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Phytochemical, In Vitro Antioxidant and Antimicrobial Activities in Aqueous and Methanol Extracts Of *Chloroxylon Swietenia* Bark.

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ABSTRACT

To investigate the phytochemical, *in vitro* antioxidant and antimicrobial activities in aqueous and methanol extracts of *Chloroxylon swietenia* bark. The antimicrobial activity was tested against bacteria as *Staphylococcus aureus, Bacillus subtilis, Echerichia Coli, Klebsiella pneumonia, Pseudomonas aeruginosa* and fungi as *Candida albicans* and *Candida tropicalis*. The antimicrobial activity was evaluated using disc diffusion methods. The *in vitro* antioxidant activity was carried out by DPPH, ABTS and hydrogen peroxide scavenging activities in aqueous and methanol extracts. The preliminary phytochemicals revealed that the presence of Tannins, Phenols, Glycosides, Phenolic compounds, Anthroquinones, Flavonoids. The antimicrobial activity of methanol extract of *Chloroxylon swietenia* bark was effective against tested organism as compared to aqueous extract. The free radical scavenging activity in methanol extract was found to be superior to aqueous extract. The obtained results concluded that the methanol extract of *Chloroxylon swietenia* bark possess potential antimicrobial and *in vitro* antioxidant activity as compared to aqueous extract. This activity might be attributed to the presence of phytochemicals in the plant.
Keywords: Phytochemicals, Antioxidant, *Chloroxylon swietenia*, Phenols, Flavonoids

Key message: *Chloroxylon swietenia* bark methanol extract own potential antimicrobial and *in vitro* antioxidant activity when compared to aqueous extract. This may be due to the phytochemical constituents present in the plant. It can be used as an antimicrobial agent.

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INTRODUCTION

Phytochemicals (from the Greek word phyto, meaning plant) are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients[1]. They protect plants from disease, damage and contribute to the plant's color, aroma and flavor. In general, the plant chemicals that protect plant cells from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack are called as phytochemicals [2]. Recently, it is clearly known that they have roles in the protection of human health, when their dietary intake is significant. More than 4,000 phytochemicals have been cataloged [3] and are classified by protective function, physico-chemical characteristics [4] and about 150 phytochemicals have been studied in detail [3].

Antimicrobials of plant origin have enormous therapeutic potential and have been used since time immemorial. They have been proved effective in the treatment of infectious diseases simultaneously mitigating many of the side effects which are often associated with synthetic antibiotics [5]. Many infectious diseases have been known to be treated with herbal remedies based on ethnobotanical knowledge. The antimicrobial efficacy of many plants is yet to be verified [6]. Plants have an almost limitless ability to synthesize aromatic substances of different functional groups, most of which are phenols or their oxygen-substituted derivatives. These compounds are known as secondary plant metabolites and have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property [7]. Keeping this in view, the current study was planned to investigate the phytochemicals, *in vitro* antioxidant and antimicrobial activities in aqueous and methanol extracts of *Chloroxylon swietenia* bark.

MATERIALS AND METHODS

Plant collection

Chloroxylon swietenia Dc. was collected from ABS botanical garden Karripatti near Salem and Kalvarayan hills. It was authenticated by Dr. V. Chelladhurai (Research Officer – Botany Central Council for Research in Ayurvedha and Siddha, Govt. of India. Retired). Nearly 3-4 kgs of bark have been collected and packed in an air tight zip lock cover.

Processing of plant bark

Collected bark of *Chloroxylon swietenia* was shade dried for 2-3 weeks. Then it was grinded in an electronic blender and packed in air tight zip lock covers.

Preparation of extraction

500gm of *Chloroxylon swietenia* bark was packed in Soxhlet apparatus for extraction, 2 liters of methanol was used as solvent. Soxhlet was kept running for 72 hours, until the solvent colour appears in the collection tube. Methanol was removed by evaporation using rota vapour at not more than 40°C. The residue was then placed in an oven at 40°C for about 48hours to remove the moisture. The resulting dried mass was then powdered, packed into a glass vial and stored in desiccators over silica gel until use.

Preliminary phytochemicals screening

Chemical tests were carried out on the alcoholic and aqueous extract using standard procedures to identify the preliminary phytochemical screening following the methodology of Harborne (1973) [8], Trease and Evans (1989) [9] and Sofowara (1993) [10].

