TOTAL PHENOLIC CONTENT, ANTIOXIDANT CAPACITY AND ANTIFUNGAL ACTIVITY OF EXTRACTS OF *CARTHAMUS TENUIS* AND *CEPHALARIA JOPPENSIS*

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Three different extracts (aqueous, ethanolic and ethyl acetate) of *Carthamus tenuis* and *Cephalaria jopprnsis* were prepared and tested for total phenolic content (TPC), antioxidant capacity and antifungal activity. Results for *C. tenuis* are meaningfully different of known findings. As for *C. joppensis*, the medicinal and biological properties of this plant were never published before. For each plant, TPC was highest in aqueous extracts and these had highest antioxidant capacity. Ethanolic extracts of both plants had strongest activity against *Rhizopus stolonifer* (black mold).

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Introduction

Plants and their products possess many health benefits and medicinal activities. One of the most important properties of plants in terms of healthy nutrition, is antioxidant capacity, where polyphenolic compounds are among the top of active antioxidants.[1] For this reason, many methods of determining antioxidant capacity were developed, and they are based on a wide variety of chemical reactions and analytical techniques.[2] Numerous studies have shown that there is a clear and strong correlation between total phenolic content of a plant and its antioxidant capacity.[3]

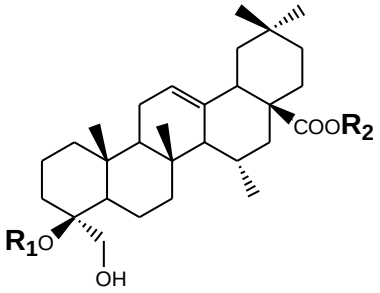
Plant extracts and essential oils are used for many other medicinal activities and medical treatments, which include anticancer, antidiabetic, antibacterial and antifungal. This last activity, antifungal, is drawing more and more research attention in the last few years due to it is immediate and practical applications for medical treatments and food storage and consumption, and its for this very reason, that many methods of testing antifungal activity were developed and published.[4] *Rhizopus stolonifer* (black mold), is one of the most common fungi and its dangers and damages can be found on almost all foods, especially bread and fruits.[5] Many studies were published with reports of new findings of possible treatments for *R. stolonifer*, and they include pure compounds from plant sources or origins (see reference 5, salicylic acid), synthetic compounds,[6] essential oils[7] and plants extracts.[8]

The genus *Carthamus* (Asteraceae) include around 47 species, where 15 of them can be found in the Middle East reagion and Western Asia.[9] Some of these species were studied for their medicinal properties, but strangely enough, *Carthamus tenuis* was very limitedly investigated, despite the fact that is very widespread in the western parts of the Middle East. Archeological studies of caves on Carmel mountain (Israel), indicate that humans used seeds of this plant, probably as food, around 48000-60000 years ago.[10] The same study found use of *Carthamus nitidus* in the Dead Sea area. In Lebanon, *C. tenuis* is used in traditional medicine to treat skin diseases (roots decoction) and hemorrhoids (roots extact).[11]

One of the earliest publications about medicinal activities of *C. tenuis* tested the TPC and antioxidant capacity of aqueous and methanolic extracts.[12] The same properties were reported in a later study, in addition to immunosuppresive activity of one of the compounds isolated from the plant.[13] All compounds that this group isolated were previously known. But the findings of these two studies are contradicting (see discussion). Methanolic extract of *C. tenuis* was also prepared by V. Kuete and his colleagues and tested for antibacterial (weak) and cytotoxic (inactive) activities.[14] They also indicate that the plant is traditionally used in Egypt to prevent abortion, to increase fertility and acts as aphrodisiac. A followup study that was conducted by authors of reference 13, showed that methnolic extract of *C. tenuis* and its fractions had immunosuppresive activity.[15] Ethanol/water (70%) extract was prepared and its TPC was found 41.8 mg g^{-1} dry extract (Folin-Ciocalteu reagent/ gallic acid method). The extract was *syriaca*),[21] and in Turkey to treat cancer (decoction of *C. specicosa*) and latex of *C. sparsipilosa* as antiseptic.[22]

