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LIMONOID 'AZADIRACHTIN' FROM *AZADIRACHTA INDICA*

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ABSTRACT

Majority of world population nearly 80 percent, in all most all nations depends on traditional medicine for their primary health care. In various phytotherapy based medical systems, native medicinal plant/herbal extracts are employed to treat various disorders and diseases. The usage of medicinal plant/herbal extracts is rapidly evolving due to their useful effects and moreover presumed with less toxic side effects. *Azadirachta indica* (Neem) is a key medicinal plant with high medicinal properties. It belongs to a family of *Meliaceae*, which is majorly cosmopolitan in distribution. For centuries, its usage in India, is very common in ethno traditional medicine and as well as other medical practices. The vegetative parts such as leaves, flowers, fruits, seeds and bark are rich source for major bio-active molecules. Hence it is referred as natural drug store or store house of phytochemicals. Its structural and chemical composition is complex in nature. Several bioactive molecules are separated, characterized, and stated with significant biological properties. However, in the present study, we assessed the distribution pattern of Triterpenes like Azadirachtin in the vegetative parts of *Azadirachta indica* (Neem) extracts through LC–MS (Liquid Chromatography and Mass Spectroscopy) spectral analysis. The results reveal the presence of major Triterpeniods like Azadirachtin

($C_{35}H_{44}O_{16}$) for the first time from the vegetative parts of *Azadirachta indica* collected from Tirumala Hills, Eastern Ghats, India.

KEYWORDS: Flavonoids, Azadirachtin**,** *Azadirachta indica*, Natural products, LC –MS.

INTRODUCTION

Majority of the global population up to 80 percent either in developed or developing nations still depends on traditional medicine for their primary health care.[1-2] Currently, usage of medicinal plant/herbal medicine for treating various disorders and diseases is quickly evolving, since they offer less or no toxic side effects.[1-2] The active components present in these Medicinal /herbal plants have been shown as capable of hindering the disease or disorder symptoms in a synergistic manner. This active substances from these Medicinal /herbal plants may consists of polysaccharides, pigments, steroids, terpenoids, flavonoids and alkaloids etc. Few studies stated that Medicinal/herbal plant extracts and purified molecules have major effects in managing various diseases and disorders.[1-10] However for the few decades, chemical agents derived from plants, termed as "phytochemicals" have gained global attention among both the public and scientific communities for their role in maintaining health and preventing disease.

Azadirachta indica is such an mportant medicinal plant, falls under the family *Meliaceae,* often known as "village pharmacy" or 'Neem tree', or nature's 'drug store' or 'store house of phytochemicals'. Therefore, it is target for various phytochemical investigations.[12-13] In nature, its distribution is in cosmopolitan, commonly found in tropics, and subtropics that covers Asia and as well as Africa.[1, 11-14] Its use is very common in almost all health practices of rural India. It's usage for centuries, in many nations, mostly in their ethno-traditional medicinal health care systems. The vegetative parts includes roots, leaves, bark, seeds and flowers have been employed to treat several acute, chronic diseases and disorders.[11-14] These vegetative parts also possess numerous phytochemicals with significant biological activities. Few active components are reported to have anti-bacterial, anti-fungal, anti-viral, antimalarial, anti-cancer and anti-inflammatory properties, while few act as insecticidal; larvicidal and very few as spermicidal.[1,11-14]

Some scientists described it possess more than 300 plus bioactive compounds, which are chemically varied in nature and present with distinctive complex structural identity.[11,14-15] Majority of these phytochemicals are differentiated into two varities, namely isoprenoids, and

non-isoprenoids. The isoprenoids classified into diterpenoids, triterpenoids, vilasinins, limonoids, and C-secomeliacins whereas nonisoprenoids classified into proteins, polysaccharides, sulphur entities, polyphenolics, dihydrochalcones, coumarins, tannins and aliphatic molecules.[1,11,14,16] Thus, it is evident that the chemical composition of *Azadirachta indica* has been studied successfully, few bio-actives are well characterized, and structurally elucidated in some varieties that are distributed in Asia and Africa and not in the Indian varieties.[11-14] Moreover, the knowledge on the chemical composition on the Indian varieties is still lacking, especially on the South Indian species from the region of Eastern Ghats. Hence the present study is undertaken to assess presence of the Triterpeniods like Azadirachtin ($C_{35}H_{44}O_{16}$) from *Azadirachta indica*.

