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ANTIMICROBIAL ACTIVITY OF LEAVES OF *CALLISTEMON LANCEOLATUS* AGAINST PATHOGENIC ORGANISMS COMPARED WITH CONTROL DRUGS

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ABSTRACT

The crude extract from the leaves of *Callistemon Lanceolatus* traditionally used in Indian system of medicines were screened against *Escerichia coli* NCIM 2931, *Proteus vulgaris* NCIM2857, *Pseudomonas aeruginosa* NCIM5029, *Staphylococcus aureus* MTCC 96, *Bacillus cereus* NCIM2155, *Bacillus subtilis* NCIM 2063 and *Bacillus megaterium* NCIM 2087 by using agar well diffusion method. *Callistemon Lanceolatus* crude extract showed significant activity against Gram positive organisms. Zone of inhibition of the extract compared with the standard antibiotics. Fresh leaves juice is active against all organisms. *E. coli, P. vulgaris, P. aeruginosa, S. aureus, B. cereus, B. subtilis* and *B. megaterium*. Crude extract active against *S. aureus.* Methanol extract only active against gram positive organisms. Chloroform extract and petroleum ether extract active against *B. megaterium* and *S. aureus.* Petroleum and chloroform ether extract are not active against *B. cereus* and *B. subtilis.*

KEYWORDS: Solvent extracts, Antimicrobial activity, Agar well diffusion method.

INTRODUCTION

Most of the drugs used in primitive medicine were obtained from plants and are the earliest and principle natural source of medicines. There is no doubt that plants are a reservoir of potentially useful chemical compounds which serve as drugs, are provided newer leads and clues for modern design by synthesis (Evans, 2002; Varier, 1995). *Callistemon lanceolatus* DC (*C. lanceolatus*) (Family: Myrtaceae) commonly known as bottle brush, is frequently cultivated throughout India in gardens as ornamental plant. Hummingbirds love the flowers, and the plant is hardier than most Bottlebrushes. Aqueous extracts of the leaves and flowers have antifungal and antibacterial activity. The extract also shows cholinesterase activity. The essential oils from leaves possess antimicrobial, fungitoxic, antinociceptive and anti-inflammatory activities (Kumar et al., 2011).

Medicinal plants form the backbone of traditional system of medicine in India. Pharmacological studies have acknowledged the value of medicinal plants as potential source of bioactive compounds (Prusti et al., 2008). Phytochemicals from medicinal plants serve as lead compounds in drug discovery and design (Chakravarthy and Gode, 1985; Ebi and Ofoefule, 2000). Medicinal plants are rich source of novel drugs that forms the ingredients in traditional system of medicines, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, bioactive principles and lead compounds in synthetic drugs (Ncube, 2008). Nowadays multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious disease (Davis, 1994; Service, 1995). In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression and allergic reactions (Ahmad et al., 1998). This situation forced scientists to search for new antimicrobial substances. Given the alarming incidence of antibiotic resistance in bacteria of medical importance (Monroe and Polk, 2000), there is a constant need for new and effective therapeutic agents (Bhavani and Ballow 2000). Therefore ,there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants (Clark, 1996 and Cordell, 2000).Several screening studies have been carried out in different parts of the world. There are several reports on the antimicrobial activity of different herbal extracts in different regions of the world (Chung et al., 2004, De Boer et al., 2004, Nair and Chanda 2004, Nair et al., 2005).

MATERIALS AND METHODS

Selection of medicinal plant for this study

Callistemon lanceolatus

Family: Myrtaceae

Parts used: Leaf

Traditional uses: *Callistemon* species are used for forestry, essential oil production, farm tree/windbreak plantings, degraded-land reclamation and ornamental horticulture, among other applications (Spencer and Lumley, 1991). In China callistemon species, especially

C. viminalis, are used in Traditional Chinese Medicine pills for treating hemorrhoids (Ji, 2009). *Callistemon* are also used as weed control (Wheeler, 2005) and as bioindicators for environmental management (Burchett et al., 2002).

