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ANTIMICROBIAL AND SYNERGISTIC EFFECT OF METHANOL EXTRACT OF *SASSUREA LAPPA* ON GRAM POSITIVE, GRAM NEGATIVE AND METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

The methanol extract from the medicinal plant *Saussurea lappa* (*S.lappa*) was tested for antimicrobial activity against gram positive organisms such as *Staphylococcus aureus* MTCC 87 and gram negative organisms like *Escherichia coli* MTTCC 41, *Proteus vulgaris* MTCC 426 (ATCC 6380), *Pseudomonas aeruginosa* 424(ATCC 25619) and *Candida albicans* MTTCC 183(ATCC 2091) and found to have significant inhibitory effect. The effect of this extract was tested against the resistant strain Methicillin resistant *Staphylococcus aureus* also. The synergistic effect in combination of the extract with antibiotics also tested and proved to have synergistic effect reducing the MIC value of the drug to two-fold. LC-MS analysis of the active fraction of this plant extract showed the presence of diaminobutyric acid, conferitin, myricetin and epigallocatechin.

KEYWORDS: *Candida albicans* MTTCC 183(ATCC 2091) and found to have significant inhibitory effect.

INTRODUCTION

Plants, especially medicinal plants have been explored continuously for therapeuticals. *Saussurea lappa* (*S.lappa*) is a medicinal plant used in ayurvedic preparations. This plant belongs to the family *Asteraceae*. The plant is also known as 'Ulpalam', 'Pakalm', 'Rucha', 'Pushkara'. It is a habitant of northern parts of India especially Himalayan region. Roots are used in medicinal preparations. The roots are bitter, sweet, thermogenic, aromatic, deodorant, digestive, stimulant, diuretic, disinfectant, antibacterial, antispasmodic, expectorant, skin stimulant and tonic (Abo et al. 1999) Most of the common pathogens develop drug resistance on continuous exposure to certain antibiotic. Multidrug resistant *Staphylococcus aureus* is one of the common among them. It is usually found in mucous membrane and human skin and extremely adaptable to antibiotics. Around 50% of infections were found to be due to MRSA in India. When some compounds are added to the common antibiotics used, it can enhance the inhibitory effect on microbial growth (Joung et al. 2010). This can reduce the quantity of antibiotic consumed. This is an important strategy in combating antibiotic resistance (Abo et al. 2000). Use of mixture of plant extracts, plant derived compounds along with antibiotic etc. is being studied at present to overcome the resistance developed by microbes.

MATERIALS AND METHOD

Dry plant material was purchased from Shimla, Himachal Pradesh, India. The plant material was identified by the Department of Botany, University of Calicut. Plant materials were washed, rinsed with distilled water and dried in air. About 10 to 20 gm of the dried material was taken and powdered using a warring blender. This powdered material was then taken for extraction with different solvents (Aqil and Ahammed 2007).

Extraction of the plant material by Bioassay- guided fractionation

In this method, plant extract was prepared using different solvents sequentially according to their increasing of order polarity. 10 gm of the powdered plant material (weight varies depending on plant material) was first mixed with the solvent of the least polarity (100ml) and the mixture was kept on an orbitory shaker at 200 rpm for 18 hrs. Supernatant was collected by filtering and evaporated to dryness. The residue obtained was weighed and stored. The residue obtained after the separation of supernatant was then mixed with solvent of next higher polarity and the process was repeated for different solvents, depending on its order of polarity. The different fractions thus collected on evaporation were dissolved in respective solvents at required concentration or in dimethylsulphoxide (DMSO) or in sterile water (if soluble). Concentration of the prepared extract was 10mg/ml (stock solution). The extract was stored under refrigeration (-20^0C) till its use for further analysis. The biological activities of the extract were tested after filter

sterilization (Liz et al. 2009).

Polarity chart

n-Hexane⟶Chloroform⟶Benzene⟶Diethylether⟶Ethyl acetate Acetone⟶Ethanol⟶Methanol⟶Water.

The percentage yield of each extract was noted and tabulated (Table 1).

Table 1: Percentage yield of plant extract prepared.

Name of the plant	Plant part used for the extraction	Solvent used	Dry mass (gm)	%yield	Extraction method used	Designated as
Saussurea	R	Methanol	3.1	14	Bioassay guided	SOL1
lappa		Ethanol	2.2	1.58	fractionation	SOL2
		Water	1.2	2.52		SOL3
		Chloroform	0.2	5.15		SOL4

Testing of antimicrobial activity

Antimicrobial activity was tested using Baur and Kirby Method (Bauer and Kirby (Bauer et al. 1966). This method was developed by Heatley et al. in 1944. Bauer and Kirby modified it and it is being used as a standard method for antimicrobial assay. Antimicrobial susceptibility tests are approved by different agencies like National committee for clinical laboratory standard (NCCLS).

