Anti-Inflammatory Approach of Cissus Quadrangularis Linn. By In-Vitro Means

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Abstract

Background: As the modern era has marked the discovery of various synthetic and semi synthetic chemical entities for aliment of diseases, so are they associated with numerous side effects. To overcome this severe side effects after chronic use and to us the synergistic combined effect of the various Phyto ingredients of the respective drug, the extracts of the crude drugs are made to use.

Objective: The objective of the study was to in vitro analyze anti-inflammatory property of *C. quadrangularis* leaves using by in-vitro three assays viz. Membrane stabilization test (Blood Serum). Inhibition of albumin denaturation (egg albumin). Inhibition of protein denaturation (bovine serum albumin).

Results: The extracts of *C. quadrangularis* is estimated The above assays simulate the exact inflammatory physiological conditions associated with arthritis and other inflammatory conditions, and hence the results shown by it can be applicable to the anti-inflammatory activity of the drug. The anti-inflammatory in-vitro assays namely Membrane stabilization test (Blood Serum), Inhibition of albumin denaturation (egg albumin) and Inhibition of protein denaturation (bovine serum albumin) were performed and showed 88%, 84% and 82% inhibition of inflammation rate respectively when compared with diclofenac as standard on invitro basis.

Conclusion: All this established a high level of guarantee that Cissus Quadrangularis an effectively control inflammation associated with various diseases or disorders and hence can serve as promising drug in the treatment of the same.

Keywords: In-vitro, semi-synthetic, *C. quadrangularis Linn*, membrane stabilization, egg albumin, bovine serum albumin, pharmacogenetic profile.

INTRODUCTION

Inflammation

Inflammation (lat. inflammare, to set on fire) is a complex biological reaction of vascular tissue to various harmful stimuli such as pathogens, damaged cells or irritants. Inflammation is a protective approach by the tissue to remove the harmful stimuli and initiate the healing process. Inflammation is a stereotyped response and is therefore considered an innate immunity mechanism, as opposed to adaptive immunity, which is specific to each pathogen.[1] Without inflammation,

wounds and infections would never heal. However, chronic inflammation can also lead to a variety of diseases, such as hay fever, atherosclerosis, rheumatoid arthritis and even cancer (e.g., gallbladder carcinoma). As such, inflammation is normally tightly regulated by the body.[2] Inflammation is classified as acute or chronic. Acute inflammation is the body's first response to noxious stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissue. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vasculature, the immune system, and various cells within the injured tissue.[3] Persistent inflammation, known as chronic inflammation, results in a progressive change in the cell type present at the site of inflammation and is characterized by simultaneous tissue destruction and healing by the inflammatory process.

***Cissus quadrangularis Linn.*[4,5]**

A climber in the family Vitaceae, is one of the most frequently used medicinal plants in Thailand and can be found throughout the country. The fresh stem and leave of C. quadrangularis are used for the treatment of hemorrhoid, menstrual disorder, scurvy and as anti-flatulence. Phytochemical studies of C. quadrangularis found several phytochemical constituents such as flavonoids, stilbene derivatives and many others, e.g., resveratrol, piceatannol, pallidol perthenocissin and phytosterols. Pharmacological studies revealed the bone fracture healing property and anti-osteoporotic effect of this plant. *C. quadrangularis* in hemorrhoid patients were very effective in the treatment of hemorrhoidal pain and inflammation as well as reducing the size of hemorrhoids. [6]

MATERIALS AND METHODS

Preparation of the extract

The pulverised and shadow dried powder of aerial parts of *C. quadrangularis* is procured from local market medicinal product Himalaya Hadjod. 10 g of plant powder were weighed and was extracted with 80 ml of petroleum ether ,80 ml of methanol ,60 ml distilled water and 20 ml of chloroform each separately using Soxhlet apparatus with temperature maintained for extraction was 55^0C. The duration of Soxhlet extraction was 3 to 4 hours. The extracts were concentrated by pouring them into clean round bottom flask and were allowed for evaporation of solvents by using distillation apparatus. Then the concentrated crude extract was stored at 40^0C in airtight bottle until further use.[7,8]

Phytochemical analysis

The methanolic and aqueous extract of *Cissus Quadrangularis* is subjected to various phytochemical analytical tests to establish the presence of different phytoconstituents present in the extract and this will help us to confirm the role of them in pharmacological activity. The result of various phytochemical analytical tests is depicted in Table 1.[9,10]

In-vitro Anti-inflammatory activity of different Extracts of *C. quadrangularis*

Membrane stabilization test

Preparation of Red Blood cells (RBCs) suspension

The principle involved here is stabilization of human red blood cell membrane by hypo tonicity induced membrane lysis.

