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IN VITRO ACTIVITY OF ASTHMA PLANT (*Euphorbia hirta* Linnaeus) LEAF EXTRACT AGAINST *Trichophyton mentagrophytes*, *Candida albicans* AND *Malassezia pachydermatis*

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ABSTRACT

Asthma plant (*Euphorbia hirta* L.), commonly known as Tawa-tawa in the Philippines, is an herbal plant that has unique properties against fungi. This study aimed to determine the *in vitro* antifungal activity of the plant's leaf crude aqueous and ethanolic extracts against *Trichophyton mentagrophytes*, *Candida albicans* and *Malassezia pachydermatis* using agar well diffusion technique. Tioconazole at 0.2 mg/ml was used as positive control. Results of the study showed a complete inhibitory effect at 100%, 75%, 50% and 25% concentrations of asthma plant leaf crude aqueous and ethanolic extracts. Crude aqueous extract has the highest mean zone of inhibition against *M. pachydermatis* and *C. albicans* at 26.67 and 17.76 mm, respectively while the crude ethanolic extract has a mean zone of inhibition against *T. mentagrophytes* at 20 mm; *C. albicans* at 19.33 mm and; *M. pachydermatis* at 21.67 mm. Qualitative phytochemical test results also revealed the presence of tannins, flavonoids, saponins and alkaloids that could be responsible for the antifungal activity of the plant leaf crude extracts against the three test organisms.

Key words: antifungal, dermatophytes, *in vitro*, secondary metabolites, Tawa-tawa

INTRODUCTION

Dermatophytosis and yeast infections are the most common fungal diseases in small animals worldwide (Mattei *et al*., 2014). Dermatophytes are confined to keratinized epithelium commonly known as ringworm that is commonly caused by *Trichophyton mentagrophytes* (Scott *et al*., 1995). The fungi are zoophilic thus close association of owners and their pets that are often asymptomatic carriers of dermatophytes can be an important source of infection (Ates *et al*., 2008). *Candida albicans*, a yeast, is a natural inhabitant of the mucous membranes of the genital, alimentary and upper respiratory tract of animals (Shepherd *et al*., 1985) while *Malassezia pachydermatis* is present on skin, lip region, interdigital area, ear canals and mucosal surface of dogs and cats (Guillot and Bond, 1999). These are opportunistic pathogens causing localized infection in immuno-suppressed patients that leads to systemic mycotic infection (Mueller *et al*., 2002).

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The availability of synthetic fungicidal agents is not always certain and local people living in rural area cannot afford to purchase them. Hence, the demand to develop cheaper and readily available herbal medicines in treating various medical conditions is increasing. Enerva *et al*. (2015) conducted a study on the cytotoxicity and antimicrobial property of *Euphorbia hirta* leaf extract. The antimicrobial analysis gave positive inhibition against *P. aeruginosa*, *S. aureus*, *C. albicans* and *T. mentagrophytes* with activity index of 0.2, 0.3, 0.4 and 0.2 respectively. Furthermore, Rao *et al*. (2010) studied the antimicrobial activity of *E. hirta* leaf ethanolic extract using agar well diffusion method. The plant's ethanolic leaf extract showed significant antimicrobial effects.

This study aims to evaluate the antifungal activity of *Euphorbia hirta* (Tawa-tawa) leaf crude aqueous and ethanolic extracts against *Trichophyton mentagrophytes*, *Candida albicans* and *Malassezia pachydermatis* using agar well diffusion technique and to profile its phytochemical contents using qualitative phytochemical tests.

MATERIALS AND METHODS

Tawa-tawa leaves collected within the premises of Visayas State University, Visca, Baybay City, Leyte were brought to the College of Veterinary Medicine for processing. Leaves were washed thoroughly with tap water, air-dried and placed in a ziplock plastic container (Galpa, 2013). Briefly, dried leaves were macerated and soaked in distilled water and ethanol at ratios of 1:2 (w:v) and 1:3 (w:v), respectively. After 48 hours, the extracts were filtered using Whatman No. 54 filter paper and was concentrated in a rotary evaporator at 60°C (Galpa, 2013). The results were called crude aqueous extract (CAE) and crude ethanolic extract (CEE), respectively. The volume, odor, color, and consistency were noted.

Aqueous and ethanolic extracts were evaluated to several qualitative phytochemical tests to determine its bioactive compounds such as alkaloid (Wagner's Test), terpenoids (Salkowski test), flavonoids (Bate-smith and Metacalf test), tannins (Ferric Chloride Test) and saponin (Froth Test) (Senguttuvan *et al*., 2014).

T. mentagrophytes from the stock cultures of the Microbiology Laboratory of the College of Veterinary Medicine, Visayas State University were subcultured onto Sabouraud dextrose agar and incubated for 7 days at 28ºC. *C. albicans* and *M. pachydermatis* on the other hand were obtained from a dog with skin disease through brush method and undergone a series of re-culturing to achieve a pure culture in Microbiology Laboratory.

