In vivo Antidiarrheal and Molecular Docking Studies of a Coumarin from the Root Extract of *Impatiens ethiopica* Grey-Wilson (Balsaminaceae)

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This is to certify that the thesis prepared by Sileshi Yimer, entitled **''*In vivo* Antidiarrheal and Molecular Docking Studies of a Coumarin from the Root Extract of *Impatiens ethiopica* Grey-Wilson (Balsaminaceae)''** and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Medicinal Chemistry complies with the regulations of the University and meets the accepted standards concerning originality and quality.

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Abstract

In vivo Antidiarrheal and Molecular Docking Studies of a Coumarin from the Root Extract of _Impatiens ethiopica_ Grey-Wilson (Balsaminaceae)

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Addis Ababa University, 2023

Diarrhea continues to be a major health problem worldwide, particularly in developing countries like Ethiopia. In Ethiopia, as in many other developing nations, traditional medicine plays a significant role in healthcare, with people relying on the therapeutic properties of plants to treat various infectious diseases. One such plant is *Impatiens ethiopica*, a member of the *Impatiens* genus traditionally used for the treatment of diarrhea. Thus, the present study was aimed to investigate the potential antidiarrheal effects of 80% hydroalcoholic root extract of *I. ethiopica* and its major constituents using both *in vivo* models integrated with molecular docking studies. Three experimental models, such as intestinal transit, intestinal secretion and castor oil-induced mice models, were employed to determine the effects of the root extract, fractions, and the isolated compound. The findings of the study revealed that the root extract significantly reduced the frequency of defecation, the weight of feces, and the onset of diarrhea at 100 mg/kg, 200 mg/kg, and 400 mg/kg. Additionally, the root extract exhibited significant reduction in intestinal fluid accumulation and gastrointestinal (GI) motility. The active root extract was then divided into four fractions based on solubility-based fractionations, utilizing successively petroleum ether, chloroform, methanol, and water solubility as the basis for separation. Of these fractions, the methanol and water fractions demonstrated a dose-dependent antidiarrheal effect, with the methanol fraction exhibiting the highest activity at a dose of 400 mg/kg. Further fractionation of

the active methanol fraction by normal-phase column chromatography led to the isolation a coumarin, coded as SY-1. The structure of SY-1 was identified as 7-hydroxy-6-methoxycoumarin (7-hydroxy-6-methoxy-2*H*-chromen-2-one), commonly known as scopoletin, on the basis of various spectroscopic techniques (ESI-MS, ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectral data). The antidiarrheal effects of scopoletin were also evaluated, demonstrating a significant reduction in intestinal fluid accumulation and gastrointestinal (GI) motility at the doses of 10 mg/kg, 20 mg/kg and 40 mg/kg. In this study, prostaglandin synthase (PGS) was chosen as a target for molecular docking analysis. Assessing scopoletin interaction with prostaglandin synthase (PGS) (COX-2; PDB ID: 4COX) revealed a promising docking score of −7.941 kcal/mol. Scopoletin demonstrated a strong binding affinity, engaging in hydrogen bonding with SER530 and pi-pi stacking interactions with TYR385 and TRP387, at specific COX-2 binding sites. These findings highlight that the antidiarrheal-like activity of *I. ethiopica* was partly attributed due to the presence of scopoletin. Overall, these results provide validation for the traditional use of *I. ethiopica* roots in the treatment of diarrhea and highlight the potential of scopoletin as antidiarrheal compound.

Keywords: *In vivo* Antidiarrheal, *Impatiens ethiopica*, coumarin, castor oil, intestinal secretion, gastrointestinal motility, molecular docking, 7-hydroxy-6-methoxycoumarin, scopoletin

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List of Abbreviations and Acronyms

^{13}C NMR	Carbon-13 nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
ADI	Antidiarrheal index
ADMET	Absorption, Distribution, Metabolism, Excretion, Toxicity
ANOVA	Analysis of variance
BMNP	Bale Mountains National Park
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
DEPT	Distortionless Enhancement by Polarization Transfer
ESI-MS	Electrospray ionization mass spectrum
FOP	Fecal out put
FT-NMR	Fourier transforms nuclear magnetic resonance
GI	Gastro intestinal
Il	Interleukin
iNOS	Inducible Nitric Oxide Synthase
LD_{50}	Lethal dose kills to 50% of exposed mice
MHz	Megahertz
MS	Mass Spectrometry
OECD	Organization for Economic Cooperation and Development
ORS	Oral Rehydration Solution
ORT	Oral Rehydration Therapy

PDB	Protein Data Bank
PGS	Prostaglandin synthase
Rf	Retardation factor
SAR	Structure activity relationship
SEM	Standard Error of Mean
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TNF-α	Tumor necrosis factor alpha
UV	Ultraviolet
WHO	World Health Organization

1 Introduction

1.1 Diarrhea Overview

Diarrhea, a widespread health concern globally, poses a significant threat, particularly to children under five years old, leading to an estimated 499,000 deaths and 688 million morbidities worldwide (Mady *et al.*, 2023; Worku *et al.*, 2023; Wolde *et al.*, 2022). Notably, sub-Saharan African and South Asian countries account for 90% of these fatalities (Worku *et al*., 2023). Ethiopia, like many sub-Saharan African countries suffers from diarrhea as a primary cause of morbidity and mortality, claiming approximately 13% of lives and affecting nearly 22% of children under five (Worku *et al.*, 2023). The 2015 Global Burden of Diarrheal Disease (GBD) report identifies malnutrition in childhood, inadequate access to clean drinking water and sanitation, and insufficient breastfeeding as significant risk factors in these regions (Abdoli and Maspi, 2018; Ugboko *et al.*, 2020).

1.2 Symptoms, Causes and Classifications

Diarrhea presents through pathological manifestations, including increased bowel movements (three or more bowel movements in a 24-hour period), elevated fecal fluidity, and potential presence of blood and mucus, resulting in electrolyte and water imbalance (Da Costa *et al.*, 2020; Araújo *et al.*, 2020). Various pathogens are identified as causative agents, including bacteria such as *Enterobacteriaceae,* enterotoxin-producing bacteria (*Vibriocholerae* and *Escherichia coli), Salmonella* species*, Shigella, Clostridium difficile, Yersinia pestis* and *Campylobacter)*), viruses (such as *rotavirus, norovirus*), parasites (including *Entamoeba histolytica*, *Giardia lamblia* and *Cryptosporidium*) (Sharma *et al.*, 2019; Araújo *et al*., 2020; Kotloff *et al.*, 2017;

Webb and Cabada, 2018; Khan *et al.*, 2023), and helminthes (such as *Ascaris lumbricoides*, *Hymenolepis nana*, *Taenia* spp., and *Trichuris trichiura*) (Gasparinho *et al.*, 2016).

Diarrhea, classified by duration as acute diarrhea (lasting less than 15 days), persistent (15 to 28 days) or chronic (lasting more than a month) has diverse etiologies: acute cases mainly stem from enteric pathogens (Mekonnen *et al.*, 2018; Adela Alemu *et al.*, 2022), while chronic are linked to bowel disorders and malabsorption syndromes (Mekonnen *et al.*, 2018; Worku *et al.*, 2023). The most common way for infectious diarrhea to spread is through fecal-oral transmission, mostly from contaminated food or drinks (Ayele *et al.*, 2023; Ugboko *et al*., 2020; Rahman *et al.*, 2015).

1.3 Treatment

Treatment for sudden onset diarrhea often requires minimal intervention (Diniz-Santos *et al.*, 2006; Ahmad *et al.*, 2021). However, severe cases may necessitate zinc supplementation, oral rehydration therapy (ORT), symptomatic management using anti-motility drugs (such as diphenoxylate, loperamide, and diloxanide, racecadotril, and atropine sulphate) and antibiotics, despite the latter's side effects (Ahmad *et al.*, 2021). Acute infectious diarrhea can be treated empirically with antibiotics to mitigate the progression of disease and reduce the severity of associated symptoms, such as fever, abdominal pain and vomiting (Diniz-Santos *et al.*, 2006). However, an increased antibiotics resistance posed challenges (Ahmad *et al.*, 2021; Diniz-Santos *et al.*, 2006)

In response to these challenges, exploring novel, diverse antidiarrheal drugs with fewer side effects becomes crucial (Abdela, 2019).

Traditional medicine offers potential solutions globally, with various herbal remedies like *Amaranthus caudatus, Coffea arabica, Balanites rotundifolia, Boscia coriacea, Cissampelos pareira, Plumbago zeylanica, Solanum hastifolium, Berberis crataegina, Cornusmas, Ecballiumelaterium, Mentha longifolia, Rhamnus cathartica, and Teucrium polium* claimed to effectively treat diarrhea (Ahmad *et al*., 2021). In Ethiopia, numerous plant families are utilized to treat diarrheal diseases, including *Malvaceae, Fabaceae, Amaranthaceae, Lamiaceae, Asteraceae, Araceae, Compositae*, *Rubiaceae*, *Zingiberaceae* (Degu *et al.*, 2022). Of these plant families, leaves are predominately used plant parts, followed by the roots, fruits, seeds, and barks, reflecting the importance of herbal medicine in managing diarrhea (Degu *et al*., 2022). Recognizing this potential, the World Health Organization (WHO) advocates for herbal medicine-based research to combat diarrheal infections (Abdela, 2019).

Specifically, some members of the genus *Impatiens,* has been traditionally used for diarrhea treatment (Singh *et al.,* 2017). Thus, the present study was aimed to investigate the potential antidiarrheal effects of compound(s) from the root extracts of *Impatiens ethiopica* using both *in vivo* model and in molecular docking studies.

1.4 The genus *Impatiens* L

The genus *Impatiens* is perennial, upright herbs with succulent-fleshy stems plants. Five key regions for diversity are identified in this genus: tropical Africa, Madagascar, Southern India and Sri Lanka, the Eastern Himalaya, and Southeast Asia (Jyosna *et al.*, 2011; Song *et al.*, 2021). They often have medium-sized, simple foliage and lovely flowers in a variety of colors, including white, pink, yellow, and orange (Pires Jr *et al.*, 2021a; Pires Jr *et al.*, 2021b). The genus *Impatiens* has a large diversity of species, belongs to the family Balsaminaceae, which is further subdivided into about 500–1000 species (Song *et al*., 2021; Pires Jr *et al*., 2021b; Szewczyk *et al*., 2016). The genus height ranges from 0.4 to 2.5 m, and they are typically found in tropical and subtropical woodlands, primarily along riverbanks. The leaves and petals are delicate and easily broken, despite having robust stems (Pires Jr *et al*., 2021b).

1.4.1 Ethnomedicinal uses

The genus *Impatiens* has a considerable importance in the Asian traditional medicine, particularly in countries such as China, India, Korea and Taiwan. The major traditional uses of the genus are depicted below in Table 1.

Table 1 Ethnomedicinal uses of the genus *Impatiens*

Scientific name	Plant part	Treatment	References
	Seeds	Suppress pain, expectorant, antidote for poisoning from fish, inflammation of the throat	(Singh *et al.*, 2017; Pires Jr *et al.*, 2021b)
	Aerial part	Articular rheumatism, beriberi, pain, swelling	(Singh *et al*., 2017)
I. balsamina	Flowers	Antipruritic, dermatitis, ant anaphylactic, antihistamine, antiplatelet activating factor	(Singh *et al.*, 2017)
	Herbs	Systemic bacterial and fungal infections, scrofulous, carbuncles, dysentery, fingernail inflammation	(Singh *et al.*, 2017)
I. textori	Whole plant	Detoxication, carbuncle, reduce blood pressure, inflammation	(Singh *et al.*, 2017)
I. emirnensis Bak	Aerial parts	Antimalarial remedy in Madagascar	(Singh *et al.*, 2017)
I. pritzellii	Rhizomes	Rheumatoid arthritis, diarrhea, acute abdominal pain	(Singh *et al.*, 2017)
I. parviflora	Herbs	Rheumatism, fractures, infections, anti-cancer herb	(Singh *et al.*, 2017)
I. tinctoria	Stem	Antifungal, wound healing, abdominal pains	(Gidamo, 2023)

Note: *I.: Impatiens*.

1.4.2 Pharmacological activities

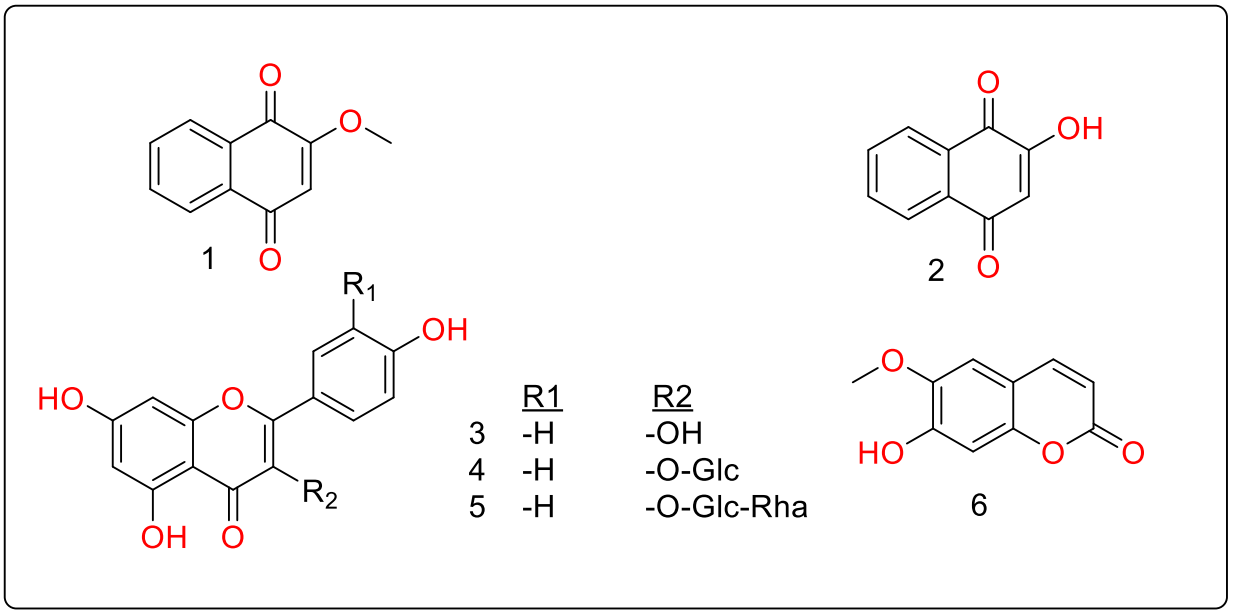
The pharmacological activities of the genus *Impatiens* are antimicrobial, antifungal, anti-inflammatory, antioxidant, antirheumatic, antipruritic, antiallergic, antitumor, antidermatitis, antiplatelet, antineurodegenerative, cytotoxic, antianxiety, antihepatic, antidiabetic and antinociceptive (Pires Jr *et al*., 2021b).

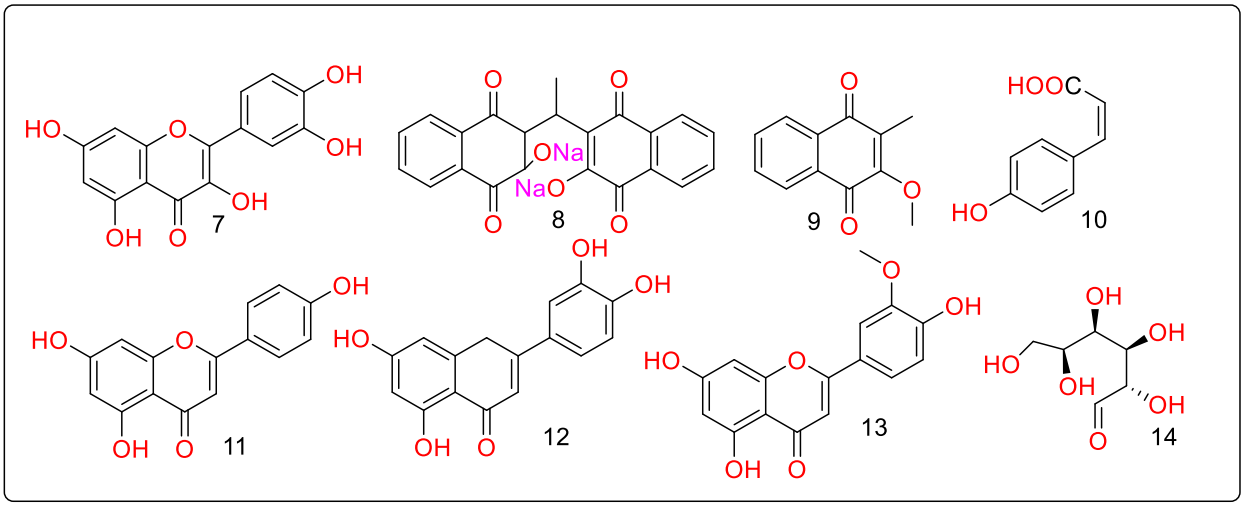
1.4.3 Phytochemistry

About 300 different compounds isolated from different member species of the genus *Impatiens* (Pires Jr *et al*., 2021b). Phenolic acids, flavonoids, coumarins, quinones, anthocyanins, tannins, saponins, phytosteroids and alkaloids are the most common (Szewczyk, 2018; Szewczyk *et al*., 2016) Table 2). Polyphenols are the main components in *Impatiens* taxa *(Pires Jr et al*., 2021a). Amongst the studied species, the most prevalent class of compounds are flavonoids (Szewczyk, 2018).

