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Research Article

IN VITRO ANTIDIABETIC ACTIVITY AND PHYTOCHEMICAL ANALYSIS OF *LUMNITZERA RACEMOSA* LEAVES

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ABSTRACT

The present study was designed to evaluate the antidiabetic property and phytochemical composition of *Lumnitzera racemosa* leaves. Mangrove plant *L. racemosa* was collected in the month of May from Bhatye beach area Ratnagiri coast, Maharashtra, India. Leaves separated, powdered and exhaustive extraction was carried out in methanol, ethanol, ethyl acetate, and pet ether solvents with increasing polarity using Soxhlet apparatus and in vitro antidiabetic activity was determined by α-amylase and α-glucosidase inhibitory assays using acarbose as standard. Phytochemical analysis was carried out as per Wagner and Bladt (1996). Results have confirmed antidiabetic activity in methanol extract which was comparable to standard acarbose followed by ethyl acetate, ethanol and pet ether extracts. The phytochemical results showed the presence of flavonoid, saponin, essential oil, alkaloid, anthraquinones, bitter principles, phenol, triterpenoids, coumarin, and tannin in the leaves.

KEYWORDS: Mangroves, *Lumnitzera racemosa*, Anti-Diabetic activity, Phytochemicals

INTRODUCTION

Diabetes mellitus (DM) is a complex and diverse group of disorders which disturbs carbohydrate, fat, and protein metabolism due to defects in both regulations of insulin secretion and insulin action. It is principally characterized by raised blood glucose level (hyperglycemia) either as a result of the pancreas does not produce enough insulin or cells do not reply to the produced insulin. Glycaemia is normally maintained by the opposing actions of the two important hormones insulin and glucagon, both of which are produced in the pancreatic islets of Langerhans. Both insulin and glucagon, pancreatic endocrine hormones, are responsible for controlling blood-glucose level within the body at an adequate level based on the body needs[1]. Therefore a therapeutic approach to treat this disorder is to decrease postprandial hyperglycemia[2]. This could be achieved by the inhibition of carbohydrate-hydrolyzing enzymes like α-amylase and α-glucosidase[3]. Both α-amylase and α-glucosidase inhibitors are the potential targets within the development of the lead compound in the treatment of diabetes[4, 5]. The ethno pharmacological survey indicates that more than 1200 plants are used traditionally for their hypoglycaemic activity[6, 7].

Plants have continuously been an exemplary source of medicines and lots of drugs available today, are directly or indirectly derived from them. Mangroves are a specialized group of salt tolerant plants that grow within the seacoast areas of tropic and sub tropic on the coastlines. Mangrove plants provide an important source for the search of novel drugs as they are stress tolerant plants and therefore synthesize a large number of phytochemicals having significant pharmacological properties like infectious diseases, diabetes and asthma[8].The bioactive compounds of mangrove plants may be used because of the potent source of modern drugs against various critical diseases[9]. A few mangrove species like *Rhizophora mucronata*[8], *Diospyros peregrine*[10], *Heritiera fomes*[11] and secondary metabolites like alkaloids[12] saponin[13], flavonoids[14], tannin[15] are used to cure diabetes.

L. racemosa, black mangrove luxuriantly grows along west coast of India. It is used for antifertility, herpes, treatment of asthma, and snake bite and for skin disorder[16]. This species was also tested for antibacterial[17], hepatoprotective, antioxidant[18, 19] and cytotoxic activities[19]. In the present study *L. racemosa* was studied for in vitro antidiabetic activity and phytochemical compositions.

MATERIALS AND METHODS

Plant Material

Mangrove plant *L. racemosa* leaves (voucher specimen bearing reference no. NIO/DOD/DIO-1466[17]) were collected in the month of May from Bhatye beach area located at 16°58’44.0691’’N and 73°17’38.7499’’E Ratnagiri coast, Maharashtra, India and identified by an expert taxonomist. The leaves were washed thoroughly under running tap water to free them from dust and other contaminants, oven dried at 40º C to remove the moisture content, grinded and resultant powder was individually sieved through a muslin cloth and used for the extract preparation.

