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Structural elucidation and evaluation of antioxidant and *In vitro* anti-hemolytic properties of polysaccharide-iron complexes from *Piper betel* and *Triticum aestivum*

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Iron is an essential trace element that the human body needs for cellular metabolism and oxygen transportation. Iron is widely used as a supplement for the treatment of iron deficiency or anemia, which can have many negative consequences. Polysaccharide iron complexes have been widely studied and shown to possess biological activities. The objectives of the current study were to prepare polysaccharide iron complexes from *P. betel* leaves and *T. aestivum*grass and to measure, characterize and assess their antioxidant activity. The results revealed that *Piper betel* polysaccharide had a high yield percentage of 2.13±0.78 percent and a polysaccharide content of 65.92±0.007%. A high iron content of 16.39% was found in PBPS-Fein comparison to 9.5% in TAPS-Fe. Characterization reveals information about structural properties. The FTIR spectra showed similarities between the polysaccharides and iron complexes, indicating that complexation did not disturb structure or functional groups. PBPS-Fe and TAPS-Fe attain thermal stability at 507.37°C and 610.28°C, respectively. The morphological images of polysaccharides revealed rough and irregular structures and the complexes were smooth and had a sheet-like structure. *Piper betel* and *Triticum aestivum* iron complex showed the highest antioxidant and anti-hemolytic activity in comparison to those of PBPS and TAPS.The complexation of polysaccharides improves their physicochemical characteristics and antioxidant activity, anti-hemolytic activity according to the study's findings. Further research has explored their biological activities and these polysaccharide iron complexes can be used as organic supplements and food fortifiers.

Keywords: Anaemia, Antioxidant, Iron content, Iron, Polysaccharide iron complex

Iron is an essential trace metal in the human body that is vital for several enzymatic processes, the most well-known of which are the transportation of oxygen and cellular metabolism[1]. Iron deficiency (ID) is a common root cause of anemia worldwide. Hence, ID and anaemia are frequently used interchangeably, and the anemia is frequently used as a substitute for IDA. Iron deficiency is speculated to be responsible for 50% of all instances of anemia[2]. Anemia is a hematological disorder and it is a global health problem that affects populations in both affluent and poor countries[3].

Oral iron supplements are needed in modern culture to treat and prevent iron deficiency in humans[4]. Oral consumption of iron complexes leads to side effects, such as nausea and vomiting[5]. When valent iron complexes enter the body, they produce free radicals, which are prone to damage the cell membrane[6].

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Natural products have always made significant contributions to the advancement of contemporary medicine and are still vital to the process of finding new drugs. *Butea monosperma* bark has been shown in earlier research to have antioxidant and antihyperglycemic effects[7]. The alcoholic extract of *Entada persuatha* demonstrated a protective effect in a model of DSS-induced mice, according to published data[8]. Similarly, ethanolic extract of *Clerodendrum infortunatum* exhibited significant antioxidant activity[9].

Polysaccharide iron complexes are usually composed of ferric ions combined with polysaccharides thereby forming a stable supplement which may probably be used as an effective iron supplement with high iron content[10,11]. These compounds are soluble and nontoxic at physiological pH values[12] Various studies exhibited that metal complexation with bioactive compounds enhances their activity and bioavailability. Amino acid-derived metal complexes exhibit various biological effects, including antitumor, antimalarial, and

antioxidant activities[13]. Additionally, zinc-genistein and Zinc- *A. paniculata* conjugated nanocomplexes demonstrated greater antioxidant activity than genistein and *A. paniculata* alone[14,15].

Piper betel leaves are rich in bioactive compounds. They belong to the family *Piperaceae*. *P. Betle* has various names in different countries. Paan is most frequently used in India, Pakistan, Nepal and Bangladesh[16]. Previously known *Piper betel* leaves have antioxidant[17,18], insecticidal and antitumour[19], neuroprotective[20], antidiabetic and anti-helmintic[21], anti-microbial[22] properties. Similar studies have been reported in *Calotropis gigantea* which a perennial herb possessing analgesic, anthelmintic, astringent, anti-inflammatory, wound healing, sedative, anti-asthmatic, antimicrobial, antioxidant, procoagulant, hepatoprotective and hypoglycemic properties[23].

Wheatgrass refers to the young grass of the common monocot wheat plant "*Triticum aestivum*"[24]. It belongs to the *Poaceae* family. *T. aestivum* grass and its extracts are reported to be high in proteins, vitamins, minerals, active enzymes, and bioactive substances such as alkaloids, glycosides, saponins, steroids, tannins, phenols, and flavonoids[25,26]. *T. aestivum* grass and its extracts possess antioxidant activity[26,27]. *T. aestivum* grass has therapeutic applications in debilitating diseases such as cancer. Due to its antioxidant properties, it aids in the management of the majority of degenerative diseases such as diabetes and cardiovascular diseases. It has been shown to be effective in the treatment of anaemia, diabetes, eczema, constipation, kidney swelling, and the common cold[25].

Khan *et al.*[28] reported presence of bioactive compounds *viz* 2,4-dihydroxybenzaldehyde in *Piper betel* followed by catechin and chlorogenic acid in *Triticum aestivum* through HPLC analysis[29]. *In silico* studies demonstrated effective binding with hepcidin, a liver-synthesized hormone that regulates iron metabolism[28]. Catechin has also been reported to possess hepatoprotective, antidiabetic and anticoagulant pharmacological properties[30].

The present study aimed to prepare *Piper betel* leaves and *Triticum aestivum* grass polysaccharide iron complex polysaccharides and compare them through characterization and antioxidant assays.

Materials and Methods

Triticum aestivum grass was collected from the Crop Research Centre, School of Agriculture, ITM University, Gwalior, Madhya Pradesh.*Piper betel* leaves were procured from village Sandalpure, Antri of Gwalior, Madhya Pradesh. All supplementary chemicals and reagents were of analytical grade and were purchased locally.

Polysaccharide extraction

Dried powders of *T. aestivum* grass and *P. betel* leaves were mixed with water at a ratio of 1:20. The extraction temperature and extraction time were set at 80°C and 6 h, respectively. The filtrate was collected and reduced to a one-fourth volume in a rotary evaporator[31,32]. Deproteinization was performed by the TCA method followed by decolorization. Polysaccharides were precipitated by the addition of anhydrous alcohol added at a ratio 1:15 and incubated at 4°C for 12 h followed by centrifugation at 4000 rpm for 15 min. Three subsequent washes with alcohol were performed for the complete removal of debris[31]. The precipitates were dried and stored at 4°C. The polysaccharides were obtained from *Piper betel* leaves and *Triticum aestivum* grass and were named as PBPS and TAPS, respectively. The extraction yield (%) was calculated as follows:

$$\text{Extraction yield (\%)} = \frac{\text{Weight of dried polysaccharide}}{\text{Weight of dried plant powder}} \times 100 \quad \ldots (1)$$

Determination of carbohydrate content and protein content

The total polysaccharide content was quantitatively estimated by the phenol sulfuric method[33]. The total protein content was evaluated by using the CBB-G250 technique with Bovine Serum Albumin as a reference at 595 nm[34].

Iron complex preparation

Dried crude polysaccharides and sodium citrate were mixed at a ratio of 2:1. The solutions were neutralized and heated to 70°C. Addition of 2M FeCl3 and NaOH led to the formation of a brown precipitate, which was subsequently centrifuged. The supernatant was dialyzed, precipitated with 95% ethanol and freeze-dried. *Piper betel* and *Triticum aestivum* polysaccharides are referred to as the PBPS-Fe complex and TAPS-Fe complex, respectively[35].

