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Original Article

Identification of Polyphenols and Evaluation of Antioxidant and α-Amylase Inhibitory Activity of Wheat Bran Extracts

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Abstract: The aim of this study was to evaluate the bioactive compounds identification and determine the potential antioxidant and alpha(*α*)-amylase inhibitory activities of Wheat Bran (WB) extract. Phytochemical analysis was done by high performance liquid chromatography (HPLC) and gas chromatography (GC) in ethanol and n-hexane extract. Antioxidant potential was determined by nitric oxide scavenging and DPPH free radical scavenging assays. HPLC analysis showed the presence of dihydroxybenzoic acid, catechin hydrate, (-) epicatechin, caffeic acid, rutin hydrate, p-coumaric acid, trans-ferulic acid, rosmarinic acid, quercetin; and kaempferol. GC analysis showed a good number of compounds present in the WB extracts. The extracts showed considerable free radicles scavenging activity in both nitric oxide scavenging and DPPH free radical scavenging assays. IC_{50} of WB ethanol extract in DPPH scavenging assay was found 39.00 µg/mL and 14.57 µg/mL for ascorbic acid. In the *in vitro α*-amaylase inhibitory activity assay, the IC_{50} value of WB ethanol extract was 61.97 µg/mL, whereas the IC_{50} value for standard drug acarbose was 35.80 µg/mL. This investigation revealed that, WB extract is a potential source of bioactive compounds which can be used as alternative supplements for natural antioxidants.

Key words: Wheat Bran; free radicals; *α*-amaylase; nitric oxide; antioxidants.

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in biological reactions as by-products of oxygen metabolism, environmental stressors and due to xenobiotic metabolism. They play significant roles in many cellular processes like signal transduction and host defense [1]. However, overproduction of these reactive species causes imbalance, leading to cellular damage and dysfunction, contributing to many immune-pathological conditions such as atherosclerosis, diabetes mellitus, inflammatory diseases, cancer, neurological diseases like Parkinson's disease, Alzheimer's disease, rheumatoid arthritis, etc. Antioxidants play a vital role in suppressing oxidative stress. They

delay or prevent the oxidation of biologically important molecules such as membrane lipids, DNA, proteins, and other molecules by inhibiting the initiation of oxidative chain reactions and subsequently minimizing the risk of many chronic diseases, including cardiovascular disease and cancer [2, 3]. Natural plant products are considered rich sources of antioxidants and flavonoids, considered mainly as polyphenolic compounds. Plant-based natural antioxidants have proven effectiveness in mitigating various degenerative diseases.

Wheat (*Triticum aestivum*) is an abundant source of dietary fiber and one of the leading cereal crops utilized for mainly human consumption and livestock feed [4]. WB (15 % of the grain) is produced during milling and reportedly contains valuable fat-soluble bioactives like tocopherols, sterols, carotenoids and steryl ferulates. These components endow WB with antioxidant properties and represent a source of natural antioxidants for disease prevention [5]. It has been shown in previous clinical trials and epidemiological studies that compounds of WB fraction possess the capability of lowering blood cholesterol and are linked to reduced risk of diseases like colon cancer, diabetes, obesity and cardiovascular disease[4]. One of the minor components of WB oil fraction is steryl ferulate, which is known as oryzanol in rice bran oil has several beneficial health effects, such as anti-diabetic properties [5], reducing serum cholesterol levels [5] and may inhibit tumorigenesis etc. [6]. High-performance liquid chromatography (HPLC) analysis of WB oil extract revealed the presence of multiple natural antioxidants including phytic acid and polyphenolic compounds such as ferulic acids. These constituents demonstrated notable antioxidant potential through several mechanisms of action. Specifically, they were shown to scavenge free radicals, thereby reducing lipid oxidation. Chelation of metal ions and activation of endogenous antioxidant enzymes were additional antioxidant effects observed. The antioxidant profile of WB oil extract and its functional components warrant further investigation into their potential health benefits and applications. [2, 7]. Phytochemicals such as tocopherols, tocoretinols, and γ-oryzanol are reported to have many beneficial health properties. γ-Oryzanol contains cycloartenol, 24-methylene cycloartanyl ferulate, campesteryl ferulate, campestanyl ferulate, β-sitosteryl ferulate and is composed of phytosterols and trans-ferulic acid esters [8]. A previous report suggests that WB contains measurable amounts of γ-oryzanol like components named steryl ferulate [9], having a contribution to reducing cholesterol absorption [10]. Though several studies showed that WB fractions have a serum cholesterol-lowering effect but, there is no previous significant report explaining the mechanism by which it exerts a serum cholesterol-lowering effect [11]. The α- amylase is a digestive enzyme found in both saliva and pancreatic juice, involved in hydrolyzing glycosidic bonds, thus breaking down insoluble starch molecules into smaller absorbable molecules such as glucose, dextrin, maltose, and maltotriose [12]. Alpha-amylase can be a good target and has been given much attention for its anti-diabetic potential for many naturally occurring medicinal plants [13]. Alpha-amylase inhibitors play a significant role in lowering

postprandial blood glucose levels [14]. It has been reported that WB has anti-diabetic potential [15], but there are no studies explaining the mechanism by which it exerts *α*- amylase inhibitory effect. This study aimed to identify the polyphenol content in WB extract through HPLC-DAD and GC-MS. The antioxidant and *α*- amylase inhibitory potential of this WB extract was also evaluated in this study.

