J. Pharm. Res. 2025;24(2):89–97

Print ISSN: 0973-7200
Online ISSN: 2454-8405

Journal of Pharmaceutical Research

ORIGINAL ARTICLE

Development and Evaluation of Anti-inflammatory Multi-herbal Extract with Indigenous Medicinal Plants

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ARTICLE INFO

Article history:
Received 24.04.2025
Accepted 16.06.2025
Published 24.06.2025

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https://doi.org/10.18579/jopcr/v24.i2.51

ABSTRACT

Ayurveda advocates the use of multiple herbs instead of single drugs, as their synergistic interactions lead to improved therapeutic benefits. The present study aimed to evaluate the combined antioxidant and anti-inflammatory effects of the ethanolic extracts of *Tamarindus indica* (TI), *Ricinus communis* (RC), *Calotropis gigantea* (CG) and *Vitex negundo* (VN), which are well known for their anti-inflammatory activity. The preliminary phytochemical screening was done, and the total phenol and flavonoid contents of the alcoholic extracts and mixtures were assessed using Folin and Ciocalteu method and aluminium chloride colourimetric method respectively. Three combinations (MX A, B and C) were developed by mixing varying quantities of the plant extracts. The antioxidant potential was screened by the in vitro DPPH radical scavenging method and the anti-inflammatory activity was evaluated by protein denaturation assay using bovine serum albumin. As a part of future evaluation, an HPTLC fingerprinting pattern using standard lupeol was also created. Phytochemical screening of all extracts showed the presence of phenolic compounds and flavonoids, which are important antioxidant and anti-inflammatory plant metabolites. All plant extracts and combinations showed good antioxidant and anti-inflammatory activity that increased with concentration. RC and MX C showed maximum antioxidant activity, with IC_{50} values of 65.62 μg/ml and 36.45 μg/ml, respectively. MXC also exhibited good anti-inflammatory activity, with IC_{50} value of 28.30 μg/ml. The IC_{50} values of the mixtures and plant extracts were comparable to the standard quercetin and diclofenac sodium. The investigation demonstrated that various antioxidant and anti-inflammatory polyherbal formulations, utilizing the alcoholic extract of these plants should provide enhanced therapeutic activity. Moreover, the HPTLC chromatogram can serve as a valuable tool for the future assessment of formulations that include these plant mixtures.

Keyword: Polyherbal formulation; Anti-inflammatory; Antioxidant activity; *Tamarindus indica*; *Calotropis gigantea*; *Vitex negundo*; *Ricinus communis*; Lupeol

INTRODUCTION

Many civilizations worldwide have used herbal medicinal products as their main source of healing. These agents, which are made from plants and plant-derived materials, contain a wealth of bioactive chemicals that may have medicinal uses[1]. Polyherbalism is an innovative concept in Ayurveda and polyherbal mixtures have been used for medicinal purposes. The medicinal and curative properties of polyherbal formulations have led to their widespread use. Ayurvedic literature has introduced polyherbalism as an approach to enhance medicinal efficacy. The active phytochemical components of individual plants are insufficient to produce the desired therapeutic effects. When multiple herbs are combined in specific proportions for formulations, therapeutic efficacy may be enhanced[2,3].

Reactive oxygen species (ROS) are thought to be the most potent inducers of inflammation, and inflammation is the cause of the majority of both acute and chronic disorders. Many studies have reported the traditional use of herbal

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species for the treatment of inflammation, especially when ROS is involved. The present study aims to demonstrate the antioxidant–anti-inflammatory effects of a preparation based on *Tamarindus indica, Ricinus communis, Calotropis gigantea* and *Vitex negundo* extracts[4].

T. indica belongs to the plant family Fabaceae (Leguminoseae) and sub-family Caesalpinioideae. It is one of the few commercialized indigenous tropical medicinal plants on the global market[5]. Presence of acids such as tartaric, acetic, and succinic acids, as well as secondary metabolites including alkaloids, flavonoids, sesquiterpenes, tannins, and glycosides, as well as gum, pectin, and sugars, have all been reported in the plant's aerial portions. *T. indica* is used as traditional medicine in most of the tropical countries. It has long been used to treat a variety of conditions, including gonorrhoea, eye disorders, inflammation, cell cytotoxicity, wound healing, malaria, fever, constipation, and abdominal pain. Every part of the plant, from root to leaf tips, serve various human needs[6].

