

In vitro activity of Indian almond (*Terminalia catappa*) leaf crude extracts against selected dermatophytes

Roxanne Joy B. Colendres and Carl Leonard M. Pradera^{1*}

ABSTRACT

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Fungal infections caused by *Trichophyton mentagrophytes*, *Aspergillus fumigatus*, and *Malassezia pachydermatis* are among the major contributors to multisystemic health problems such as dermatitis, otitis, and respiratory disorders among humans and animals. This study was conducted to determine the in vitro antifungal activity of *Terminalia catappa* leaf crude aqueous and ethanolic extracts against these fungal pathogens by measuring the zone of inhibition (ZI) using the agar well diffusion technique. Qualitative phytochemical screening tests were also performed to determine bioactive compounds present in the plant extract.

Results show that the plant's crude aqueous (CAE) and ethanolic extracts (CEE) were found to be effective against all test fungi. *M. pachydermatis* showed susceptibility towards CAE and CEE from T1 (100%), T2 (75%), T3 (50%) and T4 (25%), with the highest mean ZI of 18.33mm and 13.33, respectively. On the other hand, *T. mentagrophytes* was inhibited by CAE and CEE at T1 (100%), T2 (75%) and T3 (50%) with the highest mean ZI of 9.67mm and 10.33mm, respectively. At the same time, it was observed that *A. fumigatus* had reactive sensitivity towards CAE and CEE at T1 (100%) and T2 (75%), with the highest mean ZI of 9.33mm and 10.33mm, respectively. Moreover, phytochemical tests showed that the plant's leaf crude extracts contain alkaloids, saponins, and tannins, which could potentially inhibit fungal growth.

Keywords: Antifungal, *Aspergillus fumigatus*, bioactive compounds, *Malassezia pachydermatis*, *Trichophyton mentagrophytes*, zone of inhibition

¹College of Veterinary Medicine, Visayas State University, Baybay City, Leyte

*Corresponding Author. Address: College of Veterinary Medicine, Visayas State University, Baybay City, Leyte; Email: carl.pradera@vsu.edu.ph

INTRODUCTION

Dermatophytosis is known as the most common fungal infection in companion animals, such as dogs and cats (Moriello et al 2017). This disease is commonly caused by *Trichophyton mentagrophytes*, *Aspergillus fumigatus* and *Malassezia pachydermatis* (Mercantini et al 1995, Seyedmousavi et al 2015). As a zoonotic disease, it accounts for a high percentage of health problems among humans and animals (Mederle et al 2010, Pasquetti et al 2017). Specifically, fungal infection caused by *Trichophyton mentagrophytes* is characterized by alopecia with erythema and desquamation of epithelial cells. On the other hand, *Aspergillus fumigatus*, a saprophytic filamentous fungus, can cause localized skin infection to multisystemic diseases that can be fatal, as well as allergic response to inhaled conidia (Seyedmousavi et al 2015) while *Malassezia pachydermatis* is a lipophilic yeast that is commonly associated with otitis externa and seborrheic dermatitis in dogs and cats (Cristina and Degi 2009).

To address these dermatological issues, there are several synthetic antifungal drugs that are readily available in the market but is quite expensive and have shown to develop resistance against these pathogens (Bustamante et al 2020, Chen et al 2020). Moreover, the continuous rise of life-threatening invasive fungal infections caused by *Trichophyton mentagrophytes*, *Aspergillus fumigatus*, and *Malassezia pachydermatis* have caught the attention of medical scientists to expand antifungal drug researches (Andriole 2000). However the production of new antifungal drugs takes more time to develop and requires extensive clinical studies before they can be of general use (Roemer and Krysan 2014). Hence, ethnoveterinary products and the development of alternative herbal preparations that are safe, economical, and readily available is being explored nowadays (Stein et al 2005), one of which is Indian almond (*Terminalia catappa*) or locally known as Talisay.

