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Full Length Research Paper

Evaluation of the therapeutic and toxicological knowledge of herbalists on the most notified plants in the poison control and pharmacovigilance center of Morocco

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According to the World Health Organization, 80% of the population in developing countries is engaged in traditional medicine. Consequently, the issue of poisoning by plants is not negligible. The objective of this study is to evaluate the therapeutic and toxicological knowledge of herbalists of the most reported plants in the anti-poison center of Morocco. Field study by direct interview with 20 herbalists of the Rabat-Témara region to assess their therapeutic and toxicological knowledge of the most reported plants in the anti-poison center of Morocco, as well as the conditions of their sale, through a questionnaire. A total of 20 herbalists were accepted to participate in the study. Not all of them had a herbalist certificate and only two knew all the plants studied. The most recommended plant by herbalists to their clientele was *Atractylis gummifera*. Although the law prohibits the possession and sale of any toxic plant, the availability of these plants to the herbalists surveyed varies between 100% for *A. gummifera* and 0% for *Hyoscyamus falezlez*. None of the herbalists received notifications of cases of intoxication. Although the therapeutic knowledge of herbalists was well advanced, their toxicological knowledge was not, so we note that the majority of herbalists did not know with precision the possible side effects of the plants sold, or how they could be used safely. Although plants have real and beneficial effects, they are not devoid of side effects that can sometimes be fatal; hence the need to focus on regulation of the functions of the herbalist.

Key words: Herbalists, plants, phytotherapy, poisoning by plants.

INTRODUCTION

Since time immemorial, humans have used plants: first to feed themselves, then to heal themselves. Medication by plants is currently experiencing a veritable revival,

particularly in countries like Morocco, which is known for its great wealth of plants (nearly 42 000 species, including nearly 600 used in traditional medicine)

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(Hmamouchi, 1995). The World Health Organization estimates that 80% of the world's population uses herbal medicines for some aspect of primary healthcare. Although modern medicine is well developed almost everywhere in the world, a significant proportion of the population still relies on herbalists and traditional healers, generally poorly or not at all trained about the diagnosis of diseases and handling of herbal medicine (Bousliman et al., 2012; Bouayyadi et al., 2015).

Consequently, the proportion of intoxications linked to the use of plants is not negligible; they still constitute today a frequent cause of hospitalization in Morocco (Hamia et al., 2009). According to the latest general report of the Anti-poison and Pharmacovigilance Center of Morocco (CAPM), 197 cases of intoxication by plants and products of the traditional pharmacopoeia (PPPT) were identified in 2017, making it the ninth most common (1.17%) cause of intoxication in Morocco. Moreover, the incriminating PPPT were unknown in 27.04% of cases (Chebat, 2017). Thus, there is a need to evaluate the clinical efficacy of plants, ensure their safety, strengthen the knowledge and performance of herbalists and phytotherapists, and ensure adequate follow-up of patients. For this reason a field study was conducted with herbalists to evaluate their toxicological and therapeutic knowledge of the principal plants cited in cases of vegetal intoxication collected by the CAPM, and to determine the availability and conditions of sale of these plants.

MATERIALS AND METHODS

A transverse descriptive study was conducted by direct interview with 20 herbalists of the region of Rabat-Témara. This study took place from 01 June to 30 August 2016. A field survey by means of a questionnaire (Annex 1) was carried out. The plant objects of study were the 10 plants most reported to the Phytovigilance Unit of the CAPM. The list of these plants was retrieved during a meeting with Unit officials. The plants are reported to the CAPM by their French, Arabic, or Amazigh common names, and the center systematically specifies the scientific name of the plant according to the international binomial nomenclature. These declarations are registered in a Herbal medicines adverse events database. The herbalist participants in our study were randomly selected without distinction as to sex, age, or professional experience. The established questionnaire contained 17 questions (9 multiple-choice and 8 open questions) and covered four main topics:

- (i) Information concerning the herbalist;
 - (ii) Information on the plant, its use, and its mode of preparation;
 - (iii) Information about its availability to the herbalists and the conditions of its sale;
 - (iv) Information about the toxicity of the plant in question.
- The results obtained from this study were analyzed through the software SPSS.10.

RESULTS

After having consulted the head of the Phytovigilance Department of the CAPM, the 10 plants most reported to the Department (Table 1) are as follows: *Atractylis*

gummifera; *Papaver somniferum*; *Datura stramonium*; *Nerium oleander*; *Mandragora autumnalis*; *Rubia peregrina*; *Hyoscyamus falezlez*; *Citrullus colocynthis*; *Aristolochia longa*; *Indigo* sp. During the period of study, 30 herbalists were consulted (12 in Temara and 18 in Rabat), but only 20 agreed to participate in the study. The average age of the participants, all male, was 39 years (range: 22-60). Of the 10 plants studied, 9 were deemed poisonous plants (*A. gummifera*; *P. somniferum*; *D. stramonium*; *N. oleander*; *M. autumnalis*; *C. colocynthis*; *A. longa*, *H. falezlez*, *Indigo* sp) the one plant not deemed poisonous (*R. peregrina*) can become toxic under certain conditions of use. None of the participants was a holder of herbalist's certificate. The rest of the results will be presented according to other major axes addressed.

Information on the plant, its use, and its mode of preparation

The average number of Arabic vernacular names reported varies between one and four appellations for *D. stramonium* (Chdaq jemal; Alhayare; Habate semkala; Alghita). Apart from their therapeutic uses, 20% of the plants were used as an abortifacient, 20% for criminal intent, 15% in witchcraft, and 10% as psychoactive plants. *A. gummifera*; *N. oleander*; *R. peregrina* and *C. colocynthis* were known by all the herbalists surveyed (100%), while *H. falezlez* was known only by two (10%) herbalists. The plants most recommended to the public were *A. gummifera*; *R. peregrina*; *Indigo* sp and *C. colocynthis*. Table 1 summarizes the principal therapeutic uses of the plants studied as expressed by the herbalists consulted.

Information about its availability to the herbalists and the conditions of its sale

A. gummifera; *C. colocynthis*; *R. peregrina* were available to all the herbalists (100%) interviewed, while *H. falezlez* was not available to any herbalist. The average number in grams sold for each plant is reported in Table 1.

Information about the toxicity of the plant in question

No herbalist reported ever receiving notifications about cases of intoxication. The declared symptoms of toxicity were generally digestive and neuropsychologic. Of the toxic plants, only *A. gummifera* was recognized as toxic by all (100%) of the interviewed herbalists, while none of the surveyed herbalists (0%) knew *H. falezlez* to be a toxic plant. The toxic risks most announced to buyers were those of *A. gummifera*, *C. colocynthis* and *M. autumnalis*.

Table 1. The top ten most notified plants in the phytovigilance department of CAPM.

Arabic names	vernacular	Scientific Latin name	Main therapeutic uses announced by herbalists	Number of herbalist who knows the plant	Number of Herbalist Advising the plant to the public	Availability of the plant at the herbalist	Average number in gr sold at a time	The symptoms of toxicity announced by the herbalists	Number of Herbalist Advertising Toxicity of Toxic Plant
Adda		<i>Atractylis gummifera</i>	Skin lightening (18*); Anti acne (12*); Andabscess (3*)	20*	09 * including 8 * explain the mode of use: powder + henna skin	20*	30 g	Eye toxic (2*) + vomiting (5*) + death (16*)	20* only 08* inform about its toxic risk
Khachkhach; kharchacha; alafyoune		<i>Papaver somniferum</i>	Hypnotic for children (16*); Anxiolytic (4 *)	18*	0*	16*	07 g	Physical and psychic dependence (8*); digestive (3*); neurologicdisorders (5*)	18* only 1* informs about its toxic risk
Chdaqjemal; alhayare; habatesemkala; alghita		<i>Datura stramonium</i>	Hypnotic (10*); Antitussive (3*); Antiseptic (2*); Exciting of CNS (3*)	15*	1*	13*	06g	delirium and hallucinations (8*)	15* only 1* informs about its toxic risk
Defla; alhloya; almor		<i>Nerium oleander</i>	Antirheumatic(12*); Antiparasitic (3*); Against dermatosis(3*)	20*	2 * none of which explains the method of preparation	18*	12 g	hepatic (3*); renal (4*); several organs disorders (5*)	15* only 2* informs about its toxic risk
Bidelghoul;teryala		<i>Mandragoraaut umnalis</i>	Aphrodisiac (08*); antirheumatic(08*); to beautify the hair (2*)	13*	02 * none of which explains the method of preparation	10*	150 mg	delirium and hallucinations (10*)	12* only 4* informs about its toxic risk
Fuwwa		<i>Rubia peregrina</i>	Antianemic (13*); for hair coloring (9*)	20*	08 * including 05 * explain the preparation mode: decoction in milk	20*	50 g	00	-
Btina		<i>Hyoscyamus falezlez</i>	For weight gain (1*)	02*	00*	00*	-	00*	00*
Hdej; hantel; ferzir		<i>Citrullus colocynthis</i>	Antirheumatic(13*) Aphrodisiac (9*); hypoglycemic (4*); Antivenin (4*)	20*	05 * including 03* explain the preparation mode: put the drug underfoot	20*	80 g	delirium (05*); death (04*)	18 * only 5* informs about its toxic risk
bereztem		<i>Aristolochia longa</i>	Anticancer (13*); Anti hair loss (3*); Wound healing (2*)	16*	02 * that explain the method of preparation: mixing honey with powdered drugs	10*	50 g	No herbalist knows the symptoms	04* none* informs about its toxic risk
Nila		<i>Indigo sp</i>	A component of «khol » (11*) applied on the eyes; skin lightening (5*)	16*	08* including 04* explain the method of preparation: drug powder + soap	15*	30 g	In case of ingestion: digestive and renal disorders (02*)	03* none* informs about its toxic risk

g : grams, *Number of herbalist.

DISCUSSION

Men have always tried to use the properties of certain plants for therapeutic purposes. Evidence of the use of plants for medicinal purposes dates as far back as 60 000 years ago (Solecki and Shanidar, 1975).

