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Pharmacological effects and phenolic constituents of *Rheum wittrockii*

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Abstract: **Background**: Medicinal plants are major sources of bioactive polyphenols. The plants of *Rheum genus* contain valuable phenolic compounds which exhibit various important pharmacological activities. **Objectives**: The current study was designed to investigate the polyphenols in *Rheum wittrockii* roots through bioassays and extraction/ isolate them via chromatophilic methods. **Methods**: The roots biomass of *R. wittrockii* was extracted with methanol and fractionated into four sub-fractions, i.e. *n*-hexane, chloroform, ethyl acetate and *n*-butanol (FG1-FG4). Total Phenolic Content (TPC) assay was used to affirm the number of total phenolic contents equivalent to gallic acid. Antioxidant activities were measured via 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. α-amylase enzyme inhibition activities were performed using standard methods. Antibacterial activities were performed against Methicillin-Resistant *Staphylococcus Aureus* (MRSA) in dose dependent manner for all the fractions. The FG2 fraction was subjected to series of chromatographic separation using silica gel column to isolate the main phenolic compounds. **Results**: Chloroform fraction (FG2) showed TPC of 12.5 mg/g GAE, strong antioxidant activities i.e. 85±0.22% at dose of 100 mg/mL (IC_{50}= 6.25 mg/mL) against DPPH and also showed strong α-amylase inhibition activities with IC_{50} values of 432 µg/mL, compared to IC_{50} of standard acarbose (125 µg/mL). It was active antibacterial against MRSA at lower doses. The FG2 fraction was further subjected to various chromatographic separations to obtain three known phenolic compounds, protocatecutic acid (1), isovanilliic acid (2) and epipinoresinol (3) in appreciable quantities for the first time from this plant. The structures of all the three compounds were elucidated through FT-IR, NMR and Mass spectral studies. **Conclusion**: The present investigation concluded that the roots of *Rheum wittrockii* is a good source of phenolics. Its chloroform fraction showed potent antioxidant, anti-diabetic and antibacterial effects probably due to its three compounds isolated for the first time. The compounds are valuable pharmaceuticals with promising biological actions.

Keywords: Antioxidant; α-amylase inhibition; Antibacterial; Chloroform fraction (FG2); *Rheum wittrockii;* Total phenolic contents

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INTRODUCTION

Medicinal plants have been used as major sources of bioactive natural products for the treatment of various human diseases and scientists are keen in finding new sources of novel and known compounds of pharmacological significance (Kumar *et al.,* 2020; Gupta *et al*., 2019). Polyphenols is a group of compounds having phenol as major pharmacophore that include anthocyanins, flavonoids, phenolic acids, tannins, lignin's and coumarins etc. (Cai *et al*., 2021). These phenolics have been linked to potential health advantages such as antioxidants. Both phenolics and flavonoids are significant bioactive substances due to their benefits for human health and their potential to treat and cure many diseases (Esposito *et al*., 2019). Several phenolic compounds such as gallic acid, catechins, quercetin, kaempferol, resveratrol and rutin etc. exhibit various health-promoting activities and can be potentially used in the development of natural health supplements and functional foods. (Shang *et al*., 2022; Khattak *et al*, 2020; Park and Lee, 2021; Vidal-Casanella *et al*., 2007). The genus *Rheum* (Rhubarb) belongs to the family Polygonaceae and contains about 60 species of perennial herbaceous plants with a strong root system that are widely distributed and cultivated in Asia and Europe. Common names for plants in this genus include "Indian Rhubarb, Gilgiti Rhubarb and Small Himalayan Rhubarb (Khattak *et al*., 2020). Well-known Rheum species include *R. tanguticum R. palmatum* and *R. officinale* which have been included in several European and Asian pharmacopoeia monographs (Khattak *et al*., 2020). Traditionally, the *Rheum* plant has been used to treat gastro-intestinal disorders, hemorrhoids, diabetes measles and smallpox. Moreover, various extracts have widely been used as a purgative and anti-inflammatory agent along with having potent anti-oxidant, anti-viral, anti-bacterial, anticancer and anti-malarial effects (He *et al*., 2019). *Rheum ribes* is famous for the presence of Curcumin type of polyphenols exhibiting strong DPPH (2,2-diphenyl-1-picrylhydrazyl) and superoxide anion free radical scavenging effects (Xiang *et al*., 2020). Phytochemically,

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Rheum species contains valuable compounds such as emodin, aloe emodin, physcion, chrysophanol, rhein, emodin glycoside, chrysophanol rhein, emodin glycoside, chrysophanol glycosides and anthraquinones which exhibit various important pharmacological activities i.e., anti-oxidant anti-inflammatory, anti-malarial, anticancer, inhibition of cholesterol synthesis, anti-bacterial and anti-viral (Mohtashami *et al*., 2021). *Rheum wittrocki*, commonly known as Wittrock's rhubarb, is a perennial herbaceous plant and is native to the high-altitude regions of the western Himalayas. In Pakistan, *R. wittrocki* is found in the northern areas of Pakistan since it grows in subalpine and alpine zones at an altitude of 2700-4300 meters. It is locally known as “kahmar in Chatrali” and has been found in Dir Upper and Chitral region of Pakistan. Locally, the plant is used as traditional medicine due to its laxative and antipyretic properties (Kobylina *et al*., 2017). It is commonly known as "Da Huang" in traditional Chinese medicine and is used as purgative and anti-inflammatory (Zhuang *et al*., 2020). Owing to the greater pharmacological significance, this study was aimed to investigate the total phenolic contents, antioxidant, anti-α-amylase and antibacterial activities of different fractions of *R. wittrocki* roots and isolate the main polyphenolics for the first time. This is probably the first report on the bioassay guided fractionation and isolation of three known phenolic compounds from *R. wittrocki* roots.

