ANTIFUNGAL PROPERTIES OF *Moringa oleifera* AND *Olea europaea* OILS AGAINST *Penicillium chrysogenum* AND *Mucor circinelloides*

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SUMMARY

The search for new antifungal substances against plant pathogens continues because of the negative effects of inorganic fungicides on both plants and the environment. This study focused on assessing the antifungal activities of Moringa oleifera (Moringa) and Olea europaea (Olive) essential oils individually and in combination against two selected fungi isolated from agricultural soils obtained from different locations. Antifungal activities of both oils individually and synergistically were investigated using the food poisoning method. The phytochemical analysis of Moringa oil revealed a combination of phytochemicals while Olive oil showed only the presence of terpenoids. From the results, Moringa oil alone demonstrated strong activity against both pathogens with mycelial radial growth inhibition ranging from 2.83±0.44mm to 5.83±1.01mm. This was significantly ($p \leq 0.05$) different from the controls (17.67±3.38mm for Penicillium chrysogenum and 39.17±0.73mm for Mucor circinelloides). For Olive oil alone, the radial growth inhibition ranged from 6.83±1.92mm to 24.00±3.00mm. This was significantly different from the controls. The synergistic combination of Moringa oil and Olive oil gave results ranging from 0.00±0.00mm to 11.33±0.67mm compared to the controls. The highest percentage mycelial growth inhibition for Moringa oil was 89.79% (Mucor circinelloides, 14%) and the lowest was 71.70% (Penicillium chrysogenum, 2%). The highest percentage mycelial growth inhibition for Olive oil was 82.55% (Mucor circinelloides, 14%) and the lowest was 16.98% (Penicillium chrysogenum, 2%). The percentage mycelial growth inhibition for the synergistic combination gave a highest value of 100% (Mucor circinelloides, 14%) and the lowest was 30.19% (Penicillium chrysogenum, 2%). The results of this study revealed that M. oleifera an O. europaea oils significantly ($p \leq 0.05$) reduced the mycelial radial growth of the tested pathogens individually and synergistically. Further studies should however, be conducted to ascertain the effectiveness of these natural fungicides in vivo.

Keywords: Antifungal properties, Pathogens, Fungi, Pesticides, Essential Oils

Moringa oleifera which belongs to the family *Moringaceae* is found in tropical and sub-tropical regions. It is a drought resistant fast-growing plant native to the Indian sub-continent (Dalla, 1993). It can develop and tolerate diverse soil conditions but flourishes in a neutral to slightly acidic, well – drained soils (Radovich, 2011). *Moringa oleifera* is largely grown in India, nations in Asia particularly in South and Southeast Asia, Philippines and Indonesia, Central America, South America, Africa and various nations of Oceania where the leaves are eaten as food. It is cultivated for its leaves, fruits (seeds), roots and oil which can be used for different purposes such as food, cosmetics for hair and skin products, and for medicinal purposes (Makkar and Becker, 1996). Folk medicine utilizes crude or squashed *M. oleifera* seeds as a decoction for treating stomach torment, ulcers, poor vision, joint pain and for supporting digestion (Popoola and Obembe, 2013).

The seed extract has been found to possess good antimicrobial activity against various bacterial and fungal species (Padla *et al*., 2012; Williams, 2013; Jeon *et al*., 2014). The antimicrobial activity of the seed is also related to the presence of a short cationic protein. This protein, known as the *M. oleifera* cationic protein, causes bacterial cell damage through rapid flocculation and the fusion of cell inner and outer membranes. The seeds of *M. oleifera* have been found to be good antioxidants, able to reduce oxidative damage associated with aging and cancer (Singh *et al.*, 2009). Also, the extract of *M. oleifera* seeds has been reported to have antidiabetic properties (Al-Malki and El Rabey, 2015).