Determination of total phenolic content [11]

Total phenolic content was carried out following the Folin- Ciocalteu method by Singleton and Rossi (1965). One ml of crude extracts solution containing (20 to 80 µg/ml) was added volumetric flask. 1 ml of Folin-

Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22ºC for 5 minutes; 7.5% of 0.75 ml of sodium bicarbonate solution was added and mixed thoroughly. The samples were measured spectrophotometrically at 765 nm using spectrometer after 90 min at 22ºC. The amount of total phenolics was determined as Gallic acid and equivalent and expressed as mg GAE/g dry weight.

Determination of total Flavonoid [12]

The test solution (standard or sample), 1 mL was mixed with 0.3 ml of $NaNO_2$ (5 %, w/v) and after 5 min, 0.5 ml of $AlCl_3$ (2 %, w/v) was added. Flavonoid standard solutions of 100 µM were used. A sample was mixed and 6 minutes later was neutralized with 0.5 ml of 1 M NaOH solution. The mixture was left for 10 min at room temperature and then subjected to spectral analysis in the range of 300–600 nm against the blank, where $AlCl_3$ solution was substituted by water. A (in the 50–500-µM concentration range) was the standard of choice for the expression of results at 510 nm. Total flavonoid content was expressed as mg /g quercetin.

Antimicrobial activity

Microorganisms

Pure culture of *S. aureus, B.subtilis, E.coli, K.pneumonia, P.aeruginosa* species of bacteria and *Candida albicans* and *C. tropicalis* species of fungi were procured from the Department of Microbiology of Thanjavur Medical College, Thanjavur. These microorganisms were identified and confirmed by Microbiologists, Department of Microbiology, Thanjavur Medical College, Thanjavur.

Disc diffusion method

The agar diffusion method (Bauer et al., 1966) [13] was employed for the initial assessment of antibacterial potential of the extracts. Petri plates were prepared by pouring 20 ml of Mueller – Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) allowed solidifying for the use in susceptibility test against bacteria and fungi. Plates were dried and uniformly spread. The excess inoculums were drained and the plates were allowed to dry for 5 min. After drying, the disc with extracts were placed on the surface of the plate with sterile forceps and gently pressed to ensure the contact with the incubated agar surface. Ciprofloxacin (5µg/disc) for bacteria and Ampho-B (10µg/disc) for fungi was used as positive control. 5 per cent DMSO was used as blind control in these assays. Finally, the inoculated plates were incubated at 37 °C for 24 h bacteria and 28 °C for 72 h fungi. The zone of inhibition was observed and measured in millimeters. Each assay in this experiment was repeated three times.

***In vitro* antioxidant activity**

DPPH ASSAY

The scavenging ability of the natural antioxidants of the plant extract towards the stable free radical DPPH was measured by the method of Shimada *et al*., (1992) [14]. Briefly, 2 ml aliquot of DPPH methanol solution (25µg/ml) was added to 0.5 ml sample solution at different concentrations. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. Then the absorbance was measured at 517nm in a spectrophotometer. L-Ascorbic acid was used as the standard.

$$\text{Radical scavenging activity (\%)} = 100 - \frac{A_C - A_S}{A_C} \times 100$$

Where A_C = control is the absorbance of the control and A_S = sample is the absorbance of reaction mixture (in the presence of sample). All tests were run in triplicates (n = 3), and the average values were calculated.

Hydrogen peroxide scavenging activity assay

A modified version of the method described by Ruch *et al*. (1989) [15] was used to determine the hydrogen peroxide scavenging ability of extracts. Ten µL of extracts was dissolved in 3.4 ml of a 0.1 M

phosphate buffer (pH 7.4) solution and mixed with 600 µL of a 43 mM solution of hydrogen peroxide (prepared in the same buffer). The absorbance of these solutions was measured at 230 nm against the corresponding blank solutions. Hydrogen peroxide scavenging capacities of the extracts were calculated using the following equation:

Scavenging percentage = [(ABlank – ASample)/ABlank] × 100.

Where A_{Sample}=absorbance of reaction mixture and
A_{Blank}=absorbance of blank mixture (distilled water instead extract).

ABTS radical scavenging activity

ABTS radical scavenging activity of *Chloroxylon swietenia* bark extract and fractions was measured by the ABTS cation decolorization assay as described by Re *et al*. (1999) [16] with some modifications. The ABTS radical cation ($ABTS^{\bullet+}$) was produced by reaction of 7 mM stock solution of ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand in dark at room temperature for 12 h before use. The $ABTS^{\bullet+}$ solution was diluted with methanol to give an absorbance of 0.7 ± 0.01 at 734 nm. Plant extracts and fractions (1 ml) were allowed to react with 2 ml of the $ABTS^{\bullet+}$ solution and the absorbance was measured at 734 nm after 1 minute. L-Ascorbic acid was used as the standard.

