INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 13, No. 3, p. 306-313, 2018

RESEARCH PAPER OPEN ACCESS

Mycochemicals and bioactivities of *Aspergillus niger* and *Rhizomucor pusillus* associated with vermicast

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Key words: Mycochemicals, Anti-oxidant, Phenolics, Anti-bacterial.

http://dx.doi.org/10.12692/ijb/13.3.306-313 Article published on September 30, 2018

Abstract

This study aimed to determine the mycochemical constituents and biological activities of fungi associated with vermicast namely: *Aspergillus niger*, *Rhizomucor pusillus*. Mycochemical screening of *A. niger* and *R. pusillus* ethanolic extract and fungal spent revealed the presence of flavonoids and terpenoids. Other bioactive compounds like tannins and alkaloids were only detected in *A. niger* and *R. pusillus* ethanolic extracts, respectively. For the antibacterial activity as protectant, at 12 hours and 24 hours of incubation, the least zone of colonization of *Staphylococcys aureus* and *Escherichia coli* were recorded in plates treated with *A. niger* ethanolic extract and *R. pusillus* ethanolic extracts. Whereas, zone of inhibition at 12 and 24 hours of incubation against the bacterial pathogens were recorded in ethanolic extract of *A. niger* and *R. pusillus*. For the DPPH radical scavenging activity, among the four extracts, *A. niger* ethanolic extract recorded the highest ability to scavenge free radicals. Thus, the aforementioned fungal organisms possess antimicrobial and anti-oxidant properties which can be attributed to their mycochemical components.

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Introduction

Vermicast contains various amount of remnants of plant materials, microorganisms, microbial enzymes, hormones, micro and macro nutrients unutilized by the earthworms (Holmer, 2008; Lavelle and Martin, 1992). Accordingly, interactions between earthworms and microorganisms during the breakdown of organic matter (Dominguez, 2004). Previous researches have already revealed the presence of bacterial and fungal organisms in vermicasts (Abbasi and Ramasamy, 2001; Gajalakshmi and Abassi, 2003).

Fungi are eukaryotic microorganisms that play fundamental ecological roles as decomposers, mutualists, or pathogens of plants and animals (Blackwell, 2011). They perform a crucial role in the decomposition of plant structural polymers such as cellulose, hemi-cellulose and lignin (Hawksworth *et al.,* 1996).

The present study was undertaken to screen the mycochemical constituents and biological activities mainly the antibacterial and antioxidant activity of fungi (*Aspergillus niger* and *Rhizomucor pusillus*) associated with vermicast in their ethanolic extract and fungal spent. *Staphylococcus aureus* and *Escherichia coli*were used as the test organisms in the antibacterial assay. Elucidation of these properties would further lead to the utilization of *A. niger* and *R. pusillus* in the production of drugs.

Materials and methods

Preparation of Culture Media

Potato Dextrose Agar (PDA) and Potato Dextrose Broth were used to cultivate fungal isolates. PDA was used to sub-culture the fungal isolates and PDB was used in mycelial mat production. Thirty-nine (39) grams of PDA and twenty four (24) grams of PDB was dissolved separately in in one (1) liter of distilled water. It was boiled until homogenous mixture obtained. Approximately twenty (20) mL of PDB was dispensed into each bottle plugged with cotton and wrapped with paper and sealed with a rubber band. It was sterilized using an autoclave at 121°C, 15psi for 30 minutes.

Mycelial Mat Production

The seven (7) day old mycelial discs were prepared with 10 – mm – diameter cork borer then it was inoculated in prepared liquid media and incubated at room temperature for 4 days. Then, mycelial mat was harvested and it was air-dried for 7 days and pulverized.

Preparation of Ethanol Extracts

Twenty five (25) grams of powdered fungi was used. The weighed samples were mixed with 100 ml of 95% ethanol in a sterile flask for 48 hours. The extracts were filtered with Whatman No. 1 filter paper and subjected to rotary evaporator. Then, it was placed in amber bottles and sealed with aluminum foil to avoid exposure to light and dust (Maiquez *et al.,* 2016).

Preparation of Mycelial Spent

The liquid media used in the mycelial production was kept after the mycelia were harvested. Mycelial spent was filtered using Whatman No. 1 filter paper then it was placed in an amber bottle and stored in a refrigerator until needed.

Sub-Study I

Screening of Mycochemical Constituents of R. stolonifer and A. fumigatus ethanol extract and mycelial spent

Screening of mycochemical constituents of ethanol extract and mycelial spent of *R. stolonifer* and *A. fumigatus* associated with vermicast was carried out by following the standard methods described in Laboratory Manual for the UNESCO (1986). The various mycochemical constituents were alkaloids, cardiac glycosides, flavonoids, saponins, steroids, tannins, and terpenoids.