MATERIALS AND METHODS

Materials

The following chemicals were used in this study. Methanol, ethyl acetate; chloroform and butanol (commercial grade, local) were further purified by distillation. Deionized water, HCl and NaOH were of analytical grade (Merck, Darmstadt, Germany). The reagents such as $FeCl_3$, Folin–Ciocalteu reagent, Gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, acarbose, α-amylase, dimethyl sulfoxide (DMSO) used were purchased from Sigma Aldrich (Bulington, MA, USA) and used without purification. The culture of MRSA was obtained from ATCC (23235).

Plant collection and identification

The whole plant of *Rheum wittrockii* (50 Kg) was collected from Bony valley, District Chitral in August 2022 after identification by Dr. Abdul Khaliq Jan, Associate Processor, Chemistry Department, SBBU, Sheringal Dir upper KP. A voucher specimen was kept in the herbarium of Botany Department, SBBU, Sheringal (Accession number R-001). The root part was separated and washed with tap water to remove any debris. The material was shade dried for 30 days, chopped and ground to fine powder using electrical grinder. The grinded samples were passed through 80 mm sieve before further process to obtain 8 Kg of dried biomass (16 %).

Extraction and fractionation

8 Kg of powdered material was suspended in 20 L methanol in a closed aluminum container for 12 days with occasional shaking. The extract was filtered through Whattman filter paper (No. 42) and concentrated on rotary evaporator at 45ºC to obtain dark brown gummy crude (380 g). This crude was suspended in water-methanol mixture (1: 1, 200 mL) to form aqueous suspension and defatted batch wise with *n*-hexane to obtain FG1 fraction (22.3 g). The defatted suspension was successively extracted with chloroform, ethyl acetate and n-butanol to yield chloroform soluble fraction (FG2, 120 g), ethyl acetate soluble fraction (FG3, 39 g) and n-butanol soluble fraction (FG4,180 g) respectively.

Tests for phenolic contents

The fractions were screened for the presence of phenolics using the following tests (Tessema *et al*., 2023).

$FeCl_3$ test

Saturated solution of $FeCl_3$ (0.5 mL) was added into 0.5 mL of each extract solution (20 mg/mL in methanol) in a test tube and mixed. The appearance of deep blackish green color confirmed the presence of phenolic contents in our samples.

Shinoda test

A piece of magnesium ribbon and 1 ml of concentrated HCL was added with 2 mL of extract solution. The appearance of pink color of the solution showed the presence of flavonoids.

Lead acetate test

1 mL of saturated lead acetate solution was added to 5 mL of extract, flocculent white precipitate appeared which showed the presence of flavonoids.

Total phenolic content (TPC) assay

Folin Ciocalteu (FC) method was used in determination of total phenolic content assay using standard protocols. Gallic acid was used as standard drugs and also to obtain calibration curve. Briefly, dilutions of gallic acid in concentration of 5 mg/mL to 0.01 mg/mL were prepared in distilled water. Various dilutions of all the fractions were also prepared in 100, 50, 25, 12.5, 6.25, 3.12, 1.6 and 0.8 mg/mL concentrations. Distilled water was used as blank. To initiate the reaction, 50 µL of each sample was added into 20 µL FC reagent mixed in 430 µL of distilled water (solution 1). A solution-2 containing 50 µL of 20 % Na_2CO_3 in 450 µL of distilled water was mixed with solution 1 and incubated at 37 °C for 1 hour. Afterwards, the absorbance of reaction mixture was measured on a UV spectrophotometer at 720 nm. The TPC was calculated as mg of gallic acid equivalent per g of sample (Dominguez-López *et al*., 2023).

2, 2- Diphenyl -1-picrylhydrazyl (DPPH) radical scavenging assay

DPPH free radical scavenging assay was used to determine the antioxidant effect of our samples using standard protocols while ascorbic acid was used as standard. Briefly, various dilutions of ascorbic acid in concentration of 5

mg/mL to 0.01 mg/mL were prepared in distilled water [Ke *et al*., 2023]. Various dilutions of all the fractions were prepared in 100, 50, 25, 12.5, 6.25, 3.12, 1.6 and 0.8 mg/mL concentrations. 100 µL of each sample was added into 900 µL DPPH solution and incubated at 37 °C for 30 minutes in the dark. The absorbance was measured at 517 nm. DPPH solution in methanol without sample was used as control. The experiment was carried out in triplicate. DPPH free radical inhibition in percentage was calculated by this formula:

$$\text{DPPH Percentage inhibition} = \frac{(\Delta A517\ \text{Controle} - \Delta A517\ \text{Sample})}{A517\ \text{Controle}} \times 100$$

Average values were calculated while all tests were run in triplicate (n=3).

Standard method was used to determine half maximal inhibitory concentration (IC_{50}) which is the concentration of sample to decrease the absorbance by 50%. In order to calculate the IC_{50} value, the % inhibition was plotted against serial dilution of samples concentration. The two closed concentrations to 50 % inhibition were subjected to "Interpolation method" to determine IC_{50} values.

α-Amylase inhibitory assay

α-Amylase inhibition activities for all the samples were determined using a modified method (MeI *et al*., 2020). Briefly, the enzymatic solution was prepared by mixing 27.5 mg of α-amylase was in 100 mL sodium phosphate buffer (pH 6.7) while 6.7 mmol NaCl (20 mL) was used to keep the pH of this solution at pH 6.9. Various dilutions of each extract from a stock solution of 100 mg/mL concentration were prepared i.e., 100, 50, 25, 12.5, 6.25, 3.12, 1.6 and 0.8 mg/mL in distilled water. Similarly, various concentrations of acarbose standard solution were prepared (1000 µg/mL-0.97 µg/mL in DW). To initiate the reaction, 100 µL of each sample was combined with 100 µL of the enzymatic solution and incubated for 30 minutes at 37 °C followed by addition of 200 µL of substrate (1 % starch solution prepared in phosphate buffer, pH 6.8) and again incubated for 10 minutes at 37 °C. 200 µL of Dinitro Salicylic Acid (DNS) was added to the reaction mixture after incubation in order to produce color. The reaction was stopped by keeping each reaction vial in boiling water for 5 minutes. 2.20 mL DW was added to each sample before UV/Visible measurements. Sodium phosphate buffer solution served as control. A BMS double beam spectrophotometer (VIS 1100) was used to observe absorptions at wave length of 540 nm. The measurements were recorded in triplicate while the calculation of the percent inhibition were obtained by using the formula:

$$\text{DPPH Percentage inhibition} = \frac{(\Delta A540\ \text{Controle} - \Delta A540\ \text{Sample})}{A540\ \text{Controle}} \times 100$$

