Screening and Evaluation of Antioxidant and Cytotoxic Potential of Herbal Mixture with *Annona muricata* and *Guilandina bonduc* against Cancer lines (A 549, SiHa, HeLa) -A Systematic Study

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Abstract

Polyherbal formulations are widely utilized in ethnomedicine due to their synergistic effects and broad-spectrum bioactivity. This study aimed to evaluate the antioxidant properties, cytotoxic potential against cancer cell lines and to perform GC-MS profiling of a polyherbal mixture derived from Annona muricata leaves and Guilandina bonduc seeds. Ethanolic extracts of both plants were prepared using Soxhlet extraction and combined in a 1:1 ratio. Standardization involved phytochemical screening, determination of total phenolic content (TPC), total flavonoid content (TFC) and assessing antioxidant activity using DPPH and ABTS assays and cytotoxicity screening.

Phytochemical analysis confirmed the presence of alkaloids, flavonoids, phenolics, tannins and terpenoids. GC-MS analysis identified active bioactive constituents including 9,12-octadecadienoic acid, phytol, caryophyllene and squalene. Herbal mixture shows low cytotoxicity to normal cells and moderate cytotoxicity to cancer cells, especially A549 (lung cancer). The herbal mixture demonstrated significant antioxidant potential and moderate cytotoxicity towards cancer cell lines (A 549 and SiHa), supporting its traditional use and paving the way for further pharmacological investigations.

Keywords: *Annona muricata*, *Guilandina bonduc*, antioxidant activity, polyherbal formulation, ABTS assay, GC-MS, MTT assay, phytochemicals

Introduction

Cancer is a group of diseases characterized by the uncontrolled division of abnormal cells, which can invade surrounding tissues and can spread to distant parts of the body through metastasis. It remains a leading cause of death globally, accounting for nearly 10 million deaths in 2020[25]. The development of cancer involves a multistep process that includes genetic mutations, epigenetic changes and disruptions in regulatory pathways such as apoptosis, proliferation and angiogenesis[4]. While significant advances have been made in conventional therapies such as chemotherapy, radiotherapy and targeted therapy, their use is often limited by adverse effects on normal cells, development of resistance and high treatment costs[15]. As a result, there has been increasing interest in the exploration of natural products, particularly plant-based compounds, as potential sources of safer and more effective anticancer agents.

Numerous medicinal plants contain bioactive constituents with known antioxidant, anti-inflammatory and cytotoxic activities that may contribute to cancer prevention and therapy[14]. Among these, polyherbal formulations, combinations of multiple plant extracts, are gaining attention for their synergistic effects and reduced toxicity profiles. *Annona muricata* is known for its rich content of acetogenins, flavonoids and alkaloids, many of which possess good antioxidant and therapeutic properties[26]. *Guilandina bonduc* (syn. *Caesalpinia bonduc*), traditionally used for various ailments, is rich in diterpenoids, saponins and flavonoids, offering anti-inflammatory and antioxidant effects[7].

Reactive oxygen species (ROS) are natural byproducts of cellular metabolism, but excessive production can lead to oxidative stress, which damages cellular components such as DNA, proteins and lipids. Oxidative stress is closely associated with the pathogenesis of several chronic diseases including cancer, cardiovascular disorders and neurodegenerative conditions[17]. Antioxidants play a crucial role in neutralizing ROS and maintaining redox balance in cells. Naturally occurring antioxidants derived from medicinal plants have attracted significant interest due to their potential therapeutic applications, minimal side effects and ability to modulate signaling pathways involved in cancer development[12]. Oxidative stress is implicated in the pathophysiology of various chronic diseases, making the identification of plant-based antioxidants highly valuable. Among various antioxidant assays, the DPPH and ABTS assays are widely used due to their reliability and simplicity[19].