Olea europaea (Olive tree) belongs to the family Oleaceae which are commonly grown in the Arabian Peninsula, Canary Islands, Portugal and Southern Asia (De Alzaa *et al*., 2018). It is short, rarely exceeds the height of 8 – 15 m and it is best known for its oil, which is of great importance in the Mediterranean region in cuisines. Olive oil has many uses in pharmaceuticals, cooking, cosmetics, religious purposes, as salad dressing and used traditionally as fuel for oil lamps (De Alzaa *et al*., 2018). Old and current pharmacopoeias have included numerous preparations based on Olive oil, considering its therapeutic uses as a remedy for many maladies, with useful impacts in a significant number of the human organs and systems in all periods of life (Kiralan *et al*., 2009). Essential oils like *M. oleifera* seed oil and *O. europaea* have been reported to possess antifungal activities without negative effects on humans and the environment (Akhtar *et al*., 2013).

Mucor and *Penicillium* species are examples of fungal pathogens that cause diseases in plants. These organisms are associated with the post-harvest spoilage of fruits. *Mucor* species causes *Mucor* rot in fruits especially citrus fruits (Webber *et al.*, 2014) while *Penicillium* species cause green and blue moulds in citrus plants which is the most common post-harvest disease in citrus worldwide (McKay *et al*., 2012; Akhtar *et al*., 2013; Bhattarai *et al.,* 2013). Post-harvest fruit rot caused by these pathogens contribute to significant losses in the citrus industry during de- greening, storage, transport and marketing (Smilanick *et al*., 2006; Akhtar *et al*., 2013). Therefore, it is of great importance to find safe and environment friendly ways to control these pathogens. Apart from losses due to postharvest pathogens, toxins produced by spoilage agents could also harm humans and spoilage products could serve as vehicles for pathogens (Barth *et al*., 2009).

Despite the presence of different varieties of chemical fungicides, the search for new antifungal substances against plant pathogens continues because of the negative effects associated with the use of fungicides (Thangavelu *et al*., 2003). A great deal of attention is being directed to the development of safer and environment friendly antifungal agents against plant pathogens (Schwingshackl and Hoffmann, 2014). The aim of this study was to assess the antifungal activities of *M. oleifera* and *O. europaea* oils against selected plant pathogens individually and synergistically.

MATERIALS AND METHODS

Collection of plant samples

The wings and coats of *Moringa oleifera* seeds were removed and dried. Fine powder of *Moringa* seeds was prepared using mortar and pestle previously purchased from a market in Edo State, Nigeria. The Olive oil used in this study was purchased at a Supermarket in Edo State, Nigeria.

Collection of farmyard soil samples

Soil samples were obtained from three different farmlands. At each location, soil samples were taken at a depth of 2 – 15 cm and homogenized. These samples were transported to the laboratory for test in decontaminated bags (Aina *et al*., 2011).

Isolation of fungi

Soil dilutions were obtained by placing 1 g of each soil sample in 10 ml of sterile distilled water. Dilutions of 10^{-3}, 10^{-4} and 10^{-5} were used for isolation of fungi. An aliquot of 1 ml of the suspension of all test concentrations was mixed with pathogen-free potato dextrose agar media and the experiment was replicated thrice. The growth media were supplemented with Chloramphenicol (250 mg) to suppress the growth of bacteria before pouring into Petri dishes. The cultures were incubated for 7 days at room temperature (Waksman, 1994).

Identification of fungal isolates

The fungi were identified using the colony's morphological traits and microscopic attributes as reported by Barnett and Hunter (1972). The colony length, which comprises the length and width of the colonies, the presence or absence of aerial mycelium, color, wrinkles, furrows, and any other pigment, as well as macro morphological features were all assessed.

