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Full Length Research Paper

Omega 3-fatty acids, atorvastatin as modulators for inflammatory pattern versus diclofenac in osteoarthritis induced in experimental rats

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Antiinflammatory properties of statins and omega-3 fatty acids are well known and documented before. Present work aimed mainly to demonstrate their effects on inflammatory pattern of osteoarthritis (OA) induced in rats. Osteoarthritis was induced by single intraarticular injection of monosodium iodoacetate (MIA) in the right knee joints in a dose level of 24.6 mg/kg body weight. Omega 3 fatty acids and atorvastatin were applied topically(cream form) in a dose levels 1 g/kg and 10 mg/kg body weight respectively either individually or in combination versus diclofenac sodium in a dose level 5 mg/kg body weight for comparison. The treatment started after 24 h of OA induction, daily for 3 weeks. Collective results indicated that the drugs under study significantly decreased serum interleukine-6 (IL-6), tumor necrosis factor-α (TNF-α), C-reactive protein (CRP) and total cholesterol (TC). Joint tissue contents showed significant decrease in myeloperoxidase (MPO), matrix metalloproteinase2 (MMP2) along with an increase in tissue inhibitor metalloproteinase2 (TIMP2). Combined form of atorvastatin and omega 3 fatty acids demonstrated marked effects than their individual use as compared to Diclofenac.

Key words: Osteoarthritis, monosodium iodoacetate, atorvastatin, omega-3 fatty acids, diclofenac.

INTRODUCTION

Osteoarthritis (OA) is a chronic joint disease, widely distributed all over the world. Joint is composed of articular cartilage and its mechanical properties are due to the integrity of extracellular matrix, which consists of proteoglycan and collagens. Degeneration of the joint cartilage is the main picture of OA, beside other features like changes in synovial and subchondral bone metabolism (Martel -Pelletier et al., 2008).

The clinical features of OA include joint pain, swelling, stiffness and loss of mobility (Goldring and Goldring, 2006) which may be progressed later to characteristic pathological form (Lee, 2003). Matrix metalloproteinases (MMPs) in general facilitate the breakdown of extracellular matrix of the connective tissue. On the other hand tissue inhibitor metalloproteinases (TIMPs) act as inhibitor for MMPs. Therefore during OA pathogenesis,

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TIMP shows significant decrease (Martel-Pelletier et al., 2008).

Proinflammatory cytokines are mediators of OA where interleukin (IL-1B) and tumor necrosis factor α (TNF-α) potentiate chondrocytes to induce matrix degradation factors and activate catabolic condition (Goldring, 2000). Monosodium iodoacetate (MIA) is a glycolytic pathway inhibitor which blocks the activity of glyceraldehydes 3-phosphate dehydrogenase in chondrocytes leading to disruption of metabolism and subsequent chondrocyte death (Grossin et al., 2006).

Diclofenac is an anti-inflammatory drug and has been used in treatment of OA (Burke et al., 2006). Its long term use is commonly associated with potential risk, however, testing other medications of marked therapeutic use and free of side effects may be better clinically (Farid et al., 2010). Last years, many studies referred to the anti-inflammatory effect of statins in chronic disease beside its potential as hypocholesterolemic agent (Youssef et al., 2002). Omega-3 fatty acids (FAs) are long chain polyunsaturated FAs which must be supplemented with diet, since the human body is unable to synthesize it in significant amount (Hussein et al., 2005), and its potential as anti-inflammatory agent is documented before (Simopoulos, 2002).

Docosahexaenoic acid and eicosapentaenoic acid represent the main components of omega-3 FAs (Kris-Etherton et al., 2002). Eicosapentaenoic acid can activate the eicosanoid production in turn have anti-inflammatory and antiarteriosclerotic effect (Dwyer et al., 2004), as inhibitor of pro-inflammatory cytokines (Caughey et al., 1996). Systemic administration of these agents is well known (Raatz et al., 2009), however their application topically (with the except of diclofenac) is not reported before. The present work aimed mainly to study the therapeutic potential of the drugs under study versus Diclofenac (topically) on the inflammatory pattern of Osteoarthritis induced in joints of experimental rats.

MATERIALS AND METHODS

Animals

Adult male albino rats (150 to 200 g) were used in the present study. Animals were kept in a plastic cages at room temperature and on 12 h light-dark-cycle, were fed commercially available standard chow diet and water ad libitum. Experimental design, protocol of the study and animal handling were performed according to the guidelines of the Ethical Committee of the Faculty of Pharmacy, Zagazig University, Zagazig, Egypt.

Induction of osteoarthritis

Forty two rats as supplied from Egyptian organization for biological products and vaccine (Cairo, Egypt), were divided into 6 groups (n = 7). First group was kept as normal, the remaining groups from (2 to 6) were anesthetized by thiopental 40 mg/kg. Right knees were shaved and disinfected, received a single intra-articular injection through the patellar ligament, of 24.6 mg/kg monosodium iodoacetate (MIA) in 0.6 ml saline (Sigma Aldrich).

Experimental design

Osteoarthritic rats were divided into five groups: The first one received no drugs and kept as OA control. Four groups received specific doses in the form of topical cream application of the following drugs separately for 3 weeks, diclofenac, (Novartis Pharma AG Basel, Switzerland) 5 mg/kg body weight, atorvastatin, (MUP pharmaceutical CO Isamelia, Egypt) in a dose level 10 mg/kg body weight, omega-3 fatty acids (eicosapentaenoic acid 54.5%,docosahexaenoic acid 45.5% ,Arab Co. for Gelatin and Pharmaceutical Products, Egypt) in a dose level 1 g/kg body weight . The last group received a combination of atorvastatin and omega-3 FAs using the above mentioned doses.

Cream preparation for atorvastatin and omega-3 FAs

Atorvastatin powder was finely ground in a glass morter to form very fine powder. Cream base was added in portions to atorvastatin and mixed thoroughly.Omega-3 FA and diclofenac sodium were similarly prepared like atorvastatin and were freshly prepared before their application.

Cream base formula

Cetyl alcohol 5 g, cetomacrogol 5 g and emulsifying wax, mineral oil 20 g, glycerin 10 g,methyl paraben 0.18 g, propyl paraben 0.02 g and purified water 59.8 g.

Dose incorporation

Diclofenac, atorvastatin, omega-3 FAs, in cream form were applied daily for 3 weeks in a constant weight 0.5 g cream/200 g body weight of the rat, corresponding to 5 and 10 mg, 1 g/kg body weight respectively.

Blood and tissue sampling

At the end of 1 day (after OA induction) and 3 weeks of treatment, 2 ml blood from retro-orbital vein were collected and centrifuged for serum preparation. Serum IL-6, C-reactive protein(CRP), and TNFα were evaluated by ELISA technique, following the instruction of their corresponding kits Ray Biotech Inc., DRG International Inc., USA and Koma Biotech Inc. Korea, respectively (Banerjee et al., 2003). Serum total cholesterol (TC) was determined colorimetrically using commercially available kit, xpress Bio and Biochain, CA, USA according to Allian et al. (1974). At the end of 3 weeks, right knee joints were isolated and rinsed in ice cold saline, divided into 2 parts; the first one was stored at -80°C for subsequent measurements of myeloperoxidase (MPO), TIMP2 and MMP2 using RT PCR technique to (Pfaffl, 2001). The second part was used for histopathological examination.

Histopathological study

Each knee joint was kept in 10% formalin, 1% HNO_3 for 24 h or more till they became soft, rinsed with running water, dehydrated in alcohol series, kept in xylene, paraffin 45°C, and lastly frozen. Five-micron tissue sections were cut by Leica Microtome, stained with haematoxylin and eosin (H&E) and subjected to histopathological examination.

Table 1. Serum levels of inflammatory markers and TC after 24 h of Osteoarthritis induction (OA control) as compared to normal rats (n=7).

Biochemical parameters	Normal group	OA control
IL-6 (pg/ml)	8.3±1.1	18.9 ±5.9*
TNF-α (pg/ml)	11.2±1	28.02±2.7*
TC (mg/dl)	69±0.9	75.8 ±11.7*

Values are expressed as mean ± SD, $p < 0.05$.

Table 2. Serum levels of inflammatory markers and TC in OA rats treated with diclofenac, atorvastatin, omega-3 FAs for 3 weeks as compared to OA control group (n=7).

Biochemical parameters	Normal	OA control	Diclofenac	Atorvastatin	Omega-3	Atorvastatin + omega-3
IL-6 (pg/ml)	7.8±0.8	27.4±1.4*	13.2±0.6*$^{\neq}$	13.7±0.5*$^{\neq}$	19±1*$^{\neq a}$	10.6±0.6*$^{\neq a}$
TNF-α (pg/ml)	9.8±0.8	29.4±1.3*	15.4±0.7*$^{\neq}$	15.9±0.7*$^{\neq}$	21.3±1.2*$^{\neq a}$	12.5±0.6*$^{\neq a}$
CRP (ng/ml)	1.8±0.1	13.1±0.7*	4.6±0.5*$^{\neq}$	6.8±0.3*$^{\neq a}$	7.3±0.2*$^{\neq a}$	2.9±0.3*$^{\neq a}$
TC (mg/dl)	86±2.3	97.5±1.9*	82.8±1.9*$^{\neq}$	89.2±1.7$^{\neq a}$	73±1.9*$^{\neq a}$	90±2.9$^{\neq a}$

Values are expressed as the mean ± SD, $p < 0.05$. ≠ versus the OA control group.

Table 3. Effect of diclofenac, atorvastatin, omega-3FAs for 3 weeks on joint tissue contents of TIMP2, MMP2 and MPO against OA control in Osteoarthritis rats (n=7).

Items	Normal	OA control	Diclofenac	Atorvastatin	Omega-3	Atorvastatin and Omega-3
TIMP2	25.5±4	11.5±2.7*	33.1±6.3$^{\bullet}$	29.6±6.1$^{\bullet}$	26.7±5.8$^{\bullet}$	27.7±3.6$^{\bullet}$
MMP2	1.2±0.2	10.3±1.8*	1.2±0.1$^{\bullet}$	1.3±0.1$^{\bullet}$	3.1±0.1*$^{\bullet x}$	3.4±0.1*$^{\bullet x}$
MPO	0.1±0.02	0.9±0.1*	0.3±0.03$^{*\bullet}$	0.4±0.1*$^{\bullet}$	0.4±0.1*$^{\bullet}$	0.2±0.03$^{\bullet}$

Values are expressed as the mean ± SD, $p < 0.05$. *versus the normal group. $^{\bullet}$versus the OA control group. ^{x}versus the diclofenac-treated group.

