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ANTIBACTERIAL POTENTIAL OF A MEDICINALLY IMPORTANT PLANT *CALAMUS AROMATICUS*

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Abstract

The potential of Gallic acid as a broad-spectrum antibiotic was established by evaluating five solvent extracted samples (crude extract and its four fractions) from the shade-dried (S-D) rhizomes of *Calamus aromaticus* through disc diffusion assay wherein 0.05 mg Ciprofloxacin was used as control. The results of the study indicated that *Staphylococcus aureus* and *Citrobacter freundii* were more susceptible to crude methanol extract from the shade-dried rhizome at 2 mg.disc^{-1} (53.3% and 47.0% respectively), *Bacillus subtilis* and *Klebsiella pneumoniae* to hexane extracted fraction (50.3% and 43.0% respectively) while *Pseudomonas aeruginosa*, *Escherichia coli* and *Xanthomonas campestris* were more susceptible to ethyl acetate fraction at the same concentration (86.5%, 48.5% and 64.9% respectively). *Staphylococcus aureus* was most susceptible gram positive bacterium and *Bacillus subtilis* was comparatively more resistant. Among Gram negative bacteria, *Pseudomonas aeruginosa* showed maximum susceptibility while *Klebsiella pneumoniae* revealed more resistivity in comparison to others. HPLC analysis of the extracts revealed that Gallic acid, an illustrative affiliate of phenols, was present in higher quantity in the organic solvents-extracted samples (38.4 mg.g^{-1} and 11.3 mg.g^{-1} in ethyl acetate and butanol fractions respectively) than in the aqueous fraction which provided a justification for its role in antibacterial activities.

Key words: Antibacterial; HPLC; Gallic acid; *Calamus aromaticus*; rhizome.

Introduction

From time immemorial, medication (in the form of aromatic plants) are in vogue to cure numerous ailments (Abraham *et al*., 1998; Bakht *et al*., 2017). The basic health-care needs of roughly 70-80% of the global population depend largely on customary herbal preparations (Srivastava *et al*., 1995). *Calamus aromaticus* (Skha Waja) is cultivated on large scale in Asia since long due to its profound medicinal importance (Singh *et al*., 2011). The plant, however, does not enjoy the same status as yet in Pakistan where it is almost exclusively found as a weed in damp marshy places of District Swat in Khyber Pakhtunkhwa Province (Khan & Bakht, 2016; Khan *et al*., 2016; Khan *et al*., 2017 a and b). The name "Skha Waja" is associated with this plant in the local dialect and the herb is known for its medicinal importance in the local 'Pansara' market.

Several important biological activities such as antifungal (Lee *et al*., 2004; Lee, 2007, Khan & Bakht, 2016; Khan *et al*., 2017 b), antibacterial (McGraw *et al*., 2002; Phongpaichit *et al*., 2005; Khan *et al*., 2017 a and c), allopathic (Nawamaki & Kuroyanagi; 1996), anticellular and immunosuppressive (Mehrotra *et al*., 2003) have been attributed to the rhizomes, roots and essential oil extracted from these plant parts. It can also be used as an insecticide as its essential oil has been reported to possess antigonadal activity in insects (Koul *et al*., 1977 a and b; Saxena *et al*., 1977; Schmidt & Brochers, 1981; Mathur & Saxena, 1975).

Despite some efforts to unveil the antimicrobial properties of *C. aromaticus*, the true potential with respect to the gigantic antibacterial efficacy of the rhizomes of this plant remains largely obscured (Khan & Bakht, 2016). The order of extraction based on increasing polarity of the extracting organic solvents, to add, was for the very first time employed in this particular experiment. The exact potency of *C. aromaticus* rhizomes, thus, could have not be established without scheming such a study.

Herbal extracts are routinely quantified for the presence of phenolic compounds using HPLC (High Performance Liquid Chromatography), because the results obtained through this technique are not only accurate and reliable but also repeatable (Khan & Bakht, 2016). Different extracts from the rhizome of *C. aromaticus*, in this study, were also quantified through HPLC for the presence of Gallic acid.

As discussed earlier, this study evaluates the true antibacterial potential of this enormously promising medicinal plant. But what confers this potency to the different samples extracted from this plant? What type of extracts should further be studied in an effort to isolate commercial drug from it. This study tries to provide answers to these queries by establishing a connection between the presence of Gallic acid in an extract and the antibacterial efficacy of that particular sample.

Materials and Methods

Plant material: From District Swat of Province Khyber Pukhtoonkhwa *C. aromaticus* plants were collected, the rhizomes of which were subsequently dried in shade and then grinded.

