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GC-MS analysis and *in vivo* anti-nociceptive and antidiarrheal activities of ethyl acetate and ethanol extracts of *Buxus wallichiana* Bail

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Buxus wallichiana Bail is an important plant species in the Buxaceae family that has ethnobotanical importance and is well known for its ethnomedicinal applications. Whole plant and also parts of this plant is used as traditional medicines as depressive disorders, exhaustion, HIV, cancer, rheumatism, malaria, cardiac difficulties and skin issues because of its medicinal properties. The aim of the present study was to identify the bioactive compounds in this plant by GC-MS analysis and to evaluate the in vivo biological activities like anti-noceceptive and antidirrheal activities. GCMS analysis was performed by standard procedure. Anti-nociceptive activity was assessed by tail immersion method while antidiarrheal activity was evaluated against castor oil induced diarrhea. GCMS analysis revealed 15 natural compounds in the ethanol and ethyl acetate extract of *Buxus wallichiana* of which 8 compounds were identified by ethanol extract and 7 compounds were by ethyl acetate extract exhibiting different pharmacological activities. For anti-nociceptive activity ethanol and ethyl acetate extracts at doses of 100, 200, and 300 mg/kg were used. Maximum nociception inhibition was observed with an ethanol extract at dose of 300mg/kg, which was (12.35 ± 0.09) and (13.16 ± 0.34). For antidiarrheal activity both extracts at doses of 100, 200, and 300 mg/kg were used. At dose of 300mg/kg, both extract showed a highly substantial decrease in the total number of feces which is (11.3±1.4) on ethyl acetate and (9.4±0.58) on ethanol extract while the total number of dirrheal feces is (5.07±1.08) on ethyl acetate and (4.3±0.45) on ethanol extract. The study provides valuable pharmacognostic standards for *Buxus wallichiana* highlighting its significant potential against various diseases and its rich bioactive constituents as a source of pharmaceutical products.

Keywords: GCMS analysis, anti-nociceptive, antidiarrheal, ethanol, ethyl acetate and *Buxus wallichiana*

INTRODUCTION

From earlier civilizations, many chronic illnesses have been treated with plants. An important source of effective natural products with a wide range of chemical compositions, biological characteristics and action mechanisms are produced by plants (Farag *et al.,* 2020). Medical plants have attracted a lot of study focus due to their diverse commercial applications in medicines. The phytoconstituents found in therapeutic plants have been shown to lessen human metabolic disorders and their effects. Due to their low cost and high environmental sustainability, herbal remedies are potential replacements for synthetic compounds used in pharmaceutical products (Ng, Z. X *et al*., 2020).

The *Buxus wallichiana* plant, often known as the Himalayan tree, is an associate of the Buxaceae family and can be seen across Asia, but is most prevalent in Pakistan (Din *et al*., 2022).The Buxaceae family which consists of five genera (Buxus, Notobuxus, Sarcococca, Pachysandra and Styloceras) is a small group of predominantly monoecious evergreen shrubs. Buxus is the largest genus of this family having 156 species. The members of this family are mainly woody and only sometimes herbaceous plants (Ghasemi & Hesamzadeh

2018). In the family Buxaceae, the genus Buxus contains over 70 species that have been employed in traditional medical practices for their anticholinesterase, anti-HIV, immunosuppressive and cytotoxic properties (Saleem *et al.,* 2019).

GC-MS is an analytical approach that combines the properties of gas chromatography and mass spectrometry for recognizing various compounds in a test sample. This apparatus can discriminate between chemical combinations and identify their molecular Make up with the aid of its GC and MS components. This technique can be used to study metabolites that have high volatility, low boiling temperatures or low polarity when they are derivatized. This analytical method is extremely potent because it is utilized to conduct a 100% specific test that positively detects the existence of specific chemicals (Pramod *et al*., 2020).

Initial screening of medicinal plants relies on spectroscopic and chromatographic techniques to gather essential chemical and pharmacological data. GC-MS has gained popularity for identifying functional groups and diverse bioactive compounds within medicinal plants. GC-MS is a rapid, reliable and efficient technique capable of detecting various substances, including alkaloids, alcohols, nitro compounds, organic acids, hydrocarbons, steroids, amino acids and esters using small quantity of plants extracts (Konappa *et al*., 2020).

