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Original Article

In Vitro and *In Vivo* Anti-Inflammatory Evaluation of the Whole Plant Extracts of *Crotalaria Biflora* (L)

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ABSTRACT

Background: Several species of *Crotalaria* are cultivated and consumed across the world by the rural population for a variety of purposes that include medicine, food, green manure, fodder etc. **Objective:** The present study was aimed to evaluate the anti-inflammatory activity of different extracts of the whole plant *Crotalaria biflora* by *in vitro* and *in vivo* methods. **Materials and Methods:** The powdered material of the whole plant *Crotalaria biflora* was extracted by soxhelation with different solvents such as petroleum ether, chloroform, ethyl acetate and methanol. Stabilization of human red blood cell (HRBC) membrane is the method employed for the *in vitro* evaluation. The extracts selected based on the results of *in vitro* evaluation was further subjected to *in vivo* evaluation by carrageenan-induced rat paw oedema method. In both *in vitro* and *in vivo* evaluations, Indomethacin was used as the standard control. **Results:** Among the four tested extracts in six different concentrations subjected to the *in vitro* evaluation, the ethyl acetate and methanol extracts (1000µg/ml) showed significant activity which was selected for the *in vivo* evaluation. Among the two doses of extracts (200mg.kg^{-1} and 400mg.kg^{-1}) selected for the *in vivo* evaluation, the methanol extract (400mg.kg^{-}1) showed significant activity followed by the ethyl acetate extract in the same dose. **Conclusion:** Further studies on the phytochemicals present in these extracts may give more significant results

Key words: *Crotalaria biflora* extracts, *In vitro* anti-inflammatory evaluation, *In vivo* anti-inflammatory evaluation.

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INTRODUCTION

The first documented data of genus *Crotalaria* was done by Carolus Linnaeus in the year 1753. He described 13 species belong to this genus in his Species Plantarum. They are *C. albanand* L., *C. juncea* L., *C. lunaris* L., *C. laburnifolia* L., *C. lotifolia* L., *C. micans* L., *Crotalaria perfoliata* L., *C. quinquefolia* L., *C. sagitalis* L., *C. triflora* L., *C. villosa* L., *C. verrucosa* L. Thereafter the number of species within this genus increased and now it reaches of about 702 species in global distribution mainly in tropical Africa and Madagascar and secondary radiations in rests of the world.[1-3] The genus *Crotalaria* is very diverse and extensively distributed which includes perennial aquatic herbs, erect herbs and shrubs, terrestrial creepers as well as trees can be found in diverse habitats viz., open places, forest and grasslands, plains and hill regions. In India *Crotalaria* constitute largest legume genera represented by 116 taxa in which 52 are endemic to the country with the maximum species concentration in the peninsular region (73 species restricted to the peninsular region).[2] Several species of *Crotalaria* are cultivated and consumed across the world by the rural population for variety of purposes that include medicine, food, green manure, fodder etc.[4] The plant *Crotalaria biflora*, one among them commonly found in south India at an altitude up to 300m mean sea level, the seed of this plant is edible and consumed by rural population because of its protein content.[5,6] In our previous study,[7] the whole plant of *Crotalaria biflora* was collected from the Mekkarai, the village located near the foothills of Western Ghats, Tirunelveli District, Tamil Nadu, India. The collected material was subjected to extraction, preliminary phytochemical evaluation and spectral characterization. The present study was aimed to evaluate the *in vitro* and *in vivo* anti-inflammatory activity of the extracts of the whole plant *Crotalaria biflora*.

MATERIALS AND METHODS

Collection and extraction of plant material

The whole plant of *Crotalaria biflora* was collected from the Mekkarai, a village close to the foothills of the Western Ghats in Tirunelveli District of Tamil Nadu, India. The collected plant material was identified, authenticated and subjected to shade drying and powdering by mechanical grinding. The coarse powder thus obtained was extracted by soxhelation using the solvents such as petroleum ether, chloroform, ethyl acetate, and methanol.[7] The dried extracts thus obtained were used for the evaluation of anti-inflammatory activity.

In vitro evaluation of anti-inflammatory activity

Anti-inflammatory activity of all the prepared extracts was evaluated *in vitro* by stabilization of human red blood cell (HRBC) membrane in normal and different temperature and tonicity conditions in reference to the previous literature[8-11]. All assays were done in triplicate and the results were expressed as mean ± standard deviation.

