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RESEARCH ARTICLE

# Antimicrobial and Antioxidant Activities of *Careya arborea* Roxb. Stem Bark

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**ABSTRACT**

The present study was carried out to evaluate the antimicrobial and antioxidant activities of methanol extract of *Careya arborea* (MECA) stem barks (Myrtaceae) in various *in-vitro* systems. Antimicrobial activities of MECA were carried out using disc diffusion methods with Gram positive and Gram negative bacteria and some fungal species. MECA showed broad-spectrum antimicrobial activity against all tested microorganisms. Antioxidant and free radical scavenging activities of MECA was assessed by using 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH), superoxide anion radical, nitric oxide radical and hydroxyl radical scavenging assays. The antioxidant activity of MECA increased in a concentration dependent manner. In DPPH radical scavenging assay the $IC_{50}$ value of the extract was found to be 132.25 μg/mL. MECA was found to inhibit the nitric oxide radicals generated from sodium nitroprusside. The $IC_{50}$ value was found to be 72.54 μg/mL, whereas the $IC_{50}$ value of curcumin was 20.4 μg/mL. Moreover, the MECA was found to scavenge the superoxide generated by phenazine methosulphate (PMS) / nicotinamide adenine dinucleotide (NADH)-nitroblue tetrazolium (NBT) system. MECA was also found to inhibit the hydroxyl radical generated by Fenton's reaction, where the $IC_{50}$ value of MECA was found to be more than 1000 μg/mL and for catechin the $IC_{50}$ value was found to be 5 μg/mL. The results obtained in the present study indicate that the MECA can be a potential source of natural antimicrobial and antioxidant agents.

**Keywords:** C*areya arborea, Antimicrobial activity, Antioxidant activity, Free radical scavenging, DPPH assay*

Medicinal plants, which form the backbone of traditional medicine, have in the last few decades been the subject for very intense pharmacological studies; this has been brought about by the acknowledgement of the value of medicinal plants as potential sources of new compounds of therapeutics value and as sources of lead compounds in the drug development. In developing countries, it is estimated that about 80% of the population rely on traditional medicine for their primary health care. There arises a need therefore to screen medicinal plants for bioactive compounds as a basis for further pharmacological studies. In recent years, multiple drug/chemical resistance in both human and plant pathogenic microorganisms have been developed due to indiscriminate use of commercial antimicrobial drugs/chemical commonly used in the treatment of infectious diseases. This situation has forced scientists to search new antimicrobial substances in various sources like medicinal plants.

Reactive oxygen species (ROS), which include free radicals such as superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$) and non free-radical species such as $H_2O_2$ and singled oxygen ($^1O_2$), are various forms of activated oxygen [1-3]. The importance of free radicals and ROS has attracted increasing attention over the past decade [1]. These molecules are exacerbating factors in cellular injury and aging process [4]. ROS have aroused significant interest among scientists. Their broad range of effects on biological and medicinal systems has drawn the attention of many experimental works [5]. In living organisms, various ROS can form in different ways. Normal aerobic respiration stimulates polymorphonuclear leukocytes and macrophages, and peroxisomes appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of ROS include tobacco smoke, certain pollutants, organic solvents, and pesticides [6-8].

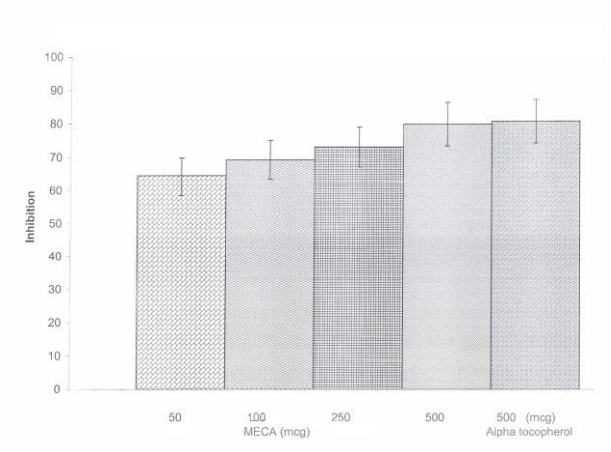


Fig 1. Inhibition (%) of lipid peroxidation of Alpha tocopherol and different doses of MECA in the linoleic acid emulsion.

