Phytochemical analysis and localization of bioactive compounds in *Prosopis juliflora*

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Certificate

This is to certify that the thesis entitled "**Phytochemical analysis and localization of bioactive compounds in *Prosopis juliflora***" submitted by Shachi Singh, ID No. 2003PH29088 for award of Ph.D Degree of the Institute, embodies original work done by her under my supervision.

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Abstract

Prosopis juliflora growing in semi arid regions of Rajasthan was selected for investigation on its bioactive compounds. Phytochemical screening of different parts of the plant revealed the presence of alkaloids, terpenoids, flavonoids, phenolics and tannins. DART (Direct analysis in real time) technique was applied to identify and study the distribution of piperidine alkaloids present in different parts of *P.juliflora.* The chemical fingerprint analysis, revealed leaf, pod and flower to be the rich source of piperidine alkaloids. The concentration and distribution of these alkaloids varied in different parts. The leaves and pod showed the largest number of alkaloid with a total of 12 different compounds, in contrast only 4 alkaloids were present in flower. Julifloridine, Prosopine, Prosopinine and Prosafrinine were ubiquitously distributed in all the alkaloid rich plant parts. Juliprosopine was pre-eminent alkaloid and was most abundant in leaf, whereas pod and flower displayed copious amounts of Julifloridine. Two new alkaloids, Prosopine and Prosopinine were identified in aerial parts of *P.juliflora,* which were further confirmed by studying their fragmentation pattern generated by LC-MS/MS. Along with the alkaloids, two carotene, three xanthophylls, two pheophytin and two fatty acids were isolated from leaf extracts, a completely new aliphatic acid was also isolated from flower extract and they were identified by UV-visible, mass, IR and NMR spectra analysis.

The alkaloid rich fraction (ARF) obtained from various parts of *P.juliflora* was assessed for their antibacterial property on several Gram-negative and Gram-positive bacterial strains. Strong antibacterial effect was shown by leaf, pod and flower extract, with MIC values ranging between 25µg/ml-100µg/ml. ARF was found to inhibit growth of multidrug resistance bacterial species and showed equivalent or greater potential to control the growth of bacteria than standard antibiotics.

Evaluation of allelopathic potential of ARF, indicated that alkaloidal extract of all tested parts markedly reduced the root growth of both dicotyledonous and monocotyledonous plant spieces. A comparative evaluation of antimitotic and cytogenotoxic properties of alkaloidal extract, showed that ARF of all plant parts caused

complete inhibition of mitotic activity and showed abnormality in cellular and chromosomal morphology. High concentrations were lethal causing cell death. New compound isolated from flower also showed antimitotic activity.

Acute and subacute toxicity study of the ARF obtained from leaf was carried out on mice by oral and intraperitoneal route. The results revealed that the LD_{50} value of the leaf extract was 630.95 mg/kg body weight for oral route and 79.43 mg/kg for intraperitoneal route. Subacute toxicity study, conducted for a period of 14 days at sublethal dose suggest that *P. juliflora* extract is not toxic when administered orally to mice at concentrations below 200 mg/kg and intraperitoneally below 10 mg/kg. Changes at high doses were observed on various physical, biochemical and hematological parameters and these changes were more pronounced for the mice receiving high doses of extract through intraperitoneal route.

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List of Abbreviations

ARF	Alkaloid rich fraction
DR	Dragondorff's reagent
PE	Petroleum ether
EA	Ethylacetate
M	Methanol
TLC	Thin layer chromatography
HPTLC	High performance thin layer chromatography
DART-MS	Direct analysis in real time mass spectrometry
HR-MS	High resolution mass spectrometry
PEG	Polyethylene glycol
HPLC	High performance liquid chromatography
LC-ESI-MS	Liquid Chromatography Electrospray Mass Spectrometry
NMR	Nuclear magnetic resonance
FTIR	Fourier transform infrared spectrophotometer
CDCl3	Deuterochloroform
TMS	Tetramethyl silane
MIC	Minimum inhibitory concentration
ZOI	Zone of inhibition
TPC	Total protein count
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvate transaminase

Chapter 1

General Introduction

Since ages, humans have relied on nature for their basic needs for the production of foodstuffs, shelters, clothing, means of transportation, fertilizers, flavors and fragrances, and, not least, medicines. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years (Fallarino, 1994; Butler, 2004).

The plant based systems continue to play an essential role in health care, and it has been estimated by the World Health Organization that approximately 80 % of the world's inhabitants rely mainly on traditional medicines for their primary health care (Farnsworth et al., 1985; Butler, 2004). About 25 % contained plant extracts or active principles derived from higher plants, and at least 119 chemical substances, derived from 90 plant species, can be considered as important drugs currently in use in one or more countries (Cragg et al., 1994; Newman et al., 2003). Of these 119 drugs, 74 % were discovered as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine (De Smet, 1997).

Natural compounds and extracts from plants have an advantage over synthetic compouds in having low human toxicity and being environmentally safe. In addition, chemical diversity of secondary plant metabolites that result from plant evolution is equal or superior to that found in synthetic combinatorial chemical libraries (Duke et al., 2000). However despite of having a wide historical background there are only a handful of plants that have been exhaustively studied for their potential value as a source of drugs. Of more than 250000 species of higher plants in the world, only about 5 -10% have been systematically investigated. This raises the prospects of obtaining novel therapeutic compounds if this vastly untapped resource could be adequately explored (Newman and Cragg, 2005).

In plants, as a result of metabolic processes, many different kinds and types of organic compounds or metabolites are produced. These metabolites are grouped into primary and secondary metabolites. The primary metabolites like chlorophyll, amino acids, nucleotides, simple carbohydrates or membrane lipids, play well defined roles in photosynthesis,

respiration, solute transport, nutrient assimilation and differentiation. The secondary metabolites differ from primary metabolites in having a restricted distribution in the plant kingdom. That is, particular secondary metabolites are often found in only one plant species or a taxonomically related group of species, whereas the basic primary metabolite are found throughout the plant kingdom (Taiz and Zeiger, 2006). The pathways involved in the biosynthesis of secondary metabolites are connected with the primary metabolite in complex manner (Fig.1).

During the past few decades, experimental and circumstantial evidence has made it clear that many secondary metabolites do indeed have functions that are vital for the fitness of a plant producing them (Wink, 1999). The main roles are:

- Defence against herbivores (insects,vertebrates)
- Defence against fungi and bacteria
- Defence against virus
- Defence against other plants competing for light, water and nutrients
- Signal compounds to attract pollinating and seed dispersing animals
- Signals for communication between plants and symbiotic microorganism (e.g N-fixing Rhizobia or mycorrhizal fungi)
- Protection against UV-light or other physical stress

These secondary metabolites have provided an invaluable resource that has been used to find new drug molecules (Johnson, 1986; Kapoor, 1990). The medicinal value of a plant can be observed from the secondary metabolites they possess which may alter certain physiological actions in the human body. The most important of these bioactive constituents of plants are terpenoids, alkaloids, tannins, flavonoids and phenolic compounds (Gurib-Fakim, 2006). Different phytochemicals have been found to possess a wide range of activities, which may help in protection against chronic diseases (Hartwell, 1982).

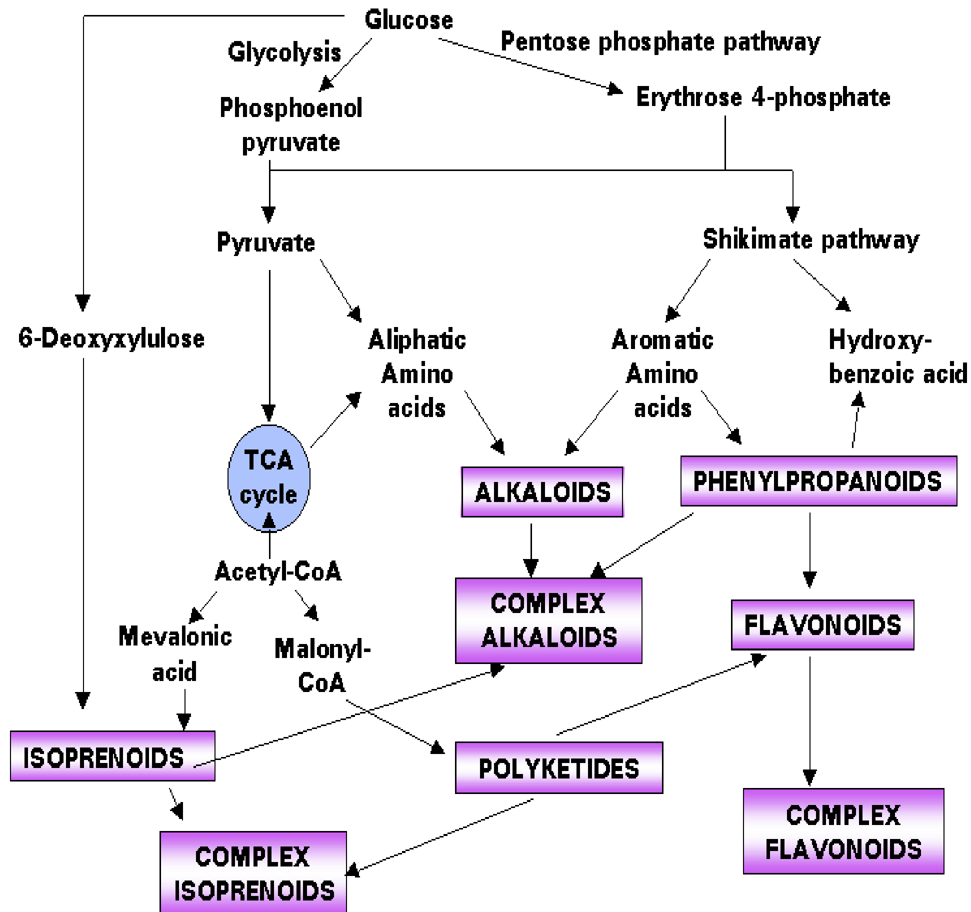


Fig 1. Main pathways leading to secondary metabolites (Gurib-Fakim, 2006)

Terpenes are commonly found in essential oils. They are widely used as insecticides and their pharmacological properties include antibacterial, antifungal, antihelmintic, antimalarial and molluscicidal (Gurib-Fakim, 2006), similarly phenolic compounds have a wide range of pharmaceutical activities such as anti-inflammatory, analgesis, antitumour, anti-HIV, anti-infective, vasodilatory, immunostimulant and antiulcerogenic (Potmeisel and Pinedo, 1995; Beissert and Schwarz, 2002). Flavonoids are known to protect the plant from UV-damaging effects and play a role in pollination by attracting animals with their colours. Recently, flavonoids have attracted interest due to the discovery of their pharmacological activities (Pietta, 2000; Brahmachari and Gorai, 2006). Alkaloids usually have a marked physiological action on humans or animals and are sometimes believed to be waste products of a nitrogen source. They are thought to play an important role in plant protection, germination and plant growth stimulation (Elisabetsky Costa-Campos, 2006). Alkaloids are pharmaceutically significant and are used as anticancerous, analgesic, antimalarial, antiarrythmic, antispasmodic, in the treatment of coughs and pain, in the treatment of gout, and as pupil dilatin (Cragg et al., 1993; Buss and Waigh, 1995; Cortes and Pazdur, 1995).

Knowledge of the chemical constituents of plants is very important, not only for the discovery of drugs and other therapeutics agents, but also in disclosing new sources of economic materials (Mojab et al., 2003). In addition, the knowledge of the chemical constituents of plants would further be valuable in discovering the actual value of folkloric remedies.

Plants are known to produce majority of secondary metabolites under stress condition (Khan and Dular, 2003), therefore in the present research work, *Prosopis juliflora*, plant of semi-arid region was chosen for the study of its pharmacological potential.

Prosopis juliflora

The genus *Prosopis* belongs to family Fabaceae, sub family Mimosoideae and contains 44 species in five sections, spread throughout southern Asia, Africa and America. *Prosopis* species exhibit an enormous variety in growth habits and in their defences (Jain and

Robert, 1964; Rastogi and Mehrotra, 1993). The genus is a fast growing, hardy and drought resistant tree, that is suitable for afforestation of arid and semiarid lands and come up well even in saline and rocky areas. As the cartilaginous inner lining of the pod does not allow the seeds to escape easily, natural regeneration through seed in the normal way is slow, but the seeds that pass out undigested in the dropping of animals sprout readily under favourable environment. Natural regeneration also takes place through root suckers.

In the Indian sub-continent, only two species of *Prosopis* are abundantly found, *P. cineraria* and *P. juliflora* in which *P. cineraria* is endemic and *P. juliflora* have been introduced (Rastogi and Mehrotra, 1993). *Prosopis* species grows predominantly in Rajasthan, Haryana, plains of Uttar Pradesh, Gujarat, North Karnataka, Tamilnadu, Madhya Pradesh, Delhi, Maharastra and also in parts of Punjab. Depending on climatic and edaphic conditions, the density of species varies from 5 to 100 trees/ha.

P. juliflora also called Vilayati kiker, Kabuli kiker, Vilayati babuls, Vilayati Khijra, is green spiny tree or shrub that was introduced into the Indian sub-continent to Sind province (Pakistan) in 1877 from South America, and later on it was introduced into many dry regions of India (Muthana and Arora, 1983). This species has since become naturalised in the arid and semi-arid parts of the country. With its wide adaptability to arid environments and its drought and disease tolerance, it has virtually exceeded all indigenous species in covering the arid and semi-arid tract. It forms thickets on marginal lands, sandy, rocky and gravely wastelands and alongside roads and railways (Muthana and Arora, 1983). *It* is now found in many states of India; Andhra Pradesh, Karnataka, Maharastra, Orissa, Punjab, Uttar Pradesh, Tamilnadu and West Bengal and in almost all districts of Haryana, Gujarat and Rajasthan (Gurumurthi et al., 1984). In all 11 districts of western Rajasthan, *P. juliflora* has spread widely, with high plant densities encountered in the districts of Pali, Jalore, Jodhpur and Nagaur (Gurumurthi et al., 1984).

P. juliflora is a small to moderate sized evergreen tree of 3-4 m height with long drooping branches and spreading crown. This species is largely represented in a shrubby

a)

b) **c)**

Fig 2. Photograph of *Prosopis juliflora* - a) whole plant b) inflorescence c) pod

state. There are straight, conical and spinescent stipules of 3-9 mm length. Leaves are bipinnate, leaflets in 13-25 pairs, oblong (3 x 1.7 mm) and dark green. Flowering spikes are axillary, hanging 6-8 cm long, cream to yellow in colour. The pods are highly variable in size and shape, mostly curved or sickle shaped, cream-coloured on ripening and indehiscent. They contain 3-25 ovoid seeds, brown to light chocolate in colour, firmly embedded in the pod (Sharma and Sharma, 1989; Shetty and Singh, 1993). It flowers twice a year, in February-March and August-September, and is a prolific seeder. The pods of autumn flowering mature by May or early June and are dispersed before the onset of the monsoon. The monsoon flowering pods mature from early November to mid December. Summer pods mature over a short period of time, whereas maturity of post-monsoon pods is staggered. In drought years, autumn flowering is very much affected, with trees often failing to flower, but the same plant flowers and fruits subsequently when there is adequate rainfall (Shetty and Singh, 1993).

Economic Importance

From early prehistoric time *Prosopis* has served as a primary source for food, fuel, shelter, weapons, tools, fibres, medicine and much other practical and aesthetic purpose (Muthana and Arora, 1983).

In western Rajasthan, *P. juliflora* is a major source of fodder, fuelwood, charcoal, timber and gums, and has established itself as a species of soil conservation and for aesthetic purposes (Rastogi and Mehrotra, 1993). It also provides utility to wildlife, where in the arid tracts of Rajasthan and Kutch, ripe fruits of this species have become the main source of nutritive food to wildlife, clusters of bushes or thickets provide hiding places for wildlife from hunters and natural enemies (Muthana and Arora, 1983).

Ripe pods are consumed by livestock in large quantities, and in drought and especially in famine conditions, the pods serve as the main protein source for cattle (Rao and Reddy, 1983). The spongy walls of the ripe pods are highly nutritive. They are a fair source of digestible protein and are important as a feed (Mahadevan, 1954). The seeds however are not digested. In America, pods after removing the seed and coarser parts are used as a

staple food, they are ground into a meal and made into cakes or used for making an alcoholic beverage, seeds are ground into powder for preparing bread (Felker et al., 1984).

The foliage can be fed to live stock both in fresh condition and as hay. The leaves are rich in plant nutrients, especially nitrogen and are therefore used as green manure. They contain nitrogen 5.6%, phosphorous 0.9%, potassium 3.11% and calcium 1.0% (Rao and Reddy, 1983).

The tree exudes a gum, which forms some what adhesive mucilage and can be used as emulsifying agent, it is also used in confectionery and employed for mending pottery (Pasiecznik, 2001). The mesquite gum is used as an adulterant and a substitute for gum Arabic, the gum is also used as culture media. The flowers are good source of honey and bark is used for tanning (Pasiecznik, 2001). Products from this plant have also been used for human consumption in bread, biscuits, sweeties, syrup and liquors (Van Den Eynden et al., 2003).

Presently 70% or more of the fuelwood demand of people in the arid and semi-arid tract of Rajasthan, Gujarat and Haryana is met by *P. juliflora*. It has a high calorific value and is preferred over indigenous species (NAS, 1980). A high quality charcoal is produced, which has virtually replaced all the other charcoal producing species in the arid zone (Vimal and Tyagi, 1986). The timber is hard, heavy, strong, close grained and does not crack. The heartwood is deep brown, while sapwood is thin and yellow, and exists in the ratio of 1:9. Strong and durable timber is obtained from the heartwood, which serves as a valuable material for agricultural implements and household furniture and utensils. The plant produces gum from February to April which can be used for sizing cloth and paper.

Use in folklore medicine

Prosopis species have been extensively used for wide range of medicinal purposes (Jain and Robert, 1964; Lewis and Elvin, 1977). The most common medicinal use of their leaves and gum is for eye ailments. The leaves, bark and gum were used as emetic and purgative 'to cleanse the system' in traditional Indian medicine and also used for diarrhea and

stomach disorders among Indian and Mexican peoples, gum dissolved in water has also been widely used as remedy for sore throat (Rastogi and Mehrotra, 1993). Macerated leaf of *P. africana* activates hormones and is also considered effective against male sterility (Igoli et al., 2003). Flowers of *P. cineraria* when mixed with sugar are eaten by women during pregnancy as a safeguard against miscarriage (Toky, 1999). The ashes removes hair when rubbed on skin (Chopra et al., 1956). *P. spicigera* is reported to be employed against snake bites and the pods and roots of this plant are stated to possess astringent properties and are used in dysentery (Kitikar and Basu, 1981). The bark of *P. spicigera* is used as remedy in rheumatism and scorpion sting, the pods and roots are used in dysentery and has anti-inflammatory activity (Chopra et al., 1956).

P. juliflora has been used as a folk remedy for catarrh, colds, diarrhea, dysentery, excrescences, flu, hoarseness, inflammation, measles, and sore throat and in healing of wounds (Duke and Wain, 1981). According to Hartwell (1971), the juice is used in folk remedies for the treatment of cancerous condition. Decoction prepared from leaf and seed extracts are used in wound healing, as disinfectant and also to treat scurvy (Tene et al., 2007). *P. juliflora* syrup prepared from ground pods has various medicinal values. It is given to children showing weight deficiency or retardation in motor development, the syrup is believed to increase lactation and it is also used for preparing various medicinal syrups, particularly for expectorants (Agroforestry tree database). Tea made from *P. juliflora* is thought to be good for digestive disturbances and skin lesions (Agroforestry tree database). Cotyledons and embryos when pulverized yield a flour rich in protein and sugar appropriate for diabetic people (Takeoka et al., 2009). In South America, preparation from fresh pods are used to treat conjunctivitis (Pasiecsnik, 2001).

Chemical investigation

Large number of chemical compounds have been isolated from different parts of *Prosopis* species. Vinaline was the first alkaloid isolated from *P. ruscifolia* by Augusto P' cerecit (1951). Sharma et al. (1964) isolated Patulitrin flavonone glycoside from *P. spicigera* which was also reported in *P. juliflora* by Wassel et al. (1972). Vitexin, a broad spectrum antibiotic, was isolated from bark of *P. ruscifolia* by Julian and Oberti (1971). The

Prosopine and Prosopinine alkaloids were isolated from *P. africana* by Ratle et al. (1966) and Khuong et al. (1972) reported 5 new alkaloid from root of this plant namely Isoprosopinine A, Isoprosopinine B, Prosophylline, Prosopine and Prosopinine.

The presence of new alkaloids from the leaves of *P. juliflora* was first reported by Ahmad et al. (1978). They isolated three alkaloids, Juliflorine, Julifloricine and Julifloridine. The structure of Julifloridine was described through spectral data. The complete structure of Juliprosopine from the leaves of this plant was deciphered by Ott. Longoni et al. (1980). Datwyler et al. (1981) isolated another alkaloid Juliprosine and proved the structure. Ahmed et al. (1989a) reported alkaloids Projuline, Projulinine, Juliprosinine and Juliflorinine from leaves of *P. juliflora*. Alkaloids 3'-oxojuliprosopine, sceojuliprosopinol, 3-oxojuliprosine and 3'-oxo-juliprosine were isolated by Nakano et al. (2004a).

Vajpeyi and Mishra (1980) reported the isolation of hexacosan-25-on-1-ol, a new keto alcohol alongwith ombuin and a triterpenoid glycoside from the bark of *P. juliflora*. From the stem bark a flavone glycosides have also been characterized as Kaempferol 4'-methylether 3-o-β-D-galactopyranoside by Vajpeyi Nee Shukla and Mishra (1981).

Bhardwaj et al. (1980) isolated, a new flavone from seeds of *P. juliflora*, Prosogerin-D (6,3,4,5-tetra methoxy-7-hydroxyflavone). Ebeid (1983) isolated a cholinesterase from *P. juliflora* seedling and the molecular weight of the isolated enzyme as estimated by a calibrated column of Sephadex G-200 was estimated as 200,000. Chemical and nutritional studies on seeds by Del Valle et al. (1983), showed that it comprised of protein 10-15%, fat 2-3%, crude fibre 20-30%, sucrose 21% and reducing sugars 2-6%, kernels contain 38% protein, 3% fat and 9% crude fibres.

Malhotra and Misra (1981a) identified new glycosides ellagic acid 4-O-α-l-rhamnosylgentiobioside and ellagic acid 4-O-rutinoside from pods of *P. juliflora*. Same group (Malhotra and Misra, 1983a) again reported from the green pods of *P. juliflora*, new glycoside of ellagic acid, (3,3'-di-O-methyl ellagic acid 4-O-(β-D-glucopyranosyluronic acid)-(1-4)-O-α-L-arabinopyranosyl-(1-6)-O-β-D-glucopyranoside) and Kaempferol,

leucocyanidin-3-O-β-D-glucopyranosidea and leucodelphinidin-3-O-β-D-glucopyranosyl-(1-4)-o-α-L-rhamnopyranoside.

From the roots of *P. juliflora* a new glycoside, 3,3'-di-O-methyl-ellagic acid 4-O-α-L-rhamnopyranoside and procyanidin have been characterized by Malhotra and Misra (1981b). Root of *P. juliflora* was also found to contain a novel tannin, glucose 1,3-diester of 3,3',5,5'-tetrahydroxy 4,4'-dimethoxy diphenic acid (Malhotra and Misra, 1981c). Malhotra and Misra (1983b), isolated from the ethanolic extract of roots two new flavanone glycoside and characterized as 3',4'-dihydroxy-5-methoxy-6-methyl flavanone, 7-O-β-D-glucopyranoside and 7,4'-dimethoxy-6,8-dimethyl-flavanone 5-O-β-D-galactopyranoside. Same group of authors (Malhotra and Misra, 1983c) again reported the isolation of a tannin from the ethanolic extract of root and characterized as 4,4'-dimethoxy-3,3',5,5'-tetrahydroxy diphenic acid 1,3-glucose ester.

Thakur and Sharma (1985), reported the isolation and characterization of allergens of *P. juliflora* pollen grains. Soni and Bisen (1988) identified some monosaccharides, disaccharides and natural gums from *P. juliflora*.

Ethanopharmacological relevance

Several compounds and extracts of *Prosopis* species have proven to be pharmacologically active. Augusto P' cerecit, (1951), reported antibacterial activity of vinaline and Iches et al. (1973) found Patulitrin to be significantly active against Lewis lung carcinoma. The Prosopine and Prosopinine isolated from *P. africana*, was reported to act on central nervous system, have antibiotic action and were used for the treatment of angina, laryngitis, rhinitis hemorrhoids, as local anaesthetic for dentistry and minor surgery (Ratle et al., 1966; Ahmad et al., 1989a).

Antibacterial activity of the ethanolic extract of *P. glandulosa* was reported by Attia et al. (1972), and *P. ruscifolia* extracts where found to be active against *Staphylococcus* (Cruse, 1959). Al-Jeboory et al. (1984) reported that alcoholic extract of *P. farcta* has a dual action of increasing and decreasing blood pressure in vivo and increases contraction of heart *in vitro*. The crude extract of *P. glandulosa* was also reported to be active against

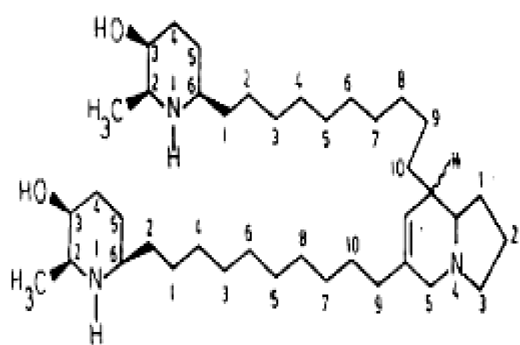
P-388 lymphocytic leukaemia and epidermoid carcinoma of nasopharynx KB (Ikram, 1983). Ahmad et al. in 1986 reported antibacterial activity of Juliflorine and same group of workers (1988) reported antimicrobial activity of an alkaloidal fraction of *P. juliflora* leaves. Aqeel et al. (1989) detected antimicrobial activity of Julifloricine isolated from *P. juliflora* and Ahmad et al. (1989b) also described the antifungal activity of some hydrosoluble alkaloids of *P. juliflora*.

El-Merzabani et al. (1979) reported the presence of cytotoxic principles in *P. juliflora*. An animal test showed that the treatment with *P. juliflora* extract could not cure the tumour bearing animal, although the mean survival time of mice was significantly increased. Hemolytic effects of alkaloidal fraction from leaves were reported by Kandasamy et al. (1989). Cytotoxic and antitumoral activity against human epithelial tumour cells (HeLa), human hepatic tumour (HepG2), and two fibroblast lineage F26 and F57 was also reported by Batatinha (1997).

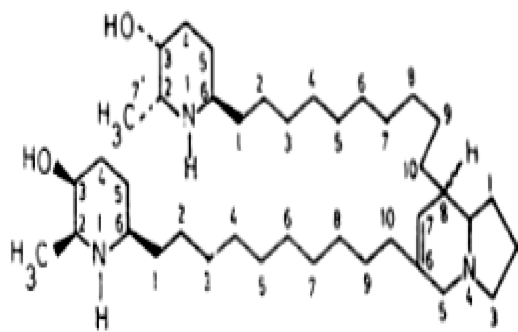
Growth inhibitory activity of alkaloids 3'-oxojuliprosopine, sceojuliprosopinol, 3-oxojuliprosine and 3'-oxo-juliprosine isolated from *P. juliflora* leaves was reported by Nakano et al. (2004a). Structure activity relationship of growth inhibitory alkaloids on germination of seedlings was also reported by Nakano et al. (2004b). Choudhary et al. (2005) reported acetylcholinesterase inhibitory potential and calcium-channel blocking activity of Juliflorine.

Thus, in view of the pharmacological importance of *P. juliflora* and its use in various herbal remedies, it was selected for investigation of its chemical constituents present in different parts of the plant, and evalution of their biological acivities on bacterial, plant and animal systems.

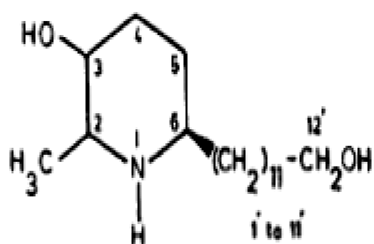
Important metabolites isolated from *Prosopis juliflora*



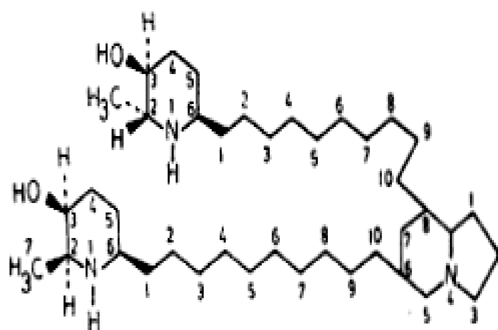
Juliflorine



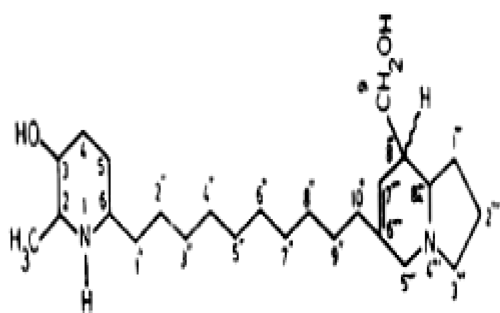
Juliflorícine



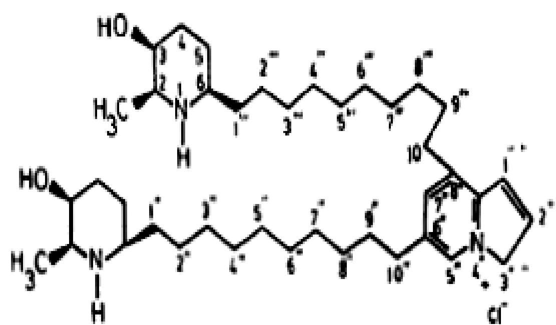
Julifloridine



Projulinine

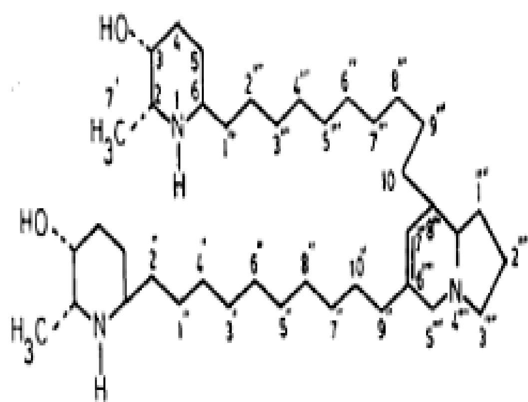


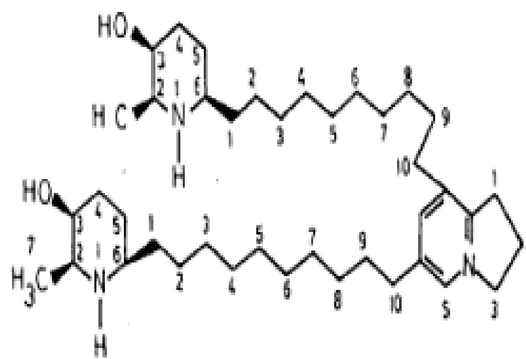
Projuline



Juliprosinine

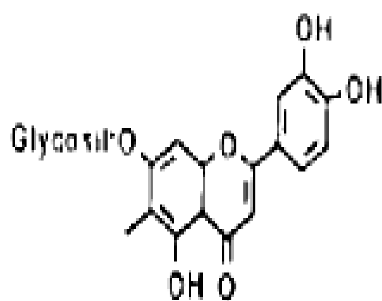
Ahmad et al., 1989a, Nakano et al., 2004a

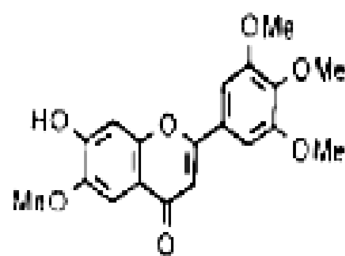




Juliflorinine

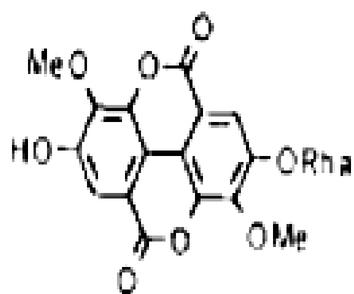
Juliprosine

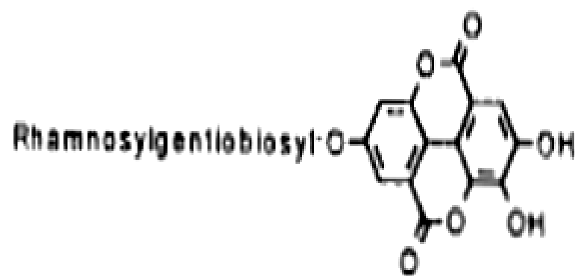




Patulitrin

Prosogerin D





Procyanidin

ellagic acid 4-O-α-l-rhamnosylgentiobioside

Ahmad et al., 1989a, Nakano et al., 2004a

Chapter 2

Phytochemical investigation of Prosopis juliflora

2.1. Introduction

Among the chemical classes present in plant species, alkaloids stand as a class of major importance in the development of new drugs because it possess a great variety of chemical structures and are responsible for pharmacological properties of the plants (Elisabetsky and Costa-Campos, 2006). Alkaloids are also used by the plants in defence mechanism against pathogens and predators (Alder et al., 2006).

Piperidine form one of the largest groups of alkaloids with piperidine core in their structures, which is derived from the amino acids lysine (Henry, 1965). Piperidine ring is a six-membered ring with no double bonds and containing one nitrogen atom.

The piperidine ring is one of the most common structural features found in biologically active agents, more than half of the known alkaloids contain piperidine rings, however, in many of these compounds, piperidine nucleus is fused to a carbocyclic or heterocyclic ring (Henry, 1965). The piperidine structural motif is present in numerous natural alkaloids. These include piperine, which gives black pepper its spicy taste (Henry, 1965). Other examples are the fire ant toxin solenopsin (Arbiser et al., 2007), the nicotine analog anabasine of the tree Tobacco (*Nicotiana glauca*), lobeline of the indian tobacco, and the toxic alkaloid coniine from poison hemlock (Henry, 1949). Thousands of piperidine compounds have been subjected to clinical and pre-clinical trials (Cragg et al., 1993).

Prosopis juliflora is reported to contain piperidine group of alkaloids, having pharmacological activity (Ahmad et al., 1989a; Aqeel et al., 1989). Therefore this plant was selected for reinvestigation of piperidine alkaloids.

Alkaloids are either widely distributed throughout the plant kingdom or are specific for orders, families and species. Within the same plant there can be variations in the pattern of alkaloid composition, which leads to variation in the activity. As reported for *Atropa baetica* (Zarate et al., 1997) and *Erythroxylum coca* (Johnson, 1995), the concentration of alkaloids varies in different parts of the same species. Therefore it becomes imperative to

analyze different parts of the plant so that full pharmacological potential of the plant could be exploited.

Several workers have extracted alkaloids from aerial parts of *Prosopis* species, such as, pharmacologically active piperidine alkaloids in *P. Africana* (Ratle et al., 1966), DNA binding alkaloids from *P. alpataco, P. argentina, P. chilensis, P. flexuosa and P. pugionata* (Tapia et al., 2000), cytotoxic alkaloids from *P. juliflora* (Hughes et al., 2005) and piperidine alkaloids of *P. juliflora* (Ahmed et al., 1989a), however there are no report on their diversity and relative distribution within different tissues of the plant. Most of the piperidine alkaloids identified in *P. juliflora* have been isolated from leaf extracts and limited work has been done to screen other parts of the plant for these alkaloids. Therefore, the present research work, aims at identification of biologically active piperidine alkaloids present in various organs of *P. juliflora* and study its diversity and relative abundance within various parts.

Along with the alkaloids, the present work also focuses on isolation of other important metabolites of *P. juliflora.*

2.2. Materials and Methods

2.2.1. Plant material

Plant material (leaf, pod, flower, root and stem) of *P. juliflora* were collected from their natural habitat in the Shekhawati regions of Rajasthan, India, during months of June and July (2009). The plant was identified with the help of standard flora by Shetty and Singh, 1993 and a voucher specimen was deposited in Dungar College herbarium, Rajasthan, India (DCH5092).

2.2.2. Sample preparation

Plant parts were washed thoroughly under tap water and then air dried under shade for two weeks and oven dried for 24 hrs at 40°C. The dried plant material was grounded to form

fine powder and filtered through sieve of 345 micron pore size. The ground plant materials were stored in a refrigerator at 4°C.

2.2.3. Solvent extraction

Plant samples were successively extracted with the help of various organic solvents (hexane, chloroform, acetone and ethanol) in the order of their increasing polarity and finally with distilled water. First of all, 50 grams of dry powder was taken in a beaker and hexane was added to it so that the plant material gets totally immersed in the solvent. This whole setup was kept for 48 hours with frequent shaking. It was first filtered with a muslin cloth, then with whatman filter paper (No.1) and finally centrifuged at 5000 rpm for 5 mins. Whole process was repeated 3 times and supernatant were collected and pooled together. Plant material left after filtration was air dried to evaporate the hexane completely and then followed by extraction with chloroform. The above process was repeated with acetone, ethanol and water and the supernatant collected were concentrated to dryness with the help of rotary evaporator (Buchi Rotavapor R-200/205) at 40 °C under different pressure conditions for the solvent. All the concentrated extracts were stored in air tight bottle at 4°C.

2.2.4. Phytochemical screening

Extracts were subjected to phytochemical screening to identify the presence of phytochemicals, according to the methods described by Harborne, 1984 with minor modifications.

Test for saponins- 300 mg of extract was boiled with 5 ml water for two minutes. Mixture was cooled and mixed vigorously and left for three minutes. The formation of frothing indicates the presence of saponins.

Test for tannins- To 5 ml of aliquot of the extract, sodium chloride was added to make it to 2% strength. Extract was filtered and mixed with 1 ml of 1% gelatin solution. Precipitation indicates the presence of tannins.

Test for Phlobatanins- 200 mg extract was boiled with 1% aqueous hydrochloric acid. Deposition of red precipitate indicates the presence of phlobatanins.

Test for phenolic compounds- 1% ferric chloride solution was added to the extract, formation of intense green, purple, blue or black colours indicates the presence of phenolic compounds.

Test for Triterpenes- 300 mg of extract was mixed with 5 ml chloroform and warmed for 30 minutes. The chloroform solution was then treated with a small volume of concentrated sulphuric acid and mixed properly. The appearance of red color indicates the presence of triterpenes.

Test for steroids- 200 mg plant material was taken in 10 ml chloroform and then filtered. In 2 ml filterate, 2 ml acetic anhydride and small amount of H_2SO_4 was added, appearance of blue green ring indicates presence of steroids.

Test for alkaloids- 200 mg plant extract was dissolved in 10 ml methanol and then filtered. In 1 ml filtrate 6 drops of Dragondroff's reagent was added. Appearance of orange precipitate indicates presence of alkaloids.

Preparation of Dragondroff's reagent
Solution A: Bismuth nitrate (0.17 g) in acetic acid (2 ml) and water (8 ml)
Solution B: Potassium iodide (4 g) in acetic acid (10 ml) and water (20 ml)
Solutions A and B were mixed and diluted to 100 ml with water

Test for flavonoides- 1 ml of dilute ammonia solution was added to the 5 ml of extract, followed by addition of few drops of concentrated sulphuric acid. The development of yellow colour indicates the presence of flavonoides.

Test for glycoside– 5 ml of extract was treated with 1 ml of glacial acetic acid containing 1 drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates the presence of deoxysugar characteristic of cardenolides.