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HRBC membrane stabilization method

An equal volume of freshly collected whole human blood from the healthy volunteers and the sterile Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, 0.42% sodium chloride/100ml distilled water) was mixed and subjected to centrifugation (3000rpm for 10min). The packed cells thus obtained was washed and reconstituted as 10%v/v suspension by using sterile isosaline (0.85% NaCl in H_2O sterilized by autoclaving). 1ml of HRBC suspension and 1ml of each test extracts in different concentration (100, 200, 400, 600, 800, 1000µg/ml) was taken in the individual tubes. Normal (HRBC suspension and Alsever solution only) and standard control (Indomethacin 100µg/ml instead of test extracts) were prepared. All the tubes were incubated for 30min at 37°C, followed by subjected to centrifugation. The supernatant was collected and its haemoglobin content was estimated spectrophotometrically (560nm) and the percentage haemolysis and protection were calculated by

$$\text{Percentage of haemolysis} = \frac{\text{OD of test}}{\text{OD of control}} \times 100$$

$$\text{Percentage of protection} = 100 - \frac{\text{OD of test}}{\text{OD of control}} \times 100$$

OD of test–Optical density of tested extracts; OD of control–Optical density of normal control

Temperature-induced haemolysis

Test extracts dissolved in isotonic phosphate buffer solution was used for the experiments. The reaction mixture contains 5ml of test extracts in different concentration (100, 200, 400, 600, 800, 1000µg/ml) and 0.1ml of 10%v/v HRBC suspension. Normal control contains saline and the standard control contains 100µg/ml of Indomethacin instead of test extracts. A set of prepared tubes was kept at 54°C for 20min. in a regulated water bath. Another set of the tube was kept at −10°C for 20min in a freezer. Then, all the tubes were centrifuged (3000rpm for 3min), supernatant was collected and the haemoglobin content was estimated spectrophotometrically (540nm) and from this the percentage inhibition of haemolysis by the tests was calculated by

$$\text{Percentage inhibition of haemolysis} = 1 - \frac{OD.2 - OD.1}{OD.3 - OD.1} \times 100$$

OD.1–Optical density of tests cooled; OD.2–Optical density of tests heated; OD.3–Optical density of normal control heated

Tonicity induced haemolysis

Test extracts dissolved in hypotonic (0.2% sodium chloride) and isotonic solution (0.9% sodium chloride) were used for the experiments. Both hypotonic and isotonic reaction mixture contains 5ml of test extracts in different concentration (100, 200, 400, 600, 800, 1000µg/ml) and 0.1ml of 10%v/v HRBC suspension. Normal control contains distilled water and the standard control contains Indomethacin 100µg/ml instead of test extracts. All the prepared tubes were kept in incubation at 37°C for 1hr and then centrifuged at 3000rpm for 3min. The supernatant was collected and its haemoglobin content was estimated spectrophotometrically (540nm) and the percentage inhibition of haemolysis was calculated by

$$\text{Percentage inhibition of haemolysis} = 1 - \frac{OD.2 - OD.1}{OD.3 - OD.1} \times 100$$

OD.1–Optical density of tests in isotonic solution; OD.2–Optical density of tests in hypotonic solution; OD.3–Optical density of normal control in hypotonic solution

In vivo evaluation of anti-inflammatory activity

Based on the results of *in vitro* evaluation, the extracts selected were subjected to *in vivo* evaluation in the experimental animals with paw oedema induced by carrageenan. The evaluation was done in reference to the previous literature.[12-14]

Healthy young adult male Albino Wistar rats of about 180–200g received from the Central animal house of Cape Bio Lab & Research Centre, Marthandam, Kanyakumari, Tamil Nadu, India, were used for the study which was approved by the institutional animal ethics committee (CBLRC/IAEC/01/01-2020). All the animal experiments were done by the guidelines of a committee for the purpose of control and supervision on experiments on animals (OECD 423). The animals selected were housed in appropriate temperature (25±1°C), humidity (55±3%) and 12h light/dark cycle and fed with commercial diet and water *ad libitum*.