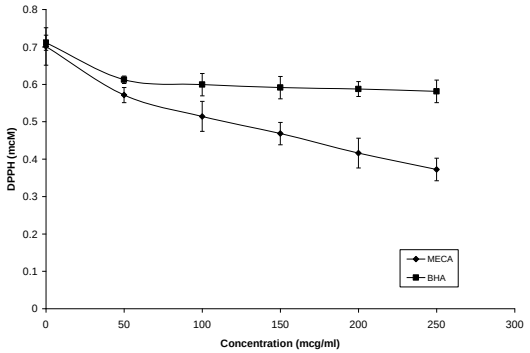


Fig 2. Free radical scavenging activity of methanol extract of Careya arborea (MECA) and BHA by 1,1-diphenyl-2-picryl hydrazyl radicals (DPPH).

*Careya arborea* Roxb. commonly known as Wild Guava belongs to the family Myrtaceae medium sized deciduous tree, bark dark grey exfoliating in thin strip. Widely available in India, Ceylon, Malay and Peninsula. The plant has been extensively investigated and chemical constituents from the barks, leaves and seeds of the plant have previously been reported to include triterpenoids [9-11] flavonoid [12], cumarin [13, 14], saponins [15] and tannins [16].

Stem bark of *Careya arborea* is traditionally used in the treatment of tumours, bronchitis, epileptic fits, astringents, antidote to snake-venom and skin disease [17]. It is also used as remedy for diarrhoea [18], dysentery with bloody stools and ear pain [19, 20]. Antipyretic [10], leech repellant, fish poison and antivenin activities were also reported in literature [21-23]. The aqueous extract of fresh root bark has been used as fish poison [15]. Pharmacological activities and mode of action of this plant is yet to be established. Based on the traditional usage and chemical constituents we selected this plant for the present study. Plant derived natural products such as flavonoids, terpenoids and steroids etc. have received considerable attention in recent years due to their diverse pharmacological properties including hepatoprotective and antioxidant activity [19, 20]. There has been growing interest in the analysis of certain flavonoids, triterpenoids and steroids stimulated by intense research in to their potential benefits to human health. One of their main properties in this regard is their antioxidant activity, which enables them to attenuate the development of tumor and inflammatory disease. Antioxidant plays an important role in inhibiting and scavenging radicals, thus providing protection to humans against infection and degenerative diseases. Realizing the fact, this research was carried out to evaluate the *in vitro* antimicrobial and antioxidant activities of methanol extract of *Careya arborea* (MECA).

## MATERIALS AND METHODS

### *Plant Materials and Extraction*

Stem bark of the plant *Careya arborea* (Family: Myrtaceae) was collected in the month of March 2004 from the Kolli Hills, Tamil Nadu, India. The plant material was taxonomically identified by Botanical Survey of India (B.S.I), Kolkata, India, and a Voucher specimen (No.GMS-3) was retained in B.S.I. herbarium. The dried powder material of the stem bark of *Careya arborea* was extracted with methanol (Yield 7.45%) in a soxhlet apparatus. The methanol extract was then distilled, evaporated and dried in vacuum. The chemical constituents of the extract were identified by qualitative analysis followed by their confirmation by thin layer chromatography.

### *Chemicals*

Ammonium thiocyanate was purchased from E. Merck, Germany. Ferrous chloride, ferric chloride, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), nicotinamide adenine dinucleotide (NADH), EDTA, butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA), α-tocopherol, ascorbic acid, quercetin, catechin, pyrocatechol, curcumin, nitroblue tetrazolium (NBT), thiobarbituric acid (TBA), 2-deoxy-2-ribose, trichloroacetic acid (TCA), phenazine methosulphate and potassium ferricyanide were purchased from Sigma Chemical Co. Ltd, USA. All other chemicals and reagents were of analytical grade.

