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Chemical characterization, antioxidant and molecular docking studies on Ajwa pulp and seed concert extracts as potential antihemolytic agent

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Abstract: Combinations of bioactive natural products often provide enhanced therapeutic effects due to synergistic interactions, though these are challenging to study with single-compound methods. *Phoenix dactylifera* var. Ajwa is a rich source of phytonutrients, and its seed byproduct also shows therapeutic promise. This study examines the phytochemical profile and antioxidant activities of combined Ajwa pulp and seed (APS) extracts through ex vivo, in vitro, and correlation analyses. GC-MS identified 73 compounds in APS, including diisobutyl phthalate (18.65%), methyl palmitate (9.66%), and methyl oleate (9.35%). Total phenolics ranged from 1079–5456 mg GAE/100g and flavonoids from 175–907 mg RE/100g. NMR confirmed α- and β-glucose in APS-M. APS-EA and APS-M inhibited oxidative hemolysis of human RBCs (>90%). APS-EA showed IC_{50} values of 1.9 mg/mL (DPPH●) and 0.45 mg/mL (ABTS●+), while APS-HX showed 69% inhibition in β-carotene bleaching. Docking studies revealed strong binding of key compounds to RBC proteins, supporting antioxidant and membrane-protective effects. Overall, APS extract is a potent antioxidant with potential for nutraceutical use.

Keywords: Combined Ajwa date extract, anti-hemolytic, antioxidant, GC-MS, fatty acids

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INTRODUCTION

Phoenix dactylifera L., or date fruit ("Khajoor" in Urdu), belongs to the Arecaceae family, comprising ~200 genera and 1,500 species. It is one of the oldest cultivated fruits, used for thousands of years (Al-Karmadi *et al.*, 2024). Among its numerous cultivars, Ajwa is a distinguished variety native to Madinah Al-Munawwarah, Saudi Arabia. Characterized by its small size, dark brown to black epicarp, and tender pulp, Ajwa dates are noted for their unique flavor and relatively softer seeds compared to other varieties, making them easier to process. In addition to their culinary appeal, Ajwa dates are revered for their medicinal significance, often regarded as both a sacred and health-promoting fruit. Extensive studies have highlighted their pharmaco-therapeutic potential, including anticancer, antitumor, antidiabetic, and anti-inflammatory effects. Notably, they exhibit strong antioxidant activity and efficient free radical scavenging capabilities (Khalid *et al.*, 2017).

Free radicals are naturally produced within the body, but their excess can cause cellular and molecular damage, leading to oxidative stress. To counteract this, both natural and synthetic antioxidants are used to enhance the body's endogenous defense against reactive oxygen species (ROS). Studies have shown that many plants and herbs contain antioxidant enzymes such as glutathione peroxidase, along with non-enzymatic antioxidants like vitamins and polyphenols (Tumilaar *et al.*, 2024). These organic antioxidants play a vital role in preventing oxidative stress-related diseases such as ischemic heart disease and atherosclerosis by inhibiting the oxidation of low-density lipoproteins (LDL). Due to synergistic effects, combinations of fruits and their seeds have demonstrated enhanced biological activity (Meghwar *et al.*, 2024). For instance, blends of pear, hemp, and pumpkin have shown increased ACE inhibition and nitric oxide (NO) production, contributing to improved vascular function and blood pressure regulation (Park *et al.*, 2019). Additionally, a nanosponge formulation incorporating *Cinnamomum pauciflorum* bark oil and cinnamaldehyde exhibited strong activity against carbapenem-resistant bacterial strains (Mitra *et al.*, 2025). The current study investigates mixed extracts of Ajwa date pulp and seed to quantify polyphenolic content, assess antioxidant potential via *in vitro* and *ex vivo* assays, analyze bioactive compounds through GC-MS, and evaluate their molecular

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interactions with RBC membrane proteins via in silico docking. While the antioxidant properties of Ajwa fruit and seeds have been previously reported, this is the first study to explore the synergistic effects of their combined extract using both *in vitro* and *ex vivo* models.

MATERIALS AND METHODS

Standards, solvents and reagents

Analytical grade chemicals were obtained from internationally renowned chemical supplier for the estimation and analysis as mentioned: BDH, England; Gallic acid (3, 4, 5-Trihydroxybenzoic acid), L-Ascorbic acid, Tween 40 (Polyoxyethylene (20) sorbitanmonopalmitate), BHT (2, 6-D-tert-butyl-p-cresol) and Potassium persulfate. (Duksan Pure Chemicals, Korea); Aluminium chloride Anhydrous, (E. Merck Dramstadt, Germany); Beta Carotene, Scharlau (Spain); Sodium Carbonate, Folin Ciocalteu's Reagent, (Alpha Aesar, Germany); (+)-Rutin Trihydrate (Avonchem, England;) Linoleic acid; (Daejung Chemicals & Metals CO., LTD, Korea);Potasium acetate, (Bioworld, USA); Chloroform, ABTS (Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)& Phosphate Buffer Saline table-ts (7.4), (Sigma-Aldrich CHEME Gmbh, USA); AAPH (2,2-Azobis 2-methyl-propionamidine) dihydrochloride, DPPH (2,2-diphenyl-1-picrylhydrazyl) and Trolox (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

Plant material

Ajwa date fruits (fully ripped) were purchased from the market of Madinah -tul -Munnawwarah, Saudi Arabia. These dates were dispatched to Karachi, Pakistan, with proper packaging and storage conditions. The specimen was submitted at Centre for Plant Conservation, Herbarium and Botanic Garden, University of Karachi for identification, bearing a voucher # 92173. The date specie and its plant parts were identified by Ms. Afsheen Athar of Department of Botany, University of Karachi.

Extraction procedure

The Ajwa dates were de-seeded and weighed separately. The seeds of Ajwa date were separated from the fruit pulp and grounded finely. Then both the pulp (fruit without seed) and powdered seeds were weighed separately and mixed on a ratio of 6:1. For extraction, 360g of Ajwa pulp and 40g seeds were mixed and sequentially extracted with *n*-hexane (Hx), ethyl acetate (EA) and methanol (M), twice at room temperature. The obtained extracts of APS (Ajwa pulp seed) were then filtered and evaporated through rotary evaporator to get concentrates of *APS-HX*, *APS-EA* and *APS-M* (*Scheme-I*). In addition, *APS-M* was further sequentially extracted twice with hot solvents mentioned in *Scheme-II* to yield hexane (*APS-MHX)*, dichloromethane (*APS-MDC)*, ethyl acetate (*APS-MEA*), acetone (*APS-MAC*) and methanol *(APS-MM)* fractions. Apart from *APS-M* and *APS-MM*, all extract and fractions were subjected to GC-MS compound identification. While NMR studies were carried out on methanolic extract *APS-M*.

GC-MS and NMR analysis

GC-MS analysis was performed on equipment of Agilent Technologies Model: *7000 GC-MS Triple Quad*. Its software was Mass Hunter workstation version B.04.00. During this analysis the electron ionization mode (ionizing potential of 70 eV) was used. Further, the column used for compound separation was Agilent column USB393752HHP-5MS 5% Phenyl Methyl Siloxane, 30m x 250µm x 0.25µm. The temperature range and rate of temperature increase was 50-250ºC and 5-8 ºC/min, respectively. Helium was used as a carrier gas with a flow rate of 16.2ml/ min and split ratio, 10:1. The sample injected by Automatic liquid sampler (ALS) and injection volume was 2.5ul. Identification of each extract and fractions were based on comparison of their retention indices (RI) calculated according to the Kovats formula, using n-alkanes (C_9-C_{33}) (Sigma-Aldrich, Germany) as standards under the same chromatographic conditions. Retention Indices were also compared with literature data available in National Institute of Standards and Technology Standard Reference Database. 1H NMR (500MHz), 2D NMR (1H-1H) COSY, NOESY, HSQC and HMBC were recorded at room temperature in CD3OD on Bruker Avance NEO 500 MHz NMR spectrophotometer. The data represented as chemical shift (δ) in parts per million (ppm) and coupling constant indicated in Hertz (Hz). The spin multiplies are mentioned as d (doublet), dd (doublet of doublet), t (triplet) and m (multiplet).

Total phenolic content

The determination of total phenolic content was carried out in accordance with (Gouveia-Figueira *et al.*, 2014) with slight modification. Briefly, 1ml of methanolic extract solution was mixed with 2.5ml of Folin-reagent (10%) followed by proper mixing. After 5min of incubation, 2.5ml of Na_2CO_3 (7.5%) was added while shaking. Later, this reaction mixture was left for 2 hours in the dark. The absorbance was measured on UV-spectrophotometer (BMS UV-2600) at 765nm against blank. Calibration curve was determined using gallic acid as standard. The values were expressed as Gallic acid equivalent i.e., GAE (mg/ 100g) of extract.

Total flavonoid content

For determining the total flavonoid content, a modified version of the (Gouveia-Figueira *et al.*, 2014) method was used. The reaction mixture consists of a sample (1ml), 0.5 ml of aluminum chloride (10%, 0.5ml), potassium acetate (1M, 0.5ml) and 4.15ml of distilled water. The absorption was immediately detected using a 415nm UV-Spectrometer with distilled water and methanol serving as a blank. The calibration curve's reference was based on rutin. The results were expressed as the Rutin Equivalent, or RE (mg/ 100g) of dry extract.

