FUDMA Journal of Sciences (FJS)
ISSN online: 2616-1370
ISSN print: 2645 - 2944
Vol. 10 No. 14, June, 2026, pp 361 – 366
DOI: https://doi.org/10.33003/fjs-2026-1014-5674

Chemical Profiling of *Piper nigrum* L. Leaves: Vitamin Quantification, Phytochemical Screening, and HPLC Identification of Major Flavonoids

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Received: 04 Aug. 2026
Accepted: 15 Aug. 2026
Published: 27 Aug. 2026

ABSTRACT
2500 g of powdered *P. nigrum* leaves were extracted by cold maceration using methanol. The crude methanol extract was subjected to fractionation by vacuum liquid chromatography, and the hexane-ethyl acetate fraction was characterized using HPLC. Phytochemical screening was carried out on the fraction, and the result revealed the presence of alkaloids, flavonoids, eugenols, steroids. Vitamin analysis was carried out on the crude extract and the result revealed Vitamin A (0.019 mg/100g), Vitamin C (15.181 mg/100g), Vitamin D (0.001 mg/100g), Vitamin E (1.355 mg/100g), Vitamin B_1 (0.163 mg/100g), Vitamin B_2 (0.360 mg/100g), Vitamin B_3 (1.250), Vitamin B_5 (2.155), Vitamin B_6 (0.361), Vitamin B_9 (0.025). HPLC analysis revealed eighteen chromatographic peaks, which were tentatively assigned to various phytochemical constituents, with quercetin (Rt. 11.050; 46.51%), as the predominant compound, followed by kaempferol (Rt. 12.166; 13.98%), with a relative low concentration of piperine (Rt 15.050; 2.80%).

Keywords: Polyherbal Formulation, Antidiabetic, Syrup, LD50, GC-MS, Diabetes Mellitus

INTRODUCTION

Although *Piper nigrum* L. has been extensively investigated, most studies have concentrated on the fruits, seeds and piperine, while comparatively fewer studies have examined the leaves. As a result, information on the chemical composition and biological properties of the leaves is still less extensive. Takooree *et al*. (2019) reported that the pharmacological importance of *P. nigrum* is largely associated with its fruits and piperine, although other parts of the plant may also contain biologically active constituents. Barman *et al*. (2025) has provided additional information on the leaves. Using liquid chromatography-mass spectrometry, the authors identified several constituents in a hydro-ethanol leaf extract, including N, N-didecyl pentanamide, piperettine, curcumin, myristicin, pipernonaline, sesamin and lupenone. The presence of these compounds indicates that the leaves contain a mixture of structurally diverse phytochemicals that may contribute to their biological activities. Studies on the biological properties of *P. nigrum* leaves have reported antioxidant, anti-inflammatory, antibacterial and anticancer activities. These effects have been associated with different classes of secondary metabolites, including alkaloids, phenolic compounds, flavonoids and terpenoids (Takooree *et al*., 2019; Barman *et al*., 2025). Barman *et al*. (2025) reported that the hydro-ethanol leaf extract inhibited the growth of MDA-MB-231, LA7 and B16-F10 cells, with MDA-MB-231 cells showing the greatest sensitivity. The extract also reduced cell migration and colony formation. Antibacterial activity was likewise observed, with a stronger effect against *Staphylococcus aureus* (Barman *et al*., 2025). These findings suggest that the biological effects of the leaves are likely to result from the combined actions of several constituents rather than from a single compound. The antioxidant activity of *P. nigrum* is of particular interest in studies involving chemically induced toxicity. Excessive production of reactive oxygen species (ROS) can disturb the balance between oxidants and endogenous antioxidant defenses, resulting in lipid peroxidation and damage to cellular components. Plant-derived phytochemicals capable of reducing oxidative stress may therefore have protective effects in experimental models of chemical toxicity. The antioxidant potential of *P. nigrum* has been linked to its diverse phytochemical composition (Takooree *et al*., 2019; Ding *et al*., 2026). In relation to the leaves, Barman *et al*. (2025) reported inhibition of intracellular ROS production together with suppression of inflammatory responses. Such findings provide a basis for further investigation of *P. nigrum* leaves in models in which oxidative stress contributes to tissue injury.

