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Identification and Quantification of the Secondary Metabolites of *Ficus nota* (Blanco) Merr. Leaf Extracts using Spectroscopic and Chromatographic Techniques

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The present report is a pioneering attempt to conduct an analysis of the secondary metabolites of Ficus nota (Blanco) Merr. leaf extracts using standard spectroscopic and chromatographic techniques. UV-vis spectroscopic method for the analysis of total phenolic and total flavonoid levels showed 95.5 ± 0.4 mg GAE/g sample and 226.5 ± 1.0 mg RHE/ g sample, respectively. These high amounts of phenolic compounds and flavonoids relate the potential of the tested extracts as an antioxidant agent. Further, GC-MS analysis revealed 18 chemical compounds and their corresponding relative amounts that can be a basis for further experiments to explore the biomedical and pharmacological properties of the Ficus nota (Blanco) Merr. leaf extracts. The identified and quantified chemical compounds were further confirmed by FTIR analysis through the presence of different chemical bonds related to the functional groups of the phytoconstituents. The current findings support the need for more investigation of the therapeutic potential of the Ficus nota (Blanco) Merr. leaf extracts that will be beneficial to human health.

Keywords: Chromatography; Ficus nota (Blanco) Merr.; Leaf extracts; Phytochemistry; Secondary metabolites; Spectroscopy.

The Philippines is known to be one of the nations in Asia having a diverse flora with a record of about 13,500 plant species in reference to the reports of Department of Environment and Natural Resources.[1] Of these recorded plants, 3500 are endemic with 1500 possessing medicinal and therapeutic values. Plants are very useful in various aspects as food resources, timber, clothing, and as herbal medicines to treat different ailments and diseases.[2] Plants were used as medicinal agents for the relief and cure of various ailments even before the Spanish regime, however, the documentation was initiated only during the Spanish era through the practices of herbolarios or mediquillos (herbal scientists). In recent years, the Philippine Department of Health approved ten medicinal plants as alternative medicines for various pharmacological applications.[3] These pharmacological properties are attributed to the occurrence of non-nutrient secondary metabolites such as alkaloids, terpenoids, phenolic compounds, tannins, flavonoids, saponins, steroids, anthraquinones, glycosides, and coumarins.[1,4] Analysis of these secondary metabolites present in plants is an important procedure to standardize and validate the applicability of medicinal plants with therapeutic properties.[5]

Secondary metabolites can be analyzed using various qualitative and quantitative techniques to identify and quantify the compounds present in a specific plant species after an appropriate extraction procedure, such as the different chemical tests based on observable changes in the tested solutions.[6] In this approach, the presence or absence stated as a negative or positive test, respectively, is based on the intensity of the color changes or precipitate formation.[7] The different functional groups related to the secondary metabolites present in the plant extract can be identified using Fourier transform infrared (FTIR) spectroscopy.[8] Total phenolic and total flavonoid contents can be measured using UV-vis spectroscopy on the basis of different colorimetric reactions between the chemical reagents used in the assay.[9] Moreover, gas chromatography-mass spectrometric (GC-MS) analysis of the extracts can be performed to identify and quantify the compounds based on molecular weight, molecular formula, peak area, and retention time.[10] These techniques are used to give important information about the phytochemical profile of a medicinal plant, especially plant species that remain untapped for their pharmacological properties.

Plant species with known medicinal properties that can be found all over the Philippines are the *Ficus* spp. that are commonly known as figs.[11] The tree parts were reported to possess bioactive secondary metabolites that can be used as traditional medication in treating various ailments. One *Ficus* tree species known to be endemic in the Philippines is tibig or *Ficus nota* (Blanco) Merr. that can grow up to 9 meters high, with large leaves and edible fruits (Figure 1).[12-13] The chemical constituents such as phenolic compounds, diols, and sitosterols of the unripe fruits were elucidated using extensive nuclear magnetic resonance (NMR) spectroscopy.[13] While a more recent phytochemical characterization of the *Ficus nota* (Blanco) Merr. leaves revealed the occurrence of secondary metabolites such as flavonoids, steroids, alkaloids, saponins, tannins, and anthraquinones.[12] The phytochemical screening results are initial information on the chemical constituents that can be isolated from the *Ficus nota* (Blanco) Merr. leaves that have potential biological and pharmacological activities. However, extensive information about the secondary metabolites present in *Ficus nota* (Blanco) Merr. is very important as this can provide baseline information for future phytochemical and pharmacological researches that will eventually develop effective and safe medicinal agents for the treatment of various illnesses. Therefore, the aim of the study is to profile the secondary metabolites present in the *Ficus nota* (Blanco) Merr. leaves through different spectroscopic and chromatographic approaches. To the best of the researchers' knowledge, no previous literatures have yet reported the identification and quantification of the secondary metabolites present in *Ficus nota* (Blanco) Merr. leaf extracts. Specifically the objectives are as follows: (1) to quantify the total phenolic and flavonoid content using UV-vis spectroscopy; (2) to identify and semi-quantify the chemical compounds using GC-MS technique, and; (3) to identify the functional groups present using FTIR spectroscopy.

