Antibacterial, Histochemical and Phytochemical Screening and Cytotoxicity Activity of Tubog, *Ficus nota* (Blanco) Merr Leaf and Fruit Extracts

Levitah C. Mapatac

Abstract

Tubog, Ficus nota (Blanco) Merr a rare tree in the Philippines and was found in Barangay Tambis, Barobo, Surigao del Sur. The present study designed to evaluate the antibacterial activities of Tubog,Ficus nota (Blanco) Merr fruit and leaves with ethanolic extracts. Preliminary phytochemical and histochemical studies were carried out. The antimicrobial activity of the fruit extract against pathogenic strains evaluated based on the zone of inhibition using paper disc diffusion method with tested bacteria of Escherichia coli ATTC 25922, Staphylococcus aureus ATTC 25923, Klebsiella pneumonia UPCC 1360, and Salmonella typhimurium UPCC 1368.The qualitative phytochemical tests exhibited the presence of common bioactive compounds including alkaloids, tannins, flavonoids, saponins, flavonoids and anthraquinones. The aqueous extract has shown high activity in ethanolic extract has shown significantly good activity when compared to standard chemotherapeutic agent Chloramphenicol. Based on the result it was concluded that Ficus nota are rich in bioactive components and exhibits antimicrobial activity against pathogens. Cytotoxicity result showed higher toxicity with the fruit compared with ficus nota leaves ethanolic extract.

Keywords: Ficus nota, phytochemical screening, cytotoxicity and histochemical test

1.0 Introduction

The importance of a country's diverse medicinal plants lies not only in their chemotherapeutic value in traditional medicine but also in their potential sources of new chemical entities for drug discovery. While the Philippine biodiversity and rich cultural traditions of plant use, scientific understanding of medicinal plants remains largely unexplored and pharmacological investigation of the Philippine flora only grown recently. For example, Vital and Rivera (2011) studied on antimicrobial, cytotoxicity and phytochemical screening of *Voacangaglobosa* (Blanco) Merr leaf extract (Apocynaceae).

Plants have long been recognized as a potent source of biologically pharmacologic active compounds, held true with various studies (Al Bayati and Al Mola, 2008; Oyetayo and Oyetayo, 2006; Cramb and Sakoe, 2003). The healing property of plants is usually linked with the presence of the secondary metabolites that which differs from one plant to another (Rios and Riceo, 2005; Oyetayo and Oyetayo, 2006).

Tubog, *Ficus nota* (Blanco) Merr are erect, up to 9 m high, or shrubby with crooked stems. In Florida, the trees are low and spreading, with trunks branching near the ground, probably

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because of having suffered occasional frost injury. No aerial roots were observed. The twigs are hollow when young, about 0.7 cm thick, and densely silky-pubescent. Older twigs are chocolate-brown, with leaf scars large and prominent. The terminal buds are 2.5 cm long, 0.7 cm thick at the base, pubescent, somewhat flattened or angular, and green to tawny. The alternative leaves are large, up to 25 cm long and 15 cm broad, ovate, the apex bluntly acute or acuminate, and the margins coarsely serrate to almost entire. The blades are variable as to the base, which is sometimes obovate and narrowed to cordate with narrow sinus, the lobes often overlapping and sometimes asymmetrical. Some blades are distinctly narrowed from the middle or above toward the rounded base. The texture is chartaceous. Hydathodes are present and densely scattered over the surface, which is pubescent only on veins above but silky-pubescent below. Venation is prominent, with 3 to 5 pairs of basal veins and 7 to 9 laterals flanking the midrib, these well elevated, widely spaced, and branched toward the margin. The petioles are up to 5 cm long, stout, silky-pubescent, rusty-scurfy, and reddish to chocolate-brown, abruptly changing to green at the base of the midrib. This species is cauliflorous, with the figs borne mostly in fascicles from the stem and branches, on pubescent peduncles up to 1.1 cm long. The sterile figs are 3.5 cm in diameter, globular to oblate-spherical, with the surface pubescent but glossy, and decorated with prominent, white flecks which are thickly scattered, the larger ones with a corky spot in the center. The figs are green when young, but change to scarlet before dropping. The umbilicus is large and somewhat depressed (Condit, 1969).

