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*Review*

## New vitality of ancient ethnomedicine in China: Review of Li ethnomedicine

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Nowadays, with the development of nature drug, herbal medicine is gaining more attention from scientists in various countries. In China, ancient ethnomedicine based on natural medicinal plants faces new opportunities and challenges. This paper examines Li ethnomedicine, which has not attracted much attention among researchers yet. Four special species, which had important significance for modern research and were very helpful for treating modern diseases, such as cancer, cardiovascular, and cerebrovascular disease, were selected for a concrete analysis from the perspective of function, economic value, developmental potential, and industrial base in order to introduce the Li ethnomedicine to the world. The development status and main problems of Li ethnomedicine were summarized and discussed for further study.

**Key words:** Li ethnomedicine, function, economic value.

### INTRODUCTION

Natural medicine appeared with the existence of human society. Before the eighteenth century, it provided the main therapeutic agents for human diseases. With the rapid development of modern medicine and the chemical pharmaceutical industry in the nineteenth century, natural medicine gradually fell by the wayside, especially in the west. In fact, the active ingredients of natural medicine or retrofitting its structure to get new chemicals have always been one of the most important sources of chemical medicine. In recent years, developed countries had shifted their research focus to the natural medicinal plants, indicating that herbal medicine is gaining more and more attention among scientists from various countries. This shift also brings new opportunities and challenges to the exploitation and development of ancient medicinal herbs in China. This paper examines Li ethnomedicine and a few special species were selected for deeper study. The development status and main problems of Li ethnomedicine are summarized and discussed for further study.

### OVERVIEW OF LI ETHNOMEDICINE

#### Representative plants of Li ethnomedicine

The Li people have lived in Hainan for more than 3,000 years and were the earliest ancestors to live on the island. As a minority population with more than 100 million people (Haofu and Wenli, 2008), the Li people have struggled with diseases for thousands of years and formed a unique set of medical theories and clinical experience using local herbs with significant regional and national characteristics, that are very different from the traditional Chinese medicine (Bingchun et al., 2007).

Most of the Li population on Hainan traditionally lived in mountainous area, which is rich in flora and fauna, with poisonous insects and snake everywhere and terrible traffic conditions (Junqing et al., 2009). As a result, the Li drugs have obvious geographical features. The Li pharmacy introduction (Mingsheng, 2008), includes 128 kinds of Li drugs; one sixth of them are for the treatment

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of bone injury, such as *Gendarussa vulgaris* Nees (Weijing et al., 2011) and *Croton crassifolius* Geiseler (Xianhui, 2009); while one tenth are for hepatitis, such as *Eudia lepta* (Spreng.) Merr (Changyu et al., 2012) and *Abrus mollis* Hance (Xiaobai et al., 2010). In addition, 8 are used to treat snakebites (e.g. *Ervatamia hainanensis* Tsiang (Xingqi, 2003)); 8 are used for cirrhosis ascites (e.g. *Brucea javanica* (Linn.) Merr. (Haifeng et al., 2012)); 7 for infertility (e.g. *Cyrtomium fortunei* J. Sm. (Junqing, 2009)); 6 are used for nephropathy (e.g. *Drynaria fortunei* (Kunze) J. Smith. (Long et al., 2005)); 4 are used for inflammation (e.g. *Ficus pumila* L.) (Junqing et al., 2009). The drugs are administered externally, orally, through fumigation and by carrying them (A kind of way to carry some particular drugs with small bag).

Recent studies have found that, besides the remarkable effect in the treatment of snakebites and bruises, Li drugs also have very significant effects as anti-inflammation and anti-tumor drugs, especially leukemia, and may provide a new therapeutic approach for cancer, hepatitis, rheumatism, and other illnesses (Caicheng, 2007).

### Historical records

The Li nationality has no words of its own, which means it is difficult to record the concrete history and development exhibition of its medicine. But it can be traced from some monographs of Traditional Chinese Medicine. "An Account of the Plants and Trees of Southern China", written by Ji Han (Jin Dynasty, 265-420 AD), is the earliest botany literature in China. The book reproduces several species such as *Aquilaria sinensis* (Lour.) Gilg, *Dalbergia odorifera* T.C. Chen, *Alpinia oxyphylla* Miq., *Areca cathecu* Linn, *Cocos nucifera* Linn, *Saccharum officinarum* L. and many others. These varieties are produced in Hainan and have the same utilization with Li ethnomedicine.

"Findings in Lingnan Area", written by Liu Xun (Tang Dynasty, 618-907 AD), described the precious animals and plants in Lingnan area. In this book, some plants used by Li people as medicine are mentioned, such as dendrobe, olives, hawkbill. In 1950s, the Li ethnomedicine attracted the attention of Chinese government. The data of Li pharmacology were gathered and arranged. "Survey of Li nation in Hainan" was published in 1954, which documented the Li ethnomedicine specifically for the first time (Caicheng, 2007). The book "200 kinds of Chinese Herbal Medicine in Hainan" edited on the base of investigation in Hainan summed up 200 kinds of Chinese herbal medicinal materials which are used frequently by people. "Dictionary of Li nation in China" (Yingbo et al., 1994) recorded about 70 kinds of Li drugs.

Recently, two specialized books of Li ethnomedicine, "Li pharmacy Introduction" and "Records of Li Folk Medicine" were edited in 2008, which summarized more

than 100 kinds of Li drugs including the ingredient, usage, dosage, and treatment (Mingsheng, 2008; Haofu, 2008). Nowadays, the government of Hainan is paying more attention to the development of Li ethnomedicine. They have made many efforts in terms of the protection, mass production, and innovation in order to help this ancient ethnomedicine regain its vital activity.

According to this review of Li ethnomedicine and the concrete analysis of special medicinal plants as follows, we can see that although Li ethnomedicine is ancient, with a history dating back 3,000 years, it still has important significance for modern research. It is very helpful for treating modern diseases, such as cancer, cardiovascular, and cerebrovascular disease (Caicheng, 2007). Some of these medicinal plants are good for people's health and can be eaten daily.

### FOUR SPECIAL MEDICINAL PLANTS

This paper selects 4 species from 128 Li medicinal plants for deeper research: *Callicarpa nudiflora* Hook. & Arn., *Nauclea officinalis*, *A. oxyphylla*, and *Dracaena angustifolia* Roxb. These four species have the common feature of high added value and economic value, as well as great exploitation potential; they are also already used in drug production. In addition, although some are used in traditional Chinese medicine, they are used in different ways for the treatment of diseases in Li ethnomedicine (Table 1).

#### *C. nudiflora* (Luohuazizhu)

*C. nudiflora*, whose leaves can be used as medicine, is harvested in the summer and autumn and can be dried into powder from the plains to hills, valleys, streams and forests or bushes at an altitude of 1200 m located in Hainan, Guangdong, Guangxi. Wuzhishan in Hainan is the most appropriate place for growing it as it has the highest medicinal value. The *C. nudiflora* has an acerbic taste that is slightly spicy and bitter. Li people use it as anti-bacterial, anti-inflammatory, and hemostatic. As early as the Qin and Han dynasties, the Li people pounded the roots and leaves of *C. nudiflora* for external use on wounds or boiled them for oral administration to treat several diseases. The Li people regard *C. nudiflora* as precious and have used it for many generations.

In modern times, the *C. nudiflora* is used as an essential herb for healing by military soldiers in China. It is found that the *C. nudiflora* is very helpful for some modern diseases such as thrombus (Ying and Guocai, 2006), and can delay the process of decrepitude (Bin et al., 1995). Today, the market demand for this medicinal material is about 3,500 tons every year, with an average price of 2,500 yuan per ton and it is estimated that the demand will increase to 5,000 tons within two years,

**Table 1.** Comparison of four medicinal plants of Li ethnomedicine

Name	<i>Callicarpa nudiflora</i>	<i>Nauclea officinalis</i>	<i>Alpinia oxyphylla</i>	<i>Dracaena angustifolia</i>
Pharmacological property	Antithrombotic (Ying and Guocai et al., 2007), anti-inflammatory (Ying and Guocai et al., 2006), anti-aging (Bin et al., 1995).	anti-inflammatory, acesodyne (Liang et al., 2011), lower blood pressure (Jian et al., 2002), anti-virus.	anti-dementia (Dehuan et al., 2002; Hongmei et al., 2012), anti-diarrhea (Xinghua et al., 2009), anti-aging (Huiling et al., 2006), neuroprotective effect (Shui et al., 2006)	Anti-inflammatory analgesic (Yuli et al., 2010), anti-thrombosis (Nian et al., 2010), stop bleeding, anti-tumor (Wenli et al., 2005)
Clinical use	Bleeding(Jie et al., 2010), infectious diseases (such as respiratory tract infections), viral hepatitis (Jinping et al., 2012)	Acute tonsillitis, acute pharyngitis, acute conjunctivitis and upper respiratory tract infection, diabetes	Hyperactivity, insomnia, Older night urine, urinary frequency, sexual dysfunction, amnesia	Bruises, chronic hepatitis, whooping cough, bronchitis, tuberculosis, chronic tonsillitis, pharyngitis
Application in Li ethnomedicine	leaves, fruits: stop the bleeding	anti-inflammatory	fruit (dry milling): stop stomach pain	resin: throat dry pain
Li ethnomedicine production	Tablet, capsule, Suppository.	Injection, extract capsule, extract tablets(Yan and Yanli et al., 2012), wine.	Soft capsule, wine.	Powdered medicine, capsule.
Economical efficiency	Huge market demand	Low cost, high market demand	Extensive use	high added value products- dragon's blood

indicating a positive market preview.

Some enterprises have already found the commercial opportunities for *C. nudiflora* and have invested in planting and production, such as Luohuazizhu suppository, which have the indications as anti-inflammatory (Ying and Guocai, 2006), for detoxification, convergence, hemostasis, bacterial infection caused by inflammation, acute infectious hepatitis, and respiratory and gastrointestinal bleeding (Jie et al., 2010).

### ***N. officinalis* (Danmu)**

The *N. officinalis* is a kind of common drug used by Li people as anti-inflammatory drug. The modern research found that the main effects of *N. officinalis* included not only anti-inflammation, but also heat-clearing, detoxicating, and analgesia (Liang et al., 2011). It is used to treat acute tonsillitis, acute pharyngitis, acute conjunctivitis, and upper respiratory tract infection (Yan and Yanli, 2012), and has demonstrated extraordinary

efficacy on diabetes mellitus (Gidado, 2004).

The growth period of *N. officinalis* is 5 to 6 years. It has strong adaptability, good resistance, and great growth ability after cutting. It grows easily and has no serious pest problems (Zhiyuan et al., 2010). The planting of *N. officinalis* in Hainan has been formed to a certain scale. The *N. officinalis* has been planted on Wuzhishan up to 1000 acres and the investment has been more than 10 million RMB since 2003.

The present productions include Danmu injections, Danmu extract tablets, and Danmu extract capsules. In daily consumption, it can be made into Danmu wine. The *N. officinalis* is one of the representative species of large-scale production in Li ethnomedicine. It has good medicinal value and is edible.

### ***A. oxyphylla* (Yizhi)**

*A. oxyphylla* mostly grows on Hainan Island, because of the warm and humid environment. Its

flowering period is about 2 to 5 months, and the maturing period is 6 months. The medicinal part is its fruit. Wildlife resources have decreased since the 1950s, and the present products are cultivated versions. *A. oxyphylla* is very good for the cardiovascular system (Shoji et al., 1984) and gastrointestinal system (Yamahara et al., 1990; Kubo et al., 1995) and has a significant effect on the treatment of allergies (Kim et al., 2002) and cancer (Chun et al., 2002). The Li people made the fruit into powder to treat stomach pain (Mingsheng, 2008). One clinical study found that *A. oxyphylla* is effective in the treatment of diarrhea (Sakai et al., 1986), enuresis, hyperactivity, and insomnia (Hengliang et al., 2002).

Meanwhile, modern research has found that it contains a high amount of taurine (Junping et al., 2002), the "smart factor", and one of the essential nutrients for the body, especially for infants' growth and educational nourishment as well as for delaying aging among the elderly (Huiling, 2006). Its isolated compounds can be used as therapeutic agents for sexual dysfunction and

amnesia (Kubo et al., 1995). Therefore, *A. oxyphylla* has a strong potential as a health food.

Due to its short growth cycle and more and increased attention from the government, more farmers are planting *A. oxyphylla*.

### ***D. angustifolia***

*D. angustifolia* is distributed only along the coastal areas of Hainan Island. It has been shown to be 8,000 years to 10,000 years old. It grows slowly and is one of the national protected species. Its resin, which is known as "Dragon's blood" is used as medicine in many places and promotes a variety of pharmacological activities, including anti-inflammatory (Yuli et al., 2010), anti-fungal (Yodosuka et al., 2000), anti-arrhythmic (Nambu et al., 1989), anti-thrombotic (Nian et al., 2010), hemostatic (Dongxu, 2001), and wound healing (Ping et al., 2000; Dongxu, 2001). In recent years, compounds with anti-tumor activity (Wenli et al., 2005) have been isolated from it; these have shown an inhibition effect on the growth of nasopharyngeal carcinoma cells (Reddy et al., 1984), liver cells, and leukemia (Yodosuka et al., 2000). The Li people use the resin to treat throat pain (Mingsheng, 2008).

### **PROBLEMS**

Based on the literature review, some problems have been identified with Li ethnomedicine. First of all, the planting of Li medicinal material is still based on scattered plantings by farmers (Wang Yeqiao, 2006), making it difficult for standardized production and large-scale industrialization due to the lack of an effective quality standard system and monitoring (Yali et al., 2009). And the sustainable use of Li medicinal resources has become another outstanding problem. Due to the lack of collection, collation, purification, and rejuvenation of the herbal resources, some species have been over-harvested and leading to destruction of their ecological environment. Many wild medicinal resources with special effects, such as periwinkle, gall wood, and the leader of maple have been rapidly depleted or have altogether disappeared. The manufacturers that use these herbs as raw materials, such as the Periwinkle medicine, cannot produce them at full capacity.

Secondly, the Li nation has no writing system. The heritage of Li drugs relies on an oral tradition between teacher and student. This way of sharing knowledge has resulted in the loss of many drugs and prescriptions. Moreover, the knowledge of Li ethnomedicine is fragmented. It has no systematic theory or drug standards. The existing national and local policies conflict with the practice of physicians using Li ethnomedicine, whose practice qualification is not recognized by the government. This situation is not conducive to the heritage and development of Li ethnomedicine.

### **SUGGESTIONS**

First of all, the comprehensive utilization of Li ethnomedicine resources needs to be strengthened. The utilization includes two aspects. The first is the comprehensive evaluation and exploration of resources in the development process, making full use of medicinal plants in a variety of ways. Studies have confirmed that the root and leaf of *Bryophyllum pinnatum* (L.f.) Oken can stop bleeding, cure swelling and pain (Surong et al., 2004); the fruit and leaf of *Amomum longiligulare* T.L.Wu contain some common active ingredients. These examples underscore the need for the comprehensive utilization of different parts of herbal medicine. The second is the transfer of useless or low efficacy ingredients in the waste into a chemical composition with medicinal value through synthesis or structural modification. The extent and level of comprehensive utilization of medicinal plants reflect the degree of scientific research and standards. China attaches great importance to comprehensive utilization of herbal and drug production drugs, such as alkaloids extracted from *Cephalotaxus* which in recent years has shown good effects on the treatment of leukemia (Yumei et al., 1995); taxol extracted from the yex, which is a strong inhibitor of tumor cells (Slichenmyer and Von Hoff, 1991); and insulin analogue from the seeds of some cucurbits (Shouyan, 1992), which is helpful for the treatment of diabetes (Zheng et al., 1996; Toshihiro et al., 2009).

And then, we should strengthen the promotion of new technologies and new methods. The development and utilization of Li ethnomedicine need new technologies and the application of new methods. The bio-pharmaceuticals developed rapidly thanks to high-tech tools during the past 10 years, especially genetic engineering, which has become an important technology for pharmaceutical products replacement. In the future, cell engineering, genetic engineering, and fermentation engineering will greatly promote the development and utilization of Li ethnomedicine.

In addition, the research into Li traditional medicine needs to be strengthened. The collation of Li medicine theory and clinical experience needs to be increased. The relevant departments, including the government of Hainan province, the Administration of Traditional Chinese Medicine of Hainan, and the Ministry of Health, should pay more attention to resource surveys and research to rescuing the Li culture as soon as possible. Specific conservation and research institutions need to be established in order to conduct further research and enhance the role of Li drugs. The protection of the ecological environment in the process of developing of the Li drugs needs to be also considered.

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## REFERENCES

- Bin X, Li L, Shangda C (1995). The Cytological Mechanism of The Chinese Medicinal Herb *Callicapa Nudiflora*. *Academic J. Guangdong College Pharm.* 11: 141.
- Bingchun G, Rongtao L, Xinquan Y, Daolin D (2007). Ethnobotany Studies on Medicinal Plants Used by Li Nationality in Wuzhishan Area of Hainan Province. *Chin. J. Ethnomed. Ethnopharm.* 4:32-34.
- Caicheng Z (2007). Overview of development status of Li drugs. *J. Med. Pharm. Chin. Minorities.* 5(5):2-4.
- Changyu B, Chaojun F, Zhanjuan C, Heping B (2012). Study on the Chemical Constituents from the stems of *Melicope ptelefolia*(Champ.ex Benth.)Hartley in Hainan. *J. Hainan Normal Univ. (Natural Science).* 01:66-70
- Chun KS, Park KK, Lee J, Kang M, Surh YJ (2002). Inhibition of mouse skin tumor promotion by anti-inflammatory diarylheptanoids derived from *Alpinia oxyphylla Miquel* (Zingiberaceae). *Oncol. Res.* 13: 37-45.
- Dehuan L, Wenqiang Z, Zhenzhen X, Weicong G, Binghui L (2002). Clinical Study on JIANNAO Mixture for Senile Vascular Dementia. *J. Fujian College Tradit. Chin. Med.* 12:40-42.
- Dongxu W (2001). Advances in studies on resin of *Dracaena cochinchinensis*. *Chin. Tradit. Herb. Drugs* 32(11):1053-1054.
- Gidado A, Ameh DA, Atawodi SE (2004). Effect of *Nauclea latifolia* leaves aqueous extracts on blood glucose levels of normal and alloxan-induced diabetic rats. *Afr. J. Biotechnol.* 4:91-93.
- Haifeng L, Weisheng Z, Ying Y (2012). Therapeutic Effect of Percutaneous Transhepatic Artery Embolism with Fructus Bruceae Oil Emulsion and Oral Use of Chinese Medicine for Late Liver Cancer. *J. Guangzhou Univ. Tradit. Chin. Med.* 05:527-531
- Haofu D, Wenli M (2008). Records of Li Folk Medicine. China Science and Technology Press, Beijing.
- Hengliang Z, Liping Z, Li C (2002). An Experimental Study on Sedative and Hypnotic Effects of Yizhiren Oral Liquid. *J. Guiyang Med. College* 2:132-135.
- Hongmei A, Bing H, Jiulin C, Zhihua Y, Miao J, Chao G, Sanli X (2012). Effects of Dihuang Yizhifang on apoptosis related gene expression in senile dementia rats induced by A $\beta$ 1-40. *Pharmacol. Clin. Chin. Mater. Med.* 28:18-20.
- Huiling D (2006). Studies of Several Traditional Chinese Medicine Extracts on Anti-aging in Mice. Dalian University of Technology, Dalian.
- Jian F, Shaoyi K, Xiang-zhou Z, Gui-lan X (2002). Studies on Anti-inflammatory Effect of Danmu Extract Tablets. *Nat. Sci. J. Hainan Univ.* 20:54.
- Jie Z, Baoquan L, Feng F, Yuping T, Wenyan L (2010). Chemical constituents from *Callicarpa nudiflora* and their hemostatic activity. *Chin. J. Chin. Mater. Med.* 35:45-47.
- Jinping C, Lin D, Weiwei G (2012). Research progress of *Callicarpa nudiflora*. *Drugs Clin.* 27:52-54.
- Junping Z, Boli Z, Yongyan W, Xiaoyu C, Ling L (2002). Effects of Qing Nao Yi Zhi Fang on Human Fetal Cerebral Neuronal Cells in Primary Culture with Oxygen Deprivation Condition. *Chin. J. Basic Med. Tradit. Chin. Med.* 8:17-20.
- Junqing Z, Shuiping D, Weili Y, Jianping T, Mingsheng L (2009). Analysis of Li medicinal plant resources in Hainan province. *J. Hainan Med. College* 15:201-205.
- Kim SH, Choi YK, Jeong HJ, Kang HU, Moon G, Shin TY, Kim HM (2002). Suppression of immunoglobulin E-mediated anaphylactic reaction by *Alpinia oxyphylla* in rats. *Immunopharmacol. Immunotoxicol.* 22:267-270.
- Kubo M, Matsuda H, Suo T, Yamanaka J, Sakanaka M, Yoshimura M (1995). Study on *Alpiniae Fructus*. I. Pharmacological evidence of efficacy of *Alpiniae Fructus* on ancient herbal literature. *Yakugaku Zasshi.* 115:852-856.
- Liang C, Na L, Yaqiong J, Gang D, Zhenzhong W, Wei X (2011). Anti-inflammatory and Analgesic Effects of Extracts from the Leaves of *Nauclea officinalis*. *Chin. J. Exp. Tradit. Med. Formulae* 17:25-27.
- Long M, Qiu D, Li F, Johnson F, Luft B (2005). Flavonoid of *Drynaria fortunei* protects against acute renal failure. *Phytother. Res.* 19(5):422-427.
- Mingsheng L (2008). Li pharmacy Introduction. People's Health Publishing House, Beijing.
- Nambu T, Huang X, Shu Y, Huang S, Hattori M, Kakiuchi N, Wang Q, Xu GJ (1989). Chronotropic effect of the methanolic extracts of the Plants of the Paris species and steroidal glycosides isolated from P. vietnamensis on spontaneous beating of myocardial cells. *Planta Med.* 55(6):501.
- Nian X, Yujuan L, Yulin D (2010). Experimental Study on Antithrombotic Effect of Dragon's Blood Extracts, Proceedings of 2010 China Pharmaceutical Conference and the Tenth Chinese pharmacists weeks.
- Ping W, Qingyun D (2000). Advances in the constituents and clinical research of the resin of the Dragon's Blood. *Strait Pharm.* 12:1-2.
- Reddy KS, Shekhani MS, Berry DE, Lynn DG, Hecht SM (1984). Afromontoside a new cytotoxic principle from *Dracaena* of romontane. *J. Chem. Soc. Perkin Trans. Org. Chem.* 5:987.
- Sakai K, Oshima N, Kutsuna T, Miyazaki Y, Nakajima H, Muraoka T, Okuma K, Nishino T (1986). Pharmaceutical studies on crude drugs. I. Effect of the Zingiberaceae crude drug extracts on sulfaganidine absorption from rat small intestine. *Yakugaku Zasshi.* 106:947-951.
- Shoji N, Umeyama A, Asakawa Y, Takemoto T, Nomoto K, Ohizum Y (1984). Structural determination of nootkatol, a new sesquiterpene isolated from *Alpinia oxyphylla* Miquel possessing calciumantagonistic activity. *J. Pharm. Sci.* 73:843.
- Shouyan Z (1992). The Exact of Insulin analogue from *Momordica charantia* Linn. *Chin. Pharm.* 5:46-49.
- Shui G (2006). Studies on Protective Effect of Protocatechuic Acid from *Alpinia oxyphylla* in PC12 Cells, Dalian University of Technology, Dalian University of Technology, Dalian.
- Slichenmyer WJ, Von Hoff DD (1991). Taxol: a new and effective anti-cancer drug. *Anti-cancer Drugs* 2(6):519-530.
- Surong C, Suhua G, Yeqing Z (2004). Pharmaceutical Research Progress of *Bryophyllum pinnatum*. *Fujian J. Tradit. Chin. Med.* 35:51.
- Toshihiro M, Takanori K, Satoshi T, Mai N, Haruka N, Eriko I, Torao I (2009). Effect of *Momordica charantia* on Adenosine Monophosphate-activated Pro-tein Kinase in Genetically Type 2 Diabetic Mice Muscle. *J. Health Sci.* 55(5):805-808.
- Wang Yeqiao YB (2006). Protection and Exploitation of the Medicinal Plant Resources in Hainan. *Chin. Wild Plant Resourc.* 25:40-43.
- Wenli M, Haofu D, Jiao W, Ling Z, Kui H (2005). Study on the New Use of Antitumor of *Dracaena cambodiana*. *J. Chin. Med. Mater.* 28:871-874.
- Wenjing W, Jun W, Gaoxiong R (2011). Study on the anti-inflammatory and analgesic effects of two kinds of *Sambucus chinensis* extracts. *West Chin. J. Pharm. Sci.* 3:247-249
- Xianhui Y, Shangwen C, Shiming D (2009). Study on the Chemical Constituents of *Croton crassifolius*. *Lishizhen Med. Mater. Medica Res.* 3:515-518.
- Xiaobai C, Yaokun G, Xiaoping W, Shihua Z, Jianling L, Ping M (2010). Effects of *Abrus mollis* on Blood Lipids and Liver Lipids in Hyperlipidemia Rats. *Chin. Pharm.* 03:202-205
- Xinghua L (2009). Studies on the Anti-diarrhea Effect of Seed *Alpiniae oxyphyllae* and the Influence of Stir-heating with Salt Solution on It, Chengdu University of Traditional Chinese Medicine, Chengdu University of Traditional Chinese Medicine, Chengdu.
- Xingqi T, Haisheng C, Congli X, Runhui L, Weidong X (2003). Studies on Chemical Constituents in the *Ervatamia hainanensis*. *Chin. J. Chin. Mater. Med.* 11:1040-1042.
- Yali G, Qin P, Minren H (2009). Ethnobotany and exploitation of medicinal plant resource in Hainan. *J. Nanjing Forestry Univ.* 33:18-20.
- Yamahara J, Yinghua L, Tamai Y (1990). Anti-ulcer effect in rats of bitter cardamon constituents. *Chem. Pharm. Bull.* 38:3053-3056.
- Yan J, Yanli L (2012). Quality Analysis Study of Fathead Tree and Three Different Formulations. *Chin. Pharm. Affairs* 26:34-36.

- Ying C, Guocai Y (2006). Study on the Anti-inflammatory and Immunologic Effects of *Callicarpa nudiflora* Hook. Et Am. Guangdong Trace Elements Sci. 8:39-42.
- Ying C, Guocai Y (2007). The *Callicarpa nudiflora* on hemorheology. Chin. Remedies Clin. 4:293-294.
- Yingbo S, Jingzhao W, Dingji L (1994). Dictionary of Li nation. Zhongshan University Press, Guangdong.
- Yodosuka A, Mimaki Y, Sashida Y (2000). Steroidal saponins from *Dracaenasurculosa*. J. Nat. Prod. 63:1237-1243.
- Yuli C (2010). The Research on Analgesic Mechanism of Dragon's Blood and Its Components - Loureirin B, Central South University for Nationalities.
- Yumei Y, Chengxiong X, Runhua S, Jiyu G, Guangjian C (1995). Studies on the Anticancer Effect of HH07A, a Derivative of Hainanensine. Acta Pharm. Sinica 1:12-16.
- Zheng P, Baoyuan L, Wangyi L, Shanwen J (1996). Characterization of the Enzymatic Mechanism of 7-Momorcharin, a Novel Ribosome Inactivating Protein with Lower Molecular Weight of 11,500 Purified from the Seeds of Bitter Gourd (*Momordica charantia*). Biochem. Biophys. Res. Commun. 1(229):287-294.
- Zhiyuan Z, Qiumei L, Qian L, Xiu-feng L (2010). Introduction of *Nauclea officinalis* cultivation techniques. J. Shanxi College Tradit. Chin. Med. 11:69-72.

Full Length Research Paper

## Antidiarrheal and antidiabetic effect of ethanol extract of whole *Ageratum conyzoides* L. in albino rat model

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*Ageratum conyzoides* L. (Asteraceae) is a medicinal herb widely used for several diseases although data are unavailable for diarrhea and diabetes. This study investigated the efficacy of orally administrated ethanol extract of *A. conyzoides* L. on diarrheal and diabetic rat models. Castor oil induced diarrheal model and gastrointestinal motility test models (barium sulfate milk and charcoal suspension) of albino rats were undertaken to measure the antidiarrheal effect. Alloxan induced diabetic model was used for antidiabetic effect. Data were analyzed by one-way analysis of variance (ANOVA) using Tukey's post hoc test for multiple comparisons. The extract reduced the severity of castor oil induced diarrhea with significant ( $p < 0.01$ ) inhibition of mean defecation numbers  $65.89 \pm 2.44$ ,  $52.1 \pm 3.21$  and  $27.13 \pm 4.67\%$  with 2.0, 1.0 and 0.5 g/kg bw of extract, respectively. The number of stools at different hours in treated group was significantly ( $p < 0.01$ ) decreased as compared to normal control and positive control group (Loperamide, 1.0 mg/kg). Distance of gastrointestinal motility was significantly ( $p < 0.05$ ) reduced to  $38.88 \pm 1.06\%$  from control distance  $57.25 \pm 1.08\%$  in barium sulfate milk model and to  $36.34 \pm 0.41\%$  from  $57.99 \pm 0.66\%$  in charcoal suspension model by the highest dose 2.0 g/kg. In a diabetic model, the blood glucose level was decreased  $17.6 \pm 1.31\%$ , by 2.0 g/kg, which was significant ( $p < 0.05$ ) compared to that ( $52.00 \pm 1.21\%$ ) of positive control (Glimepiride, 4.0 mg/kg). The results demonstrate the marked anti-diarrheal and moderate anti-diabetic role of whole *A. conyzoides* L. ethanol extract.

**Key words:** Antidiarrheal, charcoal, antidiabetic, *Ageratum conyzoides*, barium sulfate.

### INTRODUCTION

Diarrhea is one of the main causes of infant death especially in third world country (Zavala et al., 1998). Diarrhea affects the smooth life-style due to its huge discomfort, although it is not life threatening for adults (Saito et al., 2002). However, twenty percent of total children die from diarrhea before the age of five in developing countries. There are some synthetic drugs available for diarrheal treatment although most of them

have side effects like uncomfortable bowel movement, uneasiness etc. A continuous search, therefore, for an alternative treatment is still urged (Nester et al., 1998).

Diabetes mellitus is a metabolic disorder causing hyperglycemia due to partial pancreatic dysfunction (World Health Organization (WHO), 1980). It happens by inherited and/or acquired deficiency in production of insulin by the pancreas, or by the ineffectiveness of the

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insulin produced. It results either from inadequate secretion of insulin, an inadequate response of target cells to insulin, or both of these factors (Li et al., 2004). Till now, the management of diabetes is a global problem because curative treatment is yet to be discovered. However, several synthetic drugs are used to control diabetes but the modern oral hypoglycemic agents create undesirable side effects. Therefore, alternative therapy is required; a need of the hour is to shift towards the different indigenous plant and herbal formulations (Satyanarayana et al., 2006).

*Ageratum conyzoides* L. (Asteraceae) is an annual herbaceous, branched, hairy and aromatic plant with a long history of traditional medicinal uses in several countries (Abbiw, 1990; Menut et al., 1993). It is a tropical plant commonly found in West Africa, Australia, some parts of Asia and South America. It is also currently found in several tropical and sub-tropical regions including Brazil (Cruz, 1985). The plant grows commonly in waste and ruined sites as weeds in many countries (Waterhouse, 1994; Kshastriya et al., 1994).

*A. conyzoides*, except the hosting of begomovirus in Indonesia and Cameroon (Leke et al., 2012), has been widely used as herbal medication and folk remedies of different diseases. Various pharmacological and biological uses of this plant or plant-parts have been investigated by many researches. They have reported the tissue repair and collagen formation (Arulprakash et al., 2012), anticancer and antiradical scavenging (Adebayo et al., 2010), ovicidal and larvicidal (Wabo Poné et al., 2011; de Mendonça et al., 2005), antiprotozoan (Harel et al., 2011; Nour et al., 2010), anti-aflatoxinigenic and antioxidant (Patil et al., 2010), anticoccidial (Nweze and Obiwulu, 2009), haematopoietic (Ita et al., 2007), *in vitro* anti-helicobacter pylori (Ndip et al., 2007), antibacterial and wound healing (Chah et al., 2006), analgesic and anti-inflammatory (Moura et al., 2005), antitumour (Rosangkima and Prashad, 2004), gastroprotective (Shirwaikar et al., 2003) and anti-rheumatism (Chopra et al., 2002) effects of *A. conyzoides*. In Trinidad and Tobago, this plant has been used in fertility problem of female and erectile dysfunction of male (Lans, 2007). The use of this plant in human immune deficiency virus/acquired immune deficiency syndrome (HIV/AIDS) disease is also documented (Igoli et al., 2005).

*A. conyzoides* has also been proved to be used in phytoremediation of arsenic contamination (Mahmud et al., 2008). Mangesh et al. (2009) conducted a research on antidiarrheal effect of the hydroalcoholic extract of *A. conyzoides* leaves. Hypoglycaemic and antihyperglycaemic activity of leaf aqueous extract of *A. conyzoides* has been reported previously (Rahmatullah et al., 2012; Nyunai et al., 2010). However, antidiarrheal and anti-diabetic effects of whole *A. conyzoides* organic extract are yet to be studied. We investigated the antidiarrheal and anti-diabetic effects of whole *A. conyzoides* ethanol

extract in this research.

## MATERIALS AND METHODS

### Chemical and reagents

All chemicals and reagents used were of analytical grade. Absolute ethanol (99.5%), refined pure castor oil and alloxan monohydrate were purchased from Sigma-Aldrich, Munich, Germany (Cat No. A7413-10G). Barium sulfate and activated charcoal were purchased from Merck, India Limited. Alloxan monohydrate was prepared using 0.9% NaCl saline solution. Loperamide powder and glimepiride powder were kindly donated by GlaxoSmithKline, Chittagong, Bangladesh Ltd.

### Collection of plant material

*A. conyzoides* whole plant was collected from the moist hillside of the University of Chittagong, Bangladesh, during the month of 15th March to 7th April, 2010. The plant was taxonomically identified by Dr. Shaikh Bokhtear Uddin (Taxonomist and Associate Professor, Department of Botany, University of Chittagong, Bangladesh) and identification was confirmed by Sarder Nasir Uddin (Taxonomist, Bangladesh National Herbarium, Ministry of Environment and Forest, Bangladesh). A voucher specimen of the plant has been preserved, with the accession number 36073.

### Preparation of plant extract

Fresh *A. conyzoides* whole plants were washed, chopped into small pieces with chopper, air dried at room temperature ( $25 \pm 1$ )°C and ground into powder (450 g) which was left to soak in 3 L absolute ethanol for 7 days at room temperature with occasional stirring. The ethanol extract was filtered through Cheese cloth and filter paper (Whatman No. 1) and concentrated through rotary vacuum evaporator (RE200 Rotary evaporator, Bibby Sterling, UK) under reduced pressure below 50°C. The concentrated 25 g of blackish-green crude extract was collected in plastic petri dish and air dried to allow complete evaporation. The dried extract was preserved at 4°C until further use.

### Animals and diet

Six-seven weeks old wistar albino male rats weighing 180 to 200 g were obtained from the animal house of BCSIR laboratories, Chittagong. The animals were acclimatized to room temperature ( $23 \pm 0.5$ °C) with a relative humidity of  $55 \pm 5\%$  in standard wire meshed plastic cages for 4 to 5 days prior to commencement of the experiment. During the entire period of study, animals were caged individually and supplied with a standard pellet diet and water *ad libitum*. All the animals were maintained and treated according to the guidelines stipulated by the Institutional Animal Ethics Committee (AEIUC-PH-2011/06).

### Antidiarrheal assay

Antidiarrheal assay was conducted by castor oil induced diarrheal model and gastrointestinal motility test model.

### Castor oil-induced diarrhea

The method described by Shoba and Thomas (2001) was followed for this study. Twenty five wistar albino male rats were randomly divided into five equal group (n = 5) control group, positive control group and three individual treated groups. Control group received only distilled water, positive control group received loperamide 1 mg/kg as standard, and treated groups received *A. conyzoides* extract at the dose 2.0, 1.0 and 0.5 g/kg body weight, respectively. Rats were housed in separate cages having blotting paper placed below for collection of fecal matters. Diarrhea was induced by oral administration of castor oil (2 ml/rat). Extract and drugs were given orally 1 h before the administration of standard dose of 2 ml of castor oil. Diarrhea was defined as the presence of fluid material in the stool, which was stained by the absorbent paper placed beneath the cage. The number of diarrheal episodes in terms of both hard and soft pellet was counted at every hour over 5 h period for each rat. A numerical score based on stool consistency was assigned as follows: normal stool = 1, semisolid stool = 2 and watery stool = 3. Percent inhibition (PI) was calculated as follows:

$$PI = \frac{\text{Mean defecation (control group-treated group)}}{\text{Mean defecation of control group}} \times 100$$

### Gastrointestinal motility test model

This model was involved in the following two chemical assays:

1. Barium sulfate milk assay: Gastrointestinal motility test with BaSO<sub>4</sub> milk was performed according to method described by Chatterjee (1993). Briefly, rats fasted for overnight were randomly divided into five groups (n = 5). Control group received only distilled water, positive control group received commercially available anti-diarrheal drug loperamide 1.0 mg/kg, and three individual treated groups received *A. conyzoides* extract 2.0, 1.0 and 0.5 g/kg bw, respectively. Thirty minutes later, 2 ml of 10% barium sulfate suspension was administered in all groups of rats. Rats were sacrificed after 30 min. The total length of small intestine and the distance travelled by barium sulfate milk was measured and expressed as a percentage of the total length of small intestine (from pylorus to the ileo-cecal junction).
2. Charcoal suspension assay: Charcoal model was performed according to method described as BaSO<sub>4</sub> milk model. Briefly, overnight fasted rats were randomly divided into five groups (n = 5). Control group received only distilled water, positive control group received commercially available anti-diarrheal drug loperamide 1.0 mg/kg, and three individual treated groups received *A. conyzoides* extract 2.0, 1.0 and 0.5 g/kg bw, respectively. Thirty minutes later, 2ml of 10% charcoal was administered in all groups of rats. Rats were sacrificed after 30 min. The total length of small intestine and the distance travelled by charcoal was measured and expressed as a percentage of the total length of small intestine (from stomach to caecum).

### Alloxan induced antidiabetic assay

Thirty rats were randomly divided into six experimental groups (marked as group I to VI), containing five rats in each group. Diabetes was induced in five groups (group II to VI) of rats by intraperitoneal injection of alloxan monohydrate (140 mg/kg bw). After 18 h of fasting, alloxan induced diabetic rats were treated orally as: group I (normal control) received only distilled water (1 ml); group II (diabetic control) alloxan induced diabetic rats received

only distilled water (1 ml); group III (positive control) diabetic rats received reference antidiabetic drug glimepiride (4 mg/kg, purchased locally); group IV to VI (sample treated) diabetic rats treated with ethanol extract of *A. conyzoides* at the rate of 2.0, 1.0 and 0.5 g/kg bw.

### Blood collection and glucose quantification

All the animals were anesthetized with diethyl ether (Sigma-Aldrich, India), and blood was collected from cardiac vessel using disposable syringe by heart puncture method (Hoff, 2000). The blood collected from cardiac vessel was kept undisturbed in room temperature for 20 min. Serum from blood after clotting separated out and collected in a clean tube by centrifugation at 1100 g for 15 min. The level of glucose in blood samples from each of the experimental and control rat was determined spectrophotometrically at 546 nm by using glucose kit (gluco-leucolor, Sigma-Aldrich, Germany) essentially followed by glucose oxidase-peroxidase (GOD-POD) method (Trinder, 1969).

### Statistical analysis

All the values in the tests were expressed as mean ± SD (standard deviation). Data were analyzed by statistical package for social science (SPSS) software (SPSS, Version 18.0, IBM Corporation, NY) using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. The values were considered significantly different at p < 0.05.

## RESULTS

The ethanol extract showed dose-dependent inhibition of castor oil induced diarrhea in albino rats. This antidiarrheal effect in terms of defecation was significant (p < 0.01) at all the doses of extract in comparison to control group, however, the activity was less as compared to loperamide as shown in Figure 1. Highest inhibition of castor oil induced diarrheal severity was achieved 65.89 ± 2.44% at 2.0 g/kg of extract (Figure 2). The number of stools at 1st, 2nd, 3rd 4th, 5th and 6th h for ethanol extract treated group was significantly (p < 0.01) decreased as compared to control group (Figure 3). The ethanol extract showed dose-dependent inhibition of barium sulfate induced gastrointestinal motility in albino rat. This effect was significant even at lower dose of 0.5 g/kg over 30 min as compared to normal control, however, this activity was less as compared to loperamide as shown in Table 1.

The results of present study showed that the extract significantly (\*p < 0.05) reduced the gastrointestinal transit of charcoal in animal model (Table 2). The motility (57.99 ± 0.66%) of control group has been reduced mostly by the highest dose 2.0 g/kg of extract. This effect was dose-dependent and comparable to positive control loperamide (1.0 mg/kg) over 30 min study. Table 3 shows the serum blood glucose level in normal control and all experimental groups. The alloxan-induced blood glucose level was significantly (p < 0.05) decreased at the doses

**Table 1.** Effect of *A. conyzoides* L. ethanol extract of on gastrointestinal motility with barium sulfate milk model on rats.

Treatment	Dose	Gastrointestinal motility		
		Length (stomach-caecum)	Length passed by BaSO <sub>4</sub>	% motility mean±SD
Normal control (DW)	0.0 mg/kg	59.2 ± 3.11	103.6 ± 7.89	57.25 ± 1.08
Positive control (LP)	1.0 mg/kg	95.8 ± 5.89 <sup>a</sup>	29.6 ± 2.88 <sup>a</sup>	30.88 ± 1.04 <sup>a</sup>
Treated ACEx	2.0 g/kg	95.6 ± 12.79 <sup>a</sup>	37.0 ± 3.93 <sup>a</sup>	38.88 ± 1.06 <sup>b</sup>
	1.0 g/kg	103.4 ± 6.76 <sup>a</sup>	43.2 ± 3.11 <sup>a</sup>	41.77 ± 0.29 <sup>c</sup>
	0.5 g/kg	100.0 ± 7.84 <sup>a</sup>	51.8 ± 3.03 <sup>a</sup>	51.90 ± 1.01 <sup>d</sup>

DW: Distilled water; LP: Loperamide; ACEx: *Ageratum conyzoides* extract. All values are expressed as mean ± SD for 5 rats. Values with superscript <sup>a-d</sup> letters in the table for a given period of time are significantly different from each other (SPSS for windows, version 18.0, One-Way ANOVA followed by Tukey's *post hoc* test for multiple comparisons,  $p < 0.01$ ).

**Table 2.** Effect of ethanol extract of *A. Conyzoides* L. on gastrointestinal motility with 10% charcoal suspension.

Group	Dose	Gastrointestinal motility		
		Length (stomach-caecum)	Length passed by charcoal	% motility Mean±SD
Control (DW)	0.0 mg/kg	95.2±5.16	55.2±3.34	57.99±0.66
Positive Control (LP)	1.0 mg/kg	96.8±8.52 <sup>a</sup>	28.4±3.20 <sup>a</sup>	29.29±0.37 <sup>a</sup>
	2.0 g/kg	92.6±13.50 <sup>b</sup>	33.6±4.5 <sup>b</sup>	36.34±0.41 <sup>b</sup>
Treated ACEx	1.0 g/kg	96.8±10.56 <sup>c</sup>	39±4.63 <sup>c</sup>	40.26±0.42 <sup>c</sup>
	0.5 g/kg	95±14.15 <sup>d</sup>	48.2±6.87 <sup>d</sup>	50.78±0.53 <sup>d</sup>

All values are expressed as mean ± SD for 5 rats. Values with superscript <sup>a-d</sup> letters in the table for a given period of time are significantly different from each other (SPSS for windows, version 18.0, One-Way ANOVA followed by Tukey's *post hoc* test for multiple comparisons,  $p < 0.01$ ).

**Table 3.** Increase of glucose level in alloxan induced diabetic rats.

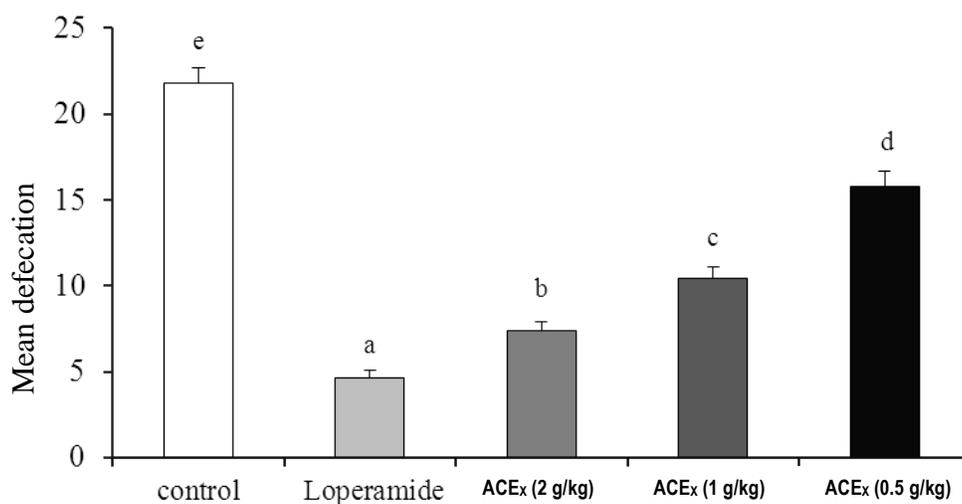
Group	Treatment	Dose	Blood glucose level (mg/dl)
G-I	Normal control (DW)	-	59.99±1.04
G-II	Diabetic control (ALXN+DW)	-	98.77±0.93 <sup>a</sup>
G-III	Positive control (ALXN+GLMP)	10 mg/kg	47.45±1.21 <sup>b</sup>
G-IV		2.0 g/kg	81.38±1.31 <sup>c</sup>
G-V	Treated (ALXN+ACEx)	1.0 g/kg	94.39±0.71 <sup>d</sup>
G-VI		0.5 g/kg	97.98±8.40 <sup>e</sup>

ALXN: Alloxan; GLMP: Glimperide; All values are expressed as mean ± SD for 5 rats. Values with superscript letters are different as follows: the values for superscript <sup>a-d</sup> different letters in the table for a given period of time are significantly different from each other, whereas, the value of superscript <sup>e</sup> letter is insignificant compared to the values of normal control and the superscript letters <sup>a-d</sup> (SPSS for windows, version 18.0, One-Way ANOVA followed by Tukey's *post hoc* test for multiple comparisons,  $p < 0.05$ ).

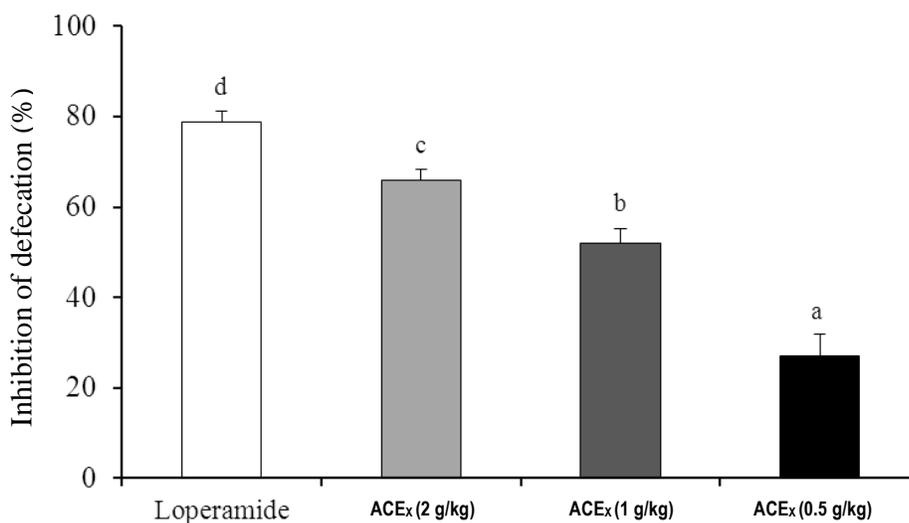
of 2.0 and 1.0 g/kg of extract. However, these effects of extract were lower than glimepiride at a dose of 4 mg/kg (Figure 4).

## DISCUSSION

In the present study, the ethanol extract of *A. conyzoides*



**Figure 1.** Effect of *A. conyzoides* ethanol extract and loperamide on castor oil induced diarrhea in terms of total no of feces in 5 h. Values are expressed as mean  $\pm$  SD for 5 rats. <sup>a-d</sup>Different letters over the bars for a given period are significantly different from each other and from the value of control<sup>e</sup> (SPSS for windows, version 18.0, One-Way ANOVA followed by Tukey's post hoc test for multiple comparisons, \*\*p < 0.01).

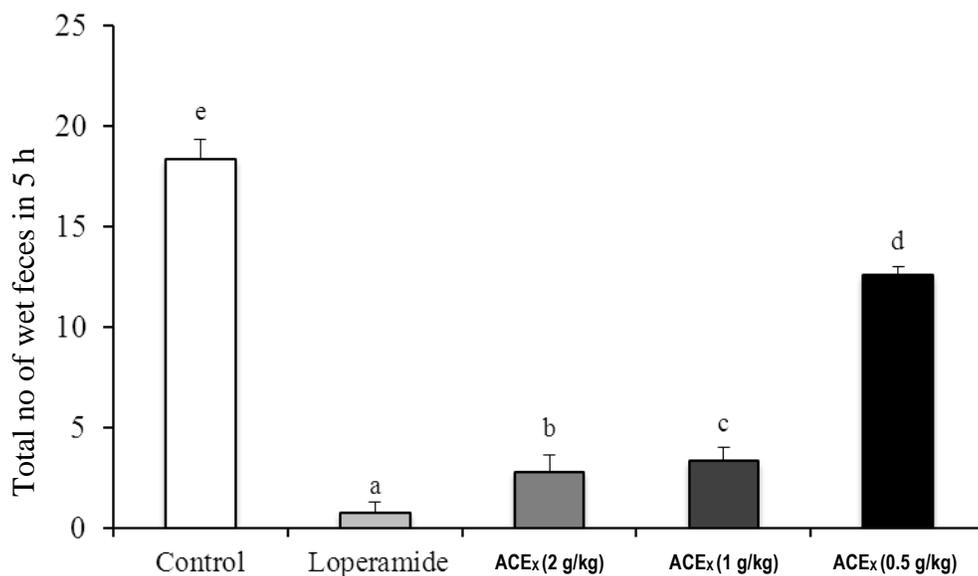


**Figure 2.** Inhibition (%) of defecation by *A. conyzoides* ethanol extract and loperamide on castor oil induced diarrhea. Values are expressed as mean  $\pm$  SD for 5 rats. <sup>a-d</sup>Different letters over the bars for a given period are significantly different from each other (SPSS for windows, version 18.0, One-Way ANOVA followed by Tukey's post hoc test for multiple comparisons, \*\*p < 0.01).