Statistical analysis

Tests were carried out in triplicate for 3 separate experiments. The scavenging activity of sample was expressed as 50% effective concentration (EC_{50}), which represented the concentration of sample having 50% of radical scavenging effect. The amount of extract needed to inhibit free radicals concentration by 50%, IC_{50}, was graphically determined by a linear regression method using Ms- Windows based graphpad Instat (version 3) software. Results were expressed as graphically / Mean ± standard deviation.

RESULTS AND DISCUSSION

Phytochemical analysis shows the presence of many medicinally important secondary metabolite types of phytoconstituents like alkaloids, coumarin, flavones, saponins, triterpenes, which indicates that the plant possesses high profile values and can be used to treat various kinds of diseases. The qualitative phytochemical investigation gave valuable information about the different phytoconstituents present in the extracts, which helps the future investigators regarding the selection of the particular extract for further investigation of isolating the active principle [17].

Table 1 Phytochemical analysis of *Chloroxylon swietenia* bark extract

S.no	Secondary metabolites	Methanol	Aqueous
1.	Tannins	+	+
2.	Steroids	+	-
3.	Saponins	-	-
4.	Alkaloids	-	-
5.	Phenols	++	+
6.	Glycosides	+	+
7.	Phenolic compounds	++	+
8.	Anthroquinones	+	+
9.	Flavonoids	+	+

(+) Presence; (-) Absence

Table 2 Quantitative analysis of Flavonoid and Total Phenol in methanol and aqueous *Chloroxylon swietenia* bark

Solvents	Total Phenol	Total Flavonoid
Methanol	21.3 ± 0.76	15.1 ± 1.04
Aqueous	13.5 ± 0.50	12.0 ± 0.50

Values are expressed as Mean ± SD for triplicates
Total phenolic content were expressed as mg/g tannic acid.
Total flavonoid content was expressed as mg /g quercetin.

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Table 1 represents the qualitative analysis of *Chloroxylon swietenia* bark extracts (methanol and aqueous). Preliminary phytochemical screening of *Chloroxylon swietenia* bark revealed that the presence of flavonoids, tannins, glycosides and phenols in both methanol and aqueous extract. Steroid, alkaloids and tannins were absent in aqueous extract. The quantitative analysis of the extract revealed that the methanol extract contained total phenol (21.3 ± 0.76) and total flavonoid (15.1 ± 1.04) while aqueous extract total phenol (13.5 ± 0.50) and total flavonoid (12.0 ± 0.50) (Table 2).

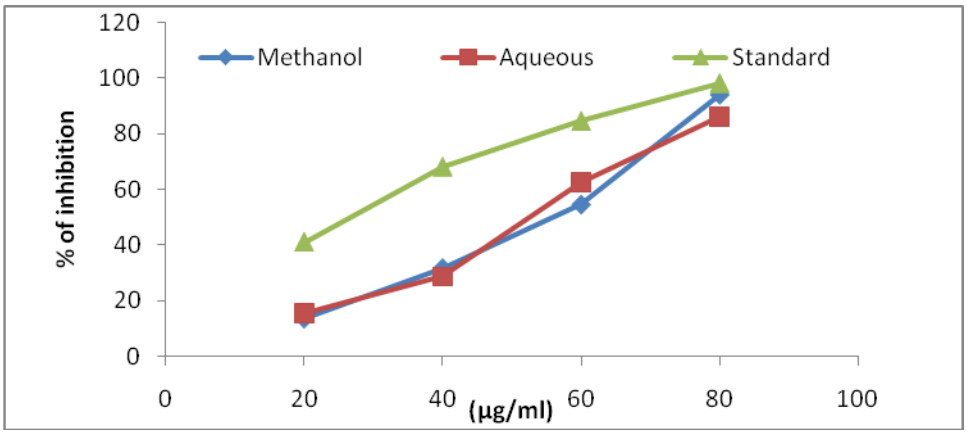
Phenolic compounds are considered to be the most important antioxidants of plant materials. They constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators. Antioxidant activity of phenolic compounds is based on their ability to donate hydrogen atoms to free radicals. The phenolic compounds are known to act as antioxidants not only because of their ability to donate hydrogen or electrons but also because they are stable radical intermediates. Interest in phenolics is increasing in the food industry because of their ability to retard oxidative degradation of lipids, thereby improving the quality and nutritional value of foods [18,19].

