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Phytochemical, antimicrobial and time-kill kinetics potentials of *Euphorbia nivulia* Buch.-Ham.: A Cholistan desert medicinal plant

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Abstract: *Euphorbia nivulia* a locally occurring plant species possesses antiseptic, analgesic and anti-inflammatory properties and is ethnopharmacologically used in various ailments like skin, ear disorders, boils, and worm infestation. Preliminary phytochemical screening showed presence of flavonoids, polyphenolics, glycosides, alkaloids, tannins and triterpenoids in (70% aqueous-ethanolic) *Euphorbia nivulia* crude extract (*En* cr) and its four fractions, i.e., hexane fraction (*En* hex), butanol fraction (*En* bt), chloroform fraction (*En* ch), and aqueous fraction (*En* aq). In current study, Agar well diffusion and time-kill kinetic assays were performed for antimicrobial activity. 300 mg/ml concentration showed maximum inhibitory zone. Highest zone of inhibition (15.5mm) was demonstrated by *En* ch fraction against *Proteus mirabilis*. *Staphyllococcus aureus* was the most sensitive bacteria against whom all fractions except *En* aq fraction were active. Maximum MIC (15.3 mg/ml) was shown by *En* ch fraction against *Proteus mirabilis*. Similarly, *En* ch fraction showed (15.1 mg/ml) remarkable MIC against *Candida albicans.* Significant higher antibacterial and antifungal activity was revealed in high concentration. Time-kill kinetics studies revealed bacteriostatic action. Noteworthy antimicrobial activity may be due to bioactive compounds of extract which may be a potential antibacterial and antifungal agent.

Keywords: *Euphorbia nivulia*, bio-active compounds, time-kill kinetics, antimicrobial activity.

INTRODUCTION

Major source of severe bacterial infections are different species of bacteria belonging to two main classes, i.e. Gram negative and Gram positive, like *Staphylococcus, Bacillus, Pseudomonas* and *Salmonella*, due to which most human beings died worldwide (Zhang *et al.*, 2016; Ahameethunisa and Hoper, 2010). After discovery of important groups of antibiotics like cephalosporins, aminoglycosides, macrolides and tetracyclines during the "golden era" in the 1960s, nowadays efficacy of these chemotherapeutics is decreasing because of increased resistance of microbes (Mayers *et al*., 2017). Treatment failures associated with multidrug-resistant bacteria has become a global concern these days (Guschi *et al*., 2015; Martin *et al*., 2015). Besides resistance, these synthetic antibiotics are costly (Li Chen *et al*., 2020) for the poor patients belonging to developing countries (Walsh and Amyes, 2004; Alder, 2005). Moreover, these synthetic products treat only one third of the infectious diseases because of resistant pathogens (Sen and Batra, 2012); and antibiotics may have serious side effects like hyper-sensitivity/allergic reactions and immune suppression (Ahmed Fouda *et al*., 2020; Freire-Moran *et al*., 2011). Due to this reason, discovery of new antibiotics is exclusively necessary. And exploration for bioactive compounds may be very helpful in this discovery in treating resistant pathogenic microbes. Today natural products are considered one of the major sources of new drug molecules (Newman and Cragg 2020); they may also serve as a model for new antibacterial drugs. Natural products may be obtained from such natural sources as bacteria, fungi, and plants (Chaudhary and Singh, 2016; Alves, 2012). Among these natural products microbes and plants constitute the major source of new drug molecules including antibiotics (Balouiri *et al.*, 2016). Natural products/compounds possess complex and diversified chemical structure. Most recently, plant and microbial extracts have become focus of research for investigation of secondary metabolites like alkaloids, glycosides, tannins, essential oils, etc., for synthesis of new potent antimicrobial molecule(s) (Mabona *et al.*, 2013; Nazzaro *et al.*, 2013; Runyoro *et al.*, 2006). In the past bioactive

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molecules of plant origin had been a unique source for majority of drugs (Bibi *et al*., 2010). Quinine from Cinchona bark and berberine from Berberis are the examples of antibiotics obtained from plants which are highly effective against certain microbes (Maridass 2008). Antibiotics of plants origin are more effective and environment friendly and less toxic (Munuswamy *et al.*, 2013; Koehn and Carter, 2005; Walsh 2003). That is the reason that people from all over the world, particularly Asians like Pakistanis use herbal medicines in primary health care system to treat infections like skin diseases, dysentery, malaria, etc. (Munuswamy *et al.*, 2013).

