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ANTIPROLIFERATIVE EFFECTS OF *CAMELLIA SINENSIS, FRANGULA ALNUS* AND *ROSMARINUS OFFICINALIS*

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Abstract - The use of medicinal plants is becoming increasingly appreciated in suppressing cancer growth and cancer prevention. In this study the antiproliferative effects of the water extracts of previously obtained ethanolic dry extracts of three different medicinal plants (*Camellia sinensis*, *Frangula alnus* from two different places and *Rosmarinus officinalis*) using cell lines derived from human cervix adenocarcinoma (HeLa cells) were investigated. The extract of *Camellia sinensis* exhibited significant cytotoxic effect against HeLa cells (IC_{50} 40.88 μg/ml). Screening in HeLa cells revealed a moderate cytotoxic effect (IC_{50} 80.26μg/ml) of the extract of *Rosmarinus officinalis,* a mild cytotoxic effect (IC_{50} 148.05 μg/ml) of the extract of *Frangula alnus* (originating in Bosnia), and the extract of *Frangula alnus* (originating in Serbia) did not show active cytotoxicity (IC_{50} > 200 μg/ml). The best antiproliferative properties are those of *Camellia sinensis*, followed by *Rosmarinus officinalis*, and the least effective was *Frangula alnus*. As regards geographic origin, the *Frangula alnus* from Bosnia possessed better antiproliferative effects than *Frangula alnus* from Serbia.

Key words: Antiproliferative effect, *Camellia sinensis, Frangula alnus, Rosmarinus officinalis.*

INTRODUCTION

Cancer is one of the major causes of death in developed countries together with cardiac and cerebrovascular diseases (WHO, 1998). Cancer can be clinically treated by surgery, radiotherapy and chemotherapy. After the surgical ablation of progressive cancer, however, metastasized tumor cells continue to progress and this is one of the causes making cancer treatment difficult (Fidler and Kripke, 1977). Ionizing radiation and most anticancer drugs damage DNA, suppress DNA replication, killing rapidly growing tumor cells, However, at the same time these treatments affect normal cells, causing serious adverse effects, such as inhibition of bone marrow functioning nausea, vomiting and alopecia (Kligerman, 1973; Medical Economics, 1998). Thus, more effective anticancer drugs with high selectivity against only malignant cells and with the ability to repress tumor metastasis are desired. As candidates for such drugs, cytotoxic, antitumor or anticancer natural products have been sought, and plant components such as *Vinca* alkaloids, taxoids, etoposide and irinotecan are now used in clinical treatments (Medical Economics, 1998).

Since ancient times, plants have been the source of medicines for the treatment of diseases. Regardless of the availability of a wealth of synthetic drugs, plants

remain an integral part of the health care in different countries, especially the developing countries. In the late 1990s, the WHO stated that a large percentage of the world's population depends on plant-based therapies to cover the needs of primary health care (Dikshit et al., 2004). Moreover, towards the end of the 20th century, plant-based OTC products, nutraceuticals and food supplements comprising complementary and alternative therapies have gained a big share in the drug market in developed countries.

Over the past decade, herbal medicines have been accepted universally, and they have an impact on both world health and international trade. Hence, medicinal plants continue to play an important role in the healthcare system of a large number of the world's population (Akerele, 1988). Traditional medicine is widely used in Serbia as well. Medicinal plants possess immunomodulatory (Agrawala et al., 2001; Pandey and Madhuri, 2006) and antioxidant properties that are related to certain aspects of anticancer activities. The use of medicinal plants is becoming increasingly appreciated in suppressing cancer growth (Waladkhani and Clemens, 1998; Orsolic and Basic, 2003) and potential cancer prevention (Pan and Ho, 2008).

Camellia sinensis (Camellia sinensis), Frangula alnus (Rhamnus frangula) and *Rosmarinus officinalis* (rosemary) have been widely used and well-documented medicinal plants for centuries (Weiss, 1988; Hansel and Sticher, 2002). However, the anticancer properties of *Camellia sinensis* and *Frangula alnus* have not been fully investigated and proven, while there are some data about anticarcinogenic properties of *Rosmarinus officinalis* (Teuscher, 2005). In this study, we investigated the antiproliferative properties of extracts of *Thea viridis, Frangula alnus* from two different places and *Rosmarinus officinalis*, using cell lines derived from human cervix adenocarcinoma.