Qualitative identification of polysaccharide iron complexes

Ten milligrams of dried PBPS and TAPS were dispersed in 2 mL of double distilled water and themixture was then stirred at room temperature for 2 h. After adding a few drops of potassium thiocyanate to the solution, the reaction phenomenon was monitored. Similarly, 10 mg of PBPS-Fe and TAPS-Fe complex was dispersed in 1MHCl and agitated for 2 h at room temperature. After adding a

few drops of potassium ferrocyanide, the reaction phenomenon was monitored and recorded[36].

Quantification of the iron content in the polysaccharide iron complexes

Ten milligrams of dried PBPS, TAPS, PBPS-Fe, and TAPS-Fe complex were dispersed in 20 mL of HCl (1 M) by stirring for 24 h to break the compounds and liberate the iron. Then, 1 mL of each solution was combined with 1 mL of hydroxylamine hydrochloride (10%) 2.5 mL of 1,10-phenanthroline (10%) and 5 mL of sodium acetate trihydrate buffer solution (pH 4.5) and the absorbance of mixture was recorded at 510 nm. The calibration curve was obtained by using standard solutions of ferrous ammonium sulfate in distilled water based on the following formula: $y = 0.0045x - 0.170$, where y = absorbance and x = conc. ($R^2 = 0.9972$)[37].

Fourier Transform Infrared Spectroscopy (FTIR)

Perkin Elmer spectra Two 105627 FT-IR was used to characterize the infrared spectra of the polysaccharides and complexes. The frequency ranged from 4000-500 cm^{-1}[,38].

Thermogravimetric analysis (TGA)

Thermogravimetric analysis was performed by using a thermogravimetric analyzer (Shimadzu's TGA- 50 series). A sample weighing between 10 to 15 mg was heated in a nitrogen environment at a rate of 10°C/min, and the flow rate was 30 mL/min[39].

High-performance liquid chromatography (HPLC)

Ten milligrams of polysaccharide and polysaccharide iron complex were dissolved in 5 mL of 2M TFA at 90°C for 3 h. followed by centrifugation for 5 min[40]. The hydrolysates were repeatedly co-evaporated with ethanol at 45°C to remove the excess acid. Then, the hydrolysates were derivatized with PMP. They were combined with a methanol solution of PMP (0.5 M) and 0.5 mL sodium hydroxide solution (0.3 M). The derivative reaction was carried out for 30 min at 70°C, followed by centrifugation at 10000 g for 5 min. To remove the excess PMP, chloroform was used. Subsequently, a membrane (0.22 μM) was used to filter the aqueous layer. The monosaccharide compositions of the polysaccharide and complex were determined from the filtrate with a Shimadzu HPLC system with RI and PDA detectors. The RP C18 column (4.6 × 250 mm, 5 μM) was used at 25°C. The mobile phase consisted of acetonitrile and phosphate buffer saline (pH 7) and was used at a ratio of 20:80 (*v/v*) with a flow rate of 1 mL/min.[41].

Scanning electron microscopy (SEM)

Scanning electron micrographs of PBPS, TAPS, PBPS-Fe, and the TAPS-Fe complex were obtained by Joel field Emission electron microscope. Each sample was observed at 5000- and 20,000-fold magnification at an accelerating voltage of 20.0 kV[42].

Antioxidant activity

1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Radical Scavenging Activity

Blois technique was used to assess DPPH free radical scavenging activity in various fractions. Aliquots of sample and extracts from 200-1000 μg/mL in distilled water and 1 mL of DPPH solution (0.1 mM in methanol) were added, shaken well, and incubated at 37°C for 30 min in the dark. The absorbance was measured at 517 nm against a suitable blank[43]. The DPPH radical scavenging activity was calculated by using the following equation:

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{(A_1 - A_2)}{A_0} \times 100 \quad \ldots (2)$$

Metal Chelating Activity

The metal chelating activity was determined using the method described by Chew *et al*[44]. A mixture of 0.2 mL plant extract at varying concentrations, 0.2 mL $FeSO_4$ (0.1 mM) and 0.4 mL ferrozine (0.25 mM) was prepared. After incubating the mixture at room temperature for 10 min, the absorbance was measured at 562 nm[44]. The metal chelating activity was then calculated using equation (i).

Reducing power assay

A mixture of 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferrocyanide was added to 1 mL of various fractions of plant extract at different concentrations (200–1000 μg/mL) diluted in distilled water. The test tubes were incubated in a water bath at 50°C for 10 min. After incubation, 2.5 mL of 10% TCA was added, and the mixture was centrifuged at 3000 rpm for 10 min. From the supernatant, 2.5 mL was collected and mixed with 2.5 mL distilled water, followed by the addition of 0.5 mL freshly prepared 0.1% $FeCl_3$. The increase in absorbance was measured at 700 nm against an appropriate blank[45].

Ferric reducing antioxidant power (FRAP)

The total Ferric Reducing Antioxidant Power was measured using a modified method by Benzie and

Table 1 — Characteristics of the crude polysaccharides of *P. betel* leaves and *T. aestivum*

Characteristics	PBPS	TAPS
Color and appearance	Dark brown powder	Pale yellow powder
Yield (%) (*w/w*)	2.13±0.56	1.6±0.12
Polysaccharide content (%) (*w/w*)	65.92±0.007	27.92±0.01
Protein content (%)	-	-
Solubility	Soluble	Sparingly soluble

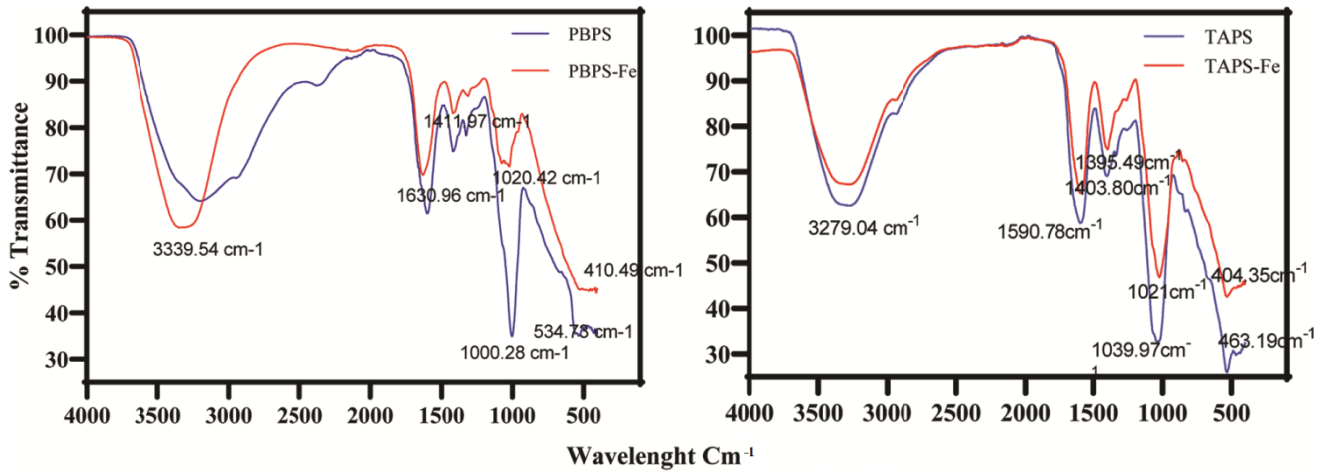

Fig. 1 — FTIR spectra of (A) PBPS and PBPS-Fe; and (B) TAPS and TAPS-Fe

Strain. A reaction mixture of plant extract (10-50 mg/mL) and freshly prepared FRAP reagent was incubated in the dark for 30 min. Absorbance was measured at 593 nm, and a standard curve using ascorbic acid was used to express the results as ascorbic acid equivalents (AAE/g)[46].

