RESEARCH PAPER

Quantitative Estimation of Phytoconstituents and LCMS Characterization of Fruits Extract of *Alstonia Scholaris* with Evaluation of Antioxidant Activity.

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ABSTRACT

Some medicinal plants have plant-based antioxidants that help to avoid the harmful impact of oxidative stress. *Alstonia scholaris* (Apocynaceae) is a tropical evergreen tree indigenous to South and Southeast Asia. The objective of the current work is to investigate the quantitative estimation of phytoconstituents and LCMS characterization of fruits extract of *A. scholaris* with evaluation of antioxidant activity. The total phenolic content is determined by Folin-Ciocalteu Reagent (FCR) technique and total flavonoid content is estimated by aluminum chloride assay from the ethanolic extract of fruits of *A. scholaris*. Additionally, the phytoconstituents of the EEAS and antioxidant activity were evaluated liquid chromatography mass spectroscopy (LCMS) analysis and 2, 2 diphenyl-1-picrylhydrazyl (DPPH) assay respectively. The total phenolic content and total flavonoid content in EEAS was found to be 400mg GAE.gm and 31.1 mg QE/gm respectively. However, the LCMS analysis revealed that EEAS showed a total of 58 phytoconstituents. In the antioxidant study, the EEAS exhibits the as compared to standard ascorbic acid IC_{50}=8.116±0.013µg/ml. After completing the study, it is concluded that EEAS possessed phenolics and flavonoid content. However, LCMS analysis revealed the other important phytoconstituents as well as plant extract also possess valuable *in-vitro* antioxidant potential. So, the evidence of the current study states that the *A. scholaris* and its isolated compounds may be a promising candidate for treating the various diseases in future.

Keywords: *Alstonia scholaris*; Total phenolic content; Total flavonoid content; Antioxidant activity; LCMS Analysis.
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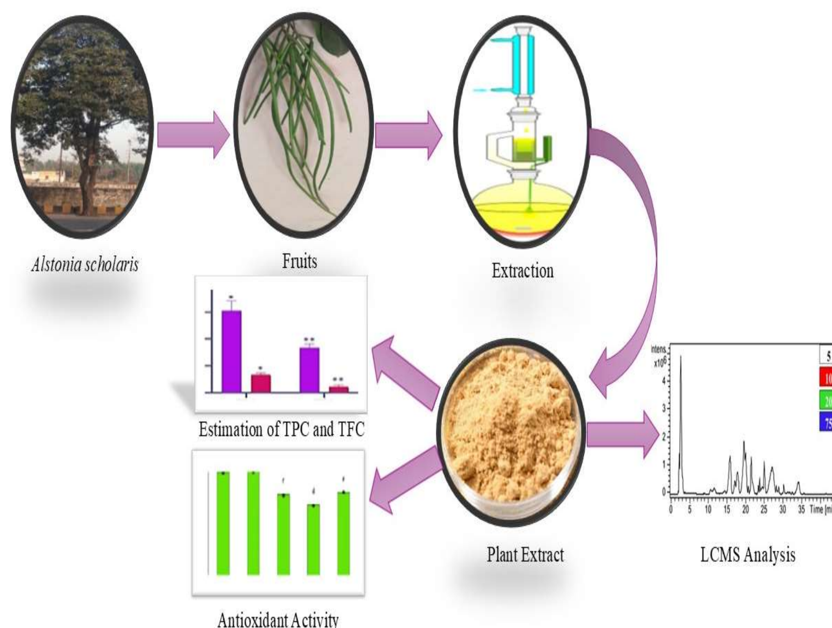
Graphical Abstract



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1. INTRODUCTION

Herbal drugs are gaining popularity among patients as they are well tolerated and have no severe side effects. But they are not considered much in therapeutic practice. Medicinal plants and their secondary metabolites are increasingly being utilized in the treatment of diseases as complementary medicine [1]. Humans have been using natural medications to treat their illnesses throughout prehistoric times. The utilization of medicinal plants originated immediately, comparable to that of animals. At that time, inadequate knowledge existed regarding the underlying causes of diseases and the appropriate medicinal remedy for treatment, leaving everything to experimentation. Over time, the rationale for utilizing specific herbal remedies in the therapy of various ailments was elucidated; hence, the application of these remedies transitioned from a scientific approach to one grounded in explanatory evidence [2]. Prior to the advent of phytochemistry in 1600 years, plants or herbs served as the primary source of preventive and therapeutic interventions. Plant derived drugs have been utilized since man has begun treating physical and mental diseases. They are a part of Traditional Medicine in various cultures of the world. Since then, medicine and medical processes have undergone development and whereas in Traditional Medicine there was a holistic approach to life with emphasis on health and maintenance of health, current Modern Medicine has a distinct emphasis on the untangling of the changes leading to disease and eliminating it [3]. Conventional medicine uses a systematic method of establishing the etiology of disease based on traditional principles that are, unfortunately, seen as old-fashioned in the modern world despite their widespread use throughout history (e.g., acupuncture, Ayurveda) [4,5]. The increasing popularity of plant-based medicines around the world has led to a huge demand for knowledge regarding the characteristics and applications of herbal remedies. According to certain theories, Ayurveda originated about six thousand years old. The objective was to promote health and longevity rather than to treat illness, and it was administered by physicians and surgeons (referred to as vaidya or Bheshaja). Currently, herbal remedies have garnered significant interest as alternative therapies effective in curing or preventing lifestyle disorders [6]. Herbs are a natural gift to humanity, maintaining health and well-being. The traditional system of treatment which finds its origin in India dates to the Vedic era and forms an extensive nutrition bank of the traditional solutions mostly from the plants [7]. Plant based drugs is the application of parts of plant like fruits, leaves, flowers, bark, seeds and roots for therapeutic use. Most of the medicines utilized in contemporary medicine are man-made. Most of the medicines currently utilized are developed from natural medicines. The most important natural sources of medications include animals, microorganisms, higher plants, and marine organism [8]. In biological organisms, reactive nitrogen species (RNS) and reactive oxygen species (ROS) like hydroxyl, nitric oxide and superoxide radicals can be responsible for DNA damage and cause the protein and lipid oxidations in cell [9]. Antioxidant mechanisms in the human body can neutralize radicals, thereby maintaining the balance between oxidation and antioxidation. Both herbal plants and food plants contain natural antioxidants. These antioxidants of natural origin, particularly polyphenols and carotenoids, possess a huge spectrum of biological action involving antiatherosclerosis, anti-inflammatory, anticancer and anti-aging [10]. These types of substances are compounds that can provide protective effects in the cell through multiple pharmacological mechanisms. Along with antioxidant effects per se, cancer-protective substances occur in most plants such as in certain vegetables and fruits and even in species and herbs commonly consumed [11]. They can be categorized into various classifications based on their chemical structure, such as carbohydrate, polyphenols, retinoids, glucosinolate (isothiocynates dithiothiols and indoles), thiols, tocopherols, trace metals and carotenoids, among others [12]. *Alstonia scholaris*, also known as the Indian Devil Tree or Saptaparni, belongs to family Apocynaceae is a tree species that holds immense significance in the field of ethnobotany, phytochemistry, and pharmacology. Native to the Indian subcontinent, this evergreen tree has long been revered for its medicinal properties and cultural importance [13]. This is an Indian native from Pakistan, Thailand, Sri Lanka, Malaysia, Solomon Island, Burma, Southeast Asia, South China, Texas, California, Florida. Northern Australia reaches a maximum height of 100 m. *A. scholaris* is an impressive deciduous tree with a broad canopy, making it an extremely coveted decorative plant in the grounds and parks [14]. The plant was originally scientifically described by Linnaeus as Echites scholaris. The generic name was, however, changed to honor the great botanist Professor C. Alston, but the species named scholaris kept reflecting its application in schools in Southeast Asia, where wood has been conventionally utilized in manufacturing blackboards and wooden slates [15]. *A. scholaris* has been utilized since ancient times in the treatment of some ailments. According to ayurveda, various parts of the plant viz. fruit, leaves, roots, and bark are employed in the treatment of various diseases. The bark possesses bitter and astringent properties, laxative, cardiotonic, stomachic, antipyretic and anthelmintics. Also used in the treatment of malarial fever, dyspepsia and abdominal disease. The extract of bark is effective as hepatoprotective, antispasmodic, immunostimulant, anticancer and antidiabetic [16] The ripen fruits are used in the treatment of epilepsy and syphilis. Also used as antiperiodic anthelmintics and tonic [17]. A polyherbal formulation namely AYUSH 64 contains the *A. scholaris*, sold and used in India for the treatment of malaria. Nearly 400 phytoconstituents have been extracted and identified from *A. scholaris*, making it the most widely recognized plants in terms of photochemistry [18]. *A. scholaris* possesses different chemical constituents like reducing sugar, flavonoids,