2.2.5. Alkaloid extraction

The alkaloid extract was obtained by an acid/basic extraction process as described by Ott-Longoni et al., 1980. The ethanol extract, as obtained from previous extraction step was fractionated between petroleum ether and water forming two layers, lower aqueous layer (F1) and upper petroleum ether layer (F2). The aqueous layer was separated and again extracted three times with petroleum ether. Aqueous layer was stirred with 0.2 N HCl for 16 h followed by filtration, with Whatmann filter paper no.1. The solution was shaken with chloroform to remove the non-basic material. The aqueous layer was basified with ammonium hydroxide until it reached pH 11, and then was extracted with chloroform. The chloroform phase was evaporated leading to the production of the Alkaloid rich fraction (ARF). ARF was again separated with methanol into chloroform insoluble and chloroform soluble fraction. The fractions were dissolved in distilled water with shaking and slightly warming at concentration of 10 mg/ml and were kept at $4^{o}C$ for further analysis.

Separation and identification of compounds at each step was checked by running different fractions on a TLC plate and observing the separated spot under UV light of 250 and 354 nm wavelengths. Presence of alkaloid in each fraction was determined by spraying dragondorff's reagent on the plates.

2.2.6. Alkaloid quantification

Alkaloid quantification was carried out by precipitating ARF with Dragendorff's reagent followed by spectrophotometric estimation of the precipitate (Sreevidya and Mehrotra, 2003).

Chemicals required:

(a) *Dragendorff's reagent (DR)-* composition mentioned in the previous section

(b) *Bismuth nitrate solution* - Bismuth nitrate stock solution was made by dissolving 10 mg $Bi(NO_3)_3{\cdot}5H_2O$ in 5 ml concentrated nitric acid and diluting to 100 ml with distilled water (D.W)

(c) *Thiourea, 3%.* - Prepared by dissolving 3 g in 100 ml D.W

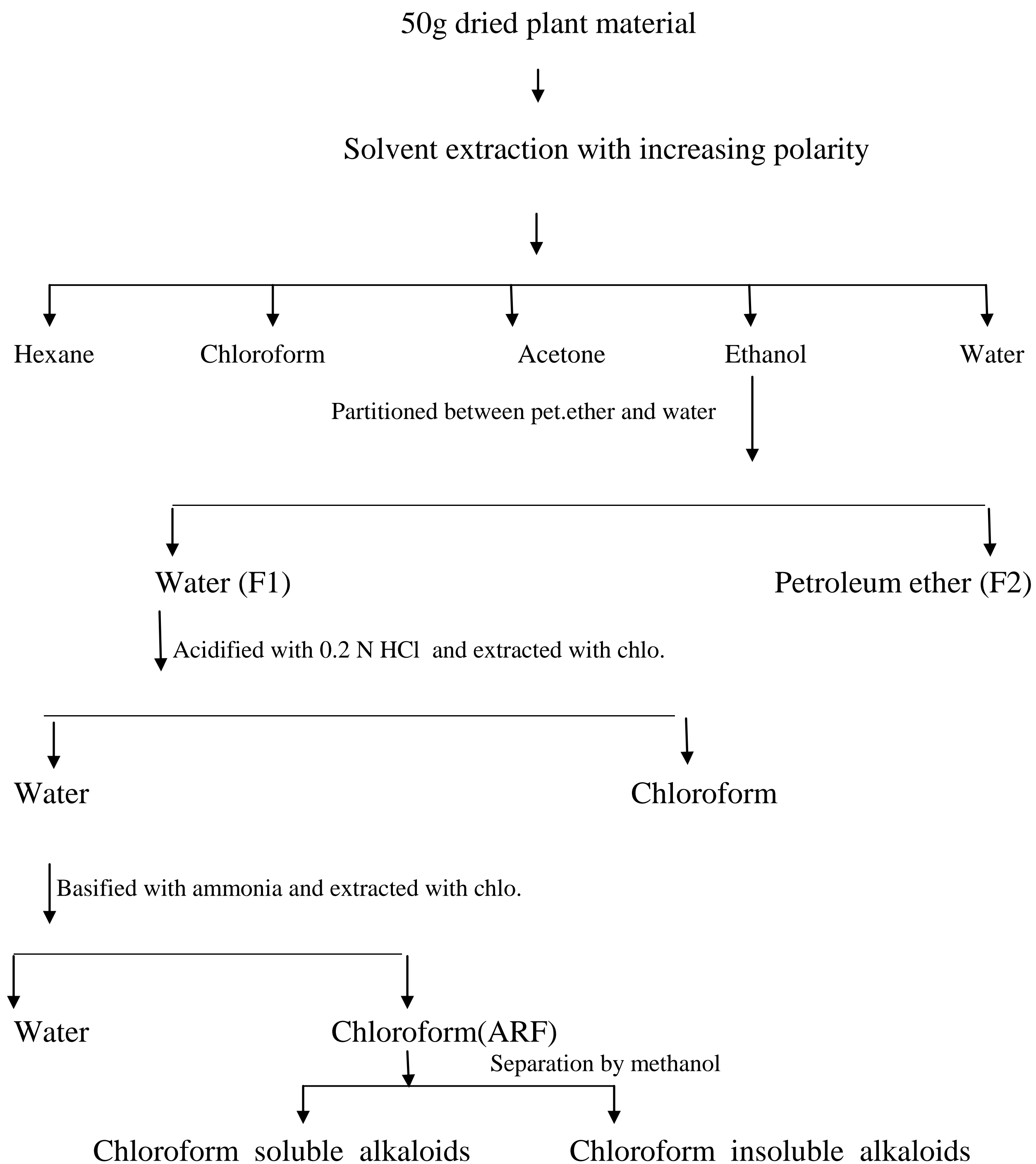


Fig 3. Schemmatic representation of solvent extraction steps

(d) *Disodium sulfide, 1%* - Prepared by dissolving 1 g in 100 ml D.W

(e) *Stock solution of alkaloid* - 10 mg of ARF was dissolved in 10 ml solvent

Procedure for Assay of Alkaloids in Plant Extracts**:** 5 ml of the ARF was taken and 2 ml of DR was added to it, the precipitate formed was centrifuged. The centrifugate was checked for complete precipitation by again adding few drops of DR. After centrifugation, the centrifugate was decanted completely. The precipitate was further washed with alcohol. The filtrate was discarded and the residue was then treated with 2 ml disodium sulfide solution. The brownish black precipitate formed was then centrifuged. Completion of precipitation was checked by adding 2 drops of disodium sulfide. The residue was dissolved in 2 ml concentrated nitric acid, with warming. This solution was diluted to 10ml in a standard flask with distilled water, 1 ml was then pipetted out, and 5 ml thiourea solution was added to it. The absorbance was measured at 435 nm against the blank containing nitric acid and thiourea. The amount of alkaloid present in the solution was calculated from the standard curve prepared with bismuth nitrate stock solution.

2.2.7. Separation of alkaloids by TLC

Chloroform soluble and insoluble fractions of the ARF were further separated by TLC (Thin layer chromatography). The chromatographic estimations were performed using following conditions: stationary phase, reverse phase (RP) C_{18} TLC plates precoated silica gel 60 F_{254} aluminium plates (20 cm×20 cm×250 µm); mobile phase, chloroform:methanol (9:1). 5 µl of ARF (10 mg/ml) were applied at one end of the TLC plate with a separation of 1 cm. The plate was dipped in the mobile phase and kept for some time till the solvent had moved $3/4^{th}$ of the plate length. Separated spots were scratched and dissolved in small amount of mobile phase solvent and kept at $4^{o}C$ for further analysis.

2.2.8. Purification of compounds from Petroleum ether fraction (F2) of leaf

The F2 fraction obtained from crude ethanol extract of leaf in the previous extraction steps, was further subjected to purification. Separation of the extract was achieved by repeated column chromatography using silica (60:120) as stationary phase and petroleum ether (PE)/ethyl acetate (EA) /methanol (M) as mobile phase. Following gradient of solvent

system was used for the initial separation, PE (100%), PE:EA (3:1), PE:ET (1:3), EA:M (3:1), M (100%). At each step of separation purity was checked by thin layer chromatography and HPTLC analysis. Identification of the compound was done by UV, NMR and MS techniques and then comparing these data with the data present in the chemical libraries (NIST database).

2.2.9. Purification of compound from aqueous fraction (F1) of flower extract

The ethanol extract obtained from flower powder was fractionated between petroleum ether and water with the help of separating funnel. The aqueous layer was basified with ammonium hydroxide until it reached pH 11, and then was extracted with chloroform to remove basic components. The resultant aqueous layer was kept in refrigerator for one month. The pale-yellow crystals, thus formed were washed with ethanol and then filtered. Following tests were performed in order to identify the compound:

- Solubility test- compound was dissolved in different polar and non-polar solvents
- Bromocresol green test- chromatogram was dipped in a solution of 0.1 g bromocresol green in 500 ml ethanol and 5 ml 0.1 M sodium hydroxide solution.
- It was subjected for mass, IR, ^{1}H and ^{13}C NMR analysis for further identification of the compound

2.2.10. HPTLC analysis

A Camag HPTLC (High performance thin layer chromatography) system containing Camag Linomat IV semiautomatic sample applicator, Hamilton syringe (100 µl), Camag TLC Scanner-3 with win CAT software version 1.3.4, were used for the identification of separated compounds on a TLC plate. Rf value and percentage area for each spot was calculated by HPTLC software. Rf could also be calculated manually by the following formula:

$$Rf = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent}}$$

2.2.11. DART-MS analysis

The DART-MS (Direct analysis in real time mass spectrometry) analysis was conducted in CDRI (Central Drug Research Institute), Lucknow. DART-MS was recorded on a JEOL-AccuTOF JMS-T100LC atmospheric pressure ionization time-of-flight mass spectrometer (Jeol, Tokyo, Japan) fitted with a DART (Direct analysis in real time) ion source. The given samples were subjected to DART source. Dry Helium was used with 4 L/min for ionization at 350°C. Extracts were analyzed in positive ion mode $[M+H]^{+}$. For the mass spectrometer, the following settings were loaded: orifice 1 set to 28V, ring lens voltage set to 13 V, ion guide RF Volt was set at 1000V and acquired m/z range was 10.0 to 1050.0, spectra recording interval was 0.4s; A solution of poly ethylene glycol (PEG600) was used for calibration. The same calibrant was also introduced at the end of each sample analysis to perform mass drift compensation. Sample were introduced into the DART He plasma using the closed end of a borosilicate glass melting point capillary tube until a signal was achieved in the total-ion chromatogram (TIC). The next sample was introduced when the TIC returned to baseline levels. Candidate molecular formulae were identified using elemental composition and isotope matching programs in the software. Tolerance in atomic mass was taken as 5mmu.

2.2.12. LC-ESI-MS/MS analysis

The LC-ESI-MS (Liquid chromatography electrospray mass spectrometry) analysis was conducted in CDRI (Central Drug Research Institute), Lucknow. It was performed on Thermo Finnigan LCQ Advantage max ion trap mass spectrometer having Finnigan Surveyor HPLC system connected to it. HPLC system contained Surveyor autosampler and Surveyor PDA detector. The column was thermo ODS-2, 250 X 4.6, 5 µm. The elution gradient was carried out with binary solvent system consisting of acetonitrile (solvent A) and 5 mM ammonium acetate buffer (solvent B) at a constant flow-rate of 0.8 ml/min. Linear gradient profile with the following proportions (v/v) of solvent A and B was applied: (10:90), (85:15), (70:30), (50:50), (80:20), (90:10), (100:0) with 5 min for re-equilibration. Injection volume was 20 µL and data was collected in the range of 210-400 nm. After the separation was achieved, separated compounds were introduced into the ESI

(Electrospray ionization) source. Capillary temperature was kept upto 275°C and spray voltage 4.5 kV. The mass spectra were scanned in the range 100-1500 Da and the maximum ion injection time was set 50 nS. All the analyses were performed in positive ion mode.

2.2.13. UV-visible spectrophotometer analysis

The UV–visible spectra were recorded on a Jasco V-630 spectrophotometer with 1 cm matched quartz cuvettes. The compounds were dissolved in the solvent (ether or methanol) in the concentration of 1mg/ml and filled in the cuvettes upto $3/4^{th}$ of its lenght. Solution was made clear and transparent with no suspended material present. If impurities were present, solution was filtered through filter of 0.45 micron pore size. The absorbances were read at 200-700 nm against the blank containing respective solvents.

2.2.14. FTIR analysis

FTIR (Fourier transform infrared spectrophotometer) spectra of the compound was recorded on Shimadzu Corporation model, IR prestige 21 (200VCE) using KBr pellet method. For preparation of KBr pellet, KBr was dried in an oven at $100^{o}C$ for 30 mins to remove any moisture. About 1% of sample was mixed with KBr and finely grounded into a homogenous mixture into a mortar. Small amount of sample was taken and made into a pellet by applying pressure upto 20,000 prf. Pellet was inserted into the FTIR sample holder and the spectrum was recorded. The spectrum was focused in the mid IR region of 400-4000 cm^{-1}.

2.2.15. NMR analysis

The NMR Analysis was conducted in CDRI (Central Drug Research Institute), Lucknow. The ^{13}C (75.4 MHz) and ^{1}H (300 MHz) NMR (Nuclear magnetic resonance) spectra were recorded on a Varian XL-300 NMR spectrometer. For ^{1}H and ^{13}C NMR, 4 mg and 30 mg of samples respectively, were disolved in an inert solvent deuterochloroform (CDCl3), or deuterated acetone The sample was sonicated or agitated to aid dissolution, and solids were removed via filtering through a plug of celite in a Pasteur pipette, directly into a thin glass

walled NMR tube of 5 mm diameter. The different chemical shifts of the proton according to their molecular environments within the molecule were measured in the NMR apparatus relative to a standard, tetramethyl silane (TMS). The intensity of the signals was integrated to show the number of protons resonating at any one frequency.

2.3. Results and Discussion

2.3.1. Solvent extraction

Solvent extraction is the primary step in isolation of phytochemicals and different solvents are responsible for extracting different groups of phytochemicals depending on their polarity (Harborne, 1984). Therefore, for preliminary screening of important phytochemicals of *P. juliflora*, a range of solvents with varying polarity were chosen for better extraction of most of the secondary metabolites. Among the five solvents that were selected (hexane, chloroform, acetone, ethanol and water), hexane was least polar and water was highest polar, whereas other solvents had intermediate polarity.

The yield of extracts obtained by extracting 50 g of plant material by various solvents is shown in Table 1. Among different solvents used, ethanol was found to be the best solvent generating highest yield from all parts of the plant. The high efficiency of ethanol can be attributed to its intermediate polarity leading to the extraction of both polar and non polar compounds (Harborne, 1984). Ethanol was followed by water and chloroform. Hexane and acetone were found to be the least effective solvents in extracting phytochemicals, which could be due to lesser amount of compounds in the plant, which could be dissolved in these solvents.

Earlier studies on *P. juliflora* have reported that leaves and pods are rich source of nutrients (Blair and Hall, 1968; Takeoka et al., 2009) and hence there are more chances of extracting metabolites from these plant parts. The result obtained in the present experiment

Table 1. Plant metabolites (in gram) extracted by various solvents from 50 gm of dry plant material

Plant parts	Solvents					
	Hexane	Chloroform	Acetone	Ethanol	Water	Total
Leaf	1.31	2.85	0.96	5.63	4.97	15.72
Stem	0.82	1.51	0.68	3.08	2.48	7.95
Pod	1.05	2.23	0.72	5.55	5.84	15.30
Flower	0.95	2.62	0.77	5.04	4.30	13.68
Root	0.74	1.79	0.56	4.04	3.35	10.48

Table 2. Phytochemicals present in the extracts, obtained from various parts of *P. juliflora*

Phytochemicals	Plant Parts				
	Leaf	Pod	Flower	Stem	Root
Tannin	+	+	-	-	++
Phlobatannin	-	-	-	-	-
Phenolics	+++	+++	+++	+	++
Flavonoid	+++	++	+++	+	++
Cardiac glycoside	-	-	-	-	-
Alkaloid	+++	+++	++	-	+
Terpenes	+++	++	+	+	++
Steroids	+++	++	+	+	+
Saponin	-	-	-	-	+

+; low concentration, ++; moderate concentration, +++; high concentration, - ; absent

follow similar pattern of metabolite distribution, as leaf and pod were found to contain highest concentration of plant metabolite (15.72 g and 15.30 g respectively), followed by flower (13.68 g), root (10.48 g) and least in stem (7.95 g).

2.3.2. Phytochemical analysis

The phytochemical screening of leaves, pod, flower, stem and root showed presence of various phytochemicals, however their concentration varied in different parts of the plants (Table 2). The extracts revealed presence of tannins, phenolics, flavonoids, alkaloids, terpenes and steroids in most parts of *P. juliflora*. Plobatannin and cardiac glycoside were absent in all the tested parts of the plant, whereas saponin was found only in roots.

Phytochemical study on *P. juliflora* has been earlier reported (Rastogi and Mehrotra, 1993) and several compounds has been identified from different parts of the plant, however in current screening step, certain important metabolites are shown to be present, not only in reported part, but also in other plant parts, such as tannin reported in root (Malhotra and Mishra, 1983c) is shown in leaves and pod, alkaloids reported in leaves (Ahmad et al., 1989a) are also evident in flower and pod. Hence these plant parts could also provide a good source for isolation of important metabolites. Phenolic compounds and flavonoids are present in most part of the plant, as mentioned in earlier reports (Vajpeyi et al., 1981; Malhotra and Mishra, 1983b).

Alkaloids were found in large concentrations in leaf, pod and flower, hence these plant parts were chosen for further extraction and purification of alkaloids.

2.3.3. Purification and quantification of alkaloids

Alkaloid purification is a multistep process (Otto Longi et al., 1980) and was achieved in the present experiment by separting the alkaloids from the nonalkaloidal components with the help of different solvents. Nonpolar components of the crude extract were removed by fractionating them with petroleum ether and water. Water fraction (F1) thus obtained, was analysized on a reverse phase TLC plate for further identification and separation of compounds. TLC analysis under normal and UV lights (Fig.4a, 4b and 4c) revealed

presence of large number of compounds in the F1 fraction, which were found to be distributed between lower and upper side of the TLC plate. TLC separates the compound based on their affinity with the mobile and stationary phase (Harborne, 1984). On a reverse phase TLC plate, polar compounds will move faster with the solvent and hence will be present on the upper side of the plate, whereas non polar compounds will bind with the stationary phase and therefore will be present on the lower side of the plate. According to the results of the TLC, it could be concluded that both polar and non polar compounds are present in F1 fraction.

Presence of alkaloid in F1 fraction was tested by application of dragendorff reagent on the plate, an orange colour confirmed alkaloids in the extract (Fig.4d). Orange colour was shown on the lower side of the reverse phase TLC plate, indicating slightly non polar nature of these alkaloids. Alkaloids are basic compounds and hence were further extracted from the F1 fraction by acid-base extraction method. After acid-base extraction, more polar and non basic materials were removed, leaving alkaloids in the extract (ARF) as shown in Fig.4e and 4f.

The ARF obtained was subjected for quantitative analysis of the alkaloids. Pod was shown to contain maximum quantity of alkaloid (0.77g), followed by leaf (0.52g) and then flower (0.43g) in 50g of dried plant material (Table 3). Earlier isolation of alkaloids from *P.juliflora* were focused on leaf extract (Ahmad et al.,1989a; Nakano et al., 2004a), however the present study shows that pod is the better source for isolation of these compounds.

Table 3. Total amount of F1 fraction and ARF (in gram) obtained from 50 g of plant material

	F1	**ARF**
Leaf	3.52	0.52
Pod	3.21	0.77
Flower	3.12	0.43

F1; aqueous fraction, ARF; alkaloid rich fraction

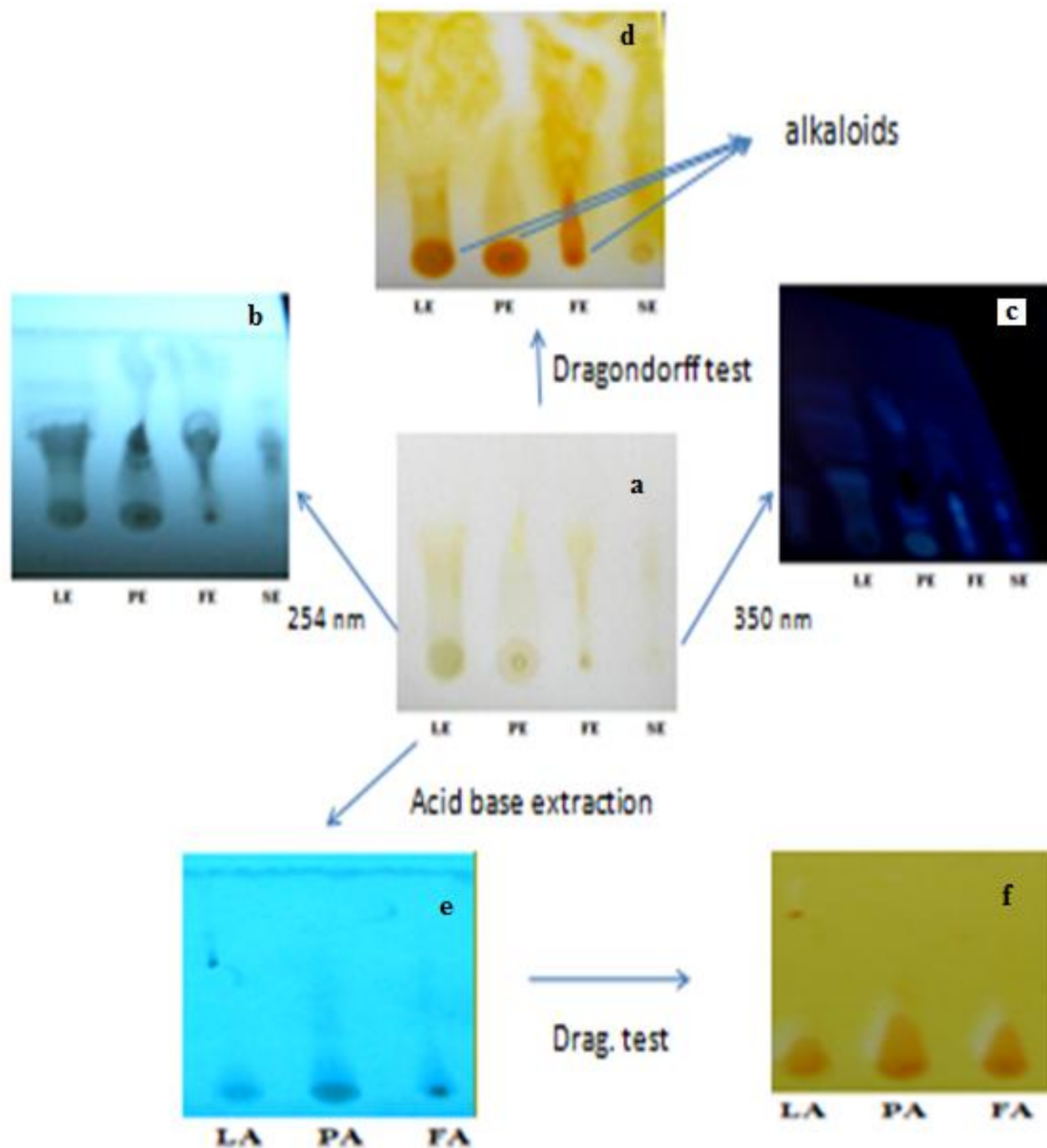


Fig 4. Schemmatic representation of ARF isolation steps - a) TLC plate under normal light b) and c) TLC plate under 254 nm and 350 nm wavelength of light showing presence of large number of compounds d) TLC plate after spraying dragondorff's reagent, showing alkaloids on the lower side of the plate e) TLC plate after acid base extraction step, showing presence of only alkaloids on the lower side of the plate f) TLC plate after dragondorff's reagent spray, showing alkaloids

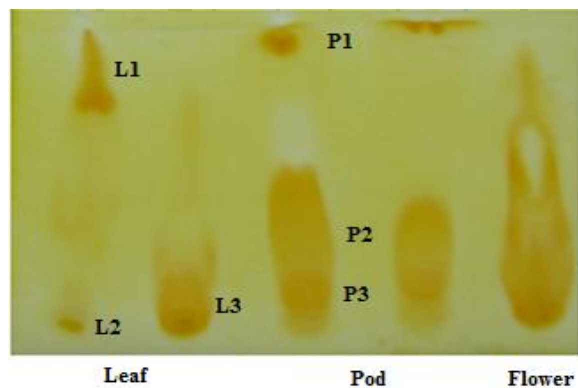
ARF was again separated with the help of methanol and chloroform into chloroform soluble and chloroform insoluble fraction. Leaf and pod ARF were separable into both fractions, whereas flower ARF was present as chloroform insoluble fraction. All the 5 fractions obtained were further separated on a TLC plate into more fractions depending on their solubility in the mobile phase (Fig.5a). Since these fractions were present in very small quantity on the plate, therefore they could not be quantified by dragandorff's precipitation test and hence were analyzed and quantified directly on the plate by HPTLC. Rf values and percentage area were calculated for the separated compounds and the results are presented in table 4.

Table 4. HPTLC analysis of fractions of ARF showing Rf values of alkaloids and their percent abundance in the extract

Leaf	%abundance	Pod	%abundance	Flower	%abundance
0.11 (L2)	10%	0.26 (P3)	15%	0.20	
		0.33		0.28	
0.83 (L1)	90%			0.51	100%
		0.44 (P2)	64%	0.59	
				0.70	
		0.60	5%		
		0.65			
		0.84 (P1)	16%		

HPTLC analysis showed that leaf chloroform soluble fraction contained 2 spots (Fig.5b), major one, with 90% abundance on the upper side (L1) and smaller spot with 10% abundance at the bottom (L2). Leaf chloroform insoluble fraction (Fig.5c) could not be separated further and thus contained only one spot (L3).

Pod fractions showed 3 spot (Fig.5d), upper spot with 16 % abundance (P1), the second spot present in the middle with 64% abundance (P2) and lower spot with 15% abundance (P3). Compounds present in the 4th and 5th spot were present in very low concentration, therefore they could not be eluted from the TLC plate and hence were left.



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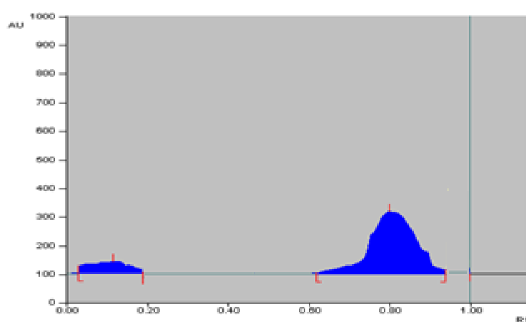

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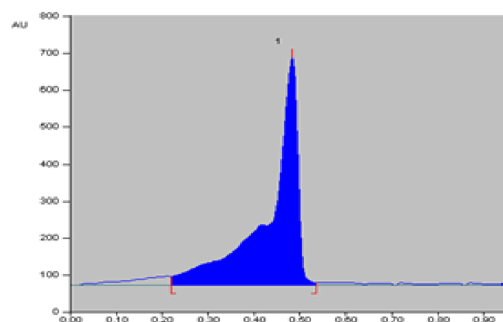

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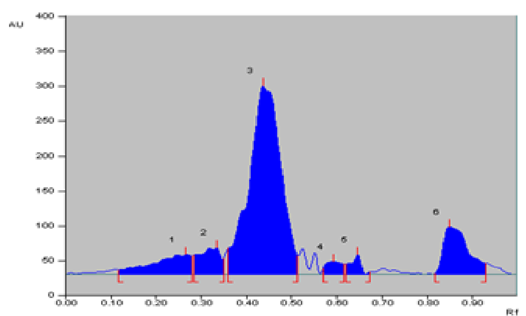

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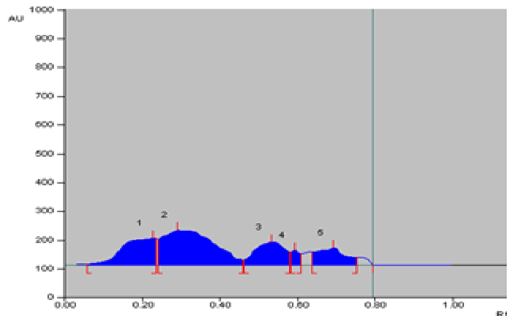


e)

Fig 5. TLC and HPTLC analysis of fractions of ARF- a) separation of compounds of ARF into various fractions on a TLC plate b) HPTLC analysis of leaf chloroform soluble fraction showing presence of 2 spot c) HPTLC analysis of leaf chloroform insoluble fraction showing presence of 1 spot d) HPTLC analysis of pod fraction showing presence of 6 spot e) HPTLC analysis of flower showing presence of a continous band

Flower extract showed one continuous band and therefore it was considered as one spot. Although HPTLC analysis showed presence of various compounds in flower ARF having different Rf values, but they were not separable and were continuous with each other (Fig.5e).

2.3.4. Identification and distribution of piperidine alkaloids in different parts of *P. juliflora*

Identification of Piperidine alkaloids in unprocessed plant material by DART-MS

Characterization of the active components from natural products has always been associated with problems of tedious and complex solvent extraction processes, which is followed by identification and quantization of bioactive components by conventional methods such as HPLC-PAD, HPLC-MS, GC-MS, etc. Although such methods have been successfully adopted to various fields of natural products and crude herbal drugs, there is an increasing requirement for more efficient and prompt analytical techniques in order to manage vast numbers of samples in short duration of time (Roscheck et al., 2009). DART-MS proves to be a better alternative of conventional method in performing quick and efficient analysis of bioactive components in real time.

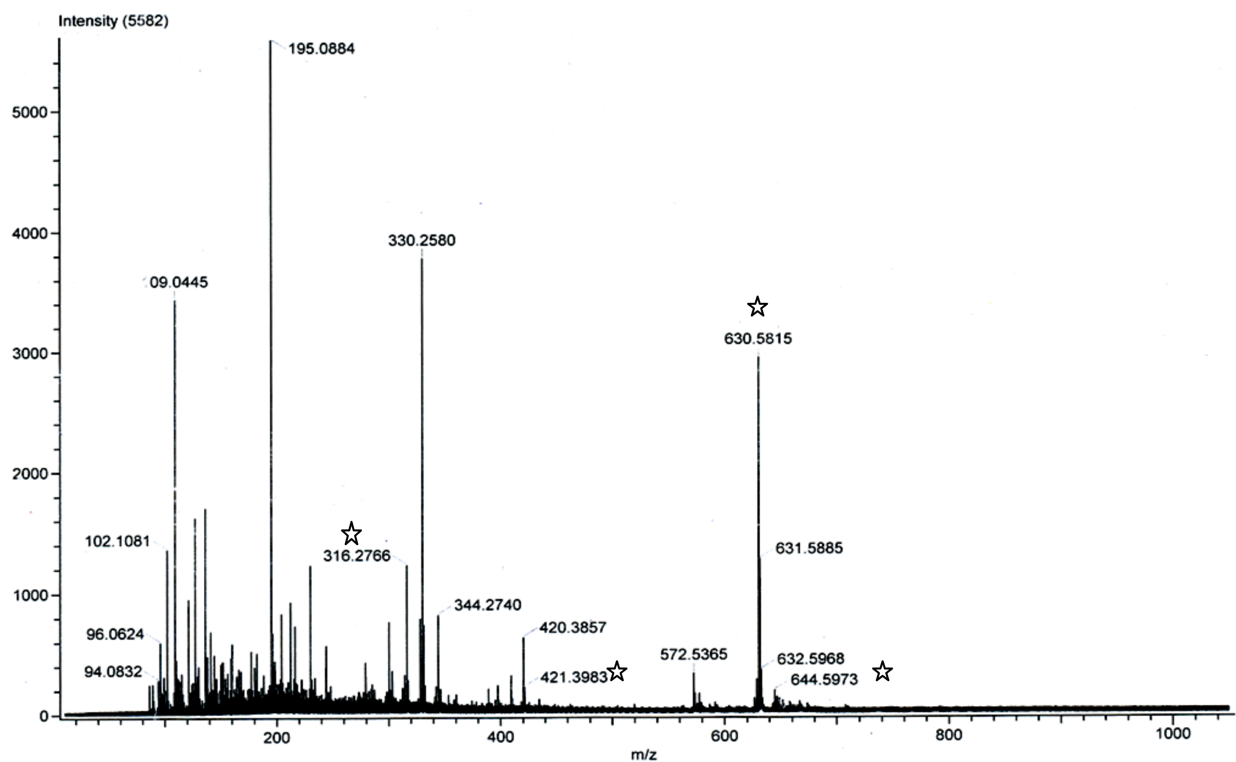
DART-MS, operating in open air, ionizes compounds (gas, liquid or solid) directly in their native condition, bypassing most steps of the analytical system, such as solvent extraction (Kubec et al., 2010). DART-MS produces $[M + H]^+$ molecular ions of most compounds, therefore simple and clear mass spectra are obtained even of multi-component samples. DART is coupled to a time of flight mass analyzer that provides selectivity and accurate elemental composition assignment through exact mass measurement. Recently many researchers have applied DART technique to directly identify chemical compounds from raw material and/or processed plant material, such as, curcumin was directly analyzed from turmeric (Kim and Jang, 2009), it was also used to determine the molecular formulae and structures of toxoid compounds in cell cultures of *Taxus wallichiana* (Banerjee et al., 2008) and hairy root of *Rauvolfia serpentina* (Madhusudanan et al., 2008).

Initial screening for the identification of piperidine alkaloids in different parts of *P. juliflora* was accomplished by directly introducing the unprocessed plant material towards the DART source. The mass spectra thus obtained, displayed piperidine alkaloids in leaf, pod and flower, whereas other tested parts of the plant (stem and root) were found to be devoid of these alkaloids (Fig.6, 7 and 8). These results are in consistent with the findings of other researchers, who have reported a high degree of qualitative and quantitative variability of piperidine alkaloids among tissues within species (Stermitz et al., 1994; Todd et al., 1995).

Direct analysis of leaf (Fig.6a) revealed presence of 12 different alkaloids. Juliprosopine (m/z $[M+H]^+$ 630.59) was shown to be the major alkaloid, whose relative abundance with respect to other alkaloids was found to be 100%. Juliprosopine a biologically active alkaloid, found in leaf of *P. juliflora* is reported to have antibacterial and antifungal activity (Ahmed et al., 1986; 1989b), plant growth inhibitory activity (Nakano et al., 2004a) antidermatophytic activity (Khan et al., 1986) and immunomodulating effect (Aqeel et al., 1992). Recently it has been reported that, Juliprosopine is a non-competitive acetylcholinesterase inhibitor and also presents a Ca2+-channel blocking activity (Choudhary et al., 2005).

Higher resolution of the peaks of leaf spectra (Fig.6b and 6c) displayed other major alkaloids with relative abundance between 40-15%, they were Julifloridine (m/z $[M+H]^+$ 300.29), Juliprosine (m/z $[M+H]^+$ 628.56), Juliprosinine (m/z $[M+H]^+$ 626.56), Prosoflorine (m/z $[M+H]^+$ 627.33), 3-oxo-juliprosine (m/z $[M+H]^+$ 625.55), Prosopine (m/z $[M+H]^+$ 316.28) and Prosopinine (m/z $[M+H]^+$ 314.28). Some other minor alkaloids observed with relative abundance less than 10% include, 3′′′′-Oxo-juliprosopine (m/z $[M+H]^+$ 644.56) , N-methyl Julifloridine (m/z $[M+H]^+$ 313.75), Prosafrinine (m/z $[M+H]^+$ 298.26) and Projuline (m/z $[M+H]^+$ 421.39) (Table 5).

Because the AccuTOF mass spectrometer accurately determines isotopic abundances and yields high resolution mass measurements from the time-of-flight mass spectrometer it was also possible to determine other isotopes of the compounds and their relative



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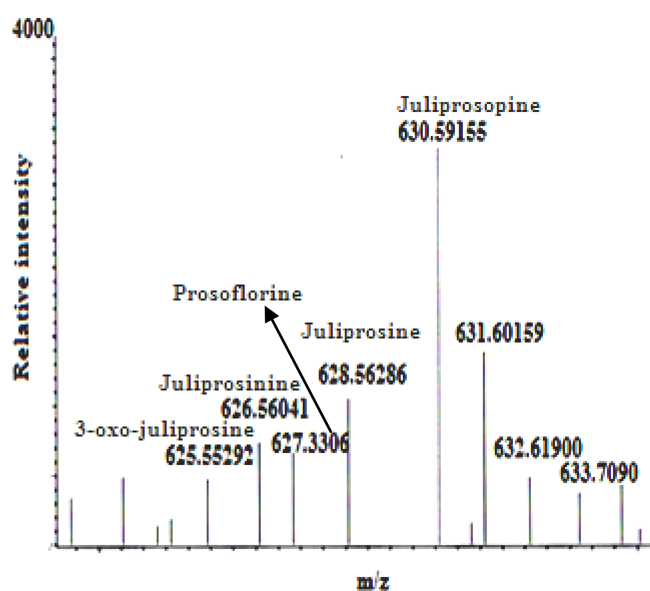

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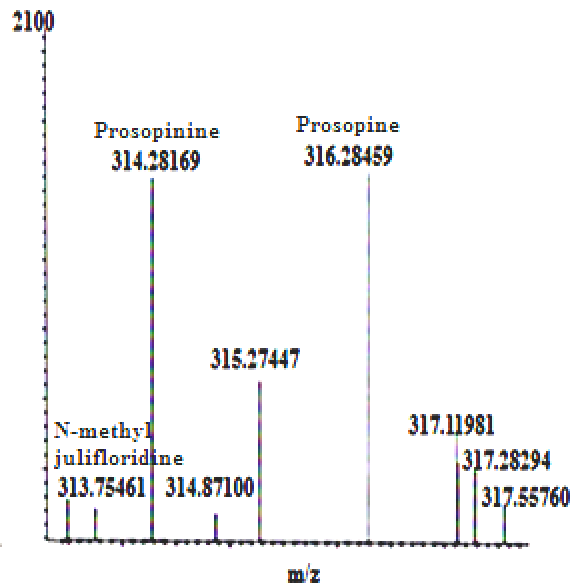


c)

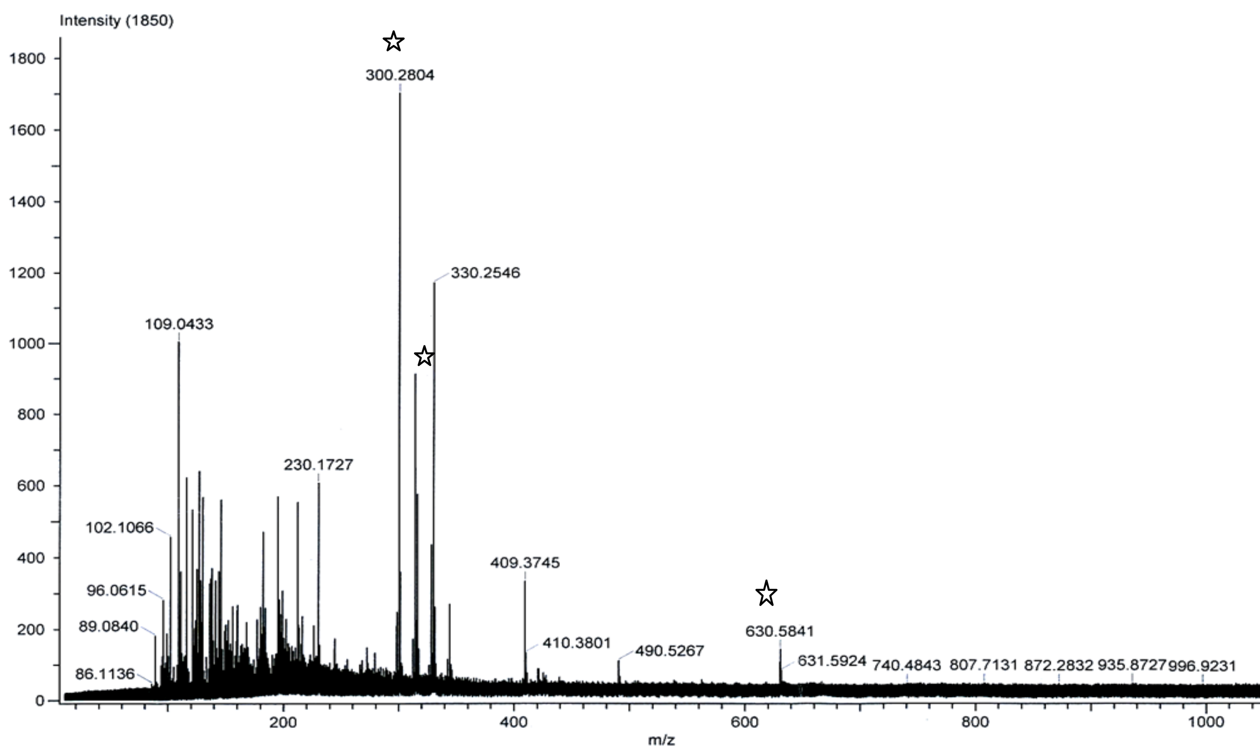
Fig 6. DART-MS analysis of leaf – a) chemical fingerprint of the compounds present in leaf showing Juliprosopine (m/z 630.5915) as major alkaloid (compounds shown by mark represent alkaloids) b) High resolution of closely related peaks showing presence of Juliprosopine, isotopes of Juliprosopine, Juliprosine, Prosoflorine, Juliprosinine, 3-oxo-juliprosine c) High resolution showing presence of Prosopine, isotopes of Prosopine, Prosopinine and N-methyl julifloridine

abundance with the help of DART-MS. Isotopic abundance of Juliprosopine was observed in leaf, showing peaks at 630.59155 (100%), 631.60159 (43%) and 632.61900 (9.1%) (Fig.6b). Two isotopes of Prosopine was also observed at 316.28459 (100%) and 317.28294 (20%) (Fig.6c). These results are in consistence with the calculated isotopic abundance (chemdraw software). An isotope of Prosopinine at 315.27447 was also observed, however its isotopic abundance was found to be 45%, irrespective of its calculated abundance to be 19.5%.

