In vivo Antidiarrheal Activity of the Extracts and a Major Compound Isolated from the Roots of *Sida ovata* Forssk

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This is to certify that the thesis prepared by Tesfa Begashaw entitled **"*In vivo* Antidiarrheal Activity of the Extracts and a Major Compound Isolated from the Roots of *Sida ovata* Forssk",** and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacognosy, complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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Abstract

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Addis Ababa University, 2024

Diarrhea is a common health issue often dealt with traditional remedies, especially in developing countries, where medicinal plants are frequently used. *Sida ovata* Forssk (*Malvaceae*) is a medicinal plant used in Nigerian folkloric medicine for the treatment of diarrhea, and in Ethiopia it is employed for managing various disorders including inflammation, wound and skin infection. However, despite its traditional uses, there is a lack of scientific evaluation regarding its claimed activities. This study aimed to assess the antidiarrheal activities of the 80% MeOH extract of *S. ovata* root and its major constituent using mice models. The hydroalcoholic extract was obtained by macerating the roots of *S. ovata* in 80% MeOH. The extract was subjected to solvent fractionation and isolation, which led to the isolation of a sterol tentatively identified as stigmasterol. Structural elucidation of the isolated compound was carried out by spectroscopic techniques (^{1}H- and ^{13}C-NMR). The antidiarrheal activity of the total extract, solvent fractions, and the isolated compound was assessed using castor oil-induced diarrhea, castor oil-induced enteropooling, and castor oil-induced gastrointestinal motility tests. Acute toxicity test results of this study indicated that the 80% MeOH root extract of *S. ovata* was safe by oral route up to a dose of 2000 mg/kg. Oral dose levels of 100, 200, and 400 mg/kg were used for the crude extract and solvent fractions, whilst doses of 25, 50, and 100 mg/kg were employed for the isolated compound. Similarly, the isolated compound appeared to be safe at the maximum dose tested

(100 mg/kg). *In vivo* antidiarrheal index of the 80% MeOH extract and solvent fractions of *S. ovata* roots increased in a dose dependent manner. The highest antidiarrheal index was observed at the maximum dose of the test substances. Among the solvent fractions, the MeOH fraction showed the highest antidiarrheal index at all dose levels, although these values are less than that of the 80% MeOH extract. Stigmasterol produced dose-dependent antidiarrheal indices with the maximum effect at 100 mg/kg. In conclusion, the present study provided evidence that the roots of *S. ovata* possess genuine antidiarrheal activity supporting the folkloric assertion of the plant.

Key words: *Sida ovata*, traditional medicine, antidiarrheal effect, stigmasterol.

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List of Abbreviations and Acronyms

^{1}H NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon thirteen nuclear magnetic resonance
AAU	Addis Ababa University
ADI	Antidiarrheal Index
ANOVA	Analysis of Variance
DEPT	Distortionless enhancement by polarization transfer
Dfreq	Delay in defecation time as percentage of negative control
DW	Distilled water
EPHI	Ethiopia Public Health Institute
FT-NMR	Fourier transform nuclear magnetic resonance
Gmeq	Gut meal travel reduction as percentage of negative control
LD_{50}	Half lethal dose
OECD	Organization for economic co-operation and development
ORS	Oral rehydration salt
ORT	Oral rehydration therapy
Pfreq	Reduction in purging frequency in the number of wet stools as percentage of negative control
R_f	Retention factor
TLC	Thin layer chromatography
TMS	Tetramethylsilane
UNICEF	United Nations Children's Fund
UV	Ultra-violet
WHO	World health organization

1. Introduction

1.1 Diarrhea

Diarrhea is a common medical condition that is characterized by increased frequency of bowel movements and passage of three or more loose or watery stools per day (Ugboko *et al*., 2021; Gidudu *et al*., 2011). Commonly, increased frequency (≥3) of defecation or increased stool weight (>200 gm/day) is a major criterion used by physicians to conclude as diarrhea (Schiller *et al*., 2016). Based on length of time, WHO (World Health Organization) categorizes diarrhea into three classes: acute, if it goes for less than 2 weeks; persistent, if it continues between 2 and 4 weeks; and chronic, if it lasts longer than 4 weeks (Florez *et al*., 2020). Based on severity it is classified as mild, if no major fluid loss or electrolyte disturbances occurred and severe, if fluid loss and electrolyte disturbances require treatment (Blaser *et al*., 2015). Based on the pathophysiologic mechanism, diarrhea is classified as osmotic, secretory, motility associated and inflammatory (Zella and Israel, 2012).

Although, this medical condition has been recognized as one of the most important health problems in ancient times, it continuing to cause severe mortality and morbidity to human beings till today (Pandhare *et al*., 2018). A major burden of diarrhea mortality is occurred among poorest people in countries of sub-Saharan Africa and South Asia (Turin and Ochoa, 2014).

Under five children who live in Africa and South-East Asia are the major victims of this condition (Yeshaw *et al*., 2020). Shortage of safe drinking water and sanitation facilities are the main contributing factors for diarrhea in these regions (Beyene and Melku, 2018). Among 15 countries of the world with high burden of diarrhea and pneumonia, Ethiopia is ranked as fifth, and diarrhea is the second leading cause of death after respiratory infections in children under 5 years (Zelelie *et al*., 2023).

Diarrhea is caused by either infectious or non-infectious etiologies. Infectious diarrhea is caused by bacterial, parasitic and viral pathogens. The major etiologic agents for infectious diarrhea caused by bacteria are *Escherichia coli*, *Salmonella* spp., *Shigella* spp. and *Vibrio cholera*. Most of the mentioned bacteria pathogens are related to food and water contamination (Hoque *et al*., 2012). The most common parasites causing diarrhea globally are *Cryptosporidium parvum* and *Giardia lamblia*. Rotavirus, norovirus, sapvirus, adenovirus and astrovirus are important viral pathogens that cause diarrhea. Worldwide, rotavirus is the most common cause of diarrhea and diarrheal mortality in infants and young children. Side effects of medications, ingestion of heavy metals, food allergies and other intestinal related diseases such as inflammation, disorders of digestive/absorptive processes are the common causes for non-infectious diarrhea (Szajewska and Mrukowicz, 2005).

Current management of diarrhea includes ORT, zinc supplement and drugs. ORT that contains dry salts with water (ORS) is used to prevent and treat dehydration during diarrhea (Rautenberg and Zerwes, 2017). As recommended by WHO and UNICEF, zinc supplementation (10–20 mg) is used to treatment acute diarrhea in childhood (Guarino *et al*., 2012). This supplement decreases the duration of diarrhea and mortality as a result of diarrhea (Teshale *et al*., 2020). Antimotility drugs such as codeine and morphine from natural opioid agonists, loperamide and diphenoxylate from synthetic opioid agonists, and atropine from muscarinic antagonists are used to reduce the duration of diarrhea in adult (Rowe and Schiller, 2020; Harig and Ramaswamy 2016). In addition, different antibiotics used empirically (Radlović *et al*., 2015). The above mentioned drugs are not free from drawback such as antimicrobial resistance, adverse effect and side effects (Diniz-Santos *et al*., 2006). In sub-Saharan Africa country, traditional medicine is highly practiced because of easily accessibility and affordability (Ahlberg, 2017).

1.2 The genus *Sida*

The genus *Sida* belongs to the family *Malvaceae* and comprises about 200 species (Subramanya *et al*., 2015). It is widely distributed in tropical and subtropical regions of the world, especially in Angola, Burundi, Congo, Eritrea, Ethiopia, Kenya, Malawi, Mozambique, South Africa, Sudan, Swaziland, Tanzania, Uganda, Zanzibar and Zimbabwe (Verdcourt, 2004), Arabia, Iran, Pakistan, and India (Dawar *et al.*, 1996). Generally, members of the genus *Sida* are herbs or undershrubs, with stellate hairs. The leaves are toothed with linear 6-8 mm long stipules. The ovary contains 5-12 cells with 1 ovule in each cell. The fruit (5-6 mm) is globose, depressed, and enclosed by the calyx. The calyx is 6-8 mm long with triangular, and acute lobes (Halde, 2011). *Sida ovata* Forssk. (Chifrig) *S. rhombifolia* L. (Gorjejit) and *S. schimperiana* Hochst. ex A.Rich. (Chifrig) are the three *Sida* species most commonly used in traditional medicine of Ethiopia (Alemneh, 2021; Zenebe *et al*., 2012).

1.2.1 Ethnomedicinal uses

Ethnomedicinal uses of the widely used *Sida* species are recorded in Table 1.

Table 1. Ethnomedicinal uses of *Sida* species

Plant name	Places of uses	Plant part	Use	Reference
S. acuta	India, Nigeria, Togo, Western Colombia, Burkina Faso	Whole plant	Asthma, renal inflammation, colds, fever, headache, ulcers and worms, fever, bronchitis, ulcer, diarrhea, dysentery, skin diseases. The paste prepared from the of leaves is mixed with coconut oil and applied on head regularly for killing dandruffs and also for strengthening hair	Karou *et al*., 2007
		Leaves	Scrotal swellings, elephantiasis, wounds, and anticancer	Rodrigues and Oliveira 2020
			Stomachache	Morilla and Demayo, 2019

Table 1. Continued

Plant name	Places of uses	Plant part	Use	Reference
S. cordifolia	India, Malaysia, Bangladesh	Whole plant	Inflammatory diseases, gonorrhea, asthma, nasal congestion, and stomatitis	Shukla *et al*., 2018
		Whole plant	Rheumatism, Parkinson's disease, fever, convulsions, urinary tract issues, neurological disorders, heart problems, diuretic, and antiviral	Srinivasan *et al*., 2022
		Root	Epilepsy, rheumatism, anorexia, fatigue, impotence, gonorrhea, cystitis, leucorrhea, urinary frequency, diabetes, diarrhea, dysentery, hemorrhoids, chronic fever, analgesic, and anti-inflammatory	Ahmed *et al*., 2018; Sivapalan, 2015
S. corymbosa	Brazil, Nigeria	Leaves	Stomachache, ulcers, wounds, and liver Disease	Rodrigues and Oliveira 2020; John-Africa *et al*., 2014
S. glutinosa	Brazil, India	Root and aerial parts	Pulmonary tuberculosis, and rheumatism	Rodrigues and Oliveira 2020; Das *et al*., 2012
S. rhombifolia	Ethiopia	Root	Diarrhea, stomach pain, digestion problem, malaria, flatulence, irritable bowel syndrome, gastritis, enteritis, hemorrhoids	Woldeyes *et al*., 2012
S. spinosa	India	Root	Fever, gonorrhea	Selvadurai *et al*., 2011
S. tuberculata	Brazil, Argentina	Whole plant	Inflammation, pain, and antimicrobial	Rodrigues and Oliveira 2020; Yamada *et al*., 2020
S. acuta, *S. cordata*, *S. cordifolia*, and *S. rhombifolia*	India		Diarrhea, leucorrhoea, gonorrhea, asthma, wheezing, fever, cold, flu, headache, weight loss, sexual strength, hair strength, hypertension, diuretic, piles, ulcer, cancer, aphrodisiac, rheumatism, urinary, venereal, skin, respiratory, and heart diseases	Singh *et al*., 2021

1.2.2 Phytochemistry

Most members of the genus *Sida* contain secondary metabolites such as alkaloids, flavonoids, and terpenoids (Muneeswari *et al*., 2019), anthraquinones (Ekpo and Etim, 2009), coumarins, ecdysteroids, tocopherols (Abat *et al*., 2017), volatile oils (Njoku *et al*., 2021), and sterols (Laili *et al*., 2022).

