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ORIGINAL ARTICLE

Mycochemicals and Bioactivities of *Rhizopus stonifer* and *Aspergillus fumigatus* associated with vermicast

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ABSTRACT
The present study revealed the mycochemical constituents, antibacterial, anti-oxidant and the cytotoxicity of R. stolonifer and A. fumigatus ethanol extracts and fungal spent. Alkaloids, terpeniods and flavonoids were detected in the all fungal extracts. For the antibacterial activity, ethanol extract of A. fumigatus and R. stolonifer as protectant and eradicant were elucidated which inhibits S. aureus and E.coli growth. In addition, for the anti-oxidant property, R. stolonifer ethanol extract shows the highest percentage of radical scavenging activity with 72.53% followed by R. stolonifer mycelial spent with 46.48% and A. fumigatus mycelial spent and ethanol extract with the percentage of 39.44% and 32.66% respectively
Keywords;*–antibacterial–anti-oxidant– eradicant – phenolics – protectant – radical scavenging*

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INTRODUCTION

Fungal organisms are ubiquitous in nature which can be found in air, soil, decaying vegetables, fruits, wastes and wastewater [1]. They serve as an active component which facilitate the biodegradation and bioconversion processes [2] One of the most utilized product of composting is the vermicast. Vermicast is the excreta of earthworms which is extremely helpful tool in neutralizing soil pH and they harness the beneficial microflora that contains vitamins, enzymes, minerals, antibiotics and growth hormones [3,4].

In this study, two fungal species isolated from vermicast namely; *Rhizopus stolonifer* and *Aspergillus fumigatus* were used to evaluate their biological activities. Fungi are known source of natural "mycochemicals" with various nutraceutical benefits such as antibiotics, and antioxidant[5]. Fungi were involved in the industrial processing of more than 10 of the 20 most profitable products used in human medicine and they also aid in disease management [6].

Recently, fungi have emerged as the new sources of antioxidants in the form of their secondary metabolites [7]. Fungi are remarkably a diverse group including approximately 1.5 million species, which can potentially provide a wide variety of metabolites such as alkaloids, benzoquinones, flavonoids, phenols, steroids, terpenoids, tetralones, and xanthones[8]. They demonstrate variety of bioactivities along with antioxidant properties and function as varied as theirstructure. They are exploited in medicine and industry and considered to be potential sources of new therapeutic agents.

Thus, this study was carried out to determine the potentials of *R. stolonifer* and *A. fumigatus* as alternative sources of natural mycochemicals, antibiotics, antioxidant and anticancer drugs.

MATERIALS AND METHODS

The study was divided into three sub-studies. Sub study I focused on the mycochemical constituents of ethanol extract and mycelial spent of *R. stolonifer* and *A. fumigatus* associated with vermicast. Sub study II dealt with the determination of antibacterial property of ethanol extract and mycelial spent of *R. stolonifer* and *A. fumigatus* associated with vermicast, sub study three determined the antioxidant

property of the fungal extracts and lastly, sub-study four, dealt with the cytotoxic activity of the tested fungi.

Preparation of Culture Media. Thirty-nine (39) grams of Potato Dextrose Agar (PDA) was dissolved in one (1) liter of distilled water. It was boiled until homogenous mixture obtained. Approximately 300 mL of the prepared medium was poured into a flask, plugged with cotton wrapped with paper and sealed with a rubber band. The medium was sterilized for 30 minutes at 121°C, 15psi. After the sterilization, the medium was allowed to cool for several minutes until ready for pour plating. Mycelia were aseptically inoculated into sterile PDA plates and it was incubated at room temperature for 7 days.

Preparation of Liquid Media. Twenty four (24) grams of Potato Dextrose Broth (PDB) was dissolved in one (1) liter of distilled water. Approximately twenty (20) mL of liquid media 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.

Preparation of Mycelial Production. The seven (7) day old mycelial discs were prepared using flame sterile 10 – mm – diameter cork borer then it was inoculated in prepared liquid media and incubated at room temperature for 4 days to allow mycelial growth. The 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 [9].

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 [10]. The various mycochemical constituents were alkaloids, cardiac glycosides, flavonoids, saponins, steroids, tannins, and terpenoids.

Sub-Study II. Determination of Antibacterial Activity.

Preparation of inoculums. Pure culture of *S. aureus* and *E. coli* were obtained from the Department of Biological Sciences in CLSU, Science City of Muñoz, Nueva Ecija. The bacteria were grown in Nutrient Agar for 24 hours and transferred to a Nutrient Broth. After 24 hours of incubation, the bacteria were adjusted to 0.5 McFarland to standardize the bacterial cell density.

Preparation of Culture Media and assay plates. Thirty eight (38) grams of Mueller Hinton Agar was dissolved in 1 liter of distilled water. It was boiled until homogenous mixture was obtained. Approximately 300 mL of the prepared medium was poured into a flask, plugged with cotton, wrapped with paper and sealed with a rubber band. The medium was sterilized for 30 minutes at 121°C, 15psi. After the sterilization, the medium was allowed to cool for several minutes until ready for pour plating. 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. Whatman 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 MH agar 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 [11].

