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Antimicrobial Sensitivity Study of the Chloroform Fraction of Aqueous Ultrasound Assisted Extract Obtained from the Fresh Leaves of *Callistemon citrinus*

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Abstract
Callistemon citrinus belongs to the family Myrtaceae is locally known as Kalki Phool generally recognised as bottle brush tree. Several studies described many traditional applications of this plant in different parts of the world. In the present study the fresh leaves juice of C. citrinus was treated by ultrasound for facilitating the extraction method in aqueous medium. After that desired phytochemicals were separated from the aqueous medium by applying fractional separation through chloroform. Phytochemical study indicated that the chloroform fraction successfully isolated some particular group of compounds such as alkaloids, flavonoids, tannins, terpinoids etc. from the wide mixing of phytochemicals observed in its aqueous extract. The crude extracts showed promising sensitivity on different Gram +ve and Gram –ve bacteria such as S. aureus, S. pyogenes, S. boydii, S. flexneri, S. sonnei and S. dysenteriae. The chloroform extract showed minimum inhibitory concentration (MIC) from 40-80 µg/ml on the above bacteria. The plant is ultimately proved a promising medicinal plant which may be used as a source of developing many promising drugs. At the same time, ultrasound treated extraction methods proved its justification in the field of phytochemical extraction.
Key words: *Callistemon citrinus, bottle brush, Kalki Phool, Green extraction, Ultrasound*

Introduction:

The plant *Callistemon citrinus* belongs to the family Myrtaceae is widely known as "bottle brush" [1] due to its cylindrical brush-like inflorescence [2]. The *C. citrinus* is locally recognized as 'Kalki Phool' in Bangladesh and widely planted for ornamental purposes. This plant is native to Australia [2] and habituated to the wet tropics, notably South America and tropical Asia [3] which is now spread all over the world including Bangladesh, India, Pakistan etc. *C. citrinus* is a slow-growing tree which may grow up to 6-15 m in height and 1.3-1.5 m in girth with sharp pointed mid-green leaves [3]. The plants have slender and drooping branches, producing showy scarlet flowers with long stamens [2]. The plants may also be termed as crimson bottle brush, red bottle brush, lemon bottle brush etc. and till to date over 132 genera and 5950 species of these plants were isolated [1]. The inflorescence of *C. citrinus* is 10-12 cm long and 4-5 cm diameter pendant spike attached on the hanging branches. Each inflorescence bears 20–50 flowers in acropetal succession. The thick evergreen leaves are slightly hard and pinnate venation lanceolate-linear shape with alternate arrangement. Different parts of the plant are reach of diversified pharmacologically active compounds which may be useful as an antidiabetic, antioxidant, anti-cholinesterase, anti-inflammatory, cardioprotective, hepatoprotective, hypolipidemic, nematicidal, larvicidal, pupicidal activity, wound healing activities etc. [4]. In the present study, chloroform fractions of the aqueous ultrasound treated

fresh leaves of *C. citrinus* were used for antimicrobial studies on some pathogenic microorganisms connected to skin disease and GIT discomfort.

The Ultrasound Assisted Extraction (UAE) method is a proven green extraction method successfully applied in several previous studies [5-9]. In this method the energy of ultrasound is used to rapture the cell wall and forcefully mix the inner components to the surrounding solvents [10-13]. Controlled application of ultrasound is suitable for rapid extraction of the crude components even in the unfavourable solvent system.

Figure 1. Leaves and flower of *Callistemon citrinus*

Table 1: Botanical Classification of bottle brush

Kingdom	Plantae
Division	Magnoliophyta
Class	Dicotyledons
Family	Myrtaceae
Genus	Callistemon .
Species	*Callistemon citrinus* Curtis

Materials and Methods

Collection of Plant Material:

