Trop J Nat Prod Res, January 2025; 9(1): 284 - 294 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Safety Evaluation, High-Performance Thin Layer Chromatography Profile, Antioxidant, Antibacterial, and Anti-inflammatory Activities of *Homalanthus macradenius* Pax & K.Hoffm Crude Leaf Extract

Rosita C. Mabolo*, Glenda Z. Doblas, Joliesa Mae S. Toledo, Reggie Y. Dela Cruz

Molecular Biology & Biotechnology, Genetics and Microbiology Division, Institute of Biological Sciences, College of Arts and Sciences, Central Mindanao University, University Town, Musuan, Maramag, Bukidnon, 8710

ARTICLE INFO

Article history:
Received 04 August 2024
Revised 23 August 2024
Accepted 25 November 2024
Published online 01 February 2025

ABSTRACT

Homalanthus macradenius is endemic to the Philippines and is one of the seven species of the genus utilised to treat various health problems in traditional medicine. This study was undertaken to evaluate the safety of *H. macradenius* leaf ethanolic extract through acute oral toxicity in Swiss albino mice per Organization for Economic Cooperation and Development (OECD) guidelines 423; determine the phytochemical constituents through High-Performance Thin-Layer Chromatography; quantify the total phenolic and flavonoid content; and to assess its bioactivities such as antioxidant, antibacterial, and anti-inflammatory potentials. Acute oral toxicity *of H. macradenius* ethanolic extract at 300 mg/kg, 2000 mg/kg, and 5000 mg/kg revealed no mortality. The HPTLC chromatogram displayed different colours of bands ranging from blue, purple, yellow, green, and orange with an Rf value of 0.10 to 0.81, which indicates the presence of different phytochemical constituents of the plant ethanolic extract. The total phenolic and flavonoid contents were 8.07±0.01 mg GAE/g and 33.86±0.03 mg QE/g, respectively. Antioxidant activity was found to be 15.68±0.02 mg AAE/g. The extract exhibited antibacterial activity with minimum inhibitory concentrations against *Enterococcus faecalis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* at 1.562±0.09 mg/mL, 12.5±0.15 mg/mL and 25±0.03 mg/mL, respectively. The percent *in vitro* COX-2 inhibitory activity showed 92.30±0.73% at 100 ppm and 60.51±4.02 % at 10 ppm with a selectivity ratio (COX-2/COX-1) of at most 1.07. Based on the findings, *H. macradenius* extract did not show oral toxicity in mice and possessed antioxidant, antibacterial, and anti-inflammatory activities, revealing its potential for future drug development.

Keywords: *Homalanthus macradenius*, Cyclooxygenase, Phenolic and flavonoid content, Acute oral toxicity.

Introduction

Plants have been used as medicinal materials since ancient times, and several are globally recognised as medicine.[1] As the World Health Organization suggests, traditional medicines and natural plant products are the main sources of primary healthcare for most people living in underdeveloped nations like the Philippines.[2] Plants have medicinal properties due to their phytochemical components, which have specific pharmacological effects on the human body.[3] Additionally, phytochemicals are recognised for their biological activity, which significantly enhances the antimicrobial effectiveness of plants.[4] Among the commonly used medicinal plants are the genus *Homalanthus* from the Euphorbiaceae family. *Homalanthus macradenius* is considered endemic in the Philippines[5,] and it is one of the seven *Homalanthus* species that have been utilised to treat various health problems in traditional medicine.

*Corresponding author. E mail: rositamabolo333@gmail.com

Tel: +09383051280

Citation: Mabolo RC, Doblas GZ, Toledo JMS, Dela Cruz RY. Safety Evaluation, High-Performance Thin Layer Chromatography Profile, Antioxidant, Antibacterial, and Anti inflammatory Activities of *Homalanthus macradenius* Pax & K.Hoffm Crude Leaf Extract. Trop J Nat Prod Res. 2025; 9(1): 284 – 294 https://doi.org/10.26538/tjnpr/v9i1.37

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

It is reported that some species of the genus show biological activities such as antimicrobial, wound-healing properties, anti-HIV, anti-protozoal, and estrogenic.[6] *H. macradenius* is typically utilised among the Manobo community in Agusan, a province in Mindanao, Philippines. The leaves cure cuts and wounds7 and alleviate diarrhoea, stomach problems, and impetigo.[8] However, there is limited information available on the biological activities of *H. macradenius;* therefore, further research is needed to explore the specific biological activities and potential medicinal uses of this species and further examine its safety. According to the Organization for Economic Cooperation and Development (OECD) guidelines, toxicological studies are essential in animals like rats, mice, guinea pigs, rabbits, and monkeys to establish the safety and efficiency of a new drug. Toxicological studies help to decide whether a new drug should be adopted for clinical use. Depending on the duration of drug exposure to animals, there are three types of toxicological studies, namely, acute, subacute, and chronic toxicological studies. This study was undertaken to analyse the phytochemical constituents through high-performance thin-layer chromatography and quantify the total phenolic and flavonoid contents of the leaf extract of *H. macradenius*. It also evaluated the safety through acute oral toxicity assay and its antioxidant, antibacterial, and anti-inflammatory activities.

Materials and Methods

Collection and Identification of Plant Sample

Samples of *H. macradenius* leaves were collected from a private farm in Barangay Kahusayan, Kitaotao, Bukidnon, Philippines, on February 12, 2024 (Figure 1). The plant sample (Figure 2) was identified and confirmed by the University Museum at Central Mindanao University, University Town, Musuan, Maramag, Bukidnon, where herbarium samples were deposited with accession number (CMU No. 00015806).

Preparation of Leaf extract

Young leaves of *H. macradenius* were collected and washed thoroughly with distilled water and air-dried for two weeks. The samples were weighed, air-dried, and pulverised using an Osterizer blender. The leaf powder (400 g) was soaked in 2L of ethanol (100%) for 72 hours. The filtrate was obtained and concentrated using a rotary evaporator at 40°C. The dried ethanolic extract of *H. macradenius* was stored at 4ºC until further use.

Figure 1: The location where the specimen was collected, a) Map of the Philippines, b) Map of Bukidnon with Municipality of Kitaotao highlighted [Photos from Google]

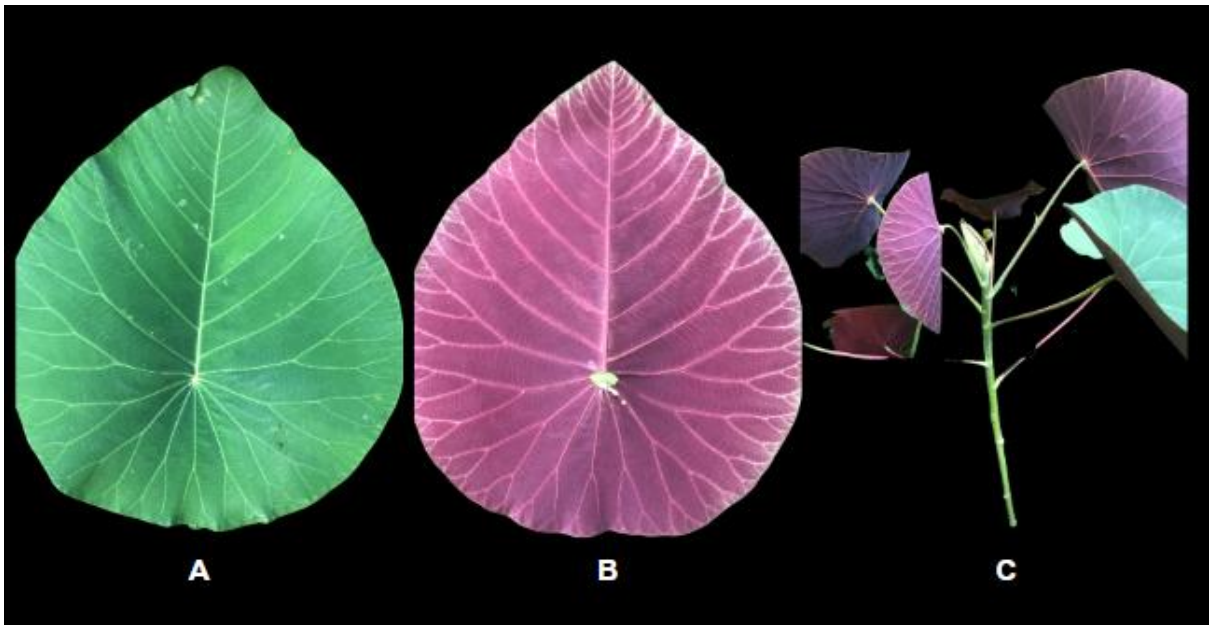


Figure 2: Actual photograph of *H. macradenius*. a) adaxial view, b) abaxial view of the leaves, and c) the stem and terminal bud of the plant

