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IN-VITRO ANTIMICROBIAL SUSCEPTIBILITY TESTING OF LEAVES METHANOL EXTRACT AND LATEX OF *EUPHORBIA HELIOSCOPIA* USING AGAR WELL DIFFUSION AND BROTH DILUTION METHODS

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ABSTRACT

Antimicrobial susceptibility testing is necessary to claim antibacterial activity of new chemicals. *Euphorbia helioscopia* has great medicinal importance because of its traditional uses and number of pharmacological activities. Various factors affect chemical composition of the plant which may ultimately modify its uses and activities. Antimicrobial susceptibility of *Euphorbia helioscopia* was tested by adopting CLSI, 2006; antimicrobial susceptibility testing guidelines. Agar well diffusion method and broth macrodilution method were used to validate antibacterial activity of standardized methanol extract and latex of *E. helioscopia*. Latex showed no antibacterial activity. *E. coli* (AI: 0.29 and MIC: 62.5 mg/mL), *S. enterica (*AI: 0.32 and MIC: 250 mg/mL), *Staph. aureus* (AI: 0.3and MIC; 250 mg/mL) showed susceptibility to leaves methanol extract. The extract was found bactericidal against *S. enterica* as MIC and MBC were the same i.e. 250 mg/mL whereas, the extract showed relatively dose dependent activity against *E. coli* i.e. bacteriostatic at 62.5 and 125 mg/mL and bactericidal at 250 mg/mL. However, extract showed bacteriostatic activity against *Staph. aureus* upto 250 mg/mL (highest dose employed).

Key words: Antimicrobial susceptibility, *Euphorbia helioscopia*, MIC, MBC.

INTRODUCTION

Bacterial resistance to currently available antibiotics has developed due to misuse of antibiotics which is an alarming situation for health care system all over the world (Fu *et al*., 2007; Abbas *et al*., 2011a). To overcome this problem, scientists are focusing on discovering effective and safe alternative sources to combat this emerged bacterial resistance (Abbas *et al*., 2010, Singh *et al*., 2010; Abbas *et al*., 2011b, Oskay, 2011; Abbas *et al*., 2011c, 2012a, 2012b; Zaman *et al*., 2012). Resistant bacterial strains have been found to show susceptibility to antimicrobials of plant origin (Tajkarimi *et al*., 2010). Since long time, the plant based products have been used to treat various ailments and now they have become part of traditional and allopathic medicine (Dubey *et al*., 2011).

Euphorbia helioscopia is an annual weed and belongs to medicinally important family *"Euphorbiaceae"*. Traditionally its leaves and stem are used as febrifuge and vermifuge, oil squeezed from its seeds has purgative action, seeds in combination with roasted pepper are effective in cholera and roots possess anthelminthic activity (Kinghorn *et al*., 1975; Webster, 1994; Nadkarni, 2002; Panda, 2004).The medicinal worth of the plant turned the research focus of number of scientists to probe into its pharmacological activities. Moreover, the plant is claimed to possess antibacterial, antifungal, antiviral, vasodepressor, phytotoxicity, antioxidant, anticancer, anti-asthmatic and molluscicidal activities (Al-Zanbagi, *et al*., 2000; Park *et al.*, 2001; Barla *et al*., 2006; Ramezani *et al*., 2008; Uzair *et al*., 2009; Nikolova *et al*., 2011; Khan *et al*., 2011; Maoulainine *et al.*, 2012; Wang *et al.*, 2012).

Pharmacological activities of the plant are due to its phytochemical constituents. *E. helioscopia* is reported to contain secondary metabolites like triterpenoids (Nazir *et al.*, 1998), diterpenoids (Yamamura *et al.*, 1981; Shizuri *et al.*, 1983; Shizuri *et al.*, 1984; Kosemura *et al.*, 1985; Yamamura *et al*., 1989), flavonoids (Kawase and Kutani, 1968; Chen *et al.*, 1979), tannins and lipids (Kosemura *et al.*, 1985). Numerous factors such as time of plant collection, place of collection, growing environment etc modify the chemical composition of the plant which ultimately affects its pharmacological actions. Thus, standardization of plant extracts is obligatory prior to proceeding for pharmacological analysis to get consistent and reproducible results. *E. helioscopia* extracts and latex have been standardized in our earlier work (Saleem *et al*., 2014 b).

