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Morphological and Molecular Characterisation of Endophytic Fungi Isolated from *Moringa oleifera* Leaves in Iraq and Chemical Analysis of Leaves Extracts Using GC-Mass

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Abstract: Endophytic fungi are found inside plants, with no disease appearance; in contrast, they improve the growth of plants spatially in limited habitats. This study aimed to isolate and characterize, morphologically and genetically endophytic fungi gathered from *Moringa oleifera* leaves, as well as detect the chemical components in the leaves using GC-Mass spectrometry. the results obtained that 59 endophytic fungi isolates were detected, with a total colonisation rate of 84.26%. These fungi belong to 8 types, where *Aspergillus* was a predominated genus with 5 species. Besides, *A. flavus* was prevalent with a colonisation rate of 27.14, and *A. niger* with a rate of 15.71; *Byssochlays spectabilis* with a rate of 28%. Moreover, the result of chemical components analysis in leaves using GC-mass showed the presence of active 30 compounds. including oleic acid, phytol, octadecenoic acid, hexadecanoic acid, methyl ester and phenol.

Key words: Endophytic fungi, *Moringa*, morphological, molecular, GC-mass.

Introduction

Fungal endophytic fungi are a special kind of symbiont organisms that invade the plant or part of it, during their growth, increases the vitality of the plant (Rosli et al., 2020). Endophytes vary and include many types, most commonly filamentous fungi; Endophytic fungi are influenced by temperature and seasons (Nahas and Hebatallah, 2019). It was recently recorded that endophytic fungi are most important micro-organisms because they produce a wide range of bioactive secondary metabolites (Ibrahim et al., 2021). The obtained compound is used in many different medical fields and pharmacology (Omomo and Bablola, 2019).

Moringa oleifera plant which is the most cultivated species of the Moringaceae family; is known for its different stimulating properties mentioned in Ayurveda (Srivastava et al., 2020). It is known for its nutritional value in tropical and subtropical countries. All parts of this plant produce phytochemicals with potential applications (Bridgemohan et al., 2002). *M. oleifera* has formidable properties and broad utilities, yet it remains unexploited, especially in areas outside its cultivation range (Liu et al .,2018).

Materials and Methods

Collecting Plant Samples and Isolating of Endophytic Fungi

The leaves, free of any disease symptoms, were collected from the green belt surrounding Hilla Governorate, which is located in the south of Baghdad,

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Iraq, for the period from the beginning of August until the end of October (2021). The fresh collected leaves were washed with tap water to get rid of dust and sterilised using ethyl alcohol (75%) for 30 seconds, then washed with distilled water three times and dried on sterile filter paper. Leaves were cut about 0.5-1 cm and then cultured on Petri dishes containing PDA medium and chloramphenicol (0.5 g/L). Four pieces with equal dimensions were placed and then wrapped with wax tape and incubated at a temperature of 25±2ºC for 7-10 days. After that, the developing colonies were isolated for diagnosis based on the phenotypic characteristics in the medium, and also depending on the taxonomic sources (Pitt, 1988; Raper and Fennel, 1965; Samson et al., 2007; Williams-Wood, 2001).

The isolated fungi were diagnosed on the basis of the general appearance of the colony, colour and microscopic characteristics. The percentage of the fungi frequency was calculated according to the following equation (Goveas et al., 2011):

$$\text{Fungi frequency (\%)} = (\text{No. of fungus isolates/total No. of isolates}) \times 100$$

Preparation of the Alcoholic Extract

The powder of *M. oleifera* leaves was used to prepare the alcoholic extract, where 100 gm of it dissolved in 500 ml of alcoholic solvent consisting of methanol + water (20:80 v/v) and placed in a soxhlet device (WTW, England) for 8 hours. Then, it was placed in a rotary evaporator (IKA, England) to extract and concentrate the solvent, under vacuum pressure at 45°C, and was dried in an electric oven (Memmert, Germany). This process was conducted in the Ministry of Science and Technology (Sultana et al., 2009).

DNA Extraction and PCR Amplification

After the isolation of endophytic fungi, they were cultured on the liquid media (PDB) and then the mycelium was collected and put in the crushing mortar. Liquid nitrogen at –180ºC was added to the mycelium which was crushed until it turned into a powder. The specific primers for region ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) for ribosomal S18 gene were used which is specific for the detection of filamentous fungi (White et al., 1990). DNA extraction was done according to Cenis (1992).

