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Antimicrobial and antioxidant efficacy of various solvent extracts of seeds and fruits rind of *Caesalpinia pulcherrima* Swartz.

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Abstract

Ethnopharmacological relevance: *Caesalpinia pulcherrima* Swartz. is an ornamental plant like a shrub or small tree. In traditional Indian medicine *C. pulcherrima* is used in the treatment of tridosha, fever, ulcer, abortifacient, emmenagogue, asthma, tumors, vata and skin diseases.
Aim of the study: In the present study, the antimicrobial and the antioxidant activities of seeds and fruits rind of *Caesalpinia pulcherrima* Swartz were investigated.
Material and methods: Hexane, chloroform, acetone, methanol and water extracts of *C. pulcherrima* were evaluated for their antimicrobial activity by agar well diffusion method. DPPH$^{\bullet}$ free radical scavenging activity, total phenolic, flavonoids and qualitative phytochemical analysis were also performed.
Results: Among the different extracts, the methanol extract showed significant antibacterial and antioxidant activities. The MIC of the methanol extract of fruits rind showed narrow MIC range (1,250 - 78 µg/ml) against all the investigated microorganisms. The methanol extract of the fruits rind showed good antioxidant activity (IC_{50} value 14 µg/ml) which is very near to the standard (ascorbic acid IC_{50} value 11.4 µg/ml).
Conclusion: The obtained results suggested that the methanol extract of the seeds and fruits rind of *C. pulcherrima* possess antimicrobial and antioxidant properties which can be used for the discovery of new bioactive natural products that may serve as leads in the development of new pharmaceuticals that address unmet therapeutic needs.
Keywords: *Caesalpinia pulcherrima,* Fabaceae/Leguminosae, antimicrobial activity, antioxidant activity, physico-chemical analysis, phenols

Introduction

Caesalpinia pulcherrima (Fabaceae/Leguminosae) is a shrub or small tree up to 5 m height, commonly known as Guleture is distributed throughout India [1]. This shrub is one of the most popular summer bloomers. From May through August this tropical looking shrub produces loads of spectacular flower clusters. The plant is used as emmenagogue, purgative, stimulant and abortifacient, also used in bronchitis, asthma and malarial fever [2]. The fruit was used to check bleeding and prevent diarrhea and dysentery, while the flowers were utilized to combat oxidative stresses [3]. According to folkloric uses, the stem has been used as an abortifacient and an emmenagogue [4, 5]. In traditional Indian medicine *C. pulcherrima* is used in the treatment of tridosha, fever, ulcer, abortifacient, emmenagogue, asthma, tumors, vata and skin diseases [6]. Antiviral, antiulcer, anti-inflammatory activity of different parts of *C. pulcherrima* has been reported [7- 9].

The use of traditional medicine is widespread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs [10]. Traditionally common people use crude extracts of plant parts as curative agents [11, 12]. Plants are known to produce antimicrobial agents as their defense mechanism; they can be considered as potential sources of new antibacterial agents. A number of antibiotics and chemotherapeutic agents of natural or semi synthetic origins are available today to fight against infections caused by various pathogenic microorganisms [13].

In searching for novel natural antioxidants, some plants have been extensively studied in the past few years for their antioxidant and radical scavenging components [14, 15] but there is still a demand to find more information concerning the antioxidant potential of plant species. Plant phenols are of interest because they are an important group of natural antioxidants and some of them are potent antimicrobial compounds [16]. DPPH (1, 1- diphenyl 2-picrylhydrazyl) has been used extensively as a free radical to evaluate reducing substances [17].

In view of the above considerations, it was thought of interest to investigate the antimicrobial and the antioxidant potential of *Caesalpinia pulcherrima.* The main goal of the present work was to assess the total phenolic and flavonoidal contents, antimicrobial activity, and antioxidant properties of various solvent extracts of the seed and fruits rind of *C. pulcherrima*.

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Material and methods

Plant collection

The fruits of *Caesalpinia pulcherrima* Swartz were collected in September, 2007 from Rajkot, Gujarat, India. The identification of this plant was done by Dr. N.K. Thakrar, of the Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India, and kept under the Voucher specimen No. PSN246.

