Introduction

Studies reveal that world population up to 80 percent relies on traditional medicine. Predominantly, the usage of medicinal plant/herbal medicine for treating various disorders and diseases is rapidly evolving and presumed with less side effects [1]. The active components existing in these Medicinal/herbal plants have been shown to efficiently hinder the disease or disorder symptoms in a synergistic manner. This active components from these Medicinal/herbal plants may comprise of polysaccharides, pigments, steroids, terpenoids, flavonoids and alkaloids etc. Earlier studies demonstrated that Medicinal/herbal plant extracts and purified molecules have significant effects in controlling and eradicating various diseases and disorders [1-8].

*A. indica* is referred as “village pharmacy” and widely employed in various health practices in rural India. It is an important medicinal plant, belongs to the family *Meliaceae*. It is distributed in cosmopolitan, indigenously covering tropics, starting from Asia to Africa [9-12]. It has numerous names, commonly called as ‘Neem tree’, or nature’s ‘drug store’ or ‘store house of phytochemicals’. Due to its wide spread and importance in ayurvedic medicine *A. indica* has been a potential target for extensive phytochemical investigations [10-11]. *A. indica* has been explored for centuries, employed in several native ethno-traditional medicinal health practices, and also its vegetative parts like, roots, leaves, bark, seeds and flowers have been used to treat various acute and chronic diseases and disorders [9-12]. These vegetative parts contain diverse range of phytochemicals with potential biological and pharmacological activities. Some of the purified bio-active compounds from *A. indica* are reported to exhibit anti-cancer, antimalarial, anti-bacterial, anti-fungal, anti-viral and anti-inflammatory properties, while few reported as insecticidal; larvicidal and spermicidal as well [9-12].

To date nearly 300 plus bioactive molecules have been reported from *A. indica*, which are chemically diverse, with their complex structures [9,12,13]. These are divided into isoprenoids, and non-isoprenoids. The isoprenoids are composed of diterpenoids,
triterpenoids, vilasinins, limonoids, and C-secomeliacins. The nonisoprenoids are composed of proteins, polysaccharides, sulphur compounds, polyphenolics, dihydrochalcone, coumarin, tannins and aliphatic compounds [9, 12, 14]. Thus, from the above reports it is clearly understood that A. indica has been effectively studied, characterized, and structurally elucidated in most African and Asian species [9-12]. However, there were no reports on the chemical composition of South Indian species especially from the region of Eastern Ghats. Hence the present study is undertaken to assess presence of the bioactive molecules like kaempferol-3-glucoside from A. indica.

Materials and Methods

Plant Collection

The germplam of A. indica was collected during the month of March, 2017 from the Tirumala hills, Eastern Ghats (Andhra Pradesh) of India, which was authenticated by native taxonomist. The collected germplasm (leaves, bark and roots) were subjected to shade dry as per described protocols [3-5]. After complete drying the germplasm was crushed and grinded in a mixer thoroughly until a fine powder is obtained.

Preparation of A. indica Extracts

Initially aqueous extracts were prepared with the collected germplasm that comprised of leaves, bark and roots using Soxhlet apparatus. Approximately 15 gram of the powder of various vegetative parts of A.indica was taken separately and packed into sterile cloth, and placed in a thimble. The round bottom flask was filled with double distilled water (200 mL), and attached to a Soxhlet extractor and condenser on a heating mantle. The side arm was insulated with glass wool. With the help of heating mantle, the water was heated to above its boiling point (120°C) making water to pass through the apparatus to the condenser, where condensation takes place and trickles into the reservoir containing the thimble. Once the water reaches the limiting point in the siphon, it further pours back into the flask, and the fresh cycle begins again. The process was performed for 72 hours. After completion, the obtained water extract was further subjected to evaporation in a distillation unit, to obtain a minimal yield of extracted plant material up to 7 to 9 mL in the falcon tubes. Later these falcon tubes were placed in boiling water bath at 60˚C to eliminate the moisture content. The extract was filtered and concentrated, and the residue was dissolved in sterile water and filtered and was kept refrigerated until use. The concentration of the extract was calculated based on the dry weight per unit volume according to the described procedures.

