

Utilizing an Extract from Endophytic Fungus, *Alternaria Alternata*, Isolated from *Ricinus Communis* L. Leaves as a Biological Agent for Controlling the Migratory Locust in Algeria

Laib Djamel Eddine

Department of Agronomy, Faculty of Sciences, 20 August 1955 University, Skikda, 21000, Algeria, djamel.skikda@gmail.com

Benzehra Abdelmadjid

Department of Agriculture and Forestry Zoology National Agronomic Institute, El-Harrach, Algiers, 16000, Algeria, benzara.a@gmail.com

Laib Imen

Department of Natural and Life Sciences, Faculty of Sciences, 20 August 1955 University, Skikda, Algeria, mina.laib@gmail.com

Aouzal Badis

BIOQUAL Laboratory, Institute of Nutrition, Food, and Agri-food Technologies (I.N.A.T.A.A.), Frères Mentouri University, 1, Ain El-Bey Road, 25000 Constantine, Algeria, badisaouzal@gmail.com

Akkal Salah

Valorization of Natural Resources, Bioactive Molecules and Biological Analysis Unit, Department of Chemistry, University of Mentouri Constantine 1, Constantine, 25000, Algeria, salah62dz@gmail.com

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UTILIZING AN EXTRACT FROM ENDOPHYTIC FUNGUS, *ALTERNARIA ALTERNATA*, ISOLATED FROM *RICINUS COMMUNIS* L. LEAVES AS A BIOLOGICAL AGENT FOR CONTROLLING THE MIGRATORY LOCUST IN ALGERIA

LAIB DJAMEL EDDINE^{1,2*}, BENZEHRA ABDELMADJID², LAIB IMEN^{3,4}, AOUZAL BADIS^{3,5} AND, AKKAL SALAH⁶

¹Department of Agronomy, Faculty of Sciences, 20 August 1955 University, Skikda, 21000, Algeria

²Department of Agriculture and Forestry Zoology National Agronomic Institute, El-Harrach, Algiers, 16000, Algeria

³Department of Natural and Life Sciences, Faculty of Sciences, 20 August 1955 University, Skikda, Algeria

⁴BIOQUAL Laboratory, Institute of Nutrition, Food, and Agri-food Technologies (I.N.A.T.A.A.), Frères Mentouri University, 1, Ain El-Bey Road, 25000 Constantine, Algeria

⁵Laboratory for Research on Biodiversity Interactions and Biotechnology, Skikda, 21000, Algeria

⁶Valorization of Natural Resources, Bioactive Molecules and Biological Analysis Unit, Department of Chemistry, University of Mentouri Constantine 1, Constantine, 25000, Algeria

Corresponding author email: djamel.skikda@gmail.com.

ABSTRACT

In this research, we aimed to explore the insecticidal potential of *Alternaria alternata*, an endophytic fungus derived from the leaves of castor oil plant. To test its insecticidal efficacy, various doses of *A. alternata* mycoextract were administered to *Locusta migratoria* L. through both topical and oral pathways. The findings revealed that the insects displayed the highest susceptibility to a concentration of 2 g/L, leading to an impressive mortality rate of 87.5 % and 93.75 % for topical and oral administrations, in the same order. The extract displayed remarkable insecticidal properties, demonstrating LC50 values of 1.09 g/L for the topical treatment and 1.129 g/L for the oral treatment. Moreover, the average survival times (LT50) for the insects were found to be 5.392 days and 6.867 days when subjected to the topical and oral treatments, respectively. The initial chemical investigation of the mycoextract unveiled the existence of different key metabolites responsible for the observed activities. Additionally, FTIR-ATR analysis indicated the presence of different groups in the mycoextract. Moreover, the GC-MS analysis identified multiple volatile insecticidal compounds in the fungal extract.

Keywords: Insecticidal activity, endophytic fungi, *alternaria alternata*, *locusta migratoria*.

INTRODUCTION

Crop losses worldwide are a result of numerous insect pests (Rahman et al., 2018) Among these pests, *L. migratoria* (Linnaeus, 1758) (Orthoptera: Acrididae), commonly known as the migratory locust, poses a significant threat due to its polyphagous nature, ecological plasticity, and wide distribution (Le Gall et al., 2019). Current control methods for this pest heavily rely on chemical pesticides, which necessitate the application of large

quantities. Nonetheless, the widespread utilization of these chemicals has resulted in a myriad of issues, including the emergence of resistance, contamination of the environment, and detrimental impacts on human well-being (Shuping et al., 2017; Berestetskiy and Hu, 2021; Zhang and lecoq, 2021).

