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Full Length Research Paper

Antihyperglycemic and antihyperlipidemic activities of ethanol extract of *Ajuga remota* Benth (Harmegusa) leaves in streptozotocin induced diabetic rats

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The leaves of *Ajuga remota* benth have been utilized traditionally for the treatment of patients with diabetes mellitus. However, its use has not been scientifically validated. The present study was therefore, aimed to assess the antihyperglycemic and antihyperlipidemic activities of ethanol extract of *A. remota* leaves in streptozotocin (STZ) induced diabetic rats. Antihyperglycemic and antihyperlipidemic activities of ethanol extract of *A. remota* leaves (AREt) were studied in streptozotocin induced diabetic rats. The effect of extract on fasting blood glucose, body weight, lipid profile, serum, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, urea, creatinine and total protein were analyzed. Glibenclamide was used as standard drug. Ethanol extract of *A. remota* leaves has showed significant blood glucose lowering effect as compared to the diabetic control group. After diabetic rats were treated with 200 and 400 mg/kg ethanol extract of *A. remota* leaves for 28 days, there were a significant decrease in fasting blood glucose, total cholesterol, triacylglycerol, low density lipoprotein cholesterol, serum alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase, and significant increase in body weight, serum total protein, high density lipoprotein cholesterol levels as compared to untreated control diabetic rats. The results of the present study showed that ethanol extract of *A. remota* leaves might be useful for management of diabetes mellitus and other associated abnormalities. The present study might support the traditional use of *A. remota* for diabetes mellitus treatment.

Key words: *Ajuga remota* benth, diabetes mellitus, streptozotocin.

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia with deranged carbohydrates, fats and proteins metabolism resulting from absolute or relative lack of insulin secretion or insulin resistance by peripheral tissues mainly the liver, skeletal muscle and adipose tissues. It is also characterized by hyperlipidemia and hyperaminoacidemia (Rao et al., 2010; Sudha et al., 2011). The long-term effects of diabetes mellitus include

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progressive development of retinopathy with potential blindness, nephropathy which may lead to renal failure, and/or neuropathy with risk of foot ulcers, amputation and features of autonomic dysfunction. People with diabetes mellitus are at increased risk of cardiovascular, peripheral vascular and cerebrovascular disease (Karimulla et al., 2011).

The global prevalence of diabetes mellitus was estimated to be 9% among adults aged 18 and above in 2014. In 2012, an estimated 1.5 million deaths were directly caused by diabetes. Currently, more than 80% of diabetes deaths occur in low- and middle-income countries. In Ethiopia, the prevalence of diabetes mellitus was 800,000 in the year 2000 and expected to reach 1.8 million by 2030 (Feleke and Enquselassie, 2005).

Modern antidiabetic drugs have their own limitations like high cost and effects such as hypoglycemia, weight gain, gastrointestinal disturbances, and liver toxicity (Dey et al. 2002). The use of plants as medicine dates back to early man. It is estimated that 70 to 80% of people worldwide rely on traditional herbal medicine to meet primary health care needs (Seifu et al., 2012.). To date, ethnobotanical information indicates that over 1200 plants are used as traditional remedies for treatment of diabetes mellitus (Fraser et al., 2007) and more than 200 pure compounds have shown blood glucose lowering activities. WHO has encouraged and recommended the use of herbs as an alternative therapy for diabetes mellitus since medicinal plants are often less expensive, easily accessible, less toxic and suitable (Ansarullah et al., 2011).

A. remota (Figure 1) is a herb that belongs to Lamiaceae family, and is locally known as 'harmegusa' or 'etse- medihanit'. It often lies on the ground and its stems grow to 40 cm high. It grows in different regions of Ethiopia at an altitude of 1600 to 2200 m, flowering from September to October (Dagne, 2009).

Phytochemical investigations of *A. remota* showed the presence of neo-Clerodane diterpenoids (Coll and Tandrón 2005), phytoecdysteroids (Kubo et al., 1983), phenolics, flavonoids, glycosides and sugars are major constituents (Debella et al., 2005). Its leaves are known to relieve stomachache, cold, fever and gonorrhea (Githinji and Kokwaro, 1993). It has been reported that *A. remota* is antimalarial (Gitua et al., 2012). Its aerial parts had some potent antimycobacterial (Cantrell et al., 1999), analgesic and antipyretic activity (Debella et al., 2005). In Ethiopia, the leaves of *A. remota* benth are used for diabetes mellitus treatment by traditional healers. The present study aimed to determine the antihyeprglycemic and antihyperlipidemic activities of 70% ethanolic leaves extract of *A. remota* benth in diabetic rats.

METHODS

Chemicals and equipment

In this study, the following drugs, reagents and instruments were used: streptozotocin (Sigma Aldrich, Germany), glibenclamide (Sanofi Winthrop Industrie, France), SensoCard glucometer and strip (77 Electronike Kft, Hungary) and 902 automated chemistry analyzer (Hitachi, Japan). All other chemicals used were analytical grade.

Collection and preparation of plant material

Fresh leaves of *A. remota* were collected from Deneba town, North Shoa about 175 km North-East of Addis Ababa in late September, 2012. The leaves were identified and authenticated by the National Herbarium (ETH) of Addis Ababa University, and voucher number 001 was given and deposited at the same institute for further reference. The dried leaves were manually grinded and the coarse powder was kept in polyethylene bags at room temperature until used for extraction.

Experimental animals

Adult male Wistar albino rats of body weight 150 to 230 g (initial) with no prior drug treatment were used for the study. Rats were purchased from Ethiopian Public Health Institute (EPHI). All the rats were acclimatized to the laboratory condition for one week before commencing the experiments and fed with pellete and tap water *ad libitum*. The animals were housed in 12 h light and dark cycle at room temperature (20 to 25°C). The experiment was performed in the laboratory of Pharmacology Department, School of Medicine, All animals employed in this study were handled as per the guidelines set by the national academies press, Washington, D.C., USA.

Extraction

The coarse powder (600 g) of *A. remota* leaves was macerated in 70% ethanol (1:10 leaves powder to solvent ratio) for 72 h with mechanical shaking twice a day. This was repeated three times until the extract gave faint or no coloration. The extract was then filtered through Whatman filter paper No.1 and the filtrate was evaporated to dryness under reduced pressure by Rota vapor and farther concentrated by water bath at 40°C. Then, the gummy residue extract was packed in air tight brown glass bottles with proper label and kept in a refrigerator at 4°C until used for the experiment.

Experimental induction of diabetes mellitus in rats

Diabetes mellitus was induced in overnight fasted rats by a single intraperitoneal injection of freshly prepared solution of streptozotocin (60 mg/kg body weight) dissolved in 0.1 M citrate buffer, pH 4.5. The negative control rats were injected with the same volume of citrate buffer only. Streptozotocin injected rats were allowed to drink 5% glucose solution overnight to overcome initial drug induced hypoglycemic mortality.

Diabetes mellitus in streptozotocin injected rats was confirmed by measuring the fasting blood glucose concentration, 72 h after injection. The rats with fasting blood glucose level above 250 mg/dL were enrolled in the study. Treatment was started on the third day after streptozotocin injection and considered as zero day of treatment.

Experimental design and treatment protocol

Rats were divided as Group I: non-diabetic or normal control,

Figure 1. Picture of *A. remota* benth.

received appropriate volume of vehicle, that is, 1% tween 80, 10 ml/kg body weight; Group II: diabetic control, received the vehicle, that is, 1% tween 80, 10 ml/kg body weight; Group III: diabetic treatment, received 200 mg/kg body weight of ethanol extract of *A. remota* leaves; Group IV: diabetic treatment, received 400 mg/kg body weight ethanol extract of *A. remota* leaves; Group V: diabetic treatment, received 600 µg/kg body weight of glibenclamide. Ethanol extract of *A. remota* leaves and glibenclamide were administered every morning for 28 days by gastric intubation with an oral gavage.

Measuring fasting blood glucose

FBG was measured with SensoCard glucometer after the collection of blood sample from the tail vein of the overnight (12 to 15 h) fasted rats on days 0, 7, 14, 21 and 28.

Determination of the weight of rats

Body weight gain or lost in each experimental rat was measured and recorded on days 0, 7, 14, 21 and 28 with triple balance.

Estimation of serum biochemical parameters

At the end of the experimental period (on day 29th), all five groups of rats were sacrificed after overnight fast, by anesthetizing with diethyl ether, and then blood was collected by direct cardiac puncture. Serum was separated after coagulated at room temperature for 30 min and centrifuged at 3000 rpm for 10 min, which was stored at -20°C until biochemical parameters were determined. Total cholesterol, triacylglycerol, high density lipoprotein-cholesterol, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, urea and creatinine were determined with 902 automated chemistry analyzer. Low density lipoprotein-cholesterol level was calculated using Friedwald equation. Serum TP was determined with A 25 BioSystems chemistry analyzer.

Statistical analysis

All the values of body weight, fasting blood sugar and serum biochemical parameters were expressed as mean ± standard error of mean (SEM) and were performed using SPSS software package Version 20.0. The values were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test.

RESULTS

Extraction

In preparation of crude ethanol extract of *A. remota* from 600 g coarse powder leaves, 11.8% (70.80 g) yield of gummy residue was obtained.