Table 2 Some common secondary metabolites of the genus *Impatiens*

Name of compounds	**Source**	**Structure**	**References**
2-methoxy-l,4-naphthoquinone	*I. balsamina*	1	(Singh *et al*., 2017)
Lawsone (2-hydroxy-1,4-naphthoquinone)	*I. balsamina*	2	(Singh *et al*., 2017)
Kaempferol	*I. balsamina*	3	(Singh *et al*., 2017)
kaemferol-3-O-glucoside	*I. balsamina*	4	(Singh *et al*., 2017)
Kaempferol-3-O-rutinoside	*I. balsamina*	5	(Singh *et al*., 2017)
7-hydroxy-6-methoxycoumarin(scopoletin)	*I. balsamina*	6	(Singh *et al*., 2017)
Quercetin	*I. balsamina*	7	(Singh *et al*., 2017)
Impatineol	*I. balsamina*	8	(Singh *et al*., 2017)
2-hydroxy-3-methoxy-1,4-aphthoquinone	*I. balsamina*	9	(Singh *et al*., 2017)
p-coumaric acid	*I. balsamina*	10	(Singh *et al*., 2017)
Apigenin	*I. textori*	11	(Singh *et al*., 2017)
Luteolin	*I. textori*	12	(Singh *et al*., 2017)
Chrysoeriol	*I. textori*	13	(Singh *et al*., 2017)
Gossypetin7-O-β-D-glucopyranoside, D-glucose	*I. scabrida*	14	(Singh *et al*., 2017)
1,2,4-trihydroxy napthalene-1-O-glucoside	*I. glandulifera*	15	(Singh *et al*., 2017)
Methyl-4-hydroxyl cinnamat	*I. bicolar*	16	(Singh *et al*., 2017)
Stigmasterol	*I. bicolar*	17	(Singh *et al*., 2017)
kaempferol 3-O-β-D-galactopyranoside	*I. bicolar*	18	(Singh *et al*., 2017)
Kaempferol-7-O-β-D-xylopyranoside	*I. bicolar*	19	(Singh *et al*., 2017)
Echinocystic acid	*I.pritzellii*	20	(Singh *et al*., 2017)


1
2
6
R1
R2
3 -H -OH
4 -H -O-Glc
5 -H -O-Glc-Rha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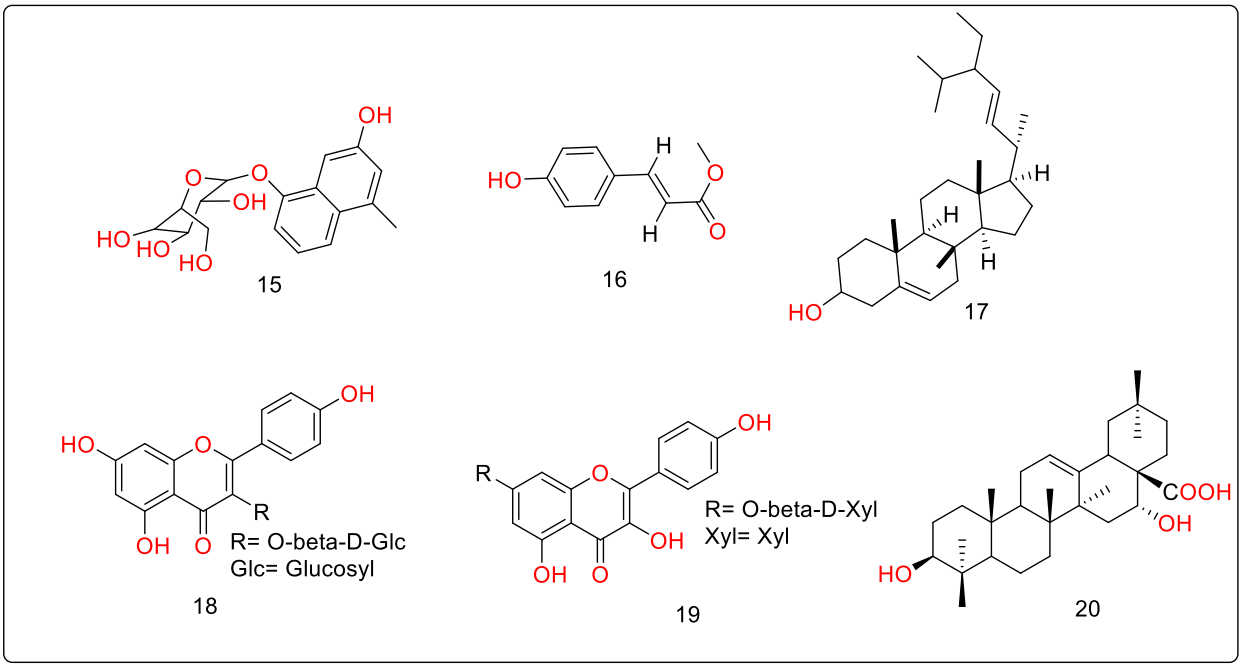


1.5 *Impatiens ethiopica* Grey-Wilson

Impatiens ethiopica Grey-Wilson, also known by its synonym *Impatiens filicornu*, is a perennial herb reaching to a height of 1.2 meters on an upright stem. It is succulent, frequently reddish-purple, leafless, and roots at the lower nodes. Leaves: 8.2 cm long, ovate, ovate-oblong, lanceolate or elliptic, glabrous to lightly pubescent, sometimes thick, dark green above, paler beneath, spirally arranged, seldom sub-opposite. Its base, which can reach a maximum length of 3.8 cm, is cuneate. The edges range from crenate to serrate, and the lower teeth occasionally resemble extensions of hair. The apex is acute-acuminate. Flowers are in sub-umbellate racemes of pink, lilac, magenta, or white; peduncle 1.5–10 cm long, thinly pubescent or glabrous (Figure 1). The plant thrives in areas that range in altitude from 1150 to 3200 meters, including shaded and moist areas in montane forests and ravines, river or stream banks, the spray zone of waterfalls, and occasionally in swamps or marshes and wet areas alongside roads (Edwards *et al.*, 2000). It grows in Ethiopia (Edwards *et al.*, 2000) particularly, the wetland areas of the

Bonche and Duda forests which are found in Gera Wereda (Moges *et al.*, 2017) and South West Ethiopia around Bebeka lowland rain forests (Friis *et al.*, 1987), and it is also found in South East Sudan on the Imatong Mountains (Friis *et al*., 1987; Edwards *et al.*, 2000). In Ethiopia, it is known by its vernacular names as 'እንሶስላ' ensosla, insosla, ensosila (Amharic) (Edwards *et al.*, 2000) and 'anshoshilla' (Afan Oromo) (Yineger *et al.*, 2008).

A B C

Figure 1 (A) Photograph of the *Impatiens ethiopica* Grey-Wilson. (B) Photograph of the root part of *Impatiens ethiopica* Grey-Wilson. (C) Crushed form of the root part. (Photograph was taken in June, 2022 by Sileshi Yimer at Gulale Botanical Garden, Addis Ababa, Ethiopia).

1.5.1 Ethnomedicinal uses

The *Impatiens ethiopica* Grey-Wilson is commonly used to treat gonorrhea, a sexually transmitted disease, in the districts of Adaba, Sinana, Dinsho, and Goba surrounding the Bale Mountains National Park (BMNP). The plant, locally referred to as "Anshoshila" (Afan Oromo), is used to cure damaged body parts with an ointment prepared from the crushed fresh roots of the plant (Yineger *et al*., 2008; Yineger, 2005).

1.6 Statement of the problem

Diarrhea remains a global health concern, causing significant morbidities and fatalities (Worku *et al.*, 2023; Wolde *et al.*, 2022), with Sub-Saharan Africa and South Asian bearing a disproportionate burden (Worku *et al*., 2023). In Ethiopia, it stands as a major causes of illness and mortality, particularly claiming approximately 13% of lives and impacting children under five years old, with an incidence rate of 22% (Worku *et al*., 2023). Beyond fatalities, diarrhea leads to severe acute malnutrition (Abate *et al.*, 2019), cognitive decline, hampers physical and scholastic development (Wolde *et al*., 2022), often linked to poor sanitation practices and contaminated water sources (Alebel *et al.*, 2018; Wolde *et al.*, 2022; Melese *et al.*, 2019).

Compounding this issue is the emergence of antibiotic-resistance bacteria strains like multidrug resistant Shigella species (one of the most causes of diarrhea), exacerbating the challenge of treating severe cases (Ugboko *et al.*, 2020). Currently therapeutic interventions for severe cases involve zinc supplementation and oral rehydration treatment (ORT) to manage excessive fluid loss and electrolyte imbalance. However, conventional drugs used to alleviate symptoms, like loperamide, difenoxin, and diphenoxylate, often entail side effects and potential drug interactions (Worku *et al*., 2023), particularly diphenoxylate risky in children due to respiratory and central nervous system complications (Casburn-Jones and Farthing, 2004; Palmer *et al.*, 1980).

Given these concerns, there is an urgent need for alternative treatment options, including exploring medicinal plants. *Impatiens* species have been well documented to possess a wide array of medicinal properties, like antimicrobial, anthelminthic, antifungal, anti-inflammatory, antioxidant, anti-rheumatic, anti-pruritic, anti-allergic, anti-tumor and anti-dermatitis activities (Singh *et al.*, 2017). Thus, this study aimed to assess the antidiarrheal potential of compound(s) isolated from the root extract of *Impatiens ethiopica*, using mice models and molecular docking

studies. This investigation seeks to contribute to identifying new, safer treatments for diarrhea, addressing the limitations associated with the current therapeutic approaches.

1.7 Significance of the study

The current investigation holds the potential to confirm the long-held belief in the effectiveness of some member species of the genus *impatiens* as a treatment for diarrhea. Through the isolation of 7-hydroxy-6-methoxycoumarin, an active compound derived from this plant, there's an exciting opportunity to pave the way for future research seeking potent antidiarrheal agents from natural sources.

7-hydroxy-6-methoxycoumarin, identified within *I. ethiopica*, emerges as a promising foundation for exploring and developing new treatments for diarrhea. The significance lies in its potential to serve as a template or starting point for the creation of novel antidiarrheal medications sourced from plants.

Furthermore, this pursuit is especially crucial given the limitations associated with current antidiarrheal drugs, which often come with side effects. By considering natural compounds like 7-hydroxy-6-methoxycoumarin, there's a chance to discover alternatives that may not carry these negative reactions, potentially offering safer and more effective treatments for managing diarrhea.

2 Objectives

2.1 General objectives

- To investigate the potential antidiarrheal effects of compound(s) from the root extracts of *Impatiens ethiopica* against experimentally induced diarrhea in mice models, alongside a molecular docking investigation.

2.2 Specific objectives

- To assess the acute oral toxicity of the 80% hydroalcoholic root extract of *I. ethiopica*;
- To evaluate *in vivo* antidiarrheal activity of the 80% hydroalcoholic root extract and its fractions of *I. ethiopica* against experimentally induced diarrhea in mice;
- To isolate compound(s) from the root extract of *I. ethiopica*;
- To characterize the structure of the isolated compound(s);
- To determine *in vivo* antidiarrheal activities of the isolated compound;
- To assess the possible molecular interactions of the isolated compound(s) and its binding affinity via molecular docking with certain proteins relevant to diarrhea.

3 Materials and Methods

3.1 Materials

3.1.1 Plant materials

The roots of *I. ethiopica* were collected from Gulale Botanical Garden, Addis Ababa, Ethiopia in June 2022. The plant was authenticated by Mr. Melaku Wondafrash, and a sample (collection number as SY-001) was stored at the National Herbarium, Department of Plant Biology and Biodiversity Management, College of Natural and Computational Sciences, Addis Ababa University (AAU) for future reference.

3.1.2 Chemicals, reagents and drug

The chemicals and reagents used in this experiment include castor oil (Amman Pharmaceutical Industries, Jordan), loperamide (Medochemie Ltd, Limassol Cyprus), activated charcoal (Acuro Organics Ltd, New Delhi), methanol (Reagent Chemical Limited, UK), chloroform (Finkem Laboratory Reagent, India), distilled water and petroleum ether. There was no further purification necessary because all of the chemicals and reagents employed were of analytical grade.

3.1.3 Instruments

Rota vapors (Heidolph Instruments GmbH and Co., Germany), UV spectroscopy (Shimadzu Spectrophotometer MultiSpec-1501, Japan), Nuclear magnetic resonance spectrometer JNM-ECS400 NMR spectrometer (JEOL Ltd., Akishima, Tokyo, Japan), Electrospray ionization mass spectrometer (ESI-MS) (JEOL Ltd., Akishima, Tokyo, Japan).

3.1.4 Experimental mice

Swiss albino mice aged 6 - 8 weeks and weighing 22 - 30 g were employed throughout this experimental activity. The mice were obtained from the animal house of the Department of Clinical Pharmacy and Pharmacology, School of Pharmacy, College of Health Science, Addis Ababa University. The mice were housed in cages and maintained under standard conditions (room temperature and humidity, 12-hour light and dark cycle). Standard pelletized food and tap water were provided ad libitum. Experimental activities were conducted in line with the internationally accepted laboratory animal use and care guideline (Rajput *et al.*, 2011) and were approved by the Institutional Review Board of the School of Pharmacy, AAU (approval code: ERB/SOP/559/16/2023) (attached in annex).

3.2 Methods

3.2.1 Extraction

The roots of *I. ethiopica* were meticulously cleaned, dried under shade, and crushed into a coarse powder using a mortar and pestle. The powdered root (500 g) underwent maceration with 80% hydroalcoholic (3 L) at room temperature for three days, with regular shaking and stirring. After 72 h, the extract was filtered using Whatman No. 1 filter paper. The marc was subjected to another three days of maceration with the same amount of solvent. The filtrates were combined and concentrated using a Rota Evaporator (Heidolph Instruments GmbH and Co., Germany) at a controlled temperature not exceeding 40 °C. The resulting concentrated filtrate, in an aqueous solution, was dried at 40 °C in an oven. The final dried hydroalcoholic extract was transferred into an amber-colored vial, weighed and kept in a refrigerator at 4 °C for further use.

3.2.2 Solubility-based fractionation

The extract was sequentially subjected to solubility-based fractionation, with petroleum ether, chloroform, methanol, and water, to yield four fractions. Initially, the extract (30 g) was sonicated in petroleum ether (150 ml); the filtrated petroleum ether solution was labeled as fraction-1. The residue underwent a similar process with chloroform (100 ml) to yield fraction-2. The subsequent step involved sonication in methanol (150 ml), resulting in fraction-3. The remaining residue underwent sonication in distilled water (150 ml) to yield fraction-4. Each fraction was dried in a vacuum oven at 40 °C, transferred into an amber-colored vial, weighed and kept in a refrigerator at 4 °C for further use.

3.2.3 Isolation of compound by column chromatography

A column was initially packed with silica gel-chloroform slurry. The methanol-soluble part (fraction-3, 2 g) was adsorbed onto silica gel (2 g) in MeOH. After drying with a Rotary evaporator, the adsorbed sample was loaded onto the column and eluted with varying $CHCl_3$ and MeOH gradients. A total of 120 fractions, each 10 ml, were collected. Specifically, fractions 37-45, eluted with $CHCl_3$ and MeOH (99:1), showed a single spot-on normal phase TLC in different solvent systems. These fractions were combined to yield SY-1, a light-yellow needle-like crystalline substance (37 mg). SY-1 was transferred into an amber-colored vial, weighed and stored in a refrigerator at 4 °C for further use.

3.2.4 Spectroscopic techniques

Nuclear Magnetic Resonance (NMR) spectra were recorded using a JNM-ECS400 NMR spectrometer (JEOL Ltd., Akishima, Tokyo, Japan) operating at 400 MHz (^{1}H) and 100 MHz (^{13}C) at room temperature. Tetramethylsilane served as an internal standard, and deteriorated chloroform was used as the solvent. The ^{1}H-NMR scanning range was 0-15 ppm, and for ^{13}C-

NMR, it was 0-205 ppm. Chemical shifts (δ) were expressed in parts per million (ppm), and coupling constants (J) in Hz. Signal multiplicities in ^{1}H-NMR signals were indicated as *s* (singlet), and *d* (doublet). The negative-mode ESI-mass spectrum was used to determine the mass.

3.2.5 Acute oral toxicity

Acute oral toxicity tests of the extract was conducted according to Organization for Economic Cooperation and Development (OECD) guideline 425 (425, 2008). A fasting mouse was given a limit dose of 2000 mg/kg on the first day, observed for any signs of toxicity in the 1st hour, and periodically for 24 h. If the mouse survived, then the same doses were administered to four more mice; thus, five mice were used for each test substance. Then, the animals were followed up for the next fourteen days.

3.2.6 Experimental design

3.2.6.1 Grouping and dosing

This study used three different antidiarrheal mice models namely: enteropooling, gastrointestinal motility, and castor oil induced diarrhea models. Mice of both sexes were randomly assigned to eight groups (negative control, positive control, and six test groups) in all models. Each group consisted of five animals (Khan *et al*., 2023). Negative controls received vehicle (10 ml/kg, distilled water) and positive controls received loperamide (3 mg/kg) in all models. The treatment groups (group III, IV and V) received different doses (100, 200 and 400 mg/kg, respectively) of the extract and fractions and (group VI, VII and VIII) received (10, 20 and 40 mg/kg, respectively) of the isolated compound (SY-1) orally which were determined based on the acute oral toxicity test guideline.

3.2.7 Determination of antidiarrheal activity

3.2.7.1 Castor oil-induced diarrhea

The method described by (Umer *et al.,* 2013) was followed for this study. Swiss albino mice of either sex were fasted for 18 h with free access to water and randomly allocated to eight groups of five animals each. Vehicle treated group received distilled water (10 ml/kg) (negative control); Group II (positive control) received the standard drug loperamide 3 mg/kg, orally. Animals in groups III, IV and V received the extract or fractions at doses of 100, 200 and 400 mg/kg, respectively and group VI, VII and VIII received (10, 20 and 40 mg/kg, respectively) of the isolated compound (SY-1) orally. One hour after treatment, diarrhea was induced by oral administration of 0.5 ml castor oil to each mouse. The animals were then housed in a separate transparent cage in which the floor is lined with white paper. The paper was changed every hour for a total of four hours. During the observational period, the onset of diarrhea, number and weight of wet stools, total number and the total weight of fecal output were recorded. Finally, the percentage of fecal output (% FOP) and diarrheal inhibition (% inhibition of defecation) were calculated by using the formulas described below.

$$\text{Percentage of fecal output (\% FOP)} = \frac{\text{Mean faecal weight of each treatment group}}{\text{Mean faecal weight of negative control}} \times 100$$

$$\text{Percentage inhibition of defecation} = \frac{\text{MO}-\text{M}}{\text{MO}} \times 100$$

Where, Mo is mean defecation of negative control and M stands for mean defecation of test sample (standard drug).

3.2.7.2 Gastrointestinal motility test by charcoal marker

In this model, mice were abstained from food 18 h with free access to water and grouped and treated as described under grouping and dosing section earlier. One hour post treatment each mouse received 0.5 ml of castor oil and, after 1 h of castor oil administration, they received 1 ml of 5% activated charcoal suspension. The animals were then sacrificed by cervical dislocation after 30 min of administering activated charcoal and the entire length of the intestine (from the pylorus to the cecum) was removed and placed lengthwise on a white paper. The distance travelled by the charcoal meal and the total length of the intestine was then measured using a calibrated ruler. The distance travelled by the charcoal meal relative to the total distance of the small intestine was expressed as a peristaltic index (PI). The peristaltic index (PI) and percentage of inhibition were calculated by using the following formula (Meite *et al.*, 2009; Ahmad *et al.*, 2021).

$$\text{Peristaltic index (PI)} = \frac{\text{Mean distance travelled by charcoal meal}}{\text{Mean length of small intestine}} \times 100$$

$$\text{Percentage inhibition} = \frac{\text{Intestinal transit by charcoal meal}(-\text{ve control} - \text{treated})}{\text{Intestinal transit by charcoal meal in the} - \text{ve control groug}} \times 100$$

3.2.7.3 Anti-enteropooling effect

Intraluminal fluid accumulation was determined using the method described by (Degu *et al.*, 2016). Animals of either sex were devoid of food for 18 h and grouped and treated, as described under grouping and dosing. After 1h, 0.5 ml of castor oil was administered orally. One hour later, the mice were sacrificed by cervical dislocation. The abdomen of each mouse was opened and the whole length of the intestine, from the pylorus to the caecum, was ligated, dissected and carefully removed. The small intestines were weighed and the intestinal contents were collected by milking into a graduated tube to measure the volume. The empty intestines were reweighed

and the difference between the two weights was calculated. Finally, the percentage of reduction of intestinal secretion and weight of intestinal contents was determined by using the following formulas (Degu *et al.*, 2016).

$$\text{Mean percentage inhibition} = \frac{\text{MVICC}-\text{MVICT}}{\text{MVICC}} \text{ x } 100$$

Where MVICC is the mean volume of the intestinal content of the control group while MVICT is the mean volume of the intestinal content of the test group

$$\text{Percentage inhibition by using MWSIC} = \frac{C-T}{C} \text{ x } 100$$

Where MWSIC is mean weight of small intestinal content while C is the mean weight of intestinal content of the control and T is the mean weight of intestine content of the test (drug group).

