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SCREENING OF PHYTOCHEMICAL CONSTITUENTS, ANTIOXIDANT AND ANTIBACTERIAL PROPERTIES OF METHANOLIC BARK EXTRACTS OF *Maclura cochinchinensis* (LOUR.) CORNER

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ABSTRACT

Maclura cochinchinensis is a traditionally used medicinal plant, aqueous bark extract of which is consumed by the Karbi tribe of Assam, India to treat jaundice. In view of its medical significance, the study was designed to see its phytochemicals, antioxidant and antimicrobial property. All the phytochemical constituents under study were tested positive. The total phenolic content and flavonoids were found to be 35.98±0.97 mg gallic acid equivalent/g and 21.52±0.44 mg Quercetin/g dry extract. The extract showed excellent DPPH antioxidant activity with IC_{50} value 46.32±0.21µg/ml. Similarly, the IC_{50} of hydroxy radical, nitric oxide radical scavenging activity and lipid peroxidation inhibition were 154.25±0.47 µg/ml, 112.19±0.22 µg/ml, 164±0.44 µg/ml and 99.50±0.35 µg/ml, 42.38±0.17 µg/ml, 119.55±0.37 for plant extract and ascorbic acid, respectively. Antibacterial study of the plant extract showed positive test to *Bacillus cereus* and *Staphylococcus aureus* with minimum inhibition concentration ranging from 125 to 500 µg/ml. Our experiments suggest that the bark-extract the *M. cochinchinensis* could be a potential source of natural antioxidant and antimicrobial agent and may be a good candidate for plant based pharmaceutical products.

KEYWORDS: ***Maclura cochinchinensis,*** **phytochemical, antioxidant, DPPH, antimicrobial.**

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INTRODUCTION

Traditionally used medicinal plants has been a source of relief in controlling different types of diseases throughout the world and found to be documented in almost all the civilization[1.] World Health Organization has estimated that more than 80% of world's population still relies on traditionally based medicine to meet their daily health care requirements[2]. Though the advancement of synthetic medicines, to a certain extent, has lifted the health care and livelihood of people, the use and importance of plants and its phytochemicals for the same has never been neglected and a large number of plants are screened for their efficacy against various diseases. Moreover, the importance of traditional medicines is finding their ways to pharmaceuticals, cosmetics, agricultures and food industries. Bacterial infection is one of the most important infectious diseases that account almost 85% of the global death from infectious diseases like acute respiratory infections, diarrheal diseases, measles, AIDS, malaria and tuberculosis[3]. A large number of antibacterial drugs which interferes with the structure or processes of bacterial growth or survival without any harm to their host are commercially available today[4]. However, indiscriminate use of commercially available drugs over the last 60 years has led to the development of multiple drug resistance capacity and has triggered a change within bacterial body which secures their survival in environments where antibiotics are present[5-8]. Because of its alarming increase of resistance capacity and reduced effectiveness of commercial drugs as well as adverse side-effects like hypersensitivity, immune-suppression and allergic reactions on the host body, there is an urgent need to develop alternative antimicrobial drugs. In addition to bacterial infections, the increase of free radicals in our body is also one of the health hazards that lead to various physiological dysfunction and diseases like diabetes, cancer, Alzheimer and several others[9-11]. Moreover, the increase in free radicals causes oxidative stress in the body leading to oxidative damages to cellular amino acids, proteins, lipids, nuclei acids and ultimately cellular death[12-13]. Although our body has innate immunity of these free radicals, these defense systems sometimes may not be sufficient enough to restore the normal redox state during oxidative stress[14]. Therefore, to restore normal redox homeostasis, additional supplementary exogenous antioxidants is required. Therefore, in view of its high source of antioxidant molecules, a higher attention has been paid by the world community to develop newer antioxidant molecules from traditionally used plants. Numerous studies all over the world have shown the importance of aromatic and medicinal plants as a source of diverse nutrient and non-nutrient molecules, many of which possess high antioxidant and effective antimicrobial properties[15-18].The effectiveness of these plants as a great source of new drugs has been recognized by the world community and necessary steps were being taken to investigate and understand the property, safety and efficacy of these plants[19]. *Maclura cochinchinensis* is one such traditionally used medicinal plant, the aqueous bark extract of which is used by the Karbi tribe of Assam, India to treat jaundice. The plant species has been found to possess antipeptic ulcer, antipyretic, antihepatitis, antiviral, antimycobacterial and anti-inflammatory activities[20-24]. Despite of its medicinal importance, no such detail studies were found regarding its phytochemicals, antioxidant and antibacterial properties. Therefore, the present study was designed to characterize the phytochemical constituents and evaluate the antioxidant and antibacterial properties of *Maclura cochinchinensis*.

