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Full Length Research Paper

Peptide hydroxamate derivatives as regulators for insulin receptor signaling and its degradation by zinc metalloprotease in diabetic rats

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Insulin-degrading enzyme (IDE) is the major zinc-metalloprotease involved in the cleavage of insulin, β-amyloid protein and mainly contributes to the pathophysiology of type 2 diabetes and Alzheimer's disease. Therefore, this enzyme is expressed as candidate target for drugs used in the management of diabetes and peptide hydroxamates have been reported recently as inhibitors for IDE. Novel synthesized peptide hydroxamic acid II containing tryptophan and a sulfonamide bond has been prepared in our laboratory. The aim of this study was to determine whether this drug could be of value in modulating diabetic states in rats. In this study, forty adult male Wistar albino rats received 20% fructose in drinking water (HFW) for six weeks to induce diabetes. Administration of the prepared compound at two dose level (5 and 10 mg/kg body weight, p.o) to diabetic rats significantly reduced IDE protein, glucagon levels, improved insulin receptor signaling (phosphorylation), insulin sensitivity and lipid profile. However, it induced certain up-regulation of IDE mRNA expression. These findings may confirm its role in the modulation of glucose homeostasis through IDE and insulin receptor signaling.

Key words: Insulin degrading enzyme, insulin receptors phosphorylation, insulin resistance, glucagon, hyperlipidemia.

INTRODUCTION

Pancreatic beta cells failure is the major contributing factor to the development of type 2 diabetes since insulin resistance of target tissues is usually associated with abnormal insulin secretion. On cellular level, the inadequate insulin signaling from insulin receptor downstream to the final substrates of insulin action may lead to multiple metabolic pathways (Iliya et al., 2016).

Insulin receptor is a hetero tetramer consisting of 2 alpha subunits and 2 beta subunits that are linked by disulphide bonds. Insulin binds to the 2 alpha subunits of insulin receptors and activates tyrosine kinase in the beta subunits. Once it is activated, tyrosine kinase promotes autophosphorylation of the beta subunits where phosphorylation of tyrosine residues is required for

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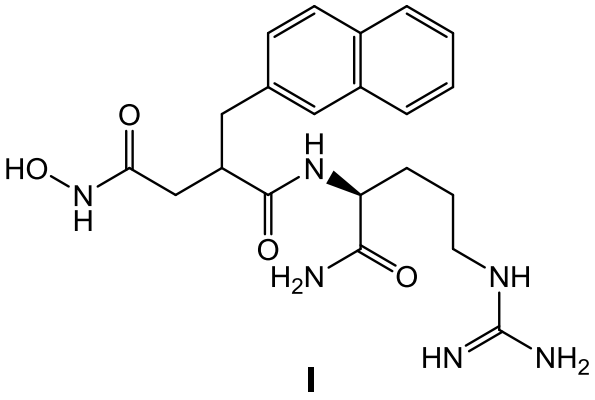
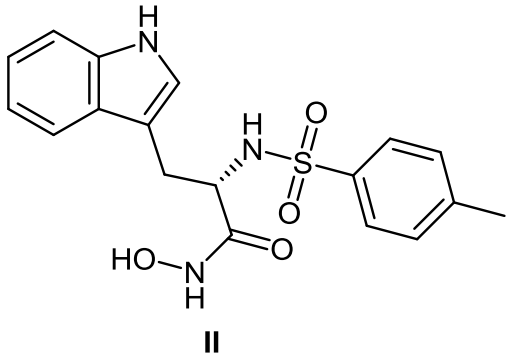


I

Figure 1. Structure of the potent and selective inhibitors of IDE.



II

Figure 2. Structure of the synthesis and antidiabetic activity of a novel peptide hydroxamic acid II.

amplification of the kinase activity. Most of the metabolic and antiapoptotic effects of insulin are mediated by the signaling pathway involving the phosphorylation of insulin receptor substrate protein and the activation of protein kinase B. In humans, rare mutation of the insulin receptor substrate-1 protein is associated with insulin resistance (Junlan and Gangjian, 2012).

Insulin-degrading enzyme (IDE) is a structurally distinctive zinc metalloprotease responsible for catabolizing insulin and other intermediate-sized peptide substrates. In principle, it should be possible to enhance the activity of insulin by inhibiting its catabolism (Deprez-Poulain et al., 2015). Thus, suitable pharmacological inhibitors of IDE may hold therapeutic value, particularly for type 2 diabetes mellitus (T2DM) and other disorders involving impaired insulin signaling (Maianti et al., 2014).

Peptide hydroxamic acids were previously reported as potent and selective inhibitors of IDE, for example, compound I showed excellent potency (ki = 2.96 ± 0.20 nM) and good selectivity (~104-fold) vis-à-vis other zinc-metalloproteases and representative member of other protease classes (Abdul-Hay et al., 2013) (Figure 1).

Herein, the synthesis and antidiabetic activity of a novel peptide hydroxamic acid II containing typtophane and a sulfonamide bond through examination of IDE gene expression and protein level as well as insulin receptor phosphorylation were reported (Figure 2).

MATERIALS AND METHODS

Chemistry

Synthesis of (S)-N-(1-(1H-benzo[d][1,2,3]triazol-1-yl)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)-4- methylbenzenesulfonamide (III)

A mixture of *p*-toluene sulfonyl chloride (0.95 g, 5 mmol) and DMAP (0.08 g, 0.65 mmol) was stirred in methylene chloride (5 ml) for 10 min. Compound II (1.8 g., 5 mmol) was dissolved in methylene chloride (5 ml) containing trimethylamine (TEA; 0.21 ml, 1.5 mmol) and the resulting solution was added to the reaction mixture. After 20 min, benzotriazole (0.72 g, 6 mmol) was added and the reaction was allowed to stir for additional 1.5 h at 25°C. Upon completion of the reaction (monitored by thin layet chromatography [TLC]), CH_2Cl_2 (50 ml) was added and the organic layer was washed with saturated Na_2CO_3 (10 ml, 3×), water (10 ml, 2×) and brine (10 ml, 1×). The organic layer was dried over anhydrous sodium sulfate and hexane (20 ml) was added. The solid separated was filtered and dried under vacuum to give the target *N*-acylbenzotriazoles (2.1 g, 92%).

Synthesis of (S)-N-hydroxy-3-(1H-indol-3-yl)-2-(4-methylphenyl-sulfonamido) propanamide (II)

To a solution of compound III (1.37.g, 3 mmol) in methylene chloride (20 ml), hydroxylamine HCl (0.42 g, 6 mmol) and TEA (0.84 ml, 6 mmol) were added. The mixture was stirred at 25°C for 1 h. The reaction was diluted with methylene chloride (40 ml), and then was washed with 6*N* HCl (3 × 10 ml), water (2 × 10 ml) and brine (1 × 10 ml). The organic layer was dried over anhydrous sodium sulfate and was filtered. The filtrate was evaporated under vacuum to afford compound II (yield 1.00 g, 90%).

Animals and ethics

Adult male Wistar albino rats (weighing 160±20 g) were housed in stainless steel cages in a controlled environment (23°C and with a 12-h light/dark cycle). All experimental procedures were performed according to the guidelines of the Animal Care and Use Committee of Zagazig University, Egypt.

Experimental design

Forty rats were randomly assigned into the following four groups, n=10, for 45 days. The first one was kept on normal rat chow diet (El-Nasr Pharmaceuticals and Chemicals Industry, Egypt), normal tap water and no treatment (Normal control group). The other three groups received normal rat chow diet, 20% fructose in drinking water (HFW) daily to induce type 2 diabetes (Mamikutty et al., 2014). It has been reported that long term fructose feeding to genetically selected albino rats result in the development of

Table 1. Primers sequence for all studied genes in the work.

Gene symbol	Primers sequence
IDE	F: 5'-GTCCTGTTGTTGGAGAGTTCCCATGTCA-3' R: 5'-GGGGAATCTTCAGAGTTTTGCAGCCAT-3'
GAPDH	F: 5'-ACCACAGTCCATGCCATCAC-3' R: 5'-TCCACCACCCTGTTGCTGTA-3'

diabetes mellitus and glomerulosclerosis [6]. One of these groups received no treatment and served as diabetic control group, while the others were co-treated orally with the synthesized peptide hydroxamic acid II containing typtophane and a sulfonamide bond in dose levels of 5 and 10 mg/kg body weight/day (Hydroxamic acid 5 group, Hydroxamic acid 10 group) for 45 days. Selection was based on a pilot study conducted in our laboratory where they produced optimal effects on the selected biomarkers without side effects. However, a higher dose of 15 mg/kg body weight/day, p.o produced deleterious effects and marked mortality.

Blood and tissue sampling

The rats were fasted overnight and supplemented with only tap water, blood samples were obtained at the baseline and at end of the experiment *via* orbital vein of anaesthetized rats. Animals were weighed before collection of blood samples. Blood was centrifuged at 4500 rpm and serum samples were immediately analyzed for glucose. The remaining serum was frozen at -80°C and stored for further analysis of lipid profile, insulin and glucagon. Rats were then killed by decapitation and the liver was excised, washed with cold saline, kept in liquid nitrogen and stored at -80°C for measurement of IDE gene expression and protein level as well as insulin receptor phosphorylation in cell lysate..

