Cytotoxic effect of thyme essential oil and Nigella saliva L extract on P815 and BSR cancer cells: Correlation with alkaline phosphatase expression

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Abstract— The anti-tumour effect of thyme (*Thymus broussonettii*) essential oil and black-seed (*Nigella saliva* L) ethyl acetate extract were first investigated in vitro using P815 and BSR cancer cell lines. The IC_{50} values ranging from 4 - 5 mg/ml for BSR cells and from 9 - 11 mg/ml for P815 cells, showed a slight sensitivity of the BSR cell line to the extracts. Carvacrol exhibited IC_{50} value of 200 ± 10 µM for P815 while vincristine (positive control) showed values IC_{50} = 4 ± 0.2 µM and 7 ± 0.25 µM for P815 and BSR cell lines, respectively. Analysis of ALP activity shows a clear increasing of this enzyme expression when the cancer cells (BSR and P815) were treated by vincristine or by Nigella extract. Conversely, thyme essential oil and carvacrol substantially reduced the expression of ALP activity. Therefore, our findings highly suggest that the repression of ALP activity by thyme essential oil and carvacrol, one of its major components, could be responsible for the regulation of P815 and BSR cancer cell growth. This study supplies new information on the possible mechanistic and a target by which thyme essential oil and pure carvacrol exert their effect on P815 and BSR cancer cells.

Keywords: Nigella extract, Thyme essential oil, P815, BSR, carvacrol; anti-proliferative, alkaline phosphatase, modulation

1 Introduction

In last decade, the investigation in medicinal plants and their pharmacological active derivatives has increased, in order to find new safe and potent drugs against cancer which continue to be a worldwide killer. Plant derived substances have recently become of great interest owing to their versatile applications [1], [2].

Plants *Thymus broussonetti* and *Nigella saliva L.* (Ranunculaceae family) has been employed for thousands years in traditional medicine. The pharmacological investigations of *Nigella saliva L* (black-seed or black cumin) extracts reveal a broad spectrum of activities including immunopotentiation, anti-histaminic, antidiabetic, anti-hypertensive, anti-inflammatory, and antimicrobial [3], [4], [5], [6]. Many of these activities have been attributed to the quinone constituents of the black-seed [7], [8]. Antitumor activity of some crude and purified components of N. sativa on Erlich ascites carcinoma, Dalton's ascites lymphoma and sarcoma 180 cells *in vitro* and *in vivo* has been reported previously [9], [10], [11]. Purified components from black-seed, thymoquinone and dithymoquinone, were cytotoxic to all cell lines, including parental and multidrug-resistant phenotype-expressing cells [9], and their action on the cell death in human colorectal cancer cells seems to be correlated with G1 phase arrest of the cell cycle, leading to apoptosis process [12].

The essential oil of thyme has also a wide spectrum of pharmacological properties, anti-inflammatory, antibacterial, antifungal, antispasmodic and antioxidant activities [13], [14], [15], [16], [17], [18], [19], [20]. We have previously shown that a crude extract (essential oil) of thyme and one of its major component, carvacrol, are capable of reducing the *in vitro* proliferation rates of human cancer lines, ovarian adenocarcinoma cell sensitive or resistant to chemotherapy [21], MCF7, and murine mastocytoma cell line (P815) [22]. Furthermore, we have also shown that black-seed extract, thyme essential oil as well as carvacrol, were effective inhibiting tumour growth and delaying mortality of tumour-bearing animal DBA2 (H2d) mice model [11], [21], [22].

The aberrant expression of Alkaline Phosphatase (ALP) activities in cancer cells has led to the suggestion that ALP isoenzymes may be involved in tumour development [23]. In general, the level of ALP activities in drug treated cancer cells was enormously enhanced with a concomitant reducing the proliferation rates [24], [25].

Based on provided information, the aim of this investigation is to evaluate whether these natural product extracts as well as carvacrol (one of major component of thyme essential oil) have anticancer activity and whether this activity is correlated to the modulation of ALP activity in tumour cell lines (P815 and BSR) used in the present study.

