Isolation And Characterization Of Baicalin, A Flavonoid From *Pterocarpus marsupium* Leaves, And Its In-Vitro Anti-Diabetic Activity

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ABSTRACT

Natural products in medicine have been gaining attention due to their potential to provide new and effective therapies for chronic diseases such as diabetes. *Pterocarpus marsupium*, a species of tree in the family Fabaceae, has been used in traditional medicine for centuries to treat diabetes, inflammation, and skin diseases. In this study, the flavonoid compound baicalin was successfully isolated and identified from the aqueous extract of *P. marsupium* leaves. The methods used in the study include column chromatography, MASS, FT-IR, 13C-NMR, and 1H-NMR spectroscopical analysis for structural characterization. Baicalin has been shown to have anti-diabetic activity by through in vitro alpha-glucosidase and alpha-amylase inhibitory assays. These results highlight the potential of baicalin as a therapeutic agent for diabetes. Further preclinical and clinical studies are necessary to fully elucidate baicalin's mechanism of action and determine its safety and efficacy in vivo. This study represents an important step in identifying and characterizing bioactive compounds from P. marsupium and highlights the potential of natural products as a source of new and effective therapies for chronic diseases such as diabetes.

Keywords: *Pterocarpus marsupium*, Baicalin, MASS, NMR, FT-IR, alpha-amylase, and alpha-glucosidase.

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INTRODUCTION

Pterocarpus is a genus of trees in the family Fabaceae, comprising around 35 species distributed in tropical and subtropical regions of Africa, Asia, and South America [1]. Pterocarpus species have a long history of use in traditional medicine to treat a variety of ailments, including diabetes, inflammation, cancer, and fever [2,3]. The Pterocarpus genus is known for containing various phytoconstituents such as flavonoids, stilbenes, coumarins, and triterpenes. The main bioactive compounds found in the Pterocarpus species are flavonoids, which have been shown to have various therapeutic properties [4,5].

Pterocarpus marsupium is a species of tree in the family Fabaceae, native to India and Sri Lanka. It is commonly known as the Indian kino tree, Malabar kino tree, or Vijayasar. For centuries, *P. marsupium* has been used in Ayurvedic medicine to treat various ailments, including diabetes, inflammation, and skin diseases [5,6]. Pterostilbene, a bioactive compound found in P. marsupium shown to have anti-diabetic, anti-inflammatory, and anti-cancer properties. Anti-diabetic activity is exerted by increasing insulin sensitivity and reducing blood glucose levels [7-11]. *P. marsupium* extracts have been used in cosmetic products for their skin-lightening and anti-aging properties [6]. Other compounds isolated are marsupin, coumarin and epicatechin [12-16].

Therefore, in an attempt to explore plant-based alternative solutions in promoting health, as well as paving the way towards our future pre-clinical and clinical studies, we aimed to isolate the active constituent from the aqueous extract of the leaves of *P. marsupium* by column chromatography. Structural characterization of the isolated compound was done by Mass, FT-IR, ^{13}C-NMR, and ^{1}H-NMR spectroscopical analysis. The isolated compound was subsequently evaluated for its antidiabetic activity in vitro.

MATERIALS AND METHODS

Sample collection and authentication

Fresh leaves of *Pterocarpus marsupium* Roxb. (Fabaceae) were acquired from the Kozhikode district of Kerala, India. The taxonomical identification and authentication of the plant were done by Dr. A. K Pradeep, Assistant Professor, Department of Botany, University of Calicut, Kerala. The voucher specimen was preserved in the laboratory, Department of Botany, University of Calicut with specimen No. 148278 for further reference.

Instruments and materials

Column chromatography was carried out using silica gel 100-200 mesh (Merck), while TLC was carried out using silica gel 60PF254. Melting points were recorded using the Kohfler melting point apparatus and were uncorrected. The IR spectra were obtained using KBr discs on Perkin Elmer FTIR spectrophotometer 1650. NMR spectra were recorded on a JEOL spectrometer at 400MHz, respectively with DMSO-d6 as solvent. Mass spectra were obtained using JEOL-Accu TOF JMS-T 100 LC Mass spectrometer.

Extraction and isolation

About 1kg of the leaf material was extracted with water by maceration processes. The extract was concentrated and the solvent was completely removed under reduced pressure and stored in desiccators for further use.

