Anti-microbial screening and cytotoxic activity of aerial part of *Thymelaea hirsuta* L. essential oil growing in south-west Tunisia

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Abstract: This study aimed to investigate the antimicrobial and cytotoxic activities of essential oil isolated by the hydro-distillation of aerial parts of *Thymelaea hirsuta*. The antimicrobial activity of the oil was evaluated against eight bacterial and three fungal pathogenic strains. The results revealed that the essential oil exhibited a moderate-to-potent anti-microbial activity against all the microorganisms tested. Gram-positive bacteria were noted to be more sensitive to the oil than gram-negative bacteria and yeasts. *In vitro* cytotoxicity evaluation against HeLa cell lines showed that the essential oil exhibited moderate cytotoxicity on human tumor cells, with a high IC_{50} value of 175µg/mL. To the author's knowledge, this is the first study reporting on the antimicrobial and cytotoxic activities of *Thymelaea hirsuta* essential oil. Overall, the results indicate that the *T. hirsuta* essential oil has a number of attractive properties that might open new promising opportunities for the control or prevention of a wide range of microbial infections and cancers and can facilitate the use of essential oils as natural preservatives against spoilage microorganisms in food systems.

Keywords: *Thymelaea hirsuta*, essential oil, antimicrobial activity, cytotoxic activity.

INTRODUCTION

Several spices and herbs have long been used for the treatment and prevention of various health conditions and diseases (Srivastava *et al*., 1996, Rao and Gan, 2014). Even in the presence of modern synthetic medicines, medicinal plants and herbs still continue to serve as possible sources for medical drugs and therapies (Tijani *et al*., 2008). In fact, the increasing concerns over the development of drug resistance to human pathogenic bacteria have fuelled the search for alternative natural sources of drugs and recent research indicates that several natural plants and herbs may hold a promise as alternative or complementary therapeutic adjuncts.

Although the precise mechanisms of action of medicinal plants have not yet been fully determined, the literature provides ample evidence that several plant and herb species represent promising rich sources of antimicrobial agents. Of particular interest, *Thymelaea hirsuta* is a perennial evergreen shrub plant belonging to the Thymeleaceae family, commonly known in Tunisia as ‘‘Methnane’’, is abundantly growing in the Mediterranean coastal plains, Sinai Peninsula and other Saharo-Arabian deserts. The aerial part of this species has traditionally been used as decoction in the treatment of diabetes and hypertension (Le Floc'h, 1983). *T. hirsuta* has been used in folk medicine for its antimelanogenesis (Kawano *et al*., 2007), antioxidant (Djeridane *et al*., 2006; Kadri *et al*., 2011), hypoglycemic and antidiabetic (El Amrani *et al*., 2009) and anti-inflammatory and antiarthritic (Azza and Oudghiri, 2015) properties. Several authors have isolated and identified daphanane diterpenes in some extracts from this plant (Brooks *et al*., 2007; Kawano *et al*., 2007; Miyamae *et al*., 2009).

Considering the growing concerns over conventional drug resistance in humans and the continuous search for efficient alternatives from natural sources, the present study was undertaken to investigate for the first time the in vitro antimicrobial activity against clinical and pathogenic microorganisms and the cytotoxic properties of the essential oil from the aerial part of *T. hirsuta* growing in South-West Tunisia.

MATERIALS AND METHODS

Chemicals, reagents and plant material

All chemicals and reagents were of the analytical gradeand purchased from Pharmacia (Uppsala, Swedeen) and Prolabo (Paris, France). The aerial parts of *T. hirsuta* were grown and collected at the flowering stage in Sidi Aïch, Gafsa, Tunisia. A Voucher specimen was confirmed by a senior A. Bekir and deposited at ISET, Sfax (Département de Génie des procédés) as Bekir 521.

Distillation of essential oil and GC/MS analysis conditions

The fresh aerial parts of *T. hirsuta* (300g) were submitted to hydro distillation for 4h using Clevenger-type

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equipment to recover the essential oils. The obtained oil was dried on anhydrous sodium sulfate to remove water, and then filtered and stored at +4°C. The samples were then analyzed by gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS) using a flame ionization detector and capillary column with HP-5MS (30m x 0.25mm, 0.25 µm) fixed to an Agilent-Technologies 6890 N Network GC system. The parameters of the GC-MS method validation were described elsewhere (Zarai *et al*., 2011).

Antimicrobial activity assay

The *T. hirsuta* essential oil samples were assayed for their antimicrobial activity against a set of ten of human-pathogenic microbial strains. The test microorganisms were selected based on their role in human skin, and oral and intestinal tracts. A slightly modified version of the protocol described by Zarai *et al*., (2011) was used for all antimicrobial experiments (agar diffusion method, determination of the minimal inhibitory concentration (MIC)**,** antibacterial assay disc-diffusion method, antibacterial assay disc-diffusion method, and antifungal assay disc-diffusion method). All experiments were carried out in triplicates.