Effect of *A. remota* leaves extract on fasting blood glucose level in diabetic rats

The effect of different doses of ethanol extract of *A. remota* on fasting blood glucose level in diabetic rats is

Table 1. Effect of *A. remota* leaves extract on fasting blood glucose level in STZ induced diabetic rats.

Groups	Fasting blood glucose level (mg/dL)				
	0 day	7th day	14th day	21st day	28th day
Normal control	97.2±5.2	99.2±3.3	94.50±3.8	98.5±3.9	97.0±5.0
Diabetic control	383.7±15.1[a]	414.7±13.2[a]	446.5±9.7[ac]	473.17±11.1[ac]	398.8±21.5 [a]
Diabetic + AREt (200 mg/kg)	386.5±16.0[a]	394.7±13.4[a]	383.3±14.9[a]	313.3±18.1[abc]	275.7±14.8[abc]
Diabetic + AREt (400 mg/kg)	381.2±24.1[a]	387.0±20.9[a]	359.2±14.2[ab]	303.5±20.1[abc]	230.2±23.1[abc]
Diabetic + Glibenclamide (600 µ/kg)	393.0±12.8[a]	336.3±21.6[ab]	328.7±15.7[ab]	266.0±14.2[abc]	207.3±29.7[abc]

The values indicate mean ±S.E.M (n=6).[a] p<0.05 compared with normal control values, [b] p<0.05 compared with diabetic control values and [c] p<0.05 compared with the initial level of fasting blood glucose level (0 day) of the rats in the respective group.

Table 2. Effect of *A. remota* leaves extract on body weight of STZ induced diabetic rats.

Groups	Body weight (g)				
	0 day	7th day	14th day	21st day	28th day
Normal control	161.1±5.4	174.1±4.7[c]	188.7±5.5[c]	202.5±5.0[c]	215.5±4.06[c]
Diabetic control	142.6±3.4	132.6±2.6[a]	122.2±2.8[ac]	118.6±4.2[ac]	114.0±3.2[ac]
Diabetic + AREt (200 mg/kg)	147.0±5.3	135.8±4.0[a]	138.9±2.5[ab]	139.2±2.6[ab]	144.6±3.2[ab]
Diabetic + AREt (400 mg/kg)	150.2±6.9	137.8±5.8[a]	144.8±3.9[ab]	147.1±4.4[ab]	149.1±4.6[ab]
Diabetic + Glibenclamide (600 µg/kg)	145.8±2.9	137.1±2.7[a]	141.4±2.7[ab]	147.4±2.6[ab]	150.1±3.8[ab]

The values indicate mean ±S.E.M (n=6).[a] p<0.05 compared with normal control values, [b] p<0.05 compared with the diabetic control group and [c] p<0.05 compared with the initial weight (0 day) of the rats in the respective group.

Table 3. Effect of *A. remota* leaves extract on lipid profile of STZ induced diabetic rats after 28 days treatment.

Groups	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)
Normal control	86.0±1.3	79.5±1.0	28.3±0.7	41.77±1.7
Diabetic control	191.2±6.3[a]	163.50±3.3[a]	18.8±0.7 [a]	139.63±6.2[a]
Diabetic + AREt (200 mg/kg)	129.5±2.1[ab]	95.67±3.7[ab]	25.2±0.4[ab]	85.20±2.3[ab]
Diabetic + AREt (400 mg/kg)	98.2±1.5[b]	80.00±2.1[b]	29.77±0.5[b]	52.50±1.6 [b]
Diabetes + Glibenclamide (600 µg/kg)	88.7±2.1[b]	69.67±2.9 [b]	35.5±0.9[ab]	39.23±2.5[b]

The values indicate mean ±S.E.M (n=6). [a] p<0.05 compared with normal control values and [b] p<0.05 compared with diabetic control values.

given in Table 1. Fasting blood glucose level of diabetic control rats were significantly (p<0.05) higher than those of normal control rats on days 0, 7, 14, 21 and 28. Fasting blood glucose level significantly decreased in diabetic rats treated with 200 mg/kg and 400 mg/kg ethanol extract of *A. remota* and 600 µg/kg glibenclamide on 21st, 14th and 7th day of treatment, respectively as compared with diabetic control rats.

Effect of *A. remota* leaves extract on body weight in diabetic rats

As shown in Table 2, the body weights of untreated diabetic control rats were significantly reduced (p< 0.05) as compared to the normal control rats. Ethanol extract of *A. remota* treated diabetic rats for 28 days significantly (p< 0.05) improved the body weight gain at 200 and 400 mg/kg body weight as compared to the diabetic control group, comparable to that of the standard at 600 µg/kg. The body weight of normal control rats significantly (p< 0.05) increased on days 7, 14, 21 and 28 as compared to the 0th day of treatment, while the diabetic control rats significantly decreased on days 14, 21 and 28 as compare to 0th day. However, the body weight of diabetic rats treated with any of the doses of ethanol extract of *A. remota* did not significantly changed from the 0th day of treatment.

Effect of *A. remota* leaves extract on serum lipid profile in diabetic rats

The level of serum lipid profile of experimental rats is shown in Table 3. Serum total cholesterol, triacylglycerol and low density lipoprotein-cholesterol increased

Table 4. Effect of *A. remota* leaves extract on serum ALT, AST and ALP of STZ induced diabetic rats after 28 days treatment.

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)
Normal control	30.0±1.1	55.8±1.35	51.833±0.8
Diabetic control	72.0±1.3[a]	94.8±3.5[a]	87.33±0.84[a]
Diabetic + AREt (200 mg/kg)	54.2±1.0[ab]	78.0±4.4[ab]	80.00±2.5[ab]
Diabetic + AREt (400 mg/kg)	47.7±2.4[ab]	75.0±3.1[ab]	72.83±1.0[ab]
Diabetic + glibenclamide (600 µg/kg)	42.8±1.6[ab]	71.5±5.5 [b]	65.83±1.2[ab]

The values indicate mean ±S.E.M (n=6). [a] p<0.05 compared with normal control values and [b] p<0.05 compared with diabetic control values.

Table 5. Effect of *A. remota* leaves extract on serum urea, creatinine and total protein of STZ induced diabetic rats after 28 days treatment.

Groups	Urea (mg/dL)	Creatinine (mg/dL)	Total protein (mg/dL)
Normal control	38.2±0.6	0.8±0.03	5.8±0.1
Diabetic control	80.0±2.5[a]	1.7±0.2[a]	4.7±0.2[a]
Diabetic + AREt (200 mg/kg)	59.3±1.7[ab]	1.3±0.1[ab]	5.7±0.1[b]
Diabetic + AREt (400 mg/kg	52.5±1.3[ab]	1.1±0.1[b]	6.0±0.1[b]
Diabetic + glibenclamide (600 µg/kg)	49.2±1.8[ab]	1.0±0.0[b]	5.9±0.1[b]

The values indicate mean ±S.E.M (n=6). [a] p<0.05 compared with normal control values and [b] p<0.05 compared with diabetic control values.

significantly (p< 0.05) in diabetic control rats as compared to the normal control rats; while, the level of high density lipoprotein-cholesterol decreased significantly (p<0.05) in diabetic control rats as compared to the normal control rats. Serum total cholesterol, triacylglycerol and low density lipoprotein-cholesterol levels significantly (p<0.05) reduced whereas that of high density lipoprotein-cholesterol significantly (p<0.05) increased with 200 mg/kg body weight and 400 mg/kg body weight ethanol extract of *A. remota* leaves treatment as compared to the diabetic rats. Treatment with 400 mg/kg body weight ethanol extract of *A. remota* leaves for 28 days reversed the aforementioned values near normal. The effect was comparable with 600 µg/kg body weight glibenclamide with the same period of treatment. A dose dependent reduction in the levels of total cholesterol, triacylglycerol, low density lipoprotein-cholesterol and increase of high density lipoprotein-cholesterol was observed with both doses of ethanol extract of *A. remota* treatment.

Effect of *A. remota* leaves extract on serum alanine aminotransferase, aspartate amino transferase and alkaline phosphatase in diabetic rats

The serum levels of alanine aminotransferase, aspartate aminotransferase and alkaline aminotransferase significantly (p<0.05) increased in diabetic control rats as compared to normal controls. Administration of 200 mg/kg body weight and 400 mg/kg body weight ethanol extract of *A. remota* for 28 days significantly (p< 0.05) reduced the activity of alanine aminotransferase, aspartate amino transferase and alkaline phosphatase in diabetic rats as compared to diabetic control group. Similar effect was also observed with 600 µg/kg glibenclamide treatment (Table 4).

Effect of *A. remota* leaves extract on serum urea, creatinine and total protein in diabetic rats

Table 5 illustrates that serum urea and creatinine levels were significantly (p<0.05) elevated in diabetic control rats as compared to the normal control rats. Whereas, a decrease in TP levels was found in diabetic control rats when compared with normal control rats. Administration of 200 and 400 mg/kg ethanol extract of *A. remota* leaves and 600 µg/kg glibenclamide significantly (p<0.05) reduced the serum urea and creatinine level as compared to the diabetic control rats. On the other hand, the level of serum total protein significantly (p<0.05) increased as compared to diabetic control rats after 28 days of treatment.

