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Evaluation of Synergistic Effect of Methanol Leaf Extract of *Cardiospermum halicacabum* and *Vitex negundo* on Inflammation and Arthritis

RAJASEKARAN AIYALU,[1] ITHYAVENTHAN SUBRAMANIAM,[2] ARULKUMARAN GOVINDARAJAN,[1] and ARIVUKKARASU RAMASAMY[1]

[1]*KMCH College of Pharmacy, Coimbatore, India*
[2]*Doctor Thangs Products, Coimbatore, India*

Methanol leaf extracts of Cardiospermum halicacabum *(MLECH) and* Vitex negundo *(MLEVN) were evaluated for acute toxicity, anti-inflammatory, and anti-arthritic activities. Phytoconstituents were isolated from these extracts by column chromatography. Both the extracts did not exhibit any sign of toxicity and mortality up to 2,000 mg.kg^{-1}. In carrageenan- induced inflammation and complete Freund's adjuvant–induced arthritis, MLECH- (100, 200, or 400 mg.kg^{-1}) and MLEVN- (100, 200, or 400 mg.kg^{-1}) treated rats showed anti-inflammatory and anti-arthritic activity compared to control groups. The flavones, luteolin, and apigenin were isolated from the MLECH and MLEVN, respectively. The treatment combination of MLECH and MLEVN in equal proportion, in both the models, showed greater reduction of inflammation than control or when MLECH or MLEVN was administered alone.*

KEYWORDS *Anti-inflammatory, anti-arthritic, flavonoids, synergistic effect*

INTRODUCTION

Cardiospermum halicacabum (CH, Sapindaceae) is a climber known to contain arachidic acid, apigenin-7-O-glucuronide, chrysoeriol-7-O-glucuronide,

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Address correspondence to Rajasekaran Aiyalu, KMCH College of Pharmacy, Kovai Estate, Kalapatti Road, Coimbatore 641048, Tamil Nadu, India. E-mail: rsekaran2001in@yahoo.co.in

luteolin-7-O-glucuronide, saponin, quebrachitol, proanthocyanidin, beta-sitosterol, and stigmosterol (17). Anti-inflammatory (22), antioxidant (7), analgesic and vasodepressant (4), antipyretic (3), antimalarial (21), diuretic (15), anti-diarrheal (20), anti-diabetic (19), and anti-ulcer (16) activities have been reported for CH. *Vitex negundo* (VN, Verbenaceae) contains flavonoids such as casticin, orientin, isoorientin, luteolin, luteolin-7-O-glucoside, corymbosin, and gardenins A and B (1). It has been demonstrated that VN was a potent anti-inflammatory (25), anti-arthritic (18), anticonvulsant (5), hepatoprotective (2), and bronchial relaxant (11). This study was taken up to assess the synergistic effect of the leaf extracts of these plants in inflammatory and arthritic animal models.

MATERIALS AND METHODS

Plant Materials and Preparation of Extracts

CH and VN were collected in November 2010 from Palakkad district, Kerala, authenticated at the Botanical survey of India, Coimbatore, and reconfirmed at St Joseph College Tiruchirappalli, Tamil Nadu, India. Voucher specimens (KMCH/Dr.TP/DST-DPRP/COG/Ch-Pal/2010/01;KMCH/Dr.TP/DST-DPRP/COG/Vn-Pal/2010/02) were deposited at the Department of Pharmacognosy, KMCH College of Pharmacy, Coimbatore, India.

Fresh mature leaves of CH and VN were cleaned to remove foreign, earthy matter and residual materials, shade-dried at room temperature (32 ± 2°C) for 10 days, pulverized to coarse powder, passed through a No. 40 mesh sieve, and extracted at 60° to 80°C successively with petroleum ether, chloroform, ethyl acetate, or methanol in a Soxhlet apparatus for 72 h. The extracts were filtered and concentrated separately under reduced pressure with an IKA Rotary evaporator (Model No RN 10 digital V, ILMAC Germany) at 40°C. Percentage yields of petroleum ether, chloroform, ethyl acetate, and methanol leaf extracts of CH and VN were 8.28%, 5.28%, 9.64%, 12.4 %, and 6.74%, 1.77%, 4.79%, and 8.4%, respectively.