Statistical analysis

Statistical analyses of data were done by Prism 5, Graph Pad, CA, USA. Results were presented as mean ± Standard Deviation (SD). Statistical differences were compared using student's t-test or one-way Analysis Of Variance (ANOVA), followed by Tukey test, considering $p < 0.05$ as statistically significant.

RESULTS

Effect of MIA after 24 h

Rats which received MIA injection demonstrated after 24 h significant increase in serum IL-6, TNFα and TC as compared to normal rats ($p<0.05$, Table 1).

Effect of atrovastatin, Omega-3 FAs and diclofenac after 3 weeks

Topical application of diclofenac induced a significant decrease of all the inflammatory markers. Omega 3 FAs and atorvastatin application showed similar decrease, while their combination demonstrated marked anti-inflammatory effect. Serum IL-6 demonstrated significant decrease following diclofenac (54.8%), atorvastatin (59.1%), omega 3 FA (45.8%), combination of the last two achieved a marked decrease (69.4%), total cholesterol illustrated also significant decrease (Table 2). Matrix metalloproteinase 2 and MPO showed significant increase along with TIMP2 decrease in OA group. Drugs application induced the reverse effects (Table 3, Figures 1 to 3).

Histopathological results

Joint tissues of normal rats exhibited normal articular surface, bone, synovium and chondrocytes (lesion score 0+) (Figure 4a). Osteoarthritic control group demonstrated intense pathological alteration in articular surfaces components (lesion score 3+) pyknotic chondrocytes, debris in articualr cavity, thickened synovial membrane by edema and inflammatory cells.

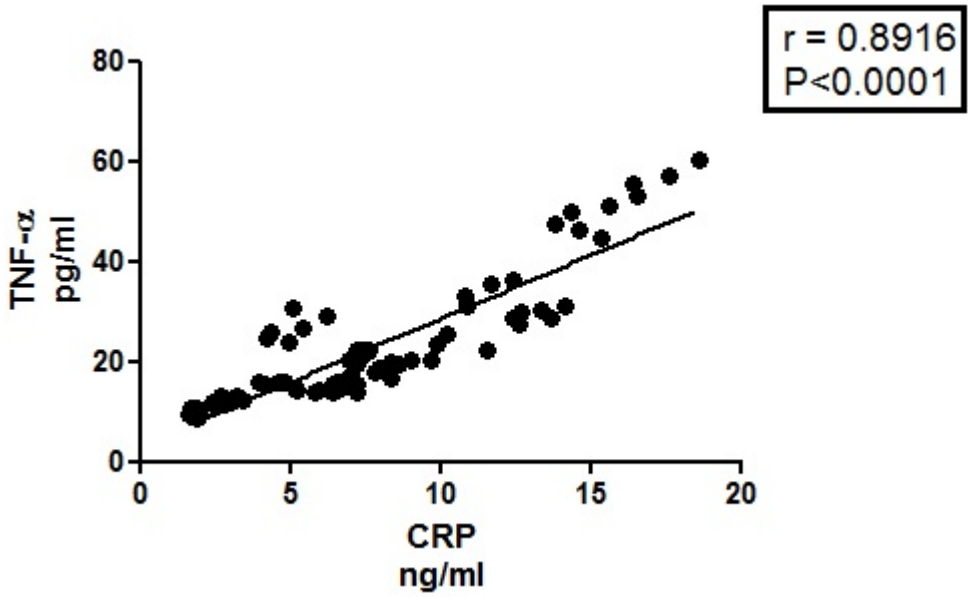

Figure 1. Correlation between CRP and TNF-α.

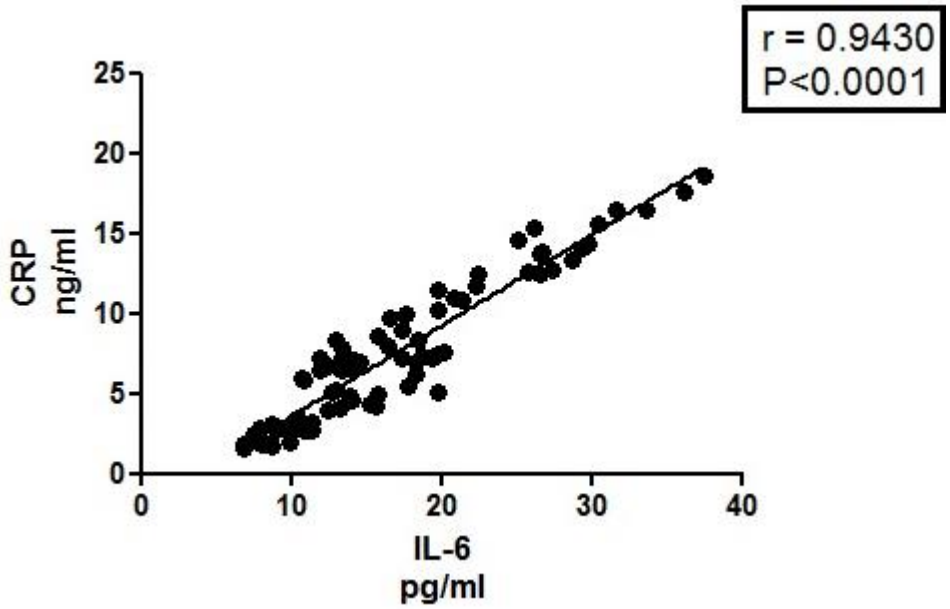

Figure 2. Correlation between IL-6 and CRP.

Moreover fragmentation of bony trabeculae with partial replacement by fibrous tissues accompanied by numerous osteoclasts (Figure 4b, c). Diclofenac treated group showed slight improvement in pathological changes (lesion score 2+). The articular surface showed loosing of chondrocytes from their lacunae, mild spindle cells proliferation together with normal synovium and articular cartilage (Figure 4d). Atorvastatin treated group had moderate improvement in OA (lesion score2+) and usually showed inflammatory cells aggregation inside the joint cavity, mild thickening in synovial membrane and early necrotic changes in bony tissues (malacia) (Figure 4e). Omega-3 FA group illustrated great improvement in OA changes of joints (lesion score 1+), softening and disorganised bone trabeculae, disorganized lacunae and absence of inflammatory cells (Figure 4f). The

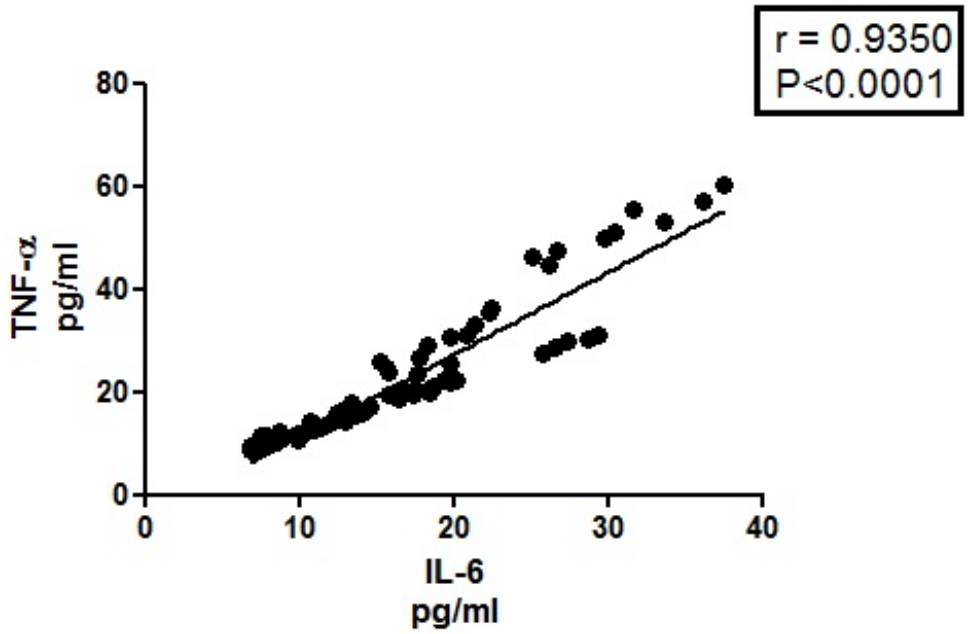

Figure 3. Correlation between IL-6 and TNF-α. Positive correlations were recorded between the inflammatory markers (CRP-TNF-α), (IL-6, CRP) and (IL-6, TNF-α).

combination treatment demonstrated great amelioration in the pathological alteration in the articular surfaces (lesion score 1+). Mild changes characterized by disorganized cartilageneous lacunae and articular cartilage, with a few pyknotic chondrocytes and normal synovial without inflammatory changes (Figure 4g).

3+------intense pathological changes in articular surfaces.
2+------moderate pathological changes in articular surfaces.
1+-----mild pathological changes in articular surfaces.
0+-----no pathological changes in articular surfaces.

DISCUSSION

Present study demonstrated that topical application of omega-3 FAs and atorvastatin either individually or in combination induced marked anti-inflammatory effect as compared to diclofenac. Histopathological examination of the knee joints of OA treated rats showed evident correlation with the biochemical findings. It was previously reported that MIA produces a rapid, technically, straight forward OA model which mimics the pathological and pharmacological features associated with human OA (Guzman et al., 2003).

In addition, it induces chemical injury and inflammation of chondrocytes or synovial membrane or both. Mechanical loading that induces OA causes the synovial cells to induce signals which can mediate the production of pro-inflammatory cytokines and cartilage degradation (Goldring and Goldring, 2007). Our histopathological findings present clear evidence of this and are in agreement with previous studies (Al-saffar et al., 2009). Induction of OA is associated with flactuation of TC. Gierman et al. (2014) indicated that hypercholesterolemia may have a role in the development of OA.

Tissue inhibitor metalloproteinase is an endogenous protease inhibitor which bind to active MMPs. MMPs derived from chondrocytes, synovium and polymorpho-nuclear leukocytes, play a major role in cartilage degradation in OA. The balance between TIMPs and MMPs is completely controlled in healthy joint, however in OA, MMP levels exceeds TIMPs leading to degradation of cartilage extracellular matrix (Alam et al., 2011).

Significant increase in MMP2 along with TIMP2 decrease in synovial fluid as observed in control group may suggest a disturbance in the balance of the enzymes, leading to high rate turnover in articular cartilage (Lee et al., 2008). Joint tissue of osteoarthritic rats demonstrated high levels of MPO. Presence of the later in syonvial fluid and neutrophils and/or macrophages within the affected joint can exaggerate the inflammatory response (Benito et al., 2005). Tissue MPO and MMP2 showed significant decrease after diclofenac treatment along with TIMP2 increase, as compared to OA control group.

