

In vitro* evaluation of pharmaceutical activities of *Teucrium ramosissimum

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ABSTRACT

Here a biological investigation of various extract from *T. ramosissimum* (Family-*Lamiaceae*), an endemic species from North Africa. Several components were extracted from the aerial parts using hexane, ethyl acetate, acetone and ethanol. These extracts were then investigated for their effect on two cancer cell lines (MCF-7 and HCT-116). Cytotoxicity of these extracts was measured using a colorimetric MTT assay. The results showed that the component extracted with ethanol, was the most cytotoxic on all cancer cells tested ($IC_{50}=6$ and $17\mu\text{g/ml}$ for MCF-7 and HCT-116 cell lines, respectively). Furthermore, these extracts were screened for their anti-inflammatory activity against 5-lipoxygenase enzyme and for their anti-Alzheimer activity against the acetylcholinesterase enzyme. All extracts (hexane, ethyl acetate, acetone and ethanol) showed a low inhibition level on 5-lipoxygenase enzyme, 19.1, 39.7, 40.6 and 23.76%, respectively (at $60\mu\text{g/ml}$). In the anti-Alzheimer activity at $60\mu\text{g/ml}$, the percentage of inhibition does not exceed 20%.

Key words: *Teucrium ramosissimum*; 5-lipoxygenase; Ach E; MCF-7; HCT-116.

INTRODUCTION

Natural plant extracts have acquired increasing interest among consumers, the minimal side effects and the effectiveness in treating some serious diseases enhanced the use of these natural products which could be an additional option to traditional medicine (Newman et al., 2003). The genus *Teucrium*, which is one of the most common species in the *Lamiaceae* family, comprises approximately 300 species, 100 of them are wide spread in the Mediterranean basin (Boulos, 2002). It has been commonly used in herbal medicine for its medicinal and biological properties such as antitumor (Rasheed et al., 1995), anti-ulcer (Galati et al., 2000), anti-inflammatory (Barrachina et al., 1995) and antibacterial activities (Monthana et al., 2009).

T. ramosissimum (*Lamiaceae*) is a perennial herb that is endemic in Tunisia flora and known as “hachichetbelgacem ben salem” (Hachicha et al., 2007). This

Mediterranean plant is useful in traditional herbal medicine for its cicatrizing properties, for the treatment of intestinal inflammation and gastric ulcer (Ben Sghaier et al., 2011a). Previous reports on the species of *T. ramosissimum* or its essential oils deal with its importance in the food and drug industries and its biological activities (Ben Sghaier et al., 2011b). Following the traditional use of this plant, we chose to study the activity of the extracts to bring more information of pharmaceutical potential for this species. The main aim of this study is to investigate, for the first time, the anti-cancer effect of *T. ramosissimum* extracts on the growth of two human tumor cell lines (MCF-7 and HCT-116) as well as the *in vitro* anti-Alzheimer (acetylcholinesterase (AChE)) and anti-inflammatory (5-lipoxygenase) activities.

MATERIALS AND METHODS

Chemicals used: All chemicals used were of analytical reagent grade. All reagents were purchased from Sigma-Aldrich-Fluka (Saint-Quentin France).

Plant material: The fresh areal parts of *T. ramosissimum* were collected in March 2011 from Orbata Mountain near of Gafsa, in the southwest part of Tunisia, and was authenticated by Dr Ridha El Mokni (laboratory of botany and plant ecology, department of life sciences, faculty of sciences of Bizerte) where a voucher specimen was deposited [LAM/52-T.ram/003]. Fresh aerial parts were dried in the dark and at room temperature, grounded to fine powder and stored at 4°C in the absence of light in a tightly closed container.

Preparation of plant extracts: The powders (9g) were extracted successively with 150ml of solvent (hexane, ethyl acetate, acetone and ethanol) by maceration for 48h and filtered with a whatman No. 1 (England) filter paper. The filtrates were then concentrated using rotary evaporation under vacuum at 35°C and then stored at 4°C.

Anti-inflammatory activity: Four extracts of *T. ramosissimum* aerial parts were examined for their 5-lipoxygenase inhibitory activity using the method cited by Bekir et al. (2013). Briefly, all extracts were dissolved in the DMSO (5%) at 60µg/ml. In a 96-well microplate, were added in order, 150µl of sodium phosphate buffer (pH 7.4), 20µl of different samples, 60µl of the substrate linoleic acid and 20µl of enzyme (500U/ml) which was mixed. After an incubation period of 10 min at room temperature, the absorbance at 234nm was recorded as $A_{(sample)}$, using a spectrophotometer (Thermofisher scientific multiskan GO). A blank experiment was also carried out applying the same procedure to a solution without the test material and the absorbance was recorded as $A_{(blank)}$. The anti-inflammatory activity of each solution was then calculated as percent inhibition according to the following equation: % inhibition = $100 (A_{(blank)} - A_{(sample)}) / A_{(blank)}$. All measurements were performed in triplicate. Nordihydroguaiaretic acid (NDGA) was used as positive control.