High-Performance Thin-Layer Chromatography

An Automatic TLC Sampler (ATS 4) and an Automatic Developing Chamber are features of the HPTLC system (CAMAG, Muttenz, Switzerland). Software for analysis included visionCATS 2.5, ADC 2, Scanner 4, TLC Visualizer, Immersion Device 3, and Plate Heater. The method was adopted from Ang *et al*. (2022) wherein on an HPTLC aluminium-backed plate (silica gel 60 F254; 20 x 10 cm; Merck), precisely 2 µL, 3µL, and 4µL of sample extracts in methanol were spotted separately under a nitrogen stream. Following sample application, the plate was developed using ethyl acetate: formic acid: water (80:10:10) as a mobile phase in a 20 x 10 cm twin trough glass chamber that had been pre-saturated. The chromatogram was then viewed in white and UV light at 254 nm and 366 nm, respectively. After that, the plate was air-dried for five minutes under the fume hood, submerged in the Natural Product (NP) reagent (immersion speed: 5 cm/s; dwell time: 1 s), and viewed under UV light at 366 nm. Natural Product (NP) reagent contains 2-aminoethyl diphenyl borate and ethyl acetate. [20] The experiment was performed in triplicate to ensure the accuracy and reliability of the results.

Total Phenolic Content Determination

The total phenolic content (TPC) of *H. macradenius ethanolic* extract was analysed following the method described by Hudz *et al*.,[9] with a slight modification of the solvent used. A 200 µL test solution was mixed with 200 µL of 10% Folin-Ciocalteu reagent and 800 µL of 10% sodium carbonate in an Eppendorf tube. The tube was inverted five

times to mix correctly. The reaction mixture was cooled to room temperature after two (2) hours of incubation and then centrifuged for 3 minutes at 11000 rpm. The absorbance at 750 nm was obtained after loading 200 µL of the reaction mixture into a 96-well microplate. The standard calibration curve was plotted using gallic acid. From the calibration curve, the amount of TPC was determined and reported in milligram gallic acid equivalents per gram dry extract (mg GAE/g. The extracts' total phenolics were estimated in triplicate samples, and the results were averaged.

Total Flavonoid Content Analysis

Total flavonoid content (TFC) in *H. macradenius* ethanolic extract was analysed with aluminium chloride reagent using the colourimetric method adapted from Koley *et al*. (2018)[10] with a slight modification in the volume of extract used. Aliquot (50 µL) of each extract was placed in a 96-well plate containing 10 µL of 10% aluminium chloride, 130 µL of 100% ethanol, and 10 µL of 1 M sodium acetate. The mixtures were incubated for 40 minutes at room temperature in the dark. The absorbance at 415 nm was measured using Spectramax. TFC was expressed as mg of quercetin equivalents per gram of dry weight (mg QE/g DW) through a calibration curve with quercetin. All samples were performed in three replicates, and the results were averaged.

Total Antioxidant Activity

The Total Antioxidant Activity (TAA) of *H. macradenius* was determined using the phosphomolybdenum assay method described by Prieto *et al*.[11] with slight modification. The test solution was mixed with 600 µL phosphomolybdenum reagent (28mM sodium phosphate, 4mm ammonium molybdate, 95% sulphuric acid), then incubated at 95°C for 90 minutes and cooled at room temperature before centrifuging for three minutes at 11000 rpm. A 200 µL reaction mixture was loaded in a 96-well microplate, and the absorbance was read at 695 nm. The calibration curve was plotted using the ascorbic acid standard. The TAA was expressed in ascorbic acid equivalents per gram (mg.AAE/g). The estimation of total antioxidant activity in the extracts was carried out in triplicate, and the results were averaged.

Acute Oral Toxicity

Acute oral toxicity assay was carried out as approved by the Institutional Care and Use Committee (IACUC) with protocol number 2024-033D.

The *Experimental Animals*

The experiment was conducted on 12 healthy Swiss albino female mice weighing 25 g to 35 g and aged 8 to 10 weeks.[12] Three (3) mice were used in each test dosage. The mice were housed in cages, acclimatised for 1 week, and maintained on a 12-h light/dark cycle at 23°C with constant humidity. Drinking water and food were provided *ad libitum*, except for the short fasting period, where only water and no food were supplied 12 hours before treatment.

The crude extract was suspended in a vehicle (10% DMSO) instead of water since the plant extract was not soluble in water. Following the fasting period, the body weight of the mice was determined, and the dose was calculated in reference to the body weight, as the volume of the extract solution given to the mice was 1 mL/kg. The administration of doses was performed according to paragraph 20 of Organization for Economic Cooperation and Development (OECD) 423 guidelines, wherein, when there is no information on a substance to be tested and for animal welfare reasons, it is recommended to use a starting dose of 300 mg/kg body weight and that the onset, duration, and severity of toxic signs determined the time interval between treatment groups. Treatment of animals at the next dose was delayed until one was confident of the survival of the previously dosed animals. After 300 mg/kg administration, a close observation was done in the experimental mice. After a day, 2000 mg/kg was also administered and observed. Paragraph 21 of OECD guideline 423 suggests that exceptionally, and only when justified by specific regulatory needs, an additional upper dose level of 5000 mg/kg body weight may be considered. This study used three (3) different dosages (300 mg/kg, 2000 mg/kg, and 5000 mg/kg), and distilled water was also administered as a normal control. Each treatment was performed in three (3) replicates, which means three (3) mice were subjected to each treatment and all the test substances were administered in a single dose by gavage using a stomach tube. After oral administration, food was provided to the mice after approximately one to two hours. The mice were observed in detail for any toxicity effect within the first six hours after the treatment period and daily for 14 days. Surviving animals were weighed, and visual observations were noted individually after 30 minutes, 4 hours, 24 hours, 48 hours, 1 week, and 2 weeks and the following parameters were observed and recorded: respiration, alertness, grooming, pain, tremors, pupils, salivation, food intake, water intake, urination, diarrhoea, skin colour, skin fur, lacrimation, convulsion, coma, hyperactivity, sleep, and mortality.[13]

Antimicrobial Susceptibility Testing

Antibacterial activity of the crude extract was tested against medically important gram-positive and gram-negative bacteria obtained from the Philippine National Collection of Microorganisms (PNCM), BIOTECH, University of the Philippines Los Baños Laguna: *Staphylococcus aureus* BIOTECH 1582, *Enterococcus faecalis* BIOTECH 10348 and *Pseudomonas aeruginosa* 1335.

Bacteria were standardised to 0.5 McFarland standard before being used in the assay. Briefly, an isolated colony from a 24–48 hours culture of the test organisms was inoculated in 10 mL Miller's Luria broth tubes and incubated at room temperature for 8–10 hours with continuous shaking. After incubation, the optical density of the cultured organisms was checked and adjusted to 0.08 to 0.13 at 625 nm in the microplate reader. Blank subtraction was done if the optical density of the blank wells was nearly close to the media wells.