Extraction and concentration of *Moringa* oil

After fine powder was obtained from dried *Moringa oleifera* seeds, the powder was soaked in hot water and allowed to stand for 4 – 5 days. After 5 days, the oil was collected from the surface and boiled until clear oil was obtained. After boiling, the oil was filtered with a fine sieve to remove any impurities. *Moringa* oil concentrations were determined by diluting an adequate quantity of the crude extract with liquid potato dextrose agar medium, followed by the addition of Tween 80 to distribute the oil in the medium and yield concentrations of 2, 6, 10 and 14% (El- Mohamedy and Abdallah, 2014).

Phytochemical analysis

Phytochemical analysis was carried out for *Moringa* oil and Olive oil as described by Ezeonu and Ejikeme (2016). The following procedures were used:

1. Anthraquinones were detected by adding benzene to an oil extract in an Erlenmeyer flask and left for 10 minutes. Ten percent ammonia solution was added and forcefully agitated for 30 seconds, and the presence of anthraquinones in the ammonia phase was indicated by the presence of pink, violet or red coloration.
2. Tannins were detected by adding a few drops of 0.1 percent Ferric chloride to the oil. A positive test was indicated by a brownish green or blue-black coloration.
3. Terpenoids were detected in the oil using a combination of Chloroform and concentrated Tetraoxosulphate (VI) acid. The presence of reddish-brown coloration indicated the presence of terpenoids.
4. Flavonoids test: An aliquot of 10 M dilute ammonia solution was added to the oil, followed by a concentrated Tetraoxosulphate (IV) acid. The presence of flavonoids was indicated by the appearance of a yellow coloration that fades when standing.
5. Test for Alkaloids: Chloroform was added to the oil in a test tube. Appearance of pink color indicates the absence of alkaloids in the oil. Appearance of brown color indicates the presence of alkaloids.
6. Steroid test: The oil was treated with acetic anhydride before being treated with concentrated Tetraoxosulphate (VI) acid. The presence of steroid was indicated by a color change from violet to blue or green.
7. Phenols were detected in the oil by adding a few drops of weak Ferric chloride solution. The presence of phenols was indicated by the production of red, blue, green or purple coloration.
8. Saponin test: The oil was poured into a test tube and aggressively shaken. Persistent froth formation indicates the presence of saponins.

Antifungal assay

Determination of mycelial radial growth inhibition

The food poisoning approach was used to test the antifungal properties of *Moringa* and Olive oils. Twenty millilitres of the medium were aseptically dispensed into each Petri dish. At the centre of each petri dish, a 9-mm diameter agar disc containing the hyphae of fungus from 7-day old cultures was placed. This procedure was repeated three times for each treatment. Plates with fungus but no plant extract were used as control. The cultures were incubated at 28°C for 7 days, with daily measurements of mycelial radial growth inhibition (El-Mohamedy and Abdallah 2014).

Determination of synergistic activities of both oils

Equal volume of 100% solution of *Moringa* oil and Olive oil were mixed. Appropriate amount of the combined oils was diluted with the molten potato dextrose agar media. Tween 80 was then added to distribute the oil in the medium to give concentrations of 2, 6, 10 and 14%

Determination of the percentage mycelial radial growth inhibition

The percentage mycelial inhibition was determined after seven days of incubation according to the equation (Elgorban *et al*., 2015): Percentage growth inhibition= R- r / R × 100

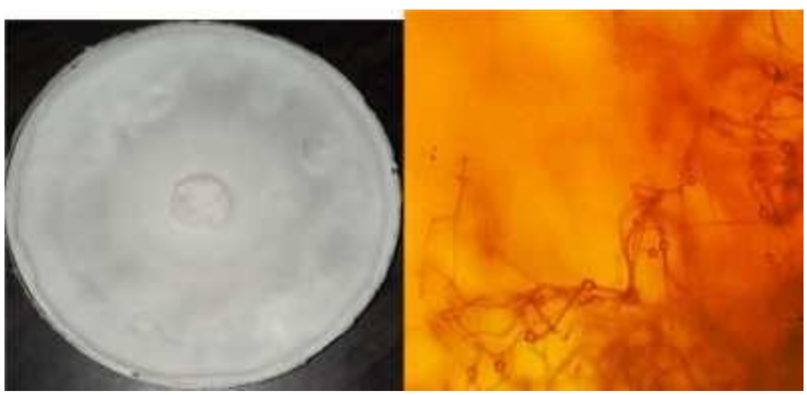
Where, R- Linear growth of fungus on control Petri dishes; r- Linear growth of fungus on Petri dishes with *Moringa* and Olive oils

