ADDIS ABABA UNIVERSITY SCHOOL OF GRADUATE STUDIES DEPARTEMENT OF BIOLOGY
(STREAM ZOOLOGY)

IN VIVO AND *IN VITRO* ANTIFERTILITY PROPERTIES OF *Vernonia amygdalina* Del.

BY

CHIROTAW AYELE

A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the Degree of Masters of Science in Biology

JULY, 2006

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Approved by examination board

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Dr. Yalemtsehay Mekonnen

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List of Abbreviations

AACHRD- African Advisory Committee for Health Research and Development

AAU- Addis Ababa University

ACh- Acetylcholine

ANOVA- One way analysis of variance

CDC- Centers for Disease Control and Prevention

DF- dead fetuses

EHNRI- Ethiopian Health and Nutrition Research Institute

F1- Fraction one

F2- Fraction two

F3- Fraction three

F4- fraction four

FSH- Follicle Stimulating Hormone

g/kg- gram per kilogram

g/l- gram per liter

IUD- Intrauterine-devices

LF- live fetuses

LH- Luteinizing Hormone

mg/ml- milligram per milliliter

MU- Mouse Uterus

µg/ml- microgram per milliliter

ηg/ml- nanogram per milliliter

NGOs- Non Government Organizations

NRC- National Resource Council

NVI- National Veterinary Institute

OAU- Organization for African Union

PRB- Population Reference Bureau

SEM- Standard Error of the Mean

SPSS- Statistical Package for the Social Sciences

STRC-Scientific and Technical Research Commission

TLC- Thin Layer Chromatography

USA- United States of America

UNICEF- United nations Childrens Fund

UNPD- United Nations Population Division

WHO- World Health Organization

W/V- Weight by volume

ABSTRACT

Rapid population growth is becoming a problem which causes sever pressure on economic, social and cultural resources. Control of fertility using traditional antifertility plants has been practiced for many years in Africa including Ethiopia. *Vernonia amygdalina* locally known as "*Girawa*" is one of the many plants used for fertility regulation in Ethiopia. In the present study the anti-implantation and abortifacient property of 95% ethanol crude extract of the leaves of the plant *in vivo* and the spasmogenic effect of its fractions in isolated mouse uterus (MU) *in vitro* were tested. The crude extract of the plant was evaluated for its anti-implantation effect a doses of 0.385, 0.5 and 1.0 g/kg body weight in mice. Its abortifacient property was also studied at doses of 1, 2 and 3 g/kg body weight. The crude extract was further fractionated to F1, F2, F3 and F4 to see the possible uterotonic property in the presence of an agonist ACh as a control. The crude extract caused a significant ($p<0.05$) reduction in mean number of implantation sites compared to the controls at a dose of 1 g/kg. A significant difference was also observed in mean number of live fetuses and survival percent between the controls and the test groups at a dose of 3 g/kg. Thus the plant showed dose dependent antifertility effect. Among the fractions tested for uterotonic activity, F3 showed a significant uterine stimulatory effect compared to the control ACh. F1 did not show any effect. But F2 and F4 showed inhibitory effect compared to the control. The results support the traditional folk use of the leaf of the plant as antifertility agent.

Key Words: *Vernonia amygdalina, in vivo, in vitro,* anti-fertility, anti-implantation, abortifacient, female mice, estrogenic, uterotonic, uterus, Ethiopia.

1. INTRODUCTION

According to World Health Organization (WHO, 2002), traditional medicine is defined as diverse health practices, approaches, knowledge and beliefs incorporating plant animal and/or mineral based medicines, spiritual therapies, manual techniques and exercises applied singularly or in combination to maintain well-being as well as to treat, diagnose or prevent illness.

1.1. The role of traditional medicine: Its benefits and challenges

Despite many achievements in human health care in the twentieth century many of the world's population in developing countries lack regular access to affordable essential drugs. For these people modern medicine is never likely to be a realistic treatment option. In contrast traditional medicine is widely available and affordable, even in remote areas. Traditional medicine is generally accessible to most people. It is important for primary health care delivery and its use is widespread in developing countries (Zhang, 2000; Tabuti, 2004).This form of medicine is more accessible to most of the population of the third world. In Uganda for example the ratio of traditional medicine practitioners to the population is between 1:200 and 1:400. This contrasts starkly with the availability of allopathic practitioners, for which the ratio is typically 1: 20,000 or less. In addition, distribution of such personnel may be uneven, with most being found in cities or other urban areas, and therefore difficult for the rural population to access (WHO, 2002).

Traditional medicine is also cheaper than modern medicine. The cost of modern medicine is increasing by modern health technology and in many cases is inappropriate to the immediate needs of people in developing countries. Traditional medicine is sometimes the only affordable source of health care especially for the world's poorest patients. For example in Ghana, Kenya and Mali research has shown that the cost of pyrimethamine/ salfadoxine antimalarial drug can cost several dollars. Yet total out-of-pocket expenditure in Ghana and Kenya is only around US$ 6 per capita per year. Conversely herbal medicines for treating malaria are considerably cheaper and may sometimes even be paid for in kind (WHO, 2002). In addition to its cheaper price traditional medicine has a wider acceptability among the people of developing countries than does modern medicine. This could be due partly to inaccessibility of modern medicine as mentioned above. But the major contributing factor is the fact that traditional medicine blends readily in to the sociocultural life of the people in whose culture it is deeply rooted. Thus, it is popular in many developing countries because it is firmly embedded with wider belief systems (Sofowora, 1982; WHO, 2002). Furthermore traditional medicine remains popular because the traditional medicine practitioners have wisely formed an important economic contract to the mutual benefit of their practice and the population they serve (Leonard, 2001).

Apart from the advantages of traditional medicine many problems must be tackled to maximize the potential of traditional medicine as a source of health care (WHO, 2002). Perhaps one of the greatest arguments against traditional medicine today is the lack of scientific proof of its efficacy. There is no thorough scientific investigation on most of the claims made by the traditional medicine practitioners (Sofowora, 1982).

In 1964, the OAU set up the Scientific and Technical Research Commission (OAU/STRC) which organized, in Dakar in 1968, the Inter-African Symposium on the Development of African medicinal plants. The symposium decided that the efficacy of herbs used by traditional health practitioners should be tested. The OAU/ STRC has thus took initiative by funding 17 research centers all over Africa in order to stimulate research in this virgin area of proof of efficacy of medicinal plants in the region. This initiative has greatly enhanced the development of medicinal plant research but there are challenges facing institutions conducting research on traditional medicine. One of the main challenges is lack of coherent national health policies and development plans that will include traditional medicine research and development, allocation of financial and other resources of traditional medicine research. In addition there is deficiency of systematic plans for developing research capacity in traditional medicine leading to lack of a critical mass of traditional medicine researchers including traditional health practitioners. Moreover weak linkage between the traditional medicine research community, health services and policy makers hindering utilization of research results in practice and lack of tools for protecting indigenous knowledge and intellectual property rights were among the problems the institutions faced (AACHRD, 2002).

