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In vitro antihepatocellular carcinoma activity of secondary metabolites of *Centaurea kilaea* Boiss.

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ABSTRACT: The aim of this study was to investigate *in vitro* cytotoxic effects of previously isolated compounds (taraxasterol, salvigenin, 3'-*O*-methyleupatorin, oleanolic acid, jaceosidin and pectolinarigenin) from *Centaurea kilaea* chloroform extract on hepatocellular carcinoma cell lines, HepG2 and Hep3B, as well as to evaluate the effect on the normal cell line, NIH3T3. *In vitro* antihepatocellular carcinoma activity of compounds was assessed by MTT method. All compounds except pectolinarigenin caused more inhibition on HepG2 rather than Hep3B. Among these compounds, it was found that Jaseosidin had the highest anticancer activity with IC_{50} values of 137.66 µg/mL and 147.66 µg/mL on the HepG2 and Hep3B cell lines, respectively. 3'-*O*- methyleupatorin showed the second highest cytotoxicity with IC_{50} values of 151.98 µg/mL and 159.24 µg/mL against the HepG2 and Hep3B cell lines, respectively. The results indicated that Jaseosidin and 3'-*O*- methyleupatorin, had the best antiproliferative activity against hepatocellular carcinoma cell lines. Also, according to our best knowledge, this study is first report on antihepatocellular carcinoma activity of Jaseosidin and 3'-*O*- methyleupatorin.

KEYWORDS: *Centaurea kilaea*; anticancer activity; hepatocellular carcinoma; jaceosidin; 3'-*O*-methyleupatorin

1. INTRODUCTION

Liver cancer is one of the major diseases that cause mortality and morbidity worldwide. It affects people's quality of life and causes society to suffer material and moral damage [1].

There are many risk factors under hepatocellular carcinoma. Viral factors such as hepatitis B and hepatitis C, excessive alcohol consumption and fatty liver are among these factors. Aflatoxins, the product of *Aspergillus* strains, are found in some dietary foods such as corn, peanuts, soybeans, are also another risk factor. Less determining factors include hereditary hemochromotosis, α-1 antitrypsin deficiency, autoimmune hepatitis and some psoriasis [2].

Hepatocellular carcinoma developing from liver cells constitutes 85-90% of primary liver cancers. Surgical treatment, regional cancer treatment, radiotherapy and chemotherapy are used in the treatment of hepatocellular carcinoma, but there is still no definitive treatment [3]. The toxic effects of chemotherapy and the resistance of cancer cells to the chemical agents used have induced the investigation of alternative treatment methods. Natural plant products may offer a solution [4]. Studies are ongoing on a number of methods such as isolation of active molecules from plants/other natural sources, synthetic chemistry, molecular modeling for drug discovery [5]. The number of scientific studies on the anticancer properties of *Centaurea* species has been increasing recently. Orabi *et al.* (2013) suggested that *C. aegyptiaca* ethanol extract had anticancer effect on the hepatocellular carcinoma cell line [6]. Sekerler *et al.* (2018) reported that chloroform extracts prepared from the aerial parts of some *Centeura* species significantly inhibited the growth of HepG2 cells [7]. Ahmed *et al.* (2014) found that the flavonoids isolated from the *C. scoparia* species had an important anticancer activity on HepG2 [8]. There are also numerous studies showing that different *Centaurea* species and their secondary metabolites have significant anticancer effects on different cancer lines [6, 9-14]. In one of these studies, it was reported that 8α-hydroxy-11α,13-dihydrozaluzanin C isolated from *Centaurea aegyptiaca* ethanol extract showed a potential cytotoxic activity against larynx carcinoma cell line [6]. Chicca *et al.* (2011) found that aguerin B from *C. deflexa* had an anti-proliferative activity against human pancreatic and colonic cancer cells [9]. In another study, Csapi *et al.* (2010) revealed that *C. arenaria*

