ISSN Print: 2664-6188
ISSN Online: 2664-6196
Impact Factor: RJIF 5.35
IJCBB 2023; 5(2): 01-07
www.biochemistryjournal.net
Received: 02-04-2023
Accepted: 06-05-2023

Akshay Milind Patil
Centre for Biotechnology Pravara Institute of Medical Sciences (DU) Loni, Tal. Rahata Dist. Ahmednagar, Maharashtra, India

Shrutkirti Shahaji Shinde
Centre for Biotechnology Pravara Institute of Medical Sciences (DU) Loni, Tal. Rahata Dist. Ahmednagar, Maharashtra, India

Dhanvarsha Pralhad Bhusari
Department of Bioinformatics & Plant Breeding, College of Agricultural Biotechnology, MPKV Rahuri, Maharashtra, India

Ganesh Bapuro Janvale
Department of Bioinformatics & Plant Breeding, College of Agricultural Biotechnology, MPKV Rahuri, Maharashtra, India

Corresponding Author:
Akshay Milind Patil
Centre for Biotechnology Pravara Institute of Medical Sciences (DU) Loni, Tal. Rahata Dist. Ahmednagar, Maharashtra, India

Identification of novel compounds using chromatographic methods and screening for protease activity and anti-inflammatory activity of *Adhatoda vasica*

Akshay Milind Patil, Shrutkirti Shahaji Shinde, Dhanvarsha Pralhad Bhusari and Ganesh Bapuro Janvale

DOI: https://doi.org/10.33545/26646188.2023.v5.i2a.42

Abstract
This investigation column eluted fractions of leaf *Adhatoda vasica* of was assessed for its phytochemical screening, column chromatography, Thin layer chromatographic studies, protease activity, anti-inflammatory activity, antidiabetic activity and antioxidant activity. Phytochemical screening reflect the presence of alkaloid, flavonoids, coumarins, terpenoids, steroids, emodins, Quinone's. Column chromatography method was used for purification of bioactive compounds. Thin layer chromatographic study was carried out by using various solvent system of different type of polarity of *n*- butanol, acetic acid and acetone. TLC profiling shows pure band at 254nm and 366 nm. The strong "proteolytic activity" also pointed out in purified fraction of eluted fraction *In vitro* anti-inflammatory activity was evaluated using albumin denaturation fraction 3,showinghighest activity 75% followed by fraction 5 (62.73%), membrane stabilization assay fraction 6 (80.23%) followed by fraction 3 (64.65) and proteinase inhibitory activity of fraction 5(88%) followed by fraction 787.68% at conc. 500 µg/ml. Aspirin (90.87%)was used as standard drug for the study of anti-inflammatory activity.

Keywords: *Adhatoda vasica,* column chromatography, TLC, protease activity, antioxidant, anti-inflammatory, antidiabetic activity, phytochemical screening

Introduction
The increase in prevalence of multiple drug resistance has shown the development of new synthetic antimicrobial, antioxidant, antidiabetic and anti-inflammatory drugs; moreover the new drug is necessary to search for new antimicrobial, antioxidative, antidiabetic and anti-inflammatory sources from alternative sources. Bioactive compounds from medicinal plant showing pharmacological activities have the potential of filling this need because their structure are different from those of the most studied plants, while those with more action may likely differ (Fabricant and Fanworth 2001) [7]. (Khateef *et al.* 2015) [1]. The leaves, flowers, fruits and roots are extensively used for treating Cold, Cough, Whooping cough, Chronic bronchitis and Asthma. It has also used considerable interest for its beneficial effects in Malaria, Dysentery, Diarrhea, Antimicrobial, Anthelmintic and Antiperiodic. Chemical constituents of *Adhatoda vasica* were isolated successfully with higher yield. The structures of isolated compounds were confirmed by the use of spectral data UV, Mass and HPLC. The extract of *Adhatoda vasica* was evaluated for Antidiabetic activity. (Bhatt *et al.* 2011) [2]. The typical bioactive compounds in plants are produced as secondary metabolites. Free radicals which have one or more unpaired electrons (superoxide, hydroxyl, peroxyl) are produced in normal or pathological cell metabolism and the compounds that can scavenge free radicals have great potential in ameliorating the diseases and pathological cells (Halliwell, 1995; Squadriato and Peyor, 1998; Gulcin *et al.*, 2001) [8-10]. Antioxidants thus play an important role to protect the human body against damage by reactive oxygen species. Screening of various bioactive compounds from plants has lead to the discovery of new medicinal drug which have efficient protection and treatment roles against various diseases (Kumar *et al.*, 2004; Sheeja and Kuttan, 2007; Mukherjee *et al.*, 2007) [11-13].

