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Research Article

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Phytochemical Investigation, Antioxidant Profiling and GC-MS Analysis of *Cajanus scarabaeoides* Seed Extracts

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Abstract

Background: *Cajanus scarabaeoides* (family: Fabaceae) is a wild species of the genus *Cajanus* and it is traditionally used in various healthcare practices.

Objective: This study aims to evaluate the seed extracts of *C. scarabaeoides* for phytochemical content, *in-vitro* antioxidant activity, and *in-silico* anti-inflammatory and anti-cancer potential.

Methods: The phytochemical content of *C. scarabaeoides* seeds was determined by measuring total phenolic contents (TPC), tannin contents (TTC), flavonoid contents (TFC), and the presence of probable compounds established by Gas chromatography-mass spectrometry (GC-MS) analysis. The antioxidant activity was analyzed by total antioxidant capacity (TAC), ferric ion reducing antioxidant power (FRAP), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assays. The molecular docking studies were carried out using AutoDock Vina.

Results: All investigated extracts of *C. scarabaeoides* showed higher TFC, followed by TTC and TPC ($p \leq 0.05$). The antioxidant potential of methanol extract was found significant in TAC, DPPH, and FRAP assays, it showed compelling proof in contrast to the other extracts with a low half-maximal inhibitory concentration (IC_{50}) for TAC (68.83 ± 2.808 µg/ml) and FRAP (53.32 ± 2.82 µg/ml). The GC-MS analysis of methanol extract identified fourteen bioactive compounds and three of them were subjected to molecular docking analysis. The molecular docking results predicted potent anti-inflammatory and anti-cancer potential.

Conclusions: The results have proven the potential antioxidant activity and a possible strong anti-inflammatory and anti-cancer activity for the methanolic seed extracts of *C. scarabaeoides*. Hence, the isolation and chemical structure identification of bioactive compounds of the methanol seed extract is being carried out.

Keywords

Cajanus scarabaeoides, Bioactive compounds, Antioxidants, GC-MS analysis, Half-maximal inhibitory concentration (IC_{50}), Molecular docking

Abbreviations

TPC: Total phenolic contents; **TTC:** Total tannins contents; **TFC:** Total flavonoid contents; **FRAP**: Ferric reducing antioxidant power assay; **DPPH:** 2, 2-Diphenyl-1-picrylhydrazyl; **TAC:** Total antioxidant capacity; **BHT:** Butylated hydroxytoluene; **GAEq:** Gallic acid equivalents; **TAEq:** Tannic acid equivalents; **QEq:** Quercetin equivalent; **ANOVA:** Analysis of variance; **DWM**: Dried

weight of the material; **WHO:** World Health Organization; **GC-MS:** Gas chromatography-mass spectrometry; **COX:** Cyclooxygenase; **PLA:** Phospholipase A; **TNF-α:** Tumor necrosis factor; **IL:** Interleukin

Introduction

Plants are enormous natural sources of bioactive compounds, which are used by humans for ages majorly as nutrients and medicines [1, 2]. The most significant of these bioactive compounds in nature include alkaloids (18%), phenolics (45%), tannins, flavonoids, steroids, and terpenoids (27%) [3, 4]. Medicinal plants and traditional medicine seem to offer an adapted solution to Indian populations [5]. People have been using medicinal plants to cure a wide range of diseases and ailments since the dawn of civilization [6]. In fact, according to the World Health Organization (WHO), 60% of the world's population, and 80% of developing countries mainly India, use medicinal plants for some aspects of primary health care [7]. Studies on already-existing bioactive natural compounds have not yet reached the necessary level, given the enormous plant diversity in the world [3]. Additionally, due to the large amount of scientific data supporting their use, medicinal plants are growing in popularity as an alternative to synthetic pharmaceuticals [8, 9].

C. scarabaeoides is a flowering herb belonging to the Fabaceae family and the genus *Cajanus*. It is locally known as "Rantur or Konda Kandi" and is widely distributed all over India, China, Bangladesh, Mauritius, Malaysia, and Madagascar [10, 11]. *C. scarabaeoides* has high drought and salinity tolerance and it is sexually compatible with cultivated pigeon pea (*Cajanus cajan*) [10]. There are several traditional uses for this plant, including the treatment of gonorrhea, smallpox, inflammatory diseases, dysentery, rinderpest, cholera, and several other conditions [12, 13]. *C. scarabaeoides* has strong antibacterial properties [14]. Three terpenes such as β-amyrin, caryophyllene-4, 5-oxide, α-amyrin and were isolated from the petroleum ether extract of the whole plant [14]. The roots of the *C. scarabaeoides* have a novel compound called epoxyflavanone named 5,7-Dihydroxy-2',4'-dimethoxy-8-(2,3-epoxy-3-methylbutyl) flavanone and two flavone glycosides were reported recently from a whole plant extract of *C. scarabaeoides* (*Cajanus* genus), namely isoorientin and Isoorientin 3' -O-methyl ether [15, 16].

C. scarabaeoides has insect resistance mechanisms like antibiosis, antixenosis, and oviposition in a non-preferential manner by its exudates and trichomes towards pathogens like phytophthora blight (*Phytophthora capsici*), pod borer (*Helicoverpa armigera*), pod wasp (*Tanaostigmodes cajaninae*), and pod fly (*Melanagromyza obtusa*) [17, 18]. In the modern world, pesticides, industrial wastes, radiation, and food additives are exposed to all creatures, including humans. This exposure causes the emergence of new diseases or increased incidence of existing ailments. As a result, incorporating natural products into the diet offering protection against mutagenesis, microbial infections, and oxidative stress lowers the risk of contracting various diseases and disorders [18, 19]. Antioxidant bioactive compounds are the focus of current research, owing to their free radicle scavenging potential, and finding novel drugs for various ailments is, therefore, crucial [20]. In this study, the phytochemical content of *C. scarabaeoides* seeds was investigated via qualitative, quantitative, and gas chromatography-mass spectrometry (GC-MS) methods and explored the possibility of its *in vitro* antioxidant properties. The *in-silico* docking studies were done to evaluate the anti-inflammatory and anti-cancer potential of the methanol seed extract constituent compounds.

Materials and Methods

Materials

Gallic acid, tannic acid, 2, 2-Diphenyl-1- Picrylhydrazyl (DPPH), ascorbic acid, butylated hydroxytoluene (BHT), Folin–Ciocalteu reagent (FC), and ferrous sulfate ($FeSO_4$) were purchased from Himedia Laboratories Pvt. Ltd, Mumbai, India. All the other chemicals used in this study were of analytical grade and obtained from commercial sources. UV/Visible spectrophotometer (Shimadzu, UV-1800, Japan) was used for reading the absorbance values.

Plant collection and identification

The mature seeds of *C. scarabaeoides* (wild species) about 3 kgs were collected from the foothill regions of Tekkali (18.6167°N 83.2333°E), Srikakulam, Andhra Pradesh, India. The plant was authenticated by the department of botany, Andhra University, Visakhapatnam, Andhra Pradesh, India. Healthy seeds were selected, and phytochemicals were extracted using four analytical grade solvents that include hexane, chloroform, ethyl acetate, methanol, and the aqueous extract with distilled water. These extracts were used for qualitative identification and quantitative estimation of phytochemicals, GC-MS analysis, and evaluation of their antioxidant properties.

Extraction with solvent

Seeds of *C. scarabaeoides* were crushed, dried in a hot air oven at 50 °C for 8 hours, and then pulverized with a laboratory-grade mechanical blender. One kilogram of crushed powder was macerated in 1000 ml of most polar analytical grade methanol (0.762; polarity value) as a menstruum for 72 hours, with occasional stirring every 2 hours [21]. After 72 hours solvent was double filtrated using cheesecloth and then with Whatman filter paper Number 1.0. The collected extract was distilled, and the dry weight of the material (DWM) was 28.0 g (2.80%).

Later, phytochemicals were separated via the polarity gradient extraction method, collected DWM was dissolved in 500 ml of distilled water in a separating funnel, and it was mixed with 500 ml of chloroform, shaken vigorously, and kept standstill until both immiscible layers appear distinctly. The chloroform fraction was collected, and it yields 0.20 % of DWM followed by ethyl acetate with a yield of 4.00%, and finally with n-hexane and which yields 2.0 % of DWM shown in the figure 1. This whole process was repeated two more times and the separated extracts were stored at -20 °C until they were used for analysis [22].

