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EVALUATION OF TOTAL PHENOLIC, FLAVANOID AND IN VITRO ANTIOXIDANT ACTIVITY OF DIFFERENT EXTRACTS OF *PIPER NIGRUM*

Tahira Foyzun *

Southeast University. Banani, Dhaka, Bangladesh.

Abstract:

The present study was carried out to evaluate the total phenolic, flavanoid and antioxidant activities of different extracts of P.nigrum seeds. The seeds of P.nigrum were extracted with methanol, ethyl acetate, chloroform and n-hexane to obtain crude methanol extract (CME), ethyl acetate extracts (CEE), chloroform extract (CCE) and n-hexane extract (CNE). Phytochemical screening showed total phenolic and flavanoid contents of different extracts. The highest phenolic and flavanoid content was found in CEE (62.88 ±1.4 mg/g GAE) and CCE (53.64 ±1.1 mg/g catechin equivalent) respectively. The antioxidant potential was evaluated in terms of DPPH radical scavenging potential and total antioxidant capacity by specific standard procedures. The DPPH radical scavenging activity of CME and CEE were better than CCE and CNE but less than that of BHT (standard). The IC_{50} value of CME, CEE, CCE and CNE were 57.5, 61, 107 and 162.5 µg/ml respectively compared to the IC_{50} value of standard BHT (3 µg/ml). The total antioxidant activity of CME was higher than the other fraction but less than that of catechin (standard). So, these results suggest the P.nigrum as a moderate medicine against free-radical-associated oxidative damage.

Key words: *phenolics, flavanoids, Piper Nigrum.*

Corresponding author:

Tahira Foyzun,

Lecturer, Southeast University.

Banani, Dhaka, Bangladesh.

Email:taniafoyzun@yahoo.com

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INTRODUCTION:

Oxidative stress is defined as imbalance between oxidants and antioxidants mainly caused by excessive free radical production in our body which are found by various processes involved in biological combustion [1]. Oxidative stress causes damage in all types of biomolecules like protein, nucleic acid, DNA and RNA [2]. Hence the balance between reactive species or free radicals and antioxidants are believed to be critical concept for maintaining a good biological system. Moreover free radicals have been implicated in the development of a number of disorders including cancer, neurodegeneration and inflammation [3, 4, 5] giving rise to studies of antioxidants for the prevention and treatment of diseases. Antioxidants act as free radical scavengers, quenchers of singlet oxygen molecule and when present in low concentrations relative to the oxidizable substrate significantly delays or reduces oxidation of the substrate.

The presence of antioxidants such as phenolics, flavanoids in plants may provide protection against a number of diseases [6]. But the high cost of natural antioxidants has led to the use of synthetic antioxidants. However studies conducted subsequently have demonstrated that synthetic antioxidants have toxic effects. As the plants produce significant amount of antioxidants to prevent the oxidative stress, they represent a potential source of new compounds with antioxidant activity. So researchers have focused their studies on plant derived natural antioxidants [7].

Black pepper is a flowering vein belonging to the family of piperaceae, cultivated for its fruits which is usually dried and used as spice and seasoning. It is considered as the king of spice among various spices and commonly used in Bangladesh. In traditional medicine, black pepper was believed to cure illness such as diarrhea, gangrene, hernia, indigestion, insect bite, liver problem, tooth decay and toothache. Moreover recent studies experimentally revealed that it has antimicrobial [8], antimutagenic [9], antioxidant activity [10] and inhalation of black pepper oil increase the reflexive swallowing movement [11].

In this study, we determined the free radical scavenging activity, total phenolic content and antioxidant level in methanol, ethyl acetate, chloroform and n-hexane extracts of *Piper nigrum* by employing many different in vitro antioxidant assays and compared the activities among the extracts.

MATERIALS AND METHODS:

Collection and Preparation of Plant Sample

The plant parts were collected from the local market of Dhaka city, Bangladesh. Then they were washed thoroughly in tap water and shade dried for several days with occasional sun drying. They were then dried in an oven for 24 hours at considerably low temperature (not more than 45^0C) for better grinding. Dry samples of fruits were ground into a fine powder in a grinding mill. The coarse powder was then stored in an air tight container and kept in cool and dry place for further use.