In Morocco the use of plants is far from negligible and it is practiced in a completely anarchic way. The Moroccan population often uses them for therapeutic purposes without taking any precautions.

Unfortunately, this enthusiasm, which is not without some hurdles and overruns imply a significant impact on plant-related poisoning (Pentel et al., 2005).

Several factors explain this often irrational and uncontrolled use for this medicine called natural medicine, firstly its reputation for safety and efficacy, its affordability compared to modern medicine, sometimes unable to treat a disease (Lehmann, 2015; Die-Kacou et al., 2009; El Hassani et al., 2013). Besides the lack of an official and codified traditional pharmacopoeia, and the lack of legislation and control determining a viable distribution system to ensure the quality of these products. In the same way, the real ignorance of the properties, the modes of use and the potential risks of the plants, by the people who sell these plants ("achaba" and "aatara"). Which failures open the way to all kinds of skidding in the collection, sale and use of plants, and also hinder the development, optimization and development of our natural resources (Soulaymani, 2010). The "Achaba" are the actual herbalists or merchant who sell the products of plant origin mainly (medicinal plants, condiments and toxic plants), but also minerals and animals or parts of animals. They play an important role at the medical level through the availability of their products and the propagation of their advice (Bellakhdar, 1997; Meziane, 2003). Unfortunately the level of knowledge and skill of some "achaba" is not all satisfactory.

Rarely prescribed by doctors, most of these products, which moreover have a very variable quality in the absence of standards of quality and control, are on free sale and in retail among herbalists.

From a legislative point of view, the profession of herbalist in Morocco is regulated by three separate laws dating all before 1960 (Soulaymani, 2010). The Dahir of February 27, 1923 relating the practice of the profession of herbalist to the provisions of the Dahir of April 12, 1916 whose second article is: "it is especially forbidden for herbalists to sell any poisonous or toxic plant".

Indeed, at many "achaba" plants and animal and mineral toxic products continue to be sold (Soulaymani, 2010), as for example in our case: *A. gummifera* which is sold by all the herbalists interviewed; *P. somniferum*; *D. stramonium*; *N. oleander*; *M. autumnalis*; *C. colocynthis*; *A. longa*; *H. falezlez* and *Indigo* sp.

In Morocco, the job-training of herbalists is usually done, oral transmission of knowledge from father to son

or from teacher to boy (Bellakhdar, 1997), therefore, knowledge in botany and herbal medicine may be lost. None of the herbalists participating in our survey has a certificate or diploma in herbalism, while the Dahir of February 19, 1960 with Article 17 stipulates that to hold and sell the plants or parts of medicinal plants, fresh or dry, the exception of plants classified in the various tables of poisonous substances, the person concerned must be provided with the herbalist's certificate and authorized under the conditions provided in Article 2 of the same Dahir.

If the legal vacuum is patent regarding the function of herbalist and the herbalism in Morocco, it is as much of the specialized and academic training in the field, evidenced by an herbalist diploma, and which allows these herbalists to have the necessary skills (knowledge of the properties, the indications and toxicities, supply, preparation of mixtures, etc.) (Soulaymani, 2010). Each plant may have more than one vernacular name, the herbalist may not know them all, and so errors in plant determination are possible. Behind the therapeutic use, it has been found in this study that other etiology can lead to intoxication by plants, such their use: as abortive; for criminal purposes; in witchcraft or as psychoactive substance.

The evolution and increasing enthusiasm of phytotherapy, during last year's, have deepened the analysis of its therapeutic efficacy and specially its toxicological aspect. This last aspect remains behind the progress of herbal medicine. Indeed, the use of traditional herbal treatments can cause therapeutic failures or accidents. This observation has been confirmed by our results, since most of the therapeutic uses reported by herbalists are described in the literature (Table 2), except in two cases. The first, *H. falezlez*, which was known only from two herbalists, one of whom reported that it is used to fatten, indication not reposted in the literature. The second case concerned *A. longa*, for which three herbalists declare that it is used against hair loss. Certainly, our herbalists have not cited all traditional uses, since a single plant may have several uses, but at least those mentioned are the main traditional uses, which have a close relationship with the mechanism of action of chemical components of the plant. Unlike the well advanced therapeutic knowledge of herbalists, toxicological knowledge remains behind the first. This is how we found that the majority of herbalists don't know exactly the manifestations of toxicity, nor the potential side effects, nor how they can be safely used, especially since the majority of the events cited are not mentioned in the literature. Table 2 summarizes the main traditional use and main symptoms of intoxication described in the literature of the top ten most notified plants in the phytovigilance department of CAPM. Moreover, these events cited by our herbalists have no scientific arguments that can be explained by the mechanisms of action of the toxic components of the plant in question.

Table 2. Main traditional uses and main symptoms of intoxication described in the literature of the top ten most-notified plants in the phytovigilance department of CAPM.

Plants	Main traditional uses	Main symptoms of the poisoning
<i>Atractylis gummifera</i> (Daniele et al., 2005; Skalli et al., 2002; Mouaffak et al., 2013).	Stop the bleedings; facilitate the deliveries; treat freckles, acne pimples and abscess	Convulsive crises ; vomitings ; fulminant hepatitis
<i>Papaver somniferum</i> (Bousliman et al., 2012 ; El Hafian et al., 2014).	Sedative of nervous system; hypnotic especially for the children	ringing in the ears; vomiting; constipation; bradycardie respiratory disorders; somnolence.
<i>Datura stramonium</i> (Flesch, 2005 ; Bhakta and Subedi, 2013; Hammiche et al., 2013).	Aphrodisiac; analgesic, hypnotic; antitussive; vulnerary.	Agitation, confusion, hallucinations, delirium, dryness of the mucous, mydriasis, tachycardia
<i>Nerium oleander</i> (Hammiche et al., (2013) ; Fakoorziba et al., 2015; Barguil and Anger, 2012 ; Flesch and Krencker, 2007).	Treatment for dermatosis ; rheumatic pains; leprosy, malaria; venereal diseases and diabetes	Digestive disorders, vision and colors disorders, atrioventricular block, bradycardia
<i>Mandragora autumnalis</i> (Passos and Mironidou-Tzouveleki, 2016); Piccillo et al., 2006); Ghourri et al., 2014) ; Gouaz, 2017).	Aphrodisiac, hypnotic, fortifying hair, used in urinary tract infections and in rheumatism	hallucinations, gastrointestinal irritation, mydriasis, tachycardia
<i>Rubia peregrina</i> (El Hafian et al., 2014 ; Gouaz, 2017 ; El Azzouzi and Zidane, 2015).	Treatment of anemia, icterus, liver disease and bowel pain; used to color hair	hepatic toxicity
<i>Hyoascyamus falezlez</i> (Hammiche and Maiza, (2006); Schmelzer and Gurib-fakim, (2008) ; Gaillard et al., 2001).	relieves pain, muscle cramps, spasms, palpitations, asthma, anxiety, treatment of cystitis	Narcotic action and important hallucinogen
<i>Citrullus colocynthis</i> (Hammiche et al., 2013 ; Benkhnique et al., 2014; Bnouham et al., 2002).	Actions: antirheumatic, purgative, anthelmintic, aphrodisiac, antidiabetic; so against venomous bites	Vomiting, abdominal pain, bloody diarrhea, confusionalstate
<i>Aristolochia longa</i> (Bouayyadi et al., 2015; Gouaz, 2017 ; Zekkour, 2008).	Cancer treatment, scorpion stings and bites, arthritis; healing wounds	Irreversible renal damage, limb paralysis, respiratory disorders
<i>Indigo</i> sp (Labiba et al., 2012; Bellakhdar, 1997).	Skin disorders, antiparasitic and antiseptic. treatment of cough and ophthalmia, is part of the constituents of Khôl	Liver injury, cardiocirculatory failure

Considering these results, we can see that the majority of herbalists, who are supposed to play a very useful role in the proper use of these products by their permanent availability in the service of the citizen, don't have the necessary and sufficient skills to advise or sell the plants. Also, the sold quantity in grams of these plants, in most cases exceeds the toxic doses described in the literature, which exposes to the risks of poisoning even more that these toxic risks are not always declared to the users, and that for most consumers, natural is synonymous to harmless, however a plant can be both useful and toxic, it is only a question of dose. Indeed, plants are considered by the population as health products and they must therefore obey, as for medicines, strict standard rules that only the specialist in herbal medicine can respond (Zeggwagh et al., 2013). Unfortunately, the legal vacuum maintains anarchy in the marketing of medicinal and toxic plants. However, the authorities must intervene to ensure the application and compliance of existing laws, to provide continuing training in herbalism; to organize awareness-raising campaigns for herbalists on the dangers of misuse of certain plants and the serious and

even fatal adverse effects that result, as well as on the growing interest in the spontaneous notification of adverse effects observed when using the plants for therapeutic purposes, which is the cornerstone of phytovigilance from which the benefit / risk ratio of plant use can be evaluated.

Conclusion

The problem of the toxicity arises for products which get through the evaluation and scientific control, although the market of these products is expanding rapidly. Many people and sometimes the medical corps consider that plants are safe and harmless because they are natural. At present, the warnings are more and more frequent and the awareness by the healthcare professionals is increasing. We hope that a sufficient attention should be given to the exercise of the profession of herbalists, because they are considered as an important link in the chain of securitization of phytotherapy. This calls for regulating a profession of herbalists in our country.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Annex 1

QUESTIONNAIRE

Herbalist N°:; Age:.... sex :..... area of residence:.....

Plant X :.....

