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RESEARCH PAPER

Antibacterial, cytotoxicity, and phytotoxicity profiles of three medicinal plants collected from Pakistan

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ABSTRACT

The purpose of present research was to examine the antibacterial, cytotoxic, and phytotoxic profiles of three important Pakistani medicinal plants viz., *Teucrium stocksianum, Chenopodium botrys,* and *Micromeria biflora*. The antibacterial, cytotoxicity, and phytotoxicity activities of samples extracted from these plants were evaluated by a modified agar well-diffusion method, brine shrimps cytotoxic assay, and *Lemna acquinoctialis*-based phytotoxic assay, respectively. Results revealed marked susceptibility of both the crude extracts and the aqueous fractions of these plants against *K. pneumonia* and *B. subtilis*. Methanolic extracts and aqueous fractions exhibited significant cytotoxicity in a concentration-dependent manner. Similarly, an outstanding phytotoxic effect was observed for the extracts/fractions. Accordingly, the extracts and fractions of the aforementioned plants possess potential antibacterial, cytotoxic, and phytotoxic effects which could be useful in the search and development of new pharmaceutical agents.

Key Words: *Teucrium stocksianum; Chenopodium botrys; Micromeria biflora;* antibacterial activity; cytotoxicity; phytotoxicity.

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Introduction

According to WHO, approximately 80 % of the world population relyon the use of traditional medicinal plants for healthcare. Essentially medicinal plants, especially herbs,are the oldest friend of human beings. It's not only providing food and shelter but also served to cure various diseases. Medicinal plants are a rich source of bioactive secondary metabolites which have the potential to be used in contemporary medicine for treatment of various difficult-to-cure diseases [1].

Teucrium stocksianum Boisssubsp. *stocksianum* belongs to family Labiatae (Lamiaceae). It has a dense compact herb which grows mostly in the hilly areas. Leaves extract of *T. stocksianum* can be used as folk's medicine for treatment of diabetes mellitus and stomach diseases. Furthermore, *T. stocksianum* aerial parts contain numerous secondary metabolites such as saponins, terpenoids, and flavonoids. In addition, *T. stocksianum* possesses antimicrobial activity against a wide range of microbes [1-3], and its aqueous/ethanolic extracts exhibit gastric cytoprotective and hepatoprotective properties [4]. On the other hand, *Micromeria biflora* (Buch.-Ham. ex D. Don) Benth

belongs to family Lamiaceae, which is found in tropical and Himalayas regions. *M. biflora*'s paste is used to cure toothache, whereas its inhaled aroma can be employed in the treatment of nose-bleeds[5]. Moreover, the plant's paste was used traditionally used as a poultice to treat wounds [5]. In addition, its juice can be taken orally and can also be inhaled to treat sinusitis. Similarly, essential oil obtained from leaves of *Micromeria biflora* Benth has been widely used to construct bacterial phylogenetic relationships [6-8].

Chenopodium botrys L. is a member of Chenopodiaceae family which is known as Jerusalem oak. Whole organs of the plant possesss aromatic odors. In the Iranian traditional medicine, the flowering aerial parts of *C. botrys* have long been used as expectorant, antiasthmatic, anticatarrhal, anticonvulsant, and tonic agents. In many cases, *C. botrys* was used as a substitutefor lavenders to keep away moths and composition of its essential oil has been reported by other researchers[9].

In addition, the aerial parts of *Chenopodium botrys* were reported to contain flavone chrysoeriol. Five flavones including salvigenin, sinensetin, and hispidulin, along with theire derivatives have been isolated from *C. botrys*[12]. Moreover, hispidulin and jaclosidin were previously isolated from *C. botrys* [13]. Accordingly, and owing to the wide range ofmedicinal uses of the three medicinal plants*T. stocksianum, M. biflora*and *C. botrys* grown in Pakistan, the present study was designed to evaluate the antibacterial, cytotoxic, and phytotoxic effects of these plants *in vitro*.

MATERIALS AND METHODS

Plant collection

Teucrium stocksianum Boisssubsp. *stocksianum, Chenopodium botrys* (Buch.-Ham. Ex D. Don), and *Micromeria biflora* L.were collected from the mountain of Razagram Khall, District Dir, Khyber Pakhtunkhwa province of Pakistan in March, 2013. The plants were identified and authenticated by Ghulam, a botanist at the Jelani Department of Botany, University of Peshawar, KPK, Pakistan. The voucher specimens no. U(PUP)-8825-8827 were placed in the herbarium of Department of Botany, University of Peshawar, KPK, Pakistan.