The leaves *Callistemon citrinus* was collected from the labelled plant from the National Botanical Garden, Dhaka, Bangladesh. Fresh and healthy leaves of *Callistemon citrinus* were washed properly by the running tap water followed by the distilled water and allowed for shade drying of the surface water [14]. Within 6 hours 100 gm of whole plants were taken in a conventional juice blender machine and made a uniform mixture by addition of distilled water q.s. to 500 ml (material solvent ratio 1:5) [10]. The juicy mixture was then passed through a 20-mesh size net for getting uniform fine particles and placed in an ultrasonic bath (Power Sonic 405) for 30 minutes of ultrasound vibration at 40^{o}C bath temperature. During the ultrasound treatment, theoretically all particles present inside the plant cell came to the aqueous media due to rapture of the cell wall. The mixture was then filtered by three layers of cloth and equally divided into two parts (Part-A and Part-B). Part-A solution was taken in a beaker and placed in a water bath for drying at 55±5^{0C}. After drying, crud extract obtained from Part-A solution was weighted, labelled and preserved in the refrigerator (5^{0C}) for further use. Part-B solution was taken into a separating funnel and mixed vigorously after adding 25 ml chloroform at room temperature 25^{0C}. Chloroform was added in the Part-B solution due to separating out some desired organic components from the aqueous solution by using its poor solubility properties as well as the wider variation of polarity index (Pi) than the water. Chloroforms have a poorer aqueous solubility (0.795 x 10^{6} µg/L water at 25^{0C}) [15] and low polarity index ($Pi_{chloroform}$ = 4.1) [16] than the water (Pi_{water} = 10.2) [17]. Low Pi of chloroform facilitated the dissolution of organic components which had a Pi close to that and at the same time the poor solubility of chloroform also made a clear layer top on the aqueous phase. Through this procedure chiefly terpenoids, flavonoids and alkaloids may be isolated from the mixture of other organic components [18]. By applying the fractional separation of chloroform may be a suitable technique for successfully separating a particular group of organics components from the aqueous mixture. After vigorous mixing with chloroform, the solution was kept undisturbed and the organic portion was collected minutely. The process was repeated three times and all chloroform extracts were added together and allowed them in the air to dry under fume hood. After drying, crud extract was weighted, labelled and preserved in the refrigerator (5^{0C}) for further use.

Extraction yield was calculated as per equation 1.

$$\% \text{ Extraction yield} = \frac{W2}{W1} \text{x } 100 \ldots\ldots\ldots \text{Equation 1 [19]}$$

Where, W1 : Weight of starting plant materials

W2: Weight of crude extract after drying

The whole procedure was repeated five times and the average value was calculated by using SPSS software for comparing yield value (%).

Phytochemical Screening Test:

Qualitative phytochemical tests for alkaloids (i.e., Dragendroff test and Mayer test), anthraquinones (i.e., addition of ammonia solution to the sample create a bright pink color in the aqueous layer), flavonoids (i.e., addition of H_2SO_4 to the sample develop a yellow color which may be disappeared on standing), glycosides (i.e., addition of glacial acetic acid, ferric chloride and H_2SO_4 create a brown ring in the interface), saponins (i.e., vigorous shaking create a stable persistent froth), steroids (i.e., addition of acetic anhydride and concentrated H_2SO_4 to the sample changes the color from violet to blue), tannins (i.e., addition of ferric chloride turns blue-black coloration of the sample), terpenoids (i.e., addition of $CHCl_3$ and H_2SO_4 to the solution make a reddish brown coloration in the interface) and vitamin C (i.e., addition of sodium nitroprusside, NaOH and HCl in the solution turns blue color) were performed as per the procedure of Allen and Harborne with slight modification and successfully applied in different previous publications [5, 7-9].

Antimicrobial study:

In the present study six pathogenic microorganisms were enrolled, two gram positive bacteria *Staphylococcus aureus* & *Streptococcus pyogenes* generally responsible for skin disease and four gram negative bacteria *Shigella boydii*, *Shigella flexneri, Shigella sonnei* & *Shigella dysenteriae* commonly responsible for GI related discomfort such as dysentery, diarrhea, abdominal cramping, rectal pain, nausea, watery diarrhea, blood, mucus, or pus in the stool etc. All microorganisms were collected from the Microbiology Lab, Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh. Nutrient agar media was used for sub-culturing bacteria at 37^{o}C and disc diffusion method [20-23] was used for studying the sensitivity of the crude extract obtained from fractional separation of chloroform from aqueous ultrasound assisted extract of fresh leaves of *Callistemon citrinus*. The filter paper discs (sensitivity discs) impregnated with the 200µg/disc, 400µg/disc and 600µg/disc of extracts were then placed on the surface of the inoculated nutrient agar with the aid of sterilized pair of forceps. Erythromycin 15µg/disc was also placed as positive control. A pre-diffusion time of 30 minutes was allowed for the extracts to diffuse from the discs into the agar medium before incubation. The degree of sensitivity of the organisms to the extracts was determined by measuring the diameter of visible zones of inhibition to the nearest millimetre.

Determination of Minimum Inhibitory Concentration (MIC):

Broth dilution method was used for the study of minimum inhibitory concentration (MIC) values of the chloroform extract obtained from ultrasound treated aqueous extract of *Callistemon citrinus* leaves. Extract samples were prepared in distilled water as per the flowing sequence 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 µg/ml and distilled water was used as control. 1 ml of each concentration was added to each 9 ml of nutrient broth containing 0.1 ml of standardized test organisms of bacterial cells. The final concentration of the above broth media was developed 100, 80, 60, 40, 20, 10 and 9.375 µg/ml. The tubes were incubated at 37^{0}C for 24 hours and level of turbidity was measured. Positive controls were equally set up by using distilled water and test organisms without extracts. The tube with least concentration of extract without growth after incubation was taken and recorded as the minimum inhibitory concentration [24].

