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Antifungal activity of essential oil from the fruits of *Ammodaucus leucotrichus* Coss. & Dur., in liquid and vapour phase against postharvest phytopathogenic fungi in apples

M. Manssouri[1], M. Znini[1], A. El Harrak[2], L. Majidi[1*]

[1]Université My Ismail, Laboratoire des Substances Naturelles & Synthèse et Dynamique Moléculaire, Faculté des Sciences et Techniques, BP 509, 52003, Errachidia, Morocco., [2]Université My Ismail, Laboratoire de Protection & Amélioration et Ecophysiologie Végétale, Faculté des Sciences et Techniques, BP 509, Errachidia, Morocco.

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ABSTRACT

The aim of this study was to evaluate the antifungal activity of essential oil (EO) from *Ammodaucus leucotrichus* fruits against three phytopathogenic fungi causing the deterioration for apples, including *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer* by using the poisoned food (PF) and the volatile activity (VA) methods. The antifungal test results indicated that the EO displayed significant potential of antifungal activity against the tested phytopathogenic fungi ($p<0.05$). In PF technique, the MICs (minimums inhibitory concentrations) were 0.5 µL/mL for *B. cinerea* and *P. expansum* and 1 µL/mL for *R. stolonifer*. Whereas, in VA assay, the complete inhibition of the mycelial growth of *B. cinerea* and *P. expansum* was observed at MIC= 0.125 µL/mL air, and that of *R. stolonifer* was observed at MIC = 0.25 µL/mL air. The overall results suggest that *A. leucotrichus* essential oil have a potential as antifungal preservatives for the control of postharvest diseases of apple.

INTRODUCTION

Apple constitutes the most important human food, serving as the primary source of calories for the majority of the world's population. To provide fruit throughout the year, fresh apples are stored after harvest. However, postharvest losses caused by fungal diseases are the major factor limiting the storage life of apples. Indeed, many pathogens including *Botrytis cinerea* (gray mold), *Penicillium expansum* (blue mold), and *Rhizopus stolonifer* (Rhizopus soft rot) reduce the market values and deteriorate the quality of fruits and render (Znini *et al.*, 2011).Traditionally, chemical treatments, by the application of synthetic fungicides, are considered to be the most effective and cheapest method of controlling postharvest diseases (Eckert and Ogawa, 1988). However, the emergence of strains of pathogens resistant to these fungicides, as well as the growing concern for human safety and the potential impact on environment largely limits their application and compels us to look for alternatives to the use of synthetic fungicides to control postharvest diseases (Suhr and Nielsen, 2003). Recently, interests have been generated in the development of safer anti-fungal agents such as plant-based essential oil sand extracts to control phytopathogens in food and agriculture industries (Gumus *et al*., 2010). Essential oils are made up of many different volatile compounds and their production by plants is believed to be predominantly a defense mechanism against pathogens and pests, and they have been shown to possess antimicrobial and antifungal properties (Znini *et al.*, 2011). They are considered as non-phytotoxic compounds, less environmental effects, and wide public acceptance (Znini *et al.*, 2011; Gumus *et al.*, 2010). *Ammodaucus leucotrichus* is the only one specie of the genus *Ammodaucus* in the *Apiaceae* family (Ozenda, 1991). It inhabits the maritime sands in the Saharan and sub-Saharan countries of North Africa, Morocco, Algeria and Tunisia, extending to Egypt and tropical Africa (Maberly, 1998).

* *Corresponding Author*
Email:lmajidi@yahoo.fr
Tel : (+212)535574497., Fax : (+212)535574485

In Morocco, which locally known as "kammûnes-sofi or ikâman", the fruits are used either by the local population as a powder or in a decoction to treat gastric-intestinal pain, gastralgias and indigestion (Bellakhdar, 1979). It is also frequently used, as an infusion, for diverse infantile diseases of the digestive apparatus: dysentery, nausea, regurgitation, vomiting. Previous studies reported that the essential oils of *A. leucotrichus* exhibited the antimicrobial activities against bacteria, yeasts and filamentous fungi (Abu Zarga *et al.,* 2013; El-Haci *et al.,* 2014). It was also reported that aqueous extracts of A. *leucotrichus* were shown to inhibit the formation of calcium oxalate monohydrate crystals and also found to potently inhibit the nucleation, growth and aggregation phases of calcium oxalate crystallization (Beghali *et al.,* 2008). Recently, we have reported the first studied of the chemical composition of essential oil of A. *leucotrichus* and its application as a green inhibitor for the corrosion of steel in 1 M HCl (Manssouri *et al*., 2015). As far as our literature survey could ascertain, antifungal activities of A. *leucotrichu* against phytopathogens causing severe diseases in apples have not previously been published. Therefore, the aim of this paper was to evaluate the antifungal properties of *A. leucotrichus* essential oil against three phytopathogens, such as *Botrytis cinerea*, *Penicillium expansum*, and *Rhizopus stolonifer* by using the poisoned food (PF) and the volatile activity (VA) methods.

