

The Phytochemical and Antimicrobial Properties of Entomopathogenic Fungi in Nueva Vizcaya, Philippines

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Abstract - Entomopathogenic fungi (EPF) are potential biocontrol agents against agricultural pests and insects. These fungi are also known to be a source of secondary metabolites and could be a potential source of antibiotic drugs in the future. This study aims to determine the phytochemical and antimicrobial properties of EPF isolated from different host insects and their larvae in the province of Nueva Vizcaya. The method employed in this study includes the collection of EPF from dead insects and their larvae, isolation and mass production of the fungi, identification of the different fungi, extraction of secondary metabolites from the fungi, phytochemical screening, and antimicrobial assay. The results revealed that the antimicrobial properties of the different EPF could be explained by their phytochemical properties. When compared to the positive control, the significantly high antifungal activities of the *Pandora neoaphidis* (EPF 1) against the *Candida albicans* can be due to the presence of sterols. Conversely, the significantly high antibacterial activities of *Beauveria alba* (EPF 5) against *Bacillus subtilis* could be due to the presence of steroids, triterpenoids, glycosides, and fatty acids. These findings indicate that entomopathogenic fungi could be a potential source of antibiotic drugs against pathogenic microorganism in the near future. To realize this, future research is highly recommended for the isolation, elucidation, and evaluation of the safety of the bioactive compounds of entomopathogenic fungi responsible for the antimicrobial activities, prior to their use in humans.

Keywords: Entomopathogenic fungi, phytochemical, antimicrobial, secondary metabolites, Nueva Vizcaya

INTRODUCTION

The recent trend in mycological research is focused on the discovery of the unknown potentials and applications of fungi. These fungi play a major role in soil ecosystem along with bacteria, protists, small invertebrates and plants, through complex trophic interactions [1]. Fungi are known to produce a vast array of secondary metabolites with biotechnological applications [2]. One of the fungal endophytes that are increasingly popular nowadays is the entomopathogenic fungi.

Entomopathogenic fungi (EPF) are fungi that grow either on the surface of the insects' exoskeleton or inside their bodies [3]. These fungi were first recognized as disease causing microorganisms in insects [4]. Furthermore, EPF are also considered as potential biocontrol agents against dengue vector *Aedes aegypti* [5], malaria vector *Anopheles stephensi* Liston and filarial

Culex quinquefasciatus Say [6] and other insect pests [7] – [11].

Secondary metabolites from EPF were found to have diverse functions and activities such as insecticidal, antibiotic, cytotoxic, and ionophoric properties [12]. The new fungal metabolites isolated from *Verticillium albo-atrum* and *Verticillium leptobactrum* showed antibacterial, antifungal, antitumor, and antiviral activities [13]. *Beuveria bassiana* also showed antibacterial property against specific bacteria [1], [2]. The EPFs *Metarhiziumanisopliae*, *Nomuraea rileyi*, and *Verticillium lecanii* produced antibiotic activity against *Bacillus* and *Saccharomyces* in the presence of insect-derived materials [14].

Most of the bacteria found in nature pose a threat to human health. These bacteria could either be gram positive or gram negative bacteria. One of these

pathogenic bacteria is *Staphylococcus aureus*. It is known to colonize the human skin, nasal cavity, and gastrointestinal tract. This gram-positive bacterium causes several skin and tissue infections such as sepsis, endocarditis, osteomyelitis, and bacteremia [15]. On the other hand, the gram-positive bacteria, *Bacillus subtilis* found on the skin, other extremities of the human body [16], and in the gastrointestinal tract [17]. *Bacillus subtilis* considered important probiotic bacteria with antimicrobial potentials in human [18]. However, these bacteria were identified to be the cause of foot odor [16]. Naturally, *Escherichia coli* exist in the gastrointestinal tract of most organisms including humans and do not harm its host [19]. However, this gram negative bacteria is known to be the cause a wide array of human diseases such as diarrhea, hemorrhagic colitis, hemolytic-uremic syndrome [20], urinary tract and bloodstream infections [21], neonatal meningitis and septicemia [22].

Aside from bacteria, human pathogenic fungi also pose a threat to human health. Of all the human pathogenic fungi, *Candida albicans* is the most widespread and responsible to numerous diseases of gastrointestinal, urogenital, oral-nasal cavity and skin [23], oral and vaginal candidiasis, and systemic infections [24].

