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RESEARCH ARTICLE

Qualitative and Quantitative Analysis and Biological Applications of *Passiflora racemosa* Brot. Leaf Methanol Extracts for Novel Insights into Pharmaceuticals

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Highlights

- The leaf extract exhibited excellent antioxidant and antidiabetic properties, since the IC_{50} values obtained were lower than the standards
- The *in vivo* acute oral toxicity on albino mice results indicated that the leaf powder and extracts are non-toxic; therefore, they are safe for biological applications
- The GC/MS analysis has also exhibited the presence of many natural antioxidants, like ascorbic acid, vitamin E, along with other bioactive compounds
- The leaf extract lacked antibacterial and antifungal potency against the tested bacteria and fungi

Abstract

Passiflora racemosa, commonly known as the passion flower, is primarily cultivated for its ornamental value. This research explores the medicinal and biological applications of the leaf extract in methanol for the first time. The toxicity test of leaf methanol extract and powdered leaf was performed in vivo on albino mice in compliance with OECD standards. The powdered-leaf and leaf methanol extract exhibited no toxicity. The antioxidant assay was conducted by the DPPH method, yielding an IC_{50} of 0.59 µg/mL, which signifies a very strong antioxidant capacity. The antidiabetic assay was accomplished using DNSA, and an IC_{50} obtained was 1.686 µg/mL, indicating a very strong antidiabetic activity. The total phenol content (TPC) was 60.127 ± 2.445 mg/g GAE, determined by the FCR reagent. The TFC was 398.215 ± 18.330 mg/g QE, determined by the $AlCl_3$ colorimetric technique. The TFC was observed to be higher compared to the TPC in the extract. The antimicrobial properties were assessed by the well-diffusion technique against pathogenic bacteria and fungi. The antimicrobial properties were not observed for the tested microorganism. The major metabolites identified by GC/MS were 9, 12, 15-Octadecatrienoic acid (Z, Z, Z), n-hexadecanoic acid, phytol, melezitose, d-ascorbic acid, and benzoic acid. Consequently, the leaves of this plant can serve as an antioxidant and antidiabetic agent. This research establishes a foundation for subsequent, more comprehensive investigations into the extraction of active metabolites for therapeutic purposes.

Keywords: *Passiflora racemosa, acute oral toxicity, antioxidant, antidiabetic, GC-MS*

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Introduction

Since ancient times, researchers have investigated the potential of plants for therapeutic use and drug discovery. Bioactive secondary metabolites are abundant in medicinal plant parts, like roots, stems, and leaves. As a result, numerous innovative medicines have been derived from plants (Sanjai et al., 2024). Many unique medicinal plants are found at various elevations in Nepal. There are also many exotic plants with significant therapeutic benefits. One such exotic herb is *Passiflora racemosa* Brot. of the *Passifloraceae* family, and native to Brazil. It has another name, the scarlet passion flower (De Barros et al., 2009). Since time immemorial, the genus *Passiflora* has been utilised medicinally to relieve anxiety (Da Fonseca et al., 2020). As noted in the literature, plants of this family have been employed as remedies for various ailments, including as a tranquilliser and an antihypertensive (Puri & Hall, 1998), as an antibacterial (Ramaiya et al., 2014), and as anticancer agents (Ricardo et al., 2019). It comprises about 750 species and 27 genera (Wiersema, 2005). This plant is cultivated in Nepal for ornamental purposes, growing outdoors and climbing up the walls of boundaries and buildings. It is distributed across many Asian countries, like Nepal, India, China, Sri Lanka, America, and Europe (Kawakami et al., 2021). The leaves consist of 3 lobes, length of about 10 cm, and the red flowers with a diameter of approximately 12 cm (Singh et al., 2001). *Passiflora* has been officially recognised as a medicinal plant by many countries in their pharmacopoeias (Sarris et al., 2012c).