Euphorbia nivulia Buch.- Ham., one of the members of *Euphorbiaceae* family is of interest to natural products researchers due to its diverse habitats and biological activities (Basak *et al.*, 2009). There is limited literature on the biological activities of *Euphorbia nivulia* (Badgujar and Mahajan, 2011). The species is widely distributed in tropical Asia, Africa, Australia and Europe. The plant is indigenous to India, Myanmar and Pakistan; in Northern and central India, it is planted as hedge plant often in dry areas and wild in arid soils (Radcliffe-Smith, 2011). Juices of leaves, bark, root, stem or latex is used traditionally for medicinal purpose. Boro community of Assam uses leaf juice in pains and boils (Basumatary *et al.*, 2004); and fleshy stem is recommended in cough (Mahajan and Badgujr, 2008). Plant latex possesses purgative properties and is used against worms (Pullaiah, 2006). Latex of leaf and root is used in skin and ear disorders, worm infection, swelling and retention of urine (Britto *et al.*, 2010). Stem is applied to bone fractures and latex possesses antiseptic properties (Kumar and Chaturvedi, 2010). Chemically, it contains tetracyclic triterpenes, diterpenes, phenolic compounds, alkaloids, cynogenic glycosides, terpenes and tannins (Badgujar and Mahajan, 2010). Pakistan is a country with diverse biological resources and a vast diversity of naturally growing plants; but only 600 to 700 species are used medicinally (Ali and Qaiser, 1986). Therefore, there is need for exploration of locally occurring species. The presence of various valuable constituents in plants has urged scientists to screen these plants for discovery of new therapeutic agents. *Euphorbia nivulia* is enrich in phytoconstituents including flavonoids and polyphenolics and a number of other constituents that may be screened for antimicrobial potential, because there are many members of Euphorbiaceae family belonging to the genus *Euphorbia* that have shown antimicrobial activities against various bacteria and fungi. Various species of *Euphorbia* possess antiseptic, disinfectant and emollient properties and are used in different skin ailments like dermatitis, psoriasis, skin rashes, itching, acne, carbuncles, boils and pustules. Antibacterial activity of *E. fusiformis* was studied against various pathogenic strains of Gram positive (*B. subtilis* and *S. aureus*) and Gram negative bacteria (*E. coli*, *K. pneumoniae*, *P. vulgaris*, *P. aeruginosa*, *S. typhii* A and *S. typhii* B). Different extracts significantly differed in their antibacterial properties with methanolic extract being very effective (Natarajan *et al.*, 2005). *E. hirta* (ethanolic extract exhibited a broad spectrum antimicrobial activity against *E. coli*, *P. Vulgaris*, *P. aeruginosa*, and *S. aureus* (*Sudhakar et al.*, *2006).* Diterpene, triterpenes and ellagic acid isolated from *Euphorbia sessiliflora* Roxb. showed moderate to strong antibacterial activity against *B. cereus*, *B. subtilis*, *M. flavas*, *M. catarrhalis*, *N. sicca*, and *C. albicans* at concentration 12.5μg/ mL (Sutthivaiyakit *et al.*, 2000). A synergistic antifungal activity against *Candida* spp. and *Cryptococcus neoformans* was displayed by cerebrosides isolated from *E. peplis* (Cateni *et al.*, 2003). No scientific validation on *E. nivulia,* a Pakistani species belonging to the Cholistan Desert of Bahawalpur region as antibacterial/antifungal agent has been done until now. So, the current study was aimed to evaluate its antibacterial/antifungal potentials against wide variety of bacteria and fungi common in the local community and the important causative microbes. Although favourable ethnopharmacological properties of *En* have been shown, but its protective potentials against microbes comparing with standard antibiotics drug have not been previously explored. In our study, the impact of increasing doses of *En* against various microbes was investigated

MATERIALS AND METHODS

Aerial parts of fresh, well grown *Euphorbia nivulia* plant were collected during the months of March and April 2015 from Hasilpur Road and adjoining areas of Bahawalpur region, Pakistan and authenticated by taxonomist, Ghulam Sarwar, Department of Botany, The Islamia University of Bahawalpur. Voucher specimen (EN-AP-05-12-041) was deposited in the herbarium of Pharmacology Research Lab, Department of Pharmacy, The Islamia University of Bahawalpur, Pakistan.

All chemicals and solvents/reagents used were of analytical grade and were purchased from Merck, Sigma-Aldrich and B.D.H. Pure cultures of the microorganisms were obtained from Microbiology Lab, Department of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur, Dr Panjwani Center for Molecular Medicine and Drug Research and H.E.J research institute of chemistry, University of Karachi, Karachi, Microbiology Lab, Department of Microbiology, FUUAST, Karachi, *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella typhi* (ATCC No. 19430), *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (AB 188), *Bacillus staphyllococcus Staphylococcus epidermidis*, *Klebsiella pneumonia (*ATCC 14), *Penicillium species*, *Aspergillus flavu1s, Aspergillus niger*, *Candida albicans* (ATCC 0383), *Proteus mirabilis,Corynebacterium xerosis Salmonella typhi para A, Shigella dysenteriae* (ATCC 72) *Bacillus cereus.* The stock cultures were maintained at 4°C.

Experimental

Preliminary phytochemical screening

Preliminary qualitative phytochemical screening of the *En* crude extract as well as its four fractions, to identify the phytoconstituents like alkaloids, glycosides, flavonoids, tannins, saponins and phenols, etc., was performed using conventional standard procedures (Younus *et al.*, 2019; WHO, 1998; Brain and Turner, 1975).

Extraction

10 kg dried powdered plant material (aerial parts) was macerated in 70% aqueous ethanol at room temperature for 15 days using cold maceration method with occasional stirring. Each time, 12 L of aqueous ethanol was used to soak the powder. The macerated mixture was filtered each three times with muslin cloth separately and then further filtration was done by Whatman Grade-1 filter paper. The filtrate was then evaporated under reduced pressure (-760mm Hg) and controlled temperature (at 45-50^{o}C) on the rotary evaporator. A thick and semisolid, dark brown gummy mass was obtained which was then placed in oven. The dried material was weighed, labeled and then stored at 4^{0}C in refrigerator in air tight container. The percentage yield was calculated. The condensed extract was used for further experimentation. Moreover, successive solvent extraction was used as previously described by Tiwari *et al.* (2011). Extracts were dried, weighed, labelled and then stored at 4°C in refrigerator in air tight containers. Aqueous ethanolic crude extract and four fractions so obtained were named as follows:

En= 70% aqueous ethanolic crude extract; *EnH*= Hexane fraction:

En Ch= Chloroform fraction; *En* Bt= Butanol fraction; *En*Aq= Aqueous fraction

Antimicrobial potential

Agar well diffusion method is widely used method to evaluate antibacterial potential of the extracts (Bibi *et al.*, 2010; NCCLS, 1993). The Nutrient agar (oxoid) plate surface was inoculated by spreading a volume of the microbial inoculum over the entire agar surface. For determination of antibacterial activity, bacterial cultures were adjusted to 0.5 McFarland turbidity standard and inoculated onto plates (diameter: 15 cm) at 37^{o}C for 18 h. For the determination of antimycotic activity, all the fungal isolates and *Candida albicans* were first adjusted to the concentration of 106 cfu/ml, and then were inoculated onto Sabroud Dextrose at 30°C for 48 h. Samples of extract, one positive control (Cefotaxime), and one negative control (DMSO) were applied to each petri plate; DMSO was used as a negative control, Cefotaxime) (500 mg) as a positive control for the bacteria and nystatin (100mg) as a positive control for the fungi. Antimicrobial activity was recorded by measuring the zones of inhibition around the wells. All experiments were performed with five replicates. % age growth inhibition was calculated by:

$$\text{Inhibition (\%)} = \left(\frac{\text{TS} - \text{SC}}{\text{PC}}\right) \times 100$$

where TS: test sample, SC: solvent control and PC : positive control (Cefotaxime for bacteria and nystatin for fungi as standard).