The purpose of this study was to discover the therapeutic ability of some plants found in the Philippines with an end goal of providing cheaper nature-based alternative medicine to the public in the midst of high-priced medicine produced by pharmaceutical companies. With the characteristic compounds present in this plant sample, this study is conducted to determine the antibacterial and cytotoxicity of Tubog, *Ficus nota* (Blanco) Merr Fruits. Furthermore, the plant extracts were subjected to preliminary phytochemical screening to analyse the possible antibacterial compounds they contain. The study provides scientific evidence on the possible use of Tubog, *Ficus nota* (Blanco) Merr Fruits to produce potential source of antibiotics.

Objectives of the Study

This study aimed to determine extracts from leaf and fruit extract of Tubog, *Ficus nota* (Blanco) Merr and its potential bioactivity.

Specifically this study aimed to:

1. Determine the bioactive compounds present in the plant through Histochemical and Phytochemical Analysis;
2. Determine the antibacterial activity of Tubog, Ficus nota (Blanco) Merr against four (4) bacteria *Escherichia coli ATTC 25922*, *Staphylococcus aureus ATTC 25923, Klebsiella pneumonia* UPCC 1360, and *Salmonella typhimurium* UPCC 1368;
3. Determine the toxicity (LC_{50}) of the plant extracts through Brine Shrimp Bioassay.

Scope and Limitation of the Study

The study was limited only on the determination of secondary metabolites of leaves

and fruit extract particularly alkaloids, steroids, flavonoids, saponins, and tannins. Histochemical plant extracts of Tubog, *Ficus nota* (Blanco) Merr fruit for antibacterial tests utilized four (4) bacteria of*Escherichia coli ATTC 25922, Staphylococcus aureus ATTC 25923, Klebsiella pneumonia* UPCC 1360, and *Salmonella typhimurium* UPCC 1368. Brine shrimp Bioassay was used for the determination of LC_{50} with a 48 hour nauplii.

Figure 1. Picture of Tubog Plant

2.0 Materials and Methods

Selection and collection of plant material

Fruits of Tubog, *Ficus nota* (Blanco) Merr were collected from Sitio Garden, Barangay Tambis, Barobo Agusan del Sur, Philippines. Preliminary identification of the plant sample was done at Department of Biology, College of Arts and Sciences, Caraga State University, Ampayon, Butuan City. Further identification and authentication with Dr Jose Vera Santos Memorial Herbarium (Philippine University Herbarium) of the Institute of Biology, University of the Philippines Diliman, Quezon City. Voucher specimen of the plant was prepared and deposited at the Institute of Biology.

Preparation of the extracts

Fruits and leaves of the plant were freshly collected, washed and cut into small pieces. For the extraction, 500 grams of fresh fruit and fresh leaves of the plant material were soaked in 95% AR ethanol (1:5) for 24 hours for leaves and 48 hours for the fruits sample. The solvent was then removed by rotary evaporation. The extract was stored inside the refrigerator until used for the laboratory analysis for phytochemical screening, antimicrobial analysis and cytotoxicity test.

Organisms and culture media

Microorganisms were obtained from culture collections of the Microbiological Research and Services Laboratory of the Natural Sciences Research Institute at the University of the Philippines Diliman, Quezon City. Organisms used were as follows: *Klebsiella pneumonia* UPCC 1360, and *Salmonella typhimurium* UPCC 1368. While *Escherichia coli ATTC 25922*, *Staphylococcus aureus ATTC 25923* were obtained from the Regional Center of the Department of Science and Technology Microbiological Laboratory, Ampayon, Butuan City. Bacterial cultures were maintained on nutrient agar (NA).

