International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.5, No.3, pp 1142-1149, July-Sept 2013

Activity Guided Isolation Of Anti-Inflammatory Compound/Fraction From Root Of Ricinus communis Linn

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Abstract: In Indian system of medicines, root of *Ricinus communis* Linn. is used to treat inflammation and liver disorders. In present study, the ME of root was prepared by soxhlet method. ME was fractionated into four fractions; explicitly, chloroform, ethyl acetate, n-butanol, and remaining aqueous fraction. The n-butanol fraction, having showed significant inhibition of inflammation, was subjected to fractionation through successive column chromatography on silica gel. For the activity assessment, each fraction or sub-fraction was submitted to bioassay system i.e. carrageenan-induced hind paw edema model for anti-inflammatory activity. Purification of n-butanol fraction gave two active pure compounds (ricinine and quercetin) and one active impure fraction (SbFr.5). This study showed for the first time that ricinine (20mg/kg) reduced significant ($p<0.05$) carrageenan induced inflammation. Dose dependent significant ($p<0.05$) inhibition of paw edema was observed with SbFr.5 (10 mg/kg, 20 mg/kg and 30 mg/kg). SbFr-5 was found to be more potent than standard drug used. This study showed that ricinine, quercetin and SbFr.5 are responsible for anti-inflammatory activity of *Ricinus communis* roots. However, further purification of sub-fraction 5 is needed which may lead to discovery of novel anti-inflammatory compound.
Key Words : *Ricinus communis*, Anti-inflammatory, Ricinine, Isolation.

1. INTRODUCTION

Inflammation can be defined as a generalized, nonspecific but beneficial response of tissues to injury. The reversible features such as pain, redness, heat and swelling are joined by a fifth and less transient feature, namely, loss of function of involved organs (1). The clinical treatment of inflammatory diseases is dependent on nonsteroidal or steroidal chemical therapeutics (2). Nonsteroidal anti-inflammatory drugs (NSAID) reduce the pain and inflammation by blocking the metabolism of arachidonic acid by cyclooxygenase enzyme (COX), and thereby the production of prostaglandin. There are certain side-effects with use of NSAIDS, like gastro-intestinal ulcers, bleeding, and renal disorders, as these drugs nonslectively inhibit both isoforms of COX enzyme (3). There are also selective COX-2 inhibitors with reduced gastric problems and side-effects but these show adverse cardiovascular effects (4). Furthermore, the use of steroidal drugs as anti-inflammatory agents is also becoming highly controversial due to their multiple side effects (5,6). Therefore, there is a need to develop

substitutes for synthetic drugs and also to search and develop new anti-inflammatory initiatives from natural sources with potent activity and reduced adverse effects.

Plants have been used by human being since ages in traditional medicine due to their therapeutic potential and the search on medicinal plants have led the discovery of novel drug candidates used against diverse diseases. *Ricinus communis* is a plant commonly found in both the tropical and temperate climates of the world (7)**.** It is a member of family Euphorbiaceae, also known as castor oil bean and castor oil plant and is cultivated throughout India for the production of castor oil, which is extracted from its seeds. The use of different parts of this plant for the treatment of various diseases in traditional or folk remedies throughout the world has been reviewed (8). In the Indian system of medicine, the leaf, root and seed oil of this plant have been used for the treatment of inflammation and liver disorders (9). The plant have been found to be hepatoprotective (10), antifilarial (11), antioxidant (12), antiasthmatic (13) and antimicrobial (14). The root of this plant is also useful as an ingredient of various prescriptions for nervous diseases and rheumatic affections such as lumbago, pleurodynia and sciatica (15). Roots of this plant showed anti-inflammatory and free radical scavenging (16), antifertility (17), antidiabetic (18), and antimicrobial activity (19).