The assay mixture contains 1.0 ml phosphate buffer (pH 7.4, 0.15 M), 2 ml hypo saline (0.25 % w/v) sodium chloride, 0.5 ml HRBC suspension (10 % v/v) with 1 ml of plant extracts of various concentrations (1.0- 6.0 mg/ml) in test solution/ 1 ml standard drug diclofenac (2.5 mg/ml) in Standard solution/ 1 ml isotonic saline in control solution were incubated at 370 C for 30 min and at the end of the incubation, the tubes were cooled under running tap water.[11] The reaction mixture was centrifuged at 3000 rpm for 20 min. The haemoglobin content in the suspension was estimated using spectrophotometer at 560 nm. The percentage hemolysis produced in the presence of isotonic saline was taken as 100 %. Percentage of HRBC membrane stabilization or protection was calculated using the following equation:[12,13]

$$\% \text{ Inhibition of haemolysis} = \frac{A1 - A2}{A1} \times 100 \quad \textbf{(1)}$$

Where:

A1 = Absorption of hypotonic buffered saline solution alone

A2 = Absorption of test sample in hypotonic solution

All extract of *C. quadrangularis* were investigated for *In-vitro* Anti-inflammatory activity by human red blood cell membrane stabilization method. Four different concentrations of extracts: 1mg/ml, 2mg/ml, 4mg/ml and 6mg/ml were used for anti-inflammatory study.[14,15]

Assay of Membrane stabilizing activity

The HRBC membrane stabilizing activity assay was carried out as reported by Saddique et al., 1989; Oyedepo et al., 2004 using 10% (v/v) Human erythrocyte suspension while Diclofenac was used as standard drugs. The assay mixtures consisted of 2 ml of hyposaline (0.25% w/v) sodium chloride, 1.0 ml of 0.15 M sodium phosphate buffer, pH 7.4, 0.5 ml of 10% (v/v) human erythrocyte suspension, 1.0 ml of drugs (standard and extracts) and final reaction mixtures were made up to 4.5 ml with isosaline.[16] To determine the anti-inflammatory activity by HRBC membrane stabilization method, the following solutions were prepared.[17]

Inhibition of protein denaturation

When BSA is heated and shows denaturation, it expresses antigens associated with a type III hypersensitivity reaction and this can be considered representative of diseases such as serum sickness, glomerulonephritis, rheumatoid arthritis and systemic lupus erythematous. Thus, the proposed assay can be applied to the investigation of drugs to treat the diseases mentioned above and others once the compounds stabilize the denaturation process.[18] This method is used to evaluate the antidenaturation or anti-inflammatory effects of natural products, as its anti-inflammatory properties are simple and inexpensive. The reaction mixture consisted of test extract in different concentrations and 1% aqueous solution of bovine albumin fraction. The pH of the reaction mixture was adjusted using a small amount of 1N HCl. The samples were incubated at 37°C for 20 minutes and then heated at 57°C for 20 minutes. After the samples had cooled, the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows:[18,19]

$$\text{Percentage inhibition} = \frac{(\text{Abs. control} - \text{Abs. sample}) \text{ X } 100}{\text{Abs. control}} \quad \textbf{(2)}$$

Inhibition of protein denaturation (bovine serum albumin)

Denaturation of tissue protein is one of the well-documented causes of inflammatory and arthritic diseases. Production of the autoantigen in certain arthritic diseases may be due to denaturation of protein *in vitro*. Agents that can prevent protein denaturation, therefore, could be worthwhile for anti-arthritis drug development. Some literatures stated that protein denaturation and macroglobulin formation cause the proteins to become antigenic, thus initiating the immune response and producing biochemical changes in connective tissue, which ultimately leads to rheumatoid arthritis.[19]

Inhibition of albumin denaturation (egg albumin)[19,20]

Test solution

5 ml of test solution consists of 0.2 ml of egg albumin and 2.8 ml of phosphate buffer saline and 2 ml of in various concentrations of extracts (100, 200, 300, 400, and 500 µg/ml).[19]

Test control solution

5 ml of test control solution consists of 0.2 ml of egg albumin and 2.8 ml of phosphate buffered saline and 2 ml of distilled water.[21]

