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ORIGINAL ARTICLE

Phytochemical, Histochemical and *In Vitro* Antimicrobial Study of Various Solvent Extracts of *Costus speciosus* (J. Koenig) Sm. and *Costus pictus* D. Don

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ABSTRACT

Objectives: Costaceae family comprises many ornamental and medicinal plants used for different diseases. This investigation includes the phytochemical, histochemical, and *in vitro* antimicrobial study of *Costus speciosus* (J. Koenig) Sm. and *C. pictus* D. Don.

Materials and Methods: Solvents such as methanol, ethyl acetate, and hexane were used to extract the leaves and rhizomes of both plants. The antibacterial study was executed using the agar well diffusion technique.

Results: Phytochemical study confirmed that alkaloids, flavonoids, quinones, and saponins were present in solvent extracts of both plants. The macromorphological studies including size, shape, texture, surface characters, and color, were analyzed. *Salmonella typhi, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus* were used for the antibacterial study. Agar well diffusion and agar disk diffusion methods were performed to determine the susceptibility of bacterial strains to various extracts of these plants.

Conclusion: Histochemical analysis revealed alkaloids, proteins, and phenols in the vascular bundles, the cortex, and epidermis of stem, root, and leaves of the plants. Inhibition zones caused by the methanol and hexane extracts showed better antibacterial activity compared to those of other extracts. Future work on the isolation, purification, and characterization of the active constituents and the elucidation of possible mechanisms can be executed.

Key words: Costaceae, *Costus pictus, Costus speciosus,* histochemical, antibacterial activity

INTRODUCTION

Plants have been used as medicine since the start of the human race.[1] These medicines were initially used as poultices, tinctures, teas, powders, *etc*.[2] Medicinal plants are familiar sources of medicine. Substantial evidence can be cited favoring herbs being used to treat diseases and restoring and fortifying body systems in the ancient systems of medicines such as Ayurvedic, Unani, and Chinese traditional medicine.[3] Antimicrobial activity is one of the most eyed usefulness in the field of herbal medicines. A measurement of determination of antibacterial activity is zone of inhibition. There is a proportionate relationship between the zone of inhibition and antibacterial activity.[4] Many plants have shown a profound antimicrobial activity. The family of Zingiberaceae comprises about 1.300 species and 52 genera spread all over Asia, tropical Africa, and the Americas.[5] In a country like India, the plant propagates in the sub-Himalayan region, central India, Maharashtra, Karnataka, and Kerala.[6] The *Costus* spp. from the family Costaceae are commonly grown as medicinal and ornamental plants.[7,8] The *Costus* spp. additionally used as a dietary supplement to manage many diseases throughout the world.[9] *Costus speciosus* (CS), commonly known as crepe ginger,[10] is an essential plant grown in India.[11] The name CS was changed recently to *Hellenia speciosa* (J. Koenig ex Smith) S. Dutta.[12,13] The pharmacological activities reported for CS are antioxidant, antibacterial, analgesic, anti-cholinergic activity, antidiabetic, anti-inflammatory, antidiuretic, antifungal, larvicidal, estrogenic activities, and anti-stress.[14,15] *Costus pictus* (CP) is another ornamental plant from the family of Costaceae. CP is also known as fiery *Costus,* insulin plant, spiral flag, and step ladder.[16,17] The rhizome and leaves show antidiuretic,

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bacterial, anti-anthelmintic, and antitumor activities.[18] It also possesses hypoglycemic and anti-inflammatory action.[19,20] The main purpose of the current study was to conduct phytochemical and histochemical analysis and evaluate the antibacterial activity of various solvent extracts of CS and CP on selected bacterial pathogens.

MATERIALS AND METHODS

Analytical-grade chemicals such as hexane, methanol, ethyl acetate, and nutrient agar were procured from Sigma-Aldrich, Germany. Other chemicals such as phloroglucinol, safranin were obtained from Loba Chemie, Sudan red III and iodine from Qualigens Fine Chemicals. Other chemicals such as sulfuric acid, hydrochloric acid, sodium hydroxide, ferric chloride, ammonium hydroxide, and acetic acid were also used.

