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RP-HPLC Analysis of Flavonoids in Marsilea Minuta L

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Abstract: ***The present study was investigated for the presence of phenolic compounds in the plant extract of Marsilea minuta L. The studies on the phenolics or flavonoids by RP-HPLC analysis showed the presence of five flavonoid compounds such as gallic acid, caffeic acid, rutin, quercetin and ferulic acid. The chromatographic separations at retention time (Rt) for gallic acid (Rt - 5.675), Caffeic acid (Rt – 9.475), Rutin (Rt -11.017), Quercetin (Rt -12.175) and Ferulic acid (Rt - 23.808). The individual flavonoid content for all the flavonoids were calculated from the corresponding calibration curve and the obtained values were compared with the standards. Hence these plant extracts with the property of phenolic compounds can be recommended for their use as an alternative anti-infective agent in natural medicine for the treatment of various diseases.***

Keywords: *Marsilea minuta*, HPLC analysis, flavonoids, retention time, standards

1. Introduction

The pteridophytes are mostly distributed in the high altitude mountainous regions such as the Himalayas, the Western Ghats and the Eastern Ghats. More than 300 species of ferns and fern allies are reported from the Western Ghats, South India [1]. The pteridophytes are long known for their medicinal and therapeutic utility. In the ancient period these plants were prescribed as herbal extract for the cure of several diseases. Theophrastus (327-287 BC) and Dioscorides (50 AD) listed many pteridophytes as a potential herbal formulation for two or more deadly disorders. Shushrutha and Charuk (700BC) in their monumental contribution on the medicinal attributes of ferns have also enormously mentioned the utility of *Marsilea minuta*, *Adiantum capillus-veneris* etc. Secondary metabolites are often restricted to a narrow set of species within a phylogenetic group. Secondary metabolites often play an important role in plant defence against herbivory [2] and other interspecies defences. Human use secondary metabolites as medicines, flavourings, and recreational drugs. It has been observed that pteridophytes are not infected by microbial pathogens which may be one of the important factors for the evolutionary success of pteridophytes and the fact that they survived for more than 350 million years [3].

The flavonoids are a large family of polyphenolic compounds synthesized by plants and structurally derived from the parent substance flavone. Flavonoids present in fruits and leafy vegetables are thought to provide potential and versatile health benefits through radical scavenging and chelating activity. The in-vitro antioxidant activities of the flavonoids are due to their ability to reduce the free radical formation and hence exhibit several biological activities. Flavonoids have been shown to inhibit certain types of cancer, dementia, cardiovascular diseases and diabetes Flavonoids may help provide protection against these diseases by contributing along with antioxidants vitamins and enzymes [4].

As per the traditional claims the plant *Marsilea minuta* has been used for astringent, hypnotic, diuretic, expectorant, aphrodisiac, anodyne, ophthalmic, constipating, strangury and dyspepsia. It is useful in psychopathy, leprosy, haemorrhoids, skin diseases, fever, insomnia and febrifuge [5]. Therefore this study was aimed to investigate the flavonoids present in the water fern *Marsilea minuta* using RP-HPLC method.

2. Review of Literature

Gallic acid (GA), Caffeic acid (CA), Rutin (RU), Ferulic acid (FA) and Quercetin (QU) are phenolic compounds. Structurally they have phenolic groups which serve as a source of readily available hydrogen atoms such that the subsequent radicals produced can be delocalized over the phenolic structure [6].

The flavonoid compounds were isolated and characterized by using thin layer chromatography (TLC), purified by preparative thin layer chromatography (PTLC) and identified using High performance chromatography (HPLC). Their structures and chemical bonds were analyzed using Ultraviolet-Visible spectrophotomery (UV), Fourier Transform-Infra Red spectroscopy (FTIR) and Nuclear Magnetic Resonance NMR (13C and 1H) techniques [7]. Two flavonoids were identified as rutin and quercetin. The isolated compounds showed a potent antioxidant radical scavenging activity, as assessed by non-physiological assays like DPPH (2, 2-diphenyl-1-picrylhydrazyl), ABTS (2, 2'-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) and FRAP (Ferric reducing antioxidant power [8].

