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In vitro bioactivity and phytochemical characterization of *Nypa fruticans.* Wurmb: a mangrove from Kerala, India

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Abstract

Nypa fruticans Wurmb is an underutilized mangrove plant in India. Mangroves generally contain more secondary metabolites to thrive in a saline environment. The present study focuses on estimation of primary metabolites like carbohydrates, proteins, lipids etc and secondary metabolites like flavonoids, phenols, tannins etc from different parts of Nypa plant such as leaf, stem, male flower, bracts, unripe endosperm and ripe endosperm. Total phenolic content (79.91±1.63 mg/g) and flavonoid contents (99.5±0.936mg/g) were high in the leaves compared to young and mature endosperm. The antioxidant activity using DPPH and SOD scavenging assay also showed very high in leaves. IC_{50} value of leaves for DPPH scavenging assay was 6.11±1.68 µg/ml and SOD scavenging assay was 11.85±1.16 µg/ml. GC-MS and LC-MS analysis of NFME of leaves identified several bioactive compounds. Brenzcatechin, 4-vinylphenol, 4-vinyl 2-methoxy-phenol, hexadecanoic acid etc were some of the compounds obtained from GC-MS analysis. In vitro study of methanolic extract for anti-diabetic, anti-arthritic, and anti-inflammatory activity were evaluated and % inhibition was high. Percentage of a-amylase inhibition of the NFME at 500 µg/ml concentration was 69.87% which showed a significant level of anti diabetic activity. Inhibition of haemolysis of erythrocytes at high temperature was 72.94% which indicated the anti inflammatory potential of NFME. Protein denaturation inhibition of methanolic extract was also significant (53.08%) that showed the anti arthritic activity.

Keywords: *Nypa fruticans*. Wurmb, Flavonoids, Phenol, GC-MS, LC-MS.

Introduction

Nypa fruticans Wurmb was reported from Muziris Pattanam wetlands of Ernakulam district of Kerala[1]. The usage of this plant is scarce in India, but used in countries like Malaysia, Nigeria, and Philippines etc. Nypa palm is a unique prostrate palm flourishes in a mangrove environment. It is the only palm considered as mangrove[2]. It yields oil, starch, sugar etc. Large amount of sap and potential for sugar production has given it the name sugar alternative having minerals and low glycaemia index[3]. *Nypa* plant is useful in many aspects as it gives variety of useful products like coconut palm. This plant can produce alcohol which can be useful as biofuel. But there is lack of scientific report on *Nypa fruticans*. Wurmb. as a fuel or alcohol producing plant and also the plant is not much familiar among common people.

Primary metabolites are the compounds essential for the plant and are directly involved in the metabolism. Carbohydrates, proteins and lipids are required for the overall development of a plant and are present in sufficient quantities in every parts of the plant. Secondary metabolites on the other hand are not directly involved in the plant metabolism but provide some defenses to the plants from external factors[4]. These defense chemicals present in plants are also known as phytochemicals. Phenols and flavonoids are largely distributed secondary metabolites in the plant kingdom. Mangroves are rich source of phenols and flavonoids and are related with the antioxidant property of plants. Many of these phytochemicals are useful to mankind by acting as drugs, pharmaceuticals, antioxidants, anti-diabetic compounds, stimulants etc. Secondary metabolites have wide range of applications in the modern world as many of the synthetic products are causing adverse results. Prasad and co-workers[5,6] reported that the antioxidant activities of the edible part of immature and mature *Nypa* fruits were related to the phenolics.

In this paper plant parts such as leaf, stem, male flower, bracts, unripe endosperm and ripe endosperm of *Nypa* plant are used to analyse the presence of primary and secondary metabolites. Many of the phenolic and flavonoid compounds were identified from the endosperm of *Nypa fruticans.* Wurmb. Some of the identified phenolics in endosperm of *N. fruticans* are chlorogenic acid, protocatechuic acid and kaempferol[6]. Total phenolic compounds present in *Nypa fruticans.* Wurmb varied between the plant parts. Leaves are the major site of stored phenolic compounds, which could be utilized as a source of substances in scavenging the radical ions and antioxidant[7]. In this paper, we conducted Antioxidant study of plant parts such as leaf, young endosperm and mature endosperm and compared the IC_{50} values. GC/MS and LC/MS analysis made easier to find several useful phytochemicals especially in the category of

flavonoids and phenols. In this paper we have investigated the GC/MS and LC/MS composition of NFME of leaves and verified the relation between various phytochemicals and their role in curing diseases. Although several studies conducted in anti-diabetic property[8,9], we tried to analyse the *in vitro* potential in curing diabetes. In this paper along with the anti-diabetic study, *in vitro* studies conducted for anti-inflammatory and anti-arthritic property.

Materials and methods

Stem, leaflets, flowers, fruits etc of *Nypa fruticans*. Wurmb were collected from the Muziris Pattanam wetlands of Ernakulam district, Kerala. These plant parts were air dried and powdered using an electric grinder and used for estimation. Analytical grade chemicals were used during experiment.

Estimation of total Carbohydrates content: Estimated by the method of Dubois et al, 1956[9]. 1gm sample was extracted using 20ml 1M H_2SO_4 by keeping in a boiling water bath at 100ºC for 1 hour. 1ml filtered sample taken in 100ml beaker and 1ml 5% phenol is added followed by 5 ml concentrated H_2SO_4 under cold condition. Absorbance was measured at 485 nm after 30 minutes and standard glucose solution was used for calibration.

Estimation of total Protein content: Proteins were estimated by Lowry's method[10]. 0.01gm sample in 10ml 2N NaOH extracted at 80ºC for 2 hours. Added cu-reagent mixture shake well continuously for 10 minutes. Then add 1ml 1:1 folin reagent, kept for 40 minutes and read absorbance at 750nm. BSA was used for calibration.