2. Materials and Methods

2.1 Chemicals and reagents

Alpha-amylase was procured from *Aspergillus oryzae*, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and Naphthyl ethylenediamine dihydrochloride was obtained from Sigma-Aldrich Chemical Co. (USA). Starch soluble (extra pure) was obtained from J.T. Baker Inc., Phillipsburg, USA. Gallic acid, 3,4-Dihydroxybenzoic acid, Catechin hydrate, Catechol, (-) Epicatechin, Caffeic acid, Vanillic acid, Syringic acid, Rutin hydrate, *p*-Coumaric acid, *trans*-Ferulic acid, Rosmarinic acid, Myricetin, Quercetin, trans-Cinnamic acid, and Kaempferol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), and ethanol were obtained from Merck (Darmstadt, Germany). *γ* –Oryzanol (98.5 %), for total sterol analysis and as standard, purchased from Sigma Chemical Company (USA). Other chemicals and reagents used were of analytical grade.

2.2 Plant collection and Preparation of WB oil concentrate

WB (*T. aestivum*) was procured from Diba Flour Mills, Narayanganj, Bangladesh. The collected WB was dried in an oven at 50 ℃ and then ground to obtain a fine powder. 500 gm of powdered bran was macerated with ethanol in a closed glass jar. It was kept in a dark place at room temperature for 5 days with occasional shaking. Then, the mixture was filtered to obtain WB ethanol extract. Finally, ethanol was evaporated with the help of a rotary evaporator operated at 50℃ with 100 rpm to obtain concentrated WB-ethanolic extract. The same process was applied to obtain WB-hexane extract. Two sticky dark-brownish crude extracts were obtained after the evaporation of the solvents. Both obtained extracts were screened for phytochemical analysis using HPLC-DAD and GC-MS methods.

2.3 HPLC-DAD Analysis

HPLC-DAD analysis was conducted using a Shimadzu system (LC-20A, Japan) consisting of a binary pump (LC-20AT), autosampler (SIL-20A HT), column oven (CTO-20A), and photodiode array detector (SPD-M20A), controlled by LC solution software. Separation was achieved on a Luna C18 (5µm) Phenomenex column (4.6 x 250 mm) maintained at 33°C.

2.3.1 Chromatographic conditions

Detection and quantification of selected polyphenolic compounds in WB ethanol extract was determined using high-performance liquid chromatography with

diode array detection (HPLC-DAD) analysis as described by Ahmed et al.[16] with minor modifications. The mobile phase consisted of 1 % acetic acid in acetonitrile (Solvent A) and 1 % acetic acid in water (Solvent B) with the following gradient elution program: 5-25 % A (0.01-20 min), 25-40 % A (20-30 min), 40-60 % A (30-35 min), 60-30 % A (35-40 min), 30–5 % A (40–45 min), and 5 % A (45–50 min).

The sample injection volume was 20µL, and the flow rate was set at 0.5 mL/min. Ultraviolet detection was set at 270 nm and used to validate the method and perform analysis. The mobile phase was filtered through a 0.45 µm nylon 6, 6 membrane filter (India) and degassed under vacuum.

2.3.2 Preparation of working standard solutions HPLC

Standard stock solutions of 16 phenolic compounds were prepared by dissolving in methanol in a 25 mL volumetric flask. The concentrations of stock solutions ranged from 4.0 to 50 µg/mL. Appropriate volumes of each stock solution were mixed and diluted serially with methanol to prepare the working standard solutions. All solutions were stored under refrigeration.

For the preparation of the calibration curve, a standard stock solution was prepared in methanol containing Gallic acid (20 µg/mL); 3,4-Dihydroxybenzoic acid (15 µg/mL); Catechin hydrate (50 µg/mL); Catechol, (-) epicatechin, rosmarinic acid (30 µg/mL each), caffeic acid, vanillic acid, syringic acid, rutin hydrate, *p*-coumaric acid, *trans*-ferulic acid, quercetin (10 µg/mL each); myricetin, kaempferol (8 µg/mL each); trans-cinnamic acid (4 µg/mL). A solution of ethanol extract of WB at a concentration of 10 mg/mL was prepared in ethanol. The samples were stored in the refrigerator.

2.4 Determination of steryl ferulates in WB n-hexan extract

Total *γ*-oryzanol-like steryl ferulate content was estimated following the method described by Kumar et al. [17, 18]. In this study, HPLC-UV (Shimadzu LC 10A system) was used coupled with Shimadzu C18 reversed-phase column (5 µm i.d, 150 mm × 4.6 mm.) and photodiode array detector (SPD-M20A), using isocratic elution with acetonitrile/methanol/ isopropyl alcohol (10:9:1 v/v/v) maintaining flow rate of 1 mL/min. The methanolic solution of the sample was injected (30 µL), and steryl ferulates were monitored at 325 nm, and *γ*-oryzanol was used as a standard for steryl ferulate quantification.

2.5 GC-MS Analysis

Phytochemical analysis of WB ethanol and n-hexane extracts was performed by GC-MS (GC-2010, Shimadzu Corporation, Kyoto, Japan) coupled with a mass spectrometer (GC-MS TQ 8030, Shimadzu Corporation, Kyoto, Japan). The inlet temperature was set at 250 ◦C and the column flow rate was 1 mL/min with Helium gas with a constant pressure of 53.5 kPa. The oven temperature was set at 50 ◦C (1 min), 200 ◦C (2 min), 300 ◦C (7 min). The temperature of the GC to MS interface

was 250 ◦C. The MS was set on a scan mode with a mass range of 50–600 m/z. The total running time for GC–MS was set for 40 min.

2.6 Anti-oxidant Assay

An antioxidant activity study was performed on WB ethanol and n-hexane extracts using nitric oxide radical scavenging assay and DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical Scavenging Assay method.