Experimental animals were divided into 7 groups of 6 each. Group I is normal control administered with saline (5ml.kg^{-1}). Group II is inflammatory control treated with carrageenan. Group III is standard control treated with Indomethacin (10mg.kg^{-1}). Group IV and V are treatment control received the ethyl acetate extract in the dose of 200 and 400mg.kg^{-1} respectively. Group VI and VII are the treatment control received methanol extract in 200 and 400mg.kg^{-1} respectively. Thirty minutes before the administration of standard drug/test extracts, 0.1ml of 1%w/v inj. carrageenan in saline (0.1ml) was administered in the sub-plantar region in the right hind paw of the animals of the entire group except normal control. The standard drug and the test extracts were administered to the animals of entire group by intraperitoneal injection except for normal and inflammatory control.

The paw volume of the injected animal was started to measure from zero hours by plethysmometrically and continued at the interval of one hour for five hours. The percentage inhibition of inflammation was calculated by

$$\text{Percentage inhibition of inflammation} = \frac{Vc - Vt}{Vc} \times 100$$

Where, Vc–mean increase in paw volume in a control group of rats; Vt–mean increase in paw volume in rats treated with test extracts

The results were expressed in mean ± SEM (Standard Error Mean) 6 experimental animals in each group. Statistical significance was assessed by ANOVA and Dunnett's test. P-values <.05 were considered significant.

RESULTS AND DISCUSSION

Table 1 shows the results of HRBC membrane stabilization assay. From the results, it was observed that the tested extracts showed a concentration-dependent rise of activity which was clearly indicated by the ethyl acetate extract (1000µg/ml) with the percentage protection of 74.11±0.26, followed by the methanol extract in the same concentration revealed percentage protection of 66.20±1.65

Stabilization of HRBC membrane in different temperature viz., heat (54°C) and cold (−10°C) conditions are presented in Table 2. The results showed a concentration-dependent rise in activity of tested extracts. From the results it was found that the methanol extract (1000µg/ml) in cold condition showed a maximum activity with the score of 78.80±1.40 percentage inhibition of haemolysis. Next to that the ethyl acetate extract in heated condition revealed a percentage inhibition of 75.20±1.30 and followed by methanol extract with the score of 64.30 ± 1.70 percentage inhibition of haemolysis. The standard drug Indomethacin (100µg/ml) showed 65.20±0.75 and 68.30±1.80 percentage inhibition of haemolysis in heated and cold condition respectively.

The results of HRBC membrane stabilization in different tonicity viz., hypotonic and isotonic condition was presented in Table 3. In this evaluation also a concentration-dependent rise of activity of extracts was observed. Maximum activity was found in ethyl acetate extract (1000µg/ml) in isotonic condition with the percentage inhibition

Table 1: Effect of test extracts on the protection of HRBC membrane.

Concentration (µg/ml)	% Protection			
	Pet. Ether	Chloroform	Ethyl acetate	Methanol
100	05.31 ± 0.28	07.45 ± 0.92	11.54 ± 0.72	8.36 ± 1.56
200	08.89 ± 0.58	11.36 ± 1.01	15.20 ± 0.14	22.51 ± 2.07
400	15.67 ± 0.67	16.52 ± 0.53	36.58 ± 1.55	31.25 ± 2.56
600	28.69 ± 1.12	35.21 ± 2.32	51.49 ± 1.95	43.23 ± 1.58
800	41.26 ± 1.76	48.29 ± 1.90	66.59 ± 0.78	63.72 ± 0.87
1000	52.22 ± 1.30	55.30 ± 1.50	74.11 ± 0.26	66.20 ± 1.65
100 (Indomethacin)	71.62 ± 1.32			

Table 2: Effect of test extracts on the protection of cell membrane in different temperature condition.

Con. (µg/ml)	% inhibition of haemolysis							
	Heat (54°C)				Cold (–10°C)			
Normal control	29.50 ± 1.52				31.32 ± 0.90			
Extracts	1	2	3	4	1	2	3	4
100	18.85 ± 0.75	21.26 ± 0.59	39.00 ± 1.60	17.27 ± 1.10	18.58 ± 0.78	20.30 ± 0.60	40.50 ± 1.10	35.50 ± 0.30
200	24.69 ± 0.85	27.57 ± 0.51	41.22 ± 0.90	26.50 ± 1.50	27.52 ± 0.88	28.81 ± 0.70	43.70 ± 1.30	42.70 ± 0.50
400	30.50 ± 1.20	33.21 ± 0.75	49.70 ± 0.83	37.80 ± 2.00	36.10 ± 0.71	34.64 ± 1.20	47.10 ± 0.80	51.90 ± 0.85
600	36.58 ± 1.90	39.20 ± 1.70	59.40 ± 1.45	45.10 ± 1.80	42.20 ± 0.10	39.59 ± 1.12	51.60 ± 0.50	63.20 ± 0.40
800	42.20 ± 1.52	44.28 ± 2.20	71.60 ± 0.70	56.42 ± 2.30	48.60 ± 0.11	44.40 ± 1.32	56.70 ± 0.70	69.60 ± 1.20
1000	47.85 ± 1.00	57.60 ± 2.08	75.20 ± 1.30	64.30 ± 1.70	51.50 ± 1.11	52.28 ± 1.15	61.50 ± 1.10	78.80 ± 1.40
100 (Std.)	65.20 ± 0.75				68.30 ± 1.80			

