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Bioefficacy of *Caesalpinia bonduc* (L.) Roxb. from Bhilai region against pathogenic clinical and MTCC microbial isolates

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Abstract

Medicinal plants as natural therapeutics are the vital source of bioactive compounds for the amelioration of several dreadful diseases since ages. The current investigation deals with antimicrobial efficacy and phytochemical analysis of *Caesalpinia bonduc* (L.) Roxb. family: Caesalpiniaceae a traditional medicinal plant, commonly known as Kantkarej. The antimicrobial bioefficacy of leaf, stem and root of *C. bonduc* were assessed against Clinical and MTCC microbial isolates of *Bacillus subtilis* (MTCC-441), *Staphylococcus aureus* (MTCC-96), *Escherichia coli* (MTCC-1687), *Klebsiella pneumoniae* (MTCC-3384) and the two fungal isolates viz., *Aspergillus niger* (MTCC-872) and *Candida albicans* (MTCC-183). Leaf chloroform extracts of *C. bonduc* emerged as the most effective extract with maximum zone of inhibition against clinical isolates of *S. aureus*, whereas maximum antifungal activity was recorded in stem chloroform extracts against clinical isolates of *A. niger*. The maximum activity index was recorded in case of leaf and stem chloroform extracts against clinical isolates of *S. aureus* and *A. niger* respectively. Whereas trivial activity was observed in case of MTCC isolates as compared to that of clinical isolates. The phytochemical analysis in leaf, stem and root of *C. bonduc* revealed the presence of phytochemicals viz., alkaloids, cardiotropins, flavonoids, glycosides, phlobatannins, phenolics, phytosterols, quinones, resins, saponins, tannins and terpenoids which might be responsible to confer antimicrobial potentiality. Thus, the present work will be a primary platform to screen out potent medicinal plant as antimicrobials useful in combating several microbial diseases with natural, less side-effects and cost-effective remedies for future generations.

Keywords: *Caesalpinia bonduc*, antimicrobial activity, clinical, MTCC, phytochemicals

Introduction

Medicinal plants are the emerging source of novel bioactive compounds possesses curative and health promoting potentialities [1]. The medicinal plants as natural remedies have been used since long back to heal several human pathogenic infections [2]. A wide array of secondary metabolites such as alkaloids, flavonoids, phytosterols, phenolics, terpenoids etc. are produced in different parts of plant such as leaf, stem and root, flower, fruit, seed and bark which possesses curative pharmacological activity [3-4]. However, these phytochemicals present in medicinal plant parts serve them for their defense mechanisms against predation by pathogenic microorganisms, insects and herbivores [5-7]. There is therefore the urgent need to look inwards for the search of efficacious medicinal plants with the aim of validating their ethno-medicinal importance and their bioefficacy testing which would stand as bedrock for antimicrobial therapy and will add to the list of potential drugs in near future.

In past few decades, the development of drug resistance among human pathogens such as *E. coli*, *K. pneumoniae*, *P. vulgaris*, *S. aureus*, *S. epidermidis* and undesirable side effects of antibiotics has become global public health concern [8]. This has necessitated the search for new antimicrobial compounds with high curative potentiality from alternative sources including medicinal plants [9]. The herbal medicines are developing since time immemorial, because these medicines are effective, easy to afford, easily available, less toxic and more acceptable in healing several pathogenic microbial diseases [10-11]. Thus, on the basis of above documentation the medicinal plants may become the foundation for the development of alternative therapy against dreadful microbial diseases [12].

In light of vast potentiality of medicinal plants as therapeutics an attempt was made to assess the bioefficacy of *Caesalpinia bonduc* (L.) Roxb. (Family: Caesalpiniaceae) common name: Kantkarej, is an annual spiny, medicinal shrub, found in tropical parts of India, Myanmar and Sri Lanka [13].

