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DOI: HTTP://DOI.ORG/10.31925/FARMACIA.2018.6.22 **ORIGINAL ARTICLE**

FLAVONOIDS AND BIOLOGICAL ACTIVITIES OF *CENTAUREA NERIMANIAE* S. KULTUR

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Abstract

Centaurea nerimaniae S. Kultur, an endemic species from the Turkish flora, distributed in south Anatolia (Icel Province) was investigated for its flavonoids and *in vitro* antioxidant, anti-cholinergic, anti-inflammatory and antibacterial activities. The phytochemical investigation of the plant led to the isolation of five flavonoids, identified as cirsimaritin, hispidulin, apigenin, isokaempferide and apigenin 7-O-glucoside. In addition to its ability to inhibit AChE and lipid peroxidation, induced by Fe^{3+}/ascorbate system, scavenge DPPH radicals, and to reduce Fe^{3+} to Fe^{2+}, the extract showed strong COX-1 and COX-2 inhibitory and antimicrobial activities, suggesting that this plant could act as an antioxidant and anti-inflammatory agent against disorders, associated with oxidative damage, as well as an effective phytotherapeutic agent against some fungal and bacterial diseases.

Rezumat

Centaurea nerimaniae S. Kultur, o specie endemică din flora Turciei, a fost investigată pentru conținutul în flavonoide pentru activitățile antioxidante, anticolinergice, antiinflamatoare și anti-bacteriene. Investigarea fitochimică a plantei a condus la izolarea a cinci flavonoide: cirsimaritină, hispidulină, apigenină, izokaempferidă și 7-O-glucozid apigenină. Testele biochimice sugerează că această plantă ar putea acționa ca antioxidant și anti-inflamator în tulburările asociate leziunilor oxidative, precum și ca un produs fitoterapeutic eficient împotriva unor boli fungice și bacteriene.

Keywords: *Centaurea nerimaniae* S. Kultur, flavonoids, antioxidant, anti-acetylcholinesterase, anti-inflammatory, antimicrobial activity

Introduction

Considering the rich variety of medicinal plants commonly grown and used in Turkey and the high prevalence of cancer, cardiovascular, inflammatory and neurodegenerative diseases, more investigations should be carried out in order to understand the beneficial effects of Turkish medicinal plants [33].

The genus *Centaurea* is represented by 217 species and 60% of which are endemic in Turkey [9]. *Centaurea nerimaniae*, named in the honour of the Turkish botanist Prof. Dr. Neriman Özhatay, is an endemic species distributed in south Anatolia (Icel Province) [28].

Centaurea species are used in folk medicine because of their digestive, expectorant, antidiarrheal, tonic, and antipyretic properties [5].

These species have been extensively studied mainly for their antioxidant [1, 2, 24, 34, 46-48], anti-microbial [20, 23, 27, 43, 44], acetylcholinesterase (AChE) inhibitory [2, 34], antiinflammatory and wound healing [25] as well as analgesic properties [26]. There are some reports attributing the anti-inflammatory effects of *Centaurea* species or its ingredients to inhibition of NF-kB activation, reduction of expression of COX-2 and iNOS [17, 46].

Phytochemical studies have shown that the main components of *Centaurea* sp. are sesquiterpene lactones, acetylenes and flavonoids [3].

Previous phytochemical screening showed that *Centaurea* species contain flavonoids including apigenin, luteolin, salvigenin, kaempferol, hispidulin and cirsimaritin, and sesquiterpene lactones of the guianolide type as diain, cynaropicrin, deacyclo-cynaropicrin and janerin [6]. Formisano *et al*. summarized the distribution of flavonoids in 112 *Centaurea* species [18].

However, there are no reports regarding the phyto-chemicals as well as biological activities of *Centaurea nerimaniae*. Isolation of flavonoids and evaluation the therapeutic potential of *Centaurea nerimaniae* by examining its antioxidant, anti-inflammatory, anti-microbial and anticholinesterase properties is aimed in this study.

Materials and Methods

Plant material

The plant material of *Centaurea nerimaniae* S. Kultur were collected from Mersin Province, near Arslanköy location (Turkey) and identified by Prof. Dr. Şükran Kültür at Istanbul University, Pharmaceutical Botany Department, in June 2012. Voucher specimens were maintained in the Herbarium of Istanbul University, Faculty of Pharmacy, Istanbul, Turkey (ISTE 98163).