3.2.7.4 *In vivo* antidiarrheal index (ADI)

Calculations were made for the delay in onset of diarrhea and purging index by comparing with control group. The *in vivo* antidiarrheal index (ADI) was then expressed according to the formula (Degu *et al.*, 2016; Ayalew *et al.*, 2022) described below;

$$\textit{In vivo} \text{ antidiarrheal index (ADI)} = \sqrt[3]{Dfreq\ x\ Gmeq\ x\ Pfreq}$$

Where Dfreq is delay in frequency defecation time or onset of diarrhea (in percent of control) obtained from castor oil induced diarrheal test which is expressed by the formula;

$$\text{Dfreq} = \frac{\text{Mean onset of diarrhea in the test group} - \text{Mean onset of diarrhea in the control group}}{\text{Mean onset of diarrhea in the control group}}\ x\ 100$$

Gmeq is gut meal travel reduction (in percent of control) obtained from charcoal meal test (% inhibition), and Pfreq is the purging frequency as number of wet stool reduction (in percent of control) obtained from castor oil diarrheal model (% inhibition of defecation).

3.2.8 Molecular docking experiment

Following early preliminary docking assessments on potential targets, molecular docking of SY-1 was implemented on the prostaglandin synthase (PGS) enzyme as a possible target for antidiarrheal in this investigation. Below is a summary of the procedures used.

3.2.8.1 Protein preparation:

The X-ray crystallography structure of *Mus musculus* prostaglandin synthase-2; cyclooxygenase-2 (COX-2) (PGS-2; PDB ID: 4COX; Chain A, B; resolution: 2.90 Å) complexed with indomethacin was downloaded from the protein data bank (www.rcsb.org). COX-2 structure consists of 604 amino acid chain sequence, complexed with indomethacin, 1-(p-chlorobenzoyl) 25-methoxy-2-methylindole-3-acetic acid (Pouplana *et al.*, 2002; Kurt *et al.*, 2022). Using Maestro V13.5 Protein Preparation Workflow module (Schrödinger 2023-1), the 3D structure of COX-2 protein in complex with indomethacin (PDBID: 4COX, chain AB) was prepared by standard procedures. This involved correcting charges, bond orders, and atom types, removing water molecules beyond 5 Å from the het group, and filling in missing side chains and loops. The OPLS4 force field was applied for energy optimization and steric hindrance removal (Madhavi Sastry *et al.*, 2013).

3.2.8.2 Ligand preparation

ChemDraw Ultra (2019) was employed to draw the structure of compound SY-1 in MDL SDfile format. Ligand preparation was carried out using the Ligprep module of Maestro V13.5,

Schrödinger Suite 2023-1, following to OPLS4 at a physiological pH of 7.0 ± 2.0. Different ionization states and stereoisomers for ligand structure were generated.

3.2.8.3 Receptor grid generation

We generated the receptor grid box to visualize the active site of the receptor for glide ligand docking. The ligand-binding site was defined by picking the proteins structure on the workspace, with a 6.0 Å radius binding site. The van der Waals radii of the receptor atoms was set with partial atomic charge scaling factor of 0.8 and partial cut-off of 0.15 to soften the receptor's nonpolar parts.

3.2.8.4 Ligand docking

Using Glide software in extra precision (XP) mode, we docked compound SY-1 into the active binding site of the prepared protein. Prior to this, we docked the co-crystallized ligand into the protein's active site to predict binding affinity and molecular interaction. Docking scores in kcal/mol were used to analyze the binding pose. All the analyses and procedures were performed using the Schrodinger suite of programs.

3.2.9 Data analysis

The statistical package for the social sciences (SPSS), version 20.0, was used to analyze the data, which were expressed as mean ± standard error of the mean (SEM). Difference between group means was analyzed with one way analysis of variance followed by Tukey post Hoc test for pairwise comparisons. A *p*-value of < 0.05 was taken as statistically significant. Molecular docking was studied by using Maestro, version 13.5 (Schrödinger 2023-1 release) software to determine drug-receptor interactions. The structures of the ligand were sketched using PerkinElmer informatics, Inc., ChemDraw professional version 19.00 software.

3.2.10 Ethical approval

Ethical approval was obtained from the Scientific and Ethics Committee of the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, Addis Ababa University with an approval code: ERB/SOP/559/16/2023.

4 Results and Discussion

4.1 Extraction yield

The maceration of dried *I. ethiopica* root (500 g) in 80% hydroalcoholic produced a dark brown sticky extract weighting 69 g, yielding 13.8% of the total weight. 80% hydroalcoholic solvent is a universal solvent due to its ability to extract a wider range of polarities, allowing for the extraction of both polar and non-polar phytochemicals, as evident by various studies (Mengesha *et al.*, 2022; Ayalew *et al.*, 2022; Birru *et al.*, 2016). Additionally, this choice of solvent was advantageous as it hinders the growth of potential microbes (Andargie *et al.*, 2022).

4.2 Acute oral toxicity

The acute oral toxicity test results of this finding revealed that 80% hydroalcoholic roots extract from *I. ethiopica* did not cause any mortality in mice within the first 24 h as well as for the subsequent 14 days, suggesting that the oral LD_{50} is greater than 2000 mg/kg.

4.3 *In vivo* antidiarrheal activities of extract and fractions

4.3.1 Effect on castor oil-induced diarrhea in mice

In this study, we induced diarrhea in mice using castor oil, known to trigger diarrhea through various mechanisms. Castor oil gets converted to ricinoleic acid in the gut, causing irritation, inflammation, and the release of inflammatory substances, like prostaglandins and histamine into the intestinal lining (Mengistu *et al.*, 2022; Mengesha *et al*., 2022). This process lead to muscle contraction, mucus secretion, and widening of blood vessels in the small intestines *(*Mengistu *et al.*, 2022; Mengesha *et al*., 2022). Ricinoleic acid binds to an enzyme called sodium potassium adenosine triphosphatase (Na+/K-ATPase), inhibiting its action. Consequently, this inhibits the reabsorption of sodium chloride and water (Ayele *et al.*, 2023; Yacob *et al.*, 2016; Rahman *et al.*,

2015). This inhibition triggers the release of nitric oxide, further aggravating diarrhea (Tadesse *et al.*, 2017). Loperamide, a common antidiarrheal drug, works by stimulating the μ-opioid receptors in the large intestine, reducing muscle movement and the release of acetylcholine. This action enhances electrolyte and water absorption while decreasing excretion (Abdela, 2019; Ayele *et al.*, 2023).

In this study, antidiarrheal effects of 80% hydroalcoholic root extract of *Impatiens ethiopica* and its fractions on castor oil-induced diarrhea in mice model is illustrated in Table 3. The results revealed that both the crude extract and its fractions significantly ($p < 0.05$) reduced the frequency, quantity, and the total weight of feces produced in mice with castor-oil-induced diarrhea in a dose-dependent manner. The 80% hydroalcoholic extract showed a significant ($p < 0.05$) antidiarrheal activity, with 75.60% of inhibition of defecation and a notably delaying onset of 98.80 min at 400 mg/kg. Interestingly, these findings were comparable to the positive control, loperamide (3 mg/kg), with a 78.49% of defecation inhibition and a 102.80 min of delayed onset. Mengistu *et al.* (2022) corroborate our findings. They found that 80% hydroalcoholic leaf extract of *Lantana camara* at 400 mg/kg inhibited defecation by 75.6%; $p<0.001$, while the positive control loperamide at 3 mg/kg shown 71.1% inhibition.

Similarly, after separating the crude extract into its fractions, the methanol and aqueous fractions emerged as the most potent, showing dose-dependent delays in diarrhea onset by 84.40 and 81.80 min, respectively, at 400 mg/kg dose. Equally, the methanol and aqueous fractions also reduced the number of wet feces output in a dose-dependent manner, with 75.60% and 70.03% percentage of inhibition of feces output, respectively, at a dose of 400 mg/kg. The results from the other fractions are presented in Table 3. These findings are also consistent with Mengesha *et*

al., (2022), where the methanol and aqueous fractions at 400 mg/kg inhibited percentage of defecation by 64.3%; $p<0.001$, and 52.9%; $p<0.05$ respectively.

Compounds from medicinal plants are known to inhibit prostaglandin synthesis, which might explain the antidiarrheal effects observed in this study. This inhibition potentially leads to increased absorption and reduced secretion of fluids and electrolytes (Bahekar and Kale, 2015; Kifle *et al.*, 2021)

Table 3 *In vivo* antidiarrheal effects of root extract and its fractions on castor oil-induced diarrhea model

Treatment types and dose	Onset of diarrhea in min	Total number of wet feces in 4 h	% Inhibition of defecation	Total number of feces output in 4 h	Total weight of wet feces (g) in 4 h	% Inhibition of feces output	Total weight of feces output (g) in 4 h
DW 10 ml/kg	33.60±6.39[a]	18.60±1.72	0[a]	18.60±1.72	0.82±0.18	0[a]	0.88±0.19
Lop. 3 mg/kg	102.80± 6.66[b]	4.00±0.31	78.49[b]	4.00±0.31	0.21±0.01	74.39[b]	0.24±0.03
Extract (mg/kg)							
IE 100	69.40±3.99[c]	6.60±0.50	64.51[c]	6.60±0.50	0.33±0.02	59.76[c]	0.37±0.03
IE 200	81.80±2.03[d]	4.80±0.66	74.19[d]	4.80±0.66	0.23±0.02	71.95[d]	0.30±0.04
IE 400	98.80±3.30[e]	4.60±0.50[a]	75.26[d]	4.60±0.50	0.24±0.03	70.73[d]	0.27±0.02
Fractions(mg/kg)							
PE 100	64.20±1.39[f]	8.00±0.32	56.98[e]	8.60±0.40	0.27±0.02	67.07[d]	0.27±0.02
PE 200	70.00±0.71[g]	6.00±0.32	67.74[e]	6.60±0.25	0.24±0.03	70.03[d]	0.25±0.01
PE 400	72.00±0.71[g]	4.60±0.40	75.26[f]	5.60±0.40	0.23±0.02	71.95[d]	0.25±0.02
CF 100	66.20±0.86[h]	7.00±0.32	62.36[e]	7.60±0.51	0.28±0.01	65.85[e]	0.29±0.02
CF 200	71.20±1.16[i]	6.00±0.45	67.74[e]	7.20±0.38	0.26±0.01	68.29[e]	0.28±0.01
CF 400	73.20±0.66[i]	5.00±0.32	73.11[d]	5.80±0.58	0.24±0.01	70.03[d]	0.25±0.01

MF 100	71.00±0.71^{j}	5.60±0.25	69.89^{e}	6.00±0.32	0.25±0.01	69.51^{e}	0.26±0.02
MF 200	75.00±0.71^{j}	4.40±0.25	76.34^{f}	6.20±0.38	0.24±0.02	70.03^{e}	0.27±0.03
MF 400	84.40±1.69^{b}	3.80±0.38	79.56^{b}	5.40±0.25	0.20±0.01	75.60^{b}	0.22±0.01
AF 100	71.00±0.71^{j}	7.00±0.45	62.36^{c}	7.80±0.58	0.26±0.01	68.29^{e}	0.28±0.03
AF 200	75.80±0.86^{j}	5.60±0.40	69.89^{c}	6.40±0.40	0.25±0.01	69.51^{e}	0.26±0.01
AF 400	81.80±1.02^{b}	5.20±0.37	72.04^{d}	6.20±0.49	0.24±0.01	70.03^{e}	0.26±0.03

All values are expressed as mean ± standard error of the mean (SEM); (n = 5); Data were analyzed by one-way ANOVA followed by Tukey post hoc test; NC: Negative control, Lop: Loperamide; IE100: *I. ethiopica* root extract (100 mg/kg), IE 200: *I. ethiopica* root extract (200 mg/kg), IE 400: *I. ethiopica* root extract (400 mg/kg), PE100: Petroleum ether fraction(100 mg/kg), PE200: Petroleum ether fraction (200 mg/kg), PE400: Petroleum ether fraction (400 mg/kg), CF100: Chloroform fraction (100 mg/kg), CF200: Chloroform fraction (200 mg/kg), CF400: Chloroform fraction (400 mg/kg), MF100: Methanol fraction (100 mg/kg), MF200: Methanol fraction (200 mg/kg), MF400: Methanol fraction (400 mg/kg), AF 100: Aqueous fraction (100 mg/kg), AF200: Aqueous fraction (200 mg/kg), AF 400: Aqueous fraction (400 mg/kg); Data followed by different letter within the same column are significantly different at $p < 0.05$.

4.3.2 Effect on castor oil-induced intestinal transit by charcoal marker

Since the majority of antidiarrheal drugs have the ability to reduce gastrointestinal motility, the charcoal meal method was selected (Tadesse *et al.*, 2017)in order to track the movement of the gastrointestinal contents during this experiment. Dose dependent inhibitory effect was observed in all types of tested doses. This effect is consistent with other reports which indicate the dose dependent antidiarrheal activity of extract and fractions of other medicinal plants (Tadesse *et al*., 2017; Feyisa *et al.*, 2020; Ayalew *et al.*, 2022). The root extracts inhibited intestinal motility by 53.89%, 60.79% and 75.88% for 100, 200, and 400 mg/kg respectively. The highest dose of the root extract (400 mg/kg) showed a significant anti-motility effect (75.88%; $p< 0.05$) as compared with 100 mg/kg of extract.

A delay in the motility of intestinal muscles and reductions of frequency of stooling promotes further absorptions of water from feces and reduces the watery nature of excrement. Based on this findings, it is likely that reductions in the propulsive movements may be attributed by the

anti-motility properties of the tested doses (Yacob *et al.*, 2016; Ayele *et al.*, 2023). This finding is further supported by the antimotility effect of the leaves of *L. camara* and, its component (Lanthadne-A) and fractions of the leaves of this plant (Tadesse *et al*., 2017).

Table 4 *In vivo* antidiarrheal effects of root extract and fractions on charcoal meal transit in castor oil-induced motility in mice model

Treatment	Dose (mg/kg)	Mean length of small intestine in cm	Mean distance travelled by charcoal marker in cm	% Charcoal meal transit (Peristalsis index)	% Inhibition
DW	10 ml/kg	50.20±2.05	45.20±2.27	90.03[a]	0
Lop.	3 mg/kg	56.60±0.97	4.80±0.51	8.49[b]	90.58
Crude extract					
IE	100	54.20±1.52	22.50±0.89	41.51[c]	53.89
IE	200	52.40±1.29	18.50±0.55	35.30[d]	60.79
IE	400	57.10±0.89	12.40±0.62	21.71[e]	75.88
Fractions					
PE	100	54.20±1.19	24.00±0.35	44.28[f]	50.81
PE	200	54.00±0.16	20.60±0.48	38.14[g]	57.63
PE	400	51.80±0.51	19.60±0.43	37.83[g]	57.98
CF	100	55.12±0.68	22.90±0.64	41.54[h]	53.85
CF	200	54.80±0.73	22.80±0.46	41.60[h]	53.79
CF	400	57.60±0.53	19.80±0.34	34.37[i]	61.82
MF	100	51.40±0.87	22.60±0.43	43.96[j]	51.17
MF	200	55.00±0.45	20.20±0.68	36.72[k]	59.21
MF	400	53.00±0.71	16.24±0.41	30.64[l]	65.96
AF	100	53.70±0.58	25.20±0.51	46.92[m]	47.87
AF	200	52.70±0.30	22.98±0.59	43.60[n]	51.57
AF	400	56.40±0.51	20.40±0.48	36.17[o]	59.82

Note: values are articulated as mean ± standard error of the mean (SEM); (n = 5); Data were analyzed by one-way ANOVA followed by Tukey post hoc test; DW: Distilled water, Lop: Loperamide; IE100: *I. ethiopica* root extract (100 mg/kg), IE 200: *I. ethiopica* root extract (200 mg/kg), IE 400: *I. ethiopica* root extract (400 mg/kg), PE100: Petroleum ether fraction(100 mg/kg), PE200: Petroleum ether fraction (200 mg/kg), PE400: Petroleum ether fraction (400 mg/kg), CF100: Chloroform fraction (100 mg/kg), CF200: Chloroform fraction (200 mg/kg), CF400: Chloroform fraction (400 mg/kg, MF100: Methanol fraction (100 mg/kg), MF200: Methanol fraction (200 mg/kg), MF400: Methanol fraction (400 mg/kg), AF 100: Aqueous fraction (100 mg/kg), AF200: Aqueous fraction (200 mg/kg), AF 400: Aqueous fraction (400 mg/kg); Data followed by different letter within the same column are significantly different at $p < 0.05$.

4.3.3 Anti-enteropooling effect

The purpose of this test is to assess the plant's antidiarrheal activity by lowering intestinal fluid accumulation. An experimental drug's effect on intestinal fluid secretion is evaluated in mice using the enteropooling (accumulation of fluid into the small intestine) approach. Watery diarrhea is caused by a large amount of water being secreted into the intestinal lumen as a result of the activation of chloride channels. This affects the inward movement of chloride ions from the cell (Teferi *et al.*, 2019). The reductions in both the volume and mass of intestinal content are the primary metrics for evaluating this activity.

All test doses of the hydroalcoholic extract of *I. ethiopica* reduced intraluminal fluid accumulation in mice compared to the vehicle treated group (Table 5). For dosages of 100, 200, and 400 mg/kg of the extract, there was a reduction in the percentage volume of small intestine content by 58.00%, 74.00% and 80.00%, respectively. The total mass of the intestinal contents was likewise significantly ($p<0.05$) reduced by the extract in a dose-dependent manner. Furthermore, a notable variation in the weight and volume of intestinal fluid between the three test doses of the root extracts was observed. For instance, when compared to doses of 100 mg/kg, the average weight and volume of small intestine content were significantly decreased at 400 mg/kg ($p <0.05$; 53.26%, 80%) respectively. The effect of this dose was found to have comparable antidiarrheal activity with 3 mg/kg loperamide ($p <0.05$; 72.82%, 88%) respectively. This conclusion was consistent with the findings of a study that examined the extract, aqueous

and methanol fractions of *Lantana camara* extract (Mengistu *et al.*, 2015). The MF reduced significantly (p <0.5) the amount of fluids in the intestine by 70.00%; 74.00% and 84.00% at tested doses of 100, 200, and 400 mg/kg respectively compared to control.

