EFFECTS OF *Withania somnifera* (L.) ROOT EXTRACTS IN MODULATING SEROTONIN LEVELS IN SWISS MICE

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented in this university or any other for the award of a degree.

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Recommendation

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DEDICATION

I hereby dedicate this thesis to my loving parents, the late Mr. James Muriuki Kabuya and Mrs. Hannah Wangari Muriuki.

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I give all the glory to God for seeing me through my studies and providing me with the grace and all the resources I have required to come this far. He has been faithful and his promises are true. My heart felt gratitudes goes to Egerton Universty for her kindness to not only give me the admission, conducive environment to carry out both coursework and project, but also extending her hand in giving me waiver of fee; God bless Egerton University. I am extremely grateful to my supervisors Dr. Lelmen Elijah and Dr. Elizabeth M. Mwangi of Egerton University under whose guidance I undertook this research and wrote this thesis. They have offered me consistent mentorship and academic guidance throughout my studies and supported me through my research. I am indebted to Biological Sciences department, Egerton University for providing the facilities I required in my research. I would like to acknowledge Mr. Dickson Dezeze of Biological Sciences department, Egerton University for according me technical assistance in the laboratory and in the fields. I also wish to register my profound appreciation to all my family members; my wife, sons, brothers and sisters without whose moral support this journey would have been very tasking.

ABSTRACT

The increase in bone complications and disorders worldwide is becoming unbearable every day, with complications of back pain and arthritis based increasingly becoming a complex clinical challenge in the society and contributing for up to 60% of the human population who suffer from bone ailments. *Withania somnifera* is widely used in the traditional medical system of India against conditions such as arthritis and rheumatism. Serotonin, on the other hand, regulates mood and general behavioral patterns. The general objective of this study was to investigate the effects of *W. somnifera* root extracts on blood serotonin levels for possible application as a natural pain relieving agent through serotonin secretion. Since more serotonin is secreted during pain, *Withania somnifera* could thus be administerd against the pain resulting from musculo-skeletal complications and as an antidote to other pain conditions. *Withania somnifera* root samples were collected from Baringo County, around Chemeron Campus, Egerton University. Following extraction with hexane, dichloromethane, ethylacetate, ethanol, methanol and distilled water, the extracts were administered to different groups of mice as room temperature obtained extracts (cold crude extracts) as well as those obtained through soxhlet (hot crude extracts). Panadol was also treated to one set of mice to act as a positive control experiment. Serotonin assay was carried out in two series to establish whether there was any effect of heat during and after extraction, on the bioactivity of the secondry metabolites. The experiment was laid out in a completely randomized design (CRD) and data entered in spreadsheets for subsequent generation of graphs and tables. Data was analyzed using GENSTAT (Version15) Software and the treatment means compared using least significant difference (LSD). The extracts from roots of *Withania somnifera* contained secondary metabolites with different concentrations (ug/mL) of alkaloids in ethyl acetate (90) and methanol (78), flavonoids in methanol (135), terpenoids in hexane (105) and dichloromethane (90), tannins in methanol (119) and phenols in methanol (38). The crude extracts proved to increase serotonin secretion in Swiss albino mice differently, some close to Panadol, with polar solvents recording higher increase (ethanol 2.5045×10^{-1}) than non-polar (n-hexane 6.78×10^{-3}) µg/mL respectively. Purification of extracts of *W. somnifera* from each solvent and identification of the actual class of compounds causing serotonin secretion were recommended.

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LIST OF ABBREVIATIONS AND ACRONYMS

Asgand	Concotion of herbal preparation for treatment containing *W. somniferas*
Ashwagadha	Dried natural roots of the plant morphologically with specific roperties.
Ayuveda	an ancient medical treatise summarisisng the hindu art of healing and prolonging life: sometimes regarded as a 5^{th} Veda.
5HT	5-Hydroxytryptamptamin (Serotinin)
ACTH	Adenocorticaltrophic hormone.
CNS	Central nervous system
DNA	Deoxyribunucleic acid
EDTA	Ethyldiaminetetramine
GABA	Gammama amino butyric acid
ICS	Receptor Induced Currents
P (SP)	Substance P a pro inflammatory neuropeptide
WHO	World Health Organisation
MVA	Mevalonic acid
SNRI	serotonin noradrenaline reuptake inhibitors
SSRI	Selective serotonin reuptake inhibitors
NSAIDs	Non- steroidal anti-inflammatorys drugs
PGs	prostaglandins
Gq11	A G-protein subtype found in serotonin receptors
DMSO	Dimryhyl sulfoxide an organic polar aprotic molecule with an amphipathicnature that is ideal for dissolving poorly soluble polar and non-polar molecules.
Diglyme,	bis(2-methoxyethyl) ether,- a solvent with a high boiling point of an organic compound dimethyl ether of diethylene glycol
Xerophyte	A plant with desert adaptations for survival

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Many plants are known to have pain-relieving properties as reported on herbal painkillers for arthritis. A review of clinical trials in the clinical journal of pain says devil's claw (*Harpagophytum procumbens*), capsaicin from hot chilies (*Capsicum* spp.), gamma-linolenic acid (GLA) from seed oils, and certain blended herbal extracts are especially good. Other broader pain-relieving benefits from these plants and two traditional favorites, white willow (*Salix* spp.) and peppermint (*Mentha piperita*). A good number of reserchers have reported commendable trends of pain treatment using *Withania somnifera* which has both protective and curative properties (Choubey *et al.,* 2013).

Withania somnifera (Solanaceae) is a xerophytic plant, found in the drier parts of Kenya, India, Sri Lanka, Afghanistan, Baluchistan and Sind. It is more distributed in the Mediterranean regions where it grows wildly on waste places and on roadsides. It is also cultivated for medicinal purposes in fields and open grounds throughout India but not much research has been done in Kenya. How ever, the plant is common in more than twelve counties including including Nairobi, Machakos, Nakuru, Makueni, Baringo Moyale among others (Beentje, 1994). The chemistry of *W. somnifera* has been extensively studied and over thirty-five chemical constituents have been identified, extracted, and isolated (Rastogi & Mehrotra, 1998). The biologically active chemical constituents are alkaloids (isopelletierine, and anaferine), steroidal lactones (withanolides and withaferins), saponins containing an additional acyl group (sitoindoside VII and VIII), and withanolides with a glucose at carbon 27 (sitoindoside IX and X) and the plant is also rich in iron. Extracts from *Withania somnifera* produce GABA-like activity, which may account for the herb's anti-anxiety effects (Mehta *et al.,* 1991). The chemo preventive effect was demonstrated in a study of its root extract on induced skin cancer in Swiss albino mice given to them before and during exposure to the skin cancer-causing agent 7,12-Dimethylbenz (a) anthracen (Prakash *et al.,* 2002). *Withania somnifera* is an ingredient in many formulations prescribed for a variety of conditions such as arthritis and rheumatism (Bone, 1996). It has also been utilized as a general tonic to increase energy, improve overall health and longevity, and prevent disease in athletes, the elderly, and during pregnancy (Bone, 1996). Given that *W. somnifera* provides protection from free radical damage in the mouse liver (Panda & Kar, 1997). Figure 1.1

below shows structures of different oxidation states of thyroid hormone in its inactive state and active state

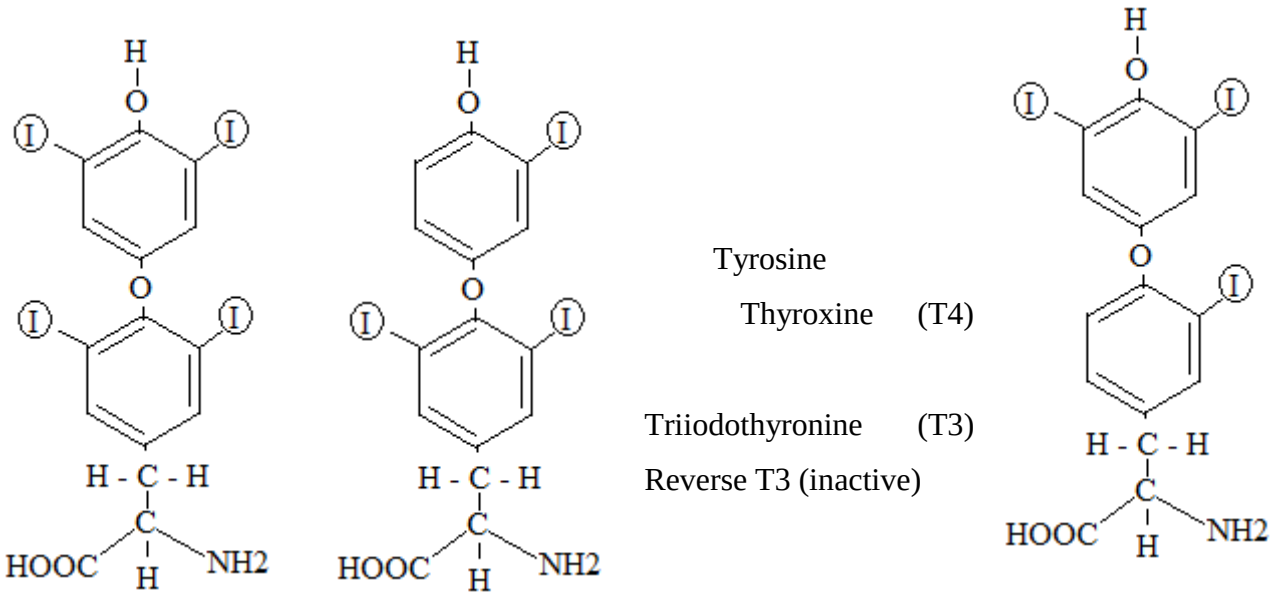

Figure 1.1: Structures of tri-iodothyronine and tetra-iodothyronine

Musculoskeletal disorders are prevalent and their impact is pervasive. They are the most common causes of severe long term pain and physical disability, and they affect hundreds of millions of people around the world. They significantly affect the psychosocial status of affected people as well as their families and care givers. At any one time, 30% of American adults are affected by joint pain, swelling, or limitation of movement (Woolf & Akesson, 2001). Serotonin (5-hydroxytryptamine) a monoamine neurotransmitter and derivative of tryptophan, is predominantly found in the gastrointestinal tract, platelets and in the central nervous system of animals. Approximately 90% of the total serotonin is located in the enterochromaffin cells in the alimentary canal, where it is used to regulate intestinal movements (Fu *et al.,* 2004). The serotonin secreted from the enterochromaffin cells eventually finds its way out of tissues into the blood where it is stored.

1.2 Statement of the Problem

Back pain and arthritis are increasingly becoming a complex clinical challenge in the present society. According to the World Health Organization (WHO), up to 60% of the human population suffers from these complications at some stage in their life time. Arthritis is characterized by the wearing of joints' soft bone, caused by failure of osteopoline, a protein responsible for calcium deposition in the osteoclasts of the bone and cartilage. This condition

predisposes an individual to other clinical disorders thus affecting negatively on their general health status. To address pain caused by musculo-skeletal disorders and providing a permanent solution has been a great challenge over generations. The present clinical remedies to these pains give a short lived relieve and therefore treatment with a longer lasting relieve from pain or total pain reduction is lacking.

Some commonly used drugs that are well-established produce some side effects from the gastrointestinal tract. As a result, every year there have been steadily increasing numbers of registered cases of drug induced physiological complications such as liver intoxication all over the world. Paracetamol is the most commonly administered pain relieving agent in the whole world. When paracetamol is taken regularly and in large doses of more than 4 g/day, there is a risk of serious side effects. Phenacetin and *N*-acetyl-*p*-aminophenol appeared to be the most satisfying of compounds. In some studies, paracetamol was characterized by high toxicity similar to acetanilide, therefore phenacetin was the first derivative to be introduced into medical practice in 1887. Figure1.3 below shows structures of some commonly used drugs in medical field as pain relirving agents. The use of these drugs is thus uneconomical in the long run and there would be required a solution to substitute them with an affordable treatment with no side effects.

If drugs could be cautiously derived from extracts of well known medicinal plants, well formulated and dose determined the problem of long term pains and joint complications would no longer be a challenge to the current and future societies. Such drugs should be derived from plants like *W. somnifera*.

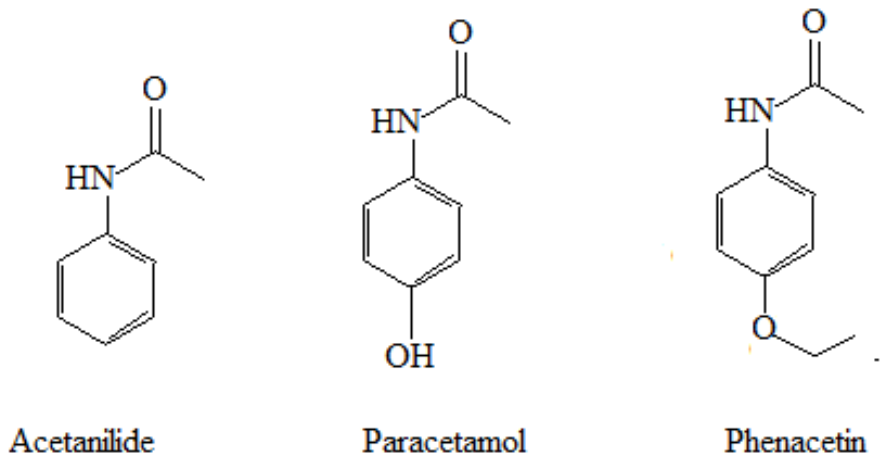

Figure 1.2: Some universal convectional alnalgesics in common use

Analgesic mixtures like Phenacetin have been associated with the development of analgesic nephropathy after a prolonged usage. In Poland for instance phenacetin was used as a component of very popular and common analgesic. In as old as 1888, 500 mg tablets of paracetamol were available over the counter in Great Britain under the trade name of Panadol, which were produced by Frederick Stearns and Co, the branch of Sterling Drugs. Paracetamol has been associated with non-specific gastrointestinal symptoms, such as nausea and vomiting, dyspepsia, abdominal pain, and bloating. Some studies with in patients with musculoskeletal pain showed that paracetamol was associated with more 'digestive adverse effects' than ibuprofen after 6–14 days of regular oral use as compared to diclofenac. Although the effects of those two drus were almost restricted to abdominal pain and some nausea as the only complications those side effects may also not be considered irrelevant. *Withania somifera*, being a potent pain modulator would probably modulate serotonin levels in the blood stream thereby reducing joint and back pains.

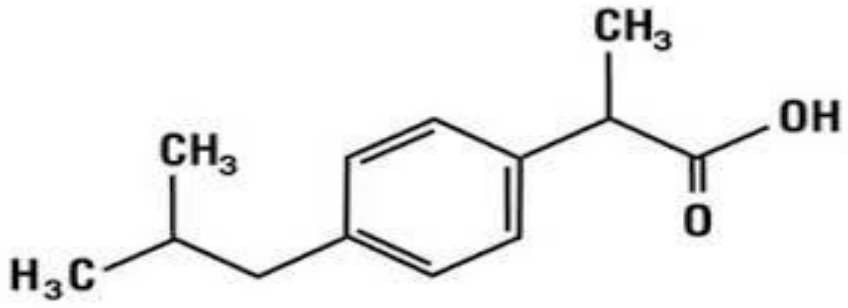

Figure 1.3: Structure of Ibuprofen

Nonsteroidal anti-inflammatory drugs (NSAIDs) are contraindicated, *e.g.*, in the case of gastric ulcers, hypersensitivity to aspirin, impairments in blood coagulation, in pregnant women, nursing mothers and children with fever accompanying a disease (Leung, 2012). Panadol administration suppresses prostaglandin production just like NSAIDs and so may have similar negative physiological setbacks as those drugs. In children metabolism of paracetamol may cause toxicity especially of the liver (hepatotoxicity). All conventional NSAIDs inhibit the conversion of arachidonic acid into prostaglandin H - PGH2. Whose commitment step is catalyzed by prostaglandin H synthase (PGHS), also termed cyclooxygenase (COX) within which isoenzymes COX-1 (PGHS-1) and COX-2 (PGHS-2) are involved (Hinz & Brune, 2002). Some enzymes have more than one function for example PGHS is a bifunctional enzyme and possesses two different enzymatic activities: cyclooxygenase and peroxidase (POX).

Prostaglandin H2 (PGH2) is a substrate for specific synthases, tissue-dependent isomerases catalyzing its further conversions into different endogenous regulators, namely: prostaglandins of the D (PGD2), E (PGE2), F (PGF2) series and prostacyclin (PGI2) and thromboxanes (TXA2 and TXB2). They all are characterized by different biological activities and many of them have anti-inflammatory properties. Thus, the action of NSAIDs, which inhibits the stage of conversion of arachidonic acid to prostaglndins and also the formation of the prostacyclin and thromboxanes as regulators, have some favorable anti-inflammatory, analgesic and antipyretic activity. Those drugs also have side effects associated with the inhibition of synthesis of particular regulators in different tissues (Dzielska-Olczak & Nowak, 2012). While traditional NSAIDs and selective COX2 inhibitors inhibit cyclooxygenase (PGHS) through competing with arachidonic acid for the active site of the enzyme paracetamol is likely to act as a factor reducing a ferryl protoporphyrin IX radical cation (Fe_4^+ =OPP*$^+$) within the peroxidase site of the PGHS enzyme. In turn, this ferryl protoporphyrin radical cation generates tyrosine radicals in the place of PGHS cyclooxygenase, which are essential for catalyzation of arachidonic acid oxidation reaction (Chakraborti *et al.*, 2010).

The prospective double-blind trial performed in patients with stable coronary disease who used paracetamol at the dose of 1 g three times a day for two weeks showed that the drug increased their blood pressure. Its effect was similar to that exerted by diclofenac and ibuprofen. Clinical studies indicate anti-aggregatory action of paracetamol in the case of parenteral administration in high doses. Paracetamol at daily doses higher than 2-2.6 g increases the risk of serious side effects in the upper segment of the digestive tract such as bleeding or perforations. Therefore, it is postulated that a long-term effect of paracetamol on the digestive tract poses higher risk to patients with osteoarthris and who require high doses of this drug for a long time.

Based on the fact that hydroperoxides of fatty acids, like PGG2 oxidize porphyrin within the peroxidase site of the enzyme, cyclooxygenase inhibition by paracetamol is difficult in the presence of high peroxide levels and thus paracetamol decreased prostaglandin synthesis ten times stronger in the brain than in the spleen going by studies by Hinz and Brune (2002). Thus, paracetamol is a preferential inhibitor of COX-2 isoenzyme. Effect of such drugs depends to a great extent on the state of environmental, oxidation or reduction (redox). The study on healthy volunteers in whom the pain was induced through electrical stimulation of the median nerve showed that analgesic action of paracetamol was completely blocked in the

group of subjects treated with paracetamol combined with tropisetron a serotonin antagonist primarily used in the prevention of chemotherapy-induced nausea and vomiting. Epidemiological data revealed that long-lasting administration of paracetamol affects blood pressure (McCrae *et al*., 2018).

Nurses health studies also presented two cohort investigations performed among younger and older women. One of them demonstrated that in patients who regularly took paracetamol (over 500 mg/24 h) are at a relative risk for development of hypertension was considerably higher than for women who did not use this drug (Graham *et al*., 2012). It is paramount noting that the risk associated with paracetamol was similar to traditional non-steroidal anti-inflammatory drugs (NSAIDs). The second cohort investigation carried out in the same study group indicated that in women who frequently used paracetamol for approximately 22 days a month, the risk of serious cardiovascular events such as heart infarction or cerebral stroke was nearly the same as after traditional NSAIDs administration. Application of paracetamol in the amount of 15 tablets or more per week is associated with the risk of cardiovascular events comparable to traditional NSAIDs (Fokunang *et al.,* 2018).

Some painkillers induce bronchial spasm in patients with Asprin asthma and are contraindicated against the strategy for treatment of pain in asthmatics in order to avoid potential bronchial spasm. In some experimental studies on animals paracetamol administered at doses twice as high as the maximum single dose demonstrated embriotoxic action (Fleetwood *et al.,* 1988). For this reason, pregnant women should be in very rare and specified conditions of a disease administer sensitively selected drugs due to possible epidemiological association between application of this drug in pregnancy and development of asthma.

The metabolism of paracetamol has been suggested to be responsible for this effect because a large amount of glutathione is used to deactivate the toxic metabolites. Lungs of the developing fetus might deplete glutathione, the main antioxidant of this organ, which can lead to oxidative stress and inflammation of the respiratory airways. In some investigations, the occurrence of wheezing breath in very small children was observed, which however, is a very weak indicator of asthma. The risk of hospitalization due to gastrointestinal events of ulceration, perforation, bleeding from the upper or lower segment of the digestive tract and has appeared to be two-fold higher in the case of taking paracetamol in combination with

traditional NSAIDs as compared to NSAIDs used in monotherapy. The problem of interaction between paracetamol and NSAIDs explains the results in relation to the additional cyclooxygenase-1(COX-1) inhibition caused by paracetamol (Graham *et al*., 2012).

The products of *W. somnifera* lack in the disadvantages reported for any convectional pain killer. The extracts from this herb have been traditionally taken as a mono-therapeutic agent or in combination with other plant species extracts where results are more improved without reported cases of physiological complications as compared to the conventional medicine. Based on the diverse distribution the world over of the plant, it means many countries could utilize their wastelands for not only pain relieving purpose by cultivation of the herb, but also development of other drugs from the same plant. The ease with which the plant can be cultivated leads to the idea of making it readily available not only to most gardens but also grow it as a potted herb and flowerbeds for curative and ornamental purposes.

1.3 Objectives

1.3.1 General objective

To investigate the effects of *Withania somnifera* (L.) root extracts in modulating the blood serotonin levels in Swiss albino mice.

1.3.2 Specific objectives

i. To determine presence and contentrations of secondary metabolites in *W. somnifera* root extracts.
ii. To determine serotonin levels in Swiss albino mice injected with root extracts of *W. somnifera* using water methanol, ethanol, ethyl acetate and dichloromethane as cold and hot extraction solvents compared to Panadol as a positive control.

1.4 Hypotheses

i. There are no significant secondary metabolites in *W. somnifera* root extracts.
ii. There is no significant difference in yield of serotonin secreted between secondary metabolites from *W. somnifera* derived through hot and cold serial extraction compared to Panadol in Swiss albino mice.

1.5 Justification

Most people who suffer from bones and joints diseases are economically and socially handicapped, with hospitals not being able to accommodate the patients. Most studies that have been carried out demonstrate serotonin production and its effects during pain conditions.

On the other hand, they also indicate that *Withania sominifera* has been traditionally used for treatment of arthritis and bone ailments. Most patients die either because of high pain intensity and lack of enough funds for proper treatment and and availability of physiologically friendly drugs. However, none of the studies has attempted to address the interaction between *Withania somnifera* and pain modulators, thus making this research important in filling that knowledge gap. Given that this study was able to evaluate the capacity of *W. sominifera* extracts to significantly increase blood serotonin levels and that *W. sominifera* has the capacity to relieve backaches then this plant may xe recommended for drug formulations with much higher confidence than routinely administered drugs The plant can conveniently be cultivated taking a short time to grow and mature, could be preserved easily in dried state and is able to grow in many geographical locations without much stress. The idra of dering pain relieving drugs from *W. somnifera* would have the outcome of reducing the high population of people that are unable to work mainly due to pain and have the capacity of getting back to their optimal activity when active extracts that are safe and have the ability to relieve pain more efficiently are discovered. In this way, they will be able to contribute to the social economic aspects in line with Kenya's vision 2030.

