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Phytochemistry, Antioxidative and Antimicrobial Efficacy of *Saussurea costus*

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ABSTRACT

This research study examines the Himalayan therapeutic edible plant *Saussurea costus* (Indian costus) which has known health benefits. Samples of the roots were gathered and verified, and the crude plant extract was made using the Soxhlet apparatus with acetone, ethanol, and water as solvents. Phytochemical studies showed that water extracts (SCW) contained the most phenols (19.36 mg/g) and flavonoids (2.41 mg/g), followed by ethanol (SCEt) and the weakest extract was with acetone (SCAc). Findings from ABTS, FRAP, as well as DPPH assays indicated highest antioxidant ability is within SCW, suggesting its use in oxidative stress-related disease treatment. Antimicrobial testing showed strong activity, and the fractions obtained were tested for the MIC value against the bacterial strains. *S. costus* showed antibacterial and antioxidative potential, aiding its therapeutic value.

Keywords: *Saussurea costus*, Column purification, Antioxidant activity, Phytochemical screening, Anti-microbial properties.

INTRODUCTION

Often referred to as Indian costus or kuth, *Saussurea costus*, Aucklandia costus, or *Saussurea lappa* belongs to a perennial herb member of Asteraceae family. This plant predominantly thrives in Himalayan region, especially in India and Pakistan, flourishing at elevations between 2,600 and 4,000 meters. *S. costus* possesses a lengthy historical background in Ayurveda, Tibetan, and Chinese medicine, valued for its therapeutic benefits. *S. costus's* roots were prized for their strong aroma and bitter taste, containing bioactive compounds like flavonoids, alkaloids, as well as sesquiterpene lactones (dehydrocostus lactone, & costunolide) considered to offer various health benefits. These include anticancer, anti-inflammatory, analgesic, antimicrobial, anti-ulcer, as well as hepatoprotective effects[1-2,4]. The plant is often utilized in treating ailments such as rheumatoid arthritis, asthma, stomach ulcers, chronic gastritis, and bronchitis[3-4]. In addition to its medicinal applications, essential oil extracted from *S. costus* roots has been utilized in perfumery along with conventional incense due to its pleasant fragrance and blending capabilities with other scents like rose and sandalwood[4]. Despite its extensive use and importance in conventional medication, *S. costus* has been classified as critically endangered species attributable to excessive harvesting as well as habitat degradation. This has

raised concerns regarding its sustainable use and conservation efforts to protect this valuable herb for future generations[1,4]. The plant's chemical profiling has uncovered a range of bioactive constituents with pharmacological characteristics encompassing anti-inflammatory as well as antioxidant actions[5]. *S. lappa* root extract reduced pain in primary dysmenorrhea treatment in comparative clinical studies[6]. Cu nanoparticles biosynthesized from *S. lappa* exhibit anti-obesity[7] and antimicrobial activity[8]. Essential oil extracted from *S. lappa* demonstrated antibacterial and antifungal activity[9]. Extracts from plant have shown ability to manage multidrug-resistant bacterial strains, such as *Acinetobacter baumannii* by modulating host immune response, highlighting its potential as complementary therapy for combating antibiotic resistance[10]. Furthermore, *S. lappa* exhibits protective effects in metal toxicity induced by metal oxide nanoparticles, such as copper oxide, thereby preventing tissue damage and toxicity[11]. *S. lappa* exhibited antiparasitic activity against *Trichinella spiralis* in rat[12]. Several compounds have been isolated from *S. costus* that include new sesquiterpenoids including 14 previously undescribed Lappanolides (A-N), exhibiting significant anti-hepatitis B virus (HBV) activity. Specifically, compounds like Lappanolides 28 and 29 were identified as potent inhibitors of HBsAg secretion with low IC_{50} values[13] while lappaterpenes isolated from root demonstrated effective inhibition of HBV secretion exploring the potential of extract to treat viral infections[14]. Medical benefits of *S. lappa* have become largely ascribed to its sesquiterpenes, especially costunolide and dehydrocostus lactone, which are major bioactive components. Dehydrocostus lactone demonstrated inhibiting activity against both HBsAg and HBeAg[14] and cardioprotective effect in doxorubicin-induced cardiotoxic rat model by inhibiting thioredoxin interacting protein reducing inflammations and oxidative stress[15]. The compounds Dehydrocostus lactone and Mokkolactone were reported to exhibit anticancer activity in gastric cancer by disrupting fatty acid synthesis through targeting ATP citrate lyase (ACLY) and inducing apoptosis[16], with Mokkolactone also showing promising inhibitory effects on EGFR L858R mutations in “non-small cell lung cancer (NSCLC)”[17]. Costunolide, another major bioactive was found to have anti-inflammatory potential in ulcerative colitis and gouty arthritis[18-19], enhanced antitumor activity in LS174T colon cancer cell lines when delivered through bilosome-based nanoparticle formulation that improved its bioavailability and solubility in water[20]. These studies underscore the immense therapeutic potential of *Saussurea costus*, with applications spanning from antiviral and anticancer therapies to antimicrobial, anti-inflammatory, and immune-boosting effects. The ongoing exploration of its bioactive compounds and their diverse mechanisms of action continues to position *S. costus* as prospective candidate for advancement of novel natural pharmaceuticals.

