CHAPTER 3

ISOLATION, CHARACTERIZATION AND ESTIMATION OF OLEANOLIC ACID FROM *ACHYRANTHES ASPERA* LINN

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3.1 Introduction to plant:

3.1.1 Introduction to family: Amaranthaceae

Amaranthaceae is a small compact family having 65 genera and 850 sps, extensively distributed in tropical and temperate region of the globe. The family is very well represented in tropical America, Africa and India. In our country Amaranthaceae is represented by 17 genera and more than 50 sps chiefly occurring in warmer regions. Members of this family are mostly herbs or shrubs. Leaves are usually hairy, inflorescence usually hairy, spike head or cymose panicle, flowers bisexual with membranous perianth, bracts and bracteoles. Perianth 5, antisepalous, ovary superior, bicarpellary, syncarpous, monolocular with a single basal ovule. Fruit is a one seeded nut or utricle, seeds have a mealy endosperm. (Kiritkar and Basu et al., 1975)

3.1.2 Introduction to genus: Achyranthes

Herbs with opposite, petiolate, entire leaves. Inflorescence a more or less slender spike, terminal on the stem and branches, the flowers at first congested and patent, finally usually laxer and deflexed; bracts deltoid or ovate, the midrib excurrent in a spine. Flowers solitary in the bracts, hermaphrodite, bi-bibracteolate. Perianth segments 4–5, 1–3(5) nerved, narrowly lanceolate, acuminate, mucronate with the excurrent midrib, indurate in fruit especially at the base. Bracteoles spinous-aristate with the excurrent midrib, the lamina forming short and free to longer and adnate membranous wings. Stamens 2–5, filaments filiform, monadelphous, alternating with quadrate to broadly quadrate-spathulate pseudostaminodes, these are simple and dentate or fimbriate, or commonly furnished with a variably developed dorsal scale; anthers bilocular. Style
slender, stigma small, truncate-capitate. Ovary with a solitary pendulous ovule, the ovary wall very thin in fruit. Entire flower with bracteoles falling with the ripening of the cylindrical seed, the deflexed bracts persistent. Endosperm copious. (Kiritkar and Basu et al., 1975)

3.1.3 Vernacular names:

Ben. Apaang, Chirchiti; Eng.: Prickly chaff flower, Rough chaff tree; Guj.: Aghede, Angheda, safed Aghedo, Anghedo; Hind.: Chirchira, Chirchitta, Latjira, Apamarg; Kan.: utranigida, uttaranee; Mal.: Kadaladi; Mar.: Aghada, Aghara; Ori.: Apamaranga, Apamargo; Punj.: Chichra, Kutri; Tam.: Chirukadaladi, Naayurivi, Nayuruvi; Tel.: Apamargamu, Uttareeni, Uttarene, Uttareni. (Ganguly et al., 2004)
3.1.4 Scientific classification:

Kingdom: Plantae (plants)
Subkingdom: Tracheobionta (Vascular plants)
Superdivision: Spermatophyta (seed plants)
Division: Magnoliophyta (Flowering plants)
Class: Magnoliopsida (Dicotyledons)
Subclass: Caryophyllidae
Order: Caryophyllales
Family: Amaranthaceae
Genus: Achyranthes
Species: Achyranthes aspera
Variety: Achyranthes aspera LINN

3.1.5 Habit and Habitat

An erect or procumbent, annual or perennial herb, often with woody base commonly found as a weed on waysides and waste places throughout India upto an altitude, 2100 m.

3.1.6 Plant description:

Main root, long cylindrical, thick, secondary and tertiary roots present, slightly ribbed, yellowish brown in colour, odour slight; taste slightly sweet and mucilaginous. Stem yellowish brown, erect, branched, cylindrical, hairy, solid, about 60 cm high. Leaves petiolate, alternate, elliptic-ovate or sub-orbicular, acute, entire, pubescent above and usually white woolly beneath. Flowers greenish-white, in small dense axillary heads or
spikes. Bracts and bracteoles persisting, ending in a spine. Seeds subcylindric, truncate at the apex, rounded at the base, black and shining. (Indian Herbal Pharmacopeia )

3.1.7 Uses Ascribed:

The plant is reported to be pungent, astringent, pectoral and diuretic. It is used as an emmenagogue, in piles and skin eruption. The decoction of the plant is useful in pneumonia and renal dropsy while the juice of the plant is used in ophthalmia and dysentery. The leaves are used in the treatment of gonorrhoea, and excessive perspiration. The extract is used for leprosy and the heated sap for tetanus. The root is astringent, the paste is applied to clear opacity of cornea, and for wound healing purposes. The root is also reported to be useful in cancer. A decoction of the root is used for stomach troubles and aqueous extract is used in bladder stones. The flowers, ground and mixed with curd and sugar, are given for menorrhagia. The flower tops are stated to be employed for the treatment of rabies. The seeds are said to be emetic and used in hydrophobia (Ganguly et al., 2004).

3.1.8 Various species of Achyranthes:

There are seven species of Achyranthes:

(1) Achyranthes aspera
(2) Achyranthes atollensis
(3) Achyranthes bidentata
(4) Achyranthes canescens
(5) Achyrathes japonica
(6) Achyranthes splendens
(7) Achyranthes mutica
3.2 Review of literature of plants:

3.2.1 Phytochemical reviews:

The plant was reported to yield a water-soluble base and chloroform soluble base. The former was earlier designated as achyranthine (Basu et al., 1957a) and was characterized as a betaine derivative of N-methylpyrrolidine-3-carboxylic acid (Basu, 1957). Later studies by Kapoor and Singh (1966) showed that water-soluble base was betaine and not achyranthine. The chloroform soluble basic fraction was shown to be a mixture of two uncharacterized alkaloidal entities (Kapoor and Singh, 1967).

The ethanol extract of the plant contained alkaloids and saponins while flavanoids and tannins were found absent (Kumar et al., 1990).

The shoot yielded a new aliphatic dihydroxyketone, characterized as 36, 47-dihydroxyhenpentacontan-4-one together with tritriacontanol (Misra et al., 1991); an essential oil; a new long chain alcohol characterized as 17-pentatriacontanol (Misra et al., 1992); four new compounds characterized as 27-cycloheptacosan-7-oleanolic acid, 16-hydroxy-26-methylheptacosan-2-one (Misra et al., 1993), 4-methylheptatriacont-1-en-10-ol and tetraccontanol-2 (Misra et al., 1996).

Various parts of the plant, viz, seeds, stem, leaves (Banerji et al., 1971) and root (Banerji and Chadha, 1970) were reported to contain ecdysterone. The chloroform extract of the stem led to the isolation of pentatriacontane, 6-pentatriacontanone, hexatriacontane and triacontane (Ali, 1993). The inflorescence was reported to contain flavanoids and alkaloids (Sinha and Dorga, 1985).
The food value of the seeds in terms of its protein quality was studied. The composition of the seeds showed close similarity to Bengal gram with a protein content of 24.8 and calorific value of 3.92/g. The hydrosylate contained the usual aminoacids. The values obtained for ten essential amino acids and cystine showed that the seed protein compared favourably with Bengal gram in its leucine, isoleucine, phenylalanine and valine content, while its tryptophan and sulphur amino acid (methionine and Cystine) content were higher than most of the pulses. It was, however, deficient in arginine, lysine and threonine as compared to whole egg protein. (Satyanarayan et al., 1964).