Biochemical measurements

Colorimetric kits (Spinreact, Spain) were used for determination of glucose, total cholesterol (TC), triacylglycerol (TAG) and high density lipoprotein cholesterol (HDL-C) levels. Low density lipoprotein cholesterol (LDL-C) was calculated from Friedewald formula: LDL-C= TC-(TAG/5+HDL-C). Enzyme-linked immunosorbent assay (ELISA) kits for rat insulin, glucagon, insulin receptor phosphorylation, and IDE were purchased from SPI Bio, Montigny Le Bretonneux, France; CUSABIO, China; Sigma Aldrich, MO, USA and Cloud-Clone Crop, assembled by USCN Life Science Inc., Wuhan, China, respectively. All biochemical analyses were done following the manufacturer's protocols.

Quantitative real time polymerase chain reaction (q-PCR) for IDE

Total RNA was isolated from the livers of animals in the studied groups using Qiagen tissue extraction kit (Qiagen, USA) and RNA was converted into cDNA using high capacity cDNA reverse transcription kit (Fermentas, USA). The primers sequence for IDE and GAPDH genes are shown in Table 1. The cDNA was subjected to amplification and analysis using the TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA) for quantitative RT-PCR. GAPDH was used as the reference gene. The assay was run on Step One™ Real-Time PCR Systemversion 3.1 (Applied Biosystems, USA) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was done using the InStat 2.04 statistical package (Graph Pad InStat). Data are presented as means ± standard deviation (SD) for six rats/group. Student's t test was used to compare means between each two groups. $P<0.05$ was considered to be statistically significant.

RESULTS

Chemistry

The target compound II was prepared according to the synthetic pathway, which is depicted in scheme 1. L-Tryptophan 1 was sulphonylated using tosyl chloride in the presence of potassium carbonate to give *N*-tosyl-L-tryptophan (2). Compound 2 was converted to its corresponding benzotriazolide 3 (90% yield) via reaction with 1*H*-benzotriazole and tosyl chloride in the presence of equivalent amount of TEA (Agha et al., 2016). Reaction of 3 with hydroxylamine HCl in the presence of TEA afforded the target compound II in 95% yield. Intermediates 2 and 3 as well as the target II were characterized by ^{1}HNMR, ^{13}CNMR, and elemental analysis.

Biochemical markers

Intake of fructose solution (HFW, 20%) induced higher adiposity, hyperglycemia, dyslipidemia, and insulin resistance. Serum glucagon, hepatic content of IDE showed significant increase along with down-regulation of IDE gene expression and a significant decrease of insulin receptor phosphorylation. Concomitant administration of hydroxamic acid peptide II at dose levels (low and high) greatly ameliorated these changes by improving insulin receptor phosphorylation and insulin sensitivity as well as IDE reduction (Tables 2 and 3). Unexpectedly, IDE mRNA level was significantly increased (Figure 3).

DISCUSSION

Rats fed on HFW in the current study developed a state of metabolic syndrome as manifested by hyperglycemia, dyslipidemia, and body weight gain (obesity). The excess

Table 2. Effect of peptide hydroxamic acid II derivative (5 or 10 mg/kg bw, p.o) daily administration along with (HFW, 20%) for six weeks on rats body weight, serum glucose and lipid profile.

Parameter	Normal control group	Diabetic control group	Peptide hydroxamic acid II derivative 5 group	Peptide hydroxamic acid II derivative 10 group
Body weight (gm)	253.6 ± 20.42	350 ± 17.03#	242.2 ± 12.42*	270 ± 13.04*
Glucose (mg/dl)	87 ± 6.5	213.4 ± 15#	76.8 ± 5.9*	81.2 ± 9.8*
TC (mg/dl)	74.2 ± 8.2	167.4 ± 2.2#	69 ± 3.48*	108.4 ± 2.7*
TAG (mg/dl)	85 ± 10.8	147.3 ± 2.2#	86.8 ± 14.8*	72.82 ± 16.29*
HDL-C (mg/dl)	41.4 ± 9	26.12 ± 2#	54.8 ± 7*	66.2 ± 4.8*
LDL-C (mg/dl)	34.92 ± 6.23	97.62 ± 18.33#	12.13± 1.11*	27.64 ± 2.1*

#Significantly different compared to normal control group at P<0.05. *Significantly different compared to diabetic control group at P<0.05.

Table 3. Effect of peptide hydroxamic acid II derivative (5 or 10 mg/kg body weight, p.o) daily administration along with (HFW, 20%) intake for six weeks on serum insulin, glucagon, hepatic insulin receptor phosphorylation and IDE.

Parameter	Normal control group	Diabetic control group	Peptide hydroxamic acid II derivative 5 group	Peptide hydroxamic acid II derivative 10 group
Insulin (µIU/ml)	2.12 ± 0.25	30 ± 2.58#	4.2 ± 0.43*	2.54 ± 0.27*
Glucagon (pg/ml)	1.8 ± 0.25	23.2 ± 1.47#	2.68 ± 0.28*	2.18 ± 0.19*
hepatic cell lysate Insulin receptor phosphorylation (µg/ml)	29.3 ± 2	1.58 ± 0.14#	20.78 ±0.93*	25.3 ± 1.4*
Liver IDE (pg/g tissue)	1.97 ± 0.1	40.1 ± 3.8#	3.19 ± 0.3*	2.18 ± 0.16*

#Significantly different compared to normal control group at P<0.05. *Significantly different compared to diabetic control group at P<0.05.

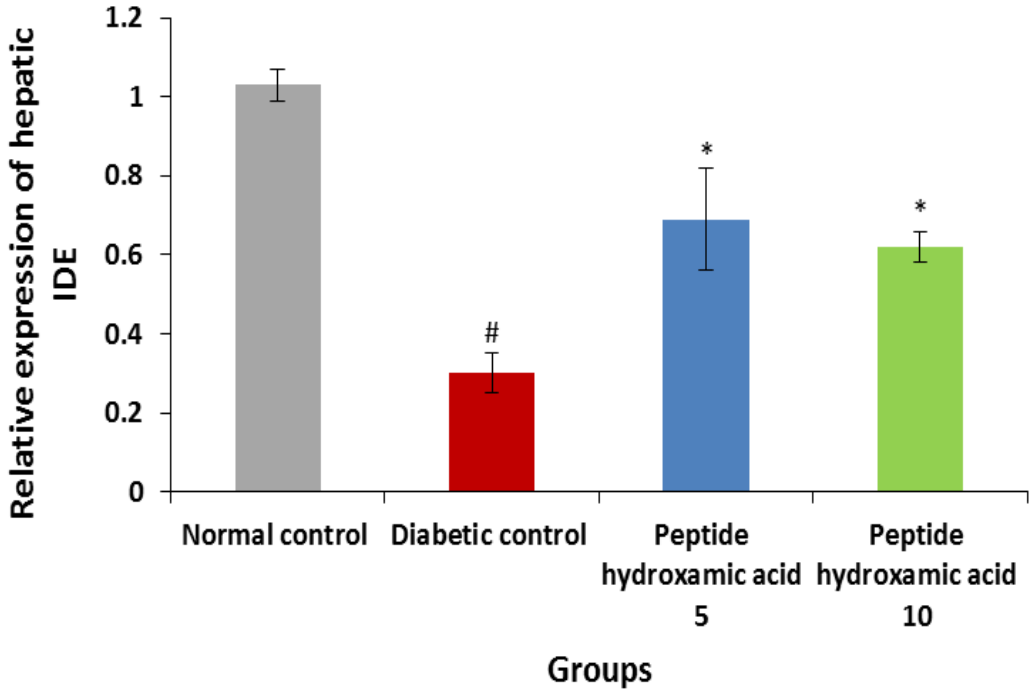


Figure 3. Effect of hydroxamic acid II derivative (5 or 10 mg/kg bw, p.o) daily administration along with (HFW, 20%) intake for six weeks on rats hepatic IDE relative expression. #Significantly different compared to normal control group at P< 0.05. *Significantly different compared to diabetic control group at P< 0.05.

calorie intake here is finally stored in the abdominal adipose tissues as TAG (Karamohamed et al., 2003) leading to the development of dyslipidemia and hyperglycemia. Adipose tissue usually acts as an endocrine organ which involves the metabolism of glucocorticoid hormone where deregulation of glucocorticoid metabolism as reported earlier may lead to obesity, dyslipidemia, hypertension, and diabetes (Kershaw and Flier, 2004).

It is generally known that sensitivity of insulin is reduced with the presence of TAG, inducing a reduction of glucose uptake by the insulin sensitive tissues leading to activation of lipolysis process, more free fatty acids, glycerol formation indeed TAG accumulation within the adipose tissue (Paschos and Paletas, 2009). These viscous cycles are repeated and more TAG is formed, leading to a hypertriglyceridemic state along with insulin resistance (elevated glucose and insulin).

Patients suffering from insulin resistance and type 2 diabetes frequently display signs of abnormal lipid metabolism, increased circulatory concentration, and elevated deposition of lipids in the skeletal muscles (McGarry, 2001). Increase in plasma free fatty acids reduces insulin-stimulated glucose uptake, whereas a decrease in plasma lipid content improves insulin activity in the skeletal muscle cells, adipocytes, and liver (Moller, 2001). Studies have shown that raising plasma fatty acids in both rodents (Kim et al., 2004) and humans (Dresner et al., 1999) abolishes insulin activation of insulin receptor substrate-1 associated PI3-kinase activity in skeletal muscles where insulin receptor substrate-1 is the most prevalent lipid associated insulin resistance and has been shown to be linked to GLUT4 translocation defects (Pessin et al., 1999).

