

**IN VITRO ASSESSMENT OF THE ANTIOXIDANT AND ACETYLCHOLINESTERASE INHIBITORY ACTIVITIES OF FICUS NOTA (BLANCO) MERR.**

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ABSTRACT

Ficus species are known to exhibit neuroprotective activity and to some extent show acetylcholinesterase inhibitory activity. The aims of the study are to assess the antioxidant activity of the crude methanolic extract of the leaves of *Ficus nota* (Blanco) Merr. using scavenging assays for hydroxyl radical, nitric oxide radical, and hydrogen peroxide, and its acetylcholinesterase (AChE) inhibitory activity using Ellman's method. Phytochemical screening revealed the presence of terpenoids, tannins, alkaloids, polyphenols, and glycosides, and the total phenolic content (TPC) was found to be 3.448 mg/g GAE. The extract showed stimulatory activity towards hydrogen peroxide. On the other hand, inhibitory activity towards nitric oxide and hydroxyl radicals was exhibited in a decreasing trend. Furthermore, AChE activity was inhibited by the extract at low concentrations. The methanolic crude extract of the leaves of *Ficus nota* (Blanco) Merr. has pro-oxidant and AChE inhibitory activities. With these, the plant may be explored as a potential pharmaceutical substance in the future.

Keywords: *Ficus*, *Ficus nota*, neuroprotective, antioxidant, acetylcholinesterase.

INTRODUCTION

Free radicals in the body are countered by antioxidant defenses which are normally found in the body. These defenses, when worn out, consequently cause damage to tissues. The hydroxyl radical, an example of a free radical, attacks nucleic acids which may cause strand breaking, cross-linking and base modifications contributing to errors in protein production and the neuronal cell malfunction and death [1].

Inhibitors of acetylcholinesterase (AChE) are pharmacologically important in the control of diseases that involve impaired acetylcholine-mediated neurotransmission [2]. Acetylcholine (ACh) is an important neurotransmitter found in the peripheral and central nervous system (CNS) involved in a number of essential processes related to behavioral activities, arousal, attention, learning and memory [3]. Dysfunction in the regulation and metabolic abnormalities of this neurotransmitter leads to neuropsychiatric disorders such as Parkinson's disease, Alzheimer's disease, Myasthenia Gravis,

schizophrenia, and Huntington's disease [4]. AChE inhibitors are composed of various classes of compounds natural, semisynthetic, and synthetic derivatives [3]. Among the natural compounds are alkaloids, terpenoids, glycosides and coumarins [5].

Ficus species, commonly known as figs, contain polyphenolic compounds and flavonoids which are responsible for potent antioxidant properties, which may help in preventing and treating different oxidative stress-related conditions such as neurodegeneration and other diseases [6]. They are also known to exhibit medicinal purposes and have shown diverse biological and pharmacological activities. They are found to have potential in the treatment of tumors, inflammatory diseases, wound healing and as antioxidants. Moreover, extracts from two species of *Ficus*: *Ficus racemosa* and *Ficus hispida* are found to increase acetylcholine (ACh) levels in hippocampi of rats [7].

The objectives of the study are to assess the antioxidant activity of *Ficus nota* (Blanco) Merr. using scavenging assays for free radicals and other reactive oxygen species, and to determine the

acetylcholinesterase (AChE) inhibitory activity using microplate assay.

MATERIALS AND METHODS

Chemicals and Equipment: All reagents used are analytical grade solvents purchased from DKL Laboratories, Inc. All the standards and reagents used to determine the antioxidant and acetylcholinesterase activity of the plant were purchased from DKL Laboratories, Inc. Manila, Philippines. Corono Electric SH-1000 Microplate Reader was employed for measuring the absorbance throughout the experiment.

Plant Preparation and Extraction: *Ficus nota* leaves were identified by the Philippine National Museum. The leaves were thoroughly washed with distilled water to remove unwanted particles and impurities and were subjected to air-drying. The dried leaves were ground into fine powder by the use of Wiley Mill apparatus. The powder was then extracted by percolation using 80% methanol for one week. The resulting mixture was concentrated using a rotary evaporator and was stored in an amber-colored bottle under 0°C until further use.

