

Research Article

Comparative Profiling of Antioxidant Mechanisms in *Averrhoa* Species Using Integrated *In Vitro* and *In Silico* Approaches

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Abstract

Reactive oxygen species (ROS) contribute to oxidative stress and are associated with the development of chronic diseases, increasing the need for effective natural antioxidants. Although *Averrhoa* species are known to contain bioactive compounds with antioxidant potential, their underlying molecular mechanisms and interspecies differences remain poorly understood due to the lack of integrative studies. In this study, we investigated the antioxidant activity and bioactive compounds of four *Averrhoa* species (*Averrhoa bilimbi*, *Averrhoa carambola*, *Averrhoa dolichocarpa*, and *Averrhoa leucopetala*) leaves using *in silico* and *in vitro* approaches. Leaf powder of *Averrhoa* spp. was macerated in 70% ethanol, then a GC-MS profiling identified 18-24 compounds in *A. dolichocarpa*, *A. bilimbi*, and *A. leucopetala*, compared to only four compounds in *A. carambola*. Subsequent network pharmacology analysis prioritized vitamin E, threonine, squalene, butanal, and neophytadiene as key bioactive compounds based on their target connectivity and association with antioxidant-related pathways. Molecular docking revealed favorable binding of vitamin E toward superoxide dismutase (SOD) and Kelch-like ECH-associated protein 1 (Keap1), suggesting potential roles in antioxidant enzyme modulation and Nrf2 pathway activation. Enzyme assays revealed that *A. dolichocarpa* exhibited the highest superoxide dismutase (SOD) (170.25 U/min/gFW) and catalase (CAT) (2.05 U/min/gFW) activities, *A. leucopetala* showed the highest ascorbate peroxidase (APX) activity (2.22 U/min/gFW), whereas *A. bilimbi* showed the strongest radical scavenging activity ($IC_{50} = 15.24 \mu\text{g/mL}$). These findings indicate that antioxidant activity in *Averrhoa* is species-dependent, involving enzyme-mediated defense and direct radical scavenging. Overall, this integrative approach provides comprehensive insight into their antioxidant mechanisms and highlights their potential for nutraceutical and pharmaceutical applications.

Keywords: Antioxidant, *Averrhoa*, Bioactive compounds, *In silico*, Enzymatic assay, Radical scavenging.

1 Introduction

Free radicals and reactive oxygen species (ROS) are continuously generated in biological systems and may increase significantly owing to factors such as ultraviolet radiation, environmental pollutants, and unhealthy

lifestyles. Excessive accumulation of ROS can lead to oxidative stress, disrupting cellular redox balance and causing damage to DNA, proteins, and lipids [1]. To mitigate oxidative stress, organisms employ enzymatic antioxidant defense systems, including superoxide dismutase (SOD), catalase (CAT), and ascorbate



peroxidase (APX), which neutralize reactive oxygen species and maintain cellular redox homeostasis. However, when ROS production exceeds the capacity of these defense mechanisms, oxidative damage occurs. Oxidative damage has been implicated in the development of various chronic diseases, including cardiovascular disorders, diabetes, and cancer [2]. Consequently, the consumption of antioxidant agents and the identification of natural antioxidant sources have become important strategies for disease prevention and the development of functional foods and pharmaceutical products.

Plants represent one of the most important sources of natural antioxidants due to their diverse repertoire of bioactive secondary metabolites. Among tropical fruit plants, species of the genus *Averrhoa* (Oxalidaceae) have attracted considerable attention because of their nutritional value and traditional medicinal uses. Two species, *A. carambola* (sweet starfruit) and *A. bilimbi* (wuluh starfruit), have been relatively well studied and demonstrate promising antioxidant activities [3], [4]. Leaf extracts of *A. carambola* have been reported to inhibit caspase-3, caspase-8, and caspase-9, as well as cytochrome P450 3A activity [4], [5]. These activities are largely attributed to the presence of phytochemicals such as flavonoids, phenolic compounds, saponins, alkaloids, tannins, and vitamin C, which contribute to strong radical scavenging capabilities [6]–[8]. Similarly, *A. bilimbi* leaf extracts have shown photoprotective effects against UV exposure [3] and moderate antioxidant activity in lipid oxidation assays such as thiobarbituric acid and ferric thiocyanate tests [9]. Its phytochemical constituents, including alkaloids, phenolics, and flavonoids, are believed to contribute to its protective effects against oxidative stress [10], [11]. Collectively, these findings support the therapeutic potential of *Averrhoa* species as sources of natural antioxidant compounds.

Although research on *A. carambola* and *A. bilimbi* has advanced considerably, studies on other members of this genus remain limited. In 2008, Rugayah and Sunarti reported two additional species, *A. dolichocarpa* and *A. leucopetala*, which are native to Papua and Gorontalo, Indonesia. These species share morphological traits similar to those of *A. carambola*, while possessing taste characteristics resembling those of *A. bilimbi* [12]. Local communities primarily utilize these fruits for culinary purposes [13], [14], and traditional knowledge suggests their potential medicinal value. However, scientific investigations of these species remain scarce, with previous studies mainly limited to basic nutritional characterization, such as protein, fat, and fiber content [15]. Given the abundance of bioactive metabolites reported in related *Averrhoa* species, it is important to determine

whether these newly described species possess antioxidant properties.

Furthermore, most antioxidant studies on *Averrhoa* species have relied primarily on chemical activity assays [3], [4], [9]. Although these assays provide useful preliminary information, they offer limited insights into the molecular mechanisms underlying antioxidant activity. Integrating phytochemical profiling with *in silico* molecular interaction analyses can provide a comprehensive understanding of how bioactive compounds interact with antioxidant-related proteins and pathways. These approaches can help identify potential molecular targets and generate mechanistic hypotheses that complement experimental antioxidant evaluations.

Therefore, this study aimed to comparatively evaluate the antioxidant potential and phytochemical profiles of four *Averrhoa* species using an integrated approach that combines GC-MS-based phytochemical profiling, *in vitro* antioxidant evaluation, and *in silico* analyses. To the best of our knowledge, this study is the first to provide a comprehensive antioxidant evaluation of *Averrhoa* spp, particularly *A. dolichocarpa* and *A. leucopetala*, using an integrative framework, enabling both a comparative assessment of antioxidant potential and mechanistic insights into the observed activities.

2 Materials and methods

An integrative experimental and computational approach was employed to evaluate the antioxidant potential of *Averrhoa* species. The workflow consisted of phytochemical profiling by GC-MS, prediction of antioxidant-related bioactivities and molecular targets, molecular docking analysis, antioxidant enzyme assays, and DPPH radical scavenging assays (Figure 1).