MATERIALS AND METHODS

Plant material collection and preparation of plant extracts

The plant was collected from Chakanibari area of middle Assam, India with the help of local people in the month of March-June, 2011. The plant was identified as *Maclura cochinchinensis* (Family Moraceae) by the Botanical Survey of India, Shillong. A voucher specimen (BR 2010-MC) was submitted for further reference. The bark of the plant was collected, washed and dried under shade for about 10 days. The dried samples were grounded into powder form by motor-driven grinder, soaked into 90% methanol for 3 days, filtered and processed for methanolic crude extraction using a Soxhlet apparatus. The solutions obtained were filtered through Whatman No. 1 filter paper and solvent evaporated to dryness at 40°C using rotary evaporator. The dry methanol extract obtained were kept in -4ºC till further use.

Phytochemical Screening

Qualitative tests of the phytochemicals from *M. cochinchinensis* were carried out following the method of Sofowora[25] and Trease and Evans[26].

Total Phenolic Contents (TPC)

The total phenolic contents of the extracts were determined by Folin -Ciocalteu method following Swain and Hillis[27] with slight modification as described by Wolfe et al.[28]. Briefly, 2 mg/ml of the extract was mixed with 5.0 ml of 10% Folin - Ciocalteu reagent and 0.5 ml of sodium carbonate (70% w/v). The mixture was vortexed for 15 sec and incubated at 40ºC for 30 min. The absorbance was measured at 765 nm using a spectrophotometer. Amount of TPC was calculated from a calibration curve of gallic acid and the results were expressed as mgGAE (gallic acid equivalent) /g dry weight of extract.

Total Flavonoids Content

Total flavonoid content of plant was determined following the method of Ordonez et al.[29]. 1 ml of 2% aluminium trichloride ($AlCl_3$) in methanol was mixed with same volume of methanolic extracts (2mg). Absorption readings at 415 nm were taken after 10 min against a blank solution consisting of a 1 ml extract and 1 ml methanol without $AlCl_3$. The concentrations of flavonoid compounds were calculated from the calibration curve of quercetin (mg/g) as standard compound and the results were expressed as mg quercetin equivalent (QNE)/g of dry extract.

Antioxidant Study

DPPH Radical Scavenging Activity

Radical scavenging activity of plant extracts was determined using DPPH (2, 2-diphenyl-1-picryl hydrazyl hydrate) following the method of Brand-Williams et al.[30]. The assay mixture consisted of 1ml of 0.1mM DPPH (in methanol), 1 ml of extract (10 to 500 µg/ml methanol) and volume made up to 3 ml with methanol. The mixture was shaken properly and allowed to stand in dark for 30 min at normal room temperature. The absorbance was measured at 517 nm against a blank solution (1 ml sample + 2 ml methanol). Ascorbic acid was used as a standard compound. The antioxidant activity of the plant extract was calculated using this equation:

$$AA\% = 100 - \{[(AB\ sample - AB\ blank) \times 100] / AB\ control\}$$

Where, AA- antioxidant activity, AB samples - the absorbance of sample and DPPH, AB blank- absorbance of sample and methanol and AB control- the absorbance of DPPH and methanol. The antioxidant activity of the extract was expressed as IC_{50}. The concentration (µg/ml) of the extract inhabiting 50% of the formation of DPPH radical is defined as IC_{50}. A lower IC_{50} value corresponds with a higher antioxidant power.