DPPH radical scavenging activity

The radical scavenging activity was accessed by the ability of antioxidant to reduce the DPPH radical. The scavenging activity was determined according to the method reported by (Gouveia-Figueira *et al.*, 2014) with some modifications. Briefly, 0.5ml of extract sample (0.1 to 4mg/ ml) was taken in separate test tubes. Later, 3.5ml of DPPH solution was added (both in methanol) and kept in dark for 30min. Note down the absorbance at 517nm taking methanol as a blank. All samples were run in triplicate and the comparative results were expressed as Ascorbic acid equivalent antioxidant capacity (AAE)g/ 100g of dry extract. While percent Radical Scavenging activity was determined by the following formula:

$$\%\,\mathrm{RSA} = \frac{\mathrm{Blank\,Absorbance\ Sample\,Absorbance}}{Control} \times 100$$

ABTS Cation De-colourization assay

The method adopted by (Gouveia-Figueira *et al.*, 2014) used with some modifications. To prepare a stock solution, 7mM of ABTS solution and 2.45mM of potassium persulfate were mixed and incubated in dark at room temperature for 16 hours to produce a dark-green colored solution. It was prepared by diluting stock solution with appropriate volumes of distilled water to get the absorbance of 0.700±0.02 at room temperature. While, the reaction mixture was prepared from 3.5ml of ABTS radical solution by adding 0.5ml of extract sample by using different concentrations. Absorbance was noted just after 6mins of incubation, at 734nm. Triplicate readings were taken for each analysis. Trolox was used as a standard and results expressed in Trolox Equivalent Antioxidant Capacity (TEAC)g/ 100g of dry extract. Percent scavenging calculated by using the following formula:

$$\%\,\mathrm{ABTS\,Scavening\ activity} = \frac{1 - A_s}{A_c} \times 100$$

Where, A_S is the absorbance value found after adding sample to the $ABTS^+$ solution, and A_C is the absorbance of the control, which includes only the $ABTS^+$ solution.

β-Carotene linoleate model assay

β-carotene bleaching assay was performed according to (Gouveia-Figueira *et al.*, 2014) with certain modifications. For this assay, β-Carotene (2mg) dissolved in 10ml of chloroform was kept in rotary evaporator to remove chloroform. Then, for the preparation of its emulsion, 40mg of linoleic acid, 400mg of Tween-40 and 100ml of distilled water were added in the evaporated sample, and sonicated in a flask for 15mins. Consequently, 4.8ml of β-Carotene emulsion was transferred in a test tube containing 0.2ml extract sample. Finally, distilled water was added to make up volume up to 5ml of the reaction mixture. Zero-time absorbance of the sample was noted as soon as the emulsion was added. Then this solution was kept in a water bath at 50 ºC for 2 hours and absorbance was noted down after every 30mins of intervals. Blank was prepared (devoid of β-carotene) for background subtraction. Antioxidant index was calculated by the following formula:

$$\mathrm{Antioxidant\ Index} = \frac{\beta - \mathrm{Carotene\ conc.\ After\ 2\ hours\ of\ assay}}{\mathrm{Initial}\ \beta\text{-}\,\mathrm{Carotene\ concentration}} \times 100$$

Data of each test recorded in triplicate while AA used as standard.

Oxidative hemolysis inhibition assay (OxHLIA)

The oxidative hemolysis inhibition assay was carried out according to the procedure used by (Miki *et al.*, 1987) with some modifications. 3ml of human blood, mixed with EDTA to avoid coagulation. This uncoagulated blood sample was centrifuged at 1500rpm to separate the plasma from HRBCs in wintrobe graduated centrifuge tubes. The ratio of plasma to HRBCs was noted down. It was followed by the washings HRBCs with phosphate buffer saline (PBS; pH 7.4) and centrifuged each time. Later, 20% suspension of HRBCs was prepared by using PBS 7.4. While, the reaction mixture consisted of 1ml HRBCs suspension, 2ml of AAPH (200mM) and 1ml of each extract. The reaction mixture was then gently shaken after fixed intervals during incubation at 37ºC for 3hours. After incubation, 8ml of PBS was added for centrifugation at 1500rpm for 5mins. Supernatant was then pipette out to record the absorbance at 540nm against the blank (PBS 7.4). Consequently, the percent inhibition was calculated by using the following formula:

$$\%\,\mathrm{Hemolysis\ inhibition} = \frac{A_{AAPH} - A_s}{A_{AAPH}} \times 100$$

Where, A_{AAPH} is the absorbance of control without extract sample. While, A_S is the absorbance of sample containing the fraction of extract and ascorbic acid was used as standard.

Molecular docking

A docking simulation was conducted to gain insight into the binding behavior of synthesized/isolated chemical compounds in the active sites of three different proteins which are spectrin, reduced human peroxiredoxin 2 and Human GPX4. The 3D crystal structures of selected proteins were retrieved from Protein Data bank. A crystal structure of spectrin (PDB ID: 5J4O with resolution 1.54 A˚), [1] Reduced human peroxiredoxin 2 (PDB ID: 7KIZ with resolution 1.70 A˚) [2] and Human GPX4 (PDB ID: 2OBI with resolution 1.55 A˚) [3] confirmed the significance, coordinated core of the structure and provided a novel mode of proteins inhibition. Therefore, the 3D structures of the proteins (PDB IDs: 5J4O, 7KIZ and 2OBI) were imported into the MOE-dock window. Moreover, water molecules were eliminated from the proteins crystal structures while their presence is unimportant to our investigation and then the proteins 3D crystal structures were protonated by MOE. The force field Amber EHT: 10 in MOE software had been employed in order to decrease the enzyme's energy. The chemical compounds structures were constructed in MOE using a builder tool, after which the 3D structure was protonated. Energy minimization was then carried out in

MOE using the force field MMFF94x. For docking simulation, the structural coordinates of proteins were chosen. Using an induced fit docking approach and triangular matching, the chemical compounds were successfully docked into the enzyme's active site. At the end the ultimate interaction pattern of protein ligand complexes was analyzed.

STATISTICAL ANALYSIS

Origin Lab was used for statistical analysis. All results were expressed as mean ±standard deviation (SD) in triplicate and were analyzed using One Way Analysis of Variance (ANOVA). The correlation between TPC, TFC, and antioxidant activity is presented by Pearson's Correlation coefficient. Results were considered statistically significant when *P*-values were below 0.05.

RESULTS

Chemical Composition and Spectrometric analysis

GC-MS

The GC-MS analysis of APS extracts and fractions lead to the identification of 73 compounds. Hexane extract showed only detection of seven compounds including methyl linoleiate (48, 43.41%) followed by methyl stearate (49, 36.22%), myristic acid (28, 14.12%) and lauric acid (21, 3.21%) (Vide table 1). Compound (21) and (26) exclusively observed in *APS-HX* while compounds (21, 26, 39, 48 and 49) are reported for first time from Ajwa extract. GC-MS of ethyl acetate extract of the APS detected nine compounds with dominating diiso butyl phthalate (37, 86.65%), lauric acid (23, 3.43%) and 5-Furandicarboxaldehyde (6, 3.16%), from ethyl acetate extract. Compound (15) and (58) exclusively eluted in *APS-EA* while 6, 15, and 58are reported for the first time from Ajwa extract. In view of the potent anti-hemolytic activity of *APS-M* extract, it was further fractionated into hexane (*APS-MHX)*, dichloromethane (*APS-MDC)*, ethyl acetate (*APS-MEA*), acetone (*APS-MAC*)and methanol *APS-MM*. GC-MS studies on *APS-MHX* fraction resulted in identification of thirty nine metabolites including methyl palmitate (39, 18.82%), and methyl linoleate acid (46, 12.7%), methyl eladiate (47, 12.7%) , methyl oleate (48,12.7%),diphenyl phthalate (60, 9.68%) and ethyl oleate (z) (54, 3.75%) as major compounds .Compound (1, 2, 3, 5, 8, 29, 31, 34, 35, 36, 38, 57) and (60) are exclusive to *APS-MHX* while compound (1-5, 7, 25, 29, 31-36, 38-39, 50, 42, 46-50, 54, 56, 57, 61, 64) are reported first time from Ajwa extract. *APS-MDC* revealed thirty-nine compounds, including methyl palmitate (39, 13.57%), ethyl palmitate (42, 13.57%), *methyl elaidate* (E) (47, 7.34%), methyl linoleate (*Z, Z*) (46, 7.32%), palmitic acid (41, 6.16%), 5 hydroxy methyl furfural (14,5.27%), *cis* -vaccenic acid (50, 3.60%), tocopherol (Vit E) (68, 3.73%) and stigmastan-3,5-diene (67, 2.04%) as major compounds. Twelve of them (9-10, 12-13, 18-19, 27, 30, 43-44, 65 and 73) were exclusively eluted in *APS-M*DC while rest of them were recurred. Compound (9-10, 12-14, 16, 18-19, 27, 30, 43-44, 65 and 71,72, 73) are reported first time from Ajwa extract. The ethyl acetate fraction, *APS-MEA*, revealed the detection of thirty-five constituents including diiso butyl phthalate (37, 25.91 %), methyl palmitate (39, 10.88%), palmitic acid (41, 10.88%), methyl linoleate (46, 7.28%), ethyl palmitate (42, 7.05%), methyl elaidate (47, 7.05%), phenyl benzoate (24, 3.73%), tocopherol (vit E) (68, 2.42%), 5- hydroxy methyl furfural (14,1.30%) vide table 1. From *APS-MAC* fraction, seventeen compounds were identified including palmitic acid (41, 22.81%), methyl linoleate (*Z,Z*) (46, 12.98%),methyl palmitate (39, 12.73%), *methyl elaidate* (E) (47, 10.33%), linoleic acid (Z,Z) (51, 8.62%), ethyl palmitate (42, 4.95%), stigmastan-3,5-diene (67,4.40%) , sitostenone (70, 4.08%), ethyl oleate (*E,E*) (53, 3.10%), stigma acetate (71, 2,60%), cholestane (63, 2.12%), ergosterene (66, 1.71%), tocopherol (vit E) (68, 2.35%).

