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Pharmacological evaluation of *Parkia speciosa* Hassk. for antioxidant, anti-inflammatory, anti-diabetic and anti-microbial activities *in vitro*

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ABSTRACT

Parkia speciosa Hassk. (Petai or Stink bean, Family Fabaceae) is a traditionally consumed medicinal plant for liver disease, oedema, kidney inflamemation, diabetes and as an anthelmintic. The phytochemical analysis of seeds of *P. speciosa* in water, methanol and hydromethanolic (1:1) extracts revealed the presence of carbohydrates, amino acid, alkaloids, saponins, tannins, flavonoids, terpenoids, glycosides, xanthoproteins and phenols. In vitro antioxidant capacity by linear regression analysis was measured by assaying DPPH radical and H_2O_2 scavenging capacities. Their respective IC_{50} values were found to be 315.75 µg/ml and 166.63 µg/ml. The hydromethanolic extract gave total phenolic, total flavonoids and FRAP values of 13.54±0.0163 mg GAE/g, 5.46±0.011 mg/g QE/g and 1.9 mM $FeSO_4$ respectively. The IC_{50} values for in vitro anti-inflammatory activities were evaluated by the following assays: lipoxygenase inhibition (IC_{50} = 493.34µg µg/ml), proteinase inhibition (IC_{50} = 1142.3 µg µg/ml) and RBC membrane stabilization (IC_{50} = 67.01 µg µg/ml) at different concentrations using aspirin as control. Studies were also carried out to assess the anti-diabetic potential by assaying the ability of the plant to inhibit pancreatic lipase and amylase activities. The % inhibition at 500 µg/ml of the plant extracts were found to be 89.5% (for lipase) and 79.2% (for amylase). Antimicrobial activity of the extracts was studied against common pathogens (*S. aureus, E. coli, P. aeruginosa* and *K. pneumoniae*). The zone of inhibition was observed using well diffusion method.

Key words: Anti-inflammatory, amylase, lipase, lipoxygenase, proteinase inhibition, *P. speciosa*.

INTRODUCTION

It is estimated that up to four billion people (representing 80% of the world's population) living in the developing world rely on herbal medicinal products as a primary source of healthcare (Bandaranayake,

2006). India has probably the oldest, richest and most diverse cultural traditions in the use of medicinal plants with an estimate of over 7500 species used by several ethnic communities (AICEP 1994; Anthropological survey of India 1994). One such plant used in Southeast Asian countries like India, Malaysia, Philippines, and Indonesia (Mitsuo and Fitriyah, 2001) is *Parkia speciosa*. It can be found in rainforests, on sandy, loamy and podzolic soils.

It also grows wildly in waterlogged locations, freshwater swamp forest and on riverbanks. Seeds of *P. speciosa* can be eaten raw, cooked or roasted. It has been used as traditional medicine to treat various ailments like diabetes, cardiovascular diseases, constipation, as a carminative, kidney pain (Gmelin et al., 1981), cancer, hepatalgia, nephritis, colic ulcers and also taken as diuretic. As *P. speciosa* (both seeds and green pericarp) is used to control blood sugar level it is believed to possess anti-diabetic effect (Suvachittanont and Pothirakit, 1988). Thus, the present investigation was undertaken to understand the potential of *P. speciosa* for antioxidant, anti-inflammatory, anti-diabetic and antimicrobial activities *in vitro*.

MATERIALS AND METHODS

Collection and preparation of samples

The raw pods of *Parkia speciosa* Hassk. were collected from Ema market, Imphal, India in May 2016. The pods were deseeded, cleaned, rinsed in distilled water, sun dried and ground into a fine powder. Crushed samples were extracted using three different solvent systems: distilled water (aqueous extract), methanol (alcoholic extract) and hydroamethanolic extract (1:1 v/v). Extraction was carried out on an orbital shaker for 24 h at room temperature. Solvents were evaporated under vacuum and the resulting extracts were stored at 4 ºC.

Fluorescence analysis

Fluorescence characteristics of the powdered seed with different chemicals were observed in daylight and ultraviolet light (Chase and Pratt, 1949).

Phytochemical screening

Phytochemical examinations were carried out for all the extracts as per the standard methodology to confirm the presence of phytoconstituents (Harborne, 1991; Khandelwal, 2009).

Detection of carbohydrates

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates as follow: *Molisch's test*: Formation of the violet ring at the junction with 2 drops of alcoholic α-naphthol solution indicates the presence of carbohydrates. *Benedict's test:* Orange red precipitate with Benedict's reagent indicates the presence of reducing sugars. *Fehling's test*: Filtrates were hydrolyzed with dil. HCl, neutralized with alkali and heated with Fehling's A and B solutions. Presence of reducing sugars was indicated by the formation of a red precipitate.

