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IN VITRO ANTIOXIDANT ACTIVITY OF *CINNAMOMUM ZEYLANICUM* LINN BARK

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ABSTRACT

There has been growing interest in the health benefits associated with natural compounds and have been demonstrated with the emphasis on antioxidants. Phenolics in fruits, vegetables, herbs and spices possess potent antioxidant, anti-inflammatory, antimutagenic and anticarcinogenic activities. The present study focused on the antioxidant activity of *Cinnamomum zeylanicum* Linn bark under *in vitro* conditions. The dried bark of *Cinnamomum zeylanicum* was extracted with water, methanol and chloroform using a Soxhlet extractor. The total phenolics content of bark as determined by Folin–Ciocalteu method and was found to be 253.33±23.09 mg/g of pyrocatechol equivalents. The extracts were tested for their radical scavenging ability against a battery of radicals such as DPPH, ABTS, H_2O_2, Superoxide anion ($O_2^{\cdot-}$), Nitric oxide (NO), Hydroxyl radical ($HO^{\cdot}$) and Ferric reducing ability. Among the three extracts used methanolic extract showed the most scavenging activity of free radicals followed by chloroform and aqueous extract. The results suggested that *Cinnamomum zeylanicum* Linn bark could serve as a potential source of antioxidant and can be used in any preparations for combating free radical mediated damage to the body.

INTRODUCTION

Oxygen is essential for survival of all on this earth. Oxygen uptake inherent to cell metabolism produces free radicals and reactive oxygen species (ROS) continuously via normal physiological processes, more so in pathological conditions. In the last decades oxidation mechanisms and free radical role in living systems have gained increased attention[1]. Reactive oxygen intermediates (ROIs) are partially reduced forms of atmospheric oxygen (O_2). They typically result from the excitation of O_2 to form singlet oxygen or from the transfer of one, two or three electrons to form a superoxide radical, ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) or a hydroxy radical ($HO^{\cdot}$), respectively. They are simultaneously degraded to nonreactive forms by enzymatic and non-enzymatic antioxidant defense mechanisms. When generation of ROS overtakes the antioxidant defense of the cell, the free radicals start attacking the cell proteins, lipids and DNA resulting in so-called oxidative stress[2].

Antioxidants protect us from diseases like cancer, Alzheimer's disease, diabetes, liver damage, atherosclerosis, inflammation, cardiovascular disorders and aging[3]. Antioxidants are substances that neutralize the harmful free radicals in our bodies. Antioxidants act as ''free radical scavengers'' and hence prevent or slow the damage done by these free radicals. Their function is as a reducing agent, which ultimately removes free radical intermediates and prevents further oxidation by being oxidized themselves. Fruits and vegetables are known as good sources of antioxidants, such as retinol (Vitamin A), ascorbic acid (Vitamin C), a-tocopherol (Vitamin E), carotenoids, flavonoids, tannins, and other phenolic compounds[4].

Herbs are widely exploited in the traditional medicine and their curative potentials are well documented. The demand for plant-based medicines, health products, pharmaceuticals, food supplement and cosmetics are increasing in both developing and developed countries, due to the growing recognition that the natural products are non-toxic, have lesser side effects and are easily available at affordable prices[5]. Many studies show that daily consumption of fruits and vegetables is associated with reduced risks for degenerative diseases like cancer and cardiovascular diseases. Some antioxidants can be synthesized within the body, but most are obtained through diet or supplements[6].

Cinnamomum zeylanicum is a small, tropical, evergreen tree belongs to the family, Lauraceae most noted for its bark, which provides the world with the commonly known spice, cinnamon. In addition to culinary uses, cinnamon has also been used to treat gastrointestinal complaints and other ailments[7,8]. Cinnamon possesses notable anti-allergenic, anti-inflammatory, anti-ulcerogenic, anti-pyretic, and anaesthetic activities[9]. Some evidence suggests that cinnamon may be effective in the supportive treatment of cancer[10], infectious diseases, and complaints associated with modern lifestyle due to its antioxidant[11], anti-microbial and anti-inflammatory effects[12].