Estimation of total Lipid content: Estimated by Barnes and Blackstock, 1973[11] method. 0.01gm sample is mixed with 2:1 chloroform- methanol reagent. Heated at 60ºC for 30 minutes. Pipetted and evaporated to dryness in a desiccator for 2 to 3 days. Conc.H_2SO_4 added and kept in a boiling water bath at 100ºC for 10 minutes. After cooling vanillin reagent was added and reading taken in spectrophotometer at 520 nm.

Estimation of total phenolic content: Estimated by Folin-Ciocalteu method[12]. The extract was mixed with 5ml of Folin-Ciocalteu reagent and after 5 minutes 7.5% (W/V) Na_2CO_3 was added, mixed for 20 seconds. Incubated at room temperature for 2 hours in the dark absorbance was measured at 740nm using UV-visible spectrophotometer. Using a six point calibration curve the total phenolics were determined by comparing the values obtained within the calibration curve of standard.

Estimation of total Flavonoid content: Flavonoids were analysed by Aluminium chloride colorimetric assay[13]. Air dried powdered sample weighed and extracted with 80% methanol. An aliquot of the extract or standard solution of catechin was added to10 ml volumetric flasks containing 4ml of distilled water. Added 0.3ml of 10% $AlCl_3$ and after 5 minutes pipette 2ml 1M NaOH and the total volume was made up to 10ml in distilled water. Absorbance was measured against the prepared reagent blank at 510nm and the total flavonoid content was obtained in relation with the values obtained for the standard.

Estimation of total Tannin content[14,15]: 0.01gm sample extracted with 10ml .05M NaOH in boiling tube placed in a water bath at 60ºC $I^{1}/_{2}$ hour. Extract centrifuged and 5 ml pipetted and to this added 1ml citrate buffer, 1ml folin reagent and 10 ml carbonate tartarate reagent. After 30 minutes and read the optical density at 760 nm in a spectrophotometer using standard tannic acid to reach the results.

GC/MS analysis of NFME of leaves: GC/ MS of methanolic leaf extract of the plant was by using Shimadzu GC-MS instrument Model Number: QP 2010S. For MS detection, Rxi-5 Sil MS used and capillary column of size 30m× 0.25mm, 0.25 µm film thickness was used. GC/MS solutions software was used for the analysis of data. Major compounds were identified by their retention times and mass fragmentation patterns using data of standards from the NIST 11, WILEY 8 libraries.

LC/MS analysis of NFME of leaves: LC/MS of methanolic leaf extract of the plant was carried out using Xevo G_2 QTOF system. Capillary voltage of 3.00 KV in the positive ion mode and 2.5 KV in the negative ion mode were used, sampling cones of 30V and extraction cones of 1KV were used in both positive and negative mode. For chromatographic separation, the column was held at 95% solvent A (0.1% acetic acid in water) and 5% solvent B (methanol) in different gradient. Optimum parameters were followed throughout the MS experiment.

Antioxidant activity - SOD Scavenging assay[16]: Fresh sample of 0.5gm ground with 3 ml of potassium phosphate buffer, centrifuged for 10 minutes and the supernatants were used for the experiment. The solution consisted of 1.2ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3ml of NBT, 0.2ml of the enzyme preparation and water in a total volume of 2.8ml. 0.2 ml of NADH added and the mixture was incubated at 30°C for 90 seconds and stopped the reaction adding of 1.0 ml of glacial acetic acid. Shaken with 4.0ml of n-butanol, kept for 10 minutes and centrifuged. Chromogen in the butanol layer was measured at 560nm in a spectrophotometer.

Antioxidant activity-DPPH Scavenging assay[17]: 0.1gm sample powder extracted with 20ml of 70% methanol placed in a shaker for 48 hours. It is evaporated in a hot water bath and 0 .01gm weighed and to this added 10 ml methanol. Different volume of extracts prepared (10, 30, 50 µl) and 1ml is taken from this and added 2 ml DPPH solution after appropriate dilution. Methanol used as the blank and DPPH in methanol used as the positive control The radical scavenging activity was calculated as follows:

Percentage Inhibition= [Absorbance of control – Absorbance of test sample)/Absorbance of control]/ × 100]

Anti-arthritic activity[18]**:** 0.05 ml different concentrations of test samples were taken mixed with 0.45 ml (0.5% w/v) BSA. Incubated the samples at 37°C for 20 minutes and then the temperature was increased by keeping the samples at 57°C for 3 minutes. After cooling, added 2.5 ml of phosphate buffer to the above solutions. The absorbance was measured using UV-Visible spectrophotometer at 416nm. The control used with 100% protein denaturation. The percentage inhibition was calculated as.

Percentage Inhibition= [Absorbance of control – Absorbance of test sample)/Absorbance of control]/ × 100]

Anti-diabetic activity[19]**:** Different concentration of the extracts taken (Stock-10mg/ml). Adding phosphate buffer of pH 6.9 volume made in to 0.5ml and phosphate buffer used as blank 0.5ml of alpha amylase (0.5mg/ml) added and incubated at 25^{o}C for 10 minutes. 1% starch solution (0.5 ml) was mixed with sodium phosphate buffer of pH 6.9 to all the tubes, and then incubated at 25^{o}C for 10 minutes. Reaction stopped by adding 1 ml of DNS and then kept in boiling water bath for 5 minutes further to room temperature. 8 ml distilled water added and read the absorbance of the solution in calorimeter at 570 nm against blank solution.