2.6.1 Nitric Oxide radical ($NO^{\cdot}$) scavenging assay

Nitric oxide scavenging activity of both ethanol and n-hexane extract was measured as described by Alam et al. and Banerjee et al. [19, 20] . In this study, the reaction mixture contains 1mL sodium nitroprusside (10mM) in phosphate buffer saline and either 1 mL extract or ascorbic acid as standard (25 µg to 400 µg/mL). The reaction mixtures were incubated at 25ºC for 90 minutes. Then, after incubation, 0.5mL of Gries-Illosovoy reagent (1 % sulphanilamide and 5 %phosphoric acid) was added and allowed to stand for 7 minutes for diazotization of nitrite ions with sulphanilamide. Then 0.5 mL naphthyl ethylene diamine dihydrochloride (0.1%) was added, resulting in the formation of a pink-colored chromophore. The reaction mixture was allowed to stand at 25ºC for 5 minutes. Finally, the absorbance of the reaction mixtures was measured at 546nm, and the percentage of scavenging activity was calculated following the equation:

$$\textbf{\% Scavenging potential} = [(Abs_{control} - Abs_{extract}) / Abs_{control}] \times 100$$

The IC_{50} was calculated to represent the concentration of the WB extract that scavenged 50 % of nitric oxide radical.

2.6.2 DPPH Free Radical Scavenging Activity

DPPH free radical scavenging potential of WB extracts was determined following the method mentioned by Alam et al. and Ahmed et al. with slight modifications[16, 19]. Serially diluted WB extracts (25µg to 400 µg/mL) were mixed with 0.5 mL methanolic solution of DPPH (0.01 %) and kept in the dark for 30 minutes at room temperature. Finally, the absorbance of the reaction mixtures was measured at 517nm, and the percentage of scavenging activity was calculated following the equation:

$$\textbf{\% Scavenging potential} = [(Abs_{control} - Abs_{extract}) / Abs_{control}] \times 100$$

The IC_{50} was calculated as the plot of inhibition (%) against the concentration of the extract to represent the concentration of the WB extract that scavenged 50 %

of DPPH free radical. This experiment was carried out in triplicate, and ascorbic acid was used as the standard antioxidant.

2.7 Alpha-Amylase Inhibitory Assay

The α-amylase inhibitory assay was performed using a modified method of Kazeem et al. [13]. Serially diluted 250 μL WB extracts (25 µg to 400 µg/mL) were mixed with 250 μL of sodium phosphate buffer containing α-amylase solution (0.5 mg/mL) and incubated for 10 minutes at room temperature. Then 250 μL of sodium phosphate buffer containing 1 % starch solution was added to the reaction mixture and allowed to stand for 10 min at 25ºC. Thereafter, 500 μL dinitrosalicylic acid (DNS) reagent was added, and the reaction mixture was incubated in a hot water bath operated at 50ºC for 5 minutes. After 5 minutes, the test tubes containing reaction mixtures were cooled to room temperature and diluted with 5 mL distilled water. Finally, the absorbance of the reaction mixtures was measured at 540 nm, and the percentage of inhibitory activity was calculated following the equation:

$$\textbf{\% Inhibition} = [(Abs_{control} - Abs_{extract}) / Abs_{control}] \times 100$$

The IC_{50} was calculated the plot of inhibition (%) against the concentration of the extract to represent the concentration of the WB extract that inhibited 50 % of α-amylase. This experiment was carried out in triplicate and Acarbose was used as the standard α-amylase inhibitor.

Statistical Analysis:

Each experiment was performed in triplicate. Statistical analysis was performed using GraphPad Prism version 9 software (GraphPad Software, San Diego, CA, USA).

3. Results

HPLC-DAD analysis of bioactive polyphenols in WB

The chromatographic separation of polyphenolic compounds that are present in the ethanol extract of WB is shown in Figure-1. The chromatogram detects and confirms the presence of 3,4-dihydroxybenzoic acid, catechin hydrate, (-) epicatechin, caffeic acid, rutin hydrate, *p*-coumaric acid, trans-ferulic acid, rosmarinic acid, quercetin and kaempferol in WB ethanol extract. All of the identified phenolic compounds are reported to have strong antioxidant properties. The content of each phenolic compound found in the ethanol extract of WB was calculated from the corresponding calibration curve, as shown in **Table 1**.

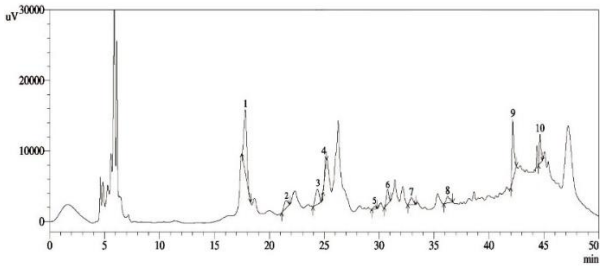

Figure 1. HPLC chromatogram of ethanol extract of WB**.** Peak: 1. 3,4-Dihydroxybenzoic acid; 2. Catechin hydrate; 3. (-) Epicatechin; 4. Caffeic acid; 5. Rutin hydrate; 6. p-Coumaric acid; 7. trans-Ferulic acid; 8. Rosmarinic acid; 9. Quercetin; and 10. Kaempferol

Table 1: Identification of polyphenolic compounds in ethanol extract of WB using HPLC-DAD

Polyphenolic Compounds	***Content (mg/100 g of the dry extract)***
3,4-Dihydroxybenzoic acid	16.03±0.15
Catechin hydrate	15.79±0.55
(-) Epicatechin	21.63±0.07
Caffeic acid	3.17±0.05
Rutin hydrate	1.01±0.04
p-*Coumaric acid*	2.86±0.07
trans-Ferulic acid	3.85±0.15
Rosmarinic acid	4.28±0.42
Quercetin	13.32±0.02
Kaempferol	3.09±0.15

3.2 Steryl ferulates in WB n-hexan extract

Total *γ*-oryzanol-like steryl ferulate content was estimated following the method described by Kumar et al [23,25] with some modifications. The chromatographic separation of steryl ferulate in *γ*-oryzanol as standard and ethanol extract of WB is shown in Fig. 2. The content of each steryl ferulate component found in the ethanol extract of WB was calculated from the corresponding calibration curve as mentioned in Table 2. Among 5 components of oryzanol [24], 24-methylene cycloartanyl ferulate (0.03±0.01 %), Campestanyl ferulate (0.22±0.01 %), and *β*-sitosteryl ferulate (0.16±0.01 %) are present.