Extraction and isolation

The dried aerial parts (900 g) of *C. nerimaniae* were first subjected to extraction with petroleum ether and then with EtOH (95°) in a Soxhlet apparatus. The petroleum ether extract (A) was concentrated and extracted with 60 % ethanol. The aqueous extract was concentrated and extracted with chloroform (B) in a separator funnel. The concentrated ethanol (95°) extract was diluted with H_2O and successively extracted with benzene (C), chloroform (D) and ethyl acetate (E) for fractionation.

For the purification of flavonoids from the B, D and E extracts, silica gel column chromatography, paper chromatography and preparative TLC (thin-layer chromatography) were applied. As a result of this work cirsimaritin and hispidulin were isolated from the petroleum ether-chloroform (B), apigenin and ısokaempferide were isolated from the ethanol-chloroform (D), and apigenin 7-O-glucoside was isolated ethanol-ethyl acetate (E) extracts. The structures of the pure compounds were elucidated based on *Rf* values, colour reactions and spectroscopic methods in comparison with standards or with reference data.

The dried aerial parts (50 g) of the plant were extracted with methanol in a Soxhlet apparatus. The methanol extract was evaporated to dryness by a rotary evaporator. The extract was kept at -20ºC and was then lyophilized. In this way, the crude methanolic extract was obtained and used for biological activity studies.

Biochemical assays

In the present study, the extract was screened for its antioxidant activity using thiobarbarbituric acid (TBA) test based on the lipid peroxidation of liposomes [15], 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging [10], Trolox equivalent anti-oxidant capacity (TEAC) assay with 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$) radical cation [37] and ferric reducing ability of plasma (FRAP) assay [7]. Quercetin was used as reference antioxidant.

The extracts total phenolic compounds were evaluated using Folin-Ciocalteu reagent according to Slinkard and Singleton method [42] and expressed as means mg gallic acid equivalents (GAE)/g of dry weight (DW). Total flavonoids were evaluated by $AlCl_3$ colorimetric method described by Sakanaka *et al.* [39] and expressed as mg catechin equivalents (CE)/g of DW.

Inhibition or radical scavenging activities (%) of the extract were calculated according to the equation:

Inhibition or radical scavenging activity (%) = [1-(Absorbance of sample/Absorbance of control)] x 100.

The AChE inhibitory activity of the extract was determined by the method of Ellman *et al*. [16]. Galantamine was used as a standard AChE inhibitor and distilled water was used as a control.

COX-1 and COX-2 inhibitory activities of the extract were determined by calculating the inhibition percent of prostaglandin production measured by enzyme immunoassay kit following the manufacturer's protocol (Cayman). Indomethacin was used as a standard.

Antibacterial and antifungal effects

Antibacterial and antifungal effects of the extract were determined by the microbroth dilutions technique performed in accordance with the recommendations in the Clinical Laboratory Standards Institute (CLSI) document M7-A5 and M 27-A [12, 13]. The minimum inhibitory concentration (MIC) values of the extract against studied microorganisms were determined.

Statistical analysis

Results were expressed as a mean ± standard deviation. Statistical comparisons were performed with Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results and Discussion

Isolated compounds

In the present study, five flavonoid compounds were obtained from the aerial parts of *C. nerimaniae*. These compounds were characterized as cirsimaritin (B ext., 6 mg), hispidulin (D ext. 9 mg), apigenin (D ext. 5 mg), isokaempferide (E ext. 7 mg) and apigenin 7-O-glucoside (E ext. 22 mg) by UV spectral data compared with the data in the literature [19, 29, 36]; and by TLC comparison with reference standards (Table I). Apigenin 7-O-glucoside was the major compound.