Acetylcholinesterase inhibition assay: The Ellman's method, as mentioned by Bekir et al. (2013), was applied for the assessment of the AChE assay. A reaction mixture of 50µl of assay buffer (0.1M di-sodium hydrogenophosphate, pH 8), 25µl of AChE (2.8U/ml), 25µl of the tested samples (hexane and ethyl acetate were dissolved in 5% DMSO; acetone, ethanol and galanthamine (positive control) were dissolved in 10% EtOH) and 125µl of the DTNB (3mM) were added in a 96-well microplate and incubated at room temperature for 15min. The reaction was initiated by addition of 25µl of the substrate acetylthiocholine iodide (ATI, 15mM). The absorbance was measured at 412nm after 10min using a spectrophotometer (Thermo fisher scientific multiskan GO).

Inhibitory activity was calculated as percent inhibition according to the following equation:

$$\% \text{ Inhibition} = 100 \times (1 - S/E),$$

- Where E and S were the respective enzyme activity without and with the test sample, respectively.
- Data presented here are the average of three replicates.

Cytotoxicity: In order to assess the anticancer activity of the *T. ramosissimum* extracts, the following human cancer cell lines were used: MCF-7 for human breast adenocarcinoma pleural effusion and HCT-116 for human colon carcinoma. The cell growth was estimated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described by Bekir et al. (2013). Cells were grown in RPMI-1640 medium at 37°C under 5% CO₂ in a humidified incubator. Cells were harvested, counted (3 x 10⁴ cells/well in 100µl) and transferred into a 96-well plate, and incubated for 24h prior to the addition of extracts. Serial dilutions of test samples were prepared by dissolving compounds in DMSO followed by dilution with RPMI-1640 medium to give final concentration at 1, 10 and 100µg/ml. Stock solutions of samples were prepared: cell lines at 90µl and samples at 10µl incubated for 48h. MTT solution at 5mg/ml was dissolved in 1ml of Phosphate Buffer Solution, and 50µl of it was added to each of the 96 wells. The wells were wrapped with aluminum foil and incubated at 37°C for 3h. The solution in each well containing media, unbound MTT and dead cells were removed by suction and 50µl of DMSO was added to each well. Then, the plates were shaken and optical density was recorded using a micro plate reader at 540nm. Positive control being doxorubicin and DMSO was used as solvent control. Controls and samples were assayed in duplicate for each concentration and replicated three times for each cell line. Extracts' cytotoxicity activity was expressed as IC₅₀, defined as the concentration of the test material required causing a 50% reduction in growth (cell number) for each cell lines.

Statistical analysis: All data were expressed as Mean ± Standard deviations of triplicate measurements. The confidence limits were set at *P* < 0.05. Correlations were carried out using the correlation and regression modes with EXCEL software.

RESULTS

Anti-inflammatory activity: Data (Table 1) showed the anti-inflammatory activity of *T. ramosissimum* extracts (aerial parts) against the 5-lipoxygenase enzyme at 60µg/ml. The best inhibiting capacity were recorded with acetone extract and ethyl acetate extract. For the others, we observed a lower inhibition.

Anti-acetylcholinesterase activity: The poorest results were obtained with the hexane and ethyl acetate extracts (Table 1), they were found to exert insignificant anti-Alzheimer effect (≤1%) at 50µg/ml. Acetone and ethanol extracts exhibited a lowinhibitory activity (5–25%) at 50µg/ml.

Table-1: Anti-acetylcholin esterase activity of *T. ramosissimum* extracts.

Extract	5-lipoxygenase inhibition		AChE inhibition	
	Dose (µg/ml)	Inhibition (%)	Dose (µg/ml)	Inhibition (%)
Hexane	60	19.10±1.66	50	n.i
Ethyl acetate	60	39.74±4.64	50	n.i
Acetone	60	40.56±5.45	50	20.0±2.3
Ethanol	60	23.70±0.69	50	8.0±3.4
NDGA	5	88.45±8.87		
Galanthamine			30	97.5±1.6

- Results are reported as the means ± SE (n=3), (*P* < 0.05), n. i.: no inhibition (inhibition≤1%).

