IN-VITRO ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF *CINNAMOMUM VERUM* (CINNAMON) AQUEOUS BARK EXTRACT IN REFERENCE TO ITS TOTAL PHENOL CONTENT AS NATURAL PRESERVATIVE TO FOOD

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ABSTRACT

The study was aimed at evaluating the antibacterial and antioxidant activity of aqueous extract of *Cinnamomum verum* (cinnamon) bark in order to prevent the spoilage and rancidity of food. The extract was tested for antibacterial activity against three gram positive (*Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecali*) and two gram negative (*Escherichia coli*, *Proteus mirabilis*) food borne pathogens. The extract proved to be broad spectrum antibacterial agent. Though the yield of total water soluble solid was less, there was remarkable reducing power, radical scavenging activity and metal chelation by the extract. Interestingly the extract had powerful protection to DNA against the dangerous hydroxyl radical which causes several kinds of damage to cell organelles. Total phenol content of the extract had significant positive correlation (P<0.05) with the antibacterial and antioxidant activity. Based on the results it can be concluded that besides the use of cinnamon as culinary spice in food, it can be alternately used as natural food preservative for its potent antibacterial and antioxidant activity.

Key words: Aqueous extract, Antibacterial activity, Antioxidant activity, DNA damage protection activity, phenolic content.

INTRODUCTION

The chemical contents of food increase due to indiscriminate use of technology and technological products. This is more prominent in case of foods due to higher use of fertilizers, pesticides, herbicides in pre-harvest period and due to chemicals and synthetic preservatives during storage of raw material, processing and packaging in post-harvest period. Even the FDA approved Aspartame (Fisher, 1989) and Mono sodium glutamate (Tadokoro *et al.*, 1974; Takasaki *et al.*, 1979) are known to have deleterious effect on health of individuals**.** The use of natural fertilizers, pesticides and food preservatives are the solution for avoiding accumulation of chemicals in food. In this direction an attempt was made to replace chemical preservatives using the traditional food ingredients having antimicrobial and antioxidant properties to preserve the food.

Spice and herbs not only are used to add aroma, taste and flavour to food, but also are used in folk medicines. Spices can increase the shelf life of the food because they inhibit growth of many microorganism through bacteriostatic or bactericidal activity and can prevent rancidity of fat through potent antioxidant activity (Snyder, 1997; Paster, 1995; Shelef, 1983; Nevas *et al*, 2004; Kim *et al.,* 1994).

Cinnamon (*Cinnamomum verum*) a common spice belonging to family Lauraceae is grown in India, Sri Lanka and Mexico. This plant is used in traditional and ayurvedic medicines. It is mostly used as a treatment for diarrohea, stomach upset, respiratory problems and as skin antiseptic (Aguilar, 1999; González, 1998; Linares *et al*., 1994, Rakshit and Ramalingam, 2010a).

The active principle compounds behind this are Volatile oils (cinnamaldehyde, eugenol, cinnamic acid, weitherhin), Mucilage, Diterpenes and Proanthocyanidins. Thus in this present study antibacterial and antioxidant activity Cinnamon was evaluated with reference to total phenolics, because there is no report on cinnamon's antibacterial and antioxidant with relation to its total phenolic content.

The objectives of the work was: To evaluate the in-vitro antibacterial activity; to find out the relationship between bacterial inhibition and total phenolic content to confirm whether bacterial inhibition was due to total phenolics; to find out the antioxidant activity and finding its relationship with total phenolic content.

MATERIALS AND METHODS

Plant material:
Cinnamon bark was purchased from local market of Vellore, Tamil Nadu, India.

Bacterial culture:

Gram positive bacteria *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis* and Gram negative bacteria *Escherichia coli*, *Proteus mirabilis* were obtained from American type culture collection, USA.

Chemicals:

Nutrient broth and Nutrient Agar were purchased from HI Media Laboratories India, Ltd. Folin–Ciocalteu reagent, gallic acid, Ferrozine and 1, 1-diphenyl-2- picrylhydrazyl (DPPH) were purchased from Sigma Chemical Company (St. Louis, MO). All reagents were of analytical grade and met the basic requirements of standard analyses.

Extraction:

Clean surface sterilised cinnamon was taken. Forty grams of it were chopped and homogenised in 200 mL of sterile distilled water in mixer. The homogenate was first filtered in mesh cloth and then by 0.25 µm pore size filter (Pall Life sciences, Bangalore). The crude extract (mg.mL^{-1}) was collected in sterile vial and stored at 4 °C until used.

