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Antibacterial potential of *Elletaria cardamomum, Syzygium aromaticum* and *Piper nigrum*, their synergistic effects and phytochemical determination

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ABSTRACT

Background: Spices are considered as rich source of bio-active antimicrobial compounds and are indispensable components of Indian cuisines since ancient times. To provide a scientific basis to traditional uses of *Elletaria cardamomum, Syzygium aromaticum and Piper nigrum*, their aqueous and organic seed extracts, isolated phytoconstituents and combinations were evaluated for antibacterial potential against gram positive (*Staphylococcus aureus, Bacillus subtilis*) and gram negative (*Escherichia coli, Salmonella typhi*) bacteria. **Methods:** Antibacterial activity of aqueous and organic seed extracts was assessed using agar diffusion assay, minimum inhibitory concentration and the effect was compared with some standard antibiotics. The presence of major phytoconstituents was detected qualitatively and quantitatively. The isolated phytoconstituents and combination of spices were subjected to disc diffusion assay to ascertain their antibacterial effect. **Results:** Organic extracts of all three spices showed good antibacterial activity against all the test strains, which was found to be comparable with standard antibiotics. Minimum inhibitory concentration for aqueous and organic seed extracts ranged from 25 to >50 mg/ml and 2 to 50 mg/ml respectively. Inhibitory activity of all the extracts was found to be increased when used in combination. This synergistic effect supports the use of these spices in combination. Quantitative phytochemical analysis showed the presence of 2.30 – 7.8% alkaloids, 5.7 – 26.2% flavonoids, 11.0 – 33.0% tannins, 0.60 – 3.8% saponins. **Conclusion:** Antibacterial efficacy shown by these spices and their combinations provides a scientific basis and thus, validates their traditional uses as homemade remedies. Isolation and characterization of different phytochemicals may further yield significant antibacterial agents.

KEYWORDS: Antibacterial activity, Minimum inhibitory concentration, Synergistic effect, Phytoconstituents

1. INTRODUCTION

Spices have been used for many centuries by various cultures to enhance the flavor and aroma of our foods as our ancestors have recognized the usage of spices in food preservation and in treatment of clinical ailments. There are several reports on development of antibiotic resistance in diverse bacterial pathogens[1]. This shift in susceptibility of pathogens to antibiotics has adversely affected its ability to successfully treat patients and therefore shifted their attention towards herbal products. At present, it has been estimated that about 80% of the world population rely on botanical preparations as medicine to meet the needs as they are considered safe and effective against certain ailments[2]. Besides, spices are known for their unique aroma and flavor derived from compounds known as phytochemicals or secondary metabolites; [3] these are antimicrobial substances that are capable of attracting benefits and repel harmful microorganisms. Antimicrobial potential of different spices is being extensively studied all over the world[4,5] but only a few studies have been carried out in a systematic manner. However, in the absence of any scientific proof of their effectiveness, the validity of these remedies remains questionable and their use locally restricted. Their systematic screening may result in the discovery of novel active compounds.

In the present study various extracts of three spices *Elletaria cardamomum* (Green cardamom) *Syzygium aromaticum* (Clove) and *Piper nigrum* (Black pepper) are screened for their antimicrobial potential and phytochemical constituents. These plants are a common household remedy against a variety of gastrointestinal disorders, e.g. indigestion, flatulence, colic pain etc.; also used as spices and condiments in foods for their flavor, aroma, and preservation; and their dried ripe fruits and essential oils have aromatic and carminative properties[6]. The essential oils of these plants have been reported to possess antimicrobial activity[7-9]. However, in folklore, seeds or their aqueous extracts are used as homemade remedies but only a little work has been done to explore them. Thus, to provide a scientific justification

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for these traditional remedies, the present study was planned to assess their antibacterial potential using aqueous and organic extracts against some clinically important bacteria. The extracts as well as their isolated phyto-constituents were assessed for their synergistic antimicrobial effect, as they are used together in Indian food preparations.

2. MATERIALS AND METHODS

2.1 Materials

All the chemicals and standard antibiotics were purchased from Hi-Media, Mumbai, India; and all the solvents used were of analytical grade. Precoated silica gel 60 F254 TLC plates and standard phytoconstituents were purchased from Merck, Germany and Sigma Chemicals, USA, respectively.