Collection, identification, and authentication of plant materials

Healthy plants of CS and CP were collected from Usha nursery, Mallapuram district, Kerala. Both the plants have deposited video accession number 722 and 723 in the Herbarium, Department of Botany Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, Maharashtra, India. The fresh leaves[21] from the plant's CP while the rhizome of CS[22] was collected, washed thoroughly, and shade dried. The sample was powdered using a laboratory mixer grinder at high speed for 5 min and was stored in a tightly-closed container for one day before being used for analysis.

Preparation of the plant extracts

Aqueous extraction: Preparation of the extracts was done by the following method. 2 g of powdered material was extracted with 50 mL of water by the maceration process. The mixture was filtered with Whatman filter paper. The filtered solution was reduced to one-fourth of its original volume (50 mL) by vacuum in a rota-evaporator at 40°C to a constant weight until the volume reached a concentration of 160 mg/mL. The solution obtained was autoclaved at 121°C and 15lb pressure and stored at 4°C for further studies.[23]

Solvent extraction (cold maceration)

The dried and powdered materials from the rhizome and leaves (2 g) were weighed accurately. The powder was macerated separately with ethyl acetate, hexane, and methanol sequentially with occasional stirring for 48 h.[24] The mixture was filtered and then reduced to one-fourth volume at 40°C with rota-evaporator and stored for further studies.[25,26]

Macro-morphological study

Macroscopic observation of the plant was carried out.[27,28] Qualitative analysis was performed on the basis of morphological and sensory properties such as size, shape, texture, surface characteristics, taste, color, odor, *etc*., was recorded.[29-31]

Histochemical study

Freehand sections of leaves, stem, rhizome and root materials were taken and treated with the respective reagent to localize the chemical constituents in the tissues. The stained sections were compared with the fresh unstained sections. The sections were mounted on a slide to be observed under a compound microscope. The mounted sections were observed under the compound microscope and were studied for various phytochemicals such as alkaloids, phenols, tannins, proteins, *etc*.[32-34]

Physicochemical tests

Various parameters such as total ash value, acid insoluble ash, water-soluble ash, sulfated ash, moisture content (loss on drying), water content, foreign organic matter, and extractive values (methanol, hexane, ethyl acetate, and water) were studied.[35-37]

Phytochemical screening:

a. Test for tannins: To about 2-3 mL extract, 2-3 drops of 5% $FeCl_3$ solution. With the formation of green or bluish-black color, the presence of tannins is indicated.[38]

b. Test for saponins (Foam formation test): To about 2 to 3 mL extract, 5 mL de-ionized water was added. Vigorous shaking resulted in persistent foam formation. It was allowed to stand for 15 min and kept for honeycomb froth, which shows saponins.[39]

c. Test for flavonoids (Shinoda test): To 1 mL extract, a few magnesium ribbon fragments and 4-5 drops of *conc.* HCl was added. The presence of flavonoids is confirmed by the formation of the pink or red.[40]

d. Test for terpenoids (Salkowwski test): In 0.5 mL extract, 2 mL chloroform along with *conc*. H_2SO_4 was added. The red-brown color at the interface indicates terpenoids.[41]

e. Test for carbohydrates (Molisch's test): 1 mL extract in addition to 1 mL of *conc*. H_2SO_4, gives a red to violet zone, which is visible at the interphase of the oil-water layers. The presence of carbohydrates and glycosides is indicated.[42]

f. The test for anthraquinone (Bontrager's test): To 1 mL of extract, 5 mL benzene was added. Further, it was shaken and filtered. 5 mL of ammonium hydroxide (10%) was added, followed by shaking the contents. A red, pink, or violet in the lower ammoniacal phase confirms the presence of anthraquinones.[43]