Most cancer cases for example where pain is amplified, diagnoses comes at an advanced stage when treatment options are limited, leading to high out-of-pocket spending, poor prognosis, and high fatality rates. Households not covered by health insurance are among the most vulnerable to health shocks, often forced to adopt coping strategies like borrowing or selling assets with potentially long-term ramifications on livelihoods (Lehmann *et al*., 2020). This research was able to address the issue of reducing health problems making most individuals self-reliant and therefore more productive. Secondary metabolites from various sources like plants, microorganisms including bacteria, actinobacteria, and fungi and its classification, production and applications in various fields (Durairaj *et al*., 2018). Since there is a constant and crucial requirement for new pharmaceutical agents to fight cancers, cardiac disorders, pests, cytotoxicity, mosquitoes, infectious diseases, and autoimmune disorders of both animals and plants as climate changes provide conditions favorable for repeated outbreaks of these diseases. New sources of bioactive secondary metabolites with novel activities must be found, if the scientific community is to put constant importance in the never ending effort to fight complications of illness. Secondary metabolites are one of the essential means of growth and defense, and are readily available for discovery (Atanasov *et al.*, 2015).

CHAPTER TWO
LITERATURE REVIEW

2.1 Ethnobotany of *Withania somnifera*

Medicinal plants offer a real substitute in developing countries for the treatment of human and animal ailments. Plant-based traditional medicine plays a key role in the development of novelties in drug discovery (Mattia, 2009). Ethnomedicine is often the single easily reached and affordable therapy available. The world market for herbal medicines based on traditional knowledge is now estimated at US$ 60 billion (Jahr *et al.,* 2013).

Plants are excellent source of secondary metabolites such as phenolics, flavonoids, alkaloids, lignans, and terpenoids. Secondary metabolites have been extensively used since ancient times and are still very popular in the treatment of various diseases and disorders (Karakaya *et al*., 2019). *Withania somnifera* is a very important plant in Ayurvedic and indigenous medical systems. It is one of the prominent medicinal plants in Indian systems of medicine, used against myriad of clinical conditions. The plant contains a range of different classes of chemical constituents such as alkaloids, steroidal lactones, and flavonoids the chemical moieties of which are responsible for various biological activities of the plant (Sheila *et al.,* 2017). It stimulates the immune system and is believed to improve memory The herb has been used in combination with others or singly, either for curative purposes or prophylactic.

A study carried out in Pakistan on the knowledge of *W. somnifera* where 55 informants were interviewed regarding detailed ethnomedicinal and sociocultural information. The study revealed that there were more than 67 medicinal plant species used to prepare 110 recipes of drugs. The major modes of herbal formulation were decoction and powdering of about 20% of each species. Three of the most active plant species determined in the study for test formulations were *Acacia modesta (*Wall), *Caralluma tuberculata (*R.Br) and *Withania somnifera* (L.) (Mattia, 2009). The plant has proved to have many immunologial advantages including antiinflammatory, antitumor, neuroprotective, antimicrobial, antistress, antidiabetic, and cardioprotective properties which are in part due to the capability of *W. somnifera* to reduce reactive oxygen species. *Withania somnifera* has been found to modulate mitochondrial function, regulate apoptosis, reduce inflammation, and enhance endothelial function due to its anti-migratory, anti-invasive and anti-metastatic effects. It inhibits growth of cultured and xenografted human breast cancer cells and prevents breast cancer development and pulmonary metastasis incidence in a transgenic mouse model (Lee *et al* .,

2015). Additionally, it has been used singly or in combination against various diseases of humans. It has also been administered as a herbal drug against Alzheimer's disease, as antiarthritic, antibacterial, anticancer, cardioprotective, stroke, hypoxia and Parkinson's disease (Nurul & Nurhuda, 2022).

2.2 The Biology of Withania somnifera

Figure 2.1: *W. somnifera*

Withania somnifera is an erect, branching, tomentose shrub, 30-15. The leaves are simple, ovate, glabrous, and up to ten centimeters long. The flowers are greenish or lurid yellow, small about one- centimeter long; few flowers (usually about five) born together in axillary, umbellate cymes (short axillary clusters). The fruits are globose berries, six millimeters in diameter, orange- red when mature, enclosed in the inflated and membranous persistent calyx (Plate 2.1).

Some of embryological features of *W. somnifera* should be of value for comparative study of related species and their phylogenetic relationships within the family. Solanaceae is mainly subdivided into three subfamilies; Cestroideae and Solanoideae, with Nicotianoideae between Cestroideae and Solanoideae. *Withania* was placed in Solanoideae, closely related to Mellissia, Aureliana and Athenaea based on chloroplast DNA, and these were all grouped in

subtribe Withaninae. Morphologically, stylarheteromorphism has been observed in *Athenaea, Aureliana* and *Withania*, supporting the molecular evidence. Dioecy has also been noted in some *Withania* species but *W. somnifera* has exclusively bisexual flowers.

It has been proven that seedlings, embryos, cotyledon, epicotyl, hypocotyl, petiole, leaves, nodes, internodes, stem, shoot tips and roots have been used in different experiments for callus induction, adventitious root induction, regeneration, differentiation, flower induction, and fruit setting (Pandey *et al.*,2017). Study of the plant bioloy in vitro has been carried out successively. In the study, composition of gelling matrix was standardized so as to develop shoot tips of *W. somnifera* and also yo realize optimization of media composition (or soilrite) for conversion of encapsulated shoot tips into plantlets. Most studies with optimized *in vitro* tissue culture conditions of *W. somnifera* have been briefly summarized recently (Singh *et al.*, 2017).

2.3 Phytochemistry of *W. somnifera*

The pharmacological effects of the root extracts of *W. somnifera* has been associated with the presence of withanolides, a group of steroidal lactones (Budhiraja & Sudhir, 1987). Its leaves are used in Ayurvedic and Unani systems for treatment of tumors and tubercular glands (Chopra, 1994). These withanolides have been isolated from the leaves of *W. somnifera* (Godfrey *et al.*, 1973), and have provefed to exhibit antibacterial, anti-fungal and antitumor activities (Devi *et al.*,1993). Advancanced studirs have given reports elucidating the chemical and pharmacological properties of *W. somnifera* (Nittala *et al.*, 1988). Laboratory analysis from those reports has revealed over 35 chemical constituents contained in the roots of *W. somnifera* (Rastogi & Mehrotra, 1998). Those biologically active chemical constituents include alkaloids steroidal lactones, saponins and withanoloides. Other benefits include the fact that *W. somnifera* is also rich in iron and so its extracts help in energy metabolism (Kulkarni & Dhir, 2008).

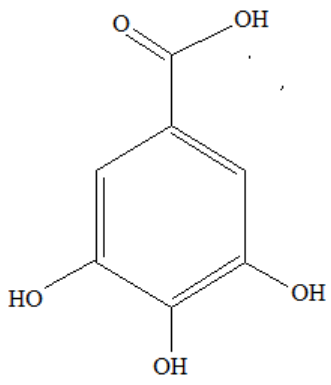

Figure 2.1: General structure of Flavonoids

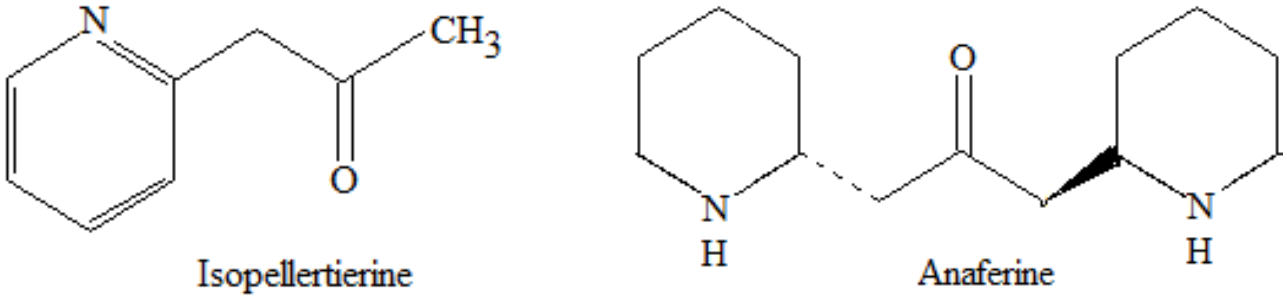

Figure 2.2: Sructures of some common alkaloids

Metabolic insight of *W. somnifera* has proved to be of significance in therapeutic world with studies showing this to be due to maximum accumulation and presence of diversified forms of withanolide. All the identified variants of withanolides became interesting for researchers due to their beneficial effects for human body (Kumar *et al.,* 2007). These analyses reveal numbers of tissue specific unique sequences, differentially expressed genes related to biosynthesis of secondary metabolites.

The roots of *W. somnifera* consist primarily of compounds known as withanolides, which are believed to account for its extraordinary medicinal properties (Dar *et al.,* 2015). Withanolides are steroidal and bear a resemblance, both in their action and appearance, to the active constituents of Asian ginseng known as ginsenosides (Chaurasiya *et al.,* 2012). Ashwagandha's withanolides have been researched in a variety of animal studies examining their effect on numerous conditions, including immune function (Mirjalili *et al.,* 2009). Chemical analysis of Ashwagandha shows its main constituents to be alkaloids and steroidal lactones. Among the various alkaloids. Withanine is the main constituent (Uddin *et al.,* 2012). The other alkaloids are somniferine, somnine, somniferinine, withananine, pseudo-withanine, tropine, pseudo-tropine, 3-a-gloyloxytropane, choline, cuscohygrine, isopelletierine, anaferine and anahydrine. Two acyl sterylglucosides viz. sitoindoside VII and sitoindoside VIII have been isolated from root. The leaves contain steroidal lactones, which are commonly called withanolides (Mirjalili *et al.,* 2009). The withanolides have C28 steroidal nucleus with a C9 side chain, with a six membered lactone ring. Twelve alkaloids, 35 withanolides, and several sitoindosides from *W. somnifera* have been isolated and studied

(Singh *et al.,* 2010). A sitoindoside is a withanolide containing a glucose molecule at carbon. Much of Ashwaganda's pharmacological activity has been attributed to two main withanolides, withaferin A and withanolide D (Rastogi & Mehrotra, 1998).

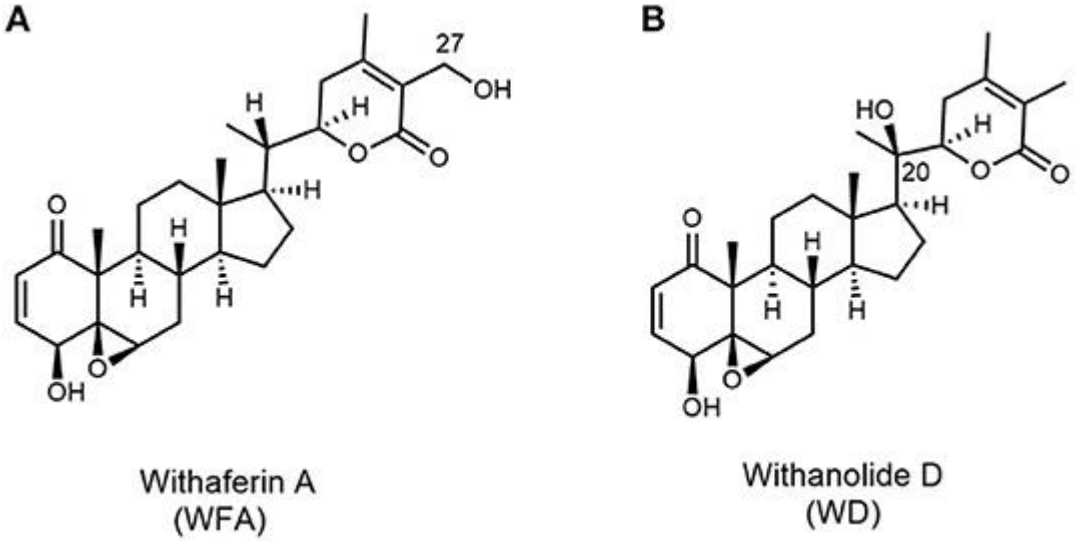

Figure 2.3: Structure of Withafrin A (A) and withanolide D (B)

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2.4 Biological activity of *Withania somnifera*

The dried roots of the plant are used in the treatment of nervous and sexual disorders. Fromchemistry point of view, the biologically active withanolides that have been studied are widely distributed in family Solanacae. Withaferin A for example is therapeutically active withanolide reported to be present in leaves. In animal studies, this secondary metabolite (withaferin A) has shown significant anticancer activity. This ability to cure is associated with withaferin A anti-migratory, anti-invasive and anti-metastatic effects. It also inhibits growth of cultured and xenografted human breast cancer cells and prevents breast cancer development and pulmonary metastasis incidence in a transgenic mouse model (Lee *at al*., 2015). Withaferin A alone and in combination with cisplatin suppresses growth and metastasis of ovarian cancer by targeting putative cancer stem cells (Kaileh *et al*., 2007).

Majority of the anticancer drugs like Vinblastine, Vincristine, and Taxol have been derived from green flora (Singh *et al.,* 2010). Examples include Geniposide derived from *Gardenia jasminoides* and Gardenia Ellis, which are members of family Rubiaceae that treats human non–smal cell lung cancer cells at a dose of IC_{50} of genipin 351.5 Mm. Colchicine from *Colchicum* genus is used in treatment of hepatocellular carcinoma at a dose rate of 10 μM. Artesunate from *Artemisia annua* , a medicinal plant in the family Asteraceae is used for treatment of chronic myeloid leukaemia at a dose rate of 2 μM (Gwozdzinski, 2018).

In research carried out on adult male Wistar rats, a mild electric shock was induced to their feet. This mild stress procedure in the experimental rats resulted in unpredictable footshock, when administered once daily for 21 days. The resulting stress on the rats produced hyperglycemia, glucose intolerance, and increase in plasma corticosterone levels, gastric ulcerations, male sexual dysfunction, cognitive deficits, immunosuppression and mental depression (Bhattacharya & Muruganandam, 2003). For the set of male Wistar rats to whish had been administered *Withania somnifera* an hour before the foot shock the experiment recorded a significantly reduced level of stress thus confirming the theory that Ashwagandha has a significant anti-stress adaptogenic effect. The chronic stress induced significant hyperglycaemia, glucose intolerance, increase in plasma corticosterone levels, gastric ulcerations, male sexual dysfunction, cognitive deficits, immunosuppression and mental depression. These chronic stress induced perturbations were attenuated by *W. somnifera* (25 and 50 mg/kg po) and by Panax ginseng (PG (100 mg/kg po), administered 1 hour before footshock for 21 days.

Panax ginseng is a plant that grows in Korea, China, and Siberia. It's considered an adaptogen, which are natural substances that are believed to stimulate the body's resistance to stressors. Panax ginseng contains many active chemicals. The mass of extract was expressed in mg per kg of the extract for every pound body weight of the animal. Further studies have also shown ashwagandha to be effective in the treatment of osteoarthritis, inflammation and stroke (Chaudhary *et al.,* 2003) and tardive dyskinesia (Bhattacharya *et al.,* 2002). Ashwagandha has been shown to be a potential antimicrobial agent, with antifungal activity against *Fusarium oxysporum f. sp. radicis-lycopersici* (Ahlem *et al.,* 2016) and moderate antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacterial strains (Ali *et al.,* 2001)

2.5 Physiological role of serotonin

The conversion of tryptophan into serotonin is presented in figure below.

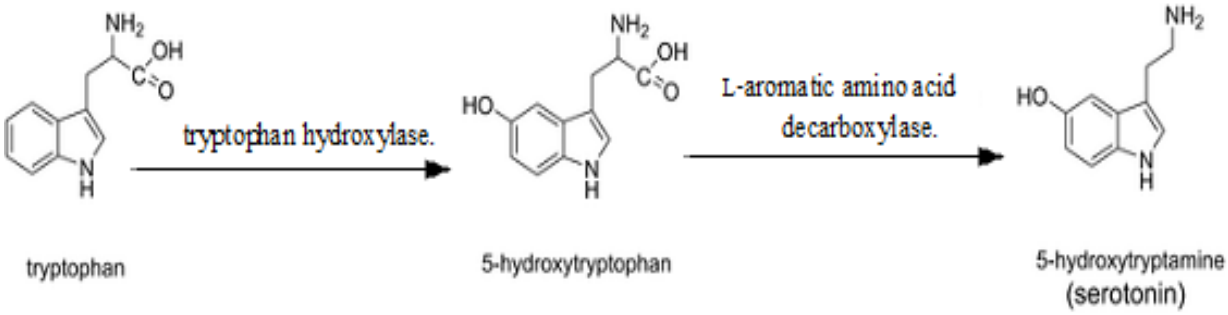


Figure 2.4: Conversion of tryptophan into serotonin

High levels of plasma serotonin have been observed to be in direct proportionality with increase in pain (Matsubara *et al.,* 1991). Although *W. somnifera* extracts have been found to reduce pain, the effect of the plant on the serotonin levels in blood has not yet been established. Reduction of plasma serotonin levels was taken as an indicator to reduction in pain up to the required standards. Substances that reduce pain were expected to reduce serotonin levels to the required amounts for positive physiological processes or at least cause reduction in serotonin levels as a course for pain reduction.

Medicinal plants are a rich source of bioactive secondary metabolites that have a wide range of medicinal uses. This is the reason why, currently, 90% of drugs come from natural or semisynthetic origins (Abdur *et al*., 2017). A research finding by Gina (2009) observes that some of the plants have known pain-relieving properties. Examples include Chamomile where Aromatherapists use chamomile essential oil to promote relaxation and pain relief. Serotonin, 5'-hydroxytryptamine (5'-HT) is an ubiquitary monoamine acting as one of the neurotransmitters at synapses of nerve cells. It has a similar chemical structure with tryptamine, dimethyltryptamine, diethytryptamine, melatonin and bufothein, all belonging to the group of indolalkylamins. It is found in nerve endings, serotonin bodies of neurons, enterochromafinne stomach cells and platelets. Its biosynthesis begins with hydroxylation of L-tryptophan. L-tryptophan is transported through the blood-brain barrier into the brain using the neutral amino acids transmitter, on which competes with other amino acids; phenylalanine, leucine and methionine. Tryptophan-hydroxylase is the first step and speed limiting factor of 5'-HT synthesis. This enzyme was found in the brain only in the serotoninergic neurons. It enables conversion of tryptophan into 5-hydroxytryptophan,

followed by the decarboxylation mediated by aromatic L-amino acid decarboxylase onto 5-hydroxytryptamine (serotonin).

Serotonin receptors are distributed throughout the CNS, with different distribution patterns for the different receptor types. Most 5-HT postsynaptic receptors are located on the subsequent neurons. The release of 5-HT is regulated by presynaptic 5-HT receptors that are located either at the soma or at the nerve endings of the serotonergic neurons (Hoyer *et al*., 2002). To come to this conclusion, studies with knockout gene for this subtype of 5'-HT1 receptor in mice was carried out. The antianxiety actions of 5'-HT1A (partial) agonists was thought to provide primarily presynaptic somatodendritic 5'-HT1A receptors that lead to reduced release of 5'-HT in terminal areas, whereas the antidepressant action of 5'-HT1A agents may have primarily provided postsynaptic 5'-HT1A receptors (De Vry, 1995). Certain 5'-HT1A agents display anti aggressive behavior, and measurement of the density of 5'-HT1A receptors in frontal cortex of suicide victims reveals that nonviolent suicide victims had a significantly higher serotonin activity, compared with controls and violent suicides (Matsubara *et al.,* 1991). Populations of 5'-HT binding sites were identified in rat brain: 5'-HT1 and 5'-HT2 sites. By the late 1950s, evidence for 5'-HT receptor heterogeneity was found in the periphery and in 1979, two distinct receptors were identified (Peroutka, 1987). In the last 20 years, seven distinct families of 5'-HT receptors have been identified.

Serotonin is synthesized in animals from the amino acid, L-tryptophan. In humans, serotonin biosynthesis from tryptophan occurs in enterochromaffin cells in the digestive tract and neurons in the central nervous system. Tryptophan is an essential amino acid that must be supplied from the diet. The quantity of tryptophan ingested daily is about 0.5–1 g, and a small part (<2%) is converted into serotonin. Daily production in humans is about 10 mg of serotonin (Dawn *et al*., 2009). Tryptophan is absorbed from the gut and enters the bloodstream where it circulates as both free and albumin-bound forms. Tryptophan is first converted to 5-hydroxytryptophan in a reaction catalyzed by tryptophan hydroxylase, which is the rate-limiting enzyme of the biosynthetic pathway. This enzyme requires tetrahydrobiopterine, oxygen, NADPH and a metal such as copper or iron for its activity. 5-Hydroxytryptophan is then converted to serotonin by L-aromatic amino acid decarboxylase, which is much more active than tryptophan hydroxylase (Villalobos & Zeng, 2018).

The 5'-HT1A receptor is the most extensively distributed of all the 5'-HT receptors in the central nervous system. It is present in high density in the cerebral cortex, hippocampus, septum, amygdala, and raphe nucleus. They were found in small amounts in the basal ganglia and thalamus as well (Mestikawy *et al.*, 1993). In the brain, 5'-HT1A receptors act as autoreceptors as well as postsynaptic receptors, involved in the inhibition of "discharge" of neurons, regulation of the production of ACTH (but not prolactin), and regulation of behavior and eating. When serotonin is bound to these specific receptors, it plays an important role in the emergence of anxiety. A large number of 5-HTR subtypes are distributed widely throughout the brain and CNS. Thus, serotonergic transmission is believed to mediate a variety of physiological functions including temperature regulation, mood, anxiety, emesis, sleep, appetite, blood pressure, and the perception of pain (Wang *et al.*, 2009).

2.6 Role of serotonin in pain modulation

Serotonin is widely distributed both at the periphery and in the central nervous system and its transport from the periphery to the CNS is prevented by the blood–brain barrier. There are actually two distinct compartments, quantitatively very unbalanced, where 5-HT can exert various effects on pain signaling mechanisms (Viguier *et al.*, 2013).

The dorsal horn of the spinal cord and the midbrain, are anatomically and functionally interconnected by a spino-bulbo-spinal loop. From the nucleus raphe magnus, the nucleus paragigantocellularis and the ventral portion of the nucleus gigantocellularis in the rostroventral medulla, serotoninergic neurons project specifically into the superficial laminae of the dorsal horn of the spinal cord where primary afferent fibers convey nociceptive signals to second order (mainly spino-thalamic) neurons (Basbaum & Kwiat, 1992).