Minimum Inhibitory Concentration (MIC) Determination

Method of Omura *et al.* (1993) with little modification was performed to determine MIC of extracts. The concentration which showed the smallest zones of inhibition was regarded as the MIC value. All experiments were performed in three replicates.

Time-kill kinetics assay/test

Time-kill kinetics of *En* crude extract as well as its four fractions was carried out following the procedure described by Tsuji (Tsuji *et al.*, 2008). Concentrations equal to MIC, twice the MIC, and four times the MIC of the extracts were prepared. Concentrations used were determined by MIC and % killing by using the formula:

Log reduction (L) = Log10 [Inoculum cfu/mL] – Log10[After treatment cfu/mL]

% Reduction = 1- 10-L × 100

STATISTICAL ANALYSIS

Graph Pad Prism Version 7.0 for Windows (Graph Pad Software Inc., San Diego, CA, USA) was used to analyze data obtained from study by using one way ANOVA followed by Dunnett's post hoc test.

RESULTS

Phytochemical screening

Results for presence of various phytochemicals like alkaloids, glycosides, flavonoids, phenols, tannins, saponins and carbohydrates in crude as well as various fractions are shown in table 1.

Antimicrobial potential of crude drug and extracts

Antimicrobial activity of the *En* crude extract as well as various fractions are shown in table 2. Dose used for all samples was 100mg/ml.

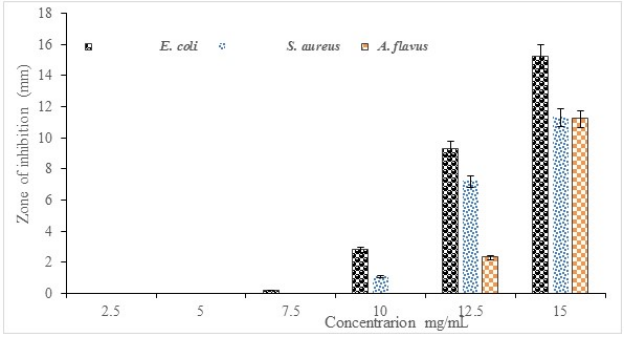

Fig. 1: MIC of *En* cr against *E. coli, S. aureus, A. flavus*

Agar well diffusion method

Maximum antibacterial activity was observed against Gram-negative bacteria. Highest zone of inhibition i.e., 15.5 mm was demonstrated by *En* ch fraction against *Proteus mirabilis* followed by zone of inhibition shown by *En* cr against *Escherichia coli*, i.e., 15.4 mm. Only *En* bt fraction was active against *Salmonella typhi*. Gram-positive bacteria also showed susceptibility against *En* cr and its fractions. *Staphyllococcus aureus (Gram-positive bacteria)* was the most sensitive species against whom all fractions except *En* aq showed remarkable antibacterial activity. A similarity in antifungal activity was observed for *En* crude extract and its fractions. Highest zone of inhibition 14.3 mm was noted by *En* ch fraction against *Candida albicans* followed by zone of inhibition exhibited by *En* bt fraction against the same species, i.e., 14.1 mm. *Aspergillus flavus* was found to share similarity in sensitivity along with *Candida albicans* against whom *En* cr and *En* hex fraction showed activity (table 2). Zones of growth inhibition around Petri plates were measured after 18 to 24 hr of incubation at 37°C for bacteria and 48 to 96 hr for fungi at 28°C. Sensitivity of microorganisms to extracts was determined by measuring size of inhibitory zones (including disk diameter) on agar surface around plates, and values <8 mm were considered as non-active.

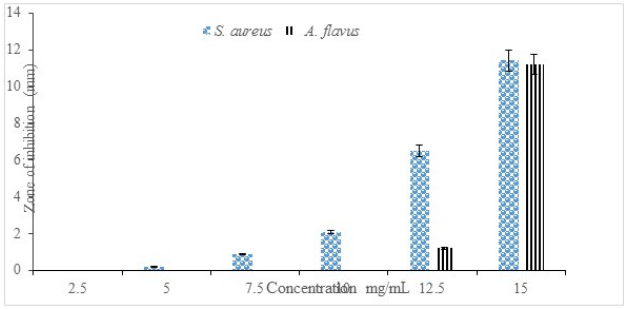

Fig. 2: MIC of *En* hex fraction against *S. aureus, A. flavus*

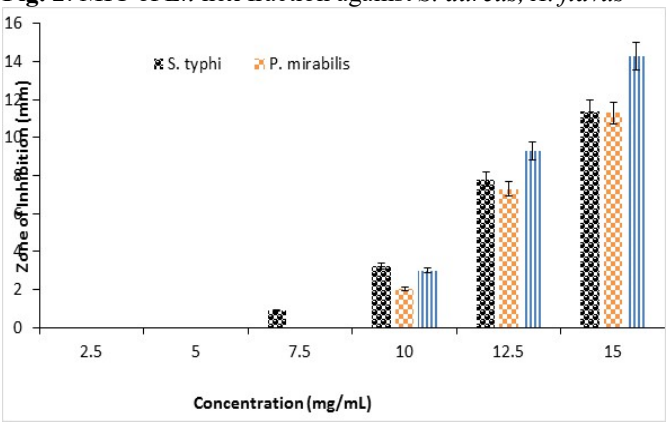

Fig. 3: MIC of *En* bt fraction against *S. typhi, P. mirabilis, C. albicans*

MIC of En crude extract and fractions

Results of MIC are shown in Table 3 and figs. 1-5. The results show that the highest MIC (15.3 mg/ml) was shown by *En* ch fraction against *Proteus mirabilis*, next to this was 15.2 mg/ml against *Escherichia coli* shown by *En* cr. Similarly, both *En* Ch and *En* bt fraction showed MIC against *Candida albica,* but *En* Ch fraction showed (15.1 mg/ml) highest MIC against *Candida albicans;* MIC for *En* cr and *En* hex was also noted against *Aspergillus flavus*.