MATERIALS AND METHODS

Plant material

Camellia sinensis is a medicinal plant from India, *Frangula alnus* is from Bosnia, *Frangula alnus* from Serbia, *Rosmarinus officinalis* is a free-growing plant from the Mediterranean.

Preparation of extracts

Extraction was done in a percolator using 70% ethanol. Low-pressure evaporation of the extract was done following extraction. A 2 l glass percolator was first lined with some cotton wool, and subsequently filled with the desired amount of precut and sifted (0.75 sift) plant which was then covered with 70% ethanol. When the extract started flowing through the faucet on the percolator the faucet was then closed and the content left to macerate for at least 16 h. Following maceration, the extract was poured out of the percolator at a speed of 2 liters per hour. The amount of the poured extract was six times the volume of the starting material (1:6 extract). The extract was then stored for the next 3 to 5 days, filtered through a series of Whatman filters and finally passed through a 0.22 μm filter (Millipore, Billerica, MA). The extract was evaporated in a rotational vacuum evaporator until a dry powder was obtained. The temperature in the evaporator was kept below 65°C under a pressure of 15-25 mbar.

Cell lines

Human cervix adenocarcinoma HeLa cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The HeLa cells were maintained in the recommended nutrition medium: RPMI 1640 medium supplemented with 100 g/L heat-inactivated (56°C) foetal bovine serum (FBS), 3 mmol/L, L-glutamine, 100 g/mL streptomycin, 100 IU/mL penicillin and 25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and adjusted to pH 7.2 with bicarbonate solution. The cells were grown in a humidified atmosphere of 5% CO_2 in air at 37°C.

Treatment of cell line

In the order to obtain water extracts, stock solutions (10 mg/mL) of the investigated original ethanolic

extracts (E1), were resuspended in physiological saline and put in the dark at room temperature for 24 h; they were shaken for 3 h. These suspensions were filtered through 0.22 μm and the obtained solutions were diluted in nutrient medium to the various working concentrations. Neoplastic HeLa (2000 cells per well) cells were seeded into 96-well microtiter plates; 24 h later, after cell adherence, five different doubly diluted concentrations of the investigated extracts were added to the wells. Because the original extracts were not totally dissolved in physiological saline, the final concentrations of investigated extracts applied to target cells were less than 12.5, 25, 50, 100 and 200 μg/mL.

Determination of cell survival

The effect of extracts on target cell survival was determined by the microculture tetrazolium test (MTT) according to Mosmann (1983) with modification by Ohno and Abe (1991), 72 h after addition of the compounds, as described earlier. Briefly, 20 μL of MTT solution (5 mg/mL phosphate-buffered saline) was added to each well. Samples were incubated for a further 4 h under the same conditions. Then, 100 μL of 100 g/L sodium dodecyl sulfate was added to dissolve formazan, the product from the conversion of MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of the absorbance of light, which was read in an ELISA plate reader (colorimeter) at 570 nm. The absorbance *(A)* at 570 nm was measured 24 h later. To determine cell survival (%), the *A* of a sample with cells grown in the presence of various concentrations of the investigated extracts was divided by the control optical density (the *A* of control cells grown only in nutrient medium) and multiplied by 100. It was implied that the *A* of the blank was always subtracted from the *A* of the corresponding sample with target cells. IC_{50} was defined as the concentration of an agent that inhibits cell survival by 50% compared with a vehicle-treated control. All IC_{50}'s were reported as a mean of two measurements, each done in triplicate.

IC_{50} were established from dose-dependent data using Graphpad Prism Ver 3.0 software.

