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Ecological Studies on Some Members of Asteraceae and their Biotechnological Applications

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ABSTRACT

This study aimed at examining the main secondary metabolites, antitumor and antimicrobial activity of the aqueous extract of some Asteraceae members naturally growing at Sharkia province. *Sonchus oleraceus, Bidens pilosa, Senecio desfontainei, Lactuca serriola, Urospermum picroides* and *Cichorium pumilum* were the selected Asteraceae members in this study. Soil texture of the habitat supporting these plants was mainly silty or sandy loam soil and pH was in the alkaline side. *B. pilosa* had the highest value of total protein content at Dyarb Negm locality, while *S. oleraceus* had lowest values at Zagazig locality. The oxidative enzyme activates of the studied species revealed that *B. pilosa* had the highest POX and PhOX at Zagazig locality compared with other localities. The antimicrobial activity was tested against six microorganisms, two funguses, *Aspergillus fumigates* and *Candida albicans*, two gram positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis* and two gram negative bacteria *Escherichia coli* and *Proteus vulgaris* using the agar disc diffusion method. The inhibitory response was concentration dependent and the MIC varied according to the treatment and microorganism type. The highest inhibition values were obtained by the plant extract of *U. picroides* and *C. pumilum* against *B. subtilis.* The cytotoxicity of some Asteraceae members revealed that viability of cells decreased with the increase of concentration of aqueous extract species and lowest viability of cells was at *L. serriola* aqueous extract, while the highest viability was at *S. oleraceus* aqueous extract.

Key words : Asteraceae members, phytochemical, antitumor, antimicrobial activity

INTRODUCTION

The family Asteraceae (Compositae) is one of the largest families, 12 sub-families and 43 tribes, distributed worldwide (Li *et al.,* 2017). Many compounds isolated, and identified associated with some bioactivity. Members of the Asteraceae have various properties : antipyretic, anti-inflammatory, detoxifying, antibacterial, wound-healing, antihemorrhagic, antalgic, anti-spasmodic and anti-tussive.

In Egypt, the family is represented by 97 genera including 230 species. Asteraceae family is commonly used in the traditional treatment of various diseases due to the presence of many phytochemicals such as alkaloids, coumarins, flavonoids, benzofurans, sterols and terpenoids. Plants had been used for medicinal purposes for many years as shown in previous studies (Abd-Rani *et al.*, 2018). These include anti-inflammatory (Formisano *et al.*, 2017), antimicrobial (Ghaderi and Sonboli, 2018), antioxidant (Babota *et al.*, 2018), anti-protozoa (Garcia *et al.*, 2017), and healing activities (Ozbilgin *et al.*, 2018). Their efficacy has been suggested to be related to their ability to promote the proliferation of keratinocytes and thus the remodelling of the extracellular matrix (Chermnykh *et al.*, 2018).

Reactive oxygen species (ROS) are natural byproducts of cellular metabolism including mitochondrial respiration, peroxisome activity, inflammatory response, etc. (Forrester *et al.*, 2018). Their excessive concentrations produce an oxidative stress. Living organisms are equipped with complex antioxidant defense systems against ROS. These protective mechanisms may be either enzymatic or non-enzymatic (Aslani and Ghobadi, 2016). Ethnomedicinal literature contains a large number of plants having polyphenols as active compounds that could help against diseases related to ROS and free radicals generation (Asif, 2015). Flavonoids and phenolic acids have been identified as the most crucial phytochemicals possessing excellent antioxidant activity (Kasote *et al.*, 2015). Polyphenolic compounds from plants are of raising interest due to their redox properties which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers and metal chelating properties (Mohankumar *et al.*, 2018).

New microbial mutations, antibiotic resistance, outbreaks of pathogenic strains, etc. are increasing. Thus, a major challenge now-a-days is to develop more efficient and affordable antimicrobial agents, especially in developing countries of the world, where a higher rate of deaths is due to infectious diseases (Ferri *et al.*, 2017).