Extraction and Solvent Evaporation

The powdered plant materials were extracted by cold extraction process. Powdered plant materials were taken in amber colored reagent bottle and soaked in 500mL of methanol, ethyl acetate, chloroform and n-hexane to obtain crude extracts. The bottle with its contents were sealed and kept for period of about 7 days with occasional shaking and stirring. The whole mixture was then filtered through cotton and Whatman no. 1 filter paper. Each of the extracts obtained were dried using a rotary evaporator under pressure at 50^0C temperature to afford crude extract known as crude methanolic extract (CME), crude ethyl acetate extract (CEE), crude chloroform extracts (CCE) and crude n- hexane extract (CNE).

Determination of Total Phenolics

Total phenolic content of different extracts of *Piper nigrum* were determined employing the method as described by Skerget *et al.*, (1965) [12] involving Folin-Ciocalteu reagent as oxidizing agent and gallic acid as standard. Firstly, 0.5 ml of plant extract or standard of different concentration solution was taken in a test tube and 2.5 ml of Folin – ciocalteu (Diluted 10 times with water) reagent solution was added into the test tube. Then 2.5 ml of Sodium carbonate (7.5%) solution was added and incubated for 20 minutes at 25^0C to complete the reaction. Then the absorbance of the solution was measured at 760 nm using a spectrophotometer against blank. A typical blank solution contained all reagents except plant extract or standard solution.

Determination of Total Flavonoids

Total flavonoid content was determined by following the procedure by Dewanto *et al.*, 2002[13]. Catechin was used as standard and the flavonoid content of the extracts were expressed as mg of catechin equivalent/gm of dried extract. Firstly, one milliliter of aqueous extract containing 0.1 g/ml of dry matter was placed in a 10 ml volumetric flask, then 5ml of distilled water added followed by 0.3ml of 5% $NaNO_2$. After 5 minutes, 0.6 ml of 10% $AlCl_3$ was added and volume made up with distilled water. The solution was mixed and absorbance was measured at 510 nm.

Antioxidant Assay

DPPH (1, 1-diphenyl-2-picrylhydrazyl) Radical Scavenging Assay:

The antioxidant activity of different extracts were determined in terms of hydrogen donating ability, using the DPPH method with a minor modification [14]. Firstly, 2 ml of methanol solution of plant extract or standard at different concentration was taken in a test tube. Then 3 ml of methanol solution of DPPH was added into the test tube. The test tube was incubated at room temperature for 30 minutes in dark place to complete the reaction. Then the absorbance of the solution was measured at 517 nm using a spectrophotometer against blank. A typical blank solution contained all reagents except plant extract or standard solution.

Determination of Total Antioxidant Capacity:

Total antioxidant capacity was measured spectrophotometrically through phosphomolybdenum method by Prieto *et al.*, (1999) [15] with some modifications.An aliquot of 0.5 ml of sample solution was combined with 3ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 1% ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95ºc for 10 minutes. After the sample had cooled to room temperature, the absorbance of aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained 3 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions. Catechin, Ascorbic acid, ∞ tochopherol can be used as standard.

RESULT AND DISCUSSION:

The plant part used in this investigation was the seeds of the *P. nigrum.* The dried coarse powder (1kg) was extracted with methanol (250ml), ethyl acetate (250ml), n-haxane (250ml) and chloroforms (250ml) separately and evaporated to dryness at 50^0C under reduced pressure to yield crude methanol extract (8.06gm), crude ethyl acetate extract (8.28gm), crude n-haxane extract (1.65gm) and crude chloroform extract (6.86gm).

Determination of Total Phenolic Content

It was reported that phenolic compounds were associated with antioxidant activity and that they play an important role in stabilizing lipid per oxidation [16]. Phenolic content of the samples were calculated on the basis of the standard curve for gallic acid. The results were expressed as mg of gallic acid equivalent (GAE)/gm of dried extracts. The values were mean of triplicate experiments and represented as mean ± STD.

