

# The Evaluation of Potential Diuretic Activity of *Melanthera Biflora* (Asteraceae) in Ethanolic Leaf Extracts in Saline-Loaded Swiss Albino Mice

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## Abstract

Diuretics are substances that increase urine production by promoting the elimination of excess water and electrolytes from the body. Although synthetic diuretics such as furosemide are widely used in the management of hypertension, edema, and renal disorders, prolonged use may lead to adverse effects including electrolyte imbalance and dehydration. This study evaluated the potential diuretic activity of the ethanolic leaf extract of *Melanthera biflora* (Asteraceae) in saline-loaded Swiss albino mice. Specifically, the study determined the extract yield, identified its phytochemical constituents, assessed its acute oral toxicity, and investigated its effects on urine output and urinary chloride concentration.

Fresh leaves of *M. biflora* were extracted using 95% ethanol through maceration. Phytochemical screening confirmed the presence of saponins, glycosides, and alkaloids, which are known to contribute to renal and diuretic activity. The extraction process produced a percentage yield of  $7.17 \pm 0.29\%$ . Acute oral toxicity testing following OECD Guideline 423 showed no mortality or observable toxic effects up to 2000 mg/kg, indicating that the extract was practically non-toxic under the conditions of the study. Based on the toxicity results, low-, mid-, and high-dose treatments were standardized at 50 mg/kg, 300 mg/kg, and 2000 mg/kg, respectively. Diuretic activity was evaluated by measuring cumulative urine volume and urinary chloride concentration over a 5-hour period following saline loading and treatment administration. Statistical analysis using one-way ANOVA revealed a significant difference in urine volume among treatment groups,  $F(4,20) = 67.89$ ,  $p < .001$ , indicating a strong treatment effect on urine output. The high-dose extract group demonstrated the highest urine volume among the extract-treated groups and showed a statistically significant increase compared with the negative control. However, its effect remained lower than that of the positive control group treated with furosemide. The results also showed a dose-dependent trend in urine output, with increasing urine volume observed from low to high extract doses. In contrast, urinary chloride concentration did not significantly differ among treatment groups,  $F(4,20) = 0.45$ ,  $p = .774$ , suggesting that the extract did not produce a measurable effect on chloride concentration under the experimental conditions.

The findings suggest that the ethanolic leaf extract of *Melanthera biflora* possesses preliminary diuretic activity primarily through increased urine output, particularly at high doses. The observed effect may be attributed to the presence of bioactive phytochemicals such as saponins and glycosides, which are associated with enhanced renal fluid excretion. However, the absence of significant changes in urinary chloride concentration indicates that the extract's electrolyte-related effects remain inconclusive. Overall,

the study provides partial support for the alternative hypothesis and supports the continued investigation of *M. biflora* as a potential plant-based diuretic agent.

**Keywords:** *Melanthera biflora*, diuretic activity, urine volume, urinary chloride concentration, phytochemicals, saline-loaded Swiss albino mice.

## CHAPTER 1

### INTRODUCTION

#### 1.1 Introduction of the Study

Diuretics are agents that increase urine output and are widely prescribed in the treatment of hypertension, edema, and kidney-related disorders. Although synthetic diuretics such as furosemide and thiazides are effective, their long-term use is often associated with adverse effects including electrolyte imbalance, dehydration, and metabolic disturbances (Fitzpatrick et al., 2022; Surma et al., 2022). These drawbacks highlight the importance of identifying safer and more sustainable diuretic agents from natural sources.

*Melanthera biflora* (Asteraceae), a medicinal herb commonly found in tropical regions, has been traditionally used for fever, inflammation, gastrointestinal ailments, and skin diseases (Effiong et al., 2019). The Asteraceae family is known to contain diverse phytochemicals such as flavonoids, alkaloids, tannins, phenolic acids, and saponins, many of which have been reported to contribute to diuretic activity (Chõng et al., 2021). For example, flavonoids enhance renal blood flow and promote natriuresis, alkaloids modulate tubular function, and saponins are recognized for their diuretic and saluretic effects (Dutta et al., 2020; Ali et al., 2023). These phytochemical properties provide a strong rationale for investigating the potential diuretic effect of *M. biflora*.

One of the widely accepted approaches to evaluate diuretic activity in experimental pharmacology is the saline-loaded model. In this method, animals are preloaded with isotonic saline solution to induce volume expansion, after which the test extract or drug is administered. This model facilitates the assessment of urine volume, electrolyte excretion, and diuretic efficiency under controlled conditions, and is often compared against standard diuretics such as furosemide (Fitzpatrick et al., 2022; Surma et al., 2022).

Therefore, this study is designed to investigate the diuretic potential of the ethanolic leaf extract of *Melanthera biflora* in saline-loaded Swiss albino mice. The diuretic activity will be evaluated primarily through urine output measurement, providing insights into the extract's pharmacological properties and its potential as a natural alternative to synthetic diuretics.

#### 1.2 Background of the Study

Diuretics promote the elimination of excess fluids and electrolytes—especially sodium and chloride—from the body by increasing urine output, thereby reducing blood volume, lowering blood pressure, and alleviating swelling (Roush, Kaur, & Ernst, 2014). They are widely used for treating hypertension, heart failure, and renal disorders. While synthetic diuretics like hydrochlorothiazide and furosemide remain primary treatments, there's growing interest in exploring safer, plant-based alternatives due to the adverse effects associated with synthetic diuretics (El-Tantawy, Temraz & Mohamed, 2024).

*Melanthera biflora* (sea daisy), belonging to the Asteraceae family, is chemically rich in bioactive phytochemical classes such as polyphenols, flavonoids, phenolic acids, sesquiterpene lactones, and triterpenes, known for their diuretic and other therapeutic actions (Rolnik and Olas, 2021). Phytochemical studies on related plants suggest that compounds like flavonoids, tannins, and phenolic acids are involved in modulating kidney function and electrolyte handling, thereby contributing to diuretic and cardiovascular

effects (Jalili & Taghadosi, 2020; El-Tantawy, Temraz & Mohamed, 2024). Among the Asteraceae, species like dandelion (*Taraxacum officinale*) are well-documented for diuretic uses, attributed partly to their high potassium content (Tanasa et al., 2025).

Considering its traditional use, taxonomic lineage, and chemical richness, the proposed study aims to evaluate the diuretic activity of an ethanolic leaf extract of *Melanthera biflora* using a saline-loaded mouse model, employing furosemide as the positive control to assess its effectiveness.

### 1.3 Conceptual Framework

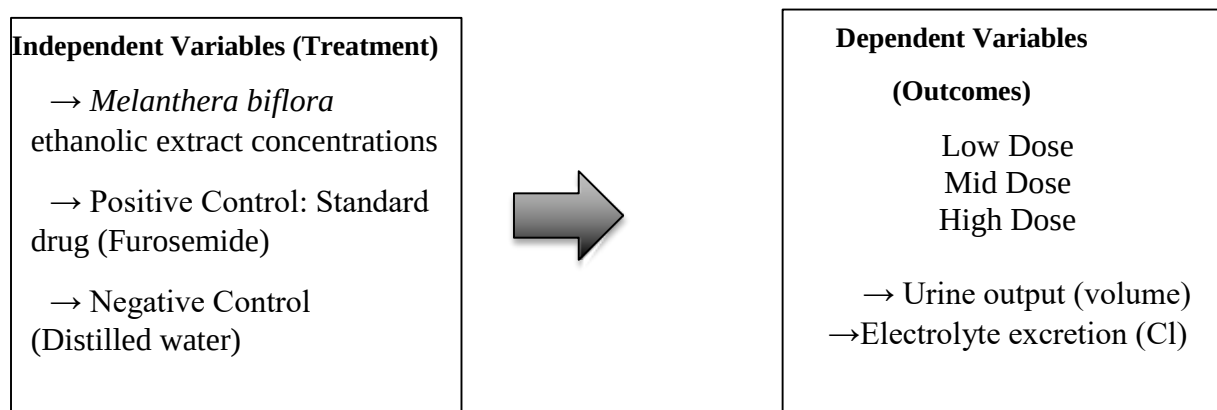


**Figure 1. Conceptual Framework of the Study**

The independent variables in this study consist of different treatments administered to saline-loaded Swiss albino mice. These treatments include three dose concentrations of *Melanthera biflora* ethanolic leaf extract: low dose, mid dose, and high dose, intended to evaluate any dose-dependent response. In addition to these experimental groups, a positive control group will receive the standard diuretic drug, furosemide, while a negative control group will receive distilled water. These variations in treatment are designed to assess the potential diuretic effect of *M. biflora* in comparison to both an established pharmaceutical and a baseline. Studies have shown that plant extracts, including *M. biflora*, have exhibited diuretic effects in animal models, which justifies the exploration of its potential as an alternative diuretic to synthetic drugs like furosemide (Raza et al., 2020). Distilled water is commonly used as a negative control in pharmacological experiments to establish a baseline and confirm that any observed effects are due to the active ingredients in the treatments, rather than the solvent or vehicle (Dutta et al., 2014; Bullecer et al., 2022).

The dependent variable is the urine output volume and electrolyte excretion (Cl), which serves as the primary indicator of diuretic activity. This measurement is commonly used in studies examining the effectiveness of diuretic treatments, as it directly reflects the renal excretion of fluids (Geleta et al., 2015; Aswini et al., 2020). By analyzing changes in urine volume across different groups, this study aims to determine whether *M. biflora* induces a statistically significant increase in diuresis, and how its effects compare to the positive and negative controls.

### 1.4 Objectives of the Study

#### General objective

The study seeks to evaluate the diuretic activity of the ethanolic leaf extract of *Melanthera biflora* in saline-loaded Swiss albino mice.

#### Specific objectives:

- 1 Determine the percentage yield of the *Melanthera biflora* leaf extract.

- 2 Conduct confirmatory tests of the phytochemical content of *M. biflora*.
- 3 Conduct acute toxicity test of the ethanolic leaf extract of *M. biflora* to determine its safety profile in mice.
- 4 Determine the specific concentrations of the crude extract as low dose, mid dose, and high dose.
- 5 Investigate the potential diuretic effect of *M. biflora* leaf extract by measuring urine output volume and electrolyte excretion.
- 6 Compare the diuretic effect of *M. biflora* leaf extract with the control drugs (furosemide and saline) using urine output volume electrolyte excretion as the primary.

### 1.5 Hypothesis of the Study

This study evaluates the potential diuretic activity of ethanolic leaf extracts of *Melanthera biflora* (Asteraceae) in saline-loaded Swiss albino mice. The following hypotheses (null and alternative) are stated in testable form to guide the experimental design and statistical analysis.

#### Null Hypotheses ( $H_0$ )

( $H_0$ ): There is no significant difference in the diuretic effect of *Melanthera biflora* extract between the control groups, positive control with furosemide and negative control with distilled water.

#### Alternative Hypothesis ( $H_1$ ):

( $H_1$ ): There is a significant difference in the diuretic effect of *Melanthera biflora* extract compared to the control groups, positive control with furosemide and negative control with distilled water.

### 1.6 Significance of the Study

This study in evaluating the potential diuretic activity of ethanolic leaf extracts of *Melanthera biflora* (Asteraceae) in saline-loaded Swiss albino mice is aimed at providing scientific evidence on a locally available medicinal plant and exploring its possible role as a natural diuretic agent that may benefit the following:

- **To the Pharmaceutical Industry:** The results of this study may contribute to the advancement of plant-based drug discovery and formulation. By establishing the phytochemical composition and pharmacological activity of *M. biflora*, the research may encourage the development of natural diuretic agents that are safer, cost-effective, and sustainable compared with synthetic drugs.
- **To the Clinical Practice:** The findings may provide healthcare professionals with baseline data on a natural alternative to conventional diuretics. Such knowledge may support the use of plant-based therapies in managing conditions like hypertension, edema, and renal disorders, while potentially reducing the risk of adverse effects associated with synthetic medications.
- **To the Community:** The study may increase awareness of the medicinal value of *M. biflora*, a plant that is abundant in Mindanao and other parts of the Philippines. Promoting indigenous and locally accessible herbal remedies may help reduce dependency on expensive pharmaceutical products and encourage the sustainable use of traditional resources for primary healthcare.
- **To the Adventist Medical Center College:** This study may enrich the academic resources of the institution by adding valuable research to its library collection. It may also inspire future student researchers to pursue pharmacological studies on Philippine medicinal plants, thereby strengthening the institution's contribution to scientific and community-oriented healthcare research.

### 1.7 Scope and Limitations of the Study

This study focuses on evaluating the diuretic activity of the ethanolic leaf extract of *Melanthera biflora* (Asteraceae) using a saline-loaded Swiss albino mice model. Only the leaves of *M. biflora* will be used for extraction with ethanol as the solvent; other plant parts, extraction solvents, or methods will not be

considered.

The experimental design will include three dose concentrations of the extract, with furosemide serving as low dose, mid dose and high dose with furosemide serving as the positive control and normal saline as the negative control. The diuretic activity will be evaluated solely based on urine output volume collected over a1, 2, 3, 4, and 5 hours, consistent with standard acute diuretic screening protocols. Measurement of urinary electrolytes (Na and K), renal biomarkers (such as sodium, potassium, creatinine, and blood urea nitrogen), or other biochemical indicators will not be conducted.

The study will also include an acute oral toxicity test following OECD Guideline No. 423 to determine the immediate safety of the extract. However, long-term administration, sub chronic and chronic toxicity studies, and histopathological examinations are beyond the scope of this study.

Practical constraints, including limited laboratory resources and sample size, may influence the extent of experimental replication and statistical precision. Furthermore, while the OECD 423 acute oral toxicity test will provide preliminary safety data, it will not substitute for comprehensive toxicological evaluations required to establish clinical safety in humans.

### 1.8 Definition of Terms

The following terms are defined in the context of this study:

**Melanthera biflora.** A medicinal plant belonging to the Asteraceae family, traditionally used for treating fever, inflammation, and skin ailments, and investigated in this study for its diuretic potential.

**Ethanollic Leaf Extract.** A concentrated solution obtained by using ethanol as a solvent to extract bioactive compounds from the leaves of *M. biflora*.

**Phytochemical Screening.** A laboratory process used to identify the presence of chemical constituents such as alkaloids, flavonoids, tannins, and saponins in plant extracts.

**Acute Toxicity Test.** An experimental procedure conducted to determine the safety and potential toxic effects of a substance after a single or short-term exposure in test animals.

**Swiss Albino Mice.** A commonly used strain of laboratory mice in pharmacological and toxicological studies due to their consistent biological responses.

