Differential antitumor effect of essential oils and their major components of *Thymus broussonettii*: relationship to cell cycle and apoptosis induction

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Summary

Thymus broussonettii, a Moroccan endemic plant, exists in two chemotypes. The aim of our study is to compare the cytotoxic activity of their essential oils and major products as well as their effect on cell cycle and apoptosis induction. The chemical composition analysis of essential oils by GC-SM revealed that the lasts are rich and diverse and the major products of the chemotypes TbA and TbE are carvacrol and thymol, respectively. The *in vitro* cytotoxic effect study against five tumor cell lines shows that TbA essential oil, rich in carvacrol, has an important cytotoxic effect, higher than that of TbE, rich in thymol. This result is confirmed by comparing cytotoxic effect of carvacrol and thymol. Furthermore, TbA EO/carvacrol and TbE EO/thymol induce cell cycle arrest at S and G_0/G_1 phases, respectively. On the other hand, carvacrol, most cytotoxic *in vitro*, was studied for its effect on solid tumor *in vivo* and apoptosis-induction. Our results show that carvacrol, administred by gavage, has an important effect on solid tumor and induce apoptosis in P815 tumor cell line.

Key words: *Thymus broussonettii*, *chemical composition, carvacrol/thymol, cell cycle, apoptosis; antitumor activity*

INTRODUCTION

Thyme is one of the most important medicinal plants used in traditionnal medecine throughout the world. It is usally used for its antispasmodic [1], antibacterial [2], antifungal [3], and antioxidant activities [4, 5]. In addition, the leafy parts of thyme and the essential oils have been used for many years as food additives to enhance flavor, aroma and as food preservative [6]. *Thymus broussonettii* is a species endemic to Morocco. Two chemotypes have been identified: one in the western part of Morocco (Aakrach), and the other in the south-western part (Essaouira). Moreover, a few studies have described the anti-inflammatory properties of this species [7] and the ability of its aqueous and ethylacetate extracts to stimulate the immune system and release stress [8].

To further exploration of the therapeutic effects associated with this plant, we try to evaluate, in comparative study, the potential antitumor activity of essential oils as well as the respective major products of two chemotypes.

MATERIALS AND METHODS

Plant material and chemicals

Two chemotypes of *Thymus broussonettii,* TbA and TbE, were collected at Aakrach-Rabat (West of Morocco) and at Essaouira (South-West of Morocco), respectively. Samples of two chemotypes were collected in June, dried in shade and flowers and leaves were used for extraction of essential oils. Carvacrol (99% purity), thymol (99% purity), DMSO (dimethylsulfoxyde), DMEM (Dulbecco's modified eagle's medium) and FCS (foetal calf serum) were purchased from Sigma (St Quentin, France).

Chemical analysis of the essential oils

The essential oils of the two chemotypes were extracted by hydrodistillation. Afterwards, they were analysed by gas chromatography-mass spectrometry (GC/MS). The spectra generated were recorded using a THERMO ELECTRON POLARIS Q Mass selective detector coupled to THERMO ELECTRON Trace GC Ultra, equipped with injector. The temperature was set at 60°C for 5 min and elevated to 250°C at 5.0°C/min, and then elevated to 300°C at 10°C/min. This temperature (300°) was held for 30 min. The total runtime was 48 min. The column used was VALBON 30 m x 0.25 mm coated with 0.25 μm thick FFAP fused silica. The MSD injection temperature was set at 250°C, with a split ratio of 100:1. Helium was used as a carrier gas and sample size was 1 μL. Scanning masses varied between 10 and 300.