MATERIAL AND METHODS

Plant material

The fruits of *A. leucotrichus* were harvested in March 2012 (full bloom) from Alnif-Errachidia (Morocco). Voucher specimens were deposited in the herbarium of the Faculty of Sciences and Technology of Errachidia (Morocco).

Table 1: Chemical composition of *A. leucotrichus* fruits essential oil from Morocco.

N[a]	Components	RI *l* [b]	RI *a* [c]	RI *p* [d]	% [e]
1	α-Pinene	936	928	1021	1.1
2	β-Pinene	978	966	1109	0.5
3	Myrcene	987	976	1158	0.2
4	3-Carene	1010	1001	1147	0.7
5	**Limonene**	1025	1019	1204	**12.5**
6	Cuminaldehyde		1210	1781	1.6
7	**Perillaldehyde**	1260	1251	1772	**73.5**
8	α-Terpinen-7-al		1267	1772	1.3
9	γ-Terpinen-7-al		1279	1854	1.5
10	Methyl perillate	1381	1372	1985	1.8
				Total identified	**94.7**
				Monoterpene hydrocarbons	15.0
				Oxygenated monoterpenes	79.7

[a]Order of elution are given on apolar column (Rtx-1);
[b]**RI *l*** = retention indices on the apolar column (Rtx-1) in literature;
[c]**RI *a*** = retention indices on the apolar column (Rtx-1) ;
[d]**RI *p*** = retention indices on the polar column (Rtx-Wax);
[e] Relative percentages of components (%) are calculated on GC peak areas on the apolar column (Rtx-1).

Essential oil isolation

The EO was prepared by hydrodistillation for 3h using a Clevenger type apparatus and analyzed by gas chromatography (GC) and gas chromatography/mass spectroscopy (GC/MS). A total of 10 components, accounting for 94.7% of the total oil, were identified (Table 1). It contained peryllaldehyd**e 7** (73.5%) followed by limonene **5** (12.5%) were the major components (Manssouri *et al*., 2015).

Fungal species and in vitro antifungal activity

Three fungal isolates causing apples rot: *B. cinerea*, *P. expansum* and *R. stolonifer* were isolated directly from rooted apples collected from different rooms in Midelt station (Morocco). All isolated fungal species were transferred to sterilized three replicates 9 cm Petri dishes containing fresh Potato Dextrose agar (PDA) medium in the presence of a quantity of streptomycin (50 mg/L) to stop the growth of bacteria. The plates were incubated at 25 ± 2 °C for 7 days and darkness. The developing fungal colonies were purified and identified up to the species level by microscopic examination through the help of the following references (Barnett and Hunter, 1972).

The antifungal activity of the essential oil of *A. leucotrichus* fruit against fungi isolated was undertaken using the poisoned food (PF) method (Rhayour *at al.,* 2003) and the volatile activity (VA) assay (Soylu *et al.,* 2010). In PF, the essential oil were dispersed as an emulsion in sterile agar suspension (0.2%) and added to PDA immediately before it was emptied into the glass Petri dishes (90×20 mm in diameter) at a temperature of 40–45°C. The concentrations tested were: 0.125, 0.25, 0.5, 1 and 2 µL/mLPDA. The control received the same quantity of sterile agar suspension (0.2%) mixed with PDA. The tested fungi were inoculated with 6 mm mycelial plugs from 7-days-old cultures cut with a sterile cork and incubated for11 day for *B. cinerea*, 7 days for *P. expansum* and 30 hours for *R. stolonifer* at 25±2°C.