It is beneficial to the tuberculosis patient (K. P. Sampath Kumar *et al.* 2010) [14]

Review of Literature

The leaves of Vasaka are rich in vitamin C, carotene and an essential oil. A study showed that *Mycobacterium tuberculosis* was inhibited by the essential oil (At a specific concentration). (K. P. SampathKumar *et al.* 2010) [14]. the flowers are slightly shown on fire and then placed on the eyelids. Regular application is said to cure eye irritation and other minor ailments. (K. P. Sampath Kumar *et al.* 2010) [14]. *In vitro* antidiabetic and anti-inflammatory activity of stem bark oh Bauhinia purpurea. At a concentration of 20 µg/ml of B. purpurea extracts petroleum ether and hexane showed a percentage inhibition 13.4% and 21.7% and for 100µg/ml extracts showed inhibition of 93.0% and 93.5%. B. purpure a extract was effective in inhibiting heat-induced albumin denaturation Maximum inhibition 81.36% was observed from petroleum ether extract and hexane extract shows 79.90% at the concentration of 250 µg/ml. Aspirin, a standard anti-inflammation drug showed the maximum inhibition 96.04% at the concentration of 250 µg/ml. The extracts also inhibited the heat induced haemolysis of RBCs maximum inhibitions 75.5% was observed from petroleum ether extract followed by methanol 75% and the aspirin standard drug showed the maximum inhibition 51.1%. *in vitro* antioxidant and anti-inflammatory activity of methanol extract of *Oxalis corniculate Linn*. Methanol extract of whole plant subjected for antioxidant and anti-inflammatory activity. (Sakat, *et al*. 2010) [24].

In vitro anti-inflammatory activity methanol extract of *Enicostema axillare*. Methanol extract of plant subjected to anti-inflammatory activity by *in vitro* methods. *In vitro* anti-inflammatory activity was evaluated using albumin denaturation assay, proteinase inhibitory activity, membrane stabilization, and anti-lipoxygenase activity at different concentrations. (G. Leelaprakash *et al*. 2011) [25]: Worked on Aspirin, Diclofenac sodium, Indomethacin were used as standard drugs Methanol Extract at a concentration range of 100-500µg/ml protects the heat-induced protein denaturation. At the concentration of 400 and 500 µg/ml, EAME showed significant inhibition of 42 and 53% of proteinase inhibitory action, but at the concentration of 100 and 200 µg/ml did not show significant ($p>0.05$) activity. Heat-induced haemolysis of erythrocyte was significantly ($p<0.05$) inhibited at the concentration of 400 and 500µg/ml. Hypotonicity-induced haemolysis and lipoxygenase activity were significantly ($p<0.01$) inhibited at the concentration range of 200-500µg/ml and 400, 500µg/ml G. (Leelaprakash *et al*. 2011) [25]

Methodology

Preparation of extract (Vandana Rawat *et al.*)

Phytochemical screening: Phytochemical studies were carried out for methanol extracts of *Adulsa (Adhatoda vasica)* leaves to detect the presence of different phytochemical constituents like steroids, terpenoids, tannins, flavonoids, saponins, glycosides, amino acids etc. by using standard procedures,

Column chromatography

1. The column 2 cm × 25 cm was packed with a solution of silica gel with water using the wet slurry method, this involve preparing a solution of silica gel with water in this case, in a beaker and subsequently adding this into the column till it is about three-fourth filled (glass wool) was pushed into the column to settle atop the packed silica gel. *6.*The solvent system of n-butanol: acetone: water 5:2:3 was poured continuously into the column and allowed to drained and about 10 fraction of 5-6 ml was collected in sterile centrifuge tube The fraction eluted on column was tested with same solvent system by TLC for the presence of active compounds. 4) Thin layer chromatography (TLC) 1. Fraction eluted on the column was subjected to TLC as per conventional one-dimensional ascending method using silica gel (60F254 MERCK) pre-coated plate, were observed under various wave length at 254nm and 366nm for band detection (Rajendra Prasad. Gujjetti *et al*. 2013) [15]. Colour of the spot and pattern were observed and RF value were calculated using formula:

$$\text{RF (Retention factor)} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by the solvent front}}$$