DISCUSSION

The present study was designed to investigate the

antihyperglycemic and antihyperlipidemic activities of ethanol extract of *A. remota* leaves in streptozotocin induced diabetic rats. Streptozotocin has been used to induce diabetes mellitus in experimental rats (Latha and Daisy 2011). A single intraperitoneal (IP) administration of 60 mg/kg streptozotocin effectively induced diabetes mellitus in rats, which was confirmed by elevated level of fasting blood glucose obtained from the tail of the rats after 72 h of injection.

The mechanism by which streptozotocin brings about diabetic mellitus includes selective destruction of insulin secreting pancreatic β-cells and minimize glucose uptake by peripheral tissues (Szkudelski, 2001). Ethanol extract of *A. remota* leaves reduced high fasting blood glucose level in streptozotocin induced diabetic rats. The mechanism antihyperglycemic of actions of ethanol extract of *A. remota* leaves might be due to an insulin mimetic effect on muscle and adipose tissues by either stimulating glucose uptake and metabolism (Daisy et al., 2010), by inhibiting hepatic gluconeogenesis (Cetto and Vázquez, 2010) and glycogenolysis (Rawi et al., 2011), by stimulation of regeneration process or increase pancreatic secretion of insulin from existing β-cells (Sharma et al., 2008) and/ or inhibition activity against α-glucosidase enzymes in small intestine which convert disaccharides into monosaccharaides for sake of absorption (Shinde et al., 2008).

The difference in the magnitude of the effect on fasting blood glucose between 400 and 200 mg/kg ethanol extract of *A. remota* leaves doses might be attributed to the higher concentration of the active component(s) responsible for more fall of fasting blood glucose in the former than the later.

Fasting blood glucose lowering effect of ethanol extract of *A. remota* leaves is similar to *Ajuga iva*, which belongs in the same family (Hilaly and Lyoussi, 2002). Phytochemical investigations of *A. remota* have reported the presence of bioactive compounds such as iridoid and flavonol glycosides and phytoecdysteroids (Kubo et al., 1983 and Manguro et al., 2006). It has been suggested that antihyperglycemic effect of ethanol extract of *A. remota* leaves extract probably attributed to these constituents through improvement of insulin level (Sundaram et al., 2012).

The loss of body weight in untreated diabetic rats is due to increased muscle wasting and catabolism of others tissue proteins (Flatt et al., 1990). However, ethanol extract of *A. remota* leaves treatment improved the body weight in diabetic rats perhaps, due to the antihyperglycemic effect which was an indication of proper glucose utilization (Pari and Satheesh, 2004), its protective effect in muscle wasting and controlling protein turn over and/or improvement in diabetes mellitus associated disorders (Oyedemi et al., 2012).

Abnormalities in lipid profile are common complications in diabetes mellitus (Gibbons, 1986). Such abnormality represents the risk factors for coronary heart diseases. Activation of hormone sensitive lipase during insulin deficiency causes an increase in free fatty acid mobilization from adipose tissue and result in synthesis of triacylglycerol in liver (Mooradian, 2009). In addition, hyperglycemia is accompanied by a rise in total cholesterol, triacylglycerol, low density lipoprotein-cholestreol and a fall in high density lipoprotein-cholesterol (Gao et al., 2009). In the present study, total cholesterol, triacylglycerol and low density lipoprotein-cholesterol levels decreased and high density lipoprotein-cholesterol increased in ethanol extract of *Ajuga remota* leaves treated diabetic rats.

The remarkable control of high serum triacylglycerol in ethanol extract of *A. remota* leaves treated diabetic rats could be due to inhibition of endogenous triacylglycerol synthesis in liver (Xie et al., 2007) or improvement in insulin level or the presence of active component(s) in ethanol extract of *A. remota* leaves that suppressed the activity of hormone sensitive lipase in adipose tissue or increased activity of hepatic lipase or lipoprotein lipase accountable for the hydrolysis of excess lipoprotein bound triacylglycerol into fatty acids (Pritchard et al., 1986).

Increased level of HDL-C in ethanol extract of *A. remota* leaves treated groups could be due to the enhancement of lecithin: cholesterol acyltransferase (LCAT) which plays a key role in incorporating the free cholesterol into high density lipoprotein which take back to the liver (Senoucia et al., 2012). LDL-C reducing effect of ethanol extract of *A.* remota leaves presumably attributed to increased expression of low density lipoprotein receptor (LDLR), which enhance low density lipoprotein particles uptake in liver from the circulation, through the depletion of intracellular cholesterol (Bursill et al., 2007).

Serum TC lowering property of ethanol extract of *A. remota* leaves could be attributed to the availability of hypocholesterolemic compounds in ethanol extract of *A. remota* leaves that may act as inhibitor for hepatic hydroxyl methyl glutaryl CoA (HMG CoA) reductase in liver, which take part in cholesterol synthesis (Kumarappan et al., 2007) or increasing the fecal content by inhibiting the absorption of cholesterol from intestine (Raederstorff et al., 2003). Isolated phytoecdysteroids and iridoid glycosides, phytochemical constituent of ethanol extract of *A. remota* leaves, from other *Ajuga* species have shown antioxidant activity (Fan et al., 2011). Thus, reduction of total cholesterol in ethanol extract of *A. remota* leaves ethanol extract of *A. remota* leaves extract treated diabetic rats might also attributed to the aforementioned phytochemical constituents by reducing lipid peroxidation by scavenging free radicals (Sharma et al., 2008).

The decrease in total cholesterol, triacylglycerol and low density lipoprotein-cholesterol and an increase in high density lipoprotein-cholesterol after 28 days treatment showed a dose dependent trend, indicating that

efficacy was proportional to the dose of ethanol extract of *A. remota* leaves. Similar hypocholestrolemia and hypotriacyleglycerol effects were observed by *A. iva* whole plant extract in streptozotocin induced diabetic rats (Hilaly et al., 2007).

The activity of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase in serum are generally indicators of liver function. In diabetic rats, the level of these enzymes are elevated due to necrosis of liver cells by the injection of STZ (Hwang et al., 1996). However, ethanol extract of *A. remota* leaves treated diabetic rats showed decreased in the activity of serum alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase enzymes that might support its hepatoprotective effect and normalization capability of impaired liver metabolism in diabetic rats.

There is increased protein catabolism with the flow of amino acids into the liver, which feeds gluconeogenesis as a result of insulin deficiency during uncontrolled diabetes mellitus. This might account for the decrease in serum total protein content in STZ induced diabetic control rats (Narendhirakannan et al., 2006). The results of the present study demonstrated that treatment of diabetic rats with ethanol extract of *A. remota* leaves caused a significant increase in serum total protein which might be attributed to an improvement in glycemic control and insulin secretion that increase protein synthesis or decrease protein degradation (Gao et al., 2009).

Negative nitrogen balance is manifested in diabetic rats associated with enhanced proteolysis in muscle and other tissues. Impaired balance of nitrogen coupled with lowered protein synthesis leads to increased concentrations of urea and creatinine in serum (Basha and Subramanian, 2011) indicating progressive renal damage in diabetic rats (Anjaneyulu and Chopra, 2004). Treatment with ethanol extract of *A. remota* leaves resulted in a considerable reduction to near normal in serum urea and creatinine level indicating the renoprotective role of ethanol extract of *A. remota* leaves or delay diabetic nephropathy development.

Hepatoprotective and renoprotective effect of ethanol extract of *A. remota* leaves might be due to phyloecdysteroids, possibly through their antioxidant activity (Krishnan et al., 2007). In conclusion, these findings demonstrated that ethanol extract of *A. remota* leaves possesses antihyperglycemic and antihyperlipidemic properties which might support the traditional claim of its use in diabetes.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Determination of bioactive constituents of *Rauwolfia vomitoria* Afzel (Asofeyeje) roots using gas chromatography-mass spectrometry (GC-MS) and Fourier transform infrared spectrometry (FT-IR)

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Rauwolfia vomitoria **Aftzel belong to the family of Apocynacea with a common name serpent wood and Igbo name as akanta. It is used traditionally to treat hypertension and rheumatoid arthritis. The present study deals with GC-MS determination and FT-IR analysis of methanolic root extract of the named plant. Exactly thirty phytochemical constituents have been identified by comparing the chromatogram peek values of unknown compounds with entries in NIST database. The major bioactive compounds were 2-Formyl-9-[β-d-ribofuranosyl]hypoxanthine (8.63%), 5-Cyclopropylcarbonyloxypenta-decane (5.58%), 1,2,5-Oxadiazol-3-carboxamide-4,4'-azobis-2,2'-dioxide (4.47%), 1-(5-Bicylco[2.2.1]heptyl)ethylamine (3.66%), 1-Adamantanemethylamine,α-methyl- (2.22%), Diphenylephrine (1.26%), Imidazole,2-amino-5-[(2-carboxy)vinyl]- (0.85%), Spiro[androst-5-ene-17,1'-Cyclobutan]-2'-one,3-hydroxy-,(3β,17β)- (0.82, and Cyclohexan-1,4,55-triol-3-one-1-carboxylic acid (0.65%). The FTIR spectrum confirmed the presence of alkyl halides, alcohols, phenols, secondary alcohols, tertiary alcohols, aromatic ethers, aldehydes, ketones, aliphatic nitro compound, aromatic organophosphorus compounds, aromatic compounds, carboxylic acid derivatives, alkenes, saturated ketones, and alkanes. Hence, this study offers bases of employing *R. vomitoria* as herbal alternative for the treatment of various diseases.**

Key words: Fourier transform infrared spectrometry (FTIR), gas chromatography-mass spectrometry (GC-MS), phytochemical, *Rauwolfia vomitoria* Aftzel.

