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Inhibitory effect of *Teucrium ramosissimum* extracts on aflatoxin B_1, benzo[a]pyrene, 4-nitro-o-phenylenediamine and sodium azide induced mutagenicity: Correlation with antioxidant activity

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Abstract

The mutagenic potential of total oligomers flavonoids (TOF), ethyl acetate (EA) and petroleum ether (PE) extracts from aerial parts of *Teucrium ramosissimum* was assessed using Ames *Salmonella* tester strains TA98, TA100 and TA1535 with and without metabolic activation (S9). None of the different extracts produced a mutagenic effect. Likewise, the antimutagenicity of the same extracts was tested using the "Ames test". Our results showed that *T. ramosissimum* extracts possess antimutagenic activity against all the tested genotoxicants (aflatoxin B_1, benzo[a]pyrene, 4-nitro-o-phenylenediamine and sodium azide) in the *Salmonella* assay systems used in this study. In addition, all extracts showed important free radical scavenging activity toward the radicals DPPH and ABTS except the PE extract.

Keywords: Ames test; Free radical scavenging activity; *Teucrium ramosissimum*

1. Introduction

The human body is continuously and unavoidably exposed to a plethora of structurally diverse chemicals with established carcinogenic activity in animal models and/or mutagenic activity in short-term tests (Maron and Ames, 1983). A characteristic of the major classes of chemical carcinogens, namely polycyclic aromatic hydrocarbons, heterocyclic amines, and aromatic amines, are that in order to express their genotoxicity and carcinogenicity, they must be metabolized to reactive intermediates that are capable of interacting covalently with DNA (Weisberger, 1999). Damage to DNA is likely to be major cause of cancer and other diseases. In recent years there has been increasing realization that several plant-derived polyphenolic compounds may possess anticancer and apoptosis inducing properties (Kilani-Jaziri et al., 2009; Mukhtar et al., 1998).

Therefore, the role of plant-derived polyphenols in chemoprevention of cancer has emerged as an interesting area of research. Plant polyphenols are natural antioxidants and most of their pharmacological properties are considered to be due to their antioxidant action (Ames et al., 1995). This is generally considered to reflect their ability to scavenge endogenously generated oxygen radicals or those radicals formed by various xenobiotics, radiation etc. However, some data in the literature suggest that the antioxidant properties of the polyphenolic compounds may not fully account for their chemopreventive effects (Gali et al., 1992). In addition, the genotoxic and mutagenic effects of vegetable extracts have been studied over the years by researchers concerned with indiscriminate consumption of such products. Damage to the users' genetic material could lead to mutagenesis and carcinogenesis as well as other toxic effects (Ramos et al., 2001). For this reason, several

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plant products have been characterized *in vitro* for their anticarcinogenic capacity due to the presence of different substances with antimutagenic activity (González-Avila et al., 2003; Ramirez-Mares et al., 1999). These substances include vitamins, carotenoids, flavonoids, and terpenoids that have been tested for antimutagenic effects in bacterial systems.

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 (Ben Sghaier et al., 2007).

In this study, we evaluated the *in vitro* mutagenic/antimutagenic potentials of *T. ramosissimum* extracts (leaf). Besides, we also determined the antioxidant capacity of *T. ramosissimum* extracts by employing various established *in vitro* systems.

2. Materials and methods

2.1. Plant material

The aerial part of *T. ramosissimum* was collected in January 2005 from the mountainous region of Gafsa in Southeast Tunisia. The plant was identified by Pr. Mohamed Chaieb (Department of Botany, Faculty of Sciences, and University of Sfax, Tunisia) according to the Flora of Tunisia (Pottier-Alaptite, 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.

2.2. Preparation of plant extracts

In order to obtain an extract enriched in total oligomers flavonoids (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 ethyl acetate, 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).

Petroleum ether and ethyl acetate extracts were obtained by Soxhlet extraction (6 h) using 100 g of the powdered leaves and 1 l of solvent. These two types of extract, with different polarities, were concentrated to dryness and each residue was kept at 4 °C. Then, the extracts were resuspended in dimethylsulfoxide (DMSO).

In the present study, three extracts were investigated. The doses of extracts we tested in the Ames test assay were 500, 250 and 50 μg/assay, for the petroleum ether extract 250, 50 and 25 μg/assay, for the ethyl acetate extract and 50, 25 and 12.5 μg/assay for the TOF extract. Whereas the doses tested in the ABTS assay were 4.5, 3.5, 2.5, 1.5 and 0.5 mg/ml and in the DPPH scavenging assay 100, 30, 10, 3, and 1 μg/ml. They are in accordance with previous investigations (Ben Mansour et al., 2007; Hayder et al., 2005; Kilani et al., 2005b), where a number of preliminary dose-finding tests involving a number of plant extracts were conducted. This means that the doses were suitable for testing the majority of the extracts, however, not necessarily all, some extracts may be toxic at one ore more of the applied doses.

2.3. Determination of total polyphenol and flavonoid contents

The polyphenol content of *T. ramosissimum* extracts was quantified by the Folin–Ciocalteau's reagent and was expressed as gallic acid equivalents (Yuan et al., 2005). Aliquots of test samples (100 μl) were mixed with 2 ml of 2% Na_2CO_3 and incubated at room temperature for 2 min. after the addition of 100 μl 50% Folin–Ciocalteau's phenol reagent, the reaction tube was further incubated for 30 min at room temperature, and finally, absorbance was read at 720 nm. Gallic acid was used as the standard for a calibration curve. Polyphenol content of the *T. ramosissimum* extracts was expressed as gallic acid equivalents.

A known volume of the extracts was placed in a 10 ml volumetric flask to estimate flavonoid content according to the modified method of Zhisten et al. (1999). After addition of 75 μl of $NaNO_2$ (5%), 150 μl of freshly prepared $AlCl_3$ (10%) and 500 μl NaOH (1N), the volume was adjusted with distilled water until 2.5 ml. After 5 min incubation, the total absorbance was measured at 510 nm (Kumar and Chattopadhyay, 2007). Quercetin (0.05 mg/ml) was used as standard for constructing a calibration curve.