Antimicrobial activities

Antibacterial activities were performed in the Micro lab of Pharmacy Department, SBBU and Sheringal. "Agar disc diffusion method" was used for determination of anti-bacterial activities against Methicillin-Resistant *Staphylococcus aureus* (MRSA, ATCC 23235) with some modifications. DMSO (dimethyl sulfoxide) was used for the preparation of sample solutions. The concentration used was 10 mg/mL of each sample. Briefly, 100 µL of bacterial suspension were inoculated on 30 ml of Tryptic Soy Agar (TSA) plates. After drying in a sterile laminar hood, at room temperature for 30 min, wells of 6 mm diameter were made by sterilized cork-borer. The various testing doses of samples (10, 20, 50, 75, 100 µl) from the stock solutions of 10 mg/mL were added in a clockwise direction from ranging keeping the last well as a negative control. Clarithromycin was used as a positive control and a standard drug. To slow down diffusion, the plates were held at room temperature. After that the plates were kept for 24 hours in the incubator at 37 ºC. Zones of inhibition were measured in mm and converted into percentage (Pham *et al*., 2017).

Statistical analysis

All the data was analyzed using Microsoft Excel (Microsoft office version 2016, Microsoft incorporation, USA) software. The data was statistically evaluated by student's T test. P values less than 0.05 and .01 were taken as significant Values and expressed as mean ± S.E.M.

Isolation of compounds

The chloroform soluble fraction (FG2) (100 g) was subjected to column chromatography using silica gel as stationary phase (normal) while the elution was carried out using *n*-hexane (100 %, FGA), *n*-hexane-$CHCl_3$ (1:1, FGB), $CHCl_3$ (100%, FGC), $CHCl_3$-EtOAc (1:1, FGD), EtOAc (100%, FGE), EtOAc-MeOH (1:1, FGF) and MeOH (100%, FGG) respectively. The fraction FGB (16 g) obtained from the solvent system *n*-hexane-$CHCl_3$ (1:1) was re-chromatographed on silica gel column, eluting with n-hexane-$CHCl_3$ and $CHCl_3$-MeOH mixtures in increasing polarity. The eluent obtained from *n*-hexane: $CHCl_3$ (6.5:3.5 and 6.0:4.0) showed single spot along with some impurities in the tailing. This semi pure compound was purified by micro column (silica gel) using *n*-hexane-EtOAc (9:1) to obtain compound 1 (protocatecutic acid, 15.3 mg). The sub-fraction FGC (19 g) obtained from the major column when eluted with $CHCl_3$ (100 %) was again loaded on another column over silica gel and eluted with mixtures of n-hexane: $CHCl_3$ and $CHCl_3$: MeOH in increasing order of polarity. The sub fractions obtained from $CHCl_3$-MeOH (9.5:0.5+DEA) showed similar pattern on TLC (a major spot with some minor spots at different Rf values). The fraction was subjected to pencil column (silica gel) while eluting $CHCl_3$-MeOH (9:1) to isolate compound 2 (isovanilliic acid, 13.9 mg) from top fraction. The FGD (5 g) sub-fraction obtained from main column while eluting with $CHCl_3$-EtOAc (1:1) on concentration showed needle like crystals from the mother liquor. The crystals were washed with *n*-hexane with slight heating several times to remove the colored impurities. The pure compound 3 was obtained and identified as epipinoresinol (35 mg).

Protocatecutic acid (1): White powder (15.3 mg); m.p. 196-197 ºC, UV/Visible (λ_{max}): 296 nm (MeOH); HR-EIMS (*m/z*): 154.117 (Calcd. for $C_6H_6O_4$, 154.026), Fragments (rel. %): 154 (M^+, 100), 137 (90), 108 (31), 58 (20), 43 (69). FT-IR ($\bar{\upsilon}_{max}$ = cm^{-1}): 3259 cm^{-1} (OH), 1700 cm^{-1} (C=O), 1605 cm^{-1} (C=C aromatic ring), 1320 and 1000 cm^{-1} (C-O). $^{13}C/^{1}H$ NMR (400-100 MHz, δ= ppm, *J*= Hz): The NMR data has been provided in results and discussion.

Isovanilliic acid (2): White powder (13.9 mg), m.p. 257-260ºC, UV/Visible (λ_{max}): 256.3-292.7 nm, HR-EIMS (*m/z)*: 167.88 (calcd. for $C_8H_8O_4$, 168.157), Fragments (rel. %): 168.2 (M^+, 100), 153.2 (94), 125.0 (17), 97.1(8), FT-IR (V_{max} = cm^{-1}): 3433 cm^{-1} (OH), 2952 cm^{-1} (CH), 1600 cm^{-1} (C=C), 1262 and 1233 cm^{-1} (C-O), $^{13}C/^{1}H$ NMR (400-100 MHz, δ= ppm, *J*= Hz): The NMR data has been provided in results and discussion.

