IJPSR (2012), Vol. 3, Issue 12 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 24 August, 2012; received in revised form 28 September, 2012; accepted 28 November, 2012

YERBA MATÉ (*ILEX PARAGUARIENSIS*) EFFECT ON HUMAN COLON NORMAL AND TUMOR CELLS

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Keywords:
Yerba maté extracts, human colon epithelial cells, human colon cancer cells, cytotoxicity, reactive oxygen species scavenging effect, IL-6

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QUICK RESPONSE CODE

IJPSR:
ICV (2011)- 5.07

Website:
www.ijpsr.com

ABSTRACT

Background: Yerba mate (*Ilex paraguariensis*) is known to possess many biological activities against diverse tissues among others alimentary tract wall.

Aim: The aims of the study were to evaluate potential toxic effects of water and ethanol (by Soxhlet extraction and shaking extraction methods) and free radical scavenging properties of Yerba mate extracts on human normal colonic epithelial and human colon carcinoma cells.

Materials and methods: NR uptake, MTT, ($DPPH^{\cdot}$) reduction and ELISA tests were used to test toxicity, anti-proliferative activity, ROS reduction and cytokine level, respectively.

Results: Depending on kind of extract, their concentration, kind of cells they influenced on and method of analysis, different toxic and stimulatory of cells viability effects were observed. Yerba mate extracts demonstrated strong free radical scavenging activity. The plant extracts also expressed immunomodulatory effects influencing on IL-6 production by normal and tumor cells. *Ilex paraguariensis* extracts stimulated normal colonic cells for the cytokine production while limited such production by human colon tumor ones.

Conclusion: Concluding, Yerba mate water and ethanolic extracts possess strong pharmacological activities against normal colonic epithelium and colon derived tumor cells. This activity may be used in health promoting endeavors and could be defined as chemopreventive factors but only with special care and after additional, thorough studies.

INTRODUCTION: Yerba mate (*Ilex paraguariensis* St. Hill. Aquifoliaceae) is one of the most popular consuming plant in South America. Traditional usage of mate is in form of beverages but in the last decade it begun to be served as candies, beers, energy drinks or creams [1].

According to the folk medicine, Yerba mate consumer goods possess many activities which strongly influence human health. They are used as stimulants against physical and mental weakness (central nervous system stimulant), and have been shown to act as hepatoprotective, choleretic, diuretic, hypocholesterolemic, anti-rheumatic, antithrombotic, anti-inflammatory, antiobesity or cardioprotective agents [1, 2]. Moreover, it has been widely used as a cancer inhibitory and antimutagenic factors acting against many carcinomas among others gastrointestinal tract pathologies [3].

Its beneficial effects are mainly attributed to its high polyphenol content. The mate is a rich source of tannins or phenylpropanoid compounds like caffeoyl derivatives and flavonoids but also xanthines, vitamins, essential oils, purine alkaloids and triterpenoid saponins [2-5]. Owning to Yerba mate biologically active components, this plant is reported to possess strong antioxidant features well as DNA-protective properties [5].

Therefore as active components of mate, after eaten, are in direct contact with alimentary tract wall, it may be considered as a potential both pro-health and anti-tumor factor in human colon. On the other hand, cytotoxic activity of plant extracts and balance between reactive oxygen species (ROS) and antioxidants are strongly important to retain overall health of the organism [5].

On account of different composition of plant extracts which depend not only on solvent used but also on extraction method and lack of effects comparison on normal and tumor cells we analyzed potential toxic effects of water and ethanol (by Soxhlet extraction and shaking extraction methods) and free radical scavenging properties of Yerba mate extracts on human normal colonic epithelial and human colon carcinoma cells.

MATERIALS AND METHODS:

Plant material: Yerba mate Rosamonte Especial dried leaves and cut twigs (*Ilex paraguariensis*) from Argentina was purchased from yerbamatestore.pl. Commercial herb samples were packed within polyethylene bags with expire time about 4 years.

