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## ***In vitro* Antiproliferative Effects of *Cotinus coggygia* Scop. on human non-melanoma and melanoma skin cancer cells**

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### **ABSTRACT**

Skin cancer is one of the most frequently diagnosed malignancies worldwide and its incidence constantly increases. The disease divides in two major subtypes: non-melanoma and melanoma skin cancer. The main drawbacks of the traditional skin cancer therapy are primary and acquired drug resistance and serious side effects due to the nonspecific treatments targeting. Despite advances in therapy strategies there is a need of new affordable natural anti-skin cancer agents, which to possess higher efficiency without causing detrimental side effects. Medicinal plants provide great possibility for the discovery of new anticancer therapeutics with preventive and treatment potential. *Cotinus coggygia* Scop. is a plant species widely applied in phytotherapy predominantly against disorders of the skin and mucosal tissues. The herb has a large range of valuable biological activities but its anticancer properties have not been thoroughly studied. The aim of the present research was to assess the antiproliferative properties of the crude leaf aqueous ethanolic extract from Bulgarian herb *C. coggygia* and its chloroformic and aqueous fractions on a panel of human skin cancer cell lines: basal cell carcinoma (TE 354.T), squamous cell carcinoma (A431) and malignant melanoma (A375) and to compare them to the cell growth inhibitory potential on normal dermal cell line (BJ). The antiproliferative capacity of the plant substances was investigated using MTT assay and microscopy cell morphology observation after 72 h cell treatment in a wide scale of concentrations. The obtained results showed that the crude extract and both fractions inhibit significant proliferation of A431 squamous cell carcinoma and A375 melanoma cells with the highest cytostatic effect registered for the aqueous fraction on A375 cells with a half maximal inhibitory concentration value of 44.33 µg/ml. *C. coggygia* exhibited no cytostatic activity towards TE 354.T basal cell carcinoma cells. The established marked slighter reduction in the growth of normal BJ cell line in comparison to cancer A375 and A431 cells was indicative for a selectivity in the antiproliferative action. It could be concluded that medicinal plant *C. coggygia* possesses promising antiproliferative properties against A375 and A431 skin cancer cell lines which will be further investigated in details.

**Keywords:** *Cotinus coggygia* Scop., antiproliferative capacity, TE 354.T basal cell carcinoma cell line, A431 squamous cell carcinoma cell line, A375 malignant melanoma cell line, BJ normal dermal cell line

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## INTRODUCTION

Skin cancer is the most common malignancy in Caucasians as the number of newly diagnosed cases constantly increases<sup>1</sup>. In addition to the exposure to UV radiation as the leading cause of skin cancer, other risk factors are the genetic susceptibility, individual characteristics as age and skin phototype, immunodeficiency, mutagens in chemicals, consumption of high-fat foods and alcohol and others. The disease is classified into two subtypes: non-melanoma skin cancer (NMSC) and melanoma skin cancer (MSC). Non-melanoma skin cancer represents one-third of all malignancies and according to World Health Organization between 2 and 3 million patients globally are diagnosed with non-melanoma skin cancer each year<sup>2</sup>. NMSC mainly includes basal cell carcinoma (BCC), which is the most frequent form of skin cancer and comprises about 80% of all non-melanoma skin malignancies, and squamous cell carcinoma (SCC), which represents about 20% of NMSC cases and could be a metastatic form. BCC is characterized by good prognosis when has been detected early and the treatment is accurate, while SCC though is usually not life-threatening could be aggressive and is able to spread to distant areas in the body. The frequency of NMSC is about 18-20 times higher than the incidence of melanoma skin cancer in white populations<sup>3</sup> but melanoma represents the most severe form of skin cancer with high metastatic potential and causing high mortality. About 287 000 cases are newly registered in 2018 and the number of death cases from melanoma amounts to above 60 000<sup>4</sup>. Around 65% of all skin malignancies deaths are a result of MSC<sup>5</sup>.