Test for alkaloids

Five milliliters of the different extracts were prepared in a beaker and 200 mL of 10% HCH_3CO_2 in C_2H_5OH was added. The mixture was filtered and the extracts was allowed to become concentrated in water bath until it reached one fourth of the original volume then concentrated NH_4OH was added. Formation of the

white precipitate or turbidity was observed for the presence of alkaloid.

Test for Cardiac glycosides: One milliliter of concentrated sulfuric acid (H_2SO_4) was prepared in the test tube. Five milliliters of different fungi extracts were mixed with 2 mL of glacial HCH_3CO_2 containing one drop of $FECl_3$. The mixture was added carefully to 1 mL of concentrated H_2SO_4 so that the concentrated H_2SO_4 was underneath the mixture. The appearance of brown ring was observed for the presence of cardiac glycosides.

Test for flavonoids

In 5 ml different extracts of fungi few drops of 1% ammonium (NH_3) solution was added in the test tube. Yellow coloration was observed for the presence of flavonoids.

Test for Saponins

A volume of 0.5 mL of different extracts were added to 10 ml distilled water and were shaken vigorously to obtain a stable persistent froth. The persistent frothing was observed for the presence of saponins.

Test for Steroids

Two milliliters of acetic anhydride were added to a 5 mL extracts of different fungi sample with 2 mL of H_2SO_4.Violet to blue or green precipitate was observed for the presence of steroids.

Test for tannins

An aliquot of 0.5 mL extracts of different fungi were added to 10 ml of distilled water on the test tube and filtered. Two ml of 5% FeCl was added to the filtered sample. The formation of brownish green to blue-black coloration was observed for the presence of tannins.

Test for Terpenoids

Five milliliters extracts of different fungi samples were added with 2 mL $CHCl_3$ in a test tube. Then, 3 mL of H_2SO_4 was added carefully to the mixture to form a layer. The formation of the reddish brown of the interface was observed for the presence of terpenoids.

Sub-Study II

Determination of the antibacterial activity of A. niger and R. pusillus

Preparation of bacterial inocula and assay plates: Pure culture of *S. aureus* and *E. coli* were were grown in Nutrient Agar for 24 hours and transferred to a Nutrient Broth. Then the bacterial cell density were adjusted to 0.5 McFarland standard. Mueller Hinton Agar (MHA) was used for the bio assay of the antibacterial potential of the fungal isolates. Approximately 15 mL of sterile MHA was poured into sterile Petri plates then allowed to cool and solidify prior to inoculation of the test organisms.

Preparation of paper disc

What man No. 1 filter paper was used as the paper disc with the use of paper puncher. The paper discs were sterilized in autoclave at 121°C, 15psi for 30 minutes.

Protectant test

The paper discs were soaked in the bacterial suspension. Then, the sterile plates with MHA were swabbed with 0.1 mL of the different extracts. The paper discs were seeded equidistantly in the plates together with the control. The plates were stored at room temperature and zone of bacterial colonization were measured using a Vernier caliper after 12 and 24 hours of incubation.

Eradicant Test

The paper discs were soaked in the different treatments. Then, the sterile plates with MH agar were poured and spreaded with 0.1 mL of the bacterial suspension using a T-rod. The discs were soaked in each treatment and seeded equidistantly in the plates. The plates were incubated within 24 hours at room temperature. The zone of inhibition was measured using Vernier caliper at 12 and 24 hours of incubation.

Sub-Study III

Determination of Antioxidant Property

The DPPH radical scavenging activity of ethanol extract and mycelial spent of *R. stolonifer* and *A. fumigatus* associated with vermicast were sent and analyzed at the Chemistry Laboratory of Center for Natural Sciences at St. Mary's University, Bayombong, Nueva Vizcaya.

DPPH Scavenging activity

The DPPH scavenging activity of the ethanol extracts and mycelial spent of *R. stolonifer* and *A. fumigatus* associated with vermicast were assayed using DPPH (Kolak *et al.,* 2006). The ethanol extracts and mycelial spent of *R. stolonifer* and *A. fumigatus* were dissolved in methanol to a final concentration of 500 ppm. A 0.1 mm DDPH in methanol was freshly prepared by diluting 1 ml DPPH stock solution (3.49 mg DPPH in 10 mL methanol) to 100 mL methanol. Then, 1 ml of the extracts and 4 mL of DPPH solution was mixed and incubated in the dark at 37° C for 30 minutes. Triplicate tests were done in each extracts. The absorbance reading was monitored at 517 nm using UV- Vis spectrophotometer (APEL-100) and the ability to scavenge the DPPH radical was calculated using the equation below:

% DPPH scavenging effect = $[(A_{control}-A_{sample})/ A_{control}]$ x 100.

