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Evaluation of some biological activities of *Trigonella hamosa* aerial parts

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The antimicrobial activity of *T. hamosa* aerial parts extract against *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus fecalis*, *Escherichia coli*, *Pseudomonas auregenosa*, *Candida albicans*, *Fusarium* sp. and *Aspergilus niger* was evaluated by disc diffusion method. The minimum inhibitory concentrations (MICs) for susceptible test microorganisms were further determined. Also the antioxidant and α-amylase inhibition activities were investigated. The extract exhibited antimicrobial activity against *C. albican, B. subtilis* and *E. fecalis;* the MICs were 5.5, 7 and 8.5 mg/ml, respectively. Antioxidant and α-amylase inhibition activities of the extract were concentration dependent and the IC_{50} values were 0.19 and 35 mg/ml, respectively. Thin layer chromatography (TLC) bioautography was used to detect the bioactive fractions of the extract. It revealed that three different fractions of different polarities exhibited antimicrobial, α-amylase inhibition and antioxidant activities. The toxicity of the extract to brine shrimp (*Artemia salina*) larvae was assessed and the LC_{50} was found to be 2.3 mg/ml. This study suggests that the extract of *T. hamosa* aerial parts could be a source of bioactive compounds of different biological activities and is safe in terms of toxicity level.

Key words: *Trigonella hamosa,* antimicrobial, antioxidant, α-amylase, toxicity.

INTRODUCTION

Medicinal plants have been the subject of concern as a source of important therapeutic drugs useful for the treatment of various diseases. The interest in drugs derived from plants is primarily attributed to the belief that green medicine is safe and dependable compared to their synthetic counterparts. A wide range of substances that can be useful for the treatment of chronic as well as infectious diseases have been obtained from plants used in traditional medicine (Harish et al., 2010; Srinivas et al., 2011). It has been frequently shown that, different parts of medicinal plants which include stems roots, leaves, flowers, seeds, etc contain bioactive compounds (Bibi et al., 2005; Rai et al., 2007; Sindhu et al., 2013). The bioactive compounds derived from plants have broad spectrum of biological activities such as antimicrobial, antiviral, antioxidant, antitumor, enzyme inhibitors, etc. (Srinivas et al., 2011; Alagesan et al., 2012; Chikezie et al., 2015). Currently, it is well known that, the indistinctive

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use of antibiotics either for the treatment of human infectious diseases or for overprotection of animal farms has led to the emergence of antibiotic resistance phenomenon among pathogenic microbes (Khan et al., 2003; Hopwood, 2007). The high incidence of multidrug resistant pathogens has threatened the effectiveness of existing antibiotics and necessitated the urgent need for the discovery of novel antimicrobial compounds possessing unique mode of action to combat these resistant microbes (Westh et al., 2004; Penner et al., 2005; Barbosa et al., 2009; Hazni et al., 2008; Kumar et al., 2008; 2010; Rawat and Upadhyaya, 2013). Antimicrobial compounds derived from plants are effective candidates for the treatment of many infectious diseases with less side effects compared to their synthetic counterparts (Mukherjee and Wahile, 2006; Mehrotra et al., 2010).

The high level of blood glucose associated with diabetes mellitus is mainly due to either the secretion of inadequate amount of insulin by pancreas or the decreased ability to respond to insulin by cells. Inhibition of enzymes involved in hydrolysis of complex carbohydrates such as α- amylase is one of the important therapeutic strategies to treat diabetes (Nair et al., 2013). Medicinal herbs contain chemical constituents with α-amylase inhibition activity and could be potential drugs for the treatment of type II diabetes (Dastjerdi et al., 2015). It has been elucidated that, the onset and progression of complications associated with diabetes may be mediated by the generation of high level of free radicals not balanced by the antioxidative defense system as in healthy individuals (Salehi et al., 2013). Therefore, antioxidants play an important role in maintenance of the antioxidant level in the body and minimize the long term complications as well (Iwai, 2008). Bioactive compounds of medicinal plants have been studied for their antioxidant activity as alternative to synthetic ones (Parejo et al., 2002).

