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In vitro: biological activity of Callistemon acuminatus Cheel flowers extracts against gram-positive and gram-negative bacteria

Manoj Kumar*
*Department of Botany, Govt. College for Women, Rewari- 123401, Haryana, India
*Email: manojgenetics@yahoo.com

Abstract - Biological activity of Callistemon acuminatus Cheel flowers extracts in different solvents (methanol, ethanol and aqueous) was carried out against seven different bacterial strains, comprising four gram-positive (Bacillus subtilis, Micrococcus luteus, Staphylococcus aureus and Streptococcus sp.) and three gram-negative (Escherichia coli, Pseudomonas aeruginosa and Salmonella typhimurium) bacterial strains by using the agar well diffusion assay (AWDA) method. The results indicated that methanol extract gave the highest yield percentage as compared to ethanol as well as aqueous extracts. Inhibition patterns were varied with the solvents as well as tested bacterial strains. Outcomes of the present study showed that Streptococcus sp. among gram-positive and E. coli among gram-negative were highly susceptible as compared to other tested bacterial strains. Minimum inhibitory concentrations (MIC) as well as minimum bactericidal concentrations (MBC) of the extracts were determined for various tested bacterial strains, exhibited a range between 12.5 to 50 mg/ml and 12.5 to 100 mg/ml respectively. The remarkable biological activity of crude extracts against tested gram-positive and gram-negative bacterial strains suggests that the flowers extract of C. acuminatus plant could be a possible source for designing and developing new broad spectrum drugs for the treatment of various infectious diseases.

Keywords - Biological activity, Callistemon acuminatus, Solvent extracts, Zone of Inhibition

1. INTRODUCTION

Antimicrobial agents are essentially important in reducing the global burden of infectious diseases [1]. However, emergence and dissemination of multidrug resistant (MDR) strain in pathogenic bacteria have become a significant public health threat as there are fewer, or even sometimes no, effective antimicrobial agents available for the infection caused by pathogenic bacteria [2,3]. Thus, in the light of the evidence of the rapid global spread of resistant clinical isolates, the need to find new antimicrobial agents is of paramount importance. However, the past record of rapid, widespread emergence of resistance to newly introduced antimicrobial agents indicates that even new families of antimicrobial agents will have a short life expectancy [4, 5].

A vast number of medicinal plants have been recognized as valuable resources of natural antibacterial compounds as an alternative that can potentially be effective in the treatment of these problematic bacterial infections. They have been used for centuries to treat infectious diseases and are considered as an important source of new antibacterial agents [6,7]. Many plants have been used because of their antibacterial traits, which are due to phytochemicals synthesized in the secondary metabolism of plant [8,9]. Plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids, phenolic compounds, and flavonoids, which have been found in vitro to have antimicrobial properties [10,11]. These natural products are typically secondary metabolites, produced by plants in response to external stimuli such as nutritional changes [6]. Approximately 80% of people in developing countries depend on natural medicines for primary health care [12,13]. Natural medicinal system plays an important role in maintaining the physical and psychological well being of majority of peoples of the World [14]. According to World Health Organization (WHO), medicinal plants would be the best source to obtain a variety of drugs [15].

Efforts toward the collection of baseline data on medicinal plants for future phytochemical and pharmacological studies and innovation are very limited. Many scientists have underlined the urgent

need for discovering new, safe, and cheap antibiotics with diverse chemical structures, novel chemical actions, and no adverse side effects in response to the emergence of resistant microbial strains to indiscriminate use of antibiotics [16-19]. Several studies have been reported to examine the antimicrobial effects of herbal plants extracts, including roots, stem, leaves or flowers [20,21]. They are widely used in the pharmaceutical industry for their remarkable structural diversity and range of pharmacological activities [22]. For the same, widespread screening of medicinal plants from the traditional system of medicine hoping to get some newer, safer, and more effective agents that can be used to fight infectious diseases [23].

