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In vitro antiproliferative, antioxidant, anti-inflammatory activities and phenolic profile of *Centaurea saligna* (K.Koch) Wagenitz

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ABSTRACT: In this study, methanol extract (CSM) and their hexane (CSH), chloroform (CSC), ethyl acetate (CSEA) and aqueous methanol fractions (CSAM) prepared from aerial parts of *Centaurea saligna* (K.Koch) Wagenitz were investigated for *in vitro* antiproliferative, anti-inflammatory and antioxidant activity. Anticancer, antioxidant, anti-inflammatory activities of these extracts were carried out by MTT, DPPH-ABTS, 5-lipoxygenase methods, respectively. Methanol extract and its fractions of *C. saligna* were evaluated for their cytotoxicity against MCF-7, A549, HeLa, HT-29, PC-3 cell lines at a concentration of 50 µg / ml. Total flavonoid and phenolic contents of extracts were detected by $AlCl_3$ and Folin-Ciocalteu methods, respectively. Analysis of phytochemical of CSEA, showing a strong anti-inflammatory activity with good antioxidant activity, was performed by LC-MS/MS. CSC exhibited the best antiproliferative activity against HeLa, HT-29, MCF-7 cell lines with 50.18%, 46.88%, 45.42% mortality, respectively. CSEA showed the highest antioxidant activity with IC_{50} values of 82.05 µg/ml and 108.4 µg/ml against ABTS and DPPH radicals, respectively. The highest total phenolic amounts have been determined in CSEA with value of 379.2 mg GAE/g extract. In the same way, the highest total flavonoid amounts have been observed in CSEA with values of 170.3 mg QE/g extract. CSEA showed strong anti-inflammatory activity with IC_{50} value of 0.10 µg/ml when compared to indomethacine as standard (22.39 µg/ml). Analysis of CSEA by LC-MS/MS revealed that the major compounds were quinic acid, 5-caffeoylquinic acid, apigenin C-hexoside-C-pentoside, p-coumaroylquinic acid, quercetin glucoside, di-caffeoylquinic acid, isorhamnetine glucoside, isorhamnetin glucuronide and isorhamnetin derivative. These results proved that CSEA has significant anti-inflammatory and antioxidant activity and CSC has good antiproliferatif activity. Also, the results demostrate that CSEA and CSC are a good source for further bioactivity-guided isolation in discovering new active antioxidant, anti-inflammatory and antiproliferative compounds.

KEYWORDS: *Centaurea saligna*; antioxidant activity; antiproliferative activity; anti-inflammatory activity.

1. INTRODUCTION

Mankind have used plants as a source of food and medicine since humanity existed. According to the report of the World Health Organization (WHO, 2002), more than 60% of the people on earth still uses herbs primarily in the treatment of many diseases, due to the bioactivity of the their phytochemicals (mainly secondary metabolites) [1].

Cancer, one of the major causes of death in the world, is a complex genetic disease characterized by uncontrolled cell growth, metastasis and angiogenesis [2]. Chemotherapy and radiotherapy among the applied cancer treatments lead to high costs and many side effects. All these side effects reduce the quality of life and discourage patients from adapting to treatment. This situation leads to the progression of cancer and related complications [3]. Therefore, there is a need to discover stronger anticancer drugs as well as being more selective and less toxic than those currently in use [4]. Various herbs with different pharmacological properties have been shown to be potent in cancer treatment because they contain antitumor compounds without causing toxicity [5].

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The idea that inflammation is an important factor in tumor progression is supported. Many cancers are caused by infection, chronic irritation and inflammation. It turns out that the environment created by inflammatory cells is an important mediator of the neoplastic process in the proliferation, survival and migration of tumors to other tissues. These views support new anti-inflammatory treatment approaches to cancer development [6]. It has been demonstrated that the underlying cause of many latent tumors is chronic inflammation that causes premalignant cells to become fully developed cancers [7].

