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CORRELATION AMONG ANTIDIABETIC POTENTIAL, BIOCHEMICAL PARAMETERS AND GC-MS ANALYSIS OF THE CRUDE EXTRACTS OF *JUSTICIA ADHATODA* L.

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Abstract

In Pakistan, the number of people with diabetes is steadily growing and adverse side effects are frequently reported for current antidiabetic therapies. *Justicia adhatoda* L. belongs to Acanthaceae family. Several biological activities are known to occur in different parts of this plant. The effects of *Justicia adhatoda* leaves on diabetes are very little known. The aim of this study was to evaluate the antidiabetic effect of different extracts on diabetic mice and to use gas chromatography-mass spectroscopy (GC-MS) to evaluate the chemical constituents present in the *Justicia adhatoda* extract.Air dried leaves of *Justicia adhatoda* (JA) were separately extracted with methanol, ethanol and ethyl acetate. The antidiabetic effect of JA-ME, JA-EE and JA-EAE was assessed separately on diabetic mice at the concentration of 200 and 400 mg/kg body weight. Fasting mean levels of blood glucose in normal, untreated diabetic and diabetic mice treated with JA-ME, JA-EE and JA-EAE was performed before and after treatment for four weeks. The results indicate thatusing the extracts for 28 days, blood glucose level reduced in all extracts. All extracts reduced high cholesterol, triglycerides, LDL, VLDL, bilirubin, ALT, ALP, AST, urea, creatinine and uric acid levels and increased HDL levels. At 400 mg/kg, the JA-ME shows the highest percentage reduction in blood glucose levels. All major components that GC-MS confirms are molecules that are biologically active indicating that certain medicinal properties may be present in the plant.

Keywords: *Justicia adhatoda*, Diabetes mellitus, Antidiabetic activity, Blood glucose level, Glibenclamide, Gas chromatography-mass spectroscopy analysis.

Introduction

Diabetes mellitus is a major disease worldwide (Palanisamy *et al*., 2011). Diabetes is a metabolic disorder due to insulin secretion and/or insulin deficiencies connected with carbohydrate, fat and protein homeostasis failure (Barcelo & Rajpathak, 2001). High blood sugar is the third biggest risk factor for premature mortality after fatalities linked to increased blood pressure and tobacco use. It has been estimated that 415 million i.e. 8.8 percent adults aged between 20 to 79 has diabetes worldwide in 2015. By 2040, this value is anticipated to increase to 642 million (10.4%) or one in ten adults (Anon., 2015).Diet, physical exercise and contemporary medications can manage diabetes mellitus (Koski, 2006). Different extracts from medicinal plants were used globally to treat diabetes. They are regarded inexpensive, less toxic and have no side effects (Gupta *et al*., 2008).

Some medicinal plants that contain toxic components like many drugs derived from the cytotoxic anticancer plants. Phytotherapeutic side effects are less common than synthetic drugs (Calixto, 2000). Diabetes management without any side effects is a challenge for the medical community. Pharmacokinetic characteristics, secondary insufficiency rates and side effects restrict medicinal treatment (Stalin *et al*., 2013). The search for new medicines continues in the treatment of diabetes despite the significant progress with hypoglycemic agents (Osadebe *et al*., 2014). Insulin and other antidiabetic agents are the available diabetes therapies. Most of the antidiabetic agents are accompanied by serious side effects (Shetti *et al*., 2012). It has been noted that medicinal plants with antidiabetic activity are a useful tool to find safer hypoglycaemic agents (Sunila *et al*., 2012).

To discover new compounds against diabetes mellitus, these plants are the primary source for the drug development. Therapeutically applied plants have bioactive composites (flavonoids, glycosides, saponins, alkaloids, phenolics, tannins and vitamins) (Ghani, 2003). Medicinal plants rely on approximately 80-85% of populations. The extracts and their active components are used to meet their primary health needs (Elujoba *et al*., 2005; Ignacimuthu *et al*., 2006).The search for powerful pharmacologically active agents in human disease treatment could play an important role (Alim *et al*., 2012).

Justicia adhatoda L. is an evergreen shrub belongs to Acanthaceae family. It is commonly used in medicine preparation. It is a prevalent tiny evergreen, sub-herbaceous bush spread throughout Pakistan, Sri Lanka India, Malaysia and Burma. Common names are Vasaka, Malabarnut, Adusa, Ardusi, Bhekkar, Adhatodai and Basak (Prajapati *et al*., 2003; Bjaj & Williams, 1995). *Justicia adhatoda* contains alkaloids, anthraquninone, flavonoids, saponins, phytosterols, triterpenoids and polyphenols (Jayapriya & Shoba, 2015). The leaves are used in Southeast Asia for wounds, diseases of the skin, headache, haemorrahge and leprosy (Adnan *et al*., 2010).

Justicia adhatoda leaves are used for cough (Lal & Yadav, 1983), for haemorrahge and urinary problem (Pushpangadan *et al*., 1995), stopping bleeding (Reddy *et al*., 1989), jaundice (Reddy *et al*., 1988), asthma and tuberculosis (Jain & Puri, 1984). The root extract of

Justicia adhatoda is used against diabetes and various disorders of liver (Bhat *et al*., 1978). In the South-East Asia, the root is used to treat malaria, tuberculosis and eye diseases and flowers are used in opthalmia for the therapy of fever, gonorrhea, cold, cough, antispasmodic, phthisis, asthma and bronchitis (Atta-ur-Rahman*et al*., 1986).

The aim of this research was to systematically evaluate and compare the hypoglycemic effect of *Justicia adhatoda* methanolic, ethanolic and ethyl acetate extracts and standard hypoglycemic agent such as glibenclamide, and to record the *Justicia adhatoda* leaf extract GC-MS profile.

Materials and Methods

Collection and identification: During the month of October 2015, the *Justicia adhatoda* fresh leaves were collected from Murree Hills, Punjab, Pakistan, and authenticated by Professor Dr. Ibrar Shinwari, Department of Environmental Sciences, International Islamic University Islamabad and Prof. Dr. Muhammad Zafar, Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan. A voucher sample of *Justicia adhatoda* with accession number 129782 was submitted for future reference to the Herbarium of Pakistan, Quaid-i-Azam University, Islamabad.

Preparation of extracts: The leaves of *Justicia adhatoda* were washed under the running water and then dried for three weeks at ambient temperature. The dried material of *Justicia adhatoda* was pulverized into powder and stored in the airtight containers for further use. Two hundred g powdered plant were macerated at room temperature for 24 h and using 1000 mm analytical grade solvents in the 2000 ml flask. To obtain the final extract, the extracts were concentrated in the rotary evaporator (Buchi, Switzerland) by vacuum evaporation. The extractswere then dried at 40 °C in a vacuum oven (Yamato, Japan). The dried extracts of *Justicia adhatoda* were collected and stored for further analysis at 4 °C in an air tight container.

Antidiabetic activity

Experimental animals: BALB/c male mice were raised in the animal house of National Institute of Health, Islamabad. The weights were between 25-35 g, eight weeks old. The animals were kept in polypropylene cages. With a light/dark cycle of 12 h/12 h, we put 6 mice per cage at 25°C. The pellets and water were given to the animals throughout the experiment. Animal handling was conducted according to internationally accepted ethical guidelines. The research protocol was endorsed by the International Islamic University's Institutional Review Board, Islamabad (Letter No. IIU (BI&BT)/FBAS-IBBC-05).