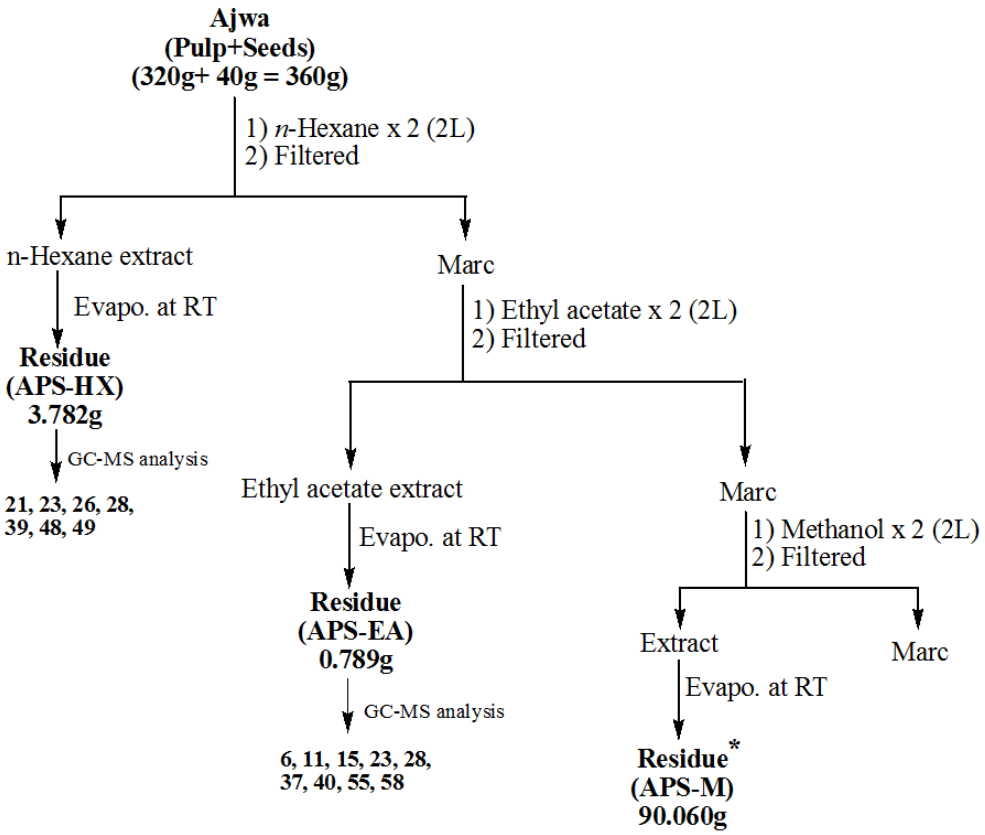
NMR

α-Glucose: ^{1}H-NMR (500MHz CD_3OD): The characteristic anomeric proton of α-glucose lie at *δ*5.10 (d, *J*= 3.60 Hz, H-1), then *δ*3.68 (d, *J*= 8.4 Hz, H-2), *δ*3.26 (m, *J*= 6.4 Hz, H-3), *δ*3.63 (t, *J*= 13.6 Hz, H-4), *δ*3.78 (m, H-5), and geminal protons *δ*3.85 (dd, *J*=11.2 and 9.2 Hz, H-6a) and *δ*3.78 (m, H-6b). The HMBC data shows correlation of *δ*3.68 (H-2 to C6), *δ*3.63 (H-4 to C2) and *δ*3.78 (H-5 to C3). The ^{1}H-^{1}H Noesy correlation shows *δ*3.26 (H-3) interact with H-5, H6a, H6b, *δ*3.63 (H-4) with H-5, H-6a, H-6b, *δ*3.78 (H-5) with H6b and *δ*3.78 (H6b) to H6a. Further COSY data indicate correlation of *δ*3.68 (H-2) to H-3, *δ*3.26 (H-3) to H-2, H-4, *δ*3.63 (H-4) to H-3, *δ*3.78 (H-5) to H-3, H-4, *δ*3.85 (H6a) to H-4 and *δ*3.78 (H6b) to H-3 and H-4.

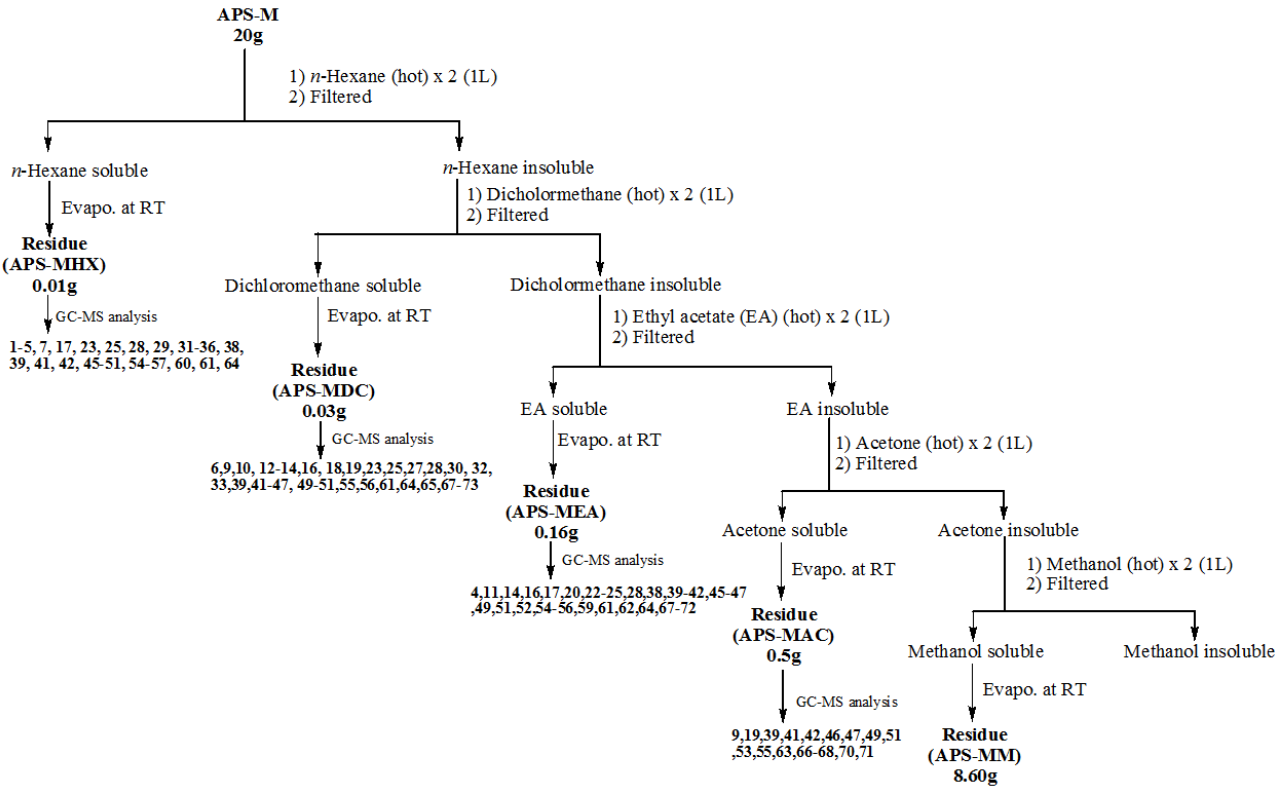
β-Glucose: ^{1}H-NMR (500MHz CD_3OD): The characteristic anomeric proton of β-glucose lie at *δ*4.47 (d, *J*= 8 Hz, H-1), then *δ*3.12 (dd, *J*= 8, 8.8 Hz, H-2), *δ*3.36 (dd, *J*= 10.4, 9.6 Hz, H-3), *δ*3.60 (t, *J*=12.4 Hz, H-4), *δ*3.30 (m, *J*= 8 Hz, H-5), and geminal protons *δ*3.99 (dd, *J*=14, 5.2 Hz, H6a) and *δ*3.48 (dd, *J*=11.2, 3.6 Hz, H6b). The HMBC correlation *δ*4.47 (H-1) to C5, *δ*3.12 (H-2) to C1, C3, C5 and *δ*3.99 (H-6a) to C5. ^{1}H-^{1}H NOESY correlation shows *δ*3.36 (H-3) interacts with H-6b and *δ*3.30 (H-5) to H-6b. Further, COSY correlation *δ*4.47 (H1) to H-2, *δ*3.36 (H-3) to H-2 and *δ*3.99 (H-6a) to H-4.