The most important target of reactive oxygen species is DNA. ROS (reactive oxygen species) causes cancer by inducing various DNA damage, including rupture of DNA strands, base modification and DNA protein crosslinking [8]. Various types of 'Reactive species' (RS) are produced in the human body and many of them are powerful oxidizing agents that can damage DNA and other biomolecules. Increased RS formation may support the development of malignancy and 'RS' occurring at normal levels may be responsible for the increased risk of cancer development in the elderly [9]. Thus, antioxidants can act in preventing cancer formation by reducing these "reactive species".

Medicinal plants have been the source of pharmaceutical bioactive compounds against various types of diseases for years [10]. Many of the *Centaurea* species have been used in conventional medicine for the treatment of antirheumatic [11-12], antimalarial [13], gastric pain, indigestion [14], kidney disease, sedative [15], diuretic, [16] cholagogue, blood cleanser, antigout [17], internal cancers [18], headache, antipyretic [19]. *Centaurea saligna*, an endemic plant for Turkey, is used in the treatment of many diseases such as astringent [20], coagulation [21], antidiabetic [22], wound healing [23] among the people. In addition, there are many biological activity experiments such as antioxidant [24], antiproliferative [25], antimicrobial [26], antidiabetic [1], antialzheimer and anti-inflammatory [27] activity on *Centaurea* species.

Centaurea species has 192 species, 114 of which are endemic to Turkey [28] and has been the subject of many chemical studies that allow the obtaining of acetylenes, flavonoids, sesquiterpene lactones and lignans as the main characteristic secondary metabolites [29]. Also, there are many essential oil studies on *Centaurea* species [30].

In this study, the antiproliferative activity and anti-inflammatory activity of *Centaurea saligna* were reported against cancer cell lines (HeLa [cervical cancer cell line], A549 [lung cancer cell line], HT-29 [colon cancer cell line], PC-3 [prostate cancer cell line]) and 5-LOX (5-lipoxygenase) enzyme, respectively, for the first time.

2. RESULTS

Table 1 summarizes the cytotoxic activity of methanol extract and fractions of *Centaurea saligna*. According to the National Cancer Institute (NCI), it has been suggested that if the cytotoxic ED_{50} (Effective Dose 50) for plant extracts is ≤ 30 µg / ml, these extracts may contain a promising rate of anticancer compounds and it would be significant to isolate the compounds from this extract [31]. In A549 cell line, the methanol extract showed moderate cytotoxic activity with cell death rate of 50.57% while CSH, CSC, CSEA and CSAM exhibited weak cytotoxic activity. In HeLa cell line, CSM showed good an antiproliferative activity with cell death rate of 59.84 % at 50 µg/ml concentration. CSC exhibited the best activity (50.18%) following CSM. As seen in the Table 1, CSM showed the best antiproliferative activity in HT-29 cell line with 53.65 % cell death rate. The cytotoxic activity of these extracts against HT-29 was, in decreasing order, CSM > CSAM > CSC > CSEA > CSH exhibited moderate cytotoxic activity against HT-29. Methanol extract and its all fractions showed a poor antiproliferative activity against the PC-3 cell line. CSC, the most active fraction tested in the MCF-7 cell line, exhibited moderate cytotoxic activity with a cell death rate of 45.42 %, CSM showed the second best activity against MCF-7 while CSH, CSEA, CSAM exhibited weak activity.

Table 1. Antiproliferative activity of various extracts obtained from aerial parts of *C. saligna.*

Ekstract and fractions	A549**	HeLa**	HT-29**	PC-3**	MCF-7**
	% death rate at 50 µg/ml concentration				
CSM*	50.57	59.84	53.65	29.01	41.05
CSH*	28.17	41.15	44.24	23.68	23.97
CSC*	33.12	50.18	46.88	27.94	45.42
CSEA*	35.89	36.94	44.74	19.42	32.39
CSAM*	32.87	37.79	49.37	10.55	27.01

*Abbreviations: CSM, CSH, CSC, CSEA, CSAM show the methanol extracts and its n-hexane, chloroform, ethyl acetate, and aqueous methanol fractions of *Centaurea saligna* respectively. **A549: Lung cancer, HeLa: Cervical cancer, HT-29: Colon cancer, PC-3: Prostate cancer, MCF-7: Breast cancer

