Anti-Proliferative Effect of Rosa Agrestis on Endometrium Cancer Cells In Vitro

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Abstract

Endometrial cancer is the most common gynaecological malignant disease. Despite of some arguments about the preference of herbal medicine to pharmaceutical drugs, because of their minimal side effects herbal medicine is proven to be effective as a single application and/or an adjuvant therapy. Rosa agretis Savi (R. agretis Savi) which belongs to Rosaceae family has been used traditionally to treat allergy, antiatopic dermatitis, and some inflammatory diseases, is commonly studied. Anti-oxidant activity is also reported. The present study was undertaken to investigate the in vitro anticancer activity of R. agrestis parts (leaves, flowers) at endometrium cancer. Extracts in a concentration of 1 µg/ml to 200 µg/ml from leaves and flowers were tested on human endometrium cell line named Ischikawa for 72 h. Cell proliferation (total cell number) and apoptotic index (flow cytometry) were evaluated for 72 h. All the results were statistically analyzed using the independent Student's t-test. All extracts decreased cell number and increased apoptotic index with dead cells in concentration and time dependent manner ($p<0.05$). When the efficiency of flowers was compared with leaves, it's clearly seen that flowers were the most effective as an anticancer agent. Besides, their effects were seemed mild in comparison to positive control as an antineopastic agent lithium chloride (LiCl). In conclusion, our study showed for the first time that R. agrestis, regardless of leaves and flowers, possessed an anticancer effect on human endometrium cells *in vitro.*

Keywords: *Rosa agrestis Savi, endometrium cancer, anticancer activity*

Introduction

Endometrial cancer is the most common gynaecological malignant disease, and the fourth most common cancer in European women, accounting for about 6% of new cancer cases and 3% of cancer deaths per year. Risk factors include the changes in the balance of female hormones in the body (estrogen/progesterone), an early age start of menstruation (before age 12) or the lately started menopause, no pregnancy, older age, obesity, hormone therapy for breast cancer, an inherited colon cancer syndrome, pollution and malnutrion [1, 2].

Traditional classification of endometrial carcinoma is based either on clinical and endocrine features (eg, types I and II) or on histopathological characteristics (eg, endometrioid, serous, or clear-cell

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adenocarcinoma) [3,4]. Type I endometrial cancers are histologically low-grade endometrioid carcinomas with a favorable prognosis and represent about 70–80% of sporadic endometrial carcinomas. These tumors are associated with unopposed estrogen exposure and usually develop in perimenopausal women, with the risk factors of obesity, anovulation, nulliparity, and exogenous estrogen exposure. They arise in a background of complex and atypical endometrial hyperplasia and commonly express estrogen (ER) and progesterone (PG) receptors. Although they are rare, mucinous adenocarcinomas are also considered within this group since they have low histopathological grade and usually express ER and PR. On the other hand, type II cancers are histologically highgrade non-endometrioid carcinomas, most frequently serous papillary, have an aggressive clinical behavior and represent about 10-20% of endometrial cancers. These tumors are unrelated to estrogen excess, and usually develop in older postmenopausal women, without any hormonal risk factors. They arise in a background of atrophic endometrium with the putative precursor lesion being endometrial intraepithelial carcinoma and occasionally endometrial polyps. Although the histological prototype for type II tumors is serous carcinomas, the less frequent clear cell carcinoma is also considered within this group [3,4].

Despite of some arguments about the preference of herbal medicine to pharmaceutical drugs due to minimal adverse effects, herbal medicine is proven to be effective as a single application and/or an adjuvant therapy [5,6]. Rosa species (Rosaceae family) are widely distributed in Turkey. In this era Rosa agretis (R. agretis), a field briar and a one of the 25 species of Rosa, is commonly studied and used as a traditional Turkish medicine. It has been used traditionally to treat allergy, antiatopic dermatitis, and some inflammatory diseases [7]. Antioxidant effect of this plant-particularly studied with leaves-was also shown before [7]. The present study was undertaken to investigate the in vitro anticancer activity of R. agrestis parts (leaves, flowers) at endometrium cancer. The anticancer activity of Rosaceae family is rarely studied, this does not include R. agretis Savi, yet the effect of R. agretis Savi on the endometrium cancer was shown in this study for the first time.

Materials and methods

Plant materials

Leaves of R. agrestis were collected in June 2000 from Istanbul (Turkey). Voucher specimens have been deposited in the Herbarium of the Faculty of Pharmacy, (ISTE) University of Istanbul (ISTE 79592). The plants were identified by Prof. Dr. Şükran Kültür, Department of Pharmaceutical Botany, Istanbul University.

Preparation of Extracts

The dried leaves (500 g) of R. agretis Savi were first extracted with petroleum ether and then with EtOH (95^{O}) in a Soxhlet apparatus. The petroleum ether extract was concentrated (A) and extracted with 60% ethanol. The ethanol extract was concentrated and extracted with chloroform (B). The concentrated EtOH extract was diluted with distilled water and extracted with benzene (C), chloroform (D) and ethyl acetate (E) successively. The E extract was dissolved in ethanol and used for assessment of anticancer activity [7].