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It is an important ethnomedicinal plant traditionally used in various therapeutic purposes such as antimicrobials, anthelmintics, antivirals, anti-inflammatory, liver disorders, antispasmodic [14]. The plant is rich in many pharmaceutically active metabolites like alkaloids, flavonoids, glycosides, phenolics, phytosterols, steroids and tannins [15-16]. Thus, in view of vast potentiality of this plant an endeavour was made to assess the antimicrobial activity and phytochemical analysis of *C. bonduc* (L.) Roxb. against Clinical and MTCC microbial isolates with a hope that this study will open a new avenue and pave the way towards identifying novel biologically active antimicrobial compounds.

Materials and Methods

Plant Materials: The different plant parts of *Caesalpinia bonduc* (L.) Roxb. (leaf, stem and root) were collected from Housing board region of Bhilai, Chhattisgarh and brought to the laboratory (Figure 1). The samples were washed under running tap water to remove extra debris and were shade dried to attain a constant weight. The shade dried plant parts were crushed with the help of pestle-mortar and finally by electric blender (Remi). The powdered material was stored in air tight container at room temperature for further use.



Fig 1: Sample collection of different parts of *C. bonduc* (L.) Roxb

Extraction Procedure: 15 g of each powdered plant material was extracted with 150 ml of chloroform, acetone, methanol and aqueous solvent sequentially on the basis of polarity index using soxhlet apparatus (Figure 2) for 8-10 h. After extraction the extract was dried in hot air oven at 40°C and the dry weight was determined using dry weight detector (Figure 3) [17-18].



Fig 2: Soxhlet apparatus for extraction of phytochemicals

Preparation of Stock Solution: The stock solution of different extracts of *C. bonduc* was prepared by dissolving it in 50% dimethyl sulfoxide with mother solvents to get a 10% stock solution. Streptomycin (standard antibacterial) and

clotrimazole (standard antifungal) was dissolved in appropriate volume of sterile distilled water to get 0.5 mg/ml and 20 mg/ml.

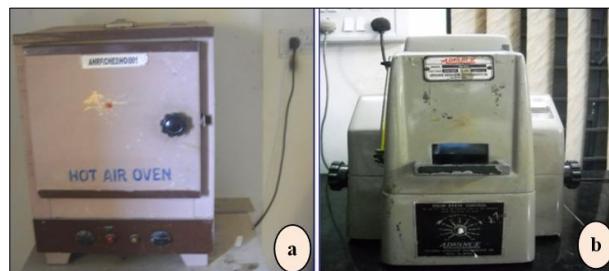


Fig 3: (a) Hot air oven and (b) Dry weight detector

Test Microorganisms: Four bacterial cultures, *Bacillus subtilis* (MTCC-441), *Staphylococcus aureus* (MTCC-96), *Escherichia coli* (MTCC-1687), *Klebsiella pneumoniae* (MTCC-3384) and the two fungal isolates *Aspergillus niger* (MTCC-872) and *Candida albicans* (MTCC-183) were procured from IMTECH, Chandigarh, India. Similarly the clinical isolates of above four bacteria and two fungi were procured from Department of Microbiology, Pt. J.L.N. Memorial Medical College, Raipur, Chhattisgarh, India.

Preparation of Bacterial Inoculums: The test organisms were maintained on nutrient agar slants. An overnight grown bacterial culture was used for inoculum preparation. One loop full of overnight growth from each bacterial culture was inoculated in 25 ml nutrient broth and incubated at 37°C for 24 h in incubator. The inoculum size of each bacterial strain was standardized by adjusting the optical density of the culture broth by adding saline suspension to a turbidity corresponding to 0.08-0.13 at 620 nm using a spectrophotometer which was equivalent to 10⁸ cfu/ ml [19].

Antibacterial Activity: The antibacterial activity of the crude extracts was determined by agar well diffusion method. 200 µl of standardized inoculums 10⁸ cfu/ml were spread on Muller Hinton Agar (Hi-media) plate. Wells were punched into the Muller Hinton Agar plate using a sterile 6 mm diameter cork borer. 20 µl of the crude extracts at 10 mg/ml were introduced into the well, allowed to stand at room temperature for about 2 h and then incubated at 37°C. Controls were set up in parallel using the solvents that were used to reconstitute the extracts. The plates were observed for the zone of inhibition after 24 h. The activity of the extracts was compared with that of streptomycin. The mean and standard error of the diameter of inhibition zones were calculated [20].