Anti-haemolytic activities

H_2O_2 induced anti-hemolytic activity

The method involved collecting whole blood, separating the red blood cells (RBCs) through centrifugation, and washing them with PBS to create a 4% RBC suspension. Plant extract (1 mL, 10 mg/mL) was added to 2 mL of the RBC suspension, followed by incubation with H_2O_2. After incubation at 37°C for 30 min, the absorbance of the supernatant was measured at 540 nm, and the percentage inhibition was calculated by using equation (ii)[47].

$$Percent\ inhibition = \frac{(Abs\ of control - Abs\ of sample)}{Abs\ of control} \times 100 \ldots . (3)$$

Statistical analysis

All experiments were carried out in three measurements. Data are expressed as mean±SE. Statistical analysis of data was performed using Graphpad prism.

Results

Extraction of polysaccharides and preparation of iron complexes

Table 1 shows the physical appearance, polysaccharide content, and yield of the plants. TAPS is pale yellow in color, while PBPS is a brown color powder. The percent yield and polysaccharide content of TAPS and PBPS were 1.6±0.18, and 2.13±0.78 and 27.92±0.01, 65.92±0.007, respectively. The physical appearance of the PBPS-Fe and TAPS-Fe complex was a reddish-brown powder.

Qualitative and quantitative identification of iron, PBPS-Fe and TAPS-Fe

When potassium ferrocyanide or potassium thiocyanate reagents were added to an aqueous solution of the PBPS-Fe and TAPS-Fe complex, the appearance did not change. In contrast, when potassium ferrocyanide was added to the PBPS-Fe and TAPS-Fe complexes in a hydrochloric acid solution, a dark blue precipitate appeared. Similarly, when potassium thiocyanate was added, a blood-red flocculent appeared.

The iron content was calculated by the spectrophotometric method. The iron content in PBPS-Fe was 16.39%, and that in TAPS-Fe was

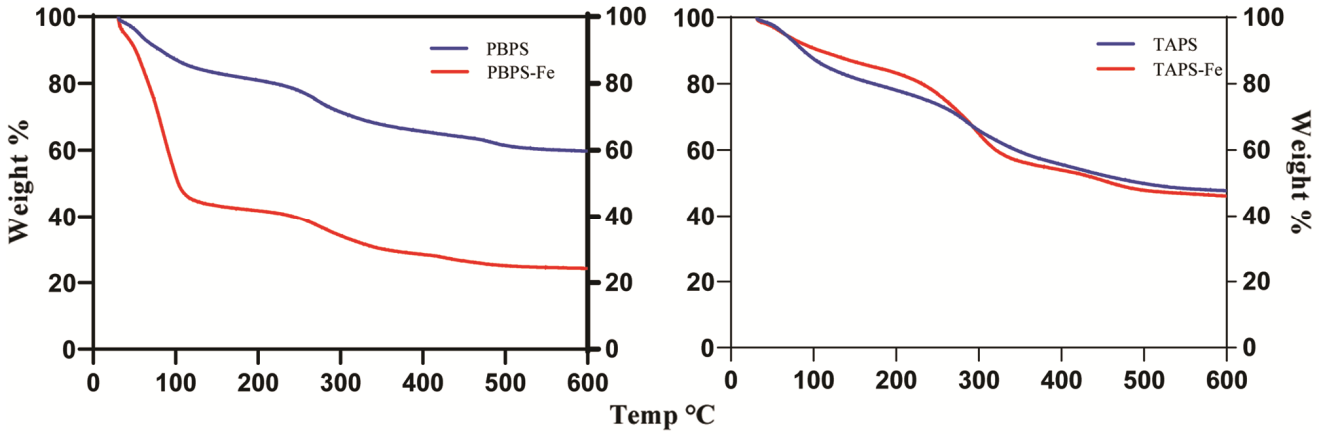

Fig. 2 — Thermogravimetry curves of (A) PBPS and PBPS- Fe; and (B) TAPS and TAPS-Fe

9.5%. Compared with TAPS-Fe, PBPS-Fe hasa high iron content.

FTIR analysis

Figure 1A and B represents the infrared spectra of *P. betel* leaves and *T. aestivum* grass polysaccharides and complexes, respectively. The strong broad absorbance bands at 3196.09 cm^{-1} and 3279.04 cm^{-1} confirmed the presence of the -OH functional group. A prominent, narrow signal at 1598.97 cm-1 in PBPS and 1590.78 cm^{-1} in TAPS indicates the presence of a carbonyl group (=CO). A strong and sharp peak at 1008.28 cm^{-1} and 1039.97 cm^{-1} in both PBPS and TAPS, respectively, indicate the presence of a primary alcohol group (-CH). The fingerprint region was showed a peak at 543.73 cm^{-1} in the *P. betel* spectrum and at 463.19 cm^{-1} in the *T. aestivum* polysaccharide IR spectrum. The migration of the absorption peaks at 1000.28 cm^{-1} and 1598.97 cm^{-1} to 1020.42 cm^{-1} and 1630.96 cm^{-1}, respectively, may be due to complexation.

TGA analysis

The stabilities of PBPS, PBPS-iron complex, and TAPS and TAPS-iron complexes were investigated by thermogravimetric analysis (Fig. 2A and B, respectively). In the current study, the temperature increased from 30 to 600°C. The rate of weight loss of PBPS was 40.28%. The rate of mass loss is stable at 571.56°C. The first weight loss of PBPS-iron complex was 52.5%, at 99.99°C followed by second weight loss which was 27.5% at 649.80°C thus final weight loss was 75.76%. The PBPS-iron complex attained thermal stability at 507.37°C. Similarly, the weight loss of TAPS was 52.60% and the rate of mass loss was stable at 580.77°C. The rate of mass loss of the TAPS-iron complex at 649.80°C was 54.16%. The TAPS-iron complex attained thermal stability at 610.28°C. The PBPS-Fe attained thermal stability at 507.37°C whereas, TAPS-Fe which attained thermal stability at 610.28°C.

HPLC (monosaccharide analysis)

Four monosaccharides were identified *i.e.* glucuronic acid, galactouronic acid, glucose, and arabinose in PBPS, and glucuronic acid, glucose, arabinose, and fucose were identified in TAPS (Fig. 3A and B). The changes in monosaccharide content and retention time between PBPS and PBPS-Fe and between TAPS and TAPS-Fe may be due to the presence of ferric ions in the monosaccharide residue chain monosaccharide.

SEM (Morphology analysis)

As shown in Figure 4A (i) PBPS had an irregular rough surface with lumps at ×5000 and PBPS-Fe had a smooth surface (Fig. 4A (i)). At ×20,000 PBPS exhibited a flocculent structure (Fig. 4B (ii)). The surface of PBPS-Fe is relatively flat and smooth in comparison to that of PBPS.As shown in (Fig. 4C (i)) the TAPS image revealed irregular lumps, while the TAPS-Fe had a smooth surface at ×5000 (Fig. 4D (i)). After 20,000-fold augmentation the polysaccharide had a flocculent structure (Fig. 4C (ii)) while in TAPS-Fe, had a flat smooth surface (Fig. 4D (ii)).

