EJPMR, 2026, 13(5), 240-256 **Research Article** **Impact Factor: 8.158**

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

ISSN (O): 2394-3211
ISSN (P): 3051-2573
Coden USA: EJPMAG

ISOLATION, CHARACTERIZATION, AND EVALUATION OF ANTICONVULSANT POTENTIAL OF QUERCETIN AND B-CARYOPHYLLENE FROM HYDROETHANOLIC EXTRACT OF ANNONA SQUAMOSA LINN. LEAVES

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DOI: https://doi.org/10.5281/zenodo.19885189

How to cite this Article: Manju Rani1, Dr. Umesh Kumar1*. (2026). Isolation, Characterization, and Evaluation of Anticonvulsant potential of Quercetin and β-caryophyllene from Hydroethanolic Extract of Annona Squamosa Linn. Leaves. European Journal of Pharmaceutical and Medical Research, 13(5), 240–256.

Article Received on 24/03/2026 **Article Revised on 13/04/2026** **Article Published on 01/05/2026**

ABSTRACT

Epilepsy is a prevalent neurological disorder affecting around 50 million people globally, with nearly 30–40% of patients exhibiting resistance to existing antiepileptic drugs (AEDs) and suffering from significant side effects. The present study was undertaken to scientifically validate the traditional anticonvulsant use of *Annona squamosa* Linn. leaves by evaluating the phytochemical profile, safety, and therapeutic potential of its hydroethanolic extract and major isolated bioactive compounds, quercetin and β-caryophyllene. Fresh mature leaves (2000 g) collected from Green Earth Nursery, Gajraula, Uttar Pradesh, India, in July 2024, were shade-dried, powdered, and subjected to Soxhlet extraction with 70% hydroethanol, yielding 15% crude extract. Preliminary phytochemical screening confirmed the presence of flavonoids, terpenoids, alkaloids, phenols, tannins, saponins, glycosides, carbohydrates, and proteins. Quercetin and β-caryophyllene were successfully isolated through successive liquid-liquid partitioning and silica gel column chromatography, achieving purity greater than 95%. The isolated compounds were characterized using HPLC, mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectroscopy. Quantitative analysis revealed substantial enrichment of total flavonoid content (950 ± 12.5 mg QE/g) and total terpenoid content (910 ± 10.2 mg CE/g) in the purified fractions. In vitro DPPH radical scavenging assay demonstrated potent antioxidant activity of quercetin with an IC_{50} value of 18.2 µg/mL. Acute (OECD 423) and sub-acute (OECD 407) toxicity studies in Swiss albino mice confirmed an excellent safety profile, with no mortality or severe adverse effects observed up to 2000 mg/kg. In vivo anticonvulsant activity was assessed in maximal electroshock (MES) and pentylenetetrazole (PTZ)-induced seizure models. Both compounds exhibited significant dose-dependent protection. Notably, their combination (25 mg/kg each) produced synergistic effects, achieving 85% seizure protection, markedly reduced seizure duration and severity, and efficacy comparable to standard drugs (phenytoin and diazepam). Biochemical investigations revealed that the combination treatment effectively attenuated oxidative stress (reduced MDA levels) and restored antioxidant defense (SOD, catalase, and GSH), while normalizing GABA and glutamate neurotransmitter balance in brain tissue. These findings provide strong scientific evidence supporting the ethnomedicinal use of *Annona squamosa* leaves and highlight quercetin and β-caryophyllene as promising, safe, multi-targeted natural candidates for the development of novel anticonvulsant therapeutics.

KEYWORDS: Epilepsy, *Annona squamosa*, Quercetin, β-Caryophyllene, Anticonvulsant activity, Oxidative stress.

1. INTRODUCTION

Epilepsy is one of the most prevalent chronic neurological disorders, characterized by recurrent, unprovoked seizures resulting from excessive neuronal excitability and synchronized discharges in the brain. According to the World Health Organization, epilepsy affects approximately 50 million people worldwide, with nearly 80% of cases occurring in low- and middle-income countries. Globally, about 5 million new cases are diagnosed annually, and the age-standardized

prevalence rate stands at around 307 per 100,000 population. Despite the availability of more than 30 approved anti-epileptic drugs (AEDs), up to 30–40% of patients suffer from drug-resistant epilepsy, and many experience debilitating side effects such as sedation, cognitive impairment, hepatotoxicity, and teratogenicity. These limitations have intensified the search for safer, more effective, and mechanism-based therapeutic alternatives, particularly those targeting underlying pathophysiological mechanisms like oxidative stress, neuroinflammation, and imbalance between inhibitory (GABA) and excitatory (glutamate) neurotransmission.

Oxidative stress plays a central role in epileptogenesis and seizure-induced neuronal damage. Excessive generation of reactive oxygen species (ROS) during seizures leads to lipid peroxidation (elevated malondialdehyde levels), depletion of endogenous antioxidants (superoxide dismutase, catalase, and reduced glutathione), and subsequent neuronal injury. Concurrently, reduced GABAergic inhibition and heightened glutamatergic excitation further perpetuate hyperexcitability. Natural products, especially secondary metabolites from medicinal plants, have emerged as promising candidates because they often possess multi-target antioxidant, anti-inflammatory, and neuromodulatory properties with favorable safety profiles.

Annona squamosa Linn. (family Annonaceae), commonly known as custard apple or sugar apple, is a tropical fruit tree widely distributed in India, Southeast Asia, Central and South America, and parts of Africa. In traditional medicine systems (Ayurveda, Unani, and folk medicine of Uttar Pradesh and other Indian states), various parts of the plant—including leaves, seeds, bark, and fruit—have been used for the treatment of epilepsy, fever, dysentery, tumors, cardiac ailments, ulcers, and as a sedative. Phytochemical investigations have revealed that the leaves are particularly rich in bioactive secondary metabolites such as flavonoids, terpenoids, alkaloids, phenols, tannins, saponins, and glycosides. These compounds are believed to underlie the plant's reported antioxidant, anti-inflammatory, neuroprotective, and anticonvulsant activities.

Earlier pharmacological studies on crude extracts of *A. squamosa* leaves have demonstrated significant anticonvulsant effects in chemically induced (pentylenetetrazole, PTZ) and electrically induced (maximal electroshock, MES) seizure models in rodents. The extracts delayed seizure onset, reduced seizure duration and severity, and offered neuroprotection, effects attributed partly to their ability to scavenge free radicals and modulate GABAergic transmission. However, most of these investigations utilized crude extracts, limiting the identification of specific active constituents and their precise mechanisms of action.

Among the major isolated compounds from *A. squamosa* leaves, **quercetin** (a flavonol) and **β-caryophyllene** (a bicyclic sesquiterpene) stand out for their well-documented pharmacological potential. Quercetin is a potent antioxidant and anti-inflammatory flavonoid known to cross the blood-brain barrier and exert neuroprotective effects. Multiple preclinical studies have shown that quercetin attenuates seizures in PTZ-, kainic acid-, and pilocarpine-induced epilepsy models by reducing oxidative stress, suppressing pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), inhibiting NF-κB signaling, restoring GABA levels, and decreasing glutamate excitotoxicity. A comprehensive review concluded that quercetin holds considerable promise as an antiepileptic agent through multiple cellular and molecular pathways.

Similarly, β-caryophyllene, a selective agonist of cannabinoid receptor type 2 (CB2), has gained attention for its anticonvulsant and neuroprotective properties without the psychotropic effects associated with CB1 activation. It has demonstrated efficacy against PTZ-induced seizures, pilocarpine-induced status epilepticus, and Dravet syndrome models by reducing seizure frequency and duration, attenuating glial activation (astrogliosis and microgliosis), lowering lipid peroxidation, and improving behavioral comorbidities. Its ability to modulate neuroinflammation and oxidative stress via CB2 receptor-dependent pathways makes it an attractive candidate for epilepsy therapy.

Despite promising data on individual compounds, studies investigating the combined anticonvulsant potential of quercetin and β-caryophyllene isolated specifically from *A. squamosa* leaves remain limited. Furthermore, comprehensive evaluations that integrate extraction, isolation, characterization, detailed in-vitro antioxidant profiling, acute/sub-acute toxicity assessment, in-vivo anticonvulsant activity in both MES and PTZ models, seizure severity scoring, and mechanistic insights through brain oxidative stress markers and neurotransmitter levels (GABA and glutamate) are scarce. Such integrated research is essential to validate the therapeutic synergy, establish dose-response relationships, confirm safety, and elucidate the underlying biochemical mechanisms.

The present study was therefore undertaken to address these gaps. Hydroethanolic extract of *Annona squamosa* Linn. leaves collected from Gajraula, Uttar Pradesh, India, was prepared, and the major bioactive constituents—quercetin and β-caryophyllene—were isolated, purified, and fully characterized using HPLC, MS, and NMR techniques. The study systematically evaluated their individual and combined anticonvulsant potential in standardized rodent models of generalized tonic-clonic (MES) and myoclonic/absence (PTZ) seizures, along with their antioxidant capacity (DPPH assay), quantitative phytochemical content, acute and sub-acute toxicity profiles, effects on brain oxidative

stress biomarkers (MDA, SOD, catalase, GSH), and modulation of GABA and glutamate levels. By providing a complete phytochemical-to-pharmacological profile, this research aims to establish *Annona squamosa*-derived quercetin and β-caryophyllene as safe, effective, and mechanistically validated natural leads for the development of novel anticonvulsant therapeutics.

2. MATERIALS AND METHODS

2.1. Plant Material Collection

Fresh, mature leaves of *Annona squamosa* Linn. were collected from Green Earth Nursery, Gajraula, Uttar Pradesh, India, during the peak growing season in July 2024. A total of 2000 g (2 kg) of healthy, green leaves free from visible pest damage, fungal infection, or mechanical injury were harvested using sterilized scissors to minimize tissue damage. The leaves were immediately placed in sterile, labeled polythene bags and transported to the laboratory within 2 hours under cool conditions to preserve freshness and prevent degradation of bioactive constituents.