2 Materials and Methods

2.1 Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and penicillin-neomycin were purchased from Sigma Chemical Co., Saint Quentin, France. The non adherent murine mastocytoma cell line (P815) as well as the adherent kidney carcinoma cell line of hamsters (BSR) were kindly provided by Prof. G. Lemaire, Institute of Biochemistry, University of Paris XI, France.

2.2 Preparation of plant extracts [11], [21].

Nigella ethyl acetate extract: (One kilogram N. sativa L. seeds was washed, dried, and crushed to a powder with an electric micronizer. The powder was exhaustively extracted with 90% ethanol at room temperature and the extract was centrifuged at 10,000 g for 15 min to remove residual solid debris. The clear supernatant was then concentrated under reduced pressure. The concentrated extract was partitioned between 10% methanol-water and n-hexane. After removing the n-hexane fraction, the aqueous layer was partitioned again with ethyl acetate.

Thyme essential oil extract: *Thymus broussonetti* was freshly collected in Akrach (the region of Rabat-Zeir, Morocco). The volatile essential oil of thyme (EOT) was prepared from dried leaves and stems using the hydro-distillation method. The essential oil was then collected after evaporation of the solvent (petroleum ether) under vacuum and stored at 4°C.

2.3 Cell lines

Cell lines were routinely cultured in complete medium RPMI 1640 supplemented with 10% fetal bovine serum (Gibco BRL, Sergy Pontoise, France), 1% penicillin-neomycin and 2% L-glutamine (complete medium). The cell viability and their morphology were examined by optic microscopy and then after le cell number were quantified by using Malassez lame for each experiment. BSR adherent cell line was first trypsinized before counting cell number.

2.4 Cellular chemosensitivity measurement

MTT assay [26]: Adherent cells (BSR) were plated out in 100 µl of complete medium at a concentration of 10^4 cells per ml and allowed to attach and grow for 24 h. 100 µl of medium containing vincristine, extracts or pure carvacrol at appropriate concentrations were then added to the cell culture. After 48 h incubation in a humidified atmosphere at 37°C, 5% CO_2, 100 µl of medium was carefully removed from each well and replaced with 20 µl MTT solution (5 mg/ml PBS). After 4 h incubation under the same conditions, the cleavage of MTT to formazan by metabolically active cells was quantified by scanning the plates at 540 and 630 nm using multi-scan apparatus. The means of three independent assays was determined to analyze the effect of drug on cancer cell line (BSR) growth. The growth inhibition rate was evaluated as percentage of parallel negative controls as follows: [(Acontrol – Aexperiment)/Acontrol)] x 100.
For the non adherent cell line, 100 µl of cultured cells (5 x 10^4 cells per ml) were plated out in flat-bottomed 96-well microtiter plates. 100 µl of medium containing vincristine, extracts or pure carvacrol at appropriate concentrations were then added immediately and plates were incubated for 48 h. Then 100 µl of medium was carefully removed from each well and replaced with 20 µl MTT solution (5 mg/ml PBS). After 4 h incubation under the same conditions, the cleavage of MTT to formazan by metabolically active cells was quantified by scanning the plates at 540 and 630 nm using multi-scan apparatus.

2.5 Determination of alkaline phosphatase (ALP) activity.

The cells (10^5 cells per ml) were seeded in triplicate into culture dishes (final volume 6 ml) and incubated for 48 h in the presence of extracts or compounds at indicated concentrations. The ALP enzymes were solubilised as described previously [Chang et 1994] [24]. Briefly, the cells from each culture dishes (6 ml final volume) were washed with PBS by using centrifugation technique at 7000 x g for 15 min. The cells were mixed with 0.5 ml of the re-suspension buffer (20 mM Tris-HCl, 1 mM MgCl2, 150 mM NaCl pH 8) and then solubilised by adding triton X-100 to a final concentration of 1%. After 20 min, the cell-free supernatant was obtained by centrifugation at 12000 x g for 15 min. The ALP activity was carried out by using 5 mM p-nitrophenyl phosphate in bicarbonate buffer (50 mM HCO-3, 0.5 mM MgCl2, pH 10) in total volume of 1 ml [27]. The reaction mixture was incubated for 2 h at 37°C and then stopped by adding 1 ml of 0.4 M NaOH. ALP activity was expressed as micromoles of p-nitrophenol produced per minute per 10^6 cells or as percentage of control.