In addition to a problem of efficacy traditional medicine has a problem of safety. People think that herbs are 'safe' and 'harmless' since they are natural and are not invented in the laboratory. But utilization of herbs may possibly expose the patient to unknown dangers (Magee and Loiacono, 2004). For example aristolochic acid and other components within herbs can cause adverse renal effects and renal toxicity (Wojcikowski *et al*., 2004).

Patients are increasingly using herbs with liver disease. But the benefit remains generally unproven and concern about adverse effects is leading to closer scrutiny of those products. Some herbal remedies used to treat liver disease may be hepatotoxic themselves (Chituri and Farrell, 2000). Apart from toxicity of herbs, there is also possible adverse reaction between herbal therapy and biomedical medications. Unfortunately most patients do not inform their health care provider that they are using herbal therapy (Magee and Loiacono, 2004). This may result in adverse reactions, some of which can be serious and fatal. The most published and potentially worst herb drug reaction is that of St John's wort (*Hypericum perforatum*) and drugs metabolized by cytochrome P450, CYP 3A4 isoenzymes. Often without the knowledge of their physicians patients use St John's wort to treat symptoms of depression. This may have varies effects. For example woman taking oral contraceptives and St John's wort may cause breakthrough menstrual bleeding. It is also reported that unexpected pregnancy may result when St John's wort is regularly used with oral contraceptives (Schwarz, 2003).

Other problem with traditional medicine is the criticism that traditional medicine lack hygiene and precise dosage. Witchcraft and the evil aspects of traditional medicine also discredit this form of medicine. A medicine is supposed to promote good health and remove physical, mental or social imbalance yet certain practices of traditional medicine are designed to bring evil (through witchcraft) to other people. Such practices are not helpful to traditional medicine and should be actively discouraged (Sofowora, 1982). Traditional health care system may also incorporate harmful traditional practices or at least believe in the importance of such practices. Thus educating the traditional practitioners in particular and the community in general is important (Addis *et al.*, 2002).

Although traditional health systems are locally accessible and culturally relevant, they must first be rendered safe. Most importantly, poor documentation, lack of standardization, and the absence of regulatory mechanisms for traditional heath care practice in many countries were seen as problems to be solved (Bodeker *et al.,* 2000).

1.2. Traditional medicine and drug discovery from plants

For centuries people have used plants for healing. Fossil records date human use of plants as medicine at least to the middle of Paleolithic age some 6000 years ago (Solecki and Shanidar, 1975) and written records about medicinal plants date back at least 5000 years (Swerdlow, 2000). Until recently, plants were important sources for the discovery of novel pharmacologically active compounds, with many blockbuster drugs being derived directly or indirectly from plants (Farnsworth, 1988; Cordell, 2000). The strong historic bond between plants and human health began to unwind in 1897, when synthetic acetyl salicylic acid (aspirin) is introduced to the world (Pierpoint, 1994). The twentieth century became a triumph for the synthetic- chemistry- dominated pharmaceutical industry which replaced natural extracts with synthetic molecules that often had connection to natural products. However, the benefits of modern drugs are felt primarily in developed countries, leaving almost 75 % of the world population without access to the modern health care products. Thus developing countries continue to rely on ethnobotanical remedies as their primary medicines (Fabricant and Farnsworth, 2001).

In technologically less developed societies like in most countries of Africa, traditional modes of thought still dominate the forms of medical practice seen in those societies

(Williamson *et al*., 1996). Traditional medicine played a crucial role in combating multiple and complex conditions affecting Africans. Because of its popularity, accessibility and affordability, more than 80% of the people in the region continued to rely on it for their health care needs (WHO, 2003). Introduction of modern medicine alone does not adequately provide for the comprehensive or integral health care needs of developing countries. Consequently in many communities the practice of simultaneous use of traditional and western medicine continues. Indeed it is always been difficult to reach poor people with development aid, particularly in health care where most resources benefit the middle classes in urban hospitals. Thus traditional medicine is often the only affordable and accessible form of health care (Naur, 2001).

Many modern drugs have their origin in ethnopharmacology (Patwardhan, 2005). A survey of pharmacopoeias of developed and developing countries was done to determine whether ethnobotanical information did indeed lead to useful drug discovery. The survey showed that from 122 compounds identified in the study, 80% of the compounds were used for the same (or related) ethnobotanical purposes. Information based on long-term use of plants by humans (ethnomedicine) likely helps to isolate safer active compounds from plants than isolating active compounds from plants with no history of human use (Fabricant and Farnsworth, 2001). Thus instead of relying on trial and error, as in random screening procedures, traditional knowledge helps scientists to target plants that may be medicinally useful (Cox and Balick, 1994). Indeed traditional medicine is a potential source of new drugs and a source of cheap starting products for the synthesis of known drugs. Some examples include reserpine from *Rauwolfia* species, viablastine from *Catharanthus roseus*

or the discovery of a contraceptive in the Zoapalte (*Montanoa tomentosa* cerv.) (Sofowora, 1982).

1.3. Plants important in reproduction and fertility control

1.3.1. *Human population growth and family planning*

According to U.S. Census Bureau estimates, world population hit the six billion in June 1999. This figure is over 3.5 times the size of human population at the beginning of the 20th century. The time required for global population to grow from 5 to 6 billion, which took 12 years was shorter than the interval between any of the previous billions (U.S. Census Bureau, 2002). In 2005 G.C., world population is estimated to be 6.5 billion. This number is expected to increase by 2.5 billion over the next 45 years, 6.5 billion to 9.1 billion in 2050. Today, 95 per cent of all population growth is absorbed by the developing world and 5 per cent by the developed world. In the case of Ethiopia total population is estimated to be 74.2 million in 2005 with current growth rate of 2.9 per cent per year. The average birth per woman is 6.14 and the contraceptive prevalence is 8.1 per cent (United Nations Population Division, 2004).

Growth of human population has been underway for thousands of years and was never a problem until recently. Currently, overpopulation led to series social and environmental problems such as poverty, overcrowded slums, crime, pollution of air, and water and depletion of the protective ozone layer (Greep, 1998). Measures that will help slow population growth are relatively less expensive than reforming our economies and

industries. Our future well-being depends on increased access to family planning and reproductive health services in developing countries and decreased consumption by people in wealthy countries (Speidel, 2000).

For millennia, men and women have made use of different methods to prevent conception. Traditional methods that date back centuries provided inspiration for the development of some of today's most reliable contraceptives including the birth control pill and the copper bearing Intrauterine-devices (IUD) (James and Kepron, 2002). Apart from means of controlling population growth rates, contraceptive agents help to improve the health conditions of woman of reproductive age. They also reduce the risk of maternal morbidity and mortality by protecting woman from giving birth while too young (Abebe *et al.,* 2003). Nowadays there are different forms of contraception both natural and artificial such as billings, temperature method, sympto-thermal method, pills, injectables, Intrauterine-devices, cup or diaphragm, condom and sterilization (CDC, 2000)

Compared to women else were in the world, women in sub-Saharan countries bear children at younger ages, have larger families and make much less family planning (Robey *et al*., 1992). Each time a woman in one of the world's poorest countries become pregnant, her risk of dying from that pregnancy is as much as 200 times greater than the risk for woman in United States or Europe. Because of the high fertility rate, poor health conditions in general and inadequate availability of medical care, the risks of pregnancy are higher in Africa than anywhere else in the world (WHO and UNICEF, 1996). Providing contraception to woman who desires it could reduce maternal deaths by as much as one-third (Population Reference Bureau, 1986). Contraceptives allow a woman to avoid

pregnancy and child-bearing once she reaches her desired family size. A mother's risk of dying climbs steadily as the number of births increases. Women who have had five or more births are 1.5 to 3 times more likely to die from pregnancy-related causes than are women who have had only two or three births (National Resource council, 1989).

