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Full Length Research Paper

In vitro evaluation of antioxidant, cytotoxic and apoptotic activities of different extracts from the leaves of *Teucrium ramosissimum* (Lamiaceae)

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The *in vitro* antioxidant, cytotoxic and apoptotic activities of *Teucrium ramosissimum* extracts were investigated. The antioxidant activities of the tested extracts were evaluated through three chemical assays which are (1): The cupric reducing antioxidant capacity (CUPRAC), (2) The reducing power (RP) and (3) The ferric reducing antioxidant power (FRAP). Total oligomer flavonoids enriched extract (TOF) showed the best antioxidant activity evaluated by the CUPRAC and FRAP assays with trolox equivalent antioxidant capacity (TEAC) values of 12.85 and 0.525 µM, respectively compared to control. Whereas ethyl acetate (EA) extract exhibited the highest reducing power when using the RP assay with a TEAC value of 2.025 µM. Furthermore, the effects of *T. ramosissimum* extracts on cell proliferation and induction of deoxyribonucleic acid (DNA) degradation, as an apoptotic marker in human leukemia cells were also examined. The cytotoxic activity revealed that the methanol extract inhibited significantly the proliferation of K562 cells (IC_{50} =150 µg/ml), however no specific DNA degradation indicating an apoptotic effect was observed.

Key words: *Teucrium ramosissimum*, antioxidant activity, cytotoxic, apoptotic, K562 cells.

INTRODUCTION

Free radicals may be defined as any chemical species that are capable of existing with one or more unpaired outer shell electrons. They are extremely reactive and generally highly unstable (Martinez-Cayuela, 1995). Reactive oxygen species, such as superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($_1O^2$), are of the greatest biological significance (Martinez-Cayuela, 1995; Schöneich, 1999). Antioxidants are substances that delay the oxidation process, inhibiting the polymerisation chain initiated by free radicals and other subsequent oxidising reactions (Halliwell and Aruoma, 1991). This concept is fundamental to biomedical, nutraceutical, food chemistry and phytochemical sciences, where synthetic antioxidants like butylated hydroxy toluene (BHT) have long been used to preserve quality of food by protecting against oxidation-related deterioration. A growing body of literature points to the importance of natural antioxidants from many plants, which may be used to reduce cellular oxidative damage, not only in foods, but also in the human body. This may provide protection against chronic diseases, including cancer and neurodegenerative diseases, inflammation and cardiovascular disease (Prior et al., 2005). Moreover, there has been increasing realization that several plant derived polyphenolic compounds may possess antimicrobial, antioxidant, anticancer and apoptosis inducing properties (Clement et al., 1998).

Therefore, the role of plant derived polyphenols in chemoprevention of cancer has emerged as an interesting area of research. To date, many anticancer drugs

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have been developed and applied by clinical doctors. However, the resistance to cancer drugs and side effects were discovered (Yang et al., 2000). For many years, the cytotoxic actions of chemotherapeutic drugs were ascribed solely to their ability to induce genotoxic damage. During the past decade, however, evidence is gradually being provided that many cancer therapeutic agents induce a cell death process known as programmed cell death, or apoptosis. Although the mechanisms by which chemotherapeutic agents can kill tumors via apoptotic pathways have been controversial, the killing of tumors through the induction of apoptosis has been now recognized as a novel strategy for the identification of anticancer drugs (Panchal, 1998; McConkey et al., 1996). In Tunisian traditional medicine, the use of *Teucrium* differs according to the species and according to the region of the country. Based on information gathered from traditional healers, herbalists and inhabitants from rural regions, *Teucrium ramosissimum* is frequently used in such diverse applications as the treatment of gastric ulcer, intestinal inflammation and particularly as cicatrisant in external use (Sghaier et al., 2007). In this study, our aim was to evaluate the antioxidant capacity of the *T. ramosissimum* extracts, using the CUPRAC, the RP and the FRAP assays. The role of these extracts in the induction of cell proliferation or apoptosis in human chronic myelogenous leukemia (CML) cell line K562 was also examined.