In vitro cytotoxic effect of essential oils, carvacrol and thymol against a panel of target cells

The cytotoxic activity was studied against following tumor cell lines: P-815 (murine mastocytoma), K-562 (human chronic myelogenous leukemia), CEM (acute T lymphoblastoid leukemia), MCF-7 (human breast adenocarcinoma) and its counterpart resistant to gemcitabine (MCF-7 gem). Cytotoxicity was measured using the colorimetric methyl tetrazolium test (MTT) assay as described and modified by Tim Mosmann [9]. The target cells were washed twice in PBS (phosphate buffer saline) and placed in 96-well microtiter plates (Bioster, Italy) at a density of $1.5.10^5$ cells/ml in 100 μl/well of culture medium (DMEM supplemented with 5% FCS and 1% of penicillin and streptomycin). Then, 100 μl of culture medium containing specified concentration of tested compounds was added in each well. After the cells exposure to serial concentrations of tested products for 48 h at 37^0C and 5% CO_2, 100 μl of medium were carefully aspirated from each well and replaced by 20 μl of MTT solution at 5 mg/ml in PBS. After incubation in the same conditions for 4 h, the plates were treated with a solution of HCl / isopropanol (24:1) to dissolve the blue intracellular formazan product. One hour later, the plates were read in a MicroELISA reader at 590 nm.

Cell cycle analysis

Cell cycle analysis was performed to evaluate the effect of our products on the distribution of tumor cells in G1, S and G2/M phases of the cell cycle. This test was performed by flow cytometry after DNA staining to reveal the total amount of DNA. Approximately, $1.5.10^6$ of K-562 tumor cells were cultured for 24 h before their incubation with TbA EO, TbE EO, carvacrol and thymol for 24 h. Cells were collected, washed with PBS, fixed with cold 70% ethanol and conserved overnight at –20 °C. Subsequently, 100μl of RNase A (1mg/ml) were added and after 30 min of incubation at 37°C, cells were stained with a solution containing 10μg/ml of

propidium iodide (PI). The samples were analysed using a FACStar plus flow cytometer (Becton-Dickinson) and WinMDI software.

Induction of apoptosis by carvacrol and thymol

P815 tumor cells were incubated with carvacrol and thymol for 24 h at 37 °C. The cells were recovered and lysed by the lysis buffer (100mM Tris HCl, 0.5M EDTA, 10% SDS (sodium dodecyl sulphate), 5M NaCl and 20mg/ml Proteinase K). Digestion was complete within 2-3 hours at 37 °C with constant agitation. About 500 μl of isopropanol was then added to the lysate and the samples were mixed until complete precipitation of DNA (10-20 min). After precipitation, DNA was transferred to a prelabeled Eppendorf tube containing 20 to 500 μl (depending on the sample size) of a solution containing 10 mM Tris HCl, 0.1 mM EDTA, pH 7.5. Complete dissolution of DNA requires several hours with constant agitation at 37 °C. Finally, the loading buffer (glycerol, xylene cyanol, bromophenol blue, ficcol, EDTA) was added and DNA is submitted to electrophoresis.

Electrophoresis

DNA samples were loaded onto a 1.5% agarose gel and DNA fragments were separated by horizontal electrophoresis (20 V for 8 h). The gel was stained with ethidium bromide and visualized under UV light (310 nm). A λ Hae III ΦX 174 was used as a molecular weight marker.

In vivo antitumor effect of carvacrol

Six-eight-week-old DBA-2 mice (Orleans, France) weighting 18–22 g were maintained under pathogen-free conditions, on 12 hours light-dark cycle. Mice were provided with sterile food and water ad libitum. P815 cells (1.10^7/100 μl) were implanted subcutaneously in the back of the mice and when the tumors reached a palpable volume, the mice were randomly divided into 3 groups of 6 mice (day 0). Daily, oral administration (gavage) of carvacrol, dissolved in vegetal oil was performed from day 0 to day 7. Each mice of group A (negative control) received only 100 μl/day of vegetal oil. Groups B and C were treated with carvacrol dissolved in 100 μl of vegetal oil by 50 mg/kg/day and 100 mg/kg/day, respectively. Mice were weighted three times a week for up to 30 days and the tumor volumes were measured. The tumor volume at day n, (TVn) was calculated using the formula: $TV=(L*W^2)/2$ where L equals the length of the tumor and W the width, as described by Yoshikawa [10].