The castor oil plant, *Ricinus communis*, a member of the Euphorbiaceae family, is documented to exhibit therapeutic properties including hepatoprotective, antidiabetic, laxative, and antifertility activities. The leaves of *Ricinus communis* have been identified as a significant source of polyphenols, primarily hydroxycinnamic acids such as chlorogenic, p-coumaric, and caffeic acids; hydroxybenzoic acids such as ellagic and gallic acids; and flavonoids such as (+)- catechin, rutin, and quercetin, which are attributed to the plant's antioxidant and anti-inflammatory properties[7,8].

The plant *Calotropis gigantea* belongs to the family Apocynaceae and is utilized alone in combination with certain medicinal herbs to treat numerous contagious diseases including a variety of inflammatory conditions like boils, rheumatoid arthritis, gout, leprosy and other disorders[9]. This medicinal plant contains a variety of phytoconstituents, including certain chemical compounds such as giganteol, α and β calotropeol, β-amyrin, giganteol, and isogiganteol, as well as alkaloids, tannins, resins, flavonoids, terpenoids, and cardiac glycosides[10].

Vitex negundo Linn, that belongs to the Verbenaceae family is a large aromatic shrub (commonly known as Nirgundi, Five leaved chaste tree) and all the parts of this plant reported to possess great therapeutic value and is employed as a remedy in various traditional systems of medicine like Ayurveda, Chinese, Siddha and Unani to treat various diseases[10]. The water extract of fresh mature leaves is used in Ayurveda medicine as anti-inflammatory, analgesic and anti-itching agent internally and externally[11]. Phytochemical studies on *Vitex negundo* Linn revealed the presence of volatile oil, triterpenes, diterpenes, sesquiterpenes, lignan, flavonoids, flavones, glycosides, iridoid glycosides and stilbene derivative[12].

Although all these plants are separately well known for their antioxidant and anti-inflammatory potential, their combined effect has not been studied. Therefore, the current study was planned to determine the combined antioxidant and anti-inflammatory potential of the polyherbal extract using suitable in-vitro methods. The study also focused on the estimation of phenolic and flavonoid compounds and also to develop an HPTLC fingerprint pattern utilizing the individual plants and the combined extract. Lupeol, a pentacyclic triterpenoid, was selected as a marker compound for the HPTLC study. Lupeol is used to reduce the inflammatory responses and also have immunomodulating properties[13]. Lupeol content is reported in all the plants selected for study like *Calotropis gigantea*[14], *Vitex negundo*[15], *Ricinus communis*[16], *Tamarindus indica*[17].

Phytochemical evaluation serves as an important tool for quality assessment, encompassing preliminary phytochemical screening, chemoprofiling, and marker compound analysis through the application of modern analytical techniques. According to the World Health Organization (2000)'s guidance, the standardization through chromatographic fingerprint analysis using suitable marker compound has become an increasingly effective and essential process in the context of polyherbal formulations[18]. Analysis by HPTLC fingerprint method has become the most potent technique for quality control of herbal medicines owing to its simplicity, flexibility and reliability and also can serve as a tool for identification, authentication and quality control of herbal medicines. It plays an important role in the quality control of complex herbal medicines[19].

MATERIALS AND METHODS

Collection and identification of plant material

Leaves of *Tamarindus indica, Ricinus communis*, and *Calotropis gigantea* were collected from the Govt. Medical College campus, Thiruvananthapuram, Kerala, and the leaves of *Vitex negundo* were obtained from Ayurveda Research Institute, Poojappura, Thiruvananthapuram. The collected specimens were examined and authenticated by Christil Lila. R, Senior Research Officer (Botany), Pharmacognosy unit, Ayurveda Research Institute, Poojappura, Thiruvananthapuram with voucher specimen numbers SKCPRC/COG/HS 2024/001 (*Ricinus communis*), SKCPRC/COG/HS 2024/002 (*Tamarindus indica*), SKCPRC/COG/HS 2024/003 (*Vitex negundo*) and SKCPRC/COG/HS 2024/004 (*Calotropis gigantea*). The herbarium was deposited at College of Pharmaceutical Sciences, Govt. Medical College, Thiruvananthapuram for later reference.

