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IN-VITRO EVALUATION OF ANTIOXIDANT ACTIVITY OF ISOLATED PHYTOCHEMICALS FROM METHANOLIC SEED EXTRACT OF ANNONA SQUAMOSA

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ABSTRACT

Annona squamosa L. (Annonaceae) seed defatted methanol extract produced Quercetin (A), 6-Methoxy isovitexin (B), Kaempferol (C), and Ferulic acid (D). The isolated compounds' structures were verified including 1H, 13C-NMR, IR, and MS spectroscopy analysis. Ascorbic acid (vitamin C) was used as the reference standard for all compounds' in vitro antioxidant properties. The UV-Visible spectrophotometer was used for all of the analysis. The study's findings suggested that each chemical had effective antioxidant activities in a concentration-dependent way.

Keywords: *Annona squamosa*, Phytochemicals, *In vitro*, antioxidant activity, ascorbic acid

INTRODUCTION

Traditionally, either in the form of pure active principles or conventional formulations, plants have been a common source of medication. It can be reasonably assumed that a significant portion of traditional therapy uses plant extracts or their active ingredients. According to a WHO survey, 80% of the world's population of more than 4,000 million relies on traditional medicines for their main health care requirements. Due to its efficiency, low incidence of adverse effects in clinical trials and affordable price, herbal medicines are gaining popularity. Drugs made from plants or their extracts are frequently given, even when the biological active ingredients are not known. Even the World

Health Organization (WHO) supports using plant-based medicines to treat various illnesses.

The family Annonaceae, which includes about 135 genera and 2300 species, includes *Annona squamosa* L., often known as sugar apple, custard apple, sweet sop, sweet apres and sitaphal **[1]**. The evergreen *A. squamosa* tree grows to a height of 3 to 8 metres. With a sum area under cultivation of forty thousand hectares, *A. squamosa* is widely grown in a number of states in India **[2]**. Different plant parts, including fruit, leaves, bark, and roots, are used to treat a range of ailments and are renowned for their therapeutic and nutraceutical benefits. The fruits are typically consumed raw or used to produce sorbet or juice drinks **[3]**. They are a high source of calcium, phosphorus, and iron **[4]**. Over the white pulp, the lustrous, brownish-black, ovoid-shaped seeds are widely dispersed. The annonaceous acetogenins, monoterpenes, sesquiterpenes, diterpenes, alkaloids, steroids, cyclopeptides, flavonoids, and essential oils have all been found in the annona squamosa plant **[5]**. Due to the existence of cyclic peptides, it has historically been employed as an insecticide, anticancer, antidiabetic, antioxidant, antilipidemic, and antiinflammatory agent **[6]**. The alcoholic seed extract may contain anticancer chemicals, according to a recent study **[7]**. Leaf extract of this plant showed antinociceptive activity **[8]**. It was discovered that roots had an anticonvulsant effect **[9]**.

EXPERIMENITAL

General experimental procedures: An electrothermal melting point apparatus was used to determine the melting points of soft glass capillaries. The IR spectra were captured using KBr pellets and an FTIR SHIMADZU 8400S spectrometer. Using TMS as the internal standard, the 1H and 13C NMR spectra were captured in $CDCl_3$ at 300 MHz and 75 MHz, respectively. Argon/Xenon was used as the FAB gas, and FAB mass spectra were captured using a JEOL SX 102/DA-6000 mass spectrometer.

Plant material: Seeds of *A. squamosa* were collected from Jaipur Rajasthan (India).

Extraction and isolation of the constituents: 48 hours were spent extracting the 1.5 kg of defatted plant material with methanol. Crude extract was produced when obtained extract was concentrated under reduced pressure. The methanolic extract was converted into slurry by being dissolved in the least amount of methanol and adsorbed on silica gel. Column chromatography was performed on the dried slurry over silica gel. The following compounds (A to D) were separated, purified, and characterised after being eluted from the

column using various solvents in order of increasing polarity.