1. Do you know the plant X? YES NO
2. The plant X is available in your shop? YES NO
3. It has another name? YES NO
4. If yes, What are the others?.....
5. What are their therapeutic uses?.....
6. It has any misuses?
7. What is the part of the plant sold?.....
8. Which is its frequency of sale?.....
9. What is the average of grams sold at the same time?
10. You recommend it to the public? YES NO
11. Do you know that it is a toxic plant? YES NO
12. If yes, and according to your knowledge, which are his symptoms of toxicity?.....
13. You inform the buyers of its toxic risks? YES NO
14. Do you explain to the buyers the mode of preparation and the dose to use?
YES NO
15. Have you already received a notification about case of toxicity due to this plant? YES NO
16. If yes, what was your requested action?.....
17. Do you have an herbalist's certificate? YES NO

Full Length Research Paper

The Mexican mistletoe *Struthanthus venetus* (HBK Blume) inhibits proliferation and synergizes antagonistic actions of Tamoxifen and Fulvestrant in breast cancer MCF-7 cells

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Mistletoes are hemi-parasitic plants widely distributed around the world, used in folk medicine to treat many diseases including diabetes, hypertension, menopausal syndrome and as complementary or adjuvant treatment for cancer. The objective of the present work was the evaluation of oestrogenic activity of hydromethanolic extract of mistletoe *Struthanthus venetus* dried leaves (StvHME), to evaluate its potential benefits in menopause and breast cancer, which have not been reported so far. Uterotrophic activity was evaluated in immature female CD1 mice, administering StvHME (10, 100 and 500 mg/kg) for three consecutive days compared to the natural hormone 17 β -oestradiol (E₂, 10 μ g/kg). Comparison of the MCF-7 positive oestrogen receptor human breast adenocarcinoma cell proliferation in the presence of StvHME (0.5, 5 and 50 μ g/ml) or E₂ (10⁻¹²-10⁻¹⁰M) related to untreated control cells was assessed using MTT cell viability assay. StvHME, produced biphasic effects in mice uteri; low doses (10 mg/kg) decreased uterine weight (15-38%; $p < 0.05$), while a higher dose (500 mg/kg) increased uterine weight (28-36%; $p < 0.05$). StvHME concentrations tested inhibited MCF-7 cell proliferation, contrasting with E₂ which increased it. StvHME (50 μ g/ml) antagonized the proliferative response to E₂ (10⁻¹² to 10⁻¹⁰ M) behaving as an anti-oestrogen. The antiproliferative response of StvHME (50 μ g/ml) showed synergism with the oestrogen antagonists Tamoxifen and Fulvestrant (ICI 182,780). Our results suggest the presence of oestrogenic and anti-oestrogenic components in the StvHME that could be acting through the oestrogen ER α and ER β receptors. Therefore, StvHME has potential utility as a complementary therapy for breast cancer.

Key words: mistletoe, *Struthanthus venetus*, MCF-7 cells, oestrogenicity, anti-oestrogenic, anti-cancer, menopause.

INTRODUCTION

Breast cancer is one of the leading causes of death among women in the world. Recently it has been

estimated that the incidence of all-cancer cases, including breast cancer, will almost double by 2030 (Bray

et al., 2012). Increased population aging due to the rise of life expectancy also increases the possibility of cancer incidence. As a result of a longer life span, the menopausal women population will also increase, coping with health disorders for a longer period (Angioli et al., 2018). During menopause, oestrogen deficiency induces vasomotor symptoms, hyperlipidemia, osteoporosis, and cardiovascular disease. Hormone replacement therapy (HRT) with exogenous hormones currently used to relieve menopausal symptoms, has proven to be effective in alleviating some of them such as hot flushes, night sweats, dyspareunia, sexual disorders, insomnia, and preventing osteoporosis (Rozenberg et al., 2013). Nonetheless, there is great controversy in recent menopausal population studies using HRT that have warned about the incidence of adverse effects, including increased risk of ischemic stroke, venous thromboembolism, and breast cancer. Because of the high risk among HRT users, the therapy is not allowed to be used for long periods, particularly in predisposed patients (NICE guideline NG23, 2015). Thus, the search for effective and safer treatments for menopause continues to be a priority.

Plants and herbs have reached an important approach as complementary or alternative treatment for many diseases and are considered to be important for the development of new strategies for both HRT and cancer. Numerous naturally occurring phytochemicals have recently gained interest as potential therapeutic breast cancer agents, which appear to directly affect oestrogen-dependent and oestrogen-independent breast cancer cell proliferation (Israel et al., 2018).

Mistletoes are widely used since ancient times in folk medicine of many cultures to treat diseases including diabetes, hypertension, cancer and to prevent menopausal syndrome (López-Martínez et al., 2013; Omeje et al., 2014). They are hemi-parasitic plants widely distributed around the world. Taxonomically, they belong to the families Misodendraceae, Loranthaceae, Santalaceae, Viscaceae among others, which are grouped in the order Santalales (Lim et al., 2016; Patel and Panda, 2014). The mistletoe *Struthanthus venetus*' taxonomic classification is: Phylum Plantae > Subphylum Magnoliophyta > Class Magnoliopsida > Order Santalales > Family Loranthaceae > Genus *Struthanthus* > Species *venetus*. This plant develops as an epiphyte hemi-parasite germinating in trunks, branches or stems of shrubs such as tulips, aralias, citrus fruits, walnuts, and trees like the casuarinas, among others (Plate 1).

The *S. venetus* is commonly known as "matapalo" as well as "injerto" (in Spanish) and is used in Mexican traditional medicine due to its anti-cough, sedative,

hypoglycaemic and antihypertensive properties (Andrade-Cetto and Heinrich, 2005; Gijón et al., 2010; Alvarez, 2003).

Recent studies have reported that long-term oral consumption of water extract from the Korean mistletoe *Viscum album* in ovariectomized rats (an oestrogen-deficient model of post-menopausal stage) alleviates menopausal symptoms and modulates glucose and lipid metabolism (Kim et al., 2015). The anti-osteoporosis effects of aqueous-methanol extracts of the Eastern Nigerian mistletoe *Lorantus micrants* have also been described (Omeje et al., 2014). Considering the oestrogens' protection mediation on bone health during women's reproductive life, these results may indicate the presence of phyto-oestrogens in the mistletoe extracts, however, it is unknown if these hemi-parasitic plants possess oestrogenic activity since this approach has not been explored.

On the other hand, anticancer activity of mistletoe extracts has been reported in human cancer cells and animal models (Varela et al., 2004, Alonso-Castro et al., 2012). The Korean mistletoe *Viscum album* is also used to treat hepatic, renal and uterine human tumour cells. In patients, an increase in health-related quality of life, remission rate, survival time, and alleviating adverse reactions to conventional breast cancer therapy has been previously described (Marvibaigi et al., 2014). The related mistletoe genuses *Scurrula* and *Viscum* have also been described to possess anticancer, antioxidant and antihypertensive properties (Lim et al., 2016).

Since, there are no previous reports about oestrogenicity and anticancer activities of *S. venetus*, the objective of the present work was to explore them using two standardized protocols: the *in vivo* uterotrophic assay in immature female CD1 mice (Kleinstreuer et al., 2016) and the MCF-7 human breast oestrogen positive cancer cell proliferation known as the E-Screen method, considered the most sensitive assay to predict oestrogenic activity since their proliferation is oestrogen dependent (Soto et al., 1995; Körner et al., 1999).

MATERIALS AND METHODS

Reagents

17 β -oestradiol (E₂; 1,3,5(10)-estratrien-3,17 β diol), Fulvestrant (ICI 182,780) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tamoxifen was a gift from ASOFARMA, (México). Cell culture reagents were obtained from Gibco (Invitrogen Corporation, Waltham, MA, USA).

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Plate 1. *Struthanthus venetus* adhered to the stem of a tulip.

Collection and preparation of the *Struthanthus venetus* hydro-methanolic extract (StvHME)

Leaves of *S. venetus* were collected during the spring in Oaxtepec, Morelos, México and registered with number 33,393 of the National Herbarium of the Biology Institute, UNAM. The material was air-dried in the shadow for 15 days, and then finely grounded with a Wiley mill (200 mesh) to powder. *S. venetus* hydro-methanolic extract was prepared from a 100 g sample of the plant, powder mixed with 200 ml of methanol-water (50:50 v/v) at room temperature (25°C) and allowed to stand for 24 h. Extractions were carried out over 5 days with brief daily manual shaking, the supernatant was removed and placed in a glass container for evaporation; the solid residue yielded 9.6 g/100 g of StvHME dry material.

Solution used for the uterotrophic and MCF-7 cell proliferation assays

A dry sample of 100 mg of StvHME was dissolved in 10 ml of 50:50 (v/v) ethanol-water solution and passed through a #1 filter paper (Whatman Inc., Hillsboro, OR, USA) to a final concentration of 10 mg of StvHME/ml (stock solution) which was stored at 4°C in amber glass vials until use. Previous to assays, the stock solution was diluted 1:10 in saline. Afterwards, work solutions were diluted with the solvent or culture medium at the appropriated concentrations.

Preliminary phytochemical screening

A sample of the stock solution (10 mg of StvHME/ml) was diluted 1:10 mg/ml with distilled water:ethanol (50:50) to get 1 mg/ml solution which was used for all phytochemical tests. To identify the

constituents present in StvHME standard methods were used (Zohra et al., 2012; Kuklinski, 2000). For each test 350 µL of StvHME sample was used, the following tests were carried out:

Frothing test: to the sample 10 ml of distilled water were added and shaken for 30 s. The mixture was then left for 30 min and observed. Formation of honeycomb froth indicates the presence of saponins.

Lieberman-Burchard test: the sample was treated with two drops of acetic anhydride, heated gently and let cool off. Then, concentrated sulphuric acid was carefully added through the walls of the test tube. The final reaction shows a brownish-red layer or ring above a greenish liquid, which indicates the presence of sterols and triterpenes.

Mayer's test: the sample was treated with 1 ml drop by drop of Mayer's reagent. The formation of a green-creamy precipitate indicates the presence of alkaloids.

Dragendoff's test: the sample was treated drop by drop with 1 ml of Dragendoff's reagent. The formation of a reddish-brown precipitate indicates the presence of alkaloids.