Antibacterial activity of the plant extract

Paper disc diffusion method was used to determine the antibacterial activity of Tubog,

Ficus nota (Blanco) Merr fruit extract. Bacteria were inoculated into Nutrient broth (NB) at 37°C for 6 hours. The turbidity of the resulting suspensions was diluted with NB to obtain transmittance of 74.3% (absorbance of 0.132) at 600 nm. The percentage is found spectrophotometrically comparable to 0.5 McFarland turbidity standards. This level of turbidity is equivalent to approximately 1.5 x 10^8 CFU/ml. These bacterial cultures were then inoculated on the surface of Mueller-Hinton agar (MHA) plates for bacteria. Subsequently, filter paper discs (6 mm diameter) saturated with extracts (25 uL) was placed on the surface of each inoculated plate. Antibiotic was used as positive control (Chloramphenicol), while solvent (95% ethanol) of the plant extracts as negative control. The tests were carried out in triplicates. The plates were incubated at 37°C for 24 hours. At the end, of incubation, zones of inhibition were measured with a transparent ruler. Zones of clearing greater than 6 mm were considered susceptible to the extracts.

Brine Shrimp Lethality Assay

Preparation of Brine Shrimp

The preparation of the artificial seawater was done by dissolving 40 grams of sodium chloride (AR) added to one liter distilled water.

Hatching the Shrimp Egg (Artemia salina)

A shallow oval-shape plastic container (35cm x 15cm x 10cm) was filled with artificial sea water prepared earlier. The container was sprinkled with minute quantity of shrimp eggs (*Artemia salina*) covered with plastic cellophane and punched with several holes and kept illuminated by a fluorescent lamp for 48 hours. After 48 hours, hatched brownish orange nauplii larvae from the illuminated container were pipette out and transferred using a micro pipette to a petri dish with shallow saline water for later administration of the treatments.

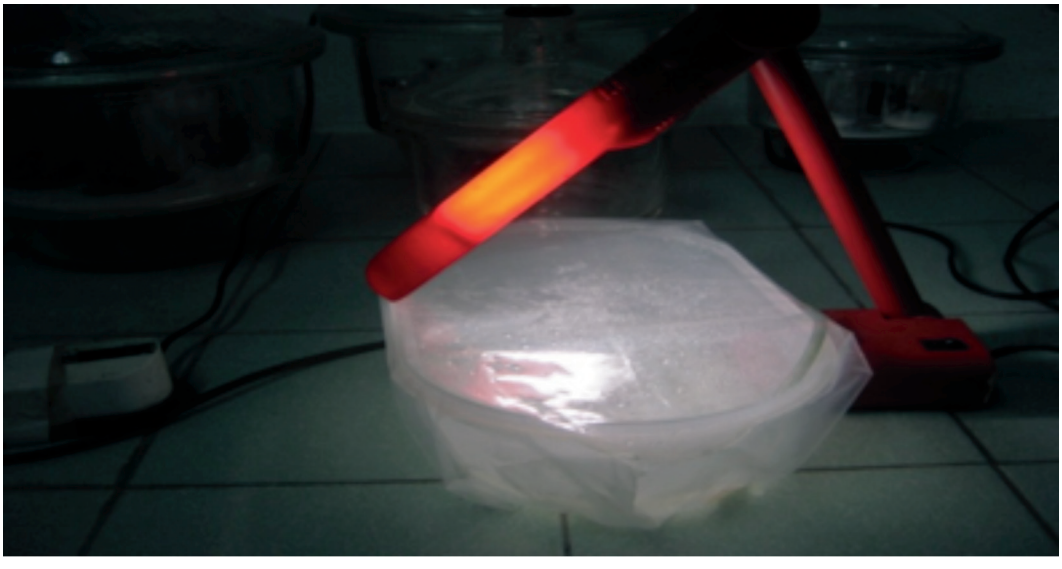


Figure 2. Preparation of Brine Shrimp Bioassay

Treatment of Brine Shrimp (Artemia salina)