Flavonoids are found in almost all plant families. Flavonoids are present in different plant parts including the leaves, stem, roots, flowers and seeds and are among the most popular anti-cancer candidates worldwide. Flavonoid derivatives have a wide range of biological actions such as antibacterial, antiviral, anti-inflammatory, anticancer and anti-allergic activities. Some of these benefits are attributed to the potent antioxidant effects of flavonoids, which include metal chelation and free-radical scavenging activities [20].

***In vitro* antioxidant activity**

DPPH assay has become quite popular in natural antioxidant studies. One of the reasons is that this method is simple and highly sensitive. DPPH is one of the few stable and commercially available organic nitrogen radicals [21]. The antioxidant effect is proportional to the disappearance of DPPH in test samples. Various methods of monitoring the amount of DPPH in the antioxidant test system have been reported: electron spin resonance spectroscopy (ESR)/plant powders, NMR / catechins and UV spectrophotometry / polyphenols [22]. However, monitoring DPPH with a UV spectrophotometer has become the most widely and commonly used method recently because of its simplicity and accuracy. DPPH shows a strong absorption maximum at 517 nm (purple). Generally, DPPH radical scavenging activity of the plant extracts is concentration dependent and a lower IC_{50} value reflects better protective action. In the present investigation methanolic extract shows the IC_{50} value is lower than aqueous extract (Figure 1). The result was comparable to that of standard ascorbic acid. The IC_{50} value of the methanol, aqueous extracts and standard ascorbic acid were found to be 51.47, 51.73 and 26.50µg/ml respectively.

Figure 1: DPPH radical scavenging activity of *Chloroxylon swietenia* bark extracts



Standard - L-Ascorbic acid
Values are means of triplicate determinations (n=3)

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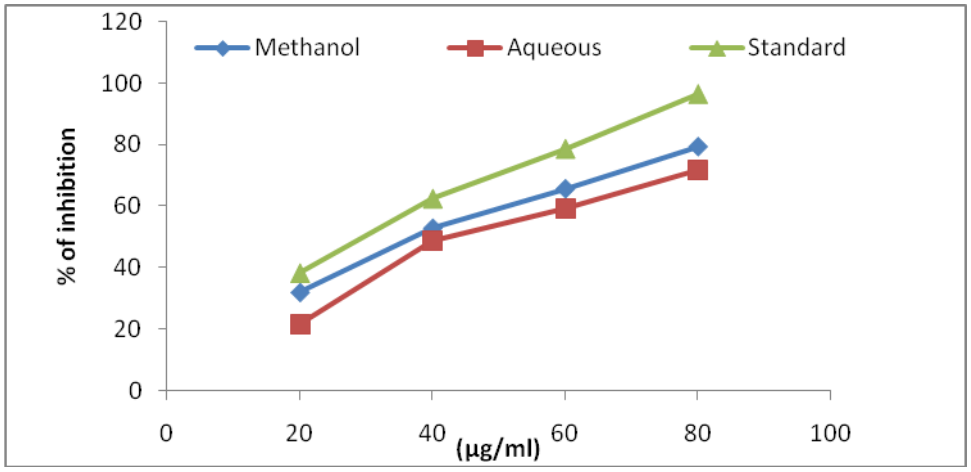
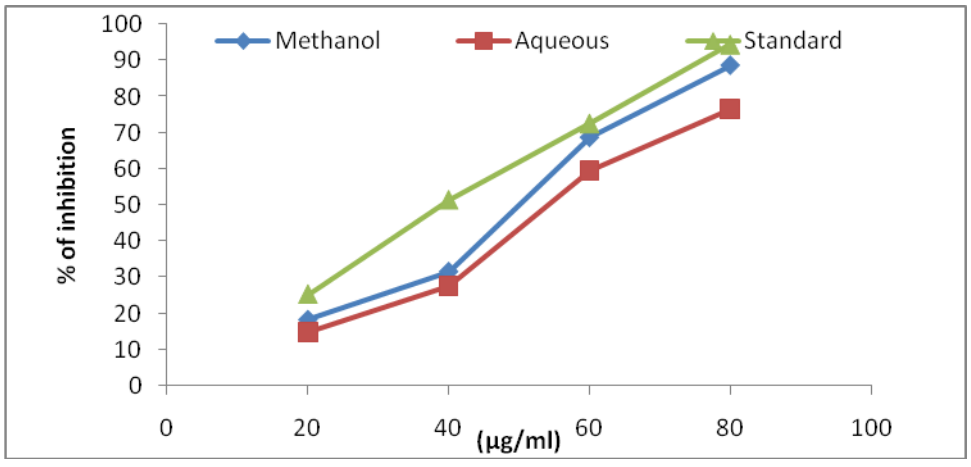
Figure 2: ABTS radical scavenging activity of *Chloroxylon swietenia* bark extracts