One of the fundamental goals of family planning is to make "every child a wanted child". Some unwanted pregnancies are aborted. Although consciously documented, many maternal deaths related to pregnancy are associated with incomplete abortions, whether self induced or induced by a trained or untrained practitioner (CDC, 2000).The major causes of maternal mortality relate to abortion are hemorrhage and sepsis, he latter is an infection that spreads the uterus to the abdominal cavity and then to the overall body. These conditions, which are caused by retained fetal or placental tissue, can lead to septic shock and death. In Addis Ababa, Ethiopia, post-abortion complications rank as the most common cause of maternal death, and are particularly common among young woman who have no other child (Kwast *et al*., 1986). As contraceptive use increases in Africa, so will family survival. Planned pregnancies, which are generally safer for the mother, produce children who are usually healthier than children from unplanned pregnancies (CDC, 2000).

Family planning has been promoted through several methods of contraception. But due to series adverse effects produced by synthetic steroidal contraceptives, attention has now been given to indigenous plants for possible contraceptive effects (Ghosh and Bhattacharya, 2004). Though oral contraceptives are highly effective means of birth control some women discontinue their use because of bothersome side effects such as acne hirsutism and weight gain. In addition oral contraceptives adversely affect thrombolysis,

carbohydrate metabolism and lipid profiles. This may be why they have been associated with an increased risk of myocardial infarction (Stampfer *et al.,* 1988; Pettiti *et al.,* 1996; Lewis *et al.,* 1997). From case control study, a three-fold increase in risk of venous thrombosis was reported for oral contraceptive users versus non users (Rosendaal *et al.*, 2003). However, the recognition that the metabolic and cardiovascular side effects associated with oral contraceptive use were dose dependent led to the development of oral contraceptive formulations containing the minimum steroid doses necessary to inhibit ovulation (Darney, 1997).

1.3.2. *Mechanism of action of antifertility plants*

Plant drugs have been used since time immemorial for their effects upon sex hormones particularly for suppressing fertility, regularizing menstrual cycle, relieving dysmennoroea, treating enlarged prostate, menopausal symptoms, breast pain and during and after childhood (Williamson *et al.,* 1996). Specific biological effects under the division of fertility regulating category are non- specific contraceptive or antifertility effects, abortifacient, uterine stimulant and uterine relaxants, labour induction and labour inhibition oxytocic and anti- oxytocic, oestrogenic and anti- oestrogenic, progestrogenic and anti- progesterogenic, ovulatory and anti- ovulatory, androgenic and anti- androgenic, spermicidal and anti- spermatogenic effects (Soejarto *at al.,* 1978). The site of action of antifertility agents in females consists of the hypothalamus, the anterior pituitary, the ovary, the oviduct, the uterus and the vagina. The Hypothalamus controls the action of the uterus via follicle stimulating hormone (FSH) and Luteinizing hormone (LH) releasing hormones. Antifertility agents may therefore exert their effort at this level either by

disrupting hormonal function of the hypothalamus and/ or the pituitary, or by interrupting the neural pathway to the hypothalamus that control the liberation of gonadotrophin-releasing hormones (Bullock *et al.*, 1995).

Early researchers in the area of female fertility regulation focused their attention to phytoestrogens following the recognition that excess ingestion of plants containing estrogenic compounds resulted in infertility in animals and humans (Williamson *et al*., 1996). Phytoestrogens are any plant compounds structurally and/ or functionally similar to ovarian and placental estrogens and their active metabolites (Patritia and Heater, 2001). They include a vast variety of structurally diverse compound. These include isoflavones found in soy, lignans found in grains, stilbenes found in the skin of grapes and fungal metabolites, for example, macrolides (Mueller, 2002). Plants with estrogenic property can directly influence pituitary action by peripheral modulation of LH and FSH decreasing secretion of this hormones and block ovulation. The decrease in LH and FSH could explain ovulation and estrous cycle blockage by some plant extracts. All substances able to inhibit this release could provoke an ovulation disruption by decreasing the number of mature follicles (Waterhoff *et al.*, 1994).

The mammalian uterus which is the main site of antifertility effects comprises outer myometrial cells which are responsible for the contraction of the uterus, inner endometrial cells which are secretary and the cervix. The physiology of the uterus and its response to oxytocic drugs differs greatly in different species. Moreover the type of motility and the threshold for the response to oxytocic drugs differs with the phase of the oesterus cycle and the stage of pregnancy (Williamson *et al*., 1996).

Several active chemical constituents accountable for uterotonic effects are discovered in various plant species from time to time. For instance two triterpenic saponins called ardisiacrispin A and B are isolated and characterized from the crude extracts of *Ardisia crispa* root. This plant root is used by Thai people for washing out dirty blood in woman suffering from menstrual pain. The isolated compounds were responsible for utero-contracting properties in treated rats (Jansakul *et al*., 1987). An active indole alkaloid compound, Yuehchukene isolated from the plant *Murraya pankalata is* used in China to regulate fertility because it has potent anti-implantation effect (Kong *et al.,* 1985).

1.3.3. *Use of antifertility plants in Ethiopia*

The use of herbs for preventive or abortive purposes of fertility control exists in Ethiopia for many centuries (Desta, 1994). Due to its significant geographical diversity and many languages, cultures and beliefs have in turn contributed to the high diversity of traditional knowledge and practices of the people, which includes the use of medicinal plants (Tewelde, 1991). Despite its significant contribution to the society, traditional medicine is given little attention in modern research and development. Thus an important aspect of sustainable use of our botanical resources is to enhance our knowledge of their biological and pharmacological effects and to characterize their chemical constituents (Dagne, 1999). Recent works in Ethiopia have shown medically important antifertility plants (Desta, 1994; Makonnen *et al.,* 1997; Yalemtsehay, 1999; Gebrie *et al.,* 2005).

