Research Article

Comparative Study of Anti-nociceptive Activity and Phenolic Content of the Ethanol Extracts of *Piper nigrum* and *Piper longum* Fruits

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ABSTRACT

The research was carried out for investigation of anti-nociceptive properties, and phenolic compound determination by HPLC-DAD method of the ethanol extracts of *P. nigrum* of two varieties (black and white pepper) and *P. longum* of two different biomes (local and foreign). The anti-nociceptive activity of the extracts was studied at different doses (250, 500 mg/kg body weight) using acetic acid induced writhing in mice and the phenolic compounds were analyzed by reverse phase HPLC method. For the evaluation of anti-nociceptive activity, the white pepper extract showed significant inhibitory effect ($P<0.001$) in comparison to the other extracts. At the dose 500 mg/kg body weight, the white pepper extract showed a significant anti-nociceptive activity in acetic acid induced writhing in mice showing 40.15% inhibition comparable to that produced by the standard drug, Diclofenac Na (49.25%). The HPLC results showed that black pepper extract contained a significant amount of catechin (114.68 mg per 100 g of dry weight) compared to white pepper and long pepper (local and foreign) (14.92, 8.51, and 26.87 mg per 100 g of dry extract, respectively). In addition, *p*-coumaric acid, rutin hydrate, and kaempferol were also determined in the extracts (black pepper-2.03, 6.82, 11.33 mg; white pepper-3.73, 12.63, 12.07 mg; long pepper local-2.17, 3.97, 14.04 mg; long pepper foreign-2.38, 5.36, 21.84 mg, respectively) in all the extracts. Therefore, the results tend to suggest that all the extracts of *P. nigrum* and *P. longum* exhibited anti-nociceptive properties and have polyphenolic compounds that might be responsible for this *in vivo* biological activity and justify its wide use in folkloric remedies.

Keywords: Acetic acid writhing model, Catechin, HPLC, Kaemferol, Rutin hydrate.

INTRODUCTION

Piper nigrum, a well-known climbing vine native to southern India and Srilanka and is cultivated everywhere in the tropical regions especially for its fruits, which are used as spices. *Piper nigrum*, known as black Pepper or in many cases white pepper, is produced from the still-green fruits of the pepper plants. Once dried, the spice is called black pepper. White pepper, however, consists of the seed of the pepper plant alone, with the darker-colored skin of the pepper fruit removed. It has a different flavor than black pepper, due to the lack of certain compounds present in the outer fruit layer of the drupe, but not found in the seed. It has anti-microbial and anti-mutagenic properties.[1,2] Inhalation of black pepper oil also increases the reflexive swallowing movement.[3]

Piper longum, known, as Long pepper is one of the most widely used Ayurvedic herbs aboriginal to northeastern and southern parts of India and Srilanka. It is used for the treatment of respiratory tract diseases like cough, bronchitis, asthma, cold, as counter-irritant and analgesic.

The genus *Piper* has high amount of phenolic compounds, being the most important the flavonoid and lignin classes.[4,5] The hydroxyl groups of phenolics are of vital importance for their biological activity: they neutralize free radicals and donate hydrogen electrons, causes metal ions to chelate in aqueous solutions and interact with proteins resulting in protein precipitation.[6-10] Antioxidant activity of phenolics offers multiple health benefits. They are capable of limiting the radicals generated by cellular metabolic activity and thus, reducing oxidative damages.[11] Flavonoids, on the other hand, are able to adhere to monocytes in the inflammatory process of atherosclerosis.[12,13] Polyphenol constituents reduce the susceptibility of low-density lipoprotein to oxidation, increasing serum antioxidant capacity.[14]

The major chemical constituents of the plants are volatile oil, resin and alkaloids viz. piperine, piperlongumine, piperlonguminine, pipperin, pippalartin, piplartine etc. It has polyphenols, which includes flavanols, flavonoids, flavandiols and phenolic acids and amides including N-isobutyleicosa-2,4-dienamide, N-Isobutyleicosa-2,4,14-trienamide, N-Isobutylocatadeca-2,4,14-trienamide, guineensine etc.[15]

Thus, in this experiment, we attempted a comparative study between the ethanol extracts of *P. nigrum* and *P. longum* fruits to investigate the anti-nociceptive activity and phenolic content through HPLC to confirm their use in folk remedy for pain, wound and other pathological conditions where free radicals are implicated.