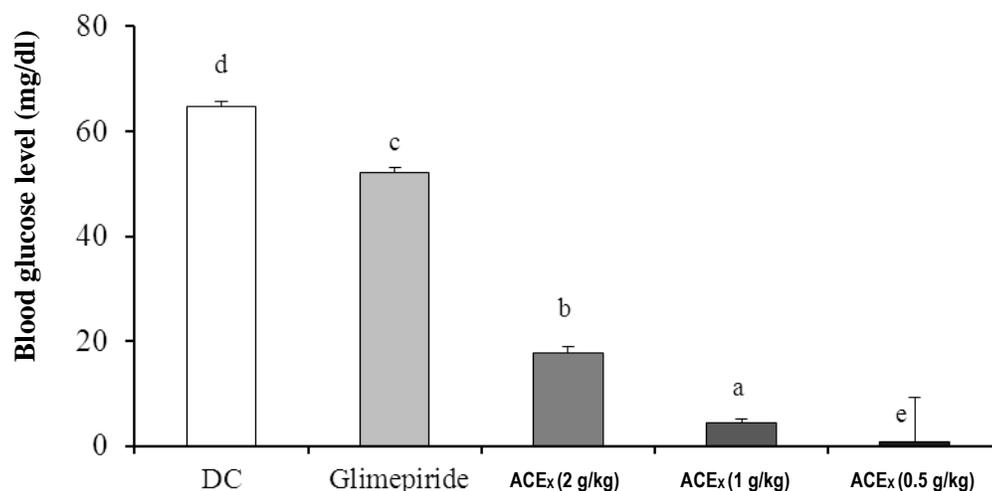
whole plant showed significant activity against castor oil, barium sulfate and charcoal induced changes of motility in gastrointestinal tract.

Castor oil induced diarrheal model is an autacid-based normal to assess antidiarrheal agent. Castor oil causes diarrhea through its active metabolite ricinolic acid which prevents fluid and electrolyte absorption

(Ammon et al., 1974; Brown and Taylor, 1996). Castor oil induced gastrointestinal hypermotility has been suggested to be indirectly mediated by the cholinergic system since it is inhibited by atropine, a known anticholinergic agent (Brown and Taylor, 1996). So the prevention of cholinergic transmission or its anticholinergic effect on gastric mucosa should be regarded as a probable



**Figure 3.** Effect of *A. conyzoides* ethanol extract and loperamide on castor oil induced diarrhea in terms of total no of wet feces in 5 h. Values are expressed as mean  $\pm$  SD for 5 rats. <sup>a-d</sup>Different letters over the bars for a given period are significantly different from each other (SPSS for windows, version 18.0, One-Way ANOVA followed by Tukey's *post hoc* test for multiple comparisons, \*\* $p < 0.01$ ).



**Figure 4.** Effect of *A. conyzoides* ethanol extracts (2.0, 1.0, 0.5 g/kg,) of on blood glucose level of alloxan-induced diabetic rats. Values with superscript letters are different as follows: the values for superscript <sup>a-d</sup>different letters over the bars for a experimental period of time are significantly different from each other, whereas, the value of superscript <sup>e</sup> is insignificant compared to the values of diabetic control (DC) and the superscript letters <sup>a-c</sup> (SPSS for windows, version 18.0, One-Way ANOVA followed by Tukey's *post hoc* test for multiple comparisons, \* $p < 0.05$ ).

mechanism of *A. conyzoides* extract (Mycek et al., 1997). Apart from this, the extract under investigation may contain certain components having affinity to  $\mu$  ( $\mu$ ) receptor, which is an opioid receptor located on the GI mucosa and relieves diarrhea when activated by an

agonist (Goodman and Gillman, 1996).

Barium sulphate increases the volume of the intestinal content by preventing the reabsorption of water. It also promotes the liberation of cholecystikinin from duodenal mucosa, which increases the secretion and motility of

small intestine and also prevents the reabsorption of NaCl and water. Barium sulphate induced diarrhea is presumed to be by osmotic properties and cholecystokinin production (Galvez et al., 1993). Further, the extract showed inhibition of peristalsis activity in normal as well as in charcoal suspension induced increase peristalsis as evident from distance traveled by charcoal suspension. This significant reduction in peristaltic activity is one of the important factors contributing to antidiarrheal activity of extract.

However, the results of this study showed that the extract reduced gastric contents and watery texture of diarrheal stools as well as gastrointestinal motility which is prevailed over other models of this study. Thus prevention of cholinergic transmission or its anticholinergic effect on gastric mucosa could be the leading mechanism for significant reduction (\*\*p < 0.01) in frequency of diarrheal episodes. Thus further phytochemical studies are required to isolate antidiarrheal component(s) from the extract to establish its exact mode of antidiarrheal activity.

The results of this study reveal that the ethanol extract of *A. conyzoides* contains pharmacologically active substances with antidiarrheal properties. These properties may explain the rationale for the effective use of the plant as an antidiarrheal agent in traditional medicine. Alloxan induced diabetes is believed to result from production of free radicals which damage the  $\beta$  cells of the pancreas (Asayama et al., 1984) and it is a useful experimental model to study the activity of hypoglycaemic agents (Szkudelski, 2001). Alloxan is a beta cytotoxin, induces "chemical diabetes" (alloxan diabetes) in a wide variety of animal species by damaging the insulin secreting pancreatic cell. The action of this diabetogenic agent is mediated by reactive oxygen species. Alloxan and the product of its reduction, dialuric acid, establish a redox cycle with the formation of superoxide radicals that undergo dismutation to hydrogen peroxide. Thereafter highly reactive hydroxyl radicals cause rapid destruction of  $\beta$  cells and resulting in a diabetic state (Szkudelski, 2001).

The possible mechanism of action of extracts could be correlated with the reminiscent effect of the reference antidiabetic drug glibenclamide that promote insulin secretion by closure of  $K^+$  -ATP channels, membrane depolarization and stimulation of  $Ca^{2+}$  influx, an initial key step in insulin secretion. Since alloxan is known to destroy pancreatic cells, the present findings appear to be in consonance with the earlier suggestion of Jackson and Bressler (1981) that sulphonylureas (for example, glibenclamide) have extra-pancreatic anti-hyperglycemic mechanism of action secondary to their insulin secreting effect and the attendant glucose uptake into, and utilization by the tissues (Jackson and Bressler, 1981).

It has been suggested that supplementation of natural antioxidants may retard or halt oxidative damage which

may lead to disease progression. Potential natural products having antioxidant effects have been proved to exert beneficial effects including diabetes mellitus (Maxwell, 1995). From phytochemical analysis, it was found that the major constituents of the *A. conyzoides* extract were mono- and sesquiterpenes, flavonoids, triterpenoids, sterols, alkaloids, coumarins, essential oils and tannins (Okunade, 2002). Over 150 plants extracts and some of their active principles including flavonoids are known to be used for the treatment of diabetes (Meiselman et al., 1976; Choi et al., 1991). Moreover, tannin-containing drug demonstrated antidiabetic activity (Klein et al., 2007). However, if the hypothesis of Marles and Farnsworth (1995) which indicates that plants which contain terpenoids and/or coumarins possess hypoglycemic activities in diabetic and normal mammals is worthwhile, it could be suggested that the hypoglycemic effect of *A. conyzoides* may be partly due to flavonoids, tannins, terpenoids and/or coumarins present in the plant (Adebayo et al., 2011; Wiedenfeld, 2011; Moreira et al., 2007; Abdelkader and Lockwood, 2011).

One or some of the other miscellaneous compounds of the plant may synergistically contribute to the hypoglycemic effect of the ethanol extract of *A. conyzoides*, a phenomenon that may validate the use of this plant in folk medicine against diabetes. More investigations are needed in order to clarify its mechanism of action.

## Conclusion

Present study justifies the traditional use of the plant in diarrhea and diabetes indicating the anti-diarrheal and antidiabetic effect of whole *A. conyzoides* extract. Since the extract is reported to contain a range of compounds, it is difficult to ascribe these observed properties to any specific group of compounds. Hence, further studies are suggested to be undertaken to conduct a structure activity relationship to better understand the mechanism of such properties of *A. conyzoides*.

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## REFERENCES

- Abbiw DK (1990). Useful plants of Ghana, Intermediate Technology Publications and Royal Botanic Gardens, Kew, London., ISBN 1853390437
- Abdelkader MS, Lockwood GB (2011). Volatile oils from the plant and hairy root cultures of *Ageratum conyzoides* L. Nat Prod. Res. 25(9):909-917.
- Adebayo AH, Jig CJ, Zhang YM, He WJ, Zeng GZ, Han HJ, Xu JJ,

- Akindahunsi AA, Tana NH (2011). A new chromene isolated from *Ageratum conyzoides*. Nat. Prod. Commun. 6(9):1263-1265.
- Adebayo AH, Tan NH, Akindahunsi AA, Zeng GZ, Zhang YM (2010). Anticancer and antiradical scavenging activity of *Ageratum conyzoides* L. (Asteraceae). Pharmacogn. Mag. 6(21):62-66.
- Ammon HV, Thomas PJ, Philips SF (1974). Effect of the oleic acid and ricinolic acid on net jejunal water and electrolyte movement. J. Clin. Invest. 53(5):374-379.
- Arulprakash K, Murugan R, Ponrasu T, Iyappan K, Gayathri VS, Suguna L (2012). Efficacy of *Ageratum conyzoides* on tissue repair and collagen formation in rats. Clin Exp Dermatol. 37(4): 418-24.
- Asayama K, Nyfeler F, English D, Pilakis SJ, Burr IM (1984). Alloxan-induced free radical production in isolated cells: Selective effect on islet cells. Diabetes 33(10):1008-1011.
- Brown JH, Taylor P (1996). Muscarinic receptor agonist and antagonist. In: Hardman LG, Limbird LE, editors. Goodman and Gilman's the pharmacological basis of therapeutics. 9<sup>th</sup> edition. New York: MacGraw, Hill. P. 141-160, ISBN 978-0-07-162442-8.
- Chah KF, Eze CA, Emuelosi CE, Esimone CO (2006). Antibacterial and wound healing properties of methanolic extracts of some Nigerian medicinal plants. J Ethnopharmacol. 104(1-2):164-167.
- Chatterjee TK (1993). Handbook on laboratory mice and rats. 1st ed. Kolkata, Department of Pharmaceutical Technology, Jadavpur University, 1993, p. 157.
- Choi JS, Yokozawa T, Oura H (1991). Improvement of hyperglycemia and hyperlipidemia in streptozocin – diabetic rats by methanolic extract of *Prunus davidiana* stems and its main component, pruning. Planta Med. 57:208.
- Chopra RN, Nayar SL, Chopra IC (2002). Glossary of Indian Medicinal Plants. New Delhi: NISCIR; 9, ISBN NO. 8172360487 9788172360481.
- Cruz GL (1985). Dicionário das plantasúteis do Brasil, 3 ed. Civilização Brasileira, Rio de Janeiro.
- de Mendonça FA, da Silva KF, dos Santos KK, Ribeiro Júnior KA, Sant'Ana AE (2005). Activities of some Brazilian plants against larvae of the mosquito *Aedes aegypti*. Fitoterapia 76(7-8):629-636.
- Galvez J, Zarzuelo A, Crespo ME, Lorente MD, Ocete MA, Jimenez J (1993). Antidiarrhoeic activity of *Euphorbia hirta* extract and isolation of an active flavonoid constituent. Planta Med. 59(4):333-336.
- Goodman A, Gillman A (1996). The pharmacological basis of Therapeutics, 9th edition, Macmillan publishers, New York, pp 924-926, ISBN 978-0-07-162442-8.
- Harel D, Khalid SA, Kaiser M, Brun R, Wünsch B, Schmidt TJ (2011). Encecalol angelate, an unstable chromene from *Ageratum conyzoides* L.: total synthesis and investigation of its antiprotozoal activity. J Ethnopharmacol. 137(1):620-625.
- Hoff Z (2000). Methods of blood collection in mouse. Lab animal technique. 29(10):47-53.
- Igoli JO, Ogaji OG, Tor-Anyiin TA, Igoli NP (2005). Traditional Medicine Practice Amongst the Igede people of Nigeria Part-II. Afr. J. Tradit. Compl. Alt. Med. 2(2):134-152.
- Ita SO, Etim OE, Ben EE, Ekpo OF (2007). Haematopoietic properties of ethanolic extract of *Ageratum conyzoides* (goat weed) in albino rats. Niger J. Physiol. Sci. 22(1):83-87.
- Jackson JE, Bressler R (1981). Clinical pharmacology of sulphonylurea hypoglycemic agents: part 1. Drugs 22(3):211-245.
- Klein G, Kim J, Himmeldirk K, Cao Y, Chen X (2007). Antidiabetes and Anti-obesity Activity of *Lagerstroemia speciosa*. Evid Based Compl. Alt. Med. 4(4):401-407.
- Kshastriya S, Sharma GD, Mishra RR (1994). Fungal succession and microbes on leaf litters in two degraded tropical forests of north-east India. Pedobiology 38:125-137.
- Lans C (2007). Ethnomedicines used in Trinidad and Tobago for reproductive problems. J. Ethnobiol. Ethnomed. 3:13.
- Leke WN, Brown JK, Lighthart ME, Sattar N, Njualed DK, Kvarnheden A (2012). *Ageratum conyzoides*: a host to a unique begomovirus disease complex in Cameroon. Virus Res. 163(1):229-237.
- Li WL, Zheng HC, Bukuru J, De Kimpe N (2004). Natural medicines used in the traditional Chinese medical system for therapy of diabetes mellitus. J. Ethnopharmacol. 92(1):1-21.
- Mangesh VT, Ashutoshpal J, Nitiness BM, Vaishali RU, Ashok VB (2009). Evaluation of antidiarrhoeal activity of hydroalcoholic extract of *Ageratum conyzoides* Linn. Res. J. Pharmacog. Phytochem. 1(1):26-29.
- Mahmud R, Inoue N, Kasajima SY, Shaheen R (2008). Assessment of potential indigenous plant species for the phytoremediation of arsenic-contaminated areas of Bangladesh. Int. J. Phytoremed. 10(2):117-130.
- Marles RJ, Farnsworth NR (1995). Antidiabetic plants and their active constituents. Phytomed. 2(2):137-189.
- Maxwell SR (1995). Prospects for the use of antioxidants therapies. Drugs 49(3):345-361.
- Meiselman HL, Halpern BP, Dateo GP (1976). Reduction of sweetness judgements by extracts from the leaves of *Ziziphus jujuba*. Physiol. Behav. 17(2):313-317.
- Menut C, Lamaty G, Amvam Zollo PH, Kuate JR, Bessière JM (1993). Aromatic plants of tropical central Africa. Part X Chemical composition of the essential oils of *Ageratum houstonianum* Mill. and *Ageratum conyzoides* L. from Cameroon. Flav Frag J. 8(1):1-4.
- Moreira MD, Picanço MC, Barbosa LC, Guedes RN, Barros EC, Campos MR (2007). Compounds from *Ageratum conyzoides*: isolation, structural elucidation and insecticidal activity. Pest Manag. Sci. 63(6):615-621.
- Moura AC, Silva EL, Fraga MC, Wanderley AG, Afiatpour P, Maia MB (2005). Antiinflammatory and chronic toxicity study of the leaves of *Ageratum conyzoides* L. in rats. Phytomedicine 12(1-2):138-142.
- Mycek MJ (1997). Harvey RA and Champe PC (editors). Lippincott's Illustrated Reviews: Pharmacology (2nd edition), Lippincott-Raven Publishers, New York. p. 40.
- Ndip RN, Malange Tarkang AE, Mbulah SM, Luma HN, Malongue A, Ndip LM, Nyongbela K, Wirmum C, Efange SM (2007). In vitro anti-*Helicobacter pylori* activity of extracts of selected medicinal plants from North West Cameroon. J. Ethnopharmacol. 114(3):452-457.
- Nour AM, Khalid SA, Kaiser M, Brun R, Abdalla WE, Schmidt TJ (2010). The antiprotozoal activity of methylated flavonoids from *Ageratum conyzoides* L. J. Ethnopharmacol. 129(1):127-130.
- Nweze NE, Obiwulu IS (2009). Anticoccidial effects of *Ageratum conyzoides*. J. Ethnopharmacol. 122(1):6-9.
- Nester WE, Robbert CE, Nancy NP, Danise GA, Martha TN (1998). Microbiology: A Human Perspective 2nd ed. Mc Graw-Hill Companies USA: 571-573.
- Nyunai N, Manguelle\_Dicoum A, Njifutie N, Abdennebi EH, Gerard C (2010). Antihyperglycaemic effect of *Ageratum conyzoides* L. fractions in normoglycaemic and diabetic male wistar Rats. Int. J. Biomed. Pharm. Sci. 4(1):38-42.
- Okunade AL (2002). Review: *Ageratum conyzoides*. Fitoterapia 73:1-16.
- Patil RP, Nimbalkar MS, Jadhav UU, Dawkar VV, Govindwar SP (2010). Antiaflatoxicogenic and antioxidant activity of an essential oil from *Ageratum conyzoides* L. J. Sci. Food Agric. 90(4):608-614.
- Rahmatullah M, Hasan SK, Ali Z, Rahman S, Jahan R (2012). Antihyperglycemic and antinociceptive activities of methanolic extract of *Euphorbia thymifolia* L. whole plants. Zhong Xi Yi Jie He Xue Bao 10(2):228-32.
- Rosangkima G, Prasad SB (2004). Antitumour activity of some plants from Meghalaya and Mizoram against murine ascites Dalton's lymphoma. Indian J. Exp. Biol. 42(10):981-988.
- Saito T, Mizutani F, Iwanga Y, Morikawa K, Kato H (2002). Laxative and antidiarrhoeal activity of Polycarbophil in Mice and Rats. Jpn. J. Pharmacol. 89:133-141.
- Satyanarayana T, Katayani BM, Latha HE, Mathews AA, Chinna EM (2006). Hypoglycemic and antihyperglycemic effect of alcoholic extract of *Euphorbia leucophylla* and its fractions in normal and in alloxan induced diabetic rats. Pharmacog. mag. 2(8):244.
- Shirwaikar A, Bhilegaonkar PM, Malini S, Kumar JS (2003). The gastroprotective activity of the ethanol extract of *Ageratum conyzoides*. J. Ethnopharmacol. 86:117-121.
- Shoba FG, Thomas M (2001). Study of antidiarrheal activity of four medicinal plants in castor oil-induced diarrhea. J. Ethnopharmacol. 76(1):73-76.

- Szkudelski T (2001). The mechanism of Alloxan and streptozotocin Action in  $\beta$  cells of the rats pancreas. *Physiol. Res.* 50(6):537–546.
- Trinder P (1969). Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Ann. Clin. Biochem.* 6:24-27.
- Wabo Poné J, Fossi Tankoua O, Yondo J, Komtangi MC, Mbida M, Bilong Bilong CF (2011). The *In Vitro* Effects of Aqueous and Ethanolic Extracts of the Leaves of *Ageratum conyzoides* (Asteraceae) on Three Life Cycle Stages of the Parasitic Nematode *Heligmosomoides bakeri* (Nematoda: Heligmosomatidae). *Vet. Med. Int.* 2011:140293.
- Waterhouse DF (1994). Biological control of weeds: Southeast Asian Prospects. Australian Centre for International Agriculture Research, Canberra, pp. 1-8.
- WHO (1980). Expert committee on diabetes mellitus: second report, World Health Organ. Tech. Rep. Ser., 646, pp.1-80
- Wiedenfeld H (2011). Plants containing pyrrolizidine alkaloids: toxicity and problems. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess.* 28:282-292.
- Zavala MA, Perez S, Perez C, Vargal R, Perez RM (1998). Antidiarrhoeal activity of *Waltheria americana*, *Commelins cuelestris* and *Alternanthera repens*. *J. Ethnopharmacol.* 61:41-47.

Full Length Research Paper

## Analgesic and anti-inflammatory activities of the ethanol extract of *Taxillus sutchuenensis* in mice

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**In this study, the analgesic activities and the mechanism of anti-inflammatory activities of 50% ethanol extract from TS (ETS) *in vivo* were investigated. Investigations were performed in mice with 2 analgesic models: acetic acid-induced writhing response and formalin-induced paw licking. The anti-inflammatory effect was tested by  $\lambda$ -carrageenan (Carr)-induced mice paw edema. These analgesic results indicated that ETS at a dose of 0.5 and 1.0 g/kg reduced the acetic acid-induced writhing responses and the licking time in the late phase of the formalin test. Moreover, Carr-induced paw edema was significantly reduced when ETS (0.5 and 1.0 g/kg) was administered 1 to 5 h after Carr injection. ETS reduced the level of malondialdehyde (MDA) in the edema paw by increasing the activities of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GRx), and catalase (CAT), in the liver and reducing tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$ , and IL-6 activities in the edema paw. This study demonstrates the analgesic and anti-inflammatory effects of ETS, thus verifying its popular use in traditional medicine.**

**Key words:** *Taxillus sutchuenensis*; analgesia, anti-inflammation, mice, malondialdehyde (MDA).

### INTRODUCTION

Inflammation is recognized as a vital process in response to the pathogenesis of various diseases, such as cardiovascular disease, cancer, atherosclerosis, arthritis, diabetes mellitus, obesity, neurodegenerative disease, heart disease, and many other life-threatening and debilitating diseases. Inflammation is the activation of the immune system caused by infection, toxins, physical injury, or chemical irritation, and is a complex process characterized by the contribution of several mediators, such as nitric oxide (NO), prostaglandins (PGs), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), prostanooids, and leukotrienes (Moncada et al., 1991; Chiu et al., 2012). Non-steroidal anti-inflammatory drugs

(NSAIDs) are one of the most commonly prescribed drugs for the treatment of inflammatory diseases (Sheeba and Asha, 2009). NSAIDs possess analgesic and anti-inflammation activity, because of the mechanism of inhibiting cyclooxygenases (COXs) for a decrease in PG production, which consequently reduces pain and inflammation. However, the NSAIDs, used clinically, are often of limited application, because of the occurrence of adverse digestive effects, most notably gastrointestinal hemorrhage, ulceration, and perforation (Wallace, 2001). Thus, developing a novel anti-inflammatory drug is crucial.

*Taxillus sutchuenensis* (TS) (Lecomte) Danser, which is

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called *Sang Ji Sheng* in Taiwan, is one species of the largest mistletoe family, Loranthaceae, which contains 75 genera and approximately 1000 species (Wilson and Calvin, 2006). This parasitical plant is capable of haustorial connections to various hosts, such as Aceraceae, Anacardiaceae, Euphorbiaceae, Fabaceae, Moraceae, Ruraceae, and Theaceae, and is widely distributed on land surfaces of the former supercontinent (Wilson and Calvin, 2006; Qui and Gilbert, 2003). For decades, the whole plant (stems and leaves) of TShas been used as herbal medicine, and has been used for the treatment of many human and animal ailments that include rheumatoid arthralgia, hypertension, obesity, cancer, and gastrointestinal tracts and wounds (Wang et al., 2008). In a previous study, the findings showed that TS possesses antioxidant, anti-inflammatory, and antiproliferative activities *in vitro* (Liu et al., 2012). However, research regarding information on the analgesic and anti-inflammatory activities of TS *in vivo* is scant.

In this study, the analgesic and anti-inflammatory activities of ETS *in vivo* were systematically investigated. Its analgesic activity was evaluated using the acetic acid-induced writhing response and the formalin test. The anti-inflammatory activity of ETS was determined using the Carr-induced paw edema model, which is a useful model for assessing inflammation. The activities of antioxidant enzymes including GRx, SOD, GPx, and CAT in the liver were subsequently determined.

## MATERIALS AND METHODS

### Plant and crude extract preparation

Plant materials were collected from Nantou County in Taiwan. They were identified and authenticated by Dr. Chao-Lin Kuo, head of the School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources (CPCR), College of Pharmacy, China Medical University. The voucher specimen (Number: CMU-CPCR-UL-096031) was deposited at CPCR. Coarse powder of TS (5 kg) was extracted 3 times at 5:5 (aqueous/ethanol). The extract was evaporated under reduced pressure by using a rota-vapor, and then stored under light protection. A yield equivalent to 15.517% of the original weight was obtained. The extract was stored in a refrigerator before use.

### Chemicals

Formalin was purchased from Nihon Shiyaku Industry, Ltd. Acetic acid,  $\lambda$ -carrageenan (Carr), indomethacin (Indo), and other chemicals were purchased from Sigma-Aldrich, Inc. (St Louis, MO, USA). The SOD, GPx, GRx, CAT, and MDA activity assay kits were purchased from Randox Laboratory Ltd.

### Experimental animals

Male Imprinting Control Region (ICR) mice (18 to 22 g) were obtained from BioLASCO Taiwan Co., Ltd. They were housed in standard cages at a constant temperature of  $22 \pm 1^\circ\text{C}$ , with a relative humidity of  $55 \pm 5\%$  and a 12 h light-dark cycle (8:00 AM to 8:00 PM) for a minimum of 1 week before the experiment. They were fed food and water *ad libitum*. The animals used in this study

were housed and cared in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Committee on Animal Research, China Medical University. The placebo groups were administered orally with 0.1 ml/10 g body weight saline. All tests were conducted under the guidelines of the International Association for the Study of Pain (Zimmermann, 1983). The required minimum number of animals and duration of observations were used to obtain consistent data.

### Acute toxicity studies

For the acute toxicity study, 10 male ICR mice (22 to 25 g) were randomly distributed. They were administered orally with doses (5000 mg/kg body weight) of TS. The animals were observed continuously for the first 4 h, and then the number of survivors was noted after 14 days of dosing.

### Acetic acid-induced writhing response

The writhing test was performed as described by Fontenele et al. (1996). The writhes were induced by intraperitoneal (i.p.) injection of 1.0% acetic acid solution (v/v, 0.1 ml/10 g body weight). Positive-control animals were pretreated with indomethacin (10 mg/kg, i.p.) 25 min before acetic acid injection. Three doses (250, 500, and 1000 mg/kg) of ETS were administered orally to each group of mice 55 min before the chemical stimulus. Five minutes after i.p. injection of acetic acid, the total numbers of writhing and stretching during 10 min were recorded.

### Formalin test

The formalin test that was administered was based on the method of a previous study (Liu et al., 2007). Nociception was induced by an i.p. injection of 20  $\mu\text{l}$  of 5% formalin in saline to the right hind paw of the mice. ETS (0.25, 0.5, and 1.0 g/kg, p.o.) was administered 60 min before the formalin injection. Indo (10 mg/kg, i.p.) was administered 30 min before nociception was induced. The control group received the same volume of saline by oral administration. The time spent with responses of licking and biting of the injected paw was taken as a response for the indicator of pain. Responses were measured for 5 min after formalin injection (early phase) and 20 to 30 min after formalin injection (late phase).

### Carr-induced mice paw edema

The anti-inflammatory activity of ETS was determined by the Carr-induced edema in the hind paws of the mice. Male ICR mice fasted for 24 h before the experiment, with free access to water. Twenty microliters of a 1% Carr suspension in saline was injected into the plantar side of the right hind paws of the mice (6 per each group) (Winter et al., 1962; Amabeoku and Kabatend, 2012). Animals were peritoneally-treated with the ETS (0.25, 0.5, and 1.0 g/kg), Indo, or normal control, 60 min prior to the injection of Carr. The paw volume was measured immediately by using a plethysmometer at 1, 2, 3, 4 and 5 h after Carr administration. The degree of swelling was evaluated by the delta volume (a-b), where a and b are the volumes of the right hind paw after and before Carr treatment, respectively. In the second experiment, the whole right hind paw tissue and liver tissue were taken at the third hour. The right hind paw tissue was rinsed in ice-cold normal saline, and immediately placed in 4 times their volume of cold normal saline, and homogenized at  $4^\circ\text{C}$ . The homogenate was then centrifuged at 12000 rpm for 5 min. The supernatant was obtained and stored at  $-80^\circ\text{C}$  in a refrigerator for MDA assays. The whole liver tissue was

immediately placed in cold normal saline and homogenized. The homogenate was then centrifuged at 12 000 rpm for 5 min. The supernatant was obtained and stored at -80°C in a refrigerator for the antioxidant enzyme (SOD, GPx, GRx, and CAT) activity assays.

#### Measurement of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 by ELISA

Serum levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Biosource International Inc., Camarillo, CA, USA), according to the manufacturer's instruction. The absorbance at 450 and 540 nm was measured on a microplate reader (VersaMax), and the concentrations of cytokines were determined as a difference in absorbance between 450 and 540 nm.

#### MDA assay

MDA was evaluated using the thiobarbituric acid-reacting substance (TRARS) method (Nakhai et al., 2007). First, the paw tissues were homogenized in buffered saline (1:4); afterward, 400  $\mu$ l of 1,1,3,3-tetraethoxypropan trichloroacetic acid (28% w/v) was added to 200  $\mu$ l of this mixture and centrifuged at 3000 g for 30 min. Thereafter, 300  $\mu$ l of the supernatant was added to 150  $\mu$ l of 2-thiobarbituric acid (1% w/v). The mixture was incubated for 45 min in a boiling water bath, and then 450  $\mu$ l n-butanol was added; the solution was centrifuged and cooled, and absorption of the supernatant was recorded in 532 nm by the microplate reader (VersaMax, Massachusetts, USA). Tetramethoxypropane was used as the standard. MDA levels were expressed as nanomoles per milligram of protein. Protein concentration was measured using the Lowry method (Lowry et al., 1951). Bovine serum albumin was used as the standard.

#### Antioxidant enzymes activity measurements

Liver tissue homogenates were collected for the estimations of SOD (Misra and Fridovich, 1972), GPx (Flohe Gunzler, 1984), GRx (Carlberg and Mannervik, 1985), and CAT enzymes (Aebi, 1984) to detect the antioxidant activities of TS. The SOD enzyme activity was determined according to the method by Misra and Fridovich. In total, 100  $\mu$ l of the tissue extract was added to 880  $\mu$ l (pH 10.2, 0.1 mM EDTA) of a carbonate buffer. Twenty microliters of 30 mM epinephrine (in 0.05% acetic acid) was added to the mixture at 480 nm for 4 min on a Hitachi U2000 Spectrophotometer. The enzyme activity was expressed as the amount of enzymes that inhibit the oxidation of epinephrine by 50%, which is equal to one unit. The total CAT activity was based on that by Aebi (1984). In brief, the reduction of 10 mM H<sub>2</sub>O<sub>2</sub> in 20 mM of the phosphate buffer (pH 7.0) was monitored by measuring the absorbance at 240 nm. The activity was calculated using a molar absorption coefficient, and the enzyme activity was defined as nmoles of dissipating hydrogen peroxide per milligram of protein per minute. The GPx enzyme activity was determined according to the method by Flohe and Gunzler (1984). The reaction mixture was composed of 500  $\mu$ l phosphate buffer, 100  $\mu$ l 0.01 M GSH (reduced form), 100  $\mu$ l 1.5 mM NADPH, and 100  $\mu$ l GRx (0.24 units). In total, 100  $\mu$ l of the tissue extract was added to the reaction mixture and incubated at 37°C for 10 min. Thereafter, 50  $\mu$ l of 12 mM t-butyl hydroperoxide was added to 450  $\mu$ l of the tissue reaction mixture, and was measured at 340 nm for 180 s. The molar extinction coefficient of  $6.22 \times 10^{-3}$  was used to determine the enzyme activity. One unit of activity is equal to the millimolar of NADPH oxidized per minute per milligram of protein. The GRx enzyme activity was determined following the method by Carlberg and Mannervik at 37°C (Carlberg and Mannervik, 1985). Fifty microliters of NADPH (2 mM) in 10 mM Tris buffer (pH 7.0) was

added in the cuvette containing 50  $\mu$ l of GSSG (20 mM) in the phosphate buffer. In total, 100  $\mu$ l of tissue extract was added to the NADPH-GSSG-buffered solution and measured at 340 nm for 3 min. The molar extinction coefficient of  $6.22 \times 10^{-3}$  was used to determine the GRx enzyme activity. One unit of activity is equal to the millimolar of NADPH oxidized per minute per milligram of protein.

#### Statistical analysis

All the data were expressed as the mean  $\pm$  standard error of mean (SEM). Statistical evaluation was conducted by one-way analysis of variance (ANOVA), followed by Scheffe's multiple-range tests. Statistical significance is expressed as \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001.

## RESULTS

### Toxicity study

The acute toxicity of ETS was evaluated in the mice at doses of up to 5000 mg/kg body weight p.o. administered for 14 days. ETS did not cause behavioral changes, and no death was observed. The oral LD<sub>50</sub> value of ETS was greater than 5000 mg/kg of body weight in the mice, and was considered to be a practically non-toxic substance.

### Acetic acid-induced writhing response

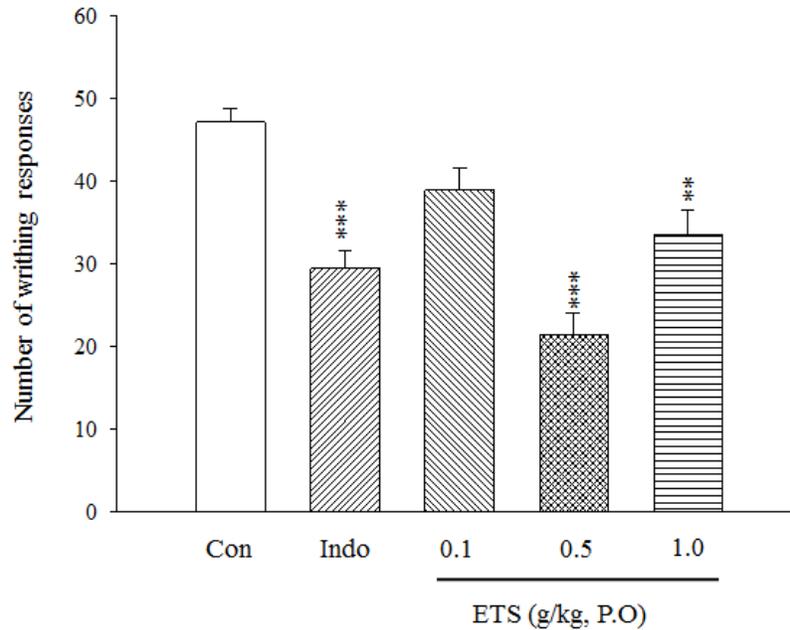
The writhing method used in this study was similar to that described in a previous study (Chao et al., 2009). The writhes were induced by i.p. injection of 1% acetic acid (10 ml/kg of body weight). ETS-treated groups received ETS (0.25, 0.5, and 1.0 g/kg, p.o.), whereas the positive control group received Indo (10 mg/kg, i.p.). The findings showed that ETS (0.5 and 1.0 g/kg) and Indo (10 mg/kg) demonstrated inhibition in this model (P < 0.01 to 0.001) (Figure 1). The inhibitory effect of acetic acid-induced writhing by ETS was similar to that produced by a positive-control Indo.

### Formalin test

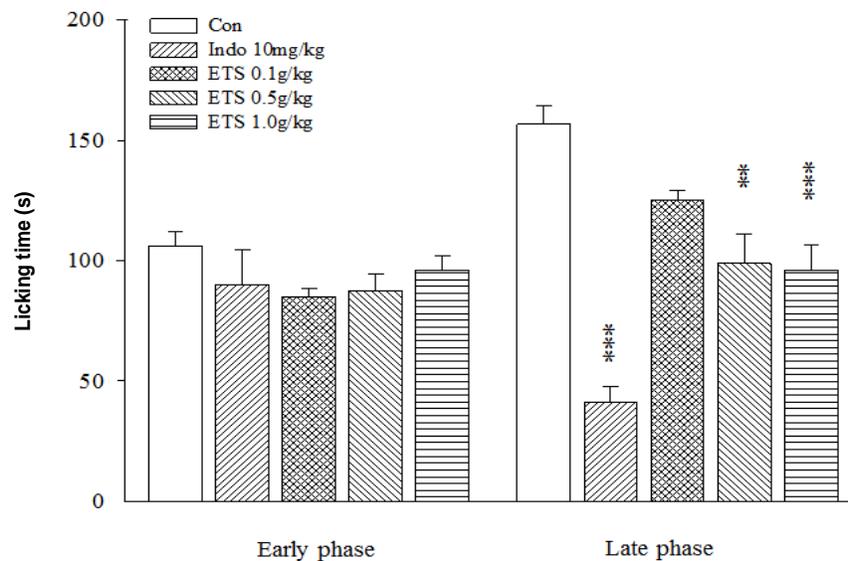
The antinociceptive activity of ETS was determined using the formalin test, as shown in Figure 2 (Dubuisson and Dennis, 1977). The results demonstrated a dose-dependent relationship of ETS in both phases of formalin-induced pain. In the early phase, no significant inhibition was generated with the doses of ETS (0.25, 0.5, and 1.0 g/kg) and Indo (10 mg/kg), compared with the control group. In the late phase, doses of 0.5 and 1.0 g/kg significantly reduced nociception. The Indo treatment also produced significant inhibition in the late phase.

### Carr-induced mice paw edema

Carr-induced mice paw edema was used to determine the



**Figure 1.** Analgesic effect of ETS and Indo on the acetic acid-induced writhing response in mice. Each value is represented as the mean  $\pm$  SEM. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared with the control (Con) group (one-way ANOVA followed by Scheffe's multiple-range test).

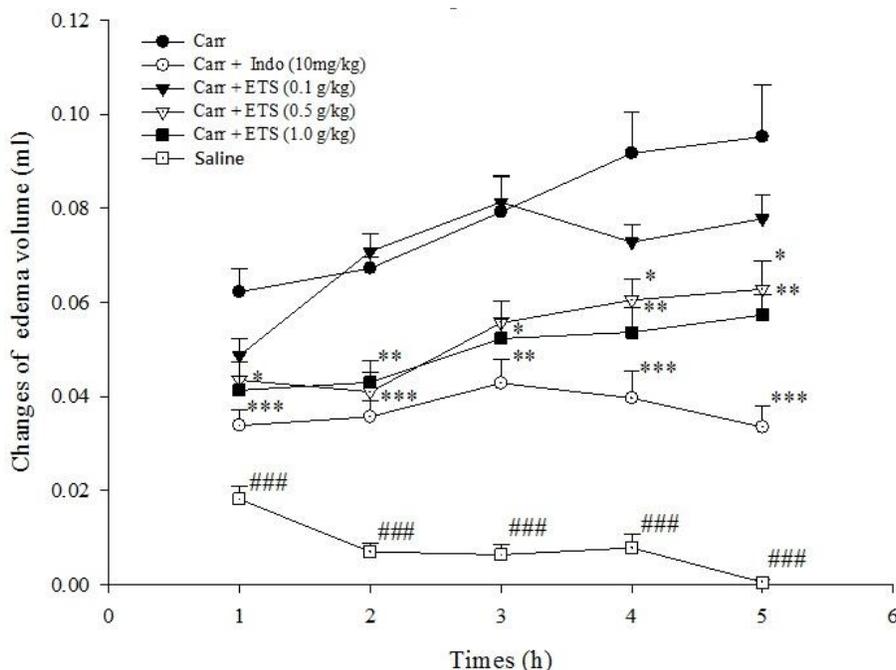


**Figure 2.** Analgesic effect of ETS and Indo on (a) early phase and (b) late phase in the formalin test on the mice. Each value is represented as the mean  $\pm$  SEM. \* $P < 0.05$ , \*\*\* $P < 0.001$ , compared with the control (Con) group (one-way ANOVA followed by Scheffe's multiple-range test).

anti-inflammatory activity of ETS (Figure 3). Carr-induced paw edema was dramatically reduced in a dose-dependent manner by administrate ETS at 1 to 5 h after Carr injection. A significant anti-inflammatory effect of Indo was also observed after the Carr injection.

### The activities of anti-oxidant enzymes

After the third hour following the intrapaw injection of Carr, liver tissues were analyzed for biochemical parameters, such as those of GPx, GRx, SOD, and CAT activities



**Figure 3.** Effects of ETS and Indo on hind-paw edema induced by Carr in mice. Each value is represented as the mean  $\pm$  SE (n = 6). \*Significant difference from the Carr group (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001).

(Table 1). Carr reduced the activities of GPx, GRx, SOD, and CAT in Carr-induced paw edema by 61.3, 40.2, 42.3, and 53.9%, respectively, compared to the control group. In the range of 0.5 to 1.0 g/kg, ETS significantly increased the activities of GPx, GRx, SOD, and CAT, compared to that observed in the Carr group, as well as the Indo group.

### MDA Level

In the control group, the MDA level in the edema paw significantly induced by Carr increased. However, MDA levels were lowered significantly after treatment with ETS at 0.5 and 1.0 g/kg. Indo, in the positive-control group, reduced the MDA level as compared to that observed in the Carr group.

### Serum TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 level

The TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 levels increased significantly in serum at the fifth hour after Carr injection (P < 0.001). However, ETS (0.5 or 1.0 g/kg) reduced the TNF- $\alpha$ , IL-1 $\beta$  and IL-6 levels in serum (P < 0.01 or P < 0.001), as well as 10 mg/kg Indo (Table 2).

## DISCUSSION

In this study, we evaluated the putative analgesic and

anti-inflammatory activities of ETS to clarify the pain and inflammation-relieving effects. Two analgesic animal models were used to identify the possible peripheral and central effects of the test substances. In the acetic acid-induced writhing response, the visceral pain model, the analgesic mechanism of abdominal writhing, was induced by acetic acid, which involves the release of arachidonic acid (AA) through COX and PG biosynthesis (Koo et al., 2006). ETS at the oral dose of 0.5 to 1.0 g/kg significantly reduced the writhing response of acetic acid-induced mice. The effect may be due to the inhibition of the synthesis of AA or its metabolites. The formalin test, which is believed to be relatively more specific in identifying pain in central and/or peripheral components, was also used in this study. The formalin test consists of 2 biphasic phases: an early phase (lasting the first 5 min) corresponding to acute neurogenic pain, and a late phase (lasting from 15 to 30 min) corresponding to inflammatory pain responses (Huang et al., 2011). ETS was administered orally (0.5 and 1.0 g/kg) to the mice, which produced dose-related inhibition late-phase pain, but did not inhibit neurogenic (early phase) pain caused by intraplantar injection of formalin, suggesting that the antinociceptive effect of ETS could be due to its peripheral action (Figure 2).

The Carr-induced mice paw edema has been well established as a valid model for evaluating NSAIDs, and has been accepted as a useful phlogistic tool for investigating the antiedematous effect of natural products (Di Rosa et al., 1971; Garcia Leme et al., 1973). Carr-

**Table 1.** Effects of ETS and Indo on changes in GPx, GR, SOD, and CAT activities were studied in Carr-induced paw edema in mice.

Group	U/mg protein			
	GPx	GR	SOD	CAT
Control	40.07 ± 2.69 <sup>###</sup>	2.87 ± 0.31 <sup>###</sup>	0.087 ± 0.005 <sup>###</sup>	8.39 ± 1.52 <sup>###</sup>
Carr	23.11 ± 2.49	1.11 ± 0.11	0.052 ± 0.003	3.87 ± 0.44
Carr + Indo (10 mg/kg)	38.79 ± 3.51 <sup>***</sup>	1.96 ± 0.24 <sup>***</sup>	0.084 ± 0.008 <sup>***</sup>	5.86 ± 0.43 <sup>**</sup>
Carr + ETS (0.25 g/kg)	24.84 ± 2.00	1.23 ± 0.16	0.059 ± 0.005	3.82 ± 0.95
Carr + ETS (0.5 g/kg)	30.23 ± 1.81 <sup>*</sup>	1.52 ± 0.16 <sup>*</sup>	0.061 ± 0.007 <sup>*</sup>	6.44 ± 0.54 <sup>**</sup>
Carr + ETS (1 g/kg)	37.44 ± 4.04 <sup>***</sup>	2.10 ± 0.29 <sup>***</sup>	0.076 ± 0.006 <sup>**</sup>	6.48 ± 0.93 <sup>***</sup>

**Table 2.** Effects of ETS and indomethacin (Indo) on changes in TNF- $\alpha$ , IL-1, and IL-6.

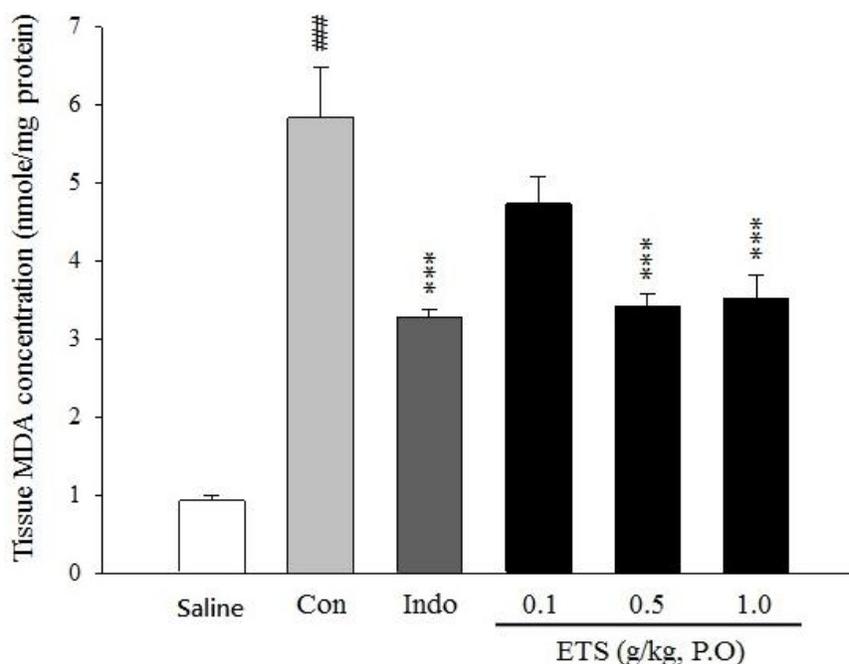
Group	Protein (ng/mg)		
	TNF- $\alpha$	IL-1	IL-6
Control	ND <sup>*</sup>	ND	ND
Carr	232.9 ± 13.8	216.9 ± 50.0	54.0 ± 13.1
Carr + Indo (10 mg/kg)	100.3 ± 9.6 <sup>***</sup>	76.6 ± 17.2 <sup>***</sup>	21.6 ± 5.1 <sup>***</sup>
Carr + ETS (0.1 g/kg)	234.2 ± 15.2	196.7 ± 30.4	50.3 ± 5.8
Carr + ETS (0.5 g/kg)	162.8 ± 14.1 <sup>**</sup>	141.1 ± 15.5 <sup>*</sup>	28.0 ± 4.5 <sup>**</sup>
Carr + ETS (1 g/kg)	124.0 ± 13.0 <sup>***</sup>	109.3 ± 17.4 <sup>**</sup>	21.0 ± 5.2 <sup>***</sup>

\*ND: Not detectable

induced inflammatory responses fall into the category of acute inflammation, which involves the synthesis or release of several inflammatory mediators including bradykinin, prostaglandins (PGs), nitric oxide, and cytokines, which further cause pain and fever (Vinegar et al., 1969). The development of edema in the hind paw following Carr injection has been characterized as a biphasic event to generate a series of inflammatory reactions (Vinegar et al., 1969). The initial phase of edema (0 to 1 h) contributes to the release of several mediators, such as histamine, 5-hydroxytryptamine (5-HT), and bradykinin (Di Rosa et al., 1971). Conversely, the second phase, in which swelling accelerates (1 to 6 h), is correlated with an elevated production of TNF- $\alpha$ , NO, and PGs (Posadas et al., 2004). In this study, it was demonstrated that ETS and indomethacin, a potent COX inhibitor, significantly inhibited the development of edema at the second phase after treatment. It suggested that the anti-edema effects of ETS are due to its inhibition of TNF- $\alpha$  or cytokine synthesis in the blood cells (Figure 3 and Table 2), as described for the anti-inflammatory mechanism of indomethacin in inhibiting the inflammatory process, induced by carrageenans (Di Rosa et al., 1971).

The inflammatory response to Carr has been linked to a rapid, mainly polymorphonuclear leucocyte (PMN) infiltration and the production of PMN-derived free radicals, as well as the release of other PMN-derived mediators (Dawson et al., 1991). The free radicals, NO and PG, are released when administering Carr for 1 to 6 h,

and the degree of paw edema was raised to the maximum level in the third hour. MDA is one of the most frequently used biomarkers for evaluating the level of oxidative stress in many systems (Nielsen et al., 1997). This compound is a reactive aldehyde and reacts with deoxyadenosine and deoxyguanosine in DNA, forming DNA adducts (Marnett, 1999; Farmer and Davoine, 2007). The excess accumulation of MDA results in a series of mutagenesis and carcinogenesis, as well as inflammatory reactions. Increasing the level of GSH, a known oxyradical scavenger, toward reduces MDA production. Endogenous GSH plays a critical role against Carr-induced local inflammation. With some pathophysiological conditions associated with inflammation or oxidative stress, these reactive oxygen species (ROS) have been proposed to mediate cell damage through various independent mechanisms, such as the initiation of lipid peroxidation, the depletion of glutathione, and the inactivation of various antioxidant enzymes. Because of the importance of the oxidative status in edema formation, the anti-inflammatory effect exhibited by the drug in this model might be related to its antioxidant properties. A comparison of the antioxidant enzymes of extract-treated mice with those of control mice showed that the activities of GRx, GPx, SOD, and CAT were significantly enhanced (Table 1). Moreover, significant decreases in the MDA level occurred with ETS treatment (Figure 4). The results suggest that ETS may act as a natural antioxidant to protect cells against the



**Figure 4.** MDA accumulation of ETS and Indo at the third hour after Carr injection in the mice edema paw. Each value is represented as the mean  $\pm$  SEM. \*\*\* $P < .001$ , compared with the Carr group (one-way ANOVA followed by Scheffe's multiple-range test).

threat of reactive free radicals and inflammatory damage.

The pro-inflammatory cytokines, principally TNF- $\alpha$  and IL-1, are small and nonstructural proteins induced at sites of local inflammation, and they initiate the cascade of inflammatory mediators by targeting the endothelium (Dinarello, 2000). The TNF- $\alpha$  and IL-1 cytokines are involved in neutrophil migration in  $\lambda$ -carrageenan-induced inflammation. These mediators are able to recruit leukocytes, such as neutrophils, as reported in several recent experimental models (Salvemini et al., 1996; Loram et al., 2007). The TNF- $\alpha$  cytokine also induces a number of physiological and pathological effects, including septic shock, cytotoxicity, and inflammation (Salvemini et al., 2003). It is capable of inducing the further release of PGE<sub>2</sub>, iNOS, and COX-2, which is suggested to have a critical role in the maintenance of the long-lasting nociceptive response (Subbaramaiah and Dannenberg, 2003). In our previous study, we demonstrated that ETS reduced LPS-induced NO production and the expression of iNOS and COX-2 in RAW264.7 cells (Liu et al., 2012). In this study, it was found that ETS significantly reduced the TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 levels in serum after Carr injection by treatment with 0.5 and 1.0 g/kg.

In conclusion, it was demonstrated that ETS exhibited anti-inflammatory activity against Carr-induced paw edema and analgesic activity against nociceptive responses triggered in mice by i.p. acetic acid or intraplantar formalin injections. The anti-inflammatory

mechanisms of ETS are considered to be closely related to the increase in the activities of antioxidant enzymes (GPx, PGx, SOD, and CAT). Furthermore, ETS had an analgesic effect in both nociceptive models. The anti-inflammatory and analgesic effects of ETS are correlated and share common molecular pathways, including the inhibition of pro-inflammatory cytokine production. Therefore, TS may act as a pharmacological agent in the prevention or treatment of diseases in which free radical formation is a pathogenic factor.

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## REFERENCES

- Aebi H (1984). Catalase in vitro. *Methods Enzymol.* 105:121–126.
- Amabeoku GJ, Kabatend J (2012). Antinociceptive and Anti-Inflammatory Activities of Leaf Methanol Extract of *Cotyledon orbiculata* L. (Crassulaceae). *Adv. Pharmacol. Sci.* 2012:862625.
- Carlberg I, Mannervik B (1985). Glutathione reductase. *Methods Enzymol.* 113:484–490.

