

# Analytical Evaluation Of Antioxidant Activity Through Gc-MS Profiling: A Comparative Study Of Phytochemical Constituents In *Alstonia Venenata* Leaf Extract

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## Abstract

**Objective:** The present study aimed to evaluate the phytochemical composition of *Alstonia venenata* (R. Br.) leaf extracts using gas chromatography–mass spectrometry (GC–MS) and to assess their in vitro antioxidant activity.

**Methods:** Leaves of *Alstonia venenata* were sequentially extracted using hexane, ethyl acetate, ethanol, and water to obtain solvent fractions of varying polarity. The extracts were analyzed by GC–MS to identify volatile and semi-volatile phytochemicals. Antioxidant activity was evaluated using DPPH radical scavenging and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) scavenging assays, and IC<sub>50</sub> values were determined.

**Results:** GC–MS analysis revealed the presence of several phytochemical classes including terpenoids, phenolic compounds, fatty acid derivatives, and alkaloids. Among the tested extracts, the ethanolic fraction exhibited the strongest antioxidant activity with IC<sub>50</sub> values of **19.97 µg/mL for DPPH** and **2.27 µg/mL for the H<sub>2</sub>O<sub>2</sub> assay**.

**Conclusion:** The findings indicate that *A. venenata* leaves contain diverse phytochemical constituents and demonstrate significant **in vitro antioxidant activity**, particularly in the ethanolic extract. Further studies are required to isolate and characterize the active compounds responsible for the observed antioxidant activity.

**KEYWORDS:** *Alstonia venenata*, GC–MS analysis Phytochemical profiling Antioxidant activity DPPH scavenging assay

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## INTRODUCTION

*Alstonia venenata* (R. Br.) is a member of the family Apocynaceae [1], and a comparatively unexplored although potentially significant to pharmacology, native to the Indian subcontinent [2, 3]. The plant is naturally distributed across several regions of South and Southeast Asia, where it has long been used in various traditional medicinal systems [4]. Its root and leaves have traditionally been used in the treatment of a variety of afflictions including fever, neurological and skin [5]. Thus, a traditionally deep-rooted and ethnobotanical awareness of medicinal properties of this plant has prompted scientific research on its phytochemistry [6].

Recent studies have demonstrated that *A. venenata* produces several pharmacologically important secondary metabolites including alkaloids, flavonoids, saponins, steroids, tannins, triterpenoids, and glycosides [7, 8]. Such substances may contribute to various pharmacological activities such as antimicrobial, neuroprotective, and anti-inflammatory [9]. Notably, echitamine as well as venenatine are alkaloids that make significant contribution to such pharmacological activities [10, 11]. These are the major therapeutic agents of the plant and *A. venenata* being a prime and promising candidate in the natural drug discovery.

## Oxidative Stress and Neuroprotection

It is the neuroprotective effects of *A. venenata* that result, in the first place, due to its ability as an antioxidant. Oxidative stress results from an imbalance between reactive oxygen species production and antioxidant defense mechanisms and is associated with the development of several neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis [12, 13]. Excess of ROS damages cell lipids and proteins, as well as, nucleic acids and hence affects the neuronal activities or causes cell death [14].

Antioxidants reduce this kind of damage by scavenging free radicals, chelating metal transition metals and preventing lipid peroxidation cascades [15]. Based on this, naturally extracted antioxidants in medicinal plants are getting more and more recognitions on their compatibility and safety. Many phytochemicals, especially the polyphenolic and flavonoid, and alkaloid groups, have very strong radical-quenching and neuroprotective properties, placing them in a position to replace the oxidative-stress-induced neurological diseases [16].

## Phytochemical Profiling Using GC–MS

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To fully comprehend the efficacy of medicinal plants, the chemical composition of the plants needs to be explained. Gas Chromatography-Mass Spectrometry (GC-MS) is considered to be one of the strongest analytical options in characterizing volatile and semi-volatile metabolites present in complicated plant samples. GC-MS combines separating ability of gas chromatography with mass spectrometry which identifies the compounds automatically, both qualitative and quantitative [17].

In this way, researchers are able to detect fatty acids, esters and terpenoids, Phenolics and alkaloids which are useful as a combination to biological activity. High sensitivity, reproducibility and specificity are the benefits of GC-MS that allow creating separate chemical fingerprints to be compared. It can be used to directly evaluate the effect of solvent selection on the extraction yield and metabolite diversity when used with solvents of varying polarity with extracts prepared by the solvents. Lipid solubility A highly polar solvent like water extracts glycosides and carbohydrates, a moderately polar solvent like ethyl acetate and ethanol extracts phenolics, flavonoids, and alkaloids, and a solvent like hexane, with low polarity, extracts lipid soluble terpenes and hydrocarbons. Comparative profiling of this nature offers a critical connection between the chemical profile of an extract and its biological activity, specifically, antioxidant behaviour.

#### Rationale of the Study

Although there is an extended medicinal background of *A. venenata*, there has been limited adoption of systematic assessment of the leaf extracts of the plant in solvents with varying polarity. Besides, it is essential to correlate its phytochemical profile with a set of quantitative antioxidant tests to support its putative neuroprotective and therapeutic potential. The determination of these relationships will be able to determine the solvent fraction with the strongest free-radical-scavenging potential as well as inform the isolation of active compounds to be used in future pharmacological development.

As there is an increasing world-wide challenge of oxidative stress-linked neurological illness and an unparalleled need to utilize plant-derived therapeutic agents, there is a timely and urgent perceptible need to conduct a sharp analytical examination of the antioxidant potential of *A. venenata*. The current study thus attempts to provide a scientific basis to its traditional uses by combining GC-MS chemical profiling with tested in vitro antioxidants, and hence the association of chemical diversity to functional bioactivity.

#### MATERIALS AND METHODS

The leaves of *Alstonia venenata* (R. Br.) were neatly gathered in Vandiperiyar in the Idukki District of Kerala in February, a month when the species was on its prime vegetative season.

##### Plant authentication

The collected plant material was authenticated by a qualified botanist from the **Department of Botany, St. Thomas College, Pala, Kerala**. A voucher specimen was deposited in the institutional herbarium for future reference.