As shown in Table 2, methanol extract of *C. saligna* and its fractions were found to possess concentration-dependent inhibitory activity against DPPH radical. A low IC_{50} value indicates the high antioxidant activity. Therefore, CSEA with IC_{50} value of 108.4 µg/ml was superior than other fractions of *C. saligna* for DPPH assay. DPPH radical scavenging activity of *C. saligna* extract and fractions decreased in the following order: CSEA > CSM > CSAM > CSC > CSH (Table 2). In ABTS assay, CSEA with IC_{50} value of 82.05 µg/ml was observed more active than other fractions of *C. saligna*. ABTS radical scavenging activity of *C. saligna* extract and fractions decreased in the following order: CSEA > CSC > CSM > CSAM > CSH (Table 1). Total phenolic amount were determined according to the equation (y=0.109x+0.2464, r^2: 0.9888) obtained from calibration curve as gallic acid equivalent (mg/g extract), while total flavonoid amount were determined according to the equation (y=0.019x+0.0001, r^2: 0.9937) obtained from calibration curve as quercetin equivalent (mg/g extract). As shown in Table 3, the content of total phenolics in extract and fractions varied from 26.10 to 379.2 mg of gallic acid equivalent per gram dried extract and the total flavonoid contents varied from 8.51 to 170.3 mg quercetin equivalent per gram dried extract. The maximum total phenolic and flavonoid levels have been observed in CSEA which showed the highest activity in DPPH, ABTS and 5-lipoxygenase inhibitory acitivity tests. CSEA exhibited very strong anti-lipoxygenase activity with IC_{50} value of 0.10 µg/ml when compared to indomethacine as standard (indomethacine IC_{50}: 22.39 µg/ml) (Table 2).

Table 2. Antioxidant and anti-inflammatory activity of various extract obtained from aerial parts of *C. saligna.*

Extracts*/ Standards	Antioxidant activity		Anti-inflammatory activity
	DPPH radical scavenging activity	ABTS radical scavenging activity	Anti-lipoxygenase activity
	IC_{50} (µg/mL)		
CSM	125.4 ± 0.42^{d}	117.2 ± 0.14^{d,e}	7.09 ± 0.21^{b}
CSH	2039 ± 3,54^{f}	264.6 ± 0.28^{f}	24.99 ± 0.01^{c}
CSC	215.0 ± 0.14^{e}	110.0 ± 0.85^{d}	23.03 ± 0.66^{c}
CSEA	108.4 ± 0.07^{c}	82.05 ± 1.49^{c}	0.10 ± 0.02^{a}
CSAM	138.7 ± 0.50^{d}	124.6 ± 0.56^{e}	19.10 ± 1.49^{c} 1
AA**	17.6 ± 0.37^{a}	14.5 ± 0.32^{a}	
Trolox**	14.54 ± 0.18^{a}	13.00 ± 0.21^{a}	
BHA**	57.15 ± 0.09^{b}	17.06 ± 0.58^{a}	
BHT**	213.6 ± 15.20^{e}	26.82 ± 1.12^{b}	
INDO**			22.39 ± 0.26^{c}

*Abbreviations: CSM, CSH, CSC, CSEA, CSAM show the methanol extracts and its n-hexane, chloroform, ethyl acetate, and aqueous methanol fractions of *Centaurea saligna*, respectively. **AA: Ascorbic acid; BHA: Butylhydroxyanisole; BHT: Butylhydroxytoluene; INDO: Indomethacine *** Each value in the table is represented as mean ± SD (n=3). The values with different letter superscripts in the same column indicate significant differences ($p<0.05$).