Determination of concentration of water soluble solid:

Five gram of cinnamon was ground in 25 mL of distilled water. The filtrate was kept for evaporation at 105 °C for 4 hours at hot air oven. The weight of the dried total soluble solid was measured after the filtrate was dry. Total soluble solid was found to be 0.0246 g.g^{-1} of cinnamon.

Antimicrobial activity by Well diffusion method:

The *in-vitro* antibacterial activity of the aqueous extract of Cinnamon (AEC) was carried out by well diffusion method (Rakshit and Ramalingam, 2010b).Sterile Nutrient agar plates were swabbed with actively growing log phase culture of respective organism. Wells around 6 mm were bored on the agar and extract 0.2 mg/mL to 0.73mg/mL were added respectively. The plates were incubated upright for twenty four hours at 37 ° C and the diameter of zones of inhibition(DZOI) was measured. Gentamicin (10 mcg) was used as positive control.

Minimum Inhibitory concentration (MIC):

MIC of the extract against each of the tested organism was determined by macro broth dilution method. Extract solutions at concentrations of 0 to 0.488 mg/mL by serial two fold dilution was prepared in peptone saline water. The tubes were inoculated with five test organism and incubated for 24 hours. The O.D value was recorded at 600 nm. The concentration at which there is no visible growth of bacteria was the MIC.

Activity Index:

The Activity Index (AI) was calculated as compared with those of their respective standard reference drugs (AI = Inhibition Zone of test sample/Inhibition zone of standard). Gentamicin (10 mcg) was used as standard drug against five bacterial cultures.

Antioxidant activity:
Reducing power

The reducing power of the extracts was evaluated by the method of Oyaizu (1986). Various concentration of the extract (0.2 mL) were mixed with 1 .0 mL of 200 mM sodium phosphate buffer (pH 6.6) and 1 mL of 1 % potassium ferricyanide. It was incubated at 50 °C for 30 min. 1 mL of 10 % trichloroacetic acid (w/v) was added and centrifuged at 4000 rpm or 10 min. 2 mL of the upper layer was mixed with 2 mL of deionised water and 0.4 mL of 0.1 % of ferric chloride. The absorbance was noted at 700 nm. The values were presented as the means of triplicate analyses. The extract concentration providing 0.5 of absorbance was reported as EC$_{50}$. Ascorbic acid was used as standard.

Metal chealtion assay:
Metal Chelating Assay:

Ferrous ions chelating activity was determined based on modified method of Dinis et al., 1994. Into tubes containing 1840 µL and 1940 µL respectively 160 µL and 40 µL of cinnamon extract and disodium EDTA was added.50 µL (0.2 mM) Ferrous chloride and 200 µL of (5 mM) Ferrozine was added to all tubes. It was incubated for 10 minutes and the final colour formed was monitored at 562nm.The chelating efficiency was compared with the

chelating activity of disodium ethylene diamine tetra acetic acid (disodium EDTA). The inhibition percentage of ferrozine –Fe^{2+} complex was calculated by the formula

%inhibition of ferrozine = [(Absorbance of control - Absorbance of Extract)/Absorbance of control]*100

Free radical scavenging activity

DPPH radical scavenging was performed based on the method of Yen and Chen (1995) with some modification. Various concentration of AEC (1 mL) were added to 4 ml of 70 % ethanol solution containing DPPH radicals (40 µg.mL^{-1}). The mixture was shaken vigorously and let to stand for 15 min at dark. The reduction of the DPPH was determined by reading the absorbance at 517nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration as:

% RSA= [(Absorbance of control-Absorbance of extract)/Absorbance of control]×100

Where control is the solution where no antioxidant has been added. The extract concentration providing 50 % of free radical scavenging activity (EC$_{50}$) was calculated from the graph of RSA percentage against extract concentration. Ascorbic acid was used as control.

Total phenol content

The extracts were diluted with the same solvent used for extraction, to a suitable concentration for analysis. Total phenolic content of extracts was assessed approximately by using the Folin–Ciocalteau phenol reagent method (Singleton *et al.,* 1999). To 200 ml of the sample extracts were added 1.0 ml of Folin–Ciocalteau reagent and 0.8 ml of sodium carbonate (7.5% w/v), and the contents were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured in a UV–Vis Spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (GAE) in µg.mL^{-1} of sample, using a standard curve generated with gallic acid.