Although antibacterial activity of *E. helioscopia* has been investigated by Uzair *et al.*,(2009) using agar well diffusion method and Bashir *et al.*,(2013) by employing disc diffusion method but only zones of inhibition were measured.

Broth dilution and agar diffusion methods are recommended for antimicrobial susceptibility testing by Clinical and Laboratory Standards Institute (CLSI; 2006).The purpose of present study was to comply with CLSI recommendations for investigating antimicrobial susceptibility of *E. helioscopia* via agar well diffusion method, for determining antibacterial activity and activity index (AI) against two gram negative and two gram positive bacteria, and broth macrodilution method, to estimate quantitatively its minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC), and bacteriostatic concentration.

MATERIALS AND METHODS

Collection of plant material: The plant was collected from the suburbs of city of Lahore, Pakistan. After identification and authentication of plant by a Taxonomist of Botany Department, Govt. College University, Lahore, Pakistan, a voucher specimen (1501) was deposited to the Herbarium. Leaves and stem were separated and dried under shade, then ground to fine powder and stored in airtight containers till extraction.

Preparation of extracts: Extract was prepared by two methods a) cold extraction (maceration) using water and methanol as solvents and b) hot sequential extraction with soxhlet using solvents in increasing order of polarity (petroleum ether, chloroform, and methanol). Solvent was evaporated on rotary evaporator and semisolid extracts were collected in the pre-weighed beakers. Leaves methanol extract and latex were selected in this study based on their *in-vitro* antioxidant activity in our earlier investigation (Saleem *et al.*, 2014 a).

Standardization of extracts: After preparation, all the extracts were subjected to standardization procedure using HPLC-RP, UV and FTIR finger prints presented in our previous work (Saleem *et al.*, 2014 a, b).

Preparation of sample dilutions: Dilutions were prepared in normal saline.

Test microorganisms: *Bacillus subtilis* [*B. subtilis*] (ATCC No. 6633), *Staphylococcus aureus* [*Staph. aureus*] (ATCC No. 25923), *Escherichia coli* [*E. coli*] (ATCC No. 25922), *Salmonella enterica* [*S. enterica*] (ATCC No. 10708) were procured from Quality Operations Laboratories (QOL), University of Veterinary and Animal Sciences, Lahore-Pakistan.

Preparation of bacterial cultures: Bacteria were grown in nutrient agar broth for 24 hours at 37 ^{o}C. Optical density (OD) of the cultures was measured at 600 nm. The cultures were diluted with media to bring OD value to 0.257 that is equivalent to turbidity of 0.5 McFarland units [10^6CFU/mL] (NCCLS, 1997).

Well diffusion method: Activity of extract was tested individually with well diffusion method (Srinivasan *et al.*, 2001, Sen and Batra, 2012). Sterilized nutrient agar media (20 mL) was poured in the petri-plates near the flame. After solidification of media, plates were streaked with bacterial culture either by swabbing, using sterile cotton swab or pouring 0.1 mL of bacterial culture and uniformly spreading with pasteur pipette. Wells of 5 mm diameter were made in each of plates with sterile cork borer (3/16´´). Each well was sealed with drop of molten media using sterile syringe. Fifty microliter of each sample was added into each well and allowed to diffuse at room temperature for 1 hour then incubated at 37 ^{o}C for 18-24 hours.The zone of inhibition (mm) was measured and activity index (AI) was calculated by diving inhibition zone of tested sample with inhibition zone of standard.The experiment was performed in triplicate.

Determination of MIC by broth macrodilution method: Serial two fold dilutions (250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, 1.95, 0.976, 0.488 mg/mL) were prepared in Nutrient Agar Broth in sterile test tubes and their OD values were measured at 600 nm. Then these tubes were inoculated with 0.1 mL of bacterial suspension and incubated at 37 ^{o}C for 18-24 hours. OD value of each test tube inoculum was measured at 600 nm on spectrophotometer. These OD values were subtracted from those obtained prior to incubation. This subtraction is important to exclude the interference in absorbance due to color of the extract. Inoculated test tubes with zero or near to zero OD value represented MIC of extract (Jorgensen *et al.*, 1999; Devienne and Raddi, 2002).