Anti-inflammatory Activity

Albumin Denaturation Assay

Method of Mizushima and Kobayashi (1968) [26] and Sakat *et al*. (2010) [24] followed by minor modification 2. The reaction mixture was consisting of test extract and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 37 ˚C HCL. 3. The sample extract were incubated at 37 ˚C for 20 min. and then heated to 51 ˚C for 20 min. 4. After cooling the sample the turbidity was measured spectrophotometrically at 660nm. 5. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follow;

$$\% \text{ inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

Membrane Stabilization Test

Preparation of Red Blood Cells (RBCs) Suspension:

Fresh whole human blood 10 ml was collected and transferred to the centrifuge tube. 2. The tubes were centrifuged at 3000 rpm for 10 min. and were washed three times with equal volume of normal saline 3. The volume of blood was measured and constituted as 10% v/v suspension with normal saline (Sakat *et al*., 2010) [24]

Heat-Induced Haemolytic

The reaction mixture 2 ml consisted of 1ml of test sample solution and 1 ml of 10% RBCs suspension 2. Instead of test sample only saline was added to the control test tube 3. Aspirin was taken as a standard drug 4. All the centrifuged tube containing reaction mixture were incubated in water bath at 56˚c for 30 min. 5. At the end of the incubation the tube were cooled under running tap water. 6.The reaction mixture was centrifuged at 2500 rpm for 5 min. and the absorbance of the supernatants was taken at 560nm.7. The experiment was performed in triplicate for all the test sample, % membrane stabilization activity was calculated by formula; (Shinde *et al*., 1999; Sakat *et al*., 2010) [27, 24]

$$\text{Percentage inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

Protein Inhibitory Action

1. The test was performed according to the modified method of Oyedepo *et al.*, (1995) [28] and Sakat *et al.*, (2010) [24].
2. The reaction mixture (2 ml) was containing 0.06 mg trypsin, 1 ml of 20 Mm Tris HCL buffer (pH 7.4) and 1 ml test sample of different concentration.
3. The reaction mixture was incubated at 37 °C for 5 min. and then 1 ml of 0.8% (W/V) casein was added.
4. The mixture was inhibited for an additional 20 min., 2 ml of 70% Perchloric acid was added to terminate the reaction.
5. Cloudy suspension was read at 210nm against buffer as blank.
6. The experiment was performed in triplicate (Leelaprakash G., Dass. Mohan 2011) [28]

Percentage protein inhibition activity was calculated by formula;

$$\text{Percentage inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

Result and Discussion

Column chromatography and TLC studies

Thin layer chromatographic studies of methanol extract of *Adulsa* was done by using silica gel F254 (MERCK) plate. solvent system n-butanol: Acetone: water (5:2:3) was used for separation of compound. fraction eluted on column chromatography showing different band pattern at 254 nm and 366 nm. Spot were characterized by Rf value under UV light.

Table 1: Column chromatography and TLC study of *Adhatoda vasica* methanol fraction with Rf value

Fraction No.	Solvent system	No. Of spot detected		Rf value	
		254 nm	366nm	254nm	366nm
1	Water	-	-	-	-
2	Water	1	1	0.70	-
3	n-butanol: Acetone: water	1	-	0.76	-
4	n-butanol: Acetone: water	1	-	0.86	-
5	n-butanol: Acetone: water	1	-	0.75	0.53
6	n-butanol: Acetone: water	1	2	0.74	0.38
7	n-butanol: Acetone: water	-	2	-	-
8	n-butanol: Acetone: water	-	-	-	-
9	n-butanol: Acetone: water	-	-	-	-
10	n-butanol: Acetone: water	-	-	-	-