The genus Callistemon is an evergreen tree or shrub, belonging from family Myrtaceae is widely used as ornamental plant. Callistemon grows upto 6-15 m in height and are commonly referred to as bottlebrush because of their cylindrical, brush like flowers resembling traditional bottlebrush (Fig. 1). This plant has attractive narrow foliage and white papery bark [24]. Leaves lanceolate sometimes broadly so, upto 7.5 cm long, with prominent vein, midrib and oil gland; flowers, crimson with dark red anthers, in 10 cm long spikes; capsules depressed-globose [25]. In India, Callistemon introduced from Australia. It is cultivated in gardens for its beautiful flower, uncommonly pretty foliage, gorgeous shade and large amount of nectar [26]. The flowers were sucked for their nectars and used to make sweet drinks. Leaves were used to cure respiratory tract infections [27]. Moreover, Callistemon is known as traditional and folk medicine has been reported to have antihelmenthic, antibacterial, antidiabetic, antiinflamentary, anticough, antibronchitis, nematicidal, larvicidal, pupicidal, antithrombotic, and antioxidant activities have been documented. It is also used as weed control and as bioindicators for environmental management. Ecologically, Callistemon is planted for forestry plantations or ornamental purposes. In India, this plant has been used by tribal communities for the treatment of gastrointestinal disorders, pain and infectious diseases [28-30].

Fig. 1: Photo of Callistemon acuminatus Cheel plant

Hence, the present study was planned to evaluate the biological activity of C. acuminatus flowers extracts with different solvents (methanol, ethanol and aqueous) against seven different bacterial strains, comprising four gram-positive and three gram-negative.

2. MATERIALS AND METHODS

2.1 Sources of bacterial strains

Bacterial strains, including both gram-positive and gram-negative obtained from M.D. University, Rohtak, Haryana and Microbial Type Culture Collection and Gene Bank Institute of Microbial Technology, Chandigarh. The bacterial

strains include Bacillius subtilis, Micrococcus luteus (MTCC106), Staphylococcus aureus (MTCC6908), Streptococcus sp. (MTCC9724), Escherichia coli DH5α, Pseudomonas aeruginosa (MTCC4673) and Salmonella typhimurium (MTCC3224) have been selected for the present investigation.

2.2 Culture of bacterial strains

The bacterial strains were propagated in the nutrient broth medium (5g/l peptone, 3g/l beef extract, 5g/l NaCl, and pH 7.0) at 37°C temperature for overnight. Slants were prepared from the isolated bacterial colonies, stored at 4°C temperature and sub-cultured in nutrient broth medium before testing the biological activity. The chemicals were purchased from Hi-media, Mumbai, India.

2.3 Preparation of plant material

Collected the fresh flowers of C. acuminatus plant and dried in the shade for one month and then ground into coarse powder with the help of mortar and pestle, and stored in airtight brown bottles at 4°C until needed for future use.

2.4 Extraction of plant material (maceration)

The shade dried 100 gm coarse powder of flowers immersed in 200 ml of different solvents (methanol, ethanol and aqueous) contained in 500 ml sterile conical flasks and covered with cotton plugs separately. It was placed aside with intermittent shaking for one week. They were first filtered with double layered muslin cloth and then through Whatman No. 1 filter paper. The march was discarded, and filtrate was subjected to evaporation by treating at 40°C in an oven to obtain a dried extract. Dried extract was stored at 4°C until used for further study [31].

2.5 Yield percentage of solvents extracts

After complete drying, yield of each extraction measured separately and extraction efficacy was quantified through calculating the weight of each extracts and yield percentage was calculated as dry weight/dry material weight ×100 [32].

2.6 Biological activity by agar well diffusion assay (AWDA) method

The biological activity of crude flowers extracts of C. acuminatus in different solvents (methanol, ethanol and aqueous) against gram-positive as well as gram-negative bacterial strains were evaluated by agar well diffusion assay (AWDA) method [32]. Diameters of the inhibitory zones were measured in millimeters (mm). For this, a well (6 mm diameter) was made with the help of a borer in cooled nutrient agar plate, overlaid with soft agar (5 ml), seeded with a target strain (~1.0 x 10^6 cfu/ml). Aliquots of the test sample (100 µl) were introduced into the well and plates were incubated at 37ºC for overnight. For each bacterial strain, dissolving solvent 10% Dimethyl Sulphoxide (DMSO) and streptomycin (50 µg/ml) were used as negative and positive controls respectively. To test the biological activity of all extracts were dissolved in 10% DMSO solvent to make a final concentration of 200 mg/ml.