Extract Preparation

Various solvent extracts were prepared from *L. racemosa* leaves as follows. 10g leaf powder was added with the 200mL solvent which was exhaustively extracted with methanol, ethanol, ethyl acetate, and pet ether solvent successively by percolation method using the Soxhlet apparatus. The extracts obtained were concentrated using rotary flash evaporator (Buchi, Japan) to get the residues and used for antidiabetic activity.

Phytochemical Analysis

Qualitative phytochemical analysis of methanol leaf extract was studied[20]. The tests for various classes of secondary metabolites were as follows.

Alkaloids: 0.2 ml sample was taken and 0.2 ml HCl was added. To this 2-3 drops of Dragendroff's reagent was added and the appearance of orange or red precipitate and turbid solution indicates the presence of alkaloids.

Carbohydrates: 0.2 ml sample was mixed with few drops of Molisch's reagent (α- naphthol dissolved in alcohol).0.2 ml sulphuric acid was added along the sides of the test tube and observed for the appearance of a purple colour ring for a positive test.

Tannins: 0.2 ml plant extract was mixed with 2 ml water and heated on a water bath for 10 minutes. The mixture was filtered and ferric chloride was added to the filtrate and observed for the dark green solution which indicates the presence of tannin.

Terpenoids: 0.2 ml plant extract was taken in a test tube with 0.2 ml chloroform. To this, concentrated sulphuric acid was added carefully to form a layer. Presence of reddish brown colour at the interface would show the presence of terpenoids.

Glycosides: 0.2 ml sample was mixed with 0.2 ml of chloroform.0.2 ml of acetic acid was added to this solution and the mixture was cooled on ice. Sulphuric acid was added carefully and the colour change from violet to blue to green indicates the presence of steroidal nucleus (Aglycone portion of glycoside).

Steroids: 0.2 ml sample was mixed with 0.2 ml chloroform. To this 0.2 ml concentrated sulphuric acid was added. The appearance of red colour in the lower layer of chloroform indicates the presence of steroids.

Saponins: To 0.5 gm extract 5 ml distilled water was added in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

Flavonoids: 0.2 ml plant extract was taken in a test tube and mixed with dilute sodium hydroxide solution. To this diluted hydrochloric acid was added. Observation of yellow solution that turns colourless later would indicate the presence of flavonoids.

Mucilage: 0.2 ml extract was taken in a test tube and 0.2 ml absolute alcohol was added and allowed to try. If the precipitation occurs then mucilage is present.

Volatile oil: 0.2 ml extract was treated with few drops of dilute hydrochloric acid. The appearance of white precipitate indicates the presence of volatile oils.

Total Flavonoid Content

Total flavonoid was determined by aluminium chloride method[21] as follows:

The 100µL extract solution was taken and added 300 µL Hexane. Then 100 µL$AlCl_3$ (10%), 100 µL Na-K tartrate and 300 µL distilled water were added sequentially.

After 30 min of incubation, the test solution was vigorously shaken and absorbance was recorded at 415 nm with a Shimadzu UV-visible spectrophotometer (UV-1800 resolution: 1nm). A standard calibration plot was generated at 415 nm using a known concentration of quercetin. The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as mg quercetin equivalent/g of sample.

HPTLC Analysis

Quantitative phytochemical analysis of leaf methanol extract was carried out by HPTLC[22] (Linomat V supplied by CAMAG) in Anchrom, R & D laboratory, Mulund, Mumbai. Details of the solvent system, extract preparation, detecting reagent and visualization of secondary metabolite classes as shown in Table (1).

