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A case study: Effects of wet cupping therapy in a male with primary infertility

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ABSTRACT

Background and purpose: Infertility is a serious health problem, resulting from the inability of a sexually active couple to achieve pregnancy in 1 year. The cost of treatment remains a challenge for majority of infertile people. Alternative therapy may be helpful in addressing the problem of infertility. The purpose of this study was to investigate the effects of wet cupping therapy in a male with infertility.
Materials and methods: The patient was a 32-year-old male with a 7-year history of infertility. Pre- and post-tests were completed by testing the man's semen and human chorionic gonadotropin in his wife's serum. The man underwent wet cupping therapy twice each month. His wife was treated with wet cupping once per month.
Results: The wife of the man became pregnant after 2 months of wet cupping therapy.
Conclusion: Wet cupping therapy can be effective in treating individuals with infertility.

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KEYWORDS

Wet cupping therapy; alternative therapy; infertility; male infertility; pregnancy; conception

Introduction

Infertility is a serious, multifaceted public health problem, defined as the inability to achieve natural pregnancy after 12 months of regular, unprotected coitus. Infertility is estimated to affect 8%–15% or approximately 50 million of reproductive-aged couples worldwide [1–3]. However, in some regions of the world, the rate of infertility is much higher, reaching approximately 30% [1,4]. Such regions with high prevalence of infertility include South Asia, sub-Saharan Africa, Middle East, North Africa, Central Asia, Central and Eastern Europe [3]. According to Mascarenhas et al. [3], one in every four couples in developing countries is affected by infertility.

Infertility is categorized into two types: primary and secondary infertility. Primary infertility, according to the World Health Organization, can be defined as the inability of reproductive-aged woman (from 15 to 49 years old) to conceive after 1 year of sexually active life [5]. Secondary infertility is the inability to conceive after a previous pregnancy [6]. The incidence of the former is higher, compared to the latter [6]. A study conducted in South-Eastern Nigeria revealed that primary infertility accounted for 65% cases of infertility, whereas secondary infertility was found in 35% [7]. About 20%–30% of infertility cases, contributing to 50% of cases overall, are due to male factors [8]. However, Ikechebelu et al. [7] reported 42% for male factors and only 26% for female factors in Nigeria, suggesting that male may have greater contribution to cases of infertility in some regions of the world. Similar findings are reported elsewhere [9,10]. These studies are contrasted with other reports, indicating that 50% of infertility cases are due to female factors [8]. This, probably, is a confirmation of reports suggesting that infertility research (including prevalence studies) is complicated due to male and female factors [3,11]. About 20%–30% of infertility cases are due to combined male and female factors [8]. Ikechebelu et al. [7] also reported a 21% combined male and female factors as possible causes of infertility in a sample of Nigerian infertile couples. About 11% cases are due to unexplained causes. Indeed, infertility is complicated and due to multiple causes, which include morphologic defects of sperm (teratozoospermia), reduced sperm count (oligozoospermia or oligospermia), motility disorder (asthenozoospermia), absence of spermatozoa in semen (azoospermia), poor

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sperm quality, testicular maldescent, testicular torsion, vas deferens, or epididymis obstruction—responsible for more than 90% of cases of male infertility [7,10,12,13]. Genitourinary tract infection due to poorly treated sexual transmitted diseases and sexual promiscuity, critical illnesses, malnutrition, genetic abnormalities, gonadotoxic oncologic therapy, environmental pollutants, chronic stress, and lifestyle changes play an integral role in occurrence of infertility [2,7,12,13].

Infertility is associated with a range of consequences, which include societal burden and psychological disorders. For example, Direkvand-Moghadam et al. [14] reported that infertility is associated with marital discord and remarriage. In some cases, more than 50% of individuals with infertility experience some form of verbal or physical abuse [9].

Despite improvement in diagnostics and treatment, unaffordability, and inaccessibility of the procedures for majority of the infertile couples are major problems. In developing countries, in particular, the deplorable state of healthcare system and the high costs of the procedures have hindered many couples from having their babies [15]. Alternative therapy has been reported to improve sperm qualities [16,17], suggesting that this treatment option may be helpful in addressing the problem of infertility. Wet cupping therapy, which is a type of alternative therapy, is the most common type of cupping therapy [1]. The therapy involves creation of a vacuum over specific, mildly punctured zones on the skin, using vacuum pumping and disposable lancets [18]. Wet cupping therapy is currently gaining recognition all over the world due to its affordability and ability to alleviate symptoms of different ailments. For example, Arslan et al. [19] reported that cupping therapy reduces upper shoulder and neck pains in office workers. The benefits of this therapy have been reported for heart diseases [20,21] and other illnesses that affect humans [22,23]. Although previous studies suggest that wet cupping therapy improves sperm quality [24], the effect of wet cupping therapy on infertility is not fully known. The implication of changes of sperm quality following wet cupping therapy on the female's ability to conceive is not completely understood. Therefore, studies investigating the role of wet cupping in achieving pregnancy will not only add useful information to the literature but also certainly provide the basis for infertile couples who wish to utilize the therapy.

The purpose of this study was to investigate the effects of wet cupping therapy on infertility in a male with primary infertility.

Methods

Ethics

The study was carried out in accordance with the Helsinki Declaration (1975, revised 1996–2013) and approved by the local ethical and research committee (IRB/Nile/CHS/PHS 0011/2018). Written informed consent was obtained from the man and his wife after they had been thoroughly explained the methodology, purpose, benefits, and possible risks of the study.

Case

The case was a 32-year-old male waiter in a university canteen. He presented with complaints of inability to get his wife pregnant and premature ejaculation since the past 7 years despite having regular and unprotected sexual intercourse. The man also had upper and lower back pain and snoring complains. The wife of the man was 29-year old. According to the man, he was confirmed infertile in a hospital, where the physician advised him to do *in vitro* fertilization. However, he could not do it because he did not have the money to finance the procedure. He had normal secondary sexual characteristics. There was no history of systemic or chronic illness. He had no history of smoking and alcohol consumption.

Pre- and post-tests were completed by the researcher by investigating the semen fluid of the man and human chorionic gonadotropin (hCG) of his wife. Serum hCG analysis was negative for pregnancy. Laboratory exam of the semen revealed decreased sperm count, low motility, and abnormal sperm morphology (see result section for sperm quality indices upon presentation). Investigation of blood sugar, testosterone, and prolactin levels did not reveal any abnormalities. Abdominal ultrasonography was essentially normal with no varicose veins or hydrocele.

The man underwent wet cupping therapy twice per month on Wednesdays after every other week. Due to the possibility of combined male and female factors in infertility, his wife was also treated with wet cupping once per month during the second wet cupping therapy of her husband. The semen fluid of the man was investigated during and after wet

cupping therapy, while hCG of his wife's serum was analyzed after the therapy period.

Spermiogram

Semen collection was done according to the recommended standards [25,26]. Semen was collected before commencement of the study. Semen sample was also collected 15 days after the first cupping therapy and 15 days after completion of the study. In each case, semen was collected after 3–4 days of abstinence from sexual activity. Collection of semen was done by masturbation into sterile sample container and delivered to the laboratory within a period of 30 minutes after ejaculation. To prevent large changes in temperature, which may affect the spermatozoa, the specimen container was maintained at a temperature of 20°C–37°C.

The semen was analyzed by a certified andrologist. Analysis and interpretation of semen was carried out according to World Health Organization (2010) guidelines [27]. The functional indices of the semen were assessed according to earlier studies [28,29]. Concentration was determined by a Makler chamber (Sefi-Medical Instruments, Haifa) [30]. Sperm morphology was analyzed according to the Tygerberg Strict Criteria [31].

The man did not take any medications during the period of the study.

hCG testing

hCG is a hormone, which is produced early in pregnancy after implantation by trophoblast cells [32]. This hormone can be detected in the maternal blood as early as 8 days after implantation and peaks at around 100,000 mlU/ml at a gestational age of 10 weeks, and thereafter declines, maintaining a concentration of around 20,000 mIU/ml throughout the period of pregnancy [32]. Detection of this hormone is highly reliable and sensitive for determination of pregnancy [32].

The level of hCG was determined from blood samples drawn from the wife before commencement of the study and after 15 days after completion of the study. hCG analysis was carried out according to Korevaar et al. [33] and Strom et al. [34]. Serum hCG was analyzed by the chemiluminescent immunometric assay, calibrated against fourth World Health Organization International Standard 75/589, on an Immulite 2000 XPi system (Siemens Healthcare Diagnostics, Deerfield, IL).

The woman did not take any medications during the period of the study.

Wet cupping therapy

Wet cupping therapy was conducted by cupping practitioner (author SD), who is a certified physician of the British Cupping Society and National Health Institute. The cupping therapy was conducted according to previous recommendation [35] with modifications. The therapy was carried out on the posterior torso. The entire back was exposed and cleaned with antiseptics. Nine points of the posterior, bilateral thoracic, lumbar and sacral areas of the spine were selected for treatment (Fig. 1). These spinal areas are implicated in regulating autonomic functions of the reproductive organs [36,37]. Sterile disposable cups measuring about 5 cm in diameter were consecutively placed at the points, mentioned above, and negative pressure was applied by cupping (vacuum) pump. After about 2–3 minutes of pumping in each point, the cups were removed. The point of the skin to which cupping pressure was applied was first punctured with 26-gage disposable lancets to a 2 mm depth. Thereafter, vacuum pumping was applied for the second time, draining about 3–5 cm^3 of blood per cupping site. The application sites were cleaned with sterile pads. As a measure against negative reactions to bloodletting or pain intolerance, an emergency physician and a nurse with emergency response kit were ready in the application room to promptly respond to negative consequences that would occur during the cupping procedure. Negative reaction did not occur during the procedure.

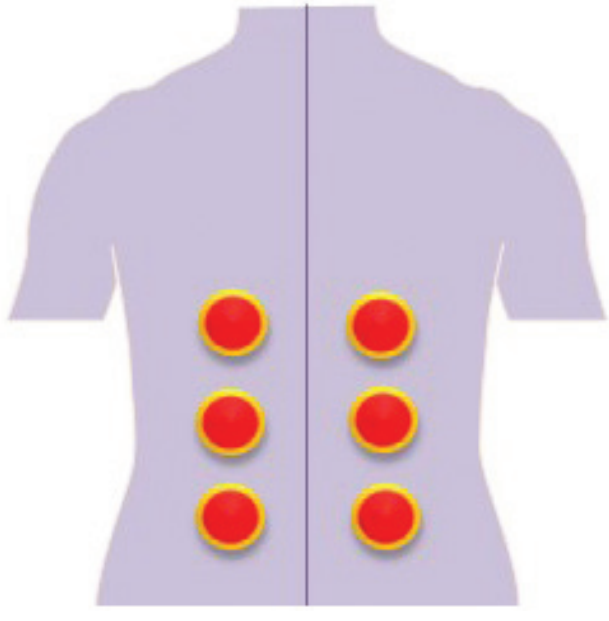

Figure 1. Posterior torso showing points on the skin where wet cupping therapy was applied.

Results

Table 1 displays the results of spermiogram indices before and after wet cupping therapy.

Before therapy, there was low volume of ejaculation, which increased up to 4 ml after therapy. The man had oligozoospermia before therapy. During therapy period, sperm count increased, and further above the threshold for normal referred to as normosphermia (Table 1). Similarly, proportion of spermatozoa with normal morphology was observed to have increased during wet cupping therapy. Upon completion of the therapy, spermiogram had normal morphology (i.e., 60% of the sperms had spermatozoa normal morphology), required for successful fertilization. Before therapy, motility was lower than normal, but increased during and after completion of therapy. Sperm vitality also increased after the period of therapy (Table 1). The semen pH did not substantially change before, during, and after completion of the therapy (range 7.1–7.7 units) (Table not shown).

Upon completion of therapy, semen sample became normozoospermic. Premature ejaculation, back pain, and snoring were present before the therapy, but disappeared after the therapy.

The wife of the man became pregnant after wet cupping therapy. The level of hCG was negative before cupping therapy, but became positive (at 49.57 mlU/ml) after therapy, indicating the presence of pregnancy.

Discussion

Infertility is a growing health problem worldwide with considerable social, medical, and financial effects [38]. Several studies have reported that couples suffer several societal issues due to infertility [9,14,38]. Furthermore, infertility negatively affects the quality of life of couples [39]. Though there are a range of infertility treatment options such as surgical (e.g., in obstructive azoospermia), medical (administration of androgens, gonadotropins, anti-estrogens, antibiotics, antioxidants, etc.), and assisted reproductive therapy (*in vitro* fertilization and intra cytoplasmic sperm injection) [40], no approach guarantees absolute effectiveness. For example, though *in vitro* fertilization and intra cytoplasmic sperm injection are the preferred approaches for addressing the problems of infertility, there is always a chance of fertilization failure, which is due to a range of factors, including sperm defects [40–42]. More importantly, the cost of assisted reproductive therapy remains a huge challenge for the majority of people, especially in developing countries [9,14]. More so, structural abnormalities of the fetus resulting from the procedure cannot be excluded [43,44].

Recent investigations have suggested that alternative therapy can alleviate the suffering of infertile couples [16,17]. For example, a recent study showed that acupuncture increases the chances of pregnancy from 26% to 43% [45]. As a type of alternative therapy, wet cupping may have positive effect on infertility, similar to that exerted by acupuncture therapy. Indeed, the results of our study indicate that wet cupping therapy can be effective in infertility treatment. Thus, wet cupping is a promising therapeutic option in alternative medicine that can potentially revert the repercussions of infertility.

The spermiogram results in this study showed improvement during the therapy and subsequently became normal after 2 months of wet cupping therapy. Also, the man's premature ejaculation also completely disappeared after therapy. Semen analysis provides essential information on male's fertility potential, and also, essential in evaluating success of treatment of infertility [38]. Indices of spermiogram, such as volume, sperm count, motility, morphology, and vitality, are crucial indicators of fertility outcome *in vivo*. These indices provide data

Table 1. Spermiogram results and ejaculation status before and after wet cupping therapy.

	Before therapy	15 days after the first therapy	15 days after the second therapy
Volume (ml)	1.5	2	4
Sperm count (million/ml)	11	19	43
Morphology (%normal)	27	43	71
Motility (%normal)	31	44	65
Vitality (%)	29	47	66
Premature ejaculation	Mild	Normal	Normal

on semen quality and fertility potential, thereby affecting the chances of conception [38,46]. For instance, adequate spermatozoa motility is required for effective penetration of the cervical mucus, transport through the female genital tract, and penetration through the corona radiata and zona pellucida before fertilization can take place [38]. For fertilization to take place, 50% of spermatozoa must have a progression rate of at least 25 μm/s, but not less than 5 μm/s at about 20°C–37°C [46]. It is recommended that sperms be checked for viability if at least 50% sperms are immotile [27,46]. Motility and viability are very important spermiogram indices for deciding on the type of treatment options for couple's infertility [27]. Thus, disordered spermatozoa motility or less than 32% motile sperms (asthenozoospermia), low sperm count (oligospermia), and abnormal morphology (teratozoospermia) can lead to infertility. A combination of the aforementioned disorders of spermiogram indices substantially decreases the fertility potential of males. The man in this study before wet cupping therapy had disorders in almost all spermiogram indices (i.e., oligoasthenoteratozoospermia). However, during therapy, these indices improved, and after therapy, his spermiogram showed a normal result (i.e., normozoospermia). Like the motility disorder, the vitality of the patient's sperm was also below 58% upon presentation, but exceeded the normal value, thus increasing fertility potential of the man.

Premature ejaculation, defined as short ejaculatory time or latency between vaginal intromission and intravaginal ejaculation due to loss of control with associated psychological distress in the partner [47], is one of the most frequent sexual disorders, experienced by infertile males. The lack of efficient treatment, in addition to poorly defined criteria for this sexual dysfunction has made premature ejaculation a critical problem that substantially interferes with infertility treatment success [48–50]. Ejaculatory latency of 20–1,200 seconds can occur in premature ejaculation, but ejaculatory latency in some healthy males may overlap with latency of infertile patients [51]. Though there is lack of widely accepted criteria, assessment of premature ejaculation basically relies on self-report, which may not be reliable at all times. Normal ejaculatory latency is believed to be around 3–6 minutes [51]. The man in this study reported impaired ejaculatory time; however, after wet cupping therapy, his ejaculation became normal as evidenced by normal spermiogram results, which resulted to wife's pregnancy. Pregnancy was confirmed by hCG analysis. But hCG test prior to wet cupping therapy showed negative result. Analysis of this hormone is recommended and used worldwide to test for pregnancy. Research data have shown that hCG test is very reliable and highly sensitive for detection of pregnancy [32].

Though female factors such as tubal occlusion and high acidity of the genital tract milieu are crucial in infertility [7], increasing reports in Nigeria and other parts of the world have shown that male factors may even play a greater role in couple's infertility [7,52–54]. For example, Ikechebelu et al. [7] reported greater contribution of male factors to infertility. This indicates the necessity of paying considerable attention to men when couples present with infertility problem in Nigeria.

The mechanisms of effects of wet cupping therapy on infertility are not exactly known, but emerging data suggest that this therapy may regulate the level of reproductive hormones. In a recent study, Abduljabbar et al. [55] reported significant changes in hormonal profile in infertile women after wet cupping therapy. Thus, the effects resulting from the application of this therapy involve stimulation of some hormones [18]. The effect of wet cupping therapy may also be associated with removal of waste from the body. Moreover, local damage of the skin and capillary vessels due to this therapy acts as a nociceptive stimulus that triggers specific regions of the nervous system [18]. Wet cupping therapy has been reported to stimulate both the peripheral [56] and autonomic nervous system functions [18].

Therefore, wet cupping therapy can amplify sexual potency of an infertile man, correct defective semen, disturbed sexual functions, and improve spermatogenesis in male, and achieve conception of healthy progeny in a female.

Conflict of Interest

The authors report that there is no conflict of interest regarding the publication of this paper.

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Hepatoprotective properties of methanol leaf extract of *Pterocarpus mildbraedii* Harms on carbon tetrachloride-induced hepatotoxicity in albino rats (*Rattus norvegicus*)

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ABSTRACT

Aim: This study evaluated the effects of methanol leaf extract of *Pterocarpus mildbraedii* Harms on carbon tetrachloride (CCl_4)-induced sub-acute hepatotoxicity in albino rats.

Methods: Fresh leaves of *P. mildbraedii* were collected in December 2016. Thirty male albino rats were randomly assigned to six groups (A–F) of five rats each. Sub-acute hepatotoxicity was induced in groups A, B, C, D, and E by intraperitoneal injection of 1 ml/kg CCl_4 in equal volume of olive oil at 3-day intervals for 12 days. Group A was given 10 ml/kg distilled water placebo and served as untreated (negative) control, groups B, C, and D were treated with 100, 200, and 400 mg/kg *P. mildbraedii* methanol leaf extract (PME), respectively, group E was treated with 100 mg/kg silymarin as positive control, while group F was given 10 ml/kg distilled water placebo and served as normal control. Treatment with PME and silymarin was done orally twice daily for 15 days. Blood samples were collected on the 15th day for evaluation of liver enzyme markers [alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase activities] and liver function (total serum protein, albumin, globulin, total cholesterol, and bilirubin), following standard procedures. Relative liver weight was calculated.

Results: Treatment with PME and silymarin significantly ($p < 0.05$) decreased the elevated ALT and AST, and thus restored hepatocellular integrity, and also ameliorated inflammatory liver enlargement in the rats given CCl_4.

Conclusion: These findings imply that treatment with PME as used in this study led to significant hepatoprotection against CCl_4-induced hepatotoxicity.

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Introduction

The liver is one of the most complex and most important organs in the human body. It plays a major role in the metabolism of food substances and detoxification of drugs and xenobiotics [1]. It is also concerned with the deamination of excess proteins, storage of iron, vitamins and glycogen, production of urea, bile, and vital enzymes in the body. The liver is constantly and variedly exposed to xenobiotics which may lead to liver damage or hepatotoxicity [2]. Toxic liver damage is a major health problem; the manifestations of which are highly variable ranging from an asymptomatic elevation of liver enzymes to fulminant liver failure. Despite notable developments in modern medicine, liver diseases remain a global health challenge [3].

Natural remedies from medicinal plants are considered to be effective and safe alternative treatments for liver diseases. Traditionally, a variety of plants have been recommended for the treatment of liver diseases [4]. The efficacy of most of these medicinal plants has not yet been scientifically validated.

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Pterocarpus mildbraedii Harms is an evergreen or semi-deciduous tree belonging to the family *Papilionaceae*. It is a medium-sized to large tree up to 35 m tall, with smooth gray or pale brown bark, and small rounded crown [5]. Two species are recognized locally, *P. mildbraedii* Harms (oha) and *Pterocarpus santalinoides* DC (nturukpa) [6]. *Pterocarpus mildbraedii* is commonly called "African rosewood." Its tender leaves are locally used as a vegetable in the preparation of soup [6,7]. The leaves are a good source of beneficial nutrients like amino acids, calcium, iron, potassium, vitamins A, B, and C, and also possess antioxidant, antibacterial, antispasmodic, and diuretic properties [8]. In South-Eastern Nigeria, leaf and stem bark extracts of *P. mildbraedii are* used for the treatment of a variety of ailments and disorders [7,9]. Studies in our laboratory and also by other researchers have shown that extracts from two species belonging to the genus *Pterocarpus* (*P. santalinoides* and *Pterocarpus erinaceus*) possess hepatoprotective activity [10,11]. The need then arose to evaluate *P. mildbraedii* for hepatoprotective activity, as it is the specie that is widely available, commonly consumed as vegetable in soups, and generally more acceptable. The present study, therefore, evaluated the effects of methanol leaf extract of *P. mildbraedii* on CCl_4-induced hepatotoxicity in albino rats.

Materials and Methods

Assay kits, chemicals, solutions, and reagents

The assay kits for evaluation of the serum enzyme activity concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and serum levels of total proteins, albumins, and total cholesterol (TC) are products of Quimica Clinica Aplicada (QCA), Spain. Total bilirubin was assayed using the Randox® bilirubin test kit (Randox Laboratories Ltd, County Antrim, United Kingdom). Methanol, silymarin, and carbon tetrachloride were sourced from Sigma-Aldrich (St. Louis, Missouri). Thiopentone sodium was obtained from Chandra Bhagat Pharma Pvt., Ltd., Mumbai, India. All other routine chemicals and reagents were of analytical grade.

Gathering of plant material, plant identification, and extract preparation

The study was carried out in 2017. Fresh leaves of *P. mildbraedii* were collected from Orifite town in Ekwusigo Local Government Area of Anambra State, Nigeria, in December 2016. The plant was identified by Mr. A.O. Ozioko, a plant taxonomist in the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka [(Voucher Specimen Number—UNH (University of Nigeria Herbarium) No. 158]. The *P. mildbraedii* leaves collected were allowed to dry under the shade, and later ground into powder. Five hundred grams (500 g) of the ground leaves were extracted with 80% methanol using the cold maceration extraction method, with intermittent shaking at 2-hour intervals for 48 hours. The extract obtained was filtered with Whatman size 1 filter paper, concentrated to dryness in a rotary evaporator (Buchi, Switzerland), and referred to as *P. mildbraedii* extract (PME).

Experimental animals

Forty-two (42) adult male albino rats (*Rattus norvegicus*) of 12 weeks of age and body weights between 220 g and 250 g were obtained from the Department of Veterinary Physiology and Pharmacology Laboratory Animal Unit, University of Nigeria, Nsukka, and used for the study. Twelve (12) of the albino rats were used to study the acute toxicity of the extract, while thirty (30) were used to study the hepatoprotective activity of PME. The albino rats were kept in stainless steel cages in a fly proof Animal House at room temperature (27°C–29°C) and allowed for 2 weeks to acclimatize. They were fed commercial pelletized rat chow (Grand Cereals Nig. Ltd, Jos, Nigeria), composed of 13% crude protein, 8% fat, 15% crude fiber, 0.9% calcium, 0.35% phosphorus, and 2,600 Kcal/kg metabolizable energy, and provided with clean water *ad libitum* all through the study. Guidelines for the use of animals for laboratory experiment were strictly adhered to [12]. The animal experimental protocol was approved by the Experimental Animal Ethics Committee of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka.

Methods

Acute toxicity test

Oral acute toxicity and median lethal dose (LD_{50}) of PME was done using the OECD Acute Toxic Class method [13]. The Annex 2b OECD test procedure with a starting dose of 50 mg/kg was followed. Twelve male albino rats were used, three rats each for testing at 50, 300, 2,000, and the limit dose of 5,000 mg/kg, respectively. The rats were fasted for 12 hours before the commencement of the test, but

drinking water was made available all through [13]. The varied doses of the extract were each dissolved in 1 ml of water and administered using an intubation cannula. The rats were observed for 14 days for any signs of toxicity.

Phytochemical analysis

Semi-quantitative phytochemical analysis was conducted to determine the phytochemicals present in PME, following the procedures described by Trease and Evans [14], and Harborne [15]. Tests for the presence of tannins, flavonoids, alkaloids, saponins, carbohydrates, glycosides, starch, sterols, and terpenes were conducted on PME. One gram (1 g) of PME was dissolved in 100 ml of distilled water in a beaker. The solution was filtered with Whatman no 1 filter paper to obtain a clear filtrate which was used to test for the presence of the phytochemicals. High levels of specific phytochemicals were scored +++, moderate levels were scored ++, low level was scored +, while phytochemicals that were absent were not scored [14,15].

Evaluation of the hepatoprotective activity of PME in albino rats

The thirty (30) male albino rats used for the hepatoprotection study were randomly assigned to six groups (A–F) of five rats each. Sub-acute hepatotoxicity was induced in groups A, B, C, D, and E by intraperitoneal injection of 1 ml/kg CCl_4 in equal volume of olive oil (50% volume/volume) at the beginning of the experiment (day 0), and at every 3 days for the next 12 days (3, 6, 9, and 12). Group A was given 10 ml/kg distilled water as placebo and served as negative control (untreated), groups B, C, and D were treated with 100, 200, and 400 mg/kg PME, respectively, group E was treated with silymarin (a standard hepatoprotective drug) at the dose of 100 mg/kg as positive control, while group F was given 10 ml/kg distilled water as placebo and served as normal control. Treatment started from day 1 of the experiment and was done orally twice daily for 15 days. Blood samples were collected from the albino rats using the orbital technique [16], on day 15 (at the end of the experiment) for serum biochemistry assay. The blood samples were allowed 30 minutes to clot. The serum biochemistry assay was done immediately upon separation of the serum from blood clot following standard procedures using Quimica Clinica Applicada® (QCA) test kits (QCA, Spain), and Randox® bilirubin test kit (Randox Laboratories Ltd, United Kingdom).

Liver damage marker enzymes, such as serum ALT and serum AST activity levels, were evaluated following the Reitman–Frankel method [17]. The ALT in the serum sample and ALT standard catalyzed the reaction of L-alanine and alpha-ketoglutaric acid to form pyruvic acid and L-glutamic acid, while the AST catalyzed the reaction of L-aspartic acid with alpha-ketoglutaric acid to form oxaloacetic acid and L-glutamic acid. These ketonic acids produced were reacted with 2-4, dinitrophenyl hydrazine to form corresponding colored hydrazone, the optical density of which was then measured and ALT/AST quantified at 505 nm wavelength using the semi-automated analyzer (Daitek Instruments Co. Ltd., Wuxi, China).

The serum ALP activity was quantified using the QCA alkaline phosphatase test kit, which is based on the phenolphthalein monophosphate method [17]. In this method, alkaline phosphatase in the serum and a standard (containing 30 IU/l alkaline phosphatase) hydrolyzed a colorless substrate of phenolphthalein monophosphate giving rise to phosphoric acid and phenolphthalein which at alkaline pH turned to pink color, the optical density of which was measured and alkaline phosphatase activity quantified at 546 nm wavelength, using the semi-automated analyzer.

Assay of serum total protein was by the direct Biuret method [18]. This procedure involved a reaction of the proteins in the serum samples and a standard (containing 5g/dl of proteins) with copper ions in the Biuret reagent in an alkaline medium, which resulted in the formation of a stable colored complex, the optical density of which was measured and the serum total protein quantified at 546 nm wavelength using the semi-automated analyzer. The serum albumin was quantified using the QCA albumin test kit, which is based on the bromocresol green method [18]. This procedure involved the reaction of the albumin in the serum samples and standard (containing 5 g/dl of albumin) with bromocresol reagent at acid pH to form a colored complex, the optical density of which was measured and albumin quantified at 630 nm wavelength using the semi-automated analyzer. The globulin levels were calculated by subtracting the serum albumin levels from the serum total protein levels [18].

The serum TC was determined using the QCA TC test kit, which is based on the enzymatic colorimetric method [19]. In this procedure, TC in the serum samples and a standard (containing 200 mg/dl of cholesterol) was enzymatically hydrolyzed by

cholesterol esterase and further oxidized by cholesterol oxidase contained in the QCA TC working reagent. The reactions led to the formation of a colored quinonic derivative, the optical density of which was measured and TC quantified at 505 nm wavelength using the semi-automated analyzer.

The total bilirubin levels in the serum samples were assayed using the Randox® bilirubin test kit (Randox Laboratories Ltd, County Antrim, United Kingdom), which is based on the Jendrassik and Grof method [20]. In this determination, the serum samples were reacted with diazotized sulfanilic acid in the presence of caffeine to produce an azo pigment, the optical density of which was measured and total bilirubin quantified at 578 nm using the semi-automated analyzer.

After the collection of blood samples, the rats were euthanized by intra-peritoneal injection of 250 mg/kg thiopentone sodium [21]. The livers were eviscerated and weighed, and the relative liver weight of individual rats was calculated.

Statistical analysis

Data obtained from the study were subjected to one-way analysis of variance, and variant means were separated *post hoc* using the least significant difference method. Statistical Package of the Social Sciences software, version 16.0, was used for the analysis. Significance was accepted at $p < 0.05$.

Results

Acute toxicity test

The varied doses of PME used for the acute toxicity test had no adverse clinical effect on the behavioral responses of the tested rats during the 14 day monitoring period. Physical observation also indicated that all the rats behaved essentially normal, with no signs of changes in the skin, fur, eyes, mucous membrane, and behavioral patterns. There was no mortality in all the treated rats. The rats tolerated the extract up to 5,000 mg/kg; therefore, the LD_{50} of the extracts is above 5,000 mg/kg ("Category 5—Unclassified" of the Global Harmonized Classification System [13].

Phytochemical analysis

Results of the phytochemical analysis showed varied degrees of bioactive phytochemicals in the PME. It showed high levels (+++) of carbohydrates, saponins, glycosides, fats and oil; and moderate levels (++) of flavonoids and tannins.