In cancer research, evaluating the cytotoxic potential of plant-derived compounds is a key step in identifying

promising anticancer agents. The MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a widely used colorimetric method for assessing cell viability and proliferation. It relies on the enzymatic reduction of MTT by mitochondrial dehydrogenases in viable cells to form insoluble formazan crystals, which can be quantified spectrophotometrically[13]. This assay provides a reliable and cost-effective means of screening the cytotoxic effects of plant extracts against both cancerous and normal cell lines, thereby offering insights into their selectivity and therapeutic potential.

This study aims to evaluate a polyherbal mixture of *A. muricata* and *G. bonduc* for their phytochemical profiles, antioxidant activity, cytotoxicity screening and phytoconstituents analysis via GC-MS.

Material and Methods

Plant Collection, Authentication and Extraction: Leaves of *Annona. muricata* and the seeds of *Guilandina. bonduc* were collected from the Nilgiri District, Tamil Nadu and authenticated by the Botanical Survey of India (BSI), Coimbatore. The plant materials were shade-dried, pulverized and extracted with 70% ethanol using the Soxhlet device. The extracts were concentrated with a rotary evaporator and combined in a 1:1 ratio for further analysis.

Preliminary Phytochemical Screening: The preliminary phytochemical screening of the standardized polyherbal extract was conducted to identify the presence of major secondary metabolites including alkaloids, flavonoids, tannins, saponins, glycosides, phenols, terpenoids and steroids. The tests were performed using standard qualitative procedures described by Harborne[5] and Kokate[8]. Briefly, small portions of the extract were subjected to specific chemical reactions: Mayer's and Wagner's tests for alkaloids, ferric chloride test for phenols, lead acetate and alkaline reagent tests for flavonoids, foam test for saponins; Keller-Killiani test for cardiac glycosides, Salkowski's and Liebermann-Burchard's tests for steroids and terpenoids and gelatin test for tannins. The appearance of characteristic color changes or precipitates was used to confirm the presence of each phytochemical class.

Determination of Total Phenolics: The total phenolic content (TPC) of the polyherbal extract was determined using the Folin–Ciocalteu method with slight modifications. Briefly, 0.5 mL of the ethanolic extract (appropriately diluted) was mixed with 2.5 mL of 1:10 diluted Folin–Ciocalteu reagent and incubated at room temperature for 5 minutes.

Subsequently, 2.0 mL of 7.5% sodium carbonate solution was added to the mixture, which was then incubated in the dark at room temperature for 30 minutes. After incubation, the absorbance of the resulting blue-colored complex was measured at 760 nm using a UV-Visible spectrophotometer. A calibration curve was prepared using standard gallic acid solutions (10–100 µg/mL) and the total phenolic content of the extract was expressed as milligrams of gallic acid equivalents (mg GAE) per gram of extract[24].

Determination of Total Flavonoids: The polyherbal extract's total flavonoid content (TFC) was determined using the aluminum chloride colorimetric method. In this assay, 0.5 mL of the ethanolic extract was mixed with 2 mL of distilled water and 0.15 mL of 5% sodium nitrite solution. After 5 minutes of incubation at room temperature, 0.15 mL of 10% aluminum chloride solution was added. Following another 6-minute incubation, 1.0 mL of 1 M sodium hydroxide was added to the reaction mixture and the final volume was adjusted to 5 mL with distilled water. The solution was mixed thoroughly and the absorbance was measured at 510 nm using a UV-visible spectrophotometer. A standard calibration curve was constructed using quercetin (10–100 µg/mL) and the flavonoid content was expressed as milligrams of quercetin equivalents (mg QE) per gram of extract[3].

Antioxidant Activity

DPPH Radical Scavenging Assay: The antioxidant activity of the polyherbal extract was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. 0.1 mM solution of DPPH was prepared in methanol and 2.0 mL of this solution was mixed with 2.0 mL of the extract at various concentrations (e.g., 10–100 µg/mL). The mixture was vortexed and incubated in the dark at room temperature for 30 minutes. The decrease in absorbance was measured at 517 nm using a UV-Visible spectrophotometer against a blank. Ascorbic acid was used as the standard reference compound. The percentage of DPPH radical scavenging activity was calculated using the formula:

$$\% \text{ inhibition} = [(A_0 - A_1) / A_0] \times 100$$

where A_0 is the absorbance of the control (DPPH without extract) and A_1 is the absorbance in the presence of the extract or standard. The IC_{50} value (concentration required to inhibit 50% of DPPH radicals) was determined from the plot of % inhibition versus extract concentration.[2]

