ORIGINAL ARTICLE

Phytopharmacognostical, genetic barcoding, and *in vitro* antimicrobial evaluation on stem bark of *Combretum decandrum* Jacq.

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Abstract

Introduction: *Atundi* or *Kara kukundi* (*Combretum decandrum* Jacq.) is an ethnobotanical plant traditionally used for the treatment of various ailments and Stem bark, is used as a substitute of betel nut for chewing. Review of literature revealed that the plant has been recently explored on pharmacognostical and analytical aspects focusing on its root, stem, and leaf, but stem bark remains unexplored. In present study, pharmacognostical, analytical, and *in vitro* antimicrobial activity of stem bark have been explored. **Materials and Methods:** All studies were carried out by the following standard protocols and statistics was applied using Microsoft Excel worksheet. **Results and Discussion:** Stem bark is brownish in color while inner surface is brown in color with splintery fibrous facture. Diagrammatic T.S. of stem bark shows cork, several layers of cortex often embedded with cluster and rosette crystal with uniseriate medullary rays. Water extractive value is 20.52 ± 2.67. Qualitative test revealed presence of carbohydrate, alkaloids, and tannin in aqueous and methanol extracts. High performance thin layer chromatography study revealed 5 peaks and 4 peaks at short and long ultraviolet respectively. The antimicrobial activity of *C. decandrum* stem bark (CDSt.Br.) aqueous extract reveals that there is considerable increase in zone of inhibition with increase in concentration. **Conclusion:** The macroscopic key identification character of CDSt.Br. is that inner surface is brown in color with splintery fibers and microscopy character is the presence of abundant cluster and rosette crystals. The antimicrobial activity of CDSt.Br. aqueous extract reveals that maximum zone of inhibition is observed at 200 mg/ml.

Key words: Antimicrobial activity, *Anukta dravya*: *Atundi*, *Combretum decandrum*, stem bark

INTRODUCTION

Atundi or Kara kukundi is an ethnobotanical plant traditionally used for the treatment of various ailments such as skin diseases, gastric troubles, and diarrhea antidote in snake bite, externally applied on wounds and the bark is used as a substitute of betel nut for chewing.[1,2]

Atundi identified botanically as *Combretum decandrum* Jacq. (*Combretum roxburghii* Spr.), a large woody sarmentose rusty pubescent climbing shrub of family Combretaceae. Young parts and spikes are brown, rusty-villous in axillary and terminal panicles with large white bracts. Leaves are simple, petiolate, obovate to lanceolate in shape. Flowers are greenish-white, pentamerous. Bracts are opposite, creamy white, leaf-like upper gradually smaller, pubescent; bracteoles sub-persistent, villous, linear, spathulate, and acuminate. Fruits are with 5 or less often with 4 papery wings.[3,4]

Review of literature revealed that the plant has been recently explored on pharmacognostical and analytical

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aspects focusing on its root, stem, and leaf. Although it is traditionally claimed for various pharmacological actions along with antimicrobial activity, the stem bark of the plant is not much explored from its pharmacognostical, analytical, and *in vitro* antimicrobial activity.[5] Hence, in this research article stem bark of the *C. decandrum* is studied for its anatomy, analytical, and antimicrobial activity.

MATERIALS AND METHODS

Collection and Authentication

Plant sample along with stem bark was collected by the first and second authors from one of its natural habitat, Gandhamardan hills, Odisha, in month of September 2016 with help of local taxonomist. Before collection, the herbarium was submitted to pharmacognosy laboratory and Botanical Survey of India for authentication. Herbarium submitted to Pharmacognosy lab of the Institute was provided with reference no. phm/6146/2014-15 and letter no. CNH/55a/2013/Tech.II/116 from Botanical Survey of India [Figure 1a-d].

