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ORIGINAL ARTICLE

Cytotoxic, Phytotoxic and Insecticidal Activities of *Chrysophthalmum montanum* (DC.) Boiss.

Chrysophthalmum montanum (DC.) Boiss.'un Sitotoksik, Fitotoksik ve İnsektisidal Aktiviteleri

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ABSTRACT

Objectives: To investigate the *in vitro* cytotoxic, phytotoxic, and insecticidal activity of *Chrysophthalmum montanum* (DC.) Boiss.

Materials and Methods: The crude methanol (80%) extract of the aerial parts of *C. montanum* was fractionated to obtain *n*-hexane, chloroform, *n*-butanol, and remaining water fractions. The crude extract and subsequent solvent fractions of the plant were evaluated for their biological activities using screening bioassays such as cytotoxicity on brine shrimp lethality, phytotoxicity against *Lemna minor* L., and insecticidal activity against *Rhyzopertha dominica* and *Tribolium castaneum*.

Results: The cytotoxicity assay revealed that the crude extract, *n*-hexane, and chloroform fractions of the plant had positive lethality with LD_{50} values of 71.51, 126.62, and 75.95 µg/mL, respectively. The extract and its fractions, except for the remaining water fraction, showed phytotoxic activity, which was expressed as percentage growth regulation in a concentration-dependent manner. *n*-hexane and chloroform fractions in particular had 100% growth inhibition (GI) at 1000 µg/mL, followed by the *n*-butanol fraction (62.6% GI) and crude extract (40.0% GI) of the plant at the same concentration. Otherwise, all samples had no insecticidal activity against *R. dominica* and *T. castaneum*.

Conclusion: This study demonstrates that *C. montanum* contains bioactive compounds related to potential biological activities such as cytotoxic and phytotoxic.

Key words: *Chrysophthalmum montanum*, Asteraceae, cytotoxic activity, phytotoxic activity, insecticidal activity

ÖZ

Amaç: Bu çalışmada, *Chrysophthalmum montanum* (DC.) Boiss.'un *in vitro* sitotoksik, fitotoksik ve insektisidal aktivitelerinin incelenmesi amaçlanmıştır.

Gereç ve Yöntemler: *C. montanum*'un toprak üstü kısmının %80 metanollü ham ekstresi *n*-hekzan, kloroform, *n*-butanol ve kalan sulu fraksiyonları elde etmek üzere ardarda fraksiyonlanmıştır. Bitkinin ham ekstre ve fraksiyonları tuzlu su karidesi letalite testinde sitotoksisitesi, *Lemna minor* L.'e karşı fitotoksisitesi ve *Rhyzopertha dominica* ile *Tribolium castaneum*'a karşı insektisidal aktivitesi gibi biyolojik tarama çalışmalarında biyolojik aktiviteleri bakımından incelenmiştir.

Bulgular: Sitotoksisite testinde ham ekstre, *n*-hekzan ve kloroform fraksiyonları sırasıyla 71.51, 126.62 ve 75.95 µg/mL LD_{50} değerleri ile belirgin letaliteye sahip bulunmuştur. Kalan su fraksiyonu hariç, ekstre ve fraksiyonların % büyüme inhibisyonu ile ölçülen fitotoksik aktivitesi konsantrasyona bağlı olarak gözlenmiştir. Özellikle *n*-hekzan ve kloroform fraksiyonları, 1000 µg/mL'de %100 büyüme inhibisyonuna sahip bulunmuş ve takiben n-butanol ve ham ekstre aynı dozda sırasıyla %62.6 ve %40.0 büyümeyi inhibe edici etkiye sahip bulunmuştur. Buna karşın, tüm örnekler *R. dominica* ve *T. castaneum*'a karşı insektisidal aktivite göstermemiştir.

Sonuç: Bu çalışma *C. montanum*'un sitotoksik ve fitotoksik biyolojik aktivite potansiyeline sahip biyoaktif bileşikler içerdiğini göstermiştir.

