Determination of the Safe Dose of Euphorbia rigida Plant Extract to be used as Biopesticide, Antimicrobial and Antioxidant with Brine shrimp lethality assay

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ABSTRACT

Euphorbia rigida Bieb. is a poisonous milk-bearing plant, which is in the form of shrubs or trees growing in tropical and temperate regions and belonging to the Euphorbiaceae family, which has nearly 100 species in our country. The Euphorbiaceae family, known for its capacity of being used as antioxidant, antimicrobial and biopecticides, has attracted great attention in recent years. In this study, the safe dose of the extracts of E. rigida Bieb. believed to have the capacity of being used in both medicinal and agricultural applications, and very common in Muğla, will be determined by using Brine shrimp lethality assay.

Key Words: *Euphorbia rigida, Brine shrimp lethality assay, Antimicrobial, Antioxidant, Biopestisit*

1 Introduction

Herbal products have been used since the Palaeolithic ages. It has been reported by the World Health Organization that 70-80% of the world population benefit from herbal products in basic health care applications (Sarışen and Çalışkan, 2005). The most important reason for the increasing interest in plant-based drugs is that although the synthesized chemical compounds used in the treatment of chronic diseases have limited effect, plant-based drugs are potentially more effective, cheaper, and have fewer side effects than synthetic drugs (Abay, 2006). As in the past, medicines obtained from plants or herbs are used in the treatment of various diseases today (Arıkan, 1992). In the modern world, human health and the environment have gained great importance. Many scientific studies point to the necessity of natural nutrition and the use of naturally derived antioxidants in order to stay healthy (Koca and Karadeniz, 2003). In addition, another factor that threatens human health is the incorrect use of chemicals in agricultural pest control. All kinds of residues of chemical pesticides used unconsciously in agricultural fields are mixed with soil, air and groundwater in various ways. Pesticides with high toxicity accumulate over time in all living creatures living in this environment and return to human through the food chain. Pesticides that return to humans by various means such as residues on the product or through the food chain threaten human health (cancer, genetic diseases etc.) (Özmen and Sümer, 2004). The main reason for refusing or returning agricultural products exported by our country is the presence of toxin or pesticide residues in our products. Increasing the export potential of our country is of great importance for our economic development. For this reason, it is very important to use herbal products for biopesticide purposes in agricultural pest control. According to a research conducted by the World Health Organization (WHO) based on some publications on medicinal plants of 91 countries, the total number of medicinal plants used for treatment purposes is around 20 000 (Çelik and Çelik, 2007). The number of medicinal plants used in our country is about 500 and it is very low compared to European countries, so it is necessary to benefit from our own natural herbal potential (Baytop, 2000). Most plant extracts have been used as topical antiseptics, or have been reported to have antimicrobial properties (Avcı et al., 2013). It is known that extracts and essential oils of naturally grown plants exhibit antibacterial and antifungal activity and provide the basis for many application areas such as antimicrobial activities, preservation of nutrients, as a biopesticide in agricultural control, pharmacy, alternative medicine and natural therapy (Çalışkan et al., 2019).