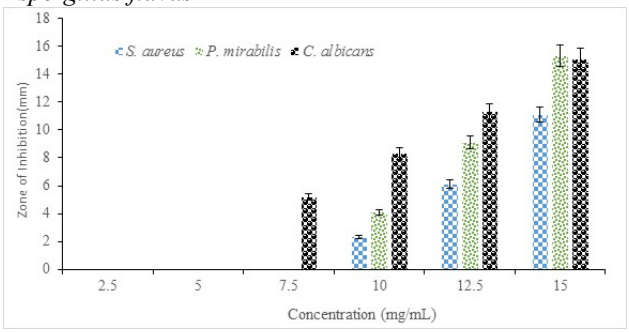

Fig. 4: MIC of *En* ch fraction against *S. aureus, P. mirabilis, C. albicans*

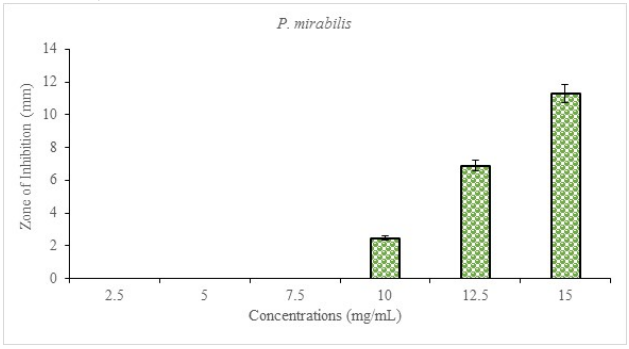

Fig. 5: MIC of *En* aq fraction against *P. mirabili*

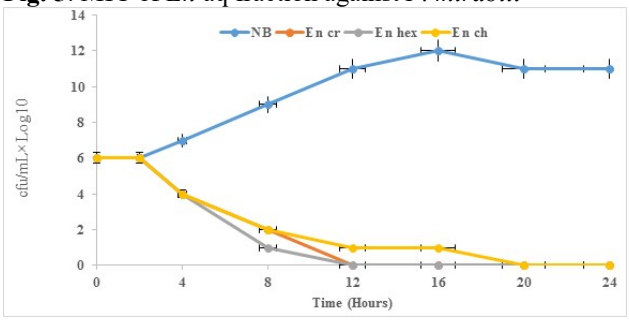

Fig. 6: Kinetics of *En* cr, *En* hex and *En* ch fraction against *S. aureus* under different treatments, where NB = nutrient broth (control)

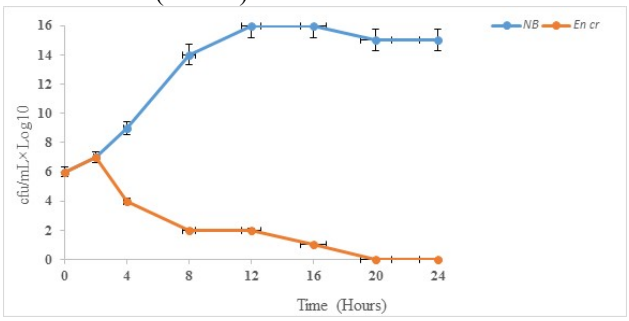

Fig. 7: Kinetics of *En cr* against *E. coli* under different treatments, where NB = nutrient broth.

Table 1: Phytochemical evaluation (*En* cr and fractions)

Test	*En* cr	*En* hex	*En* ch	*En* bt	*En* aq
Alkaloids					
Hager's test	+	-	-	-	-
Mayer's test	+	-	-	+	-
Wagner's test	+	-	-	+	-
Glycosides					
Keller-killani test	+	-	-	-	-
Tannins					
$FeCl_3$ test	+	-	-	+	+
Flavonoids					
Test with Alkal sols	+	+	-	-	-
Saponins					
Froth test	+	-	-	-	+
Phenolic Contents					
$FeCl_3$ test	+	-	-	+	+

Table 2: Antimicrobial activity of *En* cr & various fractions (Zone of Inhibition (mm) at 100 mg/ml dose)

Micro-organism	*En* cr	*En* hex	*En* bt	*En* ch	*En* aq	Cefotaxime (500 mg)
Bacteria						
Staphylococcus aureus	11.3±.48	11.1±.49	11.3±.47	11.3±.48	NZ	29.9± .5
Escherichia coli	15.4±.42*	1.9±.044	1.8±.044	NZ	NZ	26.5± .5
Bacillus subtilis	NZ	NZ	1.9±.046	1.9±.48	NZ	29.0±.58
Proteus mirabilis	1.9±.053	1.9±.054	11.2±.56	15.5±.58*	11.3±.55*	24.6±.57
Salmonella typhi	1.5±.051	NZ	11.1±.52	1.9±.055	NZ	28.6±.57
Corynebacterium xerosis	1.1±.049	NZ	1.8±.051	1.4±.052	NZ	24.5±.57
Salmonella typhi para A	1.3±.049	NZ	1.9±.056	1.1±.055	NZ	31.0±1.0
Shigella dysenteriae	NZ	NZ	1.8±.053	NZ	NZ	34.6±.57
Klebsiella pneumonia	NZ	1.9±.051	1.9±.054	1.9±.041	NZ	30.0±0.6
Bacillus cereus	1.1±.051	NZ	NZ	NZ	1.4±0.011	24.5±.57
Pseudomonas aeruginosa	NZ	1.1±.054	NZ	1.3±.053	1.1±.063	29.0±1.00
Fungi Nystatin(100mg)						
Candida albicans	NZ	NZ	14.1±.52*	14.3±.51*	NZ	27.66±.57
Aspergillus niger	NZ	0.8±.055	NZ	NZ	NZ	24.5±.57
Aspergillus flavus	11.2±.56*	11.4±.55*	1.3±.053	NZ	NZ	19.0±0.0
Penicillium sp.	NZ	NZ	NZ	NZ	NZ	19.6±.57

