Comparative evaluation of antioxidant potential of extracts of *Vitex negundo*, *Vitex trifolia*, *Terminalia bellerica*, *Terminalia chebula*, *Embelica officinalis* and *Asparagus racemosus*

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Abstract

The antioxidant potential of six important Indian medicinal plants *V. negundo*, *V. trifolia*, *T. bellerica*, *T. chebula*, *E. officinalis* and *A. racemosus* were evaluated. Extracts prepared using solvents of varying polarity were screened for their free radical scavenging and reducing capacity by chemical assays. Total phenolic content was determined in order to assess its effect on the antioxidant activity of the extract. The properties of extracting solvents affected the measured total phenolic content and antioxidant activity. Extracts of all the six medicinal plants exhibited antioxidant potential but *T. bellerica*, *T. chebula*, *E. officinalis* proved more active. The presence of antioxidant activity in the extracts showed that these plants have the potential to be an alternate source of natural antioxidants. *In vivo* study is needed for successful commercialization and to benefit the food and pharmaceutical industries.

Keywords: Free radicals, reactive oxygen species, oxidative stress, antioxidant, total phenols, scavenger

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1. Introduction

Plants and herbs have been used an important contributor to the quality of human life for thousands of years. They provide abundant natural antioxidants which are virtually important for human health. Oxidants and antioxidants in humans are maintained in balance in a normal physiological state. However, the overproduction of oxidants in certain conditions may cause oxidative stress leading to oxidative damage to biomolecules and cells. [1, 2]. Reactive oxygen species (ROS) are formed during normal cellular metabolism but when present in high concentration they become toxic. Oxidative stress or excessive production of ROS is being implicated in many diseases such as cancer, arthrosclerosis, aging, diabetes etc [3, 4]. The potential targets for ROS in cells are membrane lipids, DNA and proteins. Antioxidants are very important for human health. Natural antioxidants or other compounds such as vitamins, minerals or proteins can neutralize free radicals. Antioxidant supplementation is recommended to provide cellular protection from the deleterious effects of excessive reactive oxygen species concentration [5]. Antioxidants are our first line of defense against free radical damage and are critical for maintaining optimum health and wellbeing. The need for antioxidants becomes even
more critical with increased exposure to free radicals. Pollution, cigarette smoking, drugs, illness, stress, and even exercise can increase free radical exposure [6]. Free radical scavenger may prevent the oxidative stress by peroxidation, inhibiting free radicals and also by other mechanism they prevent diseases. In addition to endogenous antioxidant defense systems, consumption of dietary and plant-derived antioxidants appears to be a suitable alternative as the synthetic antioxidants are reported to be harmful for human health. Further, synthetic antioxidants such as butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) have restricted use in food because of their carcinogenic properties [7]. Also, the use of the most potent synthetic food antioxidant tertiary butylated hydroquinone (TBHQ) is not permitted in Japan and Europe [8]. Therefore, the search for effective, nontoxic natural compounds with antioxidantive activity has been intensified in recent years [9]. Medicinal plants contain substantial amounts phytochemicals with capacity to scavenge the free radical and can serve as a potential source of natural antioxidants [10]. The majority of antioxidant belongs to flavonoids, isoflavones, anthocyanins, coumarins, lignans, catechins and isocatechins class of phytochemicals.