MATERIALS AND METHODS

Plant material

The aerial part of *T. ramosissimum* was collected in January 2005 from the mountainous region of Gafsa in Southeast of Tunisia. The plant was identified by Pr. Mohamed Chaieb (Department of Botany, Faculty of Sciences, University of Sfax, Tunisia) according to the Flora of Tunisia (Pottier-Alapetite, 1979). A voucher specimen (Tr- 02- 05) was deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, University of Monastir, Tunisia, for future reference. The leaves were shade-dried, powdered and stored in a tightly closed container for further use.

Extraction method

Dried and powdered leaves (100 g) of *T. ramosissimum* were first defatted with petroleum ether (1 l), then extracted with chloroform (1 l), EA (1 l) and methanol (1 l) using a Soxhlet apparatus (6 h). Four different extracts were thus obtained. They were concentrated to dryness and kept at 4°C in the absence of light. In order to obtain an extract enriched in TOF, the powdered leaves were macerated in water-acetone mixture (1:2), during 24 h with continuous stirring. The extract was filtered and the acetone was evaporated under low pressure in order to obtain an aqueous phase. Tannins were partially removed by precipitation with an excess of NaCl during 24 h at 5°C and the supernatant was recovered. The latter was extracted with EA concentrated and precipitated with an excess of chloroform. The precipitate was separated and yielded the TOF extract, which was dissolved in water (Ghedira et al., 1991). The fresh leaves of *T. ramosissimum* were dried at room temperature and reduced to coarse powder. 100 g of the powdered leaves were extracted with boiling water (1 L) for 15 to 20 min. After filtration, the crude extract obtained was frozen and lyophilized, leading to the aqueous extract which was dissolved in water.

Cupric reducing antioxidant capacity assay (CUPRAC)

CUPRAC assay of the samples obtained from *T. ramosissimum* was determined according to the method of Apak et al. (2004). To test tubes containing different concentrations of extracts (1000, 750, 500, 250 and 125 µg/ml), 0.25 ml of $CuCl_2$ solution (0.01 M), 0.25 ml of ethanolic neocuproine solution (7.5 × 10-3 M), and 0.25 ml of NH_4Ac buffer solution (1 M, pH 7) were added, consecutively. The total volume was then adjusted with distillated water to 2 ml and mixed well. Absorbance against a reagent blank was measured at 450 nm after 1 h. Trolox was used as standard and total antioxidant capacity of herbal extracts was measured as µM trolox equivalent.

Reducing power assay (RP)

The RP of *T. ramosissimum* compounds was determined according to the method of Jayaprakasha et al, (2001). Sample solutions at different concentrations (1000, 750, 500, 250 and 125 µg/ml) were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide (1%, w/v, in water). After incubation at 50°C for 20 min, 2.5 ml of trichloroacetic acid (TCA) (10%, w/v, in water) were added and the mixture was centrifuged (5000 rpm, 10 min, 25°C). Supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml of ferric chloride (0.1%, w/v, in water), and then the absorbance was measured at 700 nm, using a spectrophotometer, against blanks that contained all reagents except the sample compounds. The RP tests were run in triplicate. Increase in absorbance of the reaction indicates the RP of the test samples. Trolox was used as standard and total antioxidant capacity of herbal extracts was measured as µM trolox equivalent.