Detection of alkaloids

Extracts were dissolved individually in dilute HCl and filtered. Tests for the presence of alkaloids were as follows: *Mayer's test:* Formation of a yellow coloured precipitate with Mayer's reagent indicates the presence of alkaloids. *Wagner's test*: Formation of brown/reddish precipitate with Wagner's reagent indicates the presence of alkaloids. *Dragendroff's test*: Formation of red precipitate with Dragendroff's reagent indicates the presence of alkaloids.

Detection of saponins

Foam test: Persistence of foam after 10 mins with 0.5 ml extract and 2 ml water, shaken well indicates the presence of saponins.

Detection of glycosides

Extracts were hydrolyzed with dil. HCl, and then subjected to test for glycosides. *Modified Borntrager's test*: Extracts were treated with $FeCl_3$ solution and immersed in boiling water for about 5 min. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

Detection of phytosterols

Salkowski's test: The plant extracts were treated with $CHCl_3$ and filtered. The filtrate thus obtained was mixed with few drops of concentrated H_2SO_4, shaken and allowed to stand. The appearance of golden yellow colour was indicative of triterpenes.

Detection of flavonoids

Lead acetate test: Formation of yellow colour precipitate with the extract and a few drops of lead acetate solution indicates the presence of flavonoids.

Detection of proteins and amino acids

Xanthoproteic test: Formation of yellow colour with extract and concentrated HNO_3 indicates the presence of proteins. *Ninhydrin test:* Formation of blue colour with extract and 0.25% w/v ninhydrin reagent boiled for a few minutes indicates the presence of amino acid. *Millon's test:* A reddish-brown coloration with the extract and Millon's reagent gently heated indicates the presence of tyrosine residue.

Detection of tannins

Ferric chloride test: Formation of bluish-black colour with the extract and 3-4 drops of $FeCl_3$ indicates the presence of phenols.

Detection of terpenoids

Salkowski test: Appearance of reddish brown colour with 5 ml of extract, few drops of $CHCl_3$ and 3 ml concentrated H_2SO_4 revealed the presence of terpenoids.

Total phenolic, flavonoid and FRAP activity:

Total phenolic contents were estimated according to the spectrophotometric method using gallic acid as standard (Slinkard and Singleton, 1977) and expressed in terms of gallic acid equivalent (mg of GAE/g of tissue). Aluminum chloride colorimetric method was used for determination of total flavonoids (Chang et al., 2002) and expressed in terms of quercetin equivalent (mg of QE/g of tissue). The ferric ions (Fe^{3+}) reducing antioxidant power (FRAP) method was used to measure the reducing capacity of plant extracts (Oyaizu, 1986).

Evaluation of Antioxidant Activity

DPPH radical scavenging activity*:*

Standard ascorbic acid was pipetted out into different test tubes (100-500 µg/ml). 0.1 ml solution of each dilution was taken and made up to 3 ml with DPPH (20 µg/ml). The test tubes were incubated for 10 min at room temperature. The contents of each tube were mixed well, and the absorbance was measured at 517 nm against a blank (Braca et al., 2012). The percentage inhibition of DPPH by the samples was calculated as follows.

$$\% \text{ inhibition} = \frac{O.D. \text{ of Control} - O.D. \text{ of Sample}}{O.D. \text{ of Control}} \times 100$$

..... Eq. 1

Hydrogen peroxide scavenging activity:

Plant extract in 3.4 ml phosphate buffer was added to 0.6 ml of 45 mM H_2O_2 solution and the absorbance of the reaction mixture was recorded at 230 nm against a blank containing only buffer (Ruch et al., 1989). Ascorbic acid was used as standard. The concentration of H_2O_2 (mM) in the assay medium was determined using a standard curve ($y = 0.1223x+29.62$; $R^2 = 0.586$). H_2O_2 scavenging ability was calculated as IC_{50}. The percentage inhibition was calculated as in Eq. 1.

Evaluation of *In vitro* Anti – Inflammatory Activity

Lipoxygenase inhibition:

Soybean lipoxygenase activity was assayed (Axelrod et al., 1981). The reaction contained 2.9 ml 0.1M borate buffer pH 9.0 and 50 µl 10 mM linoleic acid. The reaction was initiated by the addition of 50 µl of the soybean enzyme extract. The enzyme activity was measured by following the formation of the product, 12-HETE at 234 nm for up to 1 min. The enzyme inhibition was determined by pre-incubating the enzyme with the plant extract or standard phytochemicals prior to determining its 12-LOX activity. The percentage inhibition was calculated as in Eq. 1. IC_{50} was calculated from $y=0.0839x+8.61$; $R^2=0.788$.