The experimental set-up consisted of six treatments namely: artificial seawater (T1) as the negative control, and five doses powdered samples: 1 ppm (T2), 10 ppm (T3), 100 ppm (T4), 1000 ppm (T5), 10,000 ppm (T6). Each treatment was done in threereplicates and the treatment period lasted for 24 hours wherein 0.1gram of powdered sample of Tubog , *Ficus nota* Fruit and Seed Extract was added to the first well, shake by inverting the test tube. One (1) ml of was taken to be added to the succeeding test tubes in a tenfold dilution process.

Fifteen brine shrimp nauplii were delivered into each vial using the pipette. The vials were kept illuminated with fluorescent light during the treatment period. The treated were counted macroscopically in the stem of the pipette against a well-lighted background to determine the number of dead and alive nauplii larvae using a magnifying glass.

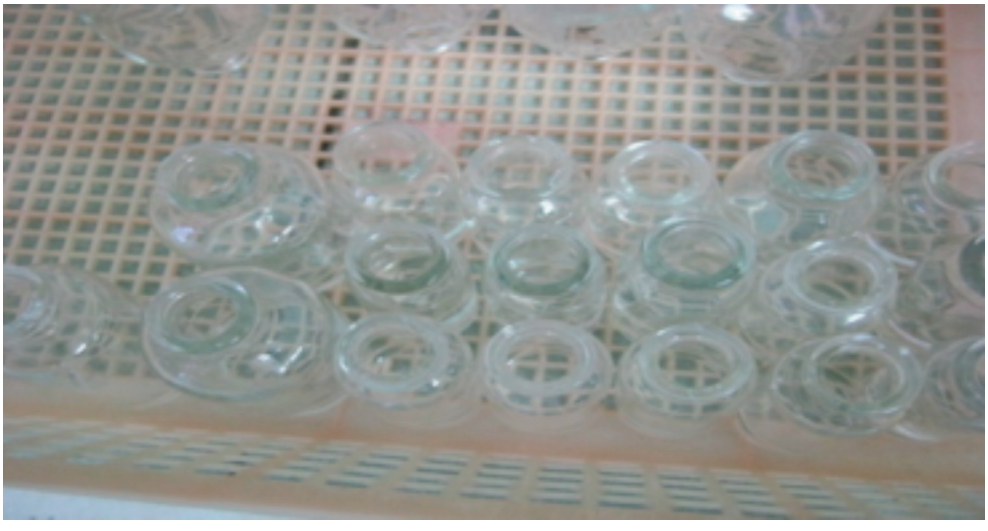


Figure 3. Process of the Tenfold Dilution

3.0 Data Analysis

Toxicity was assessed by counting the dead and alive nauplii larvae with the aid of a 3x magnifying glass and by computing the average percentage death of nauplii larvae for each treatment following this formula (Lee *et al*, 1999). And the computation of theLethal concentration (LC_{50}) is by using Probit Statistical Analysis by linear regression.

$$\%\text{Death} = \frac{\text{death in treated or control tube}}{\text{No. of treated nauplii}} \times 100$$

In cases where control deaths occurred, Abbot's formula given below was used to correct the data gathered:

$$\%\text{ Death} = \frac{\text{death in treated tube} - \text{death in control tube}}{\text{Total death}} \times 100$$