Conventional skin cancer therapy approaches include surgical intervention, cryosurgery, radiation- and chemotherapy, often accompanied by severe side effects. A primary or acquired resistance to available chemotherapeutics determines the growing interest in the discovery of novel plant-derived anti-skin cancer agents with higher efficacy and efficiency. Studies have reported numerous medicinal plants which possess promising cytotoxic potential on skin cancer cell lines and animal model systems of skin related cancers<sup>6-8</sup>. *Cotinus coggygria* Scop. (family Anacardiaceae, European smoketree) is a medicinal plant occurring from Southern Europe to Central China which is widely applied in the folk medicine against different injuries of the skin and soft tissues. Among the numerous biological properties of the plant are antimicrobial, antiseptic, wound-healing, hemostatic, anti-inflammatory, anticancer and many others<sup>9-14</sup>.

The objective of the present study was to explore the antiproliferative capacity of crude aqueous ethanolic extract from leaves of *Cotinus coggygria* and its chloroformic and aqueous fractions on human non-melanoma basal cell carcinoma TE 354.T, squamous cell carcinoma A431 and

malignant melanoma A375 cell lines. Normal dermal cell line BJ was used in the research to assess the selectivity in the action of *C. coggygia* substances.

## MATERIALS AND METHOD

### *Plant extract and fractions*

The crude aqueous ethanolic extract from leaves of *Cotinus coggygia* Scop. was produced and provided by Vemo 99 Ltd. (Sofia, Bulgaria). The extract content of specific natural components includes: (in percent of dry substance): total polyphenols, determined as catechin (from 27.0 to 32.0%); flavonoids, determined as apigenin (15.0%); flavonoids, determined as quercetin (2.0%) and others.

Fractionation of the leaf crude extract was performed by solvent–solvent partition with chloroform and distilled water. The chloroformic (nonpolar) and the aqueous (polar) fractions were then evaporated to dryness.

### *Cell lines and cultivation*

Human skin cancer cell lines TE 354.T (basal cell carcinoma), A431 (squamous cell carcinoma) and A375 (malignant melanoma) and normal skin fibroblast cell line BJ were obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Germany) in a tissue culture incubator with humidified atmosphere, 37°C and 5% CO<sub>2</sub>. Cells were cultivated as a monolayer up to 80% confluence and then were split. In the analysis were used cells in the exponential phase of cell growth.

### *MTT cell proliferation assay*

Cell proliferation of TE 354.T, A431, A375 and BJ cell lines was evaluated using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. Cells were seeded in 96-well plates at a density of  $5 \times 10^3$  cells per well and after 24 h were treated with *C. coggygia* crude leaf aqueous ethanolic extract, aqueous and chloroformic fractions at various concentrations (10, 30, 60, 90, 120, 150, 180 µg/ml) in a new medium with a final volume of 200 µl and were incubated for 72 h. Wells with untreated cells cultured in a medium for the same time period were used as controls. Cell proliferation was determined by addition of 20 µl of MTT solution (5 mg/ml) for 4 h whereafter the medium was removed and the formazan complex was solubilized in 100 µl 10% SDS, 0.01M HCl. The absorbance was measured at 570 nm using microplate reader (Thermo Scientific Multiskan Spectrum) and the percentage of cell proliferation was calculated using the following formula:

Cell proliferation (%) = (Absorbance test sample/Absorbance control) × 100

### ***Cell morphological examination***

Cell morphological alterations after treatment with *C. coggygia* extract and fractions for 72 h was observed under inverted light microscope (Carl Zeiss, Jena, Germany).

### ***Statistical analysis***

The data were presented as means ± standard error of the mean (SEM) of at least three separate experiments performed in triplicate. The half-maximal inhibitory concentration (IC<sub>50</sub>) values were calculated using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Statistical differences between untreated control and treated groups were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. The results are considered significant at values of p<0.05.

