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RESEARCH ARTICLE

Evaluation of Antioxidant Potential, Enzyme Inhibition, and Chemical Profiling through Mass Spectrometry on Bark Extract of *Berberis aristata*

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Abstract

Berberis aristata, a plant from the Berberidaceae family, is renowned for its medicinal properties. The presence of bioactive metabolites in its bark underscores its importance in traditional medicine and pharmacology. This study primarily focuses on the biological activities of the plant bark extract, specifically its antioxidant properties and alpha-glucosidase inhibition. In addition to these activities, mass spectrometry-based compound annotation was conducted to explore the metabolites present in the plant bark extract. In this study, the bark samples were collected and extracted using the cold percolation method with methanol. Subsequently, an ethyl acetate fraction was obtained from the methanol extract using a separating funnel. The methanol extract of the bark showed a total phenolic content (TPC) of 70.056 ± 2.52 mg GAE/g and a total flavonoid content (TFC) of 11.89 ± 0.16 mg QE/g. To determine the antioxidant activity of the samples, a DPPH assay was used. The ethyl acetate fraction of the bark showed potent antioxidant activity with an IC_{50} value of 188.93 ± 8.35 µg/mL, while the methanol extract of the bark showed radical scavenging activity with an IC_{50} value of 242.89 ± 4.62 µg/mL. In terms of alpha-glucosidase inhibition, the methanol extract of the bark had low enzyme inhibition activity, but the ethyl acetate fraction exhibited potential inhibition with an IC_{50} value of 112.99 ± 10.28 µg/mL. These results indicate that the plant bark extract is rich in phenolic content compared to flavonoid content. Additionally, the ethyl acetate fraction of the plant bark exhibits potent radical scavenging activity and inhibition of the digestive enzyme alpha-glucosidase. Mass spectrometric-based identification of metabolites revealed that the methanol extract of the bark contains compounds such as Palmatin, columbamine, and berberal. The presence of these metabolites further supports the medicinal use of this plant bark. These findings enhance the plant's potential in the drug discovery process in the future.

Keywords: *Berberis aristata;* Alpha-glucosidase; Antioxidant; Mass spectrometry; Metabolites

Introduction

In recent times, due to advancements in analytical tools for the purification and isolation of chemical compounds, natural products have gained recognition as promising candidates for drug development efforts [1]. Contemporary drugs often stem from natural products and derivatives, driving innovation in drug discovery through their pivotal role in uncovering cellular pathways [2, 3]. Throughout history, natural products have formed the foundation of global traditional healing systems, showcasing diverse roles such as antioxidants, antibacterials, photoreceptors, visual attractors, antidiabetic, anticancer, hypocholesterolemic, immune-stimulant and feeding repellants [4–6]. Therefore, natural products remain a prominent area of pharmaceutical research due to their

significance in lead compound discovery for critical therapeutic fields.Medicinal plants have served as crucial resources for both traditional and modern medicine worldwide, showcasing their therapeutic potential over millennia, evidenced by recorded herbal medicinal practices in civilizations such as Mesopotamia (2600 BC), Egypt (2900 BC), and Traditional Chinese Medicine [3, 7]. Medicinal plants serve as a basis for the drug discovery process therefore global studies confirm their efficacy, leading to the production of plant-based medicines [8, 9].

One of the most important medicinal plants available is *Berberis aristata* belonging to the family Berberidaceae which is native to South Asian countries like, Nepal and India [10]. In Nepal, various ethnic groups across 24 districts use *B. aristata* for its medicinal properties. This plant has a long history of being used for multiple purposes including treating a bacterial infection, anticancer agents, anti-hyperglycaemic, and antioxidants [11]. The presence of metabolites such as alkaloids, flavonoids, and polyphenolic compounds is strong evidence of the medicinal application of this plant. Although the plant has huge applications in medicine, the proper exploration and the pharmacological importance of metabolites from this plant have not been well reported. Therefore the current study focused on the antioxidant, antibacterial, and alpha-glucosidase inhibition potential of the bark of the *Berberis aristata.*

Materials and Methods

Chemicals and Reagents

All the organic solvents used in this research work were of analytical grade. Methanol (Fisher Scientific), Distilled water, Ethyl acetate, Ethanol, etc., were purchased from the local chemical suppliers. Chemicals and reagents like aluminum chloride, Folin-Ciocalteu reagent, Gallic acid, ascorbic acid, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), Quercetin (Hi-media), all were of analytical grade and were purchased from Fisher Scientific (India).