Table 5 *In vivo* antidiarrheal effects of root extract and its fractions on castor oil-induced enterpooling in mice model

Treatment	VSIC (ml)	% reduction VSIC	WSIC (g)	% reduction WSIC
NC 10 ml/kg	1.00±0.05	0[a]	1.84±0.02	0
PC 3 mg/kg	0.12±0.02	88.00[b]	0.50±0.04	72.82
Crude extract				
IE100	0.42±0.04	58.00[c]	1.46±0.05	20.65
IE200	0.26±0.02	74.00[d]	1.16±0.05	36.95
IE400	0.20±0.03	80.00[e]	0.86±0.05	53.26
Fractions				
PE 100	0.48±0.04	52.00[f]	1.58±0.04	14.13
PE 200	0.28±0.04	72.00[d]	1.34±0.05	27.17
PE 400	0.22±0.02	78.00[g]	1.02±0.06	44.56
CF 100	0.42±0.04	58.00[h]	1.70±0.05	7.60
CF 200	0.40±0.03	60.00[c]	1.28±0.04	30.43
CF 400	0.34±0.02	66.00[i]	1.02±0.06	44.56
MF 100	0.30±0.03	70.00[k]	1.38±0.06	25.00
MF 200	0.26±0.02	74.00[d]	1.16±0.05	36.95
MF 400	0.16±0.02	84.00[e]	0.92±0.04	50.00
AF 100	0.42±0.04	58.00[c]	1.46±0.04	20.65
AF 200	0.36±0.02	64.00[i]	1.30±0.03	29.34
AF 400	0.26±0.02	74.00[d]	0.96±0.02	47.82

Note: values are expressed as mean ± standard error of the mean (SEM); (n = 5); Data were analyzed by one-way ANOVA followed by Tukey post hoc test; NC: Negative control, PC: Positive control; VSIC: volume of small intestine content, WSIC: weight of small intestine content, IE100: *I. ethiopica* root extract (100 mg/kg), IE 200: *I. ethiopica* root extract (200 mg/kg), IE 400: *I. ethiopica* root extract (400 mg/kg), PE100: Petroleum ether fraction(100 mg/kg), PE200: Petroleum ether fraction (200 mg/kg), PE400: Petroleum ether fraction (400 mg/kg), CF100: Chloroform fraction (100 mg/kg), CF200: Chloroform fraction (200 mg/kg), CF400: Chloroform fraction (400 mg/kg), MF100: Methanol fraction (100 mg/kg), MF200: Methanol fraction (200 mg/kg), MF400: Methanol fraction (400 mg/kg), Aqueous fraction (100 mg/kg), AF200: Aqueous fraction (200 mg/kg), AF 400: Aqueous fraction (400 mg/kg); Data followed by different letter within the same column are significantly different at $p < 0.05$.

4.3.4 *In vivo* antidiarrheal index (ADI)

The antidiarrheal index (ADI) evaluates the combined effects of various diarrheal symptoms, including the frequency of bowel movements, the onset of diarrheal stools, and the accumulation of intestinal fluid to determine an extract's effectiveness in treating diarrhea (Andargie *et al.*, 2022; Yacob *et al.*, 2016).

Dose-dependent antidiarrheal index were observed for the fractions. The highest test dose of the extract with the highest ADI score has highest antidiarrheal activity. The antidiarrheal effectiveness of methanol fraction at 400 mg/kg was 73.54. The standard drug loperamide produced a maximum index of 113.55 as presented in Table 6.

Table 6 *In vivo* antidiarrheal indices (ADI) of 80% hydroalcoholic extract of *Impatiens ethiopica* and its fractions

Treatment	Doses mg/kg	Dfreq (%)	Gmeq (%)	Pfreq (%)	*In vivo* ADI
Extract					
	IE100	69.40	53.89	64.51	71.72
	IE200	81.80	60.79	74.19	82.63
	IE400	98.80	75.88	75.26	82.63
Fractions					
	PE100	72.00	50.81	56.98	59.29
	PE200	66.20	57.63	67.74	63.69
	PE400	71.20	57.98	75.26	67.73
	CF100	73.20	53.85	62.36	62.64
	CF200	71.00	53.79	67.74	63.72
	CF400	75.00	61.82	73.11	69.72
	MF100	84.40	51.17	69.89	67.08
	MF200	71.00	59.21	76.34	68.46
	MF400	75.80	65.96	79.56	73.54
	AF100	81.80	47.87	62.36	62.50
	AF200	75.80	51.57	69.89	64.89
	AF400	81.80	59.82	72.04	70.64
Standard drug					
	Lop.3 mg/kg	205.95	90.58	78.49	113.55

Note: Dfreq: Delay in the onset of diarrhea (min), Gmeq: Gut meal movement reduction in intestinal transit, Pfreq: Purging frequency in quantity of wet feces inhibition; Lop: Loperamide (3 mg/kg); ADI: Antidiarrheal index; IE100: *Impatiens ethiopica* root extract (100 mg/kg), IE200: *Impatiens ethiopica* root extract (200 mg/kg), IE400:

Impatiens ethiopica root extract (400 mg/kg), PE100: Petroleum ether fraction(100 mg/kg), PE200: Petroleum ether fraction (200 mg/kg), PE400: Petroleum ether fraction(400 mg/kg), CF100: Chloroform fraction (100 mg/kg), CF200: Chloroform fraction (200 mg/kg), CF400: Chloroform fraction (400 mg/kg), MF100: Methanol fraction (100 mg/kg), MF200: Methanol fraction (200 mg/kg), MF400: Methanol fraction (400 mg/kg), AF100: Aqueous fraction (100 mg/kg), AF200: Aqueous fraction (200 mg/kg), AF400: Aqueous fraction (400 mg/kg).

4.4 Structural elucidation of the isolated compound (SY-1)

The crude extract was fractionated based on solubility, and further purification was done through column chromatography using silica gel, resulted in the isolation of a light yellow needle-like crystalline substance, which was given the code name SY-1.

Compound SY-1 was isolated a light-yellow needle-like crystalline substance from the root extract of *I. ethiopica*. The Rf value of SY-1 was found to be 0.45 on normal phase-TLC using $CHCl_3$: MeOH (9:1) as a solvent system. When observed at a wavelength of 254 nm on a TLC plate, SY-1 exhibited a characteristic bluish color, which is often associated with the fluorescence emitted by coumarins. This suggested that SY-1 could potentially be a coumarin.

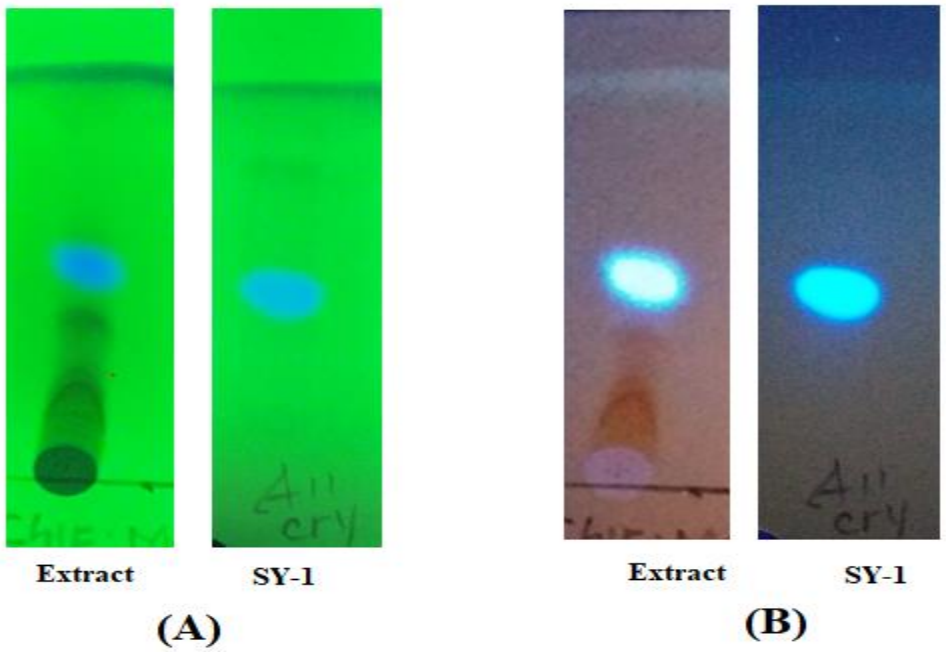


Figure 2 TLC profiles of the crude extract and the isolated compound (SY-1). A= viewed under UV light at 254 nm; B= Viewed under UV light 366 nm

The negative-mode ESI-mass spectrum (Figure 2) of SY-1 showed a pseudo molecular ion at *m/z* = 191.12 $[M-H]^-$, indicating a relative molecular weight of 192 mu for SY-1. This, along with the data obtained from the ^1H and ^{13}C-NMR analysis, led to the determination of a molecular formula of $C_{10}H_8O_4$.

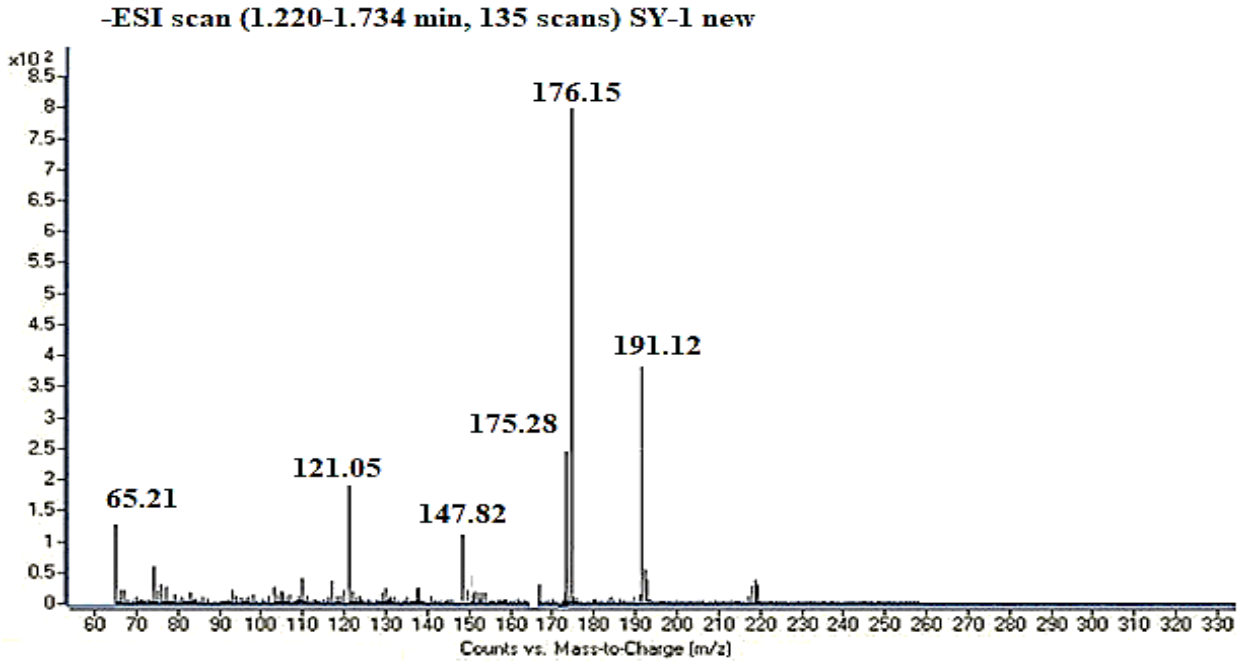


Figure 3 (-ve)-ESI-mass spectrum of SY-1

In the ^1H-NMR spectrum of SY-1, two doublet protons were observed at δ 6.20 (*J*=12 Hz; H-3) and 7.49 (*J*=12 Hz; H-4), indicating the presence of two *ortho*-coupled aromatic protons at positions H-3 and H-4 of the alpha-pyrone unit, respectively. Two singlets at δ 6.77 (H-8) and 6.85 (H-5) each integrating for 1H, indicated the existence of two *para*-assigned aromatic protons at positions H-5 and H-8, respectively. Additionally, a singlet integrating for 3H at δ 3.87 indicated the presence of a methoxy ($-OCH_3$) group in the structure of SY-1.

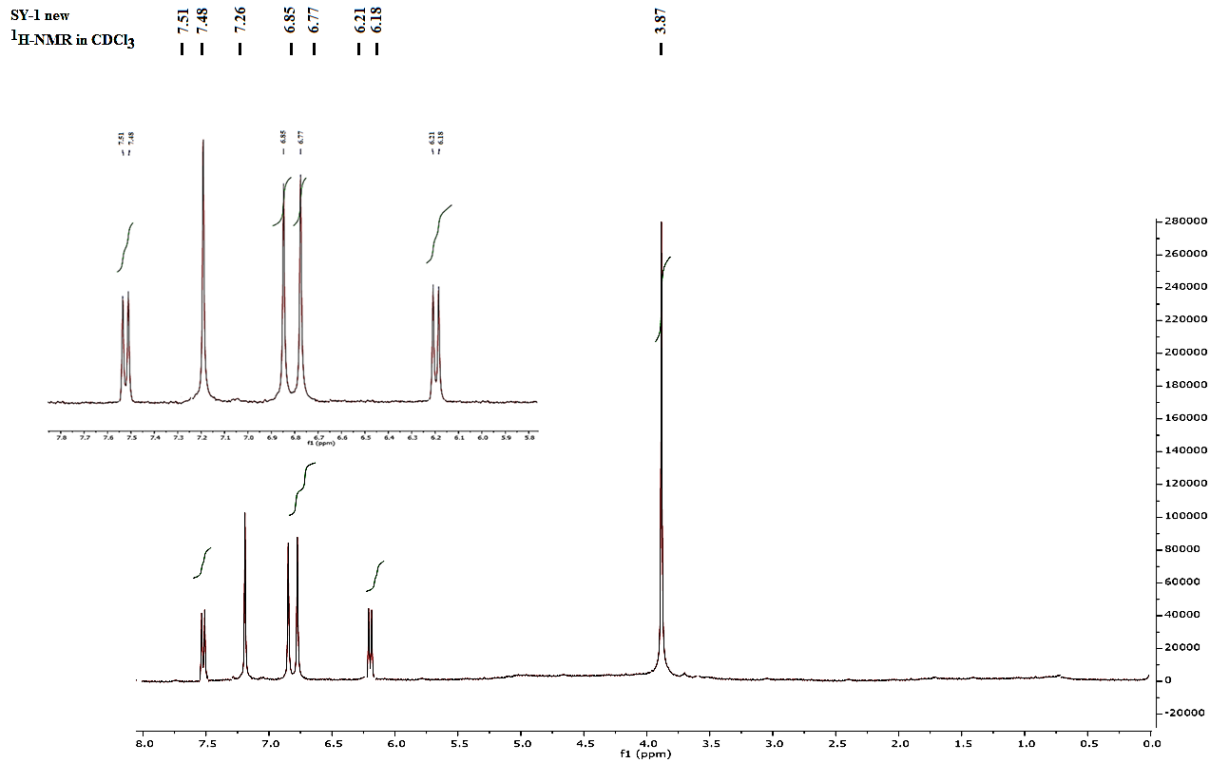


Figure 4 ^{1}H-NMR spectrum of compound SY-1 in CDCl3

The ^{13}C-NMR spectrum revealed ten carbon atoms in SY-1, as evident by ten visible signals in the spectrum. Of these, the most downfield signal resonating at δ 161.26 indicated the carbonyl carbon of the cyclic ester group in the alpha-pyrone unit. Four aromatic methine carbon signals were clearly observed in the DEPT-135 spectral data of SY-1, which are assignable to δ 103.22 (C-8), 113.47 (C-5), 107.53 (C-3) and 143.23 (C-4). A carbon signal at δ 56.44 indicated the presence of a methoxy (-OCH_3) group. Four signals resonating at δ 111.51, 144.01, 149.71 and 150.32 were assigned to the remaining quaternary aromatic carbons of C-4a, C-6, C-7 and C-1a, respectively.

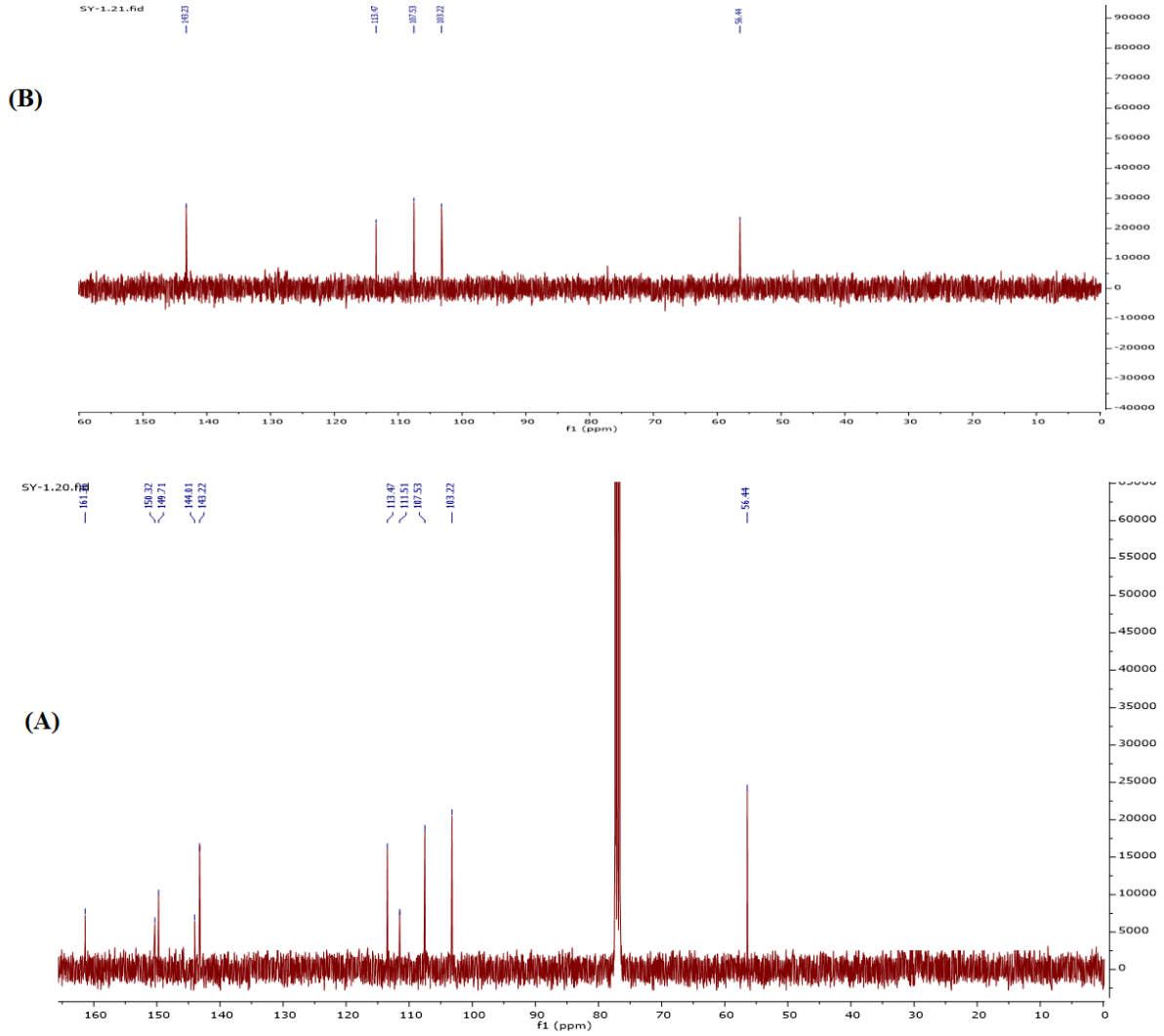


Figure 5 ^{13}C-NMR (A) and DEPT-135 (B) spectral data of compound SY-1 in $CDCl_3$.