Consequently, the search for effective alternatives to safeguard crops against *L. migratoria* has become paramount (Zhang and Lecoq, 2021). The remarkable potential of endophytic fungi

lies in their ability to reside within host plants without causing visible infection symptoms, offering a promising approach for safeguarding plants against insect pests. Within these fungi, a myriad of bioactive secondary metabolites is produced, exhibiting a wide range of activities (Kaur, 2020). As such, the focal point of this investigation revolves around evaluating the insecticidal efficacy of an extract derived from *A. alternata*, isolated from castor oil plant leaves, against *L. migratoria*.

MATERIAL AND METHODS

Material

In February 2022, we collected *R. communis* leaves from a natural vegetation community in Skikda, Algeria, from twenty plants. Subsequently, in July 2022, we also gathered *L. migratoria* insects from the same location, which are recognized as potential pests of various crops in our country (Algeria). The identification of the two mentioned species was carried out morphologically for enhanced scientific accuracy.

Methods

i. Insect Breeding

To foster the insect specimens, we utilized a breeding cage with dimensions of 50 cm³, enclosed with mesh and equipped with a sliding door for easy access. The cage was thoughtfully furnished with nesting boxes containing a blend of sterilized peat (approximately 66 %) and sand (approximately 33 %), while ensuring a constant moisture content of 10 %. To ensure a conducive environment, a 60-watt incandescent bulb provided constant lighting and maintained a temperature of 30 ± 1°C for 12 hours each day. The insects were nourished with fresh wheat leaves, which can be sprouted in trays to maintain a continuous rotation,

following the protocol outlined by Ouedraogo et al., (2002).

ii. Isolation and Identification of Endophytic *A. alternata*

After harvesting *R. communis* leaves, a modified version of Li et al., (2015) protocol was employed, transforming the entire process. The leaves underwent meticulous rinsing under flowing water to ensure the thorough elimination of any dirt particles. Subsequently, they were delicately cut into 5 mm² segments. To ensure cleanliness, these segments were briefly immersed in 75 % ethanol for one minute and then subjected to multiple distilled water washes, each lasting two minutes, to eliminate any residual ethanol.

Once dried, the segments were placed in Petri dishes that contained a modified Potato dextrose Agar medium supplemented with streptomycin (200 mg/L). This addition of streptomycin aimed to inhibit bacterial growth. The segments were then incubated at 25 °C for a period of twenty-one days to encourage the proliferation of fungal strains. Developing fungi from the segment tips were directly isolated and transferred to fresh potato dextrose Agar medium without antibiotics. Following this, the fungal isolates were cultivated at 25 °C for 3 to 6 days. Finally, microscopic examinations were carried out to identify the cultivated fungi.

iii. Production of *A. alternata* Extract

We executed the extraction process following the well-established method described by Dolatabad et al., (2017). Initially, agar discs containing *A. alternata* mycelium were inoculated into 50 ml of Potato dextrose broth Nutrient medium, and the containers were tightly closed to maintain a contamination-free environment. To ensure thorough mixing, the cultures underwent a 21 day incubation period at a temperature of 25 ± 2 °C, with regular agitation on a mechanical shaker at

a speed of 150 rpm every hour. After the 21-day incubation, the mycelia of *A. alternata* were detached from the medium using a filtration process. The produced filtrate was combined with identical volume of ethyl acetate, and then phase separation into organic and aqueous layers was allowed to occur over a period of 10 minutes.

Following this, the ethyl acetate layer underwent evaporation employing a rotavapor. The resulting mycoextract was subsequently dried in a lyophilizer until it transformed into a dry powder state.

iv. *Detection of Major Groups of Secondary Metabolites*

The identification of major secondary metabolite groups followed the methodologies outlined in Harborne (1998). Initially, the mycoextract was incorporated separately in watered-down hydrochloric acid, filtered, and then exposed to K_2HgI_4 . The Manifestation of alkaloids was evidenced by the development of a distinctive yellow deposit. For the uncovering of flavonoids a small amount of NaOH solution were added to the extract, resulting in a vivid yellow coloration. However, this coloration became transparent after the inclusion of watered-down HCl.