Table 2: Contents of steryl ferulate components in n-hexane extract of *Triticum estivum* bran.

Peaks	Standard Compounds	% of Hexane extract of WB
1	Cycloartenyl ferulate	ND
2	24-methylene cycloartanyl ferulate	0.03±0.01
3	Campesteryl ferulate	ND
4	Campestanyl ferulate	0.22±0.01
5	*β*-sitosteryl ferulate	0.16±0.01

Values are expressed as mean±RSD. RSD: Relative Standard Deviation. ND: Not Detected

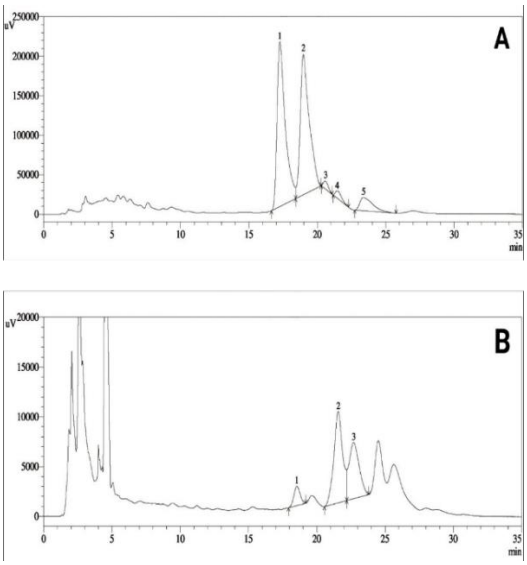

Figure 2. Chromatogram of steryl ferulate components. A) *γ*-oryzanol as standard; Peak 1. cycloartenyl ferulate; Peak 2. 24-methylene cycloartanyl ferulate; Peak 3. campesteryl ferulate; Peak 4. campestanyl ferulate Peak 5. *β*-sitosteryl ferulate B) n-hexan extract of WB; Peak 1. 24-methylene cycloartanyl ferulate; Peak 2. campesteryl ferulate; Peak 3. *β*-sitosteryl ferulate

3.2 GC-MS Analysis of WB extracts

Phytochemical analysis of both ethanol and n-hexane extract of WB was performed using GC-MS. The chromatographic separation of ethanol and n-hexane extract of WB is shown in Fig. 3 and Fig.4, respectively. The identified components are listed according to the order of their retention time. The identified components of ethanol extract of WB are listed in Table 3 and that of n-hexane in Table 4 respectively.

3.2.1 GC-MS Analysis of Triticum estivum bran ethanol extract

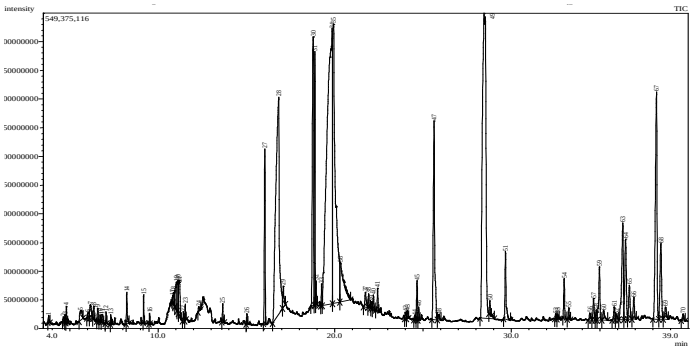

Figure 3: Chromatogram of ethanol extract of WB of GC-MS Analysis

Table 3: GC-MS Analysis of ethanol extract of WB.

SI No.	**Retention time (Min)**	**% Area**	**Tentative compound name**
1	3.805	0.09	Diglycerol
2	4.63	0.07	Cyclopentanone
3	4.742	0.08	Pyrrolidine
4	4.81	0.18	Cyclohexanone
5	5.616	0.31	2-Hydroxy-gamma-butyrolactone
6	6.03	0.13	2,5-Hexanediamine, 2,5-dimethyl-
7	6.175	0.31	Thiazole, 2-amino-5-methyl-
8	6.364	0.17	2,5-Dimethylfuran-3,4(2H,5H)-dione
9	6.584	0.15	1H-Azonine, octahydro-1-nitroso-
10	6.729	0.06	Phenol, 2-methoxy-
11	6.831	0.13	3-Butene-1,2-diol
12	7.054	0.09	Phenylethyl Alcohol
13	7.325	0.06	Methyl nicotinate
14	8.233	0.36	1-Ethyl-2-hydroxymethylimidazole

15	9.185	0.19	2-Methoxy-4-vinylphenol
16	9.518	0.06	Phenol, 2,6-dimethoxy-
17	10.82	0.07	1H-Benzotriazole, 5-methyl-
18	10.865	0.28	Apocynin
19	11.04	0.59	Beta-D-Glucopyranose, 4-O-.beta.-D-galactopyranosyl-
20	11.12	0.52	1,3,2-Dioxaphosphorinane, 2-(2-methoxyethoxy)-5,5-dimethyl-
21	11.215	0.75	d-Mannitol, 1,4-anhydro-
22	11.431	0.09	Dodecanoic acid
23	11.541	0.11	3',5'-Dimethoxyacetophenone
24	12.295	0.07	d-Glycero-d-ido-heptose
25	13.658	0.18	Tetradecanoic acid
26	15.028	0.1	Pentadecanoic acid
27	16.043	2.03	Octadecanoic acid, 3-hydroxy-2-tetradecyl-, methyl ester, (2R,3R)-
28	16.83	10.92	n-Hexadecanoic acid
29	17.089	0.33	Hexadecanoic acid, ethyl ester
30	18.784	5.23	6,9-Octadecadienoic acid, methyl ester
31	18.874	2.92	9-Octadecenoic acid, methyl ester, (E)-
32	18.944	0.21	9-Octadecenoic acid, methyl ester, (E)-
33	19.259	0.21	Methyl stearate
34	19.85	21.22	9-Octadecynoic acid
35	19.934	11.06	9-Octadecenoic acid (Z)-, hexadecyl ester