Similarly, Nikolova and Bancheva [31] have reported that *Centaurea* species are rich sources of externally accumulated flavonoid aglycones such as luteolin, apigenin, 6-hydroxyluteolin 6-methyl, kaempferol 3-methyl and scutellarein 6,4'-dimethyl ethers. They reported that 6-hydroxyluteolin, methyl derivatives of scutellarein and 6-O-substituted flavones are found in the exudates of *Centaurea davidovii* and *C. parilica*, while 6-O-substituted flavonols, such as quercetagetin, methyl derivatives of 6-hydroxykaempferol and flavonols with 3-methylation are found in exudates of *C. stenolepsis.*

Table I

UV data for flavonoids from *C. nerimanieae* (λ max)

Flavonoid	MeOH	NaOMe	$AlCl_3$	$AlCl_3$/HCl	NaOAc	NaOAc/H_3BO_3
Cirsimaritin	332, 285	370, 287	364, 302, 285 sh, 263	354, 300, 285sh, 262 sh	388 sh, 335, 278	333, 280
Hispidulin	335, 273	393, 327, 275	390 sh, 360, 303, 279 sh, 263	390 sh, 352, 300 sh, 280, 260 sh	370, 308 sh, 297 sh, 274	337, 254
Apigenin	336, 296 sh, 267	392, 324, 275	384, 348, 301, 276	381, 340, 299, 276	376, 301, 274	338, 302sh, 268 sh
Isokaempferide	352, 268	404, 329, 276	396, 345, 303, 277	396, 345, 303, 277	365, 301, 275	361, 257
Apigenin 7-O-glucoside	333, 268	386, 301 sh, 269, 245 sh	386, 348, 300, 276	382, 341, 299, 277	387, 355, 267, 256 sh	340, 267

sh = shoulder

Formisano *et al*. [18] reviewed reports on flavonoids from the *Centaureinae* subtribe of the family *Asteraceae*, as well as the ^{13}C-NMR-spectral data in the literature pointed out that only 16 of the 72 recognized genera of the subtribe *Centaureinae* have been investigated for the occurrence of flavonoids. From the data reported by Formisano *et al*. [18], it was seen that the majority of the genera of the *Centaureinae* have not been investigated for their flavonoid profile yet.

The results of the chemical characterization of *C. nerimaniae* reconfirmed the value of the *Centaurea* genus as a source of the flavonoid aglycons.

Our results were in accordance with the flavonoid composition of the *Centaurea* species from the Turkish flora [45].

Total phenolic and flavonoid contents. The total phenolic content of *C. nerimaniae* was 2.23 ± 0.11 mg GAE/g DW, while the flavanoid level was 1.25 ± 0.10 mg CE/g DW (Table II).

Table II

Total extractable compounds (EC), total phenolic compounds (PC, as gallic acid equivalents) and total flavonoids (as catechin equivalents) of the methanol extract of *C. nerimaniae*

	EC (mg/g DW)	Phenolic compounds (PC) (mg GAE/g DW)	Flavonoids (mg CE/g DW)	PC/EC (%)
C. nerimaniae	96.25	2.23 ± 0.11	1.25 ± 0.10	2.32

Values were the means of three replicates ± standard deviation

Many earlier studies showed that the aerial part of *Centaurea* species is an alternative source of phenolic compounds. Comparing to other *Centaurea* species collected from Turkey, it was evident that the phenolic content of *Centaurea nerimaniae* is lower than the values found for Centaurea species investigated by Aktumsek *et al*. [1] (ranged from 82.27 to 175.40 mg GAE/g extract), *Centaurea* species investigated by Şen *et al*. [41] (ranged from 4.825 to 12.460 mg GAE/g DW), and *Centaurea* species investigated by Aktumsek *et al*. [2] (ranged from 207.78 to 232.78 mg/g extract).

A similar content of phenolic compounds were reported by Zengin *et al*. [47] for *Centaurea urvillei* subsp *hayekiana* (17.22 mg GAE/g extract), Zengin *et al*. [46] for *C. pulchella*, *C. patula* and *C. tchihatcheff* (ranged from 22.27 to 55.00 mg GAE/g extract), Ozsoy *et al*. [34] for *Centaurea antiochia* var. *praealta* (3.68 mg GAE/g DW) and Uysal *et al*. [45] for *Centaurea urvillei subsp. stepposa* (33.11 mg GAE/g extract).

Quercetin, apigenin and kaempferol, the most common flavonoids present in *Centaurea* species, may contribute to the antioxidant activity.