Plant Collection and Preparation of Extract

The sample of *Berberis aristata* was collected from the Arghakhachi district based on local healers and ethnomedical importance. Arghakhanchi district, located in the mid-western region of Nepal, experiences a subtropical to temperate climate and features hilly and mountainous terrain (68% of the district is covered by the mountainous Mahabharat Range) with altitudes varying from 305 meters to 2,515 meters above sea level. The collected plant sample was identified in the National Herbarium and Plant Laboratories in Godawari, Nepal. After identification, the bark of the sample was scraped and collected. The collected sample was dried under shade, for one week and ground into the powder.

For the extraction process, methanol was used as the solvent. The powder of the bark of Berberis aristata was dissolved in the methanol. For this 100 grams of powder of bark was taken in the conical flask and 300 mL of methanol was also added on this. The bark powder and methanol were properly shaken to make the completely soluble metabolites in the solvents. After 48 hours, the solvent was filtrated and collected in the clean beaker. After subsequent filtration two times, the collected filtrate was placed in the rota-evaporator for the evaporation of the solvent. Thus obtained viscous extract was placed in the water bath at 40 ^{o}C for the evaporation of the remaining solvent. After complete evaporation of the solvent, the dry extract of the bark of *Berberis aristata* was obtained.

Fractionation of Methanol-Based Solvent Extract

The bark extract on methanol was further extracted using ethyl acetate as a solvent. For the fractionation, the methanol extract of bark was taken in a separating funnel with 200 mL water in it. To extract the ethyl acetate fraction, an equal volume of ethyl acetate was added to it and meticulously shaken. After a complete shake of the separating funnel, the ethyl acetate fraction was collected in the beaker. Thus obtained ethyl acetate fraction was dried using the rota-evaporator and water bath subsequently at 40 ^{o}C. Thus obtained dry extract was used to examine the biological activities.

Calculation of Total Phenolic Content

For the calculation of the total phenolic content, the methanol extract of bark of concentration 500 µg/mL was used. This test was done by following the Folin-Ciocalteu phenol assay with slight modification [12]. For the calculation of phenolic content, the calibration curve was drawn by using the different concentrations of gallic acid. For this 0.5 mg/mL concentration of gallic acid was diluted further with the distilled water and 20 µL of it was loaded in a 96-well plate in triplicate followed by the addition of the same volume of sample. To initiate the reaction in each well 100 µL, of FC reagent was added, and to stabilize the colour, 80 µL of Na_2CO_3 was added separately. After adding all the reagents the plate was placed in the dark for 25 minutes and absorbance was measured at 765 nm using a microplate reader (Synergy LX, BioTek Instruments, Inc., USA). Thus obtained result was expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g).

Calculation of Total Flavonoid Content

This test was done by following the colorimetric method with slight modification [13]. For the calculation of the total flavonoid content, the methanol extract of bark of concentration 500 µg/mL was used. For the calculation of flavonoid content, the calibration curve was drawn by using the different concentrations of quercetin. For this 0.1 mg/mL concentration of quercetin was diluted further with the distilled water and 130 µL of it was loaded in a 96-well plate in triplicate followed by the addition of 20 µL of sample. To maintain the volume of the sample 110 µL of distilled water was added to the well-containing sample. To initiate the reaction and stabilize it, 60 µL of ethanol, 5 µL $AlCl_3$, and 5 µL potassium acetate were added separately. After adding all the reagents the plate was placed in the dark for 25 minutes and absorbance was measured at 415 nm using a microplate reader (Synergy LX, BioTek Instruments, Inc., USA). Thus obtained result was expressed as milligrams of quercetin equivalent per gram of dry weight (mg QE/g).