Standard - L-Ascorbic acid
Values are means of triplicate determinations (n=3)

The ABTS assay is based on the inhibition of the absorbance of radical cation, ABTS+, which has a characteristic wavelength at 734 nm, by antioxidants (Figure 2). The principle behind the technique involves the reaction between ABTS and potassium per sulphate to produce the ABTS radical cation ($ABTS^{+}$) which is a bluegreen chromogen. In the presence of antioxidant reductant, the colored radical is converted back to colorless ABTS. The order of ABTS radical scavenging activity of the extract was almost similar to that observed for DPPH [23]. The ABTS method is one of the most often used method for the determination of total antioxidant capacity. It is based on neutralization of radical cation formed by a single-electron oxidation of a synthetic ABTS chromophore to a strongly absorbing ABTS•+ radical according to the reaction ABTS-e- → ABTS• +. A stable ABTS radical cation, which has blue-green chromophore absorption, was produced by oxidation of ABTS with potassium persulfate prior to the addition of antioxidants. The antioxidant activity of the natural products, including carotenoids, phenolic compounds and some plasma antioxidants, is determined by the decolorization of the ABTS, by measuring the absorbance at 734 nm [16]. In the present investigation methanolic extract shows the IC_{50} value is lower than aqueous extract. The result was comparable to that of standard ascorbic acid. The IC_{50} value of the methanol, aqueous extracts and standard ascorbic acid were found to be 43.75, 49.62 and 30µg/ml respectively.

Figure 3: H_2O_2 radical scavenging activity of *Chloroxylon swietenia* bark extracts



Standard - L-Ascorbic acid
Values are means of triplicate determinations (n=3)

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Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+}ions to form hydroxyl radical and this may be the origin of many of its toxic effects [24]. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. The figure 3 mentioned the hydrogen peroxide scavenging activity of the methanol and aqueous extracts of *Chloroxylon swietenia* bark at low concentration (20µg/ml) showed 18.35% and 14.87 % of scavenging activity. At high concentration (80µg/ml) showed 88.64 % and 76.49 % scavenging activity. The IC_{50} value of the methanol, aqueous extracts and standard ascorbic acid was found to be 48.90, 55.14 and 40.54µg/ml respectively.

Figure 4: Antimicrobial activity of the Methanol extract of *Chloroxylon swietenia* Dc. bark

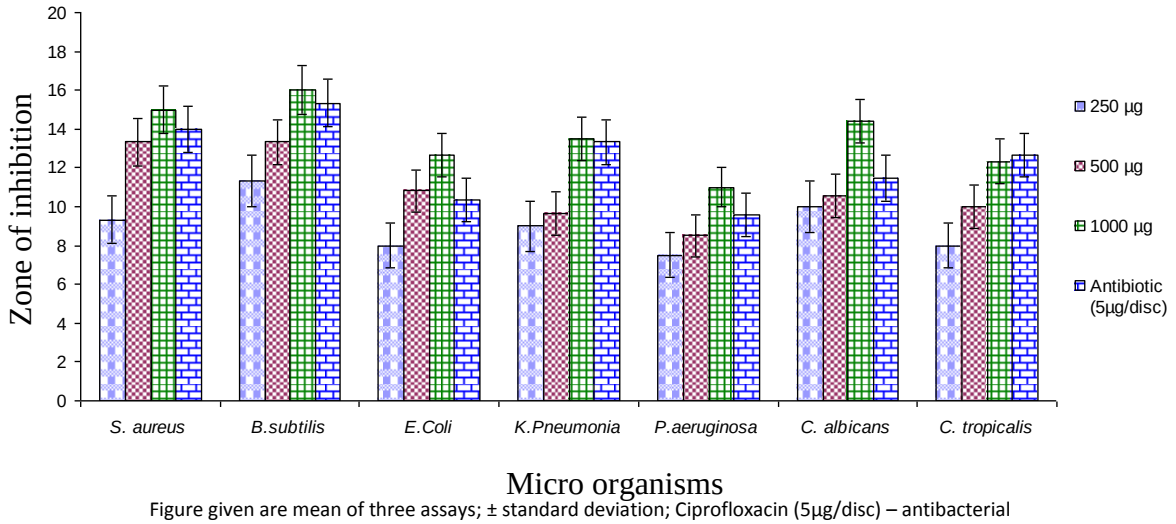

Figure given are mean of three assays; ± standard deviation; Ciprofloxacin (5µg/disc) – antibacterial drug; Ampho-B (10µg/disc) – antifungal.