Preparation of extract and fractions

Shade dried plants of *T. stocksianum*(500g), *C. botrys*(500g), and *M. biflora*(500 g) were placed in different flasks which contain 1 L solvent (methanol & water) and extracted successively with methanol (x3) and water (x3) at room temperature (cold extraction) for 7 days. The solvent extracts were concentrated under reduced pressure at 40 °C by means of rotary evaporation. Combined extracts of *T. stocksianum* (12.5 g), *C. botrys* (10.2 g), and *M. biflora* (8.5 g)were collected by concentration of solvent extract under reduce pressure at 40 ^{0}C.

Antibacterial activity

Microorganism assortment and preservation

Three selected strains of Gram-positive (*Staphylococcus epidermidis, Staphylococcus aureus*, and *Bacillus subtilis*) and two of Gram-negative bacteria (*Klebsiella pneumonia* and *Escherichia coli)* (Table 1) were obtained from the stock culture PNRL laboratories, Institute of Chemical Sciences, University of Peshawar, Peshawar, Pakistan which were obtain from human blood and stored in Mueller-Hinton agar at 4°C prior to subculture. Streptomycin was used as a standard antibiotic drug and obtains from local market of sharpaw hospital.

Table 1. The antibacterial effect of the tested medicinal plants in terms of zone of inhibition.

Bacteria	Reference	Zone of inhibition (mm)							
		NS	TSW	TSM	CBW	CBM	MBW	MBM	STD
Klebsiella pneumoniae	Clinical isolate	-	-	-	14±1.10	16±1.17	-	-	28±0.95
Bacillus subtilis	ATCC 6633	-	15±0.9	14±1.13	10±0.85	12±0.54	-	-	26±1.10
Staphylococcus aureus	ATCC 25923	-	-	-	-	-	-	-	28±1.10

Results are mean of three different experimental assays.
TSW*: Teucrium stocksianum* water extract, TSM: *Teucrium stocksianum* methanolic extract CBW: *Chenopodium botrys* water extract, CBM: *Chenopodium botrys* methanolic extract MBW*: Micromeria. Biflora* water extract, MBM: *Micromeria biflora* methanolic extract STD: Standard drug: NS; Negative strain

Antimicrobial assay against selected bacterial strains

To evaluate the antibacterial activity of extracts obtained from the Pakistani medicinal plants, a modified agar well-diffusion method was adopted using Mueller-Hinton agar (MHA) as the medium. Cultures were prepared in triplicates and were incubated at 37°C for a period of 24 to 72 h. An amount of 0.6 mL of the broth culture of the tested organism was placed in a sterile petri-dish and 20 mL of the sterile molten MHA was added. Wells were cut into the medium using 0.2 mL of each fraction while Streptomycin (2 mg/mL) was used as a standard antimicrobial agent. Inoculation was performed in an hour to ensure diffusion of the antimicrobial agent into the medium. Inoculation plates were incubated at 37°C for 24 h and the diameters of the zone of inhibition of microbial growth were measured in millimeters [10].

In-vitro cytotoxic assay

To assess the cytotoxic potential of the crude extracts and aqueous fractions of tested medicinal plants, we employed the brine shrimp cytotoxic assay as has been described in the literature[11, 12]. Briefly, test samples were prepared in various concentrations of 10, 100, and 1000 µg/mL. Brine shrimp (*Artemia salina* Leach) nauplii were hatched in a precise tank at room temperature. Then, from stock solutions, 5, 50 and 500 µg/mL were injected into 9 vials (3 vials for each dilution). Each vial confined ten shrimps and 5 mLof brine. The vials were added with a dry yeast suspension, as their food, and were incubated for 24 h under illumination. For analysis, the live nauplii were counted with the aid of a 3 x magnifying glass and the percent deaths at each dose was calculated. Data were processed with the aid of a Graph Pad to estimate LD_{50} values (LD_{50} was the mean of three replicates).

