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Exploring the therapeutic potential of a polyherbal combination for pain and inflammation

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Abstract: **Background**: Pain and inflammation are physiological responses to tissue injury and serves as a defense mechanism against tissue injury caused by various harmful stimuli. Nearly all acute and chronic diseases, are influenced by inflammatory process. Presently, available pharmacologic agents have limitations due to their adverse effects. Therefore, there has been growing attention towards alternative and combination-based therapeutic approaches aimed at attaining enhanced efficacy with minimal adverse effects. In folk medicines *Boswellia serrata, Brassica nigra, Piper longum* and *Withania somnifera* have been reported to have analgesic and anti-inflammatory effect but their efficacy in combination, has not been studied. **Objectives**: The aim of this study is to evaluate the analgesic and anti-inflammatory effects of *Boswellia serrata, Brassica nigra, Piper longum, Withania somnifera* and their combination using *in vivo* models. **Method**: The plant extracts were administered orally to experimental animals, individually and in combination, at doses of 400 and 800 mg/kg. The analgesic activity was assessed using the tail immersion, hot plate and acetic acid-induced writhing tests in Swiss albino mice, while anti-inflammatory activity was examined via carrageenan-induced paw edema in Wistar albino rats. Acute toxicity was evaluated with the doses up to 3000 mg/kg. **Results**: In the acute toxicity study no mortality was observed. All individual extracts significantly increased pain thresholds and reduced inflammation in carrageenan-induced paw edema assay in dose depended manner as compared to vehicle controls ($p < 0.05$), with the polyherbal combination producing the highly significant effects ($p < 0.001$). **Conclusion**: The obtained results suggest that each of these plants possesses analgesic and anti-inflammatory properties while their combination offers enhanced efficacy, likely due to complementary pharmacological action among the plant extracts, indicating their use as more effective herbal therapeutic alternative for pain and inflammation.

Keywords: Analgesic; Anti-inflammatory; *Boswellia serrata*; *Brassica nigra*; *Piper longum*; *Withania somnifera*

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INTRODUCTION

Pain and inflammation are important physiological responses that alert the body to injury and serve as a defensive device to counteract tissue injury compelled by several harmful stimuli (Yu *et al*., 2020; Arome *et al*., 2014). However, persistence of these mechanisms not only compromises physical wellbeing but also disrupts mental health thus reducing quality of life (Wang *et al*., 2025), and may potentially lead to progressive conditions such as autoinflammatory disorders, cancers or neurodegenerative diseases (Dinarello, 2010). Pain and inflammation are linked with cyclooxygenase (COX) enzymes, particularly COX-2, that either directly stimulate pain receptors or sensitize them to other pain-producing mediators (Arome *et al*., 2014). Activation of COX-2 causes the release of pro inflammatory mediators such as NO, PG, TNF-α, IL-1β and IL-6, which collectively promote inflammation (Farhan *et al*., 2020). Current pharmacological management, including non-steroidal anti-inflammatory drugs, acetaminophen and opioids often create significant safety risks, such as gastrointestinal adverse reactions, liver toxicity and addiction, respectively (Wang *et al*., 2025). This creates opportunities for formulating new therapeutic strategies to achieve effective pharmacological response with minimum side effects (Gao *et al*., 2021; Wang *et al*., 2025). Plant-based therapies are increasingly recognized for managing pain and inflammation (Arome *et al*., 2014). Compared to many synthetic drugs, herbal preparations deliver extensive therapeutic benefits with fewer side effects targeting multiple inflammatory pathways through the mutual action of various phytochemicals (Ghasemian *et al*., 2016; Wang *et al*., 2025). In light of these challenges, increasing attention has been directed toward medicinal plants with significant anti-inflammatory potential. Among them *Boswellia serrata* (Indian frankincense), *Brassica nigra* (Black mustard), *Piper longum* (Long pepper) and *Withania somnifera* (Indian ginseng) have been previously reported to effectively treat inflammatory disorders (Ara *et al*., 2021; Bhat *et al*., 2014; Siddiqui, 2011; Singh *et al*., 2010).

