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PHYTOCHEMICAL ANALYSIS, *IN* VITRO EVALUATION OF ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF LEAVES OF *TRICHOPUS ZEYLANICUS* SSP *TRAVANCORICUS* BURKILL EX K. NARAYANAN (TRICHOPODACEAE)

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Abstract
Free radicals are an outcome of various metabolic activities and their excess production leads to many diseases. Therefore, it is necessary to neutralize excess free radicals. Free radical scavenging activity of various extracts of *Trichopus zeylanicus* ssp *travancoricus* leaf was evaluated using different assays. Petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *Trichopus zeylanicus*ssp*travancoricus* scavenged DPPH, hydroxyl, superoxide, ABTS free radicals and increased reducing potential in a concentration dependent manner. Maximum DPPH and superoxide radical scavenging activity of methanol extract was recorded whereas ethyl acetate extract showed enhanced hydroxyl radical scavenging activity. Ethanol extract showed the highest ABTS radical cation scavenging activity. The reducing power of the *T. zeylanicus*ssp*travancoricus* was in following order: ethanol > methanol > petroleum ether > ethyl acetate > benzene. The qualitative phytochemical analysis revealed the presence of important phto constituents. These results are an indication of antioxidant potential of the extracts and may be responsible for some of the therapeutic uses of *T. zeylanicus* ssp *travancoricus*.

Key words: Arogyapachi, flavonoid, antioxidant activity, reducing power.

Introduction
The reactive oxygen species that are constantly generated in the human body cause oxidative stress. The ratio of reactive oxygen species may be increased by the factors such as drugs, chlorinated compounds, deficiency of natural antioxidants, alcohol, stress and unhealthy food. Despite naturally occurring antioxidant systems in the human body, reactive oxygen species cause lipid, protein and DNA oxidation. These damages at the molecular level may influence the etiology of diseases, such as cancer, kidney failure, atherosclerosis, diabetes, hepato toxicity neuro degenerative disorders and aging – related diseases (Strzemski *et al*., 2017). Natural antioxidants are often added in foods to prevent the radical chain reactions of oxidation by inhibiting the initiation and propagation step leading to the termination of the reaction and a delay in the oxidation process (Hossain *et al*., 2011). Reactive oxygen species, such as the superoxide anion (O_2^-) hydroxyl radicals (OH^-) and nitric oxide (NO), inactivate enzymes and damage vital cellular components, causing injury. Antioxidants may

provide resistance against oxidative stress by scavenging free radicals. Therefore, compounds with antioxidative properties may be useful in the treatment of various disorders (Parim*et al*., 2015).

Phyto consitutents are mainstay for exerting various therapeutic properties and serve as a blue print for the development of new pharmaceuticals (Hossain *et al*., 2017). Worldwide currently, there has been an augmented interest to find antioxidant compounds which are pharmacologically active and have minor or no side effects (Ntie-Kang *et al*., 2013). Medicinal plants are the greatest source of natural antioxidant. In the developing world, medicinal plants serve as a potential source of primary healthcare needs (Uddin *et al*., 2016). The antioxidant activity of medicinal plants are owing to the presence of phyto compounds such as phenolics (polyphenolics) and flavonoids to prevent the oxidative stress caused by ROS/RNS (Ntie – Kang *et al*., 2013; Uddin *et al*., 2016).
Trichopus zeylanicus ssp *travancoricus* Burkill ex K. Narayanan is a rhizomatous herb belongs to the family Trichopodaceae locally known as Arogyapachai(Tamil) and Arogyapacha (Malayalam) and literally known as the green that gives strength. In India, the species have reported as endemic to the Southern Western Ghats with a restricted distribution in Agasthyamalai Biosphere Reserve. It is reported as one of the important ethno medicinal plants. The kani tribe of Agasthyamalai has introduced various uses of this wild plant to the present medical world. They also claim that one who consumes the fruits of Arogyapachai regularly, will remains healthy, agile and disease resistant (Pushpangadan*et al*., 1988). The powdered leaves of *T. zeylanicus*ssp*travancoricus* along with stem bark of *Mangiferaindica* was used to treat venereal diseases (Ayyanar and Ignacimuthu, 2005). The indigenous tribal community in Agastyar hills traditionally uses this plant as an instant energy booster that combat fatigue (Biju*et al*., 2019). *T. zeylanicus* ssp *travancoricus* also possess several pharmacological activities and medicinal properties such as choleretic, aphrodisiac, hepato protective, mast cell stabilization (Subramaniam*et al*., 1997, 1998; 1999), adaptogenic (Singh *et al*., 2005), cardioprotective (Velavan*et al*., 2009) anxiolytic and antidepressant activity, hepato protective activity, immunomodulatory activity and anticulcer activity (Rishikesh*et al*., 2013; 2017 Bachhav and Sambathkumar 2016). The chemical investigation of aerial part, fresh leaves, dried leave and fruits were already carried out by some investigators (Vignesh and Ramasubbu, 2017; Anilan and Muthusamy 2020; Sasi and Ramasubbu, 2017). The current study planned to investigate the phytochemical and antioxidant activities of different solvent extracts prepared from the leaves of *Trichopus zeylanicus* ssp *travancoricus*.