1.4. Description and Medicinal use of *Vernonia amygdalina*

Vernonia amygdalina Del., known by its local name *Grawa* in Amharic is a shrub or a small tree usually branched near the base, 2-10m high, barks rough with dense black streaks, and grows under a range of ecological zones in Africa. It belongs to the Family Asteraceae. The plant is commonly known as bitter leaf and is a popular African vegetable (Hutchioson and Daizein, 1963 cited in Bonis *at al*., 1995; Oboh, 2005). In Nigeria the macerated leaves of the plant are used in making soup while the water extract serves as tonic drink for prevention of certain illnesses. The leaves have found relevance in traditional folk medicine as anti-helminth and antimalarial activity (Farombi, 2003). The chopped root of *V. amygdalina* is used for treatment of sexually transmitted diseases in Guruve district Zimbabwe (Kambizi and Afolayan, 2001). In South Africa the root of the plant is used for its antifertility effect and for treatment of amenorrhoea (absence of menstruation) and dysmenorrhoea (Wyk and Gericke, 2002 cited in Steenkamp, 2003).The leaves of *V. amygdalina* are crushed to make a paste to be rubbed on the body of livestock for destroying ectoparasites (Chifundera, 1998). In Ethiopia the leaves of the plant are used to treat skin wound by Zay people (Giday *et al*., 2003). It is one of the traditionally used antifertility plants in Ethiopia. Hydroalcoholic extract of the leaves was reported to be used in Ethiopia as traditional medicine for fertility regulation. Preliminary study also confirmed that the plant has antifertility effect (Desta, 1994).

Earlier investigations on *V. amygdalina* showed that purified chloroform fractions identified as vernodaline, vernolide and vernomygdine elicited cytotoxic effects in human carcinomia narsopharynx cells (Kupchan *et al*., 1969 cited in Farombi, 2003). Lutiolin,

luteoline 7-O, -β glucuronoside and lutioline 7-O, - β- glucoside flavonoid compounds isolated from the leaves of *V. amygdalina* have antioxidant activity (Igile *et al*., 1994). Several stigmastine type saponins such as vernoniosides A1, A2, B2, D3, A4, and C have been identified from the leaves of the plant (Ohigashi *et al*., 1991; Jisaka *et al*., 1992; Kamperdick *et al.,* 1992).

A growing body of literature in behavioral, ecological and pharmacological sciences suggests that animals use certain plants for the control of parasitic infection and certain related illnesses (Cousins and Huffman 2002). More than ten years ago, Huffman reported on a sick female chimpanzee that recovered her health after she sucked out the bitter pith of *V. amygdalina* (a plant not normally eaten by health chimps), but this observation was met with skepticism. However this self-medicating behavior was subsequently witnessed in other chimps. Chemical analysis revealed that the pith of *V. amygdalina* contains sesquiterpene lactones that have activity against intestinal parasites (Smith, 2002). It has been also increasingly apparent that chimpanzees in Africa and their human counterparts share strong similarities in the plants they use for the treatment of similar diseases (Cousins and Huffman, 2002).

Acute toxicity test done on crude chloroform extract of *V. amygdalina* in Swiss albino mice showed that the extract did not cause significant acute toxicity at doses less than or equal to 798 mg/kg of body weight (Animut,2002). Though detailed acute toxicity test was not done in this study, the extract was evaluated for its safety up to 5 g/kg body weight in mice prior to the study.

Figure 1.4. A photograph showing *Vernonia amygdalina* with its green foliage (The picture was taken from AAU, Science Faculty compound), September, 2005.

2. OBJECTIVE OF THE STUDY

2.1. General objective

-To test the possible anti-fertility properties of the leaves of *Vernonia amygdalina* (Del.) in *vivo* and *in vitro*

2.2. Specific objectives

2.2.1. To evaluate the effect of the leaves *V. amygdalina* on implantation and its possible abotifacient property

2.2.2. To determine whether the leaves of the plant have uterotonic property

3. MATERIALS AND METHODS

3.1. Plant material collection

The leaves of *Vernonia amygdalina* were collected in January 2005 from the Science Faculty compound, AAU, Addis Ababa. Plant sample is identified in National Herbarium of AAU and specimen is deposited in the Herbarium with specimen number CH-1.

3.2. Plant crude extraction and fractionation

The fresh leaves of the plant were collected, shade dried and ground into coarse size for preparation of the extract in Biomedical laboratory of the Department of Biology, AAU. The dried and ground leaves of the plant were mixed in 95% ethanol 1:10 ratio (W/V) in separate Erlenmeyer flasks and placed in orbital shaker (GFL, model 3020, Germany) at room temperature for 24 hours. The extract was then filtered with cotton and Whatman filter paper (15.0 cm size).The solvent was removed from the extract under reduced temperature and pressure using a Rotavapor (Buchi RF121, Switzerland). The extract was then freeze dried in a lyophilizer (Vacuubrad, GMBH Germany). From 650 g dry leaf 40 g of crude extract was obtained. The crude extract was kept in a refrigerator at -20 ^{0}C until use for the experiments.

For *in vitro* test 10 g of the crude extract was fractionated by means of column chromatography by using silica gel of particle size 0.063-0.200 mm (mesh size 70-230) and eluting with chloroform and subsequently with chloroform containing different

proportion of pure methanol. Fractions were collected and examined by thin layer chromatography and fractions having similar substances were pooled (Table 1).

From the 10 g of crude extract, 1.4, 2.1, 1.8 and 1.9 mg of Fraction 1 (F1), Fraction 2 (F2), Fraction 3 (F3) and Fraction 4 (F4) was obtained respectively.

Table 3.1 Different fractions obtained from 95% ethanol extract of *V. amygdalina*.

Solvent proportion	**Fractions before pooling**	**Fractions after pooling**
100 % Chloroform	f1 *	F1 *f1, f2
95% chloroform + 5% methanol	f2 *	
90 % Chloroform + 10% methanol	f3	F2
80% Chloroform + 20% methanol	f4	F3
50% Chloroform + 50% methanol	f5 *	F4 *f5, f6
100% methanol	f6 *	

* Pooled after TLC was made

3.3. Laboratory animal preparation

Adult virgin female Swiss albino mice weighing 25-35 g were used for all the experiments. The mice were obtained from animal house of the Department of Biology, AAU. They were kept in uniform experimental conditions of temperature, food and water exposed to 12 hours of light and 12 hours of darkness. Pellet diet and tap water were provided *ad libitum* to all mice in their respective separate cages.

3.4. Pharmacological screening

3.4.1. *Determination of anti-implantation effect of the crude extract*

Four groups of matured virgin female mice (n=5 mice/group) weighing 25-35 grams were employed for the test. Group I was control group and group II, III and IV were experimental groups. The mice were left overnight with males of proven fertility in the ratio of one male for two females. Vaginal smear was taken washing with normal saline solution using a micropipette every next morning. The wet smear of vaginal washing was dropped in a slide and was examined microscopically until the detection of spermatozoa. Day one of pregnancy was confirmed by the presence of spermatozoa in the vaginal smear. After mating group I (control) were treated orally with vehicle (0.5 ml of distilled water) and the experimental groups (groups II, III and IV) were given with the crude extract at a dose of 0.385, 0.5 and 1.0 g/kg body weight respectively suspended in distilled water from day 1 to day 7 of pregnancy. On the 10^{th} day of pregnancy the mice were sacrificed with cervical dislocation and uterine horns were inspected for number of implants (Kamath and Rana, 2002).