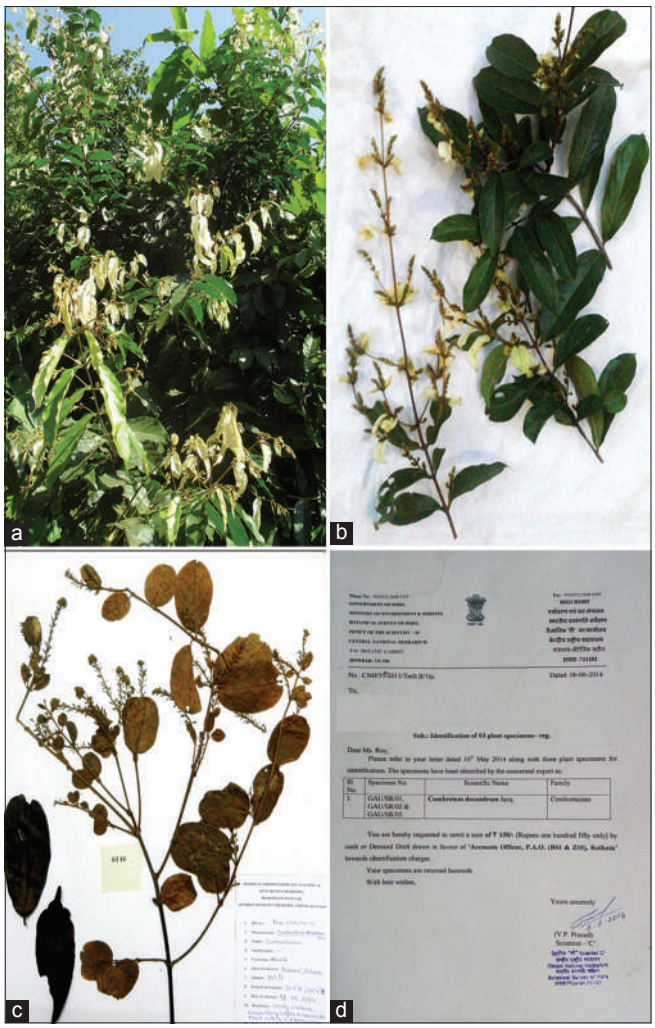


Figure 1: (a) Plant in natural habitat, (b) flowering twig, (c) herbarium phm/6146/2014-15, (d) certificate from BSI

Pharmacognostical Study

Macroscopic observations were made with naked eyes and centimeter scale was used to measure the length of stem bark cut pieces. The stem bark cut pieces were washed and transverse sections were taken cleared with choral hydrate to observe the anatomy of stem bark with help of Quasmo Binocular compound microscope. For histochemical tests, the thick transverse sections of the stem bark were exposed to Iodine, Phloroglucinol, and HCl for observation of starch grain and lignified tissue.

To obtain powder for powder microscopy, the cut pieces of shade-dried stem bark were grounded by mechanical grinder and sieved through 80# sieve. For micrometry, triplicate readings were recorded and mean value was taken into consideration along with standard mean of deviation.[6]

Genetic Barcoding

The study was carried out at Aristogene Biosciences Private Limited, Bengaluru. The DNA was isolated from fresh leaf samples using homogenizer, lysis buffer, and centrifuged. The supernatant was discarded and pellet was washed and exposed to column purification. The barcoding was based on sequence homology and phylogenic analysis. The forward primer used was: 1016_245_012_PCR_B_PRIMER_2-C05.ab1. Reverse primer was not used as the read of forward primer was quite good.

Physicochemical Parameters and Qualitative Analysis

The powder of stem bark was exposed to physicochemical, i.e., pH, loss on drying, total ash value, acid insoluble ash value, water-soluble extractive value, and alcohol soluble extractive value, protocols followed as recommended by API. For qualitative analysis, the presence of various secondary metabolites dissolved in water and alcohol extract was carried out as per reference.[7,8]

Quantification of Total Phenolic Content (Folin–Ciocalteu Reagent)

The total phenolics content of the extract was estimated according to the method described by Singleton and Rossi. The concentration of methanolic extracts solution was 10 mg/10 ml. From this solution, 1 ml was taken in test tubes and by dilution with same solvent up to 10 ml. This is stock solution. From stock solution, different concentrations were taken in different test tubes. This same procedure was used for standard. Gallic acid was used as a standard; 1 ml of Folin–Ciocalteu reagent was added in this concentration and the content of the flask was mixed thoroughly and 5 min later 4 ml of 20% sodium carbonate was added, and the mixture

