

EXTRACTION, ISOLATION AND CHARACTERIZATION OF BIOACTIVE FLAVONOIDS FROM THE FRUITS OF *PHYSALIS PERUVIANA* LINN EXTRACT

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ABSTRACT

Objective: Although *Physalis peruviana* Linn. fruit commonly known as golden berries, Cape gooseberries and wild tomato is highly esteemed by consumers all over the world, scientific reports about the fruits are scarce and there was no systemic study in the literature regarding the phytoconstituents present in the fruits of Indian *P. peruviana* L. Hence, the present study was aimed to determine the total flavonoids as well as phenolic content in the fruits of *P. peruviana* L.

Methods: The ethanolic extract of *P. peruviana* L. fruits was subjected to phytochemical screening and the total flavonoids as well as phenolic contents were estimated according to standard protocols. The extract was subjected to high-performance liquid chromatography analysis for the identification of major flavonoids and the individual flavonoids were characterized by various spectral studies.

Results: The qualitative analysis revealed the presence of biologically active secondary metabolites such as phenols, flavonoids, glycosides, sterols, saponins, tannins, lactones and alkaloids in the fruit extract. The fruit extract was also found to contain significant amounts of both phenolic compounds, as well as flavonoids such as rutin, myricetin, quercetin and kaempferol.

Conclusion: The results of the present study indicate that many compounds rather than a single component of *P. peruviana* L. fruit is responsible for its pharmacological as well as beneficial effects. The data also provide a rationale for the use of golden berries in the traditional medicine for various ailments.

Keywords: *Physalis peruviana* L., Cape gooseberry, Flavonoids, Phenols, Phytochemicals.

INTRODUCTION

Physalis peruviana L. is a widely used herb in folk medicine for various ailments. The botanical name of the plant is *P. peruviana* Linnaeus, belonging to the family *Solanaceae* and genus *Physalis* [1]. It is a native plant from the Peruvian Andes and now widely distributed throughout the tropical and sub-tropical countries. The plant has high multiplication potential as it grows in poor soils [2]. The cultivation of *P. peruviana* L. in Colombia is steadily increasing to satisfy the growing export demands, ranking it second after banana fruit exports [3].

The most distinctive feature of *P. peruviana* L. is the accrescent fruiting calyx, which enlarges to cover the fruit and hangs downwards like a lantern [2]. The common names for the *P. peruviana* L. fruits include golden berry, Cape gooseberry, and wild tomato. The fruits are succulent golden spheres the sizes of marbles [4]. The round fruit has an average diameter of 20-25 mm and an approximate weight of 4-5 g. Each fruit approximately contains 100-200 small seeds [2]. A single plant may yield 300 fruits, and carefully tended plants can provide 25-35 tons/hectare [5]. The shelf life of the fruit is 1 month while without calyx is 4-5 days [1]. The time between the initiation of germination and the first crop is approximately 9 months. The serviceable life of the plant production goes from 9 to 11 months from the time of the first harvest, since thereafter both the productivity and fruit quality decreases [2].

The pH value of the fruit pulp is around 3.76-3.86. Generally, the fruits are consumed fresh [6]. So far, no studies indicate possible adverse effects of Cape gooseberries. Unlike other fruits that must be processed close to the place of harvest, golden berry is characterized by unique storage properties. The fruits are long lasting when stored in a sealed container and kept in a dry atmosphere for several months, and they freeze well. Wu *et al.*, (2006) [7] reported that the ethanolic extract of

the fruit had higher antioxidant properties than the aqueous extract. Furthermore, the antioxidant activity associated with the fruits is due to high levels of polyphenols and significant levels of vitamin A and C. Despite wide use in folk medicine, there are only a few reports in the literature concerning *P. peruviana* L. fruit extract and its chemical composition. Recently, we have reported the anti-diabetic properties of *P. peruviana* L. fruit extract in high fat diet-low dose streptozotocin induced Type 2 diabetes in experimental rats [8]. A thorough literature survey revealed that there are no systemic reports available regarding the phytoconstituents present in the fruits of Indian *P. peruviana* L. Hence, the present study was aimed to determine the presence of biologically important flavonoids as well as total phenolic content in the fruits of *P. peruviana* L.