MATERIAL AND METHODS

Material

Bacterial cultures *P. aeruginosa, E. coli, S. aureus*, as well as *B. subtilis* of accession numbers MCC 2408, MCC 2010, MCC 2265, and MCC 3099 respectively have been gathered from the National Centre for Microbial Resources, Pune.

Collection of plant samples and their extraction

Dr. Anamika of the Department of Botany at Vardhman College Bijnor verified the *Saussurea costus* plants that were gathered locally. The roots were cut, thoroughly cleansed using normal water, and shade dried. Soxhlet extraction of powdered roots was performed using protocol of Azwanida, 2015[21]. Briefly, the powdered dried roots were packed in a Soxhlet extractor and extracted sequentially (1:25 w/v) using Acetone (Ac), ethanol (Et), and water (W) with increasing polarity at 60-80^{0}C temperature. The extracts were then filtered. Filtered extract were concentrated employing rotary vacuum evaporator as well as weighed to determine their percent yields and stored in their respective solvents at 4°Celsius until additional utilization.

Phytochemical screening of extracts

The plant extracts obtained using different solvents were analyzed to identify bioactive compounds. Standard phytochemical screening methods were employed to test for occurrence of flavonoids, saponins, glycosides, carbohydrates, alkaloids, phenolics, steroids, terpenoids, as well as tannins.

Total Phenolic content (TPC) estimation of crude

Extracts' phenolic content has been computed via a spectrophotometric method utilizing Folin-Ciocalteu reagent, following procedure outlined by Sidduraju & Becker (2003)[22]. In conclusion,

50 µL of plant extract had been diluted to final volume 1 mL and distilled water. Subsequently, 0.5 mL of Folin-Ciocalteu reagent (diluted 1:1 with water) as well as 2.5 mL of 20% sodium carbonate solution are included. Mixture was adequately combined and incubated in darkness for 45 minutes. Absorbance has been determined at 765nm. TPC (Total phenolic content), assessed using gallic acid as standard, whereas milligrams gallic acid equivalents were determined from gallic acid standard curve's linear equation. Outcomes are articulated as milligrams gallic acid equivalents (mg GAE) per gram of dry weight. The following formula was used to estimate TPC:

$$TPC = (\frac{C * V}{m})$$

Where:
C = mg/mL of Gallic acid
V = Volume of plant extract in mL
m = Plant weight in grams

