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EFFICIENT EXTRACTION OF TRITERPENOID SAPONINS FROM LEAVES OF *CLEMATIS NAPAULENSIS* DC.– AN INDIGENOUS MEDICINAL PLANT USED IN TREATMENT OF RHEUMATOID ARTHRITIS

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ABSTRACT

Clematis napaulensis DC. is a semi-creeper plant, found in China, Nepal and North-eastern part of India. The plant is being reported to be used by Chakesang tribe of Nagaland for the treatment of Rheumatoid Arthritis. The current studies is focussed on the systematic exploration of its phytoconstituents and subsequent fractionation of Triterpenoid saponins and investigatied for its anti-inflammatory. Extraction of the phytoconstituents were performed through soxhlet apparatus, examined for its phytoconsituents, further the phytoconstitutent triterpenoid saponins was recovered by solvent extraction by Column Chromatography using Chloroform : Methanol : Water as mobile phase. Presence of triterpenoid saponins was confirmed through TLC technique using Hexane : Ethyl Acetate (7.2 : 2.8) as mobile phase using Oleanolic Acid as standard. The concentration of triterpenoid saponins in the extract was quantified by spectrometric method at 548nm. The crude extract from *Clematis napaulensis* DC. was investigated for its anti-inflammatory activity by Protein Denaturation Assay and Proteinase Inhibitory Assay. The concentration of triterpenoid saponins from the extract was found to be 0.289mg/g (w/w) of sample leave powder. The research study results have demonstrated that methanol leaf extract of *Clematis napaulensis* DC. has very good anti-inflammatory activity as both protein denaturation assay and proteinase inhibition assay displayed increasing inhibition with increasing concentration.

Key words: *Clematis napaulensis* DC., Triterpenoid saponins, Inhibition of Protein denaturation Assay

INTRODUCTION

Herbal medicines have been gaining popularity worldwide. They are an integral component of alternative medical care and provide a rich source for innovative drug discovery. Knowing about the imminent threat upon the diversity of medicinal plants and hence their therapeutic values, it is ongoing research to develop more novel and effective methods to obtain compounds of therapeutic interest.[1] Of all the drugs prescribed around the world 25% of it are of herbal origin and among the 252 essential medicine list of WHO, 11% are of plant origin. But after years of overmedicating, facing resistant bacteria in the micro biome and treating the illness rather than the root of the problem, people are beginning to pay more attention to natural, herbal medicine.[2] Herbs have been known to be used around the globe to treat cardiovascular diseases, prostrate problems, depression, inflammation and weakened immune system. An important benefit associated with herbal medicine is the insignificant side effects. Scientific researches have revealed that herbal medicines are highly effective for certain diseases and conditions. Added to that with better understanding the efficacy can be vastly increase knowing the optimum doses.[3]

An important factor is the choice of the plant part which can yield maximum metabolite targeted, at optimum temperature and time of drying if the samples have to be in powdered form. Successful evaluation of biologically active compounds from plant parts are largely dependent on the sort of solvent used within the extraction system. Properties of a ideal solvent in plant extractions consists of, low toxicity, ease of evaporation at low temperature, fast physiologic absorption of the extract, preservative action.[4]

Our research study is focused on the efficient extraction of Triterpenoid saponins from medicinal plant *Clematis napaulensis* DC. Triterpenoid saponins are surface active glycosides of triterpenes (30C) that occurs naturally in flora and fauna. Their diversity, novel bioactivities of relevance to the pharmaceutical industry and usefulness as ingredients of cosmetics, food and feeding stuff has generated great interest in the study of these molecules. With the introduction of advanced methods of isolation and structure determination there has been increased research in these molecules.[5] They are ubiquitous to plants helping in their defence against biotic stresses. Till date, identified saponins from Clematis plants, includes about 70 oleanolic saponins, 50 hederagenin saponins and two gypsogenin saponins.[6]

Saponins as a whole can be classified into two groups based on the nature of the aglycone skeleton. The first group consists of the steroidal saponins, which are almost exclusively present in the monocotylednous angiosperms. The second group consists of the triterpenoid saponins, which are most common and occur mainly in the dicotyledonous angiosperms.[7]