Percentage Inhibition= [Optical density of control – Optical Density of test)/ Optical density of control}/ × 100]

Anti-inflammatory activity by Heat induced haemolysis[20]**:** 10 ml fresh whole human blood was collected and transferred to the centrifuged tubes and centrifuged at 3000 rpm for 10 min and were washed three times with equal volume of normal saline. The volume of the blood was made into 10% v/v suspension with normal saline. The reaction mixture consisted of different concentration of test and 1ml of 10% RBCs suspension, instead of test, saline was added to the control test tube. Reaction mixture were incubated in a water bath at 56^{o}C for 30 min. The reaction mixture was cooled and centrifuged at 2500 rpm for 5min and the absorbance of the supernatants was measured at 560nm.

Percentage Inhibition= (optical density of control – optical Density of test)/optical density of control}/ × 100]

Results and discussion

Nypa fruticans. Wurmb selected in this phytochemical study is one of the mangroves which has limited occurrence in Kerala. Several investigations have shown that Nypa palm has antioxidant properties and therapeutic potential and it might be due to their phenolic components[6]. Although the sap is used as a beverage[21], the leaves, stem, flowers, fruits etc are discarded.

Two types of metabolites occur in plants and primary metabolites control the growth and metabolism. The results of our investigation showed that the primary metabolites and secondary metabolites in the plant are high. Among primary metabolites, carbohydrate content is very high in the stem (566±1.527mg/g) and unripe endosperm (426±1.285mg/g). Protein content is higher in the stem (86.151±0.653mg/g) and lipid content is higher in the flower (100.868±1.99mg/g) and leaf (87.91±1.55mg/g) (Figure-1).

Phenolics and flavonoids are rich in fruits and vegetables that has potential antioxidant activities[22]. Several investigations in plants have shown that phenols and flavonoids are indicators of antioxidants. Phenols and flavonoids are the most widely distributed secondary metabolites in plant kingdom. Phenols, flavonoids and tannins are defensive metabolites in plants and can act against reactive oxygen species. Flavonoids have been found to possess anti-oxidant and anti-radical properties[23]. Thus antioxidant activity and the occurrence of flavonoids and phenols is much related.

All the parts analyzed have the content of phenols, flavonoids and tannins. Here in the above investigation, flavonoids were very high in the leaf, bract, unripe endosperm and ripe endosperm. Flavonoid content in the plant ranged from 11.34 ± 0.12mg/g in the flower to 99.5 ± 2.3mg/g in the leaf. Phenol content was also higher in the leaf (79.231 ± 1.34mg/g), bract (74.253±1.206mg/g) and unripe endosperm (56.393 ± 1.143mg/g). Tannin content was very less in all parts selected for the study (2.112 ± 1.06m/g in ripe endosperm to3.569 ± 0.112mg/g in bract) (Figure-1).

Although the sap of the inflorescence is used as a beverage[24,25], fruits and leaves are discarded. Higher antioxidant activity was also reported in other palm trees that contains high phenolics[4,5,26,27]. Methanol extract of leaves, unripe endosperm and ripe endosperm showed that DPPH scavenging activity was highest in the leaves. IC_{50} values for leaves were 6.11± 1.68µg/ml, this lower IC_{50} related with higher antioxidant activity (Figure-8), (Table-3). This DPPH scavenging activity of the leaves might be contributed by the phenolic and flavonoid compounds. IC_{50} value for SOD scavenging activity was also less in the leaves (Figure-9). Unripe endosperm showed low IC_{50} value compared to ripe endosperm for both DPPH scavenging activity and SOD scavenging activity (Figure-8 and 9) (Table-3). All results thus proved that the low IC_{50} are the reflection of high amount of phenols and flavonoids. Above results agreed with the previous report[6] that leaves are the important source of phenols, flavonoids and antioxidants.

In the present study, excellent separation of compounds occurred during GC/MS analysis of methanolic leaf extract of NF (Figure-2). After comparing the peaks with library 14 compounds obtained and that are given in the Table-1. Compounds identified are biologically active in different aspects. 2, 3-Dihydro-3, 5-Dihydroxy-6-Methyl-4H-Pyran-4-One is an Antioxidant that prevents colon cancer growth by inhibiting NF-Kappa B cell[28]. Brenzkatechin is a natural phenol and antioxidant, included in the chemical family of flavonoids. 4-Vinylphenol is a phenolic compound found in beer and vine. 4-Vinyl-2-Methoxy-Phenol and 7-Tetradecenal, (Z)- are

compounds acting as pheromones[29,30]. 9-Octadecenoic Acid (Z)- is used an emulsifying agent, emullient[31], common monounsaturated fat in human diet.

LC/MS QTOF analysis of methanolic leaf extract of NF revealed compound chromatograms for positive and negative ions. Some of the selected mass of negative ion compounds further analysed through MS/MS spectrum. Chromatograms (Figure-3-7) for fragmented ions were obtained and compared the spectral characteristics with previous references detected the occurrence of bioactive flavonoid and phenol compounds (Table-2).

Identified flavonoid catechins were widely used natural phenol and antioxidant. As flavonoids, catechins are *in vitro* antioxidants in high concentration but compared with other flavonoids, their antioxidant potential is less[32]. *p*-Coumaric acid has antioxidant properties and has potential to reduce the risk of stomach cancer[33] by reducing the formation of carcinogenic nitrosamines[34]. *p*-Coumaric acid in honey also demonstrate *in vitro* anti-inflammatory activity[35]. Chlorogenic acid is an antioxidant compound and some review articles in 2011[36] and 2014[37] report moderate blood pressure lowering effects from chlorogenic acid administration. Procyanidins are a group of proanthocyanidins occur in apples, grapes, pine bark, cinnamon, grape seeds, grape skin[38], cocoa, red vines[39] etc. Rutine is a citrus flavonoid glycoside found in many plants including buckwheat[40].