**Saline-Loaded Model.** An experimental method in which animals are administered saline solution to induce fluid overload, enabling the evaluation of diuretic effects.

**Positive Control.** A reference treatment known to produce a measurable effect, used in experiments to validate test results.

**Furosemide.** A standard loop diuretic drug used as a positive control in experimental studies to compare the effectiveness of test substances.

**Negative Control.** A treatment expected to produce no significant effect, serving as a baseline for comparison in experimental studies.

**Diuretic.** A substance that increases urine production by the kidneys, aiding in the removal of excess fluid and salts from the body.

**Diuretic Activity.** The capacity of a substance to induce diuresis, typically measured by the volume of urine excreted after administration.

**Urine Output Volume.** The measurable quantity of urine excreted within a specific period, used as an indicator of diuretic activity

**Electrolyte excretion.** The process by which the body eliminates excess electrolytes, such as sodium, potassium, and chloride, primarily through urine

## CHAPTER 2

### REVIEW OF LITERATURE AND RELATED STUDIES

This chapter reviews existing literature and studies on *Melanthera biflora* (Asteraceae), which can support potential diuretic activity. It will discuss the plant's traditional uses, phytochemical compositions, and reported pharmacological effects that can support the goal of this study—investigating its diuretic properties.

#### 2.1 Literature Review

##### 2.1.1 *Melanthera biflora* (Asteraceae)

*Melanthera biflora* is a widespread coastal plant, commonly thriving in tropical and subtropical regions. It is a resilient, fast-growing herbaceous species characterized by green leaves and small yellow flowers. This plant typically establishes itself in sandy and saline environments such as seashores, roadsides, and disturbed areas. In several traditional medicine systems, *M. biflora* is known for treating various ailments, including skin injuries, digestive issues, and infections (Michel et al., 2020).

##### 2.1.2 Taxonomy of *Melanthera biflora* (Asteraceae) Taxonomy

The taxonomic classification of *Melanthera biflora* is according to Integrated Taxonomic Information System (ITIS) – Report (2010).

**Kingdom:** Plantae

**Division:** Tracheophyta

**Class:** Magnoliopsida

**Order:** Asterales

**Family:** Asteraceae

**Genus:** *Melanthera* Rohr

**Species:** *Melanthera biflora*



**Figure 2. *Melanthera biflora* (Asteraceae) credit to Australian National Botanic Gardens**

##### 2.1.3 Morphological Description of *Melanthera biflora* (Asteraceae)

This species grows as a sprawling or trailing perennial herb, often extending up to 2 meters in height. Its stems are slender and can either trail on the ground or climb on adjacent plants. Leaves are ovate with smooth surfaces and mildly pointed tips. The inflorescence consists of small yellow flower heads, typically measuring 8–10 mm in diameter. The plant bears fruits in dense clusters, a distinguishing feature of this species. (World Flora Online 2025; iNaturalist 2025). The photo of *M. biflora* is shown in Figure 2.

#### 2.1.4 *Melanthera biflora* (Asteraceae) Habitat

*Melanthera biflora* naturally occurs along coastal regions, particularly in sandy or saline areas such as beaches, dunes, and mangrove peripheries. It also colonizes disturbed sites like abandoned fields and roadsides. As a pioneer species, it contributes to the rehabilitation of degraded coastal zones across tropical Asia and the Indo-Pacific. (National Parks Board 2025).

#### 2.1.5 *Melanthera biflora* (Asteraceae) Traditional Uses

In the Philippines, *Melanthera biflora*—locally called hagonoi or agunoi—has a history of traditional medicinal use. The leaves are commonly employed as a natural diuretic, while root infusions are taken for digestive relief. Other documented uses include remedies for stomach pain, fever, and external wounds. (Rolnik & Olas, 2021).

Beyond the Philippines, similar applications exist in other parts of Southeast Asia. In Malaysia, for instance, leaf decoctions are applied to skin infections, and shoots are consumed as part of traditional diets (GlobinMed, 2021). In Langkawi, the plant is consumed raw with condiments for medicinal or nutritional purposes. Additionally, postpartum tonics incorporating leaf extracts have been reported, while flower components are sometimes used for purgative purposes (GlobinMed 2021).

#### 2.1.6 Ethnobotanical and Pharmacologic Uses of *Melanthera biflora*

Table 1 shows how *Melanthera biflora* has been valued both in folk medicine and in scientific research. For generations, people have turned to this plant to treat everyday health problems like cuts, stomach pain, skin infections, and swelling (Effiong et al., 2019; Kumar et al., 2020). Modern studies now support these traditional practices, revealing that the plant contains compounds with antibacterial, antioxidant, and anti-inflammatory effects (Mossalam & Youssef, 2020; Kumar et al., 2021). Different parts of the plant whether it's the leaves, flowers, or even the whole plant are used for healing (Ikewuchi et al., 2020). Because of this, *M. biflora* stands out not just as a traditional remedy, but also as a promising source of natural medicine recognized by science today.

**Table 1. Ethnobotanical and Pharmacological Uses of *Melanthera biflora***

| Study Title                                                                                            | Results                                                                                                                       | Citation                                                                                                                                                                                                                                                                                                                                            |
|--------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Antibacterial activity of methanol extract of <i>Wedelia biflora</i> (syn. <i>Melanthera biflora</i> ) | Effective against <i>Bacillus cereus</i> and <i>Staphylococcus aureus</i> at high doses; no effect on Gram-negative bacteria. | Dang TTN, et al 2024. Antibacterial activity of methanol extract of <i>Wedelia biflora</i> (syn. <i>Melanthera biflora</i> ). Magna Scientia. Available from: <a href="https://magnascientiapub.com/journals/msabp/sites/default/files/MSABP-2024-0050.pdf">https://magnascientiapub.com/journals/msabp/sites/default/files/MSABP-2024-0050.pdf</a> |
| <i>Melanthera biflora</i> (syn. <i>Wedelia biflora</i> ) –                                             | Used for cuts, ulcers, swellings, gonorrhea,                                                                                  | NParks. 2025. <i>Melanthera biflora</i> (syn. <i>Wedelia biflora</i> ) – Flora Fauna Web. National Parks Board, Singapore. Available from: <a href="https://www.nparks.gov.sg/florafaunaweb/flora/2/5/2553">https://www.nparks.gov.sg/florafaunaweb/flora/2/5/2553</a>                                                                              |

|                                                                              |                                                                                                                                             |                                                                                                                                                                                                                                                                                           |
|------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Flora Fauna Web)                                                             | and urinary issues.                                                                                                                         |                                                                                                                                                                                                                                                                                           |
| <i>Melanthera biflora</i> – Useful Tropical Plants Database                  | Treats stomach pain, wounds, headaches; used as diuretic and for flatulence.                                                                | Useful Tropical Plants. <i>Melanthera biflora</i> . Available from: <a href="https://tropical.theferns.info/viewtropical.php?id=Melanthera+biflora">https://tropical.theferns.info/viewtropical.php?id=Melanthera+biflora</a>                                                             |
| Minerals, vitamins and amino acids compositions of <i>Melanthera biflora</i> | High in vitamin C, iron, calcium; contains all essential amino acids except tryptophan.                                                     | Kalu EC, et al. 2019. Minerals, vitamins and amino acids compositions of <i>Melanthera biflora</i> . Int J Biochem Res Rev. Available from: <a href="https://journalijbcrr.com/index.php/IJBCRR/article/view/510?utm">https://journalijbcrr.com/index.php/IJBCRR/article/view/510?utm</a> |
| Traditional Folk and Ethnobotanical Uses of <i>Melanthera biflora</i>        | Traditional folk applications include: raw consumption for stomach discomfort, postpartum tonic, skin infections, and natural diuretic use. | GlobinMed (2021) – “ <i>Melanthera biflora</i> Ethnobotanical Uses”; NParks (2025). Available from: <a href="https://www.globinmed.com/plant/melanthera-biflora">https://www.globinmed.com/plant/melanthera-biflora</a>                                                                   |

## 2.1.7 Members of Asteraceae with Diuretic Activity

The Asteraceae family is well known for its ethnomedicinal applications, with several species traditionally used for managing urinary and renal conditions. Diuretic activity, in particular, has been documented in different members of this family, and scientific studies have provided supporting evidence for their pharmacological properties. Table 2 shows selected Asteraceae plants with reported diuretic activity, including their observed effects and corresponding references. Presenting these studies highlights the relevance of Asteraceae species in diuretic research and provides a foundation for exploring the possible activity of *Melanthera biflora* (Michel et al., 2020; Devkota, 2022).

**Table 2. Members of Asteraceae with Diuretic Activity**

| Study Title                                                                                                          | Members of Asteraceae                                                               | Results                                                                                                                 | Citation                                                                                                                                                                                                                                                                                       |
|----------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Diuretic effect of <i>Achillea millefolium</i> L. in rats: Involvement of bradykinin B2 receptors and cyclooxygenase | <i>Achillea millefolium</i> (Yarrow)                                                | Significant increase in urinary volume and $\text{Na}^+/\text{K}^+$ excretion. Involves bradykinin B2 and COX pathways. | Souza Pd, et al. 2013. Involvement of bradykinin and prostaglandins in the diuretic effects of <i>Achillea millefolium</i> L. (Asteraceae). J Ethnopharmacol. doi: 10.1016/j.jep.2013.06.015 <a href="https://pubmed.ncbi.nlm.nih.gov/23791807">https://pubmed.ncbi.nlm.nih.gov/23791807</a>   |
| A review on phytochemical and pharmacological properties of <i>Taraxacum officinale</i>                              | <i>Taraxacum</i> spp. (Dandelion)                                                   | Diuretic activity supported by traditional use and modern studies. Comparable to furosemide in mild diuresis.           | Schütz K, et al. 2006. Taraxacum—A review on its phytochemical and pharmacological profile. J Ethnopharmacology. 107(3):313–323. <a href="https://www.sciencedirect.com/science/article/abs/pii/S0378874106003576">https://www.sciencedirect.com/science/article/abs/pii/S0378874106003576</a> |
| Comparative Review of Asteraceae and Lamiaceae Families with Diuretic Activity                                       | <i>Chamaemelum nobile</i> , <i>Chrysanthemum morifolium</i> , <i>Tagetes erecta</i> | All listed plants showed significant diuretic activity in experimental models.                                          | Michel J, et al. 2020. A Review on the Potential Use of Medicinal Plants from Asteraceae and Lamiaceae Plant Family in Cardiovascular Diseases. 2020:1349582. <a href="https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7291392">https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7291392</a>          |
| Diuretic effects of aqueous extract of                                                                               | <i>Tanacetum vulgare</i> (Tansy)                                                    | Increased urine output                                                                                                  | Lahlou S, et al. 2007. Diuretic activity of the aqueous extracts of <i>Carum carvi</i> and <i>Tanacetum vulgare</i> in normal rats. J Ethnopharmacology.                                                                                                                                       |

|                                                                                                |                                  |                                                                                                                             |                                                                                                                                                                                                                                                                                                                  |
|------------------------------------------------------------------------------------------------|----------------------------------|-----------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Tanacetum vulgare</i> L. in rats                                                            |                                  | and urinary Na <sup>+</sup> /K <sup>+</sup> . Comparable to furosemide without toxicity.                                    | 110(3):458–463.<br><a href="https://www.sciencedirect.com/science/article/abs/pii/S0944711304001580">https://www.sciencedirect.com/science/article/abs/pii/S0944711304001580</a>                                                                                                                                 |
| Ethnopharmacological potential of <i>Spilanthes paniculata</i>                                 | <i>Spilanthes paniculata</i>     | Aqueous extract increased urine volume, conductivity, Na <sup>+</sup> /K <sup>+</sup> excretion in a dose-dependent manner. | Pandey A, et al. 2021. Therapeutic and ethnobotanical aspects of medicinal plants from family Asteraceae. Research Gate.<br><a href="https://www.researchgate.net/publication/356752516">https://www.researchgate.net/publication/356752516</a>                                                                  |
| Evaluation of diuretic activity of <i>Tagetes erecta</i> in hypertensive and normotensive rats | <i>Tagetes erecta</i> (Marigold) | Induced significant diuretic and natriuretic effects in both hypertensive and normal rats.                                  | Zanovello M, et al. 2021. <i>Tagetes erecta</i> L. flowers, a medicinal plant traditionally used to promote diuresis, induced diuretic and natriuretic effects in normotensive and hypertensive rats. PubMed.<br><a href="https://pubmed.ncbi.nlm.nih.gov/34229058">https://pubmed.ncbi.nlm.nih.gov/34229058</a> |

## 2. 2 Phytochemical Content of *Melanthera biflora*

Phytochemical screening of *Melanthera biflora* has revealed the presence of several important secondary metabolite groups that underpin its reported pharmacological actions such as antibacterial, antioxidant, anti-inflammatory, and diuretic activities. The main phytochemical groups detected include Alkaloids, Flavonoids, Tannins, Saponins, Glycosides, Terpenoids, and Phenolic acids Kalu et al. (2019), Below is a discussion of how each of these phytochemicals contributes to the plant's pharmacological potential.

### 2.2.1 Alkaloids

According to Kalu et al. (2019), *M. biflora* leaves contain alkaloids such as morphine-like compounds,

methyilmorphine, dephnoline, biflorin, aromoline, and homoaromoline. These alkaloids may contribute to antimicrobial and diuretic effects through their interactions with microbial cell membranes and their influence on renal tubular processes.

### 2.2.2 Flavonoids

The same study identified flavonoids such as quercetin, kaempferol, daidzein, luteolin, salvagenin, and sinensetin. identified by Kalu et al. (2019), are known for their antioxidant and anti-inflammatory properties, help to neutralize free radicals and reduce oxidative stress, which could explain the plant's therapeutic benefits.

### 2.2.3 Tannins

Kalu et al. (2019) also reported the presence of tannins, primarily tannic acid, which may provide antimicrobial and antioxidant benefits. Tannins act by binding to microbial proteins and scavenging free radicals, contributing to cellular protection.

### 2.2.4 Saponin

Although the specific quantity was not detailed by Kalu et al. (2019), saponins were confirmed in the leaves of *M. biflora*. Saponins are known for their diuretic and cardioprotective activities, as they help regulate fluid balance and support heart function.

### 2.2.5 Glycosides

Glycosides, including compounds such as quabain, gitogenin, diosgenin, and neohegen, were detected in *M. biflora* leaves. These compounds are often associated with diuretic and cardiotonic effects, as they enhance renal excretion and improve cardiac contractility (Kalu et al., 2019).

