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Effect of solvent extraction system on the antioxidant activity and RP-HPLC based determination of phenolic and water soluble vitamins in an annual herb *Mazus pumilus*

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Abstract

The objective of the present study was to evaluate the content of water-soluble vitamins like ascorbic acid (C), thiamine (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5), pyridoxine (B6) and folic acid (B9) in an ethno-medicinally edible plant *Mazus pumilus* Burm.f. by high performance liquid chromatography (HPLC) method. The *in vitro* antioxidant activity was carried out in different solvent system of varying polarity. The 70% Aqueous (Aq.) ethanol was appeared to be the most potent solvent for extraction of antioxidant components. The total phenolic and flavonoid content were found 65.640 ± 0.130 mg/g dm (dry mass) and 18.877 ± 0.070 mg/g dm respectively in the plant. The estimation of phenolic acids, polyphenolics in the 70 % aq. ethanol extract was also carried out by HPLC method. The water soluble B vitamins ranged between 0.072 to 5.632 mg/100g dm. The results confirm that this ethno medicinally important plant is a good source of natural antioxidants which could be used for the good of human being at the same time it can be used as a vitamin supplement to our diet. The present study also gives an account of traditional significance of the plants under investigation.

Keywords: *Mazus pumilus*, Antioxidant activity, Different solvent extraction system, Phenolic and water soluble vitamins by HPLC.

1. Introduction

Plants are known to produce various antioxidant molecules. Phytochemicals associated with the antioxidant activity includes the phenolic compounds like flavonoids, flavones, isoflavones, coumarins, lignans, catechins and epicatechins^[1]. The presence of ascorbic acid and vitamins in the plant also showed potent antioxidant activity^[2]. Free radical generation is a part of normal metabolic process but the imbalance between free radicals and the antioxidant compounds present results in the etiology of various diseases. For the treatment of various free radical mediated diseases like cancer, diabetes, atherosclerosis, rheumatism etc. the antioxidant based drug formulation has been reported to be used worldwide^[3]. Synthetic antioxidants like Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) are cheaper and more efficient in dealing with the oxidative stress of living systems but a long term use results in toxic effects. Therefore search for natural antioxidants has received much attention and efforts have been made to identify natural compounds that can act as suitable antioxidants to replace synthetic ones. Since these phytochemicals are a part of the normal physiology of the plant cells they are less harmful to mankind and the deleterious effect of synthetic antioxidants can be avoided^[4].

Mazus pumilus belonging to the family Phrymaceae, is a low lying herb, found in wet grasslands, in moist waste lands. The juice of the plant is used in the treatment of typhoid^[5] and the young leaves are cooked as vegetable^[6]. A very little work relates to the antioxidant activity of this plant has been reported by Sahid *et al*^[7]. Therefore, in this study the antioxidant potential of *M. pumilus* is evaluated through quantitative antioxidant profiling. A qualitative RP-HPLC study was also conducted to identify the presence of polyphenolic compounds with antioxidant properties. The food value of the plant was also determined along with the analysis of water soluble vitamin content.

Material and Methods

Plant Materials

Live sample of *M. pumilus* were collected from various locations of Kolkata, India and

identifications were authenticated from Botanical Survey of India, Howrah. The voucher specimens were preserved in our office. One portion of the plant samples were stored at 15°C and processed for vitamin estimation. The other part was dried in shade, pulverized and stored in an airtight container to evaluate the antioxidant properties and quantitation of phenolics and polyphenolics by HPLC.

Antioxidant activity of *M. pumilus* in different solvent extraction system

Extraction of plant material

For extraction of antioxidant compounds four different solvent system was used *viz.*, benzene, chloroform, methanol and 70% ethanol. One gram plant material taken and 20 ml each solvent and extraction was achieved with agitation for 18–24 h at ambient temperature. The extracts were filtered and diluted to 25 ml and aliquot were analyzed for their total phenolic and flavonoid content, reducing power and their *in vitro* radical scavenging capacity.