Hyperglycemia observed here may be attributed to the fact that unlike glucose, fructose does not stimulate insulin secretion from pancreatic β-cell (Bray et al., 2004); additionally, the reduction of insulin sensitivity during hypertriglyceridemia may induce hyperglycemia. The present study demonstrated an increase of liver IDE protein level in rats fed on HFW (20%). This may be attributed to a direct response for a negative feedback mechanism due to an alteration of circulated insulin. However, there is no sufficient information about IDE regulation in obesity (Duckworth et al., 1998; Hulse et al., 2009). Inactivation of IDE by gene knockout induced hyperinsulinemia and insulin resistance in mice as previously reported may add further support (Wei et al., 2014). Increased glucagon level recorded in the diabetic rats may induce IDE protein as previously reported (Wei et al., 2014).

Probable biochemical effects of our compound may be mediated through different mechanisms. The first one is through enhancing glucose uptake by liver, muscle, and adipose tissue; although, this is not done in most studies. The second one may be attributed to parathyroid hormone (PTH) role where several studies indicated that PTH may elicit insulin resistance by reducing the number of glucose transporter available in cell membranes responsible for glucose uptake (Sung et al., 2012; Teegarden and Donkin, 2009). Additionally, it suppresses insulin release (Perna et al., 1990) and promotes insulin resistance in adipocytes (Teegarden and Donkin, 2009).

It may have a beneficial effect on insulin action either directly by stimulating the expression of insulin receptor, enhancing in turn insulin responsiveness for glucose transport or indirectly *via* its role in regulating extracellular calcium (Maestro et al., 2000), ensuring its influx through cell membranes and an adequate intracellular calcium pool because calcium is essential for insulin-mediated intracellular processes in insulin responsive tissues such as skeletal muscles and adipose tissues (Williams et al., 1990).

Generally, insulinemia is a result of the balance between the insulin produced and secreted by pancreatic islets β-cells and insulin removal from the plasma (insulin clearance) (Duckworth et al., 1998). Insulin clearance is fundamentally controlled by the liver and more than 50% of insulin secreted is removed by the liver after the second passage through the portal vein (Butterfield, 1970; Kotronen et al., 2008; Mittelman et al., 2000). Hepatic removal and degradation of insulin is mainly controlled by IDE (Amata et al., 2009; Duckworth et al., 1998). Every insulin-responsive cell expresses IDE mainly to oppose insulin signaling by uncoupling insulin from insulin receptor (IR) by removing or degrading insulin (Amata et al., 2009). Accordingly, hepatic IDE inhibition increased insulin sensitivity coupled with increased IR activity (Li et al., 2002; Leissring et al., 2010). Therefore, IDE expression and its action have certain role in glucose hemostasis, since IDE reduces insulinemia by activation or increasing insulin removal in the liver. Additionally, it reduces insulin sensitivity by interrupting the IR signaling pathway.

Many studies indicated that IDE expression and insulin clearance are reduced in diabetic individuals especially those having type 2 diabetes (Karamohamed et al., 2003; Kotronen et al., 2008; Slominskiĭ et al., 2009) and also in diabetic and obese rodents (Pessin et al., 1999; Moller, 2001; Matveyenko et al., 2008).

Taken in consideration that IDE expression and activity represent the major contributing factors to alterations in insulin clearance; the latter can also be affected by certain factors like the changes in renal blood flow or other physiological conditions (Duckworth et al., 1998). Finally, the observed decrease in hepatic IDE expression in the present study is mostly attributed to hypertriglyceridemia produced in subsequent to high-fat diet (HFD) intake since TAG and non esterified fatty acids are inhibitors for insulin clearance and hepatic IDE expression (Hamel et al., 2003; Kotronen et al., 2008; Yoshii et al., 2006). Another explanation may be through certain cytokines like interleukin-6 (IL-6) which are released by oversized adipose tissue might control insulin

clearance and hepatic IDE expression (Rezende et al., 2012).

Conclusion

Rats received HFD developed insulin resistance, hyperglycemia, and dyslipidemia. Hydroxamic acid peptide administration improved such metabolic disturbances as manifested by an inhibition of hepatic IDE level, increased insulin sensitivity coupled with increased insulin receptor phosphorylation.

Conflict of interests

The authors have not declared any conflict of interests.

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GC-MS analysis of esterified fatty acids obtained from leaves and seeds of *Triplaris gardneriana* Wedd.

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***Triplaris gardneriana* Wedd. belongs to the family Polygonaceae. In Brazil, the plant is known as "Pajéu". The plant has a traditional use in folk medicine for the treatment of some human diseases. In this study, it was realized for first time, the extraction and characterization by gas chromatography–mass spectrometry (GC-MS) of the fixed oils from the leaves and seeds of *T. gardneriana* collected at different times and it was evaluated the antioxidant and antibacterial activities. The esters were identified by comparing the mass spectra obtained with those of the equipment database. The antioxidant activity was evaluated by the methods of radical scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) and co-oxidation of β-carotene against linoleic acid. The antibacterial effect was evaluated by the microdilution broth method. In the fixed oil of the leaves were identified 22 compounds, totaling 61.53%. Methyl palmitate (15.14%) and methyl oleate (14.35%) were the majoritary compounds. For the sample of the seeds fixed oil, 13 compounds were identified, representing 97.81%. Among these, 10-Octadecenoic acid, methyl ester (45.53%) was identified as majoritary constituent. The oils do not show antioxidant activity, but showed moderate antibacterial activity. The fatty acid composition of the fixed oils showed differences, noting a greater variety of constituents in the leaf oil. The presence of these compounds in the studied plant is important phytochemically because it contributes to the chemical and pharmacological knowledge of this specie.**

Key words: Polygonaceae, *Triplaris gardneriana*, fixed oils, esters, Caatinga, antioxidant activity, antibacterial activity.

INTRODUCTION

The Polygonaceae family comprises 51 genera and about 1100 species, widely distributed in tropical and temperate regions. The plants belonging to this family are known to produce a large number of biologically important molecules such as alkaloids, benzenoids, carotenoids, coumarins, depsídeos, stilbenes,

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phenylpropanoids, flavonoids, lactones, lignans, quinoid, tannins, terpenoids, and xanthones. In Brazil, seven genera are of spontaneous occurrence, among them is *Triplaris*, which comprises approximately 20 species in South and Central America (Oliveira et al., 2008).

Phytochemical studies on species of genus *Triplaris* have identified the presence triterpenes, amide, phenylpropanoid glycoside, benzenoid, and flavonols simple and glycoside (Oliveira et al., 2008; Hussein et al., 2005; Macedo et al., 2015). In the folk medicine, the species are used in the treatment of malaria diarrhea, dysentery, stomach pain, enteritis, fever, sores, inflammation of the throat, skin lesions induced by leishmaniasis, linfatites, measles, cough, intestinal worms and is also used as an energetic exciting and hallucinogen (Oliveira et al., 2008). Plants of this genus showed activities such as antioxidant, cytotoxicity against the human cancer cell lines, anticholinesterase, anti-HIV, larvicidal, antimicrobial, leishmanicidal, immunomodulatory, agglutinating, antiplasmodial, antimalarial, anti-inflammatory, stimulating smooth muscle and molluscicidal activity (Schertz et al., 1960; Tan et al., 1991; Deharo et al., 2004; Estevez et al., 2007; Camones et al., 2010).

Triplaris gardneriana Wedd. is a tree that belongs to the family Polygonaceae and occurs only in the biome Caatinga and Pantanal. In brazilian northeast this specie is known as "Pajéu" and occurs in the riparian forest of the Rio São Francisco. It is used in folk medicine to treat bleeding hemorrhoids, cancer, gastritis, ulcers, cough, pain, heartburn, flu, rheumatism, bronchitis, leucorrhoea, gonorrhea and inflammation of internal organs (Cartaxo et al., 2010; Pereira et al., 2014). However, few studies of biological activities are reported for specie, only, moluscicide, stimulating smooth muscle and toxicity activities (Souza and Rouquayrol, 1974; Barros et al., 1970). Recent studies of seed extracts of *T. gardneriana* showed antibacterial, antioxidant and anticholinesterase activities (Farias et al., 2013) and extracts of the leaves showed antioxidant and photoprotective activities (Macedo et al., 2015).

Phytochemical study with extracts from the wood of this specie revealed the presence of aliphatic hydrocarbon, sitosterol, ferulatos (Braz Filho and Rodrigues, 1974) and betulinic acid. Another study on this plant, identified the volatile constituents of the essential oil of fruits, the identification of the constituents was performed by gas chromatography-mass spectrometry (GC-MS) (Carneiro et al., 2010). Recently, flavonol glycosides were identified in extracts of leaves using liquid chromatography-mass spectrometry (LC-MS) (Macedo et al., 2015).