3.4.2. *Determination of abortifacient property of the crude extract*

Four groups of mature virgin female Swiss albino mice (n =5 mice/group) weighing 25–35 grams were used for the experiment. Group I was control group and group II, III and IV were experimental groups. The mice were caged overnight with males of proven fertility and vaginal plug was examined next morning by lifting the tail for sign of pregnancy. The

day on which vaginal plug was detected was taken as day 1 of mating. On the 18 day of pregnancy group II, III and IV were given the crude extract orally suspended in distilled water at a dose of 1, 2 and 3g/kg body weight respectively. Group I were treated with the same amount of vehicle and with the same route of administration as the experimental group. All animals were observed for 48 hours for possible abortion and other clinical signs following the method of Anaga *et al*., (2004). After 48 hours the mice were sacrificed by cervical dislocation and laparotomized. Then uterine horns were inspected for number of live and dead fetuses (De Freitas *et al.,* 2005).

3.4.3. *In vitro mouse assay for uterotonic activity using extract fractions*

Non pregnant or virgin female albino mice were used for testing utero-contracting (uterotonic) activity of the different fractions of the plant. The mice were sacrificed by cervical dislocation and the uterine horns were removed and placed in a dish containing physiological De Jalon's solution of the following composition (g/l): NaCl, 9 gram; KCl, 0.42 gram; $NaHCO_3$, 0.5 gram; D-Glucose, 0.5 gram; $CaCl_2$, 0.062 gram. Approximately 1.5- 2cm long uterine strip was cut and each strip was mounted in a 25 ml thermostatically regulated organ bath containing the solution. The organ bath containing the solution was maintained at about 35 to 37^0 C and was bubbled with a mixture of air containing 95% O_2 and 5% CO_2.

The uterine strip was tied with a string to a transducer (Grass FT.03) that was connected to a Grass model 7H, Serial 42V45J polygraph (Grass Instrument Co., Quincy, Mass., USA) to record isometric contractions. A tension of 1 gm was applied to the tissue and was

allowed to equilibrate for at least 30 minutes before starting the test. Acetylcholine (ACh) was used as a stimulant to record contractions. A response curve was constructed using ACh at a final bath concentration each of 40ηg/ml, 80ηg/ml, 160ηg/ml and 320ηg/ml with a time interval of 3 min. Each time, the added ACh was left in contact with the tissue for 30 sec and then washed with De Jalon's solution. It was then left to resume its normal contraction. ACh of 80ηg/ml was selected as the control concentration that induced sub-maximal contraction of the uterine tissue.

Extract sample of the given concentration was added in the organ bath and left in contact with the tissue for 5min. The control ACh 80 ηg/ml was added at the end of the 5 min in the presence of the sample and then washed after 30 sec. After the rhythmic contraction resumed, the control ACh was added to establish the reversible contraction capacity of the tissue and also to test the extent the extract acted upon the uterine tissue. The same procedure was repeated for different samples at different concentrations. Each sample was tested at 5, 10, 15 and 20µg/ml as final bath concentration for fraction F2 and F4 and at 2, 4 6 and 8µg/ml for fraction F3. This method was adopted from previous workers (Desta, 1994 and Yalemtsehay, 1999).

3.5. Statistical data analysis

Data were analyzed by using SPSS (version 13) software. All values were expressed as the mean value ± standard error of the mean (SEM) and the level of significance was calculated by one way analysis of variance (ANOVA). This was followed by Scheffe post-hoc test for comparisons of the means of the various doses and fractions. A probability level of less than 5% ($p<0.05$) was considered statistically significant difference between test and control groups as well as among test groups for measured values.

4. RESULTS

4.1. Effect of the crude extract on implantation

Table 4.1. Anti-implantation effect of 95% ethanol crude leaf extract of *V. amygdalina* in mice.

Treatment	No. of implantation sites	95% confidence interval of the mean
Vehicle (control)	6.2 ± 0.58 *1g	5.66- 6.78
0.385g/kg bwt	6.0 ± 0.68 *1g	5.37- 6.63
0.5g/kg bwt	5.4 ± 0.51	4.89- 5.91
1 g/kg bwt	1.8 ± 0.97	0.83- 2.77

Data= Mean ±SEM, P<0.05, n=5

The mean number of implantation sites for mice that received 1g/kg body weight of the extract was significantly ($p<0.05$) lower than those in the control mice (Table 4.1).There was also statistically significant difference in mean number of implantation sites between the test groups which received 0.385 g/kg and 1g/kg body weight of the extract. But no statistically significant ($p<0.05$) difference was observed between the controls and those which received 0.5g/kg. In addition the difference observed between the test groups which received 0.5 g/kg and 1g/kg body weight of the crude extract was not statistically significant ($p<0.05$). Moreover, no statistically significant ($p<0.05$) difference was observed between the test groups which received 0.385 g/kg and 0.5 g/kg.

4.2. Abortifacient activity of the extract in mice

Table 4.2. Abortifacient effect of 95% ethanolic crude leaf extract of *V. amygdalina* in mice

Treatment	Fetuses		
	Live	Dead	Survival (%)
Vehicle (Control)	6.40 ± 0.75 *3	0.20 ± 0.20	98.00 ± 2.00 *3
1g/kg bwt	5.40 ± 0.51 *3	0.60 ± 0.40	90.46 ± 6.58
2g/kg bwt	3.00 ± 1.27	0.60 ± 0.40	50.28 ± 21.04
3g/kg bwt	1.20 ± 0.58	1.80 ± 1.60	37.00 ± 15.62

Survival % = $\frac{\text{Live fetuses}}{\text{Live +dead Fetuses}}$ X 100%

As shown in table 4.2., no statistically significant ($p<0.05$) difference was observed in the mean number of live fetuses (LF) and survival percent between the control and test groups which received 1g/kg and 2 g/kg body weight of the extract. But there was statistically significant difference between the control and the test group which received and 3g/kg body weight of the extract. The difference between the test groups which received 1g/kg and 3g/kg body weight of the extract was also statistically significant. There was also statistically significant difference in survival percent between the control and the test group which received 3g/kg body weight of the extract. But no statistically significant difference was observed in mean number of fetuses between test groups which received 2g/kg and 3g/kg body weight of the extract. In addition there was no statistically significant difference in the mean number of dead fetuses (DF) among any of the groups. Moreover, no statistically significant difference was observed in mean survival percent among the test groups.

Dose- response curve showing the mean number of live and dead fetuses in mice dosed with 1, 2 and 3g/kg body weight is shown in figure 4.1.