DNA damage protective activity

DNA damage protective activities of the extract was determined by the method of Russo *et al*. (2001) with some modification. The experiment was performed with 1 µL of pBR 322 DNA and 24 µg o the plant extract. Prior to irradiation with UV , 6 % was added. Due to exposure of UV light , H_2O_2 is photolysed to hydroxyl radicals which damage the DNA.The tubes were then placed directly on the surface of a UV trans –illuminator at 300 nm and irradiated for 15 min. All DNAs were run on 1 % agarose gel .Plasmid DNA with and without H_2O_2 and UV irradiation was also included as negative and positive control groups.

Statistical analysis

The experimental results are expressed as means ± SD of three parallel measurements. The results were processed using Microsoft Excel 2007 and the data were subjected to one way analysis of variance (ANOVA). Significant differences between groups were determined at P<0.05. To evaluate relationships between experimental parameters, results were analysed for correlation and regression, and tested for significance by student's t test (P <0.05).

RESULTS AND DISCUSSION

Antibacterial activity

Three of the bacteria used were Gram positive (*S.aureus*, *B. cereus*, *E. faecalis*) and two were gram negative (*E.coli* and *P.mirabilis*). There was no significant variation in antibacterial activity between the bacterial cultures. For *S. aureus*, the DZOI values were between 0 cm to 1.6 cm. At 0.2 mg.mL^{-1} concentration of AEC, *S.aureus* had no inhibitiory activity. Similarly for *E.coli* there was no inhibitory activity at 0.2 mg.mL^{-1} concentration. The maximum DZOI observed was against *B.cereus* and *E.faecalis*. The diameter of zone of inhibition (DZOI) was dose dependent on concentration of AEC. In general, the order of sensitiveness of AEC extract against the bacterial cultures are *B.cereus>P.mirabilis>E.faecalis> E.coli> S.aureus* (Table 1).

The Minimum Inhibitory Concentration (MIC) of AEC for different cultures were found out by broth dilution method and it was within 0.1233 mg.mL^{-1} to 0.24 mg.mL^{-1}. *B.cereus* and *E.coli* had MIC at 0.6165 mg.mL^{-1} concentration and MIC for *S.aureus* was at 0.2466 mg.mL^{-1}. Thus the sensitivity of gram negative bacteria was more than gram positive bacteria (Table 2).

The activity index calculated was highest for *E.coli* and *P.mirabilis*. High activity index was observed for all four organisms ranging from 0.80, 0.71 and 0.38. Comparing the five bacterial cultures, less activity index was