2.4. Determination of tannins

According to Nwabueze (2007), extraction of tannins in the samples was achieved by dissolving 5 g of extract in 50 ml of distilled water in a conical flask, allowing the mixture to stand 30 min with the shaking of the flask at 10 min intervals and then centrifuging it at 5000 *g* to obtain a supernatant (tannin extract). The extract was diluted to 100 ml in a standard flask using distilled water. Five milliliters of the diluted extract and 5 ml of standard tannic acid (0.01 g/l) were measured into different 50 ml volumetric flasks. One milliliter of Folin–Denis reagent was added to each flask, followed by 2.5 ml of saturated sodium carbonate solution. The solutions were made up to the 50 ml mark with distilled water and incubated at room temperature (20–30 °C) for 90 min. The absorption of these solutions were measured against that of the reagent blank (containing 5 ml of distilled water in place of extract or standard tannic acid solution) in a Spectronic Genesys 10 s, Thermo Electron Corp (Madison, WI, USA) spectrophotometer at a 760 nm wavelength. Tannin content (tannic acid equivalents) was calculated in triplicate, using the following formula:

Sample reading − blank / Standard reading − blank.

2.5. Determination of total sterol content

Twenty milligrams of each extract dissolved in 500 µl of acetone was mixed with 250 µl of a digitonin solution (2% in the alcohol 78%) and heated to 60 °C for reaching half of volume. After cooling to room temperature for 15 min, the precipitate was separated on a weighed filter (M_0). Then, the filter was washed 10 times with water, 10 times with alcohol 78%, once with acetone, once with alcohol 78%, and finally once with anhydrous ether and it was dried for 3 h at 78%. After cooling, the filter was weighed (M_f). Sterol content was expressed according to the following formula:

$$\%\text{Sterols} = (P_{steroids} / P_{extract}) \times 100, \text{where } P_{steroids} = (M_f - M_0) \times 0.25.$$

2.6. Bacterial tested strains

Salmonella typhimurium TA100 (his G46/rfa/Δuvrb/pkM101), *S. typhimurium* TA1535 (his G46/rfa/Δuvrb) and *S. typhimurium* TA98 (his D3052/bio chlD uvrb gal/rfa/pkM101), which are histidine-requiring mutants, were kindly provided by Pr. I. Felzenswalb (Instituto de Biologia Roberto Alcântara Gomes, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil) and maintained as described by Maron and Ames (1983). The genotypes of the test strains were checked routinely for their histidine requirement, deep rough (rfa) character, UV sensitivity (uvrB mutation) and presence of the R factor. They were stored at −80 °C.

S. typhimurium TA100 and TA98 strains are known to be more responsive to aflatoxin B_1 (AFB_1), benzo[a]pyrene (B[a] P) and to 4-nitro-o-phenylenediamine (NOPD) while *S. typhimurium* TA1535 strain which contains the base-pair substitution mutation hisG46 (Zeiger, 1998) is known to be more responsive to certain mutagens such as sodium azide (SA) (Maron and Ames, 1983).

2.7. Salmonella-microsome assay

The mutagenicity assay with *S. typhimurium* was performed as described by Maron and Ames (1983). The test is based on the plate incorporation method, using *S. typhimurium* test strains TA100, TA98 and TA1535 with and without an exogenous metabolic system: S9 fraction in S9 mix. The S9 microsome fraction is prepared from the livers of rats treated with Aroclor 1254 (Maron and Ames, 1983) and stored at −80 °C.

The test strains from frozen cultures were grown overnight for 12–16 h at 37 °C in the Oxoid Nutrient Broth No. 2. Various concentrations of each extract were added to 2 ml of top agar, supplemented with 0.5 mM L-histidine and 0.5 mM D-biotine, mixed with 100 µl of bacterial culture (approximate cell density 2×10^8–5×10^8 cells/ml) and then poured onto a plate containing minimum agar. The plates were incubated at 37 °C for 48 h and his+revertant colonies were counted. The influence of metabolic activation was tested by adding 500 µl of S9 mixture. Negative and positive control cultures gave number of revertants per plate that were within the normal limits found in the laboratory. Data were collected with a mean ± standard deviation of three plates (n = 3).

2.8. Antimutagenicity testing

A modified plate incorporation procedure (Lee et al., 2000) was employed to determine the effect of extracts on NOPD, B[a] P, AFB_1 and SA induced mutagenicity. In brief, 0.5 ml of phosphate buffer was distributed in sterilized capped tubes in an ice bath, then 0.1 ml of test extracts and 0.1 ml of bacterial culture (prepared as described in mutagenicity test) were added. After vortexing gently and preincubating at 37 °C for 30 min, 2 ml of top agar supplemented with 0.05 M L-histidine and D-biotine were added to each tube and vortexed for 3 s. The resulting entire was overlaid on the minimal agar plate. The plates were incubated at 37 °C for 48 h and the revertant bacterial colonies on each plate were counted. The inhibition percentage of mutagenicity (IP) induced by each mutagen, was calculated relative to the number of revertant colonies obtained in the control group treated with the mutagen alone, using the following formula:

$$\text{IP}(\%) = [1-(\text{number of revertants on test plates}-\text{SR}) \div (\text{number of revertants on control plates}-\text{SR})] \times 100.$$

Test plates: plates incubated with mutagen and extract
Control plates: plates incubated with the mutagen alone
SR: spontaneous revertants (test strains incubated in the absence of both extract and mutagen)

2.9.DPPH (2-2-diphenyl-1-picrylhydrazyl) free-radical-scavenging activity

DPPH is a free radical that has a blue-violet color when dissolved in ethanol. The loss of color indicates a radical scavenging activity. In order to measure the antioxidant activity, the DPPH free-radical-scavenging assay was carried out according to the procedure described by Fenglin et al. (2004). An aliquot of each tested compound at various concentrations (100, 30, 10, 3, or 1 µg/ml in ethanol) was mixed with 23.6 µg/ml of DPPH solution in ethanol. After incubation of the mixture for 30 min, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The scavenging activities were expressed as a percentage of the absorbance of the control DPPH solution (Yagi et al., 2002), and the radical scavenging activity was obtained from the following equation:

$$\text{Activity}(\%) = [(A_{517}\text{control} - A_{517}\text{sample}) / A_{517}\text{control}] \times 100.$$

The results were expressed as mean of at least three independent experiments. Results were expressed as percentage activity. Mean inhibiting concentrations IC_{50} were calculated by use of the Litchifield and Wilcoxon test (Galati et al., 2001). Data were collected and expressed as mean ± standard deviation of three independent experiments.