Folin's test: to the sample five drops of Folin reagent and two drops of Na₂CO₃ (7.5%) were added. A green colour indicates a few phenols, a light blue indicates moderate phenols and intense blue indicates abundance of phenols. **FeCl₃ test:** to the sample three drops of FeCl₃ solution (1%) were added; formation of a greenish-black colour indicates the presence of phenolic compounds.

Shinoda's test: to the sample some magnesium chips and 2 drops of diluted HCl (0.5 N) were added and heated gently. A pink or red

colour indicates the presence of flavonoids.

Sodium hydroxide test: to the sample 2 ml of NaOH (10%) solution were added. A yellow colour indicates the presence of flavonoids, which on adding diluted HCl (0.5 N) became colourless.

Anthocyanidins: the sample was treated with one drop of concentrated HCl and shaken. Then 0.5 ml of NaOH (20%) was added. A colourless solution indicates the presence of anthocyanidins.

Non-reducing sugars: the sample was treated with 1 ml of concentrated hydrochloric acid and gently heated. Then 1.5 mg of resorcinol were added and heated for 2 min. An orange or red colour indicates the presence of non-reducing sugars.

Grignard's test: in a glass tube with a lid, the sample was placed and 1 ml of distilled water with one drop of CHCl_3 were added. A strip of filter paper, previously soaked with picric acid solution (0.5%) and Na_2CO_3 (5%) was placed inside the tube and heated to 35°C for 24 h. A red or pink colour indicates the presence of cyanogenic glycosides.

Borntrager's test: to the sample, 5 ml of CHCl_3 were added and shaken for 5 min, filtered and then 5 ml of an ammonia solution (10%) were added shaken again, a pink, red or violet colour in the aqueous layer after shaking indicates the presence of free anthraquinone.

Filter paper strip: the sample was placed in a glass tube with a lid; a strip of filter paper previously soaked in NaOH (5%) was placed inside the tube without touching the extract, closing the lid and then heated. The presence of fluorescent spots on the paper when observed with an UV light indicates the presence of coumarins.

Ninhydrin test: to the sample three drops of Ninhydrin reagent were added and boiled for few minutes. A blue colour indicates the presence of amino acids.

Hydrolysable tannins: to the sample 3 mg of NaNO_2 and three drops of glacial acetic acid were added. A pink or brown colour indicates the presence of hydrolysable tannins.

Condensed tannins: to the sample 2 ml of butanol were added and shaken, then 0.5 ml HCl (0.5N) and heated. The blue colour when adding NaHCO_3 indicates the presence of condensed tannins.

Arnow test: 2 ml of HCl (0.5N), 2 ml of NaNO_2 (10%) and three drops of NaOH (2N) were added to the sample. The pink, orange or purple colour when adding NaOH indicates the presence of phenylpropanoids.

Carotenoids: to the sample 0.5 ml of CHCl_3 and 1 ml concentrated sulphuric acid were added and cooled. The appearance of red or blue ring at the contact zone of the two liquids indicates the presence of carotenoids.

Lactones: to the sample four drops of NaOH (10%) and two drops of concentrated sulphuric acid were added. A yellow colour indicates the presence of lactones.

Animals

All experimental studies were conducted in accordance to the Mexican National Protection Laws on Animal Protection and the

General Health Law Related to Health Research (NOM-062-Z00-1999). The animals were obtained from the animal facilities of the Faculty of Medicine of the National University of México. Immature female CD1 mice (10-15 g, 21 days old) were used to evaluate oestrogenic activity by uterotrophic assay (Kleinstreuer et al., 2016). The animals were kept in a room at constant temperature (20-22°C) with 12-12 h light-dark cycle, food and water intake were monitored maintaining standard chow (Nutricubos, Purina) and water *ad libitum*.

Evaluation of uterotrophic activity of the StvHME

The animals were weighted and distributed among groups according to a balanced Latin-square block design based on body weight (6-7 animals/group in each experiment) and randomly assigned to treatment groups. Different groups of animals were subcutaneously (s.c.) injected once a day for three consecutive days with the positive reference E_2 (10 $\mu\text{g}/\text{kg}$), or StvHME (10, 100 and 500 mg/kg), the control group (C) only received the vehicle (V; 10 ml/kg). After 24 h from the last treatment, animals were weighted, and uteri were dissected, blotted, and weighted to obtain uterine wet weight. Then the uteri were dried at 37°C for 24 h, and weighted again to obtain uterine dry weight. Uterine weights of the treated and the control groups were expressed in mg.

Cell proliferation experiments

MCF-7 cell-line culture conditions

Human MCF-7 breast cancer oestrogen receptor-positive cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). MCF-7 cells were grown in phenol red-free Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Invitrogen Corporation, Waltham, MA, USA) high glucose, supplemented with 1% (v/v) of an antibiotic-antimycotic mix (penicillin G sodium, streptomycin sulphate and amphotericin B). For all experiments, 10% (v/v) of charcoal-dextran stripped foetal bovine serum (CDFBS) hormones free was used according to a reported method (Körner et al., 1999). The cell culture was maintained in a humidified atmosphere of 5% carbon dioxide in 95% air at 37°C. The cell stocks were sub-cultured weekly at 70% confluence over a maximum of 20 passages using 0.05% trypsin-0.02% EDTA (pH 7.3). E_2 was prepared as stock solution in ethanol (0.1 M) and the StvHME stock solution (10 mg/ml) were freshly diluted in culture medium avoiding ethanol concentrations higher than 0.01% (v/v). To explore the mediation of the oestrogen receptors in the cell proliferation rate, they were treated with StvHME and co-incubated with the anti-oestrogens Tamoxifen (1×10^{-6} M), or Fulvestrant (ICI 182,780; 10 nM).

Experimental design of cell proliferation

In all the assays, 2500 cells contained in 150 μL were placed into each of the 96 wells of plastic tissue culture plates (Falcon). The cells were left to attach for 24 h, afterwards the cell culture media was substituted for the treatment. In all assays each concentration was tested in 8 replicates including control cells.

Experiments

1) A temporal course of proliferative response was explored comparing proliferation of MCF-7 cells in the presence of E_2 10^{-10} M or StvHME 50 $\mu\text{g}/\text{ml}$, and control cells incubated only with CDFBS

Table 1. Uterotrophic activities of E₂ and the StvHME in immature CD1 mice.

Group	Dose	a Body weight (g)	Uterine wet weight (mg)	b Uww Δ%	Uterine dry weight (mg)	c Udw Δ%
V	10 ml/kg	9.3 ± 0.27	11.4 ± 0.85	100	3.40 ± 0.18	100
E ₂	10 µg/kg	9.4 ± 0.24	42.8 ± 2.34	275	9.37 ± 0.52	175
StvHME	10 mg/kg	9.4 ± 0.21	7.1 ± 0.50	-38*	2.88 ± 0.13	-15*
	100 mg/kg	9.3 ± 0.20	8.9 ± 0.51	-22	3.03 ± 0.19	-10
	500 mg/kg	9.4 ± 0.49	15.5 ± 1.35	+36**	4.35 ± 0.39	+28**

V = vehicle; E₂ = 17β-oestradiol; StvHME = hydromethanolic extract of the mistletoe *Struthanthus venetus*. n/group = 13.

a Body weight after treatment. Values are given in mean ± SEM.

b Uww = uterine wet weight. **c** Udw = uterine dry weight.

T test * $p < 0.001$ (Uww) and $p = 0.027$ (Udw) vs V; ** $p = 0.017$ (Uww) and $p = 0.044$ (Udw) vs V; Δ% = represent the uterotrophic effect calculated as a percentage difference related to the V) control group = [Uww or Udw of E₂ or StvHME × 100/Uww or UdwV] – 100.

media during 3 days.

2) The proliferative response to E₂ (10⁻¹² to 10⁻¹⁰ M) compared with StvHME (0.5, 5 and 50 µg/ml); and their interaction of E₂ (10⁻¹² to 10⁻¹⁰ M) + StvHME 50 µg/ml in a 200 µL medium and the control evaluated on the sixth day of culture.

3) Groups of cells were assigned to the treatment with E₂ 10⁻¹⁰ M, StvHME 50 µg/ml, or StvHME 50 µg/ml co-incubated with the anti-oestrogens Tamoxifen (1x10⁻⁶ M), or Fulvestrant (10nM) and the control.

The proliferative effect on MCF-7 cells was evaluated using the viability MTT assay (Mosmann, 1983). At the end of the experiment, 20 µL of MTT solution (5 mg/ml) in phosphate-buffered saline (PBS) were added to the wells' medium and maintained for 4 h at 37°C. The medium was removed and the formed formazan was dissolved in dimethyl sulfoxide (Merck, Kenilworth, NJ, USA). OD was read in an UV microtiter plate reader at 492-630 nm (Stat Fax 3200, Awareness Technology, Inc. Palm City, USA). The number of cells was obtained from a calibration curve of number of cells-OD and was expressed as a percentage related to the control cells.

Statistical analysis

Numeric values presented for each experiment are the mean of at least two independent experiments. Data was analyzed with the Sigma Plot statistical package (version 2011, Jandel Corporation), using one-way analysis of variance (ANOVA) for comparisons between groups, and T student test or Dunn or Mann-Whitney tests as appropriate. Data are presented as ± standard error (SEM). *P* values below 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

This study describes for the first time, the oestrogenic and anti-oestrogenic properties of the Mexican hemiparasitic mistletoe StvHME. The response elicited by StvHME in uteri of the immature female CD1 mice showed biphasic behaviour (Table 1). StvHME with the 10 mg/Kg dose decreased significantly uterine wet and dry weights (38%, $p < 0.001$ and 15%, $p = 0.027$, respectively) related to the control group, inducing an anti-uterotrophic effect. The 100 mg/kg dose also

decreased uterine weight but the changes did not reach significance. In contrast, the 500 mg/kg dose increased significantly mice uterine wet (36%) and dry (28%) weights ($p = 0.017$ and $p = 0.044$ respectively) suggesting that high concentrations of StvHME are oestrogenic. The positive control E₂ (10 µg/kg) showed its classic significant uterotrophic effect increasing 274-275% of uterine wet and dry weights respectively ($p < 0.001$).