g. Test for cardiac glycosides (Keller-Kiliani test): A mixture of 2 mL of acetic acid added with 1-2 drops of 2% ferric chloride solution was mixed with 1 mL extract. This mixture was then introduced into another test tube that had 2 mL *conc.* H_2SO_4. The appearance of a brownish-colored ring at the interphase and cardiac glycosides are indicated in the sample.[44]

h. Test for coumarins: A test tube was made with filter paper moistened in dilute NaOH, about 1 mL of sample extract was taken. The sample was heated for 3-5 min. Further, the filter paper was examined under ultraviolet (365 nm) for yellow-colored fluorescence, which confirms the test for coumarins.[45]

i. Test for steroids (Liebermann-Burchard test): About 2 mL of acetic acid was added to 1 mL extract. After cooling the solution on an ice bath, *conc.* H_2SO_4 was added carefully. The development of violet to blue or bluish-green color confirms the test for steroids.[46]

j. Test for alkaloids: In 1 mL extract, 2 mL of 1% HCl was added, and the solution was heated. Further, 4 to 5 drops of Mayer's

reagent was added. A precipitate white or cream in color formation confirms the test for alkaloids.[47]

Antimicrobial activity studies

Microorganisms

Reference bacterial strains were obtained from the Master of Science Department of Maulana Azad College, Dr. Rafiq Zakaria Campus, Aurangabad (MS.), India. The strains comprise of *Bacillus subtilis* (ATCC 19659), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), and *Salmonella typhi* (ATCC 14028). The bacterial isolates were kept at 4˚C on an agar slant. The sample was sub-cultured for 24 h in nutrient agar at 37˚C before any susceptibility test.[48]

Media preparation

About 23 g of nutrient agar was dissolved in distilled water (1000 mL). The mixture was autoclaved for 15 min at 121˚C. Further, it was left to cool at room temperature. After cooling (about 45˚C), it was transferred into petri dishes. Each petri dish was left to cool for about 30-35 min until completely set.[49-51]

Agar disk diffusion method

Each bacterial sample was spread on sterile agar plates using a clean and pre-sterilized cotton swab. Pre-calibrated Whatman filter paper disks of about 5 mm in diameter and 2.5 µL infused capacity was prepared and sterilized. Each disk was infused with different crude extracts of concentration 40 mg/disk and then sited on bacterial pre-swabbed agar plates. Streptomycin was used as a positive control. The samples were allowed to diffuse at RT for 30 min. The diffused petri plates were kept for incubation at 35 ± 0.5˚C for 24 h. After incubation, the microbial growth was determined by measuring the diameter (mm) for inhibition zones.[52-54]

Agar well diffusion test

Each bacterial inoculum was swabbed on the sterile agar plate using a clean and a sterilized cotton swab. Wells of about 7 mm in diameter with 50 µL capacity were made into the agar plates. The initial concentration of crude extract used for the agar well diffusion method was 40 mg/mL. Due to the higher concentration of the crude extract, it was found that the zones of inhibition merged with each other. Henceforth, the concentration of the crude extracts was reduced to 20 mg/mL. Positive control (PC) (streptomycin) 30 *µg/µL*, negative control (various solvent extracts), and sample solution (all crude extracts) were added to each well and allowed to diffuse for 30 min at RT. Plates were incubated for 24 h at 35 ± 0.5˚C to determine the inhibition zones (mm) as a measure of antibacterial activity.[55,56] No statistical analysis was carried out in this study.

RESULTS

Macro-morphological study

Costus speciosus: CS is an erect succulent herb up to 3 m in height. The leaves are elliptical to oblong-lanceolate silky beneath, subsessile, thick spirally arranged with stem-clasping sheaths up to 4 cm. The rhizome of CS is tuberous, having 10-15 cm length and 1-3 cm diameter. Rhizomes are usually sub-cylindrical unbranched and covered with a brownish epidermis or cork. Small circular scars of about 2 - 4 mm in diameter are present on the upper and lower surfaces at specific intervals. Flowers are white , large, thick, cone-like terminal spikes, with bright red stripes, lips with the yellowish throat; red capsules, fruits globosely trigonous, the diameter of about 2 cm, black colored seeds, with white aril. The upper surface was marked with nodal scars circular with a residue of leaf bases, lower and lateral surfaces exhibited small circular spots of roots, or few thin rootlets fractured fibrous and fractured yellowish-brown surface. Stems nearly woody at base, unbranched, spirally twisted in the upper part. No characteristics of taste or odor (Figure 1).

