toxic compounds being produced and/or the destruction of cell membranes leading to cell death (Senthil *et al*, 2004). Tissue damage resulting from the imbalance between reactive oxygen species generating and scavenging systems (oxidative stress) has been implicated in the pathology of a number of disorders, such as atherosclerosis, ischemia-reperfusion injury, cancer, malaria, diabetes, inflammatory joint disease, asthma, cardiovascular diseases, cataracts, immune system decline, and could play a role in neurodegenerative diseases and

ageing processes (Young and Woodside, 2001). The harmful action of free radicals can, result in the death of milk secretary cells and thus lower or stop milk production.

Mammalian cells have complex defense mechanisms for radical detoxification. Antioxidants are agents which scavenge the free radicals and prevent the damage caused by them. A number of these compounds are of exogenous nature and are obtained from food. Examples include antioxidants like α-tocopherol, β-carotene, and ascorbic acid, and some micronutrient elements such as zinc and selenium (Halliwell and Gutteridge, 1998). Synthetic antioxidants like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (BHQ) can be very expensive and toxic (Moure *et al*., 2001). Providing modern healthcare to rural people is still a far-reaching goal, due to economic constraints (Grover *et al*., 2002). Hence, it is necessary to resort to forms of medicine that mostly depend on the locally available plant materials to treat various health disorders (Subhasree *et al*, 2009).The antioxidant activity of most plants is mostly due to phenolic components, such as flavonoids (Pietta, 1998), phenolic acids, and phenolic diterpenes (Shahidi and Wanasundara, 1992). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play a significant role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994). Zulu medicinal plants have been reported to have the ability to fight against free radicals (Opoku *et al*, 2002).

2.5 SCOPE OF THIS WORK

Aim

This project aims to scientifically validate the use of G.*perpensa* by traditional healers in stimulating milk production in lactating mothers.

0bjectives

- To identify and collect the plant G. *perpensa* (rhizomes).
- To screen the plant for the presence of phytochemicals.
- To investigate antioxidant activity of the plant.
- To investigate cytotoxicity of the plant.
- To investigate the stimulation of milk production activity (in lactating rats) by the plant.

CHAPTER 3

MATERIALS AND METHODS

The chapter briefly lists the major chemicals, reagents, and equipments that were used in the study. A brief description of the methods is also given. Details of the preparation of reagents and the details of the methodology are in the Appendix A and B, respectively.

3.1 Materials (see appendix A for details)

These chemicals were purchased from Sigma-Aldrich Co. Ltd (Steinheim, Germany)—2,2-Diphenyl-1-picryl-hydrazyl (DPPH), 3-ethylbenzothiazoline-6-sulfonate (ABTS), Butylated hydroxyl toluene (BHT), 4,4[1]- [3-(2-pyridinyl)-1,2,4-triazine-5,6-dryl] bisbenzene sulphonic acid (ferrozine), Butylated hydroxyl-anisole (BHA), Ascorbic acid (AA), Gallic acid, NADH, Glutathione reduced (GSH), Tris-HCl, Phenazineethosulfate, Tetrazolium bromide, Alcohol dehydrogenase, Citric acid, Ethylenediaminetetra-acetic acid (EDTA), DNA, Thiobarbituric acid (TBA), Folin-Ciocalteu Phenol reagent, Ferrous ammonium sulfate, Xanthine, Xanthine oxidase, Bovine Serum Albumin, Nitro blue tetrazolium salt, Ammonium molybdate, Linoliec acid, Lipoxidase, Coomassie Brilliant Blue G-250, Hydrogen peroxide, Lipoxygenase, σ-phthaldehyde, Sodium nitroproside, prolactin, metoclopramide, dopamine, oxytocin, stilbestrol, 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB), s-acetyl- thiocholine iodide (ASChI), Dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT salt)

Other materials and equipment include:

Organbath (Academic instrument unit)

Freeze dryer—VirTis benchtop K (Polychem Suppliers)

Spectrophotometer—Spekol 13000 (Polychem suppliers)

Centrifuge—Eppendof 5404R (Merck)

Platform shaker—Labcon (Polychem suppliers)

Sprague-Dawley Rats (Department of Biochemistry, UZ, animal house).

Artemisia salina eggs (Fish designs, Mtunzini)

Fish— (*Oreochromis mossambicus*) (Department of Zoology, UZ)

3.2 METHODOLOGY (see appendix B for details)

3.2.3 Plant collection and preparation

The plant was fleshly collected from KwaDlangezwa area (Empangeni, KZN) and was identified by the staff of the Department of Botany, the University of Zululand (UZ). Voucher specimen [MBC/01(ZULU)] was lodged in the Botany Department Herbarium, UZ. The plant material was thoroughly washed and separated into leaf, stem and rhizome. The rhizomes were chopped, dried at 50 ^{0}C ± 2 ^{0}C for 48 h, powdered (2mm mesh) and stored in sterile brown glass bottles at -4 ^{0}C until used.

3.2.4 Preparation of methanol and water extracts

The dried plant material was separately extracted (1:5 w/v) with methanol and water for 24 h by incubating the mixture on an orbital shaker (150 rpm, room temperature). The extracts were filtered through Whatman No. 1 filter paper. The methanol extract was dried under reduced pressure at 40 ^{o}C using a rotary evaporator. Aqueous filtrates were freeze-dried.

Each extract was re-suspended in the respective solvent of extraction to yield a stock solution.

3.2.5 Phytochemical screening

Chemical tests were carried out on the plant extracts using standard procedures described by Odebiyi and Sofowara (1978), and Harbone (1973) to identify the chemical constituents. The following phytochemicals were tested for: Alkaloids, Tannins, Saponins, Anthraquinones, Steroids, Flavonoids, Terpenoids and Cardiac glycosides.

3.3 ANTIOXIDANT ACTIVITY

The methanol extract was screened for antioxidant activities.

3.3.1 Free radical scavenging activity

3.3.1.1 1,1-diphenyl-2-picryl hydrazyl (DPPH) Scavenging activity

DPPH radical scavenging activity of plant extract was determined spectrophotometrically in the absence and presence of different concentrations (0-5 mg/100 ml) of plant extracts (Brad-Williams, 1995). A mixture of 2 ml plant extract and 2 ml DPPH (2 mg% in methanol) was incubated for 30 min and the absorbance read at 517 nm.

3.3.1.2 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS*) scavenging activity

ABTS* radical scavenging activity of plant extracts was determined spectrophotometrically (Re *et al,* 1999). ABTS* was generated by incubating 7 mM of ABTS and 2.45 mM potassium persulfate in the dark for 16 h. The ABTS* produced was diluted 60 times using

methanol, v/v). Various concentrations (0-5 mg/100 ml) of the extracts (1 ml) were mixed with 1 ml of diluted ABTS* and the absorbance at 734 read after 6 min incubation.

3.3.1.3 Superoxide anion (O^{-}_{2}) scavenging activity

The assay is based on the capacity of the extract to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in the riboflavin–light–NBT system (Beauchamp and Fridovich, 1971). The method used by Nagai *et al* (2001) for determination of superoxide dismutase was followed with some modification. Reaction mixture containing 0.02 ml each of sodium carbonate buffer (50 mM, pH 10.5), 0.15% bovine serum albumin, 3 mM Xanthine, 3 mM EDTA, 0.75 mM NBT and 0.02 ml of plant extract (0-5 mg/100 ml) was incubated with 0.02 ml of Xanthine oxidase (6 mU) for 20 min at 25 ºC. The production of blue formazan was monitored after the addition of 0.02 ml of 6 mM $CuCl_2$, at 560 nm.

3.3.1.4 Hydroxyl radical (^{-}OH) scavenging activity

The assay is based on benzoic acid hydroxylation method, as described by Osawa, *et al* (1997). In a screw-capped tube, 0.2 ml each of $FeSO_4$, $7H_2O$ (10 mM), EDTA (10 mM), different concentrations (0-5 mg/100 ml) of extracts, DNA (10 mM), and phosphate buffer (pH 7.4; 0.1 mol) were mixed. Then 200 µl of H_2O_2 solution (10 mM) was added. The reaction mixture was then incubated at 37 ºC for 2hr. After that, 1.0 ml TCA (2.8%) and 1.0 ml of 1% TBA were added and the mixture was boiled and allowed to cool on ice. Absorbance was read at 520 nm.

3.3.1.5 NO^- scavenging activity

Nitric oxide, produced from the spontaneous reaction of sodium nitroprusside in aqueous solution at physiological pH, reacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction (Garrat, 1964). Scavengers of nitric oxide compete with oxygen and reduce the production of nitric oxide (Badami *et al*., 2005). The 3 ml reaction mixture containing 2 ml of 10 mM sodium nitroprusside, 0.5 ml of phosphate buffer saline (pH 7.4, 0.01 M) and 0.5 ml of different concentrations (0-5 mg/100 ml) of *G. perpensa* extract was incubated at 25 °C for 150 min. Thereafter, 0.5 ml of the reaction mixture (containing nitrite) was pipetted and mixed with 1.0 ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization.Then,1.0 ml of naphthylethylenediamine dihydrochloride (0.1 %) was added, and allowed to stand for 30 min in diffused light. The absorbance was then measured at 540 nm.

3.3.2 Reducing power

The reducing power of *G. perpensa* extract was evaluated according to the method described by Oyaizu (1986). To 2.5 ml of different concentrations (0-5 mg/100 ml) of *G. perpensa* extract in methanol was added, 2.5 ml of 0.2 M phosphate buffer (pH 6.6), and 2.5 ml of 1% potassium ferricyanide $K_3Fe(CN)_6$. The mixture was incubated at 50 ^oC for 20 min, and 2.5 ml of 10% TCA was added to the mixture and centrifuged at 1000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1 %). The absorbance was measured spectrophotometrically at 700 nm. The higher the absorbance value the stronger the reducing power. Ascorbic acid (0-5 mg/100 ml) and BHA (0-5 mg/100 ml) was used as the standard.

3.3.3 Chelating Activity on Fe^{2+}

The Fe^{2+} chelating effect of the extract was measured according to the method of Decker and Welch (1990). To 0.5 ml of various concentrations (0-5 mg/100 ml) of *G. perpensa* extract in methanol, 1.6 ml of deionized water and 0.05 ml of $FeCl_2$ (2 mM) were added. After 30 s, the reaction was initiated by the addition of 5 mM ferrozine (0.1 ml). Then, the mixture was shaken and left at room temperature for 10 min. Absorbance of the mixture was measured spectrophotometrically at 562 nm. Citric acid (CA) (0-5 mg/100 ml) and EDTA (0-5 mg/100 ml) were used as standards.

3.3.4 Effect on Xanthine oxidase activity

The effect of plant extract on generation of free radicals by xanthine oxidase was determined by the production of uric acid from xanthine spectrophotometrically (Constantino *et al*., 1992).

3.3.5 Effect of extract on DNA sugar damage

Measurements of the hydroxyl radical mediated DNA damage was done according to the method of Halliwell and Gutteridge (1981) in which TBA reacting species produced from calf thymus DNA /ferrous ammonium sulphate in the presence of the plant extract was measured spectrophotometrically.

3.3.6. Effect of extract on lipid peroxidation

3.3.6.1 Lipid peroxidation induced by Fe^{2+} / Ascorbate

The antioxidant activity of the plant extract on the inhibition of oxidation induced by ascorbic acid (AA)/$FeCl_3$ was evaluated. Liver obtained from Sprague-Dawley rats (180-200 g) was

perfused and homogenized (1:10 w/v) with 120 mM KCl, 50 mM phosphate buffer (pH 7.40). After centrifugation (700 x g, 10 min, 4 ºC) the supernatant was kept at -20 ºC until use. Protein content was quantified by the Lowry *et al* (1951) method and the protein concentration of the supernatant was adjusted to 10mg/ml. The reaction mixture containing 0.1 ml of the diluted homogenate, 0.1ml Tris-HCl buffer (20 mM, pH 7), 0.1ml KCl (30 mM), and 0.1 ml of various concentration (0-5 mg/100 ml) of *G. perpensa* extract, was incubated for 5min at 37 ºC. The AA/Fe^{2+} (0.1 ml; 0.1 mM) was added to the mixture and then incubated for 30 min at 37 ºC. The products of lipid peroxidation were detected by the thiobarbituric acid method measuring the absorbance at 532 nm (Ohkowa *et al*, 1979).

3.3.6.2 Lipid peroxidation in the rat brain homogenate

Whole rat brain homogenate (0.1 ml of 10% w/v homogenized in phosphate buffer saline) and 0.1 ml of various concentration (0-5 mg/100 ml) of *G. perpensa* extract were mixed and incubated in a water bath at 35 ^{0}C for 5 min; 0.15 ml H_2O_2 (10 mM) was added to induce lipid peroxidation and the mixture reincubated at 37 ^{o}C for 15 min. Then, thiobarbituric acid reactive species (TBARS) were measured as indication of the lipid peroxide formed (Ohkowa *et al*, 1979).

3.3.6.3 Effect of extract on Lipoxygenase (lipoxidase) activity

The method reported by Mathisen *et al*. (2002) was used to determine the ability of the extract to inhibit lipoxygenase activity. *G perpensa* extract (0.5 ml, 0-50 mg/l) was mixed with 0.95 ml borate buffer (0.2 M; pH 9.0), 2.0 ml substrate solution (linoleic acid) and enzyme solution (lipoxygenase). The absorbance was immediately measured at 234 nm.

3.3.6.4 Determination of malondialdehyde (MDA)—TBARS

The method reported by Halliwell and Gutteridge (1990) was used to determine MDA. To 100 µl of the reaction mixture in test tubes, 1.5 ml of TCA (10 %) was added and incubated for 10 minutes. This was then centrifuged [3500 rpm for 20 min] and the supernatant mixed with 1.5 ml of 1% TBA. The mixture was heated in boiling water bath for 30 minutes, allowed to cool and then 2 ml of n-butanol was added. The absorbance of the butanol layer was measured at 532 nm.

3.3.7. Effect of extract on Acetylcholine esterase activity

Acetylcholine activity was estimated using acetylthiocholine iodide as substrate following the method of Ellman *et al* (1961). The fish brain was homogenized in phosphate buffer pH 8 and diluted with sufficient amount of phosphate buffer to obtain 20 mg of brain tissue per one millilitre of buffer. The tissue was centrifuged at 1000 x g for 20 min and supernatant was analysed immediately. Supernatant (0.4 ml) was used, and 2.6 ml phosphate buffer (pH 8.0) was added. Then 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB) reagent (100 µl) was added and mixed well, 5 mM s-acetyl- thiocholine iodide (ASChI) solution (20 µl) was added quickly and mixed well. The absorbance was recorded at 60 second intervals at 412 nm. The yellow colour produced by enzymatic reaction resulted in changes in absorbance. The reaction rate (R) was calculated in units of changes in absorbance per minute from the formula: $R=574xA/C$. Where R = rate of µmoles of substrate hydrolysed/min/g brain tissue, A =change in absorbance per min and C= original concentration of brain tissue (mg/ml) in the homogenate (this value remains constant at 20 mg/ml throughout the study) and percentage inhibition was calculated. Tacrine was used as the standard (0-5 mg/100 ml).

3.3.8 Total phenolic content

The total phenolic content (measured as gallic acid equivalent) was determined using the Folin- Ciocalteu reagent method as described by Kujala *et al* (2000).

3.3.9 NADH Content

A modified method for determining NADH by Stern *et al* (2002) was used to determine the concentration of NADH in the plant extract. The plant material was extracted (1:5w/v) with phosphate buffer containing 20 mM $NaHCO_3$, 100 mM Na_2CO_3 and 10 mM nicotinamide. The filtrate was heated on a dry heating block at 60 ºC for 30 min to destroy NAD^+/$NADP^+$, and quickly chilled to 0 ºC. The extract was immediately analyzed for NADH. Thereafter 1 ml of a reaction mixture containing 100 µmol Tris-HCl (pH 8), 2 µmol phenazineethosulfate, 0.5 µmol Tetrazolium bromide, and 0.2 mg alcohol dehydrogenase, 0.2 ml of the plant extract was added. The mixture was incubated for 5 min at 37 ºC and 60 µmol ethanol added. This was again incubated for 10 min at 37 ºC and the absorbance read at 570 nm. The concentration of NADH in the extract was determined from the NADH standard graph.

3.3.10 (SH) sulfhydryl content

Powdered plant material was extracted with ice cold distilled water containing 30 µmol EDTA. The SH content was measured (as reduced glutathione) fluorometrically (420 nm, activation 350 nm) using the σ-phthaldehyde condensation method of Cohen and Lyle, (1966).

3.3.11 Determination of total antioxidant capacity

The assay was based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH, according to the method of Prieto *et al* (1999). The *G perpensa* extract (0.3 ml) was combined with 3 ml of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The mixture was incubated at 95 ^{0}C for 90 min. After cooling to room temperature, the absorbance of the solution was measured at 695 nm against a blank. The anti-oxidant activity is expressed as the number of equivalents of ascorbic acid and BHT.

