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Bio-efficacy of four medicinal plant extracts and a synthetic compound: A comparative study

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Abstract
In the present study bio-efficacy of four different medicinal plant (Kalonji, betel leaves, flax seeds and miswak) and a synthetic compound p-bromo hydroxy benzophenone derivative was carried out. In the *in-vitro* antithrombolytic and thrombolytic study hexane extract of miswak exhibited significant activity with reference to stranded drug streptokinase and the a synthetic compound p-bromo hydroxy benzophenone derivative, further antioxidant activity result revealed that methanol extraction of flax seeds has highest radical scavenging activity against DPPH, NO and LPO and methanol extract of miswak have exhibited highest H_2O_2 radical scavenging activity as compared to the standard, ascorbic acid.

Keywords: Medicinal plant, Benzophenone, Antithrombolytic, Thrombolytic, Antioxidant

1. Introduction

Thrombosis is one of the peril factors of cardiovascular disease such as pulmonary emboli, strokes, deep vein thrombosis and heart attacks which lead to morbidity and mortality in many countries [1, 2]. A thrombus is the formation of a blood clot in the circulatory system which starts with platelet aggregation due to the failure of haemostasis which causes vascular blockage and it escort to a serious consequence in thrombotic diseases such as cerebral infarction, fatal significances and acute myocardial which may lead to death [3-5]. The first drug used in the anticoagulation therapy for the treatment for cerebral venous sinus thrombosis is Intravenous heparin (A linear polysaccharide with the highest charge density) because of it is safe, effective and feasible [6]. Some commonly used thrombolytic drugs to dissolve clots are alteplase, anistreplase, streptokinase, urokinase and tissue plasminogen activator (tPA) [7].

On other hand, free radical (Pro-oxidants) is a molecular species capable of independent existence that contains an unpaired electron in its atomic orbital. Oxidative stress is linked to enlarged initiation of free radicals on other hand due to decrease of antioxidant concentration [8]. The presence of an unpaired electron results in certain common properties that are shared by most radicals. Many radicals behave oxidant by either donate an electron to or accept an electron from other molecules and sometime excessive ROS are produced [9]. Up regulated of ROS levels lead to "non-specific" damage of macromolecules such as carbohydrate, proteins, DNA and lipids [10, 11]. Current many researchers have confirmed that many diseases including cancers, diabetes, cataract, arteriosclerosis, cardiovascular diseases, Alzheimer's disease, arthritis and Parkinson's disease are highly correlated with free radicals and cellular redox imbalance [12].

Since ancient times, herbal plants are considered as a healthy resource of life [13]. Herbs have been used for the treatment of many diseases as often perceived as safe because they are "natural" with no side effect [14, 15]. Nowadays people are being attack with various diseases because unhealthy food products and considerable efforts have been directed towards the discovery and development of natural products (Herbal medicines) from various plant and animal sources which have anticancer [16], antiplatelet [17, 18], antioxidant [19] anticoagulant [20-22], antithrombotic and thrombolytic activities [23] etc. in many developing

countries [24]. With this view and literature survey, in our present work we studied antioxidant, antithrombotic and thrombolytic activities with frequent comparison to different four popular medicinal plants (Table 1) products extract (Non polar and polar) and a synthesized compound p-bromo hydroxy benzophenone.

Table 1

	Nigella sativa (Kalonji seeds)	Piper betel (Betel leaves)	Linum usitatissimum (Flax seeds)	Salvadora persica (Miswak)
Kingdom	Plantae	Plantae	Plantae	Plantae
Clade	Angiosperms	Angiosperms	Angiosperms	Angiosperms
Sub clade	Eudicots	Magnoliids	Eudicots	Eudicots
Order	Ranunculales	Piperales	Manpighiales	Brassicales
Family	Ranunculaceae	Piperaceae	Linaceae	Salvadoraceae
Genus	Nigella	Piper	Linum	Salvadora
Species	N.sativa	P.betle	L.usitatissimum	S.persica