MATERIALS AND METHODS

Plant materials

Fruits of *P. nigrum* (black and white pepper) and *P. longum* (local and foreign-Indian origin) were collected

from local market of Dhaka, Bangladesh during April 2013, and identified by the experts of BCSIR (Bangladesh Council of Scientific and Industrial Research).

Sample preparation and extraction

The fruits of *P.nigrum* and *P.longum* were coarsely powdered using a grinder. Around 25 g of each of the powdered samples were soaked in 95% ethanol (250 ml) separately. They were shaken for 24 hours using an orbital shaker (VRN-480, Gemmy, Taiwan). The solutions were then filtered through whatman filter paper (Bibby RE200, Sterilin Ltd., UK) with the help of a vacuum pump. Finally, the filtrates were concentrated separately by a rotary vacuum evaporator (R-215, Buchi, Switzerland) at low temperature and freeze dried. Then the ethanol extracts of *P. nigrum* (black pepper, BP) (2.95 g) and *P. nigrum* (white pepper, WP) (2.73 g); *P. longum* (local, LPL) (2.42 g); *P. longum* (foreign, LPF) (2.58 g) were obtained respectively and stored in a cool place for further research.

Test animals & drug

Young Swiss-albino mice either sex, 3-4 weeks of age, weighing 20 - 25 g, were used for in vivo pharmacological screening. Mice were purchased from the Animal Research Branch of the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR,B). They were housed in standard environmental conditions at pharmacology laboratories, pharmacy discipline, Khulna University and fed with rodent diet and water ad libitum. Experimental protocols were in compliance with pharmacy discipline, Khulna University ethics committee on Research in animals as well as internationally accepted principles for laboratory animal use and care. The standard drug Diclofenac Na was used for this study and purchased from Square Pharmaceuticals Ltd, Bangladesh.

Antinociceptive activity

The antinociceptive activity of the crude ethanolic extracts of *P. longum* and *P. nigrum* were studied using acetic acid induced writhing model in mice.[16,17] The animals were divided into control, positive control and test groups with five mice in each group. The animals of test groups received test substance at the dose of 250 and 500 mg/kg body weight. Positive control group was administered with Diclofenac Na (standard drug) at the dose of 25 mg/kg body weight and vehicle control group was treated with 1% tween 80 in water at the dose of 10 ml/kg body weight. Test samples, standard drug and control vehicle were administered orally 30 min before intraperitonial administration of 0.7% acetic acid. After an interval of 15 min, the mice were observed writhing (constriction of abdomen, turning of trunk and extension of hind legs) for 5 min.

Chemicals

Gallic acid (GA), (+)-catechin hydrate (CH), vanillic acid (VA), caffeic acid (CA), (-)-epicatechin (EC), *p*-coumaric acid (PCA), rutin hydrate (RH), ellagic acid (EA), myricetin (MC), kaempferol (KF), and quercetin (QU) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), and ethanol was obtained from Merck (Darmstadt, Germany).

HPLC system

Chromatographic analyses were carried out on a Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) systems (Thermo Fisher Scientific Inc., MA, USA), coupled to a quaternary rapid separation pump (LPG-3400RS), Ultimate 3000RS autosamplier (WPS-3000) and rapid separation diode array detector (DAD-3000RS). Phenolic compounds were separated on a Acclaim® C18 (4.6 x 250 mm; 5µm) column (Dionix, USA) which was controlled at 30°C using a temperature controlled column compartment (TCC-3000). Data acquisition, peak integration, and calibrations were performed with Dionix Chromeleon software (Version 6.80 RS 10).

Chromatographic conditions

The phenolic composition of the ethanol extracts of BP, WP, LPL, and LPF were determined by HPLC, as described before.[18,19] Acetonitrile (solvent A), acetic acid solution pH 3.0 (solvent B), and methanol (solvent C) was used to make the mobile phase. The gradient elution program of the system was: 0 min, 5%A/95%B; 10 min, 10%A/80%B/10%C; 20 min, 20%A/60%B/20%C and 30min, 100% A. For equilibration of the column, a 5 min post run was set-up at initial conditions. A constant flow rate was maintained (1 ml/min) and the injection volume used was 20 µl. For UV detection, the wavelength program was optimized to monitor phenolic compounds at their respective maximum absorbance wavelengths as follows: λ 280 nm held for 18.0 min before switching to λ 320 nm where it was held for 6 min, and finally to λ 380 nm, which was kept constant for the rest of the analysis. The diode array detector was set at an acquisition range of 200 nm to 700 nm. The detection and quantification of GA, CH, VA, CA, and EC was done at 280 nm, PCA, RH, and EA at 320 nm, and MC, QU, and KF at 380 nm, respectively.