MATERIALS AND METHODS

Plant material and extraction procedure

The *Ficus nota* (Blanco) Merr. leaf samples were collected from San Francisco, San Antonio, Nueva Ecija, Philippines. The plant material was then authenticated at the Far Eastern University Herbarium, Manila, Philippines. The extraction procedure was based on the previously reported protocol with minor modifications.[1] In brief, leaves were chopped and washed with tap water, then rinsed with distilled water before air drying for two weeks. The dried leaves were pulverized and soaked in 95% ethanol for 48 hours. The collected extracts were filtered and concentrated *in vacuo* using a rotary evaporator and subsequently lyophilized at -20 °C in a freeze dryer. The resulting extracts were placed in an amber bottle and stored at 4 °C until use.

Total phenolic and total flavonoid content

The quantitative analysis of the total phenolic and total flavonoid content was conducted using a UV-1280 UV-vis spectrophotometer (Shimadzu, Japan). For the total phenolic content, a classic procedure with some modifications was employed.[14] A 0.5 mL of the leaf extracts was mixed with 2.5 mL of the freshly prepared Folin-Ciocalteu reagent. A 2.0 mL of the 7.5 % sodium carbonate solution was then added to the mixture, and allowed to stand for an hour for the completion

of the color formation. The absorbance value of the resulting blue colored mixture was measured at 760 nm against a reagent blank, and using gallic acid as the standard. The total phenolic content was calculated and expressed as mg GA equivalent (GAE) per gram of sample.

For the total flavonoid content of the leaf extract, a colorimetric procedure with minor modifications was employed.[15] A 0.5 mL of diluted leaf extracts were placed into test tubes containing 2.0 mL of distilled water and 0.15 mL of 5% sodium nitrite solution. After 5 minutes, 0.15 mL of aluminum chloride hexahydrate solution was added to the test solution. Then 1.0 mL of sodium hydroxide solution was added, and the absorbance value of the resulting test solution was measured at 415 nm after 15 minutes reaction time. The total flavonoid content was calculated using the standard rutin hydrate, and expressed as mg rutin hydrate equivalent (RHE) per gram of the sample.

Gas chromatography - mass spectrometric (GC-MS) analysis

Prior to the instrumental analysis, a stock solution was prepared by dissolving 1.0 mL of the leaf extracts with ethanol 5.0 mL solution. A 20.0 µL aliquot of this solution was transferred to a vial and 980.0 µL of ethanol was added to obtain a 1.0 mL sample solution. The solution was filtered using a 0.2 µm syringe filter before injection to the GC-MS instrument. The identification and semi-quantification of the secondary metabolites was accomplished using a GCMS-QP 2010 Ultra (Shimadzu, Japan) using a Shimadzu SH-I-5-MS column (30.0 m, 0.25 mm ID, 0.25 µm film thickness) and He as the carrier gas at 1.20 mL/min constant column flow. The injector port was operated at 300 °C using a splitless method of injection and the oven temperature programming was as follows: initially at 50 °C held for 3 minutes; increased to 150 °C at a rate of 10°C/min held for 5 minutes, and increased again to 290 °C at a rate of 10°C/min and held for 15 minutes. An electron ionization system at 230 °C ion source temperature and an interface temperature of 300 °C, and operated in electron impact mode for quadrupole mass spectrometer acquisition with a 7-minute solvent cut time.

FTIR spectral characterization

The FTIR analysis of the leaf extracts was performed using an FTIR spectrometer IRSpirit with Q-ATR accessory (Shimadzu, Japan). Few drops of the leaf extracts were placed on the ATR crystal and the spectra were obtained using a general procedure for the FTIR instrumentation.[16] The instrumental analysis utilized a wavelength range of 700-4000 cm^{-1}, with a resolution of 4 cm^{-1} for 20 accumulation times. A background spectrum was recorded before every sample testing, and the analysis was performed at ambient temperature.