2.7 Determination of minimum inhibitory concentration (MIC)

MIC is the concentration giving the least inhibitory activity and below which there is no further inhibition were recorded by using the broth dilution method [33]. Briefly, 1.0 ml of reconstituted extract solution at a concentration of 200 mg/ml was added to another test tube containing 1.0 ml of sterile broth so as to obtain a concentration of 100 mg/ml. One milliliter of this dilution was transferred to another test tube till the 7^{th} test tube was reached. The 8^{th} test tube did not contain any extract, but a solution of pure solvent and served as negative control. Then 1.0 ml of overnight grown cultures of each bacterial strain, adjusted at ~ 1.0 x 10^6 cfu/ml was put into each tube and thoroughly mixed by vortex mixer. Tubes were incubated at 37°C for overnight and observed the growth in the form of turbidity. The test tube with the lowest dilution with no detectable growth by visual inspection considered the MIC's value.

2.8 Determination of minimum bactericidal concentration (MBC)

MBC values were determined by removing 100 µl of bacterial suspension from the MIC positive

tube as well as one above and one below the same tube, spread on nutrient agar plates and incubated at 37°C for overnight. After incubation, plates were examined for colony growth and MBC's were recorded [34,35].

2.9 Statistical analysis

Experiments were carried out in three independent sets, each consisting of three replicates.

3. RESULTS

After drying, yield percentage (gms) of flowers extracts with various solvents (methanol, ethanol and aqueous) was measured independently and quantified the efficiency of extraction. The results of present study, methanol extraction gave highest yield percentage (9.76%) followed by ethanol (8.25%) and aqueous (7.82%) are illustrated in Table 1.

Table 1: Yield percentage of C. acuminatus flowers extracts in different solvents

Solvent	Yield percentage of extracts (gms)		
	Weight of dry powder	Weight of dry extracts	Yield percentage
Methanol	100	9.76	9.76
Ethanol	100	8.25	8.25
Aqueous	100	7.82	7.82

The obtained results showed that the tested different bacterial strains responded differently to the different solvents extracts. However, different solvents extracts exhibited biological activity against all the tested bacterial strains, comprising both gram-positive and gram-negative as shown in Fig. 2. The maximum zone of inhibition was recorded for methanol extracts against B. subtilis (24), M. luteus (25), S. aureus (20), Streptococcus sp. (26), E. coli (23), P. aeruginosa (20) and S. typhimurium (22). Ethanol extracts showed inhibition against B. subtilis (21), M. luteus (22), S. aureus (18), Streptococcus sp. (24), E. coli (21), P. aeruginosa (18) and S. typhimurium (19). Similarly, aqueous extracts produced inhibitory zones towards B. subtilis (19), M. luteus (20), S. aureus (15), Streptococcus sp. (22), E. coli (18), P. aeruginosa (15) and S. typhimurium (17). However, streptomycin used as a positive control exhibited higher inhibition as compared to different solvents extracts against B. subtilis (26), M. luteus (27), S. aureus (23), Streptococcus sp. (28), E. coli (25), P. aeruginosa (22), and S. typhimurium (24), while DMSO doesn't shows any inhibitory zones.