Qualitative methods for evaluation of phytochemicals

Hexane, chloroform, ethyl acetate, methanol, and aqueous extracts of *C. scarabaeoides* were analyzed qualitatively for the

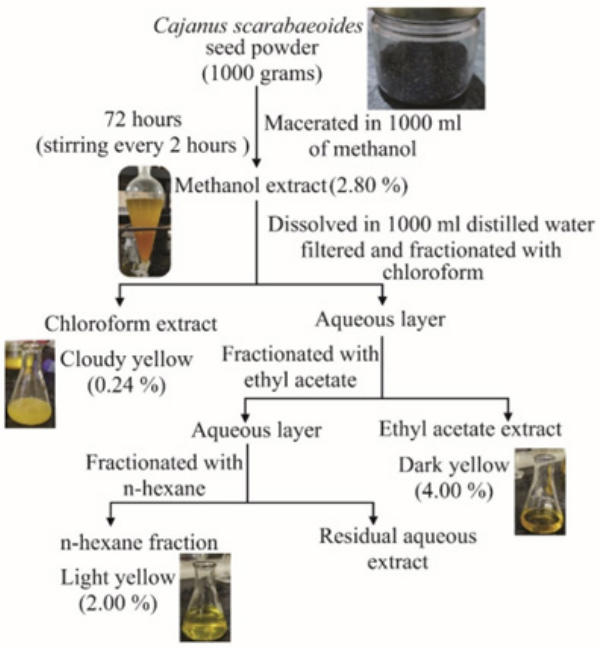

Figure 1: Schematic diagram of extraction and fractionation of *C. scarabaeoides* seed powder by using various organic solvents like hexane, chloroform, ethyl acetate, and methanol.

presence of various phytochemicals, i.e., alkaloids, tannins, flavonoids, phenols, terpenoids, cardiac glycosides, saponins, quinones, coumarins, and triterpenoids [12, 23-25]. The presence of alkaloids was determined by the Dragendorff test, tannins by Braymer's test, phenols by the ferric chloride test, flavonoids by the lead acetate test, terpenoids by the Salkowski test, cardiac glycosides by Keller-Killani test, quinones by alcoholic KOH test, coumarins by NaOH test and saponins were identified based on their foam-forming abilities.

Quantitative methods for evaluation of phytochemicals

Total phenolic content

The total phenolic content (TPC) was estimated by using the FC method [26]. This method involved mixing 0.2 mL of hexane, chloroform, ethyl acetate, methanol, and aqueous extract of *C. scarabaeoides* (10, 30, 60, 90, and 120 mg/ml) to 1 mL FC reagent and 0.8 mL 7.5 % Na_2CO_3, followed by incubation for 30 minutes. Absorption was measured at 765 nm in a UV/Vis spectrophotometer, and the results were expressed as gallic acid equivalents (GAEq) in mg/gm DWM. The gallic acid calibration curve's (20-200 µg/ml) regression equation was $y = 0.0094x + 0.116$ with an R^2 of 0.964.

Total tannin content

The TTC was measured by using the FC method [27, 28]. In this method, 1.0 mL of hexane, chloroform, ethyl acetate, methanol, and aqueous extract of *C. scarabaeoides* (10, 30, 60, 90, and 120 mg/ml) was added to 0.5 mL of FC reagent, 6.5 mL of water, and 1.5 mL of 20% Na_2CO_3 and incubated for 60 minutes. Optical density was measured at 725 nm in a UV/Vis spectrophotometer, and the results were reported as tannic acid equivalents (TAEq) in mg/gm of DWM. The regression equation for the tannic acid calibration curve (0.1-0.5 mg/ml) was $y = 1.13x + 0.007$, giving an R^2 of 0.9998.

Total flavonoid content

The total flavonoid content (TFC) was determined by using a colorimetric method with minor adjustments [29-31]. In this method, 1.0 mL of hexane, chloroform, ethyl acetate, methanol, and aqueous extract (10, 30, 60, 90, and 120 mg/ml) of *C. scarabaeoides* was mixed with 75 µL of 5% $NaNO_2$ and 1.0 mL of water. The content was incubated for 5 minutes with 75 µL of 10% $AlCl_3.H_2O$, and then it was mixed with 0.5 mL of 1M NaOH. Finally, the entire content was incubated for another 15 minutes, and absorption was measured by using a UV/Vis spectrophotometer at 510 nm [30]. The results were represented in mg of quercetin equivalents (QEq) per gm DWM. The regression equation for the quercetin calibration curve (0.1-0.5 mg/ml) was $y = 0.2292x + 0.2441$, with an R^2 of 0.9989.

Evaluation of *in vitro* non-enzymatic antioxidants

Total antioxidant capacity assay

The total antioxidant capacity of the seed extracts of *C. scarabaeoides* was estimated by mixing 0.2 mL of hexane, chloroform, ethyl acetate, methanol, and aqueous extract (100, 200, 300, 400 500 µg/ml) with 1.8 mL water and 2.0 mL of phosphomolybdate reagent and it was incubated for 90 min at 95 °C. The absorbance was read at 695 nm by using a UV/Vis spectrophotometer and results were expressed in ascorbic acid equivalents (AAEq mg/ml), using ascorbic acid and BHT as standard antioxidants [32-34]. The regression equation for the ascorbic acid calibration curve (100-500 µg/ml) was $y = 0.0006x - 0.003$, with an R^2 of 0.981.

Ferric ion reducing antioxidant Power (FRAP) Assay

The FRAP assay was performed by adding 3.0 mL of FRAP reagent to 100 µL of hexane, chloroform, ethyl acetate, methanol, and aqueous extract (100, 200, 300, 400 500 µg/ml) of *C. scarabaeoides* and it was vortexed. The absorbance was measured at 593 nm, using a UV/Vis spectrophotometer, and the results were represented in terms of FRAP Units (FU), using ascorbic acid and BHT as standard antioxidants [35, 36]. The regression equation for the ascorbic acid calibration curve (10-100 µM) was $y = 0.0118x + 0.0394$, with an R^2 of 0.9996.

DPPH radical scavenging activity

The DPPH radical scavenging activity of *C. scarabaeoides* hexane, chloroform, ethyl acetate, methanol, and aqueous extract (100, 200, 300, 400 500 µg/ml) was determined by mixing 3.0 mL of seed extract with 1.0 mL of 0.1 mM of DPPH (2,2-diphenyl-1-picrylhydrazyl) in methanol. It was incubated for 30 minutes at 37°C, and absorbance was measured at 517 nm using a UV/Vis spectrophotometer [37, 38]. The results were represented in the percentage of inhibition (I) by taking ascorbic acid and BHT as standards. The percentage of inhibition was calculated by the formula:

$$I = \left(\frac{\text{Absorbance of Control-Absorbance of Test}}{\text{Absorbance of Control}} \right) \times 100 \cdot$$

GC-MS quantification

Sample preparation

1 ml of methanol solvent was used to dissolve 100 µg (DWM) of methanolic plant extract. This dissolved solution was vigorously agitated and stirred for 10 seconds with a vortex stirrer, and then filtered through a 0.2-micron membrane filter. GC-MS analyses were performed using the clear extract.

Chromatographic separation

The GC-MS analysis of methanol extract *C. scarabaeoides* was carried out with GC-MS (Shimadzu QP 2010 Ultra GC-MS) equipment, Agilent Technologies. A DB-5MS, 5% phenyl methyl siloxane column with an inner diameter (ID) of 30 m and a film thickness of 0.25 m was treated with a volume injection of 1 µL. An auto-sampler with a typical injection mode was employed (Agilent, Santa Clara, USA). Samples were subsequently eluted using a gradient that ran for 63 minutes at a temperature gradient of 70 °C for 5 min, then raised to 310°C at a rate of 10 min.

Temperatures of 250 °C and 200 °C., respectively, were chosen for the injector and ion source. The carrier gas was helium, flowing at a rate of 1.0 mL/min. After a 4.5-minute solvent delay, mass spectra in the range of 50 to 700 m/z were obtained utilizing a full scan and monitoring mode. A comparison was made between the spectrum of the unknown component and the spectra of the known components stored in the NIST, WELLY, and TOX libraries. The name of each phytochemical in the plant extract was identified, along with its molecular weight and structure.

Molecular docking studies

The molecular docking studies were carried out for allo-aromadendrene, 2,4-Di-tert-butylphenol, and 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester identified in the methanolic seed extract of *C. scarabaeoides*. The X-ray crystal structures were obtained as .pdb files from Protein Databank (PDB) Website. The target proteins obtained in pdbqt format from ncbs were simplified by deleting the co-crystallized ligands and water molecules using BIOVIA Discovery Studio Visualizer v.4.5 (Accelrys). The energy minimization was done, and the root mean square value is ensured to be less than 0.001 Kcal/mol. The prepared ligands and target proteins were subjected to protein-ligand docking using the Autodock Vina software. The ligand-receptor affinity in Autodock Vina is estimated by an empirical scoring function, which is based on the X-score function [39, 40]. The protein-ligand interactions were visualized using the discovery studio visualizer.