observed against *S.aureus* (0.38). Comparing the five bacterial cultures, less activity index was observed against *S. aureus* (0.38) (Table 3).
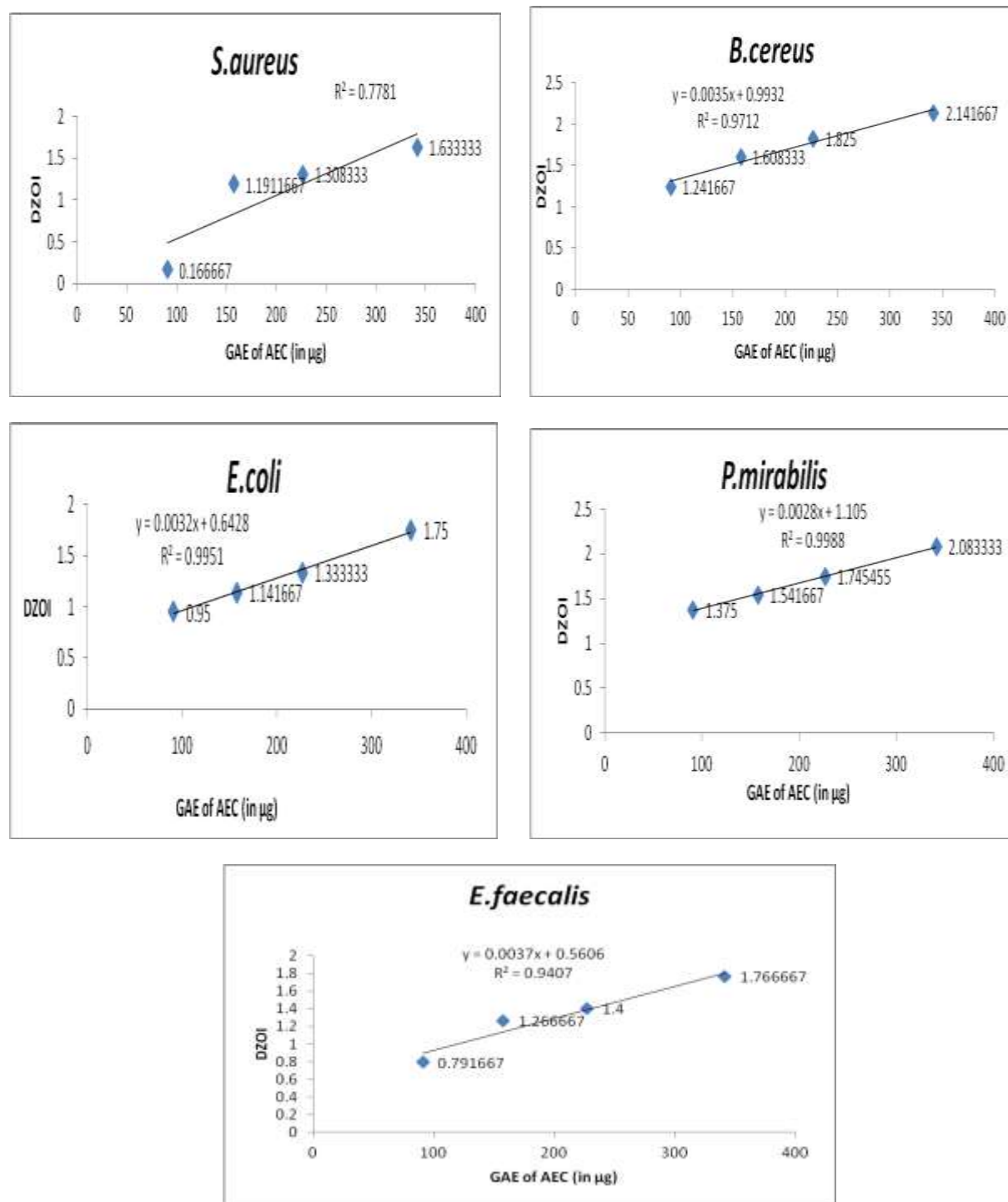

Fig.1. Relationship between diameter of inhibition zone (DZOI) and total phenolics of AEC extract for five foodborne pathogens (n=12).

Number of studies has been conducted on cinnamon oil and its application as antibacterial and antifungal agent. The main component of Cinnamon oil is eugenol (Lopez *et al*., 2007; Matan *et al*., 2006; Valverde *et al*., 2005). Eugenol has been proved to kill *L. monocytogenes, E.coli* and some antibiotic resistant bacteria (Gill, Holley, 2006;

Nostro *et al*., 2004).The other antimicrobial agent in cinnamon is cinnamaldehyde (Lopez *et al*., 2007; Valero, Giner, 2006).Thus the present study has proved that aqueous extract of cinnamon can be used as an alternate of cinnamon oil. This can be an alternate and cheapest antimicrobial agent

The Minimum Inhibitory Concentration (MIC) is cited as by most researchers as a measure of diameter of zone of inhibition corresponding to the minimum concentration of antibacterial agent by well diffusion method. The DZOI depends upon on the diffusing ability of the antibacterial agents in the agar and porosity of it. Thus Broth dilution method is a better alternate method where the hydrophilic components of the AEC can easily diffuse throughout the broth and can give an accurate Minimum concentration of the extract to behave as an bacteriostatic agent.

The activity index determines how far the extract is efficient when compared to pure standard drug like Gentamycin. The Activity Index for the AEC was lower than Gentamycin because it is a crude extract. Crude pant prepararations have generally been reported to exhibit lower antimicrobial activity than pure antibiotic substances (Navarro *et al*., 1996; Ebi and Ofoefule, 1997; Ibrahim *et al*., 1997).