- Chao J, Lu TC, Liao JW, Huang TH, Lee MS, Cheng HY, Ho LK, Kuo CL, Peng WH (2009). Analgesic and anti-inflammatory activities of ethanol root extract of *Mahonia oiwakensis* in mice. *J. Ethnopharmacol.* 125:297-303.
- Chiu YJ, Huang TH, Chiu CS, Lu TC, Chen YW, Peng WH, Chen CY (2012). Analgesic and Antiinflammatory Activities of the Aqueous Extract from *Plectranthus amboinicus* (Lour.) Spreng. Both In Vitro and In Vivo. *Evid Based Complement Alternat Med.* 2012;2012:508137. doi: 10.1155/2012/508137. Epub 2011 Sep 11.
- Dawson J, Sedgwick AD, Edwards JC, Lees P (1991). A comparative study of the cellular, exudative and histological responses to carrageenan, dextran and zymosan in the mouse. *Int. J. Tissue React.* 13:171-185.
- Dinarello CA (2000). Proinflammatory cytokines. *Chest.* 118:503-508.
- Di Rosa M, Giroud JP, Willoughby DA (1971). Studies on the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. *J. Pathol.* 104:15-29.
- Dubuisson D, Dennis S (1977). The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain* 4:161-174.
- Farmer EE, Davoine C (2007). Reactive electrophile species. *Curr. Opin. Plant Biol.* 10:380-386.
- Flohe L, Gunzler WA (1984). Assays of glutathione peroxidase. *Methods Enzymol.* 105:114-121.
- Fontenele JB, Viana GSB, Xavier-Filho J, De-Alencar JW (1996). Anti-inflammatory and analgesic activity of a water-soluble fraction from shark cartilage. *Braz. J. Med. Biol. Res.* 29:643-646.
- Garcia Leme J, Hamamura L, Leite MP, Rocha e Silva M (1973). Pharmacological analysis of the acute inflammatory process induced in the rat's paw by local injection of carrageenin and by heating. *Br. J. Pharmacol.* 48:88-96.
- Huang MH, Huang SS, Wang BS, Wu CH, Sheu MJ, Hou WC, Lin SS, Huang GJ (2011). Antioxidant and anti-inflammatory properties of *Cardiospermum halicacabum* and its reference compounds ex vivo and in vivo. *J. Ethnopharmacol.* 133:743-750.
- Koo HJ, Lim KH, Jung HJ, Park EH (2006). Antiinflammatory evaluation of gardenia extract, geniposide and genipin. *J. Ethnopharmacol.* 103:496-500.
- Liu CY, Lin YC, Deng JS, Liao JC, Peng WH, Huang GJ (2012). Antioxidant, anti-inflammatory, and antiproliferative activities of *Taxillus sutchuenensis*. *Am. J. Chin. Med.* 40:335-348.
- Liu DZ, Liang HJ, Chen CH, Su CH, Lee TH, Huang CT, Hou WC, Lin SY, Zhong WB, Lin PJ, Hung LF, Liang YC (2007). Comparative antiinflammatory characterization of wild fruiting body, liquid-state fermentation, and solid-state culture of *Taiwanofungus camphoratus* in microglia and the mechanism of its action. *J. Ethnopharmacol.* 113:45-53.
- Loram LC, Fuller A, Fick LG, Cartmell T, Poole S, Mitchell D (2007). Cytokine profiles during carrageenan-induced inflammatory hyperalgesia in rat muscle and hind paw. *Pain* 8:127-136.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Marnett LJ (1999). Lipid peroxidation-DNA damage by malondialdehyde. *Mutat. Res.* 424:83-95.
- Moncada S, Palmer RM, Higgs EA (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* 43:109-142.
- Misra HP, Fridovich I (1972). The role of superoxide anion in the autoxidation of ep phrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* 247:3170-3175.
- Nakhai LA, Mohammadirad A, Yasa N, Minaie B, Nikfar S, Ghazanfari G, Zamani MJ, Dehghan G, Jamshidi H, Boushehri VS, Khorasani R, Abdollahi M (2007). Benefits of *Zataria multiflora* Boiss in experimental model of mouse inflammatory bowel disease. *Evid. Based Complement Alternat. Med.* 4:43-50.
- Nielsen F, Mikkelsen BB, Nielsen JB, Andersen HR, Grandjean P (1997). Plasma malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-style factors. *Clin. Chem.* 43:1209-1214.
- Posadas I, Bucci M, Roviezzo F, Rossi A, Parente L, Sautebin L, Cirino G (2004). Carrageenan-induced mouse paw oedema is biphasic, age-weight dependent and displays differential nitric oxide cyclooxygenase-2 expression. *Br. J. Pharmacol.* 142:331-338.
- Qui H, Gilbert MG (2003). Lorantheaceae. *Flora of China.* 5:220-239.
- Salvemini D, Ischiropoulos H, Cuzzocrea S (2003). Roles of nitric oxide and superoxide in inflammation. *Methods Mol. Biol.* 225:291-303.
- Salvemini D, Wang ZQ, Wyatt PS, Bourdon DM, Marino MH, Manning PT, Currie MG (1996). Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation. *Br. J. Pharmacol.* 118:829-838.
- Sheeba MS, Asha VV (2009). *Cardiospermum halicacabum* ethanol extract inhibits LPS-induced COX-2, TNF-alpha and iNOS expression, which is mediated by NF-kappa B regulation, in RAW264.7 cell. *J. Ethnopharmacol.* 124:39-44.
- Subbaramaiah K, Dannenberg AJ (2003). Cyclooxygenase 2: a molecular target for cancer prevention and treatment. *Trends Pharmacol. Sci.* 24:96-102.
- Vinegar R, Schreiber W, Hugo R (1969). Biphasic development of carrageenin edema in rats. *J. Pharmacol. Exp. Ther.* 166:96-103.
- Wallace JL (2001). Pathogenesis of NSAID-induced gastroduodenal mucosal injury. *Best Pract. Res. Clin. Gastroenterol.* 15:691-703.
- Wang Y, Deng M, Zhang SY, Zhou ZK, Tian WX (2008). Parasitic loranthus from Lorantheaceae rather than Viscaceae potently inhibits fatty acid synthase and reduces body weight in mice. *J. Ethnopharmacol.* 118:473-478.
- Wilson CA, Calvin CL (2006). An origin of aerial branch parasitism in the mistletoe family, Lorantheaceae. *Am. J. Bot.* 93:787-796.
- Winter CA, Risley EA, Nuss GW (1962). Carrageenin-induced edema in hind paw of the rats as an assay for antiinflammatory drugs. *Proc. Soc. Exp. Biol. Med.* 111:544-547.
- Zimmermann M (1983). Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 16:109-110.

Full Length Research Paper

## Ameliorating effect of *Withania somnifera* on acephate administered male albino rats

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This study was performed to investigate the effects of *Withania somnifera* (family: Solanaceae) on the antioxidant status and hormonal level in acephate administered rats. Oral administration of acephate (75 mg/kg body weight/day) for 15 and 30 days caused a significant decrease in serum testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) concentration when compared with control group. Serum testosterone, LH and FSH concentration were increased in group IV and group V indicating a positive influence of *W. somnifera* on acephate administered rats. The changes in the antioxidant parameters were accompanied by an increase in testicular lipid peroxidation and reduction in glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) activity. The level of lipid peroxidation was reduced whereas GSH content, SOD and catalase activity were elevated after treatment with *W. somnifera* at the dose level of 100 mg/kg body weight/day. In conclusion, this study showed that acephate apart from being a hormonal disrupter also causes oxidative stress which contributed to reproductive toxicity in the male rats. The protective effects of *Withania* on reproductive toxicity and oxidative stress have also been shown as evidenced by a clear attenuation of acephate-induced hormonal imbalance and oxidative stress.

**Key words:** Testosterone, antioxidant, lipid peroxidation and oxidative stress.

### INTRODUCTION

Human reproductive health is a major health problem, indicating the decline in male reproductive health and increase in population of infertile males. The global magnitude of infertility is that 60 to 80 million couples suffer infertility each year and this highlights the need for research in this field to study the decline in reproductive health and effect of environmental antiandrogens (WHO, 1996).

Pesticides are the most common xenobiotics present in the environment and causing toxicity. Prolong exposure of pesticides affected the normal functioning of different organ system and produced many clinical effects (Azmi et al., 2006; Sharma and Singh, 2010). They also alter

the reproductive function by altering sperm count and sperm shape, alter sexual behavior or increase infertility in animals and human beings (Chang et al., 2004; Okamura et al., 2005; Jensen et al., 2006; Joshi and Sharma, 2011; Joshi et al., 2011).

Organophosphate pesticides belong to a group of insecticides that act by inhibiting acetylcholinesterase (AChE) activity in insects and mammals (Spassova et al., 2000; Cabello et al., 2001). Besides inhibiting cholinesterase (ChE), oxidative stress has been recently proposed as a main toxicity mechanism for organophosphorus (OPs) both in acute and chronic poisoning cases (Mostafalou et al., 2012). There have

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been increasing concerns about the effects of various organophosphate insecticides in humans and animals. These include cholinergic and non-cholinergic biological disturbances (Quistad et al., 2001; Bomser et al., 2002; Gordon and Mack, 2003). Acephate is an organophosphate insecticide used to kill insects by direct contact or ingestion and disrupts their normal nervous system functions by phosphorylating the active site of the acetylcholinesterase enzyme, rendering it inactive (Kumar, 2004). It is used worldwide because of easy availability. A number of studies were conducted on the toxicity of acephate on different organisms and indicated that it is a potent neurotoxic, mutagenic, carcinogenic, and cytotoxic compound (Singh and Jiang, 2002).

Herbal medicines have been widely used all over the world since ancient times and have been recognized by physicians and patients for their better therapeutic value as they have fewer adverse effects as compared with modern medicines (Goldman, 2001; Jasuja et al., 2012). *Withania somnifera* has been in use for over 2500 years to treat all kind of diseases and human ailments (Bhattacharya et al., 2001). Various investigators reported useful phytochemical constituents in *W. somnifera* possess antiserotogenic, anticancer, anabolic activity and beneficial effects in the treatment of arthritis, geriatric problems and stress (Asthana and Raina, 1989; Grandhi et al., 1994; Gupta and Rana, 2007; Bairwa et al., 2011; Barkatullah et al., 2013). Further, various medicinal plants were found to be strong radical scavengers, anti-inflammatory, antitumour, antistress, rejuvenating, immunomodulatory, hematopoietic and antioxidant properties (Gautam et al., 2004; Ahmad et al., 2005; Rasool and Varalakshmi, 2006; Steenkamp et al., 2013) which could compromise the medicinal use of these plants in folk medicine (Samy et al., 2013).

Since the plant is of considerable importance in traditional medicinal systems, the objective of the present study was to evaluate effects of oral administration of methanolic extract of *W. somnifera* on the hormonal level and antioxidant parameters in rats administered with acephate.

## MATERIALS AND METHODS

Acephate (Figure 1) (Chemical name: O, S-dimethyl acetylphosphoramidothioate; Trade name: Orthene; Chemical family: Organophosphate) (KR exports pvt. Ltd., Jammu, India) was used as a chemical dissolved in olive oil and administered orally via gavage.

*W. somnifera* (Registration Number - RUBL20910, Department of Botany, University of Rajasthan) used in the present study belongs to family Solanaceae and popularly known as ashwagandha. The plant was obtained from the National Institute of Ayurveda, Jaipur. Leaves and roots were powdered and extracted with 70% methanol for 24 to 36 h by soxhlet extraction method. Then, methanol was separated under reduced pressure to obtain solid mass.

This study was carried out on male albino rats breeds that have been proven fertile (*Rattus Norvegicus*) of Wistar strain weighing 150 to 200 g. They were housed in a hygienic, well-ventilated room

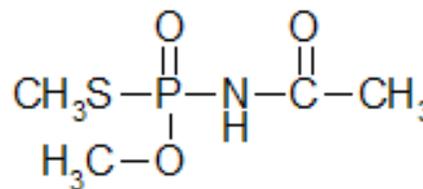


Figure 1. Acephate.

with natural light and dark cycles (12 h dark/12 h light) with relative humidity of 55±5%. They were individually housed in clean polypropylene cages (12"×10"×8") with sawdust bed and covered with stainless steel wire lids. They were fed on standard commercial pellet feed procured from Ashirwad Food Industries Ltd., Chandigarh (Punjab) and fresh water was provided *ad libitum* throughout the study. The rats were divided into 6 groups (n=6) mentioned in Table 1. All animal experiments were carried out as per the guidelines of Department of Zoology, University of Rajasthan, Ethical Committee. Experimental design are as shown in Table 1.

At the end of experimental period (30 days), the animals were weighed and euthanized under light ether anaesthesia and blood samples were collected by cardiac puncture in preheparinized tubes. Serum was separated by centrifugation at 3000 rpm and stored at -20°C to carry out biochemical parameters, FSH, LH and testosterone assay. Testis was dissected out and frozen for the biochemical estimations. They were also fixed in Bouin's fixative for at least 48 h, processed by the paraffin wax impregnation method and sections (5 µm thick) were cut using a rotary microtome. The sections were mounted on clean slides, then stained with haematoxylin and eosin (H&E) and examined by light microscopy for histopathological changes. Microphotographs of sections were taken.

The following parameters have been estimated in serum and testis-testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH) were estimated in serum through chemiluminescence in fully automatic Advia Cemtaus Immuno Assay System. Oxidative stress and antioxidant parameters, that is, lipid peroxidation (LPO) (Ohkawa et al., 1979), catalase (Claiborne, 1985), superoxide dismutase (SOD) (Das et al., 2000) and glutathione (GSH) (Carlberg and Mannervik, 1975) were performed in testis. Histopathological examination of the testis was performed by fixing them in Bouin's fixative. Further, they were cut into pieces and processed through ethanol-xylene series. The tissues were then embedded in paraffin and bee wax (3:1) (M.P. 55-62°C). Sections were cut at 5 µm thickness and stained with Harris haematoxylin and eosin (H&E).

The data obtained from the aforementioned experiments were subjected to statistical analysis. Data were represented as mean±standard error (SE). The differences were compared for statistical significance by "t- test" by using Statistical Package for Social Sciences (SPSS) software (16.0 version) and they were considered non significant at p≤0.05, significant at p≤0.01 and highly significant at p≤0.001. Graphical representation of data has been done using Microsoft Excel 2007.

## RESULTS

A significant decrease (p≤0.01 and p≤0.001) was observed in serum testosterone, FSH and LH concentration of acephate administered rats at the dose level of 75 mg/kg body weight/day for 15 and 30 days except FSH value in group II which was non-significant.

**Table 1.** Experimental design (The rats were divided into 6 groups as summarized).

Group	Treatment
I	Control received vehicle (olive oil) only
II	Acephate 75 mg/kg body weight/day for 15 days
III	Acephate 75 mg/kg body weight/day for 30 days
IV	Acephate 75 mg/kg body weight/day for 15 days, then acephate withdrawn and treated with <i>W. somnifera</i> extract (100 mg/kg body weight/day) for next 15 days, that is, from day 16 to 30
V	Acephate 75 mg/kg body weight/day + <i>W. somnifera</i> extract (100 mg/kg body weight/day) from day 1-30 (Concurrent treatment)
VI	Acephate 75 mg/kg body weight/day for 15 days, then no treatment for next 15 days (that is, from day 16 to 30)

**Table 2.** Effect of acephate and *W. somnifera* on Sex hormones levels in male albino rats.

Treatment	Serum testosterone (ng/ml)	Serum LH (mlu/ml)	Serum FSH (mlu/ml)
I Control (Vehicle only)	3.10±0.09	1.92±0.05	0.65±0.04
II Acephate for 15 days	2.58*±0.07	1.48*±0.09	0.45 <sup>ns</sup> ±0.05
III Acephate for 30 days	1.56**±0.06	0.81**±0.06	0.30**±0.04
IV Acephate from day 1 to 15+ <i>W. somnifera</i> extract for next 15 days	2.78*±0.04	1.65**±0.03	0.50*±0.02
V Acephate+ <i>W. somnifera</i> extract from day 1-30 (Concurrent feeding)	2.71*±0.08	1.60*±0.06	0.49*±0.03
VI Acephate from day 1 to 15+No treatment for next 15 days	2.68 <sup>ns</sup> ±0.10	1.54 <sup>ns</sup> ±0.11	0.48 <sup>ns</sup> ±0.04

Mean ± 6 animals, Acephate: 75 mg/kg body weight/day; *W. somnifera*: 100 mg/kg body weight/day. Groups II, III, IV, V, VI compared with I: <sup>ns</sup>p≤0.05 (Non significant); \*p≤0.01 (Significant); \*\*p≤0.001 (Highly significant).

Hormonal concentration was increased in groups IV and V indicating a positive influence of *W. somnifera* on the serum testosterone, FSH and LH in acephate administered rats (Table 2).

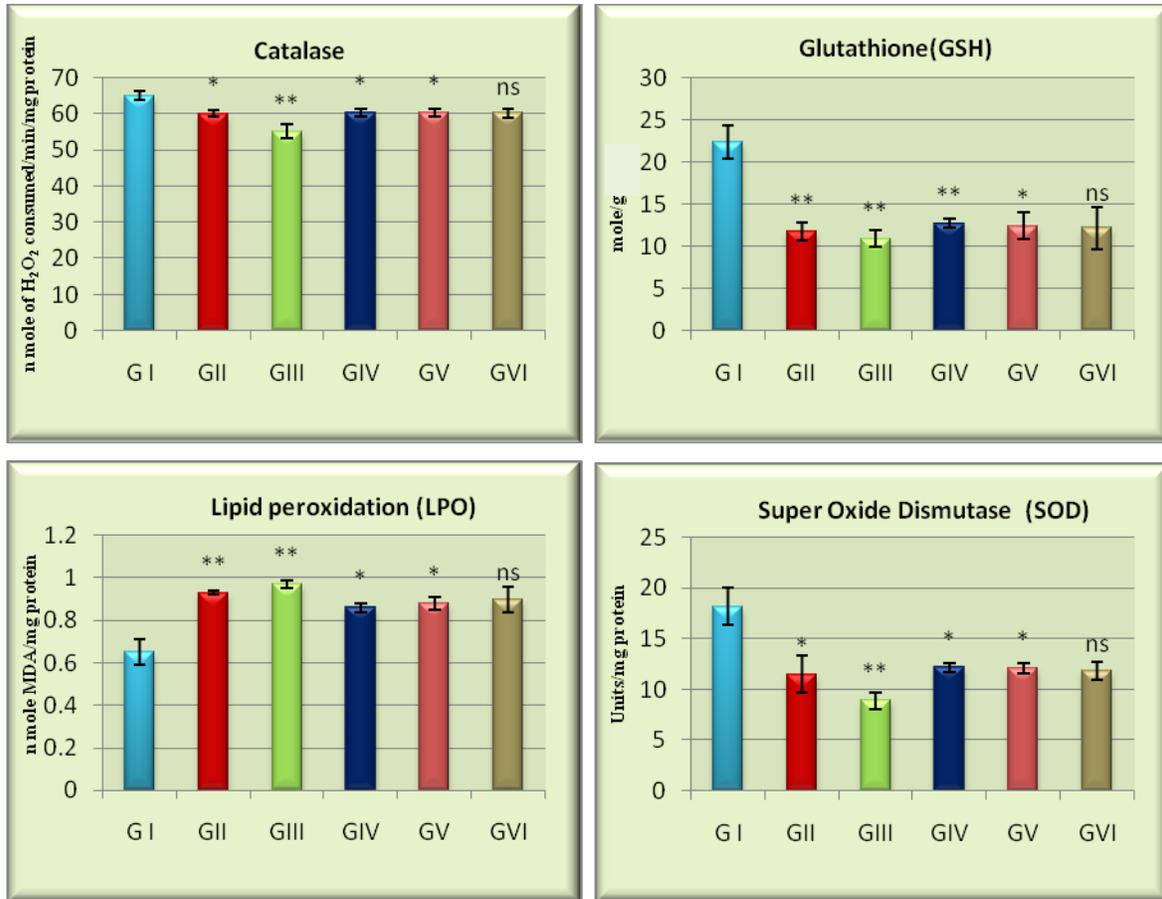
At dose level of 75 mg/kg body weight/day for 15 and 30 days, significant decrease in the catalase, SOD and GSH level of testis were noticed in acephate exposed rats in comparison to control animals. Statistically significant elevation (p≤0.01 and p≤0.001) was observed in catalase, SOD and GSH level of testis in *W. somnifera* treated rats (Figure 2). As a biomarker of lipid peroxidation, malondialdehyde (MDA) levels were measured in testis homogenates. MDA level was significantly increased in the acephate administered rats in comparison with the control one, sustaining the impaired antioxidant status of these animals. Moreover, lipid peroxidation was significantly decreased as indicated by the lower levels of MDA in testis after treatment with *W. somnifera* when compared with acephate administered rats. Non-significant decrease was observed in no treatment group (Figure 2).

The histology of testis of control rats showed normal histoarchitecture of seminiferous tubule with all successive stages of spermatogenesis. Sertoli cells were present. Irregular seminiferous tubules with inhibited spermatogenesis were seen in testis of rats treated with acephate for 15 and 30 days. Microphotograph of testis

treated with *W. somnifera* depicts slight improvement. Inter tubular space has also reached near to normal. Testis of concurrent group also maintain almost normal histoarchitecture, but not as recovered as treatment group. Lumen was filled with spermatozoa. In untreated group, testis was showing loosened tunica propria and degenerated Leydig cells. Lumen was filled with cellular debris (Figure 3).

It was found that acephate administration to rats for 15 and 30 days caused a significant reduction in the activity of catalase, SOD and GSH contents whereas an increase in thiobarbituric acid reactive substances (TBARS; measurement of LPO) activity of testis was observed. The level of LPO was reduced whereas GSH content and catalase activity were elevated after the treatment with 70% methanolic extract of *W. somnifera*. In this study, acephate administered rats had significantly lower levels of GSH contents in testis.

SOD and catalase are the major enzymes dealing with reactive oxygen species (ROS) in most cells. Both enzymes play an important role in elimination of ROS derived from the redox process of xenobiotics in the liver tissue. SOD catalyses dismutation of superoxide anions into hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Hydrogen peroxide is the end product of SOD dismutation, while degradation of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub> is catalyzed by catalase and glutathione peroxidase (GSH-Px) (Bansal and Jaswal, 2009). Since



**Figure 2.** Testicular antioxidant and oxidative stress parameters (Acephate 75 mg/kg body weight/day). Mean  $\pm$  6 animals (G II; G III; G IV; G V; G VI compared with G I); <sup>ns</sup>p<0.05 (Non significant); \*p<0.01 (Significant); G: group.

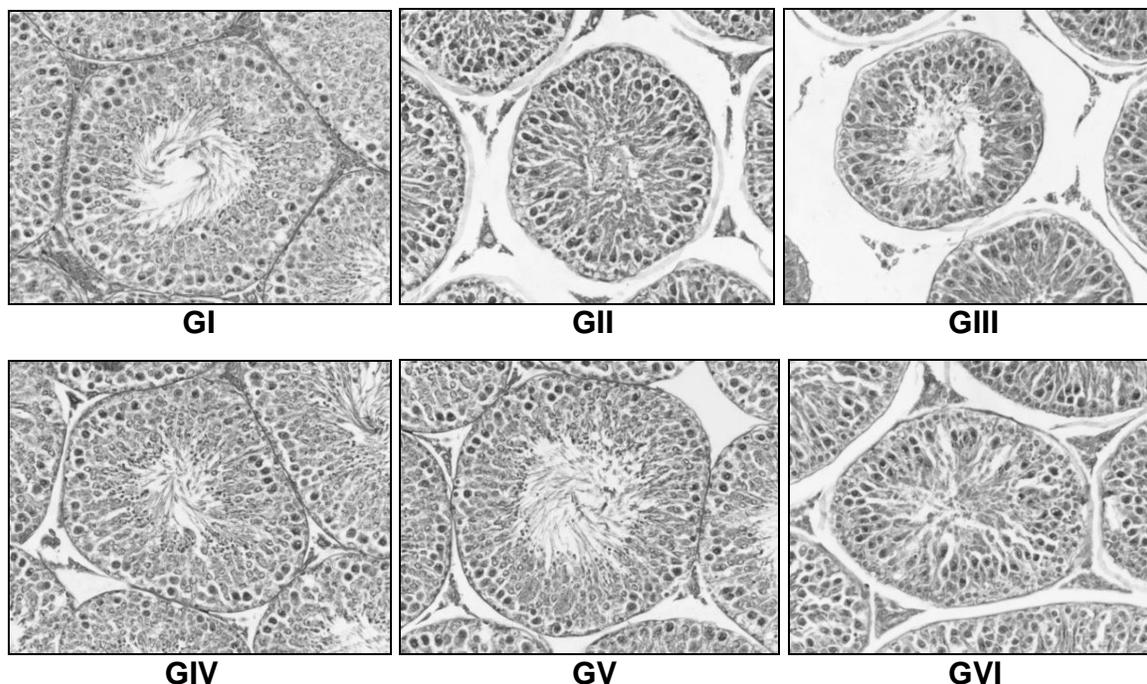
the end product of SOD is substrate for catalase catalyzation. Studies have shown that pesticides diminish the antioxidant defense system and decrease the activity of SOD and CAT, thereby elevating the lipid peroxide content.

Co-treatment with acephate and *W. somnifera* attenuated spermatogenic/testicular damage induced by acephate treatment as shown by the return of sperm count, motility and normal morphology toward normal control values. The restoration of testosterone, LH, and FSH levels to normal after administration with *W. somnifera* might have stimulated the production of quantitatively and structurally normal sperm.

## DISCUSSION

This study described that pesticides may disrupt the hormonal function of the male reproductive system and fertility. The spermatozoa, in common with all cell types have developed an elaborate antioxidant defense system consisting of enzymes such as catalase, SOD and reduced GSH that scavenge and suppress the formation

of ROS. Estimation of end products of LPO such as MDA is an index of the extent of oxidative damage to cellular structures (Sharma and Agarwal, 1996). Increased LPO is thought to be a consequence of oxidative stress which occurs when the dynamic balance between pro-oxidant and antioxidant mechanism is impaired (Nur Azlina and Nafeeza, 2007). Administration of *W. somnifera* reduced the lipid peroxidative markers in the tissues. This indicates that *W. somnifera* extract react with peroxy radicals including the inhibition of LPO chain propagation (Kulisic et al., 2004). Attenuated level of LPO in extract treated animals is suggestive of the antioxidant nature of this plant. El-Demerdash (2011) also observed that insecticide mixture resulted in a significant increase in TBARS, which might be associated with decreased levels of reduced GSH, SOD and catalase activities. Apart from enzymatic antioxidants, non-enzymatic antioxidants such as GSH play a vital role in protecting cells from oxidative damage. GSH, a reactive, intracellular, non-protein (tripeptide) thiol in living organisms, performs a key role in coordinating innate antioxidant defense mechanism. It is involved in the maintenance of normal structure of cell, probably through redox and detoxification reactions



**Figure 3.** Photomicrograph of rat testis (X200). GI: Normal testis histoarchitecture of a control rat; GII: Administered acephate for 15 days; GIII: Administered acephate for 30 days; GIV: Acephate from day 1 to 15+*W. somnifera* extract; GV: Acephate+*W. somnifera* extract from day 1 to 30 (Concurrent feeding); GVI: Acephate from day 1 to 15+No treatment for next 15 days.

(Kharb, 2010). Depletion in GSH content might be resulted from intoxication with acephate (Cereser et al., 2001). *W. somnifera* extract treated animals, showed a significantly elevated level of GSH. It is possible that extract might have reduced the extent of oxidative stress, leading to lesser GSH degradation or increase in the biosynthesis of GSH (Prasanna and Purnima, 2011). Supplementation of *W. somnifera* along with acephate (concurrent groups) decreases the free radicals may be by quenching and lowering oxidative stress (Deepa and Anuradha, 2011). Decrease in the SOD and the catalase levels in the acephate administered animals are again attributed to increased oxidative stress in these animals. It is well known that flavonoids and polyphenols are natural antioxidants but have also been reported to significantly increase SOD and catalase activities. The currently noted elevated levels of both SOD and catalase levels could be due to the influence of flavonoids and polyphenols of *W. somnifera* (Fang et al., 2002; Visavadiya and Narasimhacharya, 2005). The decrease in testosterone concentration of acephate treated rats may occur due to the reduced levels of LH (Kerr and Sharpe, 2006) as circulating LH is responsible for maintaining normal plasma testosterone concentrations. Spermatogenesis requires LH and FSH for initiation and maintenance in male rats, LH stimulates Leydig cells to secrete testosterone, normal testicular function is dependent on FSH and testosterone is absolutely required for normal spermatogenesis.

Conclusively, this study demonstrated that oral administration of *W. somnifera* extract could prevent or be helpful in reducing the complications of reproductive toxicity associated with oxidative stress.

#### REFERENCES

- Ahmad M, Saleem S, Ahmad AS, Ansari MA, Yousuf S, Hoda MN, Islam F (2005). Neuroprotective effects of *Withania somnifera* on 6-hydroxydopamine induced Parkinsonism in rats. *Hum. Exp. Toxicol.* 24:137-147.
- Asthana R, Raina MK (1989). Pharmacology of *Withania somnifera* (L) Dunal-A Review. *Indian Drugs* 26:199-205.
- Azmi MA, Naqvi SHN, Azmi MA, Aslam M (2006). Effect of pesticides residue on health and different enzyme levels in the blood of farm workers from Gadap (rural area) Karachi. *Pak. Chemospher.* 64:1739-1744.
- Bairwa GL, ND Jajuja, Joshi SC (2011). Lipid lowering and antioxidant effects of *Ammomum subulatum* seeds (Family Zingiberaceae) in cholesterol fed rabbits. *Arch Phytopathol. Plant Prot.* 44:1425-1431.
- Bansal MP, Jaswal S (2009). Hypercholesterolemia Induced Oxidative Stress Is Reduced in Rats with Diets Enriched with Supplement from *Dunaliella salina* Algae. *Am. J. Biomed. Sci.* 1:196-204.
- Barkatullah BB, Ibrar M, Ali N, Muhammad N, Rehmanullah (2013). Antispasmodic potential of leaves, barks and fruits of *Zanthoxylum armatum* DC. *Afr. J. Pharm. Pharmacol.* 7:685-693.
- Bhattacharya A, Ghosal S, Bhattacharya S (2001). Antioxidant effect of WS glycowithanolides in chronic foot shock induced perturbations of oxidative free radical scavenging enzymes and lipid peroxidation in rat frontal cortex and striatum. *J. Ethnopharmacol.* 74:1-6.
- Bomser JA, Quistad GB, Casida JE (2002). Chlorpyrifos oxon potentiates diacylglycerol-induced extracellular signal-regulated kinase (ERK 44/42) activation, possibly by diacylglycerol lipase inhibition. *Toxicol. Appl. Pharmacol.* 178:29-36.

- Cabello G, Valenzuela M, Vilaxa A, Duran V, Rodolph I, Hrepic N, Calaf G (2001). A rat mammary tumor model induced by the organophosphorus pesticides parathion and malathion possibly through acetylcholinesterase inhibition. *Environ. Health Perspect.* 109:471-479.
- Carlberg I, Mannervik B (1975). Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *J. Biol. Chem.* 250:5475-5480.
- Cereser C, Boget S, Parvaz P, Revol A (2001). Thiram induced cytotoxicity is accompanied by a rapid and drastic oxidation of reduced glutathione with consecutive lipid peroxidation and cell death. *Toxicology* 163:153-162.
- Chang HY, Shih TS, Guo YL, Tsai CY, Hsu PC (2004). Sperm function in workers exposed to N, N-dimethylformamide in the synthetic leather industry. *Fertil. Steril.* 81:1589-1594.
- Claiborne A (1985). Catalase activity In: Grenwald RA (Ed), *Handbook of methods for oxygen radical research*. CRC Press Boca Raton Florida.
- Das K, Samantha L, Chainy GBN (2000). A Modified spectrophotometric assay for superoxide dismutase using nitrite formation by superoxide radicals. *Indian J. Biochem. Biophys.* 37:201-204.
- Deepa B, Anuradha CV (2011). Antioxidant potential of *Coriandrum sativum* L seed extract. *Indian J. Exp. Biol.* 49:30-38.
- El-Demerdash FM (2011). Lipid peroxidation, oxidative stress and acetylcholinesterase in rat brain exposed to organophosphate and pyrethroid insecticides. *Food Chem. Toxicol.* 49:1346-1352.
- Fang YZ, Yang S, Wu G (2002). Free radicals, antioxidants and nutrition. *Nutrition* 18:872-879.
- Gautam M, Diwanay SS, Gairola S, Shinde YS, Jadhav SS, Patwardhan BK (2004). Immune response modulation to DPT vaccine by aqueous extract of *Withania somnifera* in experimental system. *Int. Immunopharmacol.* 4:841-849.
- Goldman P (2001). *Herbal Medicines Today and the Roots of Modern Pharmacology*. Ann. Int. Med. 135:594-600.
- Gordon CJ, Mack CM (2003). Influence of gender on thermoregulation and cholinesterase inhibition in the long-evans rat exposed to diazinon. *J. Toxicol. Environ. Health A* 66:291-304.
- Grandhi A, Majumdar AM, Patwardhan B (1994). A comparative pharmacological investigation of ashwagandha and ginseng. *J. Ethnopharmacol.* 44:131-135.
- Gupta GL, Rana AC (2007). *Withania somnifera* (Ashwagandha) A Review. *Pharmacog. Rev.* 1:129-136.
- Jasuja ND, J Choudhary, P Sharma, N Sharma, SC Joshi (2012). A Review on Bioactive Compounds and Medicinal Uses of Commiphora mukul. *J. Plant Sci.* 7:113-137.
- Jensen TK, Bonde JP, Joffe M (2006). The influence of occupational exposure on male reproductive function. *Occup. Med.* 56:544-553.
- Joshi SC, Bansal B, Jasuja ND (2011). Evaluation of reproductive and developmental toxicity of Cypermethrin in male albino rats. *Toxicol. Environ. Chem.* 93:593-602.
- Joshi SC, Sharma P (2011). Male reproductive toxicity of organophosphorous compounds: a review. *Toxicol. Environ. Chem.* 93:1486-1507.
- Kerr JB, Sharpe RM (2006). Effects and interaction of LH and LHRH agonist on testicular morphology and function in hypophysectomised rats. *J. Reprod. Fertil.* 76:175-192.
- Kharb S (2010). Antioxidant status in asymptomatic hypercholesterolemic subjects. *Gomal J. Med. Sci.* 8:153-155.
- Kulicic T, Radonic A, Katalinic V, Milos M (2004). Use of different methods for testing antioxidative activity of organo essential oil. *Food Chem.* 85:633-640.
- Kumar S (2004). Changes in rat brain regions serotonergic system following acephate poisoning. *Pest. Biochem. Physiol.* 78:140-150.
- Mostafalou S, Abdollahi M, Eghbal MA, Kouzehkonani NS (2012). Protective effect of NAC against malathion-induced oxidative stress in freshly isolated rat hepatocytes. *Adv. Pharm. Bull.* 2:79-88.
- Nur Azlina MF, Nafeeza MI (2007). Phytonutrient: Effects on Lipid Peroxidation in experimental gastritis induced by restraint stress. *Int. J. Pharmacol.* 3:254-259.
- Ohkawa H, Ohishi N, Yagi K (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 95:351-358.
- Okamura A, Kamijima M, Shibata E, Ohtani K, Takagi K, Ueyama J, Watanabe Y, Omura M, Wang H, Ichihara G, Kondo T, Nakajima T (2005). A comprehensive evaluation of the testicular toxicity of dichlorvos in Wistar rats. *Toxicology* 213:129-137.
- Prasanna GS, Purnima A (2011). Protective effect of leaf extract of *Trichilia connaroides* on hypercholesterolemia induced oxidative stress. *Int. J. Pharmacol.* 7:106-112.
- Quistad GB, Sparks SE, Casida JE (2001). Fatty acid amide hydrolase inhibition by neurotoxic organophosphorous pesticides. *Toxicol. Appl. Pharmacol.* 173:48-55.
- Rasool M, Varalakshmi P (2006). Immunomodulatory role of *Withania somnifera* root powder on experimental induced inflammation: An *in vivo* and *in vitro* study. *Vasc. Pharmacol.* 44:406-410.
- Samy AS, Mohamed H, Abdel A, Mona SM, Mona FW (2013). Antibacterial activities, chemical constituents and acute toxicity of Egyptian *Origanum majorana* L., *Peganum harmala* L. and *Salvia officinalis* L. essential oils. *Afr. J. Pharm. Pharmacol.* 7:725-735.
- Sharma P, Singh R (2010). Protective Role of Curcumin on Lindane Induced Reproductive Toxicity in Male Wistar Rats. *Bull. Environ. Contam. Toxicol.* 84:378-384.
- Sharma RK, Agarwal A (1996). Role of reactive oxygen species in male infertility. *Urology* 48:835-850.
- Singh AK, Jiang Y (2002). Immunotoxicity of acute acephate exposure in control or IL-1-challenged rats: correlation between the immune cell composition and corticosteroid concentration in blood. *J. Appl. Toxicol.* 22:279-291.
- Spassova D, White T, Singh AK (2000). Acute effects of acephate and methamidophos on acetylcholinesterase activity, endocrine system and amino acid concentrations in rats. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 126:79-89.
- Steenkamp V, Nkwane O, van Tonder J, Dinsmore A, Gulumian M (2013). Evaluation of the phenolic and flavonoid contents and radical scavenging activity of three southern African medicinal plants. *Afr. J. Pharm. Pharmacol.* 7:703-709.
- Visavadiya NP, Narasimhacharya AVRL (2005). Hypolipidemic and antioxidant activities of *Asparagus racemosus* in hypercholesteremic rats. *Indian J. Pharmacol.* 37:376-380.
- World Health Organization (1996). *The world health report WHO*, Geneva.

*Full Length Research Paper*

## A comprehensive effect of acephate on cauda epididymis and accessory sex organs of male rats

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In the present study, thirty six (36) adult male Wistar rats were divided into six groups each containing 6 Wistar rats (n = 6). Group I served as untreated control group while group II served as the positive control and group III served as the negative control. Group IV, V and VI were considered as the experimental groups. Group II received tetracyclin (28.6 mg/kg/b.wt/day), while group III received vehicle (olive oil 0.5 ml/100 g body weight/day). Group IV, V and VI were administered "acephate" and dissolved in olive oil at dose level of 25, 50 and 75mg/kg b.wt/day for 30 days, respectively. Reproductive toxicity of acephate was evaluated on the basis of weight analysis of cauda epididymis and accessory sex organs, fertility, sperm dynamics, protein content, sialic acid content and histopathological studies. There was a decrease in the weight of epididymis, ventral prostate, vas deferens and seminal vesicle. The results showed highly significant decline in sperm density and motility. Post fertility test showed 30, 60, and 80% negative results. A statistically significant increase was noticed in protein content whereas sialic acid content was decreased in the cauda epididymis and accessory sex organs. The histopathological observations also support the occurrence of toxicity being caused due to exposure of acephate. The observations are thus indicative of the reproductive toxicity caused by acephate at different dose levels in the male rats.

**Key words:** Acephate, fertility, sperm density, sialic acid, toxicity.

### INTRODUCTION

The important impact of men's reproductive health on a couple's fertility is often overlooked. Several studies have suggested that human semen quality and fecundity is declining (Aitken et al., 2004; Jørgensen et al., 2006). Environmental pollutants, occupational exposures and lifestyle have been explored as possible contributors to those changes (Homan et al., 2007). Volatile organic compounds (Wagner et al., 1990), heavy metals (Benoff et al., 2000) or xenoestrogens like some polychlorinated

biphenyls (Rozati et al., 2002), phthalate esters (Duty et al., 2003) and pesticides (Carreño et al., 2007; Joshi et al., 2011) may compromise reproductive male function. Pesticides alter the male reproductive function by altering sperm count and sperm shape, alter sexual behavior or increase infertility in animals and human beings (Figà-Talamanca et al., 2001; Sweeney, 2002; Sheiner et al., 2003; Chang et al., 2004; Presibella et al., 2005; Jensen et al., 2006; Joshi and Sharma, 2011). There have

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been increasing concerns about the effects of various organophosphate insecticides in humans and animals. In mammals, the primary site of action of organophosphate pesticides is the central and peripheral nervous system as they inhibit acetylcholinesterase (AChE), the enzyme that hydrolyses the neuro-transmitter acetylcholine (Slotkin et al., 2006; Bajgar et al., 2009). These include cholinergic and non-cholinergic biological disturbances (Bomser et al., 2002; Gordon Mack, 2003). The widespread use of organophosphates has stimulated research into the possible existence of effects related with their reproductive toxic activity (Joshi et al., 2011; Joshi and Sharma, 2011).

Acephate is an important systemic organophosphorus (OPs) insecticide with toxicity attributed to bioactivation on metabolic conversion to methamidophos which acts as an acetylcholinesterase (AChE) inhibitor (Thapar et al., 2002; Trevizan et al., 2005). It is used for control of a wide range of biting and sucking insects, especially aphids, including resistant species in fruit, vegetables, vine, and hop cultivation and in horticulture. It also controls leaf miners, lepidopterous larvae, sawflies and thrips in the previously stated crops as well as turf, mint and forestry. Acephate and its primary metabolite, methamidophos, are toxic to various species. A number of studies showed the toxicity of acephate on different organisms which indicate it as a potent neurotoxic, mutagenic, carcinogenic and cytotoxic compound (Singh and Jiang, 2002).

Little information is available about the effects of acephate on cauda epididymis and accessory sex organs of male rats. Hence, the present study is aimed to find out the effects of acephate on epididymis and accessory sex organ weight response, sperm dynamics, fertility, protein content, sialic acid content and histopathology.

## MATERIALS AND METHODS

### Chemical

Acephate (Chemical name- O, S-dimethyl acetylphosphoramidothioate; Trade name- Orthene; Chemical family- Organophosphate) (KR exports pvt. Ltd., Jammu, India) was dissolved in olive oil (0.5 ml/100 g body weight/day) and administered orally via gavage (Figure 1).

### Animal model

The present study was carried out on inbreeds proven fertile male albino rats (*Rattus norvegicus*) of Wistar strain weighing 150 to 200 g (Visweswaran and Krishnamoorthy, 2012). The animals were housed in a hygienic, well-ventilated room with natural light and dark cycles (12 h dark, 12 h light) with relative humidity  $55 \pm 5\%$ . They were individually housed in clean polypropylene cages (12" x 10" x 8") with sawdust bed and covered with stainless steel wire lids. They were fed on standard commercial pellet feed procured from Ashirwad Food Industries Ltd., Chandigarh (Punjab) and fresh water was provided *ad libitum* throughout the study.

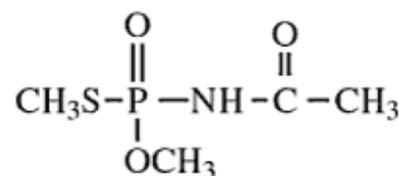


Figure 1. Acephate.

### Experimental design

The rats were divided into following groups:

- Group I: Untreated control.
- Group II: Positive control (tetracycline 28.6 mg/kg b.wt/day)
- Group III: Negative control received vehicle (olive oil 0.5 ml/100 g body weight/day) only.
- Group IV: Acephate 25 mg/kg b.wt/day for 30 days.
- Group V: Acephate 50 mg/kg b.wt./day for 30 days.
- Group VI: Acephate 75 mg/kg b.wt/day for 30 days.

### Parameters studied

Epididymis and accessory sex organs were excised blotted free of blood and weighed. The various parameters were performed by following methods:

**Organ weight:** Weight of epididymides, seminal vesicle, ventral prostate and vas deferens were recorded.

**Sperm motility:** The epididymis was removed immediately after anesthesia, and known weight of cauda epididymis was gently teased in a specific volume of physiological saline (0.9% NaCl) to release the spermatozoa from the tubules. The sperm suspension was examined within five minutes after their isolation from epididymis. The results were determined by counting both motile and immotile sperms in at least ten separate and randomly selected counting chambers of haemocytometer. The results were finally expressed as percent motility (Prasad et al., 1972).

**Sperm density:** Total number of sperms were counted using haemocytometer after further diluting the sperm suspension from cauda epididymis. The sperm density was calculated in million per ml as per dilution (Prasad et al., 1972).

**Fertility test:** The mating exposure test of all the animals was performed. They were cohabited with proestrus females in the ratio 1:3. The vaginal plug and presence of sperm in the vaginal smear was checked for positive mating. Females were separated and resultant pregnancies were noted, when dam gave birth. The number and size of litters delivered were recorded.

### Biochemical analysis

Biochemical analysis of tissues were done by following standard methods: Total protein (Lowry et al., 1951) and Sialic acid (Warren, 1959)

### Histopathology

Cauda epididymis and accessory sex organs of rats were fixed in

**Table 1.** Sperm dynamics and fertility (Acephate for 30 days).

Treatment	Sperm motility (%)	Sperm density (million/ml)	Fertility (%)
	Cauda epididymides		
Group I (untreated control group)	68.79±2.14	46.89 ±0.41	100 (+)ve
Group II (positive control) (Tetracycline)	26.59±3.28	24.12±1.98	90 (-)ve
Group III negative control (olive oil)	68.92±2.10	46.92±0.35	100 (+)ve
Group IV (25 mg)	45.67*±5.14	42.56*±0.96	30 (-)ve
Group V (50 mg)	42.69**±3.28	36.45**±1.11	60 (-)ve
Group VI (75 mg)	31.12**±5.48	28.22**±2.03	80 (-)ve

Mean ± of 6 animals. \* = P ≤ 0.01 (significant), \*\* = P ≤ 0.001 (highly significant).

**Table 2.** Tissue biochemistry (Acephate for 30 days).

Treatment	Protein (mg/g)				Sialic acid (mg/g)			
	Cauda epididymis	Seminal vesicle	Ventral prostate	Vas deferens	Cauda epididymis	Seminal vesicle	Ventral prostate	Vas deferens
Group I (untreated control group)	203.51±5.42	235.23±7.81	229.10±3.51	258.53±6.24	5.12±0.12	5.31±0.32	5.21±0.21	5.32±0.33
Group II (positive control) (Tetracycline)	247.21±2.85	275.12±8.57	262.84±8.81	286.74±5.55	3.79±1.02	2.61±0.12	3.73±0.15	2.87±0.30
Group III (negative control) (Olive Oil)	203.71±7.83	235.24±8.26	228.20±4.01	258.23±6.50	5.00±0.02	5.29±0.25	5.08±0.15	5.75±0.23
Group IV (25 mg)	234.72*±5.44	265.98*±5.02	238.16 <sup>ns</sup> ±6.20	271.58 <sup>ns</sup> ±5.33	4.21*±0.21	3.72*±0.35	4.56*±0.92	4.46*±0.30
Group V (50 mg)	242.26*±9.02	269.12*±6.70	250.94 <sup>ns</sup> ±6.21	270.07 <sup>ns</sup> ±8.25	4.11*±0.26	3.46*±0.50	3.53*±0.44	3.42*±0.54
Group VI (75 mg)	246.42**±3.13	274.71*±9.03	262.34*±9.05	286.38*±5.20	3.80*±0.34	2.63**±0.02	3.85**±0.12	2.90**±0.50

Mean ± of 6 animals. \* = P ≤ 0.01 (Significant). \*\* = P ≤ 0.001 (highly significant).

Bouin's fixative for at least 48 h, processed by the paraffin wax impregnation method and after using a rotary microtome, these were cut at 5 µm thickness and stained with haematoxylin and eosin (H&E) for light microscopic examination.

#### Statistical analysis

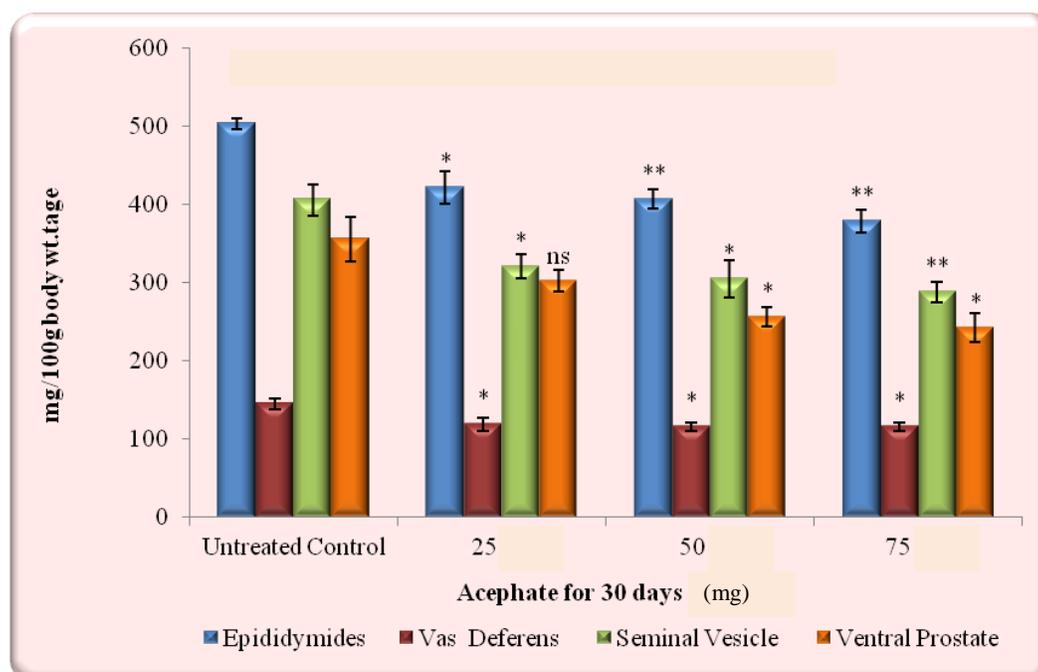
The data obtained from the above experiments were subjected to statistical analysis. Data were represented as mean ± SEM. The differences were compared for statistical significance by "t- test" by using statistical package for social sciences (SPSS) software (16.0 versions) and they were considered significant at P ≤ 0.01 and highly significant at P ≤ 0.001. Graphical representation of data

has been done using Microsoft Excel 2007.

## RESULTS

Acephate brought mark alterations in cauda epididymal weight, function and histology. The 80% negative fertility test may be attributed to lack of forward progression and reduction in density of spermatozoa and altered biochemical milieu of cauda epididymis (Joshi et al., 2003). Table 1 shows the sperm motility, sperm density and fertility (%) in untreated control, positive control, negative control and various dose level treated

groups. Group IV demonstrated significant decrease in sperm motility as well as sperm density as compared to untreated control group. Group V and Group VI showed marked reduction in both the parameters as well as fertility percentage in comparison to Group I. A gradient increase was observed in toxicity in treated group. Table 2 shows the protein and sialic acid concentration in cauda epididymis, seminal vesicle, ventral prostate and vas deferens. Group IV and V demonstrated elevation in protein level as compared to Group I in cauda epididymis and seminal vesicle. Protein concentration remained



**Figure 2.** Epididymis and accessory sex organ weight (acephate for 30 days). Mean  $\pm$  of 6 animals. \* =  $P \leq 0.01$  (Significant), \*\* =  $P \leq 0.001$  (highly significant).

unaltered in ventral prostate and vas deferens. A marked reduction in sialic acid concentration has also been observed from all the four tissues. Group VI showed highly significant increase in protein concentration in cauda epididymis as compared to untreated group while marked decrease in sialic acid concentration has been reported in seminal vesicle, ventral prostate and vas deferens. In the present study, elevation in protein (Table 2) may be due to hepatic detoxification activity which results in the inhibitory effect on the activity of enzyme or production of enzymes lost involved in the androgen biotransformation (Venkataramana et al., 2006). Another reason for elevation in protein content may be stimulation of growth proteins and RNA synthesis. Elevation in protein content caused by other insecticides has also been reported (Joshi et al., 2003; Ngoula et al., 2007).