Preparation of Yerba mate extracts for experiments: Water extract was prepared in a Department of Biochemistry, Maria Curie-Skłodowska University in Lublin. Ethanol extracts were prepared in a Department of Virology and Immunology, Maria Curie-Skłodowska University in Lublin.

1. **Water extract:** 30 g of dry Yerba mate leaves were placed in 300 ml of hot (98°C) distilled water for 30 minutes. The infusion was cool down, filtrated through filter paper (Whatman No. 2) and lyophilised.

2. **Ethanol extracts:**

   a. **Shaking extraction:** 20 g of dried leaves of Yerba mate was added to 200 ml of ethanol in flat bottom flask. The vessels were fixed in horizontal shaker and mixed with a speed of 140 c.p.m. for 72 hours in a room temperature. Extracts were then drained off under reduced pressure on Büchner funnel and rinsed with 300 ml of ethanol. Alcohol was vaporized using rotary evaporator.

   b. **Soxhlet extraction**: 15 g of dried leaves of Yerba mate was loaded into a filter paper and fixed in the main chamber of the Soxhlet extractor. To the round-bottomed flask 150 ml of ethanol was added and installed in Soxhlet extractor equipped with a condenser. Extraction was performed 7-times, 5 hours each. Alcohol was vaporized using rotary evaporator.

Dry mass of Yerba mate was dissolved in dimethyl sulfoxide (DMSO) to obtain stock solution at concentration 100 mg/ml. It was used to prepare the following working solutions: (5; 12.5; 25; 50; 75; 100; 125; 150; 175; 200 μg/ml). The final concentration of DMSO in the highest of applied plant extracts concentration (200 μg/ml) was 0.2%. DMSO concentration of 0.2% had no influence on HSF cells viability as was shown in our previous experiments.

Cell Culture: Human colon adenocarcinoma cell line HT29 (ATCC No. HTB- 38) derived from grade I tumor, was cultured in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) (Gibco TM, Paisley, UK) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Sigma, St. Louis, MO, USA) at 37°C in a humidified atmosphere with 5% CO_2. Human normal epithelial cells CCD 841 CoTr (ATCC No. CRL-1807) were cultured in RPMI 1640+DMEM (1:1) medium (Sigma) supplemented with 10% FCS at 34°C in a 5% CO_2/95% air atmosphere.

MTT Assay: Cell sensitivity to Yerba mate extracts was determined in a standard spectrophotometric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to Mosmann [6]. The MTT test is based on conversion of yellow tetrazolium salt by viable cells to purple crystals of formazan. The reaction is catalyzed by mitochondrial succinyl dehydrogenase.

After incubation with extracts, the cells grown in 96-well multiplates in 100 µl of culture medium, were subsequently incubated for 3 h with MTT solution (5 mg/ml, 25 µl/well) (Sigma, St. Louis, MO, USA). The yellow tetrazolium salt was metabolized by viable cells to formazan purple crystals. The reaction was catalyzed by mitochondrial succinyl dehydrogenase. The crystals were solubilized overnight in a 10% sodium dodecyl sulfate in 0.01 M HCl mixture. The product was quantified spectrophotometrically by absorbance measurement at 570 nm wavelength using an Emax microplate reader (Molecular Devices Corp., Menlo Park, CA).

Neutral Red (NR) Uptake Assay: The NR cytotoxicity assay is based on the uptake and lysosomal accumulation of the supravital dye, Neutral Red. Dead or damaged cells do not take up the dye [7]. The cells were grown in 96-well multiplates in 100 µl of culture medium with supplements and plant extracts at doses ranging from 25 to 250 µg/ml. Subsequently, the medium was discarded and 0.4% NR (Sigma, St. Louis, MO, USA) solution medium was added to each well. The plate was incubated for 3 h at 37°C in a humidified 5% CO2/95% air incubator. After incubation, the dye-containing medium was removed, the cells were fixed with 1% $CaCl_2$ in 4% paraformaldehyde, and thereafter the incorporated dye was solubilized using 1% acetic acetate in 50% ethanol solution (100 µl). The plates were gently shaken for 20 min. at room temperature and the extracted dye absorbance was measured spectrophotometrically at 540 nm using a microplate reader (Emax; Molecular Devices Corp.).

