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EXTRACTION, EVALUATION AND IDENTIFICATIONOF APAMARGA EXTRACT ALONG WITH ISOLATION OF ACTIVE COMPOUND RESPONSIBLE FOR ANTI-INFLAMMATORY ACTIVITY

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ABSTRACT

This study investigates the anti-inflammatory properties of Achyranthes aspera, a widely recognized medicinal plant in the Amaranthaceae family. Traditional applications include treatment for various ailments such as dropsy, skin eruptions, and as an antidote for snake bites. The primary objective was to extract, evaluate, and isolate the active compounds responsible for the plant's anti-inflammatory effects. Extraction was performed using pet ether, chloroform, and ethanol, with the highest yield obtained from the ethanol extract, which also contained significant phytochemical constituents including carbohydrates, flavonoids, phenols, saponins, terpenoids, and tannins. Quantitative analysis revealed a total phenolic content of 0.935 mg/100 mg and flavonoid content of 0.900 mg/100 mg in the ethanol extract. The anti-inflammatory efficacy of both the ethanol extract and oleanolic acid was assessed through albumin denaturation inhibition and anti-proteinase assays. Results demonstrated significant inhibition rates, with oleanolic acid achieving 93.56% and 95.90% inhibition at doses of 80 and 100 µg/kg, respectively, alongside albumin denaturation inhibition rates of 85.48% and 93.65%.

KEYWORDS: Anti-inflammatory, Phenolic, Flavonoid, Apamarga, Isolation, Dropsy, Antidote, Evaluation.

INTRODUCTION

Humankind is almost totally dependent on plants for the essentials of their existence by way of food, clothing, and shelter. Plants are also an essential source of fine chemicals, which find their applications in pharmaceutical industries across the globe. Plants have been the traditional source of raw materials and finished medicinal for many ages. A rich heritage of information on preventive and curative medicines was available even in ancient scholastic work, which was included in the Atharva Veda, Charak Samhita, Sushruta Samhita, "etc". (WHO Regional Office for the Western Pacific, 1993). The study of disease and their treatment must have also been contemporary with the dawn of human intellect.

In India, the Science of Ayurveda offered a system of medical treatment, and the majority of the illness was treated from plants. During the last few ages, much work has been done in the field of natural products. It is reported that 41% of prescriptions in the USA and 90% in Europe contain constituents from natural products which shows an increasing trend of using natural products during the period under study (**Kumar, S. *et al.*, 2017**).

Ayurveda and Siddha systems of medicine, the traditional heritage of India, include many time tested medicinal plant drugs for several diseases to which there is no answer in modern medicine till today. According to WHO, around 3.5-4 billion people across the world using herbal medicines and a major portion of traditional system of medicine involves the plant extract derived medicines, which may know as the modern-herbal medicine (**Ravishankar, B. *et al.*, 2007**).

Natural products have also been integrated into many modern compositions. Essential drugs that have come from plant sources in clinical use are cinchona, opium, ergot, rauwolfia, “etc”. All were known to healers in traditional medicine before their introduction to modem medicine. As the medicinal value of traditional Indian medicine cannot be ignored,researchers are gradually becoming interested in the identification of active principles in their extracts with intensive follow-up study (both *in-vivo* and *invitro*) of their mechanisms of action (**Yuan, H. *et al.*, 2016**).

In turn, the inflammatory response sets into motion a complex series of events, which heal and restore the injured tissue. Cell repair begins during the active phase of inflammation but usually complete after the injurious

influence has been neutralized. Damaged cells and tissues are repaired thereby. Both inflammation and repair generally serve a useful purpose. Without inflammation, bacterial infections would remain encountered, wounds would never heal, and injured tissues and organs might have permanently damaged. However, inflammation may be hazardous.(**Ravishankar, B. *et al.*, 2007**).

Material and Methods Instruments and Chemicals

1. Selection, Collection & Identification of Herbs

The medicinal plant *Achyranthes aspera* roots (100 gm) were collected locally from Bhopal,
M.P. After cleaning, plant parts were dried under shade at room temperature for 3 days and then in oven at 45°C till complete dryness. Dried plant parts were stored in air tight glass containers in dry and cool place to avoid contamination and deterioration.