2.10. Radical-scavenging activity on $ABTS^{+\cdot}$

An improved ABTS [2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt] radical cation decolorization assay was used. It involves the direct production of the blue/green $ABTS^{+\cdot}$ chromophore through the reaction between ABTS and potassium persulfate. Addition of antioxidants to the preformed radical cation reduces it to ABTS, to an extent and on a timescale depending on the antioxidant activity, the concentration of the antioxidant, and the duration of the reaction (Re et al., 1999). ABTS was dissolved in water to a 7 mM concentration. $ABTS^{+\cdot}$ was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{+\cdot}$ solution was diluted with ethanol to an absorbance of 0.7 (±0.02) at 734 nm. In order to measure the antioxidant activity of extracts, 10 µl of each sample at various concentrations (0.5, 1.5, 2.5, 3.5, and 4.5 mg/ml) was added to 990 µl of diluted $ABTS^{+\cdot}$ and the absorbance recorded every 1 min. We stop the kinetic reaction when the absorbance becomes stable. Each concentration was analyzed in triplicate. The percentage decrease of absorbance at 734 nm was calculated for each point, the antioxidant capacity of the test compounds was expressed in percent inhibition (%). The percentage scavenging of $ABTS^{+\cdot}$ was calculated by the following formula:

$$\text{Scavenging activity}(\%) = [(A_0 - A_x) / A_0] \times 100.$$

A_0 and A_x were the absorbance at 734 nm of samples without and with extract, respectively.

IC_{50} value was calculated from regression analysis. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard in comparison for the determination of the antioxidant activity of a compound.

2.10.1. Calculation of Trolox equivalent antioxidant capacity (TEAC)

A calibration curve was prepared with different concentrations (standard range 0–15 µM, final concentration) of Trolox. Trolox equivalent antioxidant capacity (TEAC) can be assigned to all samples able to scavenge the $ABTS^{+\cdot}$ by comparing their scavenging capacity to that of Trolox, a water-soluble vitamin E analog. This index is defined as the millimolar concentration of a Trolox solution whose antioxidant capacity is equivalent to 1.0 mg of the extract (Antolovich et al., 2002).

2.11. Statistical analysis

Data were collected and expressed as the mean±standard deviation of three independent experiments and analyzed for statistical significance from control. 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 $P<0.05$. The IC_{50} values and the correlation coefficients between studied parameters were demonstrated by linear regression analysis.

3. Results and discussion

3.1. Phytochemical study and metabolite content of T. ramosissimum extracts

Phenolic compounds are very important plant constituents because they exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals (Pokorny, 2001). Flavonoids are phenolic compounds, which are very effective antioxidants (Yanishlieva-Maslarova, 2001). The Folin–Ciocalteu method is a rapid and widely-used assay, to investigate the total phenolic content but it is known that different phenolic compounds have different responses in the Folin–Ciocalteu method (Kahkonen et al., 1999).

Therefore, in this work, the total polyphenol content of the extracts was expressed as gallic acid equivalents (Capecka et al., 2005) following confirmation of linearity of the response of the assay using the extract. The total flavonoid content of the *T. ramosissimum* extracts was determined by the method of Zhishen et al. (1999).

Table 1 shows the percent yield of tested extracts. The highest (5.79%) with the PE extract and the lowest (0.128%) with the TOF extract. The different tested extracts showed the presence of various quantities of tannins, coumarins, sterols and particularly, flavonoids. The total polyphenol, flavonoid, tannin and sterol contents are reported in Table 2.

In fact, the flavonoid and polyphenolic contents in 1 mg of EA extract were respectively, equivalent to 835 µg of quercetin and 306.66 µg of gallic acid. Yet the flavonoid and polyphenolic contents in 1 mg of TOF extract were, respectively, equivalents to 662.5 µg of quercetin and 315 µg of gallic acid. The percentage of sterol content in PE and EA extracts was respectively 18.6% and 12%. Moreover, the tannin content in EA extract was equivalent to 123.05 µg of tannic acid.

3.2. Mutagenic activity of extracts

In a series of experiments preceding the antimutagenicity studies, it was ascertained that the different amounts of extracts added to the indicator bacteria should not have a toxic effect on the used bacteria, and mutation frequencies do not change significantly when compared with spontaneous mutation frequencies. The results of Ames test with and without metabolic activation are reported in Table 3. None of the tested extracts induced a significant increase in the revertant number in TA98, TA100 and TA1535 strains even with or without the S9 metabolic system.

In this initial report we demonstrated that none of the evaluated extracts induced frameshift mutations on *S. typhimurium* TA98, base pair substitutions on *S. typhimurium* TA100 and TA1535. These results encourage further studies in eukaryotic models to confirm their lack of genotoxicity, above all because a number of plants are known to possess mutagenic

and toxic constituents (Ansah et al., 2005; Fernandes and Vargas, 2003).

The evaluation of bacterial mutagenicity is of particular importance as an initial test for complex mixtures because of the possibility that one or more components can be positive (Lee et al., 2005; Reid et al., 2006). On the other hand, a protective action of plants or their metabolites on genetic material has been reported, leading to its repair or to preserve its integrity (Abdelwahed et al., 2007; Ben Ammar et al., 2008; Hayder et al., 2005; Kilani et al., 2005).