The tested microorganisms are *Aspergillus fumigates* (RCMB 002008), *Candida albicans* (RCMB 005003), *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (RCMB 015, NRRL B-543), *Escherichia coli* (ATCC 25922) and *Proteus vulgaris* (RCMB 004, 1, ATCC 13315) are from the major human pathogens that attack almost all systems (beginning from mouth reaching to urinary system). They can cause a wide variety of infections ranging from superficial skin infections, dental caries to food poisoning and life-threatening infections including meningitis, endocarditis, septicemia, pneumonia and typhoid (Tong *et al.*, 2015; Misaki *et al.*, 2016; Martin and Bachman, 2018).

MATERIALS AND METHODS

All chemicals of high grade of purity were obtained from Sigma-Aldrich (St. Louis, MA, USA). All solutions were prepared with double distilled water. Analyses, qualitative and quantitative measurements involved the use of the following tools and systems : WARING COMMERCIAL Lab Blender (Dynamics Corp. of America, New Hartford, CT, USA), Hei-VAP Rotary evaporator (Heidolph, Germany).

The antimicrobial activity was done using the fungus *A. fumigates* (RCMB 002008), *C. albicans* (RCMB 005003) the bacteria of *S. aureus* (ATCC 25923), *B. subtilis* (RCMB 015, NRRL B-543), *E. coli* (ATCC 25922) and *P. vulgaris* (RCMB 004, 1, ATCC 13315).

The aerial parts of six species of Asteraceae members were collected in April, 2021 from a natural ecosystem of different sites at the Zagazig, Belbis and Dyarb Negm, Sharkia governorate, Egypt and identified by Prof. Dr. Hussien Abd El-Basset, Professor of Plant Taxonomy, Faculty of Science, Zagazig University and a voucher specimen was deposited in the herbarium of Department of Botany, Zagazig University, Egypt.

Plants were washed several times with double distilled water to remove any debris or particulates, then shade-dried at room temperature. Aerial parts were finely ground into a fine powder. The aqueous extract was prepared by soaking the plant powder in distilled water, kept in a shaker at 20°C for 24 h by continuous agitation at 100 rpm for thorough mixing. Then the extract was filtered, concentrated and stored at -4°C for further investigations. Almost 20 g of extract was obtained from 100 g of the aerial parts.

Total soluble sugars (TSS) of seven species of Asteraceae members were extracted by overnight submersion of dry tissue in 10 ml of 80% (v/v) ethanol at 25°C with periodic shaking and centrifuged at 6000 rpm. The supernatant was evaporated till completely dried then dissolved in a known volume of distilled water (Hussein *et al.*, 2014). Total protein was determined according to the method described by Lin and Wang (2014) with bovine serum albumin as a standard. An amount of 2 g of samples was grinded in mortar with 5 ml of phosphate buffer (pH 7.6) and was then centrifuged at 8000 rpm for 20 min. Thirty ml of different samples were mixed with 70 ml of distilled water then added 2.9 ml of Coosmassic Brillaint Blue solution and mixed thoroughly. The tubes were incubated for 5 min at room temperature and absorbance at 600 nm was recorded against the reagent blank. A standard curve of Absorbance (600 nm) versus concentration (µg) of protein was calculated.

Fresh leaves of six species of Asteraceae members were frozen in liquid nitrogen to a fine powder with a mortar and pestle. Plant material was homogenized in 0.005M cold phosphate buffer (KH_2PO_4, K_2HPO_4) (pH 6.5) and centrifuged at 10.000 rpm for 10 min. The supernatant was completed to a total known volume and used as enzyme source (Maric *et al.*, 2018). The assay mixture of peroxidase contained 2.3 ml of 0.1M of phosphate buffer (pH 6) at 4°C. The reaction mixture (0.5 ml) consisted of 0.01 M pyrogallol and 0.1 ml of 0.025 M hydrogen peroxide. Addition of 0.1 ml of crude enzyme extract initiated the reaction, which was measured spectrophotometrically at 420 nm (Jiang and Penner, 2015).