Estimation of total phenolic content

The total phenolic content of the different plant extracts was determined according to Folin-Ciocalteu procedure [8]. The total phenolic content was calculated as gallic acid equivalent (GAE) in mg/g dry weight of extract using the following equation based on the calibration curve $y = 0.0013x + 0.0498$, $R^2 = 0.999$, where y was the absorbance and x was the Gallic acid equivalent (mg/g).

Estimation of total flavonoid content

Total flavonoid content was estimated using the method of Ordonez *et al* [9]. Total flavonoid contents were calculated as rutin equivalent (RE) mg/ g dry weight using the following equation based on the calibration curve: $y = 0.0353x + 0.0566$, $R^2 = 0.9985$, where y was the absorbance and x was the rutin equivalent (mg/g).

Measurement of reducing power

The reducing power of the extracts was determined according to the method of Oyaizu [10]. The reducing power was calculated as ascorbic acid equivalent (AAE) in mg/ g of dry material using the following equation based on the calibration curve: $y = 0.0023x - 0.0063$, $R^2 = 0.9955$, where y was the absorbance and x was the ascorbic acid equivalent (mg/g).

Ferric reducing antioxidant power (FRAP) assay

The ability to reduce ferric ions was measured using the method described by Benzie and Strain [11]. The ability of the plant extract to reduce ferric ions of sample was the base of its antioxidant capacity. It is calculated as trolox equivalent (TE) in mg/ g dry material using the following equation $y = 0.084x - 0.168$, $R^2 = 0.996$, where y was the absorbance and x was the ascorbic acid equivalent (mg/g).

Determination of DPPH and ABTS free radical scavenging activity

The free radical scavenging activity of the plant samples was determined using the stable radical DPPH (1,1-diphenyl-2-picrylhydrazyl) [12]. The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation (ABTS⁺)-scavenging activity was measured according to the method described by Re *et al* [13]. The capability to scavenge the DPPH/ ABTS radical was calculated, using the following equation:

$$\text{DPPH/ ABTS scavenged (\%)} = \{(Ac - At)/Ac\} \times 100$$

Where Ac is the absorbance of the control reaction and At is the absorbance in presence of the sample of the extracts.

Metal chelating activity

For determination of metal chelating activity, the process of Lin *et al.* [14] was followed. The inhibition percentage of ferrozine-Fe²⁺ complex formation was calculated by using the formula given below:

$$\text{Chelating ability (\%)} = \{(Ac - At)/Ac\} \times 100$$

Where Ac is the absorbance of the control reaction and At is the absorbance in presence of the sample of the extracts.

Anti-lipid peroxidation in linoleic acid system

Anti-lipid peroxidation was assayed following method of Amabye [15], with modifications. A negative control was maintained that contained all the reagents except the extract. Inhibition of peroxidation was calculated using the formula

$$\text{Inhibition of lipid peroxidation (\%)} = \{(Ac - At)/Ac\} \times 100$$

Where Ac is the increase of absorbance of the control reaction and At is the increase of absorbance in presence of the sample of the extracts.

Estimation of phenolic acids and flavonoids in *M. pumilus* HPLC equipment

HPLC analyses were performed using Dionex Ultimate 3000 liquid chromatograph including a diode array detector (DAD) with 5 cm flow cell and with Chromeleon system manager as data processor. Separation was achieved by a reversed-phase Acclaim C18 column (5 micron particle size, 250 × 4.6 mm). 20 µL of sample was introduced into the HPLC column.

Preparation of standard solutions

The stock solution of 1 mg/ml concentration the of standard phenolics acids (gallic acid, protocatechuic acid, gentisic acid, chlorogenic acid, p-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, sinapic acid, salicylic acid and ellagic acid) and flavonoids (catechin, rutin, myricetin, quercetin, naringin, naringenin, apigenin and kaempferol) was prepared in methanol. The working solutions were prepared by diluting the standard solution with the mobile phase solvent system. The standard and working solutions were filtered through 0.45 µm PVDF-syringe filter before injecting in the HPLC instrument.