In vitro anti-arthritic activity was performed using the method of inhibition of protein denaturation. Concentrations ranging from 100µg/ml to 1000µg/ml were tested to find out the % inhibition and 50% inhibitory concentrations. A dose dependent increase in the % inhibition and anti-arthritic activity from 30.86µg/ml to 85.32µg/ml.IC_{50} value of NFME was 391.92±1.021 µg/ml (Table-4). IC_{50}value of standard drug Diclofenac was reported as 40µg/ml[36]. α- amylase inhibitory activity of NFME showed a concentration dependent increase from 25.45% to 90.56% at 100µg/ml to 1000µg/ml. The extract gave an IC_{50} value of 380.519± 3.271 µg/ml (Table-5). The standard drug Acarbose showed IC_{50} value of 325.50±4.7µg/ml[37]. The NFME of different concentrations showed haemolysis at high temperature. The results showed that the extract at higher concentrations 500µg/ml and above protect the erythrocyte membrane against lysis induced by heat. The % of inhibition varied from19.32% to85.39 % at concentration of 100 µg/ml to 1000 µg/ml (Figure-10). It is comparable with standard drug Diclofenac sodium which at 100 µg/ml offered a protection against damaging effect of heat solution[38]. Calculated IC_{50} value of anti-inflammatory activity was 303.33±4.06µg/ml (Table-4).

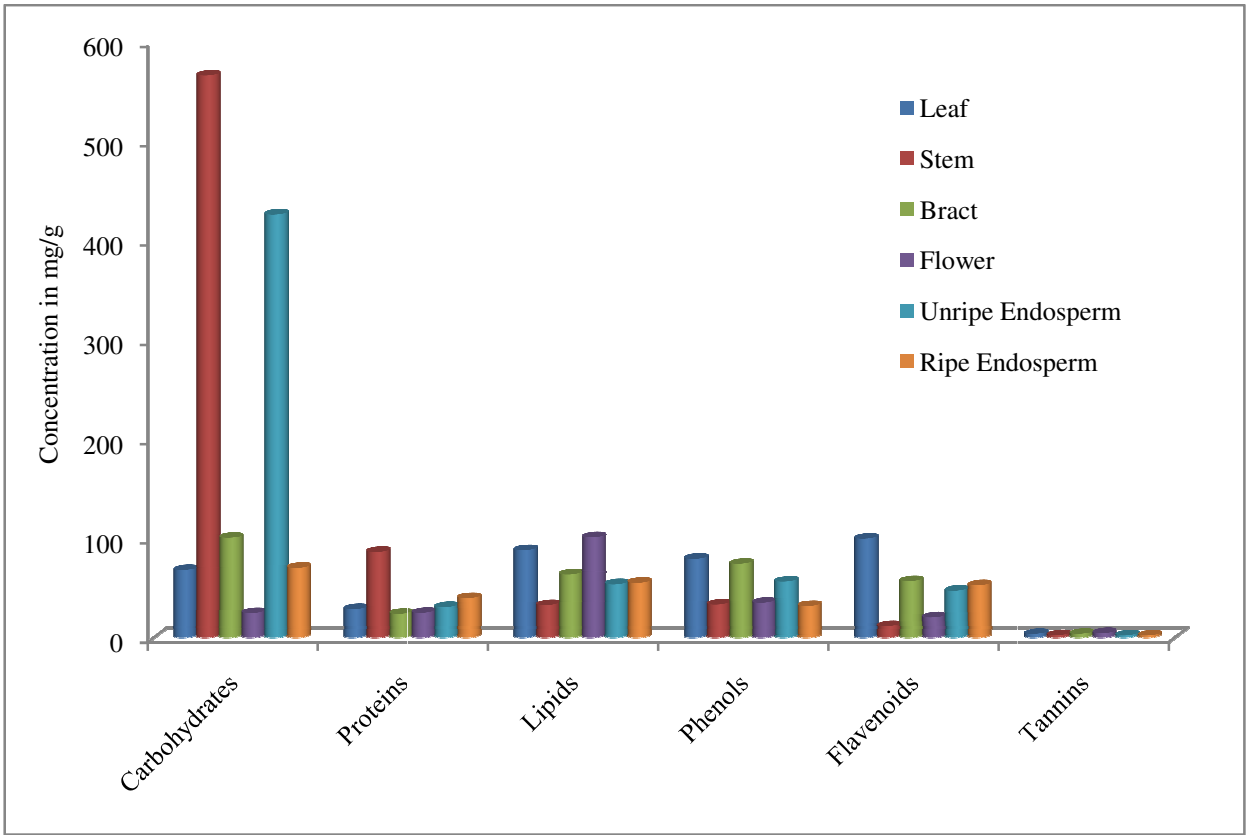

Figure-1: Concentration of Primary metabolites (Carbohydrates, Proteins and Lipids) and Secondary metabolites (Phenols, Flavonoids and Tannins)

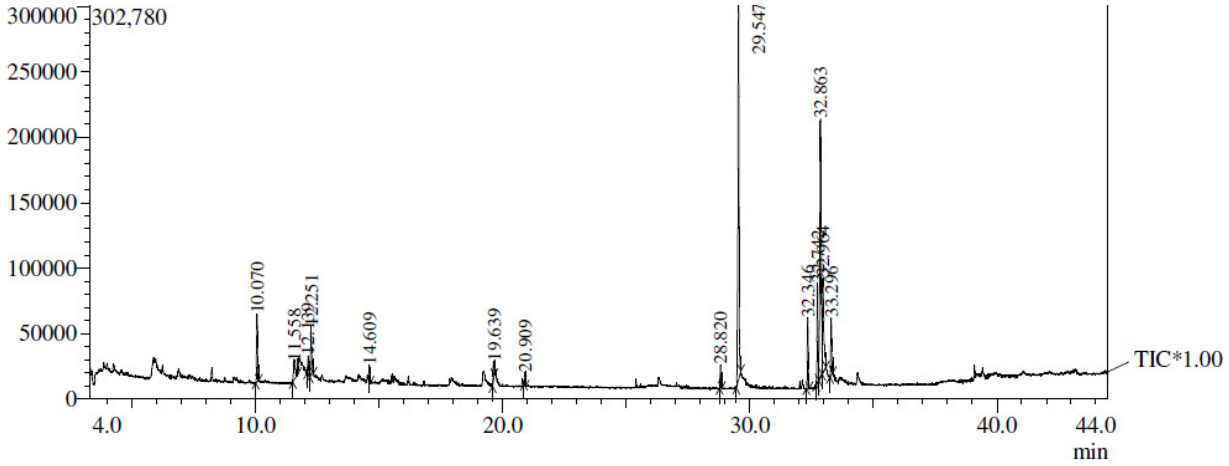

Figure-2: GC/MS spectrum of NFME of leaves.