GC-MS Analysis

This analysis was performed using Agilent (7820) USA device in the Ministry of Industry and Minerals; 1 µL of the alcoholic extract of *M. oleifera* leaves was injected using a small syringe. The organic compounds were obtained and their spectra were compared with mass spectral libraries NIST 11 L. The analysis conditions were Analytical Column: Agilent HP-5msUltra Ineit (30 m Length × 250 µm dimeter × 0.25 µm inside diameter). Injection volium 1 µL. Pressure 11.933 psi. GC Inlet Line Temperature: 25°C. Aux heaters Temperature: 310°C. Carrier Gas: He 99.99%. Injector Tempertaure: 250°C. Injection Type: Splitless and oven program was: Tempertaure Ramp1 55°C hold to 2°C, Ramp2 55°C, 180°C, 7°C/min, Ramp3 180°C to 280°C, 8°C/min, Ramp4 280°C hold to 2°C/min.

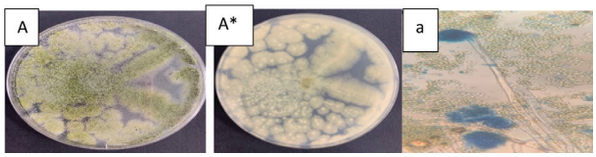
Results and Discussion

Morphological and Diagnosis Characterisation of Isolated Endophytes

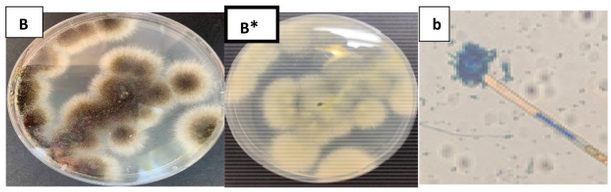
The endophytic fungi inside the plants are diverse and can produce different colours when they grow, as they are affected by temperature, type of medium and added nutrients. In this study, isolates were grown on a PDA medium for 7 days at a temperature of 25 ± 2ºC; the result detected 59 endophytic fungi isolates, all belonging to the phylum Ascomycota. The microscopic examination showed that *Aspergillus flavus* (Figure 1A) was predominant over the rest of the species with 19 isolates and a frequency of 27.14. Its colonies appeared in yellowish-green colour with transparent and septate hyphae. The colony of *Aspergillus niger* (Figure 1B) appeared black with white borders, while the hyphae were transparent and septate. In addition, *Myceliophthora spedonium* colony (Figure 1C) was white to golden yellow colour, while the hyphae were short and non-septate. On the other hand, *Aspergillus costaricensis* colony (Figure 1D) was dark black with yellow borders and the hyphae were short, transparent and non-septate. *Aspergillus terreus* colony (Figure 1E) was brown in colour with yellowish-white borders; whereas *Aspergillus caespitosus* colony (Figure 1F) appeared to be dark green in colour with white borders, and the hyphae were transparent and not septate. Moreover, *Paecilomyces* sp. colony was light yellow to light brown, whereas the colony of *Byssochlamys spectabilis* (Figure 1H) was light yellow and the hyphae were transparent and septate (Table 1).

Molecular Diagnosis

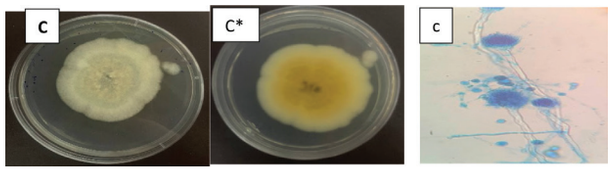
DNA extraction for all samples was carried out successfully without contamination and as evidenced by the appearance of bands. Furthermore, the results



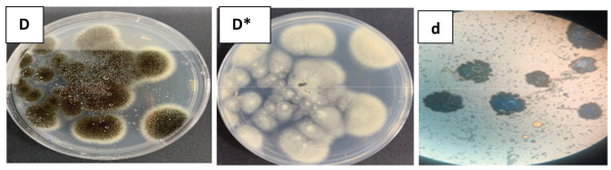
Aspergillus flavus: A colony, A* Reverse colour, a Microscopy