Preparation and fractionation of crude extract: Methanol (Analytical grade) in a volume of 5000 ml was used to soak 1 kg grinded rhizome. The solution was kept in dark for seven days at 25ºC and shaken thrice daily followed by filtration through Whatman filter paper (No.1). Another 2.5 L methanol was added to the solid

filtrate and this entire procedure was reiterated three times. The sifted solution in methanol was dried at 45ºC in a rotary evaporator (Stuart, RE 300, Bibby Sterilin Ltd., UK) which was connected to a vacuum pump. A dark vial was used to keep the semisolid crude extract at ambient temperature which was subsequently distributed in two shares. One of the two portions was stored to be further employed in the determination of antibacterial potential while the other share of the crude extract was fractionated with various organic solvents.

In order to fractionate the extract, 0.5 L of sterile distilled water was used to dissolve 50 g of crude extract and the resultant solution was afterwards dispensed in a separatory funnel. This was followed by the addition of analytical grade n-hexane in a volume of three hundred milliliter. The funnel was then agitated gently for proper mixing and separation of the two phases was achieved by allowing the solutions to settle for 15-30 minutes. The higher organic phase (n-hexane) was transferred to a beaker while more n-hexane was used to thrice fractionate the aqueous phase obtained at the bottom. The pool of n-hexane fractions, thus obtained, was dried in a rotary evaporator at 45°C under vacuum pressure. Ethyl acetate and butanol were also used to fractionate the aqueous phase in a similar fashion. Water fraction of the crude extract was obtained by drying the lower aqueous phase at the end of the process in a rotary evaporator (Fig. 1).

Preparation of media: Commercial products of Oxoid Ltd., Basingstoke, Hampshire, England *viz.*, Nutrient Agar (CM0003; to culture and grow bacterial strains), and Nutrient Broth (CM0001; to incubate and standardize the tested strains) were used in the present study. Nutrient Agar and Nutrient Broth were prepared in the required magnitudes (28 gm/L and 13 gm/L respectively) in graduated flasks. Approximately twenty milliliter Nutrient Broth was also dispensed into test tubes. The flasks and test tubes containing agar and broth were autoclaved to sterilize the media.

Afterwards, a Laminar flow hood was used to maintain a sterile environment for the pouring of agar medium into 90 mm disposable petri plates. After solidification of the medium, the petri plates were kept in an upturned position in an incubator set at 37ºC for a period of 24 hours. The unadulterated plates were, afterwards, used to culture the bacterial strains. To incubate these strains, approximately twenty milliliter Nutrient Broth was taken in flasks and placed in a shaking incubator at 37ºC overnight whereas the bacterial cultures were then standardized using the broth in test tubes.

Bacterial strains used in the present study: A list is provided in 'Table 1' regarding information of the microbes used in the study.

Antibacterial assay: In a sterile environment maintained by a laminar flow unit, an autoclaved loop was used to re-culture the bacterial strains on fresh agar plates from their stock cultures. These plates were then incubated at 37ºC for one day followed by sub-culturing of the bacteria on fresh agar plates. Afterwards, bacterial inoculum from these second streaked plates were taken in broth contained in flasks and placed in a shaking incubator set at 37ºC and 200rpm for a period of 18 hours.

The bacterial inoculums were subsequently standardized to 1-2 × 10^7 CFU ml^{-1} 0.5 McFarland Standard, and fifty microliter from each standardized inoculum was poured onto Nutrient agar plates. To achieve uniform growth of the bacterial colony, the inoculums were spread on the surface of the plates which were later placed in a laminar flow unit for 20-30 minutes.

The protocol of Disc diffusion assay elaborated by Bauer *et al*., (1966) was employed in this experiment. Three 6mm autoclaved Whatman filter paper discs were positioned on the surface of agar plates in a wedge-shaped style where a distinctive rhizome extract concentration was signified by each disc *viz.*, 0.5 mg, 1 mg and 2 mg. The positive and negative controls in the experiment were Ciprofloxacin (in a concentration of 0.05 mg) and DMSO (in a concentration of 6 $\mu l.disc^{-1}$) respectively. The agar plates inoculated with bacteria and rhizome extracts were then placed in an incubator set at a temperature of 37ºC for one day. The diameter of each zone of inhibition was documented in millimeter and the activity of each extract (in percentage) was calculated by the following formula after repeating each experiment three times:

$$\text{Percent activity} = \frac{\text{Zone of inhibition (sample)}}{\text{Zone of inhibition (control)}\}} \text{ x } 100$$

Gallic acid quantification through HPLC: A Liquid Chromatograph (Shimadzu LC-6AD) fitted with dualistic pumps, a Prominence Diode Array, PDA, Detector (Shimadzu SPD-M20A), a Communication Bus Module (Shimadzu CBM-20A) and C_{18} Diamonsil column (4.6 millimeter in diameter and 250 millimeter in length with particle size of 2.5 micrometer) was used to quantify Gallic acid in each extract. The samples were prepared in HPLC grade water and acetonitrile (Sigma Aldrich) which were also the solvents making up the mobile phase with water as 'Solvent-A' and acetonitrile as 'Solvent-B'. A nylon filter (0.45 micrometer) was used to vacuum-filter both solvents which were subsequently placed in a sonicator for the removal of air bubbles.