Determination of Radical Scavenging Activity

To determine the radical scavenging activity of methanol extract of the bark of Berberis aristata, a DPPH assay was followed [14]. This is the easiest and most reliable method to determine the radical scavenging potential of samples. For this assay, quercetin of 20 µg/mL was used as a positive control, and 50% DMSO was used as a negative control. Initially in this test, 100 µL of positive control, negative control, and samples were loaded in 96 well plates in triplicate. To initiate the reaction between the loaded samples and DPPH, 100 µL of DPPH reagent was added to each well. Then it was incubated for 30 minutes in the dark and absorbance was taken at 517 nm using a

microplate reader. The capability to scavenge the DPPH radical was calculated by using the following equation:

$$\% \text{ inhibition} = \frac{\text{Abs. of control} - \text{Abs. of sample}}{\text{Abs. of control}} \times 100$$

α-glucosidase Enzyme Inhibition Assay

The α-glucosidase inhibition potential of bark extract and ethyl acetate fraction was determined using the previously conducted method with slight modifications [15]. For this assay, p-nitrophenyl α-D-glucopyranoside was used as a substrate, and sodium phosphate buffer solution was made with pH 6.8. To a final concentration of 0.5 U/mL, 20 µL of α-glucosidase was combined with 20 µL of the samples and 120 µL of buffer solution. Then, 40 µL of 3.5 mM PNPG was added as a substrate. This reaction blend was pre-incubated for 15 minutes at 37 °C. The α-glucosidase activity was determined by measuring the p-nitrophenyl released from the hydrolysis of p-nitrophenyl α-D-glucopyranoside at 410 nm. Acarbose was used as a positive control, and 30% DMSO was used as the negative control. All experiments were performed in triplicate in a final volume of 200 µL using a microplate reader (Epoch2, BioTek, Instruments, Inc., USA). The percentage of α-glucosidase activity was calculated using the following formula.

$$\% \text{ inhibition} = \frac{\text{Abs. of control} - \text{Abs. of Extract} + \text{Enzymes}}{\text{Abs. of control}} \times 100$$

Metabolomics Methods

The study utilized high-resolution-mass spectrometry (HRMS) to analyze and characterize the secondary metabolites present in a methanolic extract of the *B. aristata* plant's bark. For the identification of the molecular formula of eluted secondary metabolites SIRIUS 4 platform was used. To prepare the sample for HRMS measurements, 0.2 milligrams of the methanolic/aqueous bark extract were dissolved in one milliliter of HPLC-grade methanol. The Bruker Compass Data Analysis 4.0 software was used to evaluate the extent of molecular ionization from the data obtained. The primary and seed ions $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[2M+H]^+$, $[2M+Na]^+$, $[M+2H]^{2+}$, and $[M+2Na]^{2+}$ were set to determine the molecular ions using Metaboscape software. The list of molecular ions was bucketed based on the observed base peak chromatogram within a retention time range of 4-32 minutes and a molecular mass range of 200-1500 m/z. The HRMS data of the bark extract showed molecular ions, retention times, and relative ion intensities. The results were exported in the mascot generic format (MGF) files. To confirm metabolites sorted by relative abundance, individual raw MS data underwent CSI: FingerID analysis in the SIRIUS 4 platform.

Statistical Analysis

To make the study more effective and accurate, all the experiments were performed in triplicates and data were presented in mean ± standard error of the mean. For the calculation of IC_{50} values GraphPad Prism software was used and all assays were processed by using Gen5 Microplate and then by MS Excel 2016.

Results and Discussion

The quantitative determination of total phenol was carried out using the Folin Ciocalteu reagent. The mechanism is based on the formation of a phosphor-tungstic-phosphomolybdic complex formed in the presence of phenolic compounds. It involves the oxidation of phenols in an alkaline solution by the yellow molybdo-tungstophosphorichetero polyanion reagent and colorimetric measurements of the resultant molybdo-tungstophosphate blue. The phenolic compounds react with FC reagent only under

basic conditions adjusted by sodium carbonate solution. Polyphenols in the *B. aristata* bark react with a specific redox reagent (FCR) to form a blue complex that exhibits a broad light absorption depending on the qualitative and quantitative composition of the phenol mixture, besides the pH of the solution that can be quantified by the UV-Visible spectrometry at 765 nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenols. Total phenolic contents (TPC) were expressed in terms of gallic acid equivalent (mg GAE/g dry weight of extract) with a calibration curve of gallic acid ($y = 0.231x$, $R^2 = 0.976$). The calibration curve of gallic acid is shown in the **Fig. 1**. All tests were carried out in triplicate and phenolic contents as gallic acid equivalents were reported as mean ± standard deviation of triplicate determination. *B. aristata* bark extract showed a TPC value of 70.056 ± 2.52 mg GAE/g. This result showed that the bark extract of *B. aristata* has abundant phenolic content.