The plant extract was dissolved in methanol and adsorbed in silica gel 60 – 120 mesh. The solvent was evaporated and loaded into a silica gel column (100 – 200), and prepared in hexane. The column was eluted with hexane followed by gradually increasing polarity with Hexane: ethyl acetate (90:10; 80:20; 70:30; 60:40; 50:50; 30:70; 20:80) and finally with 100% ethyl acetate. The column was further eluted with ethyl acetate: chloroform (95:5; 90:10; 85:15; 80:20; 70:30; 60:40; 50:50; 45:55; 40:60; 30:70; 20:80; 10:90) and finally with 100% chloroform. The column was further eluted with Chloroform: Methanol (98:2; 94:2; 92:8; 90:10; 85:15; 80:20; 70:30).

A total of 86 Fractions were collected. The pure compounds were monitored under TLC and similar fractions were observed in fractions number 59 - 68. These fractions with similar Rf values were combined and the solvent was evaporated under reduced pressure. The resulting crude materials were purified by using activated charcoal in hot methanol and the fractions are kept at room temperature overnight. The resulting solid was submitted for melting point, Mass, FT-IR, ^{13}C-NMR, and ^{1}H-NMR analysis.

Invitro antidiabetic activity

Alpha-Glucosidase Inhibition assay:

The effect of the isolated baicalin on α-glucosidase activity was determined using the α-glucosidase enzyme. The substrate solution p-nitrophenyl glucopyranoside (pNPG) was prepared in 100 mM phosphate buffer, and pH 6.8. 200 µL of α-glucosidase was pre-incubated with different concentrations (10, 20, 40, 80, 160, and 320) of the

extracts for 10 min. Then 400 µL of 5.0 mM (pNPG) as a substrate dissolved in 100 mM phosphate buffer (pH 6.8) was then added to start the reaction. The reaction mixture was incubated at 37 °C for 20 min and stopped by adding 1 mL of Na_2CO_3 (0.1 M). The yellow-colored reaction mixture, 4-nitrophenol, released from pNPG was measured at 405 nm using a UV-Visible spectrophotometer [17-19]. Voglibose was used as a positive control and the inhibitory activity of α-glucosidase was calculated using the following formula,

% Inhibition = [(Abs Control - Abs Sample) / Abs Control] x 100

Alpha-amylase inhibition assay:

The α-amylase inhibitory activity of the isolated baicalin was carried out according to the standard method with minor modification. 100 µL of α-amylase solution was mixed with different concentrations of the test, reference standard Acarbose, and control and pre-incubated at 37 °C for 15 min. Then, 100 µL of the starch solution was added to initiate the reaction, and incubation was done at 37 °C for 60 min., then 10 µL of 1 M HCl and 100 µL of iodine reagent were added to the test tubes. The absorbance of the mixture was measured at 565 nm [20-23]. α-amylase inhibitory activity was measured using the formula,

% Inhibition = [(OD of test - OD of control)/OD of test] x 100

Statistical analysis:

All data were expressed in Mean ± SD and percentage inhibition ± SD. All analyses were done in Graph Pad Prism 9 software.

RESULTS AND DISCUSSION

Isolated metabolite characterization

Aqueous extract of the leaves of *Pterocarpus marsupium* was subjected to column chromatographic isolation and 86 fractions were collected. Fractions with the same Rf value were pooled together and subjected to evaporation under reduced pressure. As a result, a pale-yellow solid compound was isolated and taken for further analysis to elucidate its structure.

The isolated constituent was a pale-yellow solid compound has a melting point of 204-206 °C and a molecular formula of $C_{21}H_{18}O_{11}$, with a molecular weight of 446.36 g/mol. The compound was then subjected to electron ionization mass spectrometry (EI-MS), which provided a molecular ion peak (M+1) at m/z 447.0, which is consistent with the molecular formula and confirms the molecular weight of the compound.