Mahdy et al. (2002) demonstrated that decreased IL-6 may be attributed to reduced cyclic adenosine monophosphate and prostaglandin (PG) production. Interleukine-10 represents a responsive anti-inflammatory agent to PG effect and subsequent suppression of the

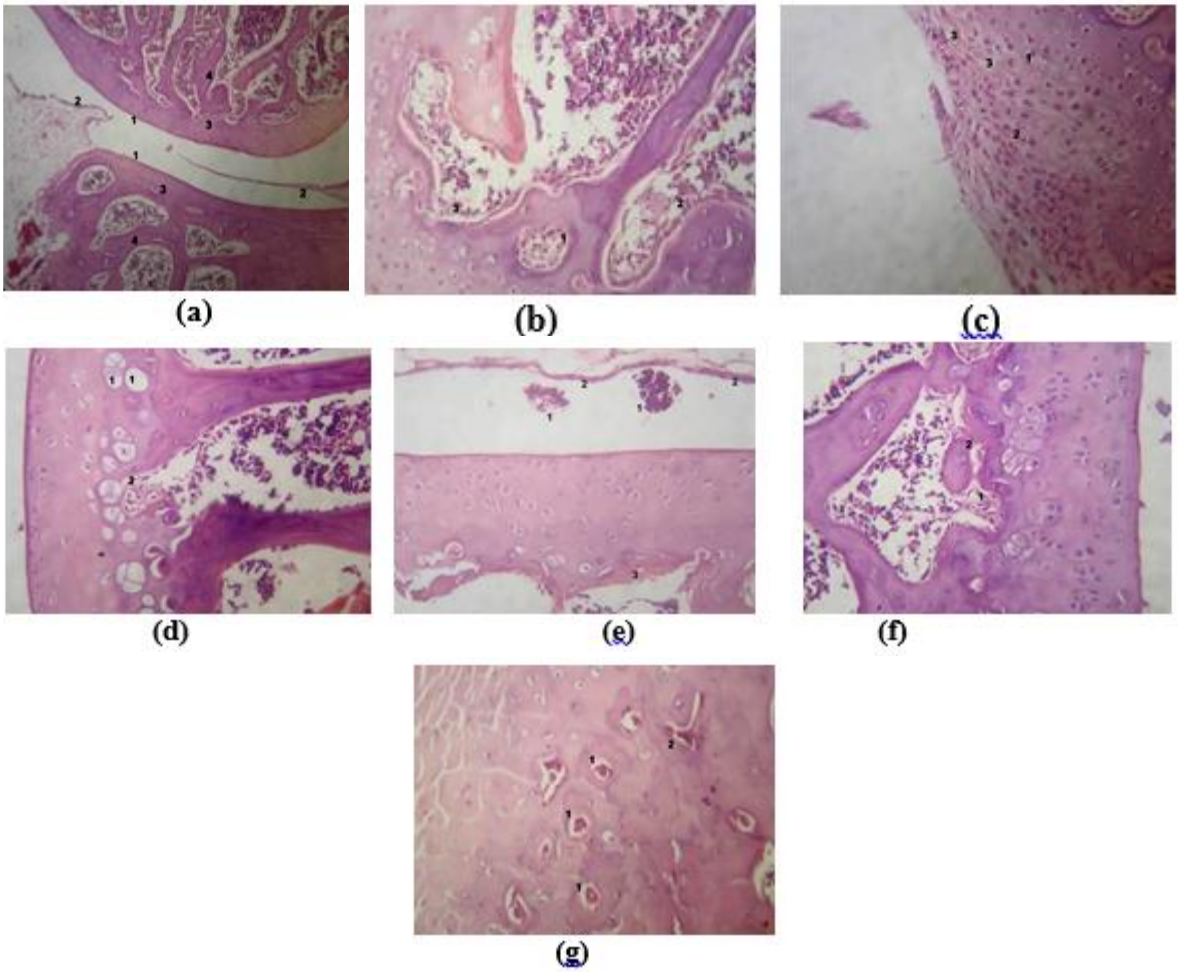

Figure 4. Photomicrographs of knee joints of normal (H&E x 300) and OA rats of the tested groups (H&Ex 1200). (a) Normal knee showing normal articular surface[1], synovium[2], chondrocyte[3] and bone[4]. (b) OA knee showing pyknotic chondrocytes[1], disorganized lacunae[2] and lysis of matrix proteoglycan with little debris[3], inside articular cavity. (c) OA knee showing fragmentation of bony trabeculae replaced by fibrous tissues[1], numerous osteoclasts[2], and inflammatory cell[3]. (d) Dic. OA treated knee showing losing of chondrocytes from lacunae[1], mild spindle cells[2], synovium and normal articular cartilage. (e) Atorvastatin OA treated knee showing aggregation of inflammatory cells in joint cavity[1], mild thickening in synovial membrane[2] and early necrotic changes malasia[3]. (f) Omega-3 FA OA treated knee showing softening of bone trabeculae[1], disorganized bone lacunae[2] and absence of inflammatory cells (g) Atorvastatin and omega-3 FA OA treated knee showing disorganized lacunae of cartilage[1], pyknotic chondrocytes[1] and disorganization of articular cartilage[2]

later resulted in IL-10 increase (Mitchell and Warner, 2006).

Non-steroidal anti-inflammatory drugs as diclofenac exhibit their action through inhibition of cyclo-oxygenase (COX) enzymes, and PG production (Barrios-Rodiles et al., 1999). COX-1 and COX-2 enzymes can be induced by cytokines as TNFα (Wahane and Kumar, 2010), although COX-1 and COX-2 were not measured. In the present study, it seems likely that they were involved and so, reduced TNF-α following diclofenac treatment may have involved COX metabolites. The current study demonstrated that topical application of atorvastatin inhibited the tested inflammatory cytokines (IL-6 and TNFα), confirming the anti-inflammatory propereties of statins that have been reported before (Maher et al., 2009).

The mechanism of action of statins in arthritis may be generated from their ability to suppress 3-hydroxy 3-methyl glutaryl coenzyme A reductase (HMG-CoA) reductase enzyme, and subsequent inhibition of isoprenoid intermediates synthesis which control many inflammatory pathways (Kwak et al., 2003). In turn cholesterol level determination may be relevant to inflammatory pattern of OA in the present study. Reduced TC here may be attributed to an inhibition of HMG-CoA and cholesterol biosynthesis (McCarey et al., 2004).

McCarey et al. (2004) demonstrated that statin can inhibit the levels of IL-6 and ameliorate endothelial dysfunction in rheumatoid arthritis. Previous study indicated that atorvastatin can shift the balance of cytokines milieu in the joints towards the production of anti-inflammatory cytokine IL-10, away from pro-inflammatory cytokines IL-6 and TNFα (Barsante et al., 2005). Topical application of omega-3 FAs significantly reduced serum IL-6, TNFα, and CRP as compared to OA control group. Omega-3 FAs can also reduce arachidonic acid metabolites and decrease the formation of pro-inflammatory compounds like leukotrienes and PGs (Chapkin et al., 1992; Joe and Lokesh, 1997).

Pischon et al. (2003) demonstrated an inverse relationship between omega-3 FAs intake and plasma level of soluble TNF receptors 1 and 2. The later encourage formation of complexes, which preserve the active trimeric form of TNF, preventing TNFα turn out into inactive monomeric forms. The receptors represent a binding protein and/or a slow release reservoir for TNF-α, indeed prolonging its half life.

Topical omega-3 FAs application resulted in hypocholesterolemic effect after 3 weeks of treatment in comparison to osteoarthritic group. Cell membrane FAs play a critical role in signal transduction where omega 3 FAs is able to modify gene expression, and change lipid level via this mechanism (Lapillonne et al., 2004). Omega-3 FAs modulate the function of sterol regulatory binding protein and peroxisome proliferation-activated receptors, both of which are involved in lipid homeostasis (Xu et al., 1999).

Yang et al. (2011) postulated that diclofenac down regulates MMP2 and MMP9 expression and their upstream enzymes of plasminogen activator urokinase and plasminogen inhibitor, both are associated with destruction of articular cartilage. Present study demonstrated also that atorvastatin significantly decreased MPO, due to an inhibition of neutrophil migration exerted Okouchi et al. (2003), and neutrophil influx to the joint of arthritic rats. This reflects a modification of tissue destruction (Joe and Lokesh, 1997). The current histopathology may present significant support to the biochemical one. Dalcico et al. (2012) reported that IL-6, IL-1 and TNFα activate the expression of metalloproteinase, so that cytokines inhibition by atorvastatin is associated with MMP2 reduction. Omega - 3 fatty acids suppresses also MMP and increases TIMP2 production via reduction of TNF-α and PGE2 (Curtis et al., 2002), while its association with atorvastatin showed significant results as compared to diclofenac treatment.

CONCLUSION

We conclude that topical application of omega 3 FAs and atorvastatin either individually or in combination induces anti-inflammatory and hypocholesterolemic effect in OA rats as compared to Diclofenac. This represents a new topical candidate for OA treatment in experimental rats but clinical trials for long term use may be recommended to confirm the present findings.

Conflict of Interests

The authors have not declared any conflict of interests.

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Neuropharmacological profile of ethanolic dried seed extract of *Persea americana* in mice

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Persea americana **Mill (Lauraceae) is a medicinal plant used traditionally in Nigeria to treat several diseases including malaria, hypertension and febrile convulsions among others. Some of these indications are related to central activity but have not been systematically evaluated. This study investigated the neuropharmacological effects and the acute toxicity profile of the ethanolic dried seed extract of *P. americana* in mice. Fresh dried grounded seed of *P. americana* was extracted with 70% ethanol. Acute toxicity (LD_{50}) profile of the ethanolic extract of *P. americana* (EEPA) at 10 to 5000 mg/kg was determined orally (*p.o.*) and intraperitoneally (*i.p.*) in mice. The EEPA was further tested for behavioral, anxiolytic, hypothermic, sedative, anticonvulsant, and anti-nociceptive activities. The LD_{50} of EEPA was determined to be ≥5000 mg/kg, *p.o.*, and 2250 mg/kg, *i.p.* The extract at 250 to 1000 mg/kg dose-dependently caused significant ($p<0.01$ to 0.001) reduction in rearing and locomotor activity, signifying central nervous system (CNS) depression; significantly ($p<0.01$) lowered normal rectal temperature showing hypothermic effect; shortened onset and increased total sleeping time of ketamine (100 mg/kg, *i.p.*), suggesting sedative activity; reduced mortality due to pentylenetetrazole, picrotoxin and strychnine, and blocked hind limb tonic extension on the electro-shock, conveying evidence of anticonvulsant activity; increased reaction time on the hot plate and inhibited acetic acid-induced writhings, indicating analgesic potential. This study reveals significant depressant effect of ethanolic extract of *P. americana* on the CNS; and manifested hypothermic, sedative, anticonvulsant and anti-nociceptive effects in mice, thus justifying its ethnomedicinal use which can also serve as a lead in drug discovery.**

Key words: Avocado, behavioral, hypnosis, anticonvulsant, nociception.

