

Use of *Ruta graveolens* L. Vegetable Extract Standardised by Furanocoumarin Content to Control *Magnaporthe oryzae* in Rice Plants

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Abstract: Blast, caused by the fungus *Magnaporthe oryzae*, is one of the most widespread diseases affecting the rice crop. The use of fungicides to combat phyto pathogenic fungi causes great environmental problems. Plant extracts have been studied as an alternative method of plant pathogen control. *Ruta graveolens* has compounds with antifungal activity, such as furanocoumarins. The objective of this study was to test the effects of various concentrations of the standardised plant extract of *R. graveolens* as well as its fractions and furanocoumarins (psoralen and bergapten) on reducing *M. oryzae* mycelial growth. The aerial part of *R. graveolens* was grinded and subjected to a characterisation process, percolation, concentration, and furanocoumarin standardisation. The standardised extract was fractionated and the fractions were subjected to high-performance liquid chromatography (HPLC) to quantify furanocoumarins. The effects of the extract, fractions, and standards against mycelial growth of *M. oryzae* were tested *in vitro*. The plant material was handled in accordance with the guidelines established by the general methods of the Brazilian Pharmacopoeia. The furanocoumarin content was the highest in the ethyl acetate fraction. The best results for the mycelial growth test were observed using the extract and the hexane and ethyl acetate fractions. The standardised herbal extract of *R. graveolens* showed antifungal activity against *M. oryzae*.

Keywords: alternative control, plant pathogens, percolation, *Oryza sativa*, antifungal activity

1. INTRODUCTION

Rice is one of the most important grains in terms of economic and social value, and is the staple food for approximately 2.4 billion people, providing 20% of the energy and 15% of the protein consumed by humans [1]. According to the National Supply Company (Conab), grain production in Brazil is expected to exceed 12 million tons in 2015, and it is estimated that its production (600 million tonnes in 2000) will increase by 40% by 2030 [2].

According to the Food and Agriculture Organization, diseases, insects, and weeds are responsible for rice yield losses of up to 25% [2]. Among the diseases that damage rice crops, blast caused by the ascomycete *Magnaporthe oryzae* is the most widespread in all regions of Brazil and has been found in more than 85 countries [3]. This disease can lead to grain yield losses of up to 100% by direct or indirect action [4]. Owing to the great economic importance of rice, *M. Oryzae* pathosystem, rice has been widely analysed at the molecular level, making this fungus a model organism for the investigation of plant diseases caused by fungi [5]. Disease control is carried out through integrated management measures consisting of genetically resistant cultivars, cultural practices, and chemical control via fungicide application. However, these measures do not totally ensure the reduction of the disease owing to various circumstances, such as extreme aggression of blast under favourable weather conditions associated with a high degree of susceptibility to the disease in some cultivars [6].

Fungicides that are used belong to three chemical groups, triazoles, strobilurins, and benzothiazoles, and tricyclazole (belonging to the class of benzothiazoles) is used most frequently. However, these methods are inefficient owing to the high genetic variability in pathogen resistance [7-8]. Another problem associated with the use of fungicides is their high toxicity when used in large quantities to ensure greater control, thereby causing harm to humans and the environment [9].

Alternative methods have been studied to control this disease. For example, the use of herbal extracts has been examined since most agents contain fungistatic and/or fungicides, which act against the

growth of pathogens [10]. However, plant secondary metabolites are influenced by various factors, including climatic and agronomic factors; accordingly, it is necessary to standardise extracts to develop products that have the quality required by the agricultural market [11].

Ruta graveolens is a plant in the Rutaceae family. It has the potential to produce furanocoumarins, which are phytoalexins that may be part of the normal constitution of the species and play a role in antifungal and antimicrobial activity [12-13]. The efficacy of the *R. Graveolens* extract in the control of pathogens has already been demonstrated in the literature, but the importance of furanocoumarins in the extract has not been studied. The objective of this study was to test the effect of various concentrations of the standardised plant extract of *R. graveolens* as well as its fractions and furanocoumarins (psoralen and bergapten) on reducing mycelial growth of *M. oryzae*.

2. MATERIALS AND METHODS

2.1. Plant Material and Processing

The dried aerial part of *R. graveolens* was obtained from a company in Goiania, Brazil. Samples were subjected to a trituration process using a Tecnal 625 Knife Mill Croton type (Piracicaba, Brazil) and the powder was characterised based on quality control analyses established in the Brazilian Pharmacopoeia, 5th ed [14]. The plant extract was obtained by the percolation extraction method using a water and alcohol solution (80%) as a solvent, and concentrated on a rota evaporator R22 IF (BUCHI). The plant extract was standardised by means of the identification of linear furanocoumarins (psoralen and bergapten) using thinlayer chromatography (TLC) and high-performance liquid chromatography (HPLC).