RESULTS

In vitro cytotoxic activity

The cytotoxic action of *Camellia sinensis, Frangula alnus* (A), *Frangula alnus* (B) and *Rosmarinus officinalis* extracts was tested against HeLa cells. The cytotoxicity (IC_{50} values in μg/ml) of the extracts against the tested cancer cells are summarized in Table 1. Fig. 1 depicts the cytotoxic curves from the MTT assay showing the survival of HeLa cells grown for 72 h in the presence of increasing concentrations of extracts.

The extract of *Camellia sinensis* exhibited a significant cytotoxic effect against HeLa cells (IC_{50} 40.88 μg/ml). The extract of *Rosmarinus officinalis* revealed a moderate cytotoxic effect screened against HeLa cells (IC_{50} 80.26 μg/ml), the extracts of *Frangula alnus* (A) displayed some mild cytotoxic effect (IC_{50} 148.05 μg/ml), and the extract of *Frangula alnus* (B) did not show active cytotoxic activity against the HeLa cell lines (IC50 > 200 μg/ml).

Light microscopy

The cytotoxic effect of extracts observed by light microscopy (Carl Zeiss inverted microscopy, with total magnification 630), at concentrations of 200 μg/ml for HeLa cells during 72 h of culturing is illustrated in Fig. 2. Figs. 2B, 2C, 2E show significant morphological changes that occurred during 72 h at different stages of cell death. The extracts damaged the integrity of the cytoplasmic membrane. In addition, in accordance with the results of the MTT test, the HeLa cells treated for 72 h with *Frangula alnus* (B) grew similar the control cells (Fig. 2 D).

DISCUSSION

We examined the antiproliferative effects of water extracts of previously obtained ethanolic dry extracts of three different medicinal plants (*Camellia sinensis, Frangula alnus* extract (A) from Bosnia and extract (B) from Serbia and *Rosmarinus officinalis)* on HeLa cells.

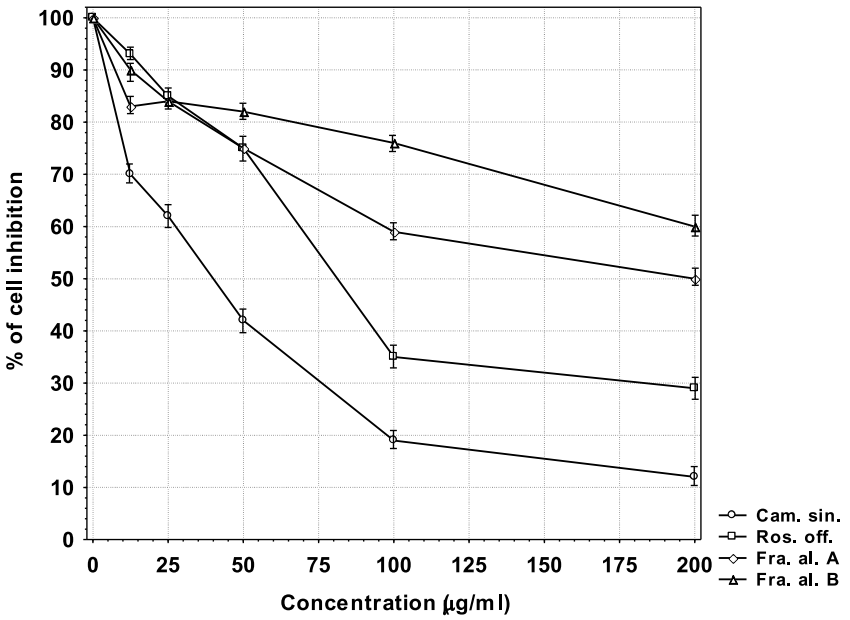

Fig. 1. Representative graph of HeLa cells survival after 72 h cell growth in the presence of increasing concentrations of investigated extracts.

