Phytochemical Screening and Antimicrobial Activity of *Terminalia catappa* L. Leaf Extract Against Potential Pathogens of Animals

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Abstract

The challenge of antimicrobial resistance prompted exploration of therapeutic products from medicinal plants. This study evaluated the antimicrobial activity of *T. catappa* leaf extract against *Staphylococcus aureus*, *Bacillus cereus*, *Bordetella bronchiseptica*, *Pasteurella multocida*, *Candida albicans*, and *Microsporum canis*. Different concentrations were used to establish the minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) of the plant extract. The extract demonstrated a broad antimicrobial spectrum by inhibiting both gram-positive and gram-negative bacteria at MIC range of 5,000 to 10,000 µg/mL. *C. albicans* was inhibited at 78.12 µg/mL and *M. canis* at 312.5 µg/mL. The MIC-MBC ratios and the MIC-MFC ratios indicate that *T. catappa* leaf extract has bactericidal action, fungicidal action to *M. canis*, and fungistatic action to *C. albicans*. Tannins, saponins and alkaloids were detected from the extract, which may be responsible for the antimicrobial activities of the plant.

Keywords: Leaf extract; Minimum bactericidal concentration; Minimum fungicidal concentration; Minimum inhibitory concentration

Introduction

Continued indiscriminate use of drugs, evolutionary adaptation and genetic mutation of pathogens have imposed a great deal of challenge for the medical community to provide safe and effective therapeutic agents. This led to a global concern that paved a way to the exploration of natural products as possible alternatives. Plants are among nature’s abundant resources that play a prominent role in the primary health care of about 80% of the world’s population (Naz et al., 2007). In several applications, medicinal plants are shown to inhibit the growth and reduce the number of many serious pathogens that have plagued mankind. Plants produce an enormous array of phytochemicals, and it is commonly accepted that a significant part of this chemical diversity is related to their defense mechanisms against wide range of pathogens (Koenraad et al., 2001). The Philippines, located in the tropics is home to several medicinal plants. One of these is *Terminalia catappa*, locally known as talisay, a large, spreading tree found along coastal environments. This tree is tolerant to strong winds, salt spray and moderate high salinity of the root zone; and it grows in freely drained, well-aerated, sandy soils (Chanda et al., 2011). Almost all of its parts can be useful in decoration (seeds), medication (seeds, leaves, bark, roots) and

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food (seeds). The leaves are used to treat dermatitis, hepatitis (Lin et al., 1997) and leprosy. The leaf, bark and roots are used for antipyretic and hemostatic purposes in India, Philippines, Malaysia and Indonesia (Poongulali & Sundararaman, 2016). The chloroform root extract of T. catappa showed antimicrobial activities against Escherichia coli and Staphylococcus aureus (Anand et al., 2015), while the methanolic leaf extracts inhibited Staphylococcus aureus, Escherichia coli, Salmonella typhi, Bacillus cereus, Zymomonas mobilis and Serratia marcescens using agar disc diffusion technique (Rajesh et al., 2015). Furthermore, chloroform fraction of the leaves showed activity against gram-negative Escherichia coli and Salmonella typhi at 500 µg/disc, while ethanol and aqueous methanol fractions were active only on Salmonella typhi at concentrations of 300 – 500 µg/disc (Mudi & Muhammad, 2011).

Several studies have been done on the antimicrobial activity of T. catappa against human pathogens and sparsely, on pathogens affecting animals. Limited studies are available that explore the antimicrobial activity of T. catappa that flourishes in the Philippine soil. To determine the antimicrobial activity of T. catappa on selective animal pathogens, the minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) of the leaf extract on Staphylococcus aureus, Bacillus cereus, Bordetella bronchiseptica, Pasteurella multocida, Microsporum canis, and Candida albicans were investigated. Qualitative phytochemical screening was done to uncover the major phytochemical groups with potential antimicrobial action.