Hepatoprotective activity of PME in albino rats

The serum ALT activity levels of rat groups B, C, D, E, and F were significantly lower ($p < 0.05$) than that of group A, and there were no significant differences ($p > 0.05$) between groups C, D, and E in their serum ALT activity levels (Table 1). The serum AST activity levels of groups C, D, E, and F were significantly lower ($p < 0.05$) than that of groups A and B, and there were no significant differences ($p > 0.05$) in serum AST activity of groups C, D, and E (Table 1). The serum ALP activity levels of group F was significantly lower ($p < 0.05$) than that of groups A, B, C, D, and E, and there were no significant differences ($p > 0.05$) in serum ALP activity between groups A, B, C, D, and E (Table 1).

There were no significant variations in the serum total protein levels among the groups, but the serum albumin levels of groups E and F were significantly higher ($p < 0.05$) than that of groups

Table 1. Effects of PME on serum enzymes of rats given sub-acute toxic doses of CCl_4.

Groups*	Mean ± standard error		
	ALT (U/l)	AST (U/l)	ALP (U/l)
Group A	102.24 ± 2.82[a]	139.73 ± 4.74[a]	242.00 ± 1.19[a]
Group B	92.25 ± 7.34[b]	138.34 ± 9.17[a]	240.68 ± 0.50[a]
Group C	65.79 ± 3.48[c]	113.01 ± 2.69[b]	239.48 ± 1.74[a]
Group D	67.82 ± 5.11[c]	109.49 ± 4.72[b]	237.90 ± 1.79[a]
Group E	64.31 ± 3.05[c]	105.49 ± 2.98[b]	233.65 ± 3.34[a]
Group F	28.07 ± 1.73[d]	61.56 ± 2.94[c]	172.02 ± 12.43[b]

*Groups. Group A—CCl_4 alone, no treatment; Group B—CCl_4 +100 mg/kg PME; Group C—CCl_4 + 200 mg/kg PME; Group D—CCl_4 + 400 mg/kg PME; Group E—CCl_4 + 100 mg/kg silymarin; Group F—No CCl_4, no treatment.

[a,b,c]Alphabetical superscripts in a column indicate significant differences between the groups, $p < 0.05$.

A, B, C, and D (Table 2). The serum globulin level of group F was significantly lower ($p < 0.05$) than that of groups A, B, C, D, and E, while the serum globulin level of group E was significantly lower ($p < 0.05$) than only that of groups A and B (Table 2).

The serum TC of group F was significantly lower ($p < 0.05$) than only that of groups A and B, and the serum TC of groups C, D, and E was lower, but not statistically different ($p > 0.05$) from that of groups A and B. There were no significant differences ($p > 0.05$) between groups C, D, and E in their serum TC (Table 3).

The serum total bilirubin of group F was significantly lower ($p < 0.05$) than only that of groups A and D, while the serum total bilirubin of groups B, C, and E was lower than that of groups A and D but not found to be statistically significant ($p > 0.05$). There were no significant differences ($p > 0.05$) between groups B, C, and E in their serum total bilirubin (Table 3).

The relative liver weight of group F was significantly lower ($p < 0.05$) than that of groups A, B, C, D, and E, while the relative liver weight of groups B and E was significantly lower ($p < 0.05$) than only that of group A. The relative liver weight of groups C and D was lower, but not statistically different ($p > 0.05$) from that of group A (Table 3).

Discussion

The result of the acute toxicity suggests that PME is not acutely toxic, and is thus considered safe for the treatment of ailments and disorders for which it is effective. This result is in agreement with the report of Ihedioha et al. [11], that methanol leaf extract of *Pterocarpus santalinoides*, a specie in the same genus *Pterocarpus*, is acutely non-toxic.

Table 2. Effects of PME on serum proteins of rats given sub-acute toxic doses of CCl_4.

Groups*	Mean ± standard error		
	Total proteins (g/dl)	Albumins (g/dl)	Globulins (g/dl)
Group A	5.64 ± 0.03	3.15 ± 0.08 [a]	2.48 ± 0.04 [a]
Group B	5.64 ± 0.05	3.23 ± 0.06 [a]	2.42 ± 0.05 [a]
Group C	5.68 ± 0.04	3.31 ± 0.04 [a]	2.37 ± 0.03 [ab]
Group D	5.57 ± 0.04	3.32 ± 0.11 [a]	2.26 ± 0.06 [ab]
Group E	5.69 ± 0.02	3.59 ± 0.02 [b]	2.11 ± 0.03 [b]
Group F	5.69 ± 0.05	3.89 ± 0.05 [c]	1.86 ± 0.04 [c]

*Groups. Group A—CCl_4 alone, no treatment; Group B—CCl_4 +100 mg/kg PME; Group C—CCl_4 + 200 mg/kg PME; Group D—CCl_4 + 400 mg/kg PME; Group E—CCl_4 + 100 mg/kg silymarin; Group F—No CCl_4, no treatment.

[a,b,c]Alphabetical superscripts in a column indicate significant differences between the groups, $p < 0.05$.

Table 3. Effects of PME on serum total cholesterol, bilirubin and relative liver weight of rats given sub-acute toxic doses of CCl_4.

Groups*	Mean ± standard error		
	Total cholesterol (mg/dl)	Total bilirubin (mg/dl)	Relative liver weight (%)
Group A	35.52 ± 3.34 [a]	0.63 ± 0.04 [a]	4.64 ± 0.16 [a]
Group B	35.23 ± 3.86 [a]	0.58 ± 0.03 [ab]	4.16 ± 0.14 [b]
Group C	28.29 ± 2.10 [ab]	0.58 ± 0.09 [ab]	4.26 ± 0.13 [ab]
Group D	33.07 ± 4.01 [ab]	0.64 ± 0.08 [a]	4.46 ± 0.16 [ab]
Group E	32.21 ± 2.32 [ab]	0.60 ± 0.10 [ab]	4.15 ± 0.11 [b]
Group F	24.10 ± 2.34 [b]	0.41 ± 0.28 [b]	3.56 ± 0.10 [c]

*Groups: Group A—CCl_4 alone, no treatment; Group B—CCl_4 +100 mg/kg PME; Group C—CCl_4 + 200 mg/kg PME; Group D—CCl_4 + 400 mg/kg PME; Group E—CCl_4 + 100 mg/kg Silymarin; Group F—No CCl_4, no treatment.

[a,b,c]Alphabetical superscripts in a column indicate significant differences between the groups, $p < 0.05$.

The findings in this study of the presence of varied levels of flavonoids, glycosides, saponins, tannins, carbohydrates, fats, and oil in PME, is in agreement with the reports of Akindahunsi and Salawu [22], and Usunobon and Igwe [23], who had earlier reported that the leaves of *P. mildbraedii* are good sources of flavonoids, tannins, saponins, and glycosides. Onyeka and Nwambakwe [24] also reported that *P. mildbraedii* leaves are rich in these phytochemicals. According to Okwu [25], these phytochemicals are commonly found in various medicinal herbs and they exhibit a wide range of biological activities.

The ability of CCl_4 to induce liver damage was evident by the significantly higher serum enzyme activity levels of ALT, AST, and ALP in all the groups that were given CCl_4 [26]. The damage to the liver hepatocytes caused by CCl_4 was evident by the alterations in the activity levels of these liver damage marker enzymes. When cell membranes of hepatocytes are damaged, enzymes, such as ALT, AST, and ALP, are released into the blood from the cytosol of hepatocytes [27]. A rise in serum transaminases is a sensitive indicator of cell membrane damage of hepatocytes. Serum activity concentrations of AST and ALT are the most commonly used biochemical markers of hepatocellular necrosis [28,29]. The finding in this study that rat groups treated with PME at all doses (B, C, and D), and those treated with 200 mg/kg and 400 mg/kg (groups C and D), respectively, had serum ALT and AST activities significantly lower than that of group A and comparable to that recorded for group E, showed that administration of PME at these doses protected the integrity of the hepatocytes, and compared favorably with silymarin which is a standard hepatoprotective drug. These findings are in agreement with the reports of Offor *et al.* [30] who recorded decreases in ALT and AST activity levels in albino rats treated with ethanol leaf extract of *P. santalinoides.* Aja et al. [31] and Ihedioha et al. [11] also recorded significant decreases in ALT and AST activity levels in albino rats in which hepatotoxicity was induced with CCl_4, and acetaminophen, respectively, and treated with leaf extract of *P. santalinoides*, a related specie in the genus *Pterocarpus*. The ability of PME to enhance hepatocellular integrity as seen in this study may be due to the presence in this extract of phytochemicals like flavonoids, glycosides, and tannins, which are known natural antioxidants [32]. Reports have shown that these phytochemicals possess the ability to reduce free radical formation and also to scavenge free radicals [32]. Carbon tetrachloride is known to induce hepatotoxicity by the generation of free radicals which induce oxidative stress [33]. Since free radicals play such an important role in CCl_4-induced hepatotoxicity, it seems logical that compounds that neutralize such free radicals may have hepatoprotective activity as a consequence of their antioxidant properties, and as such may be necessary for protection against free radical damage by CCl_4 in the liver. Flavonoids, tannins, and water-soluble glycosides also function in protection against allergies, inflammation, platelet aggregation, tumors, and hepatotoxins [25]. Other natural products that possess antioxidant properties have also been reported to protect against CCl_4-induced hepatotoxicity [34].

The lack of significant variation between the groups in their serum levels of total proteins indicates that sub-acute administration of CCl_4 as used in the study did not significantly affect total protein synthesis in the liver. This may be because the duration of the study (sub-acute) was not long enough for the total protein to be affected, as hypoproteinemia has been reported to occur mainly in chronic liver diseases [35]. The significantly lower serum albumin in the groups that were given CCl_4 is a further indication of liver dysfunction caused by CCl_4 administration [36]. Serum albumin is the major plasma protein synthesized in the liver. It is a clinically useful marker of hepatic synthetic function [29]. The liver is an important site for the synthesis of serum albumins and the administration of CCl_4 in this study adversely affected hepatic synthesis of albumins. Treatment with silymarin led to significant elevation in serum albumin level and thus enhanced hepatic synthetic activity. The significant elevation in serum globulin levels in groups A and B when compared to groups E and F is an indication of a high priority for globulin in the damaged liver [37].

Administration of CCl_4 altered the serum lipid profile by increasing the serum levels of TC of group A and others that were given CCl_4. The liver is the principal site for the formation and clearance of lipoproteins. Hence, it is not surprising that toxic liver damage can affect plasma lipid levels in a variety of ways. Cholestasis is associated with hypercholesterolemia as the major excretory pathway of cholesterol is blocked in liver disorder [38]. Treatment with PME at 200 and 400 mg/kg, and silymarin at 100 mg/kg (groups C, D, and E), slightly ameliorated this cholestasis and dyslipidemia induced by CCl_4 administration by a slight reduction of serum TC level at these doses. Adegbite and Ezekwesili

[39] recorded a reduction in serum TC level in CCl_4-induced rats treated with ethanol and aqueous leaf extracts of *Pterocarpus milbraedii*.

Treatment with PME at 100 mg/kg and 200 mg/kg (groups B and C), and silymarin at 100 mg/kg slightly enhanced hepatic clearance of bilirubin. Otuechere and Farombi [40] reported that *P. mildbraedii* methanol extract significantly reduced serum total bilirubin level in propanil-induced hepatotoxicity in albino rats. Hamza et al. [41] also recorded a significant reduction in serum bilirubin level in albino rats whose livers were damaged with CCl_4 and treated with sub-fractions of *P. mildbraedii* extracts.

The higher relative liver weight seen in group A is an indication of inflammation/degeneration which is a consequence of CCl_4-induced hepatotoxicity [42,43]. The reduction in relative liver weight after treatment with PME at all the doses used in the study (groups B, C, and D), and silymarin at 100 mg/kg (group E), suggests their amelioration of this inflammatory enlargement of the liver caused by CCl_4 toxicity.

Conclusion

Based on the results obtained from this study, it was concluded that the administration of *P. mildbraedii* methanol leaf extract to rats at the doses used in the study was protective of hepatocellular integrity, ameliorated inflammatory enlargement of the liver, and compared positively with silymarin which is a standard hepatoprotective drug. These findings imply that *P. milbraedii* methanol leaf extract possesses hepatoprotective activity against carbon tetrachloride-induced liver damage.

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Conflict of interest

The authors declared that they have no conflict of interest.

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RESEARCH ARTICLE

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Assessment of anti-nociceptive and anthelmintic activities of *Vitex Peduncularis* Wall. leaves and *in silico* molecular docking, ADME/T, and PASS prediction studies of its isolated compounds

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ABSTRACT

Background: *Vitex peduncularis* (Verbenaceae), popularly known as "Boruna," "Goda," "Ashmul gaas" (Chakma), "Korobaong" (Marma), is frequently used in the traditional medicine for the treatment of chest pain, joint ache, malarial fevers, diabetes, jaundice, urethritis, and abnormality in eyes and face.

Objective: To validate the traditional use, this study was designed to investigate the anti-nociceptive (*in vivo*) and anthelmintic (*in vitro*) activity of ethanol extract of *V. peduncularis* leaves (EEVP). Moreover, computational studies, namely, *in silico* molecular docking, absorption, distribution, metabolism, elimination (ADME)/T, and prediction of activity spectra for substances (PASS) predictions study were performed to ascertain the association of the isolated compounds for the anti-nociceptive and anthelmintic activity as well as their good oral bioavailability and safety traits.

Methods: Quantitative phytochemical study of EEVP was carried out by the established methods. The anti-nociceptive activity was determined using acetic acid-induced writhing test and formalin-induced paw-licking test in mice at the doses of 200 and 400 mg/kg body weight, whereas an aquarium worm, *Tubifex tubifex*, was used to determine the anthelmintic activity. *In silico* molecular docking study was performed using Schrödinger Maestro 10.1, whereas the SwissADME, admetSAR, and PASS online tools were used for ADME, toxicological property, and biological activity predictions, respectively.

Results: Our quantitative phytochemical study of EEVP revealed the occurrence of a substantial amount of phenolics (121.67 mg), flavonoids (97.78 mg), flavonols (39.88 mg), and condensed tannins (63.49 mg). EEVP exhibited significant and dose-dependent anti-nociceptive activity in both experimental pain models. In addition, EEVP has manifested strong anthelmintic property compared to reference standard drug levamisole. Molecular docking study showed that 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vitexin, corosolic acid, vitexilactone, and tormentic acid have the best binding affinities toward the respective enzymes [cyclooxygenase-1 (COX-1), COX-2), and tubulin]. PASS study also predicted the anti-nociceptive and anthelmintic activity of all isolated compounds. In addition, ADME/T study exhibited that all the compounds are safe and orally bioavailable from the druggable point of view.

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ABSTRACT (Continue)

Conclusion: The outcomes of our scientific investigation (*in vivo*, *in vitro*, and computational studies) significantly support the ethno-medicinal use of this plant, which might be utilized as an alternative source for the potential drug discovery.

Introduction

Vitex peduncularis Wall., belongs to the Verbenaceae family, is a moderate-sized deciduous tree with yellow flowers, and naturally grows in hill tract regions of Bangladesh, India, and many other countries. It is commonly known as "Boruna," "Goda," "Horina" (Chittagong), "Awal" (Sylhet), "Krawru" (Mogh), "Ashmul gaas" (Chakma), "Korobaong" (Marma) in Bengali, "Charaiygoda" in Hindi, and "Chang Xu Jing" in Chinese [1]. Different parts of *V. peduncularis*, such as barks and leaves, have been used in the traditional system of medicine to treat various diseases. For example, infusion of leaves and bark are being used in black water and malarial fevers [2]. Traditionally, the boiled bark is used for the chest pain and also as a drink to treat joint ache, whereas leaves are popularly known for its antibacterial property [1,3]. Leaves and barks are used for the treatment of diabetes in the hill area of Khagrachari (Bangladesh), and a decoction of the leaves is taken as a tea during the cold season in Orissa (India) [1,4]. The bark paste is used for jaundice by the Chakma people (ethnic community of Bangladesh) and also taken with water for the treatment of urethritis, and the root extract is applied externally in the affected area for the treatment of abnormality in eyes and face [1,5]. In addition, the plants of this genus have been used to treat a range of human ailments related to fungi, bacteria, insects, snakes, and poisonous spiders [1].

The preliminary phytochemical study revealed that the plant contains several categories of chemical constituents such as flavonoids, terpenoids, triterpenoids, flavones (like 4′-acetoxy-5-hydroxy-6,7-dimethoxyflavone), and iridoids. In addition, a number of phytochemicals have been isolated from this plant such as 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vitexin, corosolic acid, vitexilactone, tormentic acid, agnuside, pedunculariside, iridoid, luteolin, crisimartin, genkwanin, 3α-friedeliniol, 3β-friedeliniol, pachypodol, peduncularism, and ursolic acid [1,5–8]. Pharmacological properties, like antipyretic [1,9], antioxidant [1], anti-inflammatory [5,10], antibacterial and antifungal [11–13], and cytotoxic [1,14] activities, have been reported.

Although the plant, *V. peduncularis* has several important medicinal properties, until now, no studies have been performed to explore its antinociceptive and anthelmintic activity. For that reason, this set of studies with the use of experimental (*in vivo* and *in vitro* study) and computational [*in silico* studies such as molecular docking, absorption, distribution, metabolism, elimination (ADME)/T, and prediction of activity spectra for substances (PASS) predictions] methods was performed to investigate the antinociceptive and anthelmintic activities of this plant for the first time.

Materials and Methods

Plant material collection, identification, and ethanol extract preparation (EEVP)

Fresh leaves of *V. peduncularis* wall. were collected from Bhatiary, Chittagong, Bangladesh, in November 2017 and it was authenticated by Dr. Shaikh Bokhtear Uddin, botanist, from the University of Chittagong with a reference number (SUB 99) which has been deposited in the Herbarium of the University of Chittagong for future reference. Then, approximately 450 g of the powdered materials were soaked in 900 ml of ethanol at room temperature for 14 days with occasional stirring and shaking (Model VTRS-1, Nunes Instruments, Tamil Nadu, India). The resultant mixture was filtered through a cotton plug followed by Whatman No.1 filter paper, and the filtrate solution evaporated to yield the ethanol extract of *V. peduncularis* leaves (EEVP) which was stored in a refrigerator at 4°C for further study.

Drugs, chemicals, and reagents

Ethanol, potassium acetate, sodium acetate, sodium carbonate, acetic acid, and formalin were purchased from Merck (Darmstadt, Germany). Diclofenac sodium was obtained from Square Pharmaceuticals Ltd., Bangladesh, and Levamisole procured from ACI Limited, Bangladesh, where absorbance was measured by using UV–Vis spectrophotometer (UVmini-1240, Shimadzu, Japan). Quercetin, catechin, and Tween-80 were obtained from BDH Chemicals Ltd. Poole, UK. Gallic acid,

Folin-Ciocalteau reagent, and aluminum chloride obtained from Sigma Chemicals Co. (St. Louis, MO).

Swiss albino mice

Swiss albino mice (Male and female, 20–25 g weight approximately) were collected from Jahangir Nagar University, Savar, Dhaka 1342, Bangladesh. Mice were sheltered in polypropylene cages by maintaining suitable laboratory conditions (room temperature 25°C ± 2°C; relative humidity 55%–60%; 12 hours light/dark cycle) along with standard laboratory food and distilled water ad libitum. All the experimental works were conducted in a noiseless condition, and the animals were acclimatized to the laboratory conditions for 10 days before experimentation. This study was carried out following the internationally accepted principle for proper use of laboratory animals,' namely, the National Institutes of Health and the International Council for Laboratory Animal Science. The present study protocol was reviewed and approved by the "P&D committee" of the Department of Pharmacy, International Islamic University Chittagong, Bangladesh, with a reference number: Pharm-P&D-61/08'16-129.

Quantitative phytochemical analysis

Determination of total phenolic, flavonoid, flavonol, and condensed tannins contents

The total phenolic content of EEVP was determined using the Folin–Ciocalteu method [15] and expressed as milligrams of gallic acid equivalents (mg GAE/g dried extract), while the total flavonoid content was determined by $AlCl_3$ assay method [16] and expressed as milligrams of quercetin equivalents (mg QE/g dried extract). On the other hand, the total flavonol content was determined by using the method previously described by Kumaran and Karunakaran [17], using quercetin (QE) as a reference standard whereas total condensed tannins content was determined by using the method previously described by Sun et al. [18], using catechin equivalent (CAE) as a reference standard. All the experiments were conducted in triplicates (n = 3), and the results were expressed as mean ± standard error of mean (SEM).

Acute oral toxicity test

To investigate the possible toxicity of EEVP, acute oral toxicity test was performed based on the Organisation for Economic Co-operation and Development Guidelines [19] for the Testing of Chemicals (Test No. 420: Acute Oral Toxicity) using a limited dose of 2,000 mg/kg, body weight of the mice.

Antinociceptive activity (in vivo)

Experiment design and dosing groups

In this current study, mice were divided into four groups, and each group consisting of six mice (n = 6). Here, the control group received 1% Tween-80 in distilled water, the positive control group received standard drug, Diclofenac sodium (10 mg/kg, body weight), whereas the remaining groups were given 200 and 400 mg/kg, body weight of EEVP.

Acetic acid-induced writhing test

The acetic acid-induced writhing test was performed based on the previously reported method [20]. Thirty minutes after the administration of doses, 0.6% acetic acid was injected intraperitoneally (i.p.) into the mice. After 5 minutes of injection, the number of writhing (abdominal constrictions) was counted for 15 minutes, and the writhing responses were compared with the control group. The anti-nociceptive activity was stated as a percentage of writhing inhibition and calculated using the following formula: percent of inhibition (%) = [(Control group – Test groups) / Control group] × 100.

Formalin-induced licking test

The formalin-induced paw-licking test was performed based on the previously described method [21]. Thirty minutes after the administration of doses, 20 µl of 2.5% formalin was injected subcutaneously (s.c) into the sub-plantar region of the right hind paw of mice. In this test, pain response was reflected as indicative of the nociceptive behavior, and the total time spent in the behavioral responses to nociception, including licking/biting of the injected paw was noted. The entire time spent was noted up to 30 min where the first 5 minutes (0–5 minutes) was considered as an early phase or neurogenic phase and the last 15 minutes (15–30 minutes) considered as a late phase or inflammatory phase. The percentage of inhibition of antinociceptive activity was calculated as described in the previous method.

Anthelmintic activity (in vitro)

The anthelmintic activity of EEVP was investigated using the previously reported methods [22,23]. Here, an aquarium worm, namely *Tubifex tubifex* was used to assess the anthelmintic property of

EEVP because of its anatomical and physiological resemblance to human intestinal roundworm parasites. The aquarium worms were obtained from a shop at Chittagong, Bangladesh, and the average sizes of used worms were about 2–2.5 cm in length. This study was conducted in triplicates and randomly divided into five groups, and each group consists of 10–12 worms. In this study, the negative control group received only distilled water (Group I), the positive control group received standard drug, levamisole (1 mg/ml) (Group II), whereas the remaining groups namely Group III, IV, and V, were considered as test groups at three different concentrations of EEVP (5, 8 and 10 mg/ml) respectively. Briefly, almost 10–12 worms were taken in each petri dish in five groups, and 3 ml of the test solution of EEVP/standard drug were added. Then the starting time, paralysis time, and death time of the worms were noted very carefully.

Selection of compounds for the computational study (in silico)

3,4-dihydroxybenzoic acid [PubChem Compound ID (CID): 72], 4-hydroxybenzoic acid (PubChem CID: 135), vitexin (PubChem CID: 5280441), corosolic acid (PubChem CID: 6918774), vitexilactone (PubChem CID: 21636178), tormentic acid (PubChem CID: 73193), agnuside (PubChem CID: 442416), iridoid (PubChem CID: 453214), and luteolin (PubChem CID: 5280445) were selected based on the availability as major compounds through literature review [1,5–8] and the chemical structures of the compounds were downloaded from the PubChem database.

Molecular docking analysis

Ligand and enzyme preparations

The chemical structures of nine major representative compounds were obtained from PubChem compound repository, neutralized at pH 7.0 ± 2.0 and minimized by the LigPrep tool (force field OPLS_2005) embedded in Schrödinger suite-Maestro v 10.1. On the other hand, the 3D crystallographic enzyme structures were downloaded from the Protein Data Bank Research Collaboratory for Structural Bioinformatics PDB [24]: cyclooxygenase-1 (COX-1, PDB id: 2OYE) [25], cyclooxygenase-2 (COX-2, PDB id: 3HS5) [26], and tubulin-colchicine enzyme (PDB: 1SAO) [27]. The enzyme was prepared for a docking experiment by Protein Preparation Wizard embedded in Schrödinger suite-Maestro v 10.1 as the previously described method [28].

Glide standard precision docking

Molecular docking studies were performed to elucidate the possible mechanism of the selected compounds against COX and tubulin-colchicine enzymes for antinociceptive and anthelmintic activity. Docking experiments were carried out using Glide embedded in Maestro by standard precision scoring function as the previously described method [28,29].

ADME study and toxicological property prediction

The pharmacokinetic properties (ADME) of the compounds were determined by SwissADME (http://www.swissadme.ch/). Here, molecular descriptors such as molecular weight, Hydrogen bond acceptor, Hydrogen bond donor, LogP (Lipophilicity), molar refractivity, number of rotatable bonds, topological polar surface area, and violations of Lipinski's rule of five were calculated since orally active drugs should comply with these widely utilized drug-likeness properties to establish their pharmaceutical credibility. In addition, toxicological properties of the compounds were predicted by the admetSAR online server (http://lmmd.ecust.edu.cn/admetsar1/predict/) as toxicity is a prime issue during the drug discovery process.

PASS prediction study

The six major phytoconstituents, namely, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vitexin, corosolic acid, vitexilactone, and tormentic acid were investigated for evaluating the antinociceptive, anthelmintic, and other biological activities by using PASS online program (http://www.pharma-expert.ru/passonline/).

Statistical analysis

Results were expressed as mean ± SEM, while SPSS v20 was used for data analysis, and all comparisons were made by using a one-way analysis of variance followed by Dunnett's test. A *p*-value of less than 0.05, 0.01, and 0.001 considered as statistically significant.

Results

Quantitative phytochemical study

The result of total phenolic, flavonoid, flavonol, and condensed tannins contents are shown in Table 1. The total phenolic and flavonoid contents of EEVP were 121.67 ± 3.51 mg GAE/g and 97.78 ± 1.57 mg QE/g dried plant extract, respectively. Also, the total

Table 1. Total phenolic, flavonoid, flavonol, and condensed tannins contents of the ethanol extract of *V. peduncularis* leaves.

Tested Extract	Phenolic content (mg GAE/g dried extract)	Flavonoid content (mg QE/g dried extract)	Flavonol content (mg QE/g dried extract)	Condensed tannins content (mg CAE/g dried extract)
EEVP	121.67±3.51	97.78±1.57	39.88±0.49	63.49±3.15

Values are expressed as mean ± SEM (n = 3). EEVP = ethanol extract of *V. peduncularis* leaves; GAE = gallic acid equivalent; QE = quercetin equivalent; CAE = catechin equivalent.

flavonol and condensed tannins contents of EEVP were 39.88 ± 0.49 mg QE/g and 63.49 ± 3.15 mg CAE/g dried plant extract, respectively.

Acute toxicity test

Oral administration of EEVP up to the dose of 2,000 mg/kg did not produce any mortality, behavioral and neurological changes during the 72 hours of the observation period. Therefore, the dose up to 2,000 mg/kg was considered safe for EEVP, and the LD_{50} value of the extract was found to be higher than 2,000 mg/kg.

Antinociceptive activity

Acetic acid-induced writhing test

In the acetic acid-induced abdominal writhing test, EEVP exhibited 26.24% and 55.25% inhibition of writhing at the doses of 200 and 400 mg/kg body weight, respectively, whereas the standard drug, Diclofenac sodium (10 mg/kg body weight), displayed 70.71% inhibition as compared to the control group, and the results were statistically significant (Fig. 1).

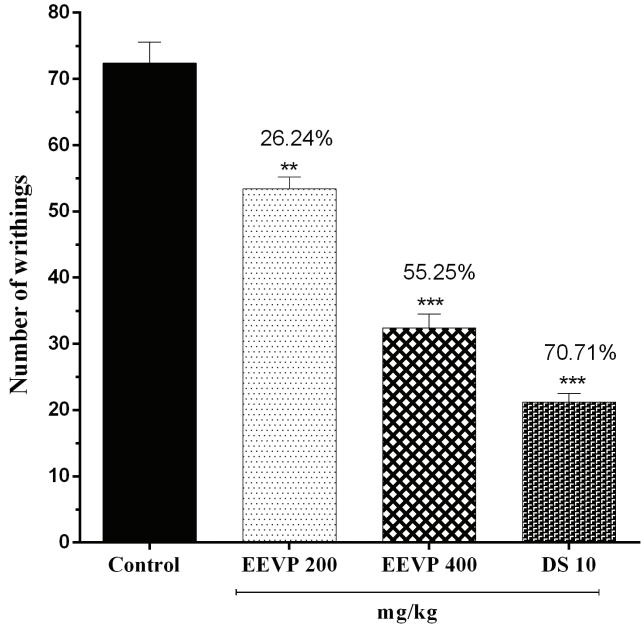

Figure 1. Anti-nociceptive effect of EEVP in acetic acid -induced writhing test in mice. Values are expressed as mean ± SEM (n=6). EEVP = ethanol extract of *V. peduncularis* leaves; DS = Diclofenac sodium as standard drug; $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the control group (Dunnett's Test).

Formalin-induced licking test

The effect of EEVP on formalin-induced paw-licking test in mice is shown in Table 2. In this study, EEVP significantly and dose-dependently inhibited the paw-licking response in both the early phase (29.55% at 200 mg/kg and 47.78% at 400 mg/kg, body weight) and the late phase (25.08% at 200 mg/kg and 42.87% at 400 mg/kg, body weight) of the formalin test which were comparable to those of the reference standard drug, Diclofenac sodium, 10 mg/kg body weight (65.12% and 71.18% in the early and late phases, respectively).

Anthelmintic activity

Results of the anthelmintic activity of EEVP are shown in Table 3. Here, EEVP showed a significant and dose-dependent anthelmintic action at concentrations of 5, 8, and 10 mg/ml. From the result, it was seen that the extract gave the shortest time of paralysis and death at the highest concentration (10 mg/ml) whereas the extract displayed the longest time of paralysis and death at the lowest concentration (5 mg/ml). Therefore, it can be concluded that the time is taken for paralysis and death decreases as the increase of concentrations of EEVP and vice versa.