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Boswellia serrata contains boswellic acid and other bioactive constituents, has long been used in the management of osteoarthritis, rheumatoid arthritis and inflammatory bowel diseases (Suchita *et al*., 2021), Similarly, *Brassica nigra* has been traditionally employed as rubefacients and in the management of respiratory ailments (Bhat *et al*., 2014). *Piper longum* enriched with piperlongumine, piperine and other alkaloids has attracted attention not only for its anti-inflammatory potential but also for its anticancer potential (Bao *et al*., 2014). *Withania somnifera* has a long history of use in traditional medicine and is well recognized for its anti-inflammatory, antioxidant, antitumor and antibacterial properties (Singh *et al*., 2010). Although these findings demonstrate that each plant exhibit therapeutic efficacy individually, but investigation on their combination was still lacking. Notably, Boswellic acids from *B. serrata* have potent anti-inflammatory property by inhibiting 5-lipoxygenases (Suchita *et al*., 2021), flavonoids present in *B. nigra* possess antioxidant and anti-trypsin effects (Alam *et al*., 2011). Piperine and piperlongumine of *P. longum* having immunomodulatory activity (Zaveri *et al*., 2010) while Withanolide derivatives from *W. somnifera* exhibit anti-inflammatory effect by inhibiting NF-κB activation (Kumar *et al*., 2015). These individual chemical components modulate different molecular pathways such as prostaglandin production, cytokine release, and oxidative stress cascades and provide a strong justification to combine them in a single or sequential process.

Based on the hypothesis, the polyherbal combination of these herbal extracts would demonstrate greater analgesic and anti-inflammatory efficacy than individual components due to their complementary mechanisms of action, the current research is aimed at preparing this poly herbal combination; and evaluating and comparing the analgesic as well as anti-inflammatory effects of each constituent, individually and in combination.

MATERIALS AND METHODS

Plant materials

Oleo-gum resin of *Boswellia serrata*, seeds of *Brassica nigra*, fruits of *Piper longum* and roots of *Withania somnifera* were purchased from local market in Karachi, and were authenticated by a taxonomist with identification number of *Boswellia serrata* (BSGR-01-21), *Brassica nigra* (BNS-02-21), *Piper longum* (PLF-03-21) and *Withania somnifera* (WSR-04-21).

Chemicals

All chemicals and solvents used in this study were of analytical grade (Merck and B.D.H.) and were purchased from local market.

Experimental animals

The experiments were performed using Swiss albino mice (20–25 g) and Wistar albino rat (150-180g), purchased from the National Facility for Laboratory Animal Research and Care, The International Center for Chemical and Biological Sciences (ICCBS), University of Karachi. To minimize variability due to sex-based differences in body weight and aggressive behavior in males only female animals were used in this study. One week before the experiment, the animals were sheltered in plexiglass enclosure under 22±2ºC temperature with humidity level 55±5% and exposed to 12h light and dark pattern. There were total 12 groups in each experiment comprising of seven animals in each group. The animals were randomly allocated to groups.

Preparation of extracts

Oleo-gum resin of *Boswellia serrata*, seeds of *Brassica nigra*, fruits of *Piper longum* and roots of *Withania somnifera* were washed and dried thoroughly in the shaded area for 2-3 days. Each plant material of 2kg was soaked in 3 liters of ethanol (used as the extraction vehicle) in a closed glass bottle, shaken occasionally for 7 days and then filtered. Rotary evaporator R-200 (Buchi, Switzerland) was used to evaporate ethanol and concentrate the filtrate under reduced pressure (4-6 mm Hg) at 40°C. The resulting extracts were used for phytochemical analysis and for evaluating analgesic and anti-inflammatory activities. The herbal combination was formulated by mixing all four crude drug extracts in equal ratio.

Percentage yield

The percentage yield of ethanol extracts of *Boswellia serrata* (oleo-gum resin), *Brassica nigra* (seeds), *Piper longum* (fruits) and *Withania somnifera* (roots) was determined using the following equation:

% yield= $W_1 / W_2 \times 100$.

Where W_1 = weight of concentrated crude extract

W_2 = weight of powdered crude drug sample

Preliminary qualitative phytochemical screening

Phytochemical study of *Boswellia serrata* oleo-gum resin ethanol extract (BSE), B*rassica nigra* seed extract (BNE), *Piper longum* fruit extract (PLE) and *Withania somnifera* root extract (WSE) were conducted to identify and confirm the presence or absence of both primary and secondary metabolites (Roopalatha and Nair, 2013; Zhran *et al*., 2023).

Acute toxicity test

For acute oral toxicity study, Swiss albino mice were selected. The ethanolic extracts of all four plants and polyherbal combination in the doses of 1000, 2000 and 3000 mg/kg were given orally and mice were monitored for the first 4h, then up to 24h to observe any change such as convulsions, grooming behavior, hyperactivity, hypothermia, sedation and mortality (Ashraf *et al*., 2018).

Methods of assessment

Analgesic potential was tested using the tail immersion, hot plate and acetic acid induce writhing test, while anti-

inflammatory activity was assessed using the carrageenan-induced paw edema model.