***Isolation of compound A as Quercetin*:** Molecule-A was obtained by using $CHCl_3$ as eluting solvent in column. After the solvent was eliminated, the resultant material was crystallised with methanol to form light yellow needles, m.p. 315-316°C. IR (KBr, cm^{-1}) 3465 (-OH, stretching), 3020 (Ar., -CH stretching), 1595, 1525 (Ar., -CC stretching), 915, 830, 790. ^1H NMR ($CDCl_3$, δppm) 12.20 (s, -OH at C5), 10.15 (s, -OH at C3), 8.89 (s, -OH at C7), 8.96 (s, -OH at C3'), 8.32 (s, OH at C4'), 7.73 (m, 2H, C2′ & C6′), 7.02 (d, 1H, 8.4, C5′), 6.30 (d, 1H, 2.5, C6 & C8). 13C NMR ($CDCl_3$, δppm) 155.92 (C2), 133.86 (C3), 177.28 (C4), 159.82 (C5), 98.23 (C6), 163.75 (C7), 92.50 (C8), 155.10 (C9), 103.76 (C10), 124.91 (C1′), 113.55 (C2′), 142.35 (C3′), 146.60 (C4′), 114.34 (C5′), 119.83 (C6′). MS (m/z): 302 (M^+), 284, 152, 105, 95 etc. Molecular formula calculated as $C_{15}H_{10}O_7$

Isolation of compound B as 6-methoxy isovitexin: Compound B was isolated when column was eluted the column with chloroform and ethyl acetate with ratio 1:1 as orange-red needles, m.p. 243-44°C. It gives positive ferric chloride test which indicates the presence of flavonoid. IR (KBr, cm^{-1}): 3460 (-O-H stretching), 3025 (Ar., -CH stretching), 1585, 1505 (Ar., -CC stretching), 905, 815, 765. 1H NMR ($CDCl_3$, δppm): 12.35 (s, 1H, -OH at C5), 10.55 (s, 1H, -OH at C3), 9.05 (s, 1H, -OH at C7), 8.75 (s, 1H, -OH at C4'), 7.25 (d, 2H, *J = 5.0 Hz*, C2', C6') and 7.05 (d, 2H, *J = 2.5 Hz*, C3', C5'), 6.75 (s, 1H, C8) 3.52 (s, 1H, - OCH_3 at C6). MS (m/z): 316 (M+), 301, 169, 122 etc. Molecular formula calculated as $C_{16}H_{12}O_7$

***Isolation of compound C as Kaempferol*:** The light yellow amorphous powder obtained when column was eluted with chloroform and Ethyl acetate in ratio 1:3. m.p. 275-276°C. The compound gave positive Shinoda and sodium hydroxide test indicated its flavonoid nature. It appeared dull yellow under UV (366 nm) light and remained unchanged in NH_3 vapours, suggested it to be a flavonol. IR (KBr, cm^{-1}): 3415, 2825, 1705, 1506, 1245, 1220 cm^{-1}. 1H NMR ($CDCl_3$, δppm) 12.16 (s, -OH at C5), 10.12 (s, -OH at C3), 8.93 (s, -OH at C7), 8.46 (s, -OH at C4'), 6.16 (d, 1H, *J = 2.3 Hz*, C6), 6.31(d, 1H, *J = 2.3 Hz*, C8), 6.82 (d, 2H, *J = 8.6 Hz*, C3′ & C5′), 7.97 (d, 2H, *J = 8.6 Hz*, C2′ & C6′). MS (*m/z*): 286, 153, 121, 93. Molecular formula $C_{15}H_{10}O_6$

***Isolation of compound D as Ferulic acid*:** Light yellow crystals obtained when column was eluted with Ethyl acetate. It showed melting point 168-169°C. IR (KBr, cm^{-1}) 3455, 1695, 1610, 1515, 1280, 945. 1H NMR ($CDCl_3$, δ ppm): 3.96 (s, 3H, C4'), 6.32 (d,