Plant extraction

The seeds and fruit rinds were separated and then washed under running tap water, air dried, homogenized to fine powder and stored in air-tight bottles. The powder of the seeds and fruits rind was extracted (10 g) successively with 100 ml each of n-hexane, chloroform, acetone, methanol and water with crude cold percolation extraction method. The extraction flasks were kept on a rotary shaker for 24 h at 120 rpm. Thereafter they were filtered through 8 layers of muslin cloth and centrifuged at 5000 rpm for 15 min. The supernatant was concentrated to dryness under reduced pressure and stored at 4°C in air-tight bottles.

Qualitative Phytochemical analysis

The preliminary phytochemical analyses (Alkaloids, Flavonoids, Tannins, Phlobatannins, Triterpenes, Steroids, Saponins, and Cardiac glycosides) were performed according to the reported method [18].

Physico-chemical analysis

Physico-chemical analyses were carried out according to reported method in The Ayurvedic Pharmacopoeia of India [19] and Vaghasiya et al. [20]. The following physico-chemical analyses; loss on drying at 105° C, total ash, water soluble ash, acid insoluble ash, Petroleum ether soluble extractive value, Ethanol soluble extractive value and Aqueous soluble extractive value were carried out.

Determination of the total phenolic content

The amount of the total phenolic content in the extracts of the different parts of *C. pulcherrima* was determined with Folin-Ciocalteu reagent method [21]. 0.5 ml sample (1 mg/ml) in 3 replicates and 0.1ml (0.5 N) Folin-Ciocalteu reagent were mixed and the mixture was incubated at room temperature for 15 min. Then, 2.5 ml saturated sodium carbonate was added and the resulting mixture was again incubated at room temperature for 30 min. The absorbance of the mixture was measured at 760 nm using spectrophotometer (Systronics: 106). The calibration curve was prepared using gallic acid (10 to 100 µg/ml) solution in distilled water. Total phenol values were expressed in terms of gallic acid (SRL, India) equivalent (mg/g of extracted compound).

Determination of the flavonoidal content

The flavonoidal content in the extracts of the different parts of *C. pulcherrima* was determined by Aluminum chloride colorimetric method [22]. 1.0 ml sample (1 mg/ml) in 3 replicates, 1.0 ml of methanol, 0.5 ml of (1.2 %) Aluminum chloride and 0.5 ml (120 mM) potassium acetate were mixed and the resulting mixture was incubated for 30 min at room temperature. The absorbance of all the samples was measured at 415 nm using spectrophotometer (Systronics: 106). The calibration curve was prepared using quercetin (5 to 60 µg/ml) solution in methanol. The flavonoidal content was expressed in terms of quercetin (SRL, India) equivalent (mg/g of extracted compound).

DPPH• free radical scavenging assay

The free radical scavenging activity of various solvent extracts of *C. pulcherrima* was measured by using 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) by the modified method of McCune and Johns [23]. The reaction mixture consisting of DPPH in methanol (0.3 mM, 1 ml) and different concentrations of the solvent extracts (1 ml) were incubated for 10 min. in dark, after which the absorbance was measured at 517 nm. Ascorbic acid (Hi Media, India) was used as positive control. The radical scavenging activity was expressed in terms of the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% (IC_{50}).

$$\% \text{ DPPH}^{\bullet} \text{ radical scavenging} = [(B - A) / B] \times 100$$

Where B is the absorbance of the blank (DPPH• plus methanol) and A is the absorbance of the sample (DPPH•, methanol plus sample).

Antimicrobial Assay

Microorganisms tested

The microbial strains used to assess the antimicrobial properties included six Gram positive, five Gram negative and four fungal strains (Table 4)**.** The investigated microbial strains were obtained from National Chemical Laboratory (NCL), Pune, India. The microorganisms were maintained on nutrient agar/MGYP (Hi Media, India) slope at 4° C and sub-cultured before use. The microorganisms are clinically important ones causing several infections and it is essential to overcome them through some active therapeutic agents.

