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COMPARATIVE PHYTOCHEMICAL AND ANTIOXIDATIVE PROFILING OF ASSAMESE CULINARY FOR DEVELOPING ANTIARTHRITIC POLYHERBAL FORMULATION

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ABSTRACT

Background: Rheumatoid arthritis (RA) is a chronic inflammatory disorder where oxidative stress plays a critical role in disease progression with severe co-morbidities. Therefore, plant-derived bioactive compounds are increasingly explored due to their safety, affordability, and multi-targeted therapeutic properties. The present study aimed to comparatively evaluate four commonly used Assamese culinary herbs, *Curcuma longa*, *Zingiber officinale*, *Piper nigrum*, and *Cinnamomum tamala* for their phytochemical profile and antioxidant potential to identify candidates for synergistic anti-arthritic formulations. **Methodology:** Ethanolic extracts of the selected herbs were analyzed for TPC, TFC, and TTC using spectrophotometric assays, and bioactive constituents were identified *via* high-resolution LC-MS. Antioxidant activity was assessed using the DPPH and ABTS radical-scavenging assays. Data were statistically analyzed using one-way ANOVA followed by Tukey's test ($p<0.05$). **Result and Discussion:** Among the tested herbs, turmeric showed markedly higher phytochemical content (TPC: 377.50±4.50 mg GAE/g, TFC: 294.29±2.18 mg CE/g, TTC: 201.25±3.14 mg TAE/g). HR-LCMS revealed characteristic compounds including curcumin (*C. longa*), 6-gingerol (*Z. officinale*), Dipiperamide E (*P. nigrum*), and eugenol (*C. tamala*). Antioxidant assays confirmed *C. longa*'s superior free-radical scavenging activity (DPPH IC_{50}: 64.83±0.49 µg/ml; ABTS IC_{50}: 145.60±0.95 µg/ml). *Z. officinale*, *P. nigrum*, and *C. tamala* extracts exhibited moderate but complementary activity profiles. **Conclusion:** The comparative phytochemical and antioxidant profiling demonstrated that *C. longa* was the most potent candidate, with *Z. officinale*, *P. nigrum*, and *C. tamala* exhibiting supportive bioactivity. The integrative evidence substantiated the rational design of synergistic polyherbal formulations targeting oxidative stress & inflammation in RA, with potential application in therapeutic development.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disorder affecting approximately 0.5-1% of the global population, with higher prevalence in women and increasing incidence in developing countries. It is characterized by persistent synovial inflammation, progressive cartilage and bone

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destruction, functional disability, and systemic complications, ultimately leading to significant morbidity, reduced quality of life, and socioeconomic burden [1]. Despite advances in therapeutic strategies, the clinical management of RA remains challenging. Non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids provide only symptomatic relief and are associated with gastrointestinal, renal, cardiovascular, and metabolic side effects. Disease-modifying antirheumatic drugs (DMARDs), such as methotrexate, require long-term administration and are limited by delayed onset, hepatotoxicity, hematological toxicity, and incomplete disease control in certain patients. Biologic therapies, although highly effective, are costly, require parenteral administration, and increase the risk of infections and malignancies, thereby limiting their accessibility and long-term sustainability in resource-constrained settings. These limitations underscore the urgent need for safer, affordable, and multi-targeted therapeutic alternatives [2]. Natural plant-derived bioactive compounds are a promising approach owing to their antioxidant, anti-inflammatory, and immunomodulatory properties, as well as favorable safety profiles and cultural acceptance. In particular, culinary herbs and spices consumed in daily diets often function as both nutraceuticals and therapeutic agents. Assam and the Northeast region of India, recognized as a biodiversity hotspot, possess a rich ethnomedicinal heritage in which local communities traditionally use dietary plants to manage inflammatory disorders. Species such as *Clerodendrum colebrookianum*, *Houttuynia cordata*, *Centella asiatica*, *Moringa oleifera*, and *Piper longum* have been used against pain and inflammation, although systematic pharmacological and mechanistic studies remain limited [3, 4]. Oxidative stress has emerged as a central pathogenic mechanism in RA, in which an imbalance between reactive oxygen species (ROS) and antioxidant defenses amplifies synovial inflammation, activates nuclear factor κB (NF-κB), and promotes the release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukins. This cascade drives cartilage degradation and bone erosion. Consequently, phytochemicals with strong antioxidant potential may play a crucial role in suppressing these processes and mitigating RA progression [5]. Among the culinary herbs of Assam, *Curcuma longa* (turmeric), *Zingiber officinale* (ginger), *Piper nigrum* (black pepper), and *Cinnamomum tamala* (Indian bay leaf) stand out for their traditional use and pharmacological promise [6]. Turmeric-derived curcumin downregulates NF-κB, suppresses NLRP3 inflammasome activation, and enhances Nrf2-mediated antioxidant defenses. Ginger constituents, including gingerols and shogaols, inhibit COX-2/5-LOX pathways, reduce NF-κB activation, and attenuate inflammatory markers such as C-reactive protein and TNF-α. Piperine from black pepper enhances curcumin bioavailability while exerting intrinsic anti-inflammatory and antioxidant effects. Indian bay leaf provides eugenol and cinnamaldehyde, which modulate cytokine signaling and scavenge ROS. Collectively, these herbs exhibit complementary mechanisms of antioxidant defense, cytokine modulation, pain attenuation, and bioenhancement, making them strong candidates for synergistic polyherbal formulations [7, 8]. Modern analytical techniques, particularly high-resolution liquid chromatography–mass spectrometry (HR-LCMS), now enable precise profiling of phytochemical constituents and their correlation with biological activity. Such approaches provide the scientific basis for evidence-based evaluation and rational design of polyherbal anti-arthritic therapeutics [9]. Moreover, given the influence of environmental factors (soil composition, seasonal variation, elevation, and agronomic practices) on phytochemical biosynthesis, place-based studies in Assam are critical for validating traditional knowledge and exploring the therapeutic potential of local resources [10]. The present study aimed to quantify TPC, TFC, and TTC and to compare the antioxidant potential of *C. longa*, *Z. officinale*, *P. nigrum*, and *C. tamala* under identical analytical conditions, which was crucial for scientific validation, formulation optimization, and quality standardization in the development of an anti-arthritic polyherbal formulation. Furthermore, the integration of HR-LCMS for these herbs was intended to standardize and quality check. It was hypothesized that these herbs, owing to their distinct yet complementary bioactive constituents, can synergistically mitigate oxidative stress and inflammatory processes implicated in RA. This study addressed the research gap by providing a scientific rationale for developing culturally acceptable, safe, and affordable polyherbal formulations that integrate traditional ethnomedicinal practices with modern phytopharmacology, systematically linking oxidative stress, antioxidant capacity, and anti-inflammatory effects.

MATERIALS AND METHODS

Reagents/chemicals

Analytical precision was ensured by using HPLC/LC-MS-grade chemicals and reagents. The organic solvents obtained were ethanol, methanol, chloroform, and n-hexane, purchased from Merck (India). Acetonitrile (LC-MS grade) and formic acid

were obtained from Sigma-Aldrich (USA). Analytical standards of gallic acid, catechin, tannic acid, and ascorbic acid were supplied by Sigma-Aldrich. DPPH, ABTS, potassium persulfate, Folin-Ciocalteu reagent, sodium carbonate, aluminum chloride, sodium nitrate, sodium hydroxide, and phosphate-buffered saline (PBS) were purchased from HiMedia Laboratories (India).

Plants Collection and Identification

Fresh rhizomes of *C. longa* L. and *Z. officinale* Roscoe, dried fruits of *P. nigrum* L., and dried leaves of *C. tamala* (Buch.-Ham.) T. Nees and Eberm. were collected from local markets and kitchen gardens across Golaghat district, Assam, India, during the post-monsoon season (September-November 2023), which corresponds to the period of optimal phytochemical abundance in these herbs. Initial identification was carried out using regional floras and authenticated by a plant taxonomist at the Department of Botany, Gauhati University. For confirmation, the specimens were compared with reference samples preserved at the institutional herbarium. Voucher specimens of each plant were prepared and deposited in the Herbarium under accession numbers: *C. longa* (GUBH20536), *Z. officinale* (GUBH20533), *P. nigrum* (GUBH20313), and *C. tamala* (GUBH20537). These voucher specimens will serve as future reference material for assessing the study's reproducibility.

The physicochemical properties of plant parts

The rough physicochemical quality of the plant material was assessed in accordance with the standard procedures specified in the Ayurvedic Pharmacopoeia of India, as advised by the Ministry of AYUSH. These tests were performed to support quality control of selected plant materials [11].