The leaves are rich in flavonoid glycosides, which are the major flavonoids in several species of this genus (Farag et al., 2016b). Various secondary metabolites have been isolated from leaves of different species. Examples include isoorientin, 4'-O-glucosylorientin, 2''-O-rhamnosylorientin, scoparin, 2''-O-rhanosyl scoparin, and 8-C-glucosyl-diosmetin (Escobar et al; 1983); cyanogenic glycoside passicoccin; cyanogenic glycosides linamarin and lotaustralian (Spencer et al;1986); pantothenic acid, ascorbic acid, C-glycosyl flavonoid 2''-xylosylvitexin, and coumarin esculetin (Ulubelen et al; 1981); flavonoid saponarin (Ulubelen et al; 1982); cyanogenic glycoside barterin (Olafsdottir et al; 1989); cyanogenic glycoside passibiflorin (Olafsdottir et al; 1997); passicapsin; cyanogenic bisglycoside 4-bi-vinosyltetraphyllin (Fischer et al; 1982); cyanogenic glycoside passibiflorin (Andersen et al; 1998); and a sulphate ester of tetraphyllin B (Jaroszewski et al; 1989). A comprehensive metabolite study of many species was conducted by Farag et al. (2016b).

Most previous studies have focused on other *Passiflora* species for isolation and biological investigation. To our knowledge, this is the first detailed study on *Passiflora racemosa* Brot. of the cultivated type in Nepal. The primary goal of this research is the identification of the secondary metabolites in the leaves and exploration of the biological applications of the leaf methanol extract, with plans for isolation of compounds. Since this is the first study on this species, it is expected to attract further research into the medicinal potential of its leaves for drug discovery, rather than merely ornamental use. Additionally, no attempts have been made to isolate compounds from the flowers, presenting another avenue for future research.

Materials and Methods

Collection of plant material

The leaves were acquired from the premises of Deerwalk School, Sifal, Kathmandu (27.7119° N, 85.3410° E). The voucher code for this plant was issued by the National Herbarium and Plant Laboratory Godavari with code number KATH-RDP0002.

Fig. 1. a) *Passiflora racemosa* Brot. b) Herbarium of *Passiflora racemosa* Brot.

Preparation of extract

The collected leaves were properly washed. They were dried in the shade for 15 days and ground into powder (1 kg) in an electric grinder. The powder was soaked in 80% methanol (solute to solvent ratio: 1:3) for three days with occasional shaking. It was first filtered through cotton and then through Whatman paper 41. The filtrate was concentrated in the rotary evaporator at 40°C and 140 rpm. The extract was preserved at 4°C until further use.

Profiling of bioactive compounds

The secondary metabolites present in the extract were qualitatively tested following the protocol mentioned in (Harborne, 1984; Joshi et al., 2021).

GC-MS analysis

The quantitative profiling of secondary metabolites in the leaf methanol extract was performed by GC/MS analysis. The instrument specification is a Shimadzu GC/MS/QP2010 Plus gas chromatograph equipped with an AOC-20i auto sampler located at the Forensic Laboratory at Nepal Academy of Science and Technology (NAST). An RTX-5MS capillary column with helium as the carrier gas was used. A primary pressure of 500-900 mm was applied. The inside oven temperature was set at 60°C, the ion source at 200°C, the interface at 280°C, and the injection temperature at 280°C, employing splitless injection mode. The pressure was maintained at 80 kPa. The total flow rate of 5.9 mL/min, the column flow rate of 2.89 mL/min, the linear velocity of 92.3 cm/s, and the purge flow rate of 3.0 mL/min were programmed. The MS acquisition mode was set to normal scanning (scan speed 2500), with a runtime of 14.67 minutes, and an m/z scan range of 40 to 500.

Determination of total phenolic content (TPC)

The amount of phenolic in leaf extract was determined by the protocol mentioned by Lu et al. (2011). Twenty microlitres of leaf methanol extract (5 mg/mL) in 50% dimethyl sulphoxide was pipetted in triplicate into a 96-well plate. To each well, 100 microlitres of FCR (diluted ten times in distilled water) was added. The plate was incubated in the dark for 15 minutes at r.t., and an initial A (absorbance) was recorded at 765 nm. Then, 80 microlitres of sodium carbonate (7.5% in D/W) was added to each well. The plate was incubated in the dark for 30 minutes, and the final A was recorded at 765 nm using a microplate reader (Biotech, USA). A standard solution of gallic acid (1 mg/mL in methanol diluted to various concentrations), also in triplicate, was processed similarly to the leaf extract. Results are expressed as mg GAE per gram. The TPC was calculated by the usual formula.