RESULTS

Total phenolic and total flavonoid content

The total phenolic and total flavonoid contents of the tested leaf extracts of *Ficus nota* (Blanco) Merr. were presented in Table 1.

Fig. 1. *Ficus nota* (Blanco) Merr. plant used in the study

GC-MS analysis of *Ficus nota* (Blanco) Merr. leaf extracts

The GC-MS analysis of the *Ficus nota* (Blanco) Merr. leaf extracts provided qualitative and semi-quantitative data for the secondary metabolites present in the leaf extract. The results showed 18 chemical compounds as illustrated in Table 2 and Figure 2. The chemical compounds were identified on the basis of retention time, molecular weight, and molecular formula using the available data in the NIST library.

FTIR analysis of *Ficus nota* (Blanco) Merr. leaf extracts

Based on the peak value in the wavelength range used in the FTIR analysis, functional groups can be determined.[17] The FTIR analysis showed significant peaks at about 3258.5 cm^{-1}, 1608.5 cm^{-1}, and 1028.8 cm^{-1}, and some small peaks 2929.9 cm^{-1}, 1245.4 cm^{-1}, and 816.4 cm^{-1}, as illustrated in Figure 3, and tabulated in Table 3.

DISCUSSION

Total phenolic and total flavonoid content

The total phenolic content of 95.5 ± 0.4 mg GAE/g sample is related to the polyhydroxyl phenolic compounds that facilitate free-radical scavenging potential of the leaf extract.[18] While the total flavonoid content of 226.5 ± 1.0 mg RHE/g sample (Table 1). These phenolic compounds and flavonoids are reported to be responsible for a significant level of antioxidant capacity in plant species.[19] The high levels of phenolics and flavonoids in the tested leaf extract revealed that *Ficus nota* (Blanco) Merr. is a source of natural antioxidants with potential pharmacological properties.

Comparison to the previous reports of the total phenolic and total flavonoid contents of plant extracts of other *Ficus* species such as: (1) *Ficus religiosa* bark extracts = 4.81 ± 1.01 mg GAE/g

Table 1. Total phenolic (mg GAE/g) and total flavonoid (RHE) contents of the leaf extracts of *Ficus nota* (Blanco) Merr.

Total phenolic content (mg GAE/g sample)	Total flavonoid content (mg RHE/g sample)
95.5 ± 0.4	226.5 ± 1.0

Table 2. Secondary metabolites identified and semi-quantified from the *Ficus nota* (Blanco) Merr. leaf extracts using GC-MS analysis

Identity based on mass spectral comparison with NIST Library Data	Retention time (min)	Relative amounts based on peak area
Ethyl diethoxyacetate	7.327	2.13%
5-Isobutylnonane or 5-butylnonane	12.416	1.10%
Anhydro-d-mannosan	12.675	1.67%
2,4-Di-tert-butylphenol	16.584	5.67%
Methyl laureate	16.871	4.03%
3-Buten-2-one, 4-(2-hydroxy-2,6,6-trimethylcyclohexyl)	20.613	0.98%
Methyl myristate	21.658	1.76%
Methyl palmitate	24.566	6.31%
Palmitic acid	24.963	6.64%
Ethyl palmitate	25.368	17.83%
Methyl elaidate	26.528	3.70%
Phytol	26.663	0.70%
Methyl stearate	26.778	1.26%
Stearic acid	27.101	1.96%
Ethyl stearate	27.431	16.58%
2,2'-Methylenebis(4-methyl-6-tert-butylphenol)	29.534	2.42%
2-Monopalmitin	30.183	17.63%
α-Monostearin	31.767	7.62%

sample and 109.15 ± 1.2 mg RHE/g sample[20]; (2) *Ficus carica* Linn. leaf extracts = 33.93 ± 0.31 mg GAE/g and 26.28 ± 0.20 mg quercetin equivalent (QE)/g sample[21]; (3) *Ficus palmata* Forssk aerial part extracts = 49.24 ± 1.21 mg GAE/g sample and 29.9 ± 1.13 mg QE/g sample[22], and (4) *Ficus sycomorus* L. leaf extracts = 1980.4/ mg GAE/g sample and 109.9/ mg QE/g sample[23] proved that *Ficus* spp. are rich source secondary metabolites that can be isolated and further developed for its possible therapeutic uses.