3.3. Antimutagenic activities of extracts

Results reported in Figs. 1, 2, 3, 4 and 5, show the protective effect of *T. ramosissimum* extracts on the reversion potential of the mutagens tested. All extracts were effective in reducing the number of frameshift mutations induced by the direct genotoxicant NOPD (10 μg/plate) and the indirect genotoxicant B[a] P (5 μg/plate) in strain TA98, as well as the base pair substitution induced by the direct acting agent SA (1.5 μg/plate) and indirect acting agent AFB1 (0.5 μg/plate) in strains TA100 and TA1535. The highest antimutagenic effect was determined in the TOF extract (70.41%), and the lowest effect (35.13%) was determined in the presence of EA extract against 10 μg/plate of NOPD in strain TA98 (Fig. 1), whereas the same extract showed a significant antimutagenic effect against SA in the TA100 assay system (83.22%) (Fig. 2). In opposite, in the presence of the TA100 assay system, against 1.5 μg/plate of SA mutagen, EA extract exhibited an inverse dose dependent effect with a higher maximal inhibition percentage of 83.22% compared to TOF extract (49.47%) which showed a dose dependent effect. In the indirect systems against AFB1, all extracts were highly efficient, a reduction of 86.36–98.54% with respect to the observed level of the positive control (Fig. 4). Moreover, EA and PE extracts showed the most important antimutagenic effect against the indirect B[a]P mutagen in the TA98 assay system in an inverse dose dependent manner (92.25%) at respectively 25 and 50 μg/plate. Whereas when using the TA98 assay system, TOF extract increased the mutagenicity induced by B[a] P (Fig. 5).

Two explanations for such protection against mutagen induced damage: one is that the plant extracts may adsorb the mutagens in a way similar to the carcinogen adsorption which has been associated with pyrrole pigments, such as hemin and chlorophyllin (Ferguson et al., 2004; Ikuma et al., 2006); another is that the extracts could induce DNA glicosylase enzymes which are capable of repairing alkylating DNA bases (Steele and Kelloff., 2005). Extracts may also inhibit microsomal enzyme activation or they may directly protect DNA from the electrophilic B[a]P epoxide, 7,8-dihydroxy 9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, a putative ultimate carcinogenic metabolite (Harris et al., 2003) and other intermediates of the mutagen. In fact several metabolic intermediates and ROS formed during microsomal enzyme activation also are capable of breaking DNA strands.

Thus, an alteration in the function of the enzyme may result in altered reaction rates and differential pathways of the metabolism of mutagens and carcinogens; in some cases, this modification provides protection against chemically induced mutagenesis. Indeed, this effect is known to play a role in the antimutagenic effect of some plant extracts (Horn and Ferrao, 2003). Moreover, anticarcinogenicity of flavonoids contributes to block the formation of carcinogen (Edenharder and Grûnhage, 2003; Ferguson et al., 2004). However, the extracts may also directly protect DNA from the electrophilic metabolite of the mutagen. In fact, flavonoids provide strong nucleophilic centers, which enables them to react with electrophilic mutagens and form adducts that may result in the prevention of genotoxic damage (Marnewick et al., 2000).

Antimutagenic activity of the tested extracts may be ascribed to flavonoids (Calomme et al., 1996), tannins (Barrato et al., 2003) and total polyphenols (Ben Ammar et al., 2008). We cannot, however, exclude the possibility that other compounds with antimutagenic properties participate in the inhibitory effect of mutagens.

3.4. Scavenging effect on DPPH radical

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples (Nagai et al., 2003; Skandrani et al., 2007). The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating ability. The DPPH radical is a stable free radical and the DPPH radical-scavenging activity was determined by the decrease in absorbance at 517 nm, due to reduction by the antioxidant (AH) or reaction with a radical species, as shown in the Eqs. (1) and (2) (Gordon, 2001).

$$DPPH^{\cdot} + AH \rightarrow DPPH{-}H + A^{\cdot} \quad (1)$$

Table 1
Phytochemical screening of extracts from *T. ramosissimum* aerial parts.

Extracts	Yield(%w/w)	Tannins	Flavonoids	Coumarins	Sterols
PE	5.79	–	–	–	++
EA	2.6	++	++++	++	+
TOF	0.128	+	++++	++	–

– = not detectable; + = low quantities; ++ = average quantities; ++++ = high quantities.
PE: petroleum ether, EA: ethyl acetate, TOF: Total oligomers flavonoids.

Table 2
Quantitative polyphenol, flavonoid, tannins and sterol contents of extracts from *T. ramosissimum* aerial parts.

Metabolites	Extracts		
	PE	EA	TOF
Total polyphenols (gallic acid equivalents)[a]	–	306.66±8	315±9
Flavonoids (quercetin equivalents)[a]	–	835±11	662.5±13
Tannins (tannic acid equivalents)[a]	–	123.05±7	–
Sterols (%)	18.6±3	12±1.5	–

PE: petroleum ether, EA: ethyl acetate, TOF: total oligomers flavonoid.
[a] Means of three experiments.

Table 3
Mutagenic study of *Teucrium ramosissimum* extracts by the *S. typhimurium* TA1535, TA100 and TA98 assay systems in the presence and absence of the metabolic activation system (S9).

		TA1535		TA100		TA98	
Extracts	Dose μg/plate	−S9	+S9	−S9	+S9	−S9	+S9
EA	25	15.5±1.5	21.5±2.5	127.5±4.5	150±2	19.5±1.5	39±3
	50	13±1	18±3	133±5	160±3	16±1	35±3
	250	14.5±1.5	15±1.5	139±5	176±4	20±1	33±2
TOF	12.5	13±1	18.5±2.5	128±1	160±2	22±1	29.5±1
	25	12±00	21.5±2.5	130.5±8.5	144±3	17.5±1.5	35±2
	50	17±2	29.5±2.5	140±5	132±3	22.5±1.5	45±2
EP	50	21±3	28.5±2.5	112.5±6	291±4	22.5±1.5	38±2
	250	23±2	31.5±2.5	105±5	186±2	28.5±4	41±1
	500	17±2	35±2.5	98.5±3	168±4	36±1.5	50±4
Spontaneous revertants		11.5±0.5	19.5±1.5	127.5±0.5	159±2	15.5±0.5	33±2
PC		421±1	438±3	883±7	1370±4	1002±6	192±5

Positive control (PC): TA100/−S9 and TA 1535/−S9, SA (1.5 μg/plate); TA100/+S9, AFB1 (0.5 μg/plate); TA 1535/+S9, 2AA (1 μg/plate); TA98/−S9 NOPD (10 μg/plate); TA98/+S9 B[a]P(5 μg/plate).

$$DPPH^{\cdot} + A^{\cdot} \rightarrow DPPH{-}A^{\cdot} \quad (2)$$

The DPPH radical-scavenging capacity was reported after 30 min reaction time for each diluted plant extract. For each sample, five concentrations (μg/ml) were tested.