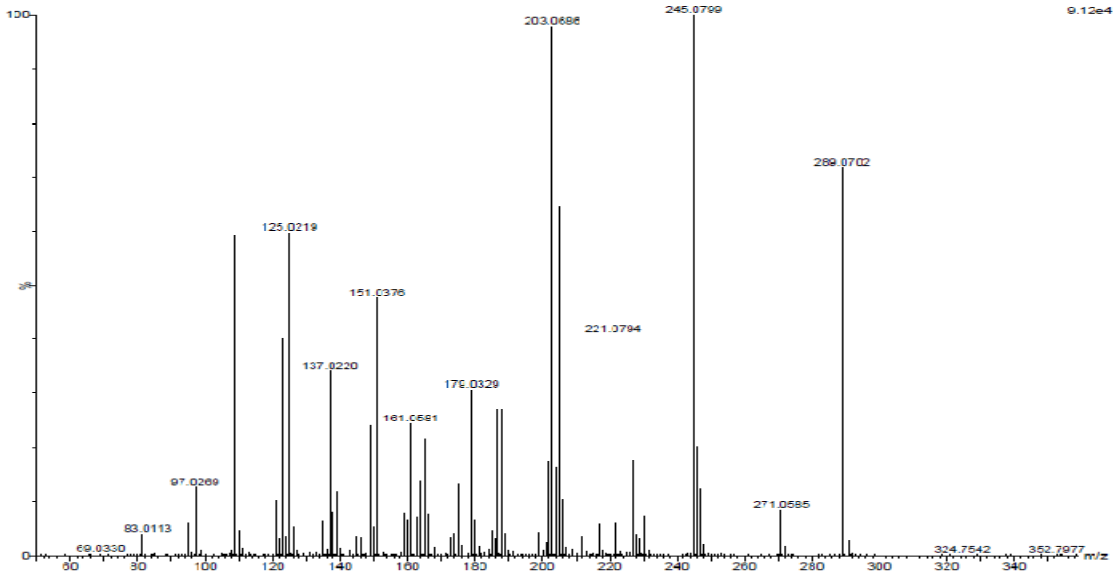

Figure-3: MS/MS spectrum of (+)-Catechin.

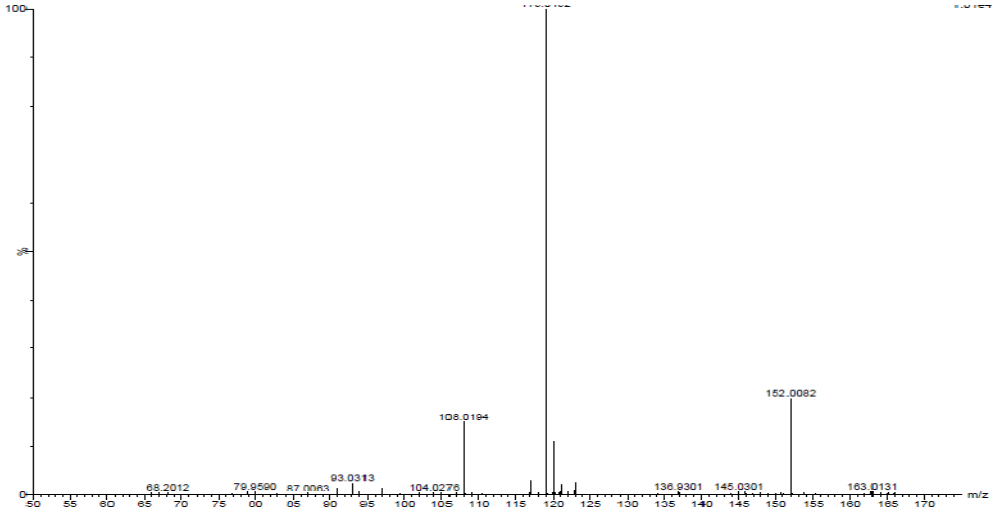

Figure-4: MS/MS spectrum of *p*-Coumaric acid.

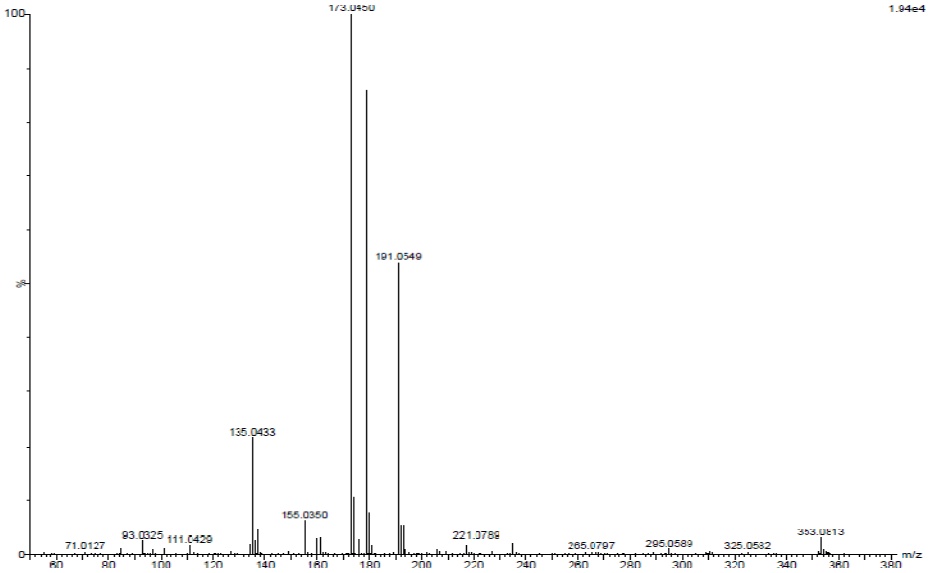

Figure-5: MS/MS spectrum of Chlorogenic acid.