Pain is thought to be a distressing sensory and emotional experience connected to tissue damage. Moreover, it is commonly brought on by unpleasant stimuli and transmitted to the central nervous system (CNS), where it is recognized. It is a method of protecting the body from harm. Despite the availability of adequate drugs, pain remains a challenging and incapacitating health problem that affects 80% of adults globally. The most prevalent condition that causes harm and injury is chronically prolonged pain that is untreated. The majority of disabilities are caused by these crippling disorders, which can also be fatal if not properly treated and monitored. The cornerstone for treating and controlling chronic disorders remains the use of conventional painkillers. Unfortunately, they come with a host of negative health effects and toxicity, such as gastrointestinal discomfort, gastric ulcers, altered kidney function, potential effects on hypertension, liver damage and platelet suppression, which can cause bleeding (Mitra *et al*., 2022).

Diarrhea is characterized by the movement of abnormal liquids or unformed stools through the digestive system, associated with elevated defecation frequency and abdominal pain. Every year, 2.5 million children die worldwide from diarrhea-associated complications, and 80% of these deaths occur in developing countries. The most popular treatment for diarrhea is oral rehydration using saline. In its guidelines for diarrheal management, the World Health Organization has acknowledged the accepted use of traditional medical practices. Natural antidiarrheal agents include kaolin, pectin, berberine and muscarinic agents in addition to morphine, codeine and paregoric (Nasrin *et al*., 2022).

MATERIALS AND METHODS

Collection of Plant

The whole plant of *Buxus wallichiana* was collected from District Bunir (KPK) Pakistan. The
plant was identified using the Flora of Pakistan and by consulting with a plant taxonomist and was mounted on the Herbarium of Qurtuba University of Science and Information Technology Peshawar under the voucher number of Nadia Bot. 240 for future reference.

Preparation and extraction of plant material

The whole plant was collected, air dried, ground into powder and stored. 100 grams of the powder underwent separate extraction with ethyl acetate and ethanol solvents (500 mL each). The extracts were filtered, concentrated and stored at 40 °C for future use, following the methodology of Kifle *et al*., (2021).

GC–MS analysis

The plant extracts underwent GC–MS analysis using a Perkin-Elmer Clarus 680 system following Konappa *et al*. (2020) procedure at PCSIR Peshawar. Perkin-Elmer Clarus 680 system equipped with a fused silica column, packed with capillary column. Pure helium gas (99.99%) was used as the carrier gas at a constant flow rate of 1 mL/min. For GC–MS spectral detection, an electron ionization energy method was adopted with high ionization energy of 70 eV (electron Volts) with 0.2 s of scan time and fragments ranging from 40 to 600 m/z. The injection quantity of 1 µL was used (split ratio 10:1) and the injector temperature was maintained at 250 °C (constant). The column oven temperature was set at 50 °C for 3 min, raised at 10 °C per min up to 280 °C and final temperature was increased to 300 °C for 10 min. The contents of phytochemicals present in the test samples was identified based on comparison of their retention time (min), peak area, peak height and mass spectral patterns with those spectral database of authentic compounds stored in the National Institute of Standards and Technology (NIST) library.

In vivo Biological activities

Experimental animals

Swiss albino mice (25-30g, both sexes) from the Veterinary Research Institute in Peshawar were acclimated to standard laboratory conditions for 4-5 days before the experiment: 12-hour light/dark cycle, (28 ± 5°C), (55 ± 5%) humidity with free access to food and water. The protocols for animal handling and utilization in experiments were obtained by the Ethical committee of Veternary Research Institute Peshawar.