Hydroxyl Radical ($OH^{\cdot}$) Scavenging Activity

Hydroxyl radical scavenging activity was measured following the method of Kunchandy and Rao[31]. 1 ml of reaction mixture consisted of 100 µl of 28mM 2-deoxyribose (prepared in 20 mM KH_2PO_4-KOH buffer) (pH 7.4), 0.5 ml plant extract (concentration range 50 to 500 µg/ml of buffer), 100 µl of 1.04mM EDTA, 100 µl 200mM $FeCl_3$, 100 µl of 1mM H_2O_2 and 100 µl of 1mM ascorbic acid was incubated at 37 ºC for 1 h. After incubation, 1 ml of 1% thiobarbituric acid and 1 ml of 2.8% trichloroacetic acid were added to the test tubes and incubated at 80 ± 1ºC for 20 min. After cooling, the color development was read at 532 nm against blank solution. Mannitol was used as a positive control. The hydroxyl radical scavenging activity of the extract is represented as % inhibition of deoxyribose degradation and was calculated as follows:

$$\% \text{ Inhibition} = 1 - (AB_{sample} / AB_{control}) \times 100$$

Where, $AB_{Control}$ is the absorbance of the control reaction and AB_{sample} is the absorbance in the presence of extracts. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration (in µg/ml) of extracts that inhibits the formation of OH^{-} radicals by 50%.

Nitric Oxide Radical Scavenging Activity

The nitric oxide scavenging activity was performed following the method of Garrat[32]. The reaction mixture consisted of 4 ml 10 mM sodium nitroprusside, 1 ml PBS (pH 7.4) and 1 ml of extract/standard (ascorbic acid) solution (10-500 µg/ml). The assay mixture was incubated at 25ºC for 150 min. After incubation, 0.5 ml of the reaction mixture was added to 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min. Thereafter, 1ml of naphthylethylenediamine dihydrochloride (0.1%w/v) was added, and the mixture was allowed to stand for 30 min at room temperature. The absorbance was measured at 540 nm. Ascorbic acid was used as a reference compound. The percentage inhibition was calculated as:

$$\% \text{ NO radical Scavenging activity} = [(AB_{Control} - AB_{sample})/AB_{Control}] \times 100$$

Where, $AB_{Control}$ is the absorbance of the control reaction and AB_{sample} is the absorbance in the presence of the extracts. The antioxidant activity of the extract was expressed as IC_{50}.

Lipid Peroxidation Inhibition Activity

Lipid peroxidation inhibitory activity was studied following the modified thiobarbituric acid reactive species assay of Ohkawa et al.[33] to measure the lipid peroxide formation using egg yolk homogenates as lipid-rich media[34]. Lipid peroxidation was induced in 0.5 ml of egg homogenate (10%v/v) by adding 1ml of plant extract/standard (concentration range 50 to 500 µg/ml) and 0.05ml of 75 mM $FeSO_4$. The mixture was incubated for 30 min at 37ºC. Then, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulphate is added and the resulting mixture vortexed and heated for 1h at 95°C. After cooling, 5.0 ml of butanol added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. Inhibition of lipid peroxidation (%) by the extract was calculated using the formula:

$$\text{Lipid peroxidation inhibitory } (\%) = [(1 - E/C) \times 100]$$

Where, C is the absorbance value of the fully oxidized control and E is the absorbance in presence of extract.