### *Antimicrobial Activity*

#### Preparation of test microorganisms

*Pseudomonas aeruginosa* (ATCC 9027, gram negative), *Escherichia coli* (ATCC 9837, gram negative), *Salmonella typi* (ATCC 43579, gram negative), *Shigella dysenteriae* (ATCC 13313, gram negative), *Vibrio cholerae* (ATCC 14033, gram negative), *Staphylococcus aureus* (ATCC 6538, gram positive), *Streptococcus pneumoniae* (ATCC 49619, gram positive), *Micrococ-*

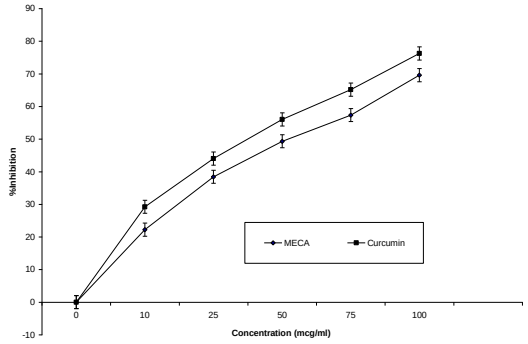


Fig 3. Inihibition of nitricoxide radical by methanol extract of *Careya arborea* ( MECA).

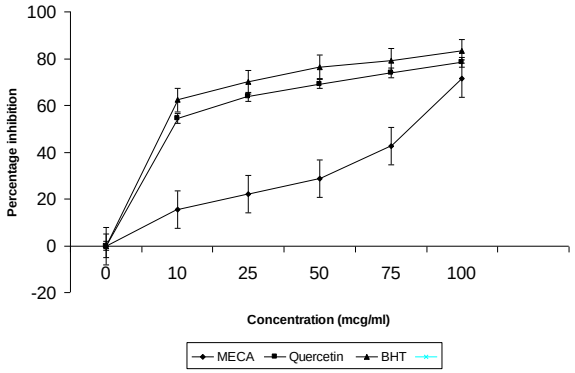


Fig 4. Superoxide anion scavenging activity of methanol extract of *Careya arborea* (MECA) and same doses of quercetin and BHT by PMS/NADH-NBT method.

*cus luteus* (ATCC 10240, gram positive) and *Staphylococcus epidermidis* (ATCC 12228, gram positive) were used for antibacterial activity. Fungal organism such as *Candida albicans* (ATCC, 10231), *Aspergillus niger* (ATCC 16404), *Aspergillus flavus* (ATCC 9643) and *Alternaria solani* (ATCC 20476) strains were also employed for determination of antifungal activity.

Bacteria and fungi were obtained from the stock cultures of Central Drugs Laboratory, Kolkata (CDL), Indian, Institute of Chemical Biology (IICB), Kolkata, and Mycology and Plant Pathology Laboratory, Kolkata, India. The bacterial and fungal stock cultures were maintained on Muller Hinton Agar slants, which were stored at 4ºC. For the purpose of antimicrobial evaluation, thirteen microorganisms were used. These bacteria were maintained on Nutrient agar base. The fungus was maintained on sabouraud-dextrose agar, which is often used with antibiotics for the isolation of the pathogenic fungi.

### *Antimicrobial Activity Determination*

Agar cultures of the test microorganisms were prepared as described [24]. Three to five similar colonies were selected and transferred with loop into 5 mL of broth. The broth cultures were incubated for 24 hours at 37$^{o}$ C. The MECA was dissolved in sterile water by magnetic stirrer. For screening, sterile, 6-mm diameter filter paper disc were impregnated with 25-200 µg of the MECA respectively. Then the paper discs placed in Mueller Hinton agar. The inoculum for each organism was prepared from broth cultures. The concentration of cultures was to $10^5$ colony-forming units ($1\times10^5$ cfu/mL). The results were recorded by measuring the zones of growth inhibition surrounding the disc. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. All data on antimicrobial activity are the average of triplicate analyses. Ofloxacin (5 µg/disc), and the antifungal compound Miconazole nitrate (40 µg/disc) were used as reference standards, as was recommended by the National Committee for Clinical Laboratory Standards (NCCLS).

### *Total Antioxidant Activity Determination*

The antioxidant activity of MECA was determined according to the thiocyanate method [25]. About 10 mg of MECA was dissolved in 10 mL water. Various concentrations (50, 100, 250 and 500 µg/mL) of MECA were added to linoleic acid emulsion (2.5 mL, 0.04M, pH 7.0) and phosphate buffer (2 mL, 0.04M, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid, 0.2804 g of Tween 20 as emulsifier and 50 mL phosphate buffer mixture was homogenized. The final volume was adjusted to 5 mL with potassium phosphate buffer (0.04M, pH 7.0). Then the mixed samples were incubated at 37ºC in a glass flask for 60 h to accelerate the oxidation process. Each 12 h, 1 mL of the incubated sample was removed and 0.1 mL of $FeCl_2$ (0.02M) and 0.1 mL of ammonium thiocyanate (30%) were added to the 1 mL aliquot that was removed from the sample.