Fig. 6 shows the dose-response curve of the DPPH radical-scavenging effect of the three tested extracts from *T. ramosissimum*. It was found that the radical-scavenging activities of all extracts increased with increasing concentration. The scavenging effect level was in the following order: TOF (88.64%)>EA (72.39%)>PE (0.91%) at a concentration of 100 μg/ml. The half-inhibition concentrations (IC_{50}) of DPPH radical in the presence of TOF and EA extracts were respectively 10.5 μg/ml and 13 μg/ml. Therefore TOF extract exhibited the highest DPPH-scavenging activity, while PE extract showed the weakest effect. A comparison with the commercial antioxidant showed that the concentration needed to obtain 88.64% DPPH radical-scavenging activity for vitamin E was 7.5 μg/ml, which was equal to the scavenging effect of 100 μg/ml of TOF extract; vitamin E had an IC_{50} value of 3.1 μg/ml. Although the DPPH radical-scavenging abilities of the extracts at the doses of 1, 3 and 10 μg/ml were significantly lower than that of vitamin E.

Extracts did show hydrogen-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. The high antiradical activity of TOF and EA extracts reflects the high phenolic content (respectively 315±9 μg and 306.66±8 μg gallic acid equivalents/ml) and flavonoid content (respectively 662.5±13 μg and 835±11 μg quercetin equivalents/ml) (Table 2).

3.5. ABTS radical cation scavenging activity

The $ABTS^{+\cdot}$ radical formed as follows $ABTS\text{-}e^{-} \rightarrow ABTS^{+\cdot}$ reacts quickly with electron/hydrogen donors to form colorless ABTS. The reaction is pH-independent. A decrease of the $ABTS^{+\cdot}$ concentration is linearly dependent on the antioxidant sample concentration, including Trolox as a calibrating standard (Van den Berg et al., 2000).

The results revealed that EA and TOF extracts present antioxidant efficiency against the $ABTS^{+}$ radical with respectively IC_{50} of 0.21 mg/ml and 0.15 mg/ml.

Decolorization of $ABTS^{+\cdot}$ also reflects the capacity of antioxidant species to donate electrons or hydrogen atoms to

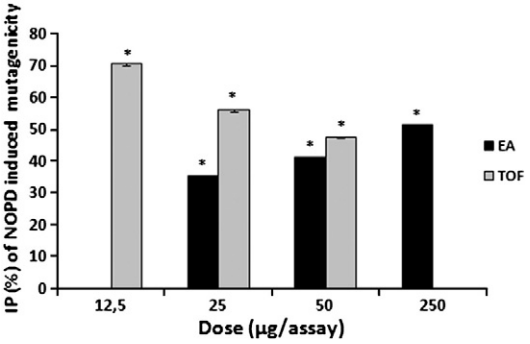

Fig. 1. Inhibitory effect of *T. ramosissimum* extracts on the mutagenicity induced by 4-nitro-o-phenylenediamine (NOPD) (10 μg/plate) in the *S. typhimurium* TA98 assay system in the absence of S9. EA (ethyl acetate), TOF (total oligomers flavonoids). Symbols represent statistical significance from control (*p<0.05). IP: Inhibition percentage.

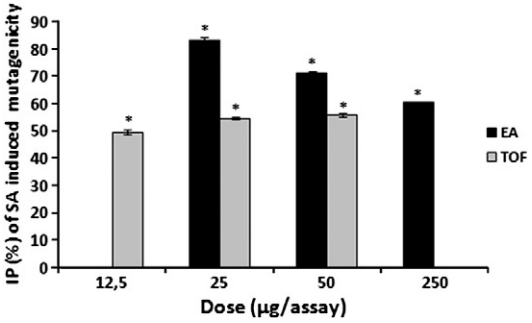

Fig. 2. Inhibitory effect of *T. ramosissimum* extracts on the mutagenicity induced by sodium azide (SA) (1.5 μg/plate) in the *S. Typhimurium* TA100 assay system in the absence of S9. EA (ethyl acetate), TOF (total oligomers flavonoids). Symbols represent statistical significance from control (*p<0.05). IP: Inhibition percentage.

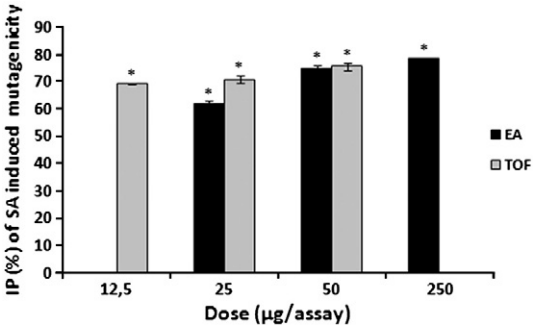

Fig. 3. Inhibitory effect of *T. ramosissimum* extracts on the mutagenicity induced by sodium azide (SA) (1.5 µg/plate) in the *S. typhimurium* TA1535 assay system in the absence of S9. EA (ethyl acetate), TOF (total oligomers flavonoids). Symbols represent statistical significance from control (*p<0.05). IP: Inhibition percentage.

