Research Paper

Bioassay Guided Isolation of Anti-Inflammatory Compounds from *Bauhinia variegata* L.: A Key Ingredient in Herbo-Mineral Formulation, Gandmala Kandan Ras

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Medicinal herb *Bauhinia variegata* L. is the main ingredient in "Gandmala Kandan Ras" (GKR) herbo-mineral medicine used in Ayurveda for the treatment of swelling, inflammation and tumors. This study aimed to isolate the anti-inflammatory compounds from the methanolic extract of aerial parts of *Bauhinia variegata*. Through bioassay-guided isolation, three known flavonoids, namely kaempferol, ombuin and quercetin were identified from methanolic extract of aerial parts of *Bauhinia variega*. The primary screening by protein denaturation method revealed a significant percentage of inhibition of protein denaturation of compounds kaempferol and quercetin. The anti-inflammatory assays against COX-1/2 enzymes showed significant anti-inflammatory activity of these two compounds, compared to the standard drug, indomethacin. Of which, quercetin as a potent non-selective inhibitor of COX1 and COX2 with IC_{50} values of 172.05±4.29 and 220.62±9.13 nM, respectively; while kaempferol showed selective COX2 inhibition with the IC_{50} of 154.86±5.60 nM. The results provided evidence that supports the Ayurvedic usage of Gandmala Kandan Ras formulation in the treatment of inflammation, which was attributed to the natural active kaempferol and quercetin. The results indicated that plant *Bauhinia variegata* could be considered as an excellent natural source of remedial medicine for inflammation.

Key words: *Bauhinia*, protein denaturation method, cyclooxygenase enzymes, inhibitory assay

Bauhinia genus belongs to the family Fabaceae, well recorded in the flora of Brazil, India, Nepal and South Africa[1,2]. Traditionally, the bark, flowers and roots of the genus *Bauhinia* are most valued in Brazil, India and South Africa for treating various alignments such as diabetes, inflammation, tumors, gastrointestinal disorders and infectious diseases[3]. In Ayurveda, an herbo-mineral formulation named "Gandmala Kandan Ras" (GKR) is widely used for patients suffering from inflammation-related conditions such as swelling, cysts and tumors. The Sanskrit technical term "Gandmala" is "scrofula," which is an inflammatory disorder indicating swelling of the neck and inflammation of lymph glands. GKR formulation mainly contains ingredients from *Bauhinia variegata* (*B. variegata*) L. and Shuddha Guggulu[4].

B. variegate L. is a semi-evergreen tree, usually called "Camel's foot creeper" in English, and "Kanchanara or Kachnar" in Sanskrit. In some parts of India, buds and flowers of *B. variegata* are used as vegetables and are cooked and eaten as a famous food named "Karalen Ki Sabji"[5]. Particularly, in the Indian tribes, the *B. variegata* has been using in the treatment of multiple conditions, including microbial

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infections, oxidative stress, chronic inflammation and cancer. Biologically, *B. variegata* has been reported for its antibacterial[6], antioxidant[6,7], anti-inflammation[3], anti-hyperlipidemic[7], anti-cancer[6], hepatoprotective properties[8], trypsin inhibitory[9] and immunomodulatory[10] activities. The broad spectrum of biological activities of *B. variegata* is mainly due to the presence of multiple bioactive compounds such as flavonoids, flavanones, bibenzyls, triterpenes, flavonol-glycosides, saponins and phenanthraquinones in its different parts[11,12].

In the current study, we aimed to investigate the anti-inflammatory agents present in aerial parts extract of *B. variegata*, one of the main ingredients of GKR formulation[4], using bio-guided isolation and screening its bioactive metabolites for anti-inflammatory activity against cyclooxygenase enzymes.

MATERIALS AND METHODS

Collection:

The aerial parts of *B. variegata* L. were collected at Seshachalam hills, Tirupati, Andhra Pradesh, India, in October 2019 and a voucher specimen (DB-SVU-2019-3626) has deposited at the Department of Botany, Andhra University, Visakhapatnam, Andhra Pradesh, India.

Extraction and bioassay-guided isolation:

Approximately 250 g of the dried aerial parts of *B. variegata* was grounded and extracted by maceration method using methanol (3×500 ml×7 d) at room temperature. All the three fractions of extract were filtered using a muslin cloth and concentrated on a rotary evaporator (Shimadzu Rotation evaporator QR 2005-S, Japan) under reduced pressure at 40° thereby provided a crude methanol extract of the aerial parts of *B. variegata* (ME) 4.5 g, 18 % w/w as black solid.