Proteinase Inhibitory Action*:*

The test was performed according to the modified method of Sakat et al., (2010). 2 ml of the reaction mixture contains 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations (100 - 500 µg/ml). The mixture was incubated at 37 ^oC for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to arrest the reaction. Cloudy suspension was centrifuged, and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in duplicate. The percentage inhibition of protein denaturation was calculated using Eq. 1. IC_{50} was calculated from $y=0.0399x+4.428$, $R^2=0.8585$.

RBC membrane stabilization activity:

Various concentrations of extract, reference sample and control were separately mixed with 1 ml of phosphate buffer, 2 ml of hyposaline and 0.5 ml of hRBC suspension. Diclofenac sodium (100 µg/ml) was used as a standard drug. All the assay mixtures were incubated at 37 ^oC for 30 min and centrifuged at 3000 rpm. The supernatant was used to estimate the haemoglobin content using a spectrophotometer at 560 nm (Azeem et al., 2010). The percentage of hemolysis was estimated assuming that the control produced 100% haemolysis.

The percentage inhibition of protein denaturation was calculated using Eq. 1. IC_{50} was calculated from y=0.1525x+39.78, R^2= 0.5502.

***In-vitro* anti-diabetic evaluation**

Alpha amylase inhibition assay:

The procedure described by Shai et al., (2010) with slight modifications was used to determine the α-amylase inhibitory activity of the fractions. A volume of 250 µl of each fraction or acarbose at different concentrations (100-500 µg/ml) was incubated with 500 µl of porcine pancreatic amylase (2 U/ml) in phosphate buffer (100 mM, pH 6.8) at 37 °C for 20 min. Thereafter, 250 µl of 1% starch dissolved in 100 mM phosphate buffer (pH 6.8) was further added to the reaction mixture and incubated at 37° C for 1 h. Dinitrosalicylate colour reagent (1 ml) was then added and boiled for 10 min. The absorbance of the resulting mixture was read at 540 nm the inhibitory activities of the fractions on α-amylase were calculated by using the following formula:

$$\% \text{ inhibition} = 1 - \frac{B-b}{A-a} \times 100 \quad \text{----- eq. (2)}$$

where 'A' is the activity of the enzyme without inhibitor, 'a' is the negative control without the inhibitor, 'B' is the activity of the enzyme with inhibitor, and 'b' is the negative control with inhibitor.

Porcine pancreatic lipase (PPL) inhibition assay: This was performed as described by Bustanji et al., (2011) with minor modification. The enzyme solution was prepared immediately before use, by suspending crude porcine pancreatic lipase powder in 0.1 M phosphate buffer (pH 7.6) (100 U/ml). The solution was then centrifuged at 2000 rpm for 10 min and the clear supernatant was recovered. Triolein (1% v/v) was used as the substrate for PLL. The plant extract (5, 12.5, 25, 100, 125, 250, 500 µg/ml) was preincubated with 200 µl of PPL solution for 5 min at 37 °C, before the addition of 800 µl triolein substrate solution. The absorbance was measured at 450 nm against blank using denatured enzyme in an ELISA reader. The denatured enzyme was prepared by boiling the enzyme solution for 5 min. Orlistat was used as a reference drug. The extract was dissolved in DMSO at a final concentration not exceeding 1% (v/v). The activity of the negative control was checked in the presence and absence of the inhibitor. The % inhibition was calculated according to the formula (2).

Mechanism of porcine pancreatic lipase inhibition: The inhibition mode of crude extract of *P. speciosa* on porcine pancreatic lipase (PPL) was assayed with increasing concentrations of the substrate Triolein (20, 40, 60, and 80 µM) in the presence and absence of two different concentrations of the extracts (100 and 200 µg/mL). The mode of inhibition was determined by Lineweaver-Burk plot.

Screening for Antibacterial activity of methanolic extract

Agar well diffusion assay:

The antibacterial activity was carried out by employing 24 h cultures of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae.* The standard medium Mueller Hinton Agar, was poured to a depth of 4 mm in a 90 mm petridish. The bacterial inoculum was prepared from an 18 h broth culture of the microbe to be tested and was standardized with sterile physiologic saline to contain 106 cfu/ml. A well (6 mm diameter) was made using a sterile cork borer. The standard drug and extracts were placed in the well. Antibacterial assay plates were for overnight incubation. Ciprofloxacin (5 µg/disc) was used as a positive control for antibacterial activity. After 24 h of incubation at 37 ^{o}C, zone of inhibition (ZOI) was observed and diameter measured.

Statistical analysis:

Data of *in vitro* assays recorded were analyzed using Microsoft Excel to determine IC_{50}. One-way analysis of variance (ANOVA) was performed and $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

Folkloric medicine from natural plant products has traditionally been used to cure a number of common ailments as they are a rich source of bioactive molecules. More intensive research involving the discovery of anti-inflammatory and anti-diabetic agents from medicinal plant sources is currently underway because of its known ability of suppressing relevant reactions of the inflammatory cascade. The present study was carried out to assess the validity of ethanomedicinal use of *P. speciosa* in the management and treatment of inflammatory disorders.