2.1 Plant Materials

Averrhoa spp. leaves were obtained from the Bogor Botanic Gardens (BBG) plant collection in West Java, Indonesia. All four *Averrhoa* species were cultivated in the BBG under comparable environmental conditions. Information regarding the origin and planting locations of the studied species is provided as follows: *Averrhoa bilimbi* originated from Java and was planted at VI.C.348 and VII.D.3-3a area; *A. carambola* originated from South Kalimantan and was planted at XV.B.IV.14-14a and XVI.I.A.39 area; *A. dolichocarpa*, an endemic species from Papua, was planted at VI.C.310a and VII.D.96-96a area; and *A. leucopetala*, an endemic species from Gorontalo, was planted at XXIV.B.79, XXIV.B.81, and XXIV.B.86 area. For each species, up

Workflow analysis

Antioxidant Mechanisms in *Averrhoa* species from in silico analysis

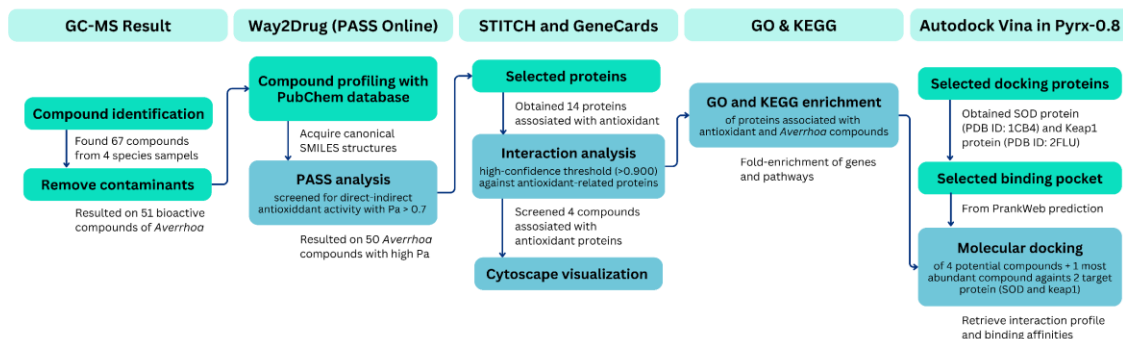


Figure 1: Workflow analysis and antioxidant mechanisms in this work.

to 150 grams of fresh leaves were harvested from healthy plants. Leaves from the 4th to the 8th position from the shoot apex were collected. The leaves were selected based on maturity, greenness, uniform green coloration, and freshness and were subsequently used for further analysis. Three biological replicates were used for each species.

2.2 Phytochemical profiling

Volatile compounds in each species were identified using gas chromatography mass spectrometry (GC-MS). Leaf powder was extracted by maceration methods in 70% ethanol for 3 consecutive 24-h periods. The extracts were then filtered using Whatman No. 1 filter paper and concentrated using a rotary evaporator at 40°C and 100 rpm for 45 mins to obtain the crude ethanolic extract. The extract was diluted to a final concentration of 1000 ppm and filtered through a 0.22 µm membrane filter prior to GC-MS analysis. A 1 µL aliquot of each sample was analyzed using a Shimadzu GCMS-QP2010S system equipped with an Agilent DB-5MS UI column. The flow rate was 15°C/min with programmed temperature from 70°C to 305°C. The carrier gas was helium, maintained at a pressure of 30.0 kPa and a total flow rate of 35.6 mL/min with a split ratio of 1:49. Compounds were matched against the WILEY229.LIB database for identification. Known contaminants such as siloxanes and plasticizers were excluded from the GC-MS results. Compounds were classified into terpenoids, phenolics, aromatics, fatty acid derivatives, and hydrocarbons based on their chemical structure and biosynthetic origin.

2.3 Preliminary in silico analysis

2.3.1 Screening of *Averrhoa* phytochemicals

in silico analysis was performed to predict their antioxidant potential and possible molecular interactions of phytochemicals identified in *Averrhoa* species. Canonical SMILES structures of the compounds were retrieved from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Biological activities, specifically related to antioxidant properties, were predicted using the PASS online tool (<https://www.way2drug.com/passonline/>). A total of 50 compounds met the criteria for having a probability of activity (Pa) greater than 0.7, as this threshold indicates a high likelihood (>70%) of exhibiting measurable biological activity.

Potential molecular interactions between *Averrhoa* compounds and antioxidant-related proteins were investigated using the STITCH database (<http://stitch.embl.de/>). A curated set of target genes was obtained from GeneCards (<https://www.genecards.org/>), including superoxide dismutases (SOD1-3), catalase (CAT), glutathione peroxidases (GPX1-4), peroxiredoxins (PRDX1-6), thioredoxins (TXN), thioredoxin reductases (TXNRD1-2), and glutathione reductase (GSR). These enzymes are key players in oxidative stress control and relevant targets for the antioxidant analysis. Compound-protein interactions were analyzed using a high-confidence threshold (>0.900) to ensure better interaction prediction. The resulting protein interaction networks were further visualized using Cytoscape 3.10.4.



2.3.2 Functional enrichment analysis

Proteins associated with *Averrhoa* compounds identified from the STITCH network were subjected to functional enrichment analysis using the DAVID Bioinformatics Resources online tools (<https://davidbioinformatics.nih.gov/>). The identified proteins were functionally annotated into three Gene Ontology (GO) categories: biological process (BP), cellular component (CC), and molecular function (MF). Statistically significant enriched GO terms in each category were visualized using bar charts. In addition, pathway enrichment analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) to identify antioxidant-related pathways, with the most significant pathways illustrated based on fold-enrichment values.

2.3.4 Molecular docking analysis

Molecular docking was performed using AutoDock Vina implemented within PyRx-0.8 to evaluate the binding interactions between selected *Averrhoa* phytochemicals and antioxidant-related enzymes. Candidate ligands were initially selected based on compound-protein interaction analysis using the STITCH database. In addition, the common compounds identified between *Averrhoa* species were also included to accommodate the comparison between major phytochemical constituents and screened compounds.

To evaluate the binding affinities of each ligand toward superoxide dismutase (SOD; RCSB ID: 1CB4) and Kelch-like ECH-associated protein 1 (Keap1; RCSB ID: 2FLU), blind and site-specific docking was performed. Because the selected protein structures lacked co-crystallized ligands to define binding pockets, potential cavities were predicted using the PrankWeb online server (<https://prankweb.cz/>). The reliability of the predicted docking poses was subsequently assessed by evaluating the occupancy of the predicted binding pocket and comparing the interacting residues with previously reported docking.

For SOD, Pocket 3 was selected as a potential allosteric site as it did not overlap with known active residues. For Keap1, Pocket 1 was selected due to its highest probability score. Docking grids were centered on the selected cavities: for SOD ($X = 3.43$, $Y = 70.69$, $Z = 10.43$) and for Keap1 ($X = 23.72$, $Y = 20.69$, $Z = 9.39$). The grid box was generated with a dimension of 60 x 60 x 60 Å and a default grid spacing of 0.375 Å.