For PhOX assay, the mixture contained 1.5 ml of 0.1 M phosphate buffer (pH 6) at 4°C. The reaction mixture (0.5 ml) consisted of 0.01 M pyrogallol. The addition of 1 ml of crude enzyme extract initiated the reaction, which was measured spectrophotometrically at 420 nm (Sanchez-Julia and Turner, 2021).

Alpha esterases (α-esterases) and beta esterases (β-esterases) were determined according to Biely (2016) using α-naphthyl acetate or β-naphthyl acetate as substrates, respectively. The reaction mixture consisted of 5 ml substrate solution (3×10^{-4}M α- or β-naphthylacetate, 1% acetone and 0.1M phosphate buffer, pH 7) and 20 µl of plant homogenate. The mixture was incubated for 15 min at 27°C, then 1 ml of diazo-blue colour reagent (prepared by mixing two parts of 1% diazoblue B and five parts of 5% sodium lauryl sulphate) was added. The developed colour was read at 600 or 555 nm for α- and β-naphthol produced from hydrolysis of the substrate, respectively.

The susceptibility tests were performed according to NCCLS recommendations (National Committee for Clinical Laboratory Standards). Screening tests concerning the inhibition zone were done following the well diffusion method (Matuschek *et al.*, 2014). The inoculum suspension was prepared from colonies grown overnight on an agar plate and inoculated into Mueller-Hinton broth (fungi using malt broth). The plates were kept at room temperature to allow the extract to diffuse into agar medium. The inhibition zone was measured after 24 h at 37°C. Controls using DMSO were adequately done. The lowest concentration of test sample that produced no visible growth of a given strain of bacteria was considered as MIC. Evaluation of cytotoxicity against HepG-2 cell line of the some Asteraceae members was investigated using Doxorubicin as a positive control and the untreated cells were the negative control. HepG-2 cells (human Hepatocellular cancer cell line) were obtained from VACSERA Tissue Culture Unit.

Dimethyl sulfoxide (DMSO), crystal violet and trypan blue dye were purchased from Sigma (St. Louis, Mo., USA). Fetal bovine serum, DMEM, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin and 0.25% Trypsin-EDTA were purchased from Lonza. Crystal violet stain (1%) : It was composed of 0.5% (w/v) crystal violet and 50% methanol then made up to volume with ddH_2O (double distilled water) and filtered through a Whatmann No.1 filter paper.

The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50 µg/ml gentamycin. All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were sub-cultured two times a week.

For cytotoxicity assay, the cells were seeded in 96-well plate at a cell concentration of 1 × 104 cells per well in 100 µl of growth medium. Fresh medium containing different concentrations of the test sample was added after 24 h of seeding. Serial two-fold dilutions of the tested chemical compound were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA) using a multichannel pipette. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO2 for a period of 24 h. The little percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the experiment. The absorbance of the plates was measured after gently shaken on Microplate reader (TECAN, Inc.), using a test wavelength of 490 nm. The 50% inhibitory concentration (IC50), the concentration required to cause toxic effects in 50% of intact cells was estimated from graphic plots of the dose response curve for each conc. using Graphpad Prism software (San Diego, CA, USA) (Gomha *et al.*, 2015).

The samples were prepared in triplicate for each analysis and the mean value was obtained. The results were expressed as the mean±SD. Significant differences between experimental treatments were determined by using one-way ANOVA followed by the Tukey's Post Hoc test using IBM SPSS Statistics v.19. The P value ≤0.05 was denoted as statistical significance level.