Anti-nociceptive Efficacy by Tail Immersion Approach

The analgesic activity of ethanol and ethyl acetate extracts from *Buxus wallichiana* was evaluated using the tail immersion test, following Chatterjee *et al*. (2015) with 25 mice divided into 5 groups, each consisting of 5 mice. This method entails immersing the last 3 cm of the mouse's tail into a water bath maintained at (55.0±0.5) °C. The mices reaction, indicated by tail withdrawal, was observed shortly after immersion. Stopwatch-recorded reaction times were taken, with each mouse serving as its own control. Control group readings were obtained at 0 and 10-minute intervals and the average of these values represented the initial reaction time. Test groups received various doses of *Buxus wallichiana* ethanol and ethyl acetate extracts (200, 300, and 400 mg/kg) orally, with a 30-minute latency period. Morphine (5 mg/kg) was administered to the standard group, while the control group received 1 ml/kg of saline solution. Reaction times for the test groups were measured and recorded at 30, 60 and 90 minutes post administration.

Antidiarrheal Efficacy via Castor Oil-Induced Diarrhea

The antidiarrheal activity was assessed according to the method used by Awouters *et al*. (1978) with slight modifications. 25 mice in 5 groups after an 18-hour fast, followed by oral castor oil administration. Parameters recorded included diarrhea onset time, total feces count and feces weight. Diarrhea inhibition percentage was calculated in the negative control group.

Percent inhibition = $\left(\frac{A-B}{A}\right)$ x 100 Where, A = mean number of defecation times caused by castor oil and B = mean number of defecation times caused by drug or extract.

RESULTS

GC–MS analysis

The GCMS analysis of *Buxus wallichiana* revealed 15 natural compounds in the ethanol and ethyl acetate extracts. A total of 15 compounds were identified from the GC-MS examination of which 8 compounds were identified by ethanol extract and 7 compounds were by ethyl acetate extracts. The bioactive compounds detected in ethanol extract are Furfural, Retinal, 2,6,10,14 Tetramethylpentadecan-3-one, Tetradecanoic acid,12-methyl-,methyl ester, 9,12,15-Docosatetraeonic acid, Hexadecanoicacid, methyl ester, 9,12-octadecanoic acid methyl ester. The chromatogram of these compounds is shown in Fig 1 and the compounds, combined with their retention time (RT), peak area and molecular formula are shown in Table 1 while propanoic Acid, Retinal, Hexadecanoic acid, 9-Octadecenoic acid, 1,2,3-propanetriyl ester, 3-mercapto-, propanoic acid,dodecyl ester, Octadecanal, 2-bromo-,9,12-octadecanoic acid methyl ester were identified by ethyl acetate extract. Their chromatogram is shown in Fig 2 and the compounds are listed in Table 2 together with their RT, peak area and molecular formula. *Buxus wallichiana* contains bioactive compounds, indicating their traditional use for various medical conditions. Isolating these phytochemicals and applying them to biological action could yield positive effects.

Table1: GCMS analysis of ethanol extract of *Buxus wallichiana*

S.No	Name of the compound	Retention Time (min)	Peak Area	Molecular Formula
1.	Furfural	5.26	1814262	$C_5H_4O_2$
2.	Retinal	7.84	3187615	$C_{20}H_{28}O$
3.	2,6,10,14 Tetramethylpentadecan-3-one	9.72	2486133	$C_{19}H_{38}O$
4.	Tetradecanoic acid,12-methyl-,methyl ester	12.04	36272356	$C_{16}H_{32}O_2$
5.	Linoliec acid ethyl ester	14.12	2392672	$C_{20}H_{36}O_2$
6.	9,12,15-Docosatetraeonic acid, methyl ester	16.52	5516842	$C_{23}H_{38}O_2$
7.	Hexadecanoicacid,methyl ester	17.26	3254536	$C_{17}H_{34}O_2$
8.	9,12-octadecanoic acid methyl ester	19.13	2545325	$C_{19}H_{34}O_2$