Serotonin is thus considered a major component of the inflammatory chemical milieu and contributes to the pain of tissue injury *via* an action on multiple receptor subtypes. Specifically, in the setting of tissue injury, the 5'-HT3 receptor mediates activation of nociceptors but does not contribute to injury-associated edema, due to localization of 5'-HT3 receptor transcripts to a subset of myelinated and unmyelinated afferents, few of which express the pro inflammatory neuropeptide substance P.(a specific protein expressed during inflammation) (Grailhe *et al.*, 2001).

Central serotonergic circuits modulate nociceptive transmission *via* a facilitatory action at spinal 5'-HT3 receptors. As such, it is clear that activation of both peripheral and central 5'-

HT3 receptors is pro nociceptive and that the contribution of peripheral 5'-HT3 receptors involves a novel complement of primary afferent nociceptors. Acute pain results from direct thermal, mechanical, or chemical activation of particular subsets of primary afferent neurons (nociceptors). Serotonin production from CNS occurs primarily in the raphe nucleus, a group of serotonergic neurons located in the posterior region of the ponsSerotonin is related to thermal, mechanical, or chemical activation of particular subsets of primary afferent neurons. The monoamines and 5-HT are the neurotransmitters chiefly implicated in the descending pain control modulatory pathway. Descending noradrenergic projections terminating in the dorsal horn of the spinal cord derive almost entirely from nuclei within the dorsolateral pontine tegmentum (Anthony & Kirsty, 2017). The persistent component of the pain response, in contrast, is associated with the production and release of multiple inflammatory factors, including neurotransmitters, eicosanoids, and protons.

Serotonin, a major neurotransmitter component of the inflammatory chemical milieu, may be released from platelets, mast cells, or basophils that infiltrate an area of tissue damage. Once released, serotonin is free to interact with a number of molecularly distinct receptor subtypes expressed by primary afferent nociceptors, including the 5'-HT3 receptor (5'-HT3R. Unlike all other known serotonin receptor subtypes, which are G-protein-coupled, the 5'-HT3R is a member of the nicotinic acetylcholine superfamily of excitatory ligand-gated ion channels. Functional homopentameric serotonin-gated channels are formed when a single cDNA encoding the 5'-HT3R-A subunit is expressed in heterologous systems. The recent identification of a second subunit gene, 5'-HT3R-B provides molecular evidence that some native 5'-HT3Rs may exist as heteromeric complexes. However, because 5'-HT3R-B subunits cannot form functional ion channels on their own, the 5'-HT3R-A subunit is believed to constitute an essential component of all serotonin-gated ion channels. Importantly, peripheral injection of serotonin evokes acute pain that is attenuated by relatively selective 5'-HT3R antagonists Some 5'-HT3R antagonists show unusual, nonlinear dose–response relationships or exhibit actions at other serotonin receptor subtypes that are found in primary afferent nociceptors (most notably, blockage of 5'-HT4R by ICS 205–930) (Wacker *et al.,* 2017). At the cellular level, this may be explained by our observation that 5'-HT3R-As are not expressed in the substance P (SP)-containing afferents that are necessary for the plasma extravasation component of neurogenic inflammation (Bardoni, 2019).

5’-HT1B receptors are present in the CNS, where they induce presynaptic inhibition and behavioral effects. However, they exhibit vascular effects as well, such as pulmonary vasoconstriction. They are present in many parts of the human brain, with the highest concentrations in the basal ganglia, striatum and the frontal cortex. The function of the receptor depends on its location. In the frontal cortex, it is believed to act as a terminal receptor inhibiting the release of dopamine. In the striatum and the basal ganglia, the 5’-HT1B receptor is thought to act as an auto-receptor, inhibiting the release of serotonin. Secondary role of 5’-HT1B receptors is to serve as controlling terminal hetero-receptors of secretion of other neurotransmitters such as acetylcholine, glutamate, dopamine, norepinephrine and γ-aminobutyric acid. In addition to the brain, this subtype was also found in cerebral and other arteries (Jin *et al.,* 1992).

Expression of 5’-HT1D is very low compared to the 5’-HT1B receptors and both receptors exhibit 63 % structural homology. 5’-HT1D receptors act as auto-receptors in the dorsal raphe nuclei, but were also found in the heart where they modulate the release of serotonin. In the central nervous system, 5’-HT1D receptors are involved in locomotion and anxiety. They induce also the vascular vasoconstriction in the brain. The 5’-HT1F receptor exhibits intermediate trans-membrane homology with several other 5’-HT1 receptors: 5’-HT1E (70 %), 5’-HT1Dα (63 %), 5’-HT1Dβ (60 %), 5’-HT1A (53 %). Despite similarities to 5’-HT1E receptors, 5’-HT1F receptors bind 5-methoxytryptamine and certain ergotamine derivatives with high affinity. The cloned human 5’-HT1F receptor couples to inhibition of adenylyl cyclase. Agonist effects of 5’-HT were antagonized completely and apparently competitively by the nonselective 5’-HT antagonist methiothepin (Adham *et al.,* 1993).

5’-HT2 receptors have three subtypes – 5’-HT2A, 5’-HT2B and 5’-HT2C, showing 46-50 % structural homology, preferably linked to Gq11 protein and increasing inositol triphosphate hydrolysis and intracellular Ca^{2+} concentration. This is the main excitatory receptor subtype among the G-protein coupled receptors for serotonin (5’-HT), although 5’-HT2A may also have an inhibitory effect on certain areas such as the visual cortex and the orbitofrontal cortex (Janssen *et al.,* 2002). 5’-HT2A receptor is expressed in many central and peripheral tissues mediating the contraction of smooth muscles. Furthermore, increased platelet aggregation and increased capillary permeability following exposure to serotonin (probably due to activation of this receptor subtype) were described (Cook *et al.,* 1994).

When a receptor is linked to neuron neurons of both, central and peripheral nervous system, it gets directly or indirectly related to pain. Activation of 5'-HT2B receptor leads to contraction of smooth muscle of stomach fundus. 5'-HT2B imunoreactivity was detected in the cerebellum, lateral septum, hypothalamus and medial part of the amygdala (Cox & Cohen, 1995). A 5'-HT2C antagonist agomelatine functions as an effective antidepressant due to its antagonism of 5'-HT2C receptors, thus causing a rise in dopamine and norepinephrine levels in certain areas of the brain (Goodwin *et al.,* 2009). 5'-HT3 receptors consist of 5 subunits arranged around a central ion conducting pore, which is permeable to sodium, potassium, and calcium ions. The binding of the neurotransmitter serotonin to the 5'-HT3 receptor opens the channel, which in turn leads to an excitatory in neurons. 5'-HT3 receptors are found on neurons of both, central and peripheral origin. Rodents have been shown to possess two functional 5'-HT5 receptor subtypes, 5'-HT5A and 5'-HT5B. However, the gene coding the 5'-HT5B subtype in humans includes stop codons making it non-functional in what results in a solitary expression of only 5'-HT5A subtype in human brain.

2.7 Role of serotonin in bone development and disorders

Bone formation is complex but the three-dimensional positioning of cells and matrices is straightforward. As in any discussion of bone formation, it is important to keep in mind the distinction between bone as a tissue (bone cells and the mineralized matrix) and bone as an organ (including several tissues such as bone, cartilage, fibrous tissue, marrow and blood vessels.

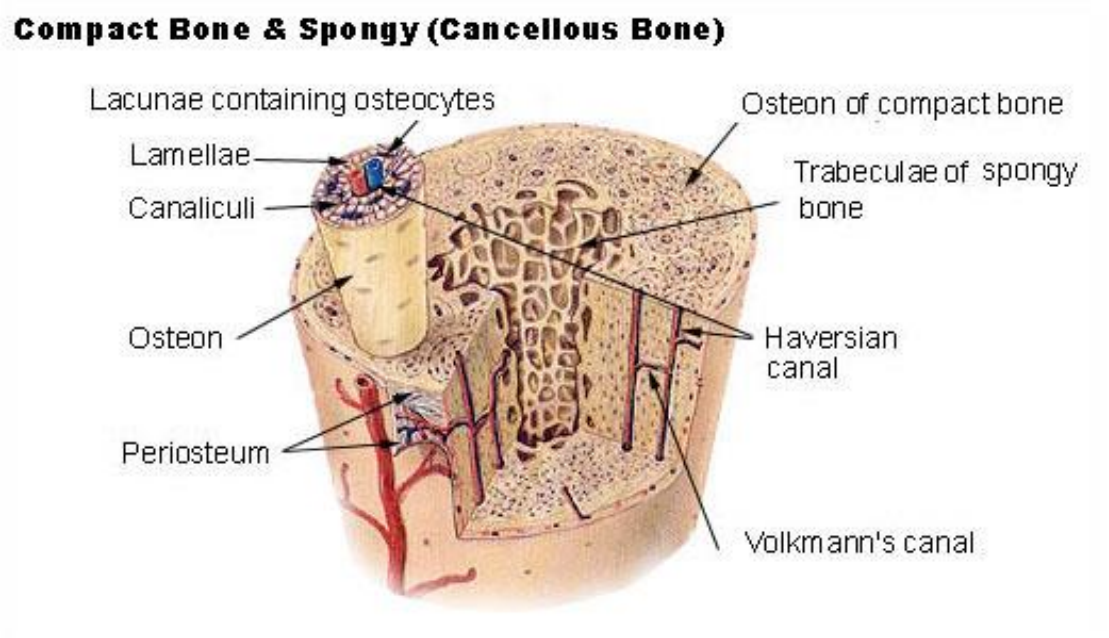


Plate 2.1: Fine structure of bone tissue showing different components

Source: U.S. National Cancer Institute's Surveillance, Epidemiology and End Results (SEER)
Normal bone develops using only 2 mechanisms:Intramembranous bone formation is mediated by the inner periosteal osteogenic layer with bone synthesized initially without the mediation of a cartilage phase.Endochondral bone formation describes the synthesis of bone on a mineralized cartilage scaffold after epiphyseal and physeal cartilage have shaped and elongated the developing organ. These mechanisms are also used in fracture and osteotomy repair with the specific mechanism dependent on the mechanical environment provided during repair. With intramembranous bone repair, mesenchymal cells differentiate along a preosteoblast to osteoblast line while endochondral bone repair is characterized by the initial synthesis of cartilage followed by the endochondral sequence of bone formation. The terms intramembranous and endochondral refer to the tissue being replaced, not to the eventual bone synthesized, which is the same in both mechanisms (Chinsamy & Hurum, 2006).

Pain alleviating properties of *Withania somnifera* extracts may be by mondulating serotonin levels that has never been established. According to research *Withania somnifera* extracts have proved to be an antidote to bone disorders. Serotonin secretion has been found to be intense during pain due to bone function impairment and therefore the need to establish the effect of the plant extracts on serotonin levels (Jinu, 2014).

Pain is the most prominent symptom in most people with bone disorders such as arthritis and is the most important determinant of disability in patients with osteoarthritis (Baar *et al.,* 1998). Self-reported persistent pain related to the musculoskeletal system has been used in a number of population-based surveys to assess the prevalence of musculoskeletal conditions (Kazis *et al.,* 1983). It affects up to 20% of adults and cause more functional limitations in the adult population in most welfare states than any other group of disorders. They are a major cause of years lived with disability in all continents and economies. In the Ontario Health Survey, for example, musculoskeletal conditions caused 40% of all chronic conditions, 54% of all long-term disability and 24% of all restricted activity days (Badley *et al.,* 1994).

In surveys carried out in Canada, the USA, and Western Europe, the prevalence of physical disabilities caused by a musculoskeletal condition repeatedly has been estimated at 4–5% of the adult population. The prevalence is higher among women and increases markedly with age. Musculoskeletal conditions are the main cause of disability among older age groups. Moreover, the pain and physical disability brought about by musculoskeletal conditions affect social functioning and mental health, further diminishing the patient's quality of life (Torgbenu *et al.,* 2017). On the other hand, *W. somnifera* is a pain reliever and may be related to serotonin hormone secretion and or serotonin receptors regulation. However, no drugs have been synthesized from *W. somnifera* nor has any relationship between its extracts and serotonin secretion been established. In this regard, this study is aimed at investigating the relationship if any between the two and subsequent synthesis of pain relieving, affordable and readily available natural drugs.

2.8 Role of serotonin in inflammations

Among pro-inflammatory mediators at the periphery such as prostaglandins, histamine, bradykinin and lactic acid, serotonin is one of the active components of the "inflammatory soup" which contributes to injury induced pain (inflammation) (Hamon & Bourgoin, 1999). Serotonin is a neurotransmitter and hormone that contributes to the regulation of various physiological functions by its actions in the central nervous system (CNS) and in the respective organ systems. Peripheral 5-HT is predominantly produced by enterochromaffin (EC) cells of the gastrointestinal (GI) tract. These gut-resident cells produce much more 5-HT than all neuronal and other sources combined, establishing EC cells as the main source of this biogenic amine in the human body. Peripheral 5-HT is also a potent immune modulator and affects various immune cells through its receptors and *via* the recently

identified process of serotonylation. Alterations in 5-HT signalling have been described in inflammatory conditions of the gut, such as inflammatory bowel disease. The association between 5-HT and inflammation, however, is not limited to the gut, as changes in 5-HT levels have also been reported in patients with allergic airway inflammation and rheumatoid arthritis (Shajib & Khan, 2015).

2.9 Current trends in pain management by most commonly used convectional drugs

All over the world, drugs have been developed and used as pain antidotes. They have been administered as a convectional way to relief pain as well as the complications that come with it during tissue trauma. Although antidepressants were not originally designed to act as analgesics, they are reported to have analgesic effects for chronic pain. Antidepressants have virtually no alteration of normal physiology of neurons *i.e.* no antinociceptive effects. However, they, are considered first-line drugs of choice for neuropathic pain (Hideaki, 2017) and treatment of disorders characterized by widespread musculoskeletal pain accompanied by fatigue, sleep, memory and mood issues (fibromyalgia). Some drugs must be used in combinations to improve their efficacy. In this regard, specific antidepressants with analgesic effects include tricyclic antidepressants, (TCA) such as fluoxetine, paroxetine and citalopram. They are limited and inconsistent, yet they have a superior tolerability profile compared with tricyclic antidepressants. which have long been used, and serotonin noradrenaline reuptake inhibitors (SNRI), which are comparatively new antidepressants. Selective serotonin reuptake inhibitors (SSRI), which are frequently used to treat depression, are not effective against chronic pain (Dharmshaktu *et al.,* 2012).

Generally, the combination of NSAIDs and paracetamol provides greater analgesia than paracetamol alone for the acute pain after orthopaedic, gynaecological and dental surgery (Thybo *et al.*, 2017). Most drugs work better in combination rather than when administered alone for example when paracetamol is used in combination with NSAID, it has a greater effect on acute analgesic activity than NSAIDs alone. Actually, greater activity has been noted for combinations of paracetamol (1,000 mg) and ibuprofen (400 mg) than that produced by combinations of paracetamol (1,000 mg) or ibuprofen (400 mg) with codeine 30 mg (Daniels *et al.,* 2011).

However, a large-scale trial showed that the combination of paracetamol (3 g daily) and ibuprofen (1.2g daily) generally produced a slightly greater effect than the same dose of

paracetamol alone, but there was no significant contrast with ibuprofen alone (MacCrimmon *et al.*, 1970). The addition of paracetamol to opioids can increase efficacy and provide an 'opioid-sparing' effect. Thus, intravenous paracetamol often lowers the required opioid dosage in acute pain but the adverse effects of opioid treatment may not be decreased.

The clinical analgesic activity of single doses of paracetamol is increased to a small, but statistically significant extent, by caffeine. Selection of drug combinations also determines how effectively they alter the body physiology for example when paracetamol is used in combination with caffeine. The effect may be an increased rate of absorption of paracetamol after dosage with caffeine. In recent years, a considerable number of papers have claimed that caffeine potentiates the hepatotoxicity of paracetamol. The studies showing greater toxicity in rats have mainly been conducted at supratherapeutic concentrations (Sato & Izumi, 1989). Paracetamol enjoyed only limited use until the 1950s, when the chemically similar, and up until then preferred analgesic, phenacetin was withdrawn because of renal toxicity.

Cognitive effects of paracetamol is almost universally acknowledged as the 'non-drowsy' painkiller, and there is no literature to support claims of associated alterations in consciousness in humans. However, there are many anecdotal reports of euphoria or sleepiness (particularly in children and the elderly the groups in which metabolism may be reduced, after paracetamol, even in the absence of pain or pyrexia (Chhaya *et al*., 2014).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study site

Samples of *Withania somnifera* were collected at Chemeron in Baringo county, Kenya and the laboratory experiments conducted at Egerton University in Nakuru county, Kenya. Experimental animals *i.e* swiss albino mice were sourced from Njoro Boys High School in Nakuru county. The Climatic and edaphic characteristics of these locations are presented in table 3.1.

Table 3.1: Climatic and edaphic characteristics of experimental site

Site	GPSco-rdinates	Rainfall (mm)	Temp. (^{o}C)	Soil type	Soil pH	Altitude(M above sea level)
Chemeron	0.4695°N; 35.9833° E	635	32	sandy loam	4.5	2361
Egerton	0.369734; 35.932779	1200	17.0-22.0	Loamy	4.6-5.5	2227

The samples were partially processed in Chemeron while final processing, extraction of secondary metabolites and their treatment to mice took place at Njoro main campus Biological Sciences laboratories.

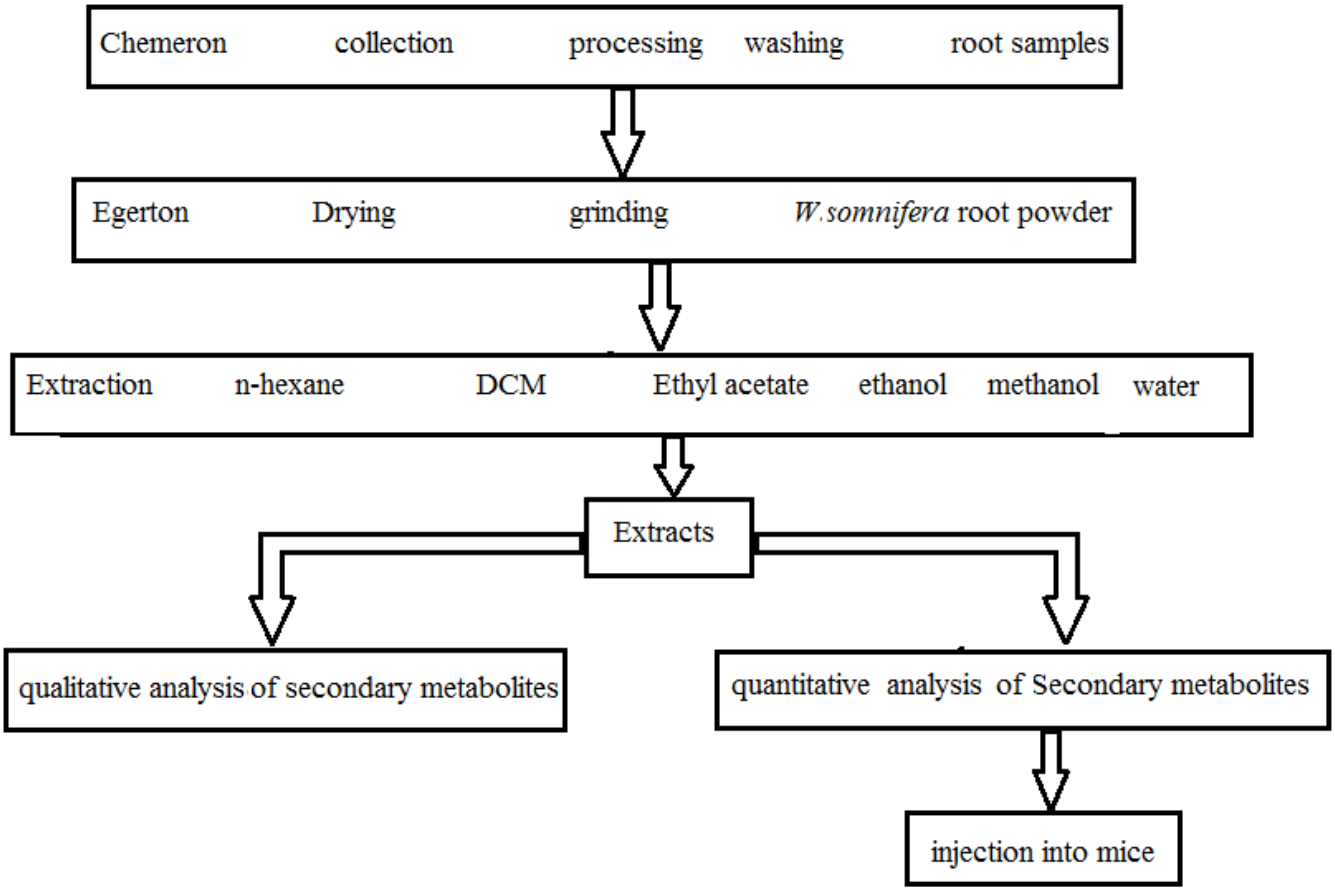


Figure 3.1: Schematic collection and processing of *Withania somnifera samples.*

Egerton University as a data collection site

The main campus of the Egerton University is located in Njoro, a small community approximately 25 kilometers (16 miles), southwest of the town of Nakuru. This is located approximately 182 kilometers (113miles), by road, northwest of Nairobi, the capital and largest city in Kenya. The coordinates of the university main campus are: 0°22' 11.0"S, 35°55' 58.0"E (Latitude:-0.369734; Longitude: 35.932779 and an altitude of 2227 merters above sea level.

Chemeron as a study site

Chemeron is about 133 km northwest of Egerton University. It is 13 km West of Marigat Town towards Kabarnet in Baringo County. It covers 450 hectares (about 1000 acres) and receives an annual rainfall of 635mm annually. The altitude of the station is 1200 m above sea level. The soil is reddish-brown, sandy loam with many stones on the surface. Chemeron River flows seasonally and traverses the region. The ground has a gentle slope in some areas

but is generally rough towards the river. Vegetation is mainly dense-bushed grassland dominated by *Acacias (A. reficiens, A. mellifera, A. tortilis* and *A. coriaceae)* and Capparaceae family comonly *Boscia angustifolia*. The sandy river valleys and banks are characterized by *Balanites aegyptiaca, Grewia bicolour* and *Tamarindus indica*. The main grasses are *Cenchrus ciliaris, Enteropogon macrostachyus, Chloris roxburghiana* and *Eragrostis superba* (Ambin *et al*., 2003).

3.2 Sample Collection

The plant species was identified by the late Professor Samwel Kavuitu Kariuki, the University cheyf taxonomist that time. God rest his soul in peace. *Withania somnifera* root samples were collected from Baringo County, around Chemeron Campus, Egerton University around noon when physiological processes of each plant was expected to be optimal. 50 healthy, mature *Withania somnifera* plants were uprooted through digging the surrounding earth to reach the tip of the taproots. Secateurs were then used to chop the plant borders and separate roots from stems. Stems and leaves were then packed in plastic bags separately for drying at Egerton university in the shade and stored for future reference in laboratory BN5. A twig carrying flowers and fruits was pressed dried and mounted as a herbarium specimen in laboratory B8 The root samples were packed in plastic bags. All the processed materials were then transported to Egerton University laboratories where they were spread in the shade to dry. After drying the root samples were gounded into powder ready for subsequent extraction with solvents. The steps followed were as described in figure 3.1.