Statistical analysis

The data generated were expressed as mean values and standard error of three (3) replications. They were then analyzed using One-way Analysis of Variance (ANOVA) procedure of SPSS version 20.0 software. Mean values were differentiated with Duncan Multiple Range (DMR) test (Ogbeibu (2005).

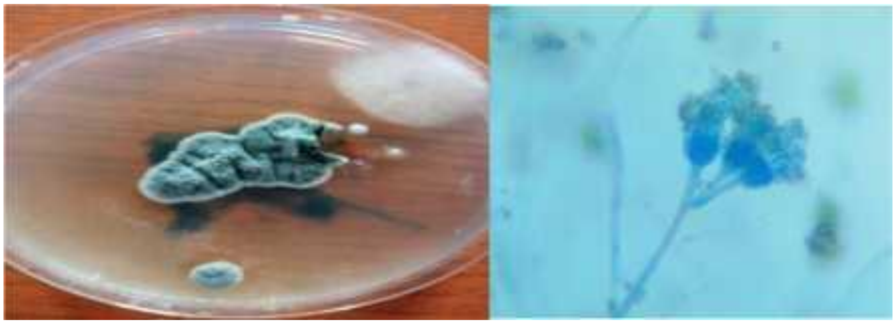
RESULTS

The phytopathogenic fungi isolated and identified in this study were *Penicillium chrysogenum* and *Mucor circinelloides*. The cultural and microscopic characteristics of the isolates are shown in Plates 1 and 2.



A **B**

Plate 1: Cultural and microscopic characteristics of *Mucor circinelloides.* A is a 7-day old pure culture of *Mucor circinelloides*,while B is photomicrograph of *Mucor circinelloides.*



A **B**

Plate 2: Cultural and microscopic characteristics of *Penicillium chrysogenum*. A is a 7-day old culture of *Penicillium chrysogenum*, while B is a photomicrograph of *Penicillium chrysogenum.*

Table 1 shows the cultural and morphological characteristics of the fungal isolates. *Penicillium chrysogenum* displayed a greenish colony with white growing edges while *Mucor circinelloides* displayed white cotton - like colony on potato dextrose agar plates. Further identification of the isolates was carried out microscopically. Colonies of *Penicillium chrysogenum* showed septate hyphae, sub globulus shape of the conidia with spherical to ellipsoidal smooth texture as shown in Plate 1B. Colonies of *Mucor circinelloides* showed non-septate hyphae and globulus shape of the conidia having an ellipsoidal texture as shown in Plate 2B.

Table 1: Cultural and morphological characteristics of the fungal isolates

Isolate	A: Penicillium chrysogenum	B: Mucor circinelloides
Morphology Description on PDA	Greenish with white edges	White cotton-like growth
Microscopic description		
Shape of conidia	Sub-globulus	Globulus
Texture of conidia	Spherical to ellipsoidal smooth	Ellipsoidal
Hyphae	Septate	Non-septate

Table 2 indicates the antifungal efficacy of Moringa oil *in vitro* against *Penicillium chrysogenum* and *Mucor circinelloides* and the controls as represented by their mycelial radial growth inhibition in millimeters. Moringa oil displayed a higher inhibitory effect against *Penicillium chrysogenum* (2.83±0.44mm) than *Mucor circinelloides* (4.00±0.76mm) at the highest concentration of 14%. These were significantly different from the control. The pathogens used were sensitive to Moringa oil even at the lowest concentration. *Penicillium chrysogenum*

(5.00±1.00mm) and *Mucor circinelloides* (4.50±0.50mm) were significantly sensitive at the lowest concentration of 2% compared to the controls (*Penicillium chrysogenum*, 17.67±3.38mm and *Mucor circinelloides*, 39.17±0.73mm).

