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Determination of Antifungal Activity of Leaf Extracts from *Hypericum* ssp. Against Plant Pathogenic Fungi *Fusarium oxysporum* and *Alternaria Alternata*

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Highlights:

- The antifungal effect of *Hypericum* ssp. was determined.
- It was determined that *F.oxysporum* was more resistant.
- Alternative control to chemical control was introduced.

Keywords:

- *Hypericum* ssp.
- *Fusarium oxysporum*
- Antifungal activity
- *Alternaria alternata*

ABSTRACT:

In this study, the antifungal activity of ethanol extracts of three plant species of *Hypericum perforatum, Hypericum scabrum* and *Hypericum origanifolium* were evaluated for against two plant pathogenic fungal species of *Fusarium oxysporum* and *Alternaria alternata*.The antifungal activities of *Hypericum* ssp. extracts (3, 15, 45, 80, 120, 200, 240, 300 ppm) were tested against fungi. Ethanol 30% as control; Commercial fungicide (1 µg/mL) was used as a positive control. All *Hypericum* ssp. plant extracts were effective in different rates against the fungi depending on the doses. In addition, IC_{50} values of the three selected plant *Hypericum* ssp. leaf extract against pathogenic fungus *F.oxysporum* and *A. alternata* were determined with of probit analysis. In terms of the IC_{50} values, the antifungal activity of *H. perforatum* plant exract against *F.oxysporum* (122.525 µg/mL) > *A.alternata* (108.484 µg/mL). On the other hand, IC_{50} values of *H. scabrum* against *A.alternata* (126.390 µg/mL) > *F.oxysporum* (113.714 µg/mL). Moreover, the IC_{50} value of *H. origanifolium* against two pathogenic fungi was calculated as *F.oxysporum* (159.931 µg/mL) > *A.alternata* (55.759 µg/mL). Results showed that *H.origanifolium* has the best fungicidal activity, with IC_{50} value of 55.759 µg/mL against *A.alternata* and *H.scabrum* 113.717 µg/mL against *F.oxysporum*. However, the highest concentrations (300 ppm) caused completely inhibition in the both fungi mycelial growth followed by lower concentrations of plant extracts. *A. alternata* were the sensitive fungal species, while the *F. oxysporum* were a more resistant to the *Hypericum* ssp. extracts. Based on the antifungal activity tests, these plants extracts this may have effective as the new natural fungicide protecting crops against fungal diseases.

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INTRODUCTION

Plant pathogenic fungi are possible for 30–45 % of known plant diseases by attacking plants and cause major losses in agricultural production (Morshedloo et al., 2015 ; Elshafie et al., 2017). Among these pathogens *Fusarium* spp. causes root rot during plant production. Fusarium root rot is the most common fungal disease affecting wheat, onions and tomato worldwide (Gibert et al., 2022). Another fungus, *Alternaria alternata* causes wilt disease in plants and brown rot in fruits such as apples (Wang et al., 2021). *Alternaria* spp. mycotoxins were detected in various naturally contaminated fruits and vegetables, such as tomato, apple, grape, blueberry, orange, lemon, mandarin, and olive. The same time the pathogen is an opportunistic that attacks different plant parts, causing leaf spots and rots (Roy et al., 2019). This effect in direct economic damages including a decrease in crop production and quality. Moreover many fungi are capable of producing mycotoxin and contamination caused by mycotoxins leads to the refusal of the crop in the market (Parry et al., 1995).

Chemical fungicides are the most efficient method in controlling fungal diseases. Use of chemical fungicides may can cause a risk to human health and organisms in the environment. Nevertheless fungicides will face important borders in the the following years due to different major concerns (Nieder et al., 2018). Plant extracts and essential oils and natural products are therefore becoming to be more acceptable and have less health risk or pollute the environment than chemical compounds and can be for this reason used as an alternate to antifungal chemicals (Jobling, 2000). Under *in vitro* study conducted by Bilici et al. (2021) was tested three different essential oils and five different doses of oils (25, 50, 75, 100, 150 µl L^{-1}) against *Fusarium oxysporum in vitro* conditions. As a result of the study, it was determined that all doses of essential oils obtained from thyme prevented the development of micellar colonies of the pathogen by 50.8-80.8%.