Preliminary Phytochemical Screening: Qualitative phytochemical tests were performed to detect the presence of active chemical compounds such as alkaloids, flavonoids, terpenoids, glycosides, polyphenols, tannins and coumarin which are known to contain AChE inhibiting activity [5,8]. Colorimetric phytochemical screening analyses were done with the methanolic crude extracts using methods which include Salkowski test for terpenoids; Bate-Smith and Metcalf, and Wilstatter test for flavonoids; gelatin test for tannins; ferric chloride test for polyphenols; Dragendorff's and Mayer's test for alkaloids; Fehling's, Benedict's, and Molisch's test for glycosides; and test for coumarins [9].

Estimation of Total Phenolic Content: The total phenolic content (TPC) of the methanolic leaf extracts was determined using the Fast Blue BB assay. Gallic acid concentrations of 0, 25, 50, 100, 250 and 500 µg/mL were prepared from a stock solution of 1 mg/mL. One milliliter of each concentration was transferred to borosilicate tubes followed by the addition of 0.1 mL of 0.1% Fast Blue BB reagent. The solution was mixed for 1 minute and 0.1 mL of 5% sodium hydroxide was added for alkalization. After 90 minutes of standing, the absorbance was read at 420 nm. The same procedure was done to the plant sample but the main difference is that each sample was analyzed with a blank

containing only the plant sample with 0.2 mL of deionized water. Blanks were used to correct for the non-phenolic contents. The TPC was expressed as gallic acid equivalent (mg/g) from the gallic acid calibration standard and GAE values of the blanks were subtracted from the GAE values of the samples [10].

Micro-Scale Antioxidant Tests: The hydroxyl scavenging assay was conducted using a standard method. One milliliter Fenton reaction mixture contained 2.8 mM 2-deoxy-2-ribose, 0.1 mM ferric chloride, 0.1 mM EDTA, 0.1 mM ascorbic acid, 1 mM H₂O₂ in 20 mM phosphate buffer pH at 7.4, and various concentrations of extracts (63-1000 mcg/mL in 95% ethanol). The reaction mixture was incubated for 1 hour at 37°C, after which, 2 mL of the reaction mixture was mixed with 0.5 mL of 2.8% trichloroacetic acid (TCA) and 0.5 mL of 1% thiobarbituric acid (TBA) to yield a final volume of 3 mL. The reaction mixture was heated in a water bath at 90°C for 30 minutes and was then cooled. The absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed in triplicates. Ascorbic acid was used as a positive control and the percentage of inhibition was calculated.

The nitric oxide scavenging ability of the fractions may be quantified according to the Griess-Illosvoy reaction [11]. The reaction mixture contained 1.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline (ph 7.4) and various concentrations of fractions, in a final volume of 3 mL. The mixture was incubated at 25°C for 150 minutes. About 0.5 mL of the reaction mixture was added to 0.5 mL of Griess reagent (1% (w/v) sulfanilamide, 2% (v/v) H₃PO₄ and 0.1% (w/v) naphthylethylene diamine hydrochloride). The absorbance was recorded at 546 nm. The reference standard used was ascorbic acid and was treated the same way as the fractions. Sodium nitroprusside in PBS (2 mL) was used as control. All tests were performed in triplicates. The percentage of nitric oxide radical inhibition was calculated.

The hydrogen peroxide scavenging ability was determined according to a previously described method with slight modifications [12]. About 0.6 mL solution of 40 mM hydrogen peroxide (H₂O₂) in phosphate buffer at ph 7.4 and different concentrations of fractions were mixed (1:1 v/v) and incubated for 30 minutes. Absorbance of H₂O₂ was recorded at 230 nm against a blank solution. All tests were performed three times and the percent inhibition of H₂O₂ was calculated.

Determination of the Acetylcholinesterase Inhibitory Activity: The microplate assay based on the Ellman's method was used to screen for the

acetylcholinesterase inhibitory effect of the methanolic crude extracts of *Ficus nota* (Blanco) Merr. For this matter, the microplate was filled with 100 μ L of 3 mM DTNB in buffer solution, 40 μ L of buffer solution, 20 μ L of 0.26 u/mL of AChE, and 20 μ L of extract in various concentrations dissolved in buffer solution containing not more than 10% methanol. The mixtures were incubated for 15 minutes at 25°C, and their absorbance was read at 412 nm. To produce a reaction, 20 μ L of 15 mM acetylthiocholine iodide was added to each of the mixtures and the absorbance read every 5 minutes for 20 minutes [13]. The same procedure was done with donepezil as the reference sample. The reactions were done in triplicate, and the percent inhibition was calculated using: % inhibition = $(E - S) / E * 100$.