CP is a perennial herb. Typically, multi-trunked or clumping stems. The stems hirsute and green near the apex, glabrous and purple toward the base, with spiral light green leaves and airy, the tissue paper-like flowers are yellow with orange-red stripes. The leaves are alternate, simple, entire, smooth surface, pinnate parallel venation, conspicuously ligulate (red-colored) large fresh-looking spirally arranged, and oblong-lanceolate being dark green above when mature, and lighter green below. The leaf's shape is narrowly elliptical with a length of 10 to 25 cm and a width up to 6 cm. The leaves have a characteristic taste and odor (Figure 2).

Histochemical study

Histochemical studies help in the determination of chemical constituents in the cells and tissues. The method can identify cellular components such as carbohydrates, proteins, nucleic acids, lipids, *etc*.[57,58] Thin sections of leaves, roots, rhizomes, and stem were taken. Further, the sections were stained using various stains, such as Sudan red III, iodine, phloroglucinol, and ferric chloride. The transverse section of rhizome leaves and stems of the plants show fibrovascular bundles. Plants are monocot commends (Figure 3). The leaves and stem show epidermis bearing simple, unlignified, thick-walled trichrome unicellular, simple, and multicellular glandular with pointed apex; central vascular bundles closed system with collenchyma on both sides. Leaves are dorsiventral on the upper epidermis. Parenchymatous cells encircle the vascular bundles in the presence of starch grains. Phytochemicals such as phenols, alkaloids, and proteins were found to be present in the epidermal tissue, the cortex, and vascular bundles of the root, stem, and leaves. The stem shows scattered lignified vascular bundles, thin-walled unlignified collenchyma, uniseriate multicellular trichrome central vascular pith primary xylem. Rhizomes are monocot with cortex showing scattered vascular bundle, thin-walled parenchymatous cells. The roots show wide vessel parenchymatous thin corks with thin-walled medulla. Central cork/pith in the epidermal region brownish with the presence of fibrous vascular bundles scattered. The primary xylem toward the center shows brownish content in the cell. The cortex is occupied in 2/3 portion surrounded by the thin-walled parenchymatous cell (Figure 4).

Physiochemical and phytochemical tests

As there is an increase in antibiotic resistance in various diseases, the alternative system medicine can be of interest. Many plants contain phytochemicals with antimicrobial properties, which could play a crucial role in therapeutic treatments.[59] The physiochemical properties such as organoleptic properties, ash value, extractive values, moisture content, foreign organic matter, and swelling index are given (Table 1). The aqueous, methanolic, ethyl acetate, and hexane extracts (HE) (Table 2) show various compounds identified. The different tests indicated alkaloids, flavonoids, quinones, and saponins. The crude extracts from leaf and rhizome samples of CP and CS did show a positive test for terpenoids and steroids. The methanolic crude extract showed a positive test for tannins. HE from both plants show chemicals like steroids, alkaloids, flavonoids, and triterpenoids, while saponins and tannins were absent. Ethyl acetate crude extract from both plants showed a negative test for saponins, while CP ethyl acetate extract indicated the presence of alkaloids.

Antimicrobial activity studies

The investigational study for antibacterial activity against various pathogenic bacterial strains using the agar disk diffusion technique was conducted. The disk diffusion method was used only for qualitative evaluation of antibacterial activity for respective crude extracts. Optimization of the antibacterial activity was carried out by using the agar well diffusion test.