Total Flavonoid content (TFC) estimation of crude

TFC of plant extracts were quantified utilizing a modified aluminum chloride colorimetric method, as outlined by Zhishen *et al.,*(1999)[23]. In this process, 250 µL of extract then diluted to 1.25 mL with distilled water, and subsequently, 75 µL of 5percent $NaNO_2$ (sodium nitrite) solution was added. After being incubated at ambient temperature for 5 min, 150 µL of 10% $AlCl_3$ solution was included and then filtered. Incorporated 500 µL of 1M NaOH and 27 µL of distilled water, mixed vigorously, and assessed the intensity of the pink hue at a wavelength of 415 nm in compared to blank reagent sample. Quercetin was taken as the standard to determine flavonoid content, and the equivalents were calculated from linear equation obtained from quercetin standard curve. Values has been articulated in milligrams of quercetin equivalents per gram of dry weight. TFC was computed using the formula:

$$TFC = (\frac{C * V}{m})$$

Where:
C = Quercetin equivalent (mg/ml)
V = Volume of the plant extract (ml)
m = Plant weight in grams

2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical inhibition assay

For antioxidant assays, all extracts were made at concentration of 1 mg/mL. DPPH free radical scavenging activity has been ascertained according to procedure described by Blois (1958)[24]. This protocol involved adding 1.5 mL of 0.1mM DPPH solution with 0.1 mL of extract. Mixture has been then mixed well as well as incubated in dark at ambient temperature for 30 minutes. Reduction in DPPH free radical concentration has been evaluated through determining absorbance at 517nm with a spectrophotometer. A reagent control consisted of DPPH solution and methanol with no extract, and all experiments were conducted in triplicates. A positive control was ascorbic acid. The % inhibition of DPPH radicals was calculated below:

$$DPPH\ inhibition\ (\%) = \left(\frac{Acontrol - Atest}{Acontrol}\right) * 100$$

Where:
Acontrol = Absorbance of the reagent control
Atest = Absorbance of the extract.

The Vitamin C equivalent antioxidant capacity (CEAC) or ascorbic acid content of each extract was ascertained utilizing standard curve generated by plotting ascorbic acid concentrations (ranging from 10-320µg/mL) against DPPH% inhibition.

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) Antioxidant assay

The ABTS assay for all extracts has been conducted following Re *et al.,*(1999)[25]. For assay, 1 mL of diluted extract was mixed with 0.1 mL of ABTS reagent. The ABTS reagent was formulated employing 7mM ABTS and 2.45mM potassium persulfate, subsequently incubated in dark at ambient temperature for 16 hours. Subsequently diluted in ethanol to bring its absorbance at 734nm to a level of 0.7. To this extract, ABTS reagent was added. This was allowed to react for 1 min in a dark chamber kept at 30°Celsius. Absorbance reading at 734nm was then done after 1 minute. A reagent control, containing ABTS solution alone without any extract, was employed for comparison. Each experiment was conducted in triplicate. Ascorbic acid served as positive control. The % inhibition of ABTS free radicals was ascertained utilizing the formula:

$$ABTS\ inhibition\ (\%) = \left(\frac{Acontrol - Atest}{Acontrol}\right) * 100$$

Where:
Acontrol = Absorbance of the reagent control
Atest = Absorbance of the extract.

The Vitamin C equivalent antioxidant capacity (CEAC), or ascorbic acid content of each extract, was ascertained from standard curve. Curve has been created by plotting percentage inhibition of ABTS against ascorbic acid concentrations (ranging from 1-5 µg/mL).

Ferric reducing antioxidant power (FRAP) assay

FRAP of each sample extract has been ascertained by a modified version of methodology delineated by Cai *et al.,*[26]. This assay evaluates extracts capacity to decrease ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). For analysis, 30 µL of each extract then mixed with 70 µL distilled water as well as 900 µL freshly prepared, pre-warmed (37°Celsius) FRAP reagent. Solution was incubated at 37°Celsius for 10 min, then absorbance has been determined at 593nm. $FeSO_4$ (Ferrous sulfate) was utilized as standard for assay. For the control, acetate buffer mixture, TPTZ solution, as well as ferric chloride in a 10:1:1 ratio was used without adding test extracts. The FRAP findings were articulated by means of µM $FeSO_4.7H_2O$ per gram of dry weight of the extract.