Table 1: Fluorescence analysis of pod powder of *P. speciosa.*

Sl No.	Experiment	Visible/Day light	UV light (365nm)
1	Powder as such	Light green	Light green
2	Powder+1N HCL	Colourless	Light green
3	Powder+Conc.H_2SO_4	Dark brown	Yellowish green
4	Powder+50% H_2SO_4	Dark brown	Fluorescent brown
5	Powder+ Conc.HNO_3	Yellow	Dark green
6	Powder+ Conc.HCl	Light yellow	Yellowish green
7	Powder+50%HNO_3	Yellow	Dark green
8	Powder+ Acetic acid	Light green	Yellow
9	Powder+ Ferric chloride	Light brown	Brownish green
10	Powder+HNO_3+NH_3	Light yellow	Green
11	Powder+ NH_3	Transparent	Light green
12	Powder+ Benzene	Light yellow	Orange
13	Powder+ Petroleum ether	Light green	Fluorescent orange
14	Powder+ Acetone	Green	Fluorescent orange
15	Powder+ Chloroform	Light green	Reddish orange
16	Powder+ Methanol	Light green	Fluorescent orange
17	Powder+ Ethanol	Light green	Fluorescent pink

Table 2: Phytochemical investigation of *P. speciosa* seed extract

Sl. No.	Phytochemical analyzed	Test performed	Result		
			80% methanol	Methanol:water (1:1)	Aqueous
1	Alkaloid test	Mayer's test	+	++	+
		Wagner's test	+	++	+
		Dragendoroff's test	+	++	+
2	Carbohydrate test	Molish's test	+	++	+
		Benedict's test	+	++	+
		Fehling's test	+	++	+
3	Saponin test	Foam test	+	++	+
4	Glycosides test	Borntrager's test	+	++	+
5	Steroid test	Salkowaski test	-	+++	+
6	Flavonoid test	Lead acetate test	+	+++	+
7	Proteins and amino acids	Ninhydrin test	+	++	+
		Xanthoproteic test	+	++	+
		Millon's test	+	++	+
8	Tannins test	Ferric chloride test	+	++	+
9	Terpenoids test	Salkowski test	+	++	+

- denotes absence, + denotes presence, + + denotes average, +++ denotes abundance of phytochemicals

Table 3: Quantitative analysis of total phenols, total flavonoids, FRAP activity

Sample	Total Phenols (mg GAE/g DW)	Total Flavonoids (mg QE/g DW)	FRAP activity (mM $FeSO_4$)
P. speciosa	13.54 ± 0.016	5.46 ± 0.011	1.9

All values are represented as mean±SD (n=3). GAE – Gallic acid equivalents; QE – Quercetin equivalents.

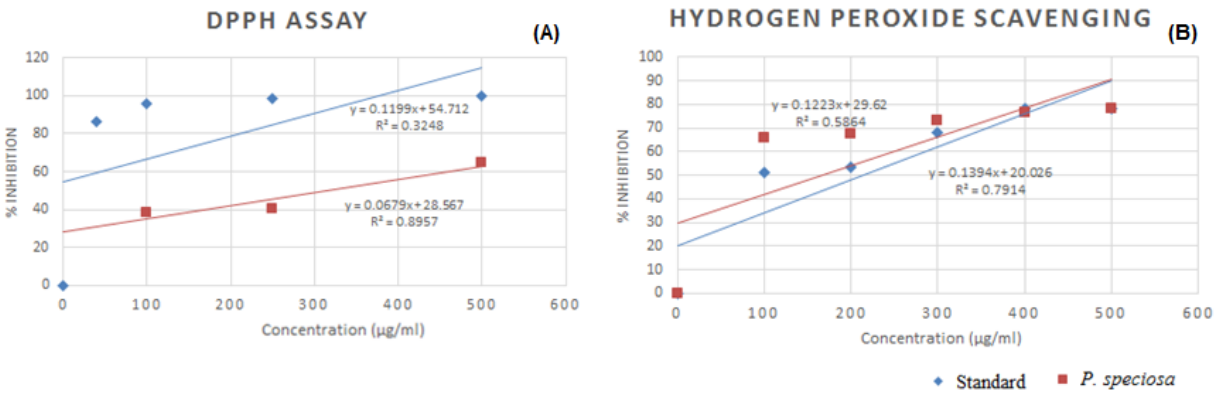

Figure1: Antioxidant activities of seed of *P. speciosa*; DPPH assay (A), H_2O_2 scavenging activity (B)

Fluorescence analysis:

The pods of *P. speciosa* produced different colours and fluorescence under UV light and day light when treated with various reagents (Table 1).