A total of 12 alkaloids were observed in pod (Fig.7a, 7b and 7c), most of the alkaloids were similar to those present in leaf, although relative abundance was different as shown in Table 6. Julifloridine (m/z $[M+H]^+$ 300.28) was found to be present in highest concentration, with 100% relative abundance. Other major alkaloid include Prosopine (52 %) and Prosopinine (36.3%). Juliprosine, Juliprosinine, Prosoflorine, 3-oxo-juliprosine, 3″″″-Oxo-juliprosopine, Prosafrinine, Projuline and Prosopinoline were present in low concentrations and their relative abundance was mostly below 10%. Isotope of Juliprosine was observed in pod (Fig.7c) at 628.55557 (100%) and 629.55939 (50%).

A total of 4 piperidine alkaloids were identified in flower (Fig.8a and 8b). Julifloridine was again the most abundant alkaloid. Along with Julifloridine (100%), Prosopine (23%), Prosopinine (24%) and Prosafrinine (16.6%) were present in considerable amount (Table 7). Flower showed 3 isotopes of Prosopine (Fig.8b), 316.28525 (100%), 317.26660 (20%) and 318.26095 (5%).

It is important to note that Julifloridine was not only the major alkaloid of pod and flower, but also the most abundant chemical compound present in pod and flower, hence pod and flower could serve as a good source of Julifloridine. However its biological activity is not reported, and hence further work needs to be done to evaluate its pharmacological potential.



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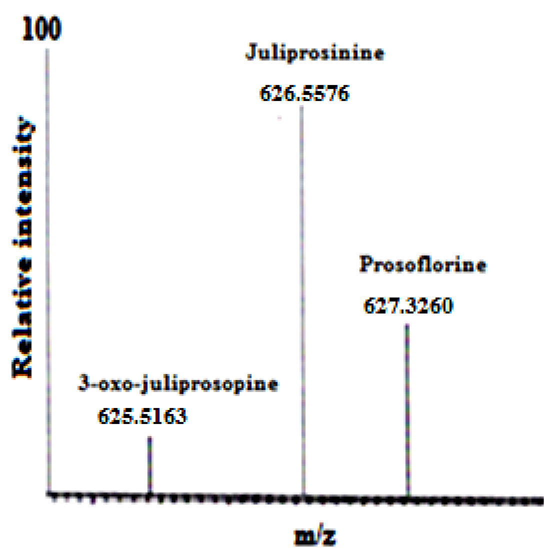
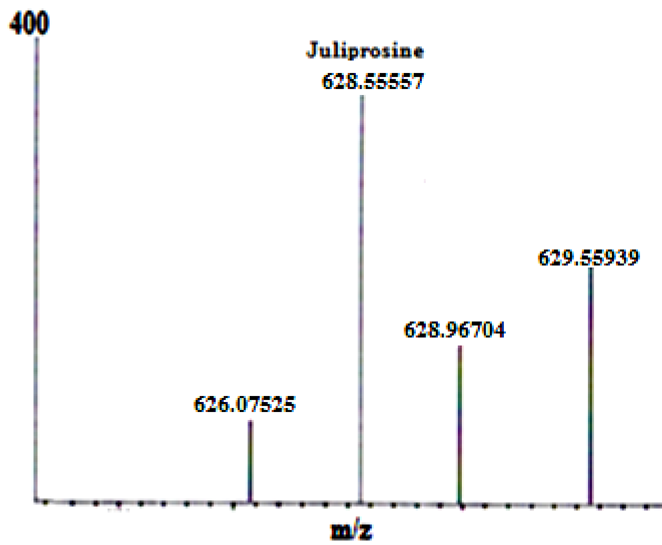
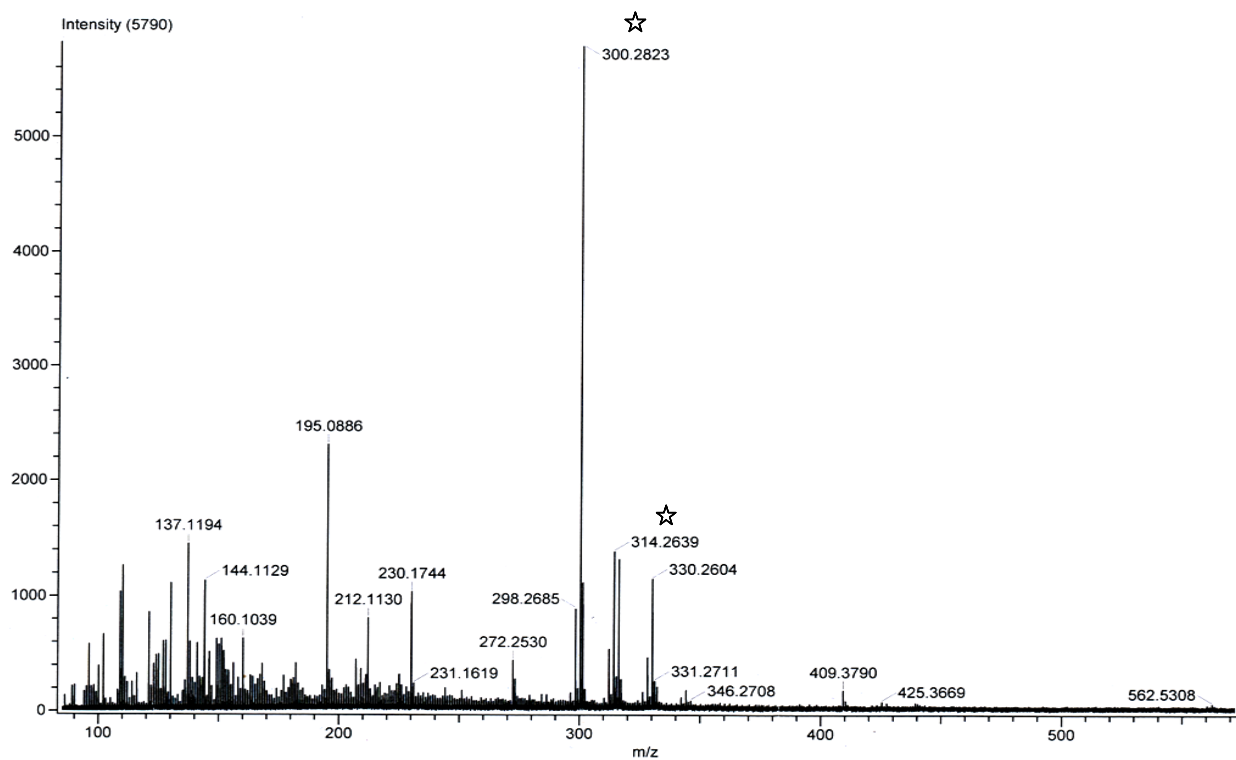




b) c)

Fig 7. DART-MS analysis of pod – a) chemical fingerprint of the compounds present in pod showing Julifloridine (m/z 300.2804) as major alkaloid (compounds shown by mark represent alkaloids) b) High resolution showing presence of Prosoflorine, Juliprosinine and 3-oxo-juliprosine c) High resolution showing presence of Juliprosine and isotopes of Juliprosine



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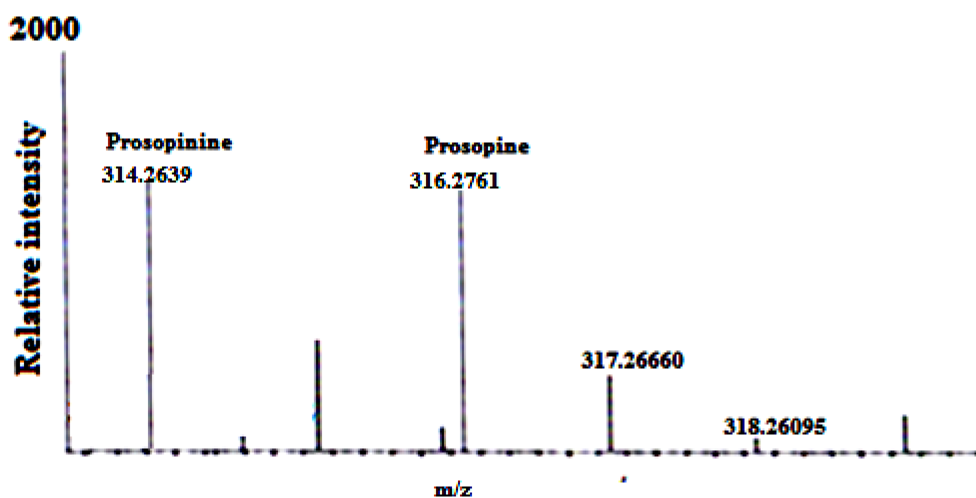


b)

Fig 8. DART-MS analysis of flower – a) chemical fingerprint of the compounds present in flower showing Julifloridine (m/z 300.2823) as major alkaloid (compounds shown by mark represent alkaloids) b) High resolution showing presence of Prosopine, Prosopinine and isotopes of Prosopine

Table 5. Peak obtained by DART-MS analysis of leaf of *P. juliflora*

Compound	Mol.formula	Exact mass $[M+H]^+$ Measured	Calculated	Difference	Relative Abundance (%)
Juliprosopine	$C_{40}H_{75}N_3O_2$	630.5915	630.5938	-0.0123	100
Juliprosine	$C_{40}H_{73}N_3O_2$	628.5628	628.5806	-0.0178	36.6
Prosoflorine	$C_{40}H_{72}N_3O_2$	627.3306	627.3315	-0.0009	23.3
Juliprosinine	$C_{40}H_{71}N_3O_2$	626.5604	626.5513	0.0091	26
3-oxo-juliprosine	$C_{40}H_{70}N_3O_2$	625.5529	625.5516	0.0013	17
3′′′′-Oxo-juliprosopine	$C_{40}H_{73}N_3O_3$	644.5636	644.5728	-0.0092	3.6
Prosopine	$C_{18}H_{37}NO_3$	316.2845	316.2852	-0.0079	35
Prosopinine	$C_{18}H_{35}NO_3$	314.2816	314.2695	0.0121	35
Julifloridine	$C_{18}H_{37}NO_2$	300.2813	300.2903	-0.0090	20
Projuline	$C_{26}H_{48}N_2O_2$	421.3983	421.3894	0.0089	4.6
Prosafrinine	$C_{18}H_{35}NO_2$	298.2674	298.2746	0.0051	4
N-methyl Julifloridine	$C_{19}H_{38}NO_2$	313.7546	313.7532	0.0014	5

Table 6. Peak obtained by DART-MS analysis of pod of *P. juliflora*

Compound	Mol.formula	Exact mass $[M+H]^+$ Measured	Calculated	Difference	Relative Abundance (%)
Juliprosopine	$C_{40}H_{75}N_3O_2$	630.5915	630.5938	-0.0123	7
Juliprosine	$C_{40}H_{73}N_3O_2$	628.5555	628.5806	-0.0067	3.5
Prosoflorine	$C_{40}H_{72}N_3O_2$	627.3260	627.3315	-0.0055	3
Juliprosinine	$C_{40}H_{71}N_3O_2$	626.5576	626.5513	-0.0039	2.3
3-oxo-juliprosine	$C_{40}H_{70}N_3O_2$	625.5163	625.5516	-0.0353	1.6
3′′′′-Oxo-juliprosopine	$C_{40}H_{73}N_3O_3$	644.5636	644.5728	-0.0092	1.4
Prosopinoline	$C_{23}H_{44}N_2O$	381.3823	380.3778	0.0045	10
Prosopine	$C_{18}H_{37}NO_3$	316.2766	316.2852	-0.0086	52
Prosopinine	$C_{18}H_{35}NO_3$	314.2883	314.2695	0.0188	36.3
Julifloridine	$C_{18}H_{37}NO_2$	300.2813	300.2903	-0.0090	100
Projuline	$C_{26}H_{48}N_2O_2$	421.3983	421.3894	0.0089	8
Prosafrinine	$C_{18}H_{35}NO_2$	298.2674	298.2746	0.0051	12

Table 7. Peak obtained by DART-MS analysis of flower of *P. juliflora*

Compound	Mol.Formula	Exact mass $[M+H]^+$		Difference	Relative Abundance (%)
		Measured	**Calculated**		
Prosopine	$C_{18}H_{37}NO_3$	316.2761	316.2852	-0.0911	23
Prosopinine	$C_{18}H_{35}NO_3$	314.2639	314.2695	0.0057	24
Julifloridine	$C_{18}H_{37}NO_2$	300.2823	300.2903	0.0080	100
Prosafrinine	$C_{18}H_{35}NO_2$	298.2685	298.2746	0.0061	16.6

Identification of the alkaloids was done by HR-MS and accurate molecular formula determination. The high resolution power of DART-MS allowed the efficient confirmation of detected molecular ions by comparison of the measured molecular mass with the corresponding theoretical molecular mass. The calculated errors for the empirical mass numbers for piperidine alkaloids as compared with their theoretical mass numbers are given in Table 5, 6 and 7. Elemental composition of the exact mass, assigned with the help of Mass Center software system was compared with the data available in chemical databases (Chapman & Hall) and were found to be similar.

A comparative analysis of the distribution pattern of these alkaloids in all 3 plant parts of *P. juliflora* is shown in Fig.9. Diversity of alkaloids in leaf and pod was high with 12 alkaloids in each part, with the difference of only two alkaloids. N-methyl julifloridine was present in leaf and absent in pod, whereas Prosopinoline was present in pod and absent in leaf. All the other alkaloids were common in both the parts, although their concentrations were different. It was observed that Juliprosopine, the main alkaloid of leaf, was present in negligible amount in pod. Other predominant alkaloids of leaf, with exact mass in the range of m/z 625-644 were also present in negligible amount in pod. Diversity of alkaloid was very low in flower, with a total of only 4 piperidine alkaloids identified. Juliprosopine and other alkaloids in the range of m/z 625-644 were completely absent. As shown in Fig.9, the relative abundance of Juliprosopine and Julifloridine was much higher as compared to other alkaloids, with the next major alkaloid having 40% relative abundance in leaf, 50% in pod and 25 % in flower.

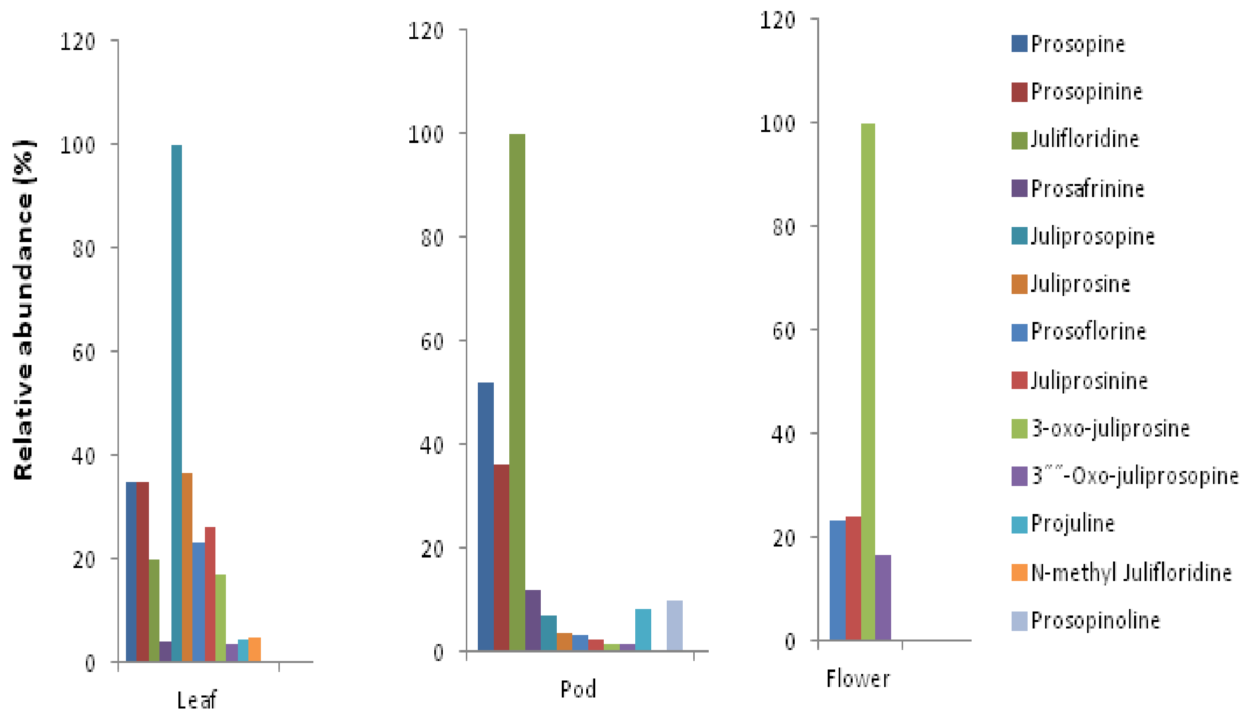


Fig 9. Relative distribution of piperidine alkaloids within leaf, pod and flower of *P. juliflora*

Two groups of piperidine alkaloids have been reported from *P. juliflora,* one group having 2 piperidine ring connected by indolizidine ring in the centre of the molecule and other without indolizidine ring, having one piperidine nucleus (Nakano et al., 2004b). Juliprosopine, Juliprosine, Prosoflorine, Juliprosinine, 3-oxo-juliprosine and 3’’’-oxo-juliprosopine identified in the present research work belong to the first group of alkaloids whereas Julifloridine, Prosopine, prosopinine, Projuline and Prosafrinine are assigned to second group. Our result clearly indicates presence of both groups of alkaloids in leaf and pod, whereas flower was shown to contain only second group of alkaloids.

DART-MS analysis of ARF

ARF was again subjected for DART analysis for qualitative and quantitative analysis of piperidine alkaloids. The mass spectra obtained for ARF (Fig.10) confirmed the presence of all the mentioned alkaloids (Table 8). They all belong to piperidine class of alkaloids which were confirmed by DART generated fragments of the compounds showing peaks at m/z $[M+H]^+$ 115 and 97 (Fig.10a) characteristic of methoxy hydroxyl of piperidine alkaloids which usually have a long alkyl chain at C-6.

Table 8. Relative abundance (in percent) of piperidine alkaloids present in ARF of leaf, pod and flower

Leaf		**Flower**		**Pod**	
Juliprosopine	100	**Prosopine**	25	**Juliprosopine**	60
Juliprosine	36.6	**Prosopinine**	28	**Juliprosine**	20
Prosoflorine	23.3	**Julifloridine**	100	**Prosoflorine**	18.6
Juliprosinine	26	**Prosafrinine**	20	**Juliprosinine**	45.2
3-oxo-juliprosine	17			**3-oxo-juliprosine**	9
3′′′′-Oxo-juliprosopine	6.6			**3′′′′-Oxo-juliprosopine**	43
Prosopine	15			**Prosopinoline**	10
Prosopinine	15			**Prosopine**	66.6
Julifloridine	37			**Prosopinine**	30
Projuline	1.6			**Julifloridine**	100
Prosafrinine	4			**Projuline**	8
				Prosafrinine	33

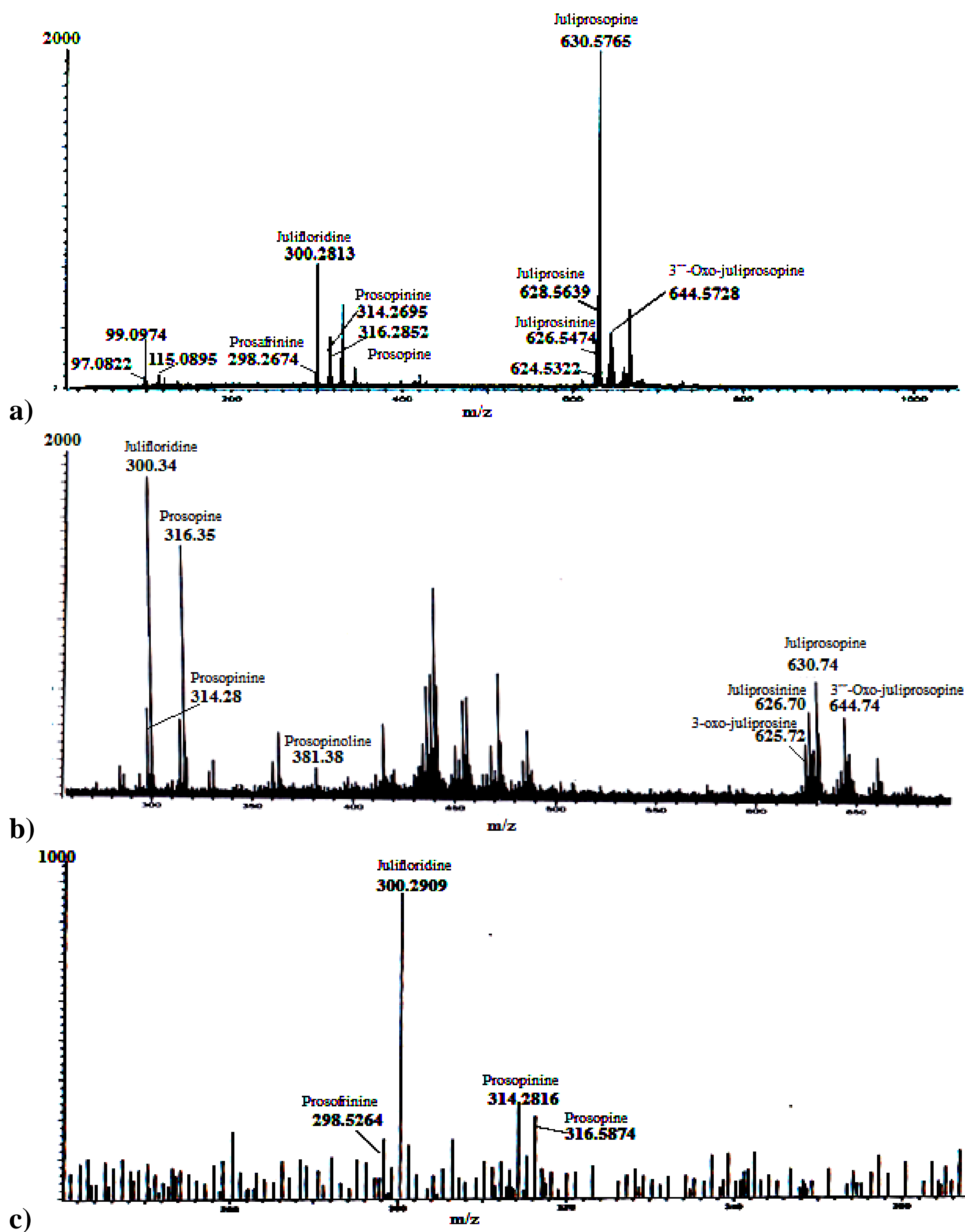


Fig 10. DART-MS analysis of ARF - a) leaf b) pod c) flower

Relative abundance of these alkaloids were however slightly different as present in the unprocessed plant parts (Table 8). Juliprosopine was found to be the major alkaloid of leaf, and Julifloridine of pod and flower, however some variations were present in the relative abundance of other alkaloids, depending upon the efficiency of each alkaloid to be extracted by the standardize procedure.

DART-MS analysis of fractions obtained from ARF

Chloroform soluble and insoluble fractions of ARF, separated by TLC were subjected to DART – MS analysis for the study of alkaloid distribution in different fractions and the results obtained are shown in Fig.11.

DART-MS analysis of L1 showed the presence of Juliprosopine, present in highest concentration (100%). L2 contained Prosopine (100%), Julifloridine (98%), Projuline (72%) and Prosopinine(30%) and some unidentified compounds. L3 showed presence of 4 compounds, in which the compound present in highest concentration was identified as Julifloridine (100%), followed by Prosopinine (65%) and Prosopine (31%).

DART-MS analysis of P1 showed presence of Juliprosopine (100%), Juliprosine (25%), Juliprosinine (12%), 3′′′′-Oxo-juliprosopine (23%) and some unidentified compounds. P2 contained unidentified alkaloids and P3 contained Julifloridine (100%).

Among the various fractions, L1 and P3 were found to contain almost pure compound, Juliprosopine and Julifloridine respectively with more than 90% concentration.

Two new alkaloids, Prosopine and Prosopinine have been identified in leaf, pod and flower. These alkaloids were earlier reported in *P. africana* (Ratle et al., 1966), however the analysis conducted by DART-MS confirmed their presence in *P. juliflora.* There exact mass and chemical composition was found to be matching with the data available in the literature (Rastogi and Mehrotra, 1993). Prosopine and Prosopinine are known to posses pharmacological property (Ratle et al., 1966) and with their identification in leaf, pod and flower of *P. juliflora*, these plant parts can be used as a source for isolation of these important metabolites.

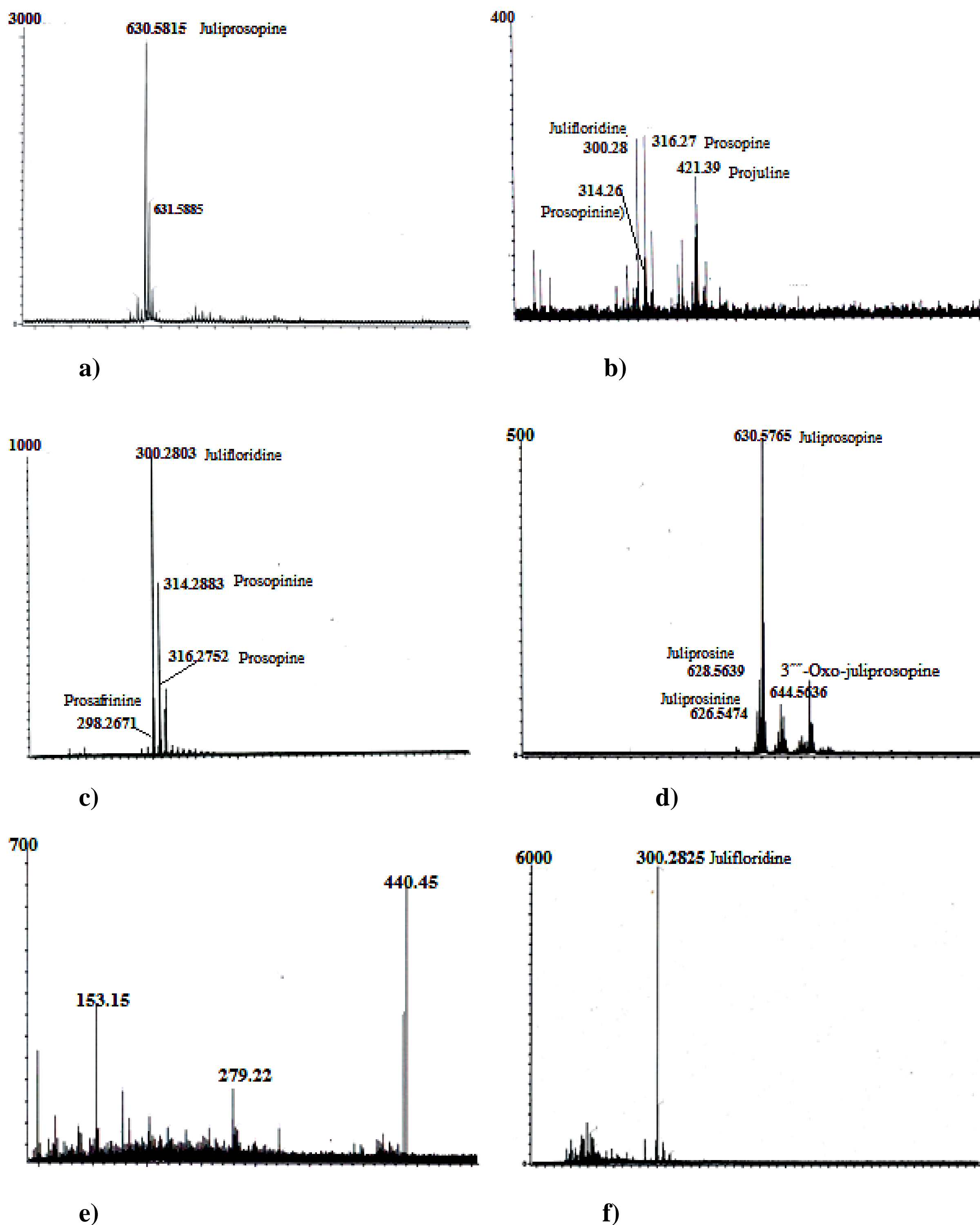


Fig 11. DART-MS analysis of fractions obtained from ARF of leaf - a) L1 b) L2 c) L3 **and pod** - d) P1 e) P2 f) P3

Identification of Prosopine and Prosopinine by LC-MS/MS analysis

Although DART-MS had identified Prosopine and Prosopinine in *P. juliflora*, further confirmation of these alkaloids was done by studying their fragmentation pattern generated by LC-MS/MS. Crude alkaloid mixture, obtained from leaf was choosen for LC-MS/MS analysis.

Prosopinine and Prosopine alkaloids were successfully chromatographed by HPLC, with a retention time of 34.57 and 36.23 min respectively, as illustrated in Fig.12. The electrospray ionizations of the Prosopinine and Prosopine alkaloids were characterized by the predominant formation of the [M+ H]+ molecular ion at 314 and 316 respectively (Fig.13). These identified compounds were further subjected to MS/MS analysis, thus generating fragments of the respective molecular ion. On the basis of the fragmentation pattern, elucidated with the help of software program (chemdraw), the product ion profile derived from precursor ion of m/z 314 and 316 was compared.

The molecular ion for Prosopinine at m/z $[M+H]^+$ 314 lead to characteristic product ions at m/z 296, 282, 214,184,158 144 and 131 (Fig.14a). The ion at m/z 296 was formed by loss of water from the molecular ion and the ion at m/z 282 was formed by loss of CH_3OH group from the heterocyclic ring. Fragment at m/z 184 aroused by removal of 2-hydroxymethyl piperidin-3-ol ring from the hydrocarbon chain and peak representing m/z 131 corresponds to 2-hydroxymethyl piperidin-3-ol ring. The ions at m/z 144, 158, 214, were formed by cleavage of hydrocarbon chains at different locations.

As shown in Fig.14b, Prosopine (m/z $[M+H]^+$ 316) fragments were observed at m/z 300, 298, 242, 214 158 and 131. The ion at m/z 300 was formed by loss of a methyl group from the parent ion and m/z 298 fragment was formed by loss of water. Fragment at m/z 131 was formed by formation of 2-hydroxymethyl piperidin-3-ol ring and the ions at m/z 242, 214 and 158 were formed by cleavage of hydrocarbon chain. The product ion spectra of both the alkaloids were found to match with the theoretically generated fragments with the help of chemdraw software, confirming the presence of these alkaloids in *P. juliflora*.

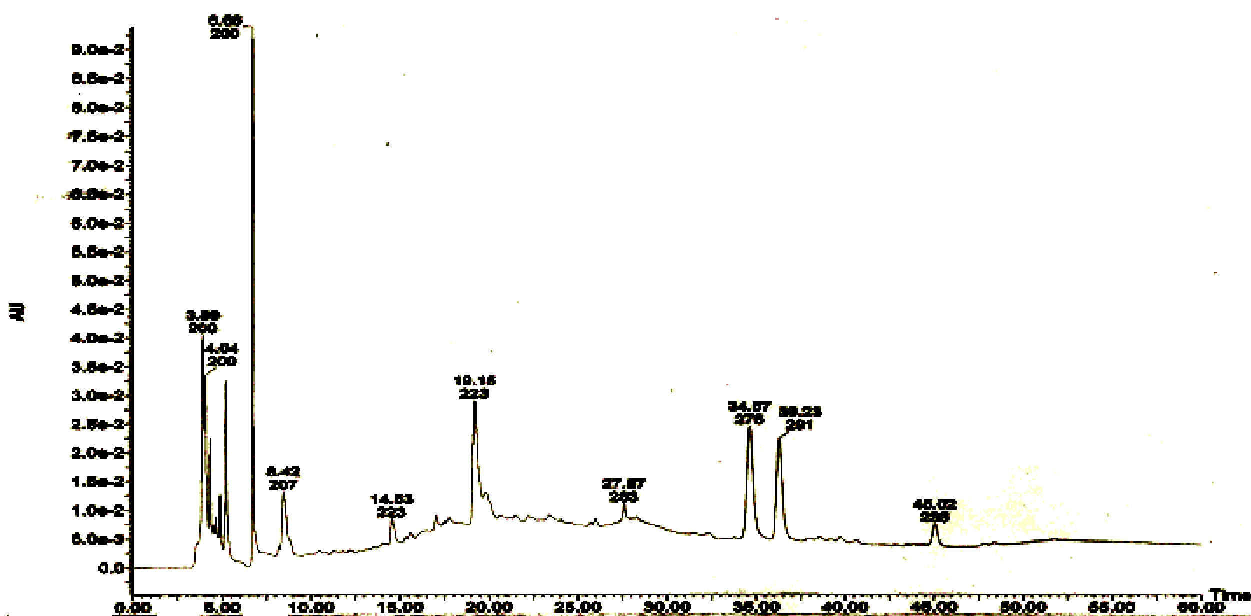


Fig 12. HPLC chromatogram of leaf ARF

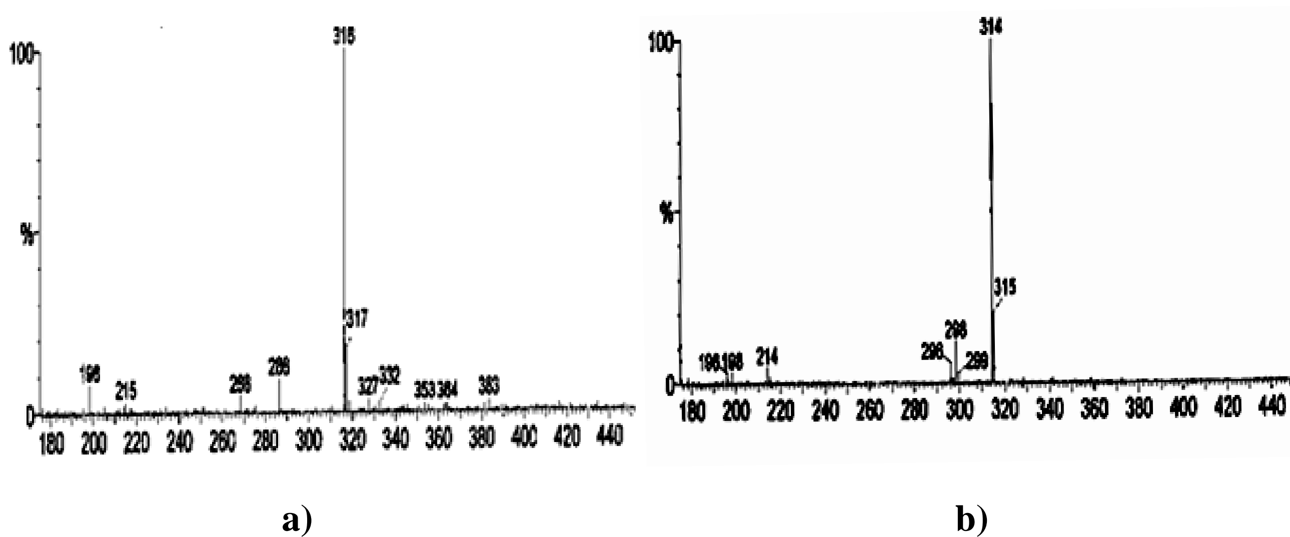
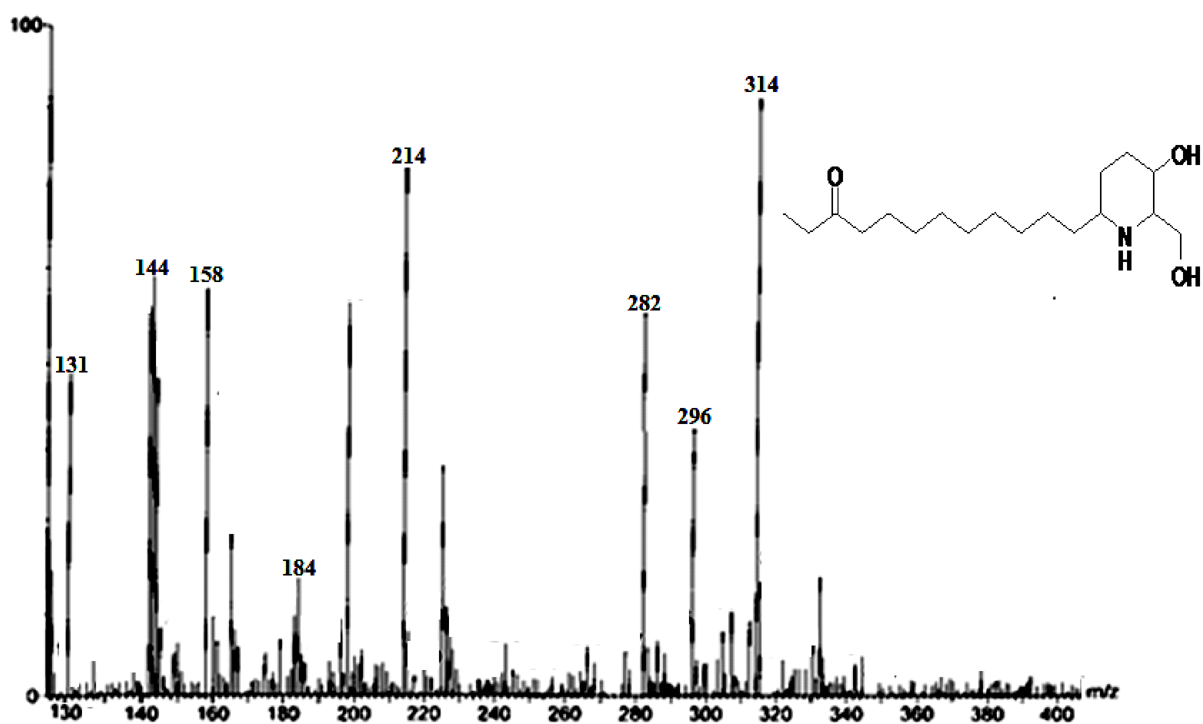
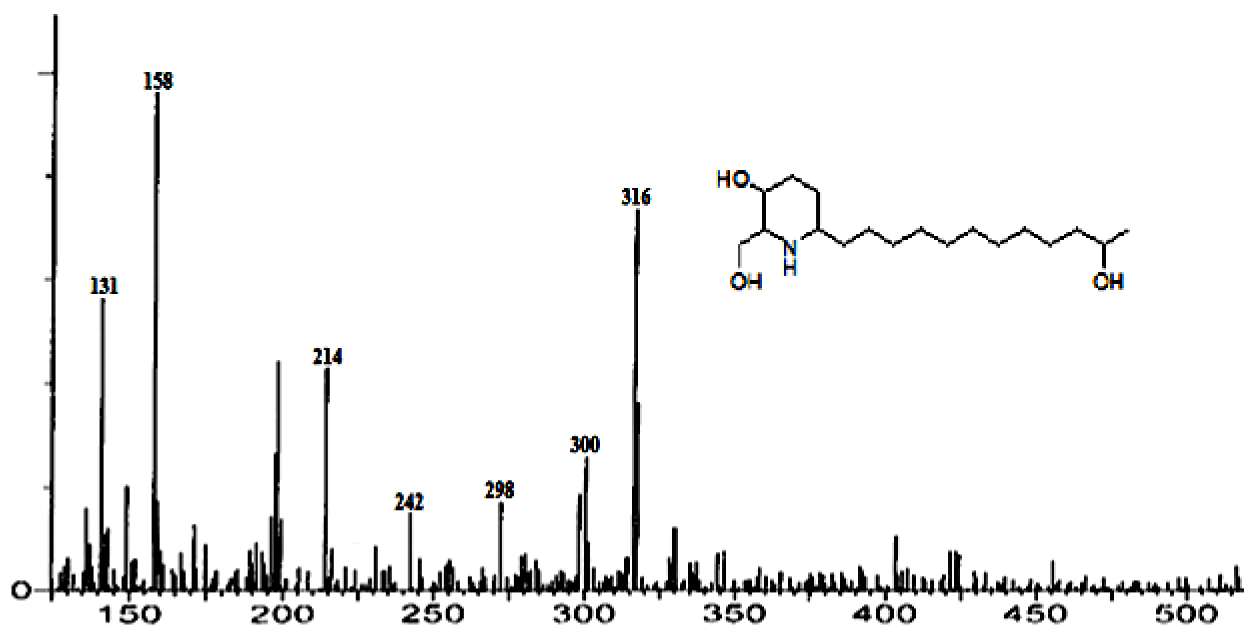


