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ANTIOXIDANT AND ANTICANCER PROPERTIES OF LEAVES AND SEED CONES FROM EUROPEAN YEW (*TAXUS BACCATA L.*)

Milena G. Milutinović[1,*] Milan S. Stanković[1], Danijela M. Cvetković[1], Marina D. Topuzović[1], Vladimir B. Mihailović[2] and Snežana D. Marković[1]

[1] *Department for Biology and Ecology, Faculty of Science, University of Kragujevac,* Radoja Domanovića No. 12, 34000 Kragujevac, Republic of Serbia

[2] *Department for Chemistry , Faculty of Science, University of Kragujevac,* Radoja Domanovića No. 12, 34000 Kragujevac, Republic of Serbia

*__Corresponding author:__ milenacurcic@kg.ac.rs; milenagen@gmail.com

Abstract: Plant extracts of the leaves and seed cones of European yew *Taxus baccata* L. (Taxaceae) were analyzed for total phenolic content, flavonoid concentrations, antioxidant and anticancer properties (cytotoxic and proapoptotic activity). The total phenolic content ranged between 8.23 and 210.01 mg Ga/g, with the IC_{50} values for antioxidant activity between 25.24 and 533.66 μg/ml. The MTT test showed that the methanolic extract of leaves had better activity on HCT-116 cells than the extract of seed cones, with IC_{50} values of 14.3 for 24 h and 4.59 for 72 h. The MDA-MB-231 cell line displayed significantly lower sensitivity to both extracts as compared to the HCT-116 cell line. Microscopic examination indicated that the extracts induced apoptosis in both cell lines. These results suggest that *T. baccata* leaves and seed cones are a potential source of phenolic compounds, especially flavonoids, as natural antioxidant, cytotoxic and strong proapoptotic substances of high value.

Key words: antioxidant activity; apoptosis; cytotoxity; *Taxus baccata* L.

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INTRODUCTION

European yew (*Taxus baccata* L.) is a species of conifers that belongs to the family Taxaceae. It is a evergreen tree or shrub up to 15 m in height. The leaves are lanceolate, up to 30 mm long and 2-3 mm broad. Female individuals of this species have seed cones containing one seed, surrounded by a fleshy shell (arillus), that assume the form of berries. All parts of the yew are poisonous, except the arillus. It inhabits forests in Europe, southwest Asia and northwest Africa (Jovanović, 1970).

Very active substances in the above ground parts of *T. baccata* are taxine alkaloids. These compounds are found in all plant parts except the arillus, and are highly toxic to mammalian,

and especially human, organisms (Wenker et al., 1998). For this reason, *T. baccata* is a little-known plant in traditional medicine. However, due to other active substances with very effective physiological activities, this plant is well known in pharmacy and medicine. The active compounds in plants of the genus *Taxus*, such as lignans, steroids and flavonoids and other phenolic components, possess antibacterial and antifungal activity (Erdemoglu et al., 2004; Krauze-Baranowska and Wiwart, 2004), enzyme inhibitory and antioxidant activity (Kucukboyaci et al., 2010), antiulcerogenic (Gurbuz et al., 2004), and anticancer activity (Sadeghi-aliabadi et al., 2003; Emami et al., 2005; Kucukboyaci and Sener, 2010).

An anticancer substance isolated from the *Taxus* species is Taxol® (Paclitaxel) was discovered in the bark of Pacific yew (*Taxus brevifolia* Peattie). The mechanism of anticancer activity is based on the suppression of mitosis by facilitating the stability of microtubules and frequency of premature centromere division (Bajić et al., 2010). It is commonly used in chemotherapy for lung, ovarian and breast cancer (Sadeghi-aliabadi et al., 2009).

Data with regard to the phenol concentration and flavonoid content, antioxidant activity of seed cones and leaves from *T. baccata* is incomplete. Thus, we determined the phenolic content and concentrations of flavonoids in leaves and seed cones in different solvents (water, methanol, acetone, ethyl acetate and petroleum ether from), and evaluated the *in vitro* cytotoxic activity of methanolic extracts on human colon and breast cancer cells, cell lines HCT-116 and MDA-MB-231, respectively. As a common therapeutic strategy for cancer is induction of apoptosis (Oltvai et al., 1993), we examined whether the methanolic extract induced apoptosis in these cell lines.