2. MATERIALS AND METHODS

Plant collection

The germplam of *Azadirachta indica* was collected in March, 2017 from the Tirumala hills, region of Eastern Ghats (Andhra Pradesh), India. Later verification was done by the native taxonomist. The collected Fresh germplasm comprising of leaves, bark and roots were subjected to shade dry as described[1,5-7], followed by appropriate crushing in a pulveriser carefully to make a fine powder.

Preparation of Plant Extracts

The pulverized powders of the germplasm consisting of leaves, bark and roots were subjected to Soxhlet extraction to make aqueous extracts. 15 gram of the powder is weighed from each part independently, nicely packed in sterile cloth, further introduced in soxhlet apparatus and extracted as per described procedures.[1,5-7] After extraction, the obtained crude extract was filtered and concentrated, and later the final residue was dissolved in sterile water, filtered and was kept in refrigerator until use. The extract concentration was obtained by calculating the dry weight per unit volume as per described procedures.[1,5-7]

LC-Mass spectral analysis

The Fresh aqueous extracts of *Azadirachta indica* were chemically fingerprinted by LC-Mass spectral analysis (SHIMADZU-LC-MS-2010A) according to the described methods.[1,5-7] The LC-MS (Liquid Chromatography and Mass Spectroscopy) experiments were conducted with the methanol and water, keeping as mobile phase, and a gradient procedure was set, using RP-C18 analytical column [240 mm× 2 cm] and the minimal flow rate was kept at 0.5 ml/min respectively. Further the extract samples were nebulized with nitrogen gas and the ion mass

(Electro Spray Ionization) of the peaks were recorded at positive mode and as well as negative mode as per described methods.[1,5-7]

RESULTS AND DISCUSSION

Several researchers widely employed various chromatographic techniques in assessing various natural or synthetic molecules that fight with various diseases and disorders.[1] Recent developments in advanced molecular biological tools like DNA sequencing, genetic engineering, gene targeting and transgenic methodologies has been showed a new path to better understand and assess the infections, diseases and disorders, which could drive for developing new age therapeutics.[17-20] Currently, to encounter diseases like cancer[17-21], and disorders like diabetes[22], several efficient drug development technologies has been established, through platforms like in silico drug designing and synthesis of novel molecules.[7,23-30] However the situations remain same. Thus alternatives are required.

Medicinal plants seems better option. In ethno-traditional medicine, medicinal plants are widely used to treat several ailments which includes diseases and disorders.[8-10] At present, usage of the medicinal plant/herbal extracts/formulations is quickly progressing, which are assumed to be minimal side effects. The active components present in this may be responsible for this outcome. The active components may be polysaccharides, pigments, steroids, terpenoids, flavonoids and alkaloids.[1,7-8] Moreover, at present studying secondary metabolites has become an active area of research, since they are probable sources for new drugs.[1,4] In majority of the studies, the plant secondary metabolites will be identified with different chromatographic techniques, by appropriate methods that include extraction, separation, purification, structural elucidation and quantification.[1-7] Initially plant germplasm comprising of vegetative parts will be collected, shade dried, lyophilized, later extracted with suitable solvents in a soxhlet extractor to remove unwanted ingredients in order to attain desired bioactive compounds. After extraction, required bio actives were separated, purified, structure elucidated and quantified with proper chromatographic techniques. Recent studies states that is an urgency to admit and introduce modern analytical tools for studying novel bioactive constituents. Moreover application of novel chemical fingerprinting approaches with analytical tools such as LC-MS, could produce quality output in short time.[27] Chromatographic fingerprinting approaches could be employed in identifying and validating various bioactive compounds that totally represent a certain plant or herb.