To identify phenolic compounds, the mycoextract was incorporated in 5 ml of distilled water, and a small amount of a 5 % $FeCl_3$ was added, leading to the development of a deep green coloration. Saponins were detected by vigorously shaking the fungal extract with distilled water and allowing it to rest for ten minutes, resulting in the establishment of a steady emulsion. Upon subjecting the fungal extract to an alcoholic $FeCl_3$ reagent, the recognition of tannins was revealed by a striking deep azure-black. This hue vanished when slightly diluted H_2SO_4 was introduced, giving way to the formation of a precipitate displaying a distinct yellowish-brown hue.

v. *FTIR-ATR Analysis of the Fungal Extract Samples*

The FTIR-ATR analysis followed the methodology outlined by Sala et al., (2020). For each sample, ATR-FTIR spectroscopy was conducted using approximately 1 mg of the mycoextract. The measurements were executed with a Cary-630 series FTIR spectrometer equipped with an ATR and a diamond (Di) IRE plate. To ensure accurate data recovery, 8 scans were performed within the spectral range of 4000 to 650 cm^{-1} , employing a resolution of 4 cm^{-1} . To minimize background interference, the spectra of individual samples underwent an automated subtraction process using resolution Pro version 5.0 program.

vi. *Analysis of Volatile Components of A. alternata Extract and Colony*

The analysis of volatile components followed established techniques, with procedures outlined by Senthilkumar (2014) for the fungal extract and Fiers (2013) for the fungal colony being adhered to. To detect volatiles emitted by the fungal colony, a fungal spore solution (2×10^7 spores/mL) was meticulously mixed with 50 μL of PDA medium in 20 mL solid-phase micro-extraction vials. These vials were securely shut to prevent contamination, placed in an inclined position for optimal exposure, and then incubated in an oven at 23 °C. Prior to the actual collection process, each micro-extraction vial underwent a 10-minute concentration step in a 25°C water bath, allowing volatile compounds to accumulate in the vial's headspace.

Following this step, SPME fibers were inserted into the solid-phase micro-extraction vials for twenty minutes to effectively obtain the volatile compounds made by the fungal hyphae. Similarly, for detecting volatiles from the *A. alternata* extract, a well-prepared solution of 1 μL fungal extract per 1 mL of methanol was

utilized. The GC-MS instrument was programmed to start at 100 °C and sustained at that temperature throughout 1 minute prior to gradually raising to 250 °C. The injection port's temperature remained steady at 250 °C, with a helium flow rate set at 1.5 mL/min, and an ionization voltage of 70 eV was utilized. Separate SPME fibers and fungal extract samples were carefully introduced into the injection port of the GC-MS machine. The Mass spectrometry scan range was adjusted to 45-450 (m/z). Subsequently, the acquired GC-MS mass spectrum data underwent comprehensive analysis using the NIST database to accurately identify the present volatile components.

vii. *Insecticidal Activity of A. alternata Extract*

In this phase, adult *L. migratoria* individuals were exposed to two types of treatments: direct spray and forced ingestion (topical and oral applications). Each treatment involved the use of 5 insects per plastic box.

To study the effects of the fungal extracts, a total of ten different doses (ranging from 0.2 to 2 g/L) were employed, and each concentration was replicated five times. As controls, five replicates were treated with distilled water. Following a 7-day observation period, the mortality rate for each treatment was determined as a percentage. To ensure accuracy, Abbott's formula (Abbott, 1925) was applied to correct the mortality rate data:

$$\text{Corrected Mortality (\%)} = \frac{\text{Test mortality (\%)} - \text{Control mortality (\%)}}{100 - \text{Control mortality (\%)}}$$

viii. *Statistical Data Analysis*

To facilitate a thorough comparison of the means of corrected mortality, we employed an ANOVA and supplemented it with Tukey's (HSD) test, adhering to a 95

% interval range. These statistical tests were chosen to effectively categorize the results into homogeneous groups, enabling a comprehensive and meaningful comparison of the collected data. To ascertain the LC50 values, we employed probit analysis on the corrected mortality data. The estimation of LC50 values was carried out employing a binomial cloglog model. This approach ensures a robust estimation of the lethal concentration required for 50 % mortality, offering a precise and reliable measurement.