36	20.34	1.98	2-Methyl-5-(2,6,6-trimethyl-cyclohex-1-enyl)-pentane-2,3-diol
37	21.733	0.3	5,5,8a-Trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene
38	21.915	0.16	S-[2-[N,N-Dimethylamino]ethyl]N,N-dimethylcarbamoyl thiocarbohydroximate
39	22.094	0.08	Glycidyl palmitate
40	22.187	0.19	11-Eicosenoic acid, methyl ester
41	22.424	0.27	3-Octene-2,5-dione, 6,6,7-trimethyl-, (E)-
42	23.996	0.1	Linoleic acid ethyl ester
43	24.117	0.11	6,9-Octadecadienoic acid, methyl ester
44	24.54	0.07	9-Octadecenoic acid, 12-hydroxy-, methyl ester, [R-(Z)]-
45	24.659	0.52	endo-2-Methyl-2-norbornanol
46	24.742	0.17	Cyclohexylmethyl S-2-(dimethylamino)ethyl propylphosphonothiolate
47	25.623	4.09	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
48	25.92	0.12	Bis(2-ethylhexyl) phthalate
49	28.453	15.48	E,E,Z-1,3,12-Nonadecatriene-5,14-diol
50	28.776	0.16	Octadecanoic acid, 2,3-dihydroxypropyl ester
51	29.664	1.04	cis-11-Eicosenamide

52	32.51	0.07	Palmitic acid vinyl ester
53	32.62	0.06	(Z)-5-(Pentadec-8-en-1-yl)benzene-1,3-diol
54	32.981	0.52	Phosphite, diisopropylmenthyl-
55	33.242	0.15	beta.-Tocopherol
56	34.437	0.13	i-Propyl 5,9,17-hexacosatrienoate
57	34.645	0.38	Vitamin E
58	34.804	0.19	Undec-10-ynoic acid, 3-methylbut-2-en-1-yl ester
59	34.979	0.98	(R)-2,7,8-Trimethyl-2-((3E,7E)-4,8,12-trimethyltrideca-3,7,11-trien-1-yl)chroman-6-ol
60	35.217	0.22	Trilinolein
61	35.817	0.24	(Z)-5-(Pentadec-8-en-1-yl)benzene-1,3-diol
62	35.96	0.12	(Z)-5-(Pentadec-8-en-1-yl)benzene-1,3-diol
63	36.304	2.39	2-Dodecen-1-yl(-)succinic anhydride
64	36.462	1.76	Campesterol
65	36.673	0.67	Ergostanol
66	36.928	0.38	Stigmasterol
67	38.208	5.4	*γ*-Sitosterol
68	38.456	1.73	Stigmastanol
69	38.72	0.31	Methoxyolivetol
70	39.718	0.13	16-Allopregnen-3.beta.,7.alpha.-diol-20-one

From these GC-MS chromatograms and peak report of WB ethanol extract, a large number of secondary metabolites have been identified that include Ethyl linolenate, phtysterols, components of oryzanol like steryl ferulate: Campesterol, Stigmasterol, *β*-sitosterol, *γ* -sitosterol, lower levels of apocynin, vitamin E, *β*-tocopherol, and so on.

3.2.1 GC-MS Analysis of Triticum estivum bran n-hexane extract.

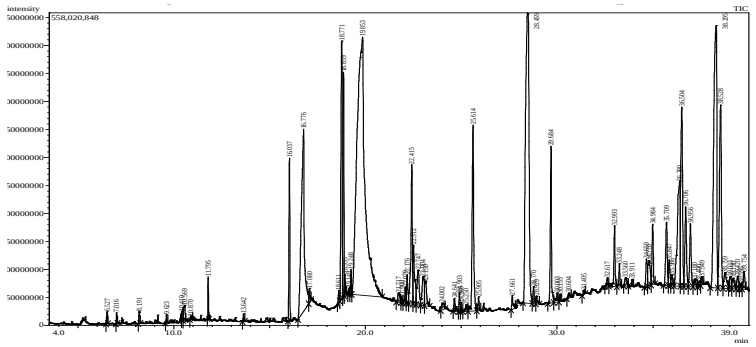

Figure 4: Chromatogram of n-hexane extract of *Triticum estivum* bran of GC-MS Analysis.

From these GC-MS chromatograms and peak report of WB n-hexane extract, a large number of secondary metabolites have been identified that include phtysterols, lower levels of *β*-tocopherol, components of oryzanol like steryl ferulate: campesterol, stigmasterol, *β*-sitosterol, *γ* -sitosterol, and so on.

Table 4: GC-MS Analysis of n-hexane extract of WB.

SI No.	**Retention time (Min)**	**% Area**	**Tentative compound name**
1	6.527	0.12	Thymine
2	7.016	0.06	Phenylethyl alcohol
3	8.191	0.12	5-hydroxymethylfurfural
4	9.623	0.07	Sulfurous acid, cyclohexylmethyl hexadecyl ester
5	10.41	0.16	Naphthalene, 1,7-dimethyl-
6	10.569	0.31	Sucrose