Standard and sample preparation

Stock solutions (100µg/ml) for each of the phenolic compounds were prepared by weighing out about 0.0050 g of the analyte into 50 ml methanol. The mixed standard solution was prepared by dilution of the standard stock solutions in methanol to give a concentration of 5 µg/ml for all the polyphenols excluding (+)-catechin hydrate, caffeic acid, rutin hydrate (4 µg/ml) and quercetin (3 µg/ml). All standard solutions were stored in the dark at 5°C and were stable for at least three months.

The calibration curves of the standards were made by dilution of the stock standards (five set of standard dilutions) with methanol to yield 1.0 - 5.0 µg/ml for GA, CH, VA, EC, PCA, EA, MC, KF; 0.5 - 4.0 µg/ml for CH, CA, RH, and 0.25 - 3.0 µg/ml for QU. The calibration curves

were constructed from chromatograms as peak area vs. concentration of standard.

A solution of BP, WP, LPL and LPF at a concentration of 5 mg/ml was prepared in ethanol by mixing with a vortex (Branson, USA) for 30 min. The samples were stored in the dark at low temperature (5°C). Spiking the sample solutions with phenolic standards was done for additional identification of individual polyphenols.

Prior to HPLC analysis, all solutions (mixed standards, sample, and spiked solutions were filtered through 0.20 µm nylon syringe filter (Sartorius, Germany) and then degassed in an ultrasonic bath (Hwashin, Korea) for 15 min.

Peak characterization and quantification

The compounds were identified by comparing with the known standard compounds using the retention time, absorbance spectrum profile and by running the samples after the addition of the known standards. The compounds were quantified by establishing calibration curves for each with the help of the known standards. Linear calibration curves for standards (peak area vs. concentration) were constructed with R^2 exceeding 0.995. Data are reported as mean ± standard deviation of triplicate independent analysis.

Statistical Analysis

Data were presented as mean ± standard deviation (S.D). Statistical analysis for animal experiment was carried out using one-way ANOVA followed by Dunnet's multiple comparisons. The results obtained were compared with the control group. *p* values < 0.05 were considered to be statistically significant (*p* indicates probability).

RESULTS

Analgesic activity

Table 1 showed the comparative anti-nociceptive activity of the different ethanolic extracts of *P. nigrum* (black and white pepper) and *P. longum* (local and foreign) on acetic acid induced writhing in mice. Among the extracts, the white pepper extract showed significant inhibitory effect, while the rest (black pepper, long pepper local and long pepper foreign) exhibited moderate inhibition (32.58, 34.09, 38.64%), respectively. White pepper extract (500 mg/kg) produced the greatest effect with 40.15% inhibition ($P < 0.01$) in comparison to the activity of the positive control, Diclofenac Na (49.25% inhibition).

Table 1: Effects of the ethanolic extract of BP, WP, LPL and LPF on acetic acid induced writhing of mice (n=5)

Treatment	Dose (mg/kg; p.o.)	Number of writhes (% Writhing)	% Writhing Inhibition
Control (1% tween 80 solution)	10 ml/kg	13.20 ± 0.74 (100)	---
Diclofenac Na	25	6.70 ± 0.49* (50.75)	49.25
Et. Extract of BP	250	10.30 ± 0.83* (78.03)	21.97
	500	8.90 ± 0.61** (67.42)	32.58
Et. Extract of WP	250	10.10 ± 0.91* (76.52)	23.48
	500	7.90 ± 0.75** (59.85)	40.15
Et. Extract of LPL	250	10.60 ± 0.72* (80.30)	29.70
	500	8.70 ± 0.64** (65.91)	34.09
Et. Extract of LPF	250	9.70 ± 0.53* (73.48)	26.52
	500	8.10 ± 0.68** (61.36)	38.64

Values are expressed as mean ± SEM (Standard Error Mean); Et.: Ethanolic; * indicates P < 0.01; ** indicates P < 0.001, one-way ANOVA followed by Dunnet's test as compared to control; n = Number of mice; p.o.: per oral.