MATERIALS AND METHODS

Chemicals

Acetone, methanol, petroleum ether, ethyl acetate and sodium hydrogen carbonate were purchased from "Zorka pharma" Šabac, Serbia. Standards of phenolic acids (gallic acid) and flavonoids (rutin hydrate), chlorogenic acid and 2,2-dyphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemicals Co., St Louis, MO, USA. The Folin-Ciocalteu's phenol reagent, 3-tert-butyl-4-hydroxyanisole (BHA) and aluminum chloride ($AlCl_3$) were from Fluka Chemie AG, Buchs, Switzerland. Dulbecco's Modified Eagle Medium (DMEM) was obtained from GIPCO, Invitrogen, USA. Fetal bovine serum (FBS) and trypsin-EDTA were from PAA (The Cell Culture

Table 1. Total phenol contents in the plant extracts, expressed as gallic acid equivalent (mg Ga/g extract) and flavonoid concentrations, expressed as rutin equivalent (mg Ru/g extract).

	leaves		seed cones	
	total phenol content	**flavonoid concentration**	**total phenol content**	**flavonoid concentration**
Water	33.38±1.12	5.88±0.14	8.23±0.91	3.96±0.82
Methanol	92.13±0.84	161.98±1.02	13.92±1.24	24.08±1.09
Acetone	170.37±1.32	64.26±0.95	30.49±1.71	26.35±1.17
Ethyl acetate	210.01±2.24	81.89±1.21	51.10±1.64	39.37±1.02
Petroleum ether	58.16±1.75	40.66±1.66	25.80±0.78	23.72±0.91

Each value in the table was obtained by calculating the average of three analyses ± standard deviation.

Company), Austria. Acridine orange (AO) was obtained from Acros Organic, New Jersey, USA. Dimethyl sulfoxide (DMSO), ethidium bromide (EB) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from SERVA, Germany. All other solvents and chemicals were of analytical grade.

Plant material

In September 2010, fresh leaves and mature cones of individual *T. baccata* female species were collected from a tree in the Dendrarium Park of the Department for Biology and Ecology, Kragujevac, Serbia. The voucher specimens of *T. baccata* L. were confirmed and deposited in the Herbarium at the Faculty of Science, University of Kragujevac. The collected leaves were air-dried in darkness at room temperature (20°C). Harvested fresh seed cones are immediately used to prepare plant extracts.

Preparation of plant extracts

The prepared plant material from *T. baccata* (10 g) was broken into small pieces (2-6 mm) using a cylindrical crusher, and extracted with water and methanol, acetone, ethyl acetate and petroleum ether. The obtained solution was filtered through Whatman, No. 1 filter paper and evaporated. The obtained extracts were stored in sterile dark glass bottles and kept in a refrigerator at + 4°C for further processing.

Determination of total phenol content and flavonoid concentrations

The phenolic content of the extracts was determined spectrophotometrically, using Folin-Ciocalteu reagent (Singleton et al., 1999). Briefly, 0.5 ml of methanolic extract solution (1 mg/ml) was added to 2.5 ml of 1:10 Folin-Ciocalteu reagent and then 2 ml of sodium carbonate (75 g/l) were added. After 15 min of incubation at 45°C, the absorbance at 765 nm was measured. The total phenolic content of the plant extract was expressed in gallic acid equivalents (GaE)/g.

The total flavonoid concentration was evaluated using aluminum chloride (Quettier et al., 2000). Samples for determination were prepared by mixing 1 ml of methanolic solution (1 mg/ml) of extract and 1 ml of aluminum chloride (20 g/l). After 1 h of incubation at room temperature, the absorbance at 415 nm was measured. The flavonoid content of the plant extract was expressed as the rutin equivalent (RuE)/g of extract (dry weight).

Evaluation of antioxidant activity

The ability of the plant extract to scavenge DPPH free radicals was assessed by the standard method (Tekao et al., 1994), suitably modified (Kumarasamy et al., 2007). DPPH (20 mg) was dissolved in methanol (250 ml) to obtain a concentration

Table 2. DPPH scavenging activity of the investigated plant extracts, presented as % of inhibition and IC_{50} values (μg/ml).

	leaves		seed cones	
	%inhibition	**IC_{50}**	**% inhibition**	**IC_{50}**
Water	66.92±1.92	533.66±9.55	38.83±1.95	> 1000
Methanol	95.59±1.74	105.41±3.12	88.83±1.41	518.51±3.19
Acetone	95.78±2.33	25.24±1.27	92.69±1.84	81.43±1.98
Ethyl acetate	95.03±1.44	29.84±1.15	83.99±0.80	180.26±1.25
Petroleum ether	78.01±1.62	438.92±4.94	36.31±1.48	> 1000