Result and Discussion:

Phytochemical extraction from *Callistemon citrinus* was probably first time done by using ultrasound treatment. A promising extraction yield was observed from Aqueous Ultrasound Assisted Extraction from fresh leaves of *C. citrinus* (16.92±2.77%) and its chloroform fraction (3.72±1.04%) were studied in the present study (Table 1). The extraction yield by using ultrasound treatment was far better than the

conventional soxhlet extraction in chloroform (2.25%), ethanol (1.75%) and water (0.5%) [25]. Qualitative phytochemical assay of ultrasound treated aqueous extract of *C. citrinus* leaves showed the presence of alkaloids, flavonoids, saponins, steroids, tannins, terpenoids etc. The presence of the similar compounds were also observed in the chloroform, ethanol and water extract studied by Krishna et. al. [25] and in methanol extract studied by Palanikumar et. al. [4]. In the present study, potent phytochemicals were separated from the chloroform fraction from aqueous extract. It was observed that chloroform extract was rich in alkaloids, flavonoids, tannins and terpinoids. Chloroform fraction was observed very effective antimicrobial sensitivity against both gram positive and gram negative pathogenic bacteria such as *S. aureus, S. pyogenes, S. boydii, S. flexneri, S. sonnei and S. dysenteriae* (Table 2). The sensitivity was proved concentration dependent and significant sensitivities were observed in case of using 20 to 40 times more concentration than the standard antibiotic erythromycin (Chart 1). From previous studies it was observed that chloroform extract provides satisfactory antimicrobial sensitivity compared to the streptomycin against *B. subtilis, B. pumilis* and *E. coli* [25]. Another studies also showed that *C. citrinus* leaves had promising antimicrobial properties against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, MRSA, *Candida albicans* by using volatile oil obtained from leaves of *Callistemon citrinus* [26]. From MIC study of chloroform fraction obtained from aqueous ultrasound treated leaves extract, it was observed that minimum 80 µg/ml concentration was required to inhibit the growth of *S. aureus* (Gram +ve), whereas, only 40 µg/ml concentration was required against Gram (–ve) bacteria *S. flexneri* (Table 4). Comparatively lower MIC were observed against other Gram (–ve) bacteria such as *S. dysenteriae* (50 µg/ml), *S. boydii* (60 µg/ml) and *S. sonnei* (60 µg/ml). Comparatively higher MIC was observed against gram positive bacteria such as *S. pyogenes* (70 µg/ml) and *S. aureus* (80 µg/ml). Results indicated that the chloroform extract from ultrasound treated aqueous leaves extract have promising antimicrobial activities against the Gram negative bacteria specially *Shigella* groups, and may be used for the treatment of dysentery related GI discomfort.

Table 1: Extraction yield of the crude extracts obtained from ultrasound treated fresh leaves of *Callistemon citrinus* and its chloroform fraction

Trial No.	Starting material (gm)	Part A: Aqueous Extract		Part B:Chloroform Fraction from Part A		
		Dry Weight of crude extract (gm)	Percentage of yield	Dry Weight of crude extract (gm)	Percentage of yield	Respective percentage of the Part A
1	50	8.9	17.8	2.1	4.2	23.60
2	50	7.8	15.6	1.7	3.4	21.79
3	50	10.2	20.4	2.5	5	24.51
4	50	6.5	13	1.1	2.2	16.92
5	50	8.9	17.8	1.9	3.8	21.35
			16.92±2.77		3.72±1.04	21.63±2.93

Table 2: Presence of phytochemicals in the crude extracts obtained from ultrasound treated fresh leaves of *Callistemon citrinus* and its chloroform fraction

Extract	Alka-loids	Anthra-quinones	Flavo-noids	Glycol-sides	Sapo-nins	Ster-oids	Tann-ins	Terpe-noids	Vita-min C
Part A	+	-	+	-	+	+	+	+	-
Part B	+	-	+	+	-	-	-	+	-

Here, Part-A: Aqueous Ultrasound Assisted Extraction from fresh leaves of *M. cordata*
Part B: Chloroform fraction from Part-A
(+) indicated presence of compound, and (–) indicated absence of compound

Table 3: Antimicrobial sensitivity of chloroform fraction of ultrasound treated aqueous *C. citrinus* leaves