Materials and Methods

Collection of plant samples

The fresh leaves of *Trichopus zeylanicus* ssp *travanconicus* Burkill ex K. Narayanan were collected from Mankkamalai, Petchiparai, Kanyakumari District, Southern Western Ghats, Tamil Nadu. The specimen collected were identified with the local flora and authenticated by Botanical Survey of India, Southern Circle, Coimbatore. The voucher specimen of *T. zeylanicus* ssp *travancoricus* (EPH: 411) was submitted in the Herbarium of Ethno pharmacology unit, Research Department of Botany, V.O. Chidambaram College, Tuticorin.
The collected leaf samples were cut into minute fragments and shade dried in anticipation of the fracture being uniform and smooth. The dried material was granulated or powdered by using blender and sieved to get uniform powder was utilized for the extraction of active constituents of the plant material.

Preliminary phytochemical screening

The qualitative tests to categorize the numerous chemical ingredients were carried out in different (petroleum ether, benezene, ethyl acetate, methanol, ethanol and water) solvent extracts of *T. zeylanicus*ssp*travancoricus* leaf using the procedures suggested by Brinda *et al.* (1981). They were tested for alkaloids, anthraquinones, catechins, coumarins, flavonoids, phenols, quinones, saponins, steroids, tannins, terpenoids, glycosides, carbohydrates, xantho proteins and fixed oil.

Estimation of total phenolics

Total phenolic content was estimated using Folin-Ciocalteau reagent based assay as previously described (Mc Donald *et al*., 2001) with little modification. To 1 ml of each extract (100 µg/ml), 5ml of Folin-Ciocalteau reagent (diluted ten- fold) and 4 ml (75 g/L) of Na_2CO_3 were added. The mixture was allowed to stand at 20°C for 30 min and the absorbance of the developed colour was recorded at 765 nm using UV-VIS spectrophotometer.1ml aliquots of 20,40,60,80,100 µg/ml methanolicgallic acid solutions were used as standard for calibration curve. The absorbance of solution was compared with gallic acid calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE g/100 g dry weight of extract).

Estimation of flavonoids

Total flavonoid content was determined according to Eom et al. (2007). An aliquot of 0.5 ml of samples were mixed with 0.1 ml of 10% aluminium chloride and 0.1 ml of potassium acetate (1M). In this mixture, 4.3 ml of 80% methanol was added to make 5 ml volume. This mixture was vortexed and the absorbance was measured spectro photo metrically at 415 nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

Antioxidant activity

Petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *T. zeylanicus* ssp *travancoricus* leaf were used to determine the *in vitro* antioxidant activity.

DPPH radical scavenging assay

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging ability of different solvent extracts of *T. seylanicus* ssp *travancoricus* leaf was determined by the method of Shen *et al*. (2010). DPPH solution of 0.1 mM was prepared using methanol. 1 mL of this prepared solution was added to 3 mL of different concentration (50, 100, 200, 400 and 800 µg/mL) of extracts. Then it was shaken vigorously and allowed to stand. After 30 min, absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10S UV: Thermo electron corporation) using Ascorbic acid as standard. Solution with lower absorbance values indicates more free radical scavenging activity. It was calculated by using the following formula.