Statistical Analysis: Means and its standard error were used to summarize the percent inhibitions. Two-factor analysis of variance determined if there are significant differences in the mean % inhibition of ascorbic acid and methanolic extract at different concentrations. Tukey's HSD was used for post hoc analysis. All statistical tests were performed using SPSS ver 20.0 P-values less than 0.05 indicate significant differences.

RESULTS AND DISCUSSION

Phytochemical Screening Test: The findings of the phytochemical screening of methanolic crude leaf extracts of *Ficus nota* are presented in table 1. The results indicate that terpenoids, tannins, polyphenols, alkaloids, and glycosides are all present in the methanolic crude leaf extracts.

Table 1: Results of the Phytochemical Screening

Phytochemical	Results
Terpenoids	+++
Flavonoids	-
Tannins	++
Alkaloids	++
Polyphenols	+++
Glycosides	+++
Coumarins	-

Legend: (-) absence; (+) weak; (++) moderate; (+++) strong

Terpenes and polyphenols are among the most studied and identified phytochemicals from plants. The presence of terpenoids and polyphenols might be responsible for the proFicus spp. such as *Ficus odorata* (Blanco) Merr. and *Ficus pseudopalma* exhibit pro-oxidant activities [14]. The presence of alkaloids is also of importance since natural alkaloids such as galantamine, physostigmine, jatrorrhizine, and cyclanolin might act as acetylcholinesterase inhibitors [15].

Total Phenolic Content: The total phenolic content (TPC) was determined using a reference standard curve of gallic acid ($y = 0.381x - 0.030$, and $R^2 = 0.983$). TPC, expressed as gallic acid equivalent (GAE), was found to be 3.448 mg/g GAE of methanolic crude extract. The result indicates that the *Ficus nota* methanolic fraction contains low amount of phenolic compounds compared to methanolic extracts of other *Ficus* species such as *Ficus sycomorus* (188.59 mg/g GAE) and *Ficus deltoidea* (245.5 mg/g GAE) [17][18]. However, some species such as *Ficus odorata* (0.925 mg/g GAE) and *Ficus benjamina* (5.31-5.73 mg/g GAE) also have low TPC [15][19].

Stimulation of Superoxide, Hydrogen Peroxide and Hydroxyl Radicals: The hydroxyl radical scavenging assay is based on the Fenton reaction where the iron-EDTA complex reacts with hydrogen peroxide to produce hydroxyl radical which attacks deoxyribose. Products of the reaction then form pink chromophores with thiobarbituric acid at low pH when heated [20].

The scavenging activity of the extract was found to be decreasing as the concentration increases. As shown in figure 1, at 125 μ g/mL, the extract showed stimulatory activity towards the hydroxyl radical. Polyphenols may exert pro-oxidant activities under certain experimental conditions [21]. Also, polyphenols that contain dihydroxyphenol and trihydroxyphenol groups may generate hydrogen peroxide [22]. Polyphenols undergo auto-oxidation and produce reactive oxygen species [23]. Furthermore, it is known that non-flavonoid phenols such as caffeic acid and derivatives of homovanillyl alcohols found in *Ficus* species are able to act as pro-oxidants in the presence of metals such as copper and iron [24][25]. Polyphenols may be responsible for the pro-oxidant activity of the extract (Figure 1).