Preparation of Fungal Inoculums: The test fungal organisms were maintained on potato dextrose agar slants. One loop full of each fungal culture was inoculated in 25 ml potato dextrose broth and incubated at 28-30°C in incubator. Stock inoculum suspensions were prepared from 7 day-old cultures grown on potato dextrose agar (Hi-media) following National Committee for Clinical Laboratory Standards [21]. Stock suspensions were adjusted to optical densities that ranged from 0.09-0.11 at 530 nm using a spectrophotometer which was equivalent to 0.9 × 10⁴ to 4.7 × 10⁴ cfu/ ml.

Antifungal Activity: The antifungal activity of the crude extracts was determined in accordance with the agar well diffusion method. The fungi were inoculated on potato

dextrose agar (Hi-media) plates. Wells were punched into the PDA plate using 6 mm diameter cork borer. 20 μ l of the crude extracts at 20 mg/ml were introduced into the well, allowed to stand at room temperature for about 2 h and the incubated at 28-30°C. Controls were set up in parallel using the solvents that were used to reconstitute the extracts. The plates were observed for the zone of inhibition after 2-3 days. The activity of the extracts was compared with that of clotrimazole. The mean and standard error of the diameter of inhibition zones were calculated [22].

Zone Size Interpretation: Test organism showing a clear zone of inhibition (ZOI) was scored as (07-10 mm) as non-inhibitory activity (Resistant), inhibition ranging (11-15 mm) was considered to be inhibitory activity (Sensitive), and greater than (16-20 mm) was considered as significant inhibitory or antimicrobial activity [23].

Activity Index: The activity index was assessed for different solvent extracts of *C. bonduc* (L.) Roxb. with the selected Clinical and MTCC isolates using standard antibiotics as streptomycin and clotrimazole. The activity index was calculated and expressed as zone of inhibition with test sample/zone of inhibition with standard antibiotics [24-25].

Qualitative Phytochemical Screening: Phytochemical analysis of the extracts of root, stem and leaf of *C. bonduc* (L.) Roxb. was carried out following [26-29].

Test for Alkaloids

(a) Mayer's Test: To 1 ml of extract, 1 ml of Mayer's reagent (1.35 g Mercuric chloride and 5 g Potassium Iodide were dissolved in 100 ml distilled water) was added. Whitish yellow or cream-colored precipitate indicated the presence of alkaloids.

Test for Cardioglycosides

5 ml of plant extract was treated with 2 ml of glacial acetic acid containing a drop of 1% FeCl_3 solution. 1 ml of conc. H_2SO_4 was layered over it. A brown ring at the interface indicates a deoxy sugar characteristic of cardioglycosides. A violet ring may appear below the ring, while in acetic acid layer, a greenish ring may form just gradually throughout thin layer.

Test for Fixed Oil and Fat

(a) Saponification Test: A few drops of 0.5 N alcoholic KOH was added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on water bath for 2 h. Formation of soap or partial neutralization of alkali indicated the presence of fixed oil and fat.

Test for Flavonoids

(a) Ferric Chloride Test: Test solution with few drops of 1% ferric chloride solution showed intense green colour in positive cases.

Test for Glycosides

(a) Legal's Test: 5 ml of the extract was treated with pyridine, sodium nitroprusside solution was added and made alkaline by using 10% NaOH. Presence of glycoside was indicated by the development of pink color.

Test for Gums and Mucilage: 5 ml extract was diluted with 10 ml of distilled water in a flask and to this 25 ml of absolute alcohol was added with constant stirring. White precipitate indicated the presence of gums and mucilage.

Test for Phytosterols

(a) Salkowski Test: To the 1 ml of prepared chloroform solution containing extract, few drop of conc. H_2SO_4 was added. Formation of brown ring indicates the presence of phytosterols.

Test for Phlobatannins: 0.5 ml of the extract was boiled with 5 ml of water and 1% HCl in a test tube for 2 min. The colour change indicates the presence of phlobatannins.

Test for Quinones

A few drops of NaOH were mixed with the plant extract and shaken vigorously. A blue green or red colour indicated the presence of quinones.