DPPH• Free Radical Scavenging Test: Free radical scavenging activity of Yerba mate extracts was measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH•) assay. This method is based on the ability of antioxidants to reduce the DPPH• stable dark violet radical (Sigma, St. Louis, MO, USA) to the yellow colored diphenyl-picrylhydrazine.

Briefly, 100 µl of DPPH• solution (0.2 mg/ml in ethanol) was added to 100 µl of extract concentrations (25–250 µg/ml) and standards. Trolox (Sigma, St. Louis, MO, USA) at increasing concentrations (1–50 µg/ml) was used as a standard for the free radical scavenging activity. After 20 min of incubation at room temperature, the absorbance of the solution was measured at 515 nm; the lower the absorbance, the higher the free radical scavenging activity of the extracts. The activity of each extract was determined by comparing its absorbance with that of a control solution (reagents without plant extract). The capability to scavenge the DPPH• radical was calculated by the following formula:

DPPH• Scavenging Effect (%) =

$$[(X_{control} - X_{extract}/X_{control}) \times 100]$$

where *X* control is the absorbance of the control and *X*extract is the absorbance in the presence of plant extract [8].

ELISA Assay: The level of human IL-6 was tested immunoenzymatically (ELISA) using commercially available kit (BenderMed Systems, Vienna, Austria) according to the manufacturer's instructions. The optical density at 450 nm with the correction wavelength of 570 nm of each ELISA sample was determined using a microplate reader (Molecular Devices Corp., Emax, Menlo Park, CA, USA). The concentration of the molecule was calculated on the basis of a standard curve. The detection limit was 1.4 pg/ml.

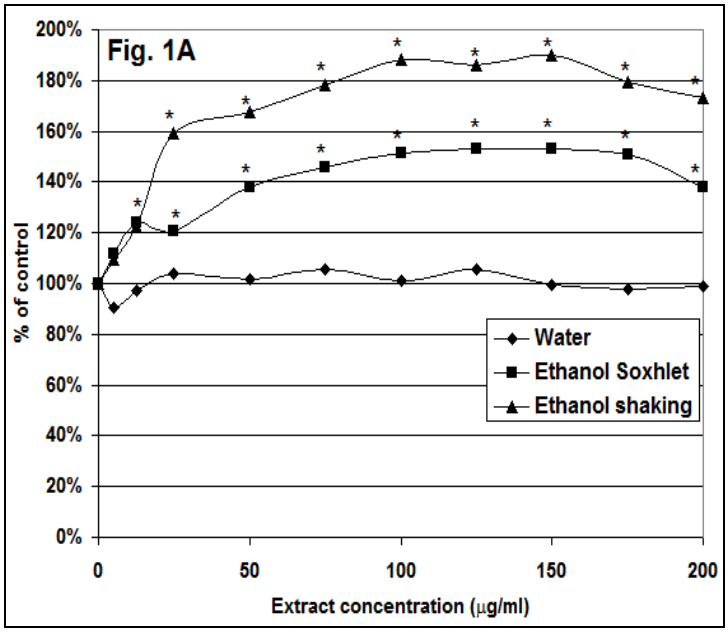
Statistical Analysis: Results are presented as means ± SD from three experiments. Data were analysed using one-way ANOVA analysis of variance with Dunnett *post hoc* test. Differences of $p \leq 0.05$ were considered significant.

RESULTS:

MTT Assay: Carrying out an analysis by MTT assay, water extract had no influence on colon tumor HT29 cell metabolism [**Fig. 1A**] but concentrations higher than 75 µg/ml significantly decreased (up to 24.8% at concentration of 200 µg/ml in comparison to the control) succinyl dehydrogenase activity in normal colonic epithelial cells (IC_{50} = 117.1 µg/ml) [**Fig. 1B**].