Barks of *Cinnamomum* plants are used as spice and herbal medicine and *Cinnamomum zeylanicum* bark contains dimeric, trimeric, and higher oligomeric proanthocyandins with doubly linked bis-flavan-3-ol units in the molecule. This class of proanthocyanidins is known to occur widely in common foods as well as the singly linked proanthocyanidins[13]. In the present study, we have evaluated the antioxidant effect of various extracts obtained from cinnamon bark under *invitro* conditions against a battery of free radicals.

MATERIALS AND METHODS

All solvents and chemicals used were of analytical grade. The bark was purchased from the local market, dried and powdered. The species was identified and a voucher specimen was deposited at the Rapinet Herbarium (BV001) at St.Joseph's College, Tiruchirappalli.

ESTIMATION OF TOTAL PHENOLS

The concentration of phenolics in the bark was determined by using Folin-Ciocalteau reagent according to the method proposed by Mallick and Singh[14] and the results were expressed as mg/g of pyrocatechol equivalents.

The bark (0.5g) was homogenized in 10X volume of 80% ethanol and was centrifuged at 10,000rpm for 20 minutes. The extraction was repeated with 80% ethanol. The supernatants were pooled and evaporated to dryness. The residue was then dissolved in a known volume of distilled water. Different aliquots 0.1 to 0.5ml were pipette out and the volume in each tube was made up to 3.0ml with distilled water. To all the tubes, 0.5 ml of Folin-Ciocalteau reagent (1N) was added and mixed. After 3 minutes, 2.0ml of 20% sodium carbonate solution was added to each tube. After mixing the tubes thoroughly, all

the tubes were kept in a boiling water bath for exactly 1 minute, and allowed to cool. The absorbance was measured at 650 nm in a spectrophotometer against a reagent blank. Standard pyrocatechol solutions (0.2-1.4ml) corresponding to 20-140µg concentrations were also treated as above. The concentration of phenols is expressed as mg/g of bark.

PREPARATION OF PLANT EXTRACTS

Fresh bark of *Cinnamomum zeylanicum* was collected and 10g of them was homogenized in 100ml of the solvents of varying polarity such as water, methanol and chloroform. The organic extracts were dried at 60°C protected from light. The residue was weighed and dissolved in dimethyl sulfoxide (DMSO) to obtain the desired concentration. Aqueous extracts were prepared fresh.

***IN VITRO* ANTIOXIDANT STUDY**

Aqueous, methanolic and chloroform extracts of *Cinnamomum zeylanicum* bark were tested for their free radical scavenging property using various *in vitro* models. All experiments were performed thrice and the results were averaged.

DPPH radical scavenging assay

The scavenging ability of the natural antioxidants of the bark towards the stable free radical DPPH was measured by the method of Mensor *et al*..[15] In brief, the bark extracts (20µl) were added to 0.5ml of methanolic solution of DPPH and 0.48ml of methanol. The mixture was allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH in methanol, without the bark extracts, served as the positive control. After 30 minutes of incubation, the discolouration of the purple colour was measured at 518nm in a spectrophotometer. The radical scavenging activity was calculated as follows:

$$\% \text{ scavenged DPPH} = \frac{Ao - A1 \times 100}{Ao}$$

where, Ao - Absorbance of control, A1 - Absorbance in the presence of bark extract

ABTS radical scavenging activity

The antioxidant effect of the bark extracts were studied using ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation decolourisation assay according to the method of Shirwaikar *et al.*,[16] ABTS radical cations (ABTS+) were produced by

reacting ABTS solution (7mM) with 2.45mM ammonium persulphate. The mixture was allowed to stand in the dark at room temperature for 12-16 hours before use. Aliquots (0.5ml) of the three different extracts were added to 0.3ml of ABTS solution and the final volume was made up to 1ml with ethanol. The absorbance was read at 745nm in a spectrophotometer and the per cent inhibition was calculated using the formula

$$\text{Inhibition (\%)} = \frac{(\text{Control} - \text{test}) \times 100}{\text{Control}}$$