Table 7 ^{1}H and ^{13}C-NMR spectral data of compound SY-1 in $CDCl_3$ and scopoletin in DMSO-d_6 (Darmawan *et al.*, 2012; Zhang *et al*., 2011).

C/H	^{1}H-NMR (ppm)		^{13}C-NMR (ppm)	
	SY-1	Scopoletin	SY-1	Scopoletin
1a	-	-	150.32	149.8
2	-	-	161.26	161.5
3	6.20 *d* (*J*=12 Hz)	6.21 *d* (*J*=9.3 Hz)	107.53	107.6
4	7.49 *d* (*J*=12 Hz)	7.92 *d* (*J*=9.3 Hz)	143.23	143.4
4a	-	-	111.51	111.5
5	6.85 *s*	7.21 *s*	113.47	113.4
6	-	-	144.01	144.1
7	-	-	149.71	150.3
8	6.77 *s*	6.78 *s*	103.22	103.2
6-OCH_3	3.87 *s*	3.87 *s*	56.44	56.5

Based on the ESI-MS and NMR spectral data, SY-1 was found to be consistent with a coumarin derivative containing one hydroxyl (-OH) and one methoxy (OCH_3) group. At the end, compound SY-1 was unambiguously identified as 7-hydroxy-6-methoxycoumarin (Figure 4), and its structure was further confirmed by comparing its spectral data with previously reported literatures (Bhatt Mehul *et al.*, 2011; Darmawan *et al.*, 2012; Zhang *et al.*, 2011).

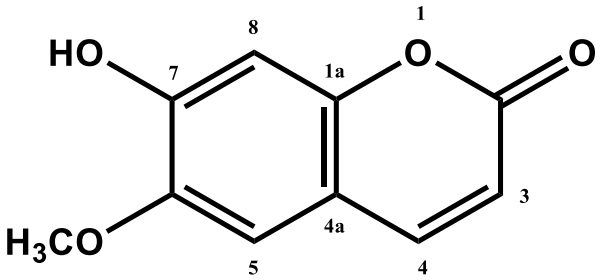


Figure 6 Chemical structure of compound SY-1.

4.5 *In vivo* antidiarrheal effect of scopoletin

4.5.1 Effects on castor oil-induced diarrhea in mice

In this study, scopoletin, found in *I. ethiopica,* was also investigated. The findings revealed that scopoletin significantly reduced the frequency, quantity, and the total weight of feces produced in mice experiencing castor oil-induced diarrhea compared to the negative control (Table 8). Additionally, scopoletin delayed the onset of diarrhea, most notably at doses of 20 mg/kg (96.60 min delay) and 40 mg/kg (99.80 min delay), without a clear dose-dependent pattern though.

Interestingly, the effect of scopoletin on reducing defecation (78.49% inhibition) was comparable to loperamide (3 mg/kg; 78.49% inhibition) at a dose of 40 mg/kg. Although scopoletin notably reduced total feces output compared to the negative control, this reduction did not follow a consistent dose-dependent trend across the three doses tested (10 mg/kg: 70.73%; 20 mg/kg: 71.95%; 40 mg/kg: 71.95%), yet it remained similar to loperamide's effect (3 mg/kg: 78.49%).

At a dose of 40 mg/kg, scopoletin had a comparable effect in mice with that of the positive control, loperamide (3 mg/kg), suggesting its potential as an antidiarrheal agent on its own or as a basis for developing more potent compounds.

Table 8 Antidiarrheal activity of scopoletin on castor oil-induced diarrhea in mice model

Treatment types and dose	Onset of diarrhea in min	Total number of wet feces in 4 h	%Inhibition of defecation	Total number of feces output in 4 h	Total weight of wet feces (g) in 4 h	% Inhibition of feces output	Total weight of feces output (g) in 4 h
DW 10 ml/kg	33.60±6.39[a]	18.60±1.72	0[a]	18.60±1.72	0.82±0.18	0[a]	0.88±0.19
Lop 3 mg/kg	102.80± 6.66[b]	4.00±0.31	78.49[b]	4.00±0.31	0.21±0.01	74.39[b]	0.24±0.03
Scopoletin(mg/kg)							
10	94.40±0.92[c]	6.40±0.40	65.59[c]	7.60±0.51	0.24±0.02	70.73[c]	0.26±0.01
20	96.60±1.28[b]	5.60±0.50	69.89[c]	6.80±0.58	0.23±0.02	71.95[c]	0.25±0.01
40	99.80±1.20[b]	4.00±0.31	78.49[b]	5.60±0.24	0.23±0.05	71.95[c]	0.24±0.02

All values are expressed as mean ± standard error of the mean (SEM); (n = 5); Data were analyzed by one-way ANOVA followed by Tukey post hoc test; NC: Negative control, Lop: Loperamide; Data followed by different letter within the same column are significantly different at $p < 0.05$.

4.5.2 Effects on castor oil-induced intestinal transit by charcoal marker in mice

Scopoletin showed a maximum anti-motility effect at 40 mg/kg dose (89.37%, $p < 0.05$), which was comparable with loperamide 3 mg/kg (90.58%, $p < 0.05$) (Table 4). Delays in intestinal muscle motility and decreased frequency of stools encourage additional water absorption from feces and lessen the watery consistency of excrement.

Table 9 Antidiarrheal effect of scopoletin on charcoal meal transit in castor oil-induced motility in mice model

Treatment	Dose (mg/kg)	Mean length of small intestine in cm	Mean distance travelled by charcoal marker in cm	% Charcoal meal transit (Peristalsis index)	% Inhibition
DW	10 ml/kg	50.20±2.05	45.20±2.27	90.03[a]	0[a]
Lop	3 mg/kg	56.60±0.97	4.80±0.51	8.49[b]	90.58[b]
Scopoletin (mg/kg)					
	10	52.56±1.10	14.50±0.76	27.58[c]	69.36[c]
	20	52.60±1.04	13.60±0.43	25.85[c]	71.28[c]
	40	52.20±0.80	5.00±0.45	9.57[b]	89.37[b]

Note: values are articulated as mean ± standard error of the mean (SEM); (n = 5); Data were analyzed by one-way ANOVA followed by Tukey post hoc test; DW: Distilled water, Lop: Loperamide; Data followed by different letter within the same column are significantly different at $p < 0.05$.

4.5.3 Anti enteropooling effect

Scopoletin at 40 mg/kg significantly ($p<0.05$; 86.00%) decreased the intraluminal fluid accumulation when compared to the negative control and showed comparable effect with the standard drug loperamide 3 mg/kg ($p<0.05$; 88.00%).

Table 10 Antidiarrheal activity of scopoletin on castor oil-induced enterpooling in mice model

Treatment	VSIC (ml)	% reduction VSIC	WSIC (g)	% reduction WSIC
NC 10 ml/kg	1.00±0.05	0[a]	1.84±0.02	0[a]
PC 3 mg/kg	0.12±0.02	88.00[b]	0.50±0.04	72.82[b]
Scopoletin				
10 mg/kg	0.26±0.02	74.00[c]	0.88±0.02	52.17[c]
20 mg/kg	0.22±0.04	78.00[d]	0.78±0.04	57.60[d]
40 mg/kg	0.14±0.02	86.00[b]	0.48±0.04	73.91[b]

Note: values are expressed as mean ± standard error of the mean (SEM); (n = 5); Data were analyzed by one-way ANOVA followed by Tukey post hoc test; NC: Negative control, PC: Positive control; VSIC: volume of small intestine content, WSIC: weight of small intestine content, IE100: SY40: Isolated compound (40 mg/kg), SY20: Isolated compound (20 mg/kg), SY10: Isolated compound (10 mg/kg; Data followed by different letter within the same column are significantly different at $p < 0.05$.

4.5.4 *In vivo* antidiarrheal index (ADI)

Scopoletin at doses of 10, 20, and 40 mg/kg showed an antidiarrheal index of 75.45, 78.36, and 88.79, respectively. More importantly, at the maximum dose scopoletin had an ADI value of 88.79, which was the highest score among the test substances. This implies the effectiveness of scopoletin in treating diarrhea.

Table 11 *In vivo* antidiarrheal index (ADI) of scopoletin from the root of *Impatiens ethiopica*

Treatment	Doses mg/kg	Dfreq (%)	Gmeq (%)	Pfreq (%)	*In vivo* ADI
Compound					
	Scopoletin 10	94.40	69.36	65.59	75.45
	Scopoletin 20	96.60	71.28	69.89	78.36
	Scopoletin 40	99.80	89.37	78.49	88.79
Standard drug					
	Lop.3 mg/kg	205.95	90.58	78.49	113.55

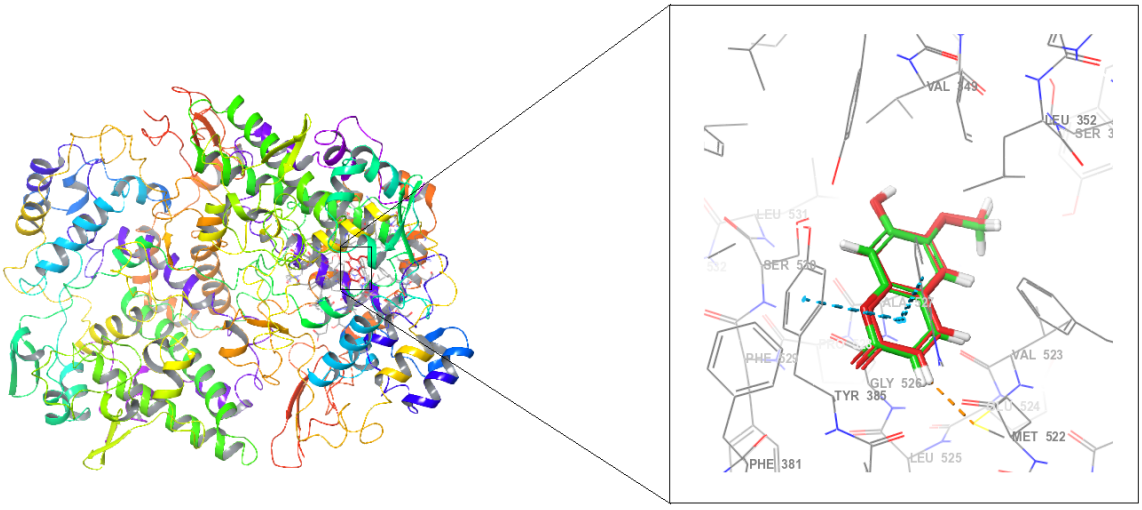
Note: Dfreq: Delay in the onset of diarrhea (min), Gmeq: Gut meal movement reduction in intestinal transit, Pfreq: Purging frequency in quantity of wet feces inhibition; Lop: Loperamide (3 mg/kg); ADI: Antidiarrheal index; SY40: Isolated compound (40 mg/kg), SY20: Isolated compound (20 mg/kg), SY10: Isolated compound (10 mg/kg).

4.6 Molecular docking analysis

The study utilized molecular docking, an essential technique for examining the potential mechanisms of biologically active compounds. Some potential antidiarrheal targets such as

prostaglandin synthase (COX-2, COX-1), TNF-α, IL-β and iNOS were screened. Based on the fitness score COX-2 was found to have favorable interactions with SY-1.

In this study, scopoletin (1) was specifically docked into the active site of the PGS enzyme (PDB ID: 4COX; Chain A, B) using Maestro V.13.5 software by Schrodinger 2023-1 Suite. Figure 7 and 8 illustrates 3D and 2D representations of scopoletin (1) docked within the active site of PGS respectively. Based on the molecular docking study, scopoletin exhibited a favorable docking score of −7.941 kcal/mol. This was attributed to its ability to engage in hydrogen and pi-pi interactions with specific residues in the binding site of the PGS enzyme, namely SER530, TRP387, and TYR385. Of these amino acid residues, scopoletin (1) establishes a hydrogen bond with the amino acid residue SER530 within the PGS enzyme's active site (Figure 7) and pi-pi stacking with TYR385and TRP387 amino acid residues. Thus, we can suggest from the molecular docking study that scopoletin (1) negatively regulates PGS.



(A) **(B)**

Figure 7 (A): 3D representation of scopoletin (1) docked within the active site of COX; (B): The 3D zoomed view of the scopoletin (1) interaction

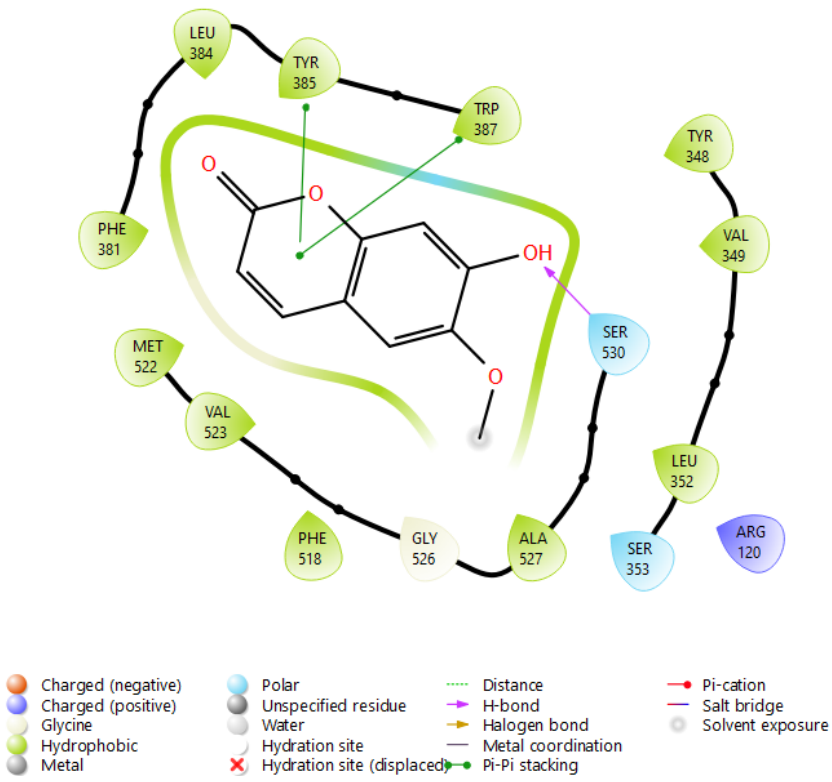


Figure 8 2 D model of scopoletin (1) showing interactions with residues at the COX enzyme.

In developing countries such as Ethiopia, people rely on medicinal plants to treat diarrhea. *I. ethiopica* is widely used in Ethiopia for treating of various illnesses. In this study, the crude extract of *I. ethiopica* exhibited antidiarrheal effects in mice induced with castor oil-induced diarrhea, as detailed in Tables 3-6. These findings align with prior reports highlighting similar antidiarrheal activity of extracts from other medicinal plants (Mengesha *et al.*, 2022; Ayalew *et al.*, 2022).

Furthermore, this study identified scopoletin for the first time in *I. ethiopica*, although it has been previously recognized in several other plants such as *Acer saccharum*, *Artemisia annua*, *Scopolia carniolica*, *Arabidopsis thaliana* (Antika *et al.*, 2022), *Ipomoea reniformis* (Convolvulaceae) (Bhatt Mehul *et al.*, 2011), and *Saussurea eopygmaea* (Saussurea) (Zhang *et al.*, 2011). It's

presence has also been reported in the *Impatiens* genus, specifically in *Impatiens balsamina* (Singh *et al.,* 2017). Scopoletin demonstrated strong antidiarrheal activity in mice, as shown in Tables 8-11.

Antidiarrheal medications often work through various mechanisms aiming to restore gastrointestinal fluid and electrolyte balance, involving targets like intestinal mu-opioid receptors, prostaglandin, serotonin, and histamine receptors, as well as ion exchangers and transporters such as the Na+/k+ exchanger (Mengesha *et al.*, 2022; Mengistu *et al*., 2022; Yacob *et al.*, 2016; Ayele *et al*., 2023; Rahman *et al*., 2015). Through molecular docking analysis, scopoletin exhibited strong binding affinity toward prostaglandin synthase (PGS) (COX-2; PDB ID: 4COX; docking score of −7.941 kcal/mol) via hydrogen bonding with SER530 and pi-pi stacking interactions with TYR385 and TRP387. This suggests that scopoletin appears to inhibit prostaglandin synthase (PGS), although experimental evidence is needed. Compounds from medicinal plants have been known to inhibit prostaglandin synthesis, potentially explaining the observed antidiarrheal effects in this study, which could lead to increased absorption and reduced secretion of fluids and electrolytes (Sakthivel *et al.*, 2022; Lee and Kim, 2015).

Notably, scopoletin possess a variety of pharmacological properties, including antibacterial, ant proliferative, antioxidant, and ant inflammatory properties (Sakthivel *et al.*, 2022; Wan Osman *et al.*, 2017; Firmansyah *et al.*, 2021). Studies have demonstrated ant inflammatory activity of scopoletin in mice with croton oil-induced edema, carrageenan-induced paw edema, and lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cells, where it reduced inflammation markers such as TNF-α, PGE2 and COX-2 expressions, among others (Lee and Kim, 2015; Sakthivel *et al.*, 2022). Hence, in this study, scopoletin is implicated at least partially responsible for the antidiarrheal activity observed in *I. ethiopica*.

5 Conclusion

The present study effectively demonstrated the *in vivo* antidiarrheal effects of *I. ethiopica* roots in mice triggered with castor oil-induced diarrhea. Scopoletin, found in the most active methanol-soluble fraction, displayed the highest antidiarrheal activity. This suggests that scopoletin is partly or fully responsible for the antidiarrheal properties of the plant. Scopoletin was observed in the molecular docking analysis to inhibit PGS, potentially leading to enhance electrolytes and water absorption, although further experimental support is necessary. The study supports the traditional medicinal usage of *I. ethiopica* in treating diarrhea and highlights scopoletin as a potential standalone antidiarrheal agent or as a foundation for developing more potent compounds.