The anti-inflammatory effects of the test ligands were determined by considering the binding energies between the ligands and the target proteins involved in the inflammatory process, which include cyclooxygenase-2 (COX-2), secretory phospholipase A_2 ($sPLA_2$), tumor necrosis factor-α (TNF- α) interleukin-6 (IL-6), and interleukin-1β (IL-1β). The anti-cancer activity was assessed by determining the binding energy between the ligands and the target proteins involved in the apoptosis process, particularly the caspase-3, caspase-9, p53, and Bax proteins.

Statistical analysis

All the recorded data were analyzed using Microsoft Excel 2022®. All the experiments were carried out in triplicate (N = 3) and the results are expressed as mean ± SE (Standard error mean). A one-way analysis of variance (ANOVA), followed by the *post-hoc* test Tukey's HSD or independent *t*-test was used where appropriate. The statistical method applied in each analysis was described in each Figure. Results were considered to be significant at $p \leq 0.05$.

Results

Table 1 summarizes the existence of phytochemical components which were qualitatively examined in seed extracts of the *C. scarabaeoides* plant using hexane, chloroform, ethyl acetate, methanol, and water. The qualitative phytochemical analysis showed that all of the mentioned extracts contained coumarins, cardiac glycosides, tannins, phenols, and flavonoids. Terpenoids and triterpenoids are present in ethyl acetate, chloroform, and methanol extracts, whereas quinones are present in all extracts except hexane. Alkaloids and saponins, however, were not identified in any of the extracts that were studied.

Figure 2 shows the percent yield, and quantitative yield of TPC, TTC, and TFC. The characterization of the phytochemicals has the potential to predict the pharmacological activity of a plant [41]. The TPC with an F-statistic is [$F(4,10) = 197132.18$, $p \leq 0.05$] of hexane, chloroform, ethyl acetate,

Table 1: Phytochemical analysis of *C. scarabaeoides* seed extracts.

S. No.	Phytochemical constituents	Solvent extracts of *Cajanus scarabaeoides* seeds				
		Hexane	Ethyl Acetate	Chloroform	Methanol	Aqueous
1.	Alkaloids	-	-	-	-	-
2.	Tannins	+	+	+	+	+
3.	Flavonoids	+	+	+	+	+
4.	Phenols	+	+	+	+	+
5.	Terpenoids	-	+	+	+	-
6.	Cardiac Glycosides	+	+	+	+	+
7.	Saponins	-	-	-	-	-
9.	Quinones	-	+	+	+	+
10.	Coumarins	+	+	+	+	+
11.	Triterpenoids	-	+	+	+	-

*+ sign = present; - sign, = absent.

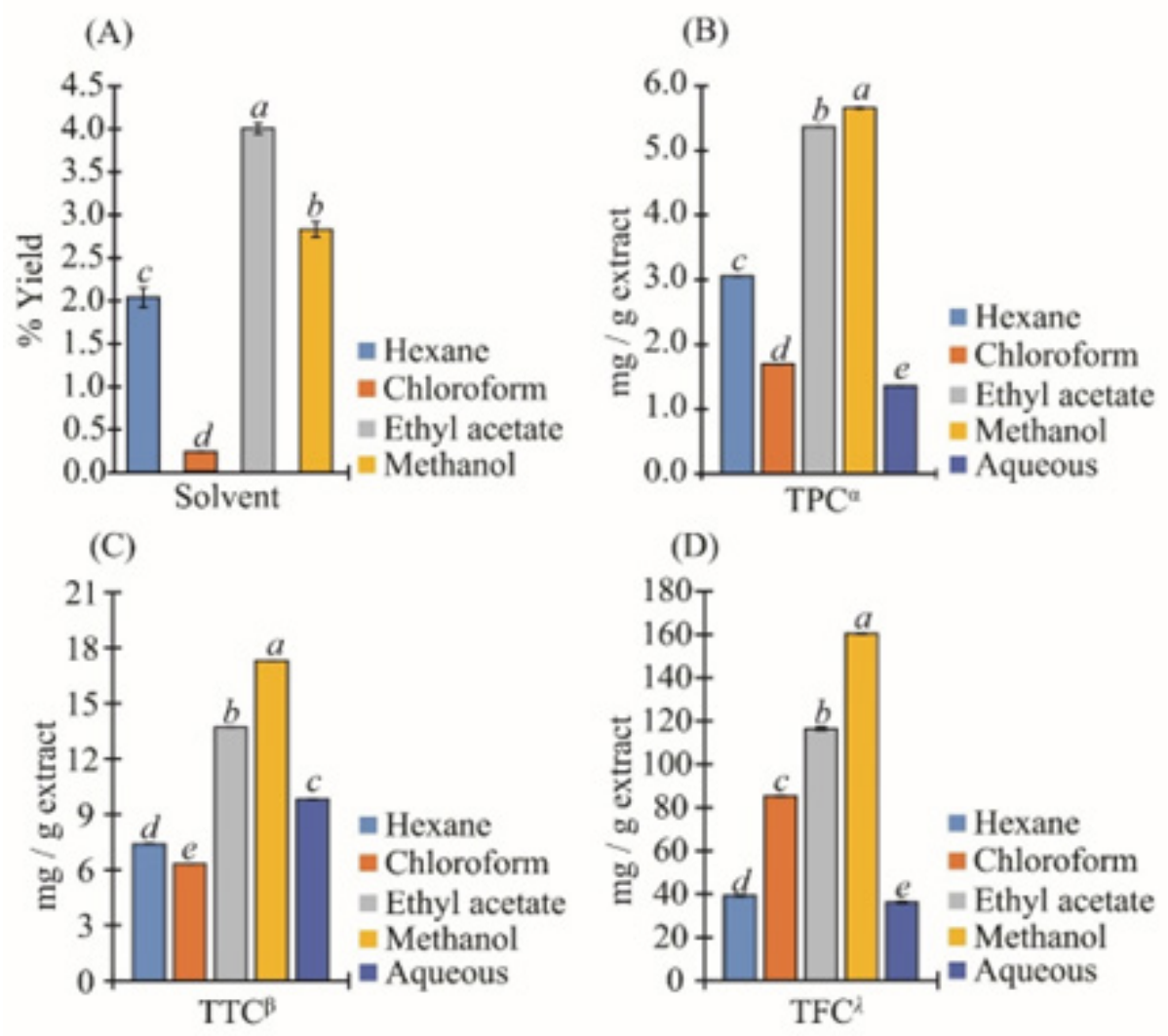

Figure 2: Quantitative phytochemical analysis of different solvent seed extracts of *C. scarabaeoides*. (A) Percentage of yield, (B) Total phenolics content, (C) Total tannins content, (D) Total flavonoid content. Values represented are the mean ± SE of three replicates ($N = 3$). Mean comparisons across the groups compared by the Tukey test *post-hoc* test and values labeled with different letters are significantly different at $p \leq 0.05$. α (mg GAEq/g extract), β (mg TAEq/g extract), λ (QEq/g extract).

methanol, and aqueous extracts are 3.06 ± 0.001, 1.7 ± 0.003, 5.37 ± 0.004, 5.66 ± 0.008, and 1.36 ± 0.003 (mg GAEq /g DWM), respectively. The Tukey *post-hoc* test of TPC samples has revealed that the mean concentrations of all extracts are significantly different from each other ($p \leq 0.05$).

The TTC with an F-statistic [$F(4,10) = 97343.69$, $p \leq 0.05$] of hexane, chloroform, ethyl acetate, methanol, and aqueous extracts are 7.43 ± 0.019, 6.02 ± 0.127, 13.7 ± 0.017, 17.3 ± 0.004, and 9.82 ± 0.017 (mg TAEq /g DWM), respectively. The Tukey *post-hoc* test of TTC samples showed a significant difference in mean concentrations of all extracts to each other ($p \leq 0.05$). The TFC with an F-statistic [$F(4,10) = 14033.92$, $p \leq 0.05$] of hexane, chloroform, ethyl acetate, methanol, and aqueous extracts are 39.33 ± 0.385, 85.25 ± 0.155, 116.42 ± 0.779, 160.47 ± 0.238 and 36.22 ± 0.394 (mg QEq /g DWM), respectively. The Tukey *post-hoc* test of TFC samples has displayed a significant difference in mean concentrations of all extracts to each other ($p \leq 0.05$).

Antioxidant activity assay of hexane, chloroform, ethyl acetate, methanol, and aqueous crude extracts was performed by total antioxidant capacity (TAC) assay, the FRAP assay, and the DPPH radical scavenging activity assay using ascorbic acid and BHT as standard drugs. The half-maximal inhibitory concentration (IC_{50}) value for each extract of *C. scarabaeoides* was calculated using linear regression of the ascorbic acid equivalents (AsEq) in µg/ml (TAC), percentage inhibition (DPPH), and FRAP units (FRAP) versus concentration on the dosage-response curve [42].