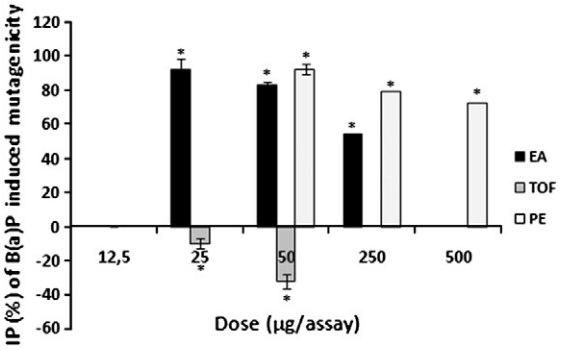

Fig. 5. Inhibitory effect t of *T. ramosissimum* extracts on the mutagenicity induced by benzo[a]pyrene (B[a]P) (5 µg/plate) in the *S. typhimurium* TA98 assay system in the presence of S9. EA (ethyl acetate), TOF (total oligomers flavonoids). Symbols represent statistical significance from control (*p<0.05). IP: Inhibition percentage.

inactivate this radical cation. In the ABTS decolorization assay, potential activity was noted at 4.5 mg/ml of all extracts studied (Fig. 7). *T. ramosissimum* is a complex mixture of several compounds, in particular phenolic compounds with a diversity of chemical structures that gives them particular properties of reaction and solubility.

Some authors have described biphasic reaction kinetics between the $ABTS^{+\cdot}$ radical and some polyphenols in foods (Van den Berg et al., 2000; Villaño et al., 2004), and we have also observed the same phenomenon when studying the scavenging capacity of all extracts tested. This reaction pattern consists of initial fast scavenging activity where the more active compounds react immediately with the radical. Reaction products are formed and together with the less reactive molecules give a second, slow reaction. Results obtained with the extracts corroborate this type of kinetic behavior in all samples and dilutions assayed. An example can be seen in Fig. 8. The first 30 min corresponds to the "fast" scavenging activity, while the final point of stabilization time (150 min) is known as the "total" scavenging activity. The magnitude also depends on the dilution assayed. In the case of TOF extract, percentage of inhibition at a stabilization time (99.8%) is higher than the percentage of inhibition at 30 min, when the dilution is 4.5 mg/ml. However, if the dilution is only 0.5 mg/ml, this percentage drops to 84.43%.

The TEAC of different extracts was also calculated. The TEAC values reflect the relative ability of hydrogen or electron-donating antioxidants of a sample to scavenge the $ABTS^{\cdot+}$ compared with that of Trolox. When referring to TEAC values, TOF seems to be the more potent antioxidant with TEAC value of 2.45 Mm. It appears that the highest antiradical activity of TOF and EA extracts reflects their high phenolic (315±9 µg and 306.66±8 µg gallic acid equivalents/ml) and flavonoid contents (662.5±13 µg and 835±11 µg quercetin equivalents/ml) (Table 2).

These results correlate very well with other investigations by Heim et al. (2002) revealing antiradical and antioxidant activities of small phenolics including flavonoids and phenolic acids. Likewise, Hagerman et al. (1998) have reported that the high molecular weight phenolics (tannins) have more ability to quench free radicals ($ABTS^{\cdot+}$) and that effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl groups substitution than the specific functional groups. On the other hand, the formation of tannin–protein complexes, both in insoluble and soluble complexes, as the result of conventional food processing, have also been shown to

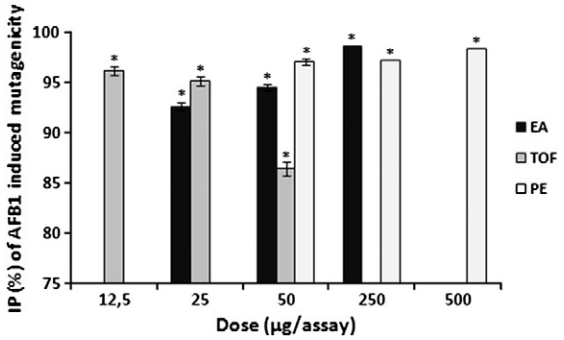

Fig. 4. Inhibitory effect of *T. ramosissimum* extracts on the mutagenicity induced by aflatoxin B1 (AFB1) (0.5 µg/plate) in the *S. typhimurium* TA100 assay system in the presence of S9. EA (ethyl acetate), TOF (total oligomers flavonoids). Symbols represent statistical significance from control (*p<0.05). IP: Inhibition percentage.

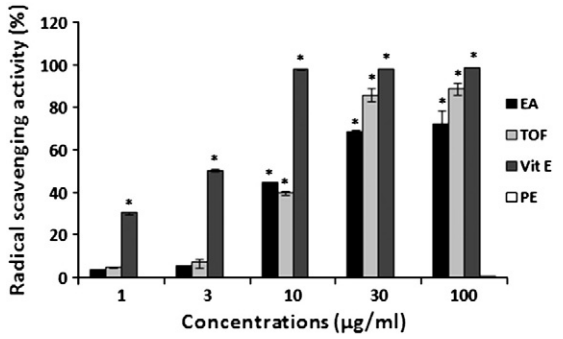

Fig. 6. Radical scavenging activity of *T. ramosissimum* extracts by DPPH method at different concentrations. EA (ethyl acetate), TOF (total oligomers flavonoids), PE (petroleum ether) and Vit. E (vitamin E). Symbols represent statistical significance from control (*p<0.05).

be potential free radical scavenger and radical sinks. Moreover, such complexes can also be suggested as one of the nutraceutical contributors to prevent the free radical mediated diseases occurring in the gastrointestinal tract (Riedl and Hagerman, 2001).