Molecular docking study for antinociceptive activity

In this study, nine major compounds of *V. peduncularis* were docked against COX-1 (PDB: 2OYE) and COX-2 (PDB: 3HS5) enzymes. Our study exhibited that 3,4-dihydroxybenzoic acid has the best binding affinity against COX-1 enzyme with the highest docking score −5.83 kcal/mol followed by 4-hydroxybenzoic acid (−5.36 kcal/mol), vitexin (−5.11 kcal/mol), corosolic acid (−3.12 kcal/mol), vitexilactone (−2.84 kcal/mol), and tormentic acid (−2.77 kcal/mol). However, three compounds, namely agnuside, iridoid, and luteolin did not show any interactions

Table 2. Antinociceptive effect of EEVP in formalin-induced paw-licking test in mice.

Treatment (mg/kg)	Licking time (s) (Mean ± SEM)			
	Early phase (0–5 minutes)	Inhibition (%)	Late phase (15–30 minutes)	Inhibition (%)
Control (0.1 ml/mouse)	40.39 ± 2.50	-	38.81 ± 2.98	-
Diclofenac Na (10)	17.22 ± 1.67***	65.12	11.18 ± 1.11***	71.18
EEVP (200)	34.79 ± 2.07*	29.55	29.07 ± 3.60	25.08
EEVP (400)	25.78 ± 1.93**	47.78	22.17 ± 0.65*	42.87

Values are expressed as mean ± SEM (n=6). EEVP = ethanol extract of *V. peduncularis* leaves; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the control group (Dunnett's Test).

Table 3. Anthelmintic activity of the ethanol extract of *V. peduncularis* leaves.

Treatment (mg/ml)	Paralysis time (min)	Death time (min)
Control (Water)	0.00	0.00
Levamisole 1	3.22 ± 0.51	6.19 ± 0.35
EEVP 5	21.19 ± 0.65***	54.18 ± 1.37***
EEVP 8	10.22 ± 0.58***	33.14 ± 0.79***
EEVP 10	5.91 ± 0.26**	21.26 ± 0.42***

Values are expressed as mean ± SEM (n = 3). EEVP = ethanol extract of *V. peduncularis* leaves. **p <0.01 and ***p <0.001 compared with standard drug Levamisole (Dunnett's test).

with the COX-1 enzyme. Alternatively, 4-hydroxybenzoic acid (−5.49 kcal/mol) was found to have the highest binding affinity to the COX-2 enzyme with the highest docking score, followed by 3,4-dihydroxybenzoic acid (−5.18 kcal/mol). However, seven compounds, i.e., vitexin, corosolic acid, vitexilactone, tormentic acid, agnuside, iridoid, and luteolin did not dock with COX-2 enzyme. The result of the docking study is shown in Table 4, and the docking figure is presented in Figures 2–4. Docking analysis of each compound of *V. peduncularis* exhibited several binding interactions between the ligands and the target enzymes. Here, 3,4-dihydroxybenzoic acid interacts with the COX-1 enzyme through two H-bonds to Thr94 and Asn515, and one pi-pi stacking interactions with His90. 4-hydroxybenzoic acid interacts with the same enzyme through the formation of two H-bonds with Ser87 and Pro514 residues, and one pi-pi stacking interactions with Phe91. Vitexin interacted with His95 through one H-bond, and one pi-pi stacking interactions, whereas Vitexilactone interacted through one H-bond with Ser87 and Tormentic acid interacted through two H-bonds with Ser87. However, Corosolic acid did not show any interaction with the COX-1 enzyme. On the other hand, 3,4-dihydroxybenzoic acid interacted with the COX-2 enzyme by forming one pi-pi stacking interactions with Trp387, while 4-hydroxybenzoic acid interacted with the same enzymatic by forming two H-bonds with Met522 and Tyr385, and two pi-pi stacking interactions with Trp387 and Tyr385.

Molecular docking study for anthelmintic activity

Results of the docking study for anthelmintic activity are presented in Table 5, and the most representative interactions between ligands and enzymes have been shown in Figures 5 and 6. Our study exhibited that corosolic acid and tormentic acid have displayed the highest and lowest binding affinity against tubulin-colchicine enzyme (PDB: 1SAO) with a docking score of −7.35 kcal/mol and −5.15 kcal/mol respectively. The ranking order of the docking score for anthelmintic action is shown below: Corosolic acid > Vitexilactone > 3,4-dihydroxybenzoic acid > 4-hydroxybenzoic acid > Tormentic acid. Here, corosolic acid and vitexilactone bind to the enzymatic pocket of the tubulin-colchicine enzyme by forming one hydrogen bond with Ser178 and Gln11, respectively. 3,4-dihydroxybenzoic acid binds with the same enzymatic pocket through the formation of two hydrogen bonds with Asn101 and Ser178, whereas 4-hydroxybenzoic acid interacts by establishing one hydrogen bond with Val315. tormentic acid binds with the same enzymatic pocket through two H-bonds with Asn101 and Ala317.

ADME study and toxicological property prediction

As stated by the Lipinski's rule of five, a compound could show drug-likeliness properties if it does not fail more than one of the following principles: (i) molecular weight not more than 500 amu; (ii) H-bond acceptors ≤ 10; (iii) H-bond donors ≤ 5; and (iv) Lipophilicity < 5. Besides, Veber et al. suggested that a compound should have the number of rotatable bonds ≤ 10 and topological polar surface area (TPSA) value ≤ 140 Å^2 for excellent molecular

Table 4. Docking score of the selected compounds with COX-1 and COX-2 enzymes for antinociceptive activity.

Compounds	COX-1 (PDB: 2OYE)			COX-2 (PDB: 3HS5)		
	Docking score (kcal/mol)	Glide e model (kcal/mol)	Glide energy (kcal/mol)	Docking score (kcal/mol)	Glide e model (kcal/mol)	Glide energy (kcal/mol)
3,4-dihydroxybenzoic acid	−5.83	−36.61	−28.9	−5.18	−26.88	−18.89
4-hydroxybenzoic acid	−5.36	−28.61	−22.33	−5.49	−28.84	−20.94
Vitexin	−5.11	−49.60	−38.35	-	-	-
Corosolic acid	−3.12	−27.97	−24.34	-	-	-
Vitexilactone	−2.84	−17.16	−16.75	-	-	-
Tormentic acid	−2.77	−29.65	−25.95	-	-	-
Agnuside	-	-	-	-	-	-
Iridoid	-	-	-	-	-	-
Luteolin	-	-	-	-	-	-

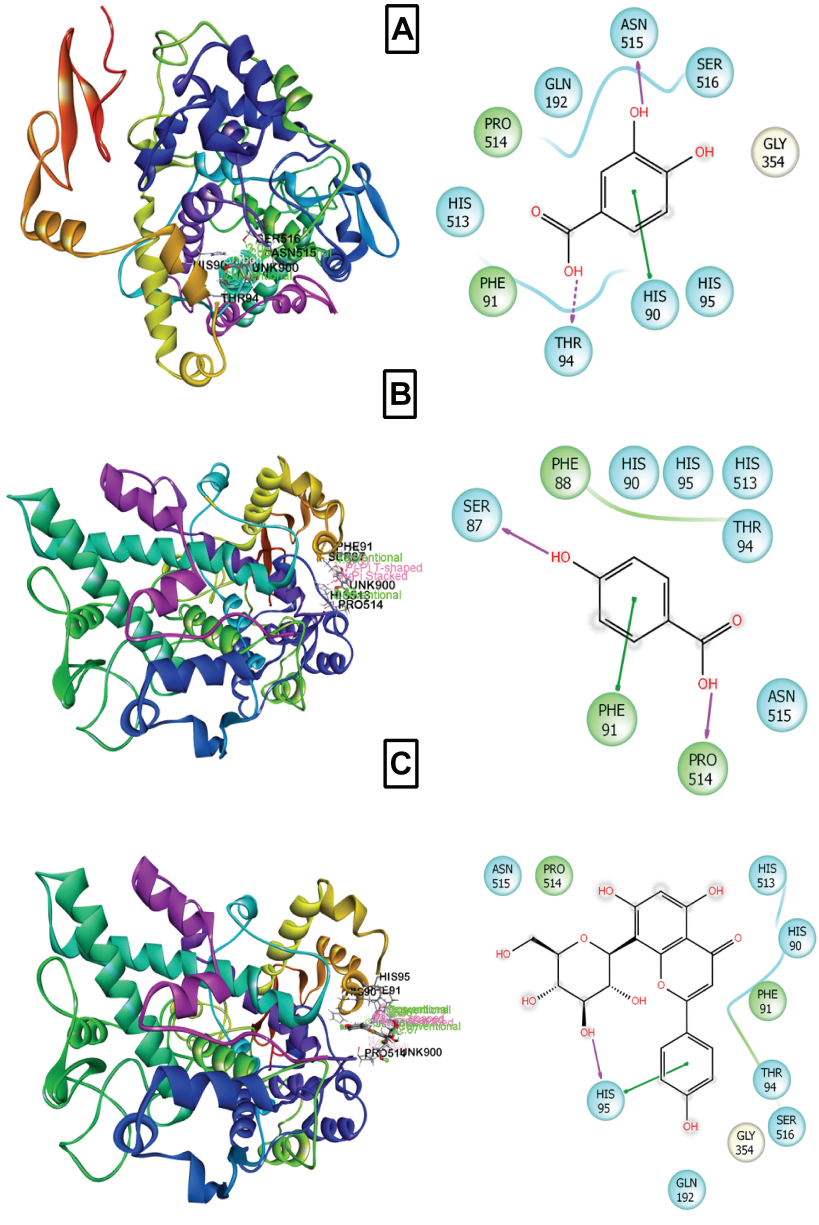

Figure 2. Best ranked pose and 2D interactions of (A) 3,4-dihydroxybenzoic acid, (B) 4-hydroxybenzoic acid, (C) Vitexin with COX-1 enzyme (PDB: 2OYE).

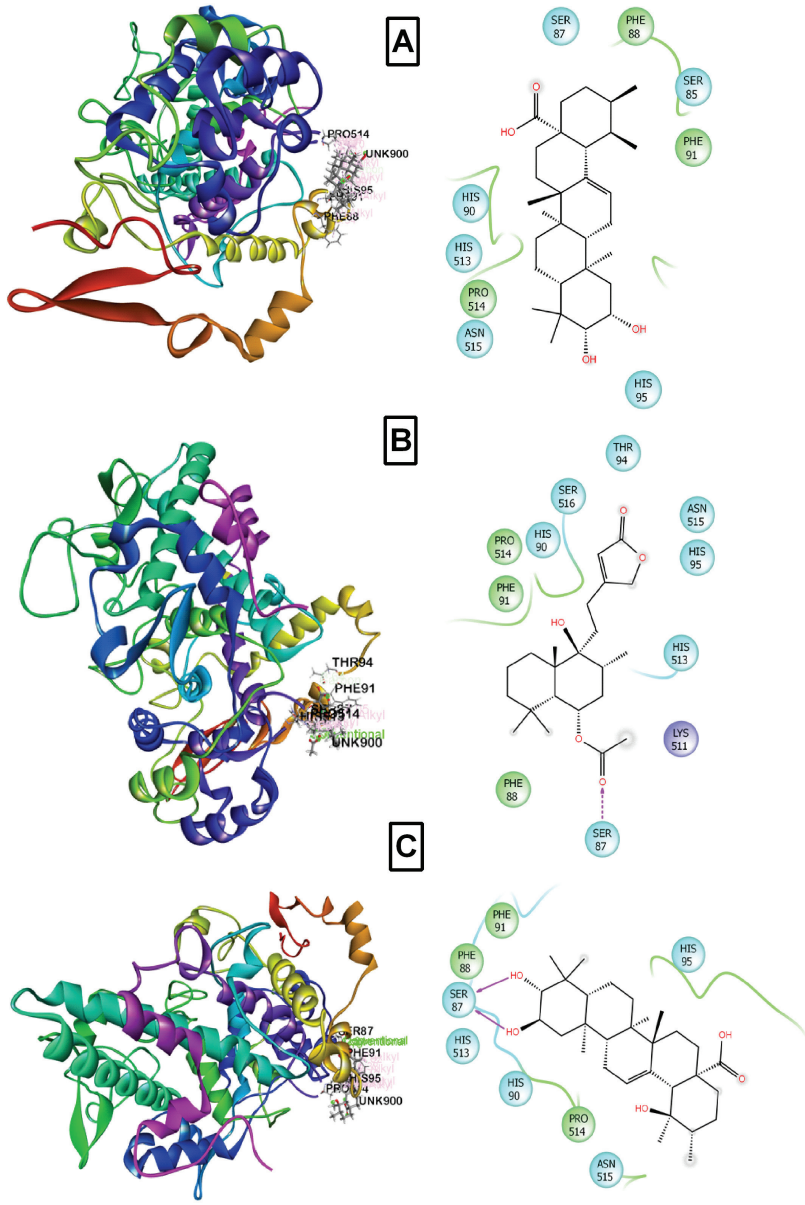

Figure 3. Best ranked pose and 2D interactions of (A) Corosolic acid, (B) Vitexilactone, (C) Tormentic acid with COX-1 enzyme (PDB: 2OYE).

flexibility of a molecule and molecular transport of drugs through membranes. Our study revealed that all of the compounds satisfied the Lipinski's rule of five and Veber's rule, which indicates that all compounds could be a good source for developing new drugs (Table 6). Again, the toxicological properties of the compounds were also predicted by the admetSAR online tool, and the results are shown in Table 7. Here, all the compounds showed non-ames toxic and non-carcinogenic properties.

PASS prediction study

Six major selected compounds of *V. peduncularis* were studied by the PASS online tool for antinociceptive, antihelmintic, and other biological activities and the potent displayed higher Pa value than Pi. This study revealed several important biological activities of the compounds such as antinociceptive, antihelmintic, antieczematic, antiseborrheic, antihemorrhagic, antineoplastic, antiprotozoal, antihypercholesterolemic, hepatoprotectant, antiulcerative, antiarthritic, antipruritic, antiseborrheic, and antimutagenic (Table 8).

Discussion

This study was carried out to explore the antinociceptive and anthelmintic activities of the EEVP in both experimental (*In vivo* and *In vitro* study) and computational (molecular docking, ADME/T, PASS

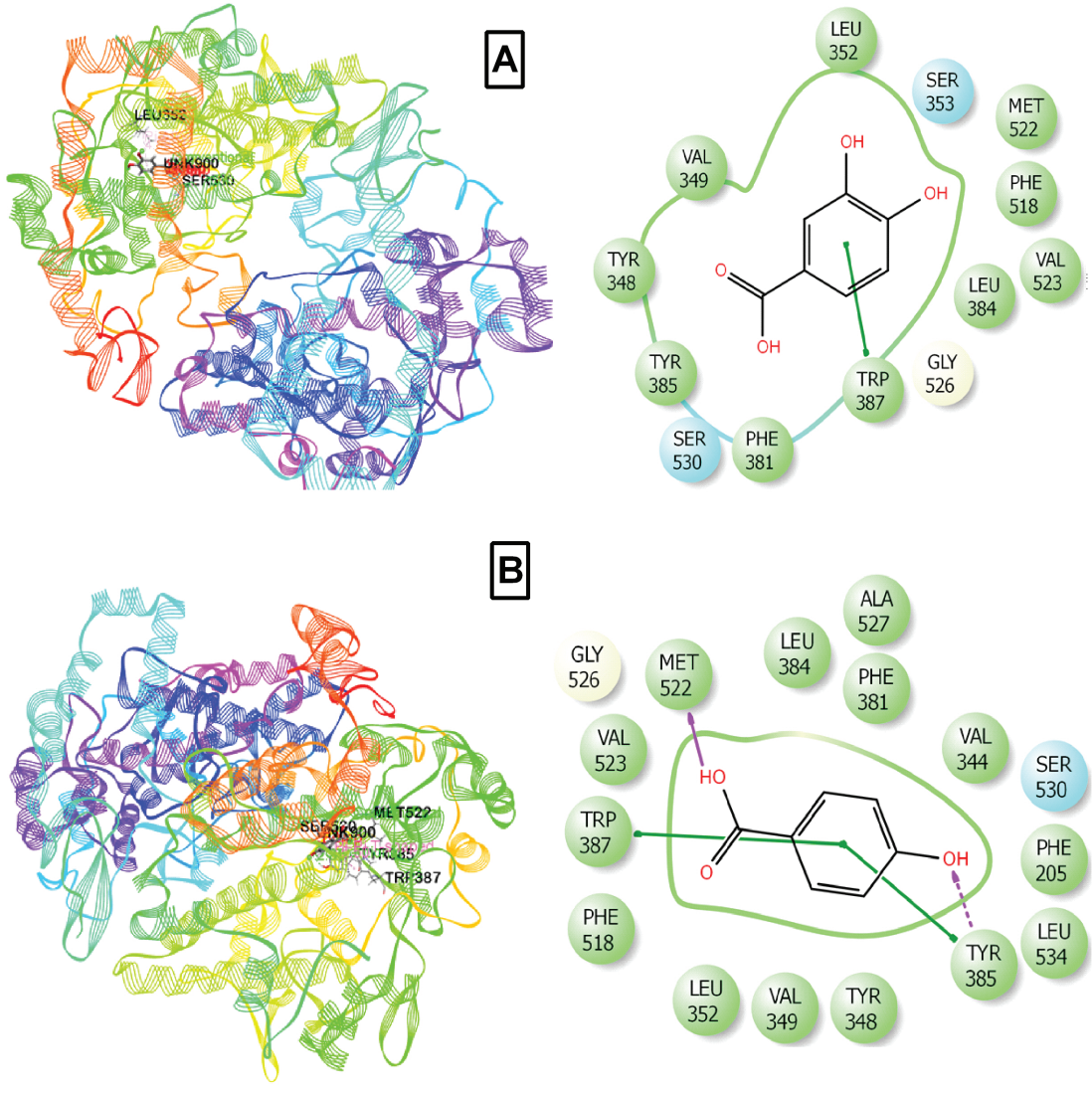

Figure 4. Best ranked pose and 2D interactions of (A) 3,4-dihydroxybenzoic acid and (B) 4-hydroxybenzoic acid with COX-2 enzyme (PDB: 3HS5).

prediction study) models. A previous phytochemical study of this plant revealed that the plant contains several categories of phytoconstituents, such as flavonoids, terpenoids, triterpenoids, flavones, and iridoids [1,5,7,8]. In addition, our quantitative phytochemical study showed that EEVP contains substantial amount of phenolic (121.67 ± 3.51 mg GAE/g dried plant extract), flavonoids (97.78 ± 1.57 mg QE/g dried plant extract), flavonol (39.88 ± 0.49 mg QE/g dried plant extract) and condensed tannins (63.49 ± 3.15 mg CE/g dried plant extract). The presence of such phytoconstituents describes the possibility of numerous pharmacological activities like anti-nociceptive, antiviral, anti-inflammatory, anthelmintic, antimicrobial, antioxidant, antidiarrheal, thrombolytic, cytotoxic, and so on. For example, phenolics compounds are responsible for anti-inflammatory, analgesic, antioxidant

Table 5. Docking score of the selected compounds with tubulin-colchicine enzyme (PDB: 1SAO) for anthelmintic activity.

Compounds	Tubulin-colchicine enzyme (PDB: 1SAO)		
	Docking score (kcal/mol)	Glide e model (kcal/mol)	Glide energy (kcal/mol)
Corosolic acid	−7.35	−48.39	−44.32
Vitexilactone	−5.88	−44.92	−37.03
3,4-dihydroxy benzoic acid	−5.78	−42.57	−32.59
4-hydroxy benzoic acid	−5.22	−28.64	−22.55
Tormentic acid	−5.15	−30.16	−27.37
Vitexin	-	-	-
Agnuside	-	-	-
Iridoid	-	-	-
Luteolin	-	-	-

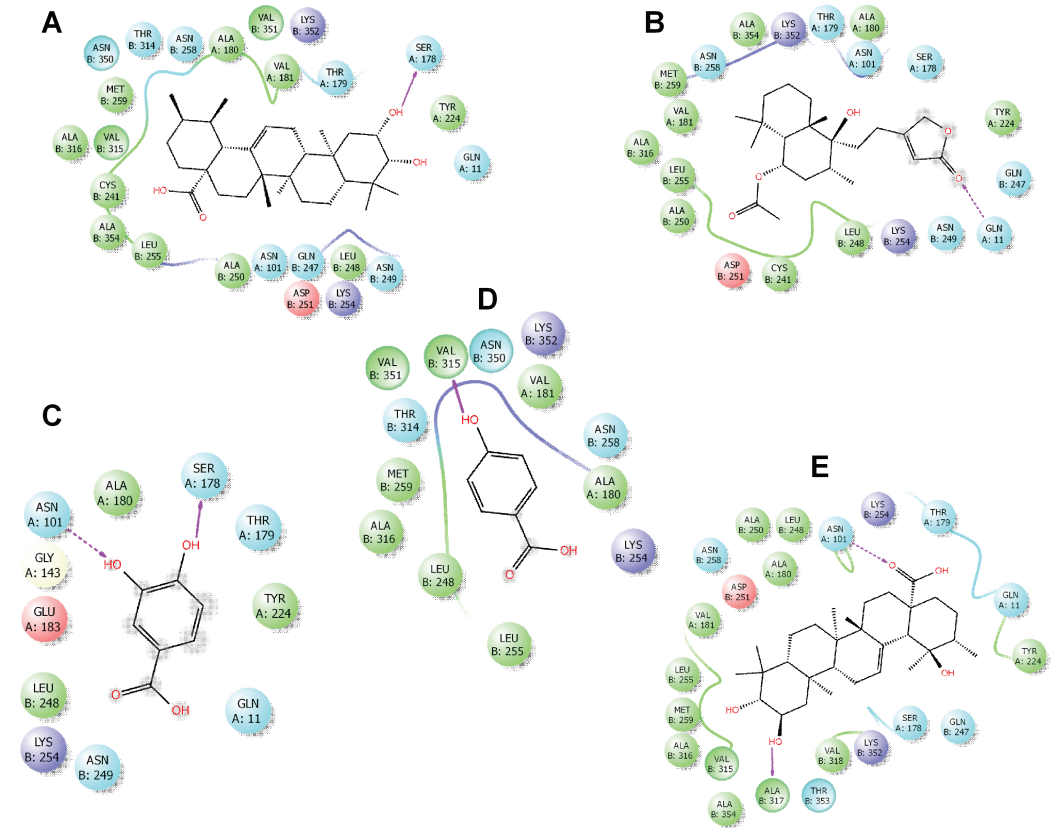

Figure 5. 2D representation of the interactions between the best pose found for (A) Corosolic acid, (B) Vitexilactone, (C) 3,4-dihydroxybenzoic acid, (D) 4-hydroxy benzoic acid, and (E) Tormentic acid with Tubulin-colchicine enzyme (PDB: 1SAO).

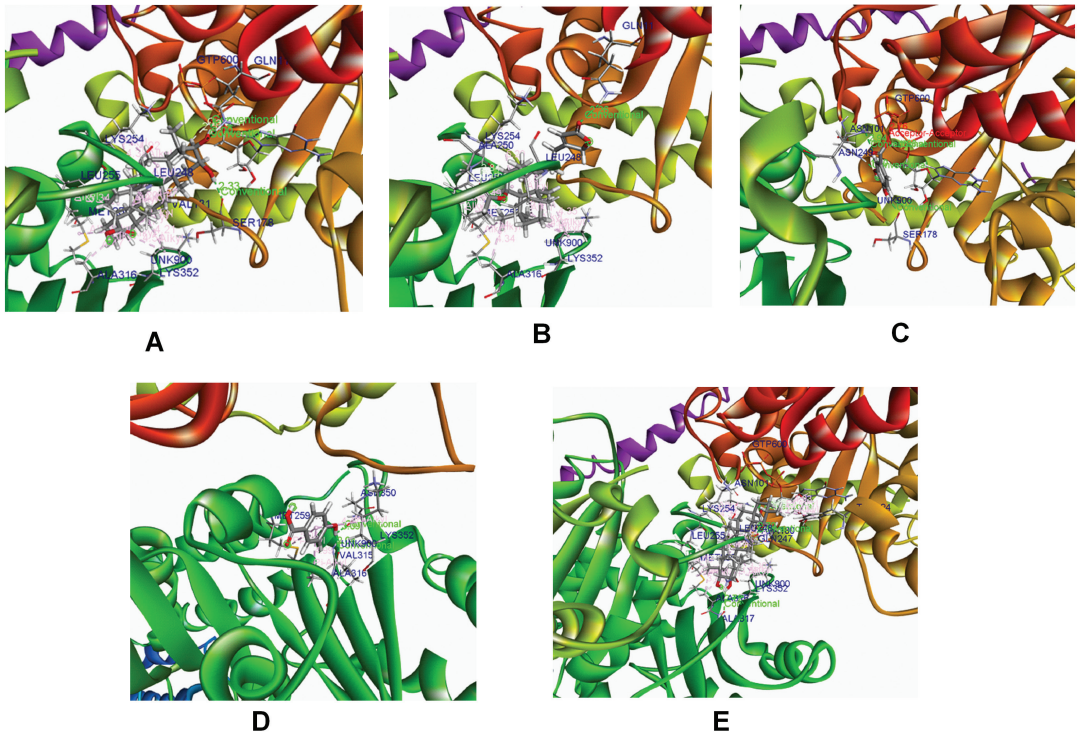

Figure 6. Best ranked pose of (A) Corosolic acid, (B) Vitexilactone, (C) 3,4-dihydroxbenzoic acid, (D) 4-hydroxybenzoic acid and (E) Tormentic acid in the binding pocket of Tubulin-colchicine enzyme (PDB ID: 1SAO).

Table 6. Physicochemical properties of the compounds for good oral bioavailability.

Compound	MW	HBA	HBD	Log P	AMR	nRB	TPSA	Lipinski's violations
Rule	**< 500**	**< 5**	**≤ 10**	**≤ 5**	**40-130**	**≤ 10**	**≤ 140**	**≤ 1**
3,4-dihydroxybenzoic acid	154.12	4	3	0.65	37.45	1	77.76	0
4-hydroxybenzoic acid	138.12	3	2	1.05	35.42	1	57.53	0
Vitexin	432.38	10	7	-0.07	106.61	3	181.05	1
Corosolic acid	472.70	4	3	5.07	138.08	1	77.76	1
Vitexilactone	378.50	5	1	3.61	104.30	5	72.83	0
Tormentic acid	488.70	5	4	4.33	139.28	1	97.99	0

MW = Molecular weight (g/mol); HBA = Hydrogen bond acceptor; HBD = Hydrogen bond donor; Log P = Lipophilicity; AMR = molar refractivity; nRB = number of rotatable bond; TPSA = topological polar surface area.

and anti-proliferative properties [30,31], whereas flavonoids are reported to have antioxidant, antibacterial, antidiarrheal, anti-nociceptive, anti-inflammatory, antiviral, anticancer, and anti-allergic activities [32–37]; iridoids have anti-inflammatory activity [5]; terpenoids are responsible for potent anti-hypertensive, antimicrobial, anti-parasitic, anti-inflammatory, nematicidal, and insecticidal properties [36,38–40] while tannins have anti-ulcerative effect and also responsible for the death of worms by selectively binding with free proteins existing in the gastrointestinal tract [16,41,42]. On the other hand, the acute oral toxicity study did not show any mortality, abnormal behavior, and neurological changes up to 2000 mg/kg dose for EEVP, which indicates that the plant extract possesses a low toxicity profile and safe for a therapeutic dose.

For the experimental evaluation of the anti-nociceptive activity of EEVP, we began our investigation with the acetic acid-induced abdominal constriction or writhing test in mice. The symptom of writhing response after inducing acetic acid (intraperitoneally in mice) causes abdominal constriction, is a well-acquainted model for the erudition of visceral pain, which undoubtedly aids to assess the peripherally reactive analgesic effect [43]. In this test, pain perception is realized by generating pro-inflammatory endogenous mediators [prostaglandins, histamine, serotonin, cyclooxygenase (COX), lipooxygenase and cytokines] in the peripheral tissue fluid influencing the acceleration of prostaglandin levels into the peritoneal cavity, which progresses the inflammatory ache by enhancing capillary permeability [44]. The agents that reduce the number of writhing response, as well as pain sensation, may be due to the interruption of prostaglandin synthesis [45]. In our study, oral administration of EEVP significantly suppressed the acetic acid-induced abdominal pain in mice, resulting in a significant analgesic activity which could be due to the demonstration of analgesic principles regulating with the prostaglandin alley, indicating that EEVP might reveal potent analgesic activity (Fig. 1).

Table 7. Toxicological property predictions of the selected compounds.

Compound	Ames toxicity	Carcinogens
3,4-dihydroxybenzoic acid	Non Ames toxic	Non-carcinogenic
4-hydroxybenzoic acid	Non Ames toxic	Non-carcinogenic
Vitexin	Ames toxic	Non-carcinogenic
Corosolic acid	Non Ames toxic	Non-carcinogenic
Vitexilactone	Non Ames toxic	Non-carcinogenic
Tormentic acid	Non Ames toxic	Non-carcinogenic

Secondly, the formalin-induced paw-licking test was performed to investigate whether the anti-nociceptive activity of EEVP is mediated from central or peripheral origin since the acetic acid-induced writhing test cannot conclude whether it produces central or peripheral effects [46]. This method involves continuing pain and exhibits a biphasic nociceptive reaction (early and late phases) mediated by two different mechanisms [47]. The early phase (0–5 minutes), which instantly begins after the injection, is because of the incessant chemical stimulation of sensory C-fibers and some opioids like drugs are highly sensitive to this severe neurogenic pain, whereas both centrally acting and anti-inflammatory drugs can inhibit the late phase (15 to 30 minuttues) which is due to inflammatory reactions [48,49]. The results of the formalin test show that EEVP significantly and dose-dependently reduced the formalin-induced nociceptive response in both phases (Table 2). Therefore, it can be concluded that the extract possesses both central and peripheral anti-nociceptive effects. However, the anti-nociceptive activity of EEVP

Table 8. Biological activities predicted for *V. peduncularis* major compounds by PASS online.