Cytotoxic activity: The *in vitro* cytotoxicity assay of *T. ramosissimum* aerial parts against two cells lines was evaluated (**Table 2**). Results showed that ethanol extract was the most active fraction on all cancer cells tested (IC_{50} were 6 and 17 μ g/ml for MCF-7 and HCT-116 cell lines, respectively). The acetone extract exhibited no significant activity against HCT-116 achieving an IC_{50} value >100 μ g/ml but it has a good activity against MCF-7 (IC_{50} =14 μ g/ml). Whereas, the hexane and ethyl acetate extracts have a moderate activity, IC_{50} values for both of them were between 21 and 62 μ g/ml.

Table-2: The cytotoxic effect of *T. ramosissimum* extracts on MCF-7 and HCT-116 cell lines.

Extracts	IC_{50} (μ g/ml)	
	MCF-7	HCT-116
Hexane	62 \pm 5	51 \pm 3
Ethyl acetate	39 \pm 3	21 \pm 1
Acetone	14 \pm 1	>100
Ethanol	6 \pm 0	17 \pm 2
Doxorubicin	0.21 \pm 0.03	0.10 \pm 0.01

- Results are reported as the means \pm SE (n=3), ($P < 0.05$).

Chemical composition of *T. ramosissimum* extracts: The composition of *T. ramosissimum* extracts for phenolics, flavonoids, tannins and anthocyanins was evaluated and presented in **Table 3**. Results showed that the amount of phenolics varied in the different extracts (3.0-42.7 GAE mg/g of dry mass). The highest quantity was in ethanol extract (42.7 \pm 1.7 GAE mg/g of dry mass), followed by acetone extract (27.7 \pm 0.9 GAE mg/g of dry mass), ethyl acetate extract (16 \pm 0.4 GAE mg/g of dry mass). Hexane extract was poor in phenolics (3 \pm 0.1 GAE mg/g of dry mass). Ethyl acetate extract possesses the most important amount of flavonoids compounds (18.4 \pm 0.7 QE mg/g of dry mass) follow-up by acetone extract (16.2 \pm 0.6 QE mg/g of dry mass), ethanol extract (9.6 \pm 0.4 QE mg/g of dry mass) and hexane extract (1.1 \pm 0.0 QE mg/g of dry mass). Concerning tannins they were abundant in ethyl acetate extract (59.1 \pm 2.2 CE mg/kg of dry extract), hexane extract (18.3 \pm 0.6 CE mg/kg of dry mass) and ethanol extract (16.0 \pm 0.9 CE mg/kg of dry mass). No tannins were detected in acetone extract. Anthocyanins were detected in small quantities in all extracts, (0.04-1.93mg cyanidin-3-glucoside equivalent/kg of dry mass).

Table-3: Chemical composition of *T. ramosissimum* extracts*.

Extracts	Phenolics (GAE) ^a	Flavonoids (QE) ^a	Tannins (CE) ^b	Anthocyanins (C3GE) ^b
Hexane	3 \pm 0.1	1.1 \pm 0.0	18.3 \pm 0.6	0.70 \pm 0.18
Ethyl acetate	16 \pm 0.4	18.4 \pm 0.7	59.1 \pm 2.2	1.93 \pm 0.07
Acetone	27.7 \pm 0.9	16.2 \pm 0.6	nd	0.04 \pm 0.08
Ethanol	42.7 \pm 1.7	9.6 \pm 0.4	16.0 \pm 0.9	1.07 \pm 0.04

- ^a: mg/g of extract; ^b:mg/Kg of dry mass; nd: not detected.
- * : These results have been found in a previous work recently published in "Analytical letters".

DISCUSSION

No data was reported in the literature regarding the anti-inflammatory activity of *T. ramosissimum* extracts (aerial parts) using the 5-lipoxygenase assay. We compared it to other species derived from the *Lamiaceae* family. For instance, the methanol extracts of three *Salvia* species (*Salvia stenophylla*, *Salvia repens* and *Salvia runcinata*) were evaluated for their anti-inflammatory activity (5-lipoxygenase assay). All extracts showed poor activity (IC_{50} >100 μ g/ml) (Kamatou et al., 2005). Furthermore, Kamatou et al. (2005) indicated that methanol/chloroform (1:1) extracts

of 16 *Salvia* species were unable to inhibit activity of 5-lipoxygenase. The exception of *S. radula* ($IC_{50}=78.81\mu\text{g/ml}$), all the extracts displayed poor anti-inflammatory activity; the IC_{50} values were in excess of $100\mu\text{g/ml}$ (Kamatou et al., 2009). Our findings revealed that the extracts of *T. ramosissimum* seem to possess similar anti-inflammatory properties than those of *Salvia* species (*Lamiaceae* family). Moreover, the potential anti-inflammatory capacity in acetone fraction might be due to the presence of phenolics compounds (27.7 ± 0.9 GAE mg/g of dry extract), act as redox 5-LOX inhibitors. The low reactivity of *T. ramosissimum* extracts (hexane and ethanol) might be related to the lower flavonoids (**Table 3**) amounts, which varied from 1.1 ± 0.0 and 9.6 ± 0.4 QE mg/g dry mass, respectively. However, recent study showed that the anti-inflammatory activity has been attributed to flavonoids amounts, among other groups of compounds (Amezouar et al., 2013). Also, correlation between flavonoids contents and anti-inflammatory activity against 5-lipoxygenase enzyme was carried out. We observed that the amounts of flavonoids in plant extracts correlated well with their anti-inflammatory activity and seems to have a notable effect on the values of % inhibition ($R^2=0.89$).