## **RESULTS AND DISCUSSION**

Medicinal plant *C. coggygia* is considered as a natural remedy for treatment of various health conditions, among which different cutaneous diseases, due to its chemical composition. It is known that plant compounds possessing immunomodulatory, anti-inflammatory and antioxidant properties have the highest potential to act as chemo-preventive agents in skin cancers<sup>15</sup> which makes *C. coggygia* an extremely suitable candidate for anticancer research. At present no data are available concerning antiproliferative effect of extracts or fractions from *C. coggygia* on skin cancer model systems.

The cytostatic properties of *Cotinus coggygia* leaf aqueous ethanolic extract and its chloroformic and aqueous fractions were studied on human non-melanoma basal cell carcinoma TE 354.T, squamous cell carcinoma A431 and malignant melanoma A375 cell lines and were compared to the non-cancerous dermal cell line BJ by MTT cell proliferation assay and cell morphology observation after treatment for 72 h in a wide range of concentrations.

The results obtained through MTT assay demonstrate considerable selective cytostatic effect of all of the tested plant substances against A431 epidermoid carcinoma cells and A375 melanoma cells (Figure 1). Statistically significant differences between control and treated groups were found. The determined IC<sub>50</sub> values of the extract, chloroformic and aqueous fractions for TE 354.T, A431, A375 and BJ cells were described in Table 1. The strongest cell growth inhibition potential was registered for the aqueous fraction of the extract towards A375 cell line with an IC<sub>50</sub> value of 44.33 µg/ml and maximal inhibition of proliferation to 26.09% at 150 µg/ml. In concern to the non-cancerous skin cell line BJ the results did not show any substantial effect on

cellular proliferation which indicates high selectivity in the anti-skin cancer cytostatic action of *C. coggygia* extract and fractions (Figure 1). *C. coggygia* exhibited no cytostatic activity towards TE 354.T basal cell carcinoma cells.

A strong reduction in the density of A431 and A375 cells monolayer as well as alterations in cell morphology including rounding and shrinkage after treatment with the plant substances were also observed. Considerable effect on the number of the adherent cells and cell morphology characteristics after *C. coggygia* exposure were noticed even in the low treatment concentrations (Figure 2).

There are limited scientific reports related to the plant anticancer cytostatic properties<sup>12,16,17</sup>. The available data on the antitumor potential of Bulgarian *C. coggygia* are very restricted. Our previous studies indicated presence of cytotoxic and cytostatic effect of the plant aqueous ethanolic leaf extract against human breast, ovarian and cervical cancer cell lines and a minor decrease in viability and proliferation of control non-tumorigenic breast cell line<sup>14,18</sup>. It was found that in breast cancer cell line the extract arrests cell cycle, induces apoptosis, exerts a significant genotoxic effect, influences the thermodynamic behavior of the cells, decreases cells clonogenic capacity and has an influence on some epigenetic processes as DNA methylation and histone modifications<sup>19,20</sup>. Another recent study detected *in vitro* antiproliferative effect of ethyl acetate extract of *C. coggygia* leaves on breast, cervical, lung, hepatocellular, colorectal and prostate cancer cells<sup>21</sup>. In regard to *in vitro* cytostatic effect of the Bulgarian plant on non-cancerous cell lines the authors reported that the extract inhibited proliferation of normal skin cell line BJ and non-tumorigenic breast cells MCF10A<sup>22</sup>.

The here-obtained results showed significant reduction of non-melanoma A431 and melanoma A375 cells proliferation and considerably weaker decrease in the proliferation rate of normal skin cells BJ after *C. coggygia* extract and fractions treatment. Malignant melanoma cell line was affected in a more pronounced degree than cutaneous squamous cell carcinoma cells. We found that the aqueous polar fraction of *C. coggygia* leaf extract possesses the highest cytostatic potential against A375 (IC<sub>50</sub>-44.33 µg/ml) and A431 (IC<sub>50</sub>-74.41 µg/ml) skin cancer cell lines. The registered effect is strongly selective which is a substantial characteristic regarding evaluation of the pharmacological potential of the plant.