Determination of Antimicrobial Activity
Micro-organisms Used

Three bacterial strains namely *Bacillus subtilis* (ATCC 11774, NCIM 2063), *Bacillus cereus* (ATCC 10876, NCIM 2156) and *Staphylococcus aureus* (ATCC BAA 1026, NCIM 2079) were used as test micro-organism. All the bacterial strains were procured from National Chemical Laboratory, Pune, India.

Disc Diffusion Method

The antimicrobial activity of each plant extract was determined following the modified Kirby-Bauer disc diffusion method[35]. Mueller Hinton Agar 2 (MHA) medium was used for the screening of antimicrobial activity. Briefly, 15 ml of molten media was poured into a sterile petriplate and allowed to solidify. The inoculum suspensions (0.1%) were swabbed uniformly and dried for 5 min. 20 µl (0.1g/ml) plant extract was sprinkled on a 6mm disc of Whatman filter paper (no. 1) and the discs were placed on the surface of medium for 5 min to allow the compounds to diffuse and incubated for 24h at 37°C. Ofloxacin (30 µg/disc) were used as a standard positive control. The test was carried out taking DMSO as a negative control. At the end of incubation, inhibition zones formed around the disc were measured with a transparent ruler in millimeter. These studies were performed in triplicate.

Minimum Inhibitory Concentration (MIC) Assay

The MIC method was applied on extracts that proved their high efficacy against microorganisms by the disk diffusion (Kirby–Bauer) method. The highest dilution of a plant extract that retained an inhibitory effect against the growth of a microorganism is known as MIC. Selected plant extracts were subjected to a serial dilution (1 - 0.05 mg) using sterile nutrient broth medium as a diluent. In a patriplate, 20 µl of an individual microorganism and 20 µl of plant extract were loaded and inoculated at 37ºC for 24 h. The highest dilution of the plant extract that retained its inhibitory effect resulting in no growth (absence of turbidity) of a microorganism is recorded as the MIC value of the extract.

STATISTICAL ANALYSIS

All the experiments were carried out in triplicate and data were expressed as ± standard error of means (SEM). Significant differences between the means of parameters were determined by student's t-test at $P < 0.05$. Statistical calculations were carried out at excel and origin software.

RESULTS AND DISCUSSIONS

Phytochemical screening of *M. cochinchinensis* extract showed positive test for all the constituents namely- alkaloids, carbohydrates, fats and oils, flavonoids, glycosides, gums, phenols, proteins, saponins, steroids and tannins (Table 1). However, glycosides (cardiac) and anthroquinones were found to be absent in the methanol extract of the plant. Similarly, out of two tests for carbohydrate, test for non-reducing sugars showed negative result. Quantitative study of phytochemicals showed high total phenolic and flavonoid content in the plant extract. The TPC and flavonoid content were found to be 35.98 ± 0.97mgGAE/g and 21.52 ± 0.44 mgQNE/g dry extract (Table 1). In a similar kind of study by Kumar et al.[36] on the antioxidant activity in some selected Indian medicinal plants, flavonoids content were found to be in the range of 23.15 ± 0.2 and 63.3 ± 0.6 mg/g of extracts. Recent studies by Shabir et al.[37] found that the phenolic contents and flavonoid content were 3.63 g/100 g dry weight and 1.19 g/100 g of dry leave weight of *Delonix regia* (Bojer ex Hook) Raf. The presence of high TPC content is always beneficial to human health, mostly due to their ability to neutralize reactive oxygen species and shows high antioxidant activity

Table 1
Qualitative analysis of the phytochemicals of M. cochinchinensis methanol extracts.

Constituent tests		Inference	TPC (mgGAE/g)	Flavonoids (mgQNE/g)
Carbohydrate	Reducing sugar	+	35.98 ± 0.97	21.52 ± 0.44
	Non-reducing sugar	–		
Gums, Fats and oils, Proteins		+		
Flavonoids, Alkaloids, Steroids, Saponin		+		
Tannins, Phenolic compounds		+		
Glycosides	Cardiac	–		
Anthroquinones		–		

TPC - total phenolic content, Result in ± SEM, + present, – absent, GAE- gallic acid equivalent, QNE- quercetin equivalent.