2.4 In vitro analysis

2.4.1 Antioxidant enzyme assay

Antioxidant enzyme activities were determined for superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) to evaluate the capacity of *Averrhoa* leaf extracts in their role in oxidative stress regulation. Leaf samples were ground in liquid nitrogen using a mortar and pestle. Approximately 250 mg of powdered leaf tissue was homogenized in 2 mL of potassium phosphate buffer (pH 7.0) supplemented with 1 mM Na-EDTA and 1% (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 10,000 rpm for 15 minutes at 4°C, and the resulting supernatant was collected and stored at -20°C until further analysis.

For SOD activity, a modified pyrogallol method was employed [16], [17]. The reaction mixture consisted of 1 mL reducing buffer solution (Tris-Cl 50 mM pH 8.2 and buffer EDTA 1 mM pH 8.0), 1 mL distilled water, and 10 µL pyrogallol 2.5 mg/mL, followed by the addition of 8 µL enzyme extract. Absorbance was measured using a spectrophotometer at 325 nm.

CAT activity was determined using the method described by Bergmeyer (1970) [18]. The reaction mixture contained 200 µL enzyme extract, 1 mL sodium phosphate buffer (pH 7.0), and 1 mL of 30% H₂O₂. The decomposition of hydrogen peroxide was monitored by measuring absorbance at 240 nm.

APX activity was measured according to the method of Nakano and Asada (1981) [19]. The reaction mixture (3 mL final volume) contained 50 mM sodium phosphate buffer (pH 7), 0.1 mM Na-EDTA, 0.5 mM ascorbic acid, 0.1 mM H₂O₂, and enzyme extract. Absorbance was recorded at 290 nm using a spectrophotometer at 1-minute intervals for 2 minutes.

2.4.2 DPPH radical scavenging assay

Leaf powder of *Averrhoa* spp. was macerated in 70% ethanol for three consecutive 24-h periods. The resulting extracts were filtered using Whatman No. 1 filter paper and concentrated using a rotary evaporator at 40°C and 100 rpm for 45 mins to obtain the crude ethanolic extract. The antioxidant activity of the ethanolic extracts was determined using the DPPH assay according to the methods described by Baliyan (2022) [20], with some modifications. The extract was made into a 100 ppm stock solution by

dissolving 5 mg in 50 mL of solvent. This stock was then diluted to obtain concentrations of 5, 10, 15, 20, and 25 ppm. Each diluted extract solution was added to 0.05 mM DPPH solution at a 1:2 ratio and incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 517 nm [21] using a blank solution that consisted of 4 mL of 0.05 mM DPPH with 2 mL of methanol. Each test was performed in triplicate for every sample. Ascorbic acid (at concentrations of 2, 4, 6, 8, and 10 ppm) was used as a positive control.

The DPPH assay evaluates the direct radical scavenging ability of phytochemicals, complementing enzymatic assays that show the activity of the intracellular antioxidant enzyme. The percentage of DPPH inhibition, present as half-maximal inhibitory concentrations (IC_{50}), was calculated using the following equation from Phachonpai *et al.*, [21]:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample solution}}{\text{Absorbance of control}} \times 100\%$$

The half inhibitory concentration (IC_{50}) values were determined to represent the concentration required to inhibit 50% of DPPH radicals. This assay reflects the capacity of phytochemicals to directly scavenge free radicals and complements enzymatic antioxidant assays that evaluate endogenous defense mechanisms.

2.4.3 Statistical analysis and visualization

One-way ANOVA followed by Tukey's post-hoc test (p -value<0.05) was performed to evaluate significant differences in antioxidant enzyme activities among *Averrhoa* spp. Additionally, principal component analysis (PCA) was conducted to examine differences in antioxidant activity among *Averrhoa* spp. using Minitab software version 19.1.1. Principal components were selected based on eigenvalues greater than 1 and a cumulative variance exceeding 80%. Visualizations of the analysis results were generated using the SRplot online tool (<https://www.bioinformatics.com.cn/srplot>).

3 Results and discussion

Here we report the comparative study for antioxidant profiling between four *Averrhoa* species with *in silico* and *in vitro* analyses. The combination of phytochemical profiling, predictive interaction analysis, molecular docking, and wet experimental validation in the laboratory to determine whether species-specific differences influence their antioxidant mechanisms. This

integrative approach enabled the correlation of compound composition with molecular and physiological antioxidant properties, which addresses the current lack of understanding across *Averrhoa* species.

3.1 Phytochemical diversity and predicted antioxidant potential of *Averrhoa* species

A total of 50 compounds has been identified across the four species of *Averrhoa*, which were used as the basis for predictive analysis to gain molecular insight on its antioxidant capacity. GC-MS profiling revealed the phytochemical composition among the plants, which were then categorized into five major classes. The complete list of *Averrhoa* compounds can be seen in Supplementary Data 1.

Classification based on chemical structure and biosynthetic origin (Figure 2) revealed that *A. leucopetala* and *A. dolichocarpa* possess a broader distribution of bioactive compound classes, including terpenoids, phenolic compounds, and aromatic compounds, whereas *A. carambola* showed minimal chemical diversity. This classification highlights the potential biological relevance of these metabolites, particularly due to the presence of functional groups associated with antioxidant mechanisms. For instance, redox-active moieties in phenolic and related compounds such as hydroxyl (-OH), carbonyl (C=O), thiol (-SH), and amine (-NH₂) groups can facilitate electron or hydrogen donation, thereby neutralize free radicals and interrupt oxidative chain reactions, ultimately enhancing antioxidant capacity [22]. Gamma tocopherol was detected in *A. dolichocarpa*, but was absent in *A. leucopetala*, *A. carambola* and *A. bilimbi* (Supplementary Data 1). In contrast, eicosane was predominantly identified in *A. carambola* and was not detected in the other species. These findings indicate species-dependent variation in both the diversity and composition of bioactive metabolites.

In addition to diversity, the abundance of representative compounds in each species was also evaluated. Compound profiling in Table 1 showed that *A. leucopetala* (24 compounds) exhibited the highest phytochemical diversity, followed by *A. dolichocarpa* (18 compounds), whereas *A. carambola* displayed a limited profile with only four detected compounds. Notably, neophytadiene was consistently detected across all four species and was particularly dominant in *A.*

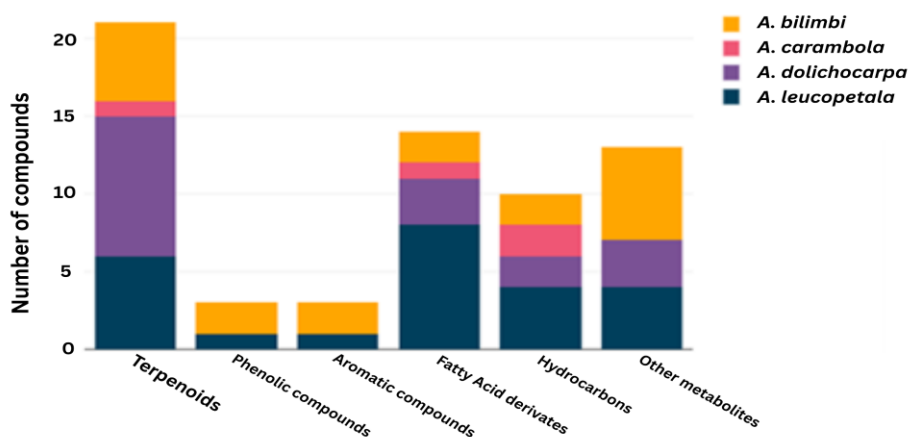


Figure 2 : Distribution of compound classes among *Averrhoa* species.