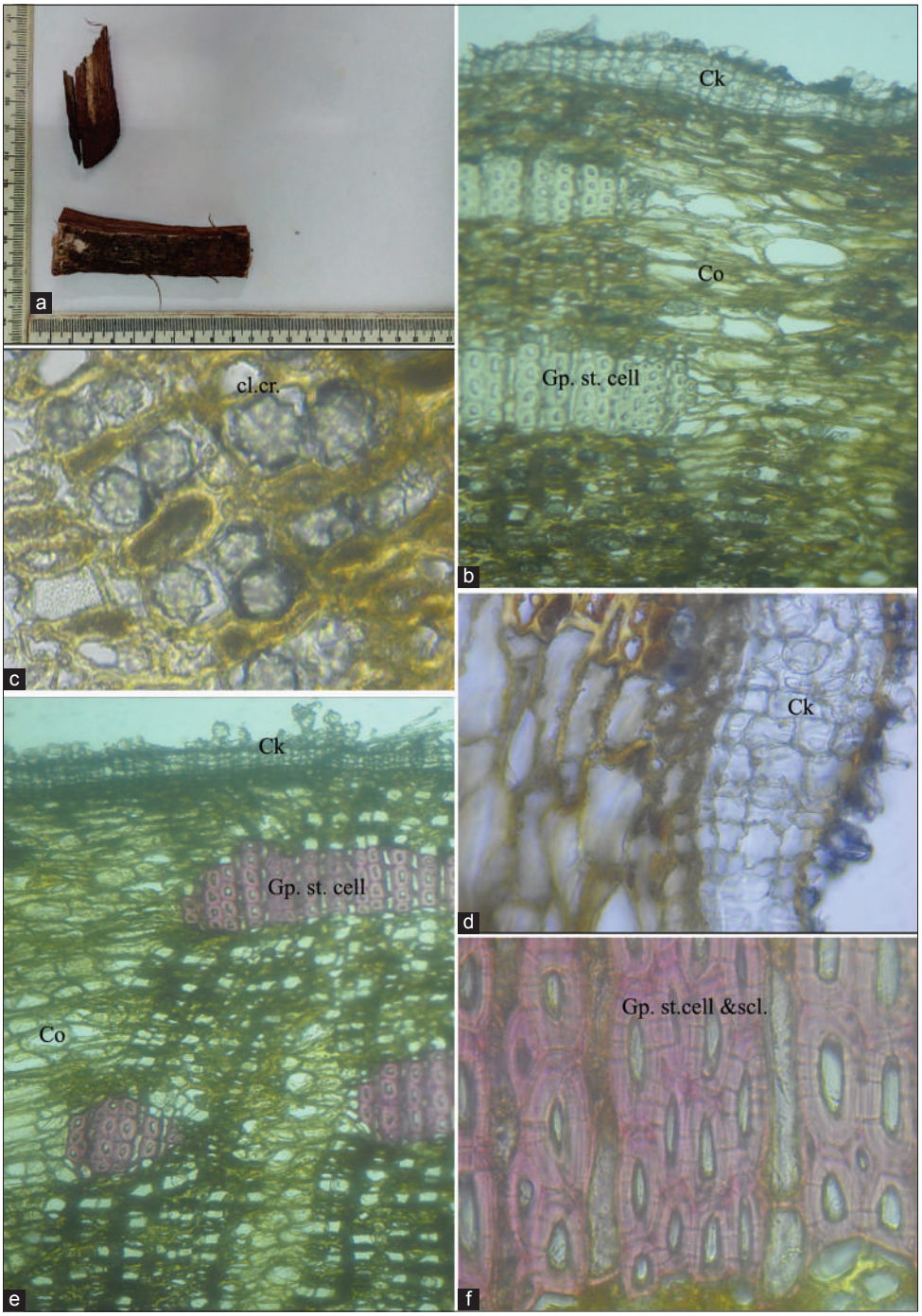


Figure 2: (a): Stem bark cut pieces, (b) T.S. of mature stem bark unstained, (c) cortex embedded with rosette and cluster crystals, (d) cork and cortex in enlarge view, (e): T.S. of stem bark stained, (f) group of lignified stone cells and sclereids. Co – cortex, Ck – cork, gr.sc.stn.cell – group of sclereids and stone cells, cl.cr. – prismatic crystal

was allowed to stand for 30 min with intermittent shaking. The absorbance of the blue color that developed was read at 765 nm in ultraviolet (UV) spectrophotometer.[9]

High Performance Thin Layer Chromatography (HPTLC) Study

Methanolic extract of stem bark was exposed to HPTLC study. The solvent system used for the study is toluene:ethyl acetate (9:1).

Chromatographic Conditions

Application mode was CAMAG Linomat V, development chamber used was of CAMAG Twin trough Chamber. Precoated Silica Gel$_{GF254}$ plates were used. Chamber Saturation was done for 30 min. Development Time was 30 min, the plate was scanned in CAMAG Scanner III with Deuterium lamp, tungsten lamp as detectors and Wincats software was used for data analysis. Spray reagent: 0.5 g of vanillin was dissolved in 100 ml sulfuric acid-ethanol (40 + 10). After