Camellia sinensis (*Camellia sinensis*) is a species of plant whose leaves are used to produce Chinese and Indian teas. It is of the genus *Camellia,* a genus of flowering plants in the family *Theaceae*. The leaves have been used in traditional Chinese medicine and other medical systems to treat asthma (functioning as a bronchodilator), angina pectoris, peripheral vascular disease and coronary artery disease. Tea extracts have become a field of interest, due to their notional antibacterial activity. The preservation of processed organic food and the treatment of persistent bacterial infections are particularly being investigated. Green tea leaves and extracts have shown to be effective against bacteria responsible for halitosis (Ming, 1992; Golender, 2003). Our results showed that *Camellia sinensis* at a concentration of around 40 µg/ml (40.88 ± 1.53) inhibited HeLa cell survival by 50% compared with a vehicle-treated control. Photometric determination of phenols by showed the presence of 24% phenols in the extract of *Camellia sinensis*.

Frangula alnus (Rhamnus frangula) is a tall deciduous shrub in the family *Rhamnaceae*. Bark for medicinal use is dried and stored for a year before use, as fresh bark is violently purgative; even dried bark can be dangerous if taken in excess (Rushforth, 1999). There are no data on the antiproliferative effects of *Frangula alnus*. According to the results of this study, *Frangula alnus* extract (A) in a concentration of around 148 µg/ml (148.05 ± 8.61) and extract (B) in concentration of more than 200 µg/ml inhibit HeLa cell survival by 50 % compared with a vehicle-treated control. Photometric determination of glucofrangulin by spectrophotometry showed that percent is 18.80% in the extract of *Frangula alnus* (A) and 14.65 % in the extract of *Frangula alnus* (B). The concentration of glucofrangulin is higher in *Frangula alnus* (A) (18.80 %) than in *Frangula alnus* (B) (14.65 %), which is in correlation with their antiproliferative effects.

Rosmarinus officinalis (rosemary) is a member of the mint family *Lamiaceae*. Rosemary may have some anticarcinogenic properties. A study where a powdered form of rosemary was given to rats in a measured amount for two weeks showed a reduc-