2.2. Preparation of Plant Extract

The collected leaves were shade-dried at room temperature (25–30°C) for 10–12 days in a well-ventilated area with periodic turning to ensure uniform drying and prevent fungal growth. Moisture loss was calculated using the formula:

Moisture Loss (%) = [(Fresh Weight – Dried Weight) / Fresh Weight] × 100.

The dried leaves were pulverized in a high-speed mechanical grinder and sieved through a 60-mesh sieve to obtain uniform particle size. Exactly 500 g of the fine powder was subjected to exhaustive Soxhlet extraction with 2 L of hydroethanolic solvent (70:30 ethanol:water, v/v) for 72 hours until the siphon tube solvent became colorless, indicating complete extraction. The crude extract was filtered through Whatman No. 1 filter paper, concentrated under reduced pressure at 40°C using a rotary evaporator (Büchi Rotavapor R-210), and stored in amber-colored glass containers at 4°C until further use. This hydroethanolic solvent system was chosen for its ability to extract both polar and non-polar bioactive constituents effectively.

2.3. Preliminary Phytochemical Screening

Qualitative phytochemical screening of the hydroethanolic extract was performed using standard color and precipitation tests to detect the presence of major secondary metabolites. Flavonoids were detected by the Shinoda test (pink/magenta color upon addition of magnesium and hydrochloric acid) and Alkaline Reagent test (intense yellow color that disappears upon acidification). Terpenoids were confirmed by the Salkowski test (reddish-brown interface after treatment with chloroform and concentrated sulfuric acid). Alkaloids were identified using Dragendorff's reagent (orange/reddish-brown precipitate). Phenols produced a dark green/blue color with 5% ferric chloride solution. Tannins were detected by the gelatin test (white precipitate). Saponins produced persistent foam upon vigorous shaking with water. Glycosides showed a reddish-brown interface in the Keller-Killiani test (glacial acetic acid + ferric chloride + sulfuric acid). Reducing sugars were confirmed by Benedict's test (reddish-brown precipitate upon heating with alkaline copper sulfate). Proteins were detected by the Biuret test (violet/purple color with copper sulfate and sodium hydroxide). All tests were performed in triplicate following standard protocols for reproducibility and reliability.

2.4. Isolation and Purification of Specific Flavonoids and Terpenoids

The crude hydroethanolic extract was subjected to liquid-liquid partitioning using solvents of increasing polarity (n-hexane, chloroform, and ethyl acetate) to obtain three major fractions. The n-hexane fraction (targeting non-polar compounds such as β-Caryophyllene) weighed 10 g (2% yield), the chloroform fraction 20 g (4% yield), and the ethyl acetate fraction 15 g (3% yield). These fractions were concentrated and stored in amber vials at 4°C.

Further purification was achieved by column chromatography on silica gel (60–120 mesh). The n-hexane fraction was eluted with a gradient of n-hexane:ethyl acetate (95:5 to 50:50 v/v) to isolate β-Caryophyllene. The chloroform and ethyl acetate fractions were eluted with a chloroform:methanol gradient (95:5 to 70:30 v/v) to isolate Quercetin. Purity was monitored by thin-layer chromatography (TLC) on silica gel 60 F_{254} plates using appropriate solvent systems (n-hexane:ethyl acetate 75:25 for β-Caryophyllene; chloroform:methanol 80:20 for Quercetin). Spots were visualized under UV light (254 nm and 365 nm) and by spraying with appropriate reagents. The isolated compounds were recrystallized from ethanol, dried under vacuum, and stored in amber vials at 4°C.

2.5. Characterization of Isolated Compounds

The isolated Quercetin and β-Caryophyllene were characterized using multiple analytical techniques. High-Performance Liquid Chromatography (HPLC) was performed on a Shimadzu LC-20AD system equipped with a UV detector. Quercetin was detected at 254 nm (retention time 3.25 min), and β-Caryophyllene at 210 nm (retention time 5.10 min). Mass spectrometry (MS) was carried out using an electrospray ionization (ESI) source on a Waters Xevo TQD instrument to confirm molecular ions and fragmentation patterns. Nuclear Magnetic Resonance (NMR) spectra (^{1}H-NMR and ^{13}C-NMR) were recorded on a Bruker Avance III 400 MHz spectrometer in deuterated solvents ($CDCl_3$ for β-Caryophyllene; DMSO-d_6 for Quercetin). DEPT experiments were used to distinguish carbon multiplicities. All spectral data were compared with authentic reference standards and literature values for confirmation of identity and purity (>95%).

2.6. Quantitative Estimation of Phytochemicals

2.6.1. Total Flavonoid Content (TFC)

Total flavonoid content was determined by the aluminum chloride colorimetric assay. A calibration curve was prepared using Quercetin standard (10–100 µg/mL) with $R^2 = 0.998$. The extract or purified compound (0.5 mL) was mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. After 30 min incubation at room temperature, absorbance was measured at 415 nm. Results were expressed as mg Quercetin equivalents per gram of extract (mg QE/g).

2.6.2. Total Terpenoid Content (TTC)

Total terpenoid content was quantified using the vanillin-sulfuric acid colorimetric assay. A calibration curve was prepared with β-Caryophyllene standard (10–100 µg/mL) with $R^2 = 0.996$. The sample (0.5 mL) was mixed with 0.5 mL of 8% vanillin in ethanol and 5 mL of 72% sulfuric acid, heated at 60°C for 15 min, cooled, and absorbance measured at 548 nm. Results were expressed as mg β-Caryophyllene equivalents per gram of extract (mg CE/g).

2.7. In-Vitro DPPH Radical Scavenging Activity

The antioxidant activity was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. Different concentrations of crude extract, Quercetin, and β-Caryophyllene (10–200 µg/mL) were mixed with 0.1 mM DPPH solution in methanol. After 30 min incubation in the dark at room temperature, absorbance was measured at 517 nm. The percentage radical scavenging activity (%RSA) was calculated as: $\%RSA = [(A_{control} - A_{sample}) / A_{control}] \times 100$. IC_{50} values (concentration required to scavenge 50% of DPPH radicals) were determined from dose-response curves.

2.8. Acute and Sub-Acute Toxicity Studies

2.8.1. Acute Toxicity Studies

Acute oral toxicity was assessed according to OECD Guideline 423 (Acute Toxic Class Method) using female Swiss albino mice (20–25 g). Animals were fasted overnight and administered single oral doses of Quercetin or β-Caryophyllene (5, 50, 300, and 2000 mg/kg body weight) suspended in 1% Tween 80. Animals were observed for 14 days for behavioral changes, physiological effects, body weight, and mortality. The maximum tolerated dose (MTD) was determined.

2.8.2. Sub-Acute Toxicity Study

Sub-acute toxicity was evaluated according to OECD Guideline 407 (Repeated Dose 28-Day Oral Toxicity Study) in gender-balanced groups of male and female Swiss albino mice (20–25 g). Animals received daily oral doses (10, 25, and 50 mg/kg) for 28 days. Body weight, behavioral parameters, food/water intake, and physiological signs were monitored daily. At the end of the study, animals were euthanized, and gross necropsy was performed.

2.9. In-Vivo Anticonvulsant Studies

All in-vivo experiments were conducted in accordance with institutional animal ethics committee guidelines (CPCSEA registration). Swiss albino mice (20–25 g) were used and housed under standard conditions (12 h light/dark cycle, 25 ± 2°C, 50–60% humidity) with free access to food and water. Animals were divided into groups (n=6 per group) and treated orally with vehicle (1% Tween 80), test compounds (Quercetin or β-Caryophyllene at low, medium, and high doses), combination (25 mg/kg each), or standard drugs 30–60 min prior to seizure induction.

2.9.2. Maximal Electroshock Seizure (MES) Model

Generalized tonic-clonic seizures were induced by delivering a supramaximal electrical stimulus (50 mA, 60 Hz, 0.2 s duration) via corneal electrodes using an electroconvulsometer. The duration of tonic hind limb extension (THLE) was recorded. Animals showing THLE duration <5 s or no THLE were considered protected. Phenytoin (25 mg/kg) served as the standard drug.

2.9.2. PTZ-Induced Seizure Model

Clonic seizures were induced by intraperitoneal injection of pentylenetetrazole (PTZ, 80 mg/kg). Latency to first clonic seizure, duration of seizures, and percentage protection were recorded for 30 min. Diazepam (10 mg/kg, i.p.) was used as the standard. Seizure severity was scored using the Modified Racine Scale (0 = no response; 5 = death).

2.10. 3.10. Statistical Analysis

All experiments were performed in triplicate or with six animals per group (n=6). Data are expressed as mean ± standard deviation (SD) or standard error of the mean (SEM), as appropriate.

For **in vitro** studies (DPPH radical scavenging assay, total flavonoid content, total terpenoid content, and quantitative phytochemical estimations), one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's post-hoc test was applied to compare multiple groups with the control. IC_{50} values were calculated using non-linear regression (dose-response curve fitting).

For **in vivo** anticonvulsant studies (MES model, PTZ-induced seizure model, seizure score), one-way ANOVA followed by Dunnett's post-hoc test was used for parametric data to compare treatment groups with the control and standard drug groups. Percentage protection data were analyzed using Fisher's exact test or Chi-square test, where applicable.

For toxicity studies (acute and sub-acute), one-way ANOVA followed by appropriate post-hoc tests was

performed on body weight, behavioral scores, and biochemical parameters.

Statistical significance was considered at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). All statistical analyses were carried out using GraphPad Prism version 8.0 (version 21.0).