Each value in the table was obtained by calculating the average of three analyses ± standard deviation. The percentage inhibition is for solutions at 1 mg/ml concentration.

of 80 μg/ml. The stock solution of plant extract was prepared in methanol to achieve the concentration of 1 mg/ml. Dilutions were made to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99, 0.97 μg/ml. Diluted solutions (1 ml each) were mixed with DPPH (1 ml). After 30 min in darkness at room temperature (23°C), the absorbance was recorded at 517 nm. The control samples contained all the reagents except the extract. The percentage inhibition was calculated using the equation: % inhibition = 100 x (A *control* – A *sample*)/A *control*), whilst IC_{50} values were estimated from the % inhibition versus concentration sigmoidal curve, using a non-linear regression analysis. The data were presented as mean values ± standard deviation (n = 3).

Cell preparation and culturing

HCT-116 human colon cancer and MDA-MB-231 human breast cancer cell lines were obtained from the American Type Culture Collection. Cells were maintained in DMEM supplemented by 10% FBS, with 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. Cells were grown in 75 cm^2 culture bottles supplied with 15 ml DMEM, and after a few passages, were seeded in a 96-well plate. All studies were done with cells at 70 to 80% confluence.

Cell viability assay (MTT assay)

HCT-116 and MDA-MB-231 cells was seeded in a 96-well plate (10 000 cells per well). After 24 h of cell incubation, the medium was replaced with 100 μl medium containing various doses of methanolic extracts in different concentrations (1-1000 μg/ml) for 24 and 72 h. Untreated cells served as a control. After 24 and 72 h of treatment, the cell viability was determined by MTT assay (Mosmann, 1983). The proliferation test is based on the color reaction of mitochondrial dehydrogenase from living cells with MTT. At the end of the treatment period, MTT (final concentration 5 mg/ml PBS) was added to each well, which was then incubated at 37°C in 5% CO_2 for 2-4 h. The colored crystals of produced formazan were dissolved in 150 μl DMSO. The absorbance was measured at 570 nm on a microplate reader. Cell proliferation was calculated as a ratio of the absorbance of the treated group divided by the absorbance of the control group, multiplied by 100 to give percentage proliferation.

Table 3. Values of antioxidant (DPPH scavenging) activity of standard substances obtained for comparison with values of extracts from *T. baccata.*

substances	% inhibition	IC_{50} μg/ml
BHA	93.37±2.5	5.39±0.31
rutin	93.71±1.1	9.28±0.27
chlorogenic acid	96.60±1.8	11.65±0.52

Average values of three analyses ± standard deviation are presented. Percentage inhibition is for solutions at 1 mg/ml concentration.

Fluorescence microscope analysis of cell death

Acridine orange/ethidium bromide (AO/EB) double staining assay was used for the determination of type of cell death (Baskić et al., 2006) by mixing 200 μl of the dye mixture (100 μl/mg AO and 100 μl/mg EB in distilled water with 2 ml cell suspensions (30 000 cells/ml) in a 6-well plate. The suspension was immediately examined and viewed under Nikon inverted fluorescent microscope (Ti-eclipse) at 400x magnification. A minimum of 300 cells was counted in every sample.

Statistical analysis

The data are expressed as the means ± standard errors (SE) from three individual experiments,

performed in triplicate for each dose. Statistical significance was determined using the Student's t-test. A p value <0.05 was considered as significant. The magnitude of correlation between variables was done using a SPSS (Chicago, IL) statistical software package (SPSS for Windows, ver. 17, 2008). The IC_{50} values were calculated from the dose curves by a computer program (CalcuSyn).

RESULTS

Total phenol content and flavonoid concentrations

The concentrations of phenols and flavonoids observed in the tested extracts are shown in Table 1. Concentrations of phenols in the different extracts of *T. baccata* leaves ranged from 33.38 to 210.01 mg/g, and in seed cones from 8.23 to 51.10 mg/g. The concentrations of flavonoids in extracts of *T. baccata* leaves ranged from 5.88 to 161.98 mg/g, and in seed cones from 3.96 to 39.37 mg/g.