Phytochemical Screening

Phytochemical screening analysis of Tubog, *Ficus nota* (Blanco) Merr Fruit was done at Chemistry Instrumentation Laboratory, Caraga State University, Ampayon, Butuan City using the

standard test by Guevarra et.al. (2005) manual for phytochemical screening.To test for alkaloids, about 0.5 g of the extract was stirred with 5 ml of 1% aqueous hydrochloric acid on a steam bath. A few drops of Dragendorff's reagent were used to treat 1 ml of the filtrate. Turbidity or precipitation with this reagent was taken as evidence for the presence of alkaloids. Exact 0.5 g of the extract was dissolved in distilled water in a test tube. Frothing which persisted on warming was taken as preliminary evidence for saponins. Also, to test for presence of tannins, about 0.5 g of the extract was dissolved in distilled water and about 10 ml of bromine water added. Decolourization of bromine water indicated the presence of tannins. Borntrager's test was used for detecting the presence of anthraquinones. In this case 0.5 g of the plant extract was shaken with benzene layer separated and half of its own volume of 10% ammonia solution added. A pink, red or violet coloration in the ammoniacal phase indicated the presence of anthraquinone. The presence of cardiac glycosides was confirmed by Liberman's test, Salkowski test and Keller-Killani test (Culei, 1982; Sofowora, 1993; Trease and Evans, 2002) and cyanogenic glycosides were carried out according to the methods described by Harborne (1973) and Trease and Evans (1983).

4.0 Results and Discussion

Histochemical Analysis on the Fruits of Tubog, Ficus nota (Blanco) Merr

Histochemical analysis of Tubog, *Ficus nota* (Blanco) Merr sections using freehand technique showed several bioactive compounds in the fruits (Table 1). The detected bioactive compounds in each part were shown at the table below;

Table 1. Results in Histochemical Tests of Tubog, Ficus nota (Blanco) Merr Fruits

Bioactive compounds	Fruits
Alkaloid	+++
Formic Acid	+++
Glycosides	+++
Saponins	+
Tannins	++

+++: (Very abundant);++: (Relatively abundant);+: (Abundant); -: (Absent)

Table 1 shows the summary of the results on the qualitative Histochemical screening of the bioactive component present in the fruit of Tubog, *Ficus nota* (Blanco) Merr. It represents the positive biologically active metabolites found in the plant studied. The test on the fruits section gives a noticeably very positive presence of the metabolites tested. Plant material containing saponins have been long used for their detergent properties (Guevarra, 2004). Alkaloids isolated from the plants are commonly found to have antimicrobial properties and maybe useful against HIV infections as well as intestinal infections associated with AIDS (McDevilt etal, 1996 and Sethi, 1979).

Formic acid

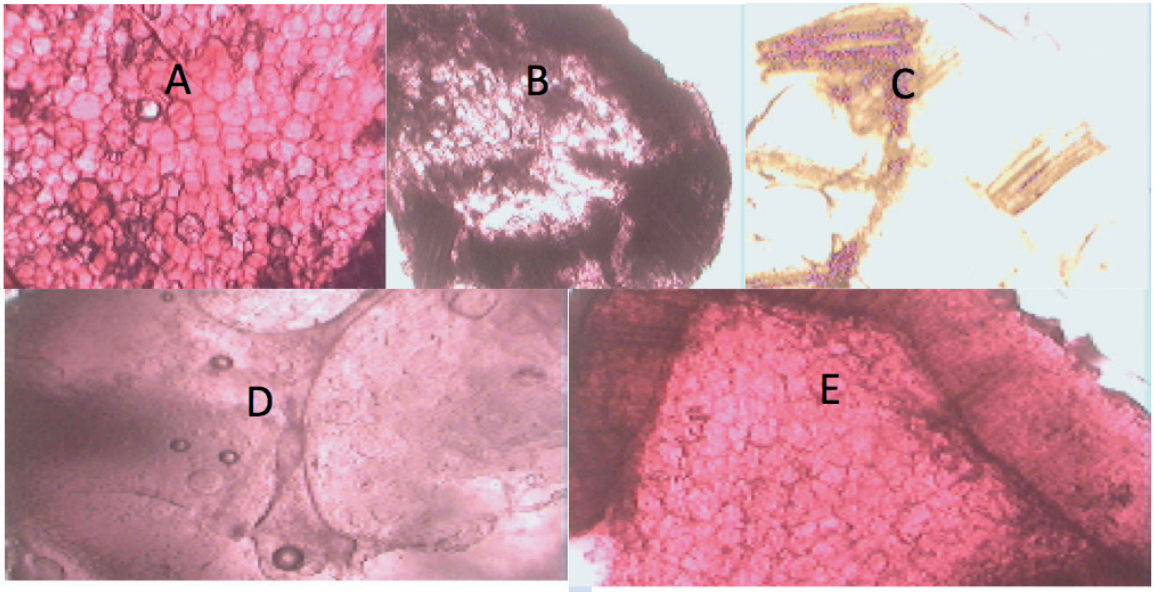
One of the major uses of formic acid is as a preservative and antibacterial agent in livestock feed. In Europe, it is applied on silage (including fresh hay) to promote the fermentation of lactic acid and to suppress the formation of butyric acid; it also allows fermentation to occur quickly, and at a lower temperature, reducing the loss of nutritional value Reutemann, W. and Kieczka, H. (2002). Formic acid arrests certain decay processes and causes the