Euphorbia rigida Bieb. is a poisonous milk-bearing plant, which is in the form of shrubs or trees growing in tropical and temperate regions and belonging to the Euphorbiaceae family, which has nearly 100 species in our country our country and is used as a poison in fishing in lakes and streams. It is prohibited because it kills all living things in the water and fish caught in this way are dangerous because they cause poisoning in humans (Tanker et al., 1998; Baytop, 1999; Ateş, 2001). E. rigida is a one or two year old plant with bluish-green colour. It has a woody storage root and a narrow spear-like stem with dense leaves. The body has simple, thick and fleshy leaves facing one another, and umbrella-shaped flowers. The fruit of the plant has a round capsule-like structure with three seeds. Its seeds are light gray or white in colour. Flowering time is between March and August and can develop at altitudes varying from sea level to 2000 m. It is seen in the cities of Tekirdağ, Canakkale, İstanbul, Amasya, Tokat, Manisa, Aydın, İzmir, Kayseri, Niğde and Antalya in Turkey (Baytop, 1998). Many Euphorbia species have been used all over the world and in Turkish culture by our people since old times for medical purposes such as laxatives, the removal of warts, the treatment of aphthae, bronchitis, cold, cough, emphysema, measles, peptic ulcer, haemorrhoids, nausea, acute mastitis, diuretics and enhancing milk secretion (Barla et al., 2007; Shi et al., 2008; Kırbağ et al. 2013; Erdoğan et al., 2012; Huang et al., 2012; Özbilgin and Çitoğlu-Saltan, 2012; Ghareeb et al. 2018; Ghosh et al., 2019: Kemboi et al., 2020). It is also known that it has been used for centuries as a colouring material (Tiryakioğlu, 2004) and as a biopesticide to preserve food stocks (Şahin et al. 2006). With antimicrobial activity studies conducted with some Euphorbia genus, it has been observed that herbal extracts of Euphorbia species have different levels of antimicrobial activity against Gr (+), Gr (-) bacteria and fungal infectious agents (Özbilgin and Çitoğlu-Saltan, 2012). Plants belonging to the Euphorbiaceae family are rich in bioactive compounds such as tiglian, ingenan and dafnan diterpenoids which have phenolic, flavoniod, especially terpenoid and steroid characteristics responsible for antioxidant effects (Tiryakioğlu, 2004; Barla et al., 2007). The same compounds are responsible for the bactericidal effects and toxic and irritating properties observed in studies with Euphorbia species (Barla et al., 2007; Shi et al., 2008; Wu et. al., 2009).

Today, resistance develops in pathogenic microorganisms and agricultural pests against many natural and synthetic compounds. For this reason, the new natural compounds obtained from plants have been tested in in vitro and in vivo conditions with biological effect tests and preferred as a biopesticide, natural antioxidant and antimicrobial source, and the interest in their applicability to humans has increased (Dülger et al., 1998; Turan-Zitouni et al., 2005a, b; Kırbağ and Zengin, 2006; Aksoy et al., 2008; Tanış et al., 2010). One of the biological tests, Brine-Shrimp test (Artemia salina larvae) is widely used as a fast and simple method in the determination of cytotoxicity of samples whose biological activities are investigated (Choudhary and Thomsen, 2001).

By using our natural resources, chemical raw materials can be obtained more cheaply and easily and an important economic contribution can be made to our country. For this purpose, the safe dose of Euphorbia rigida plant extract from the Euphorbiacea family, which has the capacity to be used as a biopesticide (Civelek and Weintraub, 2004), antimicrobial (Başaran et al., 1996; Özbilgin and Çitoğlu-Saltan, 2012) and antioxidant (Barla et al., 2007) and which is widely grown in Muğla province was tested by means of Brine shrimp cytotoxicity test (Brine shrimp lethality assay).

2 Materials and Methods

2-1 Collection of the plants and their extraction

The aerial parts of Euphorbia rigida (Bieb.) in the flowering period were collected from Muğla Sıtkı Koçman Üniversity campus (GPS:37°09'40,1"N 28°22'34"E) in Muğla at Turkey. The taxonomic identification of plant materials was confirmed and deposited in the herbarium by voucher specimen Dr. Olcay Ceylan at the Department of Biology a αMuğla Sıtkı Koçman University (Herbarium number: O0388). The samples were air-dried at room temperature and protected from direct sunlight. Seventyfive (75 g) grams of dried and powdered aerial parts of E. rigida were extracted with 2.5 L boiling water for 60 min. Decoction (aqueous phase) was filtered with a 2.5 μm filter paper (Whatman No. 42) to remove suspended particles and the extract was kept at least 3 days at -20°C and later lyophilized to obtained crude (6.72 g) extract which was stored at -20°C (Özbek et al., 2002).

2-2 Preparation of Artemia salina larvae

The SERA brand (Sera North America, Inc. Montgomeryville, U.S.A.) Artemia salina eggs (cysts) purchased from a company that sells aquarium materials were weighed to be 2 grams and then sprinkled into a 5-liter plastic, transparent, open-mouthed tank containing 2 litres of artificial sea water. The artificial sea water in the tank is continuously aerated by a double outlet air motor using double hoses. In addition, the water temperature in the tank was heated to a constant 28°C by a thermostat. The tank was left in the light for approximately 48 hours next to a desktop light source, and Artemia salina larvae (nauplii) were waited to hatch from the eggs. Experimental setup is shown in Figure (1), the image of Artemia salina eggs is shown in Figure (2), and the hatched Artemia salina larvae are shown in Figures (3 a *and* b).