Acetonitrile and water (both HPLC grade) were combined in an identical volumetric ratio (1:1) to be employed as solvent in the preparation of samples and standard solutions. A 500 ppm Gallic acid stock solution was prepared which was further diluted to 10, 20, 40, 80 and 160 ppm working solutions. A 0.45 micrometer Millipore filter was used for the filtration of each sample solution of 500 ppm concentration, and both the sample and standard solutions were afterwards subjected to centrifugation for ten minutes at 5000 rpm before their introduction into the chromatographic system.

The system was purged for the removal of air bubbles and was subjected to 2-3 blank runs. The volume of injection for both the sample and standard solutions was 20 microliter while the column temperature was adjusted to 25ºC, the combined flow rate of the pumps was maintained at 1 ml per minute, detection was done at a wavelength of 254 nanometer, and twenty five minutes were given to the system for one complete run.

Statistical calculation

Each experiment was repeated thrice and the 'mean ± standard deviation' values were calculated through Microsoft Excel 2010.

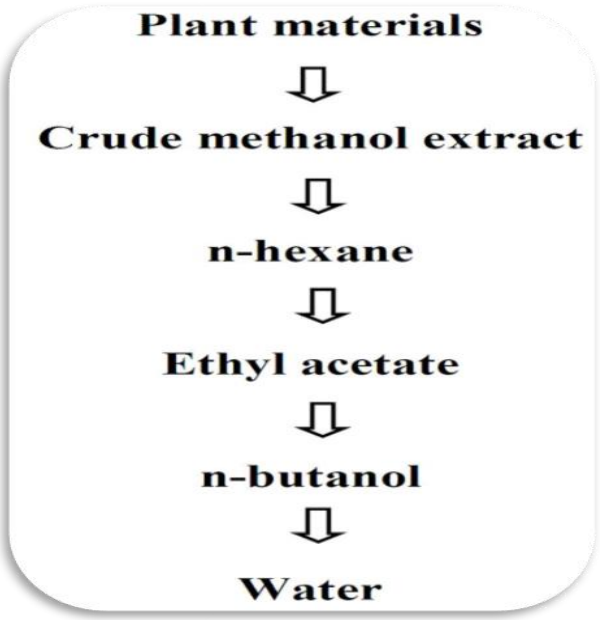

Fig. 1. Preparation and orderly fractionation of the crude extract.

Results and Discussion

Antibacterial assay: All tested concentrations of each extract were active against *Staphylococcus aureus*, except aqueous fraction which was inactive against it at 0.5 mg.disc^{-1} (Fig. 2). The most potent among the tested extracts in inhibiting the growth of this pathogen was ethyl acetate fraction with 33.3%, 43.4% and 50.1% activity at 0.5, 1 and 2 mg.disc^{-1}, respectively. Other extracts in the order of descending activity were methanol, butanol and hexane extracts which measured 53.3%, 43.9% and 43.3% activity at 2 mg.disc^{-1}, respectively. Aqueous extract, on the other hand, was the least active against *S. aureus* (23.5% and 33.3% at 1 and 2 mg.disc^{-1}, respectively). Phongpaichit *et al*., (2005); Fazal *et al*., (2012); and Kumar *et al*., (2014) reported results which are broadly in agreement with the observed effectivity of the extracts against *S. aureus*. Moreover, a similar order of activity for the extracts has been reported by Kumar *et al*., (2014), although they used different crude extracts rather than fractions of a single crude extract as employed in this study. Lin *et al*., (1999) who testified better solubility of active ingredients in organic solvents offers justification for the comparatively low effectivity of aqueous fraction.

In contrast to aqueous fraction which inhibited the growth of *Bacillus subtilis* only at the highest concentration used (2 mg.disc^{-1}), all other extracts were active against it at each of the three tested concentrations (Fig. 3). To add further, the lowest activity (21.8%) at 2 mg.disc^{-1} was also recorded for aqueous fraction. Ethyl acetate fraction, conversely, exhibited the highest activities of 31.5%, 40.6% and 50.1% at 0.5, 1 and 2 mg.disc^{-1}, respectively. It was trailed sequentially by hexane (50.3% at 2 mg.disc^{-1}), methanol (37.5% at 2 mg.disc^{-1}) and butanol (34.3% at 2 mg.disc^{-1}) extracts. Rani *et al*., (2003), Fazal *et al*., (2012), and Syed *et al*., (2014) reported similar results. Though, ethyl acetate fraction measured the highest antibacterial activity against *B. subtilis*, it was closely followed by the hexane fraction with nearly the same level of activity. Such relatively stronger antibacterial potency of the hexane extracts against this pathogen has also been reported by Fazal *et al*., (2012). Furthermore, the findings of Lin *et al*., (1999) regarding the superior ability of organic solvents to dissolve active compounds appears to be true as testified by the comparatively inferior activities of the aqueous fraction observed in this study.