2. Results and discussion

2.1 Antithrombolytic and thrombolytic activity

From the long back history, the treatment of various disease is depended on plant extract and now a days plant pharmacological activity investigation created a new era in plant derivative drugs discovery. This leads to effective remedial treatment of many diseases which changed attention towards herbal medicines. It is expected that about 30% of pharmaceuticals drugs are from plants extract derivatives [25, 26]. In our present work *in-vitro* preliminary clot lysis test were confirmed of popular medicinal plants like kalonji seeds, betel leaves, flax seeds and miswak products extract (Hexane: Non polar solvent and Methanol: Polar solvent) and a synthesized compound, p-bromo hydroxy benzophenone which showed good the antithrombolytic (Extract added before clot formation) and thrombolytic activity (Extract added just after clot formation) and Streptokinase was used as standard. The maximum clotlysis activity was frequently observed in hexane extract of miswake for the concentration 500 µg/dl, that means the hexane soluble compounds are mainly responsible for the antithrombolytic which take more than 30 minutes for delay in clot formation of blood. On other hand the same hexane extracts also showed thrombolytic activities which take 58 minutes for Clot dissolution for the concentration of 500 µg/dl as compared to the standard streptokinase which take 60-minute Clot dissolution for the same concentration. In addition, this finding help to indicate the possibility of developing novel antithrombolytic and thrombolytic compounds from hexane extract of miswak. Further needed to study and quantify the number and percentage of chemical constituents present in the hexane extract of miswak plant also help to isolate, characterize the compounds responsible for antithrombolytic and thrombolytic activity. In near future it may be implemented as a drug for the improvement of patients suffering from atherothrombotic diseases.

2.2 Antioxidant activity

The investigation of antioxidant screening revealed that some of the tested compounds showed moderate to good antioxidant activity. DPPH radical scavenging activity evaluation is a rapid and convenient technique for screening the antioxidant activities of the antioxidants.

Free radicals play a very important role in the pathogenesis of various human diseases and aging. In food products free radicals also cause damage, resulting in diminished taste and shelf life. Antioxidants are therefore protecting us against free radicals and thus save our health. The aim of our study was to explore the most potent antioxidant and examine the factors that give a picture and establish the antioxidant activity with frequent comparison to four medicinal plant extract and a synthesized compound.

All the polar and non-polar extract of medicinal plant and synthesized compound were screened for their *in-vitro* antioxidant activity by various methods such as 1,1-diphenylpicrylhydrazyl (DPPH), nitric oxide (NO), hydrogen peroxide (H_2O_2) and LPO assay which are summarized in the Tables 2-5 respectively. The investigation of antioxidant screening revealed that some of the extract showed moderate to good antioxidant activity. DPPH radical scavenging activity evaluation is a rapid and convenient technique for screening the antioxidant activities of the antioxidants and the result. The values of IC_{50}, the effective concentration at which 50% of the radicals were scavenged, were tested to evaluated the antioxidant activities. Generally, a lower IC_{50} value demonstrated greater antioxidant activity and IC_{50} values of less than 10 mg/mL usually indicated potent activities in antioxidant properties.

Methanol extract of flax seeds have shown the best antioxidant activity with IC_{50} values of 15.11 ± 0.24, 14.11 ± 0.71 and 16.82 ± 0.7 µg/mL as compared to standard ascorbic acid with IC_{50} values 13.42 ±0.38, 12.95±0.51 and 14.67±0.27 in 1,1-diphenylpicrylhydrazyl (DPPH), nitric oxide (NO) and LPO assay respectively. Further hydrogen peroxide (H_2O_2) activity method have little different scavenging activity as in this methanol extract of miswak showed good antioxidant scavenging with IC_{50} value 16.79±0.91 µg/mL as compared to standard ascorbic acid with IC_{50} values 14.45±0. Mainly, Methanol extract of flax seeds have shown the best antioxidant activity µg/mL respectively, while others showed moderate to mild radical scavenging activity in DPPH, NO, H_2O_2 and LPO methods Tables 2-5.