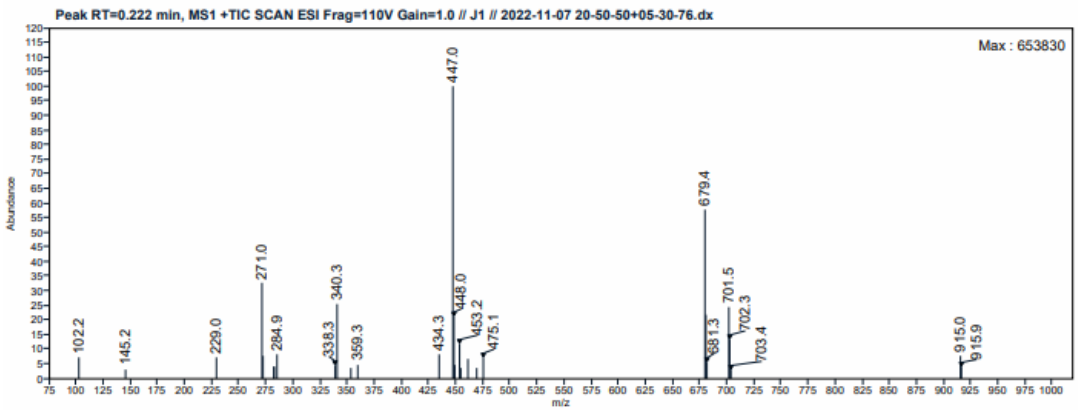


Fig. 1: Mass spectra of isolated compound Baicalin

^{1}H-NMR (DMSO-d6): δ ppm: 12.59 (1H, s, OH-5), 8.68 (1H, s, OH-6), 8.07 – 8.09 (2H, dd, H-2',6'), 7.60 – 7.61 (3H, m, H-3',4',5'), 7.02 – 7.05 (2H, d, H-3, H-8), 5.50 - 5.52 (1H, d, OH-2"), 5.30 - 5.31 (2H, d, OH-3",4"), 5.24 - 5.26 (1H, d, H-1"), 4.06 - 4.08 (1H, d, H-5"), 3.34 – 3.43 (3H, m, H-2",3",4").

The given spectral data represents the 1H-NMR spectrum of a compound dissolved in DMSO-d6. The signal at 12.59 ppm corresponds to a hydroxyl group (OH) at position 5 of the molecule. The signal at 8.68 ppm corresponds to another hydroxyl group at position 6. The two signals at 8.07-8.09 ppm correspond to the aromatic protons (H) at positions 2' and 6' of the molecule. The signals at 7.60-7.61 ppm correspond to the aromatic protons at positions 3', 4', and 5'. The signals at 7.02-7.05 ppm corresponds to the two protons on the double bond (H-3 and H-8) in the molecule. The signals at

5.50-5.52 ppm correspond to a proton on the hydroxyl group (OH) at position 2" of the sugar moiety in the molecule. The signals at 5.30-5.31 ppm correspond to two protons on the hydroxyl groups at positions 3" and 4" of the sugar moiety. The signal at 5.24-5.26 ppm corresponds to a proton on the anomeric carbon (H-1") of the sugar moiety. Finally, the signals at 4.06-4.08 ppm correspond to a proton on the hydroxymethyl group (H-5") of the sugar moiety, and the signals at 3.34-3.43 ppm correspond to the three protons on the methylene group (H-2", H-3", and H-4") of the sugar moiety. From this spectral data, it can be inferred that the compound is likely a flavonoid with sugar attached [23-25].