The defatted seeds were reported to yield a saponins in an yield of 2 per cent which was identified as oleanolic acid-oligosaccharide. The sugar moiety of the saponins was composed of glucose, galactose, xylose and rhamnose (Gopalachari and Dhar, 1952, 1958). Khastgir and associates (1958) isolated a crude sapogenin fraction from the seeds, which yielded oleanolic acid. Later, investigation led to the isolation of two oleanolic acid based saponins, saponins A (Fig 2) and saponin B (Fig 3) which were characterized as \( \alpha-\alpha\)-rhamnopyranosyl \( (1 \rightarrow 4)\)-\( \beta\)-D-glucopyranosyl \( (1 \rightarrow 4)\)-\( \beta\)-D-glucuronopyranosyl \( (1 \rightarrow 3)\)-oleanolic acid and \( \beta\)-D-galactopyranosyl \( (1 \rightarrow 28)\) ester of saponin A, respectively (Hariharian and Rangaswamy, 1970). In another study, the total saponins were hydrolyzed with acid and the genin was identified as oleanolic acid (Batta and Rangaswami, 1973). A rapid procedure for the separation of triterpenoid saponins based on partition chromatography from the plant has been described (Sarkar and Rastogi, 1960). The seeds contained hentriacontane, 10-octacosane, 10-triacosanone and 4-tritriacontanone (Ali, 1993).
The fatty oil constituent of the seed oil comprised of lauric, myristic, palmitic, stearic, arachidic, behenic, oleic and linoleic acids. (Daulatabad and Ankalgi, 1985).

The unripe fruits yielded two new saponins (C and D) (Fig 4 & 5) which were identified as \( \beta - D - \text{glucopyranosyl ester of } \alpha - L - \text{rhamnopyranosyl} (1\rightarrow 4)\beta - D - \text{glucurranopyranosyl} (1\rightarrow 3)\) oleanolic acid and \( \beta - D - \text{glucopyranosyl ester of } \alpha - L - \text{rhamnopyranosyl} (1\rightarrow 4)\beta - D - \text{glucopyranosyl} (1\rightarrow 4)\beta - D - \text{glucurranopyranosyl} (1\rightarrow 3)\) oleanolic acid (Sheshadri et al., 1981).

The chemical constituents of the root varied in different preliminary studies carried out. The root was found to contain oleanolic acid (Fig 6) as the aglycone from the saponin fraction (Khastgir and Sengupta, 1958). Both root and shoot of the plant were found to contain saponins and alkaloids but no flavanoids (Sinha and Dogra, 1985). In another study, the root of the plant was found to contain alkaloids but indicated absence of saponins and tannins (Joshi and Sabnis, 1989). In yet another preliminary chemical study, the root was reported to contain alkaloids, flavanoids, saponins, steroids and terpenoids. Glycosides were found to be absent (Agrawal et al., 1989). Isolation of \( \beta - \text{sitosterol} \) was also reported from the root. (Misra et al., 1993). Three bisdesmosidic saponins (\( \beta - D - \text{glucopyranosyl} 3 - (O - \alpha - L - \text{rhamnopyranosyl}(1\rightarrow 3)(O - \beta - D - \text{glucopyranosyloxy})\) oleanolate, \( \beta - D - \text{glucopyranosyl} 3 - (O - \beta - D - \text{galactopyranosyl}(1\rightarrow 2)(O - \beta - D - \text{glucopyranosyloxy}) \) oleanolate and \( \beta - D - \text{glucopyranosyl} 3 - (O - \beta - D - \text{glucopyranosyloxy}) \) oleanolate, 20 hydroxyecdysone, a steroid and quercetin-3-O-\( \beta - D - \text{galactoside} \) was isolated from the aerial parts of A.aspera growing in Africa, particularly in Ethiopia. (Olaf K et al, 2000). 3-Acetoxy-6-benzoyloxyapangamide (1) has been isolated from an ethyl acetate extract of the stem of Achyranthes aspera (Muhammed et al, 2005)

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Fig: Structure of Saponin A-D and Oleanolic acid

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<td>3</td>
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3.2.2 Pharmacological reviews:

3.2.2.1 General pharmacology:

The mixture of saponins isolated from the seeds caused a significant increase in force of contraction of the isolated heart of frog, guinea pig and rabbit. The stimulant effect of the lower dose (1 to 50 µg) of the saponins was blocked by pronethalol and partly by mepyramine. The effect of higher dose was not blocked by pronethalol. The saponins increased the tone of the hypodynamic heart and also the force of contraction of failing papillary muscle. The effect was quicker in onset and shorter in duration in comparison to that exerted by digoxin (Gupta et al., 1972a). the effect of saponins on the phosphorylase activity of the perfused rat heart has been investigated and compared with that of adrenalin. The saponin has been found to stimulate the phosphorylase activity of the heart and its effect was comparable to that of adrenaline (Ram et al., 1971).

The ethanolic extract of the plants (Dhar et al., 1968) and leaves (Aswal et al., 1996) were screened for preliminary biological activities. The former extract showed hypoglycemic activity in rat. It was devoid of antibacterial, antifungal, antiprotozoal, anthelmintic, antiviral and anticancer activities and effects on isolated guinea pig ileum, respiration, CVS and CNS in experimental animals. The MTD of the extract was found to be 1000mg/Kg b.w. orally in mice (Dhar et al., 1968). The leaf extract was found to be devoid of antiprotozoal and antiviral activities and effects on respiration, preganglionically stimulated nictating membrane, CVS and CNS in experimental studies. The LD₅₀ of the latter extract was >1000mg/Kg i.p. in mice (Aswal et al., 1996)
3.2.2.2 Antifertility

The alkaloidal fraction obtained from the alcoholic extract of the root bark inhibited the response of oxytocin in isolated rat uterus. This fraction did not inhibit the responses to serotonin and acetylcholine in rat uterus and to histamine in guinea pig uterus (Gupta and Khanijo, 1970).

The crude benzene extract of the stem was found to have potent abortifient effect in mice (Pakrashi et al., 1975). In an attempt to locate the active principle, various chromatographic fractions were tested for antifertility activity in female mice. The maximal activity was found to be located in the fraction eluted with 50 percent benzene in petroleum ether (Pakrashi et al., 1975b).

The benzene extract of the plant (excluding root) also revealed 66.6 per cent anti-implantation activity in female albino rats (Mathur et al., 1983). The ethanolic extract of the plant (excluding root) at a dose of 100-200 mg/Kg b.w. administered orally revealed 60 percent anti-fertility activity on early pregnancy in rats. Further, the plant also showed potent activity at secondary testing level (Prakash et al., 1987).

The methanolic extract of the root revealed 60 percent anti-implantation activity in rats while the acetone extract of the root prevented implantation in 50 percent of rats (Prakash, 1986).

The n-butanol fraction of the aerial parts prevented pregnancy in adult female rats when administered orally at a daily dose of 75 mg/Kg or more on 1-5d post coitum, but was ineffective in hamsters up to 300mg/Kg dose (Wadhwa et al., 1986).
3.2.2.3 Anti-inflammatory activity:

The water soluble alkaloid achyranthine was screened for its anti-inflammatory and anti-arthritis activity against carrageenan induced foot oedema, granuloma pouch, formalin induced arthritis and adjuvant arthritis in rats. It showed significant anti-inflammatory activity in all the four models employed but less active than phenylbutazone and betamethasone. Further, achyranthine significantly reduced the weight of adrenal gland, thymus and spleen and raised the adrenal ascorbic acid and cholesterol contents. The effects were qualitatively similar to betamethasone. All the three drugs tested reduced food intake but had no significant effect on urinary and fecal output and on mortality rate. Incidence of gastric ulcers was maximum with betamethasone and minimum with achyranthine (Neogi et al., 1969)

3.2.2.4 Diuretic activity:

Earlier studies by Bhide et al. (1958) on potassium diuretics indicated that the diuretic activity of the ash of the plant may be due to high potassium (44 percent) content. Effect of a saponin isolated from the seed on urine output in albino rats has been investigated in comparison to mersalyl and acetazolamide. The saponins in 10-20 mg/kg i.m. doses caused significant increase in urine output after 2,6 and 24 hr as compared to untreated control rats. The diuretic affect was comparable to that observed with 3mg/Kg dose of mersalyl. The optimum dose of the saponins was 10mg/Kg. After oral administration of the saponins (5-10 mg/Kg) in rats, a significant increase in urine output was observed which was comparable to that of 10mg/Kg oral dose of acetazolamide. The diuretic effect
of the saponins, like acetazolamide was associated with an increase in the excretion of sodium and potassium in the urine (Gupta et al., 1972b)

### 3.2.2.5 Antimicrobial:

The aqueous solution of the base achyranthine as well as the entire plant showed antibacterial activity against *Staphylococcus aureus*, *Streptococcus haemolyticus* and *Bacillus typhosus* (Basu et al., 1957b) while the alcoholic and the aqueous extract of the leaves showed antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* (George et al., 1947).