Compounds	Biological activities predicted by pass online	Pa	Pi
3,4-DHB acid	Antinociceptive	0.563	0.013
	Antihelmintic	0.492	0.018
	Antieczematic	0.860	0.009
	Antimutagenic	0.834	0.003
	Antihypercholesterolemic	0.612	0.013
4-HDB acid	Antinociceptive	0.533	0.021
	Antihelmintic	0.531	0.012
	Antiseborrheic	0.902	0.004
	Antieczematic	0.858	0.009
	Antimutagenic	0.813	0.004
Vitexin	Antinociceptive	0.255	0.219
	Antihelmintic	0.507	0.016
	Antihemorrhagic	0.826	0.002
	Anticarcinogenic	0.866	0.003
	Antimutagenic	0.820	0.004
Corosolic acid	Antinociceptive	0.759	0.003
	Antiulcerative	0.881	0.003
	Antiprotozoal	0.896	0.003
	Antineoplastic	0.848	0.007
	Antipruritic	0.778	0.004
Vitexilactone	Antinociceptive	0.242	0.015
	Antihelmintic	0.259	0.152
	Antineoplastic	0.752	0.018
	Antieczematic	0.691	0.049
	Antihypercholesterolemic	0.396	0.038
Tormentic acid	Antinociceptive	0.731	0.003
	Hepatoprotectant	0.975	0.001
	Antiprotozoal	0.835	0004
	Antiulcerative	0.768	0.004
	Antiarthritic	0.763	0.011

3,4-DHB acid = 3,4-dihydroxybenzoic acid; 4-HDB acid = 4-hydroxy benzoic acid; Pa = Probable activity; Pi = Probable inactivity.

might be due to the presence of several bioactive phytoconstituents such as flavonoids, terpenoids, triterpenoids, and flavones which were reported in a previous study and also substantial amount of phenolic (121.67 mg), flavonoids (97.78 mg), flavonol (39.88 mg), and condensed tannins (63.49 mg) contents have been found in EEVP during the quantitative phytochemical study. It was reported earlier by several scientific reports that many phytoconstituents such as phenolics [30], flavonoids [35,50], terpinoids [36,38], and tannins [51,52] are responsible for the anti-nociceptive activity of the plants.

Thirdly, an aquarium worm (*Tubifex tubifex*) was used to determine the anthelmintic activity of EEVP. According to the World Health Organization, 24% percent of the world's populations (more than 1.5 billion people) are suffering from helminth infections which are the root cause of developing many chronic and debilitating diseases such as elephantiasis, schistosomiasis, difficulties during pregnancy, impaired cognitive and physical development of children, and anemia [53–55]. Moreover, it may lead to bladder cancer and have long-term effects on economic productivity when it is included for livestock or veterinary world [56]. To prevent the problems made by helminths, finding a new anthelmintic agent is essential where plant-derived natural products could play a crucial role since it has no or fewer side effects and has the best compatibility with human physiology [57]. Our study concludes that EEVP possesses significant and dose-dependent anthelmintic activity at three different concentrations. This activity might be due to the presence of several phytochemicals such as flavonoids, phenols, and tannins, which were found abundantly during the quantitative phytochemical analysis of EEVP (Table 1). It was reported that flavonoids [58], tannins [59], phenolics, and terpinoids [39] might be responsible for the anthelmintic activity of the plants. Flavonoids can also inhibit the larval growth and the arachidonic acid metabolism of the worm's, which may cause the degeneration of neurons in the worm's body and result in death [58]. In addition, tannins prevent the energy generation of worms by uncoupling the oxidation phosphorylation and selectively bind to free proteins present in the gastrointestinal tract of worm's and eventually lead to death [59].

Molecular docking is the most powerful computational technique which has been widely used for the prediction of ligand-target interactions to know the binding mode of active compounds against key enzymes and also to better understand the possible molecular mechanism of various pharmacological responses [60]. Keeping in this view, a molecular docking study was carried out to comprehend the better mechanism and confirm their results with the experimental findings. In this study, nine major compounds of *V. peduncularis* namely 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vitexin, corosolic acid, vitexilactone, tormentic acid, agnuside, iridoid, and luteolin were investigated against cyclooxygenase-1 (COX-1, PDB id: 2OYE), cyclooxygenase-2 (COX-2, PDB id: 3HS5) and tubulin-colchicine enzyme (Tubulin, PDB:

1SAO) enzymes, and the docking scores obtained for all compounds have been reported in Tables 4 and 5. In the case of anti-nociceptive docking study, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vitexin, corosolic acid, vitexilactone, and tormentic acid showed the significant docking score against COX-1 enzyme among all the compounds, whereas only 3,4-dihydroxybenzoic acid and 4-hydroxybenzoic acid displayed the highest score against COX-2 enzymes. This study suggests that 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vitexin, corosolic acid, vitexilactone, and tormentic acid might be the responsible bioactive compounds for the potential anti-nociceptive activity of the EEVP. This finding is also full agreement with the various scientific studies which reported that 3,4-dihydroxybenzoic acid [61], 4-hydroxybenzoic acid [62], vitexin [63], vitexilactone [64], and tormentic acid [65] are responsible for anti-nociceptive and anti-inflammatory properties. In the case of an anthelmintic docking study, the nine compounds were docked with tubulin enzyme. Among the nine, five compounds namely corosolic acid, vitexilactone, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, and tormentic acid interacted with the tubulin enzyme and showed the promising docking scores ranging from -5.15 to -7.35 kcal/mol (Table 5). This result suggests that the five responded compounds might be responsible for the potential anthelmintic activity of the EEVP. Although further scientific studies are necessary to elucidate their mechanism of action behind such pharmacological actions.

Based on the highest score in the molecular docking study against COX-1, COX-2, and tubulin enzymes, we have selected six compounds namely 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vitexin, corosolic acid, vitexilactone, tormentic acid to explore their pharmacokinetic/drug-likeliness and toxicological properties further. Importantly, these characterizations are supposed as the crucial steps for the development of a new drug, clinical trial, and biological testing of the isolated compounds. Here, an online tool (SwissADME) was used to calculate the pharmacokinetic properties of the major compounds of *V. peduncularis* based on Lipinski's rule of five. Lipinski's rule of five stated that an orally administered drugs/compounds should have a molecular weight < 500 amu, Lipophilicity value ≤ 5, Hydrogen bond acceptor sites < 5, and Hydrogen bond donor sites ≤ 10. If any drugs or compounds violate this rule possibly will have problems with bioavailability where the total number of violation lies between 0 and 4. Moreover, Veber et al. proposed that a compound should have the number of rotatable bonds ≤ 10 and topological polar surface area ≤ 140 $Å^2$. The result of this study showed that all the compounds satisfied Lipinski and Veber rules, which indicates that these compounds are a suitable candidate for drugs and theoretically have ideal oral bioavailability (Table 6). Then, we have also determined the toxicological properties of the six selected phytocompounds using the admetSAR online tool since the safety of the compounds is an essential parameter for being a successful drug. This study exhibited that none of the compounds posed a risk of ames toxicity (excluding vitexin) and carcinogenicity (Table 7).

PASS is a computer-based online tool used for the simultaneous predictions of different kinds of biological activity based on the structure of different molecules/compounds. In this study, the molecules/compounds showing more Pa (probable activity) value than Pi (probable inactivity) are the only constituents considered as possible for a particular biological activity. Our study showed that all six compounds (3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vitexin, corosolic acid, vitexilactone, and tormentic acid) could produce antinociceptive and anthelmintic activities. In addition, they also have the possibility to yield various types of pharmacological activity like antieczematic, antihemorrhagic, antiprotozoal, antihypercholesterolemic, hepatoprotectant, antiulcerative, antiarthritic, antipruritic, antiseborrheic, and antimutagenic. This study is also consistent with previously reported scientific studies which stated that 3,4-dihydroxybenzoic acid [61], 4-hydroxybenzoic acid [62], vitexin [63], vitexilactone [64], and tormentic acid [65] are responsible for the anti-nociceptive property.

Conclusions

In brief, our study revealed that EEVP has a significant and dose-dependent anti-nociceptive and anthelmintic activities which might be due to the occurrence of a substantial amount of polyphenols and bioactive phytocompounds such as 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vitexin, corosolic acid, vitexilactone, and tormentic acid that act individually or synergistically. Our molecular docking study also revealed that the six bioactive compounds have a higher binding affinity toward the respective enzymes for the anti-nociceptive and anthelmintic properties. In addition, our ADME/T study exposed that these six compounds are safe

and orally bioavailable from a druggable point of view. PASS prediction study also presented several pharmacological activities with the anti-nociceptive and anthelmintic activity. However, further extensive studies are necessary to elucidate their exact molecular mechanism of action in animal models and subsequently on human subjects to verify its clinical efficacy as potential antinociceptive and anthelmintic agents.

Abbreviations

EEVP: Methanol extract of *Vitex peduncularis* leaves; PDB: Protein data bank; OPLS: Optimized potentials for liquid simulations; RMSD: Root-mean-square deviation; ADME: absorption, distribution, metabolism, elimination; PASS: prediction of activity spectra for substances; SPSS: Statistical package for the social sciences; SEM: Standard error of the mean; ANOVA: Analysis of variance.

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Conflict of interest

The authors declare that they have no conflict of interest

Author's contributions

Reedwan Bin Jafar Auniq, Md. Nazim Uddin Chy, and Md. Adnan collected the plant, dried, powdered, prepared the plant extract, and conceived and designed the experiments. Reedwan Bin Jafar Auniq, Md. Nazim Uddin Chy, Md. Adnan, Ajoy Roy, Mohammad Ashraful Islam, Tourna Nahrin Khan, Md. Zahid Hasan, Suhel Ahmed, and Mohammad Forhad Khan carried out experimental works, collected data, analyzed and interpreted experimental results and wrote the original draft. Md. Nazim Uddin Chy, Nazmul Islam, Muhammad Ismail Khan, Md. Alamgir Hossain, Md. Alamgir Kabir, Md Tareque Hassan Mukut, and Sabrina Islam performed the molecular docking, ADME/T, and PASS prediction study and wrote the relevant potions. Md. Nazim Uddin Chy and Md. Adnan furnished the designing of the work, checked the experimental results, and drafted the final manuscript. All authors read and approved the final manuscript.

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ORIGINAL RESEARCH

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Anti-androgenic and insulin-sensitizing actions of *Nigella sativa* oil improve polycystic ovary and associated dyslipidemia and redox disturbances

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ABSTRACT

Background/Aim: *Nigella sativa* oil has been shown to improve metabolic syndrome-associated dyslipidemia and oxidative stress. Effects of *N. sativa* oil on letrozole-induced polycystic ovary syndrome (PCOS)-associated hormonal, metabolic, redox, and lipid dysregulation in rats were investigated.

Methods: Female Sprague-Dawley rats were randomly assigned into four groups (n = 10 per group): control, PCOS, and PCOS treated with *N. sativa* oil (5 or 10 ml/kg/day) once daily *per oral* starting at day 7 after the commencement of letrozole (1 mg/kg) until day 56. Body weight and estrous cycle were recorded starting from day 7 and day 28, respectively, until day 56. Rats were dissected and serum and ovaries were collected for biochemical and histomorphological assessments.

Results: *Nigella sativa* oil (10 ml/kg/day) treatment significantly increased: number of rats undergoing regular cycle (80% vs. 20%; $p < 0.05$), average number of regular cycles (5.00 ± 0.59 vs. 0.20 ± 0.42; $p < 0.05$), appearance of corpus luteum; but reduced number of cystic follicles (7.60 ± 1.51 vs. 6.10 ± 0.19; $p < 0.05$) in PCOS rats. Body weight was also reduced by the oil in PCOS rats. However, ovarian weight, granulose, and theca cells layers showed no significant change. The oil also restored altered circulating levels of gonadotropins, steroids, and reduced fasting blood glucose. The ovarian total cholesterol, triglycerides, low-density lipoprotein-cholesterol, and malondialdehyde were markedly reduced while insulin sensitivity and ovarian high-density lipoprotein-cholesterol, superoxide dismutase, and glutathione peroxidase activities were greatly increased.

Conclusion: *Nigella sativa* oil improved polycystic ovarian-morphology and metabolic disorders via its anti-androgenic and insulin-sensitizing actions to ameliorate PCOS-associated dyslipidemia and redox disturbances.

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Dyslipidemia; letrozole; *Nigella sativa* oil; PCOS; redox

Introduction

Polycystic ovarian syndrome (PCOS) is the commonest endocrine disorder affecting about 20% women of reproductive age around the world. It is characterized by hyperandrogenism, acyclicity, polycystic ovary and metabolic disorders [1] such as insulin resistance

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(IR), obesity, and diabetes [2]. PCOS-induced up-regulation of pro-inflammatory and oxidative stress markers increases patients' risk of dyslipidemia, type 2 diabetes mellitus (T2DM), and cardiovascular disorders [3,4]. Dyslipidemia has been reported in most PCOS patients (obese/non-obese and young/old) [5].

Dyslipidemia has a prevalence of 70% in women with PCOS and has been linked with hyperandrogenism and IR. Hyperandrogenemia and IR affect lipid profile in PCOS via mechanisms relating to cholesterol metabolism, uptake, and efflux from peripheral cells. They also increase immune cells activation to release pro-inflammatory cytokines and enhance excessive production of oxidants. Therefore, elevated oxidative stress is commonly reported in PCOS [6].

PCOS is usually treated based on clinical symptoms and therapeutic schedule. Dyslipidemia is a foremost therapeutic schedule in overweight/obese PCOS women, thus weight loss is recommended [5]. However, the combined effect of IR, hyperandrogenemia, low metabolic rate, and disturbed appetite regulation makes weight loss difficult in PCOS [7]. Clomiphene citrate (CC), the first-line treatment of PCOS is associated with minor and major side effects apart from the subsection of PCOS women that are CC resistant. Other forms of treatment such as gonadotropin-releasing hormone (GnRH), metformin, letrozole, and tamoxifen are also associated with side effects [6].

Nigella sativa oil was reported to modulate lipid metabolism and redox status such as by reducing low-density lipoprotein cholesterol (LDL-C) and by increasing high-density lipoprotein (HDL-C) in IR syndrome [8]. Administration of 2.5 ml *N. sativa* oil per day in T2DM reduced total cholesterol (TC) improved glycemic control without any side effects [8,9]. The antioxidant and lipid modulating effects of *N. sativa* oil and its major active compound thymoquinone (TQ) were reported in different *in vitro* and *in vivo* disease models [10–13]. Recent studies support the hypolipidemic and redox modulating properties of *N. sativa* oil and TQ [13–17]. Here we report the beneficial effect of *N. sativa* oil in ameliorating PCOS-associated conditions (dyslipidemia and redox disturbances) *via* its anti-androgenic and insulin-sensitizing actions.

Materials and Methods

Chemicals and reagents

Essential oil (100%) of *N. Sativa*, extracted using cold pressing, was procured from Hemani International (Hemani International KEPZ, Karachi, Pakistan) and letrozole from Novartis Pharmaceuticals (Accord Healthcare, Middlesex, UK). The assay kits for thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT), cholesterol, triglycerides (TGs), HDL-C, and LDL-C are products of Cell Biolabs, Inc. (Cell Biolabs Inc, San Diego, USA). Serum sex steroids (testosterone; T and estradiol; E2) and gonadotropins [follicle stimulating hormone (FSH) and luteinizing hormone (LH)] levels were determined using enzyme-linked immunosorbent assay (ELISA) kit procured from Monobind Inc, Lake Forest, USA. Glutathione peroxidase (GPX) assay kit is from Sigma-Aldrich, St. Louis, MO, USA. Blood glucose level was determined using Glucose Roche Diagnostic glucometer strip. Other chemicals include carboxymethylcellulose (CMC), normal saline, formalin, glucose, ethanol, diethyl ether, and picric acid. The chemical reagents were of analytical grade and purchased mainly from Sigma-Aldrich except otherwise stated.

Animal ethical committee approval

The study procedure and animal handling were carried out in accordance with the Guideline for Care and Use of Laboratory Animals of the University of Ilorin and were approved by the Ethics Committee (UERC/ASN/2019/1805), Faculty of Basic Medical Sciences, University of Ilorin, Ilorin, Nigeria.

Experimental animals

Virgin female Sprague-Dawley rats (40) weighing 150.0 ± 5.0 g and aged 6 weeks old with 4–5 days regular estrus were obtained, kept, and observed for general well-being in the Animal Holding Facility of the University of Ilorin under standard housing condition (12 hours light/dark cycle at 21°C–24°C and 40%–45% humidity). They were kept in polypropylene cages and fed standard rat chow and water *ad libitum*. The animals were acclimatized for 7 days before treatment commenced.

Experimental design

The animals were randomly assigned into four groups of which n = 10 animals per group: control, PCOS, and treatment groups (*N. sativa* oil; 5 and 10 ml/kg/day). The selected doses of *N. Sativa* oil have been considered as the standard and maximum volumes of oils that could be reasonably administered to rats over a long period of time without dose/volume-related disturbances [18]. All animals were induced with PCOS, except the control group, using oral letrozole (1.0 mg/kg). Control and PCOS groups

received vehicle [10 ml/kg/day, *per oral* (*p.o.*)] and the treatment groups were given *N. sativa* oil (5 and 10 ml/kg/day, *p.o.*). Body weight and estrous cycle were monitored from day 7 and day 28, respectively, after the commencement of letrozole administration until the 56th day (Fig. 1).

PCOS induction and treatments

PCOS was induced in the experimental rats using letrozole (1 mg/kg) that was prepared in 0.5% CMC and administered to the rats, orally, once daily throughout the experimental period of 56 days. In addition, *N. sativa* oil (100%), in graded doses of 5 and 10 ml/kg/day, [18] was administered to the test groups from day 7 of the commencement of letrozole administration and 0.5% CMC *p.o.* was administered to the control group throughout the experimental period (Fig. 1). The study analysis was conducted and data were collected between August 2018 and March 2019.

Estrous cycle assessment and specimen collection

Vaginal smear cytology was carried out to determine estrous phases daily following standard procedure [19]. Rats from all the groups were fasted overnight and anesthetized with diethyl ether on day 57. Peripheral blood was collected through retro-orbital sinus puncture and serum was separated by centrifugation and stored at −20°C until biochemical analysis. Dissected right ovaries were weighed, fixed in 4% paraformaldehyde at 4°C overnight, and stored in 70% ethanol before histological processing while the left ovaries were homogenized in ice cold 1:4 w/v phosphate buffered saline and centrifuged at 4°C for 30 minutes at 3,000 relative centrifugal force. The supernatant collected was used for the estimation of antioxidant enzymes, protein content, and lipid profile.

Hormonal assays

Serum T, E2, LH, and FSH were determined using species-specific ELISA kit (Monobind Inc, Lake Forest, USA) following the manufacturer's instructions. The absorbance in each well was read at 450 nm in a microplate reader (Biocompare, CA, USA) and compared with the standard. The gonadotropins detection limit, coefficient of variations (CVs) for inter- and intra-assays were 0.134 mIU/ml, 5.2% and 8.6%, respectively, for FSH and 0.054 mIU/ml, 7.2% and 3.1% for LH. The steroids detection limit, CVs for inter- and intra-assays were 0.058 pg/ml, 5.2% and 8.6%, respectively, for T and 8.20 pg/ml, 3.1% and 7.2% for E2. All immunoassays were performed in a single batch.

Evaluation of oral glucose tolerance, fasting blood glucose, and serum insulin

Fasting blood glucose and oral glucose tolerance test (OGTT) was carried out as previously described [20]. Fasting serum insulin concentration was measured, using Accu-Bind ELISA kits procured from Monobind Inc., according to the manufacturer's

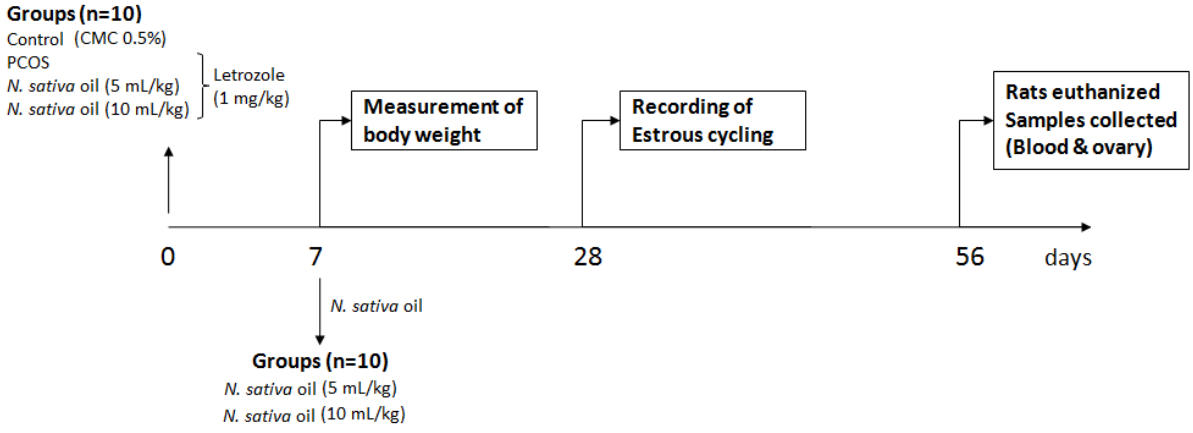

Figure 1. The experimental design. Rats were assigned into control, PCOS, and *N. sativa* oil (5 and 10 ml/kg/rat/day)-treated PCOS groups. Control received carboxymethyl cellulose (0.5% CMC *p.o.*), PCOS and *N. sativa* oil (5 and 10 ml/kg/rat/day)-treated PCOS groups received letrozole (1 mg/kg *p.o.*) prepared in 0.5% CMC throughout the experimental period. In addition, the *N. sativa* oil-treated groups were subsequently treated with *N. sativa* oil (5 and 10 ml/kg/rat/day) throughout the experimental period starting from day 7 after the commencement of letrozole. At the end of the experimental period (57th day), rats from all the groups were euthanized using diethyl ether anesthesia and blood and ovaries were collected for biochemical analysis and histopathological studies, respectively.

instructions. Quantitative Insulin Sensitivity Check Index (QUICKI) was estimated according to the following formula [21].

QUICKI = $1/[\log (I_0) + \log (G_0)]$, where I_0 is the fasting plasma insulin (microunits/ml) and G_0 is the fasting serum glucose (mg/dl).

Evaluation of ovarian lipid profile

Ovarian TGs, TC, HDL-C, and LDL-C concentrations were estimated using commercially available assay kits (ERBA Diagnostics, USA) following the manufacturer's instruction on a fully automated analyzer based on spectrophotometric principle.

Determination of ovarian antioxidant enzymes activity

CAT activity was assayed according to the previously described rapid spectrophotometric method of Cohen et al. [22] and the enzyme activity was calculated according to Aebi [23]. The enzyme activities were normalized with the protein content of each sample. CAT activity was calculated as units per milligram of protein. SOD activity was measured according to the method previously described [24] which is based on the ability of SOD to scavenge superoxide anion radical and inhibits the auto-oxidation of pyrogallol. The absorbance was measured kinetically at 420 nm, 25°C for 3 minutes. SOD activity was calculated as U/mg of protein, with 1U defined as the amount that inhibited the rate of pyrogallol autoxidation by 50%. GPX activity assay kit (Sigma-Aldrich, St. Louis, MO, USA) was used for measuring the GPX activity. It is based on the oxidation of reduced glutathione by GPX coupled to the disappearance of nicotinamide adenine dinucleotide phosphate reduced (NADPH) by glutathione reductase. GPX activity was calculated as units per gram of protein, with 1U GPX causing the formation of 1.0 μmol of nicotinamide adenine dinucleotide phosphate ($NADP^+$) from NADPH per minute.

Lipid peroxidation (LP) assay

Measurement of LP was carried out based on the TBARS method as described by Buege and Aust [25]. LP in the form of malondialdehyde (MDA) equivalent was expressed as nmol/mg protein in the sample.

Hematoxylin and eosin (H&E) staining

Ovarian tissues, fixed in 4% paraformaldehyde, were processed following the standard protocol. The tissues were sectioned (5 μm), mounted onto Superfrost Plus slides (Fisher Scientific), and stained with H&E following the standard protocol for histology. Histological examination was performed using a Nikon light microscope (Nikon, Tokyo, Japan) with an in-built camera attached to a monitor. Assessment was blind to the experimental design and performed on three sections from each group.

Statistical analysis

Using data from previously published literature [26], the sample size was estimated in the NCSS 2007 software (NCSS Statistical Software). Normalcy test was performed on the data and those that were not normally distributed were transformed before analysis using a log transformation. The statistical package, i.e., IBM SPSS Version 23 (SPSS Inc., Chicago, IL), was used for data analysis. Briefly, values presented in Tables and charts are means ± standard error of mean. Statistical differences were tested by One-way-analysis of variance with post hoc test using Fisher's least significant difference Multiple-Comparison Test. Analysis was considered statistically significant at $p < 0.05$.

Results

Nigella sativa oil restored cyclicity and improved ovarian histomorphology in PCOS rats

Control rats exhibited a normal estrous cycle of 4–5 days, ~6.75 ± 0.33 average number of complete cycles in 28 days, and all the rats (10 of 10; 100%) cycles normally (Fig. 2A–C). *Nigella sativa* oil (10 ml/kg/rat/day, *p.o.*) improved estrous cycles, increased significantly number of complete cycles in 28 days (5.00 ± 0.59 vs. 0.20 ± 0.42; $p < 0.05$), and the number of rats (8 of 10; 80%, $p < 0.05$) undergoing regular cycle when compared to the PCOS group (Fig. 2A–C). Ovarian histomorphology also showed no cytoarchitectural abnormalities in the control rats (Fig. 2D). The granulose cell layer remains markedly diminished without significant improvement in the theca interna layer but there was an increase appearance of corpus luteum in the ovary of *N. sativa* oil-treated rats (Fig. 2D). PCOS rats were completely acyclic and remain static at "pseudo diestrus" phase with significant ($p < 0.05$) decrease in the average number of complete cycles in 28 days (0.20 ± 0.42 vs. 6.75 ± 0.33; $p < 0.05$) and most of the PCOS rats (8 of 10; 80%) failed to cycle ($p < 0.05$) (Fig. 2A–C). The ovary-to-body weight ratio (%) showed no significant difference ($p > 0.05$) among the control (0.12 ± 0.03), PCOS (0.08 ± 0.08), and the treated groups (*N. sativa oil* 5 ml/kg/rat/day; 0.10 ± 0.02 and *N. sativa oil* 10 ml/kg/rat/day; 0.10 ± 0.01)

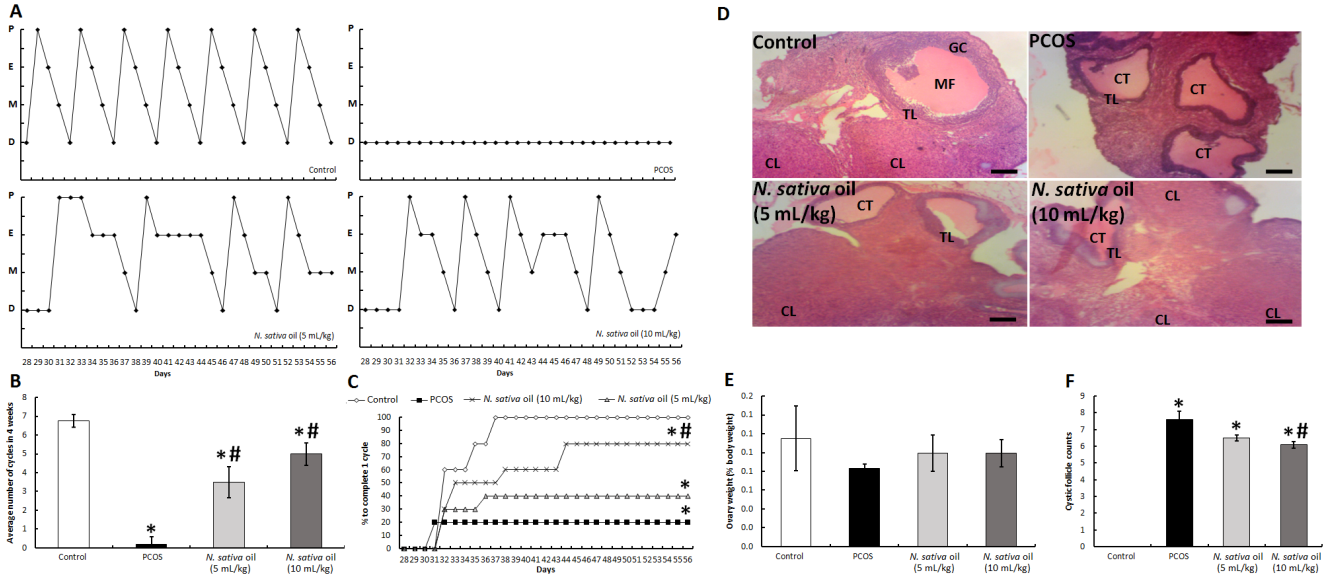

Figure 2. Estrous cycling, ovarian phenotype, ovary weight, and cystic follicle number in letrozole-induced PCOS rat treated with *N. sativa* oil. A, Estrous cycle pattern in representative females. P, proestrus; E, estrus; M, metestrus; D, diestrus. B, Average number of cycles in 4-weeks period. C, Percentage of females to complete one cycle in 4-week period. D, Histologic sections of representative ovaries from each treatment group. MF = mature follicle; GC = granulose cell layer; TL = theca interna cell layer; CL = corpus luteum; CT = cystic follicle. E, Ovary weights. F, Average number of cystic follicles/ovary. Values are means ± standard errors (n = 10). * Mean value was significantly different compared with normal control ($p < 0.05$). # Mean value was significantly different compared with the PCOS group ($p < 0.05$).

(Fig. 2E). However, PCOS rats exhibited significantly increased number of cystic follicle orientated in the periphery of the ovary (7.60 ± 1.71 vs. 0.00 ± 0.00; $p < 0.05$) and enlarged cystic follicular area characterized by a thickened theca interna cell layer and a diminished granulosa cell layer without any obvious corpus lutea in PCOS rats compared with controls. *Nigella sativa* oil (5 ml/kg/rat/day and 10 ml/kg/rat/day) reduced the number of cystic follicle significantly when compared to PCOS (6.50 ± 0.18 and 6.10 ± 0.19 vs. 7.60 ± 1.51; $p < 0.05$) (Fig. 2F).

Nigella sativa oil reduced elevated body weight in PCOS rats

PCOS group showed significant ($p < 0.05$) increase in body weight when compared to all other groups. Body weight of *N. sativa* oil treated and the reference groups were significantly ($p < 0.05$) higher compared to controls but remain similar throughout the experiment (Fig. 3).