In the case of *Klebsiella pneumoniae*, again the aqueous fraction turned out to be the least potent (19.4% and 22.1% activity at 1 and 2 mg.disc^{-1}, respectively), and it was also the only among the tested extracts which showed no activity at all at 0.5 mg.disc^{-1} (Fig. 4). Hexane fraction, on the other hand, with activities of 30.7%, 38.0% and 43.0% at 0.5, 1 and 2 mg.disc^{-1}, respectively, was the most potent in controlling the growth of this microbe. It was followed by ethyl acetate, methanol and butanol extracts which measured 41.1%, 37.1% and 34.9% activity, respectively, at 2 mg.disc^{-1}. The order of descending activity reported in the current study (Hexane fraction > Ethyl acetate fraction > Crude extract > Butanol fraction > Aqueous fraction) is not in conformity with the results reported by Fazal *et al*., (2012) wherein no activity was reported for hexane extract against *K. pneumoniae*, even though the tested organism used in both studies was the clinical isolate obtained from the same laboratory. Commercially available rhizome which is dried in sun, nonetheless, was used by Fazal *et al*., (2012) to obtain their hexane extract while the fresh rhizome used in this study was afterwards dried in shade. Sun-drying, which is the most usual form of drying for small-scale growers and herbal practitioners, is also the most cost-effective drying regime. This, however, also tends to be the most unpredictable one in terms of the medicinal quality of the preserved plant material due to the volatile nature and/or light sensitivity of many chemically active compounds. Approximately four thousand years ago, the ancient Egyptians were well aware of this fact and the discrepancy in medicinal value of sun-dried and shade-dried plant material was known to them (Heeger, 1989).

All three concentrations of each tested extract inhibited the growth of *Pseudomonas aeruginosa* (Fig. 5). Ethyl acetate fraction turned out to be the most potent (35.3%, 40.6% and 86.5% activity at 0.5, 1 and 2 mg.disc^{-1}, respectively) while aqueous fraction showed the least effectivity (19.4%, 21.4% and 27.6% activity at 0.5, 1 and 2 mg disc^{-1}, respectively) against this organism. Other extracts in the order of descending activity were crude extract (54.2% at 2 mg.disc^{-1}), hexane fraction (49.4% at 2 mg.disc^{-1}) and butanol fraction (30.4% at 2 mg.disc^{-1}). Similar results are also reported by Rani *et al*., (2003), Sabitha *et al*., (2003), Manikandan *et al*., (2010), Divya *et al*., (2011) and Fazal *et al*., (2012). The order of activity of the different extracts in the present study (Ethyl acetate fraction > Methanol extract > Hexane fraction > Butanol fraction > Aqueous fraction) is comparable to the reports of Fazal *et al*., (2012) wherein similar susceptibility to hexane extract of an identical strain of the tested organism was testified, and Lin *et al*., (1999) who argued about the unsuitability of water as a solvent of choice for bioactive compounds in comparison to organic solvents. Devi and Ganjewala (2009), conversely, conveyed absence of activity for ethyl acetate extracts from rhizome and leaves of *C. aromaticus* against *P. aeruginosa*. A different strain of the tested organism, nevertheless, was used by these investigators.

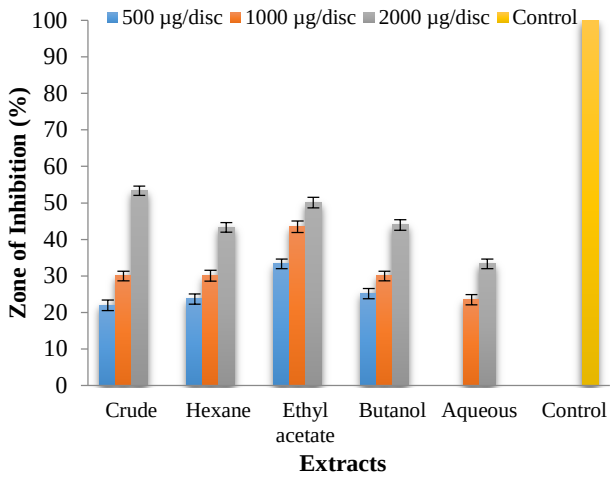

Fig. 2. Antibacterial activity (Mean ± Standard Deviation) of different extracts against *Staphylococcus aureus*.