DPPH radical scavenging activity assay was performed in order to see the ability of an antioxidant compound of the plant extract, if any. The methanol crude bark extracts showed excellent antioxidant property with percent DPPH scavenging activity of 77.55 ± 0.59% at highest concentration of plant extract (0.5 mg/ml) (Figure 1). Standard compound, ascorbic acid showed % scavenging activity 96.35 ± 0.78% at its highest concentration. No statistical significant difference was observed between the activities of plant extract and ascorbic acid at $P < 0.05$. The concentration-dependent curve of DPPH radical scavenging activity of *M. cochinchinensis* extracts compared to standard ascorbic acid suggests that the plant extracts possess high DPPH scavenging activity. The IC_{50} value for the methanolic extract was found to be 46.32 ± 0.21μg/ml whereas ascorbic acid was having much higher DPPH scavenging activity at IC_{50} 13.39 ± 0.14 μg/ml

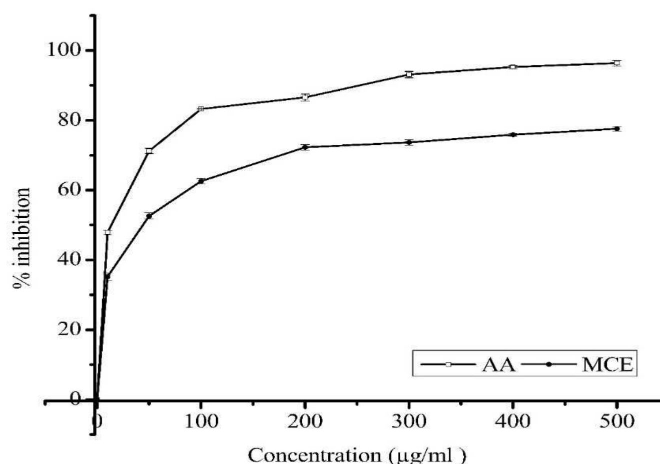

Figure 1
DPPH radical scavenging activity of methanolic extract of M. cochinchinensis.
AA-ascorbic acid, MCE- M. cochinchinensis extract.

This assay shows the abilities of the extract and standard ascorbic acid to inhibit hydroxyl radical-mediated deoxyribose degradation in a Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction mixture. The results were shown in figure 2. A dose-dependent hydroxyl radical scavenging activity was seen in the experiment. At its highest concentrations (250 μg/ml), the % inhibition values were seen to be 76.54 ± 0.87 and 62.55 ± 0.76 for standard and plant extract, respectively. The IC_{50} value for the methanol extract *M. cochinchinensis* was found to be 154.25 ± 0.47 μg/ml compared to that of standard 99.50 ± 0.35μg/ml. The hydroxyl radical scavenging activity of plant extract showed no statistical difference to the reference compound at 0.05 probability level.

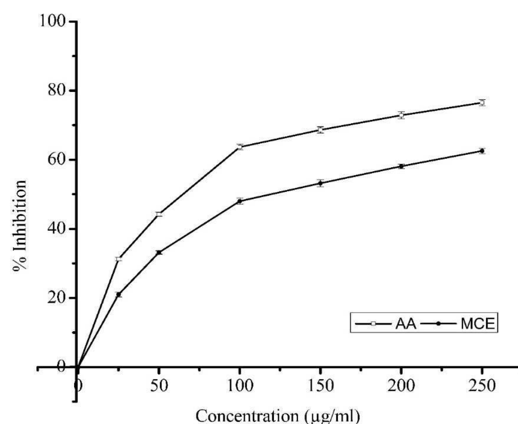

Figure 2
Hydroxy radical scavenging activity M. cochinchinensis extract (MCE). Ascorbic acid (AA) was taken as the standard antioxidant.