METHODS**Plant material**

Whole plants of *P. peruviana* L. grown in the natural environment were collected from Theni District, Tamil Nadu and identified by a plant taxonomist in CAS in Botany, University of Madras where a voucher specimen was deposited in the herbarium. Intact, fruits were carefully selected according to the degree of ripeness measured by fruit color (brilliant orange).

Preparation of fruit extract

P. peruviana L. fruits were dehusked, washed, crushed in an hot air oven at 50°C then powdered in an electrical grinder, which was then stored in an airtight brown container at 5°C until further use. The powdered fruits were delipidated with petroleum ether (60-80°C) for overnight. It was then filtered, and soxhalation was performed with 95% ethanol. Ethanol was evaporated in a rotary evaporator at 40-50°C under reduced pressure. The 100 g of dried powder of *P. peruviana* L. fruits yields 27.4% g.

Preliminary phytochemicals screening

The ethanolic extract of *P. peruviana* L. fruits was subjected to phytochemical screening for the qualitative analysis of various phytoconstituents [9,10].

Determination of total phenolic content

Total polyphenol content in the ethanolic extract of *P. peruviana* L. fruits was determined according to the Folin-Ciocalteu colorimetric method [11,12]. A standard curve was built with gallic acid reference solutions. Aliquots ranging from 2 to 10 ml of standard aqueous gallic acid solution (100 µg/ml) were pipetted into 100 ml volumetric flasks containing 70 ml of distilled water. Folin-Ciocalteu reagent (5 ml) and 10 ml of saturated sodium bicarbonate solution were added, and the volume was made up to 100 ml with distilled water. The solution was thoroughly mixed. The blank was prepared in the same manner, but without gallic acid. After 1 hr of incubation at room temperature, the absorbance was measured at 760 nm. The samples were prepared in triplicates for each analysis, and the mean value was calculated. For the determination of total phenolic content of *P. peruviana* L. fruits, aqueous solutions at the final concentration of 20 µg/ml were used; proceeding in the same manner described for the reference solutions and the total polyphenolic content was expressed as mg/g of gallic acid equivalents [13].

Determination of total flavonoid content (TFC)

TFC in the ethanolic extract of *P. peruviana* fruits was determined according to the method of Quettier-Deleu *et al.*, (2000) [14] with minor modifications. A standard curve was built with quercetin reference solutions. Aliquots ranging from 2 to 8 ml of standard quercetin ethanol extract solution (50 µg/ml) were pipetted into 25 ml volumetric flasks containing 1 ml of 2% aluminum chloride dissolved in ethanol and the volume was made up with ethanol. The blank was prepared by diluting 1 ml of 2% aluminum chloride dissolved in ethanol in a 25 ml volumetric flask with ethanol. After 1 hr at room temperature, the absorbance was measured at 420 nm. *P. peruviana* L. fruits samples were evaluated at a final concentration of 20 µg/ml, proceeding in the same manner described for the reference solutions and the TFC was calculated as quercetin equivalents (mg/g) from a calibration curve. The samples were prepared in triplicate for each analysis, and the mean value of absorbance was recorded.

High performance liquid chromatography (HPLC)-DAD system for analysis of phenolic compounds

