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Research Article

INVESTIGATION OF THE ANTIOXIDANT PRINCIPLES FROM *PSILANTHUS TRAVANCORENSIS* (WT. & ARN.) LEROY- AN UNEXPLORED TAXON OF RUBIACEAE

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ABSTRACT

The root of *Psilanthus travancorensis* (Wt. & Arn.) Leroy (Rubiaceae) is primarily used in Ayurvedic and folk medicines. It is reported to cure anaemia, cardiac diseases, skin diseases, oedema, ulcers, inflammatory swellings etc. This paper reports the phytochemical screening and isolation of an important antioxidant compound, quercetin, from *P. travancorensis* for the first time. First of all, successive extractives of stem, root and leaves were subjected to the preliminary screening for phytochemicals such as Alkaloids, Flavonoids, Glycosides, Phenols, Saponins, Tannins, Steroids, Terpenoids and Coumarins. Further, the alkaloids, flavonoids and phenols were estimated. Antioxidant activity was tested qualitatively by dot blot assay using different extracts and ethyl acetate fraction was found to be more powerful. Total antioxidant activity and reducing power of the methanolic extract was measured by DPPH (1, 1, - diphenyl-2-picryl hydrazyl) radical scavenging activity method using ascorbic acid as standard. The IC_{50} value of the extract was found to be comparable with that of ascorbic acid which is a well known antioxidant. *P. travancorensis* is observed to possess potent antioxidant activity. The secondary plant metabolite, quercetin was identified and estimated by HPLC method.

Keywords: *Psilanthus travancorensis*, Antioxidant activity, DPPH radical scavenging activity, Dot blot assay, Reducing power

INTRODUCTION

Psilanthus travancorensis (Wt. & Arn.) Leroy (Rubiaceae), a medicinal plant, as it popularly called 'Pushkaramulla', is distributed in southern Western Ghats and Sri Lanka. The plant was abundant in the Kerala forest in the past, but now very scarce[1]. Rheede has portrayed this species under the names *cherumulla* and *kattu-mulla*[2]. The root is primarily used in Ayurvedic and folk medicines. It is reported to cure anaemia, cardiac diseases, skin diseases, oedema and also diseases due to kapha and vata. A paste of the root is applied to indolent ulcers and inflammatory swellings[3,4]. Tribal people of Kerala use the root to treat rheumatic pain[5]. It is an important ingredient of Ayurvrdic formulations like Kumaryasavam, Dasamularishtam, Valiya Narayana Thailam, Dhanvantaram Ghrtham and Chyavanaprasam[6]. Comparative clinical studies on this plant are essential to substantiate its therapeutic powers. However, no systematic documentation of the phytochemical constituents of the plant has been made till date. This paper reports the phytochemical screening and isolation of an important antioxidant compound, quercetin, from *P. travancorensis* for the first time.

It has been established that oxidative stress is among the major causative factors in induction of many chronic and degenerative diseases including atherosclerosis, diabetes mellitus, Parkinson's disease, Alzheimer's disease and immune dysfunction and is involved in aging[7]. Antioxidants, both exogenous and endogenous, whether synthetic or natural, can be effective in the prevention of free radical formation by scavenging or promotion of their decomposition and suppression of such diseases[8]. There is growing interest towards natural antioxidants from herbal sources[9]. Flavonols and flavones are widely distributed secondary metabolites with antioxidants and antibacterial properties[10]. Flavonoids are polyphenolic compounds found in the fruits, vegetables, root, stem, flowers, tea and wine[11]. They are divided into seven major groups[12]. Quercetin, one of the best described flavonoids, is responsible for the colours of many fruits, flowers and vegetables. It works as anti-inflammatory, antioxidant and anticancer agents[13].

The study was designed for the phytochemical screening of *P. travancorensis* for various secondary metabolites, antioxidant studies of the plant extract, and isolation and identification of an antioxidant 'quercetin' from the root of this important medicinal plant.

MATERIALS AND METHODS

Chemicals and solvents

All the biochemicals were purchased from Sigma Chemical Company, St. Louis, MO, USA and other chemicals and solvents were purchased from SRL Chemicals, India.

Plant material

Different plant parts (root, stem and leaves) of *P. travancorensis* collected from Thiruvananthapuram District of Kerala, India were dried in shade, finely powdered and the powder was passed through 40 mesh sieve and stored in airtight containers at room temperature.

Successive extraction

About 100gms of the powder was taken in a Soxhlet apparatus and extracted successively with five solvents, viz. hexane, ethyl acetate, acetone, methanol and water. The residue was concentrated, dried and the yield was determined. The dried extracts were stored in tightly sealed dark glass containers at 5ºC for further analysis. The antioxidant activity of the ethyl acetate fraction was studied and used for further Phytochemical analysis.