To calculate the median survival time (LT50), we chose to utilize Kaplan-Meier estimators. This particular method is well-regarded for its ability to assess survival rates accurately and estimate the median time of survival for the test subjects. The median survival time is the time at which the curve crosses 0.50. Its non-parametric nature allows for a robust analysis without requiring strict assumptions about the underlying data distribution. All statistical analyses were conducted employing the XLSTAT 2019 program, which provided a robust platform for precise data analysis and interpretation.

RESULTS

i. *Efficacy of Mycoextract as an Insecticide*

The Efficacy of mycoextract as an insecticide displayed considerable variability based on the dose and time elapsed after treatment. Among the different concentrations tested, the most potent concentration against the insects was found to be 2 g/L. Remarkably, this concentration resulted in a maximum mortality rate of 87.5 % and 93.75 % after seven days for migratory locust *L. migratoria* in topical and oral treatments, respectively (as depicted in Figures 1 and 2).

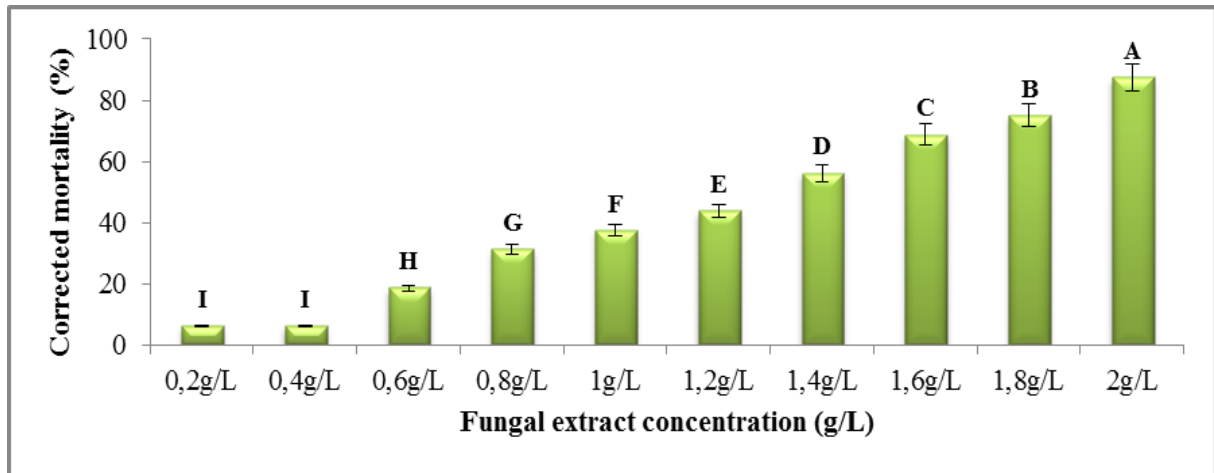


Figure 1: The corrected mortality resulting from the topical treatment of the migratory locust *L. migratoria* with *A. alternata* mycoextract.