Ferric reducing antioxidant power assay (FRAP)

Antioxidant activity of the samples was measured using the FRAP assay (Benzie and Strain, 1996).This method measures the absorption change that appears when the TPTZ (2,4,6-tri-pyridyl-s-triazine)-Fe^{3+} complex is reduced to the TPTZ-Fe^{2+} form in the presence of antioxidants. An intense blue colour, with maximum absorption at 593 nm, develops. The FRAP method gives a measure of the total antioxidant activity of the sample. The fresh FRAP reagent was prepared daily by mixing 25 ml acetate buffer (300 mM, pH 3.6), 2.5 ml TPTZ solution (10 mM) and 2.5 ml of $FeCl_3$ solution (20 mM). The reagent was warmed to 37°C for 30 min, then 50 µl volume of different concentrations of extracts (1000, 750, 500, 250 and 125 µg/ml) were added to the 950 µl of fresh FRAP reagent and the absorbance was measured after an incubation time of 30 min at 37°C. Trolox was used as standard and total antioxidant capacity of herbal extracts was measured as µM TR equivalent.

Cytotoxicity studies *in vitro*

T. ramosissimum compounds were tested *in vitro* for their antiproliferative activities against K562 (human CML) cells.

Cell culture

Human CML cell line K562 was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultivated in RPMI-1640 medium supplemented with 10% (v/v) foetal calf serum, 1% gentamycine and 2 mM L-glutamine as a complete growth medium. Cells were maintained in 25 cm^3 flasks with 10 ml of medium and were incubated at 37°C in an incubator with 5% CO_2 in humidified atmosphere. Every 3 days the cells were subcultured by splitting the culture with fresh medium.

Assay for cytotoxic activity

Cytotoxicity of *T. ramosissimum* compounds against K562 leukemia cell line was estimated by the 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells. The resulting of blue formazan product can be measured spectrophotometrically. The MTT colorimetric assay was performed in 96-well plates (Polydoro et al., 2004). Cells were seeded in a 96-well plate at a concentration of 5×10^4 cells/well and incubated at 37°C for 24 h in a 5% CO_2 enriched atmosphere. The compounds were first dissolved in 1% dimethyl sulfoxide (DMSO), then in the cell growth medium. Cells in exponential growth phase were incubated again at 37°C for 48 h with each of the tested compounds at concentrations ranging from 200 to 800 µg/ml. After that, the medium was removed and cells in each well were treated with 50 µl of MTT solution (5 mg/ml) at 37°C for 4 h. MTT solution was then discarded and 50 µl of 100% DMSO were added to dissolve insoluble formazan crystal. Optical density was measured at 540 nm. Each drug concentration was tested in triplicate. The cytotoxic effects of the extracts were estimated in terms of growth inhibition percentage and expressed as IC_{50} which is the concentration of compound which reduces the absorbance of treated cells by 50% with reference to the control (untreated cells). The IC_{50} values were graphically obtained from the dose–response curves. We determined that IC_{50} values with cytotoxicity results were more than 50% at screening concentrations.

DNA fragmentation analysis

Cell DNA fragmentation was analyzed by agarose gel electrophoresis as described by Wang et al. (2002) with slight modifications. K562 cells ($1.5\ 10^6$ cells/ml) were exposed to the extract for 24 and then for 48 h and were harvested by centrifugation. Cell pellets were resuspended in 200 µl of lysis buffer (50 mM TRIS hydrochloride (Tris–HCl), pH 8.0, 10 mM ethylene diamine tetraacetic acid (EDTA), 0.5% N-Lauryl Sarcosine Sodium Salt) at room temperature for 1 h, then centrifuged at 12 000 g for 20 min at 4°C. The supernatant was incubated overnight at 56°C with 250 µg/ml proteinase K. Cell lysates were then treated with 2 mg/ml RNase A and incubated at 56°C for 2 h. DNA was extracted with chloroform/phenol/isoamyl alcohol (24/25/1, v/v/v) and precipitated from the aqueous phase by centrifugation at 14 000 g for 30 min at 0°C. The solution recuperate was transferred to a 1.5% agarose gel and electrophoresis was carried out at 67V for 3/4 h with Tris–acetate– ethylene diamine tetraacetic acid (TAE) (Tris 2 M, sodium acetate 1 M, EDTA 50 mM) as the running buffer. DNA in the gel was colored using BET visualized under ultraviolet (UV) light (Wang et al., 2002; Kilani et al., 2008).