Phytochemical Extraction

After the initial crude quality check, the rhizomes of *C. longa* and *Z. officinale*, the fruits of *P. nigrum*, and the leaves of *C. tamala* were thoroughly washed to remove dirt and contaminants. Thereafter, the materials were individually dried in the shade at room temperature to preserve their phytochemicals, and then coarsely ground in an electric grinder. Each powdered plant sample was extracted in a Soxhlet apparatus with 500 ml of 95% ethanol in batches of 100g at 60°C for two successive days, each approximately 9 hours, to maximize recovery of bioactive compounds. Ethanolic extracts were subsequently concentrated under low pressure using a rotary evaporator and stored in airtight vessels at 4°C until further use in phytochemical and bioactivity experiments [12].

Qualitative Phytochemical Screening

The availability of the main fractions of phytoconstituents was determined through the conventional phytochemical screening methods [13]. These pre-tests provided a general overview of the chemical composition of each plant extract and revealed some details regarding their possible therapeutic significance.

QUANTITATIVE PHYTOCHEMICAL ASSAY

Total Polyphenol Content (TPC)

The TPC of each extract was determined by the Folin-Ciocalteu method and expressed as mg of gallic acid equivalents (GAE) per gram of dry extract. A calibration curve was made by using gallic acid standards ranging between 50-300 µg/ml. To each assay, 0.5 ml of sample or standard was pipetted sequentially, mixed with diluted FC reagent and 7.5% sodium carbonate, and then incubated for 30 minutes in the dark, followed by measurement at 765 nm. The samples were taken in triplicate and then analyzed using normal UV analysis methods. The standard gallic acid curve was then compared with the results thus obtained [14].

Total Flavonoid Content (TFC)

An aluminium chloride-based colorimetric assay was used to determine the flavonoid content of plant extracts, and the results were expressed as milligrams of catechin equivalents (CE) per gram of dry extract. A calibration curve was established using catechin, with concentrations between 10 and 100 µg/ml. To every concentration or standard (0.5 ml), $NaNO_2$, NaOH, and then $AlCl_3$ were added. This mixture was incubated for 10 min, and the absorbance was measured three times at 510 nm. The value of flavonoid content was determined based on the standard catechin [15].

Total Tannin Content (TTC)

By the modified Folin-Ciocalteu technique, the TTC of the extracts was reported as mg tannic acid equivalents (TAE) per gram of dry extract, compared with the tannic acid curve of calibration (10-100 µg/ml). To each sample or standard, 0.5 ml of FC reagent and 0.5 ml of 35% sodium carbonate were added, and 10 ml of distilled water was added to bring the volume to 10 ml. The absorbance was measured after 30 minutes of incubation at room temperature at 700 nm. All tests were done in triplicate, and TTC was determined with high regularity obtained using the standard curve [16].

Chromatographic Analysis

High-resolution liquid chromatography-mass spectrometry (HR-LCMS) was performed in positive ionization mode to examine the phytochemical composition of the plant extracts efficiently. Mass spectra were analyzed in an Agilent 6545 Q-TOF MS (Model G 6550A) fitted with a triple quadrupole, dual AJS electrospray ion (ESI) source. The optimal ion source settings were 35 psig, 13 L/min, and 250°C for nebulizer pressure, drying gas flow, and gas temperature, respectively, to ensure high sensitivity and accurate mass measurements. A chromatographic analysis was performed on a chromatographic system comprising an Agilent HiP autosampler (Model G4226A) and a 5.0 µl injection volume, with automated needle washing to prevent contamination during sample handling. A binary pump (Model G4220B) at a flow rate of 0.3 ml/min, and the mobile phase was composed of 95% water and 5 percent acetonitrile, keeping a stable system pressure of 1200 bar. A Model G1316C compartment precisely regulated the column temperature at 40 ± 0.8 °C. The diode array detector (DAD, Model G4212B) was used, with a response time of 2.0 sec, and automatically scanned the wavelength range from 190 to 640 nm. The annotation of major bioactive compounds with antioxidant and anti-inflammatory activities was achieved by tentatively identifying metabolites using the obtained MS data, which were compared with available metabolome databases, such as METLIN, MassBank, and NIST [17].

ANTIOXIDANT ASSAYS *IN VITRO*

DPPH Radical Scavenging Assay

The DPPH test was used to determine the antioxidant properties of the extracts, with ascorbic acid as the standard. The extracts or standard (10-100 µg/ml) were incubated with 0.1 mM DPPH solution at room temperature without light source exposure for 30 minutes. Of this, absorbance was determined at 517 nm against a methanol blank. All the measures were taken three times. The degree of DPPH inhibition was calculated, and IC_{50} values were measured to determine antioxidant potential [18].

ABTS Free Radical Scavenging Assay

The plant extracts' ability to quench the ABTS radical cation was also used to assess antioxidant activity. The reaction of 2.45 mM of potassium persulfate with 7 mM ABTS in the dark after 12 hours of incubation formed $ABTS^{\bullet+}$. Dilution with PBS was performed until the absorbance reached 0.70±0.02 at 734 nm. Plant extracts or ascorbic acid were tested at a concentration of 40 to 240 µg/ml. In each assay, 1 ml of the test sample was added to $ABTS^{\bullet+}$ (1 ml) in a test tube and left to stand at room temperature. The absorbance was recorded at 734 nm with PBS as the blank and absorbance at 734 nm in the presence of $ABTS^{\bullet+}$ as the control. Each experiment was carried out at least three times, and the IC_{50} was measured to determine antioxidant activity [19].

Statistics

Linear regression analysis was employed to calculate IC_{50} values. All experiments were carried out in triplicate, and results were expressed as mean ± SEM. The data were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test, with mean values considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSIONS

Plant Parts and Physiochemical Properties

All plant materials were within Ayurvedic Pharmacopoeial standards, ensuring the crude drug's safety, quality, and purity. The amount of extraneous matter was low (0.94-1.95%), the total ashes were low (4.72%-8.46%), and acid-insoluble ash was also low (0.48-1.36%), which shows that it was very pure. The maximum alcoholic extractive value was 13.48% in *C. tamala*, whereas the hydrophilic content was 12.38% in *C. longa*. These standards helped to prevent the use of adulterated or substandard products and provided scientific validation for traditional remedies.

Phytochemical Extraction

The ethanolic extraction of the selected plant materials yielded semi-solid aromatic masses, each with a distinct color, revealing the color properties of their natural composition. *C. tamala* exhibited the highest yield (14.48%, reddish), indicating greater solubility of its active constituents, while *C. longa* showed the lowest (11.18%, greenish). *P. nigrum* fruits with a dark black extract had a yield of 11.45%, and the rhizomes of ginger, on the other hand, yielded a reddish extract but with a slightly higher yield of 12.37%. The reddish, black, and greenish hues correspond to curcuminoids or gingerol, piperine, and chlorophyll-rich compounds, respectively. Overall, *C. tamala* demonstrated superior extractive potential among the evaluated plant species.

Qualitative Phytochemical Test

Preliminary phytochemical profiling of ethanolic extracts of *C. longa*, *Z. officinale*, *P. nigrum*, and *C. tamala* revealed a wide

variety of bioactive compounds (Table 1). Each of the four extracts was positive for alkaloids, flavonoids, phenols, glycosides, tannins, carbohydrates, and proteins, indicating a rich phytochemical composition. Interestingly, none of the four plant extracts contained saponins, suggesting that phytochemical expression varies with plant species and environmental conditions. These initial screenings were essential for ensuring the consistency and potency of herbal products by verifying the presence of active compounds.

Table 1: Phytochemicals of ginger, turmeric, black pepper, and Indian bay leaf extract

Phytochemicals	Test Performed	*Z. officinale*	*C. longa*	*P. nigrum*	*C. tamala*
Alkaloids	Dragendorff's, Hager's, Wagner's, Mayer's	+, +, +, +	+, +, +, +	+, +, +, +	+, +, –, +
Flavonoids	Shinoda, Ferric chloride	+, +	+, +	+, +	+, +
Phenols	Ellagic acid, Ferric chloride	+ ,+	+, +	+, +	+, +
Glycosides	Keller-Killiani, Legal's, Baljet	+, –, +	+, +, +	+, +, +	+, +, +
Steroids	Liebermann-Burchard, Salkowski, Sulfur	–, –, +	+, –, –	+, –, –	–, +, –
Saponins	Foam test	–	–	–	–
Tannins	Ferric chloride	+	+	+	+
Carbohydrates	Molisch's, Benedict's, Fehling's	+, +, –	+, –, +	+, –, +	+, –, +
Proteins	Biuret, Millon's	+, +	+, +	+, +	+, +