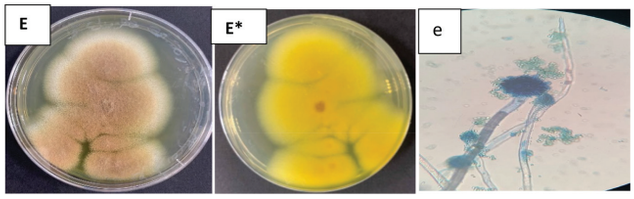
Aspergillus niger: B colony, B* Reverse colour, b Microscopy



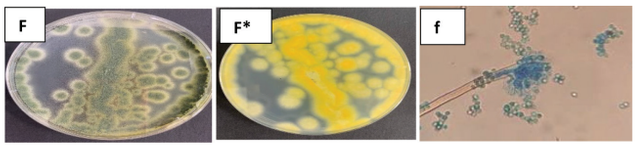
Myceliophthpra sepedonium: C colony, C* Reverse colour, c Microscopy



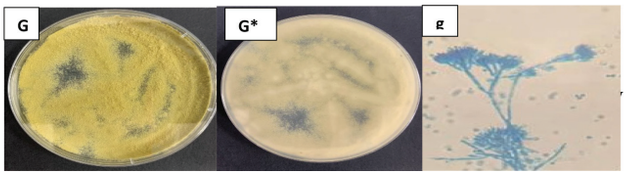
Aspergillus costaricensis: D Colony, D* Reverse colour, d Microscopy



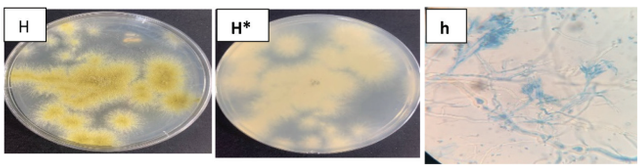
Aspergillus terreus: E Colony, E* Reverse colour, e Microscopy



Aspergillus caespitosus: F Colony, F* Reverse colour, f Microscopy



Paecilomyces sp.: G colony, G* Reverse colour, g Microscopy



Byssochlamys spectabilis: H Colony, H* Reverse colour, h Microscopy

Figure 1: Endophytic fungi on PDA medium which incubated at 25±2°C for 7 days. Magnification of each slide is 40x.

Table 1: The morphological characteristics of endophytic fungi on PDA medium after 7 days of growth

Fungus	*Colour of colony*	*Reverse colour*	*Mycelium characters*	*Topography of fungal growth*
Aspergillus flavus	Yellowish green with white borders	Off-white	Transparent and septate	The texture of the colony is velvet or woolen
Aspergillus niger	Black colony with white borders	Pale yellow	Transparent and septate	The texture of the colony is velvet
Myceliophthora sepedonium	white to golden yellow	Golden yellow	Short and non-septate	The colony like tanned leather
Aspergilluscostaricensis	Dark black colony with yellow borders	Off-white	Short, transparent and non-septate	The texture of the colony is velvet
Aspergillus terreus	Dark brown (cinnamon colour) with white-yellow borders	Dark yellow to curcomic colour	Transparent and non-septate	The texture of the colony is as powder
Aspergillus caespitosus	Dark green with white borders	Dark yellow	Transparent and non-septate	The texture of the colony is velvet
Paecilomyces sp.	Light yellow to light brown	Light yellow to green	Long, transparent and non-septate	Fast-growing, powdery colony texture
Byssochlamys spectabilis	Light yellow	Light yellow	Short and non septate	The texture of the colony velvet like penicillum colony

of measuring the purity of the extracted DNA showed that it ranged between 1.7 and 1.9 nanometers for all samples (Figure 2).

Polymerase Chain Reaction (PCR)

The specific primers ITS-1 and TIS4 were used for the 18S rRNA gene which designed by Primer plus 3 program, and the primers were checked using the BLAST tool website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The electrophoresis of PCR products detected the appearance of bands with 550 base pairs in size, depending on the DNA Marker Ladder (Figure 3). The results revealed the presence of 19 isolates of *Aspergillus flavus* with a frequency of 27.14 out of a total of 59 fungi, whereas *Aspergillus niger* had 11 isolates, with a frequency of 15.71; *Myceliophthora sepedonium* had 7 isolates with a frequency of 10. Additionally, *Aspergillus costaricensis* and *Aspergillus terreus* were found in 6 isolates each with a frequency of 8.57; *Paecilomyces sp*. and *Aspergillus caespitousus* were found in 4 isolates each and the frequency was 5.71, while *Byssochlamys spectabilis* was the lowest among all the isolated fungi with a number of 2 isolates and a frequency rate of 2.82 (Table 2).