3.3.12 Calculation of percentage inhibitory effect of *G. perpensa* extract

Unless otherwise stated, ascorbic acid, BHA and BHT were used as standards. All assays were repeated three times and the mean ± S.E reported. The inhibitory effect of *G. perpensa* extract of each parameter was calculated as:

$$\% \text{ Inhibition} = 1 - \{(A_0 - A_1)/A_0 \times 100\}$$

where, A_0 is the absorbance value of the fully oxidized control and A_1 is the absorbance of the extract. The inhibitory concentration providing 50 % inhibition (IC_{50}) was calculated from the graph of percentage inhibition against *G. perpensa* extract concentrations.

3.4 CYTOTOXICITY ASSAY

3.4.1 Brine Shrimp Lethality Screening

Brine shrimp (*Artemia salina*) lethality test was carried out using the procedure described by Meyer *et al*. (1982) and modified by McLaughlin (1991). Methanol extract of *G. perpensa* (reconstituted in 1 % DMSO in artificial seawater) was added to brine shrimp suspension to

give final concentrations of 9.2, 18.4, 29.9, 39.1 and 48.3 mg/ml (three replicates each, gallic acid and DMSO as positive and negative controls respectively). The plates were maintained under illumination and the survivors were recorded after 24 h. The percentage mortality at each concentration and control were calculated using the Abbots formula (Abbott, 1925). LC_{50} and 95% confidence intervals values were determined using the computer model (Probit Program Version 1.5) prepared by the US Environmental Protection Agency (McLaughlin *et al*., 1991).

3.4.2 MTT cell proliferation assay

Human Embryonic Kidney (HEK293) and Human Hepatocellular Carcinoma (HEPG2) cells were all grown to confluenecy in 25 cm^2 flasks. This was then trypsinized and plated into 48 well plates at specific seeding densities. Cells were incubated overnight at 37 degrees celcius. Medium was then removed and fresh medium (MEM + Glutmax + antibiotics) was added. Extracts were then added in triplicate and incubated for 4 hrs. Thereafter medium was removed and replaced by complete medium (MEM + Glutmax + antibiotics +10 % Fetal bovine serum). After 48 hours cells were subjected to the MTT assay (Mosman, 1983). Data were evaluated through regression analysis using QED statistics program and from the linear equation the LC_{50} values representing the lethal concentration for 50 % mortality.

3.5 ANIMAL EXPERIMENTS

This study was carried out after the approval from the ethical committee on animal use and care of the University of Zululand (see Appendix C).

3.5.1 Lactogenic activity

Forty female (virgin) rats weighing 200–250 g were obtained from animal house of department of Biochemistry and Microbiology at the University of Zululand. The animals were housed with male rats in a plastic cage under standard laboratory condition with 12 h dark or light cycle so that they can became pregnant. They were fed (*ad lib*) with commercial rat food (Doghouse, Pietermaritzburg) and tap water. The pregnant animals were divided into four groups consisting of control, metoclopramide treated (5 mg/kg), extract treated, and the extract plus dopamine treated groups (5 µg/kg). The extract treated group was sub divided into five sub groups that received 100, 200, 400, 800, 1600 mg/kg body weight respectively (Figure 3.1). Following birth, the litters' weights were recorded and culled to 6 litters per dam. The dams were given the extract and the drug daily for the period of six days, starting from day 4 to day 9 of lactation. On day 10 they were sacrificed for the analysis of blood samples and histological study of the mammary glands (Vogel and Vogel, 1997).

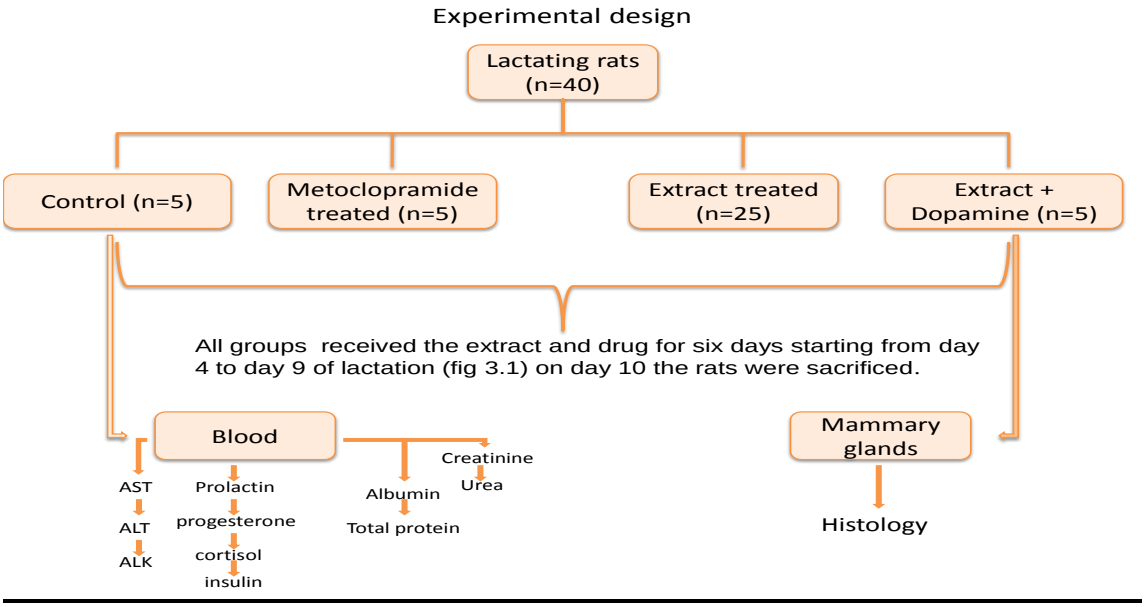


Figure 3.1Experimental design for lactogenic activity

3.5.2 Effect of oral treatment with extract on milk production

Forty lactating dams weighing 200–250 g were used for this experiment. The rats were divided into four experimental groups (see section 3.5.1 above, figure 3.1). All animals were treated daily, starting on the evening of day 4 of lactation. The extract was administered orally with a gavage syringe each day at 1800 h. Milk production was estimated 18 h and 23 h after gavage. Milk production was measured from day 5 to day 9 of lactation. Milk yield and body weight of dams and weight gain of pups were measured each day with an electronic balance. Every day during the study period, the pups were weighed at 0700 h (w1) and subsequently isolated from their mothers for 4 h. At 1100 h, the pups were weighed (w2), returned to their respective mothers and allowed to feed for 1 h. At 1200 h, they were weighed again (w3). Milk yield 18 h after the gavage was estimated as w3 – w2. Daily milk yield was corrected for weight loss due to metabolic processes in the pup (respiration, urination and defecation) during suckling. The value used was (w2 – w1)/4. This value was then multiplied by the number of suckling hours per day and added to the daily suckling gain (Sampson & Jansen 1984). Daily weight gain of pups was calculated from the pup weight at w2. Same procedure was followed for 23 h.

3.5.3 Uterine contraction studies

Twelve female non-pregnant rats weighing 200-250 g were obtained from the biomedical research unit (BRU), University of KwaZulu Natal, Durban. The animals were injected with stilbestrol (12 mg/kg) 24 hrs before they were sacrificed. The abdomens were opened and the two uterine horns from each rat were exposed avoiding stretching of uterine smooth muscles. Isolated tissues were bathed in De Jalons physiological solution maintained at

32±1^{o}C with 95 % O_2 / 5 % CO_2. Silk threads were tied at both top and bottom of the uterine strip. The bottom thread was attached to a hook connected to an aeration tube in the organ bath and the top thread was attached to isotonic transducer connected to a strain gauge coupler placed on the oscillograph. A basal tension of 1 g was applied to the segment and spontaneous contractions were measured with a myograph force transducer and physiological system. Contractile responses were expressed as milligrams of tension developed from above tension of 1 g for tissues that were not contracting spontaneously. In tissues spontaneously contracting, responses were measured by terminating the difference in peak tension that developed before (15 min before extract or oxytocin addition) and after extract or oxytocin addition to the organ bath (Moustafa *et al.*, 1999).

Oxytocin was used as standard. All assays were repeated six times and the mean ± S.E reported. The percentage contractility effect of *G. perpensa* extract of each parameter was calculated as follows:

% contractility = {(max - min force)/ Max force} x 100

where, max is the maximum force and min is the minimum force.

3.5.4 Blood analysis

The blood was obtained from the heart (by cardiac puncture) and it was analyzed by Lancet laboratories (Richards Bay) using standard pathology procedures for the measurements of prolactin, progesterone, insulin, growth hormone, cortisol, total Billirubin, conjugated billirubin, albumin, total protein, urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase(AST) and alkaline phosphatase (ALP).

3.6 Histology of mammary glands

Inguinal mammary glands were removed and were stored in 40 % formalin. Histology studies were carried out at the Vetdiagnostix Laboratories (Pietermaritzburg) by qualified pathologist having no prior knowledge to which group they belonged. This method allowed for unbiased description of the histological lesions which were present or absent in the samples.

3.7 STATISTICAL ANALYSES

The mean and standard error of the mean of three experiments were determined. Statistical analysis of the differences between mean values obtained for experimental groups were calculated using Microsoft excel program, 2003, Origin 6.0 for IC_{50} and GraphPad prism version 4. Data were subjected to one way analysis of variance (ANOVA) followed by Tukey- Kramer multiple comparison test. In all cases, p values ≤ 0.05 were regarded as statistical significant.

CHAPTER 4

RESULTS

Dried rhizomes of *G perpensa* were separately extracted with methanol and water. The methanol extracts were screened for antioxidant activity and the aqueous extract screened for lactogenic activity. This chapter gives the results that were obtained.

4.1 Percentage yield

The yield of the methanol extract obtained from G.*perpensa* rhizomes was 17.15 % (*w/w relative to dried material*) and the aqueous extract yielded 23.50 % (*w/w relative to dried material*).

4.2 Phytochemical screening

The preliminary phytochemical analysis of G*. perpensa* revealed the presence mainly of tannins, cardiac glycosides, steroids and flavonoids (Table 4.1).

Table 4.1: Phytochemical analysis of *Gunnera perpensa.*

Alkaloids	-
Tannins	++
Saponins	+
Anthraquinones	-
Steroids	++
Flavonoids	++
Phlobatannins	-
Cardiac glycosides (steroidal)	++

++ High concentration was recorded if a definite heavy precipitate observed.
+ Low concentration was recorded if the reagent produced only a slight opaqueness.
- not detected

4.3 ANTIOXIDANT ACTIVITY

4.3.1 Free Radical scavenging activity

The ability of G. *perpensa* extract to scavenge DPPH, $ABTS^{+}$, $O^{2\cdot}$-, $^{\cdot}OH$ and $NO^{\cdot}$ radicals was evaluated and the results are shown in Table 4.2. The results show that the G. *perpensa* extract significantly and dose dependently scavenged the free radicals tested, albeit to varying degree of efficiency. At a concentration of 50 mg/l, *G. perpensa* extract scavenged over 78 % of DPPH radicals with IC_{50} value of 16 mg/l, which was 2 times greater than synthetic antioxidants, BHA (36.1 mg/l) and BHT (35.9 mg/l). The extract was also found to be very effective scavenger of $ABTS^{+}$ and the activity increased in a concentration dependent manner (Table 4.2). At 50 mg/l, the extract exerted highest $ABTS^{+}$ scavenging activities of 78.45 % (IC_{50} of 9 mg/l) whereas BHA and BHT exhibited lower activities (IC_{50} of 36.7 and 31.8 mg/l respectively). The results of the scavenging ability of various concentrations of G. *perpensa* (0 - 50 mg/l) and standard antioxidants (ascorbic acid, BHA and BHT) against superoxide radical anions, hydroxyl radicals and nitric oxide radical indicate a low activity (IC_{50} values ranged between 36.7 - 50.0 mg/l). However, G. *perpensa* was found to be significantly better in hydroxyl radical scavenger than BHA, under the condition of our study. The percentage of hydroxyl radical scavenging values was 46.24 and 36.36 % for *G. perpensa* and BHA respectively. Comparing the overall free radical scavenging activities of *G. perpensa* extract and standard antioxidants against DPPH, ABTS, $O^{2\cdot-}$, $\cdot OH$ and $NO^{\cdot}$ (Table 4.2) it is noted that the extract showed the highest antioxidant activity.

Table 4. 2: Percentage scavenging of DPPH, ABTS, superoxide, hydroxyl radical and nitric oxide by G. *perpensa* rhizome extract [a] (mg/ml).

	DPPH	**ABTS**	**($O^{2\cdot}$-)**	**($^{\cdot}OH$)**	**($NO^{\cdot}$)**
Control	100.0 ± 0.02	100.0 ± 0.02	100.0 ± 0.09	100.0 ± 0.02	100.0 ± 0.01
G.*perpensa*					
1	41.49 ± 0.00	52.54 ± 0.02	10.74 ± 0.06	16.13 ± 0.02	5.76 ± 0.00
2	56.02 ± 0.02	63.61 ± 0.08	19.88 ± 0.11	32.26 ± 0.00	7.89 ± 0.00
3	68.00 ± 0.06	68.69 ± 0.08	20.51 ± 0.08	33.33 ± 0.00	9.15 ± 0.01
4	72.27 ± 0.01	71.68 ± 0.01	22.77 ± 0.04	40.86 ± 0.00	12.54 ± 0.02
5	78.77 ± 0.04	78.17 ± 0.01	25.91 ± 0.13	46.24 ± 0.00	15.25 ± 0.01
IC_{50} [b]	1.6	0.9	>5	>5	>5
AA (IC_{50})	-	-	4.49	-	-
BHA (IC_{50})	3.61	4.01	-	>5	3.67
BHT (IC_{50})	3.59	3.59	-	-	-

[a] (n =3, X ± SEM), [b]IC_{50} –inhibitory concentration, ($O^{2\cdot}$-) –Superoxide anion radical scavenging; ($^{\cdot}OH$) –Hydroxyl radical scavenging; ($NO^{\cdot}$) –Nitric oxide radical scavenging.

4.3.2 Reducing power

Figure 4.1 shows the reducing potential of G. *perpensa* extract in comparison with ascorbic acid and BHA. The results show the reducing power of the extract to be dose dependent and with apparently better activity than ascorbic acid and BHA. At the concentration of 50 mg/l, the reducing power of G. *perpensa* extract (0.641 ± 0.09) was

higher than those of ascorbic acid (0.453 ± 0.07) and BHA (0.463 ± 0.02), respectively.

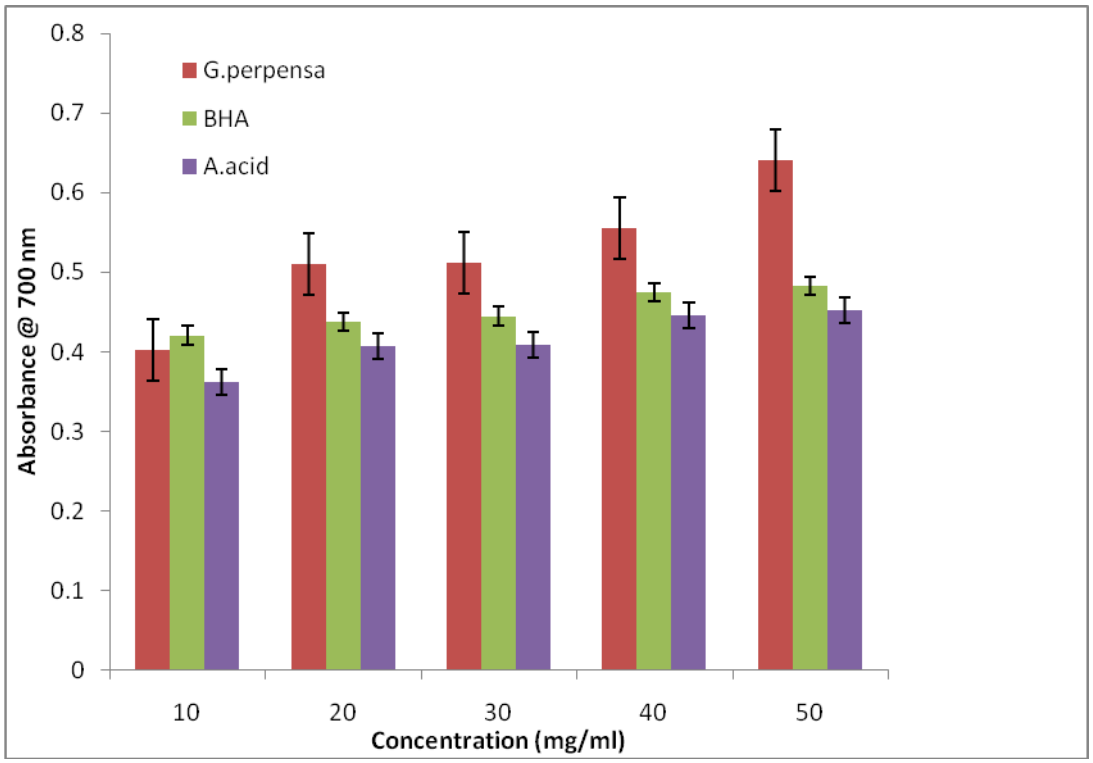


Figure 4.1: Reducing power of G. *perpensa* (GP) rhizome extract, ascorbic acid and butylated hydroxyl anisole (BHA), (mean ± SEM), (n=3).