The isolated specimens were carefully rinsed with running tap water to get rid of the dusts and debris on the surface and then a quick rinse with distilled water was taken. When the leaves were cleaned they were subsequently distributed uniformly in a shaded well-ventilated place where they were air-dried to last about fifteen days in order to have the phytoconstituents dried slowly without destruction. The completely dried product was put in the airtight containers to avoid any moisture absorption and chemical degradation. The verifiability of the plant was conducted by the Department of Botany, St. Thomas College, Pala, Kerala and a voucher specimen was attached to the institutional herbarium to be used in future..

The dry leaves were put through a mechanical grinder to fine powder and the product was taken through multiple solvent extraction in a Soxhlet apparatus. The 100 g of material extracted was separated sequentially using the solvents of decreasing polarity i.e. hexane (non-polar), ethyl acetate (moderately polar), ethanol (polar) and the distilled water (highly polar). The process of extraction cycle lasted about 12 hours till it was exhausted. The filtrates were concentrated using low pressure rotary evaporator and dried in a vacuum in order to eliminate the remaining traces of solvents. The airtight and refrigerated containers in which the residues were stored analyzed later. The hexane, ethyl acetate, and ethanol layers were present in dimethyl sulfoxide (DMSO) and then subjected to test and made the aqueous extract in the distilled water.

##### Preliminary Phytochemical Screening

Preliminary phytochemical screening was carried out using standard qualitative methods to detect the presence of major secondary metabolites including alkaloids, flavonoids, tannins, phenols, saponins, glycosides, terpenoids, carbohydrates, proteins, and starch. The qualitative phytochemical screening results are summarized in Table 1.

##### Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

###### Preparation of extracts for GC–MS analysis

The dried solvent extracts were prepared for GC–MS analysis by dissolving approximately 1–2 mg of each extract in 1 mL of HPLC-grade solvent corresponding to the extraction solvent. Hexane extracts were dissolved in hexane, ethyl acetate extracts in ethyl acetate or methanol, and ethanol extracts in methanol. The solutions were filtered through a 0.22 µm syringe filter to remove particulate matter and transferred into GC vials. A 1 µL aliquot of the filtered solution was injected into the GC–MS system for analysis.

##### GC–MS analysis

The GC–MS analysis was carried out using a Shimadzu Nexis GC-2030 instrument that was equipped with an electron impact (EI) ionization source operating at 70 eV. This setup was selected to ensure reliable and consistent ionization of the sample components. For the effective separation of compounds, an SH-I-5Sil MS capillary column with a length of 30 m, an internal diameter of 0.25 mm, and a film thickness of 0.25 µm was employed.

At the beginning of the analysis, the oven temperature was initially maintained at 70 °C. After allowing sufficient time for stabilization, the temperature was then gradually increased to a final value of 260 °C to allow proper separation of the compounds. The sample was introduced into the system using the split injection mode, and a split ratio of 10:1 was maintained throughout the run to prevent column overload and improve peak resolution.

To ensure smooth ion formation and efficient analysis, the ion source temperature was kept constant at 230 °C. At the same time, the interface temperature was maintained at 280 °C to support effective transfer of analytes from the column to the detector. A solvent delay time of 6.5 minutes was applied so that solvent-related signals would not interfere with compound detection.

Helium was selected as the carrier gas due to its inert nature and good separation efficiency. It was supplied at an inlet pressure of 62.1 kPa and allowed to flow through the column at a linear velocity of 36.8 cm/s. The mass spectral data were collected over a scan range of *m/z* 40–600, which enabled the detection of both low- and high-molecular-weight compounds.

The identification of the detected compounds was primarily achieved by comparing their mass spectral fragmentation patterns with those available in the NIST 20 standard library. In order to further improve the reliability of identification, the results were also cross-checked with an in-house alkaloid database. Key analytical information, including retention time (RT), molecular formula, molecular weight (MW), and the percentage composition of each compound, was carefully recorded and systematically arranged in tabular form for detailed interpretation and discussion.

### In Vitro Antioxidant Assays

Antioxidant capacity was evaluated using two complementary assays—DPPH radical scavenging and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) scavenging tests.

#### DPPH Radical Scavenging Activity

The working reagent in the antioxidant assay was a freshly prepared DPPH solution in ethanol in 0.1 mM. To this solution 1 mL was added with 3 mL of plant extract whose concentration had been prepared with the following concentration (12.5, 25, 50, 100 and 200 µg/mL). The mixture of the resulting reactions was thoroughly stirred and set to incubate at room temperature under dark conditions so that the mixture could saturate fully.

After incubation, absorbance of every sample at a wavelength of 517 nm was recorded using UV- visible spectrophotometer. The standard reference compound was ascorbic acid that was used in comparison. The proportion of free radical scavenging activity was then computed with the help of suitable standard formula.

In order to obtain the half-maximal inhibitory concentration (IC<sub>50</sub>) value, these percentages of inhibition were plotted against the respective extract concentrations and the regression analysis was performed in order to get the needed values.

#### Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) Scavenging Activity

Hydrogen peroxide scavenging activity was determined using a phosphate buffer system (50 mM, pH 7.4). A 1 mM hydrogen peroxide solution was prepared in the phosphate buffer. Different concentrations of plant extracts (12.5–200 µg/mL) were mixed with the H<sub>2</sub>O<sub>2</sub> solution in equal volumes. The reaction mixture was incubated at room temperature for 10 minutes, after which the absorbance was measured at 230 nm using a UV–Visible spectrophotometer with phosphate buffer as the blank. Ascorbic acid was used as the positive control. The percentage inhibition of hydrogen peroxide was calculated using the standard formula. Plant extract (taken at different concentrations) and, hydrogen peroxide solution, were added (in equal proportions) and allowed to interact with one another at room temperature to take place for 10 minutes. Incubation was followed and absorbance was measured at 230 nm with phosphates buffer as the blank and ascorbic acid as the positive control. The proper standard equation was then used to determine the peroxidase activity..

$$\% \text{ Scavenging} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100$$

• A<sub>control</sub> = Absorbance of H<sub>2</sub>O<sub>2</sub> + buffer (without extract)

• A<sub>sample</sub> = Absorbance of H<sub>2</sub>O<sub>2</sub> + extract

The results were expressed as mean (SD) and all the determinations were done in triplicate. Regression analyses were performed to find the IC<sub>50</sub> values of the various solvent extracts, which allowed the comparison of the different solvent extracts to determine the strongest antioxidant fraction.