3 Results

3.1 *In vitro* cytotoxicity

The effect of ethyl acetate extract, thyme essential oil and carvacrol was first evaluated against two cancer cell lines: adherent tumour cells BSR and non adherent tumour cells P815, by MTT assay. Vincristine was used as positive control. As shown in fig. 1(a,b), a dose dependent decrease in the growth of P815 as well as BSR was obtained with increasing concentrations of extracts. The concentrations of Nigella ethyl acetate extract (Figure 1) leading to 50% of the lytic activity (IC_{50}) were evaluated to be 9.5 ± 0.45 mg/ml and 4.5 ± 0.2 mg/ml for P815 and BSR, respectively. Also the thyme essential oil showed similar cytotoxicity (IC_{50}) which was evaluated to be 11 ± 0.4 mg/ml and 4 ± 0.23 mg/ml for P815 and BSR, respectively. Parallel, The anti-proliferative activity of vincristine on both P815 and BSR, as well as that of carvacrol on P815, were also evaluated by MTT assay in similar conditions. The IC_{50} was evaluated to be 200 ± 10 µM for carvacrol on P815 cancer cell line, which is in agreement with those found previously on chronic myeloid leukemia K562 cells [28]. As control positive, the IC_{50} for vincristine was found to be 4 ± 0.2 µM as previously reported for leukemic cell lines, CEM, HL60, U937 and K562 [29].
In order to determine whether there is a relationship between the observed cytotoxic effect and the expression of ALP activity, we used extracts and carvacrol as well as vincristine at the IC_{50} concentrations shown in (Table 1).