Despite these findings, the leaves remain less extensively characterized than the fruits and seeds. In particular, information on their vitamin composition, quantitative distribution of major phytochemicals and fraction-specific occurrence of bioactive constituents is limited. This is important because the extraction solvent and subsequent fractionation can influence which compounds are recovered and their relative concentrations. Moreover, although several studies have examined the biological activities of *P. nigrum*, investigations involving leaf-derived fractions, are still relatively limited (Takooree *et al*., 2019; Ding *et al*., 2026). The present study was therefore designed to provide additional chemical information on *P. nigrum* leaves. Vitamin analysis was carried out to establish the nutritional and secondary-metabolite composition of the leaves. The leaf extract was subsequently fractionated, with particular attention given to the hexane-ethyl acetate fraction, due to its intermediate polarity which provides favourable partitioning of secondary metabolites of intermediate polarity. Preliminary phytochemical screening and HPLC was used to characterize the fraction phytoconstituents. The study was based on the hypothesis that *P. nigrum* leaves contain a broad range of phytochemicals and vitamins and that fractionation would influence the distribution and concentration of bioactive constituents, particularly flavonoids. The findings are intended to improve the chemical characterization of *P. nigrum* leaves and provide information that may support further investigation of their pharmacological potential.

MATERIALS AND METHODS

Apparatus and equipment: Weighing balance, sintered glass funnel, vacuum pump, high speed electric grinding machine, laboratory sieve, rotary evaporator.

High-Performance Liquid Chromatography (HPLC)

Agilent 1260 Infinity High-Performance Liquid Chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, USA; equipped with a µBondapak C18 column (100 mm × 4.6 mm, 7 µm). Carrier: Acetonitrile/Water, 70:30. A 10 µL sample volume was injected and detection was performed by UV at 254 nm. The recorded pump pressure was 15 MPa. 10.00g Sample was extracted with Acetonitrile, the extract stabilized with Ethyl Acetate, put in 25ml Standard flask, and made up to the mark. 5 µL injected @ 2 mL/min flow rate).

Reagent and Chemicals

ferric chloride, acetic acid, sulphuric acid, methanol etc. all chemical used are of analytical grade obtained from Sigma Aldrich chemical

Collection and Identification of Plant sample

Fresh sample of *Piper nigrum* (leaves) were collected from a plant garden at Ireupken, Esan West Local Government Area, Edo State, Nigeria. The plant sample was identified and authenticated by Prof. H. A. Akinnibosun of the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City. A voucher specimen with a voucher number UBH-P210 was deposited at the herbarium for reference purpose.

Sample preparation

The fresh leaves of *Piper nigrum* was air-dried at ambient temperature (30 ± 2°C) for 28 days, pulverized using an electric blender and the powdered form was stored until further analysis.

Preparation of Extract

The extraction was carried out following standard methods. The powdered leaf sample (2500 g) of *Piper nigrum* was macerated in 5.0 L of methanol at room temperature. The mixture was allowed to stand for 72 hours and stirred intermittently to facilitate extraction. The mixture was filtered through Whatman No. 1 filter paper and sieved using a muslin cloth with a mesh size of 42 µm.

Vitamin Analysis

The vitamin analysis of the powered leaves of *Piper nigrum* was determined using the appropriate AOAC official methods of analysis: Vitamin A- AOAC 2001.13, C- AOAC 984.26, D- AOAC 2002.05, E- AOAC 971.30, B_1- AOAC 942.23, B_2- AOAC 940.33, B_3- AOAC 944.13, B_5- AOAC 960.46, B_6- AOAC 961.15, B_9- AOAC 2004.05

Phytochemical screening of the hexane-ethyl acetate fraction of *Piper nigrum* leaf

Glycoside (General test)

In 1 mL of glacial acetic acid, containing one drop of ferric chloride solution, 1 mL of the extract of *Piper nigrum* was dissolved. This was layered with 1 mL of concentrated H_2SO_4. Formation of a brown ring at the interface indicate the presence of glycoside.