DPPH scavenging effect (% inhibition) = $\{(A_0 – A_1) /A_0) * 100]$

where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference.

Hydroxyl radical scavenging assay

The Hydroxyl radical scavenging assay was measured using the modified method of Halliwell *et al*. (1987). Various stock solutions used in this method are EDTA (1 mM), $FeCl_3$ (10 mM), Ascorbic Acid (1 mM), H_2O_2 (10 mM) and Deoxyribose (10 mM). All the solutions were prepared in distilled deionized water.

Hydroxyl radical scavenging activity was carried out by adding 0.1 mL EDTA, 0.01 mL of $FeCl_3$, 0.1 mL H_2O_2, 0.36 mL of deoxyribose, 1.0 mL of the extract of different concentration, 0.33 mL of phosphate buffer (50 mM, pH 7.9), 1.0 mL of ascorbic acid in sequence. This mixture was kept at 37 °C for 1 h. After incubation period, 1.0 mL from the mixture was added to 1.0 mL of 10% TCA and 1.0 mL of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA) to produce the pink chromogen. It was measured at 532 nm. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Superoxide radical scavenging assay

Superoxide anion scavenging activity was measured by the method of Srinivasan *et al*. (2007). The superoxide anion radicals were generated in 3.0 ml of Tris-HCL buffer (16 mM, pH 8.0), containing 0.5 mL of NBT (0.3 mM), 0.5 mL NADH (0.936 mM) solution, 1.0 mL extract of different

concentration (50, 100, 200, 400 and 800 μg/mL), and 0.5 mL Tris-HCl buffer (16 mM, pH 8.0). An addition of 0.5 mL PMS solution (0.12 mM) was carried to start the reaction. This mixture is incubated at 25°C for 5 min and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Antioxidant activity by radical cation (ABTS+)

It was carried out by using slightly modified method of Huang *et al*. (2011). In this method, 7 mM ABTS solution and 2.45 mM potassium per sulphate were used to produce ABTS radical cation (ABTS+). This reaction mixture is kept in dark for 12-16 h at room temperature. Then this solution was diluted with ethanol to get an absorbance value of 0.70 + 0.02 at 734 nm. Then 3.9 mL of diluted ABTS+ solution is added with sample extract and is used for measuring absorabance at 734 nm by Genesys 10S UV-VIS (Thermo scientific) exactly after 6 minutes. Trolox used a standard. Resultant data were expressed as trolox equivalent antioxidant capacity (TEAC).

Reducing power

Reducing power was determined by the method of Kumar and Hemalatha (2011). In this sodium phosphate buffer (5.0 mL, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 mL, 1.0%) is mixed with 1.0 mL of the five studied concentration of extract respectively. This mixture was incubated at 50 °C. After 20 min, 5 mL of 10% trichloroacetic acid was added and centrifuged at 5000 rpm (10 min at 5°C) in a refrigerator centrifuge. After centrifugation, the upper layer (5.0 mL) was diluted with 5.0 mL of distilled water and ferric chloride and absorbance read at 700 nm.

Statistical analysis

All the experiments were estimated in triplicate determinations. The statistical analysis system SPSS was used to analyze the data.

Results and Discussion

Qualitative phytochemical screening

Petroleum ether, benzene, ethylacetate, methanol, ethanol and aqueous extracts of leaf of *T.zeylanicus* ssp *travancoricus* are qualitatively analysed for the presence of different phyto constituents and the results are presented in Table 1.

Table 1: Preliminary phytochemical screening of leaf extracts of *T. zeylanicus* ssp *travancoricus*

Bioactive components	Petroleum ether	Benzene	Ethyl acetate	Methanol	Ethanol	Water
Alkaloids	-	-	+	+	+	-
Anthraquinones	-	-	-	-	-	-
Catachins	+	-	-	+	+	+
Coumarins	-	+	-	-	-	-
Flavonoids	-	+	+	+	+	+
Phenols	+	+	+	+	+	+
Quinones	-	-	+	+	+	-
Saponins	+	+	+	+	+	+
Steroids	+	-	+	+	+	-
Tannins	+	+	+	+	+	-
Terpenoids	-	-	-	+	+	+
Sugar	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+
Xanthoproteins	+	+	+	+	+	+
Fixed oil	+	-	+	-	-	-