'+' and '–' indicate 'presence' and 'absence' of phytochemicals, respectively

Quantitative Phytochemical Screening

TPC, TFC, and TTC

Quantitative estimation of total phenolic, flavonoid, and tannin contents revealed marked variations among the four ethanolic extracts, reflecting species-specific differences in secondary metabolite composition (the absorbance of the standard in TPC, TFC, and TTC assays is shown in Table 2). *C. longa* exhibited the highest concentrations of all three bioactive groups (TPC: 377.50±4.50 mg GAE/g; TFC: 294.29±2.18 mg CE/g; TTC: 201.25±3.14 mg TAE/g), consistent with its richness in curcuminoids and other polyphenolic compounds that possess strong antioxidant and anti-inflammatory potential. *Z. officinale* showed similarly high phytochemical levels (TPC: 301.25±8.19 mg GAE/g; TFC: 262.86±0.82 mg CE/g; TTC: 188.75±5.20 mg TAE/g), attributable to the presence of gingerols and shogaols known for their redox-modulating properties. *P. nigrum* displayed moderate bioactive content (TPC: 245.00±1.90 mg GAE/g; TFC: 207.14±2.97 mg CE/g; TTC: 151.25±3.14 mg TAE/g), in line with its lower polyphenol concentration but notable for piperine and its derivatives, which enhance the bioavailability of co-administered phytochemicals. *C. tamala* showed the lowest values (TPC: 166.25±6.29 mg GAE/g; TFC: 155.70±3.77 mg CE/g; TTC: 121.25±4.78 mg TAE/g), likely due to limited extractable phenolics in leaf tissues. Overall, the results indicate that turmeric and ginger are the most phytochemically potent, whereas black pepper and Indian bay leaf play complementary and synergistic roles in potential polyherbal anti-arthritic formulations. Linear regression analysis of the calibration curves demonstrated excellent linearity for all standard compounds used for quantification. The calibration equations obtained were $y=0.008x+0.143$ ($R^2=0.964$) for gallic acid, $y=0.007x–0.031$ ($R^2=0.980$) for catechin, and $y=0.008x–0.076$ ($R^2=0.978$) for tannic acid (Figures 1-3). The high correlation coefficients ($R^2 > 0.96$) indicated a strong linear relationship between absorbance and concentration, confirming the accuracy and reliability of the colorimetric assays used to estimate total phenolic, flavonoid, and tannin contents. These results confirmed that the analytical methods employed were precise and reproducible, ensuring that the observed variations among the plant extracts genuinely reflect differences in phytochemical abundance rather than methodological error.

Table 2: Absorbance of standard in TPC, TFC, and TTC assays

TPC		TFC		TTC	
Conc.	**Std. abs. (765 nm)**	**Conc.**	**Std. abs. (510 nm)**	**Conc.**	**Std. abs. (700 nm)**
50	0.40±0.003	10	0.07±0.001	10	0.05±0.002
100	0.96±0.001	20	0.14±0.001	20	0.10±0.003
150	1.57±0.002	40	0.22±0.001	40	0.21±0.004
200	1.75±0.001	60	0.43±0.001	60	0.45±0.006
250	2.31±0.001	80	0.55±0.004	80	0.59±0.002
300	2.42±0.041	100	0.79±0.001	100	0.86±0.005

All concentrations were tested in triplicate, and absorbance values are expressed as mean ± SEM. Conc: concentration; Std. abs: Standard absorbance.

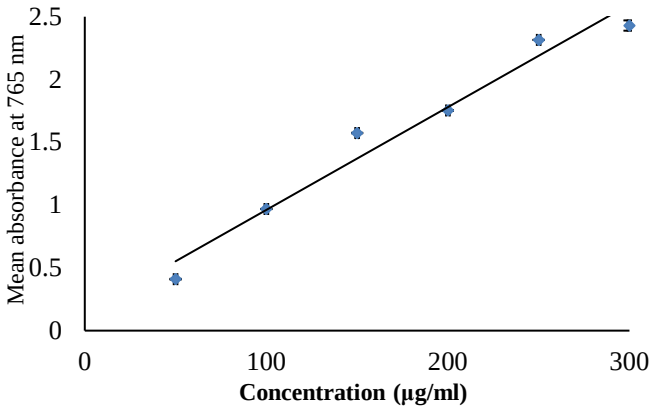


Figure 1: Calibration curve of gallic acid

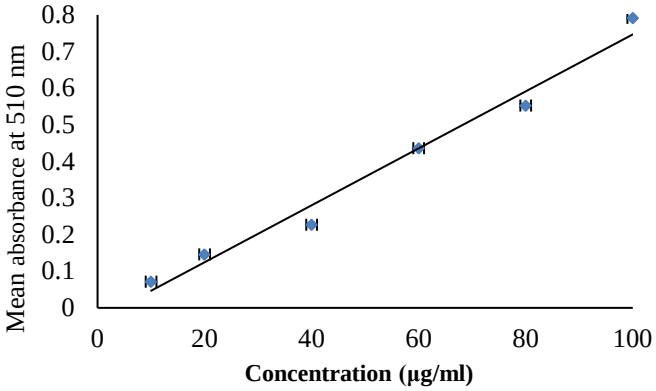


Figure 2: Calibration curve of catechin

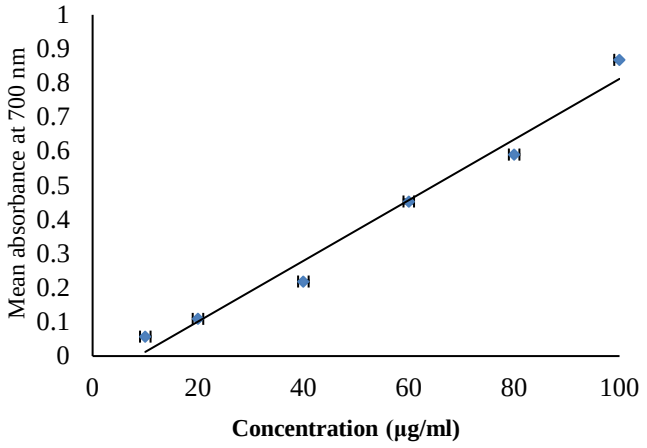


Figure 3: Calibration curve of tannic acid

CHROMATOGRAPHIC ANALYSIS

HR-LCMS Analysis of *C. longa*

Turmeric is well known for its potent bioactive compound, curcumin ($C_{21}H_{20}O_6$), with a molecular weight of 368.13 g/mol. In our HR-LCMS analysis, curcumin was clearly identified as the dominant constituent. A sharp, strong peak was observed in the positive-ion chromatogram (Figure 4) within the retention time window 10.28-10.42 minutes, indicating reliable detection under the conditions used. Mass spectrometric analysis revealed a precursor ion at m/z 368.14 (Figure 5), corresponding to the molecular ion $[M]^+$ of curcumin. Additionally, a less intense peak at m/z 369.14 was observed, which represented the protonated form $[M+H]^+$ of the molecule. Further confirmation came from tandem mass spectrometry (MS/MS), which detected a characteristic fragment ion at m/z 177.04 (Figure 6). This fragment is consistent with a bismethoxycurcumin derivative, in a doubly sodiated state $[M+2Na]^{2+}$, suggesting the loss of two methoxy ($-OCH_3$) groups, which is a typical fragmentation pattern for curcumin and its analogs. These results were consistent with the previously reported standard spectra of curcumin (Figure 7) [20], reaffirming turmeric's phytochemical richness, with curcumin as its hallmark compound.

The identification of curcumin as the major phytoconstituent in *Curcuma longa* by HR-LCMS analysis is critical for phytochemical validation and therapeutic standardization. Curcumin is the principal bioactive compound responsible for turmeric's wide range of pharmacological effects, including anti-inflammatory, antioxidant and anti-arthritic activities. The distinct retention time and strong molecular ion peak confirm the compound's purity and dominance in the extract, reflecting the chemical integrity of the plant material. The detection of specific precursor and fragment ions consistent with known curcumin fragmentation patterns further authenticated its structural identity. Moreover, the confirmation through MS/MS fragmentation provided strong evidence of analytical precision and methodological reliability. This analysis not only supports the phytochemical authenticity of *C. longa* but also ensures batch-to-batch consistency, reinforcing its suitability for use in polyherbal formulations and quality control studies.