Antioxidant activity

DPPH activity

As illustrated in Table 2, the highest DPPH scavenging activity was observed at a concentration of 1 mg/mL. The antioxidant activity of the TAPS-Fe and

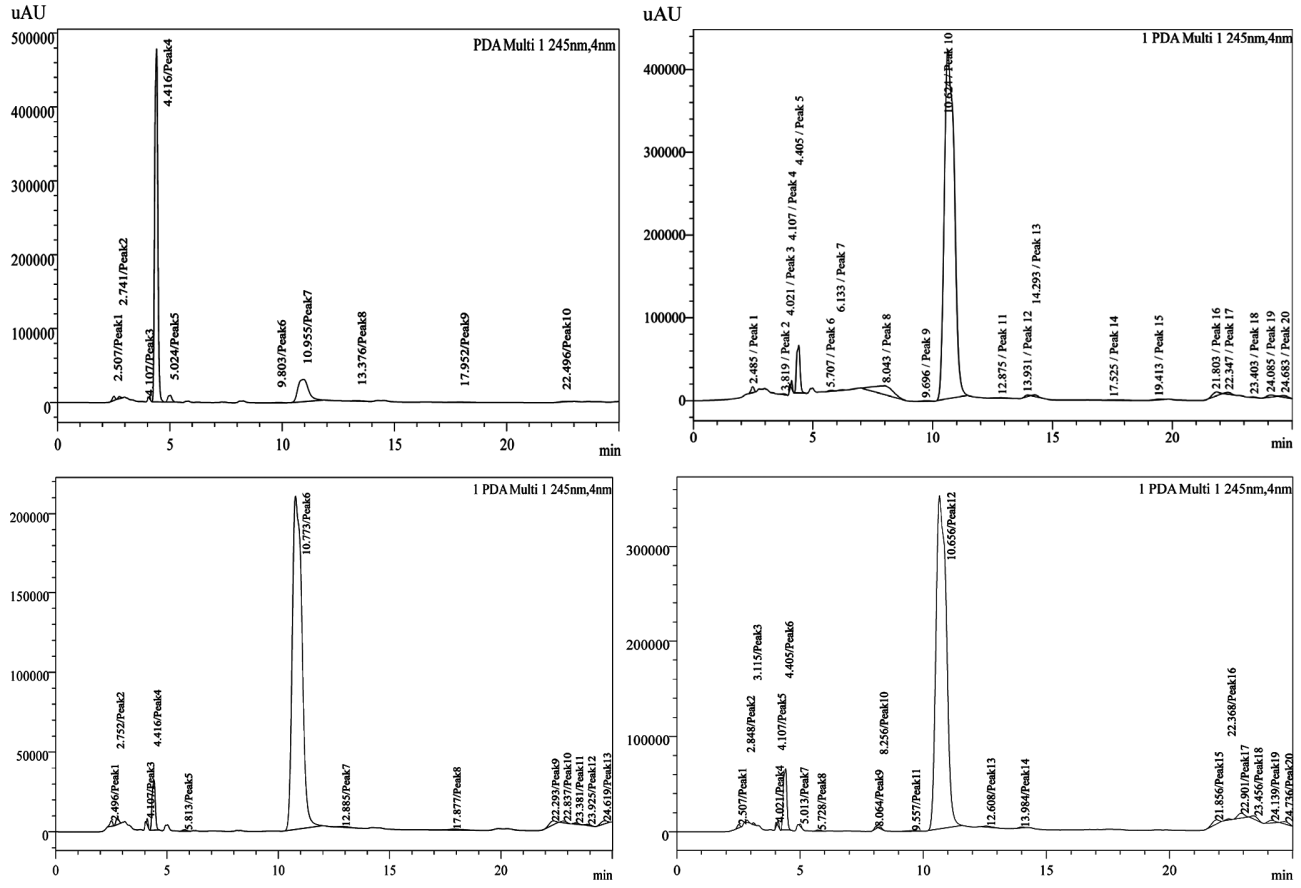

Fig. 3 — HPLC chromatograms of the (A) PBPS; (B) PBPS-Fe; (C) TAPS; and (D) TAPS-Fe monosaccharides

PBPS-Fe complexes surpassed that of their respective ligands, TAPS and PBPS. Among the tested samples, the PBPS-Fe complex exhibited the highest activity at 82.6%, followed by TAPS-Fe at 77.96%, PBPS at 57.19%, and TAPS at 54.53%, all at 1 mg/mL. Notably, the PBPS-Fe complex maintained superior activity across the concentration range of 0.2–1 mg/mL compared to TAPS, PBPS, and TAPS-Fe.

Metal Chelating Activity

Table 3 illustrates the metal chelating activity of EDTA, TAPS, PBPS, TAPS-Fe, and PBPS-Fe. The chelating activity increased with rising concentration, reaching its peak at 1 mg/mL. Notably, the TAPS-Fe and PBPS-Fe complexes exhibited greater activity compared to their respective ligands, TAPS and PBPS. The maximum chelating activities recorded were 48.42% for TAPS, 49.15% for PBPS, 55.08% for TAPS-Fe, and 53.81% for PBPS-Fe.

Reducing power assay

Table 4 presents the ferric reducing power activity of TAPS, PBPS, TAPS-Fe, and PBPS-Fe. The TAPS-Fe and PBPS-Fe complexes demonstrated significantly higher reducing capabilities, achieving 82.33±0.11% and 72.2±0.64%, respectively, compared to 37.95% for TAPS and 35.50% for PBPS.

H_2O_2 induced anti-hemolytic activity

The anti-hemolytic effects induced by H_2O_2 are presented in (Table 5). The anti-hemolytic activity increased with higher concentrations. PBPS-Fe showed the highest hemolysis inhibition at 75.54±1.74%, followed by PBPS at 71.8±0.99%, TAPS-Fe at 63.6±4.22%, and TAPS at 44.94±9.29% at a concentration of 2 mg/mL. TAPS demonstrated the lowest inhibition across the concentration range of 0.5-2 mg/mL compared to the other three.

Ferric reducing antioxidant power (FRAP)

Table 6 represent the FRAP value. FRAP value were calculated by using the standard curve equation: $y = 0.5607x + 0.03614$, $R^2=0.9808$ and were expressed as mg/g Ascorbic acid equivalent (AAE). PBPS-Fe exhibited the highest reducing activity was 411.8 mg AAE/g followed by PBPS 260.13 mg AAE/g, TAPS-Fe 161.15 mg AAE/g, TAPS 61.27 mg AAE/g. The least value was 61.27 mg AAE/g in *T. aestivum* polysaccharide.