Several studies have already been explored for the potential medicinal properties of *T. catappa*. These include but not limited to the antimicrobial, antifungal, antioxidant and even potential cytotoxic effects and phytochemical properties of the plant extracts (Espinosa Ruiz et al 2012, Terças et al 2017). In a study that was conducted by Salares and Balala (2018), it was able to demonstrate the antimicrobial activity of this plant's leaf crude ethanolic extract. Specifically, results have shown susceptibility of both gram- positive and gram-negative bacteria at 5,000 $\mu\text{g mL}^{-1}$ to 10,000 $\mu\text{g mL}^{-1}$ crude ethanolic concentration. In addition, *Candida albicans* was found to be more sensitive as compared to *Microsporium canis* at 78.12 $\mu\text{g mL}^{-1}$ minimum inhibitory concentration. Moreover, the antifungal efficacy of *T. catappa* wood and bark aqueous, ethyl acetate, and hexane extracts against several fungal pathogens have also been documented wherein 50, 100 and 150 μL of the wood aqueous extracts were found to be effective against *Aspergillus fumigatus*, *Microsporium gypseum*, *Mucor sp.*, *Penicillium sp.*, *Rhizopus sp.*, *Scopulariops sp.*, and *Sporothrix scheckii*, while the bark aqueous extracts of the same concentrations were effective against *Candida albicans*, *Ganoderma sp.*, *Sporothrix scheckii* and *Trichoderma* species. In addition, 28mm per 150 μL of the wood ethyl acetate extracts revealed a maximum inhibitory activity against *A. fumigatus*, *Scopulariops sp.*, and *M. gypseum*, while 26mm per 150 $\mu\text{g mL}^{-1}$ and 24mm per 150 μL of the bark ethyl acetate extracts showed a maximum inhibitory activity against *A. fumigatus* and *M. gypseum*, respectively

(Gandhi et al 2015). The in vitro antifungal activities of *T. catappa* and *T. arjuna* methanolic and ethanolic leaf extracts against *Aspergillus niger*, *Alternaria alternata*, *Curvularia lunata* and *Trichophyton tonsurans* were also reported (Mandloi et al 2013).

With the various studies of the promising therapeutic properties of *T. catappa* extracts, our study focused on the in vitro antifungal activity of the plant's leaf crude aqueous and ethanolic extracts against *Trichophyton mentagrophytes*, *Aspergillus fumigatus*, and *Malassezia pachydermatis*. Hence, the results of this study could provide essential information for the potential in vitro pharmacologic effects of the plant leaf crude extracts against these fungal agents by measuring its zone of inhibition and determining the presence of phytochemicals and bioactive compounds that could be responsible for the antifungal property of the plant leaf crude ethanolic and aqueous extracts. Furthermore, this study can serve as a benchmark data for further in vivo tests and clinical trials in order to formulate ethnoveterinary products with effective antifungal properties.

MATERIALS AND METHODS

Preparation of Leaf Crude Extract

Collection and Processing of Plant Material. Mature Indian almond leaves were brought and presented to the Department of Biological Sciences, Visayas State University for proper botanical identification. After which, leaves were washed thoroughly, cut into small pieces, and air-dried at room temperature until at least 80% of the moisture content was removed and they became crisp and brittle. Dried leaves were preserved in a zip lock plastic, and stored at room temperature for three days until use (Ballentes and Pradera 2019).

Extraction of *T. catappa* Leaf. This procedure was done at the Diagnostic Laboratory of the College of Veterinary Medicine, Visayas State University. Following the methods applied by Fernandez Jr et al (2013) with some modifications, dried leaves of Indian almond were pulverized in a blender before they were extracted either using distilled water or absolute ethanol as solvent systems, accordingly. About 250g of dried and pulverized leaves were soaked in a beaker containing 750mL of absolute ethanol (1:3w/v) and was left to stand on a bench for 48h. After which, the leaves were filtrated using a laboratory grade filter paper No. 54 (Whatman®) into an Erlenmeyer flask. Similarly, a separate 250g of dried leaves were soaked in a beaker containing 750mL of distilled water and was processed as above. The filtrates were identified as crude ethanolic extract (CEE) and crude aqueous extract (CAE), respectively. These were further concentrated separately in a rotary evaporator for 1-2h at 60°C and 40°C, respectively. The CEE and CAE were transferred separately to amber bottles and were left to stand at room temperature for 24h to allow solvents to evaporate. The extracts were kept under 4°C for one day until needed for the assay.