In-vitro phytotoxic assay

In vitro phytotoxicity assay of the crude extracts and aqueous fractions of tested medicinal plants against *Lemna acquinoctialis* was performed according to the protocol outlined by Saeed et al. [13]. The medium was prepared by mixing various inorganic components in 100 mL of doublydistilled water followed by addition of KOH solution to adjust the pH to 6.0-7.0, and the medium was then autoclaved at 121 ^{0}C for 15 min. Test samples (15 mg) dissolved in ethanol (1.5 mL) served as stock solution. Nine flasks (three for each dilution) were inoculated with 1000, 100, and 10 µL of the stock solution for 500, 50 and 5 ppm and the solvent was evaporated overnight under sterilized conditions. Each flask was supplemented with 20 mL of the medium. Thereafter, 10 plants each containing a rosette of three fronds were added to each flask. One other flask, supplemented with solvent was used as a control whereas a reference plant growth inhibitor (Paraquat), served as a standard phytotoxic drug. The flasks were plugged with cotton and located in growth cabinet for a week. On the 7th day, the number of fronds per flask was calculated. Results were analyzed as growth regulation in %, calculated with reference to the negative control and a Graph Pad statistical software was employed to calculate IC_{50} values.

Results

Antibacterial activity

Shown in Table 1 are results pertaining to the effect oftested plants against the bacteria. Results revealed significant susceptibility of the medicinal plants against *B. subtilis*. The methanolic extract and aqueous fraction of *Teucrium stocksianum* showed 53.84 and 57.69% activity, respectively (Figure 1a). However, the extract was not susceptible against *S. aureus*. In addition, results showed activity against *K. pneumonia* and *B. subtilis*. Furthermore, the crude methanolic extract and aqueous fractionof *T. stocksianum* exhibited 57.14 and 50.0% activity, respectively against *K. pneumonia* as depicted in Figure 1b. Similarly, crude methanolic extract and aqueous fraction of *C. botrys* displayed 46.15 and 38.46% activity, respectively, against *B. subtilis* (Figure 1b). However, the extract and the fraction were not sensitive against *S. aureus*. Results presented in Table 1 also reveal that neither the crude extract nor the aqueous fraction *M. biflora* was effective against tested bacteria.

Cytotoxic activity

Results of the cytotoxic assay of tested medicinal plants are given in Table 2. Results show that the crude extract of *T. stocksianum* exhibits a concentration-dependent cytotoxic effect (Figure 2a) with IC_{50} values of 25.20 µg/mL (Table 2), whereas the aqueous fraction of the plant showed concentration-dependent cytotoxic effect (Figure 2b) with an IC_{50} of 42.30 µg/mL. Similarly, the crude extract and its aqueous fraction of *C. botrys*, exhibited concentration-related toxicity as shown in Figures 2c and 2d with IC_{50} values of >100 for water extract and 100.10µg/mL, for methanolic extract respectively (Table 2). Likewise, the extract and aqueous fraction of *M. biflora* showed dose-dependent cytotoxic activity (Figures 2e and 2f). The calculated IC_{50} values for both were 21.50 and 10.90 µg/mL, respectively.

