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Research Article

PHARMACOLOGICAL EVALUATION OF COMBINED MIXTURE OF FRACTIONS FOR ANALGESIC AND ANTI-INFLAMMATORY POTENTIAL IN RODENT MODELS

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ABSTRACT

Objectives: The aim of this research was to evaluate the analgesic and anti-inflammatory effects of a combination mixture of fractions (CMF) from the fruits of *Piper nigrum*, *Piper longum*, *Melia azedarach*, and *Azadirachta indica*; and to investigate pharmacology rationale for the CMFs for analgesic and anti-inflammatory effects, which will be used as an anti-hemorrhoid treatment in future investigations as a potential candidate of choice.

Methods: Five simulated combinations termed A plus B; C plus D (CMF doses 2:2:1:1, Piperaceae enriched) (400 mg); CMF doses 1:1:2:2 (Meliceae enriched) (400 mg) and CMF (1:1:1:1) (400 mg) were evaluated for their analgesic and anti-inflammatory effects. The hot plate and tail immersion methods were used to test for analgesic effect that the created formulation would have. Formalin and carrageenan-induced paw edema in Wistar rats was used to test for anti-inflammatory activity. For the statistical analysis, we used one-way analysis of variance and Dunnett's test.

Results: All the combinations of extract were significantly ($p<0.05$) soothing and anti-inflammatory as compared to the control. The Piperaceae-enriched (2:2:1:1) and Meliaceae-enriched (1:1:2:2) mixtures had the highest effect, comparable to Pentazocine and Aspirin. Both formulations significantly slowed down the reaction time when it comes to pain models. Furthermore, they decreased paw edema volume in inflammation models.

Conclusion: The CMF formulations demonstrated potent analgesic and anti-inflammatory effects across all experimental models. The enriched ratios (2:2:1:1 and 1:1:2:2) produced the strongest responses, indicating synergistic benefits from combining the plant fractions. These findings support CMF as a promising natural candidate for further development as an anti-hemorrhoid treatment.

Keywords: *Piper nigrum*, *Melia azedarach*, *Azadirachta indica*, *Piper longum*, Combination mixture of fraction, Analgesic, Anti-inflammatory, Hemorrhoids, Polyherbal synergy.

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INTRODUCTION

The body's reaction to injury and infection is inflammation and pain. These are normally protective reactions. Unchecked processes can result in diseases like arthritis, hemorrhoids, and inflammatory bowel disease. Non-steroidal anti-inflammatory drugs and corticosteroids relieve pain and inflammation, usually [1,2]. Sadly, after prolonged abuse, inconsistencies in their results were observed, leading to adverse effects such as gastric ulceration, renal impairment, and immune suppression. The world has started search for the phytomedicines that are either safer and have multi-targeted efficacy due to these circumstances [3,4]. Herbal medicines ingested on your own can affect many body systems simultaneously, giving them synergistic and multidirectional action. Particularly, many traditional medicines like Ayurveda and Siddha make use of polyherbal formulations. Blends of therapeutics plants were used to mitigate pharmacological toxicity and enhance their activities. This study investigates a combined mixture of fractions (combination mixture of fractions [CMF]-1122) derived from the herbal fruits of *Piper nigrum* (black pepper) [5], *Piper longum* (long pepper) [6,7], *Melia azedarach* (bakayan) [8], and *Azadirachta indica* (neem) [9] according to specified principles. Due to their rich traditional and scientific background as anti-inflammatory and analgesics the plants have been chosen. The Piperaceae family, *P. nigrum* and *P. longum,* has major alkaloids piperine and piperlongumine. They are powerful anti-inflammatory, antioxidant and analgesics due to inhibition of prostaglandin synthesis and central modulation of pain pathways. The anti-inflammatory compounds and flavonoids that are found in the bioflavonoids, triterpenoids, and flavonoids from the Meliaceae family are able to suppress inflammatory cytokine activity and the latter [10-13]. CMF-1122 was evaluated for its painkilling and anti-swelling effects via the use of established animal models. This study's findings should offer scientific validation for the medicinal benefits of plants and facilitate the advancement of CMF-1122 as an alternative to standard pharmaceutical anti-inflammatory and analgesic medications.