Total phenolic content (TPC) and total flavonoid content (TFC)

The phenolic content was calculated from gallic acid standard curve (y =0.0073x + 0.0199; r^2=0.9991). TPC was found in the range of 1079 to 5456mg GAE/100g. *APS-EA* was most abundant in phenolic content while least amount of phenolic was showed in hexane extract (table 2).



Scheme I: Extraction and identified compounds from APS extracts. *Continued in Scheme 2.



Scheme II: Fractionation of *APS-M* extract.

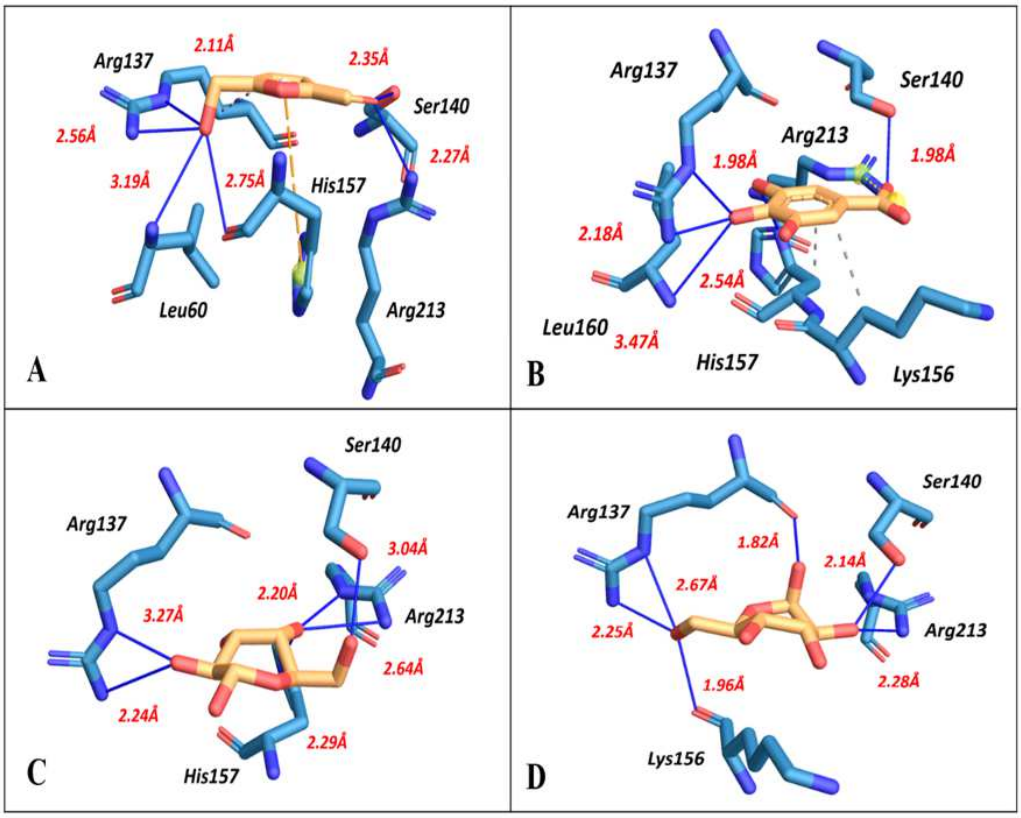


Fig. 1: Compound A (5- hydroxy methyl furfural), B (gallic acid), C (α glucose), and D (β glucose) docked with human erythrocytic spectrin (PDB ID: 5J4O). Ligands are displayed in yellow. Blue indicates the presence of hydrogen bonding. Bonds that are hydrophobic are displayed in gray.

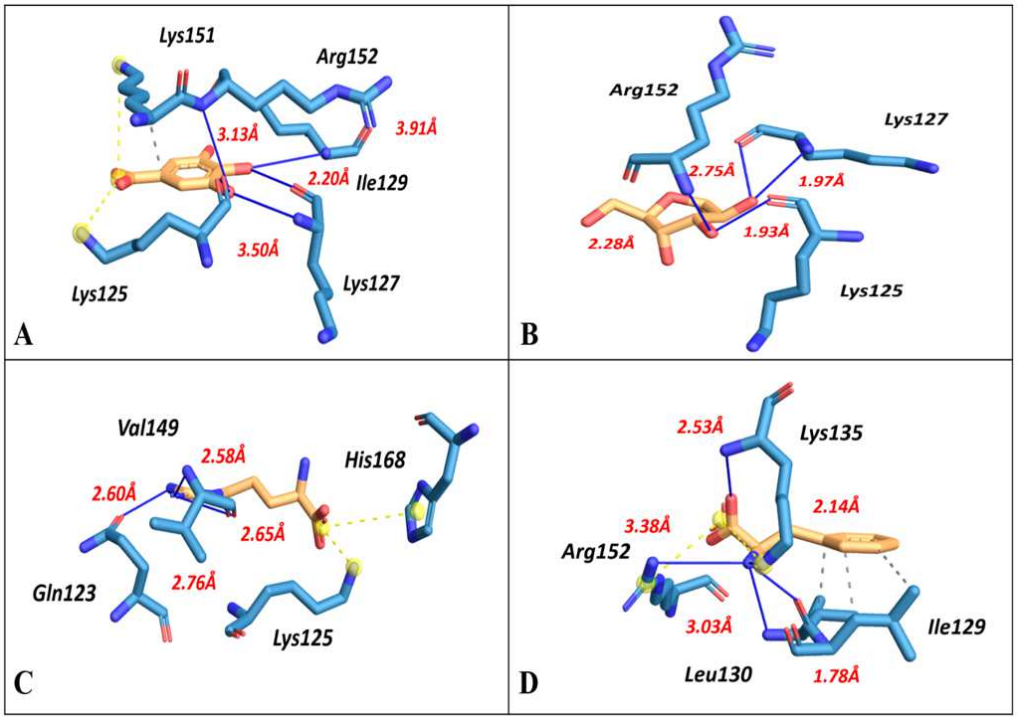


Fig. 2: Compound A (gallic acid), B (α glucose), C (Arginine) and D (Phenyl alanine) docked with Human GPX4 (PDB ID: 2OBI). Ligands are displayed in yellow. Blue indicates the presence of hydrogen bonding. Bonds that are hydrophobic are displayed in gray.

Table 1: Percent composition of phytochemicals in GC-MS studies on APS extracts and fractions