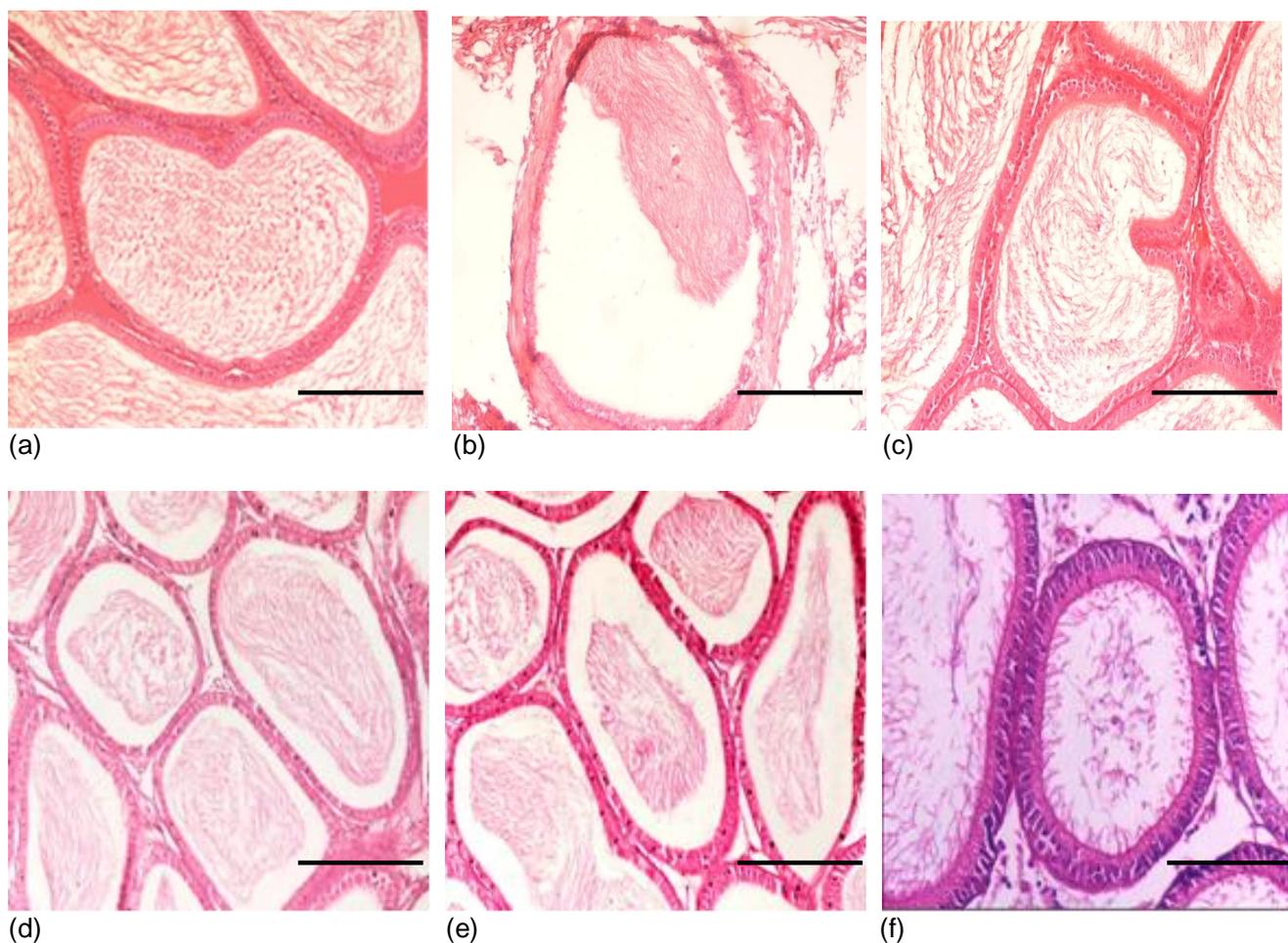
Sialic acid acts as a lubricant to facilitate the downward movement of sperm and to reduce friction among spermatozoa (Gupta, 2001). A significant decrease in the sialic acid concentration was noticed which may be due to the anti spermatogenic activity or reduced androgen production. The reduced sialic acid content of seminal vesicles caused deteriorating effects on the structural integrity of sperm cells (Bone et al., 2001).

Administration of acephate showed adverse effect on histoarchitecture of seminal vesicle with disruptive changes of muscles and connective tissue along with highly reduced secretion in the lumen (Figure 4a to d). The sex differentiation and growth of seminal vesicles are

highly dependent on androgens (Curry and Atherton, 1990), thus reduced androgen level have adverse effects on histo-architecture of seminal vesicles. Reduced epithelial folding of seminal vesicle was observed in methyl parathion treated rats compared to control (Prashanthi et al., 2006). These studies suggest that the OP pesticides may influence the semen quality by affecting the seminal vesicle functions in albino rats. Thus, obtained results collectively indicate that acephate caused toxic effects on male reproductive functions. Present study indicates limited use of such toxic insecticides to improve the quality of life for human welfare.

## DISCUSSION

Several pesticides have reduced the organ weight by affecting either hypothalamus or pituitary or both (Joshi et al., 2011; Okazaki et al., 2001). In our study, the reduction in weight of accessory sex organs (Figure 2) may be due to low availability of androgens or antiandrogenic activity of acephate (Latchoumycandane et al., 2002b). Sperm parameters such as count, motility and morphology are the key indices of male fertility (Table 1), as these are the prime markers in testicular spermatogenesis and epididymal maturation. Decline in human sperm counts and motility over the recent decades may be attributed to increased exposure to



**Figure 3.** (a-f) Photomicrographs of cauda after exposure with acephate in rats. a. Untreated control b. Positive control (Tetracycline 28.6 mg./kg b.wt./day), c. Negative control (olive oil), d. Acephate 25 mg./kg b.wt./day, e. Acephate 50 mg./kg b.wt./day, f. Acephate 75 mg./kg b.wt./day. H&E. stain, Bar = 100  $\mu$ m

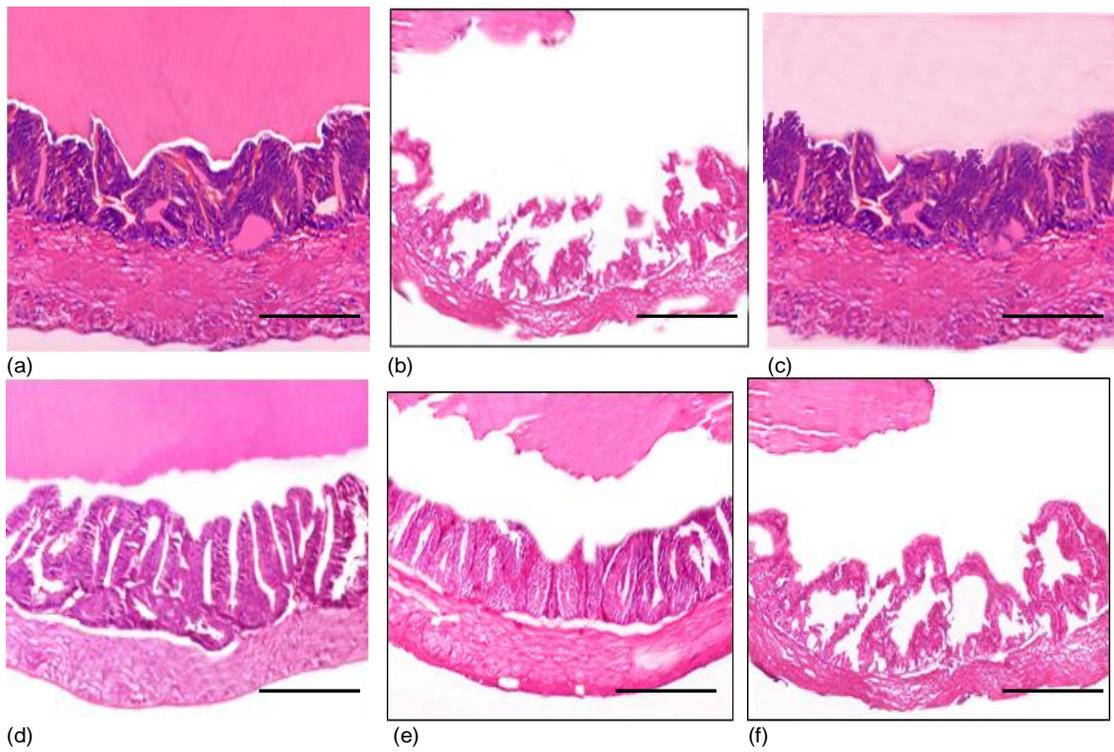
environmental endocrine disruptors like OP compounds (Yuan et al., 2010).

Epididymides are the site of differentiation, maturation and storage of spermatozoa (Yuan et al., 2010). The physiological and biochemical integrity of epididymis are dependent on androgens. Low caudal epididymal sperm density in our study may be due to alteration in androgen metabolism (Duty et al., 2003). The loss of sperm motility in the treated rats may be due to change in sperm membrane properties. Similar effect on sperm motility is also reported with other pesticides and this negative impact affects fertilizing ability of the sperm. Low fructose concentration in seminal vesicle may be another cause of low sperm motility (Bharshankar and Bharshankar, 2000). Bai and shi (2002) reported that low levels of adenosine triphosphate (ATP) content may affect sperm motility. Reduction in sperm motility may be androgen deprivation effect of acephate (Uzumcu et al., 2004).

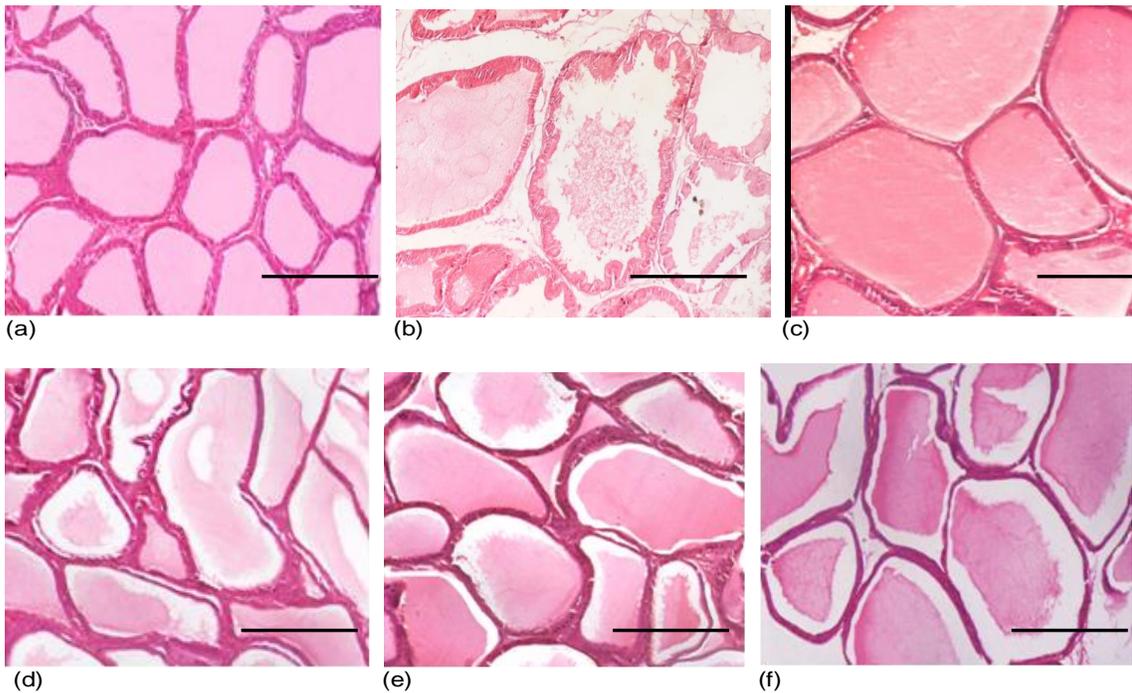
Proteins are the most important and abundant macro-

molecules playing a vital role in the architecture and physiology of the cell and in cellular metabolism (Mommson and Walsh, 1992). Sialic acid acts as a lubricant to facilitate the downward movement of sperm and to reduce friction among spermatozoa (Gupta, 2001). A significant decrease in the sialic acid concentration was noticed, which may be due to the anti spermatogenic activity or reduced androgen production. The reduced sialic acid content of seminal vesicles caused deteriorating effects on the structural integrity of sperm cells (Bone et al., 2001). The epididymis plays an essential role in male fertility, and disruption of epididymal function can lead to obstructive azoospermia.

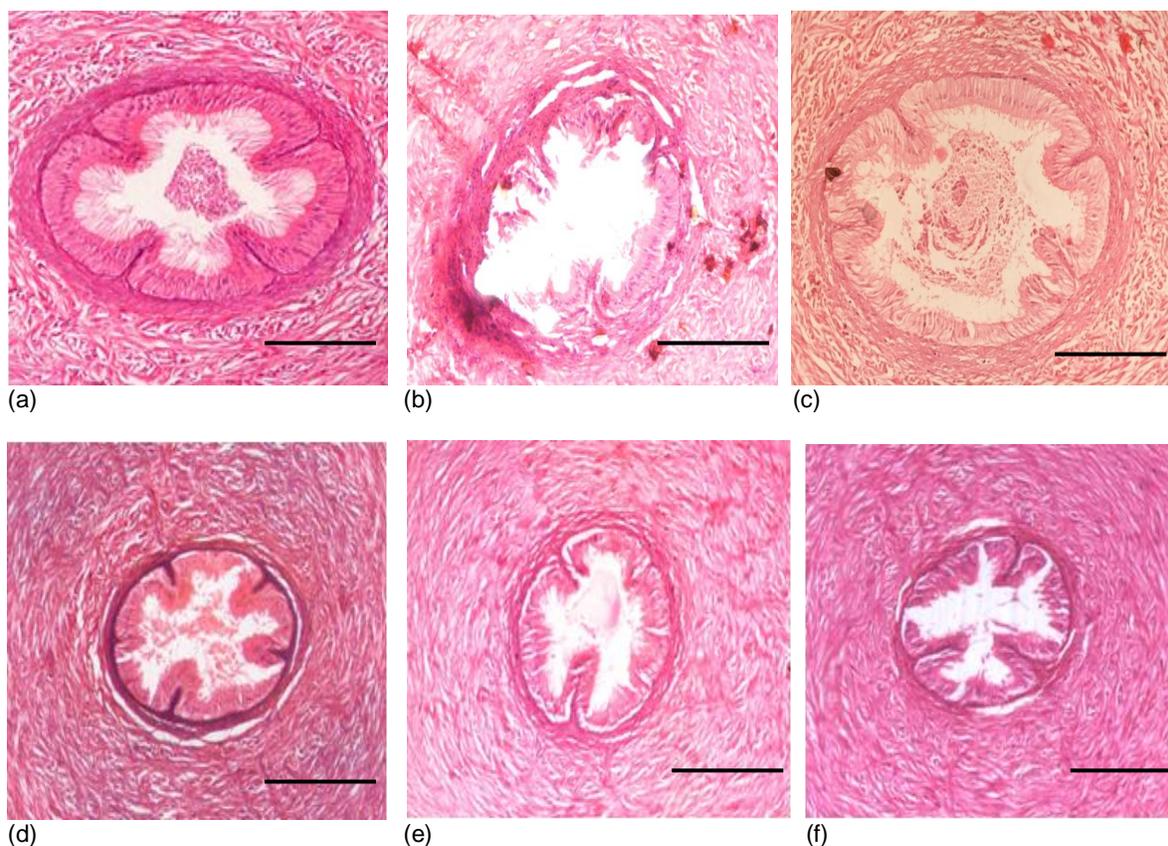
Formation and function of the epididymis is androgen-dependent. Acephate at various dose levels produced many degenerative changes in the cauda epididymis and accessory sex organs along with various pathological changes. Deficiency of androgens caused a marked reduction in tubular diameters (Figure 3a to d), regression



**Figure 4.** (a-f). Histopathology of seminal vesicle after exposure with acephate in rats. a. Untreated control, b. Positive control (Tetracycline 28.6 mg/kg b.wt/day),c. Negative control (olive oil), d. Acephate 25 mg/kg b.wt/day, e. Acephate 50 mg/kg b.wt/day, f. Acephate 75 mg/kg b.wt/day. H&E. stain, Bar = 100  $\mu$ m.



**Figure 5.** (a-f) Histopathology of prostate after exposure with acephate in rats. a. Untreated control, b. Positive control (Tetracycline 28.6 mg/kg b.wt/day),c. Negative control (olive oil), d. Acephate 25 mg/kg b.wt/day, e. Acephate 50 mg/kg b.wt/day, f. Acephate 75 mg/kg b.wt/day. H&E. stain, Bar = 100  $\mu$ m.



**Figure 6.** (a-f) Histopathology of vas deferens after exposure with acephate in rats. a. Untreated control, b. Positive control (Tetracycline 28.6 mg/kg b.wt/day), c. Negative control (olive oil), d. Acephate 25 mg/kg b.wt/day, e. Acephate 50 mg/kg b.wt/day, f. Acephate 75 mg/kg b.wt/day. H&E. stain, Bar = 100  $\mu$ m.

of epididymal epithelium, severe decline in spermatozoa number in cauda and changes in the composition of epididymal plasma. OPs significantly increased cytoplasmic vacuolation and nuclear shrinkage in the epithelial cells of the rat's ductus epididymis (Okamura et al., 2009; O'Hara et al., 2011).

Prostate plays key role in male reproduction and its secretion is essential for the normal function of spermatozoa. Decrease in weight of ventral prostate was observed after treatment with many pesticides (Joshi et al., 2011; Bian et al., 2006; Kim et al., 2005). After treatment with acephate, ventral prostate showed reduced alveoli (Figure 5a to d), with very thin and disorganized cuboidal epithelial cells lining, and lumen was filled with very little secretion. Little work is done for the effects of OPs on the histoarchitecture of ventral prostate.

Vas deferens is a thick-walled muscular tube. Mucosal lining is described to have longitudinal folds resulting into irregular outline of its lumen. These folds are believed to allow for expansion during ejaculation. Its pseudostratified epithelium may possess tuft of microvilli (stereocilia) similar to that of epididymis to absorb the excess fluid produced by the testes. Administration of acephate

caused degenerative changes in the vas deferens epithelium along with the absence of spermatozoa in the lumen (Figure 6a to d) that may be due to reduced androgen level. Structural damage to mammalian vas deferens may lead into infertility and carcinogenicity (Aziz et al., 2008).

## REFERENCES

- Aitken RJ, Koopman P, Lewis SE (2004). Fertility seeds of concern. *Nature* 432:48-52.
- Aziz N, Shah SW, Aziz RB (2008). Histological changes in male rat reproductive organs post-treated with insecticide Carbofuran Furadan. *Ann. Microscopy* 8:83-89.
- Bai JP, Shi YH (2002). Inhibition of Ca<sup>2+</sup> channels in mouse spermatogenic cells by male anti fertility compounds from *Tripterygium wilfordii* Hook. f. *Contraception* 65:441-445.
- Bajgar J, Fusek J, Kassa J, Kuca K, Jun D (2009). Chemical aspects of pharmacological prophylaxis against nerve agent poisoning. *Curr. Med. Chem.* 16:2977-2986.
- Benoff S, Jacob A, Hurley IR (2000). Male infertility and environmental exposure to lead and cadmium. *Hum. Reprod. Update* 6:107-121.
- Bharshankar RN, Bharshankar JR (2000). Relationship of seminal plasma transferin with seminal parameters in male infertility. *Ind. J. Physiol. Pharmacol.* 44:456-460.
- Bian Q, Qian J, Xu L, Chen J, Song L, Wang X (2006). The toxic effects of 4-tert-octylphenol on the reproductive system of male rats.

- Food Chem. Toxicol. 44:1355-1361.
- Bomser JA, Quistad GB, Casida JE (2002). Chlorpyrifos oxon potentiates diacylglycerol-induced extracellular signal-regulated kinase ERK 44/42; activation, possibly by diacylglycerol lipase inhibition. *Toxicol. Appl. Pharmacol.* 178:29-36.
- Bone W, Jone AR, Morin C, Nieschlag E, Cooper TG (2001). Susceptibility of glycolytic enzyme activities and motility of spermatozoa from rat, mouse and human to inhibition by proven and putative chlorinated antifertility compounds *in vitro*. *J. Androl.* 22:464-470.
- Carreño J, Rivas A, Granada A, Jose Lopez-Espinosa M, Mariscal M, Olea N, Olea-Serrano F (2007). Exposure of young men to organochlorine pesticides in Southern Spain. *Environ. Res.* 103:55-61.
- Chang HY, Shih TS, Guo YL, Tsai CY, Hsu PC (2004). Sperm function in workers exposed to N, N-dimethylformamide in the synthetic leather industry. *Fertil. Steril.* 81:1589-1594.
- Curry PT, Atherton RW (1990). Seminal vesicles: development, secretory products, and fertility. *Arch. Androl.* 25:107-113.
- Duty SM, Silva MJ, Barr DB, Brock JW, Ryan L, Chen Z, Herrick RF, Christiani DC, Hauser R (2003). Phthalate exposure and human semen parameters. *Epidemiology* 114:269-277.
- Figà-Talamanca I, Traina ME, Urbani E (2001). Occupational exposures to metals, solvents and pesticides: recent evidence on male reproductive effects and biological markers. *Occup. Med. Lond.* 51:174-188.
- Gordon CJ, Mack CM (2003). Influence of gender on thermoregulation and cholinesterase inhibition in the long-evans rat exposed to diazinon. *J. Toxicol. Environ. Health A* 66:291-304.
- Gupta RC (2001a). Depletion of energy metabolites following acetylcholinesterase inhibitor induced status epilepticus: protection by antioxidants. *Neurotoxicology* 22: 271-282.
- Homan GF, Davies M, Norman R (2007). The impact of lifestyle factors on reproductive performance in the general population and those undergoing infertility treatments: a review. *Hum. Reprod. Update* 13:209-223.
- Jensen TK, Bonde JP, Joffe M (2006). The influence of occupational exposure on male reproductive function. *Occup. Med.* 56:544-553.
- Jørgensen N, Asklund C, Carlsen E, Skakkebaek NE (2006). Coordinated European investigations of semen quality: results from studies of Scandinavian young men is a matter of concern. *Int. J. Androl.* 29:54-61.
- Joshi SC, Bansal B, Jasuja ND (2011). Evaluation of reproductive and developmental toxicity of Cypermethrin in male albino rats. *Toxicol. Environmen. Chem.* 93:593-602.
- Joshi SC, Mathur R, Gajraj A, Sharma T (2003). Influence of methyl parathion on reproductive parameters in male rats. *Environ. Toxicol. Pharmacol.* 14:91-98.
- Joshi SC, Sharma P (2011). Male Reproductive Toxicity of Organophosphorus Compounds: A Review. *Toxicol. Environ. Chem.* 93:1486-1507.
- Kim SS, Lee R.D, Lim KJ, Kwack SJ, Rhee GS, Seok JH, Lee GS, An BS, Jeung EB, Park KL (2005). Potential estrogenic and antiandrogenic effects of Permethrin in rats. *J. Reprod. Dev.* 51:201-210.
- Latchoumycandane C, Chitra KC, Mathur PP (2002b). Effects of Methoxychlor on the epididymal antioxidant system of adult rats. *Reprod. Toxicol.* 16:161-172.
- Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Mommsen TP, Walsh PJ (1992). Biochemical and environmental perspectives on nitrogen metabolism in fishes. *Cell. Mol. Life Sci.* 48:583-593.
- Ngoula F, Watcho P, Dongmo M, Kenfack A, Kamtchouing P, Tchoumboué J (2007). Effects of pirimiphos-methyl an organophosphate insecticide; on the fertility of adult male rats. *Afr. Health Sci.* 7:3-9.
- O'Hara L, Welsh M, Saunders PT, Smith LB (2011). Androgen receptor expression in the caput epididymal epithelium is essential for development of the initial segment and epididymal spermatozoa transit. *Endocrinology* 152:718-729.
- Okamura A, Kamijima M, Ohtani K, Yamanoshita O, Nakamura D, Ito Y, Miyata M, Ueyama J, Suzuki T, Imai R, Takagi K, Nakajima T (2009). Broken sperm, cytoplasmic droplets and reduced sperm motility are principal markers of decreased sperm quality due to organophosphorus pesticides in rats. *J. Occup. Health* 51:478-487.
- Okazaki K, Okazaki S, Nishimura S, Nakamura H, Kitamura Y, Hatayama K, Nakamura A, Tsuda T, Katsumata T, Nishikawa A, Hirose M (2001). A repeated 28-days oral dose toxicity study of Methoxychlor in rats based on the enhanced OECD test guidelines 407 for screening endocrine-disrupting chemicals. *Arch. Toxicol.* 75:513-521.
- Prasad MR, Chinoy NJ, Kadam KM (1972). Changes in succinic dehydrogenase levels in the rat epididymis under normal and altered physiologic conditions. *Fertil. Steril.* 23:186-190.
- Prashanthi N, Narayana K, Nayanatara A, Kumar HHC, Bairy KL, D'Souza UJ (2006). The reproductive toxicity of the organophosphate pesticide 0, 0- dimethyl 0- 4-nitrophenyl phosphorothioate methylparathion; in the male rat. *Folia Morphol. Warsz* 65:309-321.
- Presibella KM, Kita DH, Carneiro CB, Andrade AJM, Dalsenter PR (2005). Reproductive evaluation of two pesticides combined deltamethrine and endosulfan; in female rats. *Reprod. Toxicol.* 20:95-101.
- Rozati R, Reddy PP, Reddanna P, Mujtaba R (2002). Role of environmental estrogens in the deterioration of male factor fertility. *Fertil. Steril.* 78:1187-1194.
- Sheiner EK, Sheiner E, Hammel RD, Potashnik G, Carel R (2003). Effect of occupational exposures on male fertility: literature review. *Ind. Health.* 41:55-62.
- Singh AK, Jiang Y (2002). Immunotoxicity of acute acephate exposure in control or IL-1-challenged rats: correlation between the immune cell composition and corticosteroid concentration in blood. *J. Appl. Toxicol.* 22:279-291.
- Slotkin TA, Levin ED, Seidler FJ (2006). Comparative developmental neurotoxicity of organophosphate insecticides: effects on brain development are separable from systemic toxicity. *Environ. Health Perspect.* 114:746-751.
- Sweeney T (2002). Its exposure to endocrine disrupting compounds during fetal/post natal development affecting the reproductive potential of farm animals. *Domest. Anim. Endocrinol.* 23:203-209
- Thapar A, Sandhir R, Kiran R (2002). Acephate induced oxidative damage in erythrocytes. *Indian J. Exp. Biol.* 40:963-966.
- Treviz LRP, de Baptista GC, Papa G (2005). Acephate and methamidophos residues in greenhouse and in field grown tomatoes. *Hortic. Bras.* 23:38-43.
- Uzumcu M, Suzuki H, Skinner MK (2004). Effect of the antiandrogenic endocrine disruptor vinclozolin on embryonic testis cord formation and postnatal testis development and function. *Reprod. Toxicol.* 18:765-774.
- Venkataramana GV, Sandhya Rani PN, Murthy PS (2006). Impact of Malathion on the biochemical parameters of gobiid fish, *Glossogobius giuris* Ham. *J. Environ. Biol.* 27:119-122.
- Visweswaran B, Krishnamoorthy G (2012). Oxidative Stress by Tartrazine in the Testis of Wistar Rats. *IOSR J. Pharm. Biol. Sci.* 2:44-49.
- Wagner U, Schlebusch H, van der Ven H, Van der VK, Diedrich K, Krebs D (1990). Accumulation of pollutants in the genital tract of sterility patients. *J. Clin. Chem. Clin. Biochem.* 28:683-688.
- Warren L (1959). The thiobarbituric acid assay of sialic acid. *J. Bio.Chem.* 234: 1971-1975.
- Yuan C, Wang C, Gao SQ, Kong TT, Chen L, Li XF, Song L, Wang YB (2010). Effects of permethrin, cypermethrin and 3-phenoxybenzoic acid on rat sperm motility *in vitro* evaluated with computer-assisted sperm analysis. *Toxicol. In Vitro* 24:382-386.

Full Length Research Paper

## ***In vivo* assessment of antihyperglycemic and antioxidant activity from oil of linseed in streptozotocin induced diabetic rats**

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In the present study, the antihyperglycemic and antioxidant effects of oil from seeds of *Linum usitatissimum* Linn. (LO) were investigated. A streptozotocin-nicotinamide (STZ) rat model of hyperglycaemia was used to evaluate the antihyperglycemic and antioxidant properties of oil of linseed. The body weight, oral glucose tolerance test and biochemical parameters namely; glucose level, insulin level, liver glycogen content, glycosylated hemoglobin and antioxidant parameters were estimated for all treated groups and compared against diabetic control group. LO (500 and 1000 mg/kg/day) in respective groups of diabetic animals administered for 28 days reduced the blood glucose level in streptozotocin-nicotinamide induced diabetic rats. There was significant increase in body weight, liver glycogen content, plasma insulin level and decrease in the blood glucose and glycosylated hemoglobin in test groups as compared to control group. *In vivo* antioxidant studies on STZ-nicotinamide induced diabetic rats revealed decreased malondialdehyde (MDA) and increased reduced glutathione (GSH). The findings demonstrate that LO have excellent antihyperglycemic and antioxidant activities and thus have great potential as a source for natural health products.

**Key words:** Streptozotocin, *Linum usitatissimum*, fixed oil, malondialdehyde (MDA), diabetes.

### INTRODUCTION

A number of plants are being used from ancient times for the management of various disease like diabetes, hyperlipidemia, cancer, inflammation, pain etc. Many herbal products including several metals and minerals have been described for the care of diabetes mellitus in ancient literature (Nadkarni et al., 1992). Ayurveda, an ancient Indian form of medicine, deals with plants and plant extracts. This indigenous form of medicine uses the active ingredients present in plants for treating diseases

(Lewis and Elvin-Lewis, 1997). Plant drugs are frequently considered to be less toxic and free from side effects than synthetic ones (Momin, 1987). Many herbs have shown to possess hypoglycaemic action in animals and humans (Twaij and Al-Badr, 1988; Gupta, 1994).

*Linum usitatissimum* Linn. (Linaceae) is one of the important medicinal plants being used in various systems of medicine and food (Velisek et al., 1995). Human being has consumed flaxseed since the beginning of the

earliest civilizations. It was used traditionally to relieve abdominal pains and also as energy source from ancient time. Flax is considered a source of food as it contains alpha-linolenic acid lignans and polysaccharides (other than starch), all of which have positive effects in disease prevention (Rubilar et al., 2010).

Diabetes mellitus is a metabolic disorder which causes great morbidity in developed and developing countries. Diabetes was known to ancient Indian physicians as 'madumeha'. The metabolism of carbohydrates, fats and proteins are malformed in diabetes and patients with this disorder are at an increased risk of various complications like coronary heart disease, peripheral vascular disease and cerebrovascular disease (Brown, 1994; Stamler et al., 1993). It is associated with profound alterations in the plasma lipid and lipoprotein profile thereby an increased risk of premature atherosclerosis, coronary insufficiency and myocardial infarction exists (Betteridge, 1997). Accumulation of lipids in diabetes is mediated through a variety of regulatory processes, especially insulin deficiency, thereby rendering the diabetic patient more prone to hypercholesterolemia and hypertriglyceridemia [Jaiprakash et al., 1993].

The LO has been widely used in the food industries from centuries. As far as we know, the effect of LO on the blood profiles in diabetic models has not been studied. In light of these findings, we carried out this study for the evaluation of antihyperglycemic, and antioxidant potential of LO.

## MATERIALS AND METHODS

### Drugs and chemicals

The drugs and chemicals used in the study were glibenclamide (Torrent Pharmaceutical, Ahmadabad), streptozotocin, heparin (SRL, India), EDTA (Hi-media Lab. Pvt Ltd., Mumbai, India), Ellman's reagent (5,5'-dithiobis-(2-nitro-benzoic acid); DTNB), sodium sulphate, methanol, pyridine, anthrone, thiourea, benzoic acid, sodium chloride (SD Fine Chem Ltd., Mumbai, India). All the chemicals used in the study were of analytical grade.

### Isolation of oil

The dried seeds of linseed were purchased from Chaudhary Charan Singh Haryana Agriculture University, Hisar, India. The seeds were crushed and oil was extracted by hot compression method. The percentage yield of oil was found to be 35% w/w.

### Experimental animals

Healthy albino wistar rats (150 to 250 g) were procured from Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar (Haryana). The rats were housed in (Polycarbonate cage size: 29 × 22 × 14 cm) under laboratory standard conditions (25 ± 3°C: 35 to 60% humidity) with alternating light and dark cycle of 12 h each and were fed with a standard rat pellet diet (Hindustan Lever Ltd, Mumbai, India) and

water *ad libitum*. The experimental protocol was approved by Institutional Animals Ethics Committee (IAEC) and Animal care was taken as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India (Registration No. 0436).

### Induction of diabetes

Type II diabetes mellitus (NIDDM) was induced in overnight fasted animals by a single intraperitoneal injection of 50 mg/kg STZ in 0.1 M citrate buffer (pH-4.5) in a volume of 1 ml/kg body weight 15 min after the i.p. administration of 110 mg/kg nicotinamide. Diabetes was developed and stabilized over a period of 7 days. Diabetes was confirmed by the elevated blood glucose levels determined at 72 h and on 7th day after injection. Only rats confirmed with permanent NIDDM (glucose level above 250 mg/dl) were used in the study. All the animals were allowed free access to tap water and pellet diet and maintained at room temperature in plastic cages as per the guidelines of Institute of Animal Ethics committee. Blood was collected by intraocular route (Kumar et al., 2012).

### Experimental design

Rats were divided into the following groups comprising six rats in each group after the induction and confirmation of diabetes.

#### For acute antihyperglycemic model

In the acute antihyperglycemic models, the study was carried out for 4 h to check if the plant possess some antidiabetic effect.

Group 1: Normal rats.

Group 2: Diabetic control.

Group 3: Diabetic animals were administered glibenclamide (0.6 mg/kg p.o).

Group 4: Diabetic animal were administered orally 500 mg/kg/day of LO.

Group 5: Diabetic animal were administered orally 1000 mg/kg/day of LO.

#### For chronic antihyperglycemic model

In the chronic antihyperglycemic models, the animal were treated with different doses that is, 500 and 1000 mg/kg for 28 days to study the various parameters of the diabetes to confirm the antihyperglycemic activity of LO in streptozotocin induced diabetes in rats.

Group 1: Normal rats.

Group 2: Diabetic control.

Group 3: Diabetic animals were administered glibenclamide (0.6 mg/kg p.o).

Group 4: Diabetic animal were administered orally 500 mg/kg/day of LO.

Group 5: Diabetic animal were administered orally 1000 mg/kg/day of LO.

### Sample collection

#### Blood sample

In acute model, the blood was collected from retroorbital vein of the

rats previously fasted for 24 h. In chronic model, the 24 h fasted animals were sacrificed by cervical decapitation on 28th day of treatment. Trunk blood was collected in heparinized tubes. Plasma was obtained by centrifugation at 5000 rpm for 5 min to determine the biochemical parameters; glucose, insulin, cholesterol etc.

#### **Estimation of plasma glucose and cholesterol**

Plasma cholesterol and glucose level were measured by commercial supplied biological kit Erba Glucose Kit (GOD-POD Method) and Erba Cholesterol Kit (CHOD-PAP Method), respectively using Chem 5 Plus-V<sub>2</sub> Auto-analyser (Erba Mannheim Germany) in plasma sample. Glucose and cholesterol values were calculated as mg/dl blood sample.

#### **Estimation of glycosylated hemoglobin (Hb1Ac)**

Glycosylated hemoglobin was measured using commercial supplied biological kit (Erba Diagnostic) in plasma sample using Chem 5 Plus-V<sub>2</sub> Auto-analyser (Erba Mannheim Germany). Values are expressed as the percent of total hemoglobin.

#### **Estimation of insulin**

Serum insulin level was measured by an enzyme-linked immunosorbent assay (ELISA) procedure using Mercodia rat insulin ELISA kit. Briefly, the solid phase two-site enzyme immunoassay is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants 35 (epitopes) on the insulin molecule. During incubation, insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the micro titration well. After washing three times, unbound enzyme labeled antibody was removed. The bound conjugated insulin was detected by reacting with 3, 3', 5, 5'-tetramethylbenzidine. The reaction was stopped by adding acid to give a colorimetric end-point, and optical density was measured with a micro plate auto reader (Bio-tek Instrument Inc., USA) at a wavelength of 450 nm. The serum insulin is expressed as µg/l.

#### **Estimation of liver glycogen content**

Liver glycogen estimation was done by the method as described by Seifter et al. (1950) with some modifications (Seifter et al., 1950). Immediately after excision from the animal, 1 g of the liver was dropped into a previously weighed test tube containing 3 ml of 30% potassium hydroxide solution. The weight of the liver sample was determined. The tissue was then digested by heating the tube for 20 minutes in boiling water bath. The digest was then cooled, transferred quantitatively to a 50 ml volumetric flask, and diluted to the mark with water. The contents of the flask were then thoroughly mixed and a measured portion was then further diluted with water in a second volumetric flask so as to yield a solution of glycogen (3 to 30 µg/ml). Five ml aliquots of the final dilution were then pipetted into Evelyn tube and the determination with anthrone was carried out. The amount of glycogen in the aliquot used was then calculated using the following equation:

$$\mu\text{g of glycogen in aliquot} = 100 U / 1.11 S$$

U is the optical density of unknown solution. S is the optical density of the 100 µg glucose and 1.11 is the factor determined by Morris in

1948 for the conversion of the glucose to the glycogen (Seifter et al., 1950).

#### **In vivo antioxidant activity**

##### **Estimation of MDA level**

Malondialdehyde (MDA), an index of free radical generation/lipid peroxidation, was determined as described by Okhawa et al. (1979) with some modification (Okhawa et al., 1979). Briefly, the reaction mixture consisted of 0.2 ml of 8.1% sodium lauryl sulphate, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid added to 0.2 ml of blood plasma. The mixture was made up to 4.0 ml with distilled water and heated at 95°C for 60 min. After cooling the contents under running tap water, 5.0 ml of n-butanol and pyridine (15:1 v/v) and 1.0 ml of distilled water was added. The contents were centrifuged at about 3,000 rpm for 10 min. The organic layer was separated out and its absorbance was measured at 532 nm using double beam UV-Visible spectrophotometer (Systronics 2203, Bangalore, India) against a blank. MDA values were calculated using the extinction coefficient of MDA-thiobarbituric acid complex  $1.56 \times 10^5$  l/mol cm and expressed as nmol/ml.

##### **Estimation of reduced glutathione level**

The tissue sample (liver 200 mg) was homogenized in 8.0 ml of 0.02 M EDTA in an ice bath. The homogenates were kept in the ice bath until used. Aliquots of 5.0 ml of the homogenates were mixed in 15.0 ml test tubes with 4.0 ml distilled water and 1.0 ml of 50% trichloroacetic acid (TCA). The tubes were centrifuged for 15 min at approximately 3,000 rpm, 2.0 ml of supernatant was mixed with 4.0 ml of 0.4 M Tris buffer pH 8.9, 0.1 ml Ellman's reagent [5,5-dithiobis-(2-nitro-benzoic acid)] (DTNB) added and the sample shaken. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank with no homogenate. Results are expressed as µmol GSH/g tissue.

#### **Statistical analysis**

The data for various biochemical parameters were evaluated by use of one-way analysis of variance (ANOVA), followed by Dunnett's t-test using the software Sigma-Stat 3. In all the tests, the criterion for statistical significance was  $p < 0.05$ .

## **RESULTS**

### **Oral glucose tolerance test**

The effect of LO on plasma glucose level after glucose loading of 2 g/kg body weight orally to the STZ diabetic rats is expressed in the Table 1. The blood glucose level rises to a maximum in 60 min after glucose loading. The oil (500 mg/kg/day and 1000 mg/kg/day body weight) treated groups showed a significant decrease ( $p < 0.01$ ) in level of glucose as compared to control group. The oil treated group showed a marked fall in glucose level in 90 to 120 min interval.

**Table 1.** Effect of *Linum usitatissimum* oil in oral glucose tolerance test (OGTT).

Treatment	Dose (mg/kg)	Mean blood glucose concentration (mg/dl) $\pm$ SEM				
		0 min	30 min	60 min	90 min	120 min
Normal	-	80 $\pm$ 2.6	87 $\pm$ 2.8	90 $\pm$ 3.8	86 $\pm$ 2.5	83 $\pm$ 2.7
Diabetic control	-	290 $\pm$ 4.6	390 $\pm$ 5.3	413.2 $\pm$ 4.3	360 $\pm$ 2.7	331 $\pm$ 2.8
LUO	500	320 $\pm$ 2.9	332 $\pm$ 2.7	350 $\pm$ 3.0	317 $\pm$ 3.6*	288 $\pm$ 4.1**
LUO	1000	372 $\pm$ 3.6	383 $\pm$ 4.5	401 $\pm$ 4.1	360 $\pm$ 3.4**	301 $\pm$ 3.3**

Values are presented as mean  $\pm$  SEM.; n = 6 in each group. One way ANOVA followed by Dunnett's test \*\*p < 0.01 versus diabetic control; LUO: *Linum usitatissimum* oil

**Table 2.** Effect of *Linum usitatissimum* oil in STZ induced diabetic rats in acute antihyperglycemic study.

Treatment	Dose (mg/kg p.o)	Mean blood glucose concentration (mg/dl) $\pm$ SEM				
		0 h	1/2 h	1 h	2 h	4 h
Normal	-	76 $\pm$ 4.2	80 $\pm$ 3.2	77 $\pm$ 2.5	82 $\pm$ 4.1	79 $\pm$ 5.3
Control	-	340.5 $\pm$ 10.2	342 $\pm$ 11.3	346 $\pm$ 7.6	341.0 $\pm$ 6.7	332.0 $\pm$ 7.2
LUO	500	350 $\pm$ 5.7	340.1 $\pm$ 5.4	310 $\pm$ 4.5**	305 $\pm$ 3.4**	288 $\pm$ 3.6**
LUO	1000	338 $\pm$ 1.8	314 $\pm$ 1.9*	296 $\pm$ 2.3**	277 $\pm$ 2.4**	262 $\pm$ 4.9**
Glibenclamide	0.6	348 $\pm$ 7.1	328 $\pm$ 3.2**	288 $\pm$ 6.4**	260 $\pm$ 5.2**	249.0 $\pm$ 6.8**

Values are presented as mean  $\pm$  SEM; n = 6 in each group. One way ANOVA followed by Dunnett's test \*p < 0.05; \*\*p < 0.01 versus diabetic control; LUO: *Linum usitatissimum* oil.

**Table 3.** Effect of *Linum usitatissimum* oil in STZ induced diabetic rats in chronic antihyperglycemic study.

Treatment	Dose (mg/kg p.o)	Mean blood glucose concentration (mg/dl) $\pm$ SEM				
		0 <sup>th</sup> Day	7 <sup>th</sup> Day	14 <sup>th</sup> Day	21 <sup>st</sup> Day	28 <sup>th</sup>
Normal	-	80 $\pm$ 4.2	79 $\pm$ 3.2	82 $\pm$ 2.5	85.5 $\pm$ 4.1	78 $\pm$ 2.1
Control	-	380 $\pm$ 7.3	379 $\pm$ 7.6	384 $\pm$ 6.7	416 $\pm$ 7.2	410 $\pm$ 5.4
LUO	500	345 $\pm$ 7.4	299 $\pm$ 4.5**	278 $\pm$ 5.2**	245 $\pm$ 6.1**	170 $\pm$ 6.2**
LUO	1000	370 $\pm$ 5.2	286 $\pm$ 5.9**	210 $\pm$ 5.8**	190 $\pm$ 4.4**	155 $\pm$ 5.8**
Glibenclamide	0.6	344 $\pm$ 8.4	259.2 $\pm$ 7.7**	166.3 $\pm$ 4.9**	140 $\pm$ 2.6**	124 $\pm$ 1.8**

Values are presented as mean  $\pm$  SEM; n = 6 in each group. One way ANOVA followed by Dunnett's test \*\*p < 0.01 versus diabetic control; LUO: *Linum usitatissimum* oil

### Effect of LO on STZ diabetic rats in acute study

Administration of LO at a dose 500 mg/kg/day body weight p.o. to STZ diabetic rats showed reduction in blood glucose level from 350 to 288 mg/dl at 4th h. When the dose was increased as 1000 mg/kg/day then the blood glucose level decreased from 338 to 262 mg/dl which was found significant (p < 0.01) when compared with diabetic control (Table 2).

### Effect of LO on STZ diabetic rats in chronic study

In chronic study, administration of LO at the dose of 500 mg/kg body weight to STZ diabetic rats for 28 days

showed a fall in plasma glucose level from 345 to 170 mg/dl on 28th day when compared to 0 day value. LO at the dose of 1000 mg/kg/day body weight showed a significant (p < 0.01) fall in plasma glucose level from 370 to 155 mg/dl on 28th day (Table 3).

### Effect of LO on body weight

An increase in the body weight of normal rats was observed whereas the weight of diabetic control rats decreased from day 1 to day 28. LO at the dose of 500 and 1000 mg/kg/day body weight when administered to respective groups of diabetic rats showed a significant increase in body weight as compared to the diabetic

control group ( $p < 0.01$ ) (Table 4).

### Effect of LO on insulin level

Table 5 shows the level of plasma insulin in the control and experimental groups of rats. Diabetic rats showed a significant decrease ( $p < 0.01$ ) in plasma insulin compared with normal rats. Oral administration of LO at the dose of 500 mg/kg/day and 1000 mg/kg/day body weight respectively showed a significant increase in plasma insulin level in respective groups as compared to control rats.

### Effect of LO on glycosylated hemoglobin (HbA1c)

The effect of LO on HbA1c in STZ diabetic rats is shown in the Table 5. The level of glycosylated hemoglobin significantly increased ( $p < 0.01$ ) in diabetic rats as compared to normal control group. The diabetic rats when treated with LO for 28 days showed a significant ( $p < 0.01$ ) decrease in level of glycosylated Hb as compared to untreated diabetic group. The fall in glycosylated hemoglobin level was found to be dose dependent (Table 5).

### Effect of LO on hepatic glycogen

The hepatic glycogen content in diabetic rats decreased sharply as compared to control animal (Table 5). Chronic administration of LO at both the doses to diabetic rats, a significant increase ( $p < 0.01$ ) in liver glycogen content, was observed as compared to diabetic control group.

### Effect of LO on lipid profile

Table 6 shows the level of lipids in normal and tested animals. There was a significant decrease ( $p < 0.01$ ) in the level of HDL-cholesterol and a significant increase in the levels of total cholesterol and triglycerides in diabetic rats when compared to normal rats. The administration of LO reversed the level of lipids significantly ( $p < 0.05$  and  $p < 0.01$ ).

### Effect of LO on *in vivo* antioxidant parameters

The data depicted in Table 7 shows the effect of LO on plasma malonaldehyde and reduced glutathione level. Plasma MDA level was found to be significantly higher in STZ diabetic rats compared to normal rats. The LO at dose 1000 mg/kg body weight p.o significantly reduced ( $p < 0.01$ ) the level of MDA in diabetic rats. Plasma GSH level was found to be significantly lowered at both the

doses of LO in STZ diabetic rats as compared to normal rats. The chronic administration of LO at 1000 mg/kg body weight significantly increased the level of glutathione in diabetic rats.

## DISCUSSION

The aim of the study was to evaluate the antidiabetic and antioxidant potential of the LO in STZ induced diabetic rats. Diabetes mellitus causes a disturbance in the uptake of glucose as well as glucose metabolism. A dose of STZ as low as 50 mg/kg produces an incomplete destruction of pancreatic beta cells even though the rats become permanently diabetic (Kumar et al., 2012). After treatment with a low dose of STZ, many beta cells survive and regeneration is also possible (Kumar et al., 2012). Hyperglycemia generates a high level of free radicals by autoxidation of glucose and protein glycation, and oxidative stress has been reported to be a positive factor of cardiovascular complications in STZ-induced diabetes mellitus (Okutan et al., 2005). Hyperglycemia is associated with the generation of reactive oxygen species (ROS), causing oxidative damage particularly to heart, kidney, eyes, nerves, liver, small and large vessels and gastrointestinal system (Tunali and Yanardag, 2006). The increased levels of plasma glucose in STZ-induced diabetic rats were lowered by LO administration. The plasma glucose lowering activity of LO was comparable to glibenclamide, a standard hypoglycemic drug that stimulates insulin secretion from pancreatic beta cells (Tian et al., 1998). From the results of the present study, it appears that still insulin producing cells are functioning and the stimulation of insulin release could be responsible for most of the metabolic effects. It may be suggested that the mechanism of action of LO is similar to glibenclamide. The glucose lowering activity of LO may be related to both pancreatic (enhancement of insulin secretion) and extra pancreatic (peripheral utilization of glucose) mechanism.

An increase in the level of glycosylated hemoglobin (HbA1c) in the diabetic control group of rats is due to the presence of large amount of blood glucose which reacts with hemoglobin to form glycosylated hemoglobin (Chattopadhyay, 1999). Oxidative stress increases due to the activation of transcription factors, advanced glycated end products (AGEs), and protein kinase C. If diabetes is persistent for a long time, the glycosylated hemoglobin is found to increase (Sheela and Augusti, 1992). The level of HbA<sub>1c</sub> was decreased after the administration of LO 1000 mg/kg as compared to diabetic control group (\*\* $p < 0.01$ ).

In STZ induced diabetes mellitus, the loss of body weight is caused by increase in muscle wasting and catabolism of fat and proteins (Chakravarti et al., 1981). Due to insulin deficiency, protein content is decreased in

**Table 4.** Effect of *Linum usitatissimum* oil on body weight in diabetic rats.

S/No.	Treatment	Dose (mg/kg p.o)	Initial body weight (g)	Final body weight (g)	Change in weight
1	Normal	-	220±1.1	240±1.5	+20
2	Diabetic control	-	215±1.8	194±2.0	-21 <sup>a</sup>
3	LUO	500	250±2.2	255±1.0	+5
4	LUO	1000	245±1.3	255±1.4	+10 <sup>**</sup>
5	Glibenclamide	0.6	220±4.7	250±1.8	+30 <sup>**</sup>

Values are presented as mean ± SEM; n = 6 in each group. One way ANOVA followed by Dunnett's test <sup>a</sup>p < 0.01 versus normal; <sup>\*\*</sup>p < 0.01 versus diabetic control; LUO: *Linum usitatissimum* oil.

**Table 5.** Effect of *Linum usitatissimum* oil on glycosylated hemoglobin (HbA1c), hepatic glycogen and insulin in the study.

Treatment	Dose (mg/kg)	HbA1c (% of Hb)	Hepatic glycogen (mg/g wt of tissue)	Insulin (micro U/ml)
Normal	-	6±1.4	74±6.6	14±2.1
Diabetic control	-	11.3±2.4 <sup>a</sup>	27±4.5 <sup>a</sup>	7.9±1.1 <sup>a</sup>
LUO	500	9.0±2.2	47±1.8 <sup>*</sup>	10±2.0
LUO	1000	7.2±2.6 <sup>**</sup>	64±3.6 <sup>**</sup>	12±2.5 <sup>*</sup>
Glibenclamide	0.6	7.0±0.7 <sup>**</sup>	66±2.8 <sup>**</sup>	11.9±1.0 <sup>*</sup>

Values are presented as mean ± SEM; n = 6 in each group. One way ANOVA followed by Dunnett's test <sup>a</sup>p < 0.01 versus normal; <sup>\*</sup>p < 0.05; <sup>\*\*</sup>p < 0.01 versus diabetic control; LUO: *Linum usitatissimum* oil.