Table 2: Antifungal activity of *Moringa* oil on mycelial growth inhibition of two fungal isolates.

Test organism	Mycelial growth inhibition (mm) at different concentrations (%)				Control
	2	6	10	14	
Penicillium chrysogenum	5.00±1.00a	3.00±0.50a	4.50±0.50a	2.83±0.44a	17.67±3.38b
Mucor circinelloides	4.50±0.50a	5.83±1.01a	5.33±0.73a	4.00±0.76a	39.17± 0.73b

Mean ± Standard errors for all parameters were significantly different ($p < 0.05$) from the control using Duncan multiple range test.

Table 3 indicates the antifungal efficacy of olive oil *in vitro* against *Penicillium chrysogenum* and *Mucor circinelloides* as represented by their mycelial radial growth inhibition in millimiters. The olive oil showed a higher inhibitory effect against *Mucor circinelloides* (6.83±1.92mm) compared to *Penicillium chrysogenum* (7.00±0.58mm) at the highest concentration of 14 % compared to the control. The test isolates were sensitive to olive oil at the lowest concentration with sensitivity being concentration dependent. *Penicillium chrysogenum* (14.67±1.30mm) and *Mucor circinelloides* (24.00±3.00mm) were also sensitive at the lowest concentration compared to the control (*Penicillium chrysogenum*, 17.67±3.38mm and *Mucor circinelloides*, 39.17±0.73mm, respectively). Although, the antifungal activity of olive oil against *Mucor circinelloides* was significantly different from the control ($p \leq 0.05$), that of *Penicillium chrysogenum* was not significant ($p \geq 0.05$).

Table 3: Antifungal activity of olive oil on mycelial growth inhibition of two fungal isolates.

Test organism	Mycelial growth inhibition (mm) at different concentrations (%)				Control
	2	6	10	14	
Penicillium chrysogenum	14.67±1.30^{ab}	9.33±4.91^{ab}	7.33±2.40^{a}	7.00±0.58^{a}	17.67±3.38^{b}
Mucor circinelloides	24.00±3.00^{c}	15.67±3.17^{b}	7.33±0.73^{a}	6.83±1.92^{a}	39.17±0.73^{d}

Mean ± Standard errors for all parameters and the mean values differed statistically using Duncan multiple range test.

Table 4 shows the antifungal effect of the combination of *Moringa* and olive oils against *P. chrysogenum* and *M. circinelloides*. Combining the two had a higher synergistic inhibition against *M. circinelloides* compared to *P. chrysogenum*. It was observed that the total growth inhibition (0.00±0.00 mm) for *M. circinelloides* was at 14% concentration while that of *P. chrysogenum* was 5.17±1.17mm at the same concentration. The combination had a moderate inhibition on *P. chrysogenum* compared to the results of *Moringa* oil and olive oil alone. *P. chrysogenum* (11.33±0.67mm) and *M. circinelloides* (5.33±1.33mm) were sensitive at the lowest concentration of 2% compared to the control.

Table 4: Antifungal activity of the synergistic combination of *Moringa* oil and Olive oil on mycelial growth inhibition of two fungal isolates.

Test organism	Mycelial growth inhibition (mm) at different concentrations (%)				Control
	2	6	10	14	
Penicillium chrysogenum	11.33 ± 0.67^{ab}	9.50 ± 3.75^{a}	$5.17 + \pm 1.30^{a}$	5.17 ± 1.17^{a}	17.67 ± 3.38^{b}
Mucor circinelloides	5.33 ± 0.13^{b}	6.17 ± 1.00^{b}	5.00 ± 1.53^{b}	0.00 ± 0.00^{a}	39.17 ± 0.73^{c}

Mean ± Standard errors for all parameters were significant ($p < 0.05$) and the mean values differed statistically using Duncan multiple range test.