Acute toxicity test: The various extracts of *Justicia adhatoda* were tested for acute toxicity. The animals were fasted only taking water overnight. Each mouse's weight was recorded before all crude extracts were given to the mice. The animals have been split into the three treatment groups. Each group consisted of six mice. Only the vehicle was received by the control group. At different concentrations upto 5000 mg/kg body weight, the treatment groups received the methanolic extracts of *Justicia adhatoda* Food was available after the extracts were given. The animals were observed daily for two weeks (Burger *et al*., 2005).

Induction of diabetes: BALB/c male mice were fasted for 12-14 h. The fasting blood glucose levels were recorded by glucometer. After that the mice were made diabetic with alloxan monohydrate. Alloxan monohydrate was prepared prior to injection by weighing the individual weight of the animal. Thirty minutes after the administration of alloxan, food and water was given to the mice (Carvalho *et al*., 2003). The blood glucose level of each mouse has been determined from the tail after 48 h of alloxan injection. In this study, mice above 200 mg/dl with fast blood glucose were included (Gidado *et al*., 2005).

Experimental design: Fifty-four 25-35 g BALB/c male mice were housed in the aluminum cages. Water and standard chow supply ad libitum were given to the mice. Mice were acclimatized in normal laboratory circumstances for one week (25 ± 1 °C temperature, 12 h light/dark cycle and 50-60% relative humidity) prior to the start of the experiment. All the mice were randomly distributed in nine groups of six members each. Thrice a week on the alternate days, the samples were given from day one to the twenty-eighth days to the respective groups. Experimental mice were split into the groups below:

1) Group I Normal control: In this group, these were normal mice who received normal saline.
2) Group II Diabetic control: Mice administering diabetes with 150 mg/kg body weight alloxan monohydrate but receiving no treatment.
3) Group III Positive control: Diabetic mice were orally given 10 mg/kg body weight glibenclamide.
4) Group IV: Test group with oral administration of diabetic mice with *Justicia adhatoda* methanolic extract (JA-ME) 200 mg/kg body weight.
5) Group V: Test group with oral administration of diabetic mice with *Justicia adhatoda* methanolic extract (JA-ME) 400 mg/kg body weight.
6) Group VI: Test group with oral administration of diabetic mice with *Justicia adhatoda* ethanolic extract (JA-EE) 200 mg/kg body weight.
7) Group VII: Test group with oral administration of diabetic mice with *Justicia adhatoda* ethanolic extract (JA-EE) 400 mg/kg body weight.
8) Group VIII: Test group with oral administration of diabetic mice with *Justicia adhatoda* ethyl acetate extract (JA-EAE) 200 mg/kg body weight.
9) Group IX: Test group with oral administration of diabetic mice with *Justicia adhatoda* ethyl acetate extract (JA-EAE) 400 mg/kg body weight.

Oral glucose tolerance test: The mice were fasted twelve hours on the test day. They were given a dose of 5 g/kg of glucose orally. The blood glucose concentrations were 0 (before glucose injection) and 2, 4, 6 and 8 h after glucose administration. Blood samples from the tail were collected (Kumar *et al*., 2006).

Assessment of body weight: After 72 h, the body weights were measured after the mice were confirmed as diabetic. After the twenty-eight days, the mice's body weights were measured using the digital weighing balance.

Collection of blood sample: On the last day of the experiment i.e., twenty-eight days, the mice were anaesthetized by chloroform inhalation. Blood samples were collected through the abdominal aorta in the tubes under anesthesia for biochemical investigations. After centrifuge, the blood samples and serums were separated at 6000 rpm for 15 minutes. The serums were analyzed for biochemical investigations.

Determination of biochemical parameters: The blood samples were permitted to coagulate at 25°C for 45 minutes. The serum was centrifuged for 15 minutes at 6000 rpm. Serum replicates were analyzed for ALT, AST, ALP and bilirubin. The concentrations of serum glucose, TC, TG, HDL, LDL and VLDL were enzymatically measured using kits. For the assessment of liver function tests, the AMP diagnostic kits were used (LFTs) (Shah *et al*., 2013).

Gas chromatography-mass spectrometry (GC-MS) analysis: Using the (Agilent 7890A/5975C) auto sampler, GC-MS was used to conduct the chromatographic procedure. Thousand parts per million solutions in methanol, ethanol and ethyl acetate were prepared from methanolic, ethanolic and ethyl acetate plant extracts. Using the DB-5MS Column (30 meters up to 0.25 mm and 0.25µm film thickness), one µL of each sample was injected. The helium gas was used as a carrier gas at the flow rate of 1 ml/min. The analysis was conducted for 2 minutes using the initial temperature programming of 50˚C. After that, the ramp rate is up to 130˚C/min. Then the 12˚C ramp/min at 180˚C temperature. After that raised the temperature to 280˚C and retained it for 15 minutes at 3˚C/min. The temperature of the ion source was set at 250˚C and the temperature of the injection port was set at 250˚C. The entire running time was 58.5 minutes. The device was operated in electron impact mode with 70ev electron power. The scanning range of mass spectral was set at 10-1050 m/z. We interpret the GC-MS mass spectrum using the NIST database (Abirami & Rajendran, 2012).

Statistical analysis

In this study, the data obtained was presented as mean ± standard deviation. In order to determine the variability between groups, one-way AVOVA was performed by Statistix 8.1. Multiple comparisons of the Tukey's and Kruskal-Wallis tests calculated the significant differences between the *In vivo* treatment groups. The statistical significance at $p<0.05$ was set.

Results

Antidiabetic activity of *Justiciaadhatoda*

Effect of *Justicia adhatoda* extracts on oral acute toxicity study: For the determination of LD_{50} value, acute toxicity study is performed in the experimental animals. The LD_{50} determination was conducted in mice by OECD guideline 423. This study shows that no observable signs of toxicity were generated by various extracts of *Justicia adhatoda* upto the dose of 5000 mg/kg. This was verified by the absence of major changes in the behaviors of mice such as weight loss, breathing, restlessness, diarrhea and coma etc. For two weeks, no death was observed. This result confirms that the mice have an average lethal dose (LD_{50}) of more than 5000 mg/kg.

Effect of *Justicia adhatoda* extracts on oral glucose tolerance test: Fig. 1 shows blood glucose concentrations of control mice, diabetic mice caused by alloxan and diabetic mice treated by conventional medication (glibenclamide) and various extracts at different times after oral glucose administration. There was no reduction in blood glucose concentrations in normal control mice. After 2 h, there was an increase in blood glucose concentrations and remained high in diabetic control mice over the next 8 h. Diabetic mice treated with glibenclamide reduced blood sugar levels. Methanol, ethanol and ethyl acetate extracts of *Justicia adhatoda* significantly lower the blood glucose levels at 4 h and remained low at 200 and 400 mg/kg doses over the next 4 h. The methanolic leaf extract of *Justicia adhatoda*, ethanolic leaf extract of *Justicia adhatoda* at 400 mg/kg and glibenclamide lowered the blood glucose concentrations in diabetic mice considerably after 8 h of treatment.