Clematis species are a source of various conventional medicine used since the beginning of Chinese civilization.[12] Earlier studies have highlighted that some related species of genus *Clematis* such as, *Clematis tangutica*, *Clematis argenticulata*, *Clematis ganpiniana*, *Clematis terniflora* and *Clematis tibetana* were examined for its pharmacological properties such as anticancer, anti-inflammatory, analgesic, sedative and even antimicrobial.[13] It has been studied that active triterpenoid saponins has diversified pharmaceutical applications such as hepato-protective, cough therapy, anti-inflammatory, anti-bacterial and anti-tumour.[14]

Inflammation is a physiological response that protects the body from tissue injury. Acute inflammation, with exudation of fluid and plasma proteins as its main features, occurs very rapidly, and the process can last for few or several minutes to several days. Chronic inflammation occurs when the acute inflammatory process occurs repeatedly or continuously, with the process lasting for several weeks to months and even years.[15] The migration of leukocytes from the venous systems to the site of damage, and the release of cytokines, are known to play a crucial role in the inflammatory response. These chemicals cause widening of blood capillaries (vasodilation) and the permeability of the capillaries. This will lead to increased blood flow to the injured site.[16] Although inflammation is a physiological process within the body, it can manifest as symptoms such as severe pain due to rheumatoid arthritis. Standard anti-inflammatory drugs are used to alleviate these symptoms, such as non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids that inhibit the enzymes cyclooxygenase (COX) and phospholipase A2 (PLA2), respectively. However, cases have been reported that these COX inhibitors are associated to increased risk of heart attack and stroke.[17]

MATERIAL AND METHODS

Plant materials

Clematis napaulensis DC. (syn. *Clematis forestii*) commonly known as Nepal Clematis, is a native plant to China and Indian subcontinent. First reported by Hooker J Dalton in 1872, in 'The Flora of British India', it belongs to the species of flowering

plants in the buttercup family Ranunculaceae. The leave sample of *Clematis napaulensis* DC. for the proposed research study was obtained from specified location from Khonoma village in Kohima district of Nagaland.

Chemical and Reagents required

a) Methanol 99% (Molychem)
b) Silica gel 60-120 mesh (Merck)
c) Chloroform 99% (Loba Chemie Pvt Ltd)
d) n-Hexane HPLC grade (Himedia)
e) Petroleum ether 40-60ºC (Rankem)
f) Ethyl acetate 98% (Qualigens Fine Chemicals)
g) n-Butanol 99.4% (Sigma Aldrich)
h) Oleanolic acid (Biosynth)
i) Acetyl Salicylic Acid (Sigma Aldrich)
j) Bovine Serum Albumin (Himedia)
k) Metallic iodine (Qualigens)
l) Tris-HCl Buffer (Thermofisher)
m) Phosphate buffer (Thermofisher)
n) Perchloric acid (Sigma Aldrich)

Instruments and Apparatuses required

a) Vacuum Rotary Evaporator (Innovative Technologies, Guwahati Assam)
b) Hot Water Bath (Innovative Technologies, Guwahati Assam)
c) Chromatographic column (Borosil)
d) Separating Flask (Borosil)
e) Magnetic stirrer (Labline)
f) TLC Silica gel 60 F_{254}
g) TLC chamber
h) UV-VIS spectrophotometer (LMSPUV1200)
i) Triple Heating Mantle (Coslab)
j) Hot Air Oven (Biocraft Scientific Systems)

Extraction of Triterpenoid Saponins:

The leave samples of *Clematis napaulensis* DC. were harvested, cleansed thoroughly of unwanted organic debris and dried completely inside hot air oven. The powdered leave sample was prepared by grinding the dried leaves using Electric blender.
About, 200g of the sample powder was weigh and extracted in Soxhlet apparatus at 70ºC using methanol as solvent. The residue was dried and weighed; the solvent extract obtained was concentrated in Vacuum Rotary Evaporator at 50ºC at 400 mbar pressure. The extract was sealed and stored in cold storage until further use. About 42g of crude extract was recovered after drying at Hot Air Oven at 60ºC for 24 h. The crude extract was weigh and completely dissolved in 100mL of pure distilled water.
The aqueous phase was partitioned three times with Petroleum ether (50mL x 3 times) using Separating flask to remove the fat and pigments, each time recovered the aqueous phase. The aqueous phase was again partitioned with Ethyl acetate (50mL x 3 times) to remove organic matters and recovered the aqueous phase. The remaining aqueous phase is partitioned with n-Butanol (50mL x 3 times) to fractionate triterpenoid saponins. The n-Butanol fraction was subjected to 550 mbar pressure to obtain concentrated extract and recovered the n-Butanol using Rotary evaporator (B.P of n-Butanol = 118°C at 1 atm). The concentrated extract was further dried in hot water bath at 80ºC for 2 h to obtain dried residue.
About 25g of dry silica gel (60-120 particle size) was weigh and mixed well with the n-Butanol fraction in methanol and dried. Five Chromatographic Columns A, B, C, D and E were set up at absolute perpendicularity and filled with wetted silica gel with continuous stirring and gentle tapping with rubber tube to avoid formation of air space. The silica gel was filled up to 3/4 of the column height and methanol was added to cover about 1cm above the silica gel. The column was allowed to stand for 15 min to pack precisely and to exclude the air bubbles. A small quantity of non-absorbent cotton was place above the packing to prevent air transit. About 5gm each of the dried silica gel containing sample was loaded in the column. Mobile phases of 50mL each containing Chloroform: Methanol: Water in the ratio of 85:15:1.5(101.5mL), 80:20:2(100.2mL), 75:25:2.5(102.5mL), 70:30:3(103mL) and 65:30:3.5(103.5mL) and tagged as A, B, C, D and E respectively in five conical flasks. Phytoconstituents were elected with mobile phases in the respective columns. The elution start point identified by coloration and was collected 5 test tubes each containing 10mL (eg., A1, A2, A3, A4 and A5 for A column).

Thin Layer Chromatography

Oleanolic acid at a concentration of 1mg/mL was prepared from a stock solution by serial dilution as a standard reference for the identification of triterpenoid saponins. TLC plate with the dimension of 10 cm^2 was cut and six spots were made and spotted with five elute of column chromatography viz., A3, B3, C3, D3, E3 and a standard. The plate was completely dried in

hot air oven and subsequent preparation of mobile phase solvent of n-Hexane : Ethyl Acetate in the ratio of 7.2:2.8 (10mL) was done and then air dried. The dried TLC plate was kept in iodine chamber before comparative analysis and observation.

The total concentration of Triterpenoid Saponins in each sample was determined by the method as described by Wei et al., 2015 using Oleanolic Acid (OA) as standard. A standard curve for OA was prepared using the method as described by Wei et al., 2015.[18] Absorbances were measured for the samples considered for TLC viz., A3, B3, C3, D3 and E3 at 548nm wavelength. From the absorbances, the concentrations of each sample were calculated w.r.t. OA standard.

Inhibition of protein denaturation assay

The assay for Inhibition of Protein denaturation was performed with reference to the protocol followed by Mizushima and Kobayashi with change in concentration and standard.[19]

The crude extract from leave of *Clematis napaulensis* DC was considered for Inhibition of protein denaturation assay. About 1mL each of sample extract solutions were prepared at an increasing concentration of 50µg/mL, 100µg/mL, 200µg/mL, 400µg/mL, and 800µg/mL in distilled water from 1mg/mL stock solution. About 1mL of 800µg/mL acetyl salicylic acid was also prepared as positive control. About 450µL of (5% w/v) Bovine Serum Albumin in distilled water was prepared and added to all sample extract solutions and positive control. All the extract samples and positive control preparations were mixed with 1.4mL of PBS buffer. All the mixtures were incubated at 35ºC for 15 minutes in water bath followed by heating at 70ºC for 5 minutes. The mixtures were then rapidly cooled under tap water and absorbance readings were taken at 660nm. The graph of absorbance vs. concentration was plotted to check for effectiveness of crude extract with increasing concentration and Regression coefficient (R^2) was calculated. The sample extract solution absorbance at 800µg/mL was taken in triplicate to obtain the average as the final absorbance. Percentage inhibition was calculated by using the formula

% Inhibition of protein denaturation.