Preparation of extracts

The leaves were chopped into small pieces after a thorough cleaning process, dried in the shade at room temperature and then ground to a fine powder in a mechanical blender. Dried leaf powder (30 g) was packed into a soxhlet apparatus

and extracted with 500 ml of ethanol (95%) at 60-65^{0}C for 4-6 hours. The extract was filtered, and the filtrate was concentrated by distillation. Each extract was then dried, weighed, and stored at 4^{0}C.

Chemicals and reagents

All the chemicals and solvents of standard analytical grade were used for the study. Quercetin, PBS (phosphate buffered saline), Diclofenac sodium and Lupeol were purchased from Sigma–Aldrich (Mumbai-India). Bovine serum albumin and Folin-Ciocalteu (F.C) reagent were collected from HI media laboratories Pvt. Ltd Mumbai. Aluminium chloride, sodium nitrite and sodium hydroxide were obtained from Nice chemicals PVT LTD, Kochi, Gallic acid and DPPH from Sisco research laboratories PVT LTD Mumbai.

Preliminary phytochemical screening

The phytoconstituents in the extracts were determined qualitatively according to standard procedures described previously[20]. Phytochemicals tested included alkaloids, flavonoids, saponins, tannins, glycosides and terpenoids.

Total phenol content

The total phenol content was determined using the Folin and Ciocalteu method, as described by Singleton and Rossi with slight modifications[21]. Gallic acid was used as a standard and was prepared in methanol. From the stock solution (1 mg/ml), working solutions of gallic acid in concentrations of 20, 40, 60, 80, and 100 μg/ml were prepared and transferred to tubing (each in triplicate) and made up to 1ml with distilled water. 1 ml of alcoholic extract of TI, CG, RC, and VN at a concentration of 1000 μg/ml was used for the estimation. The standard and samples were mixed well with 5ml Folin-Ciocalteu Reagent (1:10 dilution), kept for 5 minutes, then treated with 4 ml 20% Na_2CO_3 solution, and after 30 minutes, absorbance was measured against the blank at 760 nm. The total phenol content for each extract was derived from the gallic acid calibration curve and expressed as mg of gallic acid equivalent/gm of the ethanolic extract (mg GAE/gm).

Total flavonoid content

The total flavonoid content was determined according to the aluminium chloride method[22]. From the quercetin standard (stock) solution in methanol containing 1000 μg/ml, working solutions of quercetin containing 10, 25, 50, 100, and 250 μg/ml quercetin were prepared. The alcoholic extracts of TI, CG, RC, and VN were tested at 1000 μg/ml concentration. Add 4 ml of water and 0.3 ml of 5% sodium nitrite solution to the test tubes and mix thoroughly. After 5 minutes, 0.3 ml of 10% aluminium chloride solution was added, and at the 6th minute, 2 ml of 1M sodium hydroxide was added. The total volume of the contents was then made up to 10 ml with distilled water and once again mixed well, and the absorbance was measured against the blank at 510 nm. The blank was prepared without the addition of aluminium chloride solution. The total phenol content for each extract was derived from the calibration curve of standard quercetin and expressed as milligram quercetin equivalent per gram of ethanolic extract (mg QE/gm).

Preparation of mixtures

To create a uniform mixture, the four extracts were taken in different amounts and constantly agitated. Table 1 shows the amounts of extracts needed to make 1 g of the polyherbal mixture. The mixtures were developed on the basis of the results of phytochemical screening and quantitative analysis.

Table 1: Composition of different mixtures

Mixture	TI (mg)	RC (mg)	CG (mg)	VN (mg)
A	500	125	125	250
B	200	200	300	300
C	250	500	125	125

HPTLC analysis

HPTLC finger printing studies were performed according to the method of Wagner and Baldt[23] and Harbone[24]. For HPTLC profiling, a CAMAG HPTLC system was utilized, which included a Linomat 5 automatic applicator with a 100 μL syringe, a twin trough plate development chamber (20X10 cm), a Camag TLC scanner, and Win CATS planar chromatography manager software.