3.6. Correlation of the different activities with the total polyphenol and flavonoid content

It is interesting to observe the correlation between the phenolic content and antioxidant activity of plant extracts, since phenolic compounds contribute directly to antioxidant activity (Duh, 1999). This study, allowed us to establish a significant correlation between studied parameters (total polyphenolic content, total flavonoid content, antimutagenic, and antiradical activities) in selected *T. ramosissimum* plant parts. This correlation was demonstrated by linear regression analysis. With reference to Fig. 9 and Fig. 10, the correlation values of the total polyphenol and flavonoid content with the different activities were satisfactory ($r>0.75$). As far as a strong correlation was detected between flavonoid and total polyphenol content on one side and inhibitory percentage of induced mutagenicity on the other side. Correlation coefficient values between flavonoid and polyphenol contents, and antimutagenic activity against NOPD, SA, AFB1 and B(a)P in strains TA98, TA100 and TA1535, were respectively 0.96, 0.89, 0.75, 0.97 and 0.99 for EA extract and 0.94, 0.85, 0.99, 0.97 and 0.99 for TOF extract. This is in accordance with results reported by some authors who described a correlation between polyphenols, flavonoids and antimutagenic activity measured by various methods (Ben Ammar et al., 2008; Calomme et al., 1996; Kilani-Jaziri et al., 2009).

When the relationship between total phenolic content and total flavonoid content of all extracts was plotted as shown in Fig. 9 (e, f), the correlation coefficient (r) between these two parameters was about 1 indicating that there is a significant positive relationship between the total phenolic and flavonoid contents of all plant extracts selected in this study.

The Folin–Ciocalteu method for the determination of polyphenolic compounds is, such as the methods of antioxidant activity determination, based on redox properties of the compounds, thus, the values could partially express the antioxidant activity. This is confirmed by the highly significant correlation between the values of Folin–Ciocalteu method and the values of individual methods for antioxidant activity. Our results prove that the content of phenolic compounds and antiradical activity correlate very well ($r=0.75$ and $r=0.86$ for EA extract and $r=0.8$ and $r=0.76$ for TOF extract) for the tested *T. ramosissimum* extracts.

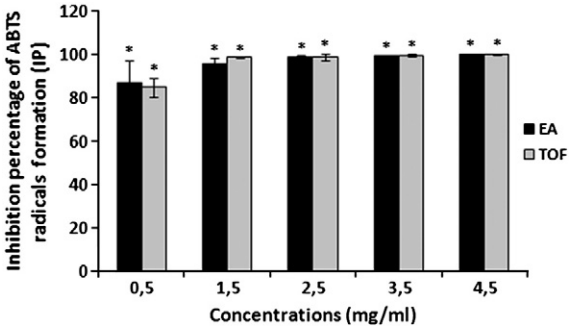

Fig. 7. Concentration-dependent ABTS free radical scavenging activity of *T. ramosissimum* extracts. EA (ethyl acetate), TOF (total oligomers flavonoids). Symbols represent statistical significance from control (*$p<0.05$).

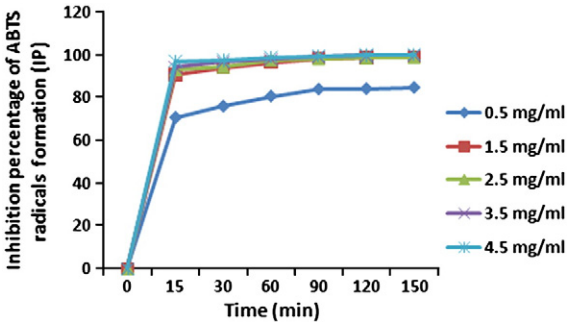

Fig. 8. Extract concentration and time-dependent ABTS free radical scavenging activity of TOF extract.

3.7. Correlation of the different activities

In a comparison of methods used in this study, all the methods showed the capability to determine the antiradical and antimutagenic activities of *T. ramosissimum* extracts. Nevertheless, the three methods are capable of prescreening antiradical and antimutagenic activities. As shown in Table 4, a direct correlation between the three methods was demonstrated by linear regression analysis. The strong correlation between the mean values of ABTS radical cation scavenging activity and antimutagenic activity ($r=0.99$ and $r=0.75$), between DPPH scavenging effect and ABTS radical cation scavenging activity ($r=0.99$ and $r=0.89$) and between DPPH inhibitory percentage and antimutagenic activity ($r=0.99$ and $r=0.93$) for TOF and EA extracts deserves detailed attention. This could be explained from the basic concept that antioxidants are reducing agents. The results suggest that the reducing ability of polyphenols seems to be an important factor dictating free radical-scavenging capacity of these compounds.

In this study, we suspect an eventual correlation between antiradical and antimutagenic effects of *T. ramosissimum* extracts. Antioxidant potential expressed by the different extracts may provide a common mechanism for inhibiting the genotoxicity of both direct and indirect tested mutagens. In fact, phenolic compounds are ubiquitous in fruit, vegetables, and nuts, several of which have been reported to be inhibitors of chemical carcinogenesis (Fiala et al., 1985; Stich, 1991). A wide range of phenolics derived from herbs and spices possesses potent antioxidant, anti-inflammatory, antimutagenic, anticarcinogenic and anti-tumor activities, which contribute to their chemopreventive potential (Surh, 2002). It has been observed that many plant polyphenols such as ellagic acid, catechins and chlorogenic, caffeic and ferulic acids act as potent