Table 1: Absorbance of gallic acid at different concentrations after treatment with Folin-Ciocaltue reagent:

Concentration (µg/ml)	Absorbance			Absorbance Mean ± STD
	a	b	c	
1	0.078	0.075	0.076	0.076 ± 0.001
2	0.176	0.171	0.181	0.176 ± 0.005
4	0.364	0.368	0.372	0.368 ± 0.004
8	0.722	0.718	0.726	0.722 ± 0.004
16	1.413	1.417	1.423	1.417 ± 0.005
32	2.758	2.752	2.764	2.758 ± 0.006

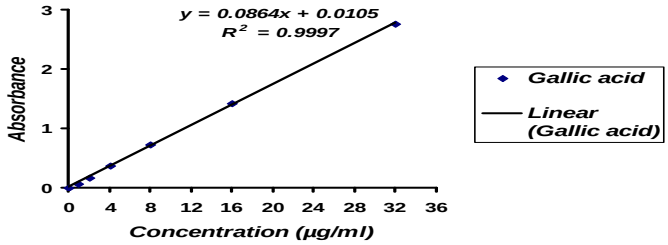

Fig 1: Standard curve of gallic acid for the determination of total phenolic content.

Table 2: Determination of total phenolic content of the crude methanolic extract (CME), crude ethyl acetate extract (CEC), crude chloroform extract (CCE) and crude n-haxane extract (CNE) of *P. nigrum*.

Sample	No. of sample	Concentration (µg/ml)	Absorbance	GAE/gm of dried sample	GAE/gm of dried sample Mean ± STD
Crude methanol Extract (CME)	1.	250	0.432	38.70	38.70±0.745
	2.	250	0.440	39.44	
	3.	250	0.424	37.95	
Crude ethyl acetate extract (CEE)	1.	250	0.692	62.88	62.88±1.420
	2.	250	0.672	61.02	
	3.	250	0.702	64.74	
Crude chloroform extract (CCE)	1.	250	0.574	51.53	52.46±1.160
	2.	250	0.576	52.09	
	3.	250	0.594	53.76	
Crude n-haxane extract (CNE)	1.	250	0.466	41.86	40.50±1.198
	2.	250	0.446	40.00	
	3.	250	0.442	39.62	

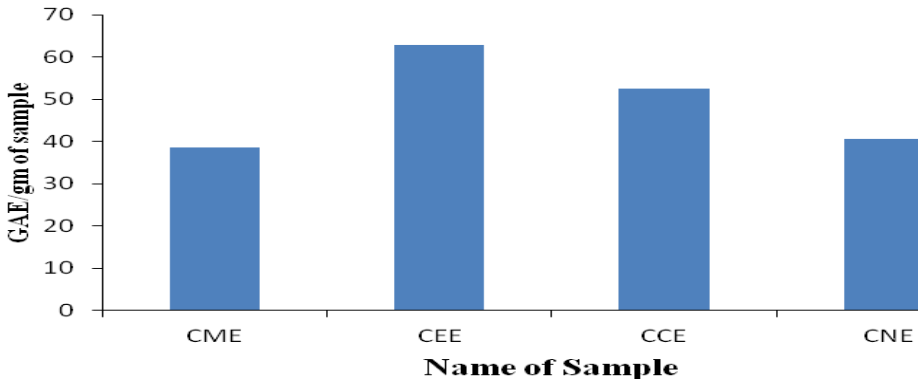

Fig. 2: Total phenolic content (mg/gm plant extract in gallic acid equivalent) of the crude methanolic extract (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE) of *P. nigrum*.

The result of the total phenolic content showed that crude methanolic extract (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE) yielded 38.70±0.745, 62.88±1.420, 52.46±1.160 and 40.50± 1.198 GAE/gm of dried sample respectively. The result demonstrated that the total phenolic content of crude ethyl acetate extract (CEE) is higher than that of crude methanolic extract (CME), crude chloroform extract (CCE) and crude n-haxane extract (CNE).

Determination of Total Flavonoids:

Quantitative determination of total flavanoids was done on the basis of a standard curve of catechin (R2=0.9967). The results were expressed as mg of catechin equivalent per gram of dried sample. The values represented the mean of triplicates ± STD of crude methanolic extract (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE).

The results showed that, total flavonoid content (TFC) of crude methanolic extract (CME), crude ethyl acetate extract (CEE) and crude n-haxane extract (CNE) were 13.674±0.745, 39.564±1.420 and 32.56±1.198 µg of GAE/gm of dried extract respectively, while the total flavonoid content of crude chloroform extract (CCE) was 53.64±1.160 µg of catechin equivalent/ gm of dried extract. These findings demonstrated that the total flavonoid content of crude chloroform extract (CCE) was higher than that of crude methanolic extract (CME), crude ethyl acetate extract (CEE) and crude n-haxane extract (CNE).