Approximately 3.0 g of ME was subjected to column chromatography (sintered disc column, 600 mm×45 mm; Product code: 6101067, Borosil, India) over silica gel (230-400 mesh, CAS No.: 112926-00-8, Merck) by employing a mobile phase hexane-ethyl acetate gradient (0-100 %) yielded eight fractions (F1-8). These fractions were concentrated in a vacuum and screened for their protein denaturation capacity against bovine serum albumin (Sigma-Aldrich, India). The fractions that exhibited prominent inhibitory action against protein denaturation were further purified by column chromatography (sintered disc column, 300 mm×18 mm; Product code: 6101062, Borosil, India) over silica gel (230-400 mesh) using mobile phase hexane-chloroform gradient (0-100 %) and thin layer chromatography (pre-coated glass silica plates, TLC-Silica gel 60 GF_{254}, CAS: 7631-86-9, Merck, Germany) to obtain the bioactive compounds. Further, ^{1}H- Nuclear magnetic resonance (NMR) and ^{13}C-NMR (Bruker Avance 400 Spectrometer, Germany), Mass (LC/MS Triple Quad Portfolio, Agilent, China) spectral analyses, melting point (m.p.) determination (M-560/565 m.p. Apparatus, Buchi, Switzerland) and CNHS (2400 CHNS Organic Elemental Analyzer, PerkinElmer, USA) analyses were applied for the structure elucidation of isolated compounds.

In vitro anti-inflammatory assays:

Preliminary screening by protein denaturation method: In the present study, *in vitro* anti-inflammatory activity of ME and its fractions (F1-8) was evaluated against bovine serum albumin protein using the protein denaturation method[13]. Briefly, the bovine serum albumin protein (1 %) was prepared using 50 mM sodium phosphate buffer (pH 6.4). To 0.2 ml of the above solution, added 0.1 ml of the tested samples at three different concentrations 100, 200 or 400 µg/ml. The final volume was adjusted to 5 ml with buffer and incubated at 37° for 20 min. The tubes were heated in a steam bath at 95° for 20 min, then cooled to room temperature. Finally, the turbidity in the cooling tubes was measured at 660 nm by Ultraviolet-Visible Spectrophotometer (Model SL 210, Elico India Ltd.). The experiment was performed in triplicate (n=3) and the quantitative value was expressed as the mean±standard deviation (SD).

Cyclooxygenase (COX-1/2) inhibitory assay: The abilities of compounds (kaempferol, ombuin and quercetin) to inhibit isoenzymes COX-1/2 were performed using (ovine/human) COX inhibitor assay kit (Cayman, No.: 560131)[3]. To 10 µl of either COX1 or COX2 added 960 µl of 0.1 M Tris-HCl buffer and one of the three concentrations of the test samples and incubated for 10 min at 37°. After 2 min, add 10 µl of 100 µM arachidonic acid, 50 µl of 1 M HCl and Ellman's reagent. The absorbance was noted spectrophotometrically at 410 nm against the blank. The percentage of inhibition was calculated with the optical density values and the IC_{50} values were determined by linear regression.

RESULTS AND DISCUSSION

The preliminary anti-inflammatory assay revealed the good potential of protein denaturation inhibition of ME, Fraction (F)3, and F5 with the percentage of protein denaturation inhibition found to be 92.76±1.36 %, 92.00±5.12 % and 70.65±1.52 %, respectively (Table 1). By using chromatography and analyses of their spectral NMR data and elemental composition, three known flavonoids, namely kaempferol (1), ombuin (2) and quercetin (3) were successfully identified from F3 and F5 two most active fractions (fig. 1). Particularly, F3 (620 mg) yielded compound 1 (100 mg) as a yellow solid and F5 (250 mg) yielded compounds, 2 (60 mg) as a pale yellow powder and 3 (50 mg) as a shiny yellow powder. At 400 mg/ml concentration, compounds 1 (92.95±1.22 %) and 3 (93.16±2.67 %) exhibited profound anti-inflammatory potency, which was substantially higher than that of compound 2 (44.65±2.70 %) (Table 1). Notably, the percentage of inhibition of compounds 1 and 3 was also very high at 200 mg/ml and substantially lower at 100 mg/ml concentration (Table 1).