ABTS Assay: The ABTS radical scavenging assay involves generating the $ABTS^+$ radical cation by mixing 7 mM ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 hours. The resulting radical solution is then diluted with ethanol or phosphate-buffered saline (PBS) to obtain an absorbance of 0.70 ± 0.02 at 734 nm. For the assay, 950 µL of the diluted $ABTS^+$ solution was mixed with 50 µL of the plant extract or standard (e.g. Trolox) at various concentrations and the mixture was incubated in the dark at room temperature for 6 minutes. The absorbance was then measured at 734 nm using a UV-Vis spectrophotometer. The percentage inhibition of the $ABTS^+$ radical is calculated using the formula:

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample[19].

Cytotoxicity Assay by MTT Method: L929 (Mouse fibroblast) cell line (normal Cells), SiHa (human cervical squamous cell carcinoma), A549 (lung cancer cell line), and HeLa (cervical cancer cell line) were obtained from the National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecco's modified eagle medium (DMEM; Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS), L-glutamine, sodium bicarbonate (Merck, Germany) and an antibiotic solution containing penicillin (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (2.5 µg/mL). The cells were cultured in 25 cm^2 tissue culture flasks and incubated at 37 °C in a humidified atmosphere with 5% CO_2 (NBS Eppendorf, Germany). Cell viability was initially assessed by direct microscopic observation using an inverted phase-contrast microscope and further confirmed using the MTT assay.

For cytotoxicity testing, a two-day-old confluent monolayer of cells was trypsinized and resuspended in 10% growth medium; 100 µL of the cell suspension containing 5×10^4 cells/well was seeded into a 96-well tissue culture plate and incubated at 37 °C in a humidified CO_2 incubator. The test sample was prepared by dissolving 1 mg of the compound in 1 mL of 0.1% DMSO using a cyclomixer, followed by filtration through a 0.22 µm Millipore syringe filter for sterility. Serial twofold dilutions of the sample in 5% DMEM were prepared to yield concentrations of 100, 50, 25, 12.5 and 6.25 µg/mL and 100 µL of each concentration was added in triplicate to the respective wells. For the MTT assay, 15 mg of MTT (Sigma, M-5655) was dissolved in 3 mL of phosphate-buffered saline (PBS) and sterilized via filtration.

After 24 hours of treatment, the medium was removed and 30 µL of the MTT solution was added to each well and incubated for 4 hours at 37 °C. The MTT solution was then removed and 100 µL of DMSO was added to solubilize the formazan crystals, with gentle pipetting. Absorbance was measured at 540 nm using a microplate reader and the percentage of growth inhibition was calculated using the formula[6]. The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples} \times 100}{\text{Mean OD of control group}}$$

GC-MS Analysis: The ethanolic extract of the herbal mixture was analyzed using Gas Chromatography-Mass Spectroscopy for the identification of the phytochemical compounds present using instrument Model -7890 A GC with 5975C with triple axis detector. The column used for the experiment is DB 5MS 30 m x 0.250mm diameter x 0.25 Micrometer thickness. The analysis was performed by injecting 2 µl of the sample with a split ratio of 5:1. Helium gas (99.9995%) was used as the carrier gas at a flow rate of 1 mL/min. The analysis was performed in the EI mode with 70 eV of ionization energy. The injector temperature was maintained at 280°C[20].