= [1- Ab2/Ab1] x 100

Where, Ab1= Absorbance by positive control and Ab2 = Absorbance by Sample extract both at 800µg/mL.

Proteinase Inhibitory Assay

The assay was performed with reference to the method of Sakat *et al*.[20] About 1mL of extract was mixed with 2 mL of (0.06mg) of Trypsin and 1mL of 20mM Tris-HCl buffer. The mixture was incubated at 37ºC for 5 minutes and then added with 0.8% (w/v) Casein. The mixture was then incubated for another 20 min, subsequently about 2mL of 70% Perchloric acid was added to stop the reaction. The solution was centrifuged and separated the supernatant solution and the absorbance was measured at 210 nm against buffer as blank. PBS was taken as control and percentage Proteinase inhibition was calculated as

% Proteinase inhibition = $100 \times (1 - A2/A1)$

Where, A1 = absorption of the control sample, and A2 = absorption of the test sample.

RESULTS

Extraction was performed in Soxhlet apparatus, and evaporated in vacuum evaporator. After evaporation about 0.210 g (w/w) was obtained for every gram of sample leave powder. Subsequently the crude dried extract was fractionated with n-butanol, which yielded 0.056 g (w/w).

TLC analysis of Column Chromatographic elutes of A3, B3, C3, D3 and E3 containing chloroform, methanol and water in varying ratios whereas all the elutes contains Triterpenoid saponins which was confirmed by comparison with standard Oleanolic acid (Fig 5).

Spectrometric analysis at 548 nm showed that the mobile phase ratio, (chloroform: methanol: water :: 70: 30: 3) in D3 column showed highest concentration 1.159mg/mL (w/v) of Triterpenoid saponins when compared with other mobile phase composition. Total concentration of Triterpenoid saponins in the elutes was found to be 289.75mg for 200g of dry powdered leave.

Protein Denaturation Assay

To determine anti-inflammatory property, protein denaturation assay was performed. The sample extract and ASA as standard positive control, spectrometric readings showed absorbance of 0.055 and 0.076 (50µg/mL), 0.089 and 0.152 (100µg/mL), 0.134 and 0.305 (200µg/mL), 0.209 and 0.610 (400µg/mL), 0.337 and 1.220 (800µg/mL) respectively as compared to buffered solution containing 5% BSA as control, shown in table 3.

Anti-proteinase activity

Diclofenac sodium as well as sample extract showed anti-proteinase activity with increasing concentration. The percentages of anti-proteinase activity at 50µg/mL were 15.89±0.1 and 21.22±0.15 and at 500µg/mL, the activities were 73.11±0.30 and 86.84±0.25 for methanol extract and standard Diclofenac sodium respectively with steady increase. The observed study result is shown in table 4.

DISCUSSIONS

Among the different extraction techniques, Soxhlet extraction has been considered as best suited for the extraction of Triterpenoid saponins considering volatility of solvent (methanol) and sample solubility, as well lower temperature extraction and moderate usage of solvent. Developing a significant and efficient method for extracting the triterpenoid saponins from *C. napaulensis* DC is performed. In future studies, the phytochemical can be extracted with ease in *C. napaulensis* DC as well as related species for its medicinal values. Methanol was considered as an appropriate solvent to extract potential denaturable phytochemicals like triterpenoid saponins. The solvent also has the ability to extract wide range phytoconstituents and efficient recovery of the extract phytoconstituents at lower temperature.

The plant leave powder comprised of non soluble crude fibers (158g out of 200g) that are separated during Soxhlet extraction with methanol at 70ºC. The fractionation process removes significant quantity of unwanted extract by use of Petroleum ether to remove fat and pigments and Ethyl acetate removing other organic matters (30.8g out of 42g). The starting aqueous phase retains the sample of target (triterpenoid saponins in this case) as it can solubilize the targeted compound. However, distilled water is a polar solvent favorable to many other metabolites. The column selectivity to triterpenoid saponins is increased by the use of appropriate mobile phase ratios. The concentration of triterpenoid saponins in elutes are identified by spectrophotometric method. The percentage yield was 0.1449% which is not negligible, considering the fact that triterpenoid saponins are secondary metabolites.