Hydrogen peroxide scavenging activity

The scavenging activity of hydrogen peroxide by the plant extracts was determined by the method of Ruch *et al*.,[17] A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Bark extracts at the concentration of 10mg/10µl were added to 0.6ml of H_2O_2 solution. The total volume was made up to 3ml with phosphate buffer. H_2O_2 in phosphate buffer without plant extract served as control. The absorbance of the reaction mixture was recorded at 230nm in a spectrophotometer. The blank solution contained phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging by the plant extracts was calculated as

$$\text{\% scavenged hydrogen peroxide} = \frac{\text{Ao- A1 x 100}}{\text{Ao}}$$

where, Ao - Absorbance of control, A1 - Absorbance in the presence of bark extract

Superoxide scavenging activity

The superoxide scavenging ability of the extracts was assessed by the method of Winterbourn *et al.*[18] Superoxide anions were generated in samples that contained in 3.0ml, 0.02ml of the bark extracts (20mg), 0.2ml of EDTA, 0.1ml of NBT, 0.05ml of riboflavin and 2.63ml of phosphate buffer. The control tubes were also set up where DMSO was added instead of the plant extracts. All the tubes were vortexed and the initial optical density was measured at 560nm in a spectrophotometer. The tubes were illuminated using a fluorescent lamp for 30 minutes. The absorbance was measured again at 560nm in a spectrophotometer. The difference in absorbance before and after illumination was indicative of superoxide anion scavenging activity.

Nitric oxide scavenging activity

The extent of inhibition of nitric oxide radical generation *in vitro* was followed as per the method reported by Green *et al.,*[19] The reaction was initiated by adding 2.0ml of sodium nitroprusside, 0.5ml of PBS, 0.5ml of bark extracts (50mg) and incubated at 25°C for 30 minutes. Griess reagent (0.5ml) was added and incubated for another 30 minutes. Control tubes were prepared without the extracts. The absorbance was read at 546nm against the reagent blank, in a spectrophotometer.

Hydroxyl radical scavenging activity

The DNA damage induced *in vitro* by hydroxyl radicals generated by hydrogen peroxide in the presence and the absence of bark extracts was quantified by the production of TBARS (thiobarbituric acid reactive substances) spectrophotometrically as per the procedure given by Elizabeth and Rao[20]. The reaction mixture contained in a final volume of 0.98ml, 28mM deoxy ribose, 0.1mM FeCl3, 0.1mM EDTA, 1mM H_2O_2, 0.1mM ascorbate and 20mM buffer. 20µl of each of bark extract was added such that the final volume was 1ml. The reaction mixture was then incubated for one hour at 37°C. After the incubation, 0.5 ml of TBA and 0.5 ml of HCl were added and heated in a boiling water bath for 20 minutes. It was then allowed to cool and the absorbance was measured at 532 nm in a spectrophotometer. The per cent TBARS produced for positive control (H_2O_2) was fixed as 100% and the relative per cent TBARS was calculated for the plant extract treated groups.

FRAP assay

Ferric Reducing Antioxidant Power (FRAP) assay is quick and simple to perform, and the reaction is reproducible and linearly related to the molar concentration of the antioxidants present. Hence an attempt was made to analyze the ferric reducing potential of the extracts and standard at different concentrations. The reductive potential of the extract was determined according to the method of Oyaizu [21]. The presence of reducers (i.e., antioxidants) causes the reduction of the Fe^{3+} ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+} concentration. The different concentrations of extracts and standard (125, 250, 500, 750 and l000 µg) in 1 ml of distilled water was mixed with 2.5 ml of phosphate

buffer and 2.5 ml of potassium ferricyanide. The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid was added to the mixture, which was then centrifuged for 10 min at 1000g. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml), and the absorbance was measured at 700 nm in a spectrophotometer. Higher absorbance of the reaction mixture indicated greater reductive potential.

Statistical analysis

The experimental results are expressed as means ± SD of three parallel measurements. The results were processed using Microsoft Excel 2007.

RESULTS

The extracts were tested for their radical scavenging ability against a battery of radicals such as DPPH, ABTS, H_2O_2, $O_2^{\cdot-}$, NO, HO^- and Ferric reducing ability. All the three extracts of *Cinnamomum zeylanicum* bark showed considerable antioxidant activity in all the *in vitro* free radical scavenging models studied.