+ present – absent

The methanol and ethanol extracts of *T. zeylanicus* ssp. *Travancoricus* leaf exhibits the presence of alkaloids, catechins, flavonoids, phenols, quinones, saponins, steroids, tannins, terpenoids, sugars, glycosides and xantho proteins. The therapeutic properties of traditional medicinal plants are owing to the presence of various classes of phytochemicals such as alkaloids, flavonoids glycosides, phenols, saponins and sterols (Oladeji*et al*., 2020).

Total phenolic content and flavonoid constant

The total phenolic and flavonoid constants of the methanol extract of *T. zeylanicus* ssp *travanconicus* leaf were found to be 1.34 $g100g^{-1}$ and 1.81 g $100g^{-1}$ respectively. Phenolics derived from various natural sources are linked to antioxidant, anti-inflammatory, antiallergic, anticarcinogenic, antihypertensive, cardio protective, antiarthritic and antimicrobial activities (Rauha*et al*., 2000; Penna*et al*., 2001). The most important groups of secondary metabolites in plants, flavonoids, are also the better sources of natural antioxidants in human diets. So, flavonoids can be used as a unique constituent in the control of different human diseases. It has polyphenolic structure, for that it become responsible for different pharmacological activities. The antioxidant effect of flavonoids is due to their hydroxyl groups, by scavenging free radicals or by chelating metal ions. The prevention of radical generation that damage the biomolecules leading to oxidative stress can be protected by flavonoids and it also gives security against the many diseases such as cancer, cardiovascular and respiratory disorders, arthritis and early ageing (Bose *et al*., 2018).

Antioxidant activity

DPPH radical scavenging activity

DPPH radical scavenging activity has been widely used to evaluate the free radical scavenging activity of antioxidants or hydrogen donors. The results of the DPPH radical scavenging activity of the leaf extracts of *T. zeylanicus*ssp*travancoricus* were shown in Fig. 1. The level of colour change is proportional to the concentration and potency of the antioxidants. Among the solvent tested in the present study, methanol extract exhibited highest DPPH radical scavenging activity. At 800 µg/mL concentration methanol extract of *T. zeylanicus*ssp*travancoricus* leaf possessed 129.36% DPPH radical scavenging activity. 50% inhibition of DPPH radical by the methanol extract of *T. zeylanicus*ssp*travancoricus* was shown at 22.88 µg/mL. The percentage of inhibition and IC_{50} value of standard ascorbic acid were 118.46% and 20.40 µg/mL respectively (Table 2). In general the radical scavenging activity of extracts could be related to the nature of phenolics and their hydrogen donating ability (Basama*et al*., 2011)