Absence of spots

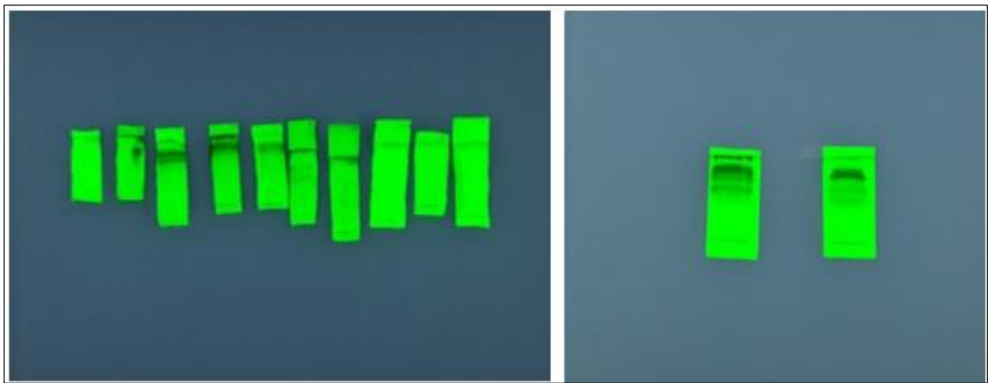

Plate 1: TLC plates of samples visualized under 254 nm of (A) column eluted fraction & (B) crude methanolic extract of Adulsa in same solvent system

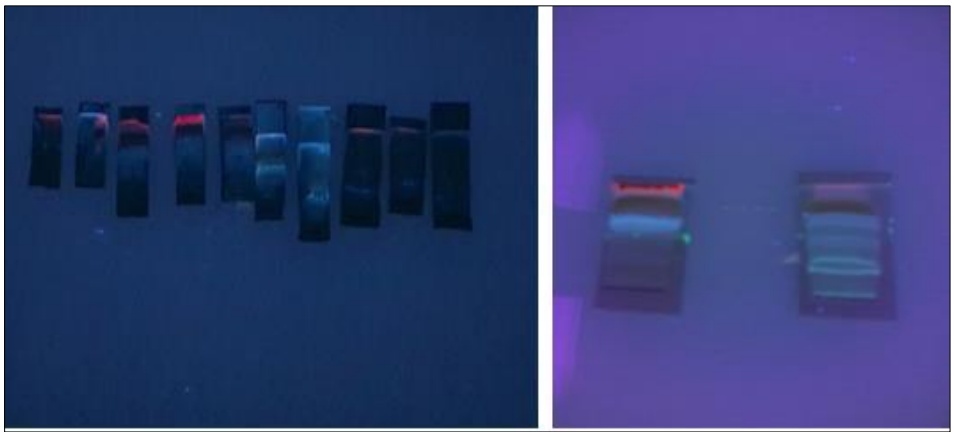

Plate 2: TLC plates of samples visualized under 366 nm of (A) column eluted fraction & (B) crude methanolic extract of Adulsa in same solvent system

Estimation of protein content of eluted fractions of column chromatography using NanoDrop spectrophotometer

Table 2: Determination of Protein concentration by Nanodrop technique

Fraction no	Protein concentration
1 (water eluted)	-0.051
2 (water eluted)	-10.972
3 (solvent eluted)	-15.441
4 (solvent eluted)	-21.124
5 (solvent eluted)	8.476
6 (solvent eluted)	13.593
7 (solvent eluted)	15.453
8 (solvent eluted)	19.275
9 (solvent eluted)	20.440
10 (solvent eluted)	26.289