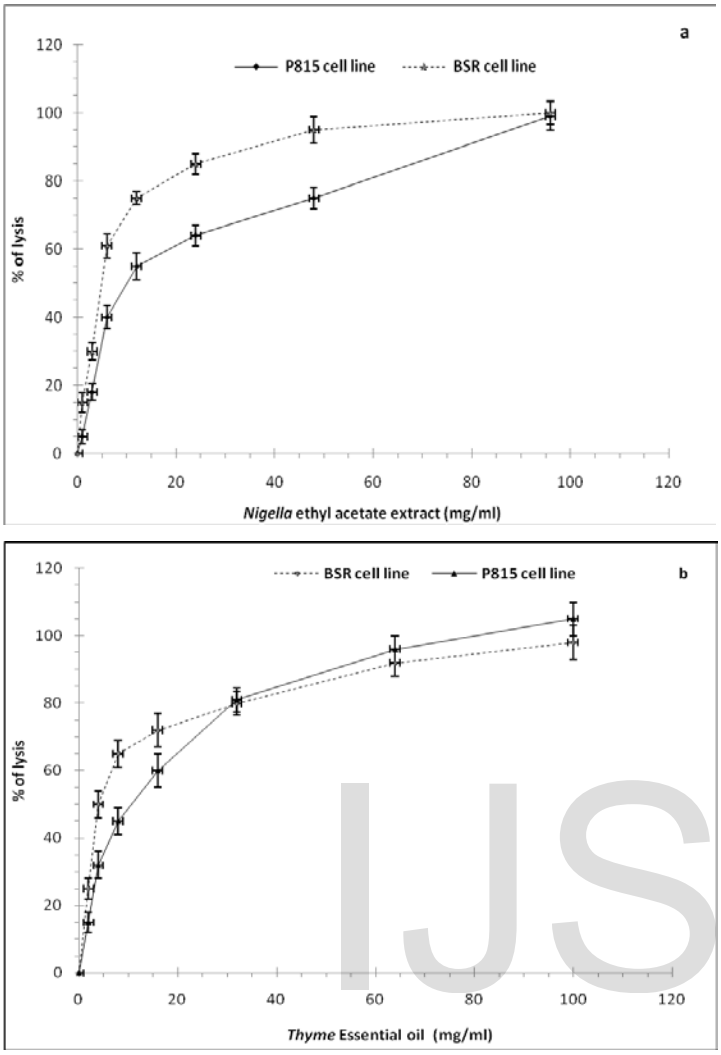

Fig 1. Effect of carvacrol on both BSR and P815 cancer cell growth. Cell lines (10^4cells/ml) were cultured in 96-well culture plates for 48 h in the absence or presence of Negilla ethyl acetate extract (a) or Thyme essential oil (b) at various concentrations. Then the cell growth was evaluated by using the MTT assay as described in the methods section. Data are the mean of triplicate measurements ± S.D.

Table 1.Cytotoxic activity of extracts and compounds on different cell lines expressed as the concentration providing 50% inhibition (IC_{50}).

Cell lines	Nigella extract (mg/ml)	Thyme essential oil (mg/ml)	Vincristine (μM)	Carvacrol (μM)
P815	**9.5 ± 0.45**	**11 ± 0.4**	**4 ± 0.2**	**200 ± 10**
BSR	**4.5 ± 0.2**	**4 ± 0.23**	**7 ± 0.25**	_

3.2 Effect on both cell growth and intracellular ALP activity.

Experiments were carried out by incubation cells (P815 or BSR) in 6-well culture plates (final volume of 6 ml, 10^5 cells/ml) for 48 h in the presence of different compounds or extracts at concentrations corresponding to their IC_{50} (see Methods section). The cell viability and their morphology were examined by optic microscopy and then after le cell number were quantified by using Malassez lame for each experiment. Than the solubilised cell extract was prepared and the ALP activity was determined as described in the methods section. By using cell counting by optic microscopy, the IC_{50} previously determined by the MTT assay (Table 1) give almost the same percentage inhibition of cancer cell lines (Table 2).

Table 2. Effect of extracts and compounds on both P815 cell viability and intracellular ALP activity. P815 cells (10^5/ml) were cultured in 6-well culture plates for 24 h. The cells were than treated with extracts or compounds at the indicated concentrations for 48 h. The cells were collected, counted and washed with PBS. Than the solubilised extract was prepared and the enzyme activities were determined as described in the methods section. Data are the mean of triplicate measurements ± S.D

P815 % of control	Vincristine (7 μM)	Nigella extract (9.5 mg/ml)	Thyme essential oil (11 mg/ml)	Carvacrol (200 μM)
Cell number	**-45 ± 4**	**-52 ± 7**	**-48 ± 5**	**-55 ± 5**
ALP activity After 48 h	**+66 ± 8**	**+46 ± 5**	**-40 ± 5**	**-60 ± 7**
Direct effect on **ALP activity**	**-4 ± 2**	**-3 ± 2.5**	**+2 ± 0.5**	**+4 ± 2**

Analysis of intracellular solubilised ALP activity shows clearly that this enzyme activity was enhanced significantly when the cells were treated by vincristine (+ 66%) or by Nigella extract (+46%), and at the same time, cell proliferation is inhibited. The abnormal increasing proportion of ALP was thought to be resulting from P815 cell stress reaction as reported previously [24], [25], [30]. Conversely, both thyme essential oil and one of its major component carvacrol were able to reduce the P815 growth cells and substantially reduced the expression of ALP activity by 40% and 60%, respectively. These results clearly indicate that the inhibition of P815 cell growth by thyme essential oil as well as by carvacrol (at IC_{50}) was not correlated to the high expression of ALP activity in response to the toxic effects as usually reported for many anticancer agents [24], [25], [30]. There was not significant direct effect on ALP (Table 2). Similar experiments were carried out on the adherent cancer cell line (BSR). All extracts and pure compounds used at their IC_{50} were able to reduce significantly the cell growth (Table 3). This decrease in cell proliferation is accompanied by an increase in intracellular ALP activity in the case of Nigella ethyl acetate extract and vincristine treatment. On the other hand, thyme essential oil and carvacrol shows a slight nega-

tive effect, but significant, on intracellular ALP activity, as for P815 cell line determined above. Also there was not direct effect on the solubilised ALP activity (Table 3).