Antioxidant activity

The antioxidant activity of different extracts from the leaves and seed cones of *T. baccata* is expressed as the percentage of inhibition and in terms of IC_{50} (μg/ml) values (Table 2). In the presented results, a lower IC_{50} value indicates higher antioxidant activity. The percentage of inhibition ranged from 66.92 to 95.78% for the leaf extracts and from 36.31 to 92.69% for seed cone extracts. The highest activity in percentages was that of acetone, methanolic and ethyl acetate extracts of leaves as well as seed cones. The IC_{50} values for the antioxidant activity of the leaf extracts ranged from 25.24 to 533.66 μg/ml, and from 81.43 to over the 1000 μg/ml for the seed cone extract. Parallel to the examination of the antioxidant activity of the extracts from the leaves and seed cones of *T. baccata*, the values for BHA (93.37%; 5.39 μg/ml), rutin (93.71%; 9.28 μg/ml) and chlorogenic acid (96.60%; 11.65 μg/ml) as standard compounds (Table 3) are presented.

Cytotoxic activity

Since the methanolic extract of leaves displayed the highest flavonoid content, it was used for

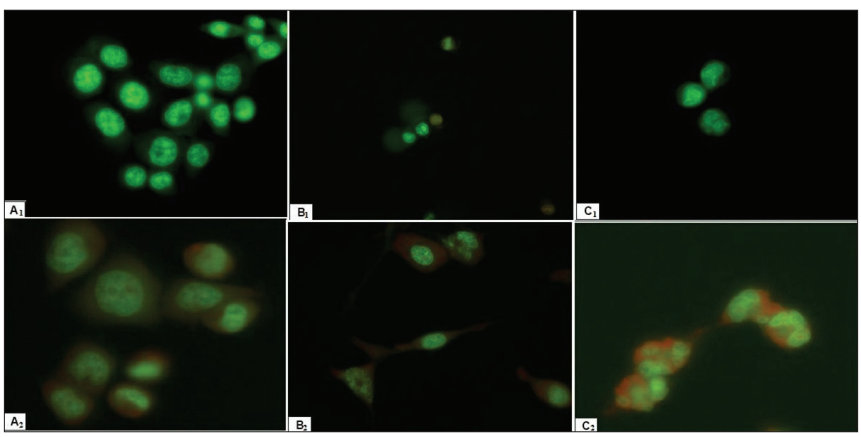

Fig. 1. Typical morphological changes of HCT-116 and MDA-MB-231 cells induced by methanolic extracts (250 μg/ml) of leaves (B_1 - HCT-116 cells: B_2 - MDA-MB-231 cells) and seed cones (C_1 - HCT-116 cells: C_2 - MDA-MB-231 cells) of *T. baccata*, after 24 h of exposure. Cells were stained with AO/EB. Untreated cells served as control cells (A_1 - HCT-116 cells: A_2 - MDA-MB-231 cells). The images were taken using fluorescence microscopy at 400× magnification.

further cytotoxicity tests. The cytotoxic activity of the methanolic extracts of *T. baccata* leaves and seed cones was analyzed on HCT-116 and MDA-MB-231 cell lines, using an MTT viability assay (Table 4). The effect of both extracts was expressed by IC_{50} (inhibitory dose that inhibits cells growth for 50%). The *T. baccata* leaf extract was cytotoxic on HCT-116 cells with IC_{50} values lower than 30 μg/ml, which was considered good cytotoxic activity for crude extracts (Suffness and Pezzuto, 1990), while the extract of seed cones showed weaker effects. The extracts did not produce significant cytotoxic effects on the MDA-MB-231 cell line.

Fluorescence microscope analysis of cell death

In order to determine whether the methanolic extracts induced apoptosis, the AO/EB method was performed. HCT-116 and MDA-MB-231 cells treated with 250 μg/ml (the concentration that induced cytotoxic effects on both cell lines) methanolic extracts of leaves and seed cones of *T. baccata* were stained with AO/EB and analyzed under a fluorescence microscope for the percentages of viable, early and late apoptotic and necrotic cells. Untreated cells are represented as the control (Fig. 1). The results obtained with AO/EB double staining are presented in Fig. 2. Compared with the spontaneous apoptosis observed in the HCT-116 control cells (early apoptotic 3.19%, 0% late apoptotic and 0% necrotic cells), treatment with the methanolic extract of leaves increased the percentages of early apoptotic cells (66.55%), late apoptotic cells (21.84%) and total number of apoptotic cells (88.90%), and necrotic cells to a lesser extent (2.39%). The methanolic extracts of seed cones induced increased percentages in early apoptotic (53.85%), slightly increased percentages in late apoptotic cells (0.43%) and an increase in the total number of apoptotic cells (54.27%).