The seeds growing on cattle dung revealed antibacterial activity against bacterial strains of *Bacillus subtilis*, *Pseudomonas cichorii* and *Salmonella typhimurium* (Sushil Kumar et al., 1997). In another study, the 80 percent ethanolic extract of the leaves and stem of the plant inhibited *Bacillus subtilis* and *Staphylococcus aureus* bacterial strains at a concentration of 25 mg/ml (Valsaraj et al., 1997).

The aqueous leaf extract in in-vivo studies showed antibacterial activity against *Proteus vulgaris* at concentration of 5000ppm. The extract was inactive against *Klebsiella aerogenes*, *Pseudomonas aeruginosa* and *Escherichia coli* (Perumal Samy et al., 1998).

The aqueous residues of another sample of the plant leaves were found devoid of any activity against *Alkaligenes viscolactis*, *Aeromonas hydrophilla*, *Cytophaga* sp., Vibrio parahaemolytica, viz *Damsela*, *Bacillus cereus* and *Streptococcus pyogenes* in addition to *Escherichia coli*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa* (Perumal Samy et al., 1999). In another study, the extract of the leaves was found to be active against the isolated bacteria *E.coli*, *S.citri* and aerobic spore formers from soft drinks (Meera et al., 1999)
The essential oil isolated from the shoot was reported to have antifungal activity against *Aspergillus carneus* at various concentrations. The oil showed 85-100 percent inhibition of the mycelial growth at concentration ranging between 1000-5000 ppm (Misra et al., 1992).

In comparative study of herbal agents used for fumigation in relation to formalin, the plant reduced the microbial colony counts in air samples considerably (Bisht et al., 1988).

The acetone, chloroform, ethyl acetate, hexane and methanol leaf extracts of *Acalypha indica*, *Achyranthes aspera*, *Leucas aspera*, *Morinda tinctoria* and *Ocimum sanctum* were studied against the early fourth-instar larvae of *Aedes aegypti* L and *Culex quinquefasciatus* Say. The larval mortality was observed after 24 h exposure. All extracts showed moderate larvicidal effects; however, the highest larval mortality was found in the ethyl acetate extract of *A. aspera*. In the present study, bioassay-guided fractionation of *A. aspera* led to the separation and identification of a saponin as a potential mosquito larvicidal compound, with LC$_{50}$ value of 18.20 and 27.24 ppm against *A. aegypti* and *C. quinquefasciatus*, respectively. (Bhagavan et al., 2008).

### 3.2.2.6 Anthelmintic:

Alcoholic extract of the plant did not show any effect on human *Ascaris lumbricoides* in vitro (kaleysa Raj, 1975). The leaf extract at a dilution of 1:5 showed 100 percent mortality against *Meloidogyne javanica* (Nandal and Bhatti, 1983).

### 3.2.2.7 Clinical Reviews:

The plant was subjected to wide clinical evaluation with special reference to its use in leprosy, bronchial asthma and fistula-in-ano. Diuretic activity could not be confirmed.
3.2.2.7.1 Leprosy:

The effect of oral decoction of *A. aspera* in the treatment of leprosy was studied (uncontrolled) in 19 patients who were found to have the positive stains smears at the S.S. Hospital, Varanasi. Fourteen patients were in stage of reaction and rest of them had active lesions but none of them was in quiescent stage. The study revealed encouraging results in both lepra reaction as well as the quiescent stage of lepromatous leprosy. Skin lesions and ulcers had tendency to subside quickly with the treatment. The bacteriological index had also improved (Tripathi et al., 1963).

3.2.2.7.2 Fistula-in-ano:

There have been a number of studies on the use of ‘Kshaarasootra’ (a medicated thread prepared by coating the latex of *Euphorbia neriifolia*, alkaline powder of *A. aspera* and *Curcuma longa*) in the treatment of fistula-in-ano. The studies revealed that the long term use of ‘Kshaarasootra’ was quite effective in treatment of various fistulous tracks (Despande and Sharma, 1973, 1976; Deshpande et al., 1966, 1975; Raghavaiah, 1976; Gangasatyam, 1981; Varshney and Tyagi, 1991). The standardization of ‘Kshaarasootra’, the methods of identification and assay of the individual constituents were studied (Dwivedi et al., 1991; Sharma et al., 1994, 1995).

The Indian Council of Medicinal Research has carried out a multicentric randomized controlled trial to evaluate the efficacy of ‘Kshaarasootra’ in the management of fistula-in-ano (265 patients) in comparison with the conventional surgery (237 patients). The trial was carried out at Bombay, Chandigarh, New Delhi and Wardha. The results have revealed that the long term outcome with ‘Kshaarasootra’ (recurrence 4 percent) was better than with surgery (recurrence 11 percent), although the initial healing time was
Review of Literature

longer (8 wk with thread and 4 wk with surgery). 'Kshaarasootra' offered an effective, ambulatory and safe alternative treatment for patients with fistula-in-ano (ICMR, 1991). 'Kshaarasootra' has also been found to give encouraging results in 5 patients of chronic non healing milk-fistula 'stannadi-vrana' with additional local application of 'jatyaditila' and oral administration of 'shigru gugglu' (two tablets t.i.d) during the course of treatment (Singh et al., 1994b)

3.2.2.7.3 Bronchial asthma:

A pilot study was carried out at the Central Research Institute for Siddha in Madras on 15 cases of bronchial asthma. The oil obtained from the root soaked in cows urine was smeared on betel leaf and administered thrice a day to these patients. In most if the cases symptoms like wheezing, gasping, dyspnoea, sneezing and cough disappeared. A fall in the total WBC and eosinophil counts and ESR was observed (Suresh et al., 1985)

3.2.3 Phytoanalytical methods:

The contents of oleanolic acid in the root of Achyranthes bidentata and Achyranthes aspera from different places of production have been determined by dual-wavelength TLC-scanning. The results showed that the content of oleanolic acid is 0.91%-1.14% in cultured A. bidentata, 0.78%-1.42% in A. bidentata natural and 0.054% in A. aspera. (Li et al., 1995). A highly selective and sensitive HPLC–ESI–MS–MS method was developed for the determination of oleanolic acid in human plasma. The oleanolic acid and glycyrrhetinic acid (internal standard) were recovered from plasma with ethyl acetate liquid–liquid extraction. The organic extracts were dried under a stream of warm nitrogen, reconstituted in mobile phase and injected into a Zorbax-Extend ODS analytical