Phytochemical screening:

Phytochemicals or phytoconstituents are defined as secondary metabolites which are produced by majority of plants possessing medicinal uses. The analysis of revealed the presence of saponins, flavonoids, terpenoids, coumarins, quinines, cardiac glycosides, xanthoproteins, steroids, phenols, carboxylic acid group alkaloids, tannins, terpenoids, glycosides, resins in varying concentrations (Table 2).

Total phenols and total flavonoid content:

Secondary metabolites such as phenols are synthesized by the pentose phosphate, shikimate, and phenylpropanoid pathways in plants (Randhir et al., 2004). Flavonoids, the most common of these are known to provide health benefits through their antioxidant activity and modulation of cell signalling pathways. The total phenolic and flavonoid contents of hydromethanolic extract of *P. speciosa* was found to be 13.54±0.016 mg GAE/g and 5.46±0.011 mg/g QE/g of fresh weight tissue respectively (Table 3). The consumption of *P. speciosa* could be attributed to the wide range of physiological properties it exhibits, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Benavente-Garcia et al., 1997; Manach et al., 2005; Middleton et al., 2000).

***In vitro* antioxidant activity**

The antioxidative activities observed in plant extracts are attributed to the different synergistic mechanisms exhibited by various polyphenolic compounds acting as free radical scavengers due to their redox properties. They play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Udegbunam et al.,2010).

FRAP assay:

Fe^{3+} reducing antioxidant power assay has been used to determine antioxidant activity as it is simple and quick and further contributed by the reproducibility of the reaction and the linear relation to molar concentration of the antioxidants. The results showed that FRAP values were higher in hydromethanolic extracts of the plants when compared to the standard ascorbic acid (Table 3).

DPPH scavenging activity:

Total antioxidant capacity of the extracts was calculated using % inhibition against concentration of ascorbic acid (y=0.1199x+54.71; R^2=0.325) (Fig 1A). At 500 µg/ml concentration, *P. speciosa* extract exhibited DPPH radical scavenging activity that was found to be 64.52±2.4 µg/ml. The IC_{50} from the graph were found to be 194.52 µg/ml (ascorbic acid, y = 0.1199x+54.712, R^2=0.3248) and 315.75 µg/ml (*P. speciosa,* 0.0679x+28.56, R^2 =0.6225) (Table 4). The results of the present investigation demonstrate that *P. speciosa* can significantly decrease *in vitro* DPPH radical concentration, thus suggesting that plant extract contains secondary metabolites with strong antioxidant activity.

H_2O_2 scavenging activity:

H_2O_2 crosses cell membranes rapidly, reacts with Fe^{2+} and Cu^{2+} ions to form hydroxyl radicals thereby leading to the generation of toxic effects (Wagner et al.,1996; Handa et al., 2006). H_2O_2 is a weak oxidizing agent and

can inactivate few enzymes and proteins directly, usually by oxidation of essential thiol (-SH) groups. Scavenging of hydrogen peroxide and its percentage inhibition in methanolic extracts of seeds of *P. speciosa* demonstrated IC_{50} values of 166.63 µg/ml (y=0.1223x+29.62; R^2=0.586) as given in the Table 4. Ascorbic acid taken as reference showed an IC_{50} value of 215.68 µg/ml (y=0.1394x+20.0; R^2= 0.7914) (Fig 1B).

Table 4: *In vitro* antioxidant and anti-inflammatory activity of *P. speciosa* seed extract

	Assay	Control			*P. speciosa*	
		Conc. (µg/ml)	% inhibition	IC_{50} (µg/ml)	% inhibition	IC_{50} (µg/ml)
***In vitro* antioxidant activity**	**DPPH scavenging activity**	100	28.6 ± 9.2	194.52	20.3 ± 1.1	315.75
		200	64.35 ±2.1		38.7 ± 7.8	
		300	64.20 ±9.8		40.19 ±6.0	
		400	72.34±2.9		55.31±3.8	
		500	80.83 ±3.4		64.52 ±2.4	
	H_2O_2 scavenging activity	100	51.01± 3.4	215.68	65.91± 3.9	166.63
		200	53.68±2.8		67.53± 7.6	
		300	67.93± 9.9		72.97± 2.3	
		400	78.21± 7.4		76.70± 6.6	
		500	78.42± 5.6		78.06± 5.7	
***In vitro* anti-inflammatory activity**	**LOX inhibiting activity**	100	45.14± 7.8	280.71	25.73± 5.5	493.34
		200	48.26± 8.3		30.24± 1.1	
		300	51.1± 10.2		32.30± 4.7	
		400	56.9± 11.4		38.6± 10.2	
	Proteinase inhibitory activity	100	3.15± 0.3	899.1	10.89± 2.4	1142.3
		200	6.01± 1.3		16.07± 5.7	
		300	10.7± 2.7		17.73± 3.1	
		400	21.3± 4.8		18.86± 1.1	
		500	29.9± 5.9		22.78± 3.6	
	RBC membrane stabilization	100	88.21± 11.2	53.75	86.54± 8.1	67.01
		200	95.26± 8.9		89.61± 10.3	
		300	96.67± 9.6		94.63± 9.8	
		400	97.44± 12.1		97.45± 6.7	
		500	99.36± 6.7		99.21± 12.6	
***In vitro* anti-diabetic activity**	**Amylase inhibitory action**	100	18.9 ± 2.4	324.18	36.84 ± 4.8	199.29
		200	30.3 ± 3.7		48.22 ± 11.1	
		300	54.2 ± 6.9		56.06 ± 9.6	
		400	69.8 ± 10.1		69.8 ± 4.5	
		500	79.2 ± 12.2		79.2 ± 9.6	
	Pancreatic lipase inhibitory action	25	68.1 ± 4.5	227.27	72.9 ± 3.5	196.61
		100	71.9 ± 3.2		72.9 ± 4.7	
		125	73.7 ± 8.9		81.2 ± 9.6	
		250	76.1 ± 9.2		88.5 ± 7.1	
		500	76.3 ± 8.6		89.5 ± 5.9	