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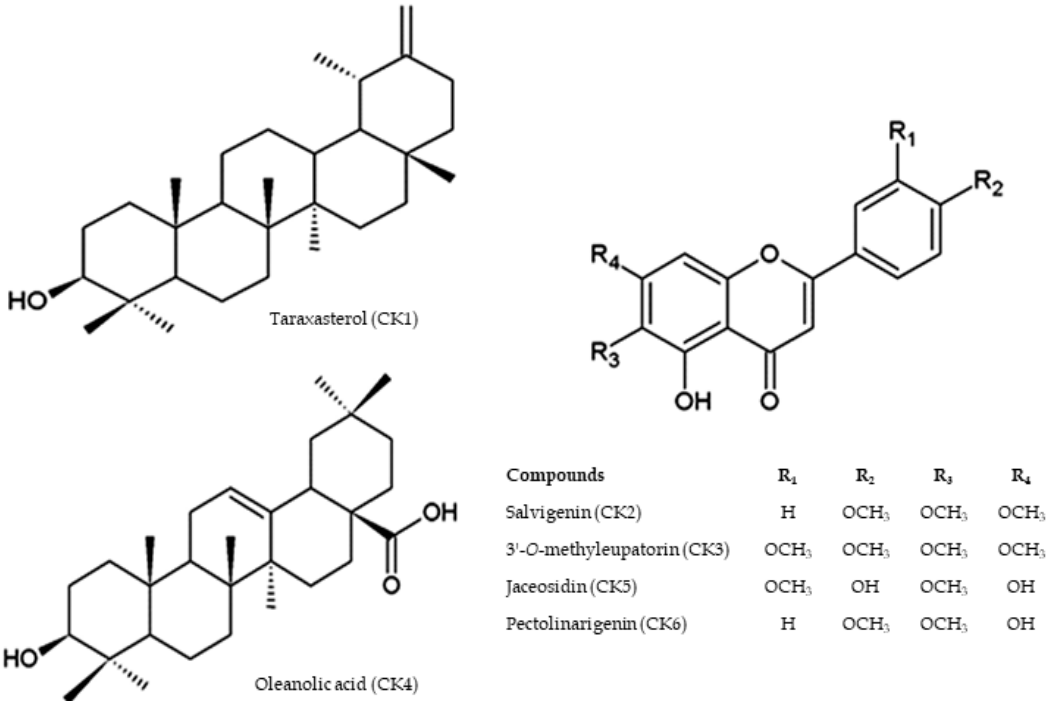
chloroform extract and its secondary metabolites (apigenin, eupatorin, arctigenin, arctiin, matairesinol and cnicin) had potent growth inhibitory activities on cervix adenocarcinoma (HeLa), breast adenocarcinoma (MCF7) and skin epidermoid carcinoma (A431) cells [10].

Based on these findings, in this study, we aimed to investigate the anticancer effect of four flavonoids, salvigenin, 3'-*O*- methyleupatorin, jaceosidin and pectolinarigenin along with two triterpenes, taraxasterol and oleanolic acid previously obtained from *Centeura kilaea* Boiss. chloroform extract by our team [Figure 1, 12] on HepG2 and Hep3B hepatocellular carcinoma cell lines. Anticancer activities of taraxasterol on HepG2 cell line, oleanolic acid on Hep3B and HepG2 cell lines and pectolinarigenin on Huh-7D12 cell line were previously reported [15-17]. However, this study is the first report on anticancer activity of taraxasterol, salvigenin, 3'-*O*-methyleupatorin, jaceosidin and pectolinarigenin against HepG2 and/or Hep3B cancer cell lines. In order to evaluate selectivity, the cytotoxic activities of these compounds on NIH3T3 fibroblast cell line, normal cancer cells, were studied.



Compounds	R_1	R_2	R_3	R_4
Salvigenin (CK2)	H	OCH_3	OCH_3	OCH_3
3'-*O*-methyleupatorin (CK3)	OCH_3	OCH_3	OCH_3	OCH_3
Jaceosidin (CK5)	OCH_3	OH	OCH_3	OH
Pectolinarigenin (CK6)	H	OCH_3	OCH_3	OH

Figure 1. Chemical structures of CK1–CK6.

2. RESULTS

The IC_{50} values of the compounds isolated from *Centeura kilaea* were given in Table 1. Jaceosidin had the highest toxicity on hepatocellular carcinoma cells when compared to other compounds. IC_{50} values of the Jaceosidin were found as 147.67 µg/mL and 137.67 µg/mL against Hep3B and HepG2 cell lines, respectively.

Table 1. IC_{50} values of compounds isolated from *Centeura kilaea.*

Compounds[a]	HepG2 cell line	Hep3B cell line
	µg/mL	
CK1	178.57	>500
CK2	165.56	>500
CK3	151.98	159.24
CK4	229.36	>500
CK5	137.67	147.67
CK6	427.35	188.47

[a] CK1, CK2, CK3, CK4, CK5 and CK6 show taraxasterol, salvigenin, 3'-*O*-methyleupatorin, oleanolic acid, jaceosidin and pectolinarigenin isolated from *Centeura kilaea.*

3'-*O*-methyleupatorin showed the second highest cytotoxic activity with IC_{50} values of 151.98 and 159.24 µg/mL against Hep3B and HepG2 cell lines, respectively. The compounds were found to be particularly effective against the HepG2 cell line. It was also found that pectolinarigenin with an IC_{50} value of 427.35 µg/mL against HepG2 cell line, and taraxasterol, salvigenin and oleanolic acid with IC_{50} values of >500 µg/mL (for each compounds) against Hep3B cell line had the lowest anticancer effects (Table 1).