The HPTLC was performed on 9.0 × 10.0 cm precoated silica gel 60 F 254 HPTLC plate (E. MERCK KGaA). The sample solution was applied as bands to the plate by CAMAG Linomat applicator fitted with 100 μl syringe. Five microliters of the sample solution and 2 μl lupeol (1000 mg/l) were spotted as bands with bandwidth of 8mm. After the application of the spots, the chromatogram was developed in a twin trough glass chamber (20×10 cm) that had previously been saturated with the solvent system n-Hexane: Ethyl Acetate (80:20). The solvent system was chosen from the literature[25]. The plate was produced vertically rising to a height of 70.0 mm. A TLC scanner with winCATS Planar chromatography manager software was used for densitometric scanning. Images were taken in visible light and UV wavelengths of 366 nm and 254 nm while the plate was in the photo-documentation chamber. The Peak numbers were detected with the height, peak area and peak densitogram. Following scanning, the plates were post-derivatized by subjecting them to an anisaldehyde sulfuric acid reagent and then drying for 20 minutes at 120 ^{0}C in an oven. Following derivatization, the plates were examined at

visible light and UV under 366 nm and 254 nm.

Antioxidant activity

DPPH inhibition in the plant extracts and mixtures was determined by the method of Brand-Williams et al.,[26,27] with some modifications. The DPPH radical scavenging assay depends upon the colour change of 1-diphenyl-2-picryl hydrazyl (DPPH) radical, which is red in colour, to 2,2'-diphenyl-1-picryl hydrazine (yellow coloured) on scavenging. The decrease in absorbance thus occurs is directly proportional to the anti-oxidant capacity of the test substance. 3 ml of 0.1 mM solution of DPPH (4 mg DPPH in 100ml ethanol) was incubated with 1 ml each of 25, 50, 100, 200, and 400 μg/ml extract in ethanol. Quercetin similar concentrations was used as the standard. The control consisted of 3 ml DPPH and 1 ml ethanol only. The percentage inhibition was calculated by the formula,

$$Percentage\ inhibition = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} X100$$

Anti-inflammatory activity

Ethanolic extracts of TI, RC, CG, and VN, as well as the reference standard (Diclofenac sodium) and control (no standard/test sample), were prepared at various quantities (25, 50, 100, and 200 μg/mL) and added to 4 mL of phosphate buffer solution (0.2 M, pH 7.4). All the tubes were combined with 1 mL of a 1 mM albumin solution in phosphate buffer, and the tubes were then incubated for 15 minutes at 37^0C. The reaction mixture was maintained at 60°C in a water bath for 15 minutes in order to produce denaturation. The turbidity was measured at 660 nm after cooling[28].

The following formula was utilized to determine the percentage inhibition of denaturation.

$$Percentage\ inhibition = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} X100$$

Statistical analysis

Values were expressed as Mean ± SD. The statistical analysis was performed by one -way analysis of variance (ANOVA) followed by Tukey's test. The p-value < 0.05 was considered statistically significant when compared with the control. The statistical analysis was made with the Graph pad prism version 8.

RESULTS

Preliminary phytochemical screening

The preliminary phytochemical analysis showed that the ethanolic extracts of leaves of TI, RC, CG, and VN contained carbohydrates, alkaloids, tannins, flavonoids and phenols. The results of the phytochemical screening are shown in Table 2.

Table 2: Results of phytochemical screening

Phytochemicals	Test	TI	RC	CG	VN
Alkaloids	Mayers test	++	+	++	+++
	Wagner's test	+++	++	+	+++
	Hager's test	++	+++	++	++
	Dragondroff's test	+++	+++	++	+++
Carbohydrates	Molisch's test	+++	+	+	+
	Benedict's test	+	+	++	+
	Fehling's test	+++	+	+	+
	Iodine test	-	-	-	-
Tannins and phenolic compounds	Ferric chloride test	+++	+	+	+++
	Lead acetate test	+	+	++	++
Phytosterols	Libbermann-Burchard test	+	-	-	-
	Salkowski test	+	-	-	-
Saponins	Foam test	-	+	-	+
Proteins and amino acids	Biuret test	-	+	-	+
	Ninhydrin test	+++	+	-	+
Flavonoids	Shinoda test	++	+	++	+++
	Alkali test	+++	+	++	++

+++ = Highly present, ++ = Moderately present, + = Present

Total phenol content

The absorbance values obtained at different concentrations of gallic acid (20, 40, 60, 80 and 100 mg/ml) were used for constructing the calibration curve. The result is expressed as Gallic acid equivalents. The total phenolic content of the extracts was calculated from the regression equation of the calibration curve (y = 0.0104x + 0.0422, R^2 = 0.9985) and expressed as mg gallic acid equivalents (GAE) per gram of extract (mg/g). The results are given in Table 3.