Figure 5: Antimicrobial activity of the aqueous extract of *Chloroxylon swietenia* Dc. Bark

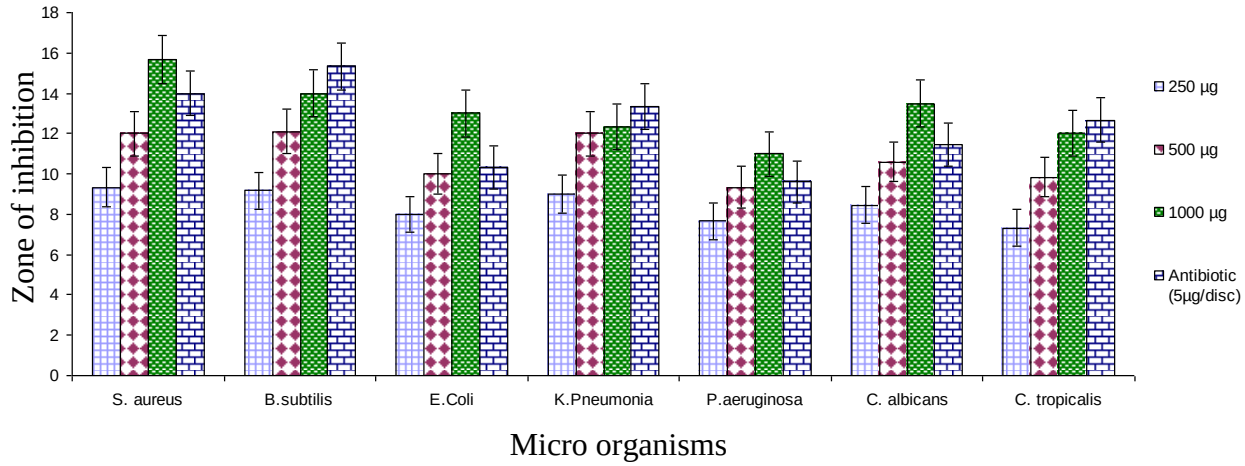

Figure given are mean of three assays; ± standard deviation; Ciprofloxacin (5µg/disc) – antibacterial drug; Ampho-B (10µg/disc) – antifungal.

Antimicrobial activity

Aqueous and methanolic extracts of *Chloroxylon swietenia* bark were screened against *S. aureus, B.subtilis, E.coli, K.pneumonia and P.aeruginosa* for bacteria, *Candida albicans and C. tropicalis* species of fungi were evaluated using the standard disc diffusion method. The solidified nutrient agar plates were swapped with the test organism and samples were impregnated. The *in vitro* antimicrobial activity of the *Chloroxylon swietenia* extract against these bacteria and fungi were qualitatively assessed by the presence of inhibition

zones. The inhibitory activities in culture media of the extracts were reported in figure 4 and 5 were comparable with standard antimicrobiotic viz. Ciprofloxacin (Bacteria) and Ampho- B (Fungi). Among the two extracts (Aqueous and Methanol), methanolic extract possess potential antimicrobial activity than aqueous extract.

The evaluation of antimicrobial potential by disc diffusion method indicated that all the bacterial tested organisms showed growth inhibition towards the plant extract, with differing sensitivity [25]. The methanol extract of pomegranate peels were more active when compared to water extract against various microorganisms [26]. These results confirmed the evidences in previous studies reported that methanol as a better solvent for more consistent extraction of antimicrobial substances from medicinal plant compared to other solvents, such as water, ethanol and hexane [27-29].

CONCLUSION

In conclusion, the results of the present study shows that the methanol and aqueous extracts of *Chloroxylon swietenia* bark which contain significant amount of flavonoids and phenols exhibits the greatest antioxidant activity through the scavenging of free radicals such as DPPH, ABTS and hydrogen peroxide which participate in various pathophysiology of diseases including diabetic, cardiovascular and ageing. Among the two extracts, methanolic extract contain rich source of phenols and flavonoids and also possess potential *in vitro* antioxidant and antimicrobial activity than aqueous extract. Overall, the plant extract is a source of natural antioxidants that can be important in disease prevention and health preservation.

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