Column purification of extracts

Fractions showing antibacterial activity were purified via flash column chromatography. Silica gel (60-120mesh, 30 g) was pre-activated and wet-packed into a column having inner diameter 18mm as well as length 300mm, using chloroform as the packing solvent. The gradient elution system used chloroform and methanol from 100% chloroform to 50% methanol, as well as rate of flow was maintained at 1 mL/minute. The column fractions then dried at ambient temperature and kept at 60°Celsius until further analysis.

Antibacterial activity and Minimum inhibition estimation (MIC) of crude and purified

The antibacterial activity of crude and fractions was performed on Müeller-Hinton agar following CLSI protocol. Müeller-Hinton agar was freshly prepared following the manufacturer's directions. Briefly, the medium was produced, autoclaved, cooled at 45-50°C, and put into flat-bottomed glass Petri dishes having diameter 100mm as well as depth 4mm. Media was brought to an ambient temperature. 0.1 mL of bacterial cultures (*P. aeruginosa, S. aureus, B. subtilis*, as well as *E. coli*) of 0.6 OD were spread on separate agar media. Discs of 6 mm diameter dipped in crude extracts and fractions were little dried and carefully placed over the plates. Agar plates were placed upside down in BOD at a temperature of 37°C for 24-72 hours. Plates were inspected for zones of inhibition, and samples that demonstrated antibacterial properties against the specified bacterial species were subjected to minimum inhibitory concentration (MIC) determination. The CLSI standard protocols for the broth dilution method was employed to assess MIC of crude as well as fractions. Müeller-Hinton broth was prepared. 0.1 ml of an overnight grown culture of OD 0.1 was added to 96 well microplates. Crude and fractions were properly diluted and added to their respective wells leaving two wells for each bacterial culture with culture only for cell control and three wells of 0.1 mL of acetone, ethanol, and water were also set for negative control. Plates were tapped or covered and incubated for 24 hours. in BOD. Absorbances were noted at 600nm and data was used to estimate MIC values. A properly diluted imipenem antibiotic standard solution was used for comparative analysis.

Statistical analysis

All experiments, including total phenolic content (TPC), total flavonoid content (TFC), and antioxidant assays, were performed in triplicate. The results are presented as mean ± standard deviation (SD), calculated using Microsoft Excel (Office 2016). Minimum inhibitory concentrations (MICs) of the fractions were determined using GraphPad Prism version 9.5.0.

RESULT AND DISCUSSION

The extraction yields of *Saussurea costus* using different solvent systems demonstrate significant variability based on the solvent employed. Water exhibited the maximum extraction yield (49.0%) of the bioactive compounds of *S. costus* due to its higher efficacy towards antioxidants and phenolic compounds[26-27]. Ethanol produced a lower yield of 42.4% but dissolved both polar as well as non-polar compounds[28-29]. Yield of acetone was the lowest at 39.2% and this is propounded to be the effect of its selective solvation tendency. It is often the case that mixed solvents such as 80% ethanol for phenolic extraction are more effective than pure solvents[26,30]. Generally, oven-dried samples yield more than air-dried samples due to better solvent penetration[26]. The phytochemical study of *S. costus*