2.2. Reagents

Ethanol (95% v/v) (Vetec), methyl alcohol (Vetec), PA-grade ethyl ether (Vetec), dichloromethane PA (Vetec), acetic acid PA (Vetec), ethyl acetate (99.5% v/v) (Neon), hexane PA (QHEMIS), butanol PA (QHEMIS), and acetonitrile (HPLC grade, JT Baker).

2.3. Thin Layer Chromatography

One gram of the powder and the plant extract were subjected to extraction with 25 mL of methanol for 30 minutes in an ultrasound bath. Aliquots of these extracts and the standards psoralen and bergapten (99% purity, Sigma Aldrich, St. Louis, MO, USA) were applied to one end of an aluminium chromatographic plate impregnated with silica gel 60F254 (Merck), using a micropipette. The chromatography plate was placed in a suitable container with a mobile phase consisting of dichloromethane and ethyl ether (1: 1.5 v/v) acidified with 2 mL of acetic acid. After elution, the plate was dried at room temperature, followed by UV visualization of the bands (Spectrolin) under ultraviolet light at 365 nm. The plant extract was also analysed by TLC to identify rutin and chlorogenic acid, according to the methods described by [15].

2.4. High-Performance Liquid Chromatography

A Waters Alliance HPLC system (Milford, MA, USA) with the e2695 separation module, equipped with a quaternary pump and diode array detector (PDA) 2998 was used, and the analysis was performed according to guideline Q2 (R1) of the International Conference on Harmonization [16]. Furanocoumarin identification was conducted by comparing the UV absorption spectrum of the sample with those of standards.

2.5. Fractionation of Vegetable Extract and Analysis of Fractions

Fractionation of the extract was conducted according to the methods by [17] with modifications using hexane, ethyl acetate, and *n*-butanol as solvents. These fractions, after being concentrated in a rotaevaporator, were analysed by HPLC to quantify furanocoumarins and were subjected to phytochemical analysis to determine the total phenolic content using the Waterman and Mole (1994)[18] method, flavonoid content (Rolim et al. 2006) [19], and tannin content using the Waterman and Mole (1987) [20] method. The octanol/water partition coefficients used for standardisation were determined following the methodology of [21].

2.6. Effect of *R. Graveolens* Extracts on the Inhibition of Mycelial Growth of *M. Oryzae*

The inhibitory effect was observed using the herbal extract of *R. graveolens* L. as well as its fractions, and psoralen and bergapten standards at the concentrations described in Table 1. The standards were

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tested in isolation and in synergy. In the synergism test, the same concentrations described in Table 1 were used in a 1:1 proportion. These standards were analysed to determine whether they are responsible for the antifungal activity of the plant extract, and their concentrations corresponded to their percentages in the extract. These, along with the extract and fractions, were added to the potato dextrose agar (PDA) culture medium to obtain the concentrations described above. After solidification of the medium, *M. Oryzae* mycelium discs of 5mm were added to the centre of the petridish and incubated for seven days at 25°C according to the methodology of [22].

Table1. Concentrations used in the mycelial growth test

Concentrations	Extract of <i>Ruta graveolens</i> L. (mg/mL)	Hexane fraction (mg/mL)	Fraction Ac. (mg/mL)	Fraction But (mg/mL)	Fraction hydromethanol (mg/mL)	Psoralen (µg/mL)	Bergapten (µg/mL)
1	0.335	0.335	0.335	0.335	0.335	0.13	0.22
2	0.675	0.675	0.675	0.675	0.675	0.53	0.92
3	1.35	1.35	1.35	1.35	1.35	2.13	3.71
4	2.7	2.7	2.7	2.7	2.7	8.52	14.87
5	4	4	4	4	4	18.70	32.65
6	5.4	5.4	5.4	5.4	5.4	34.07	59.51
7	6.75	6.75	6.75	6.75	6.75	53.25	92.94

F Ac: Ethyl acetate fraction; *F But:* butanol fraction

The control consisted of plates containing PDA medium and mycelium discs. All treatments were performed in quadruplicate. The *M. Oryzae* isolate used was 10,790, obtained from the plant pathology lab collection of Embrapa Rice and Beans.