1H, J=15 Hz, C2'), 6.95 (d, 1H, *J = 9.0 Hz*, C6), 7.14 (dd, 1H, *J = 8.0 and 2.0 Hz*, C5), 7.07 (d, 1H, *J = 2.0 Hz*, C3), 7.73 (d, 1H, *J = 15.0 Hz*, C1'). 13C NMR ($CDCl_3$, δppm): 54.98 (C4'), 108.48 (C5), 113.39 (C2), 113.78 (C2'), 122.57 (C3), 125.68 (C4), 145.81 (C1'), 146.05 (C6), 147.37 (C1), 170.36 (C3'). MS (m/z): 194 (M+), 179, 161, 133, 105, 89, 77, 51. Molecular formula $C_{10}H_{10}O_4$

Evaluation of antioxidant activity: All of the discovered compounds' antioxidant activity was evaluated using various in-vitro methodologies, including the DPPH scavenging assay, Nitric oxide radical assay, Hydroxyl radical scavenging activity, and Hydrogen peroxide scavenging activity.

The following standard and additional chemical concentrations were employed:

Standard: 5, 10, 15, or 20 µg/ml of ascorbic acid (vitamin C).

Compound A to D at 5, 10, 15, 20µg/ml

DPPH scavenging activity: To estimate the power of the compounds to scavenge free radicals, Shirwaiker et al. utilised the DPPH method **[10]**. Different concentrations of compounds produced in methanol (5, 10, 15, 20 g/ml) were combined with a 0.1 mM DPPH solution that was mixed in 95% methanol. A spectrophotometer was utilized to test the solution's absorbance at 517 nm after 30 minutes.

Nitric oxide radical activity: Nitric oxide radical activity was performed using the Garret et al. method **[11]**. Different substances were combined with 5mM sodium nitro prusside mixed in phosphate buffer saline at concentrations of 5, 10, 15, and 20 g/ml. This solution underwent a further reaction with Griess reagent after 150 minutes at 250°C incubation. The solution's absorbance was measured at 546 nm. 5, 10, 15, and 20 g/ml quantities of ascorbic acid (vitamin C) were considered typical.

Hydroxyl radical scavenging activity: It was done using the salicylic acid approach suggested by Smirnoff and Cumbes **[12]**. Salicylic acid, ferrous sulphate, and hydrogen peroxide, each at a concentration of 9 mmol/l in one millilitre, were added to one millilitre of a solution containing various concentrations of all the chemicals. A spectrophotometer was used to detect the mixture's absorbance at 510 nm after 60 minutes of incubation at 37 °C.

Hydrogen peroxide scavenging activity: Ruch *et al's* approach was used to assess each compound's ability to scavenge hydrogen peroxide **[13]**. A 0.6 ml solution of hydrogen peroxide was combined with individual component concentrations of 5, 10, 15, and 20 g/ml. At 230 nm, absorbance was measured.

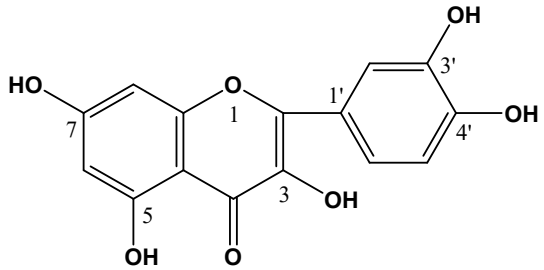
Each experiment was run three times. For the calculation, an average reading was obtained using the scavenging activity formula.

$$\% \text{ Scavenging activity } = \frac{\text{Ao} - \text{A1}}{\text{Ao}} \text{ x } 100$$