Standard solution

5 ml of standard solution consists of 0.2 ml of egg albumin and 2.8 ml of phosphate buffer saline and diclofenac 100 µg/ml. The pH of the above solutions was adjusted to 6.4 using a small amount of 1N HCl. The samples were incubated at 37°C for 20 min and heated at 70°C for 5 min denaturation's, and the results were compared with standard diclofenac sodium. After cooling, their absorbance was measured at 660 nm using pure blank. Diclofenac sodium (standard drug) was used as reference drug and treated as such for the determination of absorbance.[22]

Statistical analysis

All data were analyzed statistically using Graphpad prism V. 8.3. The descriptive data were expressed as mean ± standard error of mean. The percentage of inhibition rate between different groups was analyzed by independent sample t-test. The differences were considered to be statistically significant when $P < 0.05$.[23]

RESULTS AND DISCUSSION

Phytochemical analysis

Table 1: Phytochemical Analysis of the methanolic plant extract *cissus quadrangularis*

Sr. No	Phytochemical constituents	Test	Observation	
			Methanolic Extract	Aqueous Extract
1	Flavonoid	Ferric chloride test	Violet colour	Violet colour
2	Protein - Amino Acids	Million's reagent Ninhydrin Test	red colour purple colour	red colour purple colour
3	Tannin	Ferric chloride test Lead acetate test	Violet colour	Violet colour

4	Alkaloid	Dragendorff's test Mayer's reagent	Reddish brown ppt Reddish brown ppt	Reddish brown ppt Reddish brown ppt
5	Phenol	Ferric chloride test Lead acetate test	Dark green colour	 ------
6	Saponins	Foam test Lead acetate test	White precipitate	-------
7	Carbohydrates	Molisch's test	brownish ring at the junction of two liquids	brownish ring at the junction of two liquids

Analytical isolation and confirmation of constituents

Thin Layer Chromatography

The methanol extract was subjected to thin layer chromatography. Number of solvent systems was tried. The solvent system which shows good resolution was used. The visualization of spot was done by exposing the plate to iodine vapour using the solvent system Benzene: Methanol: Ammonia (9:0.6:0.4). Thin Layer chromatography (TLC) To support phytochemical screening, the methanol extract was subjected to thin layer chromatography.

(A) **(B)** **(C)**

Figure 1: TLC of the plant extract with different solvent. (A)- TLC separation of flavonoids: with derivatization, viewed under UV light at 254 nm. (B)- TLC separation of flavonoids: before derivatization. (C)- TLC separation of flavonoids: after derivatization with confirmatory spray agent 3% boric acid + 10% oxalic acid.

In-vitro Anti-inflammatory activity of different Extracts of *C. quadrangularis*

All extract of *C. quadrangularis* were investigated for *In-vitro* Anti-inflammatory activity by human red blood cell membrane stabilization method. Four different concentrations of extracts: 1mg/ml, 2mg/ml, 4mg/ml and 6mg/ml were used for anti-inflammatory study. The results of the membrane stabilization

assay of methanolic extract shown in Table 2.

Table 2: Percentage inhibition for HRBC assay.

Concentration	AECQ	MECQ
1000µg/ml	24.6938±0.96	29.33252±0.42
2000µg/ml	41.5136±0.36	43.30986±0.39
4000µg/ml	71.5197±0.45	79.53154±0.75
6000µg/ml	80.2052±0.85	88.16595±0.29

*Values are expressed as mean±SD (n= 3), AECQ- Aqueous extract of *cissus quadrangularis*, MECQ- Methanolic extract of *cissus quadrangularis*.