Chemicals and Instruments

Apigenin, carrageenan, complete Freund's adjuvant (CFA), diclofenac sodium, and luteolin were purchased from Sigma Aldrich, USA. Solvents were procured from Qualigens, India. Electronic spectra were recorded on a Shimadzhu UV-1700 PharmaSpec UV-Vis Spectrophotometer. IR Spectra were recorded on a Jasco instrument. ^{1}H-NMR and ^{13}C-NMR were recorded in a Brucker Avance III 500 MHz FTNMR using deuterated methanol as solvent and tetramethylsilane (TMS) as internal standard. Mass spectra were recorded in Joel GCMS GC-Mate-II.

Preliminary Phytochemical Investigations

Quality control tests for the crude leaf powder of the plants were carried out as per IP 1996 and WHO guidelines (23) to assess purity, and the leaf extracts were tested for the presence of heavy metals at Sophisticated Analytical Instrumental facility, IIT, Chennai, India

Isolation of Flavonoids by Column Chromatography

The phytochemical constituents were identified by optimization of thin-layer chromatography (TLC). TLC plates were developed and scanned at 254 and 366 nm, which showed prominent band separation in TEFM (Toluene: ethyl acetate: formic acetate: methanol 3:6:1.6:0.4). Based on the results of preliminary screening, 36 g and 33 g of crude MLECH and MLEVN were chromatographed on silica gel column (Merck 60-120 mesh, 500 g), respectively, and successively eluted with stepwise gradient elution with petroleum ether, chloroform, ethyl acetate, and methanol. A total of 200 fractions from MLECH and 440 fractions from MLEVN were collected, and each fraction was spotted on pre-coated silica gel (Merck, GF_{254}, 0.25–mm-thick) TLC plate and eluted in the solvents.

Animals

Wistar female albino mice and Wistar strains rats of either gender were obtained from the animal house of Kovai medical center research and educational trust, Coimbatore, India. The animals were kept in polypropylene cages at 25 ± 2°C, humidity (50% ± 5%), and 12-h light/dark cycles. They were fed with standard laboratory animal diet and water *ad libitum*. Animals were acclimatized to laboratory conditions, and the experiments were conducted in accordance with ethical norms approved by the Committee for the Purpose of Control and Supervision on Experiments on Animals and Institutional Animal Ethical Committee (KMCRET/DST/01/2011, dt.16/07/2011).

Acute Oral Toxicity Study

The acute oral toxicity study was carried out as per the guideline set by the Organization for Economic Co-operation and Development (14). Healthy young adult albino nulliparous, non-pregnant female mice, weighing about 20 to 30 g, were administered as a single dose (1 mL) orally using oral feeding needle with 5, 50, 300, or 2,000 $mg.kg^{-1}$ of MLECH and MLEVN in 1% w/v sodium lauryl sulfate (SLS) suspension. Animals were observed individually for the first 30 min, periodically during the first 24 h, with special attention

given during the first 4 h, and daily thereafter, for a total of 14 days to study toxicity signs.

Anti-inflammatory Activity

The anti-inflammatory activity was evaluated by the carrageenan-induced paw edema method (24). The rats weighing 100 to 130 g were divided into 11 groups, each consisting of six animals. The first and second groups received 5 mL.kg^{-1} of 1% w/v sodium lauryl sulfate as vehicle control and 10 mg.kg^{-1} (*p.o*) of diclofenac sodium as standard, respectively, for comparative pharmacological assessment. Equal proportions of MLECH at a dose of 100, 200, or 400 mg.kg^{-1} and MLEVN at a dose of 100, 200, or 400 mg.kg^{-1} in 1 % w/v SLS suspension were administered orally to the third, fourth, and fifth group and the sixth, seventh, and eighth group of rats, respectively. Combined MLECH and MLEVN at a dose of 100, 200, or 400 mg.kg^{-1} each were administered orally to the ninth, tenth, and eleventh groups of rats, respectively. After 30 min of extracts administration, 0.1 mL of 1% w/v carrageenan was injected into the right hind paw sub-plantar region of each rat. The paw volumes of control, standard, and extract treated rats were measured by water displacement plethismometer (Inco-Niviqure, Version 60.1, India) first at half an hour and then every hour until 6 h, after carrageenan administration.