NZ = no zone

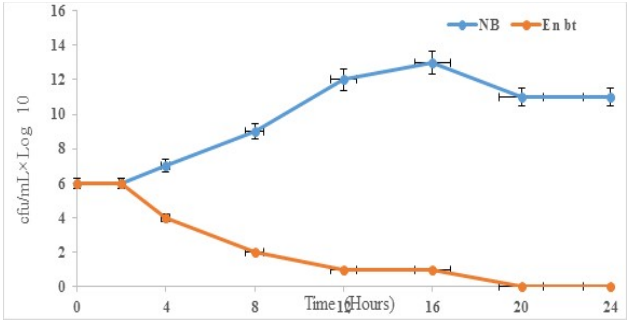

Fig. 8: Kinetics of *En* bt fraction against *S. typhi* under different treatments, where NB = nutrient broth.

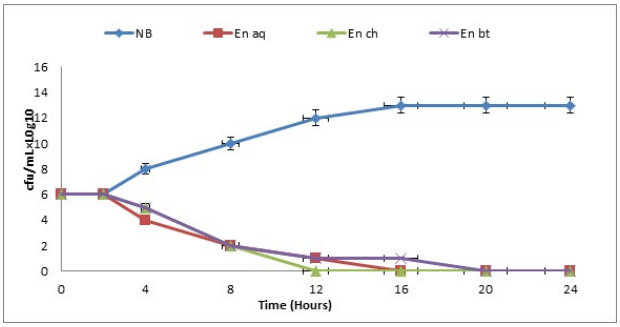

Fig. 9: Kinetics of *En* aq, *En* ch and *En* bt fraction against *P. mirabilis* under different treatments, where NB = nutrient broth.

Table 3: MIC of *En* cr & various fractions

Sample	Conc.	Micro-organisms					
		E. coli	*S. aureus*	*S. typhi*	*P. mirabilis*	*C. albicans*	*A. flavus*
En cr	1	15.2±0.51*	11.3±0.53	-	-	-	11.2±.72*
En hex	2	-	11.4±.053*	-	-	-	11.2±.058
En bt	3	-	-	11.4±.049*	11.3±.056	14.3±.054*	-
En ch	4	-	11.1±.53	-	15.3±.54*	15.1±.48	-
En aq	5	-	-	-	11.3±.59	-	-

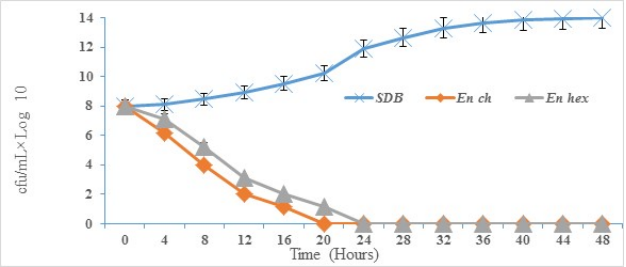

Fig. 10: Kinetics of *En* ch and *En* hex fraction against *C. albicans* under different treatments, where SDB =nutrient broth

Time-kill kinetics analysis

Results of time-kill kinetics of *En* cr as well as four fractions, i.e. *En* Ch, *En* hex, *En* Aq and *En* bt against *S. aureus*, *E. coli, P. mirabilus*, *S. typhi and C. albicans* are shown in figs. 6-10.

DISCUSSION

Globally, screening of medicinal plants for various diseases is the most interesting topic of research among researchers these days. WHO recently estimated that approximately 80% of the world population relies on plants and their extracts in various forms to treat their ailments on the basis of folk medicines prevalent in their respective traditional systems/therapies. In the present work, *Euphorbia nivulia* –Ham (70% hydro alcoholic) crude extract (*En* cr) as well as its various fractions show good activity against most of the tested bacterial and fungal strains. Results were compared with standard antibiotics. Results of preliminary phytochemical screening show the presence of different chemical constituents like glycosides, alkaloids, saponins, flavonoids, phenolics, tannins etc. **(**Younus *et al.*, 2019) (table 1). These constituents belong to important classes of secondary metabolities that offer defense against many microorganisms, insects and herbivores, etc. Exhibition of antibacterial activity by this plant species against both Gram positive and Gram negative bacteria and various fungal strains indicates the presence of several broad spectrum antibiotic bio-active molecules. It confirms that additive and synergistic effects of phytochemicals (in fruits and vegetables) may be responsible for potent bioactivity against microbes. It also explains why single antimicrobial agent cannot replace natural phytochemicals in combination to offer the benefits in a disease (Liu, 2003). Antimicrobial potentials of various species of the genus *Euphorbia* have been previously reported. For example, aqueous extract of *E. tirucalli* has revealed significant antibacterial activity for various bacterial strains, and fungi such as *Candida albicans* (Singh and Vidyasagar, 2015; Bhalodia and Shukla 2011). Some authorities have suggested that its healing property is due to action of phytoconstituents of this plant particularly flavonoids, tannins and steroids (Kane and Bhandari, 2013; Bhalodia and Shukla 2011). Different solvents were used to extract various phytochemicals depending on their solubility or polarity in the respective solvent. *En* hydro-alcoholic (70% aqueous ethanolic) extract might have higher solubility for more phytochemicals. Similarly, antimicrobial activity demonstrated by aqueous extract gives scientific evidence for plant use in treatment of diseases as per traditional system of medicines; since most traditional healers use aqueous solvent for preparation of decoctions in treatment of a disease. The above results show that activity of 70% aqueous ethanolic extract possesses significant antibacterial and antifungal activity. The study also shows the presence of different phytochemicals with biological activity that can be of valuable therapeutic index. Result of phytochemical screening in the current investigation shows that the plant is rich in flavonoids, tannins and phenolic compounds besides saponin, triterpenoids, steroids, glycosides, amino acids and proteins; these are the chemical constituents which impart pharmacological activity to the plant. The plant displayed significant antimicrobial activity against *S.aureus* and *E. coli* due to several triterpenes (Khare, 2008). Our findings show that aqueous EtOH extract of *En* was found to have high phenolic contents and flavonoids. Many flavonoids are medicinal agents effective against human diseases, like microbial infections, AIDS and cardiac diseases (Jiangrong and Jiang, 2007; Noriaki *et al.*, 2005; Carlo *et al.*, 1999; Rice-Evans & Packer, 1998); flavonoids are also used in various foods and pharmaceuticals as preservative (Slusarczyk *et al.*, 2009; Chlopcikova *et al.*, 2004).