Saponins (Frothing Test)

In a test tube, 0.5 g of extract of *Piper nigrum* was diluted with distilled water and observed for frothing. Saponin Rein weiss (supplied by Merck) was used as standard.

Alkaloids (Picric acid/Wagner's reagents)

1 mL of extract of *Piper nigrum* was transferred into two test tubes labeled A and B.
To test tube A: 2 mL of Wagner's reagents was added. Formation of a reddish-brown precipitate indicate a positive test for alkaloids.
To test tube B: 2 mL of Picric acid was added. The formation of a yellowish precipitate indicates a positive test.

Test for Phenolics compounds (Ferric chloride test)

5 mL of 90% ethanol was added to 1 mL of extract of *Piper nigrum*, followed by the addition of one drop of 10% $FeCl_3$. The formation of a pale yellow colouration indicate a positive test.

Test for Eugenols (KOH/HCl test)

To 5 mL of 5% KOH solution, 2 mL of extract of *Piper nigrum* was added. The aqueous layer was separated and filtered, followed by the addition of few drops of dilute HCl to the filtrate. Formation of a pale-yellow precipitate indicate a positive test.

Test for Steroids (Acetic anhydride and H_2SO_4 test)

To 0.5 g of extract of *Piper nigrum* in 2 mL of dilute H_2SO_4, 2 mL of acetic anhydride was added. The presence of steroid requires a colour change from violet to blue or green.

Test for terpenoids (Salkowski test)

A mixture of 5 mL of extract of *Piper nigrum*, 2 mL of chloroform and 3 mL of concentrated H_2SO_4, were carefully added down to the inner side of the wall of the test tube to form a layer. Formation of a reddish brown colouration at the interface is required for the presence of terpenoids.

Test for Flavonoids (Lead acetate test)

2 mL of extract of *Piper nigrum* was boiled in 10 mL of distilled water and filtered. To the filtrate, few drops of 10% of lead acetate solution was added. Formation of a yellowish precipitate indicate a positive result.

Test for Tannins ($FeCl_3$ test)

A mixture of 2 mL of extract of *Piper nigrum* and 10 mL of distilled water was boiled for 5 minutes and then filtered into two halves. A solution of ferric chloride ($FeCl_3$) was added to about 2 drops of the filtrate. For a hydrolysable tannin, formation of a bluish precipitate is required.

Test for Reducing Sugar

Equal volume of Fehling's solution, A and B were boiled for a minute, 1 mL of extract of *Piper nigrum* was added and boiled for 5 minutes. The formation of a brick-red precipitate indicates a positive test.

Fractionation and Isolation of Constituents of *P. Nigrum*

50 g of the methanolic extract were adsorbed onto 10 g of silica gel and subjected to vacuum liquid chromatography (VLC) using silica gel (60-120 mesh) as the stationary phase. Elution was carried out sequentially with 100% n-hexane, and n-hexane: ethyl acetate (1:1, v/v), as the mobile phases. The VLC apparatus consisted of a sintered glass funnel packed with silica gel to approximately three-quarters of its capacity. A vacuum was applied, and the funnel was gently tapped for

10-15 minutes to ensure uniform packing of the stationary phase. A disc of Whatman No. 1 filter paper, cut to fit the funnel, was placed on top of the packed silica gel before the silica gel-adsorbed crude extract was carefully loaded onto the column. Elution was performed successively with the selected solvent systems, and the eluates were collected as separate fractions. Each fraction was concentrated under reduced pressure using a water bath.