INTRODUCTION

Developing countries most especially the rural areas are constantly being inundated by infectious diseases and attempts to manage these ailments with conventional drugs have posed mankind with a lot of health consequences. Researches over the years have shown that over dependence on drugs as sole remedies for treating diseases have led to malfunction of important organs of the body such as the liver, the kidney, eyes,

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lungs and the brain. Sequel to this, attentions are now being shifted to the use of medicinal plants in the treatment of infectious diseases. The economic realities of the developing countries, where most people hardly can afford good meal, housing, clothing and the expensive pharmaceutical products for health-care, has forced many to resort to alternative complementary medicine. Again, the development of drug-resistant strains of microorganisms and autoimmune problems make it imperative for a continued search for new drugs from natural products (Vaitheeswarn and Edward, 2014). Cowan (1999) reported that plants still represent large untapped sources of structurally novel compounds that might serve as lead for the development of novel drugs. Traditional systems of medicines are prepared from a single plant or combinations of number of plants. The efficacy depends on the use of proper plant part and its biological potency which in turn depends upon the presence of required quantity and nature of secondary metabolite in a raw drug (Savithramma et al., 2010; Vinoth et al., 2011). Herbal medicines are safer than synthetic medicines because the phytochemicals in the plant extract target the biochemical pathway. These bioactive compounds that contribute to the pharmacological efficacy of medicinal plants have recently been identified with relatively expensive and often laborious techniques such as gas chromatography (GC) combined with specific detections schemes (Eisenhauer et al., 2009). The specific detection schemes could be mass spectrometry (MS) or Fourier transform infrared spectrometry (FTIR). Gas chromatography combined with mass spectroscopy (GC-MS) can identify pure compounds present at less than 1 ng biological specimen and quantification purpose (Liebler et al., 1996). The unknown organic compounds in a complex mixture can be determined by interpretation and also by matching the spectra with reference spectra (Hites, 1997). FT-IR spectroscopy has demonstrated to be a reliable and sensitive method for finding out the functional groups present in plant samples using IR region in the range of 400 to 4000 cm^{-1}. For most common plant compounds, the spectrum of an unknown compound can be identified by comparison to a library of known compounds (Griffiths and Haseth, 1986).

Nigeria is rich in diverse plant resources, therefore screening for active compounds residents in plants with effective protection and treatment of different diseases and infections should be pursued. *Rauwolfia vomitoria* belongs to the family Apocynacea and its common names include serpent wood, poison's devil pepper and swizzler stick. The plant is a shrub or tree which grows over 10 m tall of the forest and common in secondary growth throughout most of African regions. Sometimes due to the flower and the sweet scented fruits and it is sometime planted as an ornamental shrub. In Nigerian local languages, *R. vomitoria* is called asofeyeje (Yoruba), akanta (Igbo), wada (Hausa), mmoneba (Efik) and utoenyin (Ibibio) (Mecha et al., 1980; Ekutudo, 2003; Ehiagbonare, 2004). The plant is of different species: the Indian species (*Rauvolfia serpentina*) and the African species (*R. vomitoria*) (Kutalek and Prinz, 2007). The roots, root bark, leaves and stem-bark are employed in herbal medicine for the treatment of several ailments. Kutalek and Prinz (2007) reported that the plant is used in ethnomedicine against snake bites, fever and nervous disorders. They further reported that a watery solution of the bark of *R. vomitoria* can be used against such parasites as lice and scabies. *R. vomitoria* is employed in the treatment of rheumatoid arthritis (Fapojuwomi and Asinwa, 2013). Sharma (2004) added that the root of *R. vomitoria* is good for the treatment of snake bites, insect stings, nervous disorders, mania, epilepsy, intractable skin disorders such as psoriasis, excessive sweating, itching, hypertension, sedative, uterine contraction in child birth and gynecological ointment for the treatment of menopausal disorders. Prajapati et al. (2007) observed that *R. vomitoria* is a sedative, hypnotic, good for reducing blood pressure, good for treating insanity, anthelmintic, an antidote to snake venom, anti-anxiety agent and stimulant to central nervous system. A decoction of the root of the plant could be given during labour pains to increase uterine contraction. Odugbemi (2008) also reported that *R. vomitoria* (Asofeyeje) is good for the treatment of hypertension, insomnia, nervous disorder, jaundice, fever, diarrhea, dysentery, scabies, mental disorders, anthelmintics and malaria.

Reserpine is an alkaloid derived from *R. vomitoria* and is the major reason for the medicinal use of the plant in India. Reserpine is derived from the root bark of the plant and was isolated in 1952 as purified alkaloid for the treatment of hypertension. Reserpine irreversibly binds to the storage vesicles of neurotransmitters, particularly norepinephrine, serotonin and dopamine. Eventually, catecholamine depletion occurs because of the body's inability to store these neurotransmitters. Therefore, the study employed gas chromatography combined with mass spectrophotometry and Fourier transform infrared (GC-MS/FT-IR) technique to determine the phytochemicals present and the functional groups they possess, respectively.

MATERIALS AND METHODS

Collection and identification of plant

R. vomitoria roots were collected from a bush near Abia State University. The collected specimen was taken to the Forest Herbarium, Ibadan (FHI), Nigeria for proper identification. The specimen already was existing in the FHI. It has the serial No. 72.

Preparation of extract for GC-MS

The roots of *R. vomitoria* were washed to remove sand and humus, and then allowed to dry under shade. The roots were first chopped into small particles and ground to powder using a grinder. Five

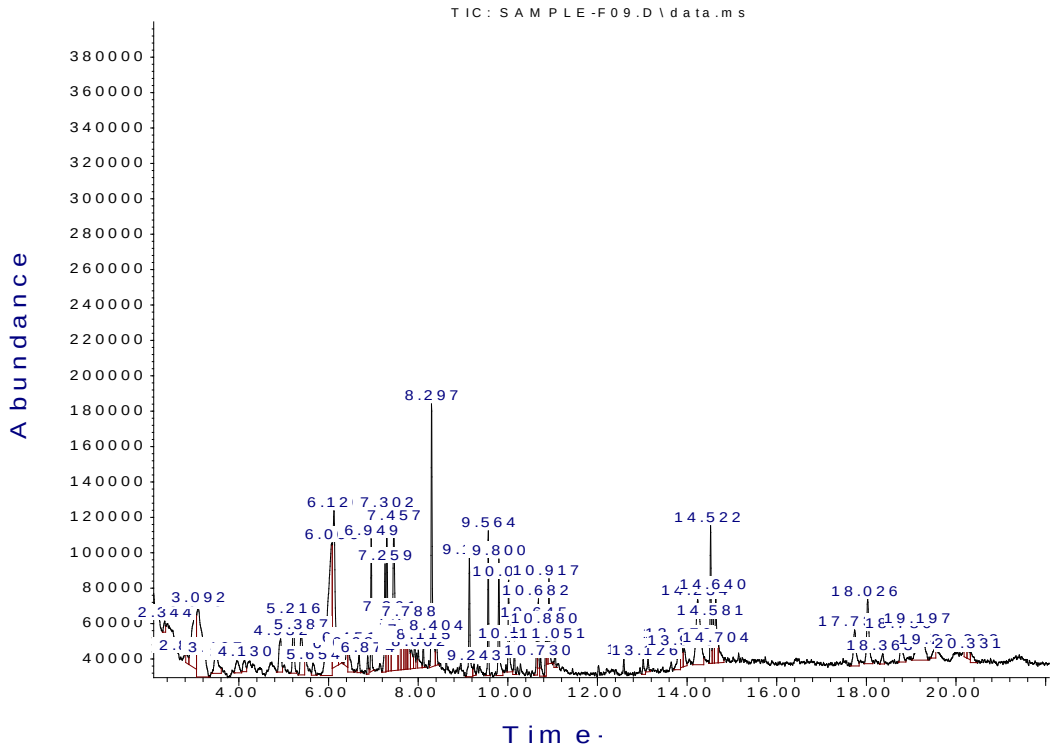

Figure 1. Mass Chromatogram of Methanolic extract of leaves of *Rauwolfia vomitoria*.

grams of the plant powder was transferred to a round bottom flask and treated with methanol in a soxhlet apparatus. The temperature was maintained between 45 and 47°C and lasted for 24 h. The extract was collected and evaporated using vacuum distillation unit. The methanolic extract so obtained was then subjected to GC-MS.

FT-IR spectroscopic analysis

The FT-IR spectra of the samples were determined using a Fourier transform infrared spectrometer (Thermo Scientific Nicolet iS5) iDI Transmission Accessory. Approximately, 2 mg of the solid sample of *R. vomitoria* was ground with 200 mg of dried potassium bromide (KBr) to form a homogenous powder, which was compressed into a thin pellet by using 15Tons hydraulic press. KBr pellet holder was then recorded for FT-IR measurement in the wave number range from 4000 to 400 cm^{-1} using 16 scan.

GC-MS analysis

GC-MS analysis on the methanolic extract was carried out using Agilent 7890A-5975C GC-MS system, employing the following conditions. HPS-Column (30×0.25 μm), operating in electron impact mode at 70 eV; carrier gas flow (a constant) = 1 ml/min. Injection volume was set at 0.5 μL, at split ratio of 10:1. The injection temperature was 250°C and ion source temperature 280°C. Oven temperature for 2 min at the initial 70°C and to 280°C at 15°C/min rate for 5 min. The mass spectra was measured at 70 eV.