4.3.3 Chelating activity

The ferrous ion chelating activity of the extract was assayed by inhibition of the formation of red-colored ferrozine and ferrous complex. Figure 4.2 shows the results that were obtained, indicating weak chelating activity. At the concentration of 5 mg/l, the extract and citric acid chelated 7.51 and 7.42 % of ferrous ions respectively, while EDTA showed moderate ferrous ion chelating activity of 14.81 %.

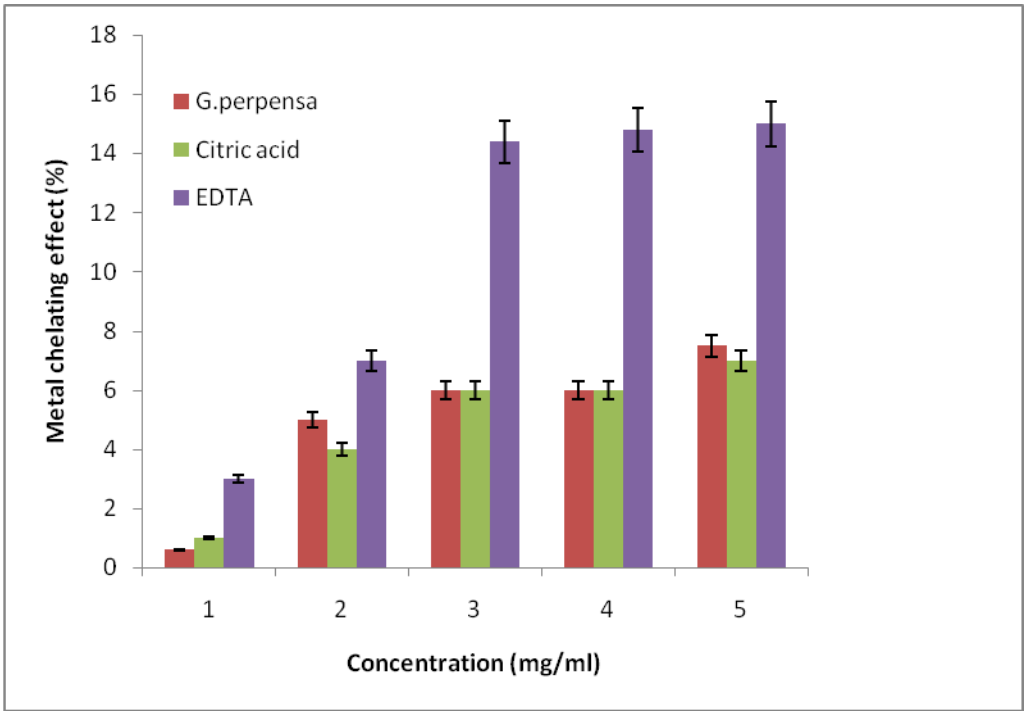


Figure 4.2: Metal chelating assay of G. *perpensa* (GP) rhizome extract, citric acid (CA) and ethylenediaminetetraacetic acid (EDTA), (mean ± SEM), (n=3).

4.3.4 Other oxidative system and cytotoxicity

Table 4.3 presents the results of the other antioxidant systems tested and the cytotoxicity of the extract. The G. *perpensa* extract had a relatively high total polyphenol and NADH contents. The extracts significantly inhibited hydrogen peroxide induced lipid peroxidation in the rat brain, and also inhibited lipoxygenase activity, and the AA/Fe^{2+} system.

The cytotoxic activity against brine shrimps, and two human cell lines, HEH293 and HEPG2 shows that the degree of lethality was directly proportional to the different concentrations of the extract.

Table 4.3 Polyphenol and NADH contents, total antioxidant capacity (TAC %), and cytotoxicity of G. *perpensa* extract and standard controls (mg/ml) [a].

	G.*perpensa*	BHA	BHT	AA	Gallic acid
Polyphenol contents					
Total phenol [b]	248±1.28	-	-	-	-
TAC (%) [c]	-	-	64	36	-
Antioxidative activity (%)					
Lipid peroxidation					
(i) Fe^{2+} / Ascorbate	48.01	22.69	-	-	-
(ii) TBARS	71.13	63.56	-	-	-
Lipoxygenase	30.00	28.27	-	-	-
NADH [d]	3.8	-	-	-	-
Cytotoxicity					
[e]LC_{50} (95% Cl)	137.62	-	-	-	11.45
[f]LC_{50} (95% Cl)	279.43				
[g]LC_{50} (95% Cl)	222.33				

[a] (Expressed as mean ± SEM), (n=3), [b] Expressed as mg gallic acid/g of dry plant material, [c] TAC- Total antioxidant capacity reactive to ascorbic acid and BHT, [d] NADH- ρm/g, [e]LC_{50} (95 % Cl)- Lethal concentrations with 50 % larvae mortality rate and 95 % confidence intervals (95 % Cl)-mg/ml, [f]LC_{50} (95 % Cl) lethal concentrations with 50% HEK293 mortality rate, [g]LC_{50} (95 % Cl) lethal concentrations with 50 % HEPG2 mortality rate (μg/ml). No appreciable amount of –SH was detected in the plant sample.

4.4. ANIMAL EXPERIMENTS

Lactating rats were administered with different concentrations of aqueous extracts of *G perpensa* and the body weights of the dams and pups measured. Milk production was

measured, and the blood was analyzed for biochemical parameters. Histology of the mammary gland was also studied.

4.4.1 Milk production

Figures 4.3 and 4.4 show the body weights of the dams and pups respectively. All pups gained weight during the study period and the rate of weight gain for the treated groups was significantly higher than that for the controls. Body weight for 18h increased from 9.18±0.15 to 12.30±0.21 g/pup for the controls and for 23 h the body weight increased from 9.25±0.11 to 12.42±0.19. For the rats receiving the extract (1600 mg/kg) the body weight for 18h increased from 10.66±0.00 to 17.90±0.27 and for 23 h the body weight increased from 11.75±0.26 to 19.99±0.24. A significant difference was observed between treated group (1600 mg/kg) ($p<0.05$) and the controls. For the dams, there was an increase in body weight (from 192±0.04 to 254±0.06), but statistically there was no significant difference.

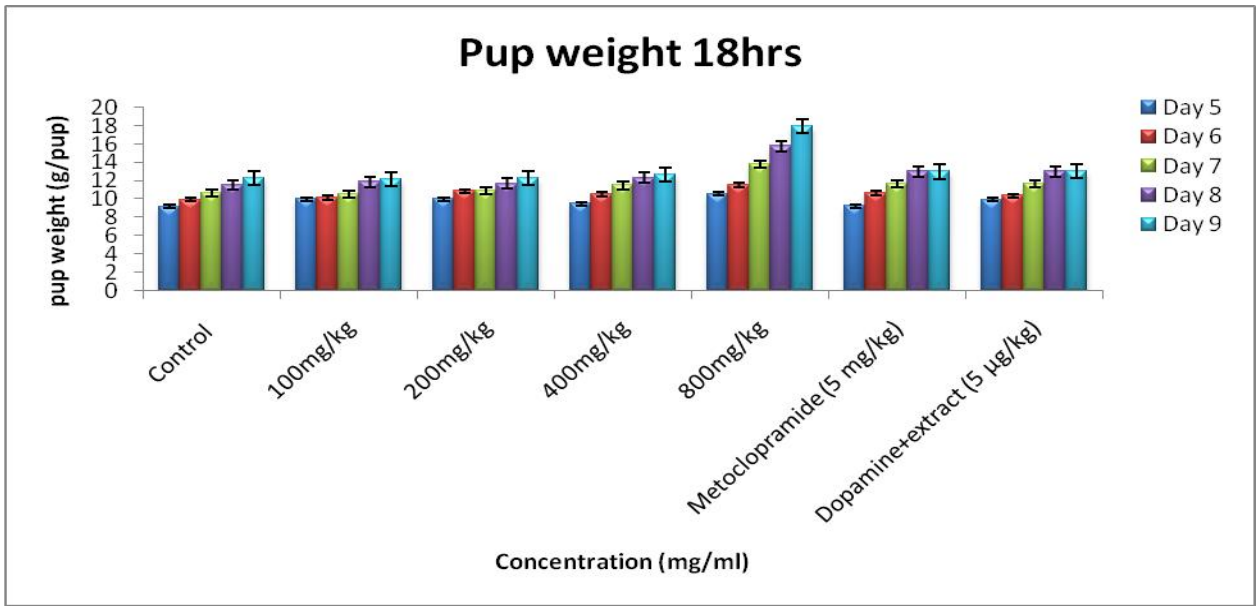


Figure 4.3a: Effect of aqueous extract of G.*perpensa* on pup weight after 18 hours (mean ± SEM), (n=5). The significant difference was not observed.

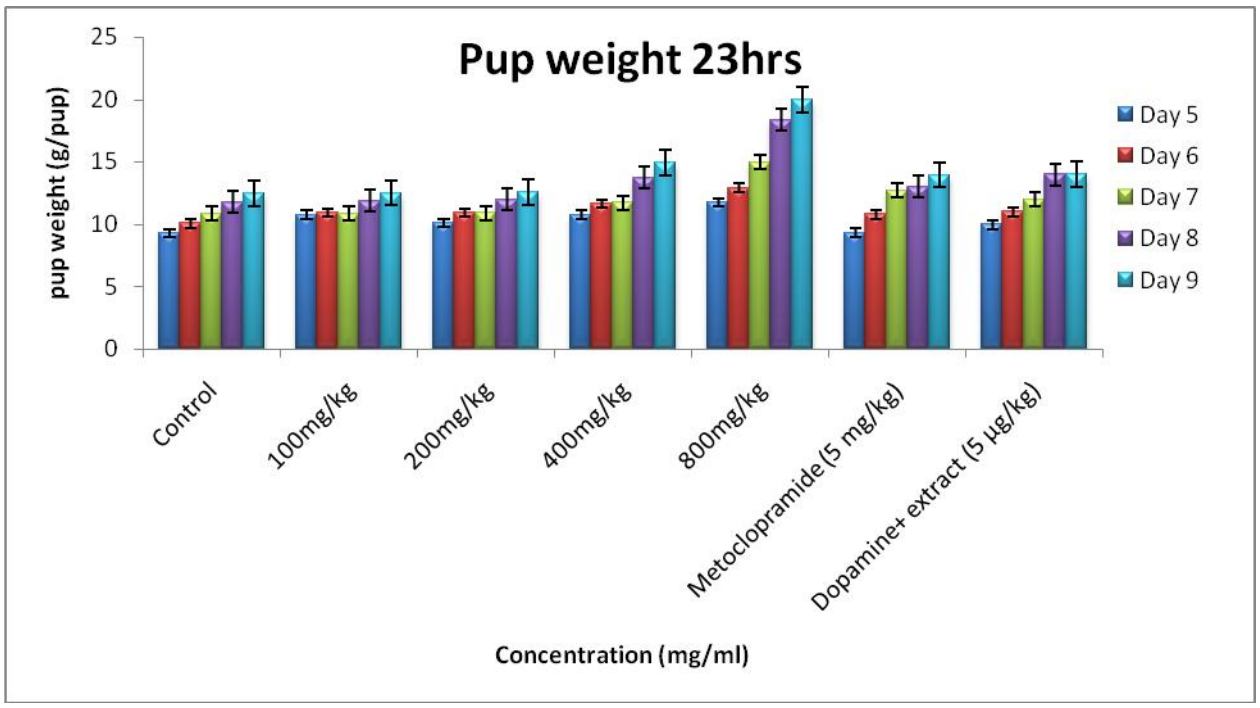


Figure 4.3b: Effect of aqueous extract of G.*perpensa* on pup weight after 23 hours (mean ± SEM), (n=5). The significant different was observed (P<0.05) at the highest concentration (800 mg/kg).

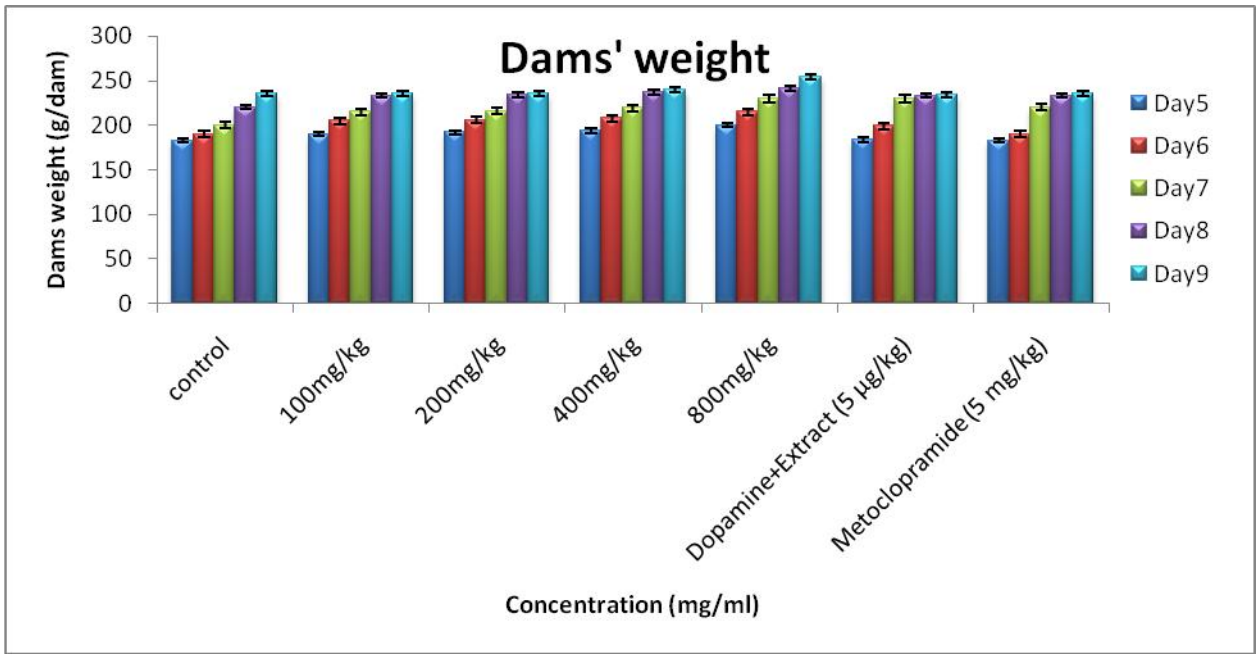


Figure 4.4: Effect of aqueous extract of G.*perpensa* on dam's weight from day 5 of lactation to day 9. The significant different was not observed, n=5.

Milk production data (Fig 4.5) 18 and 23 hours after gavage indicated that milk yield was significantly increased in the highest concentration (1600 mg.kg^{-1}) group receiving the extract. The activity was found to be dose dependant.

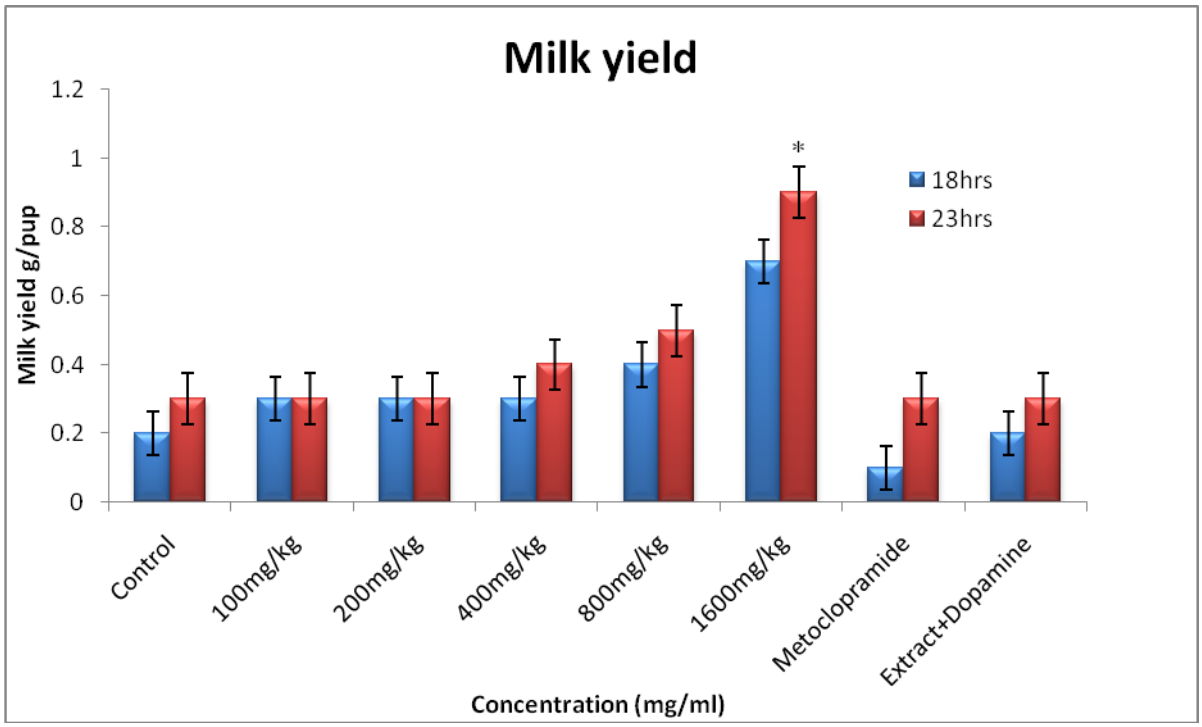


Figure 4.5: Effect of aqueous extract of G.*perpensa* on milk yield 18 hours and 23 hours after gavage (mean ± SEM), (n=5). The statistical different was observed between the control and the experiments ($P<0.05$).