Table 3: Results of total phenol and flavonoid content

Si. no	Material	Total phenol content (mg/gm GAE)	Total flavonoid content (mg/gm QE)
1	TI	86.06±1.8	169.46±0.88
2	RC	64.66±0.42	93.94±0.73
3	CG	33.40±1.00	187.56±1.35
4	VN	92.43±0.23	112.64±0.63
5	MX A	87.22±0.04	252.85±0.97
6	MX B	55.64±0.06	182.40±1.11
7	MX C	146.73±0.40	146.48±0.88

Values are expressed in Mean ± SD of triplicate readings

Total flavonoid content

The total Flavonoid content was determined by aluminium chloride colourimetric method and standard used was quercetin with concentrations of 10, 25, 50,100, 250 μg/ml. The total flavonoid content of the extracts and mixtures were estimated, and the results are shown in Table 3. The absorbance values obtained at different concentrations of quercetin were used for constructing the calibration curve. The result is expressed as quercetin equivalents. The total flavonoid content of the extracts was calculated from the regression equation of the calibration curve (y = 0.0008x + 0.0031, R^2 = 0.9984) and expressed as quercetin equivalents (QE) per gram of extract (mg/g). The total flavonoid content in the case of plant extracts was found to be CG > TI > VN > RC. Among mixtures MX A > MX B > MX C.

HPTLC analysis

For the HPTLC study, the ethanol extracts of TI, RC, CG, VN, MX C and standard lupeol was developed using the solvent system n-Hexane: Ethyl acetate (80:20) and visualized under white ray, UV 254 and 366 nm pre-derivatization and post derivatization with anisaldehyde sulfuric acid spray reagent (Figure 1). The Rf values and area percentage of different bands obtained post derivatization with anisaldehyde sulfuric acid and visualization under 366 nm are presented in Table 4. Figures 3 and 4 indicating the chromatogram and 3D overlay of densitogram. A number of 5 bands were observed in TI, CG, VN, MX C and 11 bands in RC. A spot for lupeol was obtained at Rf value of 0.48. Among extracts, the peak area for lupeol was maximum in CG. No spot of lupeol was seen in VN contrary to the literature reference. Seasonal variation in lupeol content has been reported in many plants[29,30]. The absence of spot may be due to seasonal variation. A common spot with Rf value 0.31 was observed in all the plant extracts and in the mixture when the spots are observed pre-derivatization under 366 nm, as shown in Figure 1 **(A)**.

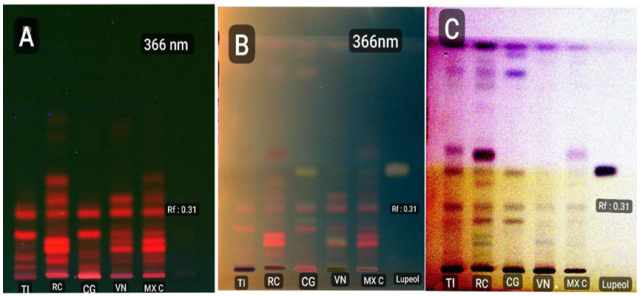

Fig. 1: HPTLC chromatogram of ethanolic extracts of TI, RC, CG, VN, MX C and lupeol (A) Visualization under 366 nm pre-derivatization (B) Visualization under 366 nm post derivatization with anisaldehyde and sulphuric acid spray reagent (C) Post derivatization and visualization under white ray