Nigella sativa oil altered circulating levels of gonadotropins and sex steroids in PCOS rats

The serum levels of T, LH, and FSH increased and E2 decreased significantly ($p < 0.05$) in the PCOS group when compared to control. Treatment with *N. sativa* oil (5 or 10 ml/kg/rat/day) significantly

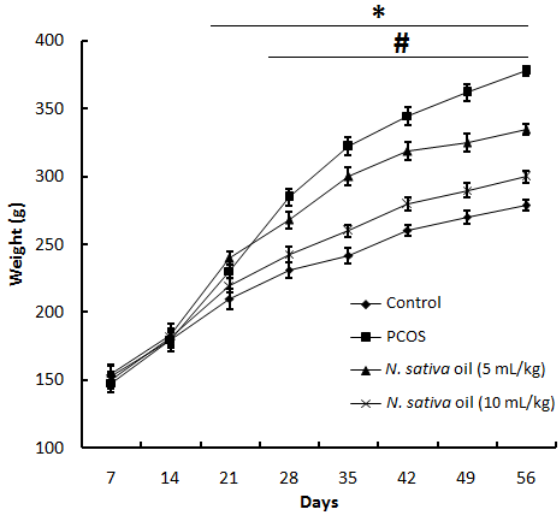

Figure 3. Body weight of letrozole-induced PCOS rats treated with *N. sativa* oil. The rats' weights were monitored at 7 days interval after letrozole administration, i.e., from the day of commencement of *N. sativa* oil treatment for 56 days. * Mean value was significantly different compared with normal control ($p < 0.05$). # Mean value was significantly different compared with the PCOS group ($p < 0.05$).

($p < 0.05$) altered serum levels of E2 (increased) and LH (decreased). However, treatment with *N. sativa* oil (10 ml/kg/rat/day) reduced serum T and

FSH levels significantly ($p < 0.05$) when compared with PCOS rats. Notably, serum levels of E2 and LH in the treated groups showed no significant ($p > 0.05$) change when compared with controls. T and LH levels remain significantly ($p < 0.05$) increased when compared with control and decreased when compared with PCOS rats (Table 1).

Nigella sativa oil improved letrozole-induced PCOS associated metabolic disturbances

PCOS group exhibited significant ($p < 0.05$) increase in serum insulin and fasting blood glucose levels with associated significant ($p < 0.05$) decrease in insulin sensitivity when compared with the control group (Fig. 4A–C). *Nigella sativa* oil significantly ($p < 0.05$) reduced fasting blood glucose and serum insulin levels associated with significant ($p < 0.05$) increase in insulin sensitivity when compared with the PCOS group. Fasting serum insulin and blood glucose levels in *N. sativa* oil-treated group remain significantly ($p > 0.05$) elevated when compared with the control. Notably, the insulin sensitivity in *N. sativa* oil (10 ml/kg/rat/day)-treated group showed no significant ($p > 0.05$) difference when compared with the control group. OGTT revealed significant ($p < 0.05$) glucose intolerance at 60 and

Table 1. Effect of 7 weeks administration of *N. sativa* oil on serum levels of LH, FSH, E2, and T` in letrozole (1 mg/kg)-induced PCOS rats.

Grouping	LH (mIU/ml)	FSH (mIU/ml)	E2 (pg/ml)	T (ng/ml)
Control	5.50 ± 1.84	0.90 ± 0.06	16.52 ± 2.11	0.05 ± 0.02
PCOS	9.00 ± 0.50*	1.27 ± 0.07*	12.23 ± 0.21*	0.98 ± 0.22*
N. sativa oil (5 ml/kg)	6.00 ± 0.52#	1.17 ± 0.05*	15.10 ± 1.55#	0.62 ± 0.14*
N. sativa oil (10 ml/kg)	6.00 ± 0.02#	1.01 ± 0.03#	15.66 ± 0.72#	0.48 ± 0.04*,#

Values are means ± standard errors ($n = 10$). LH = luteinizing hormone; FSH = follicle stimulating hormone; E2 = estrogen; T = testosterone.
*Mean values were significantly different compared with control rats at $p < 0{\cdot}05$.
#Mean values were significantly different compared with PCOS rats at $p < 0{\cdot}05$.

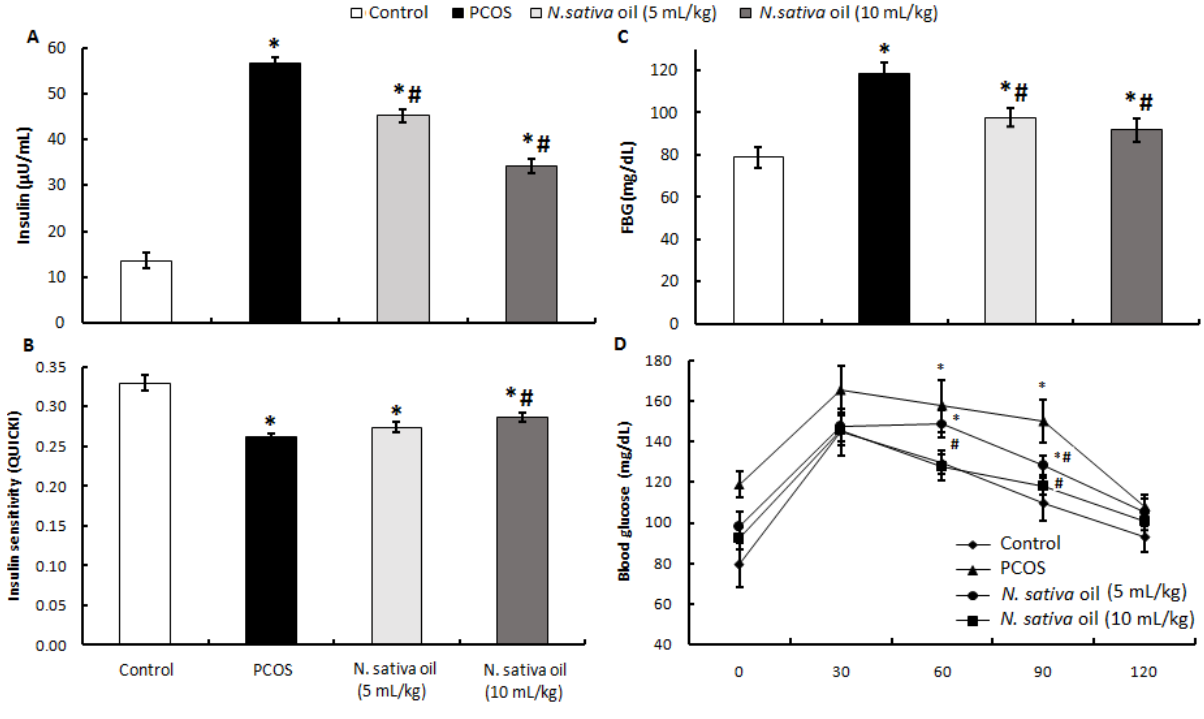

Figure 4. Serum insulin, insulin sensitivity, fasting blood glucose, and OGTT in letrozole-induced PCOS rats. A, Serum insulin concentrations. B, Insulin sensitivity. C, Fasting blood glucose concentrations. D, OGTT curve. Values are means ± standard errors ($n = 10$). * Mean value was significantly different compared with normal control ($p < 0.05$). # Mean value was significantly different compared with the PCOS group ($p < 0.05$).

90 minutes after oral glucose load (2 g/kg) was administered to the PCOS and *N. sativa* oil (5 ml/kg/rat/day)-treated groups (Fig. 4D). *Nigella sativa oil* (10 ml/kg/rat/day) enhanced glucose tolerance by lowering the blood glucose concentration at all points along the OGTT curve without a significant difference when compared to controls. *Nigella sativa* oil (5 ml/kg/rat/day) lowered the blood glucose concentration significantly ($p < 0.05$) only at the 90 minutes point of the OGTT curve (Fig. 4D).

Nigella sativa oil improved ovarian lipid profile in letrozole-induced PCOS rats

The PCOS ovarian tissue lipid profile showed a significant ($p < 0.05$) increase in TC, TG, LDL-C, and decrease HDL-C when compared with control (Table 2). *Nigella sativa* oil (5 ml/kg/rat/day) reduced TG and increased HDL-C significantly ($p < 0.05$) when compared with the PCOS group. The high dose of *N. sativa* oil (10 ml/kg/rat/day) significantly ($p < 0.05$) reduced ovarian TC, TG, LDL-C, and increased HDL-C when compared with the PCOS group. Notably, *N. sativa* oil (10 ml/kg) further reduced LDL-C more significantly ($p < 0.05$) when compared with control (Table 2).

Nigella sativa oil enhanced ovarian antioxidant status in letrozole-induced PCOS rats

PCOS group exhibited significant ($p < 0.05$) decrease in ovarian CAT, SOD, and GPX activities and an increase in LP (MDA) when compared to the control group (Fig. 5). Treatment with *N. sativa* oil significantly ($p < 0.05$) increased the ovarian SOD and GPX activities in a dose-dependent manner but CAT activities remain comparable with the PCOS group. Lipid peroxidation as assessed by the MDA equivalent was significantly ($p < 0.05$) reduced by the high dose of *N. sativa* oil (10 ml/kg/rat/day) only when compared to the PCOS group (Fig. 5).

Discussion

Nutritional and pharmacological benefits of *N. sativa* oil are commonly reported in conditions relating to pre-diabetes, IR, and T2D [14,17]. These conditions along with chronic systemic inflammation, disturbed redox status, and dyslipidemia are common co-morbidities of PCOS [6,27]. Therefore, we hypothesized that metabolic disorders relating to lipid profile and redox status that was previously shown to be modulated by *N. sativa* oil in T2D could also be ameliorated in PCOS. Till date, the modulatory properties of *N. sativa* oil on PCOS-associated dyslipidemia and redox disturbances remain unclear. Hence, investigation on the therapeutic schedule to improve lipid profile and redox status in PCOS using *N. sativa* oil could pave way for the use of this dietary oil as a supplemental functional food against PCOS-associated disorders, especially in the overweight/obese women.

Cystic ovary, acyclicity, altered ovarian thecal interna, and granulose cell layer which constitute core features of PCOS were all observed in the letrozole-induced PCOS rats except the ovary weight which showed no significant change (Fig. 1A–F). Daily administration of *N. sativa* oil (10 ml/kg/rat/day) restored uterine cyclicity in most of the animals, reduced ovarian follicular cyst, and enhanced corpus luteum appearance in a manner comparable to the control except in few details such as theca interna which is thinner in the control group. Previous studies have variously shown that letrozole, an alpha aromatase inhibitor, induced most of the endocrine and ovarian features of PCOS. Exposure to letrozole induced PCOS with striking morphological similarities to human PCOS, including a thickened theca cell layer, anovulation, and increased ovarian weight and size along with increased body weight [17,20].

Table 2. Effect of 7 weeks administration of *N. sativa* oil on ovarian lipid profiles in letrozole (1 mg/kg)-induced PCOS rats

Groups	TC (mmol/l)	TG (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)
Control	2.56 ± 0.15	0.29 ± 0.06	0.52 ± 0.03	0.28 ± 0.04
PCOS	3.25 ± 0.20*	0.52 ± 0.12*	0.33 ± 0.02*	0.41 ± 0.08*
N. sativa oil (5 ml/kg)	3.31 ± 0.44	0.37 ± 0.03#	0.54 ± 0.03 #	0.35 ± 0.05
N. sativa oil (10 ml/kg)	2.54 ± 0.24#	0.30 ± 0.03#	0.60 ± 0.04#	0.15 ± 0.02*,#

Values are means ± standard errors ($n = 10$). HDL = high-density lipoprotein; LDL = low-density lipoprotein; TC = total cholesterol; TG triglycerides.
*Mean values were significantly different compared with control rats at $p < 0{\cdot}05$.
#Mean values were significantly different compared with PCOS rats at $p < 0{\cdot}05$.

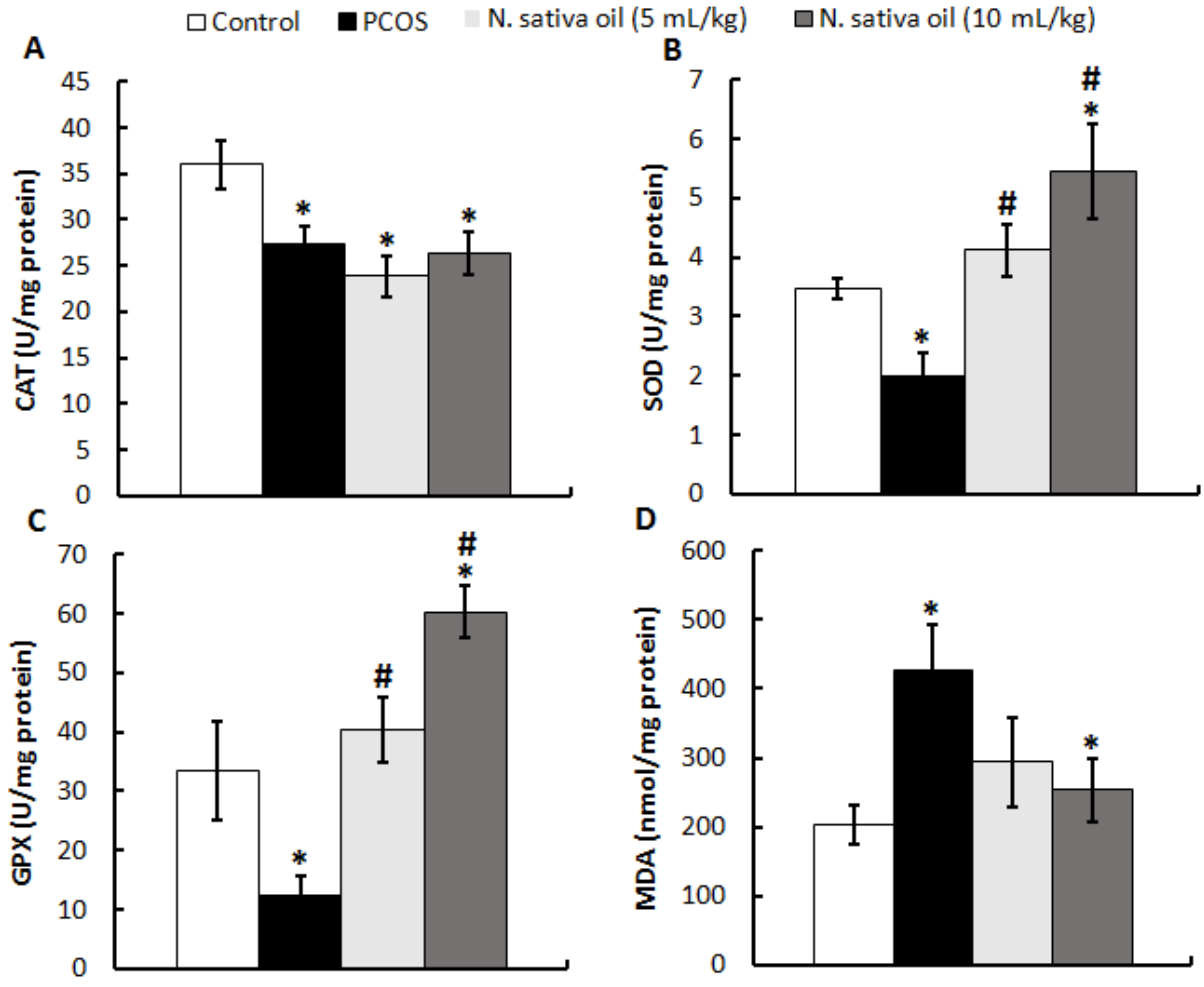

Figure 5. Ovarian antioxidant enzymes activities and LP product MDA in letrozole-induced PCOS rat treated with *N. sativa* oil. A, Ovarian tissue CAT activities. B, Ovarian tissue SOD activities. C, Ovarian tissue GPX activities. D, Ovarian tissue MDA content. Values are means ± standard errors (n = 10). * Mean value was significantly different compared with normal control ($p < 0.05$). # Mean value was significantly different compared with the PCOS group ($p < 0.05$).

Different approaches such as; prenatal and postnatal treatment with androgens, estrogen, letrozole, and antiprogestin, exposure to constant light, and genetic modification have all yielded varying characteristic features of PCOS [17,18]. However, the postnatal letrozole model, used in this study, was shown to consistently produce the cardinal features of human PCOS, i.e., hyperandrogenism, acyclicity, polycystic ovary, and metabolic disorders. More importantly, the model exhibits metabolic derangement including IR, hyperglycemia, hyperlipidemia, oxidative stress, and features of metabolic syndrome [26,28]. These features develop as a pathologic manifestation of hyperandrogenemia, a key factor in the etiology of PCOS. Letrozole inhibits its non-steroidal aromatase enzymes to prevent the conversion of testosterone and androstenedione to estradiol and estrone, respectively. These alter sex steroid hormone levels and their feedback on the hypothalamic–pituitary–gonadal (HPG) axis to distort the pulsatile secretion of GnRH from the hypothalamus—a key to establishing and maintaining normal gonadal function. Consequently, letrozole causes hormonal dysregulation, circulating and intra-ovarian hyperandrogenism, the appearance of polycystic ovarian morphology, follicular atresia, and thickened theca cell layer [29]. Letrozole model is superior in the studying of ovarian features of human-like PCOS and as such selected in the present study.

Till date, there is a limited report on the beneficial effect of *N. sativa* oil in PCOS. However, Arif et al. [30] recently employed *in vitro* and *in vivo* model systems to investigate the effect of TQ on ovarian morphology and function. They found that pretreatment with TQ in a PCOS rat model [sc. inj. of

pregnant mare serum gonadotrophin (PMSG) 20 IU followed by human chorionic gonadotropin 20 IU] significantly reduced cysts formation, increased ovulation rate, and normalize ovarian levels of key factors that influence follicular maturation. TQ is the most abundant volatile phenolic compound which has been consistently implicated for pharmacological actions of *N. sativa* oil, especially the widely reported anti-inflammatory, antioxidant, and immunomodulatory actions [30]. *Nigella sativa* oil has been shown to be a reservoir for the major phenolic compounds in *N. sativa*. Using supercritical CO_2 extraction at different conditions and hydrodistillation, a total of 47 different compounds were detected in *N. sativa* oil with TQ constituting the most abundant (33.12%–38.41%) in all fractions obtained. Dithymoquinone, thymohydroquinone, and thymol were also found in an appreciable amount in *N. sativa* oil which suggests their contribution to the therapeutic and nutritional benefits of *N. sativa* oil [31].

Hormonal dysregulation, a cardinal feature of PCOS, is well preserved in the letrozole model. Serum levels of T, LH, and FSH were markedly increased while E2 decreased in the PCOS rats. Treatment with *N. sativa* oil reduced the elevated serum levels of T, LH, FSH, and increased E2 to values comparable to control. Elevated T and LH levels were the main hormonal disorders commonly associated with human PCOS. However, elevated T, LH, FSH, and reduced E2 have been reported in the letrozole-induced PCOS model which concur with the current study [28]. Few reports have observed no change in the E2 and FSH levels; however, almost all related studies have reported hyperandrogenemia (elevated level of T) in the letrozole model of PCOS. The small variation in the previous and present study could be linked with the type of animal used, dose, duration, nature and form of the drug (letrozole) used for the PCOS induction [26,32]. Caldwell et al. [32], used two implantable pelletised continuous release letrozole (for PCOS induction in 21 days old female mice) which release as low as ~40 μg letrozole daily over a prolonged period of 90 days and they found no significant change in the serum levels of LH, FSH, and E2 contrary to our reports in this study. In a similar and earlier study, serum E2 was not altered in the PCOS rats when 21-days-old female rats were implanted subcutaneous with 90-days continuous-release pellets that release as low as ~400 μg letrozole daily over a prolonged period of 90 days [26]. While all human disease models cannot be said to be an exact replica of the human condition, the two letrozole-induced PCOS models (high dose but shorter duration using powdered letrozole and low dose but prolonged continuous release using pelletized letrozole) are consistent within their limitation.

Furthermore, our result showed that letrozole-induced PCOS exhibits IR as reflected by the elevated fasting blood glucose (FBG), insulin levels, decreased insulin sensitivity (QUICKI) and glucose tolerance (OGTT) similar to human PCOS [33]. Previous studies have also reported elevated FBG and decreased insulin sensitivity in letrozole models of PCOS except for the few studies that reported an insignificant change in the FBG and insulin sensitivity [29,34,35]. Those studies used either low dose of letrozole (as low as 200 μg/kg/day for 30 days) or used implantable pelletised continuous release letrozole which release as low as ~40–400 μg letrozole daily over a prolonged period of 90 days [26,32]. Treatment with *N. sativa* oil markedly reduced FBG and restored insulin level, insulin sensitivity, and glucose tolerance. These beneficial effects of *N. sativa* oil are commonly reported in T2D [36,37]. Enhanced insulin sensitivity and compensatory decreased insulin level ameliorate disorders relating to glucose metabolism and insulin-mediated testosterone actions. These may not be unconnected with *N. sativa* oil-induced activation of insulin receptors to improve tissue insulin sensitivity and increase peripheral utilization of glucose [38,39]. Though, further studies may be required to confirm the insulin-sensitizing actions of *N. sativa* oil in PCOS.

It has been shown that the letrozole model of PCOS develops dyslipidemia and disrupted redox balance similar to human PCOS [35,40,41]. Dyslipidemia associated with letrozole-induced PCOS manifests with elevated TC, TG, LDL-C, and reduced HDL-C or at least one of them in other studies. Studies that have reported no change in lipid profile after letrozole administration used either low dose or continuous-release models that have been previously explained [26,32]. Treatment with *N. sativa* oil improved the lipid profile by reducing the serum levels of TC, TG, LDL-C, and increasing HDL-C in PCOS rats. Hypolipidemic potential of *N. sativa* oil in metabolic syndrome has been reported in different studies [9,10,12,15]. There is no report on the hypolipidemic potential of *N. sativa* oil in the PCOS condition. This effect of *N. sativa* has been linked with the TQ via mechanisms involving regulation of hydroxymethylglutaryle-coenzyme A reductase (HMG-CoA reductase), Apo-A1,

Apo-B100, and low-density lipoprotein-receptor (LDL-R) genes to control cholesterol synthesis in the liver [42,43]. Others have also linked the hypolipidemic potential of *N. sativa* to enhance lipid metabolism due to the antioxidative action of TQ [11,12] and clofibrate actions of the nigellamin content [44].

Redox balance, as evaluated by endogenous antioxidant enzyme activities and LP products (MDA), showed that letrozole-induced PCOS exhibited marked reduction of endogenous antioxidant enzyme activities with increased level of MDA. *Nigella sativa* oil treatment significantly increased the serum SOD and GPX activities and reduced LP in PCOS rat. Excessive production of oxidants which overwhelm the endogenous antioxidant defense system gives rise to oxidative stress. Oxidative stress has been implicated in the pathogenesis of many diseases linked with inflammation [45] and PCOS is an oxidative stress/chronic systemic inflammatory disorder [46]. *Nigella sativa* oil known for its radical scavenging activity and anti-inflammatory efficacy [47,48], the antioxidant activity in PCOS-associated oxidative stress is expected. It has been shown that both the oil and derived TQ inhibit eicosanoid generation in leukocytes and non-enzymatic LP *in vitro* and none of the essential oil and the bioactive compounds (TQ, carvacrol, t-anethole, or 4-terpineol) produced prooxidant activities in the site-specific assays [49]. The oil possessed a strong antioxidant effect that cannot be achieved by adding up the activities of the few bioactive components. *Nigella sativa* oil considerably attenuated oxidative stress in the rat brain by up-regulating the activities of antioxidant enzymes to prevent pentylenetetrazol-kindled seizures better than Valproate [50]. It was also shown through *in vivo* and *in vitro* cell culture that *N. sativa* oil fractions rich in TQ are most potent in terms of antioxidant capacity and prevented toluene-induced loss of glutathione, an endogenous antioxidant, in cells and hepatic tissue [51]. Recently, Sultan et al. [13,14] showed that the fixed and essential oils of *N. sativa* enhanced serum antioxidant capacity by modulating hepatic antioxidants enzymes and boosting of immunity in streptozotocin-induced diabetic rats. Therefore, the use of *N. sativa* oil could be a potential approach in ameliorating PCOS-related hyperandrogenism, dysmenorrhea, distorted folliculogenesis, and metabolic disorder as can be exacerbated by altered redox status and chronic systemic inflammation.

From the foregoing, the biological action pathway is proposed (Fig. 6) thus, *N. sativa* oil, a proven insulin-sensitizing agent [8,9,15,52,53], could have caused marked decrease intra-ovarian and serum androgen level in the letrozole-induced PCOS rats by enhancing insulin sensitivity and suppressing compensatory hyperinsulinemia (HI) via insulin-mediated inhibition of androgen production and androgen receptor signaling. These, ultimately suppress hyperandrogenism-induced polycystic ovarian morphology and restore estradiol level thereby improve estrogen-mediated negative feedback mechanism on the HPG axis. This action of *N. sativa* oil on insulin-mediated androgen-androgen receptor signaling may be more complex and cannot be the only pathway for the *N. sativa* oil induced amelioration of PCOS features in the rat. However, this is one of the biological pathways through which *N. sativa* oil might work to improve PCOS-associated metabolic derangement while improving androgen-mediated altered ovarian features in PCOS rat.

In addition, suppression of androgen-mediated changes and enhanced estrogen level, as observed in this study following treatment with *N. sativa* oil, improves estrogen receptor-mediated activation of LDL-R activity [54]. These might be responsible for the improved lipid profile in PCOS rat treated with *N. sativa* oil. Lower serum testosterone could also down-regulate scavenger receptor B1 and hepatic lipase (HL) to reduce hepatocytes' and steroidogenic cells' uptake of HDL-C and cholesterol efflux from peripheral cells [6]. Studies have also shown that the oil and TQ-rich fractions of *N. sativa* improved dyslipidemia of metabolic syndrome by modulating the activity of HMG-CoA [47].

Moreover, anti-androgenic action and enhanced insulin sensitivity by *N. sativa* oil could have inhibited activation of inflammatory cells limiting respiratory burst and excessive production of oxidants in mononuclear and leukocytic cells (LCs) specifically and other body cells in general [6]. *Nigella sativa* oil-mediated up-regulation of antioxidant enzymes activities and inhibition of LP can confer protection on inflammatory/immune cells and other body cells to prevent oxidative stress state in the PCOS rats. Hyperandrogenemia and IR were shown to independently or jointly increase the activation of inflammatory cells to enhance oxidants production [6].

Finally, by enhancing the insulin sensitivity and suppression of androgen-mediated alterations in the ovary of PCOS rats, *N. sativa* oil improved polycystic ovarian-morphology, circulating levels of gonadotropins and sex steroids, metabolic disorders relating to lipid metabolism and redox balance.

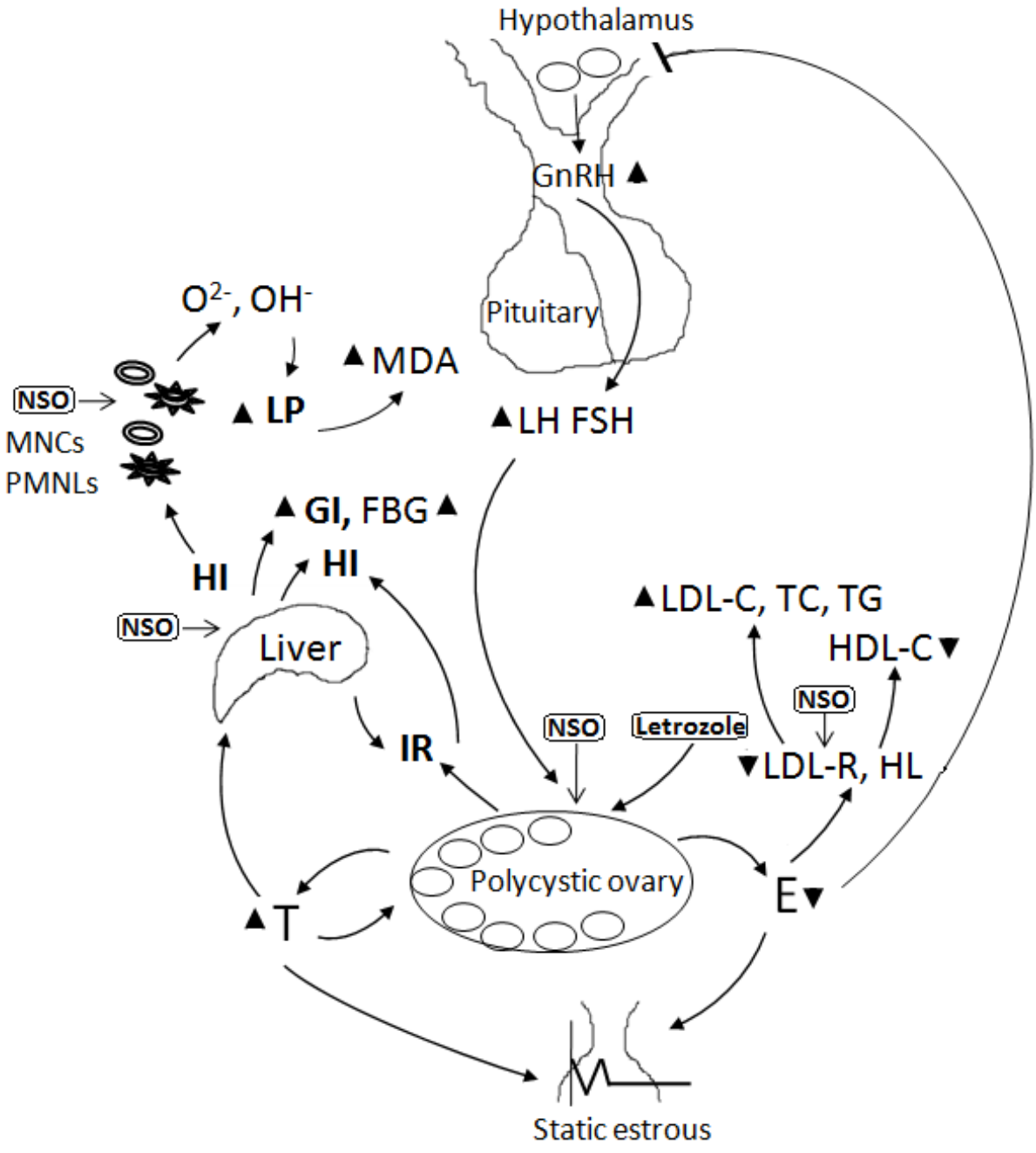

Figure 6. Proposed mechanisms of actions of *N. sativa* oil in PCOS. *Nigella sativa* oil interferes with letrozole-induced hyper-androgenemia to reduce circulating levels of testosterone (T) and increase circulating levels of estrogen (E) by alleviating letrozole-mediated inhibition of aromatase enzyme in the ovary. These modulatory properties of *N. sativa* oil restored estrous cyclicity and improved ovarian polycystic morphology. The increased level of E by *N. sativa* oil treatment restored the pulsatile release of GnRH by exerting the negative feedback effect on the hypothalamus. This improved LH and FSH hormones release to correct altered steroidogenesis in the PCOS ovary. *Nigella sativa* oil directly mediates LDL-R and HL expressions and indirectly enhances E-mediated upregulation of LDL-R and HL to ameliorate PCOS related dyslipidemia. Again, *N. sativa* oil directly and via anti-androgenic actions enhances insulin sensitivity to improve compensatory HI and correct PCOS-related altered glucose metabolism. The oil is also thought to suppress HI- and hyper-androgenemic-activations of mononuclear cells (MNCs) and polymorphonuclear LCs to control redox balance and LP via the upregulation of antioxidant enzymes' activities. All together these contribute to improve polycystic ovary, PCOS-associated dyslipidemia, and redox disturbances. ▲ = increase; ▼ = decrease; → = positive influence; —| = feedback inhibition. MDA = malondialdehyde; NSO = *N. sativa* oil; LZ = letrozole.