Table 3. Total flavonoid and phenol contents of various extracts obtained from aerial parts of *C. saligna.*

Extracts*/ Standards	TPC (mg GAE/g extract)**	TFC (mg QE/g extract)**
CSM	133.3 ± 3.77^{c}	80.04 ± 0.60^{c}
CSH	26.10 ± 0.89^{a}	8.51 ± 0.12^{a}
CSC	210.5 ± 0.69^{d}	98.38 ± 5.14^{d}
CSEA	379.2 ± 1.69^{e}	170.3 ± 2.31^{e}
CSAM	50.22 ± 3.35^{b}	48.14 ± 1,49^{b}

*Abbreviations: CSM, CSH, CSC, CSEA, CSAM show the methanol extracts and its n-hexane, chloroform, ethyl acetate, and aqueous methanol fractions of *Centaurea saligna* respectively. **Total phenolic and total flavonoid contents were expressed as gallic acid equivalent (GAE) and quercetin equivalent (QE), respectively. *** Each value in the table is represented as mean ± SD (n=3). The values with different letter superscripts in the same column indicate significant differences ($p<0.05$).

In this study, chemical compounds ethyl acetate fraction of *C. saligna* due to its more prominent antioxidant and antiinflammatory activities compared to those of the other fractions were investigated by using LC-MS/MS. Nine phenolic compounds including quinic acid, 5-caffeoylquinic acid, apigenin C-hexoside-C-pentoside, p-coumaroylquinic acid, quercetin glucoside, di-caffeoylquinic acid, isorhamnetine glucoside, isorhamnetin glucuronide and isorhamnetin derivative were detected by LC/MS-MS (Figure 1) (Table 4).

Table 4. Phenolic contents of ethyl acetate fractions of *C. saligna.*

Rt	[M-H] *m/z*	Fragments	Identification	Ref
3.8	191	173, 127	Quinic acid	[65]
6.8	353	191, 173, 127	5-caffeoylquinic acid	[65]
7.3	563	343, 387, 353	Apigenin C-hexoside-C-pentoside	[66]
8.5	337	191, 163, 145	p-coumaroylquinic acid	[66]
9.7	463	301	Quercetin glucoside	[66]
11.5	515	353, 191, 173	Di-caffeoylquinic acid	[66]
11.9	477	315, 299	Similar to Isorhamnetin glucoside	[66]
13.3	491	329, 313	Unknown similar to Isorhamnetin glucuronide (EA)	[66]
17.6	435	315, 301, 297, 151	Similar to İsorhamnetin derivative (EA)	[66]

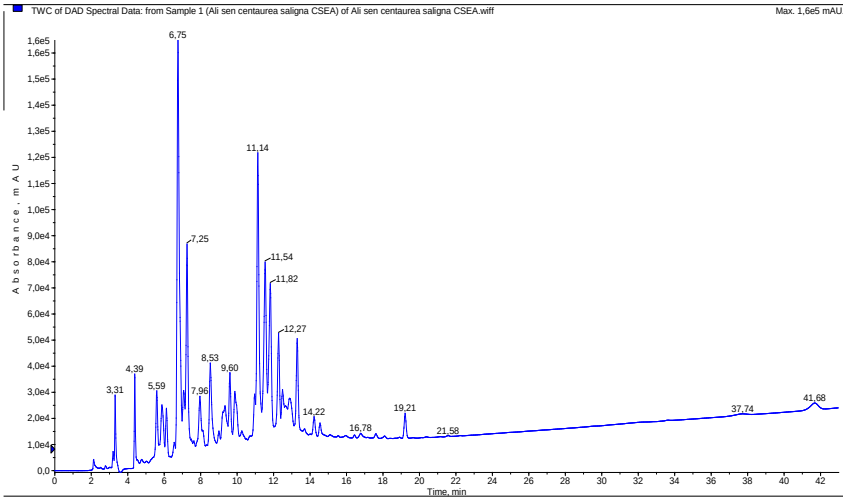

Figure 1. LC-MS/MS chromatogram of ethyl acetate fraction of *C. saligna.*

3. DISCUSSION

The study was aimed to research the phenolic compounds of active CSEA as well as the antioxidant, anti-inflammatory and anticancer potential of *Centaurea saligna* extracts. There are many articles in the literature on the antioxidant effects of the different extracts and compounds from many *Centaurea* species. The DPPH test is often used to understand the antioxidant capability of plant extracts containing polar components. DPPH, is a purple radical, when it reacts with an antioxidant, it turns into yellow (diphenyl picrylhydrazine). DPPH radical scavenging efficiency of the extracts was evaluated by using IC_{50} values. A low IC_{50} value proves that the sample has strong antioxidant activity.