The genus *Trigonella* L. comprises about 135 species and is extremely important from medicinal point of view as they are famous for their steroidal Saponins contents. Most of the work has been carried out on T. *foenum-graecum* to discover the wealth bioactive compounds present in different parts of the plant and few reports are available on bioactivity of *Trigonella hamosa* L. (Hamed, 2007; Salah-Eldin et al., 2007; Yadav and Baquer, 2014). The present work was done to evaluate the antimicrobial, antioxidant and α-amylase inhibition activities as well as cytotoxicity of the aerial parts extract of T. *hamosa*

MATERIALS AND METHODS

Plant material

The aerial parts of *T. hamosa* L. were collected in spring of 2014 from Sohag Governorate, Egypt. The plant was identified by using a voucher specimen (T-119) deposited in the herbarium in Botany Dept., Faculty of Science, Sohag University, Sohag, Egypt.

Extraction and separation

The air-dried and powdered plant (1 Kg) was extracted exhaustively with CH_2Cl_2-MeOH (1:1) at room temperature. The solvent was distilled under reduced pressure, furnishing a gummy residue (20 g). The residue was kept in sterile glass vials and stored at 4ºC until use.

Antimicrobial activity

Microbial strains

The microbial strains used during this study included: *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATTC 25923, *Enterococcus fecalis* ATCC 29212, *Escherichia coli* ATCC 8739, *Pseudomonas auregenosa* ATCC 4027, *Candida albicans* ATCC 10231, *Fusarium* sp. and *Aspergilus niger*. These microbial strains were kindly provided by staff members of microbiology lab., National Institute of Oceanography and fisheries, Alexandria, Egypt.

Preparation of inoculum

To prepare the inoculum of bacterial strains, cells were grown in nutrient broth and incubated for 24 h at 32°C. Cultures were centrifuged at 7000 rpm, washed with sterile saline and adjusted to 0.1 O.D at 600 nm and stored at 4°C until use (Cwala et al., 2011). Fungal inoculum was prepared according to Wayne (2002) and culture suspension was standardized to 0.1 O.D at 530 nm.

Disc diffusion assay

Preliminary assessment of antimicrobial activity of *T. hamosa* extract was carried out by disc diffusion method. Nutrient agar (NA) and potato dextrose agar (PDA) plates were spread with 50 µL of different bacterial and fungal cultures standardized to O.D 0.1 respectively. Sterile filter paper discs, 6 mm in diameter, were impregnated with 50 µL of *T. hamosa* aerial parts extract (100 mg/ml) or methanol, dried and placed on the NA or PDA plates previously seeded with the respective microorganism. After incubation for 24 h for bacteria and 48 h for fungi, the presence of sterile zone around each paper disc indicative of antimicrobial activity was observed (Rajauria et al., 2012; Abdel-Shakour et al., 2015).

Determination of minimum inhibitory concentration (MIC)

The MICs for susceptible microbial indicators, as indicated by disk diffusion assay, were determined. Nutrient broth medium was prepared in test tubes and sterilized by autoclaving. Water stock solutions of *T. hamosa* aerial parts extract were aseptically added to sterile nutrient broth medium to give a final concentration of 0.5 to 8.5 mg/ml. Each tube was inoculated with 10 µL of standardized inoculum of the respective test organism. Individual blanks were done for each tube which contain the growth media and extract without the inoculum. All tubes were incubated aerobically at 37°C for 24 h. The concentration at which no growth was observed in comparison with the blank tube was determined (Akinyemi et al., 2006).