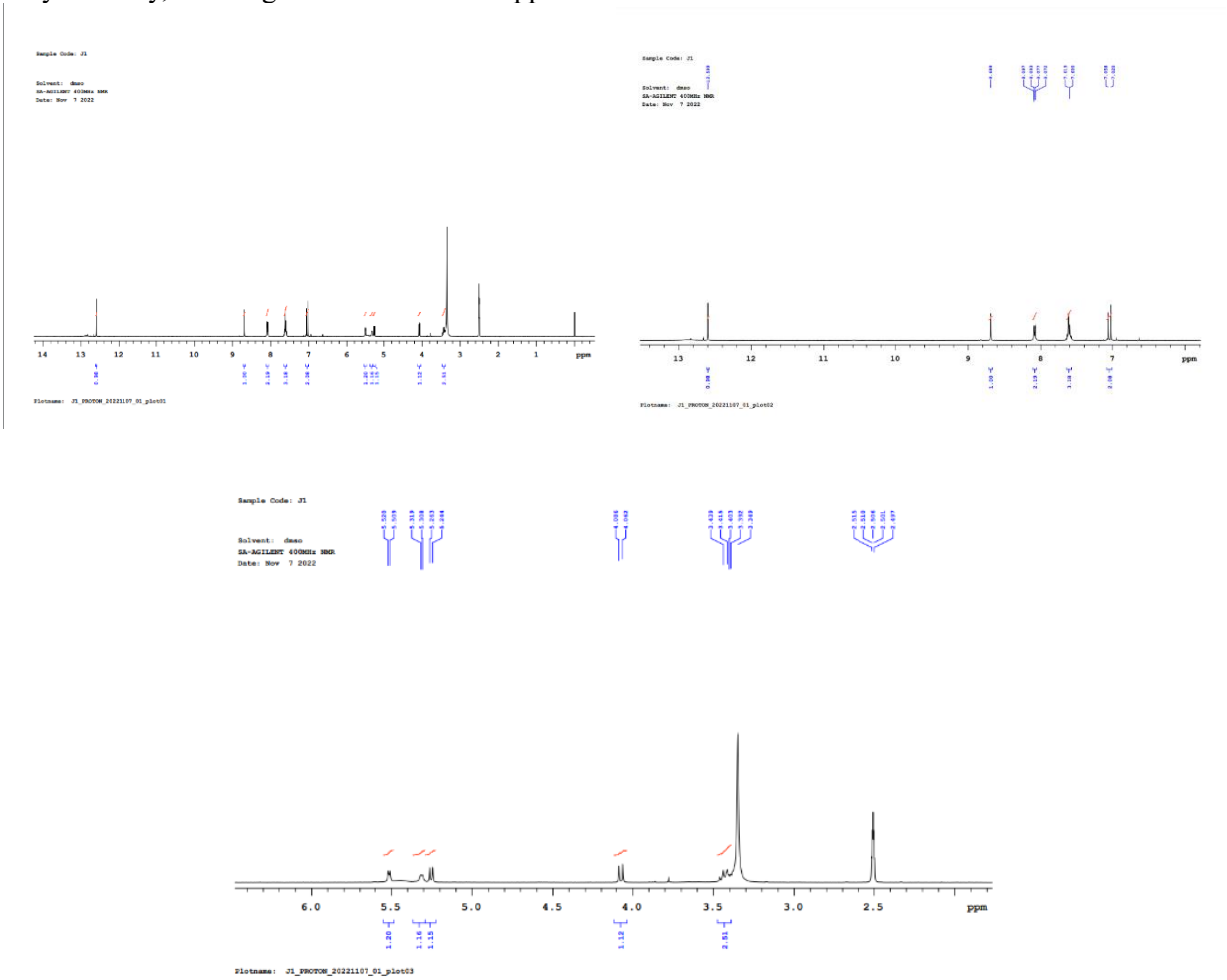


Fig. 2: ^{1}H NMR spectra of isolated compound Baicalin

^{13}C-NMR (DMSO-d6): δppm 163.52 (C-2), 106.11 (C-3), 182.53 (C4), 146.76 (C-5), 130.83 (C-6), 151.25 (C-7), 93.71 (C-8), 149.18 (C-9), 104.74 (C-10), 130.83 (C-1'), 126.36 (C-2' , 6'), 129.15 (C3' ,C- 5'), 132.03 (C-4'), 99.89 (C-1"), 72.78 (C-2"), 75.23 (C-3"), 71.29 (C-4"), 75.49 (C-5"), 170.01 (C-6")

The given spectral data represents the 13C-NMR spectrum of a compound dissolved in DMSO-d6. The signal at 163.52 ppm corresponds to the carbonyl carbon (C-2) in the molecule. The signal at 106.11 ppm corresponds to the carbon (C-3) adjacent to the hydroxyl group at position 5. The signal at 182.53 ppm corresponds to the carbonyl carbon (C-4) in the molecule. The signal at 146.76 ppm corresponds to the carbon (C-5) in the aromatic ring. The signal at 130.83 ppm corresponds to the carbon (C-6) adjacent to the hydroxyl group at position 6. The signal at 151.25 ppm corresponds to the carbon (C-7) in the aromatic ring. The signal at 93.71 ppm corresponds to the carbon (C-8) on the double bond in the molecule. The signal at 149.18 ppm corresponds to the carbon (C-9) in the aromatic ring. The signal at 104.74 ppm corresponds to the carbon (C-10) adjacent to the hydroxyl group at position 6. The signals at 130.83 ppm and 126.36 ppm correspond to the carbon atoms in the aromatic ring at positions 1' and 2'/6', respectively. The signals at 129.15 ppm and 132.03 ppm correspond to the carbon atoms in the aromatic ring at positions 3'/5' and 4', respectively. The signal at 99.89 ppm