Identification of components

Interpretation of mass spectrum obtained from GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 82,000 patterns. The spectrum of the unknown component was compared with the spectra of the known components stored in the NIST library. The name, molecular weight, molecular formula and structure of the components of the test materials were ascertained.

Identification of functional groups

The FTIR spectrum was used to identify the functional groups of the active components present in plant sample based on the peaks values in the region of IR radiation. When the plant extract was passed into FTIR, the functional groups of the components were separated based on its peaks ratio.

RESULTS

The results of GC-MS/FT-IR and biological activities of identified compounds of methanol extract of roots of *R. vomitoria* are presented in Figures 1 and 2 and Tables 1 to 3. A total of thirty compounds were identified from the methanol root extract of *R. vomitoria.* The identification of the phytochemical compounds was confirmed based on the peak area, retention time and molecular formula (Figure 1 and Table 1). According to Duke's phytochemical and ethnobotanical databases (1998), the biological activities of some of the identified compounds are presented in Table 2. The results of FT-IR spectroscopic analysis revealed the presence of certain

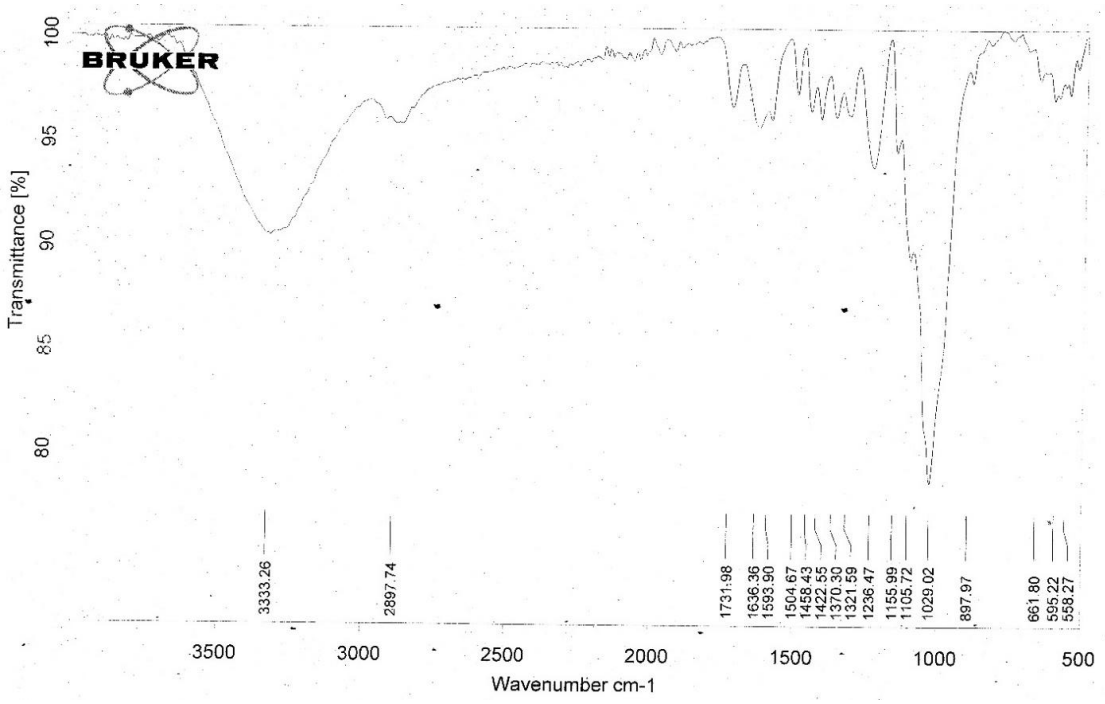

Figure 2. FTIR spectrum of methanolic extract of leaves of *Rauwolfia vomitoria*.

functional groups present in methanol extract of roots of *R. vomitoria* (Figure 2 and Table 3). The absorption at 3333.26 cm^{-1} is due to the O-H stretching of strong bond present in the extract. The band at 2897.74 cm^{-1} is due to C-H_3, C-H_2 and C-H bend of alkanes; the band at 1731.98 cm^{-1} showed saturated ketones; the band at 1636.36 cm^{-1} showed alkene C=C bend; the band at 1593.90 cm^{-1} showed carboxylic acid derivatives C=O bend; the band at 1504.67 cm^{-1} showed aromatic C=C bend; the bend at 1458.43 cm^{-1} showed aromatic organophosphorus compounds P-C bend; the bend at 1422.55 cm^{-1} showed alcohol and phenol O-H in-plane bend, the band at 1370.30 cm^{-1} showed aliphatic nitro compound N-O bending; the bend at 1321.59 cm^{-1} showed A-CH_3 bending, the band at 1236.47 cm^{-1} showed aromatic ethers C-O bend; the band between 1155.99 and 1105.72 cm^{-1} showed tertiary and secondary alcohol C-O bends; the band at 1029.02 cm^{-1} showed fluoroalkanes C-X bend; the band at 897.97 cm^{-1} showed alkenes =C-H and =CH_2 out of plane bending; the band at 661.80 cm^{-1} showed alcohol phenol bends and the bands between 595.22 and 558.27 cm^{-1} showed bromoalkane C-X bend.

DISCUSSION

The techniques of GC-MS/FT-IR have proved effective and more reliable in the determination of chemical compounds present in plants. Earlier reports elucidated the biological activities of Cyclohexan-1,4,5-triol-3-one-1-carboxylic acid, Spiro[androst-5-ene-17,1'-Cyclobutan]-2'-one,3-hydroxy-,(3β,17β), 2-Trifluoroacetoxydodecane and Adamantane methylamine,α-methyl-. Jeyadevi et al. (2013) reported that cyclohexan-1,4,5-triol-3-one-1-carboxylic acid has antibacterial activity against *Escherichia coli*, Srinivasan et al. (2014) observed that imidazole,2-amino-5-[(2-carboxyvinyl)] has antimicrobial activities, spiro[androst-5-ene-17,1'-Cyclobutan]-2'-one,3-hydroxy-,(3β,17β) possesses ant-inflammatory and antimicrobial activities; Mohan et al. (2014) reported that 2-Trifluoroacetoxydodecane, a fluorocompound has antimicrobial, anticancer and anti-inflammatory, while Xing et al. (2008) observed that 1-Adamantanemethylamine,α-methyl- otherwise known as Rimantadine, inhibits *in vitro* replication of influenza A virus isolates from each of the three antigenic sub-types (H1N1, H2N2 and H3N3) that have been found in man. From the result of the functional group analysis, it can be inferred that alkyl halides, alcohols, phenols, secondary alcohols, tertiary alcohols, aromatic ethers, aldehydes, ketones, aliphatic nitro compound, aromatic organophosphorus compounds, aromatic compounds, carboxylic acid derivatives, alkenes, saturated ketones and alkanes might be responsible for the various medicinal properties of the root of *R. vomitoria.*

Conclusion

In the present study, the FTIR and GC-MS spectral

Table 1. List of compounds identified at various retention times from methanolic extract of roots of *Rauwolfia vomitoria* by GC-MS.

S/N	RT	Compound name	MW	Formula	Area (%)
1	2.344	Cyclopropyl carbinol	72	C_4H_8O	0.28
2	3.467	1-pentanol,4-amino	103	$C_3H_{13}NO$	1.19
3	3.954	Cyclohexan-1,4,5-triol-3-one-1-carboxylic acid	190	$C_7H_{10}O_6$	0.65
4	4.932	Diphenylephrine	167	$C_9H_{13}NO_2$	1.26
5	5.216	Phenol, 2,6-dimethoxy-	154	$C_8H_{10}O_3$	1.58
6	5.387	2-Trifluoroacetoxydodecane	282	$C_{14}H_{25}F_3O_2$	1.84
7	5.654	1-(5-Bicylco[2.2.1]heptyl)ethylamine	139	$C_9H_{17}N$	3.66
8	6.066	2-Formyl-9-[β-d-ribofuranosyl]hypoxanthine	296	$C_{11}H_{12}N_4O_6$	8.63
9	6.248	1-Guanidinosuccinimide	141	$C_5H_7N_3O_2$	0.31
10	6.398	3-Nonyn-2-ol	140	$C_9H_{16}O$	0.62
11	6.451	Imidazole,2-amino-5-[(2-carboxy)vinyl]-	153	$C_6H_7N_3O_2$	0.85
12	6.874	Cyclohexanemethanol,4-ethenyl-α,α,4-trimethyl-3-(1-methylethenyl)-,[1R-(1α, 3α, 4β)]-	222	$C_{15}H_{26}O$	0.31
13	6.949	2-Propenoic acid, 1-methylindecylester	240	$C_{15}H_{28}O_2$	1.97
14	7.302	3-(Prop-2-enoyloxy)dodecane	240	$C_{15}H_{28}O_2$	2.97
15	7.457	5-Cyclopropylcarbonyloxypenta-decane	296	$C_{19}H_{36}O_2$	5.58
16	8.002	4-((1E)-3-Hydroxy-1-Propenyl)-2-methoxyphenol	180	$C_{10}H_{12}O_3$	0.47
17	8.297	Didodecylphthalate	502	$C_{32}H_{54}O_4$	5.14
18	9.142	Cyclopentaneundecanoic acid, methyl ester	268	$C_{17}H_{32}O_2$	2.38
19	9.243	Undecanoic acid	186	$C_{11}H_{12}O_2$	0.26
20	9.564	Propanamide, 3,(3,4-dimethylphenylephrine)	241	$C_{11}H_{15}NO_3S$	1.98
21	10.019	9,12-Octadecadienal	264	$C_{18}H_{32}O$	1.73
22	10.147	2-Decyn-1-ol	154	$C_{10}H_{18}O$	0.48
23	10.843	3-Decyn-2-ol	154	$C_{10}H_{18}O$	0.60
24	10.917	1-methyldodecylamine	199	$C_{13}H_{29}N$	1.33
25	13.019	Urs-12-en-28-ol	426	$C_{30}H_{50}O$	0.28
26	13.822	2, 6,10-Dodecatrien-1-ol,3,7,11-trimethyl-	222	$C_{15}H_{26}O$	1.25
27	13.929	3'Hydroxyquinalbarbitone	254	$C_{12}H_{18}N_2O_4$	0.56
28	14.234	1,2,5-Oxadiazol-3-carboxamide-4,4'-azobis-2,2'-dioxide	284	$C_6H_4N_8O_6$	4.47
29	14.522	1-Adamantanemethylamine,α-methyl-	179	$C_{12}H_{21}N$	2.22
30	14.581	Spiro[androst-5-ene-17,1'-Cyclobutan]-2'-one,3-hydroxy-,(3β,17β)-	328	$C_{22}H_{32}O_2$	0.82