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Conflict of interest

None.

Author's contribution

Abdulrazaq Bidemi Nafiu conceived, designed, participated in the animal experiments and drafting of the manuscript. Suliat Alimi, Abdussalam Babalola, Ayodele Temitope Ogunlade, Fatima Dobarako Muhammad, Abdur Raheem Adesola Idowu Abioye, and Abdul musawwir Alli oluwafuyi participated in the animal experiments, data analysis, scientific advice and revised the manuscript. Lukman Aboyeji Oyewole, Olugbenga Akinola, Joseph Olajide Olayemi, Abdulbasit Amin, Wahab Imam Abdulmajeed, Ibrahim Musa carried out some biochemical assays and contributed to the writing. Mohammad Tariqur Rahman contributed with technical support, scientific advice and revised the manuscript.

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Anti-cancer and anti-oxidative activities of Nigerian traditional medicinal plants/recipes

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ABSTRACT

Background: The claim of using medicinal plants in treating cancer without scientific proof is on the rise in the north east region of Nigeria, perhaps it is due to the perceived increase in the burden of cancer in the region. Hence, the anti-cancer and anti-oxidant activities of the extracts from Nigerian anti-cancer recipes and plants were investigated.
Methods: The four (4) recipes and the sixteen (16) single plant samples were extracted by two (2) hot and two (2) cold extraction techniques using distilled water and 95% ethanol as solvents. All extracts were investigated for anti-oxidative activities and *in vitro* anti-cancer activity on DU145 prostate and MCF-7 breast cancer cell lines by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Apoptosis studies by flow cytometry using Annexin V/propidium iodide staining, and gelatin zymography for matrix metalloproteinase (MMP-2/9) activities inhibition were conducted of the most potent selected extracts initially tested for the anticancer activity.
Results: Depending on the solvent of extraction and the extraction type, extracts exhibited varying degree of antioxidant activity. Similarly, for the anti-cancer activity, the extracts from *Calotropis procera* exhibited the most potent anti-proliferative activity on both DU145 and MCF-7 cancer cell lines with IC_{50} values ranging between 4.18 ± 0.22 and 10.32 ± 1.16 µg/ml. The extracts of *C. procera*-ethanolic Soxhlet extraction with percentage apoptosis induction of 42.27% ± 2.11% and of the recipe NCR003-ethanolic cold maceration with 40.87% ± 0.94% show the highest apoptotic activities on breast cancer cell line (MCF-7) and prostate cancer cell line (DU145), respectively, whereas the highest MMP-2/9 inhibition activities were found in the ethanolic extracts of *C. procera* on both cancer cell lines.
Conclusion: This study suggested that the extracts from the plant C. *procera* to be precise and can be a promising candidate for further development as an anti-cancer drug.

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Introduction

Cancer is a global pandemic that cuts across all race, gender, tribe, and age. It is among the major cause of morbidity and mortality [1]. Predictive projections based on the GLOBOCAN 2018 indicate that the global cancer burden has risen to 18.1 million cases and 9.6 million cancer death suggesting that one in every five men and one in every six women will develop cancer over the course of their lifetime and that one in eight men and one in eleven women diagnosed with cancer will die from their disease. Asia and African account for the higher proportion of cancer death [2]. In Nigeria, research has indicated that prostate (11.3%) and breast (22.7%) cancers are the most common cancer among men and women, respectively [2, 3]. Many risk factors are attributed to the etiology of cancer which includes unhealthy lifestyle such as smoking, alcoholism, and dietary imbalances, others include hormonal imbalance, chronic infections, chronic inflammation, and most predominantly due to random mutations arising during DNA replication [4]. In addition, the generation of free radicals that

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leads to oxidative stress is equally implicated in the etiology of cancer [5].

Presently, there is limited success in clinical therapies for cancer treatment such as radiation, chemotherapy, immunomodulation, and surgery as evident by the high morbidity and mortality rates [6]. The National Cancer Institute (NCI) is at the forefront of systematic screening of extracts from plants and other organisms for their potential anticancer activity [7]. In the last few decades, human immortal cancer cell lines have aggregated an accessible, easily usable set of biological models with which to examine cancer biology and to analyze the inherent efficacy of anticancer (natural and synthetic) drugs. Medicinal plants either single or in combination as a recipe (formulate) have a long history of use in the treatment of cancers and their related illnesses by traditional medical practitioners, which are attested to by several studies conducted on herbs under a multitude of ethnobotanical grounds [8]. Antioxidants are believed to play an important role in preventing or slowing the progression of cancer development. The mechanisms of preventing tumor progression by natural phytochemicals include inhibition of genotoxic effects, increased antioxidants and anti-inflammatory activities, inhibition of proteases and cell proliferation, aiding and evading apoptosis, as well as synchronizing signal transduction pathways [9].

The use of traditional medicinal herbs for the treatment of certain ailments including cancer is well established in Nigeria. Despite formal legislation of traditional medicine practice dating back to the 1960s and several others thereafter [10,11], facts on scientific validation of certain claims of plant potency are grossly inadequate and hence the need to embark on such validations, especially on some dreaded ailment such as cancer.

In this study, the anti-proliferative activities, as well as anti-oxidant activities of some Nigerian medicinal plants and recipes, have been primarily investigated using MCF-7 (breast) and DU 145 (prostate) cancer cell lines. The most potent extracts with high anti-proliferative activity were selected to test for apoptosis induction and matrix metalloproteinase (MMP) inhibition activity in order to evaluate for their potential to be further developed as a new anticancer drug.

Materials and Methods

Plant collection

Medicinal recipes and plants were collected based on an ethnomedicinal survey conducted by administering a semi-structured questionnaire to traditional medicine practitioners (TMPs) and herbalist in some towns of Borno, Yobe, and Gombe states of North-Eastern Nigeria. The four medicinal recipes (formulae) which are composed of different combinations of 12 individual medicinal plants and 4 single medicinal plants (not part of any recipe) that had frequent mention by the respondents and TMPs, were chosen for this study (Table 1). All plants were collected at various locations in the North-Eastern part of Nigeria, between the months of November 2013 and January 2014. They were authenticated by a plant taxonomist in the biological science department of the University of Maiduguri, Nigeria, Voucher specimen was kept at the herbarium of the Department of Biochemistry, University of Maiduguri.

Sample extraction

The collected parts of the medicinal plants were washed, dried at 50ºC in a hot air oven, and milled. The recipes and single plant samples were extracted using two extraction techniques, cold and hot extractions by using distilled water and 95% ethanol as solvents at the ratio of 1:10 w/v as the menstruum. For the cold extraction, the recipes/plants were dissolved in distilled water (aqueous cold extraction) and/or 95% ethanol [ethanolic cold maceration (EtCM)] and placed on a shaker at 200 rpm for 24 hours. Whereas hot extraction was performed by boiling in distilled water for 2 hours on a hot plate (aqueous hot extraction), and/or using Soxhlet apparatus with 95% ethanol for 24 hours [ethanolic Soxhlet extraction (EtSE)]. All the extracts were first filtered with Whatman No. 1 filters paper connected with a vacuum pump and then dried by a rotary evaporator (Buchi, Switzerland). The dried extracts were stored in a brown glass bottle at −20°C until used.

Anti-oxidative activities

Free radical scavenging activity

Free radical scavenging activity of the extracts was determined by the diphenyl picryl hydrazine method as described by [12].

Inhibition of lipid peroxidation activity

The lipid peroxidation activity of the extracts was assayed by the ferric-thiocyanate method as described by [13].

Metal ion chelating activity

The metal ion chelating activity of the extracts was assayed by the modified ferrous ion chelating method as described by [13].

Anti-cancer activities

Cancer cell lines, MCF-7 [American Type Culture Collection (ATCC) HTB-22] for breast cancer, DU145 (ATCC HTB-81) for prostate cancer, were obtained from ATCC, USA. All the cells were maintained in Dulbecco's Modified Eagle Media (DMEM) (Gibco BRL, Gaithersburg, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, USA), 100 IU/ml of penicillin, and 100 mg/ml of streptomycin (Gibco BRL, Gaithersburg, USA) and maintained at 37°C under 5% CO_2 atmosphere.

Anti-proliferative activity

The anti-proliferative activity on MCF-7 and DU145 cell lines was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as modified by [14]. Briefly, an amount of 1×10^4 cells/well of the cancer cell lines was seeded into a 96-well plate adjusted to 180 µl with DMEM, and incubated at 37°C under 5% CO_2 atmosphere for 24 hours. The cells were then treated with 20 µl of the samples at 1 mg/ml for the preliminary screening. Cells were then incubated at 37°C under 5% CO_2 atmosphere for 48 hours. After incubation, the medium was removed and the cells were washed with phosphate buffer saline (PBS) three times. Then, 100 µl of 0.5 mg/ml MTT solution in PBS pH 7.6 was added into each well and further incubated for 3 hours. After that, the MTT solution was removed and 100 µl of dimethyl sulfoxide were added into each well to dissolve the blue–violet crystals. The plates were shaken manually for 5 minutes and optical density was measured at 570 nm by a microplate Reader (GloMax-Multi Detection microplate reader, Promega USA). The percentage inhibition was calculated by

$$\% \text{ inhibition} = [100 - (A_{570} \text{ of sample} \times 100) / A_{570} \text{ of control}]$$

The extract that showed 60% and above of cell growth inhibition were selected to determine the concentration providing 50% of growth inhibition (IC_{50} value) which was calculated by the extrapolated plot from the graph between % inhibition and various concentrations (mg/ml). Extracts with the IC_{50} values of < 30 µg/ml from the anti-proliferative

Table 1. Compositions and routes of administration of Nigerian traditional plants/recipes indicated for anti-cancer treatment.

Recipe codes	Plant scientific name/ compositions	Family	Voucher specimens	Part uses	Ratio of recipes	Traditional preparations	Routes of administration	Place of collection
NCR001	*Diospyros mespiliformis Hochst* *Guiera senegalensis* J.F Gmel *Cassia singueana Delile* *Dichrostachys cinerea* W.&Arn.	Ebenaceae Combretaceae Fabaceae Mimosaceae	BCH#0051 BCH#0052 BCH00#61 BCH00#55	Twig Twig Twig Twig	Equal amount	Dissolve in water add little milk	Oral/topical	Konduga Dalori Dalori Maiduguri
NCR002	*Acacia nilotica L*. *Nigella sativa L* *Cassia alata* L.	Leguminosae Ranunculaceae Caesalpiniaceae	BCH#0053 BCH#0057 BCH#0056	Fruit Seed Root	2:1:1	Boil in water	Oral	Maiduguri Maiduguri Maiduguri
NCR003	*Diospyros mespiliformis Hochst* *Calotropis procera* (Aiton) *Combretum glutinosum Perr.ex DC*.	Ebenaceae Asclepiadaceae Combretaceae	BCH#0051 BCH00#54 BCH#0060	Leaf Root Leaf	2:1:2	Dissolve in hot water or mix with cow milk fat	Oral/topical	Konduga Maiduguri Nguru
NCR004	*Ampelosus grantii* (Barker) planch *Euphorbia balsamifera* L. *Tamarinus indica* L.	Vitaceae Caesalpineaceae Fabaceae	BCH#0059 BCH#0058 BCH#0062	Root Root Fruit	3:2:1	Boil in water	Oral	Damboa Damboa Maiduguri
	Balanite egyptica (L) Delile	Balantiaceae	BCH#0063	leaf	1	Boil in water	Oral	Maiduguri
	Luffa egyptiaca Mill	Cucurbitaceae	BCH#0065	leaf	1	Boil in water	Oral	Gombe
	Boswelia dalzielli (Hutch)	Burseraceae	BCH#0064	bark	1	Dissolve in water	Oral/topical	Damboa
	Cissampelos owariensis L	Cissampelos	BCH#0066	leaf	1	Dissolve in water	Topical/ chewing	Damboa

NCR = recipe code.

activity on both cell lines were selected for further investigation via apoptosis induction and MMP inhibition studies. The standard anticancer drug doxorubicin was used as positive controls.

Apoptosis induction by flow cytometric analysis

Fluorescence isothiocyanate (FITC) conjugated Annexin V and propidium iodide (PI) double staining method was used for this assay [15]. Briefly, cells were seed at a density of 1×10^6cells/well in a 6-well plate, and incubated in the total volume of 2 ml for 24 hours at 37°C, 5% $CO_{2.}$ Thereafter, the media was aspirated to remove death and suspended cells, a fresh media 1.8 ml was added and 200 µl of the selected extracts at IC_{50} concentration previously determined from the anti-proliferative study were used to treat the cells and further incubated for 24 hours. The cells were gently harvested by scraping and analyzed by Annexin V-FITC/PI detection kit (eBioscience, San Diego, USA.) according to the manufacturer's instruction. The cell pellet was washed with PBS pH 9.0 once and with 1× binding buffer and re-suspended in 100 µl of the binding buffer, 5 µl of FITC-conjugated Annexin-V was added and the cells were incubated in the dark at room temperature for 15 minutes. Cells were washed with 1× binding buffer and re-suspended in 200 µl of the buffer, and then 5 µl of PI was added. The cells were incubated for 5 minutes at room temperature. Data were acquired on the BD Accuri C6 flow cytometer (BD Biosciences USA) using BD Accuri C6 software. Standard anticancer drug doxorubicin was used as positive controls.

Inhibition of matrix metalloproteinase MMP-2/9 activity

The selected extracts from both cell lines were assayed for the gelatinolytic activity of MMP-2/9 inhibition by the methods described by [16]. A monolayer of cells 5×10^5 cells/well was maintained in a 6-well plate in a culture media without FBS for 24 hours, and treated with the extracts at IC_{50} concentrations and further incubated for another 24 hours. The culture supernatant was collected for gelatinolytic activities of MMP-2/9 by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis zymography using gelatin as the substrate. Briefly, 20 µl of the cell culture supernatant were suspended in non-reducing loading buffer [0.125M Tris (pH 6.8), 4% SDS, and 0.04% bromophenol blue] without prior denaturation, and run on 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, gels were washed to remove SDS and incubated twice with gentle shaking for 20 minutes each in the renaturing buffer (50 mM Tris, 5 mM $CaCl_2$, 0.02% NaN_3, and 2.5% TritonX-100). The gels were then incubated for 24 hours at 37°C in the developing buffer [50 mM Tris (pH 7.5), 5 mM $CaCl_2$, 0.02% NaN_3, and 1% Triton X-100]. Gels were stained using simply blue safe stain (Invitrogen, USA) for at least 1 hour and de-stained with distilled water for clear gel background. The gels were scan at a resolution of 300 dpi in a TIFF format by Brother MCF-J415W Scanner and the gelatinolytic bands were quantified using the Image J (National Institute of Health) software [17]. The experiment was conducted in three independent separate experiments. The potency of MMP-2/9 inhibitions of the samples was compared with the standard anti-cancer drug—doxorubicin. The percentages of MMP-2/9 inhibition in comparing to the control (the untreated systems) were calculated by the following equation;

$$\text{MMP inhibition (\%)} = 100 - [(\text{MMP content of sample}/\text{MMP content of control}) \times 100]$$

Statistical analysis

The results were presented as the mean ± standard deviation ($n = 4$). Analysis of variance with the least significant difference test was used for the analysis of test results at the significance level of p-value < 0.05.

Results

Anti-oxidative activities of the Nigerian recipe/plant extracts

The anti-oxidative activities of the extracts were investigated via free radical scavenging, inhibition of lipid peroxidation, and metal ion chelating activities of the Nigerian recipe/plant (Table 2). All the extracts from *Acacia nilotica, Boswellia dalzielli*, *Combretum glutinosum*, and *Cassia singueana* showed the best free radical scavenging and the inhibition of lipid peroxidation activities which were comparable to vitamin C [with scavenging activity (SC_{50}) of 0.03 ± 0.01 mg/ml) and vitamin E (with LC_{50} of 1.44 ± 0.30 mg/ml), respectively ($p < 0.05$), while only the ethanolic extracts of *A. grantii* gave the highest metal chelating activity, which was comparable to ethylene diamine tetraacetate (EDTA) (with MC_{50} of 0.45 ± 0.02 mg/ml) ($p < 0.05$).

Table 2. Anti-oxidant activities: free radical SC_{50}, inhibition of lipid peroxidation (LC_{50}), and metal chelating activity (MC_{50}) of Nigerian traditional anti-cancer plants/recipes extracts

Recipe/Plant codes	Extraction	Anti-oxidative activities		
		SC_{50} (mg/ml)	LC_{50} (mg/ml)	MC_{50} (mg/ml)
NCR001	AQC	0.87 ± 0.04^{d}	1.86 ± 0.17^{a}	1.29 ± 0.34^{b}
	AQH	0.69 ± 0.03^{c}	1.67 ± 0.10^{a}	1.89 ± 0.76^{b}
	EtCM	0.06 ± 0.01^{a}	1.33 ± 0.17^{a}	2.23 ± 0.98^{c}
	EtSE	0.17 ± 0.03^{b}	2.64 ± 0.98^{b}	8.83 ± 1.26^{f}
NCR002	AQC	0.48 ± 0.06^{c}	1.63 ± 0.30^{a}	2.10 ± 0.33^{c}
	AQH	2.97 ± 0.32^{f}	6.22 ± 1.37^{e}	2.10 ± 1.35^{c}
	EtCM	0.08 ± 0.01^{a}	2.52 ± 0.20^{b}	13.29 ± 0.05^{h}
	EtSE	0.16 ± 0.02^{b}	3.87 ± 0.36^{c}	24.61 ± 1.56^{k}
NCR003	AQC	0.50 ± 0.02^{c}	2.15 ± 0.13^{b}	2.03 ± 0.71^{c}
	AQH	0.52 ± 0.04^{c}	2.54 ± 0.02^{b}	2.26 ± 0.62^{c}
	EtCM	0.27 ± 0.01^{b}	2.00 ± 0.68^{a}	2.34 ± 0.56^{c}
	EtSE	0.26 ± 0.01^{b}	3.67 ± 2.24^{c}	1.79 ± 0.26^{b}
NCR004	AQC	8.62 ± 0.40^{h}	3.79 ± 0.85^{c}	2.88 ± 0.23^{c}
	AQH	6.47 ± 0.39^{g}	4.31 ± 0.49^{d}	8.96 ± 2.84^{f}
	EtCM	0.29 ± 0.01^{b}	2.90 ± 0.24^{b}	3.41 ± 0.85^{d}
	EtSE	0.35 ± 0.01^{c}	6.70 ± 2.91^{e}	2.66 ± 0.11^{c}
Diospyros mesipiliformis	AQC	1.02 ± 0.19^{d}	2.35 ± 0.21^{b}	2.19 ± 0.09^{c}
	AQH	1.10 ± 0.15^{e}	2.30 ± 0.13^{b}	2.17 ± 0.40^{c}
	EtCM	0.26 ± 0.01^{b}	1.59 ± 0.34^{a}	12.83 ± 2.12^{h}
	EtSE	0.26 ± 0.01^{b}	2.14 ± 0.42^{b}	7.52 ± 0.96^{e}
Cassia singueana	AQC	0.06 ± 0.01^{a}	1.69 ± 0.08^{a}	1.97 ± 0.42^{b}
	AQH	0.06 ± 0.00^{a}	1.80 ± 0.06^{a}	2.47 ± 0.96^{c}
	EtCM	0.03 ± 0.01^{a}	2.23 ± 0.66^{b}	2.35 ± 1.76^{c}
	EtSE	0.04 ± 0.00^{a}	1.48 ± 0.34^{a}	4.63 ± 0.87^{d}
Acacia nilotica	AQC	0.04 ± 0.03^{a}	1.35 ± 0.05^{a}	6.07 ± 0.16^{e}
	AQH	0.04 ± 0.00^{a}	1.33 ± 0.06^{a}	4.20 ± 0.55^{d}
	EtCM	0.02 ± 0.00^{a}	2.06 ± 0.02^{a}	NA
	EtSE	0.02 ± 0.01^{a}	2.01 ± 0.15^{a}	NA
Calotropis procera	AQC	2.35 ± 0.31^{f}	6.70 ± 1.29^{e}	2.25 ± 0.85^{c}
	AQH	1.20 ± 0.21^{e}	6.35 ± 0.62^{e}	3.50 ± 1.29^{d}
	EtCM	1.80 ± 0.29^{e}	3.75 ± 0.78^{c}	10.05 ± 0.57^{g}
	EtSE	0.54 ± 0.03^{c}	1.76 ± 0.32^{a}	2.30 ± 0.11^{c}
Combretum glutinosum	AQC	0.07 ± 0.01^{a}	2.58 ± 0.11^{b}	NA
	AQH	0.07 ± 0.02^{a}	1.98 ± 0.07^{a}	16.40 ± 1.48^{i}
	EtCM	0.05 ± 0.01^{a}	2.25 ± 0.84^{b}	30.23 ± 1.63^{l}
	EtSE	0.09 ± 0.01^{a}	2.30 ± 0.65^{b}	15.49 ± 1.29^{i}
Ampelosus grantii	AQC	1.25 ± 0.16^{e}	5.63 ± 0.55^{e}	4.00 ± 1.17^{d}
	AQH	2.18 ± 0.33^{f}	10.51 ± 0.04^{g}	4.58 ± 0.59^{d}
	EtCM	0.05 ± 0.01^{a}	3.22 ± 0.52^{c}	0.44 ± 0.29^{a}
	EtSE	0.03 ± 0.01^{a}	2.09 ± 0.17^{b}	0.57 ± 0.13^{a}
Boswelia dalzielli	AQC	0.08 ± 0.00^{a}	1.88 ± 0.02^{a}	7.34 ± 0.27^{e}
	AQH	0.09 ± 0.01^{a}	1.82 ± 0.06^{a}	6.05 ± 0.47^{e}
	EtCM	0.03 ± 0.00^{a}	1.86 ± 0.28^{a}	17.14 ± 6.25^{j}
	EtSE	0.03 ± 0.01^{a}	1.93 ± 0.20^{a}	15.48 ± 1.04^{i}
Luffa egyptica	AQC	1.05 ± 0.08^{d}	1.01 ± 0.80^{a}	2.58 ± 0.18^{c}
	AQH	0.61 ± 0.04^{c}	1.00 ± 0.54^{a}	3.04 ± 0.60^{d}
	EtCM	0.27 ± 0.04^{b}	2.78 ± 0.52^{b}	3.78 ± 1.57^{d}
	EtSE	0.57 ± 0.02^{c}	4.75 ± 0.84^{d}	2.18 ± 0.48^{c}
Vitamin C		0.03 ± 0.01^{a}	NT	NT
Vitamin E		NT	1.44 ± 0.30^{a}	NT
EDTA		NT	NT	0.45 ± 0.02^{a}

Note: Values are means ± standard deviations (n = 4). The subscripts $^{a\text{-}l}$ is significantly different in the same column at $p < 0.05$. AQC = aqueous cold extraction, AQH = aqueous hot extraction, EtCM = ethanolic cold maceration, EtSE = ethanolic soxhlet extraction, NA = no activity detected, NCR = recipe code, NT = not tested.

Anti-proliferative activities of the Nigerian recipe/ plant extracts

In this study, the total of 80 extracts from 4 Nigerian recipes and 16 single medicinal plants (12 as part of the recipes) were screened at first, at the concentration of 1 mg/ml, for the anti-proliferative activity on two cancer cell lines, DU145 prostate, and MCF-7 breast cancer cell lines using MTT assay. The extracts that had 60% inhibition and above (result not presented) on DU145 and MCF-7 cancer cell lines, were selected for further studies.

Table 3 showed the IC_{50} value of the previously screened extracts (extract with above 60% inhibition at the screening stage). This result revealed that the anti-proliferative activity of 8 extracts for the DU-145 prostate cancer cell line, and 4 extracts for MCF-7 cell lines gave the IC_{50} of < 30 µg/ml which are considered as 'very active' and could be considered as potential source for cancer drug according to the NCI [18, 19]. Worthy to note is that all the extracts from *Calotropis procera* showed anti-proliferative activity on both cell lines that were classified as very active compounds (IC_{50} of < 30 µg/ml), but lower than that of doxorubicin ($p < 0.05$).

Apoptosis induction of the Nigerian recipe/plant extracts

Extracts that gave the IC_{50} of < 30 µg/ml for anti-proliferative activity on DU-145 prostate and MCF-7 breast cancer cell lines were tested for apoptosis induction activity by flow cytometer using Annexin V/PI double staining method. Table 4 demonstrates

Table 3. Anti-proliferative activity (IC_{50} values in µg/ml) of the selected Nigerian traditional anti-cancer plants/ recipes extracts on the prostate (DU-145) and breast (MCF-7) cancer cell lines by MTT assay

Plants/ Recipe codes	Extraction	IC_{50} values of the extracts on cancer cell lines (µg/ml)	
		DU-145	MCF-7
NCR001	AQC	>200	>200
	AQH	>200	>200
	EtCM	>200	>200
	EtSE	>200	>200
NCR002	AQC	>200	>200
	EtCM	>200	>200
	EtSE	>200	>200
NCR003	AQC	58.66 ± 1.15^{f}	58.49 ± 1.71^{e}
	AQH	7.65 ± 0.18^{c}	62.57 ± 7.50^{e}
	EtCM	7.25 ± 0.36^{c}	42.76 ± 12.57^{d}
	EtSE	5.28 ± 0.29^{b}	56.77 ± 6.51^{e}
Diospyrosmesipiliformis	AQC	>200	NT
	AQH	>200	>200
	EtCM	>200	NT
	EtSE	NT	>200
Guierasenegalensis	AQC	>200	>200
	AQH	>200	>200
	EtCM	140.86 ± 16.00^{g}	70.71 ± 14.68^{f}
	EtSE	142.63 ± 2.14^{g}	81.37 ± 9.55^{f}
Cassia singueana	AQC	>200	>200
	AQH	>200	>200.
	EtCM	>200	>200
	EtSE	>200	>200
Dichrostachyscinerea	AQH	>200	>200
	EtCM	69.98 ± 2.98^{f}	94.09 ± 15.78^{g}
	EtSE	>200	>200
Acacia nilotica	AQC	NT	>200
	AQH	143.63 ± 5.41^{g}	>200
	EtCM	>200	>200
	EtSE	>200	54.71 ± 10.07^{e}
Cassia alata	AQC	>200	>200
	EtCM	61.37 ± 1.58^{f}	>200
	EtSE	67.83 ± 8.84^{f}	>200

(Continued)

Table 3. (Continued)

Plants/ Recipe codes	Extraction	IC_{50} values of the extracts on cancer cell lines (µg/ml)	
		DU-145	MCF-7
Calotropisprocera	AQC	5.18 ± 0.08[b]	9.27 ± 0.33[c]
	AQH	4.94 ± 0.42[b]	10.32 ± 1.16[c]
	EtCM	4.18 ± 0.22[b]	5.21 ± 0.41[b]
	EtSE	5.61 ± 0.36[b]	5.75 ± 0.49[b]
Combretumglutinosum	AQC	NT	>200
	AQH	>200	>200
Ampelosusgrantii	EtSE	>200	>200
Euphorbia balsamifera	EtCM	>200	NT
	EtSE	>200	NT
Bosweliadalzielli	AQC	>200	>200
	AQH	>200	>200
	EtCM	NT	>200
	EtSE	>200	>200
Cissampelosowariensis	AQH	>200	>200
	EtCM	NT	>200
Luffaegyptica	EtCM	10.13 ± 1.50[d]	91.24 ± 14.96[g]
	EtSE	68.56 ± 15.84[f]	72.16 ± 10.12[f]
Doxorubucin		22.74 ± 0.51[e]	10.74 ± 2.55[c]

Note: The anti-proliferative activity of the extracts that showed 60% and above of cell growth inhibition are selected to determine the concentration providing 50% of growth inhibition (IC_{50} value). AQC = aqueous cold extraction, AQH = aqueous hot extraction, EtCM = ethanolic cold maceration, EtSE = ethanolic soxhlet extraction, NCR = recipe code, NT = not tested. Values are means ± standard error of mean (n = 4). The subscripts [a-l] are significantly different in the same column at $p < 0.05$.

that all selected extracts appeared to induce apoptosis in both cancer cell lines.

The extract from NCR003-EtCM gave the highest apoptosis induction properties considering in the early apoptosis (Q1-LR), (40.87% ± 0.94%) on DU 145 cell line, whereas *C. procera*-EtCM and *C. procera*-EtSE showed 41.20% ± 0.44% and 42.27% ± 2.11%, respectively, on MCF-7 cell line ($p < 0.05$), as shown in Figure 1.

Figure 1 exhibits the density plots of DU 145 and MCF-7 cells after treatment with the NCR003-EtCM and *C. procera*-EtCM extracts at the concentration of their IC_{50} values. These two extracts not only showed the highest apoptosis induction but also superior than doxorubicin ($p < 0.05$).

MMP-2/9 inhibition activity of the Nigerian recipe/ plant extracts

Same extracts selected for apoptosis study were also used at their "active" determined IC_{50} values for the MMP studies. All the selected extracts at their concentration of IC_{50} values demonstrated the MMP-2 and MMP-9 inhibition activities on DU-145 and MCF-7 cell lines, (Fig. 2). The extracts from the plant *C. procera* appeared to have the highest MMP-2/9 inhibition on both cancer cell lines among the selected extracts. The highest MMP-9 inhibition activity on the DU-145 cell line of *C. procera*-EtSE extract was superior to doxorubicin, whereas the *C. procera*-EtCM extract was comparable to doxorubicin on the MMP-2 and MMP-9 inhibition activity on MCF-7 cell line ($p < 0.05$).

Discussion

The use of traditional medicine and by extension herbal medicine has been in practice since antiquities and different crude methods are employed in its preparation [20]. Some of these crude practices is often in tandem with different folklore wisdoms, cultures, and religions of these practitioners [21]. Mimicking these practice in this study has really affected the efficacy of these plants/recipes as the same plant or recipe sample showed a varied activity due to different extraction methods or different solvent used, this critically revealed the choice of solvent and method of preparation as key in assessing therapeutic efficacy of herbal medicine scientifically and even in traditional practice.