3.3 Extraction process

In cold extraction procedures, the vapour pressure points were considered and temperatures set for different solvents 2-5^{o}C above the individual solvents boiling point. The hot extraction process however was carried out at 100^{0}C. The secondary metabolites extraction was done at room and at high temperatures. At room temperaturesm the extracts were later concentrated using the vacuum evaporator as described in section 3.3.1 below, each time setting temperatures of the extracting solvents slightly higher than the actual boiling point of each solvent. The two extraction procedures were used in order to conclusively get the best output in the optimum conditions. This is because the solubility of a solute in a particular solvent may vary with variation in temperatures. Some metabolites require high kinetic energy to dissolve in polar solutions while others may need low kinetic energy to dissolve in the same solvent.

3.3.1 Cold extraction of plant components

To the Winchester bottle was put 500 g of ground plant material which was then soaked in 2 L of methanol for 24 hours. The extract was filtered and the Winchester bottle containing the residue refilled with fresh 2 L of distilled methanol for further 24 hours until methanol color changed no more**.** This Extraction was carried out working at room temperatures to the conclusive point of serial extraction each time using 2000mls of the solvent. Solvent recovery and thus concentration of secondary metabolites was carried out using the vaccum evaporator where temperatures of individual solvents was set each 2-5^{0}C above the boiling point of the solvent. n-hexane (68.5^o) =73^{0}C, Dichloromethane(39.6^o) =43^{0}C, Ethyl acetate(77.1^o) =80^{0}c ethanol (78.4^o) =82^{0}C methanol (64.7$^{o)}$ =67^{0}C and distilled water 100^{0}C (Bhan 2017) For hot extraction, recovery of solvents was carried out at 100^{0}C.

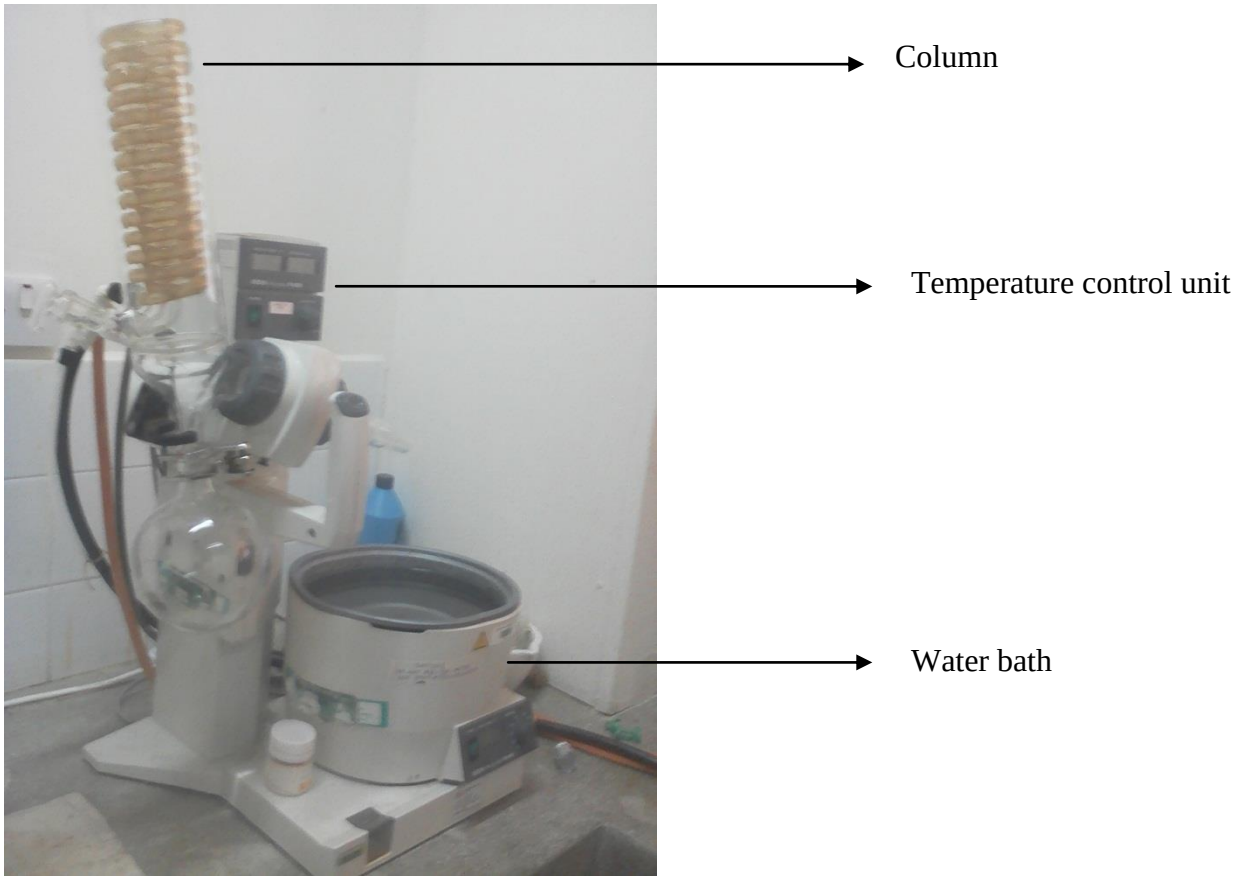


Plate 3.1: A photograph of vacuum evaporator shot inside CL9, Chemistry laboratories, Egerton University.

3.3.2 Hot extraction of plant components

All the laboratory work was carried out at Egerton University, Njoro. The samples were carried to Botany laboratories where they were dried in the shade for crude extracts using organic solvents from Chemistry laboratories. Serial extraction was carried out in order of polarity of the defined solvents and thus the extracts followed forward extraction.

Soxhlet/hot extraction

Of the root fraction of *W. somnifera* ground material,500g was grounded and placed in a Winchester bottle to which was added 1.5 liters of distilled water.. After observing that there was no colour change with distilled water, the material was dried again in the shade to evaporate water and then Distilled methanol ntroduced and the procedure for water repeated to conclusive extraction. The other four solvents extraction were also repeated the same way, ethanol, ethyl acetate, dichloromethane and finally hexane (Rastogi & Mehrotra, 1998). The residue that remained in the round bottomed flask was treated as aqueous crude extract, methanol crude extract and other solvents used crude extracts, the source of evaporation being Soxhlet apparatus. This dry powdered plant roots were successively and exhaustively extracted with hexane, dichloromethane, ethyl acetate and methanol (1.5 L each time) for twenty four hours each. The solvents were evaporated to concentrate and determine masses of crude extracts containg the secondary metabolites contained in 500g of the powder.

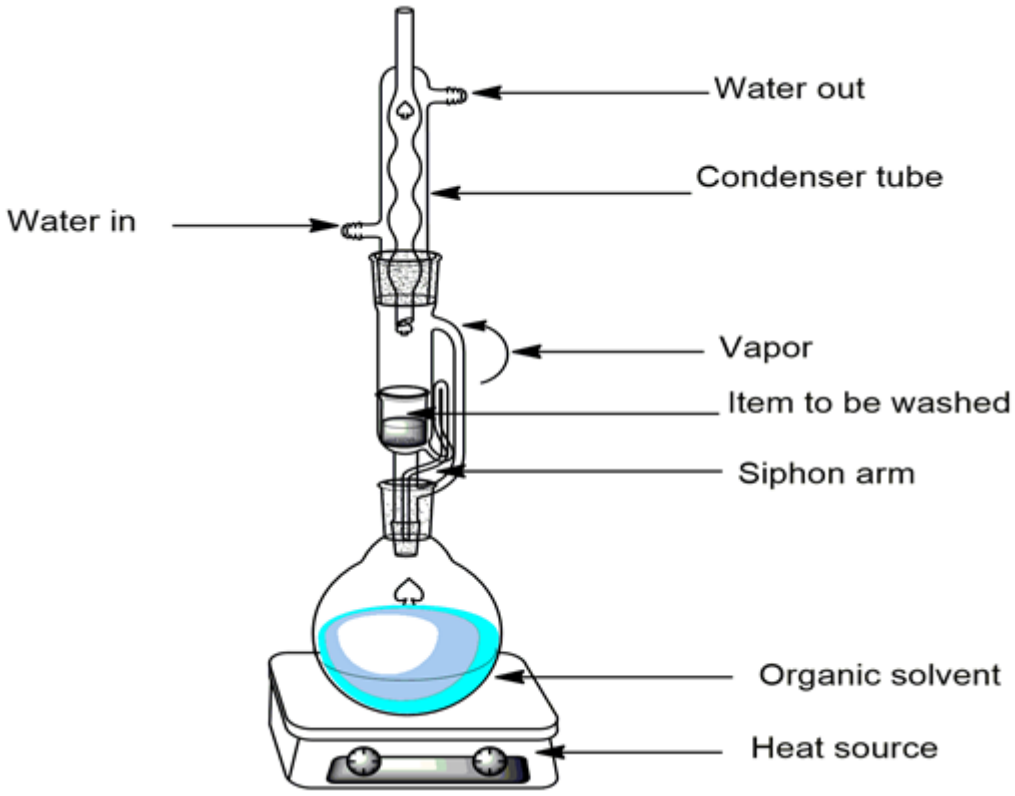


Plate 3.2: Soxhlet apparatus

A photo taken in the lab. B8 of Biological Sciences Department, Egerton University main campus during experimentation.

3.3.3 Preparation for testing for secondary metabolites

0.3731 g each, of Hexane and 0.3721g of dichloromethane crude extracts were each dissolved in 1 mL distilled water and 3 drops of Dimethyl sulfoxide (DMSO) was added to facilitate dissolution. Each sample was then tested for both presence and concentration of certain secondary metabolites.

0.3730 g of ethyl acetate 0.3705 g of methanol, 0.3695g of ethanol and 0.3729g of aquauos crude extracts were each dissolved in 1 mL distilled water and tested for both presence and concentration of certain secondary metabolites**.**

3.4 Proximate analysis of secondary metabolites

3.4.1 Alkaloids content

Withania somnifera root extracts prepared in section 3.3.3 were used for both qualitative and quantitative analysis of secondary metabolites (Madhu *et al*., 2016). To test for presence of alkaloids, 0.5 cm^3 of Dragendorff's reagent (Bismuth potassium iodide solution) was mixed with 2 cm^3 of different crude extracts where precipitation of orange colour was taken to infer the presence of alkaloids.

For quantitative analysis, the solubilized portions of the above crude extracts were each added to separate glass test tubes each tube containg 1 mL methanol and boiled for 1 hour. The extracts were filtered and concentrated to dryness. To each tube, 0.5 mL of 2M hydrochloric acid was added to the residue and washed with 1mL ether to remove fats and chlorophyll that resulted in aqueous phase. The aqueous phase was divided into portions for subsequent procedures. One of the portions was made basic with ammonia, and shaken with four portions of 20 mL of chloroform. The combined chloroform extracts were then dried with 5mL sodium sulfate and the total volume brought to 10 mL. from each tube, 3 ml aliquots was evaporated to dryness. Two reagents were prepared; oxidation reagent and a modified Erlich reagent. The oxidation reagent was made of a 30% hydrogen peroxide (containing 5 mg/ml sodium pyrophosphate for its stability. About 0.1 mL of this solution was diluted with methanol to 25 mL (this reagent was prepared fresh for each experiment). The modified Erlich reagent was prepared by dissolving 2 g of 4–

Dimethyaminobenzaldehyde in 100 mL of methanol (containing 10 mL boron trifluoride (BF_3 prepared in14% in methanol).

Absorption was determined by first adding 0.5 mL (5%) of oxidation reagent to the sample tube and the lower half of the un-stoppered tube immersed in a boiling water bath for 20–30 minutes. After 10 minutes, methanol was evaporated. Diglyme, (1 mL of 10%) and acetic anhydride (0.1 mL of 5%) were added respectively. The tube was heated again in the boiling water bath for 1 minute, 10 seconds. This was important to avoid any contamination by water, acids, or hydrogen peroxide. The tube was heated in a water bath at 55-60 °C for 4–5 minutes to develop the color. The cooled solution was diluted with acetone to 4 mL. The absorbance of the sample solution was measured at 565 nm. The blank solution was prepared by duplicating the above procedure, but omitting the sample. Concentrated samples were further diluted with acetone. For samples whose results such as record confirmation were for a reason required later, they were stoppered and stored in the dark, and a decrease in absorbance of about 1.4 per hour was allowed throughout the experiment.

3.4.2 Determination of total flavonoid content

Total flavonoid content was measured by the aluminium chloride colorimetric assay. For each of the aqueous phase solutions prepared, a reaction mixture was made that consisted of 1 mL of extract and 4 mL of distilled water, which were transferred into a 10 mL volumetric flask each. To the flask, 0.30 mL of 5 % sodium nitrite was treated and after 5 minutes, 0.3 mL of 10 % aluminum chloride was mixed. After 5 minutes, 2 mL of 1M Sodium hydroxide was treated and diluted to 10 mL with distilled water. A set of reference standard solutions of quercetin (20, 40, 60, 80 and 100 μg/ml) were prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with an UV/Visible spectrophotometer. The total flavonoid content was expressed as mg of quercetin equivalents (QE) /g of extract (Hakimuddin *et al*., 2004).

3.4.3 Sterols and terpenoids

Dissolved samples of the the different extracts were treated to a mixture of chloroform ($2\ cm^3$) and concentrated tetraoxosulphate (VI) acid ($3\ cm^3$) was added to $1\ cm^3$ of each extract to form a layer. The presence of a reddish brown colouration at the interface shows positive results for the presence of terpenoids (Ejikeme *et al.,* 2014).

To 1mL of the prepared crude extracts in section 3.3.3 a representative tube was stirred with 1mL hexane to remove most of the colouring material. The resulting solution was extracted with 10mL dichloromethane. The resultant solution was dehydrated over anhydrous sodium sulfate and filtered. 1 mL of filtrate was mixed with 0.5 mL acetic acid anhydride, followed by addition of 2 drops of concentrated sulphuric acid. Color change from green to blue indicated presence of sterols, while pink to purple indicated presence of terpenoids.

3.4.4 Saponins

To determine the presence of saponins in the plant extract, 0.5 g of aqueous, methanol, ethanol, ethyl acetate, dichloromethane and hexane plant extracts were each shaken with 10mL of water in a test-tube. Persistence of froth at the top of the test tube for more than 30 minutes indicated the presence of saponins. For quantitative estimation of saponins the test extracts prepared in section 3,3,3 were each dissolved in 80% methanol, then 2ml of Vanilin in ethanol was added and mixed well, Next, 2ml of 72% sulphuric acid solution was added, mixed well and heated on a water bath at 60^0 c for 10min, Absorbance was then measured at 544nm against reagent blank. Diosgenin was used as a standard material and the assay compared with Diosgenin equivalents.

3.4.5 Quantitative estimation of phenolic compounds

The total phenolics content in different solvent extracts was determined with the Folin-Ciocalteu's reagent. In the procedure, different solutions prepared from 3.3.3 of the W. somnifera crude extracts were mixed with 0.4 ml Folin- Ciocalteu's reagent (diluted 1:10 v/v). After 5 min 4 ml of sodium carbonate solution was added. The final volume of the tubes were made up to 10 ml with distilled water and allowed to stand for 90 min at room temperature. Absorbance of sample was measured against the blank at 750 nm using a spectrophotometer. A calibration curve was constructed using catechol solutions as standard and total phenolic content of the extract was expressed in terms of milligrams of catechol per gram of dry weight and the standard graph (Sankhalkar & Vernekar, 2016).

3.4.6 Test for tannins

To 1 cm^3 of the dissolved root crude extracts of *W. somnifera* was added 3 drops of 0.1% ferric chloride. A brownish green or a blue black colouration showed positive test (Sharma, 1981).

3.5 Experimental procedures with mice

The experimental design was carefully selected based on the variables. Extraction solvents were taken as the only variable so that any results had as a source of variation the treatments, (injection with extracts derived from different solvents of *Withania somnifera* root fraction) and the error component. Given that the animals were treated in identical cages in the same laboratory, fed from the same dietary material and source of water, a completely randomized design was decided as the best experimental model in this research (Timo, 2014).

The Swiss albino mice were grouped into sets of four mice so as to replicate and consider the mean response values for each treatment. The method of handling the animals with caution and permission took care of the animal protection act (The Prevention of Cruelty to Animals Act chapter 59 of 1962) (Lane-Petter, 1876). Swiss albino mice for running experiments in this research were obtained from Njoro High School Animal House and used for the study. The male mice were selected for their stability in hormonal concentration secretion without many variations among healthy animals, and were to be mature to realize full functionality of the organs and of the same age per treatment so that the vibrancies of the organs were relatively homogenous. The selected mice were kept in quiet and spacious cages to acclimatize while being fed *ad libitum* with standard mice pellets and water. The repeats for each treatment were four each for experiment series i and also four each for experiments in series ii.

Castration of mice was considered to be a better method where acute pain was inflicted to mice and data collected after 24 hours. However Formalin nociception test woul have considered periodical collection of data while the blood volume from each mouse is too small. The main objective of the study was to establish a relationship between serotonin levels, pain and root extracts from *W. somnifera* and so formaline nociception test was disqualified only for this research.

Experimental mice aged 8 weeks for series I and 10 weeks for series ii were castrated according to rodent surgery Standard Operating Procedures (SOP) (Tsukamato *et al.*, 2015). Animals were euthanized using isoflurane as per anesthesia SOP. Ophthalmic ointment was applied in both eyes to prevent corneal desiccation and reapplied if the animal took more than 10 minutes to castrate or as needed if the mice showed stress signs. Hair over the scrotum of the animal was removed using a clipper and loose hair removed with a sterile gauze. The

skin surface was wiped with 70% alcohol followed by 2% chlorhexidine solution or povidone-iodine solution. Each animal was placed in dorsal recumbency position. This was followed by a midline incision in the scrotum made, approximately 1 cm in length to expose the tunica and another midline incision in the tunica, slightly smaller than the incision in the skin.

A sterile gauze was soaked in a pad with sterile isotonic saline. One testicle was pushed out of the tunica, while gently raised to expose the underlying blood vessels and tubules, and rested on the saline-soaked gauze. The fat surrounding the vas deferens and spermatic blood vessels was gently removed using dry sterile gauze to facilitate cauterization. The vas deferens and spermatic blood vessels were cauterized and removed testes placed aside while checking for bleeding. Sterile cotton-tipped swabs were used to gently return any remaining tissues into the scrotum. That procedure was repeated with the other testes for every animal. The instruments were disinfected between each animal by dipping them in a hot glass bead sterilizer for approximately 30 seconds after removing any blood and debris and was let to cool completely. The castrated animals were allowed to recover in a clean cage and provided supplemental heat using a heating lamp for approximately 30 minutes and monitored until they fully recovered prior to returning them to their housing room.

Five (5) sets of four virtually healthy males and mature albino mice were used in each of the two series of experiments. The two series of experimental procedures were independent in order to observe the actual trends in physiological bevaviour of mice when extracted metabolites were treated differently. In total fourty swiss albino mice were used in this research The first set of four mice in the first series of treatments were then injected with 2 mL of *W. somnifera* root extract, methanol fraction. Subsequent groups were injected with *W. somnifera* root extract, other fractions including ethanol,dichloromethane, water, and n-hexane.

Choice of distilled water as a negative control was based on the fact that unless the duration of injection is extended, there is no toxicity at few exposuers and small doses of injection with distilled water. This has in a different reaeach been demonstrated that for rats injected with distilled water intravenous, there was no adverse effects at 11 doses of 5 mL/kg body weight of the rat similar to noral buffered saline (Shayne *et al.,* 2016). Blood was collected according to Chen (2014) with modifications.

General anesthetic of isoflurane was soaked in cotton wool and inhaled to the mice each at a time a desicator and five minutes allowed for the drug to be effective. Each mouse was then placed in right lateral recumbency and the heart gently repeatedly pressed on the left lateral thoracic wall. The area around which blood was to be collectected was disinfected by weting the skin with 70% alcohol in cotton swab. At approximately between the 5^{th} and 6^{th} ribs the small size needle (guage 21) was inserted slowly, perpendicular to the body. The plunger was slightly retracted to create a vacuum inside the syringe and the needle gently advanced until blood flash appeared in needle hub. The needle was immobilized and aspiration continued until a sufficient amount of blood was collected. Each mice was then euthanized immediately upon completion of blood collection.

The blood collected through heart puncture raged between 1mL and 1.5Ml. For centrifugation, only 1mL of blood was transferred into eppedof tubes from each mouse. Blood serotonin levels was determined over a period of 12 hours, recorded and compared for the five sets of Swiss albino mice. In the second series of experiments similar sets of mice were injected with 2 mL of *W. somnifera* root extracts that had been boiled for 10 minutes and cooled before injecting the mice with them. Although heat extractracted metabolites were subjected to high temperatutures during extraction, those metabolites were heated again to double heat treat them so that in case any high temperature sensitive once did not go without the exposure. Serotonin levels were taken and recorded, according to procedure on section 3.7. Blood serotonin levels were again determined over a period of 12 hours and recorded. The negative control set had blood serotonin levels determined without any treatment with extracts. For the positive control experiment, Panadol was used as a non-steroidal anti-inflammatory drug according to Rorry and Jamic (2016). After the experimental procedures the mice were sacrificed through anesthesia and their remains incinerated.

3.6 Bioassay animal experiments

3.6.1 Experimental animals preparation by castration for serotonin determination *Withania somnifera* extract bioassay with mice

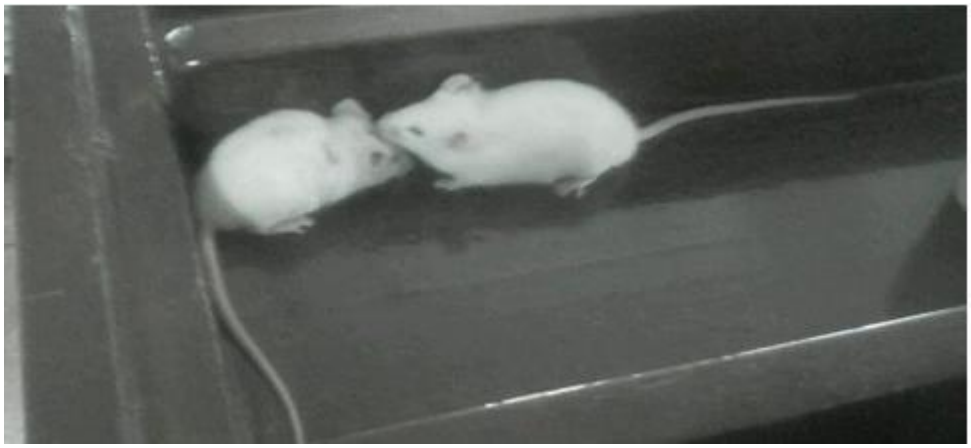


Plate 3.3: Swiss albino mice

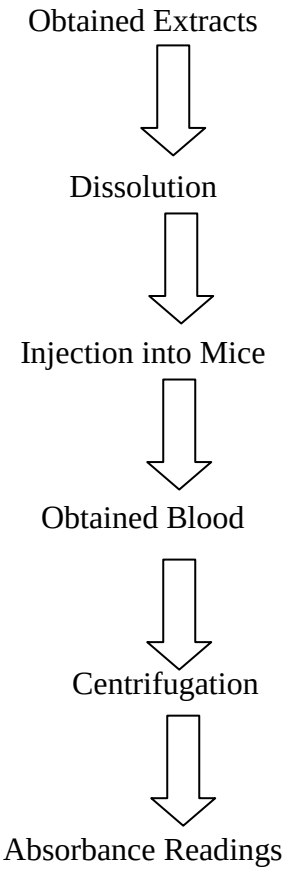


Figure 3.2: Bioassy of *Withania somnifera* extracts with swiss albino mice

Plate 3.4: A photograph of hematocrit fraction of mice blood after first treatment with EDTA and subsequent centrifugation at 1600g for 20 minutes at 0 to 4°C.