In VA assay, the Petri dishes (90×20 mm) were filled with 20 mL of potato dextrose agar (PDA) medium and then seeded with a mycelial disc (6 mm diameter), cut from the periphery of 7-days--old mycelium culture of the tested fungi. The Petri dishes (90×20 mm, which offer 80 mL air spaces after addition of 20 mL agar media) were inverted and sterile filter paper discs (9 mm in diameter) impregnated with different concentrations of essential oil (0.125, 0.25, 0.5, 1 and 2 µL/mL air) are deposited on the inverted lid and incubated for11 day for *B. cinerea*, 7 days for *P. expansum* and 30 hours for *R. stolonifer* at 25±2°C. For corresponding control equal amount of water was poured on the sterilized paper filter.

In both types of experiments, three replicate plates were inoculated for each treatment (fungi/amount) and the experiment was conducted three times and the mycelial growth was followed by measuring the diameter following two perpendicular lines passing by the centre of the dish. Fungi-toxicity of essential oil was expressed in terms of percentage of mycelial growth inhibition (I %) and calculated following the formula (Pandey *et al*., 1982).

$$I(\%) = \frac{D_t - D_i}{D_t} x100$$

with D_t=Average diameter of fungal colony in control. D_i=Average diameter of fungal colony in treatment. The lowest

concentration that completely inhibited the growth of the fungus was considered the minimum inhibitory concentration (MIC).

Transfer experiments

The fungistatic–fungicidal nature of essential oil was tested by observing revival of growth of the inhibited mycelial disc following its transfer to non-treated PDA. A fungicidal effect was where there was no growth, whereas a fungistatic effect was where temporary inhibition of microbial growth occurred.

Data analysis

The inhibitory effect of essential oil on mycelia growth was analyzed by an analysis of variance (ANOVA). Mean and standard error of data were calculated using SPSS program version 15.0 for Windows. The separation of means was done by using the least significant difference (LSD) test at $p< 0.05$.

RESULTS AND DISCUSSION

Results

The antifungal activity obtained by PF and VA techniques with different concentrations of essential oil of *A. leucotrichus* was reported in Tables 2 and 3, respectively. In both techniques, the results indicate that the inhibition of the mycelial growth of each train was significantly influenced by the essential oil concentration ($p<0.05$). Also, the percentage inhibition of mycelia growth increased with increasing amounts of essential oil for all the strains tested suggesting that this oil inhibited the growth of all the strains in a dose-dependent manner.

Table 2: Antifungal activity of *A. leucotrichus* essential oil against *B .cinerea*, *P. expansum* and *R. stolonifer* at various concentrations using PF technique.

Strain	*B. cinerea*	*P. expansum*	*R. stolonifer*
Incubation time	11 days 25 ± 2 ° C	7 days 25 ± 2 ° C	60 hours 25 ± 2 ° C
Concentration (µL/mL)			
2	100±0.00 $^{a.1}$	100±0.00 $^{a.1}$	100±0.00 $^{a.1}$
1	100±0.00 $^{a.1}$	100±0.00 $^{a.1}$	100±0.00 $^{a.1}$
0.5	100±0.00 $^{a.1}$	100±0.00 $^{a.1}$	75.00±0.00 $^{b.2}$
0.25	82.22 ±0.57 $^{b.1}$	73.33±1.85 $^{b.2}$	69.44±2.83 $^{c.3}$
0.125	65.23±4.83 $^{c.1}$	55.22±1.83 $^{c.2}$	33.23±2.32 $^{d.3}$

Mean values (± standard deviation) followed by different (column) and numbers (line) in each row indicate significant differences ($p<0.05$) by least significant difference test (LSD).

Using the PF technique, the results (Table 2 and Fig. 1) indicate that the *B. cenerea,* and *P. expansum* showed a strong sensitivity to *A. leucotrichus* essential oil at all concentrations. The inhibition rate reached 65.23% for *B. cinerea*, and 55.22% for *P. expansum* at 0.125 µL/mL, and complete inhibition effect (100%) was observed at 0.5 µL/mL, indicating that this latter concentration was the MIC of *A. leucotrichus* oil against two strains tested. Whereas it displayed moderate-high antifungal activity against *R. stolonifer*, the percentage inhibition increases moderately with the concentration reaching the complete inhibition (100%) to 1 µL/mL, indicating that MIC was 1 µL/mL against *R. stolonifer*. Moreover, it is important to know the fungitoxic nature of the essential oil against *B. cinerea* and *P. expansum* at 0.5 to 2 µL/mL and at 1 to 2 µL/mL for *R. stolonifer*. Indeed, the transfer of mycelial discs where growth inhibition was complete by essential oil into PDA medium without this oil, showed that mycelial growth of R. *stolonifer* grew after incubation for first hour, indicating a fungistatic effect of this oil against this strain (MFC >2 µL/mL).Whereas no mycelial growth was observed in *B. cinerea* and *P. expansum* after treatment with oil at 0.5 to 2 µL/mL, indicating a fungicidal effect of this oil against these strains (MFC = 0.5 µL/mL). Therefore, the plant phytopathogens studied can be classified according to their sensitivity to the oil in the following order: *B. cinerea*> *P. expansum*> *R. stolonifer*.