As detailed above, in native ethno-traditional medicine *A. indica* is broadly used in various health practices for treating several diseases and disorders.[1,11-14] Its chemical composure is quiet complex, well studied and characterized.[11] Patela et al 2016, categorized neem active ingredients into two types, specifically Isoprenoids and non-isoprenoids. In the isoprenoids category, the diterpenoids, triterpenoids and steroids were placed. The Flavonoids, carbohydrates, proteins, coumarins, hydrocarbons, fatty acids and esters, and other acids were positioned under the category of non-isoprenoids.

Next, the triterpenoids are discriminated into different categories based on the removal of carbon atom from the side chain or from the ring skeletal structure of the parent compound. The triterpenoids are separated as protolimonoids, mononortriterpenoids, dinortriterpenoids, trinortriterpenoids, tetranortriterpenoids, pentanortriterpenoids, hexanortriter-penoids, octanortriterpenoids and nonanortriterpenoids. Next, tetranortriterpenoids were separated into two groups, one as ring-intact- tetranortriterpenoids and the later as ring-seco-tetranortriterpenoids. The diterpenoids also allocated into podacarpanoids (margolone) and abeitanoids (sugiol). However, in most plants this complex chemical composure differs due to their geographical distribution, seasonal variations and other environmental factors.[4] In spite of its beneficial effects, the chemical composition of Indian *A.indica* species, dispersed in Eastern Ghats has not studied.[1] Therefore, the current study is initiated in aim to report the Triterpeniods like Azadirachtin ($C_{35}H_{44}O_{16}$) in *Azadirachta indica.*

Limonoids are a cluster of natural compounds mostly found in citrus fruits, particularly in the seeds, peels, and leaves. They are also known as triterpenoids, which are organic compounds derived from the blend of six isoprene units.[31] Limonoids exists as diverse chemical structures and display a wide range of biological activities. They have been the subject of wide research due to their prospective health benefits and pharmaceutical applications. The limonoids like limonin, nomilin, obacunone, and deacetylnomilin are mostly common. Research studies revealed that limonoids own various pharmacological properties, like anticancer, antimicrobial, anti-inflammatory, and antioxidant activities. They have shown significant effects in arresting the growth of cancer cells and inducing apoptosis (cell death) in various types of cancer that include breast, colon, and prostate cancer. Limonoids also assessed for their potential in preventing or treating diseases, like cardiovascular disorders, diabetes, and obesity.[31]

In addition to their medicinal values, limonoids also used as natural insecticides and pesticides due to their insect-repellent and pesticidal activities. Their toxic effects on certain insects and pests, made them as best alternatives to the common synthetic chemical pesticides. The limonoids have shown significant results in few studies, however further research is required to fully understand their mechanism, mode of action, potential side effects, and other therapeutic applications.[31] As always, it's desirable to check with healthcare professionals or experts before use of limonoid-rich products for medicinal purposes.

In the present study we inspected the distribution pattern of limonoids like Azadirachtin in the vegetative parts of *Azadirachta indica.* The *Azadirachta indica* germplasm were collected from Eastern Ghats (Andhra Pradesh, India), shade dried, subjected to grinding in a pulveriser and made into fine coarse powder. Next, the powdered coarse material from various vegetative parts were extracted with universal solvent, water in a soxhlet apparatus and aqueous water residues were attained. Later, these aqueous water residues were filter sterilized individually and subjected to LC-MS spectral analysis. In order to obtain chemical finger printing profile of the aqueous extracts of *Azadirachta indica*, an analytical protocol based on LC-MS (ESI) was employed. The LC-MS spectral profile data discloses the presence of Azadirachtin in the extracts, displaying with the protonated molecular ions, with respective *m/z* detected in both positive mode (Fig.2A-4A) and as well as in the negative mode (Fig. 2B-4B, Table-1). The figure (Fig. 1A) demonstrates the structural illustration of Azadirachtin ($C_{35}H_{44}O_{16}$).

Root extract

The LC-MS spectral profile of crude Aqueous root extract of *Azadirachta indica* exhibits the existence of a molecular ion peak of Azadirachtin ($C_{35}H_{44}O_{16}$) at 720.7 m/z. The protonated molecular ion peaks of Azadirachtin was recognised in positive mode (Fig.2A) and completely absent in negative mode (Fig. 2B).