**Table 6.** Effect of *Linum usitatissimum* oil on Lipid profile.

Treatment	Dose (mg/kg)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)
Normal	-	85±1.5	16±2.5	66±1.9
Diabetic control	-	232±2.4 <sup>a</sup>	43±3.1 <sup>a</sup>	37.4±1.2 <sup>a</sup>
LUO	500	177±3.6 <sup>**</sup>	29±1.6 <sup>**</sup>	44±2.1
LUO	1000	111±2.5 <sup>**</sup>	21±1.3 <sup>**</sup>	48±1.3 <sup>*</sup>
Glibenclamide	0.6	138±1.8 <sup>**</sup>	14.4±1.2 <sup>**</sup>	53±1.8 <sup>*</sup>

Values are presented as mean ± SEM; n = 6 in each group. One way ANOVA followed by Dunnett's test <sup>a</sup>p < 0.01 versus normal; <sup>\*</sup>p < 0.05; <sup>\*\*</sup>p < 0.01 versus diabetic control; LUO: *Linum usitatissimum* oil.

muscular tissue by proteolysis (Flat et al., 1990). A decrease in body weight was registered in case of STZ diabetic control group rats while in tested groups the weight loss was reversed. Fatty acid mobilisation from adipose tissue is sensitive to insulin. Insulin's most potent action is the suppression of adipose tissue lipolysis (Campbell et al., 1992). A rise in plasma insulin concentration of only 5 IU/ml inhibits lipolysis by 50%, whereas a reduction in basal insulin levels result in a marked acceleration of lipolysis (Bonadonna et al., 1990). We demonstrated that LO increased plasma insulin concentrations in diabetic rats. Insulin levels higher than those of the control group may result in inhibition of lipolysis and decreased plasma triglyceride and cholesterol levels. Some studies suggest that the antihyperglycemic action of traditional antidiabetic plant

extracts may be due in part to decreased glucose absorption *in vivo* (Gallagher et al., 2003). This mechanistic explanation may also apply to the actions of LO in lowering the triglyceride and cholesterol level.

The conversion of glucose to glycogen in the liver cells is dependent on the extracellular glucose concentration and on the availability of insulin which stimulates glycogen synthesis over a wide range of glucose concentration (Kumar et al., 2011). Diabetes reduces activity of glycogen synthase thereby affecting the glycogen storage and synthesis in rat liver and skeletal muscle (Kumar et al., 2011). Oral administration of LO 1000 mg/kg body weight significantly increased hepatic glycogen levels in STZ diabetic rats possibly because of the reactivation of the glycogen synthase system as a result of increased insulin secretion.

**Table 7.** Effect of *Linum usitatissimum* oil on antioxidant parameters (MDA and GSH).

Treatment	Dose (mg/kg)	MDA (nmol/dl)	GSH (µmol/g)
Normal	-	2.8±0.2	41.2±2.8
Diabetic control	-	5.4±0.4 <sup>a</sup>	14±1.15 <sup>a</sup>
LUO	500	3.8±0.7	22±2.4
LUO	1000 mg/kg	3.1±0.3**	34±2.1**
Glibenclamide	0.6 mg/kg	3.0±0.2**	38±1.8**

Values are presented as mean ± SEM; n = 6 in each group. One way ANOVA followed by Dunnett's test <sup>a</sup>p < 0.01 versus normal; \*\*p < 0.01 versus diabetic control; LUO: *Linum usitatissimum* oil.

## Conclusion

The present study showed that oral administration of *Linum usitatissimum* has potential antidiabetic, anti-hyperlipidemic and antioxidant effect in STZ induced diabetic rats. The potent antioxidant activity may be responsible for the antihyperglycemic and antihyperlipidemic effects. Thus the investigation reveals that *L. usitatissimum* can be used as a natural oral agent with antihyperglycemic, antihyperlipidemic and antioxidant effects.

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## REFERENCES

- Betteridge J (1997). Lipid disorders in diabetes mellitus, in: J.C. Pickup, G. Williams (Eds.), Text Book of Diabetes, Blackwell Science Publishers, London, pp. 1–35.
- Bonadonna RC, Groop LC, Zych K, Shank M, DeFronzo RA (1990). Dose dependent effect of insulin on plasma free fatty acid turnover and oxidation in humans. *Am. J. Physiol.* 259:736–750.
- Brown WV (1994). Lipoprotein disorders in diabetes mellitus. *Med. Clin. North Am.* 78:143–161.
- Campbell PJ, Carlson MG, Hill JO, Nurjhan N (1992). Regulation of free fatty acid metabolism by insulin in humans: role of lipolysis and reesterification. *Am. J. Physiol.* 263:1063–1069.
- Chakravarti BK, Gupta S, Gambir SS, Gode KD (1981). Pancreatic betacell regeneration in rats by (-) epicatechin. *The Lancet* 2:759–760.
- Chattopadhyay RR (1999). Possible mechanism of antihyperglycemic effect of *Azadirachta indica* leaf extract. *J. Ethnopharmacol.* 67:373–376.
- Flat SK, Day C, Bailey CJ, Flatt PR (1990). Traditional plant treatments for diabetes: studies in normal and streptozotocin diabetic mice. *Diabetologia* 33:462–464.
- Gallagher AM, Flatt PR, Duffy G, Abdel-Wahab YHA (2003). The effects of traditional antidiabetic plants on *in vitro* glucose diffusion. *Nutr. Res.* 23(3):413–424.
- Gupta SS (1994). Prospects and perspectives of natural plant products in medicine. *Ind. J. Pharmacol.* 26:5–9.
- Jaiprakash R, Rani NMA, Venkataraman BV (1993). Effect of felodipine on serum lipid profile in short term streptozotocin diabetes in rats, *Indian, J. Exp. Biol.* 31:283–284.
- Kumar S, Kamboj J, Sharma S (2011). In vivo Anti-diabetic and Antioxidant potential of *Psoralea corylifolia* seeds in Streptozotocin induced type-2 diabetic rats. *J. health Sci.* 57(3):1–11.
- Kumar S, Sharma S, Vasudeva N (2012). GC-MS analysis and screening of antidiabetic, antioxidant and hypolipidemic potential of *Cinnamomum tamala* oil in streptozotocin induced diabetes mellitus in rats. *Cardiovasc. Diabetol.* 11:95.
- Kumar S, Sharma S, Vasudeva N, Ranga V (2012). In vivo antihyperglycemic and antioxidant potentials of ethanolic extract from *Tecomella undulata*. *Diabetol. Metab. Syndrome* 4:33.
- Kumar S, Singh R, Sharma S, Vasudeva N (2012). Acute and chronic animal models for the evaluation of anti-diabetic agents. *Cardiovasc. Diabetol.* 11:9.
- Lewis HS, Elvin-Lewis MPH (1977). Plants affecting man's health, in: Medical Botany, Wiley, New York, pp. 217–218.
- Momin A (1987). Role of indigenous medicine in primary health care, in: Proceedings of First International Seminar on Unani Medicine, New Delhi, India, p. 54.
- Nadkarni AK (1992). *Indian Materia Medica*, vol. 1, Popular Prakashan Ltd., Bombay.
- Okhawa H, Ohishi N, Yagi K (1979). Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. *Anal. Biochem.* 95:351–358.
- Okutan H, Ozcelik N, Yilmaz HR, Uz E (2005). Effects of phenethyl ester on lipid peroxidation and anti oxidant enzymes in diabetic rat heart. *Clin. Biochem.* 38:191–196.
- Rubilar M, Gutiérrez C, Verdugo M, Shene C, Sineiro J (2010). Flaxseed as a source of functional ingredients. *J. soil Sci. Plant Nutr.* 10(3):373–377.
- Seifter S, Dayton S, Molic B, Mutwzter E (1950). The estimation of glycogen with the anthrone reagent. *Arch. Biochem.* 25(1):191–200.
- Sheela GC, Augusti K (1992). Antidiabetic effects of S-allyl cystine sulphoxide isolated from garlic *Allium sativum* Linn. *Indian J. Exp. Biol.* 30:523–526.
- Stamler I, Vaccaro O, Neaton JD, Wentworth D (1993). Diabetes, other risk factors and 12-yr cardiovascular mortality for men screened in the multiple risk factor intervention trial, *Diabetes Care* 15:434–444.
- Tian YM, Johnson G, Ashcroft JH (1998). Sulfonylureas enhance exocytosis from pancreatic b-cells by a mechanism that does not involve direct activation of protein kinase C. *Diabetes* 47:1722–1726.
- Tunali S, Yanardag R (2006). Effect of vanadyl sulfate on the status of lipid parameters and on stomach and spleen tissues of streptozotocin-induced diabetic rats. *Pharmacol. Res.* 53:271–277.
- Twajj HAA, Al-Badr AA (1988). Hypoglycaemic activity of *Artemisia herba alba*, *J. Ethnopharmacol.* 24:123–126.
- Velisek J, Mikulcova R, Mikova K, Woldie KS, Link J, Davidek J (1995). Chemometric investigation of mustard seed. *Lebenson Wiss Technol.* 28(6):620–624.

Full Length Research Paper

## Inhibitory effects of methanolic extracts of two eggplant species from South-western Nigeria on starch hydrolysing enzymes linked to type-2 diabetes

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This study sought to investigate the inhibitory effect of methanolic extract of different species of African eggplant (*Solanum melongena* L.) and (*Solanum macrocarpon*) on starch hydrolysing enzymes relevant to type-2 diabetes ( $\alpha$ -amylase and  $\alpha$ -glucosidase). The phenolic content and antioxidant properties of the eggplant varieties were also assessed. The results revealed that both extracts exhibited mild  $\alpha$ -amylase and stronger  $\alpha$ -glucosidase inhibitory activities in a dose dependent manner. Furthermore, *S. macrocarpon* exhibited stronger radicals (1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging ability, nitric-oxide (NO $\cdot$ ) scavenging ability and ferric reducing antioxidant property (FRAP) than *S. melongena*, while *S. melongena* had stronger hydroxyl (OH $\cdot$ ) radical ability. The inhibition of starch hydrolysing enzymes and antioxidant activities suggested the potential use of eggplants in the dietary management or control of postprandial hyperglycemia associated with type-2 diabetes.

**Key words:** Antioxidant activity, diabetes,  $\alpha$ -amylase,  $\alpha$ -glucosidase, *Solanum macrocarpon*, *Solanum melongena*.

### INTRODUCTION

Non insulin dependent diabetes mellitus (NIDDM) is the commonest form of diabetes which accounts for 90% of all cases. The prevalence of this disease is increasing annually and the number of diabetics is projected to rise above 300 million worldwide (Bailey and Day, 2004; Li et al., 2004). Hyperglycemia is a metabolic disorder primarily characterized by  $\beta$ -cells disorder, relative insulin deficiency, and an abnormal rise in blood sugar right after a meal (Kwon et al., 2007). Pancreatic  $\alpha$ -amylase breaks down large polysaccharides (starch) into disaccharides and oligosaccharides, before the action of  $\alpha$ -glucosidases which break down disaccharides into monosaccharides (glucose) which is readily absorbed into the blood stream.

Inhibition of pancreatic  $\alpha$ -amylase and  $\alpha$ -glucosidase is the mechanism adopted by many commercially available drugs for the management of non insulin dependent

diabetes mellitus (Krentz and Bailey, 2005). Hence, inhibition of intestinal  $\alpha$ -glucosidase which delays the absorption of glucose after starch conversion moderates the postprandial blood glucose elevation and thus mimics the effects of dieting on hyperglycemia (Bischoff, 1994). Chronic amylase inhibition may also be useful for treating type 2 diabetes and obesity (Koike, 2005). Increasing evidence in both experimental and clinical studies have shown the participation of oxidative stress in the development and progression of diabetes mellitus (Baynes and Thorpe, 1999; Ceriello 2000). This is usually accompanied by increased production of free radicals or impaired antioxidant defences (Halliwell et al., 1990; Saxena et al., 1993).

Free radicals are formed disproportionately in diabetes by glucose oxidation, non-enzymatic glycation of

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proteins, and the subsequent oxidative degradation of glycosylated proteins. Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes, increased lipid peroxidation, and development of insulin resistance (Maritim et al., 2003).

The growing number of diabetics, coupled with the harsh side effects of some synthetic drugs has led to the increasing search for alternatives, which are relatively cheap with minimal side effect (Chakraborty et al., 2002). One therapeutic approach for treating diabetes is to decrease the post-prandial hyperglycemia (Kwon et al., 2006, 2007).

Phenolic compounds are widely distributed in edible plants and have been suggested to protect against a variety of diseases (Johnson et al., 2005). Recent investigations suggest that phenolic components of higher plants may act as antioxidants or via other mechanisms prevent disease processes (Wang et al., 2000). Recent findings have also demonstrated that phenolics cross intestinal barriers and are sufficiently absorbed to have the potential to exert biological effects (Williamson and Monach, 2005).

Phenols are the most abundant antioxidants in human diets. They exhibit a wide range of biological effects including antibacterial, anti-inflammatory, anti-allergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and vasodilatory actions (Scalbert and Williamson, 2000; Middleton et al., 2000). They are capable of removing free radicals, chelate metal catalysts, activating antioxidant enzymes, reducing  $\alpha$ -tocopherols and inhibiting oxidases (Amic et al., 2003). They are also important for improving the sensory and nutritional qualities, in that they impart colors, flavors and tastes (Kim et al., 2002).

Eggplant is an economic flowering plant belonging to the family Solanaceae and widely distributed throughout the temperate and tropical regions (Eun-Ju et al., 2011). Members are mostly herbaceous plants, and the fruit is berry and the seeds have large endosperm and are grown mainly for food and medicinal purposes (Kwon et al., 2008). However, there are different types of species and selection in the genus that have diverse shape, size and colour. While information is available on phenolic contents, flavonoids, anthocyanins, antioxidants, and anti-diabetic activity of *Solanum melongena* eggplant fruit extract (Kwon et al., 2008; Scalzo et al., 2010), few information are reported for *Solanum macrocarpon* which is commonly found in southwestern Nigeria known as "igbagba pupa", and used as sauce, stew and part of salad, but there are information on the leaf part which is been used as soup condiment in Nigeria (Oboh et al., 2005; Fasuyi, 2006; Ijarotimi et al., 2010), while the aqueous extract of the fruit had been shown to be nutritious, to lower high blood pressure, to treat constipation and lower hyperlipidaemia (Chinedu et al., 2011; Dougnon et al., 2012; Sodipo et al., 2013).

Both of these species are domesticated and differ from

their wild ancestors in that they underwent artificial selection for larger fruit with less bitter flavour (Knapp et al., 2013; Grubben and Denton, 2004). This present study sought to explore further and to compare the ability of these African eggplant varieties *in vitro* ability using different models of oxidative stress. It is also expedient to investigate the mechanism of anti-diabetic effect of the methanolic extract of these species since there are dearth of information on the *S. macrocarpon*.

## MATERIALS AND METHODS

### Sample collection

Fresh eggplants (*S. melongena* L.) and (*S. macrocarpon*) (200 g) each were purchased from Erekesan main market at Akure, Ondo State, Nigeria. The identification and authentication of the samples was carried out at the Crop, Soil, and Pest management (CSP) Department of the Federal University of Technology, Akure, Nigeria. The eggplant samples were sliced into pieces, sun-dried for 7 days and milled into powder using a Waring heavy duty blender (Waring Products Division, New Hartford, Connecticut, USA), and the powder was stored in an airtight plastic container. All the chemicals used were of analytical grade, and distilled water was used for the analyses.

### Chemicals and equipment

Folin-Ciocalteu's phenol reagent, gallic acid and anhydrous sodium carbonate used were products of Fluka (Buchs, Switzerland). Quercetin and DPPH, ascorbic acid and starch were products of Merck (Darmstadt, Germany), H<sub>2</sub>O<sub>2</sub>, and Iron (II) sulphate were products of Sigma (Aldrich, USA). Iron (III) chloride 6-hydrate and trichloroacetic acid are Fisher products. All other chemicals used were purchased from Rovet Scientific Limited, Benin City, Edo State, Nigeria. The distilled water used was obtained from the Chemistry Department at Federal University of Technology, Akure. Optical absorbance was measured with a ultraviolet (UV)-Visible spectrophotometer (Model 6305; Jenway, Barloworld Scientific, Dunmow, United Kingdom).

### Sample preparation

Extraction of phenolics compounds was carried out using the modified method of Chu et al. (2002). About 20 g each of the milled samples was soaked in 80% methanolic (1:20 w/v) for 24 h. Thereafter, they were filtered through Whatman No. 2 filter paper on a Bucher funnel under vacuum. The filtrate was evaporated using a rotary evaporator under vacuum at 40°C to dryness. All samples extract were stored at -4°C.

### Extract preparation

A 0.5 g of the methanol extract of each sample was reconstituted in 50 ml distilled water, which was subsequently used for the various assays. All antioxidant tests and analyses were performed in triplicate, and results were averaged.

### Total phenol determination

The total phenol content of the sample was determined by adding

0.5 ml of the sample extract to an equal volume of water, and 2.5 ml 10% Folin-Ciocalteu reagent (v/v) and 2.0 ml of 7.5% sodium carbonate was subsequently added. The reaction mixture was incubated at 45°C for 40 min, and absorbance was measured at 726 nm (JENWAY 6305), gallic acid was used as the standard phenol (Singleton et al., 1999).

#### Determination of total flavonoid content

The total flavonoid content of the extracts of the sample was determined by the method reported by Meda et al. (2005). The volume 0.5 ml of samples and the standard quercetin was mixed with 0.5 ml methanol, 50 µl of 10% AlCl<sub>3</sub>, 50 µl of 1 mol/L potassium acetate and 1.4 ml water. The reaction mixture was incubated at room temperature for 30 min. Thereafter, the absorbance of the reaction mixture was subsequently measured at 415 nm. The total flavonoid content was calculated using quercetin as standard.

#### Ferric reducing power (FRAP)

The reducing property of the extracts was determined by assessing the ability of the extract to reduce FeCl<sub>3</sub> solution as described by (Oyaizu, 1986). A 2.5 ml aliquot was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min, and then 2.5 ml of 10% trichloroacetic acid was added. This mixture was centrifuged at 650 rpm for 10 min, and 5 ml of the supernatant was mixed with an equal volume of water and 1 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm against a reagent blank. A higher absorbance indicates a higher reducing power.

#### α-Amylase inhibition assay

The methanolic extracts volume (500 µl) and 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing Hog pancreatic α-amylase (EC 3.2.1.1) (0.5 mg/ml) were incubated at 25°C for 10 min. Then, 500 µl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) was added to each tube. The reaction mixtures was incubated at 25°C for 10 min and stopped with 1.0 ml of dinitrosalicylic acid colour reagent. Thereafter, the mixture was incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted by adding 10 ml of distilled water, and absorbance measured at 540 nm using the spectrophotometer (JENWAY 6305). The percentage (%) enzyme inhibitory activity of the aqueous extracts was calculated (Worthington, 1993).

#### α-Glucosidase inhibition assay

The volume of the methanolic extracts (50 µl) and 100 µl of α-glucosidase solution (1.0 U/ml) in 0.1 M phosphate buffer (pH 6.9) was incubated at 25°C for 10 min. Then, 50 µl of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added. The mixtures were incubated at 25°C for 5 min. Then, 2 ml of Na<sub>2</sub>CO<sub>3</sub> was added to terminate the reaction before reading the absorbance at 405 nm in the spectrophotometer (JENWAY 6305). The α-glucosidase inhibitory activity was expressed as percentage inhibition. The percentage (%) enzyme inhibitory activity of the aqueous extracts was calculated (Apostolidis et al., 2007).

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of samples})}{\text{Absorbance of control}} \times 100$$

#### 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging ability

The free radical scavenging ability of the extracts against DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical was evaluated as described by (Gyamfi et al., 1999). Briefly, an appropriate dilution of the extracts was mixed with 1 ml of 0.4 mM methanolic solution containing DPPH radicals. The mixture was left in the dark for 30 min and the absorbance was measured at 516 nm. The DPPH free radical scavenging ability was subsequently calculated with respect to the reference which contained all the reagents without the test sample.

#### Fenton reaction (degradation of deoxyribose)

The ability of the extract to prevent Fe<sup>2+</sup>/H<sub>2</sub>O<sub>2</sub>-induced decomposition of deoxyribose was carried out using the method of Halliwell and Gutteridge (1981). Methanolic extract (0 to 100 µl) was added to a reaction mixture containing 120 ml 20 mM deoxyribose, 400 ml 0.1 M phosphate buffer, 40 ml 20 mM hydrogen peroxide, and 40 ml 500 mM FeSO<sub>4</sub>, and the volume were made to 800 ml with distilled water. The reaction mixture was incubated at 37°C for 30 min and the reaction was stopped by the addition of 0.5 ml of 2.8% trichloroacetic acid; this was followed by the addition of 0.4 ml of 0.6% thiobarbituric acid (TBA) solution. The tubes were subsequently incubated in boiling water for 20 min. The absorbance was measured at 532 nm in a spectrophotometer (JENWAY6305).

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of samples})}{\text{Absorbance of control}} \times 100$$

#### Determination of nitric-oxide radical scavenging ability (NO·)

The scavenging effect of the extracts against nitric-oxide radical was measured according to the method of Mercocci et al. (1994). The reaction mixture containing 1 ml (25 mM) sodium nitroprusside in phosphate buffer saline and samples for 100, 200, 300, and 400µl, respectively were incubated at 37°C for 2 h. An aliquot after (0.5 ml) of the incubation was removed and diluted with 0.3 ml Greiss reagent (1% suphanilamide in 5% H<sub>3</sub>PO<sub>4</sub> and 0.1% Naphthylethylenediaminedihydrochloride). The absorbance of the chromophore formed was immediately read at 570 nm against distilled water as blank.

#### Statistical analysis

The results of the replicate readings were pooled and expressed as mean ± standard deviation. Student *t* test was performed and significance was accepted at  $P \leq 0.05$  (Zar, 1984). IC<sub>50</sub> (concentration of extract that will cause 50% inhibitory activity) was determined using linear regression analysis

## RESULTS

Results of this study (Table 1) showed that the phenolic compound *S. macrocarpon* eggplant extract (50.01

**Table 1.** Total phenol, Total flavonoids, and Ferric Reducing power (FRAP) of methanolic extracts of eggplant species.

Samples	Total phenols(mgGAE/100g)	Total flavonoids (mgQE/100g)	Reducing power (mg/100g)
<i>S. macrocarpon</i>	50.01±1.85 <sup>a</sup>	15.48±1.75 <sup>a</sup>	56.67±1.43 <sup>a</sup>
<i>S. melongena</i>	34.57±1.07 <sup>b</sup>	9.34±1.34 <sup>b</sup>	48.78±0.55 <sup>b</sup>

Data represent means of triplicate determinations. Values with the same letter along the same column are not significantly different ( $P < 0.05$ ). mgGAE: Milligram gallic acid equivalent; mgQE: Milligram Quercetin equivalent.

**Table 2.** IC<sub>50</sub> µg/mL of antioxidant activities and enzymes inhibition methanolic extracts of eggplant species.

Samples	DPPH-Free Radical scavenging ability	OH-Radical scavenging ability	No-Radical Scavenging ability	α-amylase inhibition	α-glucosidase inhibition
<i>S. macrocarpon</i>	727.80±0.19 <sup>a</sup>	163.08±0.28 <sup>b</sup>	551.26±0.89 <sup>a</sup>	42.66±0.24 <sup>a</sup>	71.77±0.50 <sup>a</sup>
<i>S. melongena</i>	965.25±0.02 <sup>b</sup>	91.99±0.27 <sup>a</sup>	874.12±0.42 <sup>b</sup>	40.11±0.15 <sup>a</sup>	63.24±0.30 <sup>b</sup>

Data represent means of triplicate determinations. Values with the same letter along the same column are not significantly different ( $P < 0.05$ ).

mg/100 g) is significantly ( $P < 0.05$ ) higher than *S. melongena* eggplant extract (34.57 mg/100 g). In this study, total flavonoids were evaluated in the two species. Table 1 results revealed that *S. macrocarpon* had higher total flavonoids content (15.48 mgQE/100 g) than *S. melongena* (9.34 mgQE/100 g). The ferric reducing antioxidant capacity (FRAP) of the extract reported as ascorbic acid equivalent which is the ability of the phenolic extracts to reduce Fe (III) to Fe (II); a measure of their antioxidant properties. Both species had high reducing power as shown in Table 1. It showed that *S. macrocarpon* had a significantly higher ( $P < 0.05$ ) reducing power (56.67 mg/100 g) than *S. melongena* (48.78 mg/100 g).

The interaction of eggplant extracts with α-amylase, as shown in Figure 1, revealed that both extracts had a marked inhibition of pancreatic α-amylase activity in a dose dependent manner in the range of 0-100 µg/ml. However, judging by the IC<sub>50</sub> (extract concentration causing 50% enzyme inhibition) value (Table 2), there was no significant change ( $P > 0.05$ ) in the inhibitory ability of *S. macrocarpon* and *S. melongena* extract. In the same vein, the ability of the eggplant extracts to inhibit α-glucosidase activity *in vitro* was investigated and the result is presented in Figure 2. The results revealed that both eggplant extract inhibited α-glucosidase in a dose-dependent manner (0 to 100 µg/ml). However, there was higher inhibitory ability of *S. melongena* extract on α-glucosidase than *S. macrocarpon* extract. It was observed that the extracts showed a stronger inhibition of pancreatic α-glucosidase and a mild inhibition of α-amylase as shown in Figures 1 and 2.

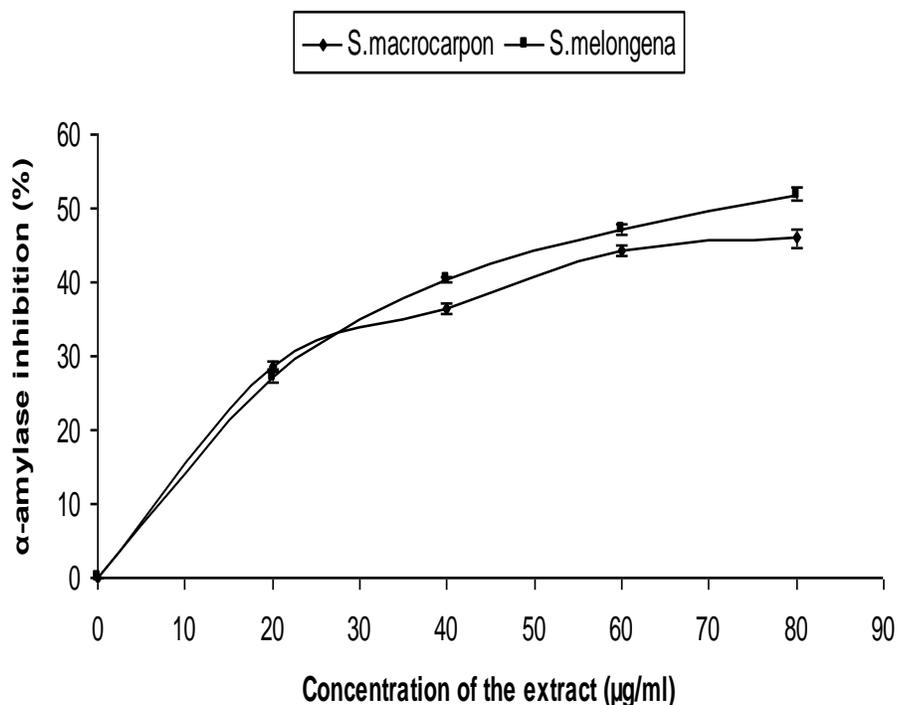
The IC<sub>50</sub> of the ability of the 80% methanolic extracts to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical is shown in Figure 3. DPPH had been used to test the free radical scavenging ability of various natural products (Brand et al., 1995). In this study, the extracts obtained from *Solanum* species exhibit lower IC<sub>50</sub> values,

indicating the higher potential as free radical scavengers. However, *S. macrocarpon* had more lower IC<sub>50</sub> value of (727.80 µg/ml) while *S. melongena* had (965.25 µg/ml). Both extract scavenged DPPH in a dose-dependent manner at all tested concentration (0 to 330 µg/ml) (Figure 3). However, the results revealed that *S. macrocarpon* had significantly higher ( $P < 0.05$ ) free radical scavenging ability from the IC<sub>50</sub> than *S. melongena*.

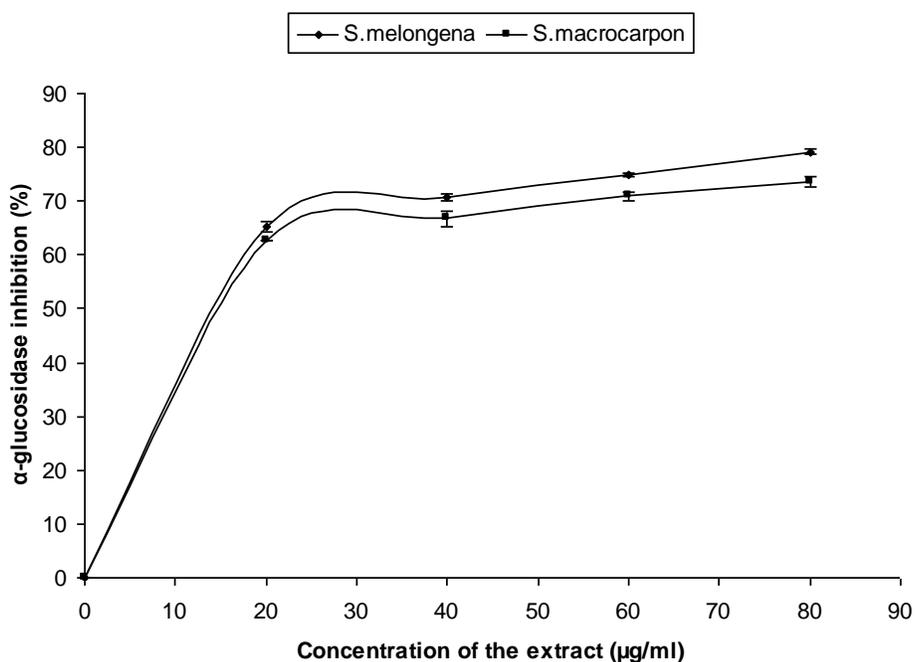
IC<sub>50</sub> values represent eggplant concentrations required to scavenge 50% free radicals as presented in Table 2; a low IC<sub>50</sub> translates to a higher antioxidant activity. Table 2 and Figure 4 showed the inhibitory effect of *S. macrocarpon* and *S. melongena* eggplant on deoxyribose degradation under different Fe<sup>2+</sup>/H<sub>2</sub>O<sub>2</sub> oxidative assault. It is noteworthy that when Fe<sup>2+</sup> or H<sub>2</sub>O<sub>2</sub> was used as the oxidants, *S. melongena* eggplant exerted a marked inhibitory effect (91.99 µg/ml) on deoxyribose degradation when compared to *S. macrocarpon* (163.10 µg/ml). This showed that individual components of the eggplants exhibited inhibitory effect on OH<sup>•</sup> radical-induced oxidative assault on deoxy-ribose degradation. The nitric oxide (NO<sup>•</sup>) radical scavenging ability of the methanolic extracts of *S. macrocarpon* and *S. melongena* is presented in Figure 5 and the IC<sub>50</sub> in Table 2. The results revealed that both extracts were able to scavenge NO<sup>•</sup> radical produced in a dose dependent manner (0 to 250 µg/ml). However, as revealed from the Figure 5, the NO<sup>•</sup> radical scavenging ability of *S. macrocarpon* at the maximum concentration exhibited inhibition at (17.38%), while *S. melongena* exhibited a 12.49% maximum tested concentration.

## DISCUSSION

Phenolic compound embraces a wide range of plant substances which possess common aromatic ring bearing one or more hydroxyl substituents. They tend to



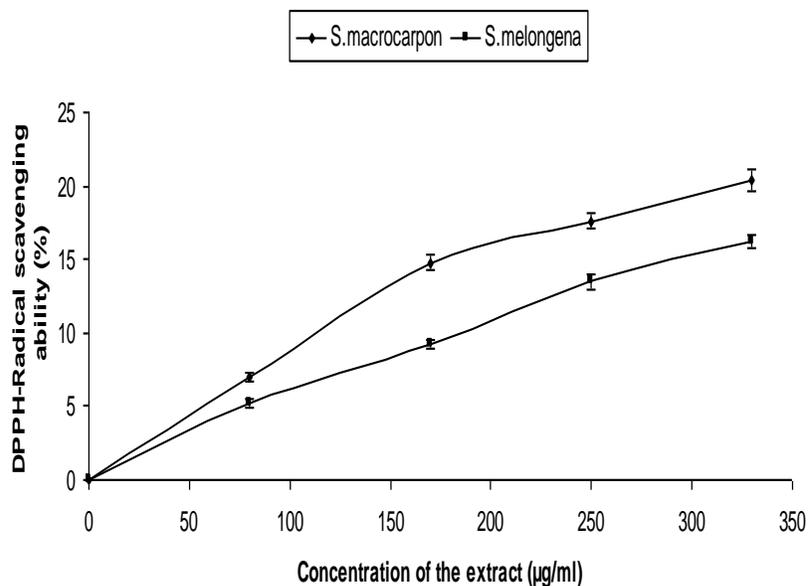
**Figure 1.**  $\alpha$ -Amylase inhibitory activity of methanolic extract of eggplant species. Values represent mean  $\pm$  standard deviation,  $n = 3$ . ( $P < 0.05$ )



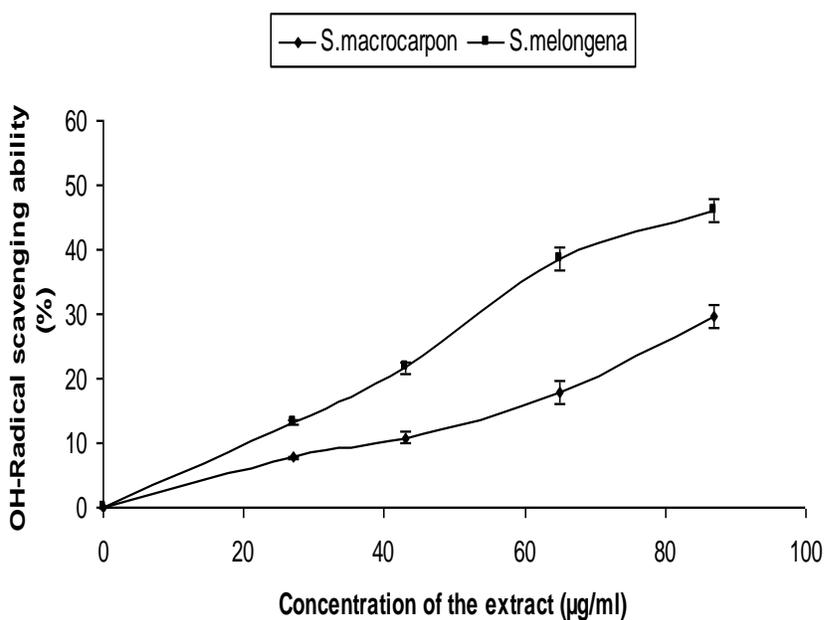
**Figure 2.**  $\alpha$ -Glucosidase inhibitory activity of methanolic extract of eggplant species. Values represent mean  $\pm$  standard deviation,  $n = 3$  ( $P < 0.05$ )

be water soluble since they most frequently occur combined with sugar as glycosides and are usually located in the cell vacuole (Galston., 1969; Materska and

Perucka, 2005). Phenolic compounds are plant-derived antioxidants that possess metal-chelating capabilities and radical-scavenging properties (Bors and Saran, 1987;



**Figure 3.** DPPH free radical-scavenging ability of methanolic extract of eggplant species. Values represent mean  $\pm$  standard deviation,  $n = 3$ . ( $P < 0.05$ )

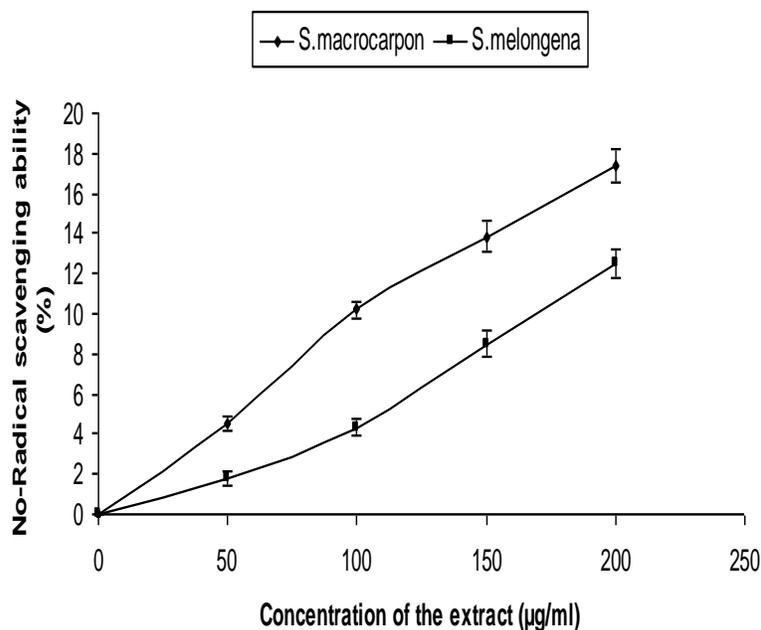


**Figure 4.** Hydroxyl radical-scavenging ability of methanolic extract of eggplant species. Values represent mean  $\pm$  standard deviation,  $n = 3$ . ( $P < 0.05$ )

Lopes et al., 1999). Phenols display a vast variety of structure which can be divided into three main classes, which are flavonoids, tannin and phenolic acids (Strube et al., 1993). Phenolic compounds known to possess high antioxidant activity are commonly found in fruits, vegetables, and herbs. The results of this study is lower than the methanolic extracts of Thailand varieties of eggplant (Akanitapichat et al., 2010), and methanol

extract of the bark and leaves of southern African medicinal plants (Steenkamp et al., 2013).

However, this results is higher than that of aqueous extracts of seed and flesh of riped and unripe *S. melongena* (Fetegbe et al., 2012), and aqueous extract of *S. aethiopicum* cultivars (Nwanna et al., 2013). These differences may be due to the complexity of these groups of compounds, method



**Figure 5.** Nitric-oxide radical-scavenging ability of methanolic extract of eggplant species. Values represent mean  $\pm$  standard deviation,  $n = 3$ , ( $P < 0.05$ )

of extraction, climatic condition and cultural practices, maturity at harvest, and storage condition (Podsdek, 2007). Singleton et al. (1999) reported that different phenolic compounds in matrix samples have different responses in the Folin–Ciocalteu method, depending on the number of phenolic groups they have. Some of the inorganic substances (hydrazine, hydroxyammonium chloride, iron ammonium sulfate, iron sulfate, manganese sulfate, and so on) may react with the Folin–Ciocalteu reagent to give elevated apparent phenolic concentrations (Prior et al., 2005). Flavonoids and other plant phenolics are especially common in leaves, flowering tissues and woody parts such as the stem and bark (Larson, 1988). Flavonoids are a class of widely distributed phytochemicals with antioxidant and biological activity. The results gotten is higher than that of (Chaira et al., 2009) on water-methanolic extracts on different common Tunisia date cultivars, also on *S. aethiopicum* cultivars (Nwanna et al., 2013) but lower than that reported by Sariburun et al. (2010) on different varieties of blackberry and ethanolic extract of *Zanthoxylum armatum* fruit (Barkatullah et al., 2013). However, the results are within the same range of flavonoids extracts of selected tropical plants (Mustafa et al., 2010). The trend in the total flavonoid contents agreed with the total phenolic contents results. The antioxidant activity of flavonoids has been linked to the number of OH groups and their arrangement on the molecule. They are constituents of plant foods that have been implicated in the reduction of cancer risk (Sies, 1986). Phenolics and flavonoids of plant origin are

reported to have potent antioxidants, and homeostatic balance between pro-oxidant and anti-oxidants is known to be important for maintenance of health as well as prevention from various degenerative diseases (Rawat et al., 2011).

Antioxidants are strong reducing agents and this is principally because of the redox properties of their hydroxyl groups and the structural relationships of any parts of their chemical structure (Eleazu et al., 2011). The ability of the extracts of both eggplants to reduced Iron (III) to Iron (II) is higher than that reported by Eun-Ju et al. (2011) on antioxidant activity of different parts of eggplant (*S. melongena* L.), on riped and unriped *S. melongena* L. (Fetegbe et al., 2012), and on plantain extracts (Shodehinde and Oboh, 2012). It is also well known that iron and iron complexes stimulate lipid peroxidation in cells (Gogvadze et al., 2003). The mechanism(s) that underlies the antioxidant activity of both eggplant extracts measured in the presence of  $Fe^{2+}$  may be credited to the ability of the extracts to scavenge radical, reduce  $Fe^{3+}$ . This showed that eggplants phytochemicals such as phenolic compounds are strong reducing agents that could act by readily neutralizing free radicals through electron donation or hydrogen atom transfer.

This study confirm work done by Know et al. (2008) also, Das et al. (2011) showed that there was a direct relationship between eggplants and their cardioprotective ability. In addition, the ripe fruits of these eggplant species may contain other compounds such as anthocyanins that give the fruits its characteristic yellow

and purple pigments and possibly additional antioxidant effect. In addition, it was found that extracts from *S. melongena* (purple colour) small size eggplant fruits with potent antioxidant activities have been shown to contain high phenolic and anthocyanin content (Nisha et al., 2009). Nevertheless, this *S. macrocarpon* eggplant consumed popularly in the southeastern Nigeria have also shown to have high antioxidant activities which must have resulted from its high phenolics and flavonoids content.

The results of enzymes inhibition is in agreement with earlier reports that plant phytochemicals (Adefegha and Oboh, 2013) are mild inhibitors of amylase and strong inhibitors of glucosidase activity (Kwon et al., 2007). Higher inhibition of glucosidase activity and mild inhibition of amylase activity of the eggplant extracts could address the major drawback of currently used glucosidase and amylase inhibitor drugs with side effects such as abdominal distention, flatulence, meteorism and possibly diarrhoea (Pinto et al., 2009). It has been suggested that such adverse effects might be caused by the excessive pancreatic amylase inhibition resulting in the abnormal bacterial fermentation of undigested carbohydrates in the colon (Kwon et al., 2007). This agrees with the findings on *S. melongena* phenolics which have been recommended as a choice diet for the management of type 2 diabetes (Pinto et al., 2009; Nickavar and Yousefian, 2009). The results of the enzymes ( $\alpha$ -amylase and  $\alpha$ -glucosidase) inhibitory assays agreed with the phenolic contents, flavonoids contents and reducing power activity of both extracts. Nevertheless, these extracts had higher inhibitory effect than that reported by (Oboh et al., 2012) on ethanolic extract of fluted pumpkin, also to what was reported on 80% acetone and ethyl acetate phenolics extract on bitterleaf (Saliu et al., 2011).

DPPH is a free radical donor that accepts an electron or hydrogen to become a stable diamagnetic molecule (Je et al., 2009). The tendencies of electron or hydrogen donation are critical factors in characterizing antioxidant activity that involves free radical scavenging (Hu et al., 2000). In addition, the trend in the results agree with the flavonoid distribution in the extracts and many earlier research articles, where correlations were reported between flavonoids content and antioxidant capacity of some plant foods (Chen et al., 2007; Shodehinde and Oboh, 2012). The inhibitory properties of the methanolic extracts to scavenge DPPH is higher than the ability of three Brazilian plants using three different extractant (Pereira et al., 2009) but lower than that gotten by Sariburun et al. (2010) on raspberry and blackberry cultivars. However this results correlate with that of Pereira et al. (2009) in which the inhibitory potency of DPPH radical was higher in methanolic extractant than aqueous extract. The oxygen molecule might produce a highly reactive oxygen species (ROS) by some exogenous factors and endogenous metabolic processes in human body.

ROS include a number of chemically reactive molecules

such as hydrogen peroxide ( $H_2O_2$ ), superoxide ( $O_2^{\cdot-}$ ), and the hydroxyl radical ( $\cdot OH$ ). The  $\cdot OH$  in the cells can easily cross cell membranes at specific sites, react with most biomolecules and furthermore cause tissue damage and cell death. Thus, removing  $\cdot OH$  is very important for the protection of living systems. As shown, the  $\cdot OH$  scavenging effect by the *S. macrocarpon* and *S. melongena* eggplants extracts, and apparently the *S. macrocarpon* eggplant extracts exhibit higher potent  $\cdot OH$  scavenging ability with consequent protection of deoxyribose damage than *S. melongena* from the  $IC_{50}$ . We may attribute this observed  $\cdot OH$  scavenging ability to possible total phenolic acids, tannin, flavonoids contents such as anthocyanins, flavonols, flavones, flavonols, flavanones (Noda et al., 2000; Kwon et al., 2008). More so, these differences could be due to the complexity of individual phenolic constituents and their concentrations (Wahle et al., 2009).

The oxidative destruction of the beta cells in the pancreas is crucial in the development of diabetes, thus radical have been shown to be effective in the management of diabetes in animal models. Diabetes can also be initiated in animals by synthetic drugs such as alloxan and streptozotocin through the production of reactive oxygen species (Oberley, 1988). Single nucleotide polymorphisms (SNP) is a component of antihypertensive drugs; it causes cytotoxicity through the release of cyanide or  $NO^{\cdot}$  which acts in cooperation with other ROS to cause neuronal damage (Oboh and Rocha, 2007). Moreso, SNP has been shown to undergo photodegradation ultimately producing nitrogen oxide ( $NO^{\cdot}$ )  $[(CN)_5-Fe]^{3+}$  and  $[(CN)_4-Fe]^{2+}$  species (Arnold et al., 1984). Likewise, it has been observed that the iron moiety of SNP may have a free iron coordination site for  $H_2O_2$ , which could trigger the generation of highly reactive oxygen species such as hydroxyl radicals ( $\cdot OH$ ) via the Fenton reaction (Graf et al., 1984). Therefore, following a short-lasting release of  $NO^{\cdot}$ , iron moiety of SNP could cause a long-lasting generation of  $\cdot OH$  radicals and oxidant stress/injury similar to that of ferrous citrate iron complexes, which may initiate a lipid peroxidation chain reaction and oxidative injury (Mohanakumar et al., 1994).

*S. melongena* extract exhibited higher inhibition of  $NO^{\cdot}$  radical produced than *S. macrocarpon*, the reason may be as a result of the synergistic activity of the phenols constituents, however this is contrary to the ability of this extract on the produced  $\cdot OH$ . Furthermore, since nitric oxide has been proposed to act as a pro-oxidant at high concentrations, or when it reacts with superoxide, forming the highly reactive peroxynitrite ( $ONOO^{\cdot}$ ) (Radi et al., 2001), we can speculate that the eggplant extracts may scavenge superoxide and peroxynitrite, and may further interact with the cyanide moiety or even the release of iron from the ferrocyanide moiety of SNP, thereby exerting a synergistic-like anti-oxidant effect. The results of this study could eventually explain the mechanism of action of eggplants in the management of postprandial

hyperglycemia and one of its long term complications.

## Conclusion

This study revealed that the antioxidative properties and inhibition of key enzymes linked to non-insulin diabetes mellitus ( $\alpha$ -amylase and  $\alpha$ -glucosidase) could be part of the mechanism through which these eggplants could manage/prevent type-2 diabetes.

## REFERENCES

- Adefegha S, Oboh G (2013). A review: Phytochemistry and mode of action of some tropical spices in the management of type-2 diabetes and hypertension. *Afr. J. Pharm. Pharmacol.* 7(7):332-346.
- Akanitapichat P, Phraibung K, Nuchklang K, Prompitakkul S (2010). Antioxidant and hepatoprotective activities of five eggplant varieties. *Food Chem. Toxicol.* 48:3017-3021.
- Amic D, Davidovic-Amic D, Beslo D, Trinajstic N (2003). Structure-radical scavenging activity relationship of flavonoids. *Croatia Chem Acta*.76(1):55-61. *amygdalina* del.) *J. food biochem* doi:10.1111/j.1745-4514.2011.00576.x.
- Apostolidis E, Kwon YI, Shetty K (2007). Inhibitory potential of herb, fruit, and fungal-enriched cheese against key enzymes linked to type 2 diabetes and hypertension. *Inno. Food Sci. Emerging Technol.* 8:46-54.
- Arnold WP, Longnecker DE, Epstein RM (1984). Photodegradation of sodium nitroprusside: biologic activity and cyanide release. *Anesthesiology* 61:254-260.
- Bailey CJ, Day C. (2004) Avandamet: Combined metformin-rosiglitazone treatment for insulin resistance in type 2 diabetes. *Int. J. Clin. Pract.* 58:867-876.
- Barkatullah BB, Ibrar M, Ali N, Muhammad NR (2013). Antispasmodic potential of leaves, barks and fruits of *Zanthoxylum armatum* DC Afr. *J. Pharm. Pharmacol.* 7(13):685-693.
- Baynes JW (1991). Role of oxidative stress in development of complications in diabetes. *Diabetes* 40:405-412.
- Baynes JW, Thorpe SR (1999). Role of oxidative stress in diabetic complications: A new perspective on an old paradigm. *Diabetes* 48:1-9.
- Bischoff H (1994). Pharmacology of alpha-glucosidase inhibition. *Eur. J. Clin. Invest.* 24:3-10.
- Bors W, Saran M (1987). Radical scavenging by flavonoid antioxidants. *Free Radic. Res. Commun.* 2:289-294.
- Brand WW, Cuvelier MC, Berset C (1995). Use of a free radical method to evaluate antioxidant activity. *LWT* 28:25-30.
- Ceriello A. (2000). Oxidative stress and glycemic regulation. *Metabolism* 49:27-29.
- Chaira N, Smaali MI, Martinez-Tome M, Ferchichi (2009). A simple phenolic composition flavonoid contents and antioxidant capacities in water-methanolic extracts of Tunisian common date cultivars (*Phoenix dactylifera* L). *Int. J. Food Sci. Nutr.* 60:316-329.
- Chakraborty R, Rajagopalan R (2002). Diabetes and insulin resistance associated disorders: Disease and therapy. *Curr Sci.* 83:1533-1538.
- Chinedu SN, Olasumbo AC, Eboji OK, Emiloju OC, Arinola OK, Dania DI (2011). Proximate and Phytochemical Analyses of *Solanum aethiopicum* L. and *Solanum macrocarpon* L. *Fruits. J. Chem. Sci.* 1(3).2231-606X.
- Chu Y, Sun J, Wu X, Liu RH (2002). Antioxidant and antiproliferative activity of common vegetables. *J. Agric. Food Chem.* 50:6910-6916.
- Das S, Raychaudhuri U, Falchi M, Bertelli A, Braga PC, Das DK (2011). Cardioprotective properties of raw and cooked eggplant (*Solanum melongena* L). *Food Funct.* 2:395.
- Doungnon TV, Bankolé HS, Johnson RS, Klotoé JR, Fernand GD, Assogba GF (2012). Phytochemical Screening, Nutritional and Toxicological Analyses of Leaves and Fruits of *Solanum macrocarpon* Linn (Solanaceae) in Cotonou (Benin). *Food Nutr. Sci.* 3:1595-1603.
- Eleazu CO, Oti E, Eleazu KC, Obi V (2012). Nutrient composition, polyphenolic contents and free radical scavenging activities of five honey samples from NRCRI, Umudike and Different locations in South Eastern Nigeria. *Afr. J. Food Sci. Technol.* 3(7):160-170.
- Eun-Ju J, Myung-Suk B, Eun-Kyung J, Young-Hong J, Seung-Cheol L (2011). Antioxidant activity of different parts of eggplant. *J. Med. Plants Res.* 5(18).4610-4615.
- Fasuyi AO (2006). Nutritional potentials of some tropical vegetable leaf meals: chemical characterization and functional properties. *Afr. J. Biotech.* 5:49-53.
- Fetegbe MA, Rocha JBT, Ibukun EO, Kade IJ (2012). A comparative study on ripe and unripe eggplant (*Solanum melongena*) as dietary antioxidant sources. *J. Med. Plants Res.* 6(18):90.
- Galston AW (1969). In prospective in phytochemistry (editors J.B Harbone and T.S Swain). Academic Press London. pp. 193-204.
- Gogvadze V, Walter PB, Ames BN (2003). The role of Fe<sup>2+</sup>-induced lipid peroxidation in the initiation of the mitochondrial permeability transition. *Arch. Biochem. Biophys.* 414:255-260.
- Graf E, Mahoney JR, Bryant RG, Eaton JW (1984). Iron catalyzed hydroxyl radical formation: stringent requirement for free iron coordination site. *J. Biol. Chem.* 259:3620-3624.
- Grubben GJH, Denton OA (2004). *Plant Resources of Tropical Africa II: Vegetables* (Leiden, Wageningen: Backhuys Publishers) 35-198.
- Gyamfi MA, Yonamine M, Aniya Y (1999). Free radical scavenging action of medicinal herbs from Ghana: *Thonningia sanguinea* on experimentally-induced liver injuries. *Gen. Pharm.* 32:661-667.
- Halliwell B, Gutteridge JM (1990). Role of free radicals and catalytic metal ions in human disease: An overview. *Meth Enzymol.* 186:1-85.
- Halliwell B, Gutteridge JMC (1981). Formation of thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts: The role of superoxide and hydroxyl radicals. *FEBS Lett.* 128:347-352.
- Hu C, Zhang Y, Kitts, DD (2000). Evaluation of antioxidant and pro-oxidant activities of bamboo Phyllostachysniger Var. Henonis leaf extract *in vitro*. *J. Agric. Food Chem.* 48:3170-3176.
- Ijartimi OS, Ekeh O, Ajayi OP (2010). Nutrient composition of selected medicinal leafy vegetables in western Nigeria. *J. Med. Food* 13(2):476-479.
- Je JY, Park PJ, Kim EK, Ahn CB (2009). Antioxidant and angiotensin-I-converting enzyme inhibitory activity of *Bambusae caulis* in Liguamen. *Food Chem.* 113:932-935.
- Johnson K, Sharp P, Clifford M, Morgan L (2005). Dietary polyphenols decrease glucose uptake by human intestinal caco-2 cells. *FEBS Lett.* 579:1653-1657.
- Kim D, Lee CY, Wrolstad THE, Acree E, Decker MH, Penner DS, Reid P, Sporns SJ, Schwartz CF (2002). *Current Protocols in Food Analytical Chemistry*, Shoemaker (Eds.). John Wiley & Sons Inc., New York. p. 101-102.
- Knapp S, Vorontsova MS, Prohens J (2013). Wild relatives of the eggplant (*Solanum melongena* L.: Solanaceae): Nwe understanding of species names in a complex group. *Plosone* 8:e57039.
- Koike D (2005). Effect of a wheat amylase inhibitor on canine carbohydrate digestion, gastrointestinal function, and pancreatic growth. *Gastroenterology* 108:1221-1229.
- Krentz AJ, Bailey CJ (2005). Oral antidiabetic agents: Current role in type 2 diabetes mellitus. *Drugs.* 65:385-411.
- Kwon YI, Apostolidis EK, Shetty (2008). In vitro studies of eggplant (*Solanum melongena*) phenolics as inhibitors of key enzymes relevant for type 2 diabetes and hypertension. *Bioresource Tech.* 9:2981-2988.
- Kwon YI, Apostolidis E, Kim YC, Shetty K (2007). Health benefits of traditional corn, beans and pumpkin: In vitro studies for hyperglycemia and hypertension management. *J. Med. Food* 10:266-275.
- Kwon YI, Vatter DA, Shetty K (2006). Evaluation of clonal herbs of *Lamiaceae* species for management of diabetes and hypertension. *Asia Pac. J. Clin. Nutr.* 15:107-118.
- Larson RA (1988). The antioxidants of higher plants. *Phytochem.* 27(4):969-978
- Li WL, Zheng HC, Bukuru J, De Kimpe N (2004). Natural medicines used in the traditional Chinese medicinal system for therapy of diabetes mellitus. *J. Ethnopharmacol.* 92:1-12.