exhibited differences that were dependent on the solvent. The acetone extract revealed the presence of alkaloids (in mild amounts), along with flavonoids, phenols, glycosides, tannins, and carbohydrates. While there are reports of acetone extract (SCAc) of *S. costus* exhibiting a strong presence of alkaloids, flavonoids possess anti-oxidizing and anti-inflammation activity[31-32]. Glycosides, tannins, and carbohydrates (Fehling's positive) were common in all of the extracts, thus providing anti-inflammatory, antimicrobial, and immunoenhancement effects[26]. All the samples were negative for steroids. This is consistent with research focused on the benefits of using water as a solvent and the pharmacological aspects of *S. costus*[26,31]. TPC of extracts was estimated from a linear curve equation obtained by plotting graph concentration as well as absorbances of gallic acid at 765nm (Fig. 1A). Water extract exhibited the highest TPC and TFC of 19.36 and 2.41 mg/g respectively, followed by ethanol extract of 15.076 mg/g TPC and 2.04 mg/g of flavonoid content and the lowest TPC and flavonoid content in acetone extract (Fig. 1). The result suggests the efficiency of water in extracting many phenolic as well as polyphenolic-like flavonoid constituents and follows other's works suggesting suitable and efficient solvents to extract phenolic compounds[26,29]. A solvent of 70% ethanol efficiently extracted phenolic from *S. costus* roots as found by Elshaer *et al.,*2022[33].

Table 1: Phytochemical screening of *S. costus* extracts

Sample	Alkaloid			Flavonoid	Phenol	Glycosides	Tannins	Carbohydrate			Saponins	Steroids
	Mayer's test	Dragendorff's test	Wagner test					Molisch	Fehling's	Benedicts		
SCAc	+	-	-	+	+	+	+	-	+	-	-	-
SCEt	-	-	-	+	+	+	+	-	+	-	-	-
SCW	-	+	+	+	+	+	+	-	+	-	-	-

SCAc: Acetone extract of *S. costus*, SCEt: Ethanol extract of *S. costus*. SCW: *S. costus* water extract.+indicates the presence and –indicates the absence of a particular phytochemical