2.2 Plant materials and extraction procedure

The seeds of different plants viz *E. cardamomum*, *S. aromaticum*, *P. nigrum* were purchased from local market in Delhi, India. All the plant materials were identified and contaminated particles were removed. The air-dried plant materials were powdered and used for extraction. Extracts were prepared using four different solvents with increasing polarity – Hexane, dichloromethane, ethanol and water. 50 g of each powdered spice was extracted/stirred in 500 ml of the solvent for 24 h with intermittent shaking. Each extracted material was vacuum filtered using Whatman filter paper and then the solvent was evaporated using rotary evaporator.

2.3 Bacterial cultures

Reference bacterial strains *viz. Staphylococcus aureus* (MTCC 3160), *Escherichia coli* (MTCC 119), *Salmonella typhi* (MTCC 531) and *Bacillus subtilis* (MTCC 121) were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. These were maintained on nutrient agar slants. All the isolates were sub cultured regularly and stored at 4°C as well as at - 80°C by making their suspension in 10% glycerol.

2.4 Inoculum preparation

A loopful of isolated colonies was inoculated into 4 ml of peptone water, incubated at 37°C for 4 h. This actively growing bacterial suspension was then adjusted with peptone water so as to obtain a turbidity visually comparable to that of 0.5 McFarland standard prepared by mixing 0.5 ml of 1.75% (w/v) barium chloride dihydrate ($BaCl_2.2H_2O$) with 99.5 ml of 1% (v/v) sulphuric acid (H_2SO_4). This turbidity is equivalent to approximately $1–2 \times 10^8$ colony forming units per ml (CFU/ml).

2.5 Qualitative Phytochemical Screening

Various phytochemicals were detected in the extracts so prepared by conducting various tests like: Wagner's test, Fehling's test, Benedict's test, Borntrager test, Biuret test, Libermann-Burchard's test, Saponification test, ferric chloride test, alkaline reagent test, Mg and HCl reduction test etc[10].

2.6 Quantitative analysis:

Alkaloids were quantitatively determined according to the method of Harborne[11]. Saponins were determined according to the method of Obadoni and Ochuko[12]. Tannin determination was done according to Van-Burden and Robinson[13]. Flavanoids were determined according to the method of Bohm et al with slight modifications[14].

2.7 Thin layer chromatography (TLC)

Identification of major phyto-constituents was further carried out by TLC using pre-coated silica gel 60 F264 plates. Different screening systems were used to obtain better resolution of the components. The developed plates were observed under visible as well as UV light (254 nm and 356 nm). Rf value of each spot was calculated as; Rf = Distance travelled by the solute/Distance travelled by the solvent.

2.8 Bioautography of extracts

Qualitatively isolated group of compounds which were subjected to thin layer chromatography, were also assessed for their antibacterial potential using agar disc diffusion assay. Alkaloids were isolated by mixing 1 g powdered sample with 1 ml of 10% (v/v) ammonia solution and extracted with 5 ml methanol for 10 min on water bath (40°C). It was then filtered through Whatman filter paper No. 1 and the filtrate was concentrated using rotary evaporator. Isolation of flavonoids was achieved by heating 1 g powdered sample with 5 ml methanol on water bath at 40°C for 10 min. The filtrate was then concentrated using rotary evaporator to 1/4th of its original volume. For saponins, one gram powdered sample was extracted with 5 ml methanol by heating on a water bath at 40°C for 10 min. The extract was filtered and evaporated to 1 ml, mixed with 0.5 ml water and then extracted thrice with 3 ml n-butanol. The n-butanol phase was evaporated and concentrated to approximately 1 ml. Tannins were obtained by treating 1 g powdered sample with 10 ml 2 M hydrochloric acid (HCl) and hydrolyzing in boiling water bath for 30 min. The solution was filtered, mixed thoroughly with 1 ml ethyl acetate, and ethyl acetate layer was then discarded. Five drops of amyl alcohol were added and shaken thoroughly. Alcoholic layer was retained and used for antibacterial testing. Glycosides were isolated by extracting 1 g powdered sample with 5 ml of 50% (v/v) methanol and 10 ml of 10% (w/ v) lead (II) acetate solution by heating on water bath at 40°C for 10 min. The filtrate was cooled to room temperature and then extracted twice with 10 ml dichloromethane/isopropanol (3:2). The combined lower phases were filtered over anhydrous sodium sulphate and evaporated to

dryness. The residue was dissolved in 1 ml dichloromethane/ isopropanol (3:2) and this solution was further used for antibacterial investigations. Sterile discs (6 mm) were saturated with all the five isolated group of compounds, air dried and used for antibacterial activity testing.