Con. – Concentration; Std. – Standard (Indomethacin); 1 – Petroleum ether; 2 – Chloroform; 3 – Ethyl acetate; 4 – Methanol

Table 3: Effect of test extracts on the protection of cell membrane in different tonicity condition.

Con. (µg/ml)	% inhibition of haemolysis							
	Hypotonic solution				Isotonic solution			
Normal control	08.30 ± 0.32				06.25 ± 0.80			
Extracts	1	2	3	4	1	2	3	4
100	07.32 ± 0.15	10.60 ± 0.61	10.06 ± 0.90	10.80 ± 0.20	10.86 ± 0.87	12.03 ± 0.40	18.50 ± 0.30	17.10 ± 0.50
200	12.19 ± 0.59	15.50 ± 0.21	31.40 ± 0.60	20.30 ± 0.10	16.32 ± 0.79	19.19 ± 0.20	27.30 ± 0.50	20.80 ± 0.80
400	17.30 ± 0.30	18.12 ± 0.55	32.60 ± 0.40	27.50 ± 0.70	20.10 ± 0.61	22.60 ± 0.20	34.40 ± 0.70	26.40 ± 0.70
600	21.20 ± 0.20	22.20 ± 0.80	43.40 ± 0.03	28.30 ± 0.80	24.20 ± 0.15	26.20 ± 0.12	44.50 ± 0.60	33.20 ± 0.80
800	25.50 ± 0.72	24.20 ± 1.23	50.20 ± 0.40	36.10 ± 0.60	28.50 ± 0.15	29.26 ± 0.24	47.70 ± 0.40	37.50 ± 0.60
1000	27.25 ± 0.70	25.10 ± 1.81	57.10 ± 0.60	51.40 ± 0.30	31.30 ± 0.11	32.81 ± 0.32	73.10 ± 0.90	46.30 ± 0.90
100 (Std.)	72.50 ± 0.70				86.8 ± 0.50			

Con. – Concentration; Std. – Standard (Indomethacin); 1 – Petroleum ether; 2 – Chloroform; 3 – Ethyl acetate; 4 – Methanol

of haemolysis of 73.10±0.90. But the same extract showed a score of 57.10±0.60 percentage inhibition of haemolysis in hypotonic condition. Next to that the methanol extract showed significant results. But it showed a maximum activity in hypotonic condition with the percentage inhibition of haemolysis of 51.40±0.30. In the isotonic condition, the same extract showed a less score of 46.30± 0.90 comparing with hypotonic of it.

From the results of *in vitro* anti-inflammatory evaluation, it was found that the ethyl acetate and methanol extract of *C. biflora* showed significant activity, particularly the ethyl acetate extract. It was also confirmed that the concentration of the extracts, the differences in the environmental conditions such as temperature and tonicity plays an important role in the findings. It is well known that normally the cell membrane plays an important role in the viability of cell. Exposure of red blood cells to diverse adverse conditions such as heat, cold and hypotonicity, induces free radicals formation which in turn activates the lipid peroxidation that makes the cell heavily susceptible to secondary damage and the released inflammatory mediators elevate the cell membrane permeability that causes leakage of serum proteins and fluids into the cells. But it can be prevented by the stabilization of cell membrane. In this study, the ethyl acetate and methanol extracts of *C. biflora* revealed a significant RBC membrane stabilization comparing with the standard drug Indomethacin. It may be due to the blocking of release of inflammatory mediators and lytic enzymes. A previous literature[15] reported that the saponins and flavonoids possess a significant effect on lysosomal membrane stabilization in both *in vitro* and *in vivo* evaluation. Our study on the phytochemical evaluation of the extracts of *C. biflora* revealed the presence saponins and flavonoids in the ethyl acetate and methanol extract[7] which may be responsible for their activity in the *in vitro* evaluation.