Table2. Results of the characterisation of *Ruta graveolens* powder

Analyses	Results
Loss during drying	10.66 ± 0.2% (m/m)
Total ash content	6.79 ± 0.04% (m/m)
Level of acid-insoluble ash	0.26% ± 0.02 (m/m)
Intumescence index	3.5 ± 0.1 mL
Particle size	Moderately coarse (355 µm)

2.7. Analysis of Results

The evaluation of mycelial growth of the fungal colonies was performed using a digital calliper to evaluate the diameters of the two orthogonal axes. Then, the radius and colony area were calculated. Statistical analysis was performed based on the average area of the colony using analysis of variance implemented in ASSISTAT version 7.7 beta program, and the averages were compared using Tukey's tests at the 5% probability level.

3. RESULTS AND DISCUSSION

3.1. Characterisation of Plant Material

The loss in weight during drying of the powder was indicative of the volatile material content of the plant and indirectly showed residual moisture content of 10.66% ± 0.2% (m/m), which is within the values set in the general methods section of Brazilian Pharmacopoeia, 5th ed. (2010), [14] indicating proper conservation and efficient drying of the material. The ash content is the total non-volatile residual substances obtained by incineration, representing the sum of the integral inorganic material in the plant sample (intrinsic ash) and adherent foreign materials (extrinsic ash). [23] The total ash content found in *R. graveolens* L. powder was 6.79% ± 0.04%, which was within the set range (i.e., 2% to 20%) proposed by the general methods of the Brazilian Pharmacopoeia, 5th ed. (2010), [14] thus confirming the quality of the plant material used in the experiment. This content is lower than that reported by Nazish et al. (2009), [23] who found a total ash content of 8.13%, potentially owing to a higher amount of adherent foreign materials in the vegetable raw material. However, the analysis of acid-insoluble ash is used to determine the level of constituents or inorganic impurities contained in organic substances. [14] According to Couto et al. (2009), [24] this analysis enables the quantification

of silica and siliceous constituents in the sample. The acid-insoluble ash content acid in the samples was $0.26\% \pm 0.02\%$ (m/m), indicating that the plant samples were not contaminated by soil and sand residues (siliceous material). A high ash content indicates the presence of non-volatile inorganic impurities that may be present as contaminants. [25] However, Nazish et al. (2009) [23] found a higher amount of acid-insoluble ash in *R. graveolens* L. powder, i.e., 2.0%, and this is still within the range established by the general methods of the Brazilian Pharmacopoeia, 5th ed. (2010), [14] which is up to 2%. The in tumescence index estimated using water and alcohol was $3.5 \pm 1.3 \pm 0.1$ mL and 0.1 mL, respectively, showing that the plant material has mucilages consisting of long-chain carbohydrates that are able to retain water and to promote the formation of gels, such as pectin and hemicellulose. [26] Based on a particle size analysis, the particles of the plant material that were predominantly retained in a 355- μ m mesh sieve represented 55.84% of all material. Based on this observation, the *R. graveolens* powder analysed in this study was classified as moderately coarse according to the specifications of the general methods of the Brazilian Pharmacopoeia, 5th ed. (2010). [14]

Fractionation of plant extract, quantification of furanocoumarins, and phytochemical analyses of the extract and fractions

Figure 1A presents the chromatographic profile of *R. graveolens* L., where bands representing the standards and the sample and their respective retention factor (Rf) values are shown. The psoralen Rf was 0.68 and that of bergapten was 0.67. These results show that the concentrated extract of *R. graveolens* L. and the powder were positive for the markers in the study.

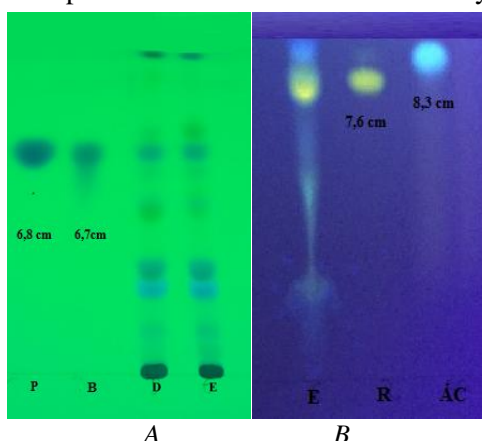


Figure1. Chromatographic profile of the *Ruta graveolens* L. plant material: A- Identification of psoralen (P) and bergapten (B) in the plant drug (D) and the extract (E); B- identification of rutin (R) and chlorogenic acid (Á.CLO) in the plant extract (EX). Source: author.