Fig 13. ESI-MS of separated fractions by HPLC - a) 316 for Prosopine and b) 314 for Prosopinine



a)



b)

Fig 14. LC-MS/MS analysis - a) Prosopinine b) Prosopine

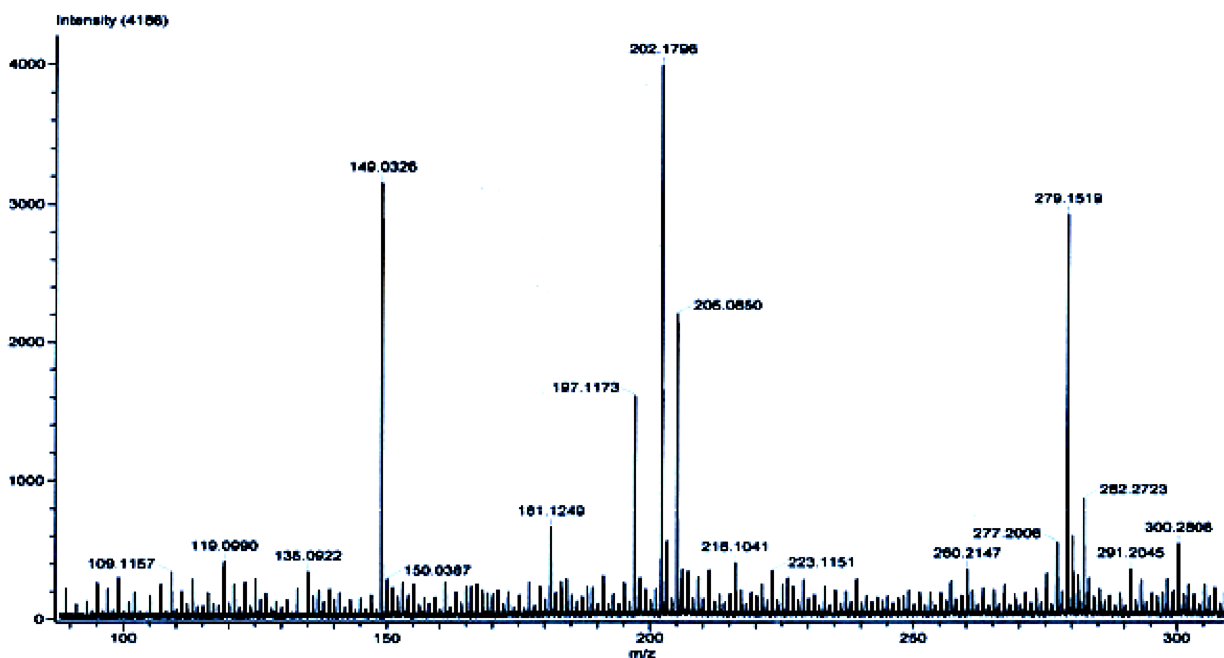
2.3.5. Identification of compounds present in Petroleum ether fraction (F2) of leaf

Direct analysis by DART-MS

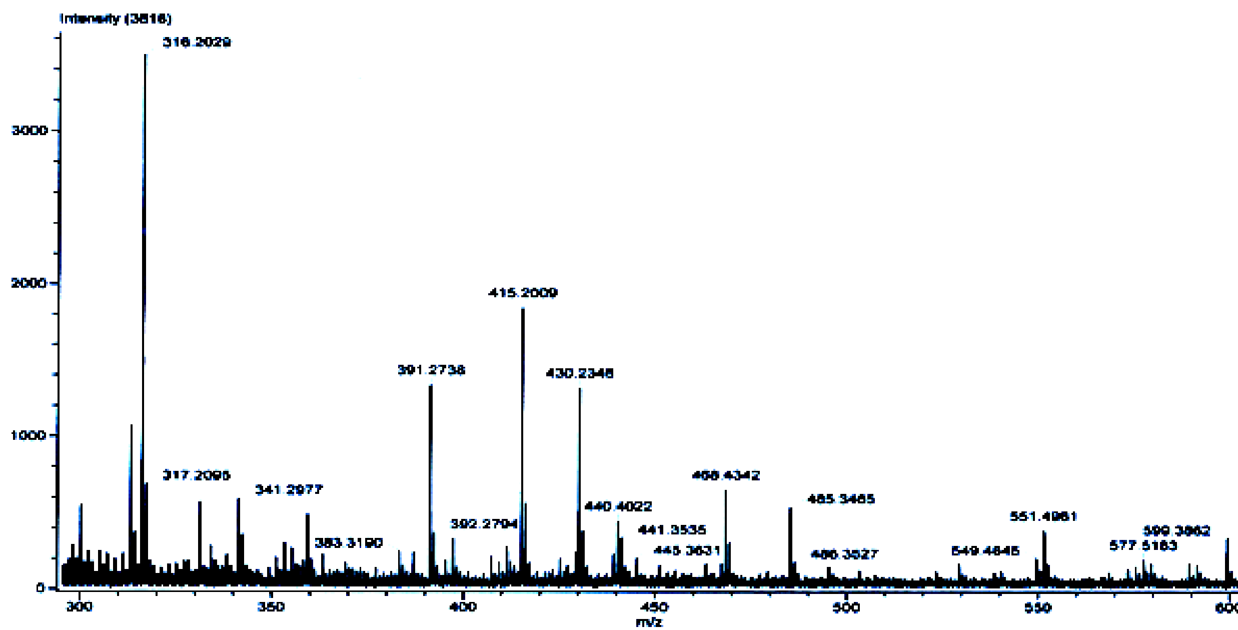
Apart from containing pharmacologically active piperidine alkaloids, leaf of *P. juliflora* is reported to contain fatty acids (Marangoni and Ali, 1988). Hence leaf extract was reinvestigated to identify these important metabolites. DART-MS analysis revealed the presence of fatty acids in the non-polar extract (F2) of *P. juliflora* leaf (Fig.15). Some of them were present as dimmers, which include propanoic acid, pentanoic acid, nonanoic acid and pentadecanoic acid. Unsaturated acid identified include, steridonic acid, linoleic acid and oleic acid. These acids are reported to be biologically active (Burr et al., 1980; Ellie and Rolfes, 2008) Along with the fatty acids, betasitosterol was also observed. There observed molecular mass was compared with the calculated mass and was found to be identical (Table 9). Molecular formula estimated for the masses were similar with the data available in the literature (NIST database).

Table 9. DART peak measurement of petroleum ether fraction (F2) of leaf

Compound	Molecular Formula	Observed mass	Calculated mass	Mass Difference
Propanoic acid dimer	$C_3H_7O_4$	149.0326	149.0814	-0.0488
pentanoic acid dimer	$C_5H_{11}O_4$	205.0850	205.0440	0.0410
Steridonic acid	$C_{18}H_{29}O_2$	277.2006	277.2168	-0.0162
α linoleic acid	$C_{18}H_{31}O_2$	279.1519	279.2324	-0.0805
Oleic acid	$C_{18}H_{35}O_2$	283.2723	283.2637	0.0086
Nonanoic acid dimer	$C_{18}H_{37}O4$	317.2095	317.2692	-0.0597
Docosanoic acid	$C_{22}H_{45}O_2$	341.2977	341.3420	-0.0443
Betasitosterol	$C_{29}H_{51}O$	415.2009	415.2940	-0.0931
Pentadecanoic acid dimer	$C_{30}H_{61}O4$	485.3465	485.3570	-0.0105



a)



b)

Fig 15. DART-MS analysis of petroleum ether fraction (F2) of leaf extract - a) m/z value in the range of 100-300 b) m/z value in the range of 300-600

Identification of the compounds separated from the petroleum ether fraction (F2) by chromatographic techniques

Phytochemical analysis of leaves, as shown in Table 2, showed presence of terpenes, which include pigment molecules. Hence leaves were further investigated for the presence of various pigments with the help of column chromatography.

The F2 extract was first separated by PE, the fraction eluted first was an orange yellow colour extract which contained 3 compounds as determined by TLC. The orange fraction was further subjected to silica gel purification using PE:EA (9:1) as eluting solvent which resulted in the isolation of 2 compounds (designated as compound 1and 2).

When the solvent system was changed to PE:EA (3:1), 12 fractions were eluted, which were again repeatedly subjected to silica gel purification. Fractions were analyzed by TLC and those containing common compounds were pooled together and eluted with a gradient of solvent system containing PE and EA. This separation resulted in isolation of various compounds of different colours , 2 yellow colored compounds (designated as compound 3 and 4), light yellow grey (designated as compound 5), one dark gray compound (designated as compound 6) and dark brown (designated as compound 7). Purity of all the compounds were checked by TLC and HPTLC analysis (Fig.16).

Fraction eluted with EA:M (3:1) gave brown colour compound which when kept in refrigerator developed white crystals (designated as compound 8). Elution with methanol did not resulted into further sepatration of compounds. This fraction when kept at $4^{o}C$ also developed white crystals (designated as compound 9).

The isolated compounds were identified based on their colour, solubility and by comparing their UV and ^{1}H NMR spectra with that of standard compounds, as available on chemical database (NIST).

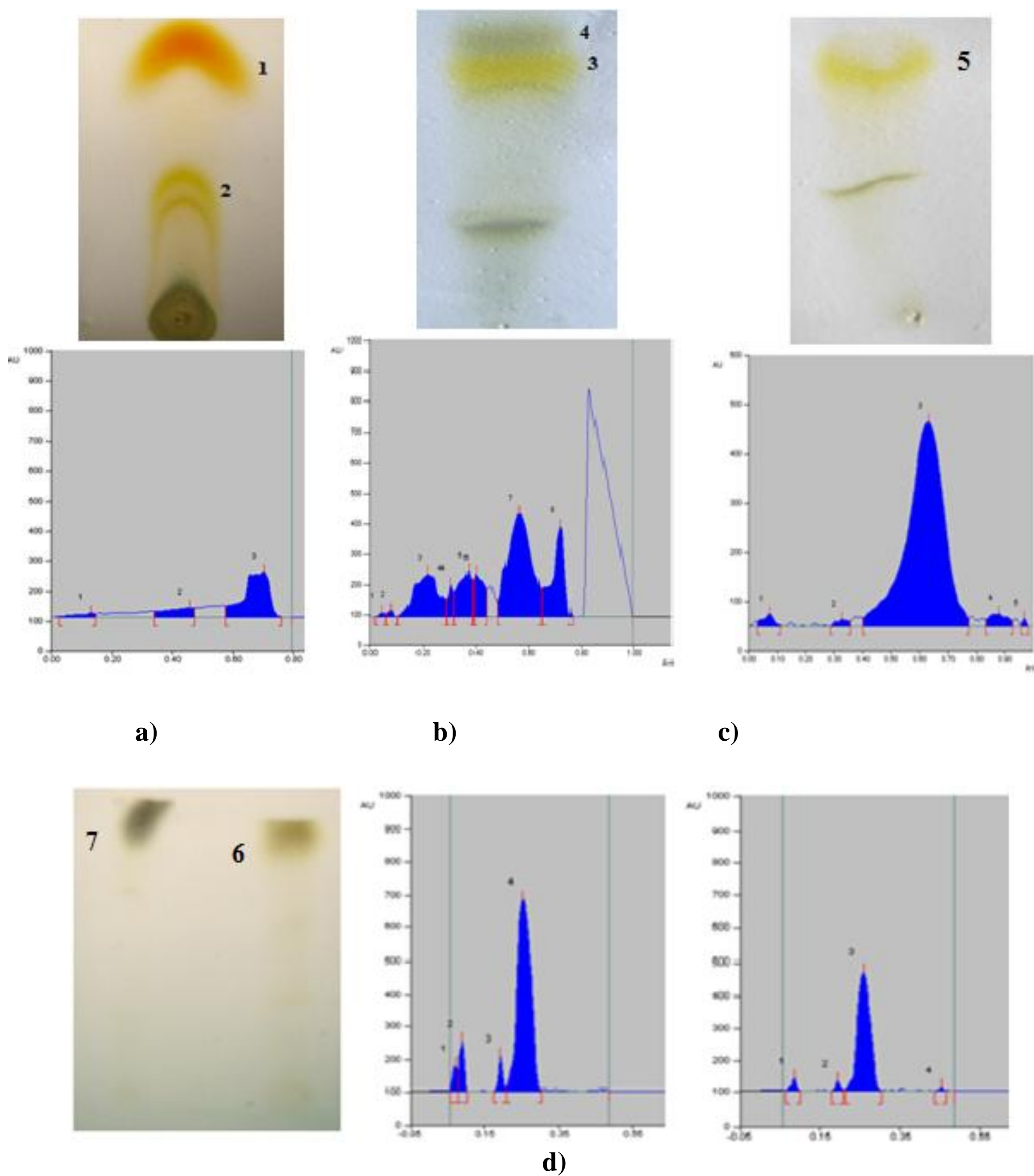


Fig 16. TLC and HPTLC analysis of compounds isolated from F2 fraction - a) Compound 1 and 2 b) Compound 3 and 4 c) Compound 5 d) Compound 6 and 7

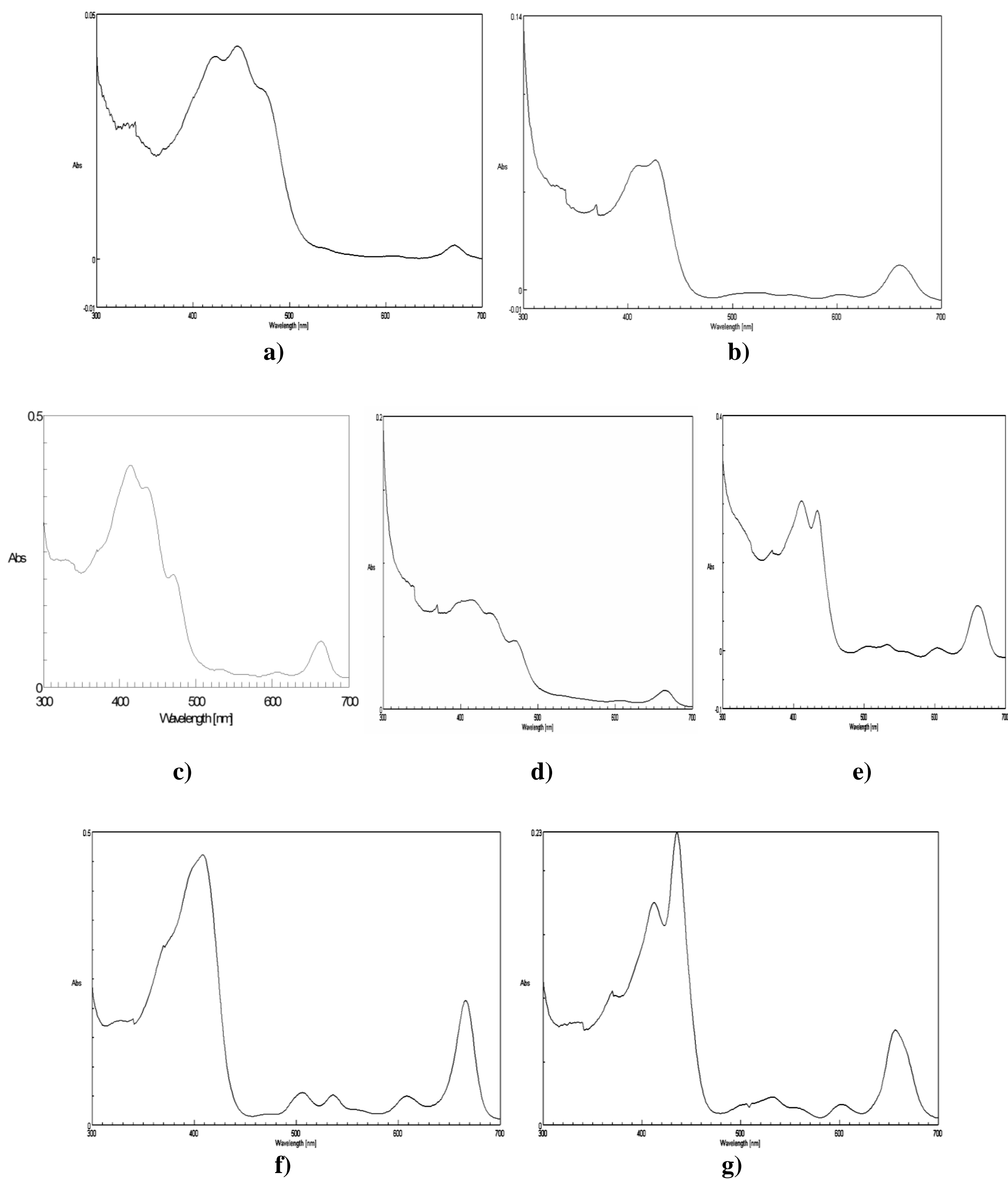


Fig 17. UV-visible absorption spectra of compounds isolated from F2 fraction - a) Compound 1 b) Compound 2 c) Compound 3 d) Compound 4 e) Compound 5 f) Compound 6 g) Compound 7

Compound 1 – was obtained as red – orange color compound, purity of the compound was checked by HPTLC analysis, which showed a single spot (Fig.16a). It was found to be soluble in more non polar solvents, such as ether and chloroform. Absorption spectra showed maximum absorption at 425, 450 and 477 nm, characteristic of beta carotene (Fig.17a).

^{1}H NMR spectra, showed signals for the olefinic region between 6.0–7.0 ppm, however the signals in this region were very weak. Strong signals of CH_3 and CH_2 of acyl chains between 0.9 and 3.0 ppm were found. Three methyl signals were observed at δ 1.25 (29, 30, 38, 39), 2.03 (31, 40) and 2.29 (19, 20, 21, 22). Protons of CH_2 group of cyclohexene was shown as a broad area between 1.59-1.97 ppm (Fig.18).

Compound 2 - was also an orange-red colour compound that appeared as a single spot on TLC plate, HPTLC analysis confirmed the purity of this compound (Fig.16a). UV-Visible spectra showed maximum absorbtion between 400-450 nm (Fig.17b). Based on the above characteristics, this compound was identified as a carotene.

It was subjected to ^{1}H NMR analysis for further identification. Proton signals were found to be identical with beta carotene, with some minor variations (Fig.19). Characteristic olefinic protons signals were observed in the region between 6-7 ppm. Signal for hydroxyl proton were observed at 5.36 ppm, which differs from beta carotene, indicating the presence of a new compound. Three signals for CH_3 protons were seen at δ 1.25 (29, 30, 38, 39), 2.03 (31, 40) and 2.29 (19, 20, 21, 22). Signal for CH_2 groups present in cyclohexene ring was spread between 1.9-1.4 ppm, with a strong signal at 1.59 ppm.

Compound 3–was obtained as yellow coloured compound and appeared as a separate spot on TLC plate (Fig.16b). The maximum absorption was found at 414, 435, 470,664 nm, which is characteristic of lutein (Fig.17c). Proton NMR showed oleifinic region between 5.8-7 ppm, whereas signal for CH_3 protons were observed at δ 0.99 (9, 8) , 1.29 (30, 40) , 2.22 (29. 30, 31, 32). Signal at 3.5 ppm corresponds to hydrogen present in CH group. Signal for CH_2 group of cyclohexene is present at 1.84 ppm and between 1.60-1.68 ppm. Strong signal of OH group was observed at 4.09 ppm (Fig.20).

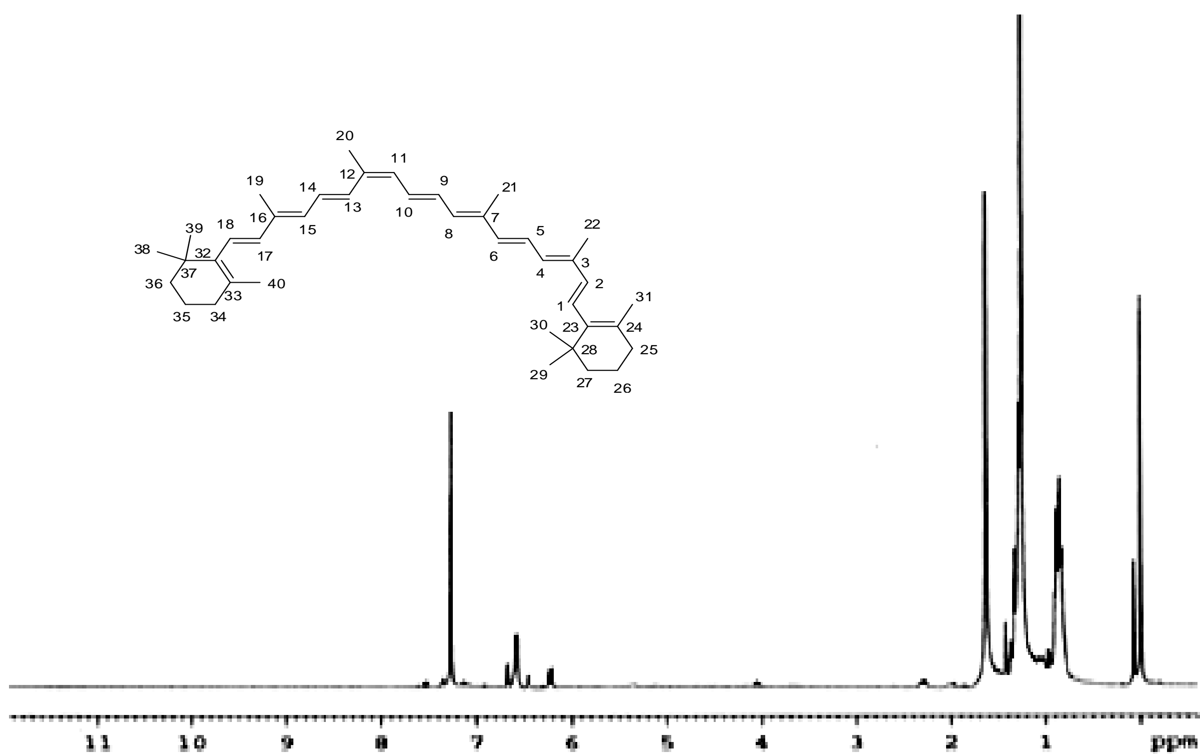


Fig 18. ^{1}H NMR spectra of compound 1 and the elucidated structure shown in the inset

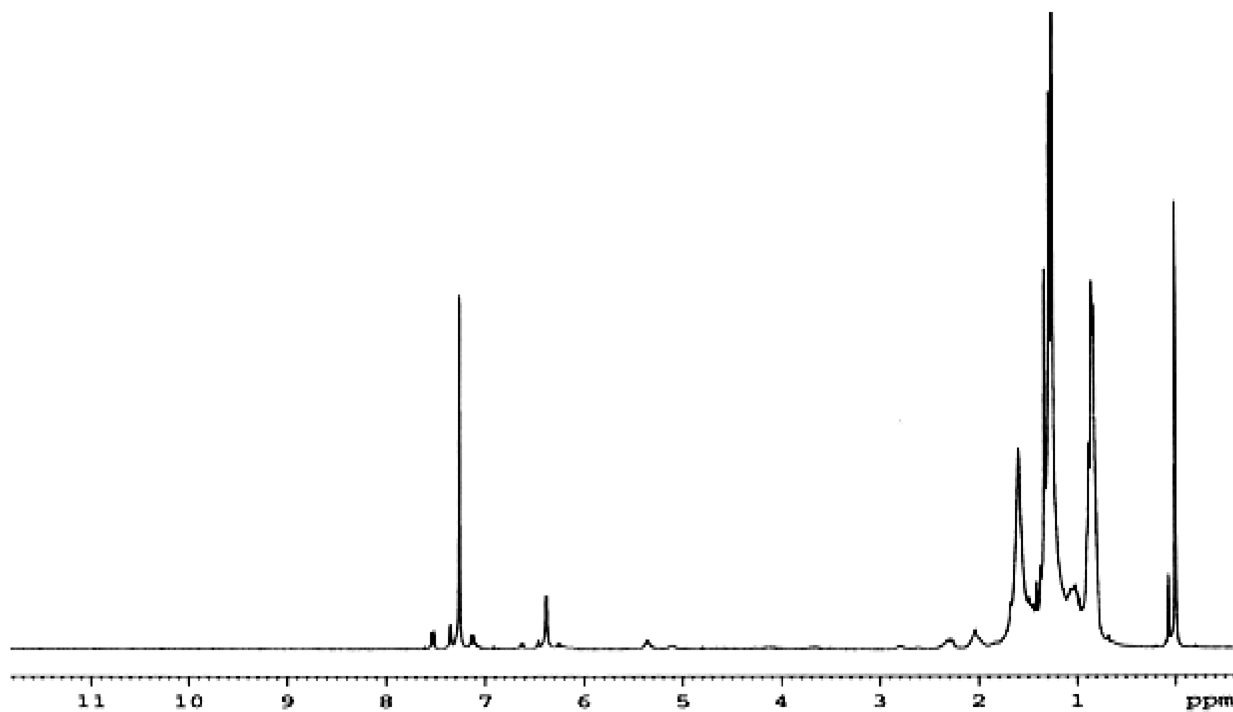


Fig 19. ^{1}H NMR spectra of compound 2

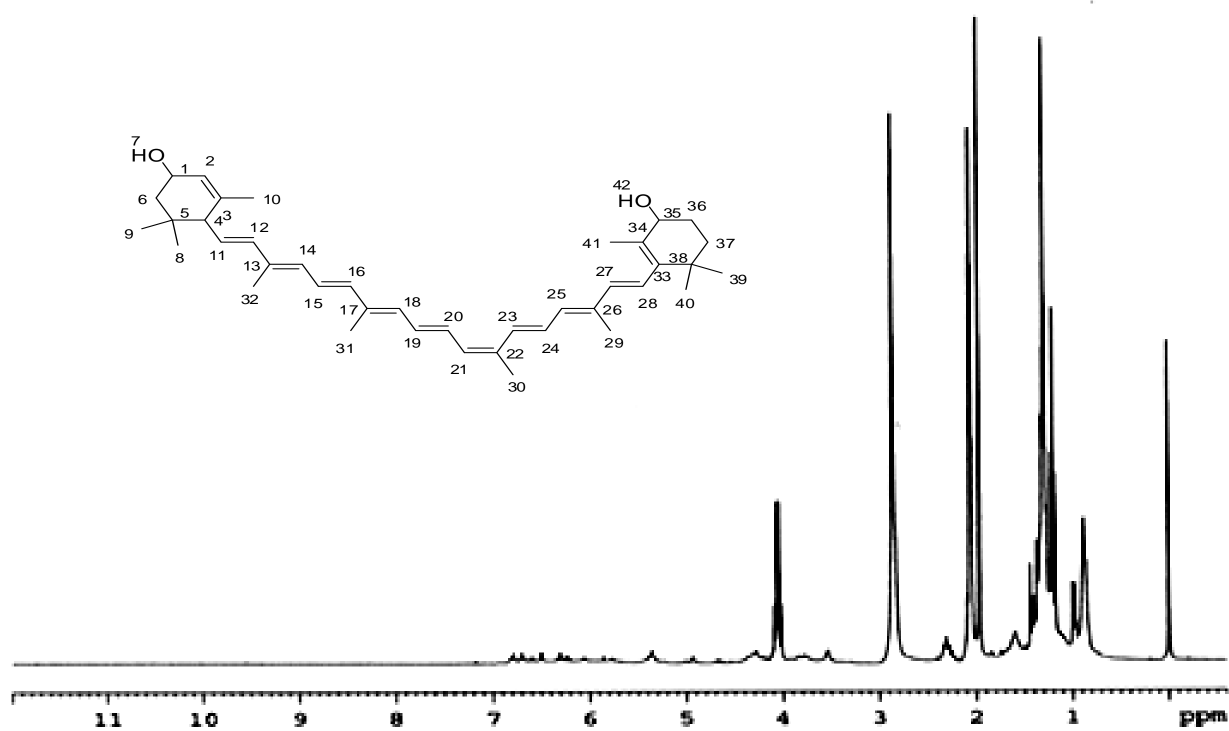


Fig 20. ^{1}H NMR spectra of compound 3 and the elucidated structure shown in the inset

***Compound 4* -** was a yellow grey coloured pigment (Fig.16b), and showed absorbtion at 418, 435, 470,664 nm similar to xanthophylls (Fig.17d). Proton NMR confirmed the presence of xanthophylls, as signal of hydroxyl group was observed at 4.14 ppm (Fig. 21). Signal of oleifinic protons were very weak, probably due to the presence of deuterium in solvent chloroform , which might have replaced the proton. Signal for CH_3 protons were observed at δ 0.88, 1.26 and 2.23, whereas Signal for CH_2 group of cyclohexene were found between 1.58-1.65 ppm. Two other strong signals at 2.17 and 2.05 ppm were observed, which indicates the presence of a different form of xanthophylls.

***Compound 5*-** was yellow in colour with almost 95% purity, as shown by HPTLC analysis (Fig.16c). and showed similar absorption spectra, as that of lutein (Fig.17e). Olefinic signals in proton NMR were observed between 6-7 ppm and signal for CH_3 protons were observed at δ 0.99, 1.24, 2.23 and signal at 3.5 ppm corresponds to hydrogen present in CH group. Signal for CH_2 group of cyclohexene at 1.84ppm and between 1.60-1.68 ppm. Strong signal of OH group were observed at 4.09 ppm. Hexadiene signal was observed at 5.04 and 5.35 ppm (Fig.22).

This compound was identified as anhydroleutin. Main difference between them is, lutein contains 2 hydroxyl group, whereas anhydrolutein contains one and this difference is evident while observing its proton signals.

Compound 6 and 7- Compound 6 was obtained as dark brownish-green compound, whereas compound 7 was obtained as grey colour coumpound. The HPTLC analysis, showed that both the compounds are present as single spot with almost 98% purity (Fig.16d). Compound 6 showed major diphasic peaks at 410 and 667 nm together with minor peaks at 505, 534 and 610 nm indicating it to be a chlorophyll derivative (Fig.17f) and compound 7 exhibited major peak at 435 nm with a shoulder of 415 nm and another major peak at 655 nm with minor peaks at 520, 560 and 598 nm (Fig.17g). These absorptions indicated that compound 6 is pheophytin a and compound 7 is pheophytin b. Further confirmations were done by analyzing their proton NMR spectra.

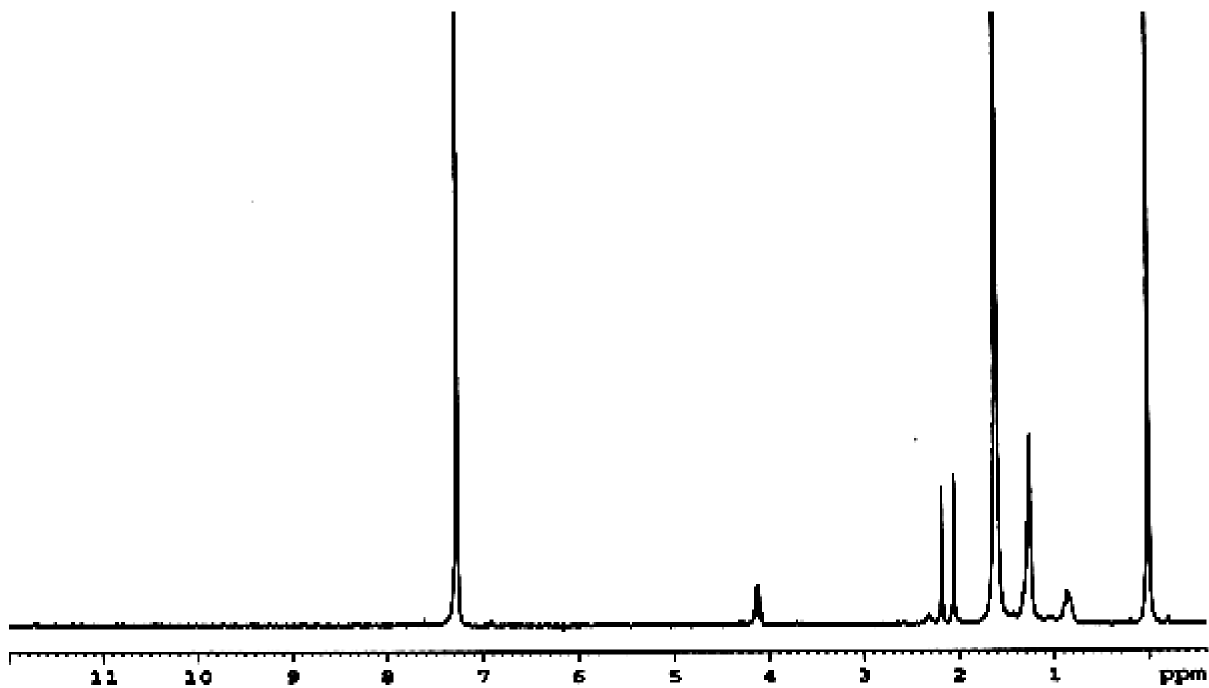


Fig 21. ^{1}H NMR spectra of compound 4

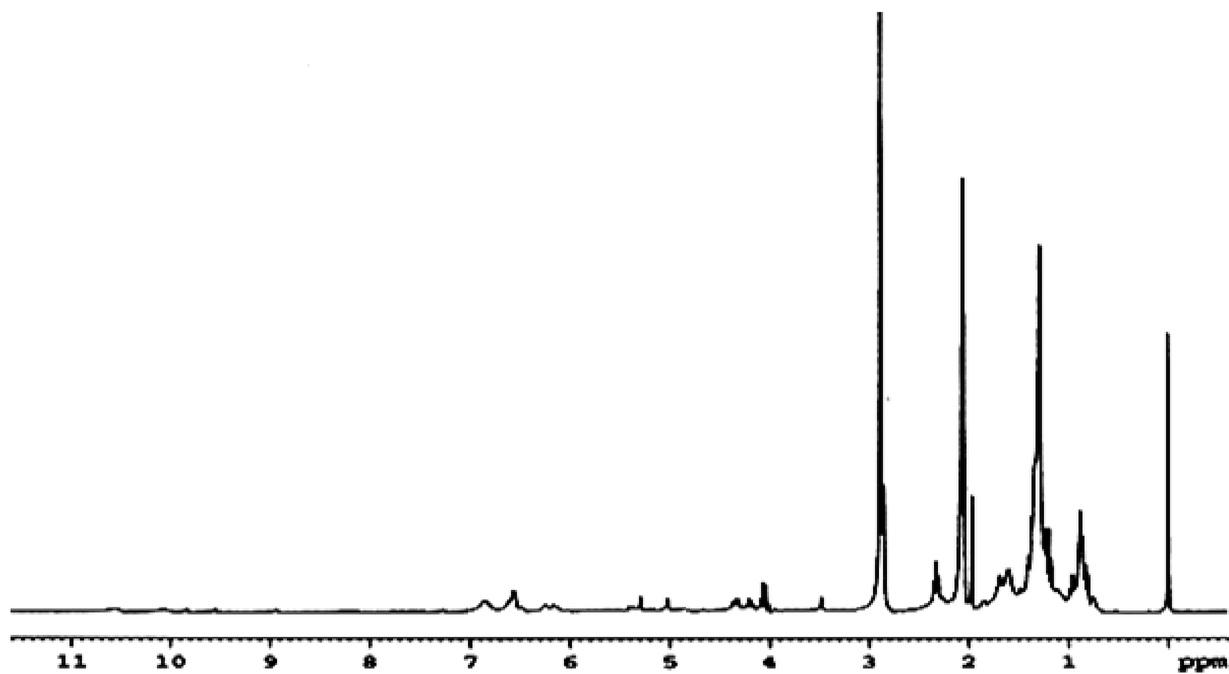


Fig 22. ^{1}H NMR spectra of compound 5

The $_1$H-NMR (Fig.23) spectrum of compound 6 exhibited signals for three aromatic methine groups [δ 9.76 (H-10), 9.52 (H-5) and 8.74 (H-20)], one conjugated vinyl group [δ 7.97 (Ha-3^1), 6.31 (H-3^2) and 6.16 (Hb-3^2)], three aromatic methyl groups [δ 3.90 (CH_3-12^1), 3.42 (CH_3-2^1) and 3.23 (CH_3- 7^1)], two methyl ester groups [δ 3.59 (OCH_3-15^2) and 3.52, (CH_3-17^3)], one ethyl group [δ 3.71 (CH_2-8^1) and δ 1.68 (CH_3-8^2)], two amino group protons [δ (N–H) 1.10 and 1.37], one methoxy group [δ 3.91 (OCH_3-15^1)], and one downfield shifted methyl group δ 1.59 (CH_3-18^1)].

Compound 7, along with the common signals of compound 6, also revealed the pheophytin b type characteristics (Fig.24), showing signals for aldehyde proton at δ 11.01 (H-7^1), 10.25 (H-5); and two aromatic methyl groups [δ 3.75 (H3-12^1), 3.27 (H3-2^1)].

In the phytyl chain, signal for intermediate CH group (3,8,13,18) at 1.65 ppm, intermediate CH_2 group (2,5,6,7,10,11,12,15,16,17) at 1.25 ppm and side chain CH_3 group (4,9,14,20) at 0.96 ppm was observed.

Coumpound 8 and 9- were identified as fatty acid, having one carboxylic acid and a saturated carbon chain, based on their proton NMR spectra (Fig.25 and 26). These acids were separated as esters due to the reaction of OH group of the solvent (ethanol) with the COOH group of the acid, showing peak at 3.79 ppm. Terminal CH_3 peak of the acid appeared at 0.88 and intermediate CH_2 peak at 1.29 ppm. Two additional peaks at 2.2 and 1.8 ppm for hydrogens present on alpha and beta carbons were also found. These compounds showed small peaks as compared to the solvent acetone at 2.07 and 2.98 ppm, due to the low quantity in the sample.

Based on the number of hydrogen present in the compounds, calculated by measuring the peak intensity of proton NMR and their masses shown in Table 9, compound 8 could be identified as Propanoic acid and compound 9 as Pentadecanoic acid.