Bark extract

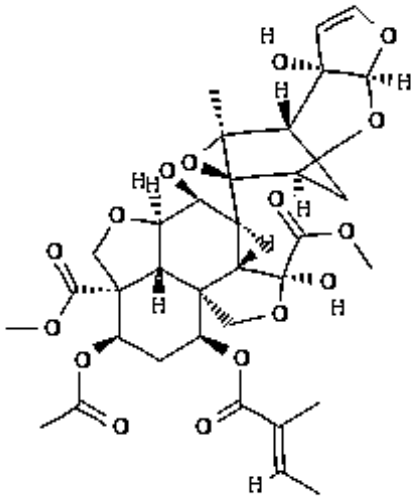
Interestingly, the bark extracts revealed presence of the bioactive molecules in both modes. The LC-MS profile of crude aqueous bark extract of *Azadirachta indica* clearly represents the presence of a molecular ion peak of Azadirachtin ($C_{35}H_{44}O_{16}$) at 720.7 m/z respectively. The protonated molecular ion peaks of **A**zadirachtin was clearly noticed in positive mode (Fig. 3A) and as well as in negative mode (Fig. 3B).

Leaf extract

The leaf extract of *Azadirachta indica* was also followed the similar too with root extract. The LC-MS data of crude aqueous leaf extract of *Azadirachta indica* also displays the presence of Azadirachtin ($C_{35}H_{44}O_{16}$) at 720.7 m/z respectively. The protonated molecular ion peaks of Azadirachtin were clearly detected in positive mode (Fig. 4A) and undetectable in negative mode (Fig. 4B). Similar results were reported in other studies as well.[32-37] Thus, the triterpeniods like Azadirachtin ($C_{35}H_{44}O_{16}$) reported in the present study were comparable with other studies.

Table 1: The distribution pattern of from various vegetative parts of *A. Indica.*

S. No	Name of the Identified Molecule in the LCMS spectra	Molecular formula	Mass (*m/z*)	Presence/Absence of molecule in the LC MS spectra of Root extract		Presence/Absence of molecule in the LC MS spectra of Bark extract		Presence/Absence of molecule in the LC MS spectra of Leaf extract	
				positive mode	negative mode	positive mode	negative mode	positive mode	negative mode
1	**Azadirachtin**	**$C_{35}H_{44}O_{16}$**	**720.7**	yes	no	yes	yes	yes	no



Azadirachtin $C_{35}H_{44}O_{16}$ (Mass 720.7 m/z)

Fig 1. The structural representation of identified bioactive molecule Azadirachtin from *Azardicta Indica.*