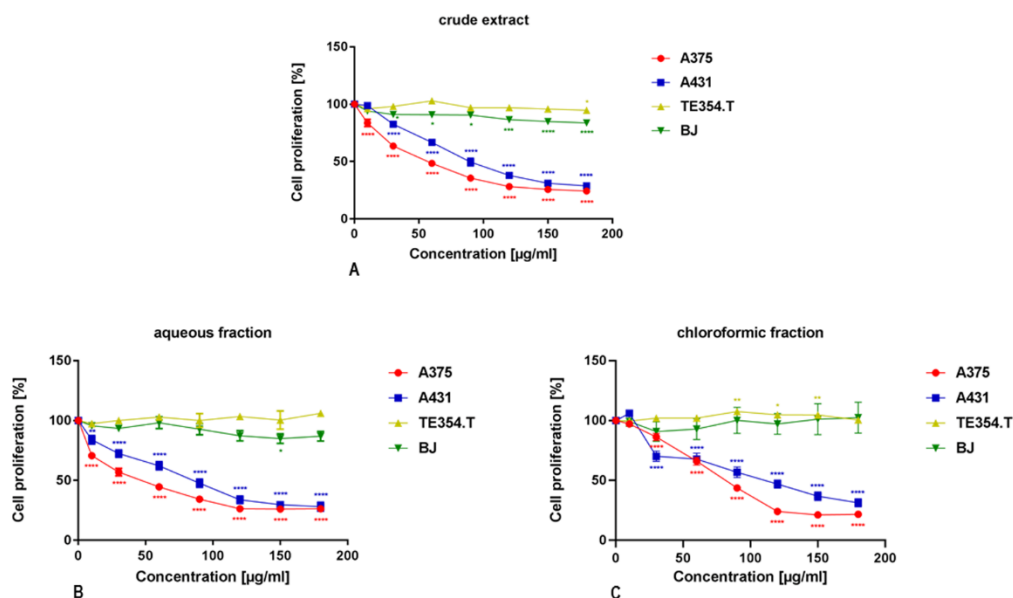
The detected in our research half maximal inhibitory concentrations are indication for strong cell growth inhibitory action when compared to the values reported in other studies in concern to the anti-skin cancer activity of different medicinal plants. Mild cytotoxic and stronger antiproliferative effects were registered for two ethanolic extracts obtained from leaves and

stems of *Artemisia absinthium* L. towards A375 cell line with IC<sub>50</sub> values of 295.4 µg/ml and 312 µg/ml at 72 h after exposure, respectively, and a weaker affection on non-malignant HaCaT cells was found with IC<sub>50</sub> values of 397.7 µg/ml and 361.8 µg/ml<sup>23</sup>. Koutsoulas et al. detected presence of cytostatic effect of methanolic *Salvia pomifera* and *Salvia fruticosa* extracts on two melanoma cell lines - A375 and Mel JuSo with IC<sub>50</sub> values of 57.95 µg/ml and 70.29 µg/ml for A375, and 63.57 µg/ml and 76.53 µg/ml for Mel JuSo cells, respectively<sup>24</sup>. Another study evaluating the antiproliferative potential of stem *Basella alba* and *Basella rubra* extracts against A431 epidermoid carcinoma cell line recorded inhibitory effect on the cell growth of the tested cells with IC<sub>50</sub> values of 3.66 mg/ml for *B. alba* and 6.87 mg/ml for *B. rubra*<sup>25</sup>.

Anti-skin cancer activity of *C. coggyria* could be attributed to some active compounds found in the plant such as gallic acid<sup>26,27</sup>, apigenin<sup>28</sup>, fisetin<sup>29</sup>, myricetin<sup>30</sup>, quercetin<sup>31</sup> and others.