Minimum inhibitory concentration was determined following the micro broth dilution method of the Clinical and Laboratory Standards Institute (CLSI) with some modifications. A 50 mg/mL extract stock solution was prepared and diluted with 5% DMSO. The solution was then serially diluted two-fold across eight dilutions, resulting in a concentration range from 25 mg/mL as the highest to 0.195 mg/mL as the lowest. Briefly, 100µL of the plant extract sample (50mg/mL) was pipetted to A1. Then, 50 µL from A1 was serially pipetted to the subsequent 7 wells (A2–A8), initially containing 50 µL of sterile broth. Then, 50 µL from A8 was pipetted and discarded so that each well contained 50 µL, after which 50 µL of the standardised bacteria were added to A1–A8, increasing the final volume to 100 µL. This process was repeated in B1, C1, and D1 for the replicates. Four controls were observed: solvent extraction (10% DMSO), ampicillin as antibiotic control, growth control (standardised inoculum), and sterility control (Miller's LB broth) (Figure 3). The plate was incubated at 37°C for 24 hours. After overnight incubation, 10 µL of alamarBlue™ (ThermoFisher) dye reagent was added to the wells and incubated further for one hour at 37°C. Colour changes in the wells were detected visually and via fluorescence and absorbance. Blue indicates no cell growth while pink indicates cellular growth.[15]

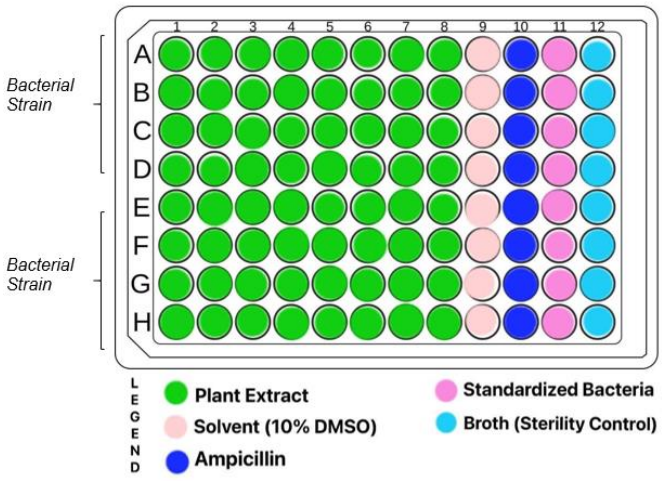


Figure 3: 96 well plate design in Minimum Inhibitory Concentration

Cyclooxygenase (COX) Inhibition Assay

The COX inhibition assay was performed following the procedure outlined by Bonner and Fry (2012) with minor modifications.[44] Initially, 5.18 mL of 100 mM Tris buffer at pH 8 was added to a clean vial. Separately, 96 µL of COX-2 and COX-1 (SIGMA-ALDRICH) enzymes (250 U/mL) and 480 µL of 20 µM Hematin (SIGMA-ALDRICH) were mixed and combined with the buffer to create the enzyme-cofactor solution. A volume of 120 µL of this enzyme-cofactor mixture was transferred into each well that had previously received 50 µL of the same buffer. Subsequently, 10 µL of 10 µg/mL and 100 µg/mL plant extracts in DMSO were added to each well. For controls, 10 µL of 4mM indomethacin (SIGMA-ALDRICH) in 100% DMSO and 5% DMSO were used as positive and negative controls, respectively. After incubating the mixture at 27°C for 15 minutes, 10 µL of 200 µM Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) (SIGMA-ALDRICH) and 10 µL of 2000 µM arachidonic acid (SIGMA-ALDRICH) were added to each well. The reaction mixture was then mixed and purged with N_2. The reaction was monitored for 3 minutes using a microplate reader, with absorbance set at 535 nm and emission at 590 nm. Fluorescence intensity was recorded every 12 seconds for 3 minutes. To determine the % inhibition, the average slope of each replicate was calculated using the formula:

$$\% \text{ Inhibition} = \frac{\text{Slope}_{\text{uninhibited}} - \text{Slope}_{\text{inhibited}}}{\text{Slope}_{\text{uninhibited}}} \times 100$$

, where the Slope uninhibited is the slope of the line from the fluorescence vs time plot of the negative control group, and the Slope inhibited is the slope of the line from the fluorescence vs time plot of the samples/positive control.[44]

Statistical analysis

All experimental measurements were carried out in triplicate and are expressed as the average of three analyses. The data were expressed as the mean ± SD. One-way analysis of variance (ANOVA) was used to express the significance of differences ($p<0.05$) between means, and the data was analysed using the Statistical Package for the Social Sciences (SPSS) program.

Results and Discussions

This study highlights the significant role of phytochemical compounds in enhancing the antioxidant, antibacterial, and anti-inflammatory properties of *H. macradenius* leaf ethanolic extract. HPTLC chromatogram results revealed the colour and location of the bands after the derivatisation with the natural product reagent, as indicated by Rf values ranging from 0.10 to 0.81 (Figure 1 & Table 1). A chromatogram with an excellent resolution of neighbouring peaks was obtained by optimising the chromatographic conditions. Different solvent systems consisting of ethyl acetate, formic acid, water, and acetic acid in various ratios were tested to separate the compounds. The best resolution of peaks was obtained from the ternary solvent solution containing ethyl acetate: formic acid: water (80:10:10) and with this solvent system or mobile phase, a preeminent separation with distinct Rf difference and a successful baseline separation of the bands was obtained.

Table 1. HPTLC profile of the *H. macradenius* ethanolic extracts using Ethyl Acetate: Formic Acid: Water (80:10:10) under 366 nm as mobile phase and Natural Product as a derivatising agent.

Range **of R_f Values**	**2µL**		**3µL**		**4µL**	
	R_f	**Color**	**R_f**	**Color**	**R_f**	**Color**
0.00 – 0.20	0.10	Blue	0.09	Blue	0.09	Blue
0.21 – 0.40	0.17	Yellow	0.17	Yellow	0.16	Yellow
0.21 – 0.40	0.26	Yellow	0.26	Yellow	0.25	Yellow
	0.30	Blue	0.30	Blue	0.30	Blue
	0.33	Yellow	0.33	Yellow	0.33	Yellow
	0.36	Dark Blue	0.36	Dark Blue	0.36	Dark Blue
0.41 – 0.60	0.42	Blue	0.42	Blue	0.42	Blue
	0.50	Orange	0.49	Orange	0.48	Orange
	0.55	Yellow	0.55	Yellow	0.55	Yellow
0.61 – 0.80	0.62	Green	0.61	Green	0.61	Green
0.81 – 1.00	0.81	Purple	0.81	Purple	0.81	Purple
	0.93	Red	0.92	Red	0.92	Red
	0.96	Red	0.95	Red	0.95	Red
	0.97	Red	0.97	Red	0.97	Red

The peaks with Rf values ranging from 0.92 to 0.97 are assumed to be the plant chlorophyll content. Different phytochemicals may be represented by bands of differing colours at different Rf values. The Rf value showed the position at which a substance is located in the chromatogram. Moreover, the densitogram obtained in this study illustrated that fourteen peaks were present in *H. macradenius* ethanolic extract when the mobile phase containing ethyl acetate: formic acid: water (80:10:10) and derivatisation with Natural Product reagen*t* was used (Figure 5). Three (3) out of the fourteen (14) peaks are said to be chlorophyll, and therefore, eleven (11) peaks are considered to be secondary metabolites of the plant extract. The peaks indicate the presence of phytoconstituents, and each peak on the chromatogram represents a component that is separated from the mixture based on its affinity for the stationary and mobile phases.

Phenolic acids and anthocyanins are responsible for the blue fluorescence and purple bands, while flavonoids show up as orange or yellow bands and chlorophylls are fluorescing brilliant red, respectively.[16,17] Based on the present result, the HPTLC chromatogram of the ethanolic extract of *H.macradenius* may contain phenolic acids, flavonoids, and anthocyanins. HPTLC analysis of *H. macradenius*

revealed the presence of phenolics and flavonoids, which is consistent with their known presence in plants of this family. This finding aligns with the previous report of Jafaar *et al.*[18], in which flavonoids and phenolic acids were detected in analysed fractions using ethyl acetate and n-butanol as solvent solutions. Quercetin and rutin were the main flavonoids identified in ethyl acetate/n-butanol fraction before hydrolysis; quercetin was the main detected aglycone in acidic hydrolysed fractions. Chlorogenic acid and caffeic acid are only detected in n-butanol fractions. Another study by Rajasudha and Manikandan[19] on *Euphorbia hirta* using acetone, chloroform, ethyl acetate, methanol, and hydro-alcohol (70% Methanol) as extraction solvents and employed for phytochemical screening reported that all the concentrates demonstrated and revealed the presence of flavonoids in all the extracts.[19] Ang *et al*. [20] using the same solvent solution, reported that the HPTLC chromatogram of the frond ethanolic extract of *D. quercifolia* using ethyl acetate: formic acid: water (16:2:2) as mobile phase and Natural Products (NP) as a derivatising reagent showed a distinctive band that may contain flavonoids, phenolic acids, and anthocyanins based on the displayed colour of the visible bands and frond ethanolic extract of *D. esculentum*, which displayed the distinctive colour of purple and blue could include phenolic acids and anthocyanin.[20]