Many phenolic-flavonoids found in plants exhibit antipyretic, analgesic, anti-inflammatory and antioxidant properties. Phenolic compounds were known to exhibit radical scavenging and antimicrobial activity [9].

Twelve phenolic compounds namely ellagic acid, catechol, gallic acid, quercetin, resorcinol, tannic acid, vanillin, salicylic acid, acetyl salicylic acid, benzoic acid, phloroglucinol and ascorbic acid were taken up for qualitative and quantitative HPLC analysis[10].

3. Materials and Methods

Marsilea minuta Linn. plants were collected from the rice fields of Mannachanallur, Tiruchirappalli in the month of October to December.

Preparation of Standards for HPLC
Standard stock solutions of gallic acid, ferulic acid, caffeic acid, rutin and quercetin flavonoids were prepared in methanol at concentrations of 2, 4, 6, 8 and 10 µg/ml and filtered through HPLC filter 0.45 mm membrane filter (Millipore). Extract 3: 25 g of whole plant powder was extracted with 15 ml 95% ethanol for 6 h. The resulting extract was suspended over water and partitioned using petroleum ether to remove waxes and impurities.

Preparation of extract
The sample was prepared according to the procedure. The extraction of known quantity of powdered sample of whole plant of *M. minuta* L. was carried out using 2 ml of fermented broth with 50 mL of 95% ethanol under 80 KHz, 45°C in ultrasonic extraction device for 30min, was repeated twice. The extract was collected and filtered; the filtrate was dried at 50°C under reduced pressure in a rotary evaporator. The dried crude extract was dissolved in the 100 ml mobile phase. After filtering through a filter paper and a 0.45 mm membrane filter (Millipore), the extract was injected into HPLC.

RP-HPLC analysis of flavonoids [11]
The extract was analyzed for flavonoids using a RP-HPLC method 17, Shimadzu Corp., Kyoto, consisting of a LC-10ATVp pump, SCL 10A system controller and a variable Shimadzu SPD- 10ATVp UV VIS detector and a loop injector with a loop size of 20 µl was used. The peak area was calculated with CLASSVP software. Reverse phase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column (250 x 4.6 mm i.d., particle size 5 µm, Luna 5 µ C-18; phenomenex, Torrance, CA, USA) at 25 °C. The gradient elution of solvent A (water-acetic acid; 25:1 v/v) and solvent B (methanol) had a significant effect on the resolution of compounds. Detection wavelength was 280 nm. Gallic acid, caffeic acid, ferulic acid, rutin and quercetin were used as internal and external standards. Phenolic acids present in each sample were identified by comparing chromatographic peaks with the retention time (Rt) of individual standards. The amount of each phenolic acid is expressed as mg/g.

4. Results

RP-HPLC method is one of the most fast and reliable method for identification of plant phenolics. Phenolic compounds can be defined as a large series of chemical constituents possessing at least one aromatic ring, bearing hydroxyl and other sub-constituents. Phenolics can be divided into two groups namely flavonoids and non flavonoids; because of the diversity and complexity of natural phenolics in medicinal plants, it is difficult to characterize every compound and elucidate its structure. However, it is not difficult to identify the major categories of phenolic compounds. The chromatographic separations of Retention time (Rt) for Gallic acid (Rt- 5.750), Caffeic acid (Rt-9.450), Rutin (Rt-10.517), Quercetin (Rt-12.400) and Ferulic acid (Rt-24.175) standard is shown in Figure -1. The content of each flavonoid was calculated from the corresponding calibration curve and presented as the mean of five determinations as shown in Table -1.

HPLC analysis of whole plant of *M. minuta* L. clearly showed the presence of flavonoids (phenolics) such as Gallic acid, Ferulic acid, Caffeic acid, Rutin and Quercetin. The chromatographic separations at retention time (Rt) for gallic acid (Rt - 5.675), Caffeic acid (Rt – 9.475), Rutin (Rt - 11.017), Quercetin (Rt -12.175) and Ferulic acid (Rt - 23.808) of ethanolic extract of *M. minuta* L. is shown in Figure-2. Individual flavonoid content for all the flavonoids were calculated from the calibration curve plotted and given in terms of its Rt along with the standards as indicated in Table -2.