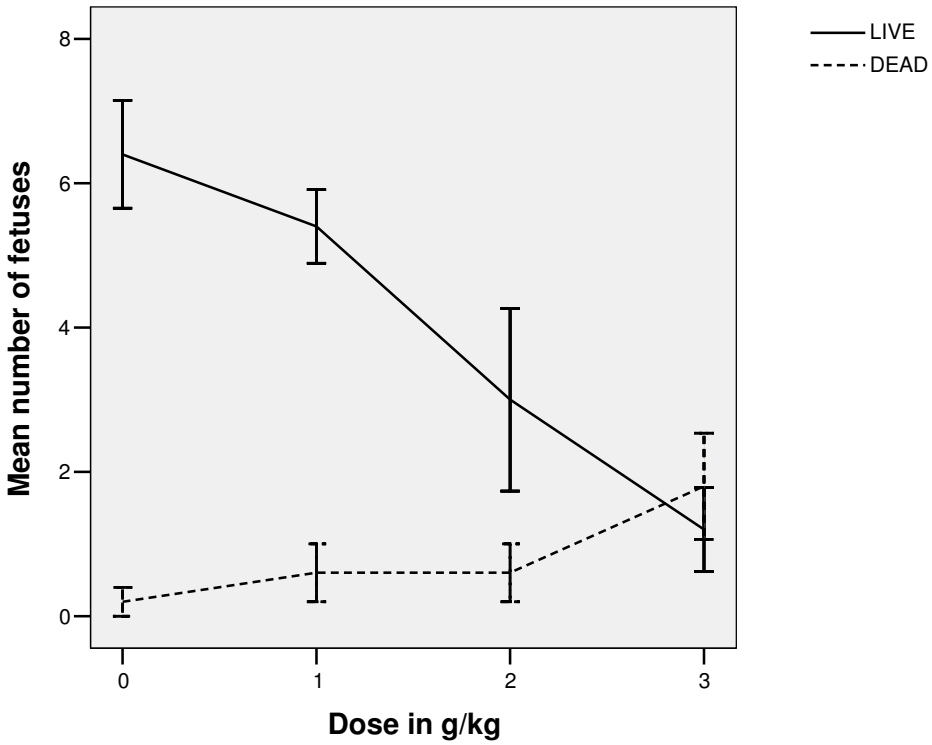

Figure 4.1: Dose- response curve (line graph) showing the mean number of live and dead fetuses in mice which received the extract at a dose of 1, 2 and 3g/kg body weight.

4.3. *In vitro* effects on mouse uterus

Isolated mouse uterine preparations were used to study the spasmogenic or contractile effect of the crude 95% ethanol extract of V. *amygdalina* and its different fractions. The dose dependent effects of the crude extract and its different fractions on mouse uterus expressed as mean % contraction ± SEM values are given in table 4.3.

As shown in the table, the mean percentage contractions recorded for the crude extract was significantly different from the control ACh alone at $p<0.05$ and $F= 22.0$. Though not statistically significant the crude extract showed uterine contraction effect more than the control ACh when the dose was increased from 80 to 120 µg/ml. But the contraction effect decreases when the dose is increased to 160 and 180 µg/ml.

The mean percentage contractions recorded for F2, F3 and F4 were significantly different from the control ACh alone at $p<0.05$ and $F= 128.7$, 61.4 and 59.5 respectively. F2 showed inhibitory effect on mouse uterus showing a dose dependent decease in inhibition as the dose increases from 5 µg/ml (61.2 %) to 10 µg/ml (93.8 %). From its minimum inhibition effect (93.8 %) at 10 µg/ml, the inhibition effect increase to 83.8 % at a dose of 15 µg/ml. Then the inhibition effect further increases to 41.6 % as the dose increases from 15 µg/ml to 20 µg/ml. On the other hand, F3 showed significant contraction effect as the dose was increased from 2 µg/ml (77.1 %) to 4µg/ml (89.3 %) and reaches a maximum mean contraction of 109.3 % at a dose of 6 µg/ml. However, with a higher dose of 8 µg/ml, the mean contraction decreases to 82.1 %. On the contrary, F4 showed a dose dependent decrease in inhibition of contraction as the done was increased from 5 µg/ml (74.0 %) to

10 µg/ml (80.1 %). The inhibition effect further decreases to 96.5 % as the dose increases to 15 µg/ml and reaches its maximum decrease in inhibition (101.8 %) at a dose of 20 µg/ml.

Dose response curve (line-graph) showing the mean percentage contraction by ACh in the presence of the crude extract and its fractions (F2, F3 and F4) at different organ bath concentration as compared to the control ACh alone on isolated mouse uterus is shown in figures 4.2, 4.3 and 4.4.

Polygraph tracing showing the effect of ACh in the absence and presence of F3 on isolated mouse uterus at different organ bath concentrations is shown in figure 4.5.

Table 4.3: The effect of 95% ethanol crude extract of *V. amygdalina* and its different fractions on ACh-induced contraction of Mouse Uterus (MU)

Type of extract	**Groups**	**Extract Concentration (µg/ml)**	**Contractile Response (%)**	**F-value**
Crude	1	80	85.3 ± 1.8 *2, 3	22.0
	2	120	101.6 ± 1.5 *4	
	3	160	96.6 ± 1.7	
	4	180	88.9 ± 1.2	
F2	1	5	61.2 ± 1.9 *2,3,4	128.7
	2	10	93.8 ± 1.8 *3,4	
	3	15	83.8 ± 2.7 *4	
	4	20	41.6 ± 3.8	
F3	1	2	77.1 ± 2.3 *2,3	59.5
	2	4	89.3 ± 1.8 *3	
	3	6	109.3 ± 3.2 *4	
	4	8	82.1 ± 1.9	
F4	1	5	74.0 ± 2.0 *3,4	61.4
	2	10	80.1 ± 1.7 *3,4	
	3	15	96.5 ± 1.3 *4	
	4	20	101.8 ± 1.6	

Control ACh = 80ηg/ml of organ bath concentration, n =5 MU for the crude extract and for each of the fractions.

Responses were expressed as % of the initial concentrations induced by spasmogenic ACh prior to the addition of the plant extracts. Data of contractile response were expressed as mean ± SEM of five mouse uterus preparations. Significant relative to the control: * $P<0.05$

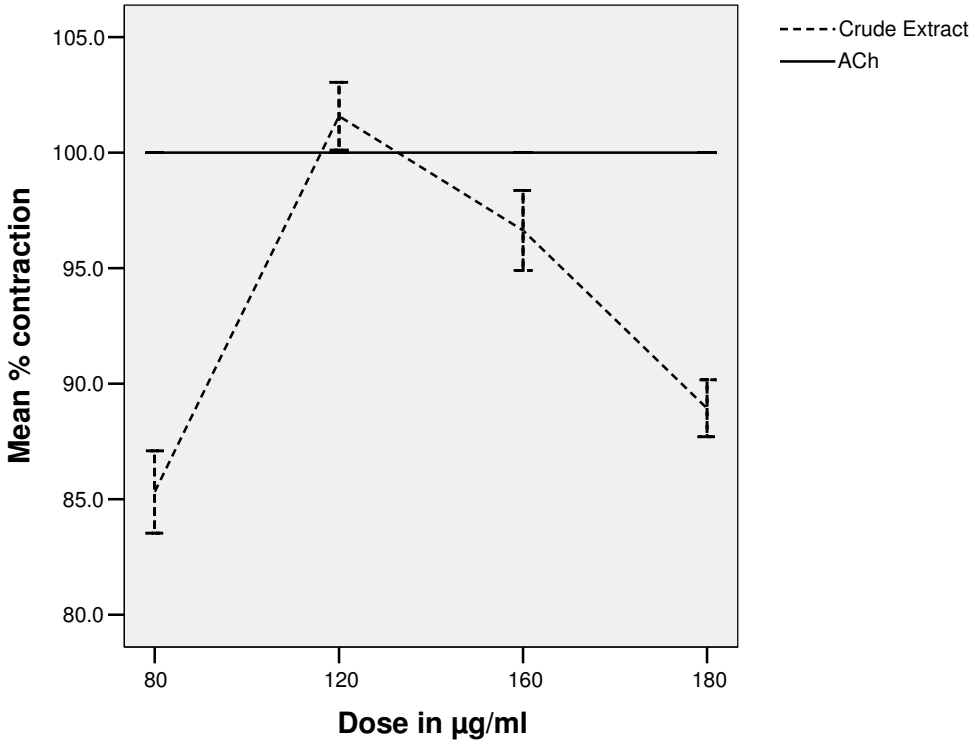

Figure 4.2. Dose response curve (line-graph) showing the mean percentage contraction by ACh in the presence of the 95% leaf ethanol crude extract of *V. amygdalina* at different organ bath concentrations as compared with the control ACh alone on isolated mouse uterus. Vertical bars represent standard errors of the mean.