Fig. 1: Experimental Setup

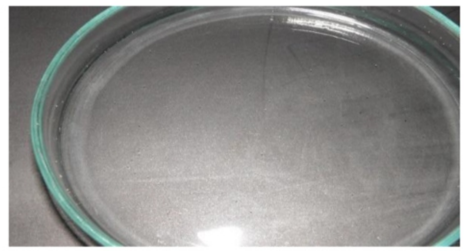

Fig 2: Artemia salina eggs

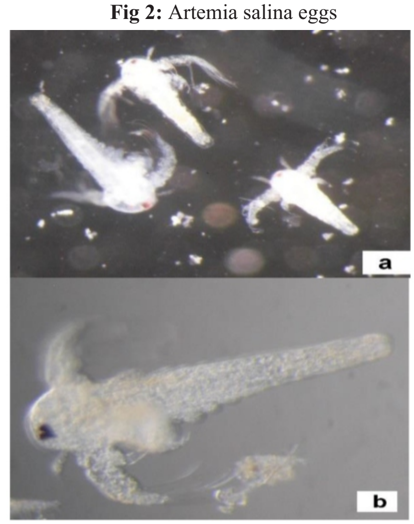

Fig. 3: Artemia salina larvae emerged from eggs (a), a single larva (b)

2-3 Brine shrimp cytotoxicity test (Brine shrimp lethality assay)

Artemia salina larvae are one of the toxicity test organisms used to determine the LC50 level. Diluted solutions of 10, 20, 30, 40 and 50 ppm prepared from the dry extract obtained from the aqueous extract of E. rigida plant, sterile artificial sea water prepared with distilled water for control purposes (35 g non-iodized rock salt, 1000 ml sterile distilled water) and 20, 100 and 200 ppm solutions of Elandor® (İmidacloprid) used as insecticide (the recommended dose in agricultural practice is 200 ppm, Öncüer, 2004) were used to test the cytotoxic effect by means of Brine Shrimp method. As a result of the study, the LC50 value was calculated (Table 1) and the toxicity levels (Brayn et al., 1997) (Table 2) were determined. The study was conducted according to Sowemimoa et al., 2007.

2-4 Cytotoxic effect determination

Stock solutions of 100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm were prepared from E. rigida plant extract with distilled water. 500 µl of each of the stock solutions were taken and put into glass test tubes adjusted to a volume of 5 ml. The top of the test tubes are completed to 5 ml with sterile artificial sea water. Thus, diluted solutions of 10 ppm, 20 ppm, 30 ppm, 40 ppm and 50 ppm concentrations where the applications would be conducted were obtained. Elandor stock solutions were diluted in a similar way, and final concentrations of 20, 100 and 200 ppm where the applications would be conducted were obtained.

Artemia salina larvae migrate in the water after hatching towards the area where the light is intense (Choudhary and Thomsen, 2001). Thus, the larvae taken from the part where the larvae were dense with the help of a micropipette together with approximately 10 µl of water were transferred into a petri dish under stereomicroscope and 30 larvae were counted. 30 living organisms were transferred to into tubes (Control) with 5 ml sterile artificial sea water, glass tubes containing E. rigida plant extract in concentrations of 10 ppm, 20 ppm, 30 ppm, 40 ppm and 50 ppm and glass tubes containing 20, 100 and 200 ppm solutions of Elandor used as insecticide. The study was carried out in 3 parallels. At the end of 24 hours under light, the numbers of live and dead larvae (defined as dead according to their immobility) were counted with the help of a magnifying glass. The obtained results were transferred to the EPA (The U. S. Environmental Protection Agency, Cincinnati, Ohio, U.S.A.) Probit Analysis Program (Version 1.5) and the LC50 value and 95% confidence lower and upper limits were calculated (EPA, 2021). LC50 values obtained were interpreted according to Brayn et al., 1997.