On the other hand, when both ethanol extracts were used, stimulation of active reductase enzymes were observed and conversion of MTT was higher than in control sample. Ethanolic extracts obtained by shaking method were stronger stimulators of metabolic activity in both normal and tumor cells than prepared by Soxhlet extraction.

The higher level of mitochondrial dehydrogenase activation (89.6% above control) was found in HT29 cells after their incubation with 150 μg/ml of extract concentration [Fig. 1A]. In normal cells the highest level of MTT conversion (46.9% above control) was observed after 125 μg/ml of extract addition. When concentrations of extracts exceeded values leading to maximal MTT reduction were used then slight, gradual decrease of reducatse enzymes activity was found [Fig. 1B].



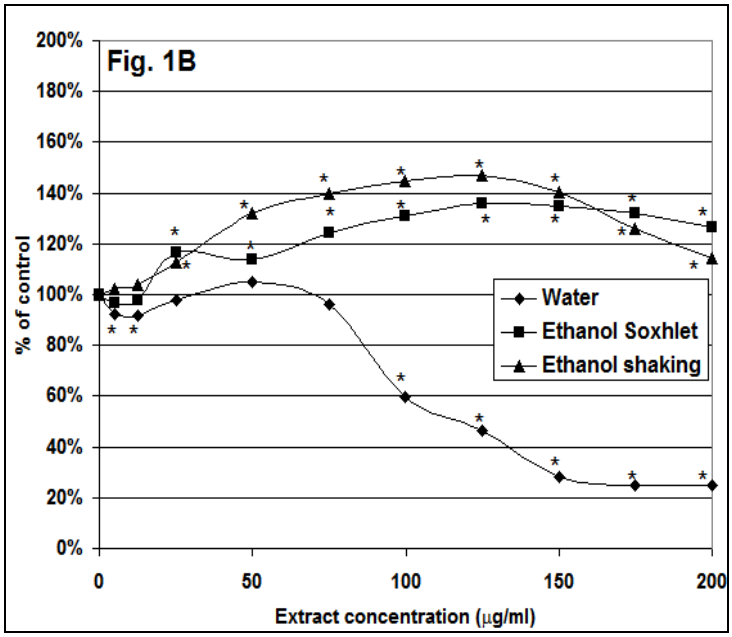
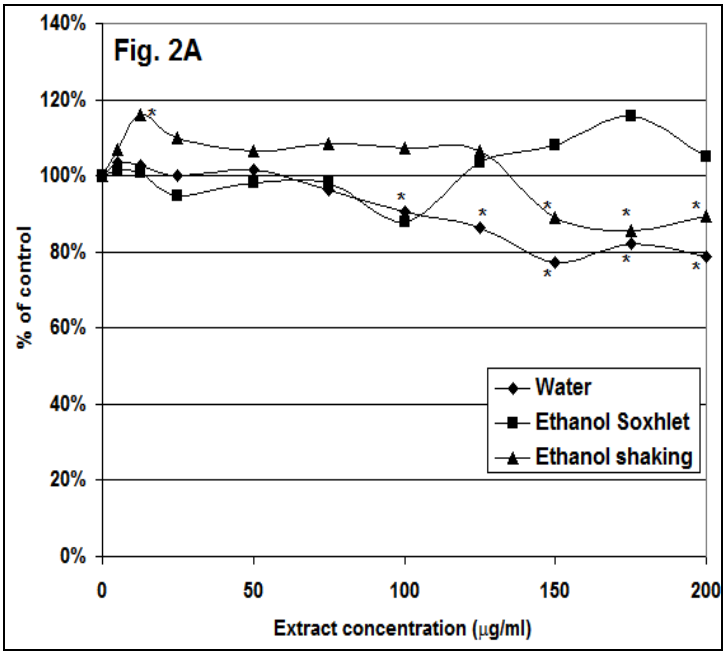


FIG. 1: THE EFFECT OF 24 H TREATMENT OF HUMAN COLON TUMOR CELLS (HT29) (A) AND HUMAN NORMAL COLONIC EPITHELIAL CELLS (CCD 841 COTR) (B) WITH WATER AND ETHANOL EXTRACTS OF *ILEX PARAGUARIENSIS* MTT ASSAY. The results are presented as a percentage of the controls, arbitrarily set to 100%. The figure shows an average of three independent experiments. $^{*}p \leq 0.05$ - culture of cells treated with plant extracts compared to a non-treated control culture