Compound 1 (Kaempferol) (fig. 1)[14] is a yellow solid, m.p.: 275-276°; (Retention factor) R_f: 0.6 (hexane:ethyl acetate 1:1); ^{1}H NMR (400 MHz, Dimethyl Sulfoxide (DMSO)-d_6): 2.27 (s, 1H, OH), 2.98 (s, 1H, OH), 3.93 (s, 1H, OH), 5.03 (s, 1H, OH), 6.35 (s, 1H, Ar-H), 6.45 (s, 1H, Ar-H), 7.12-7.13 (d, 2H, J=4 Hz, Ar-H), 7.61-7.63 (d, 2H, J=8 Hz, Ar-H). ^{13}C NMR (400 MHz, DMSO-d_6): 94.96 (C-8), 100.15 (C-6), 104.82 (C-4), 116.49 (C-12/14), 122.48 (C-10), 131.48 (C-11/15), 136.89 (C-2), 146.42 (C-1), 158.13 (C-9), 160.36 (C-13), 160.49 (C-5), 164.68 (C-7), 175.78 (C-3). CHNS analysis for $C_{15}H_{10}O_6$: calcd. C-62.94 %, H-3.52 %, found C-62.96 %, H-3.54 %. Electrospray Ionisation-Mass Spectrometry (ESI-MS): calcd. m/z for $C_{15}H_{10}O_6$: 286.05 [M], found 285.14 [(M+H$^+$), positive mode], 287.24 [(M-H$^+$), negative mode].

Compound 2 (Ombuin) (fig. 1)[15] is a pale yellow powder; m.p.: 202-203°; R_f: 0.5 (hexane:ethyl acetate 1:1); ^{1}H NMR (400 MHz, DMSO-d6): 2.97 (s, 1H, OH), 3.55 (s, 1H, OH), 3.85 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 5.03 (s, 1H, OH), 6.23 (d, 1H, J=1 Hz, Ar-H), 6.24-6.25 (d, 1H, J=4 Hz, Ar-H), 6.85-6.87 (d, 1H, J= 8 Hz, Ar-H), 7.02-7.06 (m, 2H, Ar-H). ^{13}C NMR (400 MHz, DMSO-d_6): 57.07 (C-10), 57.81 (C-17), 93.54 (C-8), 98.70 (C-6), 106.03 (C-4), 113.24 (C-15), 116.39 (C-12), 121.00 (C-16), 124.86 (C-11), 138.13 (C-2), 146.92 (C-13), 147.87 (C-1), 150.82 (C-14), 159.09 (C-9), 161.63 (C-5), 166.42 (C-7), 176.27 (C-3). CHNS analysis for $C_{17}H_{14}O_7$: calcd. C-61.82 %, H-4.27 %, found C-61.76 %, H-4.24 %. ESI-MS: calcd. m/z for $C_{17}H_{14}O_7$: 330.07 [M], found 331.63 [(M+H$^+$), positive mode], 329.20 [(M-H$^+$), negative mode].

Compound 3 (Quercetin) (fig. 1)[14] is a shiny yellow powder; m.p.: 316-317°; R_f: 0.4 (hexane:ethyl acetate 1:1); ^{1}H NMR (400 MHz, DMSO-d_6): 2.77 (s, 1H, OH), 3.01 (s, 1H, OH), 3.50 (s, 1H, OH), 3.87 (s, 1H, OH), 5.01 (s, 1H, OH), 6.19 (d, 1H, J= 1 Hz, Ar-H), 6.19-6.20 (d, 1H, J= 4 Hz, Ar-H), 6.77-6.78 (d, 1H, J= 4 Hz, Ar-H), 6.98-7.01 (m, 2H, Ar-H). ^{13}C NMR (400 MHz, DMSO-d_6): 94.81 (C-8), 99.99 (C-6), 104.66 (C-4), 116.36 (C-11), 116.67 (C-14), 122.01 (C-15), 122.19 (C-10), 137.48 (C-2), 145.68 (C-12), 147.21 (C-1), 148.73 (C-13), 157.97 (C-9), 160.34 (C-5), 164.52 (C-7), 175.62 (C-3). CHNS analysis for $C_{15}H_{10}O_7$: calcd. C-59.61 %, H-3.34 %, found C-59.62 %, H-3.34 %. ESI-MS: calcd. m/z for $C_{15}H_{10}O_7$: 302.04 [M], found 303.66 [(M+H$^+$), positive mode], 301.25 [(M-H$^+$), negative mode].