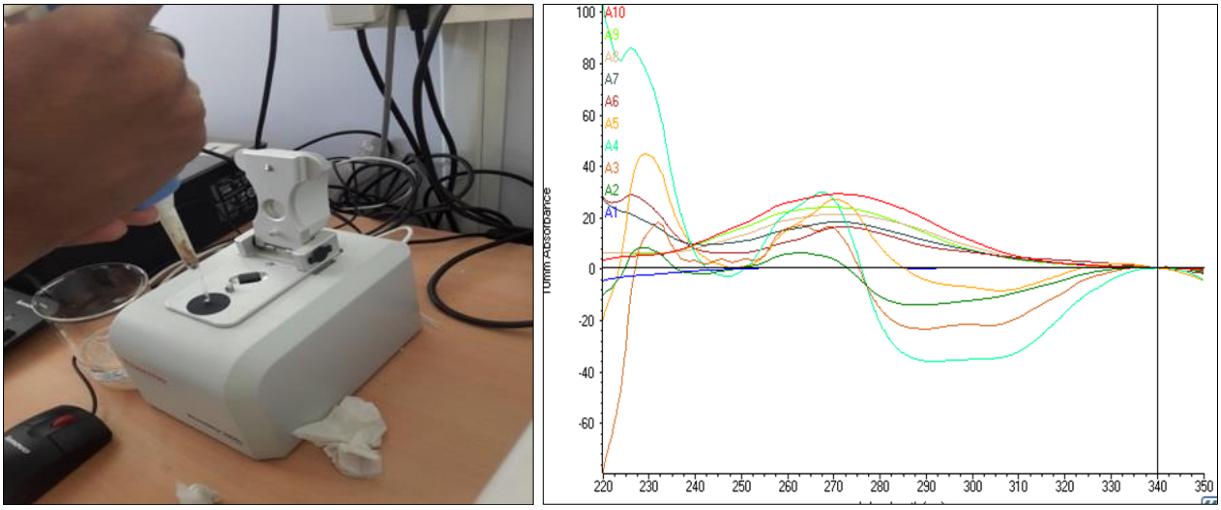

Fig 3: Determination of protein concentration of column eluted fractions of column chromatography of *Adhatoda vasica*

Phytochemical Analysis

Qualitative Phytochemical investigation for extracts of *Adulsa (Adhatoda vasica)* leaves With various specific reagent showed the presence of steroids, flavonoids, coumarous, terpenoids, terpenoids, emodins, alkaloids, phenols and Quinone's Methanolic extract of *Adhatoda vasica* leaves contain maximum number of phytoconstituents. The results of phytochemical screening were showed in the Table

Protease activity

Protease plays a vital role in many pathological processes. Arthritis, tumour invasia & metabtasis, infections and number of degenerative diseases have been linked with proteolytic enzymes. They have found extensive applications in bioremediation processes.

Protein concentration was found act by Nanodrop among this sample protein concentration was measured in fraction no 6 & 7 was checked for protease test microorganisms.

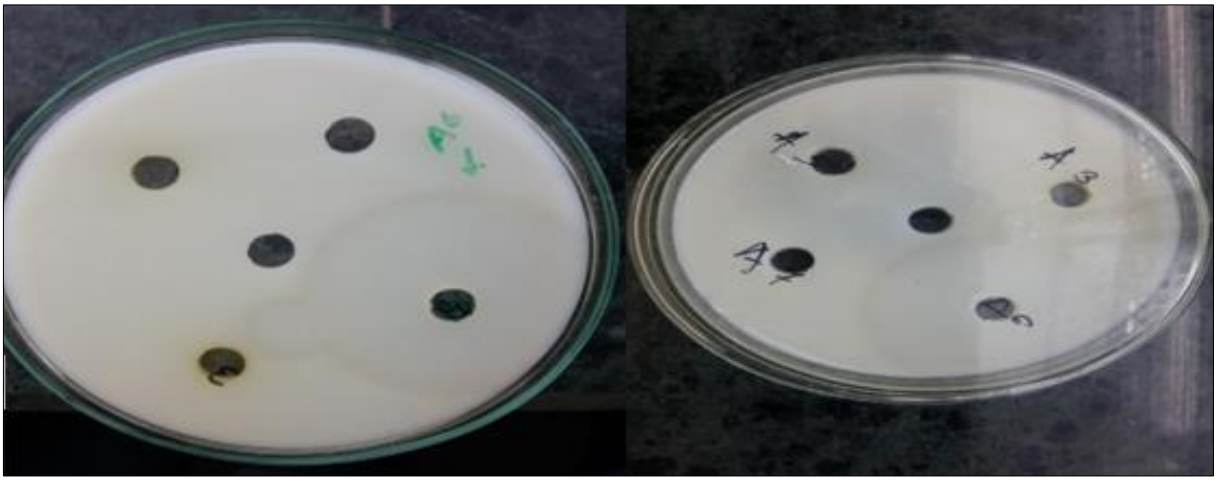

Fig 4: Protease activity on Milk agar of column eluted fraction A6 was observed

***In vitro* Anti-inflammatory activity**

Albumin denaturation assay: Denaturation of protein is a well-documented cause of inflammation. As a part investigation on the mechanism of the anti-inflammatory activity, ability of fraction it inhibit denaturation was studied. It was effective in inhibiting head induced albumin

denaturation. Maximum inhibition of fraction No 1. Maximum in albumin denaturation test inhibition of 75% was observed at µg/ml, followed by fraction 5(62.73) aspirin a standard drug show maximum inhibition 78.89% at conc.100 µg/ml