Statistical analysis

Data were collected and expressed as the mean±standard deviation of three independent experiments and analyzed for statistical significance from control and between each others. The data were tested for statistical differences by one-way ANOVA followed by Duncan's multiple comparison tests using STATISTICA (Version 6.0, Statsoft Inc.). The criterion for significance was set at $P < 0.05$. IC_{50} values, from the *in vitro* data, were calculated by regression analysis.

RESULTS AND DISCUSSION

CUPRAC assay

The cupric ion (Cu^{2+}) reducing ability of the tested extracts is shown in Figure 1. Results obtained showed that the cupric ion (Cu^{2+}) RP of different tested compounds at the same concentration (1mg/ml) exhibited the following order: TOF extract > aqueous extract > MeOH extract> EA extract> $CHCl_3$ extract> petroleum ether extract (PE) with TEAC values of, respectively 12.85, 10.95, 9.65, 4.9, 4.85 and 4.05 µM (Figure 4a). Also, a correlation between the cupric ion (Cu^{2+}) reducing ability and concentrations of all tested extracts was observed. In the same way, these compounds present a dose dependent reducing effect of Cu^{2+}. Though the reductive abilities of all extracts were significantly higher than that of Trolox. This important reducing effect can be due to the presence of natural antioxidant molecules that can trap free electrons which are the initiator of oxidation reactions.

Besides, many studies showed the existence of natural antioxidants in all parts of plants and that the typical compounds having this activity are mainly: polyphenols (Chanwitheesuk et al., 2005). According to several studies, the most property of polyphenols is their antioxidant activity and their capacity to inactivate and stabilize the free radicals through their hydroxyl groups (Njiveldt et al., 2001). In addition, the antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Parr and Bolwell, 2000). According to another report, a very positive relationship between total phenols and antioxidant activity was found in many plant species (Velioglu et al., 1998). Thus, the RP of various extracts might be due to their hydrogen-donating ability as described by Shimada et al. (1992) and to the bioactive compounds associated with antioxidant activity (Siddhuraju et al., 2002). These bioactive compounds are good electron donors and could terminate the free radical chain reactions by converting free radicals to more stable products.

RP assay

As far as RP is associated with antioxidant activity (Yen and Duh, 1993), it seems interesting to determine the RP