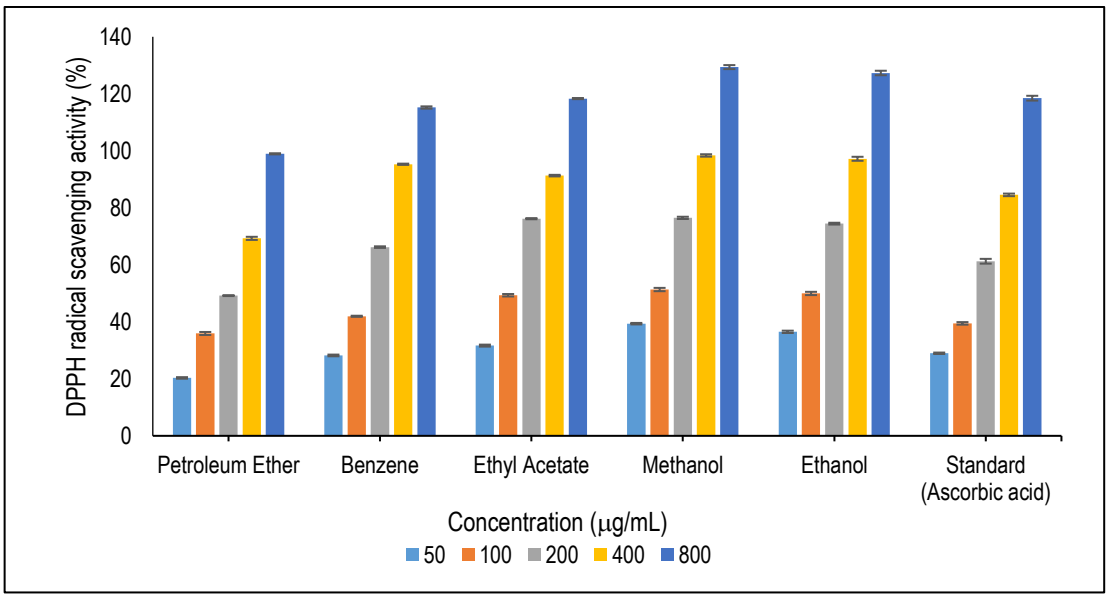

Figure 1: DPPH radical scavenging activity of *T. zeylanicus* ssp *travancoricus* leaf

Hydroxyl radical scavenging activity

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as highly damaging to almost every molecule found in living cells. This radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenic, mutation and cytotoxicity (Moskovitz, 2002). In the present study, the results of hydroxyl radical scavenging activity of different solvent extracts of *T. zeylanicus*ssp*travancoricus* leaf was shown in Fig. 2. It indicated that ethyl acetate extract of *T. zeylanicus*ssp*travancoricus* leaf exhibited 132.21% scavenging activity on hydroxyl radical at 800 µg/mL concentration. The concentration of ethyl acetate extract of *T. zeylanicus* ssp *travancoricus* needed for 50% inhibition (IC_{50}) was found to be 23.11 µg/mL. The percentage of inhibition and IC_{50} value of standard ascorbic acid were 126.73% and 22.34 µg/mL respectively (Table 2). The ability of the above mentioned extracts to quench hydroxyl radicals seem to be directly related the prevention of propagation of the process of lipid peroxidation and seems to be good scavenger of active oxygen, thus reducing the rate of chain reaction (Anusuya*et al*., 2009).