2.9 Determination of antibacterial activity by agar diffusion method

Sensitivity of different bacterial strains to various extracts was measured in terms of zone of inhibition using agar diffusion assay[15]. The plates containing Nutrient agar were spread with 0.2 ml of the inoculum and the discs loaded with sample were placed on it. The plates inoculated with different bacteria were incubated at 37°C up to 48 h and diameter of any resultant zone of inhibition was measured. For each combination of extract and the bacterial strain, the experiment was performed in duplicate and repeated thrice.

The antibacterial activity of different plant extracts was compared with five commonly employed antibiotics *viz*. gentamycin (30 µg/ disc), nalidixic acid (30 µg/disc), streptomycin (25 µg/disc), ampicillin (25 µg/disc) and tetracyclin (10 µg/ disc).

2.11 Statistical analysis

All values have been expressed as mean ± standard deviation and the comparison of the antibacterial activity of the samples with standard antibiotics was evaluated by applying t-test. P=0.05 values were considered to indicate statistically significant difference.

3. RESULTS AND DISCUSSION

3.1 Qualitative and quantitative analysis of seeds for their phytoconstituents

Qualitative phytochemical analysis showed the presence of alkaloids, flavonoids, tannins, saponins and cardiac glycosides (**Table 1**) and the data for their quantitative determination has been presented (**Table 2**). Quantitative phytochemical analysis showed the presence of 2.30 – 7.8% alkaloids, 5.7 – 26.2% flavonoids, 11.0 – 33.0% tannins, 0.60 – 3.8% saponins and glycosides. The phytoconstituents detected in the plant materials could be responsible for their antimicrobial activity though their exact mode of action is poorly understood.

Table 1: Qualitative phyto-chemical analysis of different extracts of *Elletaria cardamomum*, *Syzygium aromaticum* and *Piper nigrum*

Plants	Extract	Alkaloids	Flavanoids	Polyphenols	Glycosides	Phytosterols	Carbohydrates	Oil/fats
E. cardamomum	H	+	-	-	-	+	-	+
	D	+	-	-	+	-	-	+
	E	-	+	-	-	-	-	-
	W	-	+	-	-	-	-	-
S. aromaticum	H	+	-	-	+	+	-	+
	D	+	+	-	-	+	+	+
	E	-	+	+	-	-	+	+
	W	+	+	+	-	-	+	-
P. nigrum	H	+	-	+	+	+	-	+
	D	+	-	+	-	+	-	+
	E	+	+	+	-	+	-	-
	W	+	+	+	-	-	-	-

(+), Presence (-), Absence **H**- Hexane extract, **D**- DCM extract, **E**-Ethanolic extract, **W**-aqueous extract

2.10 Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration of the effective seed extracts was worked out by agar dilution method[16]. Nutrient agar plates containing varying concentrations (1–50 mg/ml) of different seed extracts were prepared and inoculated with 0.1 ml of the inoculum. The plates were incubated at 37°C for 24 h and the lowest concentration of the extract causing complete inhibition of the bacterial growth was taken as MIC.

Table- 2: Quantitative (Percent) phytochemical evaluation of seeds of different plants