Where Ao = Absorbance of control; A1= Absorbance of sample

RESULTS AND DISCUSSION

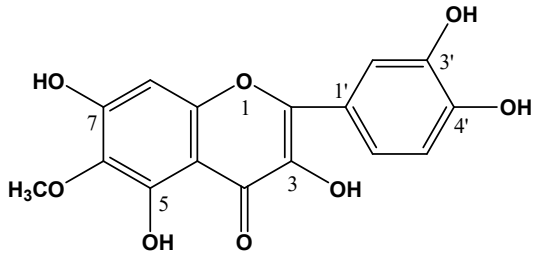
Characterization of compound A as Quercetin: Mass spectrum analysis at 302 [M+] was used to determine the chemical formula of molecule A, which is $C_{15}H_{10}O_7$. A large peak at 3465 in the IR spectra (KBr, cm^{-1}) confirmed the presence of the hydroxyl group. Along with the aromatic C=C stretching at 1595 and 1525, the aromatic C-H stretching was seen at 3020. The meta-coupled protons at C-6 and C-8 locations, respectively, were visible as a set of doublets at 6.29 (*J = 2.5 Hz*) in the 1H NMR spectra ($CDCl_3$, δppm). The proton at C-5' location was ascribed to a doublet seen at 7.04 (*J = 8.4 Hz*). At 7.72, overlapping doublets (*J = 2.5 Hz*) and quartets (*J = 2.5, 8.4 Hz*) of the protons at C2' and C6' sites were seen. 05 -OH groups connected to C5, C3, C7, C3' and C4' locations, respectively, were attributed to five singlets detected at 12.20, 10.15, 8.89, 8.96 and 8.32. The absorption at 177.28 (C4) in the 13C NMR spectrum ($CDCl_3$, δppm) revealed the existence of one carbonyl group, and these values were allocated based on the reported values [14,15]. The hydroxyl group-attached carbon atoms C3, C5, C7, C3', and C4' displayed absorptions at 133.86, 159.82, 163.75, 142.35, and 146.60, respectively. At 155.92 (C2), 98.23 (C6), 92.50 (C8), 155.10 (C9), 103.76 (C10), 124.91 (C1'), 113.55 (C2'), 114.34 (C5'), and 119.83 (C6'), additional absorptions were also noted. Quercetin was identified as molecule-A based on the aforementioned information.



Compound A

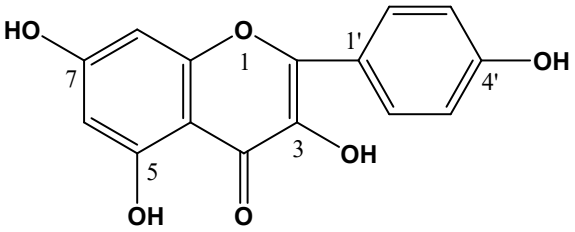
Characterization of compound B as 6-methoxy isovitexin: In the mass spectrum of molecule-B, the important molecular ion peak emerged at m/z 316 [M+]. Other significant peaks appeared at 301, 169, 122, and other locations. $C_{16}H_{12}O_7$ was calculated as the compound's molecular formula. A large peak at 3460 in the IR spectra (KBr, cm^{-1}) confirmed the presence of the hydroxyl group. Along with the aromatic C=C stretching at 1585 and 1505, the aromatic -CH stretching was seen at 3025. When protons were positioned at C2', C6' and C3', C5', respectively, the 1H-NMR spectrum ($CDCl_3$,

δppm) showed a set of doublets at 7.25 (d, 2H, *J = 5.0 Hz*, C2', C6') and 7.05 (d, 2H, *J = 2.5 Hz*, C3', C5'). 04 -OH groups connected to C5, C3, C7, and C4' positions, respectively, were attributed to four singlets detected at 12.35, 10.55, 9.05, and 8.75. For three protons at location C6, a singlet peak was seen at 3.52. Based on the aforementioned data, substance B was identified as 6-methoxy isovitexin. For the first time from this plant, component B has been isolated and characterized **[16]**.