INTRODUCTION

The use of plants in form of concoction, infusion, decoction, etc., was the order of the day until the development of scientific means of extraction and purification which have paved the way for the identification of the precise compound(s) that is/are responsible for the observed pharmacological responses (Newman and Craig, 2012).

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In recent years, secondary plant metabolites previously with unknown pharmacological activities have been extensively investigated as sources of medicinal agents. Leads can be obtained in many ways among which are the screening of natural products from plants, animals, minerals and microorganism (Fabricant and Farnsworth, 2001). Of these, plants are the most abundant, diversified and constitutive. Although the modern synthetic methods of drug discovery have revolutionized drug production, plants remain a valuable source of drugs as many important drugs used in medicine today can be traced to plants (Balunas and Kinghorn, 2005).

Persea americana (Lauraceae) commonly known as avocado, has been cultivated for its highly nutritious values since about 8000 BC, and there is evidence that it was eaten as wild fruits before then. It is believed to have originated in Mexico and Central America including the Pacific coasts of Guatemala, El Salvador, Nicaragua, Costa Rica, and Panama (Chen et al., 2008). Avocado is now widely cultivated in large quantities in Indonesia, Brazil, South Africa, Israel, USA (California and Florida) and Australia. Different species of avocado is presently found in several tropical and sub-tropical countries including Nigeria where the fruits are in high demand. In Southwest Nigeria, avocado grows wild in the forests or is planted in selected locations for various purposes including commercial, protection against wind-storms, as shade and other uses.

P. americana leaf or fruit is a popular medicinal plant used traditionally in the management of several diseases in many southwestern states of Nigeria where it is indicated for treating malaria, hypertension, rheumatism, febrile convulsions and diabetes among other uses (Owolabi et al., 2005; Anita et al., 2005; Ezuruike and Prieto, 2014). Traditional herbal practitioners recommend taking the seed (fresh or dried) to treat various ailments most importantly, diabetes, hypertension and arthritis.

Pharmacological activities reported for the leaf extract of *P. americana* include acute toxicity and anti-diarrheal effect of the chloroform-methanol extract of the leaf has been reported (Odo et al., 2014), anticonvulsant (Ojewole and Amabeoku, 2006), analgesic, anti-inflammatory and hypotensive (Adeyemi et al., 2002; Imafidon and Amaechina, 2010), and vaso-relaxant (Owolabi et al., 2005). The seed has been reported to possess antiulcer (Ukwe and Nwafor, 2005), wound healing (Nayak et al., 2008), antioxidant (Matsusaka et al., 2003; Asaolu et al., 2010; Wang et al., 2010) and hypoglycaemic activities (Okonta et al., 2007; Edem et al., 2009; Nwaogu et al., 2008; Koffi et al., 2009; Jiménez-Arellanes et al., 2013), antiprotozoal and antimycobacterial activities (Jiménez-Arellanes et al., 2013). Acute toxicity and genotoxic activity of the seed ethanolic extract was reported (Padila-Camberos et al., 2013). Phenolics from the seed of the plant showed significant activities *in vitro* antioxidant and antimicrobial assays in addition to inhibition of lipid and protein oxidation in porcine patties (Rodríguez-Carpena et al., 2011). Combination of the leaf of *P. americana*, stem and leaf of *Cymbopogon citratus* and fruit of *Citrus medical* as well as honey in ethanol and sucrose experimental model has been shown to exhibit antihypertensive potential (Dzeufiet et al., 2014).

Phytochemical studies on avocado seeds indicate various groups of secondary metabolites including phytosterols, triterpenes, fatty acids, furanoic acids, flavonol, proanthocyanidins, saponins, amino acids, polyphenols and absicic acids (Nwaogu et al., 2008; Leite et al., 2009), catechins, epicatechin, hydroxybenzoic acids, hydroxycinnamic acids and procyanidins (Rodríguez-Carpena et al., 2011). Lipids, triacylglycerol, monoenoic acids and oleic acid (regarded as an especially important functional component of avocado) were reported to account for approximately 50% of the monounsaturated fatty acids obtained from a Japanese species (Takenaga et al., 2008).

Various bioactivities of this plant seed have been reported, however, considering the rate and manner of its use in folkloric medicine, there is a need to explore other effects and presently there is no comprehensive study relating these ethnomedicinal claims to central effects. It therefore becomes imperative to investigate its possible effect on the central nervous system (CNS) as a preliminary screening step in our quest for identifying CNS-acting agents from natural products and to provide scientific basis for the various folkloric claims. In this study we set out to assess some central nervous system activities and determine the acute toxicity profile of the ethanolic dried seed of this Nigerian species.

MATERIALS AND METHODS

Plant collection, identification, authentication and preparation

Matured fruits of *P. americana* were purchased in June 2013 from the central market in Ondo Town, Ondo State, Southwest, Nigeria. Mr. Bernard Omomoh, the herbarium officer of Botany Department, Faculty of Sciences, Obafemi Awolowo University, Ile-Ife, identified and authenticated the plant. The herbarium specimen sample of the fruit and leaf was prepared and deposited with reference number IFE 17374. The fruits were allowed to ripen in the laboratory after which their seeds were removed, sliced into smaller pieces and sun-dried for 1 week before powdered into coarse sizes. The powdered seed was macerated with 70% ethanol on a mechanical shaker for 72 h. The mixture was filtered and the filtrate concentrated *in vacuo* using the rotatory evaporator. The semisolid extract obtained was further freeze-dried to yield 11.60 g (2.9%$^{w}/_{w}$) ethanolic dried seed extract of *P. americana* (EEPA) and stored in the refrigerator until use. The hydro-alcoholic (ethanolic) extract of the seed was used in this study in order to maximize the extraction process, namely, both polar and non-polar compounds present in the dried seed would be extracted.

Preliminary phytochemical screening

The EEPA was dissolved in water and screened for the presence of secondary metabolites such as terpenes, alkaloids, flavonoids,

tannins, saponins, sterols and glycosides (Mir et al., 2013).

Liquid chromatography-mass spectrometry (LC-MS) analysis of the EEPA

The LC-MS analysis of the EEPA was carried out to obtain the fingerprints of the crude extract and the sample of EEPA was analyzed at Central Analytical Facilities, Stellenbosch University, Stellenbosch, South Africa. The analysis was performed on Waters Synapt G2 Waters UPLC with PDA, detection source was by electrospray positive, Capillary voltage 3 kV, Cone Voltage 15 V and Lock mass was Leucine encephalin.

Laboratory materials

Drugs

The following drugs were used: diazepam (Valium® Roche, Switzerland), pentylenetetrazole (PTZ) (Sigma, USA), strychnine (Sigma, Switzerland, MSDS), picrotoxin , phenytoin sodium (Epanutin® Pharma-deko), acetic acid (BDH Chemical Ltd, Poole, England), diclofenac potassium (Diclogesic® Supreme), ketamine (Ketalar® Pfizer), morphine (Sigma, St. Louis, USA), normal saline (JUHEL Pharm., Nigeria).

Laboratory animals

Adult male and female albino mice (Vom strain) weighing between 18 and 25 g were obtained from the Animal House, Department of Pharmacology, Obafemi Awolowo University (OAU), Ile-Ife. The mice were caged sex-wise separately to prevent mating and pregnancy. The animals were supplied with regular food and water throughout the period of study except during experiment. Each mice was used only once for the entire period of study. The animal experiments were conducted within the period of 10.00 a.m. and 4.00 p.m. daily at ambient temperature of 30 ± 2°C. The Postgraduate Research Committee, Faculty of Pharmacy, OAU approved the study with reference number: PHA/2008/075. Guidelines on the care and use of experimental animals published by the National Institute of Health (NIH, 1985), as being implemented by the University Research Committee of OAU was complied with.

Acute toxicity studies

Acute toxicity effect of EEPA was assessed in mice using both intraperitoneal (*i.p*) and oral (*p.o.)* routes according to Lorke's method (Lorke, 1983). The method involves using thirteen animals on the whole for a rapid and economic LD_{50} estimation. For each route, the procedure was divided into 2 phases. The first phase consists of three groups (n=3) at the dose levels of 10, 100, and 1000 mg/kg. The second phase also involve four groups (n=1) at the dose levels of 1000, 1600, 2900, and 5000 mg/kg, respectively. Immediately after treatment, each mouse was placed inside the Plexiglas cage and observed for immediate effects up to 30 min and thereafter for 24 h for lethal effects culminating into death. The LD_{50} of EEPA was estimated as the geometric mean of the lowest dose causing death and the highest dose causing no death according to the formula below:

$LD_{50} = \sqrt{((A)\ B)}$ or $(A \times B)^{½}$

where A is the maximum dose producing 0% death and B is the dose producing 100% death (Lorke, 1983).

The choice of route of administration

The oral route has been reported to be unpredictable due to effect of many factors including biodegradation of active components, poor bioavailability, effect of food substances etc. (Pond and Tozer, 1984; Castel-Branco et al., 2009; Gavhane and Yadav, 2012). The choice of *i.p.* route for neuropharmacological evaluation was also supported in previous study (de Carvalho et al., 2001), hence, the *i.p*. route was chosen in this study. The working doses used in this study were 250, 500 and 1000 mg/kg, i.p. which were lower than half of the LD_{50} value estimated to be 2250 mg/kg, i.p.