Effect of *Justicia adhatoda* extracts on the blood glucose level in mice after 28 days treatment: Fig. 2 demonstrates blood glucose concentrations of control mice, diabetic mice caused by alloxan and dziabetic mice treated by conventional medication (glibenclamide) and various extracts on different days (0 to 28th day) following oral glucose administration. The levels of blood glucose of diabetic mice treated with alloxan increases when compared with the normal mice. The level of blood glucose increased from 260.67 ± 10.33 mg/dl to 273.67 ± 13.00 mg/dl on 28th day. After treatment with oral administration of JA-ME, JA-EE and JA-EAE at 200 mg/kg and 400 mg/kg, blood sugar levels were lower compared to diabetic mice. Mice's blood glucose level in JA-ME at 200 mg/kg and 400 mg/kg decreased from 277.67 ± 16.68 mg/dl to 199.00 ± 6.07 mg/dl and 299.67 ± 6.15 mg/dl to 206.50 ± 8.69 mg/dl respectively. The level of blood glucose of mice in JA-EE at 200 mg/kg and 400 mg/kg decreased from 251.83 ± 11.96 mg/dl to 166.33 ± 5.7 mg/dl and 297.17 ± 3.61 mg/dl to 198.00 ± 10.81 mg/dl respectively. The level of blood glucose of mice in JA-EAE at 200 mg/kg and 400 mg/kg decreased from 240.17 ± 20.50 mg/dl to 180.33 ± 10.19 mg/dl and 257.83 ± 9.49 mg/dl to 184.33 ± 8.06 mg/dl respectively. Glibenclamide also decreased the blood glucose level in mice after 28 days treatment (255.50 ± 10.15 mg/dl to 111.67 ± 5.47 mg/dl). After 28 days treatment, methanolic leaf extract of *Justicia adhatoda* at 400 mg/kg, *Justicia adhatoda* ethanolic leaf extract at 200 mg/kg, *Justicia adhatoda* ethanolic leaf extract at 400 mg/kg and the glibenclamide lower the blood sugar level in diabetic mice.

Effect of *Justicia adhatoda* extracts on the body weight: The body weight of normal control mice was increased from 28.67 ± 2.73g to 32.33 ± 1.75 g. The body weight of diabetic mice has been significantly reduced from 28.33 ± 2.80 g to 23.67 ± 1.21 g. All the extracts show improvement in the body weight after treatment with the extracts, when compared with the diabetic and positive treated groups except the JA-EAE at 200 mg/kg. The mice's body weight in JA-ME at 200 mg/kg slightly increased from 28.67 ± 3.50g to 29.83 ± 2.31 g and at 400 mg/kg slightly increased from 31.33 ± 2.42g to 32.67 ± 1.50 g. The body weight of mice in JA-EE at 200 mg/kg slightly increased from 30.83 ± 1.83 g to 31.33 ± 1.21 g and at 400 mg/kg slightly increased from 31.67 ± 0.82 g to 32.83 ± 1.17 g. The body weight of mice in JA-EAE at 200 mg/kg slightly decreased from 28.67 ± 1.63 g to 27.67 ± 2.80 g and at 400 mg/kg slightly increased from 27.83 ± 2.04g to 28.67 ± 2.42 g (Fig. 3).

Effect of *Justicia adhatoda* extracts on the serum lipid profile: The Alloxan induced group showed significant elevation of the levels of cholesterol, triglycerides, LDL, VLDL and decreased the level of HDL. After the 28 days treatment with the JA-ME, JA-EE and JA-EAE at 200 mg/kg and 400 mg/kg, decreased the higher levels of cholesterol, triglycerides, LDL, VLDL and significant increased in HDL level when compared to diabetic mice. Standard medication glibenclamide also decreased the total cholesterol, triglycerides, LDL, VLDL levels and increased the HDL level (Table. 1).

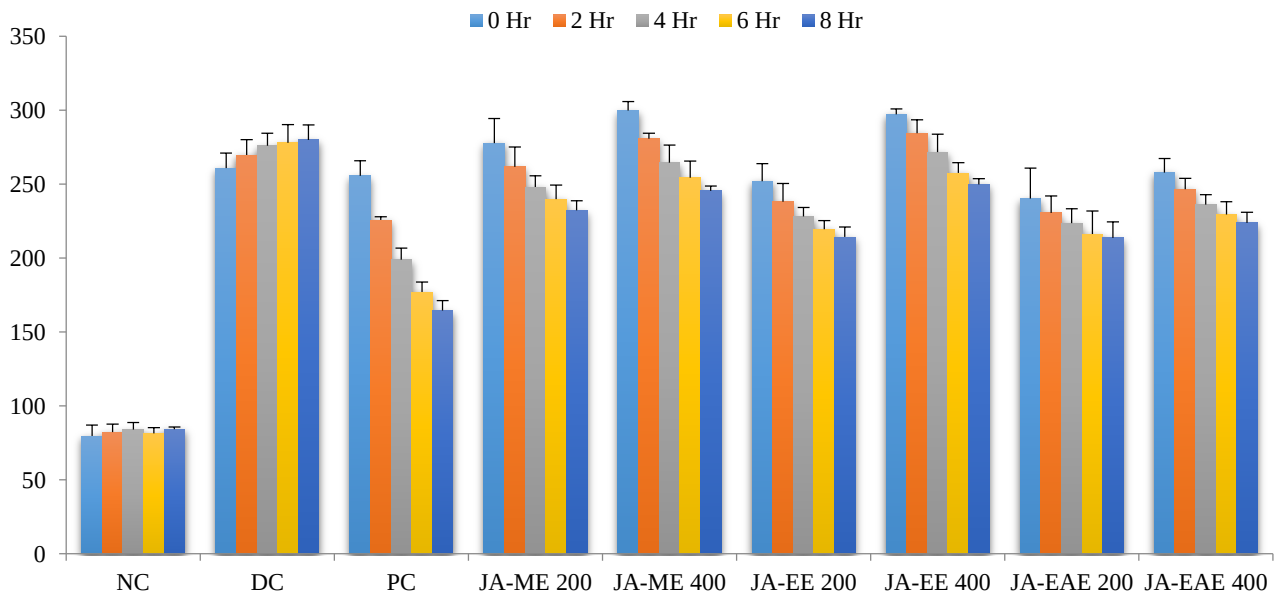


Fig. 1. Effect of *Justicia adhatoda* extracts on oral glucose tolerance test. Results are expressed as mean ± standard deviation (n=6). NC (Normal control), DC (Diabetic control), PC (Positive control), JA-ME (*Justicia adhatoda* methanolic extract), JA-EE (*Justicia adhatoda* ethanolic extract), JA-EAE (*Justicia adhatoda* ethyl acetate extract).