steroids, alkaloids, tannins, carbohydrates, leucoanthocyanins, fats, proteins, fixed oils and many others [19]. Antioxidants are vital for the prevention of oxidative damage due to free radicals present in our body. Oxidative stress (OS) has been associated with aging and etiology of many prolonged illnesses, such as cancer, diabetes, and cardiovascular diseases. Many natural sources, including plants, have been identified as rich sources of antioxidants [20]. Evaluating the antioxidant activity of *A. scholaris* fruit could add a new dimension to the search for natural antioxidant sources, making it an important study from a health promotion standpoint [21]. Characterization of the chemical constituents present in the ethanolic extract of fruits of *A. scholaris* (EEAS) is vital for understanding its medicinal properties. The ethanolic extract might contain bioactive metabolites like alkaloids, flavonoids, saponins, phenolic acids and terpinoids that possess antioxidants and other therapeutic properties [22]. By identifying and characterizing these compounds, the study can provide a deeper insight into the specific compounds responsible for the fruit's pharmacological effects, paving the way for future drug development and therapeutic applications. The research literature indicates that the various pharmacognostical and phytochemical studies has been conducted on the other parts of *Alstonia scholaris* such as leaves, bark, flower and latex except the fruits. Hence there is big gap in exploring the pharmacognostical and phytochemical studies along with fruits part of *Alstonia scholaris.* Findings from literature indicate that the fruits' part is unexplored till now. There is lack of studies that proved the quantitively estimation of phytochemicals and LCMS analysis specifically for the extract obtained from the fruit material of *Alstonia scholaris*. It is believed that fruits contain the various secondary metabolites that are responsible for treating various diseases. Furthermore, to fulfill this gap present research aimed to evaluate quantitative estimation of phytoconstituents and LCMS characterization of fruit extract of *A. scholaris* with evaluation of antioxidant activity. A figures of Whole plant of *A. scholaris* and its fruits are shown in Figure 1 (a and b).

Figure 1. (a): Whole-plant *A. scholaris*; **(b)**: Fruits of *A. scholaris*

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Good-quality chemicals and reagents were employed in the current research. Acetone, Acetonitrile, Ethanol, Petroleum Ether, Gallic acid, Quercetin, DDPH, Methanol, Aluminum chloride, Sodium nitrate, Sodium hydroxide, Sodium bicarbonate.

2.2. Apparatus, equipment and instruments

Liquid chromatography system (Waters Alliance e2695/HPLC-TQD Mass spectrometer), UV/Visible Spectrophotometer (JASCO V-630 Japan), Microplate reader (IMark BioRad), Electronic balance, Electric grinder, Soxhlet apparatus (Bionics Scientific Technology Pvt, Ltd), Rota evaporator (Buchi India Pvt., Ltd), Hot air oven (Seientech Technologies Pvt. Ltd.), Desiccator.\

2.3. Sample collection, identification and authentication

The fruit of *A. scholaris* was collected in from nearby area of Moradabad particularly from Gool Chakkar Khushalpur. This plant material was taxonomically identified. The identification of the fresh plant material was done by Dr. Sunita Garg, Former Chief Scientist and Head RHMD at CSIR-NIScPR, New Delhi. A sample

receipt of the plant has been placed under the voucher number NIScPR/RHMD Consult/2022/4125.

2.4. Powder sample preparation

After collecting the fresh fruits of *A. scholaris*, the fruits were well cleaned with normal tap water, then two times using distilled water. They were then kept dry for between ten to fifteen days at ambient temperature in the shade. The shade-dried fruits of *A. scholaris* had been

broken into little bits. After all the dried materials were completely crushed and ground into a coarsely powdered form, it was stored at room temperature until it was needed for additional research.