**Table 1: The IC<sub>50</sub> values determined by MTT assay.**

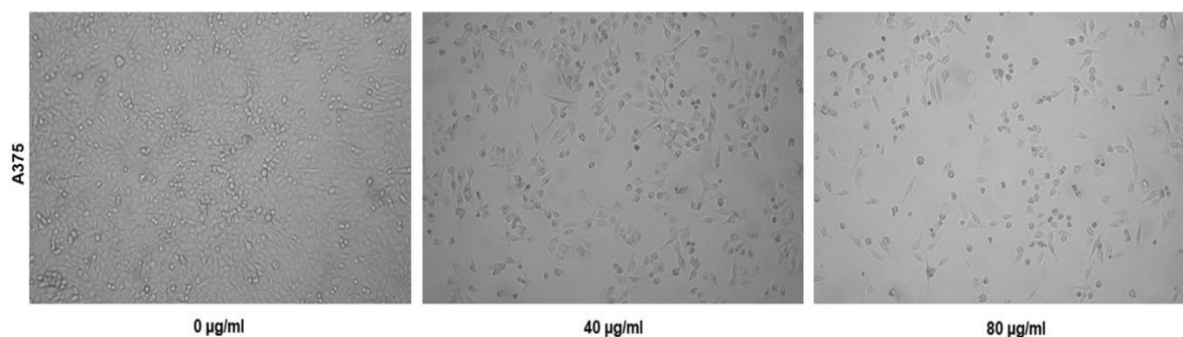
IC <sub>50</sub> [µg/ml]			
Cell line	Crude extract	Aqueous fraction	Chloroformic fraction
A375	52.25	<b>44.33</b>	71.81
A431	89.95	74.41	87.27
TE 354.T	>180	>180	>180
BJ	>180	>180	>180



**Figure 1: MTT cell proliferation assay of TE 354.T, A431, A375 and BJ cells treated for 72 h with increasing concentrations of *Cotinus coggyria* crude aqueous ethanolic extract (A), aqueous fraction (B) and chloroformic fraction of the extract (C). Error bars represent**



standard error of the mean (SEM). \*, \*\*, \*\*\* and \*\*\*\* indicate significant differences from the control group by Dunnett's test (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ ).



**Figure 2: Cell morphology alterations of A375 cells after treatment with *Cotinus coggygia* aqueous fraction of crude leaves extract in concentrations of 40 µg/ml and 80 µg/ml for 72 h compared to untreated control.**

## CONCLUSION

The present study detected that medicinal plant *Cotinus coggygia* exhibits significant highly selective *in vitro* proliferation inhibitory properties against A375 melanoma and A431 non-melanoma squamous cell carcinoma cells and the strongest cytostatic effect was registered for the aqueous fraction of the extract against melanoma cells. Future studies will be directed to more complex assessment of anti-skin cancer therapeutic potential of the plant.

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## REFERENCES

1. Khazaei Z, Ghorat F, Jarrahi AM, Adineh HA, Sohrabivafa M, Goodarzi E. Global incidence and mortality of skin cancer by histological subtype and its relationship with the human development index (HDI); An ecology study in 2018. WCRJ 2019; 6: e1265.
2. World Health Organization /online/; Available online: [https://www.who.int/news-room/q-a-detail/radiation-ultraviolet-\(uv\)-radiation-and-skin-cancer](https://www.who.int/news-room/q-a-detail/radiation-ultraviolet-(uv)-radiation-and-skin-cancer).
3. Leiter U, Garbe C. Epidemiology of melanoma and nonmelanoma skin cancer-the role of sunlight. Adv Exp Med Biol 2008; 624: 89-103.

4. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 2018; 68: 394-424.
5. Cummins DL, Cummins JM, Pantle H, Silverman MA, Leonard AL, Chanmugam A. Cutaneous Malignant Melanoma. *Mayo Clin Proc* 2006; 81(4): 500-507.
6. Kim KH, Moon E, Kim SY, Choi SU, Lee KR. Lignan constituents of *Tilia amurensis* and their biological evaluation on antitumor and anti-inflammatory activities. *Food Chem Toxicol* 2012; 50: 3680-3686.
7. Mazumder K, Biswas B, Raja IM, Fukase KA. Review of cytotoxic plants of the Indian subcontinent and a broad-spectrum analysis of their bioactive compounds. *Molecules* 2020; 25: 1904.
8. Chinembiri TN, du Plessis LH, Gerber M, Hamman JH, du Plessis J. Review of natural compounds for potential skin cancer treatment. *Molecules* 2014; 19(8): 11679-11721.
9. Matic S, Stanić S, Mihailović M, Bogojević D. *Cotinus coggygia* Scop.: An overview of its chemical constituents, pharmacological and toxicological potential. *Saudi J Biol Sci* 2016; 23(4): 452-461.
10. Pavlov D, Eftimov M, Nashar M, Tzaneva M, Ivanova D, Valcheva-Kuzmanova S. Anti-inflammatory properties of aqueous infusion from *Cotinus coggygia* leaves in rats. *Scr Sci Pharm* 2014; 1: 25.
11. Deniz FSS, Salmas RE, Emerce E, Cankaya IIT, Yusufoglu HS, Orhan IE. Evaluation of collagenase, elastase and tyrosinase inhibitory activities of *Cotinus coggygia* Scop. through *in vitro* and *in silico* approaches. *South Afr J Bot* 2020. 132: 277–288.
12. Artun FT, Karagoz A, Ozcan G, Melikoglu G, Anil S, Kultur S, Sutlupinar N. *In vitro* anticancer and cytotoxic activities of some plant extracts on HeLa and Vero cell lines. *J BUON* 2016; 21: 720-725.
13. Pollio A, Zarrelli A, Romanucci V, Di Mauro A, Barra F, Pinto G, Crescenzi E, Roscetto E, Palumbo G. Polyphenolic profile and targeted bioactivity of methanolic extracts from Mediterranean ethnomedicinal plants on human cancer cell lines. *Molecules* 2016; 21: 395.
14. Gospodinova Z, Bózsity N, Nikolova M, Krasteva M, Zupkó I. Antiproliferative properties against human breast, cervical and ovarian cancer cell lines, and antioxidant capacity of leaf aqueous ethanolic extract from *Cotinus coggygia* Scop. *Acta Med Bulg* 2017; 44: 20-25.