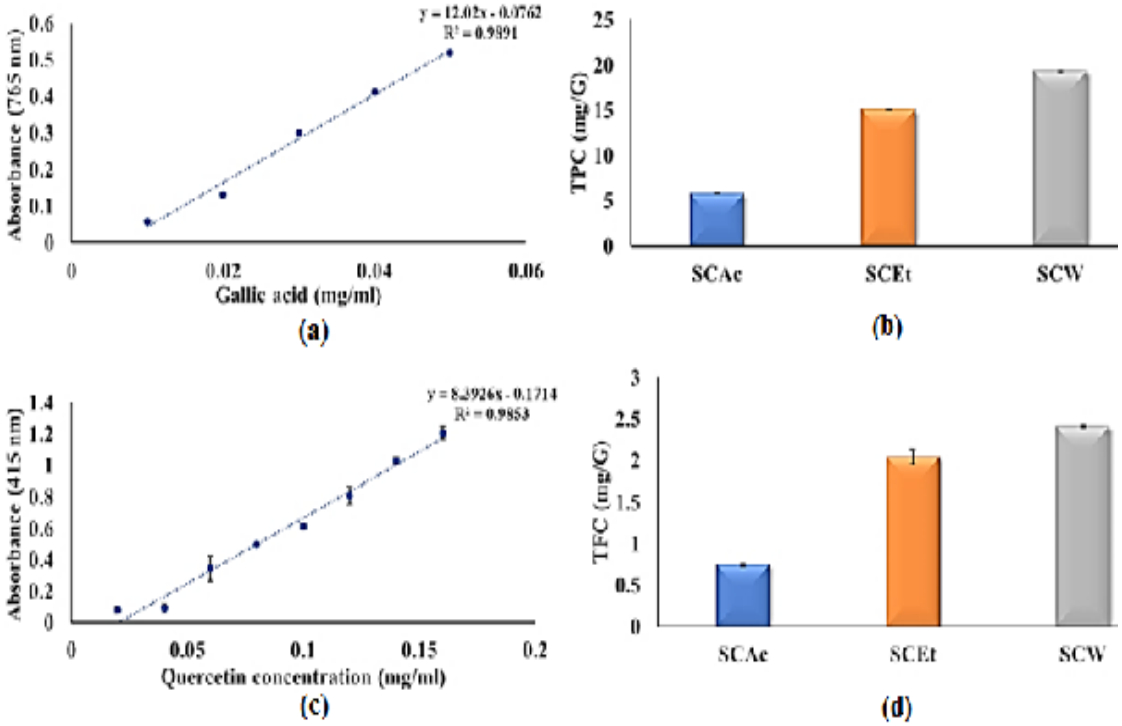

Fig. 1. Quantitative estimation of *S. costus* extracts. A: Gallic acid calibration graph, B: TPC in mg/G of dry weight of all extracts, C: Quercetin calibration graph, D: TFC of all extracts in mg/G of dry weight. Data are indicated as mean±std. dev. Where n (replicates) is 3

***Saussurea costus* extracts antioxidative capacity**

The *Saussurea costus* roots showed high antioxidant activity. Ethanolic (SCEt) and water-soluble (SCW) extracts exhibited substantial action of radical scavenging against ABTS as well as DPPH radicals, with SCW causing about 76.19% DPPH inhibition as well as 87.64% ABTS inhibition, which was again in line with high CEAC levels (225 µg/mL & 3.6 µg/mL) respectively (Fig. 2). *S. costus* root extracts are known to have a large number of flavonoids, sesquiterpene terpenes (especially dehydrocostus as well as costunolide lactone), along other phenolic compounds. These components are important for the antioxidant properties of plant[34]. In addition, *S. costus* contains specific antioxidants that scavenge free radicals thus lowering oxidative stress. According to studies, the ethanolic extract has maximum activity for inhibiting free radicals having a 0.123 mg/mL IC_{50} value for DPPH assays which confirms its potent radical scavenging activities[35]. The antioxidant properties of *S. costus* indicate that it has useful therapeutic effects in conditions that involve elevated oxidative stress levels like inflammation as well as cancer. Its ability to modulate raised reactive oxygen species levels (ROS) might account for its anti-inflammatory and anti-cancer activities[34]. The previous studies support that the antioxidative properties are correlated with phenolic compounds, and *S. costus* also exhibited a pattern of phenolic-antioxidant relationship similar to SCW and SCEt fractions[36]. The FRAP assay results for various extracts of *Saussurea costus* demonstrated remarkable differences in antioxidant capacity with the aqueous extract (SCW) having maximum value at 4057.14 µM Fe(II)/g dry wt., ethanolic extract (SCEt) having second highest value at 3657.14 µM Fe(II)/g dry wt., whereas acetone extract (SCAc) had a significantly lower value around 342.86 µM Fe(II)/g dry wt. Previous investigations have described differences in antioxidant properties of *Saussurea costus* extract, which might be related to extraction solvent chosen. In one study, ethanolic extracts exhibited considerable inhibition of oxidative stress markers consistent with the high values of FRAP as measured for SCEt and SCW in our findings[35].