Although it has been established that entomopathogenic fungi is used extensively in agriculture as a biocontrol agent, there were limited studies that delved on the use of EPF as a source of secondary metabolites and bioactive compounds for drug discovery [25] – [27]. Hence, this study was conceptualized to solve the existing health problem of the Filipinos particularly those from the province of Nueva Vizcaya.

In Nueva Vizcaya, the rates of death per 100,000 populations are caused by pathogenic microorganisms. The death rate caused by pneumonia, chronic lower respiratory diseases, tuberculosis, septicemia, and intestinal infectious diseases are 74.7, 31.5, 11.4, 3.7, and 2.8, respectively [28]. Though there were available antibiotic drugs in the market, these drugs are expensive and synthetically formulated. Furthermore, entomopathogenic fungi are readily available since the province is a haven of different insect species. The EPF used in this study were collected from the different insect species from the lowland municipalities of the province such as Bambang and Bayombong; and from the highland

municipalities such as Diadi and Santa Fe, Nueva Vizcaya.

OBJECTIVES OF THE STUDY

This study was conducted to determine the phytochemical and antimicrobial properties of entomopathogenic fungi isolated from different host insects and their larvae in the province of Nueva Vizcaya. Specifically, this study aims to determine the secondary metabolites present in the different EPF extract. Moreover, this also aims to determine the antibacterial activities of EPF against gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus megaterium*, *Micrococcus luteus*), gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio fischeri*, *Serratia marcescens*, *Klebsiella pneumoniae*), and the pathogenic fungi (*Candida albicans*).

MATERIALS AND METHOD

Reagents

The chemicals used in this study are all analytical grade including the methanol, ethanol, dichloromethane, ethyl acetate and spray reagents. The thin layer chromatography (TLC) was performed on silica gel, SGF₂₅₄ (Merck). The silica gels F254 were aluminum-backed pre-coated TLC sheets.

Collection of samples

The entomopathogenic fungi were collected from selected municipalities of Nueva Vizcaya. These samples were obtained from dead insects or from insect larvae that were colonized by the said microfungi. The collected samples were placed in clean, sterilized plastic containers and were processed in the laboratory within 24 hours.

Isolation and sub-culture of entomopathogenic fungi

The potato dextrose agar (PDA) culture media was prepared by dissolving 39 g of PDA (Hi-Media) in 1000 mL of distilled water. Using an autoclave, the media was sterilized for 15 minutes at 121 °C, 15 psi. The sterile media was then poured into the sterile petri plates under the aseptic laminar flow hood. The collected samples were placed on the solidified agar and were incubated at room temperature for 5 days until fungi emerge. To obtain a pure fungal isolate, a small portion of the agar containing the mycelia were cut and sub-cultured onto fresh PDA plates. The

purified isolates were transferred onto the fresh PDA plates and Riddell slides for the identification of the different EPF.

Mass Production of Entomopathogenic Fungi and Extraction of Secondary Metabolites

To have enough culture of the samples, the fungal colony that were grown on the PDA plates were cut into small squares (5mm²) and were subcultured on 20 mL PDA slants for one week. After incubation, 10 mL of sterile distilled water were poured on each fungal culture and were dislodged using an inoculating loop. The dislodged mycelia and spores were inoculated on 1000 mL sterile potato dextrose broth (PDB) for three to four weeks.

To extract the crude secondary metabolites of the fungal culture, the mycelial biomass and culture broth were soaked overnight with 500 mL ethyl acetate. The mixture was filtered and evaporated in water bath at 40 °C until syrupy crude extracts were obtained. To calculate the percent yield for each of the fungal isolate, the crude culture extracts and the oven dried mycelia biomass were weighed.

Phytochemical Screening

The determination of the phytochemical properties of the EPF was employed based on standard protocol [29]. This procedure was performed to determine the number and classes of metabolites present in the sample. The crude culture extracts were spotted on TLC plates. The TLC plates were then developed in 8:2 DCM:MeOH solvent system. Initially, the spots corresponding for a particular metabolite were visualized on the TLC plates under UV light at 365 nm wavelength. Different spray reagents were used on the TLC plates to determine the different classes of compounds present in the sample. Dragendorff's reagent was used to detect the presence of alkaloids while phenols, tannins, and flavonoids were detected using FeCl₃-K₃Fe(CN)₆ reagent. Vanillin-sulfuric acid reagent was used to establish the presence of triterpenes, fatty acids, and sterols. The Borntrager reagent was also used to detect the presence

of coumarins, anthraquinones, anthones, and phenols. Leibermann-Buchard's reagent was used to confirm the presence of steroids, triterpenoids, glycosides, and gallic acid. The presences of specific secondary metabolites in the crude culture extracts were identified based on the calculated Rf values. These Rf values were calculated using the equation below:

$$R_f = \frac{\text{Distance travelled by the spot}}{\text{Distance travelled by the solvent}}$$