corresponds to the anomeric carbon (C-1") of the sugar moiety in the molecule. The signals at 72.78 ppm, 75.23 ppm, 71.29 ppm, and 75.49 ppm correspond to the carbon atoms in the sugar moiety at positions 2", 3", 4", and 5", respectively. The signal at 170.01 ppm corresponds to the carbonyl carbon (C-6") in the sugar moiety [26,27].

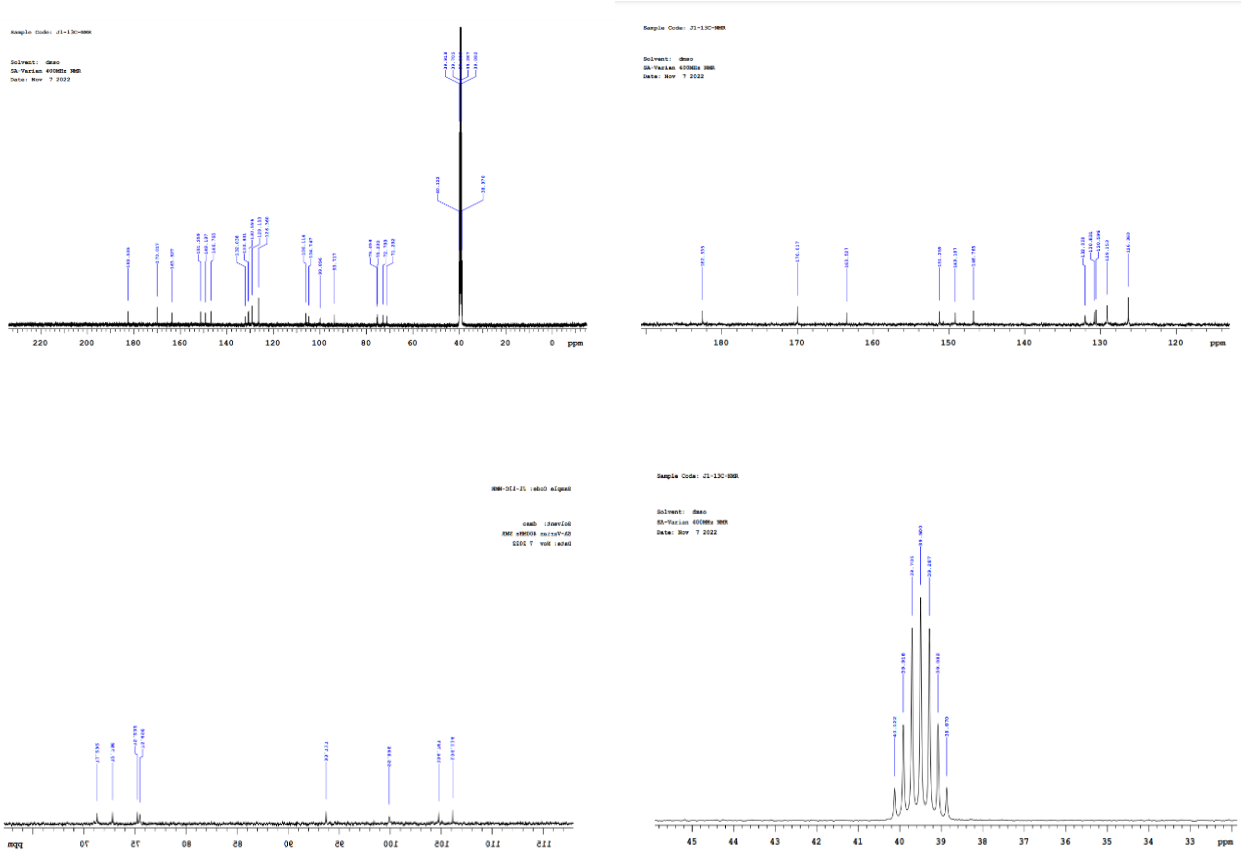


Fig. 3: ^{13}C NMR spectra of isolated compound Baicalin

FT-IR (KBr cm-1): 3483(-OH stretching), 3251 cm^{-1} (-COOH stretching); 2898 cm^{-1} (-CH stretching); 1723 (-C=O stretching); 1657 cm^{-1} (-C=O stretching); 1607cm^{-1} (-C=C stretching); 1571cm^{-1} (-CH bending); 1061 cm^{-1} (-C-O bending).