Novelty-induced behavior (NIB): Rearing and locomotion

Novelty-induced behavior was assessed as described by Onigbogi et al. (2000), which were further modified in this study. Five groups (n=5) were randomly selected. Group I was administered the vehicle (5% Tween 80, 10 ml/kg, *i.p*.). Groups II to IV were injected the EEPA (250, 500 and 1000 mg/kg, *i.p*.), respectively. Group V was injected diazepam (1 mg/kg, *i.p*.) as positive control (Gonzalez-Trujano et al., 2006). Mice in all the groups were pretreated 30 min prior to test. Each animal was placed inside the observation cage and assessed for rearing (number of times the animal stand on its hind-limbs with fore limbs in the air or against the wall) for 20 min, and locomotion (the number of lines crossed with all limbs) for the first 10 min.

Effect of EEPA on anxiolytic test

Effect of EEPA on the elevated plus-maze

Mice were randomly distributed as described in previous sections. After 30 min pretreatment, each mouse was placed in the central intersection of the elevated plus maze (EPM). The time spent in the open and closed arms as well as the number of times the animal enter each arm was recorded for 5 min (Pellow et al., 1985). The results obtained were analyzed and compared among the groups. Diazepam (1 mg/kg) was used as the positive control drug (Adeyemi et al., 2010).

Effect of EEPA on the hole board

Mice were randomly distributed as described in above section. After 30 min pretreatment, each mouse was placed in the center of the hole board. The number of head-poking demonstrated by each mouse in 5 min was recorded. The results were analyzed and compared among the groups. Diazepam (1 mg/kg) was used as the positive control drug (Takeda et al., 1998; Yadav et al., 2008).

Effect of EEPA on normal rectal temperature of mice

Five different groups (n=5) of mice were randomly selected. Group I received vehicle, groups II to IV were administered the extract (250, 500 and 1000 mg/kg, *i.p*., respectively), while group V was injected diazepam (1 mg/kg, *i.p*.) as the positive control drug (Vale et al., 1999). Rectal temperatures were initially taken before pretreatment and at 30, 60, 90 and 120 min post treatment. All rectal temperatures were measured with digital thermometer (thermoprobe) inserted 2 cm into the anus of the mouse (Al-Nagger et al., 2003; Oyemitan et al., 2008).

Effect of EEPA on ketamine-induced hypnosis

Ketamine (100 mg/kg, *i.p.*) was used to induce sleep in mice (Mimura et al., 1990). Mice in different groups (n=5) were pretreated with vehicle, extract (250, 500 and 1000 mg/kg, *i.p.*, respectively) and diazepam (1 mg/kg, *i.p.*) as positive control drug (Adeyemi et al., 2010) 30 min prior to the administration of ketamine. Each animal was observed for sleep latency (SL) or the onset of sleep (time from injection to time of loss of righting reflex); and the duration of sleep or total sleeping time (TST), that is, time from loss and recovery of consciousness.

Effect of EEPA on chemical and maximal electroshock seizure (MES) convulsion tests

Effect of EEPA on pentylenetetrazole (PTZ)-induced convulsion: Pentylenetetrazole (85 mg/kg, *i.p.*) was used to induce tonic-clonic convulsion (Swinyard et al., 1989). Five different groups (n=5) of mice were randomly selected. Group I received vehicle, groups II to IV were administered the extract (250, 500 and 1000 mg/kg, *i.p.*, respectively) and group V was injected diazepam (1 mg/kg, *i.p.*) as positive control drug (Gonzalez-Trujano et al., 2006). The anticonvulsant activity assessment was carried out 30 min prior to injection of PTZ (85 mg/kg, *i.p.*) and observed for the onset of convulsion, time of death and mortality. Animal that survived beyond 30 min post PTZ injection is assumed to be protected in this model (de Sarro et al., 1999).

Effect of EEPA on picrotoxin-induced convulsion: The experiment described in section (a) was repeated using picrotoxin (10 mg/kg, *i.p*) as the convulsant agent and diazepam (2 mg/kg, *i.p.*) as positive control. The manifestation of tonic-clonic convulsion was then assessed as earlier discussed (de Sarro et al., 1999).

Effect of EEPA on strychnine-induced convulsion: The experiment described in section (b) was repeated using strychnine (4 mg/kg, *i.p.*) as the convulsant agent, and diazepam (5 mg/kg, *i.p.*) as positive control (Shenoy et al., 1982). The manifestation of tonic-clonic convulsion was then assessed as earlier discussed (Swinyard et al., 1989). Each animal was observed for tonic-clonic convulsions and mortality. Animals that survived beyond 30 min were regarded as protected.

Effect of EEPA on MES-induced convulsion: Electroconvulsive shock was used to induce hind limb tonic extension (HLTE). The electrical stimulus (50 mA, 50 Hz, 0.2 s duration) was applied through the ear lobes by electrode clamp (transauricular-ear-clips), using an Electro-Convulsiometer. Group I was administered the vehicle, groups II to IV were injected the extract (250, 500 and 1000 mg/kg, *i.p.*), respectively, while Group V was injected phenytoin sodium (25 mg/kg, *i.p.*) as the positive control drug (Sousa et al., 2009). After 30 min pretreatment, each mouse was submitted to MES test. Protection against HLTE was taken as positive result (Pourgholami et al., 1999).

Effect of EEPA on thermal and chemical nociception in mice

Effect of EEPA on the hot plate test: Mice were randomly allocated to five groups (n=5). Mice in group I were injected vehicle; groups II to IV with EEPA (250, 500 and 1000 mg/kg respectively), and group V with morphine (10 mg/kg) as the positive control drug (Viana et al., 2003). Each mouse was dropped gently on the hot plate maintained at 55°C and the time taken for the mouse to lick the fore or hind paw or jump up on the plate was taken as reaction time, the test was carried out at 30, 60, 90 and 120 min post-treatment. The cut off time was set at 30 s (Silva et al., 2003).

Effect of EEPA on acetic acid-induced writhings: Five different groups (n=5) of mice were randomly selected. Group I received vehicle, groups II-IV were administered EEPA (250, 500 and 1000 mg/kg, *i.p.* respectively), while group V was injected diclofenac (100 mg/kg, *i.p.*) as positive control drug (Gonzalez-Trujano et al., 2006). Thirty minutes after treatment, each mouse was intraperitoneally injected 1% acetic acid (10 ml/kg) and allowed 5 min delay before assessment for up to 20 min inside the Plexiglas's cage. The number of writhings or abdominal constrictions displayed by each mouse was counted and recorded (Viana et al., 2003; Silva et al., 2003).

Statistical analysis

The results were expressed as mean±standard error of mean (SEM) and analyzed using one-way analysis of variance (ANOVA) followed by post hoc test using Dunnett's test for comparison between the treated groups and control. The anticonvulsant results were presented in percentage by comparing the % protection/ mortality in treatment groups versus the negative control group. GraphPad Instat 3 (UK) and GraphPad Prism 5 (© 1990-2003, GraphPad Software, Inc.) were used for the analysis of the results.

RESULTS

Preliminary phytochemical test

Results of the preliminary phytochemical screening confirmed the presence of alkaloids, phenols, flavonoids, saponins, terpenes, steroids, and glycosides; however, phytosterols was not detected in the EEPA sample.

LC-MS analysis of the EEPA

The fingerprints of the crude EEPA performed on the LC-MS showed the presence of numerous compounds which can be used as a reference fingerprint for other EEPA crude extract (Figure 1).

Acute toxicity studies

In the oral route, doses of EEPA up to 5000 mg/kg produce no mortality in the mice, and in the intraperitoneal route, there was mortality at 2900 mg/kg but none at 1600 mg/kg; according to Lorke (1983) the LD_{50} values were calculated to be ≥5000 and 2154 mg/kg for the oral and intraperitoneal routes, respectively.

Effect of EEPA on novelty-induced behavior

The EEPA (250, 500 and 1000 mg/kg, *i.p.*) caused significant [$p<0.01$; $F_{(4, 20)}=98$] and dose-dependent decrease in the number of rearings. Diazepam (1 mg/kg, *i.p.*) also produced significant ($p<0.01$) decrease in rearings (Figure 2A). The EEPA (250, 500, and 1000 mg/kg, *i.p.*) and diazepam caused significant [$p<0.01$; $F_{(4,}$

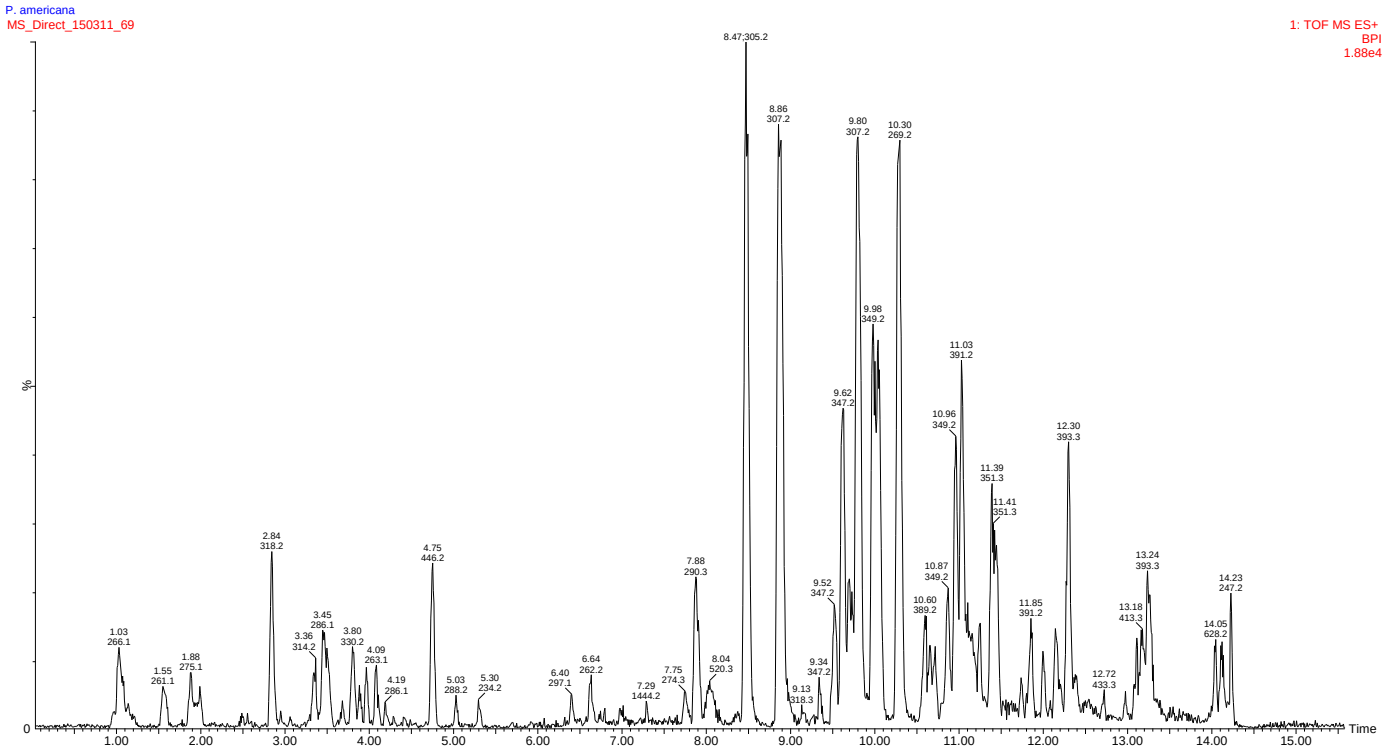

Figure 1. Chromatogram of LC-MS of the EEPA indicating numerous compounds.