Determination of total flavonoid content (TFC)

The amount of flavonoids in leaf methanol extract was determined by the protocol mentioned by Chang et al. (2020) with slight modification. Briefly, a 10% aluminium chloride ($AlCl_3$) solution in methanol was prepared. 20 µL extract and 110 µL distilled water were taken in triplicate in a 96-well plate. The initial A was measured at 415 nm. To each well, five microlitres of $AlCl_3$ solution, five microlitres of potassium acetate, and sixty microlitres of ethanol were added sequentially. The plate was incubated in the dark for 30 minutes at r.t. The final A was measured at 415 nm. 130 µL standard quercetin (0.1 mg/mL in methanol diluted to various concentrations), also in triplicate, was processed similarly to the leaf extract. Results are expressed as mg QE per gram. The TFC was calculated by the usual formula.

Determination of antioxidant activity (AOA)

To determine the *in vitro* AOA of leaf methanol extract, the protocol mentioned by Javanmardi et al. (2003) was used with slight modification. Plant extract (50 µL) of different concentrations was taken in a 96-well plate in triplicate. To each well, one hundred fifty microlitres of DPPH solution (0.1mM in methanol) (Sigma Aldrich, Germany) was added. The plate was kept in the dark for thirty minutes. The absorbance was measured at 520 nm using a microplate reader. In this study, ascorbic acid was used as a standard. The percentage scavenging activity was calculated by the formula: $\%\ \text{Scavenging} = [X_0 - X_1)/X_0] \times 100 \ldots (1)$, where X_0 = Absorbance of the control solution, X_1 = Absorbance of the extract/standard. Finally, the IC_{50} value was calculated.

Biological applications

Antidiabetic assay

To investigate the antidiabetic potential of the leaf methanol extract, the protocol by Kim et al. (2005b) was used with some

modifications. The α-amylase (0.5 mg in 1 mL of phosphate buffer at pH 6.9) was prepared as a working solution. The leaf extract (1 mg/mL) was prepared in methanol and diluted in phosphate buffer to obtain solutions of different concentrations (200, 175, 150, and 125 µg/mL). To two hundred microlitres of α-amylase, two hundred microlitres of leaf extract solution was added. It was incubated for ten minutes at 30℃. A two-hundred-microlitre starch solution (1% w/v in water) was added to each tube. It was again incubated for a further 3 minutes. The reaction was quenched by adding DNSA reagent (3, 5-dinitrosalicylic acid). The positive control was 1 mg/mL metformin without leaf extract in the mixture. The tube representing 100% enzyme activity was prepared without any plant extract. All tubes were then incubated at 85 ℃ for 10 minutes. After incubation, five millilitres of distilled water were added to each tube. Finally, the absorbance was measured at 540 nm. The inhibition rate was calculated by the formula,

$$\frac{Absorbance of\ 100\%\ Enzyme Activity - Absorbance of Sample Reaction}{Absorbance of\ 100\%\ Enzyme Activity} \quad \ldots (2)$$

Antibacterial assay

The antibacterial efficacy of the leaf methanol extract was investigated by the standard protocol mentioned in (CLSI, 2012). For this, the agar well diffusion method was used against pathogenic bacteria. The bacterial strains used were acquired from the Central Department of Microbiology, Tribhuvan University, Kirtipur. The bacteria used included *S. aureus* (ATCC-25293), *B. subtilis* (ATCC-6633), *E. coli* (ATCC-25922), and *K. pneumoniae* (ATCC-13883). First, the culture of each test organism was prepared in nutrient broth (NB). Then, it was diluted with sterile NB media to maintain turbidity at 0.5 McFarland standards (approximately 10^{6-8} CFU mL^{-1}). Then, about one hundred microlitres of the inoculum was spread on Mueller-Hinton agar (MHA) plates. 20 µL of the leaf extract solution (50 mg/mL) was loaded into each well. The plates were subsequently incubated at 37℃ for twenty-four hours. The ZOI was measured. Ampicillin (1 mg mL^{-1}) was used as the positive control, and dimethyl sulphoxide as the negative control.