Identified Compounds/ IUPAC Name	*APS-HX*	*APS-EA*	*APS-MHX*	*APS-MDC*	*APS-MEA*	*APS-MAC*	Subtotal	% Overall	RI[a]	RI[b]
1,2,3-Trimethylbenzene(1) [b,c]	-	-	0.13	-	-	-	0.13	0.02	971	1005
1-Ethyl-2-methylbenzene (2) [b,c]	-	-	0.12	-	-	-	0.12	0.02	980	969
1-Methyl-3-propylbenzene (3) [b,c]	-	-	0.06	-	-	-	0.06	0.01	1049	1042
Nonanal(4)[b]	-	-	0.07	-	0.15	-	0.22	0.04	1057	1081
p-Menthatriene (1,3,8-*p*-Menthatriene) (5)[b,c]	-	-	0.08	-	-	-	0.08	0.01	1073	1085
2,5-Furandicarboxaldehyde (6)[b]	-	3.16	-	0.03	-	-	3.19	0.53	1076	1078
Undecane(7)[b]	-	-	0.07	-	-	-	0.07	0.01	1092	1100
2-Nonen-1-ol (8)[a]	-	-	-	-	-	0.65	0.65	0.11	1096	1051
Solerone (5-Acetyldihydrofuran-2(3H)-one) (9)[a]	-	-	-	0.42	-	-	0.42	0.07	1134	1299
Succinic acid, monomethyl ester (Butanedioic acid, monomethyl ester) (10) [b,c]	-	-	-	0.42	-	-	0.42	0.07	1136	1106
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (11) [b,c]	-	0.66	-	-	0.92	-	1.58	0.26	1143	1162
5-Hydroxymaltol (3,5-Dihydroxy-2-methyl-4H-pyran-4-one) (12) [b,c]	-	-	-	0.73	-	-	0.73	0.12	1148	1188
2,5-Dihydrothiophene (13) [a]	-	-	-	0.58	-	-	0.58	0.10	1229	-
5-Hydroxymethylfurfural (14) [b,c]	-	-	-	5.27	1.30	-	6.57	1.09	1253	1252
(Z)-2-Decenal (15) [b]	-	1.4	-	-	-	-	1.40	0.23	1254	1229
5-Acetoxymethyl-2-furaldehyde (16) [b,c]	-	-	-	0.95	0.39	-	1.34	0.22	1256	1286
Capric acid (Decanoic acid) (17) [b]	-	-	0.01	-	0.22	-	0.24	0.04	1367	1387
DL-Proline, 5-oxo-, methyl ester (18) [b,c]	-	-	-	1.10	-	-	1.10	0.18	1391	1390
α-Naphthol (1-Naphthalenol) (19) [b,c]	-	-	-	0.45	-	-	0.45	0.07	1465	1473
Undecanoic acid (20) [b,c]	-	-	-	-	0.42	2.35	2.77	0.46	1491	1466
Lauric acid, methyl ester (Methyl dodecanoate) (21) [b]	3.21	-	-	-	-	-	3.21	0.54	1510	1525
4-O-β-D-galactopyranosyl-β-D-Glucopyranose(22) [a]	-	-	-	-	0.41	-	0.41	0.07	1556	-
Lauric acid (Dodecanoic acid) (23) [b,c]	0.43	3.43	0.15	0.37	1.54	-	5.92	0.99	1565	1576
Phenyl benzoate (24) [b,c]	-	-	-	-	3.73	-	3.73	0.62	1656	1626
Myristic acid, methyl ester (Methyl tetradecanoate) (25)[b,c]	-	-	0.51	0.86	0.57	-	1.94	0.32	1712	1726
Myristoleic acid, methyl ester (Methyl *cis*-9-tetradecenoate) (26) [b,c]	0.59	-	-	-	-	-	0.59	0.10	1715	1715
8-Tetradecen-1-ol acetate (27) [b,c]	-	-	-	0.40	-	-	0.40	0.07	1754	1787
Myristic acid (Tetradecanoic acid) (28) [b]	14.14	2.2	0.39	1.21	1.94	-	19.87	3.31	1758	1765
Myristic acid, ethyl ester (Ethyl tetradecanoate) (29)[b,c]	-	-	0.23	-	-	-	0.23	0.04	1786	1793
Methyl 13-methylmyristate (Methyl 13-methyltetradecanoate) (30)[b]	-	-	-	0.88	-	-	0.88	0.15	1791	1773
Octadecene(31) [b,c]	-	-	0.10	-	-	-	0.10	0.02	1793	1795
Pentadecanoic acid, methyl ester (32) [b,c]	-	-	0.88	0.97	-	-	1.85	0.31	1816	1807
Pentadecanoic acid (33)[b,c]	-	-	0.35	0.96	-	-	1.32	0.22	1823	1869
6,10,14-Trimethyl-2- pentadecanone(34)[b,c]	-	-	0.24	-	-	-	0.24	0.04	1832	1846
Palmitonitrile(35)[b]	-	-	0.22	-	-	-	0.22	0.04	1838	1857
Palmitoleic acid, methyl ester (9-Hexadecenoic acid, methyl ester) (36)[a,c]	-	-	0.15	-	-	-	0.15	0.03	1846	1885
Diisobutyl phthalate (1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester) (37)[b,c]	-	86	-	-	25.91	-	111.91	18.65	1860	1868
Pentadecanoic acid, ethyl ester (38)[b,c]	-	-	0.39	-	-	-	0.39	0.07	1876	1897
Palmitic acid, methyl ester (Methyl hexadecanoate) (39)[b,c]	1.96	-	18.82	13.57	10.88	12.73	57.97	9.66	1908	1928
Dibutyl phthalate (1,2-Benzenedicarboxylic acid, dibutyl ester) (40)[b,c]	-	2.07	-	-	1.16	-	3.23	0.54	1948	1967
Palmitic acid (Hexadecanoic acid) (41) [b]	-	-	6.16	6.16	10.88	22.81	46.01	7.67	1952	1964
Palmitic acid, ethyl ester (Ethyl Hexadecanoate) (42) [a,c]	-	-	6.02	13.57	7.05	4.95	31.60	5.27	1953	1993
Benzanilide (N-Phenylbenzamide) (43)[b,c]	-	-	-	5.56	-	-	5.56	0.94	1961	1989
Methyl cis-11-octadecenoate (44)[a,c]	-	-	-	7.54	-	-	7.54	1.27	2041	-
Margaric acid (Heptadecanoic acid) (45) [b,c]	-	-	0.02	0.41	0.21	-	0.63	0.11	2060	2038
Linoleic acid, methyl ester (Methyl (Z,Z)-9,12-octadecadienoate) (46) [b,c]	-	-	12.71	7.32	7.28	12.98	40.28	6.71	2089	2098
Elaidic acid, methyl ester (Methyl (E)-9-Octadecenoate) (47) [b,c]	-	-	12.71	7.34	7.05	10.33	37.43	6.24	2094	2085
Methyl (Z)-9-octadecenoate (48) [b,c]	43.41	-	12.71	-	-	-	56.12	9.35	2095	2085
Stearic acid, methyl ester (Methyl octadecanoate) (49) [b,c]	36.22	-	1.79	1.53	0.92	1.55	42.02	7.00	2124	2133
cis-Vaccenic acid (cis-11-Octadecenoic acid) (50) [b,c]	-	-	2.35	3.60	-	-	5.95	0.99	2135	2116
Linoleic acid*((Z,Z)*-9,12-Octadecadienoic acid) (51) [b]	-	-	2.82	3.30	4.69	8.62	19.43	3.24	2141	2134
trans-13-Octadecenoic acid (52) [b,c]	-	-	-	-	0.94	-	0.94	0.16	2148	2163
Oleic acid, ethyl ester (Ethyl *(E)*-9-Octadecenoate) (53)[b,c]	-	-	-	-	-	3.10	3.10	0.52	2158	2174
Oleic acid ethyl ester (Ethyl *(Z)*-9-Octadecenoate) (54) [b,c]	-	-	3.75	-	2.02	-	5.76	0.96	2161	2142
Stearic acid (Octadecanoic acid) (55) [b,c]	-	0.09	0.68	3.30	0.82	2.65	7.53	1.26	2167	2188
Stearic acid, ethyl ester (Ethyl Octadecanoate) (56) [b,c]	-	-	0.30	0.28	0.14	-	0.72	0.12	2191	2193
cis-13-Eicosenoic acid, methyl ester (57) [b,c]	-	-	0.49	-	-	-	0.49	0.08	2350	2321
Glyceryl palmitate (Hexadecanoic acid, 2,3-dihydroxypropyl ester) (58)[b]	-	0.96	-	-	-	-	0.96	0.16	2473	2482
Behenic acid, methyl ester (Methyl docosanoate) (59) [b,c]	-	-	-	-	0.37	-	0.37	0.06	2534	2530
Diphenyl phthalate (1,2-Benzenedicarboxylic acid, diphenyl ester) (60)[a,c]	-	-	9.68	-	0.00	-	9.68	1.61	2550	2555
Tricosanoic acid (61) [b,c]	-	-	0.77	0.54	0.34	-	1.65	0.28	2632	2615
5-amino-1H-imidazole-4-carboxamide (62) [a]	-	-	-	-	0.78	-	0.78	0.13	2712	-
Cholestan-22(26)-epoxy-3,16-dione (63) [a]	-	-	-	-	-	2.12	2.12	0.35	2713	-
Lignoceric acid, methyl ester (Methyl tetracosanoate) (64) [b,c]	-	-	0.97	0.64	0.44	-	2.04	0.34	2731	2731
Ethyl iso-allocholate(65) [a]	-	-	-	0.69	-	-	0.69	0.12	2739	-
Ergost-5-en-3-ol, acetate, (3β,24R)- (66) [a]	-	-	-	-	-	1.71	1.71	0.29	-	-
Stigmastan-3,5-diene (67) [a]	-	-	1.00	2.04	1.30	4.40	8.74	1.46	-	-
Vitamin E (α-Tocopherol) (68) [a]	-	-	0.58	3.73	2.42	2.35	9.08	1.51	-	-
Lupenone (Lup-20(29)-en-3-one) (69) [a]	-	-	0.53	0.44	0.36	-	1.33	0.22	-	-
Sitostenone (Stigmast-4-en-3-one) (70) [a]	-	-	1.00	1.30	1.08	4.08	7.46	1.24	-	-
Stigmasta-5,22-dien-3-ol, acetate, (3β) (71) [a]	-	-	-	0.52	0.93	2.60	4.06	0.68	-	-
γ-Sitosterol (Stigmast-5-en-3-ol, (3β,24S)) (72) [a]	-	-	-	0.44	0.45	-	0.89	0.15	-	-
Stigmast-5-en-3-ol, oleate(73) [a]	-	-	-	0.36	-	-	0.36	0.06	-	-
	100	100	100	100	100.00	100.00		100.00		

*Order of elution is given for column (HP-5MS); a Mass spectra were compared with literature fragmentation pattern given in National Institute of Standard Reference Data Base Number 69; b Mass spectrum and retention indices comparable with values given in literature available in NIST database; c Retention index value match with nonequivalent column; d Relative percentage with respect to the most abundant compound in the extract; RI[a] observed retention index; RI[b] literature retention index.