analysis of *R. vomitoria* root extract composed of various functional groups and variety of bioactive compounds which are responsible for many biological activities. In addition, further research is necessary to identify and purify the active compounds responsible for therapeutic activity.

Table 2. Biological activities of phytochemical compounds identified in methanol extract of roots of *Rauwolfia vomitoria*.

S/N	Compound name	Biological activity
1	1-pentanol,4-amino	Increase aromatic amino acid decarboxylase activity
2	Cyclohexan-1,4,5-triol-3-one-1-carboxylic acid	Acidifier, inhibit production of uric acid, urinary acidulant
3	3-Nonyn-2-ol	Oligosaccharide provider
4	2-Propenoic acid, 1-methylindecylester	Arachidonic acid inhibitor, Increase aromatic amino acid decarboxylase activity, inhibit production of uric acid, urinary acidulant
5	3-(Prop-2-enoyloxy)dodecane	Propecic activity
6	4-((1E)-3-Hydroxy-1-Propenyl)-2-methoxyphenol	Anticancer, antidote (emetine), cytochrome-P4502E1 inhibitor, EDRF promoter, elastase inhibitor, endothelium-derived relaxing factor promoter, enteromotility enhancer, ethanol-absorption inhibitor, fertility enhancer, enteroparasiticide, memory enhancer,
7	Cyclopentaneundecanoic acid, methyl ester	Increase aromatic amino acid decarboxylase activity, inhibit production uric acid, arachidonic acid inhibitor
8	Undecanoic acid	Acidifier, inhibit production of uric acid, urinary acidulant
9	2-Decyn-1-ol	Oligosaccharide provider
10	3-Decyn-2-ol	Oligosaccharide provider
11	Urs-12-en-28-ol	Oligosaccharide provider, decrease endothelial leukocyte-adhension, energizer, fertility enhancer, stimulate PUFA desaturase and elongase enzymes, trypsin enhancer
12	2, 6,10-Dodecatrien-1-ol,3,7,11-trimethyl-	Oligosaccharide provider
13	1-Adamantanemethylamine,α-methyl-	Catechol-O-methyl-transferase inhibitor, methyl donor, methyl-guanidine inhibitor
14	Spiro[androst-5-ene-17,1'-Cyclobutan]-2'-one,3-hydroxy-,(3β,17β)-	-

Duke's phytochemical and ethnobotanical databases

Table 3. FTIR analysis of methanolic extract of roots of *Rauwolfia vomitoria*.

S/N	Wave number (cm^{-1})	Functional group
1	558.27	C-X (Bromoalkanes, alkyl halides
2	661.80	O-H (Alcohols and phenols)
3	897.97	=C-H and =CH2 out of plane bending (alkenes)
4	1029.02	C-X (Fluoroalkanes, alkyl halides)
5	1105.72	C-O (Secondary alcohols)
6	1155.99	C-O (Tertiary alcohols)
7	1236.47	C-O (Aromatic ethers)
8	1321.59	A-CH_3 bending (Aldehydes and ketones)
9	1370.30	N-O (Aliphatic nitro compounds)
10	1422.55	O-H bending in plane (Alcohols and phenols)

Table 3. Contd.

11	1458.43	P-C (Aromatic organophosphorus compounds)
12	1504.67	C=C (aromatic compounds)
13	1593.90	C=O (carboxylic acids/derivatives)
14	1636.36	C=C (alkenes)
15	1731.98	C=O (saturated ketones)
16	2897.74	CH_3, CH_3 and CH band (Alkanes)
17	3333.26	O-H (strong bond) (Alcohols and phenols)

Conflicts of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Screening of some pyrazole derivatives as promising antileishmanial agent

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Pyrazole derivatives (I-VII) were prepared in good yields using aldol condensation followed by cyclization and were characterized by elemental analysis, IR and ^{1}H NMR spectroscopy. *In vitro* antileishmanial activity test was conducted using Alamar blue reduction method. The test revealed that the synthesized compounds (except compound IIb) exhibit better antileishmanial activity than the standard drug miltefosine and lower antileishmanial activity (except compounds III and IIIb) compared to the standard drug amphotericin B deoxycholate. Compound IIIb, phenyl pyrazoline with propanoyl side chain, 1-(3-phenyl-5-(1-phenyl-3-p-tolyl-1H-pyrazol-4-yl)-4,5-dihydropyrazol-1-yl)propan-1-one, was found to be the most active (IC_{50}= 0.0112 µgml^{-1}) than the standards miltefosine (IC_{50} = 0.3±0.04 µgml^{-1}) and amphotericin B deoxycholate (IC_{50} = 0.2±0.02 µgml^{-1}) for *Leishmania donovani*. Compound III was found to be the most active (IC_{50} = 0.28±0.03 µgml^{-1}) and has comparable antileishmanial activity to the standard miltefosine (IC_{50} = 0.3±0.04 µgml^{-1}) and amphotericin B deoxycholate (IC_{50} = 0.2±0.02 µgml^{-1}) on *Leishmania aethiopica* amastigote.

Key words: Pyrazole derivative, biological screening, antileishmanial agent.

INTRODUCTION

Leishmaniasis is a group of vector-borne diseases caused by species of the genus *Leishmania,* a compulsory intra-cellular parasite of the mammalian host cell (dos Santos et al., 2011; Luiz et al., 2012). *Leishmania* parasites exist in two forms: amastigote in the mammalian host and a flagellated promastigote in the insect vector (dos Santos et al., 2011). Clinical manifestation of leishmaniasis occur in four major forms in humans: (i) visceral, the most severe and life-threatening form; (ii) cutaneous, originating as nodules and ulcers that may persist for years; (iii) diffuse cutaneous leishmaniasis, which is a long-lasting disease due to a deficient cellular-mediated immune response; and (iv) mucocutaneous, causing permanent lesions in the mouth, nose or genital mucosa (dos et al., 2011; Luiz et al., 2012; Sa´nchez-Moreno et al., 2012). This life-threatening disease that affects about 12 million people worldwide with 1.5 million to 2 million new cases of cutaneous leishmaniasis (CL) and 500,000 new cases of visceral leishmaniasis (VL) each year is endemic in the tropical and sub-tropical regions (Desjeux,

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1996). Endemic human leishmaniasis is reported in 88countries, majority of them are low-income countries (Desjeux, 1999). East Africa is one of the world's main endemic areas for VL, and over the last 20 years has gained dramatic increase in the number of VL cases, due to a complexity of factors (Reithinger et al., 2007). Several studies have convincingly shown that malnutrition, HIV and genetic susceptibility are individually responsible for VL (Bucheton et al., 2002). The epidemiological pattern of *Leishmania* species is changing, with a tendency to urbanization and geographic expansion. Despite the high worldwide prevalence, no vaccine for Leishmaniasis and complex vector control, few advances were made in the treatment of this disease ((dos et al., 2011; Marra et al., 2012).

The difficulty to control this parasitic disease remains a serious problem mainly due to the diversity of mammalian reservoirs (wild and domestic animals), species of vectors and *Leishmania* species (dos et al., 2011). Chemotherapy for leishmaniasis is generally ineffective mainly due to the emergence of drug-resistant strains and toxicity of the therapeutic agents (Marra et al., 2012) The pentavalent antimonials compounds, such as sodium stibogluconate (pentostan) and meglumine antimoniate (glucantime) are widely used as primary therapy, but they induce toxic side effects together with drug resistance (dos et al., 2011; Braga et al., 2007).