HR-LCMS Analysis of *Z. officinale*

Gingerol is a major phytoconstituent in the rhizome of *Z. officinale*. Among many isomers, 6-gingerol ($C_{17}H_{26}O_{4)}$ is the most prominent form of gingerol with a molecular weight of 294.38 g/mol. The total chromatogram of *Z. officinale* extract showed a higher peak between RT 13.46 and RT 13.66 min (Figure 8), and a precursor ion at m/z=370.26 was selected for MS/MS analysis. The precursor ion indicated that 6-gingerol forms $[M-H+2K]^+$ adducts in the MS spectrum (Figure 9). The MS/MS fragmentation showed a peak of m/z 146.09 (Figure 10), which confirmed the phytoconstituent as 6-gingerol in $[M-H+2Na]^+$ adduct form, considering the most probable fragment occurring between C7-C8 due to its higher electron density. It is essential to mention that the ring moiety in a chemical structure

is relatively in resonance compared to aliphatic side chains. Further validation of the confirmed compound was achieved by comparing its MS2 spectrum with that of 6-gingerol (Figure 11) [21]. We observed the highest fragment at m/z 99.08 (charge 0) $[M+3H]^{3+}$, which confirmed the phytoconstituent as 6-gingerol in the $[M-H+2Na]^{+}$ form. Its precise detection authenticated the quality of the plant material, ensuring consistency in therapeutic efficacy. Additionally, matching the experimental MS fragmentation pattern with the standard spectrum further substantiated structural confirmation and analytical accuracy. This characterization not only reinforced the chemical standardization of *Z. officinale* extract but also provided a crucial reference for quality control and reproducibility in further formulation and pharmacological studies.

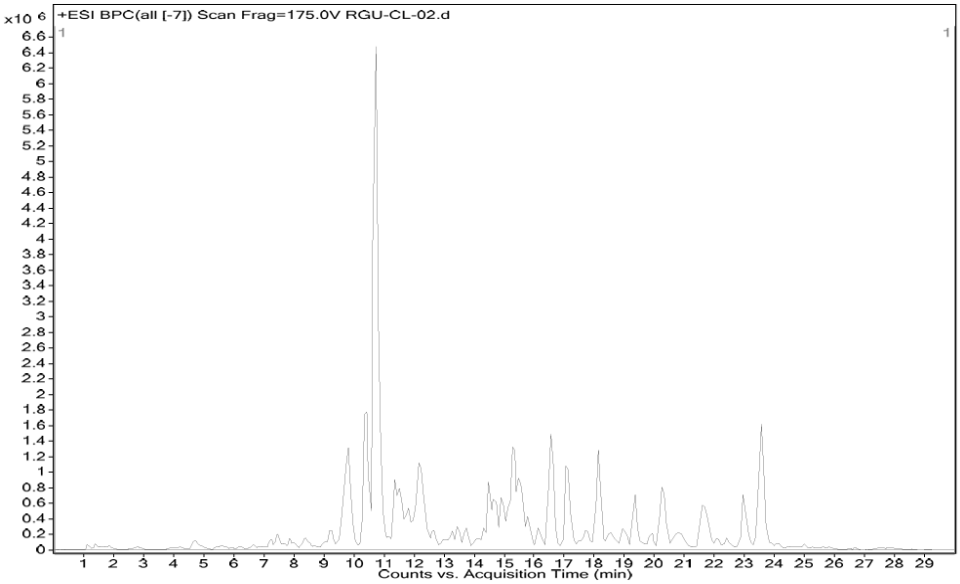


Figure 4: Total ion chromatogram of *C. longa* extract

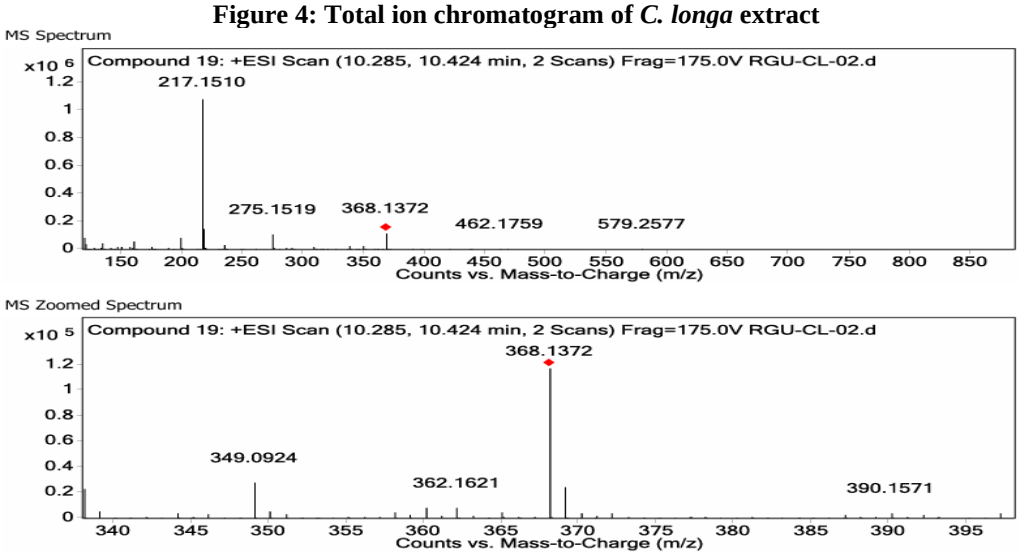


Figure 5: MS spectra of *C. longa* extract

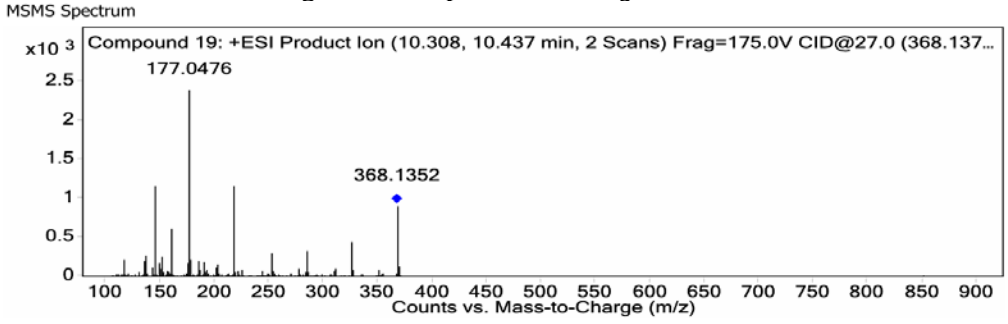


Figure 6: MS/MS spectra of *C. longa* extract

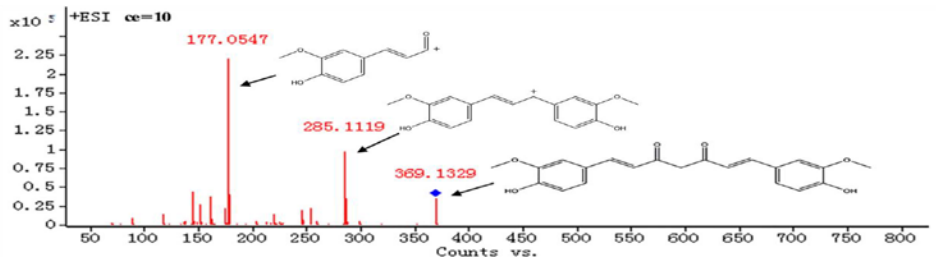


Figure 7: Standard product ion spectra of curcumin

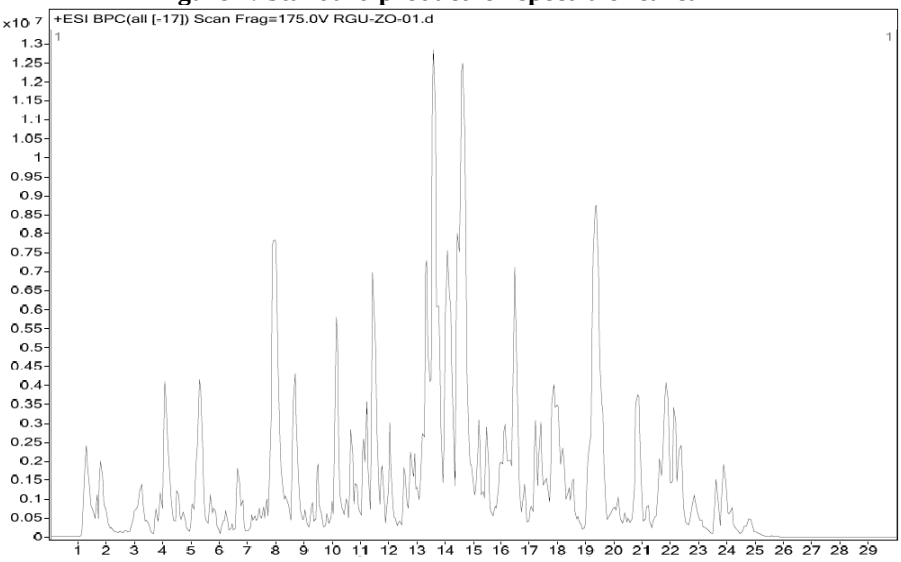


Figure 8: Total chromatogram of the *Z. officinale* extract (X-axis: Acquisition time in minutes, Y-axis: Counts)