carambola, reaching a peak area of 52.29%. This suggests that the phytochemical composition of *A. carambola* is largely dominated by this diterpenoid compound. Neophytadiene has been reported to exhibit antioxidant-related activity, including the modulation of ROS and involvement in signaling pathways such as PI3K/Akt, which are associated with cellular stress responses [23]. Beyond its antioxidant function, neophytadiene appears to be integral to plant stress response that would increase under abiotic stress conditions, such as the elicitation of methyl jasmonate, salinity, red-light exposure, and drought, suggesting its

contribution to chemical defense mechanisms and adaptability to fluctuating environments [24], [25].

Furthermore, the most abundant compounds in each species generally exhibited relatively high predicted biological activity based on PASS analysis ($P_a > 0.7$), indicating a high likelihood of functional relevance. For example, cyclopentanol showed a high predicted activity ($P_a = 0.929$) and was among the major compounds in both *A. dolichocarpa* (13.38%) and *A. bilimbi* (13.11%). Several abundant compounds, including

Table 1 : Compound profiling of *Averrhoa* species.

Species	Number of compounds	Most abundant compound			
		No	Compound Name	Peak area (%)	PASS Prediction (Antioxidant)
<i>A. leucopetala</i>	24	1	Tetratetracontane	26.49	0.926
		2	Neophytadiene	12.57	0.720
<i>A. dolichocarpa</i>	17	1	trans-Pinane (2,6,6-trimethylbicyclo[3.1.1]heptane)	22.26	0.827
		2	Cyclopentanol	13.38	0.929
<i>A. carambola</i>	4	1	Neophytadiene	52.29	0.720
		2	Eicosane	4.83	0.926
<i>A. bilimbi</i>	9	1	Neophytadiene	20.76	0.720
		2	Cyclopentanol	13.11	0.929

neophytadiene, cyclopentanol, and selected hydrocarbons, were also predicted to interact with the CYP2J enzyme. This enzyme is a major epoxigenase enzyme related to the cytochrome P450-related pathway that metabolizes arachidonic acid into epoxyeicosatrienoic acids (EETs) in the system, which can exert antioxidant effects by reducing ROS levels and overexpressing antioxidant enzymes [26], [27]. While these predictions and interpretations suggest a possible involvement in oxidative metabolism, their exact role in the antioxidant mechanism remains indirect and requires further validation.

The differences in the relative abundance of phytochemicals among *Averrhoa* species primarily reflect variations in phytochemical composition and chemical diversity (Supplementary Data 1). However, the biological significance of these differences should be interpreted with caution, as antioxidant potential is not necessarily proportional to the abundance of the compound. Indeed, some compounds present at lower abundances may still exhibit high predicted antioxidant activity based on PASS analysis, indicating that bioactivity cannot be inferred solely from abundance data.

Overall, the integration of phytochemical profiling and PASS prediction indicates that *Averrhoa* species contain abundant compounds with potential antioxidant activity, either through direct redox-active functional groups or through indirect modulation of oxidative stress-related pathways. The consistent presence and dominance of neophytadiene across species further suggest its potential role as a key contributor to the antioxidant profile of the genus.

3.2 Compound-protein interaction network and mechanistic insights

The STITCH analysis of bioactive compounds from four *Averrhoa* species against antioxidant-related proteins revealed a distinct interaction network (Figure 3). Among the 50 identified compounds, only four exhibited high-confidence interactions (score > 0.900), namely butanal peak area 1.04 in *A. bilimbi*, threonine (peak area 0.92 in *A. leucopetala*), vitamin E (peak areas 3.17 in *A. leucopetala* and 6.89 in *A. dolichocarpa*), and squalene (peak areas 3.61 in *A. leucopetala* and 8.69 in *A. bilimbi*). These findings indicate that several compounds exhibit strong predicted interactions in STITCH, supporting their biological relevance as key contributors to antioxidant potential.

In *A. leucopetala*, threonine was predicted to interact with reduced glutathione (GSH), oxygen (O_2), and hydrogen peroxide (H_2O_2), and was further connected to enzymatic antioxidants, including GPX1-4, PRDX6, TXNRD1-2, and SOD1-3. These interactions suggest a potential role of threonine in maintaining redox balance through glutathione and thioredoxin mechanisms, which are central to intracellular redox buffering. Supporting this, a previous study demonstrated that dietary threonine supplementation enhances antioxidant defense by increasing catalase, glutathione reductase, and GSH activities, while upregulating antioxidant-related pathways such as NFE2L2 signaling and gamma-glutamylcysteine ligase expression [28]. Although threonine as an amino acid is not considered a classical antioxidant and showed limited direct antioxidant activity in the PASS prediction, its association with antioxidant-related functions may arise indirectly through metabolic regulation.

The STITCH interaction network and KEGG/GO enrichment analyses linked threonine to amino acid metabolism and cellular processes involved in redox homeostasis, suggesting a supportive role in maintaining antioxidant defenses rather than acting as a direct radical scavenger. Therefore, the observed antioxidant association of threonine is likely mediated through its contribution to metabolic pathways that influence cellular redox balance.

Furthermore, vitamin E (alpha-tocopherol) demonstrated a more direct and central role within the antioxidant network. It was associated with CAT, GPX1-4, and GSR, as well as O_2 and H_2O_2 , highlighting its central role within the antioxidant network and its contribution to enzymatic detoxification pathways. It was detected in *A. leucopetala* and *A. dolichocarpa* and possesses direct antioxidant properties based on PASS analysis. Vitamin E is known as a radical scavenger that prevents lipid peroxidation and protects cellular membranes by donating hydrogen from its chromanol ring [29]-[31]. A previous study showed that pretreatment with vitamin E effectively suppresses intracellular ROS levels induced by GPX4 inhibition *in vitro* using Human Embryonic Kidney 293 (HEK293) cells [32]. In addition, it activates the Nrf2 antioxidant pathway, thereby enhancing endogenous defenses such as SOD and CAT in



response to oxidative challenges [33]. A similar tocopherol interaction pattern was also observed in *A. dolichocarpa*, suggesting a conserved antioxidant function of vitamin E across these species.