1.2.3 Pharmacological activities

Pharmacological studies have confirmed that the methanolic seed extract of *S. rhombifolia* possesses potent antitubercular and antifungal activities (Poojari., 2017), and the whole plant extracts of *S. acuta*, *S. cordata*, *S. cordifolia* and *S. rhombifolia* have antioxidant, antimicrobial, antimalarial, antiulcer, anti-inflammatory, antidiabetic, analgesic, and anticancer activities (Singh *et al*., 2021). Furthermore, different parts of extracts of *S. cordifolia, S. acuta, S. cordata* and *S. rhombifolia* have been shown to display analgesic, antidiarrheal, antidiuretic and anti-inflammatory properties (Singh *et al*., 2021; Rai *et al*., 2018; Sutradhar *et al*., 2006).

1.3 *Sida ovata* Forssk

1.3.1 Botanical description

Sida ovata Forssk. (*Malvaceae*) is a perennial subshrub, and common on wastelands. It also grows along waysides on sandy places. Flowering and fruiting stages of the plant are from August to February. *Sida ovata* is easily identified by its ovate leaves and short pedicels. The plant is erect and grows up to 50 cm high and its stem is densely clothed with short stellate hairs (Tambde *et al*., 2016). Its flowers are white to pale yellow in colour, the schizocarp is incompletely enclosed by the calyx, and the mericarps are reticulate towards the margin only (Vollesen, 1996).

It is distributed in drier parts of Africa, Arabia, Iran, Pakistan, and India (Dawar *et al*, 1996).

1.3.2 Ethnomedicinal use of *Sida ovata*

The reported ethnomedicinal uses of different parts of *S. ovata* in different parts of the world are depicted in Table 2.

Table 2. Ethnomedicinal uses of *Sida ovata*

Local name	Place of use	Parts used	Medicinal use	Reference
Dekidaero	Northwestern Tigray, Ethiopia	Leaf, Root	Inflammation, wounds, pus	Zenebe *et al*., 2012
Qirqicha	Sidama, Southern Ethiopia	Leaf, Root	Toothache	Tefera and Kim, 2019
Chifrig	Shinasha, Agew-awi, and Amhara, Northwest Ethiopia	Leaf, Root	Wound	Giday *et al*., 2007
	Butajira, South central Ethiopia	Leaf	Eczema	Gedif and Hahn, 2003
	Boricha, Southern Ethiopia		Skin, eye infection	Tamene *et al*., 2020
	Dheeraa', Arsi zone, Ethiopia	Leaf	Ear ailments	Wondimu *et al*., 2007
Engonini	Arusha city, Tanzania	Root	Stomach worms	Mollel *et al*., 2022
Bala	Gujarat, India	Root	Asthma, fever, rheumatisms, leucorrhoea	Maitreya, 2014
		Seed	sexual debility, lumbago	
Uvyaiyo, Uthundu	Ukambani region, Eastern Kenya	Leaf, Stem Stem + *Asparagus flagellaris*	Hypochondriasis Menorrhagia	Wanzala *et al*., 2016

Table 2. Continued

Local name	Place of use	Parts used	Medicinal use	Reference
Awe-apwaka	Ngai Sub county, Uganda	Leaf + *Ocimum basilicum*	Sickle cell anemia	Okello and Ssegawa, 2007
Miyar-tsanya	Wamakko, Nigeria	Leaf Root	Snake bite, swellings, dropsy pulmonary troubles, toothache	Singh and Imam, 2012
Miyar-tsanya	Madobi Town, Nigeria	Leaf	Malaria	Mukhtar *et al*., 2019
Kharanti	Mayurbhanj, India Rajasthan, Northern India	Seed Seed	Cough, fever, Lumbago	Katewa and Galav, 2005
Dabi	Saharanpur, India	Root	Sexual debility	Kumar and Singh, 2023
Miyar-tsanya	Katsina state, Nigeria	Whole plant	Diarrhea	Kankara *et al*., 2015

1.4 Statement of the problem

Despite the availability of many drugs for treating diarrhea, majority of them suffer from adverse effects like the induction of bronchospasm and vomiting by racecadotril (Tormo *et al*., 2008); intestinal constipation by loperamide (Wang *et al*., 2005), dependency and respiratory depression by morphine and its analogs (Khansari *et al*., 2013). ORT has been the mainstay of treatment of diarrhea; however it does not reduce the general stool volume and duration of diarrhea (Atia and Buchman, 2009).

Furthermore, there is a rise in antimicrobial-resistant species that cause diarrhea in Sub-Saharan African countries, particularly those due to rotavirus, adenovirus, *Shigella, Vibrio cholerae, Escherichia coli and Salmonella typhi, Giardia duodenalis, Blastocystis hominis, Entamoeba histolytica, Cryptosporidium spp., and Dientamoeba fragilis* (Mafokwane *et al*., 2023; Kariuki *et al*., 2022; Boughattas *et al*., 2017).

2. Objectives

2.1 General objective

- To investigate the antidiarrheal activity of the 80% MeOH root extract, solvent fractions of *S. ovata*, and its major constituent

2.2 Specific objectives

- To investigate the antidiarrheal activity of the 80% MeOH root extract;
- To determine the antidiarrheal activity of the solvent fractions;
- To isolate the major compound from the active fraction(s); and
- To determine the antidiarrheal activity of the isolated compound.

3. Materials and Methods

3.1. Materials

3.1.1 Plant material

Fresh roots of *S. ovata* were collected in October 2022 from Wesen Kurkur, Shewarobit, North Shoa Zone, Amhara regional State, around 225 km North of Addis Ababa. The plant material was authenticated by Mr. Melaku Wendafrash, and a voucher specimen (collection number TB-001) was deposited 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 drugs

The chemicals and reagents used were: castor oil (Amman Pharmaceutical Industries, Jordan), distilled water (Ethiopian Pharmaceutical Manufacturing Factory, Ethiopia), loperamide (Medochemie Ltd, Limassol, Cyprus), activated charcoal (Acuro Organics Ltd, New Delhi, India), methanol, *n*-hexane, chloroform (Reagent Chemical Limited, UK) and Silica gel (Lobachem, India). All chemicals used were of analytical grade and were used as received without any further purification.

3.1.3. Instruments

The following instruments were used for the experiment: Rota vapor (Buchi Rota Vapor R-200, Switzerland), UV spectroscopy (Shimadzu Spectrophotometer MultiSpec-1501, Japan), Nuclear magnetic resonance spectrometer (Bruker Avance DMx400 FT-NMR, Germany).

3.1.4 Experimental animals

Healthy Swiss albino female mice (24 - 32 g), aged 8 - 10 weeks (Saleem *et al*., 2017) were used for acute oral toxicity test. The mice were obtained from the animal house of the School of Pharmacy, AAU, and the Ethiopian Public Health Institute (EPHI). Mice were kept in well ventilated cages, maintained at room temperature, and on a 12/12 h light-dark cycle with access to standard laboratory pellet food and water *ad libitum*. The mice were left to acclimatize to the laboratory conditions for 7 days before the commencement of experiments. The tests were conducted in accordance with the internationally accepted standard guidelines (Bartram, 2001), and approved by the ethical review committee of the School of Pharmacy, AAU.

3.2 Methods

3.2.1 Extraction

Fresh roots of *S. ovata* were first cleaned, removing dust and debris, and then gently washed with water. Subsequently, they were reduced to an appropriate size, and air-dried under shade for two weeks. Then, the dried roots were grinded using a grinder, and 1000 gm. of the powdered root was macerated two times by dividing it as 500 gm and 500 gm in 80% MeOH in a powdered root, solvent ratio of 1:10, i.e. 5000 ml of 80% MeOH was used for each 500 gm with regular manual shaking for 72 h. The extract was initially filtered using nylon cloth and then Whatman filter paper no.1. The filtrate was evaporated under reduced pressure at 40 °C in a rotary evaporator set at 45 rpm to yield the total extract. The marc was further macerated for an additional 72 h and concentrated to dryness in a rotary evaporator at a temperature not exceeding 40 °C. The dried extract was stored in an amber glass bottle in a refrigerator at 4 °C until needed.

3.2.2 Solvent fractionation

The 80% MeOH root extract (35 g) were exhaustively extracted successively by maceration using solvents of increasing polarity i.e. *n*-hexane, chloroform, MeOH and water. The organic solvents were evaporated using a rotary evaporator and further dried in an oven, whilst the aqueous extract was dried in an oven at a temperature not exceeding 40 °C. The dried solvent fractions were collected, the percentage yields calculated, and stored separately in an amber glass bottles in a refrigerator at 4 °C until needed.