Anahtar Kelimeler: *Chrysophthalmum montanum*, Asteraceae, sitotoksik aktivite, fitotoksik aktivite, insektisidal aktivite

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INTRODUCTION

Medicinal plants contain various chemical constituents that have potential to use for their biological activities. Natural resources that yield valuable phytochemical products are often used in the treatment serious diseases. Moreover, folk medicines can attribute to the discovery of a large number of clinically effective compounds.

Chrysophthalmum Schultz Bip., a member of the family Asteraceae, the tribus Inulaeae[1], is represented by three species, namely *Chrysophthalmum montanum* (DC.) Boiss., *C. dichotomum* Boiss. & Heldr., and *C. gueneri* Aytac and Anderb[2] in Turkey. *C. montanum*, known as "nezle otu and tutça", is a herbaceous perennial plant mainly distributed in eastern parts of Turkey. Its aerial parts are traditionally used for the treatment of the common cold and sinusitis, as well as healing wounds on the body of human and animal in Turkey.[3-5]

To date, a few studies on the morphologic characteristics and preliminary evaluation of biologic antioxidant and antimicrobial activities have been reported on *C. montanum*.[2,3,6-10] Only one recent phytochemical study has been conducted on the isolation of sesquiterpene lactones from *C. montanum*.[11] However, there have been no other experimental studies for the scientific evaluation of phytotoxic, cytotoxic, and insecticidal effects of *C. montanum*.

The Asteraceae family has been intensively investigated in the treatment of various diseases in recent years. The family is well-known as a good source of sesquiterpene lactones, which are associated with antitumor, cytotoxic, antimicrobial, anti-inflammatory, and phytotoxic activities.[12,13] In our ongoing research on *C. montanum*, we revealed that *C. montanum* had cytotoxicity against some cancer cell lines using sulforhodamine B assays.[14] The aim of the present study was to investigate the therapeutic importance of *C. montanum,* which is relatively safe from toxic effects, for its phytotoxic, cytotoxic, and insecticidal activities by using screening bioassays.

EXPERIMENTAL

Chemicals

In the extraction and fractionation procedure, methanol, *n*-hexane, chloroform, and *n*-butanol were of analytical grade and purchased from Merck Co. (Darmstadt, Germany). Analytical thin-layer chromatography (TLC) was performed on precoated Kieselgel 60 F_{254} plates (Art. 5554, Merck). The plates were sprayed with anisaldehyde reagent [76% methanol (Merck) and 19% ortho-phosphoric acid (Riedel-De Haën, Buchs, SG Switzerland), 5% *p*-anisaldehyde (Merck)] and 20% H_2SO_4 (Merck) solution in MeOH (Merck).

Plant material

The aerial parts of *C. montanum* (DC.) Boiss. were collected from the valley of Tohma River, Akçadağ, Malatya, Turkey at the flowering stage in July 2014. The plant material was identified by one of the authors (Professor, PhD Hayri Duman). An authenticated voucher specimen (Hayri Duman 10324) was deposited in the Herbarium of GAZI, Ankara, Turkey.

Preparation of extracts

The air-dried aerial parts of *C. montanum* (500 g) were extracted four times (4x3000 mL) with 80% methanol at 25°C by stirring for 2 days. Following filtration, the combined methanol extracts were evaporated *in vacuo* at 40°C to dryness. The concentrated MeOH extract (90.8 g, CM) were further fractionated through successive solvent extractions with *n*-hexane (11x250 mL), chloroform (8x250 mL), and *n*-butanol saturated with H_2O (8x250 mL) in a seperatory funnel. Each extract, as well as its remaining aqueous phase (R-H_2O) after solvent extractions were evaporated to dryness under reduced pressure to yield an "*n*-hexane fraction" (1.7 g, CMH), "$CHCl_3$ fraction" (15.8 g, CMC), "*n*-BuOH fraction" (21.4 g, CMB), and "R-H_2O fraction" (36.4 g, CMR), respectively.

