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Phytotoxic and Cytotoxic Evaluation of Methanolic Extract of *Trifolium alexandrinum*

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Abstract: *Trifolium alexandrinum* L. native to the Mediterranean has become a major invasive plant growing in temperate, sub-tropical as well as high altitude areas. In Pakistan, *T. alexandrinum* has spread extremely rapidly, now it can be found in almost every ecological region f Khyber Pakhtunkhwa Province. Knowledge of the mechanisms why plants can successfully invade and disperse fastly is very useful in determining the invasive mechanism of this plant. The objective of this study was to evaluate *Trifolium alexandrinum* methanolic extract [TAME] against phytotoxic and cytotoxic activities. Medicinal plants, due to the presence of bioactive compounds play an extremely important role in maintaining and improving human health. Different parts of medicinal plants are used to minimize the oxidative stress, impotency, cancer, cardiac dysfunction and microbial. Traditionally it is used for the treatment of different diseases like respiratory diseases, skin diseases, depression, memory loss and effective in heath building; both in body and mind. Its oil is also used for milk improving and health. Its extracts also have immunosuppressive effects. *Trifolium alexandrinum* is a small branched tree. Dried plant were grinded and its methanolic extracts [TAME] were prepared. These methanolic extracts were evaluated for biological assays according to stander protocol *in-vitro*. The phytotoxic activity against Zea maize seed's shoot and roots germination were performed. Cytotoxic activity of plants methanolic extracts against brine shrimps. The results obtained in this study indicated that TAME did not possess significant phytotoxic and cytotoxic activities which might be due to the presence of bioactive compounds. Further studies are required to scan, isolate and purify the major bioactive compounds and to verify both *ex-vivo* and *in-vivo* assays.

Key words: *Trifolium alexandrinum* · Phytochemicals · Allelopathy · Cytotoxic · *In vitro* Assays

INTRODUCTION

Plants are natural resource of producing large number of bioactive chemical constituents in a most proficient way and with specific selectivity. Since the middle of the 19 century, different classes of bioactive compounds have been isolated and characterized. Many of these are used as the active ingredients of the modern medicine, or as the lead compounds for new drugs discovery. Several plant derived medicines, are rich in phenolic compounds such as those used in protection against coronary heart diseases and carcinogenesis [1]. Different extracts from conventional medicinal plants have been tested. Many reports have show the effectiveness or usefulness of traditional herbs against microorganisms; as a result, plants are one of the bedrocks for modern medicine to attain new principles [2].

Berseem [*Trifolium alexandrinum* L] is one of the most important leguminous forages in the Mediterranean region and in the Middle-East. Egyptian clover [*Trifolium alexandrinum* L] is considered the main winter forage crop in Egypt. It is cultivated as an animal feed and for soil improved [3]. Berseem is an annual, sparsely hairy, erect forage legume; 30 to 80 cm high. Berseem is a variable species that can be classified into four cultivars groups according to their branching behaviour and

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subsequent productivity. Berseem is a fast growing, high quality forage that is mainly cut and fed as green chopped forage. It is high quality green forage. Berseem should be cut 50 to 60 days after planting and then every 30-40 days. It is slightly less drought-resistant but does better on high moisture and alkalinic soils. It is very productive when temperatures rise after winter. Berseem clover can also be used as green manure crop. Berseem is mainly valued as a winter crop in the subtropics as it grows well in mild winter and recovers strongly after cutting. It does not grow well under hot summer conditions. It is cultivated from 35°N to the Tropics, from sea level up to 750 m [1500 m in North West Himalaya] [4]. Berseem has a shallow tap root. Its stems are hollow, branching at the base, with alternate leaves bearing 4-5 cm long x 2-3 cm broad leaflets [5]. Berseem can be mixed with 20% ground mays to provide high quality grass [6]. Berseem is an N-fixing legume. It may require rhizobium inoculation outside its native area [7].

We used the selected plant [*Trifolium alexandrinum*] for phytotoxicity and cytotoxicity. Allelopathy is the production of biochemicals by organism that influence the growth, survival and reproduction of other organisms. These biochemicals are secondary metabolites known as allelochemicals and can have beneficial [Positive allelopathy] or detrimental [Negative allelopathy] effects on the target organisms. Allelochemicals with negative allelopathic effects are an important part of plant defense against herbivory [8]. Therefore, extensive research has been conducted to develop allelopathic compounds which improve the production of agricultural ecosystems through different ways, like the use of allelochemicals from plants as pesticides instead of synthetic chemicals. Medicinal plants are screened for their allelopathic capacity [9]. There are various medicinal plants reported to have anti-cancer activity because the treatment has low cost and low side effects. The discovery and identification of new antitumor drug with low side effects on immune system has become an essential goal in many studies of immune pharmacology [10]. The search for anti-cancer drugs from plant sources started in earliest in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine and the isolation of the cytotoxic podophyllotoxines [11].