Table 2: Parameters of calibration graphs for the nine phenolic standards in this study

Peak no.	Polyphenolic Compound	Linearity range (µg/ml)	Correlation coefficients (r^2)	Detection limit (µg/ml)[a]	Quantitation limit (µg/ml)[a]	Recovery (%)[b]
1	GA	1.0 – 5.0	0.9951	0.20	0.65	97.3 ± 1.99
2	CH	0.5 – 4.0	0.9972	0.10	0.38	97.5 ± 1.81
3	VA	1.0 – 5.0	0.9948	0.21	0.72	96.4 ± 1.04
4	CA	0.5 – 4.0	0.9950	0.14	0.47	97.9 ± 1.02
5	EC	1.0 – 5.0	0.9959	0.28	0.85	98.2 ± 2.84
6	PCA	1.0 – 5.0	0.9982	0.26	0.90	102.9 ± 2.65
7	RH	0.5 – 4.0	0.9976	0.13	0.45	101.3 ± 2.90
8	EA	1.0 – 5.0	0.9990	0.29	0.92	97.2 ± 2.08
9	MC	1.0 – 5.0	0.9981	0.29	0.92	98.2 ± 3.01
10	QU	0.25 – 3.0	0.9972	0.07	0.24	100.2 ± 3.13
11	KF	1.0 – 5.0	0.9991	0.27	0.86	101.5 ± 3.54

[a]Data were expressed as mean of triplicate measurements; [b]Recovery is expressed as mean ± standard deviation carried out in ethanol extract of *P. nigrum* (Black Pepper).

Table 3: Contents of polyphenolic compounds in the ethanol extract of *P. nigrum* [black pepper, white pepper, long pepper (local & foreign)] (n=3)

Polyphenolic compounds	Content (mg/100 g of dry extract)				% RSD			
	BP	WP	LPL	LPF	BP	WP	LPL	LPF
GA	11.69	ND	ND	3.02	0.68	ND	ND	0.03
CH	ND	9.02	ND	ND	ND	1.02	ND	ND
VA	ND	2.05	ND	ND	ND	0.02	ND	ND
CA	114.68	14.92	8.51	26.87	3.71	1.59	0.08	1.21
EC	ND	ND	16.47	35.63	ND	ND	0.97	1.35
PCA	2.03	3.73	2.17	2.38	0.04	0.05	0.02	0.02
RH	6.82	12.63	3.97	5.36	0.57	1.24	0.05	0.86
EA	24.65	34.31	ND	ND	0.83	2.56	ND	ND
MC	ND	ND	ND	3.51	ND	ND	ND	0.04
QU	ND	1.42	ND	ND	ND	0.03	ND	ND
KF	11.33	12.07	14.04	21.84	0.64	1.08	0.86	1.87

ND: Not Detected

Phenolic content determination of the ethanol extracts by HPLC

Identification and quantification of individual phenolic compounds in the ethanolic extracts of BP, WP, LPL and LPF were analyzed by HPLC. The chromatographic separations of polyphenols in the standard and extracts are shown in table 2, 3 and figure 1, 2, 3, 4 and 5. The phenolic content of each of the compounds was calculated from the corresponding calibration curve and presented as a mean of the five determinations as shown in table 3.

The identity of the peaks in the sample solutions were confirmed by comparing the retention times of the phenolic compounds found in these chromatograms, with that of the retention times of the standards (figure 1). The experimental results indicated that ethanol extract of BP contained an especially high concentration of catechin (114.68 mg per 100 g of dry weight) compared to WP, LPL and LPF (14.92, 8.51, and 26.87 mg per 100 g of dry weight, respectively). The presence of *p*-coumaric acid, rutin hydrate, and kaempferol was also confirmed (BP-2.03, 6.82, 11.33 mg; WP-3.73, 12.63, 12.07 mg; LPL-2.17, 3.97, 14.04 mg; LPF-2.38, 5.36, 21.84 mg, respectively) in all the extracts. Gallic acid was found only in BP and LPF at a low concentration (11.69 mg, and 3.02 mg, respectively) while WP was the only extract showing catechin and vanillic acid (9.02 and 2.05 mg, respectively). Epicatechin, however, was detected only in the LP extracts at a reasonable concentration (LPL-16.47 mg; LPF-35.63 mg, respectively). On the other hand, quercetin was detected in WP (1.42 mg) alone, while myricetin was detected in LPF (3.51 mg). In addition, ellagic acid was present in BP and WP with WP having the greater concentration (24.65, 34.31 mg, respectively). EC was found in LPL and LPF with LPF showing a greater concentration (16.47, 35.63 mg, respectively).