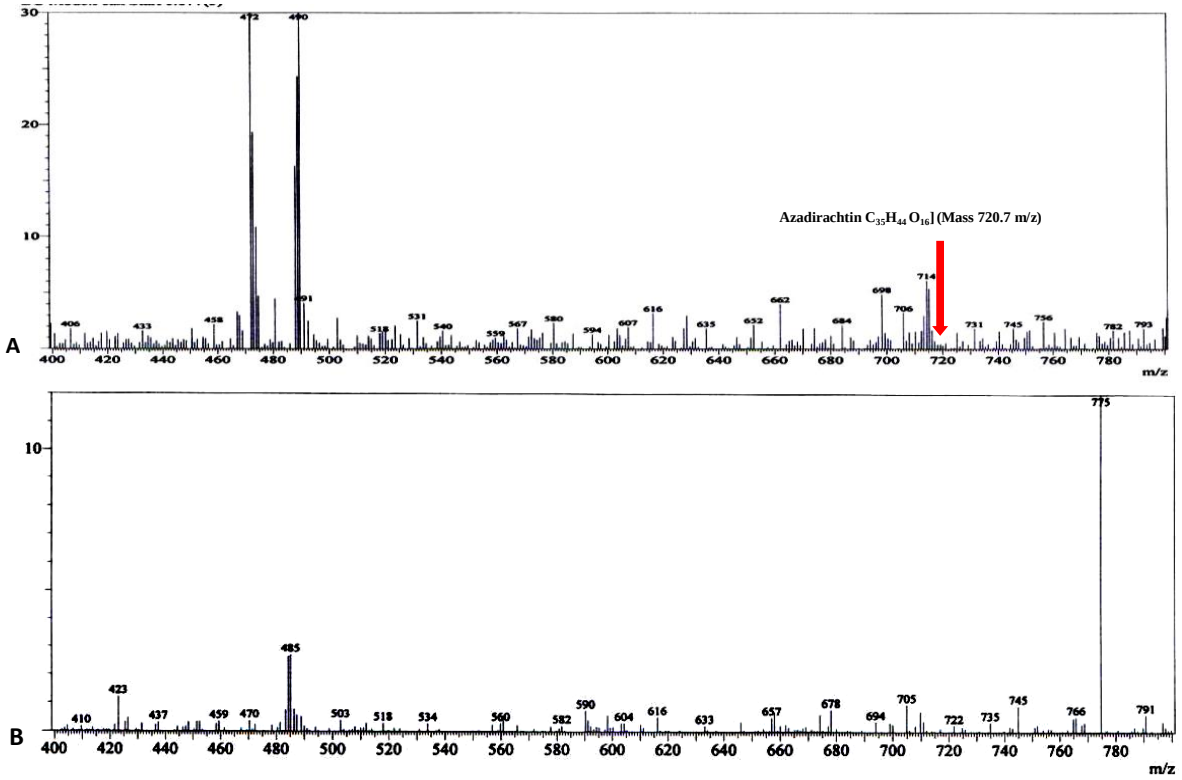


Fig 2 A (Positive mode)-B (Negative mode). The LC-MS spectral analysis (Positive mode and negative mode) of Azadirachtin from the crude aqueous root extract of *A. Indica.*