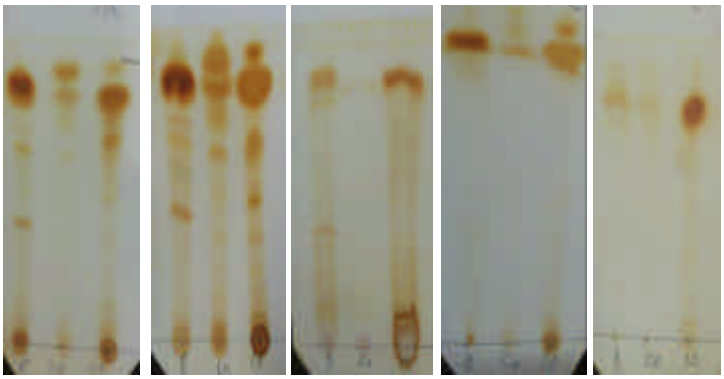
Plants	Alkaloids	Flavanoids	Tannins	Saponins
E. cardamomum	2.3 ± 0.14	5.7 ± 0.12	11 ± 0.1	0.60 ± 0.05
S. aromaticum	5.4 ± 0.22	26.2 ± 0.27	12.8 ± 0.18	3.8 ± 0.11
P. nigrum	7.8 ± 0.32	9.7 ± 0.23	33 ± 0.29	0.65 ± 0.09

3.2 Thin layer chromatography

The presence of phyto-constituents was further confirmed by thin layer chromatography and their Rf values have been presented (**Table 3, Figure 1A–E**). The components were best resolved in screening system chloroform/methanol/water (8.7: 1.1: 0.2)

Table 3: Rf values of different phyto-constituents

Plants	Alkaloid	Flavonoid	Tannins	Saponins	Glycosides
E. cardamonum	0.568, 0.667, 0.764, 0.843	0.250, 0.318, 0.477, 0.590, 0.704, 0.795, 0.909	0.063, 0.425, 0.787	0.916, 0.979	0.809, 0.880
S. aromatataticum	0.470, 0.647, 0.784, 0.862	0.068, 0.204, 0.318, 0.431, 0.613, 0.795, 0.909	0.085, 0.446, 0.808	0.895, 0.979	0.119, 0.309, 0.357, 0.476, 0.619, 0.785
P. nigrum	0.334, 0.431, 0.490, 0.568, 0.725, 0.784	0.295, 0.409, 0.545, 0.636, 0.704, 0.863	0.063, 0.127, 0.361, 0.761, 0.829	0.937	0.690, 0.785, 0.834, 0.904



A: Alkaloids, **B**: Flavanoids, **C**: Tannins, **D**: Saponins, **E**: Glycosides

Figure 1: TLC of *Elletaria cardamomum*, *Syzygium aromaticum*, and *Piper nigrum* extracts. TLC of *Piper nigrum(1)*, *Elletaria cardamomum* (2) and *Syzygium aromaticum* (3) extracts

3.3 Antibacterial activity

Different extracts of spices *Elletaria cardamomum*, *Syzygium aromaticum* and *Piper nigrum* and standard antibiotics showed significant antibacterial activity against gram positive (*S. aureus, B. subtilis*) and gram negative (*E. coli, S. typhi*) bacteria as assessed by the diameter of zone of inhibition of the extracts. (**Table 4 and Table 5**) The plants differed in their antimicrobial activity against test strains. The best antimicrobial activity was observed in the organic (hexane, DCM and ethanol) extracts of *S. aromaticum* against *S. aureus* (gram positive) with diameter of zone of inhibition as 19, 19 and 18 mm respectively. Despite similar sensitivity pattern exhibited by hexane, DCM and ethanolic extracts, the latter is the preferred choice because of its polar nature, volatility, miscibility with polar and nonpolar solvents and relatively lower toxicity. Organic extracts of *S. aromaticum* showed significant inhibitory activity against all the strains as shown in Table 4, although activity of aqueous extract was not found to be significant.

Table 4: Antibacterial activity of different extracts of *Elletaria cardamomum*, *Syzygium aromaticum* and *Piper nigrum*

Plants	Extracts	*E.coli*	*S. typhi*	*S. aureus*	*B. subtilis*	Mean	±SD
		Diameter of Zone of Inhibition (mm)					
E. cardamomum	**H**	12	9	9	10	10	1.41
	D	9	7	8	12	9	2.1
	E	8	9	10	9	9	0.81
	W	7	-	-	7	7	0
S. aromaticum	**H**	17	17	19	13	16.5	2.5
	D	15	14	19	10	14.5	3.6
	E	13	14	18	12	14.25	2.62
	W	9	9	8	7	8.25	0.95
P. nigrum	**H**	14	12	12	17	13.75	2.36
	D	15	16	14	19	16	2.16
	E	8	14	11	14	11.75	2.8
	W	8	8	7	9	8	0.81

