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Quantification of DNA damage products by gas chromatography tandem mass spectrometry in lung cell lines and prevention effect of thyme antioxidants on oxidative induced DNA damage

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ABSTRACT

Lung cancer has a high treatment cost and poor prognosis in comparison to other types of cancers. This work was involved in studying oxidative DNA base damage inhibition. Accordingly, standard carvacrol, thymol, thymoquinone with water and water-methanol extract of thyme (*Origanum vulgare* L. subsp. *hirtum* (link.) Ietswaart), thyme oil and thyme water were prepared and investigated for their efficacy to inhibit DNA oxidative damage formed by H_2O_2 in malignant lung cells (A549). The antioxidant capacity by ABTS assay was 271.73 ± 11.45 mg trolox equivalent/mL for thyme oil. HPLC analysis was carried out to determine the contents of different thyme extracts, results showing the presence of carvacrol, thymol, protocatechuic acid, caffeic acid, epicatechin and rosmarinic acid in water and water-methanol extracts while only carvacrol and thymol were found in thyme oil and thyme water. After DNA isolation from the cultured cells, the formed oxidative induced DNA damage products were analysed using GC–MS/MS. It was proven that the antioxidants in the cell culture media have succeeded to inhibit oxidative DNA base damage. Thymoquinone was shown to be the best protectant antioxidant among other antioxidants against the formation of oxidative DNA damage, whereas water-methanol extract of thyme was the best among the plant-sourced samples. Thymoquinone and thyme water-methanol extract were investigated for their efficacy on cultured healthy lung cells (BEAS-2B), and it was proven that they are efficient in protection against the oxidation of DNA of healthy lung cells too.

1. Introduction

Lung cancer has one of the highest mortality rates in human beings among other types of cancer diseases [1–3]. The most common risk factor for lung cancer is smoking [4,5]. This disease has a high incidence rate in ages higher than 50 years and still has one of the poorest prognosis rates except in the early stages of the disease [4]. Different studies have indicated the role of oxidative damage of DNA in carcinogenesis [6–12]. Thus, antioxidants have been studied and investigated widely for their protection and scavenging ability of the reactive oxygen species (ROS) which are the main cause of oxidative induced DNA damage [13,14]. Diets rich in antioxidant substances have been shown to be effective in decreasing the risk of cancer diseases including lung cancers [15,16].

Various studies have reported increased levels of DNA nucleobase damage products in lung cancer cells [1,17–19], which may suggest a potential reliable indicator of oxidative stress in organisms with potential malignancy [9]. Thus, accumulated evidence could indicate the possibility of using such damage products as potential biomarkers that may assist in the early detection of lung malignancies. Different techniques have been described for the measurement of DNA nucleobase damage products, such techniques include high performance liquid chromatography (HPLC), liquid chromatography/tandem mass spectrometry (LC–MS/MS), gas chromatography mass spectrometry (GCMS), alkaline filter elution, single cell gel electrophoresis (comet assay) and others [9,20–23].

Oxidative stress is a condition where the antioxidant defence mechanisms are imbalanced with the pro-oxidant agents like ROS where the imbalance is in favour for the pro-oxidants. The living cells are constantly exposed to damaging oxidant radicals, for this reason several defences are recruited to counteract the oxidative stress, these defences include antioxidants such as vitamin C and E and enzymes such as

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superoxide dismutase, catalase and glutathione peroxidase [24]. The contribution of natural antioxidants particularly those of phenolic nature to the defence against cancers have been studied [25,26]. However, conflicting studies have reported that some antioxidant may in fact accelerate the incidence of some types of malignancies by mechanisms other than DNA damage [27–29]. Antioxidants, as shown by literature, are thought to have appreciated activity in conditions where oxidative DNA damage is the cause of the problem. Thus, so many mutations and malignancies are still reduced as long as the DNA damage is inhibited by antioxidants. Nevertheless there are also reports on increased oxidative DNA product levels with excess of antioxidant treatment [30,31].

To investigate the inhibition of DNA damage in lung cancer cells, a human lung epithelial cell line (A549 tumour cells) [29,32] was used and the results were compared to those obtained from the experiments carried out on human bronchial epithelial cell line (BEAS-B2) [33]. The in vitro and in vivo effects of various plant extracts on tumour cell lines were reviewed in literature [25].

In this study, thyme (*Origanum vulgare* L. subsp. *hirtum* (link.) Ietswaart) was extracted and the obtained thyme oil and thyme water in addition to the prepared carvacrol, thymol and thymoquinone standard substances were investigated for their ability to inhibit the formation of oxidative DNA nucleobase damage products in malignant lung cells (A549) in accordance with healthy lung cells (BEAS-2B) which were used as control. Sample preparation and determination of oxidative induced DNA nucleobase damage products by GC–MS/MS were achieved using a modified method from those described in literature [20,34] to detect all possible oxidative DNA damage products in healthy and malignant lung cells.