Free radicals have been implicated in causing ailments such as cancer [5]. Membrane integrity is paramount for normal metabolic activity and survival of both cell and subcellular organelles, and the presence of polyunsaturated fatty acid in the membrane bi-layer confers a unique characteristic

Table 4. Apoptosis induction of the selected Nigerian traditional anti-cancer plants/recipes extracts on the prostate (DU-145) and breast (MCF-7) cancer cell lines at IC_{50} concentration (µg/ml) detected by flow cytometer with Annexin V/ PI double staining

Extract	Living		Early apoptosis		Late apoptosis		Necrosis	
	DU-145	MCF-7	DU-145	MCF-7	DU-145	MCF-7	DU-145	MCF-7
Control	86.87 ± 1.37^{d}	84.40 ± 0.80^{d}	7.90 ± 0.40^{a}	7.00 ± 0.10^{a}	3.20 ± 1.30^{a}	1.43 ± 0.27^{a}	2.00 ± 0.30^{c}	3.13 ± 0.46^{c}
Doxorubucin	54.97 ± 0.61^{a}	58.13 ± 9.15^{b}	37.23 ± 1.92^{e}	36.47 ± 6.47^{c}	7.10 ± 2.22^{b}	4.70 ± 2.30^{b}	0.77 ± 0.17^{a}	0.70 ± 0.40^{a}
NCR003-AQH	71.73 ± 9.80^{c}	71.53 ± 1.16^{c}	14.93 ± 6.93^{b}	23.23 ± 0.43^{b}	6.07 ± 3.21^{b}	1.57 ± 0.77^{a}	7.23 ± 0.15^{d}	3.67 ± 0.17^{c}
NCR003-EtCM	61.10 ± 5.71^{b}	NT	29.50 ± 2.93^{d}	NT	7.80 ± 4.30^{b}	NT	1.57 ± 0.12^{b}	NT
NCR003-EtSE	44.73 ± 5.92^{a}	NT	40.87 ± 0.94^{e}	NT	11.90 ± 5.90^{d}	NT	2.50 ± 0.95^{c}	NT
C. procera-AQC	59.30 ± 4.82^{b}	NT	31.77 ± 4.86^{d}	NT	7.43 ± 2.31^{b}	NT	1.53 ± 0.03^{b}	NT
C. procera-AQH	60.73 ± 3.35^{b}	73.90 ± 3.91^{c}	31.33 ± 2.41^{d}	20.70 ± 1.42^{b}	6.86 ± 2.14^{b}	3.97 ± 1.56^{b}	1.06 ± 0.06^{b}	1.43 ± 0.94^{b}
C. procera-EtCM	67.97 ± 2.72^{c}	55.56 ± 4.20^{b}	26.57 ± 2.53^{d}	34.70 ± 5.05^{c}	4.17 ± 0.21^{a}	7.77 ± 1.44^{c}	1.30 ± 0.10^{b}	1.93 ± 1.03^{b}
C. procera-EtSE	72.70 ± 1.04^{c}	49.63 ± 1.35^{a}	22.8 ± 1.25^{c}	41.20 ± 0.44^{d}	3.73 ± 0.18^{a}	7.63 ± 1.47^{c}	0.80 ± 0.10^{a}	1.50 ± 0.20^{b}
L. egyptiaca-EtCM	60.00 ± 2.81^{b}	48.07 ± 1.12^{a}	29.37 ± 2.18^{d}	42.27 ± 2.11^{d}	9.07 ± 4.07^{c}	8.77 ± 2.32^{d}	1.53 ± 0.93^{b}	0.87 ± 0.33^{a}

The apoptosis induction of the extracts that showed IC_{50} values < 30 µg/ml from anti-proliferative activity by MTT assay is selected. AQC = aqueous cold extraction, AQH = aqueous hot extraction, EtCM = ethanolic cold maceration, EtSE = ethanolic Soxhlet extraction, NCR = recipe code, NT = not tested. Values are means ± standard deviations (n = 4). The subscripts $^{a-l}$ are significantly different in the same column at $p < 0.05$.

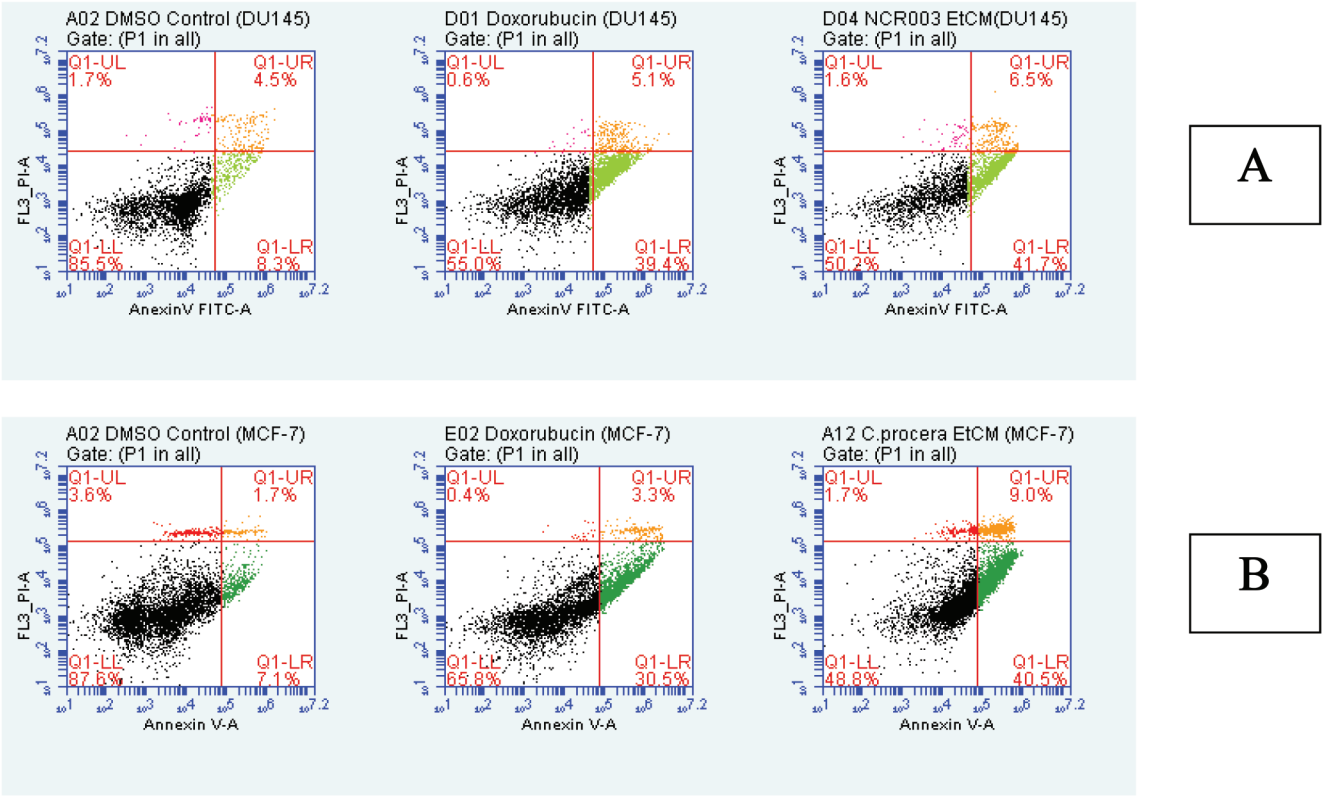

Figure 1. Apoptosis induction activity by flow cytometer using Annexin V/PI staining of the most potent extracts at IC_{50} concentration after 24 hours on DU-145 (A) and MCF-7 cell lines (B). Early apoptosis (Annexin V positive and PI negative) is Q1-LR, Late apoptosis (Annexin positive and PI positive) is Q1-UR, and Necrosis (Annexin V negative and PI positive) is Q1-UL, and living cells (Annexin V negative and PI negative) is Q1-LL.

of membrane fluidity required for normal functions [22]. Overproduction of free radicals can lead to oxidative damage [5, 23]. Most of the extracts from the Nigerian recipes/plants in this study demonstrated effective anti-oxidative activity. All extracts from *A. nilotica, B. dalzielli, C. glutinosum*, and *C.*

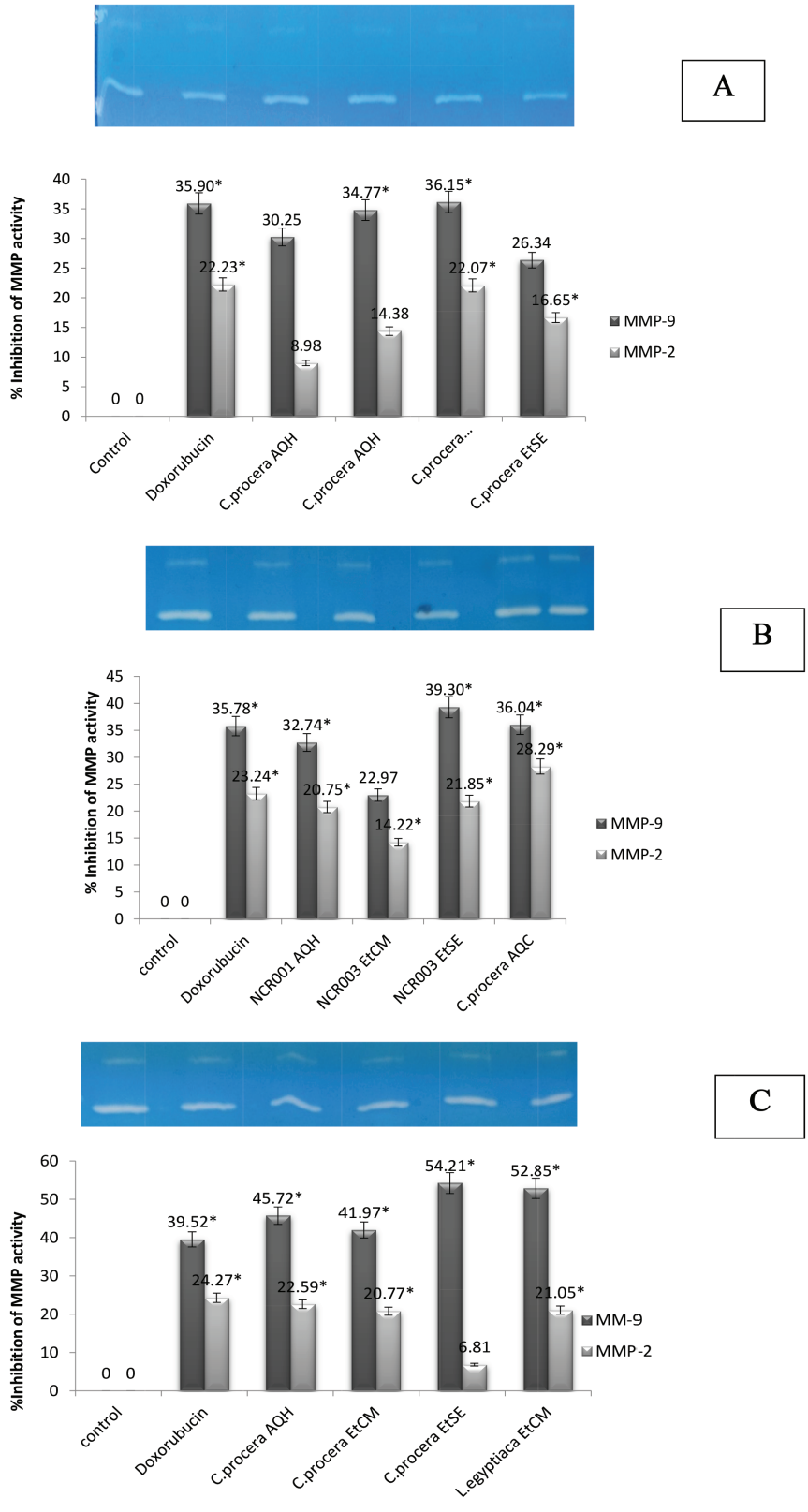

Figure 2. Gelatinolytic zymography of MMP-2/9 inhibition activity of the extracts at IC_{50} concentration on DU-145 cell line (A & B) and MCF-7 cell line (C). Values are Mean ± SE. Asterisk (*) indicate significance difference ($p < 0.05$) from negative control.

singueana plants showed the best free radical scavenging and inhibition of lipid peroxidation activities which were comparable to standards as shown in Table 2. While only the ethanolic extracts of *Ampelosus grantii* gave the highest metal chelating activity, which were comparable to EDTA. Several

research studies are increasingly showing that anti-oxidants rich in fruits, vegetables, and medicinal plants are beneficial and can slow the progression of diseases [24], synonymous to which some of these plants have exhibited, of great interest are those plants that form part of recipes.

The anti-proliferative activity of eight extracts for the DU 145 prostate cancer cell line and four extracts for MCF-7 cell lines gave the IC_{50} values of < 30 µg/ml which are considered as "very active" and could be considered as a potential source for cancer drug according to the NCI [18, 19]. Extracts from *C*. *procera* and the recipes that contained it showed remarkable anti-proliferative activity on both cell lines and hence are considered or classified as very active compounds (IC_{50} of < 30 µg/ml) despite showing activity lower than that of doxorubicin ($p < 0.05$). This outstanding anti-proliferative activity of *C*. *procera* on cancer cell lines has been reported by several researchers. For example, Marthur and co-researchers [18] found that methanolic extract from the root of *C*. *procera* inhibits the proliferation of Hep2 cancer cell line and induces apoptosis, an anti-proliferative activity that could possibly be attributed to certain compounds found in the root of *C*. *procera* such as cardenolide [25] and certain secondary metabolite such as alkaloids that have the ability to induce apoptosis and subsequent cell death [26]. The medicinal benefit of *C*. *procera* plant is also well documented in Ayurveda medicine [27]. For the group of recipes considered in this study, the anti-cancer recipe NCR003 which is composed of the medicinal plants *C*. *procera, C*. *glutinosum*, and *D*. *mesipiliformis* exhibited a "very active" activity on DU 145 cell line. It seems that the anti-proliferative activity of the NCR003 extract may be attributed to the presence of *C*. *procera* in the combination of the recipe, having shown more activity than the other plants when considering them on individual basis. Though the rationale behind the wisdom of formulating these medicinal recipes is not clear; we believe therapeutic efficacy is not just about anti-proliferation activity alone in treating diseases such as cancer but also in improving such efficacies and preventing unwanted side effect; Both *C*. *glutinosum* and *D*. *mesipiliformis* as members of NCR003 recipe exhibited a very good antioxidant activity which could perhaps be an adjuvant toward improving the therapeutic value of the anticancer active component of *C*. *procera*. The ethanolic extract of *Luffa egyptiaca M*., by cold (EtCM) extraction also showed interestingly good potentials on the DU145 human prostate cancer cell line. This plant and its derived components like the cardenolide have been reported for their anti-tumor activity [28].

All selected extracts tested at their concentration of their IC_{50} values appeared to induce apoptosis in both cancer cell lines. The extract from NCR003-EtCM gave the highest apoptosis induction properties on the DU-145 cell line, whereas *C*. *procera*-EtCM and *C*. *procera*-EtSE exhibited the same on MCF-7 cell line ($p < 0.05$). These two extracts not only showed the highest apoptosis induction but also superior than doxorubicin ($p < 0.05$). The extracts could have induced apoptosis by probably causing nuclear fragmentation, decrease of mitochondrial membrane potential, and inducing the asymmetrical distribution of phosphatidylserine residues [29]. For example, Joshi and his colleagues [30] have reported that the methanolic extract of *C*. *procera* can induce early apoptosis on human skin melanoma (SK-MEL-2) cell lines at 19.6%, whereas the standard drug paclitaxel showed 15.85% on the same cell line. This clearly underscored the importance of *C*. *procera* as a potential candidate for anticancer drug development.

Matrix metalloproteases are a family of Zn-dependent proteases that are incriminated in tumor inversion and angiogenesis, especially MMP-2 and MMP-9 [31]. The extracts from the plant *C*. *procera* appeared to be the highest MMP-2/9 inhibitor on both cancer cell lines among the selected extracts. The highest MMP-9 inhibition activity on the DU-145 cell line of *C*. *procera*-EtSE extract was superior to doxorubicin, whereas the *C*. *procera*-EtCM extract was comparable to doxorubicin on the MMP-2 and MMP-9 inhibition activity on MCF-7 cell line ($p < 0.05$).

Several studies have shown that the synthesis of MMPs can be blocked by many active compounds through interacting with molecules that direct their activities to the cell surface or inhibit their enzymatic activity [32]. Some of these compounds are derived from natural plant source such as halofuginone (an alkaloid from the medicinal plant *Dichroa febrifuga* Lour.) interferes with the TGF-β signaling pathway and inhibits bladder carcinoma metastasis by blocking MMP-2 expression[33]. Lambert and his co-researchers [34] revealed that isolated natural compound epigallocatechin gallate and other polyphenols and flavonoids from plants, herbs, and fruit sources such as long-chain fatty acids exhibited MMP inhibition activities but were far less potent than tissue inhibitors of MMPs. This shows that the MMP inhibition activity of the selected extracts may

be due to these types of aforementioned mechanisms by the secondary metabolites or phytochemicals such as alkaloids, flavonoids, and tannins, and might not necessarily be due to toxicity and subsequent cell death.

Conclusion

In this study, 80 aqueous and ethanolic extracts from ethnomedicinal surveyed recipes and plants that are used for anti-cancer treatment from North-Eastern Nigeria were investigated for their anti-proliferative and anti-oxidative activities. Among those recipe/plant extracts, the extract from *Calotropis procera* roots seemed to have the most potent anti-cancer activity including anti-proliferative activity, apoptosis induction, and MMP-2/9 inhibition on the DU 145 and MCF-7 prostate and breast cancer cell lines respectively, that can be significantly compared to the standard drug, doxorubicin. The higher anti-cancer activities of the NCR003 extracts than the other recipe extracts may be due to the presence of *C*. *procera* plant forming part of this recipe. Moreover, many of the extracts also exhibited varying degrees of anti-oxidative activities including free radical scavenging activity, inhibition of lipid peroxidation, and metal chelating, which may help to prevent many degenerative diseases such as cancer, Alzheimer's disease, Parkinson, and diabetes mellitus. This study suggested that the extracts from the plant *C*. *procera* or in combination as a recipe can be a promising candidate for further development as an anti-cancer drug.

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Conflict of interest

All the authors have read and agreed to the content of the manuscript and have declared no conflict of interest.

Authors' contributions

Abubakar Gidado and Korawinwich Boonpisuttinant were involved in the general supervision of the work, Aliyu Daja conducted the survey, Aliyu Daja and Suthamas Kanjanawongwanich did the bench-work to generate the data used in this study. Aliyu Daja developed the manuscript and again Abubakar Gidado and Korawinwich Boonpisuttinant proof-read and edit the manuscript.

List of Abbreviations

AQC = aqueous cold extraction
AQH = aqueous hot extraction
ATCC = American Type Culture Collection
DMEM = Dubecco's Modified Eagle Media
EDTA = ethylene diamine tetra acetate
EtCM = ethanolic cold maceration
EtSE = ethanolic Soxhlet extraction
FBS = fetal bovine serum
FITC = fluorescence isothiocyanate
MMP = matrix metalloproteases
MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI = National Cancer Institute
PI = propidium iodine
SDS = Sodium dodecyl sulfate
IC_{50} = Half maximal inhibitory concentration
SK-MEL-2 = Melanoma cell line
NCR = Nigerian Cancer Recipe

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REVIEW ARTICLE

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A review of the effects of medicinal plants on exercise and physical health factors in athletes

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ABSTRACT

Background: Medicinal plants have long been considered and have a long history, especially in eastern countries. Today, some of these herbs are used as sports supplements and studies have been carried out on the effects of some of these herbs on the general health and some of the functional factors of athletes and non-athletes. Some studies have confirmed the antioxidant and anti-inflammatory and regulatory effects the herbs have on body metabolism; due to the intentional and scientific use of these herbs, as well as the greater partial efficacy of some of these herbs along with exercise.

Methods: In this review paper, the effects of some of these supplements and herbal remedies (*Curcuma longa*, *Cinnamomum verum*, *Camellia sinensis*, *Zingiber officinale*, *Crocus sativus*, and *Allium sativum*) on the general health and fitness of athletes and non-athletes have been studied by using dozens of papers as well as credible sources.

Results: After searching, screening, and qualitative evaluation of the studies, at the end of the final synthesis, 88 articles were performed. Most of these studies have confirmed the antioxidant and anti-inflammatory effects of these herbs, and have also emphasized that paired with exercise these herbs have a greater efficacy.

Conclusion: Different studies have been done on the effects of herbal remedies with exercise. However, in most of these studies the protocols of exercise and physical activity were not based on specific principles and, more importantly, the dosage of these herbal remedies in human samples was the same for different individuals and not based on body weight or time and type of exercise.

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Introduction

Human use of herbal remedies goes back as far as to 60,000 years ago. Early humans knew that they were dependent on nature for both health and disease. Treatments were based on the instinct and experience of using plants and parts of the body of animals and minerals that were not part of the diet. The history of medicine in Iran dates back to the Aryan period, about 7000 BC. The first Aryan physician and surgeon, Trita, was very familiar with plants and their properties. He used plant extracts to cure diseases. There are about 8,000 species of herbs in Iran, 2,300 species of which are aromatic and medicinal, 450 species of them are sold in Iran's Medicinal herbs shops [1,2].

As well as Iran, China, and India have also been pioneers of traditional medicine and herbal medicine. The earliest discoveries related to medicinal herbs made in China include an inscription called Shinon, about 2800 BC, which describes about 1,000 medicinal species [1].

The extent and variety of climates in Iran, China, and India have made these regions of special importance in terms of the number and variety of valuable

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medicinal species in the world, so that there are hundreds of species of medicinal herbs with very effective properties in afore mentioned countries. For instance, herbs and spices, such as black pepper, herring, saffron, corcumin, licorice, poppy, nutmeg, castor, sesame, aloe Vera, ginseng, tea and many more which are introduced today [1,2].

Individuals and consumers are attracted to medicinal plants because of they have fewer side effects compared to biochemical drugs and supplements. Numerous studies have been done on medicinal herbs to produce various drugs and dietary supplements. Most of these studies have focused on the antioxidant and anti-inflammatory elements of these plants, some of the studies have shown a light on the oxidative, blood sugar, and lipid lowering properties of said plants. For example, a study by Sanktia et al. [3–5] showed that the use of garlic extract, due to its antioxidant effects, can reduce the oxidative stress caused by some diseases. In addition, other studies found that green tea extract (GTE) increased sympathetic system activity, activated catecholamines, increased fat burning, and increased insulin sensitivity [6,7].

Another study found that the daily cinnamon intake would decrease glucose and blood lipids in diabetic patients [8]. The research has also been done on the use of medicinal herbs as a sports supplement and their effects on some of the athletes' functional and health factors, supplements incorporating (*Curcuma longa*, *Cinnamomum verum*, *Camellia sinensis*, *Zingiber officinale*, *Crocus sativus*, and *Allium sativum*).

This paper will review the effect of these medicinal plants on some of the functional and general health factors of athletes and non-athletes.

Material and Methods

In this systematic review, data were collected from textbooks, databases, such as Magiran, SID, and Iran Medex, international databases of Web of Knowledge, PubMed, Scopus, and Google Scholar. To do this systematic review, different combinations of Keywords for instance: medicinal plants, phytochemical compounds, *C. longa*, *C. verum*, *C. sinensis*, *Z. officinale*, *C. sativus*, and *A. sativum*, exercise and physical health factors in human athletes and animals, also their Persian equivalents were used in the title, abstract, and keywords of the above databases. All of the information was obtained from published books or articles between 1997 and 2017. From Iranian data bases, Magiran, Sid, and Iran Medex, 1,180 Persian articles were collected from Iranian databases and 1,724 articles were collected from international databases as mentioned above. After checking for duplicates, 1,150 of the Persian articles were found to be duplicate, with their removal, 30 Persian articles remained. In addition, there were also 1,664 duplicate English articles, that with the removal, 60 English articles remained. Finally, 90 articles in total were included in this systematic review.

Search Strategy

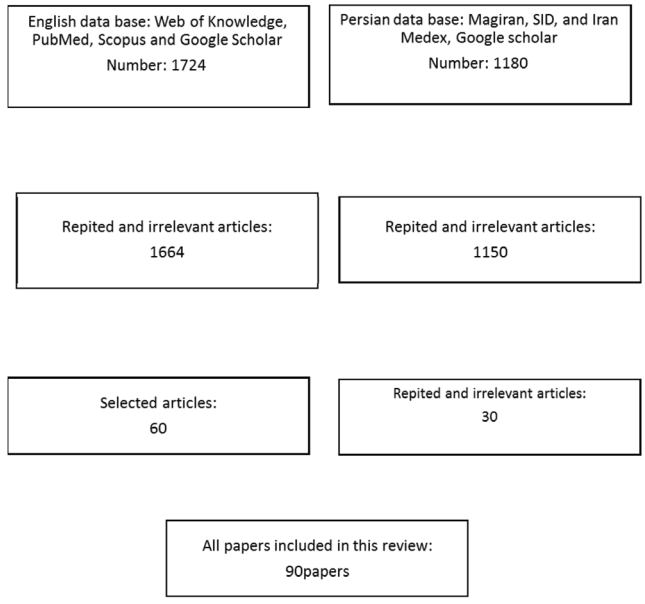

Figure 1. Flowchart of study selection.

Result and Discussion

Curcuma longa

Curcuma longa is one of the constituents of turmeric which accounts for about 0.3% of turmeric compounds. Various studies have confirmed the antioxidant effects of *C. longa* in the fight against free radicals [9,10]. *Curcuma longa* plays an important role in preventing the progression of certain diseases, such as arthritis, cancer, Alzheimer's, diabetes, and cardiovascular disease [11]. However, its most important role is to prevent cancer-causing factors and to inhibit the growth of cancer tumors [12].

In some studies, the effect of *C. longa* supplementation combined with exercise on the prevention of external threat and general health indicators was investigated. For example, some studies have shown that aerobic exercise with *C. longa* supplementation inhibits liver injury [13–15]. One of these studies is a study by Mirdar et al. [13]. In this study, 46 male rats were allocated into six groups

including; Control (C), vehicle (V), lead (L), lead and exercise (LE), lead and curcumin (LC), Lead + exercise + curcumin (LEC). Animals in training groups ran on treadmill for 8 weeks (25–64 minutes per day, 15–22 meter/minute and 0% grade). Animals in Lead, LE, LC, and LEC groups received lead acetate (20 mg/kg/body weight) and vehicle animals were given ethyl oleat (30 mg/kg/body weight) intraperitoneally for 3 days a week for 8 weeks. Rats in curcumin and LEC groups were given Curcumin (30 mg/kg/body weight), for 3 day per week for 8 weeks. IgA levels were measured by single radial immuno-diffusion method. They found that *C. longa* supplementation with exercise suppressed the effects of lead exposure in rats and may increase serum levels of immunoglobulin A [13]. Dabid Roshan et al. [14] found that recreational aerobic exercise with *C. longa* supplementation may have beneficial effects in preventing lead-induced oxidative damage [14]. In a study of *C. longa* supplementation with aerobic exercise and its effect on reducing the harmful effects of air pollution and reproduction, it was found that regular exercise with *C. longa* supplementation inhibited the harmful effects of air pollutants. However, it has no effect on improving the adequacy and quantity of reproductive indices [16].

Delecroix et al. [17] in a randomized cross-over design studied 16 elite level rugby players (age: 20.7 ± 1.4 y; height: 1.82 ± 0.06 m; mass: 89.4 ± 14.8 kg) with a weekly training volume based on rugby and gym training of 13 ± 4 hours were studied. The participants were divided into four groups: 1) dominant leg and curcumin + piperine supplementation, 2) non-dominant leg + curcumin + piperine supplementation, 3) dominant leg + placebo, and 4) non-dominant leg + placebo in a randomized and balanced way (Table 1). The supplementation was blinded: placebo versus pills containing curcumin and piperine. In the experimental condition, the participants consumed 2 g of curcumin and 20 mg of piperine, three times a day, starting 48 hours pre-exercise and continuing until 48 hours post-exercise. In the control condition, the subjects were asked to take a placebo (glucose pills) at the same time of the day. The daily supplementation was divided into three intakes, every 6 hours between 8 am and 10 pm except on the exercise day. On the exercise day, the first intake was taken 45 minutes before the exercise and the second intake was taken immediately after the exercise, and the last intake was consumed 6 hours after the second one. Fifteen days following the first session, the participants were evaluated on the other leg and in the other condition, at the same time as during the first session. The exercise induced muscle damage. Muscle function was assessed during the pre-test familiarization session, just before exercise, then immediately, 24, 48, and 72 hours following the exercise. In this study, they found that supplementation with *C. longa* before and after high-speed exercise will reduce some of the muscle damage [17]. In the study of Sciberras et al. [18], 11 male recreational athletes (35.5 ± 5.7 years; Wmax 275 ± 6 W; 87.2 ± 10.3 kg) consuming a low carbohydrate diet of 2.3 ± 0.2 g/kg/day underwent three double blind trials with curcumin supplementation, placebo supplementation, and no supplementation (control) to observe the response of serum interleukins (IL-6, IL1-RA, and IL-10), cortisol, c-reactive protein (CRP), and subjective assessment of training stress. Dosage for the present study subjects was a single dose of 500 mg of Meriva® curcumin (five tablets) with midday meal for 3 days, and then 500 mg ingested just before exercise. Samples for plasma curcumin analysis were taken before and three hours post ingestion. They found that supplementation of *C. longa* with 2 hours of cycling in men reduces inflammatory cytokines [18]. In addition, in the study of Ray Hamidie et al. [19], 36 ten weeks-old male Wistar rats (body weight, 282–390 g) were used (six rats in every group). A standard diet (Oriental Yeast, Tokyo, Japan) and water were provided *ad libitum*. The animals were randomly divided into six groups: control without eTR, curcumin 50 mg/kg-BW/day without eTR, curcumin 100 mg/kg-BW/day without eTR, control with eTR, curcumin 50 mg/kg-BW/day with eTR, and curcumin 100 mg/kg-BW/day with eTR. All animals were injected intraperitoneally (I.P), once a day for 28 days with curcumin (50 or 100 mg/kg-BW/day) dissolved in dimethyl sulfoxide (DMSO) or the same volume of DMSO (vehicle alone). The endurance exercise training (eTR) group rats swam 2 hour/day in four 30-minute bouts separated by 5 minutes of rest. Their results demonstrated that the consumption of *C. longa* in male rats combined with endurance training will increase DNA replication and increase mitochondrial biogenesis and reduce skeletal muscle damage in rodents by increasing cAMP activity [19]. However, one study found that taking *C. longa* supplementation with endurance training did not significantly change the increase in SOD and glutathione peroxidase levels in male rats. These enzymes are known as enzymatic antioxidants [20]. Studies on the effect of *C. longa* consumption on metabolic

factors have also been studied. For example, in the experimental study of Hosseini et al. [21], 28 Wistar menopausal fat rats aged 24 months were randomly divided into four groups: control, supplement, training, and training + supplement. The training program was planned for 8 weeks and three sessions per week. Each session consisted of 10 activity sets in one minute with the intensity of 50% and 2 minutes rest between the sets. In the first week, subjects started with 14 m/minute and increased their speed to 28 m/minute until the eighth week. In the supplement groups, 30 mg of curcumin solution per kilogram of body weight were injected three sessions per week for 8 weeks. Blood samples were taken after the last training session and the levels of adiponectin, chemerin, glucose, insulin and insulin resistance index (HOMA-IR= Homeostatic Model Assessment of Insulin Resistance) were measured. They found that resistance training combined with *C. longa* use effects adiponectin, chemerin, and insulin resistance, it could decrease obesity-induced inflammatory responses, and have a positive role in obese postmenopausal mice [21]. It has been shown that supplementation of *C. longa* with resistance training in patients with non-alcoholic fatty liver improves their liver function [22].