15. Katiyar SK. Green tea prevents non-melanoma skin cancer by enhancing DNA repair. *Arch Biochem Biophys* 2011; 508: 152–158.
16. Wang G, Wang J, Du L, Li F. Effect and mechanism of total flavonoids extracted from *Cotinus coggygia* against glioblastoma cancer *in vitro* and *in vivo*. *Biomed Res Int* 2015; 2015, 856349.
17. Antal DS, Ardelean F, Pinzaru I, Borcan F, Ledeti I, Coricovac D, Zupko I, Baghdikian B, Ollivier E, Soica C, Bolintineanu SL. Effects of cyclodextrin complexation on the anti-cancer effects of *Cotinus coggygia* extract and its constituents, butein and sulfuretin. *Rev Chim* 2016; 67 (8): 1618–1622.
18. Gospodinova Z, Krasteva M. *Cotinus coggygia* Scop. leaf extract exerts high but not dose- and time-dependent *in vitro* cytotoxic activity on human breast cancer cells. *Genet Plant Physiol* 2017; 7: 176-183.
19. Gospodinova ZI, Zupkó I, Bózsity N, Manova VI, Georgieva MS, Todinova SJ, Taneva SG, Ocsovszki I, Krasteva ME. *Cotinus coggygia* Scop. induces cell cycle arrest, apoptosis, genotoxic effects, thermodynamic and epigenetic events in MCF7 breast cancer cells. *Z Naturforsch* 2021; 76(3–4)c: 129–140.
20. Gospodinova Z, Krasteva M, Manova V. Effects of *Cotinus coggygia* extract on the transcriptional levels of histone deacetylase genes in breast cancer cells *in vitro*. *Am J Pharm Health Res* 2017; 5: 60-69.
21. Iliev I, Ivanov I, Todorova K, Tasheva D, Dimitrova M. *Cotinus coggygia* non-volatile fraction affects the survival of human cultured cells. *Acta morphol anthropol* 2021; 28: (1-2).
22. Iliev I, Ivanov I, Todorova K, Dimitrova M. Effects of a *Cotinus coggygia* ethyl acetate extract on two human normal cell lines. *Acta Morphol Anthropol* 2020; 27(3-4): 25–29.
23. Moacă EA, Pavel IZ, Danciu C, Crăiniceanu, Minda D, Ardelean F, Antal DS, Ghiulai R, Cioca A, Derban M, Simu S, Chioibaş R, Szuhaneck C, Dehelean CA. Romanian Wormwood (*Artemisia absinthium* L.): Physicochemical and Nutraceutical Screening. *Molecules* 2019; 24: 3087.
24. Koutsoulas A, Čarnecká M, Slanina J, Tóth J, Slaninová I. Characterization of phenolic compounds and antiproliferative effects of *Salvia pomifera* and *Salvia fruticosa* extracts. *Molecules* 2019; 24(16): 2921.
25. Kumar BR, Anupam A, Manchikanti P, Rameshbabu AP, Dasgupta S, Dhara S. Identification and characterization of bioactive phenolic constituents, anti-proliferative,

- and anti-angiogenic activity of stem extracts of *Basella alba* and *rubra*. J Food Sci Technol 2018; 55: 1675–1684.
26. Lo C, Lai TY, Yang JS, Yang JH, Ma YS, Weng SW, Lin HY, Chen HY, Lin JG, Chung JG. Gallic acid inhibits the migration and invasion of A375.S2 human melanoma cells through the inhibition of matrix metalloproteinase-2 and Ras. Melanoma Res 2011; 21(4): 267–273.
27. Subramanian V, Venkatesan B, Tumala A, Vellaichamy E. Topical application of gallic acid suppresses the 7,12-DMBA/Croton oil induced two-step skin carcinogenesis by modulating anti-oxidants and MMP-2/MMP-9 in Swiss albino mice. Food Chem Toxicol 2014; 66: 44-55.
28. Ghițu A, Schwiebs A, Radeke HH, Avram S, Zupko I, Bor A, Pavel IZ, Dehelean CA, Oprean C, Bojin F, Farcas C, Soica C, Duicu O, Danciu C. A comprehensive assessment of apigenin as an antiproliferative, proapoptotic, antiangiogenic and immunomodulatory phytochemical. Nutrients 2019; 11 (4): 858.
29. Syed DN, Afaq F, Maddodi N, Johnson JJ, Sarfaraz S, Ahmad A, Setaluri V, Mukhtar H. Inhibition of human melanoma cell growth by the dietary flavonoid fisetin is associated with disruption of Wnt/beta-catenin signaling and decreased Mitf levels. J Invest Dermatol 2011; 131(6): 1291-1299.
30. Sun W, Tao Y, Yu D, Zhao T, Wu L, Yu W, Han W. Myricetin exerts potent anticancer effects on human skin tumor cells. Trop J Pharm Res 2018; 17(6): 1067–1072.
31. Thangasamy T, Sittadjody S, Lanza-Jacoby S, Wachsberger PR, Limesand KH, Burd R. Quercetin selectively inhibits bioreduction and enhances apoptosis in melanoma cells that overexpress tyrosinase. Nutr Cancer 2007; 59(2): 258-268.



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