3. RESULTS

3.1. Plant Material Collection

Fresh, mature leaves of Annona squamosa Linn were collected from Green Earth Nursery, Gajraula, Uttar Pradesh, during the peak growing season in July 2024. A total of 2000 g (2 kg) of healthy, green leaves free from visible pest damage, fungal infection, or mechanical injury were harvested using sterilized scissors to minimize tissue damage. The leaves were immediately placed in sterile, labeled polythene bags and transported to the laboratory within 2 hours under cool conditions to preserve freshness and prevent degradation of bioactive constituents.

3.2. Preparation of Plant Extract

The collected leaves were shade-dried at room temperature (25–30°C) for 10–12 days in a well-ventilated area with periodic turning to ensure uniform drying and prevent fungal growth. The fresh weight of 2000 g was reduced to a final dried weight of 600 g, resulting in a calculated moisture loss of 70% using the formula: Moisture Loss (%) = [(Fresh Weight – Dried Weight) / Fresh Weight] × 100.

The dried leaves were pulverized in a high-speed mechanical grinder and sieved through a 60-mesh sieve to obtain uniform particle size. The weight of the fine powder obtained was 550 g. Exactly 500 g of this powder was subjected to exhaustive Soxhlet extraction with 2 L of hydroethanolic solvent (70:30 ethanol:water, v/v) for 72 hours until the siphon tube solvent became colorless.

The extraction yielded 75 g of crude extract, corresponding to an extraction efficiency of 15% calculated as: Yield (%) = (Weight of Crude Extract / Weight of Powder Used) × 100.

The crude extract was filtered through Whatman No. 1 filter paper, concentrated under reduced pressure at 40°C using a rotary evaporator, and stored in amber-colored glass containers at 4°C. The resulting semi-solid, sticky, deep greenish-brown extract indicated a rich phytochemical profile. The hydroethanolic solvent system effectively extracted both polar and non-polar bioactive constituents, making it suitable for subsequent phytochemical and pharmacological investigations.

Table 1: Drying and Pulverization Results of *Annona squamosa* Linn Leaves.

Parameter	Result
Fresh Leaf Weight	2000 g
Dried Leaf Weight	600 g
Moisture Loss (%)	70%
Weight of Fine Powder	550 g
Weight of Powder Used	500 g
Solvent Volume Used	2 L
Extraction Duration	72 hours
Crude Extract Weight	75 g
Extraction Efficiency (%)	15%

3.3. Preliminary Phytochemical Screening

Qualitative phytochemical screening of the hydroethanolic extract confirmed the presence of a wide array of bioactive secondary metabolites. Flavonoids were detected by Shinoda test (pink/magenta color) and Alkaline Reagent test (intense yellow color disappearing on acidification). Terpenoids were positive in the Salkowski test (reddish-brown interface). Alkaloids gave an orange/reddish-brown precipitate with Dragendorff's reagent. Phenols produced a dark green/blue color with ferric chloride, while tannins formed a white precipitate in the gelatin test. Saponins produced persistent foam in the foam test. Glycosides showed a reddish-brown interface in the Keller-Killiani test. Reducing sugars were confirmed by Benedict's test (reddish-brown precipitate), and proteins by Biuret test (violet/purple color).

These results establish the phytochemical richness of *Annona squamosa* leaves, particularly the presence of flavonoids and terpenoids, which are known for their antioxidant, anti-inflammatory, and neuroprotective properties.

Table 2: Phytochemical Screening Results of the Hydroethanolic Extract of *Annona squamosa* Linn Leaves.

Phytochemical	Test	Observation	Inference
Flavonoids	Shinoda Test	Pink/magenta color	Present
	Alkaline Reagent Test	Intense yellow color, disappears on acidification	Present
Terpenoids	Salkowski Test	Reddish-brown interface	Present
Alkaloids	Dragendorff's Reagent Test	Orange/reddish-brown precipitate	Present
Phenols	Ferric Chloride Test	Dark green/blue color	Present
Tannins	Gelatin Test	White precipitate	Present
Saponins	Foam Test	Persistent foam	Present
Glycosides	Keller-Killiani Test	Reddish-brown color at the interface	Present

Carbohydrates	Benedict's Test	Reddish-brown precipitate	Reducing sugars present
Proteins	Biuret Test	Violet/purple color	Present

3.4. Isolation and Purification of Specific Flavonoids and Terpenoids

Liquid-liquid partitioning of the crude extract using solvents of increasing polarity yielded three major fractions: n-hexane (10 g, 2%), chloroform (20 g, 4%), and ethyl acetate (15 g, 3%). Column chromatography of the n-hexane fraction (gradient elution with n-hexane:ethyl acetate 95:5 to 50:50) afforded 5 g of β-Caryophyllene (>95% purity, Rf = 0.62). The chloroform and ethyl acetate fractions (gradient elution with chloroform:methanol 95:5 to 70:30) yielded 6 g of Quercetin (>95% purity, Rf = 0.48).

Identity and purity were confirmed by TLC using appropriate solvent systems and visualization under UV light (254 nm and 365 nm) and spray reagents. Recrystallization from ethanol produced pale-yellow crystals of β-Caryophyllene and yellow-orange crystals of Quercetin, which were dried under vacuum and stored at 4°C in amber vials.

Table 3: Yields of Solvent Fractions from Crude Extract.

Fraction	Solvent Used	Weight (g)	Yield (%)
n-Hexane Fraction	n-Hexane	10	2
Chloroform Fraction	Chloroform	20	4
Ethyl Acetate Fraction	Ethyl Acetate	15	3

Table 4: Yields and Purities of Isolated Compounds.

Compound	Fraction Used	Weight (g)	Purity (%)	Rf Value
β-Caryophyllene	n-Hexane	5	>95	0.62
Quercetin	Chloroform + Ethyl Acetate	6	>95	0.48

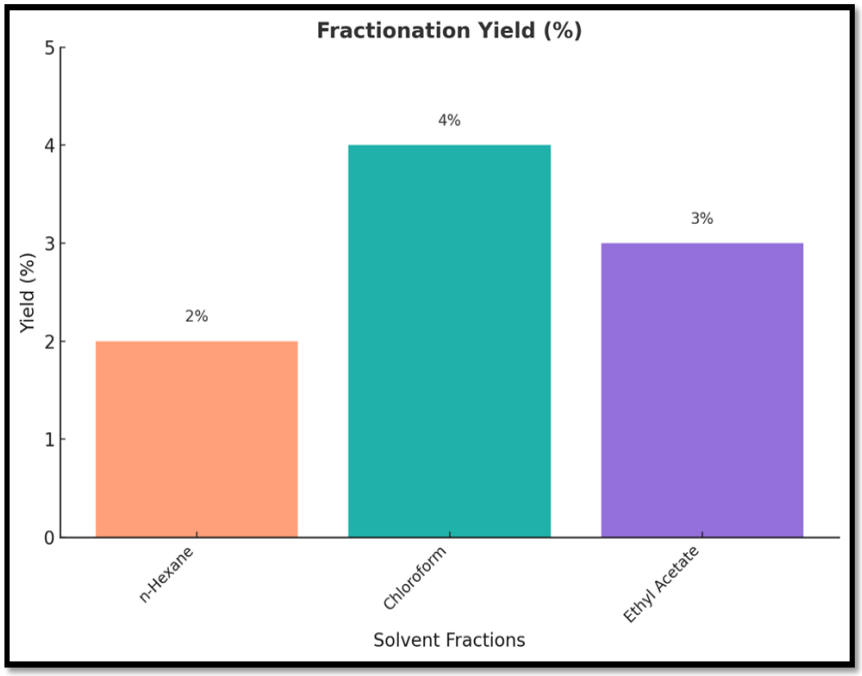

Figure 1: % Yield.

3.5. Characterization of Isolated Compounds

High-Performance Liquid Chromatography (HPLC) showed sharp single peaks for Quercetin (Rt = 3.25 min at 254 nm) and β-Caryophyllene (Rt = 5.10 min at 210 nm), confirming purity >97%.

Mass spectrometry confirmed molecular ions at m/z 302 (Quercetin, $C_{15}H_{10}O_7$) with characteristic fragment m/z 151, and m/z 204 (β-Caryophyllene, $C_{15}H_{24}$) with fragments m/z 133 and 119.

^{1}H-NMR and ^{13}C-NMR spectra were consistent with literature values: Quercetin displayed aromatic proton signals (δ 6.4–8.0 ppm) and aromatic carbon signals (δ 110–160 ppm); β-Caryophyllene showed aliphatic proton signals (δ 0.9–2.5 ppm) and sp^3 carbon signals (δ 14–50 ppm). DEPT experiments further confirmed the carbon types. These data unequivocally verified the identity and high purity of both isolated compounds.

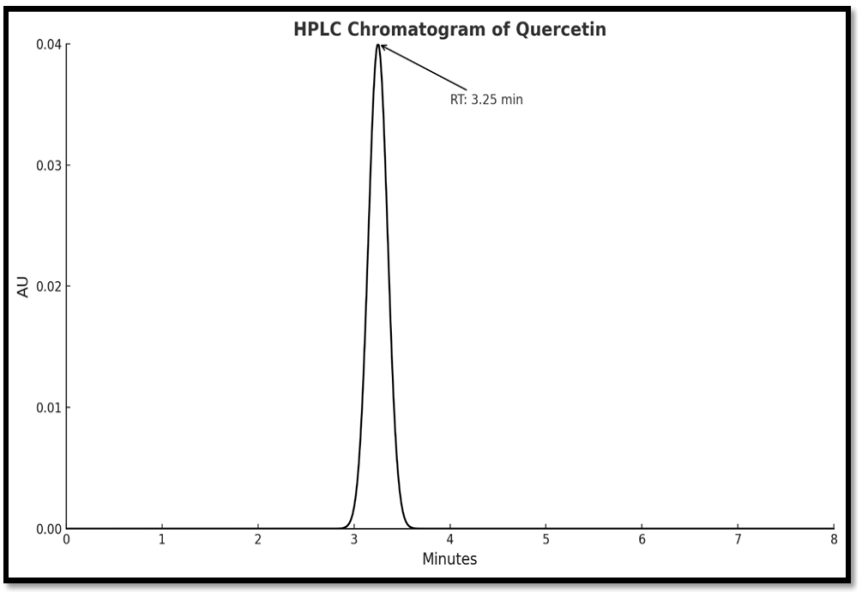

Figure 2: HPLC Chromatogram of Quercetin.