Some researchers stated that DPPH assay was associated with high phenolic content [32]. It is generally approved that the ABTS test gives more significant results than the DPPH test when samples including lipophilic, hydrophilic and highly pigmented antioxidant compounds are analyzed [33].

Zengin *et al*. (2018) determined that *C. saligna* water, methanol and ethyl acetate extracts were highly possesed DPPH and ABTS radicals scavenging activity. They proved that Trolox equivalent of water, ethyl acetate, methanol and extracts of this plant 68.43, 22.74, 67.39 mg TE/g extract, respectively [34]. Keser et al observed that water, ethanol, methanol and acetone extracts of *C. saligna* scavenged 56.48%, 47.58%, 71.96% and 21.09% of DPPH radical at 500 µg/ml concentration, respectively. Our activity results were higher than this study results [35]. Several studies have been conducted on the DPPH scavenging activity of different species of the *Centaurea* genus, such as methanol extract of *C. kurdica* (IC_{50}: 367.71 µg/ml), *C. cheirolopha* (IC_{50}:

227.45 µg/ml), *C. amanicola* (IC_{50}: 581.36 µg/ml), *C. rigida* (IC_{50}: 475.06 µg/ml), *C. ptosimopappoides* (IC_{50}: 745.13 µg/ml) [36]. *C. saligna* was found to be more active than these plants. In a study in which all extracts of *C. cyanus* showed significant activity, Anwari *et al.* found that ethanol extract of *C. cyanus* leaves and flowers had 40 and 60 µg/ml of IC_{50} value against the DPPH radical, respectively [37]. According to this study, it is seen that *C. cyanus* is more active than *C. saligna*.

Keser *et al*. found that water, ethanol, methanol and acetone extracts of *C. saligna* scavenge 94.81 %, 50.43 %, 99.94 % and 60.04 % of ABTS radical at 500 µg/mL concentration, respectively [35]. In a study of ABTS radical scavenging activity conducted by Zengin *et al*., water, ethyl acetate, methanol extracts of *C. saligna* included 157.8, 48.87, 112.98 mg TE/g extract, respectively [34] . Petroleum ether, acetone, methanol, and water extracts of the three *Centaurea* species (*C. balsamita, C. depressa* and *C. lycopifolia*) were evaluated for ABTS cation radical scavenging activity. IC_{50} value of the methanol extract of *C. balsamita, C. depressa* and *C. lycopifolia* were found 24.21, 70.08, and 89.05 µg/ml respectively. *Centaurea* extracts showed very poor activity that confirm the ABTS assay results of *Centaurea* species reported by Boğa et al [38].

Zengin *et al*. (2018) reported that total phenol contents of *C. saligna* water, ethyl acetate and methanol extracts were 30.18, 26.21 and 23.03 mg GAE/g, respectively. In the same study, it was reported that total flavonoid contents of these extracts were 25.81, 43.16 and 6.33 mg RE/g, respectively [34]. According to work of Keser *et al*., total phenolic contents of *C. saligna* water, ethanol, methanol and acetone extracts were found ranged between 35.02 and 106.27 µg GAE/g while total flavonoid contents of *C. saligna* water, ethanol, methanol and acetone extracts ranged between 395.87 and 1561.25 µg CE/g [35]. Total phenolic amount of *Centaurea calcitrapa* subsp. *calcitrapa, C. spicata* and *C. ptosimopappa* were found ranged between 17.25 and 120.50 mg GAE/g [39]. The phenol and flavonoid content of *C. depressa* were determined as 44.5 mg GAE/g, 42.25 mg QE/g, respectively [40]. Ozsoy *et al.* found that the total phenol content of *Centaurea antiochia* var. *praealta* was 3.68 mg GAE/g [27]. Total phenolic amount of methanol extract of *C. aksoyi* was revealed as 3.28 mg GAE/g extract while C. *amaena* was found as 3.62 mg GAE/g extract. Total flavovoid content of methanol extract of *C. aksoyi* and *C. amaena* were found as 6.96 and 5.83 mg QE/g extract by Albayrak *et al* [41]. Our results were higher when compared with these literature data.