Cell lines and culture condition

The essential oil was submitted to cytotoxicity effect assays to determine the proliferation rates of HeLa cells after treatment with essential oil using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Mitochondrial dehydrogenases were used to reduce the MTT yellow compound into a water-insoluble blue compound formazan, depending on the viability of cells as described by Zarai *et al*., (2011).

RESULTS

Antimicrobial assays

The *T. hirsuta* essential oil was evaluated for antimicrobial activity against ten pathogenic strains, which have not previously been reported on, including eight bacterial and three fungal species using the diameter of inhibition zone, the minimum inhibitory concentrations (MIC) and the concentration inhibiting 50% (IC_{50}). The results presented in table 1, revealed that all the microorganisms under investigation showed susceptibility to *T. hirsuta* essential oil activity, with the values recorded for inhibition zone diameters ranging from 11.4 to 26.0mm/sample (table 1). According to the results shown in table 1, Gram-negative bacteria generally showed lower levels of susceptibility than Gram positive bacteria, which could be attributed to their outer membrane that playsa significant role as a an obstacle to biomolecules (Chopra and Greenwood, 2001). *S. aureus*, which considered as the most bacteria causing food poisoning, was the most sensitive organism, exhibiting the highest mean of inhibition zone (27.0±0.8 mm) and the lowest MIC value (>400µg/mL), followed by *E. cloacae* (22.0±0.6mm; MIC >580µg/mL), *B. cereus* (19.8±0.4 mm; MIC >820µg/mL), *M. luteus* (16±0.5mm; MIC >890µg/mL), *B. subtilis* (15.4±1.0mm; MIC>970µg/mL) and *E. faecalis* (14.0±0.7mm; MIC >1120µg/mL), respectively. Gram-negative bacteria *B. cinerea* was more resistant to essential oil, displaying the lowest mean of inhibition zone (10.6±0.4mm) and the highest MIC value (>1220µg/mL), as compared to *E. coli* (11.2±1.0mm; MIC >1150µg/mL). table 2 shows that the IC_{50} values recorded for *T. hirsute* essential oil on bacteria ranged from 1650µg/ml to 2300µg/ml. The bacterial inhibition zones registered for ampicillin discs (10 µg/disc), which were used as positive controls, were noted to range between 20 and 25 mm.

The antifungal activity of *T. hirsute* essential oil presented in table 1 showed moderate inhibitory activity against all the fungi used as test strains. Among the fungal strains, *T. hirsute* was found to be more effective against the pathogenic yeast *C. albicans*, with a mean inhibition zone of 13.2±0.4 mm and a MIC value >1060, as compared to *B. cinerea* (13.2±0.4mm; MIC value >1120µg/mL) and *A. niger* (11.4±0.3mm; MIC value >1140µg/mL). The mean inhibition zones of the positive controls were noted to range from 17.0±1.80 to 31.1±1.75mm. The IC_{50} values of *T. hirsute* essential oil on fungi ranged from 2430µg/ml to 2900µg/ml. The inhibition zone recorded for *cycloheximide* (10µg/disc), which was used as a positive control for bacteria, ranged from 27 to 29 mm.

Cytotoxicity assays

The *in vitro* cytotoxic activities of the *T. hirsute* essential oil on HeLa cell survival was investigated at different dosages ranging from 01.35 to 3000.00µg/ml by the MTT assay based on cell viability. The results depicted in table 2 revealed that the essential oil exhibited a dose-dependent inhibitory effect. The IC_{50} values of the essential oil were found to be 175µg/mL. While the application the essential oil at a concentration of 750µg/ml destroyed the HeLa cells by about 80%, their use at a concentration of 3000µg/ml was noted to induce the total destruction of the cells.

DISCUSSION

Furthermore, biological activity was affected by essential oils. The findings shown in table 2 shows strong activity with inhibition zone higher than 20 mm, moderate activity with inhibition zone ranged from 12mm to 20mm, and weaker activity with inhibition zone less than 10mm. In fact, *S. aureus* has often been considered as one of the most prevalent gram-positive bacteria to induce food poisonings. It does not originate from the food itself but from the activity of humans who contaminate foods after being processed (Rauha *et al*., 2000). The resistance of Gram-negative bacteria to essential oil was previously

Table 1: Antibacterial and antifungal activity of the essential oil of *T. hirsute* using agar disc diffusion, IC_{50} and minimal inhibition concentration (MIC)

Bacteria	Diameter of inhibition	IC_{50} [b]	MIC [c]	DD [d]
Staphylococcus aureus	27.0±0.8	1650±40	>400.00	21.2±0.6
Enterobacter cloacae	22.0±0.6	1930±50	>580.00	20.4±1.1
Bacillus cereus	19.8±0.4	2128±60	>820.00	21.2±1.0
Enterococcus faecalis	16.0±0.5	2300±40	>1120.00	24.6±0.7
Bacillus subtilis	15.4±1.0	2380±50	>970.00	25.3±0.5
Micrococcus luteus	14.0±0.7	2260±30	>890.00	20.2±1.5
Escherchia coli	11.4±0.3	2900±50	>1140.00	29±0.5
Fungi				
Candida albicans	13.2±0.4	2430±30	>1060.00	27.2±1.0
Botrytis cinerea	11.8±0.2	2840±20	>1120.00	28.3±1.0
Aspergillus niger	10.6±0.4	2160±30	>1220.00	20.2±0.5