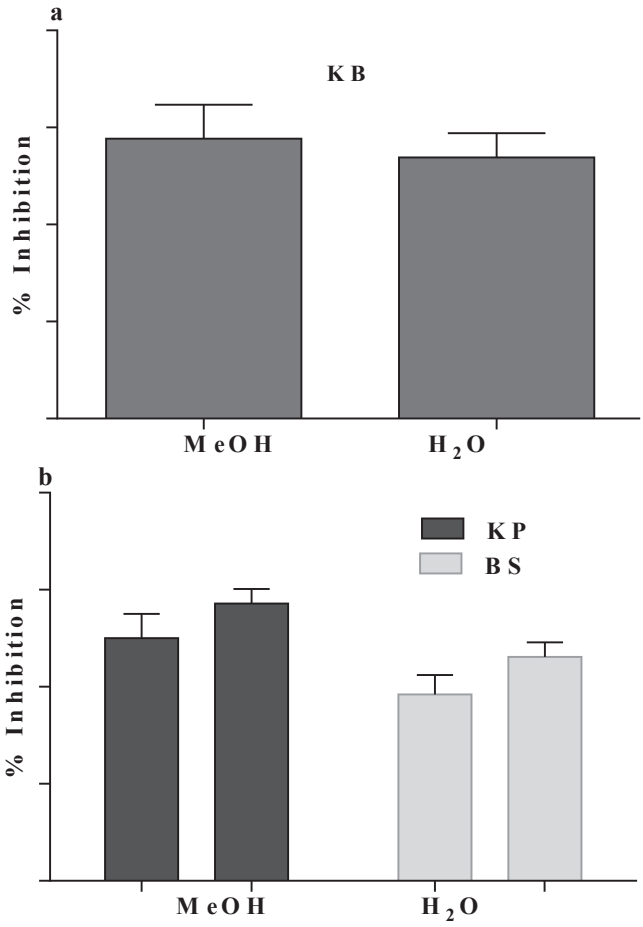

Figure 1. The percent effect of *Teucrium stocksianum* (a) against *Bacillus subtilis* and *Chenopodium botrys* (b) against *Klebsiella pneumonia* and *Bacillus subtilis*.

The % inhibitory effect is calculated with respect to the standard activity. $\% \text{ inhibition} = \frac{\text{Effect of test extract}}{\text{Effect of standard}} \times 100$

Phytotoxic activity

Listed in Table 3 are results of the phytotoxic effect of tested medicinal plants. Results show that the crude extract and aqueous fraction of *T. stocksianum* exhibited noticeable concentration-dependent phytotoxicity (Figure 3a) with IC_{50} values of 15.67 and 10.10µg/mL, respectively, as presented in Table 3. On the other hand, the crude extract and aqueous fraction of *C. botrys* showed concentration-dependent phytotoxicity (Figures 3c and 3d) with IC_{50} values of 13.60 and 12.50 µg/mL, respectively. Similarly, a concentration-dependent phytotoxicity was observed for the crude extract and aqueous fraction of *M. biflora* (Figure 3e and f) with IC_{50} values of 581.00 and 570.13 µg/mL, respectively.

Discussion

Animals and plants have been employed over the years, for the discovery of new effective antimicrobials. However, due to the evolution of resistant genes in bacteria and to the irrational uses of antibiotics, clinical microbiologists are facing the greatest challenge of multiple drug resistance against currently used antimicrobials which affected their efficacy and clinical utility and led to the development of antibiotic resistance [14]. Therefore, the discovery of new antimicrobials is the demand of the present era to cope with the challenges of microbial resistance in life-threatening infections. Antimicrobials of natural origins are believed to act on different sites and mechanisms; they, therefore, could be considered more beneficial in the prevention of bacterial resistance [15-17]. In this connection, three important medicinal plants were tested against three commonly infectious pathogens including *K. pneumonia, B. subtilis*,and *S. aureus*.

Table 2. Cytotoxic activity of tested medicinal plants.IC_{50}values of tested medicinal plants in cytotoxic activity assay.

Name of Extracts	No. shrimps	Number of surviving shrimps			IC_{50} (µg/mL)
		10 µg/mL	100 µg/mL	1000 µg/mL	
Control		Sample	Sample	Sample	------
TSW	10	7±0.57	4±0.24	0±0.00	42.30±1.52
TSM	10	6±0.66	3±0.16	0±0.00	25.20±2.89
CBW	10	9±1.97	7±0.05	2±0.00	>100
CBM	10	8±0.00	5±0.00	2±0.00	100.10±3.77
MBW	10	5±0.00	4±0.05	0±0.00	10.90±1.60
MBM	10	6±0.02	4±0.00	0±0.00	21.50±0.87

Data are mean± SEM of three independent assays. (Etoposidehas an IC_{50}= 7.4625µg/mL).