Table 4: Albumin denaturation assay of column eluted fraction of *Adhatoda vasica*

Test sample	% inhibition
Fraction 3	75 ± 0.010
Fraction 5	62.73 ± 0.006
Fraction 6	55.91 ± 0.006
Fraction 7	39.91 ± 0.006
Standard Aspirin	78.89 ± 0.010

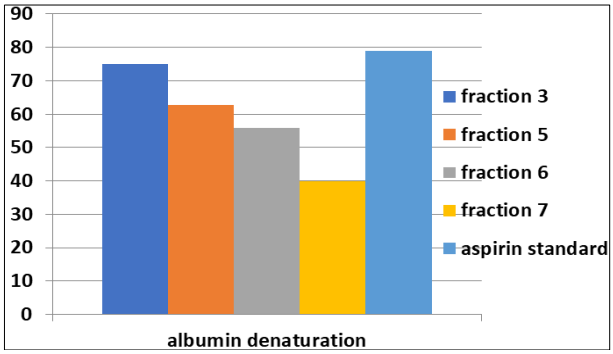

Fig 5: Albumin denaturation assay on column eluted fractions of Adulsa

Membrane stabilization assay: The HRBC membrane stabilization has been used as a method to study the *in vitro* anti-inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue Inflammation and damage upon extra cellular release. The lysosomal enzymes released during inflammation produce a various disorder. The extracellular activity of these enzymes are said to berelated to acute or chronic inflammation. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane. Extract was effective in membrane stabilization at different concentration as shown in Table,4.5) Methanolic extract was effective in membrane stabilization maximum inhibition of 80.23% was observed at 500 µg/ml, followed by fraction 3(64.65) aspirin a standard drug show maximum inhibition 80.75% at conc.100 µg/ml.

Table 5: Membrane stabilization assay of column eluted fractions of Adulsa

Test sample	% inhibition
Fraction 3	64.65 ± 0.010
Fraction 5	50 ± 0.010
Fraction 6	80.23 ± 0.008
Fraction 7	39.43 ± 0.010
Standard Aspirin	80.75 ± 0.006

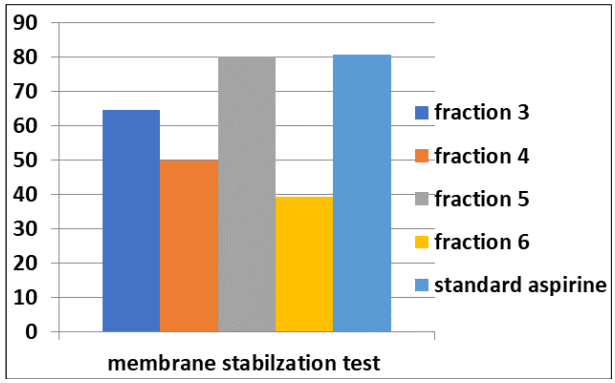

Fig 6: Membrane stabilization assay of column eluted fractions of Adulsa

Proteinase inhibitory activity

Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a rich source of proteinase which carries in their lysosomal granules many serine proteinases. It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors. *Adhatoda vasica (*Adulsa)

methanol extract exhibited significant ant proteinase activity at different concentrations as shown in Table Methanolic extract was exhibited significant proteinase inhibitory activity it shows maximum inhibition of 88% was observed at 500 µg/ml, followed by fraction 7(87.68) & fraction 3 (85.90) aspirin a standard drug show maximum inhibition 90.87% at conc.100 µg/ml.