In *A. bilimbi*, the dominant interacting compounds are squalene and butanal, which were associated with oxygen and hydrogen peroxide but lacked strong direct interactions with antioxidant enzymes. Compounds predicted to interact primarily with oxygen suggest a potential indirect antioxidant mechanism, in which antioxidant effects are mediated through ROS modulation rather than direct enzyme binding. In another study, it was reported that the six isoprene double bonds of squalene enable electron donation and free radical scavenging, thereby reducing oxidative stress [34]. This suggests that the antioxidant capacity of *A. bilimbi* may rely more heavily on direct radical scavenging and ROS neutralization, which is consistent with its strong DPPH activity observed experimentally (Table 4). Meanwhile, the association of butanal with oxygen and hydrogen peroxide remains less defined, whether it has a specific antioxidant role or lacks one. Lastly, *A. carambola* did not exhibit high confidence (> 0.900) interactions within the selected proteins. This may reflect limited *in silico* database representation of its phytochemical constituents and could be supported by experimental analysis.

The STITCH analysis highlights the limited coverage of current databases, with only four out of fifty identified compounds showing high-confidence interactions and relatively sparse network connectivity. This underscores that *Averrhoa* phytochemicals remain underexplored.

Despite the limited number of high-confidence compound-protein interactions identified by STITCH, the resulting network highlighted several proteins associated with redox regulation and antioxidant defense. To further investigate the biological significance of these interactions, the associated genes were subjected to GO and KEGG enrichment analyses. The enrichment results were largely consistent with the network-derived targets, indicating that the identified proteins participate in antioxidant-related processes.

Specifically, GO enrichment (Figure 3b) revealed significant overrepresentation of peroxidase activity ($-\log_{10} p\text{-value} = 18.939$), oxidoreductase activity (15.983), and antioxidant activity (13.907), suggesting that the proteins identified through the interaction network are primarily involved in electron-transfer reactions and ROS detoxification. In the CC category, enriched terms included the extracellular exosome and mitochondrial matrix, suggesting that antioxidant-related proteins are localized in compartments associated with redox signaling and energy metabolism. Meanwhile, the BP category highlights

glutathione metabolism and cellular responses to oxidative stress.

Consistent with these findings, KEGG analysis (Figure 3c) identified glutathione metabolism as the most significantly enriched pathway, together with peroxisome and longevity-regulating pathways. Consistently, KEGG pathway analysis (Figure 3c) identified glutathione metabolism as the most significantly enriched pathway with the highest gene count ($n = 6$). Additional enriched pathways included peroxisome and longevity-regulating pathways, as well as chemical carcinogenesis and reactive oxygen species. The enrichment of these pathways suggests that *Averrhoa* compounds may contribute not only to immediate ROS scavenging but also to broader cellular protection mechanisms and stress adaptation. Collectively, the STITCH, GO, and KEGG analyses converge on a common mechanism whereby *Averrhoa*-derived compounds may influence antioxidant defense by modulating redox-related enzymes, glutathione-dependent detoxification systems, and oxidative stress response pathways.

3.3 Molecular docking analysis and mechanistic implications

Molecular docking analysis was performed to evaluate screened compounds with protein targets associated with antioxidants, namely SOD and Kelch-Neh2 (Keap1). The ligands for this analysis were selected from STITCH results (vitamin E, squalene, butanal, and threonine) as well as the common compound (neophytadiene), enabling comparative analysis between predicted bioactive and abundant candidates. SOD protein was selected as a representative antioxidant enzyme responsible for superoxide radical detoxification, as it is widely present in plants [35] and is consistent with the subsequent enzyme activity analysis results. Meanwhile, Keap1 was chosen due to its regulatory role in the Nrf2-mediated cellular defense pathway against oxidative stress and toxicity [36], allowing verification of the indirect antioxidant mechanism. Therefore, these two proteins were selected to evaluate both the direct antioxidant enzyme modulation and the potential activation of cellular antioxidant defense pathways.

For SOD (Figure 4a), the desired molecular mode of action for the ligands was based on previous research that suggests increased activity

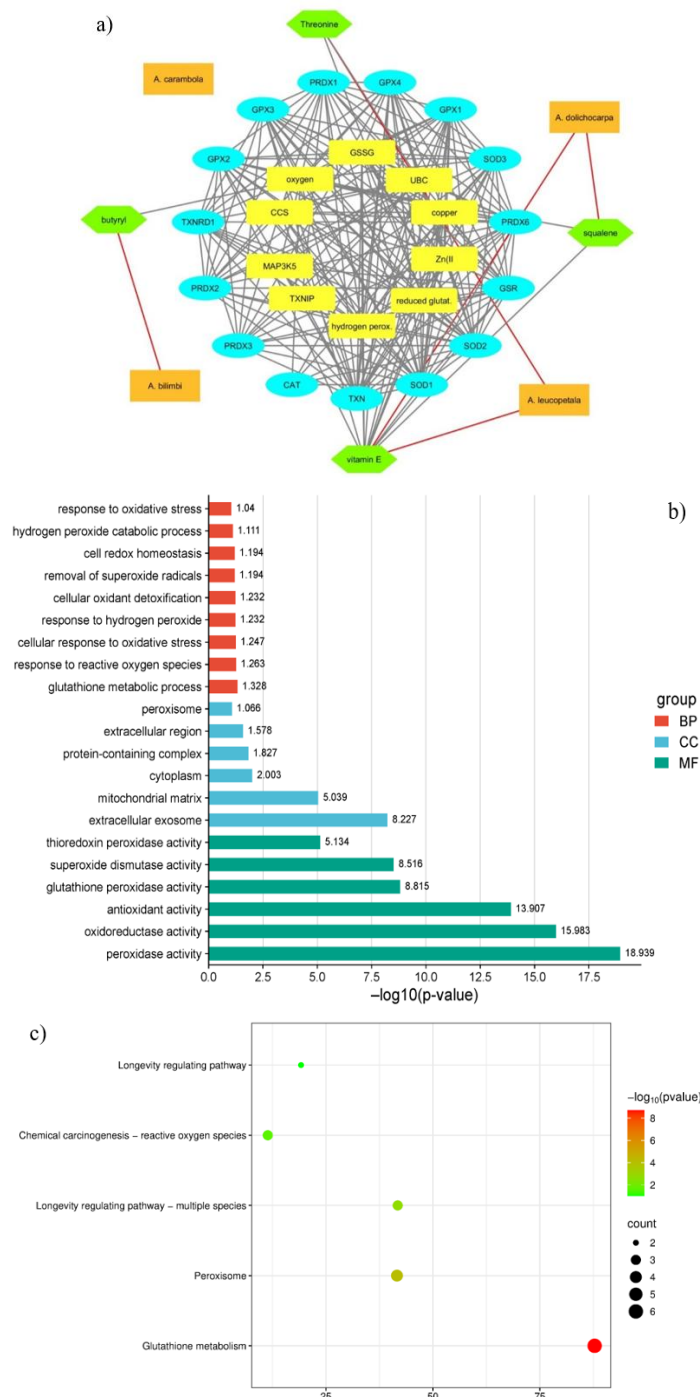


Figure 3 : Integrated network and functional enrichment analysis of antioxidant-related targets associated with *Averrhoa* compounds. (a) Compound-protein interaction network constructed using the STITCH database and visualized with Cytoscape 3.10.4. (b) Gene Ontology (GO) enrichment analysis of identified proteins across Molecular Function (MF), Biological Process (BP), and Cellular Component (CC) categories. (c) KEGG pathway enrichment analysis based on gene count and enrichment significance.