7	10.87	0.06	Alpha.-d-glucopyranose, 4-o-.beta.-d-galactopyranosyl-
8	11.795	0.3	Phthalic acid, di-(1-hexen-5-yl) ester
9	13.642	0.06	Tetradecanoic acid
10	16.037	1.67	Octadecanoic acid, 3-hydroxy-2-tetradecyl-, methyl ester (2R,3R)
11	16.776	6.76	1,2,4-Trioxolane-2-octanoic acid, 5-octyl-, methyl ester
12	17.08	0.19	Hexadecanoic acid, ethyl ester
13	18.611	0.13	N-Nonadecanol-1
14	18.771	3.99	Cyclopropanebutyric acid, 2-[(2-nonylcyclopropyl)methyl]-, methyl ester
15	18.859	2.33	9-Octadecenoic acid, methyl ester, (E)-
16	18.933	0.19	9-Octadecenoic acid, methyl ester, (E)-
17	19.149	0.07	Octadecanoic acid, 7-hydroxy-, methyl ester
18	19.248	0.23	Heptadecanoic acid, 16-methyl-, methyl ester
19	19.853	25.04	E,E,Z-1,3,12-Nonadecatriene-5,14-diol
20	21.717	0.23	5-heptylresorcinol
21	21.9	0.08	3-Cyclopentylpropionic acid, 2-dimethylaminoethyl ester
22	22.079	0.24	Glycidyl palmitate
23	22.176	0.44	11-Eicosenoic acid methyl ester
24	22.415	2.33	2-Myristynoic acid
25	22.512	1.15	Palmidrol
26	22.747	0.93	1-Butanone, 4-nitro-1-2-oxocyclohexyl
27	23.004	0.82	1-Butanone, 4-nitro-1-2-oxocyclohexyl

28	23.15	0.52	3H,8H-Dipyrrolo[1,2-a:2,1-b]imidazole-3,8-dione, hexahydro
29	24.002	0.2	Pentyl linoleate
30	24.641	0.15	Carbamic acid, 2-(dimethylamino)ethyl ester
31	24.903	0.21	Bicyclo[10.1.0]tridec-1-ene
32	24.98	0.11	Glycidyl oleate
33	25.25	0.15	Glycidyl oleate
34	25.614	3.55	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
35	25.905	0.21	Bis(2-ethylhexyl) phthalate
36	27.661	0.22	Cyclopropane, 1,1-dichloro-2,2,3,3-tetramethyl-
37	28.459	12.02	E,E,Z-1,3,12-Nonadecatriene-5,14-diol
38	28.77	0.26	Octadecanoic acid, 2,3-dihydroxypropyl ester
39	28.929	0.12	Triacontanoic acid, methyl ester
40	29.684	2.2	6-Octadecenoic acid
41	30.003	0.22	Tetradecanamide
42	30.155	0.1	Squalene
43	30.604	0.1	Alpha-tocospiro A
44	31.405	0.06	17-pentatriacontene
45	32.617	0.14	(Z)-5-(Pentadec-8-en-1-yl)benzene-1,3-diol
46	32.993	0.89	3-(1-Methylhept-1-enyl)-5-methyl-2,5-dihydrofuran-2-one
47	33.248	0.32	Beta-tocopherol
48	33.56	0.19	Propanoic acid, 3,3'-thiobis-, didodecyl ester
49	33.911	0.1	Cholesta-4,6-dien-3-ol, 3.beta

50	34.659	0.51	Vitamin E
51	34.77	0.72	1-Butanone, 1-bicyclo[4.1.0]hept-7-yl-
52	34.984	0.89	(R)-2,7,8-Trimethyl-2-((3E,7E)-4,8,12-trimethyltrideca-3,7,11-trien-1-yl)chroman-6-ol
53	35.709	1.22	4-Methyl-2,2,4-tris(4'-methoxyphenyl)pentane
54	35.847	0.44	3H,8H-Dipyrrolo[1,2-a:2,1-b]imidazole-3,8-dione, hexahydro
55	35.986	0.22	(Z)-5-(Pentadec-8-en-1-yl)benzene-1,3-diol
56	36.39	3.65	Butylphosphonic acid, ethyl neopentyl ester
57	36.504	3.65	Campesterol
58	36.706	1.58	Ergostanol
59	36.956	1.16	Stigmasterol
60	37.18	0.19	7,8-Epoxylanostan-11-ol, 3-acetoxy-
61	37.4	0.11	16-Allopregnen-3.beta.,7.alpha.-diol-20-one
62	37.549	0.17	Ergost-7-en-3-ol, (3.beta)
63	38.295	9.4	Gamma-sitosterol
64	38.528	4.2	Stigmastanol
65	38.759	0.5	Cyclopropa[5,6] stigmast-22-en-3-ol, 3',6-dihydro-, (3.beta.,5.beta.,6.alpha.,22E)-
66	39.033	0.29	Stigmasta-5,24(28)-dien-3-ol, (3.beta.)-
67	39.19	0.29	Beta-amyrin
68	39.42	0.26	Beta-sitosterol
69	39.595	0.11	4,22-Stigmastadiene-3-one
70	39.754	0.32	33-Norgorgosta-5,24(28)-dien-3-ol, (3.beta)

3.2 Antioxidant activity of WB extracts

Antioxidant activity study was performed on WB ethanol and n-hexane extracts using Nitric Oxide Radical Scavenging Assay and DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical Scavenging Assay method. IC_{50} values were calculated in comparison to the concentration of standard or bran extract required to scavenge 50 % of the free radicals.

3.2.1 Nitric Oxide Radical Scavenging Activity

In this study, Sodium nitroprusside was used, which produces nitric oxide radicals that have a strong oxidizing ability and can alter cellular structure as well as function. The scavenging activity of WB extracts on nitric oxide production was determined following previously mentioned methods [10, 19] and graphically represented in Figure 5.

Both ethanol and n-hexane extract of WB exhibited significant nitric oxide radical scavenging activity, resulting in the reduction of the NO- concentration in the assay medium. The graph shows that the scavenging activity of WB extracts on Nitric oxide production was found to increase in a concentration-dependent manner. The IC_{50} values for nitric oxide scavenging ability of WB extracts are listed in **Table 5.**

Table 5: IC_{50} values for nitric oxide scavenging ability of WB extracts and ascorbic acid as standard.