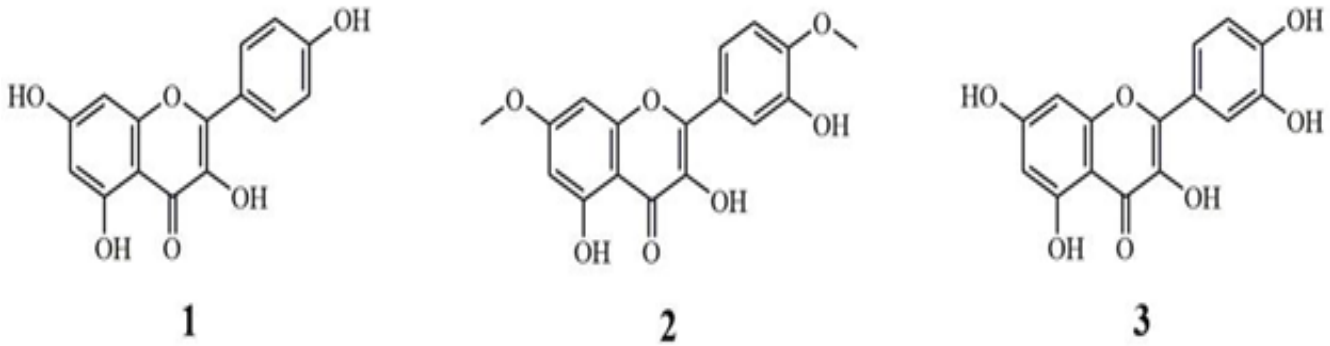

Fig. 1: Chemical representation of isolated flavonoids (1-3) from *Bauhinia variegate*
Note: 1: Kaempferol; 2: Ombuin and 3: Quercetin

TABLE 1: YIELD AND INHIBITORY EFFECTS OF METHANOL EXTRACT (ME), FRACTIONS (F1-8), AND COMPOUNDS (1-3) FROM *Bauhinia variegata* AGAINST BOVINE ALBUMIN PROTEIN DENATURATION

Sample	Yield (mg)	% Inhibition of protein denaturation*		
		100 µg/ml	200 µg/ml	400 µg/ml
ME	4500	41.24±1.97	69.38±3.62	92.76±1.36
F1	800	3.45±0.21	14.94±3.75	25.97±1.42
F2	450	9.45±1.73	19.87±2.28	27.93±1.00
F3	620	67.78±2.34	72.97±6.57	92.00±5.12
F4	250	11.02±3.10	25.93±3.52	27.90±1.76
F5	310	35.55±6.43	54.06±5.26	70.65±1.52
F6	200	6.57±1.40	19.01±1.09	27.31±0.36
F7	550	13.45±0.72	25.69±2.74	41.14±1.43
F8	140	3.45±1.66	6.86±1.72	7.67±1.72
1	100	66.10±1.87	90.60±1.29	92.95±1.22
2	60	20.92±2.59	32.95±1.63	44.65±2.70
3	50	70.11±2.41	88.88±1.30	93.16±2.67

Note: *mean±SD (n=3)

Based on their protein denaturation inhibition capability, active compounds 1 and 3 at a concentration range of 25 to 100 µg/ml were further evaluated for *in vitro* anti-inflammatory activity against COX-1/2 isoenzymes using (ovine/human) inhibitor assay kit (Table 2). The IC_{50} values were calculated against COX-1/2 through dose-response assay and the results were presented in Table 3. IC_{50} values were taken as the lowest concentration of compounds that exhibited inhibition of 50 %, relative to the inhibition control without compounds.

The IC_{50} values of compound 3 against COX1 and COX2 enzymes were noted to be 172.05±4.29 and 220.62±9.13 nM respectively, higher than that of the reference drug indomethacin (IC_{50} values: 110.35±2.91 and 84.26±4.40 nM respectively). Compound 1, on the other hand was selectively active against the COX2 enzyme with the IC_{50} values of 154.86±5.60 nM (Table 3), whereas its inhibition against COX1 at the concentration of 100 µg/ml reached 48.83±0.64 % only (Table 2). Taken together, our data revealed the potent inhibition efficiency of the compounds 1 and 3 on COX enzymes.

GKR is a herbo-mineral formulation that is well-known for its anti-inflammatory effects such as Gandamala (lymphadenopathy), Galaganda (goitre) and Arbuda (tumor)[4,5]. The general composition of "GKR" formulation is *B. variegata* (144 g), shuddha guggulu (144 g), mandur bhasma (36 g), sonth (*Zingiber officinale* rhizome, 24 g), kali mirch (*Piper nigrum*, 24 g), pippali (*Piper longum*, 24 g), shuddha parad (12 g), shuddha gandhak (6 g), tamra bhasma (18 g), sendha namak (6 g), and cow's ghee (quantity sufficient). This oral formulation (tablets) is recommended for adults (250-1000 mg per day) and children (8 mg per kg body weight per day) suffering from different types of inflammation[4].