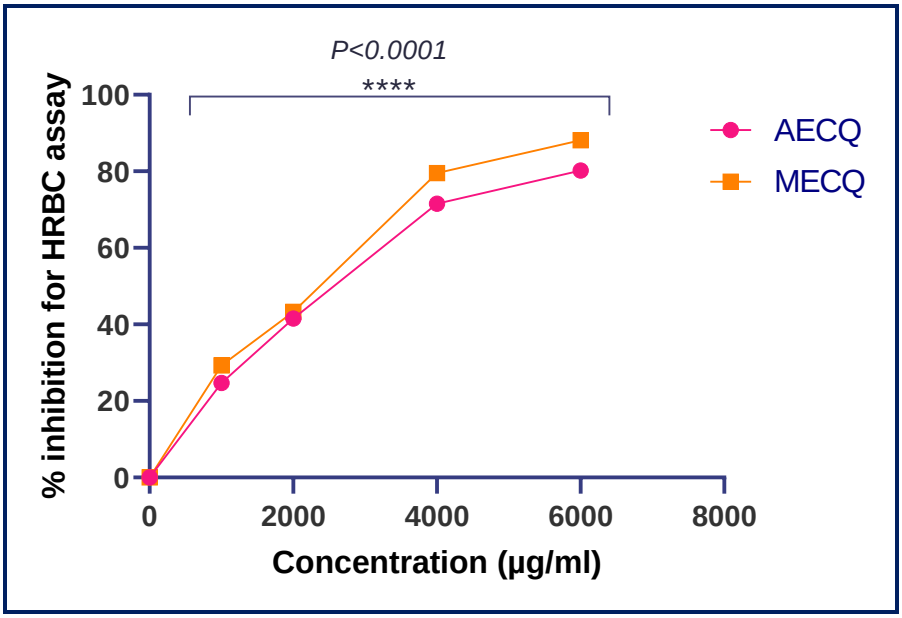

Figure 2: Percentage inhibition for HRBC assay, AECQ- Aqueous extract of cissus quadrangularis, MECQ- Methanolic extract of cissus quadrangularis.

Inhibition of Protein denaturation

When BSA is heated and it is showing denaturation, it expresses antigens associated to Type III hypersensitive reaction and this can be considered as representative of diseases such as serum sickness, glomerulonephritis, rheumatoid arthritis and systemic lupus erythematous. The reading and calculation of the percentage inhibition for BSA assay of aqueous and methanolic extract is as shown below.

Table 3: Anti-inflammatory activity of *Cissus Quadrangularis* methanolic and aqueous extract by Bovine serum method.

Sr.no	Concentration of herbal extracts	% Inhibition by Bovine serum method	
		AECQ	MECQ
1	100µg/ml	19.9541±0.26	35.63±0.16
2	200µg/ml	31.4±0.48	55.5±0.85
3	300µg/ml	44.97±0.18	64.86±.96

4	400µg/ml	60.14±0.48	73.81±0.15
5	500µg/ml	74.11±0.26	84.11±0.73

* Values are expressed as mean±SD (n= 3), AECQ- Aqueous extract of *cissus quadrangularis*, MECQ- Methanolic extract of *cissus quadrangularis*.

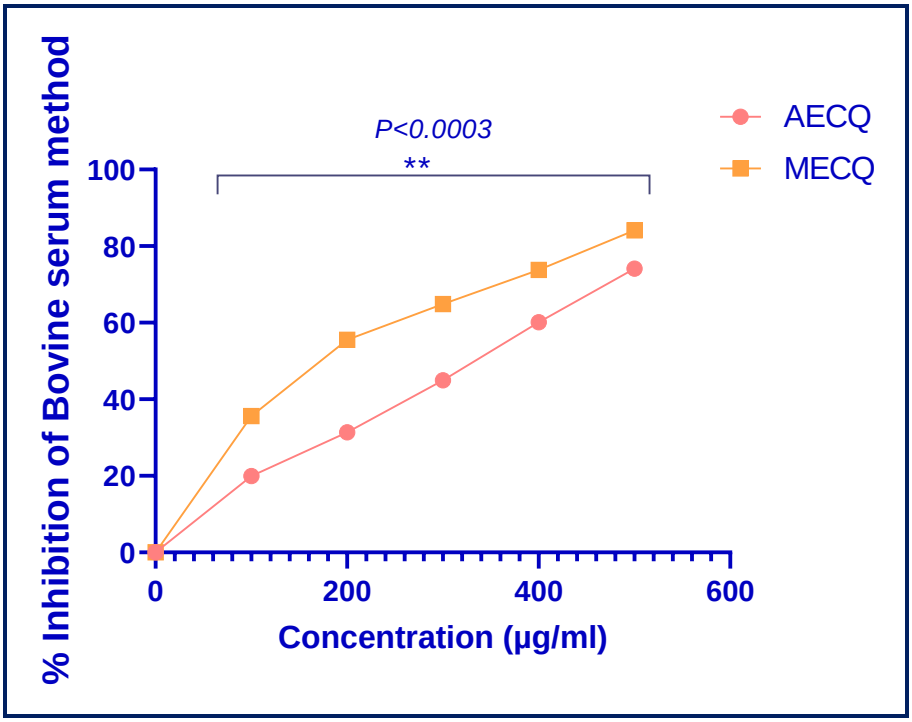

Figure 3: % Inhibition by Bovine serum method, AECQ- Aqueous extract of cissus quadrangularis, MECQ- Methanolic extract of cissus quadrangularis.