3.2.3 Compound isolation

Initially, silica gel slurry was prepared by mixing silica gel (particle size: 0.063 - 0.200 mm) with chloroform. Then, the slurry was poured into a column very slowly so that the stationary bed is even and air bubbles are avoided. Following overnight conditioning of the packed column, a total of 2 g of the most active MeOH fraction was loaded on to the column and eluted with $CHCl_3$:MeOH gradients i.e., increasing the amount of MeOH in $CHCl_3$ to afford 105 fractions. Fractions of 10 ml were collected as follows: [(1–26), $CHCl_3$ (100%)], [(26–40), $CHCl_3$:MeOH (95:5)], [(41–50), $CHCl_3$:MeOH (90:10)], [(51–70), $CHCl_3$:MeOH (85:15)], [(70–80), $CHCl_3$:MeOH (80:20)], [(81–105) MeOH (100%)]. These fractions were pooled according to their TLC profiles into 5 subfractions F1 to F5 as follows: F1 (1–29), F2 (31–59), F3 (60–69), F4 (70–80), and F5 (81–105). F4 gave a single spot-on reversed phase TLC in different solvent systems. F4 was evaporated to dryness at 40 °C to yield 20 mg of a white powder coded TB-2. TB-2 was weighed, transferred into an amber-colored vial, and stored in a refrigerator at 4 °C until used.

3.2.4 Spectroscopic techniques

NMR spectra were recorded on an FT-NMR spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C, at room temperature using $CDCl_3$ as a solvent. A region from 0 to 15 ppm for ^{1}H and 0 to 220 ppm for ^{13}C was employed for scanning. Signals were referred to internal standard tetramethylsilane (TMS). Chemical shifts were reported in ppm and coupling constants (*J*) were expressed in Hz. Multiplicities of ^{1}H NMR signals are indicated as *s* (singlet), *d* (doublet), *dd* (doublet of doublet), *t* (triplet) and *m* (multiplet).

3.2.5 Acute oral toxicity test

Acute oral toxicity tests of the extract and fractions were conducted according to Organization for Economic Cooperation and Development (OECD) guideline 425 (Saleem *et al*., 2017)). Healthy Swiss albino female mice (24 - 32 g), aged 8 - 10 weeks (Saleem *et al*., 2017) were used for acute oral toxicity test. First, one female mouse was administered with a 2000 mg/kg dose of *S. ovata* 80% MeOH root extract, then observed for any signs of toxicity in the first hour, and periodically for a day. If the mouse survived, then the same 2000 mg/kg dose of the 80% MeOH extract was administered to four 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 of animals

In all models the mice were randomly divided into five groups. One group (I) served as negative control, another group (II) served as positive control, and three groups (III, IV, and V) served as test animals. Six animals were used for each group. The test groups were treated with doses of

100 mg/kg, 200 mg/kg, and 400 mg/kg for both crude extracts and solvent fractions. These doses were determined based on previous acute toxicity study results. The LD_{50} of the root extract and solvent fractions was greater than 2000 mg/kg. Therefore, one-tenth of this dose was used as the middle dose. One-half and double of the middle dose were used as the low and high doses, respectively. The negative control group received distilled water at a volume of 10 ml/kg. Loperamide (3 mg/kg) was used as a positive control for castor oil-induced diarrhea, anti-enteropooling and anti-motility tests (Ferede *et al*., 2021). All doses were given orally using mice oral gavage (Ayalew *et al*., 2022; Yimer *et al*., 2020). The isolated compound (TB-2) was administered at doses of 25 mg/kg, 50 mg/kg, and 100 mg/kg.

3.2.7 Determination of *in vivo* antidiarrheal activity

3.2.7.1 Castor oil-induced diarrhea model

Thirty Swiss albino mice of either sex were fasted for 18 h for food with free access to water. The animals were screened initially by giving 0.5 ml of castor oil and only those 306 mice showing diarrhea were selected for the final experiment (Tadesse., 2014). One hour after taking of controls and extracts, all mice received 0.5 mL of castor oil by using mice oral gavage. The experimental animals were kept in separate metabolic cages lined with a transparent paper. The paper was changed each time after the mouse defecated. During observational period of 4 h, onset of diarrhea, number and weight of wet feces, and the total number and weight of fecal output (both diarrheal and non-diarrheal) were recorded and compared with the control group. The percentages of diarrheal inhibition, as well as the weight of wet and total fecal output, were determined according to the formulae below (Gudeta *et al*., 2020).

$$\text{Percent inhibition of diarrhea} = \frac{\text{Mean number of wet defecation (negatve control} - \text{ test group)}}{\text{Mean number of wet defecation (negatve control)}} \times 100$$

$$\text{Percent of wet fecal output} = \frac{\text{Mean weight of wet feces of each treatment group}}{\text{Mean weight wet feces of negative control group}} \times 100$$

$$\text{Percent of total fecal output} = \frac{\text{Mean fecal weight of each treatment group}}{\text{Mean fecal weight of negative control group}} \times 100$$

3.2.7.2 Castor oil-induced enteropooling model

In this model, the experimental animals were devoid of food for 18 h with free access to water. One h post treatments of their respective graded doses of the test substances, all animals received 0.5 mL of castor oil and were sacrificed 1 h later by cervical dislocation. The abdomen of each animal was then opened and the whole length of small intestine, from the pylorus to the caecum, was ligated, dissected, and carefully removed. Then, the dissected small intestine was weighed and the contents were collected by milking into a graduated cylinder and volume of contents was measured. Intestinal weight was measured before and after removing bowel contents and the difference was calculated. Percentage of intestinal secretion and weight of intestinal contents reduction were determined by using the formula given below (Belayneh *et al*., 2024).

$$\text{Percent inhibition by using MVIC} = \frac{\text{MVICC} - \text{MVICT}}{\text{MVICC}} \times 100$$

Where,

MVIC - Mean volume of intestinal content

MVICC - Mean Volume of intestinal content of control group

MVICT - Mean Volume of intestinal content of test group

$$\text{Percent inhibition by using MWIC} = \frac{\text{MWICC} - \text{MWICT}}{\text{MWICC}} \times 100$$

Where,

MWIC – Mean weight of intestinal content

MWICC- Mean weight of intestinal content of control group

MWICT - Mean weight of intestinal content of test group

3.2.7.3 Castor oil-induced gastrointestinal motility test

The mice were fasted for 18 h with free access to water. One h post treatment of the test substances, all the mice received a 0.5 mL of castor oil and, after 1 h they received 1 mL of 5% activated charcoal suspension in distilled water. Then, the mice were sacrificed by cervical dislocation just 1 h following charcoal meal. The small intestine was dissected out from pylorus to caecum and placed lengthwise on a white paper. Then, the distance travelled by the charcoal marker and the total length of the small intestine were measured. The peristaltic index and percentage inhibition were calculated by using the following formula (Sisay *et al*., 2019).

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

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

3.2.7.4 *In vivo* antidiarrheal index

In vivo antidiarrheal index (ADI) is the measure of the antidiarrheal effectiveness of the plant. It is mathematically calculated based on the formula given below (Alemu *et al*., 2022).

$$\text{ADI} = \sqrt[3]{\text{Dfreq} \times \text{Gmeq} \times \text{Pfreq}}$$

$$\text{Dfreq} = \frac{\text{Mean onset of diarrhea in (treated group} - \text{negative control group)}}{\text{Mean onset of diarrhea in the negative control group}} \times 100$$

$$\text{Gmeq} = \frac{\text{Intestinal transit by charcoal meal (control - treated)}}{\text{Intestinal transit by charcoal meal in the control group}} \times 100$$

$$\text{Pfreq} = \frac{\text{Mean number of wet defecation (negative control-test group)}}{\text{Mean number of wet feces of negative control}} \times 100$$

Where, Dfreq is the delay in defecation time as percentage of negative control,

Gmeq is the gut meal travel reduction as percentage of negative control and

Pfreq is the reduction in purging frequency in the number of wet stools as percentage of negative control.

3.2.8 Statistical analysis

Data was entered, and analyzed with the International Business Machines Corporation (IBM) statistical package for social sciences (SPSS) version 26. The data obtained in the study was tabulated, and expressed as mean ± standard errors of the mean (SEM). The statistical analysis was carried out using one-way ANOVA followed by Tukey post-hoc test to compare variations among groups. The result was considered significant when $p < 0.05$.

3.2.9 Ethical approval

Ethical approval was obtained from the Research Ethics Committee of the School of Pharmacy, College of Health Sciences, Addis Ababa University (approval code: ERB/SOP/553/15/2023).

4. Results

4.1 Yields of the extract and solvent fractions

Yields and colour of the 80% MeOH extract and solvent fractions obtained from the root extract of *S. ovata* are shown in Table 3.

Table 3. Colour and percentage yields of the 80% MeOH extract and solvent fractions of the roots of *Sida ovata*

Sample	Colour of extract	Percentage yield (w/w)
80% MeOH extract	Brown	4.5
n-Hexane fraction	Brown	0.7
Chloroform fraction	Yellowish-brown	0.8
MeOH fraction	Light brown	2.0

4.2 Acute oral toxicity

During the acute oral toxicity testing, neither the 80% MeOH extract nor the solvent fractions, caused any significant behavioral changes or mortality throughout the 14-day observation period. Similarly, the experimental animals tolerated the maximum tested dose of 100 mg/kg of the isolated compound without showing significant changes in behavior such as alertness, motor activity, breathing, restlessness, diarrhea, convulsions, coma and in appearance of the animals. However, further comprehensive toxicity studies, including sub-acute, sub-chronic and chronic toxicity assessments are recommended to establish the safety of the roots of the plant.

4.3 Antidiarrheal activity

4.3.1 Effect on castor oil-induced diarrhea in mice

The animals were screened initially by giving 0.5 ml of castor oil and only those 306 mice showing diarrhea were selected for the final experiment. After 1 hour of administration of the corresponding treatment doses, the mice received 0.5 mL of castor oil orally using oral gavage and were individually placed in a separate transparent cage with a white, non-wetting, transparent paper-lined floor. Then, the paper was changed every hour for a total of four hours.