H- Hexane extract, **D**- DCM extract, **E**-Ethanolic extract, **W**-Aqueous extract

Table 5: Antibacterial activity of some standard antibiotics

Antibiotics (µg/disc)	*E.coli*	*S. typhi*	*S. aureus*	*B. subtilis*	Mean	±SD
	Diameter of zone of inhibition (mm)					
Gen (30)	22	24	20	27	23.25	2.98
NA (30)	12	10	8	18	12	4.32
Str (25)	18	19	16	24	19.25	3.4
Amp (25)	11	10	14	12	11.75	1.7
Tet (10)	20	18	26	20	21	3.46

Gen – Gentamycin, **NA** – Nalidixic acid, **Str** – Streptomycin, **Amp** – Ampicillin, **Tet** – Tetracyclin

All the organic extracts of *P. nigrum* also showed good inhibitory activity against all the microbial strains but best inhibitory activity was shown by the DCM extract. The zone of inhibition shown by DCM extract of *P. nigrum* was found to be 15, 16, 14 and 19 mm

against *E. coli*, *S. typhi*, *S. aureus* and *B. subtilis* respectively. *E. cardamomum* extracts didn't show any significant inhibitory activity. Organic extracts of all the spices were found to possess better inhibitory activity than aqueous extracts which might be due to present of different active phytocompounds.

3.4 Minimum inhibitory concentration

All the plant extracts were checked for their minimum inhibitory concentration against all the test strains in the concentration range of 1 to 50 mg/ml. The MIC values were found to be plant and strain dependent. Better efficacy of organic extracts was further proven by the MIC studies. (**Table 6**) The minimal inhibitory concentration for organic and aqueous extracts ranged from 2 to 50 mg/ml and 35 to >50 mg/ml, respectively. The MIC values for all the hexane extracts were found to be better than other extracts against all the tested strains. The MIC value for *S. aromaticum* hexane extract was 2 mg/ml against E. coli and 4 mg/ml against *S. aureus*. This data coincide with those of Smith-palmer et al.[17] who reported strong inhibitory activity of clove oil to food-borne pathogens including *E. coli* and *S. aureus*.

Table 6: Minimum Inhibitory concentration (mg/ml) of different extracts of *Elletaria cardamomum*, *Syzygium aromaticum* and *Piper nigrum*

Plants	Extracts	*E.coli*	*S. typhi*	*S. aureus*	*B. subtilis*
E. cardamomum	H	5	42	5	35
	D	5	10	30	25
	E	25	35	>50	>50
	W	>50	>50	>50	>50
S. aromaticum	H	2	25	4	25
	D	6	25	30	35
	E	20	20	40	15
	W	35	>50	>50	>50
P. nigrum	H	2	20	5	20
	D	2	4	10	8
	E	5	10	30	10
	W	40	>50	>50	>50

H- Hexane extract, **D**- DCM extract, **E**-Ethanolic extract, **W**-Aqueous extract

All the organic extracts of *P. nigrum* were found to be effective against all the tested strains as observed by the MIC values. Karsha and Lakshmi[18] has also reported that organic extracts of black pepper possess good inhibitory activity against both gram positive and gram negative bacterial strains. According to Harold[19] the antimicrobial activity of black pepper is due to the presence of essential oil (3%), whose aroma is dominated by monoterpenes hydrocarbons: sabinene, ß-pinene and limonene. Furthermore, terpinene, a-pinene, myrcene, and monoterpene derivatives like borneol, carvone, carvacrol, 1,8-cineol and linalool are also present. The mechanism of action of terpene is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds[20]. In case of *E. cardamomum*, hexane extract exhibited good MIC against most of the bacterial strains but for other extracts it was found to be greater than 30 mg/ml.

3.5 Antibacterial activity of isolated phytoconstituents

Isolated groups of compounds demonstrated their antibacterial effect though to a lesser extent (**Table 7**). Purified alkaloids as well as their synthetic derivatives are used as medicinal agents for their various biological effects such as analgesic, antispasmodic and bactericidal[6]. Flavonoids have also been reported to possess anti-bacterial activity, which could be attributed to their ability to form complex with extracellular, soluble proteins and bacterial cell walls[21]. In case of isolated alkaloids and flavonoids, the inhibitory activity observed with all the strains was not found to be significant. Plant tannins, another class of polyphenolic compounds, results in their antimicrobial action by precipitating microbial protein[22] and this potency is governed by their concentration in the plants. In the present study, tannins isolated form all three spices showed exceptionally good inhibitory activity in terms of zone of inhibition of 14 to 19.5 mm against tested strains. Good antibacterial activity was shown by saponins, a special class of glycosides; though their concentration is much lower as revealed by quantitative analysis. The present study revealed that the crude extracts contain a number of phytoconstituents whose isolation and purification may yield significant novel antimicrobial agents.