Also, the percentage proliferation values of the compounds isolated from *Centeura kilaea* at 100 µg/mL concentration were shown as the bar graphs in the Figure 2-4. Compared to the control group (without drug exposure), all compounds showed a statistically significant inhibition against HepG2 cell line ($p<0.001$ for each compounds). Out of these compounds, jaceosidin exhibited the highest activity with an inhibition rate of 36.3% (Figure 2). Compared to the control group, three compounds, 3'-*O*-methyleupatorin, jaceosidin and pectolinarigenin displayed a statistically significant inhibition against Hep3B cell line ($p<0.01$ for each of the first two compounds, $p<0.05$ for the last compound). Among these compounds, jaceosidin demonstrated the highest activity with an inhibition rate of 33.9% (Figure 3). Finally, taraxasterol, salvigenin, 3'-*O*-methyleupatorin, jaceosidin showed a statistically significant inhibition against NIH3T3 mouse fibroblast cell line compared to the control group ($p<0.001$ for each compounds). Jaceosidin represented highest cytotoxic activity with an inhibition rate of 49.2% among these compounds (Figure 4).

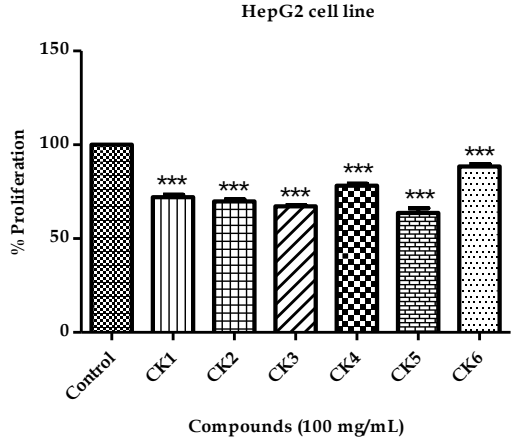


Figure 2. Effects of 100 µg/mL concentration of compounds isolated from *C. kilaea* against HepG2 cell line. CK1, CK2, CK3, CK4, CK5 and CK6 show taraxasterol, salvigenin, 3'-*O*-methyleupatorin, oleanolic acid, jaceosidin and pectolinarigenin isolated from *Centeura kilaea*, respectively. Data were presented as mean ± standard deviation (SD). ***p<0.001 versus control group.

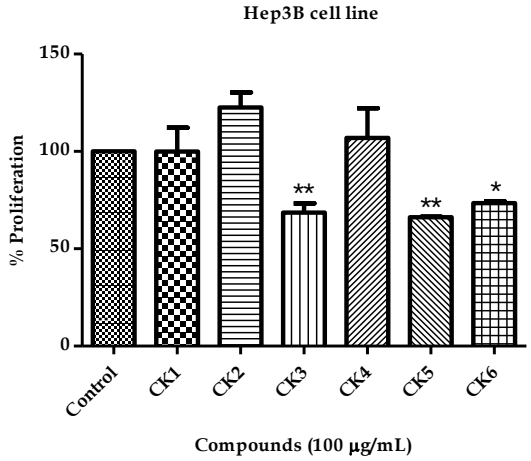


Figure 3. Effects of 100 µg/mL concentration of compounds isolated from *C. kilaea* against Hep3B cell line. CK1, CK2, CK3, CK4, CK5 and CK6 show taraxasterol, salvigenin, 3'-*O*-methyleupatorin, oleanolic acid, jaceosidin and pectolinarigenin isolated from *Centeura kilaea*, respectively. Data were presented as mean ± standard deviation (SD). *p<0.05, **p<0.01 versus control group.