Test for Resins

To 5 ml of the extract in a test tube, 5 ml of the copper acetate solution was added. The resulting solution was shaken vigorously and allowed to separate. The separation of a green coloured solution was considered as positive for resins.

Test for Saponins

(a) Foam Test: 5 ml of the extract was diluted with distilled water and made upto 20 ml. The suspension was shaken in graduated cylinder for 15 min. A 2 cm layer of foam indicated presence of saponins.

Test for Tannins and Phenolic Compounds

- Ferric Chloride Test:** 5% ferric chloride solution was added to the extract in a test tube and deep green colour was observed in positive test of tannins.
- Lead Acetate Test:** 5 ml extract was diluted with equal volume of distilled water in a test tube. 3 ml of 10% lead acetate solution was added to it. A bulky white precipitate indicated the presence of phenolic compound.

Test for Terpenoids

5 ml of the plant extract was mixed with 2 ml of chloroform and 1 ml conc. H_2SO_4 was added to form a layer. Formation of a reddish-brown coloration at the interface indicated the presence of terpenoids.

Results and Discussion

In the present study, *Caesalpinia bonduc* (L.) Roxb. (Family: Caesalpiniaceae) was explored for its phytochemical analysis and antimicrobial activity against clinical and MTCC isolates. Medicinal plants play a vital role in health care system as folklore remedy since time

immemorial. The isolated bioactive compounds from crude extracts confer biological activity and play a key role in the development of novel drugs. In the current investigation, different parts of *Caesalpinia bonduc* (L.) Roxb. viz., leaf, stem, root was successively extracted using four different solvents based on their polarity index as chloroform, acetone, methanol and aqueous. The total percentage yield of *C. bonduc* (L.) Roxb. extracts were found to be leaf (48.67%), stem (22.74%) and root (34.40%) respectively (Table 1).

Table 1: Percentage yield of *C. bonduc* (L.) Roxb. in different solvent extracts

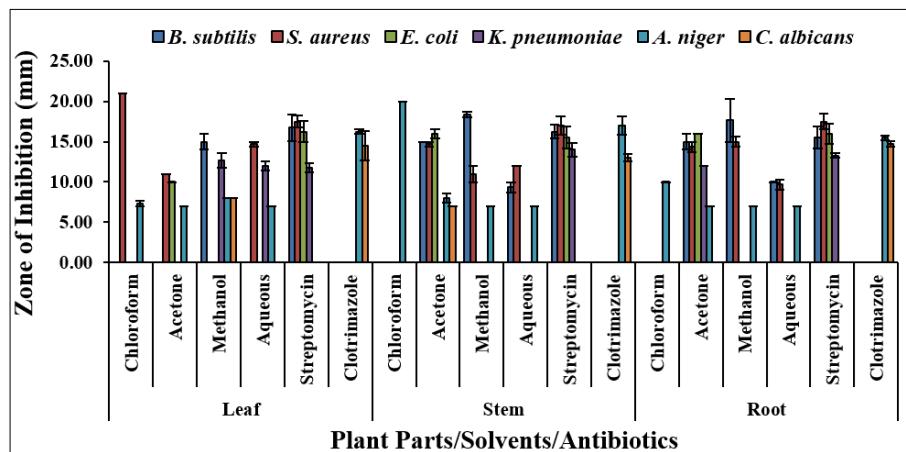
Solvents	Plant Parts		
	Leaf	Stem	Root
Chloroform	10.60	2.47	2.87
Acetone	6.00	1.87	3.13
Methanol	17.67	5.93	5.67
Aqueous	14.40	12.47	22.73
Total Percentage Yield	48.67	22.74	34.40

The highest percentage yield was recorded in methanol extract of leaf (17.67%) and aqueous extract of stem (12.47%) and root (22.73%) respectively. Whereas, lowest percentage yield was found in acetone extract of leaf (6.00%) and stem (1.87%) and chloroform extract of root (2.87%). The highest percentage yield was found in root extract in case of aqueous solvent (22.73%) as compared to other organic solvents. It might be due to the fact that water is a universal solvent and used to extract phytochemicals possessing antimicrobial activity [30-31].