2. Extraction of chemical constituents using different solvents by Soxhlet apparatus Coarsely powered roots of *Achyranthes aspera* (100 gm) was then extracted by successive extraction using different organic solvents, like defatted with petroleum ether and successively extracted with chloroform and ethanol for 36 hrs uses soxhlet apparatus. To ensure complete extraction each extract was evaporated to dryness under reduced pressure by rotary evaporator and the resulted dried residue was stored in air-tight container for further use **(Kokate, 1994, Abubakar AR, *et al.*, 2020, C.K. Kokate, 2014).**

% $yield$ = Actual yield Theoretical yield x 100

3. Phytochemical Screening

Experiment was performed to identify presence or absence of different phytoconstituents by detailed qualitative phytochemical analysis. The colour intensity or the precipitate formation was used as medical responses to tests. Following standard procedures were used **(C.K.Kokate, 2014)(Evans, 2009).**

Tests for Alkaloids

- **Dragendorff'sTest:** 1 ml of extract was taken. Alcohol was mixed and was shaken well with little drops of acetic acid and Dragendroff's reagent. The presence of alkaloids indicates by the presence of an orange red precipitate.

- **Mayer's Test:** 1 ml of extract was dissolved in acetic acid with a few drops of Mayer's reagent added to it. The presence of alkaloids was indicated by the formation of a dull white precipitate.

- **Wagner's Test:** In acetic acid 1ml of extract was dissolved. Few drops of Wagner's reagent were added. The presence of alkaloids indicated the reddish-brown precipitate.

- **Hager's Test:** 1-2 ml of extract was dissolved in acetic acid. To it 3 mL of Hager's reagent was added; the formation of yellow precipitates indicatedby the presence of alkaloids.

Test for Glycosides

- **Borntragers Test:** Dilute sulphuric acid was added to 3 ml of test solution dilute sulfuric acid was added. It was boiled for 5 minutes and then filtrate was obtained. To the cold filtrate, equal amount of benzene or chloroform was added to the cold filtrate and shaken well. Separation of organic solvent layer was obtained and then ammonia was added to it.

Test for Carbohydrates

- **Molisch'sTest:** The aqueous solution of the extract to 1 ml were mixed with few drops of Molish reagent (naphthol) and conc. H_2SO_4 (sulphuric acid) was added dropwise along the wall of the test tube. When two liquid mixes up, formation of purple colour ring at the junction occurs. It indicates the presence of carbohydrates.

- **Fehling's Test:** Equal amount of Fehling A and Fehling B solution were mixed (1ml each) and 2ml of aqueous solution of extract was added. Boil it for 5-10 minutes on water bath. Formation of reddish brown coloured precipitate due to cuprous oxide formation showsthe presence of reducing sugar.

- **Benedict's test:** In a test tube equal amount of Benedict's reagent and extract were mixed and heated for 5-10 minutes in the water bath. Depending on the amount of reducing sugar present in the test solution, appears green, yellow or red which shows the presences of reducing sugar.

- **Barfoed's Test:** In the aqueous solution of extract, 1 ml of Benedict solution was added and heated for boiling. In the presence of monosaccharides red colour indication was seen due to formation of cupric oxide.

Test for Flavonoids

- **Shinoda's Test:** A few magnesium turnings and little drops of concentrated hydrochloric acid to 1 ml of extract in alcohol were added. It was heated on a water bath. when the formation of red to pink colour occurred, indicated the presence of flavonoids.

4. Quantitative Phytochemical Estimation

6.4.1 TPC

The total phenolic content of *Apamarga* extract was examined by using the Folin- Ciocalteu Assay. The *Apamarga* extracts (0.2 mL from stock solution) were mixed with 2.5 ml. of Folin-Ciocalteu Reagent and 2mL of 7.5% sodium carbonate. This mixture was diluted up to 7 ml. with distilled water. The solutions were then allowed to rest at room temperature for two hours before being measured spectrophotometrically at 760 nm. Calibration curves were created using standard solutions of Gallic Acid Equivalent (GAE) mg/gm. Gallic aid was concentrated to 20, 40, 60, 80, and 100 µg/mL. The Folin-ciocalteu reagent is sensitive to reducing compounds

including polyphenols. They produce a blue colour upon reaction. This blue colour was measured spectrophotometrically (**Tangco *et al*., 2015)**.