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column (150 mm x 4.6 mm i.d., 5 μm), with the mobile phase consisting of methanol-
ammonium acetate (32.5 mM) (85:15, v/v) pumped at a flow rate of 1.0 ml/min, and 30% of the eluent was split into a MS system with electrospray ionization tandem mass (ESI–MS–MS) detection in negative ion mode. The tandem mass detection was performed on a Finnigan Surveyor LC-TSQ Quantum Ultra AM tandem mass spectrometer operated in selected reaction monitoring mode. The parent to product ion combinations of m/z 455.4 —> 455.4 and 469.3 —> 425.2 at 38 V 1.5 mTorr Ar CID were used to quantify oleanolic acid and glycyrrhetinic acid, respectively. The assay was validated in the concentration range of 0.02–30.0 ng/ml for oleacolic acid when 0.5 ml of plasma was processed. The precision of the assay (expressed as relative standard deviation, %RSD) was less than 15% at all concentrations levels within the tested range and adequate accuracy, and the limit of quantification was 0.02 ng/ml. The established method was applied for the pharmacokinetics study of oleanolic acid capsules in 18 healthy male Chinese volunteers with the mean values of C_{max}, T_{max}, AUC_{0–48}, AUC_{0–∞}, t_{1/2}, CL/F, and V/F of oleanolic acid after p.o. a single 40 mg dose obtained were 12.12 ± 6.84 ng/ml, 5.2 ± 2.9 h, 114.34 ± 74.87 ng h/ml, 124.29 ± 106.77 ng h/ml, 8.73 ± 6.11 h, 555.3 ± 347.7 L/h, and 3371.1 ± 1990.1 L, respectively. (Min et al., 2006). Oing et al., 2006 reported an HPLC method for the content determination of oleanolic acid in Caulis clematidis armandii after hydrolysis. Magdalena et al., 2005 reported HPTLC combined with densitometric method was used to determine the content of oleanolic acid in acetone extract from the Lamium albi flos L. The plant extract was separated on Si 60 HPTLC plates and determined with the use of two densitometric methods: measurement of fluorescence and absorbance. The content of oleanolic acid was statistically calculated.
a measurement of absorbance in 1 g of the dry *Lami albi flos* L. 1458 μg of oleanolic acid was determined (0.147%). In a fluorescence method in 1 g of the plant material 1516 μg of oleanolic acid was determined (0.149%). Wang et al., 1998 reported a HPLC method for the determination of oleanolic acid in Aralia species. Capillary electrophoretic method was developed for the assay of oleanolic acid in traditional Chinese herb *Ligustrum lucidum* and its medicinal preparation. (Huitao et al., 2000).
3.3 Materials and Methods

3.3.1 Materials

The seeds of *Achyranthes aspera* Linn were collected from the botanical garden of L.M. College of Pharmacy, Ahmedabad, Gujarat in the month of November 2006. The identity of the plant was confirmed at Department of Pharmacognosy, L.M. College of Pharmacy, Ahmedabad. After collection the seeds were washed carefully and dried to constant weight at 45°C. The seeds were stored in an air-tight container. All chemicals and solvents were of analytical grade. (E. merck, Ltd, Worli and Mumbai, India).

3.3.2 Pharmacognostical studies:

3.3.2.1 Determination of physico-chemical parameters (*API 2001*)

3.3.2.1.1 Determination of ash value:

(a) Determination of total ash:

Accurately weighed 2 gm of powdered drug was taken in a tared silica dish and it was incinerated at a temperature not exceeding 450°C until free from carbon. The sample was cooled and weighed. If a carbon free ash cannot be obtained in this way, the charred mass was exhausted with hot water. The residue was collected on an ashless filter paper and the residue and the filter paper were incinerated. The filtrate was evaporated to dryness, and ignited at a temperature not exceeding 450°C. The percentage of ash was calculated with reference to the air dried drug.
Materials and Methods

(b) Determination of acid-insoluble ash:

The ash obtained as described in the above section was boiled for 5 min, with 25 ml of dilute hydrochloric acid. The insoluble matter was collected on an ashless filter paper and washed with hot water and ignited to constant weight. The percentage of acid-insoluble ash was calculated with reference to the air-dried drug.

3.3 2.1.2 Determination of sodium and potassium.

The ash obtained in above section was dissolved in measured quantity of water and amount of sodium and potassium was estimated by flame photometer.

3.3.2.1.3 Determination of extractive values:

(a) Determination of ethanol-soluble extractives:

5 gms of coarsely powdered drug was macerated with 100 ml of ethanol (95%) in a closed flask for twenty four hrs. The flasks were shaken intermittently during six hours and allowed to stand for eighteen hours. The extract was rapidly filtered, taking precautions against loss of solvent. 25 ml of the filterate was evaporated to dryness in a tared flat bottomed shallow dish and dried at 100°C, to a constant weight. The percentage of ethanol-soluble extractive was calculated with reference to the air-dried drug.

(b) Determination of water soluble extractives:

Water soluble extractives was obtained by following the same procedure as described for ethanolic soluble extractive using chloroform water (0.25% chloroform in water) instead of ethanol.
3.3.2.2 Loss on drying:

Accurately weighed seeds (5 gms) was placed in a tarred evaporation dish. It was then dried at 105°C for 5 hrs and weighed. Drying was continued and the seed was weighed at 1 hour interval until the difference between two successive weighing correspond to more than 0.25 percent. Constant weigh was reached when two consecutive weighing after drying for 30 min and cooling for 30 min, in a desiccator, did not show more than 0.01g difference.

3.3.3 Preliminary phytochemical screening:

Seed powder was subjected to the following tests separately for the presence of phytoconstituents like alkaloids, flavanoids, sterols, saponins, lignans, coumarins, carbohydrates and tannins etc.

3.3.3.1 Test for alkaloids (Geisman, 1955; Sim, 1969):

Seed powder (1 gm) was extracted with 20 ml alcohol (95%) by refluxing for 15 min and filtered. The filtrate was evaporated to dryness. The residue was dissolved in 15 ml of H₂SO₄( 2N) and filtered. After making alkaline the filtrate was extracted with chloroform. The residue left after evaporation was tested for the presence of alkaloids with Dragen-droff’s reagent. Development of orange coloured precipitates indicated presence of alkaloids.
3.3.3.2 Test for flavanoids:

3.3.3.2.1 Shinoda test: (List et al., 1967)

Seed powder (1 gm) was extracted with 10 ml of ethanol (95%) for 15 min on a boiling water bath and filtered. To the filtrate was added a small piece of magnesium ribbon and 3 to 4 drops of concentrated sulphuric acid. Formation of red colour indicated the presence of flavanoids.

3.3.3.2.2 Fluorescence test: (Giesmann, 1955)

Seed powder (1 gm) was extracted with 15 ml methanol for 2 min, on a boiling water bath, filtered while hot and evaporated to dryness. To the residue was added 0.3 ml boric acid solution (3% w/v) and 1 ml of oxalic acid solution (10% w/v). The mixture was evaporated to dryness and the residue was dissolved in 10 ml of ether. The ethereal solution showed greenish fluorescence under UV light indicating presence of flavanoids.

3.3.3.3 Test of saponins (Evans and Evans, 2002)

3.3.3.3.1 Froth test:

Seed powder (1 gm) was vigorously shaken with 5 ml of distilled water in a test tube for 30 seconds and was left undisturbed for 20 min. Persistent froth indicated presence of saponins.

3.3.3.3.2 Hemolytic zone:

0.5 ml of blood was mixed with gelatin solution (3 gm gelatin powder dissolved in 100 ml of 0.85% NaCl solution at 60°C) and taken on a glass slide. A thick section of seed was placed on it. A clear hemolytic zone was formed around the section indicating the presence of saponins.
3.3.3.4 Test for sterols:

3.3.3.4.1 Liberman Burchard test: (Freudenberg and Weimges, 1962)

Seed powder drug (1 gm) moistened with 1.0 ml of acetic anhydride on a clean tile, were added 2 drops of sulphuric acid. The powder was mixed well and the colour gained by the powder was observed. Formation of green-blue –purple red colour indicated presence of sterols.

3.3.3.4.2 Salkowski reagent: (Robinson, 1964)

To the aqueous extract (2 ml), 2 ml of chloroform and 2 ml of concentrated H₂SO₄ were added and shaken well. Chloroform layer appear red and acid layer showed greenish yellow fluorescence.