Chemical constituents: Preliminary phytochemical screenings were performed for presence of saponins, tannins, carbohydrates, steroids, proteins, amino acids, Phenolic compounds and anthraquinone glycosides (Ali et al., 2011). Two neolignans, named callislignan A and B together with known Cmethyl-flavonoids, a lignan and pentacyclic triterpenoid esters were isolated from the leaves of *C. lanceolatus.* Previous chemical investigations of compounds from this family have revealed the presence of various types of secondary metabolites, including triterpenoids (Younes, 1975; Varma and Parthasarathy, 1975), Phloroglucinol Derivatives (Lounasmaa et al., 1977), C-methyl flavonoids (Huq and Misra, 1997) and tannins (Hanaa and Mohamed, 2002). A new triterpenoid, 30-hydroxyalphitolic acid **1**, and eight known triterpenoids, alphitolic acid **2**, lupenol**3**, 3-acetoxy-olean-18-en-28-oic acid **4**, betulinic acid **5**, ursolic acid **6**, betulinic acid 3-*O*caffeate **7**, morolic acid 3-*O*-caffeate **8**, and ursolic acid 3-*O*-caffeate **9**, were isolated from *C. lanceolatus* (Jeong et al., 2009). Substantial fractionation and purification of the EtOAc-soluble extract of the aerial parts of *C. lanceolatus* afforded six flavonoids, 4',5-dihydroxy-6,8-dimethyl-7- methoxyflavanone (1), eucalyptin (2), 8- demethyleucalyptin (3), sideroxylin (4), syzalterin (5), and quercetin (6) (Park et al., 2010). Two new flavonol glycosides, kaempferol 3-O-beta-D galacturonopyranoside and quercetin 3-O-(2''-Ogalloyl)-beta-D-glucoronopyranoside, were isolated, from leaves of *C. lanceolatus*, as well as eighteen known polyphenols (phenolic acids, flavonoids and three tannins) (Mahmoud et al., 2002).

Identification and Preservation of Plant materials

Fresh plant leaves were collected from the Nagpur area of India. The taxonomic identities of this plant was determined by the expertise of the Post Graduate Department of Botany of Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur. Specimen was labeled, numbered and noted with date of collection, the locally and their medicinal uses and their approximate dosages of administration were recorded. Plant leaves were washed with 70% alcohol and then rinsed with sterilized distilled water, air dried and stored in airtight bottles at 4^0C for further use.

Preparation of crude extract (Fresh juice)

Callistemon lanceolatus plant leaves were collected from around Nagpur region in the month of August-September. Leaves were cleaned under running potable water and cut into pieces and grounded in pestle and mortar (made up of dolerite stone) till homogenized mass was obtained. Homogenized mass was squeezed in 400 mesh nylon cloth (pore size 37 micron) to obtain crude extract. Crude extract was kept in sterilized glass bottle. All crude extract were prepared fresh and used before 2 hours.

CRUDE EXTRACTION

Aqueous extraction

Ten grams of dried powder was extracted in 100 ml distilled water for 6 h at slow heat. Every 2 h, it was filtered through 8 layers of muslin cloth and centrifuged at 5000 rpm for 15 min. The supernatant was collected. This process was repeated twice and after 6 h, the supernatant was concentrated to make the final volume one-fourth of the original volume (Shahidi Bonjar GH 2004). It was then autoclaved at 121^0C and 15 lbs pressure and then stored at 4^0C.

Solvent extraction

Ten grams of dried powder was extracted with 100 ml of each solvent (acetone, chloroform, methanol and petroleum ether) and flasks were kept on a rotary shaker at 190-220 rpm for 24h. Thereafter, it was filtered through 8 layers of muslin cloth and centrifuged at 5000 rpm for 15 min. The supernatant was collected and the solvent was evaporated to make the final volume one-fourth of the original volume (Shahidi Bonjar, G.H. 2004). It was stored at 4^0C in airtight bottles for further studies.

Bacterial cultures

The microbial strains are identified strains and were procured from the National Chemical Laboratory (NCL) Pune, India. The studied bacterial strains were *Escherichia coli* NCIM2931, *Proteus vulgaris* NCIM2857, *Pseudomonas aeruginosa* NCIM5029, *Staphylococcus aureus* MTCC96, *Bacillus cereus* NCIM2155, *Bacillus subtilis* NCIM2063, and *Bacillus megaterium* NCIM2087 these strains were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. They were sub-culured on nutrient agar for every 15 days and maintained on nutrient agar slants at 4^0C, fresh inoculums were taken for test.

Media

Hi -Sensitivity test broth (M 486) and Hi-sensitivity test agar (M 485) were procured from Hi-media Mumbai, India. The media were prepared according to the instructions given.

Screening for the antimicrobial potential of the plant leaves extracts

The antimicrobial activity of different solvent extracts was evaluated by agar well diffusion (Perez C, et al., 1990, Nair and Chanda, 2005 and Parekh, J. et al., 2007) using Hi-sensitivity test agar (M 485).