**Methodology**

**Preparation of T. catappa Ethanolic Leaf Extract**

Mature, obovate leaves of T. catappa were harvested from the coastal area of the Visayas State University, Baybay City, Leyte. The leaves were thoroughly washed to remove dirt and contaminants. Using clean scissors, the leaves were cut into small pieces and dried at room temperature until crisp and brittle. The initial and final weights of the leaves were recorded to determine the moisture content loss. About 300 g of the dried leaves were infused in 1500 mL ethanol (1:5, w/v) for 48 hours and filtered in a flask using Whatman® filter paper No. 54. This produced the crude leaf ethanolic extract, which was concentrated in a rotary evaporator at 40°C until the volume was reduced to one-third of the original volume. The extract was transferred to an amber bottle and set aside for 24 hours to allow evaporation of the remaining solvent and for chlorophyll to settle at the bottom. The separated portion of the extract was carefully pipetted out and kept in a sterile container until use.

**Test Organism**

Four strains of bacteria were obtained from the Microbiology Laboratory of the College of Veterinary Medicine, Visayas State University, Baybay City, Leyte. *Staphylococcus aureus, Bacillus cereus, Bordetella bronchiseptica* and *Pasteurella multocida* were inoculated in blood agar plates and incubated at 37°C for 24 hours. An isolated colony was then selected, Gram stained and viewed under the microscope to ascertain its purity and morphological characteristics. Once purity was confirmed, a loopful of the inoculum was streaked onto a nutrient agar and incubated at 37°C for 24 hours. From this, a bacterial suspension was prepared by inoculating Mueller-Hinton broth and incubating at 37°C until turbidity equaled to 0.5 McFarland turbidity standard. Similarly, *Microsporum canis* and *Candida albicans* were inoculated onto acidified Sabouraud dextrose agar slants and incubated for 2-7 days at 28°C. A fungal suspension of *C. albicans* was prepared by inoculating a loopful organism to Sabouraud dextrose broth, which was then incubated at
37°C until 0.5 McFarland turbidity standard was reached. About \(10^7\) spores/mL were prepared from \(M.\ canis\) by inoculating a loopful of the fungi into 10 mL of sterile water. The spore suspension was mixed, and a loopful amount was placed in a hemocytometer. The number of the conidial spores at the middle quadrant were counted at 400x magnification. Only five of the 25 squares in the middle quadrant were considered in the counting. The total number of conidia was computed using the formula of Hansen (2000):

\[
\text{Cells/mL} = \text{total cells in 5 squares} \times 50,000 \times \text{dilution factor}
\]

**Preparation of the Extract Stock Solution**

To prepare the stock solution of the \(T.\ catappa\) leaf extract, 1 mL of Mueller-Hinton broth was dispensed in 10 tubes. One mL of the leaf extract was added to the first tube and mixed, followed by the transfer of 1 mL of the extract solution to the second tube, then from the second to the third tube, and continuously in the same manner until the tenth tube. The extract concentration in the tubes ranged from as high as 10,000 \(\mu\)g/mL to as low as 19.53 \(\mu\)g/mL.

**Antimicrobial Assay**

The minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) were the parameters used in evaluating the antimicrobial activity of the extract. About 100 \(\mu\)L of the previously prepared extract stock solution from each tube was transferred to the wells of a 96-well microtiter plate. An equal amount of the prepared bacterial and fungal suspension was then added into each well, mixed and incubated at 37°C for 24 hours and at room temperature for five days for bacteria and conidial spores, respectively. Control wells were assayed simultaneously, consisting of broth (negative) and antibiotic or antifungal agent (positive). The MIC was determined as the lowest dilution of the extract without turbidity. Subsequently, wells with clear content were streaked onto Mueller-Hinton agar to determine the MBC and onto Sabouraud dextrose agar for the MFC of the leaf extract. The MBC/MFC indicates the lowest concentration of the leaf extract that kills or destroys bacteria or fungal cells.

**Qualitative Phytochemical Screening**

The \(T.\ catappa\) leaf extract was evaluated for the qualitative presence of phytochemical groups including terpenoids (Salkowski test), saponins (froth test), alkaloids (Dragendorff’s and Mayer’s test), flavonoids (Bate-Smith test), and tannins (ferric chloride test) according to the general techniques for phytochemical analysis as described by Sahira Banu and Cathrine (2015). The phytochemical components available in the extract could be responsible for the bioactivity and ethnomedical applications of the plant. Test reactions were interpreted as positive (+), indicating the presence of a phytochemical compound and negative (-), if the compound is absent or undetected (Senguttuvan et al., 2014).

**Data Analysis**

Data were expressed as the mode of MIC which selected the point among the 10 concentrations of the leaf extract that most likely showed inhibition of bacterial/fungal growth. The experiment was done in triplicates.