Regarding the quantification of furanocoumarins (psoralen and bergapten) in the plant extract of *R. graveolens* and its fractions using HPLC (Table 3), the highest levels of psoralen and bergapten were detected in the ethyl acetate fraction (1.59% and 2.93%, respectively). The hydromethanol fraction did not show any of the analysed standards. In Table 4, the phytochemistry results are shown, including the results of qualitative tests for the identification of the major classes of secondary metabolites in the plant material. [27] Phenolic compounds, such as tannins and flavonoids, may be responsible for the pharmacological activity of the species; accordingly, it is essential to determine their contents in the plant drug. As summarised in Table 4, the total quantity of phenolic compounds was higher in the ethyl acetate and hexane fractions, with contents of 23% and 11.66%, respectively. The fractionation process with less polar solvents (hexane and ethyl acetate) resulted in better separation of flavonoids, as these fractions showed contents of 8.25% and 5.75%, respectively. Yunus and Calixto (2001) [28] claimed that less polar solvents allow the recovery of free aglycones, such as flavones, flavonols, and flavanones. Additionally, the use of butanol, a more polar solvent, yields glycosides consisting of flavonoids linked to sugar groups.

Table3. Quantification of psoralen and bergapten in the extract and the fractions of *Ruta graveolens* L.

Samples	Content of Psoralen (%)	Content of Bergapten (%)
Extract of <i>Ruta graveolens</i> L.	0.71	1.19
Ethyl acetate fraction	1.59	2.93
Hexane fraction	0.17	0.42
Butanol fraction	0.07	0.12
Hydromethanol fraction	-	-

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Table4. Phytochemistry test results for the plant extract of *Ruta graveolens* and its fractions.

Test	Powder	Extract	FAC	FHex	FBut	FHD
Content of phenolic compounds	4.25% (m/v)	9.33% (m/v)	23% (m/v)	11.66% (m/v)	7.55% (m/v)	2.75% (m/v)
Content of Flavonoids	1.77% (m/v)	5.88% (m/v)	8.25% (m/v)	5.75% (m/v)	3.4% (m/v)	1.5% (m/v)
Content of tannins	-	0.05% (m/v)	1%	0.52%	1.88%	0.45%

*FAC (Ethyl acetate fraction); FHex (Hexane fraction); FBut (butanol fraction); FHD (hydromethanol fraction)

3.2. Effect of Plant Extracts of *R. graveolens* on the Inhibition of Mycelial Growth of *M. Oryzae*

Table 5 shows the *M. oryzae* colony areas after treatment application. Among the treatments, the herbal extract of *R. graveolens* and fractions of hexane and ethyl acetate showed the best inhibition results, of which the three highest concentrations decreased mycelial growth by more than 90%, with the largest concentration of 6.75 mg/mL resulting in reductions of 96.8%, 98.47%, and 98.95%, respectively (Figure 2A and 2B). The highest concentration of psoralen applied resulted in 99.12% inhibition of the fungal colony size (Figure 2A).

Table5. Colony area of *M. oryzae* subjected to different treatments

Concentration	Treatments							
	Extract	FHex	FAC	FBut	FHD	Pso	Bg	Ps + Bg
Control	1793,17 a	1284,87 a	1868,05 a	1483,24 a	1458,98 a	2025,61 a	1426,02 b	2238,38 bc
1	1689,55 b	544,02 b	1529,37 b	1255,12 a	1509,65 a	2053,23 ab	1567,47 a	2449,11 a
2	1532,85 c	254,03 c	1237,55 c	948,40 b	1554,24 a	2017,54 ab	1567,47 a	2492,71 a
3	963,81 d	147,32 c	694,93 d	968,83 b	1539,05 a	1883,04 bc	1292,86 b	2361,40 ab
4	419,88 e	19,62 d	295,12 e	822,75 bc	1573,81 a	1726,75 c	970,25 c	2262,06 abc
5	160,08 f	19,62 d	67,11 f	573,03 cd	1565,76 a	851,52 d	578,74 d	2053,94 c
6	70,89 f	19,62 d	19,62 f	403,22 d	1540,17 a	258,40 e	439,60 e	1537,83 d
7	53,76 f	19,62 d	19,62 f	337,72 d	1559,63 a	17,87 f	142,72 f	1037,08 e

*FAC (Ethyl acetate fraction); FHex (Hexane fraction); FBut (butanol fraction); FHD (hydromethanol fraction); Pso (psoralen); Bg (bergapten); Ps+Bg (psoralen + bergapten); ^{a-f} Means followed by different letters differ significantly based on Duncan's test at the 5% probability level.