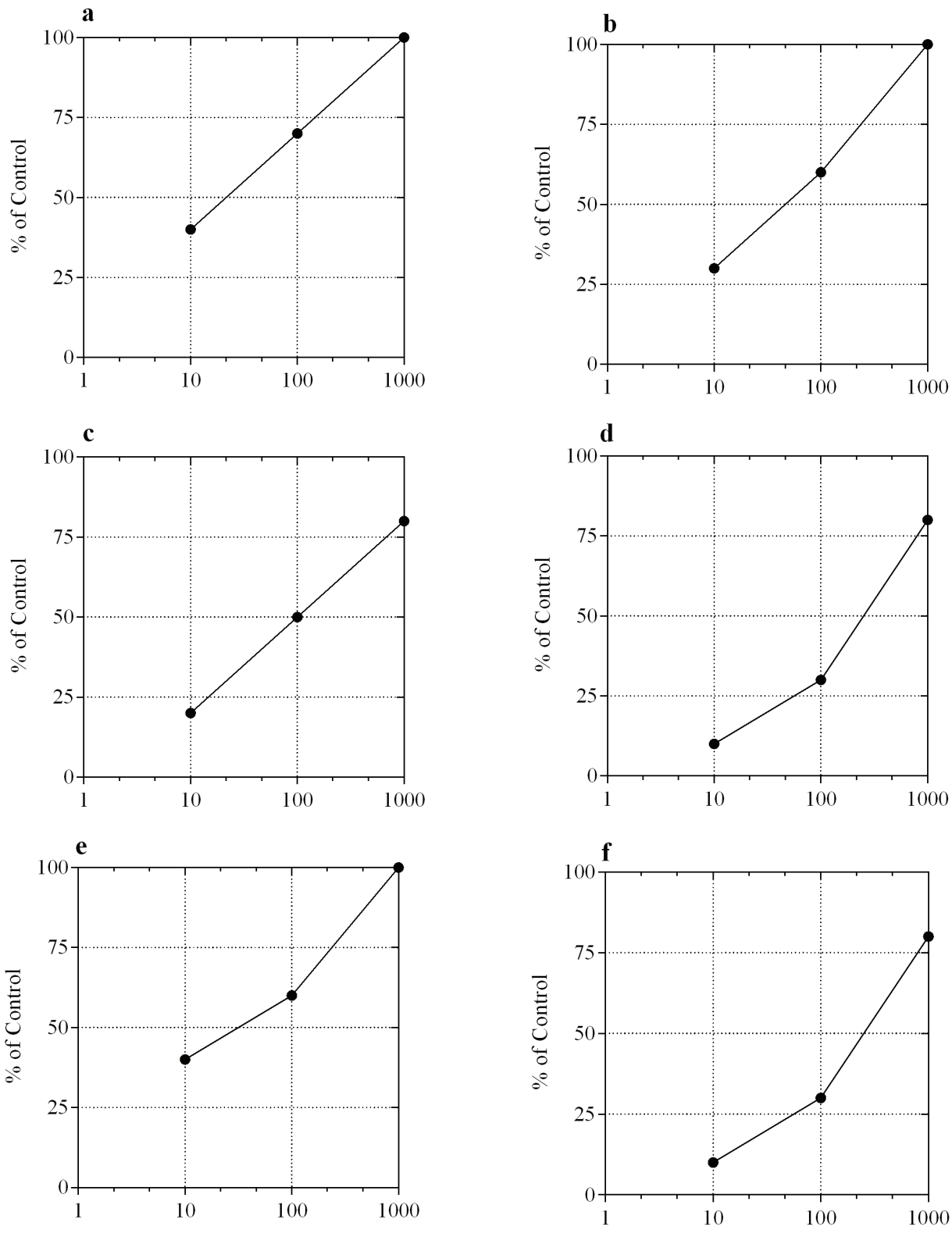

Figure 2. The percent cytotoxic effect of (a) TSM, (b) TSW, (c) CBM, (d) CBW, (e) MBM,and (f) MBW. Data shown are mean of three independent assays.