### *DPPH Radical Scavenging Activity*

The free radical scavenging activity of MECA was measured by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) using the method of Blois [26]. 0.1mM solution of DPPH in methanol was prepared and 1 mL of this solution was added to 3 mL of various concentrations of MECA and reference compound (50, 100, 150, 200 and 250 µg). After 30 min, absorbance was measured at 517 nm. Butylated hydroxy anisole (BHA) was used as a reference material. All the tests were performed in triplicate and the graph was plotted with the mean value. The percentage of inhibition was calculated by comparing the absorbance values of control and samples.

### *Nitric Oxide Radical Scavenging Activity*

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which can be measured by Griess reaction [27]. The reaction mixture (3 mL) containing sodium nitroprusside (10mM) in phosphate buffered saline (PBS) and MECA and reference com-

pound at different concentrations (10, 25, 50, 75 and 100 µg) were incubated at $25^0$ C for 150 min. Each 30 min, 0.5 mL of the incubated sample was removed. 0.5 mL of Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% $H_3PO_4$) was added to the 0.5 mL aliquot of the sample removed. The absorbance of the chromophore formed was measured at 546 nm. All the tests were performed in triplicate and the results averaged. The percentage inhibition of nitric oxide generated was measured by comparing the absorbance values of control and test. Curcumin was used as a positive control.

### *Superoxide Anion Radical Scavenging Activity*

Measurement of superoxide anion scavenging activity of MECA was done based on the method described by Nishimiki [28] with modifications. About 1 mL 156 µM nitroblue tetrazolium (NBT) solution in phosphate buffer (100 mM, pH 7.4), 1 mL 468 µM NADH in phosphate buffer (100 mM, pH 7.4) and 0.1 mL of various concentration of MECA and reference compounds (10, 25, 50, 75 and 100 µg) were mixed and the reaction was started by adding 100 µL 60 µM phenazine methosulphate (PMS) in phosphate buffer (100mM, pH 7.4). The reaction mixture was incubated at 25ºC for 5 min and the absorbance at 560 nm was measured against control samples. butylated hydroxy toluene (BHT) and quercetin were used as reference compounds. All the tests were performed in triplicate and the results averaged. The percentage inhibition was calculated by comparing the results of control and test samples.

### *Hydroxyl Radical Scavenging Activity*

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and test compound (MECA) for hydroxyl radical generated by $Fe^{3+}$-Ascorbate–EDTA–$H_2O_2$ system (Fenton reaction) according to the method of Kunchandy and Rao [29]. The reaction mixture contained, in a final volume of 1.0 mL, 100µl of 2-deoxy-2-ribose (28 mM in $KH_2PO_4$-KOH buffer, 20mM, pH 7.4), 500 µL of the various concentrations of MECA and reference compound (1, 100 and 1000 µg) in $KH_2PO_4$-KOH buffer (20 mM, pH 7.4), 200 µl of 1.04 mM EDTA and 200 µM $FeCl_3$ (1:1 v/v), 100 µl of 1.0 mM $H_2O_2$ and 100 µl of 1.0 mM ascorbic acid was incubated at 37ºC for 1 h. 1.0 mL of thiobarbituric acid (1%) and 1.0 mL of trichloroacetic acid (2.8%) were added to the test tubes and were incubated at 100ºC for 20 min. After cooling, absorbance was measured at 532 nm against control containing deoxyribose and buffer. Catechin was used as a positive control. Reactions were carried out in triplicate. The percentage inhibition was determined by comparing the results of the test and control compounds.

### *Statistical Analysis*

Experimental results were mean ± S.D of three parallel measurements. Statistical analysis was performed according to the student's *t*-test. Analysis of variance was performed by ANOVA procedure. $IC_{50}$ values for all the above experiments were determined by linear regression method. $p < 0.05$ were regarded as significant.

## RESULTS AND DISCUSSION

### *Antimicrobial Activity*

Disc diffusion methods are extensively used to investigate the antibacterial activity of natural substances and plant extracts. These assays are based on the use of discs as reservoirs containing solutions of substances to be examined. In the case of solutions with a low activity, however, a large concentration or volume is needed. The limited capacity of discs means that holes or cylinders are preferably used [30].