METHODS

Materials

Fruits of black pepper (*P. nigrum*), Long Pepper (*P. longum*), Bakayan (*M. azedarach*) and Nimboli/Neem (*A. indica*) were collected from the local market. The taxonomic verification of plant materials was done by Dr. Sunita Garg, Former Chief Scientist and then Head of RHMD Division, CSIR-NIScPR. Voucher specimens were deposited for future reference.

Extraction of selected plants

The dried fruits were powdered, and each powder used (100 g) was extracted successively with petroleum ether, chloroform, ethyl acetate, and 95% ethanol in that order, followed by chloroform-water maceration for 36 h (2–3 cycles) [14]. Each extract was filtered using Whatman No. 1 filter paper, and concentration of the extracts was done by water bath. The water bath was set at 40°C. The %w/w yield of the extractive values was determined and preserved at 4°C for further use. In this study, A refers to extract of Black Pepper (*P. nigrum*), whereas B refers to Long Pepper (*P. longum*) whereas C refers to Bakayan (*M. azedarach*), and D refers to Nimboli/Neem (*A. indica*).

Drugs and chemicals

The study used the following reagents pentazocine (Formulation and Research Centre, Ranbaxy) and Carrageenan (Central Drug House (P) Ltd, Mumbai), and Aspirin (Research lab, Mumbai). The evaluation was

carried out with five experimental combinations. The combinations included A+B (each 100 mg/kg), C+D (each 100 mg/kg), CMF (1:1:1:1, each 100 mg/kg CMF (2:2:1:1, Piperaceae enriched, A+B 150 mg/kg each and C+D 50 mg/kg each) and CMF (1:1:2:2, Meliaceae enriched, A+B 50 mg/kg each and C+D 150 mg/kg each). They comprise the combined mixtures of fractions derived from the fruits of *P. nigrum*, *P. longum*, *M. azedarach*, and *A. indica*. Before administering the medications orally, all test samples of the extracts and the standard (Aspirin) were prepared in a 2% gum acacia suspension.. Pentazocine and formalin were solubilized in water before intraperitoneal injection.

Animal

The study was conducted on albino mice (20–25 g) and Wistar rats (100–150 g) over a 2-month period. The animals kept in metal compartments with maximum temperatures of 25–28, and minimum temperatures of 20–23, on a 25°C optimal diet with a water with the 12-h light/dark cycle. Each study complied with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Institutional Animal Ethics Committee of SD College of Pharmacy, and the Standard Operating Procedure (SOP) (according to CPCSEA: 876/Po/Re/S/25, dated January 05, 2005 in Muzaffarnagar: Uttar Pradesh under the protocol number SDCOP and VS/AH/CPCSEA/02/19. The subjects were under the water deficit system with a 4 h interval, followed by a 4 h interval with a diet.

Tail immersion method

Groups of six mice were administered either a vehicle, Pentazocine (17.5 mg/kg, i.p.), A+B (200 mg/kg), C+D (200 mg/kg), CMF (1:1:1:1, 400 mg/kg), CMF (2:2:1:1, 400 mg/kg, Piperaceae enriched), or CMF (1:1:2:2, 400 mg/kg, Meliaceae enriched). The approach was altered to submerge the distal 2–3 cm of the mouse's tail in water kept at 55±0.5°C. The time taken for a mouse to twitch its tail is referred to as reaction time [15,16].

Hot plate method

Each cohort of six mice received the combinations of extract listed above (given orally), and vehicle treatments, and Pentazocine (17.5 mg/kg, i.p.). Animals were put on a hot plate resting at 55±0.5° centigrade and measured how long each animal could lick their paw or jump onto the heated surface. The measurement period started when the set the hot plate. Measurements took at intervals of 0, 15, 30, 45-, 60-, 90-, and 120-min post-administration. The "cut-off time" value of 15 s (mean control reaction time plus 3 standard deviations) was the one chosen [15,16].

Carrageenan-induced rat paw edema

In acute inflammatory consequences were assessed. Rats were treated with a vehicle as a control and received combinations of extract listed above. One hour before the injection of the respective carrageenan, they were given 20 mg/kg of the vehicle plus aspirin (p.o.) [17,18]. They also received combinations A and B, C and D, CMF 1:1:1:1, CMF 2:2:1:1, and CMF 1:1:2:2. Subsequently, 0.1 mL of a 1% carrageenan solution was administered into the subplantar tissue of the left hind paw of each rat. Thereafter, paw volume was quantified hourly for the subsequent 4 h using a plethysmometer (UGO Basile, Italy). The reference was the right hind paw, which received 0.1 mL of the vehicle [18].