RESULTS AND DISCUSSION

The soil texture was mainly silty (at sites 1 and 2) but site three was sandy loam soil (Table 1). The soil moisture percentage was the highest in the sample of site 2 with a mean of 12.7±0.83% and the lowest (1.18±0.13%) in the soil of site 3.

pH was found in the alkaline range mainly at

Table 1. Physical properties of soil supporting some Asteraceae members at three sites of Sharkia province

Locality	Soil depth (cm)	Soil texture (%)			Soil class	Soil moisture content (%)
		Sand	Silt	Clay		
Zagazig	10-30	15.0	45.0	40.0	Silty clay loam	7.94±0.34
Dyarb Negm	10-30	7.7	44.76	47.54	Silty clay	12.70±0.83
Belbis	10-30	55.0	30.0	15.0	Sandy loam	1.18±0.13

sites 1 and 2 (Table 2). The electrical conductivity (EC) ranged between 20.12 and 44.15 mS/m. The results of soil chemical properties demonstrated that calcium (Ca++) was the dominant cation in the collected soils. Besides, the soil of sites 1 and 3 prevailed by Cl^- anion with significantly higher concentrations meanwhile soil of site 2 dominated with HCO_3^- anion. The mineral elements in samples were arranged in the following order according to their concentrations : $Ca^{+2}>K^+>Na^+>Cl^->CO_3^{-2}$, respectively.

B. pilosa had the highest value of total protein content (10.6 mg/g DW) at Dyarb Negm (S2) locality, while *S. oleraceus* had lowest values (3.45 mg/g DW) attained at Zagazig (S1) locality. The carbohydrate contents of *U. picroides* recorded highest value (23.03 mg/g DW) at Zagazig (S1) locality, meanwhile *S. oleraceus* had the lowest value (9.5 mg/g DW) at the same locality. *S. oleraceus* and *B. pilosa* naturally grew at Dyarb Negem (S2) locality accumulated more carbohydrates than the same species that grew at Zagazig (S1) locality. The obvious increase of total soluble sugars in the present work at stress conditions (Dyarb Negem locality) was due to carbohydrates contribution up to 50% of the resulted osmotic potential in glycophytes (Abdel Latef and Chaoxing, 2014). Carbohydrates such as sugars (glucose, fructose, sucrose, fructans) and starch accumulated under stress probably played a leading role in osmoprotection, osmotic adjustment, carbon storage and radical scavenging (Slama *et al.*, 2015). Soluble sugars acted as an osmotic adjustment to maintain turgor or osmotic conservation factor to stabilize the cell membranes and proteins (Jini, 2017).

Plants accumulated a significant reserve of water-soluble sugars when the photosynthesis/respiration ratio was favourable (low drought), which was used for immediate growth or storage in later development (Hanan *et al.,* 2016). Total soluble protein contents of *L. serriola* increased at Zagazig site among other Asteraceae members (Fig. 1). It was claimed that the ability of *L. serriola* to adapt to a varied macro- and micro-environmental conditions in several countries was responsible for its successful establishment and proliferation (D'Andrea *et al.*, 2017). Understanding the influence of various soil moisture regimes on *L. serriola* was an essential requirement to predict its proliferation with consequent impact on development of suitable method.

B. pilosa had the highest POX and PhOX activities among the studied Asteraceae members at Zagazig (S1) and Dyarb Negm (S2) localities (Table 3). The obtained results cleared that POX activity increased in *S. desfontainei* at Belbis (S3) locality compared with that of Zagazig (S1) locality. PhOX increased in *B. pilosa* at Zagazig (S1) locality compared with that of Dyarb Negm (S2) locality. The activities of esterases (α and β) increased of *S. oleraceus* and *B. pilosa* (Fig. 2) at Dyarb Negm (S2) locality than that at Zagazig (S1). The highest value of esterases (α and β) was obtained in *C. pumilum* among the studied

Table 2. Chemical properties of soil supporting some Asteraceae members at three sites of Sharkia province