Chromatographic analysis of phenolic acids and flavonoids

HPLC analyses for the quantification of phenolic acids and flavonoids in the extract was performed following method of Seal *et al* [16] using Dionex Ultimate 3000 liquid chromatograph including a diode array detector (DAD) with 5 cm flow cell and with Chromeleon system manager as data processor. Separation was achieved by a reversed phase Acclaim C18 column (5 micron particle size, 250 × 4.6 mm). 20 µL of sample was introduced into the HPLC column. The method was validated according to the USP and ICH guidelines. The mobile phase contains methanol (Solvent A) and 0.5% aq. acetic acid solution (Solvent B) and the column was thermostatically controlled at 25 °C and the injection

volume was kept at 20 μ l. A gradient elution was performed by varying the proportion of solvent A to solvent B. Total analysis time per sample was 105 min. HPLC chromatograms were detected at three different wavelengths (272, 280 and 310 nm) using a photo diode array UV detector. Each compound was identified by its retention time and by spiking with standards under the same conditions. The quantification of phenolic acids and flavonoids in the extracts were carried out by the measurement of the integrated peak area and the contents were calculated using the calibration curve by plotting peak area against concentration of the respective standard sample. The data were reported with convergence limit in triplicate.

Estimation of water soluble vitamins in *M. pumilus*

Preparation of mixture standard vitamin solutions

The stock standard solutions of vitamin C, B1, B3, B5 and B6 and were prepared by dissolving 25 mg of the each standard in 1 ml 0.1M hydrochloric acid in 25 ml standard volumetric flask. For preparation of standard stock solutions of vitamin B9 and B2, 25 mg of the each standard were dissolved in one ml 0.1 M sodium hydroxide in 25 ml standard volumetric flask. The standard solution was stored in amber-glass bottles in the refrigerator at 4°C. The working standards were prepared by diluting with phosphate buffer (1M, pH 5.5).

Preparation of sample solution

Plant materials were washed with distilled water. The washed plant materials were cut into very small pieces, frozen in liquid nitrogen and kept at -20 °C until analysis. 1 g each of freeze-dried sample was soaked in 10 ml water and extracted with 1 ml 0.1M NaOH and 10 ml phosphate buffer (1M, pH 5.5) were added to it and kept in dark for 24 hours. The solution was first filtered through a Whatman No. 1 filter paper and the resulting filtrate was taken in a 25 ml volumetric flask and solution was topped up to the mark with HPLC grade water. The sample solution was filtered through 0.45 μ m membrane filter before injection into LC system. The stock solutions of sample were kept in a refrigerator for further use.

Chromatographic analysis of water soluble vitamins

The chromatographic analysis was carried out following the method as described by Seal *et al.* [16] with minor modifications. The mobile phase contains acetonitrile (Solvent A) and aqueous trifluoroacetic acid (TFA, 0.01% v/v) (Solvent B), the column was thermostatically controlled at 220 C and the injection volume was kept at 20 μ l. A gradient elution was performed by varying the proportion of solvent A to solvent B. Total analysis time per sample was 35 min. HPLC Chromatograms of all vitamins were detected using a photo diode array UV/detector at four different wavelengths (210, 245, 275 and 290 nm) according to absorption maxima of analysed compounds. Detection of

compound was done in same manner that followed in detection of phenolic acids and flavonoids.

The data were reported as means \pm standard error of means of three independent analyses.

Results and Discussion

Extraction yield, total phenolics and flavonoids content in *M. pumilus*

The percentage yield of the extracts of *M. pumilus* is shown in Table 1. The extraction yield of these samples varied from 1.082 % to 5.567 % (Table 1). 70 % hydro-ethanol proved to be better solvent for the plant studied. Based on our results reported here, the maximum extraction was achieved using hydro-alcohol. Hydro-alcohol has been reported to be more efficient for extraction of antioxidant over pure solvent system [17]. Antioxidant activity was maximum 80 % aq. methanol from plant materials like rice bran, wheat bran, citrus peel, coffee beans and guava leaves [18]. Methanol proved to be a better extracting solvent in case of wild leafy vegetables [19] and wild edible fruits [20]. In the present analysis, the variation in the extract yields from the plant sample differed due to different polarity of the polyphenolic or other antioxidant compounds or their association with other molecules [21]. The polarity of the solvent, nature of chemical present and parts used for extraction are the important factors that affect extraction yield [22]. The amount of the antioxidant is also affected by extraction procedure, temperature and time [23-24].