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 [11]. 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^{o} 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 l00

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 *R. stolonifer* and *A. fumigatus* Ethanol Extracts and Mycelial Spent. Presented in Table 1 are the bioactive components of R. stolonifera and A. fumigatus ethanol and fungal spent. Among which, alkaloids, flavonoids and terpenoids were present in the fungal extracts. Both *R. stolonifer* and *A. fumigatus* ethanol extracts contain alkaloids whereas the ethanol and fungal spent of *R. stolonifer* and *A. fumigatus* contains flavonoids and terpenoids. This is in accordance with [12,13] that fungi are rich sources of bioactive and novel organic compounds with interesting biological activities and a high level of biodiversity.

Table 1. Bioactive components of fungal ethanol extract and fungal spent

BIOACTIVE COMPONENTS	FUNGAL EXTRACTS			
	R. stolonifer extracts		*A. fumigatus extracst*	
	Ethanol	Fungal spent	Ethanol	Fungal spent
Alkaloids	+	-	+	-
Cardiac glycoside	-	-	-	-
Flavonoids	+	+	+	+
Saponins	-	-	-	-
Steroids	-	-	-	-
Tannins	-	-	-	-
Terpenoids	+	+	+	+

*(+) presence of bioactive compounds; (-) absence of bioactive compounds

Additionally, study of Frisvad found out that *A. fumigatus* generate many structurally and biologically diversified metabolites, that include alkaloids which is known to have in antibacterial chemotherapy [14,15]. Alkaloids are natural products of bacteria, fungi, plants and animals [16].

Similarly, flavonoids have long been used as traditional medicines with scientifically proven pharmacological benefits. Flavonoids are able to chelate free radicals immediately by donating a hydrogen atom or by single-electron transfer [17].

Thus, with the presence of the aforementioned mycochemicals is indicative of their potential antibacterial and antioxidant activities.

Antibacterial Activity of *R. stolonifer* and *A. fumigatus* Ethanol Extracts and Mycelial Spent.Antibiotics have an important role in human health. Their necessity emerged from the spread of various diseases. As a result, scientists are trying to produce and discover more antibiotics [17]. They can serve as either protectant or eradicant. Protectants or the prophylactics are used to prevent organisms from bacterial infections, and they are being applied prior to infections. Meanwhile, eradicants are used to kill the disease causing organisms while being infected. Protectant and eridicant tests were undertaken to determine the ability of *R. stolonifer* and *A. fumigatus* ethanol extracts and mycelial spent as antibacterial agent (Table 2 and Table3). The smallest the zone of colonization produced by the test organisms the greater their anti-bacterial activity as protectant while the greatest the zone of inhibition against the test pathogens the greater their eradicating activity.

Results of protectant test are shown in Table 3. At 12 hours and 24 hours of incubation, the least zone of colonization of *S. aureus* and *E.coli* were recorded in plates treated with *A. fumigatus* ethanol extract (8.44 mm for *S. aureus*; 6.30 mm for *E.coli*) and *R. stolonifer* ethanol extracts (8.19 mm for *S. aureus*: 6.23mm for *E.coli).* Meanwhile, increase of bacterial colonization was observed in plates treated with *R.*

stolonifer and *A. fumigatus* fungal spent which resulted to fully colonized plates after 24 hours of incubation. Statistically, fungal ethanol extracts were significantly lower as compared to the mycelial spent and distilled water, therefore *A. fumigatus* and *R. stolonifer* ethanol extract thus their protectant activity against *S. aureus* and *E. coli*.

Table 2. Zone of colonization of fungal ethanol extracts and mycelial spent after 12 and 24 hours of incubation against *S aureus* and *E. coli*

TREATMENTS	*S. aureus*		*E.coli*	
	12hrs	24hrs	12hrs	24hrs
R. stolonifer ethanol extract	8.19^{c}	8.28^{c}	6.23^{d}	27.54^{b}
A. fumigatus ethanol extract	8.44^{c}	8.62^{c}	6.30^{d}	10.67^{c}
R. stolonifer spent	19.16^{b}	37.58^{b}	10.51^{b}	32.42^{a}
A. fumigatus spent	14.48^{bc}	32.42^{a}	7.17^{c}	32.42^{a}
Distilled water	32.42^{a}	32.42^{a}	32.42^{a}	32.42^{a}
Streptomycin sulfate	0.00^{d}	0.00^{d}	0.00^{e}	0.00^{d}