Qualitative Phytochemical Screening of *T. catappa* Leaf Crude Extracts

T. catappa leaf crude ethanolic and aqueous extracts were individually subjected to several phytochemical tests at the Microbiology Laboratory of the College of Veterinary Medicine, Visayas State University to classify the bioactive components present. Following the methods performed by Harborne (1984) and Claustra et al (2005) with some modifications, these screening tests were conducted to detect the presence of alkaloids, flavonoids, saponins, terpenoids, and tannins in both crude ethanolic and aqueous extracts. Specifically, to screen for alkaloids, two drops of Dragendorff or Wagner's reagent were added to two mL of the test solution and the formation of orange precipitates indicate the presence of these bioactive compounds. Moreover, Bate-Smith and Metacalf Test was used to determine the presence of flavonoids. In here, one mL of the test solution was added with two drops of 80% ethyl alcohol and hydrochloric acid. After 15mins of submerging the mixture in a hot water bath, a red or violet coloration indicates the presence of leucoanthocyanins and a pink to yellow-orange coloration indicates the presence of anthocyanins, both of which are flavonoids. Froth test was used to screen for saponins. As the word implies for this test, a copious lather formation indicates the presence of saponins after adding distilled water into two mL of the test solution. Furthermore, a reddish-brown coloration confirms the presence of terpenoids via Salkowski Test after adding two drops of chloroform and concentrated sulfuric acid into two mL of the test solution. Lastly, Ferric chloride Test was used to screen for tannins in which two mL of the test solution was added with two drops of ferric chloride. After 1-2min, a color change of bluish-black and brownish-green indicate the presence of condensed tannins. The chemicals and reagents used during phytochemical screening tests were provided by the Department of Pure and Applied Chemistry, Visayas State University.

Preparation of Test Organisms

The preparation of test organisms followed standard laboratory procedures as described by Ballentes and Pradera (2019) with some modifications. *Trichophyton mentagrophytes*, *Aspergillus fumigatus*, and *Malassezia pachydermatis* were obtained from the stock cultures of the Microbiology Laboratory of the College of Veterinary Medicine, Visayas State University. *T. mentagrophytes* and *A. fumigatus* were sub-cultured onto Sabouraud dextrose agar slants and then incubated for 7 days at 27°C. On the other hand, *M. pachydermatis* was streaked onto Sabouraud dextrose agar and incubated for one day at 37°C.

Preparation of Fungal Suspensions. Inoculum suspension of each isolate of the designated fungi was prepared from 7-day-old cultures grown on Sabouraud dextrose agar slants. To dislodge the conidia of *T. mentagrophytes* and *A. fumigatus* from the hyphal mat, the fungal colonies were covered with 10mL of sterile water and the surface was probed gently using the tip of a sterile loop. Then, the conidial and hyphal fragments were transferred to a sterile tube and permitted to settle for 10min and the upper homogenous suspension was collected. Subsequently, a loopful amount of *M. pachydermatis* from the slant was moved to 10mL of Sabouraud dextrose broth with 0.2mL Tween 80 and incubated for several

hours at 37°C until the turbidity was set to 9.5 McFarland turbidity standard. This was done to provide an optical density similar to the density of a bacterial suspension at 1.5×10^8 colony-forming unit (CFU) that is used for agar well diffusion method (Fernández-Torres et al 2002).

Determination of the density of the Conidia. This step followed the method employed by Oliveira et al (2015) and Ballentes and Pradera (2019) with some modifications. From serially diluted suspension (1:10), a small amount was taken and placed on a hemocytometer to quantify the desired conidia for the assay. At low power objective, the number of conidia was totaled at the middle quadrant using only 5 of the 25 squares in the middle quadrant. Manipulation was made in the previously prepared suspension of known density to come up with the desired number of conidia by further dilution. The total number of conidia was computed using this formula:

$$\text{Cell concentration/mL} = \text{Total cell count in 5 squares} \times 50,000 \times \text{dilution factor}$$