RESULTS AND DISCUSSION

Percentage Yield of Extract

Calculations

$$\% \text{ Yield} = \frac{\text{Weight of dried extract x 100}}{\text{Weight of dried plant sample}}$$

$$= \frac{74.0 \text{ x } 100}{2500}$$

$$= 2.96\%$$

Percentage Yield of Hexane-ethyl acetate fraction

$$\% \text{ Yield} = \frac{\text{Weight of dried extract x 100}}{\text{Weight of dried plant sample}}$$

$$= \frac{9.0 \text{ x } 100}{50}$$

$$= 18.0\%$$

Table 1: Vitamin Composition of *Piper Nigrum* Leaf

S/N	VITAMIN	CONCENTRATION (mg/100 g)
1	A	0.019
2	C	15.181
3	D	0.001
4	E	1.355
5	B_1	0.163
6	B_2	0.360
7	B_3	1.250
8	B_5	2.155
9	B_6	0.361
10	B_9	0.025

The analysis of vitamins in the *Piper nigrum* leaf extract showed that it contains a variety of vitamins, including A, D, E, C, and several B vitamins (B_1, B_2, B_3, B_5, B_6, and B_9), although their concentrations varied considerably. Vitamin C was the most abundant at 15.181 mg/100 g, followed by vitamin B_5 at 2.155 mg/100 g, vitamin E at 1.355 mg/100 g, and vitamin B_3 at 1.250 mg/100 g. The other vitamins were present at lower concentrations, with B_6 at 0.361 mg/100 g, B_2 at 0.360 mg/100 g, B_1 at 0.163 mg/100 g, B_9 at 0.025 mg/100 g, vitamin A at 0.019 mg/100 g, and vitamin D at 0.001 mg/100 g. The relatively high level of vitamin C is consistent with previous findings that *P. nigrum* leaves contain appreciable amounts of ascorbic acid. Nwofia *et al*. (2013) investigated nine Nigerian varieties of piper species and reported vitamin C concentrations ranging from 157.50 to 320.20 mg/100 g. The concentration obtained in the present study (15.181 mg/100 g) was notably lower than these reported values. However, this difference should be interpreted with caution because the present study analyzed a leaf extract, whereas Nwofia *et al*. (2013) analyzed the leaves directly. Differences in extraction methods, sample preparation, geographical location, plant variety, environmental conditions, and leaf maturity could contribute to the observed variation. Their results demonstrate that vitamin content can vary within the same species, suggesting that the lower vitamin C concentration observed in the present extract may reflect characteristics of the specific plant material examined rather than generally low vitamin C levels in *P. nigrum* leaves. Peters *et al*. (2022) reported a vitamin C concentration of 150.36 ± 1.26 mg/100 g in *Piper umbellatum* leaves, which is approximately ten times higher than the 15.181 mg/100 g obtained in the present *P. nigrum* extract. As with comparisons among *P. nigrum* accessions, this difference should be interpreted cautiously because the *P. umbellatum* study analyzed prepared leaf material rather than the same extract matrix used in the present study. For the B vitamins, the present extract contained 0.163 mg/100 g of vitamin B_1, which was lower than the approximately 1.14-1.29 mg/100 g reported for Nigerian *P. nigrum* accessions by Nwofia *et al.* (2013). Similarly, the vitamin B_2 concentration obtained in the present study (0.360 mg/100 g) was lower than the approximately 0.62-0.81 mg/100 g reported for *P. nigrum* leaf accessions. In contrast, vitamin B_3 was present at 1.250 mg/100 g, which was higher than the 0.79-0.95 mg/100 g reported by the same authors. These findings indicate a different B-vitamin profile in the present extract, characterized by higher niacin but lower thiamine and riboflavin concentrations (Nwofia et al., 2013). Comparison with *P. umbellatum* further demonstrates differences in B-vitamin composition within the genus. Peters *et al*. (2022) reported concentrations of 0.12, 0.05, and 0.03 mg/100 g for vitamins B_1, B_2, and B_3, respectively, in *P. umbellatum* leaves. In comparison, the present *P. nigrum* extract contained 0.163 mg/100 g of B_1 and 0.360 mg/100 g of B_2, while its B_3 concentration of 1.250 mg/100 g was substantially higher. This variation indicates that the concentrations of individual B vitamins may differ among *Piper* species and may also be influenced by extraction and analytical procedures (Peters et al., 2022). Vitamin B_5 was detected in the present *P. nigrum* leaf extract at 2.155 mg/100 g, considerably higher than the 0.04 mg/100 g reported for *P. umbellatum* leaves by Peters *et al*. (2022). The vitamin E concentration in the present extract was 1.355 mg/100 g, which was relatively close to the 1.20 ± 0.00 mg/100 g reported for *P. umbellatum* leaves by Peters *et al*. (2022). The relatively small difference suggests that the vitamin E concentration in the present *P. nigrum* extract is comparable to that reported for another *Piper* species, although differences in sample preparation and analytical methods should be considered when making direct comparisons. Similarly, vitamin B_6 was detected at 0.361 mg/100 g, which was fairly close to the 0.30 ± 0.01 mg/100 g reported for *P. umbellatum* leaves. In contrast, the vitamin B_9 concentration in the present study (0.025 mg/100 g) was approximately half of the 0.05 ± 0.00 mg/100 g reported for *P. umbellatum* leaves (Peters *et al*., 2022). These findings further demonstrate that individual B-vitamin concentrations can vary considerably among *Piper* species, with some vitamins occurring at relatively similar concentrations and others showing substantial differences. The concentrations of vitamins A and D in the present extract were low, at 0.019