Table 4.4a Serum levels of prolactin, progesterone, growth hormone, insulin and cortisol in control and extract-treated groups (mean ± SEM), (n=5).

Groups **Parameters**	**Control Normal saline**	**Extract – treated groups**				**Extract 100mg.kg^{-1} + Dopamine 5µgkg^{-1}**	**Metoclopramide 5mg.kg^{-1}**
		100mg.kg^{-1}	**200mg.kg^{-1}**	**400mg.kg^{-1}**	**800mg.kg^{-1}**		
Prolactin (ng/ml)	0.6±20	0.6±03 NS	0.6±50 NS	0.6±07 NS	0.6±80 NS	0.6±08 NS	0.6±11 NS
Progesterone (nmol/l)	96.68±10.96	83.04±17.88 NS	107.18±17.13 NS	103.62±22.39 NS	106.64±3.63 NS	112.22±6.55 S*	124.42±23.09 S*
Growth hormone (ng/l)	0.05±04	0.05±06 NS	0.05±02 NS	0.05±07 NS	0.05±04 NS	0.05±09 NS	0.05±05 NS
Cortisol (nmol/l)	27±50	27±60 NS	27±90 NS	27±50 NS	27±38 NS	27±70 NS	27±83 NS
Insulin (µU/ml)	0.70±0.53	0.04±0.02 S*	0.08±0.06 S*	0.10±0.08 S*	0.12±0.08 S*	0.00±04 S*	0.04±0.02 S*

Not significant=NS, Significant=S* ($p<0.05$), Significant=S** ($p<0.01$), Not determined=ND.

Table 4.4b Serum levels of Alanine aminotransfrease (ALT), Aspartate amino transferase (AST), Alkaline phosphatase (ALP), bilirubin, total protein and albumin in control and extract-treated groups (mean ± SEM), (n=5).

Groups **Parameters**	**Control Normal saline**	**Extract – treated groups**				**Extract 100mg.kg^{-1} +Dopamine 5µgkg^{-1}**	**Metoclopramide 5mg.kg^{-1}**
		100mg.kg^{-1}	**200mg.kg^{-1}**	**400mg.kg^{-1}**	**800mg.kg^{-1}**		
ALT (IU/L)	87.40±34.17	68.60±5.65 NS	103±83.73 NS	104±13 NS	ND	54.20±3.14 S*	59.40±6.62 S*
AST (IU/L)	336.60±181.63	125±11 S*	188.40±40.67 NS	193.40±24.95 NS	ND	88.40±7.77 S**	129.60±30.54 S*
ALP (IU/L)	437.00±00	580.8±68.32 S**	639.00±78.49 S**	641.4±43.63 S**	ND	ND	539.4±91.52 NS
Total Billirubin (umol/l)	1.80±00	1.92±0.05 S**	1.48±0.29 S**	1.48±0.29 S**	ND	1.96±0.04 S**	1.22±0.22 S**
Conjugated Billirubin (umol/l)	1.92±0.12	1.80±12 S*	1.48±0.29 S**	1.48±0.29 S**	ND	1.64±0.16 S**	1.32±0.29 S**
Total protein (g/l)	60.60±1.69	57.20±1.56 S**	55.80±1.16 S**	65.40±1.54 S**	66.8±0.37 S**	55.00±1.30 S**	60.20±1.59 NS
Albumin (g/l)	34.60±8.39	37.60±1.75 NS	30.60±1.47 NS	39.40±0.40 NS	ND	ND	ND

Not significant=NS, Significant=S* ($p<0.05$), Significant=S** ($p<0.001$), Not determined=ND.

Table 4.4c Serum level of urea and creatinine in control and extract-treated groups (mean ± SEM), (n=5).

Groups **Parameters**	**Control Normal saline**	**Extract – treated groups**				**Extract 100mg.kg^{-1} + Dopamine 5µgkg^{-1}**	**Metoclopramide 5mg.kg^{-1}**
		100mg.kg^{-1}	**200mg.kg^{-1}**	**400mg.kg^{-1}**	**800mg.kg^{-1}**		
Urea (mmol/l)	7.86±0.02	8.76±0.53 S**	8.84±0.27 S**	7.66±0.02 S**	ND	ND	8.18±0.26 S*
Creatinine (umol/l)	68.60±0.51	67.2±4.75 NS	62.4±1.08 NS	62.6±0.24 S**	ND	ND	57.60±1.91 S**

Not significant=NS, Significant=S* ($p<0.05$), Significant=S** ($p<0.001$), Not determined=ND.

4.4.2 Blood parameters

The results of the blood analysis of hormones are presented in Table 4.4a. There was no change in prolactin, growth hormone and cortisol levels, Progesterone levels seemed to rise with the increase in the concentration of the extracts. Progesterone levels increased by 28.4 % in the group treated with metoclopramide and by 16 % in the group with extract/dopamine compared to controls. Insulin levels decreased significantly ($p< 0.05$) and this decrease was concentration dependent.

The results of liver function tests are shown in Table 4.4 b. No changes in ALT were found in all extract treated groups, but ALT was significantly lower in extract/dopamine group compared to control. AST was decreased in the group treated with 100 $mg.kg^{-1}$ extract ($p<0.05$) and extract/dopamine group compared to control. Both enzymes were significantly reduced by 32 % for ALT and by 61 % for AST compared to the control group. Bilirubin (total and conjugated) has shown a significant decrease ($p<0.001$) in all experimental groups. No changes in albumin levels were recorded (Table 4.4 b). Changes in total protein levels appeared to be concentration dependent when comparing all experimental groups. It was observed that the levels were lower at concentration of 100 and 200 mg/kg^{-1} and higher at concentration 400 and 800 mg/kg^{-1} ($p<0.00l$). Total protein was significantly lower in extract/dopamine group.

The changes in renal function metabolites are presented in Table 4.4 c. In metoclopramide group urea has shown a slight significant increase, whereas creatinine was decreased ($p<0.001$) in the groups treated with 400 mg/kg^{-1} extract and with metoclopramide.

4.4.3 Uterus contractility

The results of the uterus contractility caused by G.*perpensa* and Oxytocin are shown on (Fig 4.6a) and (Fig 4.6b). The G. *perpensa* extract and oxytocin significantly and dose dependently caused uterus contractility (see Appendix B, figure B4).

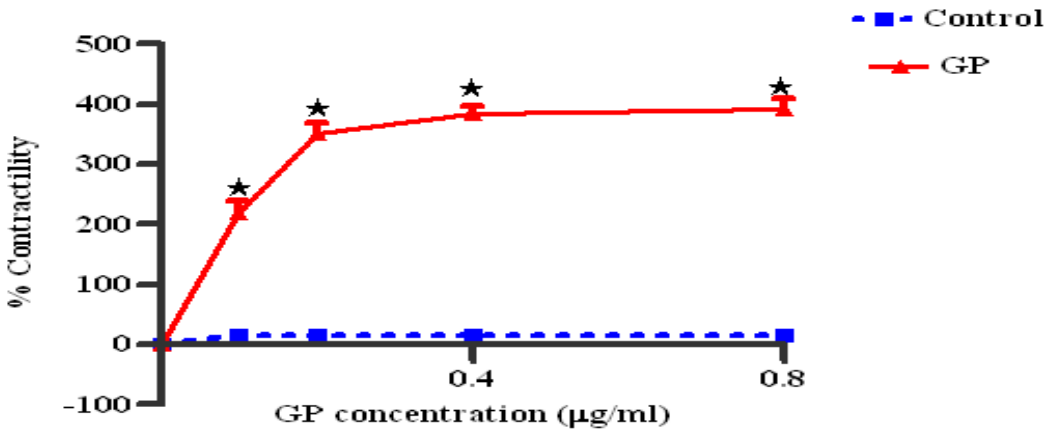


Figure 4.6a: Effect of aqueous extract of G.*perpensa* (GP) on uterus contractility, (n=6). Percentage (%) change in contractility are not absolute values.

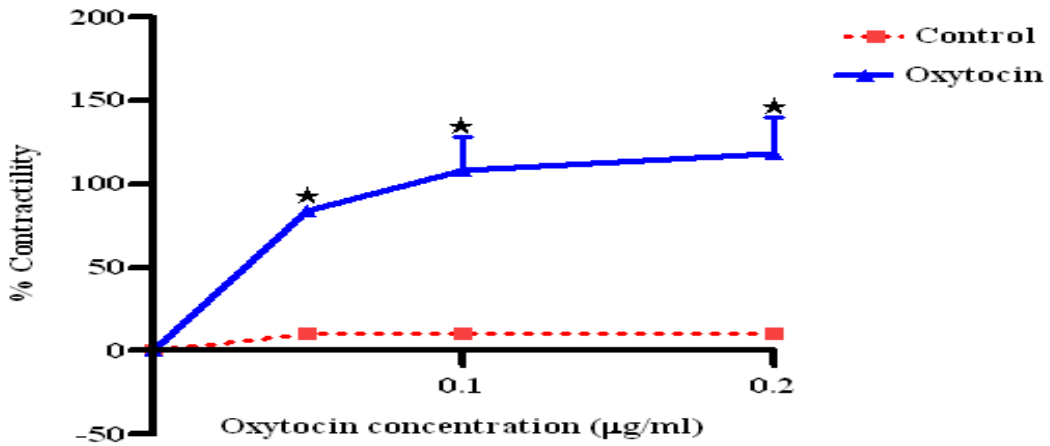


Figure 4.6b: Effect of oxytocin on uterus contractility, (n=6). Percentage (%) change in contractility are not absolute values.

4.4.4 Histology of mammary tissue

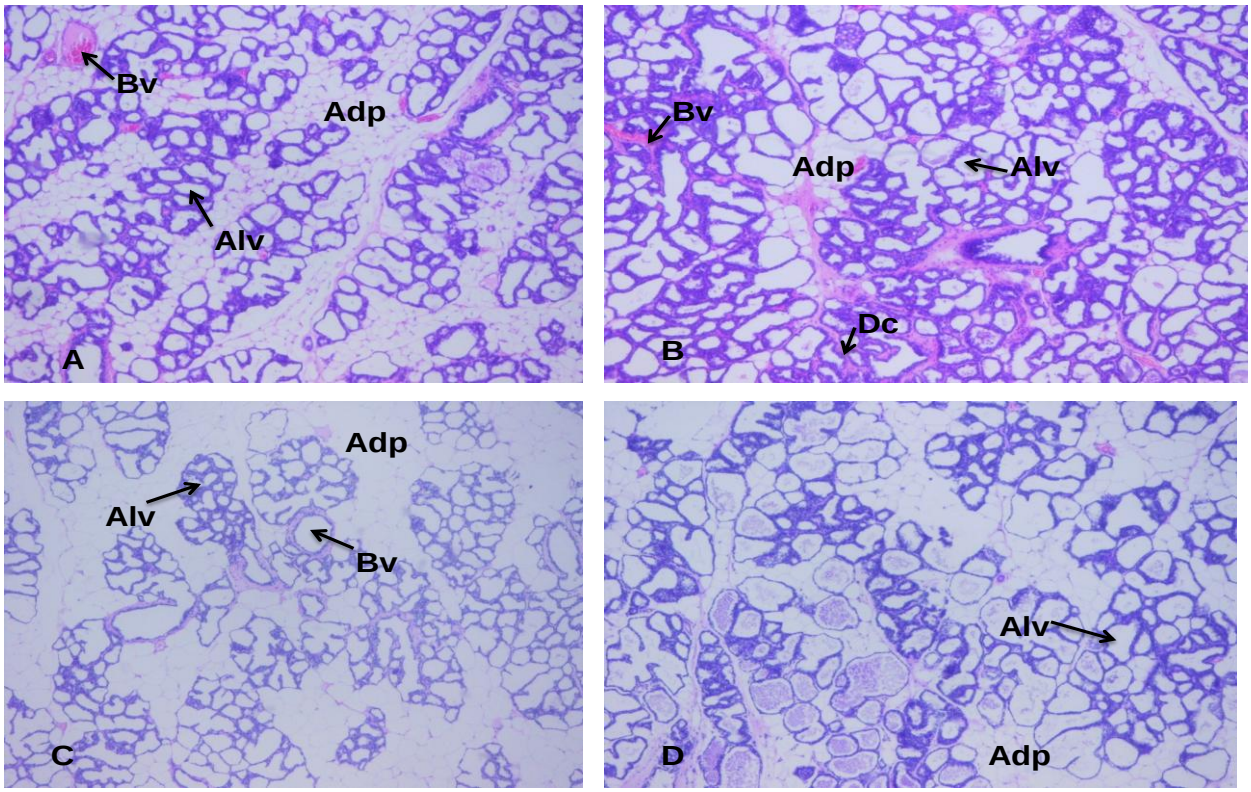
The histograms of the mammary glands are shown in figure 4.7. The aqueous extract of G*.perpensa* stimulated the lobulo-alveolar system development and its activity was found to be effective at high concentration (1600 $mg.kg^{-1}$). Figure 4.7**A** (normal, no treatment) shows very prominent alveoli which are lined by single layered flattened to cuboidal cells with small amounts of basophilic material (suspect secretory product) in their lumens. Cuboidal cells overall were the more prominent. The ducts are similarly lined by mostly single layered cells some also containing suspect secretory material. The acinar/adipose ratio seems approximately 80/20 to 90/10 in the different sections.

Figure 4.7**B** (rats treated with extract—1600 mg/kg bw) reveals very prominent alveoli lined by single layered flattened to predominantly cuboidal cells with small amounts of eosinophilic material (suspect secretory product) in some alveoli. Secretory material is most prominent in these sections. Ducts similarly lined by similar cells also containing suspected secretory material. The acinar/adipose ratio seems approximately 90/10. Mild focal areas of proliferation of epithelial cells forming small buds.

Figure 4.7**C** (rats treated with metoclopramide) shows very prominent alveoli lined by single layered flattened to cuboidal cells (possibly equal proportions) with small amounts of basophilic material (suspect secretory product). Ducts similarly lined by similar cells also containing suspected secretory material. The acinar/adipose ratio seems approximately 60/40 - alveoli seems the least prominent in this group compared to the adipose tissue component.

In Figure 4.7**D** (rats treated with dopamine and extract) prominent alveoli development was observed. Cells lined by single layered flattened to cuboidal cells with small amounts of basophilic material (suspect secretory product). Cuboidal cells seem more prominent than flattened cells. Ducts similarly lined by similar cells also containing suspect secretory material. The acinar/adipose ratio seems approximately 70/30.

Figure 4.7**E** (rats treated with dopamine alone), seems very similar to figure B, very prominent alveoli lined by single layered flattened to predominantly cuboidal cells with eosinophilic material in some alveoli were observed. Secretory material prominent in these sections. Ducts similarly lined similar cells and also containing suspect secretory material. The acinar/adipose ratio seems approximately 90/10. Mild focal areas of proliferation of epithelial cells forming small buds.