Aerial parts of this plant have been reported to have ricinine, N-demethylricinine, six flavonoid glycosides: kaempferol- 3-O-β-D-xyylopyranoside, kaempferol-3-O-β-D-glucopyranoside, quercetin-3-O- β-D-xylo pyranoside, quercetin-3-O- β-D-glucopyranoside, kaempferol-3-O-β-rutinoside, quercetin-3-O-β-rutinoside (20), as well as gallic acid, gentistic acid, quercetin, epicatechin, ellagic acid and rutin (12). Also, the seeds contain castor oil (21), ricin (23). Indole-3-acetic acid has been extracted from the roots (23). 3α-hydroxypentatriacont-14-en-26-one (ricipentatriacontanol), 3-O-benzyol-stigmast-5,22-dien-3-β,21-diol (ricinu steryl benzoate) and dipiperenoyl methyl ester methylene (ricipiperanyl ester) had also been isolated from root bark of castor bean (24).

Concerning the above-mentioned traditional uses, the current study was undertaken to assess anti-inflammatory effect of roots of *Ricinus communis* in order to verify the traditional utilization of the plant and to isolate the compound(s) responsible for its anti-inflammatory effects

2. MATERIAL AND METHODS

2.1 Plant material

Fresh roots of *Ricinus communis* Linn. were collected from I.S.F. College of Pharmacy, district Moga in August 2011. A voucher specimen (specimen number- 57486) has been deposited at the Herbarium of Punjabi University, Patiala, Punjab and specimen was examined and identified by Dr. V.K.Singhal.

2.2 Bioactivity guided fractionation and isolation

The freshly collected roots of *Ricinus communis* were washed with water, chopped, shade dried and coarsely powdered. The powder was defatted with petroleum ether (60–80^{0}C) and extracted with methanol using soxhlet extractor. The extract was dried under reduced pressure using a rotary evaporator at 40–45^{0}C. The percentage yield of the methanol extract was 6% (w/w). The crude methanol extract was suspended in distilled water (400ml) and partitioned by successive solvent extractions with chloroform (5×500 ml), ethyl acetate (5×500 ml) and n-butanol saturated with water (5×500 ml). Each extract was evaporated to dryness under reduced pressure to yield “CHLF Fr.” (7.220g), “EtOAc Fr.” (1.450g), “NBL Fr.” (8.710g) and remaining “Aqueous Fr.” (26.780g), successively.

NBL fraction (4.5g), the highest active fraction, was subjected to a silica gel (60–120 mesh size) column chromatography and eluted with the solvent systems comprising of toluene: dichloromethane (100:0, 80:20, 60:40, 40:60, 20:80, 0:100); dichloromethane: ethylacetate (100:0, 80:20, 60:40, 40:60, 20:80, 0:100) and with ethylacetate: methanol (100:0, 80:20, 60:40, 40:60, 20:80, 0:100) mixtures, total 350 fractions were collected. Following TLC monitoring, similar fractions were combined together and 5 major fractions i.e. SbFr-1 (24.7mg), SbFr-2 (43mg), SbFr-3 (340mg), SbFr-4 (28.8mg) and SbFr-5 (590mg) were obtained. Out of these subfractions, SbFr-2 showed single spot on TLC with R_f: 0.58 (Chloroform/methanol, 90:10). On crystallization, yellowish brown colored compound-RC-I was obtained from SbFr-2 and Compound-RC-II was obtained as yellow colored crystals from SbFr-4.

2.3 Phytochemical screening

The methanol extract and its various fractions and subfractions were subjected to preliminary phytochemical screening for the detection of various phytoconstituents viz. alkaloids, steroids, terpenoids, anthraquinone glycosides, flavonoids, tannins and phenolic compounds, saponins, carbohydrates, proteins and amino acids (25, 26).

2.4 Structure elucidation of isolated compounds

Structure elucidation of the RC-I and RC-II from the NBL fraction was carried out by spectral techniques; UV, ^{1}H-NMR, ^{13}C-NMR and MS. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer (chemical shift in ppm).