Sample	IC_{50} (µg/mL)
WB n-Hexane extract	55.51
WB ethanol extract	51.38
Ascorbic acid	25.64

Among 2 extracts of WB, ethanol extract has lower IC_{50} value of 51.38 µg/mL with significant nitric oxide radical scavenging potential compared to the IC_{50} value of WB n-Hexane extract.

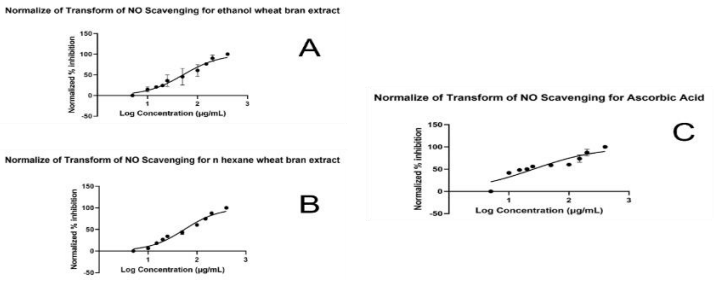

Figure 5: Nitric oxide scavenging ability of WB extracts and ascorbic acid as standard.

3.2.2 DPPH Free Radical Scavenging Activity

In the DPPH assay, 2,2-diphenyl-1-picrylhydrazyl, DPPH free radical forms a violet-colored solution, which is subsequently reduced in the presence of antioxidant to a colorless solution, 1,1-diphenyl-2-picryl hydrazine in a concentration-dependent manner. The scavenging activity of WB extracts on DPPH free radical was determined following previously mentioned methods [10, 19] and graphically represented in Figure 6.

Both ethanol and n-hexane extract of WB exhibited significant DPPH free radical scavenging activity, resulting in a colorless solution. Graphically, the DPPH free radical scavenging activity of WB extracts and Ascorbic acid as standard were found to be increased in a concentration-dependent manner. We list the IC_{50} values for the DPPH free radical scavenging potential of WB extracts in Table 6. The ethanolic and n-hexane extract of WB has an IC_{50} value of 39 µg/mL and 42.99 µg/mL, respectively, whereas the standard ascorbic acid has an IC_{50} value of 14.57 µg/mL.

Table 6: IC_{50} values for DPPH free radical scavenging ability of *T.estivum* bran extracts and Ascorbic acid as standard.

Sample	IC_{50} (µg/mL)
WB n-Hexane extract	42.99
WB ethanol extract	39.00
Ascorbic acid	14.57

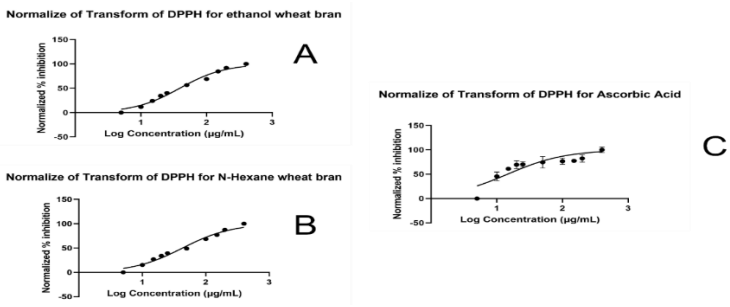

Figure 6: DPPH free radical scavenging ability of *T. estivum* bran extracts and Ascorbic acid as standard.

3.2.3 *α-amylase inhibitory activity*

Alpha-amylase inhibitory activity of the WB extracts was determined following previously mentioned methods [13] and graphically represented in Figure 7. At lower concentrations, both of the extracts showed no significant difference in the inhibition of *α*- amylase. Graphically, *α*-amylase inhibitory activity of WB extracts and acarbose as standard were found to be increased in a concentration-

depended manner. The IC_{50} values for α-amylase inhibitory potential of WB extracts are listed in **Table 7.**

Table 7: IC_{50} values for α-amylase inhibitory activity of *T. estivum* bran extracts and Acarbose as standard.

Sample	IC_{50} (µg/mL)
WB n-Hexane extract	41.45
WB ethanol extract	61.97
Acarbose	35.80

Both ethanol and n- hexane extract of WB has higher IC_{50} value of 61.97 µg/mL and 41.45 µg/mL respectively in comparison to the IC_{50} value of Acarbose (35.80 µg/mL) as standard.

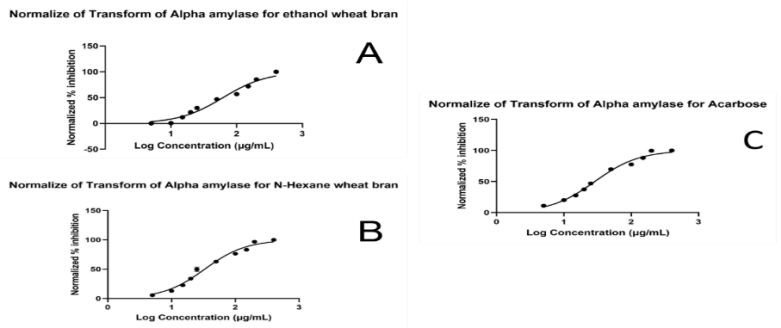

Figure 7: α-amylase inhibitory activity of WB extracts and Acarbose as standard.

4. Discussion

The aim of the study is to identify and determine the content of polyphenol in WB through HPLC and GC-MS and measure its antioxidant and anti-diabetic potential. Antioxidant activity was analyzed using nitric oxide radical scavenging assay and DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical scavenging assay on both ethanol and n-hexane WB extracts. Anti-diabetic potential of WB extract was measured by its inhibitory effect on α-amylase.