Results and Discussion

The preparation of herbal mixtures is essential to ensure their quality, efficacy and safety, particularly when evaluating their antioxidant properties and conducting GC-MS analyses. Recent studies have emphasized the importance of comprehensive phytochemical profiling and *in vitro* antioxidant assessments in the development of polyherbal formulations. For instance, Shalini and Ilango[22] conducted preliminary phytochemical studies, GC-MS analysis and *in vitro* antioxidant activity evaluations of selected medicinal plants and their polyherbal formulation, highlighting the presence of various bioactive compounds and their significant antioxidant activity. Similarly, investigations on *Nardostachys jatamansi* have demonstrated potent antioxidant properties and the successful identification of its phytochemical constituents through GC-MS analysis, reinforcing the therapeutic relevance of such plant-based products[20]. These findings underscore the necessity of integrating standardization protocols with advanced analytical techniques to validate and optimize the therapeutic potential of herbal mixtures.

Preparation of extracts: The shade-dried plant parts of *Annona muricata* and *Guilandina bonduc* were subjected to extraction using a Soxhlet apparatus with ethanol as the solvent. A standardized polyherbal mixture was prepared by combining the dried samples of both plants in a 1:1 ratio, as reported in previous literature and extracted using the same method. The percentage yield of each extract was calculated based on the dry weight of the plant material used. The physicochemical characteristics of the concentrated extracts, including their nature, color and consistency, are summarized in table 1.

Phytochemical Screening: The extract showed positive results for major phytochemical groups given in table 2. The selected extract and herbal mixture contained the major secondary metabolites such as Phenolic Compounds, Flavonoids, Tannins, Alkaloids, Steroids, Glycosides and Saponins.

Determination of Total Phenolics and **Total Flavonoids:** The mixture exhibited a total phenolic content (TPC) of 185±0.009 mg gallic acid equivalents (GAE)/g extract and a total flavonoid content (TFC) of 119.75±0.001 mg quercetin equivalents (QE)/g extract, indicating a high concentration of bioactive secondary metabolites. Phenolic and flavonoid compounds are well known for their potent antioxidant and chemopreventive properties and their abundance in the mixture suggests potential synergistic effects in scavenging free radicals and contributing to therapeutic efficacy. These findings are consistent with earlier reports emphasizing the role of polyphenolic-rich plant combinations in enhancing antioxidant potential given in table 3[10,11,23]

Table 1
Physio-Chemical parameters of the extracts

S.N.	Name of the plant	Colour of the extract	Yield of extract(%w/w)
1.	*Annona muricata*	Greenish Brown	6.8
2.	*Guilandina bonduc*	Dark Brown	5.9
3.	Herbal mixture(1:1)	Greenish Brown	6.5

Table 2
Preliminary Phytochemical Screening of the ethanolic extract of the herbal mixture

S.N.	Phytochemicals	*Annona muricata* Leaves	*Guilandina bonduc* Seeds	Herbal mixture(1:1)
1.	Phenolic Compounds	+	+	+
2.	Flavonoids	+	+	+
3.	Tannins	+	+	+
4.	Alkaloids	+	+	+
5.	Steroids	+	+	+
6.	Glycosides	+	+	+
7.	Saponins	+	+	+
8.	Terpenoids	-	-	-

(+) =Present (-) Absent

Table 3
Total phenolics and flavonoid content in plant extract

Sample	Total Phenolics(mg GAE/g)	Total Flavonoids(mg QE/g)
Annona muricata leaves	150±0.015	109.75 ±0.001
Guilandina bonduc seeds	163±0.009	103.08±0.001
Herbal mixture	185±0.009	119.75±0.001

The values in the table are expressed as TPC ± SD/ TFC ± SD (n=3) and is statistically significant** at ($p<0.05$).