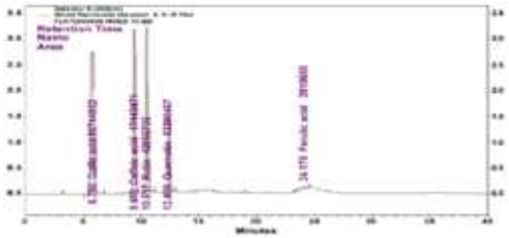

Figure 1: HPLC chromatogram of standard flavonoids

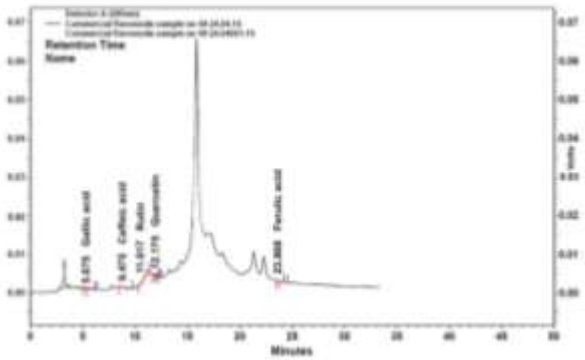

Figure 2: HPLC chromatogram of ethanolic extract of *M. minuta* L.

5. Discussion

Two flavonoids like quercetin and apigenin and 6 biflavonoids like amentoflavone, robustaflavone, 2,3-dihydroamentaflavone, hinokiflavone, 4'-O-methyl-robustaflavone and ginkgetin were separated by DPPH-HPLC experiment of *Selaginella sinensis*[12]. The characterization of bi flavonoids like Amentoflavone and Robustaflavone from *Selaginella doederleinii* by RP-HPLC method were studied [13]. Flavonoids like Ferulic acid and Caffeic acid were isolated from *Polypodium decumanum* [14].

In the same way quantification of psychotropic diterpene, salvinorin A in extracts of leaves and stems of *Salvia divinorum* showed their in the range of 0.89 to 3.70 mg/g dry weight. From the results, it is evident that the leaf extract contained high concentration of caffeic acid (5.0 mg/g of dry weight) followed by rutin (2 mg/g of dry weight) gallic acid, ferulic acid and quercetin. As these flavonoids have been of

interest for health benefits, the present analytical study proves to be a potential application to identify and quantify the phenolic compounds in the plant extracts [15].

6. Conclusion

HPLC analysis clearly showed the presence of five flavonoid compounds such as gallic acid, caffeic acid, rutin, quercetin and ferulic acid. Flavanoid compounds could be used for the treatment of albuminaris, diabetes, psoriasis, external haemarroids, blood clots, heart attacks and strokes. These compounds are also active against allergic chemicals, treat cancer cells in humans, antitumour activity against breast and liver cancer and other skin diseases. Hence, further promising effort might be taken to identify and purify the actual phytochemical component as supplementary valuable product in the form of capsules or drugs that could be availed by all the people in the future.

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Table 1: HPLC validation data for flavonoids standards

SPD10Avp (280nm)					
S.No	Retention time(Rt) sample	Area	Height	Concentration (mg/g)	Name of flavonoid
1.	5.675	6887	18	1.0	Gallic acid
2.	9.475	8134	151	5.0	Caffeic acid
3.	11.017	8493	386	2.0	Rutin
4.	12.175	80	188	Below Detection Limit	Quercetin
5.	23.808	1005	53	1.0	Ferulic acid

Table 2: HPLC validation data of ethanolic extract of M. minuta L

SPD10Avp (280nm)					
S.No	Retention time(Rt)	Area	Height	Concentration (mg/g)	Name of flavonoid
1.	5.750	56744802	2757981	10.000	Gallic acid
2.	9.450	17443471	1882880	10.000	Caffeic acid
3.	10.517	42056735	3198304	10.000	Rutin
4.	12.400	13396467	1402866	10.000	Quercetin
5.	24.175	2810655	36358	10.000	Ferulic acid