2.5 Animals

Wistar rats weighing 180–220 g of either sex obtained from Animal House, ISF College of Pharmacy, Moga, were used. The animals were fed with standard rodent pellet diet and water *ad libitum* and were housed in rooms maintained at 25±1^{0}C with a 12 h light/dark cycle following international recommendations. The animals were randomly divided into 12 groups with 6 animals in each group. All studies were performed in accordance with the guidelines for the care and use of laboratory animals, as adopted and promulgated by the Institutional Animal Care Committee, CPCSEA, India.

2.6 Preparation of test samples and dose estimation

Test samples were administered orally after suspending in 0.5% sodium carboxymethyl cellulose (CMC) suspension in distilled water. The control group animals received only vehicle. The dose of the fractions of ME and subfractions obtained by chromatographical processing were estimated based on their ratio in the corresponding fractions or subfractions. Diclofenac sodium (20mg/kg *p.o.*) was used as standard drug.

2.7 Anti-inflammatory activity

Anti-inflammatory activity was determined in Wistar rats of either sex using carrageenan induced rat paw edema method (27).The right hind paw edema was induced by sub-planter injection of 0.1ml of 1% carrageenan solution in saline. The animals were pretreated with test samples or standard one hour before the induction of inflammation. Paw volume was measured by displacement of the mercury column in a plethysmometer before and after 1hr, 2hr, 3hr, 4hr and 24hr of carrageenan injection. Reduction in the paw volume compared to the vehicle-treated control animals was considered as anti-inflammatory response. Percent inhibition paw volume was calculated using the formula-

$$\%\text{inhibition} = (1 - Vt/Vc)*100$$

Where, Vt represents mean increase in paw volume after treatment with test and standard drug and VC represents mean increase in paw volume of control group.

2.8 Statistical analysis

All values were expressed as mean ± SD. The data from animal experiments were statistically analyzed using two way ANOVA followed by Bonferroni test and $p<0.05$ was considered to be significant.

3. RESULTS AND DISCUSSION

The anti-inflammatory property of the methanol extract of *R. communis* roots has been reported earlier but no report was available for its active fraction/compound(s). Hence, the present study was performed to confirm its anti-inflammatory potential and successive isolation of bio-active fraction responsible for the same to facilitate search of novel plant based anti-inflammatory lead molecules.

Successive fractionation of ME gave four fractions, out of which NBL Fr was found to be most active (Table 1). On subsequent fractionation of NBL Fr by column chromatography, five sub-fractions were obtained which on further purification gave two pure compounds (RC-I and RC-II). On the basis of spectral analysis and comparison with reported data, RC-I and RC-II (Figure 1) were characterized as ricinine and quercetin. The NBL Fr was found to contain phenolics and flavonoids as major phytoconstituents, which were reported to have antioxidant and anti-inflammatory properties (28, 29, 30). Therefore, the NBL Fr of ME of *R. communis* had shown significant ($p<0.05$) anti-inflammatory activity in carrageenan induced paw edema animal model.

Table 1. Effect of fractions of ME of *Ricinus communis* roots against carrageenan induced hind paw edema in rats

S.no.	Group	Dose (mg/kg p.o.)	Time				
			Mean change in paw volume (ml) ± S.D. (%inhibition)				
			1hr	2hr	3hr	4hr	24hr
1	Carrageenan control	-	0.300±0.06	0.516±0.04	0.650±0.05	0.750±0.08	0.4160±0.07
2	Diclofenac sodium	20	0.175±0.06* (43.33)	0.233±0.08** (54.90)	0.250±0.05** (61.53)	0.225±0.09** (70.00)	0.200±1.0** (51.21)
3	CHLF Fr	71.00	0.250±0.05 (28.00)	0.416±0.04* (32.18)	0.466±0.08** (38.47)	0.400±0.06** (48.88)	0.266±0.05** (36.30)
4	EtOAc Fr	14.05	0.266±0.05 (11.33)	0.408±0.04* (20.93)	0.483±0.04** (25.69)	0.525±0.04** (30.00)	0.358 ±0.06 (13.94)
5	NBL Fr	86.20	0.216 ±0.04 (30.06)	0.333±0.05** (36.05)	0.383±0.07** (41.03)	0.366±0.08** (51.10)	0.233±0.05** (43.99)
6	Aqueous Fr	314.05	0.250±0.05 (16.67)	0.383±0.04* (25.77)	0.533±0.05** (32.10)	0.633±0.05** (27.80)	0.333±0.05 (19.99)