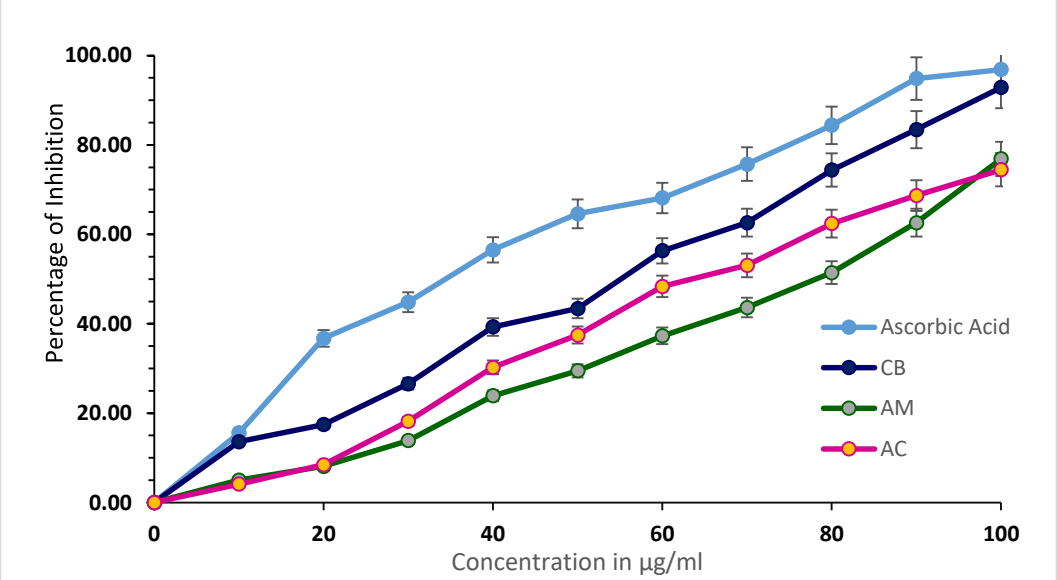

Figure 1: DPPH radical scavenging activity of the extract

Antioxidant Assays

DPPH radical scavenging activity: The DPPH assay demonstrated that ascorbic acid (standard) exhibited the highest antioxidant activity with the lowest IC_{50} value of 38.67 µg/ml, followed by *Annona muricata* 53.90 µg/ml, *Guilandina bonduc* 74.00 µg/ml and the combined extract (66.50 µg/ml) (Table 4, Figure 1). The lower is the IC_{50}, the stronger is the free radical scavenging ability. Although the combined extract did not outperform the standard or *Annona muricata* alone, it still exhibited notable antioxidant activity, suggesting that the mixture retains significant bioactivity. These results are consistent with previous studies reporting strong antioxidant effects of flavonoid- and phenolic-rich plant extracts[2,11]. All samples showed good linear correlation between concentration and RSA ($R^2 > 0.97$).

ABTS radical scavenging activity: The ABTS radical scavenging assay revealed a dose-dependent increase in antioxidant activity across all tested samples. Among them, ascorbic acid (standard) demonstrated the highest activity with an IC_{50} value of 25.23 µg/mL followed by *Annona muricata* extract (AM, IC_{50} = 28.72 µg/mL), the herbal mixture (AM + CB, IC_{50} = 36.34 µg/mL) and *Guilandina bonduc* (CB, IC_{50} = 56.57 µg/mL) (Table 4). The IC_{50} values were calculated using linear regression from the linear range of the dose-response curve, applying the equation y=mx+b as in fig. 2. The relatively lower IC_{50} values for AM and the combination extract indicate stronger antioxidant potential, supporting the hypothesis that these plant-based extracts may contain bioactive compounds with radical scavenging properties.

Cytotoxicity Assay by MTT Assay: The cytotoxic potential of the standardized polyherbal mixture was evaluated against three human cancer cell lines: A549 (lung carcinoma), HeLa (cervical carcinoma) and SiHa (cervical squamous cell carcinoma), alongside the normal fibroblast cell line L929 using the MTT assay. The extract exhibited the highest cytotoxicity against the A549 cell line, as evidenced by its significantly lower LC_{50} value of 106 µg/ml, compared to HeLa 153 µg/ml and SiHa 163 µg/ml as in figure 4.

In contrast, the LC_{50} values for the normal L929 cells were markedly higher for all three extract formulations: AC 314.73 µg/ml, AM 235.95 µg/ml and CB 215.58 µg/ml, indicating a favorable selectivity index and reduced cytotoxicity toward normal cells as in figure 3. These values were calculated using ED50 PLUS V1.0 software given in table 5. This suggests a greater susceptibility of A549 cells to the bioactive compounds within the herbal formulation. The heightened sensitivity of A549 cells may be attributed to specific molecular targets or vulnerabilities unique to lung cancer cell biology, although the precise mechanisms remain to be elucidated.