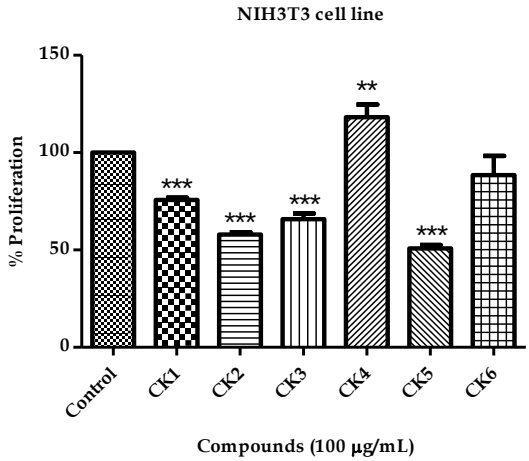


Figure 4. Effects of 100 µg/mL concentration of compounds isolated from *C. kilaea* against NIH3T3 cell line. CK1, CK2, CK3, CK4, CK5 and CK6 show taraxasterol, salvigenin, 3'-*O*-methyleupatorin, oleanolic acid, jaceosidin and pectolinarigenin isolated from *Centeura kilaea*, respectively. Data were presented as mean ± standard deviation (SD). **p<0.01, ***p<0.001 versus control group.

3. DISCUSSION

Hepatocellular carcinoma ranks fifth in cancer types in terms of morbidity and mortality, affecting about five million people worldwide each year. Systemic chemotherapy is not very effective in terms of survival, and besides, chemical resistance against drugs is one of the important difficulties in this disease. Agents such as paclitaxel, tamoxifen, octreotide or antiandrogens are completely ineffective. From this point of view, it is very important to develop new drug candidate molecules for hepatocellular carcinoma [18].

Centaurea is a genus belonging to the Asteraceae family and represented by about 205 taxa in Turkey [19-21]. *Centaurea* species contain abundant sesquiterpene lactone and flavonoids [22]. In traditional medicine, *Centaurea* species are used in the treatment of abscess, skin diseases, fever, menstrual disorders, vaginal candidiasis and also diseases of the liver, kidneys and ulcers [23, 24].

In recent years, some *Centaurea* species and their secondary metabolites have been reported to have an anticancer effect on various cell lines. Bahmani *et al.* demonstrated that *C. albonitens* extract enhanced the therapeutic effects of vincristine in leukemic cells [25]. In an another study, the anticancer effect of salograviolide A isolated from *C. ainetensis* extract was demonstrated on p-53 positive HCT-116 cell lines [26]. In a study by Alper *et al.*, effect on various cancer cell lines of *C. solstitialis* extract was investigated. According to this study, the highest cytotoxic activity was observed in the Hela cell line with an IC_{50} value of 63.18 µg/mL while the lowest cytotoxic activity was observed in the A549 cell line with an IC_{50} value of 252.5 µg/mL. However, it was reported that the IC_{50} value of 75.25 µg/mL was detected in the BEAS2b normal cell line [27]. In a study by Yirtici *et al.*, it was stated that *C. fenzlii* Reichardt extract had an inhibitory effect on the MCF-7 cell line. According to this study, *C. fenzlii* Reichardt extract on MCF-7 cell line was reported to have an IC_{50} value of 45.77 µg/mL [28].

In a study previously conducted by our team, anticancer activities of *Centaurea* species including *C. kilaea* on HepG2 cancer cell lines were investigated and it was found out that these species had an important anti-cancer activity [7]. Based on these results, in the present work, it was aimed to determine the effects of previously isolated molecules from *C.kilaea* on hepatocellular cancer cell lines such as HepG2, Hep3B, and also on NIH3T3 cell line obtained from mouse fibroblasts to evaluate the toxicity of compounds in healthy cells. According to the results, it was found that all of the isolated compounds had an inhibitory effect on the HepG2 cell line compared with the viability rates of the control group. Three out of 6 compounds, 3'-*O*-methyleupatorin, jaceosidin and pectolinarigenin showed an inhibitory effect on the growth of the Hep3B cell line. The jaceosidin, the most toxic of the isolated molecules, caused an inhibition of 36.32% in the HepG2 cell line, while leading to an inhibition of 33.86% in the Hep3B cell line. However, this compound showed toxic effect on healthy cells and caused 49% inhibition in the NIH3T3 cell line. No studies on the anticancer effects of jaceosidin against HepG2 and Hep3B cancer cell lines have been found in the literature. Although the current study is the first, there are reports that jaceosidin has significant anticancer effects

against different types of cancer, such as CAOV-3 (human ovarian cancer cell line), SKOV3 (human ovary cancer cell line), HeLa (human cervical cancer cell line), PC3 (human prostate cancer cell), U87 (human glioblastoma cell line), human bladder cancer T24 (human bladder carcinoma cell line) cell lines [29-31]. These results overlap with our current study, confirming the anticancer effect of jaceosidin.

3'-*O*- methyleupatorin was the second most effective cytotoxic constituent with IC_{50} values of 151.98 and 159.24 µg/mL against HepG2 and Hep3B cell lines, respectively. Although a study on the anticancer effects on Hela and MCF-7 cell lines of 3'-*O*- methyleupatorin has been reported in the literature [32], the anticancer activity of this compound against hepatocellular carcinoma cell lines has not been reported to date.