- Lopes GK, Schulman HM, Hermes-Lima M (1999). Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions. *Biochim. Biophys. Acta* 1472:142-152.
- Maritim AC, Sanders RA, Watkins JB (2003). Diabetes, Oxidative Stress, and Antioxidants: A Review. *J. Biochem. Mol. Toxicol.* 17(1):24-38.
- Materska M, Perucka I (2005). Antioxidant activity of the main phenolic compounds isolated from hot pepper fruits (*Capsicum annum* L.). *J. Agric. Food Chem.* 53:1730-1753.
- Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG (2005). Determination of the total phenolic, flavonoid and proline contents in Burkina Faso honey, as well as their radical scavenging activity. *Food Chem.* 91:571-577.
- Mercocci L, Maeguire JJ, Droy-Lefaiz MT, Parker L (1994). The nitric oxide-scavenging properties of Ginkgo biloba extract EGB761. *Biochem. Biophys. Res. Comm.* 201:748-755.
- Middleton EJ, Kandaswami C, Theoharides TC (2000). The effects of plant on mammalian cells: implications for inflammation, heart disease and cancer. *Pharmacol. Rev.* 52:673-839.
- Mohanakumar KP, De Bartolomeis A, Wu RM, Yeh KJ, Sternberger LM, Peng SY, Murphy DL, Chiueh CC (1994). Ferrous-citrate complex and nigral degeneration: evidence for free-radical formation and lipid peroxidation. *Ann. NY. Acad. Sci.* 738:392-399.
- Mustafa RA, Abdulhamid A, Mohamed S, Abubakar F (2010). Total phenolic compounds, flavonoids and radical scavenging activity of 21 selected tropical plants. *J. Food Sci.* 75:1.1750.
- Nickavar B, Yousefian N (2009). Inhibitory effects of six Allium species on  $\alpha$ -amylase enzyme activity. *Iranian J. Pharm. Res.* 8:53-57.
- Noda Y, Kaneyuki T, Igarashi K, Mori A, Packer L (2000). Antioxidant activity of nasunin, an anthocyanin in eggplant peels. *Toxicology* 148:119-123.
- Nwanna EE, Ibukun EO, Oboh G (2013). Nutritional contents and antioxidant activities of african garden egg (*Solanum aethiopicum*) cultivars. *Adv. Food Sci.* 35:1.
- Oberley LW (1988). Free radicals and diabetes. *Free Radic. Biol. Med.* 5:113-124.
- Oboh G, Ekperigin MM, Kazeem MI (2005). Nutritional and haemolytic properties of eggplants (*Solanum macrocarpon*) leaves. *J. Food Comp. Anal.* 18:153-160.
- Oboh G, Akinyemi AJ, Ademiluyi AO (2012). Inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase activities by ethanolic extract of *Telfairia Occidentalis* (Fluted pumpkin) leaf. *As pac J. Trop. Biomed.* 733-738.
- Oboh G, Rocha JBT (2007). Polyphenols in red pepper [*Capsicum annum* var. *aviculare* (Tepin)] and their protective effect on some pro-oxidants induced lipid-peroxidation in brain and liver. *Eur. Food Res. Technol.* 225:239-247.
- Oyaizu M (1986). Studies on products of browning reaction: antioxidative activity of products of browning reaction prepared from glucosamine. *Jap. J Nutr.* 44:307-315.
- Pereira RP, Fachineto R, Prestes A, Robson LPRL (2009). Antioxidant Effects of Different Extracts from Melissa officinalis, Matricaria recutita and Cymbopogon citrates. *Neurochem. Res.* 34:973-983.
- Pinto MDS, Ranilla LG, Apostolidis E, Lajolo FM, Genovese I, Shetty K (2009). Evaluation of anti-hyperglycemia and anti-hypertension potential of native Peruvian fruits using *in vitro* models. *J. Med. Food* 12:278-291.
- Podsedek A (2007). Natural antioxidants and antioxidant capacity of Brassica vegetables: a review. *LWT* 40(1):1-11.
- Prior RL, Wu X, Schaich K (2005). Standardized method for the determination of antioxidant capacity and phenolics in food and dietary supplements. *J. Agric. Food Chem.* 53(10):4290-4302.
- Radi R, Peluffo G, Alvarez MN, Naviliat M, Cayota A (2001). Unraveling peroxynitrite formation in biological systems. *Free Rad. Biol. Med.* 30(5):463-488.
- Rawat S, Jugran A, Giri L, Indra D (2011): Bhatt, and Ranbeer S. Rawal. Assessment of Antioxidant Properties in Fruits of *Myrica esculenta*: A Popular Wild Edible Species in Indian Himalayan Region. Evidence-Based Compl Alt. Med. Article ID 512787.
- Saliu JA, Ademiluyi AO, Akinyemi A, Oboh G (2011). *In vitro* antidiabetes and antihypertension properties of phenolic extracts from bitter leaf (*vernonia amygdalina*). *J. Food Biochem.* 36(5):569-576.
- Saxena AK, Srivastava P, Kale RK, Baquer NZ (1993). Impaired antioxidant status in diabetic rat liver. Effect of vanadate. *Biochem. Pharmacol.* 45:539-542.
- Scalbert A, Williamson G (2000). Dietary intake and bioavailability of polyphenols. *J. Nutr.* 130:2073S-2085S.
- Scalzo LR, Fibian IM, Mennella G, Giuseppe L, Rotino S, Sasso M (2010). Thermal treatment of eggplant (*Solanum melongena* L.) increases the antioxidant content and the inhibitory effect on human neutrophil burst. *J. Agric. Food Chem.* 58(6):3371-3379.
- Shodehinde SA, Oboh G (2012). Assessment of antioxidant capacity, proximate composition and inhibitory activity of unripe plantain (*Musa paradisiaca*) products on  $Fe^{2+}$  and sodium nitroprusside -induced oxidative stress *in vitro*. *J. Toxicol. Environ. Health Sci.* 4(3):46-56.
- Sies H (1986). Biochemistry of oxidative stress, *Angewandte Chemie Int.* 25:1058-1071.
- Singleton VL, Orthofer R, Lamuela-Raventos RM (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagents. *Methods Enzym.* 299:152-178.
- Sodipo OA, Abdulrahman FI, Sandabe UK, Wampana B (2013). Effects of the Aqueous Fruit Extract of *Solanum macrocarpum* L.,  $\alpha$ -solanidine and Standard Lipid Lowering Agents on Biochemical Kidney Function of Hyperlipidaemic Rats Administered Triton-X Orally for 7 Days. *Int. Res. J. Plant* 4(1):25-32.
- Steenkamp V, Nkwane O, Van-Tonder J, Dinsmore A, Gulumian M (2013). Evaluation of the phenolic and flavonoid contents and radical scavenging activity of three southern African medicinal plants. *Afr. J. Pharm. Pharmacol.* 7(13):703-709.
- Strube M, Dragstedt LO, Larsen P, Larsten JC (1993). Naturally Occurring Antitumorogens. I. Plant Phenols. The Nordic Council of Ministers: Copenhagen, Denmark.
- Wahle KWJ, Rotondo D, Brown I, Heys SD (2009). Plant phenolics in the prevention and treatment of cancer. *Functional Food and safely control by Biosensors. Bio-farms for Nutraceut.* pp.1-15.
- Wang Y, Bryant S, Tatusov R, Tatusova T (2000). Links from Genome Proteins to known 3-D Structures. *Genome Res.* 10:1643-1647.
- Williamson G, Monach C (2005). Bioavailability and bioefficiency of polyphenols in humans. Review of 93 intervention studies. *Am. J. Clin. Nutr.* 81:248-255.
- Worthington V (1993). Alpha amylase. In: *Worthington Enzyme Manual*. Worthington Biochemical Corp. Freehold, NJ, pp. 36-41.
- Zar JH (1984). *Biostatistical Analysis*, New Jersey: Prentice-Hall, 620pp. ISBN 0-13-081542-X.

*Full Length Research Paper*

## Effects of administration of carbamazepine and/or phenytoin on haematological parameters in wistar rats

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The aim of the study was to evaluate the haematological alterations following the administration of carbamazepine (CBZ) and/or phenytoin (PHE). Forty apparently, healthy male adult Wistar rats weighing between 144 and 300 g were used for the experiment. They were divided into four groups of 10 animals each. Rats in group I (controls) were given distilled water at the dose of 2 ml/kg and they served as untreated controls. Rats in groups II, III and IV were given CBZ (20 mg/kg), PHE (100 mg/kg) and CBZ + PHE (20 and 100 mg/kg), respectively. All treatments were administered orally by gavage. The regimens were given once daily for a period of eight weeks. At the end of the experiment, the rats were sacrificed and blood samples were collected for the evaluation of total erythrocyte (RBC) count, packed cell volume (PCV), haemoglobin (Hb) concentration, and platelet counts. Also, total and differential leucocyte counts were evaluated using standard laboratory procedures. There was no significant ( $P > 0.05$ ) change in the value of the PCV, Hb and platelets but the RBC decreased ( $P < 0.01$ ) in the CBZ-treated group. There were increases in lymphocytes ( $P < 0.05$ ) and neutrophils ( $P < 0.01$ ) in rats treated with CBZ. In conclusion, the administration of CBZ caused alterations in haematological parameters, the changes observed in the other treatment groups which are PHE and CBZ + PHE are not statistically significant. Haematological parameters should be strictly monitored regularly in individuals administered with CBZ and/or PHE. If there are persistent alterations, the administration of the drugs should be discontinued.

**Key words:** Carbamazepine, phenytoin, rats, oral administration, 8 weeks, haematological parameters.

### INTRODUCTION

Epilepsy is one of the frequent neurological disorders (Ashrafi et al., 2010), encompassing a group of syndromes that vary in its associated pathology and seizure types (Nair, 2003). The characteristic event in epilepsy is the seizure, which is associated with the

episodic high frequency discharge of impulses by a group of neurones (Rang et al., 2005). Phenytoin, phenobarbitone and carbamazepine are the first-line antiepileptic drugs; these first-line drugs are commonly used because of their efficacy and low cost (Misra et al., 2003). Almost

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all classes of psychotropic agents have been reported to cause blood dyscrasia, and agranulocytosis is probably the most important drug-related blood dyscrasia (Flanagan and Dunk, 2008). The rationale for combining some antiepileptic drugs (AEDs) is usually based on the presumptions concerning two aspects of efficacious treatment; the first is related to the anticonvulsant activity of the combining drugs, while the second takes into consideration the side-effects profile of the co-administered AEDs (Luszczki, 2004).

After the use of a first and a second antiepileptic drug without adequate improvement, a combination of two drugs is used (Kwan and Brodie, 2000). Successful treatment consists of finding the balance between obtaining adequate seizure control and avoiding adverse effects (Chung et al., 2005). Phenytoin (PHE) sodium is an anticonvulsant used to control 'grand mal' and psychomotor seizures (Vijay et al., 2009). Systemic administration induces anticonvulsant effect in humans and experimental animals (Ryakaczewska-Czerwińska, 2007). It exerts anti-seizure activity without causing general depression of the central nervous system, but the most significant effect of phenytoin is its ability to modify the pattern of maximal electroshock seizures (McNamara, 2006). Phenytoin blocks voltage-sensitive sodium ion channels and in this way inhibits neuronal firing in the brain (Ryakaczewska-Czerwińska, 2007). It alters potassium and calcium ion conductance, membrane potentials, concentrations of amino acids and the neurotransmitters - noradrenaline, acetylcholine and  $\gamma$ -aminobutyric acid (GABA) receptors. It paradoxically causes excitation in some cerebral neurones; a reduction of calcium permeability with inhibition of calcium influx across the membrane (Porter and Meldrum, 2007). It can cause gingival hyperplasia, agranulocytosis and aplastic anaemia deficits when given for a long time (Vijay et al., 2009).

Carbamazepine (CBZ) is a highly conventionally used antiepileptic drug, which has efficacy in attenuating picrotoxin-induced convulsion (Ali et al., 2003) and against maximal electroshock seizures (Porter and Meldrum, 2007). This may be attributed to its mechanism of action; that is, its use dependent sodium channel blockade, weak GABAergic and antiglutamatergic effects (Motohashi, 1992). CBZ is the usual drug of choice for patients with newly diagnosed partial onset seizure (Gamble et al., 2009). The rate of absorption varies widely among patients, although almost complete absorption apparently occurs in all (Porter and Meldrum, 2007). Haematological toxicity of CBZ is well documented, and a patient undergoing CBZ therapy should be carefully monitored, especially for serious adverse reactions including pure red cell aplasia (Tagawa et al., 1997).

The aim of the study was to determine the haematological alterations that may accompany the use of these drugs in male adult Wistar rats.

## MATERIALS AND METHODS

### Animals

Forty adult male Wistar rats weighing between 144 and 300 g were used for the experiment. The animals were obtained from the Animal House of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria and were housed in rat cages. The animals were fed pellets made from grower's mash (Grand Cereals, Jos, Nigeria), maize bran and groundnut cake in the ratio 4:2:1, with wheat flour serving as binder, and water was provided *ad libitum*. The animals were allowed to acclimatize for a period of two weeks before the commencement of the experiment.

### Anticonvulsant drugs

The anticonvulsant drugs used in this study were carbamazepine (CBZ) tablets (Hovid Bhd, Malaysia) at 20 mg/kg and phenytoin (PHE) capsules (BIOMEDICINE Belgium) at 100 mg/kg.

### Experimental protocols

The animals were divided at random into four groups of 10 animals each. Animals in groups 2, 3 and 4 were given CBZ (20 mg/kg), PHE (100 mg/kg) and CBZ + PHE (20 and 100 mg/kg, separately), respectively. Rats in group 1 were given distilled water at 2 ml/kg and served as untreated controls. All treatments were administered orally by gavage once daily for a period of eight weeks.

### Evaluation of the effect of CBZ and/or PHE on haematological parameters

The haematological parameters of erythrocytic mass [packed cell volume (PCV), total red blood cell (RBC) count, haemoglobin (Hb), total and differential leucocyte counts and platelet count] were analysed using an auto-analyzer (ADVIA 60<sup>®</sup> Haematology System, Bayer Healthcare, Bayer Diagnostics Europe Ltd., Chapel Lane, Swords, Co., Dublin, Ireland; manufactured in France for Bayer) in the Haematology Laboratory, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria.

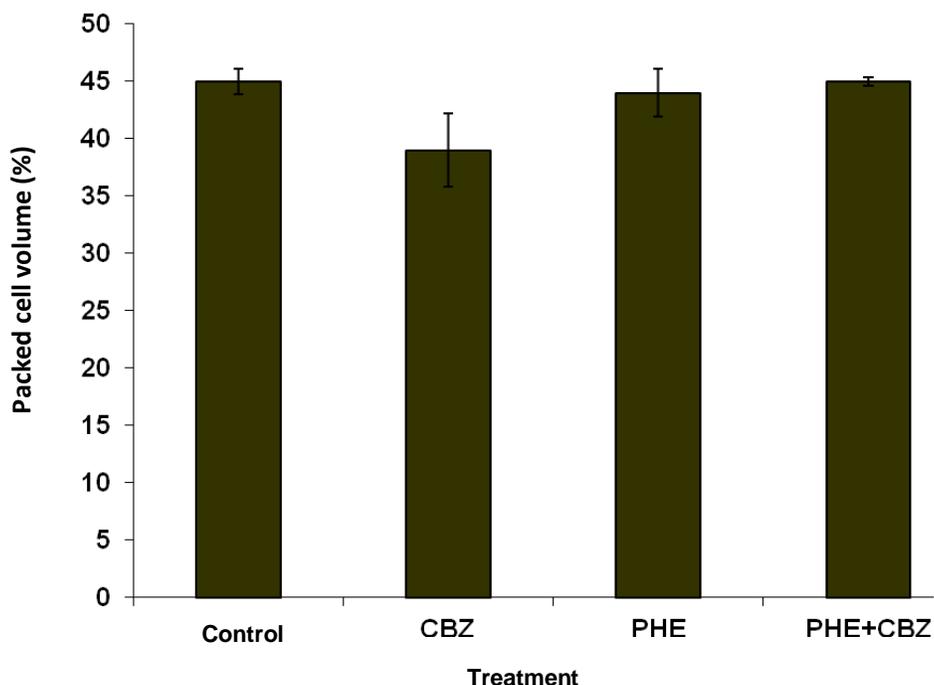
### Statistical analysis

Values obtained were expressed as mean  $\pm$  standard error of mean (SEM) and subjected to statistical analysis using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. The program used for the analysis was GraphPad Prism, Version 4.0 for Windows from GraphPad Software, San Diego, California, USA ([www.graphpad.com](http://www.graphpad.com)). Values of  $P < 0.05$  were considered significant.

## RESULTS

### Effect of treatments on the packed cell volume

There was no significant difference in the value of the PCV obtained in the CBZ, PHE and CBZ + PHE groups, compared to the control group. Also, there was no significant difference in PCV value between the drug-



**Figure 1.** Changes in the value of the PCV in the drug-treated groups upon the administration of the drugs at a dose rate of 20 mg/ka (CBZ) and 100 mg/kg (PHE) per os for a total period of 8 weeks was not statistically significant when compared with the control group.

treated groups (Figure 1).

#### Effect of treatments on red blood cell counts

The change in RBC counts obtained in the PHE and CBZ + PHE groups when respectively compared to that of the control group were not significant ( $P > 0.05$ ). The RBC counts in the CBZ group was lower ( $P < 0.01$ ) than the value recorded in the control group. There was no significant ( $P > 0.05$ ) change in RBC counts in between the drug-treated groups (Figure 2).

#### Effect of treatments on haemoglobin concentration

There was no significant ( $P > 0.05$ ) change in Hb concentrations between the experimental groups (Figure 3).

#### Effect of treatments on platelet counts in Wistar rats

There was no significant change in the platelet counts when the values obtained in the drug-treated groups were respectively compared to that of the control group. Also, the changes recorded in the counts between the drug-treated groups were not different ( $P > 0.05$ ) (Figure 4).

#### Effect of treatments on total leucocyte counts in Wistar rats

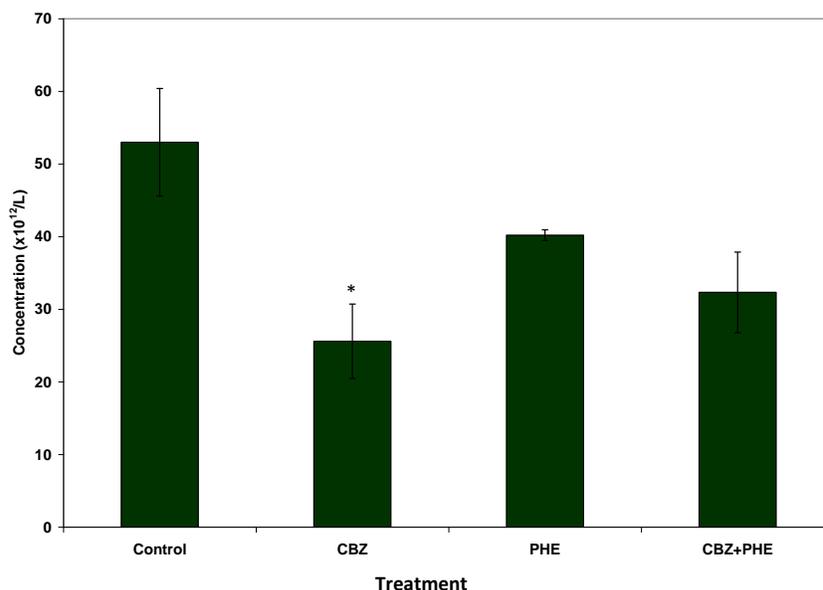
An insignificant increase was observed when the total leucocyte counts in each of the drug-treated group were compared to that of the control group. There was no significant increase in the total leucocyte counts obtained between the drug-treated groups (Figure 5).

#### Effect of treatments on neutrophil counts in Wistar rats

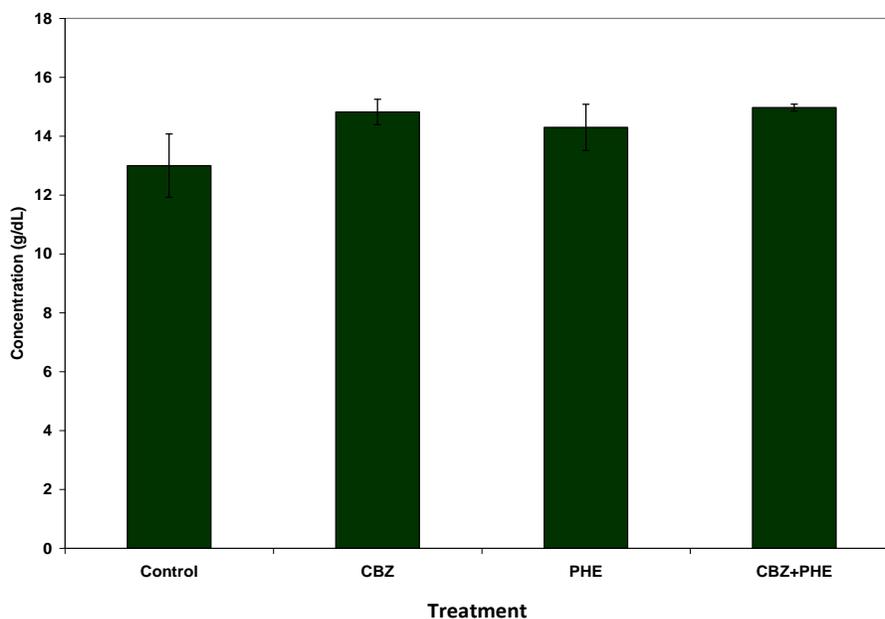
Neutrophil counts in the CBZ group rose significantly ( $P < 0.01$ ) when compared to that of the control group, but the increase in the PHE and CBZ + PHE groups was not different from that of the control group. There was no significant change in neutrophil counts obtained in between the treatment groups (Figure 6).

#### Effect of treatments on lymphocyte counts

The lymphocyte counts increased ( $P < 0.05$ ) in the CBZ group when compared to that of the control group. There was no significant ( $P > 0.05$ ) change in lymphocyte counts when the PHE and CBZ + PHE groups were



**Figure 2.** The value of RBC in the drug-treated groups decreased upon the administration of the drugs at a dose rate of 20 mg/kg (CBZ) and 100 mg/kg (PHE) per os for a total period of 8 weeks when compared with control group. \* =  $P < 0.01$  (CBZ versus control).

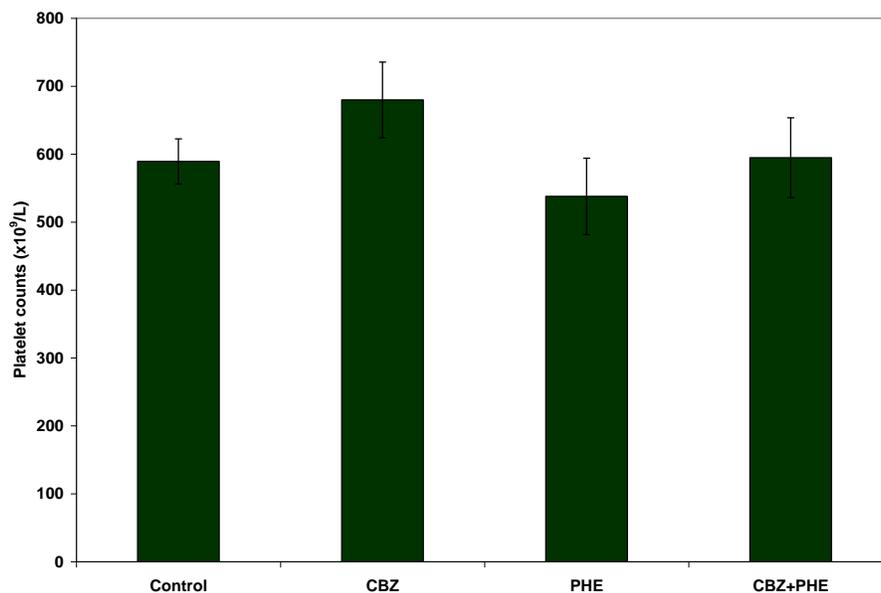


**Figure 3.** Changes in Hb concentration in the drug-treated groups upon the administration of the drugs at a dose rate of 20 mg/kg (CBZ) and 100 mg/kg (PHE) per os for a total period of 8 weeks were not statistically significant when compared with control group.

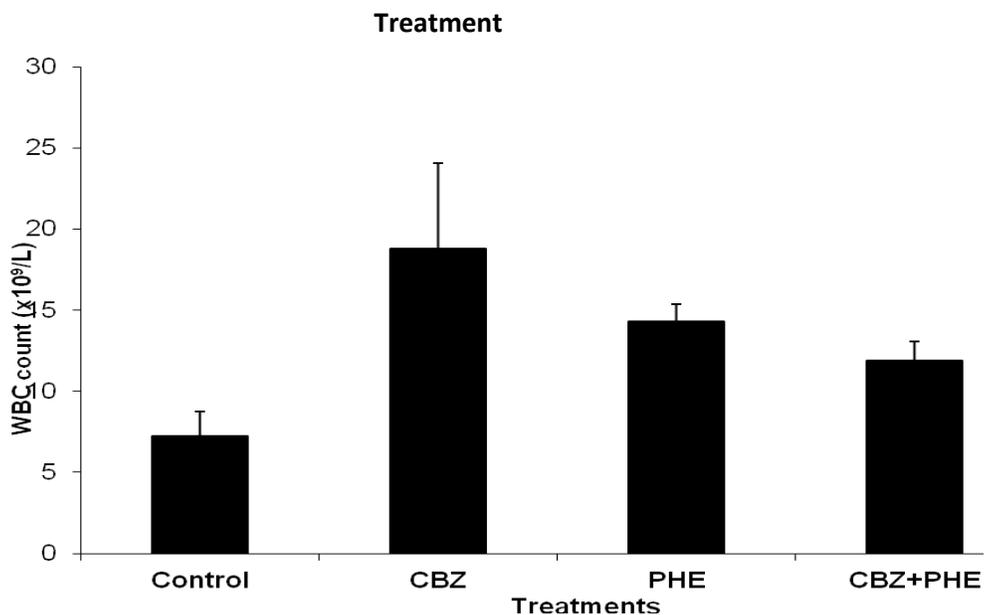
respectively compared to the control group. Similarly, no significant ( $P > 0.05$ ) changes in lymphocyte counts were recorded in between the drug-treated groups (Figure 6).

## DISCUSSION

The decrease in RBC count in the CBZ, PHE and CBZ +



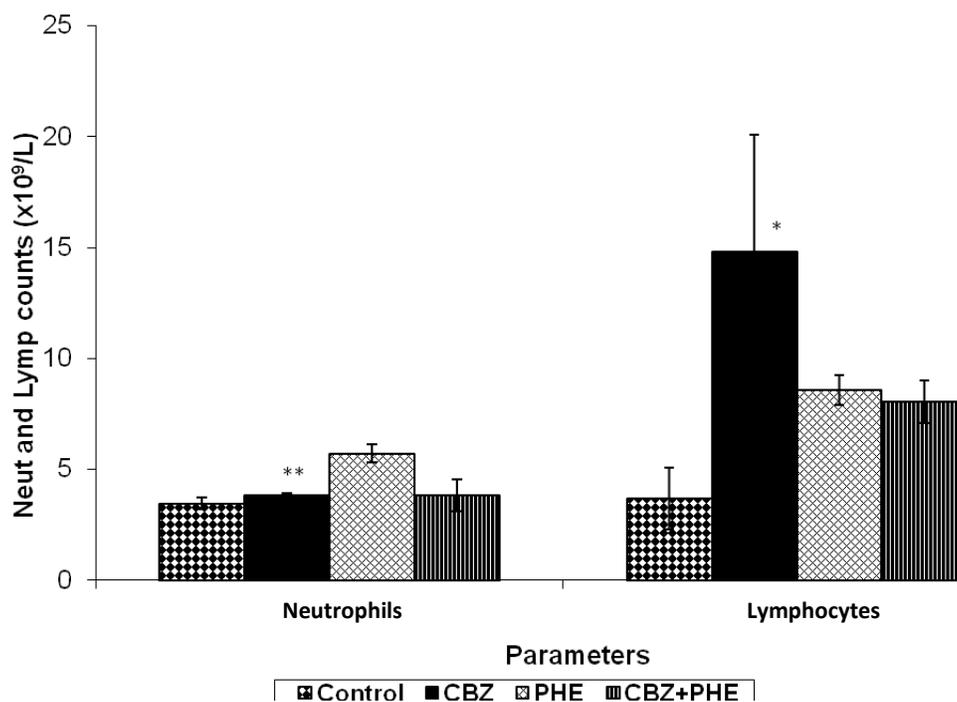
**Figure 4.** Changes in the platelet counts in the drug-treated groups upon the administration of the drugs at a dose rate of 20 mg/kg (CBZ) and 100 mg/kg (PHE) per os for a total period of 8 weeks were not statistically significant when compared with the control group.



**Figure 5.** Changes in the WBC counts in the drug-treated groups upon the administration of the drugs at a dose rate of 20 mg/kg (CBZ) and 100 mg/kg (PHE) per os for a total period of 8 weeks were not statistically significant when compared with the control group.

PHE groups agreed with the finding of Misra et al. (2003) who observed that PHE, phenobarbital and CBZ are highly toxic to the haemopoietic system. Thakur et al.

(2011) showed that decrease in RBC count in the PHE group may be due to the fact that the drug undergoes oxidative metabolism which resulted in the formation of a



**Figure 6.** The neutrophil and lymphocyte counts increased in the drug-treated groups upon the administration of the drugs at a dose rate of 20 mg/kg (CBZ) and 100 mg/kg (PHE) per os for a total period of 8 weeks when compared with the control group. \* =  $P < 0.05$  (CBZ versus control), \*\* =  $P < 0.01$  (CBZ versus control).

toxic arene oxide intermediate. This oxide covalently binds with cell macromolecules, causing cytotoxic damage, bone marrow toxicity and aplastic anaemia. The significant decrease in RBC count in the CBZ group was similar to the result obtained by Tagawa et al. (1997) who suggested that it may be due to isolated cessation of RBC production, resulting from pure RBC aplasia. However, McNamara (2006) reported that the prevalence of aplastic anaemia appears to be 1 in 200,000 patients treated with CBZ monotherapy. Therefore, the concern that aplastic anaemia may be a frequent complication of long-term CBZ therapy may remain controversial, despite the result obtained in the present study.

The non-significant decrease in RBC counts in the polytherapy group indicated a minimal effect of co-administration of the drugs on the RBCs. Drugs have been shown to cause idiosyncratic bone marrow suppression or dose-related suppression (Kaufman et al., 1996). Idiosyncratic bone marrow suppression is a life-threatening event that is not related to dose or to the duration of administration and cannot be predicted by repeated blood draws (Young, 1994; Sepkuty and Kaplan, 2004). Indeed idiosyncratic aplastic anaemia is one of the adverse drug reactions associated with all major AEDs, except gabapentine (Scheuer, 1996). The

AEDs that are primarily known to be associated with bone marrow suppression (although rare) are felbamate, CBZ, PHE, and valproate (Suchitra and Bussel, 2000).

The significant neutrophilia recorded in the CBZ-treated group may be due to cellular inflammation in the absence of an infection (Ekaidem et al., 2006). Significant increase in lymphocytes observed in the CBZ-treated group may be induced by the stimulation of formation of epoxides by the activity of cytochrome P<sub>450</sub>. The epoxides have been shown to bind covalently with macromolecules and act as haptens to stimulate immunologic actions, hence lymphocytosis (Gerson et al., 1983; Spielberg et al., 1986). Minimal effects observed with co-administration of CBZ and PHE on haematological parameters compared to the monotherapy groups may be due to the fact that CBZ can reduce the bioavailability of serum PHE (Lai et al., 1992).

## Conclusion

The administration of CBZ and/or PHE caused alterations in haematological parameters which should be strictly monitored regularly in individuals administered with CBZ and/or PHE. If there are persistent alterations, the

administration of the drugs should be discontinued.

## ACKNOWLEDGEMENT

Special thanks to the supervisory committee: Profs. J.O. Ayo and S. F. Ambali, and Dr. A. U. Zezi for having time to put me through the research processes and assisting by proof-reading the manuscript and also to Mr. Nwosu, technical staff of the Department of veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria, Nigeria.

## REFERENCES

- Ali A, Pillai KK, Pal SN (2003). Effect of folic acid and lamotrigine therapy in some rodent models of epilepsy and behaviour. *J. Pharm. Pharm* 55(3):387-391
- Ashrafi M, Hosseini SA, Abolmaali S, Biglari M, Azizi R, Farghadan M, Samadian A, Shaghafi S, Mombeini H, Saladjegheh N, Rezaei N, Aghamohammadi A (2010). Effect of anti-epileptic drugs on serum immunoglobulin levels in children. *Acta Neurol. Belgica* 110:65-70
- Chung SS, Wang N, Hank N (2005). Antiepileptic drugs: Adults. *Epilepsia*. Available at <file://localhost/E/Wiley%20InterScience%20%20JOURNALS%20%20Epilepsia-%20antiepileptic%20drugs.mht> Accessed on 15/8/2009, 5:24:04 PM.
- Ekaidem IS, Akpanabiatu MI, Uboh FE, Eka OU (2006). Vitamin B<sub>12</sub> supplementation: effects on some biochemical and haematological indices of rats on phenytoin administration. *Biokemistri* 18(1):31-37.
- Flanagan RJ, Dunk L (2008). Haematological toxicity of drugs used in Psychiatry. *Human Psychopharmacol. Clin. Experience* 23:27-41.
- Gamble CL, Williamson PR, Marson AG (2009). Lamotrigine versus carbamazepine monotherapy for epilepsy. *Cochrane Database of Systemic Reviews*, 2: 32 Available at <http://onlinelibrary.wiley.com/doi/10.1002/14651858.CD001031.pub2/pd/standad> Accessed on 11/5/2012, 4:56 am.
- Gerson WT, Fine DG, Spielberg SP, Sensenbrenner LL (1983). Anticonvulsant-induced aplastic anaemia: increased susceptibility to toxic drug metabolites *in vitro*. *Blood* 61:889-893.
- Kaufman DW, Kelly JP, Jurgelon JM (1996). Drugs in the aetiology of Kwan P, Brodie MJ (2000). Early identification of refractory epilepsy. *New Engl. J. Med.* 342:314-319.
- Lai ML, Lin TS, Huang JD (1992). Effect of single- and multiple-dose carbamazepine on the pharmacokinetics of diphenylhydantoin. *Eur. J. Clin. Pharmacol.* 43:201-203.
- Luszczki JJ (2004). Effect of lamotrigine alone or in combination with conventional antiepileptic drugs on locomotor activity in mice. *Indian J. Pharmacol.* 36(5):306-311.
- McNamara JO (2006). Pharmacotherapy of epilepsy. In: *Goodman and Gilman's the pharmacological basis of therapeutics* 11<sup>th</sup> Ed. New York: McGraw-Hill, 2006: Chapter 19.
- Misra UK, Kalita J, Rathore C (2003). Adverse drug reaction: phenytoin and carbamazepine cross reactivity: report of a case and review of literature. *Postgraduate Med. J.* 79:703-704.
- Motohashi N (1992). GABA receptor alterations after chronic lithium administration. Comparison with carbamazepine and sodium valproate. *Progr. Neuropsychopharmacol. Biol. Psychiatry* 16:571-579.
- Nair DR (2003). Epilepsy. Cleveland Clinic Foundation. Available at: <http://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/neurology/epileptic-syndrome>.
- Porter RJ, Meldrum BS (2007). Antiseizure drugs. In: *Basic and clinical pharmacology* 10<sup>th</sup> Ed, New York, McGraw-Hill, 2007: chapter 24.
- Rang HP, Dale MM, Ritter JM, Flower RJ (2007). Mechanism of action of antiepileptic drugs. In: *RANG and DALE Pharmacology*, 6<sup>th</sup> Ed, Churchill Livingstone, 2007:578-584.
- Rykcaczevska-Czerwińska M (2007). Antinociceptive effect of phenytoin in rats. *Pharmacol. Reports* 59(1):144-149.
- Scheuer ML (1996). Antiepileptic Drugs. In *Current Neurologic drugs*, Rowland LP, Klein DF (Eds.) Brunner/ Mazel, New York.
- Sepkuty JP, Kaplan PW (2004). Haematological Disorders. Available at [http://professionals.epi.com/wi/print\\_section.ph?section=haematologic](http://professionals.epi.com/wi/print_section.ph?section=haematologic). Accessed on 12/12/2011, 8:45 am.
- Spielberg SP (1986). *In vitro* analysis of idiosyncratic drug reactions. *Clin. Biochem.* 19:142-144.
- Suchitra A, Bussel JB (2000). Haematologic toxicity of Sodium. *J. Paediatric Haematol. Oncol.* 22:62-65
- Tagawa T, Sumi K, Uno R, Hagakia Y, Fujii F, Yamaguchi H (1997). Pure red cell aplasia during carbamazepine monotherapy. *Brain Dev.* 19(4):300-302.
- Thakur S, Saraswathy GR, Maheswari E (2011). Influence of vitamin C on phenytoin-induced haematotoxicity and oxidative stress in rats. *Int. J. Pharm. Res. Innovation* 2:32-39.
- Vijay P, Yeshwanth R, Bairy KL (2009). Effect of phenytoin sodium on biochemical Parameters of reproductive function in male albino Wistar rats. Available at: [www.j-pbs.org/pdf/221/JPBS081001.pdf](http://www.j-pbs.org/pdf/221/JPBS081001.pdf). Accessed on 8/2/2010.
- Young NS (1994). Agranulocytosis (conference). *J. Am. Med. Assoc.* 271(12):935.[32]

Full Length Research Paper

## Formulation and *in vitro* evaluation of antineoplastic drug loaded nanoparticles as drug delivery system

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The main aim of the present work was to formulate anti-neoplastic drug loaded polymeric nanoparticles using biodegradable polymers (Chitosan and Eudragit RS 100) by emulsion droplet coalescence method. The model drug used here is 5-fluorouracil which is a pyrimidine analogue that is mainly used to treat colonic carcinoma, under the category of anti-neoplastic drugs. Tween 20 was used as emulsifier and colloidal stabilizer. The prepared nanoparticles were evaluated for particle size, surface morphology by TEM, surface charge, drug loading and entrapment efficiency, and for drug release by diffusion. Results show that the prepared nanoparticles are in nanosize, below 1000 nm, having appropriate zeta potential values with better entrapment of drug and controlled release of drug for a period of 12 h. From the obtained formulations, EF5 was selected as best with high entrapment efficiency, optimum zeta potential, and showing more controlled release of drug.

**Key words:** Eudragit RS 100, chitosan, nanoparticles, emulsion droplet coalescence method, control release.

### INTRODUCTION

Drug delivery research is clearly moving from the micro- to the nano-size scale. Nanotechnology is therefore emerging as a field in medicine that is expected to elicit significant therapeutic benefits. The development of effective nano delivery systems capable of carrying a drug specifically and safely to a desired site of action is one of the most challenging tasks of pharmaceutical formulation investigators. They are attempting to reformulate and add new indications to the existing blockbuster drugs to maintain positive scientific outcomes and therapeutic breakthroughs (Yashwant and Deepak, 2009).

5-Fluorouracil (5-FU or 5-fluoro-2,4-pyrimidinedione) is an antimetabolite of pyrimidine analogue type, with a broad spectrum activity against solid tumors (of gastrointestinal tract, pancreas, ovary, brain, breast, etc). Due to its structure, 5-fluorouracil interferes with nucleoside metabolism and can incorporate into RNA and

DNA, leading to cytotoxicity and cell death (Zhang et al., 2008; Arias et al., 2008). Limitations are short biological half-life due to rapid metabolism, incomplete and non uniform oral absorption by dihydropyrimidine dehydrogenase and nonselective action against healthy cells. To prolong the circulation time of 5-fluorouracil and increase its efficacy, its delivery has to be modified by incorporation into nanoparticulate carriers to reduce the 5-fluorouracil associated side effects and thereby improve its therapeutic index (Li et al., 2008). Polymer systems can be to physically trap an antitumor agent and release it in a sustain form directly at the tumor site (Simeonova et al., 2003; Elvire et al., 2004). Chitosan is a linear polysaccharide of [(Yashwant and Deepak, 2009; Li et al., 2008) linked 2-acetamido-2-deoxy-D-glucopyranose, is a natural polysaccharide derived from chitin by deacetylation (Figure 1).

Chitosan is a cationic polymer, and regarded as

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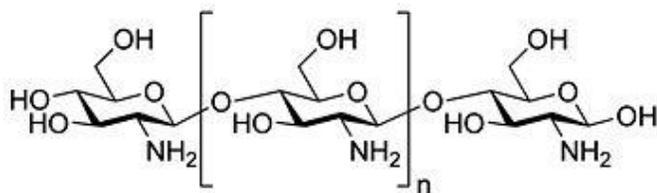


Figure 1. Structure of Chitosan.

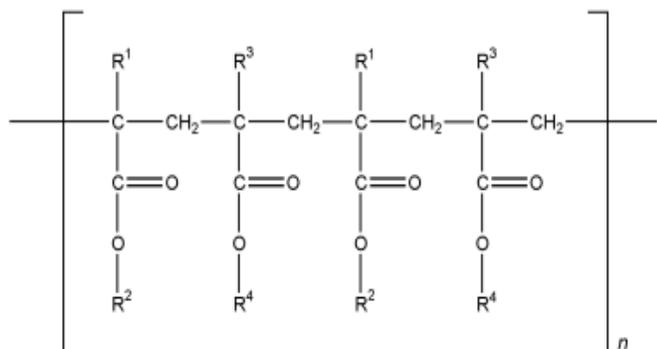


Figure 2. Structure of Eudragit RS 100.

biocompatible, biodegradable, and nontoxic (Dang and Leong, 2006; Thanou et al., 2001). Eudragit RS 100 is a copolymer of ethyl acrylate, methyl methacrylate and a low content of methacrylic acid ester with quaternary ammonium groups (Figure 2). It shows unique dissolution behavior above pH 7.0. So, it has been used as pH-sensitive polymers in various applications including enteric coating materials (Solid drug delivery vehicles). In the present work, nanoparticles of 5-Fluorouracil were prepared using chitosan, Eudragit RS 100, to deliver the drug to disease site and achieve gradual drug release (Onishi et al., 2005). This is based on the concept of localization at the disease site by nanoparticles and control release by ester hydrolysis. Secondary coating of Eudragit over 5-fluorouracil loaded chitosan nanoparticles (Ch-5-FU-NP) was used to protect the drug from acidic environment, that is, pH below 6 and shows a unique drug release above pH 6.8. By the combination of these two polymers, that is, chitosan, Eudragit targeting specific sites in the body simplifies drug administration procedures, reduce the quantity of drug required to reach therapeutic levels, decrease the drug concentration at non target sites.

The objective of this work was to deliver the 5-fluorouracil drug loaded nanoparticles as oral delivery that offers certain advantages over the current regimen of chemotherapy by injection or infusion. Oral site specific rate-controlled expected to reduce systemic side-effects and also to provide an effective and safe therapy for colon cancer with reduced dose and duration of therapy.

## MATERIALS AND METHODS

5-Fluorouracil (5Fu) was obtained from Celone Pharmaceuticals Pvt. Ltd., India. Eudragit RS 100, from Rohm Pharma (Darmstadt, Germany). Chitosan (CS, degree of deacetylation was 95.3% and was a gift sample from Indian Sea foods, Cochin). Liquid paraffin, Tween 20, and sodium chloride were obtained from SD Fine chemicals, Mumbai, India. Sodium hydroxide and sodium dihydrogen phosphate were procured from Loba chemicals Mumbai, India. All other chemicals were of analytical grade and were used without further purification.

### Experimental

#### Preformulation studies

**FTIR studies:** Drug identification was carried out by FTIR spectroscopy. The spectrum was recorded for 5-fluorouracil using spectrum BX (Perkin Elmer) infrared spectrophotometer. Samples were prepared in KBr disk (2 mg sample in 200 mg KBr) with an hydrostatic press at a force of 40 psi for 4 min. The scanning range was 400 to 4000 $\text{cm}^{-1}$  and the resolution was 4  $\text{cm}^{-1}$  (Sharma, 2011; Aslam et al., 2004).

**Differential scanning calorimetric (DSC) studies:** DSC analysis of the drug 5-fluorouracil was carried out using DSC 200F3 Maia equipped with computer analyzer. Samples (3 to 7 mg) were heated under nitrogen atmosphere on an aluminum pan at a heating rate of 10 $^{\circ}\text{C min}^{-1}$  over the temperature range of 0 to 500 $^{\circ}\text{C}$ . (Babu et al., 2006).

**X-ray diffraction (XRD) studies:** XRD patterns were traced for the 5-fluorouracil employing X-ray diffractometer (Philips PW 1729, Analytical XRD, Holland) using filtered  $\text{CuK}(\alpha)$  radiation (intensity ratio ( $\alpha_1/\alpha_2$ ): 0.500), a voltage of 40 KV, a current of 30 mA and receiving slit of 0.2 inches. The samples were analyzed over  $2\theta$  range of 5.010 to 39.990 with scanning step size of 0.020 ( $2\theta$ ) and scan step time of 1 s (Denizli et al., 1988).

#### Preparation of drug-loaded nanoparticles

Drug-loaded nanoparticles were prepared using emulsion droplet coalescence method (Conti et al., 1998). Chitosan (CS) was dissolved in 1% acetic acid and 50 mg of 5-fluorouracil in phosphate buffer saline. This solution was added to 10 ml of liquid paraffin containing 0.5% v/v Tween 20. This mixture was stirred using a homogenizer for 3 min to form water in oil (w/o) emulsion. Similarly, another w/o emulsion consisting of 1% Eudragit RS 100 in 3 M sodium hydroxide solution was prepared. Then, these two emulsions were stirred using homogenizer. As a result of coalescence of droplets, chitosan was solidified to produce nanoparticles. Eudragit RS 100 produces secondary coating over chitosan nanoparticles. The obtained 5-fluorouracil drug loaded nanoparticles were centrifuged at 3000 rpm for 60 min using Remi centrifuge and washed using ethanol and water, repeatedly to remove the remaining surfactant and liquid paraffin. Later, they were dried in air for 3 h and kept in hot air oven at 50 $^{\circ}\text{C}$  for 4 h and stored in a desiccator.

#### Transmission electron microscope (TEM)

The particle shape and morphology of the prepared 5-fluorouracil nanoparticles were determined by TEM analysis. The nanoparticles

were viewed using Philips TEM model CM200 as shown in Figure 13a, b, c, d, e and f for morphological examination.

The sample can be mounted on carbon/formvar coated copper grid or can be made of disc type with a thinned (electron transparency) central area of size 3 mm. Operating voltages are 20 to 200 kv and the resolution was 2.4 Å (Carlos, 2005).

### Zeta potential measurement of the nanoparticles

Zeta potential of the nanoparticles was determined by laser Doppler anemometry using a Malvern Zetasizer also called Doppler electro-phoretic light scatter analyzer. It is used to measure velocities and thereby zeta potential of colloid particles (Sanyogitta, 2007).

### Percentage yield

The nanoparticles were prepared by emulsion droplet coalescence method from the study of [16] and percentage yield was calculated by dividing the weight of obtained nanoparticles by the weight of calculated ingredients of nanoparticles and expressed in terms of percentage.

$$\text{Percentage yield} = \frac{\text{Practical yield of Nanoparticles}}{\text{Total Theoretical yield of Nanoparticles}} \times 100$$

### Loading efficiency

The loading efficiency was determined by centrifuging the drug-loaded nanoparticles at 5000 rpm for 30 min and separate the supernatant, and collected particles were washed with water and then subjected to another cycle of centrifugation. The amount of free 5-fluorouracil in the supernatant was assayed by UV spectrophotometer (UV-1700 Lab india) at 266 nm. The loading efficiency was calculated by using the formula (Leroux et al., 1995; Ganta et al., 2009):

$$\text{Loading efficiency} = \frac{\text{Total amount of 5 FU Nanoparticles} - \text{Free 5 FU Nanoparticles}}{\text{Total amount of 5 FU Nanoparticles}} \times 100$$

### Entrapment efficiency

The entrapment efficiency was determined using the following formula:

$$\text{Entrapment efficiency} = \frac{\text{Total amount of drug} - \text{Amount of free drug}}{\text{Total amount of drug added}} \times 100$$

### Zeta potential and polydispersity

Zeta potential and particle size are two important characteristics of nanoparticles that were determined by Zetasizer 3000 analyser system (Malvern instrument, UK) (24-25)

### In vitro drug release study

The *in vitro* drug release profile of 5-FU-Ch-NP was determined using dialysis membrane bag. 5-FU-Ch-NP (20 mg) was placed in to dialysis bag (with a molecular cut-off of 5 kDa). 5-FU-Ch-NP loaded dialysis bag was incubated in 70 ml phosphate buffer (pH 7.4). The system was maintained at 37±0.5°C with mild magnetic stirring. At appropriate time interval, 4 ml of the release media was

taken and equivalent volume of fresh phosphate buffered saline (PBS) solution was supplemented in order to keep the volume of the system identical. The sample was assayed at 266 nm by UV-Spectrophotometer (Lab India) and the cumulative percentage of drug release was calculated (Wan et al., 2009)

### In vitro release kinetics study

In order to analyze the drug release mechanism, *in vitro* release data were fitted into a zero-order, first order, Higuchi, and Korsmeyer-peppas model.