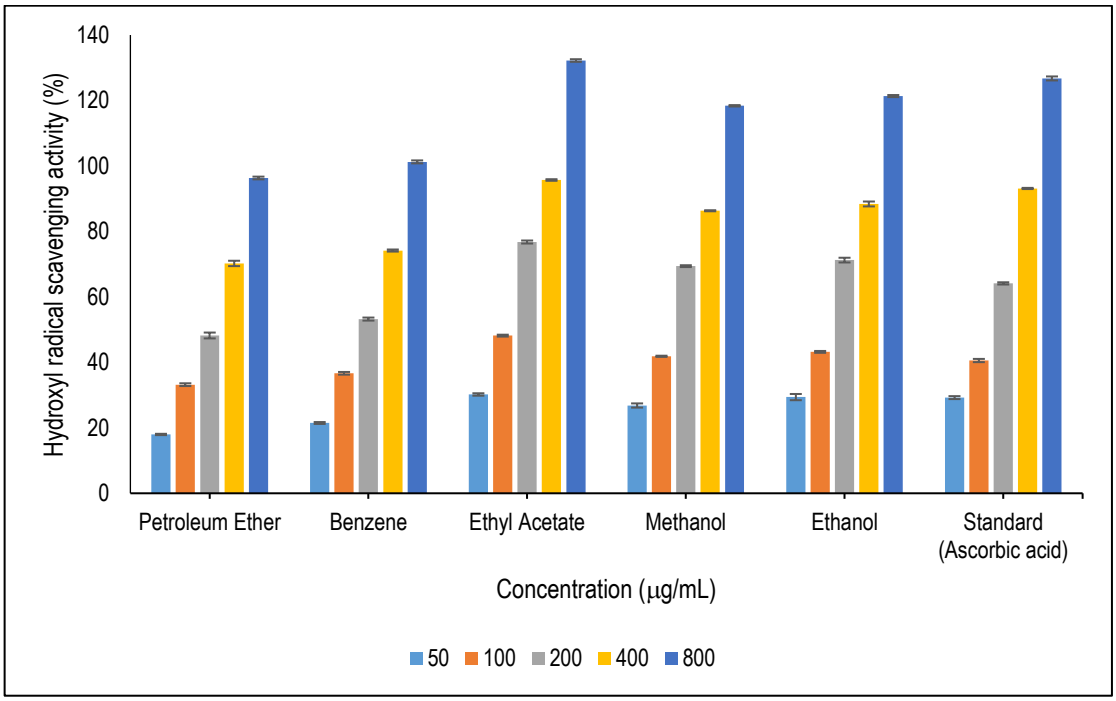

Figure 2: Hydroxyl radical scavenging activity of *T. zeylanicus* ssp *travancoricus* leaf

Superoxide radical scavenging activity

Superoxide radical scavenging activity is an antioxidant enzyme related to ROS scavengers and mediators in oxidative chain reactions. To evaluate the superoxide radical scavenging activity of antioxidants, the PMS-NADH- NBT system was employed (Suksungworn and Duangarisai 2021). In the present study, the different solvent extracts of *T. zeylanicus*ssp. *travancoricus* leaf extracts subjected to the superoxide radical scavenging activity and the results were presented in Fig. 3. It indicated that methanol extract of *T. zeylanicus*ssp*travancoricus* leaf exhibited the maximum superoxide radical scavenging activity of 141.93% with IC_{50} value of 24.68µg/mL at 800 µg/mL concentration. The percentage of inhibition and IC_{50} value of standard ascorbic acid were 112.63% and 20.45 µg/mL respectively. The radical scavenging capability of *T. zeylanicus* ssp. *travancoricus* leaf extract is possibly dependent on the number and the location of OH groups in the phenolic compounds present in the extract (Sowmya and Ananthi, 2021).

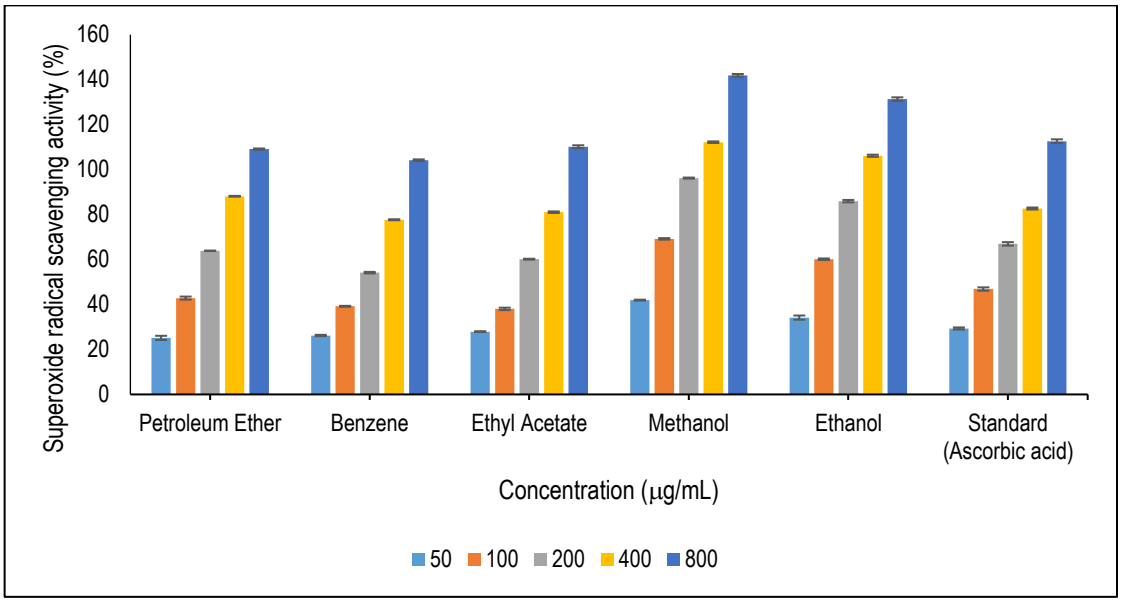

Figure 3: Superoxide radical scavenging activity of *T. zeylanicus* ssp *travancoricus* leaf