Several studies were published so far and reported medicinal/biological activities of *Cephalaria* species, where some studies investigated some species collectively and some focused on a single species. Two novel triterpene-type glycosides were isolated and characterized from *C. scoparia* in a study that investigated four *Cephalaria* species.[23] These glycosides showed notable antioxidant capacity, as well as strong antibacterial activity. A year earlier, the same research group from Turkey reported another five novel triterpene-type glycosides, that have sufficient antimicrobial activity.[24] This group continued to isolate novel triterpene-type glycosides, and in 2012 they reported the isolation and characterization of two compounds from *C. gazipashensis*.[25] But the research of this group of the *Cephalaria* genus started more than two decades ago when they reported antimicrobial and antifungal activities of three new saponins

(triterpenic glycosides), that were isolated from the methanolic extract of *C. transsylvanica*.[26] From *C. paphlagonica* they succeeded in isolation of two novel saponis, and they reported their antioxidant and antimicrobial activities.[27] Strong antibacterial activity was reported by this group for another two novel saponins (hederagenin derivatives), that were isolated from *C. davisiana*.[28] The structures of these compounds are shown in Figure 1.



1 and 2 R_1 = Rha-β(1-4)Glc-β(1-3)Rha-β(1-2)Ara-(1-
1 R_2 = Glc-β(1-6)Glc-(1- **2 R_2 =** H

Figure 1. Saponins isolated from *C. davisiana*

When researchers of this group extracted *C. balansae* with n-butanol, they isolated and characterized four new saponins, that were tested and found active as immunomodulatory, hemolytic and cytotoxic.[29] The last publication of this group concerning saponins isolated from *Cephalaria*, reported the isolation of several compounds with branched saccharide side chains.[30] These compounds are reportedly having enhanced cytotoxic activity.

In addition to saponins, the fatty acids content of *Cephalaria* plants, was studied by several groups. Eight Turkish species were analyzed (n-hexane extract) by S. Kirmizigul and her colleages, that also tested the antioxidant activity of the extracts with several methods.[31] Long chain (>14 carbons) fatty acids are dominant in these oils. A follow up study was expanded by the same Turkish group, to investigate another ten species.[32] Method of extraction as well as antioxidant capacity and results in this study were very similar to those of previous cited (ref. 31), but the fatty acids composition of the *Cephalaria* species was clearly different having shorter carbon chains. Finally, in a comprehensive study, this group tested the acetone and ethanolic extracts of twenty one different species of *Cephalaria* against *Aedes aegypti*.[33] They found that ethanolic extract was more active, and they analyzed it by chromatographical methods, and found that eight compounds had measurable activity, where Luteolin-7-*O*-β-D-glycoside (Figure 2) was most active.

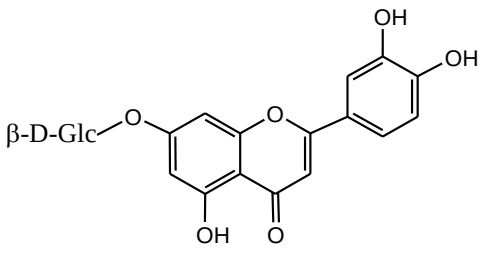


Figure 2. Structure of luteolin-7-*O*-β-D-glycoside

Essential oils of *Cephalaria* species were also studied. Ten of them were investigated by the group of S. Kirmizigul, where they analyzed the phytochemical composition of the oils.[34] They did not indicate any new compound. Recent study that extracted and analyzed the essential oil of *C. ambrosioides* also did not find new natural produtcs.[35] Another species that was extracted (90 % aqueous methanol) was *C. pastricensis*, afforded two known flavonoids, luteolin 7-*O*-glucoside and luteolin 7-*O*-arabino(1-6)glucoside, which had antioxidant activity.[36] Last study we cite here for biological activities, is comprehensive, despite the fact that it studied only *C. gigiantea* and did not report new compounds.[37]

Finally, despite being very widespread on the eastern parts of the Mediterranean basis, *Cephalaria jopponsis* was never studied before. Our wide search of published literature search could not find any study about the biological/medicinal activities of this species. The only publication we could find related to its nutrition benefits for lactating dairy cows.[38]

Experimental

Chemicals

All chemicals were purchased locally in at least analytical grade.