It is to be noted that the process of fractionation started with most polar solvent (distilled water) which can dissolve most plant analytes and then subsequently removing the non-essential constituents by taking advantage of immiscibility of separating solvents.

Analysis for percentage inhibition of protein denaturation assay had shown increasing inhibition for denaturation by sample extract as well as positive control in a buffered solution with increasing concentration. The results indicated that the plant extract has anti-inflammatory activity. The inhibition by extract at a same concentration showed lower potency than the standard; however, the extract showed increasing inhibition of protein as like standard. The potency of the sample extract can be enhanced by optimizing extraction techniques for phytochemicals responsible against inflammatory action.

Neutrophils are a reservoir for many types of serine proteinases carried in their lysosomal granules. These proteinases have been associated with arthritic conditions. Proteinase inhibitors can play a significant role in the protection against tissue damage during inflammatory responses.[21] The increasing concentration of sample extract and diclofenac sodium against casein (0.8% w/v) showed near steady increase in anti-proteinase activity by both sample extract and diclofenac sodium. The study results showed comparable anti-inflammatory activity between extract and standard.

CONCLUSION

The observation from TLC has confirmed the presence of Triterpenoid Saponins in the leave extract of *Clematis napaulensis* DC. It is also concluded from spectrometric analysis results that elute obtained from column chromatography with mobile phase having Chloroform:Methanol:Water in the ratio of 70mL:30mL:3mL showed the highest concentration of triterpenoid saponins. This particular mobile phase ratio can be considered as the primary mobile phase solvent for extracting Triterpenoid saponins from the extracts.

In vitro techniques such as inhibition of protein denaturation assay as well as Proteinase inhibition assay established the anti-inflammatory potential of *Clematis napaulensis* DC. The study results demonstrated that the extract can be used in treating the inflammatory related diseases such as Rheumatoid Arthritis, Chronic inflammations and tissue injury related to inflammations. However, more exploratory research is required to understand its mechanism towards its anti-inflammatory and other pharmacological activities.