α-amylase inhibition activity

To assess the α-amylase inhibition activity, α-amylase solution (0.5 mg/ml) and starch solution (10 mg/ml) were prepared in 0.02 M

Table 1. Antimicrobial activity of aerial parts extract of *T. hamosa.*

Test microorganism	*Mean diameter of growth inhibition zone (mm)
Bacteria	
Bacillus subtilis	14±0.6
Staphylococcus aureus	0
Pseudomonas aureginosa	0
Enterococcus fecalis	13±0.5
Escherichia coli	0
Fungi	
Candida albicans	16±1
Aspergillus niger	0
Fusarium sp.	0

* Values are presented in mean ± SD (n =3).

sodium phosphate buffer (pH 6.9) containing 0.006 M sodium chloride. The color reagent (dinitrosalicylic acid reagent) was prepared according to Miller (1959). In a test tube, 500 µL of enzyme solution was mixed with 500 µL of extract of different concentrations (20-55 mg/ml) prepared in deionized water and incubated for 10 min at 25°C. After pre- incubation, 500 µl of starch solution was added to each tube and further incubated at 25°C for 10 min. To stop the reaction, 1 ml of dinitrosalicylic acid reagent was added. The tubes were then incubated in boiling water bath for 5 min, cooled to room temperature and diluted with 10 ml of distillated water. To correct the background absorbance, individual blanks were made in which the starch solution was added after addition of the color reagent and boiling and the method was followed as described above (Suthindhiran et al., 2009; Dastjerdi et al., 2015). The inhibition activity of α-amylase was calculated as follows:

$$\text{Inhibition (\%)} = \frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100$$

Antioxidant activity

The antioxidant activity of *T. hamosa* extract was evaluated by DPPH (1, 1-Diphenyl-2-picrylhydrazyl) radical scavenging activity. DPPH solution (20 µcg/ ml) and *T. hamosa* extract of different concentrations (0.125-0.75 mg/ml) were prepared in methanol. In a test tube equal volumes of DPPH solution and extract were mixed and incubated in the dark at room temperature for 30 min. The absorbance of each concentration and the control (containing equal volumes of DPPH and methanol) was measured at 517 nm (Khalaf et al., 2008; Ravikumar et al., 2008). Radical scavenging activity was calculated according to the following equation:

$$\text{Radical Scavenging Activity (\%)} = \frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100$$

Thin layer chromatography (TLC) bioautography for bioactivity screening

Plant extract was separated using silica gel plates (GF254, Merck, Darmstadt, Germany) and developed in ethyl acetate: hexane (2:5 v/v) or ethyl acetate: hexane: methanol (3:1:1 v/v) as mobile system. After development in mobile phase, the plates were allowed to dry on air. To evaluate the antimicrobial activity of separated compound, dried TLC plate was placed on nutrient agar medium previously seeded with *Candida albicans* and incubated for 2h at 4°C. The TLC plate was removed and the culture was incubated at 37°C for 24 h. The presence of sterile zone on the media indicated the presence of active component possessing antimicrobial activity (Moncheva et al., 2002). For α-amylase inhibition activity, dry developed TLC plate was put on sterile nutrient agar medium supplemented with 0.1% starch and kept at 4°C for 2h to allow diffusion of crude extract components to the nutrient agar medium. The TLC plate was removed and the plates were sprayed with α-amylase solution (0.5 mg/ml in 0.02 M sodium phosphate buffer pH 6.9). After incubation at 37°C for 12 h, the plate was flooded with iodine solution and examined for the presence of blue colored zone indicating the presence of active component possessing α-amylase inhibition activity (Fahmy, 2016). To detect compounds with antioxidant activity, plates were sprayed with 2.54 M DPPH solution in methanol. After drying, bands exhibiting antioxidant properities appeared in yellow color on purple background (Rajauria and Abu-Ghannam, 2013). The R_f (retention factor) value of the bioactive components was calculated.

Evaluation of the toxic effect

The toxicity of *T. hamosa* aerial parts extract was assessed by brine shrimp (Artemia *salina*) larvae assay. Eggs were hatched in natural sea water at 25°C under constant aeration for 48 h. The container was continuously illuminated by normal light along the hatching experiment. Different concentrations of the extract (1to 5 mg/ml) were made in sea water and incubated with 15-30 brine shrimp larvae. After 24 h, the nauplii were examined and the mortality was determined (El-Maghraby and Shebany, 2014).