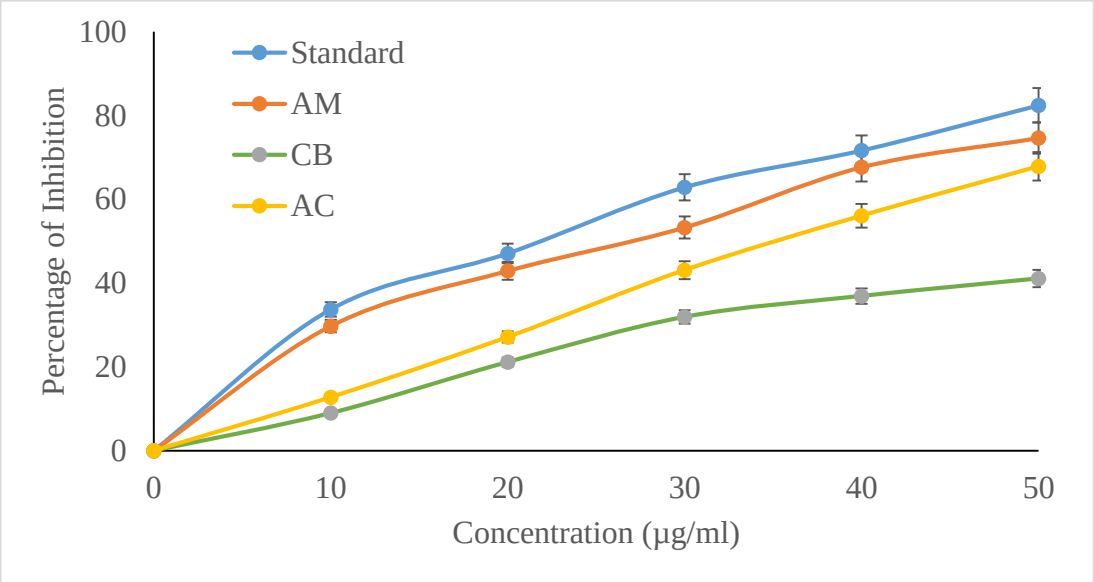

Figure 2: ABTS radical scavenging activity of the extract

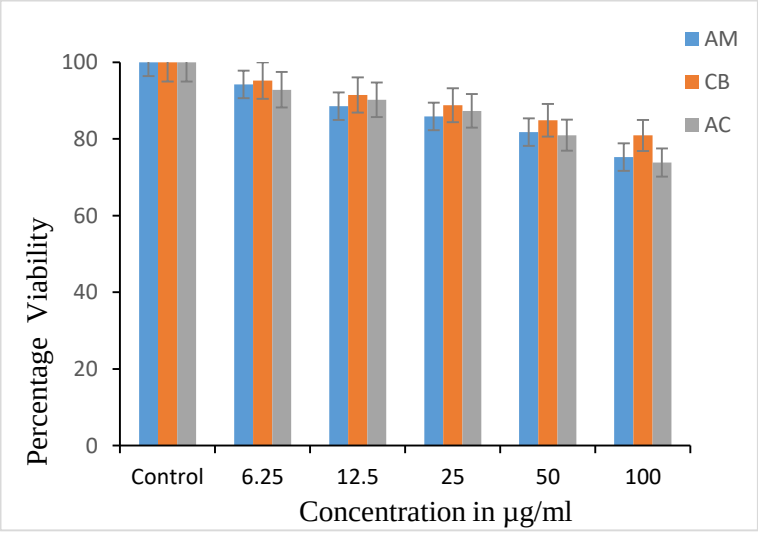

Figure 3: Cytotoxicity of ethanolic extract against L929

Further studies including apoptosis assays and gene expression profiling, are necessary to uncover the underlying pathways responsible for this differential cytotoxic response. These findings are consistent with prior research indicating that phytochemical-rich plant extracts can exert selective cytotoxic effects on certain cancer cell lines while sparing normal cells[1,16].