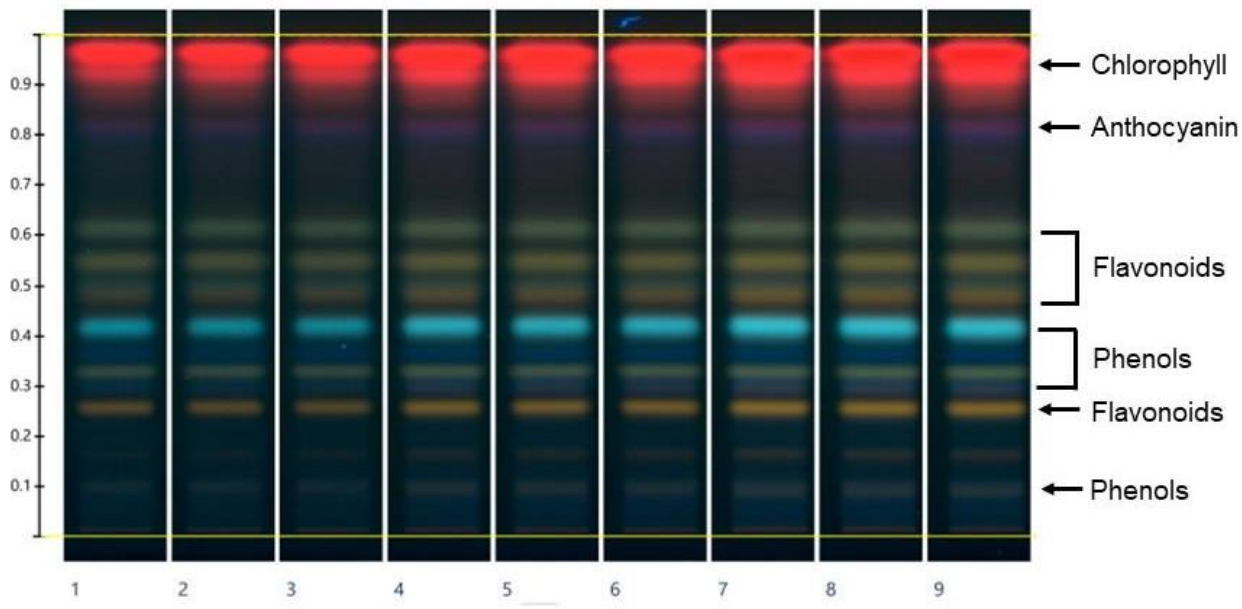


Figure 4. HPTLC Chromatogram of *H. macradenius* extracts under UV light at 366 nm. Tracks 1-3, 2µl; Tracks 4-6,3µl; Tracks 7-9, 4µl after eluting with ethyl acetate: formic acid: water (80:10:10) and derivatisation with Natural Product reagent

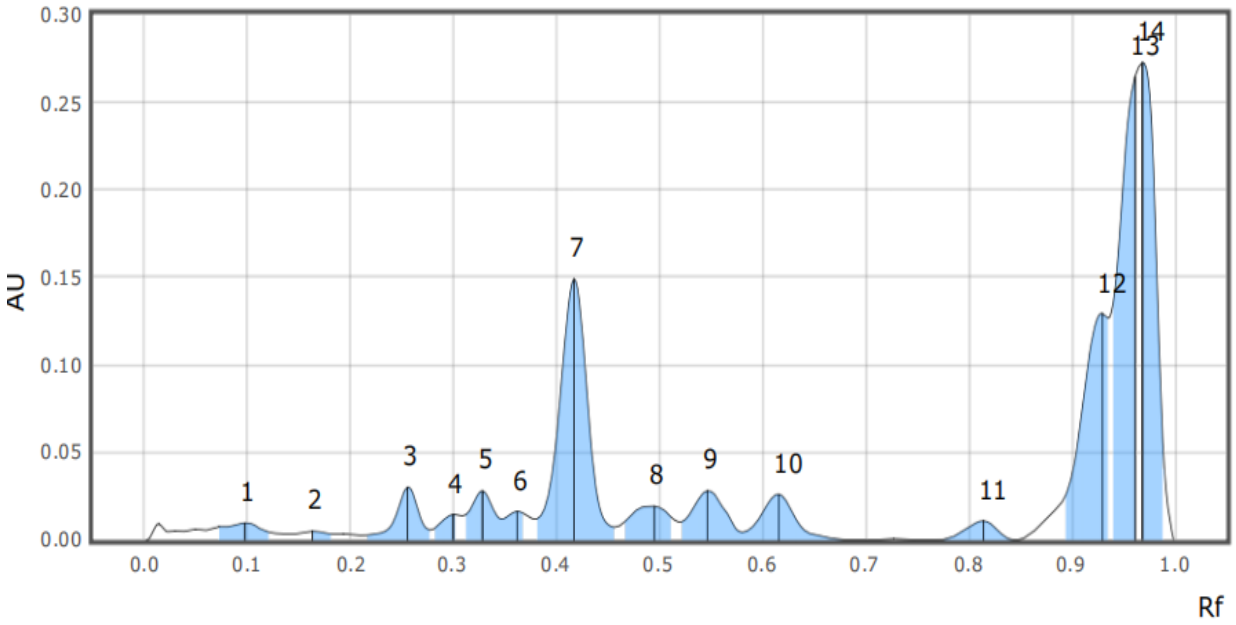


Figure 5. HPTLC Densitogram Peaks of *H. macradenius* ethanolic extract under UV light at 366 nm showing different peaks (bands) of phytoconstituents

According to Francis and Anderson[21], many HPTLC methods have been created and effectively used to separate polyphenols from other plant extract constituents as well as from one another.[21] HPTLC chemoprofile accurately and efficiently confirms the presence of constituents, and it is highly sensitive scanning densitometry for rapid chromatogram evaluation and documentation.[22] Since medicinal plants produce many secondary metabolites, it is necessary to have dependable methods for efficiently analysing and separating them. The HPTLC technique has emerged over the last 20 years as a crucial tool for the identification, quantification, and quality assessment of secondary metabolites in medicinal plants[23,] and understanding the chemical components of plants is vital because it may provide new sources of compounds and precursors for the creation of new chemical constituents that can be utilised in pharmaceuticals.[24]

Meanwhile, in the quantitative screening of the extract, the Total Phenolic content (TPC) was found to be 8.07±0.01mg GAE/g, whereas it was found that the flavonoid content of the plant extract in 1000 ppm was the same as the concentration of the TPC and TAC and was found to be 33.86±0.03mg QE/g**.** These numbers provide insightful information regarding the concentration of phenolic and flavonoids. Moreover, total Antioxidant Activity (TAA) was 15.68±0.02mg AAE/g.

The results of measuring the absorbance of gallic acid standard solutions with a concentration series of 0 - 16.5mg/mL were obtained in the form of a calibration curve with a linear regression equation, $y = 0.0645x + 0.0226$ $R^2 = 0.9959$ (Figure 6). The extract samples (Table 2) have a total phenolic content of 8.07±0.01 mg GAE/g. According to this finding, there were 8.07mg of phenolic compounds in every gram of *H. macradenius* crude leaf extract at 1000 ppm. Phenolic compounds are secondary metabolites and have been recently widely studied due to their biological effects, which could benefit human health.[26] Our study revealed that the phenolic content was lower compared to the findings of Munthe *et al*.[27], where the total phenolic contents of 276.96 mgGAE/g and 294.96 mgGAE/g, employing a plant extract concentration of 1000 ppm in the 70% ethanol extract of *P. americana* seeds.[27] Conversely, the present data indicates a lower phenolic content compared to a study on *Homalanthus nutans*, which reported a quantitative estimation of phytochemicals, specifically phenolic content, is said to be at 20 mg/g.[28] A concentration series of 0 - 25 mg/mL, the standard quercetin absorbance measurements' results were achieved as linear regression, with $y = 0.0394x - 0.0031$ and a value of $R^2 = 0.9996$ (Figure 7). Table 3 showed that the extracted sample had a total flavonoid content of 33.86±0.03 mg QE/g.