List of Microorganism enrolled in the examination	Zone of inhibition (mm)			
	Chloroform fraction of Aqueous UAE of *C. citrinus* leaves			Erythromycin
	(150 µg/disc)	(300 µg/disc)	(600 µg/disc)	(15 µg/disc)
Staphylococcus aureus (Gm +ve)	-	10.33±2.08	16.67±3.04	17.67±4.04
Streptococcus pyogenes (Gm +ve)	-	11.67±2.52	19.67±3.79	17.00±3.61
Shigella boydii (Gm -ve)	7.67±1.53	12.67±3.51	21.33±4.51	18.00±3.61
Shigella flexneri (Gm -ve)	7.33±1.53	13.67±1.53	20.67±5.51	19.67±3.79
Shigella sonnei (Gm -ve)	8.67±0.58	14.67±2.52	22.67±4.51	20.33±3.51
Shigella dysenteriae (Gm -ve)	8.33±0.58	14.33±2.52	21.33±5.03	20.67±3.21

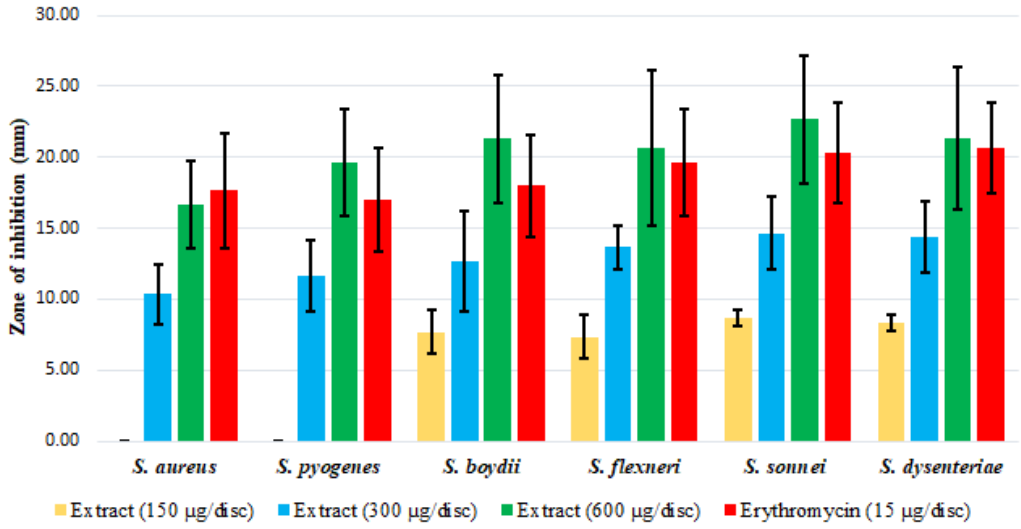

Figure 2: Comparison of dose of chloroform fraction of ultrasound treated aqueous *C. citrinus* leaves on different pathogenic microorganisms

Table 4: Determination of MIC of chloroform fraction of ultrasound treated aqueous *C. citrinus* leaves

Test organism	Turbidity*										
	Chloroform fraction from aqueous UAE crude extract										Control
	10 µg/ml	20 µg/ml	30 µg/ml	40 µg/ml	50 µg/ml	60 µg/ml	70 µg/ml	80 µg/ml	90 µg/ml	100 µg/ml	Water
S. aureus	+++	++	++	++	+	+	+	MIC	-	-	+++
S. pyogenes	+++	++	++	++	+	+	MIC	-	-	-	+++
S. boydii	+++	+++	+++	+++	+++	MIC	-	-	-	-	+++
S. flexneri	+++	++	+	MIC	-	-	-	-	-	-	+++
S. sonnei	+++	+++	+	+	+	MIC	-	-	-	-	+++
S. dysenteriae	+++	++	++	+	MIC	-	-	-	-	-	+++

Here, *(+++) dark turbidity which indicated no effects of extract on microorganisms;

(++) turbidity which indicates the mild antimicrobial effects of extract on microorganisms;

(+) light turbidity which indicates the promising antimicrobial effects of extract on microorganisms;

(-) original broth colour which indicates no growth of microorganisms.

Conclusion:

An innovative green extraction method was applied in the present study for the preparation of crude extract from the fresh leaves of *C. citrinus*. The intended method proved better in terms of extraction yield than the conventional extraction method. The extraction method was also proved faster, comparatively inexpensive and convenient. The present study also complies with some previous studies regarding antimicrobial

properties. From the present study it was established that the leaves of *C. citrinus* have some promising antimicrobial phytochemicals which may be popular for treating GI related discomfort.

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