### 2.2.6 Terpenoids

Terpenoids like linalool, germacrene,  $\alpha$ -terpineol, terpinen-4-ol, and  $\gamma$ -terpinene were also identified. These compounds are widely recognized for their antibacterial and antioxidant properties, which contribute to the plant's therapeutic profile.

### 2.2.7 Phenolic acids

The phenolic acid fraction contained compounds such as ferulic acid and vanillic acid. These acids are known to exhibit strong antioxidant and anti-inflammatory effects, which help protect cells from oxidative damage and inflammation (Kalu et al., 2019).

## 2.3 Phytochemical Contents of *Melanthera biflora* Specific to Diuretic Activity

In the context of this study, which investigates the diuretic potential of *Melanthera biflora*, the most pharmacologically relevant phytochemical groups are saponins and glycosides, with alkaloids considered secondary contributors.

### 2.3.1 Saponins

Saponins, particularly steroidal and triterpenoid saponins, are abundant in *M. biflora* and are strongly associated with diuretic activity. These compounds enhance renal excretion by promoting natriuresis and diuresis, stimulating renal blood flow, and influencing electrolyte balance. Experimental studies have shown that steroidal sapogenins such as diosgenin and hecogenin significantly increase urine volume and sodium excretion, supporting their role as natural diuretic agents (Yuan et al., 2021; Alam et al., 2020).

### 2.3.2 Glycosides

The glycoside fraction of *M. biflora*, which may include steroidal and cardiac-type glycosides, is known for both diuretic and cardiotonic effects. These compounds act by modulating  $\text{Na}^+/\text{K}^+$ -ATPase activity and

improving cardiac efficiency, which indirectly supports fluid excretion. Among these, diosgenin-derived glycosides are considered safer and more physiologically relevant for renal modulation (Patil et al., 2021).

### 2.3.3 Alkaloids

While alkaloids are present in *M. biflora*, such as biflorin and related nitrogenous compounds, their direct role in diuretic activity is less established. They may exert supportive effects by modulating renal tubular reabsorption or acting synergistically with other phytochemicals (Kumar et al., 2020). However, saponins and glycosides remain the primary bioactive constituents linked to diuresis.

Overall, the combined presence of saponins and glycosides in *Melanthera biflora* provides strong pharmacological justification for its traditional use as a natural diuretic and underpins its further evaluation through in vivo assays.

## 2.4 Diuretics

Diuretics are pharmaceutical agents that promote fluid loss from the body by increasing urine output. These drugs are commonly prescribed to treat hypertension, fluid overload, heart failure, and certain kidney disorders by facilitating the excretion of sodium and water. (Mayo Clinic Staff 2023).

### 2.4.1 Types of Diuretics and its Mechanism of Action

Diuretics are drugs that promote the excretion of water and electrolytes from the body by acting on different parts of the nephron. Each class of diuretic has a specific site of action, mechanism, and therapeutic application depending on how it influences the reabsorption of sodium, chloride, and other ions (Brater, 2021).

#### 2.4.2 Loop Diuretics

Loop diuretics act primarily on the thick ascending limb of the Loop of Henle, where they inhibit the sodium–potassium–2 chloride ( $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ ) symporter. By blocking this transporter, loop diuretics prevent the reabsorption of sodium, chloride, and potassium ions, leading to a marked increase in their excretion along with water. This produces a powerful diuretic effect, making loop diuretics such as furosemide effective in treating conditions like pulmonary edema and heart failure (Rossi & Kivela, 2021).

#### 2.4.3 Thiazide Diuretics

Thiazide diuretics act on the distal convoluted tubule, where they inhibit the sodium–chloride ( $\text{Na}^+ - \text{Cl}^-$ ) symporter. This reduces sodium and chloride reabsorption, resulting in moderate diuresis. Additionally, thiazides decrease calcium excretion, which can be beneficial in managing calcium kidney stones or osteoporosis. A commonly used thiazide diuretic is hydrochlorothiazide, often prescribed for hypertension and mild edema (Hirsch & D'Costa, 2022).

#### 2.4.4 Potassium-Sparing Diuretics

Potassium-sparing diuretics exert their effects in the collecting duct. They work either by antagonizing aldosterone receptors (as in spironolactone) or by directly blocking epithelial sodium channels (ENaC) in the tubular cells (as in amiloride). Both actions inhibit sodium reabsorption while reducing potassium excretion, thereby minimizing the risk of hypokalemia associated with other diuretics. These agents are typically used in combination with other diuretics to maintain potassium balance (Bunemann & Krebs, 2021).

#### 2.4.5 Osmotic Diuretics

Osmotic diuretics act mainly in the proximal tubule and the descending limb of the Loop of Henle. They increase the osmotic pressure within the tubular fluid, reducing water reabsorption and enhancing urine

output. Mannitol is a representative osmotic diuretic, commonly used to manage cerebral edema, acute renal failure, and elevated intraocular pressure (Tian & Zhang, 2020).

#### 2.4.6 Carbonic Anhydrase Inhibitors

Carbonic anhydrase inhibitors work in the proximal tubule, where they inhibit the enzyme carbonic anhydrase. This leads to decreased hydrogen ion secretion and increased bicarbonate excretion, which reduces sodium reabsorption and promotes diuresis. Acetazolamide is the prototype of this class and is clinically used to treat glaucoma, metabolic alkalosis, and acute mountain sickness (Berg & Nissen, 2021). All these diuretic classes differ in potency, site of action, and clinical utility, but they share the common goal of regulating fluid and electrolyte balance by modulating renal tubular transport mechanisms.

### 2.5 Furosemide

This study utilizes furosemide as the standard reference drug. Furosemide is a loop diuretic known for its potent and rapid action. It is widely used in the management of hypertension, congestive heart failure, renal disease, and pulmonary edema. Its onset of action is within 30–60 minutes (oral) and 5 minutes (IV), making it highly effective in emergency and chronic conditions (Neal & McKinley, 2021; Perazella & O'Riordan, 2021).

#### 2.5.1 Advantages of Furosemide as a Synthetic Diuretic

Furosemides is a potent loop diuretic that acts rapidly to remove excess fluid from the body, making it highly effective for conditions involving edema and fluid overload. Its primary advantage lies in its fast onset and strong diuretic effect, which is crucial in managing acute pulmonary edema, congestive heart failure, and renal insufficiency. Furosemide promotes significant excretion of sodium, chloride, and water, thereby reducing blood volume and alleviating symptoms such as dyspnea and swelling.

According to Neal and McKinley (2021), furosemide remains one of the most prescribed diuretics due to its broad therapeutic applicability and effectiveness in both acute and chronic settings. It provides symptomatic relief in patients with heart failure and cirrhosis by decreasing venous pressure and pulmonary congestion. Its dual routes of administration (oral and intravenous) allow for flexibility in clinical use, especially in emergency conditions where immediate diuresis is required. These characteristics make furosemide a cornerstone medication in the pharmacologic management of fluid retention disorders.

Furosemide's established efficacy and rapid action make it an ideal benchmark in experimental diuretic studies, allowing for a reliable comparison with new agents, including plant-based diuretics such as *Melanthera biflora* extract.

#### 2.5.2 Side Effects of Using Furosemide as a Synthetic Diuretics

Despite its therapeutic advantages, furosemide use is associated with several adverse effects that necessitate careful medical supervision. The most common include electrolyte imbalances such as hypokalemia, hyponatremia, and hypocalcemia, which can cause fatigue, muscle weakness, and cardiac arrhythmias. (U.S. Food & Drug Administration, 2024; Xue et al., 2025). Additionally, prolonged or high-dose use may lead to dehydration and hypotension, especially in elderly or volume-depleted patients. Another significant adverse outcome is hyperuricemia, which can predispose individuals to gout. Furosemide may also cause metabolic alkalosis as result of increased hydrogen ion excretion. Importantly, ototoxicity has been reported, particularly when the drug is administered rapidly via intravenous route or combined with other ototoxic agents such as aminoglycosides (U.S. Food & Drug Administration, 2024; Xue et al., 2025). These side effects underline the need for monitoring and dosage adjustment, as well as

the exploration of safer, natural alternatives like *Melanthera biflora* that could offer effective diuretic action with fewer risks.

## 2.6 Assays for Diuretic Activity

Assays for diuretic activity are experimental methods used to evaluate the potential of substances to promote urine production. Commonly, this assay involves animal mice, where parameters are measured following administration of test compounds. The saline-loaded mice model is frequently employed, in which animals are hydrated with a saline solution prior to treatment to allow assessment of diuresis (Kau et al. 1984). This model has been widely used in the literature to assess the diuretic activity of both synthetic and natural compounds, particularly plant extracts.

Recent studies have further refined this assay. For example, Wondimu et al. (2024) used Swiss albino mice and measured urine output to evaluate diuretic activity from *Erica arborea* extract. Additionally, plant extracts such as those from *Solanum lycopersicum* have been tested using similar protocols in mice, where urine output was measured to validate their diuretic activity. In the present study, distilled water will serve as the negative control and furosemide as the positive control to assess the diuretic potential of *Melanthera biflora* extract.

To provide a clearer overview, several parameters are commonly evaluated in diuretic activity assays to assess the overall effect of a test substance. These include urine volume, urine pH, and electrolyte excretion, among others, each offering insight into different aspects of renal function and diuretic potency. Collectively, these parameters help determine both the efficacy and safety profile of potential diuretic agents (Wei et al., 2022; Wondimu et al., 2024).

However, in the present study, the researchers will focus solely on urine output (urine volume) and electrolyte excretion as the primary parameter. This decision is based on the study's objective to evaluate the diuretic potential of *Melanthera biflora* extract in terms of its ability to increase urine production. By concentrating on urine volume, the study aims to provide a clear and direct assessment of diuretic activity without involving electrolyte analysis or other biochemical parameters. This approach aligns with standard preliminary screening methods used in evaluating plant-based diuretics, where total urine volume serves as a straightforward and reliable indicator of diuretic effect (Wei et al., 2022; Wondimu et al., 2024; Aswini et al., 2020).

## 2.7 Theoretical Framework

The theoretical framework of this study is anchored on the Input–Process–Output (IPO) model, which illustrates the systematic flow of the research. The framework presents how the experimental design utilizes specific inputs, undergoes a defined process, and yields measurable outputs. By organizing the study in this manner, the relationship between the administration of *Melanthera biflora* ethanolic leaf extract and its potential diuretic activity can be clearly understood. This model serves as the guide for identifying the essential components of the study and how they interact to achieve the research objectives.

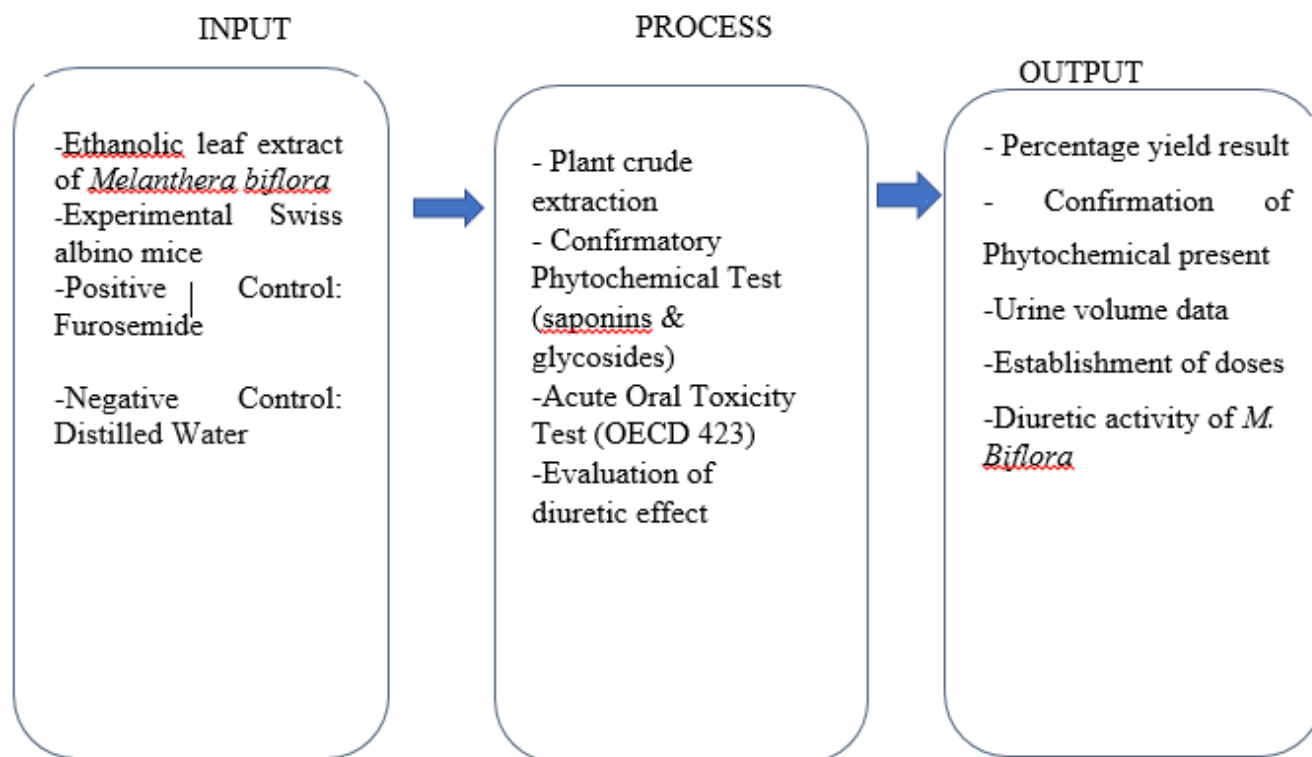


**Figure 3. Theoretical Framework (Input-Process-Output Model)**

As shown in Figure 3, the inputs of this study include the ethanolic leaf extract of *Melanthera biflora*, experimental Swiss albino mice, saline solution (negative control), and furosemide (positive control). These inputs undergo a defined experimental process consisting of plant crude extraction, confirmatory phytochemical screening (specifically for saponins and glycosides), acute oral toxicity testing following OECD Guideline 423, and evaluation of diuretic effect based on urine volume measurements.

The outputs of the study comprise the confirmation of phytochemicals present, establishment of safe and effective dosage levels, quantitative urine volume data, and the determination of the diuretic activity of *Melanthera biflora*. Altogether, this framework illustrates the logical sequence of the study, ensuring a systematic approach to evaluating the diuretic potential of *M.biflora* ethanolic leaf extract.