**Fig 4:** Antimicrobial activity (Zone of inhibition in mm, Mean \pm SE) of *C. bonduc* (L.) Roxb

The antimicrobial activity of leaf, stem and root extracts of *C. bonduc* (L.) Roxb. were assessed using agar well diffusion method on Clinical and MTCC isolates (Figure 4). The leaf, stem and root extracts of *C. bonduc* (L.) Roxb. were found almost equally effective against clinical isolates. The

maximum zone of inhibition was observed in case of chloroform extract of leaf against *S. aureus* (21.00 ± 0.00 mm) followed by methanol extracts of stem and root against *B. subtilis*. Minimum inhibition was observed by aqueous extract of stem against *B. subtilis* (9.33 ± 0.66 mm) and aqueous extract of root against *S. aureus* (9.66 ± 0.66 mm). The root acetone extract of *C. bonduc* inhibited the growth of all bacteria tested followed by stem and leaf. *E. coli* was found to be more susceptible to acetone extract of *C. bonduc* (L.) Roxb. The chloroform extract of root and stem showed no significant activity against *B. subtilis*, *S. aureus*, *E. coli* and *K. pneumoniae*. Methanol and aqueous extracts of root and stem exhibited inhibitory activity against *B. subtilis* and *S. aureus*. The acetone extract of stem showed inhibition to all the organisms except *K. pneumoniae*. The chloroform and acetone extracts of leaf showed inhibitory response against *S. aureus* and *E. coli* respectively. Whereas, inhibitory activity was recorded in methanol leaf extracts against *B. subtilis* and *K. pneumoniae* and aqueous leaf extract against *S. aureus* and *K. pneumoniae*. The chloroform extract of *C. bonduc* (L.) Roxb. stem showed maximum zone of inhibition against *A. niger* (20.00 ± 0.00 mm) followed by methanol leaf extract with zone of inhibition of 8.00 ± 0.00 mm against *A. niger* whereas, the chloroform extract of root showed maximum inhibition of 10.00 ± 0.00 mm against *A. niger*. The *C. albicans* was found to be less susceptible towards different extracts tested (Figure 5). The maximum antimicrobial activity was observed in case of organic solvents as compared to aqueous solvents although the yield was found to be maximum in aqueous solvent [32]. The decline in bioactivity of aqueous extract might be due to the excessive heating of the aqueous soluble active constituents during the extraction process which often affect biologically active substances such as flavonoids, essential oils and other heterogeneous phytoconstituents present in the extract [33].

**Fig 5:** Antimicrobial activity of *C. bonduc* (L.) Roxb. against clinical isolates "Streptomycin"- Standard antibacterial, "Clotrimazole"- Standard antifungal

The antibacterial activity of leaf, stem and root of *C. bonduc* (L.) Roxb. were assessed against MTCC microbial cultures and the acetone extract of *C. bonduc* (L.) Roxb. root showed maximum zone of inhibition against *K. pneumoniae* (14.33 ± 0.33 mm) followed by *B. subtilis* (13.66 ± 0.33 mm). The acetone extract of stem was found responsive against *K. pneumoniae*. The methanol root extract of *C. bonduc* (L.) Roxb. was found to be inhibitory towards *E. coli*. All the extracts of different parts showed fairly equal zone of inhibition against MTCC fungal cultures. However, in *C. albicans* (MTCC-183) the inhibitory response could only be

seen in stem acetone and methanol extracts of the plant under investigation (Figure 6). The results of antimicrobial activity revealed that leaf extract was found to be more inhibitory against selected microbes followed by stem and root. Similar findings were also well documented [34]. In the present study the Gram-positive bacteria were found to be more susceptible than Gram-negative bacteria towards the plant extracts tested. It is due to the fact that in case of Gram-positive bacteria the outer layer of cell wall is made up of single layer of peptidoglycan whereas, Gram-negative bacterial cell wall is made up of thick murein layer and phospholipids with

lipopolysaccharides which makes the cell wall impermeable for antimicrobial substances [35-36]. However, in case of fungal isolates *A. niger* was found to be more susceptible for the extracts as compared to that of *C. albicans*. The outcome of the results revealed that as compared to clinical isolates the

MTCC isolates showed no significant activity which justifies that MTCC isolates are more resistant towards different solvent extracts of *C. bonduc* (L.) Roxb. as that of clinical isolates.