Epipinoresinol (3): Yellow needle like crystal (35 mg), $[\alpha]_D^{26}$: +131 (Me_2CO), m.p.137-139 ºC. UV/Visible λ_{max}: 237, 281 nm, HR-EIMS *(m/z)*: 358.21 (calcd. for $C_{20}H_{22}O_6$ 358.1416), Fragments (rel. %): 358.2, (M^+, 62), 325.8 (5), 258.81 (4), 225.2 (6), 204.8 (18), 163.7 (36), 152.7 (100), 136.8 (46), 124.8 (11). $^{13}C/^{1}H$ NMR (400-100 MHz, δ= ppm, *J*= Hz): See result and discussion.

Characterization of compounds

Thin layer chromatography was carried out using pre coated TLC card (F_{254}, Merck, Darmstadt, Germany) while ceric sulphate was used as visualization reagent. The melting points were measured at Gllenkemp instrument and are uncorrected. UV/Visible spectral measurements were carried out using VIS1100 spectrophotometer (BMS, USA) while FT-IR spectra were recorded on "Thermo-Scientific Nicolet summit FTIR spectrometer, (Thermo-Fisher, USA)" with ATR assembly. Mass spectral measurements were carried out using HP-5973 (Agilent Technology, USA) in EIMS mode. NMR spectra were obtained on Bruker 400 MHz spectrophotometer (Bruker, Germany, 400 MHz for ^{1}H-NMR while 100 MHz for ^{13}C NMR).

RESULTS

Preliminary phytochemical tests for phenolics

The results of phytochemical analysis showed the presence of significant amount of phenolics in the crude as well as in chloroform fraction (FG2) of *Rheum wittrockii* roots. The *n*-hexane (FG1) and ethyl acetate (FG3) fractions showed presence of small amount of phenolics while no phenolic were detected in the *n*-butanol fraction (FG4).

Total phenolic content (TPC) measurements

Folin Ciocalteu (FC) method was used in determination of total phenolic content assay using Gallic acid as standard. The absorptions were monitored on respective wave lengths through UV/Visible spectrophotometer at 720 nm (λ_{max}). Various concentrations (5 to 0.02 mg/mL) of the standard gallic acid were used to obtain the dose dependent calibration curve (fig. 1A). It showed a steady relationship between the two variables, concentration and absorptions (R^2>0.9). Various dilutions of all the fractions FG1-FG4 (from 100-0.8 mg/mL) were prepared in distilled water to measure the dose dependent response when chelating with FC reagent. The data suggests a higher absorbance for the FG2 fraction as compared to the other fractions used showing the presence of higher amounts of gallic acid equivalent phenolics in this fraction (fig. 1b).

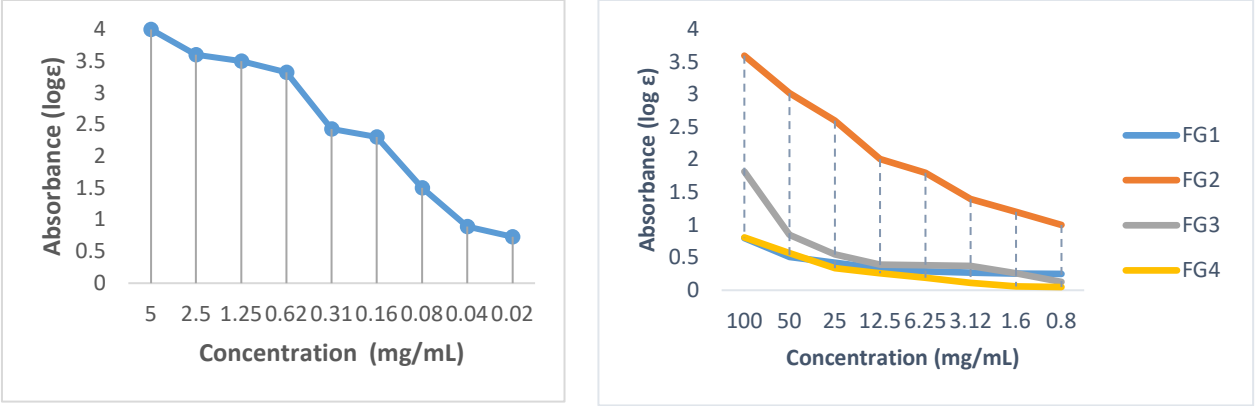
The total phenolic contents measurement against gallic acid for each of the fraction is provided in table 1. The results indicate that the FG2 fraction contains 12.5 mgGAE/g of dry weight phenolic contents while FG3 showed 10 mg GAE/g dry weight phenolics. The other extracts showed a relatively lower amount (0.4 mg) of GAE contents per g of samples.

DPPH antioxidant activities

Antioxidant activity was determined using DPPH Free radical scavenging assay. *Table*-2 illustrates results of DPPH % inhibition by different extracts of *R. wittrockii* root. All the measurements were carried out using UV/Visible spectrophotometer at 517 nm where DPPH solution served as control (without inhibitor) and methanol was used as blank while ascorbic acid was used as standard. Amongst all, the FG2 fraction exhibited the highest antioxidant activity; 85 ± 0.22 % at dose of 100 mg/mL while ethyl acetate soluble fraction exhibited 77.22 ± 0.25 % inhibition. The *n*-hexane and butanol remained less potent at the same concentrations. The IC_{50} valued obtained for chloroform fraction was 6.25 mg/mL while that of ethyl acetate was calculated as 19 mg/mL. The standard ascorbic acid showed 98 % inhibition at a dose of 5 mg/mL with IC_{50} value of 150 µg/mL against DPPH.