The brine shrimp cytotoxicity assay was considered as a convenient probe for preliminary assessment of toxicity, detection of fungal toxins, heavy metals, pesticides and cytotoxicity testing of dental materials. It can also be extrapolated for cell-line toxicity and anti tumor activity. The brine shrimp assay is very useful for the isolation of biogenic compounds from plant extracts [12]. *Trifolium alexandrinum* yet been tested for the cytotoxic compounds.

The aim of this study is to study the biological and pharmacological values of Trifolium alexandrinum and to determine the allelopathic properties and cytotoxic potency of the methanolic crude extract of *Trifolium alexandrinum.*

MATERIALS AND METHODS

Plant Collection: *Trifolium alexandrinum* was collected from District Bannu during the February 2012. Collected plant samples were dried under shadow at a room temperature for 45 days and ground it into the fine powder.

Plant Extract Preparation: A total of 200 g powder from each samples were taken and placed in the 70% commercial grade methanol [CH_3OH] and stirred well, then after passing of 72 hours the extracts were filtered by using qualitative Whatman filter paper No 1 in vise bath the filtrate was placed at 40 °C and thus the entire methanol was evaporated, so the crude extracts of the plant were obtained and stored it in the refrigerator at 4 °C for the purpose of future *in-vitro* studies.

Cytotoxic Brine Shrimp Lethality Test: Cytotoxic brine shrimp lethality test was carried out according to the protocol [13]. Two sub solutions of plant extract were prepared of 3000µg/ml, 6000 µg/ml, from stock solution by using the formula $M_1V_1=M_2V_2$. Media for Shrimp-hatching was prepared by dissolving 5 g of sea salt is in 250 ml distilled water and stirred using the magnetic stirrer for nearly about 2 hrs. Brine shrimps were hatched in two compartment rectangular tray containing sea salt saline. Eggs were sprinkled in dark compartment of tray and after 24 hrs of shrimps hatching larvae was collected by pipette from the lightened side.

Samples in test tubes are labeled as 3000µg/ml and 6000µg/ml. Drum vials were used in this bioassay. 0.5 ml of each solution [3000µg/ml, 6000µg/ml] was taken in vials and evaporated the solvents. Residues was re dissolved in saline of 2 ml. 8 shrimps were transferred to each vial and raised the volume up to 5 ml and incubate at 25–28 °C. After 24 hrs of incubation survivors were counted with help of 3× magnifying glass and calculation was done using Abbot's formula;

% Death = [Sample-control/control] × 100

Phytotoxic Bioassay: One plant seeds [Maize] were tested against the phytotoxic activity of TAM extract. This Phytotoxic test of TAM extract was performed according to the protocol of McLaughlin and Rogers [14]. Different fractions of TAM extract were prepared from the stock solution, 250µg /ml, 500µg /ml and 1000µg /ml. set the Whatman filter paper in each Petriplates. Put 5 ml of 250µg /ml, 500µg /ml and 1000µg /ml solutions is taken [With the help of a small graduated cylinder] and put on the Petri plates labeled it. Distilled water (DH_2O) was used as a control. After that keep the petriplates for drying at 40 °C for the evaporation of methanol due to its toxic property from the filter papers. Then put 5 ml DH_2O and put on each filter paper set in the Petriplates of all the three concentrated as well as in the control. Then 5 maize seeds from already washed by 1% $HgCl_2$ and socked in DH_2O seeds were placed in each Petri plates by scientific method at equal distance in the Petri plates. Then all the Petri plates were incubated in the growth room and after three days, take the first reading i.e. length of root/ radical and add the distilled water to each plate to maintain the moist condition. After the seven days, take the last reading of the seed's growth and calculate the % inhibition of growth.

Statistical Analysis: To determine various parameters of the plant extract treatment effects, one-way analysis of variance was carried by computer software SPSS 13.0. Level of significance among the various treatments was determined by LSD at 0.05% and 0.01% level of probability.