Compound B

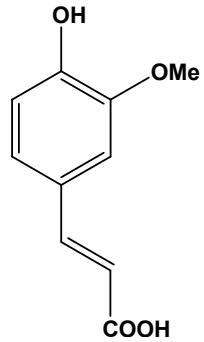
Characterization of Compound C as Kaempferol: It was separated as a yellowish-light amorphous powder having a melting point of 275-276ºC. The distinct molecular ion peak at m/z 286 [M+], which corresponds to its chemical formula $C_{15}H_{10}O_6$, was identified through analysis of its mass spectrum. The further significant fragment ions showed up at m/z 153, 121, and 93. The aglycone's usual fragmentation pattern indicated that it was a 3, 5, 7, 4'-tetrahydroxy flavone. The substance passed the sodium hydroxide and Shinoda tests for flavonoid origin. The O-H stretch peak was at 3415, the C-H stretch peak was at 2825, and the C=O stretch peak was at 1705 in the IR (KBr, cm^{-1}) spectra. Two singlet peaks for two different hydrogen at position C6 and C8 were visible in the 1HNMR spectra at 6.16 and 6.31, respectively. Aromatic protons have doublet peaks at C2' and C6', C3' and C5', at 7.97 and 6.82, respectively. 04 -OH groups connected to C5, C3, C7 and C4' locations, respectively, were attributed to four singlets detected at 12.16, 10.12, 8.93 and 8.46. The facts listed above allowed for its identification as kaempferol **[17]**.



Compound C

Characterization of compound D as Ferulic acid: The molecular ion peak for the identified phenolic compound, with the molecular formula $C_{10}H_{10}O_4$, was seen in the EI-MS at m/z 194 (M+) as base peak. The IR spectra (KBr, cm^{-1}) verifies the structure of ferulic acid with peaks at 3455 (-OH stretching), 1695 (-C=O stretching), 1280 (-C-O stretching), and 1515, 1610 (Ar., C=C). The methoxy group's distinctive signal was visible in the 1HNMR spectrum at 3.96 as singlet peak. Three aromatic protons, which correspond to the C6, C5, and C3 of the isolated compound's aromatic portion, were

also visible in the compound's spectra at frequencies of 6.95 (d, *J = 9.0 Hz*), 7.14 (dd, *J = 8.0 and 2.0 Hz*), and 7.07 (d, *J = 2.0 Hz*). The existence of two additional proton doublets with *J = 15 Hz* at 6.32 and 7.73 revealed the presence of the side chains C2' and C1', respectively, in the molecule. According to the suggested structure of ferulic acid **[18]** the 13CNMR spectra revealed the existence of 10 signals, including 6 aromatic carbon signals and 4 aliphatic chain signals (4-hydroxy-3-methoxycinnamic acid).



Compound D

Assessment of *in-vitro* Antioxidant Activity

***DPPH scavenging activity*: Figure 1** illustrates the DPPH scavenging activity of compounds A, B, C, and D and shows that % of inhibition augmented with escalating compound concentrations. The standard's maximum IC_{50} value for scavenging DPPH was discovered to be 89.67 for 20 g/ml. The compound-B concentration produced the most notable outcome when compared to the standard. Compound-D demonstrated the least effective effectiveness, scoring 35.10, 38.24, 39.87, and 39.92 for concentrations of 5 g/ml, 10 g/ml, 15 g/ml, and 20 g/ml, respectively.

***Nitric oxide radical activity*:** With higher compound concentrations, the percentages of inhibitions rose. The IC_{50} value for standard was found to be 40.09 and 60.93 for 5 g/ml and 20 g/ml, respectively. The IC_{50} value for nitric oxide scavenging was discovered lowest 27.93 for compound-D at 5 g/ml and maximum 65.49 for compound-B at 20 g/ml **(Figure 2)**.