α-Amylase inhibition activities

Collaboration curve was obtained first for the measurements of α-amylase inhibition by standard drug, acarbose in dose dependent manner. The control sample (enzyme +starch+ DNS) showed maximum absorption of 0.778 at 540 nm. A relatively non-linear curve was obtained for acarbose (R^2=0.65) (fig. 2). The IC_{50} values obtained for acarbose was 125 µg/mL. Amongst the *R. wittrocki* samples FG2 exhibited outstanding α-amylase inhibition (90 % at 100 mg/mL dose, 83 % at 50 mg/ and 59 % at 0.781 mg/mL) with IC_{50} values of 0.432 mg/mL or 432 µg/mL (table 3). This potential is comparable to the standard for acarbose which showed IC_{50} of 125 µg/mL. FG1 did not inhibit the enzyme activity while FG3 suppressed enzyme activity by 75% at 100 mg/mL, was moderately effective at 50 and 25 mg/mL and was ineffective at lower concentrations (IC_{50} = 12 mg/mL). The same pattern was observed for FG4 fraction with relatively lower IC_{50} (10 mg/mL) as compared to FG3.



A **B**

Fig. 1: A: Gallic acid standard curve; B: Absorption studies of samples against FC reagent.

Table 1: Measurements of total phenolic contents equivalent to Gallic acid.

Extract	Concentration	Gallic acid equivalent	Absorption equivalent at 720 nm	Number of milligrams of GA equivalent in 1g fraction
FG1	100	0.04	0.8	0.4
FG2	100	3.6	1.25	12.5
FG3	100	0.1	1.8	1
FG4	100	0.04	0.81	0.4

Table 2: Dose dependent DPPH (% age inhibition) activities of *Rheum wittrockii* root fractions.

Concentration (mg/mL)	%age inhibition (Mean±SEM) FG1	FG2	FG3	FG4
100	52 ± 0.32	85 ± 0.22	77 ± 0.25	67 ± 0.30
50	33 ± 0.43	81 ± 0.12	68 ± 0.21	47 ± 0.31
25	30 ± 0.61	76 ± 0.45	60 ± 0.24	27 ± 0.21
12.5	13 ± 0.21	67 ± 0.67	38 ± 0.56	24 ± 0.24
6.25	-	50 ± 0.57	33 ± 0.54	-
3.12	-	33 ± 0.21	17 ± 0.43	-
1.6	-	-	-	-
0.8	-	-	-	-
IC_{50} values	101.5 mg/mL	6.25 mg/mL	19 mg/mL	31.5 mg/Ml

Note: The absorption of control was obtained as 3. SEM is the standard error mean of three assays.

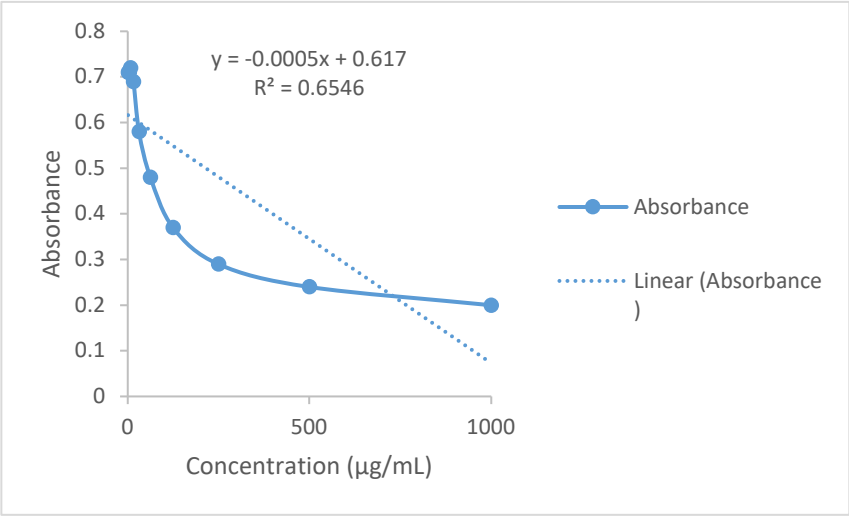


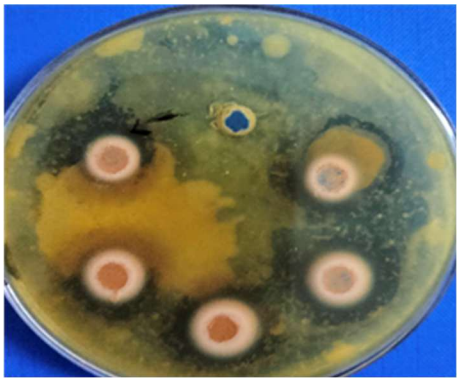
Fig. 2: Standard curve in α-amylase inhibition for Acarbose.

Table 3: Dose dependent α-amylase inhibition activities of FG1-FG4 fractions of *R. wittrocki*.

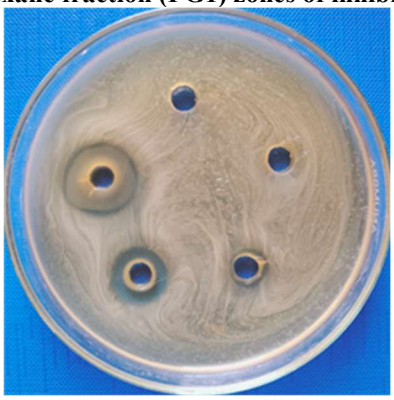
Concentration (mg/mL)	α -amylase % age inhibition			
	FG1	FG-2	FG3	FG4
100	47	90	75	80
50	--	83	68	73
25	--	80	66	67
12.5	--	73	52	52
6.25	--	70	38	38
3.125	--	66	34	34
1.562	--	60	26	33
0.781	--	59	23	29
IC_{50} =	NC	432 µg/mL	12 mg/mL	10.5 mg/mL

Note: The control absorption was obtained as 0.778. (Control= Enzyme + starch + DNS)

n-hexane fraction (FG1) zones of inhibition



Chloroform fraction (FG2) zones of inhibition



Ethyl acetate fraction (FG3) zones of inhibition

n-Butanol fraction (FG4) zones of inhibition

Standard (Clarithromycin) zones of inhibition

Fig. 3: Visual representation of zone of inhibition against MRSA by R. wittrocki samples and standard.

Table 4: Zone of inhibitions (in mm) of FG1-Fg4 against MRSA.