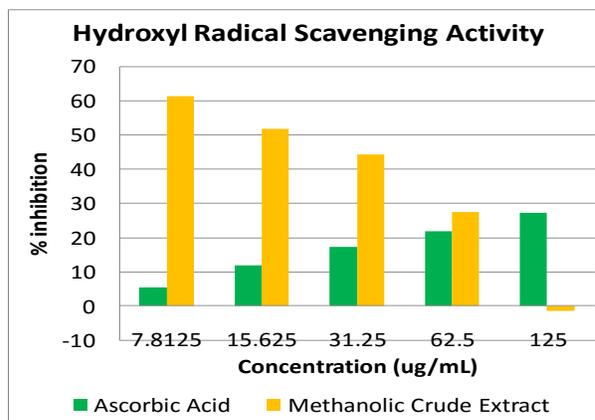


Figure 1: Hydroxyl radical scavenging activities of ascorbic acid and methanolic crude extracts

For nitric oxide radical scavenging assay, sodium nitroprusside with Griess reagent at physiological pH decomposes to produce nitric oxide radicals. Oxygen reacts with nitric oxide to produce nitrites. Nitrites are the products that are quantified using spectrophotometers. Scavengers, when added, compete with oxygen to decrease nitrite formation [26].

In the study, the nitric oxide scavenging activity of the methanolic crude extracts was determined against ascorbic acid. There is a decreasing trend in the inhibitory activity of the extract but its nitric oxide scavenging activity was higher than that of ascorbic acid at 31.25, 62.5, and 125 $\mu\text{g/mL}$. The inhibitory activity of ascorbic acid was found to be superior than that of the extract at 125 and 250 $\mu\text{g/mL}$ (Figure 2).

Based on a study, the double bonds present in monoterpenes make the molecules reactive towards ozone and nitrogen dioxide which are normally present in the air. After reaction, the alkyl radical that is formed further reacts with oxygen to form alkylperoxy radicals. Finally, the alkylperoxy radicals react with nitric oxide to form nitroketones and hydroxyl radicals [27]. This could explain the decreasing nitric oxide scavenging activity since the *Ficus nota* methanolic fraction contains terpenes.

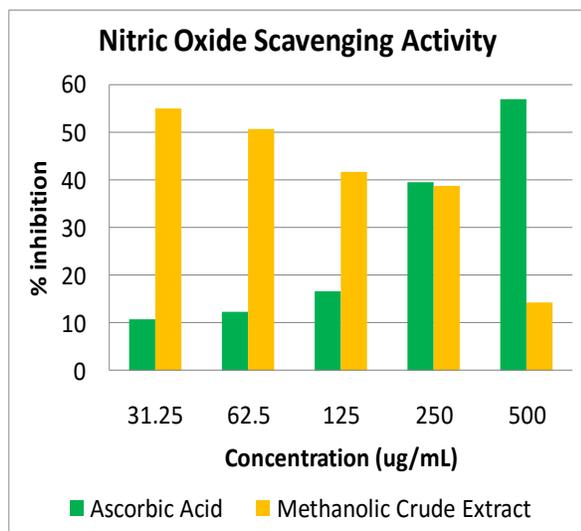


Figure 2: Nitric Oxide radical scavenging activities of ascorbic acid and methanolic crude extracts

The hydrogen peroxide scavenging activity of methanolic crude extracts was determined against that of ascorbic acid. At all concentrations, the extract showed stimulatory activity towards hydrogen peroxide (Figure 3).

Similarly, the reaction between terpenes and atmospheric ozone resulting to the production of high amounts of hydrogen peroxide is well-documented [28]. Secondary plant metabolites such as phenolic acids, and terpenoids act as both anticancer and pro-oxidant agents [29]. The pro-oxidant activity of the extract may be due to the presence of polyphenols and terpenoids.

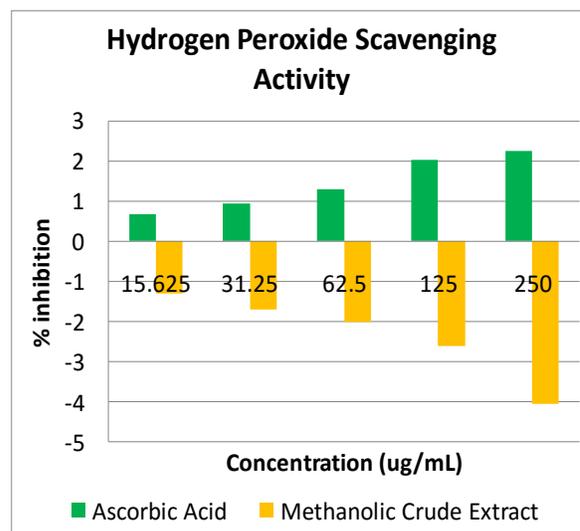


Figure 3: Hydrogen peroxide scavenging activities of ascorbic acid and methanolic crude extracts Microplate Assay