Antifungal Assay of Crude Extract

Agar Well Diffusion Technique. Following the standard laboratory procedures and as described by Ballentes and Pradera (2019), Sabouraud's Dextrose Agar (SDA) was used as culture medium for the assay. For *T. mentagrophytes*, one ml of fungal inoculum was poured into the sterile petri plates using a sterile pipette and 15mL molten SDA were added. The plates were rotated eight times each in clockwise rotation and in vertical direction to evenly distribute the agar and test organisms. Once hardened, wells were made on the agar using a borer with a diameter of 7mm. The same method was also applied for *A. fumigatus*. For *M. pachydermatis*, SDA was hardened first and the test organism was swabbed. About 0.7mL of the varying concentrations of *T. catappa* leaf crude ethanolic and aqueous extracts were placed into each corresponding well. The preparation was incubated at 27°C for 4 days for *Trichophyton mentagrophytes* and *Aspergillus fumigatus* and at 37°C for 2 days for *Malassezia pachydermatis*. After incubation, the zone of inhibition (ZI) was measured from side to side using a metric ruler (mm) and the results were compared to the standard measurement set by the Clinical Laboratory Standard Institute.

Experimental Design

The experiment was laid out in a Completely Randomized Design (CRD). There were seven treatment groups, replicated three times, with seven plastic wells in each replicate. The treatment groups are shown below:

T0₍₋₎ = Distilled water (negative control)

T0₍₋₎ = Absolute ethanol (negative control)

T0₍₊₎ = Tioconazole at 1 mg mL^{-1} (positive control)

T1 = 10µL of 100% *T. catappa* leaf crude aqueous and ethanolic extracts, respectively

- T2 = 10 μ L of 70% *T. catappa* leaf crude aqueous and ethanolic extracts, respectively
- T3 = 10 μ L of 50% *T. catappa* leaf crude aqueous and ethanolic extracts, respectively
- T4 = 10 μ L of 25% *T. catappa* leaf crude aqueous and ethanolic extracts, respectively

These concentrations were used after a pilot test was conducted prior to its final assay.

The zone of inhibition (ZI), a circular area around the spot of the antifungal drugs in which the fungal colonies do not grow (Cooper 1955, Hsu and Lockwood 1969), was measured using a metric ruler (mm) and the breakpoints for disc diffusion method recommended by the Clinical Laboratory Standard Institute was used with some modifications. Specifically, a clear zone of inhibition measuring <0.125mm, 0.25–0.5mm, and >1.00mm were considered susceptible, susceptible-dose dependent, and resistant, respectively (Rex et al 1997). Data were analyzed using Analysis of Variance (ANOVA) and significant differences (p -value:<0.05) between treatment means were determined and compared by way of Tukey's Honestly Significant Difference (HSD) using the SPSS statistical software program (Verma 2012).

RESULT AND DISCUSSION

The antifungal activities of the varying concentrations of *T. catappa* leaf crude aqueous extract (CAE) and crude ethanolic extract (CEE) are listed in Table 1. Results showed that the plant's leaf crude aqueous and ethanolic extracts were both effective against the in vitro growth of *Malassezia pachydermatis* from T1 (100%) to T4 (25%), *Trichophyton mentagrophytes* from T1 (100%) to T3 (75%), and *Aspergillus fumigatus* from T1 (100%) to T2 (50%). This has been observed by measuring its mean zone of inhibition (ZI) using these concentrations. For instance, the CAE showed a complete inhibitory effect at 100% (ZI=18.33mm), 75% (ZI=15mm), 50% (ZI=13.67mm) and 25% (ZI=9.33mm) concentrations against *M. pachydermatis*. The same result was also observed using CEE but with less sensitivity such that T1 (100%), T2 (50%), T3 (50%) and T4 (25%) yielded a mean zone of inhibition of 13.33mm, 10.67mm, 8.67mm and 8mm, respectively. The promising antifungal properties of *Terminalia catappa* shown in this study conforms with the results presented by several researches (Ahon et al 2011, Gandhi et al 2015, Rubini et al 2013). In one study, it was documented that the plant's leaf ethanolic and methanolic fractions are effective against *Aspergillus niger*, *Trichophyton tonsurans*, *Curvularia lunata* and *Alternaria alternata* with varying levels of sensitivity (Mandloi et al 2013). A similar finding was also obtained to assess the antifungal activity against *Candida albicans* using gas chromatography coupled to mass spectrometry ionization and hydrogen nuclear magnetic resonance techniques (Terças et al 2017). Moreover, the antifungal activity of the same plant extracts against *Candida albicans*, *Aspergillus fumigatus* and