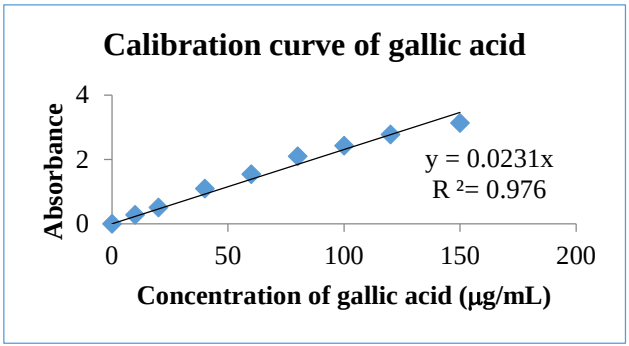

Figure 1: *Calibration curve of gallic acid*

The study conducted previously showed that the TPC content was only 11.04±2.20 mg GAE/g [16]. Therefore this study provides strong insight on the phenolic content of the bark of *Berberis aristata.*

Total Flavonoid content

The estimation of the total flavonoid present in the methanolic extract of the bark of *Berberis aristata* was according to the standard procedure involving quercetin as standard. The intensity of light absorption at 415 nm wavelength is proportional to the concentration of flavonoid and has an intense yellow fluorescence which was observed under a UV spectrophotometer. The TFC was measured by the $AlCl_3$ method, the mechanism is based on the formation of complex flavonoid-$AlCl_3$, which provides a bathochromic displacement and hyperchromic effect. $AlCl_3$ forms acid-stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavonoids. The reported TFC of the bark of *Berberis aristata* extract is expressed in terms of quercetin equivalent (mg QE/g dry weight of extract) with a calibration curve of quercetin ($y = 0.0161x$, $R^2 = 0.841$) as shown in **Fig.2**. The TFC data calculated on the bark of *Berberis aristata* is 11.89 ± 0.16 mg QE/g. The study conducted previously showed that the TFC content was only 6.08±0.50 mg QE/g [16]. Therefore this study provides strong insight on the flavonoid content of the bark of *Berberis aristata.*

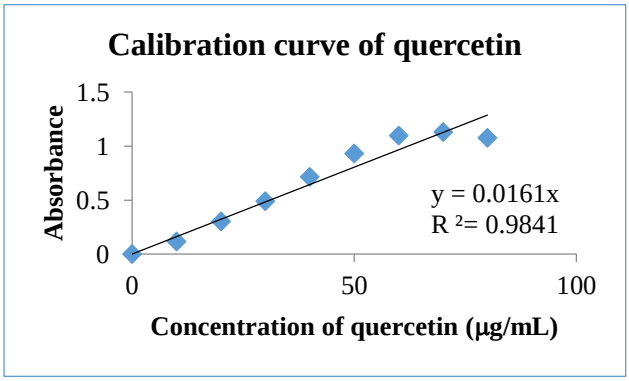

Figure 2: *Calibration curve of quercetin*

Radical Scavenging Potential

Antioxidant potential was determined by using the DPPH assay. This method is easy to adopt and is considered the most reliable method to evaluate the radical scavenging potential of the plant extract. First of all, the screening of the samples was done in which both samples possess inhibition above 50% therefore IC_{50} of the methanol extract of bark

and ethyl acetate fraction was calculated. The extract of ethyl acetate fraction of *Berberis aristata* showed the highest inhibition of 82.66% at the concentration of 500 µg/mL while bark extract showed inhibition of 76.55% at the same concentration. Among the two solvents, the ethyl acetate fraction showed the lowest IC_{50} value of 188.93±8.35 µg/mL, and bark extract showed the highest IC_{50} value of 242.89±4.62 µg/mL. Therefore among the two, ethyl acetate fraction was considered more valuable in terms of radical scavenging potential. To compare the effectiveness of the radical scavenging potential of the extract standard drugs acarbose were used. The inhibitory concentration of sample and standard is shown in **Table 1**.The standard drug acarbose has an IC_{50} value of 6.29 ± 1.02 µg/mL, which indicates the sample was effective but not as much as the standard drugs used.

Table 1: IC_{50} values of the tested sample and standard drugs.

Samples	IC_{50} (µg/mL)
Methanol bark extract	242.89±4.62
Ethyl acetate fraction	188.93±8.35
Quercetin	6.29 ± 1.02

From the previous research, it was found that the plant possesses antioxidant activity with an IC_{50} value of 67.07 ± 2.94 µg/mL [17]. So this previous research on the same plant provides strong support to the current study.