The acetylcholinesterase inhibitory activity was determined using Ellman's method. DTNB (5,5-dithiobis[2-nitrobenzoic acid]), also known as Ellman's reagent, reacts with the thiocholine that was previously released from the reaction between acetylthiocholine and acetylcholinesterase enzymes [2]. The complex that is produced from the reaction between thiocholine and Ellman's reagent is quantified using a spectrophotometer. Donepezil, a reversible acetylcholinesterase inhibitor, was used as the standard [14]. The acetylcholinesterase inhibitory activity of the methanolic crude extract was determined against that of donepezil.

The results showed that at 31.25 $\mu\text{g/mL}$, the methanolic crude extract showed higher inhibitory activity than donepezil over 20 minutes (Figure 4). However, stimulatory activity was exhibited by the extract at higher concentrations (Figure 6, 7, 8). At 62.5 $\mu\text{g/mL}$, the extract inhibited AChE specifically at 15 and 20 minutes, but stimulated the enzyme at 0, 5, and 10 minute/s (Figure 5). The data (p-value of 0.104) did not provide sufficient evidence to indicate that at least a pair of extracts is different. Therefore, the results were not statistically significant.

Natural alkaloids like galantamine, physostigmine, jatroerrhizine, and cyclanoline are known inhibitors of acetylcholinesterase [14]. Phytochemical screening of the methanolic crude extract of *Ficus nota* (Blanco) Merr. revealed the presence of alkaloids. The alkaloids in the extract may be responsible for the inhibitory activity at lower concentrations.

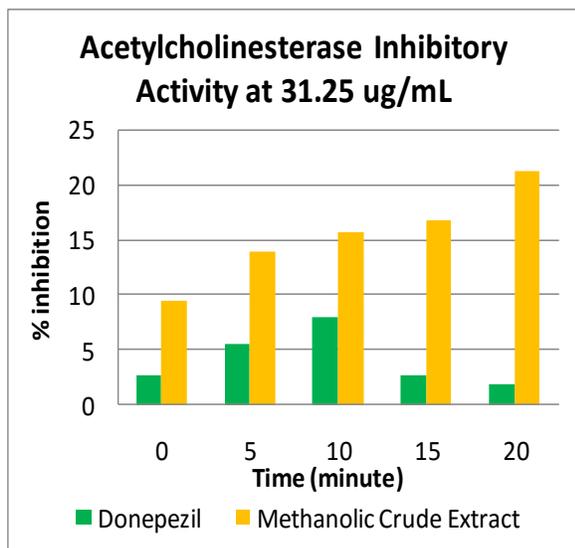


Figure 4: Acetylcholinesterase inhibitory activities of donepezil and methanolic crude extract at 31.25 ug/mL

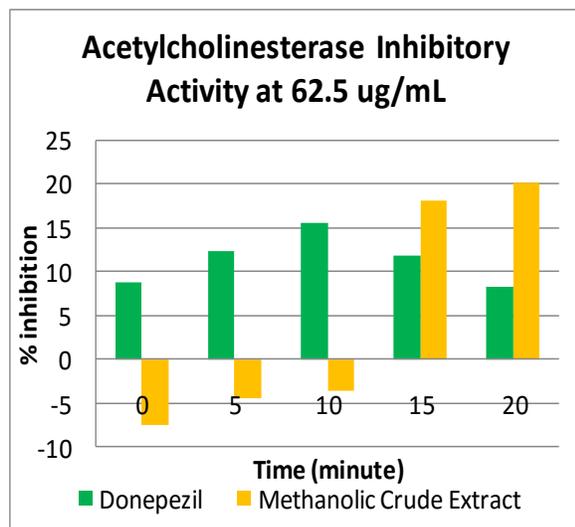


Figure 5: Acetylcholinesterase inhibitory activities of donepezil and methanolic crude extract at 62.5 ug/mL