The genus Vitex belongs to the family Verbenaceae. It includes 80 genera and about 800 species. Vitex is a genus of trees and shrubs widely distributed in the tropic and warm region. Vitex negundo Linn is a shrub quite prolific in India, occurring up to an altitude of about 1500 m. It is one of the common plants used in traditional medicine and reported to have a number of biological activities [11]. Flavonoids, iridoids, terpenes and steroids are the major classes of compounds isolated from V. negundo [12]. A large number of compounds with varying structural classifications including glucononitol, camphene, α-pinene, citral, β-caryophyllene, casicin, orientin, isoorientin, corymbosin, flavonoid glycosides, diterpenoid, triterpenoids, long chain unsaturated fatty acids, iridoid glycosides and lignin have been reported from the leaves, twigs, seeds and roots of V. negundo [13]. Although all parts of V. negundo are used as medicine in the indigenous system of medicine, the leaves are most potent for medicinal use. Decoction of the leaves of V. negundo is used as a bath in the puerperal state of women in India. The leaf extract of V. negundo has been reported to exhibit a wide range of pharmacological activities. The methanolic extract of V. negundo had been found to exhibit very potent antifeedent, anti-inflammatory, analgesic and anti-convulsant activities [13]. V. negundo is used in several commercial formulations and in the Ayurvedic System of Medicine, leaves of V. negundo is used as a drug of choice for pain, inflammation and related diseases [14, 15]. Vitex trifolia is a shrub or small tree, its leaves contains flavonoids, sterols and terpenoids. The leaves are considered useful as an external application to all rheumatic pain, sprains etc. The petroleum and ethanol extracts of leaves of V. trifolia exhibited moderate inhibiting activity against most of the tested Gram positive and Gram negative bacteria [13, 16]. Significant hepatoprotective activity of aqueous and ethanol extracts of V. trifolia in carbon tetrachloride induced liver damage of male wistar rats was also reported [17].

Terminalia chebula belongs to the family Combretaceae and is found throughout India chiefly in deciduous forests. It occurs abundantly in North India and range extends southwards at 300 to 900 m altitude [18]. Its yellowish brown fruits are included in the Indian pharmacopoeia under the category astringent. It possesses laxative, diuretic, cardio tonic and hypoglycemic properties. The dried fruit of T. chebula is used extensively in the indigenous system of medicine (Ayurvedic) for its homeostatic, antitussive, laxative, diuretic and cardio tonic activities [19]. Gallic acid, ellagic acid, tannic acid, ethyl gallate, chebulic acid, chebulagic acid, corilagin, mannitol, ascorbic acid and other compounds had been reported from T. chebula [20]. Terminalia bellirica plant is also common throughout India in the plains and lower hills, chiefly in the deciduous forests at 900 m elevation where the climate is not very dry [21]. Emblica officinalis is a medium sized tree found cultivated or growing wild in mixed deciduous forests of India ascending up to 1300 m on hills [22]. Triphala, a combination drug is a composite mixture of T. chebula, T. bellerica and E. officinalis and used in popular traditional medicine for the treatment of many chronic diseases such as ageing, heart ailments, hepatic diseases etc. [23, 24]. Fruit of E. officinalis is a rich source of phenolic compounds [25]. A. racemosus is distributed in tropical and subtropical parts of India including the Andamans and ascending in the Himalayas up to an altitude of 1500 m. The root of A. racemosus is the source of the drug ‘shatavar’ and is highly mucilaginous, antidiarrhoeal, refrigerant, diuretic, antisyneretic, nutritive, tonic, and demulcent, galactagogue, aphrodisiac and antispasmodic. The root contained proteins, saponins, carbohydrates, crude fibre, inorganic matter and mucilage. The sapogenin present in the root was identified as sarsapogenin [26, 27].
Medicinal plants have been extremely studied for their antioxidant assay. Selection of the plants in the present study was based on their use in traditional Indian Systems of Medicine (ISM). Extraction solvent significantly alter the antioxidant estimation, therefore, selection of extracting solvent of matching polarity is one of the most important factors in the extraction of antioxidants. Water, methanol and ethanol have been widely used for extraction of antioxidants.

Polar solvents and alcoholic solutions frequently provide satisfactory extraction [28, 29]. However, more than one extractant or sequential multi solvent extraction are preferred in very few cases. Herbs are traditionally ingested as hot water infusion. Recovery of antioxidant phytochemicals from plant materials is controlled by their chemical properties and distribution in plant matrix. Because of the presence of different groups of antioxidants with differing chemical behaviour in plant matrix, extract yield and resulting antioxidant activities are strongly dependent on the nature of extracting solvent as polarity of the solvent used for extraction purposes may or may not be matching. Plants extracts made with water are nutritionally more relevant and also required for clinical usages.