Among pigment molecules, two beta carotene, three xanthophylls and two pheophytin were extracted from the leaves of *P.juliflora.* These carotenoids are reported to have many physiological functions (Bjelakovic G, et al. 2004) and the most common one is

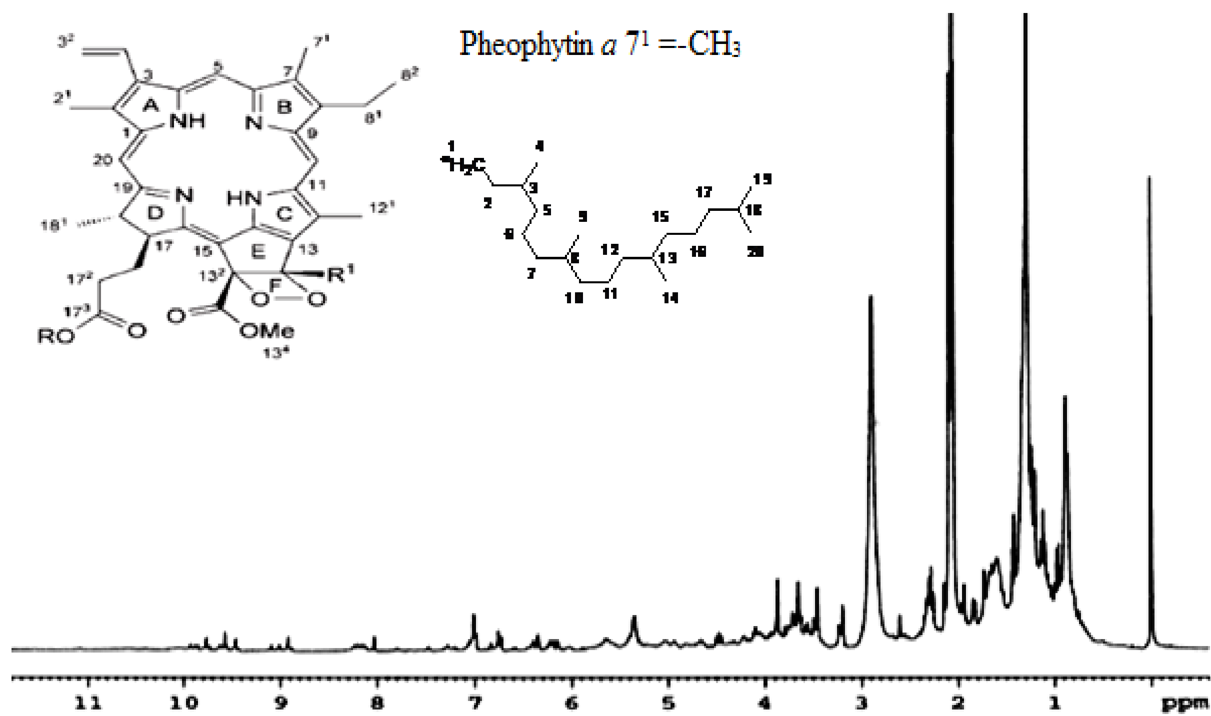


Fig 23. ¹H NMR spectra of compound 6 and the elucidated structure shown in the inset

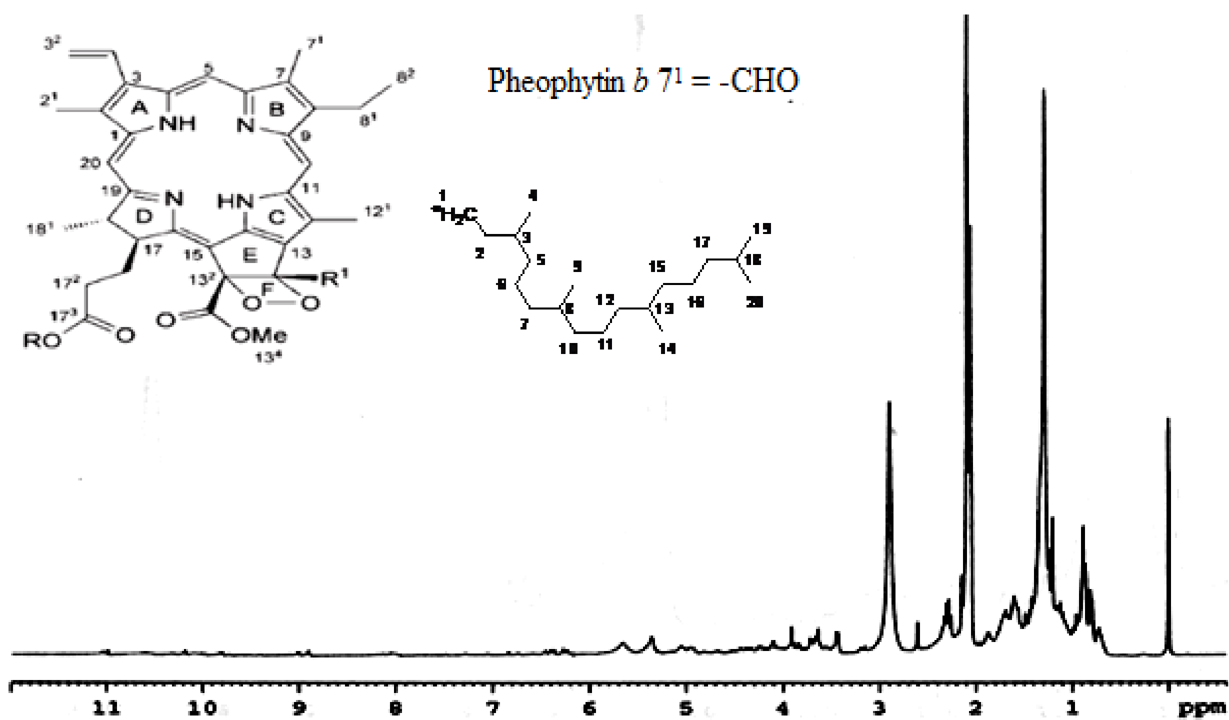


Fig 24. ¹H NMR spectra of compound 7 and the elucidated structure shown in the inset

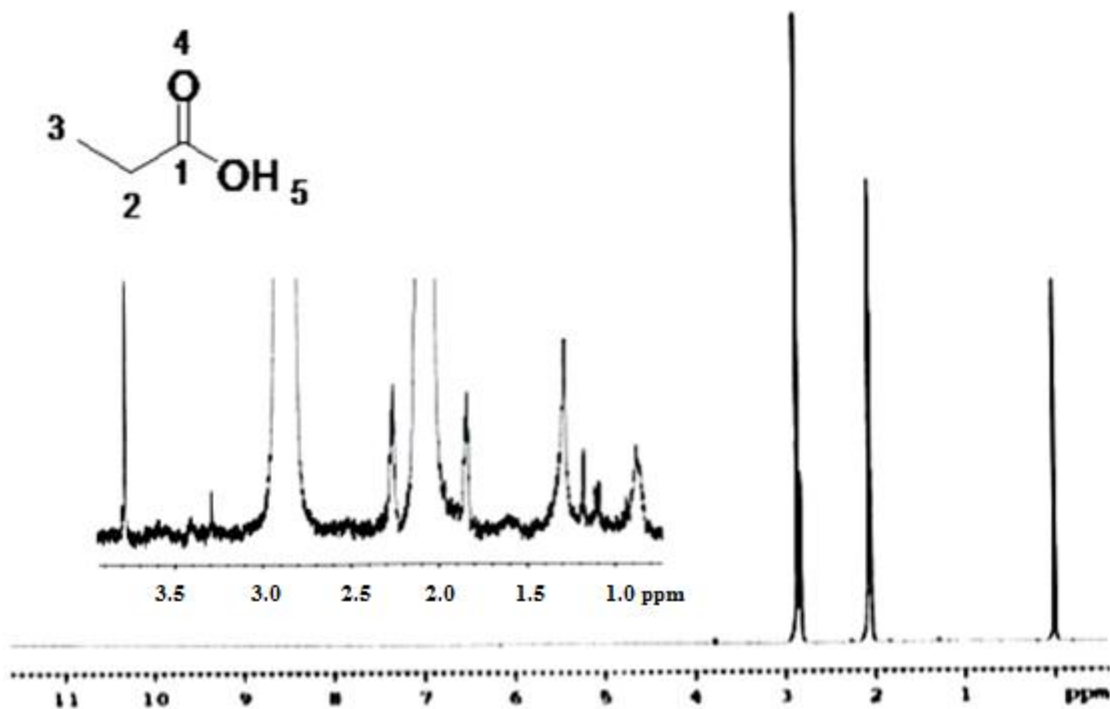


Fig 25. ^{1}H NMR of compound 8 and the elucidated structure shown in the inset (high resolution of the peaks between 1-3.5 ppm is shown in inset)

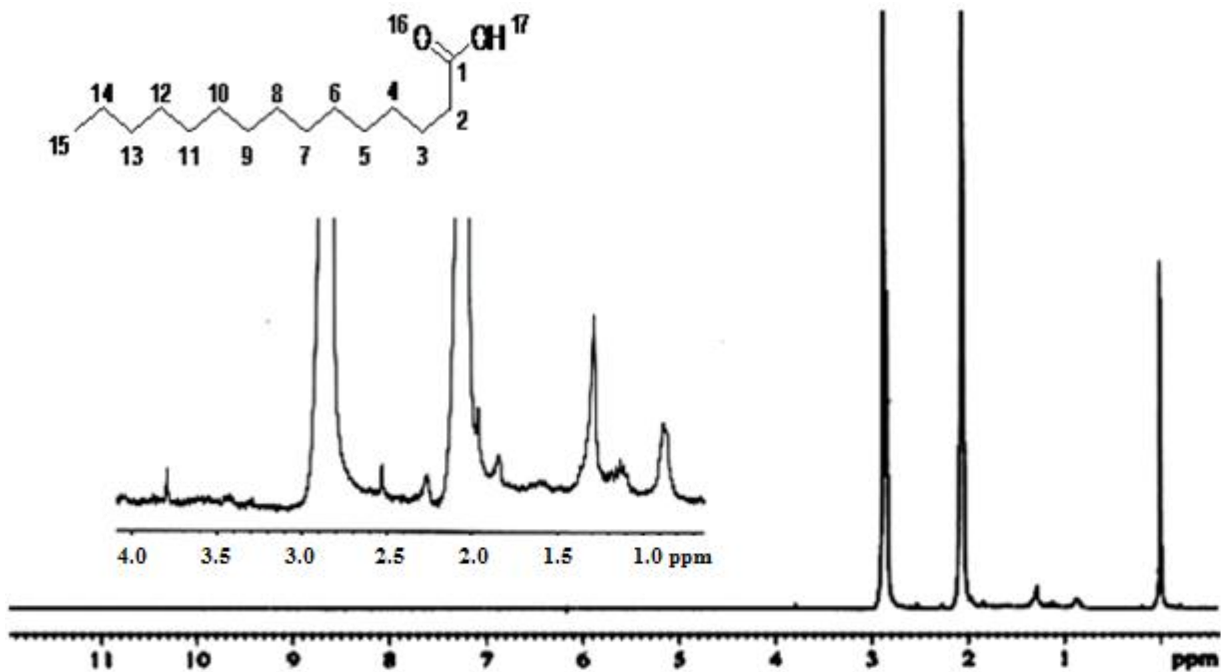


Fig 26. ^{1}H NMR of compound 9 and the elucidated structure shown in the inset (high resolution of the peaks between 1-4.0 ppm is shown in inset)

antioxidant activity (Diplock1 et al., 1998).They were identified based on their UV-visible spectra, proton NMR spectra and their colour, however their masses did not matched with any of the reported carotenoids (Fig.15). The possible reason could be presence of either some new forms of carotenoids in *P. juliflora*, or presence of modified form of the reported carotenoids, having different molecular weight.

2.3.6. Identification of compound from water fraction (F1) of flower extract

Compound 10- was extracted from the flower as yellow crystal (Fig.27a), with [M+H]+ value of 391.37319 (Fig.27b) . Literature survey did not reveal any compound identified in *P.juliflora* having similar m/z value. Therefore this compound was subjected to several chemical tests, IR and ^{1}H and $^{13}CNMR$ analysis for its identification.

Solubility test for the compound showed that crystals were soluble in methanol and insoluble in ether, indicating slightly polar nature of the compound. Bromocresol green test indicated presence of carboxylic group, as yellow spots were visible on blue background (data not shown).

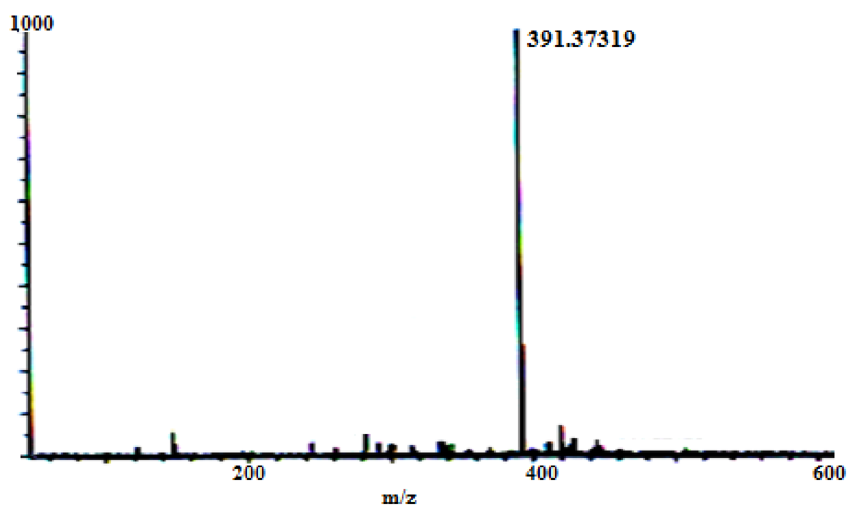
IR spectra showed presence of carboxylic acid and alkyl groups (Fig.27c). A strong, wide band for the O–H stretch in the region between 3300-2500 cm^{-1}, centered at about 3000 cm^{-1} observed. This is in the same region for the C–H stretching bands of alkyl groups. Thus a carboxylic acid shows a somewhat "messy" absorption pattern in the region 3300-2500 cm^{-1}, with the broad O–H band superimposed on the sharp C–H stretching bands. The reason of broad O–H stretch band of carboxylic acids could be attributed to the presence of hydrogen-bonded dimmers. The carbonyl stretch C=O of a carboxylic acid appears as an intense band from 1760-1690 cm^{-1}. The C–O stretch appears in the region 1320-1210 cm^{-1}, and the O–H bend is in the region 1440-1395 cm^{-1} and 950-910 cm^{-1}, although the 1440-1395 band may not be distinguishable from C–H bending bands in the same region.

Compound 10 was converted into esters by dissolving in methanol and subjected to NMR analysis, which showed the presence of an ester. ^{13}C NMR spectra showed the

presence of carboxylic group/ester at 184.39 ppm and saturated alkane (methyl) at 26.15 ppm (Fig.28a).

^{1}H NMR also showed the presence of a methyl ester at 3.71 ppm where a single peak was observed. Signal for alkanes were observed between 0.9-2.2 ppm. Terminal CH_3 peak appeared at 0.92 ppm indicating the presence of methyl protons in highly shielded environment and the signal was split up into triplet indicating presence of an adjacent CH_2 group. A triplet at 1.18 ppm again indicates presence of an intermediate CH_2 group. Two single peaks at 2.13 and 1.7 ppm were observed, which corresponds to the hydrogens present on alpha and beta carbons of the aliphatic acid. Two additional signal for alkanes were observed at 1.36 (triplet) and 1.53 (multiplet). A mulitiplet was also observed at 3.6 ppm and a single large peak at 1.92 ppm (Fig.28b). On the basis of above analysis the compound is an aliphatic acid containing branched hydrocarbon chain.

Phytochemical investigation done in the present research work, provides useful information for the comparative studies of the presence of important metabolites in different parts of the plant. These data can help us to choose appropriate plant part for extraction of medically and therapeutically important phytochemical with greater quantity.



a)

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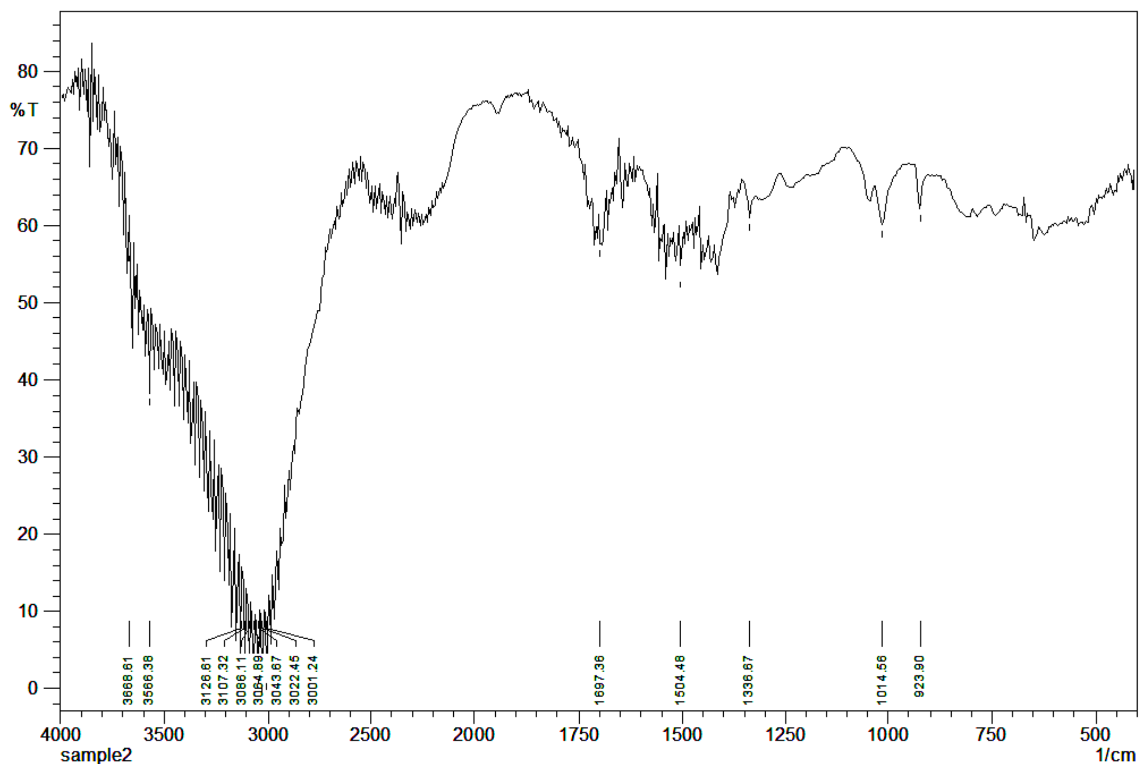
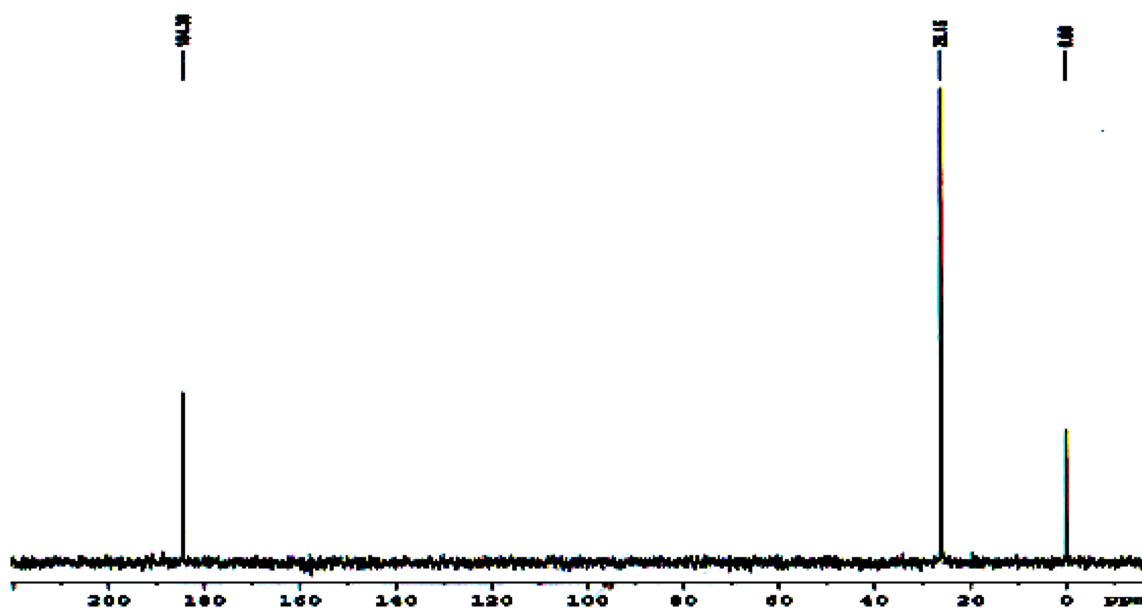
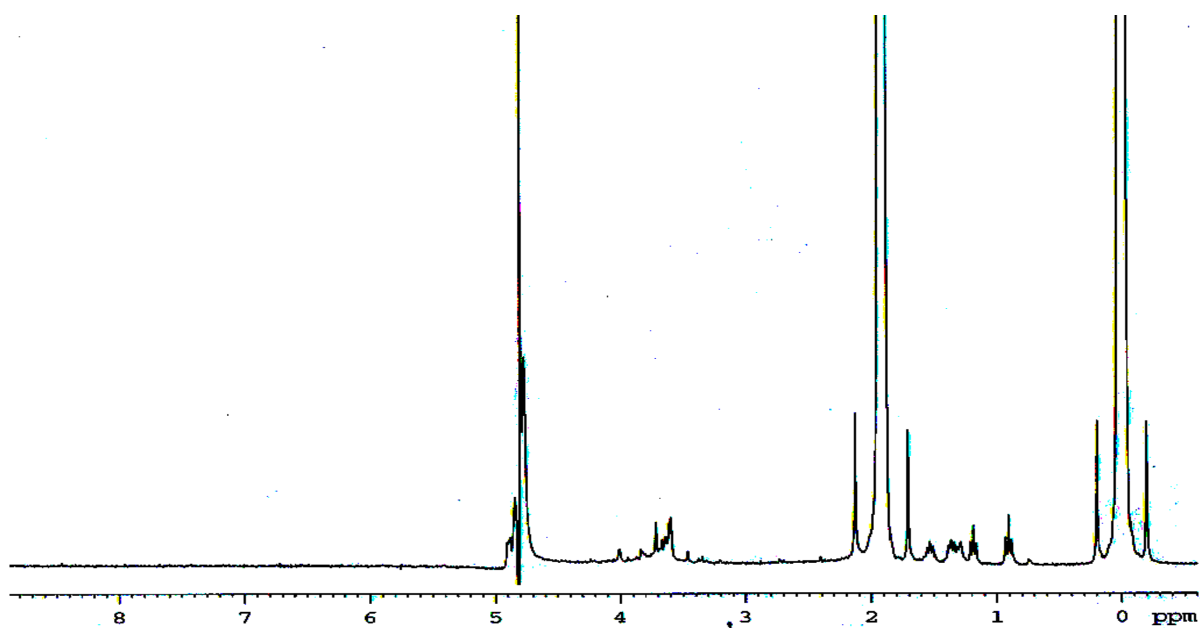


c)

Fig. 27. Analysis of Compound 10 - a) yellow crystals of compound 10 b) DART-MS of compound 10 c) IR spectra of compound 10



a)



b)

Fig 28. NMR analysis of compound 10 - a) ^{13}C NMR spectra b) ^{1}H NMR spectra

Chapter 3

Antibacterial activity of Prosopis juliflora

3.1. Introduction

Throughout the history of mankind, infectious diseases have remained a major cause of death and disability accounting for about 22% of the global disease burden (Murray and Lopez, 1997). The discovery of penicillin in the 1940s and several other antibiotics in subsequent years led to great improvements in the management of infectious diseases particularly in developed countries. However, despite this success, the increased use of antibiotics led to the inevitable development of resistance, with the effect that diseases that were hitherto thought to have been controlled by antibiotics later re-emerged as resistant infections (Mcdonald, 2006; Paterson, 2006).

At present major pathogenic bacteria that contribute the most to the global infectious disease burden such as *Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella* species *and Mycobacterium tuberculosis* are resistant to standard antibiotic therapies (Fluit et al., 2001).

This global emergence of multi-drug resistant bacterial strains has limited the effectiveness of current drugs, causing treatment failures (Hujer et al., 2006). The containment of this drug resistance requires that, new potent antimicrobial compounds be identified as alternatives to existing antibiotics. However, the current state of development of new antimicrobial drugs is not encouraging with only a few new ones being licenced in recent years (Butler, 2004). This mismatch between the slow development of new drugs and the fast emergence of resistant strains makes the future management of infectious diseases look bleak.

Much like the situation in human medicine, the use of antibiotics in agriculture, livestock and poultry has accelerated the development of antibiotic resistant strains of microbial pathogens, potentially complicating treatment for plants, animals and humans (White et al., 2002). Furthermore changing patterns of susceptibility and the availability of new antimicrobial agents require continuous updating of knowledge concerning treatment of disease caused by such pathogens.

Thus there is a need to look for alternative strategies for the management of harmful microbes. One of the possible strategies towards this objective involves the rational localization of bioactive phytochemicals which have antimicrobial activity. It is known that plant produce certain chemicals which are naturally toxic to microorganism but not to humans (Stapleton et al., 2004).

The rich chemical diversity in plants has been reported to be a promising source of antibacterial compounds (Basri and Fan, 2005; Smith et al., 2007), raising hopes of obtaining novel antibiotics that can aid the fight against drug resistant infections. Antimicrobial activity of the plants has been extensively studied (Nickell, 1959; Brantner and Grein, 1994; Springfield et al, 2003; Parekh and Chanda, 2007; Singh et al., 2011). Antimicrobial activity of various plant parts has also been observed by several workers viz. leaves of *Arbutus menziesii* (Kabadi and hammerland 1963), bark of *Fraxinus* (Jung and Hubbles, 1965), seeds of *Peganum harmala* (Ross et al., 1980) root, shoot and fruit of *C.colocynthis, C.depressus, L. barbarum and F.cretica* (Harsh, 1983).

The present work aims at screening alkaloid rich fractions (ARF) and compounds 1-10 obtained from parts of *Prosopis juliflora* against some wild type and drug resistance bacterial and cyanobaterial strains and compare their activity with standard antibiotics.

3.2. Materials and Methods

3.2.1. Preparation of test solution

A stock solution of 10 mg/ml of ARF of leaf, pod and flower and of compounds 1-10 was prepared in phosphate buffer saline (pH 7.0 ± 0.2). Stock solutions were sterilized by 0.45µ millipore filters and stored in freezer till use.

3.2.2. Test organisms and culture media

Bacterial bioassay was performed by disc diffusion method and well diffusion as described by Baeur et al., 1996, on the following test bacteria, obtained from MTCC

(IMTECH, Chandigarh*),* *Escherichia coli* (MTCC 40), *Staphylococcus aureus* (MTCC3160), *Bacillus cereus* (MTCC430), *Pseudomonas putida* (MTCC672*), Klebsiella* sp. (MTCC3384), *Salmonella* sp. (MTCC3215). Other strains such as *E. coli (ampicillin resistance), Alcaligen* sp.*, Acinetobacter* sp. were local isolates.

All the bacterial strains were cultured on nutrient media, except *E. coli* which was cultured on LB media (Luria berterni broth). All the chemicals used were of analytical grade and were of Hi-media.

Composition of Nutrient media

Peptone	5g
Beef extract	3g
NaCl	10g

Composition of LB media

Bacto-tryptone	10g
Yeast extract	5g
NaCl	8g

Compounds of both the media were dissolved in distilled water and total volume was made upto 1L, pH was adjusted to 7.5. For solidified media 2% agar was added. Media was sterilized by autoclaving.

3.2.3. Activation of bacterial strains

A loop full of the strain was inoculated in 20 ml of LB media for *E. coli* and Nutrient agar for other bacterial strains in 100 ml conical flask and incubated on a rotary shaker (125 rpm / min) for 24h to activate the strains. Media along with 2% agar was prepared and poured into petri plates of 90 mm size. 100 µl ($1x10^8$ cfu/ml) of the test strains were spread over the plates after they solidified. The experiment was performed under strict aseptic conditions.

3.2.4. Antibacterial activity test by Disc diffusion method

For the agar disc diffusion method, the test solution (10 µl) was applied on the sterile whatman filter paper disc (Hi media) of 0.5 cm size and allowed to dry for 1h. Then the disc was introduced on the upper layer of media with the bacteria. The plates were kept at room temperature for 1h so that the compound could diffuse into the media and after that they were incubated for 24h at 37°C.

3.2.5. Antibacterial activity test by Well diffusion method

For agar well diffusion method, a well was prepared in the plates with the help of a cork-borer (0.5 cm). 100 µl of the test compound was introduced into the well. The plates were kept at room temperature for 1h so that the compound could diffuse into the media and after that they were incubated for 24h at 37°C.

Antibacterial activity of the compound was determined by measuring the diameter of zone of inhibition of bacterial growth. Distilled water was used as negative control and the control activity was deduced from the test results. Standard antibiotics, chloramphenicol, ampicillin, tetracycline, streptomycin, rifampin, sulfa drug and oflaxacin (1mg/ml) were used as positive control.

3.2.6. Determination of Minimum inhibitory concentration (MIC) of test bacterial strains

MIC was determined by two-fold serial dilution method. A serial dilution of various fractions was carried out to give final concentrations between 0.5-0.0025 mg/ml. 100 µl of varying concentrations of test samples were added into the test tubes separately, containing 10 ml of standardized suspension of tested bacteria (10^8 cfu ml^{-1}). The test tubes were incubated at 37°C for 24h. Controls were used with the test organisms, using distilled water instead of the plant extract. Growth was determined by measuring O.D at 600 nm. The least concentration of the samples with no growth was taken as the MIC.

3.3. Results and Discussion

3.3.1. Antibacterial activity of ARF

In vitro evaluation of plants for antimicrobial property is the first step towards achieving the goal for developing eco-friendly management of infectious diseases of humans by search for new bio-molecules of plant origin. Considering this, alkaloid rich fractions and compounds 1-10 obtained from different parts of *P. juliflora* were tested for its antibacterial property, against nine bacterial strains, in which two were gram positive (*B. cereus and S. aureus*) and seven were gram negative bacteria (two strains of *E. coli, Salmonella* sp.*, P. putida, Klebsiella* sp.*, Acinetobacter* sp.*,* and *Alcaligenes* sp.,). Most of the selected bacteria were pathogenic and can cause diseases (Chugh, 2008).

ARF of leaf, pod and flower were found to possess significant antibacterial activity (Fig.29), whereas compounds 1-10 did not showed any inhibitory activity, as compared to oflaxacin used as positive control. ARF of all plant parts were active against most of the tested bacterial strains (Table 10) however variations were present in their activity towards different bacteria, when compared with the control (Fig.30)

In case of *E. coli*, leaf ARF was found to be most effective in inhibiting bacterial growth with 95.2% growth inhibition , which is comparable with control (100% inhibition). Leaf was followed by flower with 85.7% and least for pod with 71.4%. For all the three extracts growth inhibition was more than 50% and hence these plant parts, especially leaf could be used as an alternative to synthetic compounds used to control *E.coli*.

For *P. putida*, leaf and flower extracts have shown more promising results to control growth of this bacteria as percent inhibition was more than control causing 118.7% and 106.2% growth inhibition for leaf and flower respectively. Percent inhibition for pod was also comparable with the control with 93.7% inhibition.

Percent inhibition for *Klebsiella* and *S. aureus* was highest for leaf (76.6% and 64.5% respectively) followed by pod and flower which had equivalent activity (60% and 54.8%

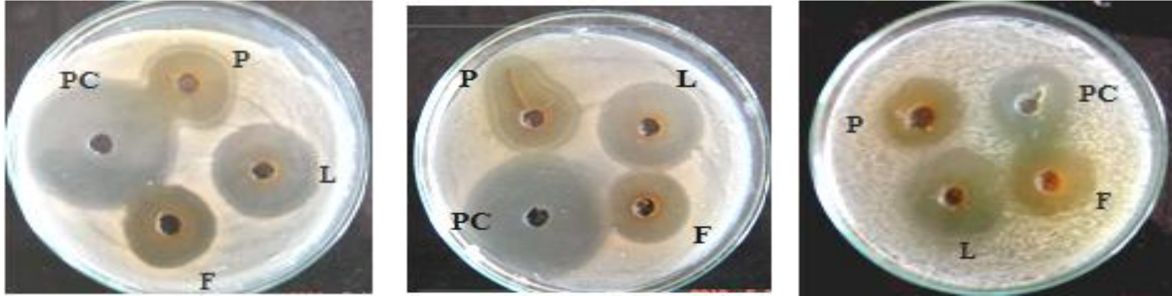
respectively). For *B. cereus* leaf and pod were equally effective with 54.8% inhibitory potential in comparison to control. Flower was less effective and its activity was below 50%.

Salmonella and *E. coli* (ampR) were most susceptible towards leaf ARF with 94.7% and 90% inhibition respectively, followed by flower (78.9% and 80%) and then pod (73.6% and 75%).

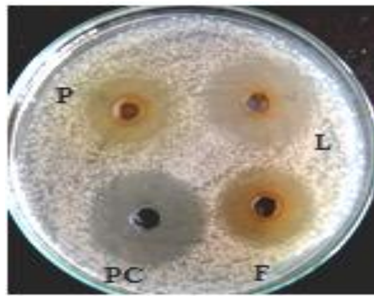
Acinetobacter and *Alcaligenes* were found to be less susceptible towards leaf and flower ARF and were inhibited significantly only by pod extract with 80% and 54.8% inhibition respectively. Leaf and flower showed less than 50% inhibition for both the bacterial strains.

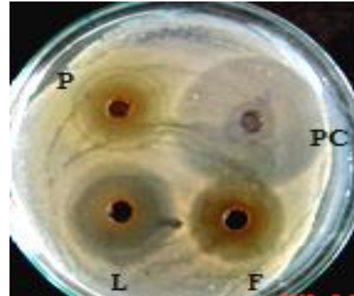
A comparative evaluation of antibacterial activity between leaf, pod and flower by measuring ZOI showed leaf extract to be comparatively more effective from pod and flower in inhibiting most of the bacterial growth except *Acinetobacter* and *Alcaligenes* strains. *Acinetobacter* and *Alcaligenes* were maximum inhibited by pod extract. Among pod and flower extract, flower was more effective for most of the tested bacterial strain (Table10)

A mixture of alkaloids from *P. juliflora* leaf extract has shown significant inhibitory effects on gram positive bacteria (Aqeel et al 1989; Kanthasamy et al., 1989). Similarly earlier studies done on purified alkaloids, Juliprosopine and Julifloricine isolated from leaf extract, showed more effectiveness on gram positive bacteria than gram negative (Ahmad et al 1988; Zainal et al 1988), however in the present experiment, both groups of bacterial strains were equally inhibited by the ARF of leaf, pod and flower, which reflects a broad spectrum antibacterial property of the alkaloidal extract and indicates presence of some other alkaloids that might be responsible for inhibition of gram-negative bacteria as shown by DART-MS analysis in Table 8.

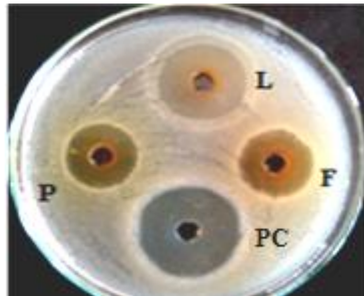


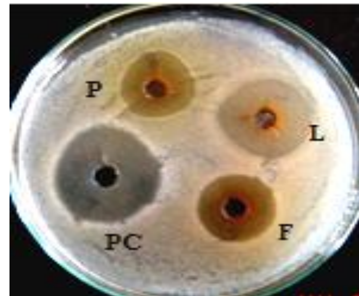
Klebsiella sp. *S. aureus* *P. putida*





E. coli *B. cereus*





Salmonella sp. *E.coli (amp R)*

Fig 29. Antibacterial activity of ARF by well diffusion method

L – leaf; P – Pod; F – Flower; PC – Positive control (Oflaxacin)

Table 10. Antibacterial activity of ARF, obtained from different parts of *P. juliflora* and standard antibiotics on tested bacterial strains (values are expressed as mean±SD, n=5)

	Zone of inhibition (cm)								
	E.coli	***P.putida***	***B.cereus***	***Klebsiella* sp.**	***S.aureus***	***Salmonella* sp.**	***E.coli (amp R)***	***Acinetobacter* sp.**	***Alcaligenes* sp.**
Leaf	2.0± 0.03	1.9±0.33	2.1±0.07	1.7±0.00	2.0±0.51	1.8±0.12	1.8±0.45	1.0±0.66	0.9±0.57
Pod	1.5±0.67	1.5±0.79	1.8±0.55	1.7±0.18	1.7±0.72	1.4±0.54	1.5±0.23	1.6±0.55	1.7±0.44
Flower	1.8±0.54	1.7±0.45	1.8±0.69	1.5±0.33	1.7±0.94	1.5±0.66	1.6±0.71	1.1±0.69	1.2±0.34
Ampilcillin	2.2±0.57	0.9±0.66	1.7±0.00	1.8±0.89	1.3±0.66	1.3±0.51	-	-	-
Chloramphenicol	2.6±0.79	1.2±0.88	1.2±0.88	2.4±0.16	1.5±0.57	1.5±0.16	2.3±0.33	-	-
Tetracyclin	2.0±0.54..	1.5±0.08	1.5±0.67	1.4±0.66	2.0±0.51	1.8±0.33	3.0±0.05	-	-
Rifampin	-	-	1.3±0.23	-	1.1±0.54	-	-	-	-
Streptomycin	1.1±0.44	1.3±0.33	0.9±0.79	1.3±0.57	-	-	-	1.7±0.67	-
Sulfa drug	-	-	1.1±0.89	1.2±0.51	1.3±0.07	1.2±0.07	-	-	-
Oflaxacin	2.1±0.68	1.6±0.54	3.0±0.66	3.1±0.44	3.1±0.88	1.9±0.66	2.0±0.88	2.2±0.51	3.1±0.16

Extract- 10 mg/ml ; Antibiotic-1 mg/ml

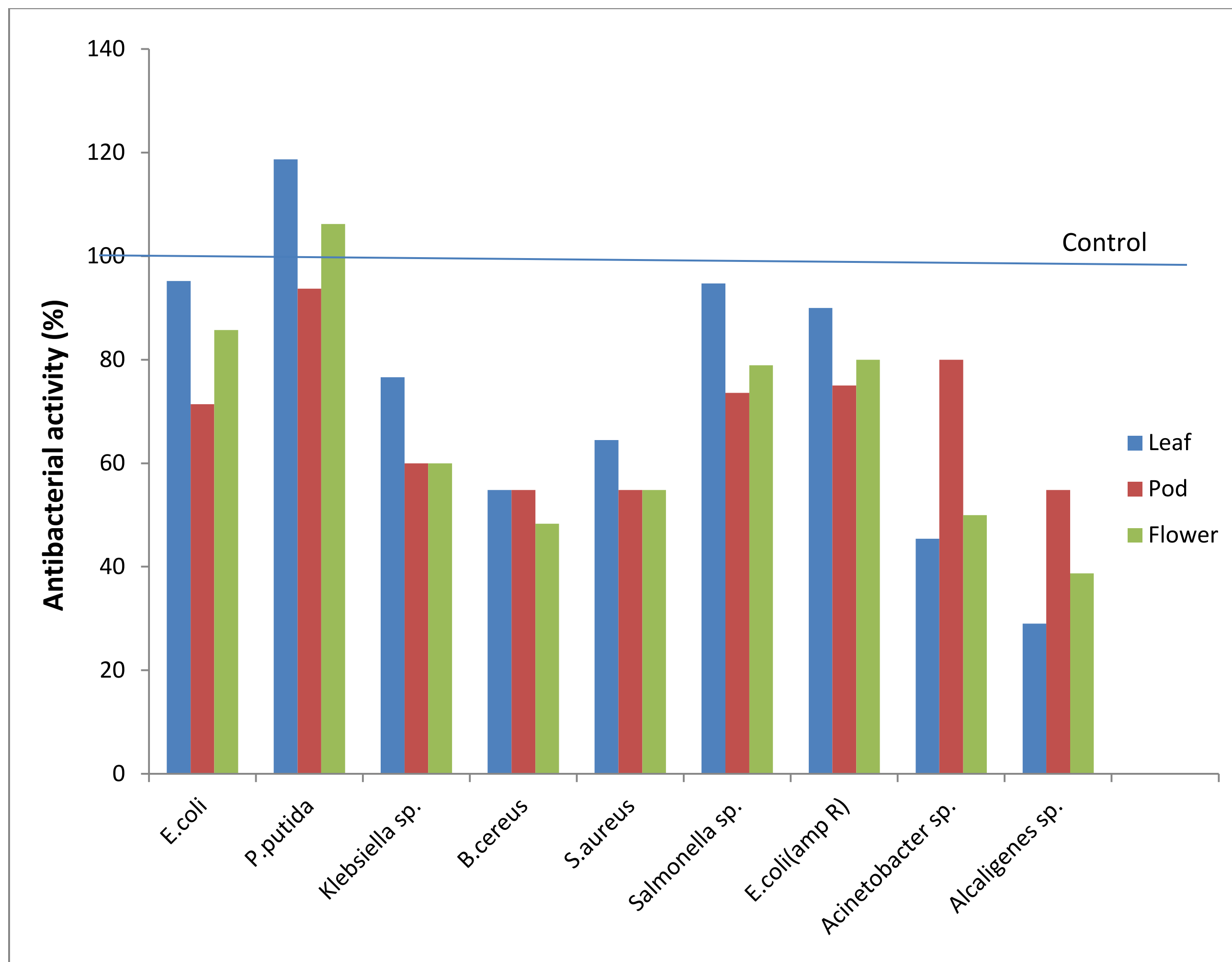


Fig 30. Comparative evaluation of antibacterial activity (in percentage) of ARF obtained from leaf, pod and flower with Oflaxacin as positive control

3.3.2. Comparison of antibacterial activity of ARF with standard antibiotics

A comparison of the efficiency of alkaloidal extract with standard antibiotics was done by disc diffusion method. Seven antibiotics, which include ampicillin, tetracycline, chloramphenicol, rifampin, streptomycin, sulfa drug and oflaxacin, were chosen. Most of the antibiotics are broad spectrum antibiotics, capable of inhibiting growth of both Gram-positive and Gram–negative bacteria, except rifampin, which is used in the treatment of mycobacterium and some Gram-positive bacterial infections (Martin, 2003).