As shown in figure 3a and figure 3b, the TAC of *C. scarabaeoides* hexane, chloroform, ethyl acetate, methanol, and aqueous extracts, the F statistic is [$F(6,14) = 17525.29$, $p \leq 0.05$], and the maximum antioxidant activity was reported for methanol extract cumulatively followed by ethyl acetate, hexane, chloroform, and aqueous extracts, which was 158.31 ± 0.472 µg/mL, 148.39 ± 0.656 µg/mL, 75.33 ± 0.872 µg/mL, 62.67 ± 0.705 µg/mL, and 59.78 ± 0.667 µg/mL, respectively compared to ascorbic acid and BHT which was 295.63 ± 1.148 and 330 ± 1.122 (AscEq µg/ml). Mean concentrations of all extracts are significantly different from each other ($p \leq 0.05$), except the mean pair of chloroform- aqueous ($Q = 3.4149$, $p = 0.2616$) ($p > 0.05$).

The IC_{50} values of TAC with an F statistic of [$F(6,14)$ = 204.33, $p \leq 0.05$] hexane (y = 0.1067x + 41.667, R^2 = 0.841), chloroform (y = 0.1222x + 26.002, R^2 = 0.995), ethyl acetate (y = 0.5239x - 8.782, R^2 = 0.956), methanol (y = 0.4686x + 17.747, R^2 = 0.962), and aqueous (y = 0.1133x + 25.779, R^2 = 0.914) extracts, respectively, were 78.1 ± 10.861 µg/mL, 196.38 ± 1.654 µg/mL, 112.2 ± 2.425 µg/mL, 68.83 ± 2.808 µg/mL, and 213.78 ± 4.10 µg/mL. The IC_{50} for mean concentrations of all extracts are significantly different from each other ($p \leq 0.05$), except the mean pairs of chloroform-aqueous (Q = 3.72, p = 0.1886), ethyl acetate-BHT (Q = 1.5401, p = 0.9214) and methanol-ascorbic acid (Q = 1.294, p = 0.9639) ($p > 0.05$).

As shown in figure 3c and figure 3d, the FRAP of *C. scarabaeoides* hexane, chloroform, ethyl acetate, methanol, and aqueous extracts, the F statistic is [$F(6,14)$ = 7437.524, $p \leq 0.05$], and the maximum FRAP activity was reported for methanol extract cumulatively followed by ethyl acetate, chloroform, hexane, and aqueous extracts, which was 92.98 ± 0.976 FU, 69.28 ± 0.043 FU, 41.09 ± 0.062 FU, 36.52 ± 0.049 FU, and 25.25 ± 0.088 FU, respectively compared to ascorbic acid and BHT which was 61.56 ± 0.051 FU and 114.47 ± 0.02 FU. The mean concentrations of all extracts are significantly different from each other ($p \leq 0.05$).

The IC_{50} values of FRAP [$F(6,14)$ = 26805.88, $p \leq 0.05$] of hexane (y = 0.1015x + 6.062, R^2 = 0.986), chloroform (y = 0.1335x + 1.056, R^2 = 0.997), ethyl acetate (y = 0.1531x + 23.348, R^2 = 0.983), methanol (y = 0.1742x + 40.712, R^2 = 0.895), and aqueous (y = 0.0676x + 4.971, R^2 = 0.796) extracts, respectively, were 432.89 ± 0.38 µg/mL, 366.62 ± 0.38 µg/mL, 174.08 ± 0.55 µg/mL, 53.32 ± 2.82 µg/mL, and 666.11 ± 0.33 µg/mL. The IC_{50} for mean concentrations of all extracts are significantly different from each other ($p \leq 0.05$).

The results of the DPPH radical scavenging test of hexane, chloroform, ethyl acetate, methanol, and aqueous extracts of *C. scarabaeoides* displayed variable levels of scavenging power. As shown in figure 3e and figure 3f, cumulative results of this experiment with an F statistic [$F(6,14)$ = 1045.174, $p \leq 0.05$], the methanolic extract had a stronger scavenging action than other extracts, with a value of 68.96 ± 0.098%, followed by chloroform, hexane, aqueous extract 67.98 ± 0.038%, 67.79 ± 0.082%, 66.96 ± 0.116%, respectively, and finally, ethyl acetate with the value of 64.04 ± 0.135% compared to the standards; ascorbic acid with the value of 75.65 ± 0.057% and BHT with the value of 69.36 ± 0.176%.

Mean concentrations of all extracts are significantly different from each other ($p \leq 0.05$), except the mean pairs of hexane-chloroform (Q = 1.7392, p = 0.8712) and methanol-BHT (Q = 3.6614, p = 0.2012) ($p > 0.05$). Hence, significant differences between extraction solvents were also found for all samples. The IC_{50} values of DPPH [$F(6,14)$ = 1392.026, $p \leq 0.05$] of hexane (y = 0.1197x + 31.89, R^2 = 0.975), chloroform (y = 0.1098x + 35.035, R^2 = 0.934), ethyl acetate (y = 0.0972x + 34.869, R^2 = 0.980), methanol (y = 0.1271x + 30.831, R^2 = 0.951), and aqueous (y = 0.1272x + 28.793, R^2 = 0.945), extracts, respectively, were 151.30 ± 0.853 µg/mL, 136.29 ± 1.454 µg/mL, 155.67 ± 1.751 µg/mL, 150.82 ± 0.424 µg/mL, and 166.72 ± 0.798 µg/mL. The IC_{50} mean concentrations of all extract pairs are significantly different from each other ($p \leq 0.05$), except hexane-ethyl acetate (Q = 4.2543, p = 0.1015), hexane-methanol (Q = 0.4423, p = 0.9999) and ethyl acetate-methanol (Q = 4.6966, p = 0.05902) ($p > 0.05$).

A linear correlation of TPC, TTC, and TFC with an antioxidant potential (TAC, DPPH, and FRAP) of plant extracts was established. From the obtained results, figure 4 shows a correlation between the TPC of various extracts with their respective antioxidant assays. TPC of hexane [$r(3)$= 0.993, p = 0.001], chloroform [$r(3)$= 0.984, p = 0.002], ethyl acetate [$r(3)$= 0.990, p = 0.001] and aqueous [$r(3)$= 0.993, p = 0.001] extracts have a strong correlation ($0.8 \leq r \leq 1.0$) with FRAP activity, whereas methanol [$r(3)$= 0.996, p = 0.0003] extract has a strong correlation with DPPH activity.

Figure 5 shows a correlation between the TTC of various extracts with their respective antioxidant assays. TTC of hexane [$r(3)$= 0.976, p = 0.004], chloroform [$r(3)$= 0.990, p = 0.001], ethyl acetate [$r(3)$= 0.999, p = 0.00004] and aqueous [$r(3)$= 0.986, p = 0.002] extracts have a strong correlation with FRAP activity, whereas methanol [$r(3)$= 0.996, p = 0.0003] extract has a strong correlation with TAC activity.

Figure 6 shows a correlation between the TFC of various extracts with their respective antioxidant assays. TFC of hexane [$r(3)$= 0.948, p = 0.014] and chloroform [$r(3)$= 0.869, p = 0.06] extracts have a strong correlation with DPPH activity, whereas ethyl acetate [$r(3)$= 0.975, p = 0.005], methanol [$r(3)$= 0.970, p = 0.006] and aqueous [$r(3)$= 0.977, p = 0.004] extracts have a strong correlation with TAC activity. Hence, cumulatively phenolic and tannin compounds have a high correlation with FRAP activity.

However, the phenolic compounds of methanol extract showed a high correlation with DPPH activity. Likewise, tannins and flavonoids have shown a high correlation with TAC activity. All the results of Pearson Correlation Analysis are significant at $p \leq 0.05$, except TFC of chloroform extract in correlation with DPPH activity where p = 0.06.

Different therapeutic plant extracts have different biological potentials, which can be determined by evaluating the chemical structure and composition of the extracts. To our knowledge, there isn't any research on GC-MS-based plant metabolic characterization that reveals the existence of different bioactive chemicals in methanolic extracts of seeds of *C. scarabaeoides*. The GC-MS analysis was thus carried out in the planned study. Methanolic extract of the *C. scarabaeoides* seeds was evaluated using GC-MS in the electron impact (EI) detection mode. Each peak was identified as a bioactive chemical by comparing its peak retention time, molecular weight, and molecular formula to those of the recognized compounds suggested by the NIST library (Table 2).