3.3.3.5 Test for tannin: (Clerk et al., 1947)

To the aqueous extract (2-3 ml), was added 1% gelatin solution containing NaCl. Heavy white precipitate indicated presence of tannins.

3.3.3.6 Test for phenolic compounds:

3 3.3.6.1 Test with FeCl₃: (Feigl, 1956)

To the methanolic extract of powdered seeds, a drop of freshly prepared FeCl₃ solution was added. Brownish green colour indicated presence of phenols.

3.3.3.6.2 Test with Folin Ciocalteu reagent: (Feigl, 1956a)

To a drop of methanolic extract of seed, a drop of Folin Ciocalteu reagent was added. Development of bluish green colour showed presence of phenols.
3.3.4 Isolation and identification of chemical marker compounds:

3.3.4.1 Isolation of crude saponins

The air-dried seeds of *A. aspera* (1 Kg) were pulverized and passed through 60 # sieve. The powder (500 gms) were defatted with petrol ether 60-80°C (500 ml x 3) using a soxhlet extractor at 50°C for 2 hrs. The solution was filtered using a Whatmann No. 1 filter paper. The petrol ether extract was discarded and the marc left was air-dried. The air-dried marc (500 gms) were extracted with boiling ethanol (4 Lit x 3) for four hours using a soxhlet extractor. The ethanolic extract so obtained was reduced under pressure to get a semisolid gum (150 gm). The semi solid gum (75 gms) was suspended in distilled water (500 ml). This suspension was extracted successively with ether (75 ml x 3) and then with n-butanol (75ml x 3). The n-butanol fraction were combined and concentrated to syrupy residue. The syrupy residue was taken in methanol (250 ml) and this was added to large volume of ether. The brown precipitates of crude saponins was filtered and dried. The precipitates were further purified by dissolving in methanol and reprecipitating it with ether. The crude saponins thus obtained were recrystallized in methanol to get a white amorphous powder (10 gms).

3.3.4.2 Isolation of oleanolic acid:

The crude saponins (5 gms) were suspended in water (250 ml) and hydrolyzed with 25%v/v hydrochloric acid for 4 hr under reflux at 100°C. The precipitates so obtained were filtered using a Whatmann No 1 filter paper and dried under reduced pressure to give a powdered mass (0.5 gm). This powdered mass was extracted with diethyl ether (100 ml) for 4 hr using soxhlet extractor at 50°C. The ethereal solution collected was
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concentrated to 50 ml. The concentrated ethereal solution was extracted with 3% methanolic KOH solution (100 ml) in a separating funnel. The methanolic solution so obtained was acidified with hydrochloric acid until the separation of maximum precipitates. The precipitates so obtained were dried in vacuum and recrystallized several times with methanol to get a white product with m.p. 302-310°C. The white product was subjected to I.R, U.V and was confirmed as oleanolic acid by comparing it with standard.

3.3.4.3 Structural confirmation of oleanolic acid:

The structure of isolated oleanolic acid was elucidated by M.P, IR, UV, Mass spectroscopy and Rf values on TLC plates. The data were compared with that of standard oleanolic acid as well as the data given in the literature. Standard oleanolic acid was obtained from RRL Jammu, India. (Table No. 2, Figure 8, 9, 10)

3.3.4.4 Determination of the purity of oleanolic acid:

The purity of oleanolic acid was determined using TLC and UV spectroscopy. The precoated silica Gel F 254 TLC plates were spotted with 4, 6, 8 and 10 µL of oleanolic acid solutions which were equivalent to 400, 600, 800 and 1000 ng of oleanolic acid respectively. The TLC plates were then developed with various solvent systems. A single spot was obtained at Rf 0.53 after derivatization with anisaldehyde sulphuric acid reagent and heating the TLC plates at 100°C for 5 minutes. Stock solutions of oleanolic acid in methanol (2mg/100 ml) were prepared and the spectrum was scanned from 200 to 400 nm using UV spectroscopy. The maximum absorbance was obtained at 212 nm. The molar absorbptivity was also calculated and compared with literature values.
3.3.5 Estimation of chemical marker compound in achyranthes aspera:

3.3.5.1 Instrument:

(a) Spotting device- Linomat IV Automatic Sample Spotter (Camag, Switzerland)

(b) Syringe- 100 μL (Hamilton)

(c) TLC chamber- Glass twin trough chamber for 20 × 10 cm plates (Camag)

(d) Densitometer- TLC Scanner 3 linked to WinCats software (Camag).

(e) HPTLC plates- 20 × 10 cm, precoated with silica gel 60 F254, 0.2 mm layer thickness.

3.3.5.2 Spotting parameters:

Start position: 15 mm from bottom edge.

Band width: 4 mm

Space between two bands: 6 mm

Spraying rate (sec/μL): 10

3.3.5.3 Chromatographic conditions:

Spotting volume: For calibration curve (4-12μL)

Amount/band: 400-1200ng (For standard curve)

Separation technique: Ascending

Developing chamber: Twin trough chamber (Camag)

Mobile phase: Hexane: Ethyl acetate:Acetic acid (10:2.5:0.5)

Chamber saturation time: 45 minutes

Temperature: 25± 2°C

Migration distance: 9 cm
3.3.5.4 **Densitometric scanning:**

Mode: Absorbance/ Reflectance  
Wavelength: 530 nm  
Lamp used: Tungsten  
Slit dimension: 3.0x0.45 mm

3.3.5.5 **Detection method:**

(a) Anisaldehyde-sulphuric acid reagent  
Anisaldehyde (0.5 mL) was mixed with 10 mL glacial acetic acid, followed by 85 mL methanol and 5 mL concentrated sulfuric acid, in that order.

(b) Derivatization- The plates were sprayed with freshly prepared anisaldehyde-sulphuric acid reagent and heated at 100°C for 5 min before scanning.

3.3.5.6 **Preparation of standard solution:**

A stock solution of standard oleanolic acid (500 µg/mL) was prepared in methanol. Standard stock solution was diluted further to obtain final concentration of 100 µg/mL oleanolic acid with methanol.

3.3.5.7 **Preparation of sample solution:**

3.3.5.7.1 **Preparation of ethanolic extract:**

Accurately weighed (5 gm) powdered seeds of *A.aspera* was refluxed with ethanol (15x3, each 1 hr). The extract was combined and filtered while hot through Whatmann No. 1 filter paper and transferred to a 50 mL volumetric flask. The volume was made upto the mark with 95% ethanol.

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3.3.5.7.2 Optimization of the acid hydrolysis of the saponins

(i) Effect of Time:
Ethanolic extract (5 ml) as prepared in 3.3.5.7.1 was transferred to four different 50 ml volumetric flasks. Hydrochloric acid (3N, 2 ml) was added to each flask. The flasks were heated under reflux for 1, 2, 3, 4 and 5 hrs respectively. The flasks were cooled and diluted up to the mark with distilled water. The contents of each flask were extracted with chloroform (7x3 ml) and the extract was transferred to (25 ml x 4) volumetric flasks respectively. The solutions of each extract were diluted up to the mark with chloroform. The solution (20 µL) was spotted on silica gel G F254 plates along with 2, 4, 6 µL of standard oleanolic acid solutions and the plates were developed and scanned as described under 3.3.5.8. The peak area was computed and the concentration of oleanolic acid obtained were calculated from the calibration curve. (Figure 18a)

(ii) Effect of Conc of Hydrochloric acid.
Ethanolic extract (5 ml) as prepared in 3.3.5.7.1 was transferred to four different 50 ml volumetric flasks. 2 ml of 2, 3, 5 and 10N hydrochloric acid was added to each flask respectively. Each of the above solutions were heated for 2 hrs under reflux. The solutions were further treated and results computed as described under 3.3.5.7.2 (i)(Figure 18b)
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(iii) Effect of Temperature:
Ethanolic extract (5 ml) as prepared in 3.3.5.7.1 was transferred to four different volumetric flask (50 ml). 2 ml of 3N hydrochloric acid solution was added to the above flasks. The solutions were heated at 25, 50, 75 and 100° C respectively for 2 hrs under reflux. The solutions were further treated and the results computed as described under 3.3.5.7.2 (i).