α-glucosidase Inhibition Assay

Both samples were screened for α-glucosidase inhibition. Among the two only the ethyl acetate fraction showed the potential inhibition at 500 µg/mL concentration. Therefore, IC_{50} values were calculated where the ethyl acetate fraction showed least IC_{50} values of 112.99 ± 10.28 µg/mL shown in **Table 2**. To compare the effectiveness of the inhibition, it was compared to the standard drug acarbose whose IC_{50} value was 344.23 ± 1.04 µg/mL. This result indicated that the ethyl acetate fraction of the bark of *Berberis aristata* is composed of multiple metabolites that possess the potential to inhibit the α-glucosidase enzyme.

Table 2: IC_{50} values of the tested sample and standard drugs.

Samples	IC_{50} (µg/mL)
Ethyl acetate fraction	112.99 ± 10.28
Acarbose (standard)	344.23 ± 1.04

Identification of Secondary Metabolites from Mass-Spectrometry

Through mass measurements and the determination of molecular formulas, the study identified and annotated several compounds found in the bioactive plant extracts. The compounds were characterized using MS2 spectra in the database search shown in **Fig 3**, where the structures of the compounds were predicted using the Sirius software. This software uses advanced algorithms and databases to analyze the mass spectrometry data, both MS1 and MS2. The compounds were prioritized for structural annotation based on their abundance in the crude extract.

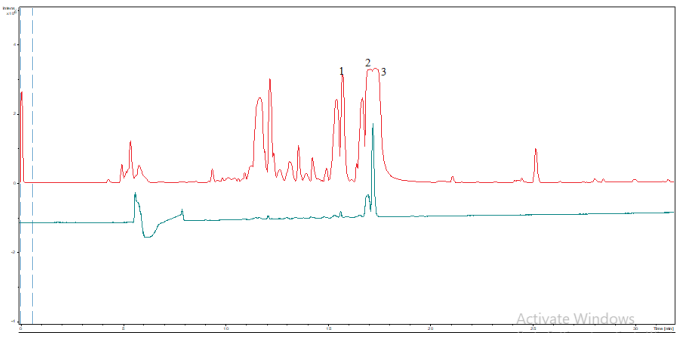

***Figure 3**: Red chromatogram: Most intense peak (base peak) in the mass spectrum of Berberis aristata bark extract. Green chromatogram: UV absorbance at 250 nm.*

Among the identified compounds, compound 1 was eluted at RT 15.73 minutes. The m/z mass of the eluted compound