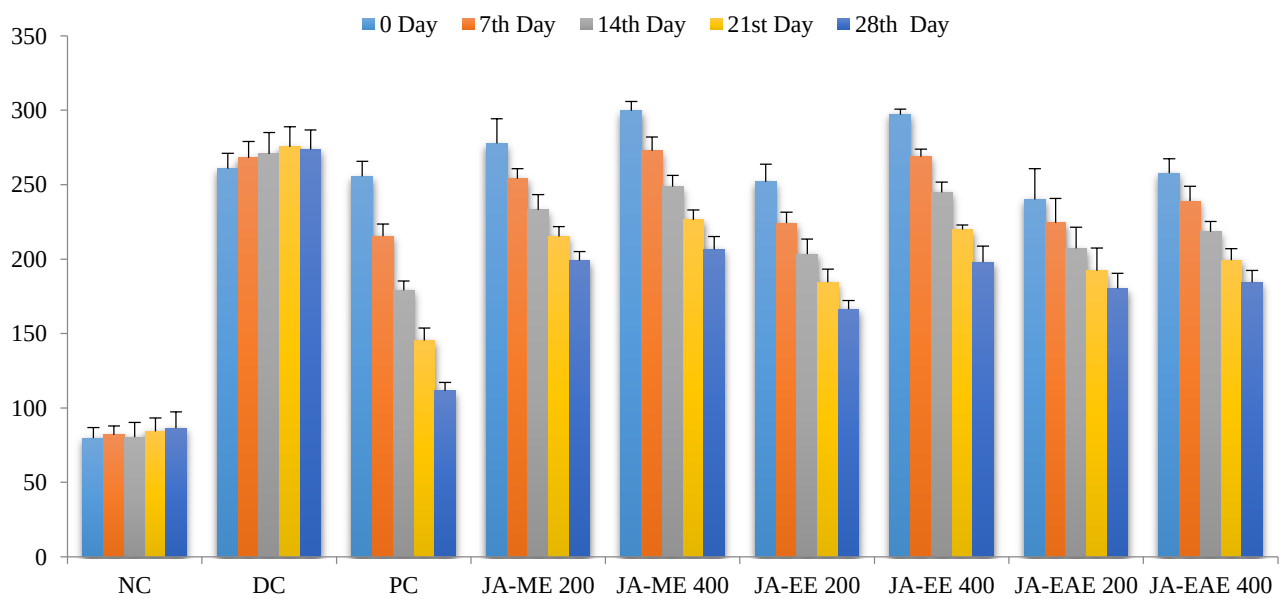


Fig. 2. Effect of *Justicia adhatoda* extracts on the blood glucose level in mice after 28 days. Results are expressed as mean ± standard deviation (n=6).

Table 1. Effect of *Justicia adhatoda* extracts on the serum lipid profile.

Groups	TC (mg/dl)	TG (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
NC	100.83 ± 5.81^{d}	91.33 ± 6.25^{c}	42.11 ± 2.87^{a}	61.83 ± 3.92^{f}	18.30 ± 1.27^{c}
DC	186.33 ± 7.14^{a}	141.17 ± 6.52^{a}	26.05 ± 2.56^{f}	136.50 ± 5.43^{a}	28.23 ± 1.30^{a}
PC	106.67 ± 6.47^{d}	97.50 ± 7.09^{c}	39.79 ± 1.45^{ab}	70.33 ± 3.98^{e}	19.50 ± 1.42^{c}
JA-ME 200	135.17 ± 2.56^{bc}	114.67 ± 3.26^{b}	33.37 ± 2.44^{de}	94.00 ± 3.58^{c}	22.93 ± 0.65^{b}
JA-ME 400	133.00 ± 3.03^{bc}	110.50 ± 3.27^{b}	36.43 ± 1.91^{bcd}	87.17 ± 3.43^{cd}	22.10 ± 0.65^{b}
JA-EE 200	133.83 ± 2.32^{bc}	111.17 ± 5.27^{b}	34.43 ± 1.63^{cde}	92.67 ± 3.73^{cd}	22.20 ± 1.05^{b}
JA-EE 400	130.67 ± 5.16^{c}	109.67 ± 3.98^{b}	37.65 ± 1.57^{bc}	85.67 ± 2.94^{d}	21.93 ± 0.79^{b}
JA-EAE 200	141.17 ± 3.54^{b}	117.33 ± 2.50^{b}	30.73 ± 1.00^{e}	107.50 ± 4.18^{b}	23.47 ± 0.51^{b}
JA-EAE 400	140.33 ± 3.67^{b}	118.67 ± 3.93^{b}	33.63 ± 2.57^{de}	104.33 ± 2.06^{b}	23.73 ± 0.79^{b}

Results are expressed as mean ± standard deviation (n=6). Means are considerably distinct (p<0.05) from each another in the column with distinct superscript (a-f) letters

Table 2. Effect of *Justicia adhatoda* extracts on the liver function markers.

Groups	Bilirubin (mg/dl)	ALT (u/l)	ALP (u/l)	AST (u/l)
NC	0.42 ± 0.03^{d}	30.67 ± 2.87^{c}	114.67 ± 5.17^{e}	26.83 ± 4.70^{e}
DC	1.73 ± 0.13^{a}	70.83 ± 6.52^{a}	278.67 ± 20.38^{a}	91.67 ± 7.03^{a}
PC	0.52 ± 0.04^{d}	35.17 ± 2.99^{c}	128.33 ± 7.11^{e}	37.33 ± 4.67^{d}
JA-ME 200	0.84 ± 0.04^{c}	44.67 ± 3.78^{b}	168.67 ± 9.10^{bcd}	44.67 ± 4.55^{bcd}
JA-ME 400	0.83 ± 0.03^{c}	43.00 ± 2.76^{b}	163.67 ± 6.50^{cd}	43.17 ± 3.76^{cd}
JA-EE 200	0.83 ± 0.04^{c}	46.83 ± 4.71^{b}	160.17 ± 8.91^{cd}	46.67 ± 1.37^{bc}
JA-EE 400	0.79 ± 0.02^{c}	47.17 ± 2.40^{b}	157.00 ± 2.09^{d}	45.67 ± 3.45^{bc}
JA-EAE 200	1.03 ± 0.10^{b}	49.17 ± 2.32^{b}	182.00 ± 5.51^{b}	51.33 ± 3.27^{b}
JA-EAE 400	0.99 ± 0.07^{b}	48.83 ± 1.94^{b}	177.00 ± 6.63^{bc}	49.17 ± 3.54^{bc}

Results are expressed as mean ± standard deviation (n=6). Means are considerably distinct (p<0.05) from each another in the column with distinct superscript (a-e) letters

Table 3. Effect of *Justiciaadhatoda* extracts on the kidney function markers.

Groups	Urea (mg/dl)	Creatinine (mg/dl)	Uric Acid (mg/dl)
NC	30.67 ± 2.73^{f}	0.94 ± 0.11^{d}	3.87 ± 0.37^{f}
DC	75.17 ± 2.86^{a}	2.17 ± 0.28^{a}	9.84 ± 0.27^{a}
PC	37.33 ± 1.86^{e}	1.14 ± 0.15^{cd}	4.11 ± 0.23^{f}
JA-ME 200	49.00 ± 2.83^{bcd}	1.43 ± 0.06^{b}	4.73 ± 0.22^{e}
JA-ME 400	45.83 ± 1.94^{d}	1.34 ± 0.07^{bc}	4.56 ± 0.15^{e}
JA-EE 200	47.17 ± 2.56^{cd}	1.49 ± 0.04^{b}	5.39 ± 0.17^{cd}
JA-EE 400	48.17 ± 4.40^{cd}	1.44 ± 0.05^{b}	4.98 ± 0.25^{de}
JA-EAE 200	54.00 ± 4.38^{b}	1.55 ± 0.07^{b}	5.94 ± 0.06^{b}
JA-EAE 400	52.33 ± 2.51^{bc}	1.54 ± 0.03^{b}	5.64 ± 0.18^{bc}

Results are expressed as mean ± standard deviation (n=6). Means are considerably distinct (p<0.05) from each another in the column with distinct superscript (a-f) letters

Effect of *Justicia adhatoda* extracts on the liver function markers: The Alloxan induced group increased the levels of bilirubin, ALT, ALP and AST. After the 28 days treatment with the JA-ME, JA-EE and JA-EAE at 200 mg/kg and 400 mg/kg, decreased the concentrations of bilirubin, ALT, ALP and AST when compared to diabetic mice. Standard medication glibenclamide also decreased the bilirubin, ALT, ALP and AST levels (Table 2).