## CHAPTER 3

### METHODOLOGY

This chapter will describe in detail the materials and methods that will be used to evaluate the potential diuretic activity of the ethanolic leaf extract of *Melanthera biflora* (Asteraceae) in saline-loaded Swiss albino mice. The methods will include the materials, research flow, extraction and characteristics of the plant, formulation, evaluation, and data analysis. The study will focus on quantitatively measuring urine output to determine whether the plant extract has diuretic potential.

#### 3.1 Materials

##### 3.1.1 Standards, Reagents, Chemicals

Ethanol (95 %) will be used as the extraction solvent for *M. biflora* leaves. For the saline-loaded model, normal saline (0.9 % NaCl) will be administered orally to impose a standardized salt and water load, thereby ensuring comparable hydration and extracellular fluid expansion across all experimental groups.

The standard diuretic drug, furosemide, will serve as the positive control due to its potent loop-diuretic effect on sodium and water excretion. Phytochemical reagents such as water for the froth test for saponins, Wagner's reagent for the alkaloid test, and glacial acetic acid, ferric chloride, and concentrated sulfuric acid for the Keller–Kiliani test for glycosides will be used for confirmatory phytochemical screening. The crude ethanolic extract of *Melanthera biflora* leaves will first be evaluated for uniform dispersion in distilled water. If the extract does not disperse evenly, a suspending agent will be incorporated to ensure consistent dosing and homogeneity. Specifically, a 0.5% w/v sodium carboxymethylcellulose (Na-CMC) solution will be used as the vehicle, as this concentration has been used to provide stable suspensions. For the electrolyte excretion analysis (Cl).

### 3.1.2 Plant Materials

Fresh leaves of *Melanthera biflora* will be collected from a coastal area in Tominobo, Iligan City, Lanao del Norte, Philippines (coordinates: 8.20244° N, 124.21056° E), where the plant naturally grows. The location was chosen due to the natural abundance of *M. biflora* in this coastal habitat, ensuring that the collected specimens are healthy and representative of the species growing under natural environmental conditions. The plant will be authenticated by a botanist at Mindanao State University–Iligan Institute of Technology (MSU–IIT), Tibanga, Iligan City, Lanao Del Norte.

After collection, the leaves will be thoroughly washed with distilled water to remove dirt, sand, and other debris. They will then be shade-dried at room temperature (~25–28°C) with a relative humidity of 50–60% until crisp and brittle. The dried leaves will be pulverized into a fine powder using a Hanabishi electric grinder, a widely available brand in Iligan City. The powdered leaves will then be sieved through a 60-mesh sieve to remove coarse fibers and standardize the particle size, ensuring efficient extraction of bioactive compounds. The sieved powder will be stored in airtight, light-resistant containers to protect against moisture, air, and light, which could degrade the extractable phytochemicals. Containers will be properly labeled with the plant name, date of collection, and date of drying, and stored at room temperature (25–28°C) until extraction.

### 3.1.3 Instruments and Apparatus

The laboratory instruments and apparatus used in this study include Erlenmeyer flasks for containing the powdered *Melanthera biflora* leaves and solvents, a maceration shaker for mixing the plant material with the extraction solvent, and a rotary evaporator for concentrating the extract under reduced pressure and controlled temperature. An analytical balance will be employed for accurate weighing of plant material and extracts, while graduated cylinders will facilitate precise measurement and transfer of liquid volumes. A centrifuge will be used to separate solid residues from the liquid extract, ensuring a clear solution for subsequent analyses. Oral gavage needles and syringes will also be used for the accurate administration of plant extracts, distilled water, and standard diuretic solutions to the experimental mice. For the electrolyte excretion analysis (Cl) while a standard titration setup, including burettes and Erlenmeyer flasks, will be utilized for argentometric chloride determination. Suppository mold for urine storage, an ice bath to maintain sample stability, and a vortex mixer for homogenizing samples will also be employed to ensure accurate biochemical analysis.

## 3.2 Research Flow

The proposed research will follow the sequence outlined in the research flow diagram, ensuring a systematic and scientifically valid approach to evaluating the diuretic potential of *M. biflora* ethanolic leaf extract.

The proposed research will follow the sequence below:

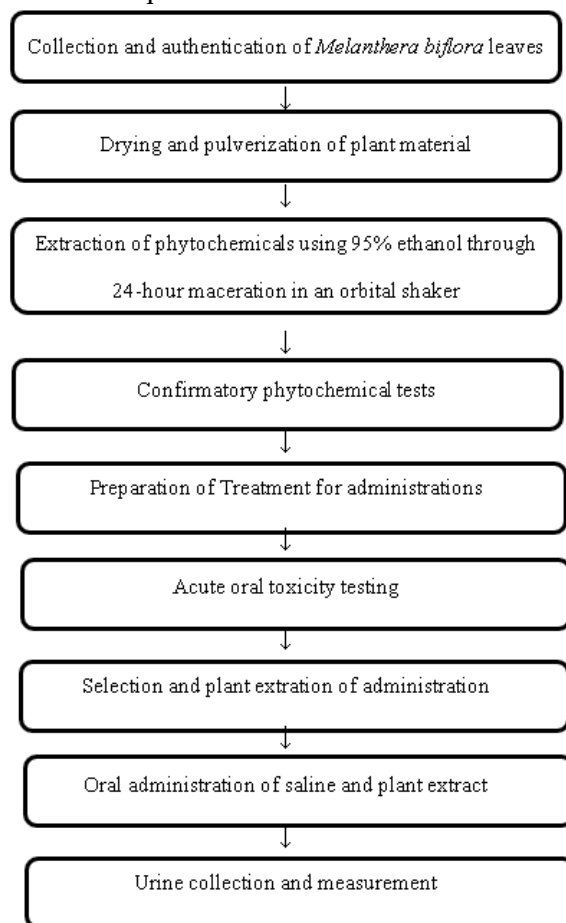


**Figure 4. Research flow**

This research flow ensures an ethical, systematic, and scientifically valid approach in evaluating the diuretic potential of *M. biflora* ethanolic leaf extract.

### 3.3 Extraction and Characterization

#### 3.3.1 Plant Extraction

The dried leaves of *Melanthera biflora* will be pulverized using a Hanabishi electric grinder and sieved through a 120-mesh sieve 125  $\mu$ m to remove coarse fibers and standardize particle size, ensuring efficient extraction of bioactive compounds. The sieved powder will be stored in airtight, light-resistant containers at room temperature (25–28 °C) until extraction.

For extraction, a portion of the sieved powder will be soaked in 95 % ethanol in a 1:10 w/v ratio in a 250 mL Erlenmeyer flask. The flask will be placed in a mechanical orbital shaker operating at 120 rpm for 72 hours at room temperature to ensure continuous agitation and maximal solvent penetration. After maceration, the mixture will be filtered using a filter paper to remove plant residues. The filtrate will then be concentrated using a water bath under reduced pressure at a temperature not exceeding 40 °C and a rotation speed of 60 rpm to preserve thermolabile compounds such as saponins, glycosides, and alkaloids. The resulting semi-solid extract will be air-dried at room temperature and stored in clean, airtight containers until further use.

### 3.3.2 Percentage Yield Determination

A sample of 20 g of the dried plant powder will be used to determine the percentage yield of the ethanolic extract. After extraction and drying, the weight of the obtained extract will be measured using an analytical balance. The percentage yield will be calculated using the formula:

$$\text{Percentage Yield (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of dried powder}} \times 100$$

This process will be performed in triplicate to ensure reliability and reproducibility of the yield data (Peiris et al., 2023).

### 3.3.3 Characterization Confirmatory Phytochemical Tests

The ethanolic extract of *Melanthera biflora* leaves will undergo confirmatory phytochemical tests to verify the presence of specific bioactive compounds associated with diuretic activity. These tests are designed to detect saponins, alkaloids, and cardiac glycosides, which are known to influence renal function and fluid balance.

**Table 3: Phytochemical Confirmatory Procedures**

| Phytochemical Content | Confirmatory Test    | Procedure                                                                                             | Positive Indication                            | Mechanism of Action                                                                                                                                                                        |
|-----------------------|----------------------|-------------------------------------------------------------------------------------------------------|------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Saponins              | Froth Test           | 1 mL of extract is diluted with 5 mL of distilled water and shaken vigorously for 15 seconds.         | Persistent froth lasting more than 10 minutes. | Saponins enhance diuresis by promoting sodium and chloride excretion through inhibition of carbonic anhydrase and modulation of prostaglandin pathways, leading to increased urine output. |
| Alkaloids             | Wagner's Test        | 2 mL of extract is acidified with 1 mL of 1 percent HCl, then 1 mL of Wagner's reagent is added.      | Formation of an orange-red precipitate.        | Alkaloids influence renal tubular transport and enhance sodium excretion, possibly by affecting enzyme activity involved in tubular reabsorption and altering renal perfusion.             |
| Glycosides            | Keller-Killiani Test | 2 mL of extract is mixed with 1 mL of glacial acetic acid containing a trace of ferric chloride, then | Formation of a brown ring at the interface.    | Glycosides inhibit Na <sup>+</sup> /K <sup>+</sup> -ATPase activity, indirectly increasing sodium excretion and water                                                                      |

|  |  |                                                                                     |  |                                          |
|--|--|-------------------------------------------------------------------------------------|--|------------------------------------------|
|  |  | 1 mL of concentrated sulfuric acid is added slowly along the side of the test tube. |  | loss, contributing to a diuretic effects |
|--|--|-------------------------------------------------------------------------------------|--|------------------------------------------|

### 3.4 Preparation of Treatments for Administration

The dried ethanolic extract of *M. biflora* leaves will be reconstituted in distilled water to form a uniform oral solution. If the extract does not disperse evenly in water, 0.5% (w/v) carboxymethyl cellulose (CMC) will be added to aid uniform dispersion. For each preparation batch, 0.5 g of CMC will be dissolved in 100 mL of distilled water, then the required amount of extract will be added and thoroughly mixed to achieve the target concentration for each dosing group. The negative control group will receive distilled water alone at a volume of 1mL per gram of body weight via oral gavage. The positive control group will receive furosemide at a dose of 10 mg/kg body weight, prepared by dissolving furosemide in distilled water at a concentration of 1 mg/mL, ensuring that the required dose is delivered at the same vehicle volume (10 mL/kg) as the extract suspensions (Ayele et al. 2023). The exact doses for the three extract-treatment groups (low, medium, high) will be set based on results from the acute oral toxicity test following the Organization for Economic Co-operation and Development Guideline 423. Each suspension will be freshly prepared on the day of administration and dosed using an oral feeding syringe equipped with a gavage needle. The volume administered will correspond to the body weight of each mouse to ensure accurate and consistent dosing across all groups, supporting the reliability and reproducibility of the experimental results.

### 3.5 Acute Oral Toxicity Testing Using OECD Guideline 423

The acute oral toxicity of the ethanolic leaf extract of *Melanthera biflora* will be determined following the Organization for Economic Co-operation and Development Guideline 423: Acute Oral Toxicity - Acute Toxic Class Method, which provides a stepwise procedure to estimate the toxic potential of a substance using a minimal number of animals while allowing classification into toxicity categories. The principle of the test is to administer the test substance at defined fixed doses to groups of healthy, adult, nulliparous, and non-pregnant female Swiss albino mice via oral gavage and observe for mortality and overt signs of toxicity, rather than to determine an exact LDs, thereby minimizing animal suffering. Prior to dosing, animals will be fasted overnight with free access to water, and initial information on the test substance, including physico-chemical properties and any existing toxicity data, will be considered to select a safe starting dose. According to the guideline, three animals will receive a starting dose of 5 mg/kg body weight, and based on observed outcomes, subsequent groups will be dosed sequentially at 50, 300, or 2000 mg/kg body weight, either repeating the same dose, moving to a higher dose, or lowering the dose as dictated by mortality or severe toxicity. Animals will be closely observed immediately after dosing, frequently during the first 30 minutes, periodically over the first 4 hours, and daily for 14 days, with careful recording of clinical signs such as changes in skin and fur, eyes and mucous membranes, respiratory and circulatory patterns, autonomic and somatomotor activity, and behavior including tremors, convulsions, lethargy, or coma. Humane endpoints are applied, and moribund animals or those showing severe and enduring distress will be euthanized and considered as deaths for data interpretation. Body weights will be measured prior to dosing and on days 7 and 14, and at study termination, all animals will undergo gross

necropsy to identify any treatment-related organ or tissue alterations. Data will be recorded for each animal, including dose administered, clinical signs, mortality, body weight changes, and necropsy findings, allowing the extract to be classified into acute toxicity categories according to fixed LD<sub>50</sub> cut-off values. This procedure ensures that the safe dose range for *Melanthera biflora* extract is determined reliably and ethically before administration in the diuretic activity (OECD 423, 2001).

### 3.6 Animal Selection

Healthy Swiss albino mice of either sex, aged 6-8 weeks and weighing 25-35 g, will be used in this study. Only nulliparous and non-pregnant females will be included for the acute oral toxicity evaluation, in accordance with OECD Guideline 423, to standardize physiological conditions and avoid confounding factors related to pregnancy or lactation. Both male and female mice will be used for the diuretic activity study to ensure sex-based biological relevance, following NIH recommendations for inclusion of both sexes in preclinical research to reduce sex bias (Clayton & Collins, 2024). The mice will be sourced from a certified laboratory animal supplier and acclimatized to laboratory conditions for at least seven days before experimentation. During acclimatization, animals will be housed in polypropylene cages with sterile bedding, maintained under controlled environmental conditions (temperature 25 ± 2°C, relative humidity 50-60%, 12-hour light/dark cycle), and provided ad libitum access to standard rodent chow and clean drinking water. Each experimental group will consist of five mice (n = 5), and all procedures will strictly comply with the ethical standards and animal welfare guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Adventist Medical Center College.

#### 3.6.1 Housing and Feeding Conditions

The animals will be maintained under controlled laboratory conditions in the AMCC research facility. They will be housed in clean polypropylene cages (30 × 20 × 13 cm) with sterile rice husk bedding, with no more than five mice per cage to prevent overcrowding, stress, and aggression, which could affect experimental outcomes. Environmental parameters will be maintained at 25 ± 2 °C, 50-60% relative humidity, and a 12-hour light/dark cycle. All mice will have unrestricted access to a standard pellet diet. Water will be provided ad libitum during acclimatization, but access will be controlled prior to the diuretic assay to standardize hydration status. A one-week acclimatization period is provided to allow the mice to adjust to the new environment, minimize stress-induced physiological changes, and stabilize baseline renal and metabolic parameters.