HPLC profiling of WB ethanol extract reveals that it contains 3,4-dihydroxybenzoic acid, catechin hydrate, (-) epicatechin, caffeic acid, rutin hydrate, *p*-coumaric acid, trans-ferulic acid, rosmarinic acid, quercetin and kaempferol, which are similar with the findings reported by Zhou et al. [2]. The content of 3,4-dihydroxybenzoic acid is highest and is 16.03±0.15 mg whereas rutin hydrate content is the lowest and 1.01±0.04 g per 100 g of dry extract among 10

identified polyphenolic compounds. The total content of selected polyphenolic compounds was 85.03 mg per 100 g of dry extract which clarifies the reason behind its antioxidant potential.

Total *γ*-oryzanol-like steryl ferulate content was estimated and among 5 components of oryzanol [21], 24-methylene cycloartanyl ferulate (0.03±0.01 %), Campestanyl ferulate (0.22±0.01 %) and *β*-sitosteryl ferulate (0.16±0.01 %) is present, and this finding is similar to the HPLC findings of Kumar et al. [17]. Kumar et al. found 3 major peaks from the HPLC chromatogram of WB oil but with a notable quantity. Previous studies of Talawar et al, [22] also reported higher content of total *γ*-oryzanol-like steryl ferulate compared to the reports of Moreau et al. [23] and Rebolleda et al. [9]. The previous report also summarized that *γ*-oryzanol-like steryl ferulate is associated with several significant beneficial health effects such as antioxidant properties, hypocholesterolemic effect etc. [24].

Phytochemical analysis of both ethanol and n-hexane extract of WB was performed using GC-MS. GC-MS chromatograms and peak report of WB ethanol extract, ethyl linolenate, phtysterols, components of oryzanol like steryl ferulate: Campesterol, Stigmasterol, *β*-sitosterol, *γ* -sitosterol, lower levels of apocynin, vitamin E, *β*-tocopherol, and many more are identified, whereas from GC-MS chromatograms of WB n-hexane extract, phtysterols, lower levels of *β*-tocopherol, components of oryzanol like steryl ferulate: campesterol, stigmasterol, *β*-sitosterol, *γ* -sitosterol, and many more are identified. The presence of oryzanol like steryl ferulate gives clarity to the reports of HPLC profiling of steryl ferulate. Between ethanol and n-hexane extract of WB, n-hexane extract contains higher amount of steryl ferulates (18.67 % area) than that of in ethanol extract (9.27 % area) which is less than half of the n-hexane extract of WB.

Reactive nitrogen species causes cellular damage and dysfunction and thus leads to tissue toxicity and inflammatory conditions. Suppression of $NO^{\cdot}$ release or scavenging of $NO^{\cdot}$ can reduce the load of reactive nitrogen species. Both extracts reduced the amount of nitrite generated from sodium nitroprusside and the scavenging potential was found to increase in concentration dependent manner.

DPPH (2,2-diphenyl-1-picrylhydrazyl), is a stable free radical and in the DPPH assay, DPPH free radical forms a violet-colored solution which is subsequently reduced to 1,1-diphenyl-2-picryl hydrazine (colorless solution) in the presence of antioxidant either due to transfer an electron or hydrogen atom to DPPH. Both ethanol and n-hexane extract of WB exhibited significant DPPH free radical scavenging activity resulting in colorless solution. DPPH free radical scavenging activity of WB extracts and ascorbic acid, as standard, were found to be increased in a concentration-depended manner. The ethanolic and n- hexane extract of WB has IC_{50} value of 39 µg/mL and 42.99 µg/mL respectively, whereas the standard ascorbic acid has IC_{50} value of 14.57 µg/mL. Lower value of IC_{50} of WB ethanol extract indicates that it contains greater quantity of phytochemicals with antioxidant properties than in the n-hexane extract. IC_{50} value of both extracts are not too high

in comparison with that of ascorbic acid, suggesting the presence of significant antioxidant potential of WB.

With the development of modern medicine, many naturally occurring medicinal plants have been identified as a good source of beneficial and nutritional health effects as well as pharmacological activities. α- amylase is one of the important digestive enzymes, found in saliva and pancreatic juice, involved in hydrolyzing glyosidic bond thus breaking down insoluble starch molecules into smaller absorbable molecules such as glucose, dextrin, maltose. Inhibitors of α-amylase delay or inhibit the breakdown of starch by blockade of site of the α-amylase. Alpha-amylase inhibitors play significant role in lowering postprandial blood glucose level. We observed that at lower concentrations, both of the extracts showed no significant difference in the inhibition of α- amylase. With the increase in the concentration of WB extracts, α-amylase inhibitory activity of *T. estivum* bran extracts and acarbose as standard were found to be increased in a concentration-depended manner. Both ethanol and n- hexane extract of WB has higher IC_{50} value of 61.97 µg/mL and 41.45 µg/mL respectively in comparison to the IC_{50} value of acarbose (35.80 µg/mL) as standard. IC_{50} value of n-hexane extracts is not too high as opposed to that of acarbose, suggesting that it can be given attention for α-amylase inhibitory potential.

5. Conclusions

HPLC-profiling showed the presence of several phenols, including ferulic acid and caffeic acid, which are associated with antioxidant and anti-inflammatory activities. Total γ-oryzanol-like steryl ferulate content was estimated, and among 5 components of oryzanol, 24-methylene cycloartanyl ferulate, campestanyl ferulate, and β-sitosteryl ferulate were identified. Both ethanol and n-hexane extract of WB exerted antioxidant activities. Out of two extracts, WB n-hexane extract displayed moderate inhibition of the α-amylase enzyme. In conclusion, the presence of a notable number of antioxidants in WB may considered suitable for addition to diet as an alternative means of preventing chronic diseases such as cardiovascular disease, cancer, and type 2 diabetes.

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Institutional Review Board Statement:

This study is only based on *in vitro* experiments. No animals were used in any experiment.

Data Availability Statement:

All data are availed in the result section of this manuscript.

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Conflicts of Interest:

The authors declared no potential conflict of interests with respect to the research, authorship, and/or publication of this paper.

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