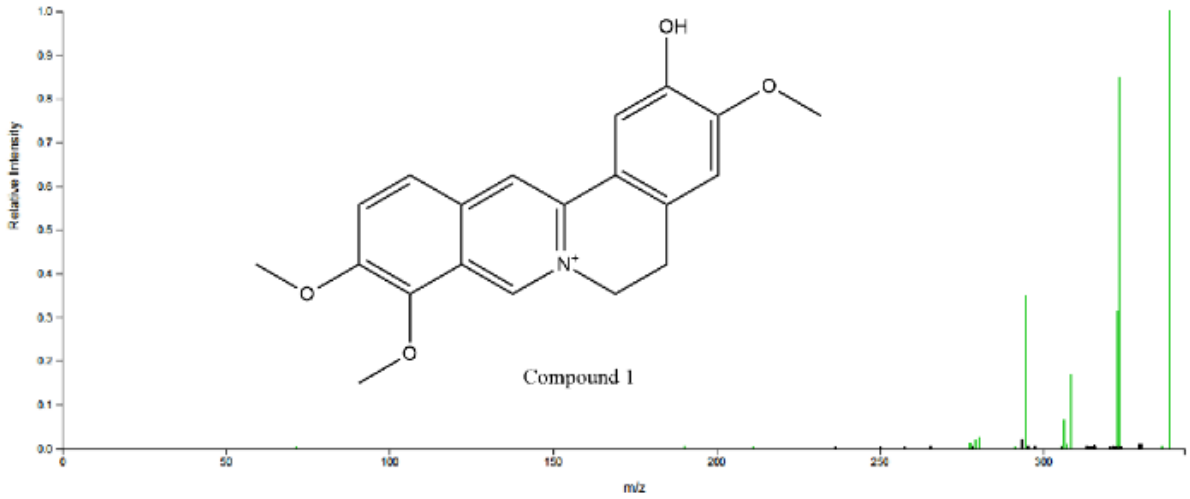

Figure 4: *The mass spectrum (MS2) of the compound 1*

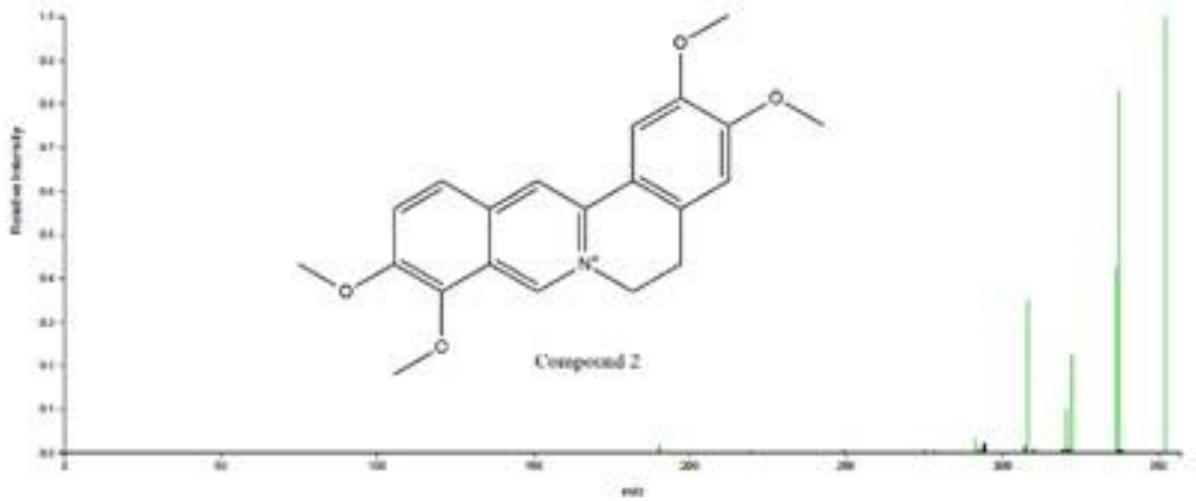

Figure 5: *The mass spectrum (MS2) of the compound 2.*

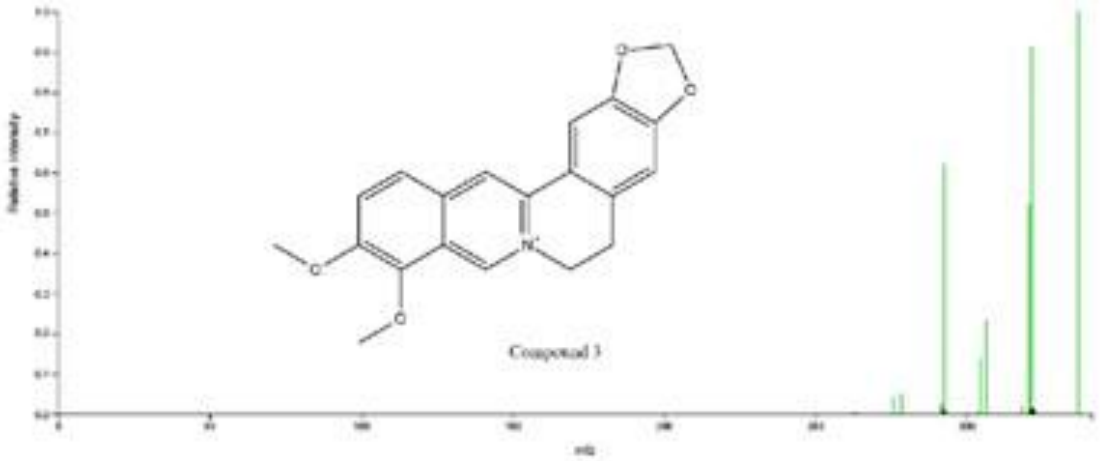

Figure 6: *The mass spectrum (MS2) of the compound 3*

was 338.13913 Da and the measured mass was 337.13186 Da. The SIRIUS platform illustrated the fragmentation pattern of compound 1 , shown in **Fig. 4** and showed $C_{20}H_{19}NO_4$ as its molecular formula, with a score of 100 %. The fragmentation of $C_{20}H_{19}NO_4$ gives four major fragments $C_{20}H_{17}NO_4$, $C_{19}H_{16}NO_4$, $C_{19}H_{15}NO_4$, and $C_{19}H_{15}NO_3$ by eliminating H_2, CH_3, CH_4, CH_4O. Furthermore, the fragmentation process

continues and the second fragment that is $C_{19}H_{16}NO_4$ becomes stable after forming three small molecules $C_{14}H_{10}O_2$, C_5H_{10}, and $C_{18}H_{14}NO_2$. Whereas the third fragment that is $C_{19}H_{15}NO_4$ becomes stable after forming $C_{17}H_{12}NO_3$, and $C_{18}H_{15}NO_3$. The last fragment that is $C_{19}H_{15}NO_3$ becomes stable $C_{18}H_{12}NO_3$. From this fragmentation pattern, the identified compound is columbamine.