Table 5: Kinetic analysis of pancreatic lipase inhibition by crude extracts of *P. speciosa*

	Velocity of enzyme activity at different concentration of substrate [S] (µM)				Vmax (µM min^{-1})	Km (µM)
	20	40	60	80		
Control	2.71	3.968	10.85	12.345	333.33	2595.34
P. speciosa	1.2	2.967	3.636	5.000	44.05	738.85

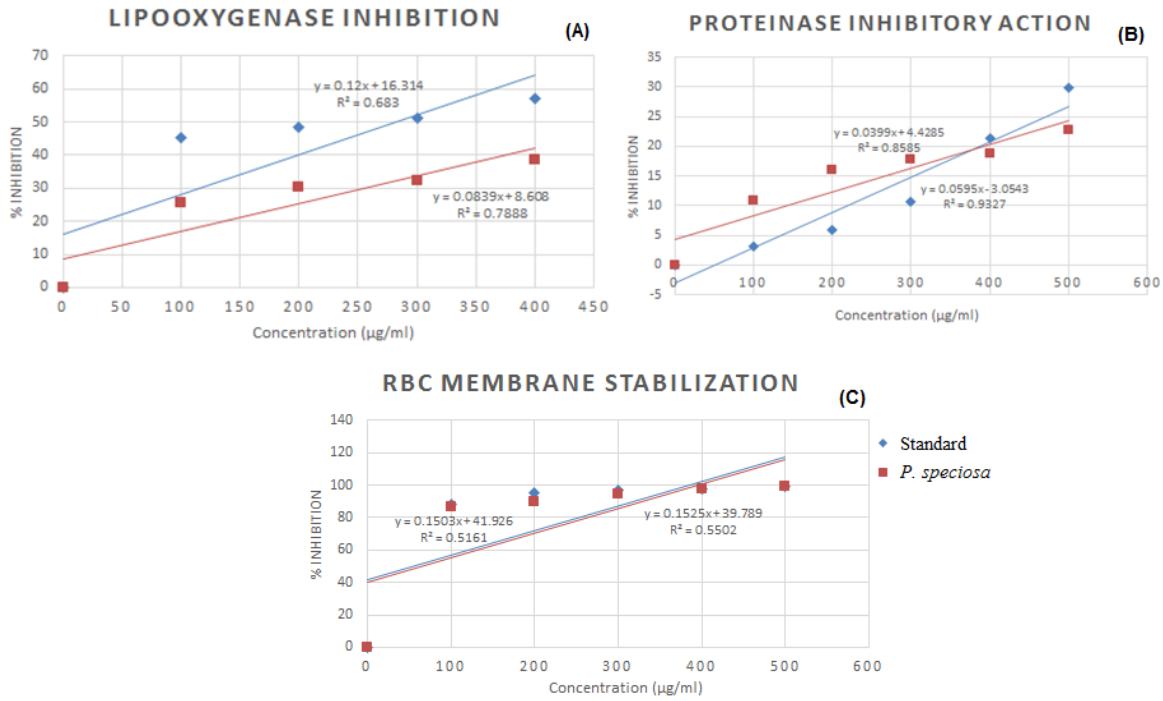

Figure 2: Anti-inflammatory activities of seed extract of *P. speciosa*; lipoxygenase inhibition assay (A), proteinase inhibitory action (B), RBC membrane stabilization assay (C).