$_{20)}$=47] and dose-dependent reduction in locomotor activity compared to the vehicle (Figure 2B).

Effect of EEPA on anxiety

The EEPA at 250 mg/kg, *i.p*. caused significant [p<0.01; $F_{(4,\ 20)}$=47] increase in the number of head pokes compared to the vehicle. At the doses of 500 and 1000 mg/kg, *i.p.* it showed a significant (p<0.05 to 0.01) decrease in the number of head pokes similarly to diazepam (1 mg/kg, *i.p*.) which also showed significant (p<0.01) decrease in the exploratory activity (Figure 3). On the EPM, EEPA at all doses did not cause a significant increase in the percentage number of entries into either of the closed or open arms when compared to the control group, showing a lack of anxiolytic effect at these doses (result not shown).

Effect of EEPA on rectal temperature of mice

Vehicle did not cause significant variation in rectal temperature up to 120 min post-treatment. The EEPA at 250 mg/kg *i.p.,* caused significant [p<0.01; $F_{(4,\ 20)}$=3.8] at 60 min, at 500 mg/kg, it caused significant [p<0.01; $F_{(4,\ 20)}$=7.3] reduction in rectal temperature at 30, 60 and 90 min post-treatment. However, the extract at 1000 mg/kg, *i.p.,* caused a significant [p<0.01; $F_{(4,20)}$=12.3] decrease in the rectal temperature of the mice compared with vehicle at 30, 60, 90 and 120 min post-treatment (Figure 4).

Effect of EEPA on ketamine-induced hypnosis

The EEPA (250, 500 and 1000 mg/kg, i.p.) significantly [p < 0.01; $F_{(4,\ 20)}$= 23] reduced SL in a dose dependent manner compared to vehicle. The EEPA at 250 mg/kg *i.p*. significantly (p<0.05) prolonged the TST induced by ketamine (100 mg/kg, *i.p*.), and at 500 and 1000 mg/kg, caused significant [p<0.01; $F_{(4,\ 20)}$=61] increase in TST compared to the vehicle. The standard drug, diazepam 1 mg/kg, *i.p.* significantly (p<0.01) reduced SL and also significantly (p<0.05) prolonged the TST compared to vehicle (Figure 5A and B).

Effect of EEPA on chemical and electric shock convulsion tests

The results of chemo-and electro-convulsion tests are summarized in Table 1. Mice in the vehicle group were not protected in all the models. The EEPA (250, 500 and 1000 mg/kg, *i.p*.) completely protected the mice against mortality and offered 100% protection against PTZ-induced convulsion. In the picrotoxin-induced convulsion test, mice in all the treated groups showed severe convulsions including tonic-clonic. However, EEPA at 500

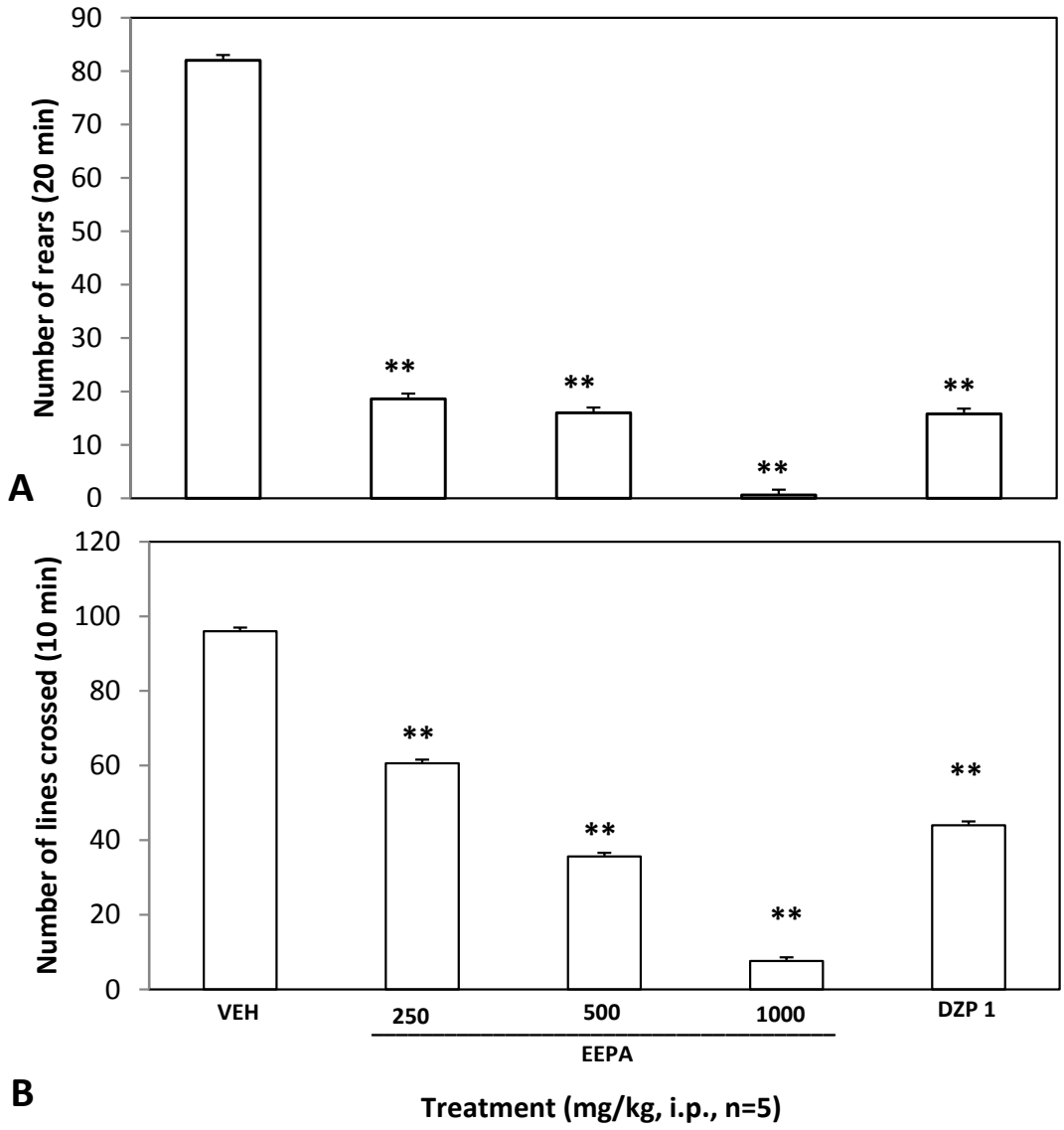

Figure 2. Effect of EEPA on rearing (panel A) and locomotor activity (panel B) in mice. Each bar represents Mean±SEM. VEH, EEPA and DZP represent vehicle, ethanolic extract of *P. americana* and diazepam respectively. **p<0.01, statistically lower than vehicle (ANOVA, Dunnett's test).

and 1000 mg/kg, *i.p.,* offered 60 and 80% protections respectively, compared to diazepam (2 mg/kg, *i.p*.) which provided 80%. Strychnine induced convulsion with severe tonic-clonic convulsion. Only EEPA at 1000 mg/kg, *i.p*. and diazepam (5 mg/kg, *i.p*.) offered 60 and 40% protections respectively. In the MES test, EEPA (500 to 1000 mg/kg, *i.p*.), and phenytoin sodium (25 mg/kg, *i.p*.) offered 100% protections against HLTE with zero mortality.

Effect of EEPA on thermal and chemical nociception

The EEPA (250, 500 and 1000 mg/kg, *i.p*.) induced significant [p<0.01; $F_{(4, 20)}$=32] increase in reaction time to thermal stimulation in mice on the hot plate test. At 1000 mg/kg, *i.p.,* EEPA increased the reaction time significantly [p<0.01; $F_{(4,20)}$=10-22] throughout the 120 min test duration. Prolongation of reaction time was significant (p<0.01) up to 90 min for 500 mg/kg., and up to 60 min at 250 mg/kg respectively. The standard drug, morphine induced significant [p<0.01; $F_{(4,20)}$=10-22] increase in reaction time compared to control up to 90 min (Table 2). The result of acetic acid-induced writhings showed that the EEPA (250, 500, and 1000 mg/kg, *i.p*.) significantly [p<0.01; $F_{(4,20)}$=63] reduced the number of acetic acid-induced writhes and offered 33, 54 and 85% analgesia compared with diclofenac (100 mg/kg, *i.p*.), which

produced 85% analgesia (Table 3).

DISCUSSION

In this study, we investigated the effect of the ethanolic extract of the dried seed of *P. americana* (EEPA) on novelty induced behaviour (NIB), anxiety, rectal temperature, sedation, convulsion and nociception in mice. The acute toxicity profile (LD_{50}) of the extract was also determined orally and intraperitoneally. The major effect of the extract was found to cause depression of the central nervous system.

Preliminary phytochemical screening of the EEPA confirmed the presence of alkaloids, phenols, terpenes, flavonoids, saponins, steroids and glycosides similar to report by Arukwe et al. (2012), suggesting that the species used in this and the other studies were the same although they were from different southern states of Nigeria. The EEPA was analysed with liquid chromatography and mass spectroscopy (LC-MS) and the chromatogram obtained showed the presence of several compounds (Figure 1) which serves as the fingerprints of the EEPA in this study.

The LD_{50} values obtained in the acute toxicity study of the ethanolic extract of the dried seed of *P. americana* were found to be ≥ 5000 mg/kg, *p.o*., and 2154 mg/kg, *i.p*., which indicates that it is non-toxic and moderately toxic through the oral and intraperitoneal routes respectively (Lorke, 1983). However, the values obtained here for the oral route contrasted sharply from that obtained in another study on ethanolic seed extract of a Mexican species, which caused a mortality of 20, 60 and 80% at doses of 500, 1000 and 2000 mg/kg, p.o., respectively (Padilla-Camberos et al., 2013). This could be due to differences in the chemical composition of the extracts occasioned by geographical and species variation (Guo et al., 2013).