## RESULTS AND DISCUSSION

### Phytochemical Composition Qualitative screening

Phytochemical Composition Qualitative screening of the *Alstonia venenata* leaf extracts showed a wide variety of bioactive secondary metabolites. They were distributed differently in the solvent systems. This is evident in Table 1 where it is noted that alkaloids, glycosides, flavonoids, phenols, carbohydrates, and proteins were always present in majority of the extracts. The ethanolic fraction was mostly composed of terpenoids, tannins, steroids, and coumarins. The extracts of the hexane and ethyl acetate had moderate amounts of alkaloids, glycosides, and saponins. All samples did not contain fixed oils, gums, and starches.

The phytochemicals were not as many in the aqueous extract and weaker reactions were observed in the various tests. The third phytochemical profile was the most broad and demonstrates the impact of solvent polarity on solubility and extraction efficacy of compounds. Dragendorff and Hagers reagents gave positive results and Salkowski test showed the presence of alkaloids in the ethanol fraction and steroids in a single test. The test of the Noller indicated only the presence of terpenoids in the ethanol extract indicating that ethanol is a good dissolve of the mid polar triterpenoid structures. Likewise, ferric chloride and lead acetate tests also showed the presence of phenolic substances and tannins in the ethanolic extract and the extract of ethyl acetate. The saponin test and ninhydrin test generated foam and purple color, respectively, which indicated the presence of saponins and amino acids within all the solvent fractions. Such data reinforce the idea of the chemical diversity of *A. venenata* leaves and suggest ethanol as the most effective solvent to extract all the active compounds.

#### GC-MS Analysis of Solvent Extracts

The GC-MS profiling provides a detailed picture of the chemical compounds of the different solvent extracts of *A. venenata*. Table 2-4 list the identified compounds, their retention time (RT) and their molecular formula, molecular weight (MW) and relative concentration (percent). The hexane extract (Table 2, Figure 1) contained eight compounds predominantly in the fatty acids, hydrocarbons and terpenoids. The major components were squalene (30.34%),  $\alpha$ -tocopherol (21.79%), and n-hexanedioic acid (13.19%). These are compounds referred to as antioxidant.

#### Phytochemical Composition

A preliminary qualitative screening of *Alstonia venenata* leaf extracts demonstrated a wide plant range of bioactive secondary metabolites, with extract solvent system ranging having significantly varying these. As shown in Table 1, alkaloids, glycosides, flavonoids, phenols, carbohydrates and proteins were also always found in most solvent fractions. Conversely, terpenoids, tannin, steroids and coumarins were mostly confined to the ethanolic extract, as an importance of solvent polarity on the extraction efficiency. Other reactions towards alkaloids, glycosides, and saponins were moderate in both hexane and ethyl acetate fractions and fixed oils, gums as well as starches were not found in any of the samples. Aqueous extract contained the lowest concentration of phytoconstituents and mostly weak reactions implying low solubility of most compounds in polar water systems.

The most diverse and the strongest phytochemical profile was presented by the ethanolic fraction. A confirmatory analyses on the presence of alkaloids with Dragendorff and Hager showed positive results, and Salkowski test showed the presence of steroids in the ethanol extract only. On the same note, Noller test also detected terpenoids only in the ethanolic fraction indicating that ethanol has a better capacity to dissolve

mid polar triterpenoid. Ferric chloride and lead acetate reaction with both ethanol and ethyl acetate were used to confirm the presence of phenolic compounds and tannins respectively. The evidence of the saponins and amino acids in all the solvent extracts was due to foam formation and purple coloration during the saponin and ninhydrin tests. All in all, these findings indicate the chemical enrichment of leaves of *A. venenata* and the utility of ethanol as the best solvent to extract the vast diversity of phytochemicals.

#### GC-MS Analysis of Solvent Extracts

In the profiling of the *A. venenata* leaf extracts, the gas chromatography-mass spectrometry (GC-MS) provided more than adequately provided environmentally informative chemical fingerprint of the extracts when the solvents varied.

The GC-MS chromatograms of the hexane, ethyl acetate, and ethanol extracts are presented in **Figures 1-3**, while the identified compounds with their retention times (RT), molecular weights (MW), and relative abundances are listed in **Tables 2-4**.

Table 2, Table 3 and Table 4 summarized the identified compounds, their retention times (RT), molecular formulas, molecular weights (MW) and relative abundances (%) of their presence in the sample.

Eight compounds were detected in the hexane extract (Table 2, Figure 1), with majority of them being fatty acids, hydrocarbons, and terpenoid derivatives. The most common ones were squalene (30.34%),  $\alpha$ -tocopherol (21.79%) and n-hexanedioic acid (13.19%). Such compounds are generally known to be antioxidants, anticancer and membrane stabilizers. The elevated levels of the lipophilic antioxidants squalene and  $\alpha$ -tocopherol indicate the abundance of antioxidants in the non-polar fraction following an essential oil-type chemical profile.

A significantly wider chemical composition was observed in the ethyl acetate extract (Table 3, Figure 2), which contained thirty-five compounds, including hydrocarbons (alkanes and alkenes), alcohols, phenolics, benzoic and cinnamic acid derivatives, fatty acids, terpenoids and an indole alkaloid derivative. The larger compounds were n-hexadecanoic acid (15.45%), compounds of benzoic acid, as well as, a few phenolic esters. The occurrence of the chemical diversity and the enrichment of the phenolic acids and benzoic derivatives, in particular, indicate that the moderately polar solvent is very adequate to extract both lipid-soluble and hydrophilic molecules to yield intermediate antioxidant potential.