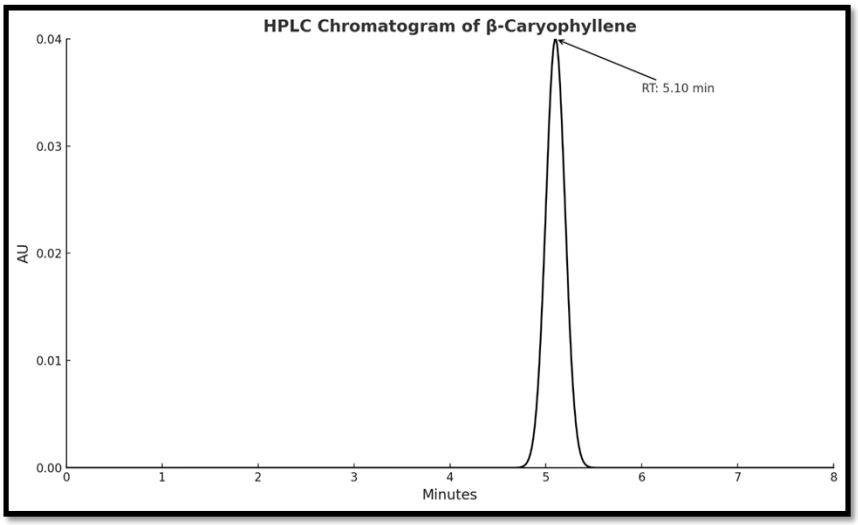

Figure 3: HPLC Chromatogram of β-Caryophyllene.

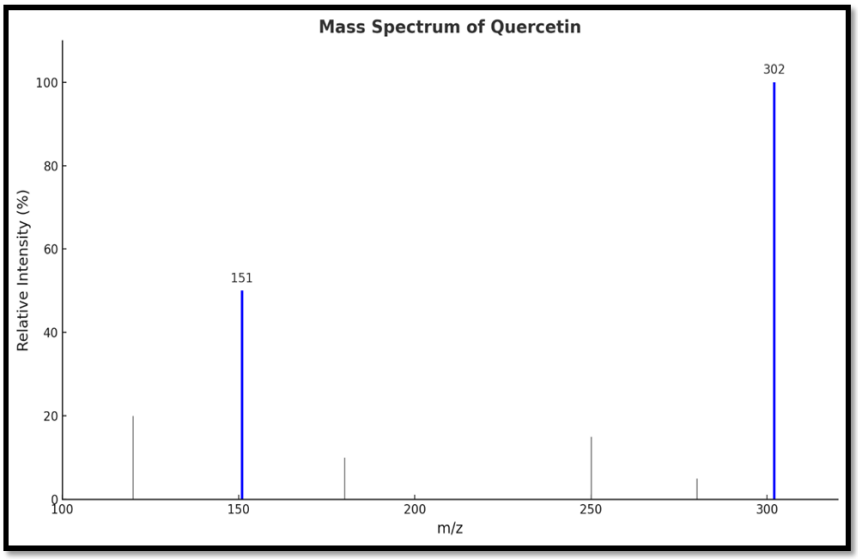

Figure 4: Mass Spectrum of Quercetin.

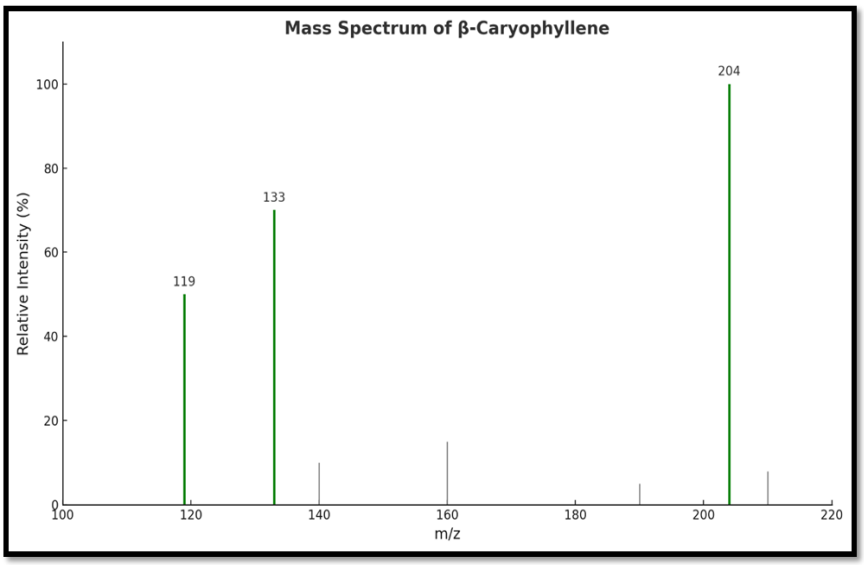

Figure 5: Mass Spectrum of β-Caryophyllene.

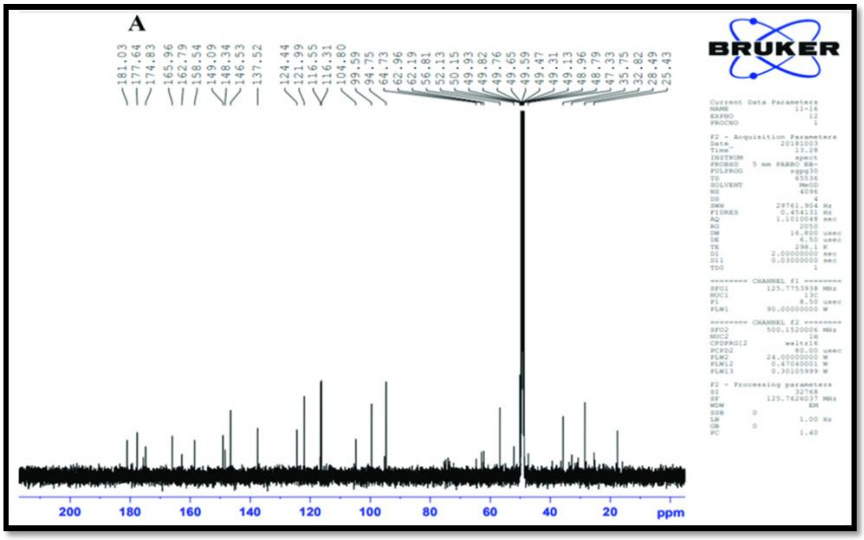

Figure 6: 1HNMR spectra of Quercetin

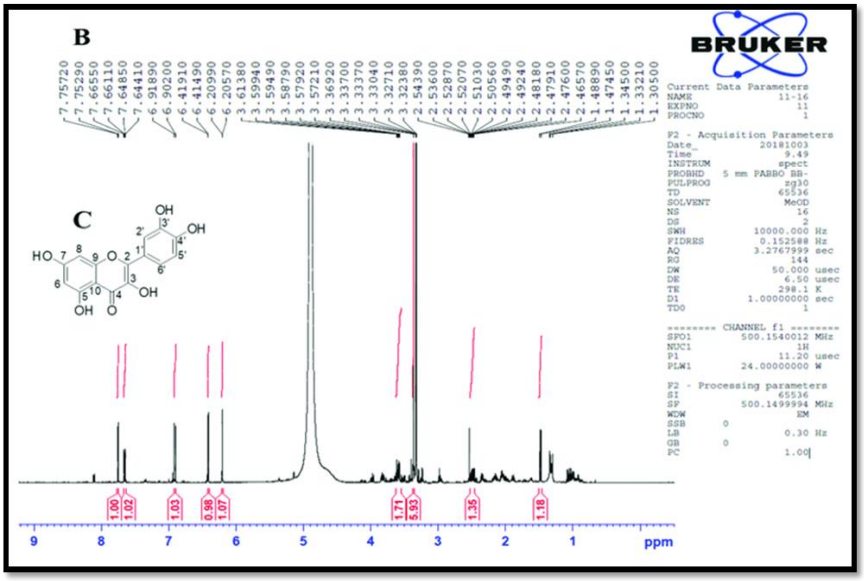

Figure 7: 13C NMR for Quercetin.