Analgesic activity

Tail immersion test

The pain-relieving effect of all four ethanol extracts and their polyherbal combination was assessed by standard procedure (Gupta *et al*., 2015). According to this procedure, about 3-5 cm of tail was submerged into water bath, controlled at temperature 50.0 ± 0.5°C. The reaction time was determined as a time taken for tail to withdraw from hot water. Observations were noted at 0, 30, 60, 90 and 120 minutes after the oral administration of extracts. For this study, 84 animals were first weighed and then divided into 12 groups, with each group consisting of seven animals. Group 1 served as the control group and received 0.5% sodium carboxymethylcellulose (Na-CMC) suspension orally. Group 2 was the standard group and received aspirin 100 mg/kg orally. Group 3, 4, 5, 6, 7, 8, 9 and 10 were treated with plant extracts BSE, BNE, PLE and WSE at doses of 400mg/kg and 800 mg/kg. Whereas group 11 and 12 received polyherbal combination (PHC) at doses of 400 and 800 mg/kg, respectively.

Hot plate test

The hot plate test was performed with some minor modification (Ariyo *et al*., 2020). The grouping of animals and treatment protocol were kept identical to tail immersion procedure. The plate temperature was kept at 55.0 ± 0.5ºC. Each animal was placed on the plate, and the latency period to response (paw licking or jumping) was noted by using a stopwatch, at 0, 30, 60, 90 and 120 minutes after treatment.

Acetic acid-induced writhing test

Acetic acid-induced abdominal writhing was assessed using the method described in literature (Ariyo *et al*., 2020). The experimental groups received same treatment as described for the earlier tests. After 30 minutes of treatment, an injection of acetic acid (10 mL/kg of 0.6% acetic acid) was administered intraperitoneally to all animals. The number of abdominal writhes after this injection was observed and recorded for 20 minutes.

Anti-inflammatory activity

Carrageenan-induced rat paw edema assay

To examine the anti-inflammatory effect of the extracts and their combination, the carrageenan-induced rat paw edema model was used (Ariyo *et al*., 2020). An intra-dermal 0.1 ml injection of 1% of carrageenan was administered on the left hind paw's plantar surface of each rat. All groups received the same treatment and dosing regimen as has been describe in hot plate test, except group 2 (standard) to whom diclofenac sodium 50 mg/kg was administered. The thickness of paw edema was measured prior the administration of inflammatory agent (0 hour) and then at 1, 2, 3, 4 and 5 hours following the induction of inflammation using a digital vernier caliper. The percentage inhibition (% inhibition) of paw edema was calculated using the following formula

% inhibition = (1- Vt / Vc) x 100

Where:

Vt = Increase in paw volume of treated group

Vc = Increase in paw volume of control group

Statistical analysis

Findings of the study are described as mean ± SEM. One way ANOVA followed by Post hoc test (tukey) were used for statistical analysis. Value of p were considered significant with $p < 0.05^a$, more significant with $p < 0.01^b$, and highly significant with $p < 0.001^c$.

RESULTS

The ethanol extract of *Boswellia serrata* produced 270 g of extract from 2 kg of plant material, giving a yield of 13.5% whereas *Brassica nigra* yielded 314 g (15.7%). *Piper longum* produces the highest yield at 396 g (19.8%), while *Withania somnifera* yielded 254 g of extract (12.7%).

Preliminary qualitative phytochemical analysis

Various phytochemical tests were conducted on the ethanol extracts of BSE, BNE, PLE and WSE, which confirm that all four ethanol extracts contain carbohydrates, flavonoids, fats and fixed oil, phenols and terpenoids. Alkaloids were present in BNE, PLE, and WSE, with BNE also exhibiting a positive result for protein and saponin. Quinone was identified only in PLE, whereas steroid was detected in BSE, BNE and WSE.

Acute toxicity result

The assessment of acute toxicity indicated that all the four extracts and their polyherbal combination were non-toxic as no fatalities were observed till the maximum dose of 3000 mg/kg. Therefore, extracts and their combination were considered safe at dose up to 3000 mg/kg.

Analgesic activity

The analgesic activity of four plant extracts and their polyherbal combinations was evaluated using three standard models: tail immersion test, hot plate test, and acetic acid induced writhing test. These approaches provide a comprehensive analysis of their effects on pain reduction.

Tail immersions test

All herbal extracts and their polyherbal combination (400 and 800 mg/kg) demonstrated a notable analgesic response ($p < 0.001$) when compared to the control at 90 minutes. Analgesic activity results from the tail immersions method are detailed in table 1, The most significant and persistent analgesic effect was found in PHC at both doses. Considering the individual plant, BSE demonstrated a strongest effect, followed by WSE, PLE and BNE. The duration of the latency period was dose dependent, with greater effect observed at 800 mg/kg compare to 400 mg/kg.