Since the major portion of date pulp consists of sugars, therefore, a correction factor had been applied. For total flavonoid content was calculated from rutin to determine standard curve (y=0.005x-0.0015; r^2=0.9995). TFC was observed in the range of 175 to 907 mg RE/100g with *APS-M* showed the highest flavonoid content (table 2).

DPPH• and ABTS•+ Radical scavenging assay

In DPPH• radical scavenging assays, both concentration dependent oxidation inhibitory activity, as well as antioxidant content, were observed (table 3). The *APS-EA* and *APS-M* extracts demonstrated robust antioxidant activity, with inhibition values of 76.36% and 59.85%, respectively. The IC50 values of *APS-EA* and *APS-M* were found to be 1.9mg/ml and 3.2mg/ml. The antioxidant content in the DPPH• assay was quantified using two standard curves: ascorbic acid (y=-3.089x + 1.0583; r^2=0.9942) and Trolox equivalent (y=-2.9392x + 1.0613; r^2=0.9992). The calculated antioxidant capacities of *APS-EA* were 26.91 mM AAE/100g and 28.31 mM TE/100g. Similarly, for ABTS•+ assay, the Trolox equivalent standard curve (y=-2.6783x + 0.8216; r^2= 0.9971) was utilized to determine antioxidant activity. The highest radical scavenging activity was observed in the *APS-EA* extract (94.13%) followed by *APS-M* (68.76%), with corresponding IC_{50} values of 0.45 mg/ml and 2.1mg/ ml, respectively.

β-Carotene Bleaching Assay

In β-carotene bleaching assay, only *APS-HX* showed significant oxidation inhibitory activity, which is 69% as indicated in table 4. While *APS- EA* and *APS-M* extracts showed activity in between 27- 35% respectively.

Oxidative hemolysis inhibitory assay (OxHLIA)

In this assay, *APS-EA* and *APS-M* extracts of Ajwa dates showed significant hemolysis inhibitory activity, i.e., 93-94.03% (table 4) in healthy Human erythrocytes. Due to solubility problem, *APS-HX* was not evaluated.

Correlation between TPC, TFC and Antioxidant activity of Ajwa extract

In table 5, strong positive correlation was observed between phenolic content, DPPH (r^2=0.8671; p<0.01) and ABTS scavenging activities (r^2=0.9280; p<0.001). While flavonoid content showed little or no correlation with DPPH and ABTS inhibitory activities with respect to solvent extract. For β-carotene bleaching assay, significant negative correlation was observed among β-carotene and DPPH• (r^2=-0.9795; p<0.001) and ABTS•+(r^2=-0.9451; p<0.001) activity. Further, significant linear correlation TPC was found with DPPH• (r^2=0.7508) and ABTS•+ (r^2=0.8586). Moreover, there was a strong correlation between DPPH• and ABTS•+ values as well (r^2=0.9813). In addition to above, significant linearity of β-carotene was also found with DPPH (r^2=0.9624) and ABTS values (r^2=0.8935).

Molecular docking studies

To conduct molecular docking investigations the software Molecular Operating Environment (MOE), was used. All 17 prepared chemical ligand structures were docked into the active site of proteins as well as carried out blind docking where binding sites are not known. Molecular docking simulations were carried using triangular matching and induced fit docking protocol in MOE-Dock module. According to the docking data, proteins binding site was occupied effectively by synthesized/isolated analogs, which stabilized the enzyme-ligand complexes.

Docking analysis of Spectrin

All compounds demonstrated a substantial effect on the human erythrocytic spectrin protein (Cutts *et al.*, 2017) with docking scores ranging from -6.39 to -4.11. For the docking investigations, a receptor with a binding site was not employed, so blind docking has been carried out. The findings demonstrated that all of these compounds formed hydrogen, hydrophobic, and van der Waals interactions to bind the protein by amino acids Lys56, Arg137, Ser140, His157, Leu160, and Arg213. The interaction pattern on the basis of protein ligand interaction pattern (PLIP) analysis is shown in fig. 1.

Docking analysis of Human GPX4

Molecular docking carried out in order to anticipate the binding interactions that would occur between the chosen chemicals and the human GPx4 target protein with the three-dimensional crystal structure retrieved from protein data bank (PDB ID: 20BI) (Scheerer *et al.*, 2007). By docking in the binding pocket cavity containing common amino acid residues such as GLN123, LYS127, LYS135, GLY128, ILE129, VAL149, ARG152, GLY154, PRO155 and THR136 selected for docking studies (Alameen *et al.*, 2022). The interaction pattern analysis of top four virtual hits which are Gallic acid, α glucose, Arginine and Phenyl alanine on the basis of PLIP analysis are shown in fig. 2.

DISCUSSION

The GC-MS spectrometric analysis on APS extracts revealed various bioactive chemical compounds. It was found that free fatty acids (FFA) and fatty acids esters (FAE) were most abundantly found (46% of the total compounds), out of which, 27% were saturated, 16% monounsaturated and 1% polyunsaturated. The highest proportion of fatty acids was found in APS-MEA (13.69%) while the lowest were in APS-MAC (5.47%). Likewise, highest FAE were observed in APS-MHX (21.91%) while lowest observed in APS-MAC (8.21%). The order of fractions as per the highest proportion of fatty acid and esters is MEA>MHX=MDC>MAC and MHX>MDC>MEA>MAC respectively. NMR evaluation of APS-M extract identified pure α- and β-glucose and showed that sugar is the dominant part of the chemical composition of Ajwa date fruit.

Table 2: Total Phenolic and Flavonoid content in APS extracts.

Sample	Total Phenolic content (TPC) (mg GAE/100g of extract)	Total flavonoid content (TFC) (mg RE/100g of extract)
APS-HX	1079 ± 5.00[a]	175.7 ± 3.84[a]
APS-EA	5456 ± 8.18 [b]	163.7 ± 2.15 [a]
APS-M	1818.25 ± 2.69 [c]	907.5 ±7.35[b]

Lower case letter in same column indicates significant difference ($p<0.05$), same letters in each column are statistically non-significant. Each value is presented as Mean ± SD (n=3). GAE= Gallic acid equivalent; RE=Rutin equivalent.

Table 3: DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ Radical Scavenging Assay

Extract	CONC.	DPPH$^{\bullet}$			ABTS$^{\bullet+}$	
	(mg/ml)	% Inhibition	AAE (mM/100g)	TEAC (mM/100g)	% Inhibition	TEAC (mM/100g)
APS-HX	0.1	4.5±0.30[a]	4.293±0.05[a]	4.637±0.17[a]	18.51±0.18[a]	9.916±0.01[a]
	0.2	6.5±0.31[b]	4.901±0.04[b]	5.283±0.10[b]	19.94±0.32[b]	10.29±0.03[b]
	0.3	7.22±0.30[c]	5.137±0.01[c]	5.521±0.08[c]	20.96±0.38[c]	10.55±0.03[c]
	0.4	7.32±0.22[d]	5.170±0.002[d]	5.555±0.22[d]	23.16±0.15[d]	11.11±0.07[d]
	0.5	7.53±0.04[e]	5.235±0.01[e]	5.623±0.14[e]	23.90±0.15[e]	11.29±0.14[e]
	1	9.18±0.04 [f]	5.755±0.04[f]	6.168±0.07[f]	35.92±0.12 [f]	14.28±0.09 [f]
	4	31.26±1.73 [g]	12.710±0.02[g]	13.449±0.12[g]	41.93±0.06[g]	15.89±0.09[g]
APS-EA	0.1	9.59±0.15 [a]	5.885±0.03 [a]	6.304±0.03 [a]	30.49±0.22 [a]	12.97±0.11 [a]
	0.2	13.10±0.18 [b]	6.990±0.012 [b]	7.461±0.13 [b]	34.01±0.14 [b]	13.87±0.11 [b]
	0.3	17.13±0.20 [c]	8.257±0.07 [c]	8.788±0.09 [c]	39.88±0.11 [c]	15.36±0.09 [c]
	0.4	20.74±1.3 [d]	9.395±0.16 [d]	9.978±0.03 [d]	47.50±0.22 [d]	17.30±0.11 [d]
	0.5	28.58±0.48 [e]	11.865±0.29 [e]	12.564±0.08 [e]	50.29±0.17 [e]	18.01±0.10 [e]
	1	41.48±0.43 [f]	15.927±0.03 [f]	16.817±0.14 [f]	82.25±0.13 [f]	26.15±0.07 [f]
	4	76.36±0.44 [g]	26.910±0.09 [g]	28.317±0.33 [g]	94.13±0.46 [g]	29.18±0.07 [g]
APS-M	0.1	11.04±0.43 [a]	6.340±0.09 [a]	6.780±0.06 [a]	17.30±0.27 [a]	9.61±0.14 [a]
	0.2	12.48±0.24 [b]	6.795±0.03 [b]	7.257±0.10 [b]	29.17±0.17 [b]	12.64±0.03 [b]
	0.3	13.41±0.47 [c]	7.087±0.09 [c]	7.563±0.04 [c]	29.77±0.05 [c]	12.79±0.07 [c]
	0.4	14.55±0.54 [d]	7.445±0.04 [d]	7.937±0.08 [d]	35.77±0.11 [d]	14.32±0.13 [d]
	0.5	16.71±0.29 [e]	8.127±0.09 [e]	8.652±0.05 [e]	39.00±0.15 [e]	15.14±0.12 [e]
	1	21.05±0.73 [f]	9.492±0.07 [f]	10.080±0.12 [f]	40.90±0.03 [f]	15.62±0.14 [f]
	4	59.85±0.99 [g]	21.712±0.25 [g]	22.873±0.11 [g]	68.76±0.05 [g]	22.72±0.04 [g]