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FIGURES AND TABLES

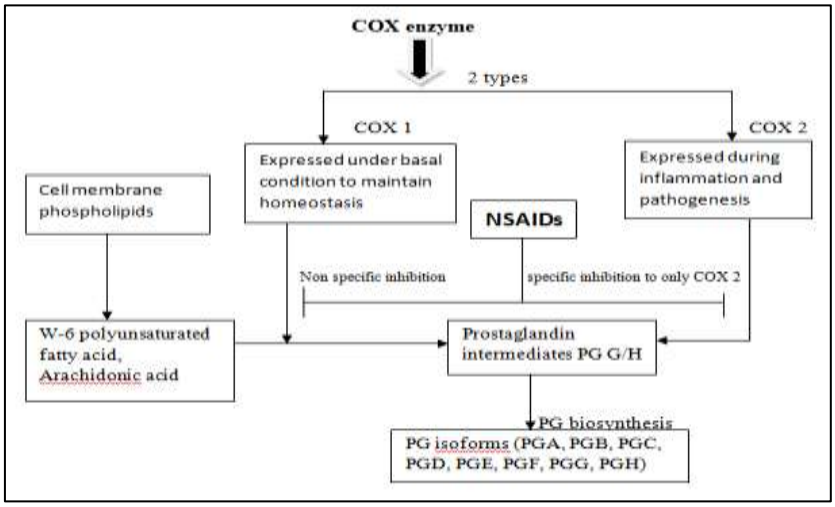

Fig 1: Targeted inhibition pathway of Cyclooxygenase enzyme action by NSAIDs

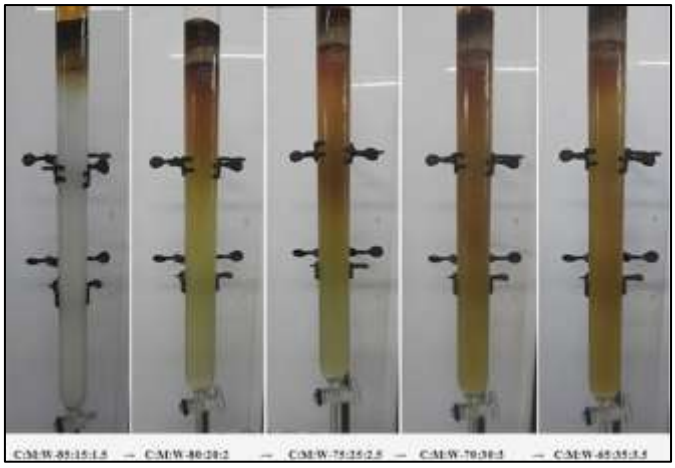

Fig 2: Column Chromatography for Triterpenoid saponins

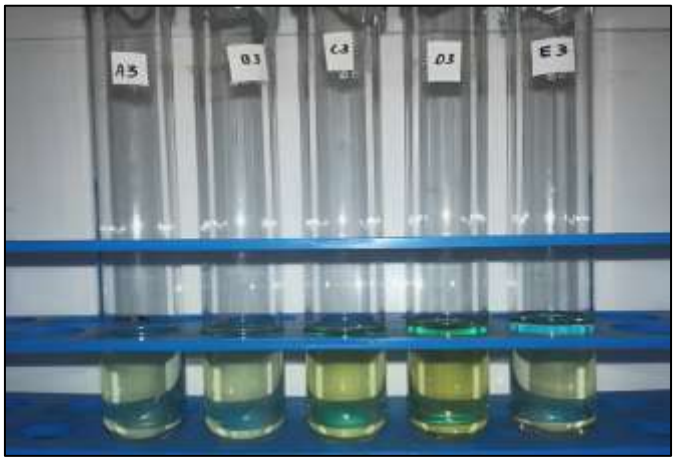

Fig 3: Sample for Absorbance measurement

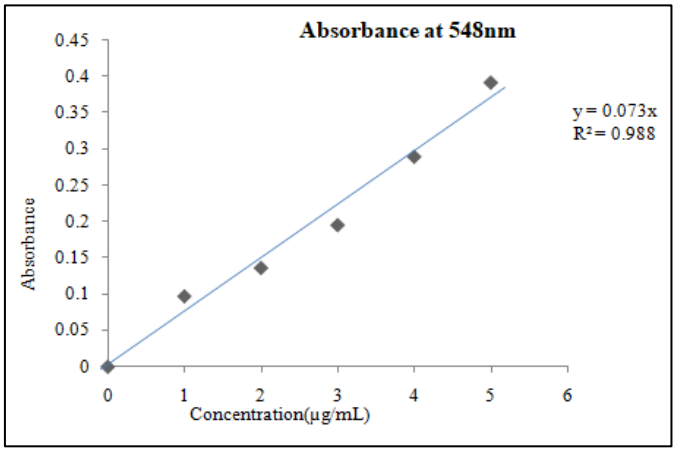

Fig 4: Standard graph of Oleanolic acid

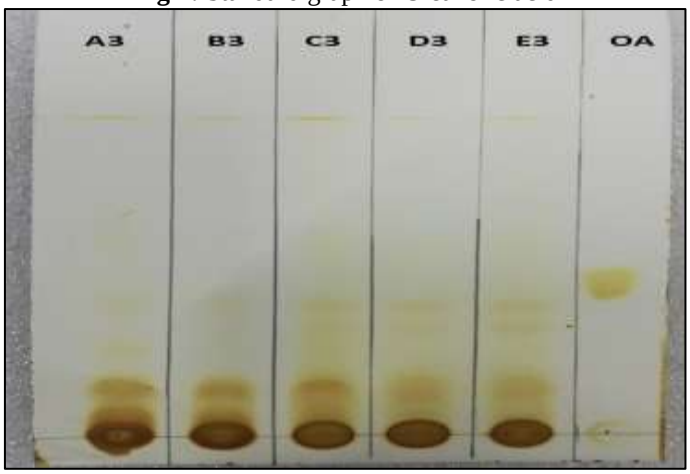

Fig 5: TLC for selected mobile phase samples

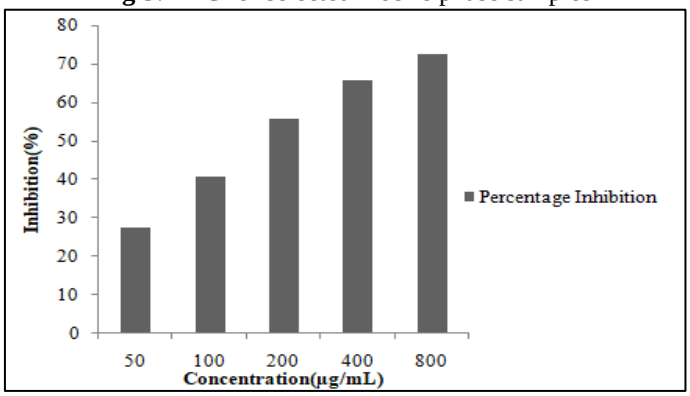

Fig 6: Percentage Inhibition of Protein denaturation by Extract compared to ASA.

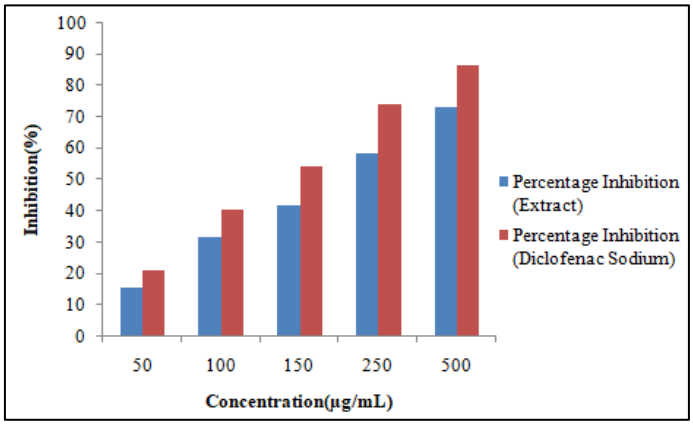

Fig 7: Comparative percentage of anti-proteinase activity

Clematis species	**Triterpenoid saponins (Structural nomenclature)**
Clematis tangutica	3-O-β-D-ribopyranosyl oleanolic acid 28-O-α-L rhamnopyranosyl-(1→4)-β-D-gluco pyranosyl-(1→6)-β-D glucopyranoside. [8]
Clematis chinensis Osbeck	3-O-beta-[(O-alpha-L-rhamnopyranosyl- (1→6)-O-beta-D-glucopyranosyl-(1→4)-O-beta-D-glucopyranosyl-(1→4)-O-beta-D-ribopyranosyl-(1→3)-O-alpha-L-arabino pyranosyl) oxy]olean-12-en-21alpha hydroxyl 28-oic acid-O-alpha-L-rhamna pyranosyl –(1→4)-O-beta-D-gluco pyranosyl-(1→6)-glucopyranosyl ester. [9]