2.5. Preparation of extract

Firstly, the completely dried up fruits powder (200g) of *A. scholaris* was defatted using 2000ml petroleum ether (60-80º) for the removal of extra fats to a period of 48 hrs. Following the marc was completely dried and extracted using 2000ml ethanol (95%v/v) in Soxhlet apparatus at 40-50ºC. The filtration process was carried out utilizing the Whatmann filter paper no 1. Following filtration, the solvent was eliminated under a lower pressure at 50°C with rota evaporator to get ethanolic extract. The percentage yield of petroleum ether and ethanolic extract of the fruit of *A. scholaris* (EEAS) were estimated and dry extract was collected and preserved in a refrigerator in an air-tight container for further studies.

2.6. Qualitative and quantitative spectrophotometric studies of phytoconstituents

Total phenolic content (TPC) and total flavonoid content (TFC) in EEAS were determined by using Folin-Ciocalteu colorimetric and Aluminum chloride colorimetric method respectively conducted by using standard procedures to identify some major phytochemical constituents like phenols and flavonoids.

2.6.1. Total phenolic content (TPC)

Typically, most of the phenolic substances are present in food products and non-edible vegetation. The phenolic compounds have been linked to a variety of potential biological impacts. Chemical compounds with an aromatic hydroxyl group (-OH) that bond directly altogether are referred to phenols or phenolics. The most fundamental class of phenols is carboxylic acid or C_6H_5OH [23] According to the quantity of phenols components in the molecular, phenols are categorized as simple phenols or polyphenols. It has been proposed that flavonoid and other phenolic compounds may help prevent the onset of cardiovascular diseases and malignancy [24] McDoland et al., 2001, described the folin-ciocalteu reagent (FCR) substance, which was utilized to estimate the phenolic compounds determination. Aqueous 40µl Na_2CO_3 (1.0M) and diluted 50µl folin ciocalteu reagent were combined with various test sample dilutions. The solution was heated on water bath at 45ºC for 15 minutes and the total phenolic content was estimated. The absorbance was measured 760nm using a spectrophotometer. The standard curve was formed using 0, 50, 100, 150, 200, 250 and 300µg/ml. The standard curve of gallic acid was prepared in ethanol: water (50:50 v/v) at concentrations 25µg/ml to 300µg/ml. Extracts from herbs or plants include phenols that are oxidized by FCR reagent and turn a deep blue color that can be noticed using an ultraviolet visible spectrophotometer. Each experiment had been done three times, and the calibration curve was established with the average absorbance measurements achieved at various concentrations of gallic acids. The TPC of the extracts was quantified as milligrams of gallic acid equivalent (GAE) per gram of dry weight sample (mg/g) [25]. The TPC in all samples was determined with the following equation 1. $C = c\frac{V}{m}$ (1).

Where C=Total Phenolic content mg GAE/g dry extract, c= gallic acid concentration from calibration curve in mg/mL, V= plant extract volume in mL and m= plant extract mass in gram.

2.6.2. Total flavonoids content (TFC)

Flavonoids are 15-carbon containing polyphenolic substances that are soluble in water. Among the most well-known flavonoids include anthocyanidins, rutin, kaempferol, quercetin and catechins. Through their antioxidant properties and pathways of cell signaling flavonoids improve life [26]. Flavonoids are a class of plant compounds that has been identified for its ability to inhibition of oxidative and hydrolytic enzymes, anti-inflammatory activity and free radical scavenging activity. Because polyphenolic substances have powerful antioxidant effects and may protect toward long-term conditions like heart issues, neurological disorders, cataracts, and several types of malignancies, there has been a recent surge in fascination with plant-based products abundant in these compounds [27]. The aluminum chloride technique was employed for assessing the flavonoids compound. Quercetin was served as the reference material in this procedure, and the concentrations of flavonoids were quantified in standings of quercetin equivalent. The quercetin calibration curve was created for this reason. In a volumetric flask add 4ml distilled water and 1ml of standard or extract solution (20, 40, 60, 80 or 100mg/l) was added. 5% $NaNO_2$ in the amount of 0.3 ml was transferred to the flask. 0.3ml of 10% $AlCl_3$ was transferred to the solution mixture. Add 2ml of sodium hydroxide (1M) and make up the volume up to 10ml with distilled water at the 6th min. The UV-Visible spectrophotometer was used to record the absorbance at 510nm. The linear equation of the calibration curve of standard was employed to quantify the total flavonoid content in term of milligram of quercetin equivalent per gram (mg QE/g) [28].

2.7. LCMS analysis

2.7.1. Liquid Chromatography-Mass Spectroscopy (LCMS) of EEAS

Liquid Chromatography Mass Spectrometry (LCMS) is fast emerging as the tool of choice for liquid chromatographers. It can be applied to biological molecules. The LCMS information can be utilized to yield information regarding the molecular weight, structure, identity and amount of specific sample constituents [29].

- *Experimental/Instrumental conditions*

- Type of samples : EEAS
- Name of column : C18 (Sunfire)
- Wavelength : 280nm
- Flow rate : 0.2 ml/minute
- Solvent System (A) : Acetonitrile
- Solvent System (B) : $HCOONH_4$ buffer

The chemical constituents of ethanolic extract of fruits of *A. scholaris* (EEAS) were determined with the help of LCMS analysis. The LC-MS analysis was performed at CSIR, CDRI Lucknow, using liquid chromatography mass spectroscopy system (ACQ-TQD#QBB1152) with C18 column (ACCUCORE C18, 150×4.6, 2.6µm). The 100mg ethanolic extract of fruits of *A. scholaris* (EEAS) was centrifuged at 12000 rpm for 10 min previous to LCMS investigation. An amount of 10µl used for the LCMS analyses. The mobile phase contains two solvents- solvent A (Acetonitrile) and solvent B (Ammonium formate). The mobile phase solvents were delivered at a flow rate of 1ml/minute in gradient elution. The temperature of the column was kept at 35°C with a pressure minimum of 0 and a maximum of 300 bar. The mass spectra were recorded in positive and negative modes between m/z 150-2000. The constituents were detected at a wavelength of 280nm. The mass fragments have been recognized through the utilization of the spectrum database for organic compounds within SDBS applications [30].