Antifungal assay

The antifungal study of leaf extract was performed similarly to antibacterial, with two different fungi, *Alternaria* and *Fusarium*, by the protocol mentioned in (CLSI, 2012). Finally, the plates were incubated at 28℃ for a week, and the ZOI was measured. Cycloheximide (50 mg mL^{-1}) was taken as a positive control, and dimethyl sulphoxide as a negative control.

Acute oral toxicity test

The toxicity test of leaf methanol extract and powdered leaf was carried out on Albino mice by OECD TG 425 Up and Down Procedure (OECD, 2002). In this method, three Albino mice of the same sex (female) were fed with a single dose of leaf extract and powder as per their body weight. After that, they were observed for fourteen days. During this observation period, close monitoring was done for the initial four hours and twenty-four hours. If any one of the three mice dies within fourteen days, then the leaf extract and powder are toxic; if not, the leaf extract or powder is non-toxic. However, if the first result is toxic, further observations must be carried out with the decreased dose. Quantitatively, toxicity is analysed based on the LD_{50} value. This means if with the leaf extract or powder, mortality is not observed with a single dose of > 2000 mg/kg body weight, the leaf extract or powder is non-toxic. If mortality is observed with a single dose of > 2000 mg/kg body weight, the leaf extract or powder is toxic, and further, repeated observation must be carried out with a decreased dose twice or thrice.

Statistical Analysis

All the experiments were performed thrice. The data were presented as mean ± standard deviation. The linear regression coefficient (R^2) values were calculated for TPC, TFC, antioxidant activity, and antidiabetic activity using GraphPad Prism 8.4 for Windows.

Results and Discussion

Phytochemical analysis

The phytochemical analysis of the leaf methanol extract indicated several secondary metabolites in the leaf extract, as mentioned in **Table 1**. Phytochemical analysis indicates the presence of nearly all common secondary metabolites except tannins, volatile oils, coumarins, and quinones.

Table 1. Phytochemical analysis of leaf methanol extract

S.N.	Phytochemical constituents	Methanol extract
1	Alkaloids	+
2	Flavonoids	++
3	Terpenoids	+
4	Polyphenols	++
5	Saponin	+
6	Tannins	-
7	Reducing sugar	+
8	Volatile oil	-
9	Coumarins	-
10	Quinone	-

Note: + means present, ++ means very good, - means absent

GC/MS analysis

The objective of this analysis was to identify the secondary metabolites in the extract. The compounds were identified on the basis of mass match with the National Institute of Standards and Technology (NIST) library database facility of the instrument. Fifteen compounds were identified, which are tabulated in **Table 2** along with their retention times, area percentages, m/z values, molecular formula, molecular mass, and associated bioactivity. The low molecular mass and highly volatile compounds (mass < 600) have been identified. The major compounds based on area % are: 9, 12, 15-Octadecatrienoic acid, (*Z, Z, Z*) (12.54 %), n-hexadecanoic acid (11.22 %), Phytol (9.79 %), Melezitose (6.50 %), d-ascorbic acid (2.52 %), and benzoic acid (2.49 %). The chromatogram has been depicted **in Figure 2**.