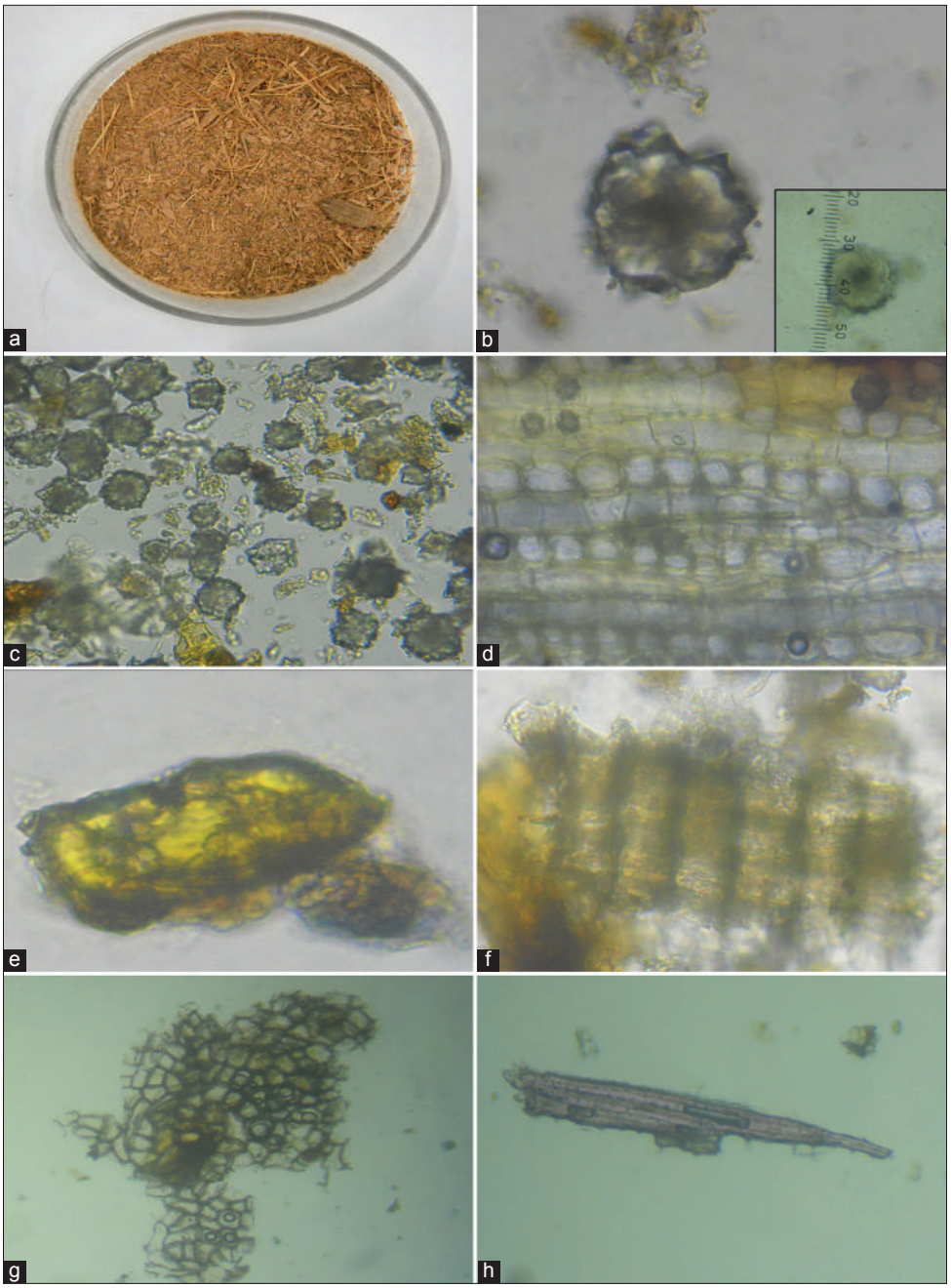


Figure 3: (a): Powder of stem bark, (b) micro measurement of rosette crystal, (c) rosette and cluster crystals, (d) phloem fibers, (e) Stone cells, (f) fibers passing through medullary rays, (g) cork cells in surface view, (h) lignified sclereide

spraying, the plate was heated at 120°C until maximum spot color intensity was reached.[10]

In Vitro Antimicrobial Activity

Ciprofloxacin hydrochloride tablet of 500 mg was taken as reference standard and prepared by taking average weight of five tablets. Powdering the tablets into mortar-pestle. From powder take approximate weight of one tablet into 100 ml volumetric flask and make up volume 100 ml with double distilled water. The sample was sonicated and prepared 5 mcg/ml standards solution by dilution method. The culture preparation was done as per standard protocol. Mueller-Hinton Agar was used as media, and for sample preparation 5 g of coarse powder was extracted with 50 ml of distilled water by subjecting it to constant heat at temperature 70°C for 4 h; filtered through muslin cloth and filtrate was centrifuged at 4000 rpm for 10 min. The supernatant was collected and used as stock solution for activity. Activity were carried out at three different concentration levels, i.e., 100 mg/ml, 150 mg/ml, and 200 mg/ml. Application volume was 100 µl. Fifteen milliliters of media were poured in the sterile Petri plate and allowed to solidify on a smooth surface. Five microliters of bacterial cultures were mixed slowly with remaining

media. The plates were then solidified and wells were made using sterile borer. Then, blank, standard, and test samples were added into, respectively, labeled wells and plate was incubated in incubator at 35°C for 24 h and observed for zone of inhibition.[7] In the agar plates, the sample marked with "2" stands for *C. decandrum* stem bark (CDSt.Br.) sample in all microbes at all concentrations and the central well is of reference standard at 100 mg/ml.[7]

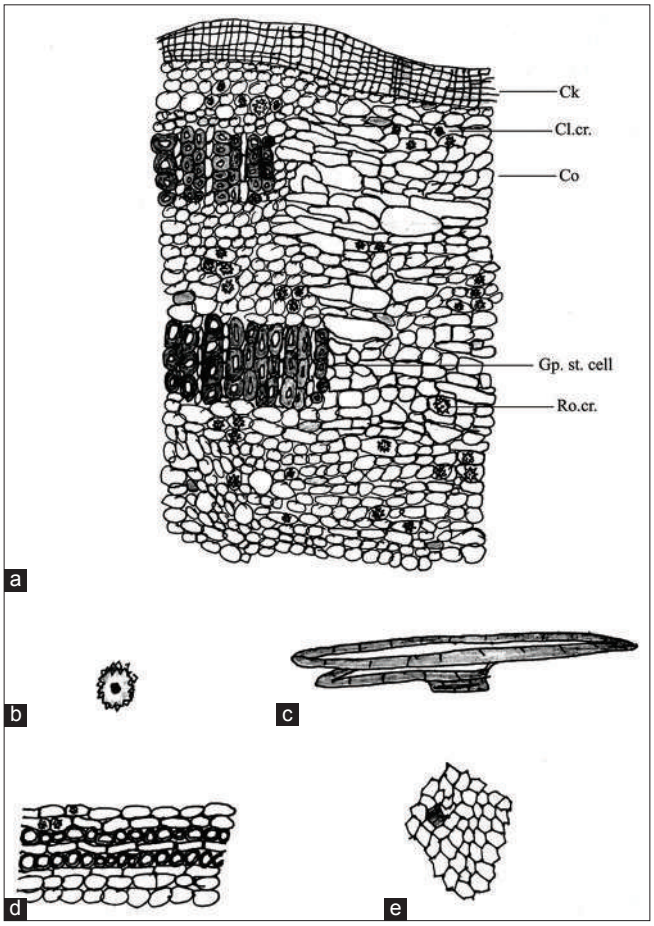


Figure 4: (a): Detailed transverse section of stem bark, (b) rosette crystal, (c) Fragment of sclereide, (d) phloem fibers, (e) fragment of cork cells in surface view. Co – cortex, Ck – cork, gr.sc.stn.cell – group of sclereids and stone cells, Cl.cr. – prismatic crystal, Ro.Cr. – rosette crystal of calcium oxalate