Where $A_{control}$ is the absorbance of the control, which is the DPPH solution without the extract, the A_{sample} is the absorbance of the test sample containing the mixture of DPPH and the ethanol and hot water extract. The synthetic antioxidant cathechin was used as positive control.

Statistical analysis

Experimental units were laid out using Completely Randomized Design (CRD) and the data gathered were analyzed using Analysis of Variance (ANOVA) and means were compared using Multiple Duncan Range Test (DMRT) at 5% level of significance.

Results and discussion

Mycochemical constituents of A. niger and R. pusillus

Fungi are a promising source of novel bioactive compounds as lead structures for medicine and plant protection (Duarte *et al.,* 2012). These compounds are responsible for different medicinal properties of the extracts. Shown in Table 1 are the screened bioactive components of fungal isolates. Flavanoids and terpenoids were present in all ethanol and fungal spent of *A. niger* and *R. pusillus*. Meanwhile, alkaloids were also detected in *R. pusillus* ethanol extracts and steroids are present only in *R. pusillus* fungal spent.

Table 1. Bioactive components of the ethanolic extracts and fungal spent of fungi.

Bioactive components	*A. niger*		*R. pusillus*	
	Ethanolic Extract	Fungal Spent	Ethanolic extract	Fungal spent
Alkaloids	-	-	+	-
Cardiac glycosides	-	-	-	-
Flavonoids	+	+	+	+
Saponins	-	-	-	-
Steroids	-	-	-	+
Tannins	+	-	-	-
Terpenoids	+	+	+	+

*(+) determines the presence of bioactive compounds; (-) absence of bioactive compounds.

This coincides with the studies of Madhusudan and Mishra, (2017); Murthy *et al.* (2011), wherein the presence of terpenoids was detected in *A. niger* and flavonoids in *Mucor* sp., respectively. Similarly,

findings of Yadav *et al.* (2014), showed the phytochemical constituents of different endophytic fungi *A. niger*, and *A. flavus* contained flavonoids and terpenes. Also, Ladoh *et al.* (2015), revealed that fungi of genus *Aspergillus*, *Penicillium*, *Trichoderma* and *Fusarium* contains flavonoids, anthroquinones, tannins, phenols, coumarins and terpenoids.

Antibacterial activity A. niger and R. pusillus Protectant

At 12 and 24 hours of incubation, the least zone of colonization of *S. aureus* and *E. coli* were recorded in plates treated with *A. niger* ethanolic extract (9.64 mm and 12.35 mm for *S. aureus*; 9.60 mm and 12.37 mm for *E. coli*) and *R. pusillus* ethanolic extracts (0.00 mm and 13.03 mm for *S. aureus*: 19.35 mm and 32.44 mm for *E. coli)*. In addition, plates treated with *A. niger* spent shows minimum colonization (10.18 mm for *S. aureus* and 12.75 mm for *E. coli*) but colonized the whole plate after 24 hours of incubation for both test organisms as presented in Table 2.

Table 2. Measurement of zone of colonization of S. aureus and E. coli in plates treated with different treatments.

Treatments	*S. aureus*		*E coli*	
	12 hrs	24 hrs	12 hrs	24 hrs
A. niger ethanolic extract	9.64^{b}	12.35^{b}	9.60^{c}	12.37^{c}
R. pusillus ethanolic extract	$.00^{c}$	13.03^{b}	19.35^{b}	32.44^{b}
A. niger spent	10.18^{b}	32.42^{a}	12.75^{c}	32.42^{a}
R. pusillus spent	32.42^{a}	32.42^{a}	32.42^{a}	32.42^{a}
Distilled water	32.42^{a}	32.42^{a}	32.42^{a}	32.42^{a}
Streptomycin sulfate	0.00^{c}	0.00^{c}	0.00^{d}	0.00^{d}

*Treatments with the same letter are not significantly different with each other at 5% level of significance.

Statistical analysis revealed that *R. pusillus* ethanolic extract was comparable with that of the Streptomycin sulfate (positive control) for *S. aureus* after 12 hours of incubation. Meanwhile, fungal ethanolic extracts were also significantly lower as compared to the spent and distilled water, therefore the inhibitory activity of *A. niger* and *R. pusillus* ethanolic extracts against *E. coli* and *S. aureus* as protectant agent. Eradicant: Table 3 presents the antibacterial activity as eradicant which revealed the absence of zone of inhibition discs with *A. niger* and *R. pusillus* spent both for *S. aureus* and *E. coli*. Whereas, zone of inhibition at 12 and 24 hours of incubation against the bacterial pathogens were recorded in ethanolic extract of *A. niger (*9.43 mm and 9.28mm in *S. aureus*) and *R. pusillus* (11.94 mm and 11.75 mm in *S. aureus;* 9.40 mm and 9.08 mm *E. coli)*. Statistical analysis revealed that the zone of inhibition produced by *A. niger* and *R. pusillus* spent were comparable to the negative control which indicates the lack of antibacterial activity in 12 and 24 hours of incubation against *S. aureus* and *E. coli*. Whereas, ethanolic extracts of *A. niger* and *R. pusillus* were significantly higher than negative control, which signifies their antibacterial activity against *S. aureus* and *E. coli,* however it is still incomparable with the positive control.