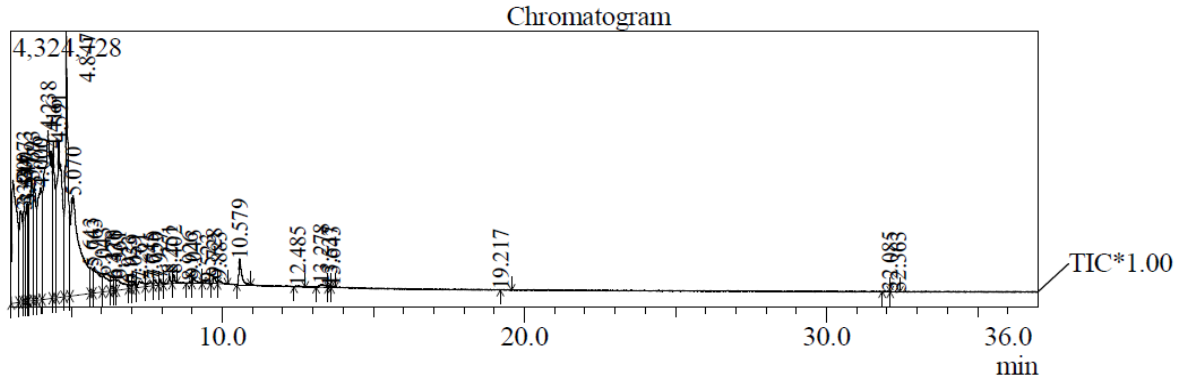

Fig. 2. GC-MS chromatogram of leaf methanol extract

Table 2. GC-MS analysis of leaf methanol extract of *Passiflora racemosa* Brot.

Peak No.	R.Time	Area%	Base m/z	Name, MF, Mass	Bioactivity
3	3.444	2.49	105.00	Benzoic acid	
4	3.547	2.52	85.05	d-Ascorbic acid ($C_6H_8O_6$, 176.12)	Natural antioxidant (Gęgotek et al., 2022)
5	3.577	0.67	135.05	2-Methoxy-4-vinylphenol($C_9H_{10}O_2$, 150.17)	Antioxidant, antimicrobial and anti-inflammatory (Asami et al, 2023)
8	4.000	6.50	58.05	Melezitose ($C_{18}H_{32}O_{16}$, 504.4)	

11	4.591	11.22	73.00	n-Hexadecanoic acid ($C_{16}H_{32}O_2$, 256.42)	Prostaglandin-E_{29}-reductase inhibitor (E. Y. Chang et al., 2016)
12	4.847	9.79	71.05	Phytol ($C_{20}H_{40}O$, 296.5)	Schistosomicide drug (De Moraes et al., 2014)
13	5.070	12.54	67.05	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	
14	5.643	0.80	43.00	cis-3-Hexenyl iso-butyrate	
25	7.927	0.12	69.00	Supraene ($C_{30}H_{50}$, 410.7,)	Investigated as an adjunctive cancer therapy (Bhilwade et al., 2019)
26	8.251	0.23	43.05	.alpha.-Tocospiro A ($C_{29}H_{50}O_4$, 462.7)	Cytotoxic against human HL60 cells by SRB assay (Yuan et al., 2014)
27	8.402	0.20	43.05	.alpha.-Tocospiro B ($C_{28}H_{48}O_4$, 448.7)	Antimycobacterial activity against Mycobacterium tuberculosis H37Rv (Wu et al., 2007)
28	8.926	0.04	57.10	Eicosane ($C_{20}H_{42}$, 282.5)	
29	9.043	0.21	137.10	.delta.-Tocopherol ($C_{27}H_{46}O_2$, 402.7)	A food antioxidant, a natural antioxidant (Food, 2015)
33	10.579	0.98	165.10	Vitamin E	Natural antioxidant (Traber & Atkinson, 2007)
35	13.278	0.23	43.05	.beta.-Sitosterol ($C_{29}H_{50}O$, 414.7)	Methyl transferase inhibitor, an anticholesterolemic drug, and an antioxidant (S. Babu & Jayaraman, 2020)

The structures of a few significant metabolites identified by GC/MS have been depicted in **Figure 3.**