RESULTS AND DISCUSSION

Macroscopy

The stem bark is flat, the cut pieces of stem bark measures about 1.9–19.5 cm × 1.2–7.5 cm outer surface is brownish yellow in color with longitudinal cracks and striations whereas the inner surface is dark brown in color with longitudinal striations and splintery fibrous fracture. On drying, the stem bark turns more brownish in color, cracked from outer side [Figure 2a].

Microscopy

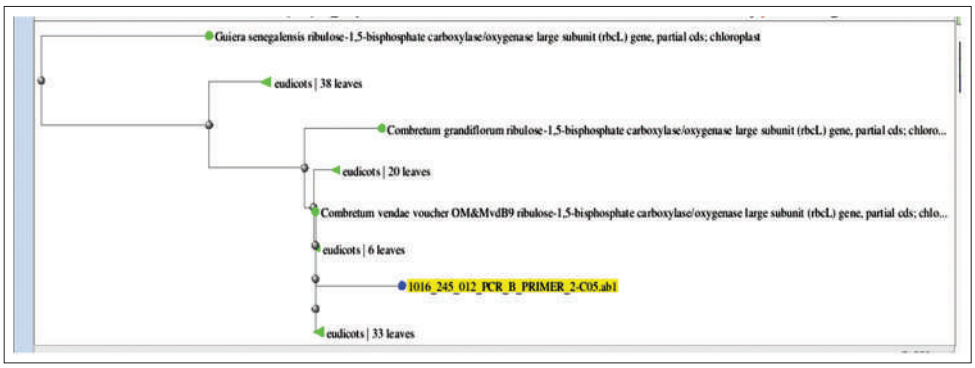
Diagrammatic T.S. of stem bark shows cork and several layers of cortex with medullary rays

Detailed T.S. of stem bark shows multi-layered outer cork, phellem consists compactly arranged tubular cells and often filled with brown content. Phellem, followed by phellogen, consists of two layers of compactly arranged tubular cells, which is differentiate outer cork and inner cortex. Phellogen is followed by phelloderm, cortical region which consists of several layers of parenchyma cells abundantly embedded with rosette and cluster crystals of calcium oxalate. Medullary rays are uniseriate filled with secondary metabolites and irregular patches of lignified group of stone cells and sclereids. Secondary phloem situated in small pockets above the group of stone cells, consist of phloem fibers and sieve elements [Figures 2b-f and 4a].

Powder Microscopy

Powder of stem bark is brownish cream with pink tinge with woody slight aromatic fragrance sweet taste initially followed by astringent and bitter taste and fibrous smooth in touch [Figure 3a].

The powder microscopy of stem bark shows the presence of ample amount of rosette crystals, cluster crystals, brown content, fragment of fiber passing through medullary rays, fragment of cork cells in surface view, isolated lignified stone cells, and lignified sclereids [Figures 3b-h and 4b-e].



Graph 1: Phylogenetic tree

DNA Barcoding

The plant samples were identified as *C. decandrum* on the basis of sequence homology and phylogenic analysis. The results are depicted in Table 1 and phylogenetic tree is depicted in Graph 1.

Physicochemical Parameters

The results of physicochemical parameters show that foreign matter is absent in stem bark other values, as mentioned in Table 2.

Qualitative and Quantitative Analysis

Qualitative test revealed the presence of carbohydrate, alkaloids, and tannin in aqueous as well as methanolic extract of stem bark. The results of tests performed are depicted in Table 2.

The total phenol content of methanolic extract of *C. decandrum* was calculated as gallic acid equivalent of phenols. The various concentration of gallic acid taken to obtain linearity

Table 1: Results obtained from blast sequencing

Description	Max score	Total score	Query cover (%)	Ident. (%)	Accession
Thiloa glaucocarpa ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1075	1075	96	99	FJ381802.1
Combretum micranthum ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1075	1075	96	99	FJ381793.1
Combretum vendae voucher OM&MvdB9 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1075	1075	96	99	EU338136.1
Combretum platypetalum voucher OM1020 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1075	1075	96	99	EU338124.1
Quisqualis indica ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1074	1074	96	99	FJ381798.1
Combretum glutinosum ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1074	1074	96	99	FJ381789.1
Combretum fragrans ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1074	1074	96	99	FJ381788.1
Combretum oxystachyum voucher OM1052 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1074	1074	96	99	EU338127.1
Combretum caffrum voucher OM&MvdB11 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1072	1072	95	99	EU338167.1
Combretum woodii voucher OM1421 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1072	1072	95	99	EU338137.1
Combretum kraussii voucher OM&MvdB36 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1072	1072	95	99	EU338134.1
Combretum platypetalum voucher OM1658 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1072	1072	95	99	EU338125.1
Combretum grandiflorum ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1070	1070	96	99	FJ381797.1
Combretum sp. A (Winter 7225) ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	1070	1070	96	99	J381791

at 765 nm absorbance is 25 µg/ml, 50 µg/ml, 75 µg/ml, and 100 µg/ml with absorbance 0.676, 1.263, 1.82, and 2.184 nm, respectively [Graph 2]. The R^2 obtained was 0.989. The absorbance was 0.108 nm and calculated concentration is 0.217 µg/ml in the methanolic extract of stem bark.