Hot plate test

The hot plate test, a standard test for evaluating analgesic activity was applied to assess the anti-nociceptive effect of herbal extracts and their polyherbal combination. The analgesic action of extracts and their polyherbal combination in the hot plate test is given in table 2.

At both 400 and 800 mg/kg, polyherbal combination produced a significant increase in latency period ($p < 0.001$) and at the dose of 800 mg/kg the polyherbal combination exhibited similar analgesic activity to aspirin (100 mg/kg). BSE and WSE manifest significant effect on dose of 400 and 800 mg/kg ($p < 0.001$). At the dose of 400 mg/kg PLE showed significant effect ($p < 0.05$) at 30 minutes, while with the increase in dose (800 mg/kg) highly significant result ($p < 0.001$) was observed from 30 minutes. However, BNE expressed their efficacy after 60 minutes on both doses.

Acetic acid induced writhing test

There was a progressive decline in the number of writhes in the writhing test in mice (Table 3). Notably, mice that received a PHC at both doses showed a substantial decrease in writhes ($p < 0.001$) compared to all other groups, although this effect was dose depended. BSE closely followed PHC showing highly significant result ($p < 0.001$) at both doses. WSE also exhibited highly significant result ($p < 0.001$) at both doses although number of writhes were higher than PHC and BSE. PLE displayed a dose depended effect, on 400 mg/kg it showed significance effect ($p < 0.05$), which increase to highly significant at dose of 800 mg/kg ($p < 0.001$). BNE extract showed no significant result at the lower dose; however, with higher dose it produced significant result $p < 0.05$.

Anti-inflammatory activity

Carrageenan-induced rat paw edema assay

The anti-inflammatory effect of low and high dose of BSE, BNE, PLE, WSE, and PHC on paw edema is given in table 4. All treatment effectively decreased inflammation at both doses. However, we observed difference in response between different doses and different extracts, higher doses exhibited more effective results, showing a dose depended effect.

The polyherbal combination (PHC) showed maximum suppression ($p < 0.001$) of paw swelling at all times (1-5 hr.) after carrageenan injection. After PHC, BSE stood out as the most effective in reducing inflammation. This was followed by WSE and PLE with good and high-moderate efficacy, respectively, while BNE exhibited only moderate efficacy.

DISCUSSION

The current study carried out the phytochemical analysis of ethanol extracts from *Boswellia serrata*, *Brassica nigra*, *Piper longum* and *Withania somnifera*. The analgesic and anti-inflammatory effects of these extracts were assessed, both as individual agents and in a polyherbal combination in the ratio of 1:1:1:1.

The percentage yields obtained from *Boswellia serrata*, *Brassica nigra*, *Piper longum* and *Withania somnifera* samples were 13.5%, 15.7%, 19.8% and 12.7% respectively. This variability can be attributed to differences in phytochemical composition between plants as well as differences in extraction variables for example solvent selection, solvent-to-solid ratio, extraction time and temperature (Sun *et al*., 2025).

The phytochemical screening of all four plant extracts showed marked differences in the presence of bioactive compounds. Alkaloids were detected in *Brassica nigra, Piper longum* and *Withania somnifera*, and responsible for anti-inflammatory effect of these plants as reported in a study by Rajput *et al*., 2022. Flavonoids, phenols and terpenoids were identified in all extracts of our study have been extensively documented for their potent antioxidants and anti-inflammatory activities (Riaz *et al*., 2023). Among all four extracts, protein and saponins were identified only in *Brassica nigra* and these have been reported to inhibit COX and LOX enzymes and reduce NO production, leading to suppression in inflammatory responses (Wijesekara *et al*., 2024). Similarly, Quinones were found only in *Piper longum,* which has a significant role in regulating important signaling pathways involved in inflammation, such as the NF-κB, mitogen-activated protein kinase, culminating in the inhibition of inflammatory processes (Krishnakumar *et al*., 2025). Steroids were present in *Boswellia serrata, Piper longum* and *Withania somnifera* and exert anti-inflammatory effect by inhibiting inflammatory mediators (Mukhopadhyay *et al*., 2023).

These findings are supported by earlier studies confirming the validity of the obtained data (Anu *et al*., 2013; Chatterjee *et al*., 2010; Danlamin *et al*., 2016; Subhashini Devi *et al*., 2014). The diverse phytochemical composition of these extracts provide a strong biochemical justification for their long-standing application in the management of inflammation, and presence of these phytochemicals highlights the multi-targeted anti-inflammatory potential of polyherbal combination of these extracts. The current study demonstrates that ethanol extracts of *Boswellia serrata, Brassica nigra, Pipper longum and Withania somnifera* as well as their polyherbal combination in the ratio of 1:1:1:1, induce marked antinociceptive and anti-inflammatory effects.