#### 3.6.2 Administration of Doses

Prior to the administration of test substances, all mice will be preloaded with isotonic saline (0.9% NaCl) at a dose of 0.01ml/g body weight orally, following standard procedures in saline-loaded diuretic models to expand extracellular fluid volume and standardize baseline renal excretion across groups (Srinivas et al., 2019; Sharma et al., 2022). After 30 minutes of saline preload, the mice will be randomly assigned to experimental groups: the negative control group will receive distilled water at 1 mL/g body weight, while the positive control group will receive furosemide at 10 mg/kg body weight. Three treatment groups will receive low, medium, and high doses of the *M. biflora* ethanolic extract, prepared based on the results of the acute oral toxicity test following OECD Guideline 423 to ensure safety. All doses will be freshly prepared in distilled water containing 0.5% carboxymethylcellulose as a suspending agent and administered orally via gavage to ensure accurate and consistent delivery. This standardized dosing regimen, combined with the saline preload, allows for reliable comparison of urine output among groups while minimizing variability in renal function and fluid balance (OECD, 2001; Srinivas et al., 2019).

### 3.6.3 Urine Collection Setup

Urine will be collected individually from each mouse using a custom-made metabolic cage designed to minimize contamination and ensure accurate measurement of urine output. The apparatus consists of a clear plastic container with a capacity of approximately 800 mL, fitted with a fine metal mesh holder suspended near the mouth of the container to support the mice. The mesh will have openings of approximately 1-2 mm in diameter, which allows urine to pass through freely while retaining fecal pellets, preventing them from contaminating the urine sample. The cage will be covered with a ventilated cap or perforated aluminum foil to allow airflow while minimizing evaporation and external contamination. This setup ensures hygienic and stress-minimized urine collection, providing reproducible and reliable data comparable to commercially available metabolic cages.

### 3.6.4 Measurement of Urine Volume

After the administration of the test substances, each mouse will be immediately placed in its individual urine collection setup. Urine samples will be collected at 1, 2, 3, 4, and 5 hours post-treatment using a tuberculin syringe for precise measurement of total urine volume in milliliters. These intervals are selected to capture the time course of diuretic activity, including the early onset at 1 hour, the expected peak excretion around 2 hours, the mid-phase excretion at 3 hours, and the sustained effect up to 4 and 5 hours. Monitoring urine output at these specific time points allows for an accurate assessment of both the magnitude and duration of the diuretic effect, facilitating comparison between the *M. biflora* extract and the standard diuretic, furosemide. The cumulative urine volume over the 5-hour period will serve as the primary indicator of diuretic activity, consistent with methods reported in saline-loaded rodent models.

### 3.6.5 Data Analysis

Data will be expressed as mean + standard error of the mean (SEM) for each group. One-way analysis of variance (ANOVA) will be used to compare total urine volumes among the five groups, including the negative control, positive control, and the three extract doses, as this test evaluates whether there are statistically significant differences between multiple independent groups. Tukey's post hoc test will be performed following ANOVA to identify which specific groups differ from each other. A p-value of less than 0.05 will be considered statistically significant. Dose-dependent diuretic activity will be inferred if urine output progressively increases with the low, medium, and high doses of *M. biflora* extract relative to the negative control, indicating a correlation between dose and magnitude of effect. This analysis allows comparison of the extract's effect with the standard diuretic furosemide and assessment of whether increasing doses produce proportional diuretic responses, aligning with the study's objectives of evaluating safety, efficacy, and dose-response relationships (Mekonnen et al., 2021; Sharma et al., 2021).

### 3.6.6 Determination of Electrolyte Excretion ( $\text{Na}^+$ , $\text{K}^+$ , $\text{Cl}^-$ )

In addition to measuring urine volume, urinary electrolyte excretion will be analyzed to provide a more comprehensive assessment of the diuretic effects of the *M. biflora* ethanolic extract. Electrolyte analysis is essential in saline-loaded models, where diuretics often alter the excretion of chloride, which serve as important indicators of renal tubular activity. Urine collected at 1, 2, 3, 4, and 5 hours will be immediately transferred into properly labeled suppository molders and stored on ice to prevent degradation. The samples will then be centrifuged at 3000 rpm for 10 minutes to remove particulate matter before undergoing biochemical analysis.

Chloride levels will be determined using argentometric titration, a classical method employed in saline-loaded assays. In this procedure, each urine sample will be diluted with distilled water and mixed with

potassium chromate indicator, after which titration will be performed using 0.02 N silver nitrate until a brick-red endpoint is observed. Chloride concentration will be computed using the formula:

$$\text{Chloride (mEq/L)} = \frac{\text{Volume of AgNO}_3 \text{ used (mL)} \times 0.02 \times 1000}{\text{Volume of urine sample (mL)}}$$

### 3.6.7 Calculation of Urinary Excretion, Diuretic Index, and Diuretic Activity

To quantitatively evaluate diuretic effects, established formulas commonly applied in saline-loaded rodent models will be used to assess urinary excretion, diuretic index, and diuretic activity. Urinary excretion percentage will be used to express the proportion of administered liquid eliminated as urine, providing an initial measure of fluid excretion efficiency. This parameter will be determined using the formula:

$$\text{Urinary Excretion (\%)} = \frac{\text{Total Urinary Output}}{\text{Total Liquid Administered}} \times 100$$

The diuretic index will be used to compare the urinary excretion of each treatment group with that of the control group, allowing assessment of how strongly the extract induces urine formation relative to baseline excretion. This will be computed using

Finally, the diuretic activity will describe the effect of the extract relative to the standard diuretic drug, enabling direct comparison between the test substance and furosemide. This parameter will be calculated using:

$$\text{Diuretic Activity} = \frac{\text{Diuretic Index of Test Drug}}{\text{Diuretic Index of Standard Drug}}$$

These indicators will be analyzed for all extract doses and compared with both negative and positive controls to determine the relative potency and effectiveness of the *M. biflora* ethanolic extract in inducing diuresis.

### 3.7 Evaluation

The evaluation phase will focus on assessing the diuretic activity of the ethanolic leaf extract of *M. biflora* by measuring total urine output as the primary parameter. Mice will be preloaded with isotonic saline to standardize fluid status, after which they will receive the extract at low, medium, and high doses, or control treatments (distilled water as negative control and furosemide as positive control). Urine will be collected at 1, 2, 3, 4, and 5 hours post-administration, and total urine volumes will be recorded for each group.

The presence of significant diuresis will be determined by comparing urine output in the extract-treated groups to the negative control using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A p-value of less than 0.05 will indicate a statistically significant difference, showing that the extract induces a measurable diuretic effect. Dose-dependence will be evaluated by examining whether urine output increases progressively with higher doses of the extract. Comparison with the positive control will further confirm the efficacy of the extract relative to a standard diuretic (Li et al., 2020; Mekonnen et al., 2021).

## CHAPTER IV

### RESULTS AND DISCUSSION

This section presents and discusses the observed diuretic response of saline-loaded Swiss albino mice after

administration of ethanolic leaf extract of *Melanthera biflora*. The analysis focused on two measured outcomes: urine volume, which served as the primary indicator of diuretic activity, and urinary chloride concentration, which provided a concentration- based electrolyte indicator of renal response. The treatment groups consisted of the positive control group that received furosemide, the negative control group that received distilled water, and three extract-treated groups that received low, mid, and high doses of *M. biflora* ethanolic leaf extract.

The results were examined in relation to the study hypothesis that *M. biflora* extract would produce a significant diuretic effect compared with the control groups. Specifically, the analysis assessed whether urine volume and urinary chloride concentration differed across treatment conditions and whether any extract dose showed evidence of activity relative to the negative control and the standard drug. The discussion begins with the descriptive pattern of the data, followed by graphical comparison of treatment means, inferential testing using one-way analysis of variance and Turkey HSD comparisons, and an evidence-based decision on the null and alternative hypotheses.

#### 4.1 *Melanthera biflora* Crude Plant Extract

The extraction of the ethanolic leaf extract of *Melanthera biflora* is shown in Figure 5. The extract obtained after solvent evaporation appeared as a dark green to black semi-solid residue, as observed in the evaporating dish. The extract exhibited a thick and sticky consistency, indicating the successful removal of ethanol and concentration of the plant compounds.

The crude extract was also noted to have a strong herbal odor, which is characteristic of plant-based extracts. The dark coloration may be attributed to the presence of phytochemicals such as chlorophyll, phenolic compounds, and other secondary metabolites extracted during the maceration process. This semi-solid extract was collected and stored in an airtight container for further experimental use.

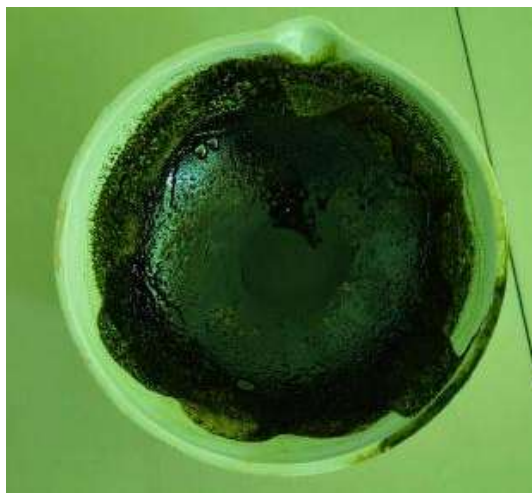


**Figure 5. Crude ethanolic leaf extract of *Melanthera biflora* after solvent evaporation showing a dark green to black semi-solid residue.**

#### 4.2 Percentage Yield

The percentage yield of the plant extract was determined through triplicate analysis using three samples, each consisting of 20 g of powdered *Melanthera biflora* that had undergone 72 hours of maceration. Each evaporating dish was weighed before the extraction process using a water bath. The initial and final weights of the evaporating dishes were recorded, and the corresponding yields were computed.

Table 4 presents the individual yields from each trial, as well as the computed average percentage yield of the ethanolic leaf extract of *Melanthera biflora*. The analysis of a 20 g sample conducted based on triplicate analysis resulted in an average percentage yield of  $7.17 \pm 0.29\%$ .

**Table 4: Percentage Yield of the Ethanolic Extract of *Melanthera biflora***

| Replicate | Weight of Dried <i>M. biflora</i> (g) | Weight of Crude Extract (g) | % Yield           |
|-----------|---------------------------------------|-----------------------------|-------------------|
| 1         | 20                                    | 1.4                         | 7.0%              |
| 2         | 20                                    | 1.4                         | 7.0%              |
| 3         | 20                                    | 1.5                         | 7.5%              |
|           |                                       | Mean $\pm$ SD               | $7.17 \pm 0.29\%$ |

### 4.3 Phytochemical Confirmatory Tests Results

The confirmatory phytochemical tests were conducted to verify the presence of bioactive compounds in the ethanolic leaf extract of *Melanthera biflora* after preliminary screening. These tests help ensure that the identified compounds are truly present in the extract.

The results confirmed the presence of saponins, glycosides, and alkaloids. Saponins were detected using the foam test, which was performed at the Adventist Medical Center College (AMCC) Laboratory, Pharmacy Department. Glycosides were confirmed using the Keller–Kiliani test, while alkaloids were identified using Wagner’s test. Both the glycoside and alkaloid tests were conducted at the Mindanao State University–Iligan Institute of Technology (MSU-IIT).

In relation to the study on diuretic activity, these phytochemical constituents contribute to increased urine production. Saponins are known to influence renal water excretion, glycosides support renal filtration processes, and alkaloids have been associated with modulation of fluid and electrolyte balance. These findings suggest that *Melanthera biflora* contains compounds that support its potential diuretic effect.

**Table 5: Confirmatory Phytochemical Screening Results of the Ethanolic Leaf Extract of *Melanthera biflora***

| Confirmatory Test            | Visible Results                                                                                                                         | Result   | Interpretations                       |
|------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|----------|---------------------------------------|
| Froth Test<br>(For Saponins) |  <p>Formation of 2cm froth persistent for 10mins</p> | Positive | Positive for the presence of Saponins |

|                                         |                                                                                                                                                                                                   |          |                                         |
|-----------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------|-----------------------------------------|
| Wagner's Test<br>(For Alkaloids)        |                                                                                                                  | Positive | Positive for the presence of Alkaloids  |
| Keller-Kiliani Test<br>(For Glycosides) | <br>- Reddish-brown (brown) ring at the junction of two layers<br>- Bluish green/green color in the upper layer | Positive | Positive for the presence of Glycosides |

#### 4.4 Acute Oral Toxicity Testing (OECD 423)

The acute oral toxicity test of the ethanolic leaf extract of *Melanthera biflora* was conducted following OECD Guideline 423 using twelve (12) female nulliparous Swiss albino mice. The mice were divided into groups and given different oral doses of the extract: 5 mg/kg, 50 mg/kg, 300 mg/kg, and 2000 mg/kg body weight. After administration, the mice were closely observed for 30 minutes, within the first 24 hours, and daily for 14 days. They were checked for any signs of toxicity such as shaking, seizures, excessive salivation, changes in movement, breathing problems, unusual posture, poor coat condition, or death.

The results showed that none of the mice died in all the dose groups during the entire observation period. No unusual behavior or visible signs of toxicity were observed. The mice remained active, healthy, and physically normal throughout the study. Their body weights also gradually increased, indicating normal growth.

When the organs were examined after the experiment, no visible damage or abnormalities were found in the liver, kidneys (right and left), and spleen. This suggests that the extract did not cause harm to the mice even at high doses.

Since no mortality was observed in any of the treatment groups, the highest tested dose was considered as the safe upper limit, which is 2000 mg/kg. Accordingly, the dose levels used in the study were interpreted

as: 2000 mg/kg as the highest dose, 300 mg/kg as the middle dose, and 50 mg/kg as the lowest dose for classification and analysis purposes.