Table 2. Phytochemical constituent of plants *Costus speciosus* and *Costus pictus*

Chemical constituent	*Costus speciosus*				*Costus pictus*			
	AE	ME	EAE	HE	AE	ME	EAE	HE
Tannins	-	+	-	-	-	-	-	-
Saponins	+	+	-	-	+	+	-	-
Flavanoids	+	-	-	-	+	+	+	+
Quinones	+	+	+	+	+	+	+	+
Glycosides	-	+	+	+	+	+	+	+
Cardiac glycosides	+	+	+	+	+	+	+	+
Carbohydrates	-	+	+	+	-	+	+	+
Terpenoids	-	+	+	+	+	+	+	+
Phenols	+	+	-	-	+	+	+	+
Coumarins	+	+	-	-	+	+	+	+
Steroids	+	+	-	-	+	+	+	+
Alkaloids	-	+	-	-	+	+	+	+

AE: Aqueous extract, ME: Methanolic extract, HE: Hexane extract, EAE: Ethyl acetate extract

Table 1. Physiochemical test results for *Costus speciosus* and *Costus pictus*

Tests	Results	
	Costus speciosus (rhizome)	*Costus pictus* (leaves)
1- Organoleptic properties a) Appearance	Buff colored powder	Light green powder
b) Color	Buff brown	Light green
c) Odor	No characteristic odor	Characteristic odor
d) Taste	No taste	Sour in taste
e) pH	7.4 ± 0.06	6.5 ± 0.08
2- Moisture content (%)	4.5 ± 0.32	10.155 ± 0.032
3- Ash value (%)	-	-
a) Total ash value	4.5 ± 0.5	14.29 ± 0.15
b) Acid insoluble ash value	0.932 ± 0.03	3.20 ± 0.060
c) Water soluble ash value	1.58 ± 0.07	8.70 ± 0.012
4- Foreign organic matter (%)	0.7 ± 0.04	0.07 ± 0.063
5- Extractive values (%)	-	-
a) Methanol	8.74 ± 0.99	17 ± 0.03
b) Ethyl acetate	5.32 ± 0.63	7.60 ± 0.02
c) Hexane	2.06 ± 0.02	6.50 ± 0.03
d) Aqueous	7.34 ± 0.63	10.25 ± 0.07
6- Swelling index	Initial (mL) 3.5 ± 0.32 Final (mL) 5.6 ± 0.13	Initial (mL) 2.5 ± 0.20 Final (mL) 6.5 ± 0.130

Agar disk diffusion method

The different solvent extracts for CS had a lower to negligible effect based on the agar disk diffusion method. However, the marketed extract of CS was highly effective against various bacterial strains. The solvent extracts of CP had an efficient antibacterial activity; however, the marketed extract shows a lesser activity comparatively. Concluding the overall results, the marketed extract of CS and different solvent crude extracts of CP was used for the agar well diffusion method. The zone of inhibition obtained for the agar disk diffusion technique is shown in (Table 3). The graphical representation of the zone of inhibition achieved using the agar disk diffusion technique is shown (Figure 5).

Agar well diffusion test

The methanolic extract (ME) showed the highest inhibition against *E. coli*. In contrast, the hexane and ethyl acetate extracts displayed comparatively low activity. A potential antibacterial activity was exerted by the hexane extract and ME against *S. aureus* with inhibition zones of 38 and 17 mm, respectively (Figure 6). However, the ethyl acetate extract showed comparatively lower activity (9 mm). The standard extract showed maximum activity against *B. subtilis*. While the hexane, ethyl acetate, and methanolic extracts, caused comparatively lower activity. Against *P. aeruginosa,* the HE shows the highest activity, while the ME, reference and ethyl acetate extract show comparatively lesser activity. The best antibacterial activity against *S. typhi* was observed with the ME comparatively with other extracts (Table 4). The aqueous extract indicated a mild activity against *S. typhi*, while no activity was recorded against other bacterial pathogens tested. The graphical representation of the inhibition zones achieved using the agar well diffusion method is shown at Figure 7.