Clematis grata	3-O-β-D-glucopyranosyl-3-β-hydroxyl-olean-12-en-28-oic acid. [10]
Clematis mundsurica	3-O-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-ribopyranosyl-(1→3)- α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl hederagenin 28-O-β-D-glucopyranosyl- (1→6)- β-D-glucopyranoside.[11]

Table 1: Triterpenoid Saponins in *Clematis species*

Table 1: Triterpenoid Saponins in *Clematis species*

Sample	**Absorbance (548nm)**	**Concentration Calculated (w/v)**
A3	0.096	1.041mg/mL
B3	0.092	1.109mg/mL
C3	0.093	1.097mg/mL
D3	0.088	1.159mg/mL
E3	0.090	1.133mg/mL

Table 2: Comparative concentrations obtained from five mobile phase elutes

Concentration (µg/mL)	**Absorbance (660nm)**		**Percentage inhibition**
	Sample Extract	**Positive Control**	
50	0.055	0.076	27.63
100	0.089	0.152	40.82
200	0.134	0.305	55.80
400	0.209	0.610	65.80
800	0.337	1.220	72.38

Table 3: Concentration vs. Absorbance of protein denaturation assay

Concentration (µg/mL)	**Percentage of inhibition**	
	Methanol Extract	**Diclofenac Sodium**
50	15.89±0.1	21.22±0.15

100	31.95±0.21	40.33±0.13
150	42.06±0.10	54.56±0.32
250	58.60±0.21	74.21±0.38
500	73.11±0.30	86.84±0.25

Table 4: Anti-proteinase activity of extract and Diclofenac sodium