2. Materials and methods

2.1. Chemicals

Acetonitrile, formic acid, HCl, trimethylchlorosilane (TMCS) and methanol in HPLC grade were purchased from Merck (Darmstadt, Germany). Pyridine anhydrous, gallic acid, hydrogen peroxide trace Select Ultra ≥30%, 5-(hydroxymethyl)uracil (5HMUra), 4,6-diamino-5-nitropyrimidine (46D5NP), 5,6-Dihydroxy methyluracil (56DHUra), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), Deoxyribonucleic acid (DNA) from calf thymus, ABTS, ethylene diamine tetra acetic acid (EDTA), catalase, NaCl, proteinase K, sodium dodecyl sulphate (SDA), thymoquinone, thymol, trolox, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), ethanol (absolute) in HPLC grade and trichloroacetic acid (TCA) were from Sigma-Aldrich (St. Louis, MO., USA). Iron(II) sulfate-7-hydrate extra pure and NaOH from Riedel-de Haen. *N*,*O*-Bis(trimethylsilyl)triflouroacetamid (BSTFA) and carvacrol were purchased from Aldrich (USA). Alloxan (Alx) was from Titan Biotech (Delhi, India). 4,6-Diamino-5-(formylamino)pyrimidine (46D5FP) from Santa Cruz Biotechnology (Texas, USA). 5-Formyluracil (5FUra) was from IS Chemical Technology (Shanghai, China). 5-(Hydroxymethyl) cytosine (5HMCyt), 5-hydroxyhydantoin (5HH), 5,6-dihydrothymine (56DHThy), 2,8-dihydroxyadenine (28DHAde), 5-hydroxy-5-methylhydantoin (5H5MH) and 2-hydroxy adenine (2HAde) were purchased from Toronto Research Chemicals (Toronto, Canada). 5,6-Dihydrouracil (56DHUra), 5-Hydroxyuracil (5HUra), 5-Hydroxycytosine (5HCyt), Thymine Glycol (ThyGly), 8-oxo-7,8-dihydroadenine (8-oxoAde), 8-oxo-7,8-dihydroguanine (8-oxoGua) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) were from National Institute of Standards and Technology (MD, USA). RPMI-1640 medium were purchased from Lonza Bioscience (Verviers, Belgium). L-Glutamine, penicillin G and streptomycin 0.05% trypsin-EDTA solution and fetal bovine serum was from Gibco; Thermo Fisher Scientific (MA, USA).

2.2. Extraction of the thyme

Thyme used in this study (*Origanum vulgare* L. subsp. *hirtum* (link.) Ietswaart) was originally cultivated in Çan/Çanakkale, Turkey and was purchased in a dried form. The type of thyme used in this study was determined in the Botany subunit, Department of Biology, Graduate School of Natural and Applied Sciences, Uludag University, Bursa, Turkey.

The leaves of dried thyme were separated from their branches and were extracted as such. Two different extraction solvents, water and methanol-water (1:1, v/v), were used to extract the plant samples using ultrasonic bath. Approximately 4 g of thyme samples were extracted with extraction solvent of 80 mL at room temperature for 120 min. The extracts were filtered and stored at 4 °C.

2.3. Thyme oil and thyme water production

The oil of thyme and its distillate water were obtained using Clevenger apparatus. 400 mL distilled water was added into a round-bottom flask containing 40 g dried thyme. Clevenger apparatus with water condenser were set over the flask. The contents of the flask were boiled for 7 h on an electric mantle. At the end, 40 mL of thyme distillate water and 1.5 mL of thyme oil were obtained. These samples were taken into glass bottles and stored at 4 °C.

2.4. Antioxidant capacity assay

The total antioxidant capacities of samples were determined by ABTS method. $ABTS^+$ was produced by reacting 20 mM ABTS solution with 2.45 mM potassium persulfate solution and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The procedure for samples were performed by adding 0.2 mL sample, 3.8 mL of ethanol and 1 mL of the $ABTS^+$ radical cation solution which was diluted with ethanol at a ratio of 1:10 and the absorbance was recorded at 734 nm against blank after 6 min. Standard curve was prepared using concentrations from $2.94.10^{-3}$ to $8.82.10^{-1}$ mM for trolox. The results were expressed as milligram of trolox equivalents (TE) per mL sample.

2.5. HPLC analysis of the extracts

An Agilent 1200 HPLC-DAD system (Waldbronn, Germany), consisting of a vacuum degasser, binary pump, autosampler and a diode-array detector was used for the analysis of the phenolic components of thyme. The injection volume was 10 µL and the flow rate was set to 0.5 mL/min. An isocratic mobile phase program composed of acetonitrile (A) and distilled water (B) was used for carvacrol, thymol and thymoquinone separations. The program of 58% A (0–20 min) was implemented. Other phenolic compounds were analyzed using a gradient mobile phase consisting of 1% aqueous formic acid (v/v) (A) and acetonitrile (B); the gradient program of 10% B (0 min), 13% B (10 min), 41.5% B (20 min), 70% B (25 min), 10% B (30 min) and 10% B (35 min) was applied.

Carvacrol, thymol and thymoquinone solutions were prepared in different concentrations for the quantitative analysis using HPLC-DAD system. Carvacrol and thymol were detected at 273 nm whereas thymoquinone at 254 nm. Other phenolic compounds were detected at 280 and 360 nm. Calculations involved the use of the least square method to produce a straight line equation where the concentration versus the peak area was used in the calibration curve.

2.6. Cytotoxic studies

2.6.1. Cell culture

Non-small cell lung cancer cell (A549, human lung epithelial cell line) and normal lung cell (BEAS-2B, human bronchial epithelial cell

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