Fungal suspensions were prepared using the method employed by (Fernández-Torres *et al*., 2002) and the guidelines of the Clinical and Laboratory Standards Institute with some modification. Four days old of *T. mentagrophytes* culture grown in Sabouraud dextrose agar slants were covered with 10 ml Sabouraud dextrose broth and was gently probed on the surface with the tip of a sterile loop to dislodge the conidia from the hyphal mat. The resulting conidia and hyphal mixture were then withdrawn and transferred to a sterile tube and allowed to settle for 10 minutes. The collection of the upper homogenous particles completed the preparation for fungal suspension.

For *C. albicans* and *M. pachydermatis*, the fungal inoculum was prepared by inoculating a loopful organism to Sabouraud Dextrose broth and incubated until turbidity is equivalent to 0.5 McFarland turbidity standard.

After gentle agitation, a small amount of the serially diluted 1:10 fungal suspension *T. mentagrophyte* was quantified on a hemocytometer. At 40x magnification, conidia

counting was done in the middle quadrant utilizing 5 of the 25 squares that resulted in a volume where the calculation of the concentration was based.

A total estimate of 104 cells per mL was needed for the assay (Fernández-Torres *et al*., 2002). Further dilution of the previously prepared suspension of known density was made to acquire the desired number of conidia. The total was computed using the formula:

Cell concentration / ml = Total cell count in 5 squares x 50,000 x dilution factor

Consequently, a loopful amount of *C. albicans* from the plate was transferred to 10 ml Sabouraud Dextrose broth and incubated for 6-12 hours at 37°C until the turbidity was set to 0.5 McFarland turbidity standard. The same manner was done to *M. pachydermatis*. One ml of the conidial suspension was seeded into sterile petri plate. Then 15ml of Sabouraud Dextrose Agar (SDA) was poured into the plate and rotated gently to allow thorough mixture of contents. The agar was allowed to solidify. After solidification, wells were made uniformly using a borer instrument and was labelled corresponding to the Tawa-tawa leaf crude extract concentration to be placed on the prepared media. Using a 1 mL syringe, each well was placed with 0.7 ml of the different concentrations of the plant extract along with control treatments. The plates containing *C. albicans* and *M. pachydermatis* were incubated at 37°C and checked after 24 hours for zone of inhibition. The plates containing *T. mentagrophytes* were incubated at room temperature and checked after 5 days for zone of inhibition. The resulting fungal growth in each plate was measured using a metric ruler (mm) from side to side of the clear area of zone of inhibition.

The experiment was laid out in a Complete Randomized Design (CRD). There were six different treatment groups, replicated three times, with six plastic wells in each replicate. $T0_{(-)}$ served as the negative control and was treated with distilled water or ethanol; $T0_{(+)}$ served as the positive control and was treated with 0.2 mg/ml of Tioconazole; and T1-T4 were treated with Tawa-tawa leaf crude aqueous and ethanolic extracts at 25%, 50%, 75% and 100% concentrations, respectively (Galpa, 2013).

The zone of inhibition was analyzed using the Analysis of Variance (ANOVA). Significant differences (P<0.05) between treatment means were compared using Tukey's Honestly Significant Difference.

RESULTS AND DISCUSSION

Table 1 shows the summary of the mean zone of inhibition (ZI) (mm) of Tawa-tawa crude aqueous and ethanolic extracts against *Trichophyton mentagrophytes*, *Candida albicans* and *Malassezia pachydermatis*. The zone of inhibition was measured using a metric ruler in millimeters (mm). At 25%, 50%, 75% and 100% CEE concentrations, the zone of inhibition revealed a complete inhibitory activity with severe reactivity against the test organisms. The same results were also achieved using CAE with the exception at *T. mentagrophytes* which yielded less reactivity. Among all treatments, $T0_{(+)}$ (0.2 mg/ml Tioconazole) produced the widest zone of inhibition and had severe reaction with complete inhibitory activity against the test organisms. A dose-dependent manner can also be observed because as the varying concentrations increases, there is a direct proportional effect in terms of zone of inhibition.

For *T. mentagrophytes*, CAE results showed that $T0_{(+)}$ (positive control) treated with

Table 1. Mean zone of inhibition (ZI) (mm) of Tawa-tawa leaf CAE and CEE against *T. mentagrophytes*, *C. albicans* and *M. pachydermatis*.

Treatments	*T. mentagrophytes* (ZI)		*C. albicans* (ZI)		*M. pachydermatis* (ZI)	
	CAE	CEE	CAE	CEE	CAE	CEE
$T0_{(-)}$ (Ethanol)	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
$T0_{(+)}$ (0.2 mg/ml Tioconazole)	22.67 ± 2.52^{d}	22.00 ± 2.65^{d}	22.67 ± 2.52^{e}	24.67 ± 1.54^{d}	30.33 ± 2.08^{c}	30.67 ± 1.15^{e}
T1 (25%)	8.33 ± 0.577^{b}	12.67 ± 0.58^{b}	10.00 ± 1.00^{b}	13.67 ± 1.54^{b}	21.00 ± 3.60^{b}	12.67 ± 1.53^{b}
T2 (50%)	9.33 ± 0.577^{bc}	13.67 ± 0.58^{b}	12.67 ± 0.577^{bc}	15.33 ± 1.00^{b}	22.33 ± 3.51^{bc}	15.00 ± 1.73^{bc}
T3 (75%)	11.00 ± 1.00^{c}	16.00 ± 1.00^{bc}	14.34 ± 1.15^{cd}	17.33 ± 0.58^{c}	24.67 ± 3.51^{bc}	18.67 ± 2.87^{cd}
T4 (100%)	13.33 ± 0.577^{c}	20.00 ± 4.00^{cd}	17.67 ± 1.52^{d}	19.33 ± 0.58^{c}	26.67 ± 3.06^{bc}	21.67 ± 2.08^{d}