3.7 Data of serotonin using Utra Violet spectrophotometer

The experiment was laid down in a completely randomized design (CRD). The spectrophotometric readings were taken for each prepared blood sample and recorded as treatment for injection with root aqueous extract, root methanol extract, root ethanol extract, root ethyl acetate extract root dichloromethane extract and root hexane extract using cuvettes. Different absorbance values taken formed the basis for mean treatment values for different extracts. The strength of absorbance was used to judge the concentration of serotonin in different blood samples.

Other studies related to 5'-HT-SO_4 research have had application of capillary electrophoresis (CE) with laser-induced fluorescence (LIF) technique which also has its limitations some of which include CE-LIF could not fulfill the expectation of becoming a routinely applied technique in clinical laboratories or pharmaceutical industry. The reasons are the relatively high cost of instrumentation, the lack of ready to use applications and the need for careful design of method development requiring skilled and experienced analyst (Szoka & Tábi, 2010).

Platelet-rich plasma was prepared from 1 mL of blood from each mouse by the Dillard technique (Dillard *et al.,* 1951), modified by the use of larger quantities of EDTA solution. The large quantites were so that blood did not have any chance of clotting before and after centrifugation and thus minimize the error in analytical data (Layssol *et al.*, 2022).

The platelets were then spun down at 1600 g for 20 minutes at 0 to 4°C. The supernatant was

discarded and the platelets stored in a deep-freezer. Serotonin was determined by a modification of the Welsbach method (Nishizawa *et al.*, 1997). Distilled water up to 1 mL was added to the button of platelets, and thoroughly suspended. 0.2 mL of a 10 % solution of $ZnSO_4 7\ H_2O$ was then added, shaken briefly and followed by addition 0.1 mL of 1 M NaOH. After vigorous shaking for one minute, the test-tubes were centrifuged at 1600 g for 15 minutes and then 0.5 mL of the water-clear supernatant was put in a test-tube. 0.15 mL of concentrated HCI added, and serotonin fluorescence checked at 295 and 540 nm using a spectrophotometer. A blank (1 mL of distilled water) and the standards (1 mL of a 0.5µg/mL solution of 5HT base) was run throughout the procedure.

3.8 Data Analysis

The data obtained was analyzed using Statistical Package of Social Sciences (SPSS) version 25 software. The means were compared using ANOVA. The data on serotonin levels in the blood plasma of Swiss albino mice in both variables were analyzed using GENSTAT Software (Version 15) and the treatment means compared using LSD. These data were also entered in spread sheets for subsequent generation of graphs and analysis of variance (ANOVA) tables to establish relationships and correlations between serotonin concentrations and *Withania somnifera* root fractions. The secondary metabolites were determined and reported as either present or absent (so their data analysis was descriptive).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Yield by different extraction solvents

The yields by different extraction solvents varied with polar solvents giving more weights than

the non polar solvents. It was observed that 1.678 g, 1.921 g, 2.542 g 4.326 g and 3.123g of hexane, dichloromethane, ethyl acetate methanol and aquaous extracts respectively were recorded in table 4.1 below

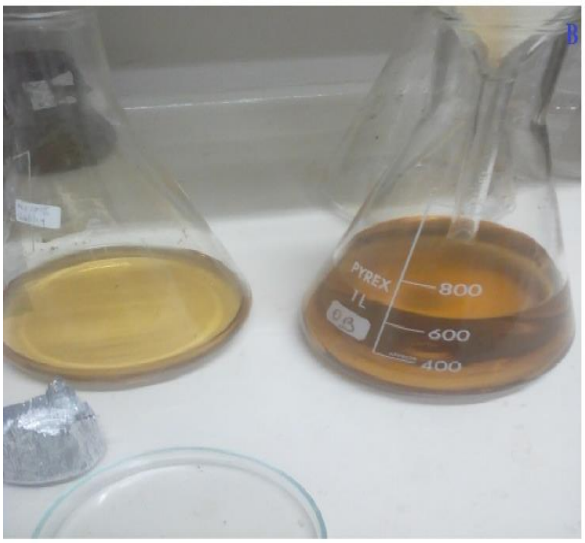
Table 4.1: Extraction yields by different solvents

Solvent	Hexane,	Dichloromethane	Ethyl acetate	Ethanol	Methanol	aqueous
Yield cold (g)	1.678	1.921	2.542	4.22	4.326	3.123
Yield hot (g)	2.354	2.922	4.232	5.54	7.412	6.824

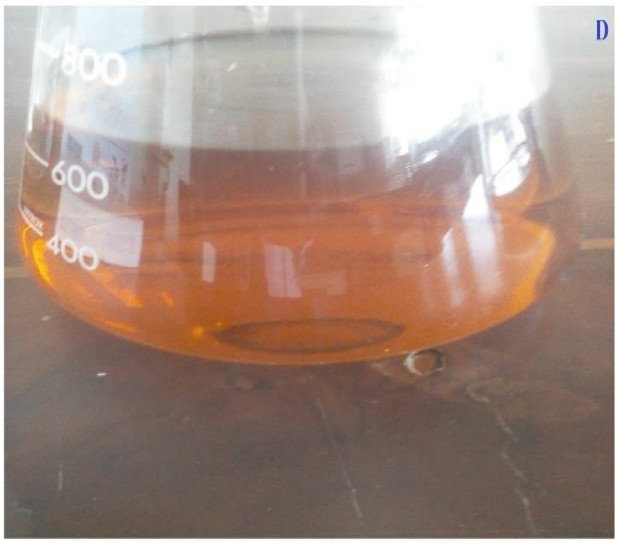
The extracts showed a trend where polsr solvents yielded comparatively higher weights than the non-polar solvents. However methanol extracts recorded a gtrater weight than the aqueous extracts , probably because the serial extraction targeted by those two solvents shared a lot of similarities. The masses yielded by the hot serial extraction also differed considerably from hexane extracts with 2.3 grams, dichloromethane 2.9, ethyl acetate 4.2 , ethanol 5.54, methanol 7.4 and 6.824grams aqueous extracts respectively. There was observed therefore an increasing trend in mass following similar time duration of extraction from non-polar to solvents with increasing polarity followed by overlaps for methanol and aqueous crude extracts. The extraction temperatures showed that hot extraction process was more effective in yield than the room temperature extraction.

Dichloromethane - black

Hexane – Green



Ethyl Acetate – Brown



Methanol – Oran

Plate 4.1: Colours of Withania somnifera extracts

4.2 Physical indicators of secondary metabolites in *W. somnifera* root extracts using cold and hot extraction methods

There were various colour differences among products of different extraction solvents after same time duration of incubation. These colors of extracts from *W. somnifera* were dichloromethane (black), hexane (brown), Ethyl-acetate (green) and methanol (orange) (Plate 4.1). The different extraction solvents resulted in different colours of extracts because they had different polarities and thus were bound to or eluted different metabolites based on metabolite polarities.

Generally, the crude extract quantity, purity, and quality greatly depend on the plant part used and the solvent used for the extraction (Jacotet *et al.*, 2018).

The results on the following page, table 4,2 shows presence or absence of various secondary metabolites in *W. somnifera* root extracts for both hot and cold (room temperature) extraction procedures**.** The cold extracts from *W. somnifera* demonstrated presence of alkaloids (methanol, aqueous and ethyl acetate), flavonoids (methanol and n-hexane), terpenoids (ethyl acetate and n-hexane), terpenoids (methanol and aqueous), tannins (aqueous and ethyl acetate) and phenols (methanol). (Table 4.2). However, the extracts lacked alkaloids (n-hexane), flavonoids (aqueous and ethyl acetate), terpenoids (methanol and aqueous), tannins (methanol and n-hexane) and phenols (aqueous, ethyl acetate and n-hexane).

The hot extracts from *W. somnifera* demonstrated presence of alkaloids (ethanol, methanol, aqueous and ethyl acetate), flavonoids (ethanol, methanol and n-hexane), terpenoids (ethanol, ethyl acetate and n-hexane), tapenoids (methanol and aqueous), tannins (aqueous, ethanol and ethyl acetate) and phenols (methanol). However, the extracts lacked alkaloids (n-hexane), flavonoids (aqueous and ethyl acetate), terpenoids (aqueous and methanol), tannins (methanol, and n-hexane) and phenols (aqueous, ethanol, ethyl acetate and n-hexane).

Table 4.2: Secondary metabolites of *W. somnifera* root extracts using cold and hot extraction techniques

Phytochemical	Aqueous		Methanol		Ethanol		Dichloromethane		n-Hexane			
	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot
Alkaloids	+	+	+	+	+	+	+	+	-	-	-	-
Flavonoids	-	-	+	+	+	+	-	-	-	-	+	+
Terpenoids	-	-	-	-	+	+	+	+	+	+	+	+
Saponins	+	+	+	+	+	+	-	-	-	-	-	-
Tannins	+	+	-	-	+	+	+	+	-	-	-	-
Phenols	-	-	+	+	_	_	-	-	-	-	-	-

Key

\+ Prencence of the indicated secondry metabolite

- Absence of the named secondary metabolite
- There were different secondary metabolites present in *Withania somnifera* root extracts samples tested. Those metabolites that included saponins, alkaloids, phenolics , Terpenoids Tannins, and Flavonoids were detected in root extracts using different extraction solvents

4.3 Total phytochemical compounds in *W. somnifera*

After establishing that secondary metabolites were distributed in different products of solvent extracts, the amounts of those different metabolites were determined using spectrophotometric analysis. Standards were first run in different concentrations so that the absorbance values of the metabolites and by extension their concentrations could be fitted easily on to the calibration curves.

4.3.1 Calibration table for quantitative analysis of alkaloids

The acornitine concentration ranged from 0 to 18 ug/Ml (appendix 20). However, the acornitine absorbance varied from 0.05 to 1.44. Absorbance versus concentration calibration curves were generated as shown on the figure (appendix 22) of gallic acid calibration curve.

4.3.2 Total phytochemical compounds in *W. somnifera* hot and cold crude extracts

Table 4.1 compares absorbance values and concentrations of different secondary metabolites obtained from different extraction solvents as hot and cold crude extracts. Absorbance values of phytochemical compounds in *W. somnifera* hot crude extracts. Appendix 4 compares absorbance values of different secondary metabolites obtained from different extraction solvents as hot crude extracts.

Table 4.3: Conentrations of phytochemical compounds (µg/mL) in *W. somnifera* hot and cold crude extracts

Metabolite	**Extraction solvent**	**Absorbance value**	**Concenttion (µg/mL)**	**absorbance**	**Concentration (µg/mL)**
		Hot		Cold	
Terpenoids	Hexane	1.450	95	1.658	105
Terpenoids	Dichloromethane	1.233	78	1.516	90
Tannins	Ethyl acetate	0.254	65	0.293	75
Tannins	Methanol	0.440	115	0.445	118
Alkaloids	Ethyl acetate	0.364	90	0.281	75
Alkaloids	Methanol	0.334	80	0.300	78
Saponinins	Methanol	0.270	77	0.280	80
Phenols	Methanol	0.124	38	0.123	34
Flavonoids	Methanol	0.334	130	0.300	115
Alkaloids	Aqueous	0.29	74	0.28	72
Saponinins	Aqueous	0.24	67	0.25	63
Tannins	Aqueous	0.39	96	0.22	56
Alkaloids	Ethanol	0.302	105	0.297	72
Flavonoids	Ethanol	0.340	88	0.322	120
Saponins	Ethanol	0.29	75	0.311	82
Tannins	Ethanol	0.480	128	0.385	95

From table 4.3, most of the secondry metabolites, alkaloids, saponins, phenols, flavonoids and tanins recorded high concentration in methanol. However alkaloids only recoded a higher concentration than methanol in ethyl acetate with 0.030 poits.

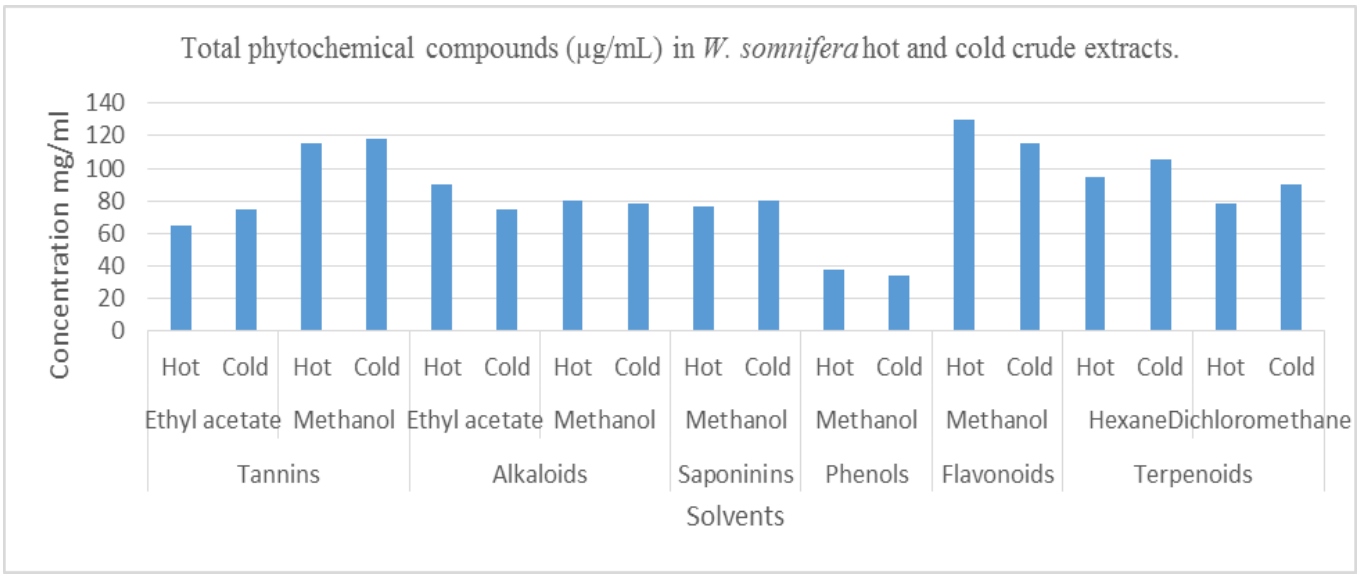


Figure 4. 1: Concentration values of secondary metabolites from different hot and cold crude extracts of root fractions of *W. somnifera.*

In a related research by Jacotet and his team the factors affecting extraction can be moderated, They investigated the impact of solvent type and plant part on the bioactive extraction process they worked with seven solvents (n-hexane, chloroform, ethyl acetate, acetone, ethanol, methanol, and water). Their research also demonstrated that different solvents extracted different concentrations of crude extracts from branches, flowers, leaves, and roots (Jacotet *et al.*, 2018).

A research carried out by Ullah *et al*. (2017) demonstrated that the extraction efficiency of acetone-water (1:1, v/v) was very high and ethyl acetate or n-hexane alone or in combinations was very low for all plant parts (branches, flowers, leaves, and roots) as compared to other solvents. The secondary metabolites are different in both physical and chemical properties.

Terpenes also called terpenoids are diverse substances generally insoluble in water. All terpenes are derived from the union of five carbon atoms that have the branched carbon skeleton of isopentane and decompose at high temperature to give isoprene. According to Gershenzon and Croteau (2012). Terpenes and their derivatives called saponins are steroid and triterpene glycosides. One group of these derivatives of terpenes called carotenoids gives the yellow, red and orange colour in some plants like carrot. *Polypodium vulgare*, *Digitalis spp*, pine and fir, peppermint plant, lemon, basil, sage, corn, *Gossypium hispida* (cotton) and wild tobacco (Kessler & Baldwin, 2001).

4.4 Results for serotonin levels

4.4.1 Difference in yield and serotonin secretion in Swiss albino mice for different extracts

Table 4.4: Serotonin secretion from mice treated with given *W. somnifera* root solvent extracts

Water		Methanol		Ethanol Absorb,		Dichloromethane		Hexane	
Absorb,	Conc.	Absorb	Conc.	Conc.	Conc.	Absorb.	Conc.	Absorb.	Conc.
1.459	0.26	2.094	0.31	2.685	0.36	1.356	0.17	0.740	0.11
1.985	0.29	2.405	0.35	2.433	0.33	1.865	0.27	0.518	0.06
1.930	0.27	1.995	0.16	2.445	0.34	1.789	0.26	0.719	0.12
1.196	0.14	2.017	0.30	2.301	0.33	1.475	0.21	0.629	0.09

From table 4.4 above, the concentration of serotonin is directly proportional to absorbance. It follows that polar solvents indeed gave comparaeively higher values rhan non polar solvents and secretion of serotonin increased with increase in polarity.

Appendix 1 also shows the different absorbance values of serotonin for the same castrated mice treated with different *Withania somnifera* root extracts using room temperature exraction procedure series ii. The values of absorbance at 295 nm obtained showed a significant difference among serotonin levels from mice due to treatments by extracts from different solvents ($F=50.2011$ $P=1.25x10^{-07}$).

Comparison among absorbance values of serotonin for mice treated with different secondary metabolites obtained from different extraction solvents as cold and hot crude extracts at 540 nm in series 1 was carried out. ($F=269.5583$ $P= 6.85x10^{-12}$). Comparison between the negative (0.244) and the positive (0.5335) controls on absorbance values and thus the concentrations showed that although Panadol was on average expected to give the best response in serotonin secretion more than the distilled water treated mice but did not imerge the best among all the W.somnifera extracts in serotonin secretion. The mean values behaved that way probably due to the fact individual animal internal factors were synchronized by both environment and diet. However, this could also be due to the number

of serotonin receptors on individual animals down regulated by Panadol and the extracts coming to a homogegous average for every mice.

Means for the other solvents were also determined and recorded. In order to constitute the Y-intercept when plotting the absorbance/concentration curve. This was so because distilled water did not have any serotonin in it and so serotonin concentration was virtually zero. Overall F value that compared the treatments with their replicates indicates there was some difference between treatments and the error component, given a higher positive treatment value than error and so the ratio greater than one. The individual columns that represented different treatments showed variations with ethanol treatment giving the highest mean followed by methanol, negative control, di- Chloromethane and finally *Withania sonifera* n-Hexane root extracts treatment.

From results on appendix 5, the mean difference comparison comparison for absorbance was determined as follows;

l.s.d (least significant difference) = t alpha/2, k\/SED where k is error degrees of freedom, and MSE is Mean squares of error

At 95 per cent confidence interval, $t_{0.005}$, 10=1.043

SED= 1.043X \/2X0.000064/4=0.00064

Ethanol	=2.466	a
Methanol	= 2.128	0. 3 38b
Water	= 1.643	0.485c
Dichloromethane	= 1.621	0.022c
Panadol	=1.396	0.225cd
n- Hexane	= 0.652	0.969e
blank	= 0.002	0.967f

Means followed by the same letters are not significantly different at 0.05 level of significance.

From this equation and given molar extinction coefficient of serotonin as 6718, different concentration values for each mice treated with an extract was calculated. Appendix 4 shows comparison of absorbance values of of serotonin for different secondary metabolites obtained from different extraction solvents as hot crude extracts in series i of experimental procedures. Absorbance values of serotonin from castrated mice with different *W. somnifera* root heat and cold extracted solvents and Panadol as a pain modulating agent taken as a positive controls.

Figure 4.8 below shows mean concentrations of serotonin for Swiss albino mice treated with different crude extracts cold from roots *of W. somnifera*

Table 4.5: Mean concentrations and absorbance of serotonin for Swiss albino mice treated with different cold solvent extracted crude extracts

Solvent	Water	ethanol	methanol	Dichloromethane	Hexane	Panadol
Concentration	0.25	0.35	0.31	0.24	0.08	0.3
Absorbance	1.643	2.466	2.128	1.621	0.652	2.119

Those concentrations and mean concentrations of serotonin varied significantly (F=23.3269 P=0.0000469). Concentrations and mean concentrations (table 4.5) of serotonin from mice treated with *W. somnifera* different solvents root extracts were obtained by multiplying concentration values by a factor of 10^{-3} which was the dilution factor including the set of mice treated with distilled water as the negative control. F-value is high indicating the model provides adequate data to show results obtained had enough justification for the treatments. The p-value was less than 0.005 indicating evidence above 95 per cent confidence of the treatments.

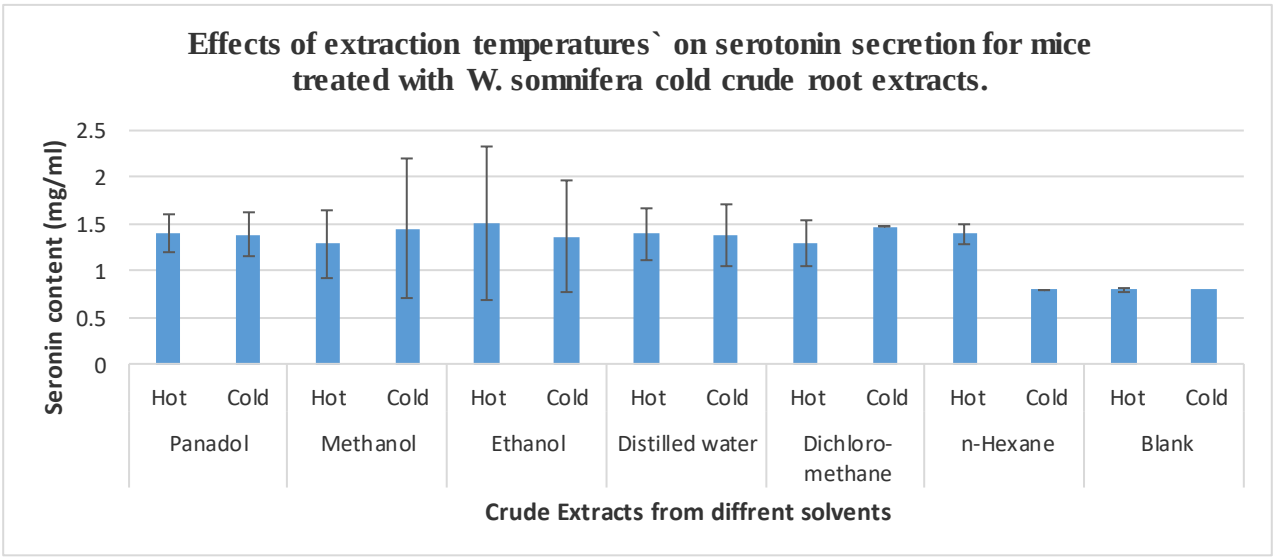


Figure 4.2: Effect of extraction temperatures on serotonin secretion.