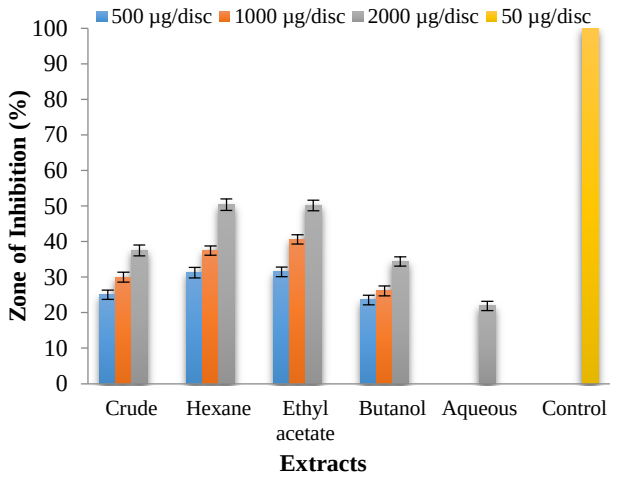

Fig. 3. Antibacterial activity (Mean ± Standard Deviation) of different extracts against *Bacillus subtilis*.

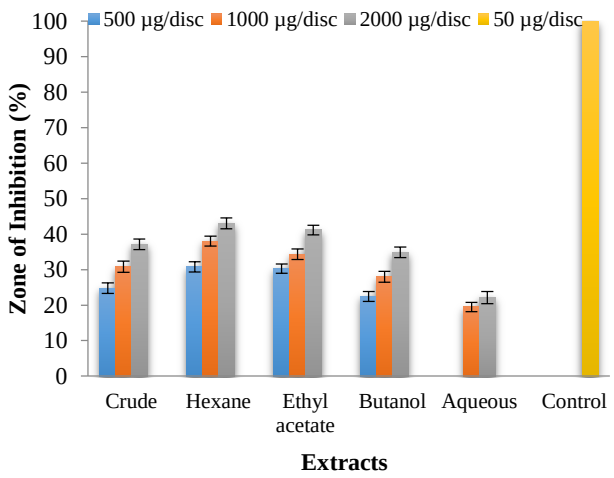

Fig. 4. Antibacterial activity (Mean ± Standard Deviation) of different extracts against *Klebsiella pneumoniae*.

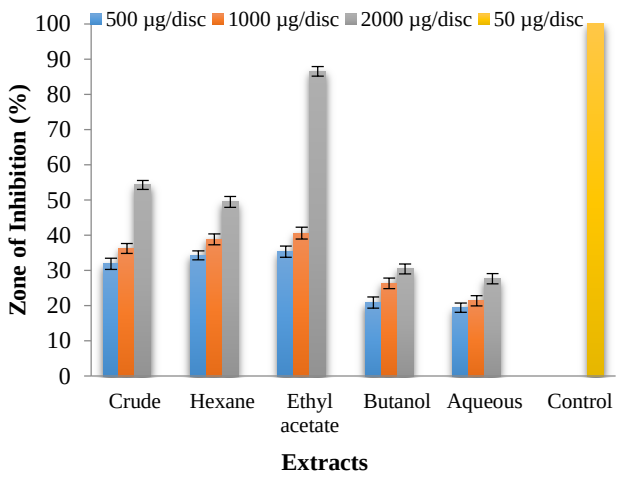

Fig. 5. Antibacterial activity (Mean ± Standard Deviation) of different extracts against *Pseudomonas aeruginosa*.

Table 1. Bacterial strains used for determination of antibacterial activities of the different solvent extracted samples from the rhizomes of *C. aromaticus*.

Microbial Species	Gram strain type	Details of the microbial strains used
Staphylococcus aureus	Possitive	ATCC # 6538
Bacillus subtilis	Possitive	Clinical isolate obtained from Microbiology Department, Quaid-e-Azam University, Islamabad, Pakistan.
Klebsiella pneumonia	Negative	Clinical isolate obtained from Microbiology Department, Quaid-e-Azam University, Islamabad, Pakistan.
Pseudomonas aeruginosa	Negative	ATCC # 9721
Escherichia coli	Negative	ATCC # 25922
Xanthomonas campestris	Negative	ATCC # 33913
Citrobacter freundii	Negative	ATCC # 8090

The present study reports susceptibility of *Escherichia coli* to all tested concentrations of each extract (Fig. 6). The least potent among the tested extracts was aqueous fraction (25.6%, 29.0% and 32.2% activity at 0.5, 1 and 2 mg.disc^{-1}, respectively) while the tested pathogen showed more susceptibility to ethyl acetate fraction (32.2%, 41.9% and 48.5% activity at 0.5, 1 and 2 mg.disc^{-1}, respectively). The ethyl acetate fraction was followed by hexane fraction, crude extract and butanol fraction which measured 45.5%, 42.1% and 32.2% activity, respectively, at 2 mg.disc^{-1}. Rani *et al*., (2003); Sabitha *et al*., (2003); Phongpaichit *et al*., (2005); Devi & Ganjewala (2009); Manikandan *et al*., (2010); Fazal *et al*., (2012); and Kumar *et al*., (2014) reported comparable results to those obtained in this study. The present study reports comparatively enhanced activities for the ethyl acetate fraction which relates to the stronger activity of rhizome ethyl acetate extracts than the same extracts from *C. aromaticus* leaves conveyed by Devi & Ganjewala (2009). Moreover, the order of effectivity of the different extracts used in the present study is similar to