6 Recommendations

Based on the findings of the current study, the following recommendations are suggested;

- Determinations of sub-acute and chronic toxicities of scopoletin,
- The use of castor oil (drug induced diarrhea), while useful in producing diarrhea in the mice, it does not fully replicate the most common cause of diarrheal symptomatology, infectious disease. Thus, additional studies in which mice models are purposefully infected with pathogenic causes of diarrhea might provide better information regarding the effects of scopoletin.
- Synthesize scopoletin based derivatives (SARs) and determination of their antidiarrheal activities,
- Separation and characterization of other compounds from the root extracts of *I. ethiopica* and determination of their antidiarrheal and antimicrobial activities,
- *In vivo* and *in silico* ADMET evaluations are suggested.

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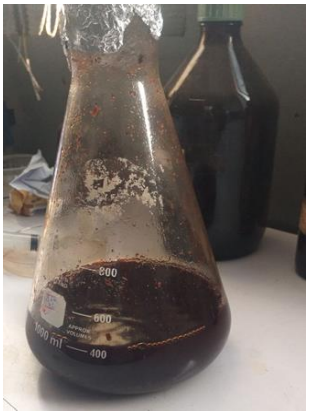
Annexes

Annex-I - Photo based activities from plant collection to compound isolation

Impatiens ethiopica Grey Wilson

Roots of *Impatiens ethiopica*

Dried roots of *Impatiens ethiopica*



Maceration

Normal phase column chromatography

Crystal structures of the compound obtained from the column (light-yellow color

Ethical clearance

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Addis Ababa University

School of Pharmacy
Ethical Review Committee

ቀን Date: December 04, 2023

ቁጥር Ref. No.: ERB/SOP/559/16/2023

To: Sileshi Yimer
School of Pharmacy

Re: Ethical Clearance

It is to be recalled that you submitted a research proposal entitled "**Anti-diarrheal activity evaluation of 80% methanolic extract of the root of *Impatients ethiopica* in mice model and isolation of its major compounds".** The committee thoroughly reviewed the proposal based on its operational guideline and found that, it fulfills all the ethical requirements stipulated in the guideline. This is, therefore, to inform you that the proposal is ethically approved for implementation.

With best regards,

Shemsu Umer (PhD)
Chairperson, ERC
School of Pharmacy
College of Health Sciences

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Draft Manuscript orginated from this thesis

Coumarin from the Root Extract of *Impatiens ethiopica* Grey-Wilson: *In vivo* Antidiarrheal and Molecular Docking Studies

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Abstract

Impatiens ethiopica has been traditionally used for the treatment of infectious diseases. The present study was aimed to investigate the potential antidiarrheal effects of the root extract of *I. ethiopica* and its major constituents using both *in vivo* models, with intestinal transit, intestinal secretion and castor oil-induced mice models. The findings of the study revealed that the root extract significantly reduced the frequency of defecation, the weight of feces, and the onset of diarrhea at 100 mg/kg, 200 mg/kg, and 400 mg/kg. The active root extract was then divided into four fractions, utilizing successively petroleum ether, chloroform, methanol, and water solubility as the basis for separation. Further fractionation of the active methanol fraction by normal-phase column chromatography led to the isolation a coumarin, 7-hydroxy-6-methoxycoumarin, commonly known as scopoletin, on the basis of various spectroscopic techniques. The antidiarrheal effects of scopoletin were also evaluated, demonstrating a significant reduction in intestinal fluid accumulation and gastrointestinal (GI) motility at the doses of 10 mg/kg, 20

mg/kg and 40 mg/kg. Scopoletin interaction with prostaglandin synthase (PGS) (COX-2; PDB ID: 4COX) revealed a promising docking score of −7.941 kcal/mol, with a strong binding affinity, engaging in hydrogen bonding with SER530 and pi-pi stacking interactions with TYR385 and TRP387, at specific COX-2 binding sites. These findings highlight that the antidiarrheal-like activity of *I. ethiopica* was partly attributed due to the presence of scopoletin. Overall, these results provide validation for the traditional use of *I. ethiopica* roots in the treatment of diarrhea and highlight the potential of scopoletin as antidiarrheal compound.

Keywords: *In vivo* Antidiarrheal, *Impatiens ethiopica*, coumarin, castor oil, intestinal secretion, gastrointestinal motility, molecular docking, 7-hydroxy-6-methoxycoumarin, scopoletin

1. Introduction

Diarrhea, a widespread health concern globally, poses a significant threat, particularly to children under five years old, leading to an estimated 499,000 deaths and 688 million morbidities worldwide (Mady *et al.*, 2023; Worku *et al.*, 2023; Wolde *et al.*, 2022). Diarrhea, classified by duration as acute diarrhea (lasting less than 15 days), persistent (15 to 28 days) or chronic (lasting more than a month) has diverse etiologies: acute cases mainly stem from enteric pathogens (Mekonnen *et al.*, 2018; Adela Alemu *et al.*, 2022), while chronic are linked to bowel disorders and malabsorption syndromes (Mekonnen *et al.*, 2018; Worku *et al.*, 2023).

Acute infectious diarrhea can be treated empirically with antibiotics to mitigate the progression of disease and reduce the severity of associated symptoms, such as fever, abdominal pain and vomiting (Diniz-Santos *et al.*, 2006). However, an increased antibiotics resistance posed challenges (Ahmad *et al.*, 2021; Diniz-Santos et al., 2006). In response to these challenges, exploring novel, diverse antidiarrheal drugs with fewer side effects becomes crucial (Abdela, 2019).

Traditional medicine offers potential solutions globally, with various herbal remedies. Recognizing this potential, the World Health Organization (WHO) advocates for herbal medicine-based research to combat diarrheal infections (Abdela, 2019). Specifically, some members of the genus *Impatiens*, has been traditionally used for diarrhea treatment (Singh *et al.*, 2017). Thus, the present study was aimed to investigate the potential antidiarrheal effects of

compound(s) from the root extracts of *Impatiens ethiopica* using both *in vivo* model and in molecular docking studies.

2. Materials and Methods

2.1. Materials

2.1.1. Plant materials

The roots of *I. ethiopica* were collected from Gulale Botanical Garden, Addis Ababa, Ethiopia in June 2022. The plant was authenticated by Mr. Melaku Wondafrash, and a sample (collection number as SY-001) was stored at the National Herbarium, Department of Plant Biology and Biodiversity Management, College of Natural and Computational Sciences, Addis Ababa University (AAU) for future reference.

2.1.2. Chemicals, reagents and drug

The chemicals and reagents used in this experiment include castor oil (Amman Pharmaceutical Industries, Jordan), loperamide (Medochemie Ltd, Limassol Cyprus), activated charcoal (Acuro Organics Ltd, New Delhi), methanol (Reagent Chemical Limited, UK), chloroform (Finkem Laboratory Reagent, India), distilled water and petroleum ether. There was no further purification necessary because all of the chemicals and reagents employed were of analytical grade.

2.1.3. Instruments

Rota vapors (Heidolph Instruments GmbH and Co., Germany), UV spectroscopy (Shimadzu Spectrophotometer MultiSpec-1501, Japan), Nuclear magnetic resonance spectrometer JNM-ECS400 NMR spectrometer (JEOL Ltd., Akishima, Tokyo, Japan), Electrospray ionization mass Spectrometer (ESI-MS) (JEOL Ltd., Akishima, Tokyo, Japan).

2.1.4. Experimental mice

Swiss albino mice aged 6 - 8 weeks and weighing 22 - 30 g were employed throughout this experimental activity. The mice were gained from the animal house of the Department of Clinical Pharmacy and Pharmacology, School of Pharmacy, College of Health Science, Addis Ababa University. The mice were housed in cages and maintained under standard conditions (room temperature and humidity, 12-hour light and dark cycle). Standard pelletized food and tap water were provided ad libitum. Experimental activities were conducted in line with the

internationally accepted laboratory animal use and care guideline (Rajput *et al.*, 2011) and were approved by the Institutional Review Board of the School of Pharmacy, AAU.

2.2. Methods

2.2.1. Extraction

The roots of *I. ethiopica* were meticulously cleaned, dried under shade, and crushed into a coarse powder using a mortar and pestle. The powdered root (500 g) underwent maceration with 80% methanol (3 L) at room temperature for three days, with regular shaking and stirring. After 72 h, the extract was filtered using Whatman No. 1 filter paper. The marc was subjected to another three days of maceration with the same amount of solvent. The filtrates were combined and concentrated using a Rota Evaporator (Heidolph Instruments GmbH and Co., Germany) at a controlled temperature not exceeding 40 °C. The resulting concentrated filtrate, in an aqueous solution, was dried at 40 °C in an oven. The final dried hydroalcoholic extract was transferred into an amber-colored vial, weighed and kept in a refrigerator at 4 °C for further use.

2.2.2. Solubility-based fractionation

The extract was sequentially subjected to solubility-based fractionation, with petroleum ether, chloroform, methanol, and water, to yield four fractions. Initially, the extract (30 g) was sonicated in petroleum ether (150 ml); the filtrated petroleum ether solution was labeled as fraction-1. The residue underwent a similar process with chloroform (100 ml) to yield fraction-2. The subsequent step involved sonication in methanol (150 ml), resulting in fraction-3. The remaining residue underwent sonication in distilled water (150 ml) to yield fraction-4. Each fraction was dried in a vacuum oven at 40 °C, transferred into an amber-colored vial, weighed and kept in a refrigerator at 4 °C for further use.

2.2.3. Isolation of compound by column chromatography

A column was initially packed with silica gel-chloroform slurry. The methanol-soluble part (fraction-3, 2 g) was adsorbed onto silica gel (2 g) in MeOH. After drying with a Rotary evaporator, the adsorbed sample was loaded onto the column and eluted with varying $CHCl_3$ and MeOH gradients. A total of 120 fractions, each 10 ml, were collected. Specifically, fractions 37-45, eluted with $CHCl_3$ and MeOH (99:1), showed a single spot-on normal phase TLC in different solvent systems. These fractions were combined to yield SY-1, a light-yellow needle-like crystalline substance (37 mg). SY-1 was transferred into an amber-colored vial, weighed and stored in a refrigerator at 4 °C for further use.

2.2.4. Acute oral toxicity test

Acute oral toxicity tests of the extract was conducted according to Organization for Economic Cooperation and Development (OECD) guideline 425 (425, 2008). A fasting mouse was given a limit dose of 2000 mg/kg on the first day, observed for any signs of toxicity in the 1st hour, and periodically for 24 h. If the mouse survived, then the same doses were administered to four more mice; thus, five mice were used for each test substance. Then, the animals were followed up for the next fourteen days.

2.2.5. Experimental design

2.2.5.1. Grouping and dosing

This study used three different antidiarrheal mice models namely: enteropooling, gastrointestinal motility, and castor oil induced diarrhea models. Mice of both sexes were randomly assigned to eight groups (negative control, positive control, and six test groups) in all models. Each group consisted of five animals (Khan *et al.*, 2023). Negative controls received vehicle (10 ml/kg, distilled water) and positive controls received loperamide (3 mg/kg) in all models. The treatment groups (group III, IV and V) received different doses (100, 200 and 400 mg/kg, respectively) of the extract and fractions and (group VI, VII and VIII) received (10, 20 and 40 mg/kg, respectively) of the isolated compound (SY-1) orally which were determined based on the acute oral toxicity test guideline.

2.2.6. Determination of antidiarrheal activity

2.2.6.1. Castor oil-induced diarrhea

The method described by (Umer *et al.*, 2013) was followed for this study. Swiss albino mice of either sex were fasted for 18 h with free access to water and randomly allocated to eight groups of five animals each. Vehicle treated group received distilled water (10 ml/kg) (negative control); Group II (positive control) received the standard drug loperamide 3 mg/kg, orally. Animals in groups III, IV and V received the extract or fractions at doses of 100, 200 and 400 mg/kg, respectively and group VI, VII and VIII received (10, 20 and 40 mg/kg, respectively) of the isolated compound (SY-1) orally. One hour after treatment, diarrhea was induced by oral administration of 0.5 ml castor oil to each mouse. The animals were then housed in a separate transparent cage in which the floor is lined with white paper. The paper was changed every hour for a total of four hours. During the observational period, the onset of diarrhea, number and weight of wet stools, total number and the total weight of fecal output were recorded. Finally, the

percentage of fecal output (% FOP) and diarrheal inhibition (% inhibition of defecation) were calculated by using the formulas described below.

$$\text{Percentage of fecal output (\% FOP)} = \frac{\text{Mean faecal weight of each treatment group}}{\text{Mean faecal weight of negative control}} \times 100$$

$$\text{Percentage inhibition of defecation} = \frac{\text{MO}-\text{M}}{\text{MO}} \times 100$$

Where, Mo is mean defecation of negative control and M stands for mean defecation of test sample (standard drug).

2.2.6.2. Gastrointestinal motility test by charcoal marker

In this model, mice were abstained from food 18 h with free access to water and grouped and treated as described under grouping and dosing section earlier. One hour post treatment each mouse received 0.5 ml of castor oil and, after 1 h, they received 1 ml of 5% activated charcoal suspension. The animals were then sacrificed by cervical dislocation after 30 min of administering activated charcoal and the entire length of the intestine (from the pylorus to the cecum) was removed and placed lengthwise on a white paper. The distance travelled by the charcoal meal and the total length of the intestine was then measured using a calibrated ruler. The distance travelled by the charcoal meal relative to the total distance of the small intestine was expressed as a peristaltic index (PI). The peristaltic index (PI) and percentage of inhibition were calculated by using the following formula (Meite *et al.*, 2009; Ahmad *et al.*, 2021).

$$\text{Peristaltic index (PI)} = \frac{\text{Mean distance travelled by charcoal meal}}{\text{Mean length of small intestine}} \times 100$$

$$\text{Percentage inhibition} = \frac{\text{Intestinal transit by charcoal meal}(-\text{ve control}-\text{treated})}{\text{Intestinal transit by charcoal meal in the}-\text{ve control groug}} \times 100$$

2.2.6.3. Anti-enteropooling test

Intraluminal fluid accumulation was determined using the method described by (Degu *et al.*, 2016). Animals of either sex were devoid of food for 18 h and grouped and treated, as described under grouping and dosing. After 1h, 0.5 ml of castor oil was administered orally. One hour later, the mice were sacrificed by cervical dislocation. The abdomen of each mouse was opened and the whole length of the intestine, from the pylorus to the caecum, was ligated, dissected and carefully removed. The small intestines were weighed and the intestinal contents were collected by milking into a graduated tube to measure the volume. The empty intestines were reweighed and the difference between the two weights was calculated. Finally, the percentage of reduction

of intestinal secretion and weight of intestinal contents was determined by using the following formulas (Degu *et al.*, 2016).

$$\text{Mean percentage inhibition} = \frac{\text{MVICC}-\text{MVICT}}{\text{MVICC}} \text{ x } 100$$

Where MVICC is the mean volume of the intestinal content of the control group while MVICT is the mean volume of the intestinal content of the test group

$$\text{Percentage inhibition by using MWSIC} = \frac{C-T}{C} \text{ x } 100$$

Where MWSIC is mean weight of small intestinal content while C is the mean weight of intestinal content of the control and T is the mean weight of intestine content of the test (drug group).

2.2.6.4. *In vivo* antidiarrheal index (ADI)

Calculations were made for the delay in diarrheal onset and purging index by comparing with control group. The *in vivo* antidiarrheal index (ADI) was then expressed according to the formula (Degu *et al*., 2016; Ayalew *et al.*, 2022) described below;

$$\textit{In vivo} \text{ antidiarrheal index (ADI)} = \sqrt[3]{Dfreq\ x\ Gmeq\ x\ Pfreq}$$

Where Dfreq is delay in defecation time or diarrheal onset (in percent of control) obtained from castor oil induced diarrheal test which is expressed by the formula;

$$\text{Dfreq} = \frac{\text{Mean onset of diarrhea in the test group} - \text{Mean onset of diarrhea in the control group}}{\text{Mean onset of diarrhea in the control group}}\ x\ 100$$

Gmeq is gut meal travel reduction (in percent of control) obtained from charcoal meal test (% inhibition), and Pfreq is the purging frequency as number of wet stool reduction (in percent of control) obtained from castor oil diarrheal model (% inhibition of defecation).

2.2.7. Molecular docking experiment

Following early preliminary docking assessments on potential targets, molecular docking of SY-1 was implemented on the prostaglandin synthase (PGS) enzyme as a possible target for antidiarrheal in this investigation. Below is a summary of the procedures used.

2.2.7.1. Protein preparation:

The X-ray crystallography structure of Mus musculus prostaglandin synthase-2; cyclooxygenase-2 (COX-2) (PGS-2; PDB ID: 4COX; Chain A, B; resolution: 2.90 Å) complexed with indomethacin was downloaded from the protein data bank (www.rcsb.org). COX-2 structure consists of 604 amino acid chain sequence, complexed with indomethacin 1-(p-chlorobenzoyl) 25-methoxy-2-methylindole-3-acetic acid (Pouplana *et al.*, 2002; Kurt *et al.*, 2022). Using

Maestro V13.5 Protein Preparation Workflow module (Schrödinger 2023-1), the 3D structure of COX-2 protein in complex with indomethacin (PDBID: 4COX, chain AB) was prepared by standard procedures. This involved correcting charges, bond orders, and atom types, removing water molecules beyond 5 Å from the het group, and filling in missing side chains and loops. The OPLS4 force field was applied for energy optimization and steric hindrance removal (Madhavi Sastry *et al.*, 2013).

2.2.7.2. Ligand preparation

ChemDraw Ultra (2019) was employed to draw the structure of compound SY-1 in MDL SDfile format. Ligand preparation was carried out using the Ligprep module of Maestro V13.5, Schrödinger Suite 2023-1, following to OPLS4 at a physiological pH of 7.0 ± 2.0. Different ionization states and stereoisomers for ligand structure were generated.

2.2.7.3. Receptor grid generation

We generated the receptor grid box to visualize the active site of the receptor for glide ligand docking. The ligand-binding site was defined by picking the proteins structure on the workspace, with a 6.0 Å radius binding site. The van der Waals radii of the receptor atoms was set with partial atomic charge scaling factor of 0.8 and partial cut-off of 0.15 to soften the receptor's nonpolar parts.

2.2.7.4. Ligand docking

Using Glide software in extra precision (XP) mode, we docked compound SY-1 into the active binding site of the prepared protein. Prior to this, we docked the co-crystallized ligand into the protein's active site to predict binding affinity and molecular interaction. Docking scores in kcal/mol were used to analyze the binding pose. All the analyses and procedures were performed using the Schrodinger suite of programs.

2.2.8. Data analysis

The statistical package for the social sciences (SPSS), version 20.0, was used to analyze the data, which were expressed as mean ± standard error of the mean (SEM). Difference between group means was analyzed with one way analysis of variance followed by Tukey post Hoc test for pairwise comparisons. A p-value of < 0.05 was taken as statistically significant. Molecular docking was studied by using Maestro, version 13.5 (Schrödinger 2023-1 release) software to determine drug-receptor interactions. The structures of the ligand were sketched using PerkinElmer informatics, Inc., ChemDraw professional version 19.00 software.