GC-MS analysis of *Ficus nota* (Blanco) Merr. leaf extracts

Based on the peak areas, the relative amounts of the identified compounds were semi-quantified. Ethyl palmitate (17.83%),

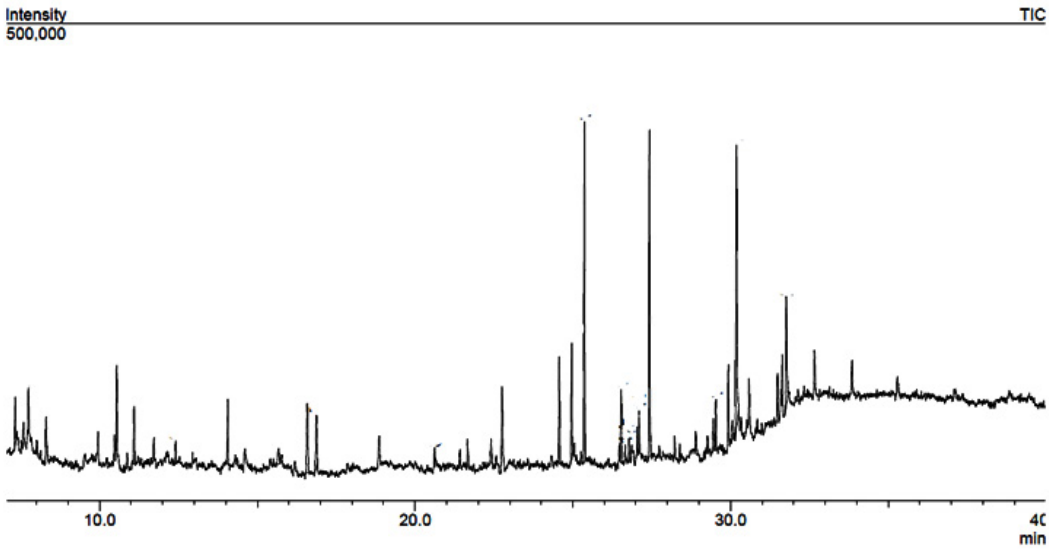

Fig. 2. Chromatogram of the *Ficus nota* (Blanco) Merr. leaf extract

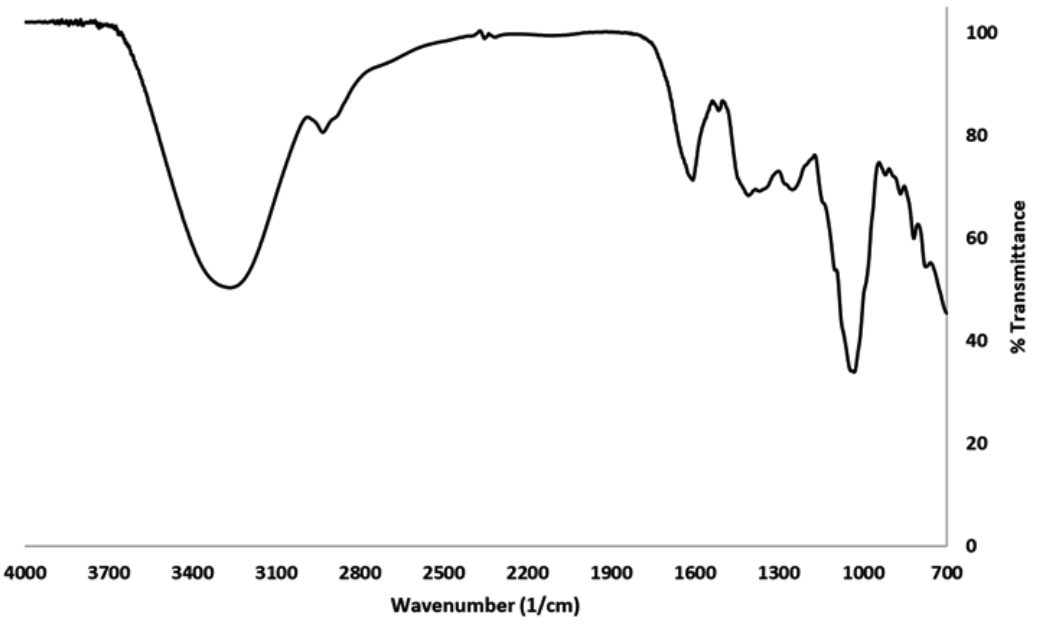

Fig. 3. FTIR spectra of the *Ficus nota* (Blanco) Merr. leaf extracts.