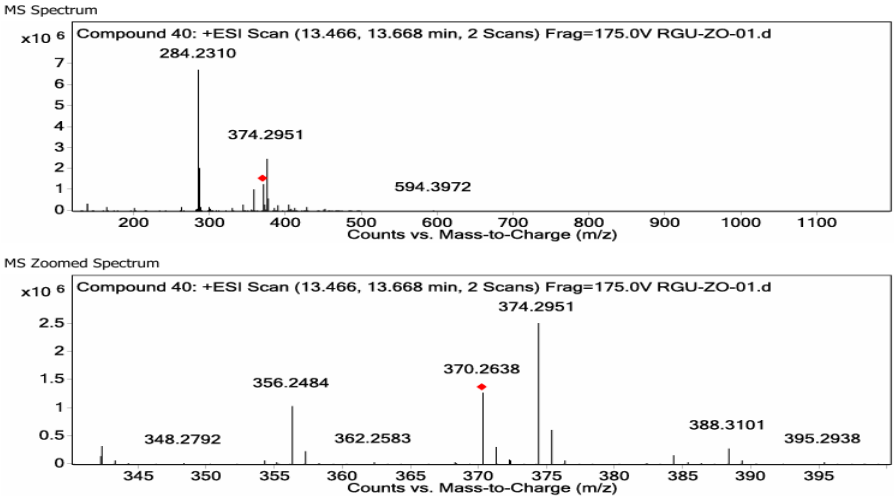


Figure 9: MS spectra of *Z. officinale* extract

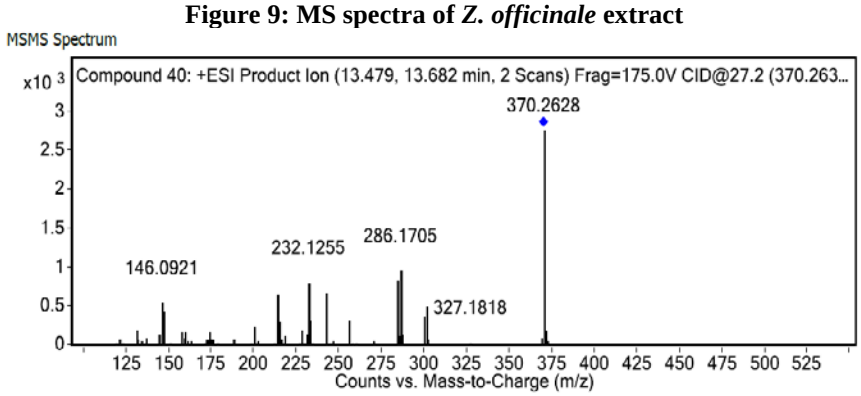


Figure 10: MS/MS spectra of *Z. officinale* extract

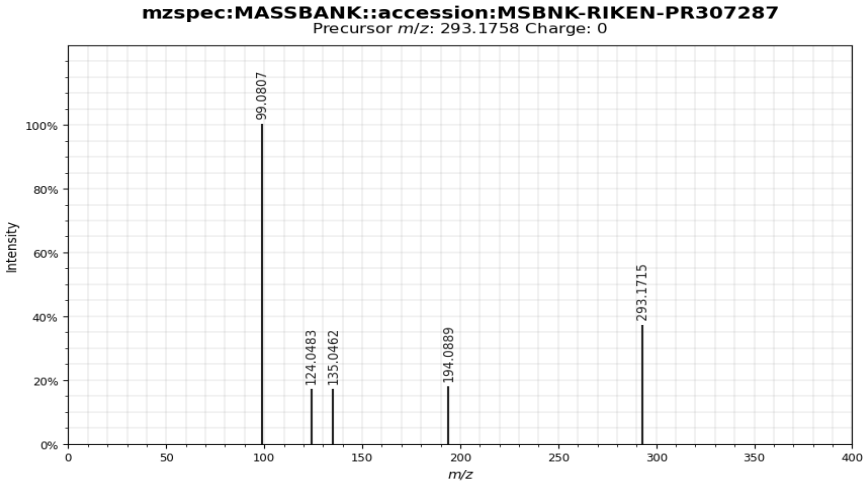


Figure 11: Standard MS/MS spectra of 6-gingerol

HR-LCMS Analysis of *P. nigrum*

P. nigrum is best known for its characteristic alkaloid piperine, often highlighted as its primary bioactive compound. Interestingly, in our HR-LCMS analysis, piperine was not the most abundant compound detected. Instead, a related compound, dipiperamide E ($C_{34}H_{38}N_2O_6$; molecular weight: 570.7 g/mol), emerged as the dominant phytochemical, as observed in the total ion chromatogram in between RT 17.57 to 17.77 (Figure 12) and mainly in its protonated form $[M+H]^+$ (Figure 13), with m/z 201.05 as one of the prominent fragments (Figure 14).

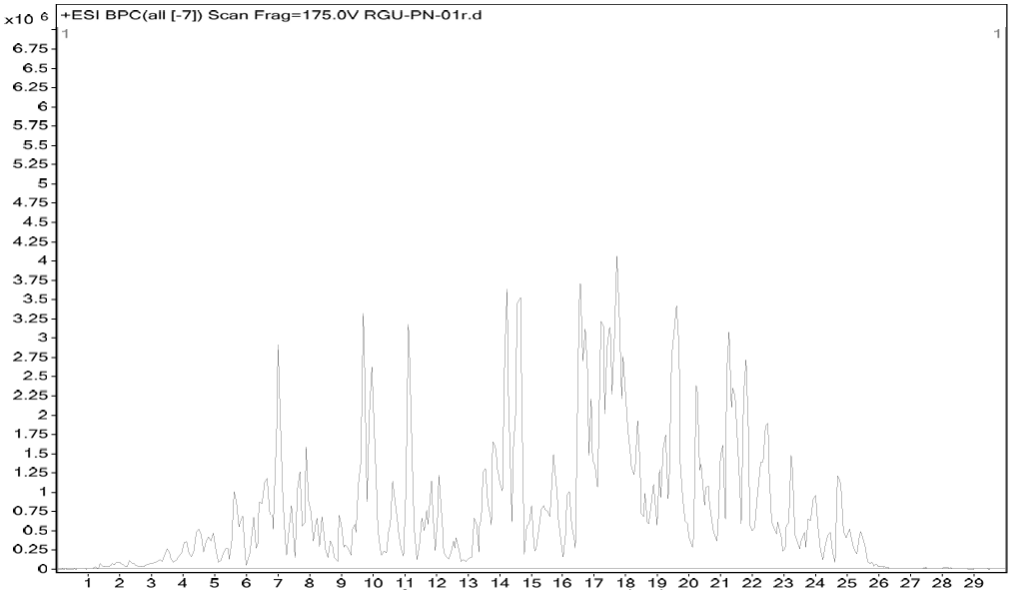


Figure 12: Total ion chromatogram of *P. nigrum* (X-axis: Acquisition time in minutes, Y-axis: Counts)

The unexpected lower signal for piperine could be influenced by several factors, including the solvent polarity used during extraction, thermal degradation of sensitive alkaloids, seasonal or geographical variations affecting the plant's phytochemical profile, or ion-suppression effects during mass spectrometric analysis. In contrast, the strong and consistent detection of dipiperamide E suggested that it may be more stable or more efficiently ionized under the analytical conditions used in this study. This finding underscores the complexity of black pepper's phytochemical profile and the importance of comprehensive profiling to elucidate the full spectrum of its bioactive potential. Nonetheless, given the anti-inflammatory pharmacological relevance of di-piperamide E (a piperamide derivative), the extract was considered for inclusion in subsequent *in vitro* antiarthritic assays to evaluate potential synergistic activity.

HR-LCMS Analysis of *C. tamala*

Eugenol ($C_{10}H_{12}O_2$, MW = 164.20 g/mol) is reported to be a major phytoconstituent in *C. tamala* leaves, along with cinnamaldehyde, cinnamic acid, and coumarin. The total chromatogram of *C. tamala* extract (Figure 15) showed a higher peak between RT 10.80 and RT 10.95 min, and a precursor ion

at m/z=203.17 (Figure 16) was selected for MS/MS analysis. The precursor ion indicated that eugenol forms [M+K]+ adducts in the MS spectrum. The MS/MS spectrum showed a peak at m/z 147.11 (Figure 17), confirming the phytoconstituent as eugenol in the $[M+H]^+$ adduct form. Other major peaks such as m/z=133.09 $[M+H]^+$, m/z=105.06 $[M+H]^+$, m/z= 161.12 $[M+2ACN+H]^+$, and 119.08 $[M+2ACN+H]^+$ were also confirmed, and validated by the standard MS2 spectrum of eugenol (Table 3, and Figure 18) [22]. From an analytical standpoint, this result demonstrated the reliability and sensitivity of the HR-LCMS method in detecting key bioactive markers. The successful comparison with a standard MS2 spectrum further ensured method accuracy and compound authenticity, strengthening the chemical characterization of *C. tamala*. In the context of a polyherbal antiarthritic formulation, the presence of eugenol justified the inclusion of *C. tamala* as it complements the anti-inflammatory actions of curcumin, gingerol, and piperine/dipiperamide E from other constituent plants. Eugenol's ability to inhibit pro-inflammatory mediators and reduce oxidative stress supports a synergistic therapeutic effect, thereby enhancing the overall efficacy, safety, and scientific validity of the formulation. The structure of principal phytoconstituents in *C. longa*, *Z. officinale*, *P. nigrum*, and *C. tamala* extracts is shown in Table 4.

