Antibacterial properties of *Piper nigrum* and *Thymus vulgaris* extracts and the safety of using them on living tissues

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Abstract: Present investigation focused on antibacterial potential of black pepper (*Piper nigrum*) and *Thymus vulgaris* extracts against four Pathogenic bacteria (*Escherichia coli O157:H7, Staphylococcus aureus* ATCC 6538, *Bacillus cereuss B-3711 Salmonella typhi)* and the safety of using them on living tissues (human tumor colon cell line and lung carcinoma cell line). From all the results we can conclude that the moderate dose not have any hazardous side effects on human cells. On the other hand, we noticed that the *Piper nigrum* or *Thymus vulgaris* has antimicrobial effect on some pathogenic bacteria. So suggested using of these spices should be performed with considering their proper concentration and more safety studies on them.

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1. Introduction

It has been shown that the human diet contains a great variety of natural mutagens and carcinogens (*Ames, 1983; Sugimura, 2000*). Still, food contains antimutagens and anticarcinogens that act to reduce or eliminate the effect of carcinogenic agents (*Stavric, 1994*). Because of the diversity and the widespread occurrence of these compounds in food (dietary fibre, vitamins A, C, E; selenium; calcium; carotenoids; polyphenolic acids; flavonoids), no human diet can be entirely free of antimutagens or anticarcinogens. Food components exhibit their antimutagenic and anticarcinogenic effect by: (1) inhibiting carcinogen activation and enhancing carcinogens detoxification; (2) scavenging DNA reactive agents; (3) suppressing the abnormal proliferation of early preneoplastic lesions; and (4) modifying some properties of the cancer cell (*De Flora and Ramel, 1988; Wargovich, 1997*).

Spices are dietary constituents consumed daily by the North African population to enhance the flavour or taste of human food. Many of them have been identified as possessing potential chemopreventive properties.

Black pepper (*Piper nigrum*) has been found to be carcinogenic in animal models (*El-Mofty et al., 1988; Shwaireb et al., 1990*). A force-feeding of Egyptian toads with black pepper led to liver tumor formation (*El-Mofty et al., 1991*). Safrole and tannic acid (constituents of black pepper) are weakly carcinogens and can be reduced by feeding for a long period with d-limonene, a pepper terpenoid (*Wrba et al., 1992*). Black pepper juice has been found to be effective against N-methylnitrosourea induced colon carcinogenesis in F344 rats (*Narisawa et al., 2000*). Carotenoids isolated from the fruits of bell pepper exhibits potent anti-tumor-promoting activity *in vivo* and *in vitro* (*Maoka et al., 2001*).

The genus Thymus comprises 215 species with *Thymus vulgaris* being one of the most important and thoroughly investigated aromatic plant.

Chemical polymorphism is characteristic to the species of Thymus; numerous chemotypes have been defined, such as carvacrol and thymol, a-terpineol, thujone, geraniol, linalool and others (*Thompson et al., 1, 1998*). Essential oils containing high amount of thymol and carvacrol were reported to possess the highest antioxidant activity (*Farag, et al., 1989; Aeschbach et al., 1994; Deighton, et al., 1994; Dapkevic˘ius, et al., 1998)*. In addition, these compounds exhibit other bioactivities, e.g. thymol is an antiseptic, while carvacrol possesses antifungal properties (*Menphini, et al., 1993*). Non-volatile antioxidants, such as flavonoids and vitamin E were also found in the extracts of *T. Vulgaris* (*Guille´n & Manzanos, 1998; Dapkevicius et al., 2002*).

Therefore essential oils of thyme can be used as natural preservative ingredients in the food industry (*Conner & Beuchat, 1984; Karapmar & Aktug, 1987; Economou, et al., 1991; Banias, et al., 1992; Shapiro, et al., 1994; Curtis, Shetty, et al., 1996; Hamss et al., 2003; Loz˘iene et al., 2007*).

Present investigation focused on antibacterial potential of black pepper (*Piper nigrum*) and *Thymus vulgaris* extracts against four Pathogenic bacteria and the safety of using them on living tissues (human tumor colon cell line).