Chloroform extract of *Centaurea stenolepis* Kerner, *C. kilaea* Boiss., *C. salicifolia, C. cuneifolia* were tested against 5-lipoxygenase enzyme by Şekerler *et al*. *C. kilaea* and *Centaurea salicifolia* showed strong anti-lipoxygenase activity with IC_{50} values of 110.0 and 97.04 µg/ml, respectively [42]. In our current study, CSEA exhibited much stronger anti-inflammatory activity than those species.

In general, the most common study methods in the search for new anticancer agents are the screening tests against a panel of cancer cell lines. In current study, MTT assay which is based on the capability of metabolically active cells to convert the pale yellow MTT dye into a spectrophotometrically measurable blue formazan product was used.

Our findings are in line with the literature on importance of *Centaurea* species as cytotoxic agents against cancer cell lines. Keser *et al*. showed that *C. saligna* methanol extract had strong antiproliferative activity. *C. saligna* water, ethanol, methanol, acetone extracts possesed 26.13, 4.90, 28.13, 8.91 µg/ml values of IC_{50} gainst MCF-7 cell line, respectively [34]. Antiproliferative activities of *C. baseri* methanol extract were found as IC_{50} values of 73.3 and 45.3 µg/ml against A549 and MCF7 cell lines, respectively [43]. Kayacan *et al*., reported that IC_{50} values of *C. nerimaniae* methanol extract was 1.42 mg/ml at 48 h for HeLa cells [44]. In a study of Ostad et al., according to the IC_{50} values obtained by *in vitro* MTT cytotoxicity assay, among the all cell line, *C. bruguierana* ssp. *belangeran* total methanol extract and fractions exhibited the lowest cytotoxic activity on the HT-29 cell line. IC_{50} value of total methanol extract and its ethyl acetate and n-butanol fractions were found > 1000 µg/ml. It was found that chlorofom fraction had value of 327 µg/ml IC_{50} on HT-29 cell line [45]. The anti-tumor effects of seven different extracts of *C. babylonica* on A549, PC-3, MCF-7 and HeLa cells were investigated by MTT assay. Except for the water extract, all six *C. babylonica* extracts showed cytotoxic activity between 72% and 90% against all tested cancer cell lines. It was found that acetone extract with IC_{50} values in the range of 3-6 µg/ml against MCF-7, HeLa and PC-3 cell lines showed good antiproliferative effects. [46].

In the current study, the phytochemical content of CSEA, which had strong anti-inflammatory activity and good antioxidant activity, was investigated by LC-MS/MS and it was observed that it contained phenolic compounds. These phenolic compounds have been reported to have significant anti-inflammatory activities in literature.

Many authors reported the quinic acid, 5-caffeoylquinic acid, apigenin, quercetin, isorhamnetin to be associated with many activities such as antioxidant, antiinflammatory and antiproliferative activities. In one study, it was observed that quinic acid esters contained in hot water extracts of *Uncaria tomentosa* (a.k.a. cat's

claw) showed anti-inflammatory activity through mechanisms that include inhibition of the pro-inflammatory transcription factor kappa B (nuclear factor) (NF-κB) [47]. Janga et al. proved that quinic acid (QA) has been shown to have anti-neuroinflammatory, radioprotection and antioxidant activities. Pre-incubation of MOVAS (the mouse vascular smooth muscle cell line) cells with Quinic acid (0.1, 1 and 10 µg/ml) for 2 hours dose-dependently inhibited protein expression of VCAM-1, TNF-α-induced mRNA and monocyte adhesion. Quinic acid inhibited TNF-α-induced phosphorylation and NK-κB activation of the MAP kinase [48]. In another experiment conducted by Lee et al., quinic acid and derivatives were tested for their inhibitory activity on nitric oxide (NO) production in an activated murine microglial cell line. The compounds significantly inhibited NO production in lipopolysaccharide (LPS) activated BV-2 cells without high cell toxicity in a microglia cell line [49].