As presented in Table 4, the 80% MeOH extract of the roots of *S. ovata* significantly delayed the onset of diarrhea, reduced the number and weight of both wet and total stools at all doses tested compared to the negative control. At a dose of 400 mg/kg, the extract showed comparable effect to that of loperamide (3 mg/kg). All the solvent fractions of the root of *S. ovata* significantly ($p < 0.05$) delayed the onset of diarrhea and stool frequency at all dose levels. However, the overall antidiarrheal effect of the chloroform fraction was less than those of the MeOH or the *n*-hexane fraction. The MeOH and *n*-hexane fractions significantly prolonged the time of diarrhea induction and decreased the frequency of stooling (number of wet feces and total number of feces) in a dose-dependent manner.

4.3.2 Effect on castor oil-induced enteropooling in mice

In the gastrointestinal enteropooling test, the 80% MeOH root extract of *S. ovata* showed significant ($p < 0.05$) reduction of both average volume and weight of intestinal contents at all tested doses compared to the negative control (Table 5). The extract showed maximum activity at the highest dose (400 mg/kg) tested, showing no statistically significant difference ($p < 0.05$) in terms of both volume of intestinal fluid and weight of intestinal contents with the positive control.

Inhibition of castor oil-induced intestinal fluid accumulation by the *n*-hexane and MeOH fractions was much higher when compared to the effect caused by the chloroform fraction.

Table 4. Effects of the 80% MeOH extract and solvent fractions of the root of *Sida ovata* on castor oil-induced diarrhea in mice

Sample	Dose	OD (min)	ANWF	ANTF	AWWF (g)	AWTF (g)	% ID	% WFO	% TFO
	DW10	23.83±1.47	10.83±0.48	13.17±0.60	1.68±0.09	1.98±0.12	----	----	----
	Lop3	124.5 ± 1.43^{acde}	1.83 ± 0.40^{ac}	4 ± 0.37^{ac}	0.06 ± 0.01^{a}	0.08 ± 0.01^{a}	83.10	3.57	4.04
80%	SO100	85.67 ± 2.12^{abde}	4.17 ± 0.31^{ab}	6.5 ± 0.43^{abe}	0.09 ± 0.01^{a}	0.12 ± 0.01^{a}	61.50	5.36	6.06
MeOH	SO200	104 ± 1.46^{abce}	3.17 ± 0.31^{a}	5 ± 0.45^{a}	0.08 ± 0.01^{a}	0.12 ± 0.01^{a}	70.73	4.76	6.06
extract	SO400	118 ± 0.73^{abcd}	2.5 ± 0.43^{a}	4.17 ± 0.40^{ac}	0.07 ± 0.01^{a}	0.1 ± 0.01^{a}	76.92	4.17	5.05
	HF100	76.83 ± 0.48^{abghijkmn}	6.17 ± 0.30^{abhijkn}	7.5 ± 0.43^{abhijkn}	0.17 ± 0.01^{aijk}	0.20 ± 0.01^{aijk}	43.03	10.12	10.10
	HF200	93.83 ± 1.33^{abhijkln}	5.17 ± 0.31^{abhijkn}	7.17 ± 0.31^{abijkn}	0.11 ± 0.01^{aijk}	0.16 ± 0.01^{aijk}	52.26	6.55	8.08
	HF400	107.5 ± 1.28^{abfgijklm}	3.33 ± 0.33^{afgijkl}	5 ± 0.37^{afijk}	0.09 ± 0.01^{aijk}	0.14 ± 0.01^{aijk}	69.25	5.36	7.07
Solvent	CF100	$36.83\pm1.07^{abfghjklmn}$	10.33 ± 0.33^{bfghlmn}	11.67 ± 0.42^{bfghlmn}	$1.45\pm0.043^{abfghjklmn}$	1.69 ± 0.07^{bfghklmn}	4.67	86.31	85.35
fractions	CF200	48.5 ± 0.77^{abfghiklmn}	9 ± 0.45^{abfghlmn}	10.5 ± 0.56^{abfghlmn}	1.20 ± 0.06^{abfghiklmn}	1.37 ± 0.09^{abfghklmn}	16.90	71.43	69.19
	CF400	$55.67\pm1.50^{abfghijlmn}$	8.67 ± 0.33^{abfghlmn}	10.67 ± 0.42^{abfghlmn}	0.93 ± 0.03^{abfghijlmn}	1.03 ± 0.18^{abfghijlmn}	19.94	55.36	52.03
	MF100	78.5 ± 0.76^{abghijklmn}	5.67 ± 0.33^{abhijkn}	6.83 ± 0.48^{abijkn}	0.16 ± 0.02^{aijk}	0.19 ± 0.01^{aijk}	47.65	9.52	9.60
	MF200	95 ± 1.59^{abfhijkln}	4.67 ± 0.33^{abijk}	6.17 ± 0.48^{aijk}	0.1 ± 0.01^{aijk}	0.15 ± 0.01^{aijk}	56.88	5.95	7.58
	MF400	$108.83\pm1.45^{abfgijklm}$	3 ± 0.37^{afgijkl}	4.5 ± 0.43^{afgijkl}	0.09 ± 0.01^{aijk}	0.14 ± 0.01^{aijk}	72.30	5.36	7.07

Values are expressed as mean ± SEM (n = 6); Analysis was performed using one way ANOVA followed by Tukey Post hoc test; OD: onset of diarrhea, ANWF: average number of wet feces, ANTF: average number of total feces, AWWF: average weight of wet feces in gram, AWTF: average weight of total feces in gram, % ID: percent inhibition of diarrhea, % WFO: percent of wet fecal output, % TFO: percent of total fecal output, DW10: distilled water 10 ml/kg (negative control), Lop3: loperamide 3 mg/kg (positive control), SO100: 80% MeOH *Sida ovata* root extract (100 mg/kg), SO200: 80% MeOH *Sida ovata* root extract (200 mg/kg), SO400: 80% MeOH *Sida ovata* root extract (400 mg/kg), HF100: *n*-hexane fraction of *Sida ovata* root (100 mg/kg), HF200: *n*-hexane fraction of *Sida ovata* root (200 mg/kg), HF400: *n*-hexane fraction *Sida ovata* root (400 mg/kg), CF100: chloroform fraction of *Sida ovata* root (100 mg/kg), CF200: chloroform fraction of *Sida ovata* root (200 mg/kg), CF400: chloroform fraction of *Sida ovata* root (400 mg/kg), MF100: MeOH fraction of *Sida ovata* root (100 mg/kg), MF200: MeOH fraction of *Sida ovata* root (200 mg/kg), MF400: MeOH fraction of *Sida ovata* root (400 mg/kg); [a] Compared with the negative control, [b] compared with the positive control, [c] compared with SO100, [d] compared with SO200, [e] compared with SO400, [f] compared with HF100, [g] compared with HF200 mg/kg, [h] compared with HF400, [i] compared with CF100, j compared with CF200, [k] compared with CF400, [l] compared with MF100, [m] compared with MF200, [n] compared with MF400; $p < 0.05$.

Table 5. Effects of the 80% MeOH extract and solvent fractions of the root of *Sida ovata* on castor oil-induced enteropooling in mice

Sample	Dose	Mean volume of small intestinal content (ml)	Percent inhibition	Mean weight of small intestinal content (g)	Percent inhibition
	DW10	0.95±0.02	----	2.35±0.04	----
	Lop3	0.13±0.02^{acdfghijklmn}	86.32	1.35±0.02^{acfghijklmn}	42.55
80% MeOH extract	SO100	0.42±0.03^{abefijkl}	55.79	1.53±0.02^{abeijk}	34.89
	SO200	0.33±0.02^{abfgijkl}	65.26	1.47±0.02^{afijkl}	37.45
	SO400	0.23±0.02^{acfgijklm}	75.79	1.4±0.03^{acfghijklm}	40.42
Solvent fractions	HF100	0.6±0.03^{abcdehimn}	36.84	1.63±0.02^{abdeijn}	30.64
	HF200	0.48±0.03^{abdehijn}	49.47	1.55±0.02^{abeijk}	34.04
	HF400	0.32±0.02^{abfgijkl}	66.32	1.53±0.02^{abeijk}	34.89
	CF100	0.78±0.03^{abcdefghklmn}	17.89	1.9±0.03^{abcdefghjklmn}	19.15
	CF200	0.67±0.02^{abcdeghmn}	29.47	1.87±0.02^{abcdefghlmn}	20.43
	CF400	0.57±0.02^{abcdehimn}	40	1.75±0.02^{abcdeghilmn}	25.53
	MF100	0.55±0.02^{abcdehimn}	42.11	1.62±0.02^{abdeijkn}	31.06
	MF200	0.37±0.02^{abefijkl}	61.05	1.57±0.02^{abeijk}	33.19
	MF400	0.3±0.03^{abfgijkl}	68.42	1.48±0.03^{abfijkl}	37.02

Values are expressed as mean ± SEM (n = 6); Analysis was performed using one way ANOVA followed by Tukey Post hoc test; DW10: distilled water 10 ml/kg (negative control), Lop3: loperamide 3 mg/kg (positive control), SO100: 80% MeOH *Sida ovata* root extract (100 mg/kg), SO200: 80% MeOH *Sida ovata* root extract (200 mg/kg), SO400: 80% MeOH *Sida ovata* root extract (400 mg/kg), HF100: *n*-hexane fraction of *Sida ovata* root (100 mg/kg), HF200: *n*-hexane fraction of *Sida ovata* root (200 mg/kg), HF400: *n*-hexane fraction *Sida ovata* root (400 mg/kg), CF100: chloroform fraction of *Sida ovata* root (100 mg/kg), CF200: chloroform fraction of *Sida ovata* root (200 mg/kg), CF400: chloroform fraction of *Sida ovata* root (400 mg/kg), MF100: MeOH fraction of *Sida ovata* root (100 mg/kg), MF200: MeOH fraction of *Sida ovata* root (200 mg/kg), MF400: MeOH fraction of *Sida ovata* root (400 mg/kg); [a] Compared with the negative control, [b] compared with the positive control [c] compared with SO100, [d] compared with SO200, [e] compared with SO400, [f] compared with HF100, [g] compared with HF200, [h] compared with HF400, [i] compared CF100, [j] compared with CF200, [k] compared with CF400, [l] compared MF100, [m] compared with MF200, [n] compared with MF400; $p < 0.05$.