HPLC analysis was performed using Shimadzu HPLC system equipped with a diode array detector. The chromatographic separations were performed on an Inertsil C18 analytical column (4.6 mm × 250 mm i.d., 5 µm). The composition of solvents and the gradient elution conditions used were described previously by Bengoechea *et al.*, (1997) [15], Schieber *et al.*, (2001) [16] and Butsat *et al.*, (2009) [17], with some modifications. The mobile phase consisted of purified water with acetic acid (pH 2.74) (solvent A) and acetonitrile (solvent B) at a flow rate of 0.8 ml/minutes. Gradient elution was performed as follows: From 0 to 5 minutes, linear gradient from 5% to 9% solvent B; from 5 to 15 minutes, 9% solvent B; from 15 to 22 minutes, linear gradient from 9% to 11% solvent B; from 22 to 38 minutes, linear gradient from 11% to 18% solvent B; from 38 to 43 minutes, from 18% to 23% solvent B; from 43 to 44 minutes, from 23% to 90% solvent B; from 44 to 45 minutes, linear gradient from 90% to 80% solvent B; from 45 to 55 minutes, isocratic at 80% solvent B; from 55 to 60 minutes, linear gradient from 80% to 5% solvent B and a re-equilibration period of 5 minutes with 5% solvent B used between individual runs. Operating conditions were as follows: Column temperature, 38°C, injection volume, 20 µl, and ultraviolet (UV)-diode array detection at 280 nm (hydroxybenzoic acids), 320 nm (hydroxycinnamic acids) and 370 nm (flavonols) at a flow-rate of 0.8 ml/minutes. Spectra were recorded from 200 to 600 nm. Phenolic compounds in the samples were identified by comparing their relative retention times and UV spectra with those of authentic compounds and were detected using an external standard method. Infrared (IR) spectral studies were carried

out in the solid state as pressed KBr pellets using Perkin Elmer Fourier transform (FT)-IR spectrophotometer in the range of 400-4000/cm. The mass spectrum of the complex was obtained using Jeol Gcmate. The ¹H nuclear magnetic resonance (NMR) and ¹³C NMR at 500.13 and 125.758 MHz were carried out respectively. The spectra were recorded without any correction for instrumental characteristics.

RESULTS AND DISCUSSION

Qualitative phytochemical analysis

The qualitative phytochemical analysis of *P. peruviana* L. fruits extract showed the presence of biologically active phyto ingredients such as phenols, flavonoids, glycosides, sterols, saponins, tannins, lactones, and alkaloids. The medicinal values of plant lie in bioactive phytochemical constituents that produce definite physiological actions on the human body and these phytochemicals are produced as secondary metabolites to defend the plant from the environment. In addition, the phenolic and flavonoid contents in the fruit extract were found to be 76.84±3.65 mg/g dry weight and 241±8.45 mg/g dry weight, respectively. Phenols are very important plant constituents because of the scavenging ability due to their hydroxyl groups [18]. Similarly, flavonoids are an important group of polyphenols widely distributed among the plant flora and containing a benzopyrone that use as antioxidants or free radicals scavengers [19]. The total phenolic and flavonoids content determined in the present study were slightly different from the earlier reports by Wu *et al.*, 2009 [20], Ahmed, 2014 [21], Rop *et al.*, 2012 [22] in which the total phenolic and flavonoids were relatively less. The maturation effects of *P. peruviana* L. fruits were classified into seven maturity states according to the surface colors of the fruits which range from dark green (Stage 0) to intense orange (Stage 6) [23]. This maturity states in turn depends on quality parameters such as soluble solids contents, titratable acidity and maturity index [23]. During early maturation, the phenol content decreased significantly with an approximate decrease of 50% between states S0 to state S3. However, during states S4-S7 no significant variations were observed. The decrease in phenolic compounds suggested that they were progressively bound to the cell walls, which is an important mechanism by which plants depend themselves against pathogen and strengthen the cell walls. The decreased in phenolic compounds could also be related to the reduction of primary metabolism in the ripe fruit, thus resulting in a lack of substrates necessary for the biosynthesis of phenolic compounds [24-27].

Identification and characterization

Four isolated compounds were identified by HPLC (Figs. 1-4) and spectral data of IR, mass, and ¹H and ¹³C NMR. HPLC analysis of the purified fraction showed that the isolated crystal components have similar retention times (approximately rutin 6.94 minutes, myricetin 11.40 minutes, quercetin 9.68 minutes and kaempferol 15.97 minutes) to the rutin, myricetin, quercetin and kaempferol standards (Figs. 5 and 6).