NR Uptake Assay: Neutral Red is the most popular cytotoxicity test providing a quantitative estimation of the viable cells number in the culture sample. In tumor cell culture water extract expressed toxic effects just from concentrations above 75 μg/ml. The lowest viability of HT29 cells was obtained after in the range of 150-200 μg/ml concentrations of the water extract addition and amounted appoximately 80% as compared to the untreated control. Similarly, ethanolic extract after shaking extraction decreased viability of cells up to approximately 86% in comparison to the control when the extracts at the concentration range 150-200 μg/ml was used. On the other hand similar concentrations of ethanolic extract but obtained by Soxhlet extraction stimulated viability of cells to the level of about 10% higher then control [**Fig. 2A**].

When normal cells were analyzed both ethanolic extracts begun to be toxic in a similar manner just from concentrations exceeded 50 μg/ml. The lowest value of colonic epithelial cells viability was found after 200 μg/ml of extract addition and was about 18% (Soxhlet extraction) and about 29% (shaking extraction) lower as compared to the control. When water extract was used increase of cells viability was observed. The highest value 36.9% above control was found as 100 μg/ml of extract was added to the normal colon cells culture [**Fig. 2B**].



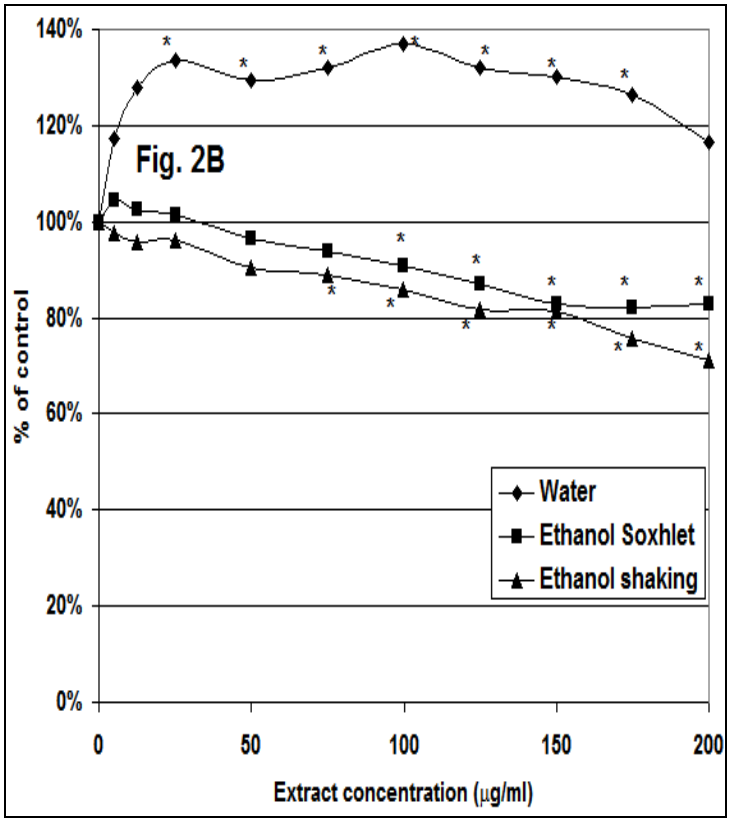


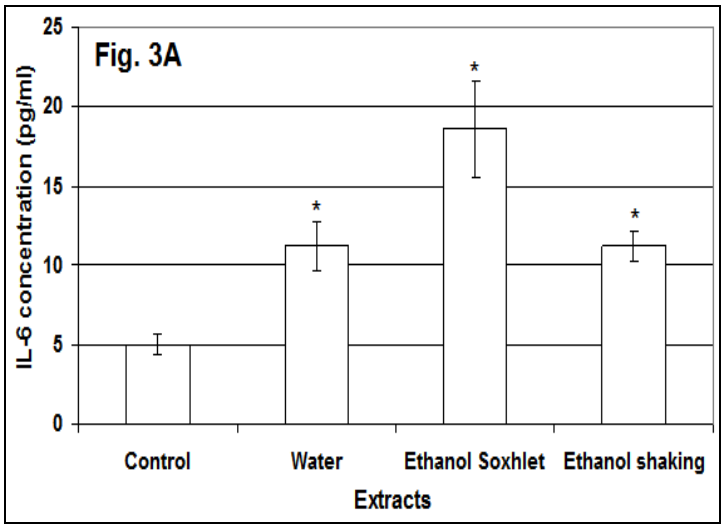
FIG. 2: THE EFFECT OF 24 H TREATMENT OF HUMAN COLON TUMOR CELLS (HT29) (A) AND HUMAN NORMAL COLONIC EPITHELIAL CELLS (CCD 841 COTR) (B) WITH WATER AND ETHANOL EXTRACTS OF *ILEX PARAGUARIENSIS*. NEUTRAL RED (NR) UPTAKE ASSAY. The results are presented as a percentage of the controls, arbitrarily set to 100%. The figure shows an average of three independent experiments. $^{*}p \leq 0.05$ - culture of cells treated with plant extracts compared to a non-treated control culture

DPPH• Free Radical Scavenging Test: DPPH• is a both radical and other radicals scavenger. It is reduced by radicals and that's why serve as an indicator of the radical nature of many biochemical reactions. Water extracts started to reduce DPPH at concentrations exceeded 50 μg/ml. The reduction gradually increased achieving the highest value 57.5% (at 200 μg/ml of the extract concentration) of scavenged radicals as compared to the control arbitrarily set to 0% of reduction. This