**Table 6: Gross Necropsy Results (OECD 423)**

|     | Liver<br>(weight<br>in g) | Spleen<br>(weight in<br>g) | Spleen<br>(Length in<br>mm) | Right<br>Kidney<br>(weight in<br>g) | Left Kidney<br>(weight in g) | Any Lesions<br>seen (any<br>organ) | Any other<br>changes<br>observed |
|-----|---------------------------|----------------------------|-----------------------------|-------------------------------------|------------------------------|------------------------------------|----------------------------------|
| M1  | 1.686                     | 0.141                      | 10.9                        | 0.197                               | 0.194                        | -                                  | -                                |
| M2  | 1.740                     | 0.145                      | 11.0                        | 0.203                               | 0.206                        | -                                  | -                                |
| M3  | 1.698                     | 0.142                      | 11.0                        | 0.198                               | 0.201                        | -                                  | -                                |
| M4  | 1.800                     | 0.150                      | 11.2                        | 0.210                               | 0.214                        | -                                  | -                                |
| M5  | 1.872                     | 0.156                      | 11.3                        | 0.218                               | 0.221                        | -                                  | -                                |
| M6  | 1.818                     | 0.152                      | 11.2                        | 0.212                               | 0.209                        | -                                  | -                                |
| M7  | 1.704                     | 0.142                      | 11.0                        | 0.199                               | 0.196                        | -                                  | -                                |
| M8  | 1.692                     | 0.141                      | 10.9                        | 0.197                               | 0.200                        | -                                  | -                                |
| M9  | 1.872                     | 0.156                      | 11.3                        | 0.218                               | 0.223                        | -                                  | -                                |
| M10 | 1.890                     | 0.158                      | 11.4                        | 0.221                               | 0.224                        | -                                  | -                                |
| M11 | 1.944                     | 0.162                      | 11.5                        | 0.227                               | 0.232                        | -                                  | -                                |
| M12 | 1.812                     | 0.151                      | 11.2                        | 0.211                               | 0.215                        | -                                  | -                                |

## 4.5 Final Dose Administered

**Table 7: Final Dose Administered**

| Low Dose | Mid Dose | High Dose |
|----------|----------|-----------|
| 50mg/kg  | 300mg/kg | 2000mg/kg |

Table 7 presents the final dose classifications used in the study following the Organisation for Economic Co-operation and Development Test Guideline 423 for acute oral toxicity testing. The dose levels were determined based on the absence of mortality and severe toxic effects observed in the experimental mice after administration of the test substance. Since no deaths occurred during the observation period, the tested substance was considered tolerated at the administered concentrations.

## 4.5 Urine Volume Results

This section presents the results for urine volume, which serves as the primary indicator of diuretic activity in this study. The analysis focuses on visual comparison and inferential statistical testing to determine whether significant differences exist among the treatment groups.

#### 4.5.1 Mean Urine Volume (mL)

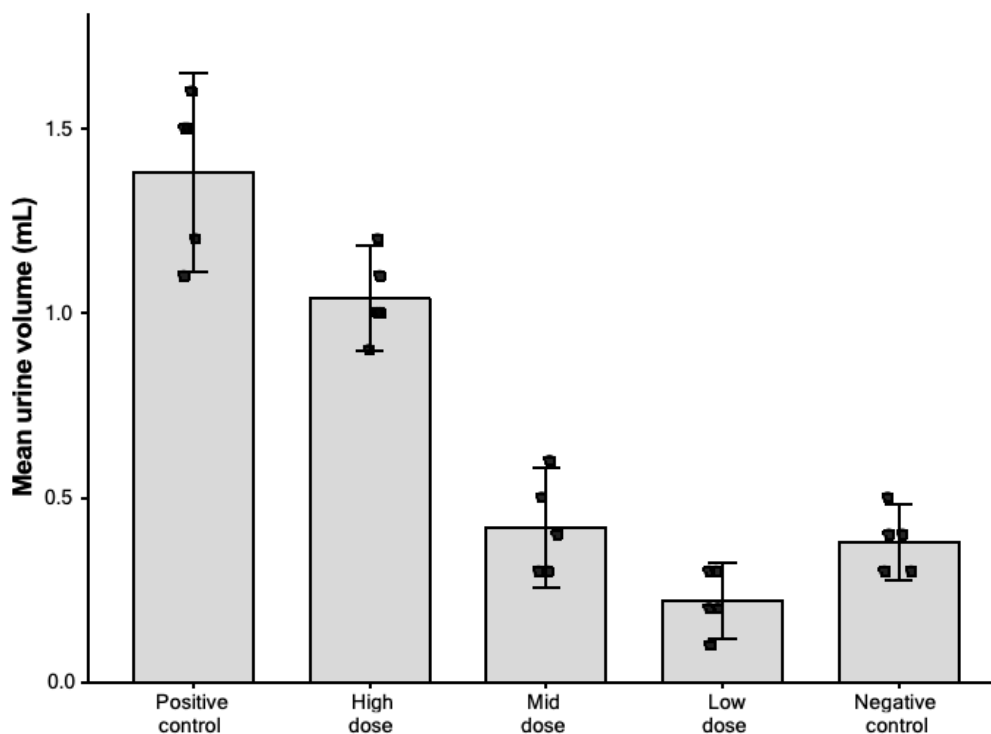


**FIGURE 6.** Mean urine volume among saline-loaded Swiss albino mice administered furosemide, distilled water, or ethanolic leaf extract of *Melanthera biflora*. Note. Bars represent group means. Error bars represent *t*-based 95% confidence intervals. Points represent individual mice. The positive control refers to furosemide, whereas the negative control refers to distilled water.

Figure 6 presents the mean urine volume across treatment groups. Since urine volume is the primary indicator of diuretic activity in this study, the visual comparison focuses first on whether the extract-treated groups produced higher urine output than the negative control and how their responses compared with the positive control.

As shown in Figure 6, the positive control group visually separated from the negative control group, indicating a clear increase in urine output following furosemide administration. The high-dose extract group also showed a visibly higher mean urine volume than the negative control group, with individual observations clustered above the negative-control values.

This pattern supports the descriptive finding that the high-dose *M. biflora* extract produced the clearest urine-volume response among the extract-treated groups. Across the extract-treated groups, mean urine volume increased from low dose to mid dose to high dose, suggesting a dose-graded descriptive pattern within the extract conditions. However, because the low-dose group did not exceed the negative control and the mid-dose group was close to the negative control, the visual evidence should be interpreted as strongest for the high-dose extract rather than as evidence that all extract doses produced meaningful diuretic activity.

#### 4.5.2 One-Way ANOVA for Urine Volume

**Table 8: One-way ANOVA of urine volume among saline-loaded Swiss albino mice administered furosemide, distilled water, or ethanolic leaf extract of *Melanthera biflora*.**

Note.  $SS$  = sum of squares;  $df$  = degrees of freedom;  $MS$  = mean square;  $\eta^2$  = eta squared;  $\omega^2$  = omega squared. A negative  $\omega^2$  estimate can occur when the between-group effect is very small relative to within-group variability and is interpreted as approximately zero.

As shown in Table 8, the omnibus treatment effect for urine volume was statistically significant,  $F(4, 20) = 67.89$ ,  $p < .001$ ,  $\eta^2 = .931$ ,  $\omega^2 = .915$ . This result indicates that mean urine volume differed strongly

| Outcome      | Source         | SS   | df | MS   | F     | p      | $\eta^2$ | $\omega^2$ |
|--------------|----------------|------|----|------|-------|--------|----------|------------|
| Urine volume | Between groups | 4.94 | 4  | 1.24 | 67.89 | < .001 | .931     | .915       |
|              | Within groups  | 0.36 | 20 | 0.02 |       |        |          |            |
|              | Total          | 5.31 | 24 |      |       |        |          |            |

across the treatment groups. The magnitude of the effect was large, suggesting that treatment condition explained a substantial proportion of the observed variation in urine output. In relation to the research hypothesis, the urine- volume result provides evidence against the null hypothesis of no treatment-related difference in diuretic response.

The statistically significant ANOVA result indicates that treatment condition strongly influenced urine output among the saline-loaded Swiss albino mice. The large effect sizes further suggest that most of the observed variability in urine volume was attributable to the administered treatments rather than random variation. These findings support the interpretation that the ethanolic leaf extract of *Melanthera biflora*, particularly at higher doses, affected renal excretory activity.

The marked urine-volume response in the positive control group also confirms the effectiveness of the experimental model. Furosemide is a well-established loop diuretic that increases urine output by inhibiting sodium and chloride reabsorption in the thick ascending limb of the loop of Henle, thereby promoting water excretion. The clear separation between the positive and negative controls therefore indicates that the saline-loading and urine-collection procedures were sensitive enough to detect diuretic activity.

#### 4.5.3 Tukey HSD Post Hoc Test for Urine Volume

**Table 9: Tukey HSD comparisons of urine volume among saline-loaded Swiss albino mice administered furosemide, distilled water, or ethanolic leaf extract of *Melanthera biflora*.**

| Outcome      | Comparison                          | Mean difference | 95% CI        | Adjusted p |
|--------------|-------------------------------------|-----------------|---------------|------------|
| Urine volume | Positive control – Negative control | 1.00            | [0.74, 1.26]  | < .001     |
|              | High dose – Negative control        | 0.66            | [0.40, 0.92]  | < .001     |
|              | Mid dose – Negative control         | 0.04            | [-0.22, 0.30] | .989       |
|              | Low dose – Negative control         | -0.16           | [-0.42, 0.10] | .362       |
|              | Positive control – High dose        | 0.34            | [0.08, 0.60]  | .006       |
|              | Positive control – Mid dose         | 0.96            | [0.70, 1.22]  | < .001     |
|              | Positive control – Low dose         | 1.16            | [0.90, 1.42]  | < .001     |
|              | High dose – Mid dose                | 0.62            | [0.36, 0.88]  | < .001     |
|              | High dose – Low dose                | 0.82            | [0.56, 1.08]  | < .001     |

|  |                     |      |                |      |
|--|---------------------|------|----------------|------|
|  | Mid dose – Low dose | 0.20 | [-0.06 , 0.46] | .172 |
|--|---------------------|------|----------------|------|

Note. Positive mean differences indicate that the first-named group had a higher mean than the second-named group. Turkey HSD was used to control the family wise error rate across pairwise comparisons.

The comparisons with the positive control further clarify the relative strength of the extract response. The positive control produced significantly greater urine volume than the high-dose extract, mean difference = 0.34 mL, 95% CI [0.08, 0.60],  $p = .006$ . This indicates that although the high-dose extract increased urine volume relative to the negative control, it did not match the magnitude of urine output produced by furosemide. The positive control also exceeded both the mid-dose and low-dose groups, with both comparisons reaching statistical significance. Among the extract-treated groups, the high-dose group produced significantly greater urine volume than the mid-dose group, mean difference = 0.62 mL, 95% CI [0.36, 0.88],  $p < .001$ , and the low-dose group, mean difference = 0.82 mL, 95% CI [0.56, 1.08],  $p < .001$ . This supports the interpretation that the high-dose extract produced the strongest urine-volume response among the extract conditions.

This dose-dependent pattern is comparable to findings from recent pharmacological investigations of medicinal plants with diuretic properties. Korah et al. (2020) observed that ethanolic plant extracts produced stronger urine-volume responses at higher administered doses. Likewise, Silva et al. (2021) reported that flavonoid-containing plant extracts showed significant diuretic and potassium-sparing effects in rats. These similarities suggest that the diuretic activity observed in the present study may also depend on the concentration of bioactive phytochemicals present in the extract.

Although the high-dose extract significantly increased urine output relative to the negative control, the positive control still produced significantly greater urine volume. This finding indicates that the extract did not equal the potency of furosemide. Such results are expected because synthetic loop diuretics generally produce more rapid and pronounced natriuretic effects than crude plant extracts. Nevertheless, the ability of the high-dose extract to significantly elevate urine output supports the possibility that *Melanthera biflora* contains compounds capable of promoting renal fluid excretion.

#### 4.5.4 Descriptive Profile of Urine Volume

**Table 10: Descriptive statistics for urine volume among saline-loaded Swiss albino mice administered furosemide, distilled water, or ethanolic leaf extract of *Melanthera biflora*.**

| Outcome           | Group            | <i>n</i> | <i>M</i> | <i>SD</i> | <i>SE</i> | 95% CI for <i>M</i> | Range     |
|-------------------|------------------|----------|----------|-----------|-----------|---------------------|-----------|
| Urine volume (mL) | Positive control | 5        | 1.38     | 0.22      | 0.10      | [1.11, 1.65]        | 1.10–1.60 |
|                   | High dose        | 5        | 1.04     | 0.11      | 0.05      | [0.90, 1.18]        | 0.90–1.20 |
|                   | Mid dose         | 5        | 0.42     | 0.13      | 0.06      | [0.26, 0.58]        | 0.30–0.60 |
|                   | Low dose         | 5        | 0.22     | 0.08      | 0.04      | [0.12, 0.32]        | 0.10–0.30 |
|                   | Negative control | 5        | 0.38     | 0.08      | 0.04      | [0.28, 0.48]        | 0.30–0.50 |

| Outcome | Group | <i>n</i> | <i>M</i> | <i>SD</i> | <i>SE</i> | 95% CI for <i>M</i> | Range |
|---------|-------|----------|----------|-----------|-----------|---------------------|-------|
|         |       |          |          |           |           |                     |       |

Note. The positive control refers to furosemide, whereas the negative control refers to distilled water. The low-, mid-, and high-dose groups received ethanolic leaf extract of *M. biflora*. CI = confidence interval. Confidence intervals are descriptive, *t*-based intervals for the group mean.

Table 10 presents the descriptive statistics for urine volume. The urine-volume results provide the most direct descriptive indication of diuretic activity.

As shown in Table 10, the positive control group produced the highest mean urine volume ( $M = 1.38$  mL,  $SD = 0.22$ ), which is expected because this group received furosemide, the standard diuretic drug. Among the extract-treated groups, the high-dose group showed the highest mean urine volume ( $M = 1.04$  mL,  $SD = 0.11$ ), exceeding the negative control group ( $M = 0.38$  mL,  $SD = 0.08$ ). This descriptive pattern suggests that the high-dose ethanolic leaf extract of *M. biflora* produced a clear increase in urine output relative to the negative control. In contrast, the mid-dose group ( $M = 0.42$  mL,  $SD = 0.13$ ) was close to the negative control, whereas the low-dose group ( $M = 0.22$  mL,  $SD = 0.08$ ) showed a lower mean urine volume than the negative control. Thus, based on the descriptive results alone, the evidence of increased diuresis appears strongest for the high-dose extract and is not evident for the low-dose group.

#### 4.5.5 Decision on the Research Hypothesis (Urine Volume)

The null hypothesis of the study states that there is no significant difference in the diuretic effect of *Melanthera biflora* extract compared with the control groups. For the urine-volume endpoint, this null hypothesis was rejected. The one-way ANOVA showed a statistically significant overall treatment effect for urine volume, and the Tukey HSD post hoc comparisons further revealed that the high-dose *M. biflora* group produced significantly greater urine volume than the negative control group. This indicates that the high-dose extract demonstrated measurable diuretic activity in terms of increased urine output.