Also, it was observed that most of the active compounds in this study were compounds with flavonoids structure. Kanadaswami *et al.* (2005) reported that the hydroxylation pattern of the B ring of flavones and flavonols such as luteolin and quercetin played an important role in anticancer activity, especially by inhibiting protein kinase activity and proliferation of cancerous cells [33]. Similarly, when the three compounds with the best activity, 3'-*O*-methyleupatorin, jaceosidin and pectolinarigenin, were compared with each other in terms of structure-activity relationship, it was observed that the presence of a 4'-OH group in the B ring of the molecule such as jaceosidin increased the anticancer activity.

In addition, some of the compounds whose activities have been investigated in the current study have been reported to have antihepatocellular carcinoma activity in previous studies. In one of these studies, Yan *et al.* (2010) found that oleanolic acid induced apoptosis in four human liver cancer cell lines (HepG2, Hep3B, Huh7 and HA22T) [15]. In another study, it was reported that pectolinarigenin exhibited cytotoxic activity with IC_{50} value of >100 µg/mL against hepatocellular carcinoma Huh-7D12 cell line [16]. Also, Bao *et al.* (2018) demonstrated that taraxasterol inhibited the growth of human liver cancer line (HepG2) [17]. In our current study, taraxasterol with IC_{50} value of 178.57 µg/mL against HepG2 cell line, oleanolic acid with IC_{50} value of 229.36 µg/mL against HepG2 cell line and pectolinarigenin with IC_{50} values of 427.35 and 188.47 µg/mL against HepG2 and Hep3B cell lines showed an anticancer activity. These results were found to be compatible with previous studies and confirm the anticancer activities of these compounds.

4. CONCLUSION

As a result, it can be said that among the all tested compounds, jaceosidin and 3'-*O*- methyleupatorin have the best inhibitory activity against hepatocellular carcinoma cells. However, further investigation, different anticancer activity methods such as XTT, WST-1 and Real Time Cell Analyzer System, is required to fully reveal antihepatocellular carcinoma effects of the compounds. It may also be useful to investigate the anticancer activity of these compounds on different cancer lines.

5. MATERIALS AND METHODS

5.1. Plant material, extraction and isolation

Anticancer activities of the compounds previously isolated by Sen *et al.* (2017) [12] were investigated in this study. Detailed information about where the aerial parts of *C. kilaea* were collected [13], obtaining chloroform extract [13], isolation of compounds from this extract [12] and determination of their structures of taraxasterol [12, 13, 34-37], salvigenin [12, 38, 39], 3'-*O*-methyleupatorin [12, 38, 39], oleanolic acid [12, 40, 41], jaceosidin [12, 38, 39], and pectolinarigenin [12, 38, 39, 42] was explained in the previous studies. In this aforementioned study, taraxasterol, salvigenin, 3'-*O*-methyleupatorin, oleanolic acid, jaceosidin and pectolinarigenin were isolated as secondary metabolites from the chloroform extract of *C. kilaea* and coded as CK1, CK2, CK3, CK4, CK5, CK6 respectively, in this study (Figure 1).

5.2. Cell culture

The human liver cancer cell lines HepG2 and Hep3B were obtained from the ATCC and maintained according to the recommendations of the ATCC at 37 °C and 5% CO2 in complete DMEM, supplemented with 100 U/mL penicillin G, 100 µg/mL streptomycin and 10% FBS.

NIH3T3 fibroblasts were cultured using DMEM (low glucose) supplemented with 100 U/mL penicillin G, 100 µg/mL streptomycin and 10% FBS. Subsequent to reaching confluence, the HepG2, Hep3B and NIH3T3 cells were detached using 0.25% trypsin-EDTA and 1×10^4 cells were seeded into the same complete medium.

5.3. *In vitro* cell proliferation assay

Cell proliferation was assessed using the MTT assay [43-45]. Cells (1×10^4 number/well) were seeded in triplicate in a 96-well plate and treated with varying concentrations of the compounds. Survival of the control groups (without drug exposure) were defined as 100%. The Cell viability were evaluated by Vybrant® MTT Cell Proliferation Assay Kit at the end of treatment for 24 h, 20 µL of MTT (5 mg/mL in PBS) was added to each well and incubated for another 4 h. The supernatant in each well was replaced with 100 µL of SDS to solubilize the MTT formazan precipitate and optical density (OD) was measured immediately at 570 nm using Epoch Microplate Spectrophotometer.

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