The most widespread phytochemical diversity was observed in the ethanolic extract (Table 4, Figure 3) which contained twenty-seven different compounds that were phenolics, terpenoids, glycosides, alkaloids, fatty acid esters and carbohydrate derivatives. Its key components are as follows:  $\beta$ -carotene (48.14%), cyclohexanetetrol (12.74%), and  $\alpha$ -tocopherol (2.79%), pointing the great antioxidant character of the extract in question. The excessive polarity and extensive solvation ability of ethanol allows it to remove the

diverse polar and semi-polar bioactives. A significant number of carotene and phenolic components offers a biochemical advantage to the strong antioxidant effects that will be realized in the latter tests. These results can be linked to previous studies which verified *A. venenata* as a staple of terpenoids and flavonoids with plenty pharmacological value.

#### DPPH Radical Scavenging Activity

The antioxidant activity measured using the DPPH radical scavenging assay is summarized in Table 5 and illustrated in Figure 4. A distinct dose-related improvement of scavenging activity was observed in all extracts as the concentration was increased (20-100 µg/mL). The ethanolic extract had the highest level of free-radical inhibition of 83.8% at 100µg/mL, then ethyl acetate (79.1%), hexane (74.3%), and aqueous extracts (68.9%). The highest level of inhibition (95.3%) was achieved with Ascorbic acid that was used as positive control.

Calculated IC<sub>50</sub> values also validated the better antioxidant efficiency of ethanol (19.97 µg/mL) relative to hexane (23.16 µg/mL), aqueous (23.00 µg/mL) and ethyl acetate (25.83 µg/mL) extracts. The smaller value of IC<sub>50</sub> of ethanolic extract implies that it has a greater number of radical-scavenging molecules that have moderate potency, including phenolics, flavonoids, carotenoids, and tocopherols. The DPPH findings correlate perfectly well with the phytochemical and GC-MS profiles, which indicated that ethanol produced the richest antioxidant compounds.

#### Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) Scavenging Activity

The hydrogen peroxide scavenging activity of the different solvent extracts is summarized in Table 6 and illustrated in Figure 5, showing a concentration-dependent increase in antioxidant activity. The ethanolic extract once again was the most active and recorded the highest inhibition of 80.5% at the concentration of 100 µg/mL followed by the ethyl acetate extract (74.9%), hexane extract (70.2%), and aqueous extracts (63.1%). Ascorbic acid which was used as the reference standard showed an inhibition of 90.8% under the same conditions. The ethanolic extract had an extremely low IC<sub>50</sub> value (2.27 µg/mL), even greater than the standard ascorbic acid (4.45 µg/mL), a formidable ability to neutralize H<sub>2</sub>O<sub>2</sub> radicals.

These congruent findings with both DPPH and H<sub>2</sub>O<sub>2</sub> tests prove that the antioxidant potential is closely related to the solvent polarity and the variety of extracted phytochemicals. The fraction with the highest free-radical scavenging capability was the ethanol fraction which was rich in carotenoids, tocopherols, and phenolics thereby making it the most bioactive extract among the tested solvent fractions.

#### DISCUSSION

It was found during this investigation that the *A. venenata* leaf extracts contained a rich spectrum of phytochemicals and a significant antioxidant activity that offers empirical evidence to the long-term history

of its use in the traditional medicine. The presence of many bioactive classes: triterpenes, phenolics, flavonoids, glycosides and fatty acid derivatives reveals that the plant has a wide pharmacological potential [25, 26]. The existence of such compounds as squalene,  $\alpha$ -tocopherol and derivatives of benzoic acids that have been demonstrated to have antioxidant, anti-inflammatory and cytoprotective effects on their own supports the ethnomedicinal assertions regarding *A. venenata* [18-20].

Despite the existence of all other solvent extracts, the ethanolic one presented the best antioxidant behavior. This observation may be attributed to the presence of antioxidant compounds such as  **$\beta$ -carotene (48.14%) and  $\alpha$ -tocopherol (2.79%)**, which are known to possess strong free-radical scavenging properties. The combined presence of multiple bioactive compounds may contribute to the observed antioxidant activity of the extract. However, possible synergistic interactions among these compounds were not specifically investigated in the present study. Carotenoids neutralize singlet oxygen and peroxy radicals and tocopherols inhibit lipid peroxidation in cell membranes [27]. These molecules in combination offer a strong two-step defense, in that, carotenoids capture the free radicals in the lipid milieu, and tocopherols stabilize the membranes against further oxidative damage by propagation [21]. The ethanol solvent is quite efficient because it is chemically intermediate in polar than other molecules, thus it can extract both lipophilic and hydrophilic molecules [28]. This observation correlates with the previous studies indicating ethanol as the most effective solvent to dissolve phenols, flavonoids and lipid soluble vitamins hence extracting with antioxidant properties.

The dose-dependent radical inhibition, which was gradual, occurred in both DPPH and hydrogen peroxide assays supporting this assertion that the active constituents in *A. venenata* effectively donate electrons or hydrogen atoms to counter the free radicals [29]. This can be explained by such patterns to compounds that have redox potential and effective free-radical scavenging capacity [22-24]. The agreement of findings in both the run assays implies that the extracts are widely antioxidant competent, which functions on the nitrogen- and oxygen-centered radicals.

The results of this research are in agreement with the previous reports of the bioactivity profile of the plant [11] that found methoxyindole alkaloids in *A. venenata* with potential neuroactive effects, and Bagheri et al. [5] found that the plant has squalene,  $\alpha$ -tocopherol, and palmitic acid among other terpenoids as potentially antioxidant and anti-inflammatory characters. Comparing extracts of the various solvent systems, the present study builds upon these findings and shows that the polarity of the solvent has a great power over the extraction yield and phytochemical diversity. Ethanol, because of its balanced solvation characteristics, gave the most chemically diversified and biologically active extract.

It is worth mentioning the presence of squalene in the hexane and ethanol fractions. Squalene, as a triterpenoid hydrocarbon, is a biosynthetic precursor of the sterols and has significant protective effects against lipid peroxy-, specifically against lipid oxidation, and as such is likely to preserve membrane integrity against oxidative stress. Similarly, benzoic acid derivatives present in the ethyl acetate fraction are also associated with antioxidant and antimicrobial activity and could be the reason why the plant was traditionally used in inflammatory and infectious disease. The presence of glycosides and flavonoids in the extract in parallel form can also divert to a greater increase in its activity as a result of the synergistic effects, in which neutralization in free-radicals and control of the oxidative enzymes, including catalase and superoxide dismutase, are involved.

