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Identification and Comparative Study of Invitro Antioxidant Potential of Fractionated Hydroalcoholic Extract of Phyllanthus Niruri Linn

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ABSTRACT

It is apparent that varieties of plants have the potential to show numerous preventive and therapeutic activities that may be relevant to the treatment of disorders caused by oxidative stress and free radicals. A variety of herbal extracts and their phyto components have been demonstrated to exