Biological Effectiveness of Plant Polyphenols under Greenhouse Conditions, Against Wilt and Root Rot Complex of Chickpea (*Cicer arietinum L.)*

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ABSTRACT

Chickpea (*Cicer arietinum* L.) production is constantly compromised by a complex of pathogens which cause wilt and root rot (WRR). Therefore, biological control and organic products have regained great importance in the last few years. In this work, polyphenols were obtained from ethanolic extracts through the ultrasound-microwave assisted technique from the plant species chinese privet leaves (Ligustrum lucidum) and moringa leaves (Moringa oleifera). A qualitative analysis through reverse phase high-performance liquid chromatography electrospray ionization mass spectrometry (RP-HPLC-ESI-MS) was conducted, so that their biological effectiveness under greenhouse conditions was determined by each group of polyphenols against *Fusarium oxysporum* f. sp. *ciceris*, *Fusarium solani*, and *Macrophomina phaseolina* which comprise the wilt and root rot complex. A complete randomized block design was established with three blocks and five treatments with nine replications each. Treatments were: Polyphenols of *Ligustrum lucidum*, polyphenols of *Moringa oleifera*, the fungicide Benomyl, the inoculated check, and the untreated check. The analysis of variance was performed and mean comparison with Duncan`s multiple range test (0.05). The results indicate that all groups of polyphenol had in their chemical composition, some compounds of known microbial activity, such as hydroxycinnamic acid, flavones, anthocyanins, catechins, and alkyphenols. Under greenhouse conditions, plants that had the lowest incidence and severity of the disease, were those treated with polyphenols from *L. lucidum* with 66% incidence, and were statistically different to the rest of the treatments.

Keywords: *Chickpea, Cicer arietinum, Wilt, Root rot, Polyphenols, Biological control, Phytopathogens.*

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Highlights of this paper

- In this work, polyphenols were obtained from ethanolic extracts through the ultrasound-microwave assisted technique from the plant species chinese privet leaves (Ligustrum lucidum) and moringa leaves (Moringa oleifera).
- A complete randomized block design was established with three blocks and five treatments with nine replications each.

1. INTRODUCTION

The area grown with chickpea (*Cicer arietinum* L.) in Mexico during the year 2016 was 66,096 ha with a production of 116,076 t. The states of Sinaloa and Sonora in the northwestern part of the country, were the main national chickpea producers providing 45 and 30%, respectively [1]. Vascular wilt caused by the fungus *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *ciceris* (Padwick) Matuo and K. Sato, is one of the most important diseases that affect chickpea. This disease is highly destructive and occurs worldwide [2]. Annual yield losses may range from 10 to 90%, but the disease could reach 100% [2] when the crop is exposed to adverse conditions, such as heat or drought stress during the reproductive stage and development of pods [3]; in Spain, it may range between 12 to 15% [4]. It is suggested that Fusarium vascular wilt includes a complex of phytopathogens similar to the wilt and root rot (WRR), that severily affects chickpea seed production. Several fungi have been reported as causal agents of the WRR on chickpea, such as *Fusarium*, *Rhizoctonia*, *Macrophomina*, and *Sclerotium* [5]. Some of the strategies used for disease management are the cultivation of resistant cultivars, crop rotation, solarization, destruction of plant debris, and use of seed-free of pathogens or treated with fungicides, although the results have been limited [6]. Control with synthetic chemicals is the method most widely used for management of diseases caused by Fusarium species; however, the use of biological products has become more important in the last few years [7]. Many investigations focus on studies about natural product formulations that have some type of biological activity against phytopathogens, with greater efficacy and and faster action. This has generated more efficient practices in a wider range of environmental conditions, pest species, and crop systems [8]. Therefore, the objective of this work was to evaluate the biological effectiveness of two groups of polyphenols obtained through ethanolic extracts, against fungi associated to wilt and root rot of chickpea under greenhouse conditions.

2. MATERIALS AND METHODS

Plant materials used for elaboration of polyphenols were obtained from leaves of *Ligustrum lucidum* W.T. Aiton and *Moringa oleifera* Lam., which were dehydrated by placing them on brown paper during two weeks at 28-30°C in the laboratory; then, they were ground up in a blender in a 150 µm Tyler RO-TAP RX-29 sieve shaker. The amount of leaves was sufficient to make up 500 g of pulverized material for each plant species. Then, the products were placed in the ultrasonic microwave reaction system XO-SM100 (Nanjing ATPIO Instrumens Manufacture Co., Ltd Company), with the amount obtained from the relationship shown in Table 1, under the following conditions: Ultrasonic (VS): power radio 20, ultrasonic on relay 10, ultrasonic off relay 3, amplitude off relay 25 and set time 20. Microwave (MV): Power radio 800, display power 0, set temp 70°C, and holding time 5. After the ultrasound-microwave process, samples were stored in an ultrafreezer at -70°C, then, a column chromatography was carried out following the methodology described by De Asmundis, et al. [9].

Table-1. Description of the relationship from the assisted extraction through ultrasound-microwave of two plant species.

Source	Ethanol	mL:1 g of sample	Relationship
Ligustrum lucidum	70% (high)	16 (high)	high x high
Moringa oleifera	70% (high)	16 (high)	high x high

The ethanolic fraction obtained was divided in glass containers and dried in an oven at 60°C, without exposure to light during 24 to 48 h. The polyphenols were recovered by scrapping them with a spatula: 2,150 mg of *L. lucidum* and 2,400 mg of *M. oleifera*. Then, they were stored in amber jars in darkness at 15-23°C. After 8 days, the polyphenols obtained were subjected to a high resolution qualitative analysis through reverse phase high-performance liquid chromatography electrospray ionization mass spectrometry (RP-HPLC-ESI-MS), according to the methodology of Ascacio-Valdés, et al. [10]. Treatments with polyphenols were prepared as follows: for *L. lucidum* 340 mg of the extract were dissolved in 400 mL of ethanol and water (8:2 v/v), while for *M. oleifera* 430 mg of the extract were dissolved with the same proportion of ethanol/water. The solutions were maintained during 24 h in a rotary shaker and then kept at 14°C until their use. The fungicide treatment consisted of one gram of Benomyl 50 WP (PROMYL) (methyl *N*-[1-(butylcarbamoyl)benzimidazol-2-yl]carbamate) which was dissolved in 1 L of water.

Strains of phytopathogens used in the evaluation. The strains used were isolated from chickpea plants with symptoms of wilt and yellowing Figure 1, collected at the Norman E. Borlaug Experimental Station in the Yaqui Valley, Sonora, Mexico (27°29.185' N, -109°56.45' W). Small root pieces with disease symptoms were disinfected with a 3% sodium hypochlorite solution, rinsed with sterile distilled water, placed in Petri plates containing potato-dextrose-agar (PDA) culture medium, and incubated at 28°C during three days. The isolation was performed by hyphal tip and the molecular identification through the polymerase chain reaction (PCR) and random amplification of polymorphic DNA, following the methodologies described by Babu, et al. [11]; Arif, et al. [12] and Jiménez-Gasco, et al. [13]. Fungal isolates were grown in 50 mL tubes with PDA at 28 ± 2°C, and once they covered most of the medium, sterile mineral oil was added in order to maintain the cultures in darkness until their use. They also were grown and maintained on PDA in sterile Petri plates in a refrigerator.

Figure-1. Chickpea plants with symptoms of wilt and root rot collected at the Norman E. Borlaug Experimental Station, in the Yaqui Valley, Sonora, Mexico.

Greenhouse trial. The greenhouse trial was established during the fall-winter 2019-2020 at the Autonomous Agrarian University Antonio Narro, located at 25º 37´N, 101°03´32¨ W, 1,786 masl, with subtropical and arid climate. Initially, four chickpea seeds per replication of cultivar Blanoro [14] were germinated in 1 L styrofoam cups with peat moss in the greenhouse at 18-29°C. Polyphenols from treatments 1 and 2 were applied with Lion

Tools® 2 L sprayer just before transplanting; three sprays were applied to the orifice made for seedling placement. After 15 and 30 days, applications were directed to the base of the stem three times in each occasion. Benomyl was also applied in the same fashion already described for treatments 1 and 2. Seedlings from treatments 1 to 4 were inoculated before transplanting by making a 2 cm incision in the roots, then, they were placed in a micro and macroconidial suspension of *F. oxysporum* f. sp. *ciceris* and *F. solani* (Mart.) Sacc. at a concentration of $1x10^8$ during 3 min. For the inoculation with *Macrophomina phaseolina* (Tassi) Goidanich, six sorghum grains previously inoculated in the laboratory following the methodology described by Pineda, et al. [15], were used for each seedling; they were placed close to the seedling roots. By the time of inoculations, fungal isolates had been stored in PDA-mineral oil and in Petri dishes with PDA for 6 months. After 25 days of emergence and seedlings were 10 cm tall, they were transplanted to black plastic bags No. 4 (volume 6 L) with 4 kg of substrate, which was 60% soil and 40% coconut fiber. The substrate was previously pasteurized at 80°C during 10 min, repeating the process three times. A total of 45 seedlings were used for the different treatments; therefore, a single seedling represented a replication. A complete randomized block design was established with three blocks and five treatments (T1: polyphenols of *Ligustrum lucidum*, T2: polyphenols of *Moringa oleifera*, T3: benomyl, T4: inoculated check, and T5: untreated check) Table 2, with fifteen replications in each block. The analysis of variance was carried out and the mean comparison with Duncan`s multiple range test (0.05). Disease incidence and severity were evaluated following the scale proposed by Carrillo [16] Table 3, and for root severity the scale proposed by Camargo [17] Table 4.

Table-2. Evaluation of several treatments to determine the antifungal effect on wilt and root rot complex of chickpea, under greenhouse conditions.

Treatments	Concentration (mg)[y]
T1 Polyphenol of *Ligustrum lucidum*	340
T2 Polyphenol of *Moringa oleifera*	430
T3 Fungicide Benomyl	1 g c.p./L water[z]
T4 Inoculated check	-
T5 Untreated check	-

Note:
[y]In 400 mL of ethanol/water 8:2 v/v.
[z]c.p. = commercial product.

Table-3. Rating scales for disease incidence and severity of wilt and root rot complex in chickpea.

	Rating	Description	Evaluation (%)
Incidence	0 - 100	Percentage of affected plants	0 - 100
Severity	0	Healthy plant	0
	1	Chlorotic plant	25
	2	Withered plant	50
	3	Infected plant and symptoms on foliage	75
	4	Dead plant	100

Table-4. Rating scale for severity on roots caused by the wilt and root rot complex of chickpea.

Rating scale	Description	Root system damaged (%)
0	Healthy roots	0
1	Root system damaged	1 – 10
2	"	11 – 15
3	"	16 – 30
4	"	31 – 50
5	Complete necrosis of the root system	51 – 75

3. RESULTS AND DISCUSSION

Different phytochemicals were identified through liquid chromatography (HPLC-MS) Table 5. The extract from *Moringa oleifera* showed flavones, anthocyanins, hydroxycinnamic acids, and curcuminoids; in the case of the

extract of *Ligustrum lucidum* flavones, alkyphenols, and hydroxycinnamic acids. The results obtained coincide with those mentioned by Saravanakumar, et al. [18], who reported that the phenolyc type compounds such as coumarins, lignins, flavonoids, and tannins are present in the defense plant system via tissue or cell wall modification, providing hardiness or rigidity. Based on the identification of polyphenols from *L. lucidum*, it is considered that they have antifungal activity by the results obtained, as well as those from *M. oleifera*. It is known that phenolic compounds have protective functions against pathogenic microorganisms [19], and that they are necessary for plant survival under stress conditions imposed by biotic and abiotic factors [20]. There are reports about the use of phenolic compounds as inhibitors of diverse pathogens, like Mendoza, et al. [21] who reported the potential of these compounds against *Botrytis cinerea* Pers. ex Fr. Therefore, the diminish of the disease observed in our study with polyphenos by the diverse treatments applied, could have been due to the presence of metabolites, such as flavonoids which have a wide range of biological activity [22]. There have been quite a number of investigations about the efficacy of plant metabolites affecting fungal growth and development. [23] found that flavonoids of *Sophora flavescens* Aiton at concentrations of 0.25, 0.5, 1, 2, and 4 g/L restrained the mycelial growth of *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *cucumerinum* J.H. Owen, and stimulative rates were enhanced with the increased concentration.

Table-5. Groups of polyphenols detected from plant extracts through high-performance liquid chromatography mass spectrometry.

Ethanolic extract	Mass	Time of retention	Compound	Family
Moringa oleifera 1:16 Ethanol 70%	304.2	18.67	1-caffeoylquinic acid	Hydroxycinnamic acids
	304.1	19.22	3-caffeoylquinic acid	Hydroxycinnamic acids
	341.0	2.28	Tartaric acid p-coumaroil	
	564.2	25.48	Quercetin 3-O-galactoside	Hydroxycinnamic acids
	310.0	32.82	Quercetin 3-O-glucoside	Flavonol
	310.0	32.82	Quercetin 4`-O-glucoside	Flavonol
	755.0	35.29	Peonidin 3-O-(6"-acetyl-galactoside)	Flavonol
	755.0	36.50	Peonidin 3-O-(6"-acetyl-glucoside)	Anthocyanins
	252.8	28.43	Delphinidin 3-O-galactoside	Anthocyanins
	540.1	51.35	Bisdemethoxycurcumin	Curcuminoids
Ligustrum lucidum 1:16 Ethanol 70%	592.8	30.55	Apigenin 6,8-di-C-glucoside	Flavones
	310.9	32.95	Cefeoyl tartaric acid	Hydroxycinnamic acids
	755.0	34.67	Quercetin 3-O-rhamnosyl-rhamnosyl-glucoside	Flavones
	346.9	39.28	5-Heptadecylresorcinol	Alkyphenols

With a 4 g/L concentration of flavonoids, the inhibition rate was 93.08%. In the therapeutic trail, plants have the biggest promotion by 60 g/L of flavonoids; the results indicated that chlorophyll content, root activity, superoxide dismutase (SOD) activity, peroxidase (POD) activity and phenylalanine ammonia-lyase (PAL) activity were increased by 17.79%, 71.55%, 48.20%, 123.80%, and 151.48%, respectively, in comparison with the control. The results of the prevention trail indicated that flavonoids of *Sophora flavescens* enhanced disease resistance of

cucumber at all the concentrations, and the best effect occurred with 80 g/L. Plant extracts and essential oils of each plant might have more than sixty compounds, and some of them could have antifungal properties. Generally, they are present in mixtures, and pathogens might be affected differentially, and by individual or mixtures at certain concentrations and proportions Montes-Belmont [24]. Tenorio, et al. [25] reported that the biocontrol activity of extracts from *Caiphora andina* Urb. and Gilg and a saponin isolated from the skin of *Chenopodium quinoa* Wild., caused an inhibition of 35% and 36%, respectively, on the fungus *Ulocladium* spp. *Ch. quinoa* showed an inhibition of 42% on *Aspergillus flavus* Link: Fr. Wang, et al. [26] indicated that the methanol extract of *Ficus sarmentosa* var. *henryi* (King) Corner has potent inhibitory activities against *Fusarium graminearum* Schwabe, *Curvularia lunata* (Wakker) Boed, *Septoria zeicola* Sout, *Botrytis cinerea* Pers.:Fr., and *Rhizoctonia solani* Kühn. Four flavonoids were isolated from this plant: eriodictyol, homoeriodictyol, dihydroquercetin, and luteolin. Contreras-Arredondo, et al. [27] found hydrolyzable and condensed tannins as active ingredients against *Fusarium oxysporum* in potato (*Solanum tuberosum* L.), which were obtained from ethanolic extracts of antelope bush flower (*Cowania plicata* D. Don.); the results indicated that the lowest inhibitory concentration 50 was 3,000 ppm, and the highest 90 was 28,000 ppm on the fungus. Murillo-Arango, et al. [28] reported that citronelal and geraniol essential oil from *Eucalyptus tereticornis* Smith had a funcididal effect on *Fusarium oxysporum* at 3 g/L concentration and a fungicidal activity at small concentrations. Two extracts obtained from *Acacia farnesiana* (L.) Willd., one hydroalcoholic and the other one aqueous, were evaluated on the fungus *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen, in terms of percentage of mycelial growth inhibition and a qualitative chemical analysis was performed; both extracts showed more than 90% mycelial growth inhibition in the first evaluation (72 hours) after the inoculation. Both extracts showed the presence of metabolites which are known to have antimicrobial activity, such as flavonoids, tannins, phenols, alkaloids, and saponins [29]. Extracts from *Amaranthus spinosus* L. caused lysis of *Phakopsora pachyrhizi* Sydow and Sydow; this plant species contains alkaloids, flavonoids, tannins, saponins, and terpenoids which can be used as botanical fungicide to inhibit spore germination Yusnawan [30]. Guerrero, et al. [31] reported that extracts from *Amaranthus viridis L.*, *Cyperus odoratus L.*, *Euphorbia hirta L.*, *Tagetes minuta L.*, *Scoparia dulcis L.*, and *Portulaca oleracea L.* affected somewhat the development of *Theobroma cacao* L. pathogens: *Moniliophthora roreri* (Cif.) H.C. Evans, Stalpers, Samson and Benny, and *Phytophthora palmivora* (Butl.) Butl. *in vitro* on all the variables studied; however, the effect of *A. viridis* and *E. hirta was noticeably superior to the other species.*

Seedlings with the lowest incidence of wilt and root rot were those treated with polyphenols from *L. lucidum* which had 66.66% Figure 2. The analysis of variance showed significant differences between polyphenols from *L. lucidum* and the rest of the treatments. The coefficient of variation was 19.1%. Incidence of WRR in seedlings treated with polyphenols from *M. oleifera* had 88.88%, and were statistically similar to the synthetic chemical check and the inoculated check. These two last treatments had a disease incidence of 100%, while in the untreated check no presence of the disease was detected. Disease severity in seedlings treated with polyphenols obtained from *L. lucidum* showed a range of 0.0 to 4.0 with an average of 1.44, while those from *M. oleifera* had a range of 0.0 to 4.0 with an average of 2.33, being statistically greater and different from the severity shown with the benomyl treatment which was not able to provide any control the disease Figure 3. Benomyl and the inoculated check had a disease incidence value of 5.0, while the untreated check had 0. The coefficient of variation was 11.1%. Significant differences were detected between treatments in root rot severity Figure 4; polyphenols obtained from *L. lucidum* showed the lowest severity with a range of 0.8 to 1.4 with an average of 1.1, polyphenols obtained from *M. oleifera* had a range of 1.6 to 2.2 with an average of 1.86, the benomyl treatment had a range of 3.7 to 5.0, in comparison with the inoculated check which showed a severity value of 5.0.

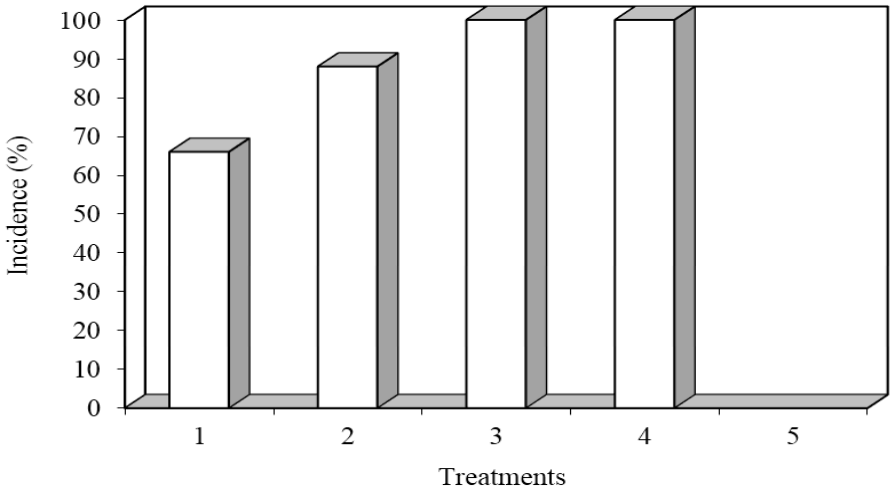

Figure-2. Incidence of wilt and root rot of chickpea seedlings inoculated under greenhouse conditions. Treatments: 1= *Ligustrum lucidum*, 2= *Moringa oleifera*, 3= Benomyl, 4= Inoculated check, 5= Untreated check.

Figure-3. Disease severity in chickpea seedings inoculated with *Fusarium oxysporum* f. sp. *ciceris*, *Fusarium solani*, and *Macrophomina phaseolina*, causal agents of wilt and root rot, after treatment with plant polyphenols.

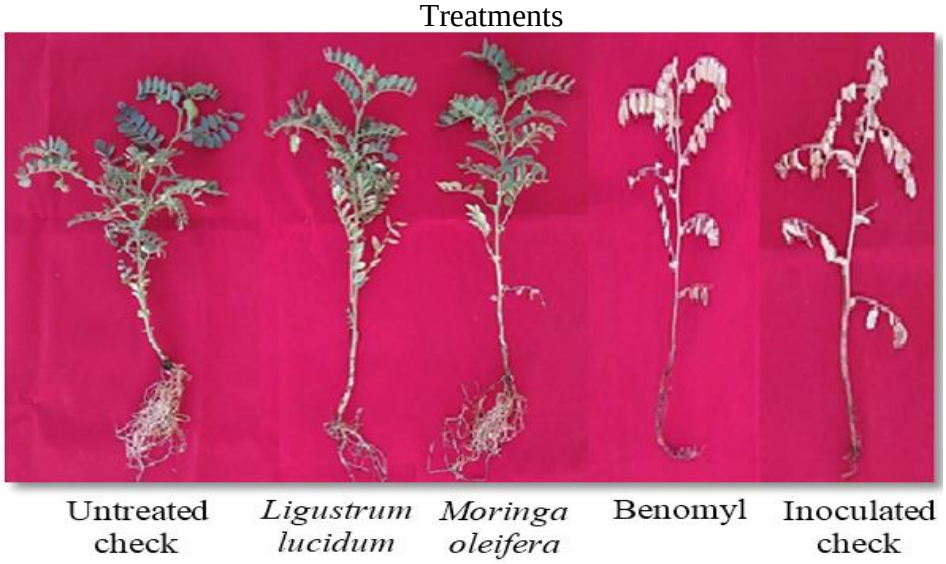

Figure-4. Disease severity in chickpea roots inoculated with *Fusarium oxysporum* f. sp. *ciceris*, *Fusarium solani*, and *Macrophomina phaseolina*, causal agents of wilt and root rot, after treatment with plant polyphenols, under greenhouse conditions.

In this study, development of the disease was high based on the severity values shown by the inoculated check and the untreated check, which had a range of 0.0 to 0.9 with an average of 0.24. The tolerance and resistance to benomyl by other phytopathogenic fungi has been reported previously, like *Botrytis cinerea* whose resistance was first reported in Holland in 1970 in isolates of ciclamen (*Cyclamen* sp. L.), after the product had been used for two years. Later, it was detected in isolates from rose (*Rosa* sp. L.) in Canada, then in Pennsylvania, USA on rose, geranium (*Pelargonium* X *hortorum* L.H. Bailey), begonia (*Begonia* X *tuberhybrida* Voss), petunia (*Petunia* sp. Juss.), cyclamen, and fuchsia (*Fuchsia hybrida* Voss) Bollen and Scholten [32]; Watson and Koons [33]; Miller and Fletcher [34]; Jarvis and Slingsby [35]; Bolton [36]; Moorman and Lease [37]. Coley-Smith [38] reported that the development of resistance to fungicides by *B. cinerea* is very important in the control of diseases that this fungus causes, primarily when benzimidazols have been used. Fungicides have been used for about two hundred years to protect plants from fungal diseases [39]. Despite the progress of chemical control against fungal diseases, the phenomenon of resistance by phytopathogens to the phytosanitary products constitutes a problem that worries the sector from the agronomic point of view. Systemic fungicides is a group of chemicals that have frequently presented such a problem; numerous cases of pathogen resistance in the field have been reported, which provoques a maladjustment in the population equilibrium from the ecological point of view. In many European, Asian, and Latinoamerican countries where this type of fungicides are used regularly, research studies are carried out with the objective to detect on time, the possible appearance of resistance in order to avoid production losses in crops of economic importance. The first research related to this topic in Cuba, was carried out when resistance to benomyl was detected in *Mycosphaerella musicola* J.L. Mulder in J.L. Mulder and Stover on banana [40]. The objective was to reduce the fungus population in order to minimize the development of resistance and to reduce the number of chemical applications. The solutions developed in Cuba since the 80´refer to the implementation of integrated management programs, which include genetic methods, biological, and cultural, combined with the application of fungicides, so that they offer a satisfactory option to reduce the selection pressure towards resistance, as a contribution to the development of sustainable agriculture. Manzo Sánchez, et al. [41] reported that according to sensitive analysis towards fungicides, ten isolates of *Mycosphaerella fijiensis* Morelet, causal agent of black sigatoka in banana, presented loss of sensitivity to benomyl, seven to propiconazol, and nine to azoxystrobin. The isolates were able to grow to concentrations that indicate the loss of sensitivity. Regarding the fungus *Venturia inaequalis* (Cook) Wint., the causal agent of apple scab, the preventive and curative activity of benzimidazols allows the scheduled application of benomyl, at 10 to 14 day intervals; this frequency has caused the appearance of resistant isolates of this fungus to the fungicide after three to four years of use in other places [42]. Research carried out in the United States, Germany, France, and Israel show that the resistance of *V. inaequalis* to benomyl is due the mutation of a single mendelian gene, and that the levels of resistance may vary depending upon the isolates [43-46]. In Mexico, the evaluation of fungicides for control of *V. inaequalis* in the Lirios region of Arteaga county in the state of Coahuila, provides evidence of inadequate protection when benzimidazol products are used, since it was observed a 26% incidence of damaged fruits, and that protective fungicides like mancozeb and captan only recorded 8% of fruits damaged [47]. For the treatment of diseases caused by *Fusarium* spp. and other fungi, systemic fungicides like benzimidazols are used, which include benomyl, carbendazim, thiabendazol, and thiophanate; however, it is probable that these fungicides also act as mutagenic agents in plants, and they might cause an increment in the level of resistance of pathogens Agrios [48]. Dane and Dalgiç [49] demonstrated the genotoxic effect of benomyl in onion roots (*Allium cepa* L.) when treated with different rates of this fungicide; anomalies were observed in the interphase and mitotic divisions of meristematic cells. These anomalies were due to the deffect of the mitotic spindle and karyokinesis without cytokinesis. Negative effects were also observed on the chromatic such as condensation and

noncondensation, and some abnormal vacuoles in the interphase. Other fungi have been studied to elucidate at the molecular level, the genes or mutations that trigger the resistance to benzimidazol compounds [50-58].

4. CONCLUSIONS

Different phytochemicals obtained from *Moringa oleifera* and *Ligustrum lucidum* were identified through liquid chromatography (HPLC-MS). The extract from *M. oleifera* showed flavones, anthocyanins, hydroxycinnamic acids, and curcuminoids; in the case of the extract from *L. lucidum* flavones, alkyphenols, and hydroxycinnamic acids. Polyphenols from these plant species reduced the incidence and severity of the Fusarium wilt and root rot of chickpea, and enhanced better plant development. The results obtained with polyphenos from *L. lucidum* are the first reported for control of a plant disease.

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