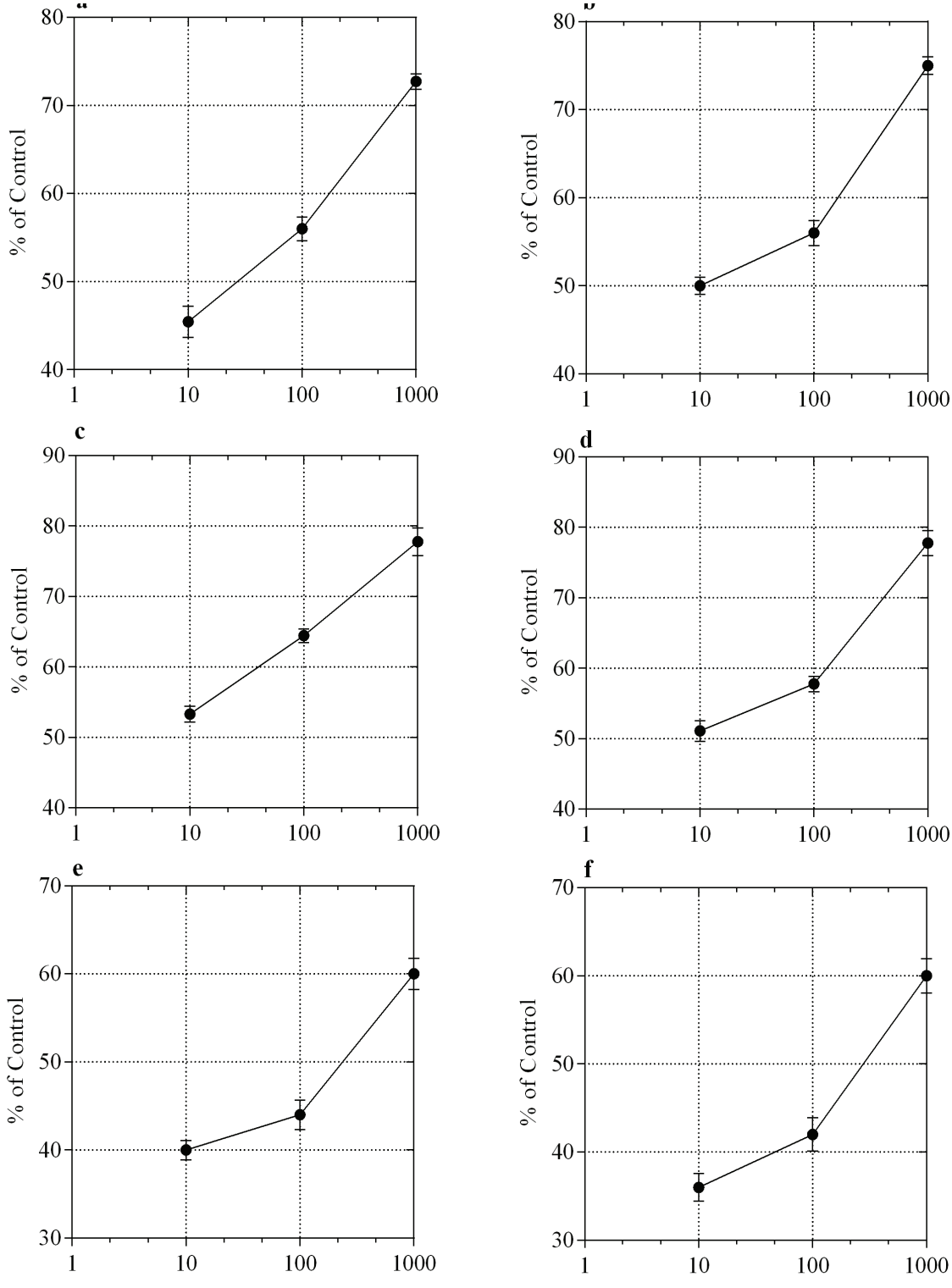

Figure 3. The percent phytoxic effect of (a) TSM, (b) TSW, (c) CBM, (d) CBW, (e) MBM, and (f) MBW. Data are reported as mean± SEM for three independent assays.

List of Abbreviations

TSW*: Teucrium stocksianum* water extract, TSM:*Teucrium stocksianum* methanolic extract; CBW:*Chenopodium botrys* water extract; CBM: *Chenopodium botrys* methanolic extract, MBW: *Micromeria biflora* water extract; MBM:*Micromeria biflora* methanolic extract. NS: Normal saline.

Table 3. Phytotoxic activity of crude methanolic and water extracts of three medicinal plants. IC_{50}values of tested medicinal plants in phytotoxicassay.

Name of Extracts	Control	Number of fronds			IC_{50} (µg/mL)
		10 µg/mL	100 µg/mL	1000 µg/mL	
		Sample	Sample	Sample	
TSW	44±0.78	22±0.97	19±1.44	11±1.01	10.10±2.01
TSM	44±0.69	24±1.76	19±1.35	12±0.86	15.67±0.83
CBW	45±1.10	22±1.97	19±1.08	10±0.78	12.50±0.48
CBM	45±1.10	21±1.57	16±1.10	10±0.95	13.60±0.45
MBW	50±0.70	32±1.57	29±1.89	20±1.67	570.13±4.20
MBM	50±0.70	30±1.92	28±1.75	20±1.23	581.00±3.17

Standard drug (Paraquat), Test samples = (15 mg).Control = Ethanol.Data are presented as mean± SEM for three independent assays. (Standard drug; Paraquat with IC_{50} = 3.142±0.09 µg/mL).