Using VA assay, the results (Tables 3 and Fig. 2) showed that the activity of the vapour of the *A. leucotrichus* essential oil was more pronounced for all strains tested. This volatile fraction completely suppressed the growth (100%) of *B. cinerea* and *P. expansum* at 0.125 µL/mL air, indicating that the MIC for both strains was 0.125 µL/mL air, whereas, the mycelial growth of *R. stolonifer* was totally inhibited at 0.25 µL/mL air (MIC value).

Table 3: Antifungal activity of *A. leucotrichus* essential oil vapour against *B. cinerea*, *P. expansum* and *R. stolonifer* at various concentrations using VA assay.

Strain	*B. cinerea*	*P. expansum*	*R. stolonifer*
Incubation time	11 days 25 ± 2 ° C	7 days 25 ± 2 ° C	60 hours 25 ± 2 ° C
Concentration (µL/mL air)			
2	100±0.00 $^{a.1}$	100±0.00 $^{a.1}$	100±0.00 $^{a.1}$
1	100±0.00 $^{a.1}$	100±0.00 $^{a.1}$	100±0.00 $^{a.1}$
0.5	100±0.00 $^{a.1}$	100±0.00 $^{a.1}$	100±0.00 $^{a.1}$
0.25	100±0.00 $^{a.1}$	100±0.00 $^{a.1}$	100±0.00 $^{a.1}$
0.125	100±0.00 $^{a.1}$	100±0.00 $^{a.1}$	82.36±0.33 $^{b.2}$

Mean values (± standard deviation) followed by different (column) and numbers (line) in each row indicate significant differences ($p<0.05$) by least significant difference test (LSD).

Moreover, after growth inhibition had been established with *A. leucotrichus* essential oil vapour, it is important to know the fungitoxic nature of the fraction volatile of this oil. Indeed, the mycelial discs, which 100% inhibition, were transferred onto PDA medium without this oil and the results showed that no mycelial growth was observed for *B. cinerea* after treatment with oil at all concentration. Also, the fungal growth of *P. expansum* was observed only after treatment with 0.125 µL/mL air. However, fungal growth of *R. stolonifer* was observed after treatment only with 0.25 µL/mL air. Thus, *A. leucotrichus* essential oil vapour showed fungicidal activity against *B. cinerea* at MFC = 0.125 µL/mL air, while against *P. expansum*, the oil exhibited fungicidal activity at MFC = 0.25 µL/mL air and fungistatic activity at MIC = 0.125 µL/mL air. For *R. stolonifer*, the fraction volatile of oil exhibited fungicidal activity at MFC = 0.5 µL/mL air and fungistatic activity at MIC = 0.25 µL/mL air. Therefore, the plant phytopathogens studied can be classified according to their sensitivity to the *A. leucotrichus* essential oil in the following order: *B. cinerea* > *P. expansum* > *R. stolonifer*.