Medicinal plants contain a wide range of intracellular microorganisms called endophytes which have compounds with biological and medical activity due to the presence of substances that confer resistance to many pathogens. *Aspergillus* spp. was isolated from many medicinal plants such as *Acacia nilotica*, *Ficus religiosa*, *Adhatoda beddomei*, *paeonia delavayi*, *Salvadora oleoides Decne*, *Sonneratia ovate*, and it was found that it contains bioactive compounds such as

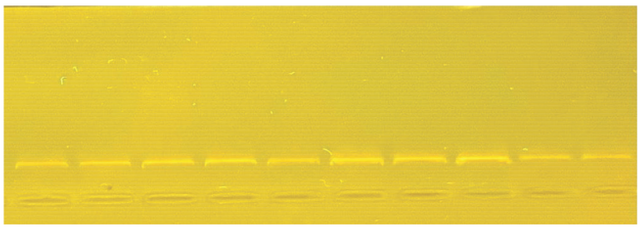


Figure 2: Extracted DNA for all samples.

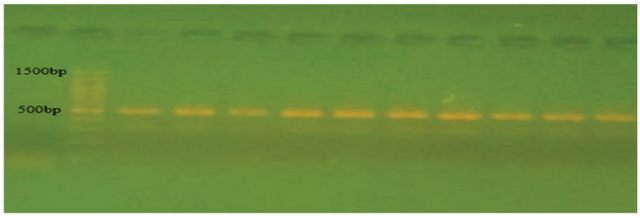


Figure 3: The electrophoresis of the PCR products.

Antidiabetic, Peptide lectin (N-acetyquinylactosamine, (N-acetylaconesDa) O-phenethylherbarin), herbarin and herbaridine, phomopoxides AG, 2,6-di-tert-butyl-p cresol, phenol-2,6bis[1,1-dimethylethyl]-4-methyl citreoisocoucoumarinol, citreoisocoumarin, and macrocarpon C (Adeleke and Babalola, 2021).

Aspergillus niger was isolated as an endophytic fungus from several plants, including the leaves of the banana plant *Musa spp.* (Zakaria and Aziz, 2018), the leaves of the oriental plane plant *Platanus orientalis* (Robl et al., 2015), the medicinal plant *Antidesma madagascariense* (Jeewon et al., 2013) and the leaves of the mango plant *Mangifera indica* (Nayak, 2015). *Aspergillus niger* belongs to the family Trichocomaceae and was found in *Moringa peregrine*, where it was isolated as an endophytic fungus. In addition, *Emericella*

Table 2: The colonisation frequency (%), accession No. in GenBank data base, and identities (%) in isolated endophytic fungi

Fungus	*Colonies No.*	*Colonisation frequency*	*Accession No. in GenBank*	*Identities*
Aspergillus flavus	19	27.14 %	KX950671.1	86%
Aspergillus niger	11	15.71 %	MN161138	83%
Myceliophthorasepedonium	7	10 %	GU966503.1	87%
Aspergilluscostaricensis	6	8.57%	MW193220.1	75%
Aspergillus terreus	6	8.57%	KY421108.1	93%
Aspergillus caespitosus	4	5.71%	MT362464.1	95/%
Paecilomyces sp.	4	5.71 %	JN227071.1	94%
Byssochlamysspectabilis	2	2.85%	MT422191.1	89%
Total	59	84.26%		

is the sexual form of *Aspergillus*, which has been described as an organism that has the ability to produce biologically active antimicrobial and anti-hepatitis C compounds and liver cancer cells; it was isolated from the Mediterranean alga *Emericella nidulans* (Haws et al., 2012). *Myceliophthora sepedonium* belongs to the family Chaetomiaceae, which is one of the largest families that possess the ability to decompose cellulose and produce biologically active secondary metabolites that have resistance to many plant diseases (Al-Dossary et al., 2021), and it is considered in this study as the first endophytic fungus isolated from *Moringa* leaves plant in Iraq. Moreover, *Paecilomyces sp.* is an endophytic fungus that provides the plant with many advantages as it increases crop productivity and gives protection to the plant from pathogens. It was also shown that the interaction between the plant and *Paecilomyces* improves the health of the plant. This genus contains secondary metabolites with different chemical compositions and various biological activities through different mechanisms (Moreno-Gavira et al., 2020); it was also isolated from the leaves of *Musa acuminata* (Cao et al., 2002). Likewise, *Aspergillus cosricensis* was isolated from *Moringa* leaves, and it was observed that it is one of the fungi that produce a lot of enzymes, and considered the first endophytic fungi isolated from the *Moringa* leaves plant in Iraq.