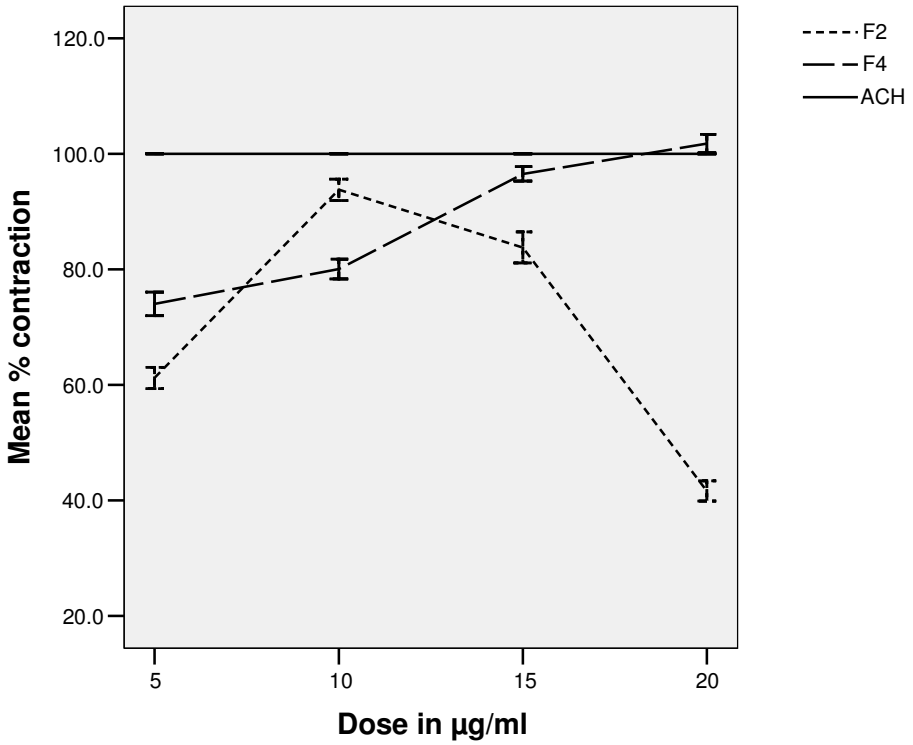

Figure 4.3. Dose response curve (line-graph) showing the mean percentage contraction by ACh in the presence of the fractions (F2, and F4) at different organ bath concentrations as compared with the control ACh alone on isolated mouse uterus. Vertical bars represent standard errors of the mean.

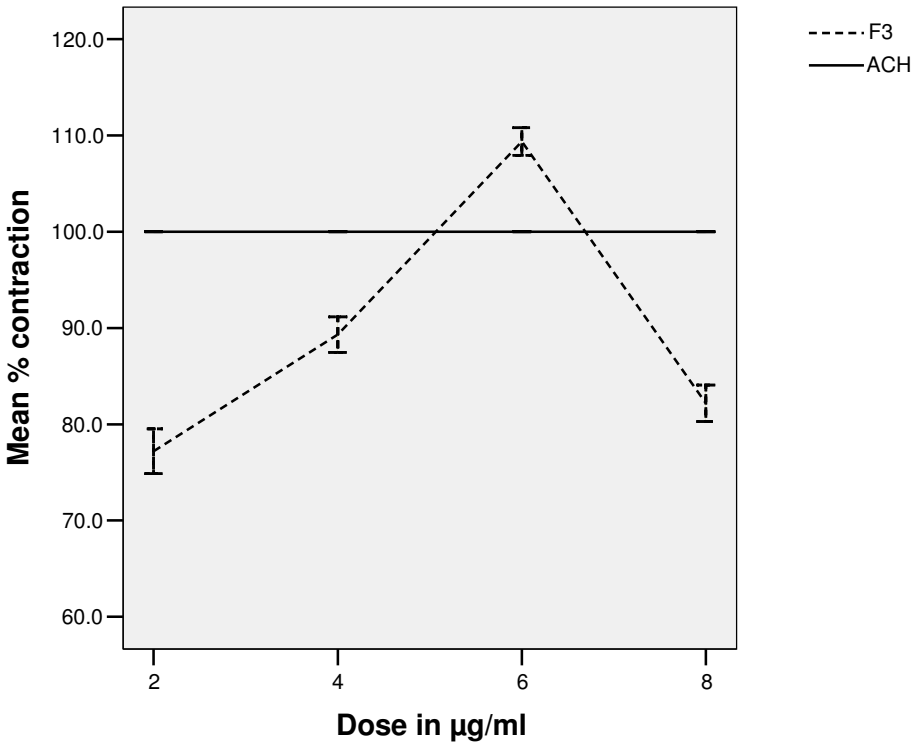

Figure 4.4. Dose response curve (line-graph) showing the mean percentage contraction by ACh in the presence of the fraction F3 at different organ bath concentrations as compared with the control ACh alone on isolated mouse uterus. Vertical bars represent standard errors of the mean. F3 showed an increased ACh-induced contraction as the dose increases from 2- 8µg/ml.

Polygraph picture

5. DISCUSSION

The results of anti-implantation experiments (table 4.1) revealed that oral administration of 95% ethanol leaf crude extract of *V. amygdalina* Del. at a dose of 1g/kg body weight showed significant decrease in number of implantation sites compared to the controls. This may indicate that the crude extract inhibited the process of implantation. The possible cause of termination of pregnancy up on oral administration of the plant extract may be due to antizygotic, antiblastocytic as well as antiestrogenic property. Plant extracts can cause endometrial alterations resulting in non-receptive endometrium and thus cause implantation failure. Nivrsarkar *et al.*, (2005) reported that extract of the leaves of *Hibiscus rosa-sinensis* caused endometrial alteration and resulted in blastocyst implantation failure in mice. It is known that administration of high levels of exogenous estrogenic substances to mice causes implantation failure (Wen-ge *et al*., 2003 cited in Simon *et al*., 2003). In mice and humans estrogen also plays a pivotal role in implantation because it participates in estrogen/progesterone balance and, therefore can affect the uterine receptivity to the embryo (Ements, 1970). Farnsworth *et al*., (1975) reported that administration of low concentrations of compounds with estrogenic activity to many species during early pregnancy resulted in rapid passage of ova through the oviducts and expulsion of the ova from the oviduct. Furthermore, degeneration of the fertilized ova while transported into the uterus too early can also decrease the number of implants and result in decreased fertility (Farnsworth, *et al*., 1975).