RESULTS AND DISCUSSION

Antimicrobial activity

The extract of *T. hamosa* aerial parts was screened for antimicrobial activity by disc diffusion method. The results obtained for the antimicrobial activity of the extract are presented in Table 1 and expressed as inhibition zone in (mm). The extract exhibited antimicrobial activity against *Bacillus subtilis*, *Enterococcus fecalis* and *Candida albicans* but no activity was observed against

Table 2. MIC values of aerial parts extract of T. *hamosa.*

Test microorganism	MIC (mg/ml)
Candida albicans	5.5
Bacillus subtilis	7
Enterococcus fecalis	8.5

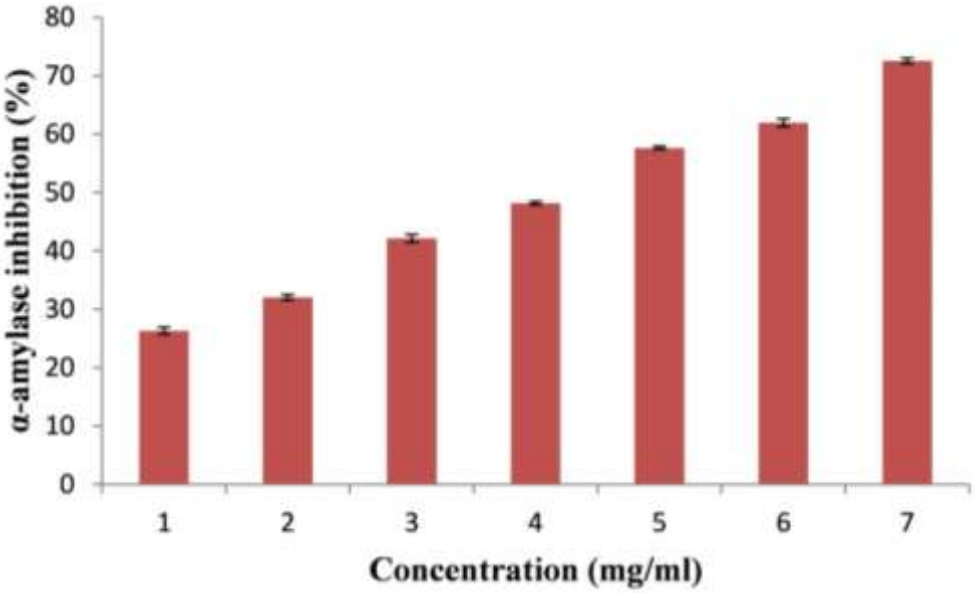

Figure 1. α- amylase inhibition activity of aerial parts extract of T. *hamosa.* Values are presented in mean ± SD (n =3).

Escherichia coli, *Pseudomonas aurigenosa*, *Staphylococcus aureus, Aspergillus niger* or *Fusarium* sp. These results suggest that the aerial parts of *T. hamosa* aerial parts possess antimicrobial activity against selected members of Gram negative bacteria, Gram positive bacteria and fungi. These results could suggest the presence of bioactive compound with broad spectrum antimicrobial activity (Aboud, 2015). Plants have been frequently reported as promising source of antimicrobial agents (Rabe and Staden, 1997; Njume et al., 2011). However, Kuete et al. (2013) studied the antibacterial activities of methanolic extract of the aerial parts of T. *hamosa* collected from Tanhat protected area (Saudi Arabia) against eight bacterial strains belonging to four species of Gram negative bacteria. They reported that the extract possesses no activity against the tested bacterial species. The production and accumulation of primary and secondary metabolites by plants is greatly affected by the environmental factors. Moreover, secondary metabolites biosynthesis by plants is the result of chemical interaction between plants and their environment, and variations in the plant metabolic profiles seem to be a direct response to changes in conditions of the plant surrounding environment (Sampaio et al., 2016). Therefore, the difference in environmental conditions could account for variation in the metabolic profile of the same plant species collected from different geographical locations.