Table 2: Results of physicochemical parameters and qualitative analysis

Parameters	Results (%W/W)	
Loss on drying	9.67±0.15	
Total ash value	15.13±1.95	
Acid-insoluble ash value	0.14±0.05	
Water extractive value	20.52±2.67	
Alcohol extractive value	18.73±2.81	
Qualitative test conducted	**Water extract**	**Alcohol extract**
Molisch's test	–ve	–ve
Fehling test	+ve	+ve
Dragendorff test	+ve	+ve
Neutral ferric chloride	+ve	+ve
Lead acetate	+ve	+ve
Seliwanoff's test	+ve	+ve
Mayer's test	+ve	–ve
Vanillin+sulfuric acid	–ve	–ve

"+ve": Presence, "–ve": Absence

Table 3: R_f values obtained at ultraviolet and visible range of CDSt.Br.

R_f at 254 nm	R_f at 366 nm	After spray (366 nm)	Visible (600 nm)
0.01	0.01	0.01	0.01
0.69	0.91	0.94	0.95
0.83	0.94	-	-
0.91	0.99	-	-
0.95	-	-	-

CDSt.Br.: *Combretum decandrum* stem bark

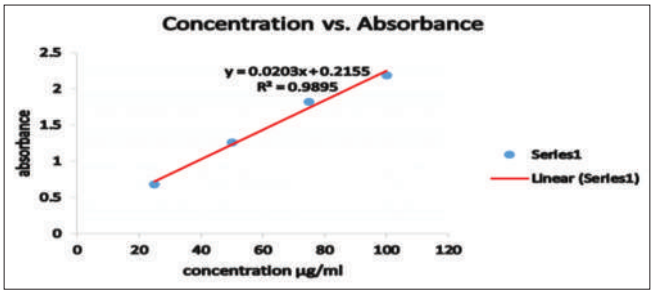
HPTLC Study

The methanol extract of stem bark shows 5 peaks, 4 peaks, and 2 peaks at UV-visible (UV-Vis) range of 254 nm, 366 nm, and 600 nm, respectively. After spraying with spray reagent leaf shows 2 peaks at 366 nm. The Rf values are mentioned in Table 3. The photos of HPTLC plate, three-dimensional graphs, and peak display at UV-Vis range are depicted in Figure 5.

Microbial Overload and *In Vitro* Antimicrobial Study

The microbial overload shows that the total bacterial count (TBC, cfu/g) was 506, and total yeast and mold count (TYMC, cfu/g) were 72. *In vitro* antimicrobial study shows significant results at 100, 150, and 200 mg/ml concentration. The values are depicted in Table 4 along with the zone of inhibition obtained for reference standard. The photographs of zone of inhibition on respective microbes are depicted in Figure 6.

The macroscopic key identification character of Combretum decandrum stem bark is that outer surface is brownish in color with smooth surface while inner surface is brown in color with splintery fibrous fracture and microscopy character of Combretaceae family showed the presence of uniseriate



Graph 2: Linear graph of Gallic acid for total phenol content

Table 4: Results of microbial overload and antimicrobial activity of aqueous extract of CDSt.Br

Name of sample/Reference standard	Name of micro-organisms			
	S. aureus	***B. subtilis***	***P. aeruginosa***	***E. coli***
Microbial overload	Occurrence			
CD St.Br.	Absent	Absent	Absent	Absent
Antimicrobial activity	Zone of inhibition in millimeter			
Ciprofloxacin 500 mg (reference standard)	38 mm	38 mm	38 mm	38 mm
CDSt.Br.				
100 mg/ml	18 mm	NZ	17 mm	19 mm
150 mg/ml	19 mm	20 mm	17 mm	19 mm
200 mg/ml	20 mm	21 mm	17 mm	20 mm

*CDSt.Br.: *Combretum decandrum* stem bark, *E. coli*: *Escherichia coli*, *Salmonella* species, *S. aureus*: *Staphylococcus aureus*, *P. aeruginosa*: *Pseudomonas aeruginosa*, *B. subtilis*: *Bacillus subtilis*

