IN VITRO ANTIOXIDANT ACTIVITY AND PHYTOCHEMICAL SCREENING OF ETHANOLIC EXTRACT OF *Tabernaemontana coronaria* (L.)

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Summary

The aim of this study was to screen for phytochemical constituents of *T. coronaria* in different solvent extraction in both qualitative and quantitative assays and to evaluate *in vitro* antioxidant activity inhibition of DPPH, FRAP, hydroxyl radical scavenging, superoxide radical scavenging, reducing power assay and nitric oxide radical scavenging activity. Results indicate that ethanolic extract of *Tabernaemontana coronaria* have marked high content of secondary metabolites among all the solvents. The total carbohydrates, phenol and flavanoid were 74.03mg/g, 98.08 mg/g, 24.28 mg/g. At (100-500µg/ml) concentration the *T. coronaria* of 500 µg/ml concentration exhibited high DPPH radical scavenging capacity using BHT as positive control. *T. coronaria* at the same concentration showed the dose dependent inhibition of FRAP, hydroxyl radical scavenging, superoxide radical scavenging, reducing power assay and nitric oxide radical scavenging activity. Collectively, our results indicate that the ethanolic extract of *T.coronaria* has the potential to scavenge free radicals and act as a good antioxidant for treating various diseases.

Keywords: oxidative stress, *T. coronaria*, phytochemical, *in vitro* antioxidant, free radicals.

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Introduction

Plants are primarily responsible for life on earth[2]. Over the past decade, herbal medicines have been accepted universally, and they have an impact on both world health and international trade[8]. *Tabernaemontana coronaria* R.Br. (syn. *Ervatamia coronaria*) is a glabrous, evergreen, dichotomously branched shrub, belonging to the family Apocynaceae. It is a shrub very common in India, grows to a height of 6-8 feet. It is distributed in upper Ganjetic plain, Garhwal, East Bengal, Assam, Karnataka, Kerala, Tamilnadu and Burma. It is a spreading, bushy, many branched shrub with elliptic oblong, wavy-margined, thin, glossy, mild to dark green leaves. It bears white, fragrant, five- petaled flowers which are 1-5 cm in diameter. The stems exude milky latex when broken. Hence it is one of the diverse plant genera commonly called 'milk wood'. It is cultivated in gardens as an ornamental plant.

This species has been extensively investigated and a number of chemical constituents such as alkaloids, triterpenoids, steroids, flavanoids, phenyl propanoids and phenolic acids were isolated from leaves, roots and stems of the plant[9]. In ayurveda, the root is acrid; bitter with a flavour; digestible; useful in Kapha, biliousness and diseases of the blood. The root has a bitter bad taste. It is aphrodisiac; tonic especially to the brain, liver, spleen and it is also a purgative[9]. The milky juice mixed with oil is rubbed on to the head to cure the pain in the eye. The milky juice is very useful in many eye infections, especially red eye. It kills intestinal worms, and its root when chewed, relieves tooth ache[15]. Herbal medicines have been proved to be powerful therapeutics for treatment of various human sufferings including Cancer, Arteriosclerosis, Ulcer, Diabetes, Kidney diseases, Liver diseases etc. A detailed investigation and documentation of plants used in local health traditions and their pharmacological evaluations can lead to development of invaluable plant drugs for many dreaded diseases[2]. The present work was undertaken to screen the phytochemical constituents and *in vitro* antioxidant activity of the plant *Tabernaemontana coronaria.*

Materials and Methods

The whole plant of *Tabernaemontana coronaria*was procured from Pathanamthitta, Kerala and was identified by the Botanical Survey of India, Tamilnadu Agricultural University (TNAU), Coimbatore and authenticated by Dr.G.V.S.Moorthy (Voucher no: BSI/SRC/5/23/09-10/Tech-987).

Preparation of the plant extract

The whole plant was air dried at 25°C for 20 days in the absence of sunlight and powdered well using a mixer and stored in an air tight container. The powdered plant material was taken and subjected to successive solvent extraction. The extraction was carried out for 16 hours with the following solvents in the increasing order of polarity. The solvents used were petroleum ether, Chloroform, Ethyl acetate, Ethanol and Water.

Phytochemical screening of *Tabernaemontana coronaria*

Phytochemical screening is done for analyzing secondary metabolites that are responsible for curing ailments. The Phytochemical screening of the plant extract was carried out by following the method of Trease and Evans; Harborne[19, 5]. The test for alkaloids was done by using Dragendroff's and Mayer's reagents. The presence of steroids was determined by using

salkowski test and flavonoids was determined by shinoda test. One ml of the extract was treated with few ml of 15% neutral ferric chloride. A dark blue or bluish black colour product shows the presence of tannins. To 1ml extract, 2 drops of freshly prepared 0.2% Ninhydrin reagent was added and heated to check the presence of proteins. A blue colour develops indicating the presence of proteins. The test for carbohydrates was done by Fehling's test and confirmed by Benedict's test. The test for cardioglycosides was done by salkowski test and saponins by frothing test. The presence of fixed oils and fats was determined by spot test and terpenoids by Lieberman's test.