Analgesic properties of our studied extracts and compound were assessed by tail immersion test, hot plate test and acetic acid-induced writhing test, the widely utilized standardized tests for evaluating analgesic properties of compounds and extracts (Abdala *et al*., 2014). In tail immersion and hot plate test, the herbal extracts and their

polyherbal combination showed significant results at both doses when compared with control, as detailed in table 1 and table 2, although persistent and pronounced decrease in pain was observed by polyherbal combination at 120 minutes indicating continues availability of drug, as piperine in the *Piper longum* increases bioavailability of other drugs (Vijayarani *et al*., 2020). The acetic acid-induced writhing test (Table 3) also demonstrated that the individual administration of ethanol extract of these plants as well as their polyherbal combination at both doses, highly reduced the number of writhes in treated animals compared with the control group. Here, the high dose of polyherbal combination exhibited phenomenal inhibition of writhing as compared to the individual herbal extracts. This effect may be attributed to the complementary effect of the phytochemicals which together target multiple pathways involved in nociception. Among the individual herbal drug extracts, *Boswellia serrata* exhibited highest inhibition of writhing, whereas minimum effect was observed with *Brassica nigra*.

This investigation also assessed the anti-inflammatory activity of extracts BSE, BNE, PLE, WSE and their PHC via the carrageenan-induced paw edema assay. Results showed that administration of the extracts at 400 and 800 mg/kg doses significantly decreased carrageenan induced paw edema progression from the initial stages to advanced stages. As mentioned in table 4, the polyherbal combination exhibited strongest anti-inflammatory activity, whereas among individual extracts BSE showed the most effective suppression of paw swelling, followed by WSE, PLE and BNE. During the assessment of analgesic and anti-inflammatory activities, the herbal extracts and their polyherbal combination showed significant results at both doses as compared with control (Table 1 -4). The results of this study show that the polyherbal combination produced effects approaching those of the reference drug, though direct equivalence cannot be claimed.

All four herbal extracts in this study produced significant, dose-dependent analgesic and anti-inflammatory effects, consistent with previous reports. In current study models, *Boswellia serrata* displayed a most effective suppression of pain and inflammation across all individual herbal extract tested and these findings are consistent with previous reports (Sharma *et al*., 2010). *Withania somnifera* produced prominent peripheral analgesic and central effects, earlier studies showed that withanolides compounds are responsible for modulating oxidative stress and cytokine production leading to a decrease in inflammation and pain (Singirala *et al*., 2025). *Piper longum* showed high to moderate efficacy and previous studies have reported that piperine is responsible for its activity (Perez Gutierrez *et al*., 2013). *Brassica nigra* exhibited the weakest efficacy among four herbs yet produced significant inhibition at higher dose due to alkaloids, flavonoids, phenols and terpenoids (Singh and Singh, 2025).

The outcomes from these different models indicate that these herbal extracts and their combination act at both central and peripheral mechanism. Increased response times in the tail-immersion and hot-plate tests indicate participation of central pathways for pain, while decrease in number of writhes in acetic acid induce test, indicates inhibition of peripheral mediators such as prostaglandins and bradykinin. The biphasic response in the carrageenan model further demonstrated that extracts and their combination not only decrease early involvement of histamine and bradykinin but also the subsequent involvement of prostaglandins and cytokines, suggesting multitarget activity of herbal extracts and their polyherbal combination (Ashagrie *et al*., 2023; Gupta *et al*., 2015).

The observed pharmacological potential of all four extracts align with their bioactive constituents, as *Boswellia serrata* contains boswellic acids, essential oils, triterpenoids and flavonoids which are responsible for its pharmacological action (Bhutada *et al*., 2017). Among these, AKBA (acetyl-11-keto-β-boswellic acid) and KBA (11-keto-β-boswellic acid) exhibited the highest anti-inflammatory potential as AKBA is a potent inhibitor of 5-lipoxygenase, causing suppression of leukotriene synthesis and β-boswellic acid by targeting microsomal prostaglandin E2 synthase-1, reduces the formation of PGE2, a basic mediator of pain and inflammation. In addition, boswellic acids suppress Cathepsin G and Human Leukocyte Elastase, decreasing neutrophil-mediated tissue injury. They also decrease pro-inflammatory cytokines (e.g., TNF-α, IL-1β) and interfere with key signaling pathways, including NF-κB, MAPK, and STAT3 (Efferth and Oesch, 2022). Collectively, these mechanisms confer the oleo-gum resin's anti-inflammatory, analgesic, and chondroprotective properties, explaining its therapeutic value in arthritis and related disorders (Bhutada *et al*., 2017).