The effects of *C. longa* have not been reported so far, and some sources have stated that daily curcumin intake of 60 to 200 mg will have no side effects, but should be avoided [23,24]. In addition, in some cases *C. longa* has been reported to induce indigestion. Researchers believe that curcumin should be taken with black pepper to better absorb *C. longa* and to eliminate this problem [25].

Cinnamomum verum

Cinnamomum, a cinnamon tree, is 5 to 7 m high. The bark of this tree is used as a medicinal plant. There are different varieties of *C. verum*, including Chinese *C. verum*, South American *C. verum*, Sri Lankan *C. verum*, Indian *C. verum*, and Ceylon *C. verum*. The most popular of which for pharmacologists is Ceylon *C. verum*. *Cinnamomum verum* contains volatile oils and it is an essential ingredient in human health, and in the treatment and prevention of certain diseases. These oils themselves contain other elements, such as eugenol, cinnamic acid, and cinnamaldehyde. Taken together, these compounds, along with other cinnamon-containing compounds, are useful in the prevention and treatment of certain diseases [26,27]. The Sstudies have shown that the use of cinnamon extract will inhibit inflammatory cytokines and prevent inflammation [28]. Another study also found that the long-term supplementation of cinnamon extract would be associated with increased antioxidant potential and stress reduction induced by an exhaustive exercise training session in male rats [29,30]. Endurance training with cinnamon supplementation in rats has been shown to decrease oxidative stress in skeletal muscle [31].

Major effects of *C. verum* on human health include antioxidant effects, prevention of the activity of bacteria that are the main cause of gastric ulcers, antimicrobial effects, and treatment of colds and upper respiratory tract infections and fever, blood sweat dilators, and help lower blood pressure, help relieve impotence [26,27].

Recently, studies have been conducted on cinnamon consumption and hypoglycemia in diabetics, which have been shown to be effective in lowering blood sugar levels [26,27]. One study found that consuming 1 g of cinnamon for 4 weeks would lower blood sugar and blood lipids in diabetics [8]. In another study, 30 diabetic women were voluntarily selected and were randomly divided into three groups: 1) Aerobic exercise; 2) aerobic exercise alongside cinnamon use; and 3) control group. Aerobic exercise took four weeks with 60% of maximum heart rate. The group 2 (exercises + cinnamon) participants received three capsules per day and each capsule contained 380 mg cinnamon. Group 3 (control group) had no aerobic exercises or cinnamon. According to results of this study, cinnamon use combined with aerobic exercise, could be beneficial in improving blood glucose and lipid profile in diabetic patients [31]. In the Badalzadeh et al. [32] study, 30 male Wistar rats (8-week-old) weighing 250–300 g were fed standard rodent laboratory diet and tap water *ad libitum*. The rats were divided into five groups (six rats in each) based on receiving regular training, exhaustive exercise, and/or cinnamon bark extract (CBE) supplementation. 1. (Con): the control rats were in the rest status and received normal diet. 2. (Con + Exst): the rats received normal diet and performed a session of exhaustive exercise. 3. (Cinn + Exst): the rats supplemented with CBE for 8 weeks and performed a session of exhaustive exercise. 4. (Train + Exst): the rats performed regular aerobic training for 8 weeks, received normal diet and performed a session of exhaustive exercise in last session. 5. (Cinn + Train + Exst): the rats performed regular training, were supplemented with CBE for 8 weeks, and performed a session of exhaustive exercise in last session. Rats in the supplemented groups (i.e., groups 3 and 5) were given 200 mg/kg/day of CBE for 8 weeks by oral gavages.

In this study, cinnamon supplementation with long-term aerobic exercise improved cardiac dynamics and improved cardiac health by affecting cardiac function by reducing serum Malone dialdehyde (MDA) levels and improving blood lipid status [32]. In the semi-experimental study, 40 male overweight students were divided into four groups, including exercise, exercise and supplementation, supplementation, and control. Six-week intensive periodic massive exercises were done in the exercise groups. In addition, three 380 mg-cinnamon capsules were administrated in the supplementation groups. Blood sampling was done from the subjects 48 hours before and after the exercise program. The results of this research have shown that 6 weeks of intense intermittent exercise combined with cinnamon supplementation will decrease serum apelin levels, which plays an important role in regulating glucose homeostasis and insulin resistance index in overweight boys [33]. In the research of Shirvani et al. [34], 16 elite cyclists aged 17 to 23 years were randomized to one of two equal groups: training (*T*, *n* = 8) and training with cinnamon powder (TC, *n* = 8). The TC group was given 5 mg/kg/day cinnamon powder, along with three main meals. The T group received an equivalent amount of placebo at the same time. Subjects in both groups cycled 80—120 km per day (75%–85% maximum heart rate) for 4 weeks. Blood samples were collected 24 hours before and 24 hours after the intervention. They found that consuming cinnamon along with vigorous endurance training in cycling men can increase the sex hormones of these men [34].

Cinnamon will not have side effects if used properly and with therapeutic doses. However, excessive use of this plant can cause side effects, such as increased heart rate, shortness of breath, increased body temperature, swelling and inflammation of the mouth, as well as tongue and gums, increased gastrointestinal motility and gastrointestinal stimulation, and increased allergic reactions. In addition, cinnamon consumption in early pregnancy is dangerous, because it causes abortion. [26,27].

Camellia sinensis

Like other medicinal herbs, green tea or *C. sinensis* is rich in flavonoids. The flavonoids in green tea are rich in a compound called Catechin. In green tea flavonoids, there are several Catechins, among which the Epigallocatechingalate (EGCG) is a potent antioxidant compound and the most abundant compound is the green tea Catechin. EGCG green tea, due to its decreasing effect on the catabolite transferase enzyme, increases noradrenaline, increases sympathetic system activity, increases lipid oxidation, and consequently decreases body fat mass [35]. Additionally, it has been shown that flavonoids in green tea increase insulin levels and improve insulin function [36]. Most studies have found that EGCG and other tea compounds are a potent antioxidant agent and help prevent development of most cancers [37,38]. It has also been shown that consumption of green tea will help maintain cardiovascular health and lower blood pressure [39]. Shimotoyodome et al. [40] divided 50 mice into five groups; 10 mice in each group. Groups were treated as follows: a low-fat diet and not exercised (LF = low fat), a high-fat diet and not exercised (HF = high fat), a high-fat diet supplemented with GTE and not exercised (GTE-HF), a high-fat diet and exercised regularly (EX-HF), or a high-fat diet supplemented with GTE and exercised regularly (GTEEX-HF). The exercise modality was treadmill running and the GTE used in this study contained 81.3% polyphenols (catechins) by weight. After 15 weeks, researchers found that mice in the green tea supplementation group were more likely to burn fat than the exercise group [40]. In a randomized, double-blind, controlled clinical trial, 132 with 107 completers, generally healthy, normally sedentary 21–65 year old men and women, with a waist circumference ≥87 cm (women) or ≥ 90 cm (men), and total cholesterol (total-C) ≥ 5.2 mmol/l at screening were selected. Eligible participants were randomly assigned to receive either 500 ml/d of a beverage providing 625 mg catechins or a control beverage for 12 weeks. Both the active and control beverages contained water, sodium chloride, artificial citrus flavoring, glucose, erythritol, and sucralose. Each 500-ml serving provided 63 kJ (15 kcal) of energy and 250 mg of sodium. The catechin beverage also contained GTE and the placebo beverage contained added caffeine to match the caffeine content of the catechin beverage (~39 mg). Subjects were instructed to consume 1,500-ml bottle per day within 30 minutes, at any time of the day, with or without food. Subjects recorded their daily consumption of the study beverage and any other caffeinated beverages. At baseline and week 12, participants underwent maximal treadmill graded exercise testing. Body composition (dual X-ray absorptiometry), abdominal fat areas (computed tomography), and clinical laboratory tests were measured at baseline. In this study, it was found that consumption of tea with exercise would result in more fat burning in the abdomen and pelvic

girdle [41]. However, in a study of professional endurance athletes, consumption of 159 mg/ kg/ body weight of GTE for 3 weeks did not affect endurance athletes' metabolism [42]. Consumption of GTE improves exercise performance and increases fat oxidation and also prevents obesity [40,43]. For example, in the study of Murase et al. [35], at experiment 1, 4-week-old male BALB/c mice were maintained at 23 _ 2°C under a 12:12-hour light-dark cycle (lights on from 0700 to 1900). At 8 weeks of age, initial measurements of endurance capacity for swimming were made, and the mice were divided into four groups (*n* = 10, 5 mice/cage). All mice were allowed unlimited access to water and a synthetic diet containing (in %) 10 (wt/wt) fat, 20 casein, 55.5 potato starch, 8.1 cellulose, 2.2 vitamins, 0.2 methionine, and 4 minerals; control animals were fed this diet alone and experimental animals had this diet supplemented with 0.2 and 0.5% GTE. The animals were maintained on their respective diets for 10 weeks. During this period, experimental mice were exercised in a pool twice a week as described below, but the non-exercise (non-Ex)-control mice were not. In the *experiment 2*, Eight-week-old mice were divided into four groups (*n* = 10, 5 mice/cage) and were allowed unlimited access to the synthetic diet used in *experiment 1*, but in this case, the experimental groups received a diet containing 0.1%–0.5% epigallocatechin gallate (EGCG) for 10 weeks. During this period, experimental mice were exercised in a pool twice a week. Feeding with GTE increased the level of β-oxidation activity in skeletal muscle. Plasma lactate concentrations in mice fed GTE had significantly decreased after exercise, concomitant with increases in free fatty acid concentrations in plasma, suggesting an increased lipid use as an energy source in GTE-fed mice. EGCG, a major component of tea catechins, also enhanced endurance capacity, suggesting that the endurance-improving effects of GTE were mediated by EGCG. The β-oxidation activity and the level of fatty acid translocase / CD36 mRNA in the muscle was higher in GTE-fed mice compared with control mice. According to these results, GTE are beneficial for improving endurance capacity [43]. Some studies have shown that consumption of GTE increases sympathetic system activity and activates catecholamines, as well as increases insulin sensitivity [44,6, 7]. Wu et al. [7] had done two-phase study. In experiment 1 (*in vivo* study), rats were divided into two groups: a control group fed standard chow and deionized distilled water and a "green tea" group fed the same chow diet but with green tea instead of water (0.5 g of lyophilized green tea powder dissolved in 100 ml of deionized distilled water). After 12 weeks of green tea supplementation, the green tea group had lower fasting plasma levels of glucose, insulin, triglyceride, and free fatty acid than the control rats. Insulin-stimulated glucose uptake of, and insulin binding to, adipocytes were significantly increased in the green tea group. In experiment 2 (*in vitro* study), a tea polyphenol extract was used to determine its effect on insulin activity *in vitro*. Green tea polyphenols (0.075%) significantly increased basal and insulin-stimulated glucose uptake of adipocytes. Their result demonstrated that green tea consumption increased insulin sensitivity in Sprague–Dawley rats and that green tea polyphenol is one of the active components. Also, Venables et al performed two studies, both with a counter-balanced crossover design. In study A, 12 healthy men performed a 30-min cycling exercise at 60% of maximal oxygen consumption ($V^{\cdot}$ O2max) before and after supplementation. In study B, 11 healthy men took an oral-glucose-tolerance test before and after supplementation. In the 24-hour before the experimental trials, participants ingested three capsules containing either GTE (total: 890 ± 13 mg polyphenols and 366 ± 5 mg EGCG) or a cornflour placebo (total: 1729 ± 22 mg. They found that consumption of GTE during moderate-intensity exercise increases fat oxidation and improves insulin sensitivity [45]. Haghighi et al. [46] found that the green tea intake along with exercise did not affect leptin levels and insulin resistance index. However, the same researchers performed a case-control study on 20 overweight or obese women between 22 and 53 years old. The women were divided into two groups: experimental group containing 11 women and control group containing 9 women. The experimental group carried out aerobic exercise and received 9-g green tea three times a day. In addition, the control group carried out the same aerobic exercise without green tea for 8 weeks. In this study, they found that consumption of green tea along with exercise would result in more fat burning than exercise alone [47]. In a study, Shimotoyodome et al. [40] found that consuming green tea with exercise would be more fat burning compared to green tea or exercise alone. In another study on 36 obese or overweight women, participants were divided into four groups (group 1 green tea; group 2 placebo; group 3 green tea plus resistance training; group 4 placebo plus resistance training). This research showed that the consumption of green tea along with exercise increased lean

mass and strength and decreased triglyceride and fat mass of abdominal and pelvic girdle [48].

In the study of Most et al. [49], 24 overweight subjects [age = 30 ± 2 years, body mass index (BMI) = 27.7 ± 0.3 kg/m^2) were given 3-day supplementation of 282 mg/day EGCG (epigallocatechin-3-gallate)**.** They reported that, green tea 3-day supplementation has been shown to increase fat metabolism and thereby reduce the level of muscle lactate concentration in overweight women. Researchers believe that green tea intake along with exercise increases fat metabolism and thereby reduces carbohydrate intake [49,50].

Zingiber officinale

Zingiber officinale or ginger is a plant root with the scientific name *Z. offinale* [51,52]. Ginger, with a 2,500-year history in Eastern medicine, has been used as an anti-inflammatory and analgesic drug for musculoskeletal disorders [53]. Ginger contains some vitamins, such as niacin (C, B_6, and B_3) and essential elements, such as potassium, magnesium, calcium, and phosphorus. In addition to those listed above, ginger contains other compounds, such as Shogaol and gingerol, which, together with some other compounds of ginger root, have pain-reducing and anti- inflammatory effects [54–58]. There have been various studies of exercise and supplementation with ginger and inflammation, and some studies have shown that ginger intake will decrease muscle inflammation 24 hours after extracurricular resistance training. However, other studies have shown that ginger consumption after extravascular exercise has no effect on reducing pain, inflammation, and interstitial water [59]. Edith et al. [60] have found that ginger consumption along with exercise did not have an effect on the inhibition of Interleukin (IL-6). In a study of the effect of ginger supplementation with resistance training on some of the oxidative stress indices in obese men, Atashak et al. [61] concluded that ginger supplementation would decrease the oxidative stress induced by resistance training. Black and O'Connor [62] in a double-blind, cross-over design 27 participants performed 24 eccentric actions of the non-dominant elbow flexors. Participants ingested a 2 g dose of ginger or placebo 24 and 48 hours after exercise. They have found that taking ginger supplements 30 minutes before riding a bike had no effect on muscle pain relief, dysfunction, or metabolic rate compared with placebo. Daryanoosh et al. [63] found that ginger supplementation before and after exercise had no effect on reducing muscle pain, but pre-race ginger supplementation would prevent elevated IL-6 and creatine kinase (CK) levels. In the Afshan et al. [64] study, 20 male athletes in a longitudinal design were randomly divided into two groups consisting of an increasing strength training group with and without ginger supplement (experimental group) and the placebo group. Experimental group consumed 3 g of ginger powder in three servings (1 g per each serving) just before three main meals. The placebo group also consumed a capsule containing 1 g of starch (placebo) in the same manner and duration as the experimental group. Blood sampling was done in three stages: before strength training, immediately after and 24 hours after strength training. They found that ginger supplementation did not affect the complete inhibition of cellular stress induced by strength training [64]. Some studies have also shown that ginger consumption combined with exercise can improve body composition. One study found that consuming 3 grams of ginger powder or 3 grams of cinnamon powder per day for 8 weeks, in healthy female athletes (13–25 years old) would cause significant changes in MDA. Furthermore, it would increase heart health and lower low-density lipoprotein (LDL) levels and also has positive changes in body composition in comparison to control group (without any ginger or cinnamon) [65]. In another study, 10 over weight men, aged 39.1 ± 3.3 years and BMI 27.2 ± 0.3 kg/m^2, were investigated. Their resting energy expenditure was measured before and 6 hours after the consumption of a breakfast meal with or without 2 g ginger powder dissolved in a hot water beverage. Subjective feelings of satiety were assessed hourly using visual analog scales and blood samples were taken fasted and for 3 hours after the breakfast consumption. Ginger had no significant effect on total resting energy expenditure. However, it had a significant effect on thermic of food, but the area under the curve was not different. Moreover, ginger was found to reduce hunger, and thus reduce body weight [66]. Despite this, excessive and irregular use of ginger can cause side effects, such as gastrointestinal disorders and damage to the internal gastric wall, and can cause stomach ulcers [67]. In addition, overuse of ginger will lead to irregularities in heart rate, restlessness, and kidney and liver disorders [53].

Crocus sativus

Crocus sativus or saffron is a plant with the scientific name Crocussativus from the Iris family. Many scholars know the origin of saffron from the slopes of the

Zagros Mountains of Iran and the lands of ancient Medes. However, today it is cultivated in most parts of Iran. The constituent of the scent of safraonal saffron, a volatile aldehyde, is formed by hydrolysis of picrocrocin compounds. Saffron is made up of three different groups of compounds: alpha-carotene, beta-carotene, and zeaxanthin, water-soluble carotenoid glycosides, such as crocin and free pigments, such as ateospatin. These compounds all have different pharmacological properties. Properties, such as antioxidant and ant-cancerous, cheerful and antidepressant, reducing the aggravating factors of seizures, helping to treat asthma, reducing spasms, preventing and reducing Alzheimer's exacerbations and Parkinson's, helping to regulate blood pressure, lower blood cholesterol and reduce atherosclerosis in heart patients, help treat iron deficiency anemia, and prevent and treat colds [68–70]. In recent years, studies have been conducted on the effects of saffron consumption and blood sugar reduction in diabetic patients with different results. In the study of Arasteh et al. [71], 30 healthy male rats were divided into three groups of ten: The test group (saffron group), the sham group (physiologic serum group), and the normal group. The test group received 50 mg/kg saffron extract through intraperitonal injection. The sham group was also intraperitoneally injected by 50 mg/kg of physiologic serum solvent for 2 weeks. Daily injection was repeated at 10 am. Blood samples were obtained before administration and on the 7th and 14th days of administration. The results showed that on the 7th day of administration the hydromethanolic extract of saffron, significantly decreased serum glucose in saffron group without having any effects on serum cholesterol and insulin levels. On the 14th day of administration, they found significant decrease of serum glucose and cholesterol levels in saffron group. In addition, after 2 weeks, the test group's serum insulin had significantly increased compared to the sham and normal groups. Hosseini et al. [72] carried out a study on the effect of saffron aqueous extract combined with resistance training on glycemic index of 36 adult male diabetic rats. They concluded that the sole consumption of aqueous saffron extract (25 mg/l daily) for 6 weeks, significantly effected the glycemic index, but supplementation with saffron aqueous supplementation paired with resistance training will have a greater effect on fasting glucose control. In a study of male Sprague–Dawley diabetic rats, 250 ±15 g in weight, and 8 weeks of age, were fed with provided pellet food for animal breeding, reproduction, and stem cells. Water was allowed freely in special 500 ml bottles. Twenty male and female rats were assigned evenly into two groups and the extract was administered orally 200 and 400 mg/kg/day, in a single dose, using intra-gastric tubes, or distilled water as vehicle. The animals were fasted overnight prior to the dosing (free access to water) and food was withheld for another 3 to 4 hours after dosing. The animals were observed for 30 minutes and 2, 4, 8, 24, and 48 hours following the administration to monitor any onset of clinical or toxicological symptoms. After that, animals were grouped into two: A) diabetic and B) non-diabetic groups. In diabetic group (A), animals were randomly distributed to five subgroups: 1) control (cn), 2) training (tn), 3) extract treatment (ext), 4) training + extract treatment (tn + ext), and 5) treated with metformin 100 mg/kg (met) and served as reference group. In non-diabetic group (B), animals were randomly distributed to four subgroups: 1) control (cn), 2) training (tn), 3) extract treatment (ext), and 4) training + extract treatment (tn + ext). Both non-diabetic and diabetic subgroups comprise 5 and 10 rats, respectively. Treated animals received intra-gastric hydroalcoholic extract of saffron at 40 mg/kg daily. After the treatment, researchers concluded that the saffron supplementation with exercise training would increase Glucose Transporter Type 4 and Activated Protein Kinase expression in these diabetic rodents [73]. In the semi-experimental study of Ghanbari-Niaki et al. [74], 44 untrained healthy men were selected from the students of Mazandaran University and divided into four groups including "water-exercise", "petal sweat-exercise", "style-exercise", and "stigma-exercise". 2-week resistance exercises consisted of 12 stations (30 seconds with 40% of a maximum repetition per station; 5 sessions a week). 500 mg Crocus sativus were daily consumed two times in the morning immediately after the exercises. Then, blood sampling was done before and 48 hours after the last session. In this research, they found that circular resistance training with saffron did not affect insulin concentrations, but saffron colonization could enhance the effect of resistance training and increase estradiol. Estradiol is a sex hormone. Decreasing this hormone indirectly causes a progressive decrease in insulin levels. Abdi et al. [75] in a semi-experimental research selected 24 men with type 2 diabetes and randomly divided them in four groups (1. control, 2. saffron extract, 3. aerobic exercises, 4. compound aerobic exercises

and saffron extract). 100 mg/day Saffron extract was used. Aerobic exercises, three days a week, for eight weeks, with 55%–70% of maximum heart rate were performed. At the end, levels of Heart-type fatty acid-binding protein (HFABP) and Troponin T were measured. The serum Troponin T increased significantly in saffron extract, aerobic exercises, and compound saffron extract-aerobic exercises groups with type 2 diabetic men (p = 0.024, p = 0.013, p = 0.005, respectively). Saffron extract consumption (100 mg/day) and aerobic exercises did not significantly influence the serum HFABP (p =0.365, p =0.188, respectively). Nevertheless, serum HFABP decreased significantly in compound saffron extract -aerobic exercises group (p = 0.003). According to the results, they concluded that, Raised cardiac Troponin T and HFABP concentration accepted as the standard biochemical markers for the diagnosis of cardiac injury. Saffron intake may beneficially protect the myocardium from injuries. Compound saffron extract-aerobic exercises can decrease levels of Troponin T and HFABP in men with type 2 diabetes. Tajik et al. [76] in a semi-experimental and applied study selected 40 sedentary women and randomly divided them into four groups: the control, saffron supplement, resistance training-placebo, and resistance training-saffron extract groups. The subjects performed resistance training protocol for 12 weeks. In addition, the supplement group received 40 mg of saffron extract per day for 12 weeks. 24 hours before and 48 hours after intervention, their blood samples were collected. The results of their study demonstrated that, the use of saffron extract in combination with resistance training will improve cardiovascular risk predictors such as CRP, LDL and insulin resistance.

Although, eating too much saffron is toxic and harmful to the kidneys. Saffron reduces appetite and causes headaches and also causes sensory disturbances, vomiting, uterine bleeding, bloody diarrhea, nosebleeds, dizziness, numbness, skin jaundice and irritations, severe flat muscles, and sometimes even death. Therefore, it is recommended that it not be used without a doctor's prescription. Experiments have shown that consuming up to 1.5 g of saffron per day for an adult is permissible, but in order to prevent further complications, it is best to consume less than this amount and excessive consumption of saffron should be avoid. In addition, saffron stimulates the reproductive system, so pregnant women should avoid eating it as it may cause abortion [68–70].

Allium sativum

Allium sativum or garlic has different ingredients. However, some studies have shown that the properties of garlic are mostly due to a sulfur compound called allicin [77]. Among the medicinal plants, garlic has been recognized as a potent antioxidant agent [77,78]. Koseoglu et al. [79] studied 17 healthy volunteers to investigate the effects of garlic supplementation on serum total antioxidant capacity and lipid parameters. Participants were administered four standardized commercial garlic tablets every day for 30 days. Blood samples were taken at day 1 [before the first administration of tablets (control) and at 3 hours after the administration of tablets], 15 and 30 days, respectively. Total antioxidant capacity (TAC), total cholesterol, LDL cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride (TG) were measured. Serum TAC significantly increased at 30 days compared with 15 days, 3 hours and control. There was also a significant increase in serum TAC at 15 days compared with 3 hours and control. Total cholesterol, LDL cholesterol, HDL cholesterol, and TG were not found to be significantly different between control, 3 hours, 15 and 30 days. The data suggest that the garlic, used as a dietary supplementation, may be beneficial in increasing the antioxidant capacity of the body [79]. In the study of Dhawan et al. [80], 20 patients of essential eypertension as diagnosed (Group I) and 20 age and sex-matched normotensive controls (Group II) were investigated. Both groups were given garlic pearls in a dose of 250 mg per day for 2 months. Blood sample of two groups were taken before and after a 2-month intervention. They found garlic supplementation has beneficial effects of reducing blood pressure and counteracting oxidative stress, and thereby, offers cardioprotection in essential hypertensives [80]. Other studies have shown that the garlic consumption is beneficial for heart patients since it reduces platelet aggregation and increases subcutaneous blood flow, decreases plasma viscosity, and decreases diastolic blood pressure [81,82]. In a double-blinded, placebo-controlled study well-trained athletes were investigated. Subjects were randomly divided to an allicin supplementation group (AS group) and a control group, and received either allicin or placebo for 14 days before and 2 days after a downhill treadmill run. They showed that supplementation of garlic before and after running on treadmill would reduce oxidative, inflammatory, cellular damage, and increase total antioxidant capacity

[83]. Another study on garlic supplementation with exercise and its effect on oxidative indexes found that the garlic supplementation would reduce the oxidative damage caused by exercise-induced DNA [84]. In the study of Shahidi et al. [85], 24 female participants were randomly selected (12 active and 12 inactive). Mean age, height, weight, and maximal oxygen consumption of subjects in the active and inactive groups were (22.1 ± 0.63 years, 162 ± 0.05 cm, 54.25 ± 7.95 kg, and 39.94 ± 8.97 ml/kg/minute) and (21.8 ± 0.98 years 165 ± 0.06 cm, 55.73 ± 5.65 kg, and 32.42 ± 5.18 ml/kg/minute), respectively. Two groups completed 14 days of garlic extract supplementation/intake (800 mg per day). Then, all the subjects were included in the contract during exhaustive exercise. Blood samples of all the participants were taken before and after the supplementation, also the third sample was taken after exhaustive activity. It was revealed that the 14-day consumption of garlic extract had no significant effect. This extract also failed to increase the damage of creatine kinase after exhaustive activities in the active and passive females. However, there was a significant difference in increase of enzyme level after exhaustive activity between the active and inactive groups. In another study, 20 male non-athletes (aged 22–26 years, body fat 16%–20% and VO2max 38–42 ml/kg/minute) were allocated in two equal supplement and placebo groups (700 mg/day garlic or dextrose for 14 days). After supplementation, all of them were participated in an aerobic exercise protocol with 75% VO2max on the treadmill for 30 minutes. The blood samples were taken in three phases (before and after the supplementation and after the exercise). The results of present study showed that a 14-day garlic supplementation had significant effect on basal triglyceride (TG) decrease, and HDL-cholesterol increase. Moreover, exercise-induced decrease of triglyceride, and LDL-cholesterol in the supplement group were significantly more in comparison age with those in the placebo group [86]. Jahangard et al. [87] were examined the effects of exhaustive running and different doses of short-term garlic supplementation on TAC and MDA in during rest and exercise induced exhaustion in male soccer players. In this study, 30 male football players (Average age: 20.8 ± 1.45 years; maximum oxygen intake 67.2 ± 5.4 ml/kg/minute and body mass index 21.5 ± 1.34 kg/m^2) were divided randomly into three homogenous groups, the placebo group and the two garlic supplementation groups given two dosages (1,200 and 2,400 mg/day). The first and second blood samples were taken in the basic state and after the Shuttle Run test and the third and fourth samples were taken after supplementation, in the basic state and after test. The results of present study showed that, garlic supplementation increased TAC and decreased MDA. Moreover, supplementation hindered significant increase in the level of MDA in male football players after the test but it failed to stop the decrease in TAC. Furthermore, the decrease of TAC level in supplementation group was significantly less than in the placebo group. Saki et al. [88] concluded that the garlic consumption would increase aerobic performance of non-athletes. In their investigation, they randomly selected and divided 20 healthy, non-athlete men into two groups: supplement (mean age, 24.2 ± 1.2 years) or placebo (mean age, 23.6 ± 2.1 years. Subjects in both groups completed the Cooper Test. Then one group received garlic supplement (in the form of capsules, 500 mg, n = 10), and the other group received placebo (in the form of capsules, 500 mg of glucose, n =10). Then, they were asked to take capsules after each breakfast and dinner for 7 days. On the eighth day, the subjects performed the Cooper Test again. They observed that the supplementation with garlic supplements for 7 days resulted in a significant difference in peak oxygen consumption compared with that of the placebo group [88]. In the study by Abraham et al. on the effect of garlic extract and its effect on Vo2max changes, they concluded that garlic supplementation with endurance training would increase Vo2max but garlic supplementation alone had no effect on Vo2max in non-athlete men [89].

Conclusion

Different studies have been done on the effects of herbal remedies combined with exercise. Most of these studies have confirmed the antioxidant and anti-inflammatory effects of these herbs, and have also emphasized the greater efficacy of these herbs along with exercise. However, in most of these studies, the protocols of exercise and physical activity were not based on specific principles and, more importantly, the dosage of these herbal remedies in human samples was the same for different individuals and not based on body weight or time and type of exercise.

Ethical approval

As this review did not involve any human or animal subjects, ethical approval was not required.

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