Table 1 summarizes the total phenolic content expressed as mg gallic acid equivalent (GAE)/ g dry mass (dm) of the extract which showed a wide variation for *M. pumilus* 31.026 – 65.640 mg GAE/ g dm. A higher phenolic content, 88 mg GAE/g dm was reported from n-butanol fraction [7]; this variation may be due to the age of the plant and other edaphic factors affecting the growth. Phenolics are plant secondary metabolites and their antioxidant activity like chelating metal ions, inactivating free radical chains are well documented [25-26]. Results of the present study showed that among all the solvent extracts; the hydro-ethanol extracts had the highest phenolic content and it decreased with decreasing polarity. Polar solvents such as aqueous methanol/ethanol are often better solubiliser of phenolic compounds as compared with absolute methanol/ethanol [18-20, 23, 24]. However, non polar solvent like ethyl acetate have also been demonstrated to extract phenolic compounds in higher amounts from onion and citrus peel [27-30].

The flavonoid content is expressed as mg rutin equivalent (RE)/ g dm. Maximum flavonoid content was observed in 70% ethanol extract (Table 1). Flavonoids show wide array of pharmacological and biochemical activities including radical scavenging properties [31-33]. The quantity of flavonoids from the solvent extract correlates to the nature of flavonoid present in the plant sample.

Table 1: Extractive capacities (% extractive yield), total phenolic content (mg GAE/ g dm) and flavonoid content (mg RE/ g dm) in *M. pumilus* in different solvent system

Parameters	Extracts of <i>M. pumilus</i> in different solvent system			
	70% aq. ethanol	Methanol	Chloroform	Benzene
Extractive Value (% extractive yield)	5.567 \pm 0.062	2.996 \pm 0.066	1.082 \pm 0.017	1.503 \pm 0.047
Total phenolic content (mg GAE/ g dm)	65.640 \pm 0.130	47.821 \pm 0.534	38.205 \pm 0.111	31.026 \pm 0.427
Total flavonoid content (mg RE/ g dm)	18.877 \pm 0.070	17.426 \pm 0.013	14.496 \pm 0.040	11.984 \pm 0.081

GAE : Gallic acid equivalent; RE : Rutin equivalent Each value in the table was obtained by calculating the average of three experiments (n=3) and data are presented as Mean \pm SEM.

Reducing property and FRAP of *M. pumilus*

Reducing property is expressed as mg ascorbic acid equivalent (AAE)/ g dm represented in Table 2. Maximum reducing activity was noted in 70% hydro ethanol (34.743 AAE/ g dm). Reducing property was also studied by FRAP and is expressed as μ mole trolox equivalent (TE)/ g dm. The results (Table 2) indicate that both hydro ethanol extract (1.856 μ mole TE/ g dm) and methanol extract (1.796 μ mole TE/ g dm) showed almost comparable results. In the reducing power assay, the presence of antioxidants in the sample reduces Fe^{+3} to Fe^{+2} which is the reflection of the antioxidant

potential of the sample. Fe^{+3} promotes free radical formation by Fenton reaction and also facilitates lipid peroxidation. The reducing property indicates that the plant sample can act as electron donors and can reduce the intermediates of lipid peroxidation processes [34]. The reducing power generally corresponds to high phenolic content [35]. The result of FRAP analysis is in congruence to the study by Shahid *et al.* [7] where the more polar n-butanol fraction showed maximum FRAP followed by chloroform and ethyl acetate fraction. The n-hexane fraction had the least FRAP value.