Trichophyton mentagrophytes has been optimized (Bognan et al 2016). However the present study also noted that the positive control, Tioconazole (1mg mL^{-1}), is the most effective among all treatment groups based on its mean zone of inhibition, and is statistically significant ($p\text{-value}<0.05$) compared to the varying concentrations of the plant's leaf crude extracts against all fungal pathogens. This means that despite the efficacy and varying reactive sensitivity of the test fungi towards *T. catappa* leaf crude solvent extracts, the positive control, Tioconazole at 1mg mL^{-1} , is still a more effective therapeutic drug. Therefore, other plant parts and solvent systems can also be explored as a more potential source of therapeutic agents which may lead in the ongoing search for antimicrobial botanicals (Manzur et al 2011).

Table 1. Zone of inhibition (mm) between each treatment group against test fungi and between crude aqueous and ethanolic extracts

Treatment	Zone of Inhibition (mm)					
	<i>Malassezia pachydermatis</i>		<i>Trichophyton mentagrophytes</i>		<i>Aspergillus fumigatus</i>	
	Aqueous	Ethanolic	Aqueous	Ethanolic	Aqueous	Ethanolic
T0(-) distilled water, ethanol	0	0	0	0	0	0
T0(+) Tioconazole (1mg/mL)	29.67±0.33 ^a	27.33±0.33 ^a	24.33±0.67 ^a	25±0.58 ^a	23±0.58 ^a	23.67±0.67 ^a
T1 (100%)	18.33±0.88 ^b	13.33±0.88 ^b	9.67±0.33 ^b	10.33±0.88 ^b	9.33±0.33 ^b	10.33±0.33 ^b
T2 (75%)	15±1 ^c	10.67±0.67 ^c	8.33±0.33 ^{bc}	9.33±0.33 ^{bc}	8 ^c	8.67±0.33 ^b
T3 (50%)	13.67±0.67 ^c	8.67±0.33 ^{cd}	8 ^c	8.33±0.33 ^c	0	0
T4 (25%)	9.33±0.33 ^d	8 ^d	0	0	0	0

n=5; Means with different letter superscript are significantly different ($p\text{-value}<0.05$).

Generally, it is also observed that the efficacy of *T. catappa* leaf crude aqueous and ethanolic extracts followed a dose-dependent manner. This means that as the concentration increases, the zone of the inhibition also increases such that, T1 (100%) has the highest mean zone of inhibition among all concentrations. Specifically, *M. pachydermatis* was shown to have higher sensitivity towards crude aqueous extracts compared to crude ethanolic extracts with T1 (100%) having the highest mean zone of inhibition (18.33mm). Inversely, *T. mentagrophytes* and *A. fumigatus* were more sensitive to the plant's leaf CEE than CAE. For *T. mentagrophytes*, the highest mean zone of inhibition was noted at T1 (ZI=10.33mm) followed by T2 (ZI=9.33mm) and T3 (ZI=8.33mm) while the highest mean zone of inhibition against *A. fumigatus* was also observed at T1 (ZI=10.33mm) followed by T2 (ZI=8.67mm).

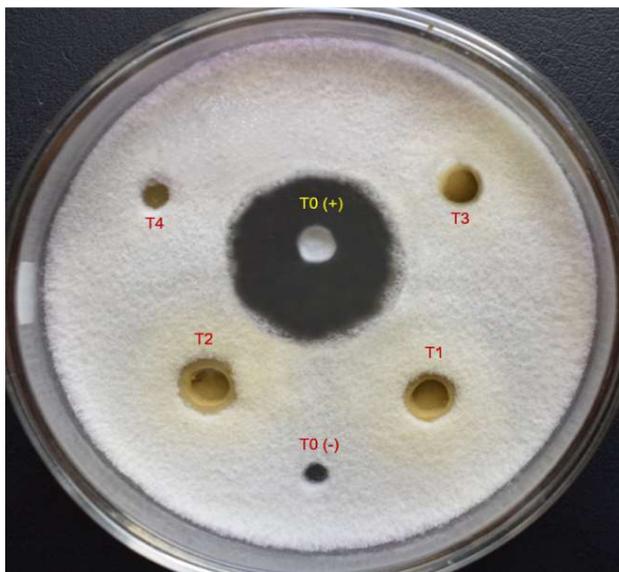


Figure 1. Zone of inhibition (mm) of *Trichophyton mentagrophytes* treated with varying concentrations of *Terminalia catappa* leaf crude ethanolic and aqueous extracts