5. DPPH

The antioxidant activity of *Apamarga* extract was measured using the DPPH. A 1 mg/ml methanol solution of extracts/standard was made.

Various concentrations of *Apamarga* extract/standard (20-100µg/ml) were produced from a 1mg/mL stock solution with 2mL of 0.1mM DPPH solution added. After overtaxing the mixture, it was allowed to remain at the room temperature for half an hour in a reasonably dark place before being measures at 517 nm with a UV spectrophotometer (Shimadzu 1700). Incubate for 30 minutes at room temperature in the dark with 3 ml of 0.1mM DPPH solution as a control. The absorbance of the control was measured against methanol (as a blank) at 517 nm. **(Athavale *et al*., 2012).**

The percentage of antioxidant activity for the sample/standard was calculated using the following formula:

% Inhibition = [(Ab of control- Ab of sample)/ Ab of control x 100]

6. Isolation of extracted active compound by HPTLC

- **Chromatography condition**

Determination of the phytochemical profile of oleanolic acid in *Achyranthes aspera* was performed by using advanced chromatographic technique like High performance thin layer chromatography (HPTLC) (Kamboj & Saluja, 2017). TLC co- chromatography was performed on ethanolic extract and standard oleanolic acid 10µl of the sample solution was spotted on the TLC plate along with the standard solution of oleanolic acid. Stationary phase consisted of TLC aluminium sheets pre coated with silica gel 60 F254, thickness 0.1mm, (10×10 cm) (Merck), Mobile phase consisted of toluene: methanol: ethylacetate (5: 0.5: 4.5).

Aliquots of each of the extracts were separately applied (Samples and standard) to the plate as 8 mm wide band with an automatic TLC applicator Linomat-V (CAMAG), 8 mm from the bottom. Densitometric scanning was performed on CAMAG scanner IV at 254.0 nm. The plates were prewashed by methanol and activated at 60 °C for 5 min prior to chromatography **(Sonia K. *et al*., 2017)**.

7. Physical characterization of isolated compound

7.1 Organoleptic Properties

Organoleptic properties of isolated compound were observed by visual observation. The organoleptic studies of oleanolic acid like general appearance like color, odor, state, etc. were observed.

7.2 Solubility study

Qualitative solubility of oleanolic acid in different solvents was determined according to USP NF, 2007. Approximately 1 mg of oleanolic acid was weighed and transferred into a 10 ml test tube and dissolved in the respective solvents (1 ml each of methanol, acetone, DMSO, chloroform and water) **(Jain and Verma 2020).**

7.2.1 pH determination

pH was determined by Electrochemical method. Digital pH meter is used to determine the pH of isolated compound. After the meter has been turned on, allowed to stabilize as necessary and properly calibrated, begin by rinsing the probe with deionized or distilled water and blotting the probe dry with lint-free tissue paper. Immerse the sensing tip of the probe in the sample and record the pH reading and Rinse the probe, blot dry and repeat step 2 on a fresh portion of sample. The two readings should agree to within the accuracy limits of the meter.

7.2.2 Rf value

TLC is a chromatographic technique used for the separation of compounds. TLC method was set up using different solvents for different compounds depending on the polarity and non- polarity of the compounds. N-hexane and ethyl acetate (7:3) were used as solvent system. R f value for each synthesized compound was calculated **(Pobłocka- Olech, L *et al*., 2018)**.

7.3 Spectral analysis by UV, IR and NMR

7.3.1 Determination of Lambda max and calibration curve

About 5mg of isolated compound (oleanolic acid) was weighed and transferred into 5ml volumetric flask (Separately). The volume was made up to 5ml using respective solvent to obtain a solution that has a concentration 1000 µg/ml. 1ml of this stock solution was taken and then diluted up to 10 ml using methanol solvent to obtain a solution that has a concentration 100 µg/ml which is standard stock solution**.**