Table 2: Reducing activity (mg AAE/ g dm), FRAP (μ mole TE/ g dm) and metal chelating activity (% inhibition/ g dm) of *M. pumilus* in different solvent system

Parameters	Extracts of <i>M. pumilus</i> in different solvent system			
	70% aq. ethanol	Methanol	Chloroform	Benzene
Reducing activity (mg AAE/ g dm)	34.743 \pm 0.133	19.529 \pm 0.121	13.370 \pm 1.255	13.019 \pm 0.487
FRAP (μ mole TE/ g dm)	1.856 \pm 0.008	1.796 \pm 0.004	1.151 \pm 0.005	0.511 \pm 0.002
Metal chelating activity (% inhibition/ g dm)	28.256 \pm 0.284	25.610 \pm 0.745	14.050 \pm 0.250	3.862 \pm 0.279

AAE : Ascorbic acid equivalent ; TE : Trolox equivalent Each value in the table was obtained by calculating the average of three experiments (n=3) and data are presented as Mean \pm SEM.

Metal chelating property of *M. pumilus*

Metal chelating ability is represented as % inhibition of metal ions/ g dm and is represented in Table 2. The hydro-ethanol extract (28.256%) showed promising metal chelating property (Table 2). Metal ions are major initiators of lipid peroxidation that results in deterioration of food [36]. The catalysis of metal ions also contributes to free radical formation that correlates with incidents of cancer and arthritis [37]. Chelating capacity of the extracts decreased with the decreasing polarity. Chelating ability can be attributed to the high phenol and flavonoid content in these extract.

Radical scavenging property of *M. pumilus*

The antioxidant capacities using DPPH and ABTS assays of various extracts from *M. pumilus* are shown in Tables 3. In the DPPH method, the free radical scavenging capacities of the plant studied ranged between 3 – 16%. In the ABTS method, the free radical scavenging capacities of the plant studied ranged between 4 – 56%. The data suggests that polar components like phenol and flavonoids of the plant studied contributed to the radical scavenging activity.

Table 3: Radical scavenging activity (% inhibition/ g dm) of *M. pumilus* in different solvent system using DPPH and ABTS

Parameters	Radical scavenging property of <i>M. pumilus</i> in different solvent system			
	70% aq. ethanol	Methanol	Chloroform	Benzene
DPPH	16.862 \pm 0.066	12.548 \pm 0.257	3.161 \pm 1.072	3.017 \pm 0.257
ABTS	56.220 \pm 0.783	43.061 \pm 0.444	28.717 \pm 0.074	4.328 \pm 0.256

Each value in the table was obtained by calculating the average of three experiments (n=3) and data are presented as Mean \pm SEM.

Lipid peroxidation of *M. pumilus*

Of the four different extracts compared maximum activity was observed in 70% hydro ethanol and methanol extract. Based on this anti-lipid peroxidation assay was studied in hydro ethanol and methanol extract and is represented in Table 4 as % inhibition of lipid peroxidation. 70% hydro-ethanol extract showed > 50% inhibition of lipid peroxidation. Similar inhibition rate was observed using n-butanol fraction [7]. Synthetic antioxidants, e.g., BHA, are added in food during processing to suppress lipid peroxidation and resulting improved food quality and stability. Synthetic antioxidants on the other hand have several side effects. The phenolic compounds and other chemical components may suppress lipid peroxidation through by inactivating the free alkyl radicals and scavenging the metal ions [38]. In addition, lipid peroxidation of cell membrane is associated with various pathological events such as atherosclerosis, inflammation and liver injury [39]. So addition of plant based phytonutrients

reduces the risk of side effects and consumption of which is also beneficial to human.

Table 4: Anti-lipid peroxidation assay (% inhibition/ g dm) of *M. pumilus* in different solvent system

Parameter	Extracts of <i>M. pumilus</i> in different solvent system	
	70% aq. ethanol	Methanol
% inhibition of lipid peroxidation	54.488 \pm 0.055	37.722 \pm 0.078

Each value in the table was obtained by calculating the average of three experiments (n=3) and data are presented as Mean \pm SEM.

Identification and quantification of different phenolic acids and flavonoids in 70% aq. ethanol extracts of *M. pumilus*

A typical HPLC chromatogram of the mixture of all standard phenolic acids and flavonoids recorded at 260 nm is presented in fig. 1.