Zone of inhibition (mm) ±SEM					
Dose	FG1	FG2	FG3	FG4	Standard
10 µL	5±0.01	6±0.5	5±0.2	4±0.13	8±0.3
25 µL	10±0.12	11.±0.18	8.±.0.12	6±0.13	11±0.4
50 µL	13±0.16	15.±0.08	13±0.16	12±0.14	15±0.43
75 µL	15±0.17	18 ±0.18	15±0.17	15±0.17	20±0.32
100 µL	16±0.18	20.±0.49	17±0.19	16±0.18	30±0.65

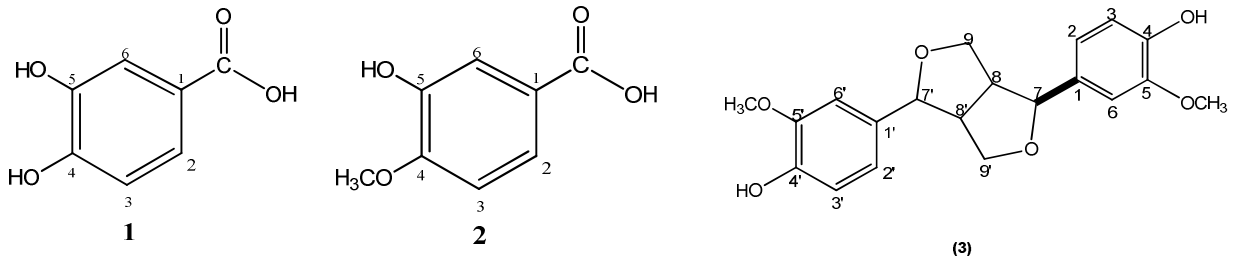


Fig. 4: Structures of compounds 1-3 from *R. wittrocki*.

Table 5: ^{13}C-^{1}HNMR data of compound 1 showing multiplicity (δ= ppm).

Carbon NO	^{13}C-NMR	Multiplicity	^{1}H-NMR (*J*= Hz)
1	120.0	-C-	--
2	114.5	-CH-	7.51 (1H, d, *J*= 8 Hz)
3	146.7	-CH-	7.61 (1H, d, *J*= 8 Hz)
4	150.4	-C-	--
5	112.3	-C-	--
6	125.2	-CH-	7.46 (1H, s)
C=O	170.2	--	--
4-OH	--	--	3.5 (1H, s)
5-OH	--	--	3.5 (1H, s)

Table 6: ^{13}C-^{1}HNMR data of compound 2 showing multiplicity (δ).

Carbon No.	^{13}C-NMR (δ)	Multiplicity	^{1}H-NMR (δ) (*J*= Hz)
1	120	-C-	--
2	114.5	-CH-	7.51 (1H, d, *J*= 8 Hz)
3	146.7	-CH-	7.61 (1H, d, *J*= 8 Hz)
4	150.4	-C-	--
5	112.3	-C-	--
6	125.2	-CH-	7.46 (1H, s)
C=O	170.6	--	--
4-OCH_3	56.1	--	3.5 (3H, s)
5-OH	--	--	3.5 (1H, s)

Antibacterial activities

The antibacterial activities of all the samples were carried out against MRSA in doses dependent manner while the standard used was clarithromycin. The MIC of standard drug was calculated prior proceeding with the samples. The drug did not showed inhibition of MRSA at 10 µL concentration however showed 11±0.4, 15±0.43, 20±0.32, 30±0.65 mm at doses of 25, 50, 75 and 100 µL. The inhibition zones of all the samples against same doses of standard have been enlisted in table 4. FG2 showed inhibition zones of 6±0.05, 11±0.18, 15±0.08, 18±0.18 and 20±0.49 at 10, 25, 50, 75 and 100 µL doses respectively and was effective at lower doses as compared to the drug (100 % at 11 µL). The other fractions showed lower inhibition as compared to standard. fig. 3 depicts visual inhibition zones of our samples and standard.

Structure elucidation of compounds

The structures of obtained compounds have been shown in fig. 4. Protocatechuic acid (1) was obtained as white powder.

Table 7: ^{13}C-^{1}H NMR data of compound (3) showing multiplicity (δ= ppm).

Carbon No.	^{13}C-NMR (δ)	Multiplicity	^{1}H-NMR (δ, *J*=Hz)
1	134.5	-C-	--
2	120.7	-CH-	6.75 (d, *J*=7.5 Hz)
3	115.3	-CH-	6.77 (d, *J*=7.5 Hz)
4	145.3	-C-	--
5	147.2	-C-	--
6	110.3	-CH-	6.97 (s)
7	86.1	-CH-	5.03 (d, *J*= 7.0 Hz)
8	54.3	-CH-	2.54 (m)
9	71.2	-CH_2-	3.84 (m)
1′	134.5	-C-	--
2′	120.7	-CH-	6.75 (d, *J*=7.5Hz)
3′	115.3	-CH-	6.77 (d, *J*=7.5Hz)
4′	145.3	-C-	--
5′	147.2	-C-	--
6′	110.3	-CH-	6.97 (s)
7′	134.5	-CH-	5.03 (d, *J*= 7.0 Hz)
8’	120.7	-CH-	2.54
9’	115.3	-CH_2-	3.84
5-OCH_3	56.1	CH_3	3.88 (s, 3H)
5’-OCH_3	56.1	CH_3	3.88 (s, 3H)
4-OH	--	OH	6.56 (s)
4’-OH	--	OH	6.56 (s)