Effect of *Justicia adhatoda* extracts on the kidney function markers: The Alloxan induced group increased the urea level, creatinine and uric acid. After the 28 days treatment with the JA-ME, JA-EE and JA-EAE at 200 mg/kg and 400 mg/kg, lower the levels of urea, creatinine and uric acid. Standard medication glibenclamide also decreased the urea, creatinine and uric acid levels (Table 3).

Gas chromatography-mass spectrometry (GC-MS) analysis of *Justicia adhatoda*: *Justicia adhatoda* ethanolic leaf extract has been selected for GC-MS analysis. Upon GC-MS analysis, the extract contained 56 chemical constituents eluted between 5.36 and 57.71 minutes (Fig. 4). These compounds belong to distinct classes of chemicals and most of them show significant biological activities. Fig. 4 demonstrates the *Justicia adhatoda* leaf extract chromatogram. Table 4 presents the compounds recognized with their peak number, retention time (RT), peak area (%), molecular formula, weight and structure. The identified major compounds were 9,12,15-Octadecatrienoic acid, (Z,Z,Z) (11.92%), Phytol, acetate (9.47%), n-Hexadecanoic acid (6.40%), β-Sitosterol (3.87%), 9,12-Octadecadienoic acid (Z,Z) (3.86%), Phytol (3.73%), Squalene (3.50%), 9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z) (3.45%), 5-Hydroxymethylfurfural (2.41%), Nonadecane (2.25%), Lethane (2.10%), Lupeol (1.92%), Phenol, 2-methyl-5-(1-methylethyl) (1.85%), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (1.71%), Hexadecanoic acid, ethyl ester (1.50%), Stearyltrimethylammonium chloride (1.48%), Neophytadiene (1.40%), Stigmasterol (1.40%), Linoleic acid ethyl ester (1.37%), Undecanoic acid, ethyl ester (1.36%), Thiirane, (methoxymethyl)- (1.34%), 1,6-Naphthyridine (1.33%), Tricosane, and 2-methyl- (1.32%).

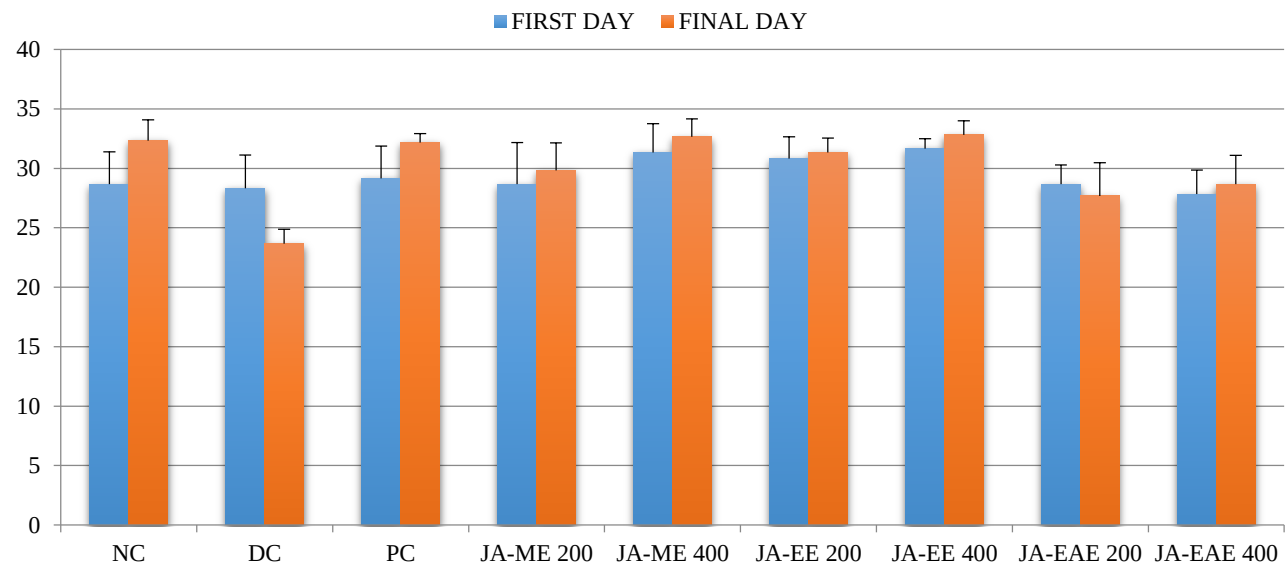


Fig. 3. Effect of *Justicia adhatoda* extracts on the body weight. Results are expressed as mean ± standard deviation (n=6).

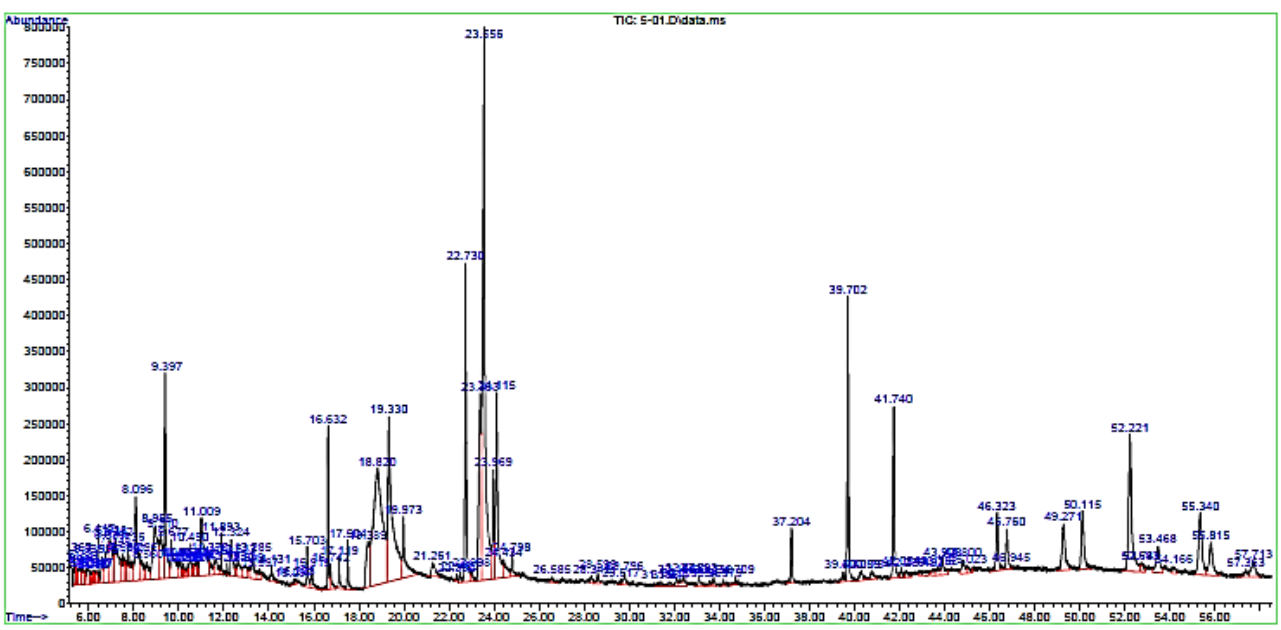


Fig. 4. GC-MS chromatogram of ethanolic leaf extract of *Justicia adhatoda*.