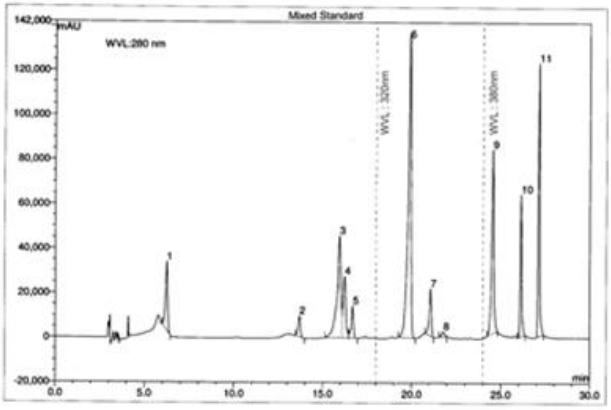

Figure 1: HPLC chromatogram of a standard mixture of polyphenolic compounds. Peaks: 1, gallic acid; 2, (+)-catechin; 3, vanillic acid; 4, caffeic acid; 5, (–)-epicatechin; 6, *p*-coumaric acid; 7, rutin hydrate; 8, ellagic acid; 9, myricetin; 10, quercetin; 11, kaempferol.

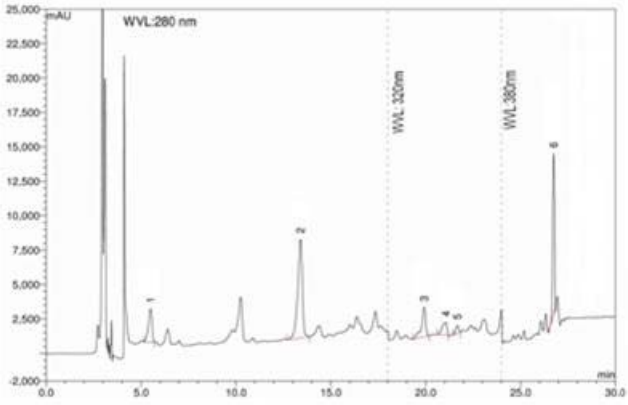

Figure 2: HPLC chromatogram of *P. nigrum* (Black Pepper). Peaks: 1, gallic acid; 2, (+)-catechin; 3, *p*-coumaric acid; 4, rutin hydrate; 5, ellagic acid; 6, kaempferol.

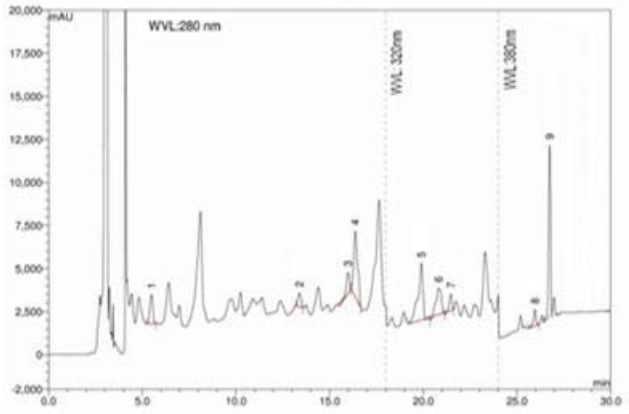

Figure 3: HPLC chromatogram of ethanol extract of *P. nigrum* (white pepper). Peaks: 1, gallic acid; 2, (+)-catechin; 3, vanillic acid; 4, caffeic acid; 5, *p*-coumaric acid; 6, rutin hydrate; 7, ellagic acid; 8, quercetin; 9, kaempferol.

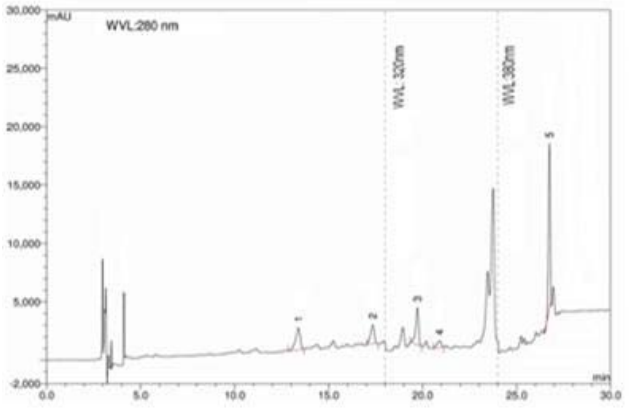

Figure 4: HPLC chromatogram of *P. longum* (local). Peaks: 1, (+)-catechin; 2, (–)epicatechin; 3, *p*-coumaric acid; 4, rutin hydrate; 5, kaempferol.