The results of the in vitro experiments are in agreement with the GC-MS data that identified the presence of both lipophilic antioxidants (carotenoids, tocopherols) and polar molecules (phenolics, terpenoid, glycosides). The two phase composition allows the formation of antioxidant activity in the aqueous and lipid cellular systems. This redundant system of defense can support the historical use of *A. venenata* in the treatment of human illnesses such as fever, epilepsy and ulcer, in which oxidative stress is an influential factor.

However, analysis of crude extract was confined in the study. Although this can be seen as an indication of the complexity of traditional preparations, further exploring this area is important in the future by isolating and characterizing individual compounds to better understand structure-activity relationships and determine which agents of antioxidants make the strongest agents. In addition, antioxidant assays in vitro in this study were done; therefore, further experiments in vivo and cell based models are important to prove the biological functionality and processes in physiological systems. Further investigation with the use of LC-MS/MS, NMR, and molecular docking could be used to learn more about molecular association and drug target possibilities. The analysis of anti-inflammatory, hepatoprotective, and neuroprotective properties also would yield to the diversification of therapeutic value of *A. venenata*. Also of paramount importance is toxicity galleries and safety studies to ascertain whether it can be given as a clinical or nutraceutical compound.

#### CONCLUSION AND FUTURE WORK

The present study demonstrated that *Alstonia venenata* leaf extracts contain diverse phytochemical constituents as identified through GC-MS analysis. Among the tested solvent extracts, the ethanolic fraction exhibited the highest in vitro antioxidant activity in both DPPH and hydrogen peroxide scavenging assays. These findings suggest that the antioxidant potential of *A. venenata* may be attributed to the presence of bioactive phytochemicals such as phenolic compounds and terpenoids. Further studies are required to isolate the

active compounds and evaluate their pharmacological potential.

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“The authors declare that they have no competing interests.”

#### Author Contributions:

All authors contributed significantly to the conception and design of the study. Data collection, analysis, and interpretation were performed collaboratively. All authors participated in manuscript preparation, reviewed the final version, and approved it for publication.

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Tables:

**Table 1. Preliminary phytochemical screening of *Alstonia venenata* leaf extracts**

Phytochemical Constituent	Test/Reagent Used	Observation	Hexane Extract	Ethyl Acetate Extract	Ethanol Extract	Aqueous Extract
Alkaloids	Hager's reagent	Yellow precipitate	–	+	+	+
Flavonoids	Shinoda test	Pink/red coloration	–	+	+	+
Phenols	Ferric chloride test	Deep blue/green colour	–	+	+	+
Tannins	Ferric chloride test	Blue-black/green colour	–	+	+	+
Saponins	Froth test	Persistent froth formation	–	–	+	+
Glycosides	Keller–Killiani test	Reddish brown ring	–	+	+	+
Terpenoids	Salkowski test	Reddish brown interface	+	+	+	–
Carbohydrates	Molisch test	Violet ring formation	–	–	+	+
Proteins	Biuret test	Violet colour	–	–	+	+
Starch	I <sub>2</sub> –KI (iodine) test	Blue-black colour	–	–	+	+

**Footnote**

Values are qualitative observations obtained from preliminary phytochemical screening of *Alstonia venenata* leaf extracts.

(+) Presence of phytochemical constituent

(-) Absence of phytochemical constituent

Hager's reagent was used for the detection of alkaloids.

The iodine (I<sub>2</sub>-KI) test produces a blue-black colour in the presence of starch.

**Table 2: GC-MS analysis of hexane of *Alstonia venenata* extract Active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) in the various extract of leaves of *Alstonia venenata***

Sl. No	Compound	Retention time	Peak area	Formula	Molecular weight
1	n-Hexadecanoic acid	18.888	13.19	C16H32O2	256
2	8,11,14-Docosatrienoic acid, methyl ester	20.529	0.77	C23H40O2	348
3	9,12,15-Octadecatrienoic acid	21.013	10.69	C18H30O2	278
4	Squalene	29.354	30.34	C30H50	410
5	Dotriacontane	30.650	7.29	C32H66	450
6	beta-Tocopherol	33.182	5.97	C29H50O2	430
7	Hexatriacontane	34.239	9.98	C36H74	506
8	dl-alpha-Tocopherol	34.796	21.79	C29H50O2	430

**Footnote:**

GC-MS analysis of *Alstonia venenata* leaf extracts using different solvent systems. RT = retention time (min); MW = molecular weight (g/mol); % = relative peak area percentage obtained from the GC-MS total ion chromatogram. Compound identification was performed by comparison of mass spectra with the NIST 20 mass spectral library with a similarity index > 90%.

**Table 3: Active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) in the various extract of leaves of *Alstonia venenata***

Sl. no	Compound	Retention time	Peak area	Formula	Molecular Weight
1	2-Undecene	6.083	0.16	C12H24	168
2	Octane, 2,4,6-trimethyl-	6.283	0.27	C10H22	142
3	1,2,3-Propanetriol, 1-acetate	8.621	5.50	C5H10O4	134
4	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	9.566	0.61	C6H8O	144
5	1-Dodecanol	11.226	0.41	C12H26O	186
6	Catechol	11.586	3.43	C6H6O2	110
7	4-Vinylphenol	12.083	1.85	C8H8O	120
8	1,2,3-Propanetriol, 1-acetate	12.535	1.04	C5H10O4	134
9	Butanoic acid	14.23	2.32	C7H12O6	192
10	2-methoxy-4-vinylphenol	14.482	2.27	C9H10O2	150
11	2-Methoxy-4-vinylphenol	14.482	0.63	C9H10O2	150
12	Methoxy beta carboline	16.151	2.27	C12H10N2O	198.225
13	n-Tridecan-1-ol	16.586	0.65	C13H28O	200
14	Phenol, 4-ethenyl-2,6-dimethoxy-	20.772	0.94	C10H12O3	180
15	1-Hexadecanol	21.532	0.62	C16H34O	242
16	1-Heptatriacotanol	22.283	1.62	C37H76O	536
17	3-Hydroxy-4,5-dimethoxybenzoic acid	24.745	7.79	C9H10O5	198