In a study conducted, *Artemisia scoparia* and its new active compound 3,5-dicaffeoyl-epi-quinic acid were reported to significantly lower TSLP, TNF-α, IL-1β and IL-6 production levels by reducing caspase-1 activity [50]. Montaal et al. isolated 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, 4,5-*O*-dicaffeoylquinic acid and 3,4,5-*O*-tricaffeoylquinic acid from *Solidago virgaurea*. One of these compounds, 3,4,5-*O*-tricaffeoylquinic acid (50 mg / kg) showed significant activity in inhibiting edema volume after 3 hours (88% of the activity of indomethacine at 10 mg / kg). Also, *Solidago virgaurea* extract and isolated compounds greatly inhibited the overproduction of the inflammatory mediators TNF-α and IL-1p [51].

In a study tested for 5-caffeoylquinic acid, overexpression of IL-8 and mRNA induced by H_2O_2 was significantly inhibited in a dose-dependent manner in the range of 0.25-2.00 mmol/l, after cells were pretreated and simultaneously treated with 5-caffeoylquinic acid. Both 5-caffeoylquinic acid (5-CQA) and caffeic acid (CA) also suppressed TNF-α-induced IL-8 secretion. At 2.00 mmol/l, caffeic acid absolutely blocked the H_2O_2- or TNF-α-induced overexpression of IL-8 in Caco-2 cells [52]. In an another experiment for the anti-inflammatory activity of 5-caffeoylquinic acid, *Coffea arabica* methanol extract and 5-caffeoylquinic acid (0.1, 0.5 and 1.0 mg/ear) were found to reduce the volume and weight of edema caused by croton oil and phenol. Treatment with *Coffea arabica* methanol extract or 5-caffeoylquinic acid reduced the activity of myeloperoxidase and N-acetyl-β-d-glucosaminides and inflammatory parameters [53].

Current epidemiological and preclinical studies provide substantial evidence in favor of the development of apigenin as a natural alternative therapy against chronic inflammatory conditions [54]. Ali *et al*. demonstrated that apigenin had multiple inflammatory targets. Based on *in vivo, in vitro*, and clinical trial studies, it has been suggested that apigenin is an effective therapeutic agent for dealing with diseases such as autoimmune disorders, rheumatoid arthritis, Alzheimer's disease, Parkinson's disease and various types of cancer [55]. A study conducted by Chiang showed that the anti-hepatoma activity of apigenin is as potent as 5-FU and its apoptotic mechanism can be mediated through the p53-dependent pathway and induction of p21 expression [56].

In one study, it was observed that quercetin exerts cytoprotective effect in normal cell cultures while inducing growth inhibition and cell death in the U138MG human glioma cell line [57]. In an experiment conducted to demonstrate the anti-inflammatory activity of quercetin, it was observed that quercetin reduced the expression of cardiovascular risk factors (SAA, fibrinogen) and human CRP in mice *in vivo*. It is thought that local anti-inflammatory and anti-proliferative effects in the aorta may contribute to the reduction of atherosclerosis [58]. Thus, these phenolic compounds (phenolic acids and flavonoids), along with other compounds, may be responsible for the activity of CSEA, which showed a particularly potent anti-inflammatory activity.

4. CONCLUSION

CSC can be an important source for obtaining compounds with antiproliferative effect, while CSEA can be a source of new bioactive compounds with antioxidant and anti-inflammatory properties. However, first of all, bioactivity-guided isolation studies are needed for the isolation of active compounds from these extracts.