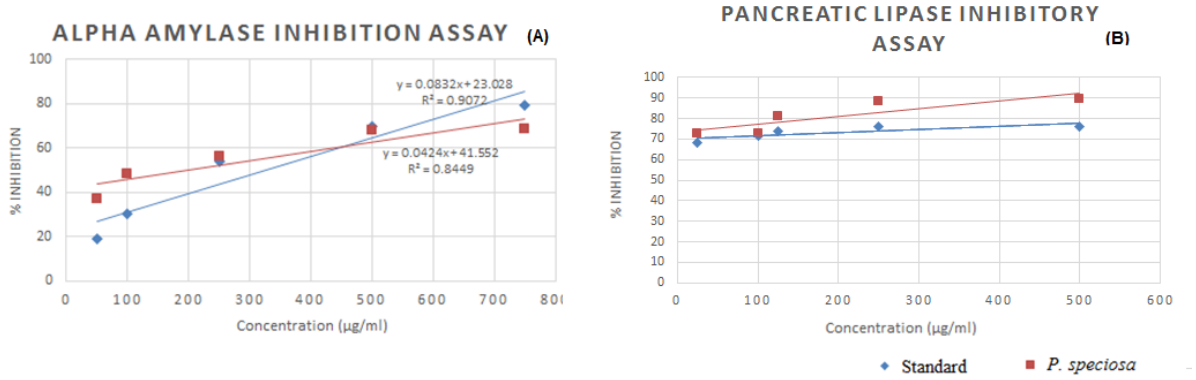

Figure 3: Anti-diabetic activities of seed extract of *P. speciosa*; alpha amylase inhibition assay (A), pancreatic lipase inhibitory assay (B).

***In vitro* anti-inflammatory activity**

Inhibition of Lipooxygenase activity:

Lipoxygenases (LOXs) comprise a family of non-heme iron-containing dioxygenases, representing the key enzymes in the biosynthesis of leukotrienes that have been postulated to play an important role in the pathophysiology of several inflammatory and allergic diseases (Ghosh,1998). In general, lipoxygenase inhibitors can bind covalently to iron or form the molecular complexes blocking access to iron (Skrzypczak-Jankun et al., 2007). The plant lipoxygenase pathway is in many respects equivalent to the 'arachidonic acid cascades' in animals. For this reason, the *in vitro* inhibition of lipoxygenase constitutes a good model for the screening of plants with anti-inflammatory potential (Abad et al., 1995). Methanolic extracts of seed of *P. speciosa* were studied at 100-500 µg/ml, and the inhibition obtained is as shown in Table 4. From these results, the strongest inhibition for all the samples was obtained at concentration of 400 µg/ml. The standard exhibited 56.9±11.4 % inhibition at a concentration of 400 µg/ml. Percentage inhibition of lipoxygenase in methanolic extracts of seed *P. speciosa* demonstrated IC_{50} values of 493.34 µg/ml (y=0.0839x+8.61, R^2=0.788) (Table 4, Fig 2A). Ascorbic acid taken as reference exhibited 280.71 µg/ml (y=0.12x+16.31, R^2 = 0.683).

Proteinase Inhibitory activity:

Activated leukocytes are widely implicated in cardio-vascular disease (CVD). Mononuclear cells are recruited to sites of vascular injury thus contributing to foam cells within atherosclerotic plaques (Mandal, 2013). Activated white blood cells discharge into the surrounding milieu reactive oxygen species (ROS) and a variety of proteolytic enzymes, particularly serine proteases (Venkatalakshmi et al., 2015). Inhibition of 22.78% and 29.9% at 500µg/ml was observed for *P. speciosa* and aspirin respectively (Table 4). The IC_{50} values as calculated from graph were found to be 899.1 µg/ml (Aspirin, y=0.0595x+3.054, R^2=0.937), 1142.3µg/ml (*P. speciosa,* y=0.0399x+4.428, R^2=0.858) (Fig 2B).

RBC membrane stabilization activity:

The erythrocyte plasma membrane resemblances to the lysosomal membrane and hence the stabilizing effect of drugs on erythrocyte membrane may correlate with its lysosomal membrane stabilizing effect (Debnath et al.,2013). The lysosomal membrane stabilization leads to the inhibition of release of the inflammatory mediators and consequent inhibition of the process of inflammation (Bhutkar and Bhise, 2012). Maximum inhibition was observed at 500 µg/ml, where aspirin and *P. speciosa* showed 99.36 and 99.21% inhibition respectively (Table 4). The IC_{50} values for the standard drug and *P. speciosa* was found to be 53.75 µg/ml (y=0.1503x+41.9, R^2=0.516) and 67.01 µg/ml (y=0.1525x+39.79, R^2=0.5502) (Fig 2C).