The methanolic crude extract of *M. cochinchinensis* showed strong inhibitory activities of nitric oxide. The plant extract and the standard compound, ascorbic acid exhibit 62.23 ± 0.77% and 88.25 ± 0.79% inhibition at its highest concentration (0.3 mg/ml) (Figure 3). The IC_{50} were found to be 112.19 ± 0.22 µg/ml and 42.38 ± 0.17 µg/ml for plant extract and ascorbic acid, respectively.

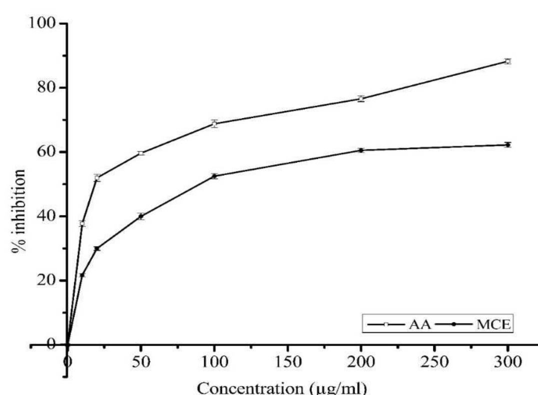

Figure 3
Nitric oxide radical scavenging activity of methanolic bark extract of M. cochinchinensis (MCE) and standard compound, ascorbic acid (AA). At P < 0.05, no significant difference.

Lipid peroxidation in foods products causes chemical spoilage in foods producing various free radicals or active oxygen such as peroxyl and hydroxyl radicals, which are directly or indirectly associated with carcinogenesis, mutagenesis, and aging[38]. In our study the antioxidant activity has been studied by the inhibition of plant extract/ascorbic acid-induced lipid peroxidation. The tested plant extracts of *M. cochinchinensis* and ascorbic acid at a highest concentration of 500 µg/ml prevented or inhibited peroxidation by 70.42 ± 0.78% and 94.72 ± 0.84%, respectively. The IC_{50} were 119.55 ± 0.37 and 164 ± 0.44 µg/ml of ascorbic acid and plant extract respectively. Recent studies by Aswathanarayan and Vittal[39] revealed that the tested plant extracts of *Rotula aquatica* and *Ancistrocladus heyneanus* at a concentration of 500 mg/ml showed an inhibition of peroxidation activity by 91.85% and 89.20%, respectively, whereas the standard compound quercetin inhibited lipid peroxidation by 97.26%. In a similar kind of experiment Keser et al.[40] on aqueous and ethanol extracts of leaves, flowers and fruits of *Crataegus monogyna* showed more than 90% inhibition of lipid peroxidation.

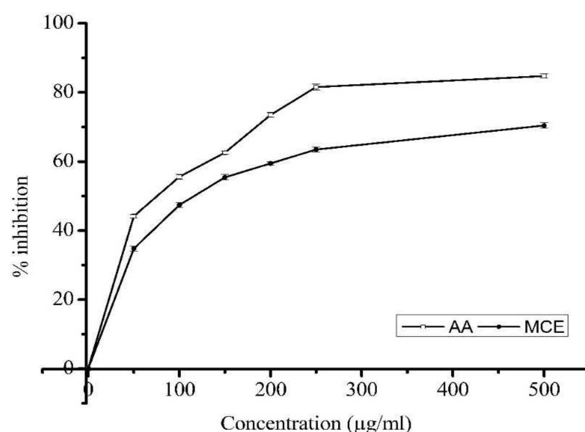

Figure 4
Lipid peroxidation inhibition activity of M. cochinchinensis extact. At the 0.05 level, the two distributions are not significantly different.