There are nearly 80 species of *Hypericum* ssp. in Turkey and this species have been used for years for treating of exterior injury and gastric ulcer (Erken et al., 2001). *H.hyssopifolium* and *H. heterophyllum* essential oils showed antifungal potential against *Fusarium* species and five groups of *Rhizoctonia solani* (El-Shahir et al., 2022). Other studies on *H. scabrum* plants showed that toxicity owing to the secondary metabolites against the microorganisms *E. coli* ATCC 8739, *C. albicans* ATCC 10231, *B. subtilis* ATCC 19659, *C. tropicalis* ATCC 750 and *S. aureus* ATCC 6538 (Dadkhah et al., 2015 ; Ergin et al., 2022).

In the presented study decreased the use of chemical fungicides by determining the antifungal activities of the leaves ethanol extract of *Hypericum perforatum*, *Hypericum scabrum* and *Hypericum origanifolium* against *Alternaria alternata* and *F. oxysporum*. For the efficacy of ethanol extracts of this plant was investigated against fungi radial growth at the *in vitro* conditions.

MATERIALS AND METHODS

Collection of Plant Samples

H. perforatum, H. scabrum and *H. origanifolium* were collected from the Amasya region in the term June–August 2018. The identification of the plants was performed by Dr. Cengiz Yıldırım and specimens were deposited at the Herbary of Ondokuz Mayıs University as OMUB-6640 (HP), OMUB-0527 (HS), OMUB-4203 (HO) respectively.

Preparation of plant extracts

The aerial parts of the *H. perforatum, H. scabrum and H. origanifolium* were dried in the shade at room temperature for about two weeks. Ultrasonic assisted extraction (UAE) technique was used to for extraction of Hypericum species. Dried Hypericum species aerial parts (5 g) were grounded. The

extraction process was applied at 35°C for 45 min with water-ethanol (7:3, v/v) solvent mixture, keeping the material to liquor (M:L) ratio as 1:30 (v/v) based on the previous results (Seyrekoğlu & Temiz, 2020). After the extraction procedure, the filtration was performed and the solvent were evaporated by rotary evaporator.

Fungal strains

The used in the study plant fungal pathogens, *F. oxysporum* and *A.alternata* were provided stock cultures from the Laboratory of Microbiology and Plant Pathology at the Suluova Vocational School University of Amasya. *A. alternata* was isolated from apple, while *F.oxysporum* was isolated from onion. These fungi are among the very significant pathogenic fungi of economic harm to plants. Fungal cultures were maintained on Potato Dextrose Agar (PDA) on petri dish (9 cm) and kept at 27±1° C.

***In vitro* antifungal activity**

The antifungal activities of *Hypericum* ssp. plant extract were determined by use of agar plate method (Nwosu & Okafor, 1995). Plant extracts were prepared at the 3, 15, 45, 80, 120, 200, 240, 300 ppm concentrations in PDA (39 gr/l) medium. Without extracts in PDA media, commercial fungicide (1 µg/mLfungicide) the recommended dose was used as a positive control and Methanol (%30) was used as a negative control. The medium was 121^{o}C 15 min. autoclaved and up to cool at 40°C and transferred to media petri plate (90 mm) (~15mL plate^{-1}). The selected fungal mycelium disc (7 mm in diameter) from 5-day fresh fungus cultures were transferred to petri plates in the middle. Then incubated at 27±1 ^{o}C (Nuve Incubator) during 10 days and were followed the growth of fungi Daily (Onaran & Yılar, 2012). The tests were done three replicate. The percentage inhibition of fungus growth due to different concentrations of plant extract was calculated as follows:

$$\text{Mycelial growth inhibition (\%)} = \frac{dc-dt}{dc} x100$$

where dc = average diameter of fungi in control, and dt = average diameter of fungi in treatment (Şesan et al. 2017).

Statistical analysis

All applications were adjusted in a entirely randomized plan. The probit analysis of the data derived in result of the experiment was performed and rates of IC_{50} were calculated. All data were calculated statistically using the SPSS22 program. Variations among concentrations and controls were compared with using DUNCAN Multiple Range Test of $p < 0.05$.

RESULTS AND DISCUSSION

Antifungal activity

It was determined from the conclusions that the inhibitive activity of *Hypericum perforatum, Hypericum scabrum* and *Hypericum origanifolium* plant extracts showed significant variation depending on the dose against the mycelial growth of tested fungi. Positive control of chemical fungicides were decreased *F. oxysporum* mycelial growth by 14% according to control but in *A.alternata*, this ratio was 1.1%. In the solvent control, ethanol had no impact on fungal growth.