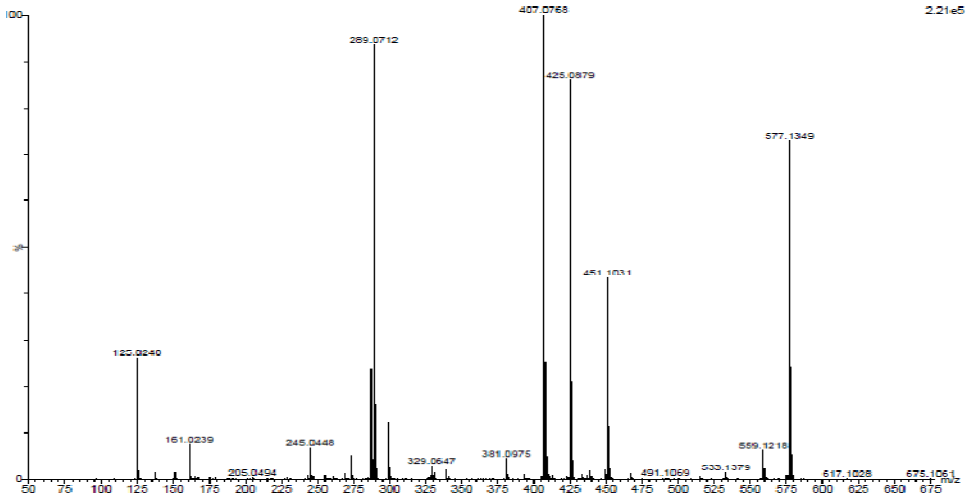

Figure-6: MS/MS spectrum of Procyanidin B2.

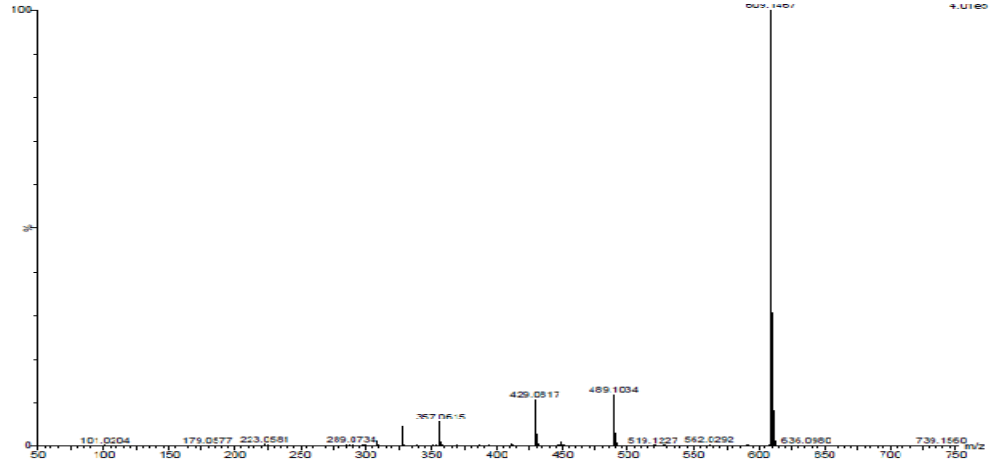

Figure-7: MS/MS spectrum of Rutine.

Table-1: Library search results

Peak#	Name	Formula	R.Time	Area	Area %
1	2,3-Dihydro-3,5-Dihydroxy-6-Methyl-4H-Pyran-4-One	$C_6H_8O_4$	10.070	137810	4.76
2	Brenzkatechin	$C_6H_4(OH)_2$	11.558	76547	2.64
3	4-Vinylphenol	C_8H_8O	12.139	32545	1.12
4	5-(Hydroxymethyl)-2-Furaldehyde	$C_6H_6O_3$	12.251	124646	4.30
5	4-VINYL-2-Methoxy-Phenol	$C_9H_{10}O_2$	14.609	21308	0.74
6	2-TERT-BUTYL-4-(1,1,3,3-Tetramethylbutyl) Phenol	$C_{18}H_{30}$	19.639	34250	1.18
7	Tridecanoic acid	$C_{13}H_{26}O_2$	20.909	24068	0.83
8	Tetradecanoic Acid, 12-Methyl-, Methyl Ester	$C_{16}H_{32}O_2$	28.820	35454	1.22
9	Hexadecanoic Acid	$C_{16}H_{32}O_2$	29.547	858749	29.66
10	Tetradecanal	$C_{14}H_{30}$	32.346	133919	4.62
11	Oxacycloheptadec-8-EN-2-ONE	$C_{16}H_{28}O_2$	32.742	225842	7.80
12	7-Tetradecenal, (Z)-	$C_{14}H_{26}O$	32.863	785643	27.13
13	9,12-Octadecadienoic Acid (Z,Z)-	$C_{18}H_{32}O_2$	32.964	294314	10.16
14	9-Octadecenoic Acid (Z)-	$C_{18}H_{34}O_2$	33.296	110462	3.81

Table-2: Characterization of Phenolic and Flavonoid compounds in NFME of leaves by LC-QTOF/MS and MS/MS data.