and 0.001 mg/100 g, respectively. Peters *et al*. (2022) reported vitamin A at 38.20 IU/100 g and vitamin D at 0.62 mg/100 g in *P. umbellatum* leaves. When the reported vitamin A value is converted to mg/100 g, the concentration obtained in the present study appears higher. In contrast, the vitamin D concentration reported for *P. umbellatum* was substantially higher than that observed in the present *P. nigrum* extract. These discrepancies may be associated with differences between the species, as well as variations in sample preparation and analytical methods (Peters *et al*., 2022).

Table 2: Phytochemical Screening of Hexane-Ethyl Acetate Leaf Fraction of *Piper nigrum*

S/N	Phytochemical	Leaf
1	Reducing sugars	-
2	Saponins	-
3	Flavonoids	+
4	Phenolic compounds	+
5	Tannins	-
6	Eugenols	+
7	Terpenoids	+
8	Steroids	+
9	Alkaloids	+
10	Glycosides	-

Phenolic compounds were detected in the hexane-ethyl acetate fraction of *Piper nigrum* leaves, consistent with previous studies on *P. nigrum* leaf extracts. Mballa et al. (2021) also identified phenolic compounds in a hydro-ethanolic extract of *P. nigrum* leaves, supporting the occurrence of this class of compounds in the species. The agreement between the present findings and previous research suggests that phenolic constituents may occur in the leaves across different extraction systems. Flavonoids were also detected in the present fraction, in agreement with Mballa et al. (2021), who reported flavonoids in *P. nigrum* leaves. Flavonoids have likewise been reported in the leaves of other *Piper* species, including *P. betle*, indicating that their occurrence is not restricted to *P. nigrum*. In addition, Odeja et al. (2026) reported flavonoid as one of the secondary metabolite found in *P. nigrum*. The positive test for alkaloids is also consistent with the established occurrence of alkaloid constituents in *P. nigrum (Yu et al., 2022)*. Mballa et al. (2021) detected alkaloids in *P. nigrum* leaves, while Yu et al. (2022) identified 36 amide alkaloid constituents in the leaves using UHPLC-MS, further demonstrating the diversity of alkaloid compounds that may occur in the leaf. Terpenoids were also detected in the hexane-ethyl acetate fraction. This differs from the findings of Mballa et al. (2021), who did not detect terpenoids in their hydro-ethanolic extract of *P. nigrum* leaves. However, terpenoids have been reported in the leaves of other *Piper* species, particularly *P. betle*, where various mono- and sesquiterpenes have been identified. The difference between the present findings and those of Mballa et al. (2021) may therefore be related to differences in species, extraction solvent, or fractionation procedures. Steroids were also detected in the present fraction, consistent with reports from other *Piper* species. Biswas et al. (2022) summarized phytochemical investigations of *P. betle* leaves in which steroidal constituents were detected. Similarly, studies of *P. guineense* leaves have reported the presence of steroid compounds, supporting the occurrence of this class of constituents among *Piper* leaves. The positive eugenol test suggests the presence of eugenol-type compounds in the hexane-ethyl acetate fraction. This observation is consistent with reports on *P. betle* leaves, in which eugenol has been identified as an important constituent by GC-MS analysis. Saini et al. (2020) reported eugenol as a major constituent of *P. betle* leaf together with various terpenoids. However, because the present observation was based on a qualitative phytochemical test, it should be interpreted as evidence suggestive of eugenol-type compounds rather than definitive identification of eugenol itself.