2. Materials and Methods

Plant material

Black peppers (*Piper nigrum*) and *Thymus vulgaris* were purchased from a Makka supermarket. The same sample of spice was used throughout the study. Spices were dissolved in distilled water at room temperature by adding the equivalent weight of spice (g) to 100 ml (50% g/ml) and other to 200 (25% g/ml) ml of distilled water and allowing it to draw for 10 min. After filtering, fresh solutions were used immediately.

Pathogenic bacteria:

Escherichia coli O157:H7, Salmonella Typhi were obtained from Microbiology laboratory, National Research Center, Cairo, Egypt *Staphylococcus aureus* ATCC 6538, *Bacillus cereuss* B-3711 was obtained from NRRL (North Regional Research Laboratory, ILIinois USA).

***In vitro* antibacterial susceptibility studies**

Disc diffusion method (*Brooks et al., 2002*) was employed for antibacterial susceptibility assay. Hundred sterilized discs of filter paper (6mm in diameter) were soaked in 1ml of aqueous decoctions of black pepper (*Piper nigrum*) and *Thymus vulgaris* for 1-2minutes and then used for screening. The concentration for each disc was 10µl or 50µl (100discs/ml of aqueous decoction of each spices or 20discs/ml). Mueller-Hinton agar (MHA) (Merck) was used as antimicrobial susceptibility test medium and Mueller-Hinton broth (MHB) (Merck) was used for preparation of inoculums. A sterile inoculating loop was touched to four-five isolated colonies of the test bacterial strains grown on MHA and used to inoculate a tube of Mueller-Hinton broth. The inoculated tube was incubated at 37°C for 24hours and standardized to match with 0.5 McFarland turbidity standards. A sterile cotton swab was dipped into the standardized bacterial test suspension and used to evenly inoculate the entire surface of MHA plates. Previously soaked discs in aqueous decoctions of *Piper nigrum* and *Thymus vulgaris* were placed on the surface of inoculated plates with sterile forceps. All plates were incubated at 37°C for 24hours. The diameters of the zones of inhibition appearing around the discs were measured to the nearest millimeter (mm) and recorded.

Examination of bacteria using transmission Electron Microscopy:

1ml of sterilized *Piper nigrum* or *Thymus vulgaris* filtrate was added to each of Nutrient agar before its solidification in sterilized Petri dishes. Then surface of the agar was streaked with *Staphylococcus aureus* (as a sample to pathogenic bacteria). Plates were incubated at 37 $\pm 2^0$C. After growth, external structure of the tested microorganism was examined using transmission Electron Microscope (JOEL- 8000cx) at National Research Centre, Egypt. Results were photocopied at the same place.

Tissue culture method to study the safety of using *Piper nigrum* or *Thymus vulgaris* on living tissues:

Tissue culture method according to *Mosmann, (1983*); *Thabrew et al.(1997*) was carried out in order to study the safety of using *Piper nigrum* or *Thymus vulgaris* on the human living tissues. Tissues were at early stages of change as a result of cancer, to easily notice the effect of *Piper nigrum* or *Thymus vulgaris* on it. Selected tissues of infected human tumor colon cell line, and lung carcinoma cell line were treated with *Piper nigrum* or *Thymus vulgaris*. This experiment was conducted in the cell cultures bioassay laboratory, at human tumor lines used for drug detection at National Research Center, Cairo, Egypt.

3. Results and Discussion

Four pathogenic bacteria organisms were used in the present study. *In vitro* antibacterial activity results of the aqueous decoction of black pepper *(Piper nigrum)* or *Thymus vulgaris* are given in table 1. The diameter of inhibitory zones recorded includes the size of filter paper disc (5 mm in diameter) and concentration of decoctions of selected spices for each disc.

In the present study, the aqueous decoction of black pepper and *Thymus vulgaris* exhibited maximum effect against *Staphylococcus aureus* (7.8 & 8.9 mm average diameter of inhibitory zone respectively) and found black pepper to be most active antibacterial agent against all bacterial strains.