Table 2: Total Phenolic Content of *H. macradenius* extract at 1000ppm

Replicate	Concentration	TPC Absorbance GAEmg/g.
1	1000 ppm	8.02
2	1000 ppm	8.05
3	1000 ppm	8.12
X ± RSD		**8.07±0.01mg GAE/g**

*x= mean value, RSD= relative standard deviation (the absolute value of the coefficient of variation)

Table 3: Total flavonoid Content of *H. macradenius* extract at 1000ppm

Replicate	Concentration	TFC Absorbanc QEmg/g.
1	1000 ppm	33.08
2	1000 ppm	33.55
3	1000 ppm	43.95
X ± RSD		**33.86±0.03mg QE/g**

*x= mean value, RSD= relative standard deviation (the absolute value of the coefficient of variation)

This result indicates that 8.07 mg of quercetin is present in every gram of *H. macradenius* crude leaf extract at 1000 ppm. The present study showed the presence of flavonoids and its derivatives. Flavonoids are compounds found naturally in plants that are thought to benefit human health. Flavonoids have been shown to have antibacterial, antiviral, anti-inflammatory, anticancer, and anti-allergic properties. A study by Bravo (1998) stated that flavonoids are highly effective scavengers of most oxidising agents, including singlet oxygen and other free radicals implicated in multiple illnesses. The principal components of medicinal plant polyphenols, flavonoid chemicals, have been extensively studied as one of many medicinal plants' most essential antioxidant elements.[29] These findings are consistent with the study of Rizk[30], highlighting the prevalence of flavonoids, particularly flavones and flavonols, within the Euphorbiaceae family. These compounds are commonly found as methyl ethers of 0- and C-glycosides. Numerous taxa within the family exhibit significant quantities of the well-known flavonols quercetin and kaempferol, along with their respective glycosides.[30]

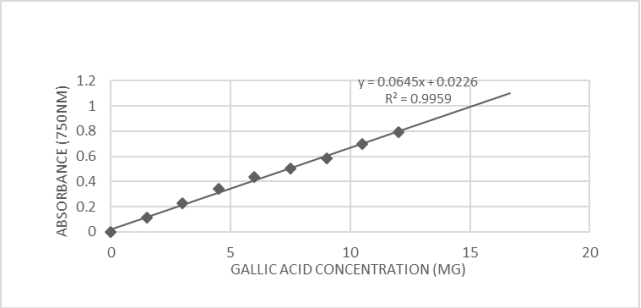


Figure 6: Calibration Curve of the Gallic Acid Standard for Total Phenolics

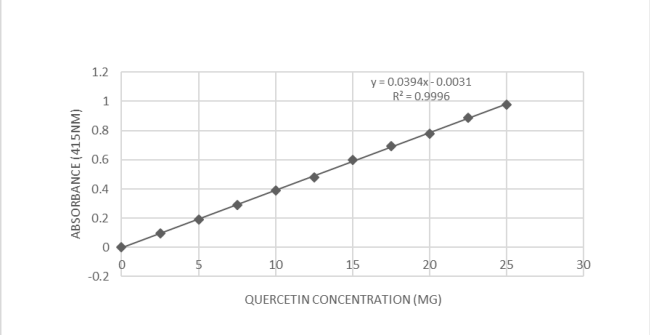


Figure 7: Calibration Curve of the Quercetin Standard for Total Flavonoids

The phosphomolybdate method is quantitative for determining the total antioxidant content (TAC), expressed as gallic acid equivalents. This approach provides a composite estimate of the antioxidant content of the ethanolic and methanolic extract's chemically varied phenolics and flavonoids.[31] This study measured TAC through ascorbic acid as standard with a concentration series of 3 - 27 mg/mL. The standard ascorbic acid absorbance measurements were achieved as linear

regression, with y = 0.0096x - 0.0109 and a value of R^2 = 0.9949 (Figure 8). Table 4 shows that the extracted sample had a total antioxidant content of 15.68±0.02mg AAE/g. The data further indicates that 15.68 mg of ascorbic acid is present in every gram of *H. macradenius* crude leaf extract at a concentration of 1000 ppm (Table 4).

Table 4: Total Antioxidant Content of *H. macradenius* extract at 1000ppm

Replicate	Concentration	TAC Absorbance mg AAEmg/g.
1	1000 ppm	15.35
2	1000 ppm	15.98
3	1000 ppm	15.72
X ± RSD		**15.68±0.02mg AAE/g**

*x= mean value, RSD= relative standard deviation (the absolute value of the coefficient of variation)

Several studies have demonstrated a link between antioxidant activity and total phenolics or flavonoid contents. Plant extracts with high phenolic content also have high flavonoid content, as observed for other plant species.[32] A study on Euphorbia's antioxidant activity, specifically *E. hirta*, demonstrated notable antioxidant activity across various conditions. The free radical scavenging ability was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The results revealed that the *E. hirta* ethanol extract at a 0.5 mg/mL concentration exhibited 61.19% ± 0.22% antioxidant activity.[33] The presence of several bioactive chemicals in plants' roots, leaves, and flowers has been shown in multiple research conducted globally.[34] The results suggested that phenolic and flavonoids may be the key contributors to *H. macradenius* antioxidant activity, based on findings in the literature for other plant extracts. Phenolic compounds are essential plant constituents with redox properties responsible for antioxidant activity, and flavonoids are secondary metabolites with antioxidant activity, the potency of which depends on the number and position of free OH groups.[35]In the oral acute toxicity study, during the 14-day experimental period, results showed no mortality and no indication of toxicity among the tested animals (Table 5).

Table 5: Summary of Gross Behavioral Observations in Mice Following Administration of Water and Plant Extract at Doses of 300 mg/kg, 2000 mg/kg, and 5000 mg/kg

Wellness Parameters	Water		300 mg/kg		2,000 mg/kg		5,000 mg/kg	
	30mins-1Hour	14Days	30mins-1Hour	14Days	30mins-1Hour	14Days	30mins-1Hour	14Days
Respiration	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Alertness	Normal	Normal	Weak	Normal	Weak	Normal	Weak	Normal
Grooming	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Pain	Absent	Absent	Absent	Absent	observed	Absent	Observed	Absent
Tremors	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Pupils	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Salivation	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Food intake	Normal	Normal	Increased	Normal	Increased	Normal	Increased	Normal
Water Intake	Normal	Normal	Normal	Normal	Increased	Normal	Increase	Normal
Urination	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Diarrhea	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Skin Color	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Skin Fur	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Lacrimation	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Convulsion	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Coma	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Hyperactivity	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Sleep	Observed	Normal	Observed	Normal	Observed	Normal	Observed	Normal
Mortality	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent

Data indicates that the one observable behaviour within the control group was sleep, occurring between 30 minutes to one-hour post-administration. This observation may be considered a typical response, given that the experiment was conducted during the daytime, and mice, being nocturnal creatures, naturally exhibit heightened activity at night, often sleeping during the day. Additionally, a 2000 mg/kg dose resulted in increased food and water intake during the one hour following administration. However, mice respiration, alertness, grooming, pupils, salivation, food and water intake, urination, mice skin colour, and fur all exhibit expected results in all groups. Moreover, observations did not detect pain, tremors, diarrhoea, lacrimation, convulsion, coma, hyperactivity, and mortality. Studying these wellness parameters in mice is essential for assessing health status, maintaining data quality, and detecting adverse effects of the administered extract at different

doses. Based on observation, treated mice showed discomfort upon receiving the plant extract doses in their mouths, likely due to the extract's distinct smell compared to water. Based on the findings, it is evident that the plant extract administered at 300 to 5000 mg/kg remains non-toxic, and the dose level of the plant extract is well-tolerated by the mice, further indicating its potential safety.

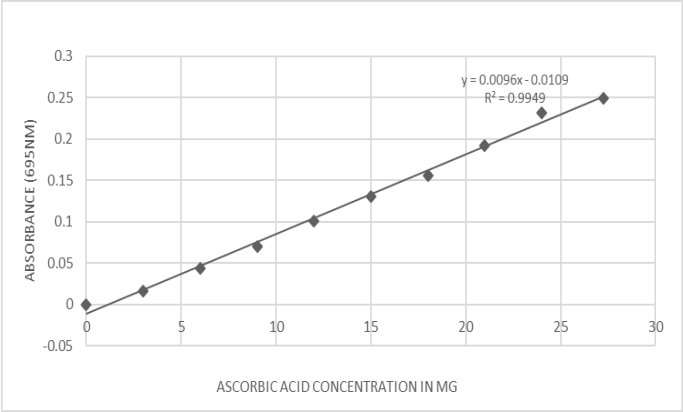


Figure 8: Calibration Curve of the Ascorbic Acid Standard for Total Antioxidant

The effects of different dosages of a test substance on the body weights of mice (Table 6) showed that the control group's mean body weight remained consistent from day 0 (23.67 ± 0.58 g) to day 14 (23.67 ± 0.58 g), indicating no significant weight change (0 ± 0.67 g). The same was observed with the 300 mg/kg group, with a mean weight of 24.0 ± 0.0 g resulting in a negligible weight change (0 ± 0.10 g) and the 5000 mg/kg group, with the mean weight remaining at 24.67 ± 0.58 g, and no significant weight change was observed (0 ± 0.38 g). Conversely, the 2000 mg/kg group exhibited a slight but statistically significant increase from 24.0±1.73 g on day 0 to 25.0 ± 1.0 g on day 13, corresponding to a weight gain of 1±1.4 g ($p<0.05$). These findings are similar to the study of Porwal *et al*.[36], where they used an ethanolic extract of *Marsdenia tenacissima* (MTE), which produced no toxic effect on the behavioural responses of the treated rats (dosed once) and observed for 14 days at a dose 5000 mg/kg.[36] Jothy *et al.*[12] also reported that the crude extract of *C. fistula* at the highest dose of 5000 mg/kg resulted in no mortalities or evidence of adverse effects, implying that *C. fistula* is nontoxic.[12]