Locality	Soil depth (cm)	pH	EC (mS/m)	Cations (ppm) (Milliequivalent/Liter)				Anions (ppm) (Milliequivalent/Liter)			OM (%)	Total N (mg/g)	Avi P (mg/kg)
				K^+	Na^+	Mg^{++}	Ca^{++}	CO_3^{--}	HCO_3^-	Cl^-			
Zagazig	10-30	9.28	20.12	0.14	0.28	0.10	0.90	_	0.18	1.82	3.28	1.37	16.02
Dyarb Negm	10-30	7.32	44.15	0.07	0.80	1.85	1.15	_	2.80	1.30	4.77	1.51	20.81
Belbis	10-30	9.27	41.31	0.22	0.44	0.50	4.50	_	0.11	0.82	1.28	0.28	44.11

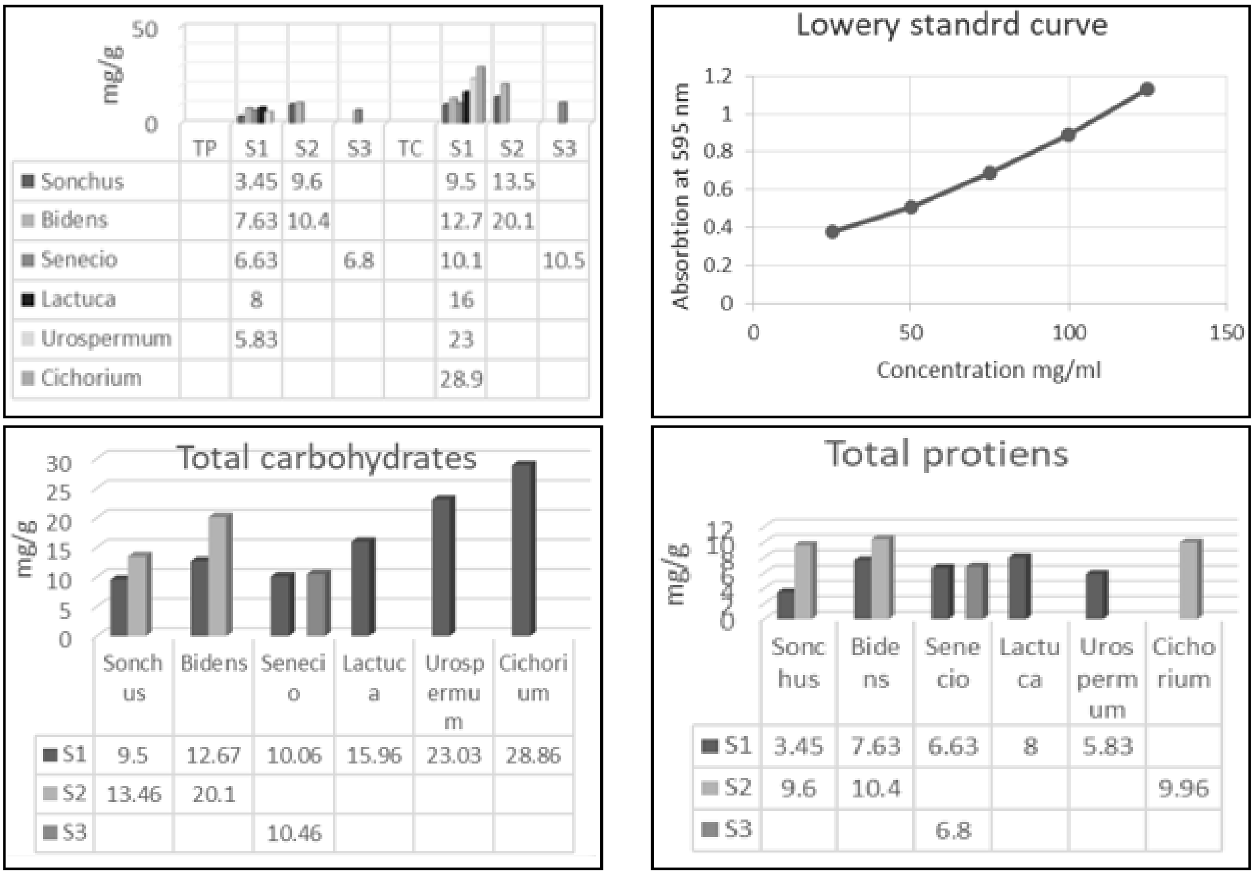

Fig. 1. Primary metabolites (mg/g DW) in some Asteraceae members at three sites of Sharkia province.