result was a equivalent to 7.99 μg/ml of the Trolox reductive activity. Ethanolic extracts possessed stronger scavenging activity as compared to the water one. Free radical scavenging action begun from concentrations 5 μg/ml (obtained by Soxhlet extraction) and 25 μg/ml (obtained by shaking extraction). Thereafter , they demonstrated similar activity which was the strongest at 200 μg/ml concentration and amounted about 73% as compared to the control. This result corresponded to the about 15 μg/ml of the Trolox activity [**Table 1**].

TABLE 1: DPPH SCAVENGING EFFECT (%). The % of reduced DPPH radical by water and ethanol extracts of *Ilex paraguariensis* is compared to the control (0% of reduction). The results were also compared to the Trolox reducing activity which was used as a standard reference of free radicals scavenger

Extract	Extract concentration (μg/ml)									
	5	12.5	25	50	75	100	125	150	175	200
Water										
% of reduction	0	0	0	0	9.23±5.35	15.89±2.02	24.45±7.10	38.44±8.00	47.79±0.89	57.47±4.76
Equivalent reduction of the extract to the following Trolox concentration	0	0	0	0	5.98	6.24	6.6	7.19	7.59	7.99
Ethanol Soxhlet extraction										
% of reduction	0.94±0.91	4.42±2.11	3.22±2.16	7.30±5.23	19.65±3.88	28.67±4.91	40.01±6.60	51.75±7.57	62.90±7.84	72.87±7.59
Equivalent reduction of the extract to the following Trolox concentration	3.01	3.59	3.39	4.07	6.12	7.61	9.50	11.44	13.29	14.95
Ethanol Shaking extraction										
% of reduction	0	0	2.36±4.09	6.97±1.14	17.46±3.33	29.81±4.57	40.74±2.84	53.64±6.96	64.67±10.55	73.00±10.44
Equivalent reduction of the extract to the following Trolox concentration	0	0	3.25	4.01	5.75	7.81	9.62	11.75	13.59	14.97

ELISA assay: In this test, only one extract concentration (12.5 μg/ml) was used which was selected as a general non toxic for cells but high enough to influence on cytokine production. As analysis was performed on normal colonic cells than all tested extracts induced IL-6 production. Ethanol extract performed using Soxhlet extraction was the most potent and the IL-6 level increased to 18.6 pg/ml when control value was 5 pg/ml [**Fig. 3A**]. On the contrary, all tested Yerba mate extracts significantly inhibited the cytokine production by tumor HT29 cells. Similarly, in this case the extract obtained by Soxhlet method was the most active and limited IL-6 level to 1.3 pg/ml while the control value was 20.3 pg/ml [**Fig. 3B**].