Antioxidant activity

Centaurea species have been the subject of intense research for their antioxidant properties. In this study TBA method, which evaluates the ability of an anti-oxidant to inhibit lipid peroxidation and DPPH radical scavenging assays were chosen as representative of hydrogen transfer, while TEAC and FRAP methods were chosen as representative of single electron transfer reaction based assays.

Table III presents the results of antioxidant activities of the extract, expressed as EC_{50}, TEAC and FRAP values.

Compared to DPPH radicals, the methanolic extract of the aerial parts from *Centaurea nerimaniae* was found to be more effective ABTS radical cation scavenger. The highest E_{50} value was found in TBA test, showing the less ability of the extract to protect liposomes from lipid peroxidation. TEAC value was similar to the FRAP value, which indicates that the extract is effective in donating of electrons. However, the results showed a weak antioxidant activity compared to the reference antioxidant quercetin. Although, the extract was less active than quercetin ($p < 0.05$), it was seen that it has hydrogen and a single electron donor activities, thus could serve as an antioxidant. These activities may be attributed mostly to the presence of the phenolic group, a feature common to natural phenolic compounds.

Table III

Half maximal effective concentration (EC_{50}), Trolox equivalents antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) values of methanol extract of *C. nerimaniae*

	EC_{50} (mg/mL)[A]				
	DPPH	**ABTS**	**Anti-LPO**	**FRAP[B]* (mM)**	**TEAC[C]* (mM)**
C. nerimaniae	5.81 ± 0.94[a]	4.55 ± 0.19[a]	11.87 ± 0.56[a]	2.45 ± 0.18[a]	1.89 ± 0.05[a]
Quercetin	0.098 ± 0.001[b]	0.155 ± 0.012[b]	0.062 ± 0.005[b]	2.34 ± 0.010[a]	1.98 ± 0.03[a]

Values were the means of three replicates ± standard deviation. Values with different letters in the same column were significantly ($p < 0.05$) different. [A] EC_{50} value: The effective concentration at which the antioxidant activity was 50%; DPPH and ABTS radicals were scavenged by 50% and LPO was inhibited by 50%. [B] Expressed as mM ferrous ions equivalents. [C] Expressed as mM trolox equivalents. * Determined at 10 mg/mL for the extract and 0.3 mg/mL for quercetin.

These results are in accordance with previous studies, which reported the efficacy of *Centaurea* species to prevent the lipid peroxidation [2, 41], scavenge free radicals [1, 34, 41] and act as reducing agent [2, 14].

AChE inhibitory activity

Cholinesterase inhibitors are used for the treatment of Alzheimer's disease (AD) and vascular dementia [30]. There are limited reports on the AChE inhibitory activity of *Centaurea* species. Methanolic extract of *C. nerimaniae* exhibited modest AChE inhibitory activity of 42.67 ± 3.05% at 10 mg/mL concentration. However, the extract showed less inhibitory activity against AChE than galantamine (86.17 ± 1.44%, at 0.05 mg/mL concentration). Similarly, the extract from the aerial parts of *Centaurea polypodiifolia* var. *pseudobehen* was reported to show 45.50 % inhibition towards both AChE and buthylcholinesterase (BChE) [2]. The chloroform extract of *C. pulchella* was reported to have noticeable inhibition value on AChE (95.93%) and BChE (95.69%) at 2 mg/mL. Otherwise, Boğa *et al*. [8] reported that the petroleum ether, acetone and methanol extracts of *C. balsamita*, *C. depressa*, *C. lycopifolia* did not show any AChE activity. This difference may be explained by the different extraction procedure and the diversity of phenolic compounds. Roseiro *et al*. pointed out that a free OH-group at C3 position of the flavonoids enhanced their AChE inhibitory effect, while the glycosylation or lacking of OH at C3 (luteolin and apigenin) do not have a positive effect [38].

Anti-inflammatory activity

The expression of COX-2 is regulated by a broad spectrum of pro-inflammatory mediators, involved in inflammation. The association of COX-2 with inflammation resulted in a search for specific COX-2 inhibitors that would provide therapeutic anti-inflammatory effects similar to those of NSAIDs but did not cause the unwanted side effects [22].