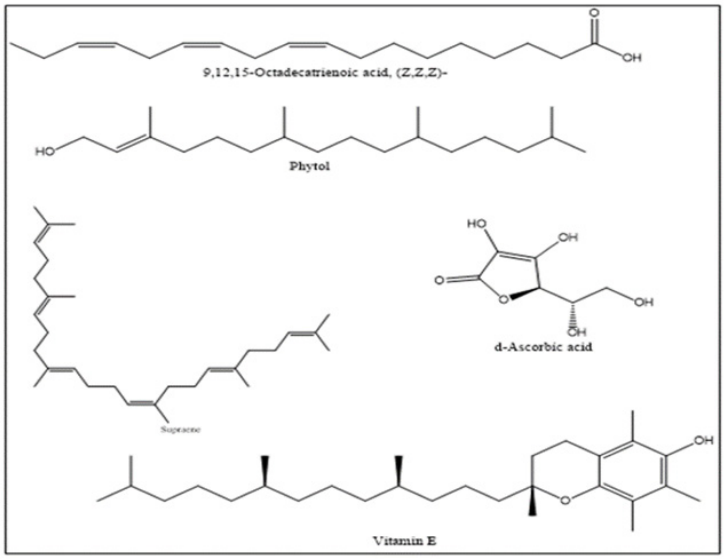

Fig. 3. Major Bioactive compounds detected in GC-MS of leaf methanol extract

TPC and total TFC analysis

The TPC was calculated from the expression, y = 0.0044x + 0.0169, R^2 = 0.9891, obtained from a standard gallic acid calibration plot, **Figure 4(a),** with TPC 60.127 ± 2.445 mg/ g GAE. Likewise, the TFC was calculated from the expression, y = 0.0036x + 0.0054, R^2 = 0.991, obtained from a standard quercetin calibration plot **Figure 4(b)** with a TFC of 398.215 ± 18.330 mg/g QE. This result indicates that the flavonoid content is higher than the phenolic content by more than 5 times, or nearly by 84.90% in the leaves. Since this is the first study on this species, we compared our results with those of other Passiflora species, including *Passiflora edulis* (TPC 24.28 ± 0.67), *Passiflora quadrangularis* (TPC 16.73 ± 0.78), and *Passiflora maliformis* (TPC 15.56 ± 0.56) (Ramaiya et al., 2014b). The TPC was found to be much higher in this species compared with the above three species. The Standard calibration curves for gallic acid and quercetin have been depicted in **Figure 4** (a) and (b), respectively.

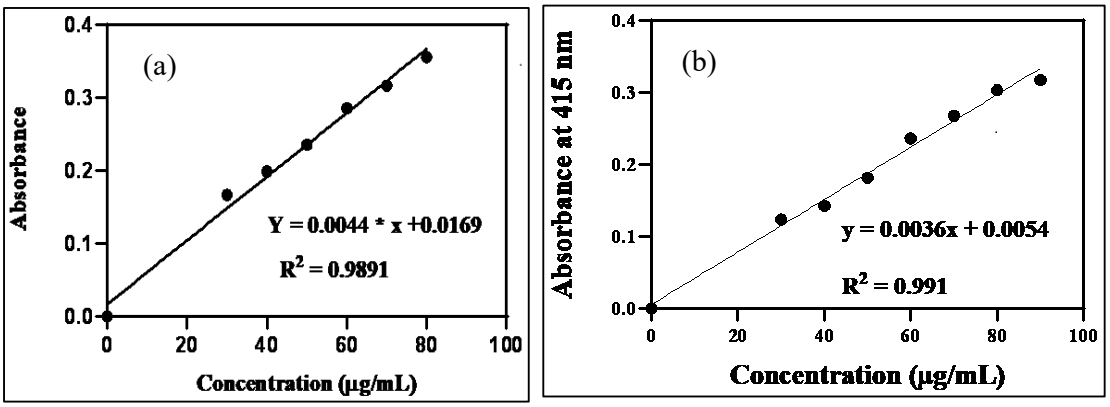

Fig . 4. (a) Gallic acid calibration curve, and (b) Quercetin calibration curve.