Table 6: Mean Body Weight of Mice Before and After Acute Oral Toxicity Testing

Body Weight (g) Treatment	Day 0	Day 3	Day 6	Day 9	Day 12	Day 14	Weight gain(g) in 14 days
Water	23.67±0.58	24±1	24±1	23±0.58	23±0.58	23.67±0.58	0±0.67
300mg/kg	24±0	24±0	24±0	23.3±0.58	24±0	24±0	0±0.10
2000mg/kg	24±1.73	24±1.73	24±1.73	24±1.73	24.6±0.58	25±1	1±1.40[a]
5000mg/kg	24.6±0.58	24±0	24±0	24.6±0.58	24.6±0.58	24.6±0.58	0±0.38

Data Provided as mean±SEM(n=3); *p<0.05 treated groups versus control group

Study from Muñoz *et al*.[37], reported that oral doses of Bignay fruit extracts ranging from 500 mg/kg to 2000 mg/kg showed no mortality.[37] As per the toxicity classification system, compounds falling between 1 and 5 g/kg are generally regarded as low-toxic, and those over 5.0 g/kg are generally regarded as non-toxic.[38]The advent of bacteria that are resistant to antibiotics presents a severe threat to world health and calls for ongoing research into new antimicrobial medicines. In this study, the antibacterial activity of *H. macradenius* crude leaf extract against *Enterococcus faecalis* BIOTECH 10348, *Staphylococcus aureus* BIOTECH 1583, and *Pseudomonas aeruginosa* BIOTECH 1335 was investigated. These bacterial strains were selected based on their clinical significance and frequency in infections linked to healthcare facilities. Common organisms known to cause infections ranging from serious bloodstream infections to urinary tract infections include *Enterococcus faecalis* and *Staphylococcus aureus.* Conversely, *Escherichia coli* and *Pseudomonas aeruginosa* are well-known for their involvement in gastrointestinal tract infections and opportunistic infections in persons with impaired immune systems, respectively. Table 7 shows that the extract exhibited antibacterial activity against *E. faecalis, S. aureus, and P. aeruginosa* with MIC values of 1.563mg/mL, 12.5 mg/mL, and 25mg/mL, respectively, based on absorbance. Moreover, the MIC values based on a colour change in plates for *E. faecalis*, *S. aureus*, and *P. aeruginosa* were 1.63 µg/mL, 6.25 µg/mL, and 0.39 µg/mL, respectively. Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that prevents the visible growth of organisms as detected by lack of visible turbidity. The clarity of the solution (absence of turbidity) indicates the inhibition of microbes. However, it was challenging to distinguish between the turbidity caused by the bacteria's development and the turbidity of the plant extract itself.[39] Therefore, aside from visual observation of the colour change, measurement through absorbance in a spectrophotometer was used to calculate the lowest extract concentration necessary to destroy the microorganisms. *P. aeruginosa* showed significant discrepancies between absorbance and colour change measurements. In this case, absorbance is generally more precise and dependable than the colour change, which can be influenced by the observer's perception and lighting conditions.

The collaborative actions of many phytochemical constituents found in the plant may be responsible for the antibacterial characteristics of the crude extracts.[28] This study also revealed the presence of phytochemical constituents within plant extracts, notably including phenols, flavonoids, and antioxidant activity. Plants contain significant quantities of polyphenols and flavonoids, which contribute to their potent antioxidant activity, consequently providing various defensive and disease-fighting properties.

H. macradenius crude leaf extract may possess antibacterial properties against various microorganisms that cause various infectious illnesses in humans. These studies validate the plant's usage in several traditional medical procedures to cure different diseases. This data also aligned with the previous antibacterial studies of the genus *Homalanthus*. The methanolic extracts of members of the genus *Homalanthus* demonstrated moderate activities against *M. fortuitum* (nervosus) and *S. epidermidis* (nervosus and novoguineensis).[40] Another study tested the ethanolic extract of *Homalanthus nutans* leaves on bacterial pathogens associated with infected skin injuries. This extract demonstrated inhibitory effects against *Pseudomonas aeruginosa* and clinical isolates of *Staphylococcus aureus* at a minimum inhibitory concentration (MIC) of 4 µg/mL.[41] The ability of the ethanolic extracts from the leaves of *H. macradenius* to inhibit the enzyme activity of cyclooxygenase (COX) *in vitro* was determined. It was found that *H. macradenius* leaf ethanolic extract at 10 ppm inhibited 60.51±1.55% of

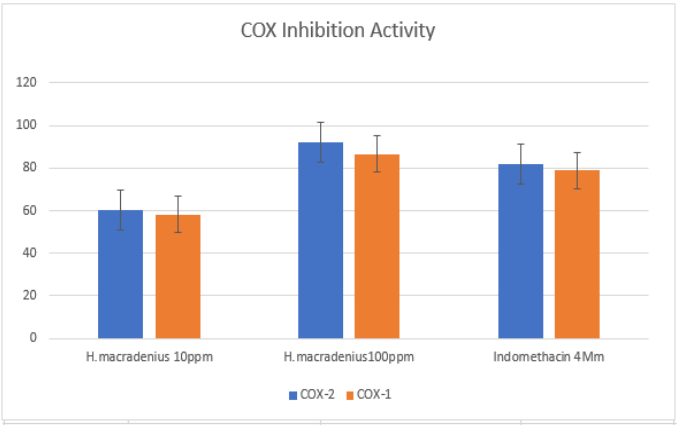
COX-2 enzyme activity and 58.1±0.63% for COX-1 (Figure 9). This is a significant activity, being above 50%. The selectivity ratio, however, was just 1.04, which means it is selective to COX-2. At a higher concentration of 100 ppm, *H. macradenius* leaf ethanolic extract inhibited 92.3±0.73% of COX-2 enzyme activity and 86.5±0.65% for COX-1. The selectivity ratio was 1.07. Anti-inflammatory drugs that inhibit more COX-2 than COX-1 are preferred because they have fewer gastrointestinal and cardiovascular side effects, and COX-2 inhibitors have also recently been used as chemotherapeutic agents.[20, 42]

Table 7: Minimum Inhibitory Concentration of *H. macradenius* Extract Based on Absorbance and Visual Color Change Observations

Bacterial Strain	MIC(Absorbance)	MIC (Color Change)
Enterococcus faecalis	1.563mg ± 0.09	1.563mg
Staphylococcus aureus	12.5mg ± 0.15	6.25 mg
Staphylococcus aureus	25mg ± 0.03	0.39mg

MIC- Minimum Inhibitory Concentration *(-) not observed

A selectivity ratio ≥ 1.00 indicates the relative selectivity of the sample extract towards the COX-2 enzyme. The ethanolic extract of *H. macradenius* exhibited a selectivity ratio of 1.04 in 10 ppm and 1.07 in 100 ppm, suggesting it is both active against COX-2 and selective for these enzymes. In comparison, indomethacin at 4 mM concentration, serving as a positive control, COX-2 inhibition was 81.96+1.47%, and COX-1 inhibition was 78.93 + 1.07%, which exhibited a ratio of 1.04. Indomethacins are non-COX-2 selective. Samples that gave ≥ 50% COX-2 inhibition and ≥ 1.00 COX-2/COX-1 ratio are considered active and COX-2 selective.[20] According to Vane (1996), medications exhibiting greater potency against COX-2 and an improved COX-2/COX-1 activity ratio are likely to possess significant anti-inflammatory effects while causing fewer adverse effects in the stomach and kidneys.[43] COX-2 selective drugs are specifically employed to alleviate acute pain following dental surgery and primary dysmenorrhea, as well as for managing conditions such as rheumatoid arthritis and osteoarthritis.[44] The results suggest that the ethanolic extract of *H. macradenius*, which exhibits potent inhibitory activity against the COX-2 enzyme, could be a valuable resource for developing NSAIDs (Non-Steroidal Anti-Inflammatory Drugs). Moreover, the plant extract represents a promising source of COX-2 selective anti-inflammatory compounds that may be developed into pharmaceutical drugs in the future.