4.3.3 Effect on castor oil-induced gastrointestinal motility in mice

As presented in the Table 6, the 80% MeOH root extract of *S. ovata* inhibited the intestinal transit of charcoal meal at all tested doses. At a dose of 400 mg/kg, the inhibitory effect of the extract was comparable with that of loperamide hydrochloride (3 mg/kg). The solvent fractions did also inhibit gastrointestinal motility of charcoal meal at all tested doses as compared to the negative control

group. Maximum effect was achieved by the 400 mg/kg MeOH fraction, which was 43.03% of the total length of the small intestine.

Table 6. Effects of the 80% MeOH extract and solvent fractions of the root of *Sida ovata* on castor oil-induced gastrointestinal motility in mice

Sample	Dose	Length of small intestine (cm)	Distance moved by the charcoal meal (cm)	Peristaltic index (%)	Percent inhibition
	DW10	55.83±0.40	45.5±0.43	81.50	----
	Lop3	56±0.37	21.83±0.60^{acdfghijklmn}	38.98	52.02
80% MeOH extract	SO100	56±0.37	28.33±0.33^{abefijkln}	50.59	37.74
	SO200	56±0.37	27.33±0.33^{abefijkl}	48.80	39.93
	SO400	56±0.37	24±0.45^{acdfghijklm}	42.86	47.25
Solvent fractions	HF100	56±0.37	31.5±0.43^{abcdefghijmn}	56.25	30.77
	HF200	56±0.37	28.33±0.33^{abefijkln}	50.59	37.74
	HF400	56±0.37	27.33±0.21^{abefijkl}	48.80	39.93
	CF100	56±0.37	37.67±0.49^{abcdefghklmn}	67.27	17.21
	CF200	56±0.37	35.67±0.33^{abcdefghklmn}	63.70	21.60
	CF400	56±0.37	32.33±0.67^{abcdeghijmn}	57.73	28.95
	MF100	56±0.37	31.17±0.60^{abcdeghijlmn}	55.66	31.49
	MF200	56±0.37	28.17±0.48^{abefijkln}	50.30	38.09
	MF400	56±0.37	26±0.26^{abcfgijkl}	46.43	42.86

Values are expressed as mean ± SEM (n = 6); Analysis was performed using one way ANOVA followed by Tukey Post hoc test; DW10: distilled water 10 ml/kg (negative control), Lop3: loperamide 3 mg/kg (positive control), SO100: 80% MeOH *Sida ovata* root extract (100 mg/kg), SO200: 80% MeOH *Sida ovata* root extract (200 mg/kg), SO400: 80% MeOH *Sida ovata* root extract (400 mg/kg), HF100: *n*-hexane fraction of *Sida ovata* root (100 mg/kg), HF200: *n*-hexane fraction of *Sida ovata* root (200 mg/kg), HF400: *n*-hexane fraction *Sida ovata* root (400 mg/kg), CF100: chloroform fraction of *Sida ovata* root (100 mg/kg), CF200: chloroform fraction of *Sida ovata* root (200 mg/kg), CF400: chloroform fraction of *Sida ovata* root (400 mg/kg), MF100: MeOH fraction of *Sida ovata* root (100 mg/kg), MF200: MeOH fraction of *Sida ovata* root (200 mg/kg), MF400: MeOH fraction of *Sida ovata* root (400 mg/kg); [a] Compared to the negative control, [b] compared to the positive control (loperamide 3 mg/kg), [c] compared with SO100 (mg/kg), [d] compared to SO200 mg/kg, [e] compared with SO400 mg/kg, [f] compared with HF100 mg/kg, [g] compared with HF200 mg/kg, [h] compared to HF400 mg/kg, [i] compared with with 100 mg/kg, [j] compared with CF 200 mg/kg, [k] compared with CF 400 mg/kg, [l] compared with MeOH fraction 100 mg/kg, [m] compared with MeOH fraction 200 mg/kg and [n] compared with MeOH fraction 400 mg/kg; $p < 0.05$.

4.3.4 *In vivo* antidiarrheal index

As shown in Table 7, *in vivo* ADI of the 80% MeOH extract and solvent fractions of *S. ovata* roots increased in a dose dependent manner. The highest ADI was observed at the maximum dose of the

test substances. At a dose of 400 mg/kg, ADI of the 80% MeOH extract (112.85%) was comparable with that of loperamide hydrochloride (3 mg/kg) (122.23%). Among the solvent fractions, the MeOH fraction showed the highest ADI at all dose levels, although these values are less than that of the 80% MeOH extract.

Table 7. *In vivo* antidiarrheal indices of the 80% MeOH extract and solvent fractions of the root of *Sida ovata*

Sample	Dose	Dfreq (%)	Gmeq (%)	Pfreq (%)	ADI
	Lop3	422.45	52.02	83.10	122.23
80%	SO100	259.50	37.74	61.50	84.45
MeOH	SO200	336.42	39.93	70.73	98.31
extract	SO400	395.17	47.25	76.92	112.85
	HF100	222.41	30.77	43.03	66.53
	HF200	293.75	37.74	52.26	83.36
	HF400	351.11	39.93	69.25	99.02
Solvent	CF100	54.55	17.21	4.67	16.37
fractions	CF200	103.52	21.60	16.90	33.56
	CF400	133.61	28.95	19.94	42.57
	MF100	229.42	31.49	47.65	70.08
	MF200	298.66	38.09	56.88	86.49
	MF400	356.69	42.86	72.30	103.39

Dfreq (%): delay in defecation time as percentage of negative control, Gmeq (%): gut meal travel reduction as percentage of negative control, Pfreq (%): reduction in purging frequency in the number of wet stools as percentage of negative control, ADI: antidiarrheal index, Lop3: loperamide 3 mg/kg (positive control), SO100: 80% MeOH *Sida ovata* root extract (100 mg/kg), SO200: 80% MeOH *Sida ovata* root extract (200 mg/kg), SO400: 80% MeOH *Sida ovata* root extract (400 mg/kg), HF100: *n*-hexane fraction of *Sida ovata* root (100 mg/kg), HF200: *n*-hexane fraction of *Sida ovata* root (200 mg/kg), HF400: *n*-hexane fraction *Sida ovata* root (400 mg/kg), CF100: chloroform fraction of *Sida ovata* root (100 mg/kg), CF200: chloroform fraction of *Sida ovata* root (200 mg/kg), CF400: chloroform fraction of *Sida ovata* root (400 mg/kg), MF100: MeOH fraction of *Sida ovata* root (100 mg/kg), MF200: MeOH fraction of *Sida ovata* root (200 mg/kg), MF400: MeOH fraction of *Sida ovata* root (400 mg/kg).

4.4 Fractionation and isolation

The 80% MeOH extract of *S. ovata* roots was subjected to solvent fractionation using *n*-hexane, chloroform and MeOH. Silica gel column chromatography of the most active MeOH fraction led to

the isolation of a white amorphus solid designated TB-2. The R_f value of TB-2 in chloroform/MeOH (4:1) was found to be 0.44 (Figure 1).

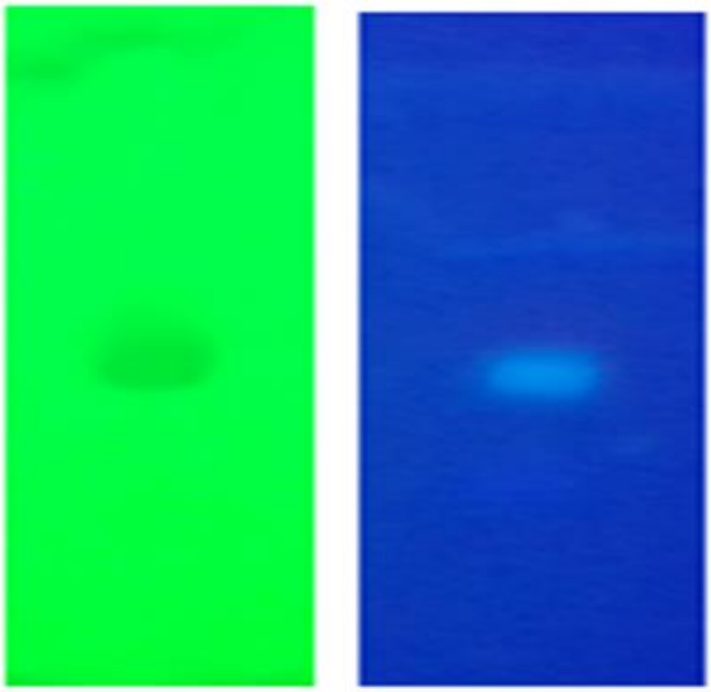

Figure 1. TLC chromatograms of TB-2 [viewed under UV 254 nm (A) and 366 nm (B); solvent system chloroform/MeOH (4:1)]

4.5 Structural elucidation of TB-2

TB-2 was characterized through the analysis of ^{1}H, ^{13}C, and DEPT-NMR spectral data. As shown in Table 8, the ^{13}C NMR spectrum of TB-2 has a total of twenty-nine signals, corresponding to twenty-nine carbon atoms. Among these, 26 carbon atoms appeared in the DEPT-135 spectrum (Figure 2B), including $6CH_3$, $9CH_2$, and 11CH groups.