The band at 3483 cm-1 indicates the presence of -OH group stretching vibration, which is associated with the hydroxyl group present in the molecule. The band at 3251 cm-1 corresponds to the stretching vibration of the -COOH group, which suggests the presence of a carboxylic acid functional group. The band at 2898 cm-1 indicates the presence of -CH stretching vibrations, which is a characteristic band for alkanes and alkenes. The band at 1723 cm-1 is associated with the stretching vibration of the carbonyl group (-C=O), which is often present in ketones and aldehydes. The band at 1657 cm-1 also corresponds to the stretching vibration of the carbonyl group (-C=O), which suggests the presence of a carboxylic acid functional group in the sample. The band at 1607 cm-1 represents the stretching vibration of the C=C bond, which suggests the presence of an unsaturated hydrocarbon or an aromatic ring. The band at 1571 cm-1 corresponds to the bending vibration of the -CH group, which indicates the presence of an alkane or alkene. The band at 1061 cm-1 indicates the bending vibration of the -C-O group, which is associated with alcohol or ether. Based on these spectral data, it can be concluded that the sample contains hydroxyl (-OH), carboxylic acid (-COOH), carbonyl (-C=O), and C=C bond functional groups [28].

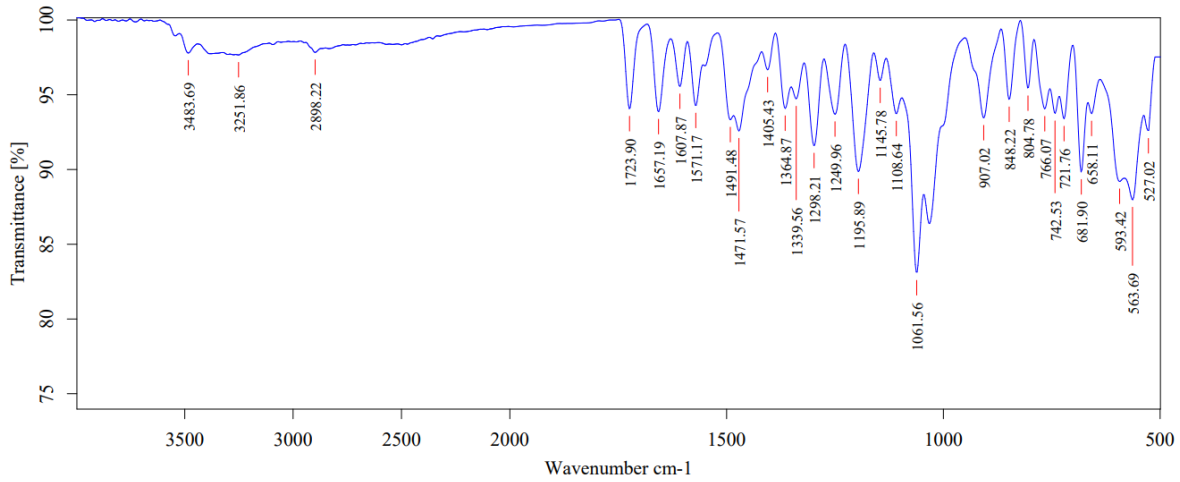
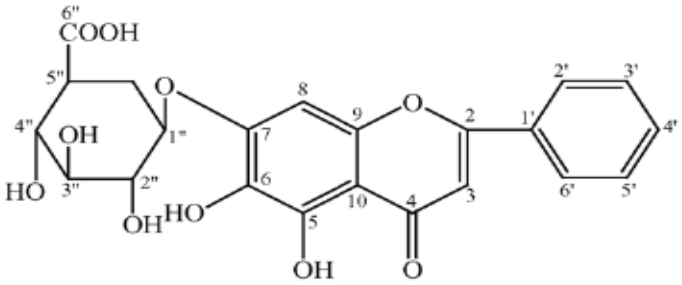


Fig. 4: FTIR spectra of isolated compound Baicalin

The compound that was separated has been recognized as Baicalin by analyzing its spectroscopic data and comparing it with information available in previous studies.