Effects of *hypericum perforatum* extracts on the mycelial growth of fungi

The fungi *F. oxysporum* and *A. alternata* were incubated on PDA medium with different concentrations of *Hypericum perforatum* extracts (Table 1). Accordding to probit analyzis, IC_{50} values against *Fusarium oxysporum* and *Alternaria alternata* of 122.523 and 108.484 µg/mL, respectively.

All concentrations of *Hypericum perforatum* extract were shown antifungal activity at different rates on fungi. This activity has also rises with the rise in the quantity of concentrations. Other concentrations except 3 and 15 ppm were decreased significantly growth of *F. oxysporum* according to control ($p < 0.05$). The decrease in 3 ppm and 15 ppm concentrations was not found to be significantly compared to the control. The concentration 300 ppm of *H. perforatum* extract were completely inhibited the growth of *Fusarium oxysporum* ($p < 0.05$). However, except for the 3 ppm concentration of extract, the other concentrations were statistically reduced the growth of *A. alternata*. The highest concentrations(240 and 300 ppm) of plant extract was observed 100% mycelium inhibition on *A. alternata.*

Table 1. Effects of *H. perforatum* Plant extracts on the Mycelial Growth of Fungi

Plant exracts	Concentrations (ppm)	*Fusarium oxysporum*		*Alternaria alternata*	
		Diameter of fungi ± standard error	Mycelial growth inhibition (%)	diameter of fungi ± standard error	Mycelial growth inhibition (%)
H. perforatum	3	7.033±0.14[a]	1.77	6.2±0.14[a]	1.5
	15	6.96±0.05[a]	2.7	5,033±0.03[b]	20.15
	45	4.23±0.25[c]	40.9	4.16±0.88[c]	33.9
	80	4.1±0.05[c]	42.7	4.10±0.57[c]	34.9
	120	3.9±0.1[c]	45.5	3.76±0.14[d]	40,3
	200	1.9±0.12[d]	73.4	1.16±0.12[e]	81.5
	240	1.03±0.25[e]	85.6	0.00±0.00[f]	100
	300	0.00±0.00f	100	0.00±0.00[f]	100
Control	Ethanol % 30	7.16±0.37[a]	-	6.30±0.11[a]	-
Positive Control	1 µg/mL	6.1±0.05[b]	14.8	6.23±0.14[a]	1.1

* Values are means of three replicates, Mean values(mean±standard error) sharing the same letter do not differ significantly by Duncan's Multiple Range Test at $p \leq 0.05$.

Effects of *Hypericum scabrum* extracts on the mycelial growth of fungi

Table 2. Effects of *H. scabrum* Plant Extracts on the Mycelial Growth of Fungi

Plant extract	Concentrations (ppm)	*Fusarium oxysporum*		*Alternaria alternata*	
		Average diameter of fungi ± SE	Mycelial growth inhibition (%)	Average diameter of fungi ± SE	Mycelial growth inhibition (%)
Hypericum scabrum	3	6.70±0.11[ab]	6.42	6.26±0.26[a]	0.6
	15	6.56±0.12[bc]	8.37	5.53±0.25[b]	12.2
	45	5.43±0.21[d]	25.6	5.00±0.28[b]	20.63
	80	4.56±0.23[e]	36.3	4.10±0.05[c]	34.9
	120	3.90±0.01[f]	45.5	3.73±0.14[c]	38.5
	200	2.23±0.12[g]	68.8	2.16±0.16[d]	65,7
	240	1.36±0.18[h]	81.8	0.00±0.00[e]	100
	300	0.9±0.03[h]	87.4	0.00±0.00[e]	100
Control	Ethanol % 30	7.16±0.37[a]	-	6.30±0.11[a]	-
Positive Control	1 µg/mL	6.1±0.05[c]	14.8	6.23±0.14[a]	1.1

*Values are means of three replicates, Mean values(mean±standard error) sharing the same letter do not differ significantly by Duncan's Multiple Range Test at $p \leq 0.05$.

In the second phase of the study *F. oxysporum* and *A. alternata* were incubated in PDA medium with different concentrations of *H. scabrum* extract. Using probit analysis, the IC_{50} value of *Hypericum scabrum* against *F. oxysporum* was determined 113,714 µg/mL while 126,390 µg/mL for *A. alternata*. The results obtained are showed in Table 2. Control and the concentration of 5 ppm plant extract showed statistically similar effects on the growth of *Fusarium oxysporum* ($p < 0.05$). The concentration 15 ppm of plant and positive control were effect similar to growth of *F. oxysporum*. The other results compared with controls, showed statistically better effect rates were found with regard to

inhibit *F. oxysporum*. The highest dose (300 ppm) of *Hypericum scabrum* extract was observed a 87.4% mycelium inhibition rate on *F. oxysporum*. Similarly, the *Hypericum scabrum* extract were also showed a similar effect on *A.alternata*. The concentrations (300 and 240 ppm) of *Hypericum scabrum* extract showed a 100% mycelium inhibition rate on *A. alternata* fungi ($p < 0.05$).

Effects of *Hypericum origanifolium* extracts on the mycelial growth of fungi

Table 3 shows the effect of extracts of *Hypericum origanifolium* on the mycelium growth of *F.oxysporum* and *A. alternata*. Using a growth inhibition assay technique, the *Hypericum origanifolium* ethanolic extracts demonstrated varying antifungal activities against *F.oxysporum* and *A. alternata* at different concentrations($p < 0.05$). The extracts showed significant antifungal activity at except 3 and 15 ppm against *F.oxysporum* growth. The greatest(100%) antifungal activity was detected at 300 ppm against *F.oxysporum* and *A.alternata*. At the same time the extracts showed significant antifungal activity at except 3 ppm against *A.alternata* growth(Figure 1).

Table 3. Effect of *H. origanifolium* Plant Extracts on the Mycelial Growth of Fungi

Plant extract	Concentrations (ppm)	*Fusarium oxysporum*		*Alternaria alternata*	
		Average diameter of fungi ± SE	Mycelial growth inhibition (%)	Average diameter of fungi ± SE	Mycelial growth inhibition (%)
Hypericum origanifolium	3	6.80±0.11^{a}	5.02	6.00±0.05^{a}	4.18
	15	6.63±0.83^{ab}	7.4	5.26±0.14^{b}	26.5
	45	5.83±0.16^{c}	18.5	3.10±0.05^{c}	56.7
	80	5.56±0.06^{c}	22.3	2.86±0.08^{cd}	60
	120	4.93±0.06^{d}	31.1	2.57±0.12^{de}	64.1
	200	2.76±0.53^{e}	61.4	2.53±0.03^{ef}	64,6
	240	2.20±0.05^{e}	69.2	2.23±0.14^{f}	68.8
	300	0.00±0.00^{f}	100	0.00±0.00^{g}	100
Control	Ethanol %30	7.16±0.37a	-	6.30±0.11^{a}	-
Positive Control	1 µg/mL	6.1±0.05bc	14.8	6.23±0.14^{a}	1.1

*Values are means of three replicates, Mean values(mean±standard error) sharing the same letter do not differ significantly by Duncan's Multiple Range Test at $p \leq 0.05$.

According to these findings, *Hypericum* ssp. of extracts showed antifungal activities against different plant pathogens. This difference may be because the different plant species were defined bioactive compounds at different rates. *Hypericum origanifolium* plants extract toxicity against the fungi *F. oxysporum* (inhibitory concentration (IC_{50})= 159.93 µg/mL) and *A. alternata* (IC_{50})=55.759 µg/mL) were calculated use of probit analysis.

All doses of *Hypericum* ssp. leaf extract when the comparison with negative control and positive control was shown antifungal effect on fungi. According to the value of the IC_{50}, generally the *Alternaria alternata* was found susceptible pathogen against *Hypericum* ssp. leaf extract according to *Fusarium oxysporum*. Generally, leaves ethanol extracts of *Hypericum* ssp. were found to potent of *in vitro* antifungal activity against tested fungi. In antifungal tests, however, *Hypericum* ssp. extracts have demonstrated better antifungal activity than the chemical fungicide in our study.

IC50 values of the three selected plant Hypericum ssp. leaf extract against pathogenic fungus F.oxysporum and A. alternata were determined with of probit analysis. In terms of the IC50 values, the antifungal activity of H. perforatum plant exract against F.oxysporum (122.525 µg/mL) > A.alternata (108.484 µg/mL). On the other hand, IC50 values of H. scabrum against A.alternata (126.390 µg/mL) > F.oxysporum (113.714 µg/mL). Moreover, the IC50 value of H. origanifolium against two pathogenic fungi was calculated as F.oxysporum (159.931 µg/mL) > A.alternata (55.759 µg/mL).