Biochemical characterization of *Tabernaemontana coronaria*

The total amount of carbohydrates and phenolic content present in the ethanolic extract of *Tabernaemontana coronaria* were determined by the standard method given by Sadasivam and Manickam[16]. The content of total flavonoid in the whole plant extract was estimated by the method of Ordon *et al*[13].

Determination of *in vitro* antioxidant activity

The reducing power of the whole plant extract was quantified according to the method of Oyaizu[14]. DPPH radical scavenging activity was adopted from those previously described with slight modifications[11]. The total antioxidant potential of sample was determined using Ferric Reducing Ability of Plasma (FRAP) by method of Benzie and strain (1996). FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue coloured Fe^{2+}-TPTZ compound from colourless oxidized Fe^{3+} form by the action of electron donating antioxidants. The hydroxyl radical scavenging activity was measured according to the method Klein *et al*[7].

Measurement of Superoxide radical scavenging activity was done using standard method of Nishikimi *et al*[12]. Superoxide anions were created in a non enzymatic PMS-NADH system through the reactions of PMS, NADH and O_2. It was analyzed by the reduction of NBT. Nitric oxide scavenging activity of the extract was determined by the use of the Griess Illosvoy reaction[3]. Reaction mixture (2ml) containing sodium nitroprusside(10mM) in phosphate buffered saline and the extracts (100-500 µg/ml) was incubated at 25^{o}C for 15 min. After incubation, 0.5ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% *N*-1-naphthylethylenediamine dihydrochloride) was added and measured at 546 nm.

Statistical analysis

The results obtained were expressed as Mean ±SD. The Statistical comparison among the groups were performed with one way ANOVA and DMRT using a statistical package program (SPSS 10.0) at $p<0.05$.

Results and Discussion

Various environmental toxicants and clinically useful drugs like acetaminophen and gentamicin can cause severe organ toxicities through the metabolic activation to highly reactive free radicals including the superoxides and reactive oxygen species[1]. Cellular systems are

protected from reactive oxygen species induced cell injuries by an array of defenses composed of various antioxidants with different function[10].

When compared with other solvents, ethanolic extract of whole plant of *T.coronaria* showed the presence of various phytoconstituent such as alkaloid, saponin, tannins, phenolic compounds, flavonoid, cardioglycosides, terpenoid, aminoacids, protein and carbohydrates are shown in table 1.

Table 1. Phytochemical Screening of *T.coronaria*

Extracts	**AL**	**SA**	**TP**	**FL**	**ST**	**CG**	**OF**	**TN**	**AP**	**CH**
Petroleum Ether	-	+	-	-	-	+	-	-	-	-
Chloroform	-	+	-	-	-	+	-	-	-	+
Ethyl Acetate	-	-	-	-	+	-	-	-	-	-
Ethanol	+	-	+	+	+	+	+	+	-	+
Water	+	-	-	-	-	+	-	-	+	-

'+' Present '_' Absent

AL-Alkaloids, CG-Cardioglycosides, SA-Saponin, OF-Oils & Fats, TP-Tannin and Phenolic compounds, TN-Terpenoids, FL-Flavonoids, AP-Aminoacids and Protein, ST-Steroids, CH-Carbohydrates.

The total carbohydrate, phenols, and flavanoid content of the ethanol extract of *T. coronaria* were studied. The total carbohydrate, phenol, flavanoid contents were found to be 74.03mg/g, 98.08mg/g and 24.28 mg/g respectively which is given in Table 2. The carbohydrates produced by plants are found to be an important source of energy for animals. Phenolic antioxidants are potent free radical terminators [18]. They donate hydrogen to free radical and hence break the reaction of lipid peroxidation at the initiation step[4]. The high potential of phenolic to scavenge free radicals may be due to the presence of many phenolic hydroxyl groups they posses [17].

Table 2. Biochemical characterization of *T. coronaria*

Particulars	**Ethanolic extract of *T. coronaria***
Carbohydrate (mg/g)	74.03±0.136
Total phenols (mg/g)	98.08±0.172
Total flavonoids (mg/g)	24.28±0.297

Values are expressed as mean ± S.D

DPPH radical scavenging activity of 79.43% which is almost near to BHT (Figure.1). DPPH free radical scavenging activity will prevent damage to DNA, hence the progression of vascular diseases. The FRAP scavenging capacity of the ethanol extracts of *T.coronaria* at five different concentrations (100-500 µg/ml) exhibited 0.27%, 0.35%, 0.47%, 0.53%, 0.64% respectively which is depicted in figure 2.