**Results and Discussion**

**Results**

**Physical Characteristics of \(T.\ catappa\) Extract during Extraction.** Air-drying of
2,000 g of *T. catappa* leaves yielded 500 g of dried leaves, constituting an approximate moisture loss of 75% (Fig. 1). The 48-hour infusion produced a filtrate with strong alcoholic odor, watery consistency and pine green color (Fig. 2). After concentrating, the extract produced strong alcoholic odor and pine green color, but the consistency became sticky. Evaporation of the remaining solvent reduced the final volume of the extract to 10 mL with watery consistency and greenish brown color.

**Antimicrobial Assay.** Table 1 outlines the MIC values of *T. catappa* against the four test bacteria. The MIC values of *T. catappa* indicate that the ethanolic leaf extract inhibited the bacteria at concentrations not lower than 5,000 µg/mL. The extract demonstrated a broad spectrum of activity by inhibiting both gram-positive and gram-negative bacteria at concentrations 5,000 µg/mL to 10,000 µg/mL. Higher doses were required to inhibit gram-negative bacteria. However, the reference antibiotics: penicillin and streptomycin with MIC of 1,250 µg/mL and 78.12 µg/mL against gram-positive and gram-negative bacteria, respectively were found to be more effective than the extract.

Interestingly, *T. catappa* extract inhibited the fungi at much lower concentrations comparable to the effective concentration of fluconazole (Table 2). *C. albicans* was more sensitive than *M. canis* and was inhibited at 78.12 µg/mL of the extract. In fact, the extract controlled *C. albicans* at the same dose as fluconazole.

Clear wells were inoculated in growth media to determine MBC and MFC. These assays show the lowest concentration at which the plant extract was able to kill the microorganisms. Figs. 3 and 4 illustrate the MBC and MFC values of the extract.

The results revealed that it would need 5,000 µg/mL of the leaf extract to effectively kill gram-positive and 10,000 µg/mL to kill gram-negative bacteria. The MBC values of the extract were generally similar to the MIC with a 1:1 ratio. However, it would only take 2,500 µg/mL of the extract to destroy *C. albicans*, the MFC was 32-folds higher than the MIC of 78.12 µg/mL. Meanwhile, the MFC for *M. canis* was 1,250 µg/mL that was four-folds higher than the MIC of 312.50 µg/mL. Generally, the fungal isolates were more sensitive to the *T. catappa* ethanolic leaf extract than their bacterial counterpart. The extract was shown to exhibit broad antimicrobial potential.
Table 1. MIC values of *T. catappa* ethanolic leaf extract against test bacteria

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Ethanol extract</th>
<th>Penicillin</th>
<th>Streptomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td>5,000</td>
<td>2,500</td>
<td>-</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>5,000</td>
<td>1,250</td>
<td>-</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>10,000</td>
<td>-</td>
<td>78.12</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>10,000</td>
<td>-</td>
<td>156.25</td>
</tr>
</tbody>
</table>

*Expressed as mode of MIC

Figure 3. MIC-MBC ratio of *T. catappa* ethanolic leaf extract against test bacteria

Table 2. MIC values of *T. catappa* ethanolic leaf extract against test fungi

<table>
<thead>
<tr>
<th>Fungi</th>
<th>MIC (µg/mL)*</th>
<th>Fluconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. canis</em></td>
<td>312.5</td>
<td>156.25</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>78.12</td>
<td>78.12</td>
</tr>
</tbody>
</table>

*Expressed as mode of MIC

Phytochemical Screening of *Terminalia catappa* Ethanolic Leaf Extract. Phytochemical screening of *T. catappa* unveiled three bioactive compounds including tannins, saponins and alkaloids (Fig. 5). These compounds are believed to be responsible for the antimicrobial action of *T. catappa* leaf extract against the test organisms. The extract produced froth formation, blue-black and brownish green coloration and reddish brown precipitate to froth test, ferric chloride test, and Wagner’s test, respectively.