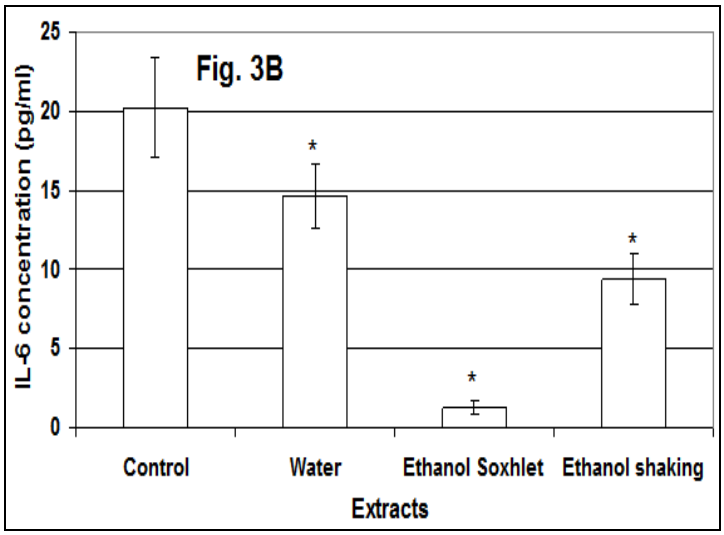


FIG. 3: IL-6 SECRETION IN CULTURE OF HUMAN NORMAL COLONIC EPITHELIAL CELLS (CCD 841 COTR) (A) AND HUMAN COLON TUMOR CELLS (HT29) (B) DURING 24 H INCUBATION WITH WATER AND ETHANOL EXTRACTS OF *ILEX PARAGUARIENSIS*. Extract concentrations was 12.5 μg/ml. ELISA test. $^{*}p \leq 0.05$ - culture of cells treated with plant extracts compared to a non-treated control culture

DISCUSSION: Although *Yerba mate* is widely used and consumed by million people all over the world it was therefore entirely justified to test it potential toxicity on tumor and normal cells of colon wall origin, antioxidant activity and immunomodulatory action based on one of the most important cytokine (IL-6) in the immunological factors network. Moreover the way of extracts preparation seems to be an important element in the pro-health activity of the plant. Herbs, generally classified as natural supplements express favorable activities and health benefits in organism but also may be related to negative or even harmful effects when prepared or used in unreliable manner [9].

Negative effects of Yerba mate consumption are usually explained as thermal lesions connected with special drinking customs [10]. However, de Andrade et al. demonstrated that there are no toxic effects, including changes in behavior for two mammalian species studied, rats and rabbits [1]. These results gave a basis for conclusions concerning safety of Yerba mate consumption in humans.

Our results are partially in agreement with such thesis. We have to, however, extend these explanation for differences in kind of destination tissue and obviously extract concentration analysis. In our opinion, based on our results, when therapeutic activity of Yerba mate is considered, lower concentrations of water extracts on normal cells should be recommended. On the other hand, when influences on tumor cells are the aim of mate action then also water extract but in high concentrations would be advisable. Based on complex array of secondary metabolites which are present in alcohol extracts they express many activities on both tumor and normal cells.

However, we found it as slightly toxic in higher concentrations (>75 μg/ml) for normal cells with no toxicity or ever stimulatory effects on cancerous cells. Therefore, their wilder application should be carefully analyzed and tested. In accordance with generally accepted principles, plant extracts limit cancer development. In the case of colon tumor it may be a little otherwise. It may be possible that colon cancer may utilize agents commonly considered as anti-tumor on its own benefit and grow faster, and metastase early.