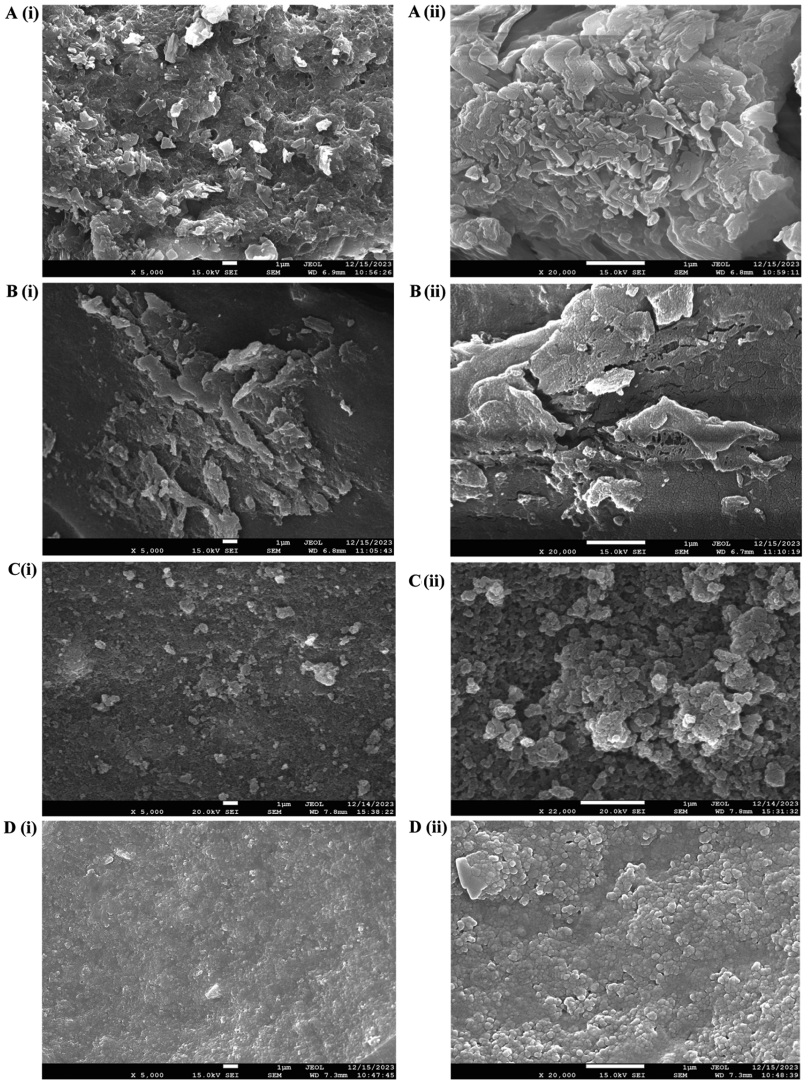

Fig. 4 — SEM images of (A) PBPS; (B) PBPS-Fe; (C) and TAPS; and (D) TAPS-Fe: (i) and (ii): ×5000 and ×20,000 magnification

Table 2 — DPPH radical scavenging activity of TAPS, PBPS, TAPS-Fe, PBPS-Fe and AA

Conc. (mg/mL)	AA	TAPS	PBPS	TAPS-Fe	PBPS-Fe
0.2	61.83±1.14	46.72±4.5	42.15±0.57	69.07±0.85	70±0.75
0.4	66.2±2.7	54.41±0.23	41.35±0.34	72.03±0.75	70.56±3.58
0.6	57.26±2.7	54.67±0.06	45.53±2.41	72.96±2.57	78.7±0.32
0.8	74.95±2.18	54.94±1.32	57.32±3.27	75.74±1.18	77.47±0.59
1	76.34±2.32	54.54±0.29	57.19±0.34	77.96±0.16	82.68±0.69

Data presented as mean±SE of three measurements

Table 3 — Metal chelating activity of TAPS, PBPS, TAPS-Fe, PBPS-Fe, EDTA

Conc. (mg/mL)	EDTA	TAPS	PBPS	TAPS-Fe	PBPS-Fe
0.2	29.67±2.98	24.28±1.02	20.89±1.26	44.07±0.98	17.27±0.08
0.4	57.9±3.18	25.72±0.34	31.16±1.31	33.05±0.49	32.29±1.2
0.6	59.6±4.65	29.66±0.80	40.08±0.72	33.62±3.18	34.38±0.08
0.8	80.22±0.75	35.56±1.48	43.94±1.43	56.22±0.75	52.37±0.16
1	81.92±1.23	48.42±0.9	49.16±3.79	55.09±0.49	53.82±0.4

Data presented as mean±SE of three measurements

Table 4 — Reducing power activity of TAPS, PBPS, TAPS-Fe, PBPS-Fe and AA

Conc. (mg/mL)	AA	TAPS	PBPS	TAPS-Fe	PBPS-Fe
0.2	87.49±0.93	38.84±0.26	35.33±0.15	77.27±0.1	65.5±2.55
0.4	88.96±0.41	40±10	36.04±0.15	76.64±0.33	66.07±1.51
0.6	89.55±0.07	39.19±0.05	36.4±0.3	80.91±0.55	68.87±1.76
0.8	89.67±0.14	37.76±0.67	35.77±0.06	82.2±0.08	70.3±2.76
1	91.18±0.5	37.95±0.05	35.5±0.15	82.33±0.11	72.2±0.64

Data presented as mean±SE of three measurements

Table 5 — H_2O_2 induced anti-hemolytic activity of TAPS, PBPS, TAPS-Fe, PBPS-Fe, AA

Conc.(mg/mL)	AA	TAPS	PBPS	TAPS-Fe	PBPS-Fe
0.5	66.04±1.46	27.86±1.17	47.93±0.33	35.9±7.38	37.65±4.98
1	72.29±2.7	30.18±1.65	68.99±15.75	46.93±9.78	45.52±0.08
1.5	71.35±5.1	36.65±1.82	60.36±0.83	59.7±3.65	56.22±1.82
2	82.08±4.7	44.94±9.29	71.8±0.99	63.6±4.22	75.54±1.74

Data presented as mean±SE of three measurements

Table 6 — Antioxidant potential (FRAP) TAPS, PBPS, TAPS-Fe, PBPS-Fe

Sample	FRAP (mg AAE/g)
TAPS	61.27±6.7
PBPS	260.13±3.09
TAPS-Fe	161.15±3.6
PBPS-Fe	411.8±7.2

Data presented as mean±SE of three measurements

Discussion

The hot water extraction method accelerates solvent permeation, polysaccharide dissolution, and polysaccharide diffusion through thermal effects[48,49]. The yield percentage was calculated by using equation (i). The yield content of PBPS was 2.13% greater than that of TAPS, which was 1.6%. Similar results were reported for 3.52% of spirulina polysaccharide extracted by using the hot water extraction technique[32]. The results revealed that the polysaccharide content of *P. betel* leaves was 65.92% and that of *T. aestivum* grass was 27.92%, as determined by the phenol sulfuric acid technique. A parallel result was obtained in tea leaves, where 64.96% of the total polysaccharide was evaluated by the phenol sulfuric acid technique[50].

Polysaccharide iron complexes were found in *Piper betel* and *T. aestivum* grass were dark brown amorphous powders. Comparable outcomes were observed for the AAPS-Fe complex and LBPIC (*Lycium* polysaccharide complex)[36,51]. Iron ions polymerize through an oxygen bridge or a hydroxyl bridge under weakly alkaline conditions after Citric acid is released. On the surface of polymerized ferric citrate, polysaccharide and iron combine to create the polysaccharide-iron complex in an alkaline environment[36].

No color changes and precipitates were observed in PBPS-Fe and TAPS-Fe. These findings strongly suggest that the iron complexes do not contain free iron ions and that the polysaccharide and iron were bonded rather than physically mixed. The bond between the polysaccharide and iron was broken by strong acid to release the iron ions[36].The iron content in PBPS-Fe was 16.39%, similar to that in the OPS-Fe complex, which was15.61%±0.04[52].