Table 3: Absorbance of catechin (standard) at different concentrations for quantitative determination of total flavonoids.

Concentration (µg/ml)	Absorbance			Absorbance Mean ±STD
	a	b	c	
31.25	0.241	0.225	0.260	0.242 ± 0.017
62.5	0.380	0.398	0.362	0.380 ±0.018
125	0.726	0.722	0.731	0.726 ± 0.004
250	1.476	1.481	1.468	1.475 ± 0.006
500	2.667	2.670	2.599	2.645 ± 0.040

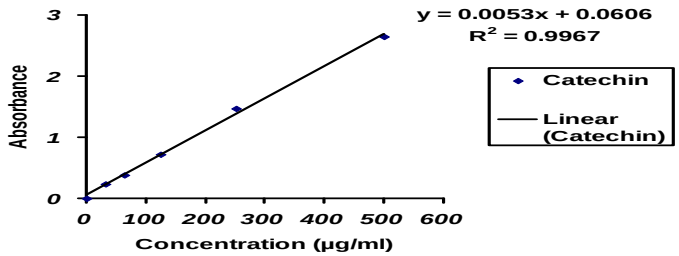

Fig 3: Standard curve of catechin for the determination of total flavonoids.

Table 4: Determination of total flavonoid content of the crude methanol extract (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE) of *P. nigrum*.

Sample	No. of sample	Concentration (µg/ml)	Absorbance	GAE/gm of dried sample	GAE/gm of dried sample Mean ± STD
Crude methanolic extract (CME)	1.	250	0.170	14.33	13.647 ±0.745
	2.	250	0.160	13.40	
	3.	250	0.158	13.21	
Crude ethyl acetate extract (CEE)	1.	250	0.450	40.37	39.564±1.420
	2.	250	0.430	38.51	
	3.	250	0.444	39.81	
Crude chloroform extract (CCE)	1.	250	0.590	53.40	53.64 ±1.160
	2.	250	0.584	52.84	
	3.	250	0.598	54.14	
Crude n-haxane extract (CNE)	1.	250	0.372	33.116	32.56 ±1.198
	2.	250	0.348	30.88	
	3.	250	0.378	33.67	

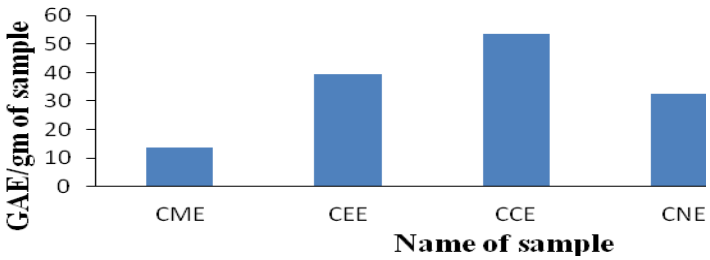

Fig 4: Total flavonoid content (mg/gm plant extract in catechin equivalent) of the crude methanolic extract (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE) of *P. nigrum*.

Total Antioxidant Activity Determination:

The assay was based on the reduction of Mo ((VI) to Mo (V) by the test agents and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. The total antioxidant activity was measured and compared among crude methanolic extract (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE) of *P. nigrum* and the reference standard catechin. The high absorbance values indicated that the sample possessed significant antioxidant activity. The results revealed that all the samples tested had moderate antioxidant activities and the effects increased with increasing concentration (table.5 and figure 3). The absorbance value of Catechin (Standard), crude methanolic extract (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE) at 600 µg/ml were 3.940±0.0011, 2.884±0.045, 2.417±0.058, 1.985±0.0021 and 1.720±0.110 respectively, which demonstrated that the total antioxidant activity of catechin (standard) is higher than that of crude methanolic extract (CME) crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE).

Table 5: Total antioxidant activity of crude methanolic extract (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE) of *P. nigrum* and catechin (standard) at different concentration.