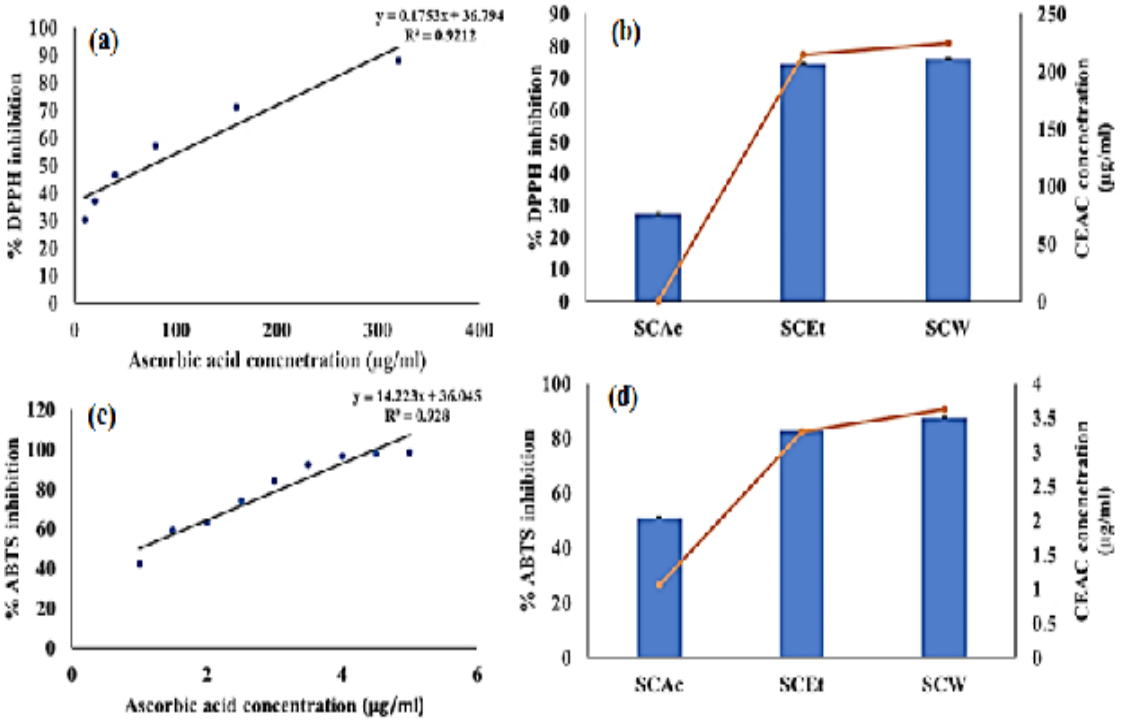

Fig. 2. Antioxidant assay (DPPH and ABTS assay) of *S. costus* extracts. A: Ascorbic acid calibration graph in *µg*/mL estimated by DPPH, B: %DPPH inhibition of *S. costus* extracts. C: Ascorbic acid calibration graph in *µg*/ml estimated by ABTS, D: %ABTS assay of *S. costus*. Data are represented as mean±std. dev. Where n (replicates) is 3.

Antibacterial activity and Minimum inhibition estimation (MIC) of crude and purified Crude extracts having potent antibacterial activities were subsequently column-purified, along fractions have been assessed for their antibacterial potency. Potent fractions having antibacterial activities were then

proceeded for MIC estimation. A total of 13 fractions were obtained: 6 for SCW, 4 from SCEt, and 3 from SCAc. Yields in the range of 0.011-0.143 g, 0.010-0.078 g, and 0.005-0.055 g were obtained from crude water, ethanol, and acetone, respectively. The highest yield was observed in fractions three of water and ethanol crude extracts, at 0.143 g and 0.078 g, respectively. The results indicate efficacy of extraction varied depending on solvent used. Differences in yields might be ascribed to varying solubility of compounds in water, acetone, as well as ethanol. Only first and second fractions of each crude were found to have antibacterial activity. SCWF1 and SCWF2 displayed the highest antimicrobial activity, particularly against *S. aureus* (1.7 cm and 1.6 cm, respectively) and *B. subtilis* (1.4 cm and 1.8 cm, respectively). Both samples also showed moderate activity against *P. aeruginosa* and lower activity against *E. coli*, which appeared to be the most resistant strain overall. SCEF2 showed slightly better activity against *E. coli* (1.4 cm) (Table 2). These findings suggest that SCWF samples have potential for further development, while SCEF and SCAcF may require optimization to enhance their antimicrobial efficacy. Methanolic extracts and oil extracted from *S. costus* were reported to have antimicrobial activity against *C. albicans* as well as *S. aureus* exhibiting MIC of essential oil as low as 3.12 µg/mL[37]. The ethanolic *S. costus* extract showed considerable activity against bacteria, with inhibition zones varying from 13-23 mm toward organisms such as *S. aureus* and *Salmonella typhi*. The higher susceptibility was reported for *Gram-positive* than *Gram-negative* bacteria[38,29]. Results from MIC table (Fig. 3(b)) indicate that SCWF1 and SCWF2 fractions exhibited activity against *Gram-positive* bacteria (with *B. subtilis* & *S. aureus* as test organisms) exhibiting MIC values between 245-307.3 µg/mL while, SCAcF1 as well as SCAcF2 has been active on *Gram-negative* bacteria. Remarkably, SCAcF2 was the most effective with a considerably low MIC (111.1 µg/mL) against *P. aeruginosa* and some action against *E. coli* (286.1 µg/mL) as well. In contrast to SCWF fractions, which did not affect *Gram-negative* bacteria, the SCAc fractions were inactive on *Gram-positive* strains. These results imply that SCWF fractions may be aimed at *Gram-positive* specific pathways, while SCAcF2 appears as a good drug for the therapy of *Gram-negative* infections, especially *P. aeruginosa*.