Baicalin

Fig. 5: Structure of isolated compound Baicalin

Invitro antidiabetic activity

Alpha - Amylase inhibition assay

The study examined the ability of baicalin, a compound isolated from *P. marsupium*, to inhibit α-amylase activity at different concentrations (10, 20, 40, 80, 160, and 360 µg/mL) in an alpha-amylase inhibition assay. The results showed that baicalin had a comparable inhibitory effect (96.05%) at 360 µg/mL on the alpha-amylase activity as the standard drug acarbose (96.01%) at a concentration of 360 µg/mL. Statistical analysis demonstrated that baicalin was significantly more effective in inhibiting alpha-amylase activity than acarbose.

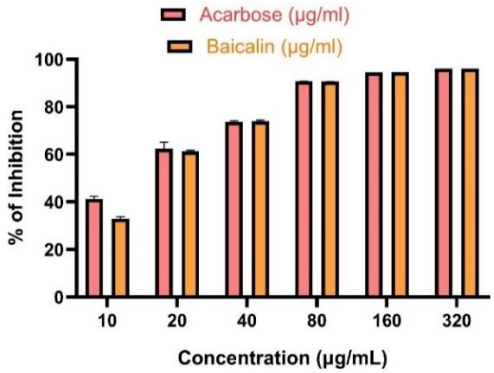


Fig. 6: α-Amylase inhibitory activity of baicalin and its comparative analysis

Alpha-glucosidase inhibition assay

The study tested different concentrations (10, 20, 40, 80, 160, and 360 µg/mL) of baicalin, an isolated compound, for its ability to inhibit α-glucosidase activity. The results indicated that baicalin had a similar inhibitory effect (95.47%) on α-glucosidase activity compared to the standard drug Volgibose (95.83%) at a concentration of 360 µg/mL. Statistical analysis revealed that baicalin was significantly effective in inhibiting α-glucosidase activity when compared to Volgibose.

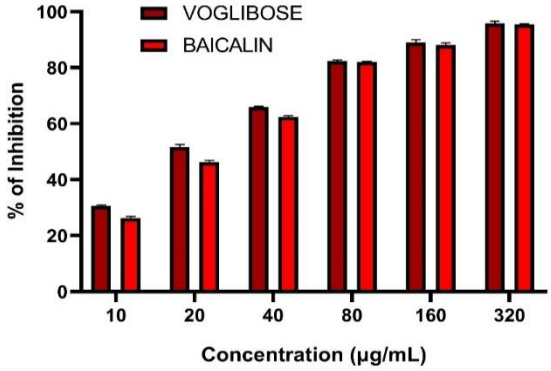


Fig. 7: α-Glucosidase inhibitory activity of baicalin and its comparative analysis

The statistical analysis confirmed that the values were presented as the mean ± standard deviation and the level of statistical significance (p) was determined using two-way ANOVA. The significance level was denoted as ***P < 0.001 when compared to the control group, indicating a very significance.

CONCLUSIONS

The isolation and identification of bioactive compounds from plant material is a challenging but important area of research. In this study, the flavonoid compound baicalin was successfully isolated and identified from the aqueous extract of *Pterocarpus marsupium* leaves. The combination of thin layer and column chromatographic techniques, as well as analytical techniques such as FTIR, NMR, and mass spectroscopy, were utilized in the isolation and identification process. This is the first time that baicalin has been isolated from the leaves of this plant species.

In the current research, the antidiabetic activity of baicalin has been demonstrated using in vitro alpha-glucosidase and alpha-amylase inhibitory assays. These results indicate the potential of baicalin as a therapeutic agent for diabetes. However, further preclinical and clinical studies are necessary to fully elucidate baicalin's mechanism of action and determine its safety and efficacy in vivo. Such studies will be important for evaluating the potential of baicalin as a treatment for diabetes and for informing its clinical use. Overall, this study represents an important step in identifying and characterizing bioactive compounds from *Pterocarpus marsupium* and highlights the potential of natural products as a source of new and effective therapies for chronic diseases such as diabetes.

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