No. of sample	Concentration (µg/ml)	Absorbance			Absorbance Mean±STD
		a	b	c	
Catechin (Standard)	600	4.000	3.823	3.996	3.940±0.0011
	300	3.676	3.680	3.678	3.678±0.0015
	150	3.494	3.486	3.440	3.473±0.005
	75	2.473	2.461	2.471	2.468±0.0005
	37.5	1.613	1.617	1.611	1.614±0.001
	18.75	0.912	0.924	0.908	0.915±0.0006
	9.375	0.426	0.442	0.416	0.425±0.0043
Crude methanolic extract (CME),	600	2.885	2.880	2.887	2.884±0.045
	300	2.000	2.102	2.014	2.039±0.0021
	150	1.835	1.847	1.831	1.838±0.0014
	75	1.756	1.754	1.784	1.753±0.005
	37.5	1.525	1.537	1.543	1.444±0.008
	18.75	1.313	1.327	1.319	1.320±0.087
	9.375	0.627	0.684	0.676	0.677±0.068
Crude ethyl acetate extract (CEE)	600	2.412	2.424	2.416	2.417±0.058
	300	1.507	1.509	1.507	1.508±0.0024
	150	0.940	0.944	0.930	0.938±0.0087
	75	0.854	0.852	0.850	0.852±0.001
	37.5	0.744	0.740	0.742	0.742±0.0012
	18.75	0.678	0.670	0.674	0.674±0.0013
	9.375	0.500	0.496	0.490	0.495±0.0065
Crude chloroform extract (CCE)	600	1.998	1.984	1.992	1.985±0.0021
	300	1.872	1.878	1.864	1.871±0.0011
	150	1.724	1.742	1.718	1.728±0.0014
	75	1.661	1.663	1.657	1.660±0.0026
	37.5	1.284	1.280	1.288	1.284±0.0024
	18.75	0.756	0.763	0.757	0.759±0.0013
	9.375	0.414	0.426	0.418	0.420±.0087
Crude n-haxane extract (CNE)	600	1.711	1.729	1.721	1.720±0.110
	300	1.550	1.565	1.557	1.557±0.0054
	150	1.497	1.483	1.490	1.490±0.06
	75	1.311	1.301	1.222	1.278±0.048
	37.5	1.121	1.212	1.012	1.115±0.1001
	18.75	0.654	0.662	0.658	0.658±0.090
	9.375	0.319	0.311	0313	0.314±0.1182

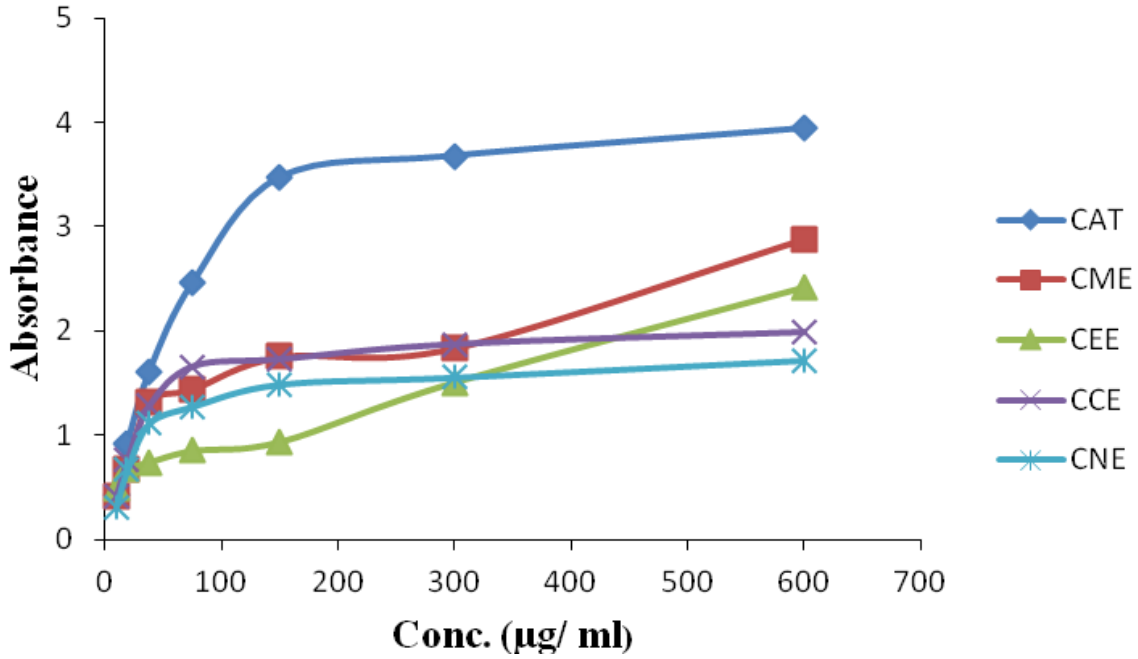

Fig 5: Total antioxidant activity of crude methanolic extract (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE) of *P. nigrum* and catechin (Standard) at different concentration.