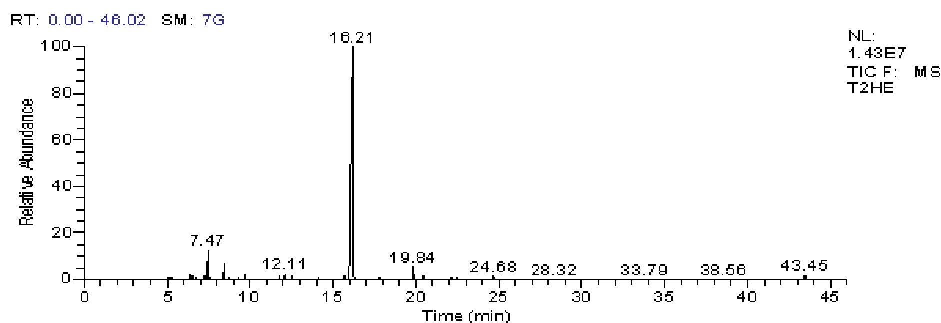
Statistical analysis

The results, expressed as means ±SEM, were analysed using an analysis of variance with a level of significance set at $p<0.05$.

RESULTS AND DISCUSSION

Chemical composition

Essential oils (EO) of two chemotypes were at first analysed by GC-MS. The results are summarized in table 1. The compounds in the number of 89 were detected in EO of TbA, whereas 106 in that of TbE. Chromatographic spectra of the two chemotype essential oils indicate that they contain essentially the same compounds but at different concentrations (fig. 1). However, the major products, which are often responsible for biological activities, are completely different. Indeed, carvacrol is the major component of TbA EO with a rate of 83.18 % and TbE EO contains thymol as a major component with a rate of 37.11%. Carvacrol is also present in TbE EO but it is 15.8 fold less abundant here than in TbA EO. Thymol ratio in TbA EO is very weak (less than 1%).



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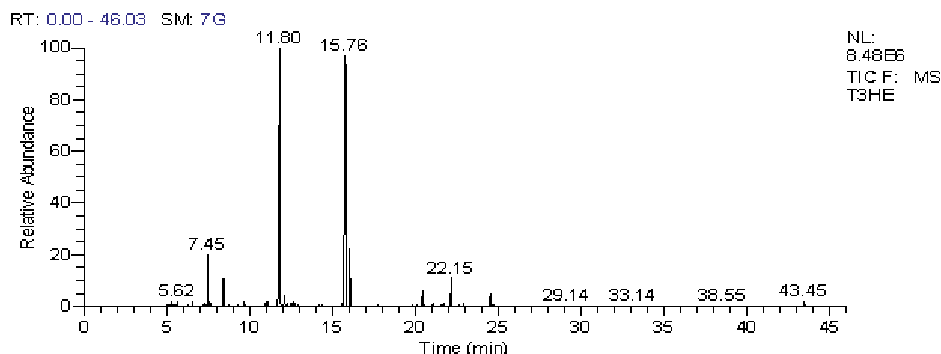

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Figure 1. Chromatographic spectra of TbA EO (A) and TbE EO (B).
Essential oils of two chemotypes: TbA (A) and TbE (B), extracted by hydrodistillation, were analysed by gas chromatography-mass spectrometry (GC/MS). Mass spectra were recorded with a THERMO ELECTRON POLARIS Q Mass Selective Detector coupled to THERMO ELECTRON Trace GC Ultra equipped with injector.