Table 1: Solvent system, extract preparation, detecting reagent and visualization for HPTLC

Classes	Solvent system	Extract preparation	Reagent	Visualization wavelength	Colour
Flavonoid	Ethyl acetate: formic acid: glacial acetic acid: water (10:0.5:0.5:1.3)	Powdered drug (1gm) in 5 ml methanol was extracted by heating on a water bath for 10 min	Anisaldehyde sulphuric acid	365	Blue
Anthragly-cosides	Ethyl acetate: methanol: water (100:13.5:10)	Powdered drug (1gm) in 5 ml methanol was extracted by heating on water bath for 10 min	10% KOH	Visible	Red, yellow
Bitter Principle	Ethyl acetate: methanol: Water (100:13.5:10)	Powdered drug (1gm) in 5 ml methanol was extracted by heating on water bath for 10 min	Vanillin sulphuric acid	Visible	Red, violet, yellow, blue brown, green
Saponin	Chloroform: acetic acid: methanol: water (6.4:3.2:1.2:0.8)	Above extract prepared, filtered and evaporated to 1 ml, mixed with 0.5 ml water and extracted with 3 ml of n-butanol	Vanillin sulphuric acid	Visible	Blue, blue violet, yellow, red brown
Triterpenes	Chloroform: methanol (9.5: 0.5)	Powdered drug (1gm) was extracted by heating under reflux for 15 min with 10 ml dichloromethane	Anisaldehyde sulphuric acid	Visible	Blue violet, red to red violet
Phenol carboxylic acid	n-butanol: acetic acid: Carboxylic acid: water (4:1:1)	Powdered drug (1gm) was extracted by heating under reflux for 15 min with 10 ml dichloromethane	$FeCl_3$	Visible	Brown, grey, black
Coumarin	Toluene: ethyl acetate (93:7)	Powdered drug (1gm) was extracted by heating under reflux for 15 min with 10 ml dichloromethane	10% KOH	365	Light blue, brown
Essential oil	Toluene: ethyl acetate (93:7)	Powdered drug (1gm) was extracted by heating under reflux for 15 min with 10 ml dichloromethane	Vanillin sulphuric acid	Visible	Red, yellow, blue, brown, green
Alkaloids	Toluene: ethyl acetate: diethyl amine (7:2:1)	Powdered drug (1gm) was moistened with 1 ml, 10% ammonia solution on water bath	Dragendroff's Reagent	Visible	Orange brown

Chromatographic Condition
Each extract was loaded on readymade fluorescent pre-coated silica gel G aluminium plate (Supplied by MERCK) and developed using appropriate solvent systems and detected. The resultant chromatograms were illuminated for the characteristic quenching or fluorescence respectively for the particular class of bioactive compounds. The plates were derivatized and heated if necessary on an HPTLC heater for the detection of compounds.

In vitro Antidiabetic Activity
Antidiabetic activity was estimated by α-amylase[23]and α-glucosidase inhibition assays[24] as follows:

α-amylase Inhibition Assay:
100 μL plant extract of various concentration and 100 μL 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl) containing α-amylase solution was incubated at 37ºC for 30 min. Then 800 μL 1% soluble starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl was added to each tube and incubated at 37° C for 15 min followed by addition of 1 mL dinitrosalicylic acid (DNSA) reagent. The test tubes were then placed in a boiling water bath, (100ºC) for 5 min and the absorbance was determined at 540 nm.

α-glucosidase Inhibition Assay:
The inhibitory activity was determined by incubating a solution of 1 ml starch substrate (2% w/v maltose) with 0.2 M Tris buffer (pH 8.0) and various concentration of plant extract for 5 min at 37°C. The reaction was initiated by adding 1 ml of α- glucosidase enzyme (1U/ml) to it followed by incubation for 40 min at 35°C. Then the reaction was terminated by adding 2 ml 6 N HCl and intensity of the colour was measured at 540 nm.