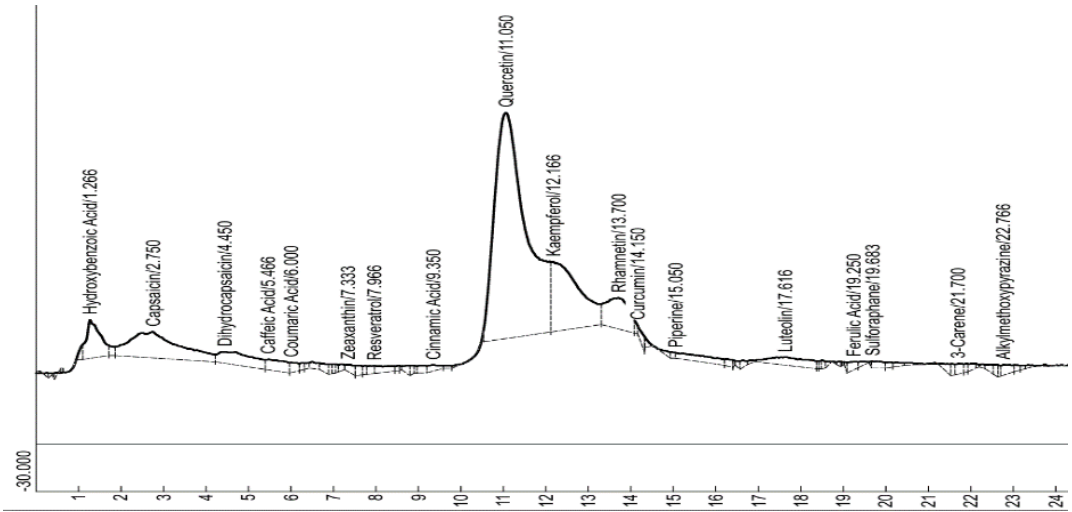

Figure 1: HPLC chromatogram of Hexane-Ethyl acetate leaf fraction of Piper nigrum

Table 3: HPLC Profile of the Hexane-Ethyl Acetate Fraction Leaves

Peak number	Retention time (mins)	Name of compound	Peak area	Area percent (%)
1	1.266	Hydroxybenzoic acid	632.2590	3.98
2	2.750	Capsaicin	1799.2280	11.33
3	4.450	Dihydrocapsaicin	547.9835	3.45
4	5.466	Caffeic acid	251.0105	1.58
5	6.000	Coumaric acid	83.7060	0.53
6	6.483	Capsanthin	109.0920	0.69
7	7.233	Zeaxanthin	117.7700	0.74
8	7.966	Resveratrol	200.1650	1.26
9	9.350	Cinnamic acid	115.0030	0.72
10	11.050	Quercetin	7383.9090	46.51
11	12.166	Kaempferol	2219.9090	13.98
12	13.700	Rhamnetin	846.6320	5.33
13	14.350	Curcumin	75.2130	0.47
14	15.050	Piperine	444.9730	2.80
15	17.616	Luteolin	643.3910	4.05
16	19.250	Ferulic acid	85.1520	0.54
17	20.500	Sulforaphane	140.3680	0.88
18	22.766	Alkylmethoxypyrazine	105.7090	0.67
		Total	15875.7880	100