Results showed that H.origanifolium has the best fungicidal activity, with IC50 value of 55.759 against A.alternata and H.scabrum 113.717 µg/mL against F.oxysporum.

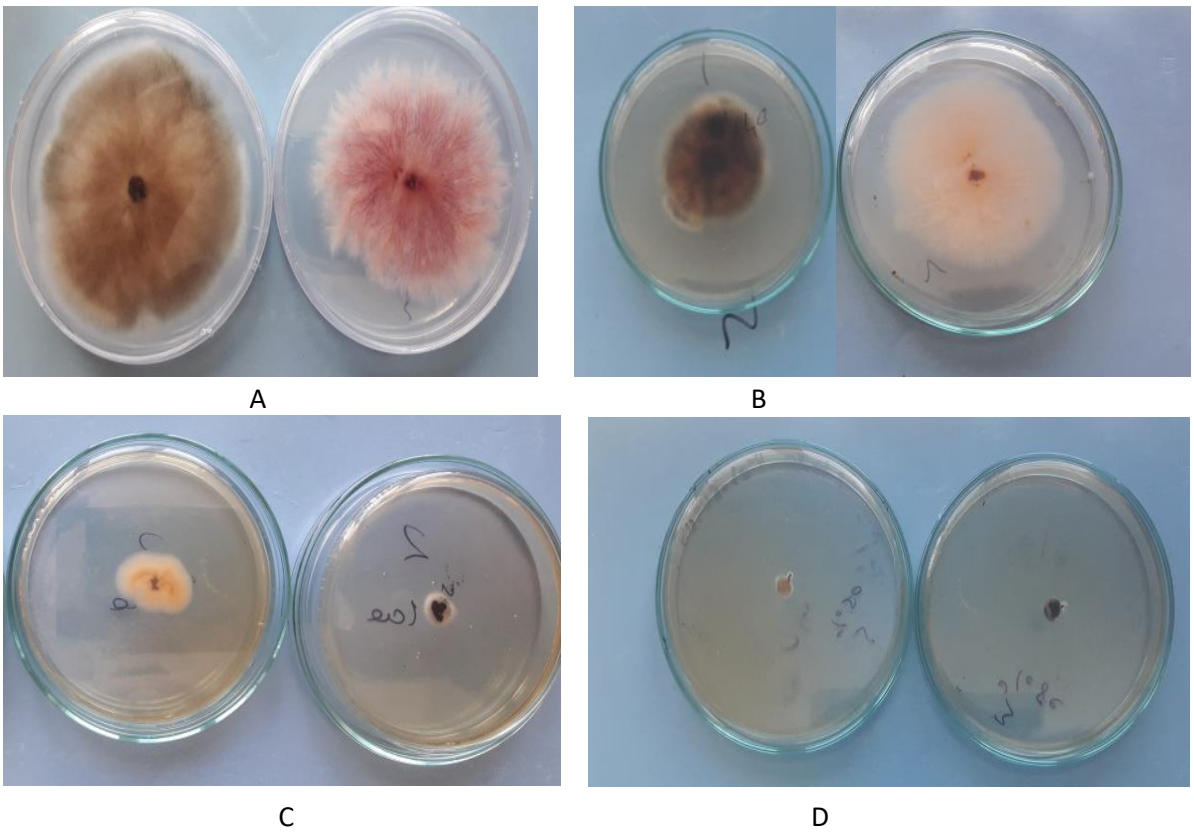

Figure 1. Antifungal Effect of *Hypericum Origanifolium* Plant Extracts on the Mycelial Growth of *F.oxysporum* and *A.alternata*(A:Control, B:120 ppm dose of *H. origanifolium*, C: 240 ppm dose of *H. origanifolium*, D: 300 ppm dose of *H. origanifolium*)