LC-Mass Spectral Analysis

The neem-extracts were subjected to chemical fingerprinting using LC-Mass spectral analysis as previously described [3]. The LC-MS (Liquid Chromatography and Mass Spectroscopy) was performed in SHIMADZU-LC-MS (Model: 2010A), using the solvents like methanol and water in combination, a gradient procedure was followed, using RP-C18 analytical column [240 mm× 2 cm] with a flow rate of 0.5 ml/min respectively. The extract samples were nebulized with nitrogen gas and the ion mass (Electro Spray Ionization) of the peaks were recorded in both positive mode and negative mode [3].

Results

Flavonoids are prominent plant secondary metabolites found in most plants. They represent with a 15-carbon molecular framework, consisting of two phenyl rings (A & B) and one heterocyclic ring (C), and commonly abbreviated as C6-C3-C6, according to the IUPAC nomenclature. Very often the flavonoid units are attached to glycosides which are generally resembling glucose. These glucosides are commonly present in most plants. Glucose is generated when glucoside is hydrolysed by means of certain chemical degeneration, or fermentation process or enzyme process. Flavonoids in combination with glucosides results in the formation of flavonol glucosides. In other words they are also termed as phenolic compounds that contain a flavonoid moiety which is O-glycosidically connected to carbohydrate moiety at the C3-position. Moreover, if the glycone group of a glycoside is glucose, then it is termed as glucoside.

In the current study we assessed the distribution pattern of flavonol glucosides like Kaempferol 3-galactoside in the vegetative parts of A. indica. The Plant germplasm were collected from Eastern Ghats (Andhra Pradesh, India), shade dried, subjected to grinding and made fine powder. Later powdered material from various vegetative parts were extracted with water (aqueous) using soxhlet apparatus and water extracts were obtained. Further, these water extracts were sterile filtrated individually and subjected to LC-MS spectral analysis. The LC-MS (ESI) was employed to acquire chemical finger print profiles of aqueous extracts of A. indica. The LC-MS spectral profile data reported the presence of kaempferol-3-glucoside in the extracts, exhibiting with the protonated molecular ion peaks, at respective m/z observed in both positive mode and as well as in the negative mode (Figure 2A, B, 4A and B) (Table 1). The figure demonstrates the structure and molecular formula of kaempferol-3-glucoside (C21H20O11) (Figure 1A).
Table 1. The distribution pattern of Kaempferol 3-glucoside from various vegetative parts of *A. indica*

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of the Identified Molecule in the LCMS spectra</th>
<th>Molecular formula</th>
<th>Mass (m/z)</th>
<th>Presence/Absence of molecule in the LC MS spectra of Root extract</th>
<th>Presence/Absence of molecule in the LC MS spectra of Bark extract</th>
<th>Presence/Absence of molecule in the LC MS spectra of Leaf extract</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Kaempferol 3-glucoside</td>
<td>C₂₁H₂₀O₁₁</td>
<td>448.3</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>

Kaempferol 3-glucoside C₂₁H₂₀O₁₁ (Mass 448.38 m/z)

Fig 1. The structural presentation of identified flavonol glucoside from *A. indica*

Root Extract

The LC-MS spectral data of crude Aqueous root extract of *A. indica* displays the presence of a molecular ion peak of kaempferol-3-glucoside (C₂₁H₂₀O₁₁) at 448.3 m/z. The protonated molecular ion peaks of kaempferol-3-glucoside was recorded in positive mode and as well as in negative mode (Figure 2A and B).

Fig 2 A (Positive mode )-B (Negative mode ). The LC-MS spectral analysis (Positive mode and negative mode ) of Kaempferol 3-glucoside from the crude aqueous root extract of *A. indica*
Bark Extract

Similar reports were noticed in bark extract too. The LC-MS spectral data of crude Aqueous bark extract of *A.indica* depicts the presence of a molecular ion peaks of kaempferol-3-glucoside ($C_{21}H_{20}O_{11}$) at 448.3 m/z. Interestingly, the protonated molecular ion peaks of kaempferol-3-glucoside were clearly recorded in positive mode and found to be completely absent in negative mode (Figure 3A,3B).

![Fig 3 A (Positive mode )-B (Negative mode ). The LC-MS spectral analysis (Positive mode and negative mode ) of Kaempferol 3-glucoside from the crude aqueous bark extract of *A. indica*](image)

Leaf Extract

![Fig 4 A (Positive mode )-B (Negative mode ). The LC-MS spectral analysis (Positive mode and negative mode ) Kaempferol 3-glucoside from the of crude aqueous leaf extract of *A. indica*](image)
The leaf extract of *A. indica* was also presented with similar findings. The LC-MS spectral data of crude Aqueous leaf extract of *A. indica* reports the presence of a molecular ion peaks of kaempferol-3-glucoside (C_{21}H_{20}O_{11}) at 448.3m/z. The protonated molecular ion peaks of kaempferol-3-glucoside were clearly noticed in positive mode and as well as negative mode (Figure 4A and B).