Microbial cultures

The following standard microbial strains were used in the present study.

Escherichia coli MTTCC 41, *Proteus vulgaris* 426 (ATCC 6380), *Staphylococcus aureus* MTCC87, *Pseudomonas aeruginosa* MTCC424(ATCC 25619) (Bendini et al., 2006), *Candida albicans* MTTCC 183(ATCC 2091), Methicillin Resistant *Staphylococcus aureus* (ATCC 43300) (Canalas et al., 2008, (Mendoza et al. 1997) These cultures were purchased from Institute of Microbial Technology (IMTECH), Chandigargh. *Klebsilla pneumonia* and *Aspergillus niger* were collected from the Department of Life Sciences, University of Calicut.

Preparation of inocula

Cultures were revived in 2 ml of sterile nutrient broth and sub cultured to fresh medium and incubated at 36^0C. Growth curve were prepared by monitoring OD at 600nm. The culture at log phase (i.e optical density 0. 5 to 0.8 or 6 x 10^5 CFU/ml) were taken for further inoculation either in the nutrient broth or plating on the nutrient agar plate.

Determination of minimum inhibitory concentration (MIC)

(Chakraborty and Mitra. 2008), (Cichewicz and Thorpe 1996)**.**

MICs for each extract against each microbial strains were determined using nutrient broth.10 ml of the medium was taken in a 50 ml conical flask and sterilized. The extract (stock diluted to 10mg/ml in DMSO) was then added to 10 ml of medium in a series of conical flasks to get a concentration of 10000, 1000, 100, 10, 0 µg/ml initially (10 times dilution). Then double dilution was used to determine the range of inhibitory activity (MIC value) of each extract. A loop-full of fresh culture at log phase (1 x 10^8 CFU/ml, turbidity equal to that of 0.5 MacFarland solution) was added to each flask so as to have concentration of 5 x 10^5CFU/ml. It was then incubated at 36^0 for 18 to 24 hrs. The OD at 600nm was monitored at different concentrations of the drug in the culture media. One tube without microorganism was taken as blank. The least concentration at which the growth of the organism was completely inhibited was recorded as MIC of that extract, comparing the OD with that of the blank. It was then confirmed by inoculating to fresh agar plate (Dugler et al. 2005). The experiment was done in triplicate and average value of MIC was then taken. It was then statistically analyzed and $p< 0.05$ was considered as significant. The experiment was repeated for different strains and extracts. The values were compared for gram positive and negative bacteria (Table 2)

Table 2: MIC obtained for the extract against the microbes tested.

Name of the plant	Extract used for the nestimatio	*Escherichia coli µg/ml*	*Pseudomonas aeuroginosa* (µg/ml)	*Klebsella pneumonia* (µg/ml)	*Proteus vulgaris* (µg/ml)	*Staphylococcus aureus* (µg/ml)	*Candida albicans* (µg/ml)	*Aspergillus niger*(µg/ml)
Saussurea lappa	**SOL1**	**156.25**	**125 .0**	**117.4**	**187. 5**	**100 .5**	**250 .0**	**350 .0**
			150.2	**130.6**	**200.5**	**125**	**275**	**400.5**
Penicillin	**SOL3**	**175**						
Streptomycin		**10**						
		40						

Table 3: Effect of the extract against MRSA.

Name of the plant extract	Extract designated as	MIC value(µg/ml)
Saussurea lappa	SOL1	156.25
	SOL3	225.0

Synergistic effect

The method was done using a 96-well micro-titre plate. In this 200µl of the sterile nutrient broth was added to each well. In the first well of series 1, ie A1, antibiotic at a concentration of 100µg/ml was added. In the well B1 sample SOL1 at a concentration of 500µg/ml was added. In the well C1 both antibiotic and SOL1 were added. The concentration of the antibiotic and SOL1 were the respective MICs or above the MIC values. All the wells were then serially diluted to next well. ie 100µl from first well has transferred to second and 100µl from second to third and so on so that a double dilution had obtained. All wells were then inoculated with culture at log phase, mixed well, covered and incubated at 36^0C for 18hrs. The growth was observed and MICs were noted and Fractional inhibitory concentrations (FICs) were calculated (Kamicker et al. 2008).