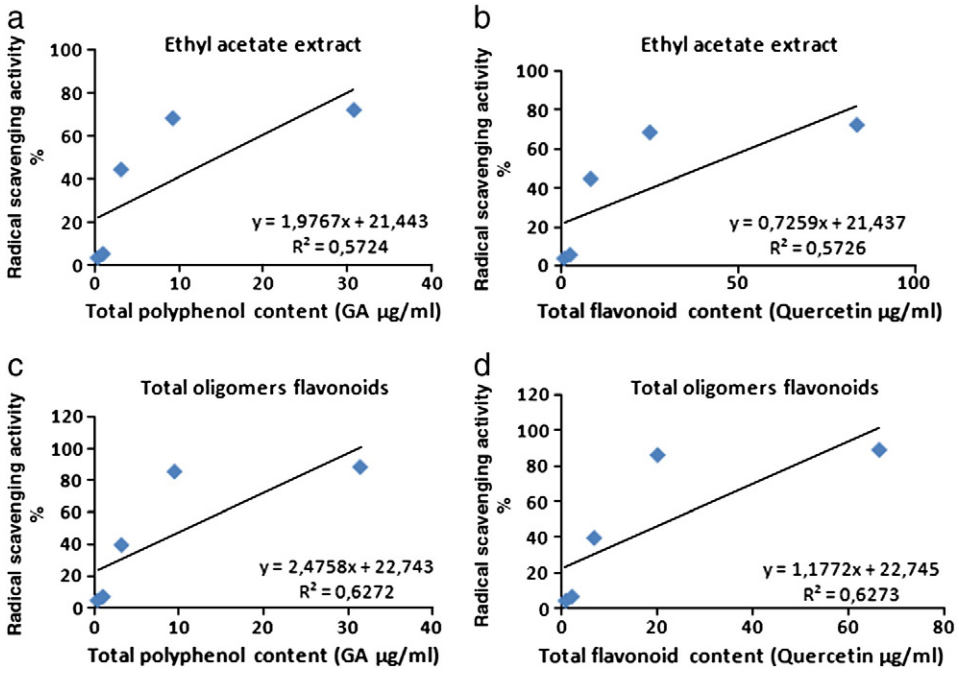

Fig. 9. Correlation between total polyphenol content (a, c), total flavonoid content (b, d) and their scavenging activity by DPPH method of ethyl acetate and total oligomer flavonoid extracts.

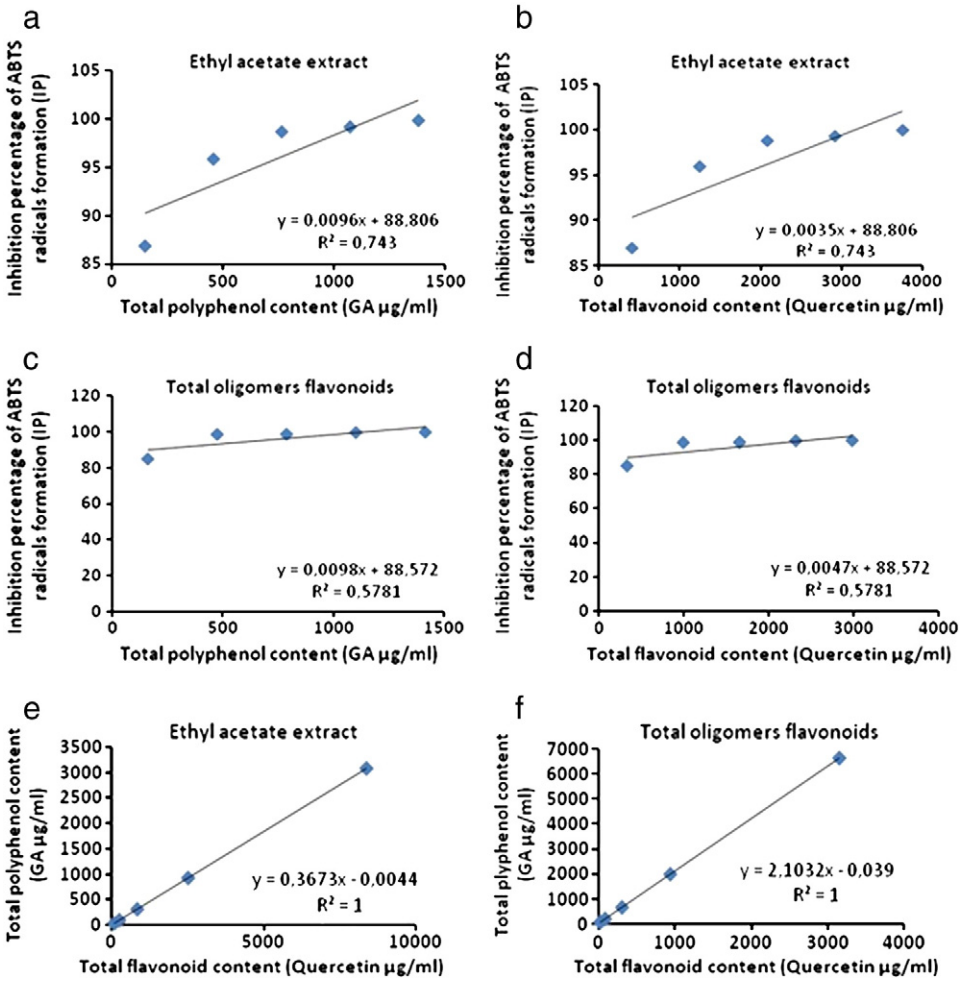

Fig. 10. Correlation between total polyphenol content (a, c), total flavonoid content (b, d) and radical scavenging activity on $ABTS^{+\cdot}$ and total polyphenol content and total flavonoid content (e, f) of ethyl acetate and total oligomer flavonoid extracts.

Table 4
Correlation coefficients, "r", for relationships between different assays.

Extracts		DPPH assay	ABTS assay	Antimutagenic activity (without S9)	Antimutagenic activity (with S9)
EA	DPPH assay	–	0.89	0.93	0.82
	ABTS assay	–	–	0.99	0.99
TOF	DPPH assay	–	0.99	0.99	0.66
	ABTS assay	–	–	0.99	0.75

antimutagenic and anticarcinogenic agents (Edenharder and Tang, 1997).

4. Conclusion

The present study has demonstrated that some *T. ramosissimum* extracts possess potent antiradical and antimutagenic activities, which could be derived from compounds such as flavonoids and polyphenols. The antimutagenic activity could be ascribed, at least in part, to their antioxidant properties but we cannot exclude other additionally mechanisms. These antiradical and antimutagenic activities could have contributed, at least partly, to the therapeutic benefits of certain traditional claims. The results presented here could be an additional argument to support the use of this species in the North African tradition medicine. Furthermore, *T. ramosissimum* extracts could give rise to antimicrobial, anti-inflammatory, anticancer and antiulcer agents and could be promising candidates for further studies designed to obtain more evidence on their components with potential chemo-preventive activity.

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