Fourier transform infrared spectroscopy (FTIR) is an important technique for functional group analysis[53]. The strong and broad absorption band at 3300–3000 cm^{-1} was attributed to -OH bonds [54,55]. The O-H stretching vibration peak and C-H stretching vibration peak were characteristic absorption peaks of polysaccharides. There was a modest peak at 1411.40 cm^{-1} and 1403.80 cm^{-1} for plant polysaccharides. The Peaks at 1651 cm^{-1} and 1420 cm^{-1}represent the asymmetric and symmetric stretching of the C=O functional group, respectively[54]. The strong and sharp peaks at 1008.28 cm^{-1} and 1039.97 cm^{-1} in both PBPS and TAPS, respectively, indicate the presence of a primary alcohol group (-CH). Peaks below 800 $cm^{-1,}$ known as the “skeletal region”, are related to carbohydrate skeletal vibrations[56]. The FTIR spectra of both TAPS and PBPS revealed similar characteristic bands (Fig. 1A and 1B).The similarities in the spectra of the polysaccharides and complexes indicate that the structure did not degrade or change appreciably during the synthesis process of the complexes[57,58].

Thermogravimetry (TG) curves were generated to represent the relationships between the sample weight and temperature, as previously described. In PBPS-Fe, weight loss at low temperatures may be due to the loss of water molecules[36]. A similar result was observed for TAPS and the TAPS-Fe complex (Fig. 2B). Compared with TAPS and PBPS, TAPS-Fe and PBPS-Fe achieved thermal stability at high temperatures (Fig 2A and B). The introduction of iron ions increases the intramolecular and intermolecular bonding and stability of the complex. Hence, the thermal stability of PBPS-Fe and TAPS-Fe is boosted by addition of iron, and the prepared complexes are stable at high temperatures[50].

The monosaccharide composition was determined as shown in (Fig. 3). PBPS was composed of glucuronic acid, galactouronic acid, glucose and arabinose (Fig. 3A) and TAPS was composed of glucuronic acid, glucose, arabinose, and fucose (Fig. 3C). The ferric ion with the residue chain of the monosaccharide is thought to be responsible for the differences in monosaccharides and retention times between PBPS and PBPS-Fe and TAPS and TAPS-Fe. Glucuronic acid and galacturonic acid have negatively charged carboxylic groups that have a strong affinity to bind with iron ions to form a more stable complex[59, 60].

SEM revealed the detailed morphology of the polysaccharides and iron complexes (Fig. 4). PBPS and TAPS showed rough and irregular surfaces while after complexation, flat and smooth surfaces were seen in images. Similar results were observed between OPS and OPS Fe (III) and between AAPS and AAPS Fe (III) obtained from onion and *Auricularia auricula*[36,52]. These morphological changes might result from the polysaccharide interactions with iron, which can alter the internal structures and, in turn, their outwards appearance[36].

Antioxidants play a crucial role in protecting organisms by mitigating oxidative damage caused by excessive free radicals. Numerous studies have identified natural polysaccharides as promising antioxidants[61]. In the present study the *In vitro* methods are employed to assess antioxidant activity, based on distinct mechanisms, to evaluate the antioxidant capacity of polysaccharides (PS) and their iron complexes (FePS).

The DPPH assay measures the ability of antioxidants to neutralize nitrogen-centered free radicals in methanol, which gives a characteristic dark purple color with an absorbance peak at 517 nm. In our study, antioxidant activity increased with rising concentrations. These results align with findings reported by Feng *et al*[62]. Notably, the iron complexes (PBPS-Fe and TAPS-Fe) exhibited superior scavenging activity compared to their respective polysaccharides (PBPS and TAPS). This enhanced activity synchronises with the observations of Huang *et al*.[63], where galactomannan-iron complex displayed greater antioxidant activity than galactomannan polysaccharide alone. The increased effectiveness may be attributed to iron complexation, which enhances the antioxidant potential. The high DPPH radical scavenging activity has also been reported in *Areca catechu* leaves[64].

The metal chelating activity of TAPS, PBPS, TAPS-Fe, and PBPS-Fe was concentration-dependent, with higher concentrations yielding greater activity (Table 3). This trend is consistent with findings from a study on the *Pyracantha* polysaccharide-iron complex[65].

Ferric reducing power is a key indicator of the antioxidant potential of natural products[66]. At a concentration of 1 mg/mL, TAPS-Fe and PBPS-Fe exhibited the highest reducing activities, whereas TAPS and PBPS showed the weakest activities (Table 4).

The FRAP assay evaluates the reducing potential by measuring how a compound donates a hydrogen atom to ferric tri-pyridyl-triazine complex, thereby disrupting the radical chain reaction[46]. The iron complexes (TAPS-Fe and PBPS-Fe) demonstrated higher FRAP values than their corresponding polysaccharides, as shown in (Table 5). Similar, FRAP activity has been evaluated in extract of *L. polyphyllum* plant[67].

The extent of hemolysis appeared to be significantly greater when red blood cells were exposed to toxic agents like hydrogen peroxide [68]. This experiment aimed to evaluate whether polysaccharide-iron complexes from *T. Aestivum* grass and *P. betel* leaves could prevent oxidative damage to the erythrocyte membrane. The polysaccharides and their complexes displayed varying patterns of anti-hemolytic activity. The results showed that TAPS, PBPS, TAPS-Fe, and PBPS-Fe exhibited strong anti-hemolytic effects in a dose-dependent manner. PBPS-Fe demonstrated the highest hemolysis inhibition at 75.54%, which was comparable to the 80% inhibition shown by the aqueous extract of *Terminalia arjuna*[69].

Conclusion

In this study, *Piper betel* and *Triticum aestivum* polysaccharide iron complexes were prepared, and physicochemical characterization confirmed that complex formation occured. The extraction yield of *P. betel* polysaccharide was high. PBPS-Fe and TAPS-Fe were reddish brown powders and soluble in water. Compared with TAPS-Fe, PBPS-Fe had a high iron content. Concisely, the results confirm that polysaccharide-iron complexes exhibit superior antioxidant activity and anti-hemolyitc activity compared to their corresponding polysaccharides. Specifically, TAPS-Fe and PBPS-Fe outperformed TAPS and PBPS in all assays conducted. This suggests that the incorporation of iron enhances the antioxidant properties of these polysaccharides. These findings underscore the potential of polysaccharide-iron complexes as effective antioxidants, contributing to the development of natural antioxidant agents for various applications and can be used as a food fortifier and organic iron supplement for the treatment of anemia.

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Conflicts of interest

All authors declare no conflicts of interest.