2.8. In-vitro antioxidant assay of EEAS by DPPH method

Antioxidants are natural and artificial substances that may have the ability to scavenging free radicals and to prevent the process of oxidation within cells produced by free radicals. Many studies are now underway to identify naturally occurring antioxidants derived from plants. Research conducted in vivo on biologically active substances derived from herbal vegetables and plants provides compelling evidence that plant components possessing antioxidant properties can provide defense towards reactive oxygen species/free radicals inside cells. The antioxidant properties of medicinal plant's phytochemicals, which are typically linked to a variety of molecules called amphipathic and together referred to as polyphenolic substances, have been linked to the plants' ability to prevent and manage illness [31]. Antioxidants lower the risk of developing a variety of illnesses, including neurodegenerative and nephrotoxicity, aging, inflammation, cancer, heart disease, liver disease and cataract. It is believed that antioxidants found in food may be able to prevent ailments caused by oxidative stress [32].

2.8.1. Preparation of 0.M DPPH solution

0.1M DPPH solution is prepared by dissolving 0.39mg DPPH dissolved in 10ml ethanol flask in a volumetric flask and makeup the final volume up to 100ml. This solution was stored in refrigerator at -20° for further analysis.

2.8.2. Preparation of extract solution

To prepare the stock solution of sample (EEAS) weighed the 10mg of EEAS and dissolved it in 10ml ethanol (1mg/ml). From this stock solution different concentration like 1, 10, 50, 100, 250, 500 and 1000µg/ml were prepared.

2.8.3. Procedure- This assay employs DPPH, a free radical. 5µl of different concentrations of (1, 10, 50, 100, 250, 500 and 1000µg/ml) of the EEAS stock solution were mixed with to 0.1 ml of 0.1 M DPPH solution in well plates. The reaction was conducted in triplicate, and a duplicate blank was made using 0.2ml of ethanol and 5µl of the chemical at various concentrations (0.78, 1.56, 3.12, 6.25, 12.25, 25 and 50µg/ml). The wells without treatment were designated control. The well plated was incubated for the next thirty minutes in darkness. The decolorization was read at 517nm after the incubation using micro plate reader (iMark, BioRad). Reaction mixture with 20µl of de-ionized water was used as Control. Ascorbic acid was standard. IC_{50} extracts were obtained from graph as concentration vs percentage inhibition. The free radical scavenging potential was represented in terms of inhibition percentage. The IC_{50} value is the concentration of sample necessary to scavenge 50% of free radical DPPH. Observations were recorded in triplicates. The IC_{50} value of EEAS provides the respective concentration in which radical scavenging activity is 50%. Graphically, IC_{50} value of EEAS and standard was calculated. The percentage inhibition was determined by applying the formula given below equation 2.

$$\%\mathrm{I} = \frac{\mathrm{AC} - \mathrm{AO}}{\mathrm{AC}} \times 100 \quad (2).$$

Where, %I= percentage inhibition, AC= Absorbance of control, AO= Absorbance of sample solution [33].

2.9. Statistical analysis

All measurements were presented in the form of the mean ± SEM (n=3). The outcomes were examined using a unifactorial analysis of variance (ANOVA) completely randomized with a 95% confidence level. Tuckey's means comparison test was used in identifying treatments with significant differences.

3. RESULTS

3.1. Assessment of total phenolic content (TPC)

Estimation of TPC in ethanolic extract of *A. scholaris* fruits (EEAS) was estimated by folin-ciocaltue reagent (FCR) method with gallic acid as standard. Various test sample dilutions were mixed with 50µl FCR and aqueous 40µl Na_2CO_3 (1.0 M). The mixture was heated on a water bath at 45°C for 15 minutes and total phenolic content was estimated. Absorbance was measured at 760 nm by a spectrophotometer. 0, 50, 100, 150, 200, 250 and 300µg/ml were used to prepare standard curve. Standard curve of gallic acid was prepared in ethanol: water (50:50 v/v) at a concentration of 25µg/ml to 300µg/ml. Absorbance measured at various concentrations of gallic acid were utilized to construct the calibration curve. The total phenolic content was quantified as a milligram of gallic acid equivalent per gram of extract (mg GAE/gm). TPC of extract was determined using the calibration curve regression equation (Y=0.0001x; R^2=988). The results of TPC in EEAS were presented in Table 1 and Figure 2.

Table 1 Estimation of total phenolic content (TPC) presented in EEAS

S.NO	Name of Plant	Total Phenolic content (mg GAE/gm)
1	*A. scholaris*	400

The data were presented as mean ± SEM;(n=3).

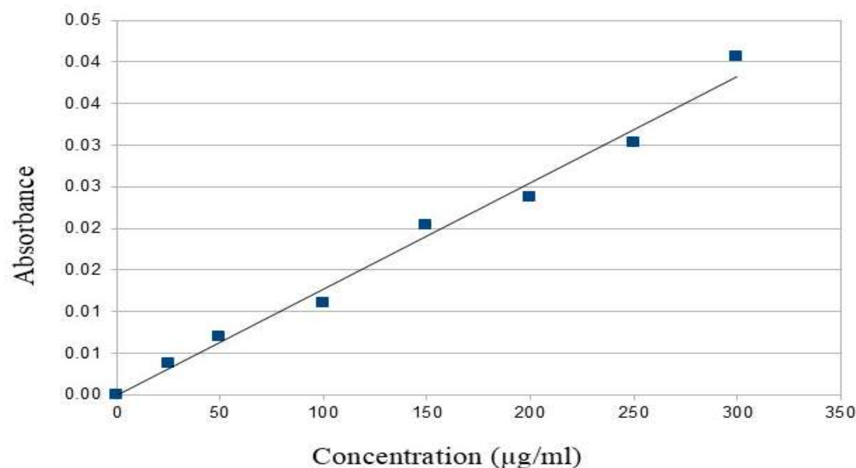

Figure 2. Standard calibration curve for determination of Total Phenolic Content (TPC)

3.2. Assessment of total flavonoid content (TFC)

The total flavonoids content in ethanolic extract of fruits of *A. scholaris* (EEAS) was measured by aluminum chloride ($AlCl_3$) assay using quercetin as standard. The Standard solution of quercetin was prepared in dilution range (10,20,40,60, 80 and 100µg/ml) and three sample dilutions were prepared (0.1, 1, 10 mg/ml). 10µl of extracts (at three different dilutions) or standard solution was taken in 96 well plates. 10 µl of 10% $AlCl_3$ solution and followed by 170µl of 60% ethanol was added to the wells. Finally, 10µl of 1M sodium acetate was incorporated into the solution within a 96-well plate and incubated for 30 minutes at ambient temperature. Suitable blanks were established for the examination. The plate was analyzed at 517 nm using a microplate reader (iMARK, BIORAD, USA). The absorbance values recorded at various concentrations of quercetin were utilized to produce the calibration curve. The total flavonoid content of the extract was measured using the regression equation of the calibration curve (Y=0.0009x; R^2=0.9999). The total flavonoid content was quantified as milligrams of Quercetin Equivalents per gram (mg QE/gm) of plant extract. The result of TFC in EEAS was tabulated in Table 2 and Figure 3.