Phytochemical analysis

The extracts of *C. montanum* (1 mg/mL) were applied to silica gel plates. The *n*-hexane and $CHCl_3$ extracts were developed with the mixture of *n*-hexane:ethylacetate (65:35) and chloroform:acetone (80:20), respectively, as mobile phases. TLC plates were evaluated under UV light at 254 and 366 nm for the determination of fluorescent compounds. Anisaldehyde reagent and 20% H_2SO_4 were sprayed on the plates to visualize the separated compounds, and then the plates were heated for 5 min at 100°C. Sesquiterpenes appeared with pink and purple coloration.

Brine shrimp lethality assay

Brine shrimp (*Artemia saline* Leach) eggs (50 mg) were sprinkled in a hatching tank (a rectangular dish 22x32 cm) half-filled with filtered brine solution. The crude extract and subsequent solvent fractions of *C. montanum* (20 mg) were dissolved in 2 mL of methanol (stock solution). The stock solutions of the extracts were diluted to 10, 100, and 1000 µg/mL concentrations in three vials. The solvent was evaporated under a fume hood by keeping overnight. After hatching (2 days), 30 shrimps were added in each vial with the volume adjusted to 5 mL using sea water. The vials were incubated at 25-27°C for 24 hours under illumination. Other vials were supplemented with solvent and reference cytotoxic drug (Etoposide: 7.46 µg/mL), which served as negative and positive controls, respectively. The number of brine shrimps that survived was counted in each vial and LD_{50} values with 95% confidence intervals were determined using Finney computer software.[15,16]

Pytotoxicity assay

The phytotoxicity assay was performed for the crude extract and subsequent solvent fractions of *C. montanum* against *Lemna minor* L.[17] The medium was prepared by mixing various constituents in 1000 mL distilled water. KOH pellets were added for the adjustment of pH at 6.0-7.0. The extracts (30.0 mg) were dissolved in 1.5 mL of methanol, which served as a stock solution. The stock solutions of the extracts were diluted to get final concentrations as 10, 100, and 1000 µg/mL (nine flasks, three for each dilution). After evaporating the solvent overnight under sterile conditions, 20 mL medium and 10 plants were added to each flask, each one containing a rosette of two fronds

of *L. minor*. Other flasks were supplemented with medium and reference plant growth inhibitor (Paraquate) as negative and positive controls, respectively. All flasks were incubated in a growth cabinet for seven days at 30˚C. The number of fronds per flasks was counted and recorded at the end of the incubation period. Growth regulation (GR) as a percentage (%) was determined using the formula given below:

The criteria indicate that the GR (%) of 0-39 for low activity,

$$\text{GR (\%)} = \frac{\text{100-}\textit{Number of the fronds in the test samples}}{\textit{Number of the fronds in the negative control}} x\ 100$$

40-59 for moderate activity, 60-69 for good activity, and >70 for significant activity were detected.

Insecticidal activity

The crude extract and subsequent solvent fractions of *C. montanum* were tested against *Rhyzopertha dominica* and *Tribolium castaneum* using impregnated filter paper.[18] The samples (200 mg) were dissolved in 3 mL of methanol and served as stock solution. The samples (1019.10 µg/cm^2) were applied to filter paper of appropriate size (9 cm or 90 mm) on petri plates using a micropipette. The plates were left for 24 hours to evaporate the solvent. The next day, 10 insects of each species were placed in each plate (test and control) using a clean brush. Permethrin (239.5 µg/cm^2) was used as positive control; methanol was used as negative control. The plates were incubated at 27˚C for 24 hours with 50% relative humidity in the growth chamber. For the calculation, the number of survivals of each species was counted and mortality (M) (%) was determined using the following formula:

$$\text{M (\%)} = \frac{\text{100} - \textit{Number of insects alive in the test samples}}{\textit{Number of insects alive in the control}} x\ 100$$

RESULTS AND DISCUSSION

In this study, we investigated the crude (80% methanol) extract and its fractions of *C. montanum* for their primary screening bioassays including cytotoxic, phytotoxic, and insecticidal activities. The cytotoxic properties of *C. montanum* were investigated at concentrations of 10, 100, and 1000 µg/mL, using etoposide as a standard. The methanol extract, *n*-hexane, and chloroform fractions of the plant had positive lethality with LD_{50} values of 71.52, 126.62, and 75.95 µg/mL against the brine shrimps, respectively (Table 1).