ABTS radical cation scavenging activity

ABTS assay can be used to determined the antifree radical scavenging activity as well as the hydrophilic and lipophilic biochemical reactions, and apply it for all types of solvent extracts to compare it with other antioxidant assays, ABTS assay depends on the presence of antioxidant reactants in the radical cation and reduction in decolourization potential property (Re *et al*., 1999; Haleshappa*et al*., 2021). In the present study, a concentration dependent assay was carried out with the extracts and the results were illustrated in Fig. 4. The ethanol extract showed potent ABTS radical cation scavenging activity in concentration dependent manner. At 800 µg/mL concentration, *T. zeylanicus* ssp *travancorius* leaf exhibited 128.09% scavenging activity in ABTS. The IC_{50} value of ethanol extract was 23.88 µg/mL. The percentage of inhibition and IC_{50} value of standard Trolox were 112.30 % and 20.32 µg/mL respectively (Table 2). Similar findings were exhibited with different standards with stronger to weaker antioxidant activity, the ABTS decolourization potential assay were shown by various medicinal plant extracts in crude nature as well as isolated fractions (Pisoschi*et al*., 2016).

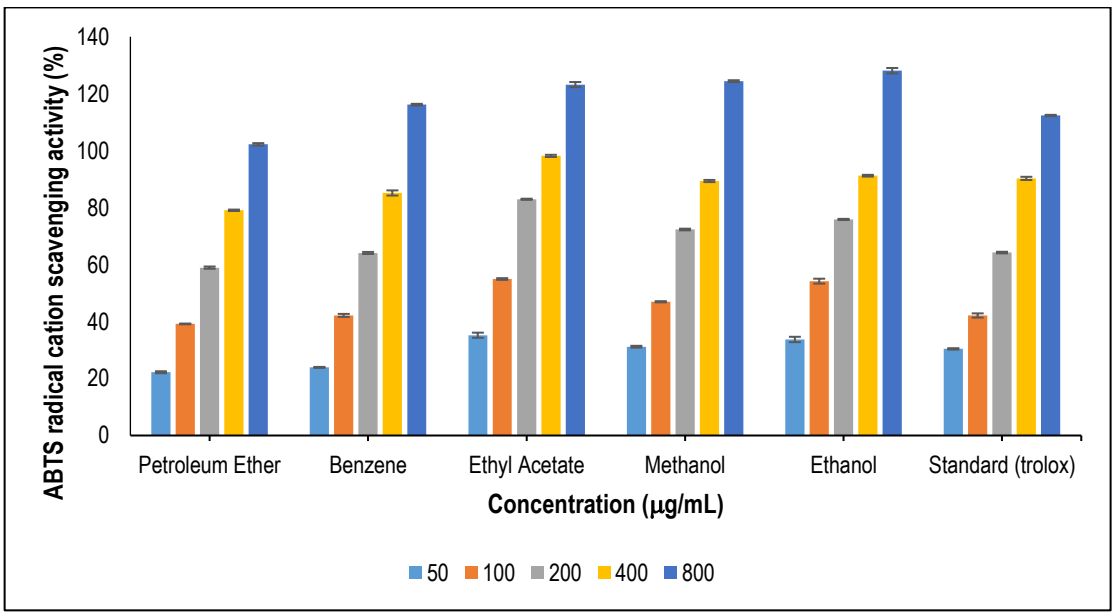

Figure 4: ABTS radical cation scavenging activity of *T. zeylanicus*ssp*travancoricus* leaf

Reducing power

The antioxidant can donate an election to free radicals, which leads to the neutralization of the radical. Reducing power assay measures the electron-donating capacity of an antioxidant (Hinne burg*et al*, 2016). Figure 5 showed the reducing ability of different solvent extracts of *T. zeylanicus* ssp *travancoricus* leaf compared to ascorbic acid. Absorbance of the solution was increased when the concentration increased. A highest absorbance indicated a higher reducing power. Among the solvent tested ethanol extract exhibited higher reducing power (0.524 OD). The reducing capacity of *T. zeylanicus*ssp*travancoricus* leaf is a significant indicator of this potential antioxidant activity.