the results reported by Kumar *et al*., (2014). In addition, butanol and aqueous fractions showed similar activities at the maximum concentration, but the effectivity of the former was comparatively greater at the two lower concentrations. An additional prominent observation was the increase in the activity of crude extract, in relation to butanol and aqueous fractions, with increasing concentration. The synergistic effect of the various solvent-soluble bioactive agents present in the crude extract offers a potential explanation for this finding and it can be further inferred that such outcome becomes more prominent with increase in concentration of these bioactive agents.

Each extract, at all the three concentrations used, was effective in controlling the growth of *Xanthomonas campestris* (Fig. 7). The tested organism was found more susceptible to ethyl acetate fraction (38.7%, 49.2% and 64.9% activity at 0.5, 1 and 2 mg $disc^{-1}$, respectively) which was closely shadowed by crude extract (61.3% activity at 2 mg $disc^{-1}$). The crude extract, in turn, was followed sequentially by hexane (51.8% activity at 2 $mg.disc^{-1}$) and aqueous (48.4% activity at 2 $mg.disc^{-1}$) fractions. Butanol fraction, on the other hand, trailed behind measuring 24.2% 28.9% and 32.2% activity at 0.5, 1 and 2 $mg.disc^{-1}$, respectively. Comparable activities of *Azadirachta indica* leaves aqueous extract against *X. campestris* have been testified by Pawar (2014). The relatively higher activities of the organic solvent extracted samples is in conformity with the results of Lin *et al*., (1999) wherein inferior effectivity was reported for water extracts in relation to the samples extracted with organic solvents. This possibility is also highlighted in the results of De Britto *et al*., (2011) who reported enhanced activities for methanol extracts of six medicinal plants against *X. campestris* in comparison to their water extracts. Presently, there is no report of any study wherein extracts from any part of *C. aromaticus* are used to determine susceptibility of *X. campestris*. The present study, hence, can be regarded as a first step in this direction.

Hexane fraction was the only one among the tested extracts which was ineffective against *Citrobacter freundii* at 0.5 and 1 $mg.disc^{-1}$ (Fig. 8). Crude extract measured highest inhibition of the tested bacterium (28.8%, 35.9% and 47.0% activity at 0.5, 1 and 2 $mg.disc^{-1}$, respectively) while hexane fraction revealed minimum activity (25.0% activity at 2 $mg.disc^{-1}$). Other extracts in descending order of activity were ethyl acetate fraction, aqueous fraction and butanol fraction which measured 39.3%, 34.4% and 34.2% activity, respectively, at 2 $mg.disc^{-1}$. The importance of evaluating *C. freundii* susceptibility to the extracts used in this study is reflected in the reported unavailability of any treatment against the infections caused by this obstinate pathogen (Badger *et al*., 1999). The synergistic effect of the various solvent-soluble bioactive agents present in the crude extract offers a potential explanation for the enhanced effectivity of the crude extracts against this bacterium. The findings of the present study can be regarded as the initial stride in an effort to find *C. aromaticus* based treatment for *C. freundii* infections as there is no record of an earlier exertion towards this end.

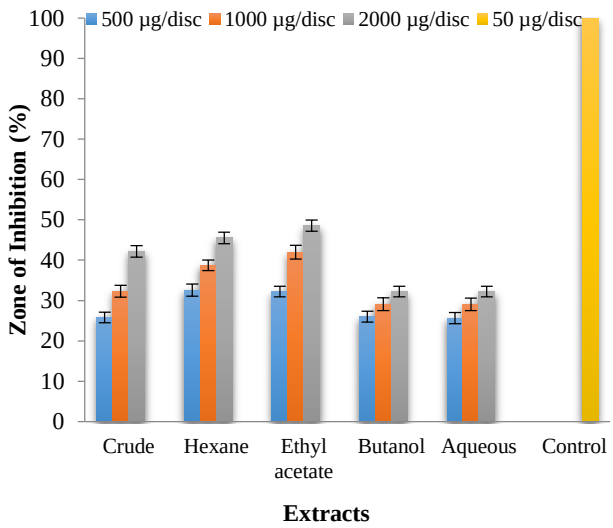

Fig. 6. Antibacterial activity (Mean ± Standard Deviation) of different extracts against *Escherichia coli*.