Aspergillus caespitosus was first isolated from the soil and also from the phloem of *Moringa peregrina* plant; it was found that the extract of *A. caespitosus* had an effect similar to the gibberellin hormone responsible for increasing the height of the plant as it increased the length of dwarf rice seedlings (Khan et al., 2014). The opportunistic fungus *Aspergillus flavus* was isolated as aendophytic fungus from *Aegilops geniculata* (Abdel-Motaal and Abou-Ellail, 2020). It was also isolated from the roots of *Chenopodium album* (Lubna et al., 2018). Additionally, *Aspergillus terreus* belongs to the Trichocomaceae family, and Rajeswari et al. (2016) study isolated this genus as an endophytic fungus from the leaves, flowers and phloem of *M. olifera*, while the fungal extract showed that it has the ability to inhibit other filamentous fungi and bacteria, as well as *A. terreus* revealed the dominance over the rest of endophytic fungi that isolated from *Zea mays* L. and soybean *Glcine max* L. *Merr* (Russo et al., 2016). Interestingly, *Byssochlamys spectabilis* is a fast-growing fungus found in soil and plants. It is resistant to low levels of oxygen, while *Paecilomyces variotii*, the sexual stage of it, produces many mycotoxins and is isolated from *Apocynaceae oleander*, *Euphorbia prostrata* and *Vernonia amygdalina* plants. It showed also a positive effect of the fungal extract on bacterial strains and cancer cells (Khiralla et al., 2016)

It is clear that filamentous fungi exhibit behave as a pathogen to the plant, as they grow on the surface as a harmful factor, or be a beneficial factor up to the point of changing the plant's resistance to all harsh environmental conditions; this is possibly due to their participation in the biological reactions that take place inside the plant, which gives the organic compounds greater effectiveness. The filamentous fungi that are found inside the plant live in a state of competition for the same host, which stimulates them to produce secondary metabolites, and this reinforces the reason for the difference in the presence of active compounds and their percentage in the leaves of the same plant during different seasons because the proportions of the presence of endophytic fungi inside it are affected by the seasons.

GC Mass Analysis

The GC-MS was measured for the alcoholic extract of *Moringa* leaves. *M. oleifera* was prepared previously by Sultana et al. (2009) and as shown in Table 3; as shown in Figure 4, 30 peaks were detected referring to the diagnosis of 30 active compounds. The most important compounds that appeared are oleic acid, phytol, 9-octadecenoic acid (stearic acid), hexadecanoic acid, methyl ester, phenol, 5-methel-2-(pyrrol-1-yl), cyclohexanone, 2,2-dimethyl-5-(3-m-ethyloxiranyl), dihydrojasmone and cyclododecane, which has been recorded as biologically active substances extracted from medicinal plants in many researches, including *Moringa* (Mishra and Patnaik, 2020).

Elmastas et al. (2006) indicated that the compound cyclohexen-1-one, 2-methyl-5-(1-methylenethenyl)-(R)- is one of the carvone compounds, which are found in aromatic plants. It contains three double bonds responsible for reducing silver nitrate. Furthermore, D-carvone was obtained from the ethanolic extract

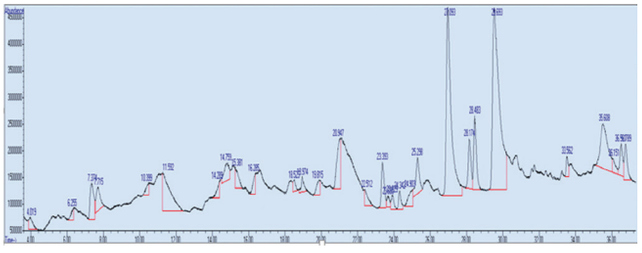


Figure 4: The chemical component obtained from the GC-MS analysis of the alcoholic extract of *Moringa oleifera* leaves.