3.3.5.7.3 Sample clean up of oleanolic acid:
Ethanolic extract (5 ml) as prepared in 3.3.5.7.1 was hydrolyzed with 3M hydrochloric acid (2 ml) under reflux on a water bath for 2 hr. The hydrolyzed solution was cooled and transferred to a 50 mL volumetric flask. The volume was made up to the mark with ethanol. The above solution (2 ml) was diluted to 10 ml with water (SI) and pH of the solution was adjusted to 4 with ammonia. This whole solution was retained on Licrosorb RP-18 column which was previously conditioned with methanol (Figure 7). After the sample loading, sample washing was done with 5% methanolic solution. Elution of the sample was done with diethyl ether. The ether layer was evaporated and the residue dissolved in 5 ml of methanol.
Figure 7: Preparation of oleanolic acid sample by solid phase extraction:

1. Prepare sample solution (S1)
   - Adjust pH to 4 with ammonia

2. Condition/equilibrating the SPEC
   - (1 ml methanol/ 1 ml water)

3. Load the sample (S1)

4. Wash with 5% methanol

5. Elute with 10 ml diethyl ether

6. Evaporate and reconstitute in methanol
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3.3.5.8 Chromatography:

Oleanolic standard solution (2.4, 6, 8, 10, 12 μL) along with 50 μL of suitably diluted samples solution were applied to 20 x 10 cm silica gel 60F254 TLC plates. The solutions were applied as 4 mm band, 6 mm apart and 10 mm from the edge of the plate, by means of Camag Linomat IV applicator. Plates were developed with hexane-ethylacetate-acetic acid (10+2.5+0.5v/v/v) in a twin trough chamber previously equilibrated (45 min) with mobile phase; the development distance was 90 mm at 25 ± 2°C and 40% relative humidity. The plates were removed from the chamber and dried in air for 5 min. The plates were sprayed with anisaldehyde sulphuric acid reagent and heated at 105°C for 5 min (pink bands were obtained). The plates were scanned densitometrically at 530 nm in absorbance/reflectance mode using tungsten lamp. Peak areas were recorded for standard oleanolic acid and the amount of oleanolic acid present in samples were calculated from the calibration plot obtained by plotting peak area against amount of standard oleanolic acid. (Table No. 3, 4 & 9, Figure No. 11, 12, 13, 14 & 15)

3.3.5.9 Validation of HPTLC method:

The method was validated according to the ICH guidelines. All results were expressed as percentages, where n represents the number of values. For statistical analysis Excel 2000 (Microsoft Office) was used. A 5% level of significance was selected.

3.3.5.9.1 Polynomial regression

The polynomial regression of an analytical method is its ability to elicit test results that are directly or by a well defined mathematical transformation, proportional to the
concentration of an analyte in sample within a given range. It is expressed in terms of
correlation co-efficient of the polynomial regression analysis. (Table No.3)

3 3.5.9 2 Precision:

Precision is a measure of either the degree of reproducibility or repeatability of the
analytical method. It is expressed as standard deviation or relative standard deviation (co­
efficient of variance). It provides an indication of random error; results should be
expressed as relative standard deviation or co-efficient of variance.

Intraday and interday precision:

Variation of the results within the same day is called intraday variation. Variation of the
results within days is called interday variation. Intraday precision was determined by
analyzing oleanolic acid standard solution as described under 3.3.5.8 for five times in
the same day. (Table No. 5)

Inter day precision was determined by analyzing oleanolic acid standard solution as
described under 3.3.5.8 daily for five days. (Table No. 5)

3 3.5.9.3 Repeatability:

Repeatability of measurement of peak area: (RSD<1% based on seven times
measurement of peak area of same spot)

Standard solution (6 μL of 100μg/ml) of oleanolic acid was spotted and developed as
described under 3.3.5.8. Area of peak was measured seven times for same spot without
changing plate position. (Table No. 6)

Repeatability of sample application: (RSD<3% based on application of equal volume of
seven spots.)
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Standard solution (6 µL) of oleanolic acid (100µg/ml) was spotted seven times on silica gel plate. The plate was developed and described under 3.3.5.8 and areas of seven spots were measured. (Table No. 7)

3.3.5.4 Accuracy:

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of an analytical method should be established on across its range.

The accuracy of the method was assessed by performing recovery studies at 3 different levels (approximately 50, 100, 125% addition of oleanolic acid). The recoveries and average recoveries were calculated. (Table No. 8)

3.3.5.9.5 Specificity:

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components (Figure 17). The peak purity of oleanolic acid in standard and in the methanolic extract of *A. aspera* was determined by comparing spectra at peak start, peak apex and peak end.

3.3.5.9.6 Limit of detection:

The detection limit is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The detection limit is usually expressed as the concentration of analyte in the sample.

According to ICH guideline it can also be calculated or performed experimentally. Different concentration of oleanolic acid standard solution in decreasing amount were spotted and analyzed as described in 3.3.5.8.
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The minimal amount spot detected with acceptable peak purity was counted as detection limit.

3.3.5.9 Limit of Quantitation

It is the lowest concentration of analyte in a sample that can be determined with an acceptable precision and accuracy under stated experimental condition. Different amount of minimum concentration of standard solution were used and determined with acceptable precision and accuracy.

3.3.5.8 Range:

The range of an experimental method is the interval between the upper and lower levels of analyte that have been demonstrated to be determined with a suitable level of precision, accuracy using the same method as proposed. The range is normally expressed in the same units as test results obtained by the analytical method.

3.3.6 Estimation of chemical marker (oleanolic acid) in seeds of Achyranthes aspera after germination:

3.3.6.1 Germination of seeds:

Accurately weighed (10 gm) of A.aspera seeds were soaked in 250 ml of distilled water in a 500 ml beaker for 12 hrs. Water was added in between to maintain the water level. The seeds were removed from the beaker after 12 hrs and transferred to a cotton cloth. Water from the beaker was poured over the cloth occasionally to keep it wet. Seeds were allowed to germinate for 48 hrs during which a notable germination was observed. Germinated seeds were removed and air dried for 3 hrs and then in oven at 50°C for 1 hr.
The seeds were pulverized and passed through 60#sieve. The powdered seeds were stored in air tight containers.

3.3.6.2 **Preparation of sample solution:**

3.3.6.2.1 **Preparation of ethanolic extract**

Accurately weighed dried and pulverized germinated seeds (5 gms) of *A. aspera* was refluxed with ethanol (20×3, each 1 hr). The extract was combined and filtered while hot through Whatmann No.1 filter paper and transferred to a 50 ml volumetric flask. The volume was made up to the mark with ethanol.

3.3.6.2.2 **Sample clean up process**

Ethanolic extract (5 ml) was treated as described under 3.3.5.7.3.

3.3.6.3 **Chromatography:**

The sample solution (25 μL) in triplicate along with 2,6,12 μL of standard oleanolic acid solution were applied to 20×10 cm silica gel 60F254 TLC plates and analyzed as described under 3.3.5.8. Peak area and peak height of all the spots were obtained, calibration curve plotted and the content of oleanolic acid in the sample solution was computed. (Table No. 10)
3.4 Results

3.4.1 Quality parameters:

*Table No. 1. Quality parameters for Achyranthes aspera seeds*

<table>
<thead>
<tr>
<th>Name of sample (Achyranthes aspera)</th>
<th>Ash values %w/w</th>
<th>Extractive values %w/w</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Ash</td>
<td>Acid insoluble</td>
</tr>
<tr>
<td>Reported Values (API 1999a)</td>
<td>NMT 17%</td>
<td>NMT 3%</td>
</tr>
<tr>
<td>Experimental Values</td>
<td>15%</td>
<td>2.5%</td>
</tr>
</tbody>
</table>

NMT= Not More Than, NLT= Not Less Than, NA= Not Available.