Discussion

Diabetes mellitus is a multi-factor disease. It affects the health, quality of life and likelihood of the patient. According to data from 2008, 230 million people worldwide have diabetes (Arumugan *et al*., 2008). Diabetes is the result of damage to Langerhan islets cells. This makes the body produce the pancreatic hypoglycemic hormone called insulin. Excessive urine, urinary glucose and high blood glucose are the main signs of diabetes (Koffi *et al*., 2009). Diabetes changes the metabolism of glucose and lipids (Rajasekaran *et al*., 2006).

Justicia adhatoda is one of the many adjuvant plants for diabetes treatment. The World Health Organization has pointed out that its complications are not only a major challenge for the future. They are also crucial in order to achieve health for all (Anon., 2008). The study strongly emphasized the need for ideal and rational use of traditional and natural medicine systems in any particular country's health care systems. This research was aimed at studying the impact of *Justicia adhatoda's* methanolic, ethanolic and ethyl acetate leaf extract on alloxan induced diabetic mice using glibenclamide as a reference drug. The results showed that all extracts of *Justicia adhatoda* had a hypoglycemic effect on the diabetic mice.

The acute toxicity research demonstrated that methanolic, ethanolic and ethyl acetate leaf extract of *Justicia adhatoda* has produced no observable toxicity indications up to a dose of 5000 mg/kg. There is lack of behavioural modifications such as paralysis, breathing, weight loss, sluggishness, restlessness, seizures and coma. Furthermore, for two weeks, no death was observed. The outcome indicates thzat there was no observable adverse effect of plant extracts. *Justicia adhatoda*'sactual median lethal dose (LD_{50}) exceeds 5000 mg/kg.

In our study, after 8 h treatment, JA-ME and JA-EE decreased the levels of blood glucose in the mice. After 28 days treatment, JA-ME and JA-EE at the 200 mg/kg and 400 mg/kg dose decreased the levels of blood glucose in the mice respectively. After 28 days treatment with all the extracts, the levels of TC, TG, LDL, VLDL, bilirubin, ALT, ALP, AST, urea, creatinine and uric acid are significantly reduced and significant increase in HDL level. Our results were consistent with the data previously published. The leaf and roots extracts of *Justicia adhatoda* was studied. Significant effects were observed on the glucose tolerance; lipid profile and animal body weight (Gulfraz *et al*., 2011). The results of *Adhatoda vasica Nees* give a protective role against diabetes which sum of its glucose lowering action (Mohan *et al*., 2014).

Higher contents of flavonoids (Oladele *et al*., 1995; Rao & Rao, 2001; Sharma *et al*., 2008), alkaloids, terpenoids (Shane-McWhorter, 2001) andsteroid glycosides (Adallu & Radhika, 2000) have been usuallyreported in anti-diabetic and anti-hyperglycemic medicinal plants. Higherconcentration of these phytoconstituents in the extract could explain its significanthypoglycemic effect, either separately or in synergy with one another. These antioxidants supposed mechanism of action was either stimulating the regeneration process or releasing pancreatic insulin secretion from existing β-cells due to mimetic impact of insulin on peripheral tissues. In addition to this mechanism, as potential antidiabetic plants, there are also other mechanisms that play an important role in reducing blood glucose levels.

By destroying pancreatic cells that secrete insulin and accelerate hypoinsulinemia and hyperglycemia, alloxan causes diabetes (Szuldelski, 2001). Alloxan causes hyperglycemia to affect pancreatic beta cells by specific cytotoxic effects (Yadav *et al*., 2002). Insulin is secreted by the unnecessary quantity of glucose in the blood. The insulin secretion stimulates the use of marginal glucose and controls glucose processing through numerous pathways (Andrew, 2000).

In diabetes mellitus, the profile of serum lipids is improved and this rise in lipids leads to coronary disease. The high concentrations of serum lipids levels are mainly due to low insulin activity owing to uninhibited lipolytic hormones activity in fat stores. Due to insulin insufficiency leading to hypertriglyceridemia, lipoprotein lipase is not launched in diabetic conditions (Pushparaj *et al*., 2007). Furthermore, insulin shortage is associated with hypercholesterolaemia. Insulin deficiency can be caused by dyslipidaemia (Murali *et al*., 2002).

Enzymes levels are mostly used for hepatic injury assessment. It is possible to measure intracellular enzymes in the serum. High AST levels specify hepatic injury caused by heart infarction, muscle damage and hepatitis virus. The alanine is converted into catalyzed glutamate and pyruvate. ALT is therefore a better parameter for identifying hepatic damage as it is more liver-specific. Higher serum enzyme levels show the loss of functional integrity of the hepatic membrane and the leakage of cells (Drotman & Lawhorn, 1978). ALP and bilirubin are also associated with the function of the liver cell. The rise in ALP is due to enhanced synthesis in the presence of enhanced biliary stress (Muriel *et al*., 1992).

In recent years, interest has risen in the research and activity of organic compounds discovered in plants. The GC-MS is an optimal method for the qualitative and quantitative evaluation of active ingredients based on plants. The variety of medicinal plants and herbs with different biological active phytochemicals can be a useful therapeutic key. Several phytochemicals have been discovered to have a broad range of activities that can assist safeguard against chronic diseases (Liu, 2003).

In our study, the *Justicia adhatoda* ethanolic leaf extract contained 56 chemical constituents eluted between 5.36 and 57.71 minutes. The major compounds identified were 9,12,15-Octadecatrienoic acid, (Z,Z,Z) (11.92%), Phytol, acetate (9.47%), n-Hexadecanoic acid (6.40%), β-Sitosterol (3.87%), 9,12-Octadecadienoic acid (Z,Z) (3.86%), Phytol (3.73%), Squalene (3.50%), 9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z) (3.45%), 5-Hydroxymethylfurfural (2.41%), Nonadecane (2.25%), Lethane (2.10%), and Lupeol (1.92%). Our findings were following the same line as revealed by other researchers. From the hydro distillation of *Adhatoda vasica (Nees.)* leaves, essential oil was obtained by GC-MS method. Eleven compounds from the oil of *Justicia adhatoda* were identified (Sarkera *et al*., 2011). In *Justicia adhatoda's* Petroleum ether extract, nine major bioactive components were identified (Jayapriya & Shoba, 2015). GC-MS analysis of methanolic leaf extracts from *Justicia adhatoda* includes the major compounds (Shukla *et al*., 2017).

Dl- α -Tocopherol has been shown as effective in various hematological disorders and malagnancies. Dl-α-Tocopherol possesses anti-oxidant, hypocholesterolemic, cancer preventive and anticoronary properties (Basu *et al*., 2014). Squalene protects the skin from UV and ionizing radiations; boost immune functions, reduces triglyceride and cholesterol levels in animal models, its supplementation in human with cholesterol lowering agents potentiates their efficacy. It also possesses anticancer properties (Kelly, 1999). Phytol compounds were present in *Justicia adhatoda* ethanolicextract. This shows its significance for the plants anticancer, antimicrobial and anti-inflammatory prospects (Kalaisezhiyen & Sasikumar, 2012). Hexadecanoic acid has antioxidant, anti-inflammatory and antimicrobial potentials (Bodoprost & Rosemeyer, 2007). B-Sitosterol prevents cancer. It also prevents angiogenesis. These sterols induced apoptosis (Rathee *et al*., 2012). Lupeol possess anti-inflammatory and anticancer activities (Saleem, 2009).