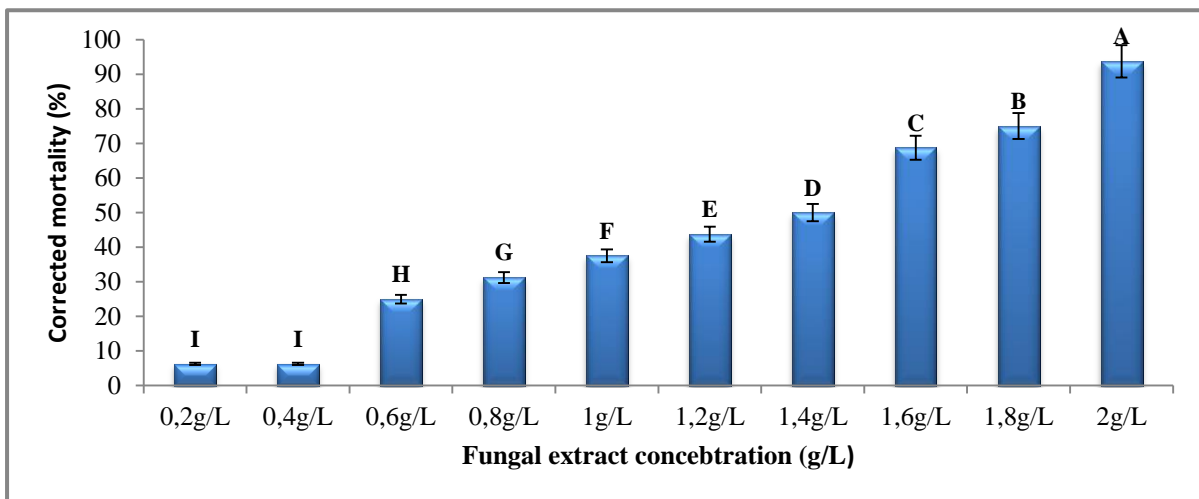


Figure 2: The corrected mortality resulting from the oral treatment of the migratory locust *L. migratoria* with *A. alternata* mycoextract.

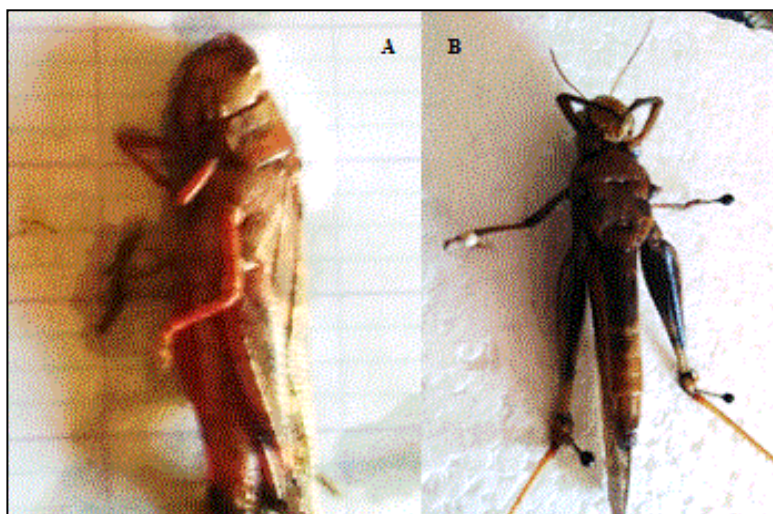


Figure 3: Impact of the endophytic fungus *A. alternata* extract on *L. migratoria*. A: topical treatment against *L. migratoria*. B: oral treatment against *L. migratoria*.

Table 1: The LC50 and LT50 values of the fungal extract

Treatment type	LC 50 of the fungal extract (g/L)	LT 50 of the fungal extract (Days)
Topical treatment	1,09	5,392
Oral treatment	1,129	6,867

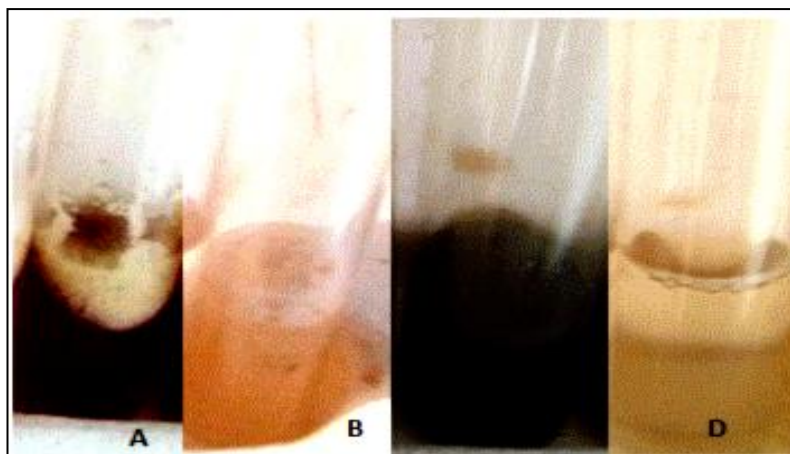


Figure 4: Preliminary chemical analysis of the fungal extract. A: Phenols test, B: Alkaloids test, C: Terpenoids test, D: Saponins test.

