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Medicinal plants as surface activity modifiers

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Surface active agents have been used in pharmaceutical formulations for different purposes, so the study of the effect of these agent on biological membranes is necessary. The aim of this study is the evaluation of aqueous extract of *Tribulus terrestris* L., *Trigonella foenum-graecum* L. and *Echium amoenum* Fisch, that contain saponins on red blood cells (RBC) as a model of biological membranes. Also some physicochemical properties of the extracts including emulsification index (E_{24}) and foam producing activity (F_h) were investigated. The aqueous extracts were prepared by maceration and then lyophilized. The different concentration of extracts in McIvan's buffer solution were incubated with RBC in different temperatures (25 and 37°C) for different time periods (15 and 30 min). The absorbance of the samples by UV spectrophotometer determmine the degree of hemolysis. In comparison of three studied extracts, *T. terrestris* L, have shown the highest hemolytic effect (12.45% in 37°C and 30 min). The values of E_{24} and F_h showed the extract of *T. terrestris* L. has the highest emulsification index (24.89%) and the highest foam producing activity (14.42 mm). The extract of *H. persicum*, with lower hemolytic effect may be preferred in pharmaceutical preparation but if the hemolytic effect was excluded, *A. dracunculus* is preferred.

Key words: *Echium amoenum*, *Trigonella foenum-graecum*, *Tribulus terrestris*, biological membrane, hemolysis.

INTRODUCTION

The Plant's saponins are naturally occurring triterpene or steroid glycosides that possess a number of biological and pharmacological activities. Saponins are found in a number of medicinal plants and are secondary metabolites of the plants. They contain either a steroidal or a triterpenoid aglycone to which one or more glycoside chains are attached. The glycoside subunits usually include glucose, galactose, glucuronic acid, xylose or rhamnose. They exhibit a range of biological properties, both beneficial and deleterious. The main biological activity ascribed to saponins is their cell membrane permeabilizing properties (Plock et al., 2001; Menin et al., 2001; Melzig et al., 2001; Seeman et al., 1973). They are natural soap like and foam-forming compounds that are widely used in foods, cosmetic and pharmaceutical preparations. The soap like charecteristic of saponins originate from their surface activities regarding the combination of hydrophilic chains (glycoside) and nonpolar aglycon moiety. Because of their foam producing property, they are widely used in the manufacture of foods, beverages and cosmetic preparations. The diversity of saponins is because of variability in the aglycone structure, the glycoside moiety and diferent combination of them. Saponins have several applications, especially in medicine, but their membrane permeabilizing activity has been of special interest. In comparison to synthetic surfactants, the natural ones have attracted more attention because of their advantages such as diverse usage as emulsifier, foaming agent, detergents and safety and ease of preparations. (Price et al., 1987; Lacaile et al., 2005). Surfactants used in pharmaceutical dosage forms of drugs are with limited absorption such as peptides or proteins to serve as penetration enhancer. A broad spectrum of surfactants is used for this purpose including Bile salts, anionic

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detergents, glycerides and lysophospholipids. Morphological and biochemical studies on membrane of absorption sites showed that surfactants enhance membrane transport followed by acute toxicity but these effects were reversible (Williams, 2004). As a result, there is a fundamental relationship between permeability enhancement activity and potential to damage human skin; furthermore, permeability enhancing activity of surfactants is not only associated with their nature, but moreover depends on other characteristics like electrical charge and polarity (Galembeck, 1998; Gould, 1996). Permeability enhancers are agents that decrease cellular layer resistance reversibly and allow the drug to pass through and between epithelial cells toward blood and lymph. Recently, enhancing drugs permeability through cellular membrane has become one of the main topics in pharmaceutical researches (Muranishi, 1990; Williams, 2004). Evaluating the permeability of enhancers using biological membranes plays an important role in developing pharmaceutical formulation. Different *in vitro* models exist for evaluation of membrane toxicity of surfactants including single cell models using erythrocytes, erythrocyte ghosts or liposomes. The erythrocyte model has been widely used as it presents a direct indication of membrane toxicity.

An additional advantage of erythrocytes model is that RBC suspension are easily prepared; furthermore, its membrane has similarities with other cell membranes (Robertis and Robertis, 1995). Therefore, in this satudy, the effects of the aqueous extract of three medicinal plants- *Trigonella foenum-graecum* L., *Echium amoenum* F.M. and *Tribulus terrestris* L. on biological membranes have been evaluated. The primary phytochemical screening has detected the presence of saponins in these plants (Price et al., 1987; Lacaile et al., 2005).