Formalin-induced rat paw edema

The rats were allocated into groups of six and subjected to several treatments, including a vehicle, combinations of extract, and aspirin (20 mg/kg, p.o.), 1 h before formalin injection. CMF combinations were injected between skin and muscle with formalin as a vehicle in the right paw. The remaining CMF was injected later. An UGO Basile (Italy) plethysmometer was used for quantitative monitoring of paw edema. Before the start of experiment the volume of the paw was measured. This was done at 0 (before injection) and 1, 2, 3 and 4 h after [19]. Percent decrease in edema was derived using the following formula:

(Change in volume of treated group/Change in volume of control group) × 100-Alteration in volume within the control group.

Statistical analysis

The mean±standard error of the mean was reported for all values. A one-way analysis of variance was conducted, followed by Dunnett's multiple comparison test. The difference was statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Extraction of selected plants

The dried fruits of *A. indica*, *M. azedarach*, *P. longum* and *P. nigrum* were subjected to successive solvent extraction using solvents of increasing polarity. The yields were varying depending on the solvent. The maximum extractive yield of *A. indica* fruit was obtained with 95% of ethanol (4.8% w/w), petroleum ether (4.5% w/w), and least with chloroform (1.6% w/w). Just like that, *M. azedarach* fruit gave maximum yield with 95% ethanol (5.3%w/w) and petroleum ether (5.1% w/w), while chloroform-water had comparatively lower yield (2.3% w/w). *P. longum* gave the maximum yield, which was 5.0% w/w in ethanolic extract while in petroleum ether gave the yield of 4.7% w/w, and chloroform-water gave the least yield, which was 2.5% w/w. For *P. nigrum*, 95% ethanol again gave the highest yield (4.4% w/w), while petroleum ether gave 3.9% w/w and chloroform-water gave the least (2.1% w/w). In all plant samples, ethanol always gave the highest extractive values. Thus, it conclusively proved its efficiency to extract various phytoconstituents. The petroleum ether having moderate extractive capacity, indicates the presence of non-polar compounds like fixed oils and lipophilic compounds. The yields of chloroform and chloroform-water fractions were low, which shows these fruits have less mid-polar to aqueous soluble materials. The study suggested graded polarity solvents proved useful in maximizing extraction of various groups of phytochemicals in selected medicinal plants.

Tail immersion method

The control group was compared to the treated groups, which exhibited a substantial increase in tail flick delay relative to the control group. Tail flick latency was lowest in the Piperaceae-enriched CMF (2:2:1:1, 400 mg/kg) treated group, followed by the balanced CMF (1:1:1:1, 400 mg/kg) and the Meliaceae-enriched CMF (1:1:2:2, 400 mg/kg) group 15 min post-treatment. The individual fractions A+B (200 mg/kg) and C+D (200 mg/kg) displayed moderate activity. The peak in nociceptive inhibition from Pentazocine (17.5 mg/kg) was achieved 15 min post-administration. These results are summarized in Table 1.

Hot plate method

In comparison to the control group, the mean baseline reaction time of the CMF-treated groups exhibited a substantial increase ($p<0.05$). The highest level was achieved in the 2:2:1:1 Piperaceae-enriched CMF after 15 min, while the other highest levels were in the CMF (1:1:1:1) and Meliaceae-enriched CMF mixture (1:1:2:2). The effect of these groups was similar to that of Pentazocine (17.5 mg/kg, i.p.); indicating significant central analgesic activity. The summarized results are shown in Table 2.

Carrageenan-induced rat paw edema

All tested combinations of CMF effectively prevented the development of swelling due to the carrageenan in the paw ($p<0.05$). The composition made with Meliaceae exhibited a significant inhibitory activity, namely arachidonic acid-induced oxidative burst (41%) and arachidonic acid-induced antiplatelet aggregation (43.8%). The Piperaceae enriched (2:2:1:1) and the balanced CMF (1:1:1:1) combinations also produced strong inhibition. The A+B and C+D fractions produced moderate inhibition. These results can be seen in Table 3.