DPPH assay

From the concentration versus % inhibition graph, an equation, y = 65.294 + 11.244, R^2 = 0.9091, was derived as depicted in **Figure 5(a)**. This equation was employed to calculate IC_{50} as 0.59 µg/mL for the leaf extract, compared to IC_{50} of 25.74 µg/mL for standard ascorbic acid, determined from the equation, y = 1.6815x + 6.7305, R^2 = 0.9885. A lower IC_{50} indicates stronger antioxidant activity. Since the IC_{50} of the leaf methanol extract is significantly lower than that of standard ascorbic acid, it suggests the leaf methanol extract acts as a very strong antioxidant. This may be supported by the higher flavonoid content in the plant extract, as shown in total flavonoid content analysis. When comparing the IC_{50} of this species with others of the same plant, such as Passiflora edulis (IC_{50} = 653.5 ± 6.1), Passiflora quadrangularis (IC_{50} = 785.2 ± 1.8), and Passiflora maliformis (IC_{50} = 456.9 ± 13.1) (Ramaiya et al., 2014b), the markedly lower IC_{50} of this species indicates superior antioxidant activity. The high antioxidant capacity of this plant may be attributed to compounds like d-ascorbic acid, 2-methoxy-4-vinylphenol, vitamin E, β-sitosterol, and δ-tocopherol, as identified through GC-MS analysis. The vivid mechanism of free radical generation and its harmful effects are depicted in **Figure 6**. Antioxidants neutralise these free radicals and protect us from detrimental effects (Tumilaar et al., 2024).

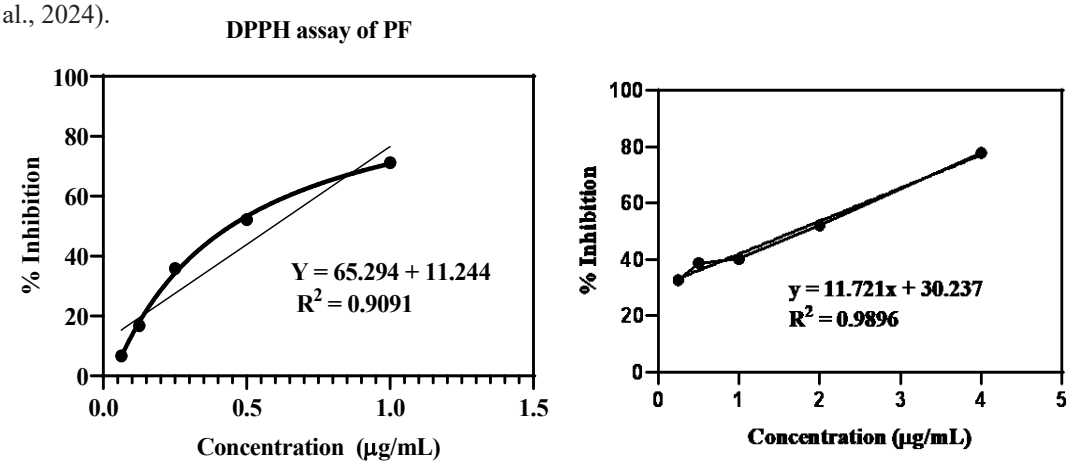

Fig. 5. (a) DPPH assay, and (b) α-amylase inhibition

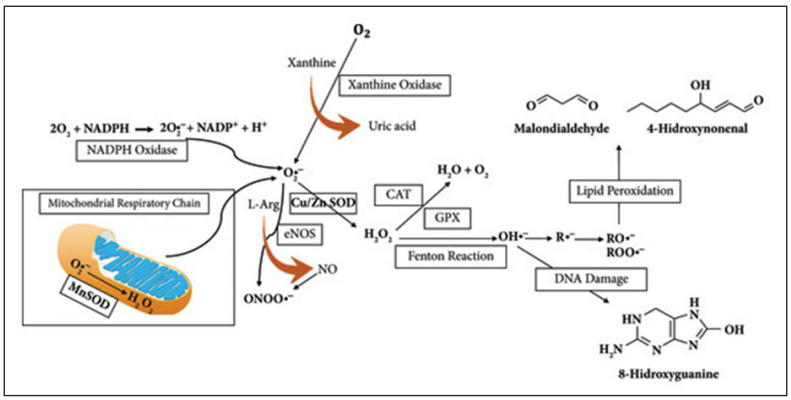

Fig. 6. Free radical generation and harmful effects (Tumilaar et al., 2024)