Compared with the spontaneous apoptosis observed in the MDA-MB-231 control cells (early apoptotic 1.47%, 0.37% late apoptotic and 0% necrotic cells), treatment with the methanolic extract of leaves increased the percentages of early apoptotic cells (43.59%). The methanolic extract of seed cones increased the percentages of early apoptotic cells (35.8%), slightly increased those of late apoptotic cells (6.05%), increased the total number of apoptotic cells (41.85%) and slightly increased the percentages of necrotic cells (2.42%).

DISCUSSION

Phenols are a very important plant constituent because of their ability to scavenge free radicals, and the phenolic content of plants may contribute directly to their antioxidant action (Tosun et al., 2009; Atolani et al., 2011; Stanković et al., 2011; Radojević et al., 2012). In ethyl acetate and

Table 4: Cytotoxic effects - IC_{50} values (μg/ml) of methanolic extracts on HCT-116 and MDA-MB-231 cell lines after 24 and 72 h exposure.

	IC_{50} μg/ml			
	after 24 h		after 72 h	
***T. baccata* extracts**	**HCT-116**	**MDA-MB-231**	**HCT-116**	**MDA-MB-231**
leaves	14.4±4.5	356.47±5.7	4.59±1.5	246.87±1.68
seed cones	49.69±7.6	> 1000	133.53±0.35	604.25±8.41

The averages of three analyses ± standard deviation are presented.

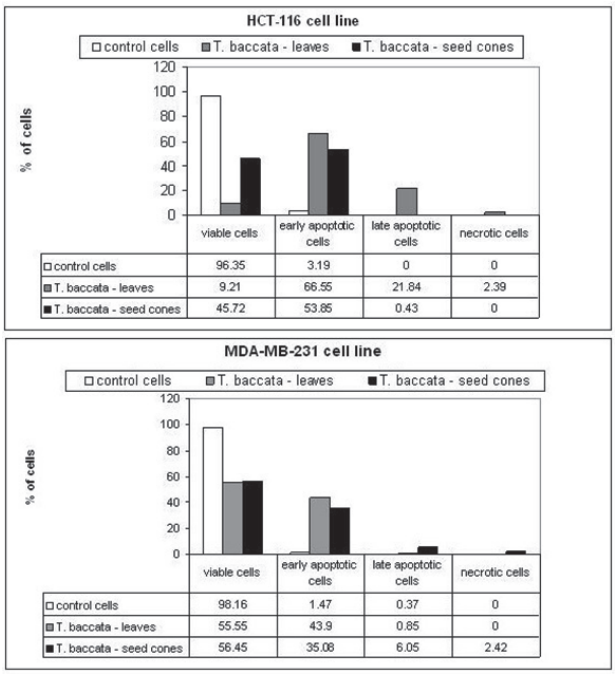

Fig. 2. Viable, apoptotic and necrotic HCT-116 and MDA-MB-231 cells as percentages of total cell number, measured by AO/EB fluorescence staining after treatment with 250 μg/ml methanolic extracts, 24 h after the treatments.

acetone extracts from leaves, very high concentrations of total phenolic compounds were measured. In the comparison of the results for leaves and seed cone extracts of *T. baccata*, it was observed that the investigated plant parts have phenolic compounds but the leaves contain very high concentrations of these compounds. We also observed that the highest concentration of phenolic compounds in the extracts was obtained using solvents of moderate polarity. In previous studies, Emami et al. (2007) confirmed the low concentration of phenolic compounds in methanolic extracts from seed cones. The highest concentration of flavonoids in the leaves was measured in the methanolic extract (161.98 mg/g), while a water extract had small amounts of flavonoids. It has been reported that the methanolic extract manifested a greater power of extraction for flavonoids from the leaves of *T. baccata*. In measuring the total phenolic content and flavonoid concentrations in the leaf and seed cone extracts, acetone and ethyl acetate extracts displayed the highest values of all the extracts.

Our data confirm the results of other researchers (Krauze-Baranowska, 2004) who analyzed the qualitative properties of flavonoids in leaf extracts and showed a number of different flavonoids in the methanolic extract. The highest concentration of flavonoids was measured in the ethyl acetate extract of seed cones (39.37 mg/g). Compared to the values obtained for the leaf extracts, seed cone extracts had a lower concentration of flavonoids.