	H.macradenius 10ppm	*H.macradenius* 100ppm	Indomethacin 4Mm
COX-2	60.51±1.55	92.3±0.73	81.96±1.47
COX-1	58.1±0.63	86.5±0.65	78.93±1.07
COX-2/COX-1	**1.04**	**1.07**	**1.04**

Figure 9: Percentage Inhibition of COX-2 and COX-1 Activity by *H. macradenius* Leaf Ethanolic Extract (10 ppm and 100 ppm) Compared to Indomethacin as a Positive Control

The observed anti-inflammatory property of *H. macradenius* in the COX inhibition assay may be attributed to phytochemicals inhibiting enzymes involved in the inflammatory process. Specifically, these phytochemicals may target key enzymes such as COX-2, thereby suppressing the production of pro-inflammatory mediators like prostaglandins.[45] This mechanism of action supports its traditional use in treating inflammatory conditions and emphasises its potential as a natural source for developing novel anti-inflammatory therapies. Recently, numerous research has shown promising data about the medicinal efficacy of herbs.[46] Certain bioactive substances found in herbs have also been demonstrated to have anti-inflammatory properties. Plants containing flavonoids are known to have good anti-inflammatory properties. The anti-inflammatory properties of flavonoids are significantly influenced by their structure. Their hydroxyl group locations are essential to imparting the characteristic, and they have a planar ring structure with unsaturation at C2–C3. Without the hydroxyl groups at the B-ring's 3' and 4' positions, the compound's anti-inflammatory properties are gone. A study by Liu *et al*. (2014) noted that numerous flavonoids reduce the expression of pro-inflammatory cytokines such as IL-6, IL-8, TNF-α, IL-1β, and monocyte chemoattractant protein-1 (MCP-1) in RAW macrophages, peripheral blood mononuclear cells, and Jurkat T cells.[47] In another study, many flavonoids can reduce inflammatory cytokines such as TNF-α, IL-6, IL-8, IL-1β, IL-17, and IFN-γ. They also efficiently reduce enzymes such as iNOS, COX-2, glucuronidase, and lysozyme. In a study by Kang *et al*. (2010), luteolin from *Lonicera japonica* Thunb. inhibited ERK1/2, c-Jun N-terminal kinases (JNK)1/2, and NF-κB pathway and also inhibited IL-6, TNF-α, and COX-2 expression.[48] Anthocyanins also show anti-inflammatory properties and reduce pro-inflammatory cytokines. Anthocyanins reduce Inflammatory Bowel Disease (IBD) by minimising the expression of IL-6, IL-9, INF-γ, MPO, TNF-α, IL-1β, IL-17A, iNOS, and COX2.[49]

Conclusion

This study marks the first investigation into the phytochemical profile, acute oral toxicity, antioxidant, antibacterial, and anti-inflammatory activities of *H. macradenius*. The findings imply that phenolics and flavonoids are present and are likely to play significant roles in the antioxidant properties of *H. macradenius*. The absence of mortality and toxicity signs following a single oral administration of *H. macradenius* crude leaf extract suggests a favourable safety profile at the tested doses. However, more investigation is necessary to evaluate its safety and effectiveness fully for possible medicinal uses. Furthermore, *H. macradenius's* antibacterial activity through MIC shows moderate

antibacterial properties against various microorganisms that cause various infectious illnesses in humans, such as *Enterococcus faecalis, Staphylococcus aureus,* and *Pseudomonas aeruginosa.* The plant also showed significant anti-inflammatory activity through *in-vitro* inhibition of cyclooxygenases. Based on the findings, *H. macradenius* extract may be safe in mice and possess antioxidant, antibacterial, and anti-inflammatory activities, revealing its future drug development potential.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

The authors express gratitude for the funding provided by the Department of Science and Technology, Science and Technology Regional Alliance of Universities for National Development (DOST-STRAND), as well as the support from the Institute of Biological Sciences, Central Mindanao University, and Tuklas Lunas Development Center, to the NSRC and TLDC Project Technical Specialist Roxan Sabasaje, Diana Rose Soliven, and Angie Rose Tuba. The authors would also like to extend their sincere appreciation to the examiners and reviewers for their dedication and constructive feedback, which greatly enhanced the quality of the paper.

References

1. Uppar V, Kiran M, Basarikatti A, Chougala M, Chandrashekharappa S, Mohan, M. Synthesis, antibacterial and antioxidant studies of 7-amino-3-(4-flurobenzoyl) indolizine-1-carboxylate derivatives. J Appl Pharm Science 2020;10:77-85 Doi: 10.7324/JABB.2021.9111
2. Vines G. Herbal harvests with a future: towards sustainable sources for medicinal plants. Plant Life Int 2004:41-42 Salisbury, www.plantlife.org.uk. Doi:9781904749004
3. Naseem U, Muhammad Z, Farhat Ali K, Shazeb K. A review on general introduction to medicinal plants, its phytochemicals and role of heavy metal and inorganic constituents. Life Sci 2014; 11(7s):520–527
4. Wintola O, Afolayan, A. The antibacterial, phytochemicals and antioxidants evaluation of the root extracts of Hydnora africana Thunb. used as antidysenteric in Eastern Cape Province, South Africa. BMC Complement Altern Med. 2015;15:307. Doi: 10.1186/s12906-015-0835-9
5. Pelser P, Barcelona J, Nickrent D. Euphorbiaceae. In: Co's Digital Flora of the Philippines. 2011; Doi: https://www.philippineplants.org/Families/Euphorbiaceae.html.
6. Wirasisya, D, Hohmann, J. An overview of the traditional use, phytochemistry, and biological activity of the genus Homalanthus. Elsevier Fitoterapia 2023; 166 Doi: https://doi.org/10.1016/j.fitote.2023.105466.
7. Dapar, M, Meve, U, Liede-Schumann, S, Alejandro, G. Ethnomedicinal plants used for the treatment of cuts and wounds by the Agusan Manobo of Sibagat, Agusan del Sur, Philippines. Ethnobot Res App 2020; 19:31. https://doi.org/10.32859/era.19.31.1-18
8. Dapar M, Alejandro G, Meve U & Liede-Schumann S. Quantitative ethnopharmacological documentation and molecular confirmation of medicinal plants used by the Manobo tribe of Agusan del Sur, Philippines. J. Ethnobiol. Ethnomed. 2020; 16(1), 1-60
9. Hudz N, Yezerska O, Shanaida M, Sedláčková V, Wieczorek P. Application of the Folin-Ciocalteu method to. Pharmacia 2019; 66(4): 209–2155. Doi. 10.3897/pharmacia.66.e38976.
10. Koley TK, Maurya A, Tripathi A, Singh BK, Singh M, Bhutia TL, Tripathi PC, Singh B. Antioxidant potential of commonly consumed underutilised leguminous vegetables. Int. J. Veg. Sci. 2018; *25*(4), 362-372.
11. Prieto P, Pineda M, Aguilar M. Spectrophotometric Quantitation of Antioxidant Capacity through the Formation of a Phosphomolybdenum Complex: Specific Application to the Determination of Vitamin E. Anal Biochem. 1999; 269,2:337-341.
12. Jothy S, Zakaria Z, Chen Y, Lau Y, Latha L, & Sasidharan, S. Acute Oral Toxicity of Methanolic Seed Extract of *Cassia fistula* in Mice. Molecules 2011; 16, 5268-5282 https://doi.org/10.3390/molecules16065268
13. Lohitasu D & Kolapalli R. Isolation and Acute Oral Toxicity studies of Araucaria heterophylla Novel Natural Polysaccharide Gum in Albino Mice. World J. Pharm. Pharm. Sci. 2016; Doi: 10.20959/wjpps201610-7752
14. Wiegand I, Hilpert K, Hancock, RW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protoc. 2008, 3, 163–175.
15. Henshaw P. Uses of Alamar Blue as an Indicator of Microbial Growth in Turbid Solutions for Antimicrobial Evaluation. 2018; Doi.org/10.7275/12222378
16. Bernardi T, Bortolini O, Massi A, Sacchetti G, Tacchini M, De Risi C. Exploring the synergy between HPTLC and HPLC-DAD for the investigation of wine-making by-products. Mol. 2019;24, 1-16.
17. Reich E, & Schibli A. Stationary Phases for Planar Separations-Plates for modern TLC LC GC.2005; 23:58-69.
18. Jaafar NS, Hamad MN, Abdulkhalik ZM, Noori ZS, Mohammad M. Phytochemical investigation And high-performance thin layer chromatography (HPTLC) identification of flavonoids and phenolic acids in *Euphorbia cyathophora* (Family: Euphorbiaceae) cultivated in Iraq. Ann Trop Med Public Health. 2020; 23, 232-.
19. Rajasudha V. & Manikandan, R. Phytochemical screening and High-performance liquid chromatography (HPLC) profile of different extracts of *Euphorbia hirta* (Linn). J Pharmacogn Phytochem. 2019; 8(1), 45-50.
20. Ang A, Sabesaje R, Barbosa G, Dela Cruz R, Mendez R, Enot, M. Cyclooxygenase (COX) AND 15-Lipoxygenase (15-LOX) Inhibitory Activity and HPTLC Profile of *Asplenium nidus*, *Diplazium esculentum*,and *Drynaria quercifolia* in Bukidnon, Philippines. Indones. J. Pharm. 2022; Doi:https://www.researchgate.net/publication/361306454.
21. Francis G, Andersen Q. Natural pigments. In Handbook of Thin-Layer Chromatography. Sherma J, Fried B, Eds.; Marcel Dekker Inc.: New York, USA 2003; 697.
22. Mukharjee PK. Quality control of herbal drugs-an approach to evaluation of botanicals 31st edition. New Delhi. Business Horizons Pharmaceuticals. Publications. 2002:183-97.
23. Bora J, Syiem D, Bhan S. Quantitative analysis of total phenolic, flavonoid. J. Pharmacog Phytother 2019;8:906-911.
24. Sonam M, Singh RP, Pooja S. Phytochemical screening and TLC profiling of various extracts of *Reinwardtia indica*. Int J Pharmacogn Phytochem. 2017;9(4):523-7.
25. Huang D, Ou B, Prior RL. The Chemistry behind Antioxidant Capacity Assays. J. Agric. Food Chem. 2005; 53(6), 1841–1856. 10.1021/jf030723c.
26. Shahidi F, Ambigaipalan P. Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects: A review. J. Funct. Foods. 2016; 18, 820–897.