Cell culture and experiment design

Human endometrial adenocarcinoma Ishikawa (Sigma no: 99040201) cell line was used in this experiment. The cell lines were cultured

in RPMI 1640 medium and supplemented with 10% fetal bovine serum, 1% L-glutamine,1% non-essential amino acids and 100 units/mL penicillin and streptomycin (Sigma Chemical Co., St Louis, Missouri). Following trypan blue exclusion assay, 1 x 105 cells per well were seeded in 24-well microtiter plates. Ishikawa cells were incubated for 24 h to allow for cell attachment on the experiment day. The cells were treated with serial concentrations of the samples. Twenty µL per well of each concentration (n:6) was added to the plates in 3 replicates to obtain final concentrations of 1,10, 100, 200 µg/mL, and LiCl at 8 µg/mL as a positive control. By these serial dilutions, the final mixture used for treating the cells contained not more than 0.1% of the solvent (ethanol), the same as in the solvent control wells. The culture plates were kept at 37°C with 5% (v/v) CO2 for 72 h. After every 24 h of incubation, total cell numbers were counted using a cell counter and flow cytometric analysis were done [8].

Apoptotic index by flow cytometry

The apoptotic index was evaluated by using flow cytometric Annexin-V-florescein isothiocyanate/ propidium iodide (Annexin-V-FITC/PI) staining. Following the instruction manual of the kit (BD Pharmingen, San Diego, CA, USA), briefly, cells were washed twice with PBS and resuspended by binding buffer containing 0.01 M HEPES, 0.14 mM NaCl, and 2.5 mM $CaCl_2$. A cell suspension (1×10^5 cells in 100 µL) in binding buffer was incubated with 5 µL of FITC-labeled Annexin V (BD Pharmingen) dye and PI for 15 min in the dark at room temperature. After incubation, the PI fluorescence and Annexin V were measured simultaneously in a BD FACS Calibur and analyzed with the instrument's operating software (CellQuest: BD Pharmingen). Data acquisition and analysis were undertaken with CellQuest and WinMDI programs [9].

Statistical analysis

The results were statistically analyzed using the independent Student's *t*-test. Data were represented as means ± standard error mean (SEM) and at least in triplicate. Results were considered significant with $p < 0.05$.

Results

Cell proliferation

The effects of flower extracts on cell proliferation

Figure 1 clearly shows that all extracts decreased cell proliferation in comparison to control group ($p<0.05$). High concentrated extract (200 µg/ml) induced highest decrease in cell number ($p<0.05$) , the efficiency was decreased from highest concentration to lowest concentration ($p<0.05$) for 72 h. LiCl decreased cell number much more higher than extract samples ($p<0.05$).

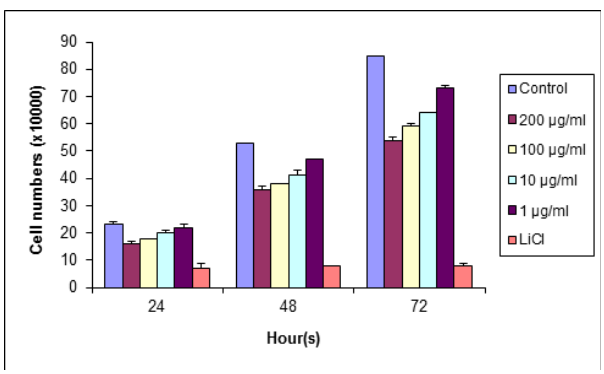

Figure 1 The effects of extracts from flowers on the proliferation of cancer cells. Data were represented as means ± SEM and at least in triplicate. LiCl, a well-known antiphyscotic drug with newly pronounced antineoplastic effects was used as positive control.

The effects of leave extracts on cell proliferation

All extracts decreased cell proliferation in comparison to control group ($p<0.05$) (Fig. 2). High concentrated extract (200 µg/ml) induced highest decrease in cell number ($p<0.05$) at the 48 th h and 72nd h, however its effect was almost same with second high concentrated extract (100 µg/ml) at the 24th h ($p>0.05$). The same situation can be also seen between low concentrated doses as 10 µg/ml and 1 µg/ml at the 24 th h. LiCl decreased cell number much more higher than extract samples ($p<0.05$).

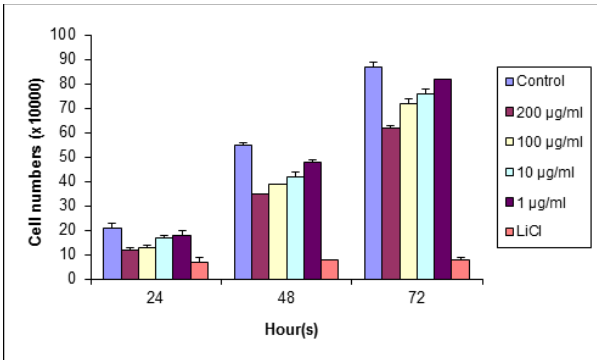

Figure 2 The effects of extracts from leaves on the proliferation of cancer cells. Data were represented as means ± SEM and at least in triplicate. LiCl, a well-known antiphyscotic drug with newly pronounced antineoplastic effects was used as positive control.