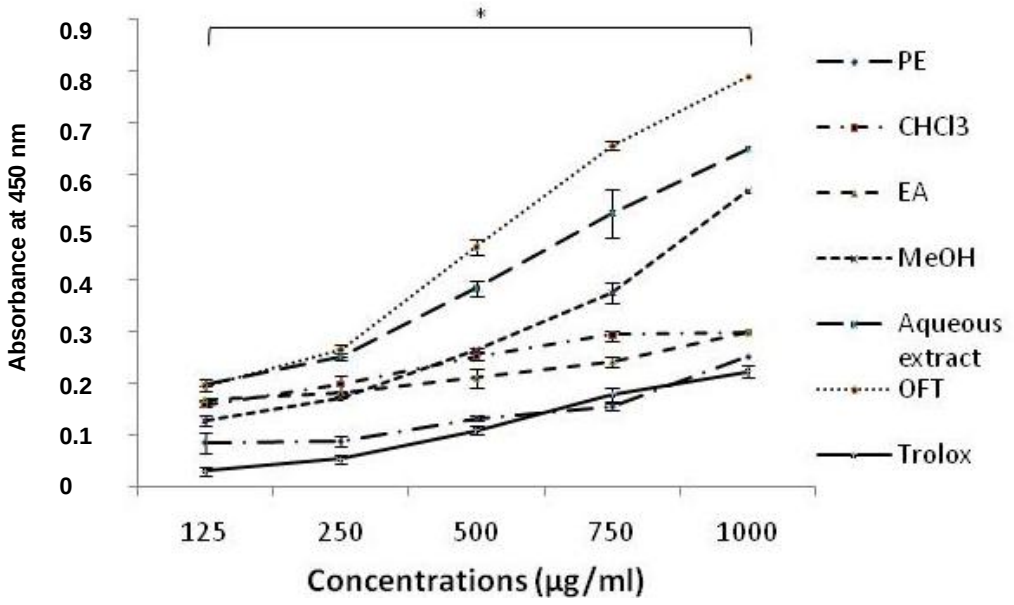

Figure 1. The Cu^{2+}-Cu^{+} reducing activity of *T. ramosissimum* extracts at different concentrations (125-1000 µg/ml) tested using CUPRAC assay. *p<0.05 (Duncan test).

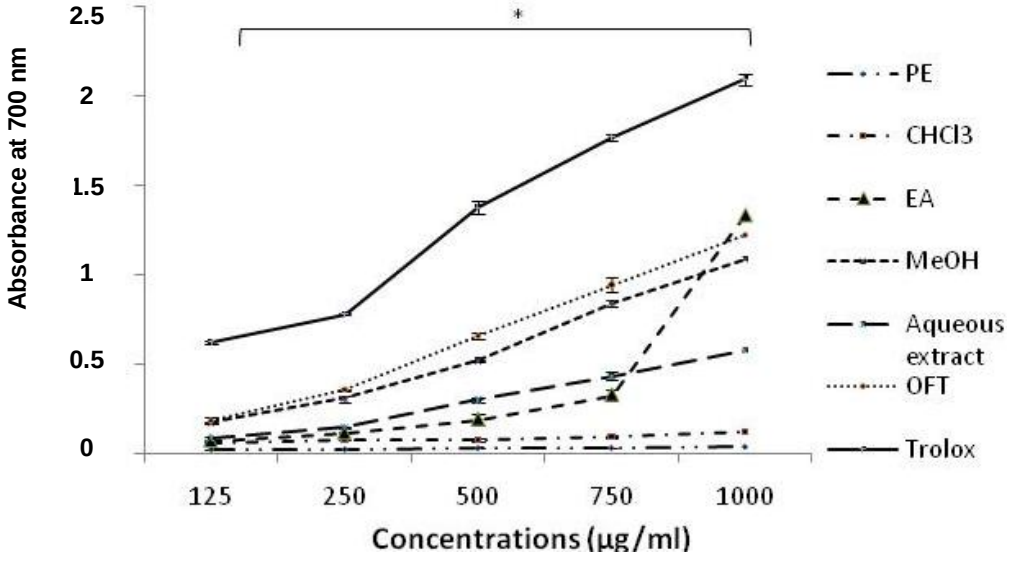

Figure 2. The Fe^{3+}-Fe^{2+} reducing activity of *T. ramosissimum* extracts at different concentrations (125 to 1000 µg/ml) tested, using RP assay. *p<0.05 (Duncan test).

of various extracts in order to elucidate a possible relationship between their antioxidant effect and their RP. As shown in Figure 2, the RP of the test samples correlated well with increasing concentrations of extracts and all the tested extracts exhibited a RP. Therefore, EA extract possesses the highest RP of iron and PE exhibited the lowest RP. The maximum of the activity was reached at the highest tested concentration (1 mg/ml), with a value of 2.025 µM equivalent of Trolox for the EA extract. Besides, PE extracts showed reducing effect of 0.05 µM equivalent of Trolox (Figure 4b). The antioxidant activity has been reported by some investigators to be concomitant with the development of RP. This property is associated with the presence of reductions that are