Table 1 : Approximate LC / EC values and confidence limits for E. rigida and Elandor according to EPA Probit Analysis Program (Version 1.5)

	Euphorbia rigida			Elandor® (İmidacloprid)		
LC/EC	Concentration Exposed	95% Confidence Limits µg/ml (ppm)		Concentration Exposed	95% Confidence Limits µg/ml (ppm)	
	µg/ml (ppm)	Lowest	Highest	µg/ml (ppm)	Lowest	Highest
LC/EC 1.00	3.772	0.00	12.147	47.895	0.002	90.700
LC/EC 5.00	8.352	0.00	18.874	71.870	0.061	114.329
LC/EC 10.00	12.759	0.00	24.179	89.232	0.425	130.414
LC/EC 15.00	16.983	0.05	28.964	103.263	1.558	143.648
LC/EC 50.00	**56.888**	**36.146**	**1831.913**	**191.437**	**131.752**	**621.028**
LC/EC 85.00	190.554	85.077	348011456.000	354.902	236.843	126261.281
LC/EC 90.00	253.645	99.947	6433705472.000	410.708	258.595	467149.125
LC/EC 95.00	387.489	126.263	%487151501312.0	509.926	293.040	3262383.500
LC/EC 99.00	857.88	194.139	%164366989.132E	765.178	367.492	125955424.0

Table 2 : Reference values used to evaluate the degree of toxicity (Brayn et al., 1997).

Toxicity Degree	LC_{50} Limits
Highly toxic	< 10 µg/ml
Toxic	10-100 µg/ ml
Harmful	100-1000 µg/ ml
Not toxic	> 1000 µg/ ml

3 Results and Discussion

According to the Brine-shrimp general toxicity test result, the LC50 value of the extract obtained from E. rigida Bieb. with water was found to be 56.89 ppm, the 95% confidence lower limit was found to be 36.15 ppm and the upper limit was found to be 1831.91 ppm (Table 1), and it was concluded that the E. rigida Bieb. plant extract was toxic according to the toxicity reference values (Table 2., Brayn et al., 1997). In addition, the LC50 (191.437 ppm) value and 95% confidence lower and upper limits (Table 1) of Elandor (Imidacloprid) used as a chemical pesticide in agricultural control are considered to be in the pest class according to the toxicity reference values (Table 2., Brayn et al., 1997) and it was concluded that plant extract of E. rigida Bieb. is more toxic and dangerous. The results we have obtained in our study are consistent with the results of many cytotoxicity studies conducted with various Euphorbia species. LC50 value of extracts obtained from all tissues of E. hirta except for flowers were found by Brine shrimp lethality test method (LC50 = 1000 ug / ml) (Huang et al., 2012). Brine shrimp lethality test was used to investigate the cytotoxicity of Euphorbia hirta. The study concluded that the LC50 of ethyl acetate and acetone decoction of plant parts were 71.15 and 92.15 µg/ml, respectively (Ghosh et al., 2019). The E. hirta extract also showed selective anticancer activity at a concentration of 100 µg/mL (Alam et al., 2016). E. microsciadia and E. szovitsii indicated the highest anti-breast cancer activities on cancer cell lines and the lowest IC50 was 59.52 µg/mL which was found for E. szovitsii against MCF-7 cell line (Asadi-Samani et al., 2019) The LD50 values of 17-acetoxyjolkinolide B and 13-hexadecanoyloxy-12-deoxyphorbol obtained from the dry roots of E. fischeriana and lathyrane diterpenoids isolated from E. nivulia were reported to have approximately the same and significant cytotoxic effect against the Colo 205, MT2 and CEM cell lines (Shi et al., 2008). IC50 values of the cytotoxic effect of compounds obtained from the dry roots of E. fischeriana against Ramos B cells are approximately 0.023 and 0.0051 µg / ml, respectively (Wang et al., 2006). The daphnane and tigliane diterpenoids isolated from the latex of E. poisonii showed a selective and potent cytotoxic effect on human kidney carcinoma (A-498) cell lines. This effect was found to be 10 000 times greater than adriamycin (Fatope et al., 1996). While the ED50 values of the compounds obtained from E. poisonii were between 2 and 4 µg / ml, they showed a weak non-selective cytotoxic effect against A-549, MCF-7, HT, A-498, PC-3, and PACA-2 cell lines. On the other hand, when its ED50 value is 15 µg / ml, it has weak but selective cytotoxic effects against prostate adinocarcinoma (PC-3) (Wang et al., 2006; Shi et al., 2008).