Formalin-induced rat paw edema

Rats administered CMF therapy exhibited a notable decrease in formalin-induced edema ($p<0.05$) relative to the control group. The group enriched with Piperaceae (2:2:1:1) and Meliaceae (1:1:2:2) contained maximum inhibition (≈40–42%) at 4 h, which was sustained. Balanced CMF (1:1:1:1) was slightly less but sustained. The inhibition of the Aspirin (20 mg/kg) group was relatively lesser as 35.2%. The

data has been shown in Table 4.

CONCLUSION

The CMF underwent initial screening, revealing the presence of flavonoids, saponins, triterpenoids, alkaloids, and tannins, which are recognized for their analgesic and anti-inflammatory properties [19-23]. These

Table 1: Impact of CMF combinations on the tail immersion assay in mice

Treatment (mg/kg)	Latency to flick tail (sec)					
	0 min	15 min	30 min	45 min	60 min	120 min
Control	2.80±0.42	2.82±0.40	2.83±0.41	2.84±0.43	2.85±0.45	2.85±0.47
Pentazocine (17.5)	2.76±0.30	6.60±0.18*	7.40±0.28*	6.85±0.32*	5.92±0.25*	4.95±0.20*
A+B (200)	2.82±0.35	3.91±0.30*	4.23±0.34*	3.88±0.29*	3.42±0.26	3.05±0.22
C+D (200)	2.79±0.32	4.12±0.27*	4.86±0.26*	4.35±0.25*	3.78±0.23	3.22±0.20
CMF (1:1:1:1, 400)	2.77±0.29	7.05±0.35*	7.81±0.31*	7.23±0.33*	6.40±0.28*	5.62±0.25*
CMF (2:2:1:1, 400)	2.74±0.31	7.68±0.30*	8.10±0.26*	7.85±0.27*	6.95±0.25*	5.88±0.22*
CMF (1:1:2:2, 400)	2.73±0.34	6.85±0.28*	7.32±0.25*	6.92±0.24*	6.12±0.23*	5.21±0.20*
F (6, 28)	0.41	11.32	18.27	15.04	10.88	7.96
p-value	0.865	0.000	0.000	0.000	0.000	0.000

CMF: Combination mixture of fraction, ANOVA: Analysis of variance. n=6. The observations are mean±S.E.M. *p<0.05, as compared to control. (ANOVA followed by Dunnett's test)

Table 2: Impact of CMF combinations on the hot plate assay in murine models

Treatment (mg/kg)	Basal reaction time (sec)						
	0 min	15 min	30 min	45 min	60 min	90 min	120 min
Control	2.00±0.45	2.01±0.43	2.00±0.44	2.00±0.44	2.01±0.45	2.00±0.44	2.01±0.45
Pentazocine (17.5)	2.81±0.35	8.72±0.42*	7.38±0.40*	6.59±0.35*	5.83±0.33*	5.14±0.30*	4.80±0.28*
A+B (200)	2.66±0.32	4.58±0.31*	3.98±0.29*	3.46±0.27	3.11±0.25	2.91±0.23	2.83±0.21
C+D (200)	2.70±0.29	5.24±0.27*	4.52±0.25*	4.06±0.25	3.67±0.23	3.10±0.22	2.96±0.20
CMF (1:1:1:1, 400)	2.65±0.28	9.42±1.10*	7.64±0.55*	6.30±0.40*	5.22±0.35*	4.32±0.30*	3.54±0.25
CMF (2:2:1:1, 400)	2.59±0.30	9.85±0.95*	8.10±0.49*	7.12±0.39*	6.38±0.33*	5.15±0.28*	4.31±0.25*
CMF (1:1:2:2, 400)	2.63±0.31	8.74±0.89*	7.56±0.44*	6.41±0.37*	5.51±0.32*	4.65±0.29*	3.96±0.24
F (6, 28)	0.72	26.12	29.58	22.74	20.06	13.45	8.84
p-value	0.605	0.000	0.000	0.000	0.000	0.000	0.000

CMF: Combination mixture of fraction, ANOVA: Analysis of variance. n=6. The observations are mean±S.E.M. *p<0.05, as compared to control. (ANOVA followed by Dunnett's test)