The ability of the bark extracts to scavenge DPPH, ABTS and H_2O_2 was quantified by a spectrophotometric assay. The extent of DPPH, ABTS and H_2O_2 scavenging by the three different extracts of *Cinnamomum zeylanicum* is presented in Figure I. The maximum scavenging effect was elicited by the methanolic extract, followed by chloroform and aqueous extract. The different extracts were screened for the extent of inhibition of *in vitro* generation of superoxide and nitric oxide. The results are presented in Figure II. The methanolic extract had maximum inhibition of superoxide and nitric oxide free radical formation followed by the inhibiting ability of chloroform and aqueous extracts. Following this, the effect of *Cinnamomum zeylanicum* bark extracts on H_2O_2 induced damage to deoxyribose was quantified as the amount of TBARS formed and the results obtained are depicted in Figure III. H_2O_2 exposure resulted in a steep increase in the extent of damage.

The effect of methanolic extract of *Cinnamomum zeylanicum* was found to be more in scavenging hydroxyl radicals, followed by the chloroform extract.

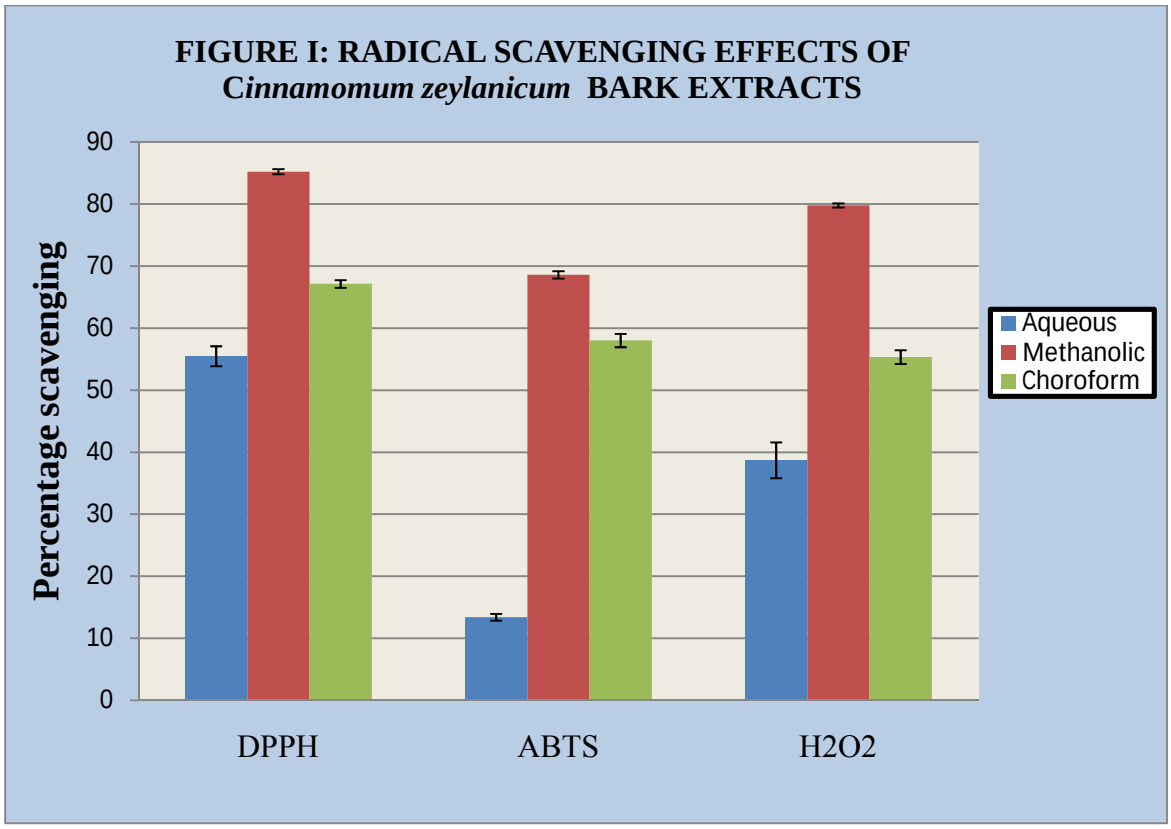


FIGURE I: RADICAL SCAVENGING EFFECTS OF
Cinnamomum zeylanicum BARK EXTRACTS
Percentage scavenging
90
80
70
60
50
40
30
20
10
0
DPPH
ABTS
H2O2
Aqueous
Methanolic
Choroform