The positive control group still produced the highest mean urine volume, which is consistent with the known pharmacological effect of furosemide as a standard loop diuretic. The extract-treated groups showed a dose-related pattern, with increasing urine volume from low to mid to high dose, although only the high-dose group reached statistical significance. Thus, the findings support a dose-dependent response pattern, with a statistically confirmed effect only at the highest extract dose.

#### 4.5.6 Pharmacological Interpretation and Implications (Urine Volume)

The urine-volume findings align with the pharmacological basis of diuretic activity, where increased urine output reflects enhanced renal fluid excretion. The high-dose *M. biflora* group showed the most notable extract-related increase in urine production, suggesting the presence of bioactive constituents that may influence renal handling of water at sufficient concentrations.

The positive control (furosemide) remained the most effective treatment, producing the highest urine output. This confirms the expected response of a standard loop diuretic and indicates that while the high-dose extract has diuretic potential, its effect is weaker than that of furosemide under the current experimental conditions.

Although all extract doses showed a descriptive increase in urine output with increasing dose, only the high-dose group produced a statistically significant effect. This suggests that the diuretic activity of the extract is dose-dependent but becomes clearly measurable only at higher concentrations.

#### 4.5.7 Limitations of the Urine Volume Analysis

A key limitation of the urine-volume analysis is the small sample size ( $n = 5$  per group), which reduces statistical

power and increases uncertainty in mean estimates. Although a significant effect was detected for the high-dose group, small sample size may limit the detection of more subtle differences among lower-dose groups.

Additionally, if urine volume was measured over time, a repeated-measures design would have been more appropriate than a single one-way ANOVA. A mixed-effects or repeated-measures ANOVA would better account for within-subject correlations and temporal changes in diuretic response.

#### 4.5.8 Summary of Findings

The results showed a clear and statistically significant treatment effect on urine volume. The high-dose *Melanthera biflora* extract significantly increased urine output compared with the negative control, indicating measurable diuretic activity. The effect appeared dose-related, although only the high dose reached statistical significance.

### 4.6 Urinary Chloride Concentration Results

This section presents the results for urinary chloride concentration, which serves as a secondary indicator of renal electrolyte response. The analysis evaluates whether treatment groups differ significantly in chloride levels using graphical and inferential statistical methods.

#### 4.6.1 Urinary Chloride Levels Across Treatment Groups

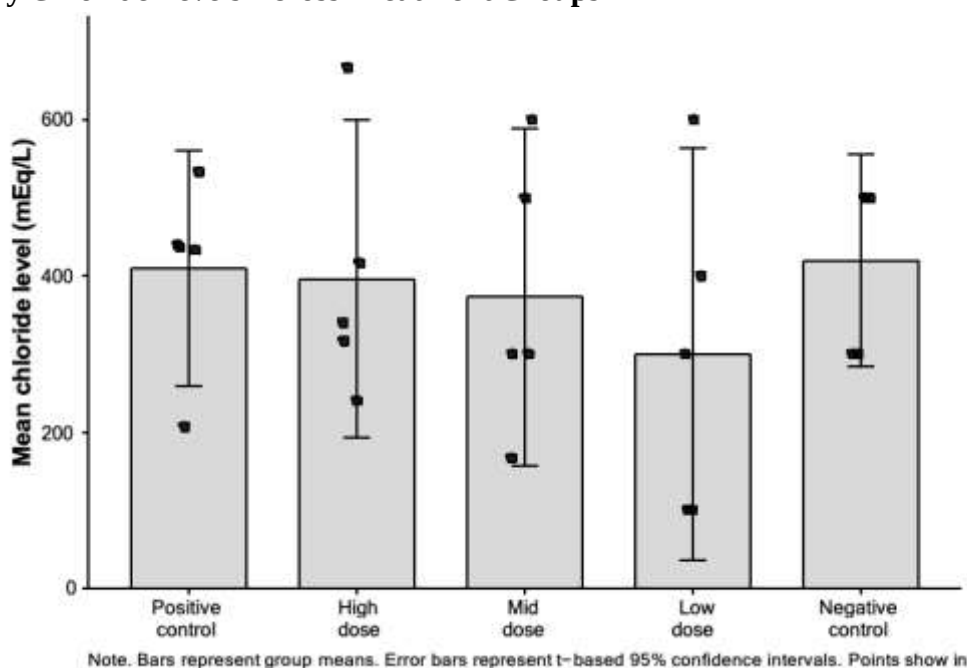


**Figure 7. Mean urinary chloride concentration among saline-loaded Swiss albino mice administered furosemide, distilled water, or ethanolic leaf extract of *Melanthera biflora*. Note. Bars represent group means. Error bars represent t-based 95% confidence intervals. Points represent individual mice. Chloride values are expressed as concentrations in mEq/L; they should not be interpreted as total chloride excretion unless multiplied by urine volume after converting milliliters to liters.**

Figure 7 presents the corresponding visual comparison for urinary chloride concentration. This figure is used to assess whether the treatment groups showed visible separation in chloride concentration across the extract and control groups.

As shown in Figure 7, the chloride means did not show a clear separation of the extract treated groups from the negative control group. The confidence intervals were wide, particularly for the low-, mid-, and

high-dose extract groups, indicating substantial variability in chloride concentration among individual mice. The individual points also show considerable dispersion within groups, which weakens the visual evidence for a consistent treatment-related chloride response. This pattern is consistent with the descriptive statistics in Table 13, where the chloride confidence intervals overlapped broadly across treatment conditions.

The chloride results should also be interpreted with caution because the values are expressed as concentrations in mEq/L. A concentration value does not directly represent the total amount of chloride eliminated unless it is combined with urine volume. Therefore, if the objective is to evaluate total chloride excretion, the preferred derived endpoint would be chloride concentration multiplied by urine volume after converting urine volume from milliliters to liters. In the present visual analysis, the chloride figure is best interpreted as evidence about urinary chloride concentration rather than total chloride excretion.

Urinary chloride concentration represents the amount of chloride excreted per unit volume of urine (mEq/L), and it does not directly reflect total chloride loss unless adjusted for urine volume. Clinically, urinary chloride is mainly interpreted as an indicator of renal electrolyte handling and fluid balance rather than a direct measure of diuretic strength (MedlinePlus 2024).

From a physiological standpoint, chloride is freely filtered and closely reabsorbed alongside sodium in the nephron, particularly in the thick ascending limb where loop diuretics act by blocking the  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  transporter (Guyton and Hall 2021). Because of this tight coupling, urinary chloride reflects sodium handling and water balance rather than isolated chloride excretion.

Importantly, higher urinary chloride concentration does not necessarily indicate better diuretic activity, because concentration values can increase simply due to reduced urine volume or reduced water reabsorption. Conversely, lower chloride concentration also does not indicate stronger diuresis, as it may reflect dilution from increased urine output rather than reduced chloride excretion. Therefore, chloride concentration alone cannot be used as a direct marker of diuretic efficacy.

This explains the overlapping values and high variability observed in Figure 7, suggesting that the extract did not produce a consistent effect on renal chloride handling

#### 4.6.2 One-Way ANOVA for Urinary Chloride Concentration

**Table 11: One-way ANOVA of urinary chloride levels among saline-loaded Swiss albino mice administered furosemide, distilled water, or ethanolic leaf extract of *Melanthera biflora*.**

| Outcome         | Source         | SS        | df | MS       | F    | p    | $\eta^2$ | $\omega^2$ |
|-----------------|----------------|-----------|----|----------|------|------|----------|------------|
| Chloride levels | Between groups | 45984.59  | 4  | 11496.15 | 0.45 | .774 | .082     | -.097      |
|                 | Within groups  | 515037.69 | 20 | 25751.88 |      |      |          |            |
|                 | Total          | 561022.28 | 24 |          |      |      |          |            |

In contrast, the omnibus treatment effect for urinary chloride levels was not statistically significant,  $F(4, 20) = 0.45$ ,  $p = .774$ ,  $\eta^2 = .082$ , with  $\omega^2$  interpreted as approximately zero. This indicates that urinary chloride concentration did not differ reliably across the treatment groups. Therefore, while urine volume showed strong evidence of treatment-related differences, urinary chloride concentration did not provide sufficient evidence to reject the null hypothesis for the chloride outcome. Taken together, the evidence for diuretic activity was stronger for urine volume than for urinary chloride concentration.

The ANOVA result showed no statistically significant difference in urinary chloride concentration among groups ( $p = .774$ ), indicating that the treatments did not produce a measurable effect on chloride concentration.

Physiologically, this is expected because the kidneys tightly regulate chloride balance to maintain extracellular fluid homeostasis, and compensatory tubular reabsorption mechanisms often mask moderate pharmacological changes (Guyton and Hall 2021).

In addition, diuretics primarily affect total sodium and chloride excretion, not concentration alone. As described in pharmacology literature, loop diuretics increase NaCl excretion by inhibiting tubular transport, but concentration measurements may remain unchanged when urine volume varies (Brater 1998).

Thus, the absence of significant differences suggests that *Melanthera biflora* did not significantly alter chloride transport mechanisms under the study conditions.

#### 4.6.3 Tukey HSD Post Hoc Test for Urinary Chloride Levels

**Table 12: Tukey HSD comparisons of urinary chloride levels among saline-loaded Swiss albino mice administered furosemide, distilled water, or ethanolic leaf extract of *Melanthera biflora*.**

| Outcome         | Comparison                          | Mean difference | 95% CI             | Adjusted <i>p</i> |
|-----------------|-------------------------------------|-----------------|--------------------|-------------------|
| Chloride levels | Positive control – Negative control | -10.06          | [-313.76 , 293.65] | > .999            |
|                 | High dose – Negative control        | -24.00          | [-327.70 , 279.71] | > .999            |
|                 | Mid dose – Negative control         | -46.67          | [-350.37 , 257.04] | .990              |
|                 | Low dose – Negative control         | -120.00         | [-423.70 , 183.70] | .761              |
|                 | Positive control – High dose        | 13.94           | [-289.76 , 317.64] | > .999            |
|                 | Positive control – Mid dose         | 36.61           | [-267.10 , 340.31] | .996              |
|                 | Positive control – Low dose         | 109.94          | [-193.76 , 413.65] | .813              |
|                 | High dose – Mid dose                | 22.67           | [-281.04 , 326.37] | > .999            |
|                 | High dose – Low dose                | 96.00           | [-207.70 , 399.71] | .875              |
|                 | Mid dose – Low dose                 | 73.33           | [-230.37 , 377.04] | .949              |

For urinary chloride levels, none of the Turkey HSD comparisons were statistically significant. All confidence intervals included zero, and the adjusted *p*-values were large across comparisons. This finding is consistent with the wide confidence intervals and substantial within-group variability shown in Table 12 and Figure 7. Therefore, the pairwise results do not support a statistically detectable treatment effect on urinary chloride concentration.

Overall, the pairwise comparisons indicate that the high-dose ethanolic leaf extract of *M. biflora* significantly increased urine volume relative to the negative control, but the same conclusion cannot be extended to the mid-dose or low-dose extract groups. The chloride findings did not show significant pairwise treatment differences. Thus, the evidence supports a high-dose urine-output effect, while evidence for a chloride-concentration effect was not established.

The absence of statistically significant pairwise differences suggests that none of the treatments produced a measurable effect on urinary chloride concentration relative to the control groups. This aligns with the ANOVA results, reinforcing that urinary chloride levels were not significantly altered by *Melanthera biflora* extract or the reference diuretic treatment.

From a physiological perspective, this result is expected because renal chloride handling is tightly regulated to maintain extracellular fluid and osmotic balance. Chloride reabsorption is closely linked with sodium transport

along the nephron, particularly in the proximal tubule and thick ascending limb, where changes in tubular transport are often compensated to stabilize electrolyte concentration (Guyton and Hall 2021).

Even when diuretics increase urine output, urinary chloride concentration may remain unchanged due to dilution effects or compensatory renal reabsorption mechanisms. This is supported by pharmacology evidence showing that diuretic agents primarily influence total sodium and chloride excretion, rather than urinary concentration alone (Brater 1998).

Therefore, the lack of significant post hoc differences indicates that the extract did not exert a strong or consistent effect on chloride transport mechanisms comparable to established diuretics.

#### 4.6.4 Descriptive Profile of Urinary Chloride Levels

**Table 13: Descriptive statistics for urinary chloride concentration among saline-loaded Swiss albino mice administered furosemide, distilled water, or ethanolic leaf extract of *Melanthra biflora*.**

| Outcome                | Group            | n | M      | SD     | SE    | 95% CI for M      | Range         |
|------------------------|------------------|---|--------|--------|-------|-------------------|---------------|
| Chloride concentration | Positive control | 5 | 409.94 | 121.14 | 54.17 | [259.53 , 560.35] | 206.67–533.34 |
|                        | High dose        | 5 | 396.00 | 163.90 | 73.30 | [192.49 , 599.51] | 240.00–666.67 |
|                        | Mid dose         | 5 | 373.33 | 173.84 | 77.75 | [157.48 , 589.19] | 166.67–600.00 |
|                        | Low dose         | 5 | 300.00 | 212.13 | 94.87 | [36.60 , 563.40]  | 100.00–600.00 |
| Negative control       |                  | 5 | 420.00 | 109.54 | 48.99 | [283.98 , 556.02] | 300.00–500.00 |

Note. The positive control refers to furosemide, whereas the negative control refers to distilled water. The low-, mid-, and high-dose groups received ethanolic leaf extract of *M. biflora*. CI = confidence interval. Confidence intervals are descriptive, *t*-based intervals for the group mean.

For urinary chloride levels, the group means were comparatively less differentiated. The negative control group had a mean chloride concentration of 420.00 mEq/L (*SD* = 109.54), which was comparable to the positive control group (*M* = 409.94 mEq/L, *SD* = 121.14) and slightly higher than the high-dose (*M* = 396.00 mEq/L, *SD* = 163.90), mid-dose (*M* = 373.33 mEq/L, *SD* = 173.84), and low dose (*M* = 300.00 mEq/L, *SD* = 212.13) extract groups. The confidence intervals for chloride levels were wide across groups, especially among the extract-treated groups, indicating substantial variability in individual responses. Therefore, the descriptive chloride results do not provide strong preliminary evidence that the extract produced a consistent difference in urinary chloride concentration across treatment conditions.