Table 1

Chemical composition of TbA EO and TbE EO.
Two essential oils are analysed by GC-MS. Products were identified by their mass spectrum using data base (Main lib, Wiley 7, PMW). The percentage of products is determined automatically measuring the AUC.

	component	retention time [min]	percentage (%)
		TbA EO	TbE EO
camphen	5.66	t	0.33
β-pinene	6.31	0.79	0.12
geraniol formate	6.59	0.38	0.35
1-octen-3-ol	6.81	0.11	t
p-menth-1,4(8)-diene	7.30	0.37	0.22
p-cymene	7.50	3.69	3.99
p-metha-1,8-diene	7.63	t	0.45
γ-terpinene	8.46	1.86	2.25
4-isopropyl-1M-2cyclohexane-1-ol	8.79	0.14	0.12
epoxylinalol	8.90	t	0
β-linalool	9.80	t	0.57
terpendiol	9.89	0.70	0
terpinene-1-ol	10.44	t	0.13
δ-3-carene	10.99	t	0.66
camphor	11.06	t	0.37
trans-2-carene-4-ol	11.17	0	t
terpineol-6-β	11.29	0	0
isoborneol	11.55	t	t
borneol	11.94	0.41	33.92
4-terpineol	12.20	0.79	1.07
linalyl propionate	12.65	0.34	0.55
isobornyl forrmate	13.73	t	t
carvacrol methyl ether	14.20	0.26	t
carvenone	14.65	t	0.10
bornyl acetate	15.59	t	0.26
thymol	15.75	0.83	37.11
carvacrol	16.20	83.18	5.26
cinerone	17.72	0.29	0.19
thujol	18.45	t	t
α-copaéne	18.52	t	t
β-bourbounene	18.81	0	t
alloromadandrene	19.58	t	0.24
transcaryophyllene	19.88	1.75	0.13
α-caryophyllene	20.91	t	t
α-muolene	21.59	t	0.19
β-cadrene	22.71	0.30	0.14
α-cadinene	22.98	t	0.28
caryophyllene oxide	24.73	0.47	0.23
germacrene D	26.33	t	0.11
murolol	26.70	t	t
isoaromadandrene epoxyde	27.15	t	t
trans-1,2-diphenylcyclobutane	29.21	0	t

t: trace less than 0.1%

Several studies have shown that the chemical composition of thyme essential oils is rich and diversified both in Morocco [11-13] and in other regions of the world [14-15]. Carvacrol, thymol and borneol are major components found in Moroccan thyme [13]. In northern California, the major constituents of thyme are thymol, carvacrol, linalool, α-terpineol and 1,8-cineole [16]. In Estonia, the major components comprise E-nerolidol, caryophyllene oxyde, myrcene, (E)-β-caryophyllene and germacrene-D [15]. *Thymus praecox* found in southern England contains, as major products, hedycaryol, linalol/lynalyl acetate or germacral-10, 4-dien-6-ol [17].

The most important factors which can be responsible for this variability of the chemical composition of thyme essential oils are climate, soil, harvest period and method of extraction and preservation. Genetic factors [17] and vegetative cycle [19-20] can also account for this variability. Despite of this great diversity, certain components are currently found in all species even though at different concentrations. This is the case of monoterpenics phenols (thymol and carvacrol), their precursors monoterpenics hydrocarbures (p-cymene and γ-terpinene), oxygenate monoterpenes (borneol and linalol), terpinene-4-ol and 1, 8-cineol [14].

In vitro cytotoxic effect of essential oils, carvacrol and thymol against a panel of target cells

The antitumor activity of TbA EO, TbE EO, carvacrol and thymol was evaluated against following five tumor cell lines: P-815, K-562, CEM, MCF-7 and MCF-7 gem. Figure 2 summarizes the obtained results. The cytotoxic effect depends on the nature of the products as well as on target cell lines. The lysis of P-815 and CEM averaged 80 and 90%, respectively, which was much higher that in any of the other target cells. On the basis of IC50 comparison, the lytic activity of TbA EO, rich in carvacrol, is higher than that of TbE EO, rich in thymol. In fact, according to tested cell line, the IC50 values (in % v/v) vary between 3.6 and 10 for TbA EO, 3.1 and 17.5 for TbE EO, 0.85 and 2.5 for carvacrol and 3.1- >22 for thymol (tab. 2). These differences seem to be related to the major component of each EO. These results have been confirmed by comparing the cytotoxicity of carvacrol and thymol showing that the IC50 of carvacrol are much lower than those of thymol.