However, no study by GC-MS identified the constituents present in the fixed oil of the specie. The fixed oils are mixtures of lipidic substances insoluble in water and soluble in nonpolar organic substances, are constituted, mainly, by saturated and unsaturated fatty acids (Matos et al., 2015). These oils have immense value for cosmetic application by the properties emollient and researches of biological acctivity reveal insecticidal potential, anti-inflammatory, laxative, antiedematogenic, fungicide and larvicidal for these compounds (Souza et al., 2006; Pereira et al., 2008; Saraiva et al., 2011; Matos et al., 2015). In this way, fixed oils characterization becomes a complementary on phytochemical characterization of the species under study.

The chemical composition of plants, as those found in fixed oils, can be influenced by many factors, and among them we can mention genetics and heredity in terms of secondary metabolites, the morphogenetic variability and ontogenetic, which is the content of the difference active substances in the different parts of the plant and during the stages of development, in addition to environmental influences, such as weather, temperature and other factors (Pereira et al., 2005; Emara and Shalaby, 2011).

The aim of this study was to investigate the phytoconstituents present in the fixed oil by GC-MS from the leaves and seeds of *T. gardneriana* collected at different times and to evaluate the antioxidant and antibacterial activities.

MATERIALS AND METHODS

Plant material

The leaves were collected in the city of Santa Maria of Boa Vista in the state of Pernambuco, Brazil in July 2013, located at 349 m elevation (08°47'59, 00 S, 039°50'42, 40 W). The botanical identity of the plant was confirmed by specialist Diogo de Oliveira Gallo. A voucher specimen of the plant was deposited in the Herbarium of the Federal University of San Francisco Valley (HVASF) under registration 21221. The seeds were obtained in the Seed Laboratory of the Reference Center for the Recuperation of the Degraded Areas of the Caatinga, registered under the LAS number 818.

Extraction

The extraction of fixed oils of the leaves and seeds of *T. gardneriana* was performed through the extraction in a Soxhlet apparatus. Aliquots of 37 g of leaves and 59 g of seed powder were placed, separately inside the apparatus containing 250 ml of hexane and heated electric blanket for two hours. Then, the mixture of oil/solvent was evaporated in a rotary evaporator to yield the crude fixed oils. The oils were weighed and their percentages were calculated based on the dry weight of the botanic material.

Saponification

The methodology used with some adaptations, was described by Matos et al. (1992). For esterification, the oils were saponified by being refluxed in 50 ml of methanol containing 1.0 g of KOH, for 30 minutes. Methanol was distilled to reduce the volume and then the volume was completed to 50 ml of water. The unsaponifiable alkaline solution was extracted with ethyl ether mixture.

Methylation of saponified fraction

The aqueous alkaline solutions were acidified to pH 2 with 10%

hydrochloric acid and the fatty acids extracted with ethyl ether. Subsequently, water was removed with anhydrous sodium sulfate and the ether distilled off. The methylation of the fatty acids was carried out by refluxing the samples for 2 min with 3 drops of concentrated hydrochloric acid in 5 ml of anhydrous methanol. The methyl esters were extracted with hexane after the addition of 10 ml of water and then the same was removed of solution with anhydrous sodium sulfate, followed by filtration.

GC-MS analysis

The substances present in the fixed oil of *T. gardneriana* were investigated on a Shimadzu QP-2010 -MS. The following conditions were used: ZB-5MS column Phenomenex Zebron (30 m x 0.25 mm x 0.25 mm); helium (99.999%) carrier gas at a constant flow of 1.1 ml/min; 1µl injection volume; injector split ratio of 1:40; injector temperature 240°C; electron impact mode at 70 eV; ion-source temperature 280°C. The oven temperature was programmed at 100°C (isothermal for 5 min), with an increase of 10°C/min to 250°C and 10°C/min to 280C. A mixture of linear hydrocarbons (C_9H_{20}–$C_{40}H_{82}$) was injected under the same experimental conditions as samples, and identification of the constituents was performed by comparing the mass spectra obtained with those of the equipments database (Wiley 7 lib and Nist 08 lib).

Evaluation of antioxidant activity

DPPH free radical scavenging assay

The free radical scavenging activity was measured using 2,2-diphenyl-1-picryl-hydrazyl ($DPPH^{\cdot}$) (Mensor et al., 2001; Almeida et al., 2011). The absorbance values were measured at 518 nm and converted into the percentage Antioxidant Activity (AA) using the following equation:

$$\% AA = [(AC - AA) / AC] \times 100$$

Where AC is absorbance of the control and AA is sample absorbance. Ethanol (1.0 ml), with solutions of the fixed oils (2.5 ml), was used as a blank. DPPH solution (1.0 ml), with ethanol (2.5 ml), was used as a negative control. The positive controls (ascorbic acid, BHA and BHT) were used as standard solutions. The values were expressed as mean ± standard deviation (SD). Assays were carried in triplicate.

Inhibition of auto oxidation of β-carotene

The β-carotene bleaching method is based on the loss of the yellow color of β-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion (Wannes et al., 2010). Ascorbic acid, BHT and BHA standards and the fixed oils were used as positive control. In the negative control, the fixed oils were substituted with an equal volume of ethanol. The absorbance was measured immediately at 470 nm. The antioxidant activity (AA%) was evaluated in terms of bleaching of β-carotene using the following formula:

$$AA\% = [1 - (A_0 - A_t) / (A_0^0 - A^0_t)] \times 100$$

Where A_0 is the initial absorbance and A_t is the final absorbance measured for the test sample, A_0^0 is the initial absorbance and A_t^0 is the final absorbance measured for the negative control (blank). The results are expressed as percentage of antioxidant activity (% AA). Tests were carried out in triplicate.

Evaluation of antibacterial activity

The antibacterial effect of the fixed oils was evaluated by microdilution broth method (CLSI, 2006). In the evaluation of antibacterial activity of the following standard strains from the American Type Culture Collection (ATCC) the following were tested: *Enterococcus faecalis* (ATCC 19433), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Salmonella enterica* (ATCC 10708), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228) and *S. aureus* resistant methicillin (MRSA).

The fixed oils of the seeds was diluted in 10 ml of ethanol 95% and the fixed oils of the leaves was diluted in pure DMSO (dimethyl sulfoxide) and methanol (1:1) to obtain the solution at concentration of 25 mg/ml. In the preparation of the inoculum, colonies obtained in Mueller-Hinton agar were used for the preparation of bacterial suspension in a 0.085% saline solution with turbidity equivalent to the tube 0,5 of the MacFarland scale. The suspension was inoculated, 100 µl in tubes containing 9.9 ml of Mueller-Hinton broth. For determination of minimum bactericidal concentration (MBC), 200 µl of Mueller-Hinton broth were distributed into 96-well microplates. Then, 200 µl of solution of samples were added to the first well and, after homogenization, transferred to the second and so forth; the following final concentrations were achieved: 12500; 6250; 3125; 1562.5; 781.2; 390.6; 195.3 and 97.6 µg/ml. Then, 10 µl of bacterial suspension were placed in 96-wells of microplate containing the sample dilution, the material was incubated at 37 °C for 24 hours. Using a replicator, aliquots were removed from the microplates after the incubation and plated on Petri plates containing Mueller-Hinton agar, followed by incubation for 24 h at 37°C. MBC was defined as the lowest concentration of the extract able to cause the death of bacteria. Thus, we considered only the results of the minimum bactericidal concentration (MBC). Tests were carried out in triplicate.

RESULTS AND DISCUSSION

After of the extraction, the yield of the fixed oils obtained from the leaves and seeds were 1.5 and 0.8%, respectively. The total ion chromatograms (TIC) are showed in Figure 1 and 2. In the Tables 1 and 2, a list of the identified compounds and their quantification in the fixed oils are presented according to their retention times and total peak area (%) of each of methylated fatty acids. The product obtained from the transesterification is showed in GC-MS analysis total ion chromatogram (TIC) in Figure 1, in which one observes the presence of 38 peaks in the chromatogram of the leaves and 15 peaks in the chromatogram seeds (Figure 2), whose comparison with the corresponding spectra of the device library allowed the identification of 22 constituents of the fixed oil of leaves totaling 61.53% (Table 1) and 13 constituents of the fixed oil from the seeds representing 97.81% (Table 2).