Results are means of three different experiments,
[a]DD: Disc Diameter of inhibition (halo size) in (mm) , E. oil 100 µg/disc,
[b]MIC: Minimum inhibitory concentration (µg/ml),
[c]IC_{50}: 50% inhibition concentration (µg/ml),
[d]DD: Disc Diameter of inhibition zone of ampicillin (10 µg/disc) and cycloheximide (10 µg/disc), were used as positive controls for bacteria and fungi, respectively.

interpreted as a lack of adaptive responses to stress and to the presence of hydrophilic outer and inner membranes, which can hinder the infiltration of hydrophobic complexes into the target cell membrane (Inouye *et al*., 2001). This membrane constitutes an effective barrier between the cytoplasm and the external environment. Due to its composition, essential oil can act to alter such structures and penetrate within the cell, leading to various alterations, such as the denaturation of proteins and enzymes, fluidity, destabilisation of the phospholipid bilayer, the disruption of the balance of K^+ and H^+ ion concentrations, and ultimate modification of the entire cell morphology, which can lead to the death of the microorganism (Holds worth and Law, 2013).

These observations are probably due to the presence of biologically active compounds in the oil, including alcohols, aldehydes, and ketones. The lipophilic nature of their hydrocarbon skeleton and hydrophilic aspect of their functional groups playa significant role in the antimicrobial action of the essential oil constituents. Therefore, it has been found that the functional chemical groups show the following ranking: phenols >aldehydes > ketones > alcohols >esters >hydrocarbons (Kalemba and Kunicka, 2003). A previous study reported that aliphatic alcohols possessed strong-to-moderate activities against several bacteria and that their activities increased with the increase of the carbon chain length (Kabelitz *et al*., 2003).

Moreover, some compounds, such as α-humulene and δ-selinene, are known to have a promising antimicrobial potential (Jakovlev *et al*., 1979; Nascimento *et al*., 2007). The resistance of Gram-negative *E. coli* bacteria could be attributed to the fact that the essential oil presumably inhibited adenosine triphosphate production from dextrose, thus disturbing the cell membrane (Gill and Holley, 2004). As shown, the presence in this essential oil of high amount of hydrocarbons could also have contributed to the inhibition of the microbial DNA gyrase (Cushnie and Lamb, 2007) and was, therefore, responsible for the antimicrobial effect. Essential oils, being rich interpenes, have previously been described to have attractive antimicrobial activity (Rahman *et al*., 2010). Thus, a synergism effect between minor and major components and interaction between the substances in the oil should also be taken into account and constitute a potential factors contributing to the antimicrobial properties described in this work (Nabavi *et al*., 2015).

Table 2: Cytotoxic activity of *T. hirsute* essential oil determined by the MTT assay

Oil (µg/ml)	% Cell viability
00.00	100.00
01.35	93.70
04.56	87.75
11.74	78.76
23.43	69.86
93.75	59.36
187.50	50.14
375.00	37.28
750.00	21.17
1000.00	13.86
1500.00	07.87
2500.00	03.85
3000.00	01.48
3500.00	00.45

The observed cytotoxicity of this essential oil could be attributed to the presence of various chemical components, including monoterpenes. The latter were previously reported to exert antitumor activities and to present a valuable source of cancer chemo preventive agents of drug discovery (Crowell, 1999). The results also showed that some other compound, including β-caryophyllene and α-humulene, displayed high cytotoxic activity against HeLa cells (Silva *et al*., 2007). The level of cytotoxicity observed for this oil could also be ascribed to the presence of mono and sesquiterpene (Legault *et al*., 2003; Wiseman *et al*., 2007, Bou *et al*., 2013). Furthermore, this high range of cytotoxicity could be explained by the synergetic effect between mixture of its main and minor constituents, which could have led to a better cellular distribution of the essential oil (Bakkali *et al*., 2008).

CONCLUSION

This study represents the first report to investigate and determine the antimicrobial and cytotoxic activities of the essential oil extracted from the aerial parts of *T. hirsuta*. The findings presented in this work indicated that this essential oil displayed a moderate to strong range of antimicrobial activity, especially against Gram-positive bacteria. The essential oil was also noted to exhibit potentcytotoxic activities. Overall, the *T. hirsuta* essential oil exhibited a number of promising properties and attributes that might provide novel opportunities for the development of antimicrobial and antitumor agents. Accordingly, further toxicological and clinical studies are needed to assess the safety of the oil for medical and therapeutic use.

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