***In vitro* anti-diabetic activity**

Alpha amylase inhibition assay:

The high prevalence of diabetes as well as its long-term complications has led to an ongoing search for hypoglycaemic agents; over the years, various medicinal plants and their extracts have been reported to be effective in the treatment of (Debnath et al., 2013). The digestive alpha amylase is responsible for hydrolyzing dietary starch (maltose), which breaks down into glucose prior to absorption. Inhibition of alpha amylase can lead to reduction in post prandial hyperglycemia in diabetic condition. The results showed that extracts of *P. speciosa* have antidiabetic activity which is compared with acarbose standard. *P. speciosa* showed a maximum inhibition of 79.2% at 500 µg/ml (Table 4). The IC_{50} of *P. speciosa* and standard acarbose were found to be 199.29 µg/ml (y=0.04242x+41.55, R^2=0.8449) and 324.18 µg/ml (y=0.0832x+23.02, R^2=0.9072) (Fig 3A). This shows that *P. speciosa* has anti-diabetic activity.

Pancreatic lipase (PPL) inhibitory assay:

Pancreatic lipase is a key enzyme in dietary triacylglycerol absorption, hydrolyzing triacylglycerol to 2-monoacylglycerol and fatty acids. It is well known that dietary fat is not directly absorbed from the intestine unless it has been subjected to the action of pancreatic lipase (Skrzypczak-Jankun et al., 2007). The polyphenolic extracts from a number of plants have been shown to be effective inhibitors of the intestinal pancreatic lipase enzyme (Harborne, 1998). A dose-dependent manner of inhibition was exhibited by the plants extract (Table 4). Maximum *in vitro* inhibitory activity exhibited by *P. speciosa* and Orlistat, the standard drug and pancreatic lipase inhibitor was found to be 89.5% and 76.3 % at 500 µg/ml respectively. The IC_{50} of *P. speciosa* and standard Orlistat were found to be 227.27 µg/ml and 196.61 µg/ml (Fig 3B).

Kinetic study of pancreatic lipase:

The mode of inhibition of the enzyme pancreatic lipase by *P. speciosa* was studied by double-reciprocal Lineweaver-Burk at 100 µg/ml as shown in Fig 4. Since both the kinetic parameters i.e., the maximal velocity of the PPL enzyme-substrate extract reaction (V_{max}) and the affinity (K_m) as determined from the double reciprocal trend lines were affected by the extract concentration, a mixed mode of inhibition was predicted. The Michaelis-Menten parameters are tabulated in Table 5. The mixed mode inhibition exhibited by pancreatic lipase means that the inhibitor binds to the enzyme whether or not the enzyme has already bound the substrate but has a greater affinity for one state or the other, thus resulting in the increase of K_m.

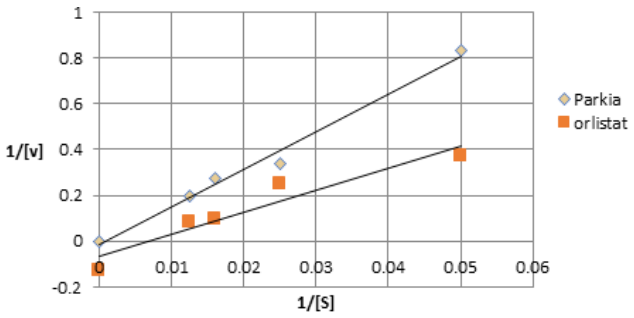

Figure 4: Lineweaver-Burke plot of *P. speciosa* extract against Pancreatic lipase

Antimicrobial Activity of Extracts:

Most of the bioactive medicinal metabolites are synthesized via plant secondary metabolic pathways during the vegetative stage of a plant's life cycle and

Table 6: Antibacterial activity of extracts of *P. speciosa*. The zone of inhibition is expressed in mm

Strain	Zone of Inhibition (mm)	
	P. speciosa	**Ciprofloxacin**
E. coli	6	7
K. pneumoniae	8	11
P. aeruginosa	9	11
S. aureus	10	20

these compounds are responsible for their therapeutic properties. In general, these compounds effectively inhibit and/or stop microbial growth via disruption of the synthesis of microbial nucleic acids, proteins and cell walls (Randhir et al., 2004). It was found that the methanolic extract of *P. speciosa* showed highest zone of inhibition for the gram positive *S. aureus* at 10 mm (Table 6).

CONCLUSION

The present study was carried out to assess the potential of *P. speciosa* Hassk. as an alternative to synthetic drugs. The hydro-alcoholic extract possesses potential antioxidant, anti-inflammatory, anti-diabetic and anti-microbial activities. The presence of high levels of phenolics and flavonoids in the sample could be responsible for its radical scavenging activities. In addition, its use in ethanomedicine has been substantiated by its ability to inhibit pancreatic amylase, pancreatic lipase, lipooxygenase and proteinase enzymes. There arises further need to explore the bioactive constituents responsible for this activity as well as to elucidate the exact mechanism of action and to extrapolate the results on animal models in order to establish possible side effects. Thus, further understanding of metabolic engineering and applying its principle to enhance the synthesis and accumulation of bioactive compounds is the need of the hour.

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