Sl. no	Compound	Retention time	Peak area	Formula	Molecular Weight
18	7-Methoxy-piperonylic acid	25.004	2.33	C9H8O5	196
19	Benzoic acid, 3,4,5-trimethoxy-	25.369	9.49	C10H12O5	212
20	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one	25.532	1.45	C11H16O3	196
21	Benzoic acid, 4-hydroxy-3,5-dimethoxy-	26.357	1.33	C9H10O5	198
22	Neophytadiene	26.914	5.18	C20H38	278
23	n-Hexadecanoic acid	29.495	15.45	C16H32O2	256
24	3,4-Difluorobenzoic acid, eicosyl ester	30.079	0.55	C27H44F2O2	438
25	1H-Indole-3-acetic acid	30.378	0.28	C13H15NO3	233
26	2-Propenoic acid, 3-(3,4,5-trimethoxyphenyl)-	30.833	0.61	C12H14O5	238
27	Heptadecanoic acid	31.358	0.38	C17H34O2	270
28	9,12-Octadecadienoic acid (Z,Z)-	32.669	2.72	C18H32O2	280
29	9,12,15-Octadecatrienoic acid (Z,Z,Z)-	32.796	11.55	C18H30O2	278
30	Octadecanoic acid	33.229	4.11	C18H36O2	284
31	2-Eicosen-5-olide	39.609	1.41	C20H36O2	308
32	Oxirane, hexadecyl-	42.995	4.88	C18H36O	268
33	Squalene	43.507	6.47	C30H50	410
34	Dotriacontane	44.72	0.56	C32H66	450
35	(R)-6-Methoxy-2,8-dimethyl-2-(4R,8R)-4,8,12-	47.291	2.02	C28H48O2	416

**Footnote:**

GC-MS analysis of *Alstonia venenata* leaf extracts using different solvent systems. RT = retention time (min); MW = molecular weight (g/mol); % = relative peak area percentage obtained from the GC-MS total ion chromatogram. Compound identification was performed by comparison of mass spectra with the NIST 20 mass spectral library with a similarity index > 90%.

**Table 4: Mass Spectrometry for Ethanol Extract**

Sl no.	Compound	Retention time	Peak area (%)	Formula	Molecular wt.
1	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	7.614	3.66	C6H8O4	144
2	5-Hydroxymethylfurfural	8.831	2.01	C6H6O	126
3	1,2,3-Propanetriol, 1-acetate	9.068	1.14	C5H10O4	134
4	Glutaric acid	9.626	0.93	C12H21ClO3	248
5	1-Amino-4-methylpiperazine	10.097	0.55	C5H13N3	115
6	Phenol, 4-propyl	10.956	1.26	C9H12O	136
7	3-Butoxypropylamine	11.880	1.61	C7H17NO	131
8	Beta-D-Glucopyranose, 1,6-anhydro	12.566	1.01	C6H10O5	162
9	Benzoic acid, 4-ethoxy-, ethyl ester	12.901	0.20	C11H14O3	194
10	1,2,3,5-Cyclohexanetetrol	14.246	12.74	C6H12O4	148
11	Quinic acid	14.573	3.20	C7H12O6	192
12	Carotene	14.938	48.14	C40H66	546

Analytical Evaluation Of Antioxidant Activity Through Gc-Ms Profiling: A Comparative Study Of Phytochemical Constituents In *Alstonia Venenata* Leaf Extract

Sl no.	Compound	Retention time	Peak area (%)	Formula	Molecular wt.
13	Benzoic acid, 3,4,5-trimethoxy	15.666	3.60	C10H12O5	212
14	Neophytadiene	16.728	0.55	C20H38	278
15	n-Hexadecanoic acid	18.979	5.80	C16H32O2	256
16	Hexadecanoic acid	19.684	0.45	C18H36O2	284
17	9,12-Octadecadienoic acid (Z,Z)	22.972	0.81	C18H32O2	280
18	9,12,15-Octadecatrienoic acid (Z,Z,Z)	23.111	4.08	C18H32O	264
19	Eicosatrienoic acid	23.656	0.66	C20H34O2	306
20	Pentadecanoic acid	30.401	0.36	C15H30O2	242
21	Squalene	35.159	0.85	C30H50	410
22	alpha-Tocopherol-beta-D-mannoside	40.499	1.41	C35H60O7	592
23	Campesterol, delta-5-Ergosterol	42.711	1.26	C28H48O	400
24	gamma-Sitosterol	45.152	2.51	C29H50O	414
25	beta-Amyrin	46.254	0.34	C30H50O	426
26	Epilupeol	47.817	0.24	C32H52O2	468
27	gamma-Tocopherol	38.913	0.65	C28H48O2	416

**Footnote:**

GC-MS analysis of *Alstonia venenata* leaf extracts using different solvent systems. RT = retention time (min); MW = molecular weight (g/mol); % = relative peak area percentage obtained from the GC-MS total ion chromatogram. Compound identification was performed by comparison of mass spectra with the NIST 20 mass spectral library with a similarity index > 90%

**Table 5: DPPH scavenging assay**

Concentration (µg/mL)	Hexane Extract (%) Scavenging	Ethyl Acetate Extract (%) Scavenging	Ethanol Extract (%) Scavenging	Water Extract (%) Scavenging	Ascorbic Acid (Standard) (%) Scavenging
20	32.5	35.6	45.1	30.3	60.8
40	46.7	52.3	57.9	43.1	72.4
60	58.1	61.4	69.3	54.8	85.7
80	67.5	71.2	75.4	62.2	90.2
100	74.3	79.1	83.8	68.9	95.3

**Footnote:**

Values are expressed as mean ± SD (n = 3). DPPH = 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay. IC<sub>50</sub> values were calculated using linear regression analysis. Ascorbic acid was used as the positive control.