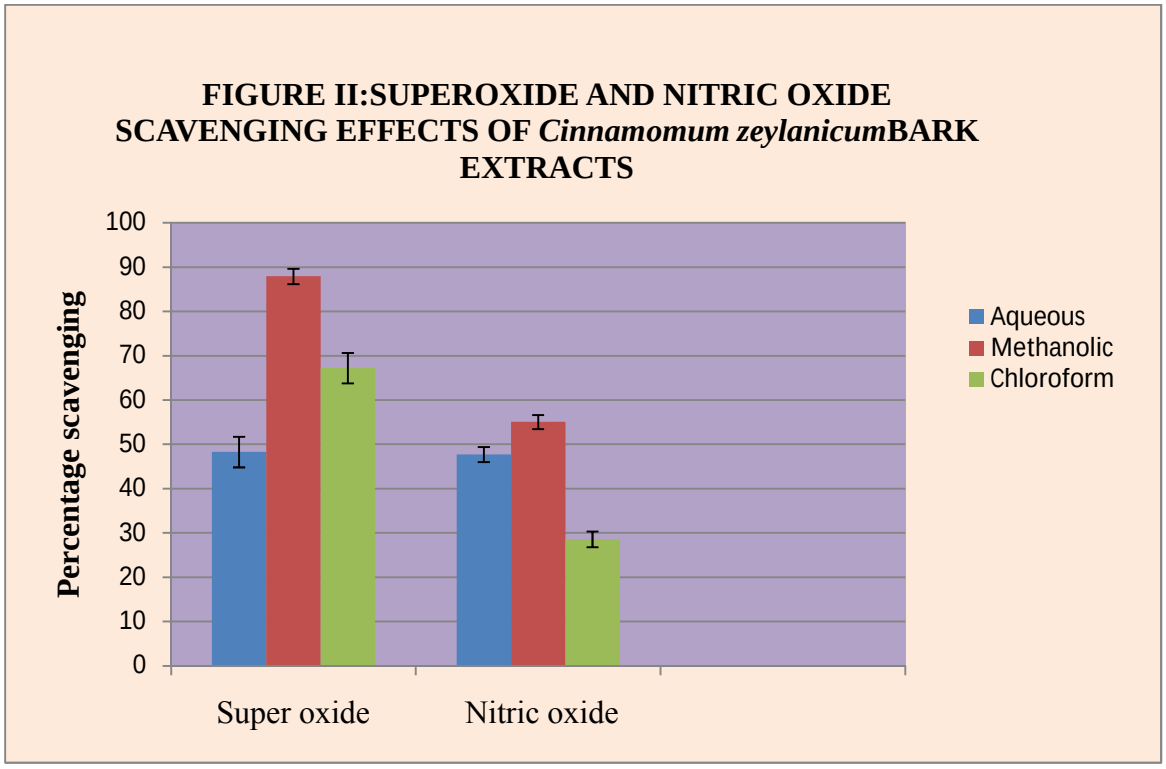
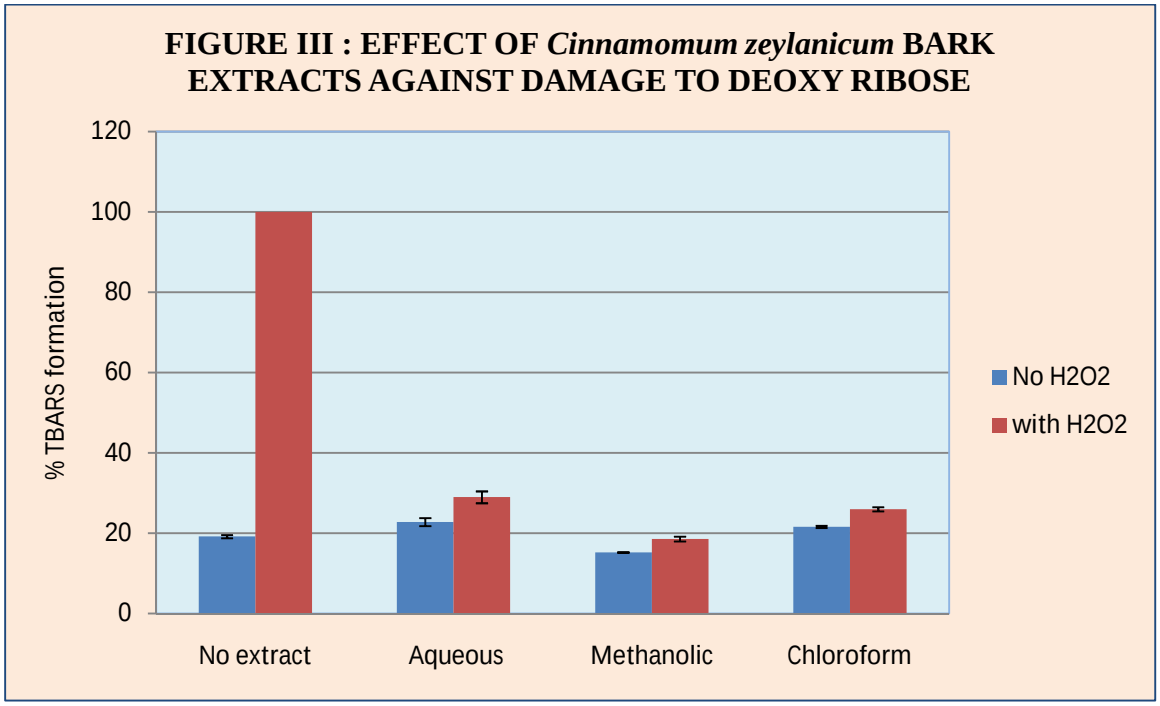