Determination of MBC and minimum bacteriostatic concentration: To determine MBC, tubes showing MIC were sub-cultured on freshly prepared nutrient agar plates. Incubated at 37 ^{o}C for 18-24 hours and growth of relevant bacteria was observed. A decrease in colony count by 99.9 % from original bacterial inoculum was taken as MBC. Plates showing bacterial growth represented minimum bacteriostatic concentration. (IIse *et al*., 1997).

RESULTS

Physical properties of pulverized leaves and extract of *E. helioscopia* were studied.

The color of leaves powder was light green, odor was pungent, extract was dark green in color with semisolid consistency. Methanolic extract was soluble in water, DMSO and all organic solvents (Fig. 1).

Well diffusion method: The antibacterial activity of latex and extract was measured in terms of zone of inhibition against *E. coli, S. enterica, Staph. aureus* and *B. subtilis* and compared with standard furazolidone

50 µg/disc as presented in Table 1. The results showed no antibacterial activity of latex against all bacteria while extract showed zones of inhibition (mm) 7 ± 0.54, 7 ± 0.56, 7.5 ± 0.52 and 0.00 against *E. coli*, *S. enterica*, *Staph. aureus* and *B. subtilis.* AI of extract in descending order was as follows: 0.32 (*S. enterica*) > 0.30 (*Staph. aureus)* > 0.29 (*E. coli)* > 0.00 (*B. subtilis).* Representative agar plates are given in Fig. 2.

Determination of MIC, MBC and minimum bacteriostatic concentration: Minimum inhibitory concentration (MIC) is defined as lowest concentrations of drug that can inhibit the visible growth. This was determined by recording OD on spectrophotometer. Minimum bactericidal concentration (MBC) and minimum bacteriostatic concentration were determined by subculturing the tubes representing MIC on agar plates. The plate showing growth of microorganism expresses the minimum bacteriostatic concentration while MBC is the lowest concentration of drug that can kill the 99.999% of original bacterial inoculum on culture plates (Henry, 2006). Extract showed four fold higher MIC against *S. enterica* and *Staph. aureus* than *E. coli* according to broth macrodilution results. MIC against *E. coli* was 62.5 mg/mL and 250 mg/mL against *S. enterica, Staph. aureus* (Table 2 and Fig. 3).The extract showed bacteriostatic activity against *Staph. aureus* at 250 mg/mL, and *E. coli* at 62.5 and 125 mg/mL. MBC of extract was 250 mg/mL against *E. coli* and *S. enterica.*

DISCUSSION

In the present study well diffusion method was adopted for determination of antibacterial activity of extracts against two Gram positive and two Gram negative pathogenic bacteria and broth macrodilution method was used for estimation of MIC against susceptible bacteria.

Disc diffusion method, agar dilution method and broth microdilution method can also be used for screening of antibacterial activity of natural compounds of hydrophilic in nature (Janseen *et al.,* 1987). The most accurate screening method for essential oils is broth dilution method with prior emulsification of oils with 0.02 % Tween 80 (Hood *et al.,* 2003).

Well diffusion method is more sensitive than disc diffusion method (Cleidson *et al.,* 2007). TLC bioautography is the latest technique, employing combinatorial chemistry and high throughput screening, used for preliminary screening of biological activities like antimicrobial, antioxidant and enzyme inhibition of natural products (Cheng and Wu, 2013).

The extract showed activity against *E. coli*, *S. enterica*, *S. aureus* while *B. subtilis* was resistant. According to Uzair *et al.,* data, *E. coli* and *S. aureus* showed resistance while *S. enterica* and *B. subtilis* were susceptible to methanolic extract of aerial parts of *E. helioscopia* (Uzair*et al.,* 2009). In another study, methanolic extract of aerial parts of *E. helioscopia* showed antibacterial activity against *E. coli* and *S. aureus* (Bashir *et al.,* 2013). Our study is consistent with Bashir *et al.,* study while contrary to Uzair *et al.,* results.

Antibacterial activity has inverse relation with AI.The extract showed greater AI (0.29) against *E. coli* as compared to *Staph. aureus* (AI; 0.30) , *S. enterica* (AI; 0.32) and *B. subtilis* (AI; 0.00).