***Hydroxyl radical scavenging activity*:** With higher compound concentrations, all compounds' percentage inhibitions of the hydroxyl radical increased. Compound-A IC_{50} value for its ability to scavenge hydroxyl radicals was determined to be 29.38, 36.41, 44.69, and 52.05 at concentrations of 5 g/ml, 10 g/ml, 15 g/ml, and 20 g/ml, respectively. At compound-B, it was discovered to be 32.10, 39.42, 47.31, and 54.71 for concentrations of 5, 10, 15, and 20 g/ml, respectively. It was discovered that the concentrations of compound-C were 27.95, 35.10, 43.82, and 50.62 for 5g/ml, 10g/ml, 15g/ml, and 20g/ml, respectively, whereas compound-D concentrations were 12.19, 13.96, 14.06, and 16.28 for 5g/ml, 10g/ml, 15g/ml, and 20g/ml, respectively. Comparing compound-B to the standard, the most effective and significant findings were found

to be 27.67, 34.50, 42.52, and 49.29 for concentrations of 5 g/ml, 10 g/ml, 15 g/ml, and 20 g/ml, respectively **(Figure 3)**.

***Hydrogen peroxide scavenging activity*:** Figure 4 displays the capacity to scavenge hydrogen peroxide in a test. The compounds with the highest levels of inhibition (20 g/ml), 75.81, 78.41, and 74.12 for compounds A, B, and C, respectively, were used. While the standard's IC_{50} value at the same concentration is 73.88 **(Figure 4)**.