Phytochemical analysis

The five successive extractives were tested for different secondary metabolites – alkaloids, flavonoids, glycosides, phenols, saponins, tannins, steroids, terpenoids and coumarins[14].

Determination of Antioxidant activity

DPPH (1, 1, - diphenyl-2-picryl hydrazyl) radical scavenging activity method

The number of antioxidant compounds present can be determined by Thin Layer Chromatographic (TLC) method. Aliquots of each of the successive extract (10µl) were applied to Merck silica gel F_{254} plates. The plates were developed with the chloroform: ethyl acetate: formic acid (5:4:1) (CEF) and ethyl acetate: methanol: water (40:5:4.4) (EMW) eluent systems and sprayed with 0.4mM DPPH solution in methanol. The spots developed were observed.

This was followed by (a) quantitative analysis by DPPH radical scavenging assay with ascorbic acid as standard[15], (b) qualitative antioxidant capacity by Dot-blot assay[16], (c) determination of total

antioxidant capacity through the formation of a Phosphomolybdenum Complex[17] and (d) reducing power by the method of Oyaizu[18].

Identification of quercetin was done by HPLC method. Sample preparation, apparatus and conditions for HPLC were according to the method of Hadjmohammadi and Sharifi[19].

RESULTS AND DISCUSSION

The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites such as alkaloids, flavonoids glycosides, phenols and steroids. The successive extracts of root, stem and leaves have revealed the presence of alkaloids, flavonoids, glycosides, phenols, terpenoids and steroids (Table 1).

Table 1: Preliminary screening of secondary metabolites from *P. travancorensis*

Secondary metabolites	Root					Stem					Leaves				
	I	II	III	IV	V	I	IIIV		III	V	I	II	III	IV	V
Alkaloids	+	+	+	+	+	-	+	+	+	+	-	-	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Glycosides	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+
Phenols	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+
Saponins	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tannin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Steroids	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Terpenoids	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
Coumarins	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

'+' Present '-' absent; I-Hexane; II-Ethyl acetate; III-Acetone; IV-Methanol; V-Aqueous

Preliminary screening tests may be useful in the detection of bioactive principle and may subsequently lead to drug discovery and development. Further, these tests facilitate their quantitative estimation and qualitative separation of pharmacologically active chemical compounds. The data of quantitative determination of secondary metabolites is tabulated (Table 2).

Table 2: Quantitative estimation of phytochemicals of *P. travancorensis*

S. No.	Secondary metabolites	Percent of secondary metabolites/dry wt.		
		Root mg/g	Stem mg/g	Leaves mg/g
1	Flavonoids	88 ± 0.942	32.83 ± 0.49	2.51 ± 0.40
2	Alkaloids	56.12 ± 0.942	29.16 ± 0.381	1.12 ± 0.181
3	Phenols	16.67 ± 1.123	8.19 ± 0.123	38.12 ± 0.913

Among the three groups of phytochemicals determined from the root, stem and leaves of *P. travancorensis,* flavonoids were found to be most abundant followed by alkaloids and phenols.

The TLC studies of the successive extractives were carried out using two different systems - chloroform: ethyl acetate: formic acid (5:4:1) (CEF) and ethyl acetate: methanol: water (40:5:4.4) (EMW). The plates were sprayed with 0.4mM DPPH solution in methanol. The spots developed were observed.

When the plates were sprayed with DPPH solution in methanol (0.4mM), the regions where substances with antioxidant capacity occurred stained yellow in the purple back ground (Fig 1 & 2). The chromatograms show that the ethyl acetate fraction gave maximum antioxidant property.

In the past few years, there has been growing interest in the involvement of reactive oxygen species (ROS) in several pathological situations. Phenolic compounds and flavonoids have been reported to be associated with antioxidant activities in biological systems, acting as scavengers of singlet oxygen and free radicals[20,21]. The DPPH radical scavenging activity of *P. travancorensis* is given in Fig.3.