Results from the phytochemical analysis of Olive and Moringa oils are shown in Table 5. For Olive oil, phenols, flavonoids, alkaloids and anthraquinones were absent while terpenoids were present. In Moringa oil phenols, flavonoids, alkaloids and saponins were present while tannins, anthraquinones and steroids were absent.

Table 5: Phytochemical analysis of Olive and Moringa oil

Phytochemical	**Inference**	
	Olive oil	Moringa oil
Pheno	-	+
Flavonoids	-	+
Alkaloids	-	+
Saponins	-	+
Terpenoids	+	+
Tannins	-	-
Steroids	-	-
Anthraquinone	-	-

+ = Present; - = Absent

Table 6 shows the percentage mycelial radial growth inhibition of Moringa oil on *Penicillium chrysogenum* and *Mucor circinelloides* at different concentrations. The highest percentage growth inhibition was 83.96 % for *Penicillium chrysogenum* and 89.79% for *Mucor circinelloides* (Moringa oil at 14%) while the lowest were 71.70% (*Penicillium chrysogenum)* and 85.11% (*Mucor circinelloides).*

Table 6: Percentage mycelial radial growth inhibition of Moringa oil on mycelial growth inhibition of two selected fungal isolates.

Test organism	Mycelial growth inhibition (mm) at different concentrations (%)			
	2	6	10	14
Penicillium chrysogenum	71.70	74.50	83.02	83.96
Mucor circinelloides	88.50	85.11	86.38	89.79

Table 7 shows the percentage mycelial radial growth inhibition of Olive oil on *P. chrysogenum* and *M. circinelloides* at different concentrations. The highest percentage growth inhibition was 72.27% for *P. chrysogenum* and 82.55% for *M. circinelloides* while the lowest was 16.98% (*P. chrysogenum*) and 38.72% (*M. circinelloides*).

Table 7: Percentage mycelial radial growth inhibition of Olive oil on mycelial growth inhibition of two selected fungal isolates.

Test organism	Mycelial growth inhibition (mm) at different concentrations (%)			
	2	6	10	14
Penicillium chrysogenum	16.98	47.17	59.24	72.26
Mucor circinelloides	38.72	60.00	81.28	82.55

Table 8 shows the percentage mycelial radial growth inhibition of the synergistic combination of both Moringa and Olive oils on *P. chrysogenum* and *M. circinelloides* at different concentrations. The highest percentage growth inhibition was 70.56% for *P. chrysogenum* and 100% for *M.circinelloides* while the lowest was 30.19% (*P. chrysogenum)* and 86.38% (*M. circinelloides)*.

Table 8: Percentage mycelial radial growth inhibition of the synergistic combination of *Moringa* oil and olive oil.

Test organism	Mycelial growth inhibition (mm) at different concentrations (%)			
	2	6	10	14
Penicillium chrysogenum	30.19	46.22	70.56	70.56
Mucor circinelloides	86.38	87.23	88.51	100

DISCUSSION

Synthetic fungicides are currently one of the most important tools in the management of plant diseases. Alternative management strategies are needed due to unfavorable public impressions of the use of synthetic pesticides, which are fungicide resistance among fungi and the high cost of developing new compounds. Plant-derived compounds have been investigated as disease management agents because they have low human toxicity, little environmental consequences, and widespread public acceptability against the selected fungal isolates (Seema *et al*., 2011; Moyo *et al*., 2012).

Essential oils and plant extracts have challenged researchers' interest in developing environmentally acceptable alternatives to synthetic fungicides for the management of fungal plant diseases (Gnanamanickam, 2002). The antifungal effects of *Moringa oleifera* seed oil and Olive oil against *Penicillium chrysogenum* and *Mucor circinelloides* were studied in this research.