The chemical composition of the fixed oil of the leaves (Table 1), with fatty acids, hydrocarbons, steroids and a triterpene, was more diverse with respect to the fixed oil of the seeds (Table 2). Comparing the chemical composition of the fixed oils, it is observed that nine esterified fatty acids methyl palmitate, palmitic acid, methyl linoleate, methyl stearate, methyl 9,11-octadecadienoate, methyl behenate, methyl cerotate, *cis*-

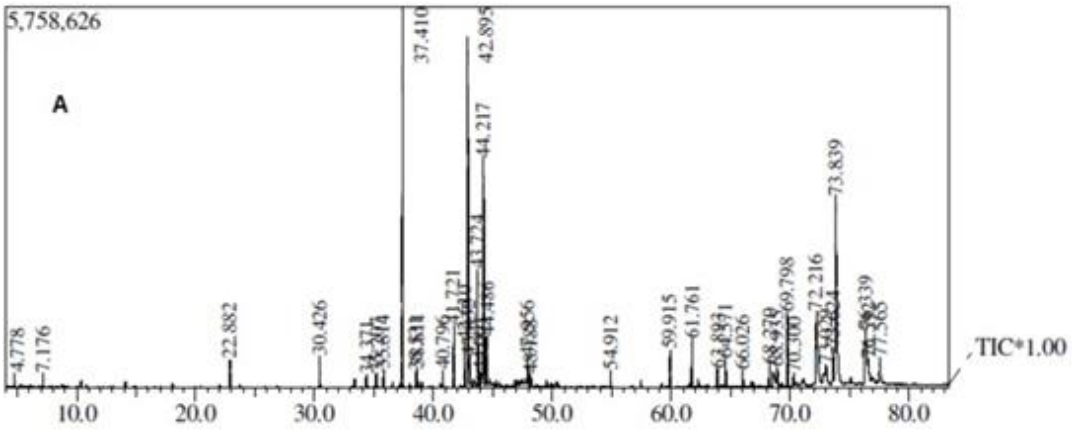

Figure 1. TIC of chemical constituents of the fixed oil from the leaves of *T. gardneriana.*

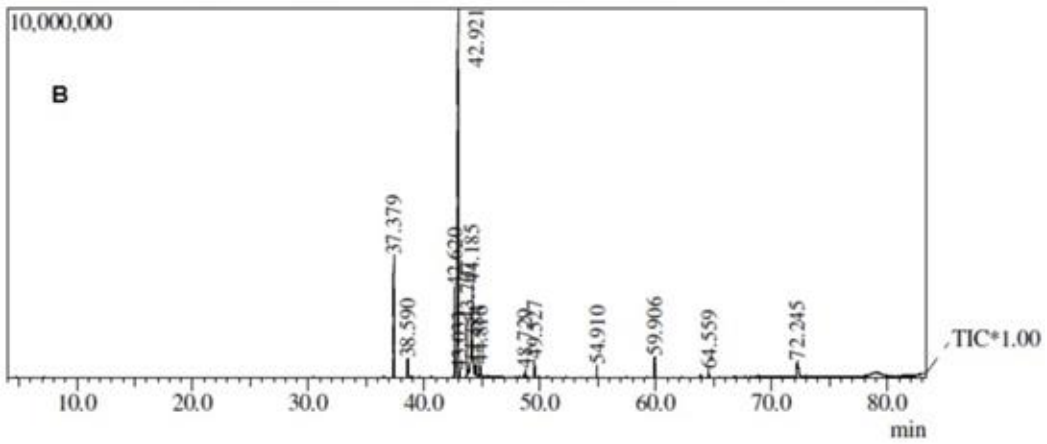

Figure 2. TIC of chemical constituents of the fixed oil from the seeds of *T. gardneriana.*

Vaccenic acid and methyl lignocerate are present both in the leaf and seed oil. The β-sitosterol was also observed in both samples of fixed oil. It was found that the content of unsaturated fatty acids in the fixed oil of leaves was only 18.77%, while 71.1% of the methyl esters identified in the seeds oil were derived from unsaturated fatty acids.

The results obtained from the identification of compounds present in fixed oils demonstrated difference in secondary metabolites as function of the different parts of the plant and the stages of development, in addition to environmental influences. The major chemical constituents of the leaves oil were methyl palmitate (15.14%), methyl oleate (14.35%). In the oil of the seeds was 10-Octadecenoic acid, methyl ester also known as methyl 10-octadecenoate (45.53%). It is found that methyl palmitate and β-sitosterol appear principally in both fixed oils analyzed. The mass spectra of methyl palmitate are shown in Figure 3 and methyl octadec-10-enoate in Figure 4.

The major compounds from the oil of the leaves and seeds, palmitate methyl (15.14%) and methyl 10-octadecenoate (45.53%), respectively, were identified in the essential oil of the fruits of *T. gardneriana* (Carneiro et al., 2010). In the fruit oil, the constituents methyl palmitate (21.67%) and methyl 10-octadecenoate (21.72%) were also the majority. It is notable that the fixed oil from the leaves showed the double of amount of methyl 10-octadecenoate compared with the oil obtained from the fruits.

The analysis presented a remarkable difference in chemical composition between fixed oils, since the leaf oil presented greater variation, and the concentration of saturated fatty acids was higher than unsaturated. There are several factors that can influence their presence, and among them we can mention the difference in the content of substances in different parts of the plant during the stages of its development and can also be related to

Table 1. Chemical constituents of the fixed oil from the leaves of *T. gardneriana.*

Peak	RT (min)	Compound	(%) GC-MS
1	4.778	NI	0.24
2	7.176	Undecane	0.30
3	22.882	Methyl laurate (C12:0)	0.89
4	30.426	Methyl myristate (C14:0)	1.05
5	34.371	NI	0.39
6	35.207	1-Octadecyne	0.39
7	35.207	NI	0.52
8	37.410	Methyl palmitate (C16:0)	15.14
9	38.531	Palmitic acid (C16:0)	0.44
10	38.611	NI	0.58
11	40.796	NI	0.53
12	41.721	NI	2.16
13	42.610	Methyl linoleate (C18:2)	1.59
14	42.895	Methyl oleate (C18:1)	14.35
15	43.032	Methyl Octadec-13-enoate (C18:1)	0.87
16	43.724	Methyl stearate (C18:0)	4.35
17	44.001	cis-Vaccenic acid	0.26
18	44.217	NI	10.31
19	44.486	Methyl 9,11-octadecadienoate (C18:2)	1.70
20	47.956	Methyl linolenate (C18:3)	1.00
21	48.188	NI	0.31
22	54.912	Methyl behenate (C22:0)	0.54
23	59.915	Methyl lignocerate (C24:0)	1.30
24	61.761	Squalene	1.87
25	63.893	Tetratetracontane	0.63
26	64.571	Methyl Cerotate (C26:0)	1.05
27	66.026	NI	0.42
28	68.270	NI	0.78
29	68.935	Methyl octacosanoate (28:0)	0.61
30	69.798	NI	3.02
31	70.300	NI	0.38
32	72.216	β-Sitosterol	6.80
33	73.029	Methyl melissicate (30:0)	0.29
34	73.624	NI	1.54
35	73.839	NI	13.96
36	76.339	β - friedelanol	6.10
37	76.592	NI	2.03
38	77.565	NI	1.30
Total	**-**	**-**	**61.53**

RT= Retention time; NI = not identified.

seasonal issues, as the plant source used in each experiment was acquired in different times and places (Nazifi et al., 2008; Emara and Shalaby, 2011).

The methyl oleate known as oleic acid, one constituent of the leaf fixed oil is an unsaturated long chain of fatty acid with 18 carbons in its structure. It is known as omega 9 essential, which participates in the metabolism in the synthesis of hormones. The methyl linoleate known as linoleic acid is a component of the two fixed oils analyzed. It is considered an omega 6, which is essential for normal cell function (Lehninger et al., 2011). Among these characteristics, oleic and linoleic acid also have the ability to reduce cholesterol levels in the blood (Erdogan et al*.,* 2014).

The fixed oils showed no antioxidant activity in any of the tested methods (Table 3). The negative outcome of

Table 2. Chemical constituents of the fixed oil from the seeds of *T. gardneriana*.

Peak	RT (min)	Compound	(%) GC-MS
1	37.379	Methyl palmitate (C16:0)	10.98
2	38.590	Palmitic acid (C16:0)	1.74
3	42.620	Methyl linoleate (C18:2)	8.57
4	42.921	Methyl Octadec-10-enoate (C18:1)	45.53
5	43.033	Methyl elaidate (C18:1)	0.58
6	43.707	Methyl stearate (C18:0)	4.66
7	44.185	cis-Vaccenic acid	15.57
8	44.484	Methyl 9,11-octadecadienoate (C18:2)	0.81
9	44.816	Stearic acid (C18:0)	1.45
10	48.729	NI	0.62
11	49.527	NI	1.57
12	54.910	Methyl behenate (C22:0)	0.99
13	59.906	Methyl lignocerate (C24:0)	1.86
14	64.559	Methyl Cerotate (C26:0)	0.53
15	72.245	β-Sitosterol	4.54
Total	-	-	**97.81**

RT = Retention time; NI = not identified.

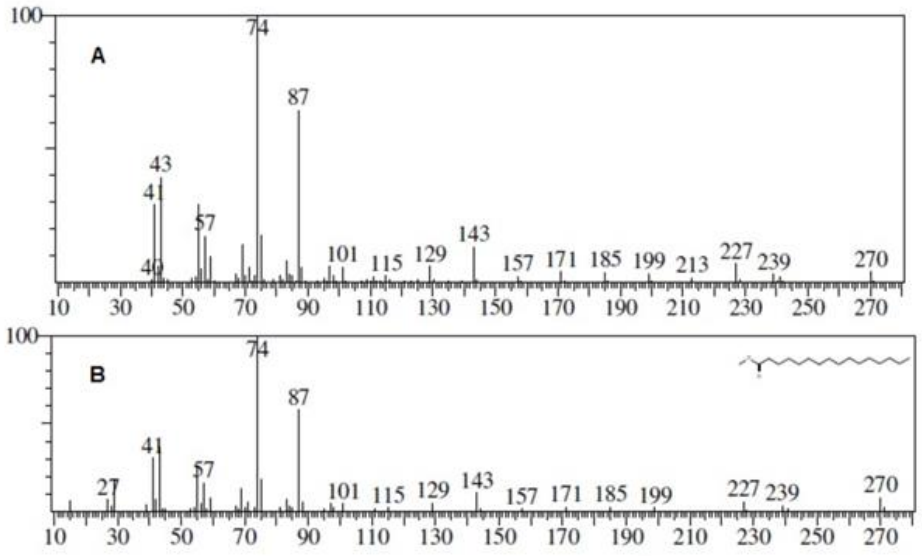

Figure 3. (A) Mass spectrum of compound methyl palmitate (15.14%) present mainly in the leaf oil. (B) Mass spectrum obtained from the library NIST08 and WILEY7.

antioxidant activity is justified in considering that lipophilic constituents are not good antioxidants. Furthermore, the presence of linoleic acid in both fixed oils, with a higher concentration in the seed oil, may have caused the oxidation of β-carotene, since free radicals formed during peroxidation of linoleic acid oxidizes the β-carotene (Alves et al., 2010).