#### Zero order kinetics

The zero order rate equation describes the systems where the drug release rate is independent of its concentration.

$$Q_1 = Q_0 + K_0 t$$

#### First order kinetics

The first order equation describes the release from a system where the release rate is concentration dependent. Kinetic equation for the first order release is as follows:

$$\text{Log } Q_t = \text{log } Q_0 + K_1 t/2.303$$

#### Higuchi model

Higuchi describes drug release as a diffusion process based in the Fick's law, square root time dependent.

$$Q_t = K_H t^{1/2}$$

#### Korsmeyer–Peppas model

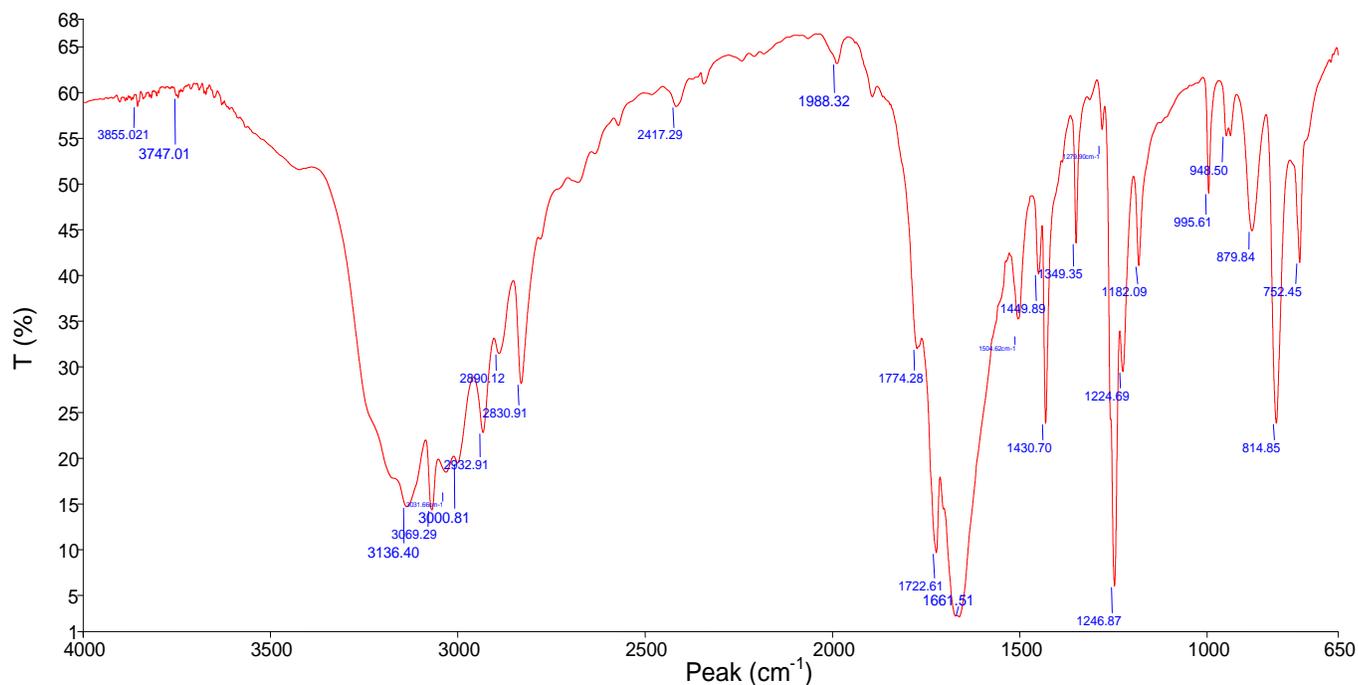
To find out the drug release mechanism, first 60% drug release data can be fitted in Korsmeyer–Peppas model which is often used to describe the drug release behavior from polymeric systems when the mechanism is not well-known or when more than one type of release phenomena is involved.

$$\text{Log } (M_t / M_\infty) = \text{Log } K_{KP} + n \text{ Log } t$$

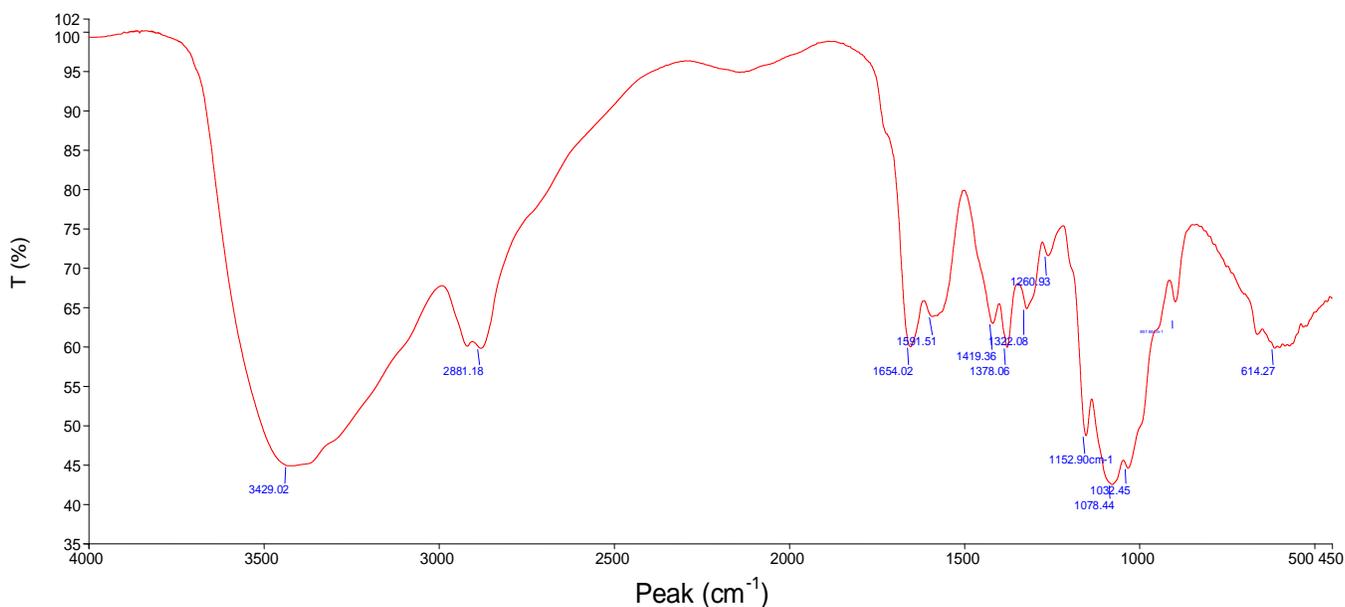
## RESULTS AND DISCUSSION

The identification of drug was studied by FTIR, DSC and XRD analysis. The FTIR spectra for pure drug 5-fluorouracil are as shown in Figure 3 and the peaks observed from the graph is evident that the absorption bands at 1661.51, 1449.89, 3136.40, 1430.70 and 1246.87 cm<sup>-1</sup> indicate the presence of C=O, C=C, N-H, C-F and C-N stretching vibrations corresponding to 5-fluorouracil, the peak at 1349.35 cm<sup>-1</sup> refers to vibration of pyrimidine compound confirming 5-fluorouracil.

Drug-polymer compatibility studies were performed by using FTIR and DSC techniques. The FTIR spectra for 5-fluorouracil and mixture of 5-fluorouracil with different polymers are as shown in Figures 4, 5, 6 and 7, respectively and the characteristic peaks observed all the characteristic absorption bands of 5-fluorouracil were



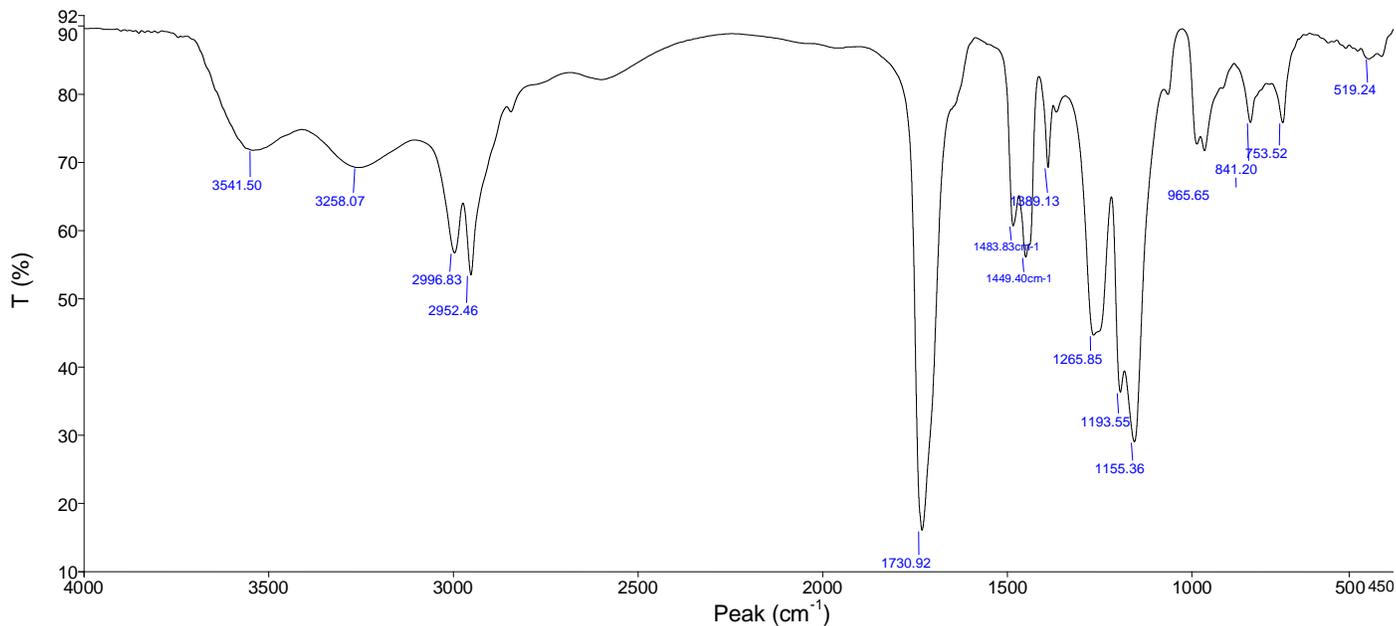
**Figure 3.** FTIR spectra of 5-FU.



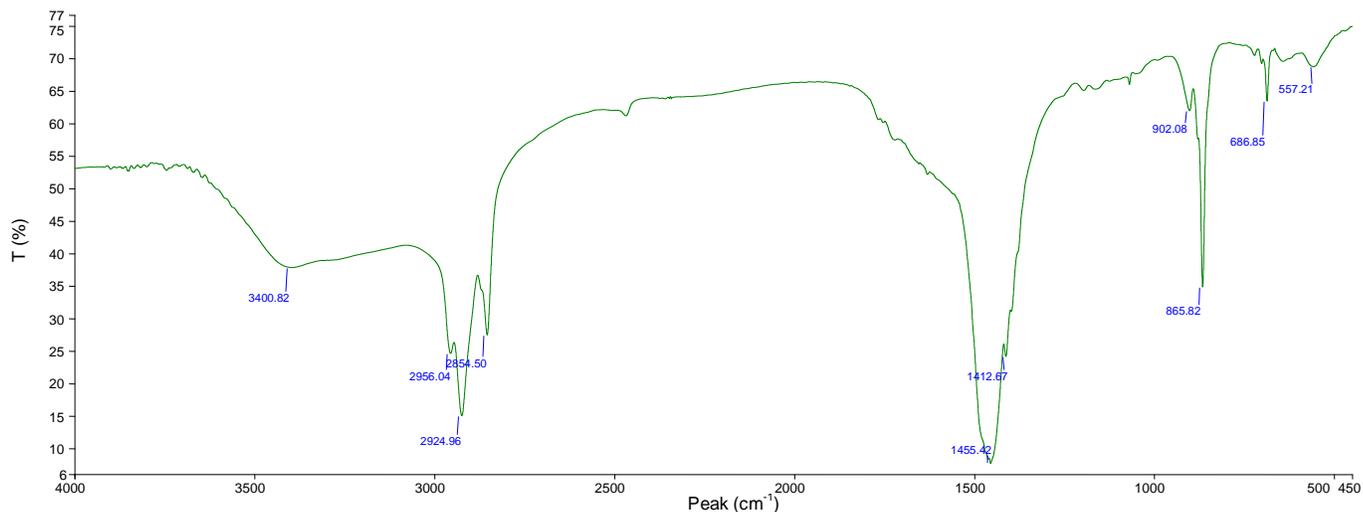
**Figure 4.** FTIR spectra of Chitosan.

diminished significantly in the finger print region of drug, which revealed that the encapsulated drug inside the polymeric core existed in an amorphous state and appearance of peaks at 2952.46 and 1412.67  $\text{cm}^{-1}$  were corresponding to the presence of Eudragit RS 100 and chitosan, respectively. All the characteristic absorption

bands of 5-fluorouracil were diminished significantly in the finger print region of drug, which revealed that the encapsulated drug inside the polymeric core existed in an amorphous state and appearance of peaks at 2952.46 and 1412.67  $\text{cm}^{-1}$  were corresponding to the presence of Eudragit RS 100 and chitosan, respectively.



**Figure 5.** FTIR spectra of Eudragit RS 100.



**Figure 7.** FTIR spectra for prepared nanoparticle formulation.

DSC thermogram of 5-fluorouracil is as shown in Figure 8. This reveals that the drug shows good thermal stability up to its melting point. The onset melting peak is about 273.47°C. It suggests that the drug is stable up to 280.13°C and undergoes degradation above that temperature.

DSC thermograms for chitosan and Eudragit RS 100 are as shown in Figures 9 and 10 and it was found that the onset of melting peak is 294.8°C and it is stable up to 321°C. The other peak observed may be due to presence

of moisture. The thermogram of Eudragit RS 100 onset melting peak was observed at 376.08°C and it shows good thermal stability up to 389.6°C. The other peak observed at 412.6°C may be due to the presence of moisture. DSC thermogram for physical mixture is as shown in Figure 11 from which it is evident that the onset melting temperature of drug was observed at 281.6°C indicating the compatibility of 5-fluorouracil with chitosan and Eudragit RS 100.

X-ray diffraction pattern of 5-fluorouracil is as shown in

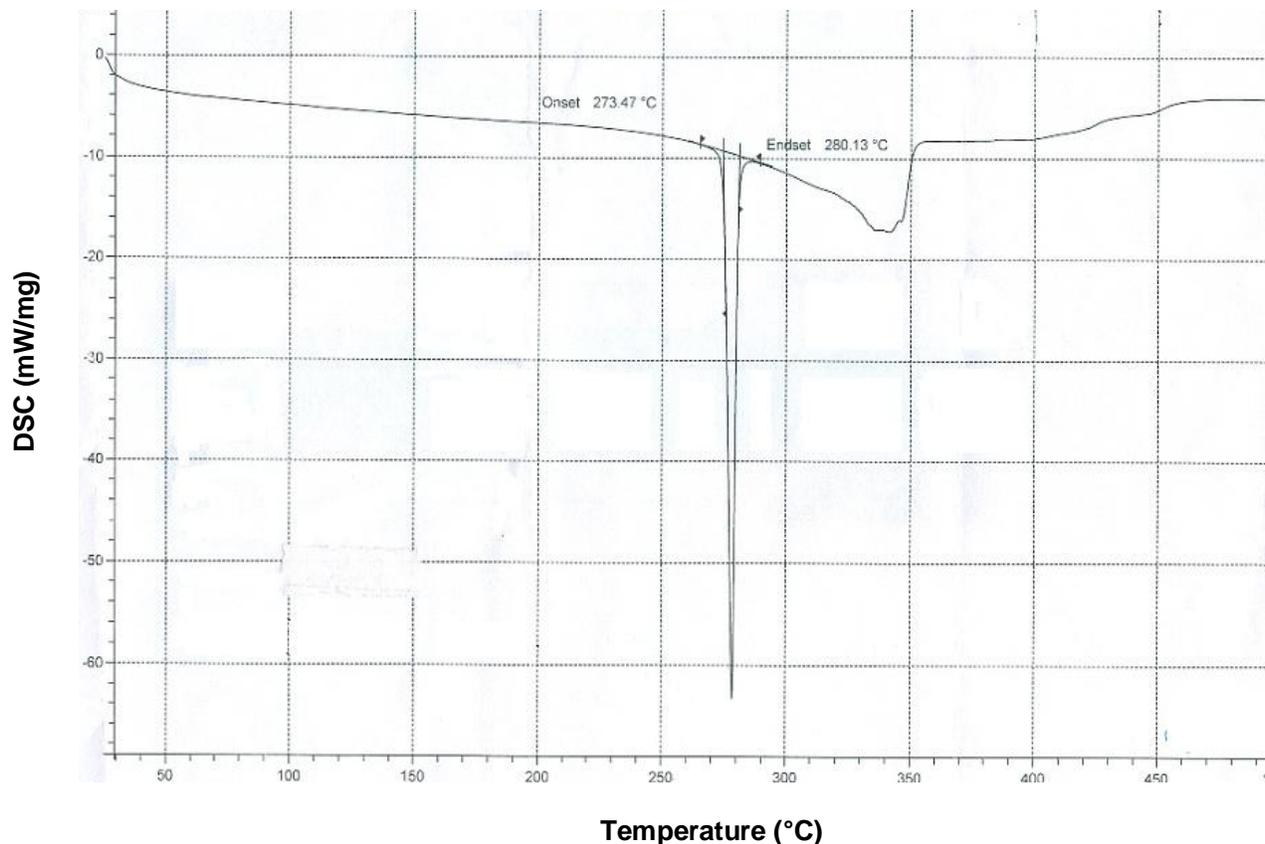


Figure 8. DSC thermograph of 5-fluorouracil (5-FU).

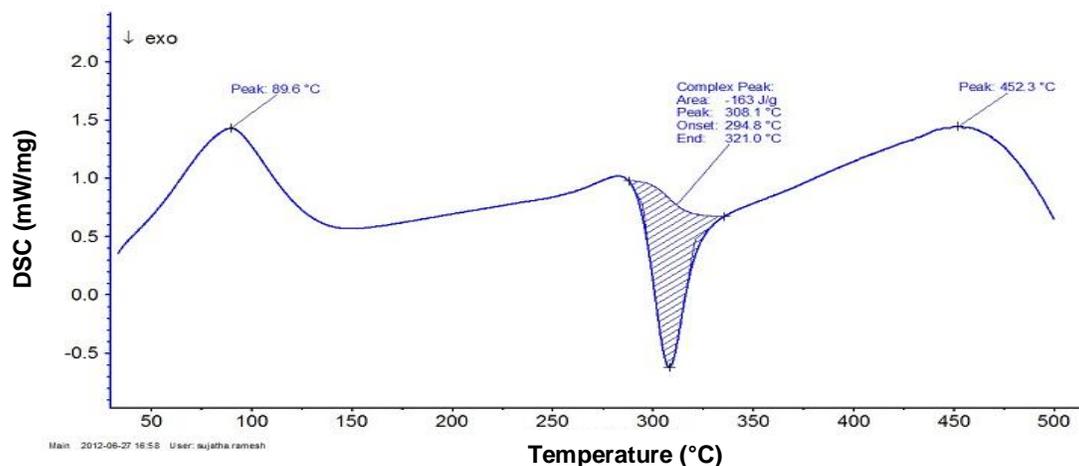


Figure 9. DSC thermograph of chitosan.

Figure 12. Sharp peak between 28.385 and 28.525° was the characteristic of 5-fluorouracil. The most intensive peaks of 5-fluorouracil were observed at 2 of 17, 29, and 32° suggesting the crystalline nature of drug.

5-Fluorouracil loaded polymeric nanoparticles were successfully prepared by emulsion droplet coalescence method using biodegradable polymers chitosan and

Eudragit RS 100. All the prepared drug loaded nanoparticles are in white and powdery appearance. The prepared nanoparticles were studied for various evaluation parameters.

Particle size and morphological characteristics for some of the prepared nanoparticles were studied by TEM analysis using Philips TEM model CM200. The

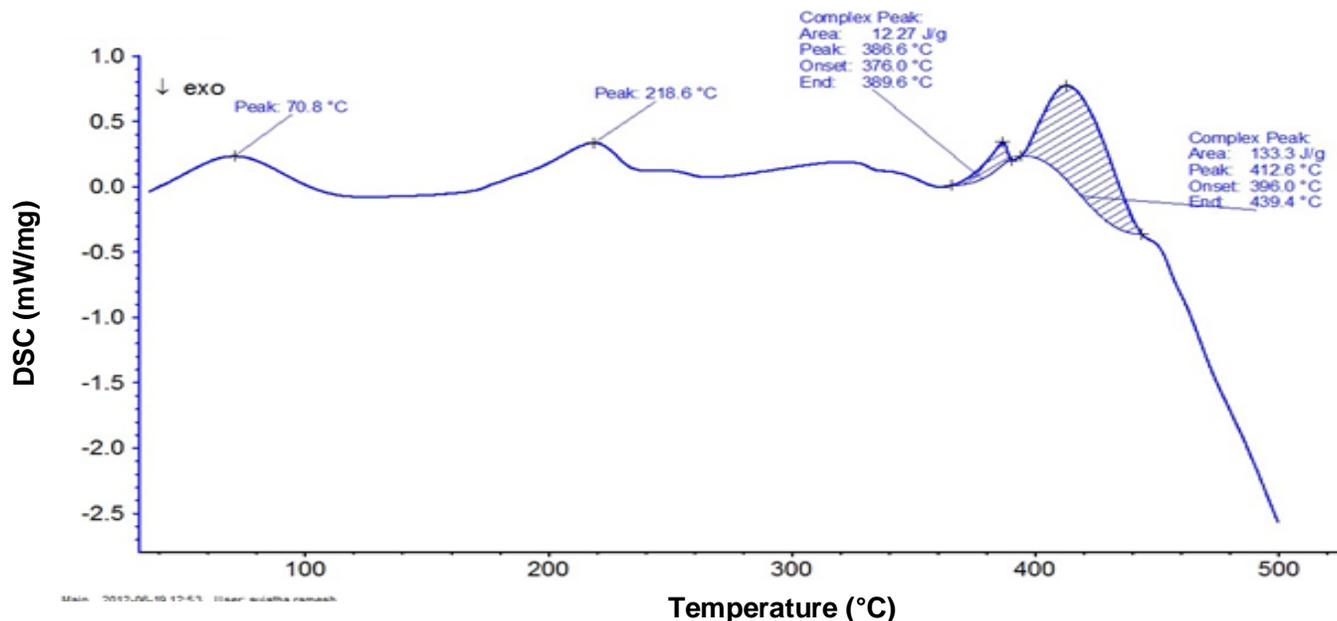


Figure 10. DSC thermogram of Eudragit RS 100.

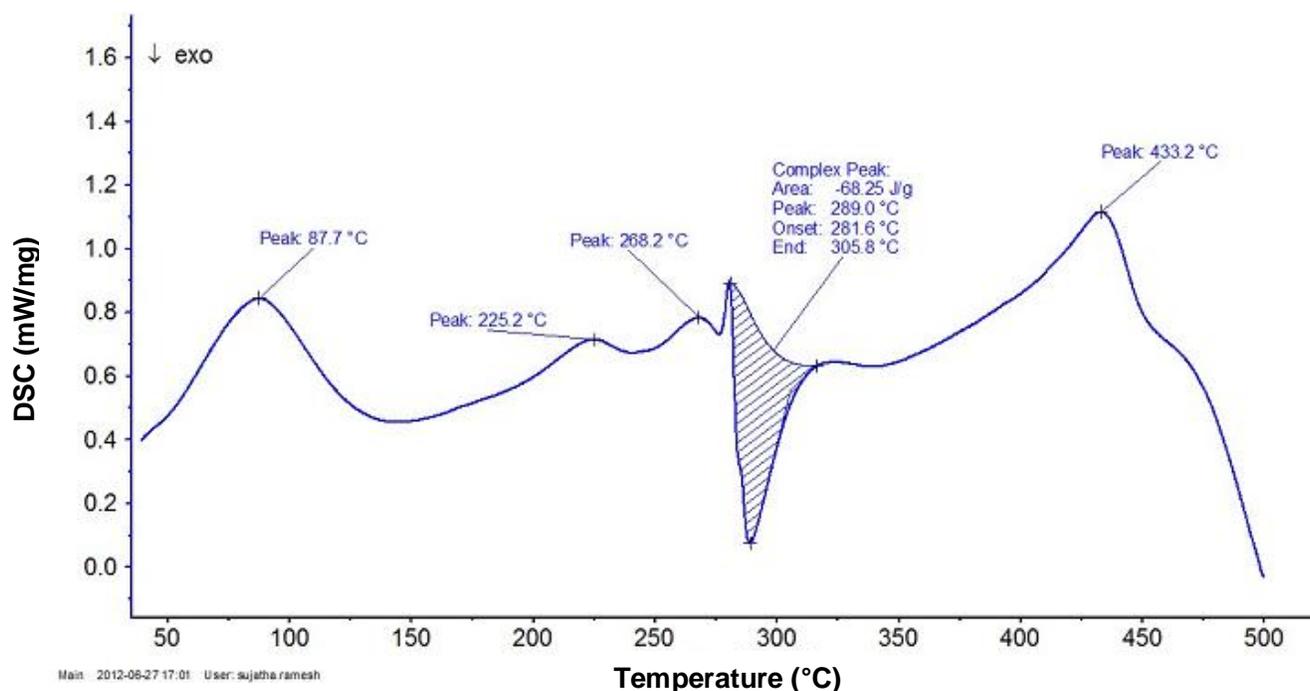


Figure 11. DSC thermogram for physical mixture 5-Fluorouracil+Chitosan+Eudragit RS 100.

corresponding images are as shown in Figure 13. They reveal that the particles of all formulations are in submicron sizes of peculiar shapes having rough or irregular surfaces; some aggregates are also observed which can be attributed to the gelling property of chitosan leading to particle-particle aggregation.

Percentage yield or particle recovery of prepared nanoparticles was calculated and the resulted values are summarized in the Table 1. The percentage yield of prepared nanoparticles was in the range of 64.76 to 87.14%. The variations in the percentage yield may be attributed to process parameters.

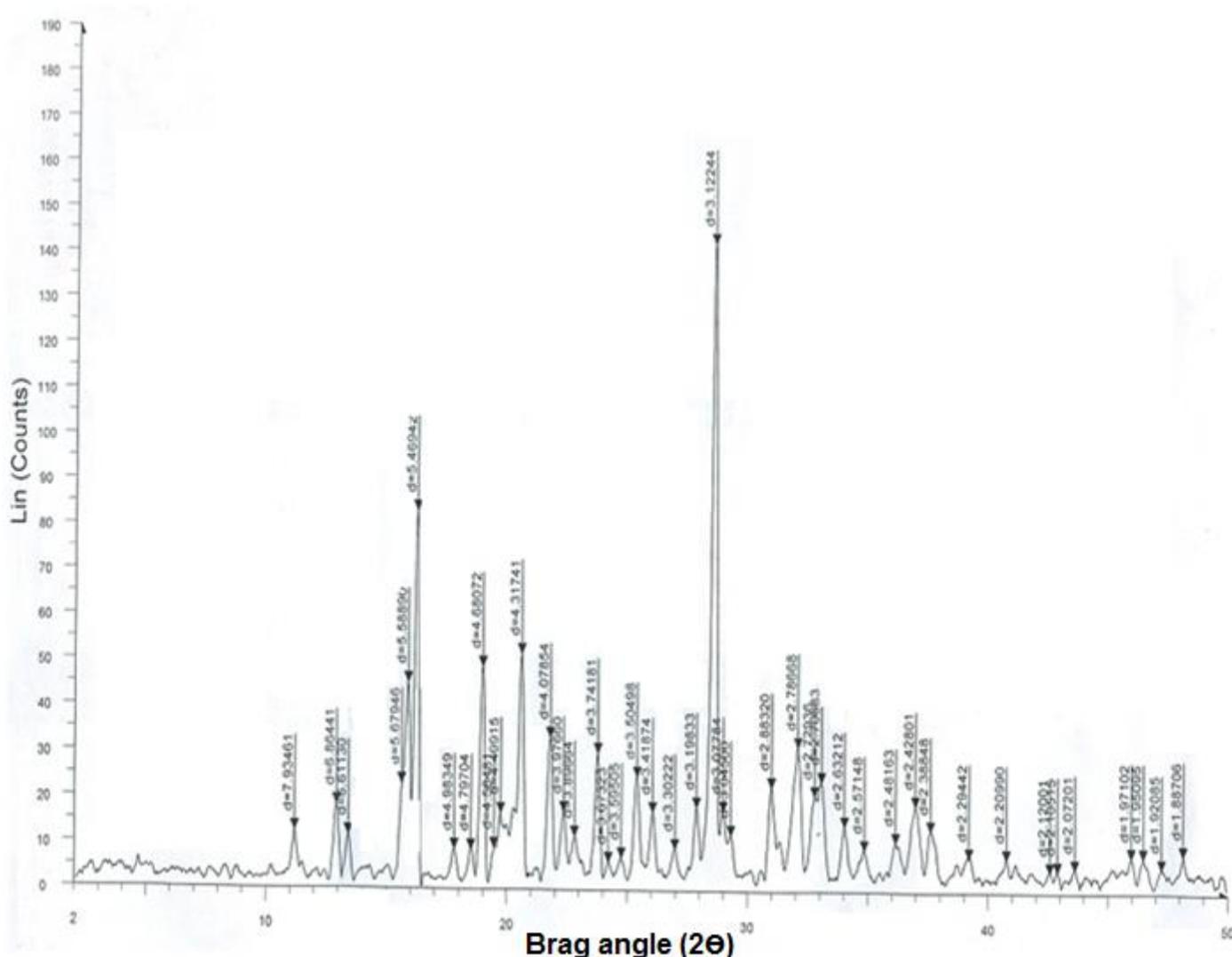


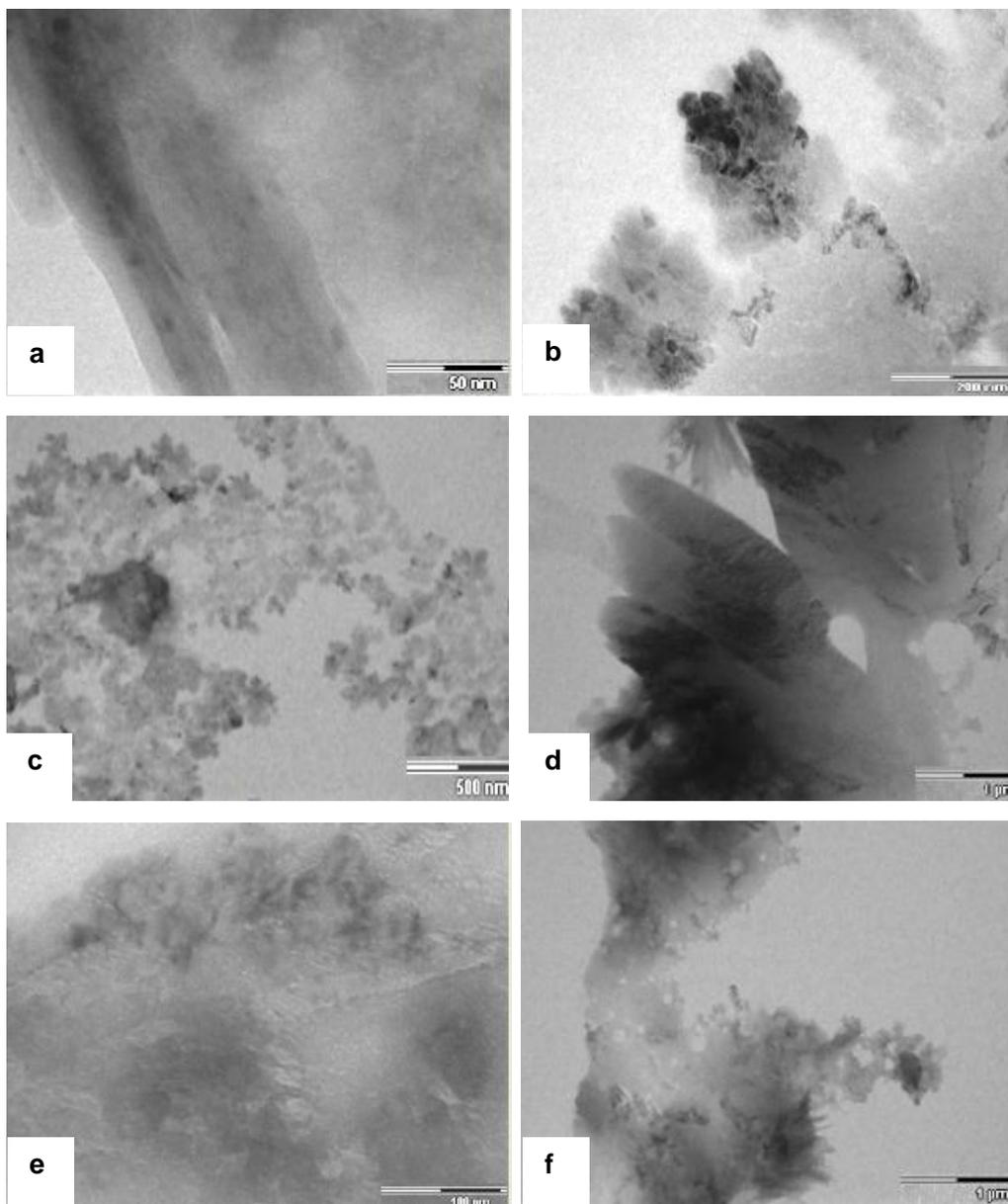
Figure 12. Schematic representation of XRD of 5-FU.

Drug loading and entrapment efficiency of each formulation was calculated and the results were summarized in the Table 2. From the Table 2, it was found that the loading efficiency of CF1 to CF6 formulations was in range of 13.17 to 19.84% and for EF1 to EF5 formulations it was found between 13.55 and 20.16%. Subsequently, the entrapment efficiency of CF1 to CF6 was found to be in range of 65.5 to 75.6% and for EF1 to EF5 formulations it was found between 64.6 and 81.6%. From these results, it was evident that the drug loading efficiency of formulation increases with the increased chitosan and Eudragit RS 100 concentration.

Average zeta potential, average diameter and polydispersity index (PDI) values of the analysed formulations were summarized in Table 2 from which it was observed that the zeta potential values were in range of  $\pm 2.46$  to  $\pm 48.4$  indicating the increasing colloidal stability of

nanoparticles with increased polymer concentration. The positive zeta potential values are representative to quaternary ammonium group present in Eudragit RS 100. The PDI values were within the range of 0.14 to 0.687. This indicates that the particles are in uniform size distribution. From the Table 2, it was observed that as polymer concentration increases, the particle size also increased. The size distribution of nanoparticles was found to be in desired range, that is, between 21 to 778.2 nm. The zeta potential and size distribution for optimized formulations are given in the Figure 14a,b and Figure 15a,b respectively.

The *in vitro* drug release study was performed by diffusion method for a period of 12 h using modified permeation apparatus. The percent cumulative drug released for each formulation was recorded and given in Table 3. The graphical representations of time versus



**Figure 13.** (a) TEM image of CF1, (b) TEM image of CF3, (c) TEM image of CF4, (d) TEM image of CF6, (e) TEM image of EF3, and (f) TEM image of EF5.

percent cumulative drug release (CDR) plots for formulations CF1 to CF6 are as shown in Figure 16, and for EF1 to EF5 in Figure 17. From the graphs, it has been shown that percent cumulative drug release of CF1 to CF6 formulations was in range of 68.28 to 80.58% and for EF1 to EF5 formulations between 61.82 and 80.94%.

The drug release study shows that the drug release from the prepared nanoparticles was found to be more controlled as the polymer concentration increases. It is evident that from the results, the drug entrapment efficiency and the particle size have a direct effect on the drug release profile of formulations. From the results, EF5 formulation having high entrapment efficiency

(81.6%) and particle size of 778.2 nm was found to have more controlled drug release as it is having high concentration of Eudragit RS 100 as secondary coating. The percent cumulative drug released for EF5 after 12 h was found to be 61.82%.

The mechanism of 5-fluorouracil release and the kinetic order of drug release from the nanoparticles were studied by fitting the *in vitro* drug release data of formulations into different kinetic models: zero order, first order, Higuchi and Korsmeyer-Peppas models. As shown in Table 4, it is evident that  $R^2$  values for zero order plots of all formulations (CF1 to EF5) were ranging from 0.939 to 0.991; for first order plots, 0.805 to 0.953; for Higuchi

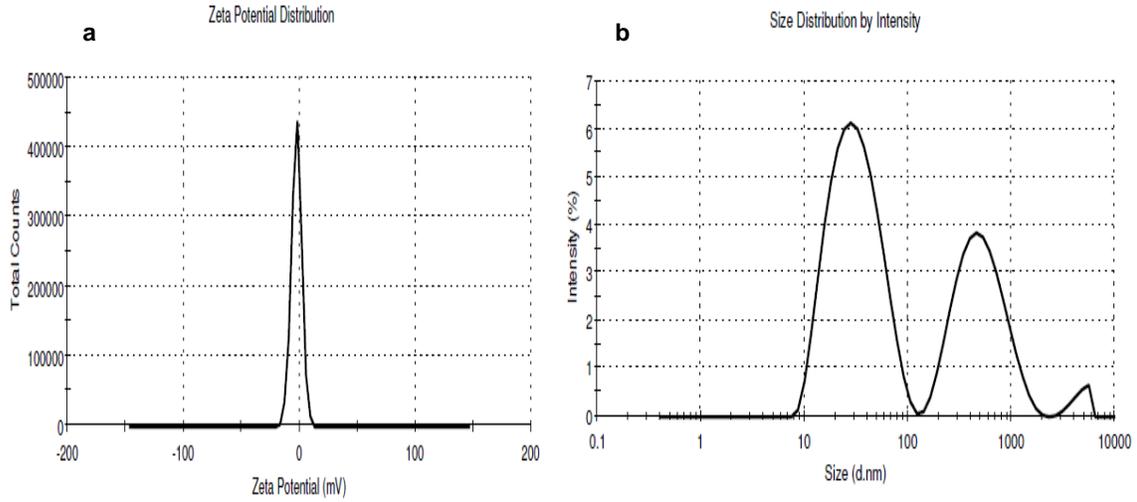


Figure 14. (a) Zeta potential of 5-FU nanoparticles (CF1) and (b) size distribution of 5-FU nanoparticles (CF1).

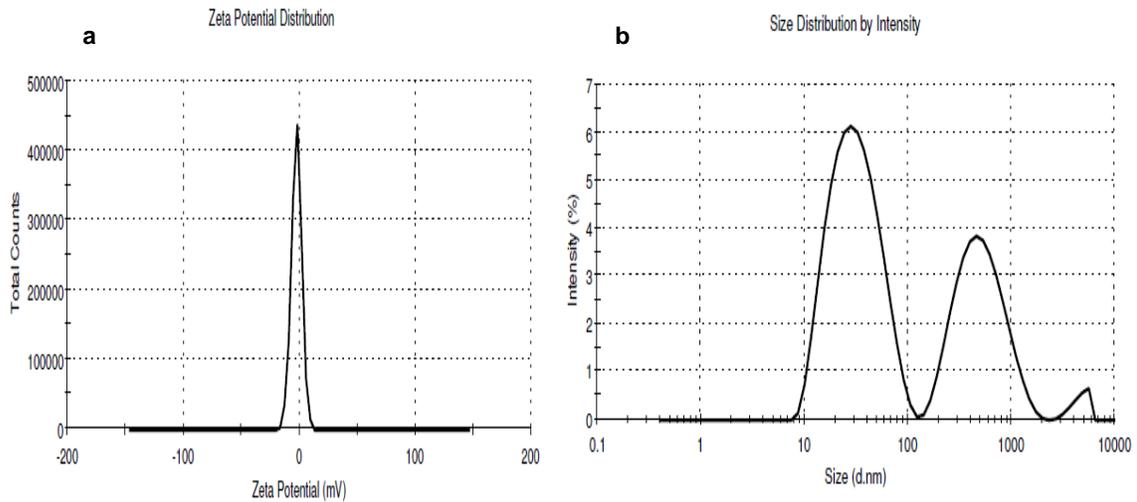


Figure 14. (a) Zeta potential of 5-FU nanoparticles (CF1) and (b) size distribution of 5-FU nanoparticles (CF1).

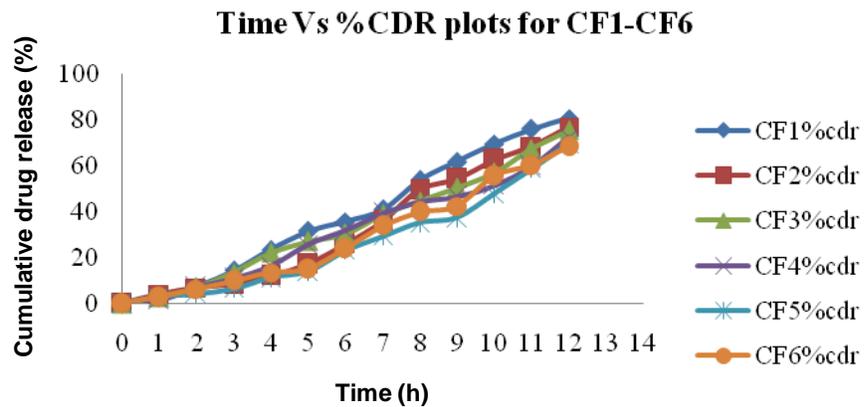


Figure 16. *In vitro* drug release of 5-FU nanoparticles of CF1-CF2 formulation.

### Time Vs % CDR plots for EF1-EF5

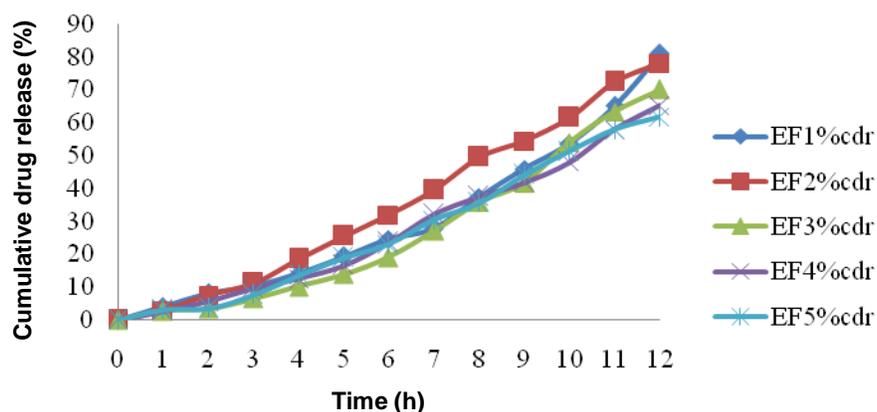


Figure 17. *In vitro* drug release of 5-FU nanoparticles of EF1-EF5 formulations.

Table 1. Formulation of 5-fluorouracil loaded nanoparticles.

S/N	Formulation code	Drug (mg)	Chitosan (% w/v)	Eudragit RS 100 (% w/v)	Tween20 (% v/v)
1	CF1	50	0.25	1.0	0.5
2	CF2	50	0.5	1.0	0.5
3	CF3	50	1.0	1.0	0.5
4	CF4	50	1.5	1.0	0.5
5	CF5	50	2.0	1.0	0.5
6	CF6	50	2.5	1.0	0.5
7	EF1	50	1.0	0.25	0.5
8	EF2	50	1.0	0.5	0.5
9	EF3	50	1.0	1.5	0.5
10	EF4	50	1.0	2.0	0.5
11	EF5	50	1.0	2.5	0.5

Table 2. Evaluation parameters of 5-fluorouracil nanoparticles.

Parameter	CF1	CF2	CF3	CF4	CF5	CF6	EF1	EF2	EF3	EF4	EF5
Practical yield	73.3	84	73.3	87.14	78.75	68.8	80.0	77.7	77.3	66.6	64.76
Unbound/Free drug (mg/50 mg)	17.25	10.8	7.51	9.5	8.5	7.2	19.75	17.7	9.8	6.8	3.9
Entrapment efficiency (%)	65.5	70.2	70.4	71.4	73.0	75.6	64.6	70.2	72.0	79.0	81.6
Loading efficiency	19.84	18.60	19.3	13.27	13.17	13.80	20.16	18.45	13.86	14.5	13.55
Average zeta potential (mv)	-2.46	6.22	-24.8	32.3	36.32	44.6	2.46	26.4	34.2	38.3	48.4
Polydispersity index (PDI)	0.67	0.32	0.22	0.47	0.31	0.56	0.14	0.22	0.37	0.44	0.68
Average particle diameter (nm)	43.33	50	100	200	300	500	21	50	100	200	778.2

plots, 0.766 to 0.904; and for Peppas equation, 0.931 to 0.998. The diffusion exponent values of Peppas plots were in range of 0.435 to 0.705. This data reveals that drug release from nanoparticles follows zero order release kinetics with non-Fickian diffusion for all formulations except CF2 and EF3 formulations which are

following zero order release with Fickian diffusion.

### Conclusion

The emulsion droplet coalescence method procedure

**Table 3.** *In vitro* diffusion studies of 5-fluorouracil drug loaded nanoparticles.

Time (h)	CF1	CF2	CF3	CF4	CF5	CF6	EF1	EF2	EF3	EF4	EF5
0	0	0	0	0	0	0	0	0	0	0	0
1	2.68	4.087	2.76	1.44	2.93	2.76	3.92	2.93	2.76	2.72	3.09
2	7.33	6.67	7.169	7.13	4.12	6.16	8.14	7.44	3.37	5.59	3.48
3	14.19	8.31	13.39	11.04	6.52	9.72	10.35	11.19	6.48	9.69	7.66
4	23.30	12.05	21.99	16.29	11.27	13.52	14.03	18.52	10.13	12.47	13.51
5	31.56	17.21	26.91	25.75	13.91	14.97	19.31	25.43	13.98	16.53	18.99
6	35.63	26.06	30.33	31.77	23.18	23.89	24.59	32.01	19.05	23.49	23.01
7	40.79	35.88	38.65	39.29	29.29	33.72	27.91	39.57	27.06	32.17	30.40
8	53.79	49.40	44.44	44.34	35.16	39.88	37.35	49.54	35.80	37.53	35.57
9	61.63	54.318	50.30	46.41	37.28	42.44	45.94	54.44	41.68	41.76	44.10
10	69.51	62.64	57.13	51.43	47.93	55.51	33.69	61.65	54.08	48.07	51.20
11	75.79	68.38	67.19	59.84	58.72	59.98	65.10	72.34	63.35	57.98	57.83
12	80.59	76.77	75.33	72.34	69.50	68.28	80.94	78.11	70.16	65.32	61.82

**Table 4.** *In vitro* diffusion studies of 5-fluorouracil drug loaded nanoparticles in various parameters derived from kinetic modeling of *in vitro* drug release studies

Formulation code	Zero order		First order		Higuchi		Kersmeyer-peppas		Release mechanism
	Slope	R <sup>2</sup>	Slope	R <sup>2</sup>	Slope	R <sup>2</sup>	N	R <sup>2</sup>	
CF1	7.199	0.991	0.063	0.949	26.21	0.881	0.474	0.993	Non-Fickian transport
CF2	6.799	0.962	0.055	0.922	24.05	0.861	0.439	0.953	Fickian transport
CF3	6.271	0.991	0.049	0.929	22.78	0.876	0.705	0.994	Non-Fickian transport
CF4	5.928	0.985	0.044	0.932	21.57	0.874	0.574	0.978	Non-Fickian transport
CF5	5.641	0.948	0.041	0.877	19.83	0.785	0.495	0.965	Non-Fickian transport
CF6	5.839	0.968	0.042	0.925	20.77	0.821	0.598	0.986	Non-Fickian transport
EF1	6.240	0.939	0.052	0.805	21.97	0.780	0.703	0.972	Non-Fickian transport
EF2	6.805	0.988	0.056	0.933	24.53	0.861	0.456	0.968	Non-Fickian transport
EF3	6.026	0.941	0.045	0.886	21.01	0.766	0.435	0.957	Fickian transport
EF4	5.495	0.975	0.038	0.931	19.60	0.831	0.585	0.991	Non-Fickian transport

was used to prepare 5-fluorouracil drug loaded nanoparticles of reproducible sizes in the range of 21 to 778 nm by addressing the effect of processing parameters. The results of the *in vitro* release profiles of the entrapped anticancer drug from chitosan-Eudragit RS 100 nanoparticles demonstrated that drug was release by a initially burst release for some formulations and combination of diffusion and slow gradual erosion of the particles. Thus, chitosan-Eudragit RS 100 containing 5-fluorouracil drug loaded nanoparticles offer an excellent potential for the control release of anticancer drugs.

Other parameters such as anticancer activity through triggering tumor cells apoptosis, histopathological studies and its pharmacokinetic parameters are under investigation.