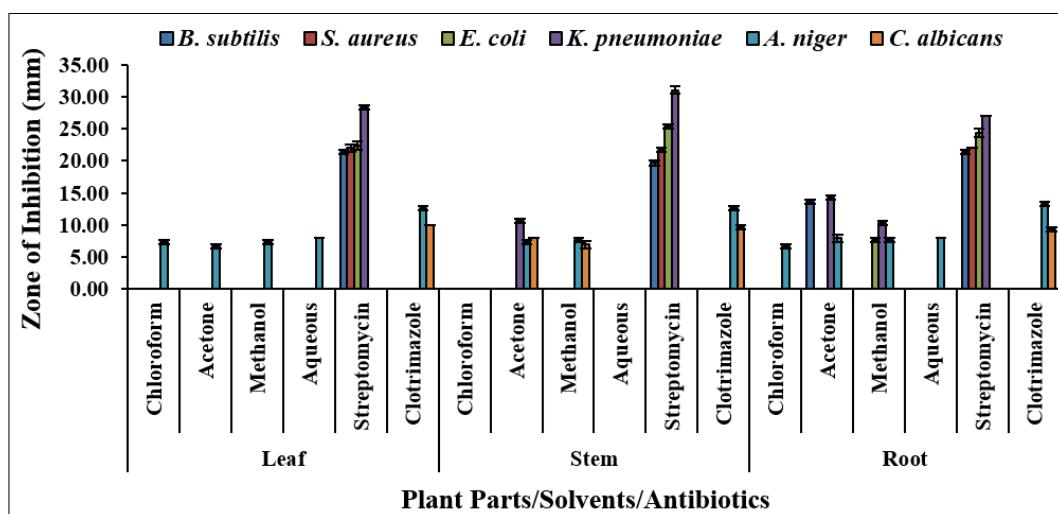


Fig 6: Antimicrobial activity of *C. bonduc* (L.) Roxb. against MTCC isolates, “Streptomycin”- Standard antibacterial, “Clotrimazole”- Standard antifungal

The activity index of *C. bonduc* (L.) Roxb. extracts against clinical microbial isolates with reference to streptomycin and clotrimazole revealed that chloroform extract of leaf of *C. bonduc* (L.) Roxb. was found to be maximum for *S. aureus* (1.20) followed by methanol (1.07) and aqueous (1.02) extracts for *K. pneumoniae*. The methanol extract of stem showed maximum activity index for *B. subtilis* (1.12)

followed by acetone extract for *E. coli* (1.03). The least activity index was observed in case of aqueous extract of stem and root for *B. subtilis* (0.57) and *S. aureus* (0.55). The highest activity index was recorded for chloroform extract of stem (1.17) and least activity index was observed for methanol (0.41) and aqueous extracts (0.41) of stem for *A. niger* (Figure 7).