Extraction of natural compounds from plants and the used extraction method are critical. It is the first step to determine the optimum extraction conditions to benefit from the determined activity and bioactive compounds. In this study, ultrasonic wave assisted extraction method was used as the extraction method. Ultrasonic wave-assisted extraction conditions of *Hypericum* species were carried out according to previous studies (Seyrekoğlu & Temiz, 2020). Extraction conditions were applied using an ethanol-water (3:7) solvent system at 30 °C for 40 min. keeping the material/liquor (M:L) ratio of 1:30. After the extraction, the mixtures were filtered on a Whatman filter paper no. 42 (125mm). The solvent was evaporated under reduced pressure in a rotary evaporator. All operations were done in triplicate. At the end of the ultrasound assisted of *H. perforatum*, *H. scabrum* and *H. origanifolium* extraction yields of 9.3 %, 5.3% and 9.3 % (w/w) were obtained respectively.In recent studies, ultrasonic wave assisted method is generally used as the extraction method. Similarly, Estevinho et al. (2021), used to ultrasonic wave assisted extraction method for the extraction of *Hypericum perforatum* L. Then 80 % ethanolic solution and only deionized water used as solvent system. The extraction of avocado using ultrasonic waves gave the better results than the maceration extraction (Arlene et al., 2015). In another study, is to produce an edible saffron extract with high quality factors (crocin, picrocrocin, and safranal) using ultrasonic wave-assisted extraction (Gazeran et al., 2016). Additionally, Ultrasonic Assisted Extraction (UAE) is a simpler, faster and more effective extraction technique than other methods. It consumes less energy, time and solvent. Thus, it has the feature of being a fast technique that produces more efficient and purer products (Syahir et al., 2020).

In this study, the exracts of the *Hypericum* ssp. which is widely grown in Turkey and collected from Amasya province, were obtained. Additionally, the antifungal acivities of different extracts of leaf parts of the plant were determined against pathogenic fungi. Previous studies have demonstrated that the genus of *Hypericum* ssp. from varied areas have varying compounds (Heshmati et al., 2018). In many researchs, it has been noticed that α-pinene and β-pinene were the main components (Khorshidi et al., 2020). Khorshidi et al. (2020) reported that, α-pinene, β-pinene, limonene, and E-caryophyllene were determined as the main compounds of *H. scabrum*. In another study, α-pinene (46.3 %) was found as the important compounds in the essential oil obtained from the flowers of the plant (Heshmati et al., 2018). In a study by Morshedloo et al. (2015) were investigated the chemical composition of *H. perforatum*. Among chemicals, 2,6-dimethyl-heptane (6.25–36.07%), ¿-pinene (5.56–26.03%), ı-cadinene (0.0–22.58%) and -cadinene (0.0–16.9%) were found as the most abundant compounds. Bertoli et al.(2015) were identified as Caryophyllene oxide the main compound of *H.origanifolium* from North of Turkey (Samsun province).This difference in bioactive compounds may be due to the fact that the same plant species was grown in different regions. Similar to our conclusions, in the previous research, plant extracts were detected to be efficient against the microorganism (Carvalho et al., 2011). The antifungal and antibacterial (Castillo et al., 2012 ; Aljubiri et al., 2021) and antiviral (Denaro et al., 2020 ; Lowe et al., 2021) activities of bioactive compounds of plants were reported from many research. In the similar studies, different research stated that the extracts of plants play a major role in controlling phytopathogenic fungi. Chahal et al. (2021) reported that extracts of *Ageratum conyzoides* L. exhibited total inhibitory effects on the mycelial growth of *Aspergillus, Alternaria, Candida, Fusarium, Phytophthora* and *Pythium* owing to the presence of various secondary metabolites. Similar studies have been carried out by Amin et al. (2022) on the antifungal activity of *Alternanthera philoxeroides* (Mart). Griseb] against *Alternaria alternata* (Fr.) Keissl., *Aspergillus flavus* Link, *Aspergillus niger* Tiegh. and *Macrophomina phaseolina* (Tassi) different fungi and they reported that crude leaf extract of *Alternanthera philoxeroides* inhibited the mycelial growth of the test fungi. Riaz et al. (2010) similarly, in our study, methanol extracts of the plant parts of *Hypericum* ssp. were found to be most effective against *Fusarium oxysporum* and *Alternaria alternata*. Similar to our results, previous reports confirmed the IC50 rates varied depending on the fungus and different plant species. Furthermore, varied plant extracts are against the pathogenicity of different fungi because secondary plant metabolites have a marked potential as a resource of effective antifungal agents (Carvalho et al., 2011). These conclusions were found to be compatible with the literature.

CONCLUSION

The leaves ethanol extract of *Hypericum* ssp. it was showed antifungal activity against *A. alternata* and *F.oxysporum*. The present study indicated that the *F.oxysporum* according to *A. alternata* is resistant against plant extract. These remarkable results form the source for future researchers proposed at a better understanding of the bioactivity of *Hypericum* ssp.plant extracts.

Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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