Antimicrobial Assay

The antimicrobial properties of the EPF were determined using the paper-disk diffusion method [30]. The crude culture extracts were tested for their inhibitory activities against gram-positive bacteria (*Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis*, *Bacillus megaterium*), gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Serratiamarscens*, *Vibrio fischeri*), and the pathogenic fungi (*Candida albicans*). The 24-hour old culture of test organisms were suspended in distilled water and the cell density were adjusted to 0.5 McFarland standard (equivalent to 1.5 x 10⁸ CFU/mL). The 6 mm diameter Whatmann paper disks previously moistened with 10mg/mL EPF extract were introduced into the culture agar plates seeded with test organisms. The culture plates were further incubated at 37° C for 24 hours. The zones of inhibition in the culture plates were measured using a digital Vernier caliper. To establish accuracy of measurement, any zone of inhibition measured for the solvent (negative control), were deducted from the zone of inhibition of the culture extracts. Streptomycin (1 mg/mL) and Nystatin (1 mg/mL) were used as positive controls for the bacteria and fungi, respectively.

RESULTS AND DISCUSSION

To ensure that the fungi were entomopathogenic, the host organisms were gathered from dead insects or dead insect larvae. The host organisms of the seven EPF are shown in Figure 1.

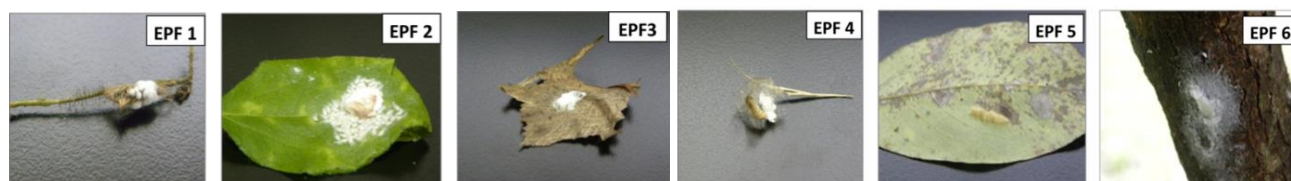


Figure 1. Host organisms of the different entomopathogenic fungi

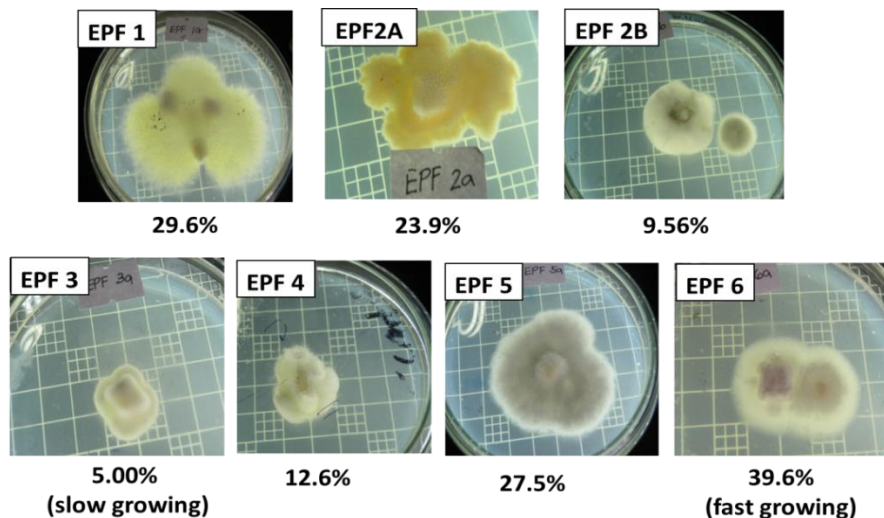


Figure 2. Fungal colony of the different entomopathogenic fungi

Figure 2 shows the fungal colony of the different EPF on the PDA media. Out of the six host insects, there were seven EPF that were present. This was determined from the fungal colonies that were isolated from the host insects. Only EPF 2 produced two fungal isolates, EPF 2A and EPF 2B.