Table 2 Estimation of Total Flavonoids content (TFC) in EEAS

S. No	Name of Plant	Total Flavonoids content (mg QE/mg)
1	*A. scholaris*	31.11

The data were presented as mean ± SEM;(n=3).

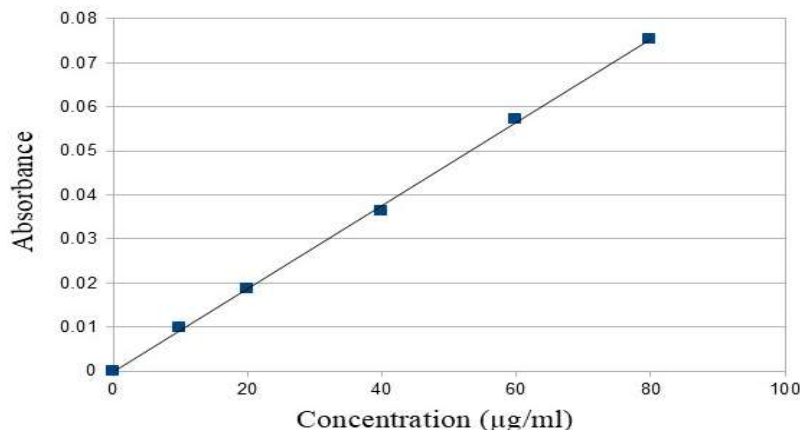

Figure 3. Standard calibration curve for determination of Total Flavonoids Content (TFC).

3.3. LCMS analysis of EEAS

The ethanolic extract of fruits of *A. scholaris* (EEAS) was subjected to LCMS for the identification of probable phytoconstituents. Several phytoconstituents were detected in positive (ESI+) and negative modes (ESI-). The lists of phytoconstituents obtained from the LCMS analysis are given in Table 3 and 4. About 38 phytoconstituents were identified in positive mode and 26 phytoconstituents identified in negative mode. The LCMS chromatograms of EEAS are shown in Figure 4 and Figure 5. The mass of the identified phytoconstituents compared with marker compound using SDBS database. The identified compounds belong to the different classes of secondary metabolites such as Alkaloids, flavonoids, phenolic compound, amino acids, carbohydrates, fat, lipid, hormones, ester, vitamin, coloring pigments and fatty acids.

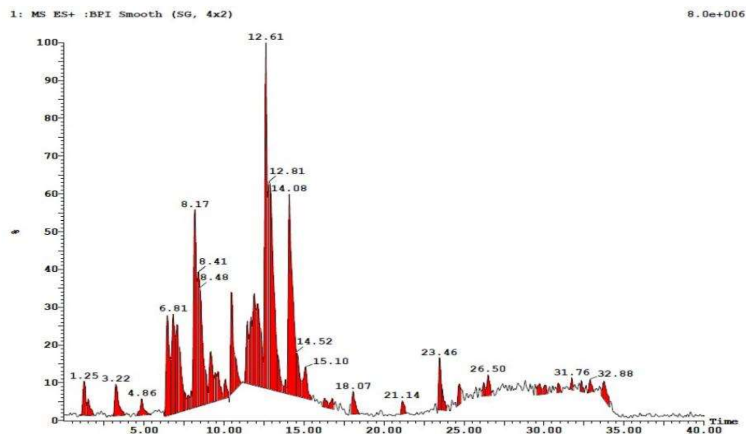

Figure 4. LCMS Chromatogram of EEAS in positive mode (ESI+).

Table 3 LC-MS profile of EEAS in positive mode (ESI+)

R. Time	Compound Name	Ion	Formula	Exact Mass	Observed Mass	Mass Diff
1.21	4-Hydroxy-3-methoxycinnamic acid	[M+H]+	$C_{10}H_{10}O_4$	194.18	195.5109	-1.3309
1.31	Pterine	Positive	$C_6H_5N_5O$	163.049	163.4313	-0.3823
3.22	Allantoin	[M+H]+	$C_4H_6N_4O_3$	158.12	158.4362	-0.3162
4.86	Kelampayoside A	Positive	$C_{20}H_{30}O_{13}$	478.44	479.4340	0.994
6.43	N-acetylneuraminic acid	Positive	$C_{11}H_{19}NO_9$	309.105	309.5842	-0.4792
6.77	Chlorogenic acid Hemihydrate	Positive	$C_{16}H_{18}O_9$	354.095	357.6852	-3.5902
6.94	Chlorogenic acid Hemihydrate	Positive	$C_{16}H_{18}O_9$	354.095	357.6111	-3.5161
7.05	N-Acetyl-Phytosphingosine	Positive	$C_{20}H_{41}NO_4$	359.303	357.6111	1.6919
7.12	N-Acetyl-Phytosphingosine	Positive	$C_{20}H_{41}NO_4$	359.303	357.6482	1.6548
7.29	N-Acetyl-Phytosphingosine	Positive	$C_{20}H_{41}NO_4$	359.303	357.7222	1.5808
8.14	(-)-Riboflavin	Positive	$C_{17}H_{20}N_4O_6$	376.138	371.6714	4.4666
8.38	(-)-Riboflavin	Positive	$C_{17}H_{20}N_4O_6$	376.138	371.7084	4.4296
8.51	(-)-Riboflavin	Positive	$C_{17}H_{20}N_4O_6$	376.138	871.7084	-495.57