Inhibition of albumin denaturation (egg albumin)

After cooling, their absorbance was measured at 660 nm using pure blank. Diclofenac sodium (standard drug) was used as reference drug and treated as such for the determination of absorbance. The percentage inhibition of protein denaturation was calculated as follows.

Table 4: Anti-inflammatory activity of Cissus Quadrangularis methanolic and aqueous extract by Egg albumin method.

Sr.no	Concentration of herbal extracts	% Inhibition by Egg albumin method	
		AECQ	MECQ
1	100µg/ml	8.27±0.48	16.47±0.24
2	200µg/ml	14.11±0.26	21.17±0.73
3	300µg/ml	20.52±0.95	34.11±0.83
4	400µg/ml	36.74±0.34	40.29±0.65
5	500µg/ml	62.35±0.49	82.35±0.91ssss

* Values are expressed as mean±SD (n= 3), AECQ- Aqueous extract of *cissus quadrangularis*, MECQ- Methanolic extract of *cissus quadrangularis*.

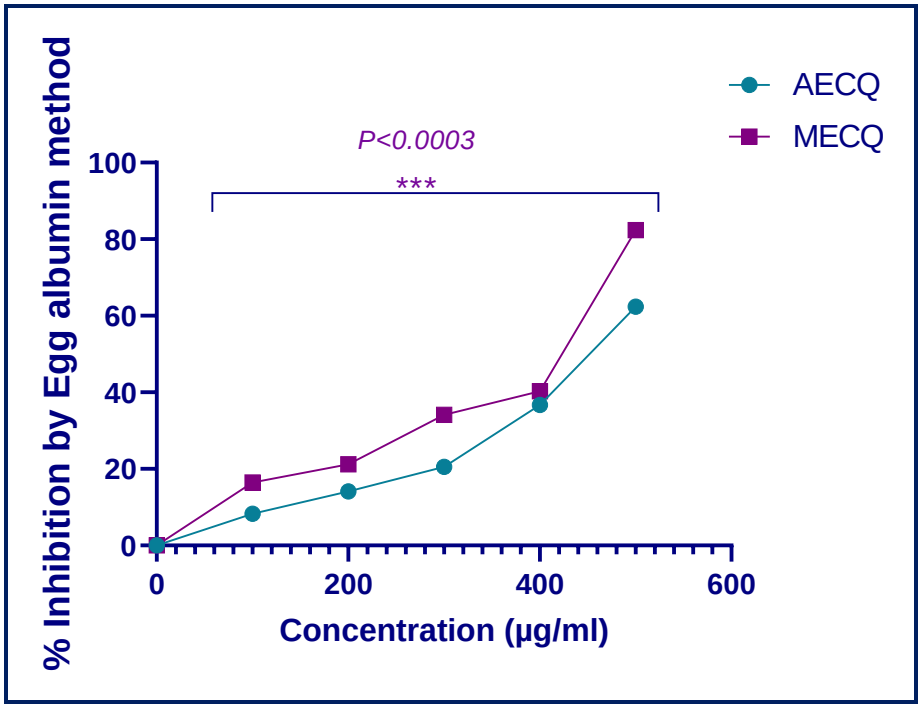

Figure 4: % Inhibition by Egg albumin method, AECQ- Aqueous extract of cissus quadrangularis, MECQ- Methanolic extract of cissus quadrangularis

DISCUSSION

The ethanolic and aqueous extracts of *Cissus Quadrangularis* is prepared from the hot Soxhlet extraction of the prior dried powder of all ariel parts of *Cissus Quadrangularis,* this extract is dried by vacuum evaporation and the obtained powder is utilized at all the steps for the preparation of various aliquots for the assays.

The phytochemical analysis of the methanolic and aqueous extract of *Cissus Quadrangularis* established the presence of different phytoconstituents present in the extract on qualitative basis and confirmed the presence of Flavonoid , Protein - Amino Acids , Tannin, Alkaloid, Phenol , Saponins and Carbohydrates as shown in Table 2.All these constituents were present on ample quantity as the results were very prominent and all of them have reported anti-inflammatory activity in isolated state .