K. pneumonia is a Gram-negative facultative anaerobic pathogen. Emergence of extensive resistance of *K. pneumonia,* similar to other *Klebsiella*,spp. has been reported by researchers. It is worth mentioning that increasing resistance to carbapenem-resistant- *K. pneumonia* has caused large scale morbidity and mortality [18]. Few therapeutic options are available for the effective treatment of infections caused by *K. pneumonia*. *C. botrys* showed significant antibacterial activity against clinically isolated *K. pneumonia*. It could therefore, be a significant natural healing agent against infections caused by the aforementioned pathogen.

B. subtilis, on the other hand, is generally considered as nonpathogenic or less pathogenic and only few cases of its infections have been reported. Therefore, little importance has been given to resistances. However, in an immune-compromised patient recurrent, septicemia has been reported due to probiotic strains of *B. subtilis* [19]. Results from this investigation revealed that *C. botrys* exhibits significant activity against *B. subtilis*. Thus, this medicinal plant could be an important therapeutic natural agent against infections caused by *B. subtilis*.

Brine shrimps cytotoxic assay is a simple but efficient tool for the assessment of cytotoxic potential of test articles [16, 20]. Cytotoxic compounds could play significant role in the treatment of various cancers [21]. Results obtained from this study show that the tested medicinal plants possess profound cytotoxic activity, and thus may be considered as new sources in the search of new drugs.

Interfering of weeds clearly decreases the quality and quantity of agricultural crops and is accountable for huge economic losses all over the world. It is estimated in US alone that weeds basis a loss of at least 12% costing nearly US$ 33 billion, while the situation is more alarming in developing countries [22]. Synthetic herbicides are broadly used for the control of weeds in agricultural sectors. However, various factors that controlled the use of synthetic herbicides include water and soil pollution, herbicide-resistant weed populations, herbicide residues and detrimental effects on non-target [23]. In recent times, more stress has been given to the natural allelelopathicchemicals (allelochemicals) from plants for weed control in crop production, especially to cope with the problem of weed resistance.

Conclusion

In summary, our findings from this study suggest that the extracts/fractions of *T. stocksianum*, *C. botrys*, and *M. biflora* display remarkable antibacterial activity against *K. pneumoniae* and *B. subtilis*, with potential cytotoxic action against brine shrimps (*A. salina* Leach) and phytotoxicity against *L. acquinoctialis*. It is, therefore assumed that, these plants could be a possible natural therapeutic modality as effective antibacterial, cytotoxic, and phytotoxic agents. However, further detailed studies are required establish the safety to get molecule(s) of lead compounds of clinical utility.

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Pakistan'dan toplanan üç tıbbi bitkinin antibakteriyel etkileri, sitotoksisite ve fitotoksisite profilleri

ÖZET

Mevcut araştırmanın amacı, Pakistan'da üç önemli tıbbi bitki olan *Teucrium stocksianum*, *Chenopodium botrys*, ve *Micromeria biflora* bitkilerinin antibakteriyel, sitotoksik ve fitotoksik profilini incelemektir. Örneklerin antibakteriyel, sitotoksik ve fitotoksik aktiviteleri sırasıyla modifiye agar difüzyon yöntemi, Brine shrimp sitotoksik yöntemi ve *Lemna acquinoctialis*'e dayanan fitotoksik yöntemi kullanılarak değerlendirildi. Sonuçlar bu bitkilerin hem özütlerinin hemde sulu fraksiyonlarının *K. pneumonia* ve *B. subtilis* suşlarına karşı belirgin şekilde duyarlı olduğunu gösterdi. Metanol özütleri ve sulu fraksiyonlar konsantrasyona bağlı olarak önemli sitotoksik aktivite gösterdiler. Benzer olarak, bu özüt ve fraksiyonların önemli derecede fitotoksik etki gösterdikleri gözlendi. Bu sonuçlara göre, özütler/fraksiyonlar yeni farmasötik ajanların gelişmesi için kullanılabilecek güçlü antibakteriyel, sitotoksik ve fitotoksik aktiviteye sahiptir.

Anahtar Kelimeler: *Teucrium stocksianum*; *Chenopodium botrys*; *Micromeria biflora*, antibakteriyel aktivite, sitotoksisite ve fitotoksisite.

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