Most published literatures in this field lay emphasis on the effect of *Moringa* and Olive oil separately against plant pathogenic fungi but not on their synergistic combination. *Moringa* oil displayed a very strong activity against *M. circinelloides* and *P. chrysogenum* compared to Olive oil. The results from this study demonstrated that *Moringa* oil at all concentrations had significant ($p \leq 0.05$) antifungal effect on the mycelial development rate of the test organisms. *Moringa* oil proved to be a better antifungal agent against *P. chrysogenum* compared to Olive oil and the synergistic combination. However, the combination of both essential oils, displayed better antifungal activity against *M. circinelloides* compared to when used singly.

The effects that *Moringa* oil had on *P. chrysogenum* and *M. circinelloides* can be attributed to the presence of phytochemicals which are naturally present in the oil. Also, because terpenoids are very lipophilic and have a low molecular weight, they might have contributed to the antifungal activity demonstrated by *Moringa* oil in this study. Terpenoids have the ability to damage cell membranes, kill cells, and stop fungus from sporulating. When compared to the complete essential oil, multiple *in-vitro* investigations suggest that terpenoids have inadequate antibacterial efficacy when applied alone (Bajpai *et al*., 2011). This indicates the important nature of other phytochemicals present in *Moringa* oil like alkaloids, saponins, phenols and flavonoids as they also have their separate use and antimicrobial effect (Tabassum and Vidyasagar, 2013).

In line with this study, El–Mohamedy and Abdallah (2014) reported that *Moringa* oil had a percentage mycelial radial growth inhibition of 100% against *Fusarium oxysporum* and *Fusarium solani* at a concentration of 2%. This same study also reported a percentage mycelial radial growth inhibition of 55.8% and 54% for *Fusarium oxysporum* and *Fusarium solani*, respectively at a concentration as low as 0.5% using *Moringa* oil.

Olive oil displayed moderate activity against *P. chrysogenum* and *M. circinelloides* compared to *Moringa* oil. The findings revealed that olive oil had a significant impact on the mycelial development rate of the chosen phytopathogenic organisms at all concentrations.

The phytochemical analysis of Olive oil showed the presence of terpenoids which have the ability to break fungal cell membranes, cause cell death, or prevent fungi sporulation. As stated earlier, terpenoids can perform these effects on fungi more effectively with the help of other phytochemicals present in olive oil. Compounds that are naturally found in olives have a possibility of being used as biopesticides. Thus, more research is needed to disclose the best method to extract and apply the antimicrobial compounds present in olive fruits. Varol *et al.* (2017) reported that Olive oil had a significant antifungal activity, with minimum inhibitory concentration of 50% against *Candida albicans* at a concentration of 2% which agrees with the results of this study.

The synergistic combination of *Moringa* and olive oil against *P.chrysogenum* and *M. circinelloides* showed a great antifungal activity. This is the first study to the best of our knowledge so far reported where this combination was used as an antifungal agent against these phytopathogens. When *Moringa* and Olive oil were used separately on *P. chrysogenum* and *M. circinelloides, M. circinelloides* had the highest percentage mycelial radial growth inhibition for both oils. When these oils were combined it gave a remarkable total growth inhibition of 100% for *M. circinelloides* at 14% concetration. This means that the synergistic combination demonstrated better antifungal properties against *M. circinelloides*.

CONCLUSION

Plant-derived antimicrobial agents that inhibit the propagation of microbial pathogens would be a far more plausible and environmentally sound method of crop protection, and will play a significant role in the development of future chemical pesticides for crop protective measures, with a focus on the control of plant diseases. According to the findings of this study, *M. oleifera* oil, olive oil and their synergistic combination demonstrated antifungal activity *in vitro* against *P. chrysogenum* and *M. circinelloides*. However, more research should be conducted to determine the efficacy of these natural fungicides *in vivo.*

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