GC-MS chromatogram represented in figure 7 of the methanol extract of *C. scarabaeoides* showed fourteen peaks indicating the presence of fourteen bioactive chemical constituents. The major compounds discovered in *C. scarabaeoides* seeds extract are Naphthalene (CAS 91-20-3), Alloaromadendrene (CAS 25246-27-9), Tetradecamethylcycloheptasiloxane (CAS 107-50-6), Methyl 5-nitro-2H-1,2,3-triazole-4-car-

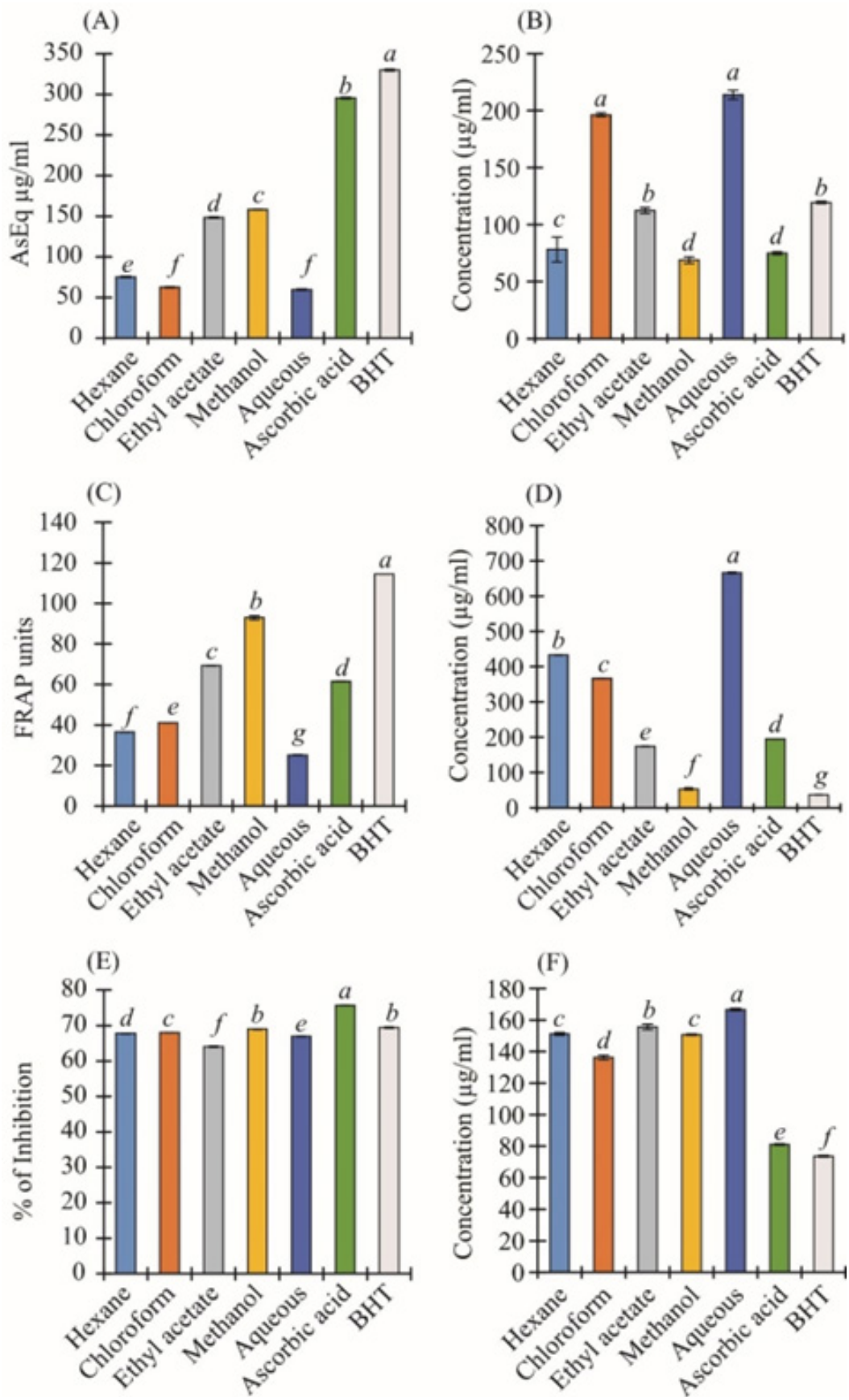

Figure 3: Antioxidant Analysis of different solvent seed extracts of *C. scarabaeoides*. (A) Total antioxidant assay, (B) IC_{50} of total antioxidant assay, (C) FRAP assay, (D) IC_{50} of FRAP assay, (E) DPPH radical scavenging assay, (F) IC_{50} of DPPH radical scavenging assay. Values represented are the mean ± SE of three replicates ($N = 3$). Mean comparisons across the groups compared by the Tukey test *post-hoc* test and values labeled with different letters are significantly different at $p \leq 0.05$.

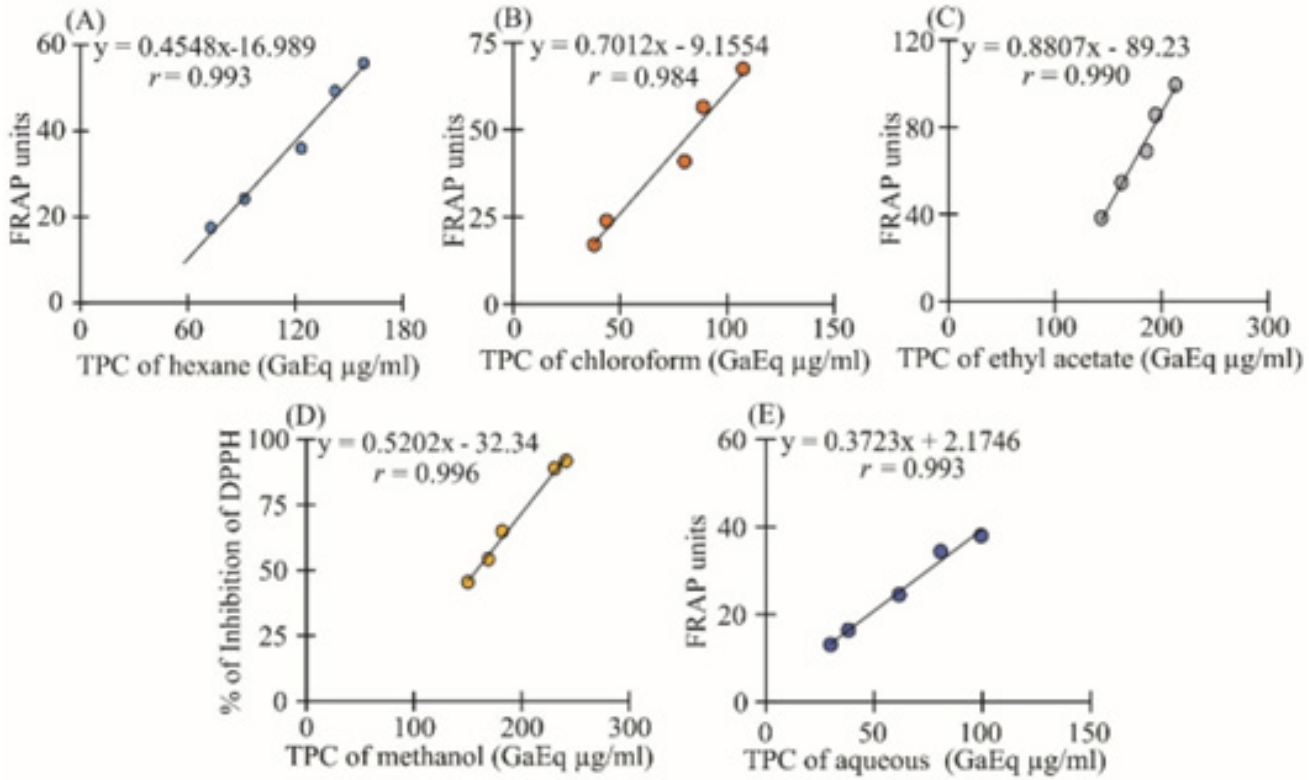

Figure 4: Scatter plots showing Pearson Correlation between (A) TPC of hexane extract and FRAP assay, (B) TPC of chloroform extract and FRAP assay, (C) TPC of ethyl acetate extract and FRAP assay, (D) TPC of methanol extract and DPPH assay, and (E) TPC of aqueous extract and FRAP assay. A two-tailed *t*-test was carried out, and the results are significant over $p \leq 0.05$.

Table 2: Compounds identified from *Cajanus scarabaeoides* Methanolic seed extract by using GC-MS.