Plant Materials

Both studied plants (aerial parts) were harvested from the wild near our laboratory in Kfar-Qari (northern Israel). The green materials were washed with distilled water and air dried for 2 weeks. The dry matter of each plant was ground into a fine powder and stored at -12 °C in sealed containers.

Extraction

500 g of plant material were stirred in 1000 mL of solvent (water, ethanol, ethyl acetate) for 24 h at 50 °C. Suspensions were allowed to cool to room temperature and filtered (Munktell quant. Grade 393) to obtain clear solutions. These were evaporated to dryness with rotary evaporator: aqueous extracts at 60 °C, ethanol and ethyl acetate extracts at 50 °C. Extracts were stored at -12 °C.

Total Phenolic Content (TPC)

TPC was determined with the method described by Kumar and Jain (with no modifications).[39] The sample mixture that contains 3 mg of extract (or standard gallic acid solutions) dissolved in 1 mL of solvent, was obtained by dilution of 0.3 g of extract in 10 mL stock solution 10 folds. Then it was added to 10 mL volumetric flask containing 8 mL of dd H_2O. After that, 1mL of Folin-Ciocalteu's reagent was added to the mixture. After 3min, 1mL of 35% Na_2CO_3 solution was added with mixing to reach the reaction system to 10 mL. The reaction mixture was mixed thoroughly and allowed to stand for 90 min at 25 °C in the dark. Absorbance of all the sample solutions against a blank was measured at 725 nm.

Table 1. TPC and antioxidant capacity of *Carthamus tenuis* and *Cephalaria jopponsis* extracts.

Plant	Total phenolic content[a,b]			Antioxidant capacity[a,c]		
	Aqueous	Ethanolic	EtOAc[d]	Aqueous	Ethanolic	EtOAc
Carthamus tenuis	31.2	17.9	6.6	48.1	36.4	21.3
Cephalaria jopponsis	26.7	18.3	8.1	41.1	30.1	20.7

[a]Average values of three tests, [b]mg of gallic acid g^{-1} of dry extract, [c]mg of ascorbic acid g^{-1} of dry extract, [d]Ethyl acetate.

Table 2. Antifungal activity of *Carthamus tenuis* and *Cephalaria jopponsis* extracts against *Rhizopus stolonifer*.

Plant	Inhibition (%)[a]					
	Aqueous extract		Ethanolic extract		Ethyl acetate extract	
	10%	20%	10%	20%	10%	20%
Carthamus tenuis	28.4	36.5	38.2	42.9	25.1	30.0
Cephalaria jopponsis	31.5	33.2	41.4	51.6	30.9	33.3

[a]Extraction solvent in each experiment was used as control and resulted in 0 % inhibition.

Calibration curve was constructed with different concentrations of gallic acid (2–12 μg mL^{-1}) as the standard and dd H_2O was used as reagent blank. The results were expressed as mg gallic acid equivalents (GAE)/g dry extract.

Antioxidant capacity

Antioxidant capacity was determined by the phosphomolybdenum method described by Sharma and Singh (with slight modifications).[40] Tested aliquot of 0.1 mL (100 mg extract) was added to 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The blank was 0.1 ml of ethanol. The tubes were capped and incubated in a boiling water bath at 95 °C for 90 min, then allowed to cool to room temperature. Absorbance of the aqueous solution of each was measured at 695 nm. The antioxidant capacity was expressed as an equivalent of ascorbic acid (mg of ascorbic acid g^{-1} of dried extract).