Determination of the antimicrobial activity

The antimicrobial assay of the extracts was carried out by agar well diffusion method [24, 25]. The media used for bacteria was Muller Hinton agar no. 2 (Hi Media, India) and for fungi it was Sabaroud dextrose agar (Hi Media, India). The tested drug was diluted in 100% DMSO at a concentration of 25 mg/ml and the antimicrobial activity was evaluated at a concentration of 2.5 mg/well. The agar was melted and cooled to 48 – 50°C and a standard inoculum (1.5 x 10^8 cfu/ml, 0.5 McFarland) was then added aseptically to the molten agar and poured into sterile Petri dishes to give a solid plate. Wells were prepared in the seeded agar plates. Each tested compound (100 µl) was introduced in the well (8.5 mm). The plates were incubated at 37° C for bacteria and 28° C for fungi 24 h and 48 h respectively. The bacterial growth was determined by measuring the the diameter of inhibition zone. For each bacterial strain DMSO was used as negative control where pure solvents were used instead of the extract. The experiment was repeated three times to minimize the error and the mean values were presented. Commercial antibiotics (Hi Media, India) were used as positive control and were evaluated for their susceptibility against the microorganisms investigated. The antibiotics studied are given in Table 5.

Determination of the Minimum Inhibition Concentration (MIC)

The end point MIC is the lowest concentration of compounds at which the tested microorganisms does not demonstrate visible growth. The broth tube dilution method utilizes relatively large amounts of the test agent and is not suitable for high throughput screening. Therefore, agar well diffusion method was used in the present study [26] to determine the MIC of the methanol extract of the different parts of *Caesalpinia pulcherrima*. The solution of the extract was 2-fold serially diluted in DMSO, in the range of 10,000 – 78 µg/ml. The MIC was defined as the lowest concentration at which visible zone of inhibition was observed.

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TABLE 1
Extractive yield (%) and Qualitative phytochemical analysis of *C. pulcherrima* Swartz

Parts Used	Extracts	Extractive Yield (%)	Alkaloids			Fla	Tan	Phl	Tri	Ste	Sap	Car
			Dra	May	Wag							
Seeds	Hexane	2.97	-	-	+	-	-	-	+	-	-	+
	Chloroform	2.35	+	-	-	-	+	-	+	+++	-	+
	Acetone	1.02	-	+	-	++	++	-	++	+	-	+
	Methanol	7.00	-	+++	++	++	+++	-	+	+	+	+++
	Aqueous	7.00	+	-	-	-	-	+	-	-	-	-
Fruits rind	Hexane	0.66	-	+	-	+	+	-	+	++	-	+
	Chloroform	0.82	-	+	-	-	+++	+	+	+++	-	++
	Acetone	2.22	+	-	+	+	+++	++	++	+	+	-
	Methanol	13.27	-	-	+++	++	+++	++	+++	-	+	++
	Aqueous	4.74	-	-	-	+	+++	+++	+++	-	+	-

Dra- Dragendroff reagent, May- Mayer's reagent, Wag- Wagner's reagent, Fla- Flavonoids, Tan-Tannins, Phl- Phlobatannins, Tri- Triterpene, Ste- Steroids, Sap- Saponins, Car- Cardiac glycosides

Results and Discussion

The presence of antibacterial substances in the higher plants is well established [27] and plants were also a good source for natural antioxidants [28]. Plants have provided a source of inspiration for novel drug compounds as plant derived medicines have made significant contribution towards human health. Phytomedicine can be used for the treatment of diseases caused by infectious diseases or those diseases caused by free radicals or it can be the base for the development of a medicine, a natural blueprint for the development of a drug.

Extractive yield

The extractive yield of the seeds and fruit rinds is given in Table 1. The extractive yield varied amongst the solvents used. In, both of the seeds and fruits rind, the methanol extracts showed highest extractive yield while the hexane and acetone extract of the fruits rind and the seeds respectively, showed minimum extractive yield. The extractive yield of the methanol extract of the fruits rind was 13.27% while that of the seeds was only 7%.