## REFERENCES

- Arias JL, Ruiz MA, Lopez-viota M, Delgado AV (2008). Poly (alkylcyanoacrylate) colloidal particles as vehicle for antitumor drug delivery: A comparative study. *Colloids Surf. Biointerfaces* 62:64-70.
- Aslam M, Fu L, Su M, Vijayamohan K, Dravid PV (2004). Novel onestep synthesis of amine-stabilized aqueous colloidal gold nanoparticles. *J. Mater. Chem.* 14:1795-1797.
- Babu VR, Sairam M, Hosamani KM, Aminabhavi TM (2006). Development of 5-fluorouracil loaded poly (a crylamide-co-methylmethacrylate) novel core-shell microspheres: *In vitro* release studies. *Int. J. Pharm.* 325:55-62.
- Carlos EA (2005). Synthesis of poly (dl-lactide-co-glycolide) nanoparticles with entrapped magnetite. 1-85. [http://etd.lsu.edu/docs/available/etd-11152005-124938/unrestricted/Astete\\_R\\_thesis.pdf](http://etd.lsu.edu/docs/available/etd-11152005-124938/unrestricted/Astete_R_thesis.pdf).
- Conti B, Monden T, Genta I, Perugini P, Decarro C, Pavanetto F (1998). Microencapsulation of cetylpyridinium chloride with a bioadhesive polymer. *Prroc. Intl. Symp.control. Release Bioactive Material.* 25:822-823.
- Dang JM, Leong KW (2006). Natural polymers for gene delivery and tissue engineering. *Adv. Drug Del. Rev.* 58:487-499.
- Denizli A, Kiremitçi M, Piskin E (1988). Subcutaneous polymeric matrix system poly (HEMA-BGA) for controlled release of an anticancer drug (5-fluorouracil): I. Synthesis and structure, *Biomaterials* 9:257-262.
- Elvire FE, Passirraani C, Colin N, Breton P, Sagodira S, Benoitte JP

- (2004). Development of novel 5FU-loaded Poly(methylidene malonate microspheres for the treatment of brain cancer, *Eur. J. Pharm. Biopharm.* 57:189-197.
- Ganta S, Paxton JW, Baguley B, Grag S (2009). Formulation and pharmacokinetic evaluation of an asulacrine nanocrystalline suspension for intravenous delivery. *Int. J. Pharm.* 367:179-186.
- Leroux JC, Allemann E, Doelker E, Gurnay R (1995). New approach for the preparation of nanoparticles by emulsification-diffusion method, *Eur. J. Pharm. Biopharm.* 41:14-21.
- Li S, Wang A, Jiang W, Guan Z (2008). Pharmacokinetic characteristics and anticancer effects of 5-Fluorouracil loaded nanoparticles. *BMC cancer* 8:103.
- Onishi H, Oosegi T, Machida Y, McGinity JW (2005). Preparation and in-vitro evaluation of chitosan microspheres containing prednisolone: comparison of simple and conjugate microspheres. *Drug Dev. Ind. Pharm.* 31:597-605.
- Sanyogitta P (2007). Novel functionalized polymers for nanoparticle formulations with anti cancer drugs. 1-200. [http://etheses.nottingham.ac.uk/316/1/Thesis\\_end\\_note-final.pdf](http://etheses.nottingham.ac.uk/316/1/Thesis_end_note-final.pdf)
- Sharma YR (2011). *Elementary Organic Chemistry*. edition.4. S. Chand publication. pp. 68-151.
- Simeonova M, Velichkova R, Ivanova G, Enchey V, Abrahams I (2003). Poly(butylcyanoacrylate) nanoparticles for topical delivery of 5-Fluorouracil. *Int. J. Pharm.* 263:133-140.
- Thanou M, Verhoef JC, Junginger HE (2001). Oral drug absorption enhancement by chitosan and its derivatives. *Adv. Drug Del. Rev.* 52:117-126.
- Wan AJ, Sun Y, Gao LP, Li HL (2009). Preparation of aspirin a aspirin and probucal in combination loaded chitosan nanoparticles and in-vitro release study. *Carbohydr. polymer* 75:566-574.
- Yashwant P, Deepak T (2009). Drug delivery nanoparticles formulation and characterization. *Drugs and Pharmaceutical Science Series, informal health care USA.* 191: 1-30.
- Zhang N, Yin Y, Xu SJ, Chen WS (2008). 5-Fluorouracil: mechanism of resistance and reversal strategies. *Molecules.* 13:1551-1569.

Full Length Research Paper

## ***In vitro* anthelmintic potential and phytochemical composition of ethanolic and aqueous crude extracts of *Zanthoxylum chalybeum* Engl.**

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Development of helminthes resistance to chemotherapeutic drugs coupled with high costs and misuse of conventional drugs call for the search of medicinal plants as alternative source of helminthes control. *In vitro* studies were conducted to determine the anthelmintic activity and phytochemical compounds of *Zanthoxylum chalybeum*. Bioassay was conducted using 70% ethanol and aqueous extracts' in serial dilutions parallel to serial dilutions of albendazole in three replicates. *Ascaris suum* model was used for the assays. Phytochemical methods and thin layer chromatography (TLC) were used for qualitative phytochemical analysis. The aqueous and ethanolic extracts of the *Z. chalybeum* inhibited 100 and 93% worm motility, respectively for 48 h post exposure. There was significant differences in motility inhibition by all dose levels' that were dose-dependent when compared with negative control ( $F_{(5, 53)} = 10.62$ ,  $P = 0.001$ ;  $R^2 = 0.92$ ). There was a significant difference in mean motility inhibition by the different methods of extraction ( $F_{(2, 53)} = 323.80$ ,  $P = 0.001$ ). The  $ED_{50}$  of ethanolic and aqueous extracts were 30.85 and 6.28 mg/ml, respectively. The potency of the plants extracts and albendazole significantly differed ( $P = 0.001$ ). The extracts contained tannins, saponins, flavonoids, alkaloids, reducing sugars, coumarins derivatives, anthracenones, and anthocyanins. TLC characterization indicated the presence of polyphenols and saponinins. In conclusion, *Z. chalybeum* root extracts showed anthelmintic potential and the phytochemical compounds present justify the plant's ethno-veterinary use and could be used as improved traditional medicines.

**Key words:** *Ascaris suum*, medicinal plants, motility inhibition, Nakasongola.

### INTRODUCTION

Helminthes infections remain a big challenge both in developed and developing countries despite being the most neglected among the healthcare systems. This is attributed to their chronic debilitating nature and the epidemiological characteristic of continuous contamination of the environment. In developing countries, the

disease may be attributed to lack of resources to regularly de-worm affected individuals in addition to development of parasite resistance to conventional drugs resulting from poor use of drugs. Moreover, parasites infections are like to increase in the face of climate change (Weaver et al., 2010; Tinsley et al., 2011). It is

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well documented that parasites undergo evolution to adapt to opportunities presented by climate change or anthelmintic use or undoubtedly as a manifestation of 'survival of the fittest' (Sargison et al., 2007; Davey et al., 2009). The different control strategies including the use of anthelmintics, grazing management and improvements in sanitation, are available for gastrointestinal nematode infections, but these control methods are associated with many problems, such as development of resistance to the currently available chemotherapeutic anthelmintic drugs (Kaplan et al., 2004; Wolstenholme et al., 2004; Melaku et al., 2013). According to Ian et al. (2007), it was concluded that "whether poor or rich livestock farmers, depending on their production systems and market conditions, the value of the animals in question may not warrant the cost of the professional veterinary care and inputs". Consequently, rural communities resort to using medicinal plants to treat symptomatic clinical signs of which they have continued to claim effectiveness. However, efficacies of the claimed potent plants have not been investigated to validate their traditional use as anthelmintics. One of these plants includes *Zanthoxylum chalybeum* Engl. (Nalule et al., 2011) called Chewing stick in West Africa.

*Z. chalybeum* commonly known as knob wood is a deciduous spiny shrub or tree up to 12 m, often growing around termite mounds and in medium to low altitudes in dry woodland or grassland, with mean annual rainfall of 750 to 1500 mm (Dharani et al., 201). *Z. chalybeum* is commonly used by the pastoralists' communities in the semi arid Uganda commonly referred to as "cattle corridor" to treat a multitude of diseases of man and livestock. The root bark is used in treatment of internal parasites in livestock, treatment of odontitis, constipation, toothache and as mouth wash in humans while the leaves are used as beverage (Tran, 2011). *Z. chalybeum* is used to treat esophageal candidiasis and the root bark is powdered and added to tea (Runyoro et al., 2006). Recently, it was reported that in Kenya, the root bark concoction is used to treat malaria (Nguta et al., 2010). The fresh twigs of the plant from East Africa are used as toothbrush, air fresheners and for skin infections (Johns et al., 1990). *In vitro* anti-measles virus activity of the plant seed (Olila et al., 2002), antibacterial and antifungal activities (Olila et al., 2001a) as well as antiviral and trypanocidal (Olila et al., 2001b) have been reported.

In the cattle corridor, the root bark is used to treat internal parasites, while the leaves are used as beverage (Katende et al., 1995). In Tanzania, the fresh leaves of the plant are pounded with leaves of *Acalypha fruticosa* and *Suregada zanzibariensis* and the resulting juice is used to treat skin infections (Hedberg et al., 1983b). The fresh twigs of the plant from East Africa are used as toothbrush, air fresheners and for skin infections (Johns et al., 1990). In Kenya, antimicrobial activity of the root bark against *Bacillus subtilis*, *Penicillium crustosum* and *Saccharomyces cerevisiae* was reported (Taniguchi et al., 1978). *In vitro* anti-measles virus activity of the plant

seed ethanol extract was demonstrated where the alkaloid skimmianine was reportedly responsible (Olila et al., 2002). Antibacterial and antifungal activities have been demonstrated (Olila et al., 2001a) as well as antiviral and trypanocidal (Olila et al., 2001b). Despite several reports on the traditional use of *Z. chalybeum*, little is known about the effectiveness or potential of *Z. chalybeum* as an anthelmintic plant. This study was therefore undertaken to determine the anthelmintic effectiveness of the ethanol and aqueous root bark extracts on gastrointestinal nematodes using *Ascaris suum* model and to determine the *Z. chalybeum* qualitative phytochemical composition.

## MATERIALS AND METHODS

### Collection of plant

The root bark of *Z. chalybeum* was collected from the Ugandan cattle corridor in Nakasongola district based on the study conducted between January and March, 2010. Sample of the plant species used were collected and identified by a plant taxonomist at the Department of Botany Herbarium, Makerere University.

### Dosage adopted by community

The amount of the plant parts used by the community was collected from five individuals. The fresh plant part amounts of each individual were weighed and the weights were recorded. The individual materials were oven dried at 60°C and thereafter re-weighed and the mean recorded. The amount of the water used by the community for extracting active ingredient was considered and this was considered in dosage determination.

### Extraction of crude plant active ingredients and extraction efficiency determination

Two hundred fifty grams (250 g) of dry plants material were macerated in 2000 ml of 70% ethanol for 72 h with intermittent shaking in duplicates. Filtration through cotton wool was done to remove coarse particles (residues) and finely through filter paper (Whatman®, England). The filtrate was concentrated on Rota-vapor type Buchi-R, Switzerland under reduced pressure at 40°C and oven-dried at 50°C. The mean yield of the duplicate samples were determined and recorded and thereafter packed into universal bottles and kept at 4°C till needed for bioassay tests.

Similarly, 250 g of fresh dried materials of the study plants were soaked in 2 L of distilled water with intermittent shaking for 72 h. Thereafter, filtering was done to remove coarse material first with cotton wool and finely with Whatman filter paper (12.5 mm). The filtrate was concentrated under reduced pressure in a rotar evaporator as earlier mentioned. The concentrated filtrates were then evaporated to dryness in an oven at 50°C and yield was recorded. The water extracts were used shortly after drying to avoid spoilage since it was not freeze dried.

### Collection and maintenance of worms

Adult worms were collected from small intestines of pigs obtained from the slaughter house in Kampala, Uganda. The adult worms were collected and transported in flask containing Goodwin's solution to the pharmacology laboratory, in the School of Veterinary

Medicine and Animal Resources, Makerere University. In the laboratory, the active worms were washed in warm water at 37°C and placed in a clean flask where they were maintained in Goodwin's solution according to Lamson and Brown (1936) and Donahue et al. (1981) till the setting of the experiment.

### Preparation of Goodwin's physiological solution

Goodwin's physiological solution was prepared from a number of chemical compounds including calcium chloride (0.20 g), glucose (5.0 g), magnesium chloride (0.10 g), potassium chloride (0.20 g), sodium bicarbonate (0.15 g), sodium chloride (8.0 g) and sodium hydrogen phosphate (0.5 g). All dissolved in 1000 ml of distilled water. Calcium chloride was added later after dissolving others and glucose was added shortly and the resultant solution was pre-warmed to 37°C before putting in the warms.

### Bioassay experimental design

In preliminary experiments, the criteria used for assessing the effects of crude plant extracts on the motility of adult *A. suum* was developed and combined in the procedures described by Kotze et al. (2004), Paolini et al. (2004) and Marie-Magdeleine et al. (2009). Motility inhibition test using *A. suum* was selected due to its suitability for use in field or laboratory settings and ease of parasite identification as well as previous reports of its application to detect resistance to both the benzimidazole and macrocyclic lactone drug groups (Gill et al., 1981).

Eighteen 250 ml conical flasks were grouped into six groups with three replicates each. To each of the three flask of group one, 100 ml of Goodwin's solution was added to act as negative control. To groups 2 to 6, serial dilutions of ethanolic crude plant extract ranging from 6.25 to 100 mg/ml for *Z. chalybeum* were added. In a parallel set up, 18 conical flasks were divided into six groups to cater for the negative control and the five level serial dilutions (concentrations from 6.25 to 100 mg/ml) of positive control (Albendazole 10%). At the end of the experiment, the procedure was repeated with serial dilutions of aqueous crude extract ranging from 4.5 to 72 mg/ml. The lowest dose level represents half of the community dose based on extraction efficiency. Stock solutions were prepared by first dissolving weighed amount of the extract in 5 to 10 ml of dimethyl sulfoxide (DMSO), then diluted by Goodwin's physiological solution to 600 ml mark to make the highest concentration (mg/ml) with the same solution. Ten average size motile adult worms were randomly placed in each of the flask. The flasks and their contents were incubated at 37°C and checked for motility at 24 and 48 h during which all the parasites in each flask were assessed for paralysis, death or motility (active) and were recorded. A motility index was calculated as the ratio between the numbers of immobile worms/total number worms in the 3 flask per concentration.

### Phytochemical screening

#### Detection of specific phytochemical compounds

The aqueous and ethanol extracts were qualitatively phytochemically analyzed using the standard methods described by Harbone (1973, 1998) and Tchamadeu et al. (2010) for the presence of alkaloids salts (Meyer's and Drangedorffs test), tannins (Styassny's reagent), saponins (foaming test), flavonoids (Shibata's reaction), reducing sugars (Fehling's tests), anthracenocides (Borntagen's reaction), coumarins (colour fluorescence under UV light), glycosides and triterpenoids (Liebermann-Burchard's test) and anthocyanosides.

### Separation of chemical groups by thin layer chromatography (TLC)

The separation of the various chemical groups was done on freshly prepared dry material plants extracts. A qualitative TLC analysis on silica gel was performed on of *Z. chalybeum* extracts in different migration systems according to Harborne (1973, 1998). Different detection agents were used with the purpose of characterizing the active principles responsible for biological effects or medicinal benefits. Vanillin-sulfuric acid reagent was employed for revealing terpenoid spots (triterpenes and saponins) while antimony trichloride was used for detection of development of blue-violet or brown spots indicative of polyphenol compounds (Wagner and Blatt, 1996). TLC analysis on silica gel was performed in chloroform/methanol (9:1 v/v), EMW (100:17:13, v/v/v) and Xylene:MeOH (4:1, v/v) with antimony trichloride in concentrated HCL and vanillin sulphuric acid sprays for detection. Chromatograms were visualized in visible light and by ultra violet (UV) irradiation (254 nm). Preparatory chromatographic glass plates were prepared by dissolving 100 g of absorbent silica gel, in 250 ml of distilled water (1:2.5) which was spread on glass plates and left *in situ* to dry overnight followed by activation by heating in the oven at 120°C for 40 min.

To a stationary phase (silica gel), extract drops were put on a mark and immersed in a preferred solvent system to wait for 40 min of mobile phase movement followed by drying and visualization under visible light and UV irradiation (254 nm) during which the relative fractions ( $R_{fs}$ ) of fluorescent spots were recorded. Thereafter, the plates were sprayed with antimony trichloride (phenolics compounds) and vanillin agents and heated at 120°C for 10 min after spraying with the reagents following standard procedures (Ciulei, 1964; Harborne, 1973; Wagner and Blatt, 1996) and the spot colors and distances moved were recorded against stationary phase.

### Data analysis and determination of ED<sub>50</sub> of the extracts

The bioassay data was analyzed by the Generalized Linear Model procedures for regression, ED<sub>50</sub> determination. Graph Pad Prism version 5.01 software (Inc San Diego, CA USA) and GenStat Release 13.2 (PC/Windows7) softwares were also used to determine the means of percent motility inhibition, regression equations ( $Y_{(1,2,3)} = A_{(1,2,3)} + C_{(1,2,3)} / (1 + \text{EXP}(-B_{(1,2,3)} \times (X - M_{(1,2,3)})))$ ) and 95% confidence intervals (CI) and to generate the dose-response curves. Two-way analysis of mean variance was carried out in GenStat Release 13.2 followed by Bonferroni post hoc *t*-test and *p*-value = 0.05 was used for significance level.

## RESULTS

### Community dosage and extraction efficiency of *Z. chalybeum* in water and 70% ethanol solvents

The mean dry weight of root bark used by the community was established to be 165.70 ± 11.88 g which was boiled in 2 L of water. The mean yields and extraction efficiency of 250 g of the plant dry material in ethanol and water solvents are shown in Table 1.

### *In vitro* anthelmintic activity of ethanolic and aqueous extracts of the selected plants

The 48 h exposure of adult *A. suum* to both ethanolic and

**Table 1.** Solvent extraction efficiency (g/250 g of dry root bark) of ethanolic and aqueous extracts of the crude plants' extracts.

Variable	Ethanolic extract (Mean ± SEM (g))	Water extract (Mean ± SEM (g))
Community adopted dry weights used	NA	165.70 ± 11.88
Extract yield (Mean ± SEM (g))	38.80 ± 4.30 <sup>b</sup>	56.67 ± 1.67 <sup>a</sup>
Extraction efficiency (%)	15.6	22.7

Comparison was done between the solvents used and values with different superscript in a row are statistically significant (P = 0.004).

**Table 2.** The dose-response effects of crude extracts of *Zanthoxylum chalybeum* on adult *Ascaris suum* motility 48 h post treatment.

Treatment	Dose (mg/ml)*	Motility inhibition (Mean ± SEM (%))	95% confidence interval of motility inhibition
Ethanolic extract	0.00	0.00 ± 0.00 <sup>a</sup>	-11.27 - 11.27
	6.25	16.67 ± 3.33 <sup>b</sup>	5.40 - 27.93
	12.50	33.33 ± 3.33 <sup>c</sup>	22.07 - 44.60
	25.0	56.67 ± 8.82 <sup>d</sup>	45.40 - 67.93
	50.0	83.33 ± 3.33 <sup>e</sup>	72.07 - 94.60
	100.0	93.33 ± 5.77 <sup>f</sup>	82.07 - 104.60
Aqueous extract	0.0	0.00 ± 0.00 <sup>a</sup>	-11.27 - 11.27
	4.5	26.67 ± 3.33 <sup>b</sup>	15.40 - 37.97
	9.0 <sup>cd</sup>	63.33 ± 6.67 <sup>c</sup>	52.07 - 74.60
	18.0	66.67 ± 3.33 <sup>d</sup>	55.40 - 77.93
	36.0	86.67 ± 3.33 <sup>e</sup>	75.40 - 97.93
	72.0	100.00 ± 0.00 <sup>f</sup>	88.73 - 111.27
Albendazole	0.00	0.00 ± 0.00 <sup>a</sup>	-8.91 - 8.91
	6.25	30.00 ± 10.0 <sup>b</sup>	17.76 - 42.24
	12.50	46.67 ± 12.20 <sup>c</sup>	34.43 - 58.91
	25.00	76.67 ± 8.82 <sup>d</sup>	64.43 - 88.91
	50.00	90.00 ± 5.77 <sup>e</sup>	77.76 - 102.24
	100.00	100.00 ± 0.00 <sup>f</sup>	87.76 - 112.24

\*Three replicates per treatment dose; Number of worms used, N = 10; <sup>cd</sup>Dose adopted by community; Goodwin's solution used in negative control. Mean values in the same treatment with different superscript in a column are statistically significant (P < 0.05).

aqueous extracts at different concentrations produced a comparable and dose-dependent reduction in motility and/or mortality to the negative and positive control groups (Table 2). A significant difference in all dose levels on motility inhibition that was dose-dependent irrespective of solvent used for extraction when compared with negative control was observed using generalized linear model; *Z. chalybeum* ( $F_{(5, 53)} = 10.62$ ,  $P = 0.001$ ;  $R^2 = 0.92$ ). There was however, no significant variation ( $P > 0.05$ ) in motility inhibition when the plant crude ethanolic and aqueous extracts were compared with the positive control, albendazole. Similarly, there was no significant interaction between methods of extraction and the dose effect on motility inhibition in ( $F_{(10,$

$53) = 1.69$ ,  $P = 0.125$ ).

### Median effective dose (ED<sub>50</sub>) and anthelmintic potency of plant crude extracts

Table 3 presents the median effective dose (ED<sub>50</sub>) of the root bark extracts and positive control while Figure 1 demonstrates the potency of the plant extracts as compared to albendazole. The results showed that the aqueous extract was more potent than the albendazole and ethanolic extract demonstrated by the shift of the graph to the left. The median effective doses of *Z. chalybeum* extracts and albendazole significantly differed

**Table 3.** The median effective doses (ED<sub>50</sub>) of the ethanol and water extracts and albendazole.

Extract/treatment	Worm motility inhibition (%)	Median effective dose (ED <sub>50</sub> ) (mg/ml)	95% CI of ED <sub>50</sub>
Ethanol	93	30.85 <sup>a</sup>	15.73 - 60.50
Water	100	6.28 <sup>b</sup>	3.79 - 10.41
Albendazole	100	15.12 <sup>b</sup>	6.95 - 32.90

Comparison was done between the ED<sub>50</sub> of solvents used and values with different superscript are statistically significant (P < 0.05).

**Table 4.** Phytochemical constituents of water and ethanol extracts of *Z. chalybeum* root bark.

Tested compound	Aqueous extract	70% Ethanolic extract
Tannins	++	++
Reducing sugars	+	++
Saponins	++	x
Alkaloid salts	++	++
Anthracenosides	++	++
Coumarin derivatives	++	++
Flavonosides	+++	+++
Steroid glycosides	++	++
Triterpenes	++	++
Anthocyanosides	++	++

(+) weakly present, (++) moderate, (+++) strongly present, (x) not tested in ethanol extract

( $F_{(2, 53)} = 323.80$ ,  $P = 0.001$ ). Thus, medicinal plant extract potency varies with the method of bioactive ingredients extraction.

#### Phytochemical constituents characterization of aqueous and ethanolic extracts of *Z. chalybeum*

Table 4 presents the qualitative phytochemical compounds detected in ethanolic and aqueous extracts of *Z. chalybeum* root bark while compounds characterization by TLC is shown in Table 5.

## DISCUSSION

The aim of this study was to determine the anthelmintic activity and phytochemical constituents of *Z. chalybeum* root bark utilized by the agro-pastoral communities in Ugandan cattle corridor. Indeed, researchers need to identify possible candidates for new drug development by pharmaceutical industries to address the drug resistance and emerging diseases.

The results of this study have shown the anthelmintic potential of the *Z. chalybeum* and support the claims by the Nakasongola agro-pastoral farmers and previous reports that this plant treats helminthes infections in livestock (Nalule et al., 2011). However, the study revealed a wide variation in the dosage adopted by the community which shows that

different individual using the plants achieves different control benefits. The community probably uses that amount due to the fear that the plant may be toxic in higher doses. Continued administration of low doses of plant crude extract to parasites may lead to the risks of development of resistance from repeated low doses exposure.

The study also revealed that the solvent used in extraction of active ingredient affect the anthelmintic activity obtained. This may be attributed to the types of compounds that are extracted depending on polarity and solubility in different solvents, and their biological effects on parasites. Other studies made similar observations (Gakuya, 2001; Costa et al., 2008) where they used different solvents for extraction observed varying bioactivity results. The study revealed that both the aqueous and ethanol extracts of the root bark of *Z. chalybeum*, significantly inhibited *A. suum* motility in a dose-dependent response by paralyzing them or causing their death with varying efficacies when compared with albendazole demonstrating the anthelmintic potential of the medicinal plants in the Ugandan cattle corridor. The variation in efficacy may also be attributed to the method of extraction, which may have yielded specific compounds, source of parasites and previous exposure to the plants. Waterman (1992) reported that plant metabolites are unstable molecules and their biological activity are dependent on their structure, physical and chemical properties. It is therefore possible that the parasite paralysis and/or death observed may have been

**Table 5.** Thin layer chromatography characterization of *Z. chalybeum* compounds under different solvent systems, sprays and observations regimes.

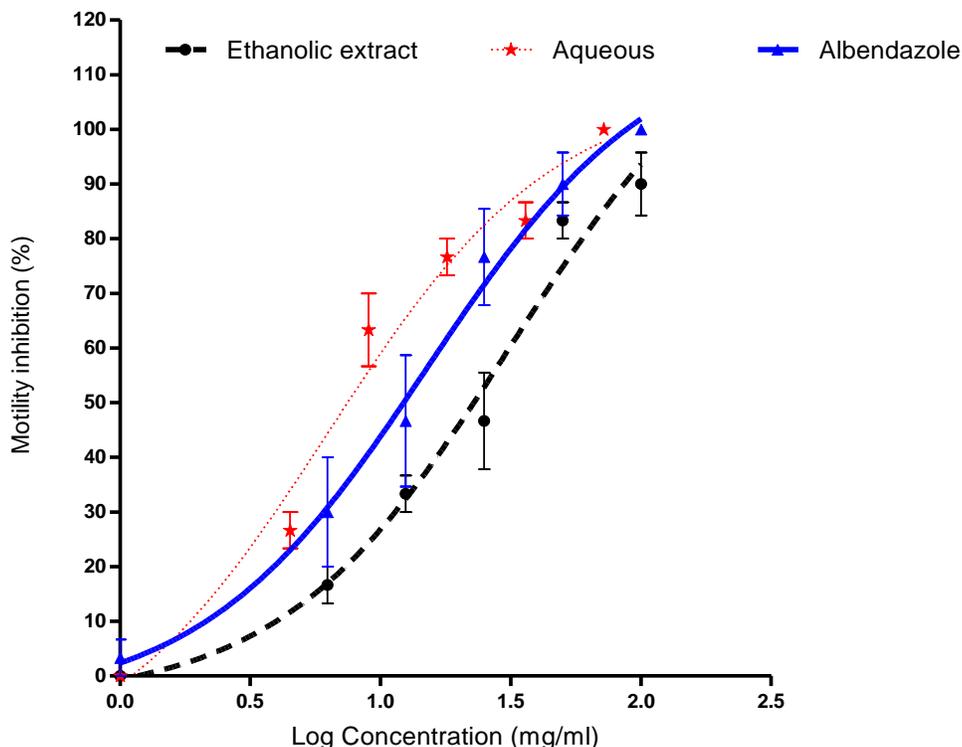
R <sub>f</sub> ×100	Chloroform/Methanol (9:1, v/v), solvent front 11.0				Ethyl acetate/Methanol/Water (100:17:13, v/v/v) solvent front 10.0				Xylene/Methanol (4:1, v/v) solvent front 10.0			
	Visible light	UV light	Antimony trichloride spray	Vanillin spray	Visible light	UV light	Antimony trichloride spray	Vanillin spray	Visible light	UV light	Antimony trichloride spray	Vanillin spray
8.0	Y	B	Y	Y	B	B	-	-	-	Y	-	-
16.7	-	O	Y	-	-	-	-	-	DY	-	-	-
20.0	-	-	-	-	-	-	-	-	-	O	-	P
22.7	Y	-	-	Y	-	-	-	-	-	-	-	-
25.0	-	LB	B	P	B	B	Y	-	-	-	-	-
35.4	-	O	-	-	OY	OY	-	OY	Y	DB	-	-
43.7	-	-	BR	-	-	-	-	-	-	-	BR	-
44.0	-	-	-	-	-	-	-	-	-	LB	-	-
50.0	-	-	-	-	-	-	-	-	-	BR	-	-
53.1	-	-	-	-	-	-	-	-	LY	-	-	-
56.4	-	-	-	-	B	B	-	-	-	-	-	-
57.6	-	-	-	-	-	-	-	BR	-	-	-	P
61.8	-	-	-	-	-	-	-	-	-	-	PK	-
62.9	-	-	-	-	-	-	-	-	-	-	-	P
64.5	-	-	-	-	-	-	-	-	-	B	-	-
75.5	-	-	-	-	-	-	-	-	-	O	-	-
84.1	-	-	-	-	-	-	-	-	LY	-	-	-
86.5	-	-	P	-	-	-	PP	-	-	-	Y	DB
87.1	-	Y	-	-	-	-	-	-	-	O	-	-
91.5	-	-	PK	PK	-	-	-	-	-	-	-	-
93.2	-	B	-	-	B	-	-	-	-	-	-	-
96.0	-	-	GY	-	-	-	-	-	-	-	-	-
97.3	-	O	-	BR	O	-	-	BR	-	-	-	-
98.5	-	-	Y	G	-	-	-	-	-	-	-	-
Total spots	2	6	8	6	6	-	2	3	4	8	7	4

Y=yellow; G=green; DG =dark green; LY=light yellow; P=purple; PK=pink; DY=deep yellow, B=Blue; O=orange; LB=Light blue, DR=deep red; R=red; BR=brown; DB=dark brown; GY=grey; V=violet. Spots at same R<sub>f</sub> with similar colours are similar or related.

attributed to plant metabolites (Table 4) like tannins, alkaloids salts and saponins among others (Makut et al., 2008). This study identified the presence of tannins, saponins, alkaloid salts,

anthracenosides, coumarin derivatives, flavonoids, steroid glycosides, triterpenes and anthocyanosides. However, other studies on *Z. chalybeum* had revealed the presence of other

specific compounds. For instance, structure elucidation using nuclear magnetic resonance (NMR), EIMS and UV spectroscopy by Aluoch (2000) who reported the presence of the benzophenanthridine



ED<sub>50</sub> Ethanolic =30.85; ED<sub>50</sub> Aqueous = 6.28; ED<sub>50</sub> Albendazole=15.12

**Figure 1.** Dose-response curves of adult *Ascaris suum* motility inhibition by ethanol and water crude extracts of *Zanthoxylum chalybeum* and albendazole, 48 h post treatment. Nonlinear regression curves of treatments are defined as;  $Y_{(1,2,3)} = A_{(1,2,3)} + C_{(1,2,3)} / (1 + \text{EXP}(-B_{(1,2,3)} \times (X - M_{(1,2,3)})))$ . Where  $Y_{(1,2,3)}$  are proportions of worm motility inhibited by ethanol, water extracts and albendazole. A is Y-value when X=0; C is the top – bottom of each curve, that is, X=0 and X= maximum; B is a rate constant expressed as reciprocal of X; M is random error and X is the dose of treatment (ethanol, aqueous and albendazole).  $A_1, A_2, A_3$  and  $C_1, C_2, C_3$  are parameter estimates for ethanol and aqueous extracts and albendazole, respectively given as 0.03, -4.90, 2.93 with s.e of, 5.93, 8.94, 6.20, respectively and 104.0, 112.7, 103.0 with s.e of 16.0, 21.3, 13.2 respectively. While, the constants  $B_1, B_2, B_3 = 3.411, 2.60, 3.333$  with s.e of 0.982, 0.979, 0.946 respectively and  $M_1, M_2, M_3 = 1.335, 0.962, 1.1672$  with s.e of 0.104, 0.114 and 0.0887 respectively. X = treatment doses (level 1 to level 6) for each treatment). Percentage variance accounted for 92.3 and standard error of observations was estimated to be 9.87. The error bars show the standard error of the percent worm motility inhibition.

alkaloids dihydrochelerythrine and chelerythrine, the 2,6-diaryl-3,7-dioxabicyclo[3.3.0]octane lignans (+) sesamin (3) and (-) asarinin (4), the quinolone alkaloid 4-methoxy-N-methyl-quinolin-2-one (5) and the triterpene. Different studies (Kato et al., 1996; Olila et al., 2002) have reported the presence of alkaloid skimmianine while another study on *Z. chalybeum* root and stem bark have reported the presence of polyphenols and their antiplasmodial and oxidant activity (Stangeland et al., 2010). It is therefore likely that these compounds could have anthelmintic activity that may have worked singly or in combination to cause the motility inhibition or paralysis of the worms that was achieved in all the studied crude extracts. Synergistic interactions have been suggested to underlie the effectiveness of phyto-medicines that lead to

better activity as well as decrease potential toxicity of some individual constituents (Kaufman et al., 1999). It has also been reported that the plant metabolites action may be additive, synergistic or antagonistic in manner acting at single or at multiple target sites (Briskin, 2000; Wynn and Fougere, 2007).

Nevertheless, it is well documented that some anthelmintic drugs like the benzimidazoles (BZD) kill the parasites by binding to a specific building block, the beta tubulin and prevent its incorporation into micro-tubules which are essential for energy metabolism (Schoenian, 2008). The benzimidazole anthelmintics are said to act by interfering with the microtubule system in *A. suum* (Barrowman et al., 1984). Thus, these compounds could have caused their effect through the same mechanism.

Paralysis of worm tissues makes them unable to feed leading to death as a result of lack of energy. It is also likely that alkaloids present in the plants could also have contributed to the paralysis and consequent death of the worms. The nematocidal activity of alkaloids had also been demonstrated when they used two rat nematodes; *Strongyloides ratti* and *Strongyloides venezuelensis* models for human nematodes (Satou et al., 2002). Alkaloids salts on the other hand are competitive antagonists at muscarinic acetylcholine receptor preventing the binding of acetylcholine and are reportedly physiologically active with sedative and analgesic properties in addition to leading to excitation of cells and neurological dysfunction (Tarnopolsky and Beal, 2001). On the other hand the saponins present in the crude extract could have caused feed refusal and starvation of the parasites leading to their death from lack of energy. Similar views on saponins effects of feeding were held by other authors who also reported that saponins kill protozoans and molluscs (Dalsgaard et al., 1990; Francis et al., 2002). It is also probable that *in vivo* paralysis lead to loss of grip of parasites on the gut wall leading to the spontaneous expulsion of parasites together with faeces.

The roles of tannins in helminths control have been documented (Athnasiadou et al., 2001a; Cenci et al., 2007; Forbey et al., 2009). The nematocidal activity of tannin extracts has also been reported with evidence of anthelmintic properties of condensed tannins by series of *in vitro* studies (Dawson et al., 1999; Athanasiadou et al., 2001a; Ademola and Idowu, 2006) and *in vivo* studies (Butter et al., 2001). Chemically, tannins are polyphenolic compounds (Bate-Smith, 1962) and synthetic phenolic anthelmintics like niclosamide and oxyclozanide are said to interfere with energy generation in helminths parasites by uncoupling oxidative phosphorylation (Martin, 1997). It is possible that tannins contained in ethanol and water extracts of *Z. chalybeum* produced similar effects. It was also suggested that tannins bind to free proteins in the gastrointestinal tract of the host animal (Athanasiadou et al., 2001b; Hoste et al., 2006) or glycoprotein on the cuticle of the parasite disturbing the physiological functions like motility, feed absorption and reproduction (Aerts et al., 1999; Githiori et al., 2006) or interference with morphology and proteolytic activity of microbes causing death (Min et al., 2003; Waghorn and McNabb, 2003). Alternatively, the presence of alkaloids salts which are physiologically active with sedative and analgesic properties could have contributed to the paralysis and consequent death of the worms. Alkaloids have been reported to be toxic resulting from their stimulatory effects that lead to excitation of cells and neurological dysfunction (Rujjanawate et al., 2003).

The study further established that the anthelmintic activity of the plant extracts varied among the increasing concentration and incubation period. Increasing effect with increasing concentration could be due to the fact that

some chemical compounds slowly get released and their effect depends on saturation of target receptors. Similar observation were made by Lullman et al. (1993) who said that the receptors get saturated with increasing dose of active ingredient that increases with incubation period. It is likely that at higher concentration all binding receptors on the worms were occupied thus leading to hyperpolarisation of membranes limiting excitation and impulse transmission, thus causing flaccid paralysis of worm muscles (Wasswa and Olila, 2006).

The extract potency (Figure 1) may be a result of synergistic action of some compounds present in different extracts. Despite the many compounds present in extracts, it is likely only a few that could be important as anthelmintics. The great variation in potencies based on the ED<sub>50s</sub> (Table 3), could be due to presence of difference in compound structure as some could have occur as an isomer or derivatives of the bigger molecules and their difference in concentration. Occurrence of several fluorescent spots in TLC at different relative fractions (R<sub>f</sub>s) within a given system under same observation conditions may be suggestive of the presence of same compounds but different molecular mass, or isomers, or derivatives of a given class of compounds and probably the same chemical structure. The appearance of the pink/purple colour under vanillin spray may be suggestive of the presence of phenolic compounds while purple staining with antimony chloride probably indicate the presence of sapogenins in CHCl<sub>3</sub>:MeOH system according to Harbone (1984). TLC characterization results typically demonstrate these minor differences in structure thus concentration. Similar observations were made by Waterman et al. (2010). It is likely that the solvent used in extraction of active ingredients could be responsible for the crude extract potency. This is in support of the observations made by Malu et al. (2009).

The compound could also be having similar functional groups though not the same but with some relationship and absorbing at different wavelength. It is also probable that the richer the plant species in bioactive compounds the more effective and potent since the compounds work in isolation or in combinations to bring about biological effects in the animal through synergy and/or additive action to enhance the medicinal value of the group of compounds. The compounds present in the extract probably justify their use by the community for health benefits.

## CONCLUSION AND RECOMMENDATION

This study results suggest that the root bark of *Z. chalybeum* have potential anthelmintic activity justifying their traditional ethno-veterinary use by the pastoral communities not withstanding under dosing revealed in the cattle corridor of Uganda that could be exploited for

sustainable livestock helminths control or reduce the risk of helminths resistance to conventional drugs.

The study has also shown that the efficacy and potency of the plant extract depend upon the solvents used to extract the bioactive ingredient although ethanol and water solvents could be used interchangeably to take advantage of preservation of ethanol and economics of aqueous solvents.

The phytochemical constituents present in the plant validates the basis for their use as anthelmintic “drugs” and direct to plant’s role in medicinal uses. However, the plant needs to be studied *in vivo* to establish the effect of digestive enzymes on efficacy in addition to determining toxicity in animals, bioavailability and bio-acceptability and safety of this noble plant species.

Over-harvesting of roots could lead to endangerment of this tree, should commercial exploitation break-even or routine used for preventive purposes on a flock-wide basis. Given that the *Z. chalybeum* roots are targeted by current users, it is warranted to assess the anthelmintic activity of leaves that would allow for less destructive harvesting to promote its conservation.

## REFERENCES

- Ademola IO, Idowu SO (2006). Anthelmintic activity of *Leucaena leucocephala* seed extract on *Haemonchus contortus*-infective larvae. *The Veterinary Record* 158:485–486
- Aerts RJ, Barry TN, McNabb WC (1999). Polyphenols and agriculture: beneficial effects of proanthocyanidins in forages. *Agric. Ecosyst. Environ.* 75:1-12.
- Aluoch AO (2000). Chemistry and biological activities of *Zanthoxylum chalybeum* and *Erythrina excelsa*. MSc thesis, University of Nairobi, Kenya.
- Athanasiadou S, Kyriazakis I, Jackson F, Coop RL (2001b). The effects of condensed tannins supplementation of foods with different protein content on parasitism, food intake and performance of sheep infected with *Trichostrongylus colubriformis*. *Brit. J. Nutr.* 86:697–706.
- Athanasiadou S, Kyriazakis I, Jackson F, Coop RL (2001a). Direct anthelmintic effects of condensed tannins towards different gastrointestinal nematodes of sheep: In vitro and in vivo studies. *Vet. Parasitol.* 99:205–219.
- Barrowman MM, Marriner SE, Bogan JA (1984). The binding and subsequent inhibition of tubulin polymerization in *Ascaris suum* (*in vitro*) by benzimidazole anthelmintics. *Biochem. Pharmacol.* 33(19):3037-3040.
- Bate-Smith EC (1962). The phenolic constituent of plants and their taxonomic significance, dicotyledons. *J. Linn. Soc., Bot.* 58:95–103.
- Briskin DP (2000). Medicinal plants and phytomedicines: Linking plant Biochemistry and physiology to human health. *Updates on phytomedicines. Plant physiol.* 124:507-514.
- Butter NL, Dawson JM, Wakelin D, BATTERY PJ (2001). Effect of dietary condensed tannins on gastrointestinal nematodes. *J. Agric. Sci.* 137:461–469.
- Cenci FB, Louvandini H, McManus CM, Dell’Porto A, Costa DM, Araújo SC, Minho AP, Abdalla AL (2007). Effects of condensed tannin from *Acacia mearnsii* on sheep infected naturally with gastrointestinal helminths. *Vet. Parasitol.* 144:132–137.
- Ciulei J (1964). *Manual for analysis of vegetables drugs.*
- Costa CTC, Bevilacqua CML, Camurça-Vasconcelos ALF, Maciel MV, Morais SM, Castro CMS, Braga RR, Oliveira LMB (2008). *In vitro* ovicidal and larvicidal activity of *Azadirachta indica* extracts on *Haemonchus contortus*. *Small Ruminant Res.* 74(1-3):284-287.
- Dalsgaard K, Hilgers L, Trouve G (1990). Classical and new approaches to adjuvant use in domestic food animals. *Adv. Vet. Sci. Comp. Med.* 35:121-160.
- Davey MW, James CE, Hudson AL (2009). Drug resistance mechanisms in helminths: is it survival of the fittest? *Trends Parasitol.* 25(7):328-335.
- Dawson JM, BATTERY PJ, Jenkins D, Wood CD, Gill M (1999). Effects of dietary Quebracho tannin on nutrient utilization and tissue metabolism in sheep and rats. *J. Sci. Food Agric.* 79:1423–1430.
- Dharani N, Rukunga G, Yenessew A, Mbori A, Mwaura L, Dawson I, Jamnadass R (2010). Common antimalarial trees and shrubs of East Africa: a description of species and guide to cultivation and conservation through use. World Agroforestry Centre (ICRAF), Nairobi, Kenya. ISBN: 978-92-9059-238-9.
- Donahue MJ, Yacoub NJ, Kaeini MR, Masaracchia RA, Harris BG (1981). Glycogen metabolizing enzymes during starvation and feeding of *A. suum* maintained in a perfusion. *J. Parasitol.* 67(4):505-510.
- Forbey JS, Harvey AL, Huffman MA, Provenza FD, Sullivan R, Tasdemir D (2009). Exploitation of secondary metabolites by animals: A response to homeostatic challenges. *Integr. Comp. Biol.* 49(3):314-328.
- Francis G, Kerem Z, Makkar HPS, Becker K (2002). The biological action of saponins in animal systems: a review. *Brit. J. Nutr.* 88:587–605.
- Gakuya DW (2001). Pharmacological and clinical evaluation of the anthelmintic activity of *Albizia anthelmintica* Brogn, *Maerua edulis* De wolf and *Maerua subcordata* De wolf plant extracts in sheep and mice. PhD Thesis, University of Nairobi, Kenya.
- Gill JH, Redwin JM, van Wyk JA, Lacey E (1991). Detection of resistance to ivermectin in *Haemonchus contortus*. *Int. J. Parasitol.* 21:771–776.
- Githiori JB, Athanasiadou S, Thamsborg SM (2006). Use of plants in novel approaches for control of gastrointestinal helminths in livestock with emphasis on small ruminants. *Vet. Parasitol.* 139:308-320.
- Harborne JB (1973). *Phytochemical Methods.* Chapman and Hall, London p. 113.
- Harborne JB (1998). *Phytochemical Methods. A guide to modern techniques of plant analysis, 3<sup>rd</sup> Edn.*, Chapman and Hall, London, p. 235.
- Hedberg I, Hedberg O, Madati PJ, Mshigeni KE, Mshiu EN, Samuelsson G (1983b). Inventory of plants used in traditional medicine in Tanzania. Part III. Plants of the families Papilionaceae–Vitaceae. *J. Ethnopharmacol.* 9:237–260.
- Hoste H, Jackson F, Athanasiadou S, Thamsborg SM, Hoskin SO (2006). The effects of tannin-rich plants on parasitic nematodes in ruminants. *Trends Parasitol.* 22(6):253-261.
- Ian SC, Khan TE, Curran MM, McCorkle MC (2007). Ethnoveterinary Medicine: Potential solutions for large scale problems? In: Wynn S.G, and Fougere (Ed). *Veterinary Herbal medicine. Library of Congress cataloging-in publication data.* ISBN: 10:0-323-02998-1. p. 695
- Johns T, Kokwaro JO, Kimanani EK (1990). Herbal remedies of the Luo of Siaya district, Kenya: establishing quantitative criteria for consensus. *Econ. Bot.* 44:369–381.
- Kaplan RM, Klei TR, Lyons ET, Lester G, Courtney CH, French DD, Tolliver SC, Vidyashankar AN, Zhao Y (2004). Prevalence of anthelmintic resistant cyathostomes on horse farms. *J. Am. Vet. Med. Assoc.* 225:903–910.
- Katende AB, Birnie A, Tengnas B (1995). *Useful Trees and shrubs for Uganda. Identification, propagation and management for Agricultural and livestock communities. Technical Handbook No. 10.* Regional Soil Conservation Unit, RSCU, 1995
- Kato A, Moriyasu H, Ichimaru H, Nishiyama Y, Yuma FD, Nganga JN, Mathenge SG, Ogeto JO (1996). Isolation of alkaloidal constituents of *Zanthoxylum umbarene* and *Zanthoxylum chalybeum* using ion-pair HPLC. *J. Nat. Prod.* 59:316-318.
- Kaufman PB, Cseke LJ, Warber S, Duke JA, Brielmann HL (1999). *Natural Products from Plants.* CRC Press, Boca Raton, FL
- Kotze AC, Clifford S, O’Grady J, Behnke JM, McCarthy JS (2004). An *in vitro* larval motility assay to determine anthelmintic sensitivity for human hookworm and *strongyloides* species. *Am. J. Trop. Med. Hyg.* 71(5):608-616.
- Lamson PD, Brown HW (1936). *Methods of testing the anthelmintics*

- properties of asacriciedes. Am. J. Hyg. 23:85-103.
- Lullman HK, Morh, Bieger D (1993). Colour Atlas of pharmacology, Theme medical publisher, Inc. New York, pp. 52-98.
- Makut MD, Gyar SD, Pennap GRI, Anthony D (2008). Phytochemical screening and antimicrobial activity of ethanolic and methanolic extracts of leaf and bark of *Khaya senegalensis*. Afr. J. Biotechnol. 7(99):1216-1219.
- Malu SP, Obochi GO, Edem CA, Nyong BE (2009). Effect of methods of extraction on phytochemical constituents and antibacterial properties of *Tetracarpidium conophorum* seeds. Global J. Pure Appl. Sci. 15(3):373-376.
- Marie-Magdeleine C, Hoste H, Mahieu M, Varo H, Archimede H (2009). *In vitro* effects of Cucurbita moschata seed extracts on Haemonchus contortus. Vet. Parasitol. 161:99-105.
- Martin RJ (1997). Modes of action of anthelmintic drugs. Vet. J. 154(1):11-34.
- Melaku A, Bogale B, Chanie M, Fentahun T, Berhanu A (2013). Study on utilization and efficacy of commonly used anthelmintics against gastrointestinal nematodes in naturally infected sheep in North Gondar, North-Western Ethiopia. Afr. J. Pharm. Pharmacol. Vol. 7(12):679-684.
- Min BR, Barry TN, Attwood GT, McNabb WC (2003). The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. Anim. Fed. Sci. Tech. 106:3-19.
- Nalule AS, Mbaria JM, Olila D, Kimenju JW (2011). Ethnopharmacological practices in management of livestock helminthes by pastoral communities in the drylands of Uganda; Livestock Res. Rural Dev. 23(2):11
- Nguta JM, Mbaria JM, Gakuya DW, Gathumbi PK, Kiama SG (2010). Antimalarial herbal remedies of Msambweni, Kenya. J. Ethnopharmacol. 128(2):424-432.
- Olila D, Olwa-Odyek, Opuda-Asibo J (2002). Screening extracts of *Zanthoxylum chalybeum* and *Warburgia ugandensis* for activity against measles virus (Swartz and Edmonston strains) in vitro. Afr. J. Health Sci. 2(1):2-10.
- Olila D, Opuda-Asibo J, Odyek-Olwa (2001b). Antibacterial and antifungal activities of extracts of *Zanthoxylum chalybeum* and *Warburgia ugandensis*, Ugandan medicinal plants. Afr. J. Health Sci. 1:66-72.
- Olila D, Opuda-Asibo J, Odyek-Olwa (2001a). Bioassay-guided studies on the cytotoxic and *in vitro* trypanocidal activities of a sesquiterpene (Muzigadial) derived from a Ugandan medicinal plant (*Warburgia ugandensis*). Afr. J. Health Sci. 1:12-15.
- Paolini V, Fouraste I, Hoste H (2004). In vitro effect of three woody plants and sainfoin extracts on 3<sup>rd</sup>-stage larvae and adult worms of three gastrointestinal nematodes. Parasitol. 129(01):69-77.
- Rujjanawate C, Kanjanapothi D, Panthong A (2003). Pharmacological effect and toxicity of alkaloids from *Gelsemium elegans* Benth. J. Ethnopharmacol. 89(1):91-95.
- Runyoro DKB, Ngassapa OD, Matee MIN, Joseph CC, Moshi MJ (2006). Medicinal plants used by Tanzanian traditional healers in the management of *Candida* infections. J. Ethnopharmacol. 106:158-165.
- Sargison ND, Jackson F, Bartley DJ, Wilson DJ, Stenhouse LJ, Penny CD (2007). Observations on the emergence of multiple anthelmintic resistance in sheep flocks in the south-east of Scotland. Vet. Parasitol. 145:65-76.
- Satou T, Koga M, Matsuhashi R, Koike K, Tada I, Nikaido T (2002). Assay of nematocidal activity of isoquinoline alkaloids using third stage larvae of *Strongyloides ratti* and *S. venezuelensis*. Vet. Parasitol. 104:131-138.
- Schoenian S (2008). Understanding anthelmintics (dewormers). Small Ruminant Info Series. Western Maryland Research & Education Center, University of Maryland Cooperative Extension. Available at: <http://www.sheepandgoat.com/articles/anthelminticswork.html>.
- Stangeland T, Wangensteen H, Katuura E, Lye KA, Paulsen BS (2010). Antioxidant and anti-plasmodial activity of extracts from three Ugandan medicinal plants. J. Med. Plants Res. 4(18):1916-1923.
- Taniguchi M, Chapya A, Kubo I, Nakanishi K (1978). Screening of East African plants for antimalarial activity. Chem. Pharm. Bull. 26:2910-2913.
- Tarnopolsky MA, Beal MF (2001). Potential for creatine and other therapies targeting cellular energy dysfunction in neurological disorders. Ann. Neurol. 49:561-574.
- Tchamadeu MC, Dzeufiet PDD, Kouambou NCC, Azebaze AGB, Allard J, Girolami JP, Tack, I, Kamtchouing P, Dimo T (2010). Hypoglycaemic effects of *Mammea africana* (Guttiferae) in diabetic rats. J. Ethnopharmacol. 127:368-372.
- Tinsley RC, York JE, Everard ALE, Stott LC, Chapple SJ, Tinsley MC (2011). Environmental constraints influencing survival of an African parasite in a north temperate habitat: effects of temperature on egg development. Parasitology 1-10.
- Tran G (2011). Knob wood (*Zanthoxylum chalybeum*). Feedipedia.org. A programme by INRA, CIRAD, AFZ and FAO. Accessed from <http://www.feedipedia.org/node/156>
- Waghorn GC, McNabb WC (2003). Consequences of plant phenolic compounds for productivity and health of ruminants. Proceedings of the Nutrition Society 62:383-392.
- Wagner G, Blatt S (1996). Plant drug analysis, a thin layer chromatography, atlas, 2<sup>nd</sup> Ed, CS Pringer Verlag, pp. 352-355.
- Wasswa P, Olila D (2006). The in vitro Ascaricidal activity of selected indigenous medicinal plants used in ethno- veterinary practices in Uganda. Afr. J. Tradit. Complement Altern. Med. 3:94-103.
- Waterman C, Smith RA, Pontiggia L, DerMarderosian A (2010). Anthelmintic screening of Sub-Saharan African plants used in traditional medicine. J. Ethnopharmacol. 127(3):755-759.
- Waterman PG (1992). Role for secondary metabolites in plants. Ciba Foundation symposia 171:255-275.
- Weaver HJ, Hawdon JM, Hoberg EP (2010). Soil-transmitted helminthiases: implications of climate change and human behavior. Review Article. Trends Parasitol. 26(12):574-581.
- Wolstenholme AJ, Fairweather I, Prichard R, von Samson-Himmelstjerna G, Sangster N (2004). Drug Resistance in Veterinary Helminthes. Trends Parasitol. 20:469-476.
- Wynn SG, Fougere BJ (2007). Introduction: Why use herbal medicine. In: Wynn SG, Fougere BJ (Eds). Veterinary Herbal medicine. Library of Congress cataloging-in publication data. p695. ISBN: 10:0-323-02998-1.

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