According to the results of the antibacterial activity (Table 4), fixed oils from the seeds and leaves showed the same activity against *S. aureus, S. epidermidis,* and *S. aureus* resistant methicillin (1562.50 µg/ml). The oil of the seeds showed moderate inhibition against *E. faecalis, K. pneumoniae* (781.35 µg/ml) and the oil of the leaf was also effective against *E. faecalis* (781.35 µg/ml), with weak inhibition against *S. enterica* (3125.00 µg/ml).

According to the results, the difference in the chemical

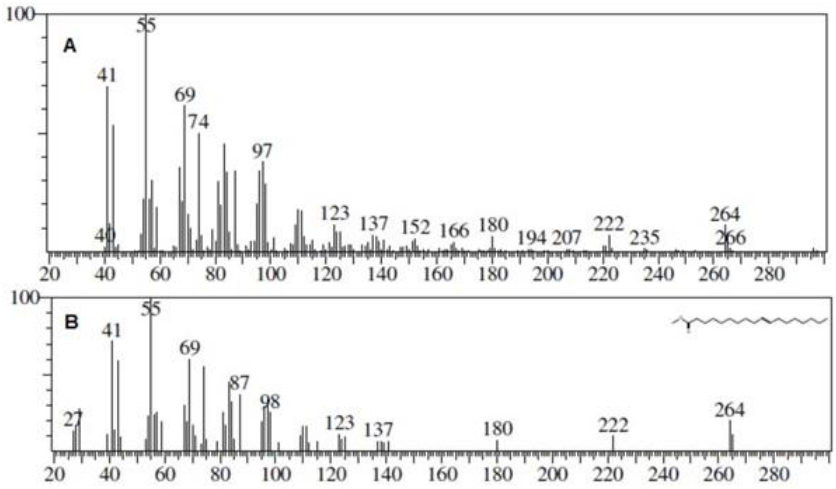

Figure 4. A) Mass spectrum of the major compound methyl octadec-10-enoate (45.53%) present in the seed oil. B) Mass spectrum obtained from the library NIST08.

Table 3. Antioxidant activity of fixed oil of the leaves and seeds of *T. gardneriana.*

Oils / Standards	DPPH (% AA)	β-carotene (%AA)
OF	-12.37±12.59	-1.39±1.38
OS	-101.19±16.78	-0.14±2.09
Ascorbic acid	93.39±0.88	8.50±0.75
BHA	67.63±8.41	19.15±8.63
BHT	73.56±1.83	25.14±15.0

Values are presented as mean ± SD (n=3). AA%= percentage of antioxidant activity; OF= fixed oil of the leaves; OS= fixed oil of the seeds; BHA (butylated hydroxyanisole); BHT (butylated hydroxytoluene).

Table 4. Antibacterial activity of fixed oil of the leaves and seeds of *T. gardneriana.*

Microorganisms	Fixed oil seeds	Fixed oil leaves
Staphylococcus aureus	1562.50	1562.50
Staphylococcus epidermidis	1562.50	1562.50
Enterococcus faecalis	781.35	781.35
Escherichia coli	-	-
Klebsiella pneumoniae	781.35	-
Salmonella enterica	-	3125.00
Staphylococcus aureus resistant methicillin (MRSA)	1562.50	1562.50

Values MBC expressed as µg/ml.

composition of oils obtained from different parts of the plant has not interfered in results of biological activities, because both oils showed moderate bacterial activity and showed no antioxidant activity.

Conclusion

This study described for the first time the identification and quantification of components obtained from fixed oil from the leaves and seeds of *T. gardneriana*. The fatty acid composition of the fixed oils showed differences, noting a greater variety of constituents in the leaf oil. In the leaves major constituents were methyl palmitate and metyl oleate, in the seeds oil was the methyl octadec-10-enoate. Neither of the oils showed antioxidant activity, but both exhibited moderate antibacterial activity. Therefore,

even though the fixed oils do not have antioxidant activity, the class of fatty acids present in the fixed oils has important physiological functions for our body and can be an alternative for pharmaceutical applications. The presence of these compounds in the studied plant is important phytochemically because contribute to the chemical and pharmacological knowledge of this specie.

Conflicts of Interests

The authors have not declared any conflicts of interests.

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Phytochemical and pharmacological screenings of organic crude fractions of *Maesa acuminata*

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The present study is aimed to investigate phytochemical and pharmacological activities of crude organic fractional extracts of *Maesa acuminata* (MA). Preliminary test for phytoconstituents shows the presence of alkaloid, glycoside, steroids, tannins, saponins and reducing sugars. Fractionation was done by ethanol (leaf: ELMA), chloroform (bark: CBMA) and n-hexane (leaf: HLMA). The crude fractions were then subjected for antioxidant, anti-inflammatory and membrane stabilizing, antimicrobial and analgesic activities. In the antioxidant test performed by 1,1-dipheny-picrylhydrazyl (DPPH) assay, the CBMA and HLMA significantly scavenged DPPH radicals, where HLMA at 100 µg/ml inhibited DPPH by 57.01% and the standard, ascorbic acid (AA) by 95.47%, respectively. In the disc diffusion antimicrobial sensitivity test, all the crude fractions of MA produce zones of inhibition between 8 to 18 mm. ELMA showed prominent antimicrobial activity than the CBMA and HLMA, where it strongly inhibited *Staphylococcus aureus* with the inhibition zones by 18 mm at 500 µg/disc. Minimum inhibitory concentration (MIC) detected with the CBMA, ELMA and HLMA were between 61.25 and 250 µg/ml for the test microorganisms. In the anti-diarrheal activity the HLMA and CBMA in oral dose (p.o.) showed 62.79 and 61.62% of percent inhibition of defection as compared to standard, loperamide (93.00%). Additionally, the fractional extracts of MA also possess anti-inflammatory, membrane stabilization and analgesic activities. In conclusion, *M. acuminata* may be a good source of therapeutic components.

Key words: Antioxidant, antimicrobial, *Maesa acuminata*.

INTRODUCTION

Plants are the backbone of life on earth and an essential resource for the well-being of mankind. One-quarter of all

prescription drugs come directly from plants or their derivatives. Additionally, four out of five people around the world today rely on plants for primary health-care (Loya et al., 2009). Notably, modern pharmacopoeia still contains at least 25% drugs derived from plants and many others which are synthetic analogues built on prototype compounds isolated from plants. In this context, medicinal plants are considered as rich resource of ingredients which can be used in drug development and synthesis. World Health Organization (WHO) enlisted approximately 21,000 important medicinal plants. Besides, these plants are playing a crucial role in the development of human cultures from the beginning of life. About 80% of the populations of many developing countries still use traditional medicines for their health care. On the other hand the resistance to the conventional drugs in a regular basis stimulating the search of natural medicines.

Maesa acuminata DC. (Family: Myrsinaceae) is a tree and its leaf juice is given to the children with symptoms of diarrhoea by the Marma and Chakma in Chittagong hill tracts in Bangladesh. Moreover, its juice is also used in cuts by the Marma (Yusuf et al., 2009). By considering the potentiality of this plant as a source of drugs, a systemic investigation was carried out to screen the phytochemical and pharmacological activities of *M. acuminata.*

MATERIALS AND METHODS

Plant collection and identification

For the investigation, the bark and leaves of *M. acuminata* was collected from the Chittagong district in Bangladesh in the month of June and July. The plant material was identified by the taxonomist (without voucher specimen), from Bangladesh Forest Research Institute Herbarium (BFRIH), Chittagong, Bangladesh.

Extraction and fractionation

Approximately 10 h hot extraction (with 95% ethanol) was carried out by using a Soxhlet extractor (Quickfit, England) after drying (temperature not exceeding 50°C) and pulverized of the plant materials. Extracts were then filtered through a cotton plug followed by Whatman filter paper (no. 1) and finally, concentrated by evaporating the solvent below 75°C. The yield of the crude MA leaves and bark extracts were 4.966 and 5.067%, respectively.

Solvent-solvent partitioning was done using the protocol described by Islam et al. (2014). The extracts were subjected to fractionation using n-hexane (leaf: 2.94 out of 3.724 gm), ethanol (leaf: 0.72 g; bark: 1.68 g), and chloroform (bark: 2.56 out of 4.307 g).

Preliminary screening for phytoconstituents

Findings of the preliminary phytochemical screenings done according to Sultana et al. (2014) are given in the Table 1.