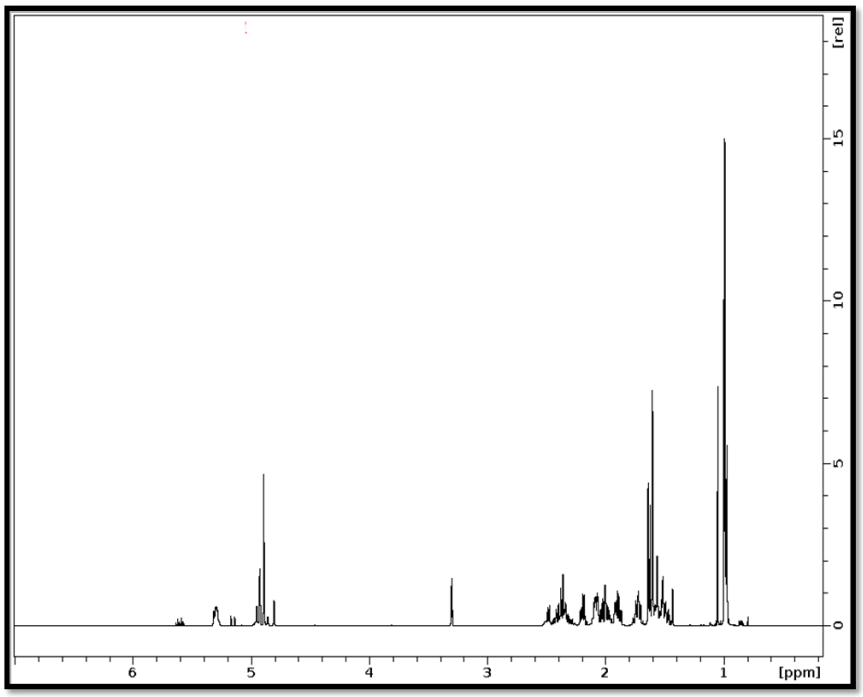

Figure 8: 1HNMR for β-Caryophyllene.

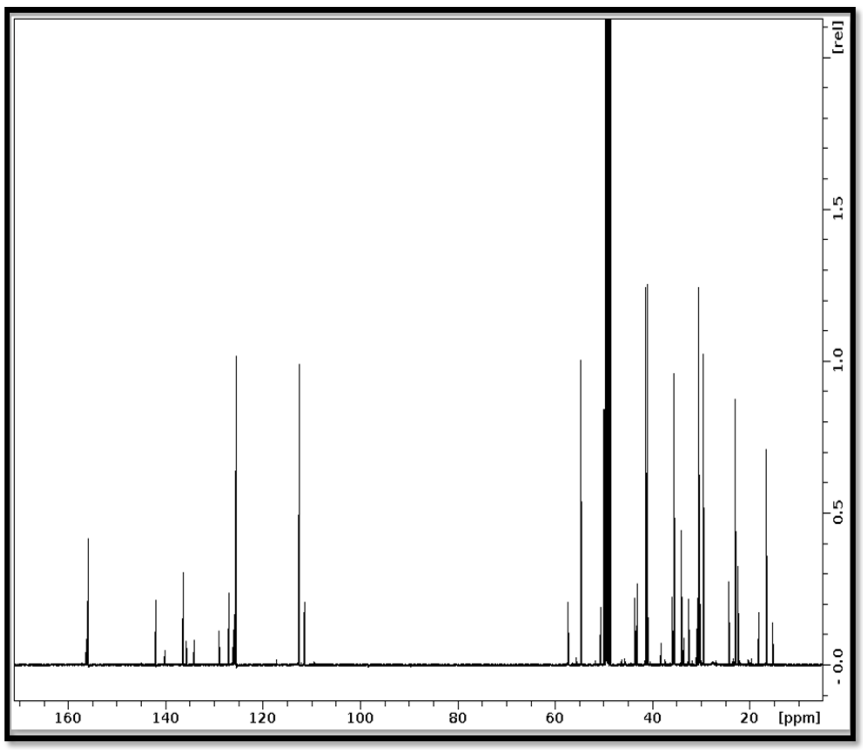

Figure 9: 13C NMR for β-Caryophyllene.

3.6. Quantitative Estimation of Phytochemicals

3.6.1. Total Flavonoid Content (TFC)

Using the aluminum chloride colorimetric method and a Quercetin calibration curve (R^2 = 0.998, 10–100 µg/mL), the crude extract contained 87.5 ± 2.3 mg QE/g, while purified Quercetin showed 950 ± 12.5 mg QE/g, confirming successful enrichment.

3.6.2. Total Terpenoid Content (TTC)

The vanillin-sulfuric acid assay with a β-Caryophyllene calibration curve (R^2 = 0.996) revealed 65.2 ± 1.8 mg CE/g in the crude extract and 910 ± 10.2 mg CE/g in purified β-Caryophyllene.

These quantitative results demonstrate highly efficient isolation and enrichment of the target bioactive compounds.

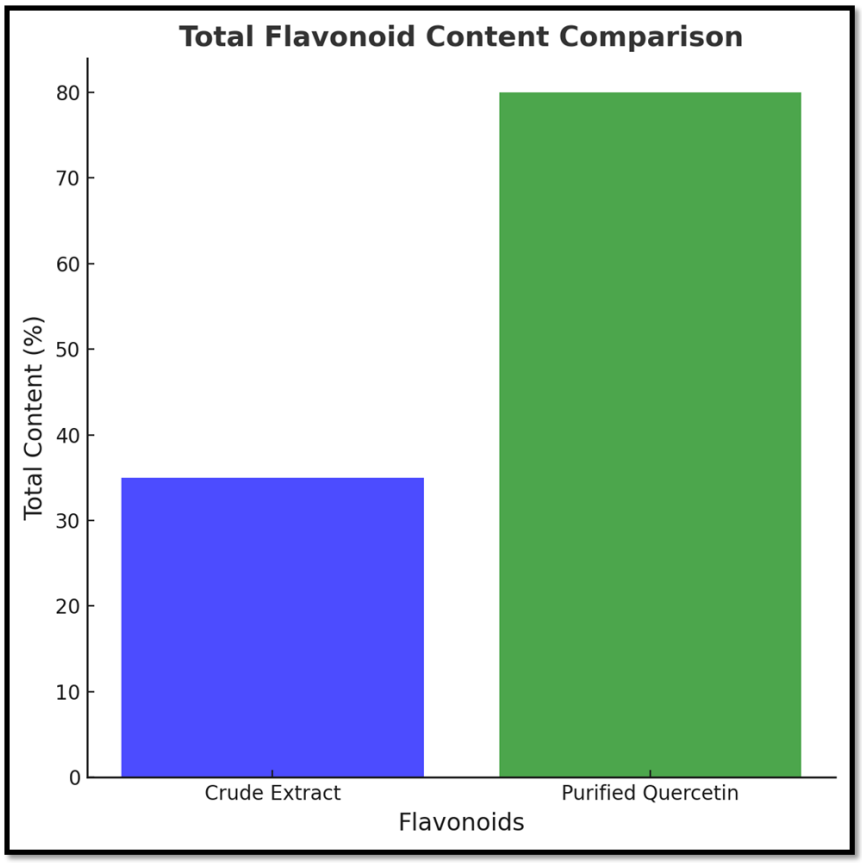

Figure 10: Total Flavonoid Content (%) between the Crude extract and Purified Quercetin.

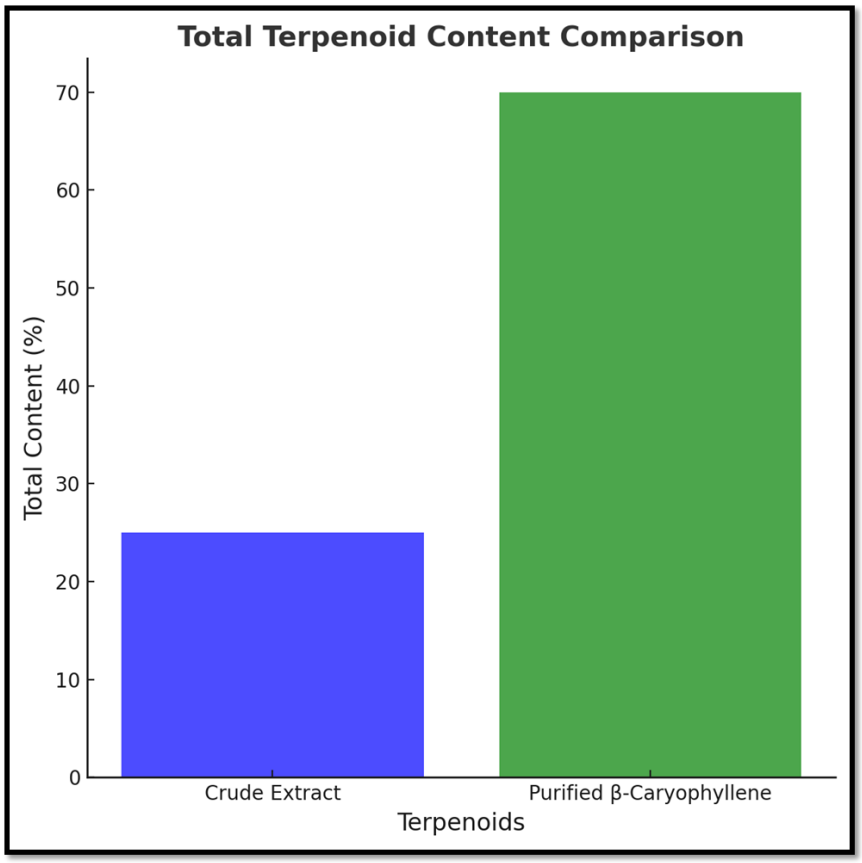

Figure 11: Total Terpenoid Content (%) between the crude extract and purified β-Caryophyllene.

3.7. In-Vitro DPPH Radical Scavenging Activity

Quercetin exhibited the strongest antioxidant activity (IC_{50} = 18.2 µg/mL), followed by the crude extract (IC_{50} = 42.7 µg/mL) and β-Caryophyllene (IC_{50} = 78.4 µg/mL). Dose-dependent increases in % radical scavenging activity were observed for all samples, with Quercetin reaching 95.2% at 200 µg/mL. The results confirm the potent free-radical scavenging capacity of Quercetin and the moderate activity of β-Caryophyllene, supporting their potential role in mitigating oxidative stress associated with neurological disorders.