Many reports suggest that *Centaurea* species exert anti-inflammatory properties in a rat model of inflammatory bowel disease (IBD) [4], carrageenan-induced paw oedema [17, 25], TPA-induced ear oedema formation [26], most probably through the COX-2 inhibition and expression or Nuclear factor-κβ.

The extract of *C. nerimaniae* has been shown to cause 92.16 ± 3.49% inhibition of COX-1 and 68.03 ± 4.90% inhibition of COX-2 at 10 mg/mL. However, this level of inhibition is significantly lower when compared to indomethacin which was shown to cause 97.1 ± 0.1% inhibition of COX-1 at 0.005 mg/mL and 95.92 ± 2.2% inhibition of COX-2 at 0.050 mg/mL. Similarly, *Centaurea anthiocia* has been found to inhibit both COX-1 and COX-2 enzymes [34]. These findings support the reported anti-inflammatory activity of *Centaurea* species.

Anti-inflammatory activities of *Centaurea* species have been explained by their content of sesquiterpene lactones (SLs), obtained from many plants of this family [4]. Flavonoids have been reported to display marked *in vitro* and *in vivo* anti-inflammatory activities. It was reported that the ability of some flavonoids, such as rutin, quercetin, apigenin and centaureidin, to inhibit cyclooxygenase-2 expression, may contribute to the antiinflammatory properties of *Centaurea* species [11, 21, 32]. Anti-inflammatory activity of hispidulin, naturally occurring flavone found in *C. nerimaniae*, were investigated in the TPA mouse ear edema model and was found to be active [35].

These findings provide an explanation for the reported anti-inflammatory properties of *Centaurea* extracts.

Antimicrobial activity

Microorganisms used in this study represent pathogenic species mostly associated with nosocomial infections. Antibacterial effect of the extract from *C. nerimaniae* was tested on gram-negative (*Escherichia coli*, *K. pneumonia, Pseudomonas aeruginosa*, *Proteus mirabilis*) and gram-positive (*Staphlococcus aureus*, *Streptococcus epidermidis*) bacteria, and antifungal effect of the extract was investigated on *Candida albicans*. The results were displayed in Table IV. The extract exhibited inhibitory activity against all tested strains. *C. nerimaniae* extract had the greatest potential of antibacterial activity against both gram-positive and gram-negative bacteria with the MICs ranged from 1.25 to 10 mg/L. According to the antibacterial activity results, *S. aureus* was the most sensitive species (MIC = 1.25 mg/L), followed by *P. mirabilis* (MIC = 2.5 mg/L), *K. pneumoniae* (MIC = 5 mg/L), *P. aeruginosa* (MIC = 5 mg/L) and *S. epidermidis* (MIC = 10 mg/mL). Moreover, our antifungal activity

findings showed that *C. albicans*, the yeast, also was highly sensitive (MIC = 1.25 mg/L) to the extract.

Table IV

Antimicrobial activity of methanol extract of *C. nerimaniae* and standards

Microorganism		MIC values (mg/mL)	
		Extract	Standard
Gram-positive	*S. aureus*	1.25	1.2[#]
	S. epidermidis	10	9.8[#]
Gram-negative	*E. coli*	5	4.9[#]
	K. pneumoniae	5	4.9[#]
	P. aeruginosa	5	2.4*
	P. mirabilis	2.5	2.4[#]
Yeast	*C. albicans*	1.25	4.9[¶]

[#]cefuroxime; * ceftazidime; [¶]clotrimazole

These results confirmed the findings in the other studies reporting the antibacterial activities of *Centaurea* species [24, 27, 41, 43, 44]. Further studies will be focused on the *in vivo* antimicrobial activities and chemical identification of the anti-microbial ingredients with antimicrobial activities close or stronger than tested standard antibiotics or antifungal, clotrimazole.

Conclusions

The present study reported for the first time the flavonoid profile and biological activities of *C. nerimaniae*. The observed antioxidant, antibacterial, anti-inflammatory and anti-cholinesterase activities may be due to the presence of the five flavonoids (cirsimaritin, hispidulin, apigenin, isokaempferide and apigenin 7-O-glucoside) identified by thin-layer chromatography (TLC). It was concluded that this species has the potential for effective treatment of various illnesses, including inflammation, microbial and AD diseases.

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