**Table 6. Hydrogen peroxide scavenging activity of *Alstonia venenata* leaf extracts**

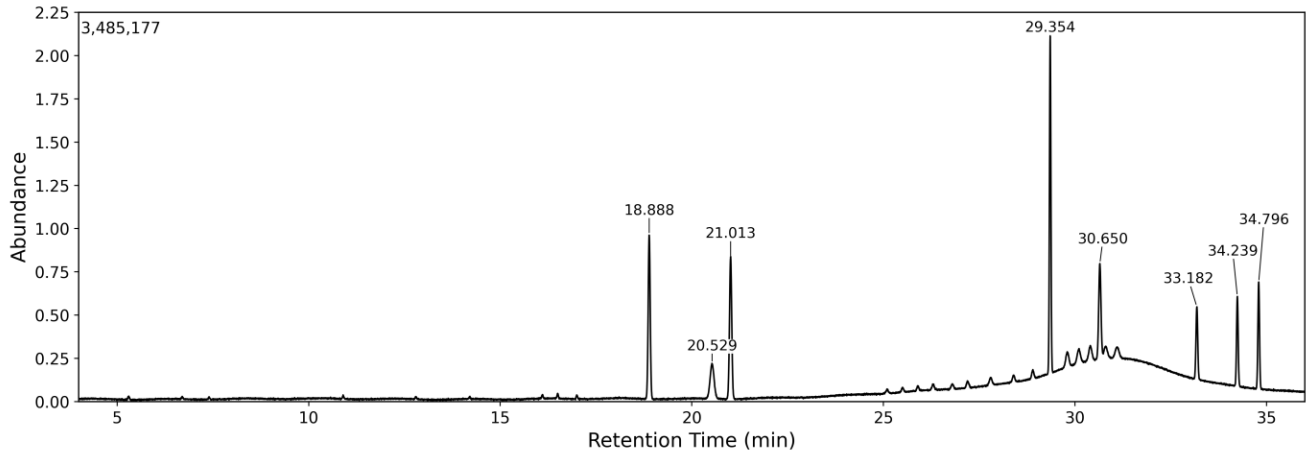
Concentration (µg/mL)	Hexane Extract (%) Scavenging	Ethyl Acetate Extract (%) Scavenging	Ethanol Extract (%) Scavenging	Water Extract (%) Scavenging	Ascorbic Acid (Standard) (%) Scavenging
20	25.4	30.2	45.1	20.8	60.2
40	39.1	43.8	57.6	35.3	70.8
60	51.3	56.1	69.2	45.7	80.4
80	61.5	66.3	74.4	54.2	85.6
100	70.2	74.9	80.5	63.1	90.8

**Footnote:**

Values are expressed as mean ± SD (n = 3) from three independent experiments. IC<sub>50</sub> values were calculated using linear regression analysis. Ascorbic acid was used as the positive control.

**Figures**

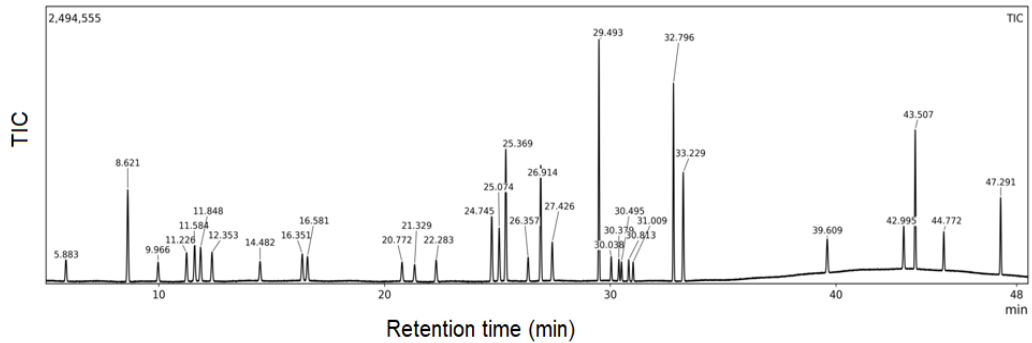
Analytical Evaluation Of Antioxidant Activity Through Gc-Ms Profiling: A Comparative Study Of Phytochemical Constituents In *Alstonia Venenata* Leaf Extract



**Footnote:**

Values are expressed as *mean* ± *SD* (*n* = 3). *H<sub>2</sub>O<sub>2</sub>* = hydrogen peroxide scavenging assay. Ascorbic acid was used as the positive control

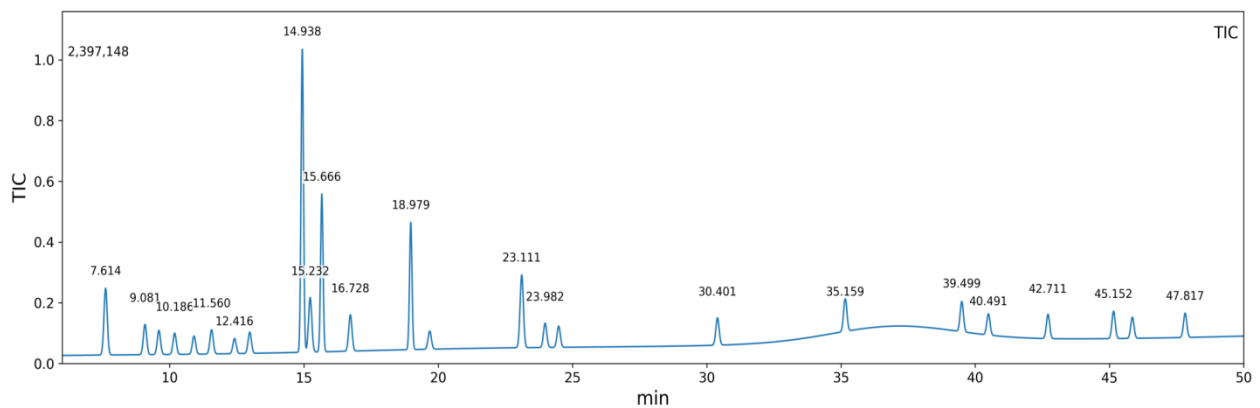
**Figure 1: Representative chromatogram (GC-MS) of hexane extract of *Alstonia venenata* leaves**



**Footnote:**

Representative **total ion chromatogram (TIC)** obtained from GC-MS analysis of the **ethyl acetate extract** of *Alstonia venenata* leaves. The *x*-axis represents retention time (min) and the *y*-axis represents total ion current (abundance).