Table 8. ^{1}H and ^{13}C NMR data of TB-2 in $CDCl_3$

	^{1}H-NMR (δ, ppm)		^{13}C-NMR	
	TB-2	Stigmasterol (Marliyana *et al*., 2021)	TB-2	Stigmasterol (Lolok *et al*., 2023)
1	1.84 *m*	1.84 *m*	36.91	36.70
2	1.82 *m*	1.83 *m*	29.69	29.70
3	3.54 1H, *m* (J = 4.0; 4.0; 4.0 Hz)	3.51 1H, *m* (J = 4.5; 4.4; 3.8 Hz)	71.82	71.90
4	2.2 *m*	2.3 *m*	45.89	42.30
5	----------	--------	140.79	140.79
6	5.37 1H, *t*, (J = 4 Hz)	5.31 1H, *t*, (J = 6.1 Hz)	121.70	121.70
7	1.98 *m*	1.97 m	31.90	31.90
8	1.5 *m*	1.46 *m*	29.22	29.20
9	0.9 *m*	0.92 *m*	50.20	50.20
10	---------	--------	36.15	36.11
11	1.5 *m*	1.50 *m*	25.39	24.34
12	2.00 *m*	2.00 *m*	39.76	39.80
13	---------	---------	42.30	40.40
14	1.00 *m*	1.01 *m*	56.85	56.90
15	1.60 *m*	1.56 *m*	24.34	24.34
16	1.70 *m*	1.72 *m*	28.24	28.90
17	1.20 *m*	1.15 *m*	56.06	56.00
18	0.70 *s*	0.69 *s*	12.02	12.00
19	1.00 *s*	1.01 *s*	19.05	19.00
20	2.1 *m*	2.06 *m*	40.45	39.80
21	1.00 (*m*)	1.02 *m*	23.10	23.10
22	5.18 *dd* (J =16.0; 8.0 Hz)	5.17 *dd* (15.2; 8.7 Hz)	138.29	138.4
23	5.04 *dd* (16.0; 8.0 Hz)	5.03 *dd* (15.15; 8.7 Hz)	129.32	129.30
24	1.50 *m*	1.54 (*m*)	51.25	51.20
25	1.6 *m*	1.55 (m)	33.99	34.10
26	0.90 *m*	0.85 (m)	21.10	21.10
27	0.8 *m*	0.80 (m)	21.10	21.10
28	1.4 *m*	1.44 (m)	25.39	25.30
29	0.8 *m*	0.81 (m)	12.02	12.00

Upon close examination of the ^{1}H and ^{13}C-NMR spectral data, it was proposed that TB-2 is a sterol. Specifically, the presence of an oxymethine carbon at position 3 in the sterol structure was indicated by a distinctive signal at a chemical shift of δ 71.82 in the ^{13}C-NMR spectrum of TB-2. Furthermore, the presence of two double bonds in compound TB-2 was apparent, as three distinct CH signals at chemical shifts δ 121.70, δ 129.32, and δ 138.29 were identified from the DEPT-135 spectrum. These signals corresponded to three olefinic CH carbons linked to positions C-6, C-23, and C-22, respectively. Additionally, an olefinic quaternary carbon appeared at δ 140.79 in the ^{13}C-NMR spectrum. The remaining carbon chemical shifts are summarized in Table 8.

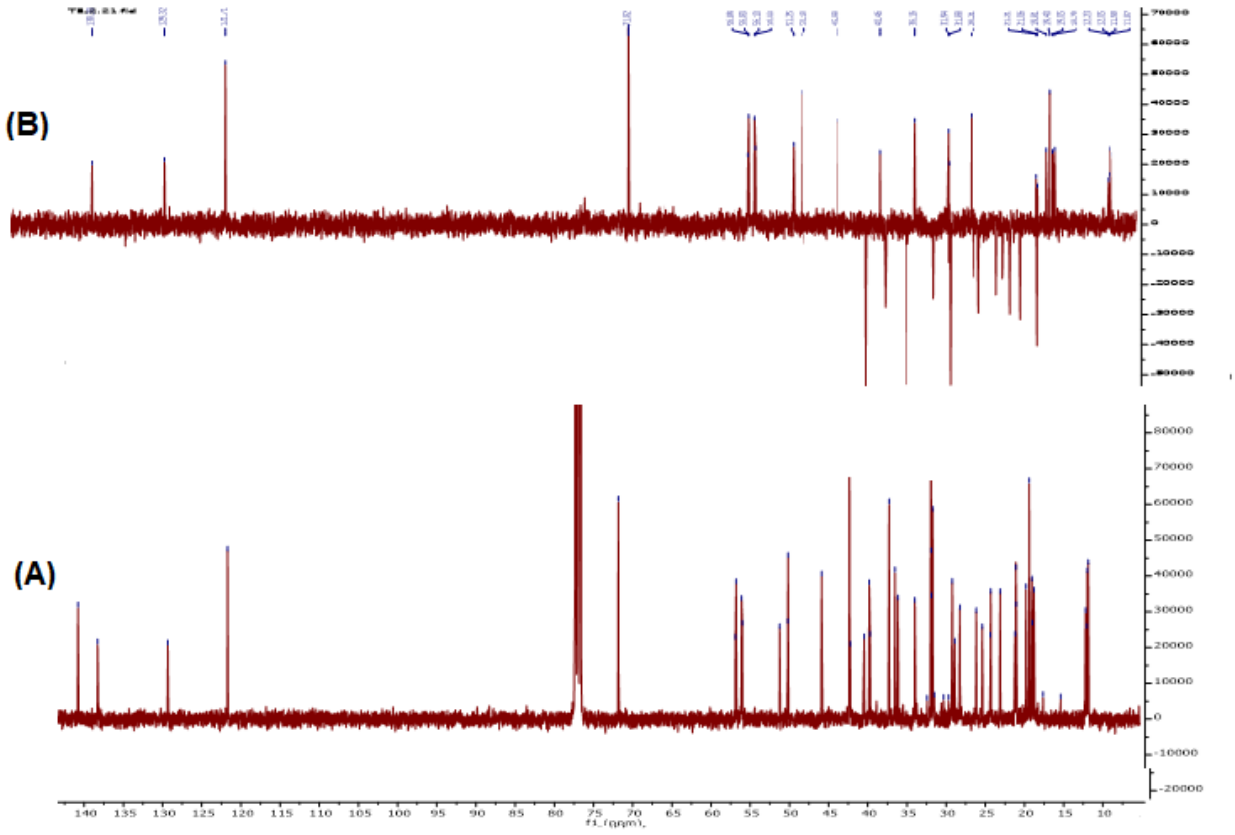

Figure 2. ^{13}C NMR (A) and DEPT-135 (B) spectra of TB-2.

The sterol nature of compound TB-2 was further supported by its ^{1}H-NMR spectrum (Figure 3). A distinctive oxymethine proton of sterol at position 3 was clearly identified by a signal resonating at δ 3.54 (1H, *m*). Moreover, three olefinic methine protons were observed at δ 5.04 (1H, *dd*, *J* =16.0, 8.0 Hz), δ 5.18 (1H, *dd*, *J* = 16.0, 8.0 Hz), and δ 5.37 (1H, *d*, *J* = 4.0 Hz) in the ^{1}H-NMR spectrum, corresponding to H-23, H-22, and H-6, respectively. The assignment of a *trans*-configuration for the double bond between C-22 and C-23 was based on the *J*-coupling constant (*J* = 16 Hz). The remaining protons were assigned as detailed in Table 8.

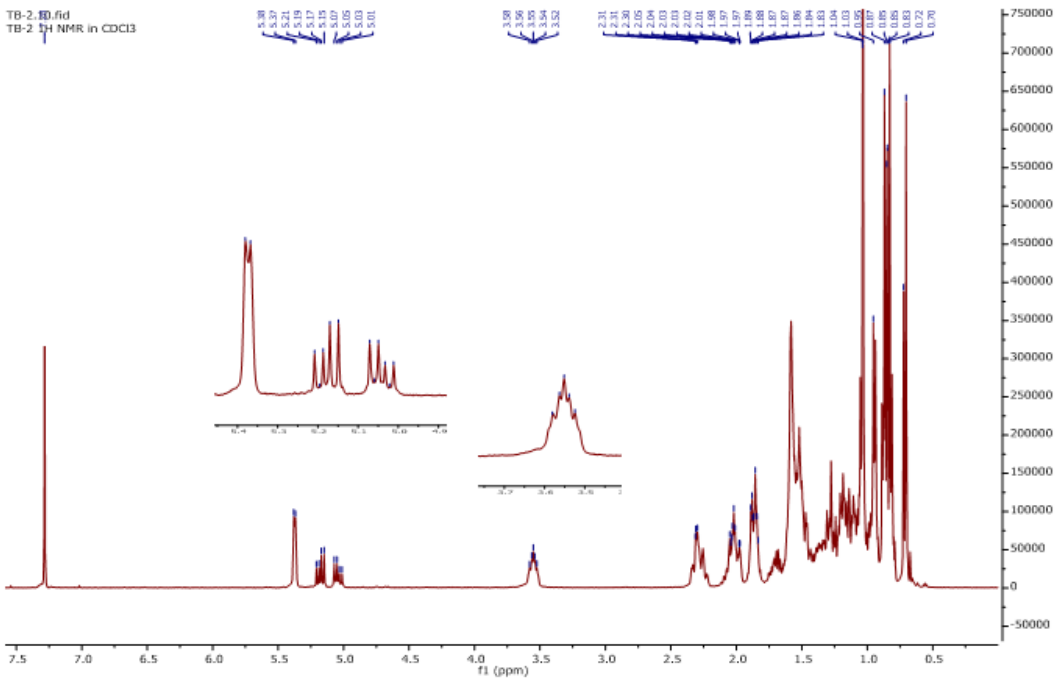

Figure 3. ^{1}H NMR spectrum of TB-2

Therefore, TB-2 was tentatively identified as stigmasterol by analyzing its ^{1}H, ^{13}C, and DEPT-135 NMR spectral data, as well as by comparing its NMR data with those reported in literature for the same compound (Lolok *et al*., 2023; Marliyana *et al*., 2021).