Screening for antioxidant activity (DPPH scavenging assay)

The antioxidant activities of the crude extracts were determined as per Islam et al. (2016). Stock solutions of the plant extracts were prepared in ethanol with a concentration range from 10 and 100 µg/ml. To the diluted sample (0.3 ml), 2.7 ml of 0.004 % DPPH ethanolic solution was added. Then the contents were mixed properly, allowed to stand at dark for 30 min to complete the reaction and absorbance was taken using a spectrophotometer at 517 nm. A similar concentration of ascorbic acid (AA) served as the positive control, while only 0.3 ml vehicle was added to DPPH solution for negative control (NC). The blank contained no sample. The DPPH radical scavenging potential was calculated using the following equation: % inhibition of DPPH radical = $[(A_{br} – A_{ar})/A_{br}] \times 100$ where, A_{br} is the absorbance of DPPH free radicals before reaction and A_{ar} is the absorbance of DPPH free radicals after reaction.

Screening for *in-vitro* anti-inflammatory and *ex vivo* membrane stabilizing activity (EAL and HRBC assays)

The anti-inflammatory (EAL; *in vitro*) and membrane stabilization (HRBC; *ex vivo*) tests of plant extracts were carried out according to Hossain et al. (2013). In anti-inflammatory test, 1% egg albumin (EAL) was constituted in phosphate buffer saline solution (PBS, pH 7.4), while for membrane stabilization test, fresh human RBC was reconstituted as 10% suspension human red blood cell reconstitution (HRBC) in isosaline (0.9% NaCl, pH 7.4). The assay mixture contains 1 ml phosphate buffer (pH 7.4, 0.15 M), 2 ml hypo-saline (0.36%). 0.5 ml of EAL/HRBC (125 - 500 µg/ml) was mixed with 0.5 ml of test sample. Acetyl salicylic acid (ASA) and distilled water (DW) were taken as positive and negative controls (NC). After incubation at 37°C for 30 min, the reaction mixtures were centrifuged and supernatant was collected for spectrophotometric analysis at 560 nm. Activity was measured by the following equation.

Inhibition (%) = 100 - [(absorbance of test solution) ÷ (absorbance of control) × 100]

Screening for antimicrobial activity (Disc diffusion assay)

The anti-bacterial action of the crude fractions were screened by the disk diffusion method described in Bauer et al. (1966). All the apparatus used were sterilized by autoclaving at a temperature of 121°C and a pressure of 15 Ibs/sq.inch for 15 min. The test was conducted with 500 µg/disc of crude fractions against 10 pathogenic bacteria (Gram positive: *Lactobacillus casei*, *Lactobacillus coryniformis*, *Bacillus cereus, Bacillus azotoformans* and *Staphylococcus aureus*; Gram negative: *Pseudomonas aeruginosa*, *Escherichia coli, Salmonella typhi*, *Vibrio cholerae* and. *Klebsiella pneumonia*) listed in Table 4.

In addition, anti-fungal activity was done in 7 fungal species (not shown). Microorganisms were collected as pure subculture from Microbiology Laboratory, Department of Pharmacy, BGC Trust University, Chittagong, Bangladesh. Azithromycin (AZ) and fluconazole were taken as standards in this context. The test

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organisms were maintained on nutrient agar slants and were sub-cultured prior to this study. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in millimeters (mm).

Minimum inhibitory concentrations (MIC) determination (Micro-dilution test)

The MIC of the crude fractions were determined by 'micro-dilution technique' (Bauer et al., 1966) in broth medium (Hi Media Laboratories Ltd., India). Briefly, a half-fold dilution from 7.81 to 1000 µg/ml was performed in this occasion. Then the turbidity due to growth of organisms was visually observed by naked eye.

Screening for anti-diarrheal activity (Castor oil-induced diarrheal model)

The anti-diarrheal activity of the crude extracts was determined by the method described in Nur et al. (2015). Briefly, the animals were all screened initially by giving 0.4 ml of castor oil and only those showing diarrhea were selected for the final experiment. Swiss mice (25-30 g of body-weight; 2 months old; both sexes) were divided into control, positive control and two test groups containing five mice in each. Control group received 1% tween-80 (10 ml/kg o.p.). The positive control group received loperamide (3 mg/kg o.p.); test groups received the CBMA and HLMA extracts at a dose of 500 mg/kg (p.o.). Each animal was placed in an individual cage, the floor of which was lined with blotting paper, which was changed every hour. Diarrhea was induced by oral administration of castor oil (0.4 ml) to all mice, 30 min after the above treatments. During the observation period (4 h), the latency period (first diarrheal defecation time) and frequency (number of defecation) were counted manually. Percent inhibition of defecation in mice was calculated by using the following equation:

$$\% \text{ inhibition} = \{(Mo-M)/Mo\} \times 100$$

Where, Mo = mean defecation of control and M = mean defecation of test sample.

Screening for antinoceptive activity (Hot plate test)

The antinoceptive activity of the crude fractions of MA was determined by the method described in Sultana et al. (2015). Diclofenac-Na (DFN) was used as standard. Young Swiss mice of either sex (body weight: 18-24 g) were divided into four groups, 5 animals in each. Group I served as control (DW, p.o.). Group II served as standard and were given DFN (9 mg/kg, i.p.). Group III and IV were treated with CBMA and HLMA at 500 mg/kg (p.o.), respectively. The animals were individually placed on the hot plate maintained at 55±2°C for 15 min after their respective treatments. The response time was noted as the time at which animals reacted to the pain stimulus either by paw licking or jump response, whichever appeared first (latency). Moreover the number of times jumped and number of times of the paw licked was noted as a pain stimulus for 2 min. The cut off time for the reaction was 15 s. The greater the latency period and the lesser the paw licking or jump response, the more is the positive activity by the test extracts.

Screening for anti-atherothrombotic activity (Clot lysis test in human blood)

The thrombolytic activity of the extract was evaluated by the method of Prasad et al. (2006) using streptokinase (SK) 1500000 I.U and ethanol as standard and negative controls, respectively. Briefly, blood was collected from 10 healthy volunteers and distributed into pre-weighed (W_1) micro-centrifuge tubes (0.5 ml/tube) and incubated at 37°C for 45 min and then weight (W_2) was taken. The weight of clotted blood (ΔW) was taken by subtracting the pre-weight and the weight of clotted blood containing tube. Then 100 µl test samples were added to the clot containing tubes marked. Each crude sample was tested for three doses (125, 250 and 500 µg). Similarly, 100 µl of streptokinase (15,00,000 U/vial/10ml) and 100 µl of ethanol were added to the controls marked tubes. Then all the tubes were incubated at 37°C for 90 min. After incubation, fluid released was removed carefully without disrupting the clot, and tubes were again weighed for getting the weight variation among the pre-weight and final weight (W_3) that was achieved for clot lyses (thrombolysis).

Statistical analysis

Values are mean ± SD (standard deviation) and percentage forms. The data were analyzed by means of analysis of variance (ANOVA) followed by *t*-Student–Newman–Keuls's as post-hoc test (except antimicrobial) using the GraphPadPrism software (version 6.0) with 95% confidence interval at $p < 0.05$.

RESULTS AND DISCUSSION

Preliminary phytochemical study revealed the presence of alkaloids, glycosides, tannins, flavonoids in HLMA, while glycosides, alkaloids, flavonoids, tannins and reducing sugars in ELMA, and alkaloid, glycoside, steroids and reducing sugar in CBMA, respectively (Table 1).

In the DPPH$^{\bullet}$ assay, HLMA at100 µg/ml showed 57.01% radical scavenging capacity, while CBMA produced 55.77%. At all concentrations both ELMA and CBMA produced lower inhibition than AA. However, there was a dose-response relationship in extracts and AA. The IC_{50} calculated for HLMA, CBMA and AA are 101.12, 80.27 and 20.01 µg/ml, respectively (Table 2).

In the EAL (*in vitro*) anti-inflammatory test, 500, 250 and 125 µg/ml of CBMA showed mean inhibition of protein denaturation 85.15, 73.05 and 26.17%; whereas, HLMA by 62.25, 47.25 and 18.36% and ELMA by 46.25, 32.25 and 26.17%, respectively. The standard drug, ASA exhibited inhibition of protein denaturation by 96.09, 73.83 and 70.31% with the concentrations of 500, 250 and125 µg/ml, respectively (Table 3).

Otherwise, in the *ex-vivo* membrane stabilization test, 500 µg/ml of CBMA inhibited hemolysis by 24.47%, while, the HLMA by 43.42%. In this occasion, ELMA (55.25%) was found to be more active than the CBMA and HLMA. At 250 µg/ml ASA produced percent inhibition by 65.20% (Table 3).

ELMA at 500 µg/disc strongly inhibited *S. aureus* (18 mm) followed by *S. typhi* (15 mm), *K. peneumonia*, (14 mm), *L. casei* (14 mm), *V. cholera* (12 mm), *L. coryniformins (11 mm), P. aeruginosa,* (11 mm), *B.*

Table 1. Phytoconstituents found in the plant extracts.

Phytoconstituent		Alkaloids	Glycosides	Steroids	Tannins	Falvonoids	Saponins	Reducing sugars	Gums
Consequence	ELMA	+	+	-	+	+	+	-	-
	HLMA	+	+	-	+	+	-	-	-
	CBMA	+	+	+	-	-	-	+	-

*+ = Presence; - = Absence; number indicates number of test performed. CBMA: Chloroform bark extract of *M. acuminata;* HLMA: n-hexane leaf extract of *M. acuminata*; ELMA: Ethanol leaf extract of *M. acuminata*.