Targeting the allosteric site offers several advantages, including bypassing ATP competition, stabilizing the protein-ligand complex, and potentially reducing toxicity, while also modulating overall activity without disturbing the catalytic site [37].

Based on the result (Table 2), vitamin E exhibited the strongest binding affinity (-6.9 kcal/mol), followed by squalene (-6.7 kcal/mol) and neophytadiene (-5.5 kcal/mol), whereas threonine and butanal showed comparatively weaker interactions. Although vitamin E

and squalene displayed similar docking scores, vitamin E formed a more favorable interaction profile through hydrogen bond formation with ASP11 and ASN51, in addition to extensive hydrophobic interactions within the allosteric pocket. Furthermore, vitamin E demonstrated greater binding-site similarity with the key allosteric residues reported previously [35]. The binding profile of vitamin E aligns with the elevated SOD activity observed in *A.*

Table 2 : Molecular docking results against the Superoxide Dismutase protein (1CB4) from AutoDock Vina.

No	Ligands	Binding Affinity (kcal/mol)	Interaction Residues
1	Neophytadiene	-5.5	CYS6, VAL7 , LYS9 , ASN51 , GLY54, CYS144 , GLY145 , VAL146
2	Vitamin E	-6.9	CYS6, VAL7 , LYS9 , GLY10, ASP11*, ASN51* , GLY54, CYS55, CYS144 , GLY145 , VAL146
3	Squalene	-6.7	VAL7 , LYS9 , GLY10, GLN15, ASN51 , GLY145 , VAL146
4	Butanal (Butyryl)	-3.2	VAL5, CYS6, VAL7 , GLY49, ASP50, ASN51* , GLY145 , VAL146 ,
5	Threonine	-4.1	CYS6, VAL7* , LYS9 , ASN51* , GLY145 , VAL146*

Note: Residues typed in bold indicate resemblance to the allosteric binding predicted by PrankWeb, while the asterisk symbol indicates a residue with conventional hydrogen bond interaction.

Table 3 : Molecular docking results against Kelch-Neh2 protein (2FLU) from AutoDock Vina.

No	Ligands	Binding Affinity (kcal/mol)	Interaction Residues
1	Neophytadiene	-6.1	THR80, GLY364, LEU365, ALA366, GLY367 , ARG415, ILE416, GLY417, VAL418 , GLY462, VAL463, VAL465 , GLY464, GLY509, GLY511, VAL512 , CYS513 , ALA556, LEU557, GLY558, ILE559 , THR560 , GLY603, VAL604, GLY605, VAL606
2	Vitamin E	-7.9	LEU365, ALA366, GLY367 , VAL369 , CYS368 , VAL418 , GLY419 , VAL420 , VAL463, GLY464, VAL465 , ALA466 , VAL467 , ALA510, GLY511, VAL512 , CYS513 , VAL514 , LEU557, GLY558, ILE559 , THR560 , VAL561 , VAL604, GLY605, VAL606 , ALA607 , VAL608
3	Squalene	-7.7	THR80, GLY364, LEU365, ALA366, GLY367 , ARG415, ILE416, GLY417, VAL418 , GLY419 , VAL420 , GLY462, GLY464, VAL465 , ALA466 , VAL467 , GLY509, ALA510, GLY511, VAL512 , CYS513 , VAL514 , ALA556, LEU557, GLY558, ILE559 , THR560 , VAL561 , GLY603, VAL604, VAL606 , ALA607
4	Butanal (Butyryl)	-3.1	THR80, GLY364, LEU365, ARG415*, ILE416, GLY462, GLY509, ALA556, GLY603
5	Threonine	-4.6	GLY364, LEU365, ALA366, ARG415, ILE416*, GLY417, VAL463, LEU557, GLY558, GLY603, VAL604*, GLY605

Note: Residues typed in bold indicate resemblance to the pocket site predicted by PrankWeb, while the asterisk symbol indicates a residue with conventional hydrogen bond interaction.

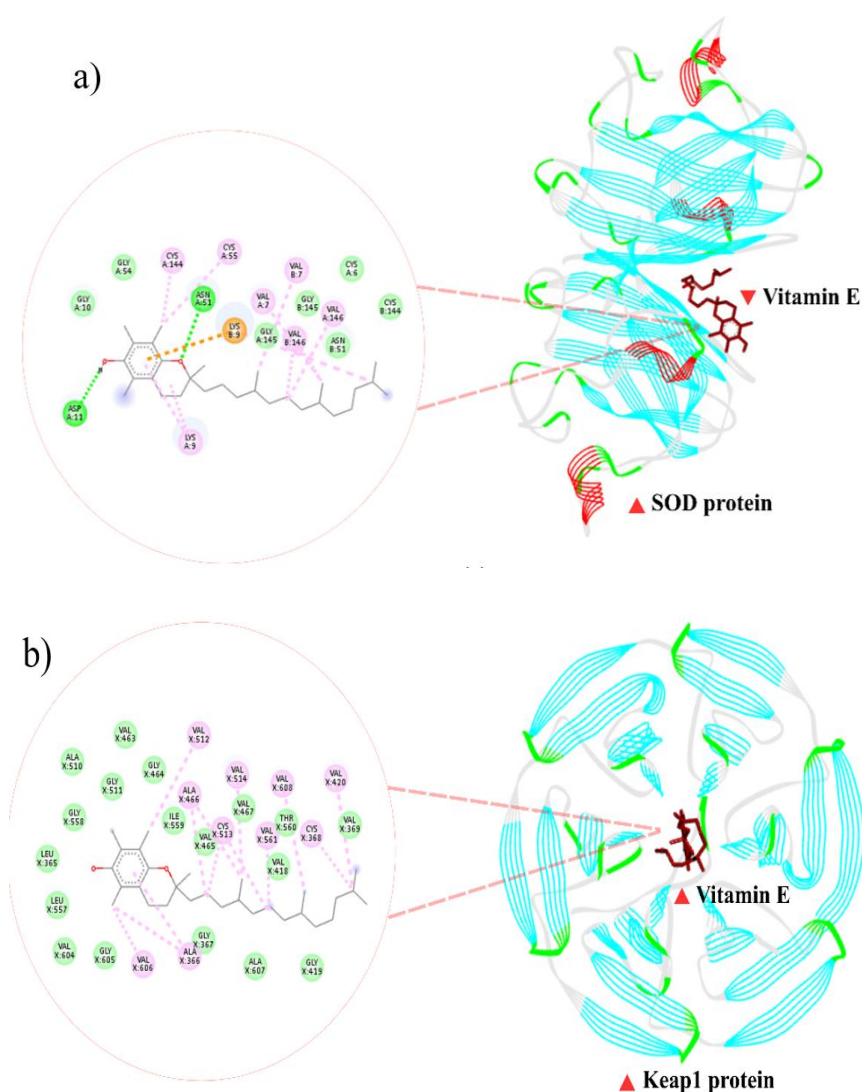


Figure 4 : Molecular docking visualizations of representative *Averrhoa*-derived compounds were visualized using Discovery Studio BIOVIA. (a) Interaction of vitamin E with the allosteric site of SOD. (b) Interaction of vitamin E with the active site of Keap1. Colored circles indicate interaction types with amino acid residues: light green (van der Waals), green (hydrocarbon), and pink (alkyl interactions).

dolichocarpa and *A. leucopetala*, supporting a mechanistic link between tocopherol presence and enzymatic antioxidant performance.