Most of the bacterial and the fungi species were inhibited by the antimicrobial activity as it is shown in Table 1. In this study, nine different bacterial and four fungi species were used to screen the possible antimicrobial activities of methanol extract of *Careya arborea* stem bark. MECA showed broad spectrum of activity against all the bacterial strains at the tested concentration of 25-200 µg/disc was summarized in Table 1. MECA showed antimicrobial activity against all tested bacterial and fungal stains at the concentration of 50 µg/disc. Ofloxacin (5 µg/disc) and Miconazole nitrate

Table 1. Antimicrobial activity of methanol extract of *Careya arborea* (MECA) stem bark, ofloxcin and miconazol nitrate on selected bacterial and fungal strains.

| Microorganism | Diameter of inhibition zone (mm) | | | | | |
|---|---|---|---|---|---|---|
| | MECA (µg/mL/disc) | | | Standards (µg/mL/disc) | | |
| | 25 | 50 | 100 | 200 | (Ofl) 5 | (Mic) 40 |
| *Pseudomonas aeruginosa* | 7 | 9 | 13 | 16 | 22 | - |
| *Escherichia coli* | - | 8 | 11 | 14 | 26 | - |
| *Salmonella typi* | 8 | 12 | 15 | 18 | 24 | - |
| *Shigella dysenteriae* | 7 | 9 | 11 | 14 | 21 | - |
| *Vibrio cholerae* | - | 7 | 9 | 13 | 20 | - |
| *Staphylococcus aureus* | - | 7 | 9 | 12 | 14 | - |
| *Streptococcus pneumoniae* | - | 7 | 10 | 13 | 22 | - |
| *Micrococcus luteus* | 7 | 9 | 10 | 13 | 19 | - |
| *Staphylococcus epidermidis* | - | 7 | 9 | 12 | 23 | - |
| *Candida albicans* | - | 7 | 10 | 14 | - | 21 |
| *Aspergillus niger* | - | - | 7 | 11 | - | 19 |
| *Aspergillus flavus* | - | - | 7 | 10 | - | 19 |
| *Alternaria solani* | - | 7 | 10 | 14 | - | 22 |

Ofl: Ofloxacin (5 µg/mL/disc); Mic: Miconazole (40 µg/mL/disc), (-): inactive.
The results are the mean values of triplicate tests repeated three times after 24-72 h of inhibition at 37ºC.

(40 μg/disc) were used as positive controls for bacteria and fungi, respectively.

### *Antioxidant Activity*

In this study the antioxidative activity of the MECA was measured using ammonium thiocyanate method. This method was used to measure the peroxide level during the initial stages of lipid oxidation. The antioxidant activity of MECA might be due to hydroperoxides, inactivation of free radicals or complexing with metal ions, or combinations thereof. This good antioxidant activity of MECA might be attributed to the presence of phytochemicals, such as flavonoids and biflavones [31]. Fig 1 illustrates the antioxidative activities of various concentrations of MECA (50, 100, 250 and 500μg/mL). MECA at 50, 100, 250 and 500 μg/mL showed antioxidant activities in a concentration dependent manner and had 64.53, 69.27, 73.04 and 79.93% inhibition respectively on lipid peroxidation of linoleic acid system. MECA at 500 μg/mL showed 79.93% inhibition, which is more or less equal to the antioxidant activity of 500 μg/mL of α-tocopherol (80.73%). The $IC_{50}$ value of MECA on lipid peroxidation was found to be 36.58 μg/mL. The results indicate that methanol extract of *Careya arborea* significantly ($p$<0.05) inhibits linoleic acid peroxidation. The antioxidative activity of the stem bark of *Careya arborea* may be due to the reduction of hydroperoxides, inactivation of free radicals, chelation of metal ions or combinations thereof.

### *DPPH Radical Scavenging Activity*

The DPPH radical is considered to be a model of a lipophilic radical. A chain reaction in lipophilic radicals was initiated by lipid autoxidation. The radical scavenging activity of MECA was determined from the reduction in the optical absorbance at 517 nm due to scavenging of stable DPPH free radical. Positive DPPH test suggests that the samples are free radical scavengers. The scavenging effects of MECA and BHA on DPPH radical are compared and shown in Fig 2. MECA had significant scavenging effects on the DPPH radical and the effects increased with increasing concentration in the range 50-250 μg/mL. Compared with that of BHA, the scavenging effect of MECA was lower. The $IC_{50}$ value of MECA on DPPH radical scavenging assay was found to be 132.25 μg/mL. The results were found statistically significant ($p$<0.05).