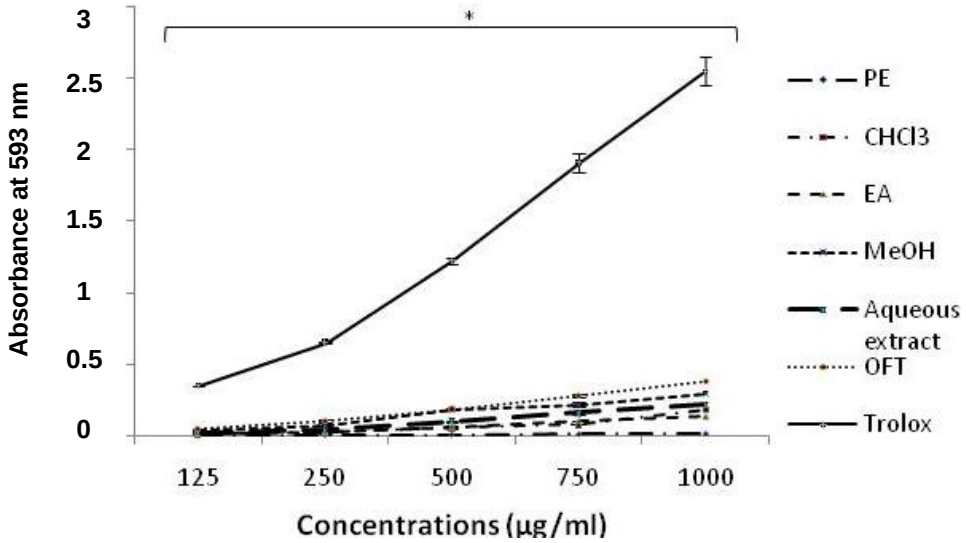

Figure 3. The Fe $^{3+}$-Fe $^{2+}$ reducing activity of *T. ramosissimum* extracts at different concentrations (125 to 1000 µg/ml) tested using FRAP assay. *p<0.05 (Duncan test).

reported to be terminators of free radical chain reaction (Duh, 1998). Moreover, Gordon (1990) reported that the antioxidant action of reductones is based on breaking the radical chain by donation of a hydrogen atom. Chen and Ahn (1998) found that natural phenolics including quercetin, rutin, catechin and caffeic acid acted as Fe^{2+} chelators.

FRAP assay

FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+}–TPTZ) complex and producing a coloured ferrous tripyridyltriazine (Fe^{2+}–TPTZ) (Benzie and Strain, 1996). Generally, the reducing properties are associated with the presence of compounds which exert their action by breaking the free radical chain by donating a hydrogen atom (Duh et al., 1999). According to Benzie and Strain (1996), the reduction of Fe^{3+}–TPTZ complex to blue-coloured Fe^{2+}–TPTZ occurs at low pH. Figure 3 depicts the Fe^{3+}-Fe^{2+} transformation investigated in the presence of PE, $CHCl_3$, EA, MeOH, aqueous and TOF extracts using the potassium ferricyanide reduction method. The reducing activity of the different extracts mentioned above increased with increasing sample concentration. Therefore, at the same concentration of 1mg/ml, PE, $CHCl_3$, EA, MeOH, aqueous and TOF extracts reduce the iron ion by 0.027, 0.275, 0.192, 0.41, 0.325 and 0.525 µM equivalent of Trolox, respectively (Figure 4c). Polyphenolic contents of all the sample extracts appear to function as good electron and hydrogen atom donors and therefore should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products. Similar observation between the polyphenolic constituents in terms of dose dependent and RP activity have been reported for several plant extracts including tea (Amarowicz et al., 2004; Yen and Chen, 1995; Zhu et al., 2002).