The minimum inhibitory concentrations of T. *hamosa* aerial parts extract for susceptible microorganisms tested are shown in Table 2. The MIC values against *Candida albicans*, *Bacillus subtilis* and *Enterococcus fecalis* were 5.5, 7 and 8.5 mg/ml, respectively.

α-amylase inhibition activity

The inhibitory effect of *T. hamosa* aerial parts extract against α- amylase enzyme was investigated. The inhibitory activity of the extract was observed at 20, 25, 30, 35, 40, 45 and 50 mg/ml. The extract showed a significant α- amylase inhibition activity under *in vitro* conditions. The inhibition activity showed a concentration-dependent manner. The highest concentration (50 mg/ml) of the extract tested showed a maximum inhibition of 72.64% on the activity of α-amylase and the lowest concentration (20 mg/ml) exhibited minimum inhibition percent of 25.9% (Figure 1). The IC50 was calculated as 35 mg/ml. Bioactive compounds exhibiting α-amylase inhibition activity constitute one of the most important

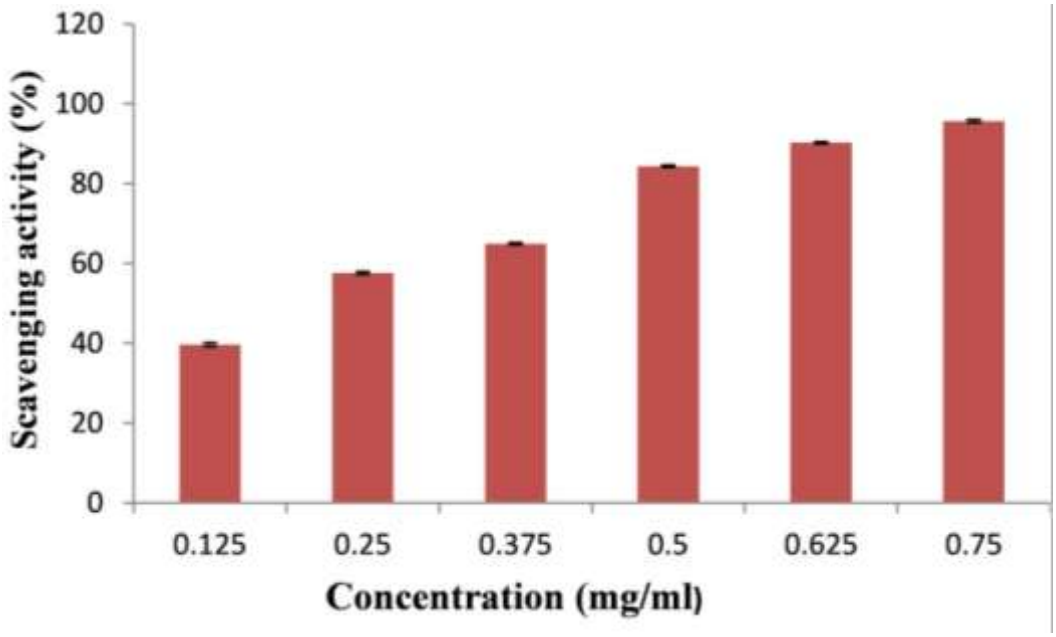

Figure 2. DPPH scavenging activity of aerial parts extract of *T. hamosa.* Values are presented in mean ± SD (n =3).

families of compounds with anti-diabetic activity. These compounds have great advantage and useful for the treatment of noninsulin diabetes mellitus (Cheng and Fantus, 2005; Upadhyay and Ahmad, 2011). Synthetic α-amylase inhibitors such as acarbose and miglitol induce gastrointestinal side effects; therefore, α-amylase inhibitors derived from medicinal herbs could be safer than their synthetic counterparts with lower negative effects (Dastjerdi et al., 2015). More than 1,200 plant species of plants used in traditional medicine have been reported to possess antidibetic activity. The search for novel bioactive compounds from these herbs could lead to potent inhibitors for enzymes involved in the development of diabetes (Narkhede, 2012).