8.65	DSS (Chemical Shape Indicator)	Positive	$C_{16}H_{20}N_2O_8$	368.121	371.6714	-3.5504
9.13	Sinapoyl malate	Positive	$C_{15}H_{16}O_9$	340.079	341.7008	-1.6218
9.27	Docosanoic acid from Rapeseed	Positive	$C_{22}H_{44}O_2$	340.334	341.6268	-1.2928
10.46	Chlorogenic acid Hemihydrate	Positive	$C_{16}H_{18}O_9$	354.095	353.6521	0.4429
10.77	trans-Zeatin-riboside	Positive	$C_{15}H_{21}N_5O_5$	351.154	353.7261	-2.5721
11.45	Raffinose	Positive	$C_{18}H_{32}O_{16}$	504.095	505.6521	0.4429
11.69	Chlorogenic acid Hemihydrate	Positive	$C_{16}H_{18}O_9$	354.095	353.6521	0.4429
11.89	DSS (Chemical Shape Indicator)	Positive	$C_{16}H_{20}N_2O_8$	368.121	371.6344	-3.5134
12.13	DSS (Chemical Shape Indicator)	Positive	$C_{16}H_{20}N_2O_8$	368.121	371.6714	-3.5504
12.61	Solasodine	Positive	$C_{27}H_{43}NO_2$	413.329	413.7043	-0.3753
12.81	Solasodine	Positive	$C_{27}H_{43}NO_2$	413.329	413.7413	-0.4123
12.92	Puerarin	Positive	$C_{21}H_{20}O_9$	416.11	413.7413	2.3687
13.09	Puerarin	Positive	$C_{21}H_{20}O_9$	416.11	413.7043	2.4057
13.22	Solasodine	Positive	$C_{27}H_{43}NO_2$	413.329	413.7413	-0.4123
14.04	Sinapoyl malate	Positive	$C_{15}H_{16}O_9$	340.079	339.6288	0.4502
14.11	Sinapoyl malate	Positive	$C_{15}H_{16}O_9$	340.079	339.6658	0.4132
14.21	5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranosyl 5'-monophosphate	Positive	$C_9H_{15}N_4O_8P$	338.062	339.6288	-1.5668
14.35	beta-Nicotinamide mononucleotide	Positive	$C_{11}H_{15}N_{2}O8P$	334.056	339.6288	-5.5728
14.42	N-6-(delta-2-Isopentenyl) adenosine hemihydrate	Positive	$C_{15}H_{21}N_5O_4$	335.159	339.7026	-4.5436
14.52	Nicotinic acid mono nucleotide	Positive	$C_{11}H_{14}NO_9P$	335.04	337.7047	-2.6647
15.14	5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranosyl 5'-monophosphate	Positive	$C_9H_{15}N_4O_8P$	338.062	337.5938	0.4682
21.14	Quercetin	Positive	$C_{15}H_{10}O_7$	302.24	303.4013	1.1613
23.46	Adenosine-3',5'-cyclicmonophosphate	Positive	$C_{10}H_{12}N_5O_6P$	329.052	329.6386	-0.5866
23.53	Adenosine-3',5'-cyclicmonophosphate	Positive	$C_{10}H_{12}N_5O_6P$	329.052	329.6016	-0.5496
26.50	Safranine	Positive	$C_{20}H_{19}N_4$	315.16	313.7283	1.4317
29.30	Cyanidin-3-O-(6''-O-(E-p-coum)-2''-O-(beta-xylopyranosyl)-beta-glucopyranoside)-5-O-beta-glucopyranoside trifluoroacetate salt	Positive	$C_{41}H_{45}O_{22}$	889.24	888.2758	0.9642
31.76	Cyanidin-3-O-(6''-O-(E-p-coum)-2''-O-(beta-xylopyranosyl)-beta-glucopyranoside)-5-O-beta-glucopyranoside trifluoroacetate salt	Positive	$C_{41}H_{45}O_{22}$	889.24	888.2758	0.9642

The data were presented as mean ± SEM;(n=3).

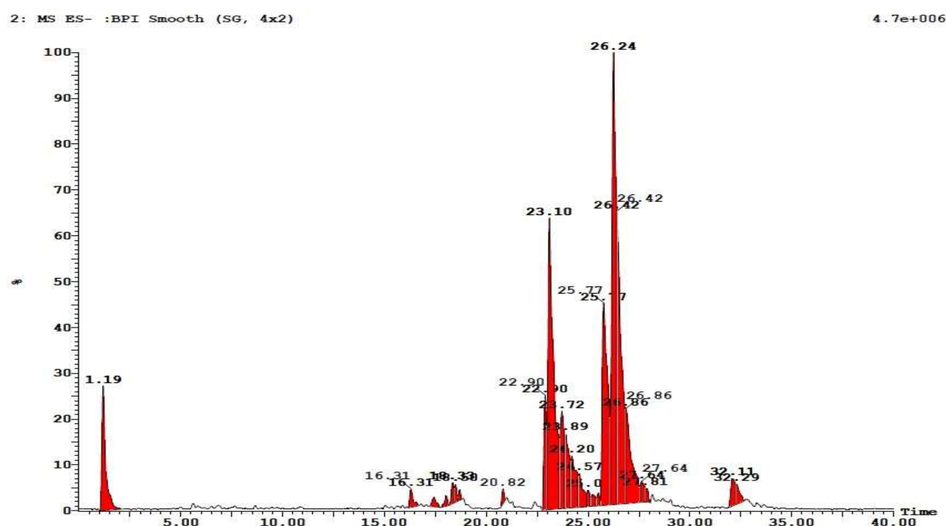

Figure 5. LCMS Chromatogram of EEAS in negative mode (ESI-).