Amphotericin (AmBisome) is now the treatment of choice. Its failure in some cases to treat visceral leishmaniasis (*Leishmania donovani*) has been reported in Sundar (Sundar et al., 2007); but this may be related to host factors such as co-infection with HIV or tuberculosis rather than parasite resistance. Miltefosine (Impavido) is a new drug for visceral and cutaneous leishmaniasis. Paromomycin drug is thought to be an inexpensive and effective treatment for leishmaniasis (Mueller et al., 2007).

Pyrazole derivatives were found to possess various important biological activities, such as antibacterial (Samir et al., 2011; Nilesh and Manish, 2011), antiinflammatory (Adnan et al., 2008; Lingaiah et al., 2011), antioxidant (Ramesh and Chetan, 2011) ACE inhibitory (Marco et al., 2010), anti-cancer (Hai-Jun et al., 2010), MAO-B inhibitory (Nesrin et al., 2007), antidepressant (Mohamed et al., 2009), antiviral (Guiping et al., 2008), antimycobacterial (Ramaiyan et al., 2010; Daniele et al., 2008), antileishmanial (Bernardino et al., 2006; Naresh et al., 2006), and antimalarial (Katiyar et al., 2005; Cunico et al., 2006) activities.

These reports have been useful for biologist, chemists and pharmacists engaged in the development of new drugs and/or synthetic routes from pyrazoline derivatives. Pyrazoline derivatives were reported to possess significant *in vitro* anti-leishmanial activity (Bekhit et al., 2014). This has prompted the synthesis and investigation of safe, effective and cheap antileishmanial agent from pyrazoline derivatives containing phenyl or thiophenyl moiety in this research laboratory.

METHODOLOGY

^{1}H NMR spectra were recorded in Bruker Avance DMX400 FT-NMR spectrometer and IR spectra using Shimadzu 8400SP Spectro-photometer. For melting point and elemental analysis, Eelectro thermal IA9100 hot storage melting point apparatus and Perkin Elmer 2400 elemental analyzer were respectively used. Haemocytometer was used for counting leishmania parasites. Purity of the reaction products were checked by means of thin layer chromatography (TLC) using silica gel plate with fluorescent indicator, melting points, IR and ^{1}H NMR spectra.

Chemicals and reagents

Acetophenone, 2-acetylthiophene and hydrazine hydrate (Sigma Aldrich), ethanol, glacial acetic acid, propanoic acid, hydrochloric acid, KOH, absolute methanol, acetonitrile, chloroform, ethyl acetate, benzene, sodium citrate, distilled H_2O, dimethyl sulfoxide (BDH, England), alamar blue, RPMI 1640 were used throughout the experiments.

Parasite strain

L. donovani, a leishmanial parasite that causes visceral leishmaniasis in Africa and *L. aethiopica* the leading cause of cutaneous leishmaniasis in Ethiopia were used for the antileishmanial testing.

Standard drugs

Amphotericin B deoxycholate (Fungizone®, E R Squibb, UK) and miltifosine/hexadecylphosphocholine (A G Scientific, San Diego, CA, USA) were used as standard drugs in the determination of the antileishmanial activity of the synthesized compounds.

Synthesis of target compounds

The intermediate α, β unsaturated ketones (II and III) were synthesized by aldol condensation of 1-phenyl-3-p-tolyl-1H-pyrazole-4-carbaldehyde I with 2-acetylthiophene and acetophenone in alcoholic KOH. The target thienyl and phenyl pyrazolines (Figures 1 and 2) were synthesized by cyclization of the intermediate α, β unsaturated ketones (II and III) with hydrazine hydrate in ethanol or the appropriate aliphatic acid (Tuha et al., 2014)

Culture conditions

L. donovani and *L. aethiopica* were cultured in tissue flasks containing RPMI 1640 medium supplemented with 10% HIFCS and 100 IU penicillin and 100 μgml^{-1} streptomycin solution at 26°C (Tariku et al., 2010; Habtemariam, 2003; Seifert et al., 2010).

Stock solution and working concentration preparation

All the compounds tested (II, IIa, IIIa, IIb, III, IIIb, IIc) were dissolved in DMSO to a final concentration of 1 $mgml^{-1}$. Both test and standard solutions were serially diluted to appropriate

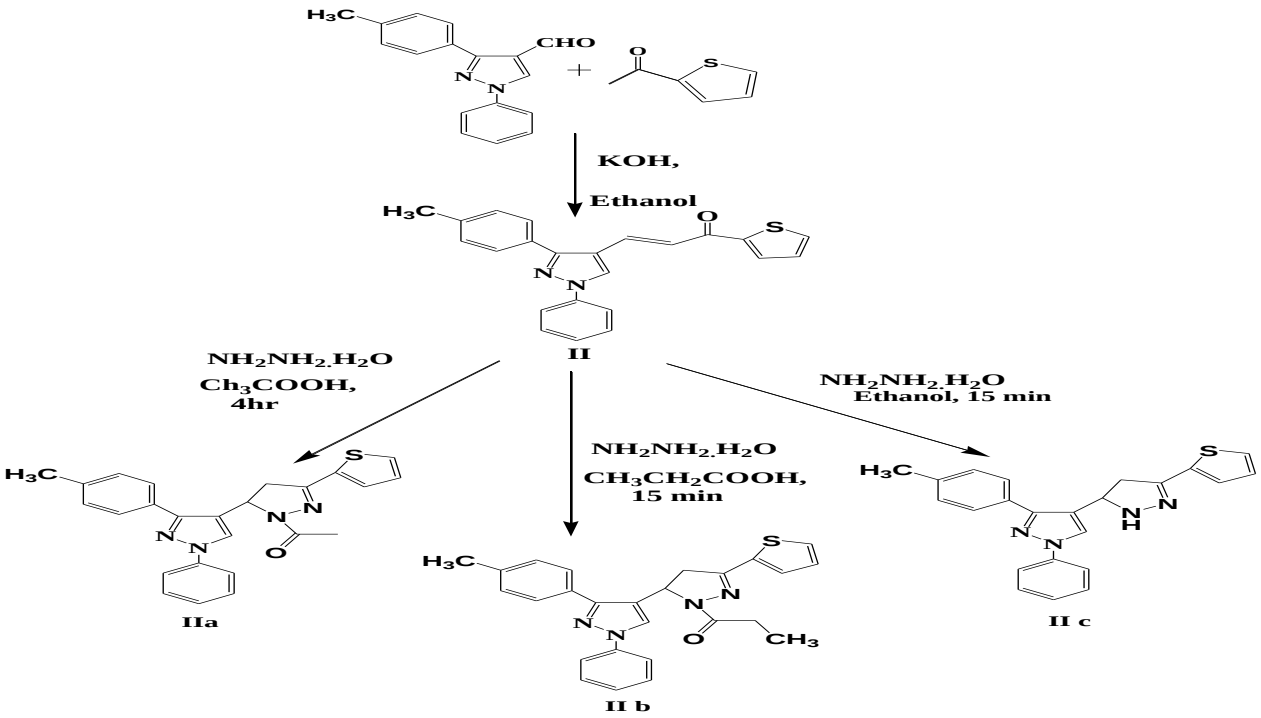

Figure 1. Scheme of synthesis of intermediate α, ß unsaturated ketone (II) and thienyl pyrazoline derivatives.

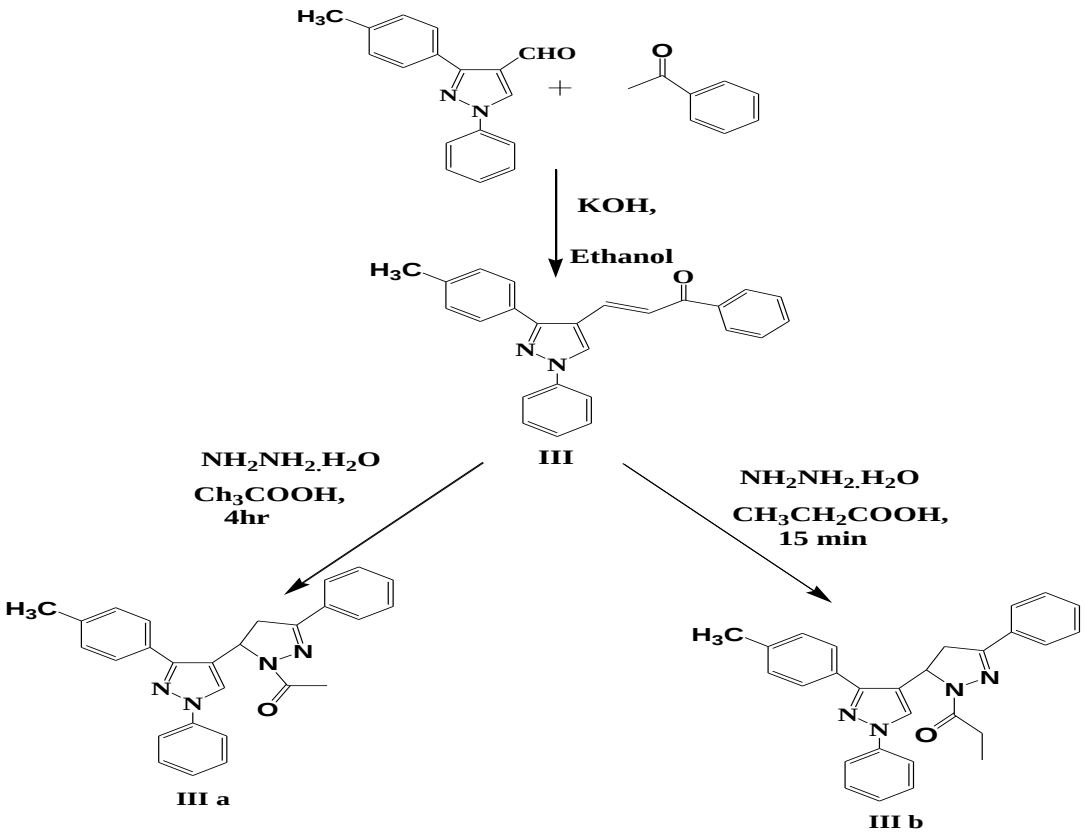

Figure 2. Scheme of synthesis of intermediate α, ß unsaturated ketone (III) and phenyl pyrazoline derivative.