Anti-arthritic Activity

Freund's adjuvant–induced arthritis method was adopted for the evaluation of anti-arthritic activity (10). The rats weighing 150 to -200 g were divided into 11 groups, each consisting of six animals. Arthritis was induced to groups 1 to 11 by injecting a 0.1 mL (0.1% w/v) suspension of killed *Mycobacterium tuberculosis* bacteria homogenized in liquid paraffin into the left hind paw of rats, where group 1 was considered as arthritic control. Group 2 received diclofenac sodium and served as reference standard. Groups 3 to 5 received 100, 200, and 400 mg.kg^{-1} of MLECH, and groups 6 to 8 received 100, 200, and 400 mg.kg^{-1} of MLEVN, respectively. Group 9 received MLECH and MLEVN (100 mg.kg^{-1} + 100 mg.kg^{-1}); group 10 received MLECH and MLEVN (200 mg.kg^{-1} + 200 mg.kg^{-1}); and group 11 received MLECH and MLEVN (400 mg.kg^{-1} + 400 mg.kg^{-1}), respectively. Extracts and diclofenac sodium were suspended in 1% w/v sodium lauryl sulfate and administered orally using rat oral feeding needles, 30 min before adjuvant injection, and continued up to day 21. The rat paw volume of control and treatments were measured on days 4, 8, 14, and 21 using water displacement plethismometer (Inco-Niviqure, Version 60.1, India). The body weight of rats in all groups was recorded before treatment and on day 21.

On day 22, blood was withdrawn through retro-orbital vein puncture of all groups by anaesthetizing the animals with diethyl ether and the biochemical parameters *viz* hemoglobin (Hb) content, White blood corpuscles (WBC) count, erythrocyte sedimentation rate (ESR) and red blood corpuscles (RBC) count were analyzed (8).

Statistical Analysis

Data expressed as mean ± SEM were analyzed by one-way analysis of variance and means compared by Dunnett's test ($p < 0.05$).

RESULTS

Preliminary Phytochemical Investigations

The leaf powders of CH and VN were in compliance for the quality control tests as per IP 1996 and WHO guidelines. Phytochemical tests confirmed the presence of carbohydrates, flavonoids, fixed oils and fats, proteins and amino acids, phenolic compounds, and tannins in both the extracts of CH and VN. While alkaloids were present in VN extract, phytosterol was present in CH extract. The heavy metal analysis of the MLECH and MLEVN revealed the presence of 0.053, 0.002, 0.042, and 0.061 ppm of As, Pb, Cd, and Hg, respectively, and they were within the USP limits of 0.1 ppm (each).

Isolation and Characterization of Flavonoids

Fractions (108 to 140) of MLECH showed a single spot with same Rf value of 0.70 on TLC, and hence they were pooled together, evaporated to dryness, and purified to yield a pale yellow solid (CH1, 230 mg; mp 331°C), and the structure was elucidated on the basis of UV shift reagents, FTIR, ^{1}H NMR, ^{13}C-NMR, and mass spectra that led to the identification of luteolin with molecular formula $C_{15}H_{10}O_6$ and molecular weight of 286.2 g.mol^{-1}.

Fractions (372 to 377) of MLEVN showed a single spot with the same Rf value of 0.67 on TLC, and hence they were pooled together, evaporated to dryness, and purified to yield light yellow solid (VN1, 248 mg; mp 345°C), where the structure was elucidated on the basis of UV shift reagents, FTIR, ^{1}H NMR, ^{13}C-NMR, and mass spectra that led to the identification of apigenin with molecular formula $C_{15}H_{10}O_5$ and molecular weight of 270.2 g.mol^{-1}.