feed to retain its nutritive value longer, and so it is widely used to preserve winter feed for cattle. In the poultry industry, it is sometimes added to feed to kill *E. coli* bacteria (Garcia et al, 2007; Griggs 2005).

Glycosides

Glycosides are a class of molecules in which, a sugar molecule is bonded to a "non-sugar" molecule. Glycosides play important roles in our lives. Many plants store medicinally important chemicals in the form of inactive glycosides. The non-sugar portion contains the biochemically active properties of medical interest. Once the glycoside is split into its two components (sugar and non-sugar parts), the non-sugar component is now free to exert its chemical effects on the body. For example, digitalis is a glycoside that when ingested, causes the heart to contract (pump) more forcefully. This is useful in medicine, where heart failure is present (Barvadiya et al,. 2013).

Figure 4. Histochemical Positive Results on Tubog, Ficus nota (Blanco) Merr. (a) Test for Alkaloids (+++); (b) Test for Formic Acid (+++); (c) Test for Glycosides; (d) Test for Saponins (+); Test for Tannins (++).



Alkaloids were very abundant in the epidermis, cortex and vascular bundles Figure 1 (A). Formic acid and glycosides were very abundantly present in the vascular bundle, epidermis and cortex (B and C) while Saponins was abundantly present in the epidermis and less abundant in cortex and vascular bundles (D). Tannins were relatively abundant in the vascular bundle and epidermis and less abundant in the cortex (E).

Phytochemical screening of Tubog, Ficus nota (Blanco) Merr Fruits

Phytochemical screening of the crude ethanolic extracts of Tubog, *Ficus nota* (Blanco)

Merr was done using test tube and different tests using different reagent. Results (Table 2) shows the bioactive component present in the Tubog, *Ficus nota* (Blanco) Merr fruits and leaves extract using the phytochemical screening for the presence of alkaloids, anthraquinones, tannins, flavonoids, steroids and saponins. No quaternary bases and amine oxide were detected in both fruits and leaves of Tubog, Ficus nota (Blanco) Merr. Phytochemicals such as alkaloids, anthraquinones, tannins, flavonoids, steroids and saponins were present in both fruits and leaves Tubog, *Ficus nota* (Blanco) Merr.

Table 2. Results in Phytochemical Tests on crude ethanolic extract of Tubog, Ficus nota (Blanco) Merr

Phytochemicals	**Tubog, *Ficus nota* Fruits**	**Tubog, *Ficus nota* Leaves**
Alkaloids	+	+
Flavonoids	+	+
Steroids	+	+
Saponins	+	+
Tannins	+	+
Anthraquinones	+	+
Quaternary Bases & Amine Oxide	-	-

+: Present -: Absent

Isolation of pure, pharmacologically active constituents from plants remains a long and tedious process. For this reason, it is necessary to have methods available which eliminate unnecessary separation procedures. Chemical screening is thus performed to allow localization and targeted isolation of new or useful constituents with potential activities. The procedure for the conduct of Phytochemical Screening was obtained from the book entitled "A Guidebook to Plant Screening: Phytochemical and Biological".