Values with lower case letter in each extract category in respective column indicate significant difference ($p<0.05$). Each value is presented as Mean ± SD (n=3)

Table 4: β-carotene bleaching assay and *ex vivo* oxidative hemolysis inhibitory activity

Sample	β-Carotene Bleaching Assay % (4mg/ml)	Oxidative Hemolysis Inhibitory Assay % (4mg/ml)
APS-HX	69.14 ± 0.06 [a]	-
APS-EA	27.76 ± 0.07 [b]	93.24 ± 0.15 [a]
APS-M	35.42 ± 0.23 [c]	94.03 ± 0.10 [b]
AA (Control)	44.84 ± 0.12 [d]	90.32 ± 0.04 [c]
BHT (Control)	59.75 0.08 [e]	-

Lower case letter in same column indicates significant difference ($p<0.05$), same letters in each column are statistically non-significant. AA concentration = 0.2mg/ml, NDT= Not Determined. Antioxidant activity of extract and values taken at time intervals of half hour till 3hrs. Hemolysis inhibitory activity, calculated after 3hrs of incubation. Each value is presented as Mean ± SD (n=3).

Table 5: Pearson's correlation coefficient between Polyphenolic content, antioxidant activity and APS extracts.

	TPC	TFC	DPPH	ABTS	β- Carotene
TPC	1				
TFC	-0.36995	1			
DPPH	0.8671**	0.1388[ns]	1		
ABTS	0.9280***	0.0019[ns]	0.9891***	1	
Carotene	-0.7558*	-0.328	-0.9795***	-0.9451***	1

*$p<0.05$; **$p<0.01$; ***$p<0.001$; ns=non-significant

Sugars are despite being energy boosters and having low glycemic index (specifically fructose), enhance bioactivity of certain compounds, improve cell-cell signaling, support stability as well as protection of biological membrane including RBCs (Lemos *et al*., 2011, Strzyz, 2019). The phytochemical investigation on combined extract of Ajwa date pulp and seed revealed significantly higher polyphenolic and flavonoid contents as compared to previous studies i.e., APS-EA (5456mg GAE/100g) and APS-M (907mg RE/100g) respectively. Earlier reports on the phenolic content of Ajwa date fruit range from 2.69mg to 466mg GAE/100g (Alqarni *et al*., 2019, Nematallah *et al*., 2018, Abdelbaky *et al*., 2023) while phenolic content of Ajwa seed has been reported as 7.80mg to 3932mg GAE/100g (Hussain *et al*., 2019, Abdelbaky *et al*., 2023). Regarding flavonoid content, Ajwa date fruit has been reported to contain 2.8mg to 1300 RE mg/100g (Ismail Hamad *et al*., 2015, Abdelbaky *et al*., 2023) while in Ajwa seeds, 4.54mg to 2956 RE mg/100g was reported (Khalid *et al*., 2017, Hussain *et al*., 2019). However, exceptions exists; (Nematallah *et al*., 2018) reported higher TPC and TFC content in aqueous acetone/ethanolic fraction of Ajwa date fruit i.e., 184.18mg GAE/g and 31.25mg RE/g. Another study on unmatured Ajwa date seed found TPC and TFC levels as 77mg GAE/g and 58mg CE/g (Alshammari *et al*., 2024). In continuation, aqueous acetone extract of pulp and peels (separated) eluted with absolute ethanol reportedly provided high polyphenolic content i.e., 184and 144mg GAE/g of extract (Nematallah *et al*., 2018). Similarly, TPC and TFC of ethanol extract of Ajwa fruit provided 93mg GAE/g and 71mg QEC/g respectively (Zihad *et al*., 2021), might be due to difference in the sample type, collection time as well as extraction technique. Among the individual APS extracts, the highest TPC content was found in APS-EA extract while lowest was observed in APS-HX. In terms of flavonoids, the highest TFC was found in APS-M. It is generally acknowledged that the polarity of the extraction solvent plays a critical role in chemical extraction and isolation, as well as the type of compounds extracted. The literature indicates variability in the quantitative analysis of phenolics in Ajwa date pulp and seeds, with values affected by the choice of extraction solvent. Such as, in phenolic content in methanolic extract of Ajwa pulp ranges from 5.78 to 291mg GAE/100g (Alqarni *et al*., 2019, Arshad *et al*., 2019) while ethanolic extracts of Ajwa pulp show phenolic content in between 224 to 650mg GAE/100g. Likewise, in Ajwa pits, methanolic extracts reported phenolic content between 7.8 to 1204mg GAE/100g and ethanolic extracts ranged from 3154 to 3932mg GAE/100g (Ahmed *et al*., 2016, Khalid *et al*., 2017). As for flavonoid content, methanolic and ethanolic extract of Ajwa pulp contain 46.7 to 46.98mg CE/100g and 42 to 64.31mg RE/100g, respectively (Alqarni *et al*., 2019, Ahmed *et al*., 2016), while in Ajwa pits, flavonoid content was ranges from 530mg CE/100g in methanolic extract to 1897 to 2956mg CE/100g in ethanolic extracts (Khalid *et al*., 2017, Ahmed *et al*., 2016). The hydroalcoholic extract of Ajwa pulp showed 11.65mg to 23.97mg GAE/g and 6.66mg to 10.28mg RE/g phenolic and flavonoid content respectively (Abdelbaky *et al*., 2023). The antioxidant of APS extracts showed that the hexane extract, APS-HX, showed the lowest potency in terms of activity and content, both in DPPH● and ABTS●+ assay. Ascorbic acid (1mg/ml) was used as positive control and 96% oxidation inhibitory activity was witnessed (not shown in table). Concentration dependent radical scavenging activity of ascorbic acid was not performed as it reached a stable plateau irrespective of concentration as reported in earlier studies (Lin *et al*., 2014). Further, no significant difference found in the values of antioxidant content calculated through two different calibration curves. i.e. ascorbic acid and trolox. Limited literature is available on the radical scavenging activity of Ajwa fruit and showed either low scavenging activity or at higher sample concentration (Kadum *et al*., 2019, Shahbaz *et al*., 2022). However, a study conducted on effect of chilling and storage on phenolic content and antioxidant capacity of Ajwa date fruit suggested comparable IC50 values (Samad *et al*., 2016). Further, aq. acetone extract of Ajwa pits reportedly showed lesser EC50 value at 0.153mg/ml (Shahbaz *et al*., 2022). Similarly, lower IC50 values (18.6 to 125µg/ml) are reported on Ajwa fruit extracted with ethanol (Kadum *et al*., 2019, Zihad *et al*., 2021). The radical scavenging mechanism of DPPH● and ABTS●+ is through single electron transfer (SET) and hydrogen atom transfer reaction (HAT) respectively. The discoloration of oxidizing solution after reduction of radical ion is measured which is proportional to the concentration of radicals being scavenged (Bibi Sadeer *et al*., 2020). The outstanding antioxidant potential of APS-EA and APS-M extracts indicates the presence of plentiful amount of free radical scavengers such as polyphenolic, terpenes, and phytosterols. Moreover, we have also observed that antioxidant assays are very much specific to the type of antioxidant content been evaluated. For instance, the antioxidant activity in DPPH● and ABTS●+ assays are more synchronous towards phenolic content ($p<0.01$) than flavonoid content, same as reported by (Rejeb *et al*., 2020). The reason might be the presence of certain specific compounds which are giving more observable antioxidant values in each of these antioxidant assays. In β-carotene bleaching assay, the extract rich in non-polar constituents, i.e., APS-HX, showed significant activity. This may indicate that unsaturated and polyunsaturated fatty acids play pivotal role in neutralizing and delaying the oxidative effect of pro-oxidant species. By far β-Carotene assay is not reported in literature on Ajwa date fruit separately or in combination of pulp and seed. However, work on other date fruit varieties had been reported and stated much higher antioxidant activity, i.e., 95-98% (Anjum *et al*., 2012). The reason behind significant antioxidant activity of APS-HX could be the