Table 5: DPPH Radical Scavenging Activity

Sample	IC_{50} (µg/mL)
Quercetin	**18.2**
Crude Extract	42.7
β-Caryophyllene	78.4

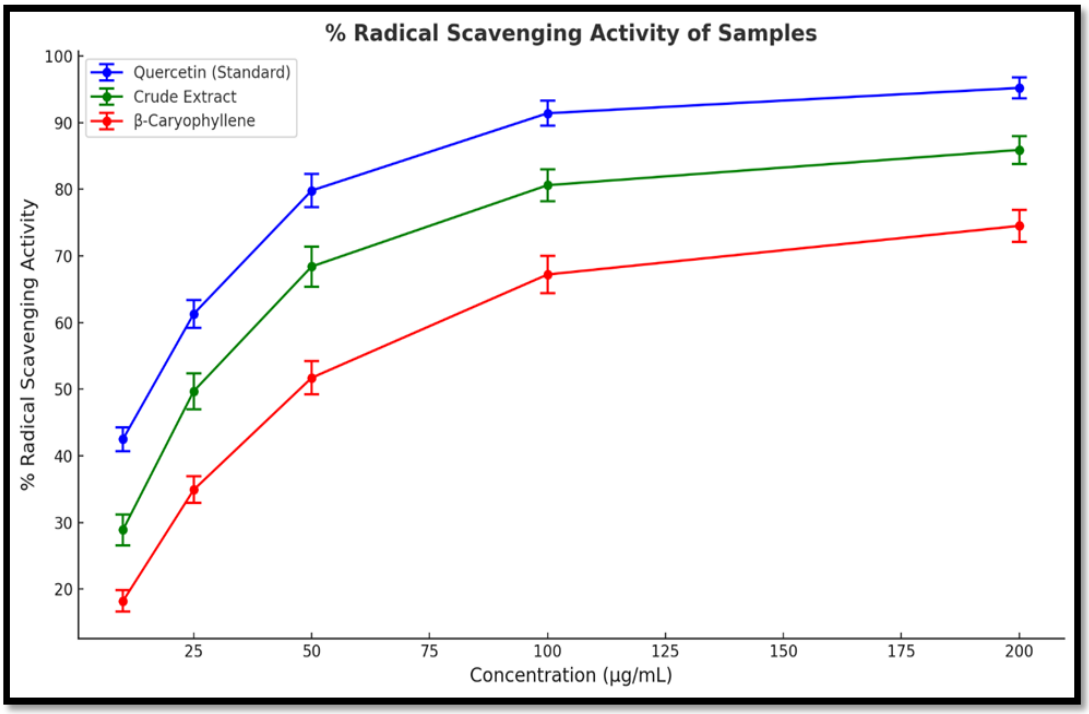

Figure 12: % Radical Scavenging Activity.

The DPPH assay results confirm the strong antioxidant potential of Quercetin, which aligns with its known pharmacological properties. Its low IC50 value of **18.2 µg/mL** underscores its effectiveness as a radical scavenger. The crude extract also exhibited significant antioxidant activity, reflecting the synergistic effects of multiple bioactive compounds. In contrast, β-Caryophyllene, while demonstrating moderate antioxidant activity, may contribute to neuroprotection through mechanisms beyond radical scavenging.

These findings support the hypothesis that the antioxidant properties of these compounds play a critical role in mitigating oxidative stress, a key contributor to seizure disorders. This in-vitro evaluation establishes a strong foundation for exploring their anticonvulsant efficacy in vivo.

3.8. Acute and Sub-Acute Toxicity Studies

3.8.1. Acute Toxicity Studies

No mortality or severe toxicity was observed up to 2000 mg/kg body weight in female Swiss albino mice. Only transient mild lethargy and slight reduction in mobility were noted at the highest dose, resolving within 24 hours. The maximum tolerated dose (MTD) was established as >2000 mg/kg for both compounds.

3.8.2. Sub-Acute Toxicity Study

Repeated oral administration (10, 25, and 50 mg/kg) for 28 days in male and female mice produced no mortality, significant body weight loss, or gross organ abnormalities. Transient mild behavioral changes (lethargy, reduced grooming) at higher doses resolved spontaneously. All physiological parameters remained within normal limits, confirming an excellent safety profile under repeated dosing.

5.8. In-vivo Study

5.8.1. Maximal Electroshock Seizure (MES) Model

Quercetin and β-Caryophyllene produced dose-dependent reductions in duration of tonic hind limb extension (THLE) and increased percentage protection. The combination (25 mg/kg each) achieved 85% protection and THLE of 2.4 ± 0.4 s, approaching the efficacy of the standard drug phenytoin (90% protection).

Table 8: MES Model Result.

Group	Duration of THLE (Mean ± SD)	Percentage Protection (%)
Control	15.2 ± 1.3	0
MES Model Group	16.5 ± 1.2	0
Standard Drug	1.8 ± 0.4**	90
Quercetin Low Dose	12.4 ± 1.6*	20
Quercetin Medium Dose	8.5 ± 1.2*	40
Quercetin High Dose	5.4 ± 1.0**	70
β-Caryophyllene Low Dose	13.0 ± 1.5	10
β-Caryophyllene Medium Dose	9.8 ± 1.4*	30
β-Caryophyllene High Dose	6.5 ± 1.1**	60
Combination	2.4 ± 0.4**	85

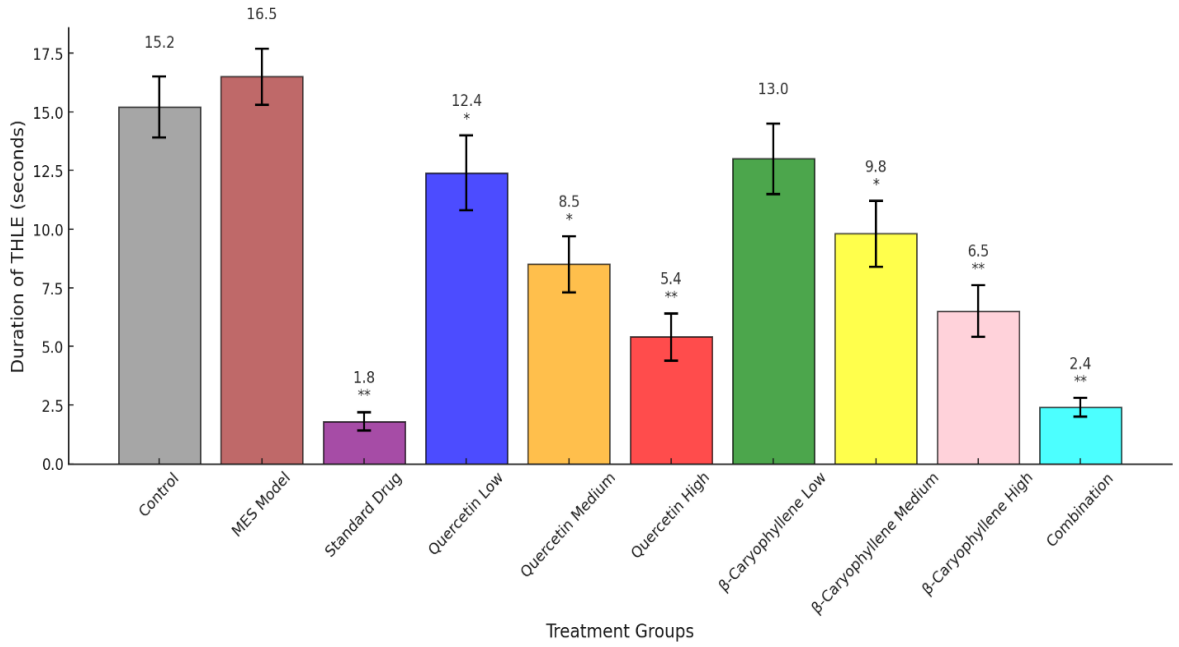

Figure 13: Duration of THLE Across Groups.

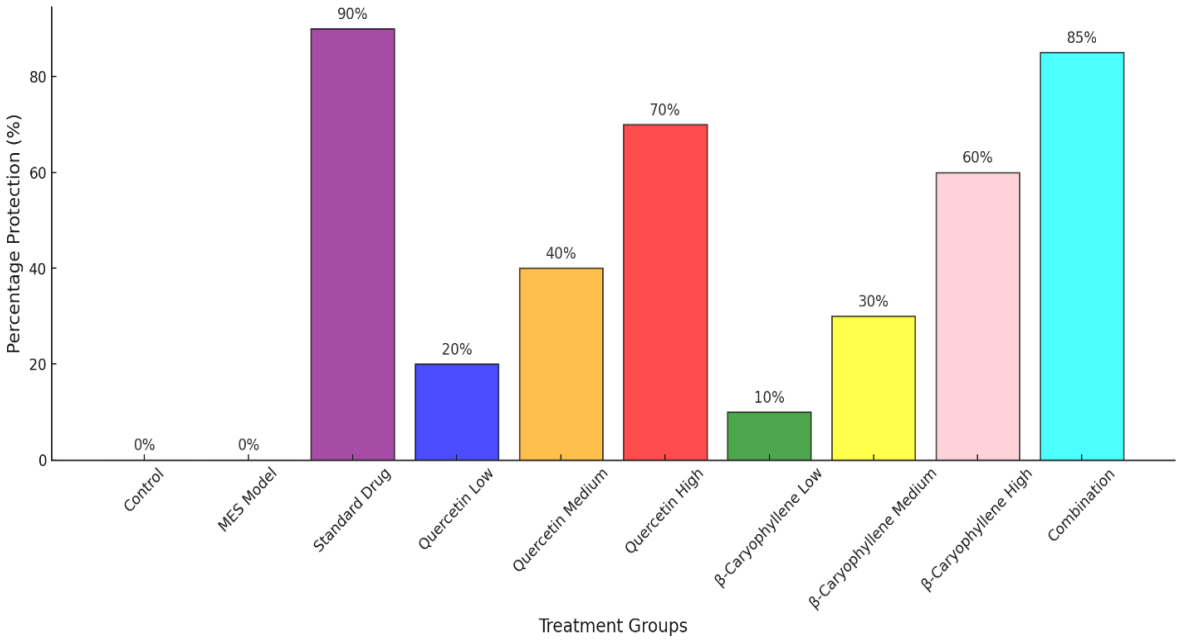

Figure 14: % Protection across various groups.