This non-toxic effect per oral of this extract may be used to justify the use of this plant in ethnomedicine for managing various ailments. The effect of EEPA on novelty-induced behaviour is suppression of rearing and locomotor activities (Figure 2). Locomotion and rearing are considered horizontal and vertical locomotor activities respectively, thus an increase in these activities signifies excitatory while a decrease indicates inhibitory or sedative effect (Zapata-Sudo et al., 2010). The results obtained here are similar to that reported for ethyl acetate extract of *Ipomoea stans,* which significantly reduced spontaneous motor activity, protected against PTZ-induced convulsion and enhanced the hypnotic effect of pentobarbital in mice (Herrera et al., 2007). The EEPA induced a motor depressant effect, indicating a possible skeletal muscle relaxant and sedative effect (Adeyemi et al., 2006). Inhibitory effect in the CNS is caused either by the augmentation of GABA inhibitory effect by binding to the $GABA_A$ receptor like the benzodiazepines (e.g. diazepam), or antagonizing the effect of glutamate by blocking glutamate receptors such as NMDA (e.g. felbamate), AMPA (e.g. topiramate), kainite, glycine, or the metabotropic receptors (Rang et al., 2007).

The elevated plus-maze (EPM) has been adequately demonstrated to be a satisfactory model for testing anxiolytic effect of drugs even when the agent does not act via the benzodiazepine receptors (Söderpalm et al., 1989). The EEPA at all doses used did not show anxiolytic effect on EPM model because there was no significance ($p>0.05$) increase in the time spent in the open arms and number of entries which could be due to the sedative effect of the extract at the doses used in this study. Previous report revealed that high doses of sedatives cause depression of all activities including exploratory activity (Hellion-Ibarrola et al., 1999). A further anxiolytic test of the extract on the hole-board model (Figure 3) reveals a positive anxiolytic effect at the lowest dose (250 mg/kg, *i.p*.) as it increased the level of exploratory activity (number of head dips) when compared to the control group. However, EEPA at 500-1000 mg/kg, *i.p*., and diazepam (1 mg/kg, *i.p*.) caused significant reduction in head-dips in this study possibly due to their sedative effect. Sedative agents have been variously reported to decrease exploratory activities on the EPM and on the hole board, but did not preclude them from exhibiting anxiolytic activity (Parka et al., 2005).

The extract (1000 mg/kg, *i.p*.) significantly ($p<0.01$) reduced the rectal temperature throughout the 120 min time interval compared to the control (Figure 4). The hypothalamus has been reported to regulate body temperature, and CNS-depressants such as hypnotics, general anaesthesia and benzodiazepines normally lower body temperature at relatively low doses (Vale et al., 1999). The result obtained in this study suggests that the extract may be causing reduction in body temperature mainly through the hypothalamus which has been widely linked to body temperature regulation (Bhattacharya et al., 2003; Nagashima, 2006).

The EEPA at all doses significantly ($p<0.05$-0.01) decreased SL and increased TST induced by ketamine in a dose dependent manner (Figure 5A and B) comparable to diazepam (1 mg/kg, *i.p.*). Reduction in SL and prolongation of TST indicate sedative effect (Hellion-Ibarrola et al., 1999), which may involve enhancement of neurotransmission of GABA in the CNS (Sivam et al., 2004) by potentiating the inhibitory influence of GABA. Sedatives are known to induce deep rest in humans and it has been shown that when patients have sufficient sleep, circadian variation in blood pressure and heart rate is significantly decreased (Rowlands et al., 1980) and by inference may contribute to its hypotensive activity.

Adverse effects of available anticonvulsant drugs make treatment difficult hence the demand for new anti-convulsants is increasingly necessitated and one of the approaches to searching for new antiepileptic drugs is

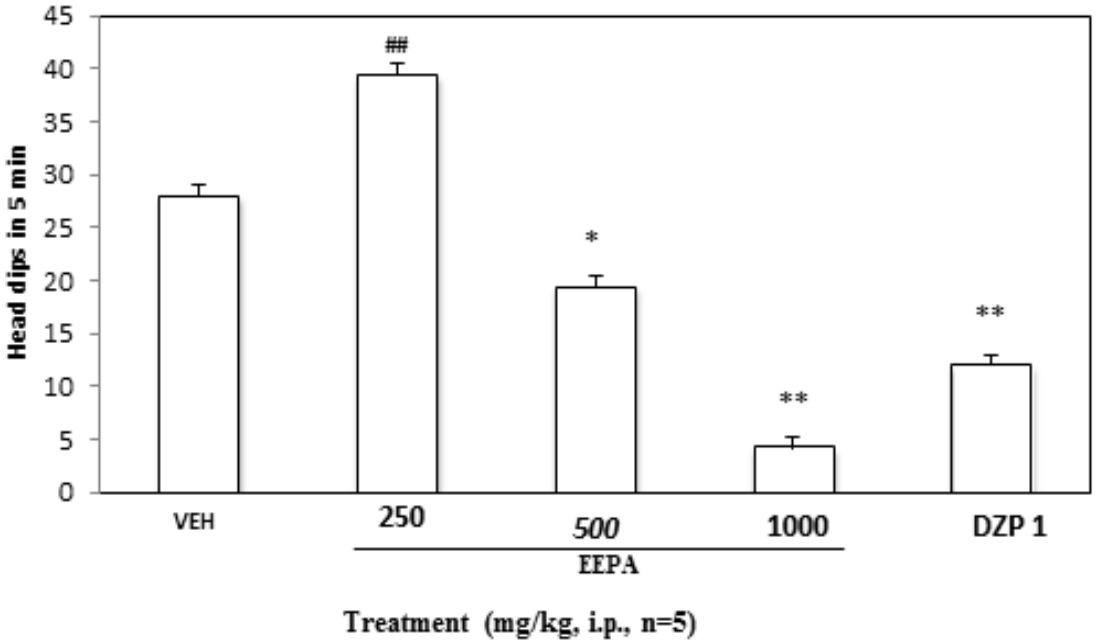

Figure 3. Effect of EEPA on head dips in mice. VEH, EEPA and DZP represent vehicle, ethanolic extract of dried seed of *P. americana* and diazepam respectively. *p < 0.05, **p<0.01; statistically lower than vehicle, ##p<0.01, statistically higher than vehicle (ANOVA, Dunnett's test)

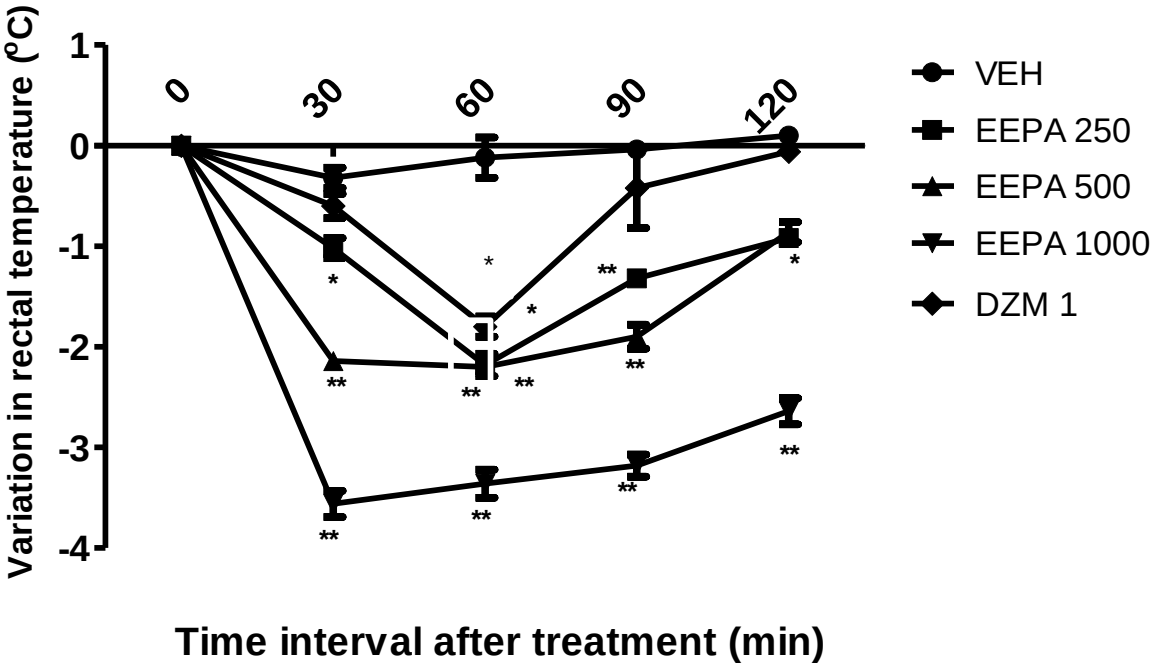

Figure 4. Effect of EEPA on normal rectal temperature of mice. Each value represents Mean±SEM. VEH, EEPA and DZM represent vehicle (5%Tween 80), ethanolic extract of dried seed of *P. americana* and diazepam respectively. N=5 for all groups. *p<0.05, **p<0.01; statistically lower than vehicle (ANOVA, Dunnett's test).

the investigation of naturally occurring compounds, thus we evaluated the effect of EEPG on seizures induced by PTZ, picrotoxin, strychnine and maximal electroshock (MES) in mice (Table 1). The results indicate that the EEPA produced significant anticonvulsant effect against PTZ, strychnine, picrotoxin and MES induced seizures. In