Another identified compound was Compound 2 eluted at RT 16.94 minutes. The m/z mass of the compound was 352.15488 Da and the measured mass was 351.14760 Da. The SIRIUS platform illustrated the fragmentation pattern of compound 2， shown in **Fig. 5** and showed $C_{21}H_{21}NO_4$ as its molecular formula, with a score of 100 %. The compound with molecular formula $C_{21}H_{21}NO_4$ undergoes fragmentation to form three major fragments $C_{20}H_{18}NO_4$, $C_{20}H_{17}NO_4$, and $C_{20}H_{17}NO_3$ by eliminating CH_3, CH_4, and CH_4O. Furthermore, the fragmentation process continues and the first fragment that is $C_{20}H_{18}NO_4$ becomes stable after forming two small molecules $C_{18}H_{15}NO_3$, and $C_{19}H_{16}NO_2$. Whereas the second fragment that is $C_{20}H_{17}NO_4$ becomes stable after forming $C_{19}H_{13}NO_4$, $C_{18}H_{13}NO_3$, $C_{17}H_{11}NO_3$, $C_{16}H_{10}NO_2$, and $C_{15}H_8NO$. The last fragment that is $C_{20}H_{17}NO_3$ does not undergo a further fragmentation. From this fragmentation pattern, the identified compound is Palmatin.

Among the identified compounds, compound 3 has an m/z value of 336.12 Da and its actual mass measurement was found 335.116 Da. This compound was eluted at RT 17.30 minutes. From the SIRIUS the confirmed molecular formula is $C_{20}H_{17}NO_4$ with the score 100%. The formula $C_{20}H_{17}NO_4$ undergoes fragmentation (shown in **Fig. 6**) to form three major fragments $C_{19}H_{14}NO_4$, $C_{19}H_{13}NO_4$, and $C_{19}H_{13}NO_3$ by losing three molecules CH_3, CH_4, and CH_4O. The last fragment does not undergo further fragmentation but the first and second fragments are unstable ions that were stable after forming $C_{16}H_8NO_4$, $C_{17}H_{10}NO_3$, $C_{18}H_{12}NO_2$, $C_{17}H_{12}NO_2$, $C_{17}H_{11}NO_2$ and $C_{18}H_{13}NO_3$ respectively. From the SIRIUS platform, the identified name of this compound was Berberal.

Conclusions

This study provides scientific justification for the traditional use of *Berberis aristata* in treating disorders such as diabetes and other severe infections. The ethyl acetate fraction demonstrated the highest efficacy in free radical scavenging activity, with an IC_{50} value of 188.93±8.35 µg/mL, followed by the bark extract with an IC_{50} values of 242.89±4.62 µg/mL. However, the bark extract exhibited limited α-glucosidase inhibition activity, while the ethyl acetate fraction showed strong inhibition with an IC_{50} value of 112.99 ± 10.28 µg/mL, compared to the standard Acarbose with an IC_{50} value of 344.23 ± 1.04 µg/mL.Mass spectrometry revealed the presence of various compounds, including Palmatin, Berberal, and columbamine, which may significantly contribute to the drug discovery process due to their diverse biological activities. This study offers valuable insights for further exploration of bioactive compounds, their associated biological properties, and potential mechanisms of action.

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Authors Contribution Statement

Keshab Bhattarai: Methodology, Investigation, Formal analysis, Data curation, Writing-original draft preparation, **Indira Pandey:** Methodology, Investigation, Formal analysis, Data curation,

Writing-original draft preparation, **Khaga Raj Sharma:** Conceptualization, Resources, Funding acquisition, Writing-review and editing, supervision

Conflicts of Interest

The authors do not have any conflict of interest pertinent to this work.

Data Availability Statement

The data that support the findings of this study can be made available from the corresponding author, upon reasonable request.

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