Peak No.	R. Time (min)	Peak area%	Molecular formula	Molecular weight	Name
1.	13.865	1.02	$C_{10}H_8$	128	Naphthalene
2.	20.926	0.15	$C_{15}H_{24}$	204	Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene- (Alloaromadendrene)
3.	22.18	0.13	$C_{14}H_{42}O_7Si_7$	518	Cycloheptasiloxane,tetradecamethyl- (Tetradecamethylcycloheptasiloxane)
4.	22.851	0.09	$C_4H_4N_4O_4$	172	Methyl5-nitro-2H-1,2,3-triazole-4-carboxylate
5.	23.29	0.44	$C_{14}H_{22}O$	206	2,4-Di-tert-butylphenol
6.	25.33	4.44	$C_{13}H_{16}$	172	1-Phenyl-1-heptyne
7.	26.554	0.33	$C_{27}H_{48}O_3Si_2$	476	Pregn-5-en-20-one, 3,16-bis[(trimethylsilyl)oxy]-, (3.beta.,16.alpha.)-
8.	28.74	0.10	$C_{15}H_{30}O_2$	242	Tridecanoicacid,12-methyl-, methyl (12-Methyltridecanoic acid methyl ester)
9.	31.19	0.17	$C_{16}H_{32}O_2$	256	Pentadecanoic acid, methyl ester
10.	31.922	0.16	$C_{16}H_{22}O_4$	278	1,2-Benzenedicarboxylicacid,bis(2-methylpropyl)ester (Diisobutyl phthalate)
11.	33.656	26.59	$C_{17}H_{34}O_2$	270	Hexadecanoic acid, methyl ester
12.	35.302	1.31	$C_{18}H_{36}O_2$	284	Hexadecanoicacid, ethyl ester
13.	37.669	61.06	$C_{19}H_{34}O_2$	294	6,9-Octadecadienoicacid, methyl ester
14.	38.596	4.01	$C_{19}H_{38}O_2$	298	Heptadecanoicacid,16-methyl-, methyl ester

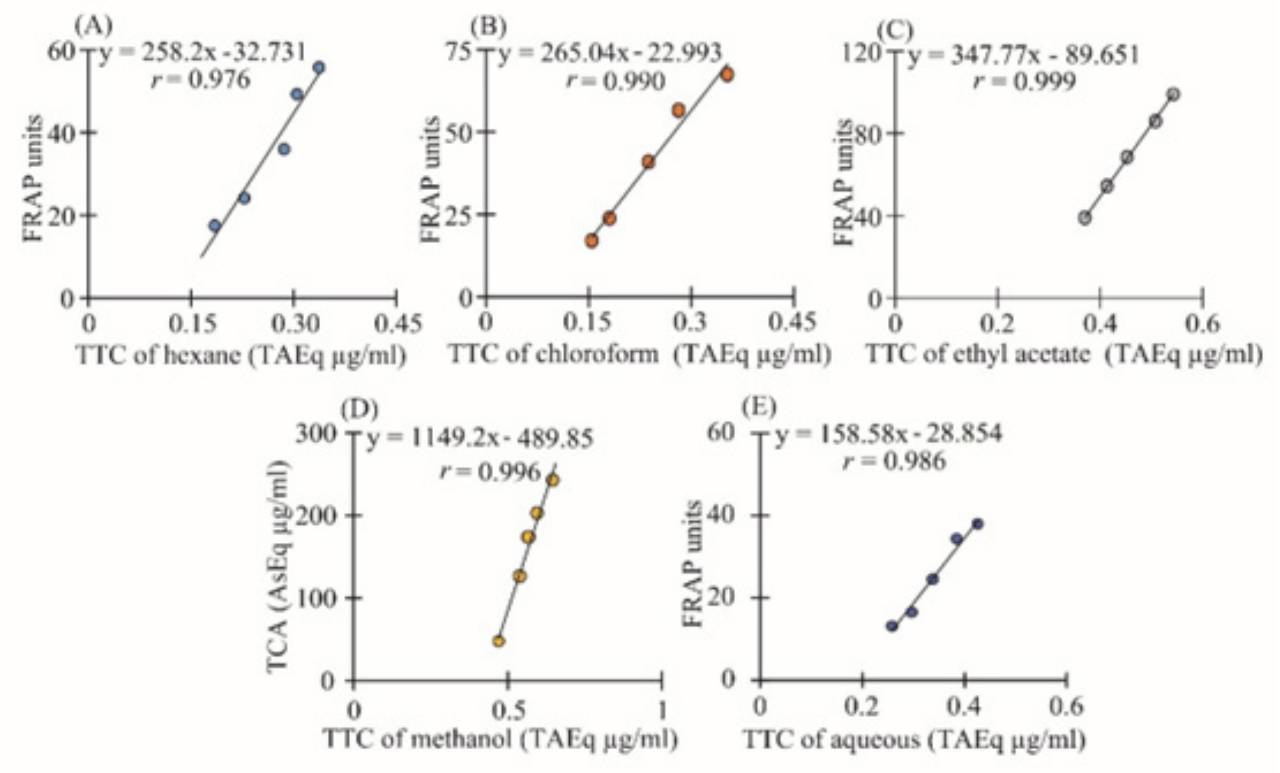

Figure 5: Scatter plots showing Pearson Correlation between (A) TTC of hexane extract and FRAP assay, (B) TTC of chloroform extract and FRAP assay, (C) TTC of ethyl acetate extract and FRAP assay, (D) TTC of methanol extract and TAC assay, and (E) TTC of aqueous extract and FRAP assay. A two-tailed *t*-test was carried out, and the results are significant over $p \leq 0.05$.

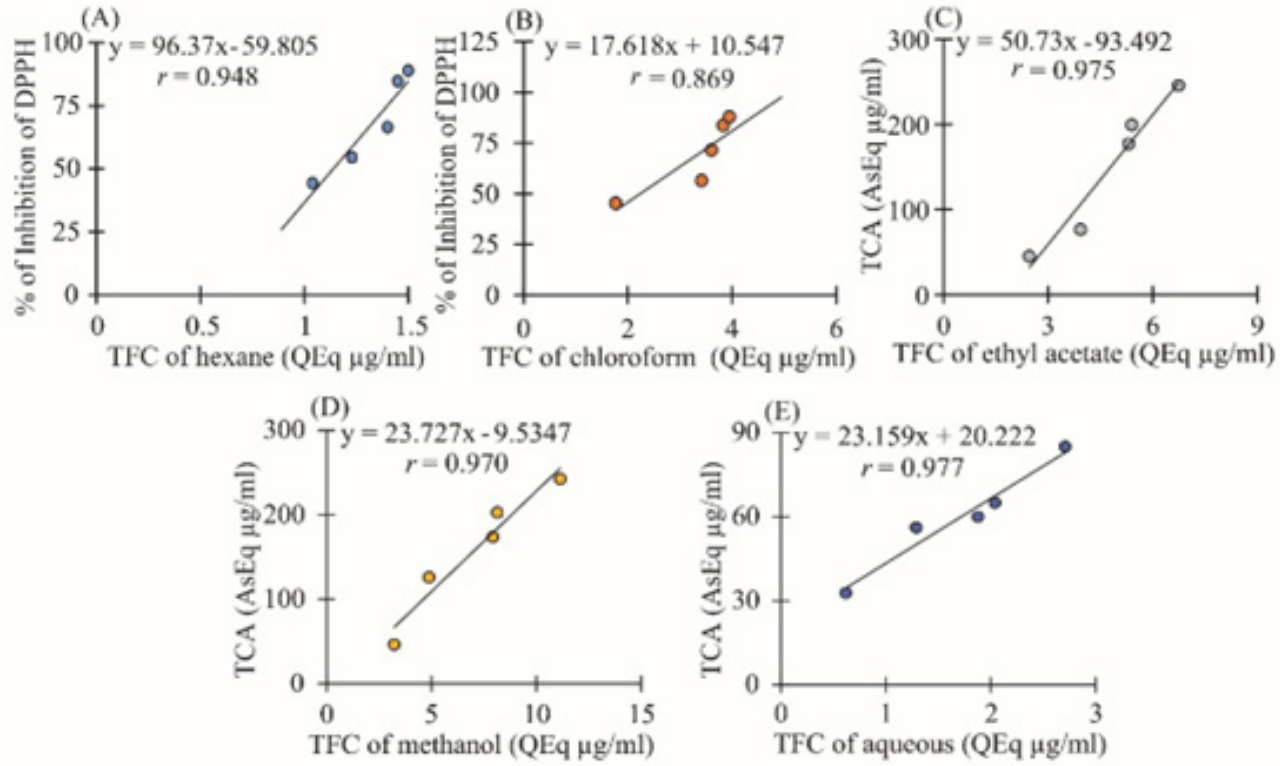

Figure 6: Scatter plots showing Pearson Correlation between (A) TFC of hexane extract and DPPH assay, (B) TFC of chloroform extract and DPPH assay, (C) TFC of ethyl acetate extract and TAC assay, (D) TFC of methanol extract and TAC assay, and (E) TFC of aqueous extract and TAC assay. A two-tailed *t*-test was carried out, and the results are significant over $p \leq 0.05$.

boxylate (CAS 524036-06-4), 2,4-Di-tert-butylphenol (CAS 96-76-4), 1-Phenyl-1-heptyne (CAS 14374-45-9), Pregn-5-en-20-one, 3,16-bis[(trimethylsilyl)oxy]-, (3.beta.,16.alpha.)-, 12-Methyltridecanoic acid methyl ester (CAS 5129-58-8), Pentadecanoic acid, methyl ester (CAS 7132-64-1), 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (CAS 84-69-5), Hexadecanoic acid, methyl ester (CAS 112-39-0), Hexadecanoic acid, ethyl ester (CAS 628-97-7), 6,9-Octadecadienoic acid, methyl ester (CAS 56599-55-4), Heptadecanoic acid, 16-methyl-, methyl ester (CAS 5129-61-3). Among the identified compounds the highest percentage of peak area was observed for 6,9-Octadecadienoicacid, methyl ester, which is about 61.06%.