Antibacterial properties of plants extracts were reported in numerous studies. Thymus has exhibit distinctive antibacterial activity mainly due to the presence of phenolic compounds, thymol and carvacrol *(Loziene et al., 2007*).

Food pathogens were selected as target microorganisms for the preliminary antibacterial screening of black pepper and thyme extracts in the present study (Table 1). The antibacterial activity of the *Piper nigrum* extracts depended on the sensitivity of bacteria. *S. aureus* and *E. coli*, were the most sensitive, while, *B. cereus* was less sensitive and *S. typhimurium* was resistant to the *Piper nigrum*. As it can be expected the dose of 50 µl was more efficient for four percent. Findings of the present study are similar to those reported by *Masood et al. (2006*). They observed the overall aqueous decoction of black pepper was the most bacterial-toxic exhibited 75% antibacterial activity as compared to aqueous decoction of bay leaf (53.4%) and aqueous decoction of aniseed (18.1%), at the concentration of 10µl/disc.On the other hand, *B. cereus*, and *S. aureus* were the most sensitive to the *Thymus vulgaris* extracts, while *E. coli*, *S. typhimurium* were resistant to the *Thymus vulgaris* except 4% *Thymus vulgaris* has effect on *S. typhimurium* with the both doses.

Lozˇien_e et al. (2007) reported that antimicrobial effects of the extracts, although being selective in terms of pathogenic bacteria and comparatively weak bactericides can also contain some potential for practical applications as a complementary property, e.g., in designing hurdle food preservation technologies.

Table (1): Antibacterial activities of black pepper (Piper nigrum.), and Thymus vulgaris extracts against some pathogenic bacteria

		B. cereus	*E. coli*	*S. typherium*	*Staph.aureus*
Piper nigrum 1%	50	0.0	5.9± 0.1	0.0	6.6 ± 0.1
2%	10	0.0	5.7± 0.3	0.0	5.9± 0.2
3%	50	0.0	6.1± 0.0	5.7± 0.0	6.7± 0.0
	10	0.0	5.9± 0.2	0.0	6.1± 0.0
4%	50	0.0	6.4± 0.2	6.2± 0.1	7.3± 0.2
	10	0.0	6.1± 0.1	5.9± 0.1	6.4± 0.2
	50	7.1± 0.2	6.8± 0.0	6.9± 0.3	7.8± 0.1
Thymus vulgaris 1%	10	6.4 ± 0.1	6.3± 0.3	6.5± 0.2	7.1± 0.3
2%	50	6.2 ± 0.2	0.0	0.0	6.1± 0.1
	10	5.7 ± 0.1	0.0	0.0	5.9±0.0
3%	50	6.4 ± 0.1	0.0	0.0	6.3±0.2
	10	6.1 ± 0.0	0.0	0.0	6.1±0.2
4%	50	7.0 ± 0.0	0.0	0.0	7.5±0.1
	10	6.2± 0.0	0.0	0.0	6.4 ± 0.1
	50	7.2±0.2	0.0	6.5 ± 0.1	8.9 ± 0.1
	10	6.9±0.1	0.0	6.2±0.2	7.7±0.2

Scanning and transmission Electron Microscopy examination for bacteria treated with *Piper nigrum or Thymus vulgaris*:

Transmission imaging microscopy was carried out at National Research Center, Cairo, Egypt. Results of examination showed the morphological shape of *S.aureus* (as a sample for pathogenic strains photo 1, control) and the analyzing of bacterial cell wall and the deformity of the external structure which led to changes in the cells from spherical clusters to non-specific shape blocks after treatment with 3% of *Piper nigrum* (Photo 2) or *Thymus vulgaris* (Photo 3). This results are agree with the results in table (1).