The principal bioactive constituents of *W.somnifera* are withanolides (Withaferin A, Withanolide A, Withanone, Withanolide D), withanosides, withanamides, flavonoids and alkaloids, which are collectively responsible for its pharmacological efficacy (Singirala *et al*., 2025) Withaferin A exerts potent anti-inflammatory effect through, inhibition of NF-κB, COX-2, and pro-inflammatory cytokines. The alkaloids are responsible for analgesic, neuroprotective, and antispasmodic actions, whereas withanamides and flavonoids further increase antioxidant defense (Lerose *et al*., 2024). These constituents act by modulating inflammatory mediators (TNF-α, IL-1β, IL-6, prostaglandins, nitric oxide), suppressing NF-κB and MAPK signaling pathways and inhibiting protein denaturation, providing significant benefits in the management of pain and inflammation (Kumar *et al*., 2015).

Table 1: Analgesic activity of mono and their polyherbal combination, by the tail immersion test

Group N=7	Treatment	Dose mg/kg	Reaction time in second (sec) mean ±SEM				
			0 min	30 min	60 min	90 min	120 min
Control	0.5%Na-CMC	10	1.32±0.04	1.32±0.06	1.50±0.06	1.45±0.04	1.38±0.07
Standard	Aspirin	100	1.38±0.07	2.04±0.13^{c}	3.02±0.06^{c}	3.87±0.04^{c}	3.49±0.00^{c}
Test	BSE	400	1.38±0.12	1.98±0.01^{c}	2.35±0.02^{c}	2.97±0.00^{c}	2.75±0.01^{c}
		800	1.32±0.07	2.01±0.01^{c}	2.82±0.02^{c}	3.52±0.03^{c}	3.43±0.11^{c}
	BNE	400	1.35±0.07	1.43±0.07	1.68±0.02^{b}	1.89±0.01^{c}	1.65±0.02^{b}
		800	1.48±0.05	1.58±0.01	1.81±0.01^{c}	2.15±0.01^{c}	2.03±0.02^{c}
	PLE	400	1.44±0.06	1.44±0.11	1.72±0.01^{c}	2.07±0.02^{c}	1.93±0.03^{c}
		800	1.34±0.06	1.65±0.11	1.92±0.01^{c}	2.43±0.01^{c}	2.27±0.02^{c}
	WSE	400	1.38±0.07	1.55±0.11	1.97±0.01^{c}	2.55±0.01^{c}	2.31±0.01^{c}
		800	1.38±0.07	1.81±0.01^{c}	2.15±0.02^{c}	2.86±0.01^{c}	2.65±0.02^{c}
	PHC	400	1.35±0.09	2.16±0.02^{c}	2.53±0.02^{c}	3.44±0.04^{c}	3.44±0.04^{c}
		800	1.31±0.09	2.38±0.04^{c}	3.04±0.03^{c}	3.97±0.02^{c}	3.97±0.01^{c}

Significant; ^{a} = $p < 0.05$, more significant; ^{b} = $p < 0.01$, highly significant; ^{c} = $p < 0.001$

Table 2: Analgesic activity of mono and their polyherbal combination, by hot plate test

Group N=7	Treatment	Dose mg/kg	Reaction time in second (sec) mean ±SEM				
			0 min	30 min	60 min	90 min	120 min
Control	0.5%Na-CMC	10	8.30±0.05	8.65±0.31	8.29±0.07	7.66±0.22	8.25±0.34
Standard	Aspirin	100	7.90±0.02	15.11±0.36^{c}	17.37±0.61^{c}	20.43±0.21^{c}	19.40±0.22^{c}
Test	BSE	400	8.75±0.23	14.59±0.06^{c}	16.33±0.30^{c}	16.97±0.04^{c}	15.07±0.38^{c}
		800	9.03±0.19	15.97±0.25^{c}	16.77±0.44^{c}	17.10±0.26^{c}	15.70±0.26^{c}
	BNE	400	8.65±0.29	9.23±0.21	10.33±0.11^{c}	12.17±0.28^{c}	11.57±0.37^{c}
		800	8.57±0.27	9.43±0.40	12.00±0.15^{c}	14.30±0.17^{c}	13.07±0.35^{c}
	PLE	400	7.27±0.21	10.17±0.42^{a}	12.47±0.37^{c}	14.40±0.20^{c}	13.30±0.16^{c}
		800	8.03±0.21	11.00±0.24^{c}	13.17±0.24^{c}	14.80±0.15^{c}	14.10±0.22^{c}
	WSE	400	7.88±0.23	10.92±0.30^{c}	13.67±0.21^{c}	15.00±0.33^{c}	14.07±0.19^{c}
		800	8.40±0.37	13.10±0.13^{c}	15.57±0.08^{c}	17.33±0.20^{c}	16.20±0.17^{c}
	PHC	400	8.88±0.22	16.85±0.18^{c}	18.70±0.32^{c}	19.13±0.22^{c}	19.08±0.33^{c}
		800	8.72±0.17	18.75±0.24^{c}	20.48±0.21^{c}	21.13±0.37^{c}	21.03±0.20^{c}