Based on the results of *in vitro* evaluation, the ethyl acetate and methanol extracts were selected for the *in vivo* evaluation in the paw oedema induced by administering the carrageenan to the experimental animals. The results showed that in case of Group I animals (normal control), there was no significant difference between the initial paw volume and the paw volume in each hour of observation. But, in case of Group II (inflammatory control) animals, the paw volume was in elevation in each hour of observation. The first-hour observation of Group III (standard control) animals revealed a slight elevation of paw volume from its initial level. However in subsequent observations, it was reduced constantly and ended in the minimum in 5th hour (last) observation. Similar results were found in the animals received ethyl

Table 4: Effect of *Crotalaria biflora* extracts in paw volume in carrageenan-induced paw oedema in rat.

Group	Paw volume (ml)					
	0h	1h	2h	3h	4h	5h
I	0.40 ± 0.25	0.57 ± 0.14	0.54 ± 0.13	0.52 ± 0.05	0.51 ± 0.07	0.46 ± 0.06
II	0.49 ± 0.02	0.54 ± 0.03	0.69 ± 0.03	0.71 ± 0.02	0.70 ± 0.02	0.68 ± 0.01
III	0.49 ± 0.01	0.52 ± 0.02	0.50 ± 0.02	0.48 ± 0.03	0.43 ± 0.02	0.42 ± 0.02
IV	0.46 ± 0.02	0.51 ± 0.01	0.48 ± 0.02**	0.43 ± 0.02**	0.42 ± 0.02**	0.40 ± 0.01**
V	0.45 ± 0.02	0.53 ± 0.02	0.50 ± 0.02**	0.49 ± 0.02***	0.47 ± 0.02***	0.41 ± 0.01***
VI	0.45 ± 0.09	0.48 ± 0.11	0.46 ± 0.12***	0.44 ± 0.18***	0.41 ± 0.15***	0.38 ± 0.21***
VII	0.47 ± 0.08	0.50 ± 0.06	0.48 ± 0.02***	0.46 ± 0.03***	0.45 ± 0.04***	0.43 ± 0.01***

Group I–Normal control; Group II–Inflammatory control; Group III–Standard control; Group IV–Ethyl acetate extract 200mg.kg^{-1}; Group V–Ethyl acetate extract 400mg.kg^{-1}; Group VI–Methanol extract 200mg.kg^{-1}; Group VII–Methanol extract 400mg.kg^{-1}

Table 5: Percentage inhibition of inflammation produced by *Crotalaria biflora* extracts and standard drug in carrageenan-induced paw oedema in rat.

Group	% inhibition					
	0h	1h	2h	3h	4h	5h
III	3.71 ± 0.15	1.85 ± 0.09	27.30 ± 1.70	33.25 ± 1.26	38.90 ± 1.91	41.28 ± 2.58
IV	5.31 ± 1.40	6.52 ± 1.81	28.65 ± 2.82***	37.18 ± 0.34***	38.95 ± 1.58***	36.41 ± 1.35***
V	7.45 ± 0.36	2.48 ± 0.76	25.96 ± 1.80***	32.86 ± 1.50***	33.12 ± 1.20***	36.65 ± 1.33***
VI	4.5 ± 1.64	6.70 ± 1.53	27.24 ± 1.36***	34.30 ± 1.77***	34.56 ± 1.28***	35.71 ± 2.72***
VII	7.15 ± 0.60	10.35 ± 1.48	30.74 ± 1.18***	40.31 ± 0.45***	42.29 ± 1.06***	39.59 ± 1.35***

Group III–Standard control; Group IV–Ethyl acetate extract 200mg.kg^{-1}; Group V–Ethyl acetate extract 400mg.kg^{-1}; Group VI–Methanol extract 200mg.kg^{-1}; Group VII–Methanol extract 400mg.kg^{-1}

acetate and methanol extracts (Group IV-VII). From the results, it was found that both methanol and ethyl acetate extracts in the dose of 400mg.kg^{-1} showed significant activity comparing with the standard control drug, Indomethacin. (Tables 4 & 5).