*Means with the same letter are not significantly different with each other

For the eradicant test (Table 4), results revealed the absence of zone of inhibition of *S. aureus* and *E.coli* when treated with *R. stolonifer* and *A. fumigatus* fungal spent. On the other hand, zone of inhibition at 12 and 24 hours of incubation against the bacterial pathogens were recorded in ethanol extract of *A. fumigatus* (with zone of inhibition measuring 13.60 mm in *S. aureus;* and11.70 mm for *E.coli*)and *R. stolonifer* (with zone of inhibition measuring 12.84 mm in *S. aureus;*. 8.19 mm for*E. coli).* Statistical analysis revealed that the zone of inhibition produced by *R. stolonifer* and *A. fumigatus* 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, ethanol extract of *R. stolonifer and A. fumigatus* 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 fungal ethanol extracts and mycelial spent after 12 and 24 hours of incubation against *S. aureus* and *E. coli*

TREATMENTS	*S. aureus*		*E. coli*	
	12hrs	24hrs	12hrs	24hrs
R. stolonifer ethanol extract	12.84^{b}	10.46^{c}	8.19^{c}	7.61^{c}
A. fumigatus ethanol extract	13.60^{b}	13.00^{b}	11.70^{b}	10.64^{b}
R. stolonifer spent	0.00^{c}	0.00^{d}	0.00^{d}	0.00^{d}
A. fumigatus spent	0.00^{c}	0.00^{d}	0.00^{d}	0.00^{d}
Distilled water	0.00^{c}	0.00^{d}	0.00^{d}	0.00^{d}
Streptomycin sulfate	26.73 ^{a}	25.82 ^{a}	25.17 ^{a}	24.00^{a}

*Means with the same letter are not significantly different with each other

Results of the present study coincides with various studies. Fungal strain *Aspergillus* species exhibited high antimicrobial activity and all the fungal extracts including *Aspergillus* species were active and were able to inhibit bacteria with different spectrum through the production of metabolites at varying degrees depending on the producing strain, extracting solvents, and the testing microbes. Antimicrobial activity *R. stolonifer* against *A. niger, A. oryzae, C. albican, P. digitatum, F. oxysporum,P. aeruginosa, E. coli,* and *S. aureus*[18,19].

Similarly, Demain & Fang stated that large number of fungal have been found to have antimicrobial activity [20]. Several studies also revealed the antibacterial activity of *Aspergillus* species against *S. aureus a*nd *E. coli* [21,22]. Findings in the study of Abdelkareem [23] showed that *A. fumigatus* from soils can produce antimicrobial compounds.

This can also be attributed to the presence of flavonoids, alkaloids and terpenoids in *R. stolonifer* and *A. fumigatus* ethanol extracts*.* Alkaloids are utilized in the development of antibacterial drugs. In addition alkaloids such as berberine exert both direct antibacterial and anti-virulence effects [24,25]. Also, alkaloids are present in ethanol extract of *A. fumigatus* have been reported to increase the antibacterial activity in antibiotics [26].

Antioxidant Activity of *R. stolonifer* and *A. fumigatus*. Antioxidants have recently become the topic of interest as radical scavengers, which inhibit the free radical mediated processes. Antioxidants are

tremendously important substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress.

A number of fungi, in particular mushrooms, have been known to possess good antioxidant activity [27]. However, much work still needs to be done to explore filamentous fungi for antioxidant activity, and the results shown by *R. stolonifer* and *A. fumigatus* support this contention.

Table 5 presented the radical scavenging activity of *R. stolonifer* and *A. fumigatus* ethanol extract and mycelial spent. *R. stolonifer* ethanol extract shows the highest percentage of RSA with 72.53% followed by *R. stolonifer* mycelial spent with 46.48% and *A. fumigatus* mycelial spent and ethanol extract with the percentage of 39.44% and 32.66% respectively.

Radical scavenging activity of *R. stolonifer* and *A. fumigatus* can also be attributed to the presence of flavonoids and terpenoids. In 2010, first reported the on antioxidant activity of *A. fumigatus* with 89.8%, 70.1%, and 74.2% scavenging effect for DPPH radical, ferrous ion and nitric oxide ion, respectively [28]. The best-described antioxidant property of flavonoids derives from its ability to directly scavenge the reactive oxygen species [29].

Also, phenolic compounds are considered to be the main secondary metabolites in plants, mushrooms, and fungi responsible for their antioxidant activity [31,32,33].Also, terpenes are reported to have a unique antioxidant in their interaction with free radicals [33].

Table 4. Free radical scavenging activity (%) of ethanol extracts and mycelial spent of *R. stolonifer* and *A. fumigatus*.

TREATMENTS	RADICAL SCAVENGING ACTIVITY (%)
R. stolonifer ethanol extract	72.53%
A. fumigatus ethanol extract	32.66%
R. stolonifer mycelial spent	46.48%
A. fumigatus mycelial spent	39.44%
Cathecin (Positive control)	76.06%

With all the findings of the present study, *R. stonifer* and *A. fumigatus* can be potential sources of natural heath remedy. However, further studies on the isolation of its bioactive compounds must be done since it is known that such filamentous fungi also produce mycotoxins which can also be dangerous to one's health.

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