Phytochemical analysis

The hexane extract of the seeds showed only trace amount of triterpenes, cardiac glycosides and alkaloids (Wagner's). The chloroform extract showed the presence of steroids and trace amount of tannins, triterpenes, cardiac glycosides and alkaloids (Dragendroff). The acetone extract showed the presence of flavonoids, tannins, triterpenes and trace amount of steroids, cardiac glycosides and alkaloids (Mayer's) while the methanol extract exhibited the presence of all phytoconstituents except phlobatannins while the aqueous extract only contained phlobatannins and alkaloids (Dragendroff) in very trace amount. The hexane extract of the fruits rind showed the presence of flavonoids, tannins, triterpenes, cardiac glycosides, alkaloids (Mayer's test) and steroids in very trace amount. Tannins, steroids, cardiac glycosides were present in the chloroform extract while phlobatannins, triterpenes and alkaloids (Mayer's test) were present in very trace amount. The acetone extract showed the presence of all phytoconstituents except cardiac glycosides. The methanol extract showed the presence of all phytoconstituents except steroids (positive for Wagner's test). Tannins, phlobatannins and triterpenes were present in aqueous extract while flavonoids and saponins were present in very trace amount (Table 1).

Physico-chemical analysis

Physico-chemical (proximate) parameters may enhance the efficacy of the crude powder against various ailments. Crude powder of the seeds and fruits rind of *C. pulcherrima* was subjected to various proximate parameters, the results of which are shown in Table 2. The fruits rind had the highest value of loss on drying (6.84%) indicated more moisture content. Water soluble ash and aqueous soluble extractive value were also the maximum in fruits rind (15.55 %). While total ash, petroleum ether soluble extractive yield and ethanol soluble extractive yield were the maximum in the seeds. The acid insoluble ash value was almost absent.

TABLE 2
Physico-chemical parameters of crude powder of *C. pulcherrima*

Parameters	Plant parts	%
Loss on drying	Seeds	5.37
	Fruits rind	6.84
Total Ash	Seeds	4.25
	Fruits rind	3.65
Water soluble ash	Seeds	2.35
	Fruits rind	2.78
Acid insoluble ash	Seeds	0.05
	Fruits rind	-
Petroleum ether soluble extractive value	Seeds	3.73
	Fruits rind	0.35
Ethanol soluble extractive value	Seeds	9.75
	Fruits rind	8.54
Aqueous soluble extractive value	Seeds	11.39
	Fruits rind	15.55

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TABLE 3
Quantitative estimation of total phenols, flavonoids and DPPH• free radical scavenging activity (IC_{50} value) of various extracts of seeds and fruits rind of *C. pulcherrima*

Parameter	Part used	Extracts				
		Hexane	Chloroform	Acetone	Methanol	Aqueous
Total phenols (mg/g)	Seeds	ND	13.9	230.3	205.5	46.2
	Fruits rind	183.5	90.7	624.6	606.9	221.5
Flavonoids (mg/g)	Seeds	ND	9.55	6.7	2.90	0.31
	Fruits rind	7.5	14.6	9.8	6.5	2.6
DPPH• (ug/ml)	Seeds	ND	500	20.5	29	290
	Fruits rind	36	47	15	14	22

ND = Not done

Total phenolic and flavonoidal content
The antioxidant activity of the phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. In addition, they have a metal chelating potential [29]. Flavonoids are a group of naturally occurring compounds widely distributed, as secondary metabolites in the plant kingdom. These flavonoids have been reported to possess antioxidant and antiradical properties [30].

The total phenolic and flavonoidal content of the seeds and fruits rind are shown in Table 3, in which the extractive solvents of both parts revealed that, the total phenolic content was more than the flavonoidal content. Furthermore, the solvent extracts of the fruits rind had more phenolic content than that of the seeds; while the maximum content being in acetone extract, closely followed by methanol extract. The same trend was observed in the seeds extracts. The flavonoidal content also was more in the fruits rind as compared to that of the seeds, while maximum content being in the chloroform extract. A number of studies have focused on the biological activities of the phenolic compounds, which are the potential antioxidants and free radical-scavengers [29, 31]. In the present study, the acetone and the methanol extracts contained the maximum amount of phenolics. Hence this may be a good source for phenolic compounds. Many studies have shown that natural antioxidants in plants are closely related to their biofunctionalities such as the reduction of chronic diseases and inhibition of pathogenic bacterial growth, which are often associated with the termination of free radical propagation in biological systems [32]. The phenolic compounds are also thought to contribute directly to the antioxidant activity. Yen et al. [33] and Duh et al. [34] suggested a correlation between phenolic content and antioxidant activity.