Figure 1. *Costus speciosus* (A) flowers (B) rhizomes (C) whole plant of *C. speciosus*

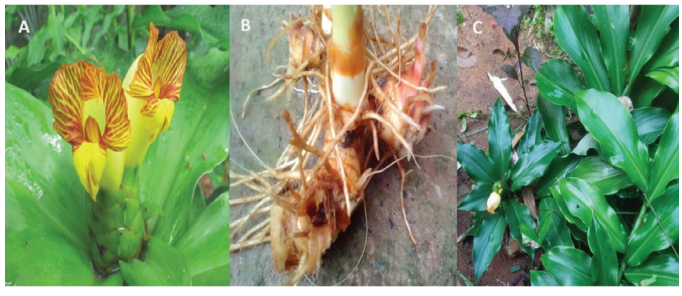

Figure 2. *Costus pictus* (A) flowers (B) roots (C) whole plant of *C. pictus*

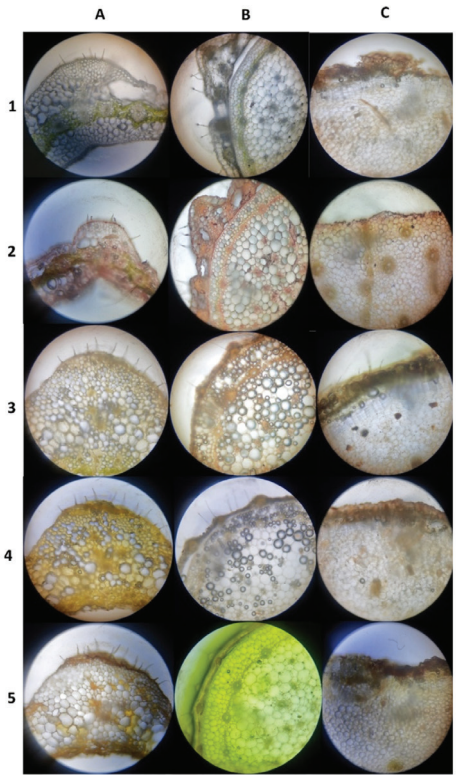

Figure 3. Histochemical studies on (A) leaves (B) stem (C) rhizome of *Costus speciosus* (1) unstained section (2) test for fixed oils, volatile oils (3) test for lignified tissues (4) test for proteins (5) test for phenols

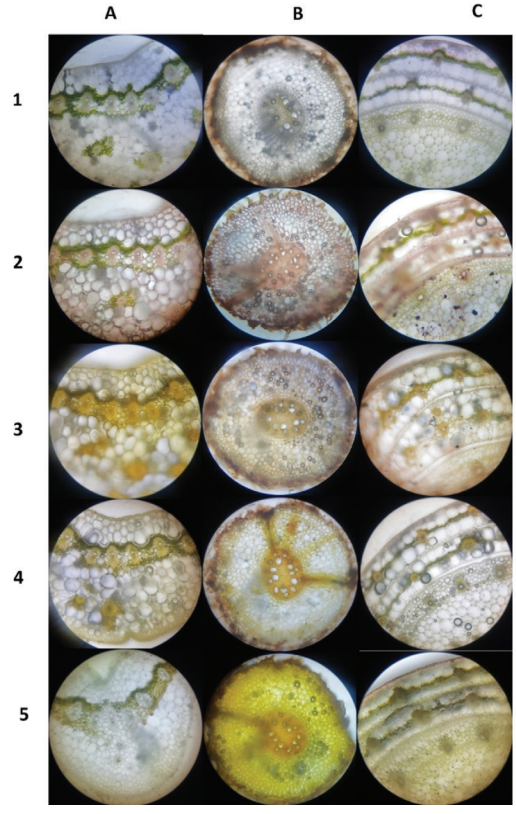

Figure 4. Histochemical studies on (A) leaves (B) roots (C) stem of *Costus pictus* (1) unstained section (2) test for fixed oils, volatile oils (3) test for lignified tissues (4) test for proteins (5) test for phenols