Current investigation also supports previous findings that the antimicrobial activities have a direct relation to increasing the concentration (%) of the extract (s) (Bhalodia and Shukla, 2011). Results also support previous findings that ethanolic/alcoholic extracts display

higher antimicrobial activity than water extracts (Al-Hashimi, 2012). Furthermore, it has been reported that large number of different phytochemicals such as phenolic compounds and its derivative compounds, fatty acid, terpenes, flavonoids and others present in ethanolic extract and various fractions can target multiple sites of bacterial cells (Oonmetta-aree *et al*., 2006; Burt, 2004). Phenolic compounds have several mechanisms of action against microbes, including change in microbial cell membrane permeability through accumulation of hydrophobic groups disrupting the membrane integrity and leakage of intracellular components and eventually cell lysis (Wu *et al*., 2016; Cowan., 1999). Phenolic compounds can also affect functions of enzymes like synthesis of proteins, DNA and RNA. Terpene compounds like phytol, lupeol and amyrin, present in the extract may have disrupted microbial cell membrane.

MIC and bactericidal concentrations do not describe the time course of a drug's antimicrobial activity against bacteria. A few antimicrobial agents demonstrate concentration dependent killing over a wide range of concentrations, while others show maximum killing at concentrations near MIC. High drug concentrations (about 10- fold higher than MIC) are required to prevent resistant bacterial subpopulations selection.

Time-kill test is most appropriate method for bactericidal or fungicidal effect determination. It is a strong tool for obtaining information about dynamic interaction between anti-microbial agent and microbial strain. Time-kill test reveals a time-dependent or a concentration-dependent antimicrobial effect. It has been well standardized for bacteria, and described in M 26-A document of CLSI (Wayne, 1999). These tests are also often used as basis for *in vitro* investigations into pharmacodynamic drug interactions. They typically provide descriptive (qualitative) information on pharmacodynamics of antimicrobials (Craig and Ebert, 1990). The time-kill kinetics profile of *En* cr as well as four fractions against *S. aureus, E. coli, P. mirabilis, S. typhi* and *C. albicans* is shown in figs. 6-10. Time kill kinetic profile against *S. aureus* was noted for *En* cr and fractions *En* hex and *En* ch for time intervals 4, 8, 12, 16 and 24 hours (fig. 6). Time kill kinetic exhibited by *En* cr was also noted against *E. coli* for time intervals of 02, 04, 08, 12, 16 and 24 hours. It was found that in the initial hours it increased very well but in between 08-12 hours it remained somewhat same, however maximum activity was shown at time interval of 24 hours (fig. 7). Time kill kinetic profile for *En* bt against *S. typhi* was noted for same time intervals as for *E. coli*. Similarly time kill pattern was shared by *S. typhi*, that was observed for *E. coli* (fig. 8). The time-kill kinetics profile of *En* bt, ch and aq fractions against *P. mirabilus* is shown in fig. 9. The trend shows that a gradual increase pattern in efficacy against growth reduction at time intervals of 4, 8, 12, 16 and 24 hours exists for these three fractions. Time kill kinetic profile displayed by *En* hex and *En* ch was noted down against test organism *C. albicans* for time intervals 04, 08, 12, 16, 20, 24 and 48 hours, respectively. In the initial hours up to 24 hours activity increased to its maximum value. This activity was remained same for next 24 hours i.e., at 48 hours (fig. 10).

CONCLUSION

The study shows that *Euphorbia nivulia* (70% hydro alcoholic) crude extract (*En* cr) as well as its various fractions exhibit good antimicrobial activity against most of the tested bacterial and fungal strains; this may help to discover new class of antibiotics that could be used as selective agents for treatment and control of infectious diseases. Our investigation may open the possibility for use of locally occurring medicinal plant species in drug development for human consumption, possibly for the treatment of skin, gastrointestinal, urinary tract, typhoid fever and respiratory tract infections. Furthermore, the study validates ethno-pharmacological uses of the plant. However, investigation for isolation, purification and structure elucidation of antimicrobial bio-active constituent(s)from the crude extract as well as various fractions of this plant species is suggested.

REFERENCES

Ahameethunisa AR and Hopper W (2010). Antibacterial activity of *Artemisia nilagirica* leaf extracts against clinical and phytopathogenic bacteria. *BMC Complement. Altern. Med.,* **10**(1): 6.

Ahmed NJ, Fouda MI, Fouda DI and Foudah AI (2020). The adverse effect reporting for the most commonly used antibiotics. *J. Pharm. Res. Int.,* **32**(8): 22-28.

Alder JD (2005). Daptomycin: A new drug class for the treatment of Gram-positive infections. *Drugs Today (Barc),* **41**(2): 81-90.

Al-Hashimi AG (2012). Antioxidant and antibacterial activities of *Hibiscus sabdariffa* L. extracts. *Afr. J. Food Sci.,* **6**(21): 506-511.

Ali SI and Qaiser M (1986). A Phytogeographical Analysis of the Phanerogames of Pakistan and Kashmir. *Proc. R. Soc. Edinb. Biol. Sci.,* **89**(4): 89-101.