3.4.2 Identification of isolated compound:

Thin Layer Chromatography of oleanolic acid showed that both isolated and standard oleanolic acid gave similar spot at Rf 0.53. These spots were visible after derivatization with anisaldehyde sulphuric acid. A pink band was observed under visible light.

Identification parameters like TLC, M.P, UV, (Figure 8) IR spectroscopy (Figure 9) and MS (Figure 10) of isolated compound was compared with standard oleanolic acid and values are shown in Table No.2.
Results & Discussion

Figure 8: UV spectrum of Oleanolic acid
Figure 9: IR spectrum of Oleanolic acid
Figure 10: Mass spectrum of oleanolic acid

Sample Name: OA-456.7
Acquired By: DHARMESH BHATT

Data Acquired: 11:13:55 AM: 10/24/2008
Volume: 5
Injection Volume: OA-456.7
Data File: OA-456.7.lic
Method File: MAB-ESI.lic
Data Processed: 10/24/2008 11:18:08 AM

MS Spectrum Graph
Polarity: Pos
Peak No 1 Ret. Time 0.433 Averaged D06-MP/OA-456.7.lic

MS Spectrum Graph
Polarity: Neg
Peak No 2 Ret. Time 0.333 Averaged D06-MP/OA-456.7.lic

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Table No. 2: Data of M.P, UV and IR spectroscopy of isolated oleanolic acid

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Literature values (Huang et al., 2001)</th>
<th>Experimental values</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.P</td>
<td>310</td>
<td>308</td>
</tr>
<tr>
<td>UV λmax (methanol)</td>
<td>212 nm</td>
<td>218.4 nm</td>
</tr>
<tr>
<td>IR (KBr, Vmax, cm⁻¹)</td>
<td>3450(OH), 1705(CO), 2930, 1450, 1250, 820</td>
<td>3402(OH), 1689.53(CO), 2939.31, 1458.08, 1274.86,</td>
</tr>
<tr>
<td>LC-MS</td>
<td>m/z 455 M+</td>
<td>m/z 455 M+</td>
</tr>
</tbody>
</table>

3.4.3 Estimation of oleanolic acid in Achyranthes aspera by HPTLC method

3.4.3.1 Calibration curve of oleanolic acid

The calibration data for standard oleanolic acid was obtained (Table No. 3 and Table No. 4). The calibration curve was also obtained by plotting concentration Vs Peak area and Peak height (Figure 11 & 12, 13 & 14,15)
Table No.3 Calibration data of oleanolic acid concentration Vs mean peak area

<table>
<thead>
<tr>
<th>Sr no</th>
<th>Concentration of oleanolic acid(ng/spot)</th>
<th>Mean peak area ±S.D N=5</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200</td>
<td>2160.78 ± 51.49</td>
<td>2.38</td>
</tr>
<tr>
<td>2</td>
<td>400</td>
<td>4015.54 ± 160.26</td>
<td>3.68</td>
</tr>
<tr>
<td>3</td>
<td>600</td>
<td>5359.84 ± 208.54</td>
<td>3.92</td>
</tr>
<tr>
<td>4</td>
<td>800</td>
<td>6626.16 ± 189.46</td>
<td>2.85</td>
</tr>
<tr>
<td>5</td>
<td>1000</td>
<td>7508.58 ± 336.39</td>
<td>4.39</td>
</tr>
<tr>
<td>6</td>
<td>1200</td>
<td>8021.92 ± 277.90</td>
<td>3.46</td>
</tr>
</tbody>
</table>

Figure 11: Calibration curve of oleanolic acid standard (Mean peak area Vs Conc in ng/spot)
Table No.4 Calibration data of oleanolic acid concentration Vs mean peak height

<table>
<thead>
<tr>
<th>Sr no</th>
<th>Oleanolic acid(ng/spot)</th>
<th>Mean peak height ±S.D</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200</td>
<td>187.86±4.27</td>
<td>2.27</td>
</tr>
<tr>
<td>2</td>
<td>400</td>
<td>327.36±8.25</td>
<td>2.52</td>
</tr>
<tr>
<td>3</td>
<td>600</td>
<td>388.00±7.72</td>
<td>1.99</td>
</tr>
<tr>
<td>4</td>
<td>800</td>
<td>449.82±10.55</td>
<td>2.34</td>
</tr>
<tr>
<td>5</td>
<td>1000</td>
<td>493.64±17.27</td>
<td>3.49</td>
</tr>
<tr>
<td>6</td>
<td>1200</td>
<td>503.54±11.19</td>
<td>2.21</td>
</tr>
</tbody>
</table>

Figure 12: Calibration curve of oleanolic acid standard (Mean peak height Vs Conc in ng/spot)

\[ y = -0.000x^2 + 0.752x + 58.58 \]
\[ R^2 = 0.992 \]
Figure 13: Detector response (Peak Area) Vs. Oleanolic concentration ranging from 200-1200ng/spot by HPTLC.
Results & Discussion

Figure 14: Detector response (Peak height) Vs. Oleanolic concentration ranging from 200-1200ng/spot by HPTLC.
Results & Discussion

Figure: 15 Calibration data of oleanolic acid (Conc ranging from 200-1200 ng/spot) by HPTLC

<table>
<thead>
<tr>
<th>Substance: oleanolic acid</th>
<th>Dimension: ng p.dev.range: 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Track</td>
<td>Rf</td>
</tr>
<tr>
<td>1</td>
<td>0.53</td>
</tr>
<tr>
<td>2</td>
<td>0.53</td>
</tr>
<tr>
<td>3</td>
<td>0.53</td>
</tr>
<tr>
<td>4</td>
<td>0.53</td>
</tr>
<tr>
<td>5</td>
<td>0.53</td>
</tr>
<tr>
<td>6</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Substance: oleanolic acid | p.dev.range: 5% |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Rf</td>
</tr>
</tbody>
</table>

No result for this substance available.

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3.4.4 Validation of HPTLC method:

3.4.4.1 Polynomial regression

A calibration curve of oleanolic acid was obtained by plotting the peak area of oleanolic acid against concentration of oleanolic acid. It was observed that the response for various concentration of standard oleanolic acid were polynomial in the range of 200-1200ng/spot. The correlation co-efficient was found to be 0.9996 and RSD ranged from 2.38-4.39%. (Table No. 3)

The polynomial regression equation : \( y = -0.0038x^2 + 11.211x + 84.586 \). Where \( x = \) concentration of oleanolic acid and \( y = \) peak area

3.4.4.2 Precision:

The interday and intraday co-efficient of variance ranged from 2.38-4.39% and 1.02-3.71% respectively. (Table No. 5)

Table No. 5 Data for Interday and Intraday Precision for oleanolic acid

<table>
<thead>
<tr>
<th>Concentration (ng/spot)</th>
<th>Inter day Precision (n=5)</th>
<th>Intra day Precision(n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak area (Mean ± S.D)</td>
<td>%CV</td>
</tr>
<tr>
<td>200</td>
<td>2160.78 ± 51.49</td>
<td>2.38</td>
</tr>
<tr>
<td>400</td>
<td>4355.54 ± 160.26</td>
<td>3.68</td>
</tr>
<tr>
<td>600</td>
<td>5309.84 ± 208.54</td>
<td>3.92</td>
</tr>
<tr>
<td>800</td>
<td>6626.16 ± 189.46</td>
<td>2.85</td>
</tr>
<tr>
<td>1000</td>
<td>7648.58 ± 336.39</td>
<td>4.39</td>
</tr>
<tr>
<td>1200</td>
<td>8021.92 ± 277.90</td>
<td>3.46</td>
</tr>
</tbody>
</table>
Results & Discussion

3.4.4.3 Repeatability:

(a) RSD for repeatability of measurement of peak area and peak height based on seven times measurement of the same spot was found to be 0.38 and 0.52 respectively (Table No. 6).