References

1 Danielson BG, Structure, chemistry, and pharmacokinetics of intravenous iron agents. *J Am Soc Nephrol*, 15 Suppl 2 (2004) S93.
2 managers WHOJAgfp, Iron deficiency anemia. assessment, prevention, and control. (2001) 47.
3 Organization WH, *The world health report 2002: reducing risks, promoting healthy life*, World Health Organization, 2002.
4 Green R, Charlton R, Seftel H, Bothwell T, Mayet F, Adams B, Finch C & Layrisse M, Body iron excretion in man: a collaborative study. *Am J Med*, 45 (1968) 336.
5 Johnson CA, Rosowski E & Zimmerman SW, A prospective open-label study evaluating the efficacy and adverse reactions of the use of Niferex-150 in ESRD patients receiving EPOGEN. *Adv Perit Dial*, 8 (1992) 444.
6 Eichbaum Q, Foran S & Dzik S, Is iron gluconate really safer than iron dextran? *Blood*, 101 (2003) 3756.
7 Sharma N & Garg V, Antihyperglycemic and antioxidant effect of hydroethanolic extract of *Butea monosperma* bark in diabetic mice. *Indian J Biochem Biophys*, 49 (2012) 55.
8 Gupta G, More AS, Kumari RR, Lingaraju MC, Pathak NN, Kumar D, Kumar D, Mishra SK & Tandan SK, Protective effect of alcoholic extract of stem of Entada pursaetha in dextran sulphate sodium-induced colitis in mice. *Indian J Biochem Biophys*, 52 (2015) 147.
9 Zohmachhuana A, Vabeiryureilai M, Meitei Thangjam N, Lalrinzuali K, Senthil Kumar N & Kumar A, Antioxidant efficacy and cytotoxicity of ethanol extract of Clerodendrum infortunatum against different cell lines. *Indian J Biochem Biophys*, 58 (2021) 572.
10 Floyd RA & Lewis CA, Hydroxyl free radical formation from hydrogen peroxide by ferrous iron-nucleotide complexes. *Biochemistry*, 22 (1983) 2645.
11 Oates PS & Morgan EH, Defective iron uptake by the duodenum of Belgrade rats fed diets of different iron contents. *Am J Physiol*, 270 (1996) G826.
12 Kudasheva DS, Lai J, Ulman A & Cowman MK, Structure of carbohydrate-bound polynuclear iron oxyhydroxide nanoparticles in parenteral formulations. *J Inorg Biochem*, 98 (2004) 1757.
13 Ancy RP, Thilaga G, Sanguni G & Gowri M, Evaluation of Antioxidant and Anticancer Activity of Amino Acid Derived Schiff Bases and their Metal Complexes–A Review. *Indian J Biochem Biophys*, 60 (2023) 437.
14 Gade R, Dwarampudi LP, Swaroop AK, Reddy DPK & Rao NS, Synthesis, spectral characterization and free radical scavenging activity of zinc-genistein nano complex. *Indian J Biochem and Biophys*, 61 (2024) 497.
15 Dwivedi MK & Singh PK, *In vitro* larvicidal, antioxidant, and DNA binding activity of biosynthesized zinc oxide nanoparticles conjugated with Andrographis paniculata aqueous extract. *Indian J Biochem Biophys*, 60 (2023) 611.
16 Guha P, Nandi SJEORTiB, Analytics, Industrial Applications & Production B, Essential oil of betel leaf (*Piper betle* L.): A novel addition to the world food sector. (2019) 149.
17 Johri S, Khan N & Khan N, Phytochemical screening, biochemical estimations and spectroscopic analysis of various extracts of *Piper betel* leaves. *Int J Pharma Res Health Sci*, 5 (2017) 1939.
18 Jaiswal S, Patel M, Saxena D & Naik SN, Antioxidant properties of *Piper betel* (L) leaf extracts from six different geographical domain of India. *JBET*, 1 (2014) 18.
19 Gundala SR & Aneja R, Piper betel leaf: a reservoir of potential xenohormetic nutraceuticals with cancer-fighting properties. *Cancer Prev Res (Phila)* 7 (2014) 477.
20 Chan EWC & Wong SK, Phytochemistry and pharmacology of three Piper species: An update. *IJP*, 1 (2014) 534.
21 Shah SK, Garg G, Jhade D & Patel N, Piper betle: Phytochemical, pharmacological and nutritional value in health management. *Int J Pharm Sci Rev Res*, 38 (2016) 181.
22 Nouri L & Mohammadi Nafchi A, Antibacterial, mechanical, and barrier properties of sago starch film incorporated with Betel leaves extract. *Int J Biol Macromol*, 66 (2014) 254.
23 Sharma M, Kumar Delta A & Kaushik P, Phytochemistry and Pharmacology of *Calotropis gigantea*—An update *Indian J Biochem Biophys*, 59 (2022) 611.
24 Alitheen NB, Oon CL, Keong YS, Chuan TK, Li HK & Yong HW, Cytotoxic effects of commercial wheatgrass and fiber towards human acute promyelocytic leukemia cells (HL60). *Pak J Pharm Sci*, 24 (2011) 243.
25 Afroz RD, Nurunnabi ASM, Hossain MZ, Khan MI, Parvin S & Rahman H, Study on effects of wheatgrass (Triticum aestivum) juice on serum triglyceride of experimentally induced hypercholesterolaemic male Long Evans rat. *J Dhaka Medical College*, 21 (2012) 197.

26 Johri S & Khan N, *In vitro* antioxidant and antihaemolytic potential of *Triticum aestivum* grass *Int J Complement Alt Med*, 9 (2017) 00310.

27 Das A, Raychaudhuri U & Chakraborty R, Effect of freeze drying and oven drying on antioxidant properties of fresh wheatgrass. *Int J Food Sci Nutr*, 63 (2012) 718.

28 Khan N, Johri S & Paul N, Inhibitory activity of polyphenolic compound extracted from *P. betel* and *T. aestivum* with hepcidin for Iron deficiency anemia: An *in silico* approach. *Indian J Biochem Biophys*, 60 (2023) 448.

29 Johri S & Khan N, Green approach using *T. aestivum* and *P. betel* in treatment of haloperidol and phenyl hydrazine induced anemia in rats. *Health Sci*, 10 (2021) 94.

30 Ganeshpurkar A & Saluja A, The pharmacological potential of catechin. *Indian J Biochem Biophys*, 57 (2020) 505.

31 Zhang N, Chen H, Ma L & Zhang Y, Physical modifications of polysaccharide from Inonotus obliquus and the antioxidant properties. *Int J Biol Macromol*, 54 (2013) 209.

32 Wang B, Liu Q, Huang Y, Yuan Y, Ma Q, Du M, Cai T & Cai Y, Extraction of Polysaccharide from Spirulina and Evaluation of Its Activities. *Evid Based Complement Alternat Med*, 2018 (2018) 3425615.

33 Dubois M, Gilles KA, Hamilton JK, Rebers Pt & Smith F, Colorimetric method for determination of sugars and related substances. *Anal Chem*, 28 (1956) 350.

34 Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72 (1976) 248.

35 Shi J, Cheng C, Zhao H, Jing J, Gong N & Lu W, *In vivo* anti-radiation activities of the Ulva pertusa polysaccharides and polysaccharide-iron(III) complex. *Int J Biol Macromol*, 60 (2013) 341.

36 Liu T, Liu T, Liu H, Fan H, Chen B, Wang D, Zhang Y & Sun F, Preparation and Characterization of a Novel Polysaccharide-Iron(III) Complex in Auricularia auricula Potentially Used as an Iron Supplement. *Biomed Res Int*, 2019 (2019) 6416941.

37 Pitarresi G, Tripodo G, Cavallaro G, Palumbo FS & Giammona G, Inulin-iron complexes: a potential treatment of iron deficiency anaemia. *Eur J Pharm Biopharm*, 68 (2008) 267.

38 Huang G, Chen Y & Wang X, Extraction and deproteinization of pumpkin polysaccharide. *Int J Food Sci Nutr*, 62 (2011) 568.

39 Yuan S, Dong PY, Ma HH, Liang SL, Li L & Zhang XF, Antioxidant and Biological Activities of the Lotus Root Polysaccharide-Iron (III) Complex. *Molecules*, 27 (2022).