For Keap1 (Table 3), vitamin E (-7.9 kcal/mol) and squalene (-7.7 kcal/mol) exhibited comparable binding affinities within the Kelch domain, the region responsible for Nrf2 recognition and sequestration [36]. The binding pocket used in this study was initially predicted using the PrankWeb server, which identified the central ligand-recognition region as the most probable binding site. Subsequent docking analysis confirmed that the selected

ligands occupied this predicted pocket. Although vitamin E and squalene showed similar docking scores, vitamin E demonstrated greater similarity to the reference binding site, interacting with 17 key residues compared to 15 residues for squalene. Notably, Gly364, one of the interacting residues identified in the present study, has previously been reported as a residue involved in Keap1 ligand recognition [36]. While several other interacting residues differed from those reported in previous studies, they were located within the same Kelch-



domain binding region, supporting the biological relevance of the predicted binding poses. In contrast, neophytadiene exhibited a lower binding affinity (-6.1 kcal/mol) and shared fewer residues with the reference binding site, whereas butanal and threonine displayed weaker interactions and more limited occupancy of the predicted binding pocket.

Vitamin E was selected as the primary docking visualization as it demonstrated the most favorable interaction profile. This selection was based on its strong binding affinity, hydrogen-bond formation, and high structural similarity to the reference binding site. This compound was predicted to be capable of occupying the Keap1 binding pocket (Figure 4b), potentially interfering with Keap1-Nrf2 association and facilitating Nrf2 activation. This further suggests their potential to disrupt Keap1-Nrf2 interactions, leading to the release of Nrf2 and activation of antioxidant response genes. Such a mechanism is supported by previous studies, where the Keap1 protein is thought to be the key target due to its tether Nrf2 [36]. Collectively, these results indicate that

Averrhoa compounds may exert antioxidant effects through allosteric enzyme modulation of SOD and transcriptional regulation via Keap1-Nrf2 signaling

3.4 In vitro antioxidant enzyme activity

The activities of key antioxidant enzymes, such as SOD, CAT, and APX, were evaluated across four *Averrhoa* species to assess their endogenous protective capacity against oxidative stress. The antioxidant enzyme analysis in Figure 5 shows that *A. dolichocarpa* exhibited the highest SOD ($170.25 \pm 1.22 \text{ U min}^{-1} \text{ gFW}^{-1}$) and CAT ($2.05 \pm 0.09 \text{ U min}^{-1} \text{ gFW}^{-1}$) activities, while *A. leucopetala* had the strongest APX activity ($2.22 \pm 0.52 \text{ U min}^{-1} \text{ gFW}^{-1}$). On the other hand, the lowest SOD level was observed in *A. carambola* ($7.74 \pm 1.22 \text{ U min}^{-1} \text{ gFW}^{-1}$), whereas *A. bilimbi* had the lowest for CAT ($0.96 \pm 0.05 \text{ U min}^{-1} \text{ gFW}^{-1}$) and APX ($1.64 \pm 0.28 \text{ U min}^{-1} \text{ gFW}^{-1}$). Additionally, principal component analysis further grouped *A. dolichocarpa* and *A. leucopetala* into a cluster together by their elevated enzyme activity, which can be seen in Supplementary Data 2. This showed *A. dolichocarpa* and *A. leucopetala* superiority in enzymatic defense, consistent with higher SOD, CAT, and APX activities. These results suggest that the two endemic species possess stronger endogenous antioxidant capacities and greater tolerance to environmental stress compared to *A. bilimbi* and *A. carambola*.

In plants, ROS are generated as byproducts of normal aerobic metabolism, where they act as signaling molecules in various biological processes. However, under biotic and abiotic stress conditions, ROS levels can increase dramatically, potentially causing oxidative

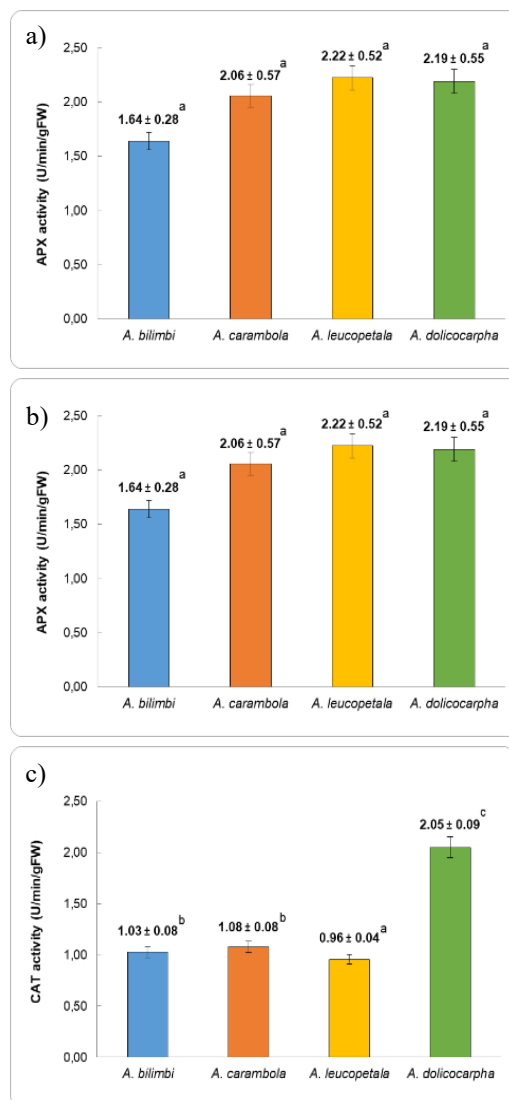


Figure 5 : Antioxidant enzyme activity in *Averrhoa* spp. leaves: (a) superoxide dismutase (SOD), (b) ascorbate peroxidase (APX), and (c) catalase (CAT). Error bars indicate standard deviation (n=3) and different superscript letters within the species indicate significant differences among *Averrhoa* spp. (Tukey's test, p -value < 0.05).

damage if not effectively managed [38]. To preserve redox balance, plants employ the enzymatic antioxidant SOD, which transforms superoxide radicals ($\text{O}_2^{\bullet-}$) into H_2O_2 and oxygen, while APX and CAT convert H_2O_2 into water to avert cellular damage [38]-[40]. In addition to enhancing stress tolerance, these enzymes exhibit pharmacological

properties such as antiaging, antiradiation, and antitumor effects by reducing ROS-induced toxicity [1]. Therefore, evaluating the activities of SOD, APX, and CAT is crucial for assessing plant resilience to environmental stressors and for determining their antioxidant potential in therapeutic contexts [39]-[42]. The ANOVA test showed significant differences in CAT (p -value < 0.05), with *A. dolichocarpa* forming a distinct group. A similar result was found in SOD activity, with Tukey's test clustering *A. carambola* and *A. bilimbi* together (lowest), *A. leucopetala* separately (intermediate), and *A. dolichocarpa* in the highest group.