Note: MnSOD = Manganese superoxide dismutase, CAT = Catalase, GPX = Glutathione peroxidase, eNOS = Endothelial Nitric oxide synthase, NADPH = Nicotinamide Adenine Dinucleotide phosphate, L-Arg = L- Arginine

Biological applications

Antidiabetic assay

The α-amylase inhibition assay indicates the antidiabetic potential of the leaf extract. It is measured by IC_{50}, which is calculated using the equation y = 11.721x + 30.237, with R^2 = 0.9896, derived from the calibration curve of the standard (**Figure 5 b)**. This assay demonstrates concentration-dependent inhibition, meaning higher concentrations result in greater inhibition. The IC_{50} for the methanol extract is 1.686 µg/mL, compared to the standard, metformin, which is (IC_{50} = 19.4 ± 1.4 mM). The lower the IC_{50}, the greater the potential of the extract as an antidiabetic agent. Because the IC_{50} of the extract is very low, approaching that of the standard, it exhibits strong antidiabetic potential and warrants further in *vivo* studies and isolation of active metabolites. Diabetes has become a global health concern, affecting people of all ages after the age of 30. It results from excessive blood sugar levels due to uncontrolled hydrolysis of starch into sugar by the enzyme α-amylase. Several phenolics and flavonoids in the plant extracts inhibit α-amylase activity, thereby lowering blood glucose levels. Consequently, there is an ongoing search for plant-based antidiabetic agents with fewer side effects than synthetic drugs.

Antibacterial activity and Antifungal activity

The methanol extract did not exhibit ZOI against the tested pathogenic bacteria at the tested concentrations. The positive control exhibited ZOI measuring 30 mm, 22 mm, 33 mm, and 27 mm for these bacteria, respectively. This suggests that the leaf methanol extract has no antibacterial effect on these bacteria. The leaf methanol extract did not exhibit antifungal activity against the tested fungi, indicating the lack of antifungal efficacy of the leaf extract. This represents the inaugural antibacterial and antifungal investigation on this plant species. Further studies are required on a large number of pathogenic bacteria and fungi.

Acute oral toxicity test

The in *vivo* acute toxicity assessment on Albino mice demonstrated that neither the leaf powder nor the methanol extract displayed toxicity, as reported in **Table 3** with LD_{50} values. Therefore, it is safe for biological applications. This is the first toxicity test on the plant of this species.

Table. 3. LD_{50} values for the leaf powder and extract

Sample	LD_{50} value
Leaf powder of *Passiflora racemosa* Brot.	>2000 mg/kg BW
Leaf extract of *Passiflora racemosa* Brot.	>2000 mg/kg BW

Conclusions

The leaf extract exhibited excellent antioxidant and antidiabetic properties, since the IC_{50} values obtained were lower than the standards. The in *vivo* acute oral toxicity on albino mice results indicated that the leaf powder and extracts are non-toxic; therefore, they are safe for biological applications. The TPC and TFC were 60.127 ± 2.445 mg/g GAE and 398.215 ± 18.330 mg/g QE, respectively, indicating a higher flavonoid content than phenolic content, reflecting excellent antioxidant activity. The GC/MS analysis has also exhibited the presence of many natural antioxidants, like ascorbic acid, vitamin E, along with other bioactive compounds mentioned in **Table 2**. The leaf extract lacked antibacterial and antifungal potency against the tested bacteria and fungi. The leaves of this plant can be used as a natural antioxidant and antidiabetic agent. Further in *vivo* studies are required for biological applications and the isolation of bioactive secondary metabolites from the leaves of this species.

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Author contributions

Ram Darash Pandey performed the experiment, analysed the data and graph, and wrote the manuscript. Susan Joshi designed the whole work setup, supervised and finalised the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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