Despite the lack of information about mistletoe oestrogenic activity, our results are in accordance with those reported by Pattanayak and Mazumder (2009) who also described weak oestrogenic activity of the hydroalcoholic extract of the large bushy parasitic plant *Dendrophthoe falcata*, (Loranthaceae family). Also, when the extract was administered with ethynyl oestradiol, it showed a low anti-oestrogenic activity in immature ovariectomized rats (Pattanayak and Mazumder, 2009). However, to confirm oestrogenic or anti-oestrogenic effects of StvHME in uteri, it is necessary to assess these effects using other *in vivo* models and longer treatments, particularly to have information about its potential utility in hipo-oestrogenic conditions like menopause.

On the other hand, StvHME displayed an anti-proliferative effect on breast cancer MCF-7 cells, behaving in an opposite way to E₂. The temporal course effect of exposure to 10⁻¹⁰ M of E₂ and 50 µg/ml of StvHME on MCF-7 cells is shown in Figure 1. The inhibitory proliferative effect of 50 µg/ml StvHME was detected after the first day and maintained in the same magnitude range along the treatment ($p < 0.01$) without dose or time dependency (Figure 1). Meanwhile the E₂ positive (10⁻¹⁰ M) control showed opposite proliferative effects with respect to StvHME after 3 days with high significance ($p < 0.001$).

The effect of different concentrations of StvHME compared with E₂ (10⁻¹² to 10⁻¹⁰ M) on MCF-7 cells is showed in Figure 2. E₂ displayed a dose dependent proliferative response mean while, StvHME in all the

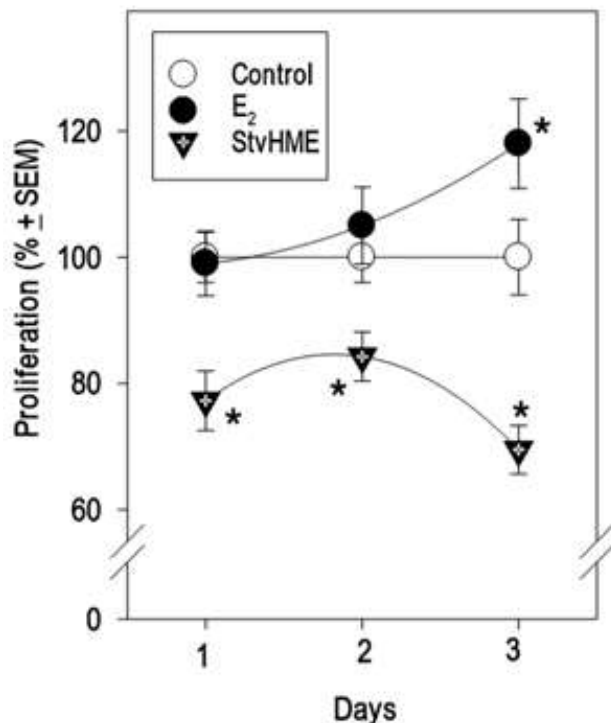


Figure 1. Temporal course effect of E₂ 10⁻¹⁰ M and 50 µg/ml of StvHME on MCF-7 cells (StvHME vs control, **p* < 0.01; StvHME vs E₂, **p* < 0.001).

evaluated concentrations (0.5, 5 and 50 µg/ml) significantly decreased MCF-7 cell proliferation (*p* < 0.05), and it is of note that the 5 and 50 µg/ml concentrations induced decreases of the same magnitude in relation to the control (*p* < 0.05, Dunn's method). Additional to the inhibitory effect of StvHME on MCF-7 cell proliferation, the extract (50 µg/ml of StvHM) was capable to abolish the proliferative response induced by E₂ (10⁻¹² to 10⁻¹⁰ M) acting as anti-oestrogen.

Anti-cancer activity of the mistletoe genera *Scurrula* and *Viscum* has previously been described and attributed to the presence of antioxidant phytoconstituents such as quercetin, which confers protection against cancer and neurodegeneration (Lim et al., 2016). Diverse constituents have been described among other mistletoe species including: lectins and viscotoxins which exert anti-tumor effects by inducing cell cycle arrest, increasing apoptosis, inhibiting angiogenesis, and potentiating immune responses (Osadebe and Omeje, 2009). However, the phytochemistry of the genus *Struthanthus* has been scarcely studied. In the Brazilian hemi-parasite plants *Struthanthus marginatus* and *Struthanthus concinnus* (Loranthaceae) terpenoid compounds have been recently identified (Leitão et al., 2013). Preliminary phytochemical screening of the StvHME extract indicated that it contains saponins, phenols, flavonoids tannins, phenylpropanoids, and lactones (Table 2). In México,

reports about the effect of mistletoes in cancer are scant (Alonso-Castro et al., 2011; Rios et al., 2001; Rivero-Cruz et al., 2005; Waizel et al., 1994). *Phoradendron reichenbachianum* (Loranthaceae) from which moronic acid and a tetracyclic triterpene was isolated, has been described with cytotoxic activity (Rios et al., 2001) and also the Mexican hemiparasitic plant *Phoradendron robinsonii* (Loranthaceae) has been described to contain a flavonoid with antimycobacterial activity (Rivero-Cruz et al., 2005).

In order to know the possibility of oestrogenic agonistic properties of StvHME as indicated in the uterotrophic assay with 500 mg/kg dose, an experiment was performed comparing the effect of the oestrogen antagonists Tamoxifen and Fulvestrant on the response to StvHME 50 µg/ml in MCF-7 cells. Both antagonists increased significantly (*p* < 0.05) the inhibition of cell proliferation elicited by StvHME (Figure 3). Considering that breast cancer is the most common type of cancer among women worldwide with an increasing incidence and mortality during the last two decades (Kolak et al., 2017), and the fact that StvHME shows a noticeable anti-proliferative activity in the MCF-7 breast oestrogen positive cancer cells, such action is of great importance since it can be potentiated by oestrogen antagonists Tamoxifen and Fulvestrant which are used clinically. This Could indicate this extract may be useful as a

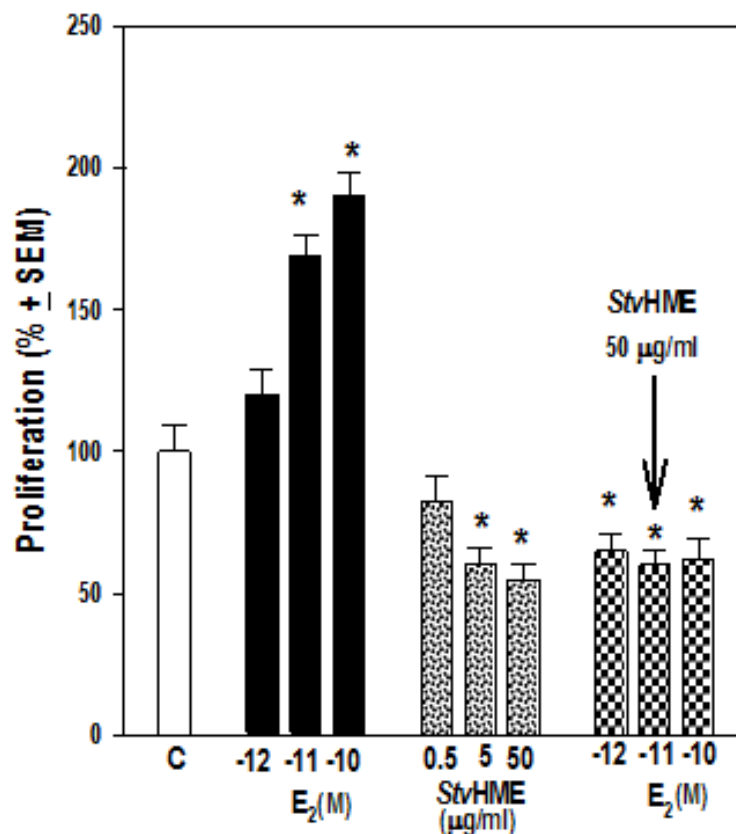


Figure 2. The effect of E₂ (10⁻¹² to 10⁻¹⁰ M), StvHME (0.5, 5 and 50 µg/ml) and the interaction of E₂ (10⁻¹² to 10⁻¹⁰ M) + StvHME (50 µg/ml) on MCF-7 cells. * $p > 0.05$

Table 2. Screening of phytochemical components of StvHME.

Component	Result	Positive control/Standard
Saponins	++	n.d.
Terpenes/steroids	-	Dexamethasone
Alkaloids	-	Berberine
Phenols	+++	Green tea
Flavonoids	++	Quercetine
Anthocyanidins	-	Cyanidin-3-glucoside
Non-reducing sugars	-	Saccharose
Cyanogenic glycosides	-	n.d.
Anthraquinones	-	n.d.
Cumarins	-	4-hydroxycumarin
Aminoacids	-	L-arginine
Tannins condensed	-	Guazuma <i>sp</i> (proanthocyanidine b and c)
Tannins hydrolyzed	++	Green tea
Phenylpropanoids	++	Noradrenaline
Carotenoids	-	Lycopene
Fatty acids	-	Corn oil
Starches	-	Starch
Lactones	++	n.d.

Highly positive: +++; positive: ++; weakly positive: +; - negative. n.d. = not determined.