Table 1 presents the percentage of mycelial colonization of the different EPF on PDA. The results showed that the biomass of the EPF and the weight of their extracts gave different percentage of mycelial colonization of EPF on PDA. This indicates that EPF 6 was fast growing fungi as compared to the rest of the EPF. Conversely, EPF 3 was slow growing fungi.

Based on the microscopic image of the morphological characteristics of the different EPF, six out of the seven EPF were identified by an expert mycologist as shown in Table 2. One of the fungi, EPF 3 was not identified because the morphological characters of the said EPF did not match the description in the taxonomic key, suggesting that this could be a new species of EPF. Moreover, EPF 3 is short-lived EPF and prone to contamination prior to its complete colonization of the culture media.

The phytochemical properties of the seven EPF are based on the presence of secondary metabolites in their crude extracts. The results are presented in Table 3. The results revealed that only *Isariasinclairii* (EPF 6) contained alkaloids. Studies showed that alkaloids have antibacterial, antioxidant and cytotoxic [31],

anti-ulcer [32], anticancer and anti-neuroinflammatory activities that could be used in the treatment of cognitive disorders [33].

Table 1. Percentage of mycelia colonization of the different entomopathogenic fungi on the potatodextrose agar media

Code	Biomass (mg)	Weight of extract (mg)	Percent colonization of mycelia
EPF1	2300	680	29.6 %
EPF 2A	2270	540	23.9 %
EPF 2B	2510	240	9.56 %
EPF3	2400	120	5.00 %
EPF4	2700	340	12.59 %
EPF5	2070	570	27.54 %
EPF6	2800	1110	39.64 %

Table 2. Identity of the different entomopathogenic fungi based on microscopic and morphological characters

Entomopathogenic fungi	Name
EPF 1	<i>Pandora neoaphidis</i>
EPF 2A	<i>Fusarium solani</i>
EPF 2B	<i>Beauveria bassiana</i>
EPF 3	Unknown
EPF 4	<i>Metarhiziumanisopliae</i>
EPF 5	<i>Beauveria alba</i>
EPF 6	<i>Isariasinclairii</i>

Table 3. Summary table for the presence of secondary metabolites in the six entomopathogenic fungi

Secondary Metabolites	EPF 1	EPF 2A	EPF 2B	EPF 3	EPF 4	EPF 5	EPF 6
Alkaloids	-	-	-	-	-	-	+
Phenols, Tannins, Flavonoids	-	-	-	-	-	-	-
Steroids, Triterpenoids, Glycosides	-	-	+	-	+	+	+
Gallic acid	-	-	-	+	-	-	-
Fatty acids	-	-	-	-	-	+	-
Triterpenes	-	-	+	-	+	-	-
Sterols	+	+	+	+	+	-	-

Legend: (+) present (-) absent

The other EPFs such as *Beauveriabassiana* (EPF 2B), *Metarhiziumanisopliae* (EPF 4), *Beauveriaalba* (EPF 5), *Isariasinclairii* (EPF 6) contained the secondary metabolites steroids, triterpenoids, and glycosides. Steroids exhibit multiple pharmacological and physiological activities in living organisms [34] such as antibacterial and antifungal [35] - [36], antioxidant [37], anti-inflammatory[38], cytotoxic [39], and anticancer activities [40].

Surprisingly, only EPF 3 and *Beauveriaalba* (EPF 5) contained gallic acid and fatty acids, respectively. Some studies revealed that gallic acid possess antibacterial activity especially in the treatment of skin and soft tissue infections[41]. Moreover, gallic acid showed antidepressant activity [42], and strong free radical scavenging and antimutagenic effects in both in vitro antioxidant and antimutagenic assays [43].