The chemical and biological properties of a plant extract are significantly influenced by the solvent system that is used for extraction. Choice of solvent for extraction of plant material depends on solubility and type of compounds intended to isolate –(Cheok et al 2012, Rebey et al 2012). Ethanol is considered as an ideal solvent for initial extraction because almost all of the antifungal bioactive compounds that can be extracted using this solvent are aromatic or saturated organic –(Das et al 2010, Turkmen et al 2006). That is why, *M. pachydermatis* is more susceptible towards the plant's leaf crude aqueous extract while *T. mentagrophytes* and *A. fumigatus* have higher reactive sensitivity by using crude ethanolic extract. Since both solvent systems (ethanol and distilled water) used in this study are polar in nature (Barbouchi et al 2020), there is a higher chance to extract almost similar phytochemical compounds (Cowan 1999) as shown in Table 2. These bioactive compounds may be responsible for the antifungal activities of the plant extracts –(Cordell 2011). Specifically, tannins are known to inhibit the extracellular microbial enzymes and deprive the substrates and metal ions required for microbial growth (Cowan 1999) while alkaloids and saponins are not only capable of complexing extracellular proteins but also of breaking the microbial membranes causing cell death (Lalitha and Venkataraman 1991, Wang et al 2020, Zhang et al 2006). The present study was conducted using qualitative analysis of the bioactive compounds only, and more sophisticated methods are suggested to be performed to justify these claims.

Table 2. Qualitative phytochemical tests of *T. catappa* leaf crude aqueous and ethanolic extracts

Phytochemicals	Tests	Descriptions	Reactions	
			CAE	CEE
Terpenoids	Salkowski Test	Red brownish coloration	-	-
Saponins	Froth Test	Formation of froth or bubbles	-	++
Tannins	Ferric chloride Test	Bluish black and brownish green coloration	+++	++++
Flavonoids	Bate-smith and Metacalf Test	Red to violet or pink to yellow orange coloration	-	-
Alkaloids	Wagner's Test	Orange to reddish brown precipitates	+++	++++

Legends: CAE: crude aqueous extract
 CEE: crude ethanolic extract
 ++++ Maximum amount
 +++ Good amount
 ++ Moderate amount
 + Low amount
 -Absent

CONCLUSIONS

In general, both the crude ethanolic (CEE) and aqueous (CAE) leaf extracts of *Terminalia catappa* were shown to have promising antifungal properties against *Trichophyton mentagrophytes*, *Aspergillus fumigatus* and *Malassezia pachydermatis* based on the observed zone of inhibition. More specifically, *T. mentagrophytes* were susceptible to T1 (100%), T2 (75%), T3 (50%) and T4 (25%), while *A. fumigatus* were susceptible at T1 (100%), T2 (75%) and T3 (50%). Additionally, T1 (100%) and T2 (75%) of the plant's leaf crude extracts were effective against *M. pachydermatis*. Moreover, the CAE were able to draw out tannins and alkaloids while the CEE got saponins, tannins and alkaloids. However, a higher mean zone of inhibition exhibited by the plant's leaf crude ethanolic extract compared to its crude aqueous extract would indicate that the former solvent system is more effective in extracting potential antifungal agents against *M. pachydermatis*. Being said, the crude ethanolic extract is also more effective in extracting potential bioactive compounds against *T. mentagrophytes* and *A. fumigatus*. For further related studies, different methods in the application of extract may be employed in antifungal assay as well as determination of the Minimum Inhibitory Concentration (MIC). Furthermore, using of other solvent systems in extracting the bioactive compounds can also be recommended.

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