Table 7: Antibacterial activity of isolated group of phyto-constituents

		E.coli	*S. typhi*	*S. aureus*	*B. subtilis*
		Diameter of Zone of Inhibition (mm)			
E. cardamomum	Alk	-	-	-	-
	Fla	-	-	9	10
	Tan	17	17	15	14
	Sap	10	10	8	9
	Gly	8	11	10	10
S. aromaticum	Alk	-	-	8	-
	Fla	10	8	12	13
	Tan	19	18	14	15
	Sap	10	12	8	11
	Gly	10	12	11	10
P. nigrum	Alk	-	-	-	-
	Fla	-	10	8	9
	Tan	18	15	19.5	15
	Sap	9	10	13	9
	Gly	11	11	12	9

Alk – Alkaloids, **Fla** – Flavanoids, **Tan** – Tannins, **Sap** – Saponins, **Gly** - Glycosides

3.6 Synergistic antibacterial activity

Combined antimicrobials are preferred as microbial tolerance is less likely to develop against substances having more than one type of modes of action[23]. It was thus necessary to check the antimicrobial

activities of these spices in combinations as used in conventional cooking or salad dressing. All the plant extracts were tested for their susceptibility against all the bacterial strains on one to one basis in a mixture of 1:1 ratio. The diameter of zone of inhibition of different combinations of plant extracts is shown in **Table 8**. Combinations of the spices in several cases demonstrated synergistic or additive effects on microorganisms. Inhibitory activity of *E. cardamomum* was found to be increased when it was used in combination with any other spice extract. Individual aqueous extracts didn't show any inhibitory activity, but in combination the activity was found to be drastically increased as observed by the zone of inhibition. The zone of inhibition for all three spice aqueous extract was found to be 11.5, 19 and 11 against *E. coli*, *S. typhi* and *S. aureus* respectively. According to Cain *et al.*[24] synergistic activity suggests different mode of actions of the combining compounds. These results support the use of these three spices in combination instead of use in isolation.

Table 8: Synergistic antibacterial effect of the mixture of different extracts of *Elletaria cardamomum*, *Syzygium aromaticum*, and *Piper nigrum* (1:1 ratio)

Combination of Plant Extracts		*E.coli*	*S. typhi*	*S. aureus*	*B. subtilis*
		Diameter of Zone of Inhibition (mm)			
E. cardamomum	**H**	9	12	17	7
+ *S. aromaticum*	**D**	15	10	9	10
	E	13	14	15	15
	W	7	7	7	7
E. cardamomum	**H**	10	20	9	12
+ *P. nigrum*	**D**	19	11	11	8
	E	13	14	12	11
	W	7	7	-	10
S. aromaticum	**H**	7.5	15	11	11
+ *P. nigrum*	**D**	14	16.5	17	9
	E	-	7	15	12
	W	10.5	9	9	9
E. cardamomum	**H**	10	14	14	16
+ *S. aromaticum*	**D**	9	13	17	13.5
+ *P. nigrum*	**E**	13.5	13.5	14	12.5
	W	11.5	19	11	10

H- Hexane extract, **D**- DCM extract, **E**-Ethanolic extract, **W**-Aqueous extract

4. CONCLUSION

In conclusion, the spices alone or in combination possessed good inhibitory activity against all the tested bacterial strains. Although aqueous extract showed good inhibitory activity in combination, which support their use together in traditional food preparations. The presence of most general phytochemicals might be responsible for their therapeutic effects. These individual constituents of the extracts can be isolated, and further characterization can be done to explore the potential of these antimicrobial agents present in the extracts, as well as to combat against drug resistant microorganisms, a large library of novel compounds is required. Natural products from plants may give us a solution to this alarming problem.

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