Calculation of 50% Inhibitory Concentration (IC_{50}):
Half-maximal Inhibitory concentration (IC_{50}) is the concentration of the substance required to inhibit a biological process such as an enzyme, cell, cell receptor or microorganism by half. The IC_{50} value was calculated by non-linear regression analysis of % inhibition recorded for different concentrations of test substances/standard. The relative activity of the sample was determined by comparing the IC_{50} value of a sample with the standard. Higher the IC_{50} value, lower will be the relative activity in comparison to standard and vice-versa. Acarbose was used as the standard inhibitor. All the tests were performed in triplicate. Percentage inhibition (I %) was calculated by:

$$\% \text{ inhibition of} = (Abs_{control} - Abs_{sample}) / (Abs_{control}) \times 100$$

Where, $Abs_{control}$ = absorbance of the solution without extract
Abs_{sample} = absorbance of the solution with extract and enzyme solution

RESULTS

Both qualitative and quantitative analysis have confirmed the presence of flavonoid, saponin, essential oil, alkaloid, anthraquinones, bitter principles, phenol, triterpenoids, coumarin, tannin, steroids and carbohydrates classes of secondary metabolites in *L. racemosa* leaves (Table:2). Total flavonoid content was found to be 1.186 mg QE/g (Fig:1).

The HPTLC chromatogram has shown 10 bands of flavonoid (Rf 0.10 to 0.82) and saponin (0.08 to 0.91), 9 bands of essential oil (0.06 to 0.84) and 7 bands of bitter principle (0.14 to 0.93) and alkaloid (0.20 to 0.72). The anthraquinone (0.06 to 0.71), phenol (0.09 to 0.48) and triterpenoids (0.29 to 0.63) have shown 5 band each while 4 bands in coumarin (0.03 to 0.16) and 3 in tannin (0.12 to 0.54) (Table:3).

The α-amylase assay has shown dose-dependent activity for all the extracts (Table:4). As the concentration increases, % inhibition increased (Fig:2). Among the extracts, methanol extract recorded highest % of inhibition (84.47%) for the highest concentration which was comparable to standard acarbose (89.39%). The second highest % inhibition was observed in ethyl acetate extract (77.25%) followed by ethanol (68.34%) and pet ether (54.82%). Among the extracts methanol showed best IC_{50} (0.65±0.009 mg/ml) followed by ethanol (3.064±0.02 mg/ml), ethyl acetate (3.077±0.01) and pet ether (4.074±0.05 mg/ml). Similar results were recorded in α-glucosidase assay for all the extracts (Table:5). In this methanol extract recorded best % inhibition (78.02%) and IC_{50} (0.035±0.03 mg/ml) followed by ethyl acetate, ethanol and pet ether (Fig:3). However, the % inhibition and IC_{50} value for acarbose was 87.77% and 0.249±0.353 mg/ml respectively.

DISCUSSION

The medicinal properties of the plant depend upon its phytochemical composition. The mangroves have shown diverse classes of secondary metabolites therefore possess several medicinal properties like antimicrobial[25], antiviral activities[26], antioxidant[27], and anti-inflamatory[28]. The antidiabetic properties of mangrove plants are related to alkaloid, flavonoid, saponin and terpenoids[29]. In view of this, the phytochemical analysis of *L. racemosa* was carried out. The qualitative and quantitative analysis of *L. racemosa* leaves have indicated the presence of flavonoid, saponin, essential oil, alkaloid, anthraquinones, bitter principles, phenol, triterpenoids, coumarin, tannin, steroids, and carbohydrates. The medicinal properties of plants are related to the quality and quantity of secondary metabolites present in it. The present HPTLC analysis have indicated the presence of more number of flavonoid, saponin, essential oil, alkaloid, bitter principles and moderate number of phenol, anthraquinones, triterpenoids, coumarin, tannin secondary metabolites in *L. racemosa* leaves. This suggests that *L. racemosa* leaves are rich in flavonoid, saponin, essential oil, alkaloid and bitter principles classes of phytochemicals. Similarly mangrove species like *Sonneratia apetala*[30] and *S. alba*[31] are found rich in these secondary metabolites. Therefore it is suggested that mangroves are rich sources of flavonoid, saponin, essential oil, alkaloid, bitter principles classes of secondary metabolites. In the present study, we have prepared leaf extract using various solvents and evaluated antidiabetic potential by α-amylase and α- glucosidase inhibition assays. Both the assays have shown best antidiabetic activity in methanol extract followed by ethyl acetate, ethanol, and pet ether solvents. The antidiabetic activity in methanol extract was comparable to standard acarbose. These results indicates that *L. racemosa* leaves possess antidiabetic potential. These results further indicate that methanol is the best solvent to extract antidiabetic principles for the leaves of the plant.