In the present report, antioxidant potential of six important medicinal plants V. negundo, V. trifolia, T. bellerica, T. chebula, E. officinalis and A. racemosus was determined with non-polar (hexane, chloroform), semipolar (ethyl acetate) and polar (methanol, n-butanol and water) solvents. Although, there have been numerous researches on crude extraction and bioactive compounds of potential plants for natural and possibly economic and effective antioxidants to replace the synthetic ones. These sources particularly leaves, bark and roots have not been received much attention as antioxidant sources as compared to the other plants/fruits. The aim of the present research work was to determine the best solvent for extraction of antioxidants from six important Indian medicinal plants and to make a comparative evaluation of the antioxidant potential of the extracts. Plant materials of six medicinal plants were extracted with five solvents hexane, chloroform, ethyl acetate, n-butanol and water using conventional extraction method. Total phenols in the extracts were determined and antioxidant potential of the extracts was assayed using chemical assays. The result would be useful for the optimization of the antioxidant yielding fractions for development of up scaling process from these medicinal plants.

2. Material and Methods

Plant material and chemicals:

Leaves of V. negundo, V. trifolia, barks of T. bellerica, T. chebula, fruits of E. officinalis and roots of A. racemosus were collected from the herbal garden of Directorate of Medicinal and Aromatic Plants Research (DMAPR), Boriavi, Anand, Gujarat, India during the year 2011. Analytical grade solvents hexane, chloroform, ethyl acetate, methanol and n-butanol were purchased from Sigma Research Laboratories (SRL), Mumbai, India. Reagent grade trichloroacetic acid (TCA), ammonium persulphate, gallic acid, Folin-Ciocalteau reagent, sodium carbonate, potassium ferricyanide, ferric chloride, ascorbic acid were also obtained from Sigma Research Laboratories (SRL), Mumbai, India.

Preparation of extract:

Plant materials were divided into small pieces and dried at room temperature under shade. Further, the powder of suitable mesh size of dried plant materials was prepared using an electric grinder and powder samples were used for preparation of the extracts. The powder samples of plant materials were extracted with methanol at room temperature for 24 hrs. This procedure was repeated for five times and the solvent was removed from the combined extract under vaccum. A part of the methanol extract obtained was dissolved in water and sequentially extracted with hexane, chloroform, ethyl acetate and n-butanol to obtain hexane, chloroform, ethyl acetate, n-butanol and aqueous extracts respectively.

Measurement of total phenolics:

Total phenolics in the extracts were quantified using Folin-Ciocalteau reagent and expressed as gallic acid equivalent [30, 31]. Extract (0.5ml) was mixed with Folin-Ciocalteau reagent (0.5ml) and distilled water (7.5 ml). The resulting mixture was incubated at room temperature for 10 minutes. Thereafter, sodium carbonate solution (1.5ml, 20%) was added to the mixture and further incubated in a water bath for 20 min at 40°C. The absorbance of the solution was measured using a UV-visible spectrophotometer at 755 nm. A calibration curve was prepared by plotting concentration vs absorbance of gallic acid as standard. Total phenolics in the extract samples were quantified from the calibration curve and were
expressed as gallic acid equivalent (GAE) per gm of dry extract.