*p < 0.05 - significant from the control value. **p < 0.001 - significant from the control value.

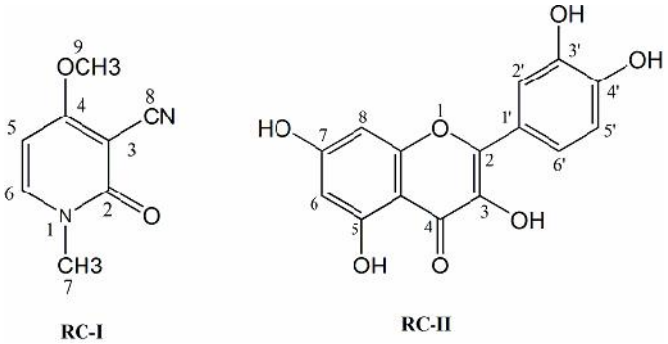

Figure 1: Structure of compound RC-I and RC-II

Compound, RC-I, was obtained as yellowish brown crystals having melting point: 200-202^{0}C, from SbFr-2 by process of crystallization. Positive qualitative test for nitrogen as detected by Lassaigne method confirmed the presence of elemental nitrogen. The IR spectrum data also revealed the absorption bands characteristics of cyano group (2222 cm^{-1}), amide group (1630 and 1637 cm^{-1}) and aromatic ring (1537 cm^{-1}). The molecular formula, $C_8H_8O_2N_2$ of this compound was determined by ESI-MS spectrum with [M+H] at m/z 165 and [M+Na] at m/z 187. RC-I when subjected to ^{1}H NMR, exhibited two single-proton downfield doublets at δ 7.92 and 6.24 ppm corresponding to H-6 and H-5, respectively. Also, two three-proton upfield singlets at δ 3.95 and 3.44 ppm were obtained (Table 3). Due to amide group, a singlet peak was obtained at δ 3.44 ppm, which was slightly upfield, indicating the presence N-CH_3 group. The other singlet corresponds to the –OCH_3 group which was present at 4th position. In ^{13}C-NMR spectrum of RC-I, the amide carbon appeared at δ 172.25 (C-2), N-CH_3 (C-7) carbon appeared very much upfield at δ 36.68 ppm. Peaks at δ 160.84, 114.00, 86.50 and 57.05 ppm correspond to C-4, C-3, C-7 and C-9, respectively. Peaks at δ 145.15 and δ 93.32 ppm represented to C-6 and C-5 respectively, as confirmed by DEPT NMR. From the above data, RC-I was identified as 4-methoxy-1-methyl-2-oxo-1,2-dihydro-pyridine-3-carbonitrile which was reported as ricinine (31).

Table 2. Effect of sub-fractions/compound of n-butanol fraction of ME of *Ricinus communis* roots against carrageenan induced hind paw edema in rats