Table 2: Antibacterial activity and ZOI of fractions against selected bacterial species

Name of Sample	Zone of Inhibition (cm)			
	S. aureus	*B. subtilis*	*P. aeruginosa*	*E. coli*
Imipenem	1.7	1.2	1.8	1.9
SCWF1	1.7	1.4	1.2	0.5
SCWF2	1.6	1.8	0.9	0.6
SCEF1	1.1	0.5	0.3	0.9
SCEF2	1.4	0.4	0.2	1.4
SCAcF1	1.3	0.5	1.2	0.7
SCAcF2	1	0.6	1.2	0.6

SCAc: Acetone extract of S. costus, SCEt: Ethanol extract of S. costus. SCW: Water extract of S. costus, F1 and F2 indicate fractions.

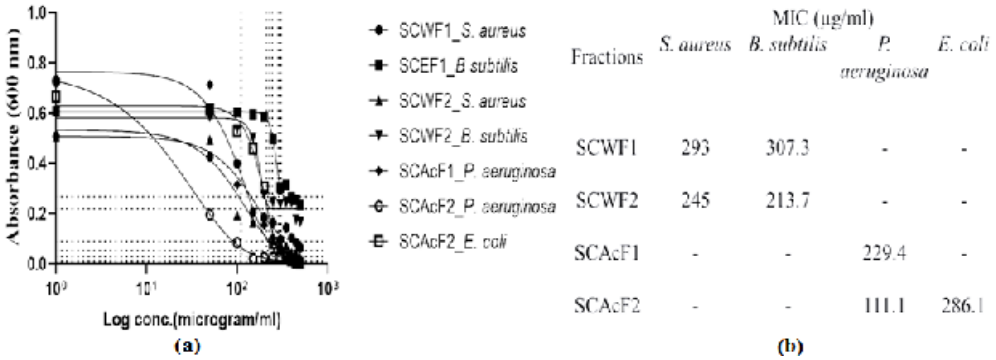

Fig. 3. Minimum inhibitory concentrations for fractions. A: MIC graph of all the fractions against the selected bacterial species. B: MIC table indicating their values against specific bacteria

CONCLUSION

The study provides evidence concerning the considerable phytochemical, antioxidant, and antibacterial activities of *Saussurea costus*, thereby explicating its suitability as a possible source of bioactive agents for diverse uses. Results from preliminary screening of phytochemicals indicated several secondary metabolites like alkaloids, flavonoids, tannins, and phenolics presence which are probably responsible for the biological activities observed. The antioxidant tests performed demonstrated plant's tendency to effectively inhibit free radicals which suggests that plant might be utilized in oxidative stress-related problems treatement. In addition, antimicrobial assessment also showed the plant's effectiveness against certain microbial strains consistent with its ethnomedicinal application. The results substantiate conventional uses of *Saussurea costus* and point to the need for more detailed research regarding its marketability.

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Conflict of interest

Nil

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