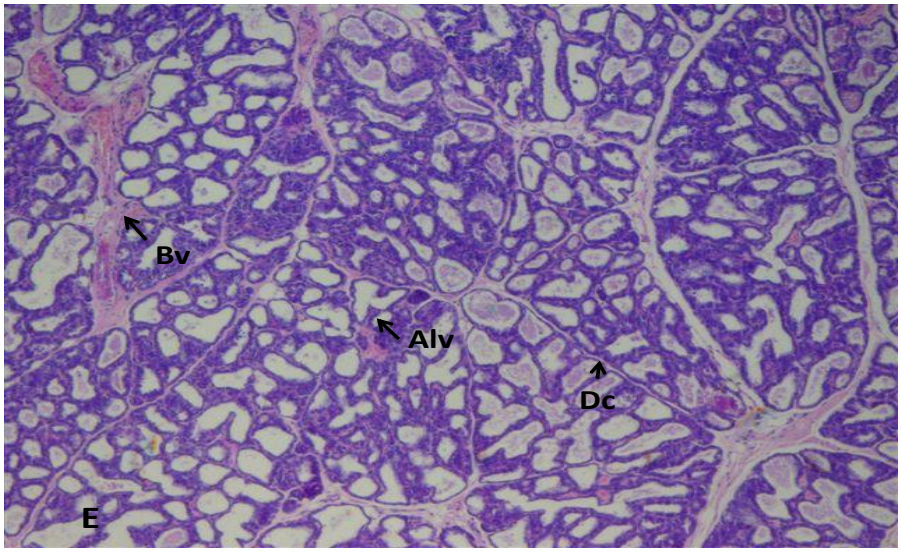


Figure 4.7a,b,c,d,e: Sections of the mammary glands from lactating rats 14-20 weeks old treated with saline, extract, dopamine + extract, and metoclopramide. (A) Mammary gland from lactating rat receiving 0.9 % NaCl, showing little alveolar development and the abundance of mammary adipose cells. (B) Mammary gland from lactating rat receiving 1600 $mg.kg^{-1}$ extract, showing ducts branching into ductile, with well defined alveolar development (compared to A,C and D) and fewer adipose cells. (C) Mammary gland from lactating rat receiving metoclopramide 5 $mg.kg^{-1}$, showing little alveolar development and the abundance of adipose cells. (D) Mammary gland from lactating rat receiving dopamine 5 $\mu g.kg^{-1}$+ extract 100 $mg.kg^{-1}$, showing little alveolar development and fewer adipose cells. (E) Mammary gland from lactating rat receiving dopamine 5 $\mu g.kg^{-1}$, showing little alveolar development and very little adipose cells Magnification A-D x 4. Adp, adipose cells; Bv, blood vessel; Avc, alveolar cells, Dc, ductile.

4.5 Effect of extract on Acetylcholinesterase activity

The results obtained from the assay of the effect of *G perpensa* extract on acetylcholinesterase activity are presented in Figure 4.8. The activity of *G.perpensa* to inhibit acetylcholinesterase was found to be 23 % and is dose dependent, while tacrine showed 68 %.

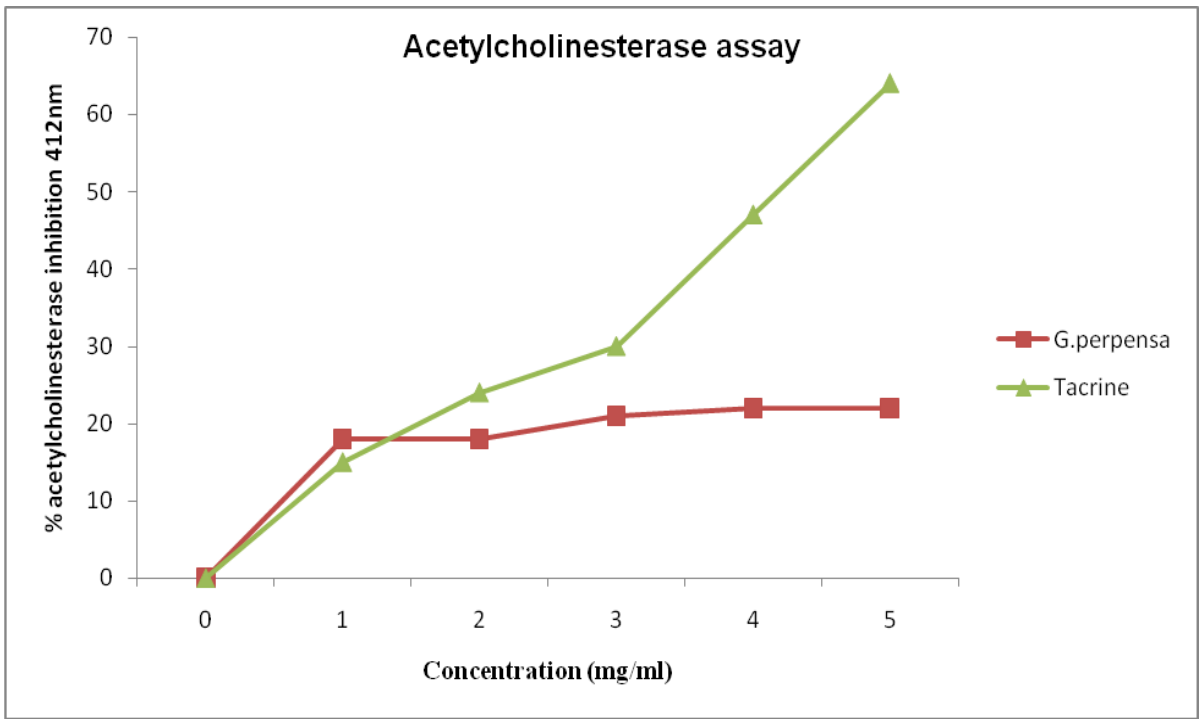


Figure 4.8: Effect of G.*perpensa* and tacrine on acetylcholinestarase, (n=3).

CHAPTER 5

DISCUSSION OF RESULTS

Milk is the food of the baby (infant)—an investment of the mother in the growth and maturity of the offspring. It is produced by the mammary glands of the lactating mother for the suckling child. Milk production/secretion is a very complicated process; it undergoes so many changes most of them are unacknowledged (Neville*, et al,* 2002). It is acknowledged that for milk to be successfully produced and delivered to the infant the alveoli cells have to develop to be able to hold more milk. Mammary glands can be induced biochemically by abnormal expression level of circulating hormones (Gudjonsson, *et al* 2002) or from a mechanical change in the tension of mammary stroma (Provenzano, *et al* 2008).

Milk secretion begins after birth due to decrease in circulating progesterone level and the presence of prolactin, which controls alveologenesis and milk protein production. Laminin and collagen in myoepithelial basement membrane interacting with beta-1 integrin on epithelial surface again, is important in this process (Streuli, *et al* 1995). Their binding ensures correct placement of prolactin receptors on basal lateral side of alveoli cells and directional secretion of milk into lactiferous ducts (Streuli, *et al* 1991). Suckling of the baby stimulates the release of hormone oxytocin which also causes contraction of the myoepithelial cells. In this way of combined control from extracellular matrix and systemic hormones, milk secretion can be reciprocally amplified so as to provide enough nutrition for the baby (Zarzynska and Motyl, 2008). However, free radicals may modulate the outcomes. Thus the presence of antioxidants may have led to neutralization of the free radicals.

5.1 Antioxidant activity of *G perpensa*

Free radicals are known to cause various diseases in living tissues. Oxidative stress and more specifically, lipid peroxidation, is known to play an important role in the pathophysiology of non-communicable diseases (Manuel *et al*., 2001) and fatigue syndrome (Vecchiet *et al*, 2003). Even though pregnancy is not a disease, it is often accompanied by a high-energy demand of many bodily functions and an increased oxygen requirement (Lachilli, *et al* 2001; Upadhyaya, *et al*. 2005). This triggered aerobic environment should primarily be responsible for raised oxidative stress in pregnancy. Oxidative damage might be prevented or limited by antioxidants. It is apparent that *G perpensa* is a good free radical scavenger, and it could act as a primary antioxidant effectively against free radicals and thus be considered a good source of natural antioxidant in preventing lipid peroxidation and protection from oxidative damage (Moure *et al*., 2001).

Hydroxyl radicals are, among the ROS, the most reactive and predominant radicals, generated endogenously during aerobic metabolism (Waling, 1975). Owing to their high reactivity, they have a very short half-life and can only be effectively scavenged at high concentrations (Rathee *et al*., 2007). In addition, nitric oxide**,** an essential bioregulator required for several physiological processes like immune response, smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and control of blood pressure, has been implicated in several pathological conditions, including cancer (Palmer *et al*., 1987). Accordingly, the ability of G. *perpensa* to scavenge these radicals has been evaluated.

The reducing capacity of many plant extracts has been correlated with their antioxidant activity (Pin-Der-Duh, 1998). In addition, reducing properties have been associated with the presence of reductants, which exert antioxidant action by breaking the radical chains through hydrogen atom donation (Gordon, 1990; Pin-Der-Duh, 1998). Furthermore, reductones have also been reported to prevent peroxide formation by reacting with certain precursors of peroxide (Rathee *et al*, 2007). It is thus likely that certain compounds present in G. *perpensa* may act as primary and secondary reductants thereby inhibiting lipid peroxidation.

Transition metals can arouse lipid peroxidation by generating hydroxyl radicals through Fenton reaction and hasten lipid peroxidation by lipid hydroperoxides into peroxyl and alkoxyl radicals (Zhao *et al*., 2006). Most effective pro-oxidants in food and drugs are ferrous ions (Yamaguchi *et al*., 1988). Chelating agents which form a bond with metal (Gordon, 1990) are effective as secondary antioxidants because they decrease redox potential, thereby stabilizing the oxidized form of the metal ion. *G perpensa* seems to have weak chelating activity.

Plant constituents that have been reported to be free radical scavengers and anti-lipoperoxidative include polyphenol (Maisuthisakul *et a*l., 2007; Adedapo *et al*., 2009), reduced glutathione (Bhatia and Jain, 2004) and NADH (Stern *et al*., 2002). NADH therapy is known to have beneficial effects in patients with chronic fatigue syndrome (Santaella *et al*, 2004). Polyphenols have antioxidant and iron-chelating properties and can combat oxidative stress. G. *perpensa* extract had a relatively high total polyphenol and NADH contents (Table 4.3) indicating a potential for anti-lipoperoxidative activity. The extracts significantly inhibited hydrogen peroxide induced lipid peroxidation in the

rat brain, and also inhibited lipoxygenase activity, and the AA/Fe^{2+} system. Apparently, G. *perpensa* is a good free radical (especially the peroxy type) scavenger.

5.2 Milk production

The floristic and ethnobotanic aspects of lactogenic plants have been studied extensively (Nacoulma-Ouedraogo 1996; Bailey and Day, 2004; Wynn and Fougere, 2007); However, little is known about their biological activities**.** In this study G.*perpensa* was investigated for its activity on milk production. To measure milk production from rats is difficult; weight of the pups is normally used (Sampson and Jansen 1984). Our results of milk yield (Fig 4.5), indicate that the extract of G.*perpensa* significantly (P<0.05) stimulated milk production in treated animals. Literature indicates that plants like *Hibiscus sabdariffa L* (Okasha *et al*, 2008) and *Acacia nilotica* ssp *adansonii* (Lompo-Ouedraogo, *et al.* 2004) exhibit their lactogenic activities through stimulation of prolactin production. Other plants like *Foeniculum vulgare* (fennel) and *Acacia nilotica* ssp *adansonii* increase milk production by causing development of breast tissue (Lompo-Ouedraogo, *et al.* 2004). Even though *G perpensa* extracts stimulated milk production in the presence of prolactin antagonist (dopamine), it had no observable effect on the levels of prolactin. Apparently the activity of G.*perpensa* on milk production is not by stimulating the lactogenic hormone (prolactin).

After parturition, prolactin induces lactation by direct stimulation of the synthesis of milk proteins in the epithelial cells and indirect stimulation of the proliferation of secretory cells. However, growth hormone (GH) is needed to support milk production when prolactin is reduced (Flint *et al*. 1992). In this study, the levels of GH and prolactin did

not significantly change in the presence of the plant's extracts. It is observed also that *G.perpensa* does not significantly affect the levels of progesterone and, cortisol.

It has been reported that tissues are less sensitive to insulin stimulation during lactation, and the increase in glucose concentration could be due to an increase in milk production during lactation (Regnault *et al*., 2004). It is apparent from the results that insulin levels were significantly decreased ($p<0.05$) and the decrease was concentration dependant.

It has been claimed that there is a decrease in the plasma protein levels during late pregnancy. This decrease could be attributed to a decline in the globulin (especially α1 and γ-fractions) causing increase in albumin/globulin ratio rather than a change in albumin level (El-Sherif and Assad, 2001). The higher serum albumin concentration recorded in this study for treated rats during lactation**,** compared to the non-treated rats is in agreement with other researchers (El-Sherif and Assad, 2001).

The enzymes, ALT and AST, are known as liver function indices in clinical diagnosis (McLean, 1962). ALT and AST levels in this investigation have shown a tendency to be elevated with the increase in the concentration of the plant extract. Okasha *et al* (2008) reported such increases in the enzymes when *Hibiscus Sabdariffa L* was administered to rats. On the other hand, both enzyme levels were significantly decreased ($p<0.001$) in the groups with metoclopramide by 4 fold for AST and by 1.6 fold for ALT. AST was decreased by 2.5 fold in the extract/dopamine group compared to controls. ALP levels were elevated ($p<0.001$) in the experimental groups by 32 to 46 %. AST level was found to be high even in the control group**;** this might be due to a transient infection because

when the extract was introduced it started to decrease. *G perpensa* slightly increases the levels of liver function enzymes ALT, AST and ALP.

Doornenbal *et al*. (1998) reported the serum urea and uric acid levels to be higher in lactating cows, compared to non-lactating cows. The results reported here are similar to those in the literature. *G perpensa* has antioxidant properties, this is also confirmed by the stimulated level of billirubin which is involved in the endogenous antioxidant system of the body (Elliott and Elliott, 2005).The change could be considered as an indicator of its participation. The results from the blood analysis seem to suggest that the extract of *G.perpensa* stimulates milk production through some means other than hormonal effect.

G.*perpensa extracts* stimulated uterus contraction and the activity was directly proportional to the concentration ($p<0.001$). The uterine contraction activity of *Gunnera perpensa (Haloragacea*e) has been reported (Khan *et al*, 2004). It is apparent that *G perpensa* exerted its effect in milk yield through ejection by stimulating the contraction of myoepithelial cells which surround the epithelial cells of the alveoli and finer ducts.

Acetylcholinesterase (AChE) is known to degrade acetylcholine. AChE inhibitors play an important role in nervous system disorders owing to their potential as pharmacological and toxicological agents. AChE inhibitors are useful in the treatment of myasthenia gravis (Srikumar *et al.,* 2004), Alzheimer disease (Perry *et al,* 1978), Parkinson's disease (Ceravolo *et al*, 2006), Most indirect acting acetylcholine (Ach) receptor agonists work by inhibiting the enzyme acetylcholinesterase. The resulting accumulation of acetylcholine causes continuous stimulation of the muscles, glands, and central nervous system (Purves *et al*, 2008). The extract of G.*perpensa* was found to inhibit the

activity of acetylcholinesterase (fig 4.8). It is apparent that the mechanism of action of G.*perpensa* to cause muscle contraction is through the inhibition of acetylcholinesterase activity.

The extent to which milk production/milk flow would have been influenced by the aqueous extracts of *G perpensa* would be impossible to judge from the histological findings alone but since physiological milk yield was determined, the histology results provide the evidence that G.*perpensa* stimulate alveolar development. It is concluded that G.*perpensa* increase milk production by causing alveolar development and the contraction of myoepithelial cells leading to milk ejection. *G.perpensa* contained saponins, tannins and flavonoids. These compounds could have contributed to the observed (anti-oxidant, lactogenic) activity of the plant.

5.2 Cytotoxic activity

The cytotoxicity of G. *perpensa* extract was determined using the brine shrimp *(A. salina)* lethality assay and MTT assay. Table 4.3 shows the result of the cytotoxicity of G. *perpensa* extract and the control (gallic acid) after 24 h exposure. The degree of the brine shrimp lethality and cell lines was found to be directly proportional to the different concentrations of the extract, with lethal concentration (LC_{50}) of 137.62 mg/ml). The level of toxicity to two human cell lines (HEK293 and HEPG2) was 279.43 µg/ml and 222.33 µg/ml respectively. The cytotoxicity of *G.perpensa* on monkey vero cells was reported to be 250 µg/ml (Veale *et al,* 1989). In toxicity evaluation of plant extracts by brine shrimp lethality bioassay, LC_{50} values lower than 1000 µg/ml are considered bioactive (Meyer *et al*., 1982). It is apparent that, when dilution in the bloodstream is

taken into account, G.*perpensa* should be regarded as non-toxic. Its estimated concentration in the bloodstream was 4.6 µg/ml (Veale *et al,* 1989). However, it is advised that *G.perpensa* be used medicinally with caution. In addition to these cell viability results, it has also been found that *G.perpensa*, significantly augments the initial response of the uterus to oxytocin, and therefore must be considered to have the potential to cause uterine hyperstimulation and its associated toxicity.