Compounds	Rt (min)	Molecular formula	[MS]- m/z	[MS-MS]- m/z of fragment ions
(+) - Catechin	3.108	$C_{15}H_{14}O_6$	289.0702	125.0219, 137.0220, 151.0376, 161.0581, 179.0329, 203.0686, 221.0794, 245.0799, 271.0585, 289.0702
P-Coumaric Acid	3.239	$C_9H_8O_3$	163.0131	119.0492, 145.0301
Chlorogenic Acid	3.274	$C_{16}H_{18}O_9$	353.0813	135.0433, 191.0549
Procyanidin B2	2.560	$C_{30}H_{26}O_{12}$	577.1349	289.0712,407.0768,425.0879, 451.1031, 577.1349
Procyanidin B3/B4/B5	2.560	$C_{30}H_{26}O_{12}$	577.1349	289.0712, 407.0768
Rutine	4.013	$C_{27}H_{30}O_{16}$	609.1467	179.0577

Table-3: IC_{50} values for DPPH assay and SOD assay.

Sample	IC_{50} for DPPH (µg/ml)	IC_{50} for SOD (µg/ml)
Nypa fruticans- Leaves	6.11±1.68	11.85±1
Nypa fruticans- Unripe Endosperm	152.53±4.01	146.83±2.04
Nypa fruticans- Ripe Endosperm	342.75±3.7	371.88±3.07

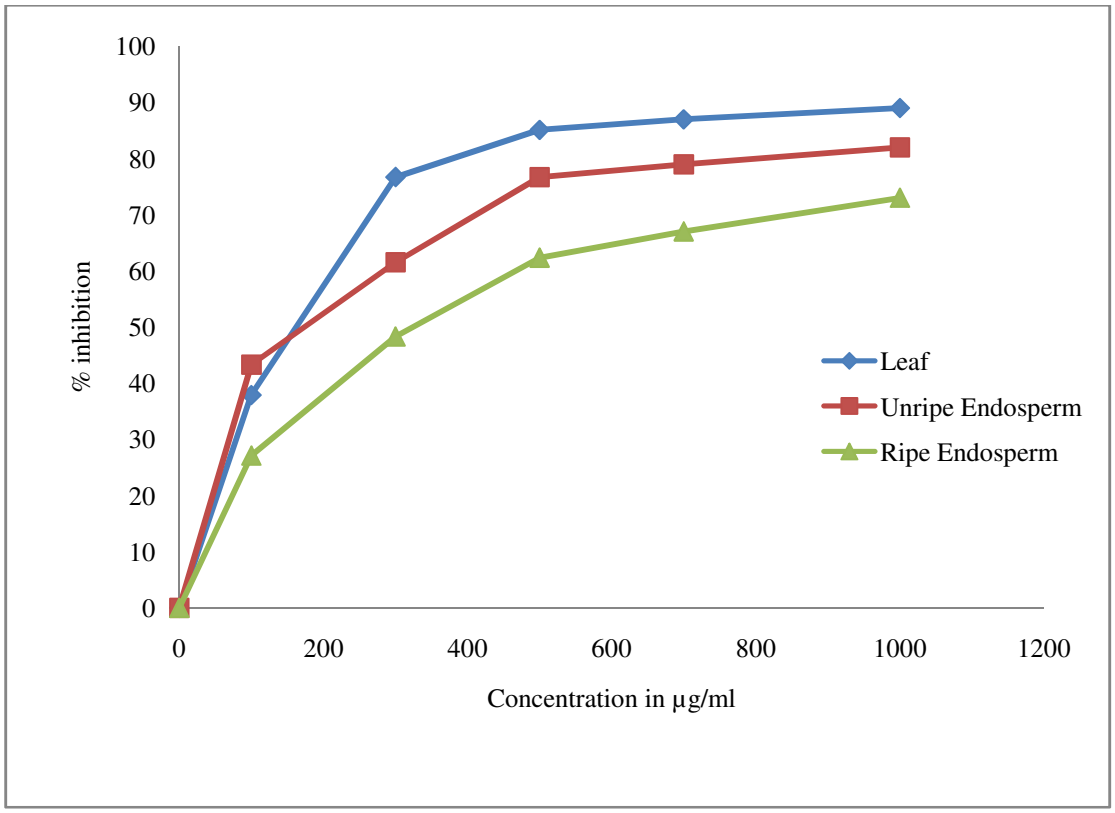

Figure-8: Antioxidant activity by DPPH scavenging assay.