DPPH• free radical scavenging activity
There are different methods for the estimation of the antioxidant activity. DPPH• free radical is commonly used as a substrate to evaluate the antioxidant activity; it is a stable free radical that can accept an electron or hydrogen radical to become a stable molecule. The DPPH• scavenging activity of the different solvent extracts of the seeds and fruit rind was different (Table 3). Comparing the five solvent extracts of the seeds and fruits rind, revealed that, the scavenging activity was more in the fruits rind than the seeds extract. The IC_{50} value of acetone and methanol extract of the fruits rind (IC_{50} value - 15 and 14 µg/ml respectively), was as good as that of the standard ascorbic acid (IC_{50} value - 11. 4 µg/ml); the other 3 extracts also showed a low IC_{50} values. In the seeds extracts, only the acetone and the methanol extracts showed a low IC_{50} values. The acetone and the methanol extracts of the fruits rind showed the maximum phenolic content and also a good antioxidant activity. It is also appeared that the extracts having more phenolics exhibited greatest antioxidant activity suggesting that the high scavenging activity may be due to hydroxyl groups existing in the phenolic compounds and the chemical structure that can provide the necessary component as a radical scavenger. Similar results were reported by Nuutila et al. [35], Yen et al. [33] and Saravana Kumar et al., [36].

Antimicrobial activity
The antimicrobial activity of various solvent extracts of the seeds and the fruits rind of *C. pulcherrima* against six Gram positive, five Gram negative and four fungal strains are shown in Table 4 and the antimicrobial activity of these organism with different antibiotics is shown in Table 5. Amongst the four solvents, the acetone and the methanol extracts of both the seeds and the fruits rind showed better antimicrobial activity. The Gram positive bacteria were more susceptible than Gram negative bacteria. All the solvents extracts of both the seeds and the fruits rind showed a feeble anti-fungal activity. The most susceptible bacteria was *M. flavus*.

From the above results, it can be stated that irrespective of the part and the solvent used, *C. pulcherrima* showed better antibacterial activity than antifungal activity. The antimicrobial activity of the fifteen studied strains against standard antibiotics is shown in Table 5. The antimicrobial activity of some of the solvent extracts was comparable with that of the standard antibiotics.

From the results of antimicrobial activity, the most active extract was subjected to the evaluation of minimum inhibitory concentration (MIC). The minimum inhibitory concentration of the methanol extract

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TABLE 4
Antimicrobial activity of various solvent extracts of seeds and fruits rind of *C. pulcherrima*

Part used	Extracts	Zone of inhibition (mm)														
		Gram positive bacteria						Gram negative bacteria					Fungus			
		Se	Bc	Bs	Sa	Mf	Cr	Kp	Pv	St	Ea	Pa	Ct	Ca	Tb	Cn
Seeds	Hexane	-	10	10	12	11	-	15	-	-	11	-	-	-	-	12
	Chloroform	-	10	-	10	17	-	20	12	-	11	11	-	-	10	13
	Acetone	18	14	19	17	23	16	19	16	12	15	16	13	12	10	11
	Methanol	16	13	13	16	21	15	18	16	12	13	15	-	11	-	11
Fruits rind	Hexane	15	13	14	16	20	15	18	17	12	13	14	-	12	-	10
	Chloroform	14	14	13	16	20	15	18	17	12	12	14	11	14	10	11
	Acetone	18	18	18	19	22	17	18	17	11	11	20	-	-	11	11
	Methanol	18	18	17	20	21	18	17	16	11	11	19	-	-	11	11