40 Shang H, Chen S, Li R, Zhou H, Wu H & Song H, Influences of extraction methods on physicochemical characteristics and activities of *Astragalus cicer* L. polysaccharides. *Process Biochem*, 73 (2018) 220.

41 Wu H, Shang H, Guo Y, Zhang H & Wu H, Comparison of different extraction methods of polysaccharides from cup plant (*Silphium perfoliatum* L.). *Process Biochem*, 90 (2020) 241.

42 Tang Y, Xiao Y, Tang Z, Jin W, Wang Y, Chen H, Yao H, Shan Z, Bu T & Wang X, Extraction of polysaccharides from *Amaranthus hybridus* L. by hot water and analysis of their antioxidant activity. *Peer J*, 7 (2019) e7149.

43 Esmaeili MA & Sonboli A, Antioxidant, free radical scavenging activities of Salvia brachyantha and its protective effect against oxidative cardiac cell injury. *Food Chem Toxicol*, 48 (2010) 846.

44 Chew YL, Goh JK & Lim YY, Assessment of *in vitro* antioxidant capacity and polyphenolic composition of selected medicinal herbs from Leguminosae family in Peninsular Malaysia. *Food Chem*, 116 (2009) 13.

45 Oyaızu M, Studies on product of browning reaction prepared from glucose amine. *Japan J Nutr*, 44 (1986) 307.

46 Benzie IF & Strain JJ, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem*, 239 (1996) 70.

47 Ramchoun M, Sellam K, Harnafi H, Alem C, Benlyas M, Khallouki F & Amrani S, Investigation of antioxidant and antihemolytic properties of *Thymus satureioides* collected from Tafilalet Region, south-east of Morocco. *Asian Pac J Trop Biomed*, 5 (2015) 93.

48 Jiang YY, Yu J, Li YB, Wang L, Hu L, Zhang L & Zhou YH, Extraction and antioxidant activities of polysaccharides from roots of *Arctium lappa* L. *Int J Biol Macromol*, 123 (2019) 531.

49 Duan M, Shang H, Chen S, Li R & Wu H, Physicochemical properties and activities of comfrey polysaccharides extracted by different techniques. *Int J Biol Macromol*, 115 (2018) 876.

50 Zhou H, Wang Z, Ma H, Wang S, Xie W, Chen Y, Xie C, Guo A, Wang C & Zheng M, Characterisation and antioxidant activity of polysaccharide iron (III) complex in Qingzhuan Dark Tea. *Food Sci Technol*, 42 (2022) e119421.

51 Shi S, Feng J, Liang Y, Sun H, Yang X, Su Z, Luo L, Wang J & Zhang W, *Lycium Barbarum* Polysaccharide-Iron (III) Chelate as Peroxidase Mimics for Total Antioxidant Capacity Assay of Fruit and Vegetable Food. *Foods*, 10 (2021).

52 El-Saied MA, El-Saadany SS, Hefnawy HT & El-Sayed AI, Preparation, Characteristics And Bioactivity of Polysaccharide Iron Complex. *NVEO*, 8 (2021) 1488.

53 Gao X, Qu H, Gao Z, Zeng D, Wang J, Baranenko D, Li Y & Lu W, Protective effects of Ulva pertusa polysaccharide and polysaccharide□iron (III) complex on cyclophosphamide induced immunosuppression in mice. *Int J Biol Macromol*, 133 (2019) 911.

54 Zhu Y, Li Q, Mao G, Zou Y, Feng W, Zheng D, Wang W, Zhou L, Zhang T, Yang J, Yang L & Wu X, Optimization of enzyme-assisted extraction and characterization of polysaccharides from *Hericium erinaceus*. *Carbohydr Polym*, 101 (2014) 606.

55 Ren L, Hemar Y, Perera CO, Lewis G, Krissansen GW & Buchanan PK, Antibacterial and antioxidant activities of aqueous extracts of eight edible mushrooms. *Bioact. Carbohydr Diet Fibre*, 3 (2014) 41.

56 Wiercigroch E, Szafraniec E, Czamara K, Pacia MZ, Majzner K, Kochan K, Kaczor A, Baranska M & Malek K, Raman and infrared spectroscopy of carbohydrates: A review. *Spectrochim Acta A Mol Biomol Spectrosc*, 185 (2017) 317.

57 Chi Y, Li Y, Zhang G, Gao Y, Ye H, Gao J & Wang P, Effect of extraction techniques on properties of polysaccharides from *Enteromorpha prolifera* and their applicability in iron chelation. *Carbohydr Polym*, 181 (2018) 616.

58 Chaidedgumjorn A, Toyoda H, Woo ER, Lee KB, Kim YS, Toida T & Imanari T, Effect of (1-->3)- and (1-->4)-linkages

of fully sulfated polysaccharides on their anticoagulant activity. *Carbohydr Res*, 337 (2002) 925.

59 Gyurcsik B & Nagy LJCcr, Carbohydrates as ligands: coordination equilibria and structure of the metal complexes. *Coord Chem Rev*, 203 (2000) 81.

60 Li Y, Wang X, Jiang Y, Wang J, Hwang H, Yang X & Wang P, Structure characterization of low molecular weight sulfate Ulva polysaccharide and the effect of its derivative on iron deficiency anemia. *Int J Biol Macromol*, 126 (2019) 747.

61 Lin X, Ji X, Wang M, Yin S & Peng Q, An alkali-extracted polysaccharide from Zizyphus jujuba cv. Muzao: Structural characterizations and antioxidant activities. *Int J Biol Macromol*, 136 (2019) 607.

62 Feng G & Zhang XF, Production of a codonopsis polysaccharide iron complex and evaluation of its properties. *Int J Biol Macromol*, 162 (2020) 1227.

63 Huang C, Tao Y, Li M, Zhang W, Fan Y & Yong Q, Synthesis and Characterization of an Antioxidative Galactomannan(-) Iron(III) Complex from Sesbania Seed. *Polymers (Basel)*, 11 (2018).

64 Sharaf S, Chinchu J, Rahitha Devi S & Prakash Kumar B, Identification of bioactive compounds with anti-inflammatory potential in the methanolic fruit extract of *Areca catechu* L. (Palmaceae). *Indian J Biochem Biophys*, 58 (2021) 464.

65 Li WF, Ma HH, Yuan S & Zhang XF, Production of Pyracantha Polysaccharide-Iron(III) complex and its biologic activity. *Molecules*, 26 (2021).

66 Xu Y, Cai F, Yu Z, Zhang L, Li X, Yang Y & Liu G, Optimisation of pressurised water extraction of polysaccharides from blackcurrant and its antioxidant activity. *Food Chem*, 194 (2016) 650.

67 Dhyani A, Nautiyal BP, Yadav VK & Nautiyal MC, Variation in morphological, biochemical and antioxidant properties of *Lilium polyphyllum*. *Indian J Biochem Biophys*, 60 (2023) 237.

68 Naim M, Gestetner B, Bondi A & Birk Y, Antioxidative and antihemolytic activities of soybean isoflavones. *J Agric Food Chem*, 24 (1976) 1174.

69 Hebbani AV, Reddy VD & Nallanchakravarthula V, *In vitro* anti-hemolytic activity of *Terminalia arjuna* (Roxb.) Wt. and Arn. bark powder aqueous extract. *Indian J Adv Chem Sci*, 3 (2014) 102.