Identification of volatile phytoconstituents by GC-MS: The GC-MS analysis of the standardized herbal mixture revealed the presence of 39 distinct phytochemical constituents, as presented in table 6 and visualized in figure 5. Among these, the predominant compounds identified were 9,12-octadecadienoic acid (22.78%), n-Hexadecanoic acid (9.43%), 9,12-octadecadienoic-2-hydroxy-1-ethyl ester (9.42%), vaccenic acid (8.72%), ethyl citrate (7.65%) and α-tocopherol (4.55%). These bioactive compounds are widely recognized for their antioxidant, anti-inflammatory and therapeutic potentials, aligning with the observed bioactivities of the extract[18]. Specifically, α-tocopherol, a potent form of vitamin E, plays a crucial role in protecting cellular structures against oxidative damage by scavenging free radicals. Similarly, unsaturated fatty acids such as linoleic acid derivatives and vaccenic acid have been reported to modulate lipid metabolism and exhibit chemopreventive effects[9,21].

The identification of these compounds was accomplished by comparing their mass spectral fragmentation patterns, retention times and molecular ion peaks with those archived in the NIST-08 Mass Spectral Library Database. This widely accepted spectral repository enabled accurate characterization by matching the unknown spectra with reference compounds based on a similarity index threshold (≥90%).

The chromatographic resolution and detector sensitivity allowed for the precise discrimination of even minor constituents. Moreover, the use of electron impact ionization (EI) in GC-MS provided detailed and reproducible fragmentation patterns, further enhancing the reliability of compound identification. The presence of such diverse classes of phytoconstituents including fatty acid esters, aliphatic acids, vitamins and organic acids, highlights the pharmacological potential of the herbal formulation. These compounds may act synergistically to exert free radical scavenging and cytoprotective effects, contributing to the antioxidant and anticancer activities demonstrated *in vitro*.

Table 4
IC_{50} value of the plant extracts against free radicals

Sample	IC_{50} of DPPH Assay in µg/ml	IC_{50} of ABTS in µg/ml
Ascorbic Acid	38.67	25.23
Annona muricata leaves	53.90	28.72
Guilandina bonduc seeds	74.00	56.57
Herbal mixture	66.50	36.34

Table 5
LC_{50} Values of herbal extract against various cancer cell lines and normal cell line (L929)

S.N.	Cell Lines	LC_{50} of the Herbal mixture in (µg/ml)
1.	L929	314.73
2.	SiHa	163
3.	HeLa	153
4.	A549	106

Table 6
Identified compounds from ethanolic extract of herbal mixture by GC-MS analysis

S.N.	Compound Name	Molecular Formula	Molecular weight	Retention time	Probability percentage
1	9,12-octadecadienoic acid	$C_{18}H_{32}O_2$	280	37.2	22.78
2	n-hexadecanoic acid	$C_{16}H_{32}O_2$	256	34	9.43
3	9,12-octadecadienoic-2-hydroxy-1-ethyl ester	$C_{21}H_{38}O_4$	354	46.03	9.45
4	ethyl citrate	$C_{12}H_{20}O_7$	276	27.7	7.65
5	vaccenic acid	$C_{18}H_{34}O_2$	282	37.35	8.72
6	α-tocopherol	$C_{29}H_{50}O_2$	430	53.14	4.56
7.	Caryophyllene	$C_{15}H_{24}$	204	23.25	2.46
8.	Phytol	$C_{20}H_{40}O$	296	36.54	2.24
9.	Squalene	$C_{30}H_{50}$	410	47.72	1.3