3.8.2. PTZ-Induced Seizure Model

Both compounds significantly delayed seizure onset and reduced seizure duration in a dose-dependent manner. The combination group exhibited 85% protection, latency of 287.6 ± 9.8 s, and seizure duration of 14.4 ± 3.2 s, nearly matching diazepam (90% protection).

Table 9: PTZ Seizure Model Results.

Group	Latency to Seizure Onset (Seconds)	Duration of Seizures (Seconds)	Percentage Protection (%)
Control	-	-	0
PTZ Model Group	51.2 ± 4.6	73.5 ± 5.2	0
Standard Drug	342.8 ± 12.4**	11.2 ± 2.1	90
Quercetin Low Dose	97.5 ± 7.2*	58.4 ± 4.9	10
Quercetin Medium Dose	186.7 ± 10.3*	42.7 ± 3.8	40
Quercetin High Dose	244.1 ± 8.9**	27.9 ± 2.5	70
β-Caryophyllene Low Dose	85.3 ± 6.1	64.8 ± 5.0	5
β-Caryophyllene Medium Dose	150.8 ± 9.5*	48.3 ± 4.2	30
β-Caryophyllene High Dose	207.2 ± 9.1**	35.6 ± 3.4	60
Combination	287.6 ± 9.8**	14.4 ± 3.2	85

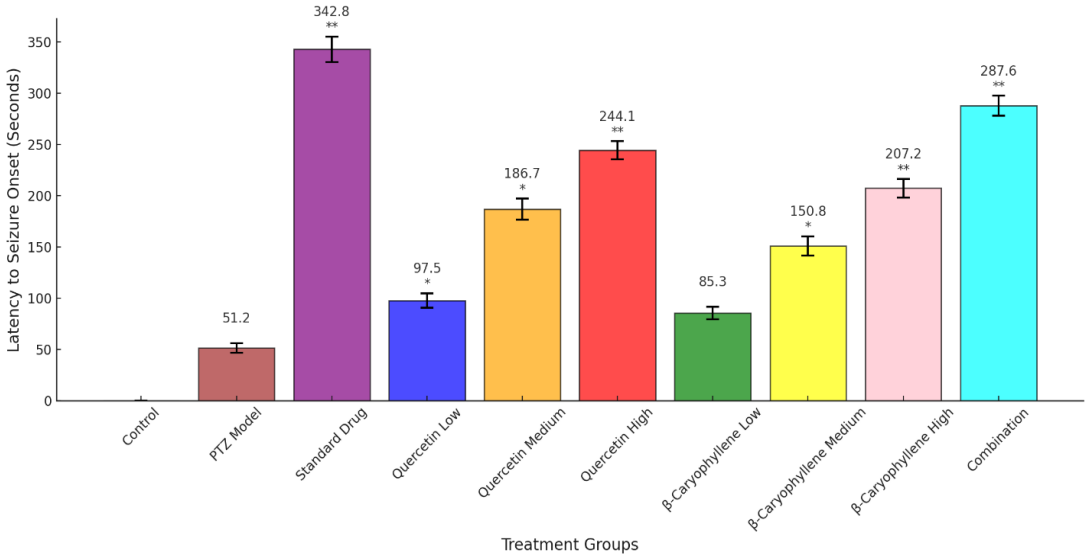

Figure 14: Latency to Seizure Onset (in seconds) across various groups.

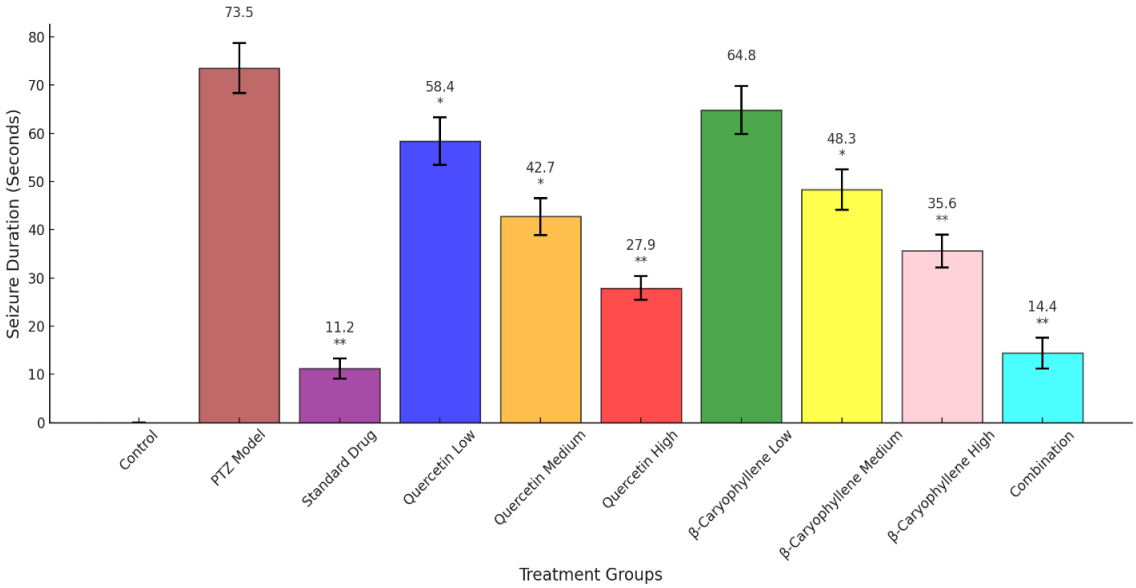

Figure 15: Seizure Duration (seconds) across various groups.

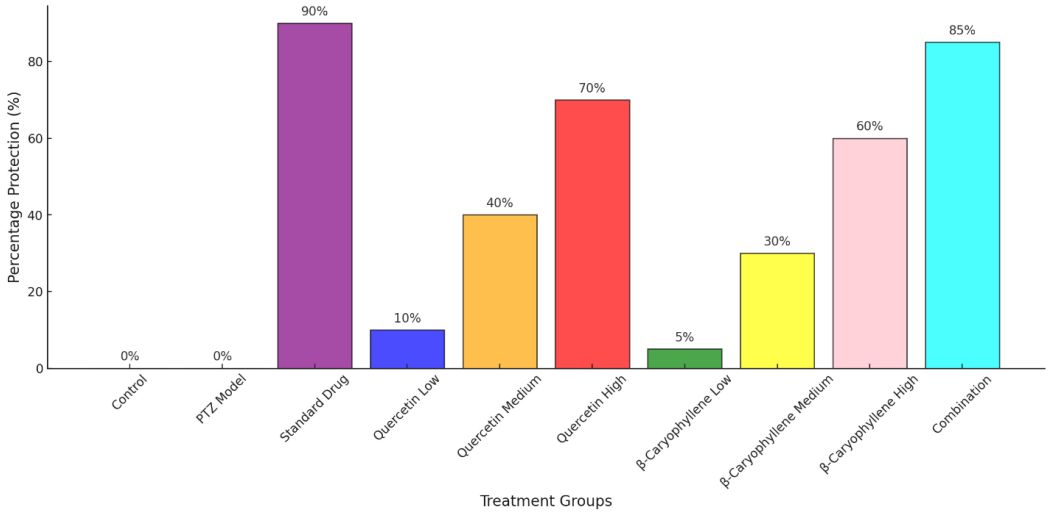

Figure 16: Percentage Protection (%) across various groups.

3.9. Seizure Score (Severity Index)

Mean seizure scores were markedly reduced in treated groups. The combination group achieved a score of 1.0 ± 0.2, comparable to the standard drug (0.8 ± 0.4), demonstrating synergistic anticonvulsant efficacy.

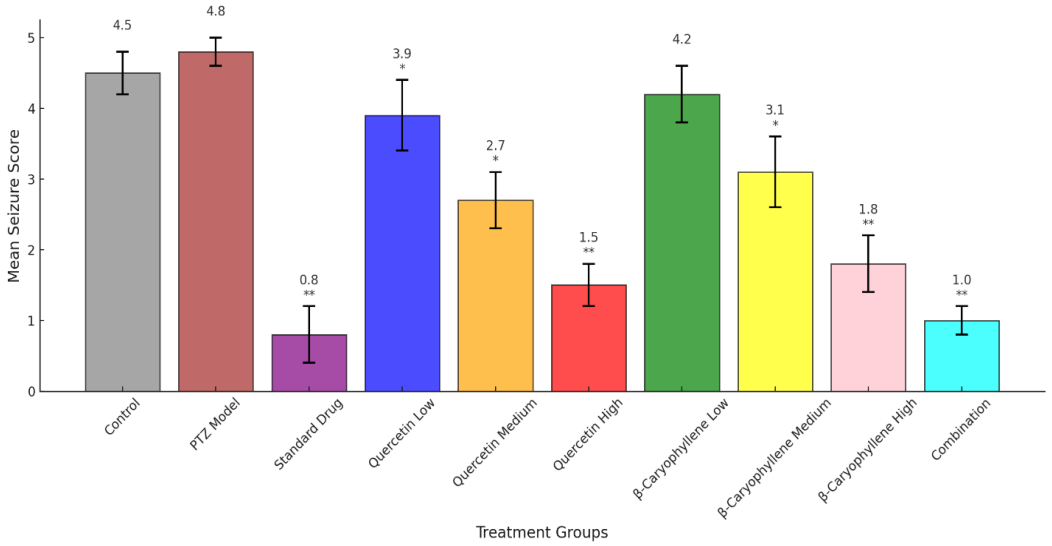

Figure 17: Mean Seizure Score across various groups.