S.no.	Group	Dose (mg/kg p.o.)	Time				
			Mean change in paw volume (ml) ± S.D. (%inhibition)				
			1hr	2hr	3hr	4hr	24hr
1	Carrageenan control	-	0.300±0.06	0.516±0.04	0.650±0.05	0.750±0.08	0.4160±0.07
2	Diclofenac sodium	20	0.175±0.06* (43.33)	0.233±0.08** (54.90)	0.250±0.05** (61.53)	0.225±0.09** (70.00)	0.200±1.0** (51.21)
3	SbFr-3	6.9	0.225±0.06 (25.00)	0.325±0.04** (37.01)	0.383±0.06** (41.07)	0.483±0.05** (35.60)	0.316±0.04* (24.03)
4	SbFr-5	10	0.200±0.08* (33.33)	0.300±0.05** (41.86)	0.333±0.05** (48.76)	0.35±0.05** (53.33)	0.250±0.05** (39.90)
5	SbFr-5	20	0.183±0.05* (40.00)	0.266±0.02** (49.01)	0.300±0.05** (53.84)	0.316±0.02** (58.66)	0.233±0.02** (43.99)
6	SbFr-5	30	0.166±0.02** (46.66)	0.216±0.02** (58.82)	0.233±0.05** (64.61)	0.216±0.02** (72.00)	0.200±0.05** (51.92)
7	RC-I	10	0.233±0.07 (23.33)	0.316±0.07** (39.21)	0.383±0.07** (41.53)	0.433±0.07** (42.66)	0.333±0.07 (19.99)
8	RC-I	20	0.183±0.02* (39.00)	0.300±0.05** (41.46)	0.366±0.05** (44.61)	0.383±0.05** (48.93)	0.300±0.05* (27.28)

*p < 0.05 - significant from the control value. **p < 0.001 - significant from the control value.

Table 3. ^{1}H and ^{13}C-NMR chemical shifts for RC-I and RC-II in DMSO-d6 at 400MHz

Assignment		RC-I		RC-II	
^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
	C2		172.25		146.19
	C3		114.00		135.59
	C4		160.84		175.49
H5	C5	6.24 (d)	93.32		160.66
H6	C6	7.92 (d)	145.15	6.18 (d)	98.09
H7	C7	3.44 (s)	36.68		163.65
H8	C8		86.50	6.36 (d)	93.13
H9	C9	3.95 (s)	57.07		156.11
	C10				102.94
	C1’				122.11
H2’	C2’			7.74 (d)	114.85
	C3’				144.69
	C4’				147.22
H5’	C5’			6.88 (d)	115.20
H6’	C6’			7.57 (dd)	119.87

Table 4: UV spectra of RC-II

Solvent/Reagent	λ_{max} (nm)		λ_{max} (nm) Shift		Interpretation
	Band I	Band II	Band I	Band II	
MeOH	373	255	_	_	Flavanol (free 3-OH)
MeOH-$AlCl_3$	432	266	+59	+11	Presence of 3-OH (with or without 5-OH)
MeOH-$AlCl_3$/HCl	428	265	+55	+10	Presence of 5-OH

Compound, RC-II, was obtained as yellow crystals. The UV spectrum (Table 4) in methanol gave absorbtion maxima at 373 nm (band I) and 255 nm (band II) indicated the flavone moiety with free hydroxyl group at C-3. The 59 nm bathochromic shift (373-432 nm) of band I after adding $AlCl_3$ indicated the presence of 3-OH with or without 5-OH. After addition of $AlCl_3$/HCl, the batochromic shift of band I by 55 nm (373-428 nm) confirmed the presence of 5-OH group. The ^{1}H-NMR spectrum showed protons at aromatic regions from 6- 8 ppm, and strong hydrogen bonding at δ 12.32 ppm (Table 3). These spectral observations suggested a quercetin nucleus. In the ^{13}C-NMR spectrum, the most downfield signal appeared at δ 175.49 ppm, was characteristic of carbonyl carbon (C-4). The four downfield signals at δ 163.65, 160.66, 144.64 and 147.22 ppm were assigned to C-7, C-5, C-3' and C-4' bearing hydroxyl group, respectively. The C-3 signal appeared at δ 135.59 ppm. The appearance of signals typical of C-6 and C-8 at δ 98.04 and 93.13 ppm further confirmed presence of hydroxyl group at C-5 and C-7 position in the ring A. The resonance of C-2', 5' and 6' at δ 114.85, 115.20 and 119.87 ppm further supported that hydroxylation of ring B at position 3' and 4'. The signals belong to C-2, C-9, C-10 and C-1' were also observed at 146.19, 156.11, 102.94 and 122.11 ppm, respectively. The ^{13}C-NMR spectra revealed 15 carbon signals typical of flavonoid nucleus. Thus the compound was identified as quercetin. This was further confirmed with characteristic peaks of quercetin as reported earlier (32, 33).