MATERIALS AND METHODS

The medicinal plants- *Tribulus terrestris*, *Trigonella foenum-graecum, Echium amoenum* were collected from Kerman province of Iran. The aqueous extract was prepared in our laboratory. All the materials used were of pharmaceutical grade except otherwise mentioned. Sodium chloride, di-sodium hydrogen phosphate, citric acid (monohydrate), di-sodium phosphate, and liquid paraffin were purchased from Merck (Germany). Drabkin's agent was supplied from Chimi-Daru (Iran). McIlvaine's buffer was prepared as follows:

Solution I, containing 21.000 g of citric acid (100 mM) and 8.775 g of sodium chloride (150 mM) made up to 1000 ml with deionized water. Solution II, containing 28.400 g of di-sodium hydrogen phosphate (200 mM) and 8.775 g of sodium chloride (150 mM) made up to 1000 ml with deionized water. These two solutions were mixed and the pH was adjusted to 7.0. The pH of final buffer solution was measured by electrical pH-meter (TWT Metrohm, Germany).

Erythrocyte suspension

Human blood was collected from healthy individuals with hematocrit around 46.7%. The heparinized blood was centrifuged at 3000 rpm for 10 min (Hermle 230 ZA, Germany) and the plasma was removed. The erythrocytes were washed three times with McIlvaine's buffer. Afterward, an erythrocyte suspension with 12% hematocrit was achieved and kept in 4°C for the experiments (Gould et al., 2000).

Membrane permeabilizing activity

A suspension of erythrocyte (200 µl) with an equal volume of one extract within a micro-tube was incubated for the determined period at 25 and 37°C. After incubation, the mixtures were spun in a microcentrifuge at 3000 rpm for 35 s (Spectrafuge 161M, England), and 200 µl of the supernatant was added to 3 ml of Drabkin's reagent. The amount of hemoglobin released was determined by the absorbance of samples in 540 nm wavelength using spectrophotometer (Shimadzu, 3100, Japan). The positive control consisted of 200 µl of uncentrifuged mixture of erythrocyte suspension and 200 µl of the buffer, which were added to 3 ml of Drabkin's reagent to obtain complete hemolysis. The negative control, measured the level of spontaneous hemolysis, included 200 µl of the buffer mixed with 200 µl erythrocyte suspension and after centrifugation for 35 s, 200 µl sample of supernatant was added to 3 ml of Drabkin's reagent. The amount (percent) of hemolysis for each sample was calculated by dividing sample absorbance by the positive control absorbance (complete hemolysis) multiplied by 100 (Gould et al., 2000) which was calculated using the following equation:

$$Hemolysis\ (\%) = \frac{Sample\ Absorbency}{Blank\ Absorbency} \times 100$$

Emulsification index (E_{24})

For detection of emulsification index (E_{24}), 5 ml of liquid paraffin was added to 5 ml of different concentrations of aqueous extracts in a graduated tube and vortexed at high speed for two minute. The emulsion stability was determined after 24 h. The E_{24} was calculated by measuring the emulsion layer formed (Carrillo et al., 1996).

Foam formation activity (F_h)

Different concentrations of tested extracts were dissolved in 5 ml disodium phosphate buffer and shaked with vibrator for 5 s. The samples put aside at 25°C for 1 min. The foam formation activity (F_h) was measured by foam height in graduated cylinder (Dehghan et al., 2008).

RESULTS AND DISCUSSION

The results of hemolysis induced by aqueous extracts are presented in (Figures 1 and 2). In order to compare the hemolytic effects of all extracts, the concentration of each extract needed to induce 50% haemolysis was determined. Results of E_{24} and F_h are presented in (Figures 3 and 4), respectively. Although, the exact mechanism of surfactants hemolysis is not fully known, it is proposed that it may be absorbed through penetration of the cell membrane by altering its permeability to