- **Lambda max**

From the above stock solution (oleanolic acid) 1.0 ml sample was transferred into a 5 ml volumetric flask and the volume was made up to mark with solvent to prepare a concentration of 20 µg/ml. The sample was scanned by UV-VIS Spectrophotometer in the range of 200- 400 nm for isolated compound, using methanol solvent as a blank. The wavelength corresponding to the maximum absorbance (max) was found **(Kumbhar and Salunkhe 2013).**

- **Preparation of calibration curve**

The prepared stock solution was further diluted with solvent to get working standard solution of 5, 10, 15, 20, 25 and 30µg/ml of oleanolic acid to construct Beer's law plot for the pure drug, the absorbance was measured, against solvent as blank. The standard graph was plotted by taking concentration of drug on X-axis and

absorbance on Y-axis in the concentration range of 5-30µg/ml (**Behera *et al.*, 2012).**

RESULTS AND DISCUSSION

1. Preparation and extraction of plant materials

Extraction of *Achyranthes aspera* roots

Shade dried powdered material was extracted in petroleum ether, chloroform and ethanol by using Soxhlation method for 48 hours, which was then filtered and dried using vacuum evaporator at 40°C and then the % yield of the plant material was determined.

2. Percentage of yield of crude extract by maceration process

Table 01: Yield percentage of *Apamarga* crude extracts.

S. No	Plant name	Solvent	Theoretical weight	Yield (gm)	% yield
1	*Apamarga*	Pet ether	289.12	1.64	0.56%
2		Chloroform	300.0	1.26	0.42%
3		Ethanol	298.35	6.22	2.08%

In phytochemical extraction the percentage yield is very crucial in order to determine the standard efficiency of extraction for a specific plant, various sections of the same plant or different solvents used. The yield of extracts received from the *Achyranthes aspera* was 0.56 % in petroleum ether, and 0.42 % in chloroform and 2.08 % in ethanol.

Table 02: Showing % yield of the crude extract of *Achyranthes aspera* extracts.

S. No.	Solvent	Weight of the powdered material (gm)	Volume of solvent (ml)	Weight of the extract (gm)	% Yield* (W/W)
1.	Pet. Ether	150	500	4.025	2.68 %
2.	Chloroform	150	500	33.635	22.42 %
4	Ethanol	150	500	48.276	32.18%

a. Preliminary phytochemical screening

Experiment was performed to identify absence or presence of different phytoconstituents by detailed qualitative phytochemical analysis. Medical reactions to testing were based on colour intensity or precipitate formation. The following standard methods were used **(Kokate *et al.*, 2000).**

Table 03: Phytochemical testing of *Achyranthes aspera* extract.

S. No.	Experiment	Presence or absence of phytochemical test		
		Pet. Ether extract	Chloroform extract	Ethanolic extract
1.	**Alkaloids**			
1.1	Dragendroff's test	-	+	+
1.2	Mayer's reagent test	-	+	+
1.3	Wagner's reagent test	-	+	+
1.3	Hager's reagent test	-	+	+
2.	**Glycoside**			
2.1	Borntrager test	-	-	+
2.2	Killer-Killiani test	-	-	+
3.	**Carbohydrates**			
3.1	Molish's test	-	+	+
3.2	Fehling's test	-	+	+
3.3	Benedict's test	-	+	+
3.4	Barfoed's test	-	+	+
5.	**Flavonoids**			
5.1	Shinoda's Test	+	+	+
6.	**Tannin and Phenolic Compounds**			
6.1	Ferric Chloride test	-	+	+
+6.2	Gelatin test	-	+	+
7.	**Saponin**			
7.1	Froth test	+	+	+
8.	**Test for Triterpenoids and Steroids**			
8.1	Salkowski's test	+	+	+

Qualitative phytochemical screening of the roots extracts of *Achyranthes aspera* revealed that carbohydrates, flavonoids, phenols, saponins, terpenoids and tannins were present in ethanolic extracts.

b. Quantitative analysis

Preliminary phytochemical tests on crude extracts indicated the existence of phenolics and flavonoids in plant material. To assess their level, assays were done for total phenolic (TPC) and total flavonoid content (TFC).

i. Total Phenolic content (TPC) estimation Table 04:- Standard table for Gallic acid.