The phytotoxicity of the investigated samples on *L. minor* was observed to have dose-dependent activity because low activity was detected in the *n*-hexane fraction with 12.5 and 18.7% inhibition at 10 and 100 µg/mL, respectively. Moderate phytotoxic activity was found in the methanol extract (40.0% inhibition) at 1000 µg/mL. Good phytotoxic activity was found in the chloroform fraction (68.7% inhibition) at 100 µg/mL and *n*-butanol fraction (62.6% inhibition) at 1000 µg/mL. Significant phytotoxic activity was shown in the *n*-hexane and chloroform fractions of the plant; 100.0% inhibition for each fraction at 1000 µg/mL (Table 2).

Table 1. Cytotoxic activities of the extract and fractions from *C. montanum*

Samples	No of survivors from 30 shrimps			LD_{50} (µg/mL)
	10 µg/mL	100 µg/mL	1000 µg/mL	
CM	25	10	06	71.52
CMH	18	23	06	126.62
CMC	15	17	00	75.95
CMB	22	22	17	-
CMR	27	26	21	-

Standard drug: Etoposide (LD_{50}=7.46 µg/mL)

Table 2. Phytotoxic activities of the extract and fractions from *C. montanum*

Samples	Growth regulation (%)		
	10 µg/mL	100 µg/mL	1000 µg/mL
CM	0	0	40.0
CMH	12.5	18.7	100.0
CMC	0	68.7	100.0
CMB	0	0	62.6
CMR	0	0	0

Standard drug: Paraquate (0.015 µg/mL)

Table 3. Insecticidal activities of the extract and fractions from *C. montanum*

Samples (1019.10 µg/cm^2)	*Tribolium castaneum*		*Rhyzopertha dominica*	
	Mortality (%)	Insecticidal activity	Mortality (%)	Insecticidal activity
CM	0	NO	0	NO
CMH	0	NO	0	NO
CMC	0	NO	0	NO
CMB	0	NO	0	NO
CMR	0	NO	0	NO

Reference insecticide: Permethrin (239.5 µg/cm^2)

The methanol extract and fractions of *C. montanum* were also screened for their insecticidal effects against *R. dominica* and *T. castaneum* using permethrin as a standard drug. There were no insecticidal effects on all samples against *T. castaneum* and *R. dominica* (Table 3).

The brine shrimp lethality assay is not specific for any particular physiologic effects. However, the cytotoxic effect of the natural constituents on the shrimp larvae was especially correlated with their anticancer potentials. This preliminary method,

which has been developed for screening, fractionation, and monitoring of physiologically active natural products, is clearly a more rapid, inexpensive, and general bioassay.[16] Moreover, phytotoxic and insecticidal constituents are mostly important to develop natural herbicides and insecticides that are safe, cost effective, and user-friendly for the environment.[19]

According to our results, the *n*-hexane and chloroform fractions of *C. montanum* were found as promising samples due to having cytotoxicity on brine shrimp. In our recent study, *n*-hexane and chloroform fractions of the plant also exhibited cytotoxicity on selected cancer cell lines.[14] In addition, our findings demonstrate that *n*-hexane and chloroform fractions of *C. montanum* possess significant phytotoxicity against *L. minor*. Our preliminary phytochemical detection using TLC showed that sesquiterpenes were as prominent components in the bioactive chloroform fraction of the plant.

CONCLUSION

In summary, the present study firstly depicts the potential of the extracts of *C. montanum* on biologic activities such as cytotoxicity against brine shrimp and phytotoxic effects, which indicate that the plant might be considered as a new potential source in the research of new drugs. Accordingly, further investigations to identify the responsible bioactive compound(s), principally sesquiterpenes, are ongoing on *C. montanum*.

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