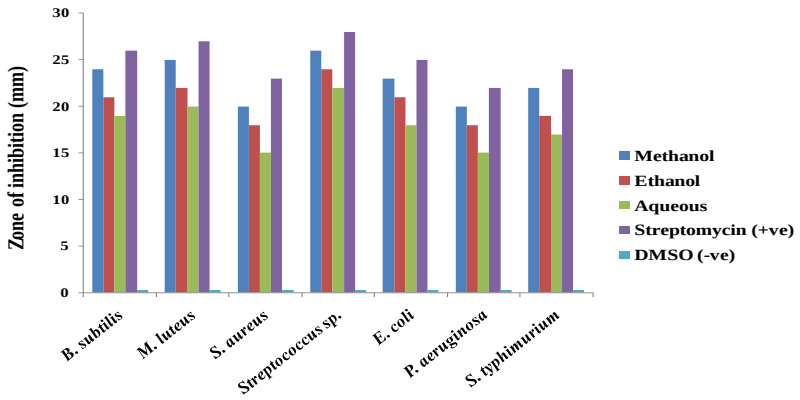

Fig. 2: Biological activity of C. acuminatus flowers extracts

MIC for methanol, ethanol and aqueous flowers extracts are shown in Fig. 3. In the present study, methanol extracts exhibited 12.5 mg/ml against B. subtilis, M. luteus, Streptococcus sp., E. coli and S. typhimurium; 25 mg/ml against S. aureus and P. aeruginosa. Samples of ethanol extract possessed 12.5 mg/ml against B. subtilis, M. luteus, Streptococcus sp. and E. coli; 25 mg/ml against S. aureus, P. aeruginosa and S. typhimurium. Similarly, aqueous extracts showed 12.5 mg/ml against only Streptococcus sp.; 25 mg/ml against B. subtilis, M. luteus, E. coli and S. typhimurium; 50 mg/ml against S. aureus and P. aeruginosa

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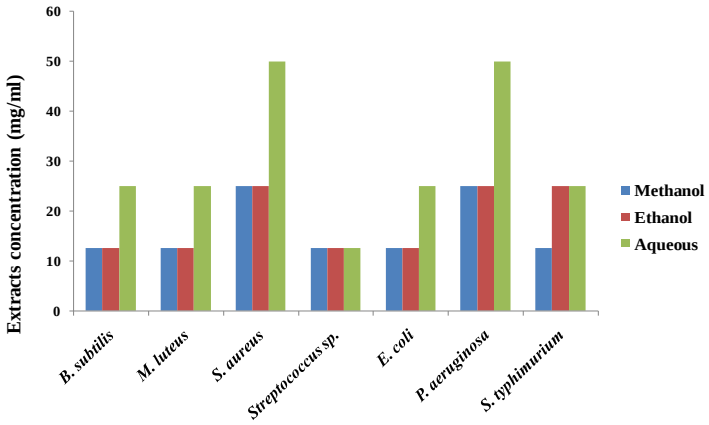

Fig. 3: MIC (mg/ml) values of C. acuminatus flowers extracts

The results of MBC values of methanol, ethanol and aqueous flowers extracts are presented in Fig. 4. Methanol extracts exhibited 12.5 mg/ml against only Streptococcus sp.; 25 mg/ml against B. subtilis, M. luteus, E. coli and S. typhimurium; 50 mg/ml against S. aureus and P. aeruginosa. Ethanol samples possessed 25 mg/ml against B. subtilis, M. luteus, Streptococcus sp. and E. coli; 50mg/ml against S. aureus, P. aeruginosa and S. typhimurium. Similarly, aqueous extract exhibited 25 mg/ml against only Streptococcus sp.; 50 mg/ml against B. subtilis, M. luteus, E. coli and S. typhimurium; 100 mg/ml against S. aureus and P. aeruginosa.