The previous studies on the chemical and biological properties have identified multiple secondary metabolites from *B. variegata* (e.g. flavonoids, flavanones, triterpenes, saponins, etc.)[11,12] and shuddha guggulu (guggulusterone, naringenin, myrrhanol, etc.) [16-20], which were noted for their anti-inflammatory activities. Besides, anti-inflammatory studies were also well-established for other GSK-included plants and multiple key chemical constituents were reported comprising ginger and gingerol derivatives (from sonth, (*Zingiber officinale* rhizome))[21] and piperine derivatives (from kali mirch (*Piper nigrum*) and pippali (*Piper longum*))[22-24]. On the other hand, mandur bhasma is well-acknowledged as an iron supplement[25] and shuddha parad and shuddha gandhak act as detoxifying agents[26]. Tamra bhasma is used as an adjuvant and sendha namak (rock salt) offers trace minerals in Ayurveda formulations[27,28].

With this in mind, we investigated the main chemical constituents present in *B. variegata* that are responsible for the anti-inflammatory actions. The *in vitro* preliminary screening of ME and its fractions (F1-8) revealed its aptitude to treat inflammation of ME and fractions F3 and F5. The bioassay-guided isolation of these two fractions has yielded three known secondary

metabolites (1-3) (fig. 1). This finding provides new insights into the phytochemical profile of *B. variegata*.

COXs are key enzymes that catalyse the production of prostaglandins and thromboxanes, which are commonly involved in the regulation of inflammation, from arachidonic acid[29]. Thus, routes of non-selective COX1 and COX2 inhibition are widely chosen for the treatment of inflammation[30,31]. Our *in vitro* tests have justified the potent inhibition against COX1/2 enzymes of compounds 1 and 3 (Table 1), of which compound 3 acts as a non-selective COX inhibitor and compound 1 is selective COX2 inhibitor (Table 2 and Table 3). This observation is in accordance with the previous study, which uncovered the significant inhibition against the COXs and 15-LOX enzymes of the leaf extract of *B. variegata*[3]. Taken together, the overall anti-inflammatory activity exhibited by compounds 1 and 3 obtained from the ME extract of *B. variegata* was significant, although lower than that of the standard drug, indomethacin. These findings further supported the folklore usage of the herbo-mineral formulation GKR in the treatment of inflammation.

To conclude, the present work provides the first evidence for the presence of anti-inflammatory compounds from *B. variegata*, the main ingredient in herbo-mineral formulation, GKR. First, we reported the bioassay-guided isolation of compounds 1-3 from the aerial parts of *B. variegata* that possessed significant *in vitro* inhibition against COX-1/2. The key metabolites responsible for anti-inflammatory activities were noted to be compounds 1 and 3 by acting against COX1/2 proteins. The results provided evidence that supports the Ayurvedic use of the GKR formulation. Also, these findings suggested that plant *B. variegata* can be a good natural source of remedial medicine for inflammation.

TABLE 2: PERCENTAGE INHIBITION OF COMPOUNDS 1 AND 3 AGAINST CYCLOOXYGENASE ENZYMES

Sample	Percentage inhibition at different concentration*			
	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml
Cyclooxygenases 1 (COX1) inhibitory assay				
1	16.33±3.00	28.31±3.10	42.45±1.68	48.83±0.64
3	39.29±0.70	48.86±1.03	61.71±2.64	69.18±2.30
Indomethacin	41.64±0.45	62.29±1.22	68.34±2.99	83.92±2.27
Cyclooxygenases 2 (COX2) inhibitory assay				
1	40.78±0.59	52.72±0.83	60.23±3.47	66.33±3.06
3	34.31±0.97	43.23±1.79	53.26±0.71	66.08±2.77
Indomethacin	48.93±0.95	62.77±1.40	79.98±1.20	88.33±2.39

Note:*mean±SD (n=3)

TABLE 3: IC_{50} VALUES OF COMPOUNDS 1-3 ON COX1/2 ENZYMES

Sample	IC_{50} values (nM)*	
	COX1	COX2
1	-	154.86±5.60
2	-	-
3	172.05±4.29	220.62±9.13
Indomethacin	110.35±2.91	84.26±4.40

Note: *Mean±SD values (n=3), "-" indicates not found up to 100 µg/ml concentration

Conflict of interest:

The authors have no conflict of interest to declare.

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