Acute Toxicity Study

Since no sign of toxicity, *viz*. lethargy, jerk, convulsion, or mortality was observed up to 2,000 mg.kg^{-1} for MLECH and MLEVN, one-twentieth,

one-tenth, and one-fifth doses of 2,000 mg.kg^{-1} (100, 200, 400 mg.kg^{-1}) were used for anti-inflammatory and anti-arthritic activity study.

Anti-inflammatory Activity

Inflammation produced by carrageenan administration (*p.o.*) was revealed by increase in water volume displacement in the plethysmometer. Administration of diclofenac sodium showed inhibition of inflammation from 1 h onward, compared to control group. Single and combined doses of MLECH and MLEVN inhibited carrageenan-induced inflammation from 2 h onward compared to the control group. MLECH at 200 mg.kg^{-1} showed better inhibition than the groups treated with MLECH at 100 mg.kg^{-1}, and MLECH at 400 mg.kg^{-1} exhibited greater inhibition than MLECH at 200 mg.kg^{-1} and MLECH 100 mg.kg^{-1}, respectively at 6 h. MLECH at 200 mg.kg^{-1} showed higher inhibition than MLECH at 100 mg.kg^{-1}, and MLEVN at 400 mg.kg^{-1} reduced inflammation greater than MLEVN at 200 mg.kg^{-1} and 100 mg.kg^{-1} doses from 3 h onward. Treatment of the combination of MLECH and MLEVN in equally proportional doses showed greater reduction of inflammation (from 3 h onward) than control and the extracts individually. In tested individual and combined doses, MLECH and MLEVN exhibited dose-dependent activity in the inhibition of inflammation, and its action was noticed from 2 to 6 h. Anti-inflammatory effect of the combined MLECH and MLEVN (400 + 400 mg.kg^{-1}) at equal proportion was almost equal to that of standard drug diclofenac sodium (10 mg.kg^{-1}; Table 1).

Anti-arthritic Activity

In FA-induced arthritis, inflammation development was observed in two phases. In the first phase, edema formation was noticed, and inflammation reached peak effect on day 8. In the second phase, slow increase in the inflammation was noticed on day 14 and drastically increased on day 21. In the present study, injection of FA showed gradual increase in the inflammation up to day 21. Increase in the paw volume and joint swelling confirmed the induction of arthritis in animals. Treatment of MLECH (100, 200, or 400 mg.kg^{-1}), MLEVN (100, 200, or 400 mg.kg^{-1}), and combination of MLECH and MLEVN in equally proportional doses and diclofenac sodium in FA-induced arthritis rats reduced inflammation when compared to arthritic control group on day 21. Arthritic rats receiving a combination of MLECH and MLEVN at equal proportion showed better inhibition than those administered individual doses of MLECH or MLEVN (Table 2). The dose-dependent effect of MLECH and MLEVN was noticed in individual as well as combined doses. Freunds adjuvant–induced hematological perturbations such as increase in WBC, increase in ESR, decrease in RBC, and decrease in

TABLE 1 Evaluation of Anti-Inflammatory Activity of Methanol Leaf Extracts of *Cardiospermum halicacabum* and *Vitex negundo* by Carrageenan-Induced Rat Hind Paw Edema Method ($n = 6$)