It was observed that ARF and antibiotics had an almost equivalent antimicrobial activity for most of the tested bacterial strains and in some cases ARF were found to be superior then some of the tested antibiotics (Table 10).

Among the tested antibiotic, oflaxacin was found to be the most effective in inhibiting all the bacterial strains with zone of inhibition (ZOI) in the range of 1.6-3.1 cm. Rifampin was least effective with ZOI in the range of 1.1-1.3 cm and inhibiting growth of only two bacterial strains (*Klebsiella* and *S.aureus*). It was followed by Sulfa drug effective on four bacteria (*Klebsiella, B.cereus, S.aureus* and *Salmonella*) and forming ZOI in the rage of 1.1-1.3 cm. Streptomycin was effective on five bacteria (*E.coli, P.putida, Klebsiella*, *B.cereus* and *Acinetobacter*) with ZOI, 0.9-1.7 cm. Ampicillin was active on most of the bacterial strains with ZOI, 0.9-2.2 cm except *E.coli* (ampR), *Acinetobacter* and *Alcaligenes.* Chloramphenicol with ZOI, 1.2-1.6 cm and Tetracycline with ZOI, 1.2-2.0 cm were also effective on most of the strains except *Acinetobacter* and *Alcaligenes* (Table 10). Hence it was observed that except oflaxacin none of the antibiotics was able to inhibit the growth of all the tested bacterial strains, however the growth of all the bacterial strains were remarkably inhibited by the alkaloid fractions, suggesting greater potential of these plant parts to control bacterial growth as compared to the standard antibiotics.

Infections caused by multi-drug resistance bacterial species are among the most difficult to treat with conventional antibiotics (Cohen, 1992; Muroi and kubo, 1996; Nikaido, 2009). Therfore in the present work ARF were tested for their ability to control multidrug resistance bacteria. As discussed previously, all the tested bacterial strains

except *Klebsiella* were found to be resistant towards one or more antibiotics. Among them few bacterial strains were found to be multidrug resistant. *Acinetobacter* was resistant towards ampicillin, chloramphinecol, tetracycline, rifampin, and sulfa drug whereas *Alcaligenes* was resistant towards ampicillin, chloramphinecol, tetracycline, rifampin, streptomycin and sulfa drug. A known *E.coli* ampicillin resistant strain was found to be not only resistant towards ampicillin but also towards rifampin, streptomycin and sulpha drug. It was observed that except Oflaxacin, none of the antibiotic was able to inhibit the growth of all multidrug resistant bacterial strain whereas leaf, pod and flower extracts were effective in inhibiting the growth of all these strains (Table 10), suggesting greater potential of these plant parts to control the growth of drug resistant microbes.

3.3.3. Minimum inhibitory concentration

The MIC values showed variation in the antibacterial effect of all the tested ARF (Table 11). The minimum inhibitory concentration required to inhibit the growth of bacteria ranged from 25-100 µg/ml. Leaf ARF was found to have lower MIC vaules as compared with pod and flower. The least MIC value for leaf extract was observed against *Klebsiella E.coli, P.putida* and *S.aureus,* (25 µg/ml), followed by *E.coli* (amp R) and *B.cereus* (50 µg/ml) and then *Alcaligenes* and *Acinetobacter* (100 µg/ml). The pod extract showed least MIC for *Klebsiella, S.aureus, Acinetobacter and Alcaligenes* (50 µg/ml) followed by *E.coli, P.putida, E.coli* (amp R) and *Salmonella,* (75 µg/ml) and highest for *B.cereus (*100 µg/ml) The flower extract exhibited the least MIC for *Klebsiella, E.coli,* and *S.aureus* (50 µg/ml), followed by *P.putida, B.cereus,* and *Salmonella* (75 µg/ml) and highest in *E.coli*(amp R), *Alcaligenes* and *Acinetobacter* (*1*00 µg/ml). MIC values of ARF of *P.juliflora* in the range of 25-100 µg/ml was found to be comparable with the reported MIC values of some important standard antibiotics on resistant bacterial strains (Torres et al., 1993).

Variation in the MIC values indicates variability in the presence and distribution of alkaloids within the tested plant parts. Variation in the alkaloids in different plant parts have been shown in previous chapter and could be probably responsible for difference in antibacterial activity.

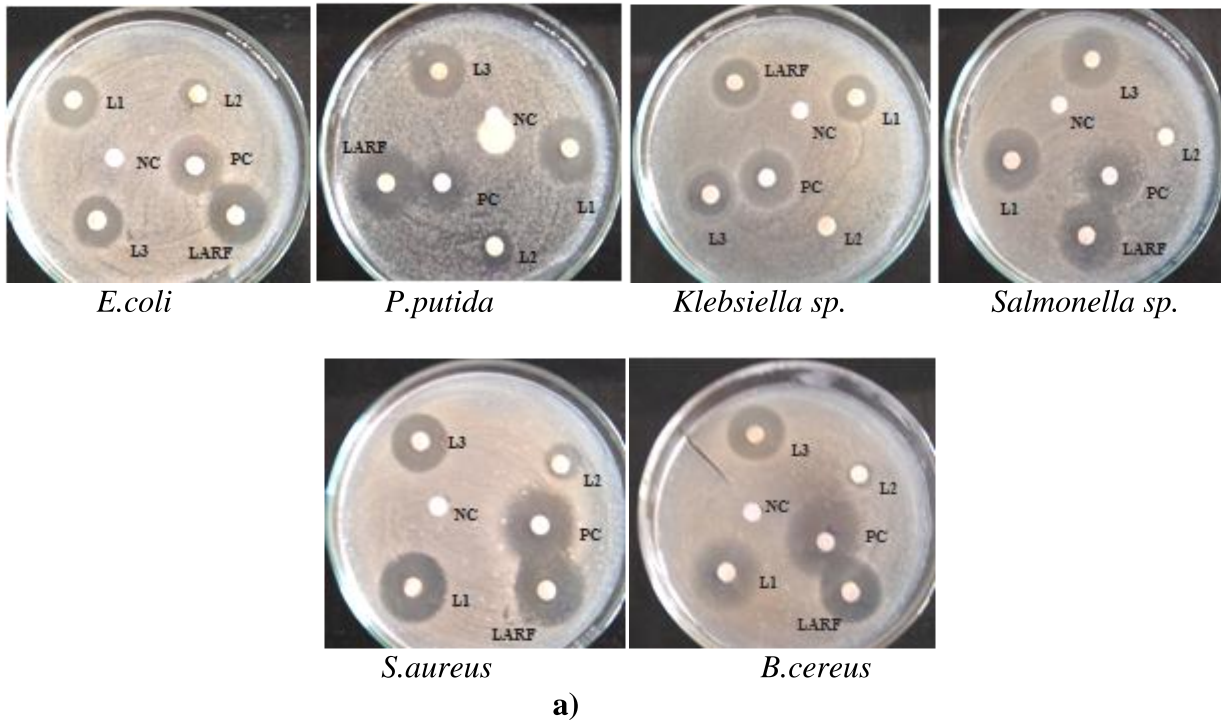
Table 11. Minimum concentration of the ARF required to inhibit the growth of tested bacterial strains

	MIC(µg/ml)		
Microorganism	**Leaf**	**Pod**	**Flower**
E.coli	25	75	50
P.putida	25	75	75
B.cereus	50	100	75
Klebsiella sp.	25	50	50
S.aureus	25	50	50
Salmonella sp.	50	75	75
E.coli(amp R)	50	75	100
Acinetobacter sp.	100	50	100
Alcaligens sp.	100	50	100

3.3.4. Synergistic effect of the alkaloids

In order to prove synergistic effect of the alkaloids, ARF were further separated into various fractions by thin layer chromatography and tested for its bioactivity using the disc diffusion assay. Results obtained revealed higher antibacterial activity of crude alkaloid mixture than its partially purified fractions (Fig.31). Synergistic effect of alkaloids was more significant in case of pod extract as compared to leaf extract as large variation in antibacterial activity between crude extract and its fractions were observed (Table 12).

Among the 3 fractions obtained for leaf ARF (described in previous chapter), it was found that L1 and L3 were effective in inhibiting the growth of all the tested bacterial species with ZOI in the range of 1.0-1.7 and 0.8-1.6 cm respectively, whereas L2 showed very low antibacterial activity against few bacterial strains (*E.coli, P.putida and S.aureus*) with ZOI 0.7 and 0.8 cm. Zone of inhibition due to L1 and L3 was found to be equivalent with that of ARF (1.4-1.7) and was slightly low only against *E.coli* and *P.putida* (Table 12).



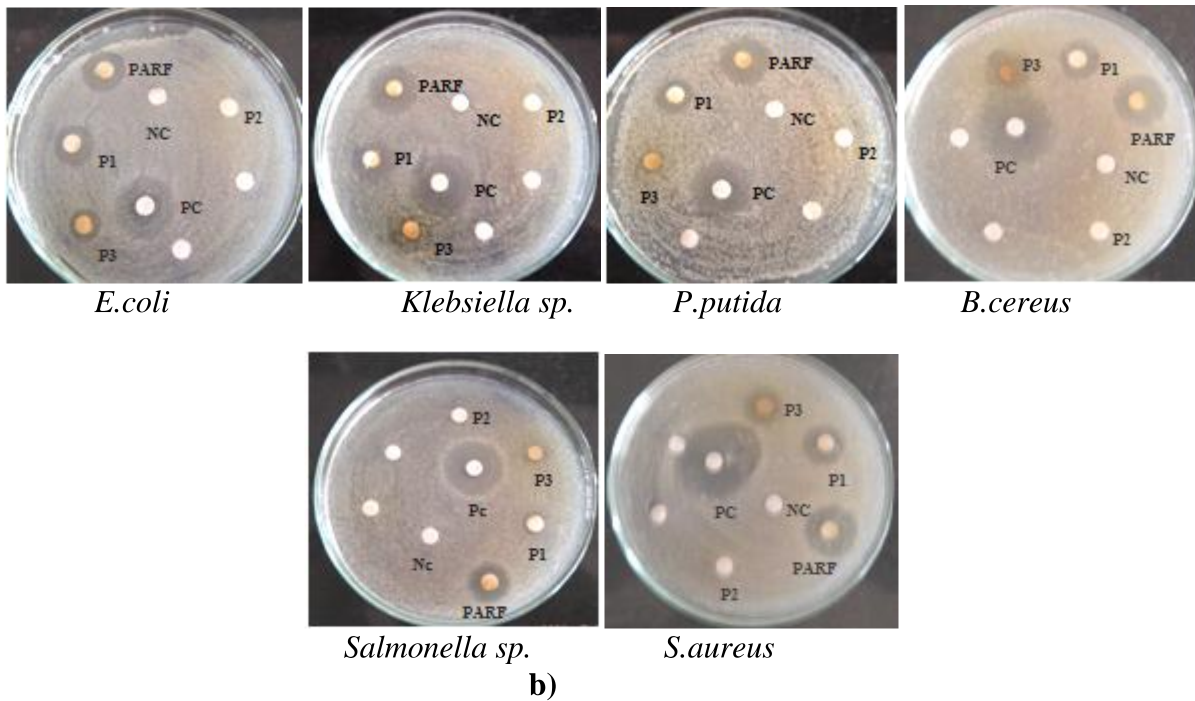


Fig 31. Synergistic activity of plant extracts- a) leaf b) pod

LARF-leaf alkaloid rich fraction; PARF- pod alkaloid rich fraction; NC-negative control; PC-positive control

Table 12. Comparative analysis of the Antibacterial activity of ARF with its fraction(values are expressed as mean±SD, n=5)

	Zone of Inhibition							
	E.coli	***P.putida***	***B.cereus***	***Klebsiella sp.***	***S.aureus***	***Salmonella sp.***	***Acinetobacter sp.***	***Alcaligen sp.***
LARF	1.6±0.79	1.7±0.88	1.6±0.88	1.4±0.16	1.7±0.57	1.7±0.16	1.5±0.66	1.4±0.57
L1	1.4±0.54.	1.5±0.08	1.5±0.67	1.4±0.66	1.7±0.51	1.6±0.33	1.0±0.51	1.1±0.75
L2	0.7±0.03	0.7±0.23	-	-	0.8±0.54	-	-	-
L3	1.4±0.44	1.4±0.33	1.5±0.79	1.3±0.57	1.4±0.89	1.6±0.89	0.8±0.34	1.1±0.69
PARF	1.2±0.68	1.3±0.54	1.5±0.66	1.4±0.44	1.4±0.88	1.0±0.66	1.3±0.55	1.5±0.44
P1	1.1±0.57	0.9±0.66	0.9±0.00	1.0±0.89	1.0±0.66	-	0.8±0.23	0.8±0.34
P2	-	-	-	-	-	-	-	-
P3	0.8±0.54.	-	1.0±0.89	0.7±0.66	0.9±0.51	-	-	0.7±0.54

Extract : 10 mg/ml

In case of pod, crude alkaloid mixture was more effective than its fraction P1, P2 and P3 and was efficient in inhibiting growth of all the tested bacterial strains. Among the fractions, P1 was found to have highest antibacterial activity (ZOI 0.8-1.1) and was effective against all bacterial strain except *Salmonella* sp., followed by P3 (ZOI 0.7-1.0) which was effective against *E.coli, B.cereus, Klebsiella, S.aureus* and *Alcaligenes sp*. P2 did not showed any antibacterial activity (Table 12).

DART-MS results, as shown in previous chapter revealed the chemical composition of these fractions. L1 and P2 were found to contain almost pure compounds, L1 contained Juliprosopine and P2 contained Julifloridine in highest concentration and could be the main compound responsible for antibacterial activity of these fractions, however their activity was less than crude fraction.

Even among various fractions, those fractions containing more alkaloids were comparatively more effective than those containg lesser alkaloids. L3 containing Julifloridine, Prosopine and Prosopinine as major constituent, was found to be more effective than P2 having Julifloridine as major constituent and L2 containing Prosopine and Prosopinine. This observation leads to the conclusion that alkaloid Julifloridine, Prosopine, Prosopinine are less effective when separated from each other than present together in alkaloid mixture (positive synergism). The results support the work of Batatinha et al., 1997 who have also reported pure alkaloids to be less toxic as compared to alkaloidal extract, suggesting synergistic effect of these alkaloids.

The results also proves antibacterial activity of Julifloridine, which has earlier not been reported. In P2 fraction, more than 90% of the compound is Julifloridine and hence its antibacterial activity could be directly related to Julifloridine, however it is slightly less effective than Juliprosopine, as evident by formation of smaller zone of inhibition.

Research has shown that some secondary metabolites of plants with no intrinsic antimicrobial activity are useful in sensitizing bacterial cells to antimicrobial agents (Stapleton, 2004). These compounds are believed to play a role in the plant's defence against infection by working in synergy with intrinsic antimicrobials. It has therefore been

suggested, that such compounds can potentially be used to improve the efficacy of antibiotics against bacterial pathogens. In the present experiment, the alkaloids, present in the alkaloidal mixture (Table 8) may be responsible for enhancing the activity of antimicrobial alkaloids.

Antibacterial test conducted in the present work shows that leaf, pod and flower extracts of *P. juliflora* have antibacterial property and also have the potential to inhibit antibiotic resistance bacterial strains. This property can be exploited to control drug resistant pathogens and use the compounds present in its plant parts as natural antimicrobials.

Chapter 4

Allelopathic, antimitotic and cytogenotoxic activity of Prosopis juliflora

4.1. Introduction

Due to increasing numbers of herbicide-resistant weed biotypes and environmental concerns about the safety of synthetic herbicides, considerable effort has been put into designing alternative weed-management strategies and reducing dependence on synthetic herbicides (Teerarak et al., 2010). In view of this, large number of natural compounds of plant origin has been screened for potential allelopathic activity (Comes, 2005; Pheng et al., 2010). Allelopathy involves secretion of allelochemicals into the environment to inhibit germination or growth of surrounding vegetation. Allelochemicals are metabolic by-products of certain plants that are reported to cause growth inhibition in other plant species by affecting physiological processes such as respiration, cell division, and water as well as nutrient uptake (Loomis and Hayes, 1996; Timbrell, 2002; Craig et al., 2011).

Allelochemicals are present in almost all parts of the plants, like leaves, stems, flowers, fruits, seeds and roots (Putnam, 1988). They are often water-soluble substances that are released into the environment through root exudation, leaching and decomposition of plant residues (Aminidehaghi et al., 2006). It is also found that allelochemicals that inhibit the growth of one species at certain concentrations might in fact stimulate the growth of the same or different species at other concentrations (Narwal, 1994).

Prosopis juliflora, a widely distributed species throught out the world was found to exert some allelopathic pressure on its surrounding vegetation through decomposition of plant parts or plant leachates or root exudates. Consequently, the release of allelochemicals into the soil inhibited seed germination and establishment of vegetation. Goel et al., (1987) reported that the leaf litter of *P. juliflora* has allelopathic properties. L-tryptophan, syringin, and lariciresinol were isolated from leaf leachates as candidates for allelopathy in *P. juliflora* (Nakano et al., 2001, 2002). Growth inhibitory alkaloids, 3′′′′-oxo-juliprosopine, secojuliprosopinal, and a mixture of 3-oxo-juliprosine and 3′ -oxo-juliprosine, juliprosopine and juliprosine have also been isolated from the leaf extracts of *P. jliflora* (Nakano et al., 2003, 2004a). However, most of these studies were concentrated on leaf leachates and exudates and the plant growth inhibition from other parts of the plant have

not been reported. Also the site of action of the allelochemicals has not been worked out in detail at cellular level.

Cell growth in plant depends upon normal mitotic processes, in which DNA synthesis, mitosis and cytokinesis occur. Cell division is a continuous process that occurs in plant meristematic regions (Singh, 2002). Mitotic activity, alterations in the mitotic phase, and individual cell aberrations are key parameters by which plant growth may be evaluated. Cytogenetic assays for evaluation of these parameters are commonly carried out using the Allium test. Characterized by rather homogenous meristematic cells, very large chromosomes and only sixteen chromosome number, the *Allium cepa* species (common onion) is ideal for use in bioassays (Havey, 2002). *Allium* has proven a rapid, reliable, and inexpensive system by which the toxic effects of various chemical compounds may be monitored for potential environmental pollution effects (Andrade et al., 2008; Leme and Marin-Morales, 2008; Seth et al., 2008). It has been widely used for detection of cytostatic, cytotoxic and mutagenic properties of different compounds, including anticancer drugs of plant origin (Kura´s et al., 2006).

Aerial parts of *P. juliflora* are used in various herbal remedies (Hartwell, 1982) and the alkaloids are found to be responsible of its pharmacological activity (Ahmed et al., 1988), but due to the lack of information about their genotoxicity, it is important to evaluate the effects of its compounds on genetic alterations. The results obtained by Allium test could be useful in correlating the genotoxic effect of *P. juliflora* compounds on *A. cepa* with that of mammalian cells as it is reported that Allium test shows good correlation with mammalian test systems (Fiskesj¨o, 1988; Grant 1994; Smakakincl et al., 1996; Fiskesj¨o, 1997; Yi & Meng 2003)

In the present chapter, the inhibitory activity of alkaloid rich fraction obtained from different parts of *P. juliflora* is evaluated. Onion bulbs and mung seeds were chosen as representative of monocotyledonous and dicotyledonous species. The study also reports antimitotic and cytotoxic activity of ARF and compound 10 (isolated from flower) with the help of Allium test.

4.2. Materials and methods

4.2.1. Test Plant species

Two plant species were selected for evaluation of growth inhibitory potential, onion bulbs (*Allium cepa*) and mung seeds (*Vigna radiate*).

4.2.2. Allelopathic activity test on *Allium cepa*

Small bulbs (1.5–2.0 cm in diameter) of the common onion, *A. cepa,* ($2n = 16$) were taken. Prior to initiating the test, the outer scales of the bulbs and the dry bottom plate were removed without destroying the root primordia. They were carefully unscaled and cultivated on the top of test tubes filled with 50 ml of the ARF in the concentrations of 25 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml, water was used as control. For each sample, a series of six bulbs were grown. Test tubes were incubated in the dark at 24°C for up to 7 days, after which the root lengths were measured.

4.2.3. Allelopathic activity test on *Vigna radiate*

Healthy uniform mung seeds were taken. Before germination, they were surface sterilized with 1% sodium hypochloride for 20 mins, then rinsed with distilled water for several times to remove excess of chemical. Seeds were first presoaked in distilled water for 2h and then soaked in different concentration of test solution (25 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml), control were treated with distilled water. The sterilized seeds were evenly placed on two layers of filter paper in sterilized petidish, with 10 seeds per petri dish. The Petri dishes were placed in growth chamber (24°C and 70% humidity and continuously dark). Treatments were arranged in a completely randomized design with three replications. Seeds were considered germinated upon radicle emergence. Germination was determined by counting the number of germinated seeds over a 5-day period and radical length was measured using a millimeter ruler.

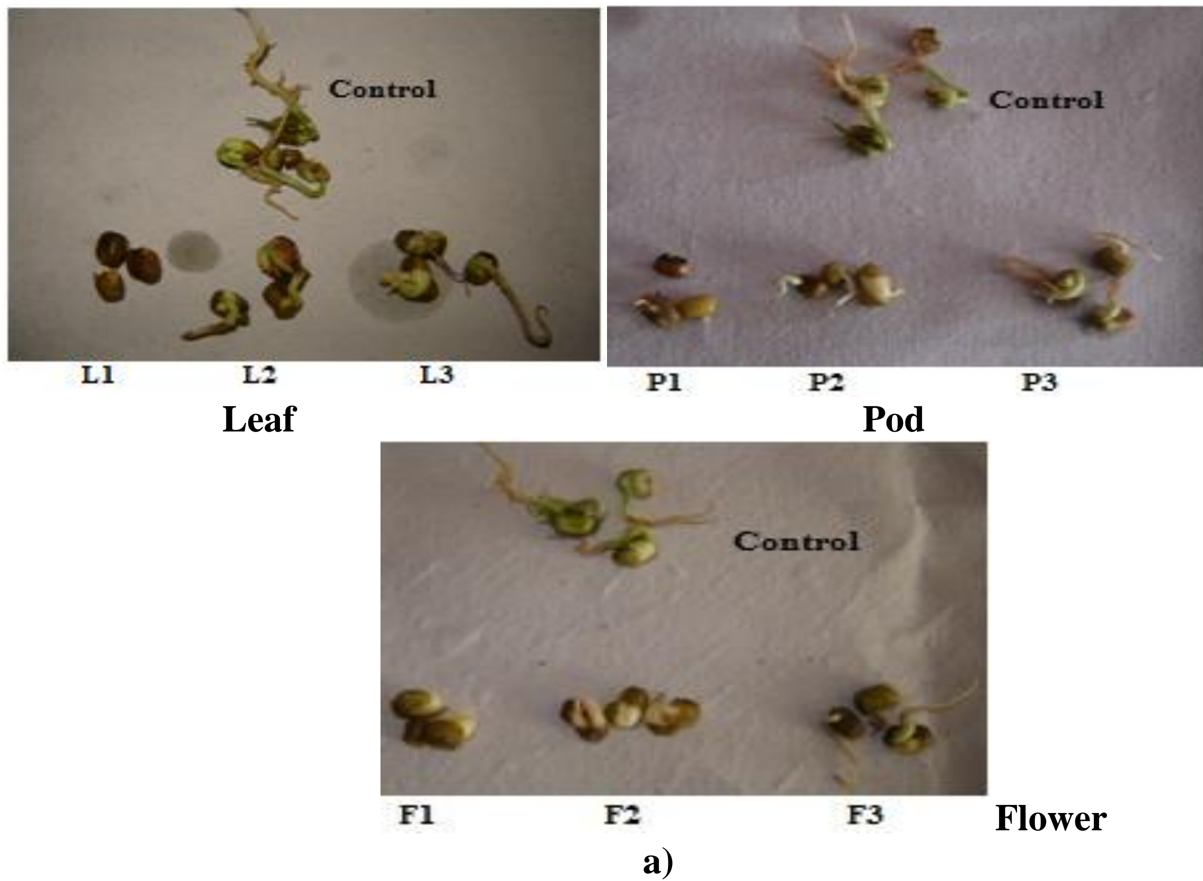
4.2.4. Allium test

Antimitotic and cytotoxic activity of ARF obtained from various parts of *P. juliflora* and of compound 10 isolated from flower of *P. juliflora* was studied by the Allium test. The Allium test was performed according to the method described by Fiskesj¨o, 1988. The roots of *A. cepa* were grown in distilled water in 200mL Erlenmeyer flasks under laboratory conditions (dark, 24°C). After reaching a length of 3 cm (±0.5 cm), roots were incubated in the ARF of leaf, pod and flower at 10, 50, 100 and 150 µg/mL concentrations and of compound 10 at 4 and 8 mg/ml concentrations. Pure water was used as control. The root tips were collected after 6, 12, 24 and 48h of incubation. For each concentration five root tips from three analogous onions were taken. Root tips were hydrolyzed in 1 N HCl, followed by squashing in 2% acetocarmine stain in 45% acetic acid. The squash preparation was observed under the microscope. Chromosome morphology and their changes were observed and mitotic index was calculated. Changes in cellular and chromosomal morphology were photographed under a light microscope (Olymphus BX41).

4.3. Results and Discussion

4.3.1. Allelopathic effect

A significant inhibition of root growth was observed after treatment of mung seeds and onion bulbs by ARF of all the tested plant parts (Fig.32). All the tested concentrations of the extracts were found to inhibit root growth in a dose dependent manner (Table 13). Fig.33 shows percentage inhibition of root growth by the extract as compared with the control. It was observed that even the lowest concentration (25µg/ml) of the leaf, flower and pod extracts used in the experiment was able to inhibit root growth by 33.3%, 22.8%, 35.4% in case of onion and 34.6%, 60.9% and 23.3% for mung seeds respectively. 100 µg/ml concentration completely inhibited (100%) the seedling growth of both bioassay



b)

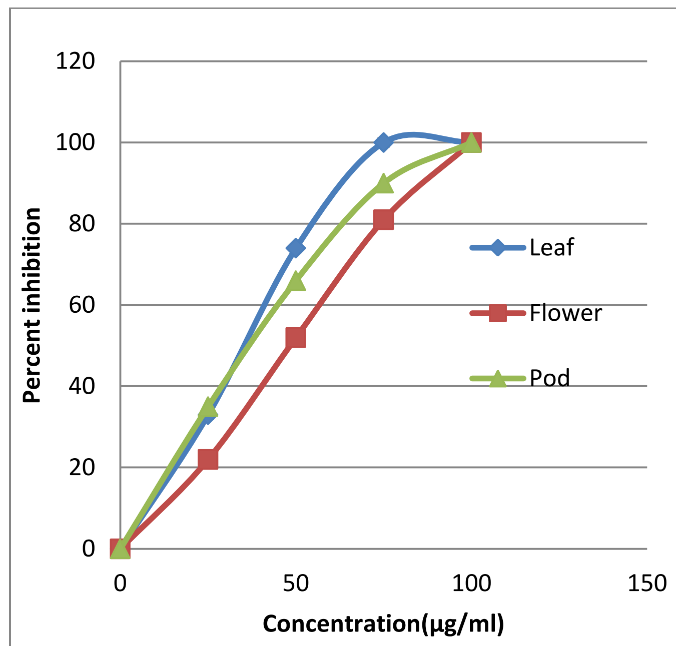
Fig 32. Allelopathic activity of ARF on root length - a) mung seeds and b) onion bulb. L1/P1/F1- 75 µg/ml; L2/P2/F2 - 50 µg/ml; L3/P3/F3 -25 µg/ml; Control - 0 µg/ml

species. Significant growth inhibition for low concentrations indicates strong allelopathic potential of the alkaloidal extracts.

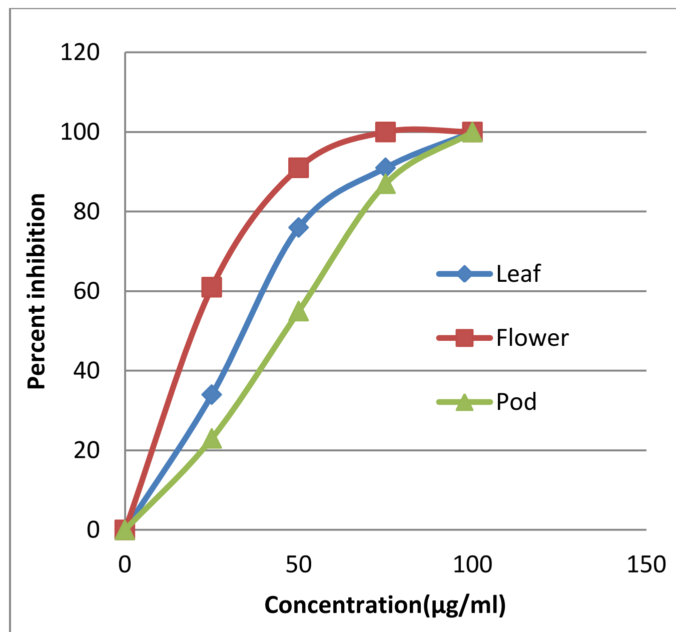
Table 13. Effect of ARF on average root length of onion bulb and mung seeds (values are expressed as mean±SD)

	Onion root length (cm)			Mung root length (cm)		
Concentration (µg/ml)	**Leaf**	**Flower**	**Pod**	**Leaf**	**Flower**	**Pod**
0	5.2±0.9	5.2±0.9	5.2±0.9	2.2±0.7	2.2±0.7	2.2±0.7
25	3.5±0.5	4.1±0.7	3.4±0.6	1.4±0.6	0.9±0.3	1.7±0.5
50	1.4±0.5	2.5±0.3	1.8±0.5	0.8±0.3	0.2±0.0	1.0±0.4
75	-	1.0±0.2	0.5±0.2	0.2±0.3	-	0.3±0.1
100	-	-	-	-	-	-

The results show variation in the allelopathic effect of all the tested ARF. It can be concluded that in case of monocotyledonous plant, leaf extract was more effective in reducing root germination as compared to pod and flower extracts, whereas in dicot plant it was found that the germination was inhibited more by flower extract, followed by pod and least by leaf extract (Table 13). It was interesting to observe that percent reduction of mung root length by flower extract was highest as compared to other tested combinations. Lowest concentration of ARF of leaf and pod caused 34.6% and 23.3% reduction of mung seeds respectively, whereas 60.9% reduction was observed for flower extract (Fig.33). Flower extract completely inhibited (100%) the growth at 75µg/ml concentration, whereas no growth was observed at 100 µg/ml in case of leaf and pod extract. The variation in the allelopathic effect could be due to the variability in the presence and distribution of alkaloids, as described in earlier chapter. Difference between dicot and monocot species, may also be attributed to variation in phytochemicals that results in variation in response.



a)



b)

Fig 33. Percent inhibition of root length by increasing ARF concentration as compared with control - (a) onion bulb and (b) mung seeds

4.3.2. Antimitotic and Cytogenotoxic activity of ARF

Cytotoxicity of the extracts at cellular and chromosomal level was evaluated by exposing meristematic root cells of *A. cepa* to different concentrations (10-150 μg/ml) of ARF of leaf, pod and flower for 48h and comparing the changes in the cellular activity with the control.

Mitotic activity in the control root tips was normal throughout the tested time period, with almost 90% of the cells undergoing mitotic division (Fig.34a-34e). Particular phases were distinguished by taking into account the condensation of chromatin. During prophase (Fig.34a), chromatin appeared in the form of a tangled mass inside a nucleus, followed by further condensation (Fig.34b). During metaphase, chromosomes formed the equatorial plate (Fig.34c) and during anaphase, each of the sister chromosomes shifted to the opposite cell pole (Fig.34d). Telophase began when chromosomes reached the poles and it was preceded by cell plate formation (Fig.34e). After the cycle was finished, the chromosomes elongated and returned to the interphase state.

The results presented in Table 14, indicated that ARF of all the plant parts reduced the percentage of cells undergoing normal mitotic process, thus causing a significant decrease of mitotic index as compared with control. Mitotic index measures the proportion of cells in the M-phase of the cell cycle and its inhibition could be interpreted as cellular death or a delay in the cell proliferation kinetics (Rojas et al., 1993).

Cytogenotoxic activity was also evaluated by observing cellular and chromosomal damage caused by ARF. The results obtained from cytogenetic analysis revealed chromosomal abnormalities and extensive cell death at higher concentration. The results are in consistent with the earlier cytoxicity study of *P. juliflora* by Batatinha et al., 1997 reporting cytotoxic and antitumoral activity of alkaloids against human epithelial tumour cells (HeLa), human hepatic tumour (HepG2), and two fibroblast lineage F26 and F57, Hughes et al., 2005 have also reported cytotoxic effect of alkaloid extract on cell cultures of glial cell, likewise significant hemolysis of erythrocytes by alkaloids of *P. juliflora*, was

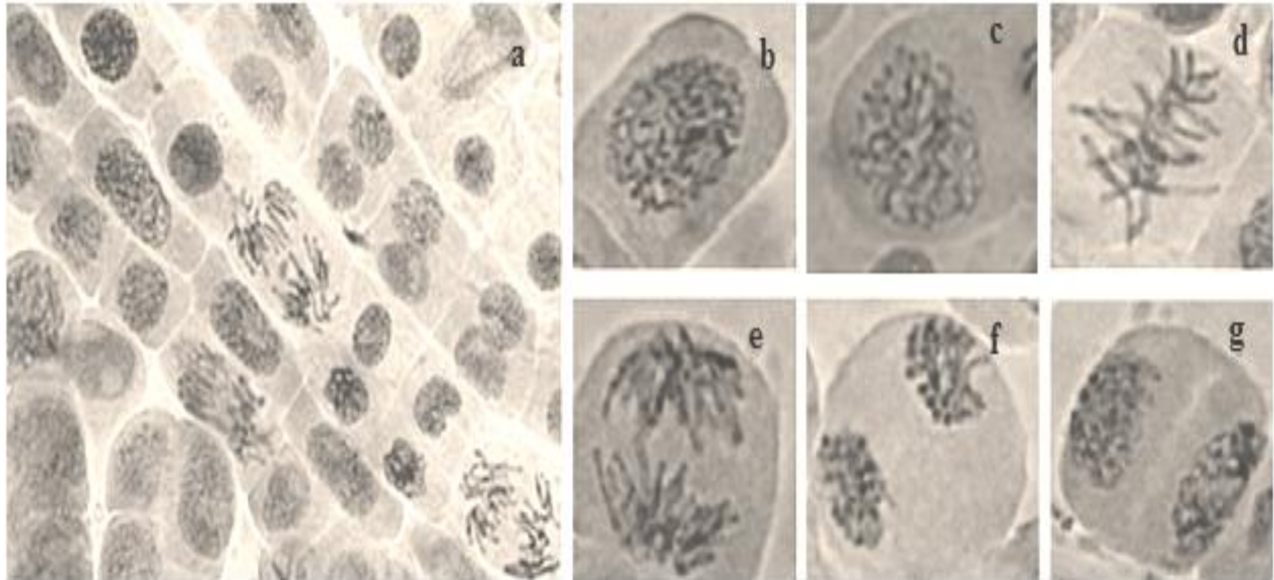


Fig 34. **Microphotographs of control meristematic cells of *Allium cepa*** - a) dividing cells at different mitotic phases, b) early prophase, c) late prophase , d) metaphase, e)anaphase, f) telophase, g) cell plate formation. a) 40x, b), c), d), e) f) and g) 100x

Table 14. Mean Mitotic index values (in percent) of the cells treated with different concentrations of ARF of leaf, pod and flower at different time interval (values are expressed as mean±SD, n=6)

Time	10(μg/ml)	50(μg/ml)	100(μg/ml)	150(μg/ml)
Leaf				
0	90.2±5.1	90.2±5.1	90.2±5.1	90.2±5.1
6	86.3±4.3	64.5±5.8	13.5±3.4	6.7±1.3
12	55.5±3.3	12.7±2.4	0	0
24	34.6±3.6	0	0	0
48	0	0	0	0
Pod				
0	90.2±5.1	90.2±5.1	90.2±5.1	90.2±5.1
6	87.6±7.4	67.3±6.7	41.0±5.8	32.8±4.9
12	81.1±6.2	25.5±3.5	20.7±3.5	8.3±3.3
24	54.7±5.5	12.6±1.3	2.5±0.4	0
48	38.9±3.9	3.4±1.8	0	0
Flower				
0	90.2±5.1	90.2±5.1	90.2±5.1	90.2±5.1
6	78.3±6.6	75.9±6.4	56.8±4.8	49.0±5.2
12	54.5±7.5	43.6±5.9	17.7±2.5	15.4±3.0
24	64.9±4.3	18.6±1.7	3.6±0.6	0
48	37.4±3.5	4.8±0.2	0	0

reported by Kandasamy et al., 1989. However all the earlier reports are on *in vitro* systems and were mostly focused on leaf alkaloidal extract. The present research work evaluates antimitotic and cytogenotoxic effect of these alkaloids in an in vivo system. Furthermore alkaloids of other parts of the plant, pod and flower have also been found to possess cytotoxic activity and shown to have a comparative toxic potential with the leaf.

4.3.3. Antimitotic and Cytogenotoxic effect of varying concentrations of ARF of leaf on root cells of *Allium cepa*

All concentrations used in the experiment caused inhibition of mitotic activity proportional to the time of incubation (Table 14). Slightly decreased mitotic index values resulted after the 6h treatment with leaf extract concentrations of 10 and 50 µg /ml, but did not lead to total inhibition of cell division. When the concentrations were increased to 100 and 150 µg/ml, there was significant reduction of mitotic index values for the same time period, which finally decreased to 0 after 12h. Complete loss of cell division for 10 and 50 µg /ml concentrations was observed at 48h and 24h respectively. Ihibition of cell division at concentration as low as 10 µg /ml, indicates strong antimitotic activity of leaf ARF.