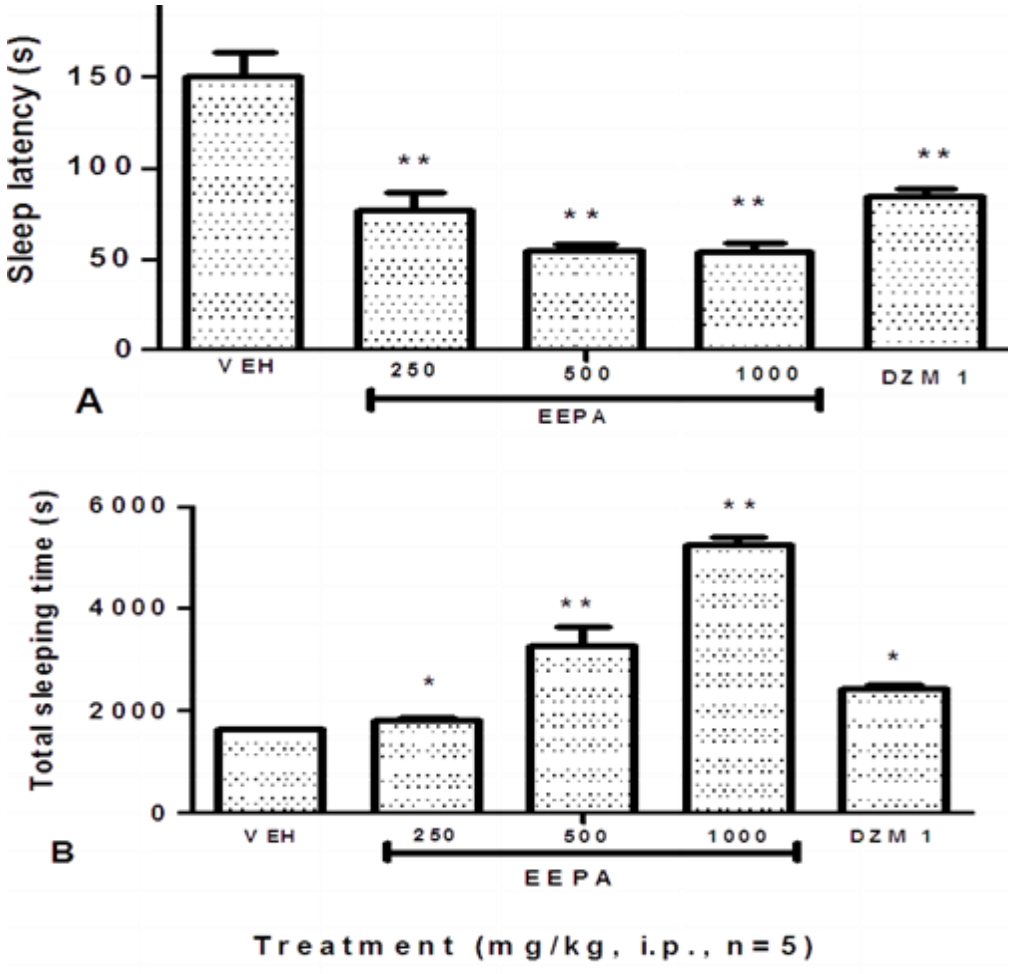

Figure 5. Effect of EEPA on Sleep Latency (panel A) and Total Sleeping Time (panel B) of ketamine-induced hypnosis in mice. Each bar represents Mean±SEM. VEH, EEPA and DZM represent vehicle (5% Tween 80), ethanolic extract of *P. americana* and diazepam respectively. *p<0.05, **p<0.01, statistically different from vehicle (ANOVA, Dunnett's test)

the PTZ model, EEPA (250, and 1000 mg/kg, *i.p*.) delayed the onset of clonic convulsions, decreased the duration of tonic convulsions and protected the mice from death comparable to the standard anticonvulsant agent, diazepam (1 mg/kg, *i.p*.). The extract at all doses used and diazepam in this study offered 100 % protection, signifying the involvement of $GABA_A$-benzodiazepine pathway in the mediation of this activity (Olatokunboh et al., 2009). PTZ-induced seizure is analogous to petit-mal type of seizures and human generalized seizures (Löscher and Schmidt, 1988). The mechanism by which PTZ is believed to exert its action is by acting as an antagonist at the $GABA_A$ receptor complex (Ramanjaneyulu et al., 1984). On the other hand, drugs that reduce T-type Ca^{2+} currents, such as ethosuximide can prevent seizures induced by PTZ (Karunakar et al., 2009). Drugs that are effective against petit-mal seizures reduce T-type calcium currents, and drugs that enhance GABAA-BZD receptor mediated neurotransmission such as benzodiazepines and phenobarbitone (McDonald and Kelly, 1995) can prevent these types of seizures. Studies have shown that activation of N-methyl-D-aspartate receptor (NMDA) is also involved in the initiation and generalization of PTZ-induced seizures (Nevis and Arnolde, 1989). The EEPA at 500 and 1000 mg/kg, *i.p.* offered 60 and 80% protections, respectively while diazepam (2 mg/kg, *i.p*.) offered 80% protection. Picrotoxin is a potent antagonist of the $GABA_A$ receptor, and it binds at the β_2/β_3 subunits of the receptor to effectively block the chloride channel, resulting in a post-synaptic neuron that is more easily excitable and prone to hyper-excitability (McDonald and Kelly, 1995). The extract profoundly delayed the onset and inhibited strychnine–induced seizures in a dose-dependent manner. The only dose of the extract that offered protection in this model provided 60% protection compared to diazepam (5

Table 1. Effect of EEPA on chemically-induced and MES convulsion tests.

Treatments (i.p.) [n = 5]	Effect of EEPA on the % protection against:			Effect of EEPA on MES-induced HLTE
	PTZ (85 mg/kg)	Picrotoxin (10 mg/kg)	Strychnine (4 mg/kg)	MES
Vehicle	0	0	0	NP
EEPA 250 mg/kg	100	0	0	NP
EEPA 500 mg/kg	100	60	0	Protected
EEPA 1000 mg/kg	100	80	60	Protected
Diazepam 1 mg/kg	100	0	0	NT
Diazepam 2 mg/kg	NT	80	0	NT
Diazepam 5 mg/kg	NT	NT	40	NT
Sodium phenytoin 25 mg/kg	NT	NT	NT	Protected

Each value represents percentage of animals that survived beyond 30 min post injection of convulsant agent. EEPA: Ethanolic dried seed extract of *P. americana*; NT: not tested; NP: not protected; PTZ: pentylene tetrazol; MES: maximal electric shock; and HLTE: hind limb tonic extension.

Table 2. Effect of EEPA on the reaction time of mice on the hot plate.

Treatment (i.p.) [n=5]	Reaction time (s), Mean±SEM after			
	30 min	60 min	90 min	120 min
Vehicle	5.68± 0.22	9.70 ±1.09	10.10±0.38	10.72±0.47
EEPA 250 mg/kg	13.26±0.37**	15.44±2.16*	12.34±0.95	9.460 ± 1.13
EEPA 500 mg/kg	16.4±1.32**	18.94±1.27**	16.38±2.92*	13.22±1.95
EEPA 1000 mg/kg	23.94±1.09**	26.72±0.84**	23.94±0.69**	18.74±0.86**
Morphine 10 mg/kg	16.40±2.18**	21.20±0.94**	25.20±1.16**	13.60±0.37

Vehicle is 5% Tween 80 and EEPA is the ethanolic extract of dried seed of *Persea americana* respectively. The reaction time is the time it takes the mouse to lick its paws. *p<0.05; **p<0.01, statistically different from vehicle (ANOVA, Dunnett's test).

mg/kg, *i.p.*), which offered 40% protection. Strychnine has been shown to act by directly antagonizing glycine at spinal cord and brainstem, thus increasing spinal reflexes (Olatokunboh et al., 2009). It could be suggested that EEPA may be exerting its anticonvulsant effect through augmentation of glycine transmission in the spinal cord and brain stem. The EEPA at 500 and 1000 mg/kg, *i.p.* successfully blocked the HLTE induced by the MES which was comparable to the standard drug (phenytoin sodium, 25 mg/kg, *i.p.*) suggesting potential antiepileptic activity against generalized tonic-clonic and partial seizures which might be acting probably through blockage of Ca^{++} or Na^{+} or both (Hegde et al., 2009). Anticonvulsant activity of the aqueous leaf extract of *P. americana* from Nigeria has been reported earlier (Ojewole and Amabeoku, 2006), suggesting that the active component(s) may be present in the leaf and seed of the plant.

The extract at all doses used significantly (p<0.01) prolonged the reaction to thermal stimulation on the hot plate test, which was comparable to morphine (10 mg/kg, *i.p.*), a standard opioid agonist (Table 2). Our findings demonstrated significant (p<0.01) activity of EEPA in the hot-plate model in a dose dependent manner which has been linked to central mediation. The pain inhibition effect of the extract, at the highest dose (1000 mg/kg, *i.p.*), was significant (p<0.01) throughout the period of observation (120 min), whereas the standard drug, morphine (10 mg/kg, *i.p.*) reached its peak effect at 90 min after which its effect became negligible. Acetic acid-induced writhing test was used to confirm the peripheral analgesic activity of the EEPA. The EEPA at all doses used significantly (p<0.01) inhibited acetic acid-induced writhes in mice (Table 3). The extract at 250, 500 and 1000 mg/kg, *i.p.* offered 33, 55 and 86% analgesia respectively compared to diclofenac's 86%, signifying peripheral analgesic activity (Silva et al., 2003). The analgesic result obtained here corroborates earlier report on the analgesic activity of the leaf extract (Adeyemi et al., 2002). Prostaglandins induce abdominal constriction by activating and sensitizing the peripheral chemo-sensitive nociceptors which are mostly responsible for causing inflammatory pain (Delevalcee et al., 1980). The results obtained from the hot plate and acetic acid-induced writhings suggested that the EEPA may mediate these anti-nociceptive activities centrally and peripherally, thus providing rationale for the ethnomedicinal use of the seed of this plant in the management of pain and rheumatism.

Further studies to isolate the active principles in this plant is imperative which can lead to discovery of novel

Table 3. Effect of EEPA on acetic acid-induced writhings in mice.

Treatment (i.p.), n=5	Number of writhes (Mean±SEM) in 30 min	% Analgesia
Vehicle	84.20±6.80	0.00
EEPA 250 mg/kg	56.40±4.11**	33.02
EEPA 500 mg/kg	38.00±2.81**	54.87
EEPA 1000 mg/kg	12.00±1.14**	85.75
Diclofenac 100 mg/kg	12.20±1.77**	85.51

Vehicle is 5% Tween 80, EEPA is ethanolic extract of *Persea americana* respectively. ***p* <0.01, statistically lower than vehicle (ANOVA, Dunnett's test).

and potent drugs that will be invaluable in mitigating myriads of diseases ravaging mankind.

Conclusion

It is concluded that major effect of the ethanolic dried seed extract of *P. americana* is depression of the central nervous system which also manifested significant hypothermic, sedative, anticonvulsant and analgesic activities in mice; thus, providing scientific basis and justification for the ethnomedicinal uses of the plant in addition to serving as a clue in the discovery of novel and useful therapeutical agent.

Conflict of interests

The authors have not declared any conflict of interest.

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