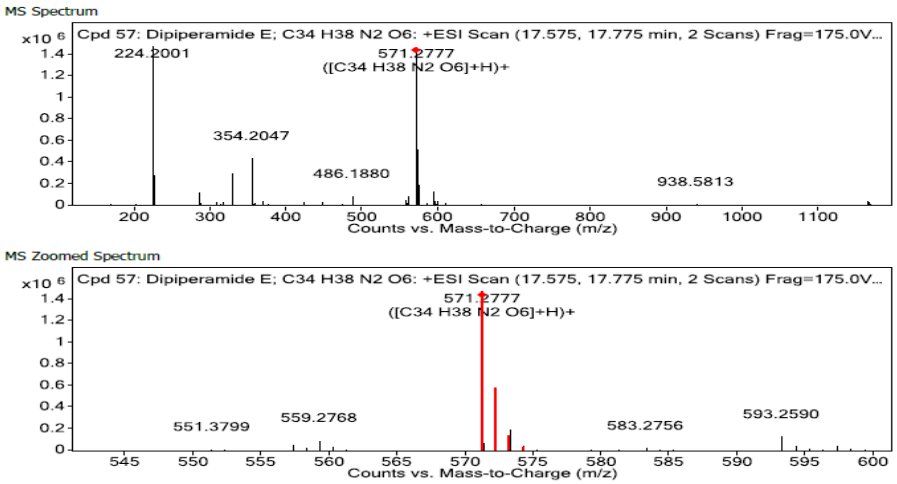


Figure 13: MS spectra of *P. nigrum*

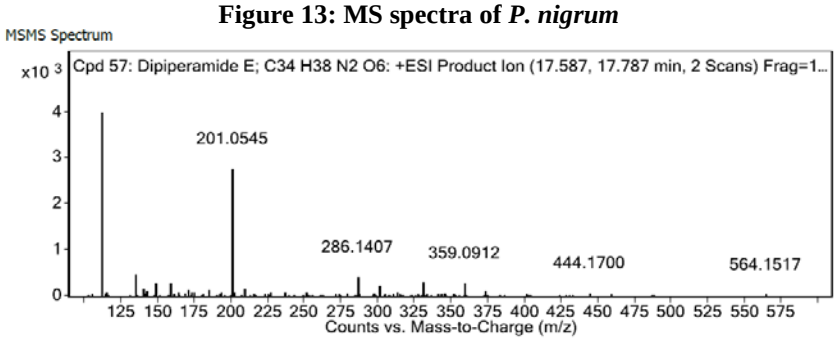


Figure 14: MS/MS spectra of *P. nigrum*

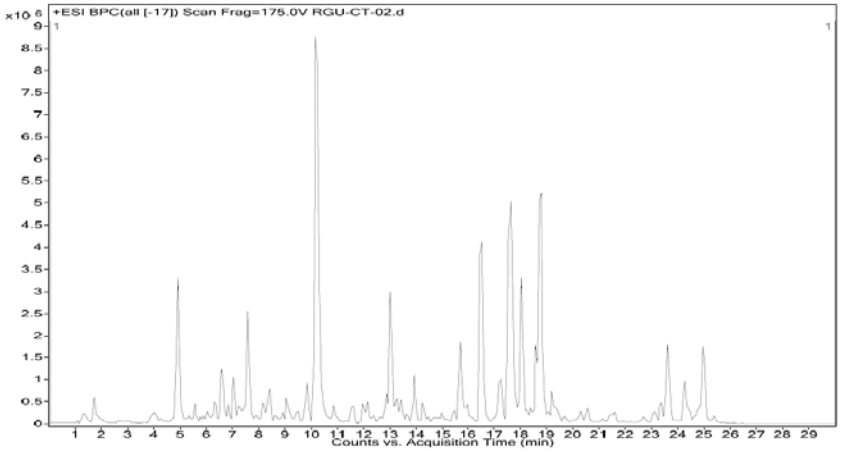


Figure 15: Total chromatogram of the *C. tamala* extract

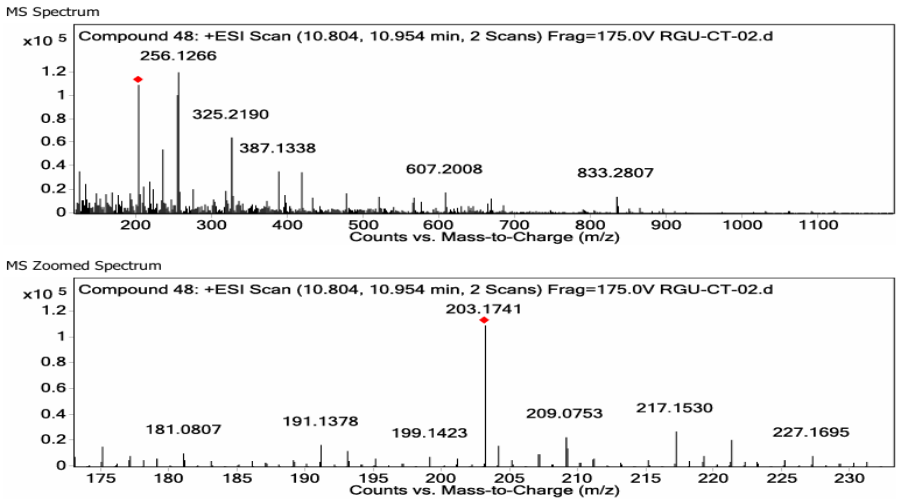


Figure 16: MS spectra of *C. tamala* extract

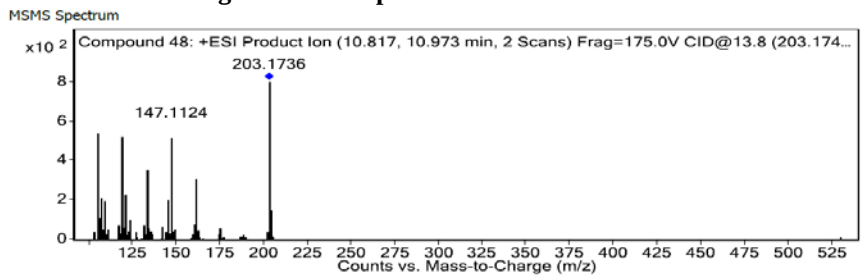


Figure 17: MS/MS spectra of *C. tamala* extract

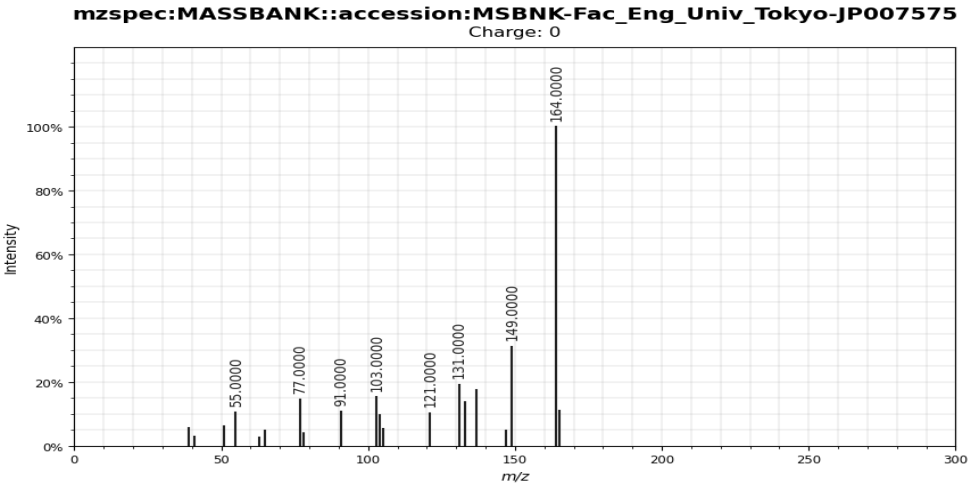


Figure 18: Standard MS/MS spectra of Eugenol

Table 3: Tandem peak list for 6-gingerol obtained in HR-LCMS

m/z	z	Abund
105.0662	1	540.57
107.0816	1	208.93
109.0973	1	196.59
119.0819	1	520.54
121.0974	1	224.7
133.0975	1	356.73
145.0954	1	199.7
147.1124	1	515.01
161.1294	1	304.32
203.1736	1	801.09