Antifungal activity

Agar plates were prepared according to Sanders, with slight modifications.[41] To prepare 20 petri agar plates, 400 mL of distilled water were heated to 60 °C and 9.2 g of potato agar was added to them. The suspention was stirred until clear solution was obtained, then it was poured into the plates and allowed to cool to room temperature. Agar plates were not stored but used immediately.

Antifungal assay was performed according to Salhi *et al*. with only changing the fungus and the plants.[42] *Rhizopus stolonifer* was grown on whole wheat bread and extracted with water. The center of each Petri dish was inoculated with 5 mm diameter disc of fungal mycelium, taken from pure culture (7 days old).Then, all inoculated dishes were incubated at 25 °C for 6 days and the radial mycelial growth was measured. The antifungal activity of each extract was calculated in terms of inhibition percentage of mycelia growth by using the following formula:

$$\% \text{ Inhibition} = [(dc - dt)/dc] \times 100$$

Where *dc* is the average increase in mycelia growth in control and *dt* is the average increase in mycelia growth in treated samples with extracts

In all experiments the control was the extraction solvent and we performed the antifungal tests using two concentrations for each extract: 10 % and 20 % (w/w).

Statistical analysis

All measurements were repeated three times and resutls introduced in the next section are average values.

Results and discussions

TPC (total phenolic content) and antioxidant capacity are shown in Table 1. Similarly, antifungal activity results are shown in Table 2.

Our TPC and antioxidant capacity tests of *C. tenuis* are notably different of previous studies. They are higher than the findings of Alali and his colleagues,[12] and with no agreement with the results of A. A. El-Hela and his colleages.[13] It is very important to notice that the reportings of these two groups are contradicting. While Alali *et al*. report that the aqueous extract has higher TPC than the methanolic (27.8 and 16.2 mg g^{-1} of dry extract, respectively), El-Hela *et al*. reported that methanolic extract had higher TPC than aqueous extract (65.8 and 18.2, respectively).

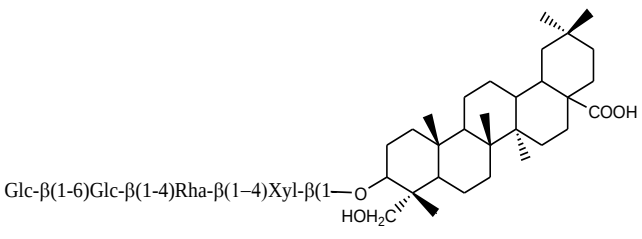


Figure 3. Structure of antifungal transsylvanoside A from *C. transsylvanica* (ref. 50).