Quercetin has already been reported to have anti-inflammatory activity (34). Hence, it supports the anti-inflammatory activity of n-butanol fraction of ME of *R. communis*. Ricinine, on biological evaluation also has shown the anti-inflammatory activity with 48.93% maximum inhibition of paw volume at 4th hr in comparison to control untreated group of animals (Table 2). Present study reports isolation and anti-inflammatory potential of ricinine from roots of *R. communis* for the very first time.

Among the sub-fractions, SbFr-5 has shown the most potent anti-inflammatory activity against carrageenan challenged animals. The activity was dose dependent and at 30 mg/kg significant ($p<0.001$) inhibition (72.00% at 4th hr) of increase in paw volume in comparison to untreated group was observed. The percentage inhibition was at par with inhibition caused by standard (Diclofenac sodium 20 mg/kg, 70%). This indicated presence of other phenolic compounds, which are more active than quercetin, ricinine and the standard drug in SbFr-5. However, SbFr-5 could not be further purified. TLC profile of SbFr-5 has shown the presence of four spots in toluene:ethylacetate:formic acid (50:40:10 v/v/v) solvent system, one of them was quercetin but in minor quantity. Hence, the major responsible compound for the anti-inflammatory property of *R.communis* is not only quercetin and ricinine, but there is mixture of other phenolic compounds present in SbFr.5.

The hind paw edema which follows intraplantar carrageenan injection involves a complex and time-dependent synthesis/ release of a plethora of different inflammatory mediators. It is believed to be a biphasic event. The early phase (1–2 h) is mediated by the release of histamine, bradykinin, serotonin and nitric oxide, while the late phase (3–5 h) is the result of the release of kinins and mainly prostaglandins (35, 36, 37, 38). Late phase of inflammation is also due to the formation of pro-inflammatory prostanoids and nitric oxide (synthesized by the inducible nitric oxide synthase isoform) (39). NSAID are known to reduce carrageenan-induced hind paw edema in rat (40). Most reports suggest that these drugs preferentially inhibit the 'late' phase response presumably by inhibiting the inducible COX-2 isoform which is believed to be responsible for the generation of pro-inflammatory prostanoids in the later stages of inflammation (41).

It seems likely that the 'late' phase inhibitory effect of NBL Fr, SbFr-5 and ricinine depends upon inhibition of hind paw prostanoids formation presumably by an effect on COX-2 activity, the predominant isoform at this stage in the response (42). Interestingly, oral administration of NBL Fr, SbFr-5 and RC-I also reduced the 'early' phase response. Showing that the anti-inflammatory effects were due to inhibition of synthesis of various inflammatory mediators of 'early' and 'late' phase.

4. CONCLUSION

The present investigation revealed that NBL Fr, SbFr-5, ricinine and quercetin of ME of *R. communis* roots showed anti-inflammatory activity against carrageenan induced paw edema in rats. Among tested fractions and compounds, SbFr-5 seems to be very potent since its effect is at par with Diclofenac sodium. So, there is a scope to explore this highly active fraction which may result in a new lead molecule for drug discovery.

5. ACKNOWLEDGMENT

We express our sincere thanks to Management and Shri Parveen Garg, Honarable Chairman, ISF College of Pharmacy, Moga, India, for providing necessary facilities and financial support for this study. We also express our gratitude to Dr. Ashish Baldi, Principal, ISF College of Pharmacy, Moga, India, for his valuable healthy criticism.

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