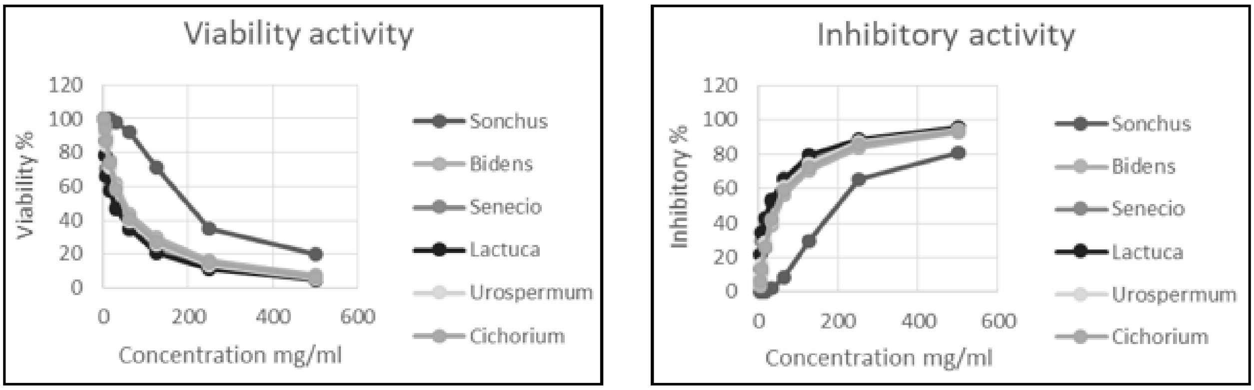

Fig. 2. Viability and inhibitory effects of activity of some Asteraceae members' aqueous extract against *Hepatocellular carcinoma* cells compared to WI38 normal cell.

Inhibitory activity against *Hepatocellular carcinoma* cells was detected under these experimental conditions with IC50 **:**

Sonchus oleraceus : IC50 = 197±5.13 µg/ml.
Bidens pilosa : IC50 = 51.7±2.31 µg/ml.
Senecio desfontainei : IC50 = 28.6±0.97 µg/ml.
Lactuca serriola : IC50 = 25.9±0.96 µg/ml.
Urospermum picroides : IC50 = 44.9±1.74 µg/ml.
Cichorium pumilum : IC50 = 45.7±2.19 µg/ml.

Asteraceae members at Dyarb Negm (S2) locality.

Stress increased levels of ROS in the plant (Huang *et al.*, 2019). To avoid damage caused by these excess ROS, plants had developed elaborate mechanisms to manage them. Enzymes played an important role in lowering the ROS levels and helping avoid oxidative

Table 3. The activity of some antioxidant enzymes (O. D. units/min/g FW) in some Asteraceae members at three sites of Sharkia

Species name	Habitats											
	Zagazig (S1)				Dyarb (S2)				Belbis (S3)			
	POX	PhOX	Esterase		POX	PhOX	Esterase		POX	PhOX	Esterase	
			α	β			α	β			α	β
Sonchus oleraceus	9.0±0.95	634.3±105.6	58.6±5.50	33.16±2.21	20.36±1.58	1283.33±101.2	139.33±5.03	40.33±3.12	-	-	-	-
Bidens pilosa	29.0 ± 2.68	2990±135.27	84.3±5.13	37.83±2.56	30.83±3.13	2280±192.87	103.66±4.72	54.5±1.30	-	-	-	-
Senecio desfontainei	22.6±0.87	1126.6±83.26	130±7.81	52.5±2.0	-	-	-	-	81.46±0.9	673.33±102.2	81.33±6.1	28.5±1.2
Lactuca serriola	16.3±1.24	920±55.67	209.3±8.32	49.03± 2.95	-	-	-	-	-	-	-	-
Urospermum picroides	16.36±1.19	866.6±112.39	92.0±6.08	28.5±1.73	-	-	-	-	-	-	-	-
Cichorium pumilum	-	-	-	-	39.43±3.41	1513.3±136.5	247.66±19.7	82.00±4.89	-	-	-	-