Antioxidant activity

The antioxidant activity of *T. hamosa* aerial parts extract was examined by DPPH scavenging activity at 0.125, 0.250, 0.375, 0.5, 0.625 and 0.750 mg/ml. It showed a concentration dependent DPPH scavenging activity. The gradual increase in extract concentration resulted in a linear increase in DPPH scavenging activity and exhibited 95.7% activity at 0.75 mg/ml (Figure 2). The IC50 was calculated as 0.19 mg/ml. It is evident from the results that the extract possesses promising antioxidant activity. The DPPH method is the most common for measuring the radical scavenging activity of plant extracts due to rapidness, simplicity and independence of sample polarity (Marinova and Batchvarov, 20110). In the presence of compounds capable of donating hydrogen atoms, the violet color of DPPH molecule is lost and the absorbance at wavelengths around 520 nm is decreased (Molynex, 2004). The free radical scavenging activity of T. *hamosa* aerial parts extract indicates the presence of efficient antioxidant compound(s) in terms of hydrogen atom donating ability

TLC bioautography

For TLC bioautography of antimicrobial and α-amylase inhibition activities, the TLC plates developed with ethyl acetate: hexane (2:5 v/v) were placed on nutrient agar plates previously inoculated with *Candida albicans* and sterile nutrient agar plates supplemented with starch, respectively. The results showed that only one band (R_f= 0.3) exhibited antimicrobial activity (Figure 3a) and one band (R_f= 0.19) showed α-amylase inhibition activity (Figure 3b). Regarding the TLC bioautography for antioxidant activity, when the TLC plate was developed with ethyl acetate: hexane (2:5 v/v), the fraction exhibiting antioxidant activity failed to migrate (Figure 3c), whereas, when the plate was developed with ethyl acetate: hexane: methanol (3:1:1 v/v) the fraction exhibiting antioxidant activity was detected at R_f = 0.79 (Figure 3d.). The fraction exhibiting antioxidant activity is characterized by yellow color in purple background as the delocalized spare electron of the DPPH molecule which give its deep violet color is transferred to hydrogen proton donated by the antioxidant compound(s) present in the extract leaving the yellow color of the picryl group (Molyneux, 2004). DPPH has been frequently used to screen the

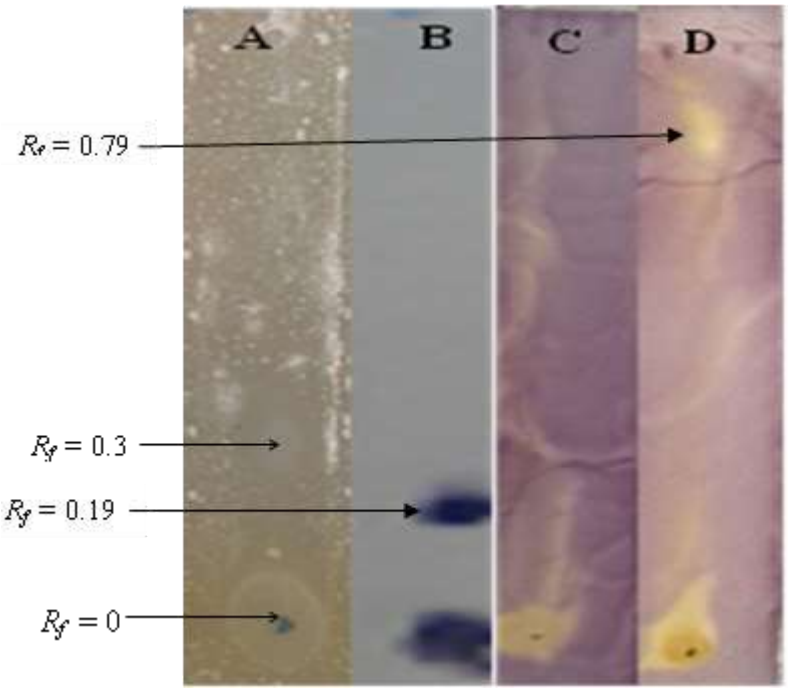

Figure 3. TLC bioautography for antimicrobial (A), α-amylase inhibition activity (B) and antioxidant activity (C and D).

antioxidant activity of plant extracts (Jaime et al., 2005; Gu et al., 2009; Bhagavathy et al., 2011). These results suggest that the bioactive compounds exhibiting antimicrobial, α-amylase inhibition and antioxidant activities have different polarities.