Table 2: The *in-vitro* antioxidant activity in DPPH method

Compounds	Conc. (µg/ml)				
	25	50	75	100	IC_{50}
KH	67.57 ± 1.08	69.36 ±0.75	74.24± 1.27	79.35 ±1.00	16.49 ± 1.01
KM	68.68 ± 0.21	73.67 ±0.38	75.65 ±0.61	79.23 ±0.61	16.11 ± 0.51
BH	52.87 ±1.74	58.57± 1.11	61.75± 0.98	65.96 ±1.59	23.91 ± 1.48
BM	55.39 ± 0.94	57.89 ±1.44	60.99 ±0.83	66.77 ±1.16	25.66 ± 0.48
FH	71.44±0.31	74.45 ±0.52	76.23±0.61	81.64±0.81	16.32 ± 0.48
FM	74.64± 0.22	77.36 ±0.41	81. 68±0.51	83.79 ±0.69	15.11 ± 0.24
MH	62.78 ± 1.39	66.56 ±1.15	71.45± 1.51	75.45 ±0.89	17.75 ± 1.09
MM	71. 46 ±0.13	75.5 ± 0.38	77.36 ±0.40	81.45± 0.62	15.24 ± 0.54
p-BHB	57.35 ± 1.11	62.46 ±1.18	66.25 ±0.81	70.36 ±0.88	18.75± 0.71
Ascorbic acid	81.81±0.08	82.34±0.33	84.45±0.39	86.26±0.45	13.42 ±0.38
Blank	-	-	-	-	-

(-) Showed no scavenging activity. Values were the means of three replicates ± SD.

Table 3: The *in-vitro* antioxidant activity in nitric oxide (NO) method

Compounds	Conc. (µg/ml)				
	25	50	75	100	IC_{50}
KH	69.48±0.81	72.36 ± 1.01	75.67±1.33	80.57 ± 1.21	15.58 ± 0.89
KM	69.46±0.11	74.78 ± 0.27	79.36 ±0.46	81.26 ± 0.62	16.21 ± 0.84
BH	60.86±1.56	63.57 ± 1.22	67.85± 1.32	72.68 ± 0.73	20.36 ± 0.97
BM	62.86±1.59	63.68 ± 1.28	69.86 ± 1.39	72.68±0.77	21.78 ± 0.99
FH	71.67±0.21	76.58 ± 0.38	80.75±0.59	82.75 ± 0.71	15.79 ±0.81
FM	76.78±0.22	81.58 ± 0.41	82.46 ± 0.53	84.68±0.72	14.11 ± 0.71
MH	60.11±1.32	65.67±1.57	69.88±0.71	72.74±1.05	17.38±0.83
MM	73.57±0.21	77.47 ± 0.31	80.48 ± 0.51	83.57±0.67	14.58±0.51
p-BHB	70.59±0.78	72.48 ± 0.88	76.47 ± 1.01	80.63 ± 1.31	15.99 ± 0.50
Ascorbic acid	83.44±0.15	84.47±0.32	87.22±0.47	89. 36±0.75	12.95±0.51
Blank	-	-	-	-	-

(-) Showed no scavenging activity. Values were the means of three replicates ± SD

Table 4: The *in-vitro* antioxidant activity in hydrogen peroxide (H_2O_2) method

Compounds	Conc. (µg/ml)				
	25	50	75	100	IC_{50}
KH	64.68±1.08	67.28±1.22	69.34±0.52	72.57±0.62	16.36±0.53
KM	60.11±1.28	62.99 ±.13	67.21±1.01	71.58±1.51	18.13±0.71
BH	51.76±1.18	4.68 ± 0.89	58.47±0.98	63.65±0.67	24.99±1.07
BM	52.85±1.19	55.75±0.89	59.35±0.99	64.31±0.69	24.65±1.08
FH	56.99±1.05	60.62±1.12	63.63±1.41	67.36±1.48	20.12±1.01
FM	63.25±1.03	66.3±1.22	68.42±0.53	71.25±0.62	16.11±0.48
MH	62.55±0.21	65.36±0.58	67.55±0.61	70.55±0.71	18.21±0.21
MM	66.47±0.21	69.55±0.41	72.65±0.52	77.35±0.75	16.79±0.91
p-BHB	51.55±0.81	54.66±1.32	57.74±0.72	62.75±1.01	21.45±0.36
Ascorbic acid	75.67±0.15	77.35±0.27	81.22±0.58	85.24±0.64	14.45±0.21
Blank	-	-	-	-	-

(-) Showed no scavenging activity. Values were the means of three replicates ± SD