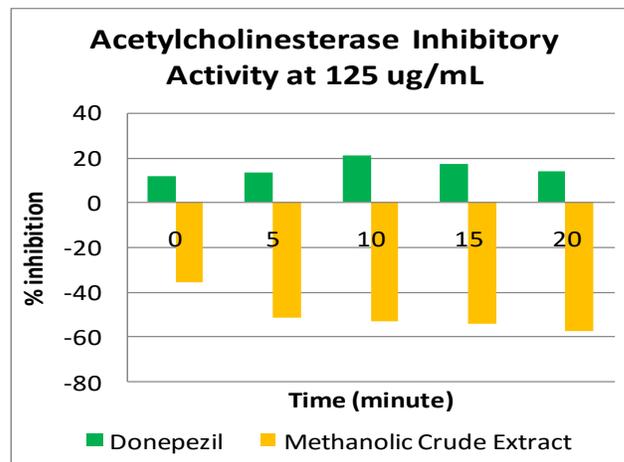


Figure 6: Acetylcholinesterase inhibitory activities of donepezil and methanolic crude extract at 125 ug/mL

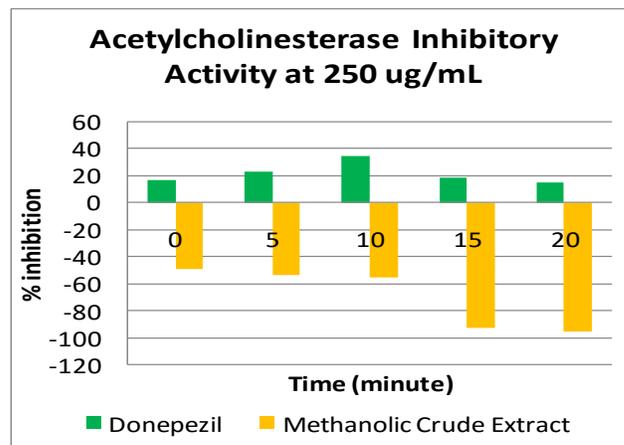


Figure 7: Acetylcholinesterase inhibitory activities of donepezil and methanolic crude extract at 250 ug/mL

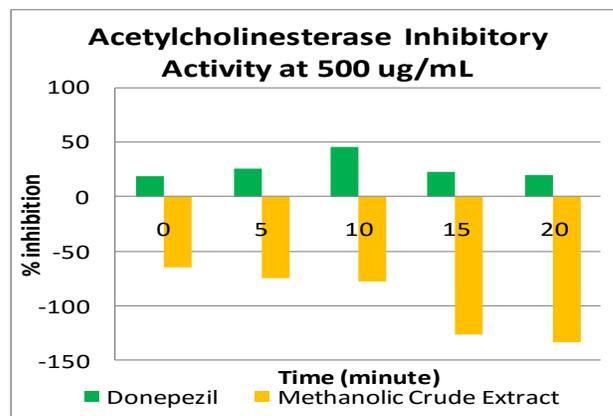


Figure 8: Acetylcholinesterase inhibitory activities of donepezil and methanolic crude extract at 500 ug/mL

CONCLUSION

The findings of the phytochemical screening indicated the presence of terpenoids, tannins, alkaloids, polyphenols and glycosides. The methanolic crude extracts showed stimulatory or pro-oxidant activity towards hydrogen peroxide. Although in a decreasing trend, the extract showed inhibitory activity towards nitric oxide. Ascorbic acid was found to be superior at 125 and 250 µg/mL only. Similarly, inhibitory activity towards hydroxyl radical was exhibited by the extract in a decreasing trend. In contrast, the extract showed stimulatory activity at 125 µg/mL. In the microplate assay, the results revealed that there is inhibition of AChE at

low concentration specifically at 31.25 µg/mL over 20 minutes.

For both micro-scale antioxidant and microplate assay, the p-values obtained by statistical analysis are all less than 0.05. Thus, the results of the study are statistically insignificant. However, the medical significance of pro-oxidant activities should not be underestimated.

Ficus nota can be developed as a new agent useful in the treatment of cancer due to its stimulatory activity towards hydrogen peroxide, hydroxyl radicals, and nitric oxide.

CONFLICTS OF INTERESTS

All authors have none to declare.

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