The IR spectrum of the rutin is shown in Fig. 7. IR values: 3483 OH (bonded), 2931 CH stretch, 1669 C=O, 1504 C=C aromatic, 1348 C-O-C, 1204 C-O-C, 1043 C-O-C. The mass spectrum of rutin is illustrated in Fig. 8. The rutin compound gave a fragments peaks at [M+H]⁺=611, [M+H]⁺-Rhamnose=465, [M+H]⁺-Rhamnose-Glucose=303. This corresponds to C₂₇H₃₀O₁₆. The NMR data of the compound depicting the structure of rutin [28] were as follows ¹H-NMR (Fig. 9) (500 MHz, DMSO-d₆), δ ppm: 3.40-3.72 (m, 12H of sugar moieties), 3.86 (d, 1H-Rham), 1.03 (3H, d, CH₃-Rham), 4.06-4.83 (4H, H-1 Glu), 5.32 (1H, d, H-6), 6.18 (1H, d, H-8), 6.42 (1H, d, H-5'), 7.85 (1H, m, H-2', H-6'); the ¹³C NMR (Fig. 10) spectrum (500 MHz, DMSO-d₆) showed 29 carbon signals which indicated the presence of 15 carbon signals due to the flavonol skeleton. The spectrum of the isolated compound, revealed the presence of one methyl carbon (δC=18.26 ppm) of rhamnose, one methylene carbon (δC=67.36 ppm), 15 methene carbons and 10 quaternary carbons. In the aliphatic region of ¹³C NMR, 12 carbon resonances are assigned for a rutoside moiety among which the most downfield signals at 102.16

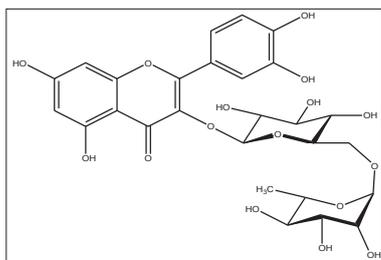


Fig. 1: Rutin

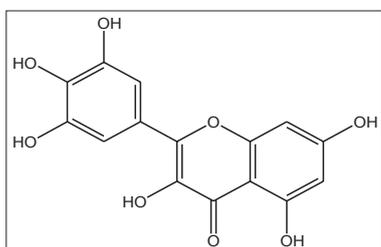


Fig. 2: Myricetin

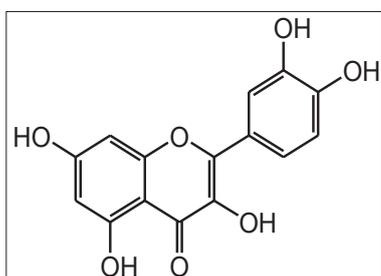


Fig. 3: Quercetin

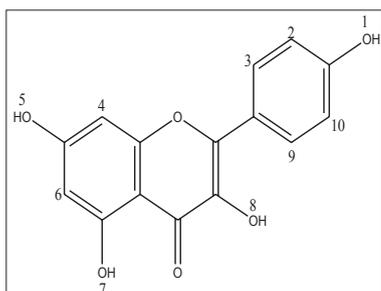


Fig. 4: Kaempferol

and 101.38 are assigned for the two anomeric carbons C1''' and C1'' of rhamnose and glucose, respectively [29].

Myricetin

The IR spectrum of the myricetin is shown in Fig. 11. The band at 3384/cm is assigned to a free OH bond vibration. The bands around 1678/cm and 1631/cm are assigned to the stretching vibration of the C-O group. The band at 1593/cm is denoted for the stretching vibration of C-C in the hexatomic ring. The band around 1536/cm is assignable to an aromatic group and the bands 1359/cm, and 1165/cm are assigned to the C-O-C vibration. The mass spectrum of myricetin is illustrated in Fig. 12. The peak at 318 corresponds to M⁺ ion of the isolated compound myricetin the compound gave a fragmentation peaks at [M+H]⁺=319 or M⁺=318, [M+H-H₂O]⁺=301, [M+H-H₂O-CO]⁺=273, [M+H-CO]⁺=291, [M+H-2CO]⁺=263 This corresponds to C₁₅H₁₀O₈. The NMR data of the compound depicting the structure of myricetin [29,30] were as follows ¹H NMR (500 MHz, DMSO-d₆) δ ppm: 6.23 (2H, s, H-2', 6'), 5.63 (2H,