### *Nitric Oxide Radical Scavenging Activity*

It is well known that nitric oxide has an important role in various types of inflammatory processes in the animal body. In the present study, crude extract of the stem bark was checked for its inhibitory effect on nitric oxide production. Fig 3 illustrates the percentage inhibition of nitric oxide generation by MECA. Curcumin was used as a reference compound. The concentration of MECA needed for 50% inhibition was found to be 72.54 μg/mL whereas 20.4 μg/mL was needed for curcumin. The results were found statistically significant ($p$<0.05).

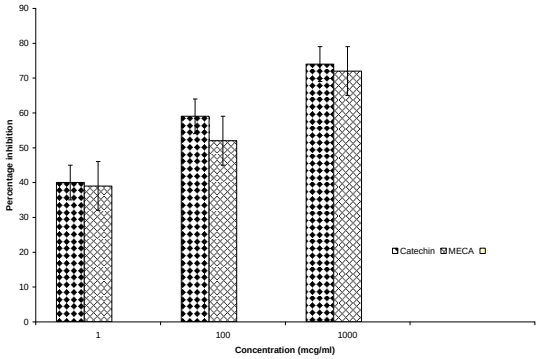


Fig 5. Hydroxyl radical scavenging activity of methanol extract of *Careya arborea* (MECA).

### *Superoxide Anion Radical Scavenging Activity*

Superoxide anions indirectly initiated lipid oxidation as a result of superoxide and hydrogen peroxide serving as precursors of singlet oxygen and hydroxyl radicals [32]. Robak and Glyglewski [33] reported that the antioxidant properties of flavonoids are effective mainly via the scavenging of superoxide anion. MECA was found to possess good scavenging activity on superoxide anions at all the tested concentration. MECA at concentrations ranging from 10 to 100 μg/mL inhibited the production of superoxide anion radicals by 15.55 to 71.32 %. MECA showed strong superoxide radical scavenging activity. The results are given in Fig 4. The $IC_{50}$ value of MECA on superoxide radical scavenging activity was found to be 94.17 μg/mL, whereas the $IC_{50}$ value of BHT and quercetin were found to be 22.77 and 31.58 μg/mL respectively. The results were found statistically significant ($p$<0.05).

### *Hydroxyl Radical Scavenging Activity*

Hydroxyl radicals are the major active oxygen species causing lipid oxidation and enormous biological damage [34]. Ferric-EDTA was incubated with $H_2O_2$ and ascorbic acid at pH 7.4. Hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-2-ribose into fragments that on heating with TBA at low pH form a pink chromogen [35, 36]. When MECA and reference compound catechin were added to the reaction mixture they removed hydroxyl radicals from the sugar and prevented the degradation. The results are shown in Fig 5. The MECA was capable of reducing DNA damage at all concentrations. Catechin used as a standard, was highly effective in inhibiting the oxidative DNA damage. The $IC_{50}$ value of MECA on hydroxyl radical scavenging assay was found to be 1342.53 μg/mL. The results were found statistically significant ($p$<0.05).

The production of free radicals and the activity of the scavenger enzymes against those radicals, such as superoxide dismutase (SOD) are correlated with the life expectancies [37]. Polyphenols, tannins and flavonoids are very valuable plant constituents in the scavenging

action due to their several phenolic hydroxyl groups [38]. The exact constituents of MECA which show free radical scavenging action are unclear. However, the phytoconstituents like polyphenol, fiavonoids and triterpenoids present in the plant extract may be responsible for antimicrobial, antioxidant and free radical scavenging activities.

The result of this study show that the *in vitro* antimicrobial and antioxidant activities of MECA. From the above studies we suggested that MECA could be used as a easily accessible source of natural antioxidant and as a possible food supplement or in pharmaceutical industry and also it may be extensively used for the treatment of some degenerative diseases such as cancer, inflammatory, liver disorder etc. Therefore, it is suggested that further work should be performed on the isolation and identification of antioxidant components in MECA. This could ultimately lead to the inclusion of this compound(s) in different antioxidant pharmaceutical formulation.

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