Groups	Dose ($mg.kg^{-1}$)	Rat paw volume (mL) (mean ± SEM)							
		Initial	30 min	1 h	2 h	3 h	4 h	5 h	6 h
Control	0.1 mL	0.176 ± 0.006	0.183 ± 0.002	0.251 ± 0.107	0.428 ± 0.018	0.566 ± 0.117	0.656 ± 0.022	0.676 ± 0.123	0.743 ± 0.115
Diclofenac sodium	10	0.171 ± 0.144	0.188 ± 0.003	0.205 ± 0.004[a]	0.225 ± 0.002[a]	0.248 ± 0.014[b]	0.243 ± 0.003[b]	0.253 ± 0.003[b]	0.266 ± 0.003[b]
MLECH	100	0.176 ± 0.002	0.193 ± 0.002	0.246 ± 0.004	0.283 ± 0.005[a]	0.346 ± 0.004[b]	0.388 ± 0.001[a]	0.480 ± 0.003[b]	0.440 ± 0.002[b]
MLECH	200	0.173 ± 0.002	0.190 ± 0.005	0.236 ± 0.003	0.271 ± 0.006[a]	0.311 ± 0.005[b,d]	0.345 ± 0.004[b,k]	0.355 ± 0.004[b,n]	0.373 ± 0.003[b,j]
MLECH	400	0.178 ± 0.003	0.191 ± 0.005	0.220 ± 0.002	0.248 ± 0.003[a,c]	0.260 ± 0.002[b,e,f]	0.276 ± 0.003[b,l,f]	0.283 ± 0.004[b,i]	0.302 ± 0.006[b,i]
MLEVN	100	0.175 ± 0.004	0.187 ± 0.003	0.235 ± 0.004	0.278 ± 0.003[a]	0.330 ± 0.006[b]	0.386 ± 0.003[a]	0.415 ± 0.005[b]	0.430 ± 0.011[b]
MLEVN	200	0.178 ± 0.006	0.198 ± 0.012	0.250 ± 0.123	0.282 ± 0.006[a]	0.291 ± 0.004[b,d]	0.320 ± 0.009[b,m]	0.331 ± 0.113[b]	0.356 ± 0.010[b]
MLEVN	400	0.180 ± 0.115	0.193 ± 0.011	0.233 ± 0.006	0.256 ± 0.008[a]	0.265 ± 0.004[b,g]	0.310 ± 0.022[a]	0.295 ± 0.005[b,o]	0.316 ± 0.008[b,p]
MLECH+MLEVN	100 + 100	0173 ± 0.004	0.190 ± 0.005	0.225 ± 0.010	0.266 ± 0.009[a]	0.310 ± 0.012[b]	0.350 ± 0.007[b]	0.388 ± 0.008[b]	0.410 ± 0.007[b]
MLECH+MLEVN	200 + 200	0.181 ± 0.005	0.196 ± 0.007	0.231 ± 0.012	0.258 ± 0.006[a]	0.268 ± 0.006[b,h]	0.290 ± 0.010[b]	0.301 ± 0.012[b]	0.313 ± 0.013[b,q]
MLECH+MLEVN	400 + 400	0.183 ± 0.010	0.200 ± 0.013	0.220 ± 0.014	0.235 ± 0.017[a]	0.248 ± 0.015[b]	0.260 ± 0.010[b]	0.265 ± 0.011[b]	0.241 ± 0.016[b]

Note: Data are mean ± SEM. Means not followed by a superscript are not different compared to control

[a] $p < 0.05$, treatment groups *vs* control.

[b] $p < 0.001$, treatment groups *vs* control.

[c] $p < 0.05$, CH 400 *vs* CH 100.

[d] $p < 0.05$, CH 200 *vs* CH 100; VN 200 *vs* VN 100.

[e] $p < 0.01$, CH 400 *vs* CH 200.

[f] $p < 0.001$, CH 400 *vs* CH 100.

[g] $p < 0.01$, VN 400 *vs* VN 100.

[h] $p < 0.05$, CH + VN (200+200) *vs* CH 200.

[i] $p < 0.01$, CH 400 *vs* CH 200.

[j] $p < 0.001$, CH 200 *vs* CH 100.

[k] $p < 0.01$, CH 200 *vs* CH100.

[l] $p < 0.001$, CH 400 *vs* CH200.

[m] $p < 0.05$, VN 200 *vs* VN100.

[n] $p < 0.01$, CH 200 *vs* CH 100.

[o] $p < 0.001$, VN 400 *vs* VN 100.

[p] $p < 0.01$, VN 400 *vs* VN 100.

[q] $p < 0.05$, CH + VN (200 + 200) *vs* CH + VN (100 + 100).