**Discussion**

The chromatographic techniques are widely employed in studying the natural or synthetic molecules that fight with various diseases and disorders. Recent advances in modern molecular biological tools like DNA sequencing, genetic engineering, gene targeting and transgenic methodologies demonstrated a new path to better understand and analyze the infections, diseases and disorders, which provide new options for developing new age therapeutics [15-18]. Currently, to combat diseases like cancer, and disorders like diabetes, several efficient drug development technologies are developed, through programs like in silico drug designing and synthesis of novel molecules [15-24]. However the problems still persist. Hence the discovery of alternative medicines are necessary.

Medicinal plants look as better option. As in ethno-traditional medicine, medicinal plants have been widely used to treat a variety of diseases and disorders [6-8]. Presently, usage of the medicinal plant/herbal extracts/formulations is quickly progressing, and assumed to have less side effects. One valid reason may be that the active ingredients present in this may be responsible for this effect. The active ingredients could be polysaccharides, pigments, steroids, terpenoids, flavonoids and alkaloids [1,5,6]. Moreover, screening these secondary metabolites has become an active field of study in the present circumstances, since they are potential sources for unique drugs [1,3]. Primarily these plant metabolites will be separated by various chromatographic techniques, by employing appropriate procedures, such as extraction, separation, purification, structural elucidation and quantification1-5. Initially various vegetative parts of the plant germplasm were collected, shade dried, lyophilized, further extracted with appropriate solvents by employing soxhlet extractor to separate bioactive compounds. After extraction, desirable bioactive compounds were separated, purified, structure elucidated and quantified by employing appropriate chromatographic techniques. Recent studies states that there is an urgency to adopt and implement modern analytical tools for investigating bioactive compounds. Moreover usage of new chemical fingerprinting methods with analytical tools like LC-MS, which can produce quality results in short time. So modern chromatographic fingerprinting methods could be employed in identifying and validating various molecules that completely represent a particular plant or herb.

As mentioned earlier, in native ethno-traditional medicine *A. indica* is widely in various health practices. Its ingredients were well studied and characterized [11]. Patela et al 2016, classified neem components into two categories, namely Isoprenoids and non-isoprenoids. In the isoprenoids category the diterpenoids, triterpenoids and steroids were placed. The Falavanoids, coumarins, carbohydrates, proteins, hydrocarbons, fatty acids and esters, and other acids were placed under the category of non-isoprenoids. Further, the triterpenoids are divided into many types based on the removal of carbon atom either from the side chain or from the ring skeletal structure of the parent compound. The triterpenoids are further classified as protolimonoids, mononortriterpenoids, dinonortriterpenoids, trinortriterpenoids, tetratonriteterpenoids, pentanonritriterpenoids, hexanonritriterpenoids, octanortriterpenoids and nonanortriterpenoids. Furthermore tetranortriterpenoids were divided into two groups, namely ring-intact- tetranortriterpenoids and ring-seco-tetranortriterpenoids. The diterpenoids were further divided into two groups, such as podacarpanoids (margolone) and abeitanoids (sugiol). But the chemical composition in plants differs due to their geographical distribution, seasonal variations and other environmental factors [3]. In spite of its therapeutic importance, the chemical composition of *A.indica* species, distributed in Eastern Ghats has not been studied in detail. Therefore, the present investigation is carried out with an aim to report the flavonol glucosides like kaempferol-3-glucoside (C_{21}H_{20}O_{11}) in *A. indica*. Thus, in the present study *A. indica* germplasm comprising of leaves, bark and roots has been collected, shade dried, subjected to grinding and made fine powder. Next, these powders were extracted with water in soxhlet apparatus and water extracts were obtained. Later the water extracts were sterile filtrated and sent to LC-MS spectral analysis. The respective molecular mass of the kaempferol-3-glucoside (C_{21}H_{20}O_{11}) have been recorded from various extracts with their positive and negative ion electrospray mass spectra (ESI-MS),and well as with their corresponding protonated and deprotonated pseudo-molecular ion peaks (Figure 2-4, Table 1).