Se - *Staphylococcus epidermidis* ATCC12228; Bc - *Bacillus cereus* ATCC11778; Bs - *Bacillus subtilis* ATCC6633; Sa - *Staphylococcus aureus* ATCC 29737; Mf - *Micrococcus flavus* ATCC10240; Cr - *Corynebacterium rubrum* ATCC14898; Kp - *Klebsiella pneumoniae* NCIM2719; Pv - *Proteus vulgaris* NCTC8313; St - *Salmonella typhimurium* ATCC23564; Ea - *Enterobacter aerogenes* ATCC13048; Pa - *Pseudomonas aeruginosa* ATCC27853; Ct - *Candida tropicalis* ATCC4563; Ca - *Candida albicans* ATCC2091; Tb - *Trichosporon beigelii* NCIM3404; Cn - *Cryptococcus neoformans* NCIM3542

TABLE 5
Antimicrobial activity using standard antibiotics

Microorganisms	Antibiotics														
	Cb	I	Ca	Cf	Cj	Ak	T	Pc	M	C	At	Fu	Ny	Ap	Kt
S. epidermidis	17	39	25	24	22	17	25	11	18	24	14	ND	ND	ND	ND
B. cereus	10	36	18	19	20	12	17	17	-	10	11	ND	ND	ND	ND
B. subtilis	20	37	25	30	35	15	21	17	16	16	14	ND	ND	ND	ND
S. aureus	22	25	19	14	21	14	19	22	16	15	16	ND	ND	ND	ND
M. flavus	32	37	24	22	30	21	27	28	10	27	28	ND	ND	ND	ND
C. rubrum	22	24	20	17	28	17	20	19	16	15	15	ND	ND	ND	ND
K. pneumoniae	32	35	17	25	28	22	30	27	-	36	33	ND	ND	ND	ND
P. vulgaris	11	16	15	19	-	11	15	16	-	18	14	ND	ND	ND	ND
S. typhimurium	22	16	18	15	17	15	18	14	-	16	12	ND	ND	ND	ND
E. aerogenes	18	19	16	12	-	17	11	12	-	13	-	ND	ND	ND	ND
P. aeruginosa	22	26	16	29	-	22	10	18	-	13	14	ND	ND	ND	ND
C. tropicalis	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	19	14	-
C. albicans	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	18	13	20
T. beigelli	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	17	15	-
C. neoformans	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	27	21	17	24

ND = Not done; - means no zone of inhibition observed, Cb = Carbenicillin; I = Imipenem; Ca = Ceftazidime; Cf = Ciprofloxacin; Cj = Cefaclor; Ak = Amikacin, T = Tetracycline; Pc = Piperacillin; M = Methicillin; C = Chloramphenicol; At = Azithromycin, Fu = Fluconazole; Ns = Nystatin; Ap = Amphotericin- B; Kt = Ketoconazole

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of the seeds and the fruits rind of *C. pulcherrima* is shown in Table 6. The MIC of *C. pulcherrima* ranged from 10,000 – 78 µg/ml. In the seeds and the fruits rind extracts, *K. pneumoniae* showed the least MIC value (< 78 µg/ml) while *C. albicans* showed MIC value > 10,000 µg/ml.

TABLE 6
Results of Minimum Inhibitory Concentration of methanol extract of *C. pulcherrima*

Microorganisms	MIC (µg/ml)	
	Seeds	Fruits rind
S. epidermidis	312	156
B. cereus	2,500	625
B. subtilis	1,250	1,250
S. aureus	5,000	1,250
C. rubrum	5,000	1,250
K. pneumoniae	< 78	< 78
P. vulgaris	312	312
S. typhimurium	5,000	78
P. aeruginosa	5,000	1,250
C. tropicalis	2,500	1,250
C. albicans	10,000	10,000
T. beigelli	5,000	2,500
C. neoformans	1,250	10,000

Conclusion

Plant based antimicrobials have enormous therapeutic potential, they can serve the purpose without any side effects that are often associated with the synthetic antimicrobials. It is hoped that this study will lead to the establishment of some results, that could be used for further investigation to isolate the active compounds from the promising extracts, which could be used to formulate new and more potent antibacterial and antioxidant drugs from natural origin.

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