FIGURE II:SUPEROXIDE AND NITRIC OXIDE
SCAVENGING EFFECTS OF Cinnamomum zeylanicumBARK
EXTRACTS
Percentage scavenging
100
90
80
70
60
50
40
30
20
10
0
Super oxide
Nitric oxide
Aqueous
Methanolic
Chloroform

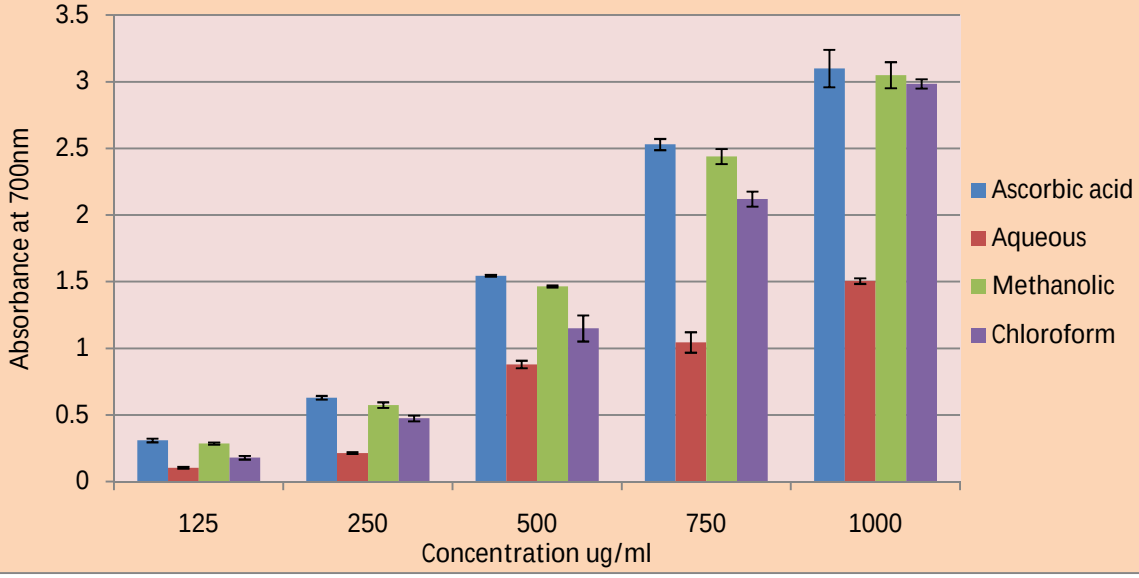
Methanolic extract showed maximum inhibition of all the free radicals tested than other two extracts. Moreover, the DPPH, ABTS, H_2O_2 and superoxide scavenging potential of methanolic extract was found to be more (85%, 68%, 79%, and 87% respectively) than for NO scavenging (55%) potential. Considerable ability of the bark extracts to scavenge hydroxyl radical was noticed and expressed in terms of percentage of TBARS formation. The percentage of TBARS formation in the presence of methanolic extract (18.6%) was lesser than in the presence of other two extracts.