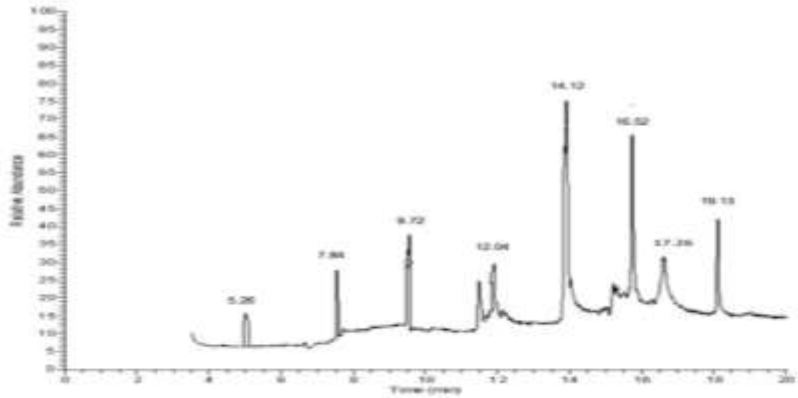

Figure 1:GCMS chromatogram of ethanol extract of *Buxus wallichiana*

Table 2:GCMS Study of ethyl acetate extract of *Buxus wallichiana*

S.No	Name of the compound	Retention Time (min)	Peak Area	Molecular Formula
1.	Propanoic Acid	7.05	2267785	$C_3H_6O_2$
2.	Retinal	9.16	2583436	$C_{20}H_{28}O$
3.	Hexadecanoic acid	11.02	27104953	$C_{16}H_{32}O_2$
4.	9-Octadecenoic acid, 1,2,3-propanetriyl ester	14.73	4635382	$C_{57}H_{104}O_6$
5.	Propanoic acid, 3-mercapto-, dodecyl ester	15.62	34760228	$C_{15}H_{30}O_2S$
6.	Octadecanal, 2-bromo-	17.13	23872235	$C_{18}H_{35}BrO$
7.	9,12-octadecanoic acid methyl ester	19.64	25305842	$C_{19}H_{34}O_2$