CHAPTER 6

CONCLUSION

The relationship between antioxidants and prevention of chronic degenerative diseases is the interest of many researchers and the restricted use of synthetic antioxidants, such as BHA and BHT, has led to the search for natural antioxidants from plants. Growing evidence indicates that reactive oxygen species are responsible for exercise-induced protein oxidation and contribute highly to muscle fatigue (Powers *et al*., 2004). Thus, the treatments that reverse muscle fatigue may be acting through mechanisms that scavenge reactive oxygen species. Even though the detailed mechanism of its effectiveness in inducing labour and facilitating the expulsion of the placenta or relief of menstrual pains has not yet been known the potential antioxidants capability and weak toxic effect of *G.perpensa* has been established in this study. It is apparent that *G.perpensa* contains compounds that might improve the action of natural dietary antioxidant. The relatively high total phenol and NADH contents and the significant total antioxidant capacity, expressed as the percentage of BHT, could contribute to *G.perpensa* antioxidant properties.

G.perpensa is not only an antioxidant, but it also stimulates milk secretion. The mechanism of action is possibly through the stimulation of muscle contraction through acetylcholine receptors. Furthermore, *G.perpensa* enhances alveolar development. It is apparent that the antioxidant properties, the ability to stimulate milk production/secretion and the relatively weak toxic activity of the plant may contribute to its use in folk medicine.

6.1 SUGGESTION FOR FUTURE STUDIES

The bioactivity of *G perpensa* has been established in this study. It is suggested that

- Isolation and characterization of active compound(s) be investigated.
- Mechanism of action of the active compound should be elucidated.
- Option for preparation of herbal medicinal products and applications in the homeopatic medicine should be considered.

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Appendix A

Reagent Details

A.1 Dragendroff's reagent

0.85 g of Bismuth nitrate was dissolved in 40 ml of distilled water and 10 ml of glacial acetic acid, followed by addition of 8 g potassium iodide dissolved in 20 ml of water.

A.2 Mayer's reagent

1.358 g of silver chloride was dissolved in 60 ml of distilled water and poured into a solution of 5 g of KI in 10 ml of distilled water and sufficient water was added to make 100 ml.

A.3 Sulphanilic acid reagent

0.33 % of sulphanilic acid was prepared in 20 % glacial acetic acid.

A.4 De Jalons physiological solution preparation

NaCl, 9.0 g; KCl, 0.42 g; $NaHCO_3$, 0.5 g; $CaCl_2.2H_2O$, 0.06 g, Glucose, 0.5 g were dissolved individually and the solution made up to 5 L.

A.5 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate)

7mM of ABTS* was prepared in water and equivalent amount of 2.45 mM potassium persulfate was added; the mixture was incubated at room temperature in dark for 16h which resulted in the production of radical cation ABTS*. The ABTS* was diluted (1:60 methanol, v/v).

A.6 Glutathione preparation

0.1-10 µg/ml was dissolved in ice cold distilled water containing 30 mµmoles/ml EDTA.

A.7 Preparation of Artificial Sea Water

The pH of natural seawater was taken and found to be 7.8 at a temperature of 28-30 ^{o}C. 30.0 g of non-iodized coarse salt was added to warm water to produce a pH of 7.8. This was used as the artificial seawater.

A.8 Phosphate buffer (pH 7.4)

40 ml of 0.2 M of potassium hydroxide was prepared and mixed with 50 ml of 0.2 M potassium dihydrogen phosphate. The mixture was made up to 100 ml.

A.9 Tris-buffer (pH 7.0)

7.88 g of Tris-HCL, 2.79 g of EDTA and 10.227 g of NaCl was dissolved in distilled water and the solution made up to 1 L.

A.10 Sodium borate buffer (pH 8)

2.473 g of boric acid was dissolved in 1000 ml distilled water. 2.012 g of sodium borate dissolved in 250 ml of distilled water. Then 166.67 ml of sodium borate was mixed together with 1000 ml of boric acid.

A.11 TBA

50 ml of glacial acetic acid and 50 ml of distilled water was mixed and 1 g of TBA was added into the solution and the solution was made up to 100 ml with distilled water.

A.12 Borate buffer (0.2M, pH 9.0)

6.18 g of boric acid was suspended in 300 ml of water. The pH was adjusted to 8.0 with 50 % NaOH solution and the final pH was adjusted to 9.0 with 10 % NaOH solution and was made up to 500 ml with water.

A.13 Linoleic acid (substrate) preparation

0.05 ml of linoleic acid was mixed with 0.05 ml of 95 % undenatured ethanol. The mixture was mixed gently until into an emulsion, with low stirring water was added up to 50 ml. For assay 5.0 ml of the above was diluted to 30 ml with borate buffer.

A.14 Formalin

1.75 g of sodium dihydrogen orthophosphate ($NaH_2PO_42H_2O$) and 3.25 g of di-sodium hydrogen orthophosphate (Na_2HP0_4) was dissolved in 25 ml of boiling water. 50 ml of 40 per cent formalin was added and the resulting mixture was made up to 400 ml with distilled water.

APPENDIX B

Details of Methodology

B.1 Phytochemical screening

Test for Alkaloids: 0.5 ml of plant extract was mixed with 5 ml of 1% aq.HCL. The mixture was stirred on the steam bath and filtered, 1 ml of filtrate was mixed with Mayer's reagent and another 1 ml of the filtrate was mixed with Dragendroff's reagent and the turbidity or precipitation was an indication of the presence on alkaloids.

Test for tannins: 5 g of plant extract was mixed with 10 ml of water and the mixture was stirred and filtered. 2 ml of filtrate was mixed with 0.1 %$Fecl_3$ solution (few drops). Blue-black, green or blue-green precipitate (precipitate) was an indication of the presence of tannins.

Test for saponins: 0.5 g of plant powder was boiled with10 ml of water and filtered. The filtrate was allowed to cool and was shaken vigorosly. The mixture was allowed to stand 15-20 minutes. The froth was an indication of the presence of saponins.

Test for anthraquinons;

a) **Free anthraquinone:** 0.5 g of plant powder was dissolved in little distilled water and shaken with 5 ml of benzene, filtered and 5 ml of 10 % ammonia solution was added to the filtrate and shaken. A pink color in the ammonia layer was taken as evidence for the presence of free anthraquinons.
b) **Combined anthraquinone:** about 0.5 g of plant powder was dissolved in little distilled water and boiled with ferric chloride solution and 5 ml of hydrochloric

acid for 10 minutes. It was filtered hot, cooled and then filtered and treated with 5 ml of 10 % ammonia solution was added to the filtrate and shaken. A pink color in the ammonia layer was taken as evidence for the presence of combined anthraquinone.

Test for flavonoids

(a) Lead acetate test; 1 ml of extract was mixed with 10 % of lead acetate. Reddish-brown coloration (or precipitate) was an indication of the presence of flavonoids.

(b) Ferric Chloride test; 1 ml of extract was mixed with 10 % of ferric chloride. Dark brown (dirty brown precipitate) was an indication of the presence of flavonoids.

(c) Sodium Hydroxide test; 1 ml of extract was mixed with 1 ml of diluted sodium hydroxide. Golden yellow precipitate an indication of the presence of flavonoids.

Test for steroids: 2 ml of acetic anhydride was added to 0.5 g extract of each sample with 2 ml H_2SO_4. The color changed from violet to blue or green in some samples indicating the presence of steroids.

Test for Cardiac glycoside

(a) Lieberman's test; 0.5 ml of extract was mixed with 2 ml of acetic anhydride, 1 ml of H_2SO_4 was also added in the presence of ice. Color change from violet to blue or green indicates the presence of steroidal nucleus i.e. the aglycone portion of the cardiac glycosides.

(b) Salkowski test; 0.5 ml of extract was mixed with 2 ml of chloroform, 2 ml of concentrated H_2SO_4 was also added in the presence of ice. Redish-brown color at the interface indicated the presence of steroidal nucleus i.e. the aglycone portion of the cardiac glycosides.

(c) Keller-killiani test*;* 0.5 ml of extract was dissolved in 2 ml of glacial acetic acid containing 1 drop of 10 % ferric chloride solution underlayed with 1 ml of H_2SO_4.

Brown ring at the interphase indicated the presence of a deoxy sugar characteristics of cardenoids, a violet ring appeared below the brown ring also indicated the presence of cardiac glycosides and the greenish ring appeared from just above the brown ring (i.e. in the acetic acid layer) and gradually spread throughout this layer indicated the presence of cardiac glycosides.

B.2 Antioxidant activity

B.2.1 Assay of 2,2-diphenyl-1-picryl hydrazyl (DPPH), 2 mg/100 ml was mixed with 2 ml of CH_3OH extract (0-5 mg/100 ml) after 1h the absorbance (517 nm) was measured against CH_3OH (Brad-Williams, 1995).

B.2.2 Assay of 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), 7 mM of ABTS (10 mg) was prepared in H_2O (3 ml) with potassium persulfate (2.45 mM) incubated at room temperature in the dark for 16h and then diluted (1 ml ABTS* : 60 ml CH_3OH). 1 ml of ABTS was mixed with 1 ml of CH_3OH extract (0-5 mg/100 ml) after 6 minutes the absorbance (734 nm) was measured against CH_3OH (Re *et al*, 1999).

B.2.3 Assay of superoxide radical (O^-_2) scavenging activity*:* The assay was based on the capacity of the extracts to inhibit the photochemical reduction of nitroblue

tetrazolium (NBT) in the riboflavin–light–NBT system (Beauchamp & Fridovich, 1971). The method used by Martinez et al, (2001) for determination of superoxide dismutase was followed after modification. To test tube(s) 0.02 ml each of the following, Bovine serum albumin 0.15 %, 3 mM Xanthine, 3 mM EDTA, NBT (0.75 mM), extract (0-5 mg/100 ml) and 0.48 ml sodium carbonate buffer (pH 10.5) was mixed together and incubated at 25 ^{0}C for 20min and 0.02 ml Xanthine oxidase (6 µM) was added then the mixture was incubated at 25 ^{0}C for 20 min and 0.02 ml $CuCl_2$ (6 Mm) was added. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 20 min. Ascorbic acid was used as the standard.

B.2.4 Assay of hydroxyl radical ($^-$OH) scavenging activity: the assay is based on benzoic acid hydroxylation method, as described by, Osawa, et al (1997). In a screw-capped tube, 0.2 ml of FeSO4.7H_2O (10 mM) and 0.2 ml EDTA (10 mM) were added. Then 0.2 ml extract (0-5 mg/100 ml), 0.2 ml DNA (10 mM) and 1 ml phosphate buffer (pH 7.4, 0.1 mol.) were added. Finally, 200 µl of an H_2O_2 solution (10 mM) was added. The reaction mixture was then incubated at 37 ^{0}C for 4 h (internal mixing). After this, 1 ml of TCA (2, 8%) and 1 ml of TBA (1%) were added and the mixture was boiled for 10 min and allowed to cool on ice. Absorbance was determined at 520 nm and Inhibition of lipid peroxidation by the extract was calculated.

B.2.4 Assay of NO^- scavenging activity: 2 ml of sodium nitropruside (10 mM), 0.5 ml of phosphate buffer saline (0.01 M; pH 7.4) was mixed with 0.5 ml extract (0-5 mg/100 ml) and incubated at 25 ^{0}C for 150 min. 0.5 ml of reaction mixture pipetted into different test tubes, 1 ml sulphanilic acid reagent (0.33% in 20 % glacial acetic acid) was added

and incubated at room temperature for 5 min; 1 ml 1-naphthylamine (5 %) was added and allowed to stand for 30 min in diffused light. The absorbance was determined spectrophotometrically at 540 nm. (Garrat, 1964)

B.2.5 Reducing power: The method reported by Oyaizu (1986) was used to measure reducing power of plant extract (0-5 mg/100 ml). 1 ml of CH_3OH extract was mixed with 2.5 ml (0.2 M) of phosphate buffer (6.6 pH) and 1 % potassium ferricyanide (2.5 ml) then incubated at 50 ^{0}C for 20 min. 10 % Trichloroacetic acid (TCA) was added to the mixture, the mixture was centrifuged (1000 rpm : 10 min). 2.5 ml of supernatant was mixed with 2.5 distilled H_2O and 0.5 ml of ferric chloride ($FeCl_3$) (0.1 %). The absorbance (700 nm) was measured against CH_3OH (Oyaizu, 1986). BHA and ascorbic acid were used as standards.

B.2.6 Chelating Activity on Fe^{2+}: The method reported by Decker and Welch (1990) used to measure the chelating activity of plant extracts on $Fe^{2+.}$ 1 ml of plant extract (0-5 mg/100 ml) was diluted with 3.75 ml of deionize water. This was mixed with $FeCl_2$ (2 mM, 0.1 ml) and 4, 4 ^{1}- [3-(2-pyridinyl)-1, 2, 4-triazine-5, 6-dryl] bisbenzene sulphonic acid (ferrozine) (5 mM, 0.2 ml), after 10 min the absorbance was measured. Ethylenediaminetetra-acetic acid (EDTA) and citric acid were used as standards.

B.3 Determination of total phenolics: The total phenolics in the extract were determined using Folin- Ciocalteu method as described by Kujala et al. (2000). To each sample solution (1.0 ml) and the standard (gallic acid) was added to 5 ml of Folin-Ciocalteu (Sigma-Aldrich) and 4 ml Sodium carbonate(7% w/v) and shaken. The solution was allowed to stand for 30 min in the dark at room temperature, after which

absorbance was measured at 765 nm using a spectrophotometer. The amount of total phenolics was expressed as gallic acid equivalent (GAE) in milligram per gram dry plant extract.

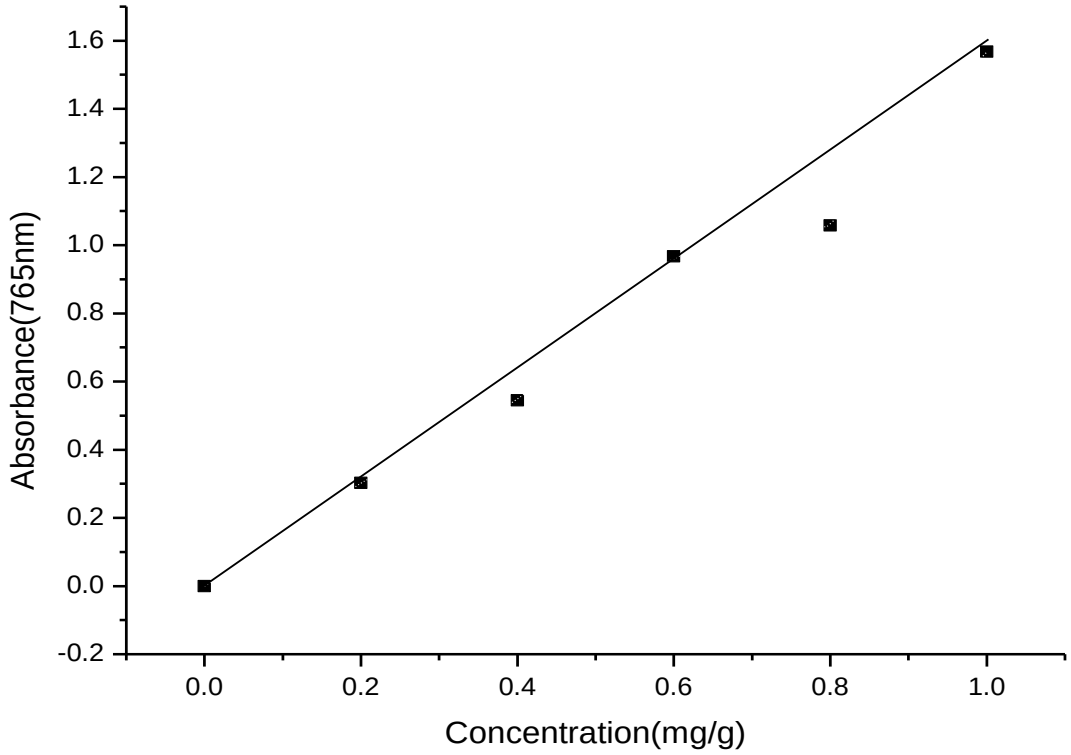

Figure B1: The standard graph of gallic acid.

B.4 Xanthine oxidase: Different concentration of plant extract was prepared in methanol (0-5 mg/100 ml). 1ml of Xanthine (21 µM) and 0.5 ml of plant extract were mixed together in one test tube and was incubated for 5 min at 20 ^{o}C, 0.2 ml of the enzyme (Xanthine oxidase, 10 units) was added into the test tubes and the mixture was incubated for 10 min at 20 ^{o}C and the absorbance was measured at 295 nm, methanol was used as the blank.

B.5 DNA sugar damage: Different concentration of plant extract was prepared in methanol, 0.3 ml of plant extract (0-5 mg/100 ml), 0.5 ml DNA (1 mg/ml of 0.15 M Nacl), 0.5 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml ferrous ammonium sulfate (4.8 mM) was mixed into the appropriate test tubes and was incubated for 1h at 37 ^{o}C with shaking. The lipid peroxide formed was measured as MDA.