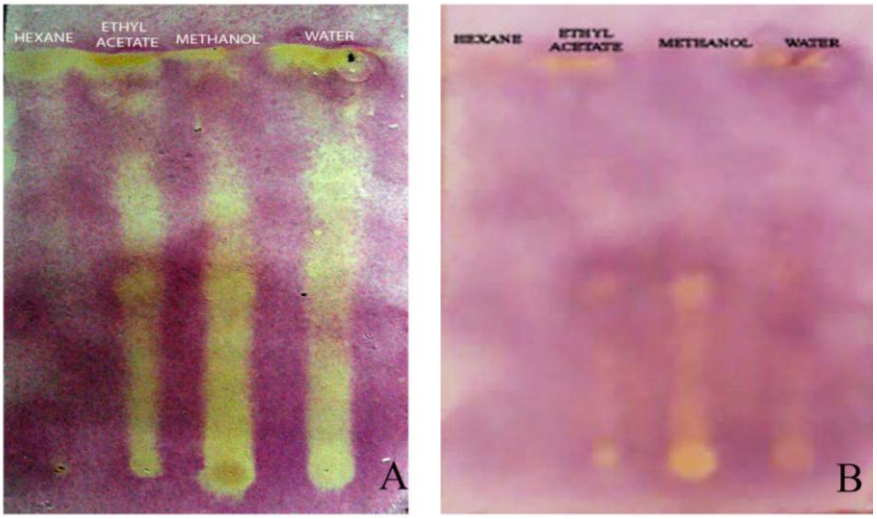

Fig. 1 & 2: Chromatogram of thin layer chromatography plates with CEF (left) and EMW as eluent systems (right)

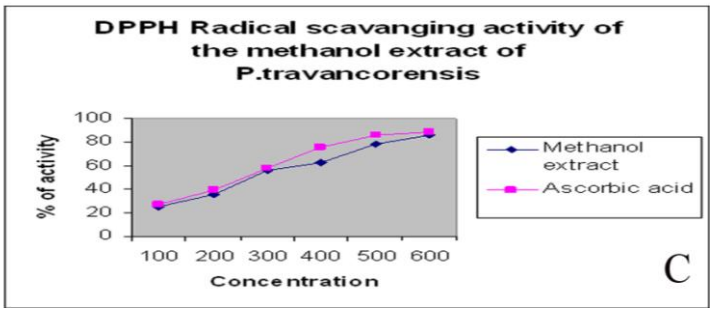

Fig. 3: DPPH radical scavenging activity of methanol extract of *P. travancorensis*. Values are the average of duplicate experiments and represented as mean ± standard deviation.

The activity was increased by increasing concentration of the sample extract. The antioxidant activity assay is based on the activity of DPPH, a stable free radical, to decolourize in the presence of antioxidants. The IC_{50} value of the extract was 61.55 µg/ml, comparable to that of ascorbic acid (IC_{50} value - 56.87 µg/ml), which is a well known antioxidant.

The results of Dot-blot assay showed coloured spots where the aliquots of different extracts were placed in row. The purple area on the plate indicates no free radical scavenging or antioxidant activity and the yellow areas indicate antioxidant activity. The more intense the yellow colour, the greater the antioxidant activity (Fig.4). This assay also shows that ethyl acetate extract showed maximum antioxidant property.

Total antioxidant capacity of the *P. travancorensis* extract, expressed as the number of gram equivalent of ascorbic acid is shown in Table 3.

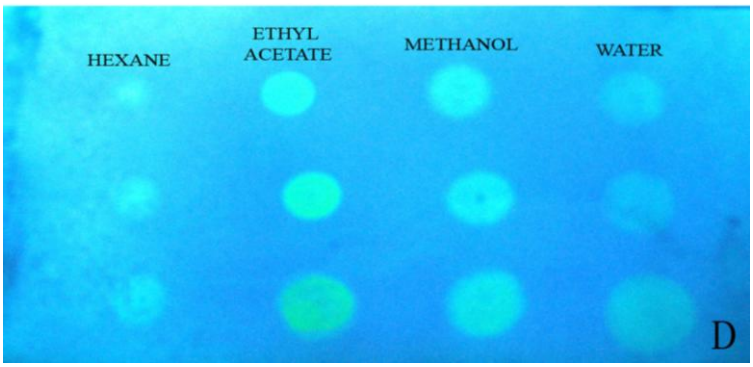

Fig. 4: Scan of dot blot test of a thin layer chromatography sprayed with 0.4Mm DPPH.

Table 3: Total antioxidant capacity of the root extract of *P.travancorensis*

Concentration (µg/ml)	Equivalent to ascorbic acid
100	0.39 ± 0.16
200	1.45 ± 0.12
400	1.52 ± 0.14
600	2.72 ± 0.18
800	3.47 ± 0.19