Alkaloids isolated from the plants are commonly found to have antimicrobial properties and maybe useful against HIV infections as well as intestinal infections associated with AIDS (McDevilt et al, 1996 and Sethi, 1979).

Flavonoids, (a large group of naturally occurring plant phenolic compounds including flavones, isoflavonoids, neoflavonoids, flavonols) also known as nature's tender drugs, possess numerous biological/pharmacological activities. Recent reports of antiviral, antibacterial, antifungal, antioxidant, anti-inflammatory, antiallergenic, antithrombic, anticarcenogenic, hepatoprotective, anticholesterolemia and cytotoxic activities of flavonoids have generated interest in studies of flavonoid-containing plants. The presence of flavonoids in Tubog, *Ficus nota* fruit extract may confirm their folkloric use as potential antibacterial agent.

Steroids are a type of organic compound that contains a characteristics arrangement of four cycloalkane rings that are joined to each other. Steroids use in medicine as anti-inflammatory

agent that acts during inflammation. Hundreds of distinct steroids are found in plants, animals, and fungi. Tubog, *Ficus nota* (Blanco) Merr is potential source of anti-inflammatory agent since it contain steroids in both fruits and leaves. Saponins are a class of chemical compounds, one of many secondary metabolites found in natural resources, with saponins found in particular abundance in various plant species. More specifically, they are amphipathic glycosides grouped, by the soap-like foaming they produce when shaken in aqueous solutions. Saponins are being promoted commercially as dietary supplements and nutriceuticals.

Tannins are polymeric phenolic phenolic substances capable of tanning leather or precipitating gelatin from solution, property known as astringency (Cowan, 1999). The astringency from the tannins is what causes the dry and pucker feeling in the mouth following the consumption of ripened fruit or red wine. Tannins have shown potential antiviral, antibacterial and antiparasitic. Studies conducted by Pieters and Vlietinck (2005) on anthraquinones found out that it contain antiparasitic, bacteriostatic, antidepressant, and antimicrobial properties (Cowan, 1999).

Brine Shrimp Bio Assay on ethanolic extract of Tubog (leaves and fruit), Ficus nota (Blanco) Merr

Table 3. Results of Brine Shrimp Lethality Assay on crude ethanolic extract of Tubog, Ficus nota (Blanco) Merr

Plant	% Mortality of Different Concentrations				LC_{50}, 24h
	10ug/ml	100ug/ml	1000ug/ml	10,000ug/ml	ug/ml
Tubog, *Ficus nota*100 (Fruits)	75.33	68.67	64.67	52.48	
Tubog, *Ficus nota* (Leaves)	100	66.67	57.8	53.33	304.07

Table 3 shows the level of toxicity of the Tubog, *Ficus nota* leaves extract against nauplii larvae at concentrations of 1, 10, 100, 1000, 10000 ppm (µg/ml). The mortality rate of nauplii larvae respectively are 40%, 53.33%, 57.8%, 66.67% and 100% which shows high affinity with its lethal concentrations of 304.07 µg/ml which implies that it is moderately toxic. The level of toxicity of the Tubog, *Ficus nota* fruit extract against nauplii larvae at concentrations of 1, 10, 100, 1000, 10000 ppm (µg/ml). The mortality rate of nauplii larvae respectively are 55.33%, 64.67%, 68.67%, 75.33% and 100% which shows high affinity with its lethal concentrations of 52.48 µg/ml which implies it is highly toxic.