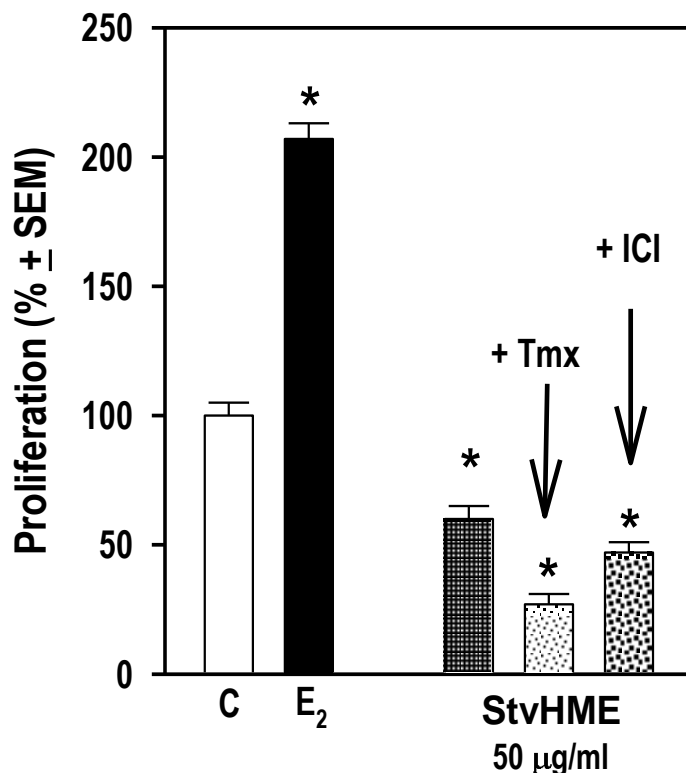


Figure 3. Effect of E₂ (10-10M) and StvHME (50 µg/ml) alone, and concomitantly with Tamoxifen (Tmx) or Fulvestrant (ICI 182,780 ; ICI). Control vs E₂, * $p < 0.01$; StvHME vs StvHME + Tmx, * $p < 0.05$; StvHME vs StvHME + ICI, * $p < 0.05$

complementary therapy in breast cancer patients treated with oestrogen antagonists, particularly in cases where tumour behaviour is aggressive, and there is intolerance to the side effects produced by toxicity of currently used drugs. Future studies in this area should focus on characterizing the effects of StvHME with more detail in long term *in vivo* studies, assessing possible toxicity, and determining selectivity in different cell lines.

In summary, our results show that StvHME in mice produced biphasic actions: at low doses an anti-oestrogenic effect, and at high doses, modest oestrogenic activity. More work is in progress to confirm possible oestrogenic effect of StvHME, to know its potential use during hypo-oestrogenic conditions. The remarkable fact is that StvHME on MCF-7 cells behaved only as an anti-oestrogen. The anti-proliferative properties of the StvHME against MCF-7 human adenocarcinoma breast cancer cells are clear and indicate that the extract could contain oestrogenic and anti-oestrogenic components, probably acting through the oestrogen ER α and ER β receptors. These results provide the basis to encourage further pharmacological and chemical characterization of the mistletoe *S. venetus*, to support its use as phytotherapeutic agent as a promising source of novel bioactive compounds against breast oestrogen-positive

cancer.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

StvHME, hydro-methanolic extract from the mistletoe *Struthanthus venetus* dried leaves; **E₂**, 17 β -oestradiol; **MCF-7**, Michigan Cancer Foundation-7; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **ER α** , oestrogenic receptor alpha; **ER β** , oestrogenic receptor beta; **ATCC**; American Type Culture Collection; **DMEM**, phenol red-free Dulbecco's modified Eagle's medium; CDFBS, charcoal-dextran stripped foetal bovine

bovine serum; **EDTA**, ethylenediaminetetra acetic acid; **PBS**, phosphate-buffered saline; **OD**, optical density; **ANOVA**, analysis of variance.

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Full Length Research Paper

Protective effect of ethanolic extract of *Cucurbita maxima* (PUMPKIN) leaf on acetaminophen-induced acute liver toxicity

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Ethanolic extract of dried leaves of *Cucurbita maxima* (Pumpkin) were screened for their phytochemical composition. The *in vitro* antioxidant property was determined by assessing the free radical (DPPH) scavenging activity. Twenty rats divided into four groups were used for this study with group 4 pre-treated with the extract and later intoxicated with 2 g/kg single dose of acetaminophen. The hepatoprotective effect of the extract was determined by measuring the liver function parameters, liver antioxidant enzyme activities and the rats liver histological micrograph. The ethanolic extract was found to be a rich source of bioactive compounds and showed a direct variation in *in vitro* free radical (DPPH) scavenging property. DPPH scavenging property increases as the concentration of the extract increases from 0.03 to 0.12 mg/l (8.9 - 64.2%) but dropped sharply to 52.2% at a concentration of 0.5 mg/l. A 400 mg/kg daily pre-treatment (for seven days) with ethanolic leaf extract of the plant was able to offer protection to the hepatic cells of the rats. This was evidenced in the significant ($p < 0.05$) reduction of the activities of alanine aminotransferase (ALT) from 117.30 ± 57.50 to 31.26 ± 11.22 μ /l and alkaline phosphatase (ALP) from 209.80 ± 67.00 μ /l to 172.00 ± 30.31 μ /l, significant ($p < 0.05$) increase of the activities of glutathione peroxidase (GPx) from 115.60 ± 10.03 to 235.45 ± 43.52 μ /mg, superoxide dismutase (SOD) from 0.02 ± 0.01 to 0.09 ± 0.05 U/mg and catalase (CAT) from 2.50 ± 2.60 to 10.23 ± 5.05 U/mg in the test group when compared with the negative control. Also, the lobular architecture of the hepatocytes was well-preserved in the test group. Based on the experimental results obtained here, *C. maxima* has an important role in medicine as it plays a role in scavenging free radicals, stimulating activities of antioxidant enzymes and preserving the liver architecture, thereby protecting the liver against acetaminophen-induced liver toxicity.

Key words: *Cucurbita maxima*, hepatoprotection, oxidative stress, free radical-scavenging, hepatocytes.

INTRODUCTION

The liver is the largest organ of the body which is involved in the metabolism and excretion of unwanted

compounds, which may be exogenous (e.g. drugs and poisons) or of endogenous origin (e.g. steroid or

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catecholamine hormones and haem groups). It performs a wider range of biochemical functions than any other organ (Reed, 2009). It is connected with most of the physiological processes, which include growth, immunity, nutrition, energy metabolism and reproduction (Mayuresh et al., 2014).

Paracetamol or acetaminophen is an active metabolite of phenacetin. Acetaminophen (APAP) is an analgesic and antipyretic substance used in the production of the drug paracetamol. It is well tolerated, lacks many of the side effects of aspirin and is available over-the-counter, so it is commonly used for the relief of fever, headache and other minor aches and pain (Vidhya and Bai, 2012). Although, safe at therapeutic doses, APAP had been found to cause severe liver injury (Erica and Emily, 2014). Mitchell et al. (1973) reported that APAP overdose is the predominant cause of acute liver failure in the United States and that toxicity begins with a reactive metabolite that binds to proteins. These findings indicated that acetaminophen was metabolically activated by cytochrome P₄₅₀ (CYP) enzymes to a reactive metabolite that depleted glutathione (GSH) and covalently bonded to protein. It has also been shown by James et al. (2009) that replenishing glutathione (GSH) prevented the toxicity. The mechanism of acetaminophen toxicity is by a complex sequence of events that include but not limited to CYP metabolism to a reactive metabolite which depletes glutathione and covalently binds to proteins, loss of glutathione with an increased formation of reactive oxygen and nitrogen species in hepatocytes undergoing necrotic changes, increased oxidative stress, associated with alterations in calcium homeostasis and initiation of signal transduction responses, causing mitochondrial permeability transition, mitochondrial permeability transition occurring with additional oxidative stress, loss of mitochondrial membrane potential, loss of the ability of the mitochondria to synthesize ATP and loss of ATP which leads to necrosis, (Mitchell et al., 1973; Jack et al., 2009). The reactive metabolite was found to be *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is formed by a direct two-electron oxidation (Dahlin et al., 1984). It was shown that NAPQI is detoxified by glutathione (GSH) to form an acetaminophen-GSH conjugate. After a toxic dose of acetaminophen, total hepatic GSH is depleted by as much as 90%, and as a result, the metabolite covalently binds to cysteine groups on protein, forming acetaminophen-protein adducts (Mitchell et al., 1973). Depletion of GSH which is an intrinsic antioxidant is capable of introducing peroxidation of cell membrane lipids, regeneration of reactive oxygen free radicals and hepatocellular fatty regeneration with centriolobular necrosis of the liver. The cellular damage is due to the failure to eliminate a toxic metabolic intermediate of the drug known as NAPQI (Vidhya and Bai, 2012; Reed, 2009).

Treatment of paracetamol overdose is based on replenishment of antioxidant thiols to supplement the role

of glutathione (Reed, 2009). Silymarin is a standardized extract obtained from the seeds of *Silybum marianum* containing approximately 70 to 80% of the silymarin flavonolignans and approximately 20 to 30% chemically undefined fraction, comprising mostly polymeric and oxidized polyphenolic compounds. It has been developed into a standard hepatoprotective drug. It is therefore, imperative to identify other plants with potential hepatoprotective effects to help prevent severe damage of the hepatocytes in cases of accidental/intentional over dosage.

Vegetables serve as indispensable constituents of the human diet, supplying the body with minerals, vitamins and certain hormone precursors, in addition to protein and energy (Aja et al., 2010). Leafy vegetables have been found to boost the concentration of red blood cells and significantly increase the serum activity of AST in experimental animals (Ezekwe et al., 2013). Focus on plant research has increased all over the world and a large body of evidence has been collected to show immense potential of medicinal plants for treatment purposes or for the production of drugs (Dahanukar et al., 2001; Olamide and Mathew, 2013; Udochukwu et al., 2015). Their use in ethnomedicine for the management of ailments stem from the presence of phytochemicals (Aja et al., 2010). *Cucurbita maxima* possess some bioactive compounds which make the possibility that the extract of the leaves may have antioxidant and anti-hepatotoxic activities (Shahlah et al., 2013; Alamgir et al., 2016). *Cucurbita* is a genus of herbaceous vines in the gourd family, *Cucurbitaceae* also known as cucurbits (Chakravarthy, 1982). Commonly known as the pumpkin, the plant is called "Ugbogulu" by the Igbo speaking areas of Nigeria. It is broadly grown for consumption as condiment and for therapeutic use (Lindhorst, 2007) and widely used like food and in folk medicine around the world (Perez, 2016). This work was carried out to ascertain the protective effect of ethanol leaf extract of *C. maxima*, a vegetable commonly used in traditional medicine and local diets, on acetaminophen-induced acute liver toxicity in albino rats.