The correlation between antibacterial activity and total phenol content are shown in Fig.1.The R^2 values were between 0.9988 and 0.7781, and decreased in the following order:*P.mirabilis>E.coli>B.cereus>E.faecalis >S.aureus.*

Total phenol content

Though the yield of total water soluble plant material was low, there was good antioxidant activity,indicating the effectiveness of the antioxidant compound present in the crude extract.Phenolics are plant secondary metabolites important in chelation of redox- active metal ions,scavenging action of free radicals, prevention of hydrogen peroxide conversions into reactive oxyradicals.Table 4 summarize the total phenolic compounds expresssed as gallic acid equivalent(GAE), which varied between 7.68±0.053 µg.mL^{-1} to 52.375±0.025 µg.mL^{-1} of crude extract of 20 µg.mL^{-1} to 120 µg.mL^{-1}, respectively (Table 4).

Reducing power activity

According to the Oyaizu's (1986), the conversion of Fe^{3+} to Fe^{2+} was measured by the Perl's Prussian blue complex colour formation. Fig 2 shows the reducing power of AEC as a function of its concentration that reduces Ferric (Fe^{3+}) to (Fe^{2+})ion through the donation of an electron. The resulting Fe^{2+}ions was measure at 700 nm. Thus due to the donation of electron to reactive free radical species, there is termination of the free radical chain reaction. The reducing power of the extract was significantly ($p< 0.05$) not much lesser than ascorbic acid. At 250 µg.mL^{-1},the reducing power was very less as compared to ascorbic acid. At 1mg. mL^{-1} concentration, AEC and ascorbic acid had almost the same reducing activity of 0.202 ± 0.0144 and 0.22033± 0.023587respectively. The results were in agreement with the EC_{50} of AEC and Ascorbic acid (Table 5). In general cinnamon extract can be used as the natural alternate antioxidant than ascorbic acid. Fig 3. shows the correlation of the reducing power capacity of AEC with its total phenol content.
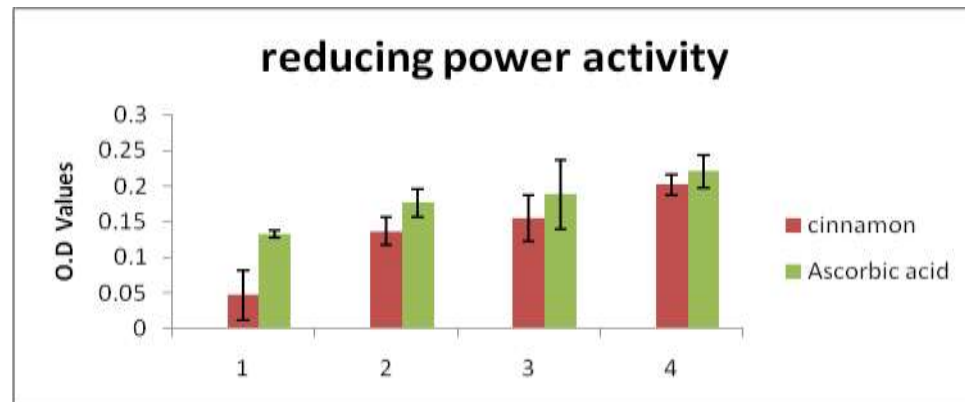

Fig 2. Reducing power under different concentrations 1). 0.25 mg.mL^{-1}, 2). 0.5 mg.mL^{-1},3).0.75 mg.mL^{-1}4).1.0 mg.mL^{-1}of AEC

Free radical scavenging activity

DPPH after forming DPPH radical forms purple colour which changes to yellow colour compound after reacting with an antioxidant. The deceased absorbance at 517 nm determines the effeciancy of reducing power of the