**Antioxidant Activity Assay**

**DPPH (2, 2-diphenyl-1-picryl hydrazyl) radical scavenging assay:**
The antioxidant activity of the plant extracts was determined using the DPPH free radical scavenging method with some modifications [32]. DPPH solution (0.1mM litre-1) was prepared in methanol. Stock solution (1mg ml-1) of extracts and the standard antioxidants (ascorbic acid, BHT, BHQ and Trolox) were also prepared in methanol. Working concentration of the standard antioxidants and extracts were prepared using appropriate dilution. Freshly prepared DPPH solution (1 ml) was added to standard antioxidants and extracts solution (3ml). The mixture was incubated in the dark for 30 min and thereafter, absorbance was recorded at 517 nm against the blank. For control, DPPH solution (1 ml) was mixed with methanol (3 ml) and used for measuring the absorbance value. The decrease in absorbance of DPPH solution on addition of test samples in relation to the control was used to calculate the antioxidant activity in terms of percentage inhibition of DPPH radical. The capability of scavenging DPPH radical (%RSA) was calculated using the following equation as described by Lee et al [33]

\[
\text{Radical scavenging percentage} = \left( \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \right) \times 100
\]

The IC50 values of the standard antioxidants as well as the plant extracts corresponded to concentration inhibiting fifty percent of initial concentration of DPPH and values were obtained from the graph of the percentage inhibition versus the concentration. Obtained IC50 values of the standards and extracts were verified experimentally.

**ABTS radical assay:**
ABTS radical cation decolorization assay was performed as described by Re et al [33]. ABTS cation was generated by the reaction between ABTS (7 mM) and potassium persulfate (2.45 mM) at room temperature in the dark for 12–16 h before use. ABTS solution was diluted with methanol to get a final absorbance of 0.7 ± 0.02 at 734 nm. For measuring the decrease of absorbance of blue-green colour of cation generated, ABTS solution (990 µl) was mixed with test solution (10 µl). The reduction in absorbance was recorded after 6 min and compared with the control.

**Reducing power assay:**
The total reducing power of standard antioxidants and extracts were determined as described by Oyaizu [34]. Different concentrations of extracts were mixed with distilled water (2.5 ml), phosphate buffer (2.5ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5ml, 1%). The resulting mixture was incubated at 50ºC for 20 min in a water bath. After cooling, trichloroacetic acid (2.5ml, 10 %) was added to the mixture. The upper layer of solution (2.5ml) was taken and mixed with distilled water (2.5 ml) and ferric chloride (0.5ml, 0.1%). The absorbance was measured using a UV-visible spectrophotometer at 700 nm. The increasing absorbance value was interpreted as increased reducing activity [35]. The extract concentration providing absorbance value of 0.5 (EC50) was calculated from the graph.

**3. Results and Discussion**

Solvent polarity plays a key role in increasing phenolic solubility [36]. Phenolic compounds such as BHT, propyl gallate etc. are known to be effective antioxidants. Phenolic compounds have been known to act as antioxidants not only because of their ability to donate electrons also because of their stable radical intermediates which can effectively prevent the oxidation at cellular and physiological level [37]. In the present study five solvents with different polarity were used and they can be arranged as following (starting from more non-polar solvent): hexane< chloroform< ethyl acetate< n- butanol< methanol< water. Even though it is unclear whether active compounds are active against free radicals after being absorbed and metabolized by cells in the body, radical scavenging assays have gained acceptance for their capacity to rapidly screen materials of interest [38]. Antioxidant activity of plants is well correlated with the content of their phenolic compounds [39]. The most commonly used antioxidant methods DPPH. And ABTS.+ were used to assess the free radical scavenging activity of the different extracts.

**Extract yield of plant material:**
The extraction yield varied depending on the type of extraction solvent and extract yield of plant materials of V. negundo, V. trifolia, T. bellerica, T. chebula, A. racemousus and E. officinalis has been described in Figure 1. For V. negundo and V. tripholia leaves, extract yield was maximum for aqueous extract and minimum for chloroform extract and it varied in the following order: aqueous> methanol> hexane> ethyl acetate> chloroform. In case of T. chebula and T.
bellerica bark extract, aqueous extract had highest yield. The extract yield for T. chebula and T. bellerica varied in the following order: aqueous > methanol > n-butanol > ethyl acetate > hexane > chloroform. For fruits of E. officinalis, yield of methanol extract was highest followed by aqueous and n-butanol extract. The extract yield varied in the following order: methanol > aqueous > n-butanol > ethyl acetate > hexane > chloroform. For A. racemosus, methanol was used for preparation of the extract.