From analysis of serotonin concentration values there was no significant difference due to temperatures at which secondary metabolites were extracted. As such the higher yielding solvents were then tested to check on solvent extraction variations as shown in figure 4.3 below.

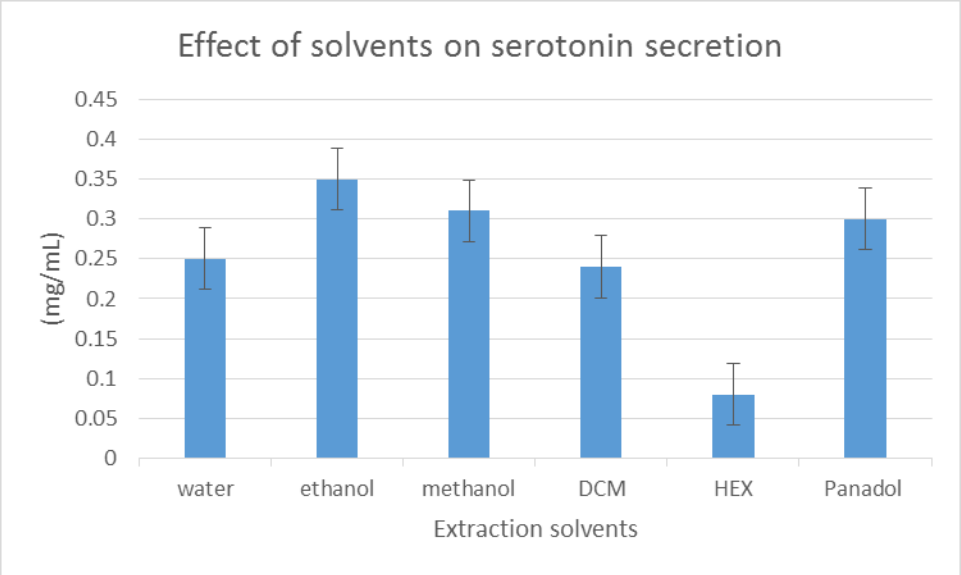


Figure 4.3: Effects of solvents on serotonin secretion

Figure 4.3 shows comparison of values of serotonin content in mice treated with different solvent fractions of W. somnifera crude extracts.

Withania somnifera ethanol root extracts had the highest absorbance values of serotonin (2.466) and thus concentration ($3.67X10^{-3}$ µg/mL) followed closely by methanol root extracts, then Panadol, negative control (distilled water treatment), DCM (Dichloromethane) and finally hexane (HEX) root extracts at the wavelength of 295 nm. The solvents so used could have targeted different metabolites other than those that evoke serotonin secretion and its subsequent recruitment to the platelets. Those metabolites could also have had effect on positive down regulation of serotonin receptors thus low serotonin absorbance and concentrations as was shown by ethyl acetate, dichloromethane and hexane extracts.

The mean concentrations of serotonin from different treatments showed mice treated with Panadol (0.5335) to have had at least significantly lower absorbance value compared to mice treated with some of the *Withania somnifera* different root extracts as demonstrated in table 4.3. This shows that Panadol either inhibits serotonin concentrations in blood and so the platelets or evokes negatively its release from the centers of its production through down regulation of serotonin receptors.

Serotonin concentration mean from ethanol extract (with absorbance of 2.466) was the highest indicating it was able to recruit serotonin to the site of tissue trauma more effectively than the other root extracts and even the positive control Panadol. This high absorbance value is an indicator of high concentration. After heat-treatment of the two best performing extracts, it was observed that methanol extracts performed better (1.568) than ethanol (1.118)

regarding serotonin concentrations release and thus the best performing extract after heat treatment. The corresponding absorbance value of serotonin was recorded as 0.062 indicating that the secondary metabolites increasing serotonin concentrations are usually polar.

4.4.2 Serotonin levels for mice treated with different secondary metabolites

Comparison of concentration means of serotonin for mice treated with different secondary metabolites obtained from different extraction solvents as cold crude extracts ($F=238.6161$ $P=3.03x10^{-10}$) appendix 1, the values calculated from serotonin absorbance at 540 nm. The concentration values were calculated as a factor of $x10^{-3}$.

Key;

Water = concentrations of serotonin from mice treated with water (negative control).

Ethanol = concentrations of serotonin from mice treated with ethanol extract.

Methanol = serotonin concentrations for mice treated with methanol extract.

Di- Chloromethane= serotonin concentrations for mice treated with DI- Chloromethane extracts.

n-Hexane= individual serotonin concentrations for mice treated with n-Hexane root extracts.

The F- value so calculated was significantly high and so treatments were important.

P-value was found to be too low explaining that even at minimum levels the data could still produce evidence and so valid was the model.

4.4.3 Absorbance levels of serotonin based on the different concentrations

Key

P---------- Positive control where Panadol was administered to mice after castration.

M ----------boiled *W. somnifera* methanol root extracts treated to a group of castrated mice.

E ----------boiled *W. somnifera* ethanol root extracts treated to a group of castrated mice

D----------boiled *W. somnifera water* root extracts treated to a group of castrated mice

4.5 Effect of temperatures during extraction process.

Serotonin levels in Swiss albino mice were determined for mice injected with root extracts of *W. somnifera* using n-hexane, methanol and water as heat treated crude extraction solvents and recorded in appendix 9.

4.5.1 Absorbance values of serotonin for mice treated with different secondary metabolites

The extracts used in treatment of swiss albino mice was to compare absorbance values and thus concentrations of serotonin for mice treated with different solvent based secondary metabolites. The extracts were also biased as hot and cold crude extracts. Panadol as a positive control treatment showed that F calculated was 3.751963, P-value 0.041274 and F critical 3.490295.

4.5.2 Absorbance values for serotonin at 540nm recorded from blood preparations of the test mice.

Withania somnifera root extracts were able to produce physiological effects to the castrated mice where the parameter of consideration, the serotonin levels were modulated differently based on both the distilled water and Panadol as the negative and positive controls respectively.

We tested the hypothesis that;

1. Heated root extracts of *W. somnifera* has no significant differencences in serotonin concentrations produced in castrated test mice compared to the positive control (Panadol).
2. Heated root extracts of *W. somnifera* has significant differencences in serotonin concentrations produced in castrated test mice compared to the positive control (Panadol) .

Since F- calculated (1.32) was much lower than F-critical (3.49) we failed to reject H_0 and conclude that there was no significant difference among serotonin absorbance values for *Withania somnifera* heat-treated root extracts and the positive control. Since F-calculated (283.62) was much higher than F-critical (3.48), we rejected H_0 and concluded that there were significant differences in serotonin yield from different *Withania somnifera* root extracts.

We wanted to test whether

1. There was any significant difference among treatments after heating
2. The positive control had any variation from the treatments.

Hypothesis;

H_0; The means were equal for boiled ethanol and methanol *Withania somnifera* root extracts before and after boiling.

$H_{1;}$ At least any two values were not equal in absorbance after extracts were boiled.

4.5.3 Absorbance values for serotonin at 295nm

Appendix 6 shows absorbance values for serotonin at 295nm recorded from blood preparations of the test mice treated with Panadol, and each of *W. somnifera* hot extracted methanol crude extracts, hot extracted ethanol crude and hot extracted water crude extracts. Distilled water blank was also included to mark the y-intercept of the curve.

Theoretically, under high temperatures, plant tissues are softened and the weak interactions affect the cell membranes. As a result, phenolic compounds can be easily extracted into the solvent (Shi & Pohorly, 2003). However, a prolonged extraction time at 80°C decreases the extraction yield because the high temperature causes the oxidation and degradation of the desired compounds (Jamian, 2014). Conversely, by keeping the temperature at a minimum level for a maximum extraction time period of 2 hours produced the highest yields (Sultana, 2013). Hence, a prolonged exposure of the sample in the solvent, allowed sufficient time for the desired compounds to migrate into the solvent. Temperatures are a critical factor for consideration because it affects plants physiological processes and even the yield of metabolites in harvested plant fractions.

Plant secondary metabolites that influence biological activities otherwise referred to as biologically active compounds usually occur in low concentration in plants. In order to obtain those natural products an extraction technique that is able to obtain extracts with high yield and with minimal changes to the functional properties of the extract is always required (Quispe *et al*., 2008). Several studies have reported variations in the biological activities of extracts prepared using different extraction techniques. Therefore, it is necessary to select the suitable extraction method as well as solvent based on sample matrix properties, chemical properties of the target metabolites, efficiency of the extraction method and desired properties of metabolites of interest. (Hayouni *et al.,* 2007). In conventional extraction, heat is transferred through convection and conduction from the surface, where, the extraction ability of solvents depends mainly on the solubility of the compound in the solvent, the mass transfer kinetics of the product and the strength of solute/matrix interaction with corresponding limitations on heat and mass diffusion rate and Ultrasound assisted solvent extraction is a process that uses high intensity, high frequency sound waves and solvents to extract targeted compounds from various matrices. Physical and chemical properties of the materials subjected to ultrasound are altered due to the propagation interaction of sound waves as they

disrupt the plant cell walls, thereby, facilitating release of extractable compounds and enhancing mass transport of solvent from the continuous phase into plant cells (Ishida *et al.*, 2001). For the extracts which were heat treated by boiling for ten minutes, the sets of castrated swiss albino mice were treated to the extracts and observed for serotonin secretion as series two of the trials.

Appendix 8 shows the figure with the differences between absorbance values of serotonin at 295nm and 540 nm. The differences were used to fit onto the calibration culves to generate concentrations of serotonin. From absorbance values differences of serotonin at 295 and 540nm, serotonin concentration was calculated as;

Concentration of serotonin = (A295nm- A 540nm) x176.215x0.1x10/6718 where;

A = absorbance,

176.215 = molecular weight of serotonin,

4= theoretical nominal sampling,

0.1= constant for the approximate weight of hematocrit,

10 = dilution factor,

6718 = molar absorptivity for serotonin at 295nm.

4.5.4 Concentrations of serotonin calculated from absorbance values of serotonin at 295nm and 540 nm.

Table 4.6 below shows the concentrations of serotonin calculated from absorbance values of serotonin at 295nm and 540 nm series ii

Table 4.6: Concentrations of serotonin derived from absorbance at 295nm and 540 nm.

F Calculated = 12.99992 F tabulated 3.055568 p- value = 9.1E-05

Blank	Panadol	Methanol	Ethanol	Water
1.0490×10^{-4}	1.1122×10^{-1}	1.4920×10^{-1}	2.1257×10^{-1}	7.9740 x 10-3
1.0490×10^{-4}	2.0320×10^{-1}	1.2297×10^{-1}	2.5045×10^{-1}	3.6722×10^{-3}
3.2520×10^{-3}	1.9683 x 10-1	1.4175×10^{-1}	2.0407×10^{-1}	8.8133×10^{-3}
2.0984×10^{-4}	1.1856 x 10-1	1.0377×10^{-1}	2.0460×10^{-1}	5.7707×10^{-3}

LSD

Ethanol	0.217923	0.022031		A
Panadol	0.157453	0.049307	0.06047	B
Methanol	0.129423	0.020354	0.02803	C

Water	0.006558	0.002312	0.122865	D
Blank	0.000918	0.001557	0.00564	D

Means followed by the same letters are not significantly different at 0.05 level of significance.

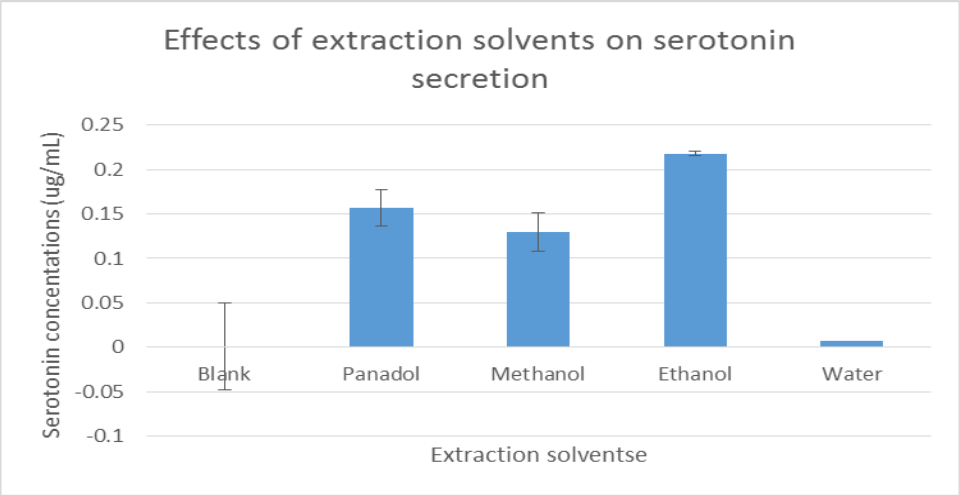


Figure 4.2: Effect of heat treatment of secondary metabolites on serotonin concentration for mice treated with different solvent extracted fractions of W. somnifera

The findings by Mitani *et al.* (2006) provided evidence of naturally occurring 5'-HT-SO_4 in human plasma and their findings opened up new possibilities for monitoring minor 5'-HT metabolism pathways in the peripheral blood stream. The findings suggested that 5'-HT-SO_4 could potentially be employed as a biomarker of certain disease severity and antidepressant treatment efficacy similarly to 5-HIAA. It has been established that serotonin O-sulphate is present in the same body fluids as other 5'-HT metabolites. The key limitations of such methods in the past have included the lack of knowledge on CNS-specific site of 5'-HT-SO_4 appearances and the fact that monitoring of 5'-HT-SO_4 was only possible in the CSF. Thus, 5-HIAA had many more advantages from a feasibility and convenience standpoint. Whether the 5'-HT-SO_4 found in plasma indeed has CNS origin or there exists a possible explanation of the elevated or lowered sulphate levels observed in the recent research, investigations are needed to explain these findings with the prevailing evidence and taking into account conclusions regarding doubtful CNS origin of 5'-HT-SO_4 so that there exists a potential tool to monitor central serotonergic metabolism in the peripheral blood stream (Tyce *et al.*, 1985).

In a different set up, serial extraction of *W. somnifera* root fraction was carried out and treated to mice yielding absorbance values at 295 nm results as demonstrated in appendix 4.1 and 4.2. The results showed that absorbance values of serotonin for mice treated with cold

serial extracted crude extracts at 295nm were significantly differenent for solvents with different polarities. Appendeces 9 and 10 show absorbance values of serotonin for mice treated with cold serial extracted crude extracts at 540 nm in the first series of experiments, appendeces 12 and 13 confirmed the trend where polar solvents had better results of serotonin secretion absorbance values in both series i and ii.

The figure on appendix 9 Shows the effect of serial extraction of secondary metabolites on serotonin concentration as absorbance values at 540nm indicating that specific secondary metabolites associated with serotonin secretion were more highly concentrated in polar solvents.

The difference between absorbance values corresponding to same positions of the two tables were used to calculate the different serotonin concentrations.

There was a significant difference among absorbance values for mice treated with different serial extracted cold extracts (F-20.70032 P=4.91E-05).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

Presence of secondary metabolites in *W. somnifera* root extracts was detected from root extract samples tested. Those metabolites included saponins, alkaloids, phenolics tannins saponins, Terpenoids and Flavonoids detected in root extracts using different extraction solvents. The serotonin levels in Swiss albino mice injected with root extracts of *W. somnifera* using n-Hexane, methanol, ethanol, di-chloromethane and water as cold extraction solvents (weights of the extracts) were determined. The difference in yield based on serial extraction and serotonin secretion in mammals for different solvents on bases of polarity of extracting solvents was established. Serotonin levels in Swiss albino mice injected with root extracts of *W. somnifera* using n-hexane, methanol and water as heat treated crude extraction solvents was determined. The results obtained from this research showed more activity of serotonin secretion from suiz albino mice treated with *W. somnifera* root extracts of high polarity, which decreased with decrease in polarity. This justifies the traditional administration of the herb *W. somnifera* through extractions using water as a solvent. However, the results also indicated lower yield of serotonin in mice treated with root fraction of *W. Somnifera* heat extracted solvents. This indicates that there are some active metabolites lost during heat extraction of root fraction of *W. somnifera* during heat extraction process. However, the order of heat extraction by serotonin yield indicated methanol to be the best solvent followed by ethanol and water taking second and third places respectively.

Serial extraction was important as it yielded different weights and colours of metabolites from different extraction solvents. This indicated that the targeting of specific groups of metabolitets are possible by either retention or maximal withdrawal when a specific solvent has been applied. The weights recorded during serial extraction were diverse and based on solvent polarities with more polar solvents producing higher yields with polar metabolites than their non polar counterparts and the reverse true for non polar secondary metabolites. The weights increased with increase in polarities both in cold and hot extraction.

As far as serotonin secretion was concerned, methanol extract produced the highest absorbance values followed by ethanol, distilled water and finally Panadol respectively which served as an indicator of changing trends of serotonin concentrations.

5.2 Recommendations

i. It is recommended that purification of extracts of W. somnifera from each solvent be carried out in order to identify the actual molecules present in each solvent that brings about bioactivity extract.

ii. Separation of the secondary metabolites to identify the actual class of compounds responsible for serotonin increase.

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APPENDICES

Appendix A: Analysis variance (ANOVA) of absorbance values of serotonin for mice treated with different solvent fractions of *W. Somnifera* cold crude extracts using different solvents as treatments associated with the lsd run series i.

ANOVA

Source of variation	Sum of Squares	Df	Mean Square	F	Sig.
Solvents	.146	4	.037	54.800	.000
Error	.010	15	.001		
Total	.156	19			

Comparisons of absorbance values of serotonin for mice treated with different solvent fractions of *W. Somnifera* cold crude extracts using different solvents as treatments associated with the lsd run series i.

Dependent Variable: absorbance

LSD

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	-1.6405000*	.1516236	.000	-1.959049	-1.321951
	C	-2.4640000*	.1516236	.000	-2.782549	-2.145451
	D	-2.1257500*	.1516236	.000	-2.444299	-1.807201
	E	-1.6192500*	.1516236	.000	-1.937799	-1.300701
	F	-.6495000*	.1516236	.000	-.968049	-.330951
B	A	1.6405000*	.1516236	.000	1.321951	1.959049
	C	-.8235000*	.1516236	.000	-1.142049	-.504951
	D	-.4852500*	.1516236	.005	-.803799	-.166701
	E	.0212500	.1516236	.890	-.297299	.339799
	F	.9910000*	.1516236	.000	.672451	1.309549
C	A	2.4640000*	.1516236	.000	2.145451	2.782549
	B	.8235000*	.1516236	.000	.504951	1.142049

	D	.3382500*	.1516236	.039	.019701	.656799
	E	.8447500*	.1516236	.000	.526201	1.163299
	F	1.8145000*	.1516236	.000	1.495951	2.133049
D	A	2.1257500*	.1516236	.000	1.807201	2.444299
	B	.4852500*	.1516236	.005	.166701	.803799
	C	-.3382500*	.1516236	.039	-.656799	-.019701
	E	.5065000*	.1516236	.004	.187951	.825049
	F	1.4762500*	.1516236	.000	1.157701	1.794799
E	A	1.6192500*	.1516236	.000	1.300701	1.937799
	B	-.0212500	.1516236	.890	-.339799	.297299
	C	-.8447500*	.1516236	.000	-1.163299	-.526201
	D	-.5065000*	.1516236	.004	-.825049	-.187951
	F	.9697500*	.1516236	.000	.651201	1.288299
F	A	.6495000*	.1516236	.000	.330951	.968049
	B	-.9910000*	.1516236	.000	-1.309549	-.672451
	C	-1.8145000*	.1516236	.000	-2.133049	-1.495951
	D	-1.4762500*	.1516236	.000	-1.794799	-1.157701
	E	-.9697500*	.1516236	.000	-1.288299	-.651201

*. The mean difference is significant at the 0.05 level.

Key;

Blank = concentration replications of distilled water to calibrate Y-axis.

Water = concentrations of serotonin from mice treated with water (negative control)

Ethanol = concentrations of serotonin from mice treated with ethanol extract.

Methanol = serotonin concentrations for mice treated with methanol extract.

di- Chloromethane = serotonin concentrations for mice treated with di- Chloromethane extracts.

n-Hexane = individual serotonin concentrations for mice treated with n-Hexane root extracts.

The F- values so calculated were significantly high and so treatments were important.

P-value was found to be too low explaining that even at minimum levels where experiment would have evidence of being rejected.

Analysis of concentrations of serotonin for mice treated with different secondary metabolites obtained from different extraction solvents as cold crude extracts at 540 nm using cold solvent extracted secondary metabolites as treatments series i.

SUMMARY								
Groups	*Count*	*Sum*	*Average*	*Variance*				
0.001	3	0.007	0.002333	1.33E-06				
1.459	3	5.111	1.703667	0.19405				
2.685	3	7.179	2.393	0.006384				
2.094	3	6.417	2.139	0.053188				
1.356	3	1792.34	597.4467	1064850				
0.74	3	1.866	0.622	0.010137				

Analysis of variance (ANOVA) for concentrations of serotonin for mice treated with different secondary metabolites obtained from different extraction solvents as cold crude extracts at 540 nm using cold solvent extracted secondary metabolites as treatments series i.

ANOVA								
Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*		
Treatments	888275.1	5	177655	1.001014	0.457679	3.105875		
Error	2129700	12	177475					
Total	3017975	17						

Beer Lamberts equation; A= Emcl where;

A= Absorbance

Em = Molar extinction coefficient

C= Concentration

L = path length of 1 cm.

Analysis of concentrations of serotonin for mice treated with *Withania somnifera* cold crude extracts mice at 540nm series i

SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
0.005	3	0.021	0.007	0.000001		
0.187	3	0.542	0.180667	0.000277		
0.009	3	0.015	0.005	0.000013		
0.01	3	0.022	0.007333	1.23E-05		
0.009	3	0.023	0.007667	1.63E-05		

Comparison of absorbance values of different secondary metabolites obtained from different extraction solvents as hot crude extracts.

Absorbance values of serotonin at 540nm series i

Panadol		Methanol		Ethanol		Distilled water		Dichloromethane		n-hexane		Blank
Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	
1.43	0.830	0.957	2.351	1.887	1.986	1.328	1.436	1.356	0.021	0.740	0.020	0.000
1.58	0.587	0.199	0.660	0.202	0.738	1.988	1.977	1.865	0.023	0.518	0.024	0.034
1.102	0.288	0.439	1.299	0.235	0.777	1.563	1.230	1.789	0.019	0.719	0.022	0.000
1.472	0.429	0.899	1.962	0.271	0.972	1.608	1.778	1.475	0.028	0.629	0.019	0.000
1.43	0.830	0.957	2.351	1.887	1.986	1.328	1.436	1.356	0.021	0.740	0.020	0.000

Serotonin absorbance values at 295 and 540 nm for mice from cold extracting solvents series i.