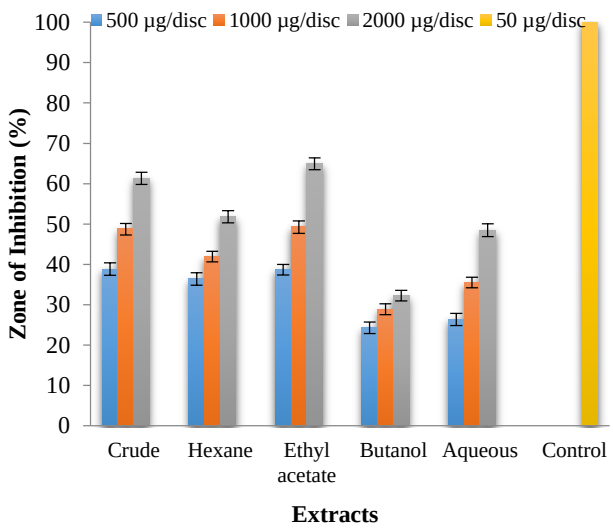

Fig. 7. Antibacterial activity (Mean ± Standard Deviation) of different extracts against *Xanthomonas campestris*.

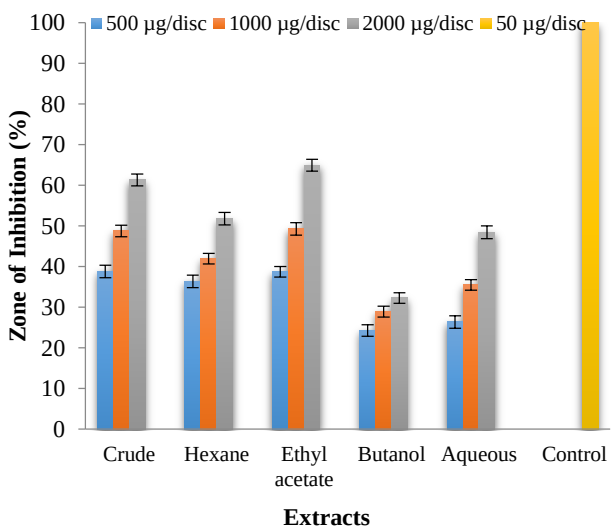

Fig. 8. Antibacterial activity (Mean ± Standard Deviation) of different extracts against *Citrobacter freundii*.

Gallic acid quantification: The regression equation and standard curve was obtained by plotting peak areas of the various Gallic acid dilutions with respect to retention-time (RT) against their corresponding concentrations. The subsequent equation was utilized to calculate Gallic acid quantity (in ppm) in each extract:

$$y = 46598\ x + 62967;\ R^2 = 0.996$$

In the aforementioned equation, 'y' represents the sample peak area while 'x' denotes Gallic acid quantity (in ppm) in that discrete sample. Additionally, standard mathematical equations were employed to convert ppm concentrations of Gallic acid thus obtained to mg per gram dry extract. The retention time for Gallic acid was calculated as 15.4 ± 0.2 minutes. Furthermore, the Gallic acid peak in each sample was specified by retention time and further testified by an appraisal of the UV spectra of both standard Gallic acid and Gallic acid in the sample.

The highest Gallic acid concentration (38.4 $mg.g^{-1}$ dry extract) was observed in ethyl acetate fraction (Fig. 9) which was shadowed by butanol fraction (11.3 $mg.g^{-1}$ dry extract). Aqueous fraction, on the other hand, revealed the lowest amount of the analyte (7.20 $mg.g^{-1}$ dry extract).

Gallic acid (3,4,5-trihydroxybenzoic acid) is a constituent of various vegetation wherein it occurs in an unbound state (Condrat *et al*., 2011). It is an illustrative affiliate of phenols (Singh *et al*., 2011) and is apparently a compelling anti-bacterial mediator (Ravn *et al*., 1989). The precision and consistency of HPLC (High Performance Liquid Chromatography) are the main reasons for its frequent use in quantifying polyphenols from herbal extracts (Khan & Bakht, 2016). The retrieval and solubility of polyphenols is affected not only by the chemical arrangement of plant extracts but also by the respective polarity of employed solvent. The cumulative quantity and rate of extraction, therefore, is profoundly affected by the choice of solvent. Imitated by the differing action of oxidation encouraged analyte degradation and solubilization, temperature and extraction time represent some additional dynamics contributing to phenolics retrieval (Robards, 2003). A proficient extraction protocol development ensuring the constancy of extracted polyphenols, hence, is also of paramount significance. Differential extraction of Gallic acid affected by the choice of solvent was also evident in this particular study.