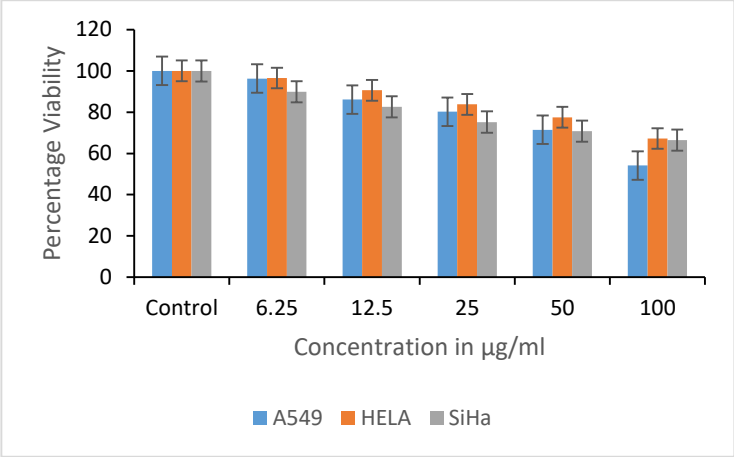

Figure 4: Cytotoxicity of ethanolic extract against various cancer cell lines

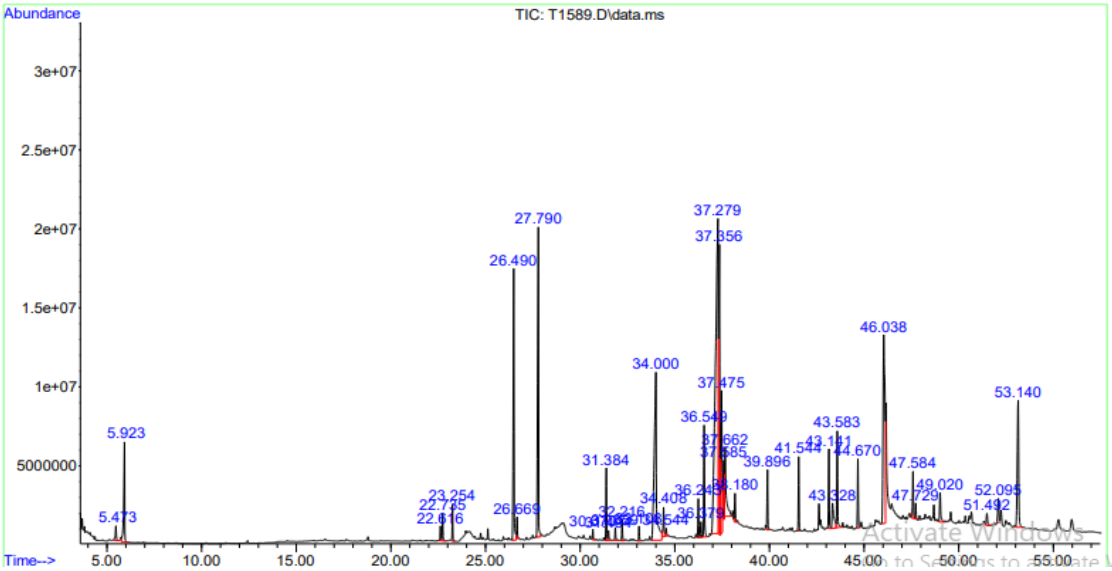

Figure 5: GC-MS Chromatogram of ethanolic extract of herbal mixture

The comprehensive phytochemical profiling thus substantiates the therapeutic relevance of the mixture and provides a chemical basis for further pharmacodynamic and pharmacokinetic evaluation.

Conclusion

The standardized polyherbal formulation comprising of *Annona muricata* leaves and *Guilandina bonduc* seeds demonstrated significant antioxidant activity and cytotoxic potential against A549, HeLa and SiHa cancer cell lines. GC-MS analysis revealed the presence of 39 phytoconstituents, with major compounds such as 9,12-octadecadienoic acid, n-hexadecanoic acid and α-tocopherol, many of which are known for their antioxidant, anti-inflammatory and anticancer properties. These findings collectively suggest that the herbal mixture possesses promising therapeutic potential and may serve as a natural source of bioactive compounds.

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