Antidiabetic activity was recorded in *S.alba*[32], *Acanthus ilicifolius*, *Bruguiera sp*., *Xylocarpus granatum*[14], *B. racemosa* and *Rhizophora sp.*[14, 33] mangrove species. The antidiabetic potential of plants is related to the presence of alkaloid[12], flavonoid[14], saponin[13, 34], phenolic compound[33] and tannin[15] classes of phytochemicals. Since *L. racemosa* found rich in these principles, therefore antidiabetic potential can be related to alkaloid, flavonoid, saponin, phenolic compound classes of secondary metabolites.

Table 2: Phytochemical constituents of *L. racemosa* leaves

Phytochemical test	Results
Alkaloid test (Dragendroff's test)	**+ve**
Carbohydrate test (Molisch's test)	**+ve**
Tannin test	**+ve**
Terpenoid test	**+ve**
Glycoside test	**+ve**
Steroid test	**+ve**
Saponin test	**+ve**
Flavonoid test	**+ve**
Protein estimation	**-ve**
Mucilage test	**-ve**
Volatile oils	**-ve**

Table 3: Secondary metabolite profile of methanol extract of *L. racemosa* leaves

Alkaloid

Peak	Retention factor	Height	Area	Assigned substance
1	0.2	59.6	1488.8	Unknown
2	0.3	14.4	244.9	Unknown
3	0.34	82	2105.6	Autogenerated
4	0.46	306.4	6355.7	Autogenerated
5	0.54	394.9	8816.5	Unknown
6	0.62	336.2	11203.7	Unknown
7	0.72	641.7	26842.8	Autogenerated

Anthraglycosides

Peak	Retention factor	Height	Area	Assigned substance
1	0.06	88.6	2176.7	Autogenerated
2	0.24	86.4	4080.8	Autogenerated
3	0.44	140.1	4286.9	Autogenerated
4	0.52	35.5	619.6	Autogenerated
5	0.71	114.5	2870.6	Autogenerated

Bitter Principle

Peak	Retention factor	Height	Area	Assigned substance
1	0.14	11.1	172.5	Autogenerated
2	0.26	90.1	4560.4	Unknown
3	0.44	146.7	4940.2	Unknown
4	0.55	212.7	4822.5	Unknown
5	0.75	39.9	1020.5	Unknown
6	0.84	169.6	6115.7	Autogenerated
7	0.93	210	6651.4	Unknown

Coumarin

Peak	Retention factor	Height	Area	Assigned substance
1	0.03	160.9	1331.5	Unknown
2	0.06	432.1	5948.3	Unknown
3	0.1	759.6	14724.3	Unknown
4	0.16	395.3	9176.2	Autogenerated

Flavonoid

Peak	Retention factor	Height	Area	Assigned substance
1	0.1	21.4	434.4	Unknown
2	0.17	23.5	350.6	Unknown
3	0.23	81.3	2291.9	Autogenerated
4	0.32	84	1160.9	Unknown
5	0.35	67	1209.8	Unknown
6	0.38	159.9	7535.7	Unknown
7	0.49	66.2	2199.2	Unknown
8	0.62	25.7	772.2	Autogenerated
9	0.7	42.4	774.9	Autogenerated
10	0.82	60.4	1084.2	Autogenerated