Table 3. Disc diffusion method results

Microorganism	Zone of inhibition (in mm)											
	Costus speciosus						*Costus pictus*					
	PC[a]	AE[b]	ME[c]	HE[d]	EAE[e]	SE[f]	PC	AE	ME	HE	EAE	SE
Staphylococcus aureus	33	-	-	5	-	12	33	-	-	-	11	12
Bacillus subtilis	30	-	-	-	-	15	3	-	80	-	12	13
Pseudomonas aeruginosa	28	-		-	-	18	25	-	70	-	10	1
Escherichia coli	26	-	-	-	-	9	26	-	80	-	80	9
Salmonella typhi	-	-	-	-	-	9	-	-	14	60	60	9

[a]PC: Positive control, [b]AE: Aqueous extract, [c]ME: Methanolic extract, [d]HE: Hexane extract, [e]EAE: Ethyl acetate extract, [f]SE: Standard extract

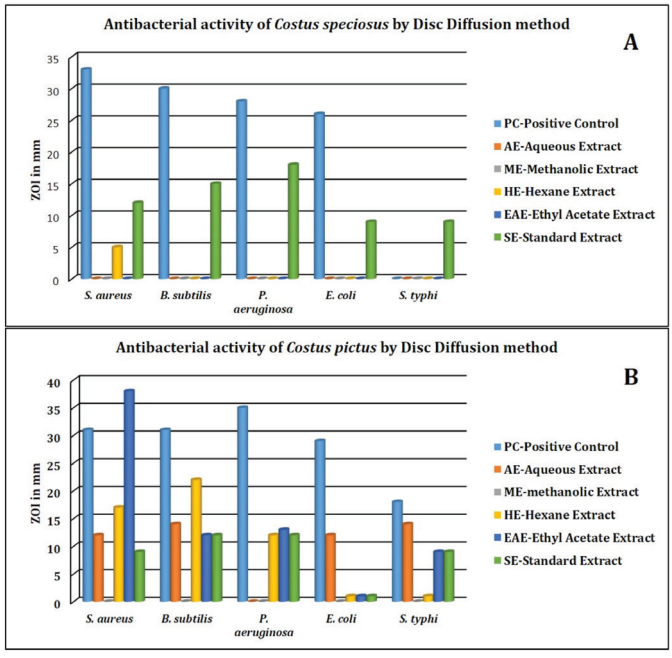

Figure 5. Graphical representation of the zone of inhibition achieved by disc diffusion method

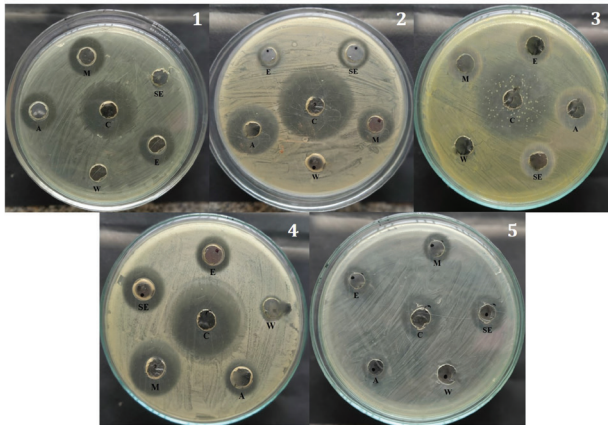

Figure 6. Zone of inhibition for agar well diffusion (1) *Escherichia coli* (2) *Pseudomonas aeruginosa* (3) *Staphylococcus aureus* (4) *Salmonella typhi* (5) *Bacillus subtilis*

A: Hexane extract, C: Positive control, E: Ethyl acetate extract, M: Methanolic extract, SE: Standard extract, W: Aqueous extract