Safety of using Piper nigrum or Thymus vulgaris on human tissues:

Tissue culture experiment was carried out to study the safety of using *Piper nigrum* or *Thymus vulgaris* on human tissues. Results showed that *Piper nigrum* or *Thymus vulgaris* has not any harmful effect on all types of cells studied. Tissues were selected when it is at the early stages of change as a result of cancer, so they are very sensitive and easy to see the effect of the tested substance on it. The effect should reach 99% of 100 ppm to prove that it has a harmful effect. Results showed that the least effect of *Piper nigrum* and *Thymus vulgaris* was on colon tissues reaching 66.8% per 100 ppm and 23.5% respectively, whereas it was 0% for lung tissues (Tables 2,3). These results agree with *Sertel et al*., (2011) who reported that the Thyme essential oil inhibits human HNSCC cell (head and neck squamous cell carcinoma) growth. Based on pharmacogenomic approaches, novel insights into the molecular mode of anticancer activity of thyme were presented. Hamss *et al.,* 2003 found that bell pepper and black peppers possess antimutagenic activity in the Drosophila wing spot test. The results, indicate that the mechanism may involve the inhibition of mutagen formation and direct interaction with electrophilic species.

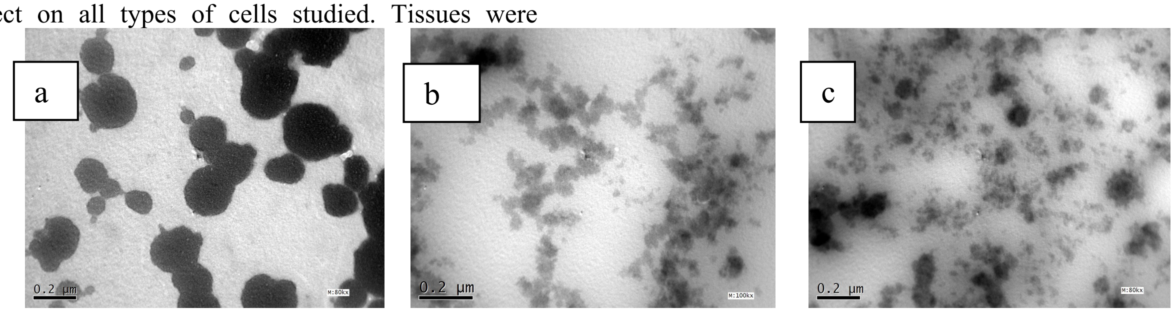

Photo (1): (a) Show the general shape of *Staphylococcus aureus* ATCC6538 (control), (b) treated with 4% black pepper, (c) treated with 4% *Thymus vulgaris* using transmission electron microscope where cell of *S. aureus* was analyzed (hollowed out) and appears in the Photo (b&c) change in the wall.

Table (2): Cytotoxic activity test against the human tumor cell line HCT116 [Colon cell line]

Sample Code	**LC_{50}** (µg/ml)	**LC_{90}** (µg/ml)	Remarks
Piper nigrum	-------------------	-------------------	**66.8% at 100ppm**
Thymus vulgaris	-------------------	-------------------	**23.5% at 100ppm**
DMSO			**1% at 100ppm**
Negative control			**0 %**

LC_{50}: Lethal concentration of the sample which causes the death of 50% of cells in 48 hrs
LC_{90}: Lethal concentration of the sample which causes the death of 90% of cells in 48 hrs

Table (3): Cytotoxic activity test against the human tumor cell line A549 [Lung carcinoma cell line

Sample Code	LC_{50} (µg/ml)	LC_{90} (µg/ml)	Remarks
Piper nigrum	--------	------	% at 100ppm
Thymus vulgaris	------	------	% at 100ppm
DMSO			5% at 100ppm
Negative control			0 %

LC_{50}: Lethal concentration of the sample which causes the death of 50% of cells in 48 hrs
LC_{90}: Lethal concentration of the sample which causes the death of 90% of cells in 48 hrs

While, *Rahimifard et al., (2009)* conclude that the all studied essential oils and extracts of *Thymus vulgaris* and *Zataria multiflora* may have cytopathologic effect on Vero, Hep2, Hela in specific concentrations..

From all the results we can conclude that the moderate dose not have any hazardous side effects on human cells. On the other hand, we noticed that the *Piper nigrum* or *Thymus vulgaris* has antimicrobial effect on some pathogenic bacteria. So suggested using of these spices should be performed with considering their proper concentration and more safety studies on them.

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