**Total phenol content (TPC):**

Total phenol content expressed as gallic acid equivalent (mg /g GAE) in different extracts of V. negundo, V. trifolia, T. bellerica, T. chebula, A. racemosus and E. officinalis has been summarized in Table 1. For V. negundo, V. trifolia, T. bellerica, A. racemosus and E. officinalis have been prepared using reference and their IC50 values were found to be 4.2, 4.3, 1.7 and 2.1 µg mL⁻¹ respectively, these values are comparable to their reported IC50 values [42, 43]. Results of DPPH assay of different extracts of V. negundo, V. trifolia, T. bellerica, T. chebula, A. racemosus and E. officinalis have been summarized in Table 1. For V. negundo and V. trifolia, ethyl acetate extract had the lowest IC50 value followed by methanol, aqueous and chloroform extract. IC50 value of ethyl acetate extract of V. negundo (4.2 µg mL⁻¹) was found to be comparable to the IC50 value of ascorbic acid and BHT and comparatively lower than reported value for ethanol extract of V. negundo leaves extract [44]. All the four extracts of T. chebula and T. bellerica exhibited very low IC50 value. For T. chebula, the IC50 varied in the following order: aqueous (1.6 µg mL⁻¹) < methanol (2.0 µg mL⁻¹) < n-butanol (2.4 µg mL⁻¹) < ethyl acetate (2.7 µg mL⁻¹). These values were comparable to the IC50 of standard antioxidants such as trolox and BHA. However, in case of T. bellerica extracts, IC50 values varied in the following order: n-butanol (2.7 µg mL⁻¹) < ethyl acetate (2.8 µg mL⁻¹) < methanol (3.1 µg mL⁻¹) < aqueous (3.5 mL⁻¹). IC50 value of ethyl acetate extract of T. chebula bark was observed to be lower than the IC50 values reported for the methanol extract of the fruits of T. chebula and T. bellerica. Also for E. officinalis, IC50 values comparable to standard antioxidants were recorded for n-butanol (1.5 µg mL⁻¹), methanol (1.6 µg mL⁻¹) and aqueous extracts (4.1 µg mL⁻¹). For A. racemosus extracts very high IC50 values (methanol extract: 614 µg mL⁻¹, n-butanol extract: 348.6 µg mL⁻¹) were in agreement to the values reported by Dohare et al [45].
Table 1: Total phenol content (TPC) and IC50 values of extracts of V. negundo, V. trifolia, T. chebula, T. bellerica, E. officinalis and A. racemosus in DPPH, ABTS and reducing power assay (RPA)

Free radical scavenging activity by stable ABTS radical cation:
In the ABTS assay, standard antioxidants ascorbic acid, BHT, BHQ and trolox had IC50 corresponding to 42.7, 116.5, 64.4 and 47.4 µg mL−1 respectively. Here also, IC50 value of V. negundo, V. trifolia, T. bellerica, T. chebula, A. racemosus and E. officinalis exhibited similar trend as in DPPH assay (Table 1). Ethyl acetate of V. negundo, V. trifolia exhibited lowest IC50 value and hexane extract exhibited the maximum IC50 value. In ABTS assay for IC50 value of five V. negundo leaves extract, the following order of scavenging activity in terms of IC50 value was reported by Tiwari et al [46]: total ethanolic>methanolic>water>ethyl acetate>hexane extract. For T. chebula, aqueous extract had lowest IC50 value and it varied in the following order: aqueous (29.2 µg mL−1) < methanol (34.5 µg mL−1) < n-butanol (48.5 µg mL−1) < ethyl acetate (56.5 µg mL−1). Also for T. bellerica, the IC50 values were found in the following order: n-butanol (38.8 µg mL−1) < ethyl acetate (40.8 µg mL−1) < methanol (42.9 µg mL−1)
ml-1)< aqueous (52.6 µg mL-1). Although, IC50 concentration of T. bellerica extract had higher values than the corresponding values of T. chebula extracts but the trend was found to be similar as observed in DPPH assay. Also the IC50 values of T. chebula and T. bellerica extracts were comparable to the four standard antioxidants. For E. officinalis, ethyl acetate (29.8 µg mL-1) and n-butanol (39.6 µg mL-1) extracts had IC50 lower than the corresponding values for all the four standard antioxidants. As observed in DPPH assay, here also, IC50 values of A. racemosus extracts were found to be very high.