It showed strong UV/Visible absorptions at 295 nm in MeOH while its FT-IR spectrum revealed the presence of hydroxyl group at 3259 cm^{-1}, aromatic rings at 3040 cm^{-1} with overtones at 2000 cm^{-1}, carbonyl group at 1700 cm^{-1} (C=O group), vinylic group at 1400 cm^{-1} and carboxy at 1000 cm^{-1} (C-O vibrations). The HR-EIMS of the compound (1) showed molecular ion peak at 154 *m/z* which was consistent to the formula $C_7H_6O_4$. The ^{13}C-NMR spectrum showed peaks for all the carbon including carbonyl carbon at δ 170.2, aromatic carbon atoms having phenolic OH groups showed peaks at δ 146.1 (C-3) and 150.7 (C-4) while the other ring carbons appeared at δ 120.0 (C-1), 114.5 (C-2), 146.7 (C-3) and 125.2 (C-6) respectively. The ^{1}H-NMR spectrums showed three types of peaks at the aromatic region. The aromatic protons showed doublets at δ 7.51 (*J*= 8 Hz, H-2), δ 7.61 (*J*= 8 Hz, H-3) and singlet at δ 7.46 (H-6) shows the presence of ortho as well meta coupled protons. The hydroxyl protons showed a broad singlet at δ 3.5 table 5). The MS and NMR results, in comparison with the literature, confirm compound (1) as protocatecheuic acid (Xu *et al*., 2023).

Isovanillic acid (2) was also obtained as white powder and showed UV absorption at 292 nm while its FT-IR showed the presence of various functionalities at different wave numbers i.e. 3433 cm^{-1} (OH), 3000(-OCH_3), 2952 cm^{-1} (aromatic C-H starching), 1600 cm^{-1} (C=O group), 1600 cm^{-1} (aromatic C=C with overtones at 2000 cm^{-1}) and 1000 cm^{-1} (C-O vibrations). The HR-EIMS of the compound 2 showed molecular ion peak at 168.066 *m/z* which was calculated for the formula $C_8H_8O_4$ 168.042. The ^{13}C-NMR spectrum showed peaks at δ 146.1 and 150.7 of ring carbons (C-4, C-5) having hydroxyl or alkoxyl group. The peak at δ 170.6 revealed the presence of carbonyl group, while the peak at δ 56.1 showed the presence of methoxy group. The ^{1}H-NMR spectra showed peaks aromatic protons at δ 6.93 to 7.60 and peaks at δ 5.33 and 9.52 revealed that the ring is substituted by hydroxyl and carboxyl group. A three protons singlet at δ 3.90 showed the presence of a methoxy group (table 6)**.** The above data was similar to the data reported in the literature for isovanillic acid (2) (Kolodziejczyk-Czepas and Liudvytska, 2021).

Epipinoresinol (3) was obtained as needle crystals from the FGD sub-fraction of main column while eluting with $CHCl_3$-EtOAc (1:1) The crystals were washed with worm *n*-hexane repeatedly until obtained as white powder. The FT-IR spectrum of this compound showed the presence of OH, OCH_3, CH, C=O, C=C and C-O functional groups at 3433, 3000, 2952, 1600, 1400 and 1233 cm^{-1}. The molecular ion peak in HR-EIMS papered at *m/z* 358.14 which was consistent with the formula $C_{20}H_{22}O_6$ and the daughter ions at *m/z* 358.9 $[M]^+$, 326.9 $[M\text{-}OMe]^+$, 205.9 $[M\text{-}ArCHO\text{-}H]^+$, 163.9 $[M\text{-}ArCHCH{=}CH_2]^+$ were analogous to the MS data described previously for epipinoresinol (Xiang *et al*., 2011). Further, the ^{13}C-NMR spectrum showed the presence of aromatic carbons with double intensities at δ 134.5 (C-1 and C-1′), 120.7 (C-2, C-2′), 115.3 (C-3, C-3′), 145.3 (C-4, C-4′), 147.2 (C-5, C-5′) and 110.3 (C-6, C-6′).The absorption for C-7, C-7′carbons appeared at δ 86.1, C-8, C-8′ at δ 54.3 while for the C-9 (CH_2), the ^{13}C-NMR showed peak at δ 76.9. The ^{1}H-NMR spectrum showed three different peaks in the aromatic

regions of double intensities at δ 6.97 (s, CH- H-6,6′), 6.75 (d, CH, *J*=7.5 Hz, H-2,2′) and 6.77 (d, CH, *J*=7.5 Hz H-3,3`). The other protons of furan moiety showed absorptions at δ 5.03 (d, *J*= 7.0 Hz, H-7, 7'), 2.54 (m, H-8, 8`) and 3.84 (m, H-9,9`). The six protons singlet peak at δ 3.88 was assigned to the two separate methoxy group protons (C-5,5′) while the two protons intensity singlet peak was assigned to the two hydroxyl groups at C-4 and 4′ (table 7). Both ^{13}C and ^{1}H-NMR data was in agreement to the known compound epipinoresinol (3) reported from *Galium verum* as described in literature (Bradic *et al*., 2021; Muro-Villanueva *et al*., 2023).

DISCUSIONS

The roots crude extract was fractionated through the application of various solvents to identify the bioactive fraction which revealed that significant amount of phenolics was present in chloroform (FG2) and ethyl acetate (FG3) fractions. The results indicate that the FG2 fraction contains 12.5 mgGAE/g of dry weight phenolic contents while FG3 showed 10 mg GAE/g dry weight phenolics. Previous studies suggest a greater amounts of TPC (92.82 mgGAE/g) in *R. emodi's* roots (Sánchez-Rangel *et al*. 2013). The methanolic extracts from roots of *R. palmatum* and *R. australe* contained 20 and 6.85 mg GAE/1g dry weight phenolic (Pham *et al*., 2017) while *R. raphonticum* roots ethanoic extract contained 1.115 mg GAE/g dry weight of TPC (Sokol-letowska *et al*., 2009).