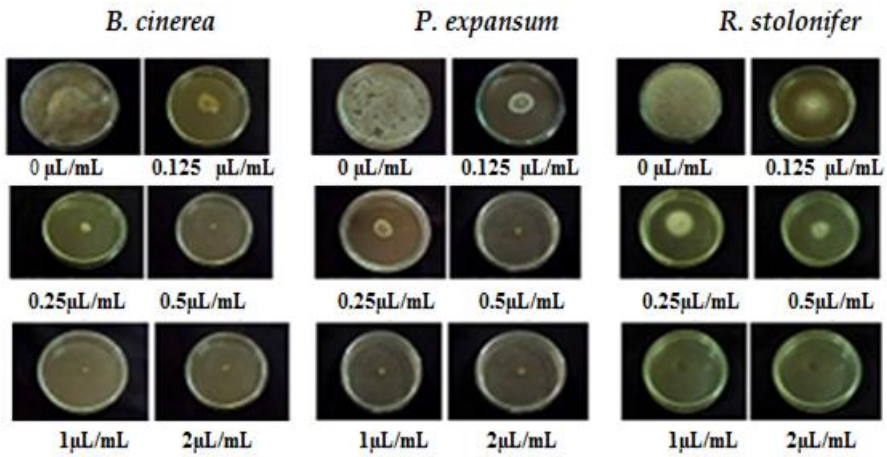

Fig. 1: Effect of various concentrations of the essential oil of *A. leucotrichus* on mycelial growth of three isolated fungal strains.

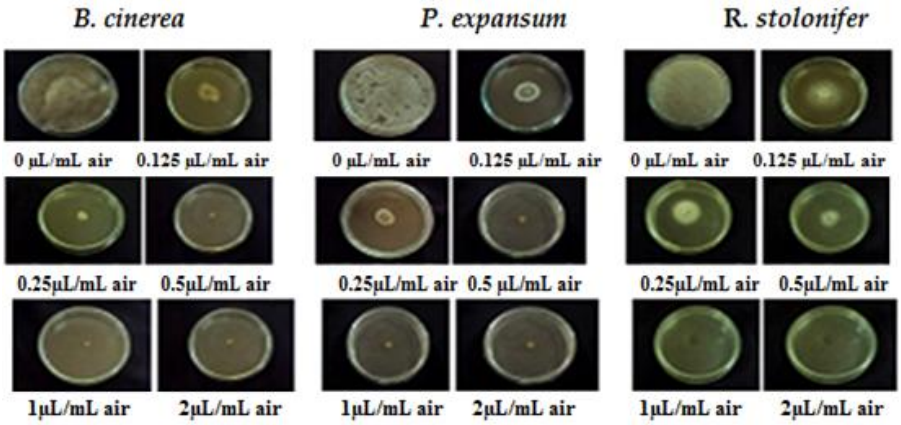

Fig. 2: Effect the essential oil vapour of *A. leucotrichus* at various concentrations on mycelial growth of three isolated fungal.

DISCUSSION

In the present study, *A. leucotrichus* essential oil showed antifungal activity against the mycelial growth of all the phytopathogenic fungi studied. The MICs were 0.5 µL/mL and 0.125 µL/mL air for *B. cinerea* and *P. expansum* using PF and VA techniques, respectively. Whereas, for *R. stolonifer*, the MICs were 1 µL/mL and 0.25 µL/mL air using PF and VA techniques, respectively. These results were in accord with those previously reported by et El-Haci et al. which the essential oil of *A. leucotrichus* possessed the most potent activity against filamentous fungi, with MIC values 0.25-0.50 µL/mL for the mycelium growth of *Fusarium oxysporum* and *Aspergillus flavus* (El-Haci *et al.*, 2014).

The activity of *A. leucotrichus* essential oil is probably related to its high levels of oxygenated monoterpenes (79,7%), especially its major constituent perillaldehyde (73.5%). Indeed, Zambonelli *et al.*, (1996) previously concluded that the high activity of oxygenated monoterpenes directed against pathogens results from their interference with enzymatic reactions during cell-wall synthesis, causing changes in cell permeability by disrupting lipid packing and changes to membrane properties and functions. Also, it is reported that perillaldehyde-rich oils are used as food additives for flavor and in perfumery to add spiciness (El-Haci *et al.*, 2014) and perillaldehyde has a sweet mint like odor and originates from essential oils which have been labeled "generally recognized as safe" by the United States Food and Drug Administration (Tolouee *et al.*, 2010). Furthermore, it is, also, reported that perillaldehyde exhibits antifungal activities against and air borne microbes and spoiling-fungi infection of fruits. Indeed, it may be used as an alternative substance to control spoiling-fungi infection *in vitro* and *in vivo* of cherry tomatoes (*Solanum lycopersicum* L.). The mycelial growth of the tested fungi was totally inhibited at 0.5 and 0.08 mL/L perillaldehyde in the air at contact and vapor conditions, respectively. Thus, perillaldehyde is suitable for development into a natural food preservative (Tian *et al.*, 2015a). Perillaldehyde was also tested for its influence on microbial count in air by vaporizing with an air washer. The highest antibacterial activity was observed when perillaldehyde was sprayed which was reduced the germ count by 53% (Sato *et al.*, 2006). Perillaldehyde showed also, notable antifungal activity against *A. niger*, a known cause of grape spoilage, with a minimum inhibitory concentration (MIC) and a minimum fungicidal concentration (MFC) of 0.25 and 1µl/ml, respectively. The accumulation of mycelial biomass was also inhibited by perillaldehyde in a dose-dependent manner,