Molecular docking studies were carried out for three out of fourteen molecules identified by GC-MS analysis of the methanolic seed extract of *C. scarabaeoides*. The molecules studied are allo-aromadendrene, 2,4-Di-tert-butylphenol, and 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester. The binding energies between the ligands and the targets studied are presented in table 3. The results predicted that all the molecules studied might contain good anti-inflammatory and anti-cancer potential. Allo-aromadendrene bound with COX-2 (8.4 kcal/mol) and PLA2 (7.2 kcal/mol) with the highest binding energies, strongly indicating its possibility as an arachidonic acid pathway inhibitor. 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester also strongly bound with COX-2 (8.1 kcal/mol) and PLA_2 (6.6 kcal/mol) suggesting another possible arachidonic acid pathway inhibitor. 2,4-Di-tert-butylphenol showed relatively less binding energy towards COX-2, but better binding energy towards PLA_2. The molecules studied also showed good binding energies with inflammatory mediators involved in other than the arachidonic acid pathway, i.e., IL-6, IL-1β, and TNF-α. All the molecules studied also demonstrated good anti-proliferative potential. 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (5.7 kcal/mol) had the strongest binding energy with proapoptotic executioner protein Bax followed by 2,4-Di-tert-butylphenol (5.5 kcal/mol) and allo-aromadendrene (5.3 kcal/mol). The binding energy for the other proapoptotic protein caspase 3 is greatest for 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester, and allo-aromadendrene strongly inhibited caspase-9 (6.0 kcal/mol) and p53 (5.8 kcal/mol). 2,4-Di-tert-butylphenol and 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester had equal binding energies for p53 (5.4 kcal/mol).

Discussion

C. scarabaeoides belongs to the clade "angiosperms" and angiospermous plants have the unique ability to synthesize a significant number of secondary metabolites or organic compounds with high structural density. These phytochemicals have accumulated in plants, which has led to an understanding of how to produce desired phytoconstituents with the potential to counteract multi-resistant free radicals [43, 44]. It is becoming more important to focus on traditional herbal medicinal plants from effective scientific viewpoints as chronic diseases increasingly become the leading causes of morbidity and mortality worldwide. The majority of chronic diseases, such as cardiovascular disease, diabetes, and other conditions, are brought on by several etiological variables that also cause the afflictions they are linked with [8, 45]. Multiple targets would need to be addressed when treating a complicated chronic condition, and herbal medicines, which are chemically complex combinations with numerous major and minor constituents, are frequently able to address multiple potential targets. Therefore, it is preferable to address several targets at once with a variety of active principles in a balanced and unique way [46-48].

Although there are numerous sophisticated methods for determining phytochemicals, traditional qualitative assays are most widely used for the initial phytochemical screening of plants [49]. From the obtained results of qualitative and quan-

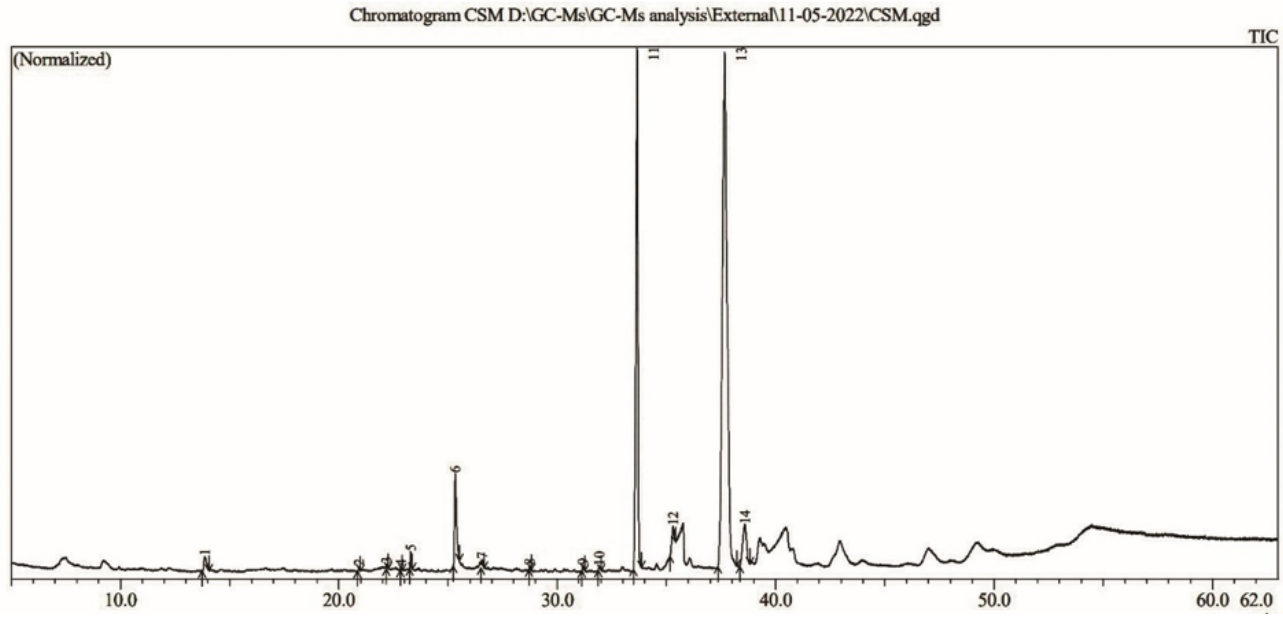

Figure 7: GC-MS Chromatogram of Methanolic seed extract of *Cajanus scarabaeoides*.

titative screening, it is evident that *C. scarabaeoides* is widely rich in flavonoids. A significant difference in TPC, TTC, and TFC between extraction solvents was found for all samples ($p \leq 0.05$). Many plants contain flavonoid compounds, which have antioxidant, antibacterial, and anti-inflammatory properties [50, 51]. By suppressing or inactivating antiproliferative, carcinogens, cell cycle arrest, and triggered apoptosis, flavonoids are helpful against cancer cells [52-54].

They can prevent oxidative stress, which is the root cause of many degenerative diseases, from generating too many free radicals. It was reported that according to several antioxidant studies, flavonoid compounds can have antioxidant activity [55, 56]. Since *C. scarabaeoides* is rich with TFC content, consumption of its seeds can prevent the negative effect of oxidative stress. Recent studies have revealed that several flavonoids and related polyphenols significantly increase the ability of herbal plants to scavenge oxidants [57]. Numerous plant extracts could be tested for antioxidant activity using the FRAP, DPPH, and TAC methods [55, 58]. Several antioxidant methods were used to measure the antioxidant capacity of the whole plant solvent extracts of *C. scarabaeoides* and their association with TPC, TTC, and TFC, however, there is no information on seed extracts of *C. scarabaeoides*, specifically [59, 60].

From the obtained results, crude methanolic extract of *C. scarabaeoides* showed promising *in vitro* antioxidant activity in a concentration-dependent manner. The highest antioxidant activity expresses the lowest IC_{50} [61]. The IC_{50} is inversely proportional to the antioxidant capacity of a compound, and hence the greater the sample's antioxidant activity, the higher the absorbance value. Similarly, the methanolic extract of *C. scarabaeoides* showed the lowest IC_{50} for TAC and FRAP compared to other extracts. Hence, it is significant that the plant extract's scavenging activity is above average, which, taken together, can demonstrate its significant protective effect against diseases in humans with adequate *in-vivo* research.