Table 1. Antipromastigote and antiamastigote activity (IC_{50} µgml^{-1}) of the test compounds and reference standards.

Test compound	Activity IC_{50} (µgml^{-1})	
	Antipromastigote	Antiamastigote
Compound II	3.1143	1.84 ± 0.08
Compound IIa	2.0730	1.08 ± 0.14
Compound IIb	6.5310	1.29 ± 0.24
Compound IIc	0.1673	2.24 ± 0.34
Compound III	0.0422	0.28 ± 0.03
Compound IIIa	1.3076	2.861 ± 0.16
Compound IIIb	0.0112	4.22 ± 0.03
Miltefosine	3.1911	0.3 ± 0.04
Amphotericin B deoxycholate	0.0460	0.2 ± 0.02

IC_{50}: values indicate the effective concentration of a compound required to achieve 50 % growth inhibition in µg/ml.

concentrations using complete media. The test compounds were prepared by three fold serial dilutions from 10 µgml^{-1} to 0.04 µg ml^{-1}. Amphotericin B deoxycholate and miltefosine which were used as a positive control for comparison of the antileishmanial activities of the test compounds, were also made in three fold serial dilutions (Foroumadi et al., 2005)

Biological activity test

In vitro antipromastigote assay

Promastigote forms of *L. donovani* and standard drugs Amphotericin B deoxycholate and miltefosine were used for the assay. 3×10^6 promastigotes of *L. donovani* in 100 µl were seeded to each well in a 96 well flat bottom plate. Various dilutions (10, 3.33, 1.11, 0.37, 0.12, and 0.04 µgml^{-1}) of test compounds were added to the parasites. The tests were done in duplicates. Some of the wells contained only the standard drugs and served as a positive control. The media and DMSO alone were used as a negative control. The plates were kept at room temperature. After 24 h, 20 µl of Alamar blue (12.5 mg of resazurin dissolved in 100 mlof distilled water) (Yang et al., 2010) was added to each of the wells. Absorbance of the resulting mixture was measured after 48 h at a wavelength of 540 and 630 nm using Enzyme Linked Immuno Sorbent Assay (ELISA) plate reader (Al-Nasiry et al., 2007). A quantitative colorimetric assay using the oxidation-reduction indicator Alamar Blue was developed to measure cytotoxicity of the synthesized compounds against the protozoan parasite *Leishmania donovani*. The Alamar Blue assay permits a simple, reproducible and reliable method for screening antileishmanial drugs (Judith and Dietmar, 2001; Shimony and Jaffe, 2008; Nakayama et al., 1997).

***In vitro* antileishmanial activity on *L. aethiopica* amastigotes**

In a 96-well microtitre plate, test substances were serially diluted to final test concentrations of 0.04 to 10 µgml^{-1} in 50 µl culture medium and 50 µl suspensions of axenic amastigotes containing 2×10^7 cells/ml were added to each well. Contents of the plates were then incubated in humidified atmosphere containing 5% CO_2 at 31°C for 72 h. After 68 h of incubation, 10 µl of fluorochrome resazurin solution (12.5 µg dissolved in 100 ml of PBS, pH=7.2) was added into each well and the fluorescence intensity was measured after a total incubation time of 72 h using 37 Victor 3 Multilabel Counter at excitation wavelength of 530 nm and emission wavelength of 590 nm. The IC_{50} (µgml^{-1}) values for each extract were evaluated from sigmoidal dose-response curves using computer software Graph pad prism 3.0 and values expressed as mean + standard [SD] of triplicate experiments with each test concentration in duplicate. Assays with standard antileishmanial drugs and negative controls (medium alone and 1% DMSO) were also performed to have reference values. Also the background fluorescence intensity of each extract, essential oil and reference drug were measured (Habtemariam, 2003).

Data analysis

The IC_{50} values for synthesized compounds tested for their *in vitro* antileishmanial activity were evaluated from sigmoidal dose-response curves using non linear regression software (GraphPad Prism®; GraphPad Software, Inc., San Diego, CA).

RESULTS AND DISCUSSION

Biological assays

In vitro antipromastigote activity

The antipromastigote assay of the synthesized compounds was carried out according to the method described in the experimental part. The results obtained were analyzed and IC_{50} (µgml^{-1}) for each test compound was calculated using Graph pad prism software (Table 1). The result revealed that the synthesized compounds except compound IIb possess better antileishmanial activity than the standard drug miltefosine which has IC_{50} value 3.1911 µgml^{-1}. However, synthesized compounds except for compounds III and IIIb exhibited lower antileishmanial activity compared to the standard amphotericin B deoxycholate (IC_{50} = 0.0460 µgml^{-1}). Compound IIIb, the phenyl pyrazoline with propanoyl side chain, was found to be the most active (IC_{50} = 0.0112 µg ml^{-1}) compound as compared to the standard miltefosine (IC_{50} = 3.1911 µg ml^{-1}) and amphotericin B deoxycholate

(IC_{50} = 0.0460 µg ml^{-1}). Compared to study done by Vikramdeep et al. (2014) and Manuel et al. (2012), this research reveal that the phenyl pyrazoline derivative compound III and IIIb have better antileishmanial activity with IC_{50} value of 0.0422 and 0.0112 µgml^{-1}, respectively. This might be due to the formation of hydrogen bonding between its carbonyl group and backbone of certain receptor active site in the former compound III, and the presence of propanoyl group in the latter compound IIIb, may play a role in the interaction with vital important biochemical process. The thienyl pyrazoline derivative, 1-phenyl-4-(3-(thiophen-2-yl)-4,5-dihydro-1H-pyrazol-5-yl)-3-p-tolyl-1H-pyrazole (compound IIc) the non-substituted compound IIc (IC_{50} = 0.1673 µgml^{-1}) seems to affect positively the biological activity leading to the better antileishmanial activity when compared with ethanyol (CH_3CO-) compound IIa and propanoyl (CH_3CH2CO-) compound IIb substituted compound and study done by Pinheiro et al. (2012).

The phenyl derivative displayed better activity than the corresponding thienyl derivatives for leishmania activity. Regarding the thienyl derivatives of the pyrazolines, activity decreased with the increase in the carbon number of aliphatic substitution at pyrazoline N_1 from H to propanoyl group. However, the activity increased with increasing the length of the side chain in the phenyl pyrazolines. This could be attributed to the associated increase in hydrophobicity of the compounds that increases hydrophobic interaction with the molecular target site.

In vitro antiamastigote activity

The antiamastigote assay of the synthesized compounds, standard drug miltefosine and amphotericin B deoxycholate on *L. aethiopica* was carried out according to the method described in the experimental part. The results obtained were analyzed and IC_{50} for each test compound was calculated using Graph pad prism software (Table 1). The result showed that the synthesized compounds except for compounds III (IC_{50} = 0.28±0.03 µgml^{-1}), possess lower antileishmanial activity than the standard drug miltefosine and amphotericin B deoxycholate that have IC_{50} value 0.3±0.04 and 0.2±0.02 µgml^{-1}, respectively. Compound IIIb which exhibited the highest antipromastigote activity, has shown the least antiamastigote activity, while compound III has almost comparable activity with the standard drug miltefosine and amphotericin B deoxycholate.

Conclusion

Seven pyrazole derivatives were synthesized using aldol condensation and subsequent cyclization reactions in a good yield (71.39 to 95.20%). The compounds were purified with recrystallization method and were characterized by elemental microanalysis, IR, and ^1H NMR spectroscopy. *In vitro* antileishmanial activity was conducted using Alamar blue reduction method and the results revealed that synthesized compounds showed better antileishmanial activity than the standard drug miltefosine. But all the synthesized compounds except for compounds III and IIIb exhibited lower antileishmanial activity compared with the standard amphotericin B deoxycholate.

Moreover, the phenyl pyrazolines showed better antileishmanial activity compared with the thienyl pyrazolines and their activity increased with increased number of carbons in the side chain. Compound IIIb, 1-(3-phenyl-5-(1-phenyl-3-p-tolyl-1H-pyrazol-4-yl)-4,5-dihydropyrazol-1-yl)propan-1-one phenyl pyrazoline, is found to be the most active (IC_{50} = 0.0112 µgml^{-1}) and this compound could represent a fruitful matrix for the development of antileishmanial agents that would deserve further derivatization and investigation. Among seven synthesized compounds, compounds III is found to be the most active (IC_{50} = 0.28±0.03 µgml^{-1}) and has comparable antileishmanial activity to the standard miltefosine and amphotericin B deoxycholate *on L. aethiopica* amastigotes.

Conflict of Interests

The authors have not declared any conflict of interests.

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