The ethyl acetate fraction revealed the maximum amount of Gallic acid, followed sequentially by butanol and aqueous fractions. Henceforth, ethyl acetate and butanol can be justly backed as the solvents of choice for the extraction of phenolics, precisely Gallic acid, from *C. aromaticus* rhizomes. Water, on the other hand, cannot be aptly used for the same purpose as indicated by the findings of the present study. Gallic acid recovery from different plant parts has also been conveyed to be fittingly attained in organic solvents by numerous investigators such as Karamać *et al*., (2005); Singh *et al*., (2010); Condrat *et al*., (2011); Deshmukh & Prabhu (2011); Singh *et al*., (2011); Gupta *et al*., (2012); and Sharma & Singla (2013). Furthermore, the quantification statistics of HPLC offers sturdy foundation for the more vigorous antibacterial potential of organic solvent-extracted samples evidenced through the findings of antibacterial bioassay utilized in the present study.

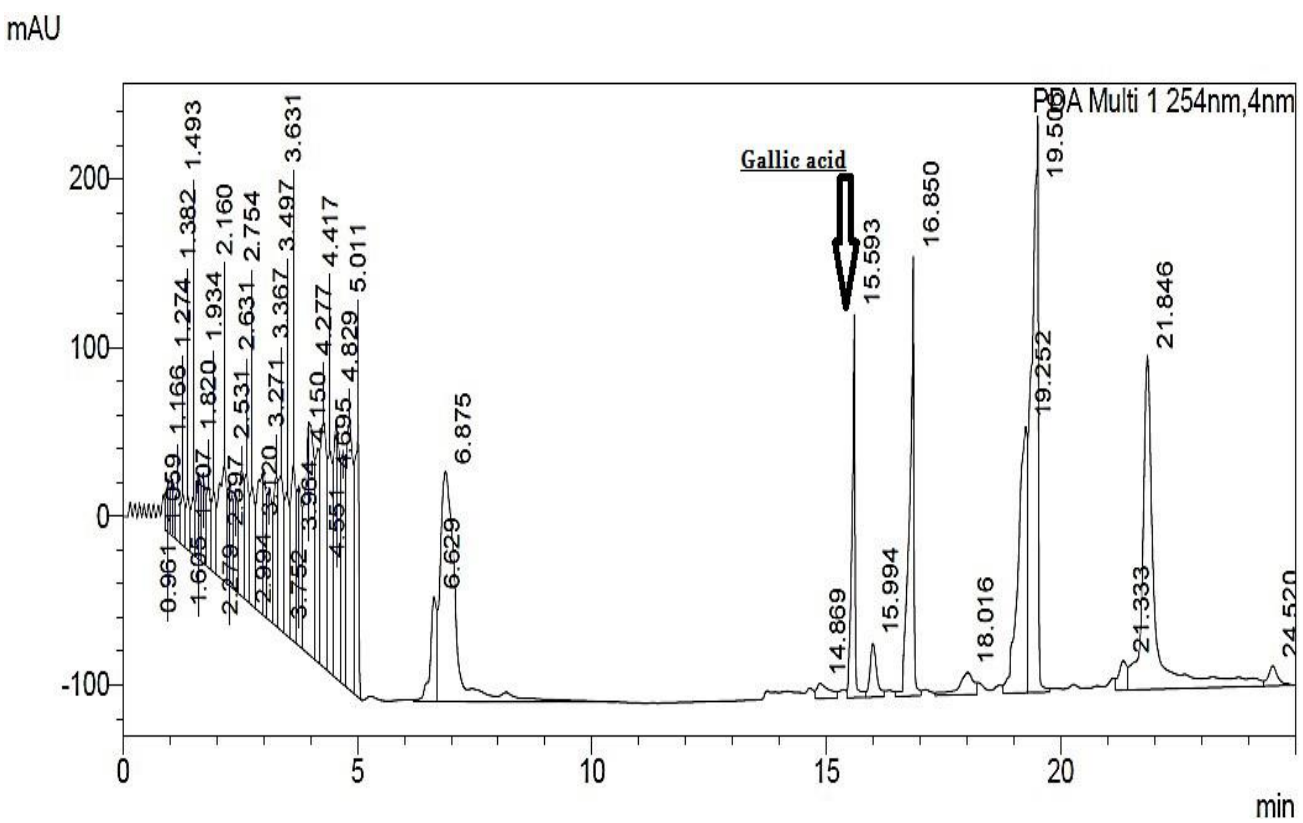

Fig. 9. HPLC chromatogram of ethyl acetate extracted sample from shade-dried rhizome.

Conclusions

The present study strongly supports the suitability of organic solvents for the extraction of phenolics, Gallic acid in particular, from *C. aromaticus* rhizomes. Water, conversely, cannot be aptly used for the same purpose as indicated by the findings of the present study. Additionally, HPLC quantification data provided a strong justification for the enhanced activities of organic solvent mediated extracts as maximum Gallic acid concentration was revealed in such extracts. This concordance, furthermore, stalwartly advocates the potential of Gallic acid as a broad spectrum antibacterial agent. We recommend further studies on the organic solvents extracted samples for the isolation of active ingredients to be used in commercial drugs. Moreover, the *In vivo* toxicity and efficacy studies of these extracts are also needed.

Acknowledgement

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