Table 4 LC-MS profile of EEAS in negative mode (ESI-)

R. Time	Compound Name	Ion	Formula	Exact Mass	Observed Mass	Mass Diff.
1.16	Scopoletin	Negative	$C_{10}H_8O_4$	192.042	191.5148	0.5272
1.23	Galactinol Dihydrate	Negative	$C_{12}H_{22}O_{11}$	342.116	341.6638	0.4522
18.33	isorhamnetin-3-rutinoside	Negative	$C_{28}H_{32}O_{16}$	624.169	618.0964	6.0726
20.83	6-Phosphogluconic acid Barium salt hydrate	Negative	$C_6H_{13}O_{10}P$	276.024	277.6895	-1.6655
22.87	Riboflavin-5′-monophosphate sodium salt hydrate	Negative	$C_{17}H_{21}N_4O_9P$	456.104	455.9591	0.1449
23.07	gamma-Linolenic acid	[M-H]-	$C_{18}H_{30}O_2$	278.43	279.7616	-1.3316
23.10	L-saccharopine	Negative	$C_{11}H_{20}N_2O_6$	276.132	279.7616	-3.6296
23.28	6-Phosphogluconic acid Barium salt hydrate	Negative	$C_6H_{13}O_{10}P$	276.024	279.7616	-3.7376
23.38	gamma-Linolenic acid	[M-H]-	$C_{18}H_{30}O_2$	278.43	279.7616	-1.3316
23.51	Riboflavin-5′-monophosphate sodium salt hydrate	Negative	$C_{17}H_{21}N_4O_9P$	456.104	455.9961	0.1079
23.58	Riboflavin-5′-monophosphate sodium salt hydrate.	Negative	$C_{17}H_{21}N_4O_9P$	456.104	455.9221	0.1819
23.72	Kaempferol-3-Glucuronide	Negative	$C_{21}H_{18}O_{12}$	462.079	455.9591	6.1199
23.89	Riboflavin-5′-monophosphate sodium salt hydrate	Negative	$C_{17}H_{21}N_4O_9P$	456.104	455.9591	0.1449
24.03	Riboflavin-5′-monophosphate sodium salt hydrate	Negative	$C_{17}H_{21}N_4O_9P$	456.104	455.9221	0.1819
24.23	Kaempferol-3-Glucuronide	Negative	$C_{21}H_{18}O_{12}$	462.079	455.9591	6.1199
25.73	2'-Deoxyinosine	Negative	$C_{10}H_{12}N_4O_4$	252.085	255.7111	-3.6261
25.77	D-Glucosamine-6-phosphate sodium salt	Negative	$C_6H_{14}NO_8P$	259.045	255.7851	3.2599
25.90	alpha-D-glucose-1-phosphate dipotassium salt dihydate	Negative	$C_6H_{13}O_9P$	260.029	255.7481	4.2809
26.21	acacetin	Negative	$C_{16}H_{12}O_5$	284.068	281.7966	2.2714
26.31	Xanthosine	Negative	$C_{10}H_{12}N_4O_6$	284.075	281.7966	2.2784
26.38	gamma-Linolenic acid	[M-H]-	$C_{18}H_{30}O_2$	278.43	281.7966	-3.3666
26.65	Luteolin	Negative	$C_{15}H_{10}O_6$	286.047	281.7596	4.2874
26.86	acacetin	Negative	$C_{16}H_{12}O_5$	284.068	281.7966	2.2714
26.93	acacetin	Negative	$C_{16}H_{12}O_5$	284.068	281.7966	2.2714
27.61	acacetin	Negative	$C_{16}H_{12}O_5$	284.068	281.7966	2.2714
32.08	Eriodictyol	Negative	$C_{15}H_{12}O_6$	288.063	283.7577	4.3053

The data were presented as mean ± SEM;(n=3).

3.4. *In-vitro* antioxidant activity of EEAS by DPPH method

In-vitro antioxidant potential of EEAS was determined by DPPH inhibition method. In this assay, ascorbic acid was taken as the reference antioxidant. According to the findings achieved from the research, antioxidant property (DPPH scavenging) was detected in ethanolic extract of fruits of *A. scholaris* (IC_{50}=145.3±0.091µg/ml), as compared to standard ascorbic acid (IC_{50} = 8.116±0.013µg/ml). 145.3µg of the sample ethanolic extract of fruits of *A. scholaris* was found equivalent to 8.116µg of reference ascorbic acid. The data of antioxidant activity was represented in Figure 6 and 7.

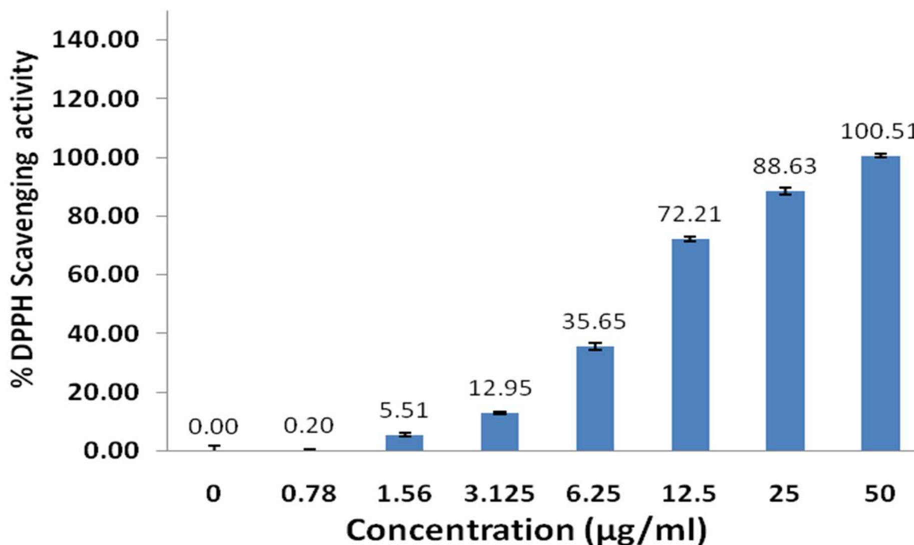

Figure 6. Percentage of antioxidant activity of Ascorbic acid.