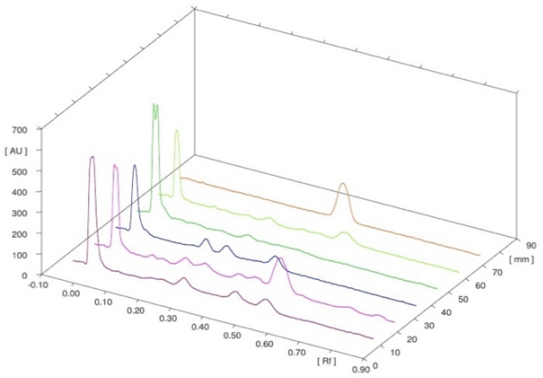

Fig. 2: 3-D overlay of densitogram of ethanolic extracts of TI, RC, CG, VN, MX C and lupeol

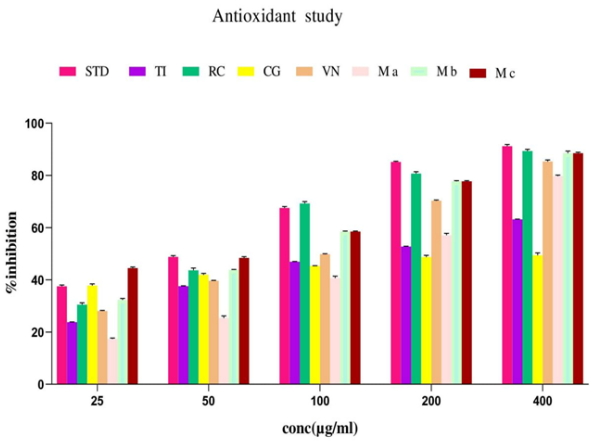

Fig. 3: DPPH radical scavenging activity of standard quercetin, ethanol extract of TI, RC, CG, VN and MX A, MXB and MXC [All values are represented as Mean ± SEM, n = 3. p<0.5, considered significant when compared to control group. Data were analyzed by using One-way ANOVA followed by Tukey's test. The analysis showed statistically significant difference with ***p< 0.001.]

Table 4: Results of HPTLC study- Data showing the number of peaks with Rf value and area percentage of plant extracts and lupeol post derivatization under 366 nm. Rf values of lupeol and common spot is highlighted

Peak	TI		RC		CG		VN		MX C		LUPEOL	
	Rf	A %	Rf	A %	Rf	A %	Rf	A%	Rf	A %	Rf	A%
1	0.05	62.44	0.05	32.88	0.02	51.76	0.01	43.35	0.02	59.45	0.48	100
2	0.19	2.03	0.13	1.67	0.19	2.58	0.04	45.52	0.12	1.90		
3	0.31	11.31	0.17	1.47	0.24	12.47	0.15	3.28	0.31	9.10		
4	0.48	12.72	0.24	6.71	0.31	14.03	0.22	2.25	0.48	3.64		
5	0.57	11.50	0.31	7.78	0.48	19.16	0.31	5.59	0.55	25.91		
6			0.40	5.59								
7			0.48	7.04								
8			0.57	31.45								
9			0.61	1.39								
10			0.76	2.37								
11			0.84	1.66								

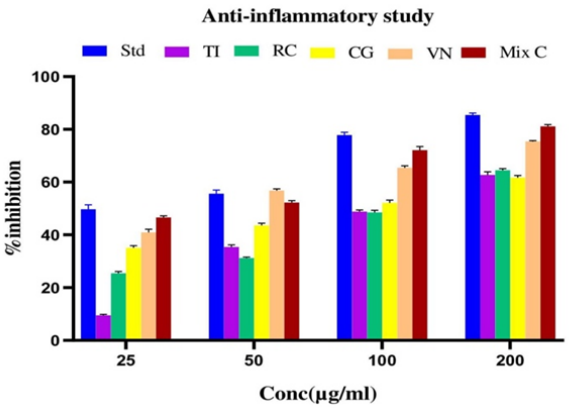

Fig. 4: Anti-inflammatory activity of standard Diclofenac sodium, ethanol extract of TI, RC, CG, VN and MX C [All values are represented as Mean ± SEM, n = 3. p<0.5, considered significant when compared to control group. Data were analyzed by using One-way ANOVA followed by Tukey's test. The analysis showed statistically significant difference with *** p< 0.001.]