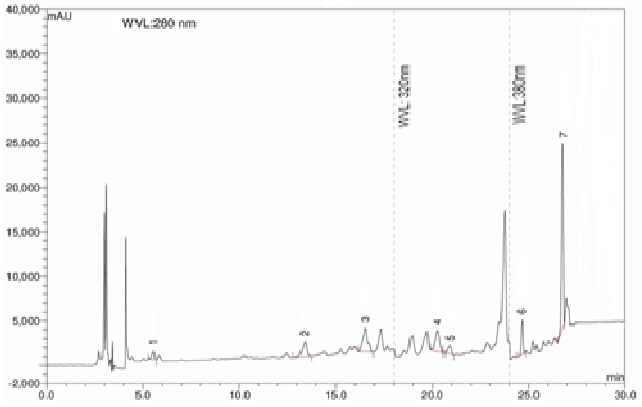

Figure 5: HPLC chromatogram of ethanol extract of *P. longum*. Peaks: 1, gallic acid; 2, (+)-catechin; 3, (–)-epicatechin; 4, *p*-coumaric acid; 5, rutin hydrate; 6, myricetin; 7, kaempferol.

DISCUSSION

Acetic acid induced writhing model in mice was used to evaluate the antinociceptive activity of the ethanolic extracts of *P. nigrum and P. longum* fruits. Percentage inhibitions of cyclooxygenases and lipoxygenases were evaluated to check the peripheral anti-nociceptive activity of the plant extracts, while the central analgesic properties were mediated via the inhibition of central pain receptors.[20] Acetic acid-induced writhing and hot-plate test methods are useful techniques for the evaluation of peripherally- and centrally-acting analgesic drugs, respectively.[21] Deraedt and his coworkers used radioimmunoassay to quantify the prostaglandins by examining the peritoneal exudates of rats obtained after intraperitoneally injecting acetic acid.[22] Results of the acetic acid induced writhing test concluded that the ethanol extract of white pepper exhibited high inhibitory effect, while the rest (black pepper, long pepper local and long pepper foreign) showed moderate inhibition for evaluation of antinociceptive properties, respectively.

HPLC analysis was performed to justify the relationship between antinociceptive activity and the polyphenolics compounds. Catechin, *p*-coumaric acid, rutin hydrate, ellagic acid and kaempferol were determined in the ethanolic extracts (BP, WP, LPL, LPF) through HPLC. Earlier studies have shown that catechin, *p*-coumaric acid, rutin hydrate, ellagic acid and kaempferol encompass effective anti-nociceptive activities.[23-25] In addition, gallic acid, vanillic acid and quercetin were present in some of the plant extracts and these three compounds had shown high anti-nociceptive activity.[26-28]

HPLC is a separation technique that includes a binary solvent system containing acidified water and polar organic solvent developed specifically to find the concentrations of polyphenolic compounds.[29,30] The method was validated using ICH guidelines.[31] Linearity ranges, correlation coefficients, detection limits, quantitation limits, and recovery were determined using standard polyphenolic compounds (Table 1).

Standard mixture solutions of eleven phenolic compounds were tested to determine the linearity between the standard mixture concentrations and peak areas. Linearity was evaluated at five calibration points for standards at the concentrations, 1.0 – 5.0 µg/ml excluding CH, CA, RH (0.5 - 4.0 µg/ml) and QU (0.25 - 3.0 µg/ml) with three measurements per calibration point. All the standard polyphenolic compounds exhibited good linearity over the determined range with correlation coefficients (r^2) between 0.995 and 1.000. Detection and quantitation limits of these standard polyphenols were in the range of 0.07-0.29 and 0.24–0.92 µg/ml, respectively. The recovery tests of all compounds range within 95.0 - 104%.

CONCLUSION

The results of the present study revealed that the ethanol extracts of *P. nigrum* and *P. longum* possess antinociceptive properties with white pepper showing prominent inhibition activities. The antinociceptive effects may be due to the presence of phytoconstituents like flavonoids, phenolics and might be responsible for its activity. The phenolic compounds are of great interest in

pain remedies. HPLC study could be used to identify and quantify the polyphenolic compounds in any medicinal plant extracts. These results also justify the use of *P. nigrum* and *P. longum* fruits in folk medicine to treat pain, swelling and similar health complications. However, a more extensive study would be necessary in determining the exact mechanism for the anti nociceptive properties of the extracts and their active compound(s).

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