MATERIALS AND METHODS

Plant materials, silymarin and acetaminophen

The plant material is *C. maxima* (pumpkin) leaf. The drug, acetaminophen was a research support from Emzor Pharmaceutical Ltd, Lagos while Silymarin is a branded drug (Sylibon 140) from Micro Laboratory Ltd, India.

Sample collection and preparation

Plant materials were collected in and around Keffi, in Nasarawa state, North Central Zone of Nigeria. The leaves were identified at the University of Ibadan Herbarium, in the Department of Botany and were assigned the voucher number UIH-22682. The leaves were rinsed in water to remove dust and sand particles, and then

dried under room temperature for fourteen (14) days. The dried leaves were then pulverised using Waring laboratory blender. Absolute ethanol (99.9%) from Sigma Chemical Company, London was used to extract the bioactive ingredients from the leaves.

Preparation of extracts

Pulverised plant material was extracted with ethanol by soaking 100 g of the ground samples in 500 ml of absolute ethanol (ratio 1:5 weight to volume) for 48 h. The extract was filtered using muslin cloth and then concentrated by heating in a water bath and stored in airtight containers.

Animal models

Male Wistar albino rats weighing between 120 and 140 g were used for the study. These rats were purchased from the animal house of the National Veterinary Research Institute (NVRI), Vom in Plateau State. They were housed in clean, well ventilated metal cages in the animal house of the Department of Zoology, Nasarawa State University, Keffi. The animals were kept under 24 h light/dark cycling. They were allowed access to unlimited food and water supply and allowed to acclimatize for two weeks before the commencement of the study. All the animals were marked for identification, and their respective weights recorded. The animals were first fed with the chow (feeds) and intubated with the plant material.

Administration of extracts and intoxication of the animals

Twenty albino rats were divided into four groups of five animals each. Group 1 (normal control) received feed and water only, group 2 (the standard control) received feed, water and a pre-treatment with Silymarin (400mg/kg), group 3 (negative control) received feed and water, while group 4 (test group) received feed, water and pre-treatment with ethanol leaf extract of the vegetable, 400 mg/kg for seven days. On the eighth day, the animals in groups 2, 3 and 4 were fasted for up to seven hours, followed by intoxication by oral administration of 2 g/kg acetaminophen and the animals were sacrificed after nine hours.

Animals sacrifice, collection and preparation of samples

At the end of the experimental period, the animals were anaesthetised. Blood samples were collected by cervical decapitation into plain tubes. Serum was collected by centrifuging at 3000 rpm for 10 min.

Preparation of liver homogenate

After bleeding, the livers were carefully removed, trimmed of extraneous tissues and rinsed in ice-cold 1.15% KCl. The livers were then blotted dry, two grams (g) was weighed and homogenized in 8 ml of ice-cold phosphate buffer (100 mM, pH 7.4). The homogenate were then centrifuged first at 6,000 rpm for six 6 min to remove nuclear debris after which the obtained supernatant were centrifuged at 10,000 rpm for twenty min (20 min) to obtain the post-mitochondrial supernatant (PMS), using a refrigerated centrifuge. This was used for the assay of the antioxidant enzymes (super oxide dismutase, catalase and glutathione peroxidase).

Biochemical analysis

Qualitative phytochemical screening of the leaf extract was carried

out using standard procedures of the Association of Analytical Chemist (2006) to identify the phytochemicals. The free radical scavenging activity of the plant extracts against DPPH radical was by a slightly modified spectrophotometric method previously described by Afolayan et al. (2014). The serum alkaline phosphatase activities of the experimental animals were estimated using the method of King (1965b). The determination of aspartate aminotransferase and alanine aminotransferase were carried out using the method of King (1965a). The total protein was estimated using the colorimetric method of Lowry et al. (1951). Total bilirubin was determined using the method of Malloy-Evelyn (1937). Superoxide dismutase activity was determined by its ability to inhibit the auto-oxidation of epinephrine and determined by the increase in absorbance at 480 nm as described by Sun and Zigma (1978). The catalase activity was determined according to the method of Beers and Sizer (1952) as described by Usoh et al. (2005) by measuring the decrease in absorbance at 240 nm due to the decomposition of H₂O₂. Determination of glutathione peroxidase (GPx) activity was by the method of Lawrence and Burk (1976). Histopathological study on the liver tissues was carried out using the haematoxylin and eosin stain as described by Bancroft et al. (2013).

Statistical analysis

The data obtained were statistically analysed by analysis of variance (ANOVA). Groups were compared using the least significant difference (LSD) at P<0.05.

RESULTS AND DISCUSSION

The ethanol extract of the leaf of *C. maxima* was found to be rich in bioactive constituents as seen in Table 1. Ethanol leaf extract of *C. maxima* was found to exhibit a concentration-dependent free radical scavenging potential from 0.03 to 0.12 mg/l, but sharply decreased at a concentration of 0.5 mg/l (Figure 1). Acetaminophen at a single dose of 2 g/kg caused significant increase (p<0.05) in the activities of AST, ALT and ALP in the serum of the rats in the negative control as compared to those in the normal control group. The intoxication decreased the total protein and albumin concentration of the rats in the negative control. However, the pre-treatment with the 400 mg/kg ethanol leaf extract of *C. maxima* for seven day prior to intoxication with acetaminophen led to marked decrease (p<0.05) in the activities of ALT and ALP, and increased concentration of the total protein and albumin in the serum as shown in Tables 2 and 3.

A single 2 g/kg oral administration of acetaminophen to the rats caused significant decrease of the activities of SOD and GPx in the negative control as compared to the normal control while the administration of the extract caused significant increase in the activities of the SOD, CAT and GPx of the test animals (Table 4).

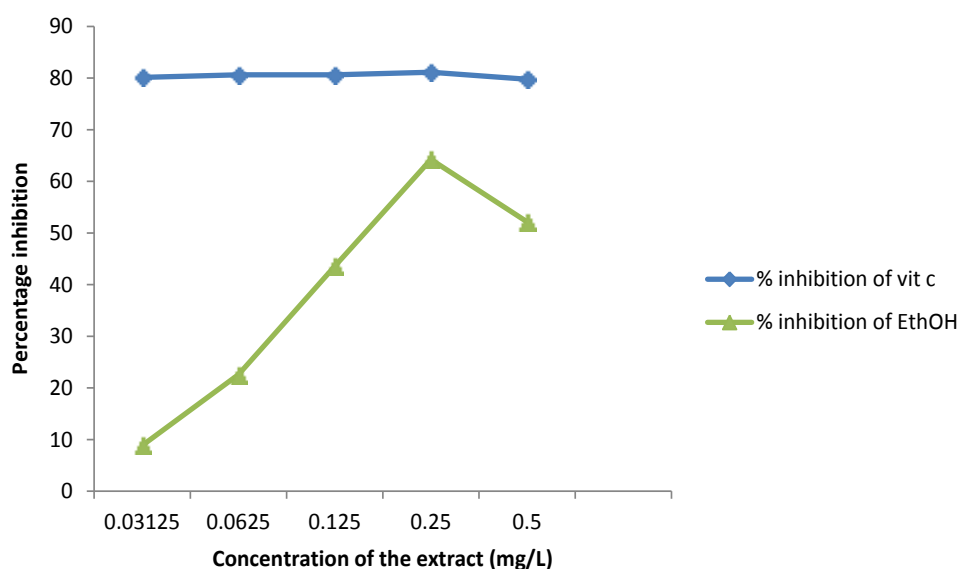
DISCUSSION

The ethanol extract of *C. maxima* leaf had a direct variation on the free radical (DPPH) scavenging property

Table 1. The qualitative phytochemical compositions of ethanol leaf extracts of *C. maxima*.

Parameter	Result
Tannin	+
Flavonoids	+
Phenols	+
Cardiac glycosides	+
Triterpenoids	+
Sterol	+
Terpenoids	+
Balsam	+

+: Indicates presence; -: indicates absence.

**Figure 1.** The free radical scavenging activity of ethanol leaf extract of *C. maxima* using ascorbic acid as standard.

with increase in concentration of the extract from 0.03 to 0.12 mg/l (8.9 to 64.2%) and dropped sharply to 52.2% at a concentration of 0.5 mg/l. This free radical scavenging property may be due to the presence of flavonoids and phenols (Table 1) which are good antioxidants. The methanol extract of the plant has been reported to have reasonable *in vitro* antioxidant potentials (Alamgir et al., 2016). However, the ascorbic acid had more DPPH-scavenging (*in vitro* antioxidant) potential than the ethanol leaf extract of *C. maxima* at the concentrations stated above.

The aminotransferase are abundant in the liver and are released into the blood stream following hepatocellular damage, making them sensitive markers of liver damage (Al-Mamary, 2002; Sarvesh 2012). 2 g/kg single dose acetaminophen caused the perturbation of the liver as evidenced in the significantly ($p < 0.05$) raised activities of

ALT, AST and ALP. This is in consonance with the work of Prabu et al. (2011) and Ekor et al. (2006), which reported liver damage as a result of the administration of 2 g/kg of acetaminophen in albino rats. Therefore, a marked increase in the serum ALT and AST activities is indicative of liver damage. Serum levels of aminotransferase are used as an indicator of damage to the liver structural integrity because these enzymes are cytoplasmic in location and are released into the circulating blood only after structural damage (Okedirani et al., 2014). The present study provides evidence that the pre-treatment of rats, with a 400 mg/kg per day with ethanol leaf extract of *C. maxima*, for seven days, was able to offer protection to the hepatic cells of the rats against toxicity and oxidative stress arising from a 2 g/kg oral intoxication with acetaminophen over nine hours (9 h). The pre-treatment with the leaf extract led to

Table 2. The effect of pre-treatment with 400 g/kgbw ethanol leaf extract of *C. maxima* on the serum activities of AST, ALT and ALP of rats intoxicated with 2 g/kg single dose of acetaminophen.