Cell viability assay

The relationship between concentration of extracts and their antiproliferative effect on K562 cells was investigated by MTT assay. MTT is a yellow water-soluble tetrazolium salt. Metabolically active cells are able to convert the dye to water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring. Cells were treated with extracts of *T. ramosissimum* at concentrations ranging from 50 to 1000 µg/ml, for 48 h at 37°C, and then IC_{50} values were determined. As shown in Table 1, all tested extracts inhibited at various levels (good to moderate), the proliferation of the malignant cells. The strongest cytotoxic effect was obtained with MeOH extract against K562 cells (IC_{50} 150 µg/ml), followed by $CHCl_3$, TOF, EA, aqueous and PE extracts with respective IC_{50}s of 200, 550, 600, 800 and 925 µg/ml. The antiproliferative effect of the *T. ramosissimum* extracts revealed that they inhibited cell-line proliferation in a concentration-dependent manner. Since it is well known that different cell lines might exhibit different sensitivities to a cytotoxic compound. In fact, many authors have studied the antiproliferative effects of plant extracts and they found comparable IC_{50}values (some IC_{50} values were superior to 200 to 350 µg/ml) (Ben et

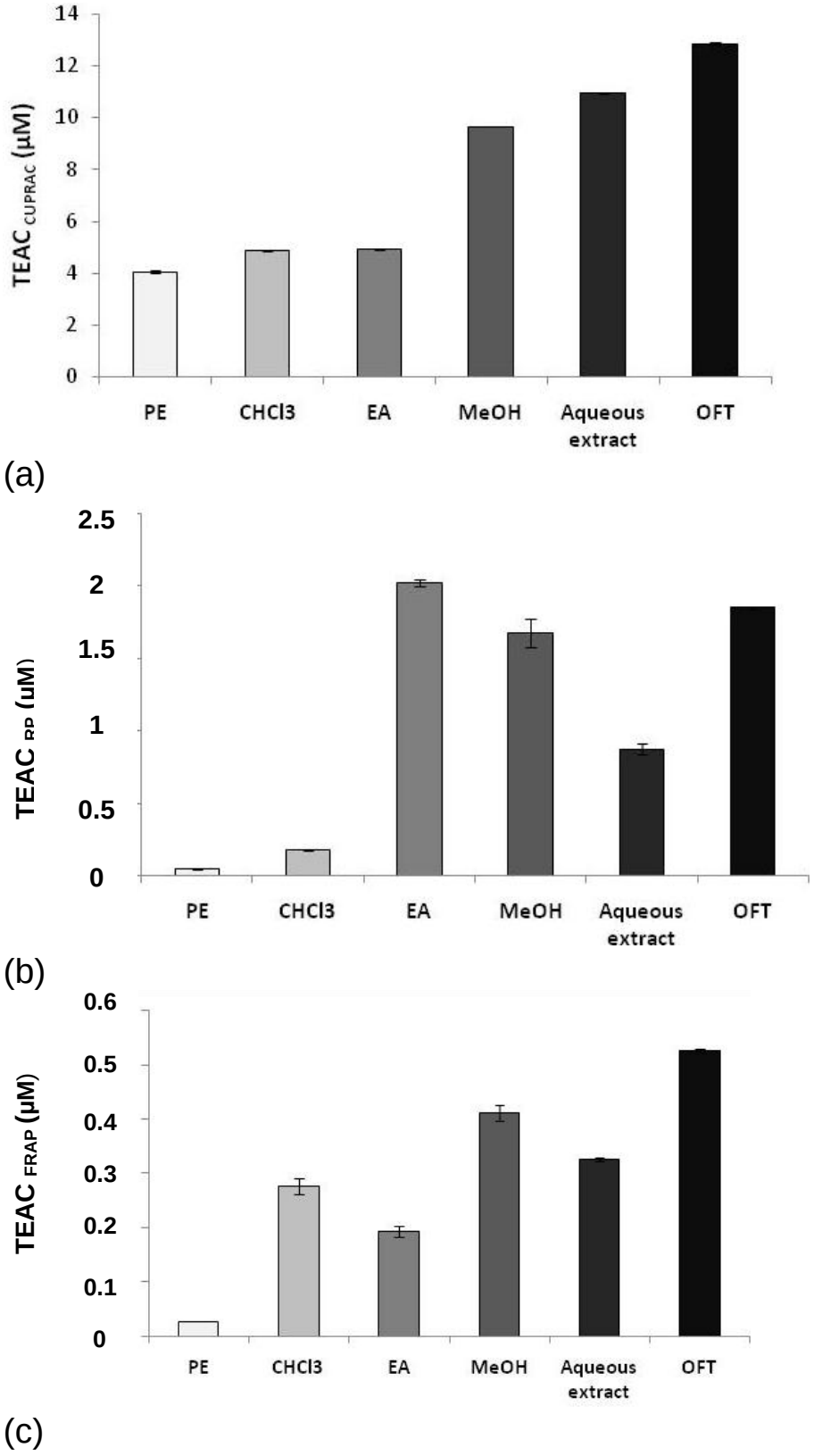

Figure 4. The trolox equivalent antioxidant capacities of various antioxidant extracts calculated with respect to the (**a)** CUPRAC, (**b)** RP and (**c)** FRAP methods.

Table 1. IC_{50} (µg/ml) values for extracts of *T. ramosissimum* against K562 leukemia cell lines.

Compound	Activity on proliferation of cell line K562[a]
PE	925±6.5
$CHCl_3$	200±2.75
EA	600±4.65
MeOH	150±2.15
Aqueous extract	800±5.95
OFT	550±3.85

[a] Results are means ± standard deviation of duplicate analysis of three replications. PE extract (petroleum ether extract) , $CHCl_3$ extract (chloroform extract), EA extract (ethyl acetate extract), TOF extract (Total oligomers flavonoids).

al., 2008).