Table 2

IC50 (in % v/v) 10^{-3} of the products against the different target cell lines.
Tumor cells were stimulated by specified concentrations of the tested compounds; TbA EO, TbE EO, carvacrol and thymol for 48 h at 37^{0}C and 5% CO_2. Lysis was determined using MTT assay as described in the Materials and Methods section and the IC50 (i.e. the concentration at which 50% of the lytic activity was reached) was calculated. The results are the mean ±SEM of two different experiments.

Product	P-815	CEM	K-562	MCF-7	MCF-7 gem
TbA EO	4.7	3.6	10	10	8.9
TbE EO	8.5	3.1	13.5	15.4	17.5
Carvacrol	1.2	1.2	1.2	2.5	0.85
Thymol	3.1	6.9	>22	22	>22

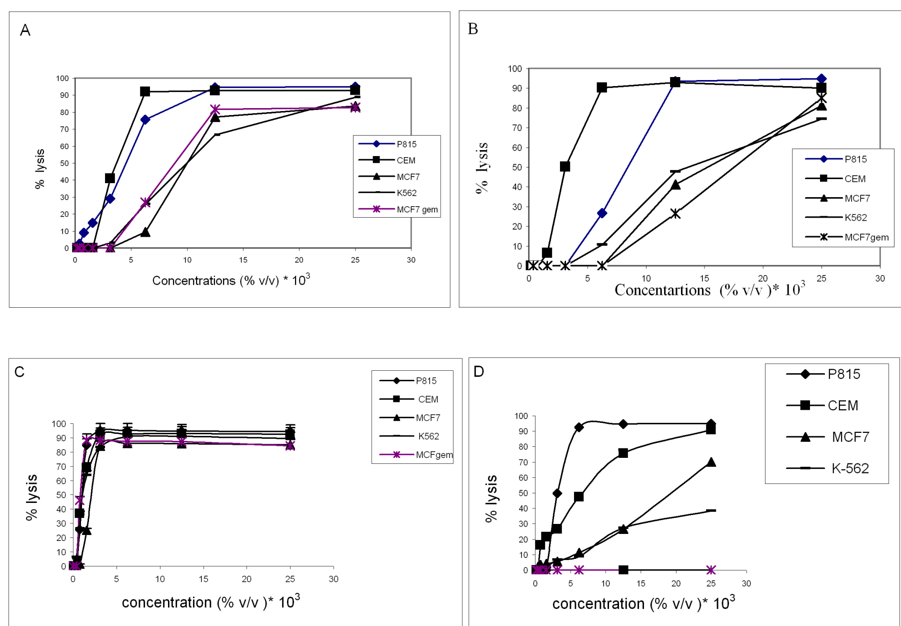

Figure 2. Cytotoxic effect of TbA and TbE EO, carvacrol and thymol against different tumor cell lines. Tumor cells were washed by centrifugation and incubated in 96-well microtiter plates at a density of $1.5x10^5$ cells/ml in 100 μl/well of culture medium. Then, cells were stimulated by specified concentrations of the tested compounds; TbA EO (A), TbE EO (B), carvacrol (C) and thymol (D) for 48 h at 37^0C and 5% CO_2. Lysis was determined using MTT assay as described in the Materials and Methods section. The results are the mean ±SEM of two tests.

These results agree with those of others who showed that essential oil of thyme can have preventive effect against cancer by means of antioxidant properties of its components [21-23]. Our results support those reported earlier that showed that carvacrol has an important *in vitro* antitumor effect against tumor cell lines like Hep-2 [24], B-16 [25] and A-549 [26]. They are also in agrement with those of Karkabounas et al., describing the antiproliferative activity of carvacrol with IC50 of 90 μM and 67 μM for 24 h and 48 h of cell incubation, respectively [27] and those of Zeytinoglu et al., who reported that carvacrol inhibits growth of myoblast cells even after activation of mutated N-ras oncogene [28].