TABLE 2 Evaluation of Anti-arthritic Activity of Methanol Leaf Extracts of *Cardiospermum halicacabum* and *Vitex negundo* on Rats by Freund's Adjuvant–Induced Arthritis Method

Groups	Dose ($mg.kg^{-1}$)	Rat paw volume (mL) (mean ± SEM)				
		Initial	Day 4	Day 8	Day 14	Day 21
Arthritic control	0.1 ml	0.873 ± 0.006	1.233 ± 0.210	1.735 ± .026	1.8166 ± 0.070	2.900 ± 0.148
Diclofenac sodium	10	0.865 ± 0.011	1.250 ± 0.088	1.183 ± 0.094*	1.100 ± 0.051**	0.956 ± 0.014***
MLECH	100	0.886 ± 0.009	1.316 ± 0.060	1.583 ± 0.065	1.550 ± 0.056	1.483 ± 0.047**
MLECH	200	0.856 ± 0.003	1.266 ± 0.033	1.533 ± 0.033	1.483 ± 0.047	1.350 ± 0.042**
MLECH	400	0.881 ± 0.003	1.283 ± 0.030	1.500 ± 0.044	1.416 ± 0.047	1.366 ± 0.049**
MLEVN	100	0.890 ± 0.002	1.400 ± 0.057	1.683 ± 0.054	1.550 ± 0.034	1.500 ± 0.085**
MLEVN	200	0.8600 ± 0.005	1.366 ± 0.049	1.566 ± 0.084	1.483 ± 0.030	1.333 ± .0666**
MLEVN	400	0.8483 ± 0.037	1.350 ± 0.061	1.516 ± 0.060	1.416 ± 0.030*	1.450 ± 0.061**
MLECH+MLEVN	100 + 100	0.883 ± 0.003	1.350 ± 0.042	1.566 ± 0.055	1.500 ± 0.073	1.416 ± 0.060**
MLECH+MLEVN	200 + 200	0.865 ± 0.004	1.300 ± 0.051	1.500 ± 0.115	1.433 ± 0.055	1.283 ± 0.087**
MLECH+MLEVN	400 + 400	0.880 ± 0.003	1.250 ± 0.105	1.366 ± 0.049*	1.283 ± 0.079	1.200 ± 0.073**

Note: Data are mean ± SEM. Means not followed by a superscript are not different from control.
*, **, ***Different from control at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Hb, were altered both by individual as well as combined doses of MLECH and MLEVN (Table 3).

DISCUSSION

Isolated compounds from fractions of MLECH and MLEVN, CH1, and VN1 under UV light at 254 nm exhibited yellow on exposure to ammonia vapor and confirmed the presence of flavonoids with free 5-OH and free 4‘ OH group. Methanol fraction of the MLECH and MLEVN gave pinkish red on reaction with ferric chloride, magnesium ribbon and HCl gave pinkish-red, vanillin and HCl gave pinkish-red color, and alpha naphthol gave negative response. These reactions confirmed the presence of flavonoid (aglycone) compound with 5,7-dihydroxy system in the A ring.

UV spectra in MeOH showed two absorption bands from 240 to 280 nm and 300 to 350 nm, confirming the presence of flavones: Band I for absorption of Ring A (Benzoyl system) and Band II for absorption of Ring B (Cinnamoyl system). A bathochromic shift observed with strong base $NaOCH_3$ confirmed the presence free 4-OH in Ring B. No decrease in intensity of signal observed confirmed free 4-OH in Ring B. 7-OH group in Ring A was confirmed since bathochromic shift was observed with NaOAc for both CH1 & VN1. Presence of *ortho*-3,4-di OH in CH1 and absence of *ortho*-3,4-di OH in VN1 confirmed by NaOAc+H_3BO_3 and AlCl3+HCl. UV spectrum of MeOH + NaOAc underwent hypsochromic shift of Band I after addition of H_3BO_3, which was corroborated by band I hypsochromic displacement observed on MeOH + $AlCl_3$ after addition of HCl for CH1. No significant shift was observed with $AlCl_3$-HCl, confirming the absence of *ortho*-3’,4’-di-OH in VN1.