Toxic effect

Preliminary assessment of the toxological properties of *T. hamosa* extract was carried out by brine shrimp larvae assay. The percentage of mortality of brine shrimp larvae exposed to different concentrations of *T. hamosa* aerial parts extract is illustrated in Figure 4. At 1 mg/ml concentration, the extract displayed no toxicity, whereas, all the nauplii were killed at 5 mg/ml of the extract and the LC50 was 2.3 mg/ml.

Natural products from medicinal plants which have several pharmacological and biological activities could possess toxological properties as well. Therefore, toxicity assessment is important to achieve safe use of these products. Brine shrimp larvae assay is the most suitable for the evaluation of toxicity of plant extracts due to simplicity, rapidness and low requirements (Hamidi et al., 2014).

According to Meyer and Clarkson who proposed a toxicity indexes for the assessment of the toxicity of plant extracts and stated that, plant extracts with LC 50 greater than 1 mg/ml are considered nontoxic; *T. hamosa* aerial parts extract is nontoxic to brine shrimp larvae (Meyer et al., 1982; Clarkson et al., 2004).

Moreover, it has been reported that brine shrimp larvae are more sensitive than cell culture and the toxic doses range from 10 to 100 greater than that of cell culture (Solis et al., 1993). This could suggest the safe use of *T. hamosa* aerial parts extracts for human use.

Conclusion

Our results indicated that the extract of *T. hamosa* aerial parts contains bioactive compounds of different biological activities such as antimicrobial, antioxidant and α-amylase inhibition activities which could be useful for pharmaceutical industries and is not toxic. Further studies are required for identification of these bioactive compounds.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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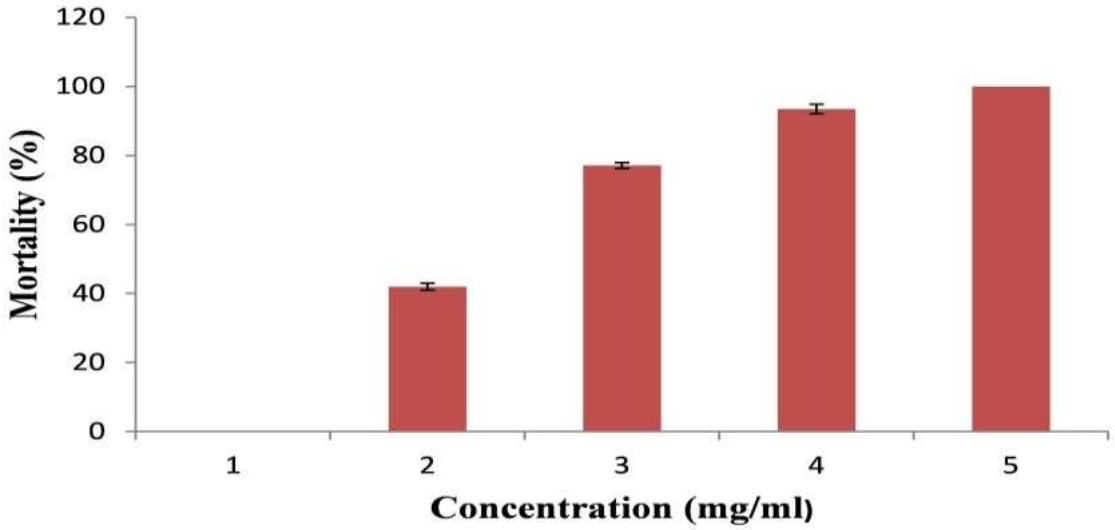

Figure 4. Percentage of mortality of brine larvae exposed to *T. hamosa* aerial parts extract. Values are presented in mean ± SD (n =3).

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