Eighteen chemical constituents were tentatively assigned by HPLC analysis. The HPLC chromatographic profile of the *Piper nigrum* sample revealed several detectable peaks corresponding to different retention times. Piperine was assessed by comparison with an authentic piperine reference standard. Other peaks were tentatively assigned based primarily on chromatographic retention-time comparison. Prominent peaks were tentatively associated with flavonoid constituents, including quercetin and kaempferol; however, the apparent partial overlap of these peaks limits the confidence of their individual peak-area estimates. The relative integrated peak areas for the peaks assigned to quercetin and kaempferol were approximately 46.51% and 13.98%, respectively. These values represent chromatographic area percentages and should not be interpreted as definitive concentrations. Other compound assignments, including capsaicin, capsanthin, curcumin and sulforaphane, were not considered definitive because independent reference-standard or LC-MS/MS confirmation was unavailable. In the *Piper nigrum* leaf fraction, quercetin was considerably more abundant at 46.51% compared with kaempferol at 13.98%, giving a quercetin-to-kaempferol ratio of approximately 3.33:1. The higher abundance of quercetin could contribute significantly to the biological activity of the fraction, as both quercetin and kaempferol are known to possess antioxidant and anti-inflammatory properties, although their effects may vary depending on the specific biological target. These flavonols have been reported to influence inflammatory mediators and signaling pathways through different mechanisms (Tian *et al*., 2021; Iyekowa *et al*., 2024). Therefore, the relatively high proportion of quercetin may contribute to the antioxidant and anti-inflammatory potential of the fraction. However, the observed 3.33:1 quercetin-to-kaempferol ratio should not be regarded as a definitive therapeutic ratio, since the present study did not directly evaluate the biological effects of this specific quercetin/kaempferol combination. Experimental evidence indicates that combinations of flavonoids may produce synergistic effects, suggesting that the biological activity of the fraction may depend not only on the predominance of quercetin but also on the combined presence of both flavonols (Banjarnahor and Artanti, 2014). Therefore, further biological and dose-response studies are required to establish the therapeutic significance of the quercetin-to-kaempferol ratio observed in the present study.

A limitation of the HPLC analysis was the incomplete availability of the original instrumental documentation and raw chromatographic data. Consequently, complete verification of all chromatographic operating parameters and independent confirmation of every compound assignment was not possible. In particular, compounds assigned solely on the basis of retention-time comparison should be regarded as tentative. Definitive confirmation would require analysis against authentic reference standards under identical conditions and/or complementary techniques such as LC-MS/MS. Similarly, the relative peak-area estimates for partially overlapping quercetin- and kaempferol-associated peaks should be interpreted cautiously.

CONCLUSION

This study revealed that the leaves of *Piper nigrum* contain a diverse range of phytochemical constituents, including alkaloids, phenolics, eugenols, steroids, terpenoids, and flavonoids. The leaves also contained notable amounts of vitamins A (0.019 mg/100 g), D (0.001 mg/100 g), E (1.355 mg/100 g), B_3 (1.250 mg/100 g), B_5 (2.155 mg/100 g), and B_6 (0.361 mg/100 g). HPLC analysis of the hexane-ethyl acetate fraction revealed several chromatographic components, with quercetin as a prominent constituent and piperine detected at 1.99%. These findings demonstrate the chemical diversity of *P. nigrum* leaves and provide a basis for further investigation of their nutritional and medicinal potential. However, the phytochemical screening was qualitative, while the HPLC findings require further validation and quantitative assessment using authentic reference standards. Future studies should therefore focus on the isolation and structural characterization of the major constituents, validated quantitative analysis, investigation of their biological activities, and evaluation of their safety and pharmacokinetic properties before any conclusions are drawn regarding their potential application in drug development.

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