Antioxidant activity

The antioxidant activity of TI, RC, CG, VN, MX A, MX B and MX C was determined by the DPPH radical scavenging assay. The percentage DPPH radical scavenging activity of the standard quercetin and the plant extracts at concentration of 25, 50,100, 200, 400 μg/ml was performed in triplicate and the results were expressed as mean ± standard deviation. The results are shown in Table 5, and Figure 3. IC_{50} values of TI, RC, CG, VN, MX A, MX B, MX C and standard quercetin was found to be 213.43, 65.26, 355.55, 123.78, 190.90, 83.28, 36.45 and 34.70 μg/ml, respectively. The anti-oxidant activity in the case of plant extracts followed the order RC > VN > TI > CG and in the case of mixtures, MX C > MX B > MX A.

Anti-inflammatory activity

Denaturation of tissue proteins may be the cause of the production of autoantigens in certain arthritic diseases. Therefore, tissue protein denaturation is a marker for inflammatory and arthritic diseases and agents that can prevent protein denaturation, therefore, would be possible candidates for anti-inflammatory drug development.

The anti-inflammatory activity of TI, RC, CG, VN and MX C was determined by the protein denaturation assay. The percentage denaturation of protein activity of standard diclofenac sodium and the plant extracts at concentrations of 25, 50,100 and 200μg/ml was performed in triplicate and the results were expressed as mean ± standard deviation. The results are shown in Table 6, and Figure 4. The IC_{50} values of standard diclofenac sodium, TI, RC, CG, VN, MX C and sodium was found to be 10.7, 134.7, 127.6, 16.29, 35.80, and 28.30 μg/ml, respectively. The anti-inflammatory activity in the case of plant extracts followed the order VN> RC > TI > CG.

DISCUSSION

Inflammation constitutes a physiological response of tissue to injury, infection, irritation, or the presence of foreign substances. Anti-inflammatory drugs are crucial due to their wide therapeutic potential and their utility in numerous diseases such as arthritis, lupus erythematosus, pemphigus, and rheumatic fever, as well as in other disorders associated with pain, fever, and inflammation. Individual herbs are insufficient to achieve the desired therapeutic effect. A composition of multiple herbs in a specific ratio, the therapeutic effect improves, while reducing toxicity[31].

The plant materials for the study, *Tamarindus indica, Ricinus communis, Calotropis gigantea* and *Vitex negundo*, were selected on the basis of their ethnomedicinal use in inflammatory conditions.

Table 5: Results of anti-oxidant activity by DPPH radical scavenging assay of plant extracts, mixtures and standard Quercetin

Conc(μg/ml)	Percentage inhibition (MEAN±SD)							
	Standard Quercetin	TI	RC	CG	VN	MX A	MX B	MX C
25	37.75±0.52	23.76±0.12	30.5±0.7	37.8±0.65	28.1±0.2	17.4±0.3	32.3±0.6	44.5±0.4
50	48.76±0.49	37.58±0.13	43.63±0.9	41.9±0.55	39.6±0.2	25.4±0.8	43.8±0.2	48.4±0.5
100	67.52±0.57	46.92±0.13	69.26±0.7	45.3±0.12	49.8±0.2	40.7±0.7	58.5±0.2	60.8±0.2
200	85.16±0.28	52.73±0.15	80.7±0.7	48.7±0.71	70.3±0.3	57.1±0.7	77.7±0.3	78.5±0.3
400	91.16±0.65	63.18±0.07	89.3±0.7	49.4±0.92	85.3±0.59	79.7±0.4	88.5±0.8	90.5±0.4

Values are expressed in Mean ± SD of triplicate readings

Table 6: Results of anti-inflammatory activity by protein denaturation assay of plant extracts, mixture C and standard Diclofenac

Con. (μg/ml)	Percentage inhibition					
	Standard	T I	R C	C G	V N	MX C
25	49.7±1.7	9.55±0.4	25.4±0.7	35.1±0.8	40.9±1.2	46.6±0.6
50	55.6±1.47	35.4±0.8	31.2±0.4	43.6±0.8	56.8±0.6	52.2±0.8
100	77.8±1.1	48.4±0.6	48.5±0.8	52.1±1.1	65.4±0.8	72.1±1.4
200	85.4±0.8	62.7±1.2	64.5±0.6	61.7±0.8	75.4±0.3	81.1±0.7

Values are expressed in Mean ± SD of triplicate readings

From the literature, the ethanolic extracts of these plants have potent antioxidant and anti-inflammatory property so ethanol was used as the solvent for extraction. The phytochemical screening of the extracts revealed the presence of flavonoids, phenolic compounds and tannins and alkaloids in all the plant extracts. Minute presence of phytosterols was detected in TI and saponins in RC and VN.