It is the first time that the anti-Alzheimer property was reported for *T. ramosissimum* extracts. These results were a preliminary screening of the inhibitory effect of these extracts on AChE. All extracts were found to exert insignificant or low anti-Alzheimer effect. The low potency against AChE could be attributed to the low alkaloids content in these extracts. This is in agreement with a previous study on cholinesterase inhibitors from natural plant, which was demonstrated that alkaloids were the most active compounds against AChE (Orhan et al., 2009). However, some extracts of plants derived from the *Lamiaceae* family, have been reported as having lower or moderate AChE inhibitor ability, such is the case of total and aqueous extracts from the *Melissa officinalis* (Hajimehdipoor et al., 2013). These extracts exhibited low (low: 5–25%; moderate: 25–50%) inhibitory activity at a concentration of $300\mu\text{g/ml}$. The ethanol extracts from the following plant species: *Melissa officinalis*, *Mentha suaveolens*, *Salvia officinalis*, *Lavandula angustifolia* and *L. pedunculata* were also evaluated for their anti-acetylcholinesterase activity at a concentration of $500\mu\text{g/ml}$ (Ferreira et al., 2006). All these plants derived from the *Lamiaceae* family. Except for *L. angustifolia* Miller (moderate inhibitory activity: $26.6\pm 9.5\%$), all the ethanolic extract showed trace or no effect on AChE (16.4 ± 5.4 - $19.3\pm 3.9\%$). Also, the methanolic and hexane extracts of two aromatic plants derived from *Lamiaceae* family: *Ballotanigra* and *Hyssopus officinalis* were searched for their activity towards AChE enzyme at concentrations of $100\mu\text{g/ml}$ (Wszelaki et al., 2010). The methanolic extract of *B. nigra* and *H. officinalis* indicated poor AChE inhibitory activity (6.5 ± 5.1 and $5.2\pm 8.2\%$, respectively), while the hexane extracts contained moderate inhibitory activity (26.7 ± 2.4 and $29.6\pm 2.3\%$, respectively).

To our knowledge, the inhibitory activity of these extracts on MCF-7 and HCT-116 cell lines has never been reported before. However, cytotoxic activity of this plant was studied against another cell line K562 (leukemic cell line) and it was reported that all the tested extracts exhibited no significant activity (IC_{50} value $>100\mu\text{g/ml}$) (Ben Sghaier et al., 2012). Among these extracts, a trend of increase in activity was observed from non-polar extract (hexane) to polar extract (ethanol). The cytotoxic activity was found maximum in ethanol extract, which may be explained by the ability of ethanol to dissolve maximum of compounds than other solvents selected. The acetone extract contained specific compounds that could be much more active on the MCF-7 cell line.

Based on the bibliographical review, it is possible to hypothesize that the significant activity in ethanol extract could be due to the presence of flavonoids that are naturally occurring phenolic compounds found in plants. Bibi et al. (2012) reported that the potential anti-cancer capacity in aqueous fraction against MCF-7 cell lines might be due to the presence of phyto constituents like saponins and flavonoids either individually or synergistically. Previous studies demonstrated that the cytotoxic activity of plant extracts was interrelated with the high phenolics compounds content and antioxidant activities. We found also a good correlation between phenolics contents and cytotoxic activity on MCF-7 and HCT-116 cell lines. The correlation coefficients R^2 were 0.941 and 0.669, respectively. We also observed good correlations between anticancer property on MCF-7 cell line and DPPH and ABTS assays (R^2 0.78 and 0.73, respectively). Furthermore, anticancer activity on HCT-116 cell line correlates with DPPH and ABTS assays (R^2 0.999 and 0.997, respectively), confirming that anticancer and antioxidant properties of *T. ramosissimum* extracts are likely attributed to their phenolics content.

CONCLUSION

Thus *T. ramosissimum* showed promising anti-cancer properties agent given the effect on cancer cells *in vitro*. The acetone and ethanol extracts gave a good anti-cancer activity, making these two extracts an important target for the isolation and characterization of the phyto-compounds responsible for this biological activity.

Conflict of interest: The authors declare that there is no conflict of interest.

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