The molecular mechanism involved in carvacrol-induced or thymol-induced cytotoxicity is not known. Despite the high resemblance of their structure, carvacrol is more cytotoxic than thymol as revealed by IC50 values (tab. 2). The relationship beween molecular structure and cytotoxic activity of thymol or carvacrol remains to be established. In fact, although carvacrol and thymol significantly reduced the level of DNA damage induced in K-562 cells by the strong oxidant H_2O_2 [29], only carvacrol inhibited mutagenicity induced by 4-nitro-o-phenylenediamine [30].

Furthermore, the nature of target cells may be involved in the differential sensitivity of cells to these components. Whether the mutation of p53 in CEM and P-815 target cell lines is involved in their higher sensitivity to tested products remains to be established. In addition, differential sensitivity of MCF-7 and MCF-7 gem to the cytotoxic effect of thymol may be associated to the level of the subunit of ribonucleotide reductase R1 [31].

Effect of TbA EO, TbE EO, carvacrol and thymol on the cell cycle

In order to investigate the molecular mechanism of the cytotoxic activity of our products, their effect on cell cycle progression was examined by flow cytometry after DNA staining to reveal the distribution of cells in the cycle phases. The results revealed that TbA EO and its major product, carvacrol, stopped the cell progression in S phase, whereas TbE EO and its major product, thymol, stopped it in the G_0/G_1 phases (tab. 3). These results suggest that the cytotoxic activiy induced by TbA EO and carvacrol may involve a cell cycle arrest in S phase while TbE EO and thymol may cause G_0/G_1 phase arrest. On the other hand, the four products revealed a sub-G_1 peak witch is an indicator of the onset of apoptosis [32].

Table 3

Effect of the two essential oils, carvacrol and thymol on the cell cycle.
Cell cycle analysis was performed by flow cytometry after DNA staining to reveal the total amount of DNA. Approximately, $1.5x10^6$ of K-562 tumor cells were cultured for 24 h before their incubation with TbA EO, TbE EO, carvacrol and thymol for 24 hours. The samples were analysed using a FACStar plus flow cytometer and the WinMDI software. The results are the mean ±SEM of two tests.

Component	Cell-cycle distribution (percentage)		
	G0/G1	S	G2/M
Control	48.71	44.53	6.76
TbA EO	17.32	82.68	0.0
TbE EO	51.6	36.1	12.3
carvacrol	20.9	79.1	0.0
thymol	62.9	5.6	31.5

Although the relationships between some phenols and the cell cycle have been described in the litterature, to our knowledge, no study was done using carvacrol [33-35]. The molecular mechanim of carvacrol-induced S-phase arrest is not known. Recent study has shown that the induction of S-phase arrest by the natural polyphenol guggulsterone correlated with a decrease in the levels of cyclin D1 and cdc2 as well as a concomitant increase in the levels of cyclin-dependent kinase inhibitor p21 and p27. Whether the effect of carvacrol on cell cycle involves the modulation of these proteins remains to be elucidated.

Induction of apoptosis by carvacrol and thymol

The induction of apoptosis in cancer cells is recognised as a valuable tool for cancer treatment [36]. In order to establish if the cytotoxic activity of carvacrol and thymol is associated with apoptosis induction, DNA fragmentation was analysed after exposure of P-815 cells to these products for 24h (fig. 3). The endonucleolytic DNA cleavage was checked by agarose gel electrophoresis and revealed fragments of 180-200 bp characteristics of apoptosis in the case of carvacrol stimulation. However, no apoptosis induction was observed following thymol stimulation. Taken together, these results permit to conclude that carvacrol and not thymol inhibit the proliferation of P-815 tumor cells through the induction of apoptosis. These results are in agreement with those of Bimczok et al. who reported the reduced lymphocyte proliferation measured after carvacrol exposure [37]. In adition they complete those of Stammati et al. who suggest an involvement of apoptosis of Hep-2 cells following exposure to carvacrol, carvone and cinnamaldehyde basing on the morphological analysis of cells [24]. The IC50 results reported by Stammati et al., however, are different from ours. This difference may be related to the nature of target cells used. Taken together, our results demonstrate for the first time that the major component of the essential oils TbA EO and TbE EO, carvacrol and thymol respectively, have different cytotoxic mechanisms since carvacrol and not thymol can induce apoptosis. This difference may explain the differential sensitivity of tumor cells to these isomers as well as to TbA EO and TbE EO.