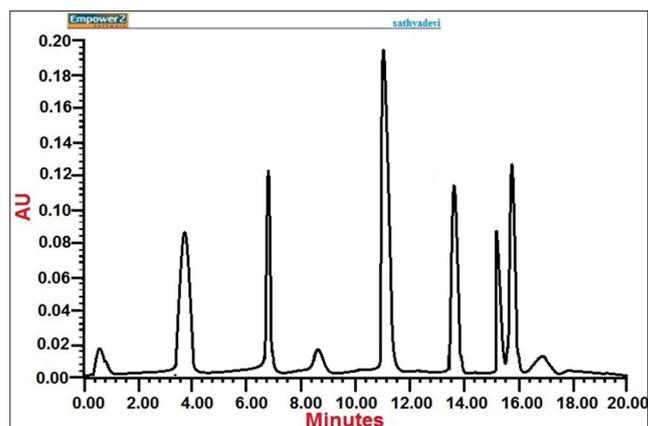


Fig. 5: High performance liquid chromatography analysis standard of rutin, myricetin, quercetin and kaempferol

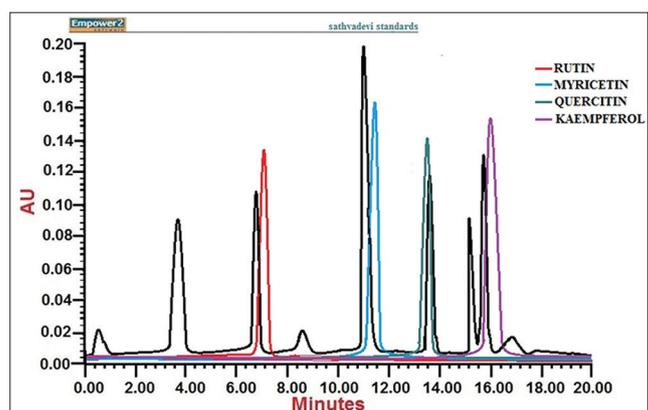


Fig. 6: High performance liquid chromatography analysis of rutin, myricetin, quercetin and kaempferol

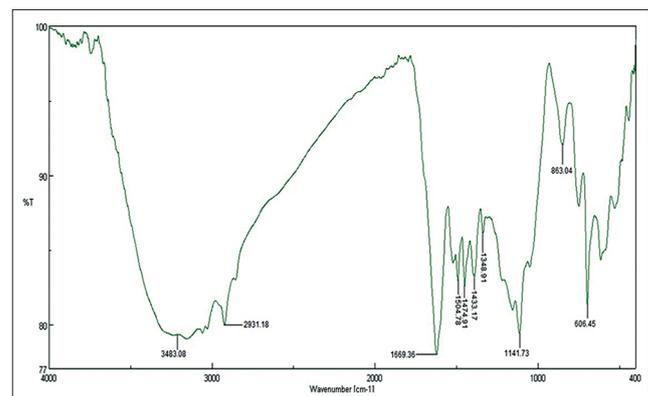


Fig. 7: Infrared spectra of rutin

d, H-8), 4.96 (5H, H) (Fig. 13). ¹³C NMR (500 MHz, DMSO-d₆) δ ppm: 182.65 (C-4), 168.73 (C-7), 165.03 (C-5), 161.26 (C-9), 148.97 (C-2), 145.90 (C-3', 5'), 136.63 (C-3), 126.49 (C-1'), 105.50 (C-2', 6'), 104.98 (C-10), 97.28 (C-6) (Fig. 14).

Quercetin

The IR spectrum of the quercetin is shown in Fig. 15. The absorption around 3350/cm is due to the presence of phenolic hydroxyl groups in the compound. The intense absorption band at 1673/cm is due to the presence of ν_(C=O). The band around 1489/cm is due to the occurrence of the aromatic group in an isolated compound. The mass spectrum of quercetin is illustrated in Fig. 16. The quercetin compound gave a