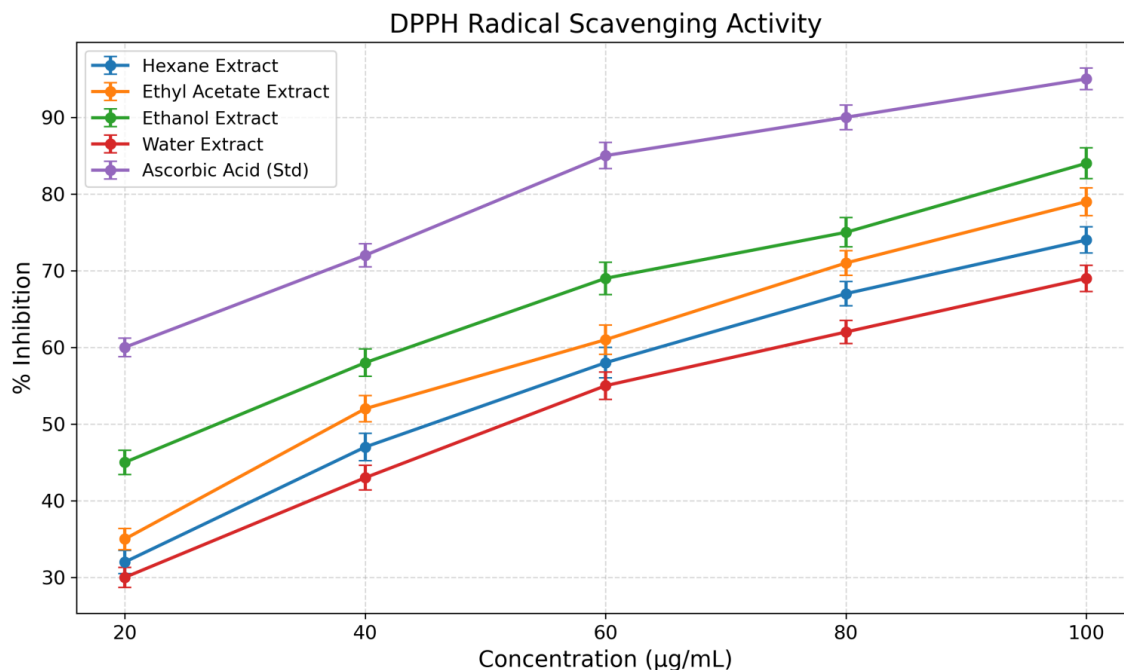
**Figure 2: GC-MS total ion chromatogram of the ethyl acetate extract of *Alstonia venenata* leaves showing major detected compounds.**



**Footnote:**

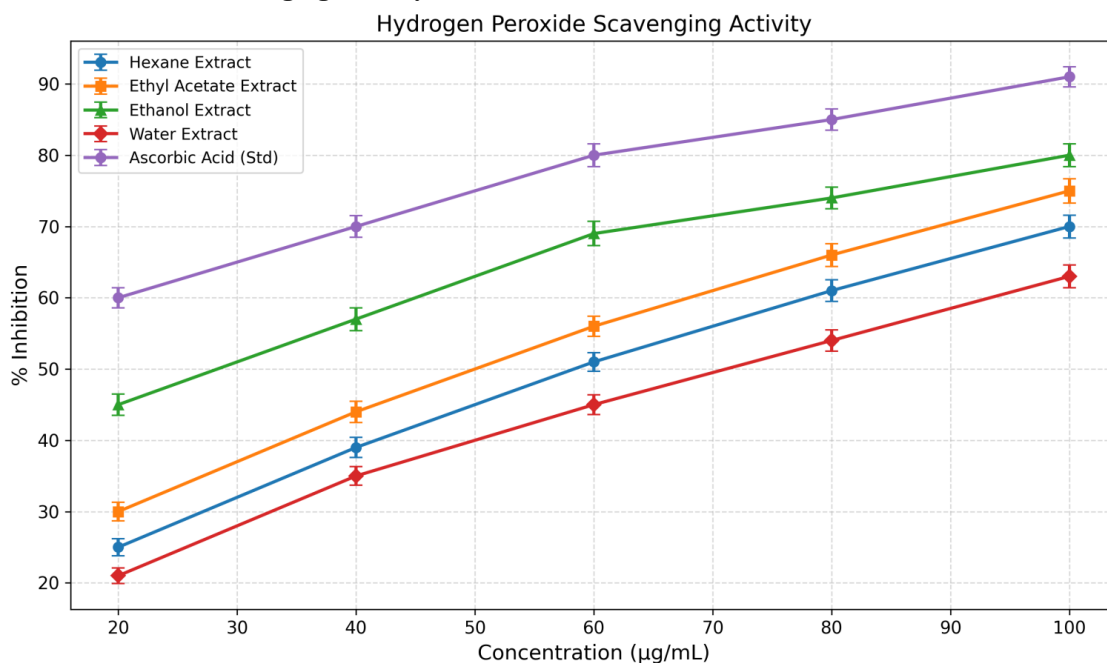
Representative **total ion chromatogram (TIC)** obtained from GC-MS analysis of the **ethanolic extract** of *Alstonia venenata* leaves. The *x*-axis represents retention time (min) and the *y*-axis represents total ion current (abundance).

**Figure 3: Representative chromatogram (GC-MS) of ethanol extract of *Alstonia venenata* leaves**



**Footnote:** Dose-dependent percentage inhibition of DPPH radicals by different solvent extracts of *Alstonia venenata* leaves and the standard ascorbic acid. Each data point represents mean  $\pm$  SD ( $n = 3$ ).

**Figure 4: DPPH Radical Scavenging Activity**



**Footnote:** Dose-dependent percentage inhibition of hydrogen peroxide ( $H_2O_2$ ) by different solvent extracts of *Alstonia venenata* leaves and the standard ascorbic acid. Each data point represents mean  $\pm$  SD ( $n = 3$ ).

**Figure 5:  $H_2O_2$  Scavenging Activity**