antioxidant. The DPPH free radica scavenging activity at vaious concentration and EC_{50} values of AEC are presented in Fig 3. and Table 5 respectively. With the addition of AEC into DPPH solution caused a rapid change of colour indicating the excellent scavenging capacity of the extract. The extract possessed dose dependent antioxidant activity. The antioxidant activity of the plant material strongly correlates with the total phenol activity (Fig. 4). Therefore, the free radical scavenging activity of the AEC appear to be due to the phenolic compound (Table 5). According to Sindhu and Emilla, 2004 Eugenol compared to cinnamaldehyde exhibited faster reaction and greater intensity of change of purple color to yellow.
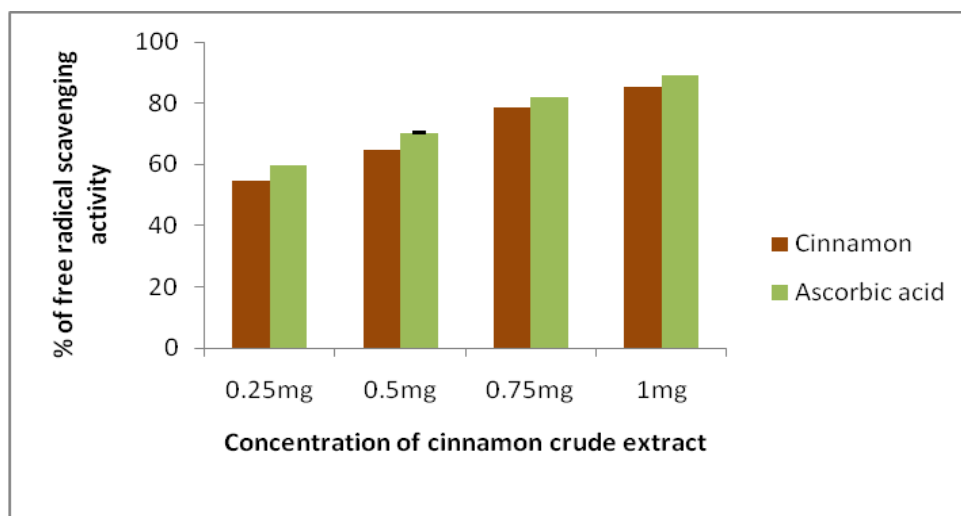

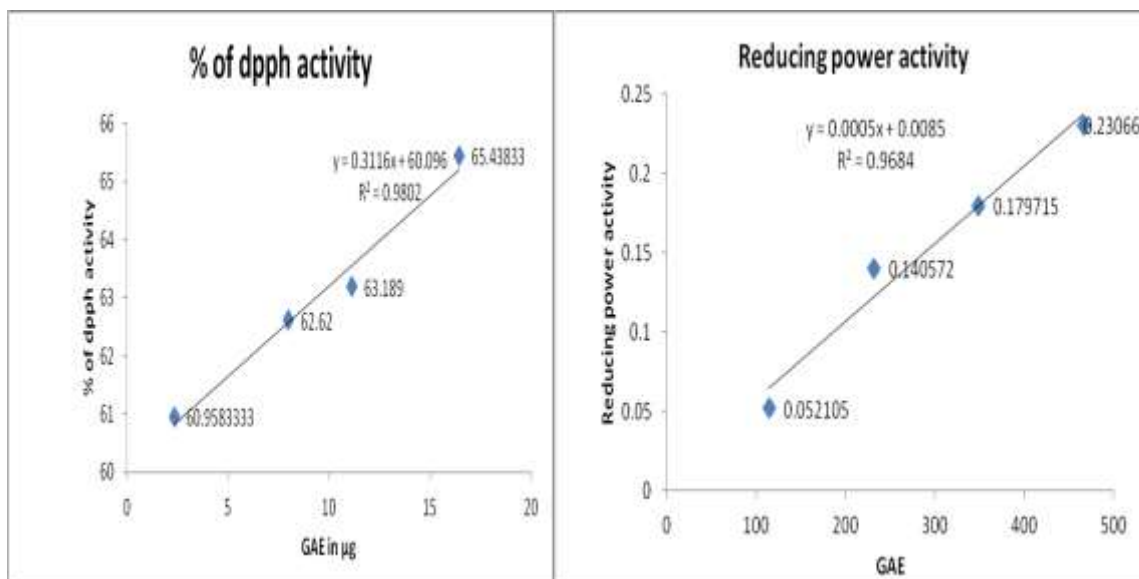Fig 3. The DPPH free radical scavenging activity at vaious concentration of AEC.

Fig 4. Relationship between DPPH free radical scavenging activity, reducing power and total phenolics of AEC extract (n=12).

The EC_{50} values of AEC extract for DPPH radical scavenging assay was higher than that of Ascorbic acid , whereas the EC_{50} of Reducing power of AEC was lower than standard Ascorbic acid.

A correlation coefficient was established by linear regression analysis between the phenolic content and their activity in each assay. The correlation coefficients of DPPH radicals scavenging capacity, and reducing power activity with the phenolic content were 0.9802 (p > 0.05) and 0.9684 (p > 0.05), respectively.