Tannin

Peak	Retention factor	Height	Area	Assigned substance
1	0.12	180.1	4311.4	Unknown
2	0.26	39.7	737	Unknown
3	0.54	17.2	107	Unknown

Phenol

Peak	Retention factor	Height	Area	Assigned substance
1	0.09	69.2	1633.2	Unknown
2	0.15	57.1	694.4	Autogenerated
3	0.31	202.1	3690.2	Unknown
4	0.41	14.1	355.3	Autogenerated
5	0.48	64.1	1703.5	Autogenerated

Saponin

Peak	Retention factor	Height	Area	Assigned substance
1	0.08	0.12	1227.7	Autogenerated
2	0.23	0.26	863.4	Unknown
3	0.31	0.39	2324.7	Unknown
4	0.42	0.48	6174.3	Autogenerated
5	0.55	0.69	3934	Autogenerated
6	0.66	0.68	445.2	Autogenerated
7	0.69	0.72	2221.3	Autogenerated
8	0.78	0.8	450.8	Unknown
9	0.83	0.87	4754.1	Unknown
10	0.91	0.96	877.3	Autogenerated

Triterpenoid

Peak	Retention factor	Height	Area	Assigned substance
1	0.29	61.6	1261.1	Unknown
2	0.35	20	459.7	Unknown
3	0.43	160.7	4008.5	Unknown
4	0.52	106	3266.9	Unknown
5	0.63	37.1	908.9	Autogenerated

Essential oil

Peak	Retention factor	Height	Area	Assigned substance
1	0.06	13.9	163.5	Unknown
2	0.1	11.2	78.2	Unknown
3	0.14	240.7	5378.3	Unknown
4	0.22	52.7	727.1	Unknown
5	0.25	194.8	5967.6	Unknown
6	0.35	30.47	40017.1	Unknown
7	0.93	286.5	13151.1	Unknown
8	0.68	435.3	35648.8	Unknown
9	0.84	549.6	28750.8	Unknown

Table 4: The percent inhibition of α-amylase by methanol, ethanol, ethyl acetate and pet ether extracts of *L. racemosa* leaves at varying concentrations

Acarbose			Methanol		Ethanol		Ethyl acetate		Pet ether	
Conc. (mg/ml)	% inhibition	IC_{50}	% inhibition	IC_{50}	% inhibition	IC_{50}	% inhibition	IC_{50}	% inhibition	IC_{50}
0.5	50.37±0.292	0.095±0.007	45.75±0.276	0.65±0.009	15.79±0.313	3.064±0.022	8.37±0.421	3.077±0.016	8.68±0.049	4.074±0.052
1.0	57.10±0.165		49.74±0.362		36.13±0.571		11.59±0.126		22.2±0.064	
2.0	70.25±0.160		66.62±0.552		41.13±0.313		26.47±0.399		28.45±0.107	
3.0	80.25±0.113		78.14±0.156		50.0±0.229		51.92±0.231		46.22±0.217	
4.0	88.10±0.037		81.55±0.329		59.13±0.416		73.12±0.337		51.43±0.123	
5.0	89.39±0.087		84.47±0.208		68.34±0.225		77.25±0.358		54.82±0.177	

Table 5: The percent inhibition of α-glucosidase by methanol, ethanol, ethyl acetate and pet ether extracts of *L. racemosa* leaves at varying concentrations