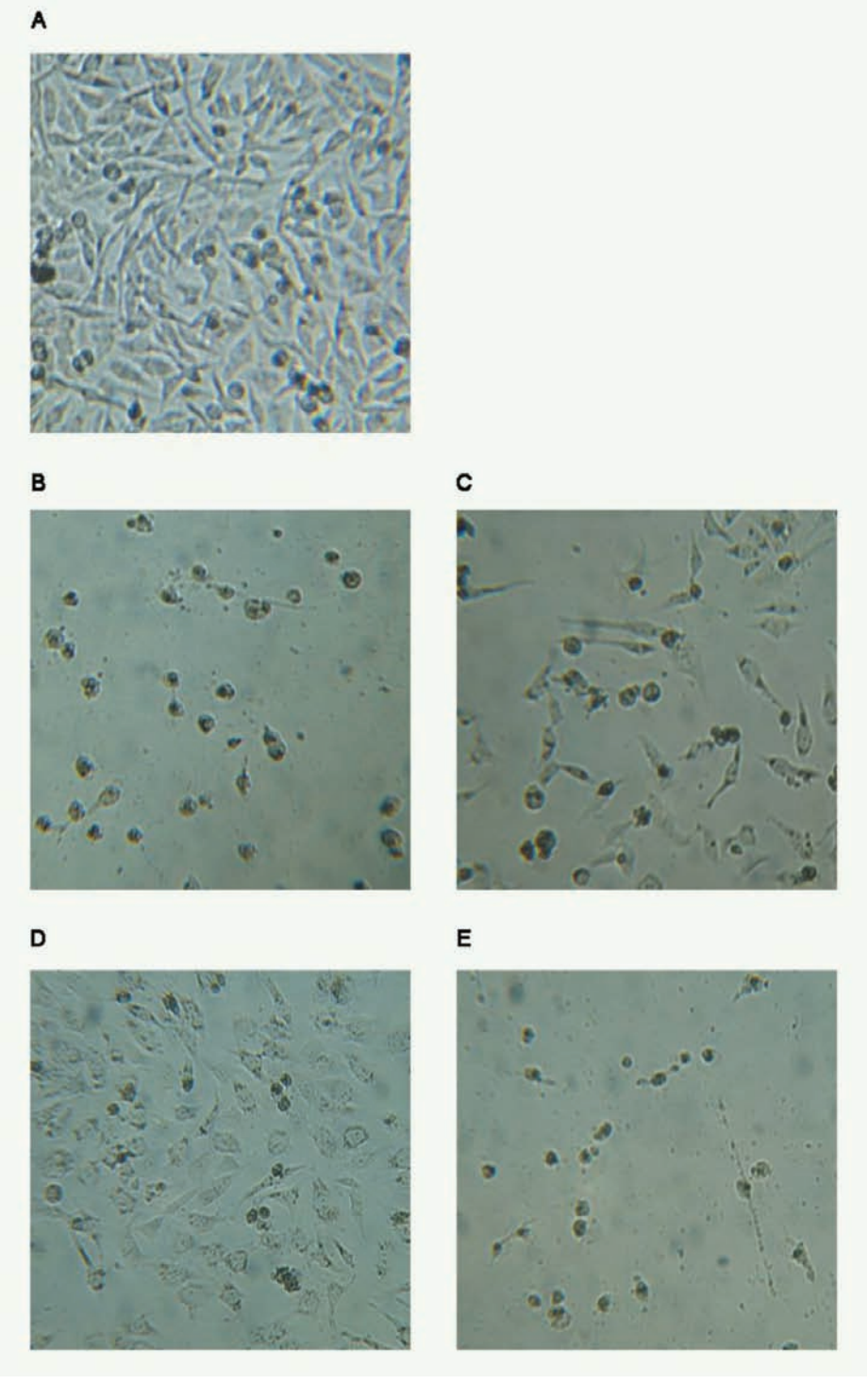

Fig. 2. Light microscopy of HeLa cells cultured with or without the 200 μg/ml of extracts: A) control; B) *Camellia sinensis;* C) *Frangula alnus* (A); D) *Frangula alnus* (B) and E) *Rosmarinus officinalis* as described in Materials and Methods and photographed 72 h after addition of drugs (Magnification 12.5X, 1.6X, 6.3/0.2).

Table 1. Concentrations of extracts that induced a 50% decrease in HeLa cell survival

Extracts	HeLa IC_{50}* (μg/ml)
Camellia sinensis	40.88 ± 1.53
Frangula alnus (A)	148.05 ± 8.61
Frangula alnus (B)	> 200
Rosmarinus officinalis	80.26 ± 1.06

Note: *IC_{50} values were obtained from the filtered extract suspensions, as described in the Materials and Methods. IC_{50} values were expressed as the mean ± SD determined from the results of the MTT assay in three independent experiments.

tion in the binding of a certain carcinogen by 76 %, and it greatly reduced the formation of mammary tumors (Teuscher, 2005). The results of a study suggest that carnosic acid, found in rosemary, may protect the brain from free radicals, lowering the risk of strokes and neurodegenerative diseases, and is anti-inflammatory (Mengoni et al., 2011). Carnosol is also a promising cancer chemoprevention and anticancer agent (Johnson, 2011). The results of this study showed that *Rosmarinus officinalis* in a concentration of around 80 μg/ml (80.26 ± 1.06) inhibits HeLa cell survival by 50% compared with a vehicle-treated control. Rosemary contains a number of potentially biologically active compounds, including antioxidants carnosic acid and rosmarinic acid. Other bioactive compounds include camphor (up to 20% in dry rosemary leaves), caffeic acid, ursolic acid, betulinic acid, rosmaridiphenol and rosmanol. Rosemary antioxidant levels are closely related to soil moisture content (National Non-Food Crops Centre, 2006).

Medicinal plants maintain the health and vitality of individuals, and have the potential to improve various diseases, including cancer. This study investigates the antiproliferative effects of extracts of *Camellia sinensis, Frangula alnus* (A – from Bosnia and B – from Serbia) and *Rosmarinus officinalis* on HeLa cells. Obtained results show that the best antiproliferative properties are those of *Camellia sinensis*, then *Rosmarinus officinalis*, and lastly *Frangula alnus*. Depending on the geographic origin, *Frangula alnus* from Bosnia possessed better antiproliferative effects than *Frangula alnus* from Serbia.