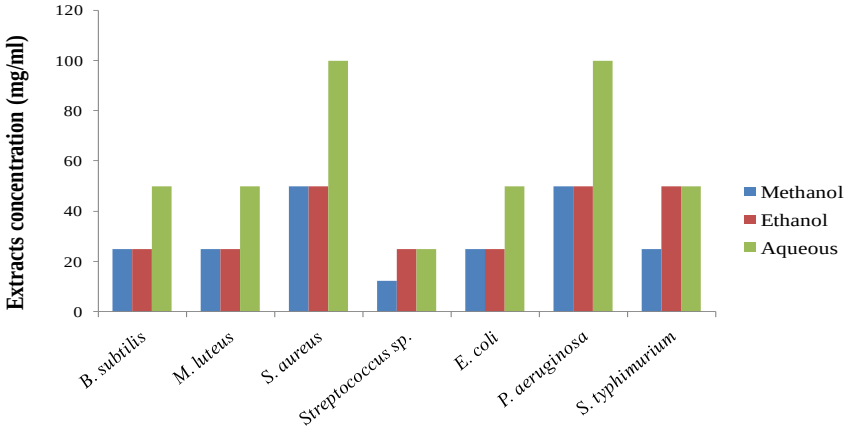

Fig. 4: MBC (mg/ml) values of C. acuminatus flowers extracts

4. DISCUSSION

Natural biological agents have been more popular due to their efficacy against antibiotic resistant microorganisms [36]. A number of various plant species, as sources of medicinal compounds have continued to play a dominant role in the maintenance of human health since ancient times and medicinal plants continued to be an important therapeutic aid for alleviating the ailments of humankind [37]. Today, there is a renewed interest in traditional medicine and increasing demand for more drugs from plant source. This revival of interest in plant derived drug is mainly due to the current widespread belief that "green medicine" is safe and more dependable than the costly synthetic drugs, many of which have adverse side effects [38]. This situation provided impetus to the search for new antimicrobial substances from various sources like medicinal plants [39]. Synthetic drugs are not only expensive and inadequate for the treatment of diseases but also often with adulterations and side effects. Therefore, it is necessary to search the novel infection fighting strategies for controlling the microbial infections [37, 40, 41]. Many studies have been undertaken with the aim of determining different antimicrobial and phytochemical constituents of medicinal plants and using them for the treatment of microbial infections as possible alternatives to synthetic chemical drugs against which many infectious microorganisms have become resistant [42-46]. A number of medicinal plants described in Ayurveda still need to be testified, according to the modern parameters to ensure their activity and efficacy. Drugs used in Ayurveda are mostly prepared by extraction with water. Therefore healers may not be able to extract all the active compound(s) [47]. The yield percentage of medicinal plant extracts which contain the bioactive metabolites vary considerably with plant species and the method or solvent used for extraction. Also, factors like age of the plant and polarity of solvent used may have affected the yield percentage [48]. In the present study, methanol solvent extract gave highest yield of extraction followed by ethanol and aqueous. The various solvents extracts exhibited inhibitory activity against all the tested seven different bacterial strains including both gram-positive and gram-negative with different degrees. Among the different solvents, methanol extract was found to be most effective in comparison to other solvents. Similar findings have been reported by Cock [27], where methanol extract of Callistemon was to have highest zone of inhibition and extracts are known to be more active against gram-positive as compared to gram-negative tested bacterial species. Moreover, in another study, reported that at an initial screening concentration of 1.67 mg/ml, alkaloids from C. citrinus inhibited bacterial growth significantly. However, P. aeruginosa was less susceptible to antibacterial effect of the alkaloids and ampicillin as compared to S. aureus [49]. Similarly, flowers extract of C. citrinus with ethanol exhibited remarkable antibacterial activity against gram-positive and gram-negative bacteria [50]. It is well known that gram-positive bacteria are more susceptible to antibiotics than gram-negative bacteria. This is attributed to structural variations observed in the cell envelope between both types of microorganisms [51]. In contrast, a study reported C. linearis extracts showed strong antibacterial activities against tested gram-negative bacteria [52].

In the present investigation, samples of methanol, ethanol and aqueous extracts produced MIC and MBC values a range between 12.5 to 50 mg/ml and 12.5 to 100 mg/ml respectively against the tested different bacterial strains. However, in another study, MIC was identified for the C. citrinus extracts most potent with lowest value 0.025 mg/ml against S. aureus and 0.21 mg/ml for P. aeruginosa. Crude extracts had a bactericidal action against S. aureus with an MBC of 0.835 mg/ml. In addition, alkaloids from C. citrinus had bacteriostatic effect against both P. aeruginosa and S. aureus bacterial strains [49]. Oyedeji et al. [53] reported MIC values of C. viminalis oils showed the lowest values 0.08 mg/ml against S. aureus and highest 5.00 mg/ml for P. aeruginosa and S. marcescena.

5. CONCLUSION

Evaluation of biological activity of medicinal plants is not only to find out the scientific rationale for their usage, but also to contribute in the global scientific efforts toward exploring new antibiotics and antimicrobial drugs to eradicate the growing phenomenon of multi-drug resistant

microorganisms. In the present study, flowers extracts of C. acuminatus plant with various solvents possesses significant inhibitory activity against tested both gram-positive as well as gram-negative bacteria and the results in agreement to a certain degree with the traditional uses of this plant. The methanol extract gave the highest yield percentage as compared to ethanol and aqueous extracts. Newer antimicrobials from the flowers extracts of C. acuminatus plant could also be of commercial interest to pharmaceutical companies and research institutes in designing and developing new broad spectrum drugs for the treatment of various infectious diseases.

COMPETING INTERESTS

The author declared that he has no competing interests.

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