Cytotoxic activities of extracts obtained from four species belonging to the Euphorbia genus (E. fischeriana, E. tirucalli, E. humifusa and E. antiquorum) on the development of human liver carcinoma cells (BEL-7402) and human lung cancer cells (A-549) were investigated with the inhibition test method in in vitro conditions. Extracts are considered effective at the lowest IC50 = 30 µg / ml dose. Chloroform extracts obtained from E. tirucalli and E. antiquorum showed strong cytotoxic activity in both human cancer cell lines. Chloroform and ethylacetate extracts obtained from E. fischeriana showed significant cytotoxic activity only on human lung cancer cell (A-549) (Wang et al., 2006).

Diterpenoid compounds in E. paralias extracts have high molluscoidal activity on Biomphalaria alexandrina. The antifeedant activity of E. paralias was tested against Spodoptera littoralis third stage larvae with the disc leaf method, a traditional test. Some compounds (at a dose of

1000 mg / ml) showed an antifeedant activity against insects by an average of 66.8% and 45.8% (Abdelgalil et al., 2002; Shi et al., 2008). Strong molluscoidal activity of the aqueous extracts of E. pulcherima and E. hirta plants and partially purified latex against Lymnaea acuminata (LC50 = 40% and 80%) was observed (Shi et al., 2008; Özbilgin and Çitoğlu-Saltan, 2012; Huang et al., 2012). E. hirta Linn latex powder was evaluated against the freshwater snails Lymnaea (Radix) acuminata and Indoplanorbis exustus in pond (Alam et al., 2016; Ghosh et al., 2019).

The petroleum ether fraction of E. hirta showed strong larvicidal activity in the third stage larvae of Anopheles stephensi, which is known as the malaria vector in cities. The flavonol glycoside afzelin, quercitin and myricitin isolated from E. hirta at different concentrations showed an inhibition effect on the proliferation (Anti-malarial) of Plasmodium falcifarum (Wang et al., 2006; Huang et al., 2012; Özbilgin and Çitoğlu-Saltan, 2012; Alam et al., 2016; Ghosh et al., 2019).

It is reported that latex belonging to Euphorbia species is used against wireworms in corn and soybean cultivation and also for nematode control in tomatoes, potatoes and peppers. It is reported that the species belonging to Cuphea genus from the Euphorbiaceae family and the plant extract called Euphorbia lagascae are effective against corn worm, wireworm and grayworm in tomato, potato, bean and clover; it has also been reported that the same extracts can be an alternative to methyl bromide applications with an effective rate of 97% on nematodes that damage potato plants (Civelek and Weintraub, 2004). E. orientalis L. was found containing bioactive compounds that have essential antibacterial and antioxidant activities. Therefore, the possibility of using Euphorbia species and E. rigida extract as an antiseptic for sterilization or disinfection of large and inanimate surfaces can be explored (Avcı et al. 2013; Metin and Bürün, 2021).

According to the brine-shrimp general toxicity test result, the LC50 value of the extract obtained from Euphorbia rigida Bieb. with water is 56.89 ppm, which supports its usability as a biopesticide in agricultural control as an alternative to chemical pesticides such as Elandor, which we used for comparison in the same study. However, studies on the systematic toxicity and safety assessment of Euphorbia species are very few. In the studies conducted, only the organs that it affects (targets) toxically and their side effects have been emphasized (Huang et al., 2012).

4 Conclusion

Antibacterial, antifungal and antiviral properties of Euphorbia extracts are well known, so it could mean that they can be used as additives or a disinfectant for inanimate surfaces in the pharmaceutics industry. By investigating the chemical contents of various extracts obtained from E. rigida plant, determining the optimum conditions and identifying and isolating the compounds responsible for the activity in in vitro and in vivo conditions as a new and alternative cytotoxicity (anticancer) and biopesticide agent, it has been shown that it can be used as a raw material in pharmaceutical and industrial (cleaning and sterilization) activities.

According to the results of this research, we can suggest that the extract obtained from E. rigida plant with water up to 57 ppm can be used as an alternative to chemicals used as biopesticide. When extract of E. rigida is used, great care should be taken while using it because it can cause damage on cells and chromosomes (Metin and Bürün, 2021). Hence, through more detailed and comprehensive studies, its usability for medical and biopesticide purposes should be investigated.

Disclosure statement

No potential conflict of interest was reported by the authors.

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