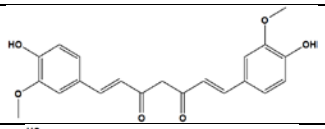
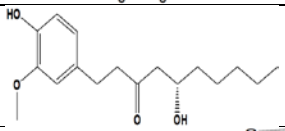
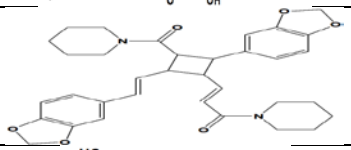
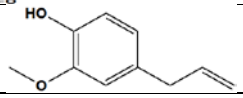
ANTIOXIDANT ASSAYS

DPPH Radical Scavenging Assay

Against *C. longa*, *Z. officinale*, *P. nigrum*, and *C. tamala* extracts, regression equations of the standard were y=0.732x+14.00 (R^2=0.994) (Figure 19), y=0.741x+12.71 (R^2=0.989) (Figure 20), y=0.720x+15.94 (R^2=0.987) (Figure 21), and y=0.694x+15.65 (R^2=0.984) (Figure 22). The regression modes of the extracts were y=0.653x+7.666, R^2=0.987 (*C. longa*) (Figure 19); y=0.634x+5.417, R^2=0.979 (*Z. officinale*) (Figure 20); y=0.596x+6.905, R^2=0.977 (*P. nigrum*) (Figure 21), and y=0.579x+2.924, R^2=0.98 (*C. tamala*) (Figure 22). The good linearity of the calibration curves (R^2>0.97)

confirmed the method's reliability and reproducibility, establishing a strong analytical foundation for further formulation work.

Table 4: Structure of principal phytoconstituents in *C. longa*, *Z. officinale*, *P. nigrum*, and *C. tamala* extracts

Phytoconstituents	Structure
Curcumin	
6-Gingerol	
Dipiperamide E	
Eugenol	

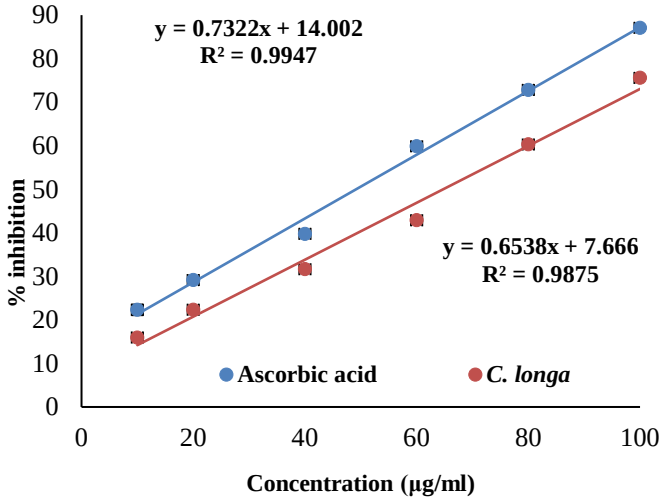


Figure 19: DPPH scavenging activity of *C. longa*

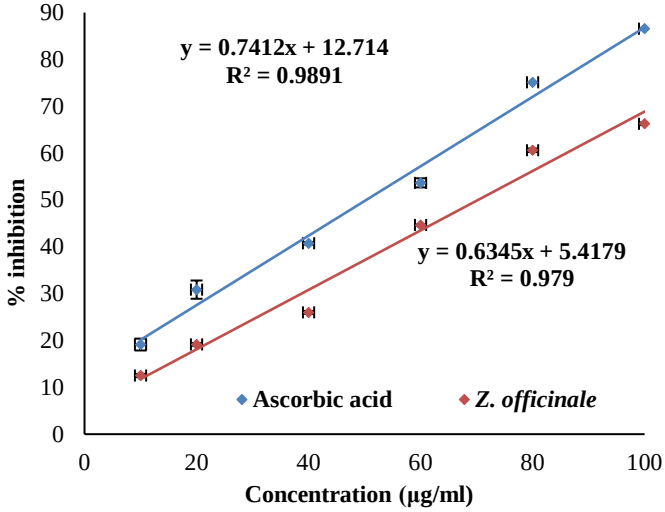


Figure 20: DPPH scavenging activity of *Z. officinale*

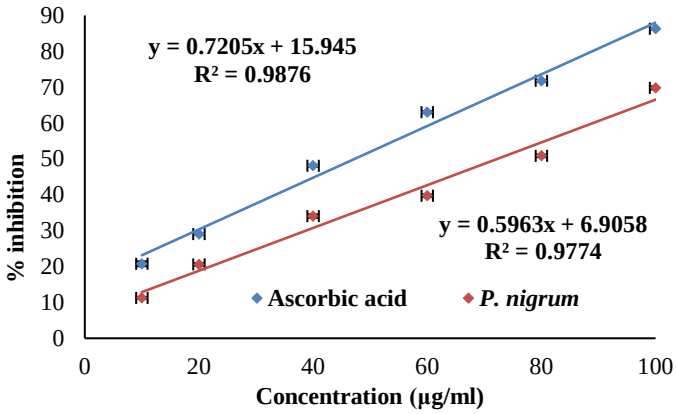


Figure 21: DPPH scavenging activity of *P. nigrum*

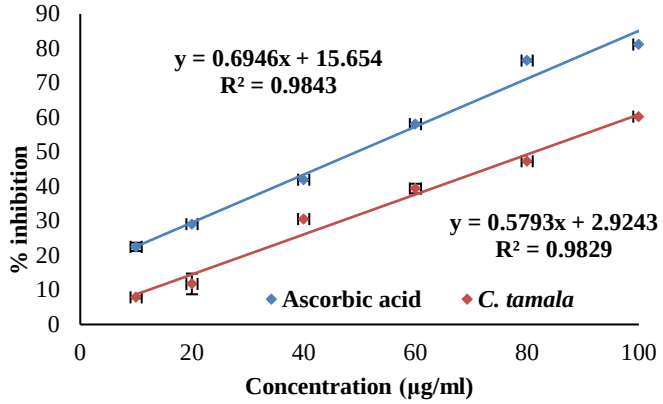


Figure 22: DPPH scavenging activity of *C. tamala*

Moreover, the statistical comparison revealed a significantly lower IC_{50} value for ascorbic acid ($p<0.05$) across all extracts, indicating the highest radical-scavenging potential (Table 5). Among the plant extracts, *C. longa* and *Z. officinale* showed statistically similar antioxidant activity ($p>0.05$), while *P. nigrum* had comparable potency to *Z. officinale* but lower than *C. longa* extract. *C. tamala* displayed a significantly higher IC_{50} value than all other samples, signifying the weakest DPPH scavenging ability. The overall order of activity was Ascorbic acid>*C. longa*>*Z. officinale*>*P. nigrum*>*C. tamala*.

ABTS Free Radical Scavenging Assay

The standard calibration curves were linear, with R^2 values ranging from 0.988 to 0.992, confirming the assays' reliability. The regression analyses for ascorbic acid versus *C. longa* and *Z. officinale* were y = 0.355x + 4.009 (Figure 23) and y = 0.362x + 1.231 (Figure 24), respectively. *P. nigrum* and *C. tamala* recorded the same regression of y =0.361x+2.765 (Figure 25 and Figure 26). The respective regression equations of the plant extracts are *C. longa*: y=0.321x-3.261 (R^2=0.986) (Figure 23), *Z. officinale*: y=0.316x-3.152 (R^2=0.986) (Figure 24), *P. nigrum*: y=0.280x-5.925 (R^2=0.980) (Figure 25), and *C. tamala*: y=0.230x-1.385 (R^2=0.977) (Figure 26).

A similar IC_{50} pattern was observed in the ABTS assay, with ascorbic acid showing a markedly lower IC_{50} than all the extracts ($p<0.05$), confirming its superior antioxidant activity. Among the extracts, *C. longa*, *Z. officinale*, and *P. nigrum* showed no significant difference ($p > 0.05$), indicating comparable ABTS radical-scavenging capacities. *C. tamala* again exhibited the highest IC_{50}, indicating significantly weaker antioxidant potential. However, the order of activity followed the same trend as Ascorbic acid>*C. longa*>*Z. officinale*>*P. nigrum*>*C. tamala* emphasizing the polyphenol-rich nature of the extracts and their prospective contribution to the antiarthritic formulation through oxidative stress modulation. Furthermore, DPPH and ABTS assays confirmed that *C. longa* and *Z. officinale* possess potent electron- and hydrogen-donating capacities, underscoring their significant contribution to the overall antioxidant and potential antiarthritic efficacy of the polyherbal formulation. In the ABTS assay, however, the overall higher IC_{50} values indicated a comparatively reduced reactivity of the extracts toward the charged $ABTS^{+\bullet}$ radicals compared to the DPPH assay. This difference could be due to variations in solubility, radical accessibility, and the polarity of antioxidant constituents, as the DPPH system primarily favors lipophilic interactions.