An important goal of our future studies will be to investigate the antiproliferative effects and anticancer properties of other medicinal plants and royal jelly which is used in Serbian folk medicine for different purposes.

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REFERENCES

Agrawala, S.K., Chatterjes, S., and *S.K. Misra* (2001). Immunepotentiation activity of a polyherbal formulation "IMMU-21". *Phytomedica*. **2**, 1-22.

Akerele, O. (1988). Medicinal plants and primary health care: an agenda for action. *Fitoterapia*. **59**, 355-363.

Dikshit, A., Shahi, S.K., Pandey, K.P., Patra, M. and *A.C. Shukla* (2004). Aromatic plants a source of natural chemotherapeutants. *Nat. Acad. Sci. Letters*. **27**, 145-164.

Fidler, L. and *M. Kripke* (1977). "L Jand" *Science*, **197**, 893-895.

Golender, L. (2003). History of Tea, *Botanics.* http://www.go127.com/HistoryTeaBotanics.html.

Hansel, R. and *O. Sticher* (2002). *Pharmakognosie, Phytopharmazie, Springer-Verlag, Heidelberg, Germany*, 58-65.

Johnson, J.J. (2011). Carnosol: A promising anti-cancer and anti-inflammatory agent. *Cancer Letters*. **305**, 1-7.

Kligerman, M.M. (1973). Cancer Medicine, ed by Holland J.F., Frei, E III, Lea & Febiger, Philadelphia. 541-565.

Medical Economics (1998). Physicians Desk Reference, 52nd ed., Medical Economics Co, Montvale, 1875-1889.

Mengoni, E.S., Vichera, G., Rigano, L.A., Rodriguez-Puebla, M.L., Galliano, S.R., Cafferata, E.E., Privetta, O.H., Moreno, S. and *A.A. Vojnov* (2011). Suppression of COX-2, IL-1β and

TNF-α expression and leukocyte infiltration in inflamed skin by bioactive compounds from *Rosmarinus officinalis* L. *Fitoterapia*. **82**, 414-421.

Ming, T.L. (1992). A revision of Camellia sect. Thea. *Acta Botanica Yunnanica*. **14**, 115-132.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*. **65**, 55-63.

National Non-Food Crops Centre. (2006). NNFCC Project Factsheet; Assessment and Development of the Supply Chain to Deliver Rosemary Antioxidants to the Food and Pharmaceutical Industries (Defra), NF0609.

http://www.nnfcc.co.uk/publications/nnfcc-project-factsheet-assessment-and-development-of-the-supply-chain-to-deliver-rosemary-antioxidants-to-the-food-and-pharmaceutical-industries-defra-nf0609.

Ohno, M. and *T. Abe* (1991). Rapid colorimetric assay for the quantification of leukemia inhibitory factor (LIF) and interleukin-6 (IL-6). *J. Immunol. Methods*. **145**, 199-203.

Orsolic, N. and *I. Basic* (2003). Immunomodulation by water-soluble derivative of propolis (WSDP) a factor of antitumor reactivity. *J. Ethnopharmacol*. **84**, 265-273.

Pan, M.H. and *C.T Ho* (2008). Chemopreventive effects of natural dietary compounds on cancer development. *Chem. Soc. Rev*. **37**, 2558-2574.

Pandey, G. and *S. Madhuri* (2006). Medicinal plants: better remedy for neoplasm. *Indian Drugs*. **43**, 869-874.

Rushforth, K. (1999). Trees of Britain and Europe. *Collins Wildlife Trust Guides*. 175-176.

Teuscher, E. (2005). *Medicinal Spices (I ed.). Medpharm, Stuttgart*. 243-252.

Waladkhani, A.R. and *M.R. Clemens* (1998). Effects of dietary phytochemicals on cancer development. *Int. J. Mol. Med*. **1**, 747-753.

Weiss, R.F. (1988). *Herbal Medicine; Arcanum, A.B., Ed.; Beaconsfield Publishers, Beaconsfield, U.K*. 22-28.

World Health Organization (1998). "World Health Statistics Annual 1996", *World Health Organization, Geneva*. 116-121.