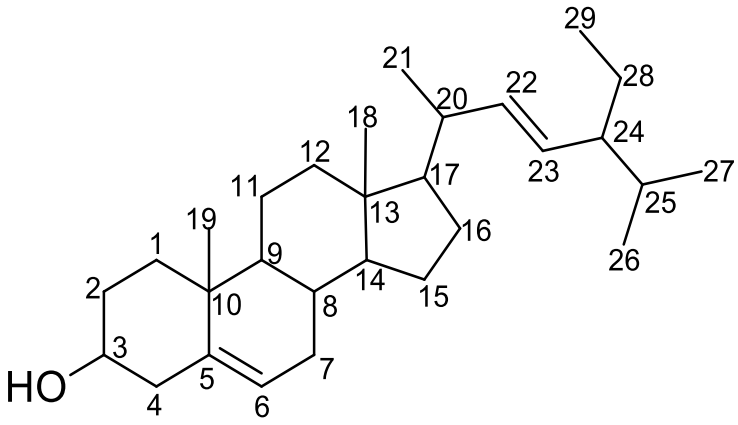

Figure 4. Structure of stigmasterol

4.6 Antidiarrheal activity of stigmasterol

Compared to the mice in the negative control group, the onset of diarrhea was significantly delayed in stigmasterol treated mice similar to those mice in the loperamide group (Table 9). Moreover, antidiarrheal activity was in a dose-dependent manner. At a dose of 100 mg/kg, percent inhibition of stigmasterol was comparable to that of the reference drug.

Water and electrolytes are accumulated in the intestinal loop of mice due to castor oil stimulation. As seen in Table 10, stigmasterol was not as potent as the loperamide. However, it caused a significant and dose-dependent reduction in the volume and weight of small intestinal content (Table 10).

Table 11 depicts that stigmasterol reduced intestinal charcoal meal propulsion (% transit) in mice compared to the control mice. In particular, stigmasterol at a dose of 100 mg/kg significantly

retarded gastrointestinal distance traveled by the charcoal (22.83 cm) meal in mice, similar to the mice receiving loperamide (21.83 cm), compared to the mice receiving vehicle.

Table 9. Effect of stigmasterol on castor oil-induced diarrhea in mice

Sample	OD (min)	ANWF	ANTF	AWWF(g)	AWTF(g)	% ID	% WFO	% TFO
DW10	23.83±1.47	10.83±0.48	13.17±0.60	1.68±0.09	1.98±0.12	-------	-------	-------
Lop3	124.50±1.43^{ade}	1.83±0.40^{ae}	4.00±0.37^{a}	0.06±0.01^{a}	0.08±0.01^{a}	83.10	3.57	4.04
SS25	102.67±1.71^{abcd}	5.67±0.42^{abcd}	5.83±0.31^{a}	0.12±0.01^{a}	0.19±0.01^{a}	47.64	7.14	9.60
SS50	118.00±0.86^{ae}	2.83±0.31^{ae}	5.17±0.48^{a}	0.08±0.01^{a}	0.13±0.01^{a}	73.87	4.76	6.57
SS100	122.67±1.02^{ae}	2.33±0.42^{ae}	4.17±0.40^{a}	0.07±0.01^{a}	0.1±0.01^{a}	78.49	4.17	5.05

Data is expressed as mean ± SEM (n = 6); Analysis was performed using one way ANOVA followed by Tukey Post hoc test; OD: onset of diarrhea, ANWF: average number of wet feces, ANTF: average number of total feces, AWWF: average weight of wet feces in gram, AWTF: average weight of total feces in gram, % ID: percent inhibition of diarrhea, % TFO: percent of total fecal output, DW10: distilled water 10 ml/kg (negative control), Lop3: loperamide 3 mg/kg (positive control), SS25: stigmasterol (25 mg/kg), SS50: stigmasterol (50 mg/kg), SS100: stigmasterol (100 mg/kg); [a] compared with negative control (DW=10 ml/kg), [b] compared with the positive control, [c] compared with SS100, [d] compared with SS50, [e] compared with SS25; $p < 0.05$.

Table 10. Effect of stigmasterol on castor oil-induced enteropooling in mice

Sample	Mean volume of small intestinal content (ml)	Percent inhibition	Mean weight of small intestinal content (g)	Percent inhibition
DW10	0.95±0.02	--------	2.35±0.04	--------
Lop3	0.13±0.02^{ade}	86.32	1.35±0.02^{ae}	42.55
SS25	0.48±0.04^{abcd}	49.47	1.75±0.07^{abcd}	25.53
SS50	0.32±0.03^{ae}	66.32	1.48±0.03^{ae}	37.02
SS100	0.22±0.02^{ae}	76.84	1.37±0.02^{ae}	41.70

Data is expressed as mean ± SEM and percent; Analysis was performed using one way ANOVA followed by Tukey Post hoc test; DW10: distilled water 10 ml/kg (negative control), Lop3: loperamide 3 mg/kg (positive control), stigmasterol SS25: stigmasterol (25 mg/kg), SS50: stigmasterol (50 mg/kg), SS100: stigmasterol (100 mg/kg); [a] compared with negative control (DW = 10 ml/kg), [b] compared with the positive control, [c] compared with SS100, [d] compared with SS50, [e] compared with SS25; $p < 0.05$.

Table 11. Effect stigmasterol on castor oil-induced gastrointestinal motility in mice

Sample	Length of small intestine (cm)	Movement of charcoal meal (cm)	Peristaltic index (%)	Percent inhibition
DW10	55.83±0.40	45.50±0.43	81.50	--------
Lop3	56.00±0.37	21.83±0.60[ade]	38.98	52.02
SS25	54.33±0.67	27.67±0.67[abcd]	50.93	37.51
SS50	54.33±0.80	24.50±0.43[abe]	45.09	44.67
SS100	55.67±0.33	22.83±0.40[ae]	41.00	49.69

Data is expressed as mean ± SEM and percent; Analysis was performed using one way ANOVA followed by Tukey Post hoc test; DW10: distilled water 10 ml/kg (negative control), Lop3: loperamide 3 mg/kg (positive control); SS25: stigmasterol (25 mg/kg), SS50: (50 mg/kg), SS100: stigmasterol (100 mg/kg), [a] compared with negative control, [b] compared with the positive control, [c] compared with SS100, [d] compared with SS50, [e] compared with SS25; Negative control: DW10, positive control: Lop 3; $p < 0.05$.

The ADI values of stigmasterol were 83.93, 109.25 and 117.44 at doses of 25, 50, and 100 mg/kg, respectively. These results indicate that the stigmasterol produced dose-dependent antidiarrheal indices with the maximum effect at 100 mg/kg (Table 12).

Table 12. *In vivo* antidiarrheal indices of stigmasterol

Sample	Dfreq (%)	Gmeq (%)	Pfreq (%)	ADI
Lop3	422.45	52.02	83.10	122.23
SS25	330.84	37.51	47.64	83.93
SS50	395.17	44.67	73.87	109.25
SS100	414.77	49.82	78.49	117.44

Dfreq (%): delay in defecation time as percentage of negative control, Gmeq (%): gut meal travel reduction as percentage of negative control, Pfreq (%): reduction in purging frequency in the number of wet stools as percentage of negative control, ADI: antidiarrheal index, Lop3: loperamide 3 mg/kg (positive control); SS100: stigmasterol 100 mg/kg, SS50: stigmasterol 50 mg/kg, SS25: stigmasterol 25 mg/kg.

5. Discussion

Plant-based medicines have been studied for the exploration of new antidiarrheal agents with forward-looking efficacy and safety (Kifle *et al*., 2021). Traditionally *S. ovata* is used for the treatment of diarrhea without scientific validation of its safety and efficacy. Therefore, it is vital to properly assess the safety and efficacy profiles of medicinal plants that are used in traditional medicine. Numerous studies have scientifically approved traditionally used antidiarrheal plants by assessing the effects of these plants on gastrointestinal motility, water, and electrolyte secretion using animal models (Ferede *et al*., 2021). The medicinal value of a plant extract depends on the amount and purity of chemical constituents, which can be affected by different factors, such as climate condition, soil nutrient, method of preparation, and parts of the plant used (Gudeta *et al*., 2020).

In the present work, acute toxicity study revealed that the 80% methanol root extract of *S. ovata* was safe as no sign of overt toxicity was observed at the limit test dose of 2000 mg/kg in mice. At this dose, mortality and delayed toxicity were not observed in the 14 -day follow-up period. As per the OECD guidelines, the LD_{50} value of the extract was greater than 2000 mg/kg in mice (Rameshwar *et al*., 2023). Overall, the current findings demonstrated that at the tested doses, the root extract of the plant was tolerable and safe following oral administration, which validates the safe use of the plant in traditional settings.

80% Methanol was used for the preparation of the total extract since it has an extended polarity index, which makes it suitable for extracting a wide range of compounds with different polarities. Furthermore, solvent fractionation was carried out to determine the fractions with the highest activity, which will make it easier to isolate the bioactive phytochemical constituent(s).

In the present study, antidiarrheal activity was investigated using castor oil-induced diarrhea, enteropooling and gastrointestinal motility tests in mice. In all the three models employed, castor oil was used as diarrhea-prompting agent, whilst loperamide was employed as a positive control because it effectively counteracts the effects of castor oil owing to its anti-motility and anti-secretory properties. It is widely known that castor oil is metabolized into ricinoleic acid in the gut, which in turn induces irritation and inflammation of the intestinal mucosa. This leads to prostaglandin release, which eventually increases gastrointestinal motility, net secretion of water and electrolytes (Gaginella and Phillips, 1975; Ammon *et al*., 1974; Pierce *et al*., 1971). Furthermore, castor oil inhibits intestinal Na^+/K^+-ATPase activity, decreases normal fluid absorption and activates adenylate cyclase or mucosal cAMP-mediated active secretion and nitric oxide (Abdela, 2019). Based on the findings of this study, both the 80% MeOH extract and solvent fractions protected the experimental animals from diarrhea perhaps by interfering with prostaglandins synthesis or activity.

The animals were screened initially by giving 0.5 ml of castor oil and only those 306 mice showing diarrhea were selected for the final experiment (Tadesse *et al*., 2014).

In spite of the absence of a precise definition of diarrhea, feces weight and consistency are frequently taken into account. Concerning this, the number of loose or wet feces was documented in this study (Ayalew *et al*., 2022). In the castor oil-induced diarrheal model, the 80% MeOH extract as well as the methanol and hexane fractions of *S. ovata* roots significantly delayed the time of diarrheal onset, decreased the frequency of defecation and weight of feces at all tested doses. The chloroform fraction, on the other hand, showed activity only at higher tested doses. In this model, the effect of the highest tested dose (400 mg/kg) of the methanol and hexane fractions was comparable with the effects of the 80% MeOH extract.