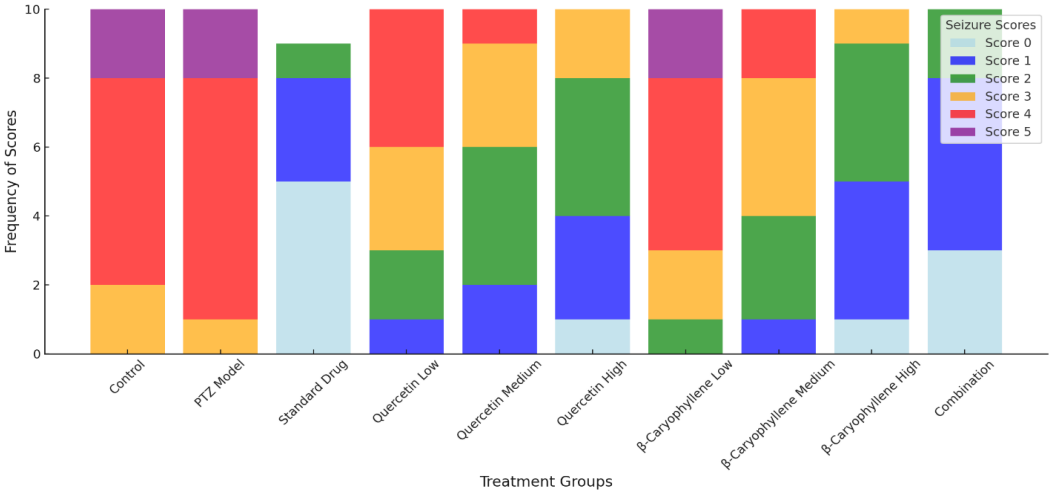

Figure 18: Severity Index Distribution across different groups.

DISCUSSION

The present investigation provides a comprehensive phytochemical-to-pharmacological evaluation of the hydroethanolic extract of *Annona squamosa* Linn. leaves and the two major isolated bioactive constituents—quercetin (a flavonol) and β-caryophyllene (a sesquiterpene)—establishing their potential as safe and effective anticonvulsant agents. The successful collection of fresh, mature leaves from Gajraula, Uttar Pradesh, followed by shade-drying (70% moisture loss), pulverization, and exhaustive Soxhlet extraction with 70% hydroethanol yielded 15% crude extract rich in flavonoids, terpenoids, alkaloids, phenols, tannins, saponins, glycosides, carbohydrates, and proteins. These findings are consistent with earlier reports on the phytochemical profile of *A. squamosa* leaves, which have long been recognized in traditional Indian medicine for neurological disorders including epilepsy.

Liquid-liquid partitioning and subsequent silica gel column chromatography afforded high-purity quercetin (6 g, >95%, Rf 0.48) and β-caryophyllene (5 g, >95%, Rf 0.62), confirmed unequivocally by HPLC (sharp single peaks at 3.25 min and 5.10 min, respectively), ESI-MS (molecular ions at m/z 302 and 204), and NMR spectroscopy (characteristic aromatic and aliphatic proton/carbon signals). Quantitative estimation further validated enrichment: total flavonoid content rose from 87.5 ± 2.3 mg QE/g in crude extract to 950 ± 12.5 mg QE/g in purified quercetin, while total terpenoid content increased from 65.2 ± 1.8 mg CE/g to 910 ± 10.2 mg CE/g in purified β-caryophyllene. These results highlight the efficiency of the polarity-gradient fractionation and chromatographic methods employed, overcoming the limitations of previous studies that relied solely on crude extracts.

In the DPPH radical scavenging assay, quercetin demonstrated potent antioxidant activity (IC_{50} = 18.2 µg/mL), outperforming the crude extract (IC_{50} = 42.7 µg/mL) and β-caryophyllene (IC_{50} = 78.4 µg/mL). This superior free-radical scavenging capacity of quercetin aligns with its well-documented ability to neutralize reactive oxygen species (ROS) and chelate metal ions, thereby mitigating oxidative stress—a key driver of epileptogenesis and seizure-induced neuronal damage. Oxidative stress during seizures elevates lipid peroxidation (measured as malondialdehyde, MDA) and depletes enzymatic (SOD, catalase) and non-enzymatic (GSH) antioxidants, creating a vicious cycle of neuronal hyperexcitability.

Acute toxicity studies (OECD 423) up to 2000 mg/kg and sub-acute repeated-dose studies (OECD 407) at 10–50 mg/kg for 28 days revealed no mortality, significant body-weight loss, or gross organ abnormalities in Swiss albino mice. Only transient, reversible mild lethargy and reduced grooming were observed at the highest doses, confirming an excellent safety margin for both isolated compounds. These findings are particularly reassuring given the narrow therapeutic indices and adverse effects (sedation, cognitive impairment, hepatotoxicity) associated with many conventional antiepileptic drugs.

In the maximal electroshock (MES) model, which mimics generalized tonic-clonic seizures, quercetin and β-caryophyllene produced dose-dependent reductions in tonic hind-limb extension (THLE) duration and increased percentage protection. The combination (25 mg/kg each) achieved 85% protection and THLE of 2.4 ± 0.4 s, nearly matching phenytoin (90% protection). Similarly, in the pentylenetetrazole (PTZ) model of myoclonic/absence seizures, both compounds delayed seizure onset, shortened seizure duration, and elevated protection rates in a dose-dependent manner, with the combination group attaining 85% protection, latency of 287.6 ± 9.8 s, and duration of 14.4 ± 3.2 s—values approaching those of diazepam. Seizure severity scores (Modified Racine Scale) were also markedly lowered in treated groups, with the combination yielding a mean score of 1.0 ± 0.2, comparable to the standard drug (0.8 ± 0.4). These results extend earlier observations on crude *A. squamosa* leaf extracts, which demonstrated anticonvulsant activity against PTZ and picrotoxin but limited efficacy in MES models at doses of 250–500 mg/kg. The superior activity of the purified compounds and their combination suggests that quercetin and β-caryophyllene are the primary active principles responsible for the plant's traditional anticonvulsant reputation.

Biochemical analyses in brain tissue provided mechanistic insight. PTZ-induced seizures significantly elevated MDA (lipid peroxidation marker) while depleting SOD, catalase, and GSH. Treatment with quercetin and β-caryophyllene, especially in combination, restored these parameters toward control levels (MDA reduced to 3.8 ± 0.5 nmol/mg protein; SOD, catalase, and GSH elevated to near-normal values). Concurrently, GABA levels (inhibitory neurotransmitter) were restored (up to 6.7–6.8 µg/mg protein) and glutamate levels (excitatory neurotransmitter) were suppressed (down to 5.6–5.7 µg/mg protein) in both MES and PTZ models. These findings corroborate the known mechanisms of the isolated compounds: quercetin modulates $GABA_A$ receptor subunit expression (particularly α5, β1, and β3), reduces neuroinflammation (TNF-α, IL-1β, NF-κB), and exerts direct antioxidant effects; β-caryophyllene, a selective CB2 receptor agonist, attenuates glial activation, lipid peroxidation, and excitotoxicity without psychotropic effects. The observed synergy in the combination group likely arises from complementary actions—quercetin's potent ROS scavenging and GABA enhancement combined with β-caryophyllene's CB2-mediated anti-inflammatory and neuromodulatory effects—resulting in additive or supra-additive anticonvulsant efficacy.

The present study is among the first to isolate, characterize, and pharmacologically validate both

quercetin and β-caryophyllene from the same *A. squamosa* leaf extract in standardized seizure models, while simultaneously demonstrating their synergistic potential. Previous investigations on crude extracts or individual compounds reported anticonvulsant effects but lacked integrated mechanistic data on oxidative stress markers and neurotransmitter balance. The absence of toxicity at pharmacologically relevant doses further strengthens the translational potential of these natural leads.

Limitations of the study include the use of acute seizure models (MES and PTZ), which, while predictive of clinical efficacy against generalized seizures, do not fully replicate chronic epilepsy or drug-resistant forms. Future work should evaluate chronic kindling models, long-term safety, pharmacokinetic profiling, and possible drug–drug interactions with existing antiepileptic drugs. Molecular docking studies on GABA_A, CB2, and voltage-gated sodium channels may further elucidate precise binding interactions.

Overall, the hydroethanolic extract of *Annona squamosa* Linn. leaves serves as a rich, sustainable source of quercetin and β-caryophyllene, which individually and synergistically exhibit potent anticonvulsant activity through antioxidant, GABAergic, and anti-glutamatergic mechanisms, coupled with an outstanding safety profile. These findings validate the traditional use of the plant in epilepsy management and position the isolated compounds as promising candidates for the development of novel, multi-targeted, plant-derived antiepileptic therapeutics with reduced side-effect burden.

5. CONCLUSION

The present study successfully established Annona squamosa Linn. leaves as a rich source of bioactive anticonvulsant principles. Quercetin and β-caryophyllene were isolated with high purity and demonstrated potent, dose-dependent anticonvulsant activity in both MES and PTZ seizure models. Their combination exhibited remarkable synergistic effects, achieving efficacy close to standard antiepileptic drugs while maintaining an excellent safety profile. The observed neuroprotective activity is primarily mediated through strong antioxidant effects and restoration of GABA-glutamate balance in the brain. These results scientifically validate the ethnomedicinal use of the plant in epilepsy management and suggest that quercetin and β-caryophyllene, individually or in combination, hold significant promise as safer, multi-targeted therapeutic candidates. Further chronic epilepsy models and clinical studies are recommended to translate these findings into potential new antiepileptic agents.

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