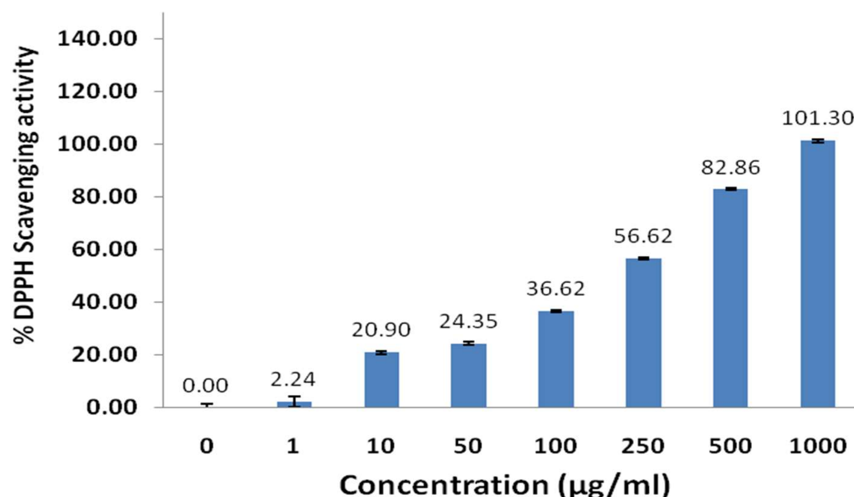

Figure 7. Percentage of antioxidant activity of *A. scholaris.*

4. DISCUSSION

Medicinal plants have significant functions in our day-to-day life to cure numerous diseases and disorders. Medicinal plant research indicates the acknowledgment of the efficacy of numerous herbal products. Plants are a significant source of natural commodities and drugs since Ayurveda one of the oldest systems of medicine used in India. In developing nations like India various plant-based medicine, also referred to as herbal medicine are employed [34]. *A. scholaris* of family Apocynaceae known as Indian devil tree has been utilized as folklore medicines [35]. The various parts of plant may have various phytochemical compounds, which may be responsible for various pharmacological effects of each part. Plants yield numerous significant compounds like phenolics and flavonoids which contain antioxidant [36]. Phenolics and flavonoids protect against free radicals and control different oxidative reactions that naturally take place. In addition, they are utilized for protecting food quality primarily through prevention of oxidative deterioration of lipid constituents [37]. The phenolic compounds are significant phytoconstituents with redox activity, thereby conferring antioxidant potential because hydroxyl groups in such compounds. They are also thought to be responsible for contributing a significant part of the antioxidant capacity in numerous plants. The TPC in EEAS was measured from

regression equation (Y=0.0001x; R^2=0.999) of calibration curve and reported as mg GAE/g of dry weight of sample. The TPC present in EEAS was found to be 400mg GAE/gm. Flavonoids represent a significant category of phenolic chemical compounds exhibiting a wide range of chemical and biological activities, especially radical scavenging and antimicrobial activity. Flavonoids are found to occur universally in plants and exert positive health benefits. Investigations on flavonoid derivatives have established antibacterial, antiviral, anti-inflammatory, anticancer, and anti-allergic activity. As quantitative basis determination, flavonoid content in some selected plant extracts was quantified using aluminum chloride in a colorimetric assay [38]. Total Flavonoid content of ethanolic extract of fruit of *A. scholaris* (EEAS) was quantified by aluminum chloride using a colorimetric assay. Total flavonoid content (TFC) in EEAS was derived from the regression equation of the calibration curve (Y= 0.0009x; R^2= 0.999) and was defined mg QE/g dry weight of sample. The flavonoid content in EEAS was found to be 31.11mg QE/gm. Liquid chromatography Mass Spectroscopy (LCMS) is a method for identification, quantization and mass determination of a broad range of semi-volatile or non-volatile organic and inorganic compounds in a mixture. LCMS is another strong tool for the analysis of complex botanical extract [39]. In the current LCMS study total 58 phytocompounds were identified in positive and negative mode. The detected compounds belonged to different groups. The LCMS of EEAS revealed the different peaks at different retention times. The weight of the identified compounds against marker compounds with SDBS database. Plant antioxidant activity is largely because they contain potent phytoconstituents. This may be caused by more than just a high percentage of major constituents, but also due to the occurrence of other constituents in trace amounts [40]. The Plant natural antioxidants are the ones that inhibit or prevent oxidative stress. In this research, free radical scavenging percentage was evaluated. The decrease in DPPH radical absorbance results from the relationship among antioxidant molecules and the radical, facilitating radical scavenging by hydrogen transfer [41]. Here, antioxidant activity of *A. scholaris* fruit extracts was assessed using DPPH radical scavenging assay. The ascorbic acid was used as a standard antioxidant agent. From results yielded by the research, antioxidant activity (DPPH scavenging) was found in ethanolic fruit extract of *A. scholaris* (IC_{50}=145.3±0.091μg/ml), when compared with standard ascorbic acid (IC_{50}=8.116±0.013μg/ml). The lower the IC_{50} value, the more effective is the compound at scavenging DPPH and this signifies greater antioxidant activity.

5. Conclusion

Phenolic and flavonoids groups are greatly responsible for antioxidant potential. The phenolic and flavonoid content related to the antioxidant potential of a medicinal plant. In ethanolic extract of fruit of *A. scholaris* (EEAS), the total phenolic, total flavonoid and antioxidant activity present significantly. The LCMS profile of the ethanolic extract of fruits of *A. scholaris* evaluated 58 compounds in both positive and negative mode. The outcome of the current research revealed that the fruit of *A. scholaris* may be the high source of natural antioxidants due to their phenolic, flavonoid contents and their outstanding scavenging activity on DPPH. To our best knowledge, current study was the first attempt to assess the potential of the ethanolic fruit extract of *A. scholaris* (EEAS) to function as antioxidant agents. Thus, a valid antioxidant assessment system must incorporate various antioxidant action tests to consider multiple pathways of antioxidant potential. The evidence obtained from this research work supports that *A. scholaris* would be good for the development of formulation for treating various diseases.

6. ABBREVIATIONS

EEAS	Ethanolic Extract of *Alstonia scholaris*
TPC	Total Phenolic Content
TFC	Total Flavonoid Content
LCMS	Liquid Chromatography Mass Spectroscopy
IC_{50}	Half Maximal Inhibitory concentration
DPPH	2,2-diphenyl-1-picrylhydrazyl
GAE	Gallic Acid Equivalent
QE	Quercetin Equivalent

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Author contribution

Vivek Kumar: data curation, formal analysis, writing–original draft preparation, visualization, investigation. **Asheesh Kumar Gupta:** conceptualization, methodology, resources, writing–review and editing, supervision. All authors read and approved of the final manuscript.

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Conflicts of interest
The authors declare that they have no conflict of interest.

Ethical approval
None to declare

Data Availability
This research paper includes all generated and analyzed data. The data are available on reasonable requests.

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