Moreover, several studies have demonstrated that extracts from several herbal medicines had an anticancer potential. For instance, Yano et al. (1994) reported that water-soluble ingredients of Sho-Saiko-To inhibited the proliferation of KIM-1 human hepatoma cells and KMC-1 cholangiocarcinoma cells with IC_{50}values of, respectively, 350 and 240 µg/ml. The cell-type specificity observed in the tested extracts is likely to be due to the presence of different classes of compounds such as flavonoids and total ploy phenols, as reported for other classes of compounds (Cragg et al., 1994). Besides, some studies have shown that flavonoids are able to influence a variety of cell function by modulating cell signaling (Musonda and Chipman, 1998), altering proliferation and cytotoxicity in cancer cell lines (Kuntz et al., 1999). Also, flavonoids show cytotoxic effects on various human cell lines, for example leukemia cells (Larocca et al., 1990; Hirano et al., 1994) and ovarian cancer cells (Benavente-Garcia et al., 1997).

Electrophoretic analysis of DNA fragmentation

The fragmentation of DNA extracted from K562 cells (1.5×10^6 cells), was detected after exposing cells to extracts for 24 and 48 h, and cell viability was evaluated. Examination of electrophoretic profiles of DNA extracted from the treated cells, revealed no ladder formation characteristic of apoptosis comparing to control cells. We suppose that the growth inhibitory effect revealed by *T. ramosissimum* extracts against K562 cells did not act through the induction of programmed cell death. However, the induction of cell death in cancer cells would be a great benefit for cancer chemotherapy. There are three mechanisms of cell death necrosis, apoptosis and autophagie. Our study allowed as to eliminate the induction of apoptotic death in cells treated by our tested extracts as far as we did not obtain the DNA ladder profile characteristic of apoptotic cell death. However, we can hypothesize that cytotoxicity induced by our extracts towards the Human CML cell line K562 should be the result of either a necrotic effect or autophagie process.

In conclusion, *T. ramosissimum* extracts appear to contain compounds with antioxidant and cytotoxic properties. As antiproliferative activity has become a new therapeutic target in cancer research, it appears reasonable to suggest that *T. ramosissimum* may have potential as an agent of chemotherapeutic and cytostatic activity in Human CML cell line. However, further investigation of its activity, *in vivo*, is necessary to elaborate and exploit this promise.

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