Table 4. Agar well diffusion method results

Microorganism	Zone of inhibition (mm)					
	PC[a]	*Costus speciosus* (SE[f])	*Costus pictus*			
			AE[b]	ME[c]	HE[d]	EAE[e]
Staphylococcus aureus	31	12	-	17	38	9
Bacillus subtilis	31	14	-	22	12	12
Pseudomonas aeruginosa	35	12	-	12	13	12
Escherichia coli	29	-	-	11	10	10
Salmonella typhi	18	14	-	10	9	9

[a]PC: Positive control, [b]AE: Aqueous extract, [c]ME: Methanolic extract, [d]HE: Hexane extract, [e]EAE: Ethyl acetate extract, [f]SE: Standard extract

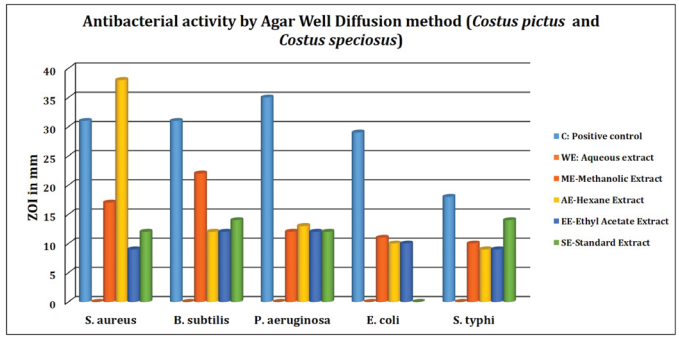

Figure 7. Graphical representation of the zone of inhibition achieved by agar well diffusion method

DISCUSSION

The histochemical and phytochemicals investigation of CS and CP reveals alkaloids, phenols, proteins, saponins, tannins, anthraquinones, and flavonoids. The literature[60-62] reveals that presence of these phytochemicals is responsible for having curative activity against several microbes. Therefore, CS and CP extracts were evaluated for antimicrobial activity and ensuring promising results as antimicrobial agents (Tables 3, 4). The comparative study carried out gives an idea about the antibacterial potential of the plant crude extracts. The standard and crude extracts show inhibitory activity against the examined

bacterial strains. *B. subtilis* was found to be more resistant compared with other bacterial strains, while *P. aeruginosa* and *S. typhi* were found to be more susceptible toward the crude and standard extracts. The negative control used in the study does not show any markable antibacterial activity against the selected pathogenic strains. Extracts of both plants that showed inhibition zone diameters of >10 mm were considered active. In according to this, both extracts are better antibacterial agents and, therefore, using these plants as antibacterial agents has been validated.

CONCLUSION

The pharmacognostic study of CS and CP gave important information concerning the morphology of crude drugs. They can be used for the authentication of CS and CP among all *Costus* spp. The adulteration and purity of these drugs can also be determined. The microscopic character, physicochemical, and phytochemical screening parameter studies help set standards for these crude drugs. Significantly, fewer data was available on the histochemical study of both the plants CS and CP; henceforth, it was carried out elaboratively. Histochemical studies revealed alkaloids, flavonoids, carbohydrates, and terpenoids in the leaf, stem, and roots. The phytochemical screening study also confirmed chemical components such as alkaloids, flavonoids, carbohydrates, phenols, glycosides, and terpenoids. Up to date, the antibacterial potential and comparative study against specified bacterial pathogens were not reported. Besides this, the novelty of the current work is to differentiate the activity between Gram-positive and Gram-negative bacterial strains. The potential increase of 2 folds in the antibacterial activity for HE (CP) against *S. aureus* was observed, however, ME (CP) shows an equivalent activity as PC. The result indicates that both plants show potential antibacterial activity ensuring bioactive compounds useful in primary healthcare. Further work is needed on the isolation, characterization, and purification of the active constituents and understanding the possible mechanism of action as an antibacterial agent.

Ethics

Ethics Committee Approval: No Ethics Committee approval required.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: A.S.B., Design: A.S.B., Data Collection or Processing: S.S.S., Analysis or Interpretation: B.A.Y., Literature Search: S.S.S., B.A.Y., Writing: S.S.S.

Conflict of Interest: No conflict of interest was declared by the authors.

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