presence of unsaturated fatty acid and terpenes. These compounds compete with β-carotene, to quench lipid peroxyl radical generated by oxidation of linoleic acid in aqueous emulsion (Bibi Sadeer *et al*., 2020), which otherwise discolour β-carotene due to the breakage of π-conjugation in between carbon atoms. Hence, the antioxidant activity is accessed by measuring delay in the rate of β-carotene bleaching which indicate the radical scavenging ability of the test sample (Bibi Sadeer *et al*., 2020). Erythrocytes with oxidative damage are a hallmark of a number of disorders, including Type-2 diabetes, obesity, hypertension, and various cardiovascular issues. Particularly, the role of RBC eNOS in regulating the mechanisms of cardiovascular has already been established (Massaccesi *et al*., 2020). Catalase, glutathione peroxidase, superoxide dismutase, and other potent antioxidant mechanisms are present in RBCs (Melo *et al*., 2024). Their alteration could disturb the redox balance by boosting the production of pro-oxidizing molecules up to an oxidative stress state (Melo *et al*., 2024). Erythrocyte hemolysis caused by AAPH serves as a useful experimental model for investigating membrane damage brought on by free radicals and for assessing the antioxidant activities of different antioxidants. The latter during thermal degradation results in a constant production of peroxyl radicals (ROO), the main oxidant species responsible for cell hemolysis caused by both lipid peroxidation and protein oxidation (Niki, 1990). When AAPH is used as an initiator in aqueous solutions at physiological temperature (37 °C), an alkyl radical (R•) is produced, and when this radical is exposed to oxygen, it transforms into the appropriate peroxyl radicals (ROO•). AAPH has a half-life of approximately 175 hours at 37 °C in neutral water and produces radicals at a rate of 1.3 106 [AAPH]/s (Banerjee *et al*., 2008). These peroxyl radicals cause a series of processes known as lipid peroxidation when they oxidize polyunsaturated lipids in RBC membranes. This lipid peroxidation leads to hemolysis (the release of hemoglobin) and intracellular K+ ions, which cause the RBC membrane to rapidly deteriorate and lose its integrity. This approach has the benefit of producing radicals without biotransformation or enzymes involvement since the azo molecule decomposes thermally. Water-soluble radical scavengers efficiently remove the radicals from the aqueous phase before the peroxyl radicals generated by AAPH damage the erythrocyte membranes. As a result, the membranes are protected from oxidative harm. However, lipid-soluble antioxidant scavenges radicals mostly from inside the membranes thus preventing erythrocyte injury. Our studied also confirmed that combine extract is potent in dose dependent manner with a maximum effect is produced at 4mg/ml in preventing AAPS induce hemolysis. In earlier studies, oxidative hemolysis inhibitory activity of Moroccan dates was reported on RBCs obtained from rabbit, at much lower percent, 0.44-33.84% (Bammou *et al*., 2016). Recently, the protective effects of Ajwa fruit and seed extract against heat and hypo-saline-induced RBCs membrane hemolysis were also investigated (Anwar *et al*., 2022) and showed 58-63.84% in methanol extract. In addition to this, literature suggested role of Ajwa dates in the maintenance of hemoglobin levels in young and pregnant anemic women (Ali *et al*., 2020) further confirming the role of Ajwa dates in RBC protection and maintenance. It is evident that APS extracts are an amalgam of water soluble and lipid soluble antioxidant and provide broader antioxidant spectrum. It is well known that free fatty acids (FFA) and fatty acids esters (FAE) play a dual role in indirect ROS scavengers and oxidation propagation, both of which are fully dependent on the existence of double bonds. They also have the ability to regulate oxidative and inflammatory stress pathways (Nagy *et al*., 2017). Contrarily, many in vitro and in-vivo studies have shown that PUFA can scavenge ROS and RNS by acting as a sacrificial antioxidant, or by oxidizing itself in order to protect another antioxidant (Frenoux *et al*., 2001, Ambrozova *et al*., 2010). Some flavonoids, such quercetin, have been shown to have anti-hemolytic activity in the past, and the high flavonoid content of the studied extracts may be the cause of their potent effect. Although in this study, detection of individual phenolic and flavonoid has not done but a vast literature revealed the presence of these phenolics and flavonoids in Ajwa. According to reports (Anosike *et al*., 2019), flavonoids have the innate potential to stop the hemolysis of RBCs brought on by free radicals. In addition, it was discovered that including vitamin C and trolox into the RBC medium significantly decreased the likelihood of hemolysis in human RBCs that were maintained at a cold temperature (Czubak *et al*., 2017). The mechanisms behind the antioxidant activity of polyphenols remain a topic of active debate (Dai *et al*., 2010) and require further research to be fully understood. The ability of these substances to partition in cell membranes and the resulting restriction on membrane fluidity, however, have been theorized to sterically inhibit free radical transit and hence lessen the kinetics of free radical events. Their aromatic or phenolic ring have the intrinsic ability to donate their H+ to free radicals and converting themselves into radical intermediates, stabilize by resonance delocalization of electrons (Dai and Mumper, 2010). Besides, phytosterols and terpenes are also known to interact with free radicals and disrupt the chain reaction. Considering above, the overall effect of APS extract might be due to synergism between these bioactive scaffolds, the same concept is reported with other plants and their parts (Meghwar *et al*., 2024). Pearson's correlation was also accessed (table 5) and strong positive correlation was observed between phenolic content, DPPH ($r2=0.8671$; $p<0.01$) and ABTS scavenging activities ($r2=0.9280$; $p<0.001$). While flavonoid content showed little or no correlation with DPPH and ABTS inhibitory activities with respect to solvent extract. This

could be due to quantitative difference in polyphenolic contents with respect to different solvent extracts i.e., APS-EA contains the most phenolic content while APS-M found to be rich in flavonoid content. Nevertheless, a number of past investigations have demonstrated a favorable association between phenolic and flavonoid content and DPPH/ABTS activities. But in our study, as mentioned earlier, values of DPPH● and ABTS●+ percent inhibition is more aligned with phenolic content than flavonoid contents, then it could be said that flavonoids radical scavenging potential is less detected by DPPH● and ABTS●+assays which is in consensus with (Saeed *et al*., 2012). On contrary, some studies show strong correlation of flavonoid with DPPH/ABTS as compared to phenolics (Nickavar *et al*., 2012). As far as β-carotene bleaching assay, significant negative correlation was observed among β-carotene and DPPH● ($r2=-0.9795$; $p<0.001$) and ABTS●+($r2=-0.9451$; $p<0.001$) activity. As β-carotene assay involved working with emulsified system, the compounds which are lipophilic can stay in the hydrophobic layer and thus can produce antioxidant effect which is in our case confirmed by high oxidation inhibitory activity of APS-HX i.e., 69%. This is the reason why polar standard compounds such as butylated hydroxytoluene (BHT) and ascorbic acid showed less antioxidant activity as compared to the extract. The negative correlation among β-carotene, polyphenolic and antioxidant activity compound is also confirmed by several other studies (Pekkarinen *et al*., 1999). Furthermore, a strong linear correlation TPC was found with DPPH● ($r2=0.7508$) and ABTS●+ ($r2=0.8586$). Additionally, a robust correlation was noted between DPPH● and ABTS●+ values ($r2=0.9813$). A significant linear relationship was also found between β-carotene and DPPH ($r2=0.9624$) as well as ABTS values ($r2=0.8935$). The pronounced membrane protective effect of APS extracts motivated us to perform molecular docking studies on selected chemical compounds derived from Ajwa date fruit. Two target proteins were chosen; human erythrocytic spectrin (membrane protein of RBC) and human GPX4 (antioxidant enzyme). These proteins were selected to further investigate the underlying mechanism of antihemolytic activity of APS extract. Significant binding interactions observed with selected compounds suggests a potential mechanism for erythrocytic membrane stability through spectrin and GPX4. Thus, it might be suggested that the overall antioxidant effect is might due to synergistic mechanism of these fatty acids, phenolic acid, flavonoids and sugars. The individual bioactivity of these chemical constituents had already been established (Elshafie *et al*., 2023) supporting the diverse biological activities of Ajwa date extracts.

CONCLUSION

This study presents the first comprehensive evaluation of the phytochemical composition and antioxidant activity of the combined Ajwa pulp and seed (APS) extracts. Among the solvent fractions, the ethyl acetate extract (APS-EA) exhibited the highest phenolic content and antioxidant activity, followed by the methanolic (APS-M) and hexane (APS-HX) extracts. *Ex vivo* assay demonstrated significant protection by APS extracts against oxidative hemolysis of human red blood cells, with antioxidant activity decreasing in the order: APS-EA > APS-M > APS-HX. DPPH radical scavenging activity, measured using both AAE and TEAC standard methods, yielded comparable results, suggesting either method is suitable for antioxidant quantification. GC-MS profiling of the APS extracts and methanolic fractions revealed a diverse range of bioactive compounds, including saturated and unsaturated fatty acids, phytosterols, and terpenes. Notably, NMR analysis of APS-M confirmed the presence of pure α- and β-glucose. These sugars, along with other metabolites such as fatty acids, may contribute to membrane protection, as evidenced by their significant anti-hemolytic activity. *In silico* docking studies further supported these findings, showing favorable interactions between NMR-characterized glucose isomers and reported phytochemicals with RBC membrane proteins and antioxidant enzymes. The antioxidant and anti-hemolytic effects of APS extracts appear to result not only from polyphenols but also from synergistic interactions among various phytoconstituents, enhancing overall therapeutic efficacy. The observed anti-hemolytic potential highlights the pharmacological relevance of APS extracts, particularly in the context of hematological disorders such as hemolytic anemia. However, further in-depth biological studies are needed to confirm these effects and explore their possible cardioprotective properties.

Ethical declaration

The human blood samples were obtained on consent of healthy volunteers, approved by Ethical Review Board, Hamdard University, Ref# AEC-18-01.

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Conflict of interest

There is no conflict of interest.

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