Table 3: GC-MS analysis for the alcoholic extract of *Moringa oleifera* leaves

Library/ ID	*Area %*	*R.Time*	*Peak*
Ethylbenzene	0.86	4.022	1
2-Furancarboxaldehyde, 5-methyl-	0.59	6.253	2
Trans- Linalool oxide (furanoid)	2.74	7.378	3
Trans- Linalool oxide (furanoid)	1.95	7.719	4
N-Methoxy-N-methylactamide	0.7	10.395	5
4H-Pyran-4-one, 2,3- hydro-3,5-di	5.94	11.595	6
2-Pentanone, 4-(2,6,6- trimethyl-2 cyclohexenyl	0.85	14.290	7
4-Flourobenzyl alcohol	1.84	14.763	8
Benzenemethanol, 3-fluoro-	1.31	15.377	9
2-pentenoic acid, 4-methyl-, methy	1.09	16.285	10
3-chloropropionic acid, heptadecyl ester	0.60	18.526	11
Dihydrojasmone	0.90	18.971	12
Phenol, 5-methyl-2-(pyrrol-1-y1)	0.99	19.812	13
Furo [2,3-c]Pyridine, 2-methyl-	4.05	20.947	14
Benzeneacetonitrile, 4-hydroxy-	1.11	22.516	15
Cyclohexanone, 2,2-dimethyl-5- (3-m ethyloxiranyl)-, [alpha. (R*) ,3. A lpha.]- (.+-.)-	2.60	23.396	16
1,2- Epithio-3-hexanol	0.59	23.698	17
3-Methyl-2-(2-oxopropyl) furan	0.76	23.925	18
6-octan-1- ol,3, 7-dimethyl- ,propa noate	0.95	24.341	19
3,4,5- Trimethyle- 1H- Pyrano [2,3-c]py	1.50	24.899	20
Hexadecanoic acid, methyl ester	2.62	25.256	21
Octadecanoic acid	19.32	27.093	22
10-Octadecenoic acid	2.92	28.171	23
Phytol	4.02	28.483	24
Cyclododecane	26.44	29.693	25
Di-n-octyl phalate	0.90	33.560	26
Oleic acid	6.82	35.612	27
14-pentadecenoic acid	0.98	36.151	28
1-Pyrrolidinebutanoic acid, 2-[(1, 1-dimethylethoxy)carbonyl]-. alpha. -nitro-, 2,6-bis(1,1-dimethlethyl	1.76	36.520	29
Silane, diethylpentadecyloxy(3-phe nylpropoxy)-	2.31	36.794	30

of *Moringa* leaves with a percentage of (1.36%), in addition to 26 other substances (Abdel-Daim et al., 2020). The alcoholic extract of *Moringa* leaves also showed the presence of 100 active substances, including phenol, 2-methoxy, Mantol, 2-Hexenedioic acid, squalane, 9-octadecenoic acid (z)-, 2,3-diydroxyprop and oleic acid (Kadhim and AL-Shammaa, 2014).

Previously, 11 compounds were diagnosed in the alcoholic extract of the leaves of *Moringa olifera*, the most compounds were methyl(11e)-11-octadecenate with a percentage of 30.1, oleic acid cis- at a rate of 19.16% and methyl-14-methyl pentadecanoate with a percentage of 17.67%; all of these compounds have hypocholesterolemic activity (Igwe et al., 2015). Moreover, this study detected the presence of 4H-Pyran-4-one,2,3-hydro-3.5-di with a percentage of 5.94%, whereas in another study this compound was obtained from the aqueous extract of *Moringa* leaves with a percentage of 8.98%, which is considered as an antioxidant agent (Bhalla et al., 2021).

Dihydrojasmone, one of the derivatives of Jasmonates, is considered a natural protection mechanism in the plant; it is non-toxic, non-mutagenic and has positive effects against botanical aphids (Paprocka et al., 2018). On the other hand, cyclododecane is a substance found in the leaves of *Moringa* with a percentage of 26.44%, and also in *Ficus asperifolia* Miq, with a percentage of 9.9% with other compounds. Cyclododecane is an important vegetable source in the detergent industry (Lawal et al., 2016). The appearance of the active substances during the chromatographic analysis depends on the type of solvent, type of device, the heating program, and the time of picking for the plant, as it increases at one time and decreases at another time, and all of them showed that the leaves of *M. oleifeira* are rich in effective compounds that need further studies to use it in several applications in various fields.

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