FIGURE III : EFFECT OF *Cinnamomum zeylanicum* BARK EXTRACTS AGAINST DAMAGE TO DEOXY RIBOSE



The reducing power of *Cinnamomum zeylanicum* bark extracts and the reference compound, ascorbic acid increased steadily with increasing concentration (Fig. IV). The reducing powers (absorbance at 700 nm) of *Cinnamomum zeylanicum* bark extracts were 1.5±0.021, 3.05±0.09, 2.98±0.035 respectively for aqueous, methanol and chloroform and 3.1±0.014 for ascorbic acid standard at a dose of 1 mg showing that bark extracts can act as electron donors and can react with free radicals to convert them to more stable products and thereby terminate radical chain reactions. All the three extracts showed lower reducing power when compared to the standard ascorbic acid. Among the three extracts, methanolic extract showed maximum reducing power than aqueous and chloroform extracts. These findings indicate that the three kinds of extracts contained some quantities of compounds with high reducing power.

FIGURE IV: REDUCING POWER OF *Cinnamomum zeylanicum* BARK EXTRACTS AND ASCORBIC ACID



DISCUSSION

Many synthetic drugs protect against oxidative damage, but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplements and traditional medicines. Hence tremendous input is made worldwide, in search of the antioxidant rich herbal plants to combat diseases.

Antioxidant effects of essential oils from rosemary (*Rosmarinus officinalis),* clove (*Syzygium aromaticum*) and cinnamon (*Cinnamomum zeylanicum*) were determined on hazelnut and poppy oils. The antioxidant activity of the essential oils was determined by measuring peroxide values (meq O_2/kg oil) at regular intervals. Amongst the investigated essential oils, the cinnamon oil was the most effective on retarding lipid oxidation of crude oils, which was followed by clove and rosemary oils[22].

Total phenolic compounds and antioxidant potential of *Hedychium spicatum* were analyzed for 16 different natural populations located in Uttarakhand (west Himalaya) for promotion as health or medicinal food. All the free radical scavenging assays (ABTS, DPPH and FRAP) showed significant ($p < 0.05$) correlation with total phenolic compounds. Total phenolic compounds showed a significant relationship ($p < 0.05$) to altitude[23]. The effect of cinnamate, a phenolic compound found in cinnamon bark and

other plant materials, on lipid metabolism and antioxidant enzyme activities in rats fed a high cholesterol diet has been studied and indicated that cinnamon suppresses lipid peroxidation via the enhancement of hepatic antioxidant enzyme activities [24].

The activities of 23 selected essential oils from vegetable materials, in inhibiting the copper-catalyzed oxidation of human-low-density lipoproteins (LDL) were determined *in vitro*. Total phenol content of essential oils gave a correlation with LDL antioxidant activity of $r = 0.75$. The activity of each phenolics compound could play a role in protecting LDL against oxidation if the substance is absorbed by the body[25].

With support of above studies, ability of *Cinnamomum zeylanicum* extracts in effectively scavenging hazardous free radicals reveals the strong antioxidant potential of the bark.

CONCLUSION

In the present study, the phenolic content of *Cinnamomum zeylanicum* bark was found to be high which might have responsible for its antioxidant and free radical scavenging activity in the *in vitro* study models. Thus our results were congruent with the findings of others. Further studies can be designed to prove the antioxidant activity of *Cinnamomum zeylanicum* bark in experimental animal models and also an attempt can be made to analyze the phenolic antioxidants present in it.

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