Discussions

In developing countries, synthetic drugs are not only expensive but also inadequate for the treatment of diseases and often associated with adulterations and side effects (Shariff, 2001). Extraction of bioactive compounds from medicinal plants could provide effective means of controlling bacterial and fungal infections. The current study successfully...
Figure 4. MIC-MFC ratio of *T. catappa* ethanolic leaf extract against test fungi

Figure 5. *Phytochemical screening of T. catappa leaf extract.* a) froth formation (arrow) indicates saponin in the froth test, b) blue-black color indicates tannin in the ferric chloride test, and c) reddish-brown precipitate indicates alkaloids in the Wagner's test

demonstrated the broad spectral activity of *T. catappa* leaf extract against potential pathogens in animals. Generally, the antimicrobial activity of bioactive compounds from plants is due to their ability to reach a site of action in the microbial cell such as the electron transport chain (ETC) and ATPases that are embedded in the plasma membrane or mitochondria (Naz et al., 2007). The site of action of some phytochemicals, however, depends on their chemical structures and properties (Simoes et al., 2009). Electron transport chains are composed of membrane-associated proteins that are arranged in order of their increasing $E_0'$ values and function in an integrated fashion to carry electrons from the primary electron donor to the terminal electron acceptor (Madigan et al., 2015). The electron transport reactions establish a proton motive force that drives ATP synthesis. Once inside the membrane, the bioactive compounds may inhibit the ETC by interfering with the flow of electrons. The ATPase synthase has also been demonstrated as a good molecular target for drugs in the treatment of various infections and the regulation of...
energy metabolism in microbes (Berger et al., 2002). In addition, many of the phytochemical compounds undergo nonspecific interactions with proteins resulting to protein inactivation and loss of function (Mason & Wasserman, 1987).

Sensitivity of the bacteria to the leaf extract greatly varies, with the gram-positive bacteria easily inhibited at lower doses than the gram-negative bacteria. It is widely recognized that gram-negative bacteria are generally less susceptible to antimicrobial products than gram-positive. This is mainly due to their cell walls that have more resistant barrier to entry (Simoes et al., 2009). The cell walls of gram-negative bacteria have an outer membrane composed of structural lipopolysaccharides, which render the cell wall impermeable to lipophilic solutes. In addition, gram-negative bacteria have an inherent overexpressed or multiple efflux pumps that prevent the intracellular accumulation of antibacterial agents (Demeterio et al., 2015). On the other hand, the cell walls of gram-positive bacteria are lined by an outer peptidoglycan layer, which is a weak barrier (Dubey et al., 2011). These differences in the cell walls confer different properties to the cell, in particular responses to external stresses, including heat, UV radiation and antibiotics (Mai-Prochnow et al., 2016). The morphological difference influences the reaction of bacteria to antibacterial agents in T. catappa. Conversely, there are two suggested types of pharmacokinetics for a potential antifungal agent in plant extracts (Hawser & Islam, 1999). One is by disrupting the integrity of the cell membrane and consequently, inhibiting hyphal formation. The other is by inhibiting cytochrome P 450 demethylase, squalene epoxidase, RNA and DNA synthesis resulting to the inhibition of the budding process of the organism.

Furthermore, the MIC-MBC ratios of the extract against bacteria were proportional (Fig. 3). However, the MFC of the leaf extract in C. albicans was 2,500 µg/mL, about 32-folds higher than the MIC. In M. canis, MFC was 1,250 µg/mL or about four-folds higher than its MIC value (Fig. 4). The ratio of the MIC-MBC has been used in inferring the mode of action of a new drug. For instance, Constable et al. (2016) define a bacteriostatic agent as having a large MIC-MBC ratio, while bactericidal agents have a small ratio (<4-6). At longer exposure, however, bacteriostatic agents may deliver bactericidal effects. This concept is in agreement with the FDA (2007) standard whereby a 1:1 to 1:2 MIC-MBC ratio considers the drug as bactericidal against a specific pathogen. Craig (2002) proposed that the saturation of the killing rate occurs at low multiples of the MIC (<4-5 times of the MIC). Drug concentrations above these values do not kill microbes faster or more extensively.

In the present study, T. catappa ethanolic leaf extract has bactericidal activity against the test bacteria at 5,000 µg/mL to 10,000 µg/mL, fungicidal to M. canis at 1,250 µg/mL to 10,000 µg/mL, but fungistatic to C. albicans at 2,500 µg/mL to 10,000 µg/mL. The antimicrobial activities of T. catappa are attributed to the phytochemicals that are active against a wide range of microorganisms including fungi, yeasts and bacteria. Kloucek et al. (2005) reported that T. catappa was among the plants with promising antibacterial properties indicating the potential for discovery of antibacterial principles. The leaves showed the broadest spectrum of action against bacteria with minimum inhibitory concentrations ranging from 0.25 mg/mL to 16 mg/mL.