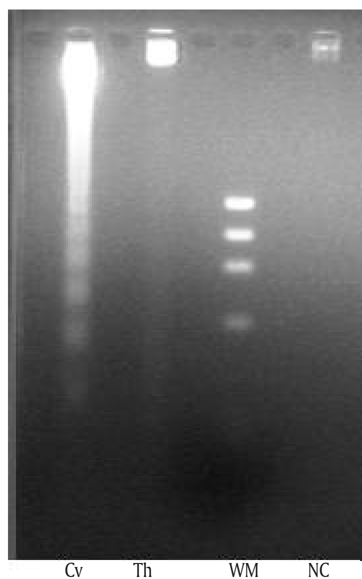

Figure 3. Effect of carvacrol and thymol on apoptosis induction.
Carvacrol and thymol-induced DNA fragmentation was detected by agarose gel electrophoresis of DNA isolated from P815 tumor cells. P815 cells were incubated with carvacrol (Cv) and thymol (Th) for 24 h. DNA of untreated cells was used as negative control (NC).

In vivo antitumor effect of carvacrol

The P815 tumor-bearing DBA-2 mice were used to investigate the cancer cell death-inducing effect of the major component of TbA EO, carvacrol. Experiments were carried out by oral administration (gavage) of carvacrol dissolved in vegetal oil to 6-8 week old mice for 7 days. The tumor volume was measured for up to 30 days (Figure 4). Interestingly, during the first 18 days there was no statistical difference ($p<0.94$) in the volumes of the tumors in all the groups of mice, including the control group (0.4 to 0.5 ± 0.1 cm^3). However, after 18 days, the tumor volume quickly increased in non treated group reaching 1.5 cm^3 at 23rd day while a decrease was observed for treated groups. It is not worthy that this decrease occurred more rapidly in the group C treated with 100mg/kg/day than the group B who received 50mg/kg/day ($p<0.05$ at day 21th). Interestingly, the reduction of tumor volume was associated with a decrease in weight loss and an increase of mice survival. The *in vivo* antitumor activity of carvacrol has not been clairly described in the litterature. In this study, we report for the first time that the gavage of carvacrol for successive seven days significantly reduced tumor volume, body weight loss and delayed mortality (data not shown). These results complete those of Karkabounas who recently reported that carvacrol induced 30% decrease of 3,4-benzopyrene carcinogenic activity *in vivo* [27].