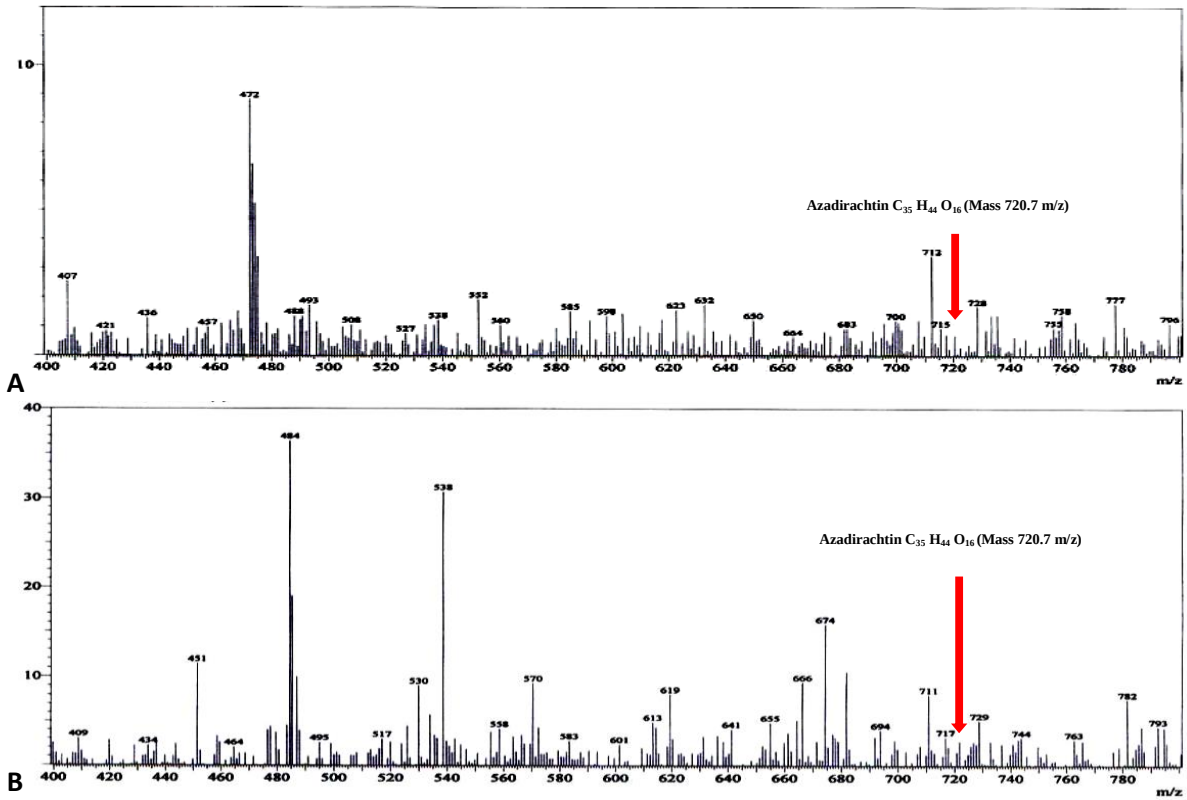


Fig 3 A (Positive mode)-B (Negative mode). The LC-MS spectral analysis (Positive mode and negative mode) of Azadirachtin from the crude aqueous bark extract of *A. Indica.*

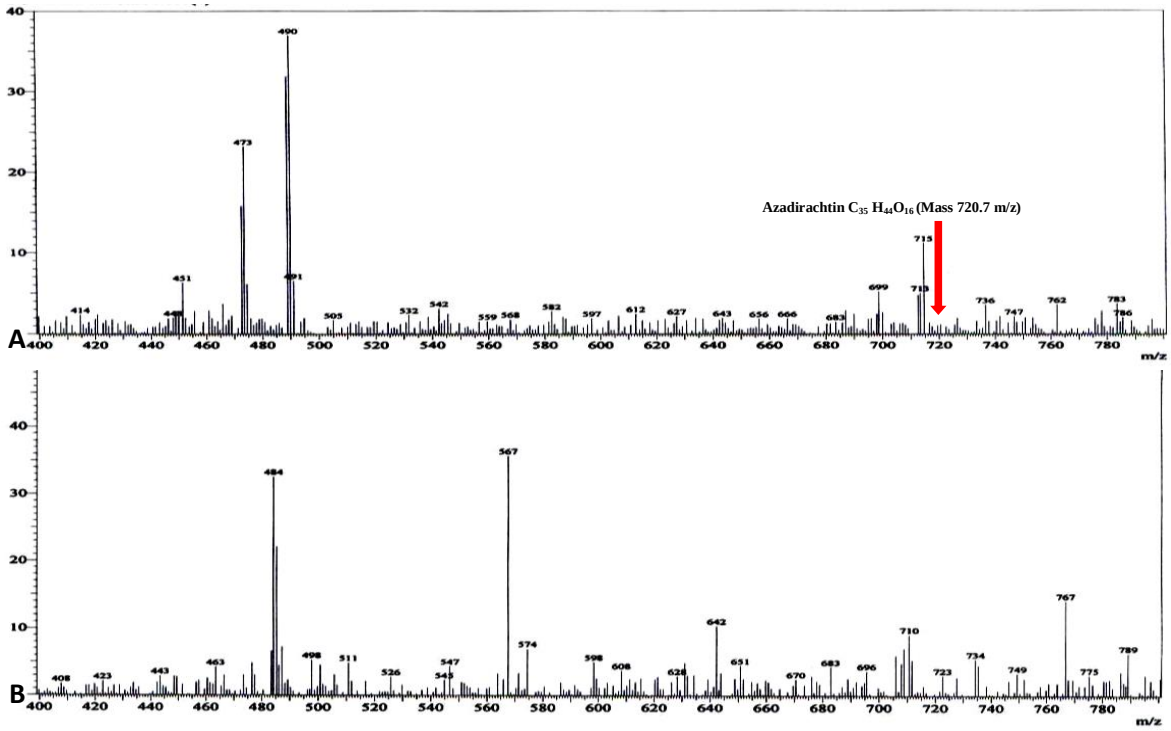


Fig 4 A (Positive mode)-B (Negative mode). The LC-MS spectral analysis (Positive mode and negative mode) Azadirachtin from the of crude aqueous leaf extract of *A. Indica.*

CONCLUSIONS

The current study for the first time, confirms the presence of Triterpeniods like Azadirachtin (C_{35} $H_{44}O_{16}$) from various vegetative parts of *Azadirachta indica* collected from Tirumala hills, Eastren Ghats, India.

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