Table 3. Effect of extracts and compounds on both BSR cell viability and intracellular alkaline phosphatase activity. BSR cells (10^5/ml) were cultured in 6-well culture plates for 24 h. The cells were than treated with extracts or compounds at the indicated concentrations for 48 h. The cells were trypsinized, collected, counted and washed with PBS. Than the solubilised extract was prepared and the enzyme activities were determined as described in the methods section. Data are the mean of triplicate measurements ± S.D

BSR % of control	**Vincristine** (7 µM)	**Nigella extract** (9.5 mg/ml)	**Thyme essential oil** (11 mg/ml)	**Carvacrol** (200 µM)
Cell number	**-51 ± 5**	**-47 ± 7**	**-52 ± 5**	**-55 ± 5**
ALPactivity After 48 h	**+45 ± 4**	**+36 ± 5**	**-15 ± 4**	**-35 ± 5**
Direct effect on **ALP activity**	**+5 ± 2**	**+3 ± 1**	**-2 ± 0.5**	**+3 ± 1.5**

4 Discussion

Based on our previous investigation, the *in vitro* effect of essential oil of *Thymus broussonetti* as well as extracts of *N. sativa L.* seeds were found to exert a significant degree of cytotoxicity against various tumor cell lines. Furthermore, we have also shown that these extracts were able to reduce significantly the tumour growth and delaying mortality of tumour-bearing animal DBA2 (H2d) mice model [11], [21]. It was proposed, owing to their lipophilic nature, that plant oil volatile appears to accumulate in the microbe cell membrane and increase their permeability, resulting in leakage (outflow) of enzymes and metabolites [31], [32]. Recent study suggests that tumour-derived ALP regulates tumour growth in advanced prostate cancer [33]. Nevertheless, the coupling mechanism between proliferative or anti-proliferative effects of thyme essential oil as well as ethyl-acetate Nigella extract and the intracellular target is not known and may differ in different cell lines. Thus, we investigate the effect of these extracts and carvacrol, one of the major components of plant *Thymus broussonetti* essential oil, on the growth pattern of P815 and BSR cells and the intracellular ALP expression.

All extract fractions used here exhibited a significant cytotoxic effect against the tumour cell lines (P815 and BSR) confirmed by both methods, MTT test and by cell count (Table 1 and 2). Our results agree with previous research in which the Nigella ethyl acetate fraction as well as thyme essential oil were found to exhibit a strong growth inhibitory effect on all malignant cells tested [11], [21].

Interestingly, we report here for the first time, that the cytotoxic effect of these extracts and pure compounds used is related not only to the intracellular ALP expression but also to the nature of the tumour cell lines. In fact, The intracellular ALP activity was enhanced when the P815 as well as BSR cells were treated by vincristine (positive control) and Nigella ethyl acetate extract and this enhancement was accompanied by a significant decrease in the cell number, which is in full agreement with the literature data concerning the induction of ALP expression correlates well with the growth inhibition pattern of cancer cells under the influence of different cytotoxic agents [24], [25], [29]. On the other hand, The intracellular ALP activity behaved differently when the P815 and BSR cells were treated by thyme essential oil and pure carvacrol, showing a significant repression of intracellular ALP activity with a concomitant high decreasing cell number (Table 2 and 3). Therefore, our findings highly suggest that the repression of ALP activity by thyme essential oil and carvacrol, one of its major components, could be responsible for the regulation of P815 and BSR cancer cell growth. It has been reported that carvacrol showed an anti-cancer effect, mainly by inducing apoptosis phenomenon [22], [34], [35], [36] and in that regard it would be of great interest to know whether ALP activity is involved in the modulation of cancer cell growth.

5 Conclusion

In the present study, we found out, that thyme essential oil and one of its major components, carvacrol, have an anti-proliferative effect resulting in inhibition of P815 and BSR cell growth. Furthermore, this inhibition of cell growth seems to be correlated to the negative modulation of ALP activity. This study supplies new information on the possible mechanistic and a target by which thyme essential oil and pure carvacrol exert their effect on P815 and BSR cancer cells.

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