(b) RSD for repeatability of sample application of peak area and peak height on seven measurements of the same spot was found to be 1.05 and 0.70% (Table No. 7)

Table No. 6 Data for repeatability of measurement of peak area and peak height for oleanolic acid: (conc =600ng/spot)

<table>
<thead>
<tr>
<th>No. of measurements</th>
<th>Height</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>395.5</td>
<td>5176.6</td>
</tr>
<tr>
<td>2</td>
<td>393.5</td>
<td>5156</td>
</tr>
<tr>
<td>3</td>
<td>392.2</td>
<td>5146</td>
</tr>
<tr>
<td>4</td>
<td>391.2</td>
<td>5132</td>
</tr>
<tr>
<td>5</td>
<td>390.5</td>
<td>5128</td>
</tr>
<tr>
<td>6</td>
<td>390.2</td>
<td>5126</td>
</tr>
<tr>
<td>7</td>
<td>389.7</td>
<td>5122</td>
</tr>
<tr>
<td>Mean</td>
<td>391.8</td>
<td>5140.9</td>
</tr>
<tr>
<td>%CV</td>
<td>0.52</td>
<td>0.38</td>
</tr>
</tbody>
</table>
Table No. 7 Data of repeatability of sample application for oleanolic acid: (Conc = 600ng/spot)

<table>
<thead>
<tr>
<th>No of measurements</th>
<th>Height</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>399.5</td>
<td>5199.3</td>
</tr>
<tr>
<td>2</td>
<td>395.6</td>
<td>5159.0</td>
</tr>
<tr>
<td>3</td>
<td>400.0</td>
<td>5055.1</td>
</tr>
<tr>
<td>4</td>
<td>396.0</td>
<td>5144.2</td>
</tr>
<tr>
<td>5</td>
<td>397.2</td>
<td>5158.1</td>
</tr>
<tr>
<td>6</td>
<td>395.0</td>
<td>5224.3</td>
</tr>
<tr>
<td>7</td>
<td>391.8</td>
<td>5125.4</td>
</tr>
<tr>
<td>Mean</td>
<td>396.4</td>
<td>5152.2</td>
</tr>
<tr>
<td>%CV</td>
<td>0.70</td>
<td>1.05</td>
</tr>
</tbody>
</table>

3.4.4 Accuracy (% recovery)

Accuracy of analysis, in terms of systemic error involved, was determined by calculating recovery of oleanolic acid by addition method at 3 levels of the calibration curve. The % recovery of oleanolic acid was found to be 100.57% which is satisfactory (Table No. 8)
Results & Discussion

Table No. 8: Recovery study of oleanolic acid by proposed HPTLC-densitometry method.

<table>
<thead>
<tr>
<th>Amount of marker present, ng</th>
<th>Amount of marker added, ng</th>
<th>Amount of marker found(^a), ng ± S.D.</th>
<th>% Recovery</th>
<th>Avg. recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>442</td>
<td>240</td>
<td>680.72 ± 0.32</td>
<td>99.81 ± 0.29</td>
<td>100.57%</td>
</tr>
<tr>
<td>442</td>
<td>400</td>
<td>850.47 ± 0.41</td>
<td>101.01 ± 0.38</td>
<td></td>
</tr>
<tr>
<td>442</td>
<td>500</td>
<td>1010.99 ± 0.77</td>
<td>100.89 ± 0.69</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) mean ± S.D (\(n = 3\))

3.4.4.5 Specificity:

It was observed that the other phyto-constituents present in the seed samples did not interfere with the peak area of oleanolic acid. Therefore, the method was specific (Figure 16). The HPTLC spectrum of standard oleanolic acid and oleanolic acid in seed sample were comparable with correlation co-efficient of 0.999 (Figure 17).
Results & Discussion

Figure: 16 HPTLC chromatogram of standard and sample oleanolic acid: (A-D chromatogram of standard and E-H chromatogram of sample extract of oleanolic acid)
Figure 17: Overlay HPTLC spectrum of standard oleanolic acid and sample extract of oleanolic acid
3.4.5 Optimization of acid hydrolysis:

Optimization of the acid hydrolysis condition is indispensable before development of quantification method. As shown in Figure 18 the acid hydrolysis of the saponins using 3 M HCl at 100°C for 2 hr provided the maximum yield of oleanolic acid.

3.4.5.1 Optimization of time:

The optimum time of hydrolysis products at different time interval (Figure 18a). As shown in the figure the formation of oleanolic acid reached the maximum after 2 hr and subsequent heating under the hydrolysis condition resulted in the loss of oleanolic acid.

3.4.5.2 Optimization of temperature:

The maximum amount of oleanolic acid was obtained using a hydrolysis temperature of 100°C

3.4.5.3 Optimization of acid concentration:

Lower acid concentration (1 and 2N HCl) required longer time to achieve the maximum concentration of oleanolic acid, where as higher acid concentration (5 and 10N HCl) produced artifacts which reduced the yield of oleanolic acid. (Figure 18b)
Results & Discussion

Figure 18: Effect of time a) and concentration b) of acid hydrolysis of the crude saponins of Achyranthes aspera on the oleanolic acid yield. Hydrolysis condition: a) 3 N HCl for 2 hr. b) 3N HCl at 100°C
3.4.6 Estimation of oleanolic acid in the seed samples of Achyranthes aspera.

The fresh seed sample powders of Achyranthes aspera was analyzed as described under 3.3.5.8. Chromatograms obtained from oleanolic acid standard and from methonolic seed extract of A. aspera are shown in Figure 19. A photograph of the chromatographic plate is shown in Figure 18. The amount of oleanolic acid was computed from calibration curve.(Figure 11). The amount of oleanolic acid in the fresh seed samples were found to be $0.343 \pm 0.0071\%$w/w. (Table No. 9)

Figure: 19 TLC Photograph of oleanolic standard solution(Track 1-5 containing 200-1000ng/spot of oleanolic acid standard respectively)
3.4.7 *Estimation of oleanolic acid in the germinated seed samples of Achyranthes aspera.*

Accurately weighed (10 gm) of *A. aspera* seeds were germinated and processed as described under 3.3.6.1. Ethanolic extract of *A. aspera* seeds (5 gms) were prepared followed by sample clean up process as described under 3.3.6.2. This sample was analysed as described under 3.3.6.3. The amount of oleanolic acid was computed from calibration curve (Figure 11). The amount of oleanolic acid in the germinated seed samples were found to be $0.625 \pm 0.018\%$w/w. (Table No. 10)

**Table No 9 : Estimation of oleanolic acid in seed samples of *Achyranthes aspera*:**

(50 µL of the sample solution was spotted)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean Peak area ± S.D. (n=3)</th>
<th>Average amount of oleanolic acid (ng/spot) ± S.D.</th>
<th>Average %w/w of Oleanolic acid ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh seeds of <em>A. aspera</em></td>
<td>6080.47 ± 127.26</td>
<td>687.08 ± 14.38</td>
<td>0.343 ± 0.0071</td>
</tr>
</tbody>
</table>
Table No. 10: Estimation of oleanolic acid in the seed samples of *Achyranthes aspera* after germination (25 µL of the sample solution was spotted)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean Peak area ± S.D. (n=3)</th>
<th>Average amount of oleanolic acid (ng/spot) ± S.D.</th>
<th>Average %w/w of Oleanolic acid ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germinated seeds of <em>A.aspera</em></td>
<td>5535.93 ± 159.65</td>
<td>625.548 ± 18.04</td>
<td>0.625 ± 0.018</td>
</tr>
</tbody>
</table>
3.5 References


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References


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