Group	AST (u/l)	ALT (u/l)	ALP (u/l)
Normal control	41.90±3.30	27.90±8.20	86.30±58.80
Standard control	42.80±10.80	17.90±3.10	167.30±107.70
Negative control	67.40±26.40 ⁺⁺	117.30±57.50 ⁺⁺	209.80±67.00 ⁺⁺
Cucurbita maxima (400 mg/kg)	72.18±42.92 ^a	31.26±11.22 ^{***a}	172.00±30.31 ^{**}

Values are mean ± SD of five (5) results, * and ** show values with significant increase and decrease respectively, compared to the negative control while ⁺⁺ and ⁻ indicate values with significant increase and decreases respectively as compared to the normal control. ^aSignificant difference from standard control. AST = aspartate aminotransferase, ALT = alanine aminotransferase, ALP = alkaline phosphatase.

Table 3. The effect of pre-treatment with 400 m/kg ethanol leaf extract of *C. maxima* on the serum concentrations of total protein, albumin, and bilirubin of rats intoxicated with 2 g/kg single dose of acetaminophen.

Groups	T. Protein (g/dl)	Albumin (g/dL)	T. Bilirubin (mg/dl)	D. Bilirubin (mg/dl)	Ind. Bilirubin (mg/dl)
Normal control	75.10±5.70	18.67±5.00	12.50±2.70	5.30±1.40	5.90±0.70
Standard control	76.80±10.50	4.70±2.30	9.00±5.20	6.50±2.90	4.00±3.30
Negative control	62.80±1.20	4.00±1.42 ⁻	16.40±4.20	4.30±2.50	7.20±1.90
<i>C. maxima</i> (400mg/kg)	64.334±4.65	6.78±1.67 [*]	11.92±7.08	3.40±2.05	8.52±6.65

Values are mean ± SD of five (5) results, *show values with significant increase as compared to the negative control. ⁻ indicates values with significant decreases as compared to the normal control.

significant ($p < 0.05$) decrease of the serum activities of ALT and ALP of the animals. However, the decrease in the serum activities of AST and ALT of the rats pre-treated with these extracts was significantly lower ($p < 0.05$) than that of Silymarin treated group. The activities of the liver antioxidant enzymes, SOD and GPx were significantly reduced ($p < 0.05$) in negative control group (Table 3). The activity of CAT was also reduced, although the reduction was not statistically significant ($p > 0.05$). This is an indication of oxidative stress in the liver. Disrupted hepatic lobular architecture of the rats was also observed (Plate 1C). All these alterations were seen in the negative control (Group 3) as compared to the normal control, group 1. The toxicant also altered the concentration of protein (total protein and albumin) in the serum of the rats, which could be as a result of the binding of NAPQI to proteins or the effect of NAPQI on the protein synthesizing/metabolizing ability of the liver. A marked rise in the serum activity of ALT, reduction in total serum protein and abnormal increase in serum bilirubin had been reported in hepatotoxicity (Olamide and Matthew, 2013; Olorunnisola et al., 2011; Martin and Friedman, 1992). A decrease in total protein and album shows that the liver's ability to synthesis protein (example albumin) has been impaired, hence indicative of liver damage. NAPQI is an oxidative product of acetaminophen metabolism that binds covalently to the sulphhydryl groups of proteins, resulting in cell necrosis and lipid peroxidation induced by decrease in glutathione in the liver causing

hepatotoxicity (Kanchana and Mohammed Sadiq, 2011). Results from the present study provide evidence of the induction of oxidative stress nine hours following acute acetaminophen intoxication. The induced oxidative stress as found in this study is evident in the significantly ($p < 0.05$) decreased activities of the SOD, CAT and the GPx of the animals in the negative control group as compared to the normal control group. Ekor et al. (2006) reported that after seven hours, following paracetamol (PCM) intoxication, there was a rise in GST activity, indicating increased GST-catalysed conjugation of PCM toxic metabolite NAPQI with GST leading to the depletion of cellular GSH level. Histological profile of the livers of the rats in the negative control group showed a poorly preserved hepatic lobular architecture, sharply demarcated hepatocyte, necrosis and exhibited periportal sinusoidal congestion (Figure 2) which is a confirmation of liver injury.

The activities of CAT, SOD and GPx increased significantly at 95% confidence level by the actions of the ethanol extract of *C. maxima* leaf. Jain and Pathak (2012) also reported the hepatoprotective activity of methanol extracts of *C. maxima* seeds against paracetamol-induced hepatotoxicity. Catalase, superoxide dismutase and glutathione peroxidase are the primary intracellular defence mechanism to cope with increased oxidative stress, eliminating superoxide anion and hydrogen peroxide that may oxidise cellular substrates thereby preventing free radical chain reactions (Ekor, et al., 2006).

Table 4. The effect of pre-treatment with 400 g/kg ethanol leaf extract of *C. maxima* on the activities of the liver antioxidant enzymes of rats intoxicated with a 2g/kg single dose of acetaminophen.

Samples	SOD (U/mg)	CAT (U/mg)	GPx (μ /mg)
Normal control	0.05 \pm 0.01	3.00 \pm 2.30	397.00 \pm 100.00
Standard control	0.20 \pm 0.40	20.90 \pm 14.90	184.60 \pm 23.60
Negative control	0.02 \pm 0.01 ^ˉ	2.50 \pm 2.60	115.60 \pm 10.03 ^ˉ
<i>Cucurbita maxima</i>	0.09 \pm 0.05*	10.23 \pm 5.05*	235.45 \pm 43.52*

Values are mean \pm SD of six (5) results, *values with significant increase as compared to the negative control, while ^ˉindicates values with significant decreases respectively as compared to the normal control.

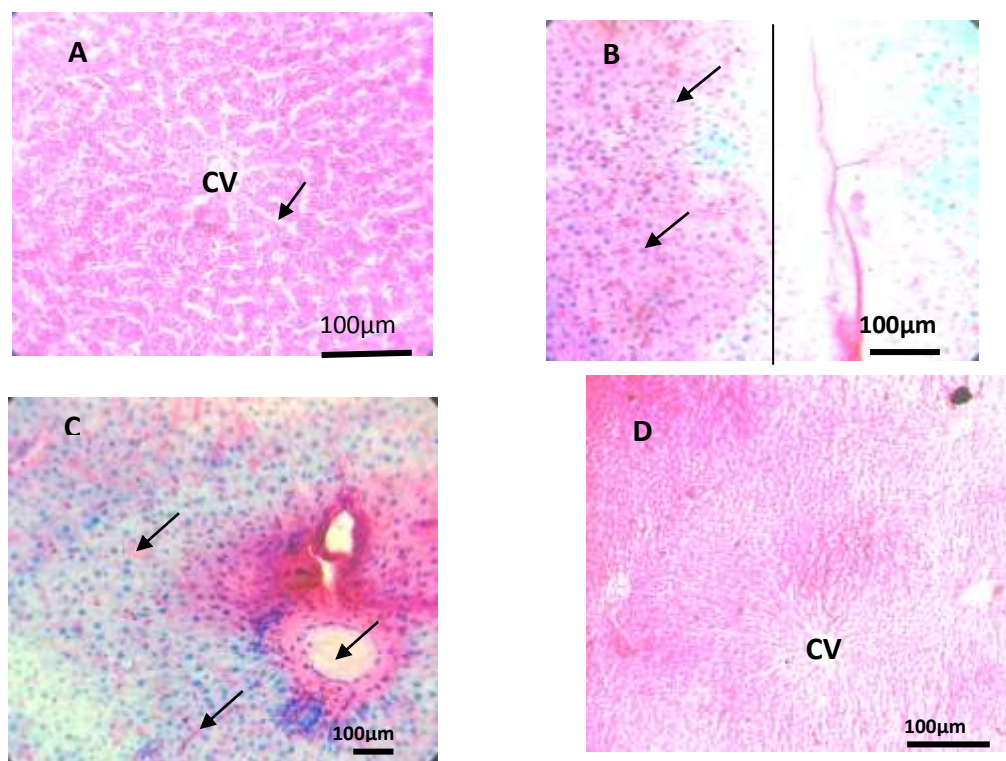


Figure 2. The histological micrograph of the rats' hepatocytes. A: The normal control, the section shows a well-preserved hepatic lobular architecture, with normal appearing cords hepatocytes interspersed by hepatic sinusoids (arrow). A normal appearing central vein (CV) is also seen. B: The negative control, section show a poorly preserved hepatic lobular architecture exhibiting peri-portal sinusoidal congestion and sharply demarcated hepatocyte necrosis as seen on plate B (arrows). C: The standard control, the liver section showed a well-preserved hepatic lobular architecture with sharply demarcated patchy areas of hepatocyte necrosis (right of image) and sinusoidal congestion (arrows). D: The test animal, section shows a well-preserved hepatic lobular architecture, with normal appearing hepatocytes and a minimal diffuse chronic inflammatory infiltrate. CV = central vein.

The induction of higher activities of these antioxidant enzymes is suggested for the protection of the livers by reducing oxidative stress on the organ. All these protections may be due to the antioxidant properties of the plants, which stem from its phytochemical components. Prerona et al. (2011) posited that the potent hepatoprotective activity of *C. maxima* aerial parts

against CCl_4 induced hepatic damage may be due to its antioxidant activity and free radical scavenging property. However, it is not known whether the health benefits are the result of individual phytochemicals, the interaction of various phytochemicals, the fibre content of plant foods or the interaction of phytochemicals and the vitamins and minerals found in the same foods.

Conclusion

The vegetable was found to be a potential antioxidant and offered protection to the hepatic cells. Therefore, it can be a good source of raw materials for the production of medicine/drugs for the prevention and treatment of liver and associated diseases. The vegetable is therefore recommended in diets.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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