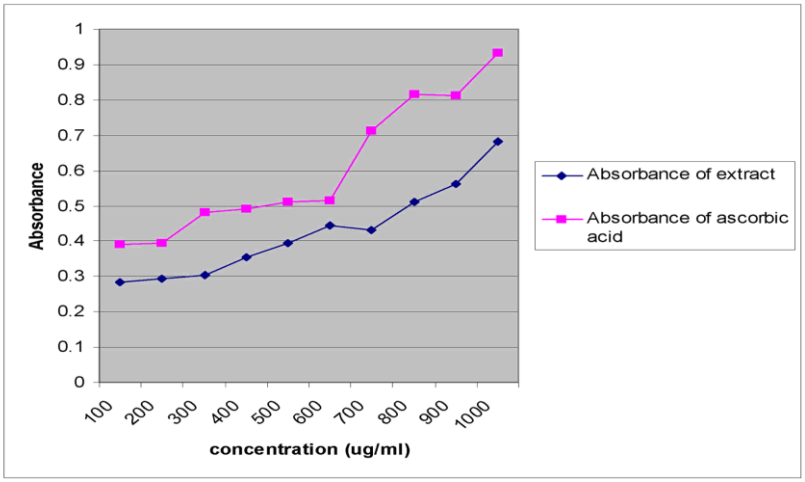

Fig. 5: Reducing power of methanol extract of *P. travancorensis*. Values are the average of duplicate experiments and represented as mean ± standard deviation.

The phospho molybdenum method of determining total antioxidant capacity was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate complex with a maximal absorption at 695 nm. Tanaka *et al*.,[22] have observed a direct correlation between antioxidant activity and reducing power of certain plant extracts. The reducing properties are generally associated with the presence of reductions[23] which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom[24].

Figure 5 shows comparative reductive capabilities of ascorbic acid and plant extract. The plant extract showed remarkable reducing property, which is increased with increase in concentration of the extract.

Determination of quercetin from the root was performed under optimum condition (30ml of 45% aqueous methanol, containing 1.85M HCl refluxed for 2hours at 84^{0}C) using HPLC at 370nm. Identification of quercetin was performed by its retention time, spiking with the standard and comparison of its UV spectrum with the standard. Typical chromatogram of quercetin and *Psilanthus* root extract are shown in fig. 6 & 7. Concentration of quercetin in *P. travancorensis* estimated by standard addition methods and was 199.6 mg/Kg tissue.

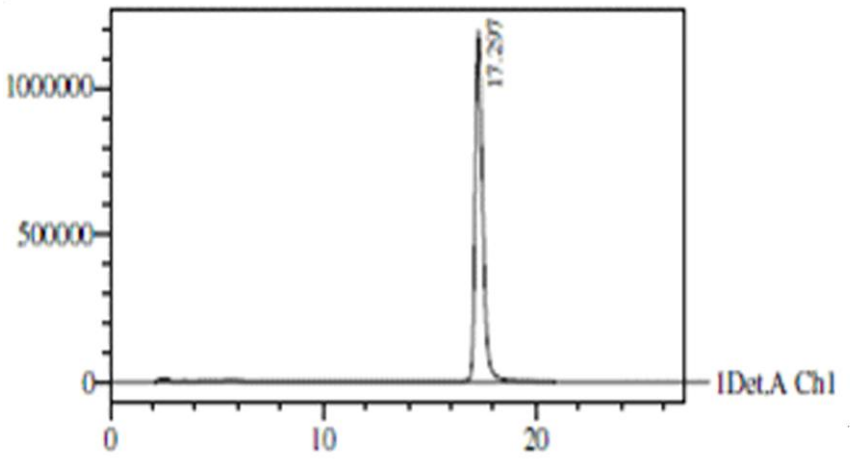

Fig. 6: Chromatogram of standard solution of quercetin

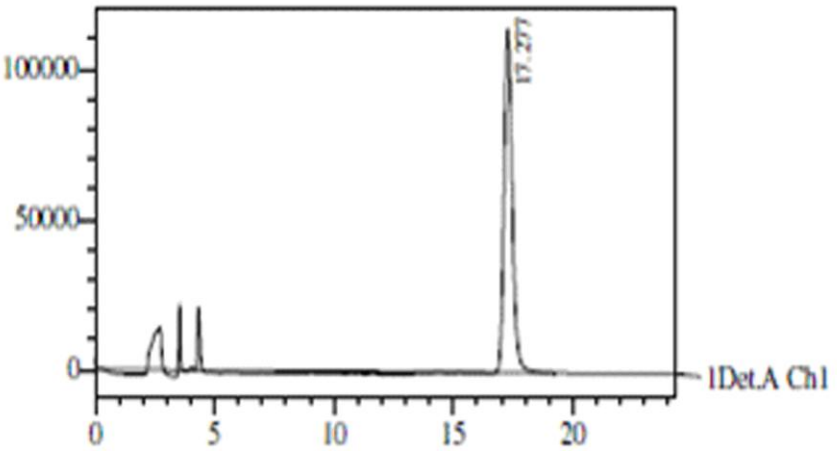

Fig. 7: Chromatogram of *P.travancorensis*

P. travancorensis is observed to possess potent antioxidant activity. However, a systematic and scientific documentation of secondary metabolites has been conspicuous by its absence. The present study attempts to bridge this gap in secondary metabolites' research by identifying and isolating an antioxidant compound – quercetin.

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