Acarbose			Methanol		Ethanol		Ethyl acetate		Pet ether	
Conc. (mg/ml)	% inhibition	IC_{50}	% inhibition	IC_{50}	% inhibition	IC_{50}	% inhibition	IC_{50}	% inhibition	IC_{50}
0.5	48.29±2.79	0.249±0.353	49.30±0.396	0.035±0.037	16.38±0.221	3.01±0.041	7.17±0.471	3.227±0.005	7.88±0.563	3.837±0.021
1.0	54.66±4.28		56.81±0.124		36.87±0.305		10.87±0.399		21.74±0.517	
2.0	69.16±2.21		64.42±0.312		39.89±0.409		33.79±0.437		29.85±0.563	
3.0	80.39±2.34		72.56±0.241		49.25±0.289		45.66±0.344		43.69±0.448	
4.0	84.83±2.93		76.89±0.447		61.54±1.318		64.66±0.489		55.62±0.587	
5.0	87.77±2.58		78.02±0.344		68.87±0.374		76.17±0.327		57.91±0.541	

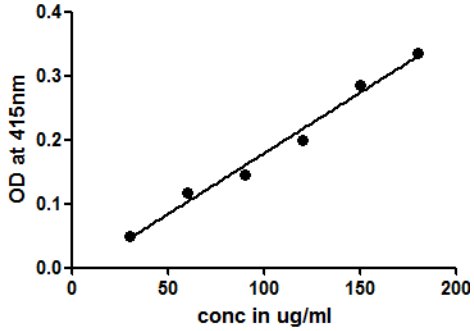

Fig 1: Quercetin standard graph

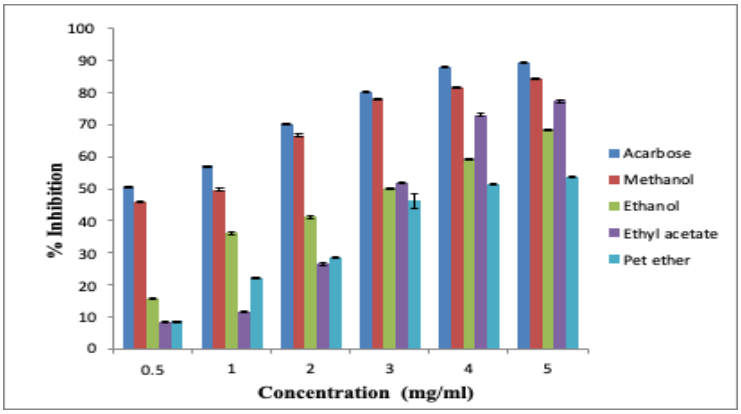

Fig 2: Inhibitory effect of α-amylase by *L. racemosa* leaves extracts at various concentrations

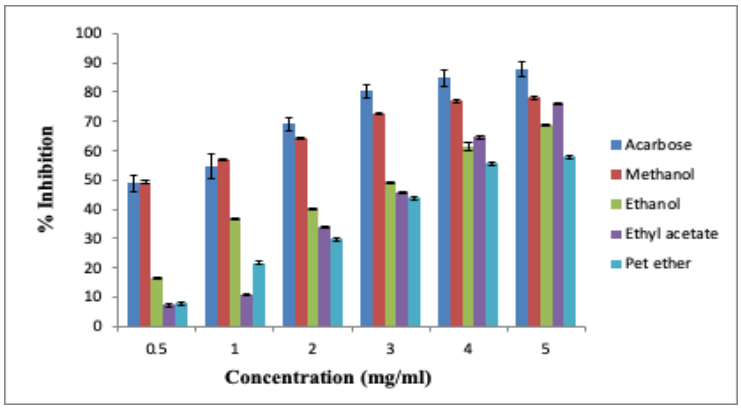

Fig 3: Inhibitory effect of α- glucosidase by *L. racemosa* leaves extracts at various concentrations

CONCLUSION

The present finding reveals that *L. racemosa* leaves are found rich source of flavonoid, saponin, essential oil, alkaloid, anthraquinones, bitter principles, phenol, triterpenoids, coumarin, and tannin. The antidiabetic potential observed in *L. racemosa* leaves may be related to the presence of flavonoid, saponin and alkaloid in it. As the plant has shown significant inhibition activity, further compound isolation, purification and characterization of bioactive compound which is responsible for the inhibiting activity has to be done for usage of an antidiabetic agent.

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