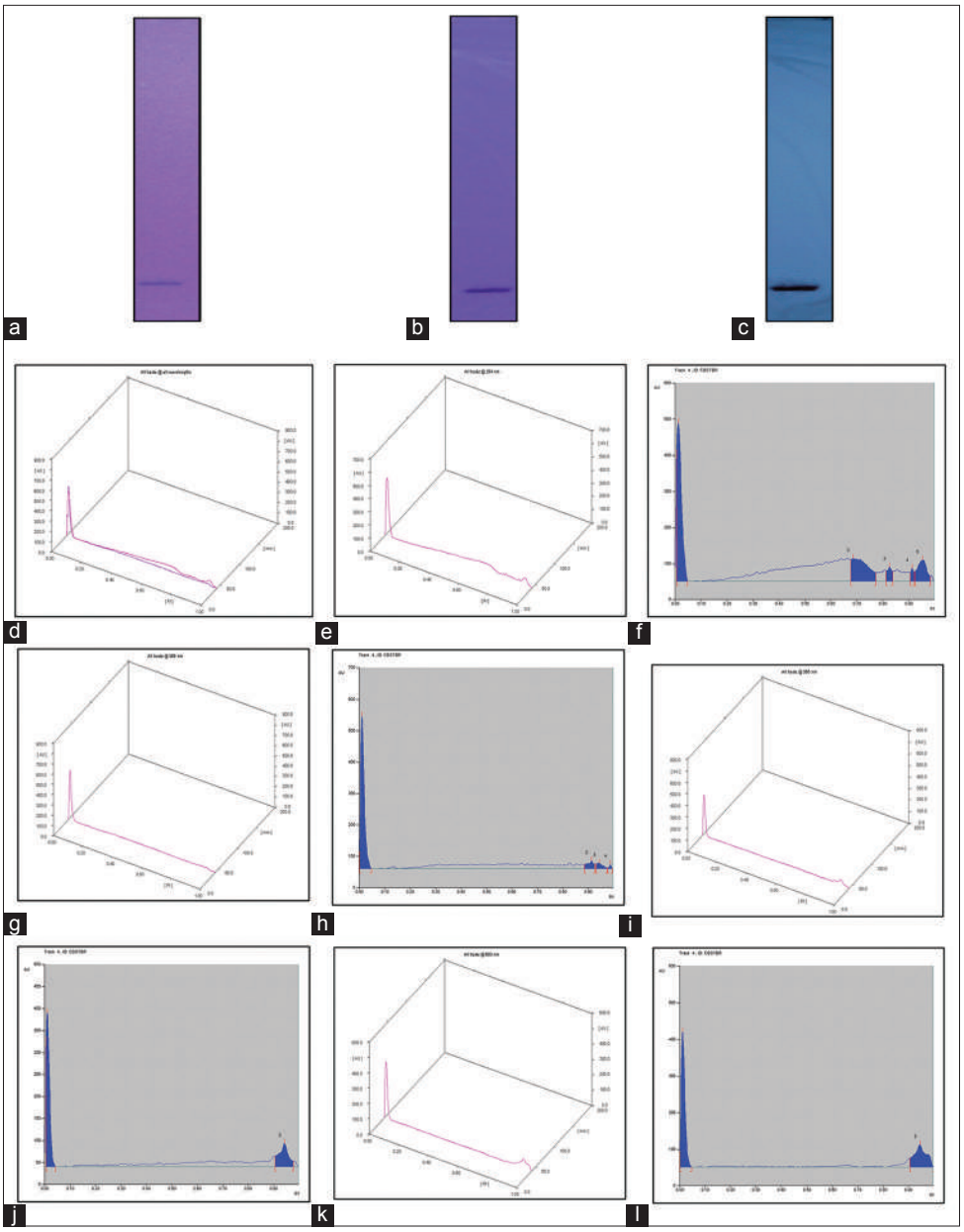


Figure 5: (a): High-performance thin-layer chromatography (HPTLC) plate of stem bark at 366 nm (before spray), (b): HPTLC plate of stem bark at 366 nm (after spray), (c): HPTLC plate of stem bark at 600 nm, (d) all tracks at ultraviolet-visible range, (e) three-dimensional (3D) of tracks at 254 nm, (e) peak display at 254 nm, (g) 3D tracks at 366 nm before spray, (h) peak display at 366 nm before spray, (i) 3D tracks at 366 nm after spray, (j) peak display at 366 nm after spray, (k): 3D tracks at 600 nm, (l) peak display at 600 nm

medullary rays and combretum genus is the secondary phloem fibers are chambered and filled with small sized cluster crystals of calcium oxalate.[11] The physicochemical parameters reveal the absence of foreign matter which indicated good collection practice, the methanol and aqueous extractive yield was high using maceration method indicating high amount of hydrophilic and hydrophobic moiety present in plant. DNA barcoding is one among the latest technology which is powerful enough to help in framework for identifying specimens along with the traditional methods,[12] as DNA markers are environmentally stable and specific, they have gained wide popularity in quality control and standardization of medicinal plant materials.[13] The DNA sequence obtained from rbcL (larger subunit of ribosome) was used for analysis and compared with the existing database available from NCBI GenBank applying BLAST sequencing method shows significant match with thiloa glaucocarpa ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit with accession number FJ381802.1 (rbcL) gene depicting correct genetic identification of *C. decandrum*. The total bacterial (TBC, cfu/g) and TYMC (TYMC, cfu/g) values obtained are under prescribed limit as mentioned in Ayurvedic Pharmacopoeia of India, i.e. TBC not more than 100,000 cfu/g and TYMC not more than 1000 cfu/g.[7] The antimicrobial