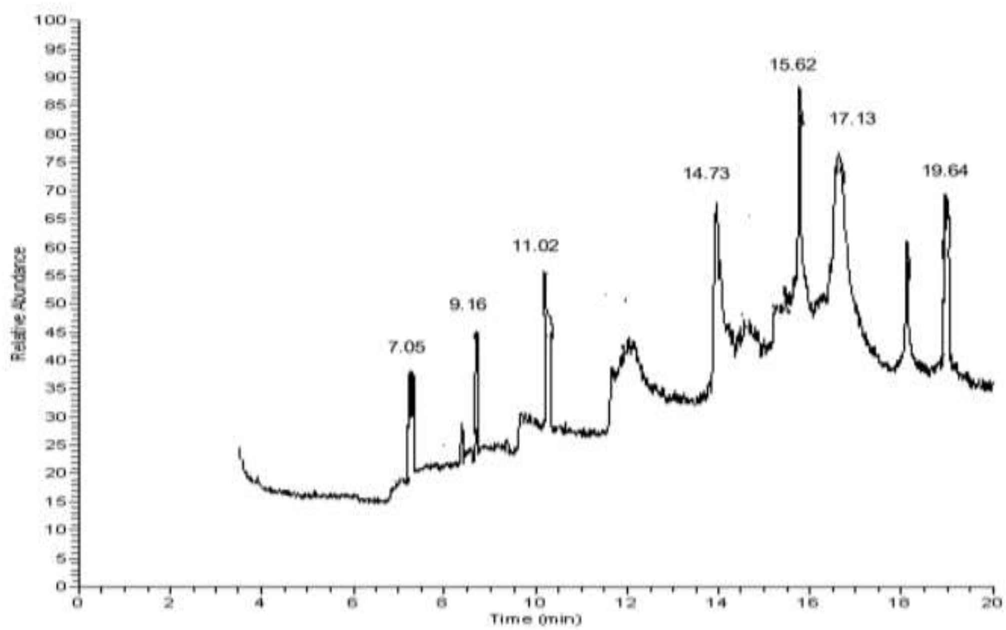

Figure 2: GCMS chromatogram of ethyl acetate extract of *Buxus wallichiana*

Anti-nociceptive activity

The results indicated that the ethyl acetate extract of *Buxus wallichiana* exhibited moderate anti-nociceptive activity at doses of 200 mg/kg, registering a reaction time of (10.0±0.15) after 30 minutes. At 300 mg/kg the modest effect recording a reaction time of (10.62±0.03) after 30 minutes. Compared to the control group the extract at all doses significantly prolonged the reaction time. The ethanolic extract demonstrated significant dose-dependent anti-nociceptive activity, with a reaction time of (10.05±0.21) at 100 mg/kg after 30 minutes and (10.18±0.24) at 200 mg/kg after 60 minutes, in comparison to the negative control group. However, its effect was lower than that of the standard drug Morphine which exhibited the highest response at a dose of 10 mg/kg with reaction times of (15.32±0.07), (14.25±0.05) and (12.53±0.02) after 30, 60 and 90 minutes respectively. The maximum inhibition of nociception in response to thermal stimuli was observed with the ethanol extract at a higher dose of 300 mg/kg, recording reaction times of (12.35±0.09) after 30 minutes and (13.16±0.34) after 90 minutes, although still less effective than the standard drug Morphine.

The statistical analysis was performed using one way ANOVA followed by dunnet's t-test. (Values are mean ± SEM. n=5, in each group *p<0.05, **p<0.01, Level of significance) is used to analyze the results and the results has been summarized in Table 3 and showed on Fig 3.

Table3. Anti-nociceptive activity of *Buxus wallichiana*

Treatment	Dose (mg/kg)	Reaction Time		
		30 min	60 min	90 min
Normal saline	1mL/kg	4.53±0.06	5.24 ±0.05	6.32±0.6
Morphine	10 mg/kg	15.32±0.07**	14.25±0.05**	12.53±0.02**
Ethyl acetate	100 mg/kg	8.23±0.21	7.42 ±0.08	9.35±0.32
	200 mg/kg	10.0±0.15*	8.52±0.08	8.21± 0.06
	300 mg/kg	10.62±0.03*	6.32±0.06	7.73±0.07
Ethanol	100 mg/kg	10.05±0.21*	9.32±0.06	11.23 ±0.18
	200 mg/kg	8.16±0.12	7.23 ±0.10	10.18 ±0.24*
	300 mg/kg	12.35±0.09**	10.40±0.43	13.16±0.34**