Figure 1. Graph of the Mortality Rate of Nauplii larvae between the Fruit and Leaves Extract of Tubog, Ficus nota (Blanco) Merr

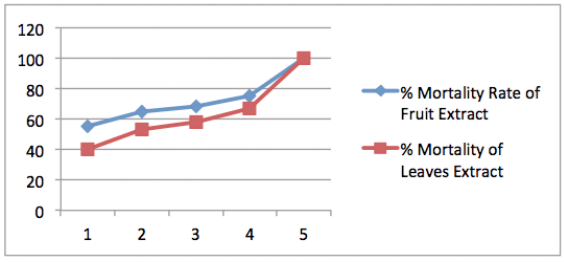


Figure 1 shows the graph of mortality rate of nauplii larvae when applied with the fruit and leaves extract of Tubog, *Ficus nota* (Blanco) Merr. The graph shows the comparison of the mortality and this show that the fruit extract (blue) has high mortality rate compared with tubog leaves extract (red).

Antibacterial Assay of Tubog, Ficus nota Fruit (Blanco) Merr

The antibacterial activity of ethanolic extracts of fruits tested into four (4) bacteria was shown at the table below. All test extracts were able to inhibit two bacteria under study, namely, *E. coli* and *S. aureus*. On the other hand, *K. pneumonia* and *S. typhimurium*were not inhibited by the plant extracts. Of the plant extracts tested, highest zone of inhibition with computed microbial index of 35 in *E. coli* followed by 33 in *S. aureus*. No zone of inhibition was noted in *K. pneumonia and S. typhimurium*. The inhibition of the positive control, chloramphenicol was comparable to those of the plant extracts. The solvent used as negative control exerted no effect against the microorganisms which suggest the effectiveness of the plant extracts (Table 4).

Table 4. Summary of Results in Antibacterial Tests of Tubog, Ficus nota Fruit (Blanco) Merr

Bacteria	Fruits (mm) Zone of Inhibition
Escherichia coli ATTC 25922	35
Staphylococcus aureus ATTC 25923	33
Klebsiella pneumonia UPCC 1360	-
Salmonella typhimurium UPCC 1368	-
Chloramphenicol	9.3
Ethanol	-

<10 mm, inactive: 14-19 mm, active ;10-13 mm, partially active ;>19 mm, very active; -: no activity

5.0 Conclusion and Recommendation

The results of this study demonstrate the antibacterial activity of Tubog, *Ficus nota* Fruit (Blanco) Merr as potential source of antibiotics against four (4) bacteria *Escherichia coli* ATTC 25922, *Staphylococcus aureus* ATTC 25923, *Klebsiella pneumonia* UPCC 1360, *Salmonella typhimurium* UPCC 1368. Histochemical screening of *Ficus nota* (Blanco) Merr fruits showed high amount of alkaloids, formic acid, glycosides, saponins and tannins. As to antibacterial screening, the plant extracts reveals that the fruit of Tubog, *Ficus nota*

(Blanco) Merr have active antibacterial property to the strains tested.

Phytochemical screening result revealed that both leaves and fruits contained secondary metabolites such as alkaloids, anthraquinones, tannins, flavonoids, steroids and saponins may bring about its potent source of antibiotics. This study provides scientific evidence on the traditional medicinal use of Tubog, *Ficus nota* (Blanco) Merr Fruits in the Philippines. From the cytotoxicity test through brine shrimp assay the fruits were found to be highly toxic with $LC_{50}$of 52.48 *u*g/ml while the leaves were found to be non-toxic with LC_{50} of 304.07 ug/ml. In view of the results of the study, the following recommendations were given:

1. To determine the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of the plant extracts and some other plant extracts because it might be found out that at minimal concentrations a greater inhibition will be exhibited by the plant's extract.
2. The extracts of fruits and leaves of Tubog, *ficus nota* (Blanco) Merr will be subjected to Mutagenicity and Clastogenicity test.
3. Further analysis on the compounds that can be found in the fruits and leaves of the sample.

6.0 References

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