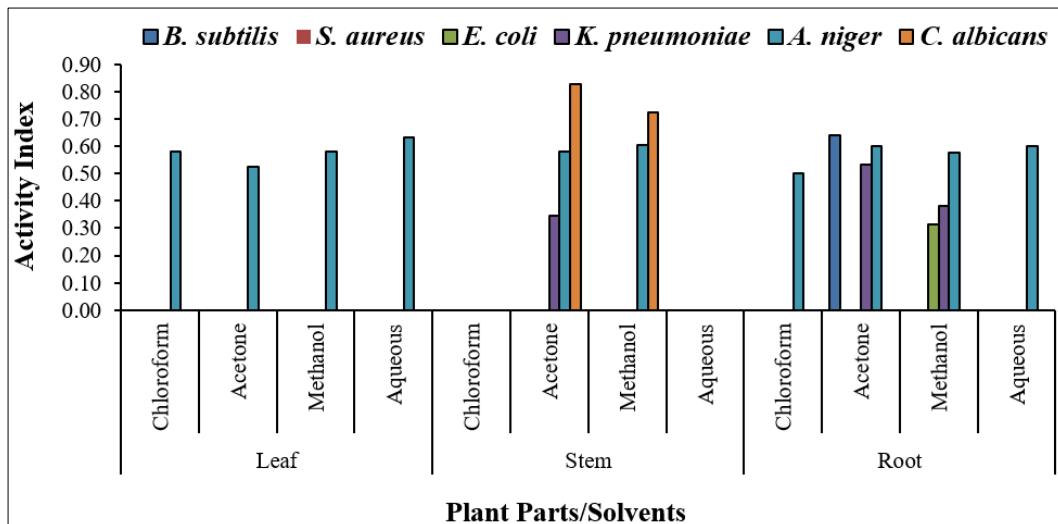


Fig 7: Activity index of *C. bonduc* (L.) Roxb. against clinical isolates with streptomycin and clotrimazole

The highest activity index of *C. bonduc* (L.) Roxb. against MTCC microbial isolates with reference to streptomycin and clotrimazole was noticed in acetone extract of root (0.64) for *B. subtilis* and the least activity index was observed in case of methanol root extract as (0.31) for *E. coli*. The maximum activity index of 0.63 was recorded in case of aqueous extract of leaf and least activity index recorded in chloroform extract of root as 0.49 against *A. niger* (MTCC-872). The stem acetone and methanol extracts recorded activity index of 0.82 and 0.72 in case of *C. albicans* (MTCC-183) (Figure 8). The

activity index of leaf, stem and root of *C. bonduc* (L.) Roxb. was recorded against standard antibiotics. The highest activity index was recorded in chloroform extract of leaf and acetone extract of root in clinical isolates of *S. aureus* and *B. subtilis* (MTCC-441), respectively with respect to streptomycin. In case of fungus, the highest activity index was recorded in chloroform extract and acetone and methanol extract of stem in clinical isolates of *A. niger* and *C. albicans* (MTCC-183), respectively with respect to clotrimazole.

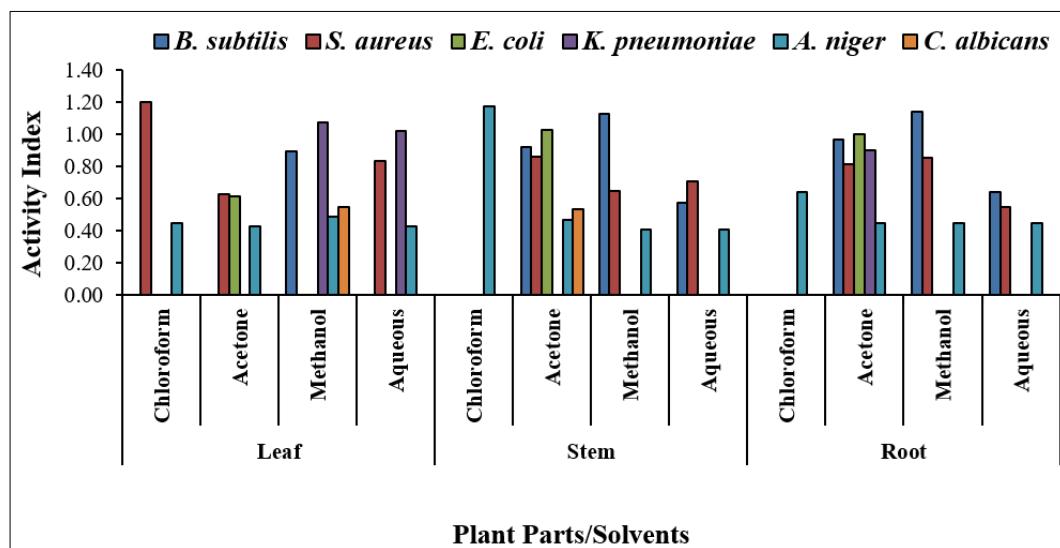


Fig 8: Activity index of *C. bonduc* (L.) Roxb. against MTCC isolates with streptomycin and clotrimazole