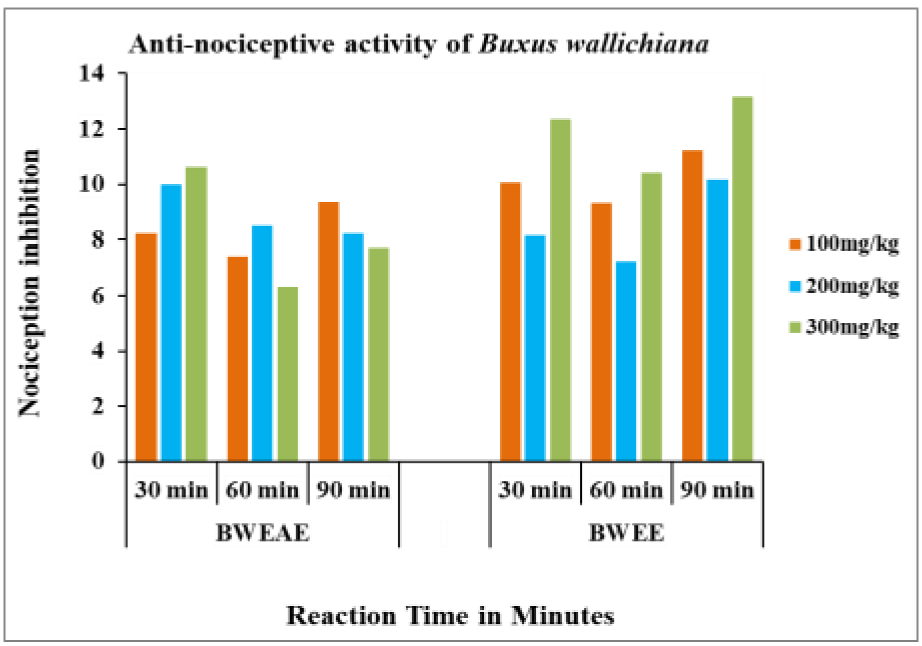

Figure 3: Nociception inhibition of thermal stimulus

Castor oil-induced diarrhea

Buxus wallichiana was tested for anti-diarrheal activity in mice using the castor oil-induced diarrheal technique. The test groups were given ethyl acetate and ethanol extracts of *Buxus wallichiana* at doses of 100, 200 and 300 mg/kg orally, whereas the positive control group was given castor oil **+** loperamide at a dose of 5 mg/kg and the negative control group was given castor oil **+** saline at a dose of 2 mL/kg orally. Diarrhea was observed in all of the animals in the testing groups following castor oil administration for 4 hours. The standard drug loperamide (5 mg/kg, p.o) made a markedly higher decrease in defecation and diarrhea, the total amount of feces is (8.0±0.74) while the total number of diarrheal feces at the standard drug loperamide is (3.00±0.64). In terms of defecation and number of wet feces (diarrhea), ethyl acetate and ethanol extracts of *Buxus wallichiana* have proven to be efficacious upon castor oil-induced diarrhea in mice at all testing doses. Both extracts demonstrated a substantial dose-dependent reduction in the total number of defecations and diarrhea at doses of 200 and 300 mg/kg but the impact of both extracts was as strong as loperamide at higher dosages of 300 mg/kg. At a greater dose of 300mg/kg, the ethyl acetate extract of *Buxus wallichiana* considerably prevented diarrhea. It has also showed a highly substantial decrease in feces at a dose of 300 mg/kg, the total number of feces is (11.3±1.4) and the total number of diarrheal feces is (5.07±1.08). A moderate effect was detected at the dose of 200 mg/kg in which the total number of feces is (15.4±2.4) and the total number of diarrheal feces is (7.3±0.74). However, ethyl acetate failed to exhibit a noteworthy effect at 100 mg/kg. Detailed results listed in Table 4, Fig 4(a) and Fig 4(b).

DISCUSSION

GCMS analysis

Gas Chromatography-Mass Spectrometry is a crucial analytical method used to identify chemicals in plant samples, particularly in studying the chemotaxonomic composition and biologically active constituents of medicinal plants (Awaludin et al. 2020). GC-MS analysis is crucial for separating phytochemical substances in plants, identifying hundreds of compounds that traditional phytochemical screening cannot identify (Olivia et al. 2021). Furfural, a product of lignocellulosic degradation, is a key flavor compound in foods (Mohammadi *et al*., 2019). Retinal, also called retinaldehyde, is a type of vitamin A and a unique component in chicory (Suleiman, 2020). Hexadecanoic acid is known for its flavor and antibacterial, cosmetic, perfumery, hypercholesterolemic and lubricant properties. Octadecanal, 2-bromo exhibits anti-inflammatory and anti-apoptotic effects. 9,12,15 octadecatrienoic acid is associated with hypocholesterolemic, anti-inflammatory, cancer preventive, nematicide, hepatoprotective, insectifuge, antihistaminic, anticoronary, antiarthritic, anti-acne and antieczemic activities. Methyl ester and hexadecanoic acid demonstrate nematicide, antioxidant, pesticide, antiandrogenic, and flavor properties. Lastly, 9,12-octadecadienoic acid offers hypocholesterolemic, anti-inflammatory, hepatoprotective, cancer preventive, nematicide, insectifuge, anti-eczemic, antihistaminic, anti-acne, anti-arthritic, anti-coronary and insectifuge effects (Pawar et al. 2023). The results of our study are related to those of Moussa & Almaghrabi (2016) who found 9,12-octadecanoic acid methyl ester, hexadecanoicacidand methyl ester from the *Peganum harmala* plant. Mohammadi et al. (2019) detected Furfural and Propanoic Acid, while Suleiman, (2020) find out Retinal by GCMS analysis which is related to our study. Similar reports were also presented by Pawar et al. (2023) who detected Hexadecanoic acid, 9,12,15 octadecatrienoic acid, Octa-decanal, 2-bromo, methyl ester, 9,12-octadecadienoic acid and Octa decanoic acid, ethyl ester which are similar to our findings. The findings indicate that *Buxus wallichiana* has a variety of bioactive compounds. As a result, it has been identified that *Buxus wallichiana* has phytopharmaceutical significance.