Preparation of inoculum – A loopful of culture was inoculated from the stock slant culture in 5 ml of Hi-sensitivity test broth and broth was incubated at 35 ± 0.5^0C in incubator for 18-20 hrs. After incubation a loopful of actively growing culture was inoculated into

10 ml of Hi-sensitivity broth. Broth was incubated at 35±0.5^{0}C for 6-8 hours. This culture was used for the inoculation of Hi-sensitivity test agar plates.

Preparation of Hi-sensitivity test agar medium
Hi-sensitivity test agar medium was prepared as per instructions of manufacturer. Required amount of agar medium was melted and 25 ml of molten medium was distributed in test tubes (25x150 mm). Medium was autoclaved at 15 lb. for 20 min. After autoclaving, medium was maintained at 45-50^{0}C in constant temperature water bath.

Inoculation of medium with test organism
0.5 ml of 6-8 hours old test organism is transferred to petridish of 100mm size (Sterilized in oven at 180^{0}C for 1 hr.) using sterile micropipette. Hi-sensitivity test agar medium maintained at 45-50^{0}C was poured and mixed properly to ensure uniform distribution of organism with medium. Seeded plates are allowed to set at room temperature.

Preparation of agar well for fresh leaves juice
10 mm borer was used to prepare wells in agar. Four wells per plate at four equidistant corners were made. A 100 µl crude extract (fresh leaves juice) was transferred by micropipette per well. Plates were immediately kept at 4^{0}C in refrigerator for 1 hr. for the good diffusion of extract and then shifted to 35±0.5^{0}C in incubator (Venkatesan, D. et al., 2009). Zone of inhibition was measured after 24 hours of incubation by zone scale.

Preparation of agar wells for different solvent extracts
5 mm borer was used to prepare wells in agar. Four wells per plate at four equidistant corners were made.

A 50 ul solvent extract was transferred by micropipette per well. Plates were immediately kept at 4^{0}C in refrigerator for 1 hr. and them shifted to 35^{0}C±0.5^{0}C in incubator. Zone of inhibition was measured after 24 hours of incubation for each bacterial strain, controls were maintained in which pure solvents were used instead of the extract. The control zones were sustracted from the test zones and the resulting zone diameter is obtained.

RESULTS AND DISCUSSION

Table 1: Results of antimicrobial activities of fresh leaves juice and solvent extracts of *Callistemon lanceolatus* leaves and compared with standard antibiotics.

Sr. No. Microorganisms	Zone of inhibition in millimeter										
	Leaves extracts						Standard antibiotics				
	FJ	WE	AE	CE	ME	PE	Am^{30}	Cf^{30}	Co^{25}	G^{50}	T^{30}
1. *Escherichia coli*	17	---	---	---	---	---	32	29	24	17	22
2. *Proteus vulgaris*	16	---	---	---	---	---	---	23	31	20	24
3. *Pseudomonas aeruginosa*	20	---	---	---	---	---	14	36	24	34	22
4. *Staphylococcus aureus*	17	11	16	10	16	12	31	23	20	16	17
5. *Bacillus cereus*	22	---	---	---	10	---	15	27	---	23	24
6. *Bacillus subtilis*	16	---	11	---	11	---	31	50	36	40	32
7. *Bacillus megaterium*	16	---	12	11	12	10	29	46	24	23	33

Key: FJ—Fresh juice of leaves; WE—Water extract; AE—Acetone extract; ME—Methanol extract; CE-Chloroform extract; PE—Petroleum ether extract; Am[30]--Amoxycilin; Cf[30]–Ciprofloxacin; Co[25]–Cotrimaxazole; G^{50}-Gentamicin; Tetracycline-T.[30]

Antibacterial activity of different solvent extracts of leaves of *Callistemon lanceolatus (CL)*, zone of inhibition in millimeter (mm).