Table 5: The *in-vitro* antioxidant activity of in lipid peroxidation method

Compounds	Conc. (µg/ml)				
	25	50	75	100	IC_{50}
KH	70.93±0.86	73.62±1.05	76.75±1.39	81.62±1.25	16.84±0.93
KM	67.92±0.88	69.83±1.37	73.91±0.93	78.87±1.19	17.69±1.03
BH	61.49±1.53	62.22±1.22	68.98±1.32	73.67±0.72	20.91±0.95
BM	44.15±0.86	45.45±1.12	48.78±1.22	51.88±1.34	25.61±0.59
FH	59.85±1.27	62.85±1.14	66.65±1.04	69.79±1.52	18.50±0.71
FM	76.43 ±0.7	80.72 ± 0.8	83.23 ± 0.58	86.62 ± 0.5	16.82 ± 0.7
MH	62.85±1.15	67.72±1.53	72.86±1.37	77.34±0.69	17.92±1.13
MM	71.81±0.34	74.75±0.58	77.62±0.68	82.82±0.84	16.73±0.50
p-BHB	62.81±1.15	67.72±1.53	72.82±1.37	77.33±0.69	17.95±1.13
Ascorbic acid	78.46±0.19	79.36±0.29	83.86±0.63	87.88±0.69	14.67±0.27
Blank	-	-	-	-	-

(-) Showed no scavenging activity. Values were the means of three replicates ±S D

Table 6: Anti- thrombotic activity

	Conc. (µg/dl)	Delay in clot formation in minutes									
		STD	KH	KM	BH	BM	FH	FM	MH	MM	p-BHB
1	0	1	1	1	1	1	1	1	1	1	1
2	100	2	2.5	2	2.5	2	7	3.5	5	3	8
3	200	2.5	7	5.4	3	6.2	13	12	8	2.5	11
4	300	*	7.5	8	6.5	9.5	19	16	14	5	20
5	400	*	9	10	13	11	27	22	28	18	29
6	500	*	11	13	17	15	30	27	*	20	21
7	600	*	19	13	25	20	*	*	*	*	*

* More than 30 minutes

Table 7: Thrombotic activity

	Conc. (µg/dl)	Clot dissolution time in minutes									
		STD	KH	KM	BH	BM	FH	FM	MH	MM	p-BHB
1	0	*	*	*	*	*	*	*	*	*	*
2	100	*	*	*	*	*	*	*	*	*	*
3	200	*	*	*	*	*	*	*	*	*	*
4	300	*	*	*	*	*	*	*	*	*	*
5	400	65	*	*	87	86	74	86	60	70	72
6	500	60	86	83	78	79	69	75	58	69	65
7	600	45	80	80	70	73	58	70	55	60	57

*More than 90 minutes

3. Materials and methods

3.1 Plant material

Fresh herbal plant materials were purchased from Super Market, Mysore. Four medicinal plants were studied: Nigella sativa (Kalonji seeds), Piper betel (Betel leaves), Linum usitatissimum (Flax seeds) and Salvadora persica (Miswak). The collected plant material was air dried at room temperature (20±2ºC) and protected from light. Dried plant material was cut and stored in tightly sealed dark containers until needed.

3.2 Solvents and reagents

All solvents and reagents were purchased from Sigma Aldrich Chemicals Pvt Ltd. Melting points were determined on an electrically heated VMP-III melting point apparatus. The FT-IR spectra were recorded using KBr disc on FT-IR Jasco 4100 infrared spectrophotometer. ^{1}H NMR spectra were recorded using Bruker DRX 400 spectrometer at 400 MHz with TMS as an internal standard. Mass spectra were recorded on LC-MS/MS (API-4000) mass spectrometer. Further, elemental analysis of the compounds was performed on a Perkin Elmer 2400 elemental analyzer.

3.3 Preparation of Plant Extracts

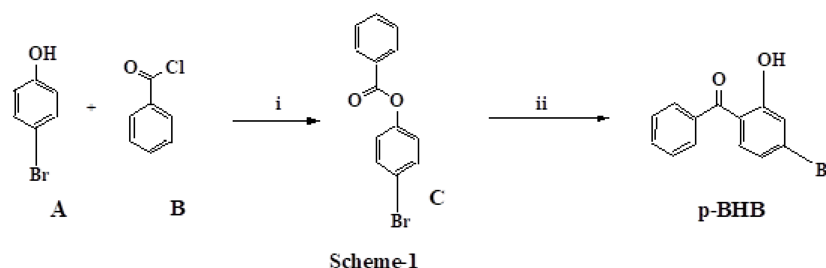
Medicinal plants can be used fresh or dried. Drying is the most common method for post-harvest preservation, and must be accomplished as soon as possible after harvesting, to maintain the quality and to prevent any contamination and phytochemical deterioration.