Tail immersion method

Pain is caused by various processes and can be neuropathic, inflammatory or physiological. The tail immersion experiment evaluates the anti-nociceptive characteristics of drugs by assessing latency time in the supraspinal and spinal systems, which is linked to the central analgesic effect of the medicines (Gao *et al*., 2021). The tail immersion-induced nociception paradigm is a widely used technique for determining the anti-nociceptive properties of plant extracts, and identifying opioid-like analgesics from marginal pain relievers (Li et al. 2021). *Buxus wallichiana* anti-nociceptive potential was assessed in Swiss albino mice for central pharmacological action. GCMS analysis of *Buxus wallichiana* revealed various bioactive compounds potentially contributing to its therapeutic properties in pain management. The findings of our study align with a study by Bilal et al. (2023), where an ethyl acetate extract of *Iris albicans* at a dose of 200 mg/kg displayed a similar moderate effect. Similar results were reported by Shahriar et al. (2018) and Asante et al. (2019) for the ethanol extract of *Citrus assamensis* and *Vernonia amygdalina* leaf extracts at doses of 100 and 200 mg/kg. The results were also accordance with the findings of More *et al*. (2021)

Antidirrheal activity by castor oil induced dirrheal method

Diarrhea is a condition characterized by frequent watery bowel movements exceeding three times within 24 hours often due to an imbalance in intestinal absorption and secretion mechanisms (Tagne *et al*., 2019). Castor oil induces diarrhea due to its ricinoleic acid metabolite. When castor oil encounters lipase in the duodenum, it releases ricinoleic acid, which remains poorly absorbed in the small intestine and leads to inflammation and irritation of the intestinal lining, triggering the release of autocoids and prostaglandins. These substances stimulate intestinal mobility, alter electrolyte permeability and promote hypersecretion, ultimately causing diarrhea (Mehesare et al. 2019).

Table 4: Antidiarrheal activity of *Buxus wallichiana* extracts.

Treatment	Conc. (µg/ml)	Total number of feces	% Inhibition of Defecation	Total number of diarrheal feces	%Inhibition of diarrhea
Castor oil + Saline	2 mL/kg	21.3±1.5	----	13.06±1.05	-----
Castor oil + Loperamide	5 mg/kg	8.0±0.74**	62.44 %	3.00±0.64**	77.03 %
Ethyl acetate	100	17.5±1.06	17.84 %	10.04±1.3	23.12 %
	200	15.4±2.4*	27.70 %	7.3±0.74*	44.10 %
	300	11.3±1.4**	46.95 %	5.07±1.08**	61.18 %
Ethanol	100	14.0±0.65	34.27 %	8.4±1.4	35.68 %
	200	12.3±1.7**	42.25 %	6.08±0.78*	53.45 %
	300	9.4±0.58**	55.87 %	4.3±0.45**	67.08 %