In *Justicia adhatoda* leaf extract, these biological activities of compounds support the plant's medicinal application. The study found substantial bioactive compounds in *Justicia adhatoda's* leaf extract. These compounds are identified in the plant as the basis for further biological and pharmacological studies to determine the plants potential health benefits.

Table 4. Phytoconstituents identified by GC-MS in the ethanol leaf extract of *Justicia adhatoda*.

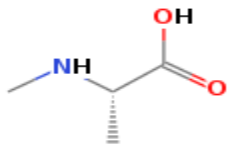
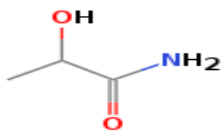
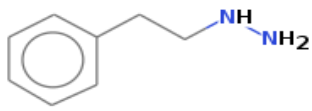
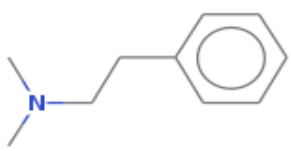
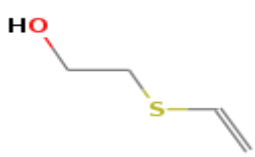
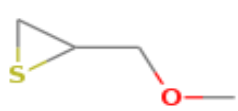
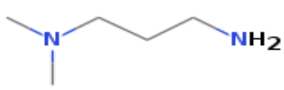
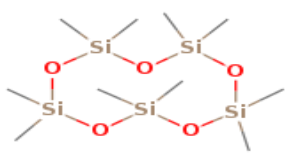
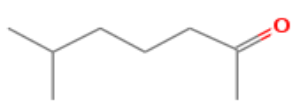
Sr. No.	RT	Compound	Molecular formula	Molecular weight	Peak area %	Structure
1.	5.364	L-Alanine, N-methyl	$C_4H_9NO_2$	103	0.31	
2.	5.629	Propanamide, 2-hydroxy-	$C_3H_7NO_2$	89	0.38	
3.	5.724	Phenelzine	$C_8H_{12}N_2$	136	0.33	
4.	5.939	Benzeneethanamine, N,N-dimethyl	$C_{10}H_{15}N$	149	0.49	
5.	6.595	2-Hydroxyethyl vinyl sulfide	C_4H_8OS	104	0.49	
6.	6.879	Thiirane, (methoxymethyl)-	C_4H_8OS	104	1.34	
7.	6.935	1,3-Propanediamine, N,N-dimethyl-	$C_5H_{14}N_2$	102	1.19	
8.	7.125	Stearyltrimethylammonium chloride	$C_{21}H_{46}ClN$	348	1.48	
9.	7.737	Cyclopentasiloxane, decamethyl	$C_{10}H_{30}O_5Si_5$	370	0.38	
10.	7.800	2-Heptanone, 6-methyl-	$C_8H_{16}O$	128	0.52	

Table 4. (Cont'd.).

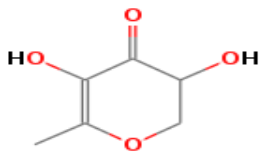
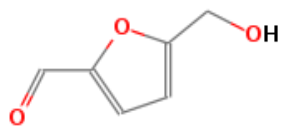
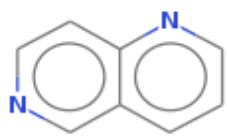
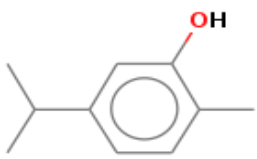
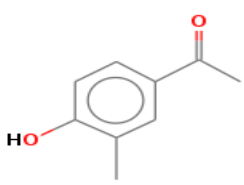
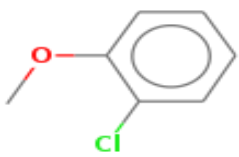
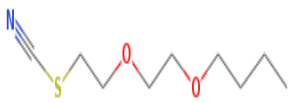
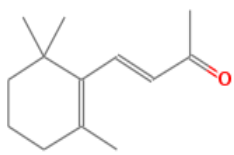
Sr. No.	RT	Compound	Molecular formula	Molecular weight	Peak area %	Structure
11.	8.096	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	$C_6H_8O_4$	144	1.71	
12.	8.563	1,3-Propanediamine, N-(3-aminopropyl)-N-methyl	$C_7H_{19}N_3$	145	0.41	
13.	8.955	5-Hydroxymethylfurfural	$C_6H_6O_3$	126	2.41	
14.	9.210	1,6-Naphthyridine	$C_8H_6N_2$	130	1.33	
15.	9.397	Phenol, 2-methyl-5-(1-methylethyl)	$C_{10}H_{14}O$	150	1.85	
16.	9.677	4-Hydroxy-3-methylacetophenone	$C_9H_{10}O_2$	150	1.24	
17.	10.490	Benzene, 1-chloro-2-methoxy-	C_7H_7ClO	142	0.45	
18.	11.009	Lethane	$C_9H_{17}NO_2S$	203	2.10	
19.	11.370	Trans-β-Ionone	$C_{13}H_{20}O$	192	0.36	

Table 4. (Cont'd.).

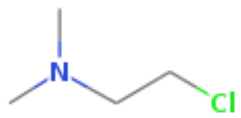
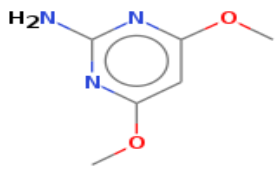
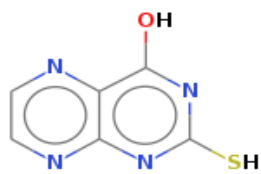
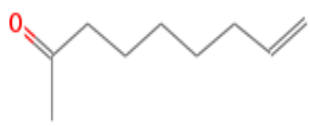
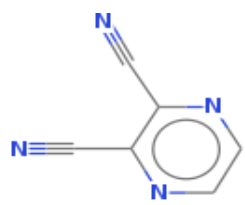
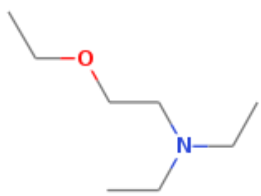
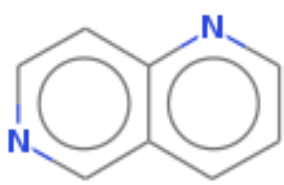
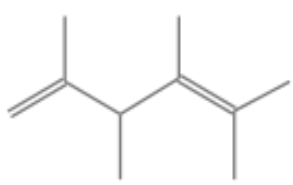
Sr. No.	RT	Compound	Molecular formula	Molecular weight	Peak area %	Structure
20.	11.635	Ethanamine, 2-chloro-N,N-dimethyl-	$C_4H_{10}ClN$	107	0.49	
21.	11.893	2-Amino-4,6-dimethoxypyrimidine	$C_6H_9N_3O_2$	155	0.29	
22.	12.324	4-Hydroxy-2-mercapto pteridine	$C_6H_4N_4OS$	180	0.60	
23.	12.613	8-Nonen-2-one	$C_9H_{16}O$	140	0.50	
24.	12.947	2, 3-Pyrazinedicarbonitrile	$C_6H_2N_4$	130	0.40	
25.	13.285	2-Ethoxyethyl,diethylamine	$C_8H_{19}NO$	145	0.58	
26.	13.597	1, 6-Naphthyridine	$C_8H_6N_2$	130	0.28	
27.	14.131	1,4-Hexadiene, 2, 3, 4, 5-tetramethyl	$C_{10}H_{18}$	138	0.29	

Table 4. (Cont'd.).