Table 6: Proteinase denaturation of column eluted fractions of Adulsa

Test sample	% inhibition
Fraction 3	85.9 ± 0.02
Fraction 5	88 ± 0.022
Fraction 6	63.62 ± 0.008
Fraction 7	87.68 ± 0.025
Standard Aspirin	90.87 ± 0.02

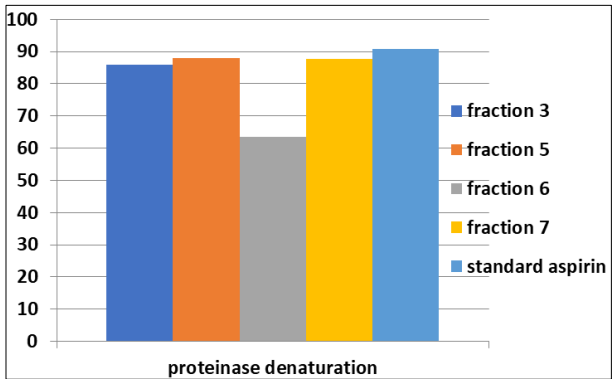

Fig 7: Proteinase denaturation of column eluted fractions of Adulsa

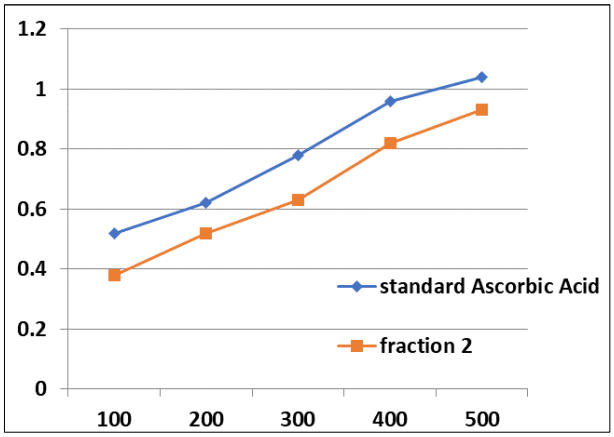

Fig 9: Reducing power assay of column eluted fraction of *Adhatoda vasica* where absorbance was taken at 700 nm on spectrophotometer.