completely inhibiting mycelial growth at 1µl/ml (Tian *et al.*, 2015b). These authors reported that the lipophilic nature of perillaldehyde interacts with the cell membrane of *A. niger* as a first step in its antifungal activity. The electrical conductivity in the cell suspensions increased above that of the control at all times when perillaldehyde was added, indicating that the permeability of the cell membrane was compromised (Tian *et al.*, 2015b).When perillaldehyde accumulates at the cytoplasmic membrane, changes in the membrane integrity occur, such as expansion (swelling) of the membrane, which may result in a destabilization of the membrane and subsequent ion leakage (Ultee *et al.*, 2002). Another assumption is that perillaldehyde probably acted as a signal that triggered the transcription processes of some specific "fungicidal" genes in the fruits (Panahirad *et al.*, 2012). Further experiments indicated that perillaldehyde activated a membrane-active mechanism that inhibits ergosterol synthesis, increases membrane permeability (as evidenced by extracellular pH and conductivity measurements), and disrupts membrane integrity, leading to cell death (Tian *et al.*, 2015a). In addition, the components in lower amount may also contribute to antifungal activity of essential involving probably some type of synergism with the other active compounds.

In this paper, we report a comparison of the two methods for testing antifungal activity of *A. leucotrichus* essential oil in order to determine which method produces the most reliable results. The PF and VA methods appeared to give an indication of the susceptibility of specific fungi. According to the results obtained, VA method in disc volatilization has better antifungal activity against the pathogens tested than that in liquid phase observed in the PF assay. Similarity behavior was observed with perillaldehyde which its vapor-phase efficacies on mycelial growth were greater than the contact-phase efficacies (Tian *et al.*, 2015a).This result indicates that the substances in direct contact method were less efficient than that in the disc volatilization method. The efficacy of essential oils in vapour state was probably attributable to the direct deposition of essential oils on lipophilic fungal mycelia together with an indirect effect via adsorption through the agar medium (Zani *et al.*, 1991). These results are in agreement with those reported in the literature (Zani *et al.*, 1991 ; Sikkema, 1993).

The essential oils are complex mixtures of many different aromatic components with various degrees of lipophilicity and relative hydrophilicity given by the presence of constituents with polar functional groups (Sikkema *et al.*, 1992). When added to a medium, the essential oils distributes more or less into the aqueous phase depending on its relative hydrophilicity.

Hence, an essential oil with constituents with low water solubility should dissolve little in aqueous medium, and consequently should show a weak activity. These essential oils, however, showed very good activity when assayed by VA and this might related to their high volatility (Zani *et al.*, 1991). These observations suggest that the physical and chemical properties (solubility and volatility) can have considerable effect on the in vitro antimicrobial activity (Tullio *et al.*, 2007).

CONCLUSION

Essential oil is one of the most promising groups of natural compounds for the development of safer antifungal agents. In continuation with our interest in antifungal substances from plant sources, we performed preliminary studies with *A. leucotrichus* which revealed significant antifungal activity against three phytopathogenic fungi causing severe diseases in apple such as *B. cinerea*, *P. expansum* and *R. stolonifer*. The results demonstrate that this essential oil is effective for inhibition or control of phytopathogens and so could be used as potential alternative to synthetic fungicides for the protection of apples from phytopathogenic fungi. The use of *A. leucotrichus* oil in vapour phase could have additional advantages such as efficacy without requiring direct contact resulting in ease of application and no alteration in organoleptic properties of the edible material/food. With respect to above-mentioned data, our findings demonstrate that *A. leucotrichus* oil vapour may be considered as a potential agent for preventing microbial mediated food spoilage. A further study in vivo condition is warranted to confirm the antifungal activity of *A. leucotrichus*, which may be used for preservation and/or extension the shelf life of raw and processed food.

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