The dried plant material was coarsely crushed into small pieces of 2-5 mm using a cylindrical crusher. About 100 g of dry powder of the resin of plant was taken in a soxhlet apparatus and subjected to extraction of solvents, first from non-polar solvent (hexane) and then with a polar solvent (methanol).

The extract samples were kept at 4°C for further assays. The extracts were filtered through a filter paper (Whatman, No. 1) and then they were concentrated by removing the solvent using rotary evaporator instrument and stored under refrigeration in glass flasks tapered with screw plastic lid and marked these samples as Kalonji Hexane extract (KH), Kalonji Methanol extract(KM), Betel leaves Hexane extract (BH), Betel leaves Methanol extract (BM), Flax seeds Hexane extract(FH), Flax seeds Methanol extract (KH), Miswak Hexane extract (MH) and Miswak Methanol extract (MM).

3.4 Preparation of (4-bromo-2-hydroxy-phenyl)-phenyl-methanone

The starting material phenyl benzoate (C) was synthesized by benzoylation of p-bromo phenol (A) with benzoyl chlorides (B) in the presence of 10% sodium hydroxide solution. The reaction mass was stirred for 2–3 h at 0°C. After the completion of the reaction, the oily product was extracted with ether layer and dried over anhydrous sodium sulphate, and the solvent was evaporated under pressure. Then, (4-bromo-2-hydroxy-phenyl)-phenyl-methanone (p-BHB) were synthesized by the Fries rearrangement of phenyl benzoate (0.001 mol) with aluminium chloride (0.002 mol). The mixture was blended and then heated to 150–170°C under without-solvent condition for about 2–3 h. Then the reaction mixture was cooled to 0°C and quenched with 6 N hydrochloric acid in the presence of ice water. The reaction mixture was stirred for about 2–3 h, filtered, and the solid (p-BHB) obtained was recrystallized with methanol by the slow evaporation method to obtain good crystals. (Scheme-1).



Scheme 1: Synthesis of (4-bromo-2-hydroxy-phenyl)-phenyl-methanone (p-BHB)

Reaction conditions and yield:
(i) Aq. NaOH, stirring 0-5 ^{O}C for 2-3 h, yield: 80-90%,
(ii) Anhy. $AlCl_3$, 150-170 ^{O}C for 2-3 h, yield: 80%.

Spectral data of compound p-BHB
Infrared (IR) (KBr) vmax (cm–1): 1635 (C=O), 3520–3640 cm^{-1} (OH). ^{1}H NMR (400 MHz) ($CDCl_3$): δ 6.7–7.8 (m, 8H, Ar–H), 10.1 (bs, 1H, OH). (LC-MS) m/z: 279 (M+1). Anal. Calcd. for $C_{13}H_9BrO_2$ (278), C, 56.34; H, 3.27%. Found: C, 56.31; H, 3.18%

3.5 Pharmacology
3.5.1 Antithrombolytic and thrombolytic activity
Experiments for antithrombolytic and thrombolytic activity were carried as reported earlier [27, 28]. Different concentrations of plant extracts (non polar: hexane, polar: methanol) and synthetic compound were added in clean micro centrifuge tubes containing 0.5 ml of blood and Streptokinase was used as standard. In first test tube only normal saline (N.S.) was added and taken as blank. Extract of four medicinal *plant source and synthetic compound* was added in increasing order in tubes (Table 2). Time was noted before each addition. The volume of each tube was made upto 1ml. For antithrombotic activity reactants (extract solution / SK/ NS) was added before clot formation (immediately after taking blood in tubes) and for thrombolytic activity, the reactants were added just after clot formation. All reactions have been maintained at 37°C in water bath. The experiment was done in triplicates (Table 2 and 3)

3.5.2 Antioxidant activity
Plant Extracts along with a synthetic compound (p-BHB) were subjected biological activity. All extracts and compound were tested for *in-vitro* antioxidant property by DPPH, nitric NO, H_2O_2 and LPO methods (Table 2, 3, 4 and 5).