Conclusion Phytochemical screening reflect the presence of alkaloid, flavonoids, coumarins, terpenoids, steroids, emodins, Quinone's. Column chromatography method was used for purification of bioactive compounds. Thin layer chromatographic study was carried out by using various solvent system of different type of polarity of *n*- butanol, acetic acid and acetone. TLC profiling shows pure band at 254nm and 366 nm. The strong "proteolytic activity" also pointed out in purified fraction of eluted fraction *In vitro* anti-inflammatory activity was evaluated using albumin denaturation fraction 3, showing highest activity 75% followed by fraction 5 (62.73%), membrane stabilization assay fraction 6 (80.23%) followed by fraction 3 (64.65) and proteinase inhibitory activity of fraction 5(88%) followed by fraction 787.68% at conc. 500 µg/ml. Aspirin (90.87%)was used as standard drug for the study of anti-inflammatory activity. *In vitro* antidiabetic activity was performed using Alfa amylase inhibition assay. Highest activity were showed in fraction 4 (79.05%) and fraction 5 (77.05%) at conc. 500 µg/ml.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Khateef. World Journal of Pharmacy and Pharmaceutical Sciences, 2015, 4(06).
2. Meenakshi Bhatt, Manoj Gahlot. Phytochemical Investigation And Antidiabetic Activity of Adhatoda Zeylanica; Asian Journal of Pharmaceutical and clinical Research. 2011, 4(2), ISSN – 0974-2441
3. Bhatt *et al*. Asian J Pharm Clin Res. 2011;4(2):2730.
4. Mohammad Shahriar. The Experiment, Phytochemical Screening Thrombolytic Activity of The leaf Extracts of Adhathoda vasica. 2013 Feb;7(4):438-441.
5. Sci. & Cult. 77 (5–6) 217-221 (2011) Mode of Action of Killing of Microorganisms with Vasaka Leaf Extract
6. Kirithikar KR, Basu BD, An ICS. Indian Medicinal Plants. International book distributors, Dehradun, India. 1995;1:371-372.
7. Fabricant DS, Fansworth NR. The value of plants used in traditional medicine for drug discovery. Environ. Health Persp. 2001;109:69-75.
8. Halliwell B. How to characterizen antioxidant: An update. Biochem. Socie. Symp. 1995;61:85-91.
9. Squadriato GL, Peyor WA. Oxidative chemistry of nitric oxide: The role of superoxide, peroxynitric and carbon dioxide. Free Rad. Biol. Med. 1998;25:392-403.
10. Gulcin L, Oktay M, Kufrevioglu IO, Aslan A. Determination of antioxidant activity of Lichen *Cetraria islandica* (L.) Ach. J. Ethnopharm. 2004;79:325-329.
11. Kumar RA, Sridevi K, Kumar NV, Nanduri S, Rajagopal S. Anticancer and immunostimulatory compounds from *Andrographis paniculata*. J. Ethnopharm. 2004;92:291-295.
12. Sheeja K, Kuttan G. Activation of cytotoxic T lymphocyte responses and attenuation of tumour growth *in vivo* by *Andrographis paniculata* extract and andrographolide. Immunop. Immunotoxicol. 2007;29:81-93.
13. Mukherjee PK, Kumar V, Houghton PJ. Screening of Indian medicinal plants for acetylcholinesterase inhibitory activity. Phyto. Res. 2007;21:1142-1145.
14. Sampath Kumar KP. Indian traditional herbs *Adhatoda vasica* and its Medicinal application J. Chem. Pharm. Res. 2010;2(1):240-245.
15. Rajendra Prasad Gujjetti, Estari Mamidala. phytochemical screening and thin layer chromatographic studies of Aervallanata r4oot extract. IJIRSET, 2013, 2(10), ISSN 2319-8753
16. Sanjeetkumar Monalisa Sarangi. A tool of biotechnology for isolation of bioactive compounds from medicinal plant. Int. J Pharm. Sci. Rev. Res. 2013;18(1):126-132.
17. Sharma PC, Yelne MB, *et al*. Database on Indian medicinal plants used in Ayurveda, vol.1 New DELHI, India: published by central council for research in Ayurveda and Siddha (CCRAS), Department of Indian system of Medicine and homeopathy, Ministry of Health and Family Welfare (Govt. of India); c2000.
18. The Wealth of India A dictionary to Indian raw materials and industrial products. First Supplement Series (Raw Materials). New Delhi: India, National Institute of Science Communication and Information Resources (NISCAIR), CSIR, 2003, 4: (J-Q).
19. Thakur RS, Puri HS, Husain A. Major Medicinal Plants of India. Lucknow, India: Published by Central Institute of Medicinal and Aromatic Plants (CIMAP); c1989.
20. Gupta A, Khamkar P, Chaphalkar SR. immunomodulatory and anti-inflammatory activity of aqueous extracts of leaf, stem and root of Ficusreligiosa on human whole blood and peripheral blood mononuclear cells. Int. J of medicine and pharmaceutical research. 2014b;2:732-739.
21. Gupta A, Chaphalkar SR. Use of flow cytometry to measure the immunostimulatory activity of aqueous extract of *Jasminum auriculatum*. International Journal of Current Advanced research. 2015a;4:87-91.
22. Gupta A, Chaphalkar SR. Flow cytometry-based assay of formulation from *Syzygium cumini* in human whole blood and gylcosylated red blood cells. Journal of Pharma research. 2014b;3:265-270.
23. Gupta AK, Tandon N, *et al*. Quality standards of Indian medicinal plants\ volume 3. New delhi, India: Published by Indian council of medical research (ICMR); c2005.

24. Sakat S, Tupe P, Hule A, Juvekar A. Anti-inflammatory potential of flavonoid fraction of Tamarindus indica Linn (seeds). Planta Medica. 2010 Aug;76(12):SL_20.
25. Leelaprakash G, Dass SM. *In vitro* anti-inflammatory activity of methanol extract of Enicostemma axillare. International Journal of Drug Development and Research. 2011 Jul;3(3):189-96.
26. Mizushima Y, Kobayashi M. Interaction of anti-inflammatory drugs with serum proteins, especially with some biologically active proteins. Journal of Pharmacy and Pharmacology. 1968 Mar;20(3):169-73.
27. Shinde UA, Phadke AS, Nair AM, Mungantiwar AA, Dikshit VJ, Saraf MN. Membrane stabilizing activity-a possible mechanism of action for the anti-inflammatory activity of Cedrus deodara wood oil. Fitoterapia. 1999 Jun 1;70(3):251-7.
28. Oyedepo DO, Gifford P. Breaking financial hardship. Dominion Publishing House; 1995.