Interestingly, the extract displayed a fungistatic action against C. albicans. This could be because the yeast is a biofilm former and a filamentous fungi (Hawser & Douglas, 1995). Biofilms are highly structured communities of microorganisms that are either surface associated or attached to one another and are enclosed within a self-produced protective extracellular matrix. The biofilm formation confers antimicrobial resistance of some fungi and bacteria as they protect the microorganisms from the environment, resist physical and chemical stress, and enable...
metabolic cooperation and community-based regulation of gene expression (Ramage et al., 2012).

The growing interests in crude extracts might be a more important way to use medicinal plants than have been realized in Western medicine, since plants contain numerous secondary metabolites which interact with pathogens in nature simultaneously (Izhaki, 2002). Phytochemical screening of *T. catappa* crude ethanolic leaf extract in this study revealed three phytochemical compounds such as tannins, saponins, and alkaloids which is consistent with the findings of Masuda et al., (1999) where alkaloids, reducing sugars, saponins, tannins, resins and steroids were isolated from ethanol fraction of *T. catappa*. Xin-Hui and Ling (2004) found that *T. catappa* seed contains 51.2% fixed oil, Catappa oil, with 54% olein, palmitin, and 46% stearin and the bark contains tannin. Phytochemical analysis yielded saponin, saponin glycosides, steroid, cardiac glycoside, tannins, volatile oils, phenols and balsam (gum). The phytochemical studies on *T. catappa* bark and leaves demonstrate the presence of tannins and flavonoid glycosides (Kloucek et al., 2005). These phytochemicals act on multiple biochemical targets of the bacterial cell and are usually responsible for the antibacterial properties of the plant (Simoes et al., 2009). Plants contain secondary metabolites, which are the most valuable phytochemicals of plant secondary metabolism, and possess sufficient chemical or structural complexity (Bhatia, 2015). Most antimicrobial secondary metabolites have relatively broad spectrum of activity (Panda & Rath, 2012). Antifungal activity is also attributed to the presence of these secondary metabolites (Terças et al., 2017).

Moreover, the ability of the phytochemical compounds to disrupt cellular barrier is the key to their inhibitory actions against pathogens. Saponins are known to disrupt the permeability of the bacterial outer membrane. Glycoside saponins might induce pore-like structures which change the membrane permeability associated with alterations in the ionic homeostasis between intracellular and extracellular compartments. They can also interfere with the energy metabolism through interaction with catabolic enzymes and the electron transport chain (Khan et al., 2006). The formation of lipid A-saponin complexes might promote antibiotic uptake to inherently resistant bacterial cells (Arabski et al., 2009). Tannins are a large group of polyphenolic compounds, which have received attention in recent years due to their claimed ability to cure a variety of diseases (Panda & Rath, 2012). Tannins form complexes with proteins through nonspecific forces such as hydrogen and covalent bonding and hydrophobic effects (Stern et al., 1996). The ability therefore of tannins to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and other molecules is their probable antimicrobial modes of action (Cowan, 1999). Alkaloids are heterocyclic nitrogen compounds that can interact with the bacterial cytoplasmic membrane, intercalate with DNA, and inhibit efflux pumps (Khan et al., 2006; Markham et al., 1999). The antimicrobial action of alkaloids could be throughout intercalation with cell wall and DNA constituents. Finally, phytochemicals act through different mechanisms from that of conventional antibiotics. The possibility of synergistic effects from the interaction of the compounds in the extract places medicinal plants like *T. catappa* a potential source of new drugs.

**Conclusion**

The current study revealed the potential antimicrobial activity of *T. catappa* leaf extract against various pathogens in animals due to the presence of bioactive phytochemical compounds including tannins, saponins and alkaloids. The urgent need to discover new drugs that are effective against a wide range of pathogens drives the necessity
for screening medicinal plants. From the obtained results, the leaf extract is worthy of further investigation to prove its efficiency as an antimicrobial alternative for bacterial and fungal infections in animals.

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