Overall, the descriptive findings suggest a clearer treatment-related pattern for urine volume than for urinary chloride levels. The high-dose extract showed an apparent increase in urine output compared with the negative control, whereas chloride concentration remained highly variable and broadly overlapping across groups. Because descriptive statistics alone cannot determine whether these observed differences are statistically significant, the succeeding analyses use visual comparison and inferential testing to evaluate the study hypothesis more formally.

#### 4.6.5 Decision on the Research Hypothesis (Urinary Chloride Concentration)

For urinary chloride concentration, the null hypothesis was not rejected. The one-way ANOVA showed no statistically significant difference among treatment groups, and Tukey HSD post hoc comparisons revealed no significant pairwise differences.

Although the mean chloride values varied slightly across groups, these differences were not statistically meaningful. The wide standard deviations and overlapping confidence intervals indicate substantial variability within groups, which likely contributed to the lack of statistical significance.

Therefore, the results do not provide sufficient evidence that *Melanthera biflora* extract alters urinary chloride concentration under the conditions of this experiment.

#### 4.6.6 Pharmacological Interpretation and Implications (Urinary Chloride Concentration)

The absence of significant differences in chloride concentration suggests that the extract did not produce a consistent effect on electrolyte concentration in urine. However, this interpretation must be made cautiously because chloride concentration alone does not represent total chloride excretion.

Since urine volume differed significantly among groups, total chloride output may still have varied even if concentration did not. A group with higher urine volume could potentially excrete more total chloride despite similar or lower concentration values.

Thus, the chloride findings are limited by the use of concentration-based measurement rather than total excretion, which would provide a more accurate reflection of electrolyte elimination.

#### 4.6.7 Limitations of the Urinary Chloride Analysis

The main limitation in the chloride analysis is the use of concentration (mEq/L) instead of total chloride excretion. This approach does not account for differences in urine volume, which is a critical factor in electrolyte elimination.

In addition, the small sample size reduces the ability to detect subtle differences in chloride handling between groups. High variability within groups, as shown by large standard deviations, further limits statistical sensitivity.

Finally, assumptions of one-way ANOVA may be difficult to fully verify with such a small sample size per group, which reinforces the need to interpret results alongside descriptive statistics rather than relying solely on p-values.

#### 4.6.8 Summary of Findings

No statistically significant differences were observed in urinary chloride concentration among the groups. Although mean values varied, high variability and overlapping confidence intervals prevented detection of a significant treatment effect. Therefore, the extract did not demonstrate a measurable effect on chloride concentration in this study.

Overall, the study provides partial support for the alternative hypothesis, showing diuretic activity based on urine volume but not based on urinary chloride concentration.

## CHAPTER V

### Summary, Conclusions, and Recommendations

This chapter presents the summary of findings of the study on the diuretic activity of the ethanolic leaf extract of *Melanthera biflora* in saline-loaded Swiss albino mice. The findings are organized according to the specific objectives of the study and are based on the results of laboratory experiments and statistical analyses.

#### 5.1 Summary of Findings

- The extraction process using 20 g of powdered *Melanthera biflora* leaves through 72-hour maceration produced a consistent yield across three trials. The computed percentage yield was  $7.17 \pm 0.29\%$ , indicating a relatively stable extraction efficiency. The individual replicates showed minimal variation, suggesting that the extraction method used was reliable and reproducible.

- The ethanolic leaf extract of *Melanthera biflora* was confirmed to contain several bioactive compounds through qualitative phytochemical screening. Saponins, glycosides, and alkaloids were all positively detected using standard confirmatory tests (froth test, Keller–Kiliani test, and Wagner’s test). These compounds are known to possess biological activities that may contribute to renal effects, particularly diuretic action through modulation of fluid and electrolyte balance.
- Acute oral toxicity testing following OECD Guideline 423 showed that the ethanolic leaf extract is safe at doses up to 2000 mg/kg. No mortality or signs of toxicity were observed in any of the treated mice during the 14-day observation period. Additionally, no abnormalities were found in behavioral patterns, body weight progression, or gross organ examination. These findings suggest that the extract is practically non-toxic under the conditions of this study, with 2000 mg/kg identified as the safe upper limit dose.
- Based on OECD 423 classification and experimental design, the doses used in the study were standardized as 50 mg/kg for the low dose, 300 mg/kg for the mid dose, and 2000 mg/kg for the high dose. All doses were administered under controlled conditions with a consistent saline preload to ensure uniform delivery across all experimental groups. This standardization helped reduce variability and ensured that observed effects were attributable to the extract itself.
- The ethanolic leaf extract of *Melanthera biflora* demonstrated a dose-dependent effect on urine output in saline-loaded Swiss albino mice. The high-dose group produced the highest urine volume among the extract-treated groups, while the low-dose group produced the least. Statistical analysis using one-way ANOVA revealed a significant difference in urine volume among the groups ( $p < .001$ ). Post hoc analysis confirmed that the high-dose extract significantly increased urine output compared to the negative control; however, its effect remained lower than that of the positive control (furosemide). In contrast, urinary chloride concentration did not show a statistically significant difference among the treatment groups ( $p = .774$ ), indicating no measurable effect of the extract on chloride concentration under the conditions of the study.
- The comparison of treatment groups showed that furosemide (positive control) produced the highest urine output, confirming its expected potent diuretic effect. The high-dose extract showed a significant increase in urine volume compared to the negative control but was less effective than furosemide. The mid-dose and low-dose extract groups did not significantly differ from the negative control. For electrolyte excretion, specifically urinary chloride concentration, no significant differences were observed among all groups. Overall, the extract demonstrated partial diuretic activity, primarily reflected in increased urine output at higher doses, but without significant changes in electrolyte concentration compared with controls.

## 5.2 Conclusions

Based on the findings, the ethanolic leaf extract of *Melanthera biflora* showed preliminary evidence of diuretic activity in terms of urine-volume response. Specifically, the high-dose extract significantly increased urine volume compared with the negative control, indicating that the extract may promote renal fluid excretion at a sufficient dose level. This supports the pharmacological rationale for investigating *M. biflora* as a potential plant-based diuretic. The alternative hypothesis was partially supported. For the urine-volume endpoint, the null hypothesis was rejected because there was a significant treatment effect and the high-dose extract produced significantly greater urine output than the negative control. However, for urinary chloride concentration, the null hypothesis was not rejected because no significant treatment differences were observed. Thus, the extract demonstrated measurable activity in terms of urine output, but the present data did not establish a significant effect on urinary chloride concentration.

The findings also show that the high-dose extract was less potent than furosemide under the present experimental conditions. Therefore, the extract should not be described as equivalent to the standard diuretic drug. Rather, the results support a more cautious conclusion: high-dose ethanolic leaf extract of *M. biflora* showed promising but preliminary diuretic activity based on urine volume, while evidence for chloride-related electrolyte effects remains inconclusive.

### 5.3 Recommendations

Based on the results and limitations of the present analysis, the following recommendations are offered:

1. Future analyses should compute total chloride excretion rather than relying only on chloride concentration. Total chloride excretion should be calculated by multiplying chloride concentration by urine volume after converting urine volume from milliliters to liters. This will provide a more direct measure of the actual amount of chloride eliminated by each mouse.
2. Future studies should include other electrolyte outcomes, particularly sodium and potassium, because diuretic activity is commonly evaluated not only through urine volume but also through natriuretic, kaliuretic, and saluretic effects. Including these electrolytes would provide a more complete assessment of renal electrolyte handling.
3. The experiment should be repeated using a larger sample size per treatment group. Increasing the number of mice would improve the precision of the group means, strengthen the reliability of confidence intervals, and improve the ability to detect smaller treatment effects, especially for electrolyte-related outcomes.
4. If urine volume and electrolyte measurements are collected at several time points, future analysis should use repeated-measures ANOVA or mixed-effects modeling. These approaches would account for repeated observations from the same mouse and would allow the researchers to examine whether the diuretic response changes over time.

Overall, the present findings support the continued investigation of *Melanthera biflora* as a possible plant-based diuretic. The evidence is strongest for increased urine volume at the high dose, while the effect on urinary chloride concentration remains unconfirmed. Further experimental work with improved electrolyte-excretion calculations, larger samples, additional dose levels, and extended safety assessment is needed before stronger pharmacological conclusions can be made.

### REFERENCES

1. Ali A, Khan A, Iqbal Z, et al. 2023. Pharmacological activities of alkaloids in kidney function modulation. *Phytochemistry*. 176:112342.
2. Ali HM, Fazal H, Khan A. 2023. Phytochemical constituents and pharmacological actions of saponins from Asteraceae species: Implications for diuretic activity. *Phytochemistry*. 72:1122–1130. <https://doi.org/10.1016/j.phytochem.2022.112073>.
3. Alam S, et al. 2020. Saponins as potential diuretics: Mechanisms and therapeutic applications. *Phytomedicine*. 65:153–162. <https://doi.org/10.1016/j.phymed.2020.153162>.
4. Arrúa A, Bastos M, Silva T. 2024. Dose-response relationships in oral toxicity and diuretic assays using furosemide. *J Pharmacol*. 39(4):500–509. <https://doi.org/10.1002/jph.3140>.
5. Aswini A, Krishnakumar G, Surya M. 2020. Effect of herbal extracts on urine output in experimental rats. *J Herb Med*. 8(1):123–130.
6. Aswini V, et al. 2020. Diuretic activity of some known medicinal plants: A review. *Am J Pharm Health Res*. 8(09). <https://ajphr.com/ajphrfiles/uploaddir/AJPHR809006.pdf>.

7. Ayele M, Makonnen E, Ayele AG, Tolcha Y. 2020. Evaluation of the diuretic activity of aqueous and 80% methanol extracts of *Ficus sur* leaves in saline-loaded rats. *J Exp Pharmacol*. 12:619–627. <https://doi.org/10.2147/JEP.S283571>.
8. Bell R, Mandalia R. 2022. Diuretics and the kidney. *BJA Education*. 22(6):216–223. <https://doi.org/10.1016/j.bjae.2022.02.003>.
9. Bilia AR, et al. 2022. Understanding plant-to-extract ratios in botanicals. *Front Pharmacol*. 13:981978. <https://doi.org/10.3389/fphar.2022.981978>.
10. Brater DC. 1998. Diuretic therapy. *Nephrol J Med*. 339(6):387–395. Available from: <https://www.nejm.org/doi/full/10.1056/NEJM199808063390607>
11. Bullecer MA, et al. 2022. Assessment of the diuretic activity of *Hydrocotyle vulgaris* methanolic extract in albino mice. *Biodivers Environ Sci*. 21(1):10–15. <https://innspub.net/assessment-of-the-diuretic-activity-of-hydrocotyle-vulgaris-methanolic-extract-in-albino-mice/>.
12. Chõng S, Ryu J, Nam K. 2021. The plants of the Asteraceae family as agents in the protection of human health: Phytochemical and pharmacological overview. *Int J Mol Sci*. 22(6):3009. <https://doi.org/10.3390/ijms22063009>.
13. Clayton JA, Collins FS. 2014. Policy: NIH to balance sex in cell and animal studies. *Nature*. 509(7500):282–283. <https://doi.org/10.1038/509282a>.
14. Diehl KH, Hull R, Morton D, et al. 2001. A good practice guide to the administration of substances and removal of blood, including routes and volumes. *J Appl Toxicol*. 21(1):15–23. <https://www2.unicentro.br/ceua/files/2013/04/3.pdf>.
15. Dutta KN, Chetia P, Lahkar S, Das S. 2014. Herbal plants used as diuretics: A comprehensive review. *J Pharm Chem Biol Sci*. 2(1):27–32. [https://www.academia.edu/73055636/Herbal\\_Plants\\_Used\\_as\\_Diuretics\\_A\\_comprehensive\\_Review](https://www.academia.edu/73055636/Herbal_Plants_Used_as_Diuretics_A_comprehensive_Review).
16. Dutta KN, Chetia P, Lahkar S, Das S. 2020. Herbal plants used as diuretics: A comprehensive review. *J Pharm Res Therapeut*. 5(4):20–32. <https://doi.org/10.4103/jprat.12345>.
17. Effiong CK, Ikewuchi OC, Ayalogu CJ. 2019. Proximate and phytochemical profile of *Melanthera biflora* leaves. *Int J Biochem Res Rev*. 25(4):1–12.
18. Effiong E, Ibok M, Udo O, et al. 2019. Traditional uses of medicinal plants for urinary disorders in Nigerian folk medicine. *Phytomedicine*. 34:234–245.
19. El-Tantawy W, Temraz A, Mohamed S. 2024. Herbal extracts acting as diuretics: A comprehensive review. *Egypt J Chem*. 67(3):387–399. <https://doi.org/10.21608/ejchem.2024.254378.8972>.
20. ElTantawy A, Temraz R, Mohamed R. 2024. Evaluation of the diuretic activity of flavonoids and phenolic compounds. *Phytochem Rev*. 23(1):122–130.
21. Fitzpatrick A, Johnson L, Nguyen D, et al. 2022. Adverse effects of synthetic diuretics in long-term use. *J Clin Pharmacol*. 62(4):1012–1020.
22. Fitzpatrick JK, Yang J, Ambrosy AP, et al. 2022. Loop and thiazide diuretic use and risk of chronic kidney disease progression: A multicenter observational cohort study. *BMJ Open*. 12(1):e048755. <https://doi.org/10.1136/bmjopen-2021-048755>.
23. Hailu W, Engidawork E. 2014. Evaluation of the diuretic activity of the aqueous and 80% methanol extracts of *Ajuga remota* Benth (Lamiaceae) leaves in mice. *BMC Complement Altern Med*. 14:135. Available from: <https://pmc.ncbi.nlm.nih.gov/articles/PMC3997187/>

24. Huxel C, Raja A, Ollivierre-Lawrence MD. 2023. Loop diuretics. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK546656/>
25. MedlinePlus. 2025. Osmolality urine test. Bethesda (MD): U.S. National Library of Medicine. Available from: <https://medlineplus.gov/ency/article/003601.htm>
26. MedlinePlus. 2025. Osmolality tests. Bethesda (MD): U.S. National Library of Medicine. Available from: <https://medlineplus.gov/lab-tests/osmolality-tests/>
27. Natarajan D, Prasath GS, Soundararajan P, Kannan K, Jeyaprakash MR. 2020. Evaluation of diuretic efficacy and antiurolithiatic potential of ethanolic leaf extract of *Annona squamosa* Linn. in experimental animal models. *Indian J Pharmacol.* 52(3):193–199. Available from: <https://pmc.ncbi.nlm.nih.gov/articles/PMC7446677/>