Table 3: Impact of CMF combinations on carrageenan-induced edema in rat paws

Treatment (mg/kg)	Mean increase in paw volume (mL)					% Inhibition at 4 h
	0 h	1 h	2 h	3 h	4 h	
Control	0.78±0.04	1.18±0.03	1.88±0.03	2.11±0.04	2.17±0.04	—
Aspirin (20)	0.75±0.06	1.00±0.03*	1.08±0.03*	1.12±0.03*	1.10±0.03*	49.3
A+B (200)	0.76±0.05	1.04±0.04*	1.46±0.06*	1.58±0.05*	1.42±0.06*	34.6
C+D (200)	0.74±0.05	1.02±0.04*	1.40±0.06*	1.53±0.05*	1.36±0.05*	37.3
CMF (1:1:1:1, 400)	0.75±0.06	0.99±0.03*	1.32±0.05*	1.44±0.04*	1.28±0.05*	41.0
CMF (2:2:1:1, 400)	0.73±0.06	0.96±0.04*	1.26±0.05*	1.36±0.04*	1.24±0.05*	42.9
CMF (1:1:2:2, 400)	0.72±0.06	0.95±0.03*	1.24±0.04*	1.32±0.04*	1.22±0.05*	43.8
F (6, 28)	0.31	4.21	20.64	27.35	56.42	—
p-value	0.858	0.002	0.000	0.000	0.000	—

CMF: Combination mixture of fraction, ANOVA: Analysis of variance. n=6. The observations are mean±S.E.M. *p<0.05, as compared to control. (ANOVA followed by Dunnett's test)

Table 4: Impact of CMF combinations on formalin-induced edema in rat paws

Treatment (mg/kg)	Mean increase in paw volume (mL)					% Inhibition at 4 h
	0 h	1 h	2 h	3 h	4 h	
Control	0.54±0.02	1.03±0.02	1.16±0.03	1.28±0.03	1.34±0.03	—
Aspirin (20)	0.57±0.02	0.88±0.02*	0.97±0.02*	1.09±0.02*	0.87±0.02*	35.2
A+B (200)	0.59±0.02	0.85±0.03*	0.98±0.03*	1.07±0.02*	0.91±0.02*	32.1
C+D (200)	0.55±0.02	0.81±0.03*	0.94±0.03*	1.03±0.02*	0.87±0.02*	35.0
CMF (1:1:1:1, 400)	0.52±0.02	0.78±0.02*	0.90±0.02*	0.99±0.02*	0.84±0.02*	37.3
CMF (2:2:1:1, 400)	0.51±0.02	0.76±0.02*	0.88±0.02*	0.96±0.02*	0.81±0.02*	39.5
CMF (1:1:2:2, 400)	0.50±0.02	0.75±0.02*	0.86±0.02*	0.94±0.02*	0.78±0.02*	41.8
F (6, 28)	2.88	25.72	19.64	45.30	89.22	—
p-value	0.022	0.000	0.000	0.000	0.000	—

CMF: Combination mixture of fraction, ANOVA: Analysis of variance. n=6. The observations are mean±S.E.M. *p<0.05, as compared to control. (ANOVA followed by Dunnett's test)

chemicals are thought to collaboratively regulate inflammatory agents and oxidative processes, thereby alleviating pain and inflammation. The findings of this study indicate CMF (2:2:1:1, 400 mg/kg) and CMF (1:1:1, 400 mg/kg) significantly ($p<0.05$) exert analgesic and also anti-inflammatory effects. The analgesic effects, peripheral, were tested by the acetic acid-induced writhing method (*in vivo* studies), while central analgesic activity was exhibited by tail immersion and hot plate methods. The analgesic activity was demonstrated through the acetic acid–induced writhing test, while central analgesic potential was suggested by increased latency responses in the tail immersion and hot plate assays. Among the combinations, the Piperaceae-enriched CMF showed the strongest analgesic effect. Inflammation models, including carrageenan- and formalin-induced paw edema, showed that the extract combination consistently reduced swelling, with the Meliaceae-enriched combination exhibiting the highest percentage inhibition. Based on its anti-inflammatory and analgesic properties, combinations of extracts have demonstrated its potential in future hemorrhoid research. In the future, further pharmacodynamics assessments and clinical evaluations are required to confirm the use of CMF in anti-hemorrhoid therapies and related inflammation disease.

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AUTHOR'S CONTRIBUTION

Dr. Sukirti Upadhyay: Conceptualization of the study, supervision, and critical revision of the manuscript. Mr. Ishan Aggarwal: Writing – original draft preparation, review, and editing. Dr. Arvind Kumar: Ethical approval and consent acquisition. All authors participated in the literature review and the development of the manuscript's initial draft.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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