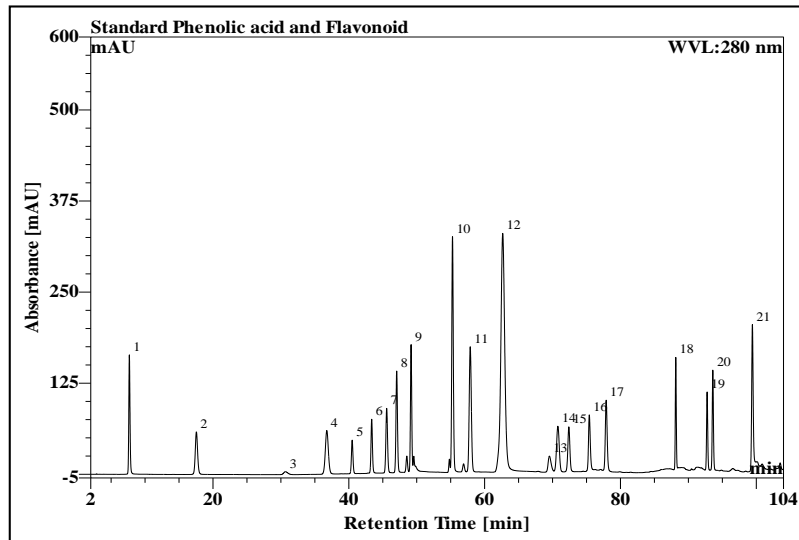


Fig 1: Chromatographic separation of standard phenolics and flavonoids. 1. Gallic acid; 2. Protocatechuic acid; 3. Gentisic acid; 4. p-hydroxybenzoic acid; 5. Catechin; 6. Chlorogenic acid; 7. Vanillic acid; 8. Caffeic acid; 9. Syringic acid; 10. p-Coumaric acid; 11. Ferullic acid; 12. Sinapic acid; 13. Salicylic acid; 14. Naringin; 15. Rutin; 16. Ellagic acid; 17. Myricetin; 18. Quercetin; 19. Naringenin; 20. Apigenin; 21. Kaempferol.

The 70% aq. ethanol extract of *M. pumilus* revealed the presence of syringic acid, ferulic acid, ellagic acid, naringin and naringenin at 260 nm as presented in table 5. The consumption of this plant would be useful for the treatment of

various chronic and neurodegenerative diseases and cancer due to the presence of these phenolics and polyphenolics in various amount [16, 40-42].

Table 5: Quantification of phenolic acids and flavonoids in 70% aq. ethanol extract of *M. pumilus* (mg/ 100g dm) by HPLC

Phenolic acids and flavonoids	Amount mg/100gm dry plant material	Phenolic acids and flavonoids	Amount mg/100gm dry plant material	Phenolic acids and flavonoids	Amount mg/100gm dry plant material
Gallic acid	ND	Caffeic acid	ND	Rutin	ND
Protocatechuic acid	ND	Syringic acid	0.244 ± 0.001	Ellagic acid	0.099 ± 0.003
Gentisic acid	ND	p-Coumaric acid	ND	Myricetin	ND
p-hydroxybenzoic acid	ND	Ferulic acid	0.185 ± 0.004	Quercetin	ND
Catechin	ND	Sinapic acid	ND	Naringenin	
Chlorogenic acid	ND	Salicylic acid	ND	Apigenin	ND
Vanillic acid	ND	Naringin	1.078 ± 0.005	Kaempferol	ND