B.6 Determination of total antioxidant capacity: 0.3 ml extract (0-5 mg/100 ml) was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95 ^{0}C for 90 min; there after the mixture was allowed to cool to room temperature, the absorbance of the solution was measured at 695 nm. The antioxidant activity was expressed as the number of equivalents of ascorbic acid and BHT.

B.7 Lipid peroxidation induced by Fe2+ / Ascorbate (AA/Fe^{2+}): The liver was obtained from the rat and it was quickly weighed and perfuse with phosphate buffer. The liver was homogenized (1:10w/v) 700 x g for 10 min. the supernatant was collected and kept at -20 ^{o}C. The supernatant was used to determine the protein. The supernatant protein was adjusted to 10 mg/ml with 50 mM phosphate buffer (pH 7.4). 0.1 ml diluted homogenate, 0.1 ml tris-HCl (20 mM; pH 7),0.1 ml KCl (30 mM), 0.1 ml plant extract (de-ionized water for control) was mixed and incubated at 37 ^{o}C for 5 min. 0.1 ml AA/Fe^{2+} (0.1 mM) was mixed and incubated at 37 ^{o}C for 30 min (intermediate mixing). Lipid peroxides were measured as MDA. 100 µl was transferred to other test tubes and 1.5 ml of TCA was added mixed after 10 minutes, the mixture went for centrifugation [3500 rpm for 20 min]. The supernatant was mixed with 1.5 ml of 1 % TBA was added. The mixture was heated in boiling water bath for 30 minutes; allowed

to cool then 2 ml of n-butanol was added. The absorbance was measured at 532 nm using butanol layer.

B.8 Lipid peroxidation assay in rat brain homogenate: Rat brain homogenate (0.1 ml of 10% v/v) and 0.1 ml of extracts (0-5 mg/100 ml) were added and the mixture was incubated in a water bath at 35 ^{0}C for 5 min; 0.15 ml H_2O_2 (10 mM) was added to induce lipid peroxidation and the mixture reincubated for at 37 ^{0}C for 15 min. Lipid peroxides were then determined as MDA. 100 µl was transferred to other test tubes and 1.5 ml of TCA was added mixed after 10 minutes, the mixture went for centrifugation [3500 rpm for 20 min]. The supernatant was mixed with 1.5 ml of 1 % TBA was added. The mixture was heated in boiling water bath for 30 minutes; allowed to cool then 2 ml of n-butanol was added. The absorbance was measured at 532 nm using butanol layer.

B.9 NADH: A modified method for determining NADH by Stern *et al* (2002) was used to determine the concentration of NADH in the plant extract. Phosphate buffer (containing 20 mM sodium dihydrogen carbonate, 100 mM sodium carbonate and 10 mM nicotinamide) was used to extract plant powder (1 g in 5 ml). The supernatant was divided into two portions. The NAD^+/$NADP^+$ of one portion was destroyed by incubating extract at 60 ^{0}C for 30 min on a dry heating block and promptly chilling to 0 ^{0}C. Both (heated and unheated) extracts were combined with 1 ml of reaction mixture (10 µM Tris-HCl, pH 8, 2 µM Phenazineethosulfate, 0.5 µM Tetrazolium Bromide and 0.2 µg alcohol dehydrogenase). The mixture incubated for 5 min at 37 ^{0}C; 0.1 ml ethanol (600 µM) was added the mixture was reincubated for 10 min at 37 ^{0}C. The absorbance was measured at 570 nm and the concentration was determined using the standard graph. NADH (10-80 ρM) was used as the standard.

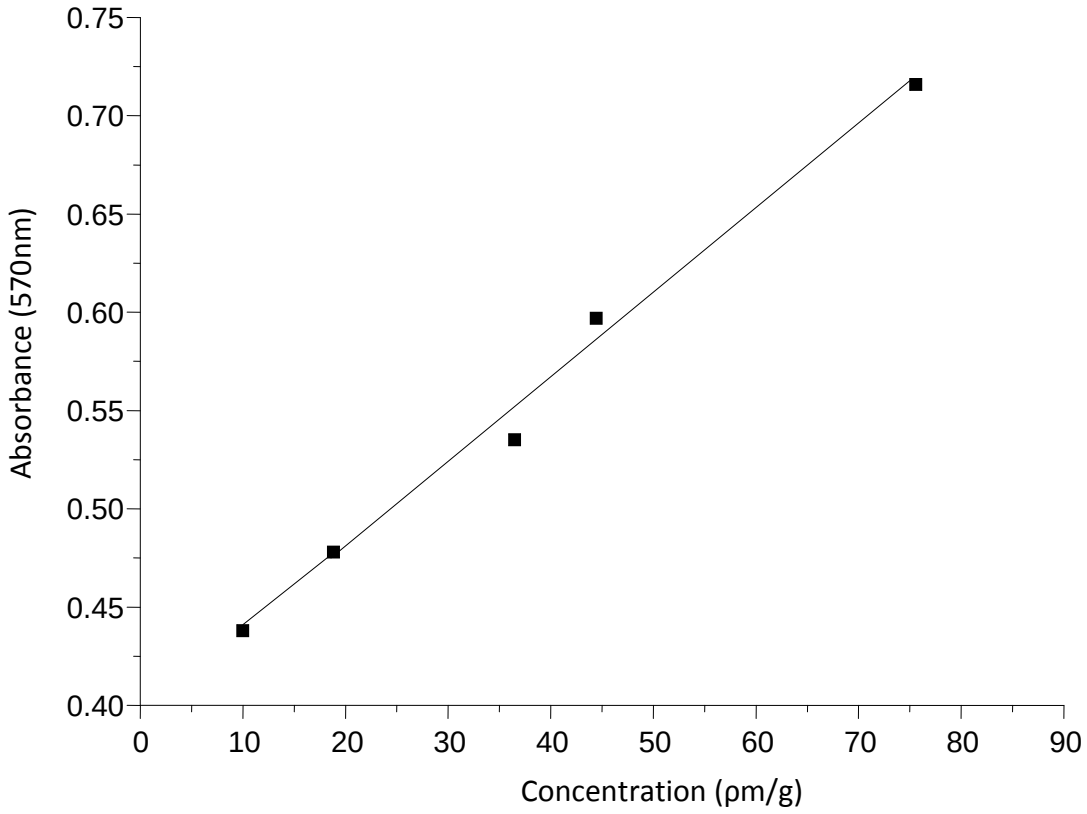

Figure B2: The standard graph of NADH

B.10 Lipoxygenase: 0.95 ml Borate buffer (0.2 M; pH 9.0), 2 ml substrate solution (0.05 ml linoleic acid and 0.05 ml 95 % ethanol) and 0.5 ml extract (0-5 mg/100 ml) were mixed followed by addition of 0.05 ml enzyme solution (500 units/3 ml). The increase in absorbance was measured spectrophotometrically at 234 nm, the inhibitory effects of the plant extract was calculated as for lipid peroxidation.

B.11 Effect of extract on Acetylcholine esterase activity: Acetylcholine activity was estimated using acetylthiocholine iodide as substrate following the method of Ellman *et al* (1961). The fish was pitched and brain was carefully removed using a scissor and

forceps. The removal of the brain was achieve by making median incision in the dorsal side of the skull and excising brain free by cutting the optic nerves an spinal cord. Care was taken to avoid any blood clots. At the same time, it was made sure that all of the brain tissues were removed and weighed. The brain was homogenized in 2 ml of 0.01 M phosphate buffer pH 8. The ground brain tissue was then diluted with sufficient amount of phosphate buffer to obtain 20 mg of brain tissue per one millilitre of buffer. The tissue was further homogenized, and centrifuged at 1000 x g for 20 min and supernatant was analysed immediately. 0.4 ml of the supernatant was used. Then 2.6 ml of phosphate pH,8.0 buffer was added. Then 100 µl of DTNB reagent was added and mixed well. 20 µl of 5mM s-acetyl- thiocholine iodide (ASChI) solution was added quickly and mixed well. The absorbance was recorded at 60 second intervals. The yellow colour produced by enzymatic reaction resulted in changes in absorbance. The reaction rate was calculated in units of changes in absorbance per minute. Tacrine was used as the standard (0-5 mg/100 ml).

The rate was calculated using. R=574xA/C. Where R = rate of µmoles, of substrate hydrolyzed/min/g brain tissue, A =change in absorbance per min and C= original concentration of brain tissue (mg/ml) in the homogenate (this value remains constant at 20 mg/ml throughout the study) and percentage inhibition was calculated as following: $1 - \frac{\text{Sample absorbance}}{\text{Control}} \times 100$

B.12 SH determination: powdered plant material was extracted (1:5 w/v) with EDTA solution (ice cold water containing 30 µM/ml EDTA) and filtered. 2 ml of extract (0-5 mg/100 ml) was mixed with 0.5 ml phosphate buffer (0.1 M, pH 8.0) and 0.1 ml of 1% σ-

phthaldehyde. The mixture was allowed to stand at room temperature for 15-20 min. The fluorescence was measured at 420 nm (activation at 350 nm). A standard graph for GSH (0.1-10 µg) was plotted (Figure B3) and,-SH content of the sample was estimated from the graph.

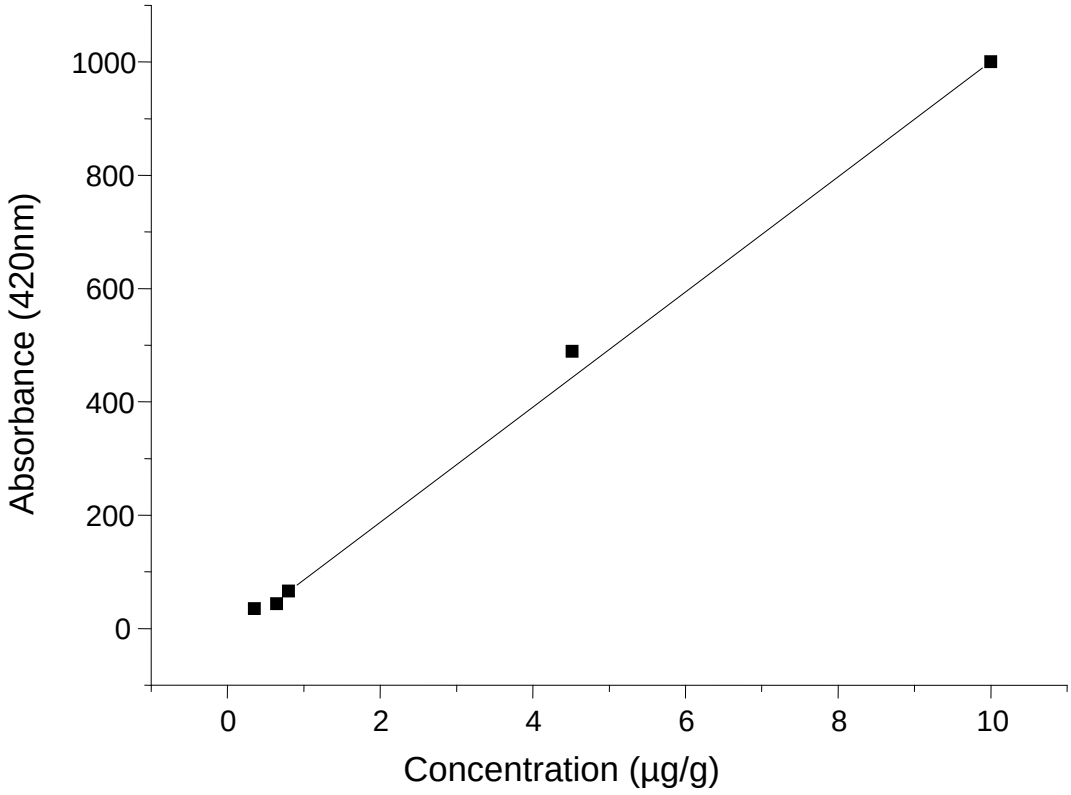

Figure B3: The standard graph of GSH

B.13 Cytotoxicity assay

B.13.1 Brine Shrimp Test: The brine shrimps *Artemisia salina* eggs were obtained from Fish designs (Distributor of live ornamental fish and products), Mtunzini, South Africa.

B.13.2 Hatching of the Brine Shrimps: The brine shrimps *Artemisia salina* eggs were mixed with artificial seawater and left to stand for about 15-20 minutes. The mixture was pour into an inverted glass bottle, with the base cut off and made-up to 2 l with artificial seawater. Aeration supplied through an open airline, with a weighed down airstone and the glass bottle was wrapped with black sheet. The hatching water was kept at a temperature of 27 to 30 ^{o}C, using a 100 w incandescent light bulb hanged over the hatching glass bottle, and left for 18 to 24 h for hatching of the eggs. The hatched *nauplii* were observed under the microscope for mobility and were transferred to a bowl in order to secure a large surface for oxygenation of the water and the bioassay performed.

B.13.3 The Brine Shrimp Assay: The brine shrimp test was carried out using the method of Meyer *et al*, 1982. Ten shrimps (counted on a slide) were introduced into the breakers containing different concentrations of *G.perpensa* (0-5 mg/100 ml) in methanol solution and made up to 5 ml volume with the artificial sea water. Different concentration was used. Each beaker was shaken lightly to ensure a homogeneous test solution and was left at room temperature. The controls were prepared with degassed distilled water and methanol solution without extract to which shrimp larvae were added. Each test was performed in triplicate. The beakers were maintained under illumination and the survivors were recorded after 24 h, criteria for death was loss of locomotive action of nauplii. The percentages death at each concentration and control were determined.

B.13.4 MTT cell proliferation assay

Human Embryonic Kidney (HEK293) and Human Hepatocellular Carcinoma (HEPG2) cells were all grown to confluenecy in 25 cm^2 flasks. This was then trypsinised and plated into 48 well plates at specific seeding densities. Cells were incubated overnight at 37 degrees celcius. Medium was then removed and fresh medium (MEM + Glutmax + antibiotics) was added. Extracts were then added in triplicate and incubated for 4 hrs. Thereafter the medium was removed and replaced by complete medium (MEM + Glutmax + antibiotics +10 % Fetal bovine serum). After 48 hours cells were subjected to the MTT assay (Mosman, 1983). At end of incubation period (48 hours), medium from cells in multiwell plate were removed. 200 µl of MTT solution as well as 200 µl of medium to each well containing cells were added. Multiwell plate was incubated at 37 ^0C for 4 hours. There after medium and MTT solution was removed from wells. 100/200/400 µl of DMSO was added to each well (stops reaction and dissolves insoluble formazan crystals. The plate was read in a plate reader at 570 nm (Mosman, 1983). Data were evaluated through regression analysis using QED statistics program and from the linear equation the LC_{50} values representing the lethal concentration for 50 % mortality.

B.14 *In vivo* study

B.14.1 Lactogenic activity: Forty rats (Sprague-Dawley) weighing 200–250 g were obtained from animal house of department of biochemistry and microbiology at the University of Zululand. The animals were housed with male rats in a metal cage under standard laboratory condition 25 ^0C with 12 h dark or light cycle so that they can became pregnant. They were feeding with food and water ad libitum. Following the birth, the litters' weights were recorded and culled to 6 litters per dam. The forty lactating

rats were randomly divided into four main groups (control, metoclopramide-treated, extract-treated and extract plus dopamine-treated groups). Control, metoclopramide-treated and extract plus dopamine treated groups consisted of five rats each (n=5), while the extract-treated group was sub-divided into five sub-groups of five rats each (n=5). Accordingly, the extract was administered in five different doses: 100, 200, 400, 800 and 1600 $mgkg^{-1}$. All groups had received the extract and the drug for six days starting from day 4 to day 9 of lactation (Vogel and Vogel, 1997). The extract and the drugs were administered orally except for dopamine which was injected intraperitoneally. The animals were then euthanized on day 10 using diethyl ether and the syringe was used to remove the blood from the heart.

B.14.2 Effect of oral treatment with Gp extract on milk production: Forty female rats (Sprague-Dawley) weighing 200–250 g were obtained from animal house of department of biochemistry and microbiology at the University of Zululand. The animals were housed with male rats in a plastic cage under standard laboratory condition 25 ^{0}C with 12 h dark or light cycle so that they can became pregnant. They were feeding with food and water ad libitum. Following the birth, the litters' weights were recorded and culled to 6 litters per dam. The forty lactating rats were randomly divided into four main groups (control, metoclopramide-treated, extract-treated and extract plus dopamine-treated groups). Control, metoclopramide-treated and extract plus dopamine treated groups consisted of five rats each (n=5), while the extract-treated group was sub-divided into five sub-groups of five rats each (n=5). Accordingly, the extract was administered in five different doses: 100, 200, 400, 800 and 1600 $mg.kg^{-1}$. All groups

had received the extract and the drug for six days starting from day 4 to day 9 of lactation (Vogel and Vogel, 1997). The extract and the drugs were administered orally except for dopamine which was injected intraperitoneally. Milk production was estimated 18 h and 23 h after gavage. Milk production was measured from day 4 to day 9 of lactation. Milk yield and body weight of dams and weight gain of pups were measured each day with an electronic balance. Every day during the study period, the pups were weighed at 0700 h (w1) and subsequently isolated from their mother for 4 h (Sampson and Jansen 1984). At 1100 h, the pups were weighed (w2), returned to their mother and allowed to feed for 1 h. At 1200 h, they were weighed (w3). Milk yield 18 h after the gavage was estimated as w3 – w2. The daily milk yield was corrected for weight loss due to metabolic processes in the pup (respiration, urination and defecation) during suckling. The value used was (w2 – w1)/4. This value was then multiplied by the number of suckling hours per day and added to the daily suckling gain (Sampson and Jansen 1984). The daily weight gain of pups was calculated from the pup weight at w2. Same procedure was followed for 23 h.