Thin Layer Chromatography of the methanol extract conducted with various solvent systems and simultaneously visualization of spot with Rf value 0.16, 0.23, 0.32, 0.40, 0.51, 0.52 and 0.64 using the solvent system Benzene: Methanol: Ammonia (9:0.6:0.4) is achieved. The results revealed the presence of various phytochemical constituents including alkaloids, flavonoids, amino acids, carbohydrates, saponins, tannins and phenolic compounds. However, methanol extracts exhibited more concentrated phytochemicals when compared to that of aqueous extracts of the plant, especially alkaloids and amino acids, which were enriched in methanolic extracts. These findings are in accordance with Shabi Ruskin *et al.,* 2014 who showed that phenol, alkaloids, tannins and flavonoids were present in the ethanolic extract of *C. quadrangularis*, while saponin and carbohydrates were absent.

Flavonoids were better separated with n-butanol: ethyl acetate: water (1:2:3) solvent system. TLC separation followed by post-development derivatization of the TLC plate with 3% boric acid + 10% oxalic acid spray revealed the presence of flavonoids in the plant extracts at Rf value of 0.66. Thereby, the presence of flavonoids was confirmed by post-development derivatization of the TLC plate. In the present study, after derivatization, the flavonoid spot could be further visualized when the plates were viewed under UV light at 254nm, which was denser in methanol extract of *C. quadrangularis* when compared to methanol extract.

The TLC profiling results showed that methanol extract of *C. quadrangularis* are rich in the presence of flavonoids and phenolic compounds. The present study is focused on In-vitro Anti-inflammatory activity of different Extracts of *C. quadrangularis*, accordingly three different assays were planned for establishing the anti-inflammatory activity. The Membrane stabilization test on human red blood cell membrane by hypo tonicity induced membrane lysis is carried out that readily confirmed the excellence of the methanolic extract with 88.16 % inhibition while that of aqueous extract with 80.20 % inhibition and diclofenac was taken as standard .Similarly Inhibition of albumin denaturation is performed to understand the invitro anti-inflammatory activity ,where BSA is heated to denaturation, that expresses antigens associated to Type III hypersensitive reaction and this can be considered as representative of diseases such as serum sickness, glomerulonephritis, rheumatoid arthritis and systemic lupus erythematous. The results demonstrated an 84.11 % and 74.11% inhibition at concentration of 500 µg/ml with methanolic and aqueous extract, assuring the perfect control of inflammation in various forms. Lastly, Inhibition of albumin denaturation (egg albumin) is estimated and is found to be 82.35% and 62.35% inhibition at concentration of 500 µg/ml with methanolic and aqueous extract in comparison to diclofenac as standard. Thus *C. quadrangularis* extract has proved to be highly potent in controlling various inflammatory activities involving Cyclooxygenase, vascular exudates, Plasma cascade systems, Vascular changes, etc. Causing the most uncomfortable and painful conditions in the body due to Acne vulgaris, Rheumatoid Arthritis, Asthma, Autoimmune diseases, Coeliac disease, Chronic prostatitis, Glomerulonephritis, Hypersensitivities, Inflammatory bowel diseases, Pelvic inflammatory disease, Reperfusion injury, Sarcoidosis, Transplant rejection, Vasculitis, Interstitial cystitis etc. All the. The future scope is to formulate the most standardized and quantized dosage form of *C. quadrangularis.*

CONCLUSION

The present study revealed the various Phyto ingredients present in i.e., Flavonoids, alkaloids, terpenes, glycosides, Saponins, Tannin and Phenols are present in the methanolic as well as aqueous extracts of *Cissus Quadrangularis*, the separation of these ingredients was achieved by TLC and also conferred by the various sprays. The methanolic as well as aqueous extracts of *cissus quadrangularis* was subjected to three different anti-inflammatory assays based on the literature review. The anti-inflammatory invitro assays namely

Membrane stabilization test (Blood Serum), Inhibition of albumin denaturation (egg albumin) and Inhibition of protein denaturation (bovine serum albumin) were performed and showed 88%, 84% and 82% inhibition of inflammation rate respectively when compared with diclofenac as standard on invitro basis. All this established a high level of guarantee that *Cissus Quadrangularis an* effectively control inflammation associated with various diseases or disorders and hence can serve as promising drug in the treatment of the same.

CONFLICT OF INTEREST:

I have no conflicts of interest regarding this investigation**.**

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