27. Munthe S, Riskainto R, Juvi D & Novia, D. Antioxidant, Total Phenolic, and Total Flavonoid of 70% Ethanol Extract of Avocado Seeds (*Persea americana* Mill.). Pharmacogn J. 2023;15,4,599-605. Doi:10.5530/pj.2023.15.126
28. Aloi S, Vaurasi V, Va'ai S, Latu FA, Imo T, Amosa P. A Pilot Study of the Phytochemical Composition of Ethanolic Extracts from Eight Samoan Medicinal Plants. Int. J. Sci. Res. 2019;9(10): 239-244
29. Bravo L. Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. Nutr Rev. 1998; 56(11):317–333. Doi: 10.1111/j.1753-4887.1998.tb01670.x
30. Rizk, AF. The Chemical Constituents and Economic Plants of the Euphorbiaceae. Bot. J. Linn. 1987;94, 293-326.
31. Lallianrawna S, Muthukumaran R, Ralte V. Gurusubramanian, G., & Kumar, N. Determination of total phenolic content, total flavonoid content and total antioxidant capacity of *Ageratina adenophora* (Spreng.) King & Rob.2016; Doi: https/www.sciencevision.org
32. Ramadeep K & Geoffrey P. Antioxidant activity in different fractions of tomatoes. Int. Food Res. 2005;38,487-494
33. Sharma N, Samarakoon KW, Gyawali R, Park YH, Lee SJ, Oh SJ, Lee TH, Jeong DK. Evaluation of the antioxidant, anti-inflammatory, and anticancer activities of *Euphorbia hirta* ethanolic extract. Molecules. 2014 15;19(9):14567-81.
34. Trang NT, Thao HX, Ahn BT. Biochemical Composition, Polyphenol and Flavonoid Content, Antibacterial Activity of Leaf and Flower Extracts of *Clerodendrum paniculatum* Distributed In Thua Thien Hue Province, Vietnam. Trop J Nat Prod Res. 2024; 8(9):8275-8281
35. Soobrattee MA, Neergheen VS, Luximon-Ramma A, Aruoma OI, Bahorun T. Phenolics as potential antioxidant therapeutic agents: Mechanism and actions. Mutat. Res.-Fund. Mol. Mutagen. 2005; 579:200–213. doi: 10.1016/j.mrfmmm.2005.03.023.
36. Porwal M, Khan NA, Maheshwari KK. Evaluation of Acute and Subacute Oral Toxicity Induced by Ethanolic Extract of *Marsdenia tenacissima* Leaves in Experimental Rats. Sci. Pharm. 2017; 85, 29. Doi.org/10.3390/scipharm85030029
37. Muñoz M, Alvarado U, Reyes J, Watanabe K. Acute oral toxicity assessment of ethanolic extracts of *Antidesma bunius* (L.) Spreng fruits in mice. Toxicol Reports. 2021;8,1289-1299
38. Piyachaturawat P, Tubtim C, Chuncharunee A, Komaratat P, Suksamrarn A. Evaluation of the acute and subacute toxicity of a choleretic phloracetophenone in experimental animals. Toxicol. Lett. 2002;129(1–2):123–132. doi: 10.1016/S0378-4274(02)00006-1.
39. Phuyal N, Jha PK, Raturi PP, Rajbhandary S. In Vitro Antibacterial Activities of Methanolic Extracts of Fruits, Seeds, and Bark of *Zanthoxylum armatum* DC. J Trop Med. 2020; 2803063. Doi: 10.1155/2020/2803063.
40. Volken MC. Biological and phytochemical investigations of Euphorbiaceae from Papua New Guinea (Doctoral dissertation, ETH Zurich) 1999.
41. Frankova A, Vistejnova L, Merinas-Amo T, Leheckova Z, Doskocil I, Wong Soon J, Kudera T, Laupua F, Alonso-Moraga A & Kokoska L. *In vitro* antibacterial activity of extracts from Samoan medicinal plants and their effect on proliferation and migration of human fibroblasts. Ethnopharmacol.2020;.113220
42. Rodrigues P, Bangali H, Hammoud A. COX 2-inhibitors; a thorough and updated survey into combinational therapies in cancers. Med Oncol. 2024;41(1):41. https://doi.org/10.1007/s12032-023-02256-7
43. Vane JR. Introduction: Mechanism of action of NSAIDs. Rheumatology, 1996; 35, 1-3.
44. Bonner A, Fry M. Development of a fluorescence-based assay to detect cyclooxygenase inhibitory activity of δ - lactone derivatives.22nd Annual Argonne Symposium for Undergraduates 2012; Central States Incorporated, Argonne National
45. Patrignani P, Tacconelli S, Sciulli M, Capone, M. New insights into COX-2 biology and inhibition. Brain Res. Rev. 2005; 48(2), 352-359.
46. Liu Z, Li Z, Bai J, Meng D, Li N, Pei Y, Hua P. Anti-inflammatory diterpenoids from the roots of *Euphorbia ebracteolata*. J Nat Prod. 2014; 25;77(4):792–9.
47. Primiani CN, Sari DR, Krisnamurti GC, Pujiati P, Setiawan MA. Anti-Inflammatory Potentials of *Elaeocarpus sphaericus* Schum Fruit Compounds by Molecular Docking Approach. Trop J Nat Prod Res. 2022; 6(10):1663-1669
48. Kang O, Choi J, Lee J, & Kwon D. Luteolin isolated from the flowers of *Lonicera japonica* suppresses inflammatory mediator release by blocking NF-κB and MAPKs activation pathways in HMC-1 cells. Molecules. 2010; 15, 385–398
49. Salaritabar A, Darvishi B, Hadjiakhoondi F, Manayi A, Sureda, A, Nabavi, S, Bishayee A. Therapeutic potential of flavonoids in inflammatory bowel disease: A comprehensive review. World J. Gastroenterol. 2017; 23, 5097–5114.