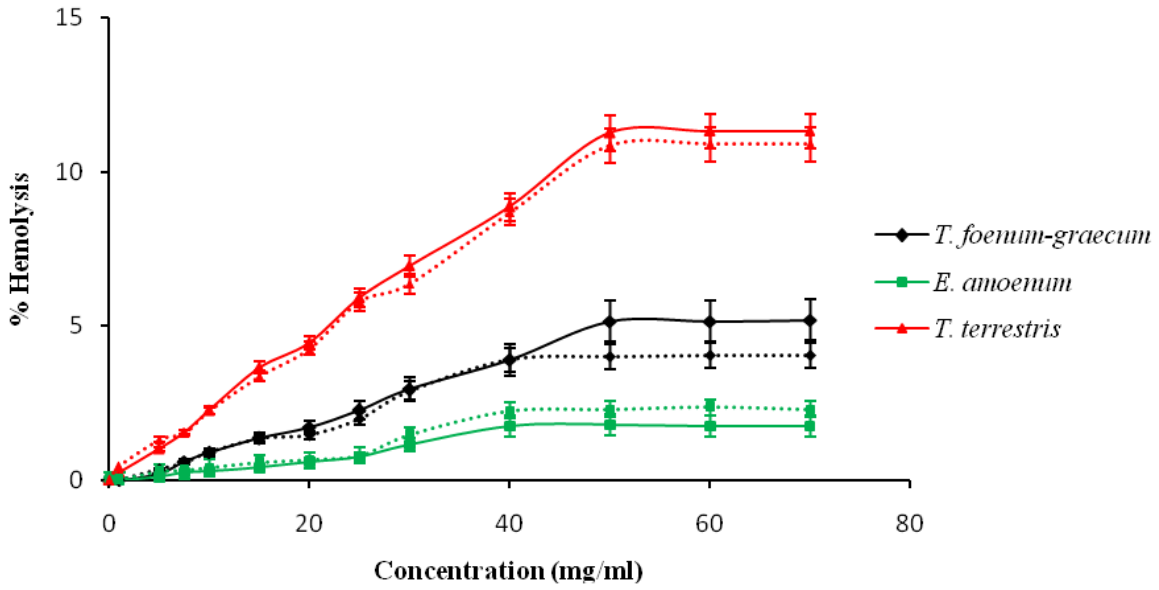

Figure 1. Comparison of percent of hemolysis induced by *T. terrestris(red), T. foenum-graecum(black), E. amoenum(green)* Aqueous extracts within 15 min (solid line) and 30 min (dotted line) at 25℃ (Mean ± SD , n = 3).

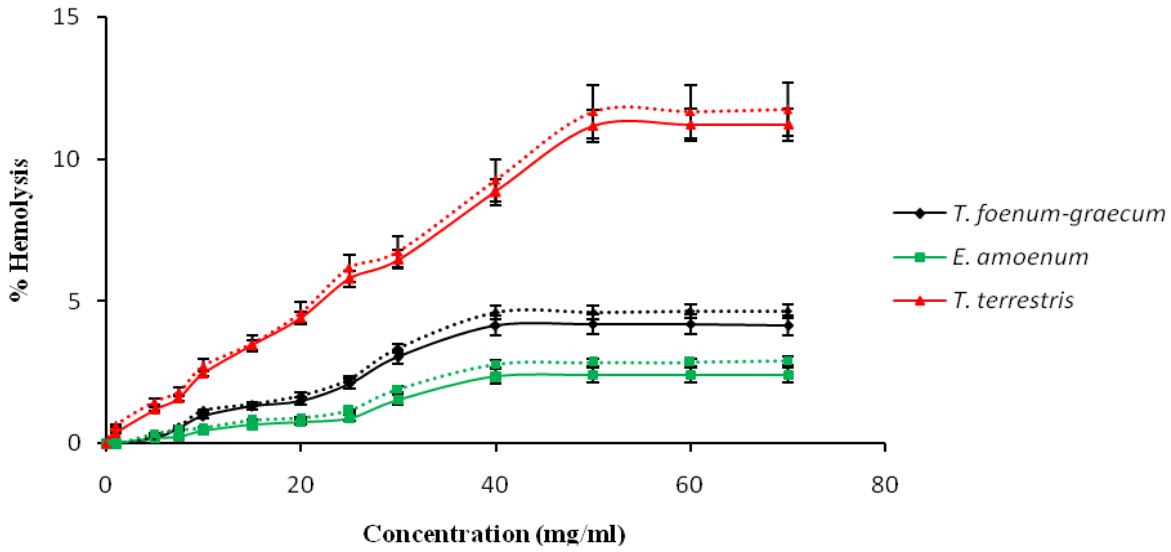

Figure 2. Comparison of percent of hemolysis induced by *T. terrestris(red), T. foenum-graecum(black), E. amoenum(green)* Aqueous extracts within 15 min (solid line) and 30 min (dotted line) at 37℃ (Mean ± SD, n = 3).

change the osmotic pressure, which in turn causes the cellular lysis (Dehghan et al., 2008). Biological membrane consists of a lipid bilayer which is stabilized by non-covalent bonds among acyl groups and ionic bonds between polar heads and aqua. The hemolysis is due to RBC membrane destruction which resulted from lysis of membrane lipid bilayer. As this hemolysis relates to concentration and type of surfactant, this model can be used for assessment of surfactants strength (Swenson, 1992). Regarding the probable toxicity of synthetic surfactants, screening for finding natural ones are being considered. Among diverse natural surfactants, saponins with especial characteristics have been more prefered. The results here demonstrate that hemolytic activity of aqueous extracts of tested plants would be increased as temperature rises which can be attributted to the liquid feature and fluidity of the lipid bilayer (Figures 1 and 2). Then, some parts of the membrane can easily move