Infrared spectra of both CH1 and VN1 showed strong absorption bands from 3,150 to 3,400 cm^{-1}, sharp absorption peaks at 1,680 cm^{-1} and around 1,162 cm^{-1}, confirming the presence of OH group (more probably the phenolic OH group), α,β- unsaturated carbonyl group (conjugated carbonyl), and C-O-C stretching, respectively.

Proton NMR of CH1 showed a broad singlet at δ 4.6 due to 5-OH proton; broad singlet at δ 4.9 due to 3′ and 4′ OH; and singlet at δ 6.56 due to proton at third position of ring C. Singlet at δ 6.24 was due to Ar-H of 5′ position of ring B; singlet at δ 6.88 was due to H-6 and H-8 protons; and doublets at 6.92 and 7.4 were due to H-6′ and H-2′ protons of ring B, respectively. ^{13}C NMR of compound CH1 showed signals at δ 182 (C-4),171 (C-7), 164 (C-2, C-5),161 (C-9), 149 (C-5′), 148(C-4′), 128(C-1′), 122(C-2′), 118 (C-3′), 115 (C-6′), 103 (C-10), 118 (C-3), and δ 98 (C-6 & C-8) for the respective carbon skeleton of luteolin. Mass spectra showed M+ ion at m/z 286.2 and peaks at 152 and 134, suggesting the cleavage of flavone ring. These data were in agreement with the reported spectral literature suggesting the presence of luteolin.

TABLE 3 Effect of Hematological Parameters in Adjuvant-Induced Arthritis in Rats ($n = 6$)

Groups	Dose (mg.kg^{-1})	WBC ($\times 10^3$ cells/ mm^3)	RBC count ($\times 10^6$/mm^3)	Hb (%)	ESR (mm/h)
Normal control	5 mL.kg^{-1} 1% SLS	6.36 ± 0.742	7.01 ± 0.130	14.80 ± 0.103	13.05 ± 0.081
Arthritic control	0.1 mL	8.07 ± 0.987	5.77 ± 0.462	11.23 ± 0.674	14.96 ± 0.485
Diclofenac sodium	10	7.12 ± 0.603**	6.83 ± 0.393**	14.52 ± 0.954**	13.59 ± 0.228**
MLECH	100	8.06 ± 0.484ns	6.10 ± 0.519*	12.30 ± 0.529*	14.38 ± 0.550*
MLECH	200	7.82 ± 0.646*	6.41 ± 0.353**	13.16 ± 0.291*	14.29 ± 0.563*
MLECH	400	7.55 ± 0.440*	6.55 ± 0.239**	13.87 ± 0.353*	14.15 ± 0.250**
MLEVN	100	8.02 ± 0.854*	6.02 ± 0.666ns	11.73 ± 0.497*	14.42 ± 0.194*
MLEVN	200	7.88 ± 0.155*	6.32 ± 0.115*	12.93 ± 0.263**	14.36 ± 0.356*
MLEVN	400	7.60 ± 0.528**	6.63 ± 0.249**	13.50 ± 0.178**	14.21 ± 0.647**
MLECH+MLEVN	100 + 100	7.89 ± 0.603**	6.38 ± 0.353**	13.05 ± 0.176**	14.18 ± 0.342**
MLECH+MLEVN	200 + 200	7.68 ± 0.844**	6.52 ± 0.173**	13.78 ± 0.318**	14.05 ± 0.638**
MLECH+MLEVN	400 + 400	7.42 ± 0.882**	6.91 ± 0.118**	14.21 ± 0.231**	13.94 ± 0.467**

ns, not significant.

Note: Data are mean ± SEM.

*, **Significant at $p < 0.05$ and $p < 0.01$, respectively.