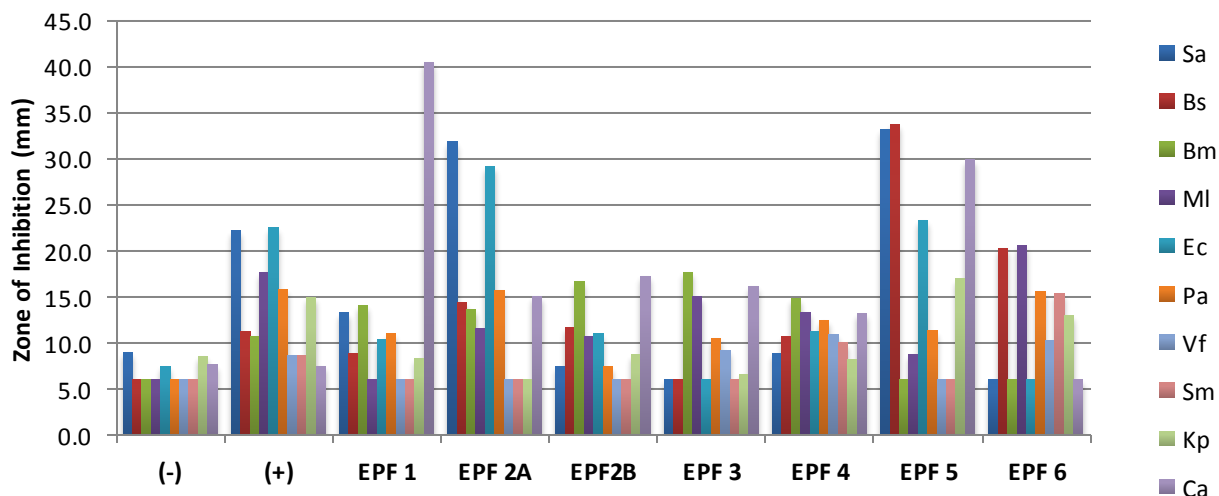
Conversely, fatty acids exhibit antibacterial, antifungal, and antioxidant activities [44] – [45]. Fatty

acids are found to enhanced cerebral blood flow, white and gray matter integrity, and improved cognitive functioning[46].

The results also showed that triterpenes are present in *Beauveriabassiana* (EPF 2B) and *Metarhiziumanisopliae* (EPF 4). Triterpenes have antibacterial, antiviral cytotoxic [47] and chemopreventive activities [48]. Triterpenes also displays hypoglycaemic activities functioning as insulin sensitizers and insulin substitutes in insulinresistant cells [49].

Table 3 further revealed that sterols are present in *Pandora neoaphidis*(EPF 1), *Fusariumsolani* (EPF 2A), *Beauveriabassiana* (EPF 2B), EPF 3, and *Metarhiziumanisopliae*(EPF 4).Sterols had been reported to have antibacterial [31], antioxidant, antidiabetic, and hypolipidemic activities [50].

Figure 3 shows the results of the zones of inhibitions of the different EPF against the different test organisms.



Legend: Sa (*Staphylococcus aureus*), Ec (*Escherichia coli*), Ca (*Candida albicans*), Ml (*Micrococcus luteus*), Sm (*Serratia marcescens*), Bs (*Bacillus subtilis*), Bm (*Bacillus megaterium*), Vf (*Vibrio fischeri*), Pa (*Pseudomonas aeruginosa*), and Kp (*Klebsiella pneumoniae*).

Figure 3. Mean zones of inhibition of the different entomopathogenic fungi against the test organisms

The microbiological assay showed that there are significant differences between the positive control and the entomopathogenic fungi extracts [$F(7, 72) = 2.87, p < .01$]. As shown in Figure 3, most of the fungi exhibited high activities against the test organisms when compared to the positive control. However, the results disclosed that most of these antimicrobial activities are not statistically significant. Surprisingly, only *Pandora neoaphidis* (EPF 1) displayed remarkably the highest antifungal activity against *Candida albicans* when compared to the positive control ($t(2) = 13.0395, p < .01$). Similarly, when compared to the positive control, only *Beauveria alba* (EPF 5) exhibited significantly the highest antibacterial activity against *Bacillus subtilis* ($t(2) = 26.105, p < .001$).

CONCLUSION AND RECOMMENDATION

The antimicrobial activities of the different entomopathogenic fungi could be explained by their phytochemical properties. The significantly high antifungal activities of the *Pandora neoaphidis* (EPF 1) against the *Candida albicans* can be due to the presence of sterols. Furthermore, the significantly high antibacterial activities of *Beauveria alba* (EPF 5) against *Bacillus subtilis* could be due to the presence of steroids, triterpenoids, glycosides, and fatty acids. Thus, entomopathogenic fungi could be a potential source for antibiotic drugs against pathogenic microorganism in the near future.

Since this study only explored on the phytochemical and antimicrobial properties of entomopathogenic fungi, the researcher highly recommends for the isolation, elucidation, and evaluation of the safety of the bioactive compounds of entomopathogenic fungi responsible for their antimicrobial activities, prior to their use in humans.

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