POX – Province peroxidase and PhOX – Phenol oxidase.

stress (Hossain *et al.*, 2015). Antioxidant enzymes such as POX and PhOX reduced the levels of superoxide and hydrogen peroxide in stressed plants (Abd-Elgawad *et al.*, 2016). In addition, (Das *et al.*, 2016) reported that PhOX and POX were the most effective antioxidant enzymes in preventing cell damage. These two enzymes revealed a great importance in regulating H_2O_2 intracellular levels (Halder *et al.*, 2018). Over-expression of the POX gene in plants improved protection against oxidative stress (Hossain *et al.*, 2014). The obtained data showed that PhOX activity was increased by salinity levels at Dyarb Negm locality. For that reason, it was supposed that PhOX and POX were probably had equal importance in the detoxification of H_2O_2 under salinity stress. The present results are in agreement with many reports suggesting that PhOX activity, coordinated with POX activity for playing a central protective role under salinity (El-Beltagi *et al.*, 2020). Significant role of PhOX was suggested in plant development processes (Yaish *et al.*, 2015), which was involved in scavenging of H_2O_2 production in chloroplasts (Habibi, 2014). PhOX activity in drought or simultaneously in salinity and drought stress was enhanced (Bilal *et al.,* 2020) who found that salinity increased total PhOX activity in explants of soybean under salinity stress. The enhancement of PhOX activity by salinity was also observed in rice (Khan *et al.,* 2016), pea (El-Araby *et al.*, 2020) and mulberry (Al-Kharusi *et al.,* 2019). Peroxidation of membrane lipids was an indication of membrane damage and leakage under salt stress conditions (Kumar *et al.*, 2015).

Ketoconazole was used as a control for fungus (Table 4), while Gentamycin was used as a control for bacterial tested organisms. The highest inhibition zone values (10 mm) were obtained by the plant extract of *U. picroides* and *C. pumilum*.

The cytotoxicity of some Asteraceae members' aqueous extract was investigated using Doxorubicin as a positive control, and the untreated cells were the negative control (Fig. 2). The viability of cells decreased with the increase of concentration of aqueous extract of the tested species. The lowest viability of cells was at *L. serriola* aqueous extract, while the highest viability of cells was at *S. oleraceus* aqueous extract.

Table 4. Antimicrobial activity of some Asteraceae members' aqueous extract (Inhibition zone in mm)

Tested microorganisms	*Sonchus oleraceus*	*Bidens pilosa*	*Senecio desfontainei*	*Lactua serriola*	*Urospermm picroides*	*Cichoriu mpumilum*	Control
Fungi							**Ketoconazole**
Aspergillus fumigates (RCMB 002008)	NA	NA	NA	NA	NA	NA	17
Candida albicans RCMB 005003 (1) ATCC 10231	NA	NA	NA	NA	NA	NA	20
Gram positive bacteria							**Gentamycin**
Staphylococcus aureus ATCC 25923	NA	NA	NA	NA	NA	NA	24
Bacillus subtilis RCMB 015 (1) NRRL B-543	NA	8	NA	8	10	10	26
Gram negatvie bacteria							**Gentamycin**
Escherichia coli ATCC 25922	NA	NA	NA	NA	NA	NA	30
Proteus vulgaris RCMB 004 (1) ATCC 13315	NA	NA	NA	NA	NA	NA	25

NA–No activity.

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