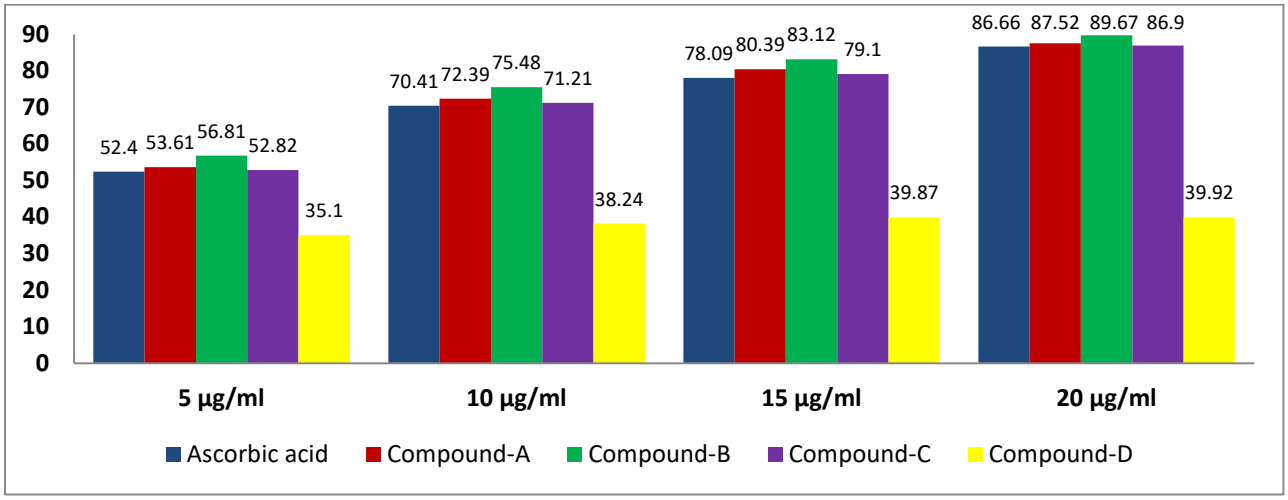


Figure 1: DPPH scavenging activity

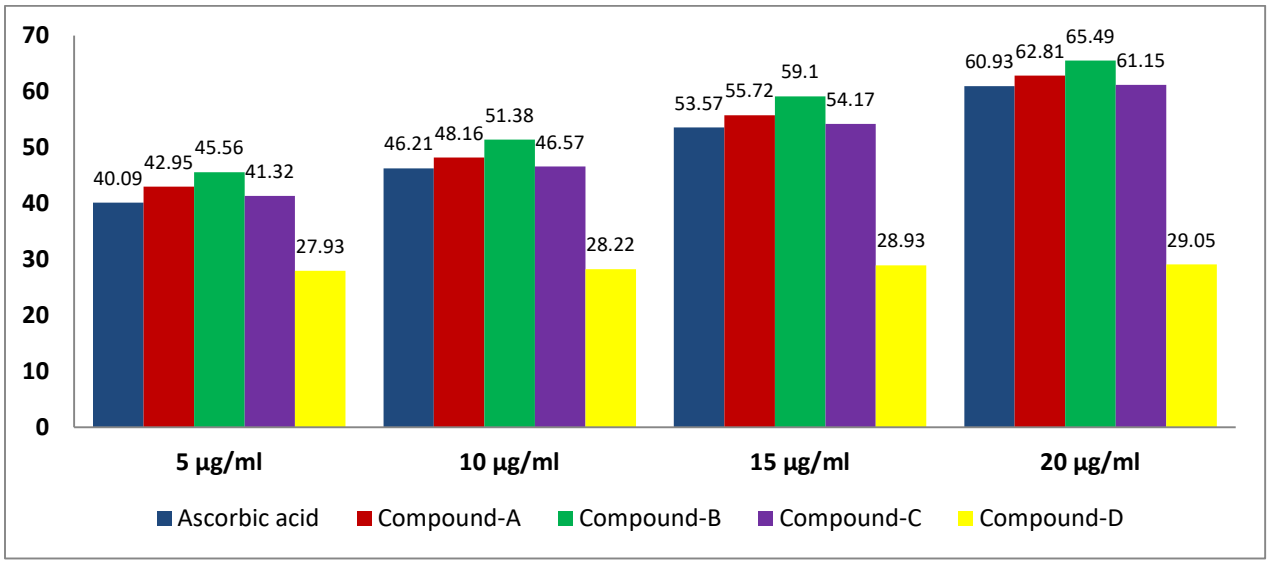


Figure 2: Nitric oxide radical activity

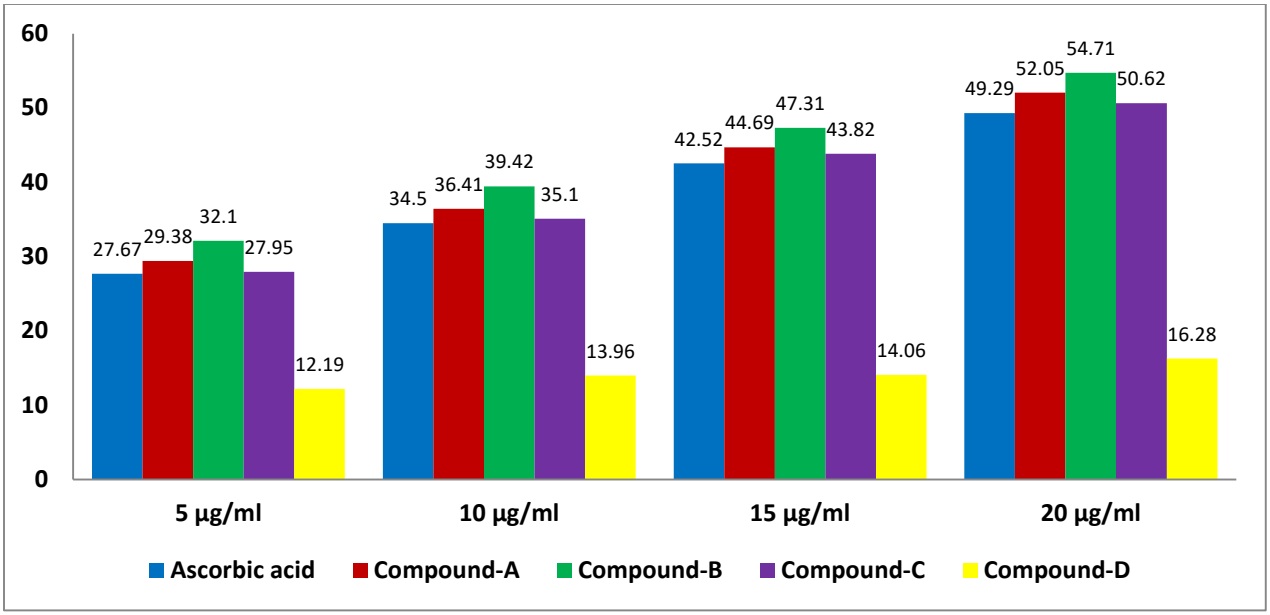


Figure 3: Hydroxyl radical scavenging activity

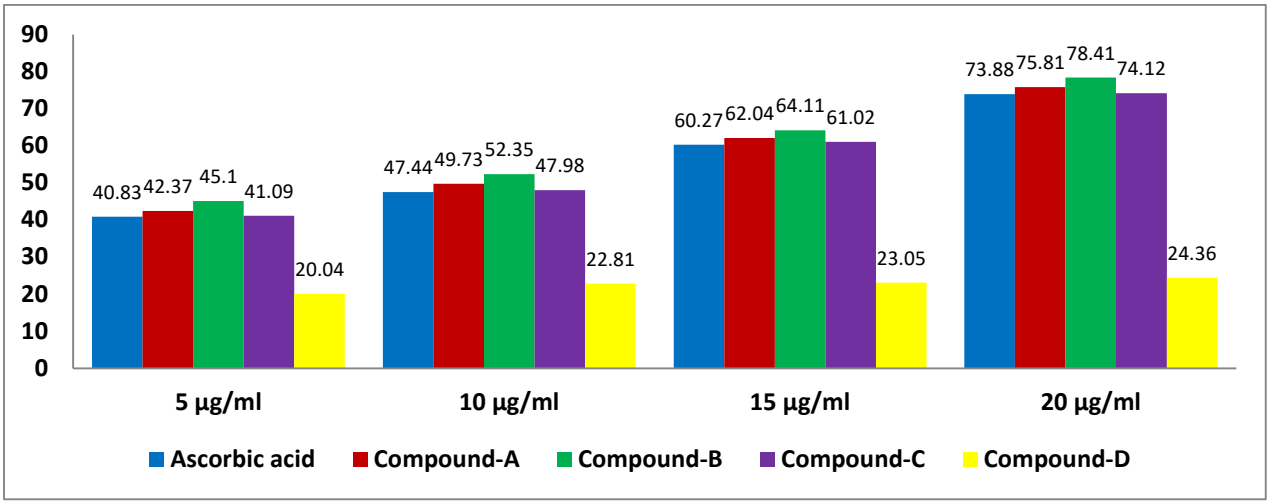


Figure 4: Hydrogen peroxide radical scavenging activity

Reactive oxygen species (ROS), which cause oxidative reactions, can have harmful consequences on a biological system. Antioxidants operate as a defence mechanism against these effects. Numerous human ailments, including cancer, diabetes, and neurological diseases, are primarily brought on by oxidative stress. Antioxidants from plant origin are useful for managing those diseases because they have the capability to scavenge free radicals, which can stop their detrimental effects and halt the spread of oxidation.

CONCLUSION

Many naturally occurring chemical compounds have been extracted from plants

and are used as sources when developing new drugs. The results of the investigation lead to the conclusion that compound A, B, and C exhibit higher scavenging activities than other compounds and vitamin C. According to in-vitro tests, isolated active components may be a substantial source of antioxidants that can help stop the progression of different oxidative stressors.

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