Blank	Water		Panadol		Ethanol		Methanol		di- Chloromethane		n- Hexane	
	295	540	295	540	295	540	295	540	295	540	295	540
0.001	1.459	0.217	1.430	0.830	2.685	0.399	2.094	0.300	1.356	0.202	0.740	0.110
0.001	1.985	0.295	1.580	0.587	2.433	0.362	2.405	0.311	1.865	0.277	0.518	0 .077
0.003	1.930	0.287	1.102	0.288	2.445	0.364	1.995	0.358	1.789	0.266	0.719	0.107
0.003	1.196	0.178	1.472	0.429	2.301	0.342	2.017	0.297	1.475	0.219	0.629	0.093
0.002	1.643	0.244	1.396	0.5335	2.466	0.367	2.128	0.317	1.621	0.241	0.652	0.062

Key;

Blank; Absorbance values of standard blank replicates, which was distilled water.

Water; Absorbance values for mice castrated and treated with distilled water

Panadol; Absorbance values for mice castrated and treated with Panado.

Ethanol; Values of absorbance given by mice treated with Ethanol extracts.

Methanol; Mice absorbance values from methanol extracts.

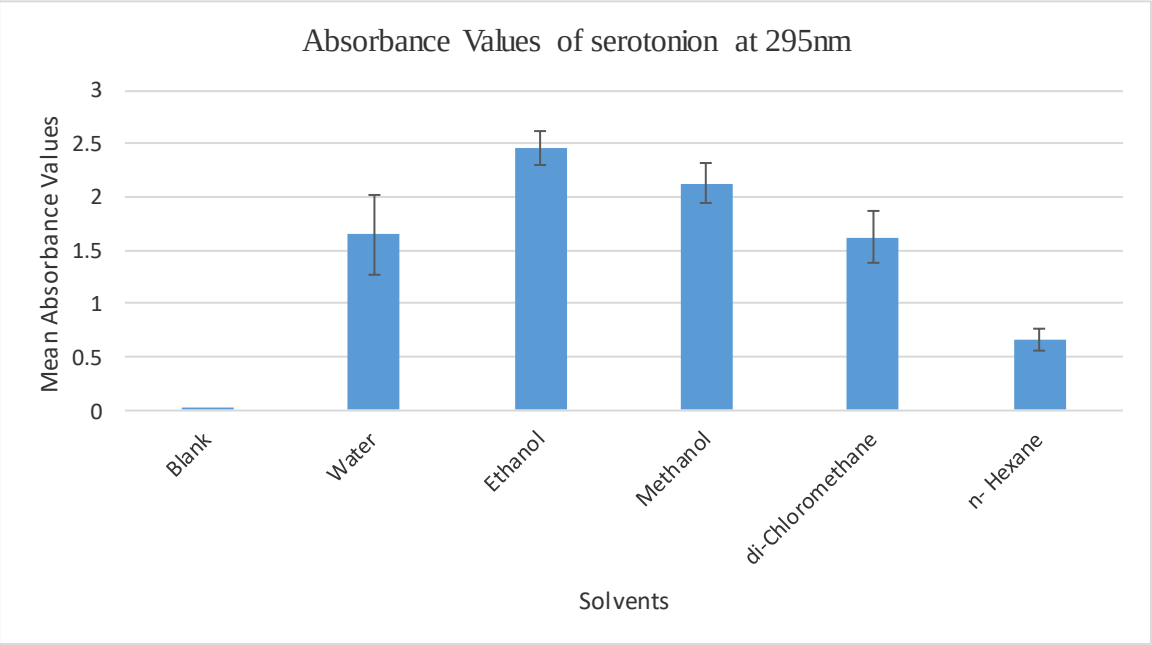
Di- Chloromethane; Di- Chloromethane extract mice treated absorbance values.

n- Hexane; n- Hexane extract mice treated absorbance values.

Absorbance values for serotonin at 295nm recorded from blood preparations of the test mice with first column distilled water labled blank series i.

Blank	Water	Ethanol	Methanol	di-Chloromethane	n- Hexane
0.001	1.459	2.685	2.094	1.356	0.740
0.001	1.985	2.433	2.405	1.865	0.518
0.003	1.930	2.445	1.995	1.789	0.719
0.003	1.196	2.301	2.017	1.475	0.629
Am=0.002	Bm=1.643	Cm=2-466	Dm=2.128	Em=1.621	Fm=0.652

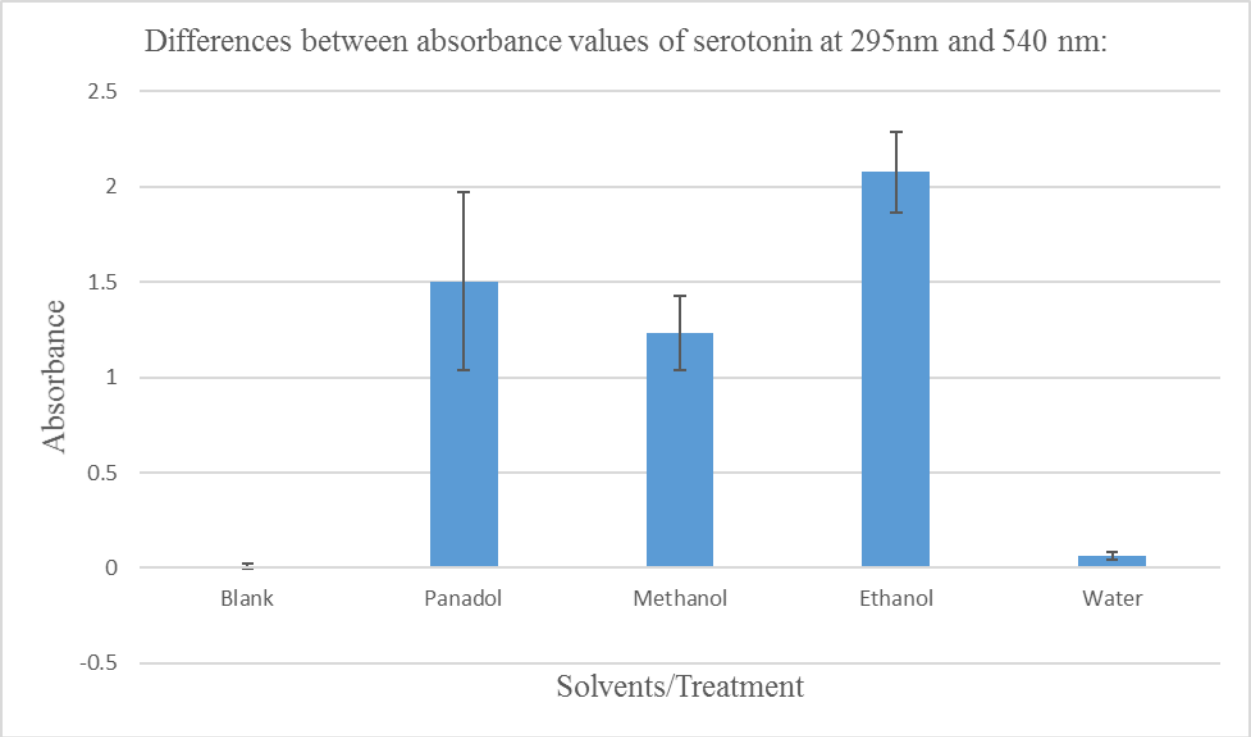
Comparison of absobance values of serotonin in mice treated with different



The table showing the differences between absorbance values of serotonin at 295nm and 540 nm series i

Blank	Panadol	Methanol	Ethanol	Water
0.001	1.069	1.422	2.026	0.076
0.001	1.937	1.172	2.387	0.035
0.031	1.876	1.351	1.945	0.084
0.002	1.130	0.989	1.95	0.055

Absorbance values differences of serotonin at 295 and 540nm



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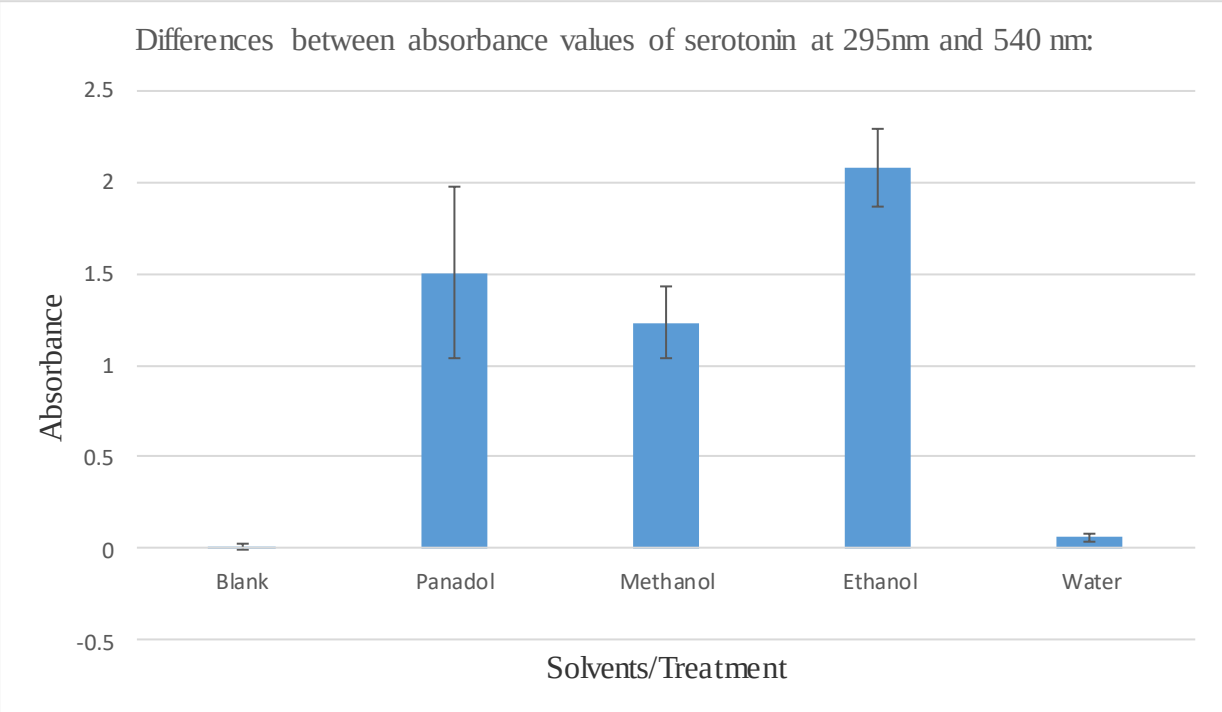
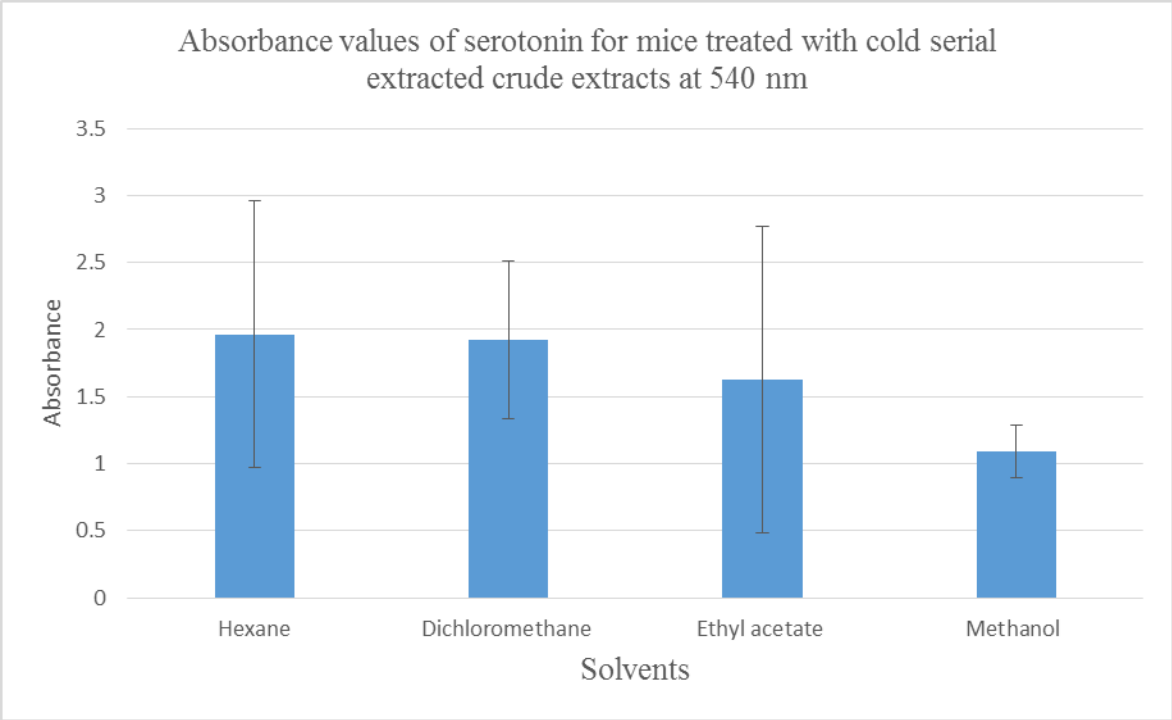
Absorbance values differences of serotonin at 295 and 540nm



Absorbance values of serotonin for mice treated with cold serial extracted crude extracts at 540 nm series i

Hexane	Dichloromethane	Ethyl acetate	Methanol
1.743	1.857	1.498	1.010
0.647	1.851	2.646	0.981
2.754	1.284	0.071	0.985
2.713	2.705	2.303	1.379

Effect of serial extraction of secondary metabolites on serotonin concentration as absorbance values at 540nm



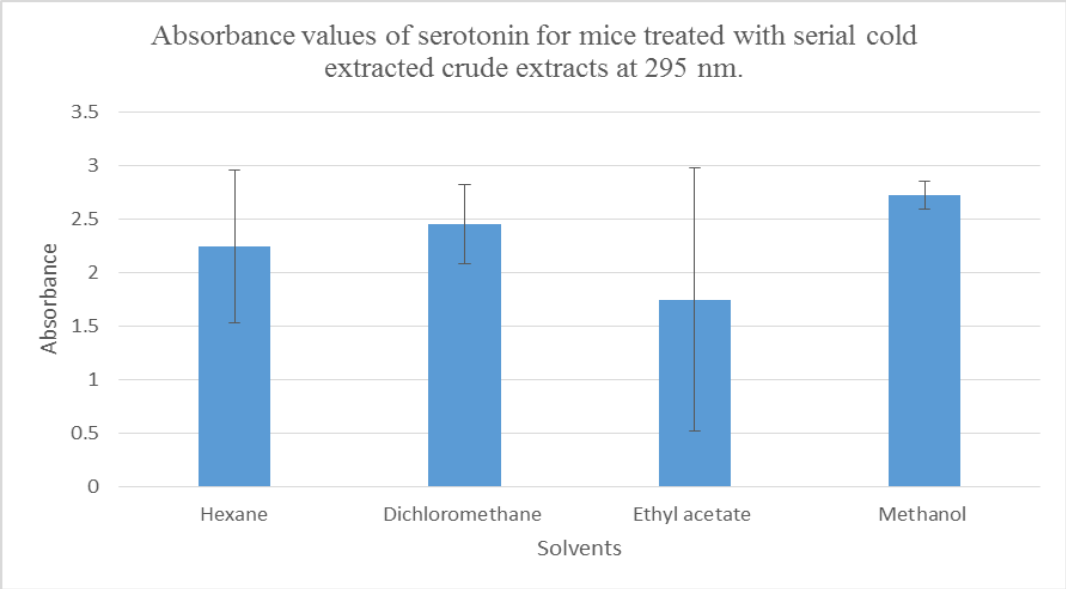
Differences in absorbance values of serotonin for mice treated with cold serial extracted crude extracts at 295nm and 540nm series i

Hexane	Dichloromethane	Ethyl acetate	Methanol
0.083	0.730	0.045	1.770
0.817	0.149	0.030	1.825
0.148	0.014	0.014	1.541
.066	0.122	0.372	1.147

Absorbance values of serotonin for mice treated with serial cold extracted crude extracts at 295 nm series ii

Hexane	Dichloromethane	Ethyl acetate	Methanol
1.826	2.587	1.543	2.780
1.464	2.000	2.676	2.806
2.902	2.343	0.085	2.765
2.779	2.872	2.675	2.526

Effects of extraction solvents on absorbance values of serotonin at 295nm



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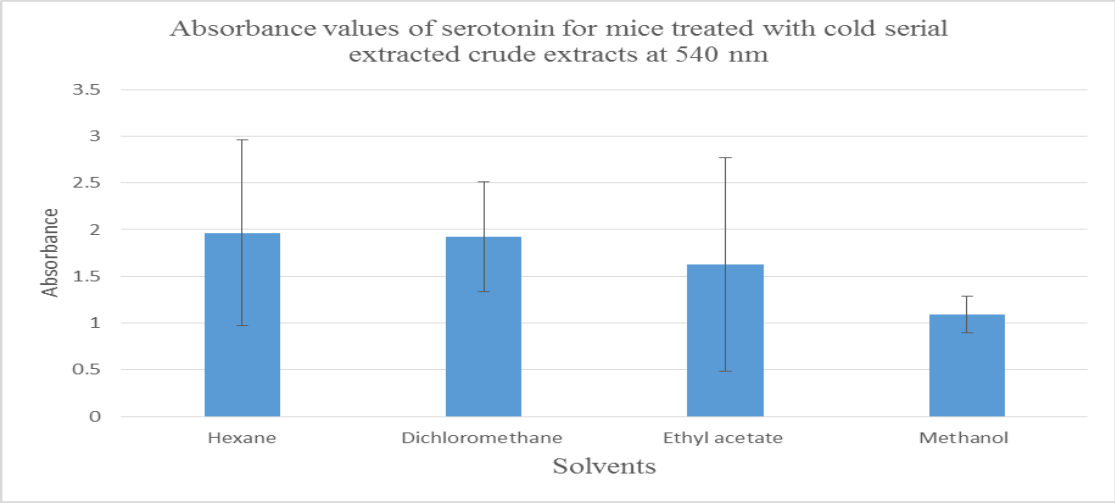
2.754	1.284	0.071	0.985
2.713	2.705	2.303	1.379

Effect of serial extraction of secondary metabolites on serotonin concentration as absorbance values at 540nm



Analysis of variance (ANOVA) of absorbance values for serotonin at 295nm

ANOVA Table						
Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Treatments	888275.1	5	177655	1.001014	0.457679	3.105875
Error	2129700	12	177475			
Total	3017975	17				

Analysis of variance (ANOVA) for concentrations of serotonin for mice treated with cold crude extracts of *Withania somnifera* at 540nm series ii

ANOVA TABLE						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatments	2.152531	2	1.076265	3.383848	0.080193	4.256495
Error	2.862536	9	0.31806			
Total	5.015066	11				

Tests of between- subject effect

Dependent Variable: Absorbance values

Source	Type III Sum of Squares	D.f	Mean Square	F	Sig.
Corrected Model	2.153[a]	2	1.076	3.384	.080
Intercept	13.822	1	13.822	43.458	.000
Treatment	2.153	2	1.076	3.384	.080
Error	2.863	9	.318		
Total	18.837	12			
Corrected Total	5.015	11			

R Squared = .429 (Adjusted R Squared = .302)

Comparison of absorbance values of serotonin for the best two cold solvent extracted products (ethanol and methanol) with Panadol as the positive control

Multiple Comparisons

Dependent Variable: Absorbance values

LSD

(I) Solvents extracts	(J) Solvents extracts	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
E	M	-.44975	.398785	.289	-1.35187	.45237
	P	.58475	.398785	.177	-.31737	1.48687
M	E	.44975	.398785	.289	-.45237	1.35187
	P	1.03450*	.398785	.029	.13238	1.93662
P	E	-.58475	.398785	.177	-1.48687	.31737
	M	-1.03450*	.398785	.029	-1.93662	-.13238

Based on observed means.

The error term is Mean SqSuare (Error) = .318.

*. The mean difference is significant at the .05 level.

Differences between absorbance

Comparison of serotonin absorbance values among mice treated with root extracts of Withania somnifera as a difference between 295nm and 540 nm series ii

Multiple Comparisons

Dependent Variable: Differences in Absorbance values LSD

(I) Test mice	(J) Test mice	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
	22	-.18760	.590745	.755	-1.43992	1.06472
11	33	-.13860	.590745	.817	-1.39092	1.11372
	44	.09360	.590745	.876	-1.15872	1.34592
	11	.18760	.590745	.755	-1.06472	1.43992
22	33	.04900	.590745	.935	-1.20332	1.30132
	44	.28120	.590745	.641	-.97112	1.53352
	11	.13860	.590745	.817	-1.11372	1.39092
33	22	-.04900	.590745	.935	-1.30132	1.20332
	44	.23220	.590745	.699	-1.02012	1.48452
	11	-.09360	.590745	.876	-1.34592	1.15872
44	22	-.28120	.590745	.641	-1.53352	.97112
	33	-.23220	.590745	.699	-1.48452	1.02012

Based on observed means.

The error term is Mean Square (Error) = .872.

Test of between- subject effets

Dependent Variable: Differences in Absorbance values

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	13.305[a]	4	3.326	55.300	.000
Intercept	19.089	1	19.089	317.353	.000
Treatments	13.305	4	3.326	55.300	.000
Error	.902	15	.060		
Total	33.296	20			
Corrected Total	14.207	19			

R Squared = .936 (Adjusted R Squared = .920)

Mean absorbance comparison table for different extraction solvents

Multiple Comparisons

Dependent Variable: Differences in Absorbance values LSD

(I) Extraction Solvents	(J) Extraction Solvents	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
A	B	-1.49425*	.173421	.000	-1.86389	-1.12461
	C	-1.22475*	.173421	.000	-1.59439	-.85511
	D	-2.06825*	.173421	.000	-2.43789	-1.69861
	E	-.05375	.173421	.761	-.42339	.31589
B	A	1.49425*	.173421	.000	1.12461	1.86389
	C	.26950	.173421	.141	-.10014	.63914
	D	-.57400*	.173421	.005	-.94364	-.20436
	E	1.44050*	.173421	.000	1.07086	1.81014
C	A	1.22475*	.173421	.000	.85511	1.59439
	B	-.26950	.173421	.141	-.63914	.10014
	D	-.84350*	.173421	.000	-1.21314	-.47386
	E	1.17100*	.173421	.000	.80136	1.54064
D	A	2.06825*	.173421	.000	1.69861	2.43789
	B	.57400*	.173421	.005	.20436	.94364
	C	.84350*	.173421	.000	.47386	1.21314
	E	2.01450*	.173421	.000	1.64486	2.38414
E	A	.05375	.173421	.761	-.31589	.42339
	B	-1.44050*	.173421	.000	-1.81014	-1.07086
	C	-1.17100*	.173421	.000	-1.54064	-.80136
	D	-2.01450*	.173421	.000	-2.38414	-1.64486

Based on observed means. The error term is Mean Square (Error) = .060.

*. The mean difference is significant at the .05 level.