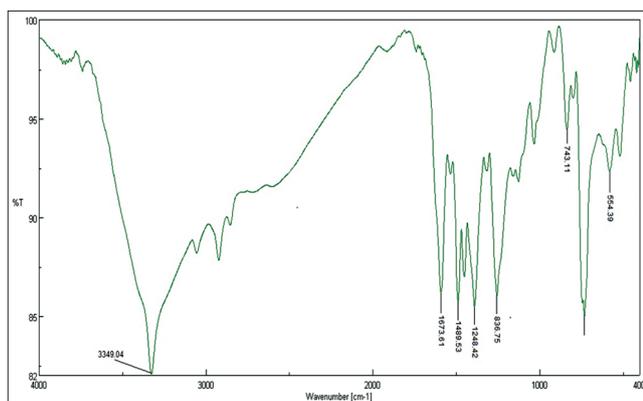


Fig. 15: Infrared spectra of quercetin

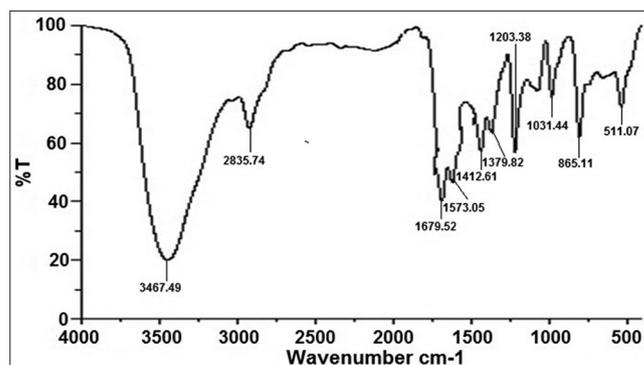


Fig. 19: Infrared spectra of kaempferol

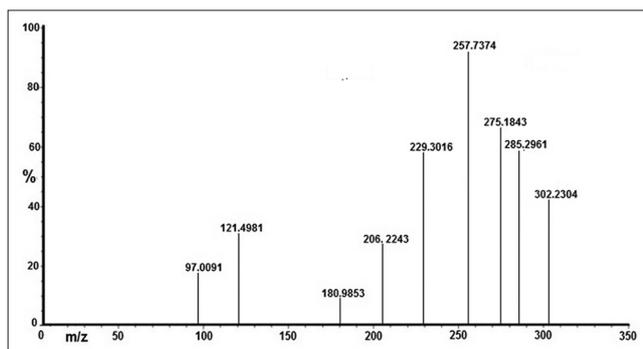


Fig. 16: Mass spectrum of quercetin

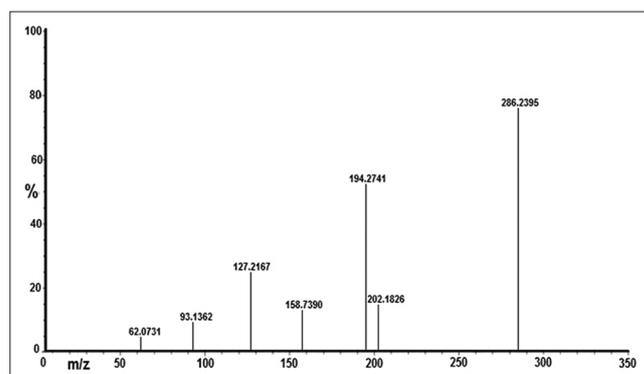


Fig. 20: Mass spectrum of kaempferol

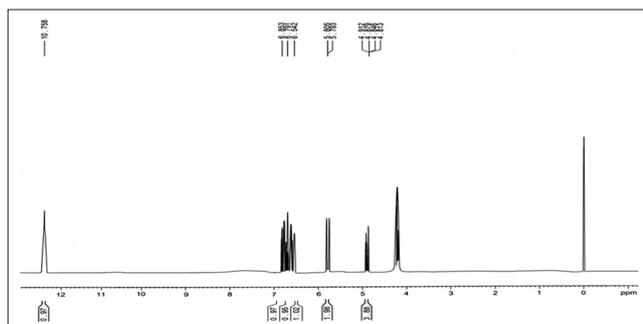


Fig. 17: ¹H nuclear magnetic resonance spectra of quercetin

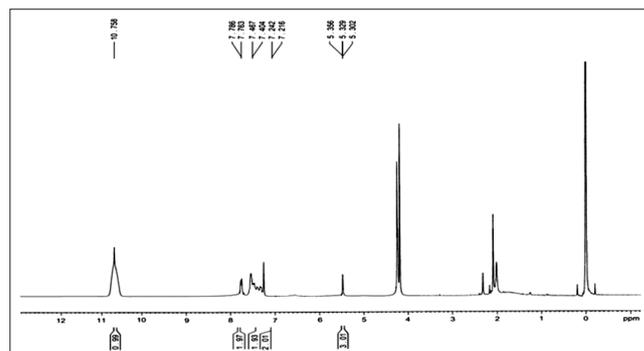


Fig. 21: ¹H nuclear magnetic resonance spectra of kaempferol

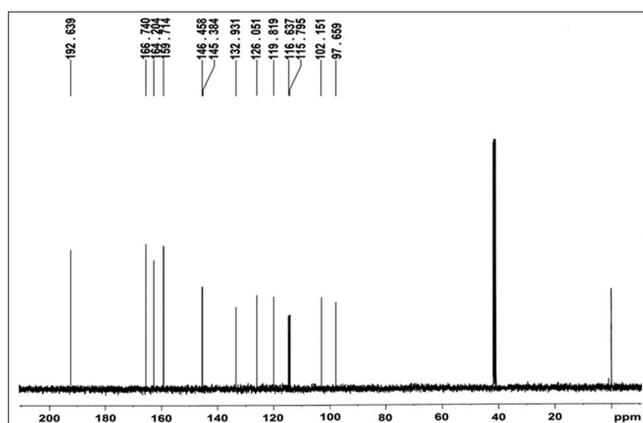


Fig. 18: ¹³C nuclear magnetic resonance spectra of quercetin

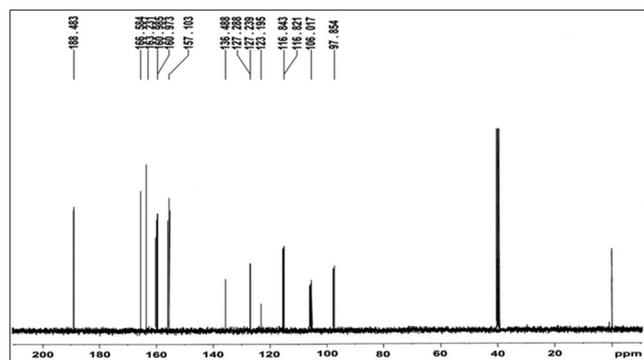


Fig. 22: ¹³C nuclear magnetic resonance spectra of kaempferol

the carbonyl group (C=O) and the absorption band at 1573, 1412/cm denotes the presence of aromatic ring. Moreover, the band around 2835/cm corresponds to the presence of C-H stretching. The mass spectrum of kaempferol is illustrated in Fig. 20. A 100% base peak [M]⁺ for compound, was observed at m/z 286 in the mass spectrum indicating the compound as kaempferol. The molecular formula was inferred from ¹H, ¹³C-NMR and mass spectrometry. Based on spectral evidence, the structure of the compound was decided to be kaempferol (C₁₅H₁₀O₆, 286.2). ¹H NMR (DMSO-d₆, 500 MHz) δ: 5.38 (1H, H-6), 7.28 (2H, H-3' and H-5'), 7.45(1H, H-8) and 7.78 (2H, H-2' and H-6') (Fig. 21). ¹³C NMR (500 MHz, DMSO-d₆) δ: 97.85 (C-6), 106.01 (C-10), 116.84 (C-3' and C-5'), 123.197 (C-1'), 127.28 (C-2' and C-6'), 136.48 (C-3), 157.10 (C-9), 160.97 (C-5), 163.67 (C-7), 166.58 (C-4) (Fig. 22).

CONCLUSION

The results of the present study established the presence of biologically active phytochemicals in the fruit extract. The data also suggested that the fruit extract contain significant amounts of rutin, myricetin, quercetin and kaempferol. Thus, it may be concluded that the fruits of *P. peruviana* L. has great potential for producing healthy and highly nutritive products. The present study also advances the perspectives for utilization of gooseberries for potential management of diet linked chronic diseases such as diabetes and its associated complications.

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