0.2 mg/ml Tioconazole has the highest mean zone of inhibition of 22.67 mm. This finding has a high significant difference among the means of varying Tawa-tawa concentrations: T1 (25%), T2 (50%), T3 (75%) and T4 (100%) with mean zones of inhibition of 8.33 mm, 9.33 mm, 11.00 mm and 13.33 mm, respectively. This implies that the varying concentrations from T1 to T4 are less reactive to the organism as compared to the positive control. While in CEE, $T0_{(+)}$ (positive control) also had the highest mean zone of inhibition of 22.00 mm which gave a highly significant difference among the treatment means of T1 (12.67 mm), T2 (13.67 mm) and T3 (16 mm). However, its effect is comparable with T4 (100%) with a mean zone of inhibition of 20 mm.

The varying concentrations of Tawa-tawa leaf CAE and CEE were both effective against *C. albicans* but the highest mean zone of inhibition was exhibited by the positive control (0.2 mg/ml Tioconazole). Results showed that it is significantly different compared to T1 (25%), T2 (50%), T3 (75%) and T4 (100%). Statistically, this means that the effect of 0.2 mg/ml Tioconazole is incomparable with the varying concentrations of crude aqueous and ethanolic extracts against *C. albicans*.

Among the three organisms tested for zone of inhibition, *M. pachydermatis* has the most sensitivity towards CAE and CEE. As for CAE, even if $T0_{(+)}$ (0.2 mg/ml Tioconazole) has the highest mean zone of inhibition (30.33 mm) among all treatments, its effect is statistically comparable with T2 (ZI= 22.33 mm), T3 (ZI= 24.67 mm) and T4 (ZI= 26.67 mm).

Extract solvent is one of the most significant factors affecting the chemical composition and biological activity of plant extracts. In this study, crude ethanolic extract rendered a more consistent antifungal activity compared to crude aqueous extract because nearly all of the identified plant components active against fungi are aromatic or saturated organic compounds (Turkmen *et al*., 2006; Das *et al*., 2010). Hence, it is very easy for ethanol to penetrate the cellular membrane of the plant's cells. Therefore, the bioactive components of plants are easily extracted by ethanol. This makes it an ideal solvent for initial extractions

(Cowan, 1999). Since both water and ethanol are polar, there is a tendency to extract similar phytochemicals as recorded in this study.

The qualitative phytochemical test results (Table 2) revealed that Tawa-tawa leaf CAE and CEE contain bioactive compounds namely tannins, flavonoids, saponins and alkaloids which could be effective against the test organisms. These phytochemicals are known to possess a diverse pharmacologic activity against many types of microorganisms. Alkaloids rank among the most efficient and therapeutically significant plant substances, hence they are widely used in medicine for the development of drugs (Harborne, 1998). They are generally toxic to micro-organisms (Mattei *et al*., 2014) by inhibiting spore germination and mycelial growth significantly substantiating their fungicidal potentials (Iwu *et al*., 1999). It has also been shown that saponins are active antifungal agents by disrupting the membrane integrity of fungal cells that leads to fungal apoptosis (Iwu *et al*., 1999; Lee and Lee, 2015). Flavonoids also act by inhibiting DNA and RNA synthesis, energy metabolism, and cytoplasmic membrane function of microorganism (Galpa, 2013) and tannins have been reported to hinder the tertiary structure of proteins by directly binding with the proteins thus effectively inhibit the function of ABC transporters that enables the fungal pathogens resistant to the administered drug (Ansari *et al*., 2013).

In conclusion, Tawa-tawa leaf crude aqueous and ethanolic extracts possess antifungal activity against *T. mentagrophytes*, *C. albicans* and *M. pachydermatis* because of its bioactive compounds such as saponins, tannins, flavonoids and alkaloids. The results can serve as a benchmark for further pharmacological study of the said plant extract.

Table 2. Qualitative phytochemical tests of Tawa-tawa leaf CAE and CEE.

Phytochemicals	Tests	Reactions		Descriptions
		CAE	CEE	
Alkaloids	Wagner's Test	+	+++	Reddish brown precipitate
Flavonoids	Bate-smith and Metacalf Test	++	++	Red or violet color
Tannins	Ferric chloride Test	+++	++	Blue-black and brownish green color
Saponins	Froth Test	+	+++	Froth formation
Terpenoids	Salkowski Test	-	-	Red-brownish green

Legend: ++++ Maximum amount
+++ Good amount
++ Moderate amount
+ Low amount
- Absent

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