Flavonoids, generally referred as di-syphenyl propanes or C6-C3-C6 ring structures, which are found in large numbers in most plants and *A.indica* is one among them. Flavonoids are major classes of bioactive compounds, with wide range of biological and physiological activities [25,26]. Typically, flavonoids are attached to a variety of hydroxyl groups. In majority of the cases flavonoids will appear in conjugated forms or their hydroxyl groups associated with one or more sugar residues. Frequently they will be associated with carboxylic acids, amines, lipids and also connected with phenols. Studies reveal that flavonoids exhibit antioxidant activity, which is based on their linked structure, and also act as reducing agents, hydrogen-donating antioxidants and quenchers of singlet oxygen [26-28]. kaempferol 3-glucoside is an important flavonoid which has clinical importance. It is also known as astragalin and have been reported in several medicinal plants like *Cuscuta chinensis*, *Camellia sinensis*, *Cassia alata*, and *Centella asiatica* etc [29]. It has wide range of pharmacological activities like anti-inflammatory, antioxidant, neuroprotective, cardioprotective, anti-obesity, anti-osteoporotic, anti-cancer, anti-ulcer, and anti-diabetic properties. It regulates various pathways, by targeting various transcription factors like NF-κB, TNF-α, and TGF-β1; enzymes like iNOS, COX-2, PGE2, MMP-1, MMP-3,
MIP-1α, COX-2, PGE-2, HK2, AChE, SOD, DRP-1, DH, PLCc1, and GPX; kinases like JNK, MAPK, Akt, ERK, SAPK, IxkBa, PI3K, and PKCζ2; cell adhesion proteins like E-cadherin, vimentin PAR-2, and NCAM. It also plays a key role in regulating various apoptotic and anti-apoptotic proteins like Beclin-1, Bcl-2, Bax, Bcl-xL, cytochrome c, LC3A/B, caspase-3, caspase-9, procaspase-3, procaspase-8, and IgE. It also regulates various inflammatory cytokines like SOCS-3, SOCS-5, IL-1β, IL-4, IL-6, IL-8, IL-13, MCP-1, CXCL1, CXCL-2, and IFN-γ [29]. However, there were no reports its presence in South Indian species of Azadirachta indica, especially from the region of Eastern Ghats. Thus, in the current study various vegetative parts of A. indica were assessed to identify the flavonol glucosides like kaempferol-3-glucoside (C_{15}H_{20}O_{11}), using new age chemical fingerprinting tools like LC-MS.

The structural representation of kaempferol-3-glucoside (C_{15}H_{20}O_{11}) has been displayed in Figure 1A along with the molecular formula. The LC-MS spectral data of various vegetative parts of crude aqueous extracts of A. indica clearly demonstrated the presence of flavonol glucosides like kaempferol-3-glucoside (C_{15}H_{20}O_{11}), in both positive mode and negative mode (Figure 2-4). Kaempferol-3-glucoside (C_{15}H_{20}O_{11}) was identified in all the extracts assessed (Table 1). The data revealed the presence of kaempferol-3-glucoside (C_{15}H_{20}O_{11}) in the spectral data and the molecular ion peaks was observed at 448.3 m/z respectively. The identified kaempferol-3-glucoside (C_{15}H_{20}O_{11}) was observed in either positive mode or negative mode or complete absence of in vegetative aqueous extracts. In root extracts of A. indica the protonated molecular ion peaks of kaempferol-3-glucoside (C_{15}H_{20}O_{11}) was observed in both positive mode (Figure 2A) and as well as in negative mode (Figure 2B). Interestingly, incase of bark extract the protonated molecular ion peaks of kaempferol-3-glucoside (C_{15}H_{20}O_{11}) was clearly noticed in positive mode and completely absent in negative mode (Figure 3A and B). The leaf extract spectral data showed the presence of kaempferol-3-glucoside (C_{15}H_{20}O_{11}) both in positive and negative mode (Figure 3A and B). Moreover, the flavonol glucosides like kaempferol-3-glucoside (C_{15}H_{20}O_{11}) reported in the present study were correlated with other studies that demonstrated the presence of these molecules [29-30].

Conclusion
Thus from the above study based on the qualitative analysis we conclude that presence of flavonol glucosides like kaempferol-3-glucoside (C_{15}H_{20}O_{11}) has been reported from various vegetative parts of A. indica collected from Tirumala hills, Eastren Ghats. However further studies are required for in depth quantitative analysis.

References