Alves IFR, Ferreira J, Dias V, Teixeira A, Martins and Pintado M (2012). A review on antimicrobial activity of mushroom (*Basidiomycetes*) extracts and isolated compounds. *Planta Med,* **78**(16): 1707-1718.

Badgujar SB and Mahajan RT (2010). Characterization of milk clotting cysteine protease of *Euphorbia nivulia* Buch. -Ham. Latex. *Green Farming (New Series)*, **1**(6): 645-648.

Badgujar SB and Mahajan RT (2011). Bioprospecting of *Euphorbia nivulia* Buch. Ham. *Inter. J. Phytopharmacol.,* **2**(2): 37-42.

Balouiri M, Sadiki M and Ibnsouda SK (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. *J. Pharm. Anal.,* **6**(2): 71-79.

Basak SK, Bakshi PK, Basu S and Basak S (2009). Keratouveitis caused by *Euphorbia* plant sap. *Indian J. Ophthalmol.,* **57**(4): 311-313.

Basumatary SK, Ahmed M and Deka SP (2004). Some medicinal plant leaves used by Boro (tribal) people of Golapara district, Assam. *Nat. Prod. Rad.*, **3**(2): 88-90.

Bhalodia NR and Shukla VJ (2011). Antibacterial and antifungal activities from leaf extracts of *Cassia fistula* L.: An ethnomedicinal plant. *J. Adv. Pharm. Technol. Res.,* **2**(2): 104.

Bibi Y, Nisa S, Waheed A, Zia M, Sarwar S, Ahmed S and Chaudhary MF (2010). Evaluation of Viburnum foetens for anticancer and antibacterial potential and phytochemical analysis. *Afr. J. Biotechnol.,* **9**(34): 5611-5615.

Brain KR and Turner TD (1975). The practical evaluation of phytochemicals. Wright Science Technical, Bristol, pp.56-64.

Britto AJ, Sujin RM and Mahesh R and Dharmar K (2010). Ethnomedicinal wisdom of the Manavalakuruchi people in Kanyakumar district, Tamilnadu. *Int. J. Appl. Biol. Pharm.,* **1**(2): 25-30.

Burt S (2004). Essential oils: Their antibacterial properties and potential applications in foods a review. *Int. J. Food Microbiol.,* **94**(3): 223-253.

Cateni F, Zilic J, Falsone G, Scialino G and Banfi E (2003). New cerebrosides from *Euphorbia peplis* L.: antimicrobial activity evaluation. *Bioorg. Med. Chem. Lett.,* **13**(24): 4345-4350.

Chlopckiova S, J Psotova, P Miketova, J Sousek, V Lichnovsky and V Simanek (2004). Chemo protective effect of plant phenolics against anthracycline induced toxicity on rat cardiomyocytes. *Phytother. Res*., **18**: 408-413.

Cowan MM (1999). Plant products as antimicrobial agents. *Clin. Microbiol. Rev.,* **12**(4): 564-582.

Craig WA and Ebert SC (1990). Killing and regrowth of bacteria *in vitro*: A review. *Scand J. Infect Dis. Suppl*, **74**: 63-70.

Di Carlo G, Mascolo N, Izzo AA and Capasso F (1999). Flavonoids: old and new aspects of a class of natural therapeutic drugs. *Life Sci.,* **65**(4): 337-353.

Freire-Moran L, Aronsson B, Manz C, Gyssens IC, So AD, Monnet DL and ECDC-EMA Working Group. (2011). Critical shortage of new antibiotics in development against multidrug-resistant bacteria Time to react is now. *Drug Resist Updat,* **14**(2): 118-124.

Guschin A, Ryzhikh P, Rumyantseva T, Gomberg M and Unemo M (2015). Treatment efficacy, treatment failures and selection of macrolide resistance in patients with high load of *Mycoplasma genitalium* during treatment of male urethritis with josamycin. *BMC Infect Dis.,* **15**(1): 40.

Jiangrong L and Jiang Y (2007). Litchi flavonoids: Isolation, identification and biological activity. *Molecules*, **12**(4): 745-758.

Jyothi TM, Shankariah MM, Prabhu K, Lakshminarasu S, Srinivasa GM and Ramachandra SS (2008). Hepatoprotective and antioxidant activity of *Euphorbia tirucalli. IJPT,* **7**(1): 25-0.

Kane SR and Bhandari SV (2013). *In vitro* phytochemical investigation, characterization & antimicrobial study of latex of *Euphorbia tirucalli* Linn. *Int. J. Chemtech. Res.,* **5**(5): 2344-2346.

Khare CP (2008). Indian medicinal plants: An illustrated dictionary. Springer Science & Business Media, pp.900.

Koehn FE and Carter GT (2005). The evolving role of natural products in drug discovery. *Nat Rev. Drug Discov.,* **4**(3): 206-220.

Kumar GP and Chaturvedi A (2010). Ethnobotanical observations of Euphorbiaceae species from Vidarbha region, Maharashtra, India. *Ethnobotanical Leaflets*, **2010**(6): 674-680.

Li F, Chen L, Bao Y, Zheng Y, Huang B, Mu Q, Feng C and Wen D (2020). Identification of the priority antibiotics based on their detection frequency, concentration, and ecological risk in urbanized coastal water. *Sci. Total. Environ.,* **747**(10): 141275.

Liu RH (2003). Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *Am. J. Clin. Nutr.,* **78**(3): 517S-520S.

Mabona U, Viljoen A, Shikanga E, Marston A and Van Vuuren S (2013). Antimicrobial activity of southern African medicinal plants with dermatological relevance: From an ethnopharmacological screening approach, to combination studies and the isolation of a bioactive compound. *J. Ethnopharmacol.,* **148**(1): 45-55.

Mahajan RT and Badgujar SB (2008). Phytochemical investigations of some laticiferous plants belonging to Khandesh region of Maharashtra. *Ethnobotanical Leaflets*, **12**(1): 1145-1152.

Maridass M and De Britto AJ (2008). Origins of plant derived medicines. *Ethnobotanical Leaflets*, 2008(**1**): 44.