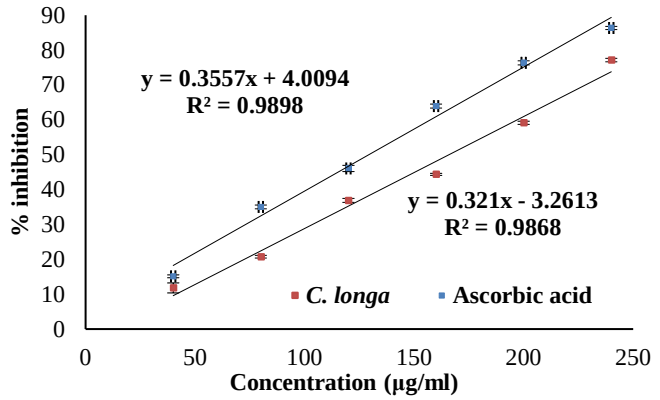


Figure 23: ABTS scavenging activity of *C. longa*

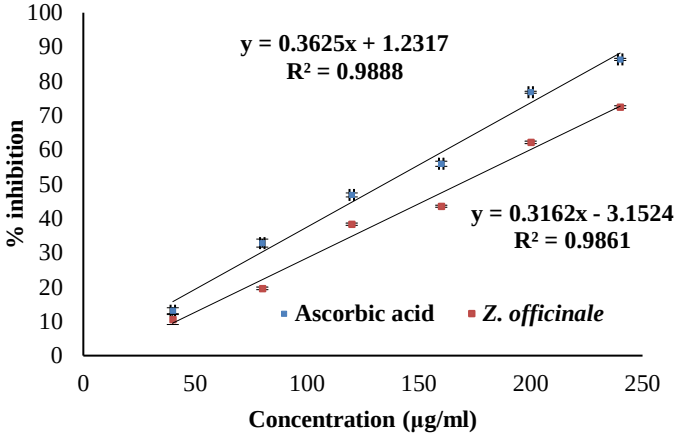


Figure 24: ABTS scavenging activity of *Z. officinale*

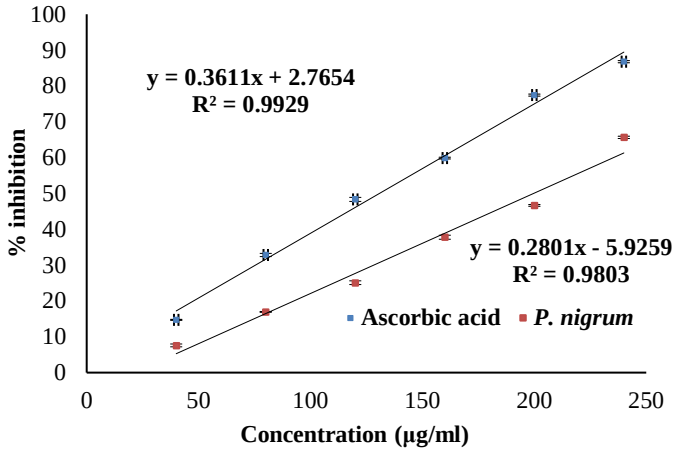


Figure 25: ABTS scavenging activity of *P. nigrum*

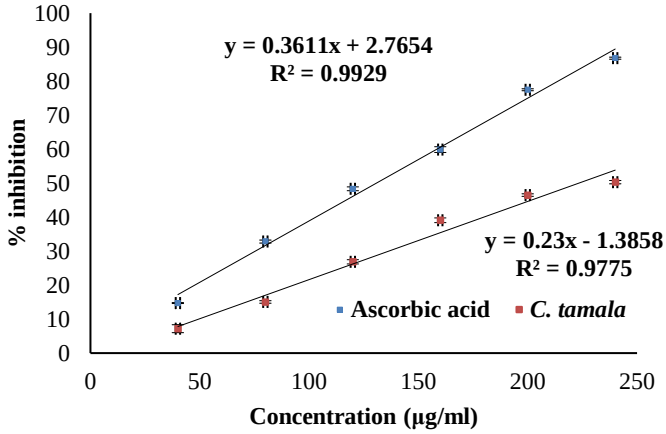


Figure 26: ABTS scavenging activity of *C. tamala*

Oxidative stress plays a pivotal role in the initiation and progression of RA by amplifying synovial inflammation, activating NF-κB, and promoting cartilage and bone damage. Antioxidant defenses are often impaired in patients with RA, creating a redox imbalance that exacerbates cytokine release and joint destruction. Establishing a clearer mechanistic link among oxidative stress, antioxidant restoration, and clinical improvement was essential to justify the therapeutic relevance of antioxidant-rich botanicals and to inform the design of optimally effective polyherbal anti-arthritic interventions. Further, each plant's antioxidant potential can vary considerably depending on its geographical origin, harvest period, drying method, and extraction procedure. Therefore, re-evaluating antioxidant activity under standardized experimental conditions ensured that all extracts used in the formulation were chemically active, comparable, and compatible in their free-radical quenching capacities. This ensured batch-to-batch consistency, supported biological rationale for combination, and provided baseline data for correlating *in vitro* antioxidant strength with subsequent anti-arthritic activity.

Table 5: IC_{50} values of standard and extracts in DPPH and ABTS assays

Test	IC_{50} (µg/ml)		IC_{50} (µg/ml)		IC_{50} (µg/ml)		IC_{50} (µg/ml)	
	Ascorbic acid	Turmeric	Ascorbic acid	Ginger	Ascorbic acid	Black pepper	Ascorbic acid	Indian bay leaf
DPPH	49.18±0.40	64.83±0.29	50.32±0.78	70.32±0.29	47.30±0.31	72.30±0.39	49.49±0.41	81.30±0.85
ABTS	129.55±0.5	145.60±0.60	134.72±0.66	148.25±0.54	130.84±0.33	157.41±0.36	130.84±0.43	211.37±0.62

In our study, the IC_{50} values of standard diclofenac and individual plant extracts were evaluated using egg albumin, BSA, and anti-proteinase assays. Diclofenac exhibited the highest antiarthritic potential with the lowest mean IC_{50} (59.00 ± 0.57 µg/ml), followed by *C. longa* (79.89 ± 0.43 µg/ml), *Z. officinale* (89.41 ± 0.79 µg/ml), *P. nigrum* (103.22 ± 0.51µg/ml), and *C. tamala* (125.28±0.70 µg/ml). Subsequently, the positive higher-order interactions among *C. longa*, *Z. officinale*, *P. nigrum*, and *C. tamala* (Coefficient = 3687.16) were confirmed, indicating synergistic relationships at the multi-component level, in which the combined effect of all four constituents enhanced the antiarthritic response beyond what each constituent contributed individually [23].

CONCLUSION

Given the limitations of currently available antiarthritic medications, this study represents a novel and significant contribution to the ethnopharmacological literature of North-East India. It establishes a scientific foundation for developing a polyherbal antiarthritic formulation comprising *Curcuma longa*, *Zingiber officinale*, *Piper nigrum*, and *Cinnamomum tamala*. Among these, *C. longa* exhibited the highest potential as an antiarthritic herb, as evidenced by its superior total phenolic content (TPC), total flavonoid content (TFC), total tannin content, and antioxidative capacity. The comparative potency order observed for the other herbs *Z. officinale*, *P. nigrum*, and *C. tamala* provides a rational basis for subsequent *in silico* and *in vitro* optimization of polyherbal combinations.

Furthermore, the integration of HR-LCMS analysis ensured a standardized phytochemical characterization, confirming the presence of key bioactive constituents: curcumin (*C. longa*), 6-gingerol (*Z. officinale*), dipiperamide E (*P. nigrum*), and eugenol (*C. tamala*). Interestingly, the study identified dipiperamide E as the predominant compound in *P. nigrum* instead of the commonly reported piperine, possibly due to solvent effects, seasonal variation, and agro-climatic factors. In summary, this study provides a valuable scientific base for selecting and combining these herbs to create an effective antiarthritic formulation.

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FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Nilutpal Hazarika conducted the laboratory experiments, collected, analyzed, and interpreted the data, and wrote the manuscript. Bipul Nath supervised the study and assisted with critical analysis and manuscript preparation. Subhashis Debnath helped in the oversight of the research and the development of the manuscript. Every author agreed to the final manuscript.

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