Apoptotic index

The effects of extracts on apoptotic index

Table 1 clearly shows that all extracts regardless of flower or leave increased apoptotic index in comparison to control group ($p<0.05$). High concentrated extract (200 µg/ml) induced highest increase in apoptotic index and the number of dead cells among extract samples ($p<0.05$). The efficiency was changed in a concentration dependent manner ($p<0.05$) for 72 h. LiCl increased apoptotic index and the number of dead cells much more higher than extract samples ($p<0.05$).

Table 1. *The apoptosis evaluation both for leaves and flowers at the 72nd h*

	Flower extracts at the 72nd h*			Leave extracts at the 72nd h*		
	Alive	**Apoptotic**	**Dead**	**Alive**	**Apop-totic**	**Dead**
Control	96±1	3±1	1±1	95±1	2±1	3±1
200 µg/ml	70±1^{a}	11±1^{b}	19±1^{a,b}	75±1^{a,b}	9±1^{a,b}	16±1^{a,b}
100 µg/ml	75±1^{a,b}	8±1^{a,b}	17±1^{a,b}	79±1^{a,b}	8±0^{a,b}	13±1^{a,b}
10 µg/ml	79±1^{a}	5±1^{b}	14±1^{a,b}	84±1^{a,b}	6±1^{a,b}	10±0^{a,b}
1 µg/ml	84±1^{a,b}	4±0^{b}	12±1^{a,b}	87±1^{a,b}	6±1^{a,b}	7±1^{b}
Lithium chloride	63±1	15±2	22±1	66±1	10±1	24±1

*Data were represented as mean (%) ± SEM and at least in triplicate.

^{a} Statistically significant ($p<0.05$) in comparison to the control group.

^{b} Statistically significant ($p<0.05$) in comparison to lithium chloride.

Discussion

Our study showed for the first time that R. agrestis, regardless of leaves and flowers, showed anticancer effect on human endometrium cells in vitro. All extracts decreased cell number and increased apoptotic index with dead cells in concentration and time dependent manner. When the efficiency of flowers was compared with leaves, it's clearly seen that flowers were the most effective as an anticancer agent. Besides, their effects were seemed mild in comparison to an antineopastic agent LiCl that its efficiency was proven at these cells in our previous reports [8,9].

Study by Bitis et al. isolated and made characterisation of seven flavonoids, the levels of total phenolics, flavonoids and proanthocyanidins, and the antioxidant activity of the leaf extract of R. agrestis Savi [7]. They showed that the R. agrestis Savi leaf

extract exhibited significant antioxidative activity as measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) (EC(50) = 47.4 microg mL(-1)), inhibited both beta-carotene bleaching and deoxyribose degradation, quenched a chemically generated superoxide anion in vitro and showed high ferrous ion chelating activity. In addition, they also determined reactivity towards 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) radical cation and ferric-reducing antioxidant power (FRAP) values which were equivalent to 2.30 mM L(-1) Trolox, the water soluble alpha-tocopherol analogue, and 1.91 mM L(-1) Fe(2+), respectively. They concluded that t he high antioxidant activity of the extract appeared to be attributed to its high content of total phenolics, flavonoids [diosmetin, kaempherol, quercetin, kaempherol 3-glucoside (astragalin), quercetin 3-rhamnoside (quercitrin), quercetin 3-xyloside and quercetin 3-galactoside (hyperoside)] and proanthocyanidins [7]. When pubmed research was done with the word "rosa agrestis", 6 publications were found and 5 of 6 including Bitis et. al. [7] studied one of the flavonoid named astragalin metioned above both in vivo and in vitro at the deseases of osteoarthritis, mastitis and the detection of macrophage response and epitelial cell response because of its' anti-inflammatory activity [7, 10-13].

Flowers were commonly studied, flowers were seemed to be neglected, therefore no eligible data was found for flowers [7, 10-13]. In the light of detailly explained rich content of leaves from previous studies [7,10-13], their anticancer activity may be explained through anti-oxidant and anti-inflammatory pathway.

Human endometrium cell line Ishıkawa belongs to type I endometrium cancer. However, clinical heterogeneity-molecular classification of patients into different subtypes based on genetic or epigenetic characteristics-should be taken into account [3,4]. This means that our results regardless of the lack of in vivo and human study can not be reflected whole treatment scenerio for endometrium cancer.

In conclusion, despite of some arguments about the preference of herbal medicine to pharmaceutical drugs, because of their minimal side effects herbal medicine is proven to be effective as a single application and/ or an adjuvant therapy [5,6]. R. agretis may be excepted as a part of promising herbal medicine for the treatment of endometrium cancer and also other cancer types following more life quality because of low toxicity.

Conflict of interest
None declared.

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