There was no significant difference in antidiarrheal effect between the methanol and hexane fractions, indicating that both nonpolar and polar secondary metabolites present in the plant contribute to the antidiarrheal activity. The present results are concordant with other studies, where the methanol and hexane fractions displayed comparable inhibition of castor oil induced diarrhea (Alemu *et al*., 2022; Degu *et al*., 2016).

In the castor oil-induced enteropooling test, all the solvent fractions produced a significant but variable reduction in intestinal fluid accumulation with the chloroform fraction showing much weaker effect than those of the methanol and hexane fractions confirming that the most active components of the plant mainly reside in the methanol and hexane fractions. However the effects of the solvent fractions were not as strong as that of the 80% MeOH extract. The reduced anti-enteropooling activity observed for the solvent fractions may have been the presence of highly active polar compounds that were not extracted by the organic solvents or the absence of synergy in the solvent fractions.

In the small intestinal transit test, the charcoal meal technique was designated to monitor the movement of the gastrointestinal content because the reduction of gastrointestinal motility is one mechanism by which numerous antidiarrheal agents can act (Tadesse *et al*., 2017). All the solvent fractions suppressed the propulsion of charcoal marker in a dose-dependent manner. This suggests that the extracts act on all parts of the intestine. Among the solvent fractions, the methanol fraction demonstrated better inhibitory effect on gastrointestinal motility than the hexane and chloroform fractions. However, percentage inhibition of propulsion of charcoal marker by the 80% MeOH extract was superior to that of the methanol fraction.

The antidiarrheal index (ADI) is a measure of a combined effect of the three independent parameters of diarrhea, namely, purging frequency, onset of diarrheal stools, and intestinal

motility. If a test substance shows high ADI values, it is regarded as an effective antidiarrheal agent (Desta *et al*., 2022). In this study, ADI was increased in a dose dependent manner. All the tested extracts attained a maximum ADI value at the highest dose of 400 mg/kg. Among the solvent fractions, the methanol fraction showed the highest ADI value although its effect was not as strong as the 80% MeOH extract. It can therefore be concluded that the most active antidiarrheal principle(s) of *S. ovata* root reside in the polar fraction.

In view of the strong antidiarrheal activities of the methanol fraction of *S. ovata* root, this fraction was further subjected to phytochemical investigation. Isolation and purification of the major compound was achieved by silica gel column chromatography. Identity of the major compound was established tentatively as the 3β-sterol, stigmasterol (**1**), by means of its ^{1}H and ^{13}C NMR spectral characteristics.

In the present study, stigmasterol displayed a significant ($p < 0.05$) antidiarrheal effect against castor oil-induced diarrhea, reduced volume and weight of small intestinal content, and decreased gastrointestinal motility in mice in a dose dependent manner. In particular, at a dose of 100 mg/ml, the effect of stigmasterol in prolonging the onset of diarrhea, and retarding gastrointestinal motility was comparable to that of loperamide (3 mg/kg).

Perusal of the literature unveils that stigmasterol is endowed with potent pharmacological effects such as anticancer, anti-osteoarthritis, anti-inflammatory, anti-diabetic, immunomodulatory, antiparasitic, antifungal, antiviral, antibacterial, antioxidant, and neuroprotective properties (Bakrim *et al*, 2022). Abiyana (2021) demonstrated that stigmasterol has potent antibacterial activity with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 3.9 μg/mL and 7.81 μg/mL, respectively, against *Escherichia coli*, which is one of the most frequently identified organisms that cause bacterial diarrhea. Stigmasterol was also shown to have a strong

activity (MIC = 12.5 μg/mL) against methicillin-resistant *Staphylococcus aureus* (MRSA), the causative agent of enterocolitis characterized by watery diarrhea that leads to severe dehydration (Yusuf *et al*., 2018). It is therefore very likely that the antibacterial activity of stigmasterol contributes to its antidiarrheal effect. To the best of our knowledge this is the first report on the antidiarrheal activity of stigmasterol.

6. Conclusion

The results of the present study revealed that the 80% MeOH root extract of *S. ovata* is endowed with a promising antidiarrheal activity. This provides the rationale for the use of the root extract of *S. ovata* as an antidiarrheal drug by traditional healers. Moreover, the *n*-hexane, chloroform and MeOH fractions showed varying degree of antidiarrheal activity, with the MeOH and *n*-hexane fractions demonstrating superior activity in all the three models of diarrhea used in the present study. This is an indication that both nonpolar and polar compounds are responsible for the antidiarrheal activity of *S. ovata* root. Stigmasterol isolated from the most active MeOH fraction prolonged the onset of diarrhea, and retarded gastrointestinal motility suggesting its major role for the antidiarrheal effect of the plant. The antidiarrheal effect of stigmasterol observed in the current study may have been coupled with its reported potent activity against diarrhea causing bacteria make this plant sterol a suitable candidate for antidiarrheal drug development. Overall, the present findings provide a scientific evidence for the folkloric use of *S. ovata* roots in the treatment of diarrhea.

Recommendations

Based on the findings of the present study the following recommendations are suggested:

- Sub-acute, sub-chronic and chronic toxicity study of the 80% MeOH extract need to be conducted to assess the long term safety profile of the extract.
- Further pharmacological studies should be done to determine the detailed antidiarrheal mechanism(s) of action of the extracts and stigmasterol.
- Use stigmasterol as a key drug lead compound for the development of safe, potent, and cost-effective antidiarrheal drugs.

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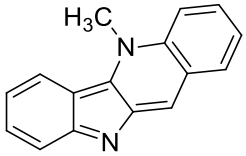
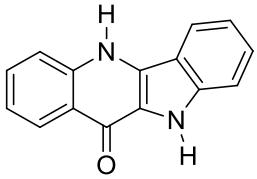
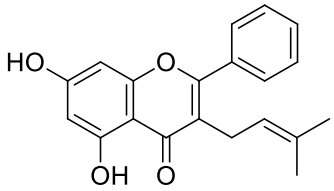
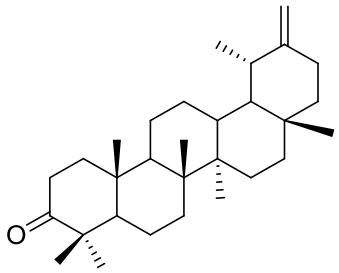
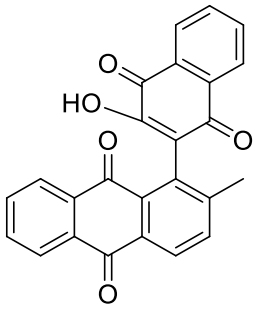
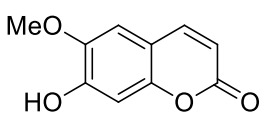
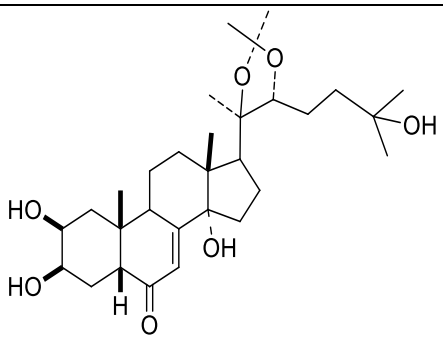
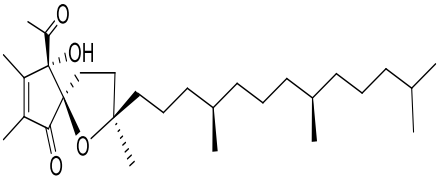
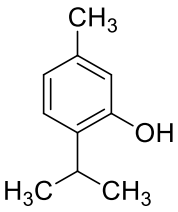
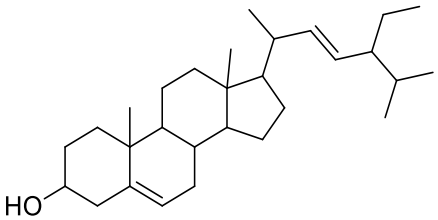
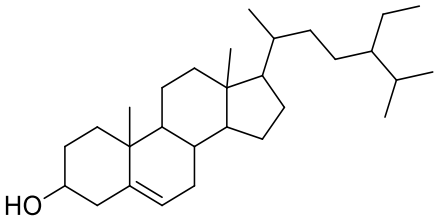
Appendices

Appendix 1. Structures of some compounds isolated from different parts of genus *Sida*

Name of the compound	Class of the compound	Structure	Refference
cryptolepine	Alkaloid		Aminah *et al*., 2021
Quindolinone	Alkaloid		Chaves *et al*., 2017
5,7-dihydroxy-3-isoprenyl flavone	Flavonoid		Aminah *et al*., 2021
Taraxasterone	Terpenoid		Aminah *et al*., 2021
Buildiaquinone	Anthraquinones		Ekpo and Etim, 2009
Scopoletin	Coumarins		Aminah *et al*., 2021

Appendix 1. continued

Name of the compound	Class of the compound	Structure	Reference
20-Hydroxyecdysone-20, 22-monoacetonide	Ecdysteroids		Aminah *et al*., 2021
α-tocospiro B	Tocopherols		Aminah *et al*., 2021
Thymol	Volatile oils		Njoku *et al*., 2021
Stigmasterol	Sterol		Laili *et al*., 2022; Das *et al*., 2011
β-sitosterol	Sterol		Das *et al*., 2011

Appendix 2. *Sida ovata* plant in its natural habitat

Photographed by Tesfa Begashaw around Wesen Kurkur, Shewarobit, Cenral Ethiopia- October, 2022

Appendix 3. Some photographs taken during laboratory work







Appendix 4. Ethical clearance

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Addis Ababa University

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School of Pharmacy
Ethical Review Committee

ቀን Date: September 04, 2023

ቁጥር Ref. No.: ERB/SOP/553/15/2023

To: **Tesfa Begashaw**
School of Pharmacy

Re: Ethical Clearance

It is to be recalled that you submitted a research proposal entitled "**Analgesic, Anti-Inflammatory, Anti-diarrheal Activities of the 80% Methanol Extract and Compound Isolation from The Root of *Sida ovata* FORSSK".** 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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