Table 3. FTIR peak values and functional group assignment for *Ficus nota* (Blanco) Merr. leaf extracts

Wavenumber (1/cm)	Bonds	Intensities	Functional group	Remarks
3258.5	O-H	Strong broad	Hydroxyl group	Hydroxyl group in alcohols or phenols
2929.9	C-H	weak	Alkane	C-H bond of alkanes
1608.5	C=O	strong	Carbonyl	Carbonyl stretching
1245.4	C-O	weak	C-O	C-O bond of esters
1028.8	C-O	Strong sharp	C-O	C-O bonds of alcohols, esters or phenols
816.4	C-H	weak	Alkane	C-H stretching of alkanes

2-Monopalmitin (17.63%), and Ethyl stearate (16.58%) were the most abundant compounds, while phytol (0.70%) was the least among the compounds identified (Table 2). The GC-MS analysis revealed the presence of secondary metabolites such as esters (Ethyl diethoxyacetate), aliphatic compound (5-Isobutylnonane), sugar derivatives (anhydro-d-mannosan), fatty acid esters (Methyl laureate, Methyl myristate, Methyl palmitate, Ethyl palmitate, Methyl elaidate, Methyl stearate, Ethyl stearate, 2-Monopalmitin, and á-Monostearin), phenolic compounds [2,4-Di-tert-butylphenol and 2,2'-Methylenebis(4-methyl-6-tert-butylphenol)], ketone [3-Buten-2-one, 4-(2-hydroxy-2,6,6-trimethylcyclohexyl)], fatty acids (Palmitic acid and Stearic acid), and terpene (phytol) (Figure 2). Previous literature reports highlighted the different pharmacological properties of the chemical compounds that were identified in *Ficus nota* (Blanco) Merr. leaf extracts such as the anhydro-d-mannosan as analgesic,[24] phytol in the reduction of the risk of cardiovascular diseases,[25] palmitic and stearic acid as antimicrobial agents,[26] 2,4-Di-tert-butylphenol as inhibitory agents against diabetes-related enzymes,[27] and fatty acids and fatty acid esters with different pharmacological properties.[28-29] The GC-MS data obtained in this study complements the findings of the phytochemical analysis of *Ficus nota* (Blanco) previously reported in the literature.[12] GS-MS is an indispensable analytical instrument for analysis of secondary metabolites in plant extracts due to its high accuracy, high precision, high sensitivity and high resolution, however, the technique is limited only to the analysis of volatile and thermally stable compounds.[30] Therefore, the instrumentation is not suitable for secondary metabolites that have high boiling points and high polarities.

FTIR analysis of *Ficus nota* (Blanco) Merr. leaf extracts

FTIR analysis of the *Ficus nota* (Blanco) Merr. leaf extracts showed the presence of compounds containing various functional groups such as hydroxyl groups for alcohols or phenols, C=O bonds for carbonyl containing compounds, C-O bonds for esters, alcohols, or phenols, and aliphatic stretching for alkanes (Figure 3).[17] The FTIR results showed characteristic peaks that can be related to the chemical structures of the bioactive secondary metabolites identified and quantified in *Ficus nota* (Blanco) Merr. leaf extracts using various spectroscopic and chromatography techniques (Table 3).

CONCLUSIONS

The present study revealed that the *Ficus nota* (Blanco) Merr. leaf extracts are a rich source of secondary metabolites that have pharmacological properties for potential therapeutic applications. The *Ficus nota* (Blanco) Merr. leaf extracts contain high amounts of total phenolics and total flavonoids that can be explored for its antioxidant properties. GC-MS analysis showed the presence and quantity of 18 phytoconstituents that possessed corresponding pharmacological properties. However, due to the limitations of GC-MS, only those compounds that are volatile were analyzed. In this regard, liquid chromatography-mass spectrometry (LC-MS) may be explored to identify and quantify other chemical compounds present in the *Ficus nota* (Blanco) Merr. leaf extracts. Moreover, FTIR results revealed the presence of functional groups related to the compounds identified and quantified using UV-vis spectroscopy and GC-MS. The use

of different spectroscopic and chromatographic techniques are gold standard analytical strategies that can be used for qualitative and quantitative analysis of secondary metabolites. The findings of this study warrant further investigation on the potential pharmacological properties of *Ficus nota* (Blanco) Merr. leaf extracts and open opportunities for the development of medicinal agents from natural resources.

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Conflict of Interest

The author(s) do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials

Permission to reproduce material from other sources

Not Applicable

Authors' Contribution

Renalyn Mae E. Diaz – conceptualization, proposal, methodology, data analysis, writing – original draft, funding acquisition**;** Darwin F. Reyes – conceptualization, methodology, data analysis, writing – original draft, writing – review and editing, funding acquisition, supervision

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