Table 2. Antioxidant activity of the crude fractions of *M. acuminata* and standard.

Concentration (µg/ml)	Percent inhibition of DPPH radical		
	HLMA	CBMA	AA
100	57.02	55.78	95.47
80	49.69	51.80	94.41
60	42.61	51.06	91.93
40	37.52	49.57	84.09
20	30.68	39.88	62.32
10	23.60	31.18	45.21
IC_{50} (µg/ml)	101.12	80.27	20.01

Values are percentage of inhibition of $DPPH^{\bullet}$. CBMA, chloroform bark extract of *M. acuminata;* HLMA, n-hexane leaf extract of *M. acuminata*; AA, ascorbic acid; IC_{50}, half minimal inhibitory concentration.

Table 3. Anti-inflammatory and membrane stabilizing activities of the crude fractions of *M. acuminata* and controls.

Conc. (µg/ml)	% inhibition of hemolysis				% inhibition of protein denaturation			
	CBMA	ELMA	HLMA	ASA	CBMA	ELMA	HLMA	ASA
125	18.42	20.36	27.63	62.68	26.17	26.17	18.36	70.31
250	22.38	35.25	33.42	65.20	73.05	32.25	47.25	73.83
500	24.47	55.25	43.42	67.65	85.16	46.25	62.25	96.09

Values are percentage inhibition. CBMA, chloroform bark extract of *M. acuminata;* ELMA, ethanol leaf extract of *M. acuminata*; HLMA, n-hexane leaf extract of *M. acuminata*; ASA, acetyl salicylic acid.

azotoformans (10 mm), *E. coli* (9 mm) and *B. cereus* (8 mm). However, the HLMA and CBMA were inactive against the test strains (Table 4). In the MIC test it was depicted that the ELMA inhibited the growth of *S. aureus* significantly at the dose of 61.25 µg/ml then followed by 72.5, 182.5, 250, 160.5, 220.5, 162.5, 125, 72.5 µg/ml against *L. casei, L. coryniformins, B. cereus, B. azotoformans, E. coli, P. aeruginosa, K. peneumonia, S. typhi, V. cholerae*. AZ (30 µg/disc) produced zones of inhibition within 12 to 21 mm. Although fluconazole (zones of inhibition between 17 to 26 mm) was active against all the test fungi but, crude fractions were found inactive for all of them (*Candida albicans, Pityrosporum ovale*, *Trichophyton sp., Cryptococcus neoformans, Aspergillus niger, Rhizopus oryzae, Aspergillus fumigatus*) (data not shown) (Table 4).

In the castor oil induced diarrhoeal mice, the CLMA and HLMA at 500 mg/kg (p.o.) showed 61.63 and 62.79% inhibition of defecation compared to standard, loperamide (3 mg/kg; p.o.) with 93% inhibition of defecation. Though, CBMA increased the number of defecation, it significantly ($p < 0.05$) increased the latency (92.20 ± 4.21) as compared to DW (Table 5).

In antinoceptive activity study by hot-plate method, both CLMA and HLMA significantly ($p < 0.05$) increased latency after 30 min of an oral administration of 500 mg/kg. However, CLMA was found better than HLMA, despite of prominent activity of DFN (Table 6).

In the *ex-vivo* thrombolytic activity test, after addition of 100 µl SK, the clots showed 81.48% clot lysis. On the other hand clots when treated with 100 µl DW (negative control) showed only negligible lysis (2.25%). All the crude fractions produced significant ($p < 0.05$) anti-thrombosis activity when compared to the DW group.

Table 4. Antibacterial activity of crude fractions of *M. acuminara* and standard.

Test microorganisms	Zone of Inhibition (mm)				MICs (µg/ml)
	CBMA (500 µg/disc)	ELMA (500 µg/disc)	HLMA (500 µg/disc)	AZ (30 µg/disc)	
L. casei	NI	14	NI	18	72.5
L. coryniformis	NI	11	NI	12	182.5
B. cereus	NI	7.33	NI	15	250
S. aureus	NI	18	NI	21	61.25
B. azotoformans	NI	10	NI	20	160.5
E. coli	NI	9	NI	16	220.5
P. aeruginosa	NI	11	NI	21	162.5
K. pneumonia	NI	14	NI	15	125
S. typhi	NI	15	NI	17	72.5
V. cholera	NI	12	NI	17	125

CBMA, chloroform bark extract of *M. acuminata;* HLMA, n-hexane leaf extract of *M. acuminata*; ELMA, ethanol leaf extract of *M. acuminata*; AZ, Azithromycin; NI, No. inhibition; Zones of inhibition less than 8 mm were considered as poor and were discarded.

Table 5. Anti-diarrheal activity of crude fractions of *M. acuminata* and controls.

Treatment groups	Dose (kg; p.o.)	Latent time	% inhibition of defecation	No. of faeces
DW	10 ml	4.20 ± 0.84	16.28	14.40 ± 3.36
Loperamide	3 mg	183.40 ± 6.88*	93.00*	4.20 ± 1.92*
CBMA	500 mg	92.20 ± 4.21*	61.63*	6.60 ± 1.52*
HLMA	500 mg	84.20 ± 5.10*	62.79*	6.40± 2.07*

*p < 0.05 compared to DW (control); values are expressed as mean ± SD (n=5). CBMA, chloroform bark extract of *M. acuminata*; HLMA, n-hexane leaf extract of *M. acuminata*; DW, distilled water.

Table 6. Antinoceptive activity of the crude fractions of *M. acuminata* and controls in *Swiss* mice.

Treatment groups	Dose (kg)	Mean latency (s)	
		Initial	After 30 min
Control (1% v/v tween 80)	10 ml	4.50 ± 0.41	6.25 ± 0.20
DFN	9 mg	4.87 ± 0.18	22.05 ± 0.49*
CLMA	500 mg	5.33 ± 0.20	10.75 ± 1.10*
HLMA	500 mg	5.36 ± 0.16	10.58 ± 0.36*

*p <0.05 compared to the control group; values are mean ± SD (n=5). CBMA, chloroform bark extract of M. acuminata; HLMA, n-hexane leaf extract of *M. acuminata*; DFN, diclofenac-Na

However, the activity was prominent to HLMA (45.63 ± 10.32%) followed by CBMA (35.64 ± 3.69%) and ELMA (26.35 ± 8.19%) (Table 7).

Polyphenols, especially the flavonoids are known for their antioxidant and cytoprotective activities (Bhullar and Rupasinghe, 2015). Our data revealed that both ELMA and HLMA contain flavonoids, thus the highest antioxidant activity of HLMA and better anti-inflammatory activity of ELMA and HLMA may be plugged with the flavonoids present in the extracts. However, the CBMA containing alkaloids and glycosides may be link to its antioxidant as well as membrane stabilization potentials.

Essential oils, especially the diterepenes those have active hydroxyl groups in their structure are capable to kill bacteria (Carvalho et al., 2011). In this study, we found ELMA has promising antibacterial activity. However, HLMA did not show this kind of effect. *Salmonella*, *Shigella* and *E. coli* are most well-known pathogens involve in diarrhea (Nur et al., 2015). However, in this study, both HLMA and CLMA were found inactive against the tested pathogenic strains, including *Salmonella* and *E. coli*, thus other activity mechanism may be involved in

Table 7. Anti-atherothrombosis activities of crude fractions of *M. acuminata* and controls.

Treatments	Concentration/tube containing 500 µl of blood	Percentage of clotlysis
DW	100 µl	2.26±8.23
SK	100 µl	81.48±2.021*
CBMA	500 µg/100 µl	35.64±3.69*
ELMA	500 µg/100 µl	26.35±8.19*
HLMA	500 µg/100 µl	45.63±10.32*

*p <0.05 compared to DW (control); values are mean ± SD; CBMA, chloroform bark extract of *M. acuminata*; ELMA, ethanol leaf extract of *M. acuminata*; HLMA, n-hexane leaf extract of *M. acuminata*; DW, distilled water; SK, streptokinase.

their anti-diarrheal activity. Saponions and flavonoids are evident to impart the neurobioogical effect in experimental animals (Musa et al., 2006). The antinoceptive activity of CLMA and HLMA may be an attribute of the presence of such types of phytochemicals. Otherwise, thrombus formation inside the blood vessel is thought to be one of the major consequences for cardiac failure and many other heart diseases (Viles-Gonzalez et al., 2004). Notably, HLMA exhibited better anti-thrombosis activity than the other crude fractions.

CONCLUSION

A plant provides the main ingredients of medicines in traditional systems and is a source of inspiration for several major pharmaceutical drugs. The present work carried out with the ethanol (ELMA), chloroform (CBMA) and n-hexane (HLMA) extracts of *M. acuminata* represents statistically significant (p <0.05) anti-oxidant activity, anti-inflammatory and membrane stabilizing activity, anti-diarrheal, analgesic and anti-atherothrombosis activities in comparison to the respective control groups. Otherwise, the ELMA showed good action in anti-bacterial and anti-fungal activities. The results obtained in the present study are in agreement to a certain degree with the traditional uses of the plants estimated. The obtained results could be helpful for further study prior to isolate and more advanced non- and/or pre-clinical trials with the responsible phytochemicals in *M. acuminata*. Again, we are concluding with a recommendation for further research with this medicinal plant.

Conflict of Interests

The authors have not declared any conflict of interests.

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