2.2.9. Ethical approval

Ethical approval was obtained from the Scientific and Ethics Committee of the Department of Pharmaceutical Chemistry and Pharmacognosy, School of Pharmacy, Addis Ababa University with an approval code ERB/SOP/559/16/2023.

3. Results and Discussion

3.1. Extraction yield

The maceration of dried *I. ethiopica* root (500 g) in 80% methanol produced a dark brown sticky extract weighting 69 g, yielding 13.8% of the total weight. 80% hydroalcoholic solvent is a universal solvent due to its ability to extract a wider range of polarities, allowing for the extraction of both polar and non-polar phytochemicals, as evident by various studies (Mengesha *et al.*, 2022; Ayalew *et al.*, 2022; Birru *et al.*, 2016). Additionally, this choice of solvent was advantageous as it hinders the growth of potential microbes (Andargie *et al.*, 2022).

3.2. Acute oral toxicity

The acute oral toxicity test results of this finding revealed that 80% hydroalcoholic roots extract from *I. ethiopica* did not cause any mortality in mice within the first 24 h as well as for the subsequent 14 days, suggesting that the oral LD_{50} is greater than 2000 mg/kg.

3.3 *In vivo* antidiarrheal activities of extract and fractions

3.3.1 Effects on castor oil-induced diarrhea in mice

In this study, we induced diarrhea in mice using castor oil, known to trigger diarrhea through various mechanisms. Castor oil gets converted to ricinoleic acid in the gut, causing irritation, inflammation, and the release of inflammatory substances, like prostaglandins and histamine into the intestinal lining (Mengistu *et al.*, 2022; Mengesha *et al*., 2022). This process lead to muscle contraction, mucus secretion, and widening of blood vessels in the small intestines *(*Mengistu *et al.*, 2022; Mengesha *et al*., 2022). Ricinoleic acid binds to an enzyme called sodium potassium adenosine triphosphatase (Na+/K-ATPase), inhibiting its action. Consequently, this inhibits the reabsorption of sodium chloride and water (Ayele *et al.*, 2023; Yacob *et al.*, 2016; Rahman *et al.*, 2015). This inhibition triggers the release of nitric oxide, further aggravating diarrhea (Tadesse *et al.*, 2017). Loperamide, a common antidiarrheal drug, works by stimulating the μ-opioid receptors in the large intestine, reducing muscle movement and the release of acetylcholine. This

action enhances electrolyte and water absorption while decreasing excretion (Abdela, 2019; Ayele *et al.*, 2023).

In this study, antidiarrheal effects of 80% hydroalcoholic root extract of *Impatiens ethiopica* and its fractions on castor oil-induced diarrhea in mice model is illustrated in Table 3. The results revealed that both the crude extract and its fractions significantly ($p < 0.05$) reduced the frequency, quantity, and the total weight of feces produced in mice with castor-oil-induced diarrhea in a dose-dependent manner. The 80% methanol extract showed a significant ($p < 0.05$) antidiarrheal activity, with 75.26% of inhibition of defecation and a notably delaying onset of 98.80 min at 400 mg/kg. Interestingly, these findings were comparable to the positive control, loperamide (3 mg/kg), with a 78.49% of defecation inhibition and a 102.80 min of delayed onset. Our finding is supported by related findings reported for the antidiarrheal activity of extract of other medicinal plants (Mengistu *et al*., 2022; Abdela, 2019).

Similarly, after separating the crude extract into its fractions, the methanol and aqueous fractions emerged as the most potent, showing dose-dependent delays in diarrhea onset by 84.40 and 81.80 min, respectively, at 400 mg/kg dose. Equally, the methanol and aqueous fractions also reduced the number of wet feces output in a dose-dependent manner, with 75.60% and 70.03% percentage of inhibition of feces output, respectively, at a dose of 400 mg/kg. The results from the other fractions are presented in Table 3. These findings are consistent with previous reports on the antidiarrheal activity of fractions of other medicinal plants (Mengesha *et al.*, 2022; Ayalew *et al.*, 2022).

Compounds from medicinal plants are known to inhibit prostaglandin synthesis, which might explain the antidiarrheal effects observed in this study. This inhibition potentially leads to increased absorption and reduced secretion of fluids and electrolytes (Bahekar and Kale, 2015; Kifle *et al*. 2021).

Table 1 *In vivo* antidiarrheal effects of root extract and its fractions on castor oil-induced diarrhea model

Treatment types and dose	Onset of diarrhea in min	Total number of wet feces in 4 h	% Inhibition of defecation	Total number of feces output in 4 h	Total weight of wet feces (g) in 4 h	% Inhibition of feces output	Total weight of feces output (g) in 4 h
DW 10 ml/kg	33.60±6.39[a]	18.60±1.72	0[a]	18.60±1.72	0.82±0.18	0[a]	0.88±0.19
Lop. 3 mg/kg	102.80± 6.66[b]	4.00±0.31	78.49	4.00±0.31	0.21±0.01	74.39[b]	0.24±0.03
Extract (mg/kg)							
IE 100	69.40±3.99[c]	6.60±0.50	64.51	6.60±0.50	0.33±0.02[ac]	59.76[c]	0.37±0.03
IE 200	81.80±2.03[d]	4.80±0.66	74.19	4.80±0.66	0.23±0.02[ac]	71.95[d]	0.30±0.04
IE 400	98.80±3.30[e]	4.60±0.50[a]	75.26	4.60±0.50	0.24±0.03[ac]	70.73[d]	0.27±0.02
Fractions(mg/kg)							
PE 100	64.20±1.39	8.00±0.32	56.98	8.60±0.40	0.27±0.02	67.07	0.27±0.02
PE 200	70.00±0.71	6.00±0.32	67.74	6.60±0.25	0.24±0.03	70.03	0.25±0.01
PE 400	72.00±0.71	4.60±0.40	75.26	5.60±0.40	0.23±0.02	71.95	0.25±0.02
CF 100	66.20±0.86	7.00±0.32	62.36	7.60±0.51	0.28±0.01	65.85	0.29±0.02
CF 200	71.20±1.16	6.00±0.45	67.74	7.20±0.38	0.26±0.01	68.29	0.28±0.01
CF 400	73.20±0.66	5.00±0.32	73.11	5.80±0.58	0.24±0.01	70.03	0.25±0.01
MF 100	71.00±0.71	5.60±0.25	69.89	6.00±0.32	0.25±0.01	69.51[e]	0.26±0.02
MF 200	75.00±0.71	4.40±0.25	76.34	6.20±0.38	0.24±0.02	70.03[e]	0.27±0.03
MF 400	84.40±1.69[b]	3.80±0.38	79.56	5.40±0.25	0.20±0.01	75.60[f]	0.22±0.01
AF 100	71.00±0.71	7.00±0.45	62.36	7.80±0.58	0.26±0.01	68.29	0.28±0.03
AF 200	75.80±0.86	5.60±0.40	69.89	6.40±0.40	0.25±0.01	69.51	0.26±0.01
AF 400	81.80±1.02	5.20±0.37	72.04	6.20±0.49	0.24±0.01	70.03	0.26±0.03

All values are expressed as mean ± standard error of the mean (SEM); (n = 5); Data were analyzed by one-way ANOVA followed by Tukey post hoc test; NC: Negative control, Lop: Loperamide; IE100: *I. ethiopica* root extract (100 mg/kg), IE 200: *I. ethiopica* root extract (200 mg/kg), IE 400: *I. ethiopica* root extract (400 mg/kg), PE100: Petroleum ether fraction(100 mg/kg), PE200: Petroleum ether fraction (200 mg/kg), PE400: Petroleum ether fraction (400 mg/kg), CF100: Chloroform fraction (100 mg/kg), CF200: Chloroform fraction (200 mg/kg), CF400: Chloroform fraction (400 mg/kg), MF100: Methanol fraction (100 mg/kg), MF200: Methanol fraction (200 mg/kg), MF400: Methanol fraction (400 mg/kg), AF 100: Aqueous fraction (100 mg/kg), AF200: Aqueous fraction (200 mg/kg), AF 400: Aqueous fraction (400 mg/kg); Data followed by different letter within the same column are significantly different at $p < 0.05$.

3.3.2. Effect on castor oil-induced intestinal transit by charcoal marker

Since the majority of antidiarrheal drugs have the ability to reduce gastrointestinal motility, the charcoal meal method was selected (Tadesse *et al.*, 2017)in order to track the movement of the gastrointestinal contents during this experiment. Dose dependent inhibitory effect was observed in all types of tested doses. This effect is consistent with other reports which indicate the dose dependent antidiarrheal activity of extract and fractions of other medicinal plants (Tadesse et al., 2017; Feyisa *et al.*, 2020; Ayalew et al., 2022). The root extracts inhibited intestinal motility by 53.89%, 60.79% and 75.88% for 100, 200, and 400 mg/kg respectively. The highest dose of the root extract (400 mg/kg) showed a significant anti-motility effect (75.88%, $p < 0.01$), as compared with 100 mg/kg of extract.

A delay in the motility of intestinal muscles and reductions of frequency of stooling promotes further absorptions of water from feces and reduces the watery nature of excrement. Based on this findings, it is likely that reductions in the propulsive movements may be attributed by the anti-motility properties of the tested doses (Yacob *et al.*, 2016; Ayele *et al.*, 2023). This finding is further supported by the antimotility effect of the leaves of *L. camara* and, its component (Lanthadne A) and fractions of the leaves of this plant (Tadesse *et al*., 2017).

Table 2 *In vivo* antidiarrheal effects of root extract and fractions on charcoal meal transit in castor oil-induced motility in mice model

Treatment	Dose (mg/kg)	Mean length of small intestine in cm	Mean distance travelled by charcoal marker in cm	% Charcoal meal transit (Peristalsis index)	% Inhibition
DW	10 ml/kg	50.20±2.05	45.20±2.27	90.03[a]	0
Lop.	3 mg/kg	56.60±0.97	4.80±0.51	8.49[b]	90.58
Crude extract					
IE	100	54.20±1.52	22.50±0.89	41.51[c]	53.89
IE	200	52.40±1.29	18.50±0.55	35.30[d]	60.79
IE	400	57.10±0.89	12.40±0.62	21.71[e]	75.88
Fractions					
PE	100	54.20±1.19	24.00±0.35	44.28[f]	50.81
PE	200	54.00±0.16	20.60±0.48	38.14[g]	57.63
PE	400	51.80±0.51	19.60±0.43	37.83[g]	57.98
CF	100	55.12±0.68	22.90±0.64	41.54[h]	53.85
CF	200	54.80±0.73	22.80±0.46	41.60[h]	53.79
CF	400	57.60±0.53	19.80±0.34	34.37[i]	61.82
MF	100	51.40±0.87	22.60±0.43	43.96[j]	51.17
MF	200	55.00±0.45	20.20±0.68	36.72[k]	59.21
MF	400	53.00±0.71	16.24±0.41	30.64[l]	65.96
AF	100	53.70±0.58	25.20±0.51	46.92[m]	47.87
AF	200	52.70±0.30	22.98±0.59	43.60[n]	51.57
AF	400	56.40±0.51	20.40±0.48	36.17[o]	59.82

Note: values are articulated as mean ± standard error of the mean (SEM); (n = 5); Data were analyzed by one-way ANOVA followed by Tukey post hoc test; DW: Distilled water, Lop: Loperamide; IE100: *I. ethiopica* root extract (100 mg/kg), IE 200: *I. ethiopica* root extract (200 mg/kg), IE 400: *I. ethiopica* root extract (400 mg/kg), PE100: Petroleum ether fraction(100 mg/kg), PE200: Petroleum ether fraction (200 mg/kg), PE400: Petroleum ether fraction (400 mg/kg), CF100: Chloroform fraction (100 mg/kg), CF200: Chloroform fraction (200 mg/kg), CF400: Chloroform fraction (400 mg/kg, MF100: Methanol fraction (100 mg/kg), MF200: Methanol fraction (200 mg/kg), MF400: Methanol fraction (400 mg/kg), AF 100: Aqueous fraction (100 mg/kg), AF200: Aqueous fraction (200 mg/kg), AF 400: Aqueous fraction (400 mg/kg); Data followed by different letter within the same column are significantly different at $p < 0.05$.

3.3.3. Anti-enteropooling effect

The purpose of this test is to assess the plant's antidiarrheal activity by lowering intestinal fluid accumulation. An experimental drug's effect on intestinal fluid secretion is evaluated in mice using the enteropooling (accumulation of fluid into the small intestine) approach. Watery diarrhea is caused by a large amount of water being secreted into the intestinal lumen as a result of the activation of chloride channels. This affects the inward movement of chloride ions from the cell (Teferi *et al.*, 2019). The reductions in both the volume and mass of intestinal content are the primary metrics for evaluating this activity.

All test doses of the hydroalcoholic extract of *I. ethiopica* reduced intraluminal fluid accumulation in mice compared to the vehicle treated group (Table 5). For dosages of 100, 200, and 400 mg/kg of the extract, there was a reduction in the percentage volume of small intestine content by 58.00%, 74.00% and 80.00%, respectively. The total mass of the intestinal contents was likewise significantly (p<0.05) reduced by the extract in a dose-dependent manner. Furthermore, a notable variation in the weight and volume of intestinal fluid between the three test doses of the root extracts was observed. For instance, when compared to doses of 100 mg/kg, the average weight and volume of small intestine content were significantly decreased at 400 mg/kg (p <0.05; 53.26%, 80%) respectively. The effect of this dose was found to have comparable antidiarrheal activity with 3 mg/kg loperamide (p <0.05; 72.82%, 88%) respectively. This conclusion was consistent with the findings of a study that examined the extract, aqueous and methanol fractions of *Lantana camara* extract (Mengistu *et al.*, 2015). The MF reduced significantly (p <0.5) the amount of fluids in the intestine by 70.00%; 74.00% and 84.00% at tested doses of 100, 200, and 400 mg/kg respectively compared to control.

Table 3 *In vivo* antidiarrheal effects of root extract and its fractions on castor oil-induced enterpooling in mice model

Treatment	VSIC (ml)	% reduction VSIC	WSIC (g)	% reduction WSIC
NC 10 ml/kg	1.00±0.05	0[a]	1.84±0.02	0
PC 3 mg/kg	0.12±0.02	88.00[b]	0.50±0.04	72.82
Crude extract				
IE100	0.42±0.04	58.00[c]	1.46±0.05	20.65
IE200	0.26±0.02	74.00[d]	1.16±0.05	36.95
IE400	0.20±0.03[fg]	80.00[e]	0.86±0.05	53.26
Fractions				
PE 100	0.48±0.04	52.00[f]	1.58±0.04	14.13
PE 200	0.28±0.04	72.00[g]	1.34±0.05	27.17
PE 400	0.22±0.02	78.00[h]	1.02±0.06	44.56
CF 100	0.42±0.04	58.00[i]	1.70±0.05	7.60
CF 200	0.40±0.03	60.00[i]	1.28±0.04	30.43
CF 400	0.34±0.02	66.00[j]	1.02±0.06	44.56
MF 100	0.30±0.03	70.00[k]	1.38±0.06	25.00
MF 200	0.26±0.02	74.00[l]	1.16±0.05	36.95
MF 400	0.16±0.02	84.00[m]	0.92±0.04	50.00
AF 100	0.42±0.04	58.00[n]	1.46±0.04	20.65
AF 200	0.36±0.02	64.00[o]	1.30±0.03	29.34
AF 400	0.26±0.02	74.00[p]	0.96±0.02	47.82

Note: values are expressed as mean ± standard error of the mean (SEM); (n = 5); Data were analyzed by one-way ANOVA followed by Tukey post hoc test; NC: Negative control, PC: Positive control; VSIC: volume of small intestine content, WSIC: weight of small intestine content, IE100: *I. ethiopica* root extract (100 mg/kg), IE 200: *I. ethiopica* root extract (200 mg/kg), IE 400: *I. ethiopica* root extract (400 mg/kg), PE100: Petroleum ether fraction(100 mg/kg), PE200: Petroleum ether fraction (200 mg/kg), PE400: Petroleum ether fraction (400 mg/kg), CF100: Chloroform fraction (100 mg/kg), CF200: Chloroform fraction (200 mg/kg), CF400: Chloroform fraction (400 mg/kg), MF100: Methanol fraction (100 mg/kg), MF200: Methanol fraction (200 mg/kg), MF400: Methanol fraction (400 mg/kg), Aqueous fraction (100 mg/kg), AF200: Aqueous fraction (200 mg/kg), AF 400: Aqueous fraction (400 mg/kg); Data followed by different letter within the same column are significantly different at $p < 0.05$.

3.3.4. *In vivo* antidiarrheal index (ADI)

The antidiarrheal index (ADI) evaluates the combined effects of various diarrheal symptoms, including the frequency of bowel movements, the onset of diarrheal stools, and the accumulation of intestinal fluid to determine an extract's effectiveness in treating diarrhea (Andargie *et al.*, 2022; Yacob *et al.*, 2016).

Dose-dependent antidiarrheal index were observed for the fractions. The highest test dose of the extract with the highest ADI score has highest antidiarrheal activity. The antidiarrheal effectiveness of methanol fractions at 400 mg/kg was 82.63. The standard drug loperamide produced a maximum index of 113.55 as presented in Table 4.

Table 4 *In vivo* antidiarrheal indices (ADI) of 80% hydroalcoholic extract of Impatiens ethiopica and its fractions

Treatment	Doses mg/kg	Dfreq (%)	Gmeq (%)	Pfreq (%)	*In vivo* ADI
Extract					
	IE100	69.40	53.89	64.51	71.72
	IE200	81.80	60.79	74.19	82.63
	IE400	98.80	75.88	75.26	82.63
Fractions					
	PE100	72.00	50.81	56.98	59.29
	PE200	66.20	57.63	67.74	63.69
	PE400	71.20	57.98	75.26	67.73
	CF100	73.20	53.85	62.36	62.64
	CF200	71.00	53.79	67.74	63.72
	CF400	75.00	61.82	73.11	69.72
	MF100	84.40	51.17	69.89	67.08
	MF200	71.00	59.21	76.34	68.46
	MF400	75.80	65.96	79.56	73.54
	AF100	81.80	47.87	62.36	62.50
	AF200	75.80	51.57	69.89	64.89
	AF400	81.80	59.82	72.04	70.64
Standard drug					
	Lop.3 mg/kg	205.95	90.58	78.49	113.55

Note: Dfreq: Delay in the onset of diarrhea (min), Gmeq: Gut meal movement reduction in intestinal transit, Pfreq: Purging frequency in quantity of wet feces inhibition; Lop: Loperamide (3 mg/kg); ADI: Antidiarrheal index; IE100: *Impatiens ethiopica* root extract (100 mg/kg), IE200: *Impatiens ethiopica* root extract (200 mg/kg), IE400: *Impatiens ethiopica* root extract (400 mg/kg), PE100: Petroleum ether fraction(100 mg/kg), PE200: Petroleum ether fraction (200 mg/kg), PE400: Petroleum ether fraction(400 mg/kg), CF100: Chloroform fraction (100 mg/kg), CF200: Chloroform fraction (200 mg/kg), CF400: Chloroform fraction (400 mg/kg), MF100: Methanol fraction (100 mg/kg), MF200: Methanol fraction (200 mg/kg), MF400: Methanol fraction (400 mg/kg), AF100: Aqueous fraction (100 mg/kg), AF200: Aqueous fraction (200 mg/kg), AF400: Aqueous fraction (400 mg/kg).