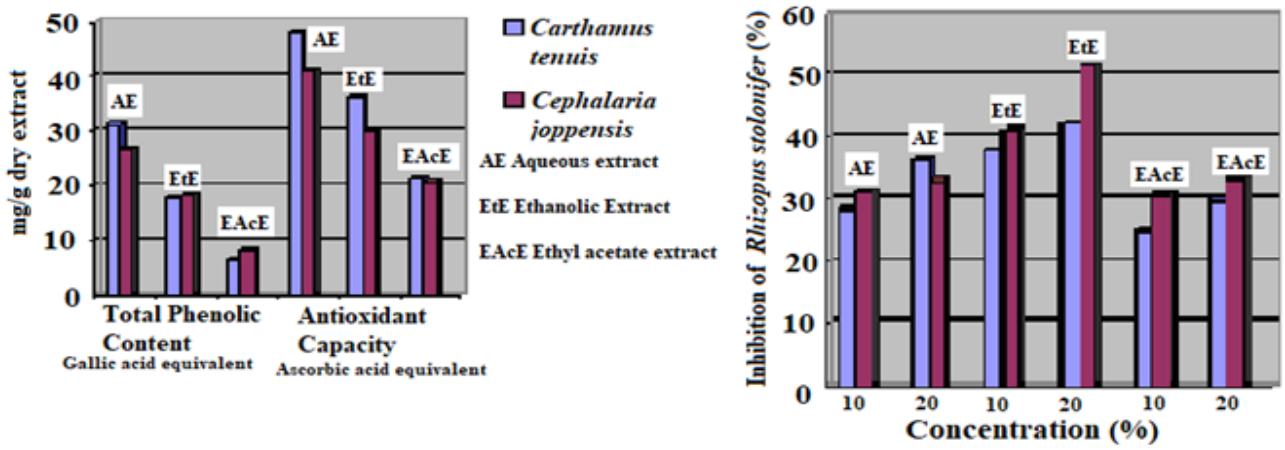


Figure 4. Total phenolic content, antioxidant capacity and inhibition of *Rhizopus stolonifer* of *Carthamus tenuis* and *Cephalaria joppensis*

Accordingly, their antioxidant tests resulted in contradicting findings, whereas Alali *et al*. measured 162.9 μmol g^{-1} of dry aqueous extract and 61.8 μmol g^{-1} for methanolic extract, El-Hela *et al*. reported 163.9 μmol g^{-1} for methanolic extract and 29.8 μmol g^{-1} for aqueous extract. The contradiction between these reports is even stranger based on the fact that both groups used exactly same methods to determine TPC (Folin-Ciocalteu reagent and gallic acid) and antioxidant capacity (ABTS and Trolox) of the extracts. Our findings are more consistent with those of Alali *et al*. in terms of that the aqueous extract had highest TPC and antioxidant capacity, even though we did not prepare methanolic extract but ethanolic.

The follow up study of the Egyptian research group (reference 13) that investigated the immunosuppressive activity of the matholic extract of *C. tenuis*,[15] discovered that this activity is due to the presence of choline in the extract. Despite the fact that this group did not isolate new natural products, and the immunosuppressive of plant derived choline is well known,[43] it is important to take this activity into account and possibly utilize it for future medicinal uses. Despite the fact that authors of reference 17 underestimated the global total number of *Carthamus* (25 instead of 37), and they concluded that this genus is not suitable as oil source, they actually contradict archeological evidences that this genus was used for oil production in the near east.[44] The use of this oil remains unclear.

The number of novel saponins that were isolated and characterized by the group of S. Kirmizigul from Turkey is outstanding (references 23-30). These compounds were found active in many medicinal/biological tests, especially antibacterial and cytotoxic activities. Based on the fact that saponins are known for their high cytotoxic effect,[45] and that they can be important prodrugs,[46] we find these dicoveries of very high impotance for drug development.

Comparison of our TPC and antioxidant capacity of the extracts of *C. tenuis* with those of Alali and his colleagues (ref. 13) reveal the fact that our results are slightly higher. As far as we can explain this, the difference might emerge from two reasons: our plant collection area is wetter than the harvest area of F. Alali in Jordan, and, seasonal variation. Our harvest was done in late March, while authors of reference 13 do not indicate their harvest time. Our plant materials were collected in the time when TPC is highest according to many published studies.[47]

Our antifungal activity findings are relatively high. All extracts had antifungal activities, when ethanolic extracts were most active, and that of *C. joppensis* is the highest. Even compared with reportedly very active extracts of plants with well known antifungal activity,[48,49] our findings are easily comparable. But also if we compare our results with the findings of S. Kirmizigul and her colleages (ref. 26), that tested the antifungal activity of three pure triterpenoid glycosides isolated from *C. transsylvanica*, our results are higher. These compounds have saponin typical structure with carboxylic acid residue. The structure of one of these compounds is shown in Figure 3.[50] To summarize our results, we present them in Figure 4.

Suggestions for further research

Both the plants, *Carthamus tenuis* and *Cephalaria joppensis* were very limitedly studied or not at all in terms of medicinal activities. Research of these plants should be widely expanded. The very limited studies so far focused on total phenolic content (with contradictions) and antioxidant capacity. Other properties such as antibaterial, antidiabetic, anticancer … etc. should be investigated. The antifungal activity of both plants is remarkable. This property should be studied in depth. The knowledge of the chemical compositions of both plants is very limited or does not exist. Both plants should be analyzed for active known, but especially for novel natural products.

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