The exact mechanism of free radical scavenging by different extracts of leaf of *T. zeylanicus*ssp*travancoricus* is not known. However, the phytochemical analsysis of *T. zeylanicus*ssp*travancoricus* leaf has shown the presence of phenols and flavonoids and their concentration increased with the increase in the amount of extracts. Therefore, the free radical scavenging and antioxidant activities of *T. zeylanicus* ssp *travancoricus* leaf may be due to the presence of various polyphenols and flavonoids.

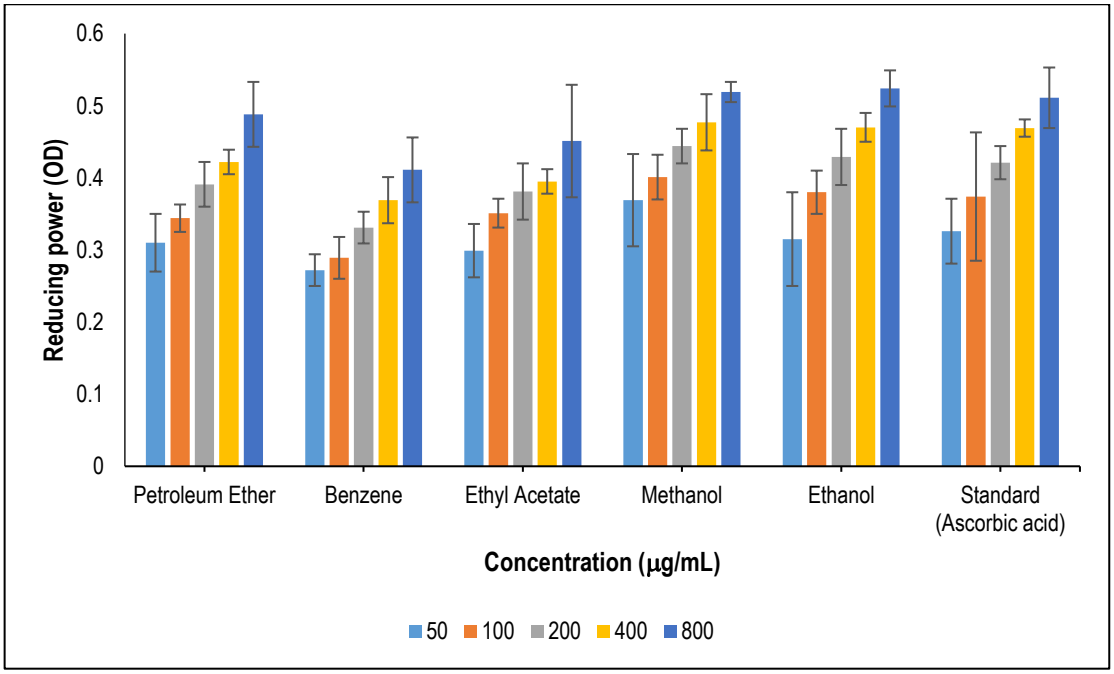

Figure 5: Reducing power of *T. zeylanicus*ssp*travancoricus* leaf

Table 2: IC_{50} values of different solvent extracts of *T. zeylanicus* ssp *travancoricus* leaf

Solvent	IC_{50} (µg/mL)			
	DPPH	Hydroxyl	Superoxide	ABTS
Petroleum ether	17.38	16.45	19.88	18.56
Benzene	19.89	18.16	18.35	21.56
Ethyl acetate	20.08	23.11	19.34	22.12
Methanol	22.88	20.45	24.68	22.78
Ethanol	21.56	21.56	23.48	23.88
Ascorbic acid	20.45	22.34	20.45	-
Trolox	-	-	-	20.32

Conclusion

The leaves of *T. zeylanicus* ssp *travancoricus* which contain a high amount of flavonoids and phenolic compounds, exhibit high antioxidant and free radical scavenging activities. The *in vitro* assays demonstrated that this plant extract is a noteworthy resource of an innate antioxidant, which may be

help in preventing the development of diverse oxidative stress. Although, in the present findings indicate the *T. zeylanicus*ssp*travancoricus* possesses antioxidant property, further research on the isolation and formulation of active ingredients from the leaves should be conducted for its therapeutic applications.

Disclosure statement

The authors declare that there are no potential conflicts of interest.

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