3.4. Structural elucidation of the isolated compound (SY-1)

The crude extract was fractionated based on solubility, and further purification was done through column chromatography using silica gel, resulted in the isolation of a light yellow needle-like crystalline substance, which was given the code name SY-1.

Compound SY-1 was isolated a light-yellow needle-like crystalline substance from the root extract of *I. ethiopica*. The Rf value of SY-1 was found to be 0.45 on normal phase-TLC using $CHCl_3$: MeOH (9:1) as a solvent system. When observed at a wavelength of 254 nm on a TLC plate, SY-1 exhibited a characteristic bluish color, which is often associated with the fluorescence emitted by coumarins. This suggested that SY-1 could potentially be a coumarin. The negative mode ESI-mass spectrum (Figure 2) of SY-1 showed a pseudomolecular ion at *m/z* = 191.12 $[M-H]^-$, indicating a relative molecular weight of 192 mu for SY-1. This, along with the data obtained from the ^1H and ^{13}C-NMR analysis, led to the determination of a molecular formula of $C_{10}H_8O_4$.

In the ^{1}H-NMR spectrum of SY-1, two doublet protons were observed at δ 6.20 (*J*=12 Hz; H-3) and 7.49 (*J*=12 Hz; H-4), indicating the presence of two *ortho*-coupled aromatic protons at positions H-3 and H-4 of the alpha-pyrone unit, respectively. Two singlet at δ 6.77 (H-8) and 6.85 (H-5) each integrating for 1H, indicated the existence of two *para*-assigned aromatic protons at positions H-5 and H-8, respectively. Additionally, a singlet integrating for 3H at δ 3.87 indicated the presence of a methoxy (-OCH_3) group in the structure of SY-1.

The ^{13}C-NMR spectrum revealed ten carbon atoms in SY-1, as evident by ten visible signals in the spectrum. Of these, the most downfield signal resonating at δ 161.26 indicated the carbonyl carbon of the cyclic ester group in the alpha-pyrone unit. Four aromatic methine carbon signals were clearly observed in the DEPT-135 spectral data of SY-1, which are assignable to δ 103.22 (C-8), 113.47 (C-5), 107.53 (C-3) and 143.23 (C-4). A carbon signal at δ 56.44 indicated the presence of a methoxy (-OCH_3) group. Four signals resonating at δ 111.51, 144.01, 149.71 and 150.32 were assigned to the remaining quaternary aromatic carbons of C-4a, C-6, C-7 and C-1a, respectively.

Based on the ESI-MS and NMR spectral data, SY-1 was found to be consistent with a coumarin derivative containing one hydroxyl (-OH) and one methoxy (OCH_3) group. At the end, compound SY-1 was unambiguously identified as 7-hydroxy-6-methoxycoumarin (Figure 4), and its structure was further confirmed by comparing its spectral data with previously reported literatures (Bhatt Mehul *et al.*, 2011; Darmawan *et al.*, 2012; Zhang *et al.*, 2011).

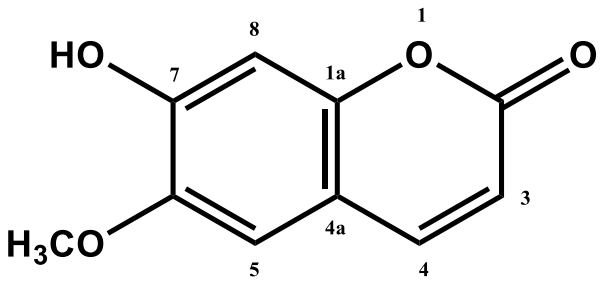


Figure 9 Chemical structure of compound SY-1.

3.5. *In vivo* antidiarrheal effect of scopoletin

3.5.1. Effects on castor oil-induced diarrhea in mice

In this study, scopoletin, found in *I. ethiopica,* was also investigated. The findings revealed that scopoletin significantly reduced the frequency, quantity, and the total weight of feces produced in mice experiencing castor oil-induced diarrhea compared to the negative control (Table 5). Additionally, scopoletin delayed the onset of diarrhea, most notably at doses of 20 mg/kg (96.60 min delay) and 40 mg/kg (99.80 min delay), without a clear dose-dependent pattern though.

Interestingly, the effect of scopoletin on reducing defecation (78.49% inhibition) was comparable to loperamide (78.49% inhibition) at a dose of 40 mg/kg. Although scopoletin notably reduced total feces output compared to the negative control, this reduction did not follow a consistent dose-dependent trend across the three doses tested (10 mg/kg: 70.73%; 20 mg/kg: 71.95%; 40 mg/kg: 71.95%), yet it remained similar to loperamide's effect (3 mg/kg: 78.49%).

At a dose of 40 mg/kg, scopoletin had a comparable effect in mice with that of the positive control, loperamide (3 mg/kg), suggesting its potential as an antidiarrheal agent on its own or as a basis for developing more potent compounds.

Table 5 Antidiarrheal activity of scopoletin on castor oil-induced diarrhea in mice model

Treatment types and dose	Onset of diarrhea in min	Total number of wet feces in 4 h	%Inhibition of defecation	Total number of feces output in 4 h	Total weight of wet feces (g) in 4 h	% Inhibition of feces output	Total weight of feces output (g) in 4 h
DW 10 ml/kg	33.60±6.39^{a}	18.60±1.72	0^{a}	18.60±1.72	0.82±0.18	0^{a}	0.88±0.19
Lop 3 mg/kg	102.80± 6.66^{b}	4.00±0.31	78.49^{b}	4.00±0.31	0.21±0.01	74.39^{b}	0.24±0.03
Scopoletin(mg/kg)							
10	94.40±0.92^{c}	6.40±0.40	65.59^{c}	7.60±0.51	0.24±0.02	70.73^{c}	0.26±0.01
20	96.60±1.28^{b}	5.60±0.50	69.89^{c}	6.80±0.58	0.23±0.02	71.95^{c}	0.25±0.01
40	99.80±1.20^{b}	4.00±0.31	78.49^{b}	5.60±0.24	0.23±0.05	71.95^{c}	0.24±0.02

All values are expressed as mean ± standard error of the mean (SEM); (n = 5); Data were analyzed by one-way ANOVA followed by Tukey post hoc test; NC: Negative control, Lop: Loperamide; Data followed by different letter within the same column are significantly different at $p < 0.05$.

3.5.2. Effects on castor oil-induced intestinal transit by charcoal marker in mice

Scopoletin showed a maximum anti-motility effect at 40 mg/kg dose (89.37%, $p < 0.05$), which was comparable with loperamide 3 mg/kg (90.58%, $p < 0.05$) (Table 6). Delays in intestinal

muscle motility and decreased frequency of stools encourage additional water absorption from feces and lessen the watery consistency of excrement.

Table 6 Antidiarrheal effect of scopoletin on charcoal meal transit in castor oil-induced motility in mice model

Treatment	Dose (mg/kg)	Mean length of small intestine in cm	Mean distance travelled by charcoal marker in cm	% Charcoal meal transit (Peristalsis index)	% Inhibition
DW	10 ml/kg	50.20±2.05	45.20±2.27	90.03[a]	0[a]
Lop	3 mg/kg	56.60±0.97	4.80±0.51	8.49[b]	90.58[b]
Scopoletin (mg/kg)					
	10	52.56±1.10	14.50±0.76	27.58[c]	69.36[c]
	20	52.60±1.04	13.60±0.43	25.85[c]	71.28[c]
	40	52.20±0.80	5.00±0.45	9.57[b]	89.37[b]

Note: values are articulated as mean ± standard error of the mean (SEM); (n = 5); Data were analyzed by one-way ANOVA followed by Tukey post hoc test; DW: Distilled water, Lop: Loperamide; Data followed by different letter within the same column are significantly different at $p < 0.05$.

3.5.3. Anti enteropooling test

Scopoletin at 40 mg/kg significantly ($p<0.05$; 86.00%) decreased the intraluminal fluid accumulation when compared to the negative control and showed comparable effect with the standard drug loperamide 3 mg/kg ($p<0.05$; 88.00%).

Table 7 Antidiarrheal activity of scopoletin on castor oil-induced enterpooling in mice model

Treatment	VSIC (ml)	% reduction VSIC	WSIC (g)	% reduction WSIC
NC 10 ml/kg	1.00±0.05	0[a]	1.84±0.02	0[a]
PC 3 mg/kg	0.12±0.02	88.00[b]	0.50±0.04	72.82[b]
Scopoletin				
10 mg/kg	0.26±0.02	74.00[c]	0.88±0.02	52.17[c]
20 mg/kg	0.22±0.04	78.00[d]	0.78±0.04	57.60[d]
40 mg/kg	0.14±0.02	86.00[b]	0.48±0.04	73.91[b]

Note: values are expressed as mean ± standard error of the mean (SEM); (n = 5); Data were analyzed by one-way ANOVA followed by Tukey post hoc test; NC: Negative control, PC: Positive control; VSIC: volume of small intestine content, WSIC: weight of small intestine content, IE100: SY40: Isolated compound (40 mg/kg), SY20: Isolated compound (20 mg/kg), SY10: Isolated compound (10 mg/kg; Data followed by different letter within the same column are significantly different at $p < 0.05$.

3.5.4. In vivo antidiarrheal index (ADI)

Scopoletin at doses of 10, 20, and 40 mg/kg showed an antidiarrheal index of 75.45, 78.36, and 88.79, respectively. More importantly, at the maximum dose scopoletin had an ADI value of 88.79, which was the highest score among the test substances. This implies the effectiveness of scopoletin in treating diarrhea.

Table 8 *In vivo* antidiarrheal index (ADI) of scopoletin from the root of Impatiens ethiopica

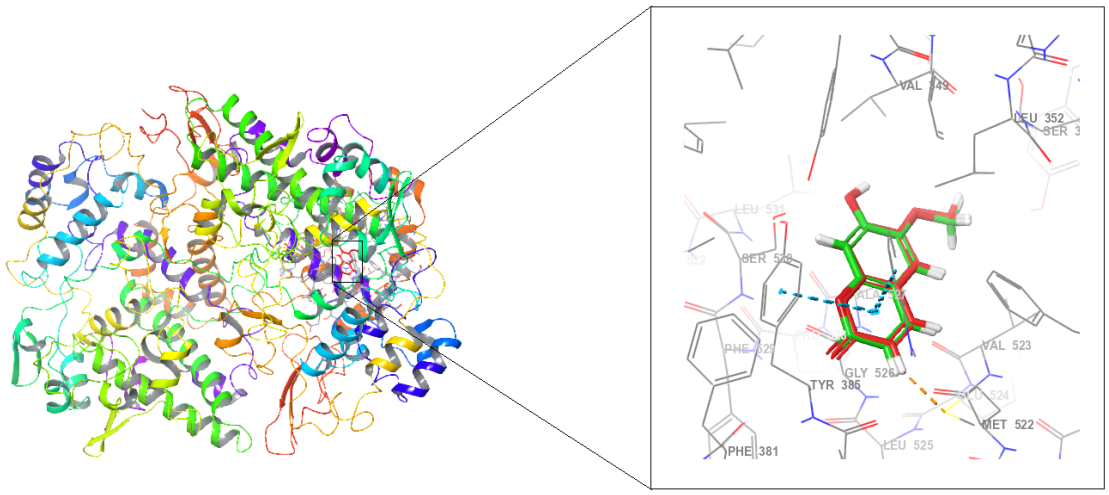
Treatment	Doses mg/kg	Dfreq (%)	Gmeq (%)	Pfreq (%)	*In vivo* ADI
Compound					
	Scopoletin 10	94.40	69.36	65.59	75.45
	Scopoletin 20	96.60	71.28	69.89	78.36
	Scopoletin 40	99.80	89.37	78.49	88.79
Standard drug					
	Lop.3 mg/kg	205.95	90.58	78.49	113.55

Note: Dfreq: Delay in the onset of diarrhea (min), Gmeq: Gut meal movement reduction in intestinal transit, Pfreq: Purging frequency in quantity of wet feces inhibition; Lop: Loperamide (3 mg/kg); ADI: Antidiarrheal index; SY40: Isolated compound (40 mg/kg), SY20: Isolated compound (20 mg/kg), SY10: Isolated compound (10 mg/kg).

3.6. Molecular docking analysis

The study utilized molecular docking, an essential technique for examining the potential mechanisms of biologically active compounds. Some potential antidiarrheal targets such as prostaglandin synthase (COX-2, COX-1), TNF-α, IL-β and iNOS were screened. Based on the fitness score COX-2 was found to have favorable interactions with SY-1.

In this study, scopoletin (1) was specifically docked into the active site of the PGS enzyme (PDB ID: 4COX; Chain A, B) using Maestro V.13.5 software by Schrodinger 2023-1 Suite. Figure 7 and 8 illustrates 2D and 3D representations of scopoletin (1) docked within the active site of PGS respectively. Based on the molecular docking study, scopoletin exhibited a favorable docking score of −7.941 kcal/mol. This was attributed to its ability to engage in hydrogen and pi-pi interactions with specific residues in the binding site of the PGS enzyme, namely SER530, TRP387, and TYR385. Of these amino acid residues, scopoletin (1) establishes a hydrogen bond with the amino acid residue SER530 within the PGS enzyme's active site (Figure 7) and pi-pi stacking with TYR385and TRP387 amino acid residues. Thus, we can suggest from the molecular docking study that scopoletin (1) negatively regulates PGS.



(A) **(B)**

Figure 10 (A): 3D representation of scopoletin (1) docked within the active site of COX; (B): The 3D zoomed view of the scopoletin (1) interaction

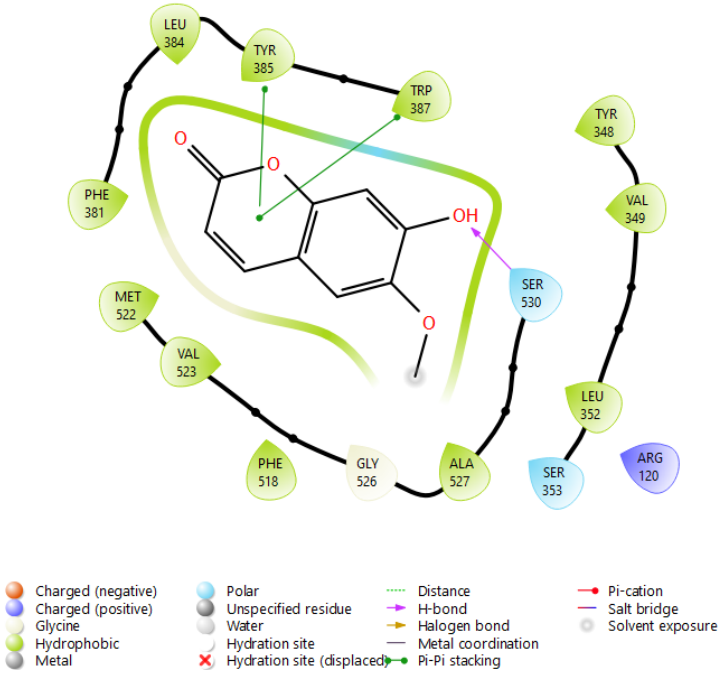


Figure 11 2 D model of scopoletin (1) showing interactions with residues at the COX enzyme.

In developing countries such as Ethiopia, people rely on medicinal plants to treat diarrhea. *I. ethiopica* is widely used in Ethiopia for treating of various illnesses. In this study, the crude extract of *I. ethiopica* exhibited antidiarrheal effects in mice induced with castor oil-induced diarrhea, as detailed in Tables 1-4. These findings are align with prior reports highlighting similar antidiarrheal activity of extracts from other medicinal plants (Mengesha *et al.*, 2022; Ayalew *et al.*, 2022).

Furthermore, this study identified scopoletin for the first time in *I. ethiopica*, although it has been previously recognized in several other plants such as *Acer saccharum*, *Artemisia annua, Scopolia carniolica, Arabidopsis thaliana* (Antika *et al.,* 2022)*, Ipomoea reniformis* (Convolvulaceae) (Bhatt Mehul *et al.*, 2011), and *Saussurea eopygmaea* (Saussurea) (Zhang *et al.*, 2011). Its presence has also been reported in the *Impatiens* genus, specifically in *Impatiens balsamina* (Singh *et al.,* 2017). Scopoletin demonstrated strong antidiarrheal activity in mice, as shown in Tables 5-8.

Antidiarrheal medications often work through various mechanisms aiming to restore gastrointestinal fluid and electrolyte balance, involving targets like intestinal mu-opioid receptors, prostaglandin, serotonin, and histamine receptors, as well as ion exchangers and transporters such as the Na+/k+ exchanger (Mengesha *et al.*, 2022; Mengistu *et al.*, 2022; Yacob et al., 2016; Ayele *et al.*, 2023; Rahman *et al*., 2015). Through molecular docking analysis, scopoletin exhibited strong binding affinity toward prostaglandin synthase (PGS) (COX-2; PDB ID: 4COX; docking score of −7.941 kcal/mol) via hydrogen bonding with SER530 and pi-pi stacking interactions with TYR385 and TRP387. This suggests that scopoletin appears to inhibit prostaglandin synthase (PGS), although experimental evidence is needed. Compounds from medicinal plants have been known to inhibit prostaglandin synthesis, potentially explaining the observed antidiarrheal effects in this study, which could lead to increased absorption and reduced secretion of fluids and electrolytes (Sakthivel *et al.*, 2022; Lee and Kim, 2015).

Notably, scopoletin possess a variety of pharmacological properties, including antibacterial, ant proliferative, antioxidant, and ant inflammatory properties (Sakthivel *et al*., 2022; Wan Osman *et al.*, 2017; Firmansyah *et al.*, 2021). Studies have demonstrated ant inflammatory activity of scopoletin in mice with croton oil-induced edema, carrageenan-induced paw edema, and lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cells, where it reduced inflammation markers such as TNF-α, PGE2 and COX-2 expressions, among others (Lee and Kim, 2015;

Sakthivel *et al*., 2022). Hence, in this study, scopoletin is implicated at least partially responsible for the antidiarrheal activity observed in *I. ethiopica*.

4. Conclusion

The present study effectively demonstrated the *in vivo* antidiarrheal effects of *I. ethiopica* roots in mice triggered with castor oil-induced diarrhea. Scopoletin, found in the most active methanol-soluble fraction, displayed the highest antidiarrheal activity. This suggests that scopoletin is partly or fully responsible for the antidiarrheal properties of the plant. Scopoletin was observed in the molecular docking analysis to inhibit PGS, potentially leading to enhance electrolytes and water absorption, although further experimental support is necessary. The study supports the traditional medicinal usage of *I. ethiopica* in treating diarrhea and highlights scopoletin as a potential standalone antidiarrheal agent or as a foundation for developing more potent compounds.

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