RESULTS

Cytotoxic activity: % Death: *Trifolium alexandrinum* preliminary screening of plants extract through cytotoxicity provides helpful information about the antitumor activity. Cytotoxic effect of *Trifolium alexandrinum* methanolic extract [TAME] was measured against brine shrimps growth as shown in Table 1. After complete hatching shrimps were transferred into glass test tube already contained saline of sea salt and extract of various concentration of the plant. After 24 hrs the effects of extract of different concentration was noted and found that the brine shrimp survival is inversely proportional of the concentration of the plant extract.

Cytotoxic Activity of *Trifolium alexandrinum*

Table 1: % survival of brine shrimps in the presence of various concentration of plant extract

Control	Total	Live	Death	% of Death
	8	7	1	12.5
3000µg /ml	8	3	5	62.5
6000µg /ml	8	1	7	87.5

After 3 Days Roots/shoots Growth Readings)

Table 2: Growth readings of shoots/roots after 3 days in the absence and presence of various concentrations of *Trifolium alexandrinum* extract

Treatments	Roots	Shoots
Control	0.55cm	1.52cm
Extract 100µg/ml	1.33cm	2.24cm
Extract 500µg/ml	2.56cm	3.45cm
Extract 1000µg/ml	3.85cm	4.13cm

After 7 days roots/shoots growth readings)

Table 3: Growth readings of shoots/roots after 7 days in the absence and presence of various concentrations of *Trifolium alexandrinum* extract

Treatment	Roots	Shoots
Control 13.13cm	10.04cm	
Extract 100µg/ml	14.33cm	11.24cm
Extract 500µg/ml	15.56cm	12.45cm
Extract 1000µg/ml	17.85cm	14.13cm

From Table 1, it is clear that at 3000µg/ml 38% survival and 62% death is occurred. While, in 6000µg/ml 13% survival and 87% death is occurred. The order of % age of *Trifolium alexandrinum* methanolic extract is shown in Table 1.

Phytotoxic Activity: To study the phytotoxic activities of the "*Trifolium alexandrinum* methanolic extracts" [TAME], 1000µg/ml concentration of the samples was used. The obtained results show that 1000µg/ml concentration of the crude extracts [TAME] slightly inhibits the growth of shoot and roots [Hypocotyls and radicals] of the maize seeds as compared to the control, as shown in the Table 2 and 3 So have the phytotoxic activities but not significant.

DISCUSSION

For the treatment of different human aliments medicinal plants play its basic role due to the presence of bioactive compounds in them, such as the treatment of inflammation, oxidative stress, heart diseases and cancer throughout the world since long. Different parts of the medicinal plants are used mostly for the treatment of various diseases because of their fewer side effects as

compare to the synthetic drugs. Local herbal system is commonly used world widely. In the same way Pakistan is also a rich country in medicinal plants which are locally used in folk medicines for the treatments of various diseases like infections, cardiovascular diseases, diseases of digestive system and skin diseases etc by local healers.

Phytotoxicity of medicinal plants are also very important because of its growth inhibition of weeds and other unwanted plants. The phytotoxic results obtained from TAME showed that TAME inhibit the growth / germination of roots and shoots of maize but not up to a significant level. TAME as compare to the other medicinal plants methanolic extracts, such as significant phytotoxic results were found by Kordali *et al*. [15], reported that essential oils and phenolic compounds inhibits the growth of roots and shoots [16], also found that water extract of *Withania somnifera* and *Datura alba* have some bioactive compounds which inhibit significantly the growth of shoots and roots of *Rumex dentatus L.*

Data revealed that the order of % age death of brine shrimps was recorded. Our result showed that the brine shrimp survival is inversely proportional to the concentration of the extract used. Kanegusuku *et al*. [17] reported organic fraction of *Rubus imperialis* [C.] showed more cytotoxicity [18] studied that methanolic fraction of *Arceuthobium oxycedri* exhibited 100% cytotoxicity for brine shrimps at high dose which are in friendship to our result.

The result of present study evaluated the folk use of this medicinal plant and suggested that methanolic extract possess some bioactive constituents having anticancer activities that can be the central point of new drugs having anticancer and shielding role against different pathogens.

CONCLUSIONS

This research report concludes that TAME showed slightly phytotoxic effect against the germination of radicals [Roots] and hypocotyls [Shoot] of maize seeds, but not significant.

TAME showed its effectiveness for the DPPH free radicals scavenging i.e. they may be used as good antioxidants. TAME are also used as cytotoxic effect.

Suggestions: The results obtained from the study of TAME indicate their usefulness from medicinal point of view, therefore; further qualitative analysis of TAME are needed to indicate the bioactive compounds present in them. To explain and summarize the possible mechanism of action involved in these bioassays. Further works are needed to isolate and purify the bioactive compounds present in TAME.

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