The plants and mixtures were studied for their phenolic compounds and flavonoids, which are important phytoconstituents in managing free radical generation and inflammation. The high content of these phytochemicals in the plants and extracts provided a preliminary understanding of the use of a polyherbal formulation developed from these plant extracts. The total phenolic content of the plant extracts followed the order VN > TI > RC > CG and in the case of mixtures, MX C > MX A > MX B. The total flavonoid content in the case of the plant extracts was found to be CG > TI > VN > RC, and among the mixtures the activity is in the order MX A > MX B > MX C.

Results from the antioxidant activity studies indicated that the plant extracts and the mixtures possessed significant antioxidant activity. The DPPH radical scavenging activity of the extracts and mixtures was compared with that of standard quercetin. The extract and mixtures showed a concentration dependent increase in activity. There was a noticeable increase in the percentage inhibition with the dose. The results of in vitro antioxidant and anti-inflammatory studies also supported the phytochemical investigations.

The plant extracts and Mixture C were subjected to in vitro anti-inflammatory activity by the protein denaturation assay method. It was also revealed that the plant extracts and the mixture possessed a significant anti-inflammatory activity. So, it can be concluded that the plant extracts in combination, can be utilized as a potential source of natural antioxidants in various antioxidant and anti-inflammatory formulations.

CONCLUSION

Inflammation is a condition associated with many diseases, and medicinal plants have been used since time immemorial for the effective management of inflammation and its related illness. The current study aimed to develop a herbal mixture suitable for incorporation into various polyherbal antioxidant and anti-inflammatory formulations using alcohol extracts of the leaves of *Tamarindus indica, Ricinus communis, Calotropis gigantea*, and *Vitex negundo*, all the plants are well recognised for their anti-inflammatory properties in traditional medicine.

Polyherbalism, employing synergism, offers benefits unattainable with single herbal formulations. Phenolic compounds and flavonoids in the plants and mixtures were considered as important phytoconstituents for managing free radical generation and inflammation. The high content of these phytochemicals in the plants and extracts provides a preliminary understanding of the utilization of a polyherbal formulation developed from these plant extracts. The results of in vitro antioxidant and anti-inflammatory studies also supported the phytochemical investigations. In essence, this study offers a comprehensive exploration of the phytochemical composition and potential therapeutic benefits of the examined medicinal plants and polyherbal mixtures. These findings validate the traditional uses of these plants and suggest promising avenues for further research in developing novel therapeutic agents. The study showed a

strong relationship between the anti-inflammatory effects of the plant extracts and their phenolic and flavonoid content, as well as their antioxidant properties. Specifically, mixture C had the highest phenolic contents, which correlated with superior antioxidant and anti-inflammatory activities compared to the other mixtures tested.

The complexity and variability of plant chemical constituents complicate the establishment of consistent quality and efficacy. High-Performance Thin-Layer Chromatography (HPTLC) of the plants and mixtures with lupeol as a standard compound was used to develop a reference profile to ensure the quality standard of both raw materials and finished products. However, further studies, including clinical trials, are required to fully elucidate their efficacy and safety for therapeutic applications, paving the way for their integration into modern medicine.

Abbreviations

CG- *Calotropis gigantea*, RC- *Ricinus communis*, TI- *Tamarindus indica*, VN- *Vitex negundo*, MX- Mixture, IC_{50}- Half maximal inhibitory concentration, DPPH- 2,2-Diphenyl-1-picrylhydrazyl, HPTLC- High performance thin layer chromatography

Acknowledgement

The authors would like to acknowledge Care Kerala Ltd, KINFRA Park, Koratty, Kerala, India for providing facilities to carry out the HPTLC study.

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