3.5 Radical scavenging activity (DPPH assay)

In addition to enzymatic antioxidant defenses, plants also possess the capability of neutralizing free radicals through electron or hydrogen donation. To evaluate these properties, the antioxidant potential of *Averrhoa* leaf extracts was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. All four *Averrhoa* species demonstrated strong free radical scavenging activity, with IC₅₀ values below 50 µg/mL (Table 4). Among them, *A. bilimbi* exhibited the lowest IC₅₀ (15.24 µg/mL), followed by *A. leucopetala* (18.09 µg/mL), *A. dolichocarpa* (22.50 µg/mL), and *A. carambola* (32.70 µg/mL). According to the classification by Souhoka *et al.*, [43], all these values fall within the "very strong" antioxidant category (< 50 µg/mL). The DPPH results suggest that *A. bilimbi* exhibits the highest inhibition percentage compared to the other species, likely due to its abundance of compounds capable of donating electrons or hydrogen atoms to neutralize free radicals. Previous studies have reported IC₅₀ values of 2.03-4.87 µg/mL for *A. bilimbi* leaf extract [44] and 20.35 µg/mL for its fruit extract [3], whereas while *A. carambola* leaf extract shows a broader activity range (4.19 µg/mL to 75.43 ± 1.64 µg/mL [45], [46]. Consistent with these findings, our data confirm that *A. bilimbi* exhibits the strongest DPPH scavenging activity, whereas *A. carambola* shows the weakest. Notably, *A. leucopetala* and *A. dolichocarpa* demonstrated comparably high antioxidant

activity relative to the well-studied species, and to the best of our knowledge, this study represents the first report of DPPH scavenging activity for these two species.

The differences in DPPH radical scavenging activity among *Averrhoa* species may be associated with variations in their phytochemical composition revealed by GC-MS analysis. *A. leucopetala*, which contained the highest number of compounds, exhibited strong antioxidant activity, suggesting that a diverse phytochemical profile may enhance antioxidant effectiveness. *A. carambola* exhibited the highest IC₅₀ value, indicating the lowest antioxidant activity among the studied species. This finding is consistent with its relatively volatile profile, as only four compounds were detected by GC-MS. In addition, *A. carambola* showed lower activities of the antioxidant enzymes SOD, CAT, and APX compared with the two endemic species, *A. dolichocarpa* and *A. leucopetala*. The combination of lower enzymatic antioxidant defense and reduced phytochemical diversity may have contributed to its weaker radical-scavenging capacity. Interestingly, *A. bilimbi* exhibited the strongest antioxidant activity (IC₅₀ = 15.24 µg mL⁻¹), despite having fewer detected compounds than *A. leucopetala* and *A. dolichocarpa*. This finding suggests that antioxidant capacity is influenced not only by the number of metabolites present but also by their possible synergistic interactions between compounds. Since GC-MS primarily detects volatile and semi-volatile constituents, the antioxidant activity may also be influenced by non-volatile phenolic and flavonoid compounds that were not captured by this analysis. A lower number of interactions was observed for *A. carambola*, although the factors underlying this pattern were not investigated in the present study.

The superior antioxidant performance of *A. leucopetala* and *A. dolichocarpa* suggests that antioxidant efficacy is influenced by the specific composition and functional roles of bioactive compounds, rather than their abundance alone. A similar pattern has been reported in bamboo, where only a subset of genotypes showed high antioxidant activity, and the predominant bioactive compound differed among genotypes, indicating that phytochemical variation may contribute to differences in antioxidant performance [47].

Furthermore, the differences in antioxidant enzyme activity may be associated with the ecological origins of the species. *A. bilimbi* originates from Java, *A. carambola* originates from South Kalimantan, *A. dolichocarpa* and *A. leucopetala* are endemic species from Papua and Gorontalo, respectively [48]. The

Table 4 : IC₅₀ values of *Averrhoa* spp.

Sample	IC ₅₀ (µg/mL)	Category
Ascorbic acid (control)	11.51	Very Strong
<i>A. bilimbi</i>	15.24	Very Strong
<i>A. carambola</i>	32.70	Very Strong
<i>A. leucopetala</i>	18.09	Very Strong
<i>A. dolichocarpa</i>	22.50	Very Strong



environmental conditions of each plant's origin may impose distinct abiotic stresses that favor the evolution of enhanced antioxidant protective systems. The higher antioxidant enzyme activities in these species may therefore represent adaptive traits that improve tolerance to environmental fluctuations and oxidative stress.

4. Conclusion

This study addressed the limited understanding of antioxidant mechanisms in *Averrhoa* species through an integrated computational and experimental approach. All four species exhibited antioxidant potential, with neophytadiene identified as a common compound. *A. leucopetala* and *A. dolichocarpa* emerged as the most promising species, primarily through direct enzymatic interactions, whereas *A. bilimbi* showed strong radical-scavenging activity despite limited enzyme-target interactions. In contrast, *A. carambola* displayed comparatively lower activity, which may reflect database limitations rather than the inactivity of its phytochemicals. These findings highlight *A. leucopetala* and *A. dolichocarpa* as promising sources of nutraceutical antioxidants, supported by both biochemical assays and *in silico* validation. Overall, antioxidant efficacy appears to depend more on the underlying mechanism than on compound abundance, with different species employing complementary antioxidant strategies. Future studies should focus on compound isolation, *in vivo* validation, and bioavailability assessment to confirm efficacy and further elucidate the molecular mechanisms underlying their antioxidant and therapeutic effects.

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Author Contributions

W.A.S.T.: conceptualization, research design, writing-reviewing and editing, funding acquisition; A.M.: research design, data analysis, writing-reviewing and editing; A.T.S.; H.W.P A.D.C.S. and R.R.: data curation: investigation, data analysis, writing the original draft; F.D.: methodology, data curation, data analysis; W.U.M.: writing-reviewing and editing, data analysis, data curation; N.I.M.I.: writing-reviewing and editing.

Conflicts of Interest

The authors declare no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors utilized the Paperpal tool to enhance the language and readability of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online [here](#).

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