Figure 1. DPPH Radical Scavenging Assay

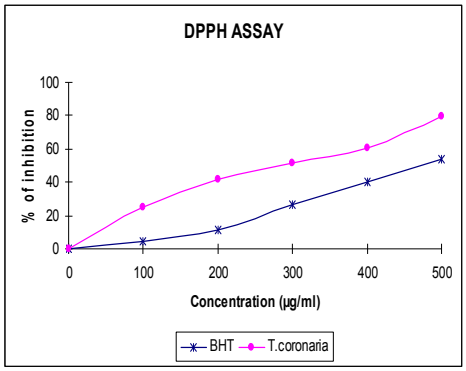
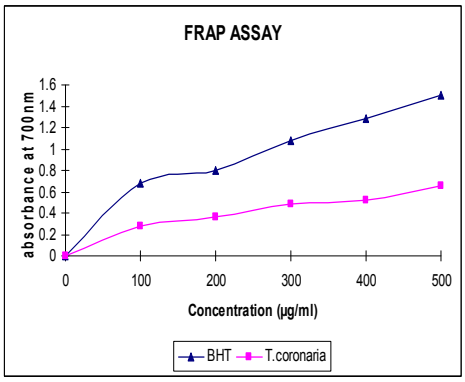


Figure 2. FRAP Assay



Hydroxyl radical are reported to cause oxidative damage to various biomolecules like DNA, lipids and proteins[6]. The hydroxyl radical scavenging capacity (Figure 3) of *T. coronaria* was compared with those of standard BHT and the values were found to be almost similar. The ethanolic extract of *T.coronaria* was found to be an effective superoxide anion scavenger to scavenge the superoxide anions as compared to standard BHT which showed 73.33% at the concentration of 500µg/ml (figure 4). The nitric oxide radical scavenging capacity of the ethanol extract of *T.coronaria* exhibited 11.6%, 38.56%, 57.66%, 80.06% and 83.43% at five different concentrations (100-500µg/ml) as depicted in figure 5. Nitric oxide inhibitors have been shown to have beneficial effects on some aspect of inflammation and tissue damage which can be seen in inflammatory diseases. For the measurements of the reducing ability, the Fe^{3+}-Fe^{2+} transformation was investigated in the presence *T.coronaria* extract. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging[20]. The reducing power activity of *Nerium indicum* leaves extract increased with increasing dosage.At a concentration of 500µg/ml the reducing power of *T. coronaria* expressed an absorbance of 0.67% which was almost near to the standard thiobarbituric acid (TBA) were shown in figure 6.

Figure 3. Hydroxyl Radical Scavenging Assay

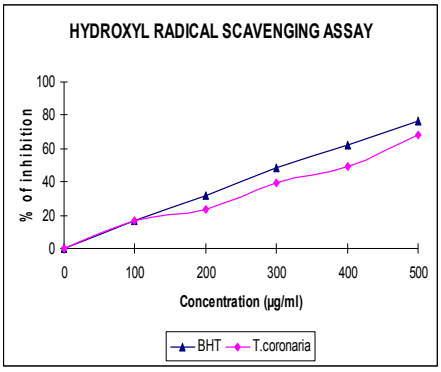


Figure 4. Super Oxide Radical Scavenging Assay

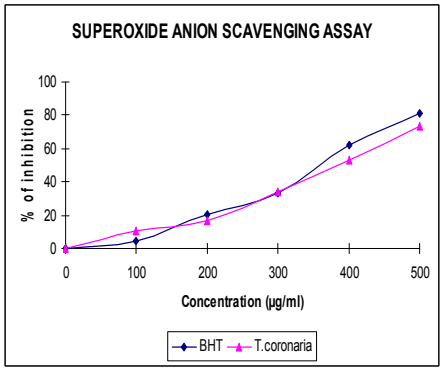


Figure 5. Nitric Oxide radical Scavenging Assay

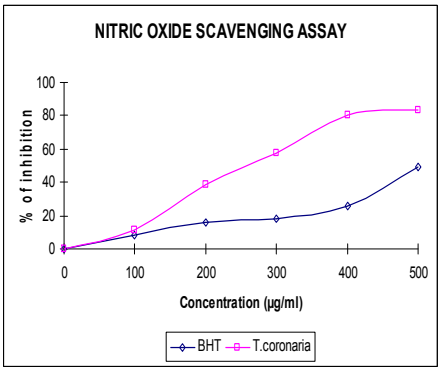
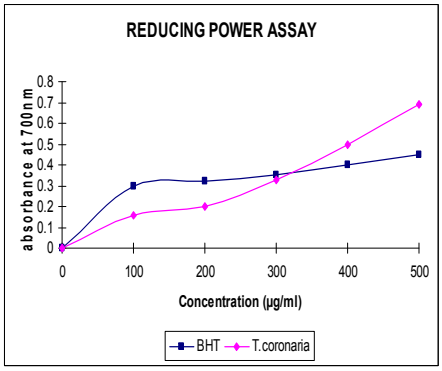


Figure 6. Reducing Power Assay



Hence, the observed *in vitro* antioxidant activity may be because of these phytoconstituents, which needs further investigation**.**

Conclusion

The results of the study clearly indicate that ethanolic extract of *T.coronaria* possess powerful *in vitro* antioxidant activity. The encouraging results of *T.coronaria* with the various *in vitro* antioxidant tests proved the plant has DPPH, FRAP, hydroxyl radical scavenging, superoxide radical scavenging, reducing power assay and nitric oxide radical scavenging activity. Hence, it is worthwhile to isolate and elucidate the bioactive principles that are responsible for the antioxidant activity that is underway.

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