GC-MS method used for metabolomic studies served as the analytical platform for the current research. It is highly sensitive, and economical, and offers the high resolution required to separate the constituent parts of a complex biological combination. These benefits have led to an increase in its application in plant metabolite profiling [62]. For the observation of functional groups and the identification of numerous bioactive chemicals found in plants, GC-MS has been extensively used [63, 64]. Alkaloids, flavonoids, organic acids, amino acids, and other chemicals found in plant extracts can be accurately identified using the GC-MS approach [65]. GC-MS analysis found several beneficial bioactive compounds that support the idea that methanol extract of *C. scarabaeoides* seeds could be used to make pharmaceutical nutraceuticals that act as strong antioxidants to cure a variety of human diseases and accompanying repercussions.

Among the identified bioactive component, alloaromadendrene is an essential oil that acts protective against oxidative stress, and it has been proven that this molecule delay aging in *Caenorhabditis elegans* [60]. Tetradecamethylcycloheptasiloxane is a major volatile component and nutraceutical of oils obtained from several plants [66, 67]. 2,4-Di-tert-butylphenol is a lipophilic phenol and it has antioxidant, anti-inflammatory, and antifungal properties [68, 69]. 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (Diisobutyl phthalate) inhibits MG-63 cells proliferation [70].

Apart from these compounds, several fatty acid methyl esters (FAMEs) like methyl pentadecanoate, n-hexadecanoic acid methyl ester, ethyl palmitate, etc were detected in GC-MS of *C. scarabaeoides* seeds, which have nutraceutical, steroidal and estrogen-mimicking properties. Hence, the physiological and biochemical properties of the major components identified from the secretion of *C. scarabaeoides* through GC-MS analysis range between nutraceutical, antioxidants, anti-inflammatory, steroidal mimicking compounds, plasticizer, antifungal properties, ectoparasiticide properties, etc.

As per the molecular docking results, the arachidonic acid pathway inhibitory potential of allo-aromadendrene, and 1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester is apparent (Table 3, Figure 8). This is the first computational analysis that gives an insight into the potential anti-inflammatory and anti-cancer effects of methanolic extracts of *C. scarabaeoides*. The two most significant areas of global scientific interest are pain and inflammation since they are the most common symptoms of several diseases that afflict millions of people [71, 72]. Inflammation is the initiating response of all types of pain whether it is acute or chronic, peripheral, or central [73]. Since many of the analgesics and anti-inflammatory drugs that are

Table 3: The ligand-target protein binding energies.

Target protein	Ligand		
	Allo-aromadendrene (kcal/mol)	2,4-Di-tert-butylphenol (kcal/mol)	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (kcal/mol)
COX-2 (4COX)	8.4	5.9	8.1
PLA2 (1DB5)	7.2	6.6	6.6
IL-6 (4NI9)	5.1	5.3	5.1
IL-1β (4G6J)	5.4	5.7	5.2
TNF-alpha (2AZ5)	5.7	5.5	5.1
Bax (6EB6)	5.3	5.5	5.7
Caspase-3 (3DEI)	5.4	5.2	5.5
Caspase-9 (2C2Z)	6.0	5.1	5.1
p53 (4HJE)	5.8	5.4	5.4

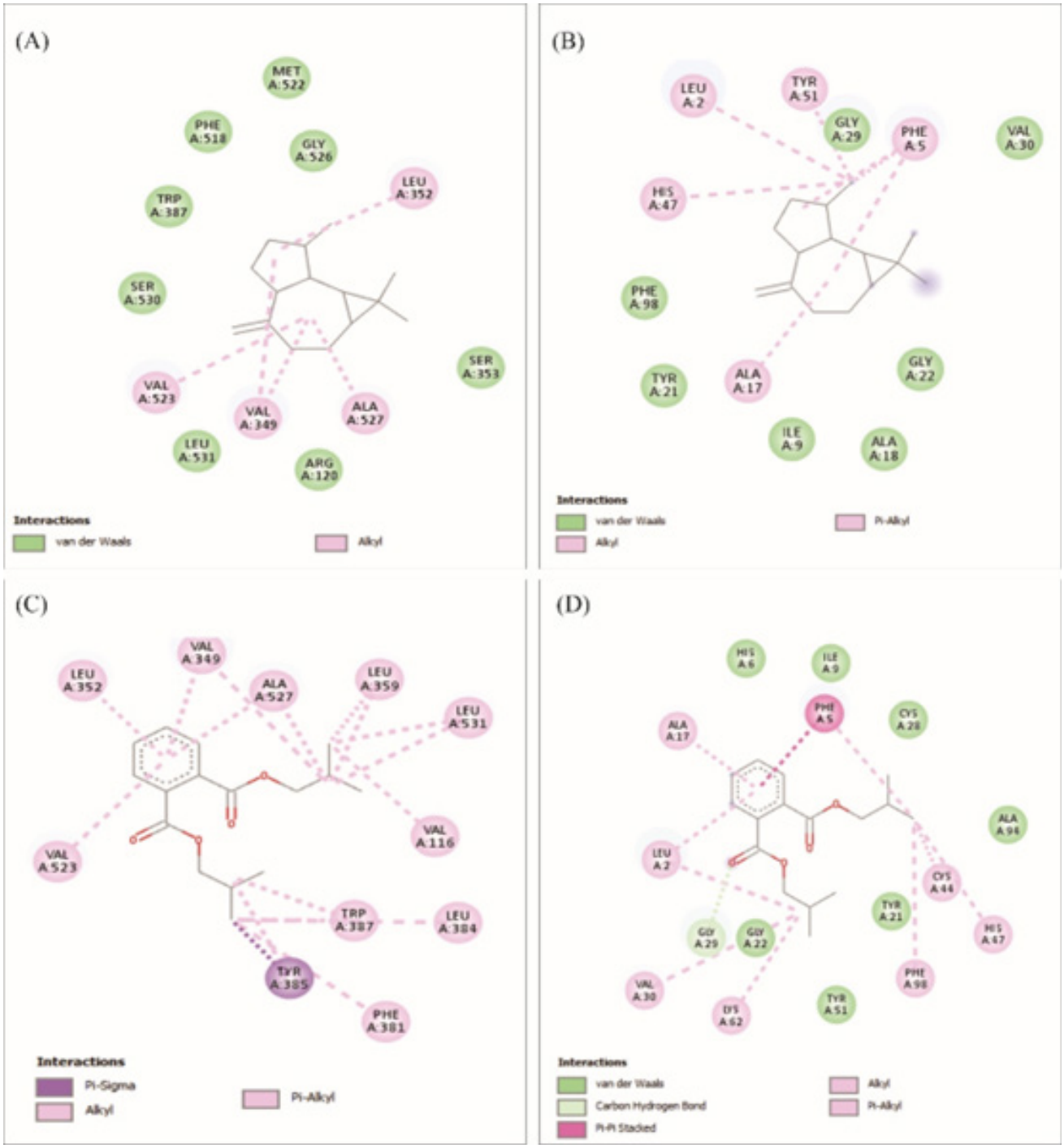

Figure 8: Molecular docking studies of ligands on COX-2 and $sPLA_2$ crystal proteins. (A). The 2D structure shows the best binding pose of allo-aromadendrene on the X-ray crystal structure of COX-2 (PDB ID: 4COX). (B). The 2D structure shows the best binding pose of allo-aromadendrene on the X-ray crystal structure of PLA_2 (PDB ID: 1DB5). (C). The 2D structure shows the best binding pose of 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester on the X-ray crystal structure of COX-2 (PDB ID: 4COX). (D). The 2D structure shows the best binding pose of 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester on the X-ray crystal structure of COX-2 (PLA_2 (PDB ID: 1DB5).

currently available have a variety of negative side effects, such as bleeding, gastrointestinal ulcers, renal abnormalities, etc., alternative therapies for the treatment of inflammation and pain are being developed all over the world [74, 75]. *C. scarabaeoides* is used during pregnancy to reduce leg pain and inflammation as well as to provide the woman with more energy after birthing [76]. Hence, *C. scarabaeoides* seeds might be a promising source of bioactive compounds to combat pain and inflammation.

Conclusion

The present study revealed the richness of phytochemicals in seeds of *C. scarabaeoides* with high total flavonoids, tannins, and phenolic content. The presence of these phytochemicals may explain *C. scarabaeoide's* significant antioxidant activity, which could explain its therapeutic effect on the biological system, as described in traditional medicine against a variety of

diseases. The molecular docking findings indicate that methanolic seed extract of *C. Scarabaeoides* may be used to inhibit the arachidonic acid pathway enzymes COX-2 and PLA_2, which in turn helps in designing new herbal-based anti-inflammatory and anti-cancer molecules. Further investigation is needed to isolate the constituent compounds from the methanolic seed extract of *C. Scarabaeoides* and evaluate their pharmacological efficacy against various conditions by *in vitro* and *in vivo* methods.

Conflicts of Interest

The authors declare no conflicts of interest.

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