Reducing power assay:
The reducing power of an extract may serve as a significant indicator of its potential as an antioxidant and reducing power activity was calculated in terms of EC50 concentration. Ascorbic acid, BHT, BHQ and trolox had EC50 concentration 35.5, 59.8, 31.3 and 65.9 µg mL-1 respectively. For V. negundo, EC50 varied in the following order: ethyl acetate (40.1 µg mL-1) < methanol (161.5 µg mL-1) < aqueous (193 µg mL-1) < chloroform (561.7 µg mL-1). Ethyl acetate extract of V. trifolia (215 µg mL-1) had the lowest EC50 concentration followed by methanol (381.7 µg mL-1), chloroform (477 µg mL-1), aqueous (549.5 µg mL-1) and hexane (605 µg mL-1). For T. chebula, aqueous extract had lowest EC50 value and it varied in the following order aqueous (27.1 µg mL-1) < methanol (37.9 µg mL-1) < n-butanol (44.9 µg mL-1) < ethyl acetate (49.4 µg mL-1). Also for T. bellerica, the EC50 values were found in the following order: n-butanol (43.1 µg mL-1) < ethyl acetate (53.0 µg mL-1) < methanol (64.2 µg mL-1) < aqueous (66.7 µg mL-1). For E. officinalis, n-butanol (33.9 µg mL-1) had the lowest EC50 concentration followed by ethyl acetate (35.5 µg mL-1), methanol (52.7 µg mL-1), aqueous (71.7 µg mL-1), chloroform (101.3 µg mL-1) and hexane (113.6 µg mL-1). For A. racemosus, here also, EC50 concentration was found to be very high.

Correlation between antioxidant activity and phenolic content:
Different phenolic compounds may show different antioxidant activities depending on their structure as well as synergistic or antagonistic effect of other compounds present in the extract [47]. Correlation analysis were performed to determine the relationship between the TPC of the extracts and corresponding 1/IC50 values obtained in DPPH, ABTS and reducing power assay (Figure 2). Significant positive correlation between TPC and 1/IC50 values of DPPH, ABTS and reducing power assay was established with estimated coefficient of

![Figure 1: Extract yield of different fractions of V. negundo, V. trifolia, T. chebula, T. bellerica and E. officinalis](image-url)
**Figure 1 (A):** Correlation between TPC and IC₅₀ value of extracts in DPPH assay

**Figure 2 (B):** Correlation between TPC and IC₅₀ value of extracts in ABTS assay

**Figure 2 (C):** Correlation between TPC and IC₅₀ value of extracts in reducing power assay
determination R2 values 0.72, 0.91 and 0.79 respectively. The high correlation between TPC and antioxidant activity suggested that the phenolic compounds contributed significantly to the antioxidant capacity of studied medicinal plants.

Conclusions
Extraction yield varied significantly, depending on the extraction solvent and plant material used. Also TPC and free radical scavenging activity varied depending on the polarity of the solvent used. The results of the present study indicated the presence of compounds in the extracts possessing low (A. racemosus), moderate (V. negundo and T. trifolia) and high (T. chebula, T. bellerica and E. officinalis) antioxidant activity. Also the lowest value IC50 values of extracts of T. chebula, T. bellerica and E. officinalis were below the maximum permissible level (200 ppm) for the synthetic antioxidants [8].

Conflict of Interest: None

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4. References


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