The antimicrobial activity of methanolic crude bark extracts of *M. cochinchinensis* was tested against *Bacillus subtilis, Bacillus cereus* and *Staphylococcus aureus*. Incubation of all the tested micro-organisms at a concentration of 2 mg/ml of plant extract showed positive test to only two bacterial strains namely *B. cereus* and *S. aureus*. *S. aureus* was seen to be more susceptible bacterium compared to *B. cereus.* Others did not show susceptibility and were found to be resistant against the methanol extract of the plant. The zone of inhibition (ZOI) at the tested concentration was found to be 11.81 ± 1.74 mm and 5.62 ± 1.34 mm for *S. aureus* and *B. cereus*, respectively (Figure 5). The reference drug, ofloxacin at tested concentration of 30 µg/disc had wider zone of inhibition compared to the test plant extract. The zones of inhibition for the drug were 12.92 ± 0.37 mm and 15.49 ± 0.54 mm for *B. cereus* and *S. aureus*, respectively. The MIC values of both the micro-organisms ranged from 125 to 500 µg/ml. In a similar type of experiment Rajamurugan et al.[41] found that the ethanolic flowers extract of *Tecoma stans* showed a good antimicrobial activity with wider zone of inhibition.

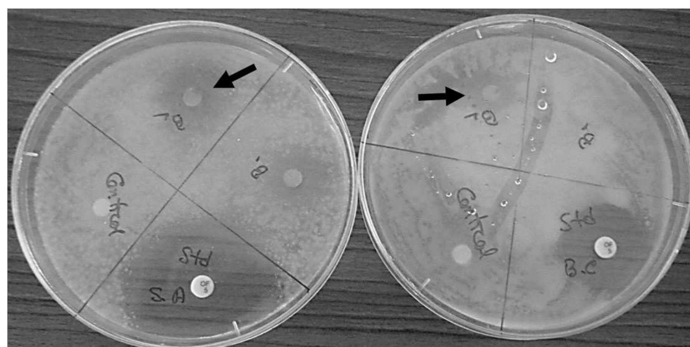

Figure 5
The determination of the zone of inhibition (in mm), SA- Staphylococcus aureus, BC- Bacillus cereus. Black arrow indicated the zone of inhibition.

Twelve medicinal plants, namely *Abrus precatorius* L, *Caesalpinia pulcherrima* Swartz*, Cardiospermum halicacabum* L, *Casuarina equisetifolia* L, *Cynodon dactylon* (L.) *Pers, Delonix regia* L, *Euphorbia hirta* L, *Euphorbia tirucalli* L, *Ficus benghalensis* L, *Gmelina asiatica* L, *Santalum album* L and *Tecomella undulata* (Sm.) Seem was studied by Parekh et al. to see the antibacterial property against 5 medically important

bacterial strains and found that the ZOI were in the range of 8 mm to 24 mm[42]. In another experiment Palombo and Semple investigated the antibacterial activity of 39 traditional Australian medicinal plants and the zones of inhibitions were found to be in the range of 6 to 14 mm[43]. Similarly, More and colleagues showed good antibacterial property in all the eight traditionally used medicinal plants with ZOI ranging from 2.2 to 16.5 mm with MIC values of 25.0 mg/ml to 0.8 mg/ml[44].

CONCLUSION

Over the few decades, there is a paradigms shift of using plant based therapies in the treatment and alleviation of many diseases. Studies have shown that plant based drugs are safe, cost effective and non-toxic compared to the synthetic and semi-synthetic products. Our result has shown that the methanol extracts of *Maclura cochinchinensis* possessed prominent in vitro antioxidant and free radical scavenging activity. Moreover, good antimicrobial activity against certain strains of micro-organism can further substantiate the use of the plant in medicine. A good correlation could be seen between the amount of total phenolic contents and the antioxidant property of the test plant. Hence, a detail study in understanding mechanism and the activity of *Maclura cochinchinensis* has a wider scope in the field of medicine and could play an important role in the prevention of oxidative dependent diseases.

CONFLICT OF INTEREST

The authors have no conflicts of interest.

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