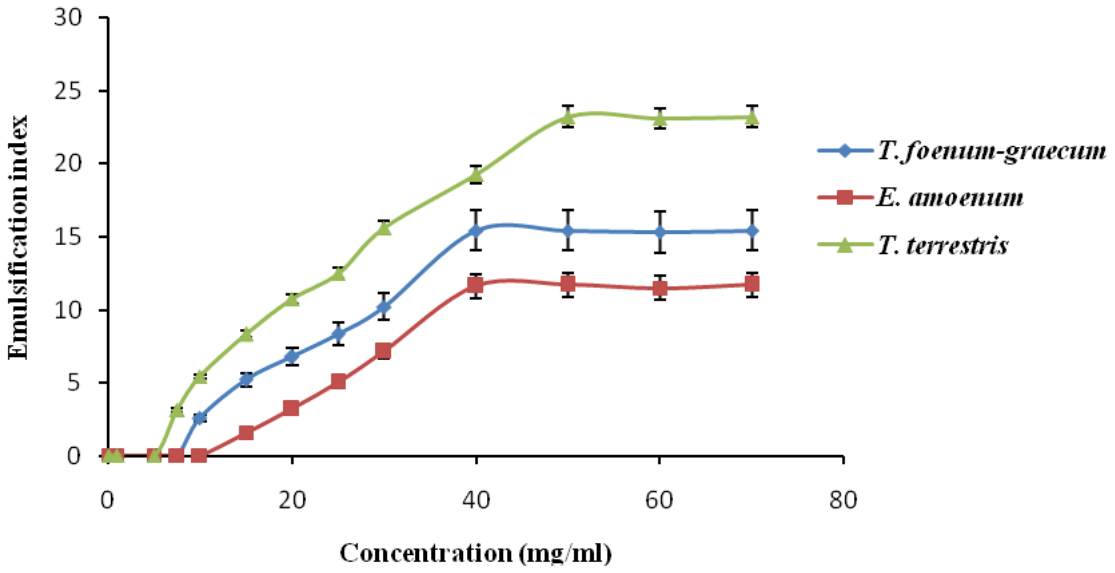

Figure 3. Emulsification index at different concentrations of *T. terrestris, T. foenum-graecum, E. amoenum* Aqueous extracts (Mean ± SD, n = 3).

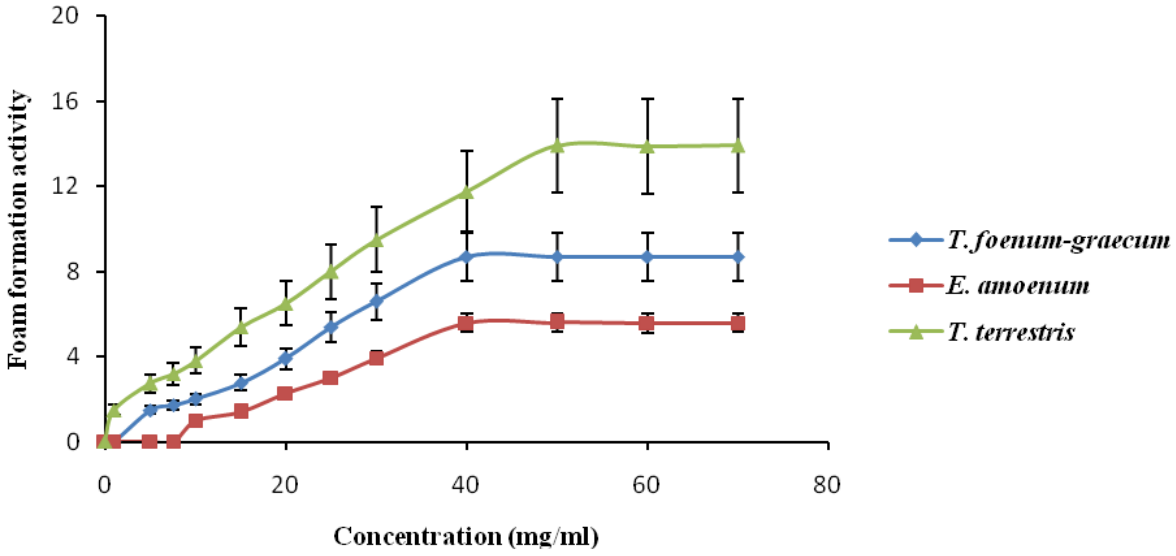

Figure 4. Foam formation activity at different concentrations of *T. terrestris, T. foenum-graecum, E. amoenum* Aqueous extracts (Mean ± SD, n = 3).

all through the surface and membrane phospholipids change to jelly in temperatures lower than physiologic temperature. This property of phospholipids stabilizes the membrane and increases its resistance (Kleszczynska et al., 2005).