Proton NMR of VN1 showed a broad singlet at δ 4.6 due to 5-OH proton, broad singlet at δ 4.9 due to 3′ and 4′ OH, and singlet at δ 6.62 due to proton at third position of ring C. Doublet at δ 6.48 was due to Ar-H of 5′ position of ring B; singlet at δ 6.23 is due to H-6 and H-8 protons; and doublets at 6.96 are due to H-6′ protons of ring B, respectively. ^{13}C NMR of compound CH1 showed signals at δ 1 δ 98 (C-6 & C-8), 182 (C-4), 164 (C-5, C-7), 161 (C-2), 158 (C-4'), 141 (C-9), 128(C-1', C-2' & C-6'), 115(C-3' & C-5'), and δ 102(C-3 and C-10) for the respective carbon skeleton of apigenin. Mass spectra showed M+ ion peak at m/z 270.2 and peaks at 152 and 118, suggesting the cleavage of flavone ring. These data were in agreement with the reported spectral literature suggesting the presence of apigenin.

In the assessment of the anti-inflammatory activity of natural and synthetic compounds, carrageenan-induced inflammation was used as standard model, since it released inflammatory and pro-inflammatory mediators including prostaglandins, leukotrienes, histamine, bradykinin, and TNF-α in a biphasic response. First phase starts with the release of histamine, serotonin, and kinins after the injection of phlogistic agent in the first few hours. The prostaglandins released in second phase a slow reacting substance, which reaches peak at 3 h. Second phase is sensitive to both the clinically useful steroidal and nonsteroidal anti-inflammatory agent.

In this study, injection of carrageenan increased the paw volume when compared to control, which confirmed the induction of inflammation. Administration of MLECH and MLEVN had potential inhibitory effect on carrageenan-induced edema from 2 to 6 h. This effect supported its inhibitory action on inflammatory mediators. The combination of both the doses of extracts showed higher inhibitory effect on carrageenan-induced inflammation. In tested individual and combined doses, MLECH and MLEVN exhibited dose-dependent activity suggesting that both MLECH and MLEVN may act synergistically in both early and late phases of inflammation.

CFA-induced arthritis is the most widely used chronic test model in which the clinical and pathological changes are comparable with that of human rheumatoid arthritis. Freund's adjuvant model was chosen since it developed chronic swelling in multiple joints with influence of inflammatory cells with erosion of joint cartilage and bone destruction, mainly due to the release of inflammatory mediators. CFA-induced polyarthritis is associated with an immune-mediated inflammatory reaction, and rat is unique in developing polyarthritis after CFA treatment; hence, they were used for the study (12). In first phase, irritant nature of the adjuvant induced inflammation, and hence measurement of 3- to 5-day effect of treatment represented the efficacy on inflammation of primary lesions such as edema and soft-tissue thickening. In the second phase, reduction in body weight is due to immunogenic responses. In the present study, administration of MLECH and MLEVN showed inhibition of inflammation on day 4 in individual and combined doses, which showed its ability to prevent primary lesions. MLECH

or MLEVN individually produced inhibition on inflammation and secondary lesions on days 14 and 21. Combined higher doses of MLECH and MLEVN elicited synergistic anti-arthritic effect greater than the lower combined doses.

Information related to the pathology of arthritis was obtained during the study from hematological parameters. It was reported that in arthritic conditions, moderate increase in WBC count occurred due to an IL-1B-mediated rise in the respective colony stimulating factors. The present study revealed that MLECH, MLEVN and diclofenac sodium treatments tend to normalize the WBC. Anti-arthritic effect of MLECH and MLEVN was supported by characteristic hematological alterations such as increased Hb, RBC, and decreased ESR. It is proposed that the reduction in the Hb count during arthritis resulted from reduced erythropoietin levels, a decreased response of the bone marrow erythropoietin and premature destruction of red blood cells and increase in ESR due to formation of endogenous protein, globulin, and fibrinogen. The combined administration of MLECH and MLEVN showed decrease in WBC and ESR and increase in RBC and Hb in a dose-dependant manner. Among the administered combined doses of MLECH and MLEVN, the higher doses exhibited better synergistic effect than the lower combined doses.

The anti-inflammatory and anti-arthritic activity of CH and VN may be due to the presence of OH groups at third or/and fourth position in B ring and at fifth position of A ring of the isolated flavonoids apigenin and luteolin (13). Therefore, anti-inflammatory and anti-arthritic activities of MLECH and MLEVN may be attributed to the presence of apigenin (6) and luteolin (9).

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