Analysis of the absorbance differences (DIF) of serotonin for castrated mice series ii

Multiple Comparisons

Dependent Variable: absorbance

LSD

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
DIFA	DIFB	-1.509750*	.173433	.000	-1.87941	-1.14009
	DIFC	-1.240250*	.173433	.000	-1.60991	-.87059
	DIFD	-2.083750*	.173433	.000	-2.45341	-1.71409
	DIFE	-.069250	.173433	.695	-.43891	.30041
DIFB	DIFA	1.509750*	.173433	.000	1.14009	1.87941
	DIFC	.269500	.173433	.141	-.10016	.63916
	DIFD	-.574000*	.173433	.005	-.94366	-.20434
	DIFE	1.440500*	.173433	.000	1.07084	1.81016
DIFC	DIFA	1.240250*	.173433	.000	.87059	1.60991
	DIFB	-.269500	.173433	.141	-.63916	.10016
	DIFD	-.843500*	.173433	.000	-1.21316	-.47384
	DIFE	1.171000*	.173433	.000	.80134	1.54066
DIFD	DIFA	2.083750*	.173433	.000	1.71409	2.45341
	DIFB	.574000*	.173433	.005	.20434	.94366
	DIFC	.843500*	.173433	.000	.47384	1.21316
	DIFE	2.014500*	.173433	.000	1.64484	2.38416
DIFE	DIFA	.069250	.173433	.695	-.30041	.43891
	DIFB	-1.440500*	.173433	.000	-1.81016	-1.07084
	DIFC	-1.171000*	.173433	.000	-1.54066	-.80134
	DIFD	-2.014500*	.173433	.000	-2.38416	-1.64484

*The mean difference is significant at the 0.05 level.

The difference between absorbance values corresponding to same positions of the two tables.

Tests of Between-Subjects Effects **(** absorbance differences at 295nm and 540 nm) experiment series ii

Dependent Variable: concentration differences

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	.148[a]	4	.037	60.772	.000
Intercept	.212	1	.212	349.471	.000
Solvents	.148	4	.037	60.772	.000
Error	.009	15	.001		
Total	.369	20			
Corrected Total	.157	19			

R Squared = .942 (Adjusted R Squared = .926)

Anova: Single Factor

Multiple Comparisons of absorbances among solvents used in extraction of secondary metabolites from roots of *W. somnifera* series ii

Dependent Variable: Absorbance value differences LSD

(I) Extraction solvents	(J) Extraction solvents	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Dichloromethane	Ethyl acetate	.13850	.211757	.525	-.32288	.59988
	Hexane	-.02475	.211757	.909	-.48613	.43663
	Methanol	-1.31700*	.211757	.000	-1.77838	-.85562
Ethyl acetate	Dichloromethane	-.13850	.211757	.525	-.59988	.32288
	Hexane	-.16325	.211757	.456	-.62463	.29813
	Methanol	-1.45550*	.211757	.000	-1.91688	-.99412
Hexane	Dichloromethane	.02475	.211757	.909	-.43663	.48613
	Ethyl acetate	.16325	.211757	.456	-.29813	.62463
	Methanol	-1.29225*	.211757	.000	-1.75363	-.83087

	Dichloromethane	1.31700*	.211757	.000	.85562	1.77838
Methanol	Ethyl acetate	1.45550*	.211757	.000	.99412	1.91688
	Hexane	1.29225*	.211757	.000	.83087	1.75363

Based on observed means.

The error term is Mean Square (Error) = .090.

*. The mean difference is significant at the .05 level.

LSD Comparisons for serotonin concentrations from castrated mice treated with different solvents root extracts of W. somnifera series ii

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Blank	Panadol	-.1565345900*	.0182717203	.000	-.195479840	-.117589340
	methanol	-.1285045900*	.0182717203	.000	-.167449840	-.089559340
	ethanol	-.2170045900*	.0182717203	.000	-.255949840	-.178059340
	Water	-.0056396400	.0182717203	.762	-.044584890	.033305610
Panadol	Blank	.1565345900*	.0182717203	.000	.117589340	.195479840
	methanol	.0280300000	.0182717203	.146	-.010915250	.066975250
	ethanol	-.0604700000*	.0182717203	.005	-.099415250	-.021524750
	Water	.1508949500*	.0182717203	.000	.111949700	.189840200
methanol	Blank	.1285045900*	.0182717203	.000	.089559340	.167449840
	Panadol	-.0280300000	.0182717203	.146	-.066975250	.010915250
	ethanol	-.0885000000*	.0182717203	.000	-.127445250	-.049554750
	Water	.1228649500*	.0182717203	.000	.083919700	.161810200
Ethanol	Blank	.2170045900*	.0182717203	.000	.178059340	.255949840
	Panadol	.0604700000*	.0182717203	.005	.021524750	.099415250
	methanol	.0885000000*	.0182717203	.000	.049554750	.127445250
	Water	.2113649500*	.0182717203	.000	.172419700	.250310200
Water	Blank	.0056396400	.0182717203	.762	-.033305610	.044584890
	Panadol	-.1508949500*	.0182717203	.000	-.189840200	-.111949700
	methanol	-.1228649500*	.0182717203	.000	-.161810200	-.083919700
	ethanol	-.2113649500*	.0182717203	.000	-.250310200	-.172419700

*. The mean difference is significant at the 0.05 level.

Analysis for serotonin absorbance values for mice treated with hot solvent extracted secondary metabolites including Panadol as the positive control (Anova single factor).

ANOVA

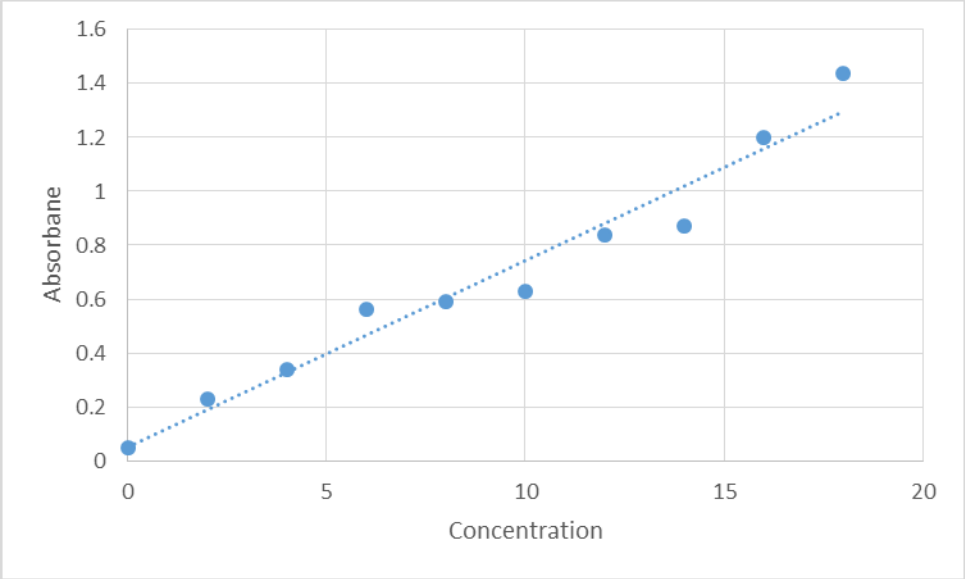
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.001603	3	1.000534	3.751963	0.041274	3.490295
Within Groups	3.200035	12	0.26667			
Total	6.201637	15				

Appendix 33: Standard calibration tables for quantitative analysis of secondary metabolites

Calibration tables for quantitative analysis of alkaloids using acornitine as the standard.

Acornitine concentration	0	2	4	6	8	10	12	14	16	18
Absorbance	0.05	0.23	0.34	0.56	0.59	0.63	0.84	0.87	1.20	1.44

Appendix 34: Acortine absorbance versus concentration (*Mcg/mL*) calibration curve.



Quacetine concentration calibration curve

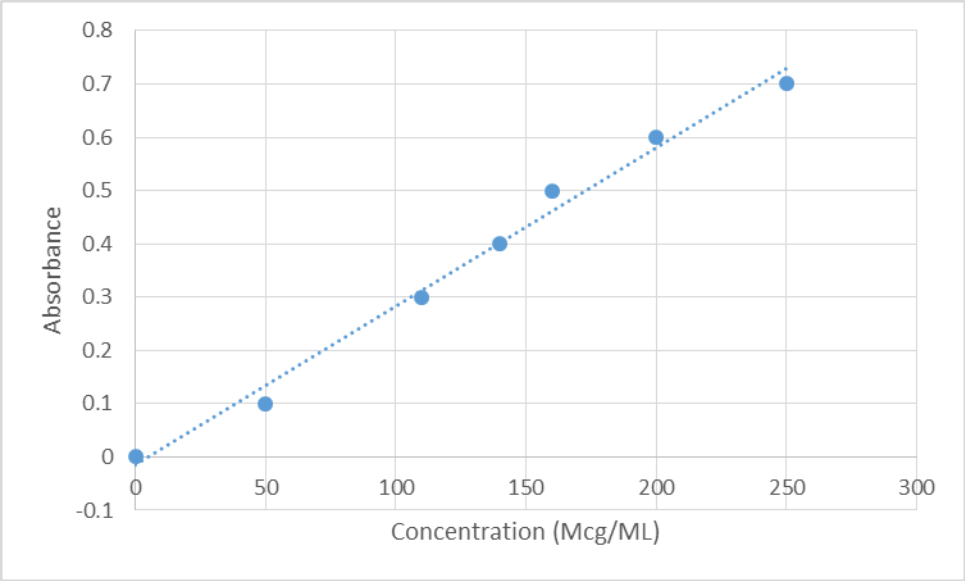
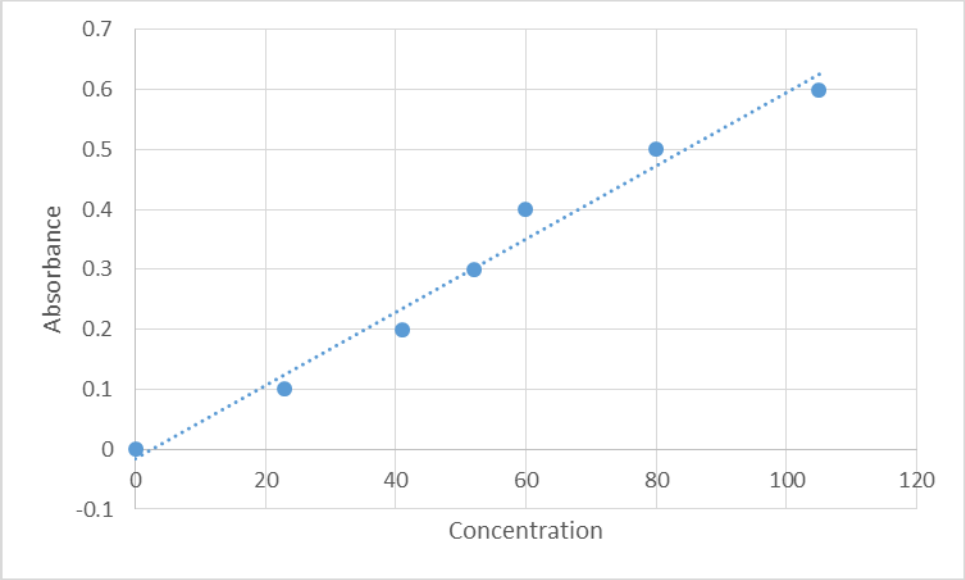
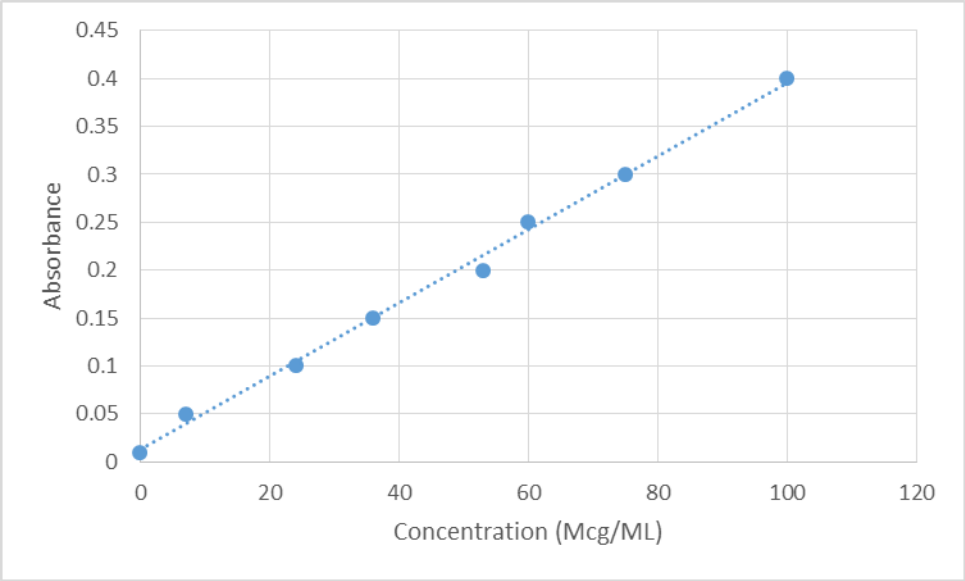


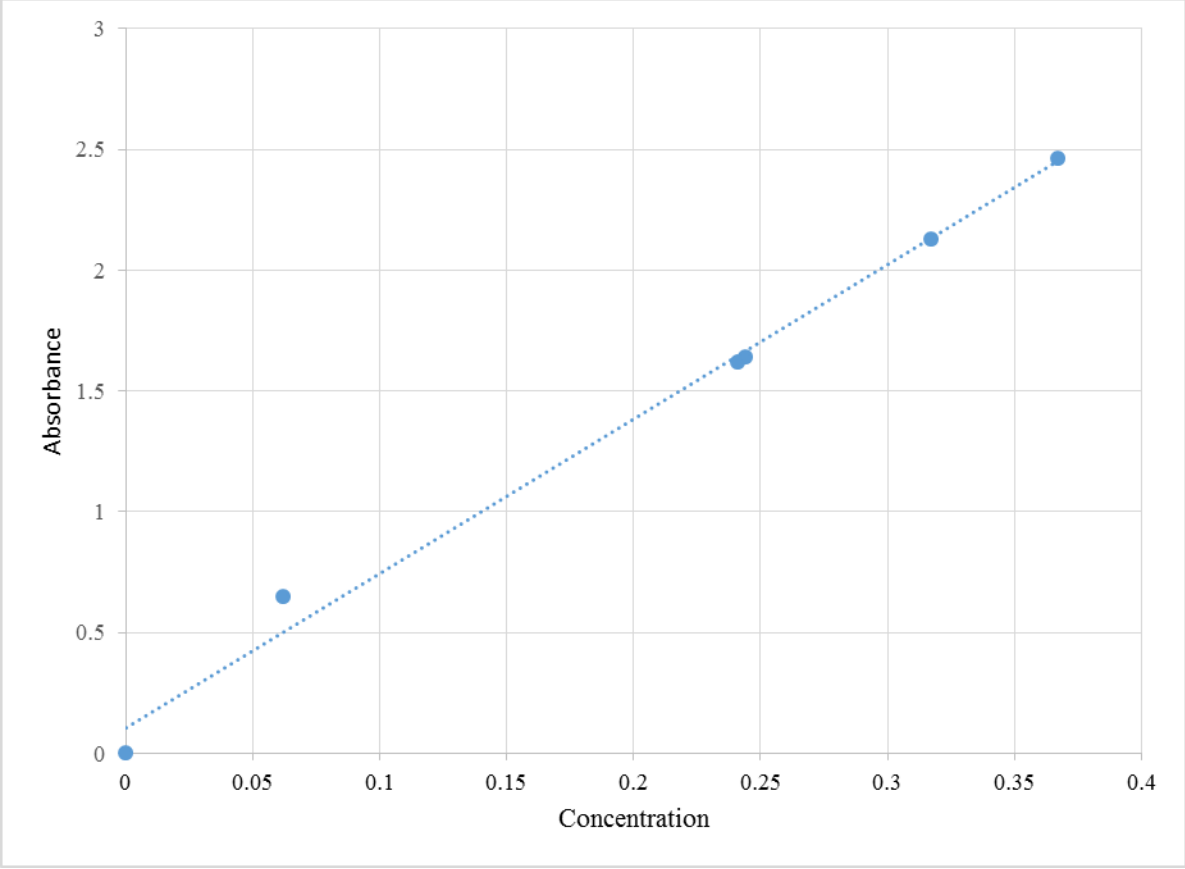
Figure showing total phenolic content calibration curve



Gallic acid calibration curve



Absorbance values for serotonin at 295 nm as was run through the spectrophotometer to provide the calibration curve for serotonin concentration determination.



Appendix B: Journal Publication

Original Research Article DOI: 10.18231/2320-1924.2018.0013

Effects of withania somnifera root extracts on serotonin secretion in suiz albino mice

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Abstract
Pain is a common manifestation of presence of disease or physical injury in humans. High levels of serotonin in the plasma has been associated with pain. This study aimed at determining the effect of Withania somnifera root extracts on serotonin levels in Suiz albino mice. W. somnifera root samples were collected and ground to small pieces. Ethanol, n-butanol, xylene and methanol were used to extract metabolites from the ground root materials using hot extraction technique. The mice were injected with the extracts separately and serotonin levels determined over a period of 12 h at 2 h intervals. Serotonin concentration was determined using Beer Lamberts method. Light absorbance by the extracts varied significantly ($F = 50.2011$ $P = 1.25x10^{-07}$. The concentrations of serotonin from mice injected with the metabolites from the selected solvents varied significantly ($F = 23.3269$ $P = 0.0000469$. In addition, there was no significant difference in light absorbance values obtained by Panadol, methanol and ethanol extracts. ($F = 3.7178$ $P = 0.089$). Extracts from W. somnifera have the ability to reduce serotonin levels in plasma. Further studies on the mechanisms involved in reducing serotonin levels by W. somnifera in blood need to be carried out. There is need for mass production of metabolites from W. somnifera.

Keywords: Withania somnifera, Root extracts, Serotonin, Suiz albino mice.

Introduction
Serotonin (5-hydroxytryptamine) is a monoamine neurotransmitter. It is a derivative of tryptophan (Kauray et al., 2013). Serotonin is predominantly found in the gastrointestinal tract, platelets and in the central nervous system of animals. Approximately 90% of the total serotonin is located in the enterochromaffin cells in the alimentary canal, where it is used to regulate intestinal movements (Maheswari and Manisha, 2015). The serotonin secreted from the enterochromaffin cells eventually finds its way out of tissues into the blood where it is stored (Rai et al., 2016).

High levels of plasma serotonin have been observed to be in direct proportionality with increase in pain (Abeer et al., 2016). However, since W. somnifera extracts have been found to reduce pain, the effect of the plant on the serotonin levels in blood has not yet been established. Reduction of plasma serotonin levels will be taken as an indicator to reduction in pain up to the required standards (Sivamani et al., 2014). Substances that reduce pain will be expected to reduce serotonin levels to the required amounts for positive physiological processes or at least reduction of serotonin levels as a course for pain reduction (Khan et al., 2015).

Withania somnifera (Solanaceae) is a xerophytic plant, found in the drier parts of Kenya, India, Sri Lanka, Afghanistan, Baluchistan and Sind and is distributed in the Mediterranean regions where it grows wildly on waste places and on road sides (Umadevi et al., 2016). It is also cultivated for medicinal purposes in fields and open grounds throughout India but not much research has been done in Kenya. The chemistry of W. somnifera has been extensively studied and over thirty-five chemical constituents have been identified, extracted, and isolated (Giri, 2013). The biologically active chemical constituents are alkaloids (isopelletierine, anaferine), steroidal lactones (withanolides, withaferins), saponinscontaining an additional acyl group (sitoindoside VII and VIII), and withanolides with a glucose at carbon 27 (sitoindoside IX and X) (Wadhwa et al., 2015). W. somnifera is also rich in iron. Research conducted at the Department of Pharmacology, University of Texas Health Science Center indicated that extracts of Ashwagandha produce GABA-like activity, which may account for the herb's anti-anxiety effects (Durg et al., 2016). The chemopreventive effect was demonstrated in a study of ashwagandha root extract on induced skin cancer in Swiss albino mice given ashwagandha before and during exposure to the skin cancer-causing agent (7,12-dimethylbenz (a) anthracene) (Kedia and Chattarji, 2018).

W. somnifera - Dunal (locally known as Ashwagandha) is widely used in Ayurvedic medicine, the traditional medical system of India. It is an ingredient in many formulations prescribed for a variety of conditions such as arthritis and rheumatism (Jayanthi et al., 2014). The plant has also been utilized as a general tonic to increase energy, improve overall health and longevity, and prevent disease in athletes, the elderly, and during pregnancy (Nabeel et al., 2013). It has been reported that W. somnifera exerts significant effect on the endocrine system. Based on the observations, that W. somnifera provides protection from free radical damage in the mouse liver; studies were conducted to determine the efficacy of W. somnifera in regulating thyroid function (Vidvashankar et al., 2014). The treatment significantly increased the serum levels of 3, 3'5-tri-iodothyronine (T3) and tetra-iodothyronine (T4), while the hepatic concentrations of glucose 6-phosphatase activity and hepatic iodothyronine 5'-monode-iodinase activity did not change significantly (Alam et al., 2015).

Back pain and arthritis are increasingly becoming a complex clinical challenge in the present society. According to the World Health Organization (WHO), up to 60% of the

Appendix C: Reseach permit

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY & INNOVATION

Ref No: AC 3165

Date of Issue: 12th April, 2019

RESEARCH LICENSE

This is to Certify that Mr.. Enoch Kiprop Tonui of Egerton University, has been licensed to conduct research in Egerton
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Appendix D: Biosafty approval

UNIVERSITY OF NAIROBI
FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY ANATOMY AND PHYSIOLOGY

P.O. Box 30197,
00100 Nairobi,
Kenya.

Tel: 4449004/4442014/ 6
Ext. 2300
Direct Line. 4448648

REF: FVM BAUEC/2020/252

Mr. Muriuki Benson Githaiga,
Egerton University
Dept. of Biochemistry and Molecular Biology
08/01/2020

Dear Mr. Muriuki Githaiga

RE: Approval of Proposal by Biosafety, Animal use and Ethics committee

Effects of Withania somnifera root extracts in modulation serotonin levels in mice.

Mr. Muriuki Githaiga SM14/3081/2011.

We refer to your proposal submitted to our committee for review and your application letter dated 13th December 2019. We have reviewed your application for ethical clearance for the study entitled **Effects of *Withania somnifera* root extracts in modulation of serotonin levels in mice.**

The numbers of mice to be used in the study, *Withania somnifera* dose levels and serotonin evaluation protocol meets minimum standards of the Faculty of Veterinary medicine ethical regulation guidelines.

We have also noted that a registered veterinary surgeon will supervise the work.

We hereby give approval for you to proceed with the project as outlined in the submitted proposal.

Yours sincerely,

Dr. Catherine Kaluwa, BVM, MSc, Ph.D
Chairperson,
Biosafety, Animal Use and Ethics Committee,
Faculty of Veterinary Medicine,
University of Nairobi.