Significant; ^{a} = $p < 0.05$, more significant; ^{b} = $p < 0.01$, highly significant; ^{c} = $p < 0.001$

Table 3: Analgesic activity of mono and their polyherbal combination, by acetic acid writhing test

Group N=7	Treatment	Dose mg/kg	Number of writhes in 20 mins mean ±SEM
Control	0.5%Na-CMC	10	25.67±1.80
Standard	Aspirin	100	8.67±1.48^{c}
Test	BSE	400	11.33±0.95^{c}
		800	8.33±0.99^{c}
	BNE	400	24.00±0.37
		800	21.00±0.58^{b}
	PLE	400	21.17±0.40^{a}
		800	19.17±0.40^{c}
	WSE	400	16.67±0.49^{c}
		800	10.83±0.48^{c}
	PHC	400	9.67±0.56^{c}
		800	7.17±0.60^{c}

Significant; ^{a} = $p < 0.05$, more significant; ^{b} = $p < 0.01$, highly significant; ^{c} = $p < 0.001$

Piper longum contains numerous bioactive compounds, most notably alkaloids (piperine, piperlongumine, piperlonguminine), lignans (sesamin), volatile oils (caryophyllene, pinene, limonene) and flavonoids (quercetin, kaempferol) (Ara *et al*., 2021).

Piperine is primarily responsible for pharmacological actions, including anti-inflammatory, antioxidant and analgesic effects (Perez Gutierrez *et al*., 2013). Other bioactive constituents, like Piperlongumine and piperlonguminine, lignans, volatile oils, collectively mediate effects on pain and inflammation by inhibiting COX-2, NF-κB, TNF-α, IL-1β, and MMPs, thereby suppressing prostaglandin and cytokine release, although the analgesic response is driven by TRPV1 desensitization and COX-2 inhibition (Jo *et al*., 2024).

Table 4: Anti-inflammatory activity of mono and their polyherbal combination, by carrageenan-induced rat paw edema assay

Group	Treatment	Dose mg/kg	Thickness of paw (mm) mean ±SEM					
			0 Hr.	0 Hr.	0 Hr.	0 Hr.	0 Hr.	0 Hr.
Control	0.5%Na-CMC	10	4.08±0.02	4.87±0.02	5.05±0.02	5.17±0.01c	5.09±0.02	4.81±0.03
Standard	Diclofenac sodium	50	4.10±0.02	4.20±0.02^{c}	4.29±0.02^{c}	4.30±0.03^{c}	4.20±0.04^{c}	4.15±0.02^{c}
Test	BSE	400	4.11±0.37	4.27±0.02^{c}	4.36±0.02^{c}	4.40±0.04^{c}	4.34±0.04^{c}	4.26±0.01^{c}
		800	4.07±0.02	4.22±0.03^{c}	4.30±0.02^{c}	4.33±0.03^{c}	4.23±0.02^{c}	4.19±0.07^{c}
	BNE	400	4.09±0.01	4.34±0.01^{c}	4.43±0.07^{c}	4.52±0.03^{c}	4.47±0.01^{c}	4.33±0.02^{c}
		800	4.11±0.02	4.34±0.03^{c}	4.42±0.10^{c}	4.48±0.02^{c}	4.66±0.06^{c}	4.34±0.03^{c}
	PLE	400	4.12±0.02	4.31±0.01^{c}	4.41±0.03^{c}	4.48±0.02^{c}	4.43±0.01^{c}	4.33±0.03^{c}
		800	4.07±0.02	4.24±0.02^{c}	4.33±0.03^{c}	4.38±0.02^{c}	4.35±0.04^{c}	4.24±0.03^{c}
	WSE	400	4.09±0.02	4.25±0.04^{c}	4.35±0.01^{c}	4.40±0.02^{c}	4.33±0.03^{c}	4.25±0.02^{c}
		800	4.08±0.01	4.23±0.02^{c}	4.33±0.01^{c}	4.37±0.02^{c}	4.31±0.04^{c}	4.23±0.03^{c}
	PHC	400	4.10±0.02	4.21±0.04^{c}	4.31±0.01^{c}	4.33±0.03^{c}	4.25±0.02^{c}	4.19±0.03^{c}
		800	4.07±0.02	4.18±0.01^{c}	4.25±0.02^{c}	4.28±0.01^{c}	4.19±0.03^{c}	4.15±0.03^{c}