The hemolytic activity of the aqueous extracts is increased in a dose-dependent manner (Figures 1 to 2). On the basis of Fick's diffusion law, flux from a membrane is proportional to concentration gradient, so by increasing the concentration of saponin, it diffuses intra membrane until reaching a concentration which leads to membrane destruction and hemolytic effects (Kleszczynska et al., 2005). In addition the hemolytic activities of saponins are associated with their chemical composition. Saponins with steroid aglycone have shown more haemolytic activity than those with triterpenoid aglycones (Takechi and Tanaka, 1995). These activities are also associated with increasing the number of monosaccharide and the complexity of their glycidic moieties (Santos et al., 1996), acyl residues or the epoxy

construction system (Oda et al., 2000). The presence of fatty acids could also favor interactions between the saponin and membrane cholesterol promoting the hemolysis. The glycoside moieties of saponins in addition may have an effect on hemolytic activity. The number of chains influences both hemolytic activity and membrane permeability. Woldemichael et al. (2001) reported that saponins possessing two suger chains induce less hemolytic activity than those containing one (Tragner and Csordas, 1987). Opposing to this examination, Yamasaki et al. (1987) showed that increasing the amount of sugar side chains increased the membrane permeability for calcium ions (Araki and Rifkind, 1981).

In view of all reports, the permeabilizing effect may be caused by the combination of target membrane composition, the type of the saponin side chain(s) and the nature of the aglycone (Woldemichael and Wink, 2001). This hemolytic activity will be increased in higher temperature because of more fluidity and permeability of the membrane (Kleszczynska et al., 2005). The results obtained in the present study showed that hemolytic activities of the extracts increased proportional to the latency of incubation (Figures 1 to 2). It was reported that increasing the incubation time of erythrocytes and surfactants would promote hemolysis (Yamasaki et al., 1987). The surfactant concentration, temperature and duration of incubation time affect the membrane permeability and hemolysis which may be due to micelle formation from surfactant and membrane phospholipids bilayer (Francis et al., 2002). Another feature of this study was to evaluate the membrane toxicity of samples. Any agents which have the ability to destruct the erythrocytes membrane can have similar effects on other cells membranes, evaluating erythrocytes membrane compatibility is a simple way for determination of surfactant toxicity. In the present study hemolysis was observed by increasing incubation period and temperature. In 70 mg/ml and temperature of 37°C *T. terrestris* extract caused 11.7% of erythrocytes destruction, while *E. amoenum* and *T. foenum-graecum* extracts caused 2.9 and 4.7% respectively. A further potential property of surface active agents is their capability in induction and stabilizing emulsions. Emulsifying index is associated with surface tension and capability in micelle production.

In this study the emulsifying index of the tested extracts has revealed significant difference from each other (Figure 3) ($p<0.05$). Saponins have detergent like or surfactant properties because they contain both hydrophilic and lipophilic components. Among these extracts, the extract of *T. terrestris* has the most emulsifying index. These differences between the plants may be due to quantity or quality of their saponin contents. In this study, the increase in concentration of plant extracts leads to promoting in emulsions constancy; although, this property was not the same in all samples (Figure 3). The results of the study show all the tested extracts particularly *E. amoenum* in lower concentrations (<20 mg/ml) have less hemolytic activity and the concentration of a plant extract for pharmaceutical usage is a critical point. The hemolytic activity of *E. amoenum* in concentrations above cmc increases significantly, in contrast to synthetic surfactants such as polysorbates (Dehghan et al., 2008). According to the hemolytic data and emulsifying index, extract of *E. amoenum* had the least toxicity and the best properties for emulsification to be used in formulations. Foam formation activity of surfactants is a propriety which is used to compare the detergency properties of detergents. Foam production and constancy depends on the type and concentration of surfactants, our results show that extract of *T. terrestris* had more capability to produce foam (Figure 4).

Nowadays there is great attention with regards to the effect of surfactants on absorption. Among the extracts, with respect to safety, *E. amoenum*, with low hemolytic effect may be preferred in pharmaceutical preparation but if the hemolytic effect is not considered, the use of *T. terrestris* is preferred.

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