5. MATERIALS AND METHODS

5.1. Plant material

Aerial parts of plant were collected at their flowering period from the Patnos district of Ağrı, Turkey in August 2018 and kept in a dark and cool place until extraction. Plant was identified by Dr. Ahmet Doğan, a

botanist of the Faculty of Pharmacy, University of Marmara. Voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, Marmara University (MARE No: 21857).

5.2. Extraction

About 15 g of dried aerial parts of *Centaurea saligna* were extracted with 8×200 ml MeOH, using an ultrasonic bath. After filtration and evaporation, the methanol extract (CSM) was dissolved in 30 ml 60% aqueous methanol, and subjected to solvent-solvent partition between n-hexane (5×50 ml), chloroform (3×50 ml), and ethyl acetate fractions (2x50 ml). The n-hexane, chloroform, ethyl acetate and aqueous methanol fractions of *Centaurea saligna* acquired by this method were coded as CSH, CSC, CSEA and CSAM, respectively. All extracts were stored at 4 °C for further analysis.

5.3. DPPH radical scavenging activity

DPPH radical scavenging capacity of each extract was applied by the method of Zou et al [59].

5.4. ABTS radical-scavenging activity

ABTS radical cation scavenging activity assay was determined according to the method described by Zou et al. [59].

5.5. *In vitro* anti-lipoxygenase activity

The anti-lipoxygenase activity was evaluated with minor modifications according to the method described by Phosrithong et al. and Yıldırım et al. [60, 61].

5.6. Determination of total phenolic contents (TPC)

Total phenolic amount of *Centaurea saligna* extracts were measured using Folin–Ciocalteau reagent and determined according to method of Gao et al. and Yıldırım et al. [61, 62].

5.7. Determination of total flavonoid contents (TFC)

Total flavonoid content was applied following a method by Zhang *at al*. and Yıldırım et al. [61,63].

5.8. Cell growth inhibition analysis

5.8.1 MTT Experiment

Antiproliferative effects of the extracts were tested against *in vitro* five different human cancer cell lines (Hela, MCF-7, PC-3, A549 and HT-29) by MTT ([3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide]) assay. Briefly, a limited number of human cancer cells (5000/well) were seeded on a 96-well microplate. The cells were kept overnight to adhere to the base. On the second day, the medium was removed and 200 µl of new medium containing test substances (50 µg/mL) was added. After a 72 hour incubation period, viable cells were tested by adding 200 µl of 5 mg/ml MTT solution. During the 4 hour period, MTT transforms into purple colored formazan crystals in the presence of mitochondrial reductase enzyme.

The medium was removed and precipitated crystals were dissolved by shaking with 100 µl of DMSO for one hour. Finally, the absorbance of the reduced MTT solution was read at 545 nm in a microplate reader along with control cells not applied to extract [64]. The percentage growth inhibition was calculated using following equation 1;

$$\%\text{cell inhibition} = 100-\{(At-Ab)/(Ac-Ab)\} \times 100 \quad \text{Eq. 1}$$

Where,
At= Absorbance value of extract
Ab= Absorbance value of blank
Ac=Absorbance value of control

5.9. Profile of bioactive compounds

LC-MS/MS analysis was performed using an Absciex 3200 Q trap MS/MS dedector. Experiments were conducted with a Shimadzu 20A HPLC system connected to an Applied Biosystems 3200 Q-Trap LC-

MS/MS instrument equipped with an ESI source operating in negative ion mode. A GL Science Inertsil ODS - 3 250 × 4.6 mm, 5 µm particle size, analytical column performing at 40° C has been used for chromatographic isolation. The solvent flow rate was kept constant at 0.5 ml/min. PDA detector was used for detection. The mobile phase for the elution gradient was prepared as (A) acetonitrile: water: formic acid (10:89:1, v/v/v) and (B) acetonitrile: water: formic acid (89:10:1, v/v/v). The composition of B was increased from 10% to 100% in 40 min. Analyst 1.6 software collected and processed the LC-ESI-MS/MS data.

5.10. Statistical analysis

Data are presented by calculating mean±standard deviations and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests in GraphPad Prism 5. The differences between the means were considered significant at the $p<0.05$ level.

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