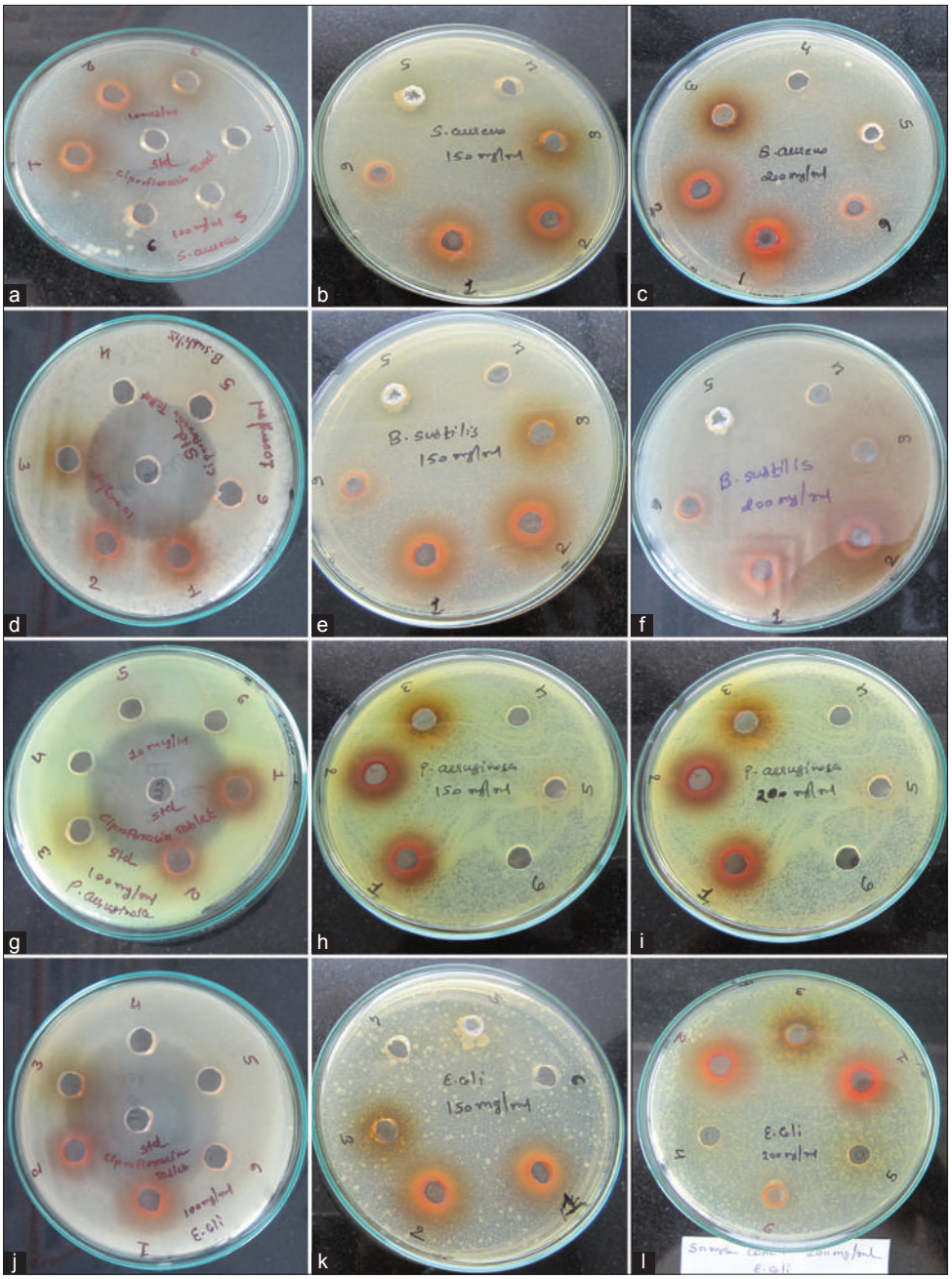


Figure 6: (a) Agar plate against *Staphylococcus aureus* with reference standard (100 mg/ml), (b) agar plate against *S. aureus* (150 mg/ml), (c): Agar plate against *S. aureus* (200 mg/ml), (d) agar plate against *Bacillus subtilis* with reference standard (100 mg/ml), (e) agar plate against *B. subtilis* (150 mg/ml), (f) agar plate against *B. subtilis* (200 mg/ml), (g) agar plate against *Pseudomonas aeruginosa* with reference standard (100 mg/ml), (h) agar plate against *P. aeruginosa* (150 mg/ml), (i) agar plate against *P. aeruginosa* (200 mg/ml), (j) agar plate against *Escherichia coli* with reference standard (100 mg/ml), (k) agar plate against *E. coli* with reference standard (150 mg/ml), (l) agar plate against *E. coli* with reference standard (200 mg/ml)

activity of CDSt.Br. aqueous extract reveals that there is considerable increase in zone of inhibition with increase in concentration. Review reveals that gallic acid and tannin content are chemical moiety responsible for antibacterial activity,[14,15] results of qualitative and quantitative analysis for total phenol content show that the presence of tannin and gallic acid equivalent value is 0.217 µg/ml which might be one of the contributing factors responsible for antibacterial activity of stem bark.

CONCLUSION

Morphologically Combretum decandrum stem bark can be identified by brown color of inner surface and splintery fracture and microscopy character is the presence of abundant cluster and rosette crystals. The results obtained from physicochemical parameters and HPTLC can help in further standardization by providing information on degree of purity for the drug. DNA barcoding is one of the new

technologies which can be helpful for correct identification of plant species along with known traditional system of taxonomy. The concentration of total phenol is 0.217 µg/ml in methanolic extract of stem bark. The antimicrobial activity of CDSt.Br. aqueous extract reveals that there is considerable increase in zone of Inhibition with increase in concentration.

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