DPPH Radical Scavenging Activity:

DPPH antioxidant assay is based on the ability of 1, 1 diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a visible deep purple color [17]. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the change in absorbance and percentage of scavenging activity is calculated. The activity was increased by increasing the concentration of the sample extract.

The antioxidant activity of the crude methanolic extracts (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE) of *P. nigrum* were evaluated by DPPH radical scavenging assay. In 1'-1' diphenyl picryl-hydrazyl radical scavenging assay, anti free radical activity of crude methanolic extract is higher than that of other extract. Fig 4. shows the dose response curve at DPPH radical scavenging activity of crude methanolic extract (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE) compared with a standard antioxidant BHT (standard). At a concentration of 250 (µg/ml) the scavenging activities of crude methanolic extract (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE) were 89.09%, 85.50%, 66.92% and 54.68% respectively while at the same concentration that of the standard BHT was 93.57%. IC_{50} values of crude methanolic extract (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE) were 57.5, 61.5, and 107.5 and 162.5 (µg/ml) respectively compared with the standard BHT with a IC_{50} value of 3 (µg/ml).

Table 6: DPPH radical scavenging activity of the crude methanol extract (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE) of *P. nigrum* and BHT (Standard) at different concentrations.

No. of sample	Concentration (µg/ml)	% of scavanging			% of scavanging Mean± standard	IC_{50} (µg/ml)
		a	b	c		
BHT (Butylated Hydroxy Toluene)	250	93.54	93.64	93.54	93.57±0.023	3
	125	92.40	92.50	91.90	92.27±0.349	
	62.5	88.10	88.09	87.90	88.03±0.112	
	31.25	81.22	82.22	82.20	81.88±0.225	
	15.625	70.94	70.96	70.96	70.95±0.001	
	7.8125	66.70	66.72	66.90	66.74±0.009	
	3.90625	53.40	53.34	53.36	53.37±0.002	
	1.953125	42.46	42.58	42.56	42.54±0.003	
Crude methanolic extract (CME)	250	89.09	89.07	89.11	89.09±0.001	57.5
	125	77.96	77.98	78.02	77.99±0.03	
	62.5	52.93	52.87	53.07	52.96±0.108	
	31.25	26.68	27.08	27.06	26.94±0.225	
	15.625	10.78	10.60	10.96	10.78±0.125	
	7.8125	6.40	6.20	6.32	6.30±0.054	
	3.90625	3.67	3.59	3.57	3.61±0.08	
	1.953125	0.26	0.16	0.36	0.26±0.068	
Crude ethyl acetate extract (CEE)	250	85.55	85.45	85.50	85.50±0.025	61
	125	72.32	72.42	72.52	72.42±0.015	
	62.5	50.62	50.64	50.54	50.60±0.0035	
	31.25	32.95	32.85	32.90	32.90±0.005	
	15.625	22.83	22.87	22.87	22.86±0.0019	
	7.8125	14.68	14.88	14.99	14.85±0.04	
	3.90625	14.32	14.34	14.36	14.34±0.001	
	1.953125	7.42	7.44	7.44	7.44±0.001	
Crude chloroform extract (CCE)	250	66.87	66.97	66.93	66.92±0.023	107.7
	125	53.80	53.90	53.85	53.85±0.002	
	62.5	32.80	32.85	32.85	32.84±0.0017	
	31.25	14.80	14.88	14.86	14.85±0.002.5	
	15.625	7.86	7.80	7.84	7.84±0.003	
	7.8125	5.67	5.63	5.61	5.64±0.002	
	3.90625	3.33	3.27	3.21	3.27±0.01	
	1.953125	0.99	0.90	1.00	0.96±0.002.5	
Crude n-haxane extract (CNE)	250	54.73	54.63	54.67	54.68±0.008	162.5
	125	47.31	47.37	47.33	47.48±0.002	
	62.5	43.40	43.38	43.44	43.40±0.001	
	31.25	41.90	41.92	41.98	41.94±0.001	
	15.625	36.70	36.66	36.80	36.72±0.003	
	7.8125	17.77	17.79	17.73	17.76±0.002	
	3.90625	5.48	5.44	5.44	5.45±0.005	
	1.953125	0.88	0.72	0.68	0.76±0.005	