Significant; ^{a} = $p < 0.05$, more significant; ^{b} = $p < 0.01$, highly significant; ^{c} = $p < 0.001$

Table 5: Percentage inhibition of anti-inflammatory activity of mono and their polyherbal combination, Carrageenan-induced rat paw edema assay

Group	Treatment	Dose mg/kg	Percentage inhibition %				
			1 Hr.	2 Hr.	3 Hr.	4 Hr.	5 Hr.
Ctrl	0.5%Na-CMC	10	-	-	-	-	-
Standard	Diclofenac sodium	50	87.37%	80.41%	81.65%	90.10%	93.15%
Test	BSE	400	80.42%	74.66%	73.20%	77.11%	79.09%
		800	81.26%	76.71%	76.42%	83.91%	84.32%
	BNE	400	68.42%	64.86%	60.72%	62.52%	67.27%
		800	71.58%	67.81%	66.31%	67.66%	69.32%
	PLE	400	77.05%	71.06%	67.69%	69.65%	72.50%
		800	78.32%	72.60%	71.36%	72.31%	76.36%
	WSE	400	79.37%	72.77%	71.52%	75.79%	77.45%
		800	80.21%	73.97%	72.89%	76.62%	78.64%
	PHC	400	86.08%	78.35%	78.90%	85.15%	87.67%
		800	86.08%	81.44%	80.73%	88.12%	89.04%

Brassica nigra contains glucosinolates (sinigrin), which yield allyl isothiocyanate (AITC) and sulforaphane (Regitha *et al*., 2023). In addition, flavonoids, phenolic acids, tannins, alkaloids, terpenoids, fatty acids and essential micronutrients are also present in this plant (Bhat, 2021). Collectively, these phytochemicals produce anti-inflammatory effects by inhibiting cyclooxygenase activity, decreasing the release of pro-inflammatory mediators and stabilizing lysosomal membranes (Alam *et al*., 2011). Therefore, when administered together as a polyherbal combination these extracts are likely to work in synergy, providing greater efficacy than individual extracts. By simultaneously targeting different but connected pathways through the interaction of multiple phytochemicals of all four species, the polyherbal combination fortifies the anti-inflammatory and analgesic effects producing a stronger therapeutic effect, overall.

The current study aligns with the *previous findings* of the increased therapeutic potential of polyherbal combination in comparison with single-herb effect as Deciga-Campos *et al*., 2021 reported that a mixture of *Syzygium aromaticum* with *Rosmarinus officinalis* exhibited amplifying analgesic and anti-inflammatory effect. Similarly, When *Boswellia serrata* was co-administered with *Piper longum* by Vijayarani *et al*., 2020 as anti-inflammatory agents, the combination increased the inhibition of paw edema compared to the individual extract.

Study limitation

The current study is, however, not devoid of limitations. The study was conducted on a single rodent species and on female gender only. Lack of chemical marker standardization is another limitation of the study. Future research may be directed to assessing efficacies of these extracts using other related models of different species and in humans; exploring of molecular mechanisms in further details, and carrying out clinical trials to validate efficacy and safety in human population.

CONCLUSION

This study demonstrated significant analgesic and anti-inflammatory activities of ethanolic extracts of *Boswellia serrata*, *Brassica nigra*, *Piper longum*, and *Withania somnifera* in experimental models. Notably, the polyherbal combination of these plants exhibited the highest efficacy, likely due to complementary interactions among their phytoconstituents.

The findings validate the traditional use of these plants in managing pain and inflammation as well as reveal the amplified therapeutic potential of polyherbal combination as effective alternatives or complements to conventional drugs. This polyherbal combination could be a novel approach for both preventing and treating inflammatory diseases. Future studies may focus on phytochemical standardization and isolation of bioactive compounds, the mechanistic validation (cytokine profiling, COX/LOX assays), pharmacokinetic studies and clinical evaluations of this combination (safety studies to support future clinical translation).

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Author's contributions
MA performed the experiments, analyzed the data, and wrote the original draft. SGB contributed to the analysis and interpretation of the data. UF contributed to drafting the manuscript. FM participated in collecting, assessing, and interpreting the data. NM provided substantial intellectual input during the drafting and revision of the manuscript. ZAM conceived and designed the experiment.

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Data availability statement
The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical approval
All the experiments in this study were carried out in compliance with the guideline from handling and use of Laboratory animals that was granted by Bio-ethical committee of the University of Karachi, with approval No. IBC KU -446/2024.

Conflict of interest
The authors declare no conflict of interest.

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