B.15 Uterus contraction: Sprague-Dawley rats weighing 200-250 g (non-pregnant) used in this study were obtained from BRU in the University of KwaZulu Natal. Animals were fed with food and water ad libitum. Quiescent uterine were removed from animals killed by decapitation. Unless otherwise noted, animals received a single injection of stilbestrol (12 mg/kg) 24 h before death. 2-3cm segments of split uterine tissue were placed in 50 ml organ bath. Isolated tissue was bathed in a De Jalons physiological buffer (pH 7.4) maintained at 32±1 ^{0}C with 95 % O_2/5 % CO_2. The buffer contained

NaCl (45.0/5 Litre), KCl (2.1/5 Litre), $NaHCO_3$ (2.5/5 Litre), $CaCl_2.2H_2O$ (0.3/5 Litre) and glucose (2.5/5 Litre). A basal tension of 1 g was applied to the segment and spontaneous contractions were measured with a myograph force transducer and physiological system. Contractile responses were expressed as milligrams of tension developed from above tension of 1 g for tissues that were not contracting spontaneously. In tissues spontaneously contracting, responses were measured by terminating the difference in peak tension that developed before (15 min before extract addition) and after extract addition to the organ bath.

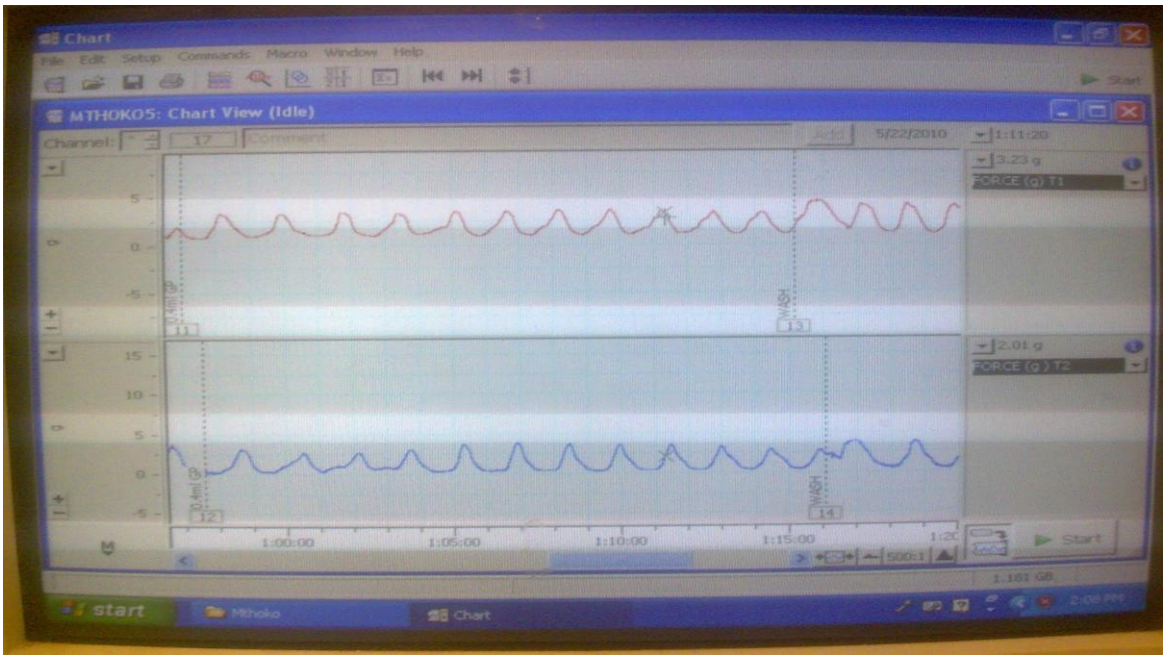

Figure B4. Contraction of uterus caused by the plant extract.

APPENDIX C

Ethics Committee
Faculty of Science and Agriculture
University of Zululand
C/O Mr L Vivier
Department of Zoology
University of Zululand
Private Bag 1001
KwaDlangezwa
3886
Tel: 035 – 902 6741
Email: lvivier@pan.uzulu.ac.za

19/01/10

To whom it may concern

ETHICS EVALUATION OF RESEARCH PROJECT PROPOSAL

This letter serves to confirm that **MBC SIMELANE** (Student No **20054768**), registered for a MSc Degree in the Department of Biochemistry and Microbiology at the University of Zululand, in accordance with appropriate rules submitted a research project proposal to the Ethics Committee of the Faculty of Science and Agriculture at the University of Zululand. The research project will investigate: **THE LACTOGENIC ACTIVITY OF *GUNNERA PERPENSA* – A ZULU MEDICINAL PLANT**. Based on the research protocol stipulated, this committee could find no reason from an ethical standpoint to reject the proposed research.

Yours sincerely

Mr L Vivier
Chairperson
Ethics Committee
Faculty of Science and Agriculture
University of Zululand

Appendix D

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Full Length Research Paper

In vitro antioxidant and cytotoxic activity of *Gunnera perpensa* L. (Gunneraceae) from South Africa

M. B. C. Simelane[1], O. A. Lawal[2], T. G. Djarova[1] and A. R. Opoku[1]*

[1]Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa 3886, South Africa.
[2]Department of Chemistry, University of Zululand, KwaDlangezwa 3886, South Africa.

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***Gunnera perpensa* L. (Gunneraceae) is a medicinal plant used by Zulu traditional healers to induce labor, expel the placenta after birth and to relief menstrual pains. Phytochemical screening of the rhizomes revealed the presence of alkaloids, flavonoids, steroids, saponins, tannins and glycosides. Methanol extracts of *G. perpensa* exhibited strong scavenging of 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) and 3-ethylbenzothiazoline-6-sulfonate (ABTS), but showed poor (< 50%) radical scavenging of nitric oxide, superoxide and hydroxyl radicals. At a concentration of 5 mg/100 ml, the extract was able to inhibit lipid peroxidation of the whole rat brain homogenate (71.13%) and lipoxygenase (30%) activity. The plant extract also contained reduced form of nicotinamide adenine dinucleotide (NADH, 3.8 pm/g), total phenol (248.45 mg/g) and traces of sulfhydryl groups (SH). The total antioxidative capacity was 36% relative to ascorbic acid (AA) and 64% relative to butylated hydroxyl toluene (BHT). The cytotoxicity of the extract (LC_{50}) to brine shrimp larvae was 137.62 mg/ml. It is apparent that the antioxidant activity of *G. perpensa* contributes to its effectiveness in folk medicine.**

Key words: *Gunnera perpensa*, gunneraceae, antioxidant activity, cytotoxicity.

INTRODUCTION

The genus *Gunnera* (Gunneraceae) is the only member of the family with about 45 - 50 species, that occurs naturally in Central and Southern Africa, New Zealand, Indonesia, Philippines, Hawaii, Mexico, Central and South America. In Africa, it is represented only by *Gunnera perpensa* L. (Mendes, 1978; Bergman et al., 1992). In the flora of South Africa, *G. perpensa* L., also known as *Ugobho* (Zulu) and *Iphuzilomlambo* (Xhosa) is a wetland perennial herb that grows (up to 1 m) in shallow water around the edge of pools in marshy areas or along streams. It is endemic to KwaZulu-Natal and Western Cape Provinces of the country (van Wyk and Gericke, 2000). *G. perpensa* has long been used in traditional medicine by the Zulus and Southern Sotho as a remedy to initiate labour, facilitate the expulsion of placenta and and clearing of the womb after birth (Veale et al., 1992; Hutchings et al., 1996; Kaido et al., 1997; van Wyk and Gericke, 2000; Ngwenya et al., 2003). Decoctions of the root or rhizome have been used for rheumatic pains, cold, wound-dressing, stomach aliments, menstrual pains, and to treat female infertility and male impotence (Iwalewa et al., 2007). Infusions of the root have also been used topically and taken orally for psoriasis, while, tinctures have been used for urinary stones (van Wyk et al., 1997, 2000).

Little is known about the chemical constituents of *G. perpensa*, although, an early study reported the presence of a bitter principle, celastrin (van Wyk and Gericke, 2000). However, pyrogallol, succinic acid, lactic acid, trimethyl ether of ellagic acid glucoside and venusol have been isolated from the aqueous extract of the dry rhizomes of this specie (Khan et al., 2004). In addition, 1,4-benzoquinones derivatives and *trans*-phyt-2-enol have also been secluded from the dichloromethane and methanol extracts of the leaves and stems and aerial parts of this plant, respectively (Drewes et al., 2005). Biological and pharmacological studies of various extracts and

*Corresponding author. E-mail: aropoku@panzulu.ac.za. Tel: +27359026099, +27725574397. Fax: +27359026568.

In vitro antioxidant and cytotoxic activity of *Gunnera perpensa* L. (Gunneraceae)

Simelane, M.B.C[1], Lawal, O.A[2], Djarova, T.G[1], Opoku, A.R[1#]

[1]Department of Biochemistry and Microbiology, University of Zululand, KwaDlangezwa 3886, South Africa
[2]Department of chemistry, University of Zululand, KwaDlangezwa 3886, South Africa
[#] Corresponding author- e-mail: aropoku@panzulu,ac.za

Introduction

➢The genus *Gunnera* L. is the only member of the family (Gunneraceae) with about 45-50 species, which occurs naturally in Central and Southern Africa, Madagascar, the Philippines, Hawaii, Mexico, Central and South America . In Africa, however, it is represented by *Gunnera perpensa* L. only [1] .

➢*G. perpensa* has long been used in traditional medicine by the Zulus and Southern Sotho as a remedy to initiate labour, facilitate the expulsion of placenta and clearing of the womb after birth[6,10] .

➢Decoctions of *G. perpensa* roots or rhizomes are also used for dysunia, rheumatic pains, dyspepsia, cold and stomach aliments, relief of menstrual pains and to treat female infertility and male impotence[5].

➢Biological and pharmacology studies of various extracts and isolated compounds from the plant confirmed antibacterial[8], antifungal [2]and uterotonic [9]activities, and wound healing

➢Considering the extensive utilization of *G. perpensa* in Zulu traditional medicine, the present study, was undertaken to investigate the *in vitro* antioxidative activity and cytotoxicity of *G. perpensa* rhizomes

Figure 1: *Gunnera perpensa* L.

Figure 2: Rhizomes of *G. perpensa*

Materials and methods

❖Plant materials of *Gunnera perpensa* were collected from KwaDlangezwa area of KwaZulu-Natal, and identified at the Department of Botany, University of Zululand, KwaDlangezwa. A voucher specimen was deposited at the University Herbarium

❖*G. perpensa* rhizomes were extracted exhaustively with methanol and the extract concentrated to dryness under reduced pressure to yield dried methanolic extract (17.15%)

❖Methods prescribed by Harbone[4], Odebiyi and Sofowora[7] were used to screen the plant extract for phytochemicals.

❖Total phenolic was determined using Folin-Ciocalteau reagent, total capacity antioxidant assay was based on the reduction of Mo (VI) to Mo (V). While,

❖The *in vitro* antioxidative activity of *G. perpensa* was determined using different methods

❖Brine shrimp (*Artemia salina*) lethality test was carried out using the procedure described by McLaughlin and modified by Setzer *et al*

Results and discussion

Phytochemical screening of *G. perpensa* rhizomes revealed the presence of alkaloids, flavonoids, steroids, saponins, tannins and glycosides

Table 1: Free radical scavenging activities of *G. perpensa* rhizome extract (IC_{50})

	G. perpensa	AA	BHA	BHT	CA	EDTA
DPPH	1.6	-	3.61	3.59	-	-
ABTS	0.9	-				
(O_2-)	>5	4.49	-	-	-	-
(·OH)	>5					
NO	>5	-	3.67	-	-	-

IC_{50} values in mg/100ml;

AA -ascorbic acid; BHA- butylated hydroxyl anisole; BHT- butylated hydroxyl toluene CA -citric acid; EDTA- ethylenediaminetetraacetic acid

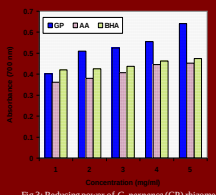

Fig 3: Reducing power of *G. perpensa* (GP) rhizome extract, ascorbic acid (AA) and butylated hydroxyl anisole (BHA). Each value is mean ± S.E ($n = 5$)

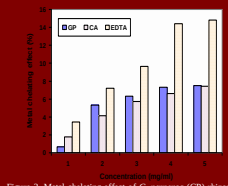

Figure 3. Metal chelating effect of *G. perpensa* (GP) rhizome extract, citric acid (CA) and ethylenediaminetetraacetic acid (EDTA). Each value is mean ± S.E ($n = 5$)

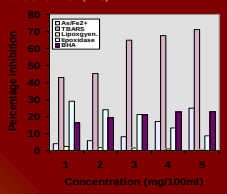

Figure 4: Lipid peroxidation induced by Ascorbate/ Fe^{2+}, Thiobarbituric acid reactive species, lipoxygenase and lipoxidase activities of *G. perpensa* against BHA

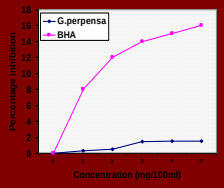

Figure 5: Xanthine oxidase of *G. perpensa* against BHA

❑*G. perpensa* exhibited strong free radical scavenging with 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) and 3-ethylbenzothiazoline-6-sulfonate (ABTS) radicals, reducing power and metal chelating activities (Figures 3&4), but showed poor (<50%) radical scavenging for nitric oxide, super oxide and hydroxyl radicals (Table 1), and ascorbic acid/Fe^{2+} oxidative system

❑ Its inhibited DNA sugar damage, lipoxygenase, lipoxidase and Xanthine oxidase activities.

❑ The plant contained NADH (3.8 ρm/g), polyphenols (0.01mg/g) and sulfhydryl group

❑The total antioxidant capacity of the plant was 36% relative to ascorbic acid and 64 % relative to Butylated hydroxyl toluene (BHT)

❑The cytotoxicity of the extract (LC50) to brine shrimp larvae was 138.79 (73.03-2251.21)mg/100ml .

Conclusion

During strenuous exercise (like childbirth), the muscle oxygen consumption increases tremendously to as high as 100-200 times compared to the normal resting conditions. Oxidative stress plays an important role in the etiology of fatigue and antioxidant treatment might be a valuable therapeutic approach. Gp contains NADH, polyphenols and SH compounds, which have the ability to scavenge reactive oxygen species suggesting that antioxidant activity may be an important mechanism of action of Gp, contributing to its use by traditional healers.

Acknowledgements

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APPENDIX E

Research Questionnaire

Interview of Traditional Healers

Date: Questionnaire No.

Name of the Interviewer:

Particulars of the area

GPS reading:

Name of the Area:

Name of the Village (Precise place):

Sociodemographic data

Gender: **Age:**

Male		15-24	
Female		25-34	
		35-44	
		45-54	
		55-64	

Plant Species particulars

Zulu name:

Plant 1:_____

Plant 2:_____

Plant 3:_____

Plant 4:_____

Scientific name:

Plant 1:_____

Plant 2:_____

Plant 3:_____

Plant 4:_____

English name:

Plant 1:_____

Plant 2:_____

Plant 3:_____

Plant 4:_____

Source of plant material:

Collected from the wild	
Cultivated (home-garden)	

What are the other uses of the plant?

_____

_____

Plant usage and collection

Question	**Usage**
Which part(s) used?	
Are the plants sold?	
In which state are the plants sold? (fresh or dry)	
If collected from the wild, when? (season)	
Any specific time of collection during the day?	
What places does the plant prefer to grow in? (wetland, dry land, forests, old fields, as weeds among the plants	

Preparation Method:

a) How is the medicine taken (e.g. by mouth or as enema)?

b) How is the medicine prepared?

Storage Method:

Dosage:

a) What is the dosage (e.g. one cup three times a day)?_____

_____

b) For how many days is the medicine taken?_____

_____

c) Are there any known side effects? _____

_____

d) Where did the knowledge come from (e.g. grandmother, relative)?

_____

_____

Age Group:

Infants	
Children	
Adults	