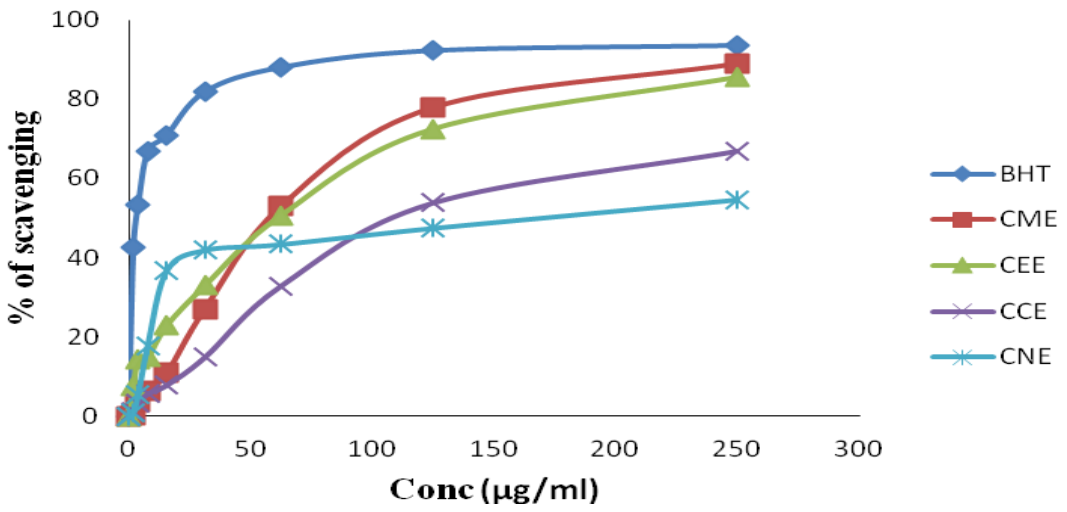

Fig 6: DPPH radical scavenging activity of crude methanol extract (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE) of *P. nigrum* and BHT (Standard).

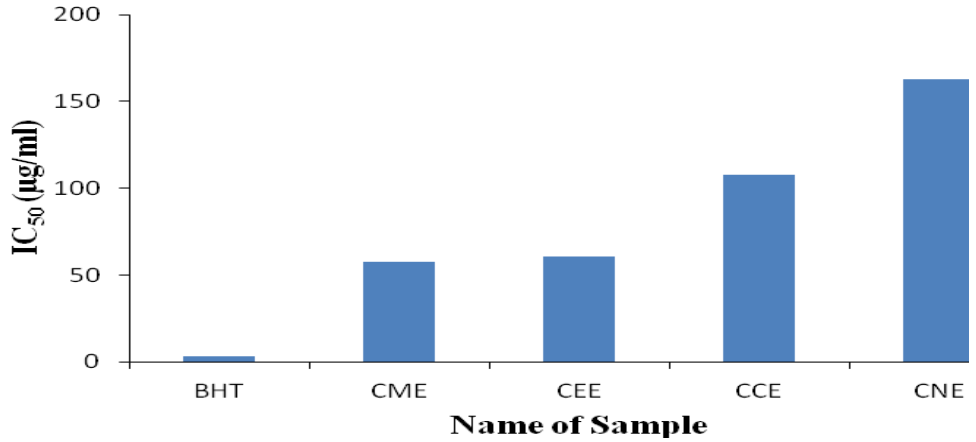

Fig 7: IC_{50} (µg/ml) values of crude methanol extract (CME), crude ethyl acetate extract (CEE), crude chloroform extract (CCE) and crude n-haxane extract (CNE) of *P. nigrum* and BHT (Standard) for DPPH radical scavenging activity.

CONCLUSION:

The study affirms the total phenolic, flavanoid and in vitro antioxidant potential of different extracts of *P. nigrum* seeds. To my knowledge, this is the first report demonstrating the comparison of antioxidant activity among the different extracts of *P.nigrum*. All the extracts show moderate antioxidant activity. But further studies are required to clarify the in vivo potential of this plant.

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