In general, water extracts may be considered as health beneficial on normal colon wall cells but when toxicity on tumor cells is considered it still needs further study.

In pathophysiology of many diseases as well as in remaining a good health, the correct balance between production and consumption of reactive oxygen species which are connected with oxidative stress has to be kept [11, 12]. *Ilex paraguariensis* is a plant which is widely used to treat gastrointestinal disorders mainly due to its eupeptic and choleretic properties [11]. These capacities are, on the other hand, closely related to antioxidant, anti-inflammatory and immuno-modulatory activities.

We found differences in IL-6 production by tumor and normal cells after their incubation with Yerba mate extracts. Arçari et al., revealed that hepatic IL-6 expression levels were reduced after Yerba mate extract treatment. They speculated that mate extracts reduce NF-κB and, thus influence on genes encoding inflammatory proteins such as IL-6 or TNF-α [13]. Their experiments aimed at evaluation of Yerba mate extracts, among others, on inflammatory markers in mice with high fat diet-induced obesity. They found differences in such markers expression in tested groups.

In our study we observed reduction of IL-6 level in colon tumor cell line (HT29) culture after its incubation with Yerba mate extracts. On the other hand, plant extracts stimulated the cytokine production by normal cells. We suppose that the mechanism may be similar to that described by Arçari *et al*. Moreover, it is known fact that normal and tumor cells differ not only in their morphology and growth pattern but especially in reactivity on microenvironmental conditions. Therefore, Yerba mate extracts proved to be pro-inflammatory factors in normal conditions but also anti-inflammatory when affect on pathologic tissue. We suppose that in further tests such extracts may be analyzed in terms of their chemopreventive properties.

Phytochemical analysis revealed that major constituents demonstrating antioxidant activities are present in Yerba mate. They consists of polyphenols like caffeoyl derivatives, flavonoids, triterpenoids, triterpenoid saponins or tannins [4, 11, 13].

In our study we found strong Yerba mate extracts' free radical reducing activity. Testes were performed using DPPH scavenging method. The DPPH is a stable radical species which is insensitive to side reactions connected with polyphenols activity like metal ion chelation or enzyme inhibition [11]. This analysis allows to estimate to what degree ROS-mediated adverse reactions in organism may be limited after, e.g. plant extracts application.

In particular, it regards to lipid peroxidation, DNA damage or protein degradation. Yerba mate extracts are rich in polyphenolic compounds and that's why may function as a health preventing plant especially based on its antioxidative and free-radical reducing activities. In our study we measured "total" antioxidative action of Yerba mate but according to Leonard et al. analyses it is currently known that *Ilex paraguariensis* affect hydroxyl and superoxide radicals level both in chemical and culture conditions [5]. It is especially important because they, in free radical group, demonstrate the most potent disadvantageous effects on cells and tissues.

Our results are also in agreement with Markowicz Bastos *et al*., observations who showed that ethanolic extracts form green Yerba mate are excellent DPPH scavengers [14]. This property may be expressed both as free radical reducing activity or as free radical appearance inhibitory factor in tissue micro-environment. However, it has to be kept in mind that plant derived polyphenols demonstrate their modulatory activities depending on both their local concentration and type of tissue or cell culture model.

Generally agents which influence on cellular metabolism or enhance detoxification could be defined as chemopreventive factors. Yerba mate partially satisfy this criterion but cytotoxic activity against broader group of cancers have to be performed.

ACKNOWLEDGMENTS: We would like to express our thanks to Mateusz Koryciński, BSc for preparing water extract of Yerba mate and Józef Kaczor, Ph.D., for his help in ethanol extracts preparation.

We would also like to thank the "Academic Society of Biotechnology Students", which has granted us a plastic laboratory equipment we used in the study which was a prize in the scientific competition.

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How to cite this article:
Frant M, Czajka M and Paduch R: Yerba Maté (*Ilex paraguariensis*) Effect on Human Colon Normal and Tumor Cells. *Int J Pharm Sci Res*. 3(12); 4730-4737.