MIC of extract in ascending order was as follows: 62.5 mg/mL (*E. coli*) >250 mg/mL (*S. aureus* and *S. enterica*). It indicated high potency of extract against *E. coli* as compared to *S. aureus and S. enterica*. Extract showed bacteriostatic activity at 62.5 and 125 mg/mL and MBC at 250 mg/mL against *E. coli.* The action of extract on *S. enterica* was bacterical at 250 mg/mL, on the other hand the same concentration (250 mg/mL) of extract exhibited bacteriostatic activity against *S. aureus.* One drug could be bacteriostatic at low concentration and bacterical at high concentration, so this is not an absolute term. Although, *E. coli, S. enterica, and S. aureus* showed susceptibility to extract but inhibition zones were significantly less as compared to standard antibiotic disc. *E. coli* was the most susceptible bacteria with lowest AI (0.29) and MIC value (62.5 mg/mL) among all tested microorganisms.

Table 1. Antibacterial activity of latex and leaves methanol extract of *E. helioscopia* against pathogenic bacteria

Groups	*E. coli*		*S. enterica*		*S. aureus*		*B. subtilis*	
	ZI	**AI**	**ZI**	**AI**	**ZI**	**AI**	**ZI**	**AI**
Standard	24		22		25		25	
Extract	7 ± 0.54	0.29	7 ± 0.56	0.32	7.5 ± 0.52	0.30	0.00	0.00
Latex	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

ZI = Zone of inhibition measured in mm, AI = Activity index, Standard = Furazolidone 50 µg/disc.

Table 2. Optical density values of leaves methanol extract at 600 nm to determine MIC.

Sr. No.	Concentrations (mg/mL)	*E. coli*	*S. enterica*	*S. aureus*
1	Control	0.629 ± 0.00	0.73 ± 0.01	0.26 ± 0.01
2	0.488	0.69 ± 0.01	0.88 ± 0.14*	0.32 ± 0.02*
3	0.976	0.77 ± 0.18*	0254 ± 0.01*	0.27 ± 0.01
4	1.95	0.70 ± 0.18*	0.50 ± 0.17*	0.21 ± 0.01*
5	3.9	0.62 ± 0.12	0.45 ± 0.01*	0.19 ± 0.01*
6	7.81	0.32 ± 0.00	0.24 ± 0.00*	0.18 ± 0.00*
7	15.62	0.34 ± 0.01*	0.15 ± 0.04*	0.13 ± 0.00*
8	31.25	0.28 ± 0.00*	0.11 ± 0.00*	0.12 ± 0.00*
9	62.5	0.00*	0.07 ± 0.00*	0.12 ± 0.00*
10	125	0.00*	0.04 ± 0.00*	0.09 ± 0.00*
11	250	0.00*	0.01 ± 0.01*	0.01 ± 0.01*

* P is < 0.05 when compared with control value

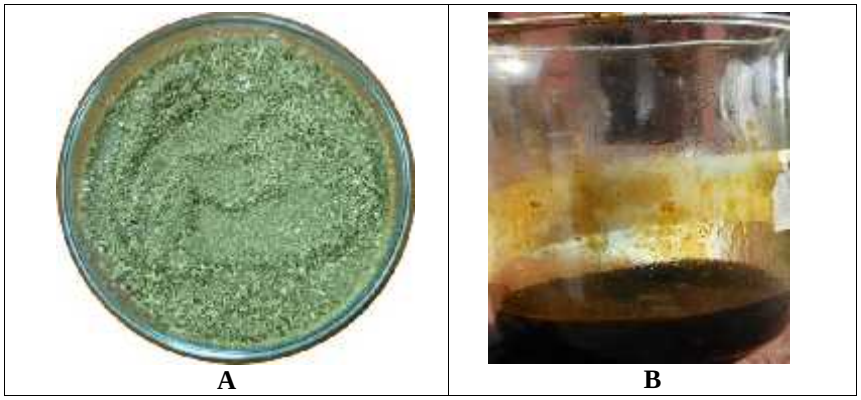

Fig. 1.Physical properties of *E. helioscopia.*
A = Pulverized leaves B = Methanol extract