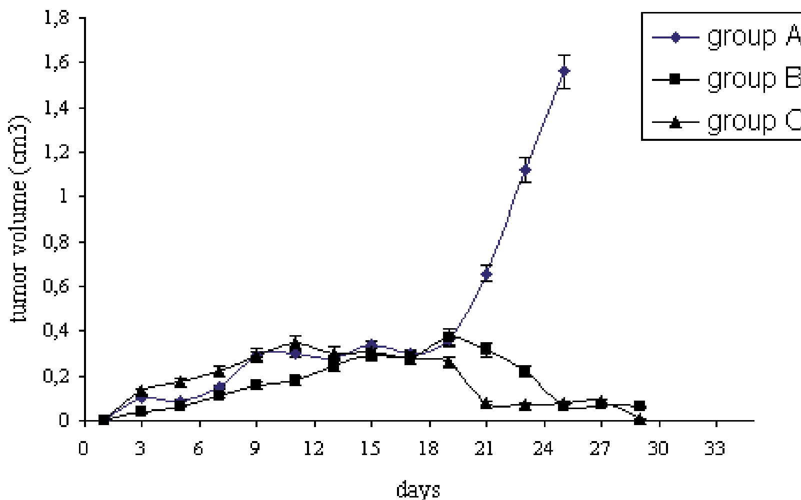

Figure 4. In vivo antitumor effect of carvacrol.
Experiments were carried out by oral administration (gavage) of carvacrol dissolved in vegetal oil to 6-8 week-old mice for 7 days. Each mouse of group A (negative control) received 100 μl/day of vegetal oil only. Groups B and C were treated by 50 mg/kg/day and 100 mg/kg/day of carvacrol dissolved in 100 μl of vegetal oil, respectively. Three times a week up to day 30, mice were weighted and the tumor volume was determined by measurement of the length (L) and width (W) of the tumor. The tumor volume at day n (TVn) was calculated as $TV=(L^*W^2)/2$. The results are the mean $\pm$SEM of two tests.

CONCLUSIONS

The aim of our study was to evaluate the potential antitumor effect of essential oils of two chemotypes of *Thymus broussonettii,* endemic species of Morocco, as well as that of their major components, carvacrol and thymol. Chemical analysis reported that the two essential oils have a rich and diverse chemical composition. The *in vitro* cytotoxic effect against five tumor cell lines shows that the chemotype TbA essential oil and its major product, carvacrol, are widly more cytotoxic than the chemotype TbE essential oil and its major product, thymol. The effect of these two compounds was also studied on the cell cycle and it was indicated that TbA EO or carvacrol caused S phase arrest while TbE EO or thymol caused G_0/G_1 phase arrest. On the other hand, using DNA fragmentation test, carvacrol and thymol were studied for their effect on apoptosis-induction and it has been shown that DNA fragmentation (180-200 bp) was detected with carvacrol and not thymol for 24h. Finally, carvacrol has an *in vivo* antitumor effect wich is more pronounced in the group treated with 100 mg/kg/day. This activity was associated with a decrease of body weight loss and an increase of survival (data not shown). Our contention, therfore, is that carvacrol may represent a novel approach in the treatment of certain forms of cancer and that its potential antitumor role needs to be investigated further.

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RÓŻNE DZIAŁANIA PRZECIWNOWOTWOROWE OLEJKÓW ETERYCZNYCH *THYMUS BROUSSONETTII*. ZWIĄZEK Z CYKLEM KOMÓRKOWYM I INDUKCJĄ APOPTOZY

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Streszczenie

Istnieją dwa chemotypy *Thymus broussonettii,* marokańskiej rośliny endemicznej. Celem pracy było porównanie działania cytotoksycznego ich olejków eterycznych i głównych związków, a także ich wpływu na cykl komórkowy i indukcję apoptozy. Analiza składu chemicznego olejków eterycznych dokonana za pomocą GC-MS dowiodła, że są one bogate i zróżnicowane, a głównymi składnikami chemotypów TbA i TbE są odpowiednio karwakrol i tymol. Analiza działania cytotoksycznego przeprowadzona na pięciu liniach komórek nowotworowych *in vitro* wykazała, że olejek eteryczny z TbA, bogaty w karwakrol, ma działanie cytotoksyczne, większe niż TbE, o dużej zawartości tymolu. Te wyniki zostały potwierdzone przez porównanie działania cytotoksycznego samego karwakrolu i tymolu. Co więcej, zarówno olejek eteryczny z TbA jak i karwakrol indukują hamowanie cyklu komórkowego w fazie S, natomiast olejek eteryczny TbE i tymol hamują rozwój komórek w fazie G_0/G_1. Z drugiej strony karwakrol, najbardziej cytotoksyczny *in vitro*, analizowano pod kątem działania na guz lity *in vivo* oraz indukcji apoptozy. Wyniki pokazują, że karwakrol podawany dożołądkowo ma istotny wpływ na rozwój guza litego i indukcję apoptozy w linii komórkowej guza P815.

Słowa kluczowe: *Thymus broussonettii*, *skład chemiczny, karwakrol/tymol, cykl komórkowy, apoptoza, działanie przeciwnowotworowe*