S. No.	Concentration (µg/ml)	Absorbance
1.	20	0.149
2.	40	0.185
3.	60	0.201
4.	80	0.235
5.	100	0.278

ii. Total Phenolic Content in extract Table 05: Total Phenolic Content

S. No	Absorbance	TPC in mg/gm equivalent of Gallic Acid
1	0.141	56 mg/gm
2	0.185	
3	0.193	

Table 06: Whole Flavonoid Content of *Apamarga* extract.

Extracts	Total Flavonoid content (mg/gm equivalent of rutin)
Methanol	17.16

***In vitro* Antioxidant Assays**

In the current investigation, the in vitro anti-oxidant activity of *Apamarga* extract was evaluated by DPPH radical scavenging activity. The results are summarized in Tables.

c. HPTLC

The results from HPTLC chromatogram for *Achyranthes aspera* extract were analyzed at 254 and 366nm. The extract evidenced 2 bands with Rf value of 0.23. The bands with Rf value of 0.23 was identified as presence of oleanolic acid as compared with standard band. The present HPTLC method for the quantification of oleanolic acid in ethanolic extract revealed as simple, accurate and precise.

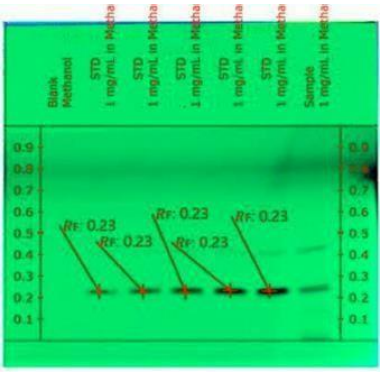

Figure 2: Chromatogram obtained from separation of plant extract and visualized under UV light of wavelength 254 nm.

d. Physical characterization of isolated compound

Table 07: Organoleptic properties of isolated compound (Oleanolic acid).

Drug	Organoleptic properties	Observation
Oleanolic acid	Color	White
	Odor	Odour less
	Appearance	Solid powder
	State	Solid

An evaluation of the isolated compound organoleptic qualities, including Appearance, color, odor, and state, was conducted. Oleanolic acid was discovered to have a white color to it when tested. Oleanolic acid has an odorless and has a solid state powder form, according to research conducted on it. Oleanolic acid exhibited the same appearance, color, odor and state as the I.P. requirements for these characteristics.

i. Solubility study

Table 08: Solubility study of Oleanolic acid.

Drug	Solvents	Observation/Inference
Oleanolic acid	Water	Slightly soluble
	Ethanol	Freely Soluble

	Methanol	Soluble
	Chloroform	Soluble
	DMSO	Freely soluble

The solubility of Oleanolic acid was determined in various non-volatile or volatile liquid vehicles such as Dimethyl sulfoxide, methanol, ethanol, chloroform, and water shown in Table 19. From the results, it was observed that the drug is freely soluble in Dimethyl sulfoxide and ethanol and soluble in methanol and chloroform.

ii. pH determination

Table 09: pH of Oleanolic acid.

S. No	Drugs	References	Observed
1.	Oleanolic acid	7.0-7.2	7.1

The digital pH meter used to determine the pH of a substance. The pH of the Oleanolic acid was found to be 7.1, which is well within the limits of the drug specification.

CONCLUSION

The extraction of *Achyranthes aspera* roots was performed in pet ether, chloroform and ethanol. The highest yield was found in the ethanol extract. Qualitative chemical tests were conducted on all the extracts and the maximum chemical constituents were observed in ethanolic extract i.e. carbohydrates, flavonoids, phenols, saponins, terpenoids and tannins. Quantitative analysis conducted in ethanolic extract and the total total phenolic and flavonoid content was observed as 0.935mg/ 100 mg and 0.900mg/ 100 mg, respectively.

Isolation of the active constituent was performed with the help of TLC and HPLC and the spot was observed on the HPTC and the RF value was 0.34. Physical characterization of the isolated compound (oleanolic acid) was performed and the solubility was found in ethanol, DMSO, and dimethyl formamide. The melting point was found to be at 308°C. The structure of the isolated compound was investigated with spectral analysis like UV, IR and NMR. All the spectral data revealed that the isolated compound was oleanolic acid.

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