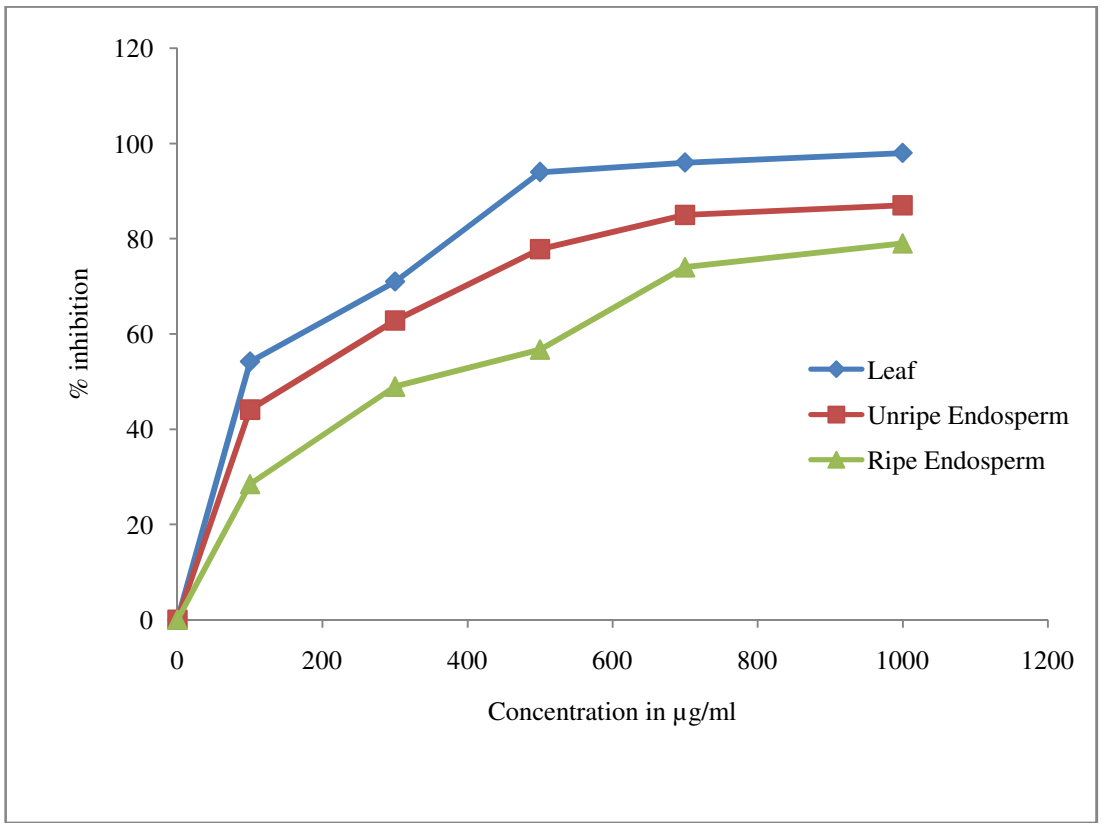

Figure-9: Antioxidant activity by SOD scavenging assay.

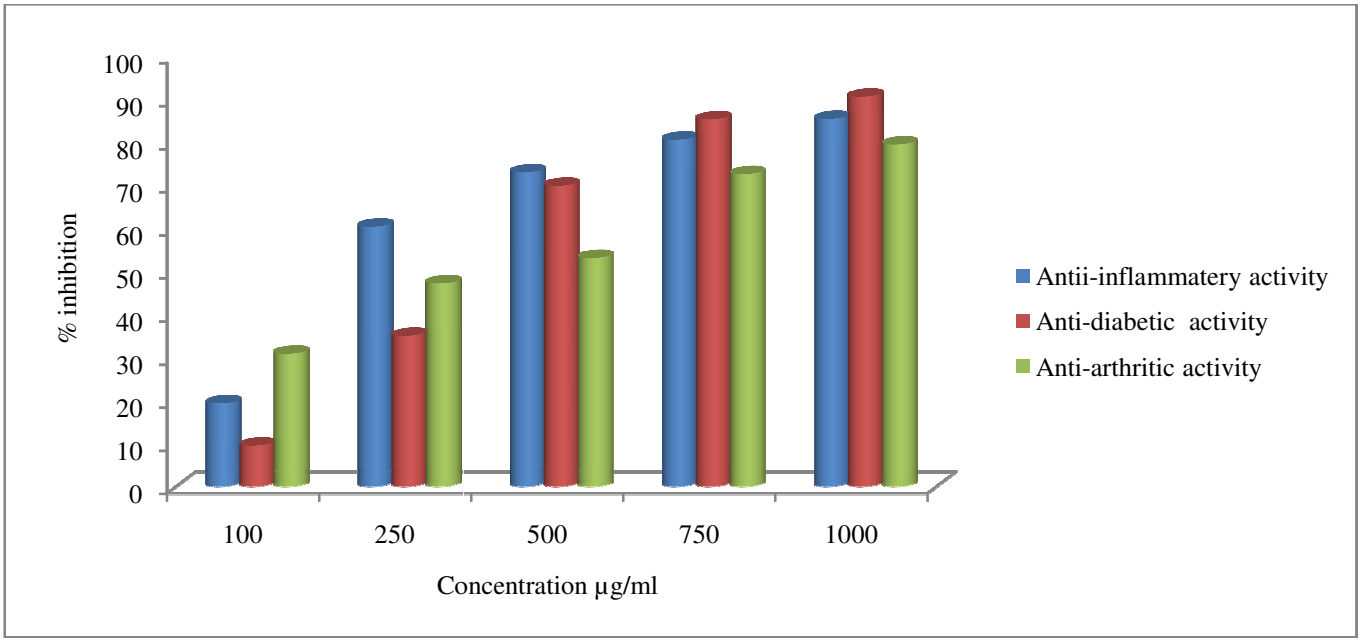

Figure-10: Anti-inflammatory, Anti-diabetic and Anti-arthritic activity of NFME of leaves.

Table-4: IC $_{50}$ values for Anti-inflammatory, Anti-diabetic and Anti-arthritic activity of NFME of leaves.

Sample	IC $_{50}$ Value for Anti-inflammatory Activity	IC $_{50}$ Value for Anti-diabetic Activity	IC $_{50}$ Value for Anti-arthritic Activity
Methanolic Leaf Extract of *Nypa fruticans*	303.33±1.06µg/ml	380.519± 1.271µg/ml	391.92±1.021µg/ml

Conclusion

Present study supports the folkloric and local use[41] of this plant in relieving pain and diabetes as the plant is a potential source of phenols, flavonoids and tannins. Many of the phenolic and flavonoids compounds reported here in LC/MS analysis and GC/MS analysis are the components of drugs and antioxidants. However Nypa palm is not considered as a valuable plant in many of the countries. Acclimatization of this plant in kerala is very important as it can protect our coastal areas from environmental hazards and it can be utilized for other purposes like extraction of drugs, Nira[42,7], natural fuels[43], vinegar[7].

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