Phytochemicals such as alkaloids, flavonoids, saponins and tannins etc are present in the plants which provide defensive system against microorganisms. The present study focused on medicinally important bioactive phytochemicals in various solvent extracts of leaf, stem and root of *C. bonduc* (Table 2). Phytochemical analysis of explored plant extracts revealed the presence of varied phytochemicals such as alkaloids, cardiaoglycosides, flavonoids, resins, saponins and tannins etc were recorded from different parts. Leaf, stem and root are significantly important part of the plants where many physiological activities take place. The medicinal plants contain several bioactive phytochemicals such as alkaloids, cardiotropin glycosides, flavonoids, glycosides, fixed oil and fats, phlobatannins, gums, mucilage, tannins, quinones and

terpenoids^[37]. These phytochemicals make a defensive line in the plants against predation by microorganisms, insects and herbivores which are responsible for antimicrobial activity. In the present study the phytochemical analysis revealed the presence of several phytocompounds in different parts of *C. bonduc* (L.) Roxb. such as alkaloids, cardiotropin glycosides, flavonoids, glycosides, phlobatannins, phenolics, phytosterols, quinones, resins, saponins, tannins and terpenoids etc. Similar findings were also documented^[38]. The preliminary screening of *C. bonduc* (L.) Roxb. would thus be meaningful as it will open a new avenue for further investigations of certain bioactive principles responsible for the antimicrobial efficacy.

Table 2: Phytochemical analysis of the extracts of *C. bonduc* (L.) Roxb. in different solvents

Phytochemicals	Different solvent extracts of various parts of <i>Caesalpinia bonduc</i> (L.) Roxb.											
	Leaf extract				Stem extract				Root extract			
	C	A	M	Aq	C	A	M	Aq	C	A	M	Aq
Alkaloids	-	-	-	+	-	+	+	-	-	+	+	-
Cardioglycosides	+	+	-	+	+	+	+	+	+	-	+	+
Fixed Oil & Fats	+	-	+	-	+	-	+	-	-	-	+	-
Flavonoids	-	+	+	+	-	+	+	+	-	+	+	+
Glycosides	+	+	+	+	+	-	+	+	+	+	+	+
Gum & Mucilage	-	-	-	-	+	-	-	-	+	-	-	-
Phlobatannins	+	+	+	+	-	+	+	+	-	+	+	+
Phenolics	-	-	+	+	-	+	+	+	-	+	+	+
Phytosterols	-	-	+	+	+	+	+	+	+	+	+	+
Quinones	-	+	+	-	+	+	+	+	-	+	+	-
Resins	-	+	+	-	-	-	+	-	-	-	-	-
Saponins	-	-	+	+	-	-	+	+	-	+	+	+
Tannins	+	+	+	+	-	+	+	+	-	+	+	+
Terpenoids	-	-	+	+	+	+	+	+	+	+	+	+

“C”: Chloroform, “A”: Acetone, “M”: Methanol, “Aq”: Aqueous, “-”: Negative, “+”: Positive

Conclusion

The present research investigation was focused on the assessment of antimicrobial and phytochemical analysis in different solvent extracts of leaf, stem and root of *C. bonduc* (L.) Roxb. against four bacterial and two fungal clinical and MTCC isolates. The results revealed that the maximum activity index was recorded in leaf chloroform, root and stem methanol extracts against clinical isolates of Gram-positive organisms viz., *S. aureus* and *B. subtilis*, respectively, whereas in case of fungus maximum activity index was recorded in stem chloroform extracts against clinical isolates of *A. niger*.

However, no significant activity was recorded against MTCC isolates as compared to clinical isolates. The above findings are in accordance with the literature which revealed that the medicinal plants have tremendous therapeutic properties and alternatives to synthetic antibiotics in near future. Thus, in light of vast potentiality of *C. bonduc* (L.) Roxb. against pathogenic microbes the present study will provide primary platform for the development of antimicrobial phytomedicines. However, the crude extracts if purified can pin pointedly focus on the bioactive compound possessing antimicrobial potentiality with better, less side-

effects and cost-effective novel drug development which would be the future perspective of the proposed work.

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