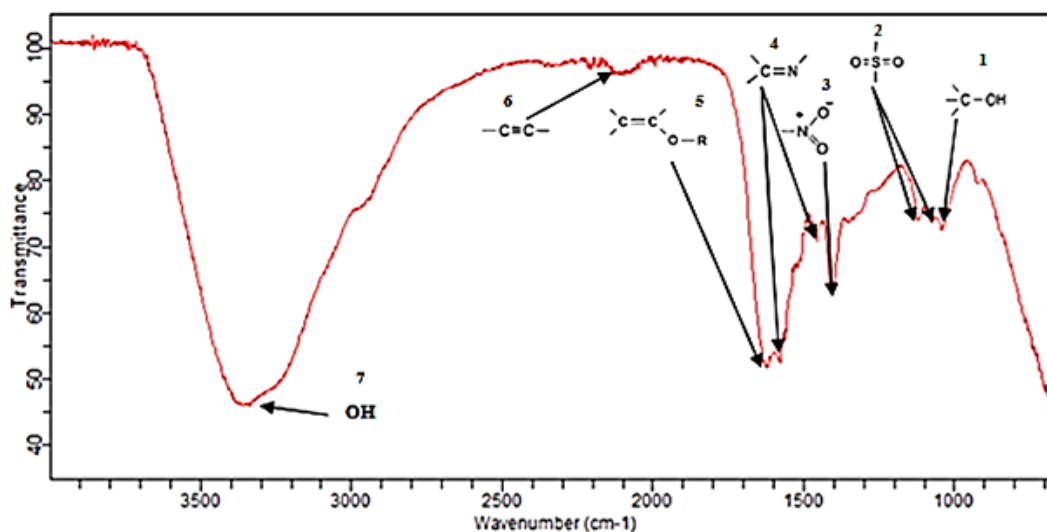


Figure 5: The ATR-FTIR spectroscopy analysis of the *A. alternata* extract. 1: alcohol (1000–1260 cm^{-1}), 2: sulfone (1100–1150 cm^{-1}), 3: nitro (1250–1400 cm^{-1}), 4: imine (1480–1690 cm^{-1}), 5: enol ether (1600–1660 cm^{-1}), 6: alkyne (2100–2300 cm^{-1}), and 7: hydroxyl (3300–3500 cm^{-1}) groups.

In the figures, capital letters were employed to signify significant differences in insecticidal activity between various extract concentrations on different days, with a significance level of 0.05. These letters served as a visual representation of the statistical significance of the data, thereby facilitating the comparison and interpretation of the results.

Furthermore, the treated locusts exhibited evident changes, such as reduced motor activity, heightened defecation, abnormal water loss evident as diarrhea, a noticeable shift in pigmentation of their bodies to a red color, and the manifestation of a dark hue in the abdomen (Figure 3).

Table 2: Volatile secondary metabolites of both fungal extract and colony

Volatil compound	Formula	Molecular weight (g/Mol)	Acquired Chromatogram	Activity	References
Phénol, 3,5-bis (1,1-diméthyléthyl) -	$C_{14}H_{22}O$	206			
Phénol, 2,6-bis (1,1-diméthyléthyl) -4-méthyl-	$C_{15}H_{24}O$	220		Insecticidal	(Zhao et al., 2020)
2,6-Di-tert-butyl-4-méthyl-phénol	$C_{15}H_{24}O$	220			
1,3-pentanediol, 2,2,4-triméthyl-	$C_8H_{18}O_2$	146			(Gerber, 2015)
2,3,4,4a, 5,6,7,8-octahydro-1H-naphtalène-1,8a-diol	$C_{10}H_{18}O_2$	170			(Herbst, 1966).

A. alternata extract displayed notable toxicity against the insects, resulting in LC 50 values of 1.09 g/L for topical treatment and 1.129 g/L for oral treatment. Additionally, the mean survival times (LT 50) were recorded as 5.392 days for *L. migratoria* under topical treatment and 6.867 days for *L. migratoria* under oral treatment (Table 1). Upon conducting the initial chemical analysis of the mycoextract, the presence of alkaloids, phenols, and terpenoids was confirmed (Figure 4). The ATR-FTIR spectroscopy analysis of the *A. alternata* extract detected a range of functional groups (Figure 5).