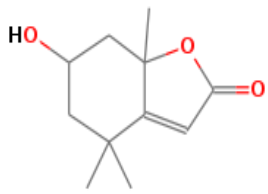
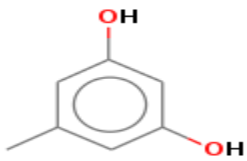
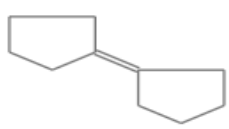
Sr. No.	RT	Compound	Molecular formula	Molecular weight	Peak area %	Structure
28.	15.703	6-Hydroxy-4, 4, 7a-trimethyl-5, 6, 7, 7a-tetrahydrobenzofuran-2(4H)-one	$C_{11}H_{16}O_3$	196	0.53	
29.	15.913	Orcinol	$C_7H_8O_2$	124	0.38	
30.	16.632	Neophytadiene	$C_{20}H_{38}$	278	1.40	
31.	16.739	2-Hexadecene, 2, 6, 10, 14-tetramethyl	$C_{20}H_{40}$	280	0.35	
32.	18.389	Undecanoic acid, ethyl ester	$C_{13}H_{26}O_2$	214	1.36	
33.	18.820	Phytol, acetate	$C_{22}H_{42}O_2$	338	9.47	
34.	19.330	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	6.40	
35.	19.973	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	1.50	
36.	21.261	Bicyclopentylidene	$C_{10}H_{16}$	136	0.43	
37.	22.730	Phytol	$C_{20}H_{40}O$	296	3.73	

Table 4. (Cont'd.).

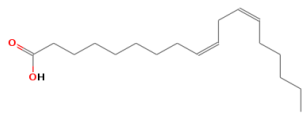
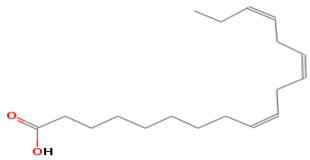
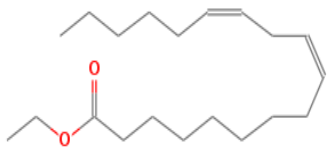
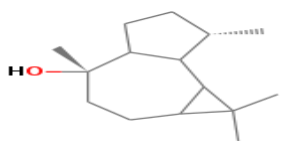
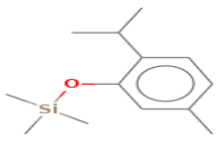
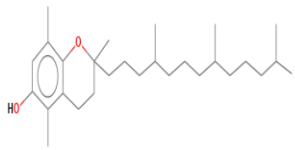
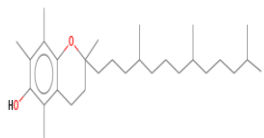
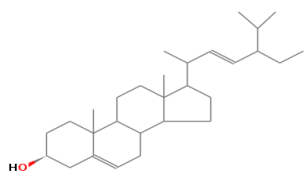
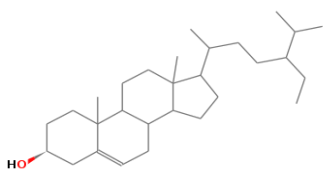
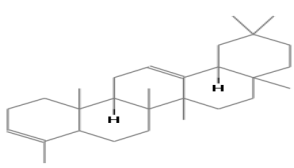
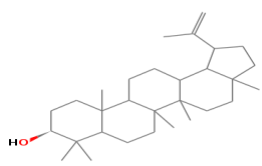
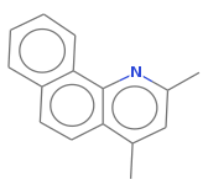
Sr. No.	RT	Compound	Molecular formula	Molecular weight	Peak area %	Structure
38.	23.383	9,12-Octadecadienoic acid (Z,Z)	$C_{18}H_{32}O_2$	280	3.86	
39.	23.556	9,12,15-Octadecatrienoic acid, (Z,Z,Z)	$C_{18}H_{30}O_2$	278	11.92	
40.	23.969	Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	308	1.37	
41.	24.115	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)	$C_{20}H_{34}O_2$	306	3.45	
42.	24.434	Ledol	$C_{15}H_{26}O$	222	0.59	
43.	24.798	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	312	0.35	
44.	37.204	Eicosane	$C_{20}H_{42}$	282	0.69	
45.	39.702	Squalene	$C_{30}H_{50}$	410	3.50	
46.	41.740	Nonadecane	$C_{19}H_{40}$	268	2.25	
47.	43.908	Thymol, TMS derivative	$C_{13}H_{22}OSi$	222	0.43	

Table 4. (Cont'd.).

Sr. No.	RT	Compound	Molecular formula	Molecular weight	Peak area %	Structure
48.	44.800	β – Tocopherol	$C_{28}H_{48}O_2$	416	0.35	
49.	46.323	Octacosane	$C_{28}H_{58}$	394	1.13	
50.	46.760	dl- α –Tocopherol	$C_{29}H_{50}O_2$	430	0.68	
51.	49.271	Tricosane, 2-methyl-	$C_{24}H_{50}$	338	1.32	
52.	50.115	Stigmasterol	$C_{29}H_{48}O$	412	1.40	
53.	52.221	β -Sitosterol	$C_{29}H_{50}O$	414	3.87	
54.	53.468	24-Noroleana-3,12-diene	$C_{29}H_{46}$	394	0.86	
55.	55.340	Lupeol	$C_{30}H_{50}O$	426	1.92	
56.	57.713	Benzo[h]quinoline, 2,4-dimethyl	$C_{15}H_{13}N$	207	0.57	

Conclusion

Results indicate that the medium lethal doses (LD_{50}) of all the three extracts exceeded 5000 mg/kg, suggesting that under the observable situation, the extracts are not toxic.The blood glucose level in all the extracts decreases by taking the extracts for 28 days.Compared to the reduced dose (200 mg/kg), the higher JA-ME concentration (400 mg/kg) showed important antihyperglycemic activity in diabetic mice. The reduced dose of JA-EE (200 mg/kg) relative to the greater dose (400 mg/kg), showed important antihyperglycemic activity in diabetic mice. The higher dose JA-EAE (400 mg/kg) showed significant antihyperglycemic activity in diabetic mice compared to the lower dose (200 mg/kg). After 28 days of treatment, all extracts decreased elevated TC, TG, LDL, VLDL, bilirubin, ALT, ALP, AST, urea, creatinine and uric acid concentrations and increased HDL concentrations. The methanol extract (400 mg/kg) showed the greatest decrease in blood glucose concentrations and capacity of *Justicia adhatoda* extracts to reduce blood glucose concentrations probably owing to antioxidant elements such as flavonoids. All major parts of the *Justicia adhatoda* extract confirmed by GC-MS are molecules that are biologically active. This shows that some medicinal properties may be present in the plant. This medicinal plant can therefore be a good candidate to be used as an alternative diabetes therapy. However, extensive research on the fractions of the extracts must be carried out in order to identify pharmacologically active compounds in order to elucidate the action mechanism and to guarantee its safety and to determine the suitable dose.

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