ND : Not detected

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

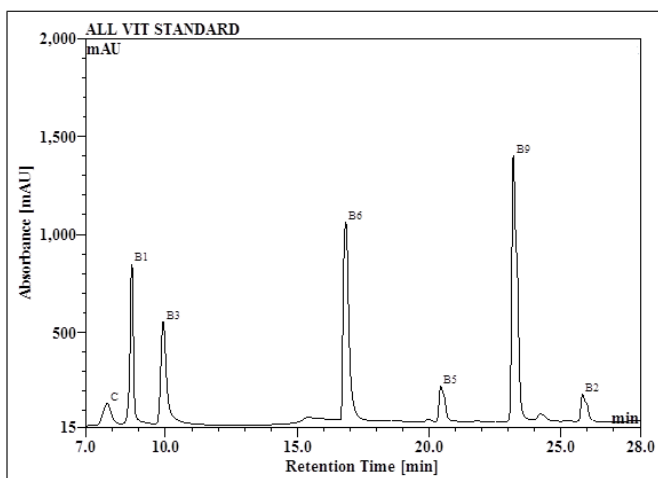


Fig 2: Chromatographic separation of standard water soluble vitamins (C) Ascorbic acid; (B1) Thiamine; (B3) Niacin; (B6) Pyridoxine; (B5) Pantothenic acid; (B9) Folic acid; (B2) Riboflavin).

Quantification of water soluble vitamins in *M. pumilus*

A typical HPLC chromatogram of the all standard vitamin

mixture recorded at 275 nm is presented in fig. 2. The vitamin content is depicted in table 6 represented as mg/ 100g dry plant material. The HPLC chromatogram (fig. 3) showed the presence of different water soluble vitamins in the plant extract. Maximum amount of vitamin B5 (pantothenic acid) is noted *M. pumilus* (5.632 mg/100g). It is an essential component of CoA required in fatty acid metabolism (FAO/WHO 2001). Vitamin C was quantified in moderate amount (0.677 mg/ 100g), which is a known antioxidant present in high amount in citrus fruits. Thiamine (B1) is essential for carbohydrate metabolism and nerve cell function [43]. Amount of B1 quantified is (0.181 mg/100g). Thiamine content is comparable to that in some common vegetables like cauliflower (0.073 mg/100g), beans (0.132mg/100g) and spinach (0.076mg/100g) [44]. Amount of riboflavin (B2) quantified was (0.419 mg/100g) which is higher than that of some common vegetables like spinach (0.24 mg/100g), green beans (0.12 mg/100g, potato (0.023 mg/100g) [44]. Vitamin B3 (0.343 mg/100g), B6 (0.388 mg/100g) and B9 (0.072 mg/100g) is also found in good amount. Vitamin B3 plays an important role in DNA repair and fat metabolism [43]. Folic acid (B9) plays an important role in DNA synthesis and repair [43]. It has been observed that vitamin B6 possess strong

antioxidant activity in linoleic acid based lipid peroxidation. Vitamin B1, B2 and B9 though acted as prooxidants at initial stages of lipid peroxidation but acted as strong antioxidant in later stages [45]. Based on the above data it is suggested that *M. pumilus* act as a good source of food preservative at the same time a considerable good source of vitamin B.

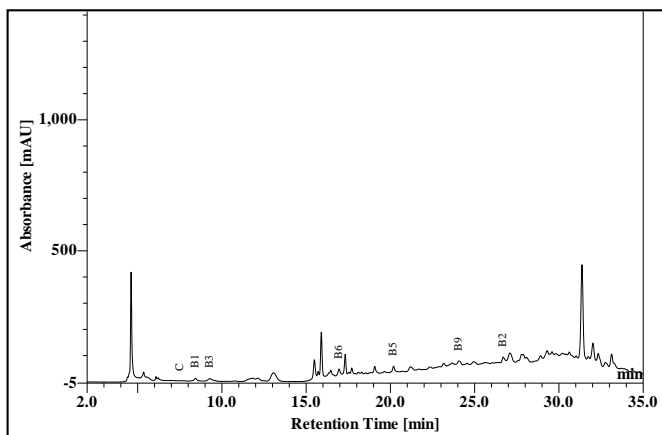


Fig 3: Chromatographic separation of water soluble vitamins in *M. pumilus*

Conclusion

Based on the above findings it can be ascertained that *M. pumilus* can be exploited as a source of natural antioxidant and vitamin B. Further, the knowledge of antioxidant and nutraceutical potential of the leaf tissue will be useful in designing strategies that will maximize the effective and practical utilization of *M. pumilus*.

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