3.5.2.1 DPPH radical scavenging activity
In the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity the hydrogen atom or electron donating ability of the compounds was measured from the bleaching of the purple-colored methanol solution of DPPH. The spectrophotometric assay uses the stable radical DPPH as a reagent. 1 ml of various concentrations of the test compounds (25, 50, 75, 100 and 100 mg/ml) in methanol was added to 4ml of 0.004% (w/v) methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against blank at 517 nm. The percent of inhibition (I%) of free radical production from DPPH was calculated by the following equation

% Of scavenging = [(A control – A sample) / A blank] x 100 (1)

Where A control is the absorbance of the control reaction (containing all reagents except the test compound) and A sample is the absorbance of the test compound. Tests were carried at in triplicate.

3.5.2.2 Nitric oxide (NO) scavenging activity
NO scavenging activity was measured by slightly modified methods of Green *et al*. NO were generated from sodium nitroprusside. 1 ml of sodium nitroprusside (10 mM) and 1.5 ml of phosphate buffer saline (0.2 M, pH 7.4) were added to different concentrations (25, 50, 75 and 100 mg/ml) of the test compounds and incubated for 150 min at 25^{0}C and 1 ml of the reaction mixture was treated with 1 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromatophore was measured at 546 nm. Nitric oxide scavenging activity was calculated using equation 1.

3.5.2.3 Hydrogen peroxide (H_2O_2) scavenging activity
The H_2O_2 scavenging activity of the test compound was determined according to the method of Ruch *et al*. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). 25, 50, 75 and 100 mg/ml concentrations of the test compounds in 3.4 ml phosphate buffer were added to H_2O_2 solution (0.6 ml, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. The percentage of scavenging activity of H_2O_2 was calculated using equation 1.

3.5.2.4 Lipid peroxidation (LPO) scavenging activity
In LPO inhibitory activity egg lecithin (3 mg/ml phosphate buffer, pH 7.4) was sonicated in an ultrasonic sonicator for 10 min to ensure p oper liposome formation. Test samples (100 ml) of different concentrations (25, 50, 75, 100 mg/ml) were added to the liposome mixture (1 ml); the control was without test sample. LPO was induced by adding ferric chloride (10, 400 mM) and L-ascorbic acid (10ml ml, 200 mM). After incubation for 1 h at 37°C the reaction was stopped by adding hydrochloric acid (2 ml, 0.25 N) containing trichloroacetic acid (150 mg/ml), thiobarbituric acid (3.75 mg/ml) and butylated hydroxy anisole (0.50 mg/ml). The reaction mixture was subsequently boiled for 15 min, cooled, centrifuged at 1000 rpm for 15 min and the absorbance of the supernatant was measured at 532 nm and compared with that of ascorbic acid. For all the above antioxidant methods, experiments were done in triplicate and the average is taken, the % inhibition at different concentration was calculated by the following formula

% Inhibition = (1-vt/vc) x 100, where, Vt= mean absorption of test compound, Vc = mean absorption of control. The IC-50 value was derived from the % inhibition at different concentration.

4. Conclusion

The main objectives of the study are the quantification of four different medicinal plant source extracts and also method of synthesis of p-bromo hydroxy benzophenone derivative with its characterization and all the sample were screened for antithrombolytic, thrombolytic and antioxidant activities. An *in-vitro* thrombolytic model was used to check the clotlysis effect of plant extracts (non polar: hexane, polar: methanol) and synthetic compound along with streptokinase as a positive control. Among them, the hexane extract of miswak showed good significant percent of the clotlysis in both antithrombolytic and thrombolytic activities with reference drug streptokinase. Also, the plant extracts and the synthetic compound are used to determined antioxidant activity. The specific assays and a comparison of the antioxidant activity results obtained from the different assays employed. The result revealed that methanol extraction of flax seeds has shown highest radical scavenging activity as compared to the standard, ascorbic acid by DPPH, NO and LPO methods. Whereas, methanol extraction of miswak have shown highest radical scavenging activity as compared to the standard, ascorbic acid by H_2O_2 method. It's worthwhile the medicinal plant extract of flax seeds and miswake is used for future development of new drugs against many more pathological conditions.

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