Apart from loss of mitotic activity, changes in chromosome and cellular morphology were observed with increasing time and concentration of ARF. Toxic symptoms were visible with lowest concentrations of ARF (10 µg /ml) after 6h, showing abnormal mitotic phases with thickened chromosomes (Fig.35a). These chromosomes were found either clustered together or scattered in the cell. Stickiness was observed between and within chromosomes (Fig.35b and 35c) which increased with the increase in concentrations and time period. The presence of chromosome stickiness could be due to the effect of the chemical compounds on the physico-chemical properties of DNA, protein or both including formation of complexes with phosphate groups in DNA, DNA condensation and formation of inter- and intra chromatid cross links (G¨om¨urgen et al., 2005).

Interphase nuclei after incubation at the lowest concentrations did not differ from the structures of the nuclei of control cells, however, differences were observed at high concentrations. Alkaloid extracts at higher concentration caused an extensive cell death,

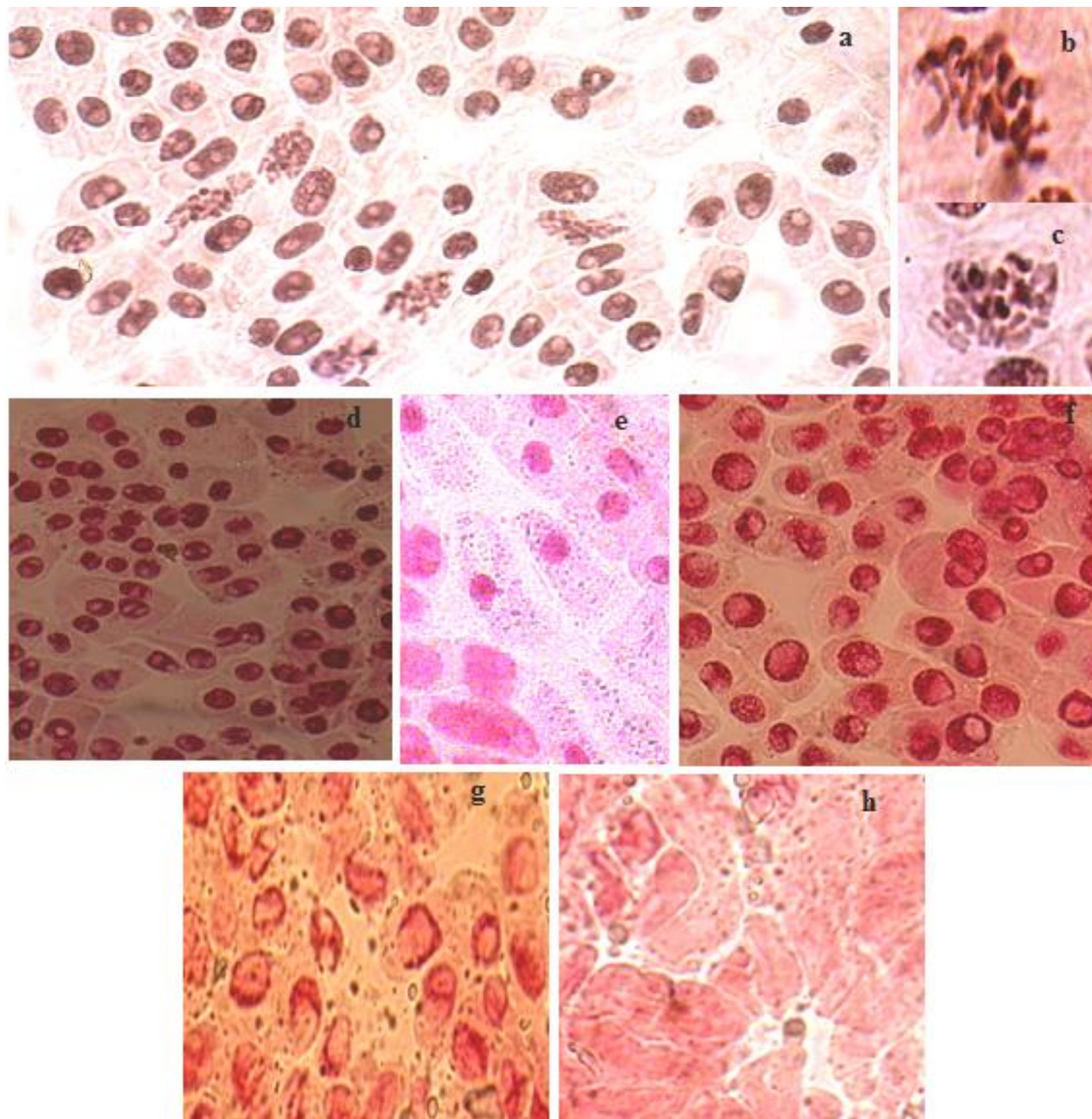


Fig 35. **Microphotographs of meristematic cells of *Allium cepa* after incubation in 100 µg/ml solutions of ARF of leaf -** a) low mitotic activity following 6h of incubation in ARF, showing condensed and contracted chromosomes with abnormal metaphase b) stickiness between chromosomes c) scattered chromosomes with stickiness within chromosome d), e) and f) cells after, 12h incubation in ARF d) contraction in nucleus e) start of autolysis showing nuclear and cytoplasm degradation f) vacuolization in nucleus g) and h) cells after 24h incubation in ARF with complete autolysis in cytoplasm and nucleus (necrosis). b) and c) 100x and others 40x

probably due to necrosis rather than apoptosis. Incubation for 12h in ARF of 100 and 150 µg /ml concentration caused the appearance of the nuclei with strongly condensed chromatin (Fig.35d). Increase in treatment duration (24h) caused vacuolization of nuclei and autolysis in the cytoplasm (Fig.35e), with some cells showing nuclear disintegration (Fig.35f). Further increase in incubation time (48h) caused complete nuclear and cytoplasmic degradation (Fig.35g).

4.3.4. Antimitotic and Cytogenotoxic effect of varying concentrations of ARF of pod on root cells of *Allium cepa*

Pod extract showed a decrease in mitotic activity with increasing concentrations and time period (Table 14). Lowest concentrations of pod (10 µg/ml) did not showed significant reduction in mitotic activity up to 12h. After 12h mitotic index value showed a gradual decrease till 48h, however complete loss of activity was not observed at this concentration. Increasing the concentration to 50 µg /ml caused significant decrease in mitotic index (3.4) within 6h of treatment and the mitotic index drastically decreased with increasing time. Complete loss of mitotic activity was observed at 100 and 150 µg /ml concentration within 48 and 24h of treatment respectively. Pod extract was found to be comparatively less toxic than leaf extract, as complete loss of activity (at 48h of incubation) could be achieved at 100 µg/ml in case of pod extract as compared to 10 µg/ml for leaf extract.

Cells were normal at lowest concentration (10 µg /ml), with increase in concentration and time period, prophase chromatin condensed to form chromosomes but was unable to undergo metaphase and the chromosomes were scattered throughout the cell (Fig.36a). The cells incubated with higher concentrations (100 and150 µg/ml), showed complete loss of mitotic activity beyond 12h of incubation and the nuclear material was found to be disintegrated with the cellular boundary still intact (Fig.36b). Binucleate cells were also visible in some cases (Fig.36c). This disintegration of nuclear material continued with the increase in time (Fig.36d).Within 48h nuclear material was completely lost with the cellular boundary still maintained, forming a ghost cell (Fig.36e).

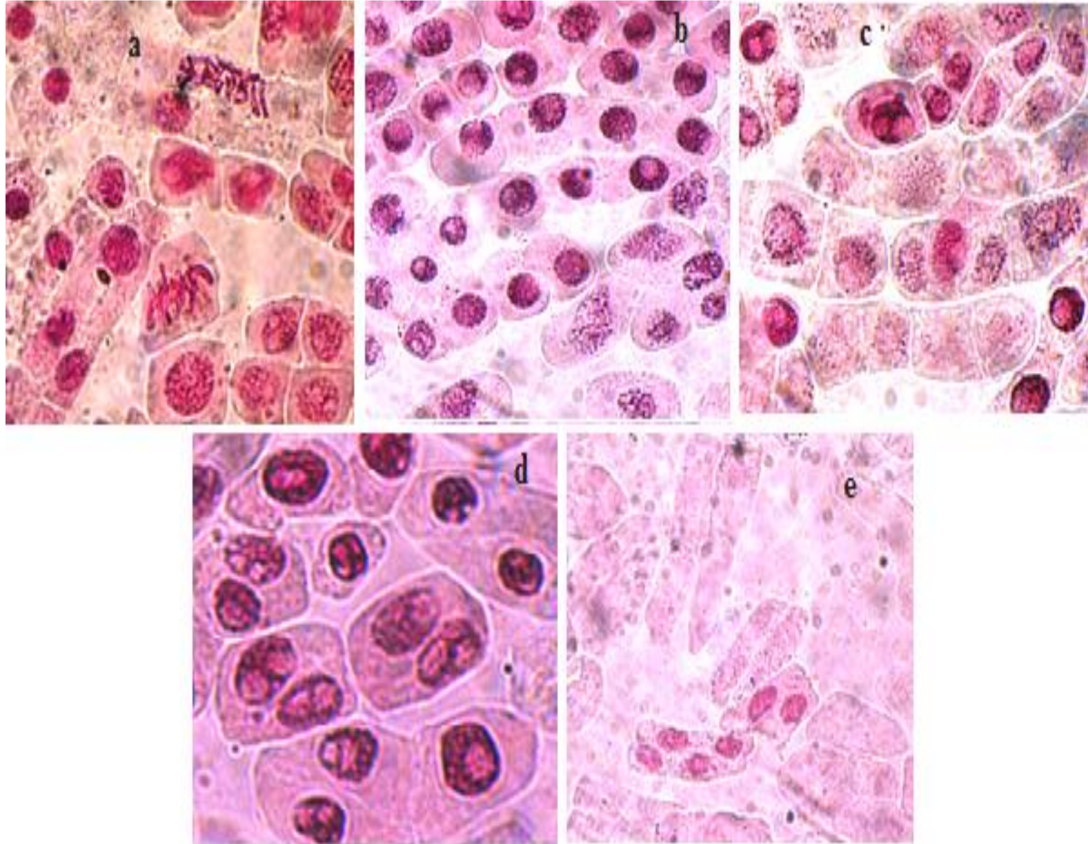


Fig 36. Microphotographs of meristematic cells of *Allium cepa* after incubation in 150 µg/ml solutions of ARF of pod - a) low mitotic activity following 6h of incubation in ARF with abnormal mitotic phases b) cells after 12h incubation in ARF showing nuclear degradation c) cells after 24h incubation in ARF d) binucleate cells e) cells after 48h incubation in ARF with the appearance of ghost cells. 40x

Two important morphological changes were observed in the genetic material, scattered chromosomes and binucleated cells. Scattered chromosomes are formed due to improper alignment of chromosomes and binucleated cells due to prevention of cytokinesis or cell plate formation. Since the microtubules have been implicated for proper alignment of chromosomes and for cell plate formation (Rieder and Salmon, 1998), it could be assumed that these alkaloids could have an inhibitory effect on microtubule formation.

Silva et al., 2007 while studying the toxicity of pod extract of *P. juliflora* on astrocytes reported a strong contraction of the cellular body and condensation of the nuclear chromatin of cultured astrocytes, which varies from the current observations. The variation in both studies might be due to difference in the cells used for testing, as in present work in vivo plant system was used whereas Silva et al., 2007 used *in vitro* animal cells, second difference could be variation in the chemical composition of the extract.

4.3.5. Antimitotic and Cytogenotoxic effect of varying concentrations of ARF of flower on root cells of *Allium cepa*

Mitotic index decreased with increasing concentration and time period with complete loss of mitotic activity at high concentrations of 100 and 150 µg /ml within 48 and 24h respectively (Table 14). Lower concentration also showed significant decrease of mitotic activity after 12h, however it did not cause complete inhibition up to 48h. Flower extract was also found to be less toxic than leaf extract.

Changes in cellular and chromosomal morphology appeared depending upon time of incubation and concentration of ARF. In cells incubated with lower concentration of ARF (50 µg/ml), normal prophase, metaphase, anaphase, telophase and cell plate formation was observed till 6h of treatment (Fig.37a). After further increase in time (12h), mitotic activity was reduced, however normal mitotic phases were still observed in few cells with no change in chromosome morphology (Fig.37b). At higher concentrations (150 µg/ml) most of the cells were found to be at interphase with the appearance of granular cytoplasm (Fig.37c). Further increase in time caused cellular swelling (Fig.37d), followed by

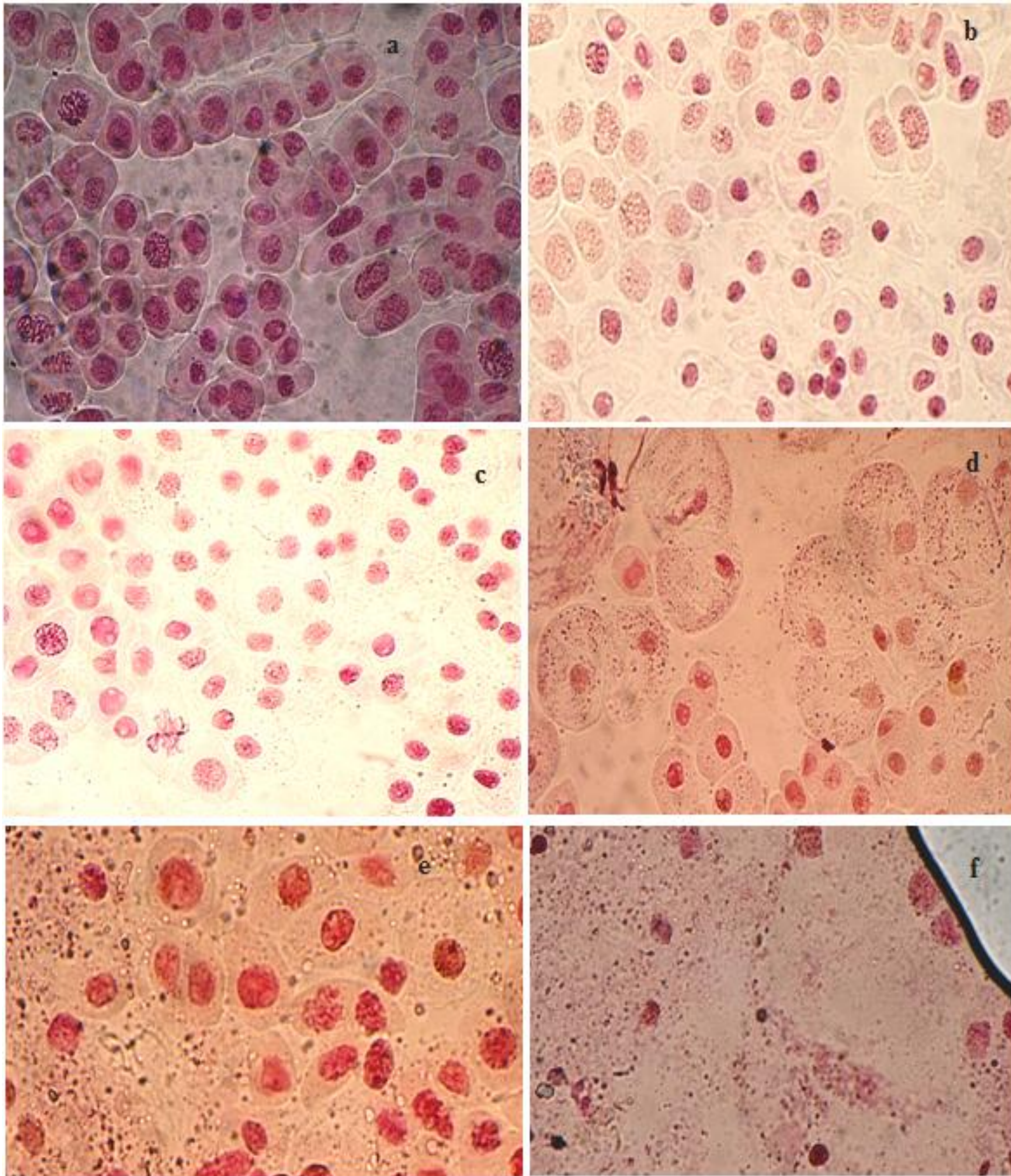


Fig 37. Microphotographs of meristematic cells of *Allium cepa* root after incubation in 150 µg/ml solutions of ARF of flower - a) cells after 6h incubation showing normal dividing cells b) and c) cells after 12h b) Low mitotic activity c) cells with granular cytoplasm showing start of necrosis d) after 24h incubation swollen and granular cells are seen e) and f) cells after 48h showing cellular degradation followed by nuclear disintegration. 40x

necrosis, i.e, bursting of the cells with the release of cytoplasmic content along with the nucleus (Fig.37e), followed by nucleus disintegration (Fig.37f).

Comparitive evaluation of the cytotoxic activity reveales variation in the activity between leaf, pod and flower. This variation could be due to variability in the chemical composition of the ARF of all the tested plant parts. Chemical composition of ARF is given in previous chapter, which shows presence of two groups of alkaloid in leaf ARF and only one group in flower ARF. On the basis of the Allium test, it could be concluded that both groups of alkaloids play a role in the antiproliferative effect. Cytotoxic characteristic of Juliprosopine has been established in earlier studies (Batatinha et al.,1997) and hence toxicity of plant parts containing Juliprosopine as its main alkaloid (as in leaf) could be explained, however other alkaloids may also contribute synergistically towards total toxicity, especially Julifloridine which is present in highest concentration in pod and flower, as these plant parts are also cytotoxic even if they contain low concentration of Juliprosopine (as in pod) or are totally devoid of it (as in flower).

4.3.6. Antimitotic and cytogenotoxic activity of Compound 10

The new compound (compound 10) isolated from flower extract was also found to show antimitotic activity. Both the concentrations used in the experiment caused inhibition of mitotic activity depending on the time of incubation. Lower concentration (4 mg/ml) was found to be less effective in reducing cell division and caused significant decrease in mitotic index value only after 48h as compared to control. When the concentration was increased upto 8 mg, there was significant reduction of mitotic index values proportional to the time period, which finally decreased to 0 after 48h (Table 15).

Table 15. Mean Mitotic index values (in percent) of the cells treated with 4 mg and 8 mg of the compound 10 at different time period (values are expressed as mean±SD, n=6)

Time	4 mg	8 mg
0 h	94±5.3	94±5.3
6 h	92±6.5	70±4.4
12 h	91±3.6	10±1.2
24 h	88±5.9	2±1.4
48 h	61±3.3	0

Changes in chromosome and cellular morphology were observed with increasing time and concentration. At lower concentration (4 mg/ml), partial c-mitosis (Colchicine like mitosis), full c-mitosis, with partially functional spindles and completely normal mitotic

phases were seen in the various cells of the same root-tip between 6 to 48h time period (Fig.38). Partial c-mitosis with stickiness between chromosomes were observed at early stages (Fig.38a and 38b). A full c-mitosis with complete spindle inactivation and c-pairs in most of the cells was observed only after 12h of treatment with 4 mg/ml (Fig.38c) concentration and after 6h for 8 mg/ml concentration. After 12h incubation with 4 mg/ml concentration, typical X-shaped c-pairs (c-pairs were found to generally separate and diverge widely), characteristic of colchicine treatment (Witkus and Berger, 1944), were observed, along with all the intermediate shapes (Fig.38d).

According to Steinegger and Levana (1947), the elastic repulsion between the two chromatids is responsible for the peculiar X-shaped appearance of the c-pairs. When stickiness is acting in different regions of the chromosome body, the shape assumed by the c-pairs is the result of two forces, viz. elastic repulsion localized at the centromere and attraction between the chromatids at the sticky regions.

C-pairs, scattered over the cell and presenting a normal development to the division of the centromere and the production of tetraploid cells, are regular feature in the material treated for 12h with 4 mg/ml concentration of the compound (Fig.38e and 38f). After the 24h treatments this normal course of the c-mitosis was found only in a few cases. Generally new forces seem to enter into action during c-metaphase, causing distributed c-mitosis, the peculiarity of which is a partition of the c-pairs in two polar groups (Fig.38i), similar pseudo-anaphases with chromosomes lacking the customary polarization and having their arms extending in all directions (Fig.38g), were visible. In addition to the different distributions of c-pairs described above, interphases with c-pairs clumped in the centre of the cell (Fig.38k) were also observed, together with the intermediate transitions after 48h of treatment.

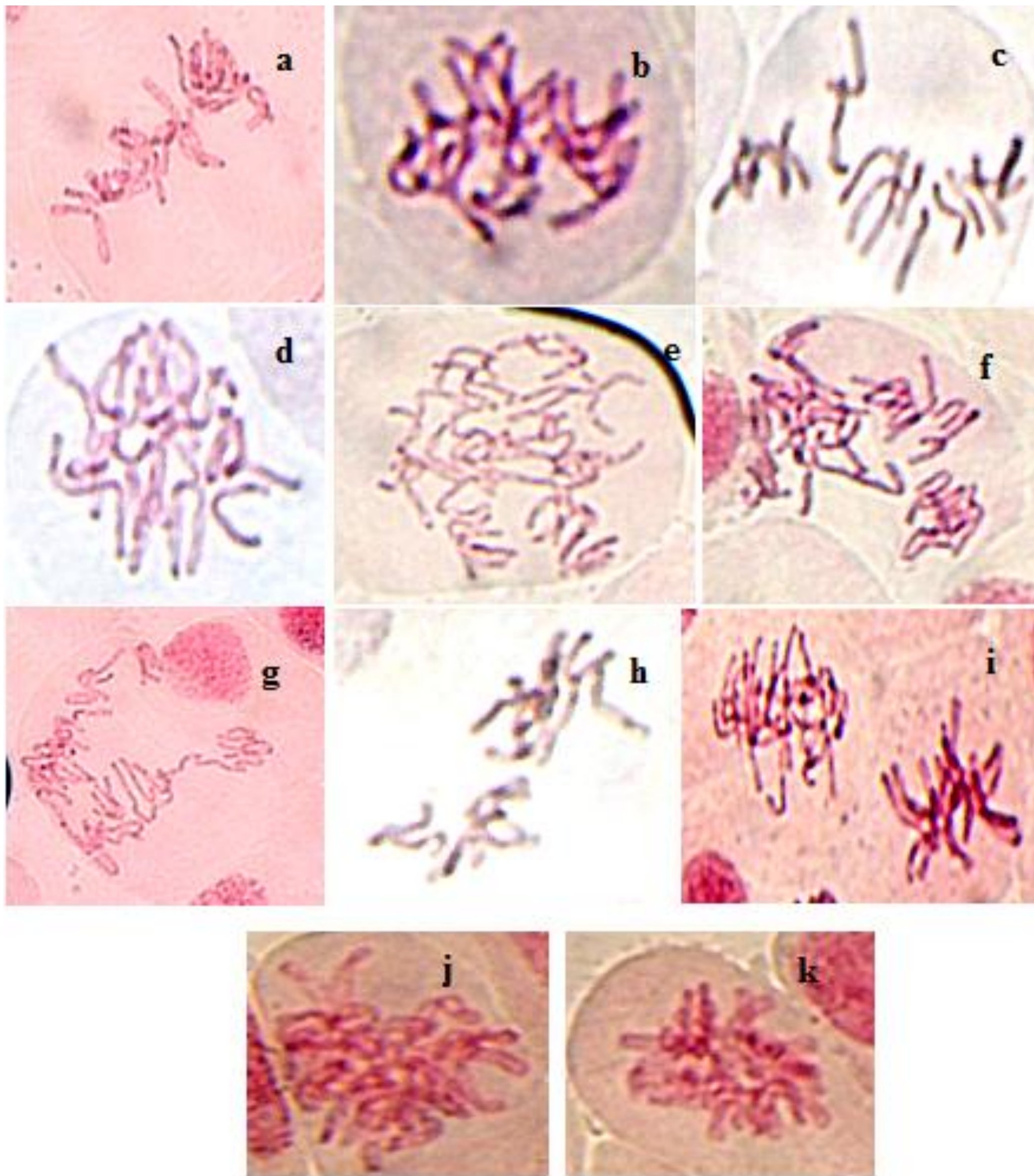


Fig 38. Chromosomal abnormalities in root cells of *Allium cepa*, seen after treatment of Compound 10 at concentration of 4 mg/ml within 48h - cells after 6h: a) Partial c-mitosis b) Partial c-mitosis sticking of the cell **cells after 12h:** c) C-mitosis d) Stickiness between c-mitosis X shaped **cells after 24h:** e) and f) Tetraploid g) Psuedoanaphase h) and i) Division in groups **cells after 48h:** j) and k) clumping in the center. 100x

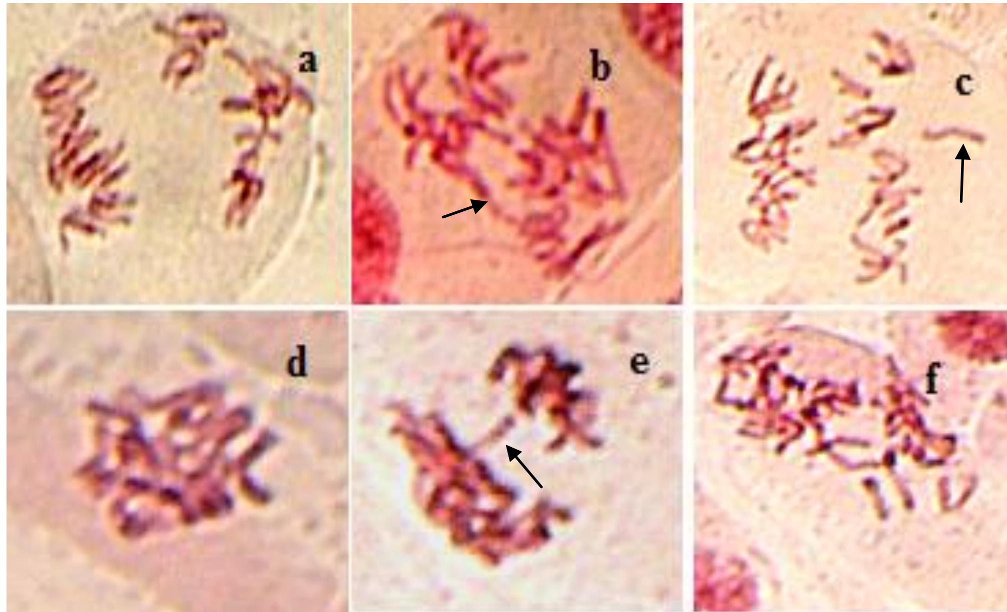


Fig 39. Chromosomal abnormalities in root cells of *Allium cepa*, seen after treatment of compound 10 at concentration of 8 mg/ml within 12h - cells after 6h: a)Multipolar anaphase b) chromosomal bridge c) lagging chromosome d) clumping of chromosome in the centre e) unequal division with chromosomal bridge **cells after 12h:** f) framentation of chromosomes. 100x

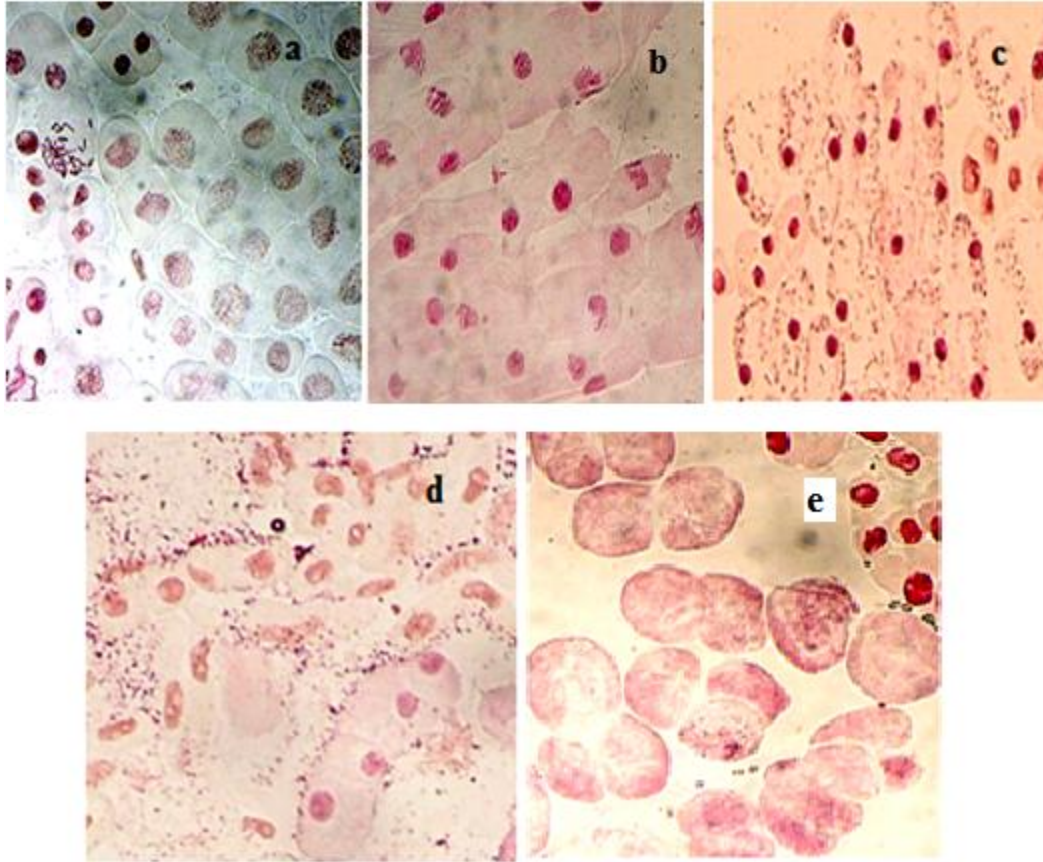


Fig 40. Cytotoxic effect of Compound 10 on root cells of *Allium cepa* seen after treatment with 8 mg/ml concentration within 48 h - a) cells after 6h showing chromosomal abberations b) and c) cells after 12h showing nuclear disintrigation and initiation of cellular autolysis d) cells after 24h showing incresed autolysis e) cells after 48 h showing formation of ghost cells. 40x

Higher concentration of the compound 10 (8 mg/ml) was found to have more damaging effect on the root tip cells. After 6h, c-mitosis, distributed c-mitosis, multipolar

anaphase (Fig.39a), psuedoanaphase, stickeness between chromosome, clumping of c-metaphase in the center of the cell (Fig.39d), misdivision during c-metaphase and c-anaphase (Fig.39e) and lagging chromosomes (Fig.39c) were observed. In some cells large bridges joining the two telophase nuclei appeared (Fig.39b). At increased time period of 12h, breakages in the chromosomes were visible (Fig.39f).

The induction of bridges between chromosomes could be attributed to chromosome breaks, stickiness and breakage and reunion of the broken ends. The stickiness prevented the separation of daughter-chromosomes and thus they remained connected by bridges (Kabarity et al., 1974; Badr et al., 1992). Large number of vagrant chromosomes and c-anaphases indicates that compound 10 acts as a potent spindle inhibitor. In the present study, a few cells with fragments of chromosomes were observed indicating the clastogenic effect of test compound as suggested for cypermethrin compound in *A. cepa* (Saxena et al. 2005).

After 24h of treatment the toxic symptom appeared in whole cell (Fig.40). Autolysis in the cytoplasm of the cell was visible, which continued with increasing time, causing complete dissolution of the cytoplasm, leaving behind only nucleus (Fig.40c and 40d). In some cells, nuclear material was found to be disintegrated with the cellular boundary still intact (Fig.40b). This disintegration of nuclear material continued with the increase in time. Within 48h nuclear material was completely lost with the cellular boundary still maintained forming a ghost cell (Fig.40e).

For many years, laboratories from all over the world have been working on finding effective remedy for tumors, up to now the most severe disease of our civilization (Kuras´, 2006). Among many therapeutic plants that are objects of interest, the compounds from *P. juliflora* seemed particularly important, as shown in our results. According to the obtained results, one can conclude that both alkaloids and compound 10 play a role in the antiproliferative and cytotoxic effect on *A. cepa* cells and these results could be useful in correlating with the mammalian system as it is reported that Allium test shows good correlation with mammalian test systems (Fiskesj¨o, 1988; Grant 1994) and extracts of this

plant have also been reported to have cytotoxic activity on human cell lines (Batatinha, 1997).

Other major interest in *P. juliflora* is to use the compounds present in its plant parts as natural herbicides. Evidence of the role of allelopathy in weed control has been cited in many publications (Chou and Kuo, 1986; Chung and Miller, 1995; Laosinwattana et al., 2009). Our results show phytotoxic alkaloids in different parts of the plant, hence the plant could be used as potential herbicide. Our Allium studies revealed that ARF alters mitotic activity and induces chromosomal aberrations in root tips. The Allium test for cytogenetic studies allows for a more detailed insight into the modes of alellopathic action. Ultimately, these results may be useful for development of *P. juliflora* extracts to be used as a natural herbicide.

Chapter 5

Acute and subacute, oral and intraperitoneal toxicity of Prosopis juliflora in mice

5.1. Introduction

Herbal plants are generally perceived as being natural, healthful and free from side effects (Akerele, 1993). However, plants contain many active ingredients that can provoke adverse reactions when used improperly (Sofowora, 1982). Many medicinal herbs are therapeutic at one dose and toxic at another, therefore it is required to know the toxicity level of a herbal extract (Calixto, 2000). Unfortunately, the subtle side effects of many traditional herbs are not generally known (Calapai, 2008)

Prosopis juliflora is known to have medicinal properties and is used in folk medicine. Leaf extract is reported to contain large number of pharmacologically active metaboloties. The extract is used in various herbal remedies, however despite of its use in the treatment of ailments, toxicities associated with the leaf extract is also reported (Dollahite and Anthony, 1957; Baca et al., 1967; Figueiredo et al., 1995). Most of the earlier studies done to evaluate toxicity potential of its extract were focused on *in vitro* systems. Hence it is important to perform a more detailed invivo toxicity studies of the plant extract as a measure of safety for the continued use of them.

Cells and tissues do not exist independently in vivo but communicate with and are interdependent on neighboring tissues. Therefore it is essential to test the medicinal herb in vivo in order to get a whole picture of the physiological microenvironment. Several animal based models have been developed, which help in screening toxicity of a substance. Rodents are said to be the best model as the results obtained after testing on them can be correlated with the human beings (Mounnissamy et al., 2010).

In screening drugs, determination of LD_{50} dose (the dose which has proved to be lethal to 50% of the tested group of animals) is usually an initial step in the assessment and evaluation of the toxic characteristics of a substance and the information obtained from these studies is useful in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity, and occasionally, revealing delayed toxicity (Akhila et al., 2007). Acute toxicity studies may also aid in the selection of starting doses for Phase 1 human studies, and provide information relevant to acute overdosing in

humans (Turner, 1965). Subacute, subchronic and chronic toxicity tests are performed to further identify and characterize the specific organs affected by the tested compound after repeated administration.

To accomplish the objective, we carried out an acute (LD50) and sub-acute toxicity (14 day) study of *P. juliflora*. Since most of the reported pharmacologically active metabolites are present in leaf ARF, hence it was selected for the in vivo study. The primary concern was to determine how safe the extract of *P. juliflora* might be after oral and interaperitoneal administration to mice.

5.2. Material and methods

5.2.1. Animals

Swiss albino mice aged 6–7 weeks, weighing about 30–35 g, were obtained from the Experimental animal facility of Birla Institute of Technology and Science, Pilani, India. The experimental procedures were approved by the Animal Experimental Ethics Committee of our institute [Ethical number: IAEC/RES/12/02)] before commencing the study. The animals were fed standard laboratory diet, given water *ad libitum* and maintained under laboratory conditions of temperature 22 ± 1°C, relative humidity 14 ± 1% and 12 h light and 12 h dark cycle. The mice were acclimatized to the laboratory conditions for at least five days prior to commencement of the experiments. The animals were allowed free access to both food and drinking water throughout the experimental periods.

5.2.2. Sample preparation

A stock solution of 500 mg/ml solution of ARF was prepared in phosphate buffer saline (pH 7.0 ± 0.2) and was sterilized by 0.45 µ Millipore filters.

5.2.3. Acute toxicity (LD_{50})

The acute toxicity study for calculation of LD_{50} value was carried out using the method of Tainter and Miller (Randhawa, 2009) and degree of toxicity was evaluated by the method of Hodge and Sterner, 1949.

An approximate toxicity dose was initially determined in a pilot study by a so called 'staircase method' using a small number of animals (2 each dose) and increasing doses of extracts (Loomis and Hayes, 1996). Six doses were then chosen for the determination of oral and intraperitoneal (i.p) acute toxicity in mice.

A total of 70 mice were randomly allotted to one control and six treatment groups, having 10 mice in each group. The extract in each case was administered orally in doses: 250, 500, 750, 1000, 1500 and 2000 mg/kg body weight and intraperitonially in doses: 20, 30, 40, 50, 100 and 500 mg/kg body weight. Control group was injected phosphate buffer saline. Following administration of the extract, the animals were observed for 24h continuously for appearance of signs of toxicity. The number of deaths that occurred was recorded. The toxicological effect was assessed based on mortality, which was expressed as an LD_{50} value. The LD_{50} value was calculated as 50% mortality during the 24h period.

5.2.4. Sub Acute toxicity

The animals were randomly divided into five groups, one control and four treatment groups of six mice each. A sub-lethal doses of 50, 100, 200 and 500 mg/kg body weight for oral and 1, 5, 10 and 20 mg/kg body weight for i.p was used and was administered once every 24 hours for 14 consecutive days. The animals were kept under close observation throughout the experimental periods for an appearance of sings of toxicity. Mortality and body weight changes were recorded. After the administration of last dose all treated and control mice groups were fasted overnight. Urine samples were collected for urinalysis. Blood was taken via cardiac puncture for hematological and biochemical studies after anesthetizing the animal with diethyl ether. Serum was separated by centrifugation for 10 minutes at 3000 rpm and stored at -20°C until serum chemistry analysis. All animals were sacrificed by cervical decapitation and examined for any abnormalities in the organ.

Body weight

Body weights were measured at the initiation of treatment and on the day of sacrifice.

Observation of Mice Behaviour

During the whole experimental period the animals were observed for behavioural display, which include excitation, depression, reflexes, muscular weakness, salivation, diarrhea, food and water in take and any local injury.

Urinalysis

During the last week of treatment, urinalysis was conducted with fresh urine to determine specific gravity, sugar, ketone, blood, pH, total protein count (TPC), albumin and urobilinogen contents using a Multistix.

Hematological estimation

Haematological studies were carried out on the day of collection. Total count of RBCs and WBCs, differential count of WBCs , platelet count were calculated with the help of heamocytometer as per the method described by Schalm et al. 1975 by using 0.015% Toludine blue as a diluting fluid, in 1:200 ratio in case of RBC and in ratio 1:20 in case of WBC counting. The results were expressed in millions/mm^3

Biochemical estimation

Biochemical measurements of the serum were carried out on following parameters: Serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvate transaminase (SGPT), cholesterol, creatinine, tiglycerides, glucose and urea. The analysis was done using biochemical kits (Spinreact Kits and Infinite Liquid STAT) and an automated biochemial autoanalyzer. All the chemicals used in the analysis is presented in Table 16.

Table 16. Reagents used in biochemical estimation

SGOT	R1	TRIS pH 7.8	80 mmol/L
	Buffer	Lactate dehydrogenase(LDH)	800U/L
		Malate dehydrogenase(MDH)	600U/L
		L-Aspartate	200mmol/L
	R2		
	Substrate	NADH	0.18 mmol/L
		α -Ketoglutarate	12mmol/L
SGPT	R1	TRIS pH 7.8	100 mmol/L
	Buffer	Lactate dehydrogenase(LDH)	1200U/L
		L-Alanine	500mmol/L
	R2	NADH	0.18 mmol/L
	Substrate	α -Ketoglutarate	15mmol/L
UREA	R1	TRIS pH 7.8	80 mmol/L
	Buffer	α Ketoglutarate	6 mmol/L
	R2	Urease	3750 U/L
		Glutamate dehydrogenase(GLDH)	6000 U/L
	Enzymes	NADH	
			0.32 mmol/L
CREATININE	R1	Picric acid	17.5 mmol/L
	R2	Sodium hydroxide	0.29 mol/L
CHOLESTEROL		Phosphate buffer, pH	90 mmol/L
		Phenol	26 mmol/L
	R	Cholesterol esterase(CHE)	1000U/L
		Cholesterol oxidase(CHOD)	300U/L
		Peroxidase(POD)	650 U/L
		4-Aminophenazone(4-AP)	0.4 mmol/L
TRIGLYCERIDE		Phosphate buffer, pH 7.5	50 mmol/L
		p-Chlorophenol	2 mmol/L
	R	Lipoprotein lipase (LPL)	150000 U/L
		Glycerol kinase(GK)	500 U/L
		Glycerol-3-oxidase(G(4-AP_	3500 U/L
		ATP	0.1 mmol/L
			0.1 mmol/L
GLUCOSE	R	Phosphate buffer, pH 7.0	170mmol/L
		Glucose oxidase	15000 IU/L
		Peroxidase	1500 IU/L
		4-aminoantipyrine	0.28 mmol/L
		Phenol	16mmol/L

The procedure applied for biochemical estimation, was followed as per the instructions of the manufacturer of the kits.