Promising antioxidant activities with IC_{50} valued of 6.25 mg/mL for FG2 (85 %) and 19 mg/mL for FG3 (77 %) were obtained in this study. The higher antioxidant activities are due to the presence of promising amounts polyphenolic compounds in *R. wittrockii* chloroform and ethyl acetate fractions. The nature and amounts of phenolic compounds are responsible for antioxidant activity. As previously described, the chloroform extract of *R. ribes* extracts exhibited greater antioxidant activities at 50 mg and 100 mg doses (91.09 and 93.14 %DPPH) (Bajracharya and Gupta, 2021). According to our results, the chloroform fraction of *R. wittrockii* shows the highest antioxidant activity by inhibiting DPPH 85±0.22% at dose of 100 mg/mL.

In the whole world the Diabetes Mellitus (DM) is the most common types of disease and about 173 M people have been diagnosed with DM with a projection of 366 million in 2030 (Tan, TE and Wong, 2023). In DM conditions, the blood glucose level increases resulting in low insulin secretion. To treat DM, the inhibition of enzymes that are involved the carbohydrates hydrolysis such as α- amylase and α- glucosidase is necessary (Eruygur *et al*., 2024). Our results provide a good insight into the antidiabetic potential that is associated with the phenolic contents present and further available for isolation of potential polyphenolic compounds that possess strong antidiabetic activities. In this study, the *R. wittrocki* sample FG2 exhibited outstanding α-amylase inhibition (90 % at 100 mg/mL dose, 83 % at 50 mg/mL) with IC_{50} values of 432 µg/mL Previously, extract from many plants showed remarkable α-amylase inhibition such as the *Tamarindus Indica* (90 %), *Vaccinium myrtillus* (75 %), *Rosmarinus officinalis* (75 %), *Securidaca longepedunculate* (45 %) etc. (Tran *et al*., 2014). The *R. turkestanicum* ethyl acetate fraction enriched in phenolic contents showed potent α-amylase inhibitory potential (IC_{50}= 46.4 µg/mL) while *R. tanguticum* root extract showed strong α-amylase inhibitory activities with IC_{50} of 42 µg/mL (Mohtashami *et al*., 2023). It has been estimated that there are 21 naturally occurring flavonoid including luteolin, amentoflavone, daidzein and luteolin 7-o-glucoside which are the strongest inhibitors of the α- amylase (Zhao *et al*., 2021). Some previous studies reported the methanolic extract of *R. palmatum* and *R. undulatum* showed 90-95 % inhibition of *S. aureus* with IC_{50} values in range of 125 to 250 g/mL (Yue *et al*., 2022). Owing to greater biological potential of FG2 fraction, it was further selected for isolation of pure compounds which resulted in isolation of protocatechuic acid (1), isovanilliic acid (2) and epipinoresinol (3) for the first time from this plant. The structures of all the three compounds were elucidated through FT-IR, NMR and Mass spectral studies The UV/Visible absorptions that arise from the electronic transitions provided evidences of the presence of aromatic rings in the compounds Akash *et al*., 2020). The FT-IR spectra of all the compounds revealed the presence of various functional groups that are responsible for their potent antioxidant as well as α-amylase inhibition. These include hydroxyl/phenolic groups, aromatic functionalities, carbonyl functional groups, vinylic and carboxy groups (Wongsa *et al*., 2022). The HR-EIMS is a spectrometric technique useful in obtaining the molecular ion that corresponds to the molecular mass of the compound. The daughter ion peaks are useful fragments that show the successful loss of neutral molecules from the parent ion and provide the specific details about the structure of compounds (Selby *et al*., 2024). Lastly, the NMR spectra are useful in determination of total number of carbons, hydrogen, their multiplicity, connections, stereochemistry and structure backbone (Huang *et al*., 2024). All these techniques were useful in determination of the structural features of these isolated compounds.

Protocatechuic acid known as PCA is a well-known active antioxidant component in Chinese traditional medicine and widely distributed in plants such as *Stenoloma chusanum*, *Cibotium barometz* etc. Various studies suggest its antioxidant role, especially against DPPH, ferric ion chelation power, OH radical scavenging as well as superoxide scavenging activities (Kakkar & Bais, 2014). The extraordinary antioxidant activities of this compound render it a suitable pharmaceutical against diabetic, cancer and different metabolic syndrome (Cadena_Iniguez *et al.*, 2024). PCA is also a well-known inhibitor of glucosidase

and amylase and shows postprandial hypoglycemic capabilities in diabetic rats in vivo (Ding *et al.*, 2024). Isovanilliic acid is a potent antioxidant and antibacterial agent against various pathogenic bacteria especially *E. coli*. It is cytotoxic and genotoxic to *E. coli* and degrade the cellular proteins (Matejcyk *et al.*, 2024). This compound has also been tested as potent α-glucosidase inhibitor (Giang *et al*, 2024). Epipinoresinol is a well-known antiproliferative lignin found in medicinal plants and has the potential of inhibiting reactive oxygen species, shows antidiabetic as well as strong antimicrobial potential (Solyomvary *et al.*, 2017). In conclusion, the study reveals the potential antioxidant, amylase inhibition and antimicrobial activities of *R. wittrocki* fractions due to the presence of these compounds.

CONCLUSION

The present investigation concluded that the roots of *Rheum wittrockii* are a good source of phenolics where maximum phenolics exist in its chloroform soluble fraction. The same fraction demonstrated potent antioxidant, anti-diabetic and antibacterial effects against DPPH, α-amylase and MRSA comparable to standard drugs used. The potency of this fraction prompted us to further investigate it for isolation of individual compounds. Three compounds were obtained for the first time from this plant using optimized isolation procedures via chromatography while their structural features were obtained using spectral techniques. The compounds are known and are valuable addition to plant natural products with promising biological actions.

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Data availability statement
All data generated or analysed during this study are included in this published article.

Ethical approval
The study was conducted under the ethical approval (Ref No. SBBU/IEC/24-12) from Institutional Ethical Committee, Shaheed BB University Sheringal, Dir (U), KP, Pakistan.

Conflict of interest
The authors declare no conflict of interest.

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