All values are expressed as mean ± SEM for 6 animals in each group; *P<0.05, **P<0.01 compared

CONCLUSION

In the present study, the whole plant of *Crotalaria biflora* was collected. After identification and authentication, the collected material was dried, powdered and extracted by soxhelation with solvents such as petroleum ether, chloroform, ethyl acetate, and methanol. All the dried extracts thus obtained were evaluated for the anti-inflammatory activity by *in vitro* and *in vivo* methods. Based on the results of *in vitro* evaluation, the ethyl acetate and methanol extracts were selected for the *in vivo* evaluation. In this evaluation, comparing with the standard drug, the methanol extract showed a significant activity followed by the ethyl acetate extract. A detailed study on the phytochemicals present in these extracts in the future may give more significant results.

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CONFLICTS OF INTEREST

None.

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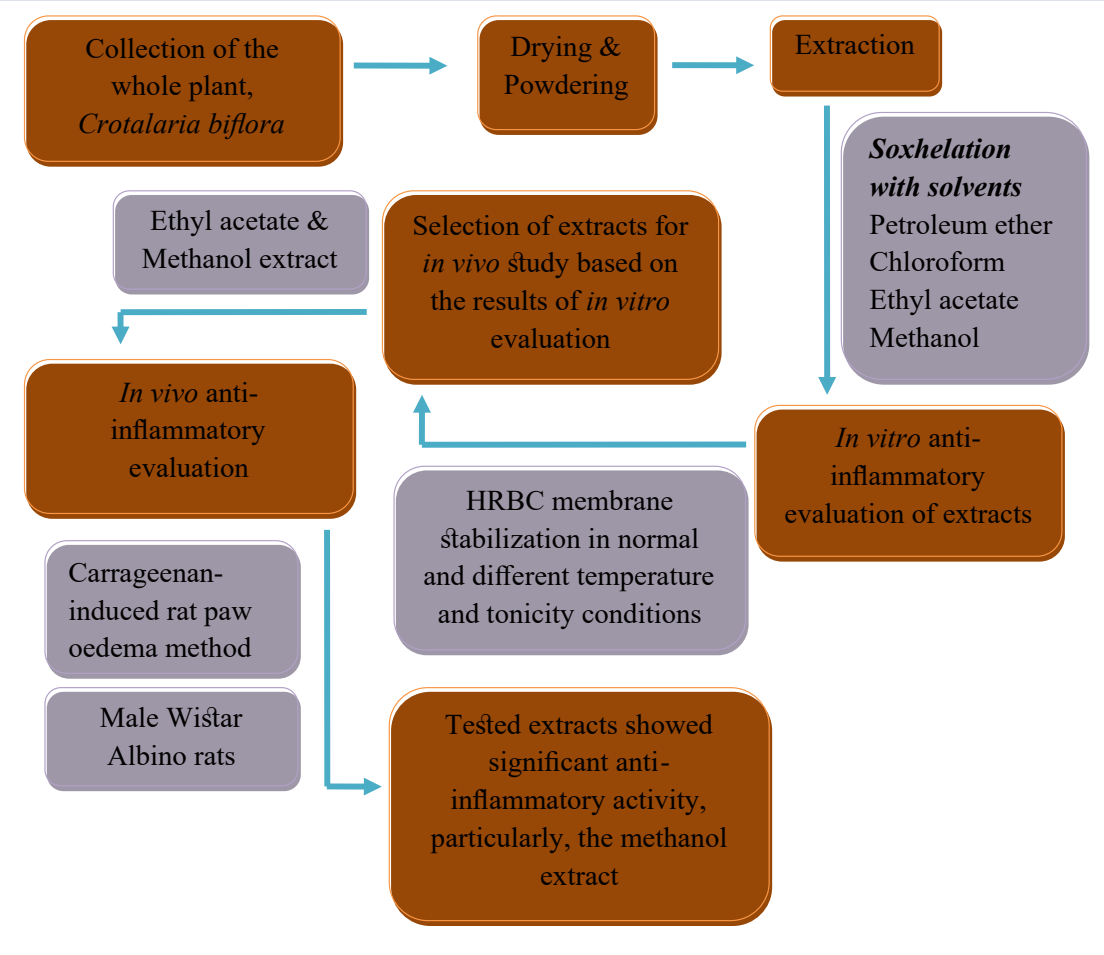
GRAPHICAL ABSTRACT



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