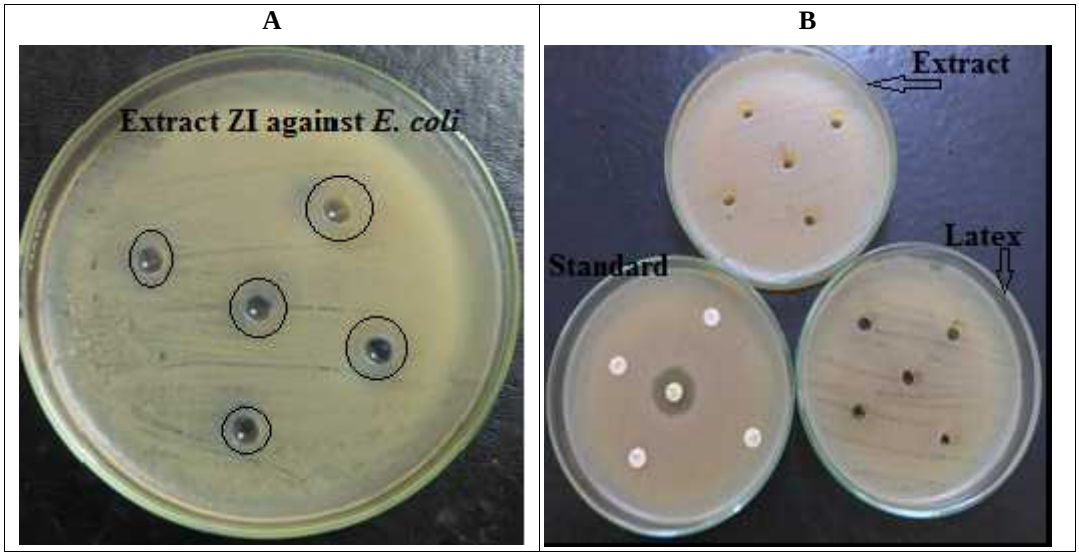

Fig. 2. Agar plates containing pathogenic bacteria incubated with latex and leaves methanol extract of *E. helioscopia.*
A = Representing antibacterial activity against *E. coli* incubated with extract, B = Representing standard plate and without antibacterial activity plates (*B. subtilis* incubated with extract and latex)

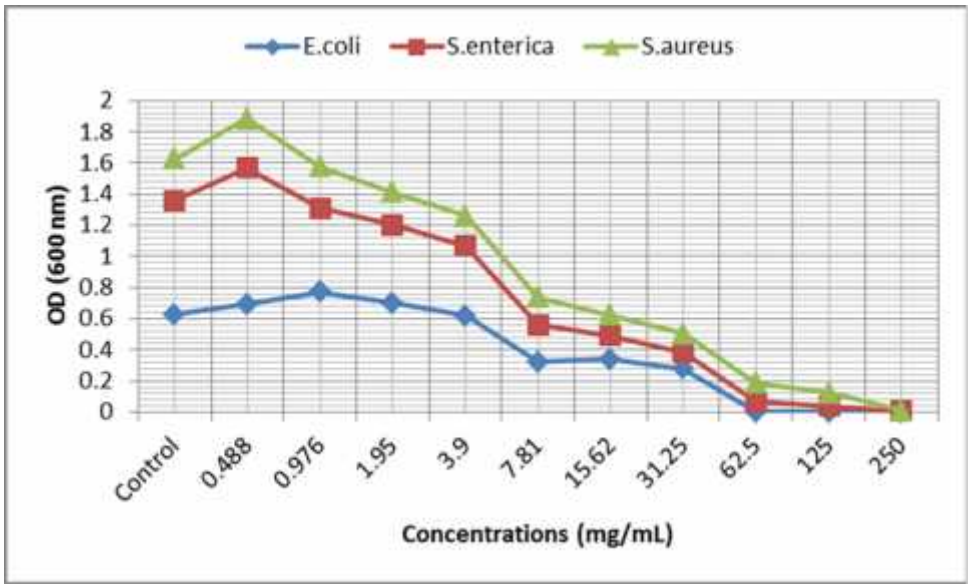

Fig. 3. Optical densities (OD) of different concentrations of extract at 600 nm against *Escherichia coli (E. coli), Salmonella enterica (S. enterica) and Staphylococcus aureus* (*S. aureus*).

Conclusion: Latex was devoid of antibacterial activity against the selected bacteria. *E. coli, S. enterica, and S. aureus* appeared susceptible to leaves methanol extract while *B. subtilis* showed resistance. *E. coli* was more susceptible among all.

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