Postmortem examination and collection of tissue samples

Mice which died during the experiment and the mice which were sacrificed on last day of experiment in the confined disinfected laboratory were subjected to post mortem examination for gross and histopathological alterations. Post mortem necropsy findings were made by systemic approach (i.e. gross changes in organ size, shape and any visible lesions). Detailed post mortem lesions from all animals were recorded. For histopathological examination, selected organs were immediately fixed in 10% buffered formalin and processed for histology with H&E staining.

Organ weights

At necropsy, all organs were carefully examined macroscopically and the brain, lungs, heart, liver, kidneys, testis (males) and ovaries (females), were weighed relative to total body weight.

Histopathological Investigation

Preparation of tissue for histology**:** The method described by Carleton (1967) was adopted for tissue preparations.

*Tissue processing***:** The tissue obtained after sacrificing the treated and control mice were trimmed to size and fixed in 10% formal-saline (formalin 100 ml, sodium chloride 8.5 g, water 900 ml). Using the tissue processor, the tissues were dehydrated using graded concentrations of ethanol as described below:

- 70% alcohol was used to dehydrate the tissue for 1h twice
- 90% alcohol was used to dehydrate the tissue for 1h twice
- Absolute alcohol was used to dehydrate the tissue for 1h twice

Tissue were cleared in xylene by transferring them into equal volumes of alcohol (absolute) and xylene for 1h, in order to avoid tissue distortion due to sharp transition from alcohol to xylene. The tissues were then passed through two changes of xylene for 1h each.

Embedding: The tissues were embedded in paraffin wax at 55ºC for 2h each. This infiltration was carried out in two changes of paraffin wax for 2h each. The tissues were later blocked out using L-shaped metal molder and subsequently mounted on a wooden block and trimmed to size for sectioning.

Sectioning: Using rotary microtome, the tissue blocks were cut into ribbons of 6-8 µm thickness each. The cut sections were picked with a brush onto a slide. The section on the slide was floated in 20% alcohol and then in warm water bath to allow for proper straightening. The sections were mounted on albumenized slides. They were stained with haematoxyline and eosin stain (Luna, 1968). The H & E stained slides were observed under microscope and lesions were recorded.

5.2.5. Statistical analysis

Numerical data were presented as means ± standard deviation. The data was analyzed by a one-way analysis of variance (ANOVA) and followed by the Least Significant Difference test. $P < 0.05$ was considered a significant difference (Mcleod, 1970).

5.3. Results and Discussion

5.3.1. LD_{50} values

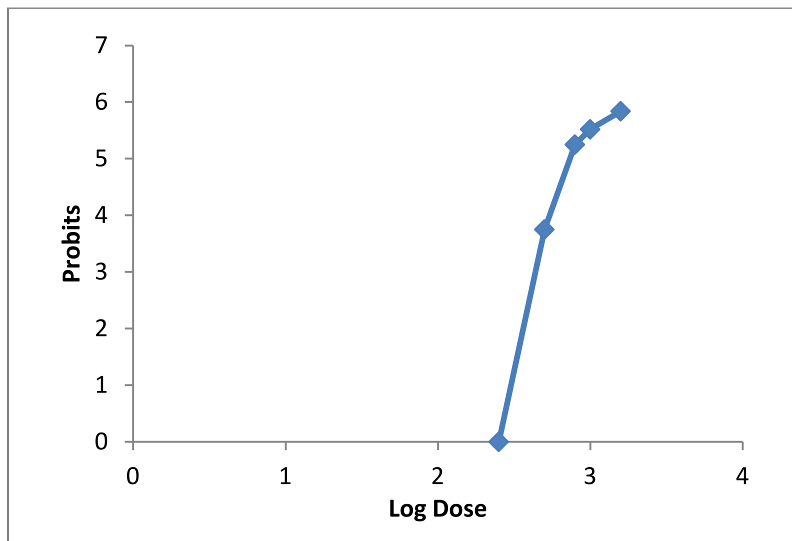
The toxicity of plant extract was examined by applying oral as well as interaperitoneal routes.There was no death of mice recorded, following oral administration of the aqueous extract up to the dose of 250 mg/kg. High doses of 750, 1000, 1500 and 2000mg/kg caused 60, 70, 80 and 90% mortality respectively. Intraperitoneal administration of the extract at a dose of 20 mg/kg caused no death, while the higher doses of 30, 40, 50,100, 500 mg/kg caused 20, 30, 40, 60 and 90% of total mice death, respectively (Table 17). Probit analysis

showed 630.95 mg/kg as LD_{50} for oral route and 79.43 mg/kg for i.p route at probit value of 5 (Fig. 41).

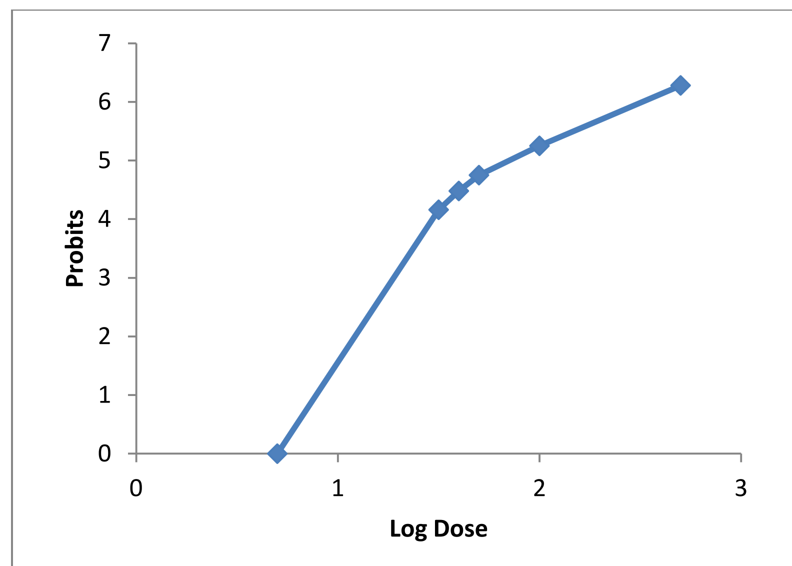
Results show that the extract is non toxic when administered via oral route and moderately toxic if taken via interaperitoneal route. LD_{50} values obtained for oral and interaperitoneal route shows large variation, which may be due to difference in the rate of absorption. The rate of absorption during oral administration is reported to dependent on many factors, such as the chemical nature of the drug (acidic or basic), degree of solubility, degree of ionization and the rate of gastric emptying (Waynforth, 1980), which might reduce the toxicity of the extract.

Table 17. **Determination of LD_{50} values via oral and interaperitoneal administration of the extract in mice by Miller and Tainter method**

Group	Oral Dose (mg/kg)	Log Dose	No.of Deaths	%Deaths	Probits	i.p Dose (mg/kg)	Log Dose	No.of Deaths	%Deaths	Probits
1.	250	2.4	0	0	-	20	0.7	0	0	-
2.	500	2.7	1	10	3.75	30	1.5	2	20	4.16
3.	750	2.9	6	60	5.25	40	1.6	3	30	4.48
4.	1000	3	7	70	5.52	50	1.7	4	40	4.75
5.	1500	3.2	8	80	5.84	100	2	6	60	5.25
6.	2000	3.3	9	90	6.28	500	2.7	9	90	6.28



a)



b)

Fig 41. Probit analysis for calculation of LD_{50} value - a) oral and b) interaperitoneal route

5.3.2. Subacute toxicity values

Physical parameters

Oral and interaperitoneal administration of plant extract in low doses (200 and 10 mg/kg body weight respectively) did not produce any significant changes in the behaviours as compared to the control group, indicating non toxic nature of the extracts upto these doses. Abnormal mice behavior was observed only with the highest concentrations of extract administered via both the routes (500 mg/kg (oral), 20 mg/kg (i.p)) which include hyperactivity, aggressiveness, loss of appettite, abnormal sitting posture, dehydration abdominal contraction and abnormal sexual behavior. Animals treated with high concentration via i.p route showed reduction in fur and skin appeared cyanotic. Behavioral changes were more pronounced at higher concentrations of i.p route as compared to oral route.

Table 18. Effect of oral and interaperitoneal administration of the extract for 14 days on body weight of mice

Group Body weight (g) Means ± SE (n=6)

Dose (mg/kg) Oral	Before treatment	After treatment	Dose (mg/kg) i.p	Before treatment	After treatment
0	35.35±1.32	36.35±0.67	**0**	36.05±1.33	35.98±0.87
50	34.67±1.36	35.22±0.36	**1**	35.73±1.51	36.42±1.43
100	34.73±1.53	35.67±1.33	**5**	37.70±1.36	36.73±2.15
200	36.52±0.35	36.26±2.33	**10**	33.50±1.87	30.59±2.35*
500	36.40±1.63	35.19±2.33	**20**	33.35±1.67	29.10±2.46*

Mice in the experimental groups were given extract daily for 14 days, whereas those in the control group received an equal volume of pure water. SD=standard deviation ;* p values were <0.05 (significant) for each the experimental groups compared to the control group.

Reduction in body weight are used as an indicator of adverse effect of drugs and chemicals (Teo et al., 2002), however in our experiment, oral treatment of the extract at low doses (50 and 100 mg/kg) caused a slight increase in body weight, and the higher doses produced no adverse effect on the body weight of the mice, indicating non toxic nature of the extract via oral route. Mice treated with high doses of extract (10 and 20 mg/kg) via i.p route showed slight reduction in body weight (8.9% and 12.7% respectively) as compared to control (Table 18).

Hematology and serum biochemistry

The hematological profiles of the experimental and control group mice are summarized in Table 19. No significant change in the values of RBCs, WBCs and % differential WBCs count of experimental animals were observed in oral treatment and up to 10 mg/kg in case of interaperitoneal route when compared to that of control group. A slight decrease in TRBC count was observed in mice treated with highest dose (20 mg/kg) via i.p route. This result is in consistent with the results of Kandasamy et al., 1989, who have reported *in vitro* hemolytic activity of high concentrations of *P. juliflora* leaf alkaloids on rat and human erythrocyte. Lowering of TRBC count in the treatment groups, may be responsible for the cyanotic colour of the mice, as body cell would be deprived of oxygen due to loss in RBC's.

Biochemical components in serum were determined after treatment and compared to that of control mice. Table 20 show values for various serum biochemical parameters. Sugar, urea, creatinine, cholesterol and triglyceride levels were within the range as compared with the control, whereas only transaminases (SGOT and SGPT) were raised at higher doses of extract, 500 mg/kg for oral and 10 and 20 mg/kg for i.p administration.

In humans, the majority of substances administered are eliminated by a combination of hepatic metabolism and renal excretion. The liver is prone to xenobiotic-induced injury because of its central role in xenobiotic metabolism, its portal location within the circulation, and its anatomic and physiologic structure (Manjrekar et al., 2008). The first sign of damage to the liver is an increase in liver enzyme, SGOT and SGPT levels in the

Table 19. Hematological parameters in mice after 14 days of oral and interaperitoneal treatment of leaf extract (values are expressed as mean±SD, n=6)

Dose (mg/kg)	TLC (x 103cmm)	TRBC (x106/cmm)	Platelet count (x 105/cmm)	WBC differential counts (%)			
				Lymphocyte	Monocyte	Neutrophil	Eosinophil
Oral							
0	5.79±0.51	6.90±0.49	2.90±0.37	62.34±2.56	1.04±0.82	34.35±2.80	2.01±0.75
50	6.12±0.44	6.51±0.89	2.53±0.35	60.54±1.45	1.02±0.75	36.22±2.94	2.40±0.89
100	5.82 ±0.42	6.32±1.63	2.43±0.94	59.30±2.51	1.21±0.52	37.64±2.32	2.11±0.98
200	5.62±1.67	5.67±1.43	2.61±0.75	58.55±2.80	1.34±0.75	36.54±3.08	2.04±0.82
500	5.27±2.14	5.23±2.36	1.97±2.33	50.32±1.75	1.02±0.88	46.32±1.51	2.36±0.75
i.p							
0	6.10±1.17	7.00±0.43	2.42±1.02	62.67±2.43	1.50±0.86	33.45±2.37	2.38±0.52
1	6.24±0.43	6.94±0.28	2.01±0.84	62.50±2.88	1.23±0.63	34.62±2.43	2.10±0.67
5	6.05±0.36	5.87±0.15	2.35±0.79	65.79±3.08	1.48±0.94	31.67±2.71	1.90±0.43
10	5.79±2.40	5.85±1.51	2.20±0.61	59.80±2.07	1.20±0.48	37.01±2.37	2.25±0.89
20	5.10±2.33	4.46±0.84*	1.52±1.68	53.26±1.67	1.56±0.42	45.54±2.33	2.05±0.75

Mice in the experimental groups were given extract daily for 14 days, whereas those in the control group received an equal volume of pure water. SD=standard deviation ;* p values were <0.05 (significant) for each the experimental groups compared to the control group.

Table 20. Serum biochemical analysis of mice treated with leaf extracts via oral and interaperitoneal route for 14 days (values are expressed as mean±SD, n=6)

Dose (mg/kg)	Sugar (mg/dl)	Urea (mg/dl	Creatinine (mg/dl)	SGOT (IU/l)	SGPT (IU/l)	Cholesterol (mg/dl)	Triglyceride (mg/dl)
Oral							
0	68.33±4.88	24.23±2.10	0.81±0.05	35.55±6.90	28.14±6.07	184.50±6.87	165.25±7.44
50	64.83±3.43	17.33±2.90	0.75±0.03	29.73±5.76	29.52±6.83	184.03±3.45	160.88±9.06
100	71.24±5.59	25.80±2.73	0.78±0.15	34.21±8.07	31.63±5.50	182.57±7.33	165.36±10.70
200	60.56±7.88	26.14±2.32	0.73±0.04	40.62±5.49	40.77±5.47	189.89±5.89	161.47±5.98
500	65.03±7.92	19.66±2.88	0.79±0.02	110.33±7.92*	101.68±5.73*	184.08±3.05	168.66±5.67
i.p							
0	70.86±6.01	27.56±1.87	0.82±0.03	37.30±7.44	30.10±6.90	188.33±0.90	165.95±8.55
1	66.09±3.69	28.44±1.75	0.80±0.04	32.06±5.89	35.52±8.80	183.24±5.23	167.05±1.45
5	71.12±7.84	25.20±2.00	0.79±0.02	41.87±6.83	39.20±10.39	185.36±6.03	169.40±5.48
10	69.43±4.00	26.07±3.66	0.77±0.11	51.71±10.12*	58.45±8.07*	190.78±4.88	171.32±2.20
20	59.97±5.49	13.58±2.67	0.79±0.05	135.04±16.29*	129.56±11.44*	187.12±1.40	164.35±1.08

Mice in the experimental groups were given extract daily for 14 days, whereas those in the control group received an equal volume of pure water. SD=standard deviation ;* p values were <0.05 (significant) for each the experimental groups compared to the control group

blood (Manjrekar et al., 2008) and the increased levels of these enzymes, shows some adverse effect of high doses of the extract on the liver.

Urinalysis

The urinalysis was negative for glucose, ketonic bodies, red blood cells, urobilinogen in the control and treatment groups, however albumin and TPC was slightly more in mice treated with highest dose of i.p route (Table 21). pH of the urine was 8-8.5 in all the treatment and control groups.

Kidney is susceptible to toxicants as high volume of blood flows through it and it filters toxins which can concentrate in the kidney tubules (Atici et al., 2005). Urine protein testing helps to evaluate and monitor kidney function, and to help detect and diagnose early kidney damage and disease. Kidneys filter the urine from the blood and retain large molecular weight compounds, such as protein in it. However, when kidney gets damaged proteins are passed on into the urine, which can be detected by simple tests. In the present experiment, a positive test for albumin and total protein indicates the adverse effect of higher doses of the extract on kidney functioning.

Gross findings

Necroscopic examination showed that gastrointestinal track and stomach were empty and there was no fat deposition in entire body which could be due to in appetence and hence the probable reason of body weight loss. Testicles and prostrate gland were slightly enlarged in the mice treated with highest dose of the extract via i.p route.

Organ weight

Selected relative organ weight data are summarized in Table 22. No significant change was observed in the organ weight of mice treated with the extract as compared with the control.

Table 21. Urinalysis of mice after 14 days treatment of leaf extract via oral and interaperitoneal route

Dose(mg/kg)	pH	Sugar	Ketone	Specific gravity	Blood	Albumin	TPC	Urobilinogen
Oral								
0	8.5	-	-	1.02	-	+	++	-
50	8.5	-	-	1.02	-	+	++	-
100	8.0	-	-	1.02	-	+	++	-
200	8.5	-	-	1.02	-	+	++	-
500	8.0	-	-	1.02	-	++	++	-
i.p								
0	8.5	-	-	1.02	-	+	++	
1	8.5	-	-	1.02	-	+	++	
5	8.0	-	-	1.02	-	+	++	
10	8.5	-	-	1.02	-	+	++	
20	8.5	-	-	1,02	-	++	+++	

Mice in the experimental groups were given extract for 14 days , whereas those in the control group received an equal volume of pure water.

Table 22. Effects of oral and interaperitoneal administration of extract for 14 days on mice organ weights (values are expressed as mean±SD, n=6)

Group Organ weight (g / 100 g of body weight)

Dose(mg/kg)	Liver	Lungs	Heart	Spleen	Kidney	Brain
Oral						
0	1.268±0.02	0.184±0.05	0.143±0.01	0.109±0.02	0.287±0.03	0.370±0.03
50	1.128±0.12	0.178±0.07	0.140±0.02	0.101±0.04	0.298±0.03	0.374±0.13
100	1.222±0.16	0.174±0.08	0.144±0.04	0.107±0.00	0.285±0.02	0.373±0.10
200	1.124±0.14	0.175±0.10	0.140±0.02	0.101±0.06	0.292±0.04	0.375±0.11
500	1.225±0.09	0.180±0.09	0.141±0.06	0.103±0.04	0.288±0.05	0.369±0.08
i.p						
0	1.231±0.05	0.181±0.05	0.142±0.02	0.108±0.02	0.289±0.04	0.371±0.07
1	1.128±0.10	0.179±0.12	0.143±0.05	0.111±0.03	0.293±0.07	0.374±0.12
5	1.233±0.16	0.176±0.06	0.144±0.09	0.100±0.05	0.290±0.07	0.368±0.06
10	1.129±0.06	0.177±0.09	0.145±0.10	0.104±0.04	0.296±0.04	0.376±0.16
20	1.226±0.18	0.178±0.04	0.142±0.06	0.106±0.07	0.289±0.05	0.373±0.18

Mice in the experimental groups were given extract for 14 days , whereas those in the control group received an equal volume of pure water.

p values were >0.05 (non-significant) for each the experimental groups compared to the control group

Histopathology

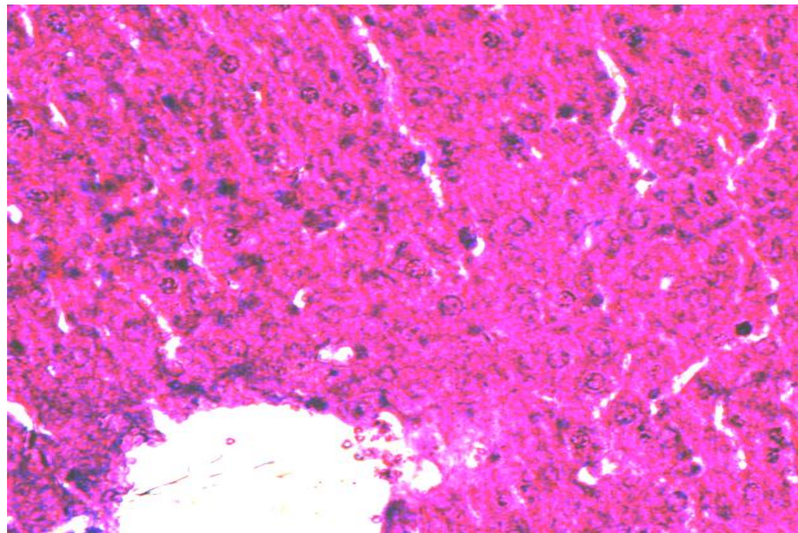
Histopathological investigations of the organs were done based on changes in morphological, behavioral and biochemical parameters. According to these parameters, three organs were selected, liver, kidney and testis for the present study.

The liver of control mice, as examined by light microscope revealed the same normal hepatic structure, essentially formed of hepatic lobules. Each lobule is made up of radiating plates, strands of cells forming a network around a central vein (Fig.42a). The liver strands are altering with narrow sinusoids. These sinusoids have irregular boundaries composed of only a single layer of fenestrated endothelial cells and large irregularly phagocytic cells, which are known as Kupffer cells. Outside the hepatic lobule at certain angles, lie the portal areas of connective tissue each including a hepatic portal vein, a branch of hepatic artery and a bile ductile. Liver sections of mice treated with highest dose via both the routes revealed priliminary damaging effects on liver cells. Initial stages of ballooning degeneration were seen in hepatocytes with granular cytoplasm. Ballooned cells are typically two to three times the size of adjacent hepatocytes and are characterized by a whispy cleared cytoplasm on H&E stained sections. The swollen hepatocytes compressed the hepatic sinusoidal walls, leading to reduced blood flow in hepatic sinusoids and the infiltration in portal vein was observed. Hepatic lobular parenchymal architecture was well maintained (Fig.42b and 42c).

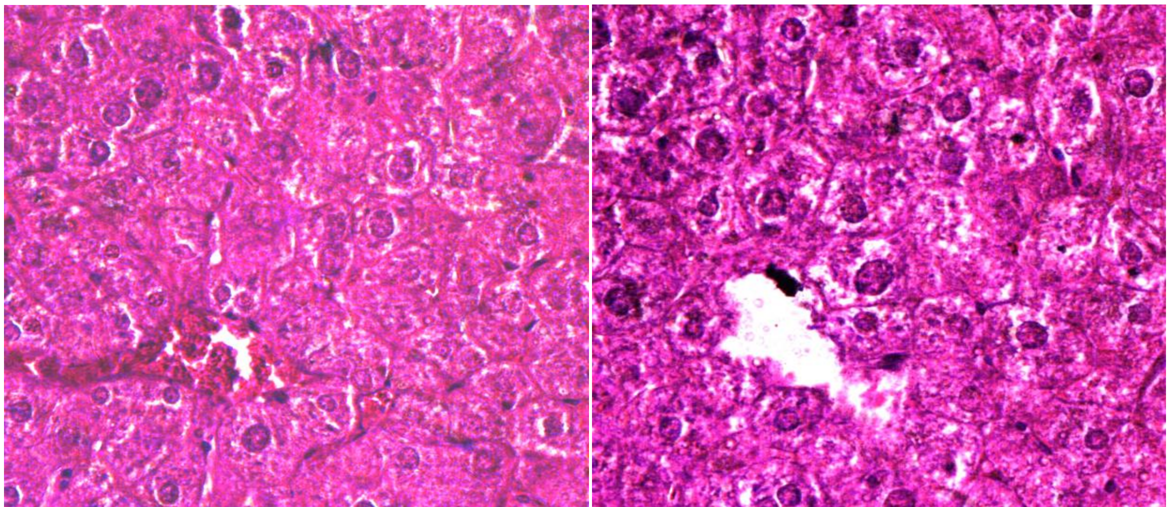
The photomicrograph of the control kidney section show normal histological features. The section indicated a detailed cortical parenchyma and the renal corpuscles appeared as dense rounded structures (Fig.43a). The kidney sections from the treated groups revealed some varying degree of distortion and disruption in microanatomy of the renal cortex, when compared to the control group in 20 mg/kg treated mice via interaperitoneal route, however as compared with liver, kidney damage was less pronounced. Focal hydropic changes were observed at some places. Tubular linning showed enlargement of the cells with eosinophilic granular cytoplasm. No glomerular damage was found in any of the groups (Fig.43b).

The control sections of the testis showed normal histological features with the cross section of the convoluted seminiferous tubules showing stratified epithelium which consists of two distinct populations of cells; the spermatogenic cells and the sertoli cells. The leydig cells within the supporting tissues in the interstitial spaces between the tubules were all visible. (Fig.44a). The treatment sections of the testis showed some histological changes at higher i.p dose, that were at variance with those obtained in the control. There was breakage in the seminiferous tubules lining causing distortion in their arrangement. Focal sloughing of sertoli cells lined within the lumen of seminiferous tubules was observed. Mass degeneration of interstitium showing oedamatus changes were also evident (Fig.44b and 44c).

The results suggests that *P. juliflora* extract is not toxic when orally administered to mice at concentrations below 200 mg/kg and intraperitoneally below 10 mg/kg. Animal study of *P.juliflora* extract was done as it is used in folk medicine and detail has been mentioned in general introduction (chapter 1), therefore it is important to find out safety dose of herbal extract . This toxicity evaluation is also important because the present study shows strong antibacterial , antimitotic and cytotoxic activity of the extract and the future study of my work includes testing these therapeutic ability of the extracts on animals and for this we need to have the safety data of the extract.

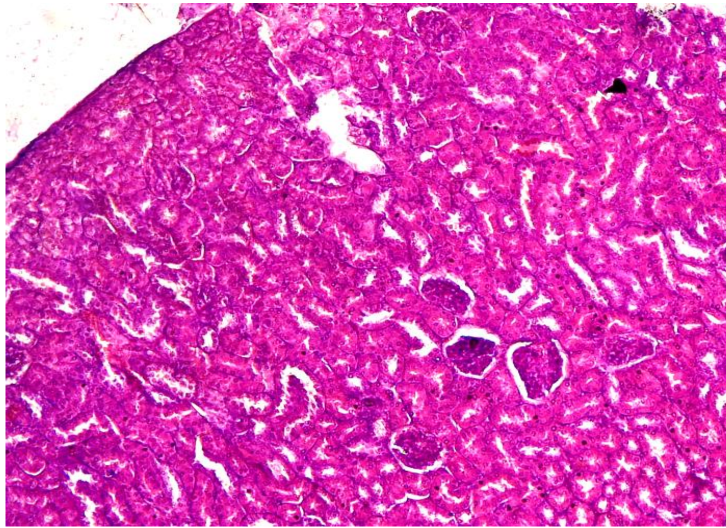


a)

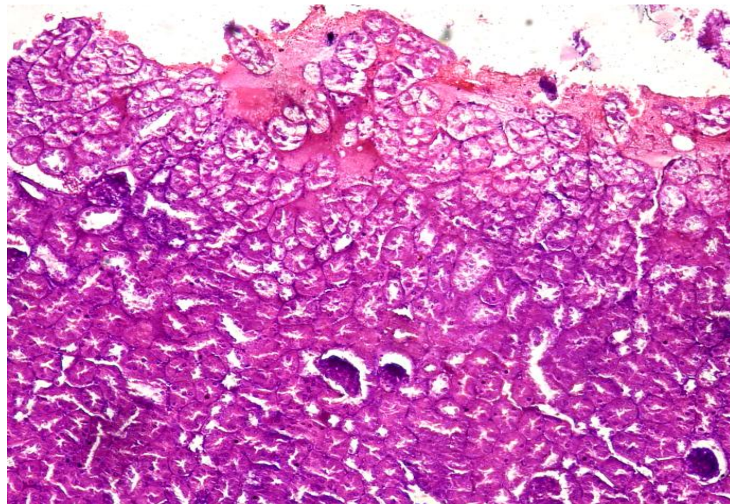


b) **c)**

Fig 42. Liver histology of mice - a) control b) cells treated with 500mg/kg of leaf extract via oral route showing ballooning degeneration of cells, compressed hepatic sinusoidal walls and infiltration in portal vein c) cells treated with 20mg/kg of leaf extract via interaperitoneal route showing more pronounced ballooning degeneration of cells and compressed hepatic sinusoidal walls. 40x

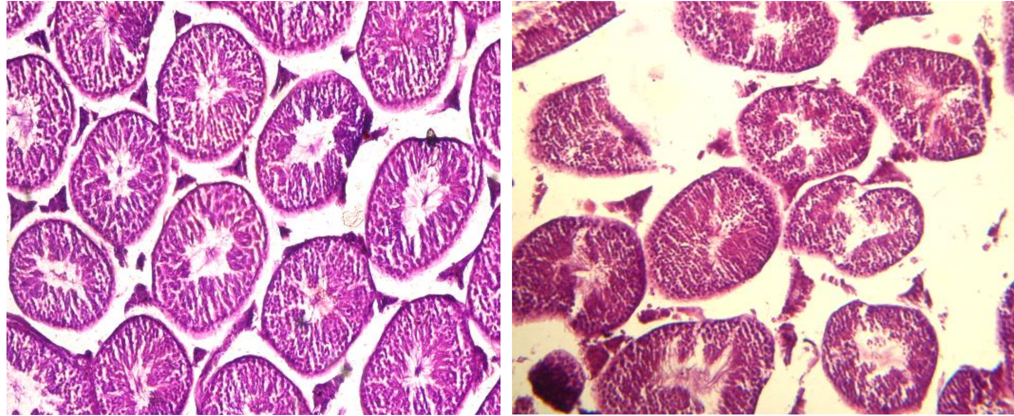


a)

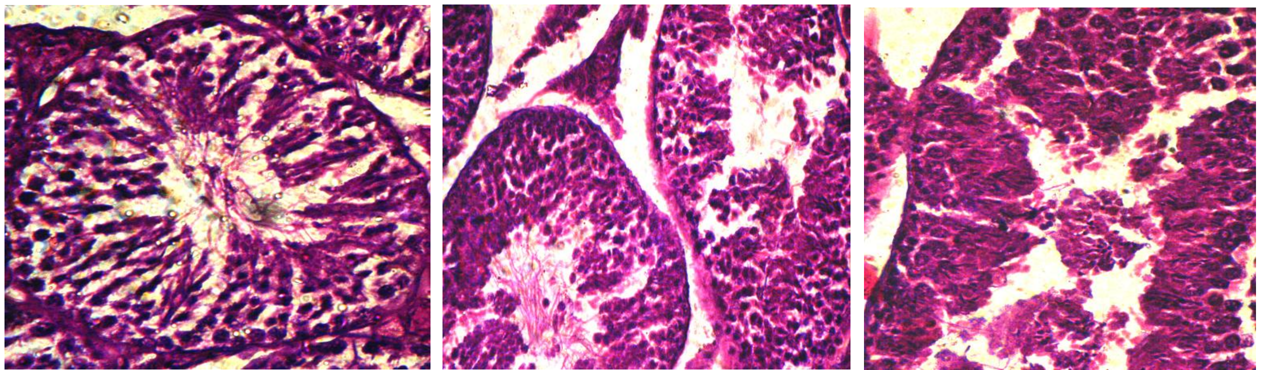


b)

Fig 43. **Kidney histology of mice -** a) control b) cells treated with 20mg/kg of leaf extract via interaperitoneal route showing hydropic changes in tubules. 40x



a) b)



c) d) e)

Fig 44. Testis histology of mice - a) control b) cells treated with 20mg/kg of leaf extract via interaperitoneal route showing distortion of semineferous tubules. 40x c) control semineferous in 100x d) and e) treated semineferous tubules in 100x

Summary

The present research work identifies piperidine alkaloids and other important metabolites present in different parts of *P.juliflora* and evaluate their biological activity. Important results obtained include:

- Extraction of plant metabolites with the help of organic and inorganic solvents resulted in isolation of various phytochemicals in different parts of the plant. Phytochemical analysis revealed presence of tannins, phenolics, flavonoids, alkaloids, terpenes and steroids in most parts of *P. juliflora.* Concentration of metabolite was highest in leaf and pod, followed by flower, root and stem. Alkaloids were present in large concentration in leaf, pod and flower.

- Alkaloids were extracted from alkaloid rich plant parts by acid-base extraction method and were obtained as alkaloid rich fraction (ARF). ARF quantification showed presence of 0.77g alkaloid in 50g of pod, 0.52g in 50g of leaf and 0.43g in 50g of flower. They were further separated into different fractions by TLC and quantified by HPTLC. Three fractions of leaf L1, L2 and L3, three fractions of pod, P1, P2 and P3 and one fraction of flower was obtained.

- DART-MS was performed for profiling piperidine alkaloids expressed in various parts of *P.juliflora*. Abundance of these alkaloids was observed in leaf, pod and flower, whereas stem and root were completely devoid of them. Qualitative and quantitative variationwere observed between different parts of the plant. Leaf and pod were found to be the richest source of piperidine alkaloid, 12 in each part, followed by flower, with 4 alkaloids. Juliprosopine was present in highest concentration in leaf, whereas, Julifloridine in pod and flower. Prosopine, Prosopinine, Julifloridine and Prosafrinine were present in all plant parts. Among the various fractions, L1 and P3 were found to contain Juliprosopine and Julifloridine in almost pure form, showing more than 90% concentration in the fraction. Identification of the alkaloids was done by HR-MS and

accurate molecular formula determination and comparing them with the data available in literatures.

- Two new alkaloids, Prosopine and Prosopinine were identified in leaf, pod and flower by DART-MS analysis. Further confirmation of these alkaloids was done by studying their fragmentation pattern generated by LC-MS/MS.

- A range of fatty acids, from short chain length to long chain length were observed in leaf by DART technique and several compounds (1-9) were isolated from the leaves of *P.juliflora.,* which include, two carotene, three xanthophylls, two pheophytin and two fatty acid (propanoic and pentadecanoic acid). Compound 10, an aliphatic acid was isolated from flower extract. They were identified based on their colour, UV-visible spectra, IR spectra and NMR spectra.

- ARF was subjected for antibacterial evaluation against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas putida, Klebsiella* sp., *Salmonella* sp., *E.coli (ampicillin resistance), Alcaligen* sp. and *Acinetobacter* sp. ARF of leaf, pod and flower were found to possess significant antibacterial activity as compared to standard antibiotics and were found to be more effective than antibiotics in inhibiting the growth of multidrug resistant strains. Leaf extract was comparatively more effective than pod and flower in inhibiting most of the bacterial growth except *Acinetobacter* and *Alcaligen* strains. Maximum inhibition of *Acinetobacter* and *Alcaligen* was shown by pod extract. The MIC values showed variation in the antibacterial effect of all the tested ARF and ranged from 25-100 µg/ml. Leaf ARF was found to have lower MIC vaules as compared with pod and flower and among bacterial species *Klebsiella* was shown to have lowest MIC. Studies on the synergistic effect of the alkaloids revealed higher antibacterial activity of crude alkaloid mixture than its partially purified fractions.

- ARF of leaf, pod and flower were shown to possess strong allelopathic potential on mung seeds and onion bulbs. All the tested concentrations were found to significantly

inhibit root length. Complete inhibition of root growth was observed at 75µg/ml concentration for leaf extract, whereas flower and pod extracts showed complete inhibition of growth at 100 µg/ml concentration.

- ARF of all the plant parts reduced the percentage of cells undergoing normal mitotic process at all the tested concentrations. Leaf extract completely inhibited cell growth at 50 µg/ml and pod and flower at 100 µg/ml within 24h of treatment. Effect of ARF on cellular and chromosomal level was evaluated by allium test which revealed chromosomal abnormalities and extensive cell death at higher concentrations.

- Compound 10 isolated from flower extract showed antimitotic activity at concentration of 4 mg/ml. Microscopic examination revealed formation of c-mitosis. At higher concentration (8mg/ml) cellular and chromosomal damage was seen.

- The acute and subacute toxicity of ARF was tested on mice model by oral and interaperitoneal routes of administration. LD_{50} value calculated for leaf ARF on mice was 630.95 mg/kg body weight for oral route and 79.43 mg/kg for i.p route. Subacute toxicity study (14 day period) showed that the extract is not toxic when administered orally to mice at concentrations below 200 mg/kg and intraperitoneally below 10 mg/kg. High doses (500 mg/kg for oral route and 20 mg/kg for i.p route) were found to alter some of the tested parameters, which include: abnormal mice behavior, reduction in body weight, decrease in TRBC count, increase in SGOT and SGPT levels, slight increase in albumin and TPC and histopathological changes in liver, kidney and testis.

- Thus the present research work proves the scientific basis of the of *P.juliflora* use in various folk medicines and helps in evaluating the pharmacological potential of different parts of the plant.

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Future scope of work

- Variation in the presence and distribution of alkaloid indicates differential regulation of metabolism of cells located in different parts. Therefore future research should define the regulatory mechanism for alkaloid accumulation in the plant.
- Future work includes complete purification of all the alkaloids, especially Prosopine and Prosopine , which are new alkaloids and testing their activity on biological systems
- Since these alkaloids are showing wide range of pharmacological activity, therefore future work also includes testing other biological activity of the alkaloids
- Testing the antibacterial ability of the extracts on animal system

Papers

1. Singh, S., Swapnil and Verma, S.K. 2011. Antibacterial properties of Alkaloid rich fractions obtained from various parts of *Prosopis juliflora.* Int. J. Pharma Sci. Res. 2:114-120.
2. Singh, S. and Verma, S.K. 2012. Application of direct analysis in real time mass spectrometry (DART-MS) for identification of an epiphytic cyanobacterium, *Nostoc* sp. Anal. Lett. (accepted)
3. Singh, S. and Verma, S.K. 2012. Allelopathic activity of alkaloid rich fraction obtained from leaf, pod and flower of *Prosopis juliflora* on plants and cyanobacterial species. Allelopathy J. (accepted)

Conferences

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3. Singh, S. and Verma, S.K. 2009. Antiinflammatory effect of phycocyanin on arachidonic acid induced inflammation in rabbit eye. Emerging Trends in Life Sciences Research. BITS, Pilani, p45.
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5. Singh, S., Singh, P. and Verma, S.K. 2007. *Invitro* antibacterial activity and phytochemical analysis of some Indian desert plants. International Symposium on Applied Phycology and Environmental biotechnology, BITS, Pilani.
6. Singh, S. and Verma, S.K.2007. Evaluation of *invitro* antioxidant capacities of phycocyanin and phenolic compounds from cyanobacterial strains. International Symposium on Applied Phycology and Environmental biotechnology, BITS, Pilani, p75.

Biography of Prof. Sanjay Kumar Verma

Prof. Sanjay Kumar verma has done M.Sc in Genetics from Banaras Hindu University (BHU), Varanasi with specialization in cyanobacteria and applied phycology and completed his Ph.D in the area of Environmental Biotechnology from the same University. He worked as post-doctoral research fellow at University of Hyderabad in the area of microbial and molecular genetics. Prof, verma has handled several research projects related to bioremediation and biodegradation of toxic industrial waste, development of recombinant bacterial biosensor for environmental applications, funded by Bhabha Atomic research Center (BARC), University Grants Commission (UGC), Council of Scientific and Industrial research (CSIR) and Department of Science and Technology (DST). Currently he is working as Professor , Department of Biological Sciences, Birla Institute of Technology and Science, Pilani. His major research interest lies in molecular biology and environmental biology and environmental biotechnology.

Biography of Mrs. Shachi Singh

Mrs. Shachi Singh completed her M.Sc in Botany from Banaras Hindu University, Varanasi and M.E in Biotechnology from Birla Institute of Technology and Science, Pilani. She has being working for his Ph.D, as a research fellow at BITS, Pilani from 2005-2011. She was awarded BITS fellowship, Junior research fellowship and Senior research fellowship from CSIR during this period. She was involved in teaching of various course of Department of Biological Sciences, BITS, Pilani. She has published research articles in international journals and presented papers in various national and international conferences.