Fractional inhibitory concentration (FIC index) was calculated using the formula

FIC index = MIC of drug in combination/ MIC of drug alone + MIC of extract in combination /MIC of extract alone (Table 4).

Table 4: FIC INDEX of different extracts in combination with antibiotics against Staphylococcus aureus and Escherichia coli.

Microorganism tested	Extract used in combination (µg/ml)	MIC of antibiotic alone (µg/ml)	MIC of antibiotic in combination (µg/ml)	MIC of extract alone (µg/ml)	MIC of extract in combination (µg/ml)	FIC★
Staphylococcus aureus	SOL1	20	4.5	150	35	0.458
Escherichia coli	SOL1	15	4.0	250	50	0.492
MRSA	SOL1	100	20	156.25	40	0.424

★ FIC INDEX < 0.5, synergy; 0.5-.75 partial synergy; 0.76 -1.0 additive; 1–4 indifference; and >4 antagonism.

LC-MS analysis

The following procedure was used for LC-MS analysis. Column used: C-18 Probe used: APCI (Atmospheric pressure chemical ionisation) Mode used: Positive (Which gives M+1 value Negative (Which shows M-1 value).

RESULTS AND DISCUSSION

Methanol extract of *Saussurea lappa* showed synergistic effect on all the microbes tested with FIC index < 0.5 including MRSA. Different kinds of diseases have been treated with plant remedies since ancient times and it is still continuing. It showed synergistic effect on *Escherichia coli* and *Staphylococcus aureus* along with gentamicin and penicillin. SOL1 extract also showed a MIC of 100µg/ml and 117.4µg/ml against *Staphylococcus aureus* and *Kelbsiella pneumonia* respectively. The antimicrobial effect of this extract was irrespective of gram positive and gram negative organisms. It showed inhibitory effect against *Candida albicans* and *Aspergillus niger* also. The continuous use of antibiotics is the common cause to develop drug resistance against a particular antibiotic (Mazumdar et al. 2005). The development of drug resistance by microbes and other emerging diseases increases the demand of screening medicinal plants to extract antimicrobial agents (Dugler et al. 2005). Drug interaction between a known antibiotic and a bioactive plant extract or component is a novel concept and can be beneficial or deleterious. The enhancing effect on antimicrobial activity of plant extracts were tested while it is used along with standard antibiotics.

The anti-inflammatory effect of the sesquiterpene compound, cynaropicrin, isolated from this plant was reported earlier (Chao-Mei et al. 2007). This plant has reported to be used in several indigenous systems of medicines for the treatment of diseases such as asthma, ulcer and stomach problems (Adhami et al. 2007). (Ahmed and Arina, 2001) reported the antimicrobial activity of *Saussurea lappa (*Ahemed and Arina 2001). Adwan et al. 2009 reported the synergistic effect of various plant extracts (*P. guajava, R. officianali*s) with known antibiotics (Penicillin, gentamicin) against different microbes like MRSA and MSSA (Adwan et al., 2008). Berberin, an alkaloid derived from different plant sources has shown synergistic effect when used with norfloxacine, with an increase in its antimicrobial activity by 16-fold against MRSA (Laura et al. 2004). LC-MS analysis of the active fraction of this plant extract showed the presence of diaminobutyric acid, conferitin, myricetin and epigallocatechin[Figures 1, 2, 3, 4 and 5]. The compounds like diaminobutyric acid, conferitin and myricetin were reported to have different biological activities. Diaminoburytic acid is an antimicrobial agent with inhibitory effect on the enzymes of cell wall synthesis. The results indicate that *Saussurea lappa* is source of natural remedy against infectious diseases.

Compounds like diaminobutyric acid (DABA), myricetin, confertin, epigallocatechin were detected in

the methanol extract of *Saussurea lappa*. Diaminobutyric acid is an antimicrobial peptide with inhibitory effect on cell wall synthesis of microorganisms (Igor et al. 2006). As per the report of Barlodi et al. 2004, DABA has enzyme inhibitory activity also (PASS). The high level antimicrobial activity of *S. lappa* observed against most of the organisms tested here may possibly be due to this compound. The antimicrobial activity of DABA against resistant strains has also been reported by Barlodi et al. 2004 (PASS). Myricetin was another compound detected in the extract of *Saussurea lappa*. Annrita et al. (2005) reported that epigallocatechin gallate (EGCg), the main polyphenol component of green tea, has several antibacterial properties including the inhibition of biofilm formation by microorganisms. Saussuria lappa (Thara et al 2012).

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