The GC-MS analysis of both the mycoextract and colony identified the identification of insecticidal volatile compounds, including phenol, 3,5-bis(1,1-dimethylethyl), phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl, 2,6-di-tert-butyl-4-methylphenol, 1,3-pentanediol, 2,2,4-trimethyl, and 2,3,4,4a,5,6,7,8-octahydro-1H-naphthalene-1,8a-diol (Table 2).

DISCUSSION

Endophytic fungi play a crucial role in enhancing the resistance of host plants against insect pests by producing diverse bioactive compounds (El-Sayed et al., 2020). Various insects have been observed to be affected by the toxic and deterrent properties of non-volatile secondary metabolites, such as alkaloids, phenols, terpenoids, and tannins (Kortbeek et al., 2019; Taye and Borkataki, 2020). On the other hand, saponins exhibit multiple insecticidal properties, acting as deterrents, molt disruptors, and regulators of insect growth (Hussain et al., 2019). The presence of phenols, tannins, alkaloids, and saponins in the fungal extract was confirmed through FTIR-ATR analysis, which detected hydroxyl, imine, and alcohol groups (Sharaf et al., 2013; Veiga et al., 2018; Lv et al., 2021).

In terms of volatile secondary metabolites with insecticidal properties,

sulfones, alkyne derivatives, nitro groups, and enol ethers are crucial precursors in the production of numerous insect-killing compounds (Yu et al., 2016; Soderlund, 2012; Nishiwaki, 2020; Liu et al., 2015). Furthermore, volatile compounds with insecticidal potential, including naphthalene and analogs of 1, 3-pentanediol, 2,2,4-trimethyl, and 2,4-di-tert-butylphenol, have been previously reported (Zhao et al., 2020; Gerber, 2015; Herbst, 1966). Upon applying the fungal extract, notable signs of intoxication were observed in *L. migratoria*, including reduced motor activity, intense defecation, and diarrhea-like water loss, which could be attributed to the effects of *A. Alternata* extract (Kemassi et al., 2018).

The brick color observed in the cuticle of the treated insects likely arises from an overproduction of β -carotene. Meanwhile, the occurrence of dark pigmentation in the digestive tract can be attributed to the specific presence of melanin (Yang et al., 2019; Chauvin, 1956). The dark black color in the abdomen following treatment may result from excessive melanization triggered by intestinal toxicity, leading to impaired nervous function due to the harmful effects on the central nervous system. Additionally, it could be a result of distension, cellular necrosis, and a physiological reaction in an attempt to heal intestine wounds. The inability of the locusts to absorb and assimilate nutrients eventually leads to protein and lipid deficiency, which ultimately results in the insects' death (Shamim et al., 2014).

Previous studies investigating the insecticidal properties of secondary metabolites generated by endophytic fungi have yielded promising results. For instance, when a combination containing mycoextract and spore suspensions of the endophytic fungus *Isaria fumosorosea*, isolated from castor oil plant leaves, was applied, it resulted in an impressive 80 % maximum mortality rate among adult *A. obtectus* after 72 hours (Laib et al., 2020a).

Similarly, an extract from *Trichoderma* sp., also isolated from *R. communis* leaves, exhibited a mortality rate of 56.52 % among adult *L. migratoria* after 72 hours when applied at a concentration of 0.4 g/L (Laib et al., 2020b). Moreover, the extract of *A. niger* isolated from the same plant exhibited its highest efficacy against insects at a concentration of 2 g/L, leading to a remarkable death rate of 86.95 % and complete eradication through both contact and ingestion treatments (Laib et al., 2022).

These findings highlight the potential of endophytic fungi as valuable sources of diverse metabolites with insecticidal activity against *L. migratoria*. Consistent with these studies, the current investigation further demonstrates that the endophytic fungus *A. alternata* also possesses insecticidal properties, adding to the growing evidence supporting the potential of endophytic fungi as effective alternatives for pest control.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interests related to this article

AUTHOR'S CONTRIBUTIONS

Laib Djamel Eddine: investigation, methodology, Writing original draft, Benzehra Abdelmadjid: project administration, supervision, validation, Laib imen: Review and editing, Aouzal Badis: Data curation, formal analyses, Akkal Salah: Funding acquisition, resources, software.

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