Metal chelation assay

Spectophometric determination of meta chelation:of the dark purple colour formed due to the ferrozine – Fe $^{2+}$ complex is decreased by the metal chelator compounds that exist in the reaction mixture. The absorbance at 562

nm is indirectly proportional to the concentration of the extract. AEC had 35% less metal chelation activity compared to Na$_2$EDTA at 100 µg/mL concentration (Table 6).

Table 1. Diameter (cm) of Zone of inhibition (DZOI) at different concentrations of Aqueous extract of Cinnamon (AEC).

	0.2 mg	0.343 mg	0.490 mg	0.735 mg
S. aureus	0	1.0	1.3	1.6
E. coli	0	1.1	1.3	1.7
B. cereus	1.2	1.6	1.8	2
P. mirabilis	1.3	1.5	1.7	2
E. faecalis	0.8	1.0	1.3	1.7

Table 2. Minimum Inhibitory concentration (MIC) by serial double dilution method.

Test organism	Concentration (in mg.mL^{-1})
S. aureus	0.2466
E. coli	0.06165
B. cereus	0.06165
P. mirabilis	0.1233
E. faecalis	0.1233

Table 3. Activity index (AI) of test organism.

Test organism	Activity index
S. aureus	0.38 ± 0.0247
E .coli	0.80 ± 0.0198
B. cereus	0.71 ± 0.0429
P. mirabilis	0.80 ± 0.0193
E. faecalis	0.70 ± 0.0263

Table 4. Total phenol content expressed as the Gallic acid equivalent (GAE).

concentration(µg.mL^{-1})	GAE
20 µg.mL^{-1}	7.68±0.053
40 µg.mL^{-1}	11.75 ± 0.08
60 µg.mL^{-1}	27.375±0.1405
80 µg.mL^{-1}	36.75±0.17
100 µg.mL^{-1}	43± 0.025
120 µg.mL^{-1}	52.375±0.025

Table 5. EC$_{50}$ values (mg/mL) obtained in the antioxidant assays.

Assay	EC$_{50}$ values of AEC	EC$_{50}$ values of Ascorbic acid
DPPH radical scavenging activity	0.321	0.053
Reducing power	10.05	14.16

Table 6. Metal chelation assay of AEC and Disodium EDTA at 0.1 mg.mL^{-1} concentration.

Concentration	Cinnamon	Disodium-EDTA
0.1 mg	39.05 ± 0.17	75.22 ± 0.05

DNA damage protective activity

The electrophoresis pattern of DNA after UV-photolysis of 6% H$_2$O$_2$ in the absence and presence of the AEC is shown in Fig. 5. 50 µg of both extract was tested for their protective activity. pBR322 DNA without UV and H$_2$O$_2$ treatment acted as the control (Lane 1). Lane 2 represented irradiated control with extract and lane 3 with treated irradiated sample.UV induced photolysis of H$_2$O$_2$ caused total destruction of the DNA (lane 3). AEC dispayed a signiicant protective activity in comparision with irradiated control. Phenolic compounds present in Cinnamon might directly quench ·OH by donating hydrogen atom or electron, and therefore protecting the supercoiled plasmid DNA from ·OH dependent strand breaks (Singh *et al*., 2009).
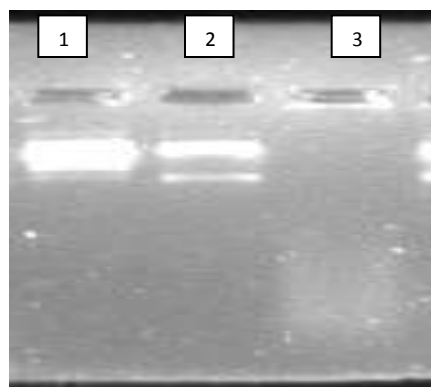
Conclusion

The results indicate that AEC is a good broad spectrum antibacterial agent and antioxidant due to its high content of phenolic compounds. It was concluded that Cinnamon bark bears diverse therapeutic potential in aqueous extract form and posses compounds which might be exploited to develop precursor of drugs and as natural preservatives. This can prevent bacterial spoilage and rancidity of food and thus increase the shelf life of food. It can be used as a natural food additive by food industries.

Fig 5. Effect of AEC at 50 μg concentration on the protection of DNA against OH·radicals generated by photolysis of H_2O_2. Lane 1: untreated DNA (control);lane 2:AEC + H_2O_2 + DNA; Lane 3 3% H_2O_2 +DNA Samples in lanes 2 and 3 were irradiated.

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