But when the dose was decreased to 0.5 g/kg and 0.385 g/kg body weight there was no significant difference in implantation sites as compared to the control. This may show that

the effect of the extract on implantation depends on the dose. This result is in agreement with the finding of Golam Sadik *et al*., (2001) who showed that ethanolic leaf and stem extract of *Pergularia daemia* when administered orally to mice inhibited implantation depending on the dose. Similar finding was reported by Montanari and Bevilacqua, (2002).They showed that administration of hydroalcoholic leaf extract of *Maytenus ilicifolia* orally to mice resulted in a reduced rate of implantation of embryos. Gebrie *et al.*, (2005) also reported that the methanolic root extract of *Rumex steudelii* when administered orally to pregnant rats resulted in significant decrease in number of litters. It is not always the case that medicinal plants result in anti-implantation effect. For example administering 70% ethanolic extract of the aerial parts of *Ruta graviolens* L. orally to mice did not cause preimplantation embryonic loss and did not affect implantation (De Freitas *et al.,* 2005).

Administration of the crude extract at a dose of 3g/kg body weight caused significant change in the number of live fetuses and fetal survival percent indicating the possible abortifacient activity of the extract. This result is in agreement with previous finding Ojukwu, 1982 cited in Awe *et al.*, (1999) who showed that the methanol extract of the fresh leaves of *V. amygdalina*, when administered to pregnant mice caused abortion within 24 hours. Golam Sadik and coworkers, (2001) also reported that oral administration of ethanol extract of both the stems and the leaves of *Pergularia daemia* showed abortifacient property in mice.

In the present study termination of pregnancy was accompanied with vaginal bleeding in some of mice after 24 hours of treatment. This result is in agreement with Al-Dissi, *et al*., (2001) who reported that administration of aqueous extract of the leaves of *Inula viscosa* to

rat showed abortifacient activity, accompanied with vaginal bleeding in some of the rats which received the extract.

In the present study the plant crude extract demonstrated both anti-implantation and abortifacient properties. This result agrees with the finding of Badami *et al.,* (2003) who reported that oral administration of ethanol extract of the powdered root of *Derris brevipes* variety *coriacea* showed both abortifacient and antiimplantation effect in rats. The result of Golam Sadik and coworkers, (2001) also supports the present finding. But it is possible that plant extracts which have effect on implantation may not have abortifacient property. On the other hand, plant extracts having abotifacient effect may not have effect on implantation (Williamson *et al.*, 1996).

The ethanol crude extract of the leaves of *V. amygdalina* on isolated mice uterine tissues resulted in significant variation contractile effect. Though not statistically significant the crude extract showed uterotonic activity greater than the control ACh. This result is in agreement with previous report of uterotonic property of *V. amygdalina* (Desta, 1994).

But fractionating the crude extract resulted in a strong contractile effect by one of the fractions (F3). This may be because the fractions contain fewer chemicals than the crude extract had. The presence of more substances in the crude extract may decrease the force of contraction due to the fact that the different phytochemicals may interact one another and some of them may be antagonistic hence the tension may not be maintained. This type of inhibitory effect in uterine muscles was showed by fractions F2 and F4.Thus in the crude extract the inhibitory effect of substances in F2 and F4 may antagonize the stimulatory

effect of F3 resulting in decreased contraction. Similar result was obtained by other workers showing that crude extracts can result in limited contraction of rat uterus (Uguru *et al.*, 1998).

Out of the fractions tested for their uterotonic effect, F3 showed significant stimulation of uterine contraction compared to the control ACh. Uterine contractility stimulated by certain plant extracts is mainly based on their oxytocic property or due to their estrogenic activity (Solloff, 1979; Uguru *et al*., 1995; Uguru *et al*., 1998 and Yalemtsehay, 1999). An abotifacient type of antifertility effect can be produced by compounds that stimulate uterine contractility (Farnsworth *et al.,* 1975). Therefore; uterine contractions showed by F3 in the present study might be due to estrogenic or oxytocic-like properties. According to this result F3 of the crude ethanol extract *V. amygdalina* may contain pharmacologically active chemical substances capable of stimulating smooth muscle of mouse uterus and the potential of being constituent of antifertility. This result is in agreement with Yalemtsehay, (1999) who reported that when the fresh leaf ethanol extract of *Moringa stenopetala* was tested in Guinea-pigs and mice uterus tissues showed oxytocic-like property. Although the plants are different they could show similar properties.

Another study by Tafese and coworkers, (2005) on water and ethanol extract of the leaves and roots of *Leonotis ocymifolia*, showed that both the water and ethanol extract of the root as well as the ethanol extract of leaf showed significant increase in uterine contraction compared to the control acetylcholine.

But F2 and F4 significantly inhibited ACh induced uterine contraction. This may be explained by the presence of varies compounds which may block the receptor sites of ACh stimulation (Uguru *et al*., 1998).

Desensitization is commonly observed *in vitro* experimental studies of isolated tissue preparations (Perry, 1982). Uguru and associates, (1998) showed that some of the hot methanol extracts of *Monechma ciliatim* produced desensitization of isolated rat uterine preparations after prolonged exposure to higher doses. This was also true in the present study which was commonly observed at doses greater than 180µg/ml for the crude extract. Fractions F2 and F4 showed the effect at doses greater than 20µg/ml final bath concentration. F3 resulted in desensitization in relatively very low doses (in doses greater than 10µg/ml).As this could be the possible explanation, however, higher doses may also have other mode of action that needs further experimentation.

6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

In the present study the possible anti-implantation and abortifacient effects of 95% crude ethanol extract *V. amygdalina* in *vivo* and the utero-contracting property of its different fractions *in vitro* has shown that

- The plant crude extract inhibits the process of implantation depending on the dose
- The plant crude extract has dose dependent abortifacient effect
- Fractionating the crude extract resulted in F3 which has uterine stimulating or oxytocic property that was dose dependent
- In conclusion, the results of the study suggest that ethanolic leaf extract of *V. amygdalina* has antifertility and oxytocic-like activity. This finding may support the traditional use of the plant as abortifacient in rural parts of Ethiopia.

6.2. Recommendations

- Possible mechanism of action of the plant in comparison with estrogenic, antiestrogenic, progestational and antiprogestational compounds which have significant effects on reproductive and related tissues should be investigated.

- Pharmacological screening, further fractionation and isolation of active ingredient and structural elucidation and further pharmacological investigation on this active ingredient should be done
- Pharmacokinetic studies on the active ingredient(s)
- Attention should be given by government, NGOs, researchers etc. to systemic evaluation of medicinal plants in order to develop safe, effective, affordable and accessible products since majority of our population depend on medicinal plants.

However, the present study had the following limitations

- No attempt was made to see the antifertility effect of the different fractions *in vivo*
- Positive controls were not used for *in vivo* experiments

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Declaration

I, the undersigned, declared that this thesis is my own original work, has not been presented for a degree to any other university and that all sources of materials used for the thesis have been duly acknowledged.

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