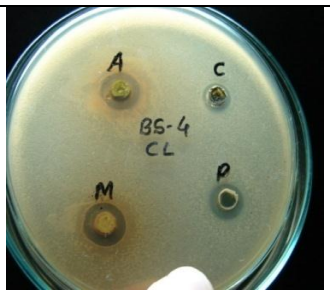
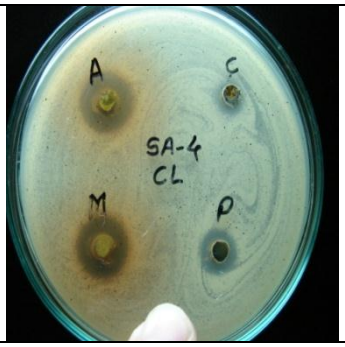


Figure-1:
Activity against *Bacillus subtilis*
Acetone extract (A)-11 mm
Chloroform extract (C) -
Methanol extract (M)-11 mm
Petroleum ether extract (P) -



Figure—2
Activity against *Staphylococcus aureus*
Acetone extract (A)-16 mm
Chloroform extract (C)-10 mm
Methanol extract (M)-16 mm
Petroleum ether extract (P)-12 mm

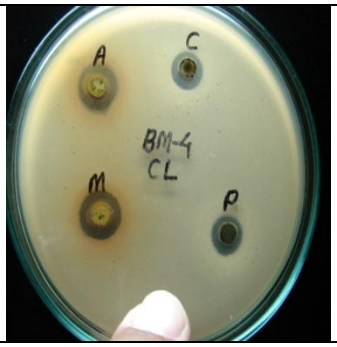


Figure--3
Activity against *Bacillus megaterium*
Acetone extract (A)-12 mm
Chloroform extract (C)-11 mm
Methanol extract (M)-12 mm
Petroleum ether extract (P)-10 mm

The extracts prepared from *Callistemon lanceolatus* leaves using different solvents showed varying degree of antimicrobial activity against organisms selected for the study. When we compared the activity of aqueous extract with fresh leaves juice, the fresh leaves juice is more active. Fresh leaves juice is active against all organisms. *E. coli, P. vulgaris, P. aeruginosa, S. aureus, B. cereus*, *B. subtilis* and *B. megaterium*. Crude extract active against *S. aureus.* Methanol extract only active against gram positive organisms. Chloroform extract and petroleum ether extract active against *B. megaterium* and *S. aureus.* Petroleum and chloroform ether extract are not active against *B. cereus* and *B. subtilis.* Water as the solvent but in our studies we found that plant extracts in organic solvent (Acetone, Chloroform, Methanol and Petroleum ether) provided more consistent antimicrobial activity compared to those extracted in water.

All the organisms are susceptible to Amoxycillin-Am[30], Ciprofloxacin-Cf[30], Gentamicin-G[50], Cotrimaxozole-Co[25] and Tetracycline-T.[30] *Proteus vulgaris* is found to be resistant to Amoxycillin-Am[30] and *Bacillus cereus* found to be resistant to Cotrimaxozole-Co.[25]

The extractive values are useful to evaluate the chemical constituents present in the crude drug and also help in estimation of specific constituents soluble in a particular solvent (Thomas S. et al., 2008). Preliminary phytochemical analysis indicated the presence of steroids, terpenoids, alkaloids, fatty acids, flavonoids, phenolic compounds and carbohydrates. The methanolic extract was also screened for anti-inflammatory activity.

Inflammation is a common phenomenon and it is a reaction of living tissues towards injury (Mohamed STK. et al., 2011). Carrageenan induced inflammation is a useful model for the estimation of anti-inflammatory effect (Ratheesh M. et al., 2007). The development of oedema in the paw of the rat after the injection of carrageenan is due to the release of histamine, serotonin, prostaglandin and the like (Mohamed STK. et al., 2011), (Georgewill OA. Et al., 2010), (Georgewill OA and Georgewill UO. 2010). *C. lanceolatus* methanolic leaf extract showed significant (P<0.05) anti-inflammatory activity at doses of 200 and 400 mg/kg. This significant anti-inflammatory of *C. lanceolatus* methanolic leaf extract at the dose of 400 mg/kg was comparable with diclofenac sodium. The presence of bioactive constituents as indicated above may be responsible for anti-inflammatory activity. However, the main active constituents responsible for the activity should be isolated from the plant.

In conclusion, pharmacognosic parameters could be useful to detect the authenticity of this medicinally useful plant. Furthermore, the methanolic extract of the leaves have potent anti-inflammatory activity (Kumar S. et al., 2011).

CONCLUSION

Callistemon Lanceolatus crude extract showed significant activity against Gram positive organisms. Several flavonoids, triterpenoids, tannins, phenolic compounds have been isolated from its leaves. *C. lanceolatus* shows various types of activities such as free radical scavenging activity, antimicrobial activity and herbicidal activity. The broad therapeutic potentials of *C. lanceolatus* may be due to the presence of the investigated active chemical constituents.

Furthermore other parts of the plants are to be exploited for further pharmacological investigations along with the establishment of mechanism and chemical constituents responsible for the respective therapeutic potential.

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