Martin I, Sawatzky P, Liu G and Mulvey MR (2015). STIs and sexual health awareness month: Antimicrobial resistance to Neisseria gonorrhoeae in Canada: 2009-2013. *Can. Commun. Dis. Rep. Wkly,* **41**(2): 35.

Mayers DL, Sobel JD, Ouellette M, Kaye KS and Marchaim D (Eds.). (2017). Antimicrobial drug resistance: Clinical and epidemiological aspects (Vol. 2). Springer. Dordrecht Heidelberg London, **1**(2): 681-1347.

Munuswamy H, Thirunavukkarasu T, Rajamani S, Elumalai EK and Ernest D (2013). A review on antimicrobial efficacy of some traditional medicinal plants in Tamilnadu. *J. Acute. Med.,* **2**(2): 99-105.

Natarajan D, Britto SJ, Srinivasan K, Nagamurugan N, Mohanasundari C and Perumal G (2005). Anti-bacterial activity of *Euphorbia fusiformis:* A rare medicinal herb. *J. Ethnopharmacol.,* **102**(1): 123-126.

Nazzaro F, Fratianni F, De Martino L, Coppola R and De Feo V (2013). Effect of essential oils on pathogenic bacteria. *Pharmaceuticals*, **6**(12): 1451-1474.

Newman DJ and GM Cragg (2020). Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. *J. Nat. Prod.*, **83**(3): 770-803.

Noriaki K, K Yukari, I Kazuya, M Kyo and F Tokio (2005). *In vitro* antibacterial, antimutagenic and anti-Influenza virus activity of caffeic acid phenyl ethyl esters. *Biocont. Sci*., **10**(4): 155-161.

Omura S, Pyl DVD, Inokoshi J, Takahashi Y, Takeshima Y (1993). Pepticinnaminsnew farnesyl-protein transferase inhibitors produced by an actinomycete I. Producingstrain, fermentation, isolation and biological activity. *J. Antibiot.,* **46**: 222-228.

Oonmetta-aree J, Suzuki T, Gasaluck P and Eumkeb G (2006). Antimicrobial properties and action of galangal (*Alpinia galanga* Linn.) on *Staphylococcus aureus*. *LWT Food Sci. Technol.,* **39**(10): 1214-1220.

Pullaiah T (2006). Encyclopedia of world medicinal plants, Regency Publication, New Delhi, **2**: 917.

Radcliffe-Smith A (1986). No. 172. Euphorbiaceae. Flora of Pakistan. *Tropicos.org. Missouri Botanical Garden*, web site: <http:// www.tropicos.org/Name/12800166>

Rice-Evans C and L Packer (1998). *Flavonoids in Health and Disease*. Marcel Dekker, New York, USA, pp.1-533.

Runyoro DK, Matee MI, Ngassapa OD, Joseph CC and Mbwambo ZH (2006). Screening of Tanzanian medicinal plants for anti-Candida activity. *BMC Complement. Altern. Med.,* **6**(1): 11.

Sen A and Batra A (2012). Evaluation of antimicrobial activity of different solvent extracts of medicinal plant: *Melia azedarach* L. *Int. J. Curr. Pharm. Res.*, **4**(2): 67-73.

Singh PS and Vidyasagar GM (2015). Antifungal screening of 61 folkloric medicinal plant extracts against dermatophytic fungi *Trichophyton rubrum*. *J. Appl. Pharm. Sci.,* **5**(5): 38-40.

Slusarczyk S, Hajnos M, Skalicka-Wozniak K and Matkowski A (2009). Antioxidant activity of polyphenols from *Lycopus lucidus* Turcz. *Food Chem.,* **113**(1): 134-138.

Sudhakar M, Rao CV, Rao PM, Raju DB Venkateswarlu Y (2006). Antimicrobial activity of *Caesalpinia pulcherrima, Euphorbia hirta* and *Asystasia gangeticum. Fitoterapia*, **77**(5): 378-380.

Sutthivaiyakit S, Thapsut M and Prachayasittikul V (2000). Constituents and bioactivity of the tubers of *Euphorbia sessiliflora*. *Phytochemistry*, **53**(8):947-950.

Tiwari P, Kumar B, Kaur M, Kaur G and Kaur H (2011). Phytochemical screening and extraction: A review. *Int. Pharm. Sci.,* **1**(1): 98-106.

Tsuji BT, Yang JC, Forrest A, Kelchlin PA and Smith PF (2008). *In vitro* pharmacodynamics of novel rifamycin ABI-0043 against *Staphylococcus aureus*. *J. Antimicrob. Chemother.,* **62**(1): 156-160.

Walsh C (2003). Where will new antibiotics come from? *Nat. Rev. Microbiol.,* **1**(1): 65-70.

Walsh FM and Amyes SG (2004). Microbiology and drug resistance mechanisms of fully resistant pathogens. *Curr. Opin. Microbiol.,* **7**(5): 439-444.

Wayne PA (1999). National Committee for Clinical Laboratory Standards (NCCLS). Performance Standards for Antimicrobial Susceptibility Testing. 9th International Supplement, pp.M100-S9.

World Health Organization (1998). Quality control methods for medicinal plant materials. World Health Organization.

Wu Y, Bai J, Zhong K, Huang Y, Qi H, Jiang Y and Gao H (2016). Antibacterial activity and membrane-disruptive mechanism of 3-p-trans-coumaroyl-2-hydroxyquinic acid, a novel phenolic compound from pine needles of *Cedrus deodara,* against *Staphylococcus aureus*. *Molecules*, **21**(8): 1084.

Younus M, Hasan MM, Abbas K and Sarwar G (2019). Pharmacognostic and physicochemical screening of *Euphorbia nivulia* Buch.-Ham. *Pak. J. Pharm. Sci.,* **32**(3): 1111-1119.

Zhang Y, Liu X, Wang Y, Jiang P and Quek S (2016). Antibacterial activity and mechanism of cinnamon essential oil against *Escherichia coli* and *Staphylococcus aureus*. *Food Control*, **59** (7): 282-289.