Table 3. Zone of inhibition of *S. aureus* and *E. coli* after 12 and 24 hours of incubation.

Treatments	*S. aureus*		*E coli*	
	12hrs	24 hrs	12 hrs	24hrs
A. niger ethanolic extract	9.43^{b}	9.28^{b}	0.00^{c}	0.00^{c}
R. pusillus ethanolic extract	11.94^{b}	11.75^{b}	9.40^{b}	9.08^{b}
A. niger spent	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}
R. pusillus spent	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}
Distilled water	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}
Streptomycin sulfate	23.99^{a}	23.86^{a}	26.34^{a}	25.88^{a}

*Treatments with the same letter are not significantly different with each other at 5% level of significance.

This coincides with the study of Madsuhudan and Mishra (2017), that *A. niger* crude extract, contains significant pathogen inhibiting compounds in both Gram positive and Gram negative bacterial strains. In addition, according to Durairaj *et al.* (2015), *A. niger* exhibited excellent antimicrobial activity against both bacterial species and fungi. The same results were obtained by Kalyani and Hemalatha (2017).

The ability of both fungal extracts to prevent bacteria from colonizing and their inhibitory activity might be due to the bioactive compounds present in the extracts like alkaloids and flavonoids.

Radical scavenging activity of A. niger and R. pusillus

Free radical scavenging property of antioxidants delays or inhibits cellular damage (Halliwell, 1995). Table 4 presents the radical scavenging activity (RSA) of the ethanol extracts of *A. niger*, *R. pusillus* and their fungal spent.

Among the four extracts, *A. niger* ethanolic extract was recorded as the treatment having the highest ability to scavenge free radicals with 73.03% followed by *R. pusillus* ethanolic extract with 71.86% and *A. niger* spent and lastly *R. pusillus* spent with the percentage of 42.50% and 29.96%, respectively.

Table 4. Free radical scavenging activity (%) of fungi *A. niger* and *R. pusillus* in their ethanolic extract and fungal spent.

Treatments	% Radical Scavenging Activity
Aspergillus niger ethanolic extract	73.03%
Rhizomucor pusillus ethanolic extract	71.86%
A. niger spent	45.20%
R. pusillus spent	29.96%
Cathecin (Positive control)	72.86% / 76.06%

*Abs DPPH-1.874; Wavelength 517 nm using Spectrum lab 752s UV Vis spectrophotometer; Concentration: 1000 nm.

In the previous study, it has been reported by Yadav *et al.* (2014), that *Aspergillus niger* strain showed the highest antioxidant activity ranging from 50% to 80%. In addition, findings of Hameed *et al.* (2017), showed that *Mucor* strains proved to be rich sources of antioxidants and secondary metabolites, which could be used in the development of nutraceutical and natural antioxidants.

Also, from the study of Nitya *et al.* (2011), the antioxidant activity of endophytic fungi isolated from *Lobelia nicotianifolia*. Meanwhile, from the study of Yen and Lee (1996), reported that *Aspergillus* sp. possessed a high antioxidant up to 78% just like *Penicillium*. Ishikawa (1995), reported that some fungi, especially *Penicillium* and *Aspergillus*, might produce antioxidants. This was confirmed by the results of this study.

Potential of the fungal extracts to scavenge free radicals are in correlation with their secondary metabolites present like terpenoids, tannins and flavonoids. Recent studies have shown that these compounds have anti-oxidative property. This coincides with the findings of Gulcin (2007) and Hajdu *et al.* (2007), that terpenes act as primary and secondary antioxidants. Flavonoids is also a compound which exhibits anti-oxidative, free radical scavenging (Yao *et al.,* 2004). In addition, tannins also have antioxidants property as reported by Koleckar *et al.* (2008).

Conclusion

Based from the results of the present study, *A. niger* and *R. pusillus* contains several bioactive compounds like alkaloids, terpenoids, flavonoids and tannins. *R. pusillus* ethanolic extract and *A. niger* ethanolic

extract possess antibacterial potential as protectant and eradicant against *E. coli* and *S. aureus*. Also, high DPPH radical scavenging activity of the aforementioned fungi were elucidated.

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