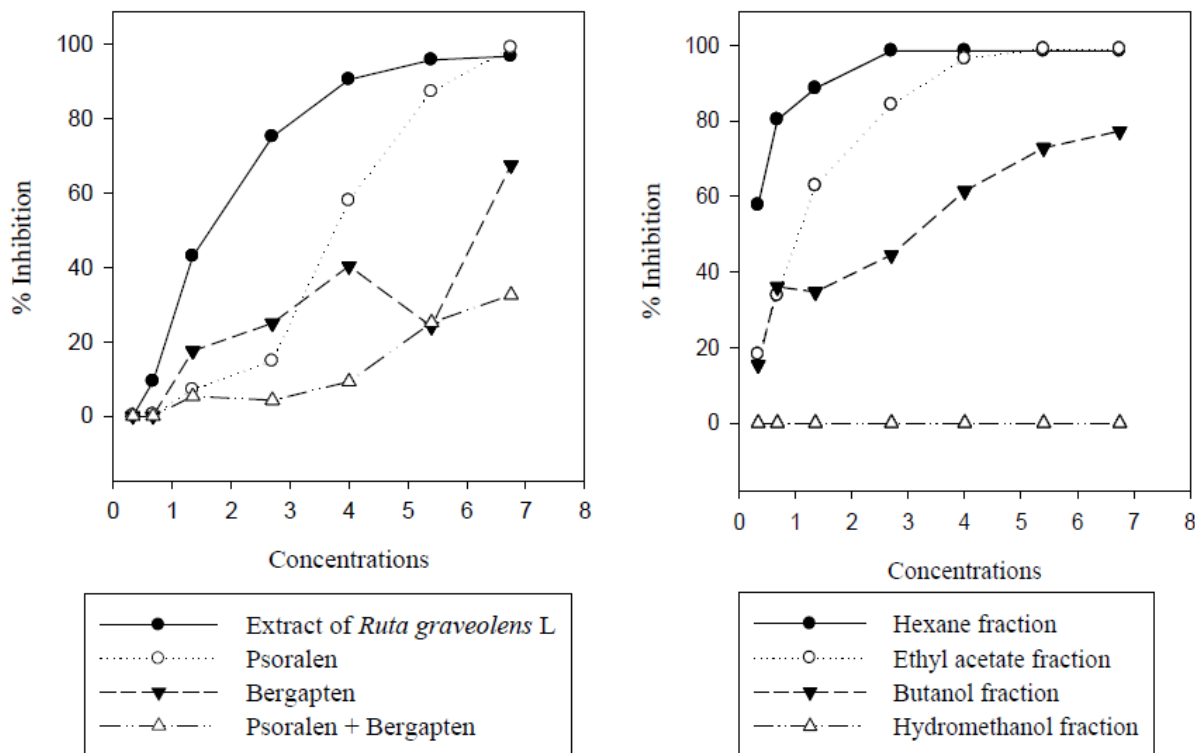


Figure2. Rate of inhibition of mycelial growth of *M. oryzae*: A- subjected to treatment with the extract of *Ruta graveolens*, psoralen, psoralen + bergapten, and bergapten; B- subjected to treatment with the fractions of the *R. graveolens* extract.

Based on the results, psoralen and bergapten showed a significant reduction in mycelial growth, where the best psoralen results were observed for the three highest concentrations, 18.70 mg/mL, 34.07 mg/mL, and 53.25 mg/mL, which resulted in inhibition levels of 57.95%, 87.24%, and 99.12%, respectively. For bergapten, a concentration of 92.94 mg/mL resulted in the highest inhibition rate, i.e., 67.53% (Figure 2A). The inhibitory effect was higher for psoralen than bergapten, and this can be explained by the octanol/water partial coefficient, which is a measure of the affinity to water or oil for hydrophilic or lipophilic solutions (Kow). The Kow was 0.79 for bergapten (an affinity associated with polar solutions) and 1.30 for psoralen (consistent with a polar solutions), which favours better permeability of the fungal membrane.

Garcia et al. (2012) [29] examined the antifungal activity of oil and plant extracts on *Sclerotiniasclerotiorum* and found that the *R. graveolens* extract results in a 25% inhibition of mycelial growth of *S. sclerotiorum*. Celoto et al. (2008) [30] found that the hydroethanolic extract of rue results in 2.3% inhibition of mycelial growth of *Colletotrichumgloeosporioides* when incorporated at a concentration of 20% on PDA culture medium. Among the factors influencing the levels of active ingredients in plants, genetic factors, cultivation conditions, the harvesting process, and material processing are particularly important. [31]

Furanocoumarins influence fungi in various ways, including cytoplasmic granulation, disruption of cell contents, disruption of the plasma membrane, and inhibition of fungal enzymes, which causes inhibition of germination as well as reduced mycelial growth. [32]

The fractions that showed the best results in the mycelial growth test had the largest psoralen and bergapten levels, suggesting that they collaborate in the *R. graveolens* extract to exert an antifungal effect. This action may be synergistic with other plant metabolites. The presence of phenolic compounds also contributed to the antifungal effect; the most active fractions against the fungus were those with the highest levels of phenolic compounds.

Wintola and Afolayan (2011) [33] stated that the amount of phenolic compounds in an extract is influenced by the solvent used in the extraction; it was possible to observe that the more nonpolar solvents were optimal for extraction. According to the literature, the hydroxyl groups present in phenolic compounds can form hydrogen bridges with enzymes involved in microbial metabolism, inactivating and inhibiting fungal biomass development. [34, 35]

Among the phenolic compounds present in *R. graveolens* extract were chlorogenic acid and rutin (a flavonoid) (Figure 1B), and a previous study showed inhibitory activity of *Penicilliumexpansum*, *Fusariumoxysporum*, and *Mucorpiriformis*[36] with respect to mycelial growth, indicating that these phenolic compounds are promising candidates for inhibitory activity.

Phenolic compounds as well as proteins and essential oils may inhibit cell wall biosynthesis components, destroying the membrane and obstructing the entry of nutrients; they may also inhibit the biosynthesis of proteins and fungal amino acids, affect sphingolipid biosynthesis, interfere with the transport of electrons, and prevent the integrity of the cell. Chemical antifungal agents function by similar mechanisms, but excess amounts can accumulate and have negative effects on consumers. [37, 38] The cell wall of fungi is composed of polysaccharides, e.g., β -glucans (β 1,3 glucan and β 1,6 glucan), α -glucans (α 1,3 glucan and α 1,4 glucan), chitin, and mannan. [39] A study carried out by Fujikawa et al. (2009) [40] has shown that the appressorium wall and germ tube of *M. oryzae* consist of β 1,3 glucan, chitin, and mannan; however, α 1,3 glucan is present only in the wall of the appressorium of this fungus.

The passage of the soluble fractions in the cell wall of *M. oryzae* with ergosterol present caused the disorganization of cellular components and a reduction in mycelial growth. Ergosterol is a lipo soluble component that is considered one of the essential constituents of the plasma membrane of fungi, presenting itself as a good indicator of fungal growth, and it may change according to the environment and the metabolites produced. [41] It acts in the increased membrane microvilli and participates along with phospholipids in the regulation of the trans-exchange membrane; its depletion alters membrane fluidity, interfering with the uptake of nutrients, which results in the inhibition of fungal growth and yields morphological changes that result in cell necrosis. [42-43] The cell wall of *M. oryzae* also contains essential hydrophobins consisting of moderately hydrophobic extracellular proteins that have several functions in the cellular process of phytopathogenic fungi, such as adhesion, sporulation, pathogenesis, fungal growth, and development. [44] They show differences in hydrophobicity from

standard values and are classified into class I and II hydrophobins. The highly lipo soluble class I is very stable, while class II is less stable and can be easily dissociated with ethanol and 2% sodium dodecyl sulfate. [45]

These hydrophobins act at the hydrophobic-hydrophilic interface at the surface of the hyphae where the inner hydrophilic side is in contact with cells and prevents the loss of water, and the hydrophobic outer part prevents the wetting process. [46-47]

4. CONCLUSION

The *R. graveolens* L. extract, which contains linear furanocoumarins (psoralen and bergapten), is a potent inhibitor of *M. oryzae* growth *in vitro*. The fractionation process led to the identification of the best fraction of the extract (i.e., the hexane fraction), which resulted in the greatest reduction in mycelial growth of *M. oryzae*, and the specificity of the substances responsible for the activity was related to the characteristics of the solvent used in fractionation.

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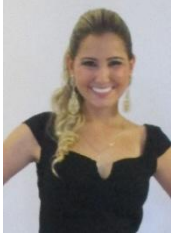
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