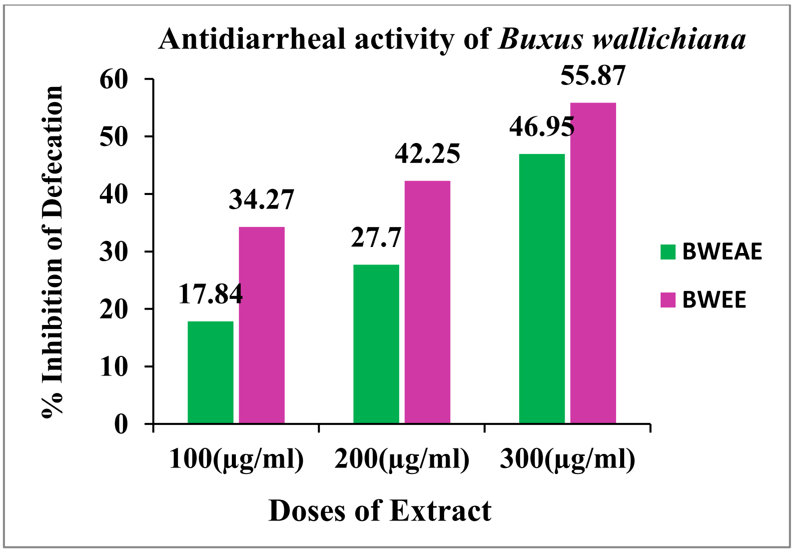

Figure 4 (a): % Inhibition of Defecation

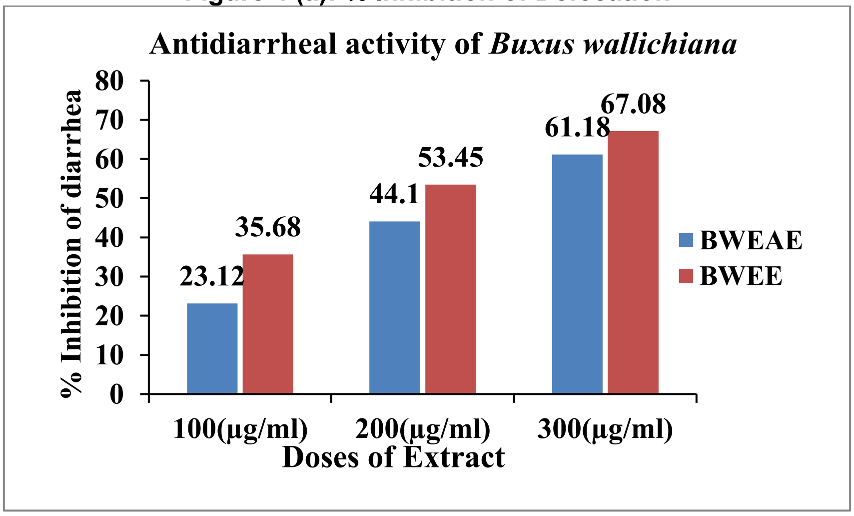

Figure 4 (b): %Inhibition of diarrhea

The anti-diarrheal investigation typically involves monitoring parameters such as the onset time of diarrhea, stool frequency, consistency, and duration when castor oil is employed to induce diarrhea. Loperamide, a known anti-diarrheal agent, acts by enhancing electrolyte permeability at the intestinal mucosal level and increasing cholecystokinin secretion in the duodenum, which, paradoxically, leads to hypersecretion and impedes fluid reabsorption (Ismail et al. 2021). Our results agree with Rahman et al. (2018) who examine antidiarrheal activity from the *Gendarussa vulgaris* leaves *abyssinica*, Alemu et al. (2022) from the roasted seed of coffea Arabica, Mengesha et al. (2022) from the acacia seyal and Worku et al. (2023) from the root of Verbascum sinaiticum. Our findings concur with Shukla & Gahlot (2020), Odenigbo et al. (2020), Doe et al. (2019) and Zehra et al. (2019). All of them indicate a highly significant result from the leaves and stem ethanol extract of *Bauhinia vahlii*, leaf extracts of *Rauwolfia vomitoria*, seed extract of *Cola nitida*and ethanol extract of *Fragaria ananassa* and *Actinidia deliciosa* fruit respectively. The result at the dose of 200 mg/kg is slightly different as ethanol show a moderate effect in term of the total number of diarrheal feces which is (6.08±0.78). A similar result reported by (Shahid et al.2023) used an ethanol extract of *Persicaria barbata* and showed a moderate effect of ethanol. But at 100 mg/kg, ethanol had no substantial impact. The bioactive compounds detected in *Buxus wallichiana* could be responsible for medicinal benefits., Rahman et al. (2019) from the *Hemigraphis alternata* leaves, Kifle et al. (2021) from *Hagenia*

CONCLUSIONS

A highly composite profile of phytoconstituents was revealed by GC-MS analysis of *Buxus wallichiana* extract. Hence, it is concluded that the phytocompounds found in *Buxus wallichiana* extract may have great potential for use in medicine to treat a variety of diseases in people. This research underscores the potential anti-noceceptive and antidiarrheal properties of ethyl acetate and ethanol extracts from *Buxus wallichiana* and suggests further investigation for therapeutic applications.

Supplementary materials

Not applicable

Author contributions

All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

All of the data is included in the article/Supplementary Material.

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Conflict of interest

The authors declared that present study was performed in absence of any conflict of interest.

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