The highest neutralization of the DPPH radical was produced by the acetone, methanolic and ethyl acetate extracts, while petroleum ether and water extracts produced lower inhibition. The greatest capacity to neutralize DPPH radicals was measured in the acetone and ethyl acetate extracts of the leaf, which neutralized 50% of free radicals at comparatively small concentrations, 25.24 and 29.84 μg/ml, respectively.

In comparing the values of flavonoid concentration and antioxidant activity of the extracts from the leaves, a deviation was observed. The methanolic extract of leaves contained a high concentration of flavonoids, but had lower antioxidant activity than the acetone and ethyl acetate extracts. These data indicate that the acetone and ethyl acetate extracts, apart from flavonoids, contain different phenolic compounds and not only the content but also the chemical properties of the flavonoids contribute to the antioxidant activity.

In the literature, there are very little data on the antioxidant activity of *T. baccata* leaf and seed cone extracts. Most data describe the antioxidant and other biological activities of individual components derived from *T. baccata*

(Erdemoglu et al., 2004; Kucukboyaci et al., 2010). Authors who have examined antioxidant activity have generally only prepared extracts using polar solvents (Serteser et al., 2009). In our analysis of the leaves and seed cones of *T. baccata*, we tested the extracts obtained using five solvents of different polarity. The results indicate that the use of moderately polar solvents allows a very effective extraction of phenolic compounds, and a high antioxidant activity in the obtained *T. baccata* extracts. This is indicated by the fact that the ethyl acetate and acetone extracts had a high value of phenolic components and the greatest ability of DPPH radical inhibition. Although acetone and ethyl acetate extracts, apart from flavonoids, contain different phenolic compounds, our data suggest that besides content, the chemical properties of flavonoids contribute to the antioxidant activity of the extracts. In comparing the percentages and IC_{50} values of antioxidant activity with the values for standard substances analyzed in our studies (BHA, rutin and chlorogenic acid), the acetone and ethyl acetate extracts from the leaves and acetone extract from the seed cones of *T. baccata* showed the strongest capacity for neutralization of DPPH radicals.

Due to the highest flavonoid concentration (161.98 mg/g) in the leaves extract from *T. baccata*, methanolic extracts were used for the investigation of cytotoxic activity. There is a correlation between a reduced risk of cancer and the consumption of vegetables and parts of plants rich in flavonoids (Ferguson et al., 2004; Park et al., 2008). Our data showed that the methanolic extract of seed cones, which has lower flavonoid concentrations, also has lower cytotoxic effect than the flavonoid-rich extract of leaves.

The different cytotoxic effects of methanolic extracts from different parts of the same plant (leaves and seed cones) are due to their chemical composition and relative content of biologically active substances. Considering the values of IC_{50}, the methanolic extract of the leaves has a better cytotoxic effect than that of seed cones. According to the American National Cancer Institute (NCI), the criteria for good cytotoxic activity for crude extracts are values lower than 30 μg/ml (Suffness and Pezzuto, 1990). The methanolic extract of *T. baccata* leaves, which exhibited the highest cytotoxic potential on HCT-116 cells with an IC_{50} of 14.43 and 4.59 μg/ml are in line with NCI criteria. The finding from this study showed that the extracts were more potent on the HCT-116 than on MDA-MB-231 cell line. The type and origin of the investigated cells affect their different sensitivity to treatments. These cell lines are from different organs and malignant origin (primary origin as a HCT-116 or metastasis as a MDA-MB-231) (Kaczmarek et al., 2008).

Many chemotherapeutic drugs and folk medicinal plants exert their anticancer effect by inducing cell apoptosis (Lai et al., 2008; Yan-Wei et al., 2009). Treated HCT-116 and MDA-MB-231 cells showed morphological changes such as reduction in size and cell volume, cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies as compared to untreated control cells, indicating that the methanolic extracts induced apoptosis. Our results support previous studies, which showed that flavonoids are responsible for the induction of apoptosis in cancer cells (Park et al., 2008).

CONCLUSIONS

T. baccata can be considered a rich natural source of polyphenolic compounds with very good anticancer properties and strong antioxidant activity. The antioxidant and anticancer activities of

extracts from *T. baccata* depend on the plant part and solvent used for extraction, as well as on the chemical properties of the active substances present in the extracts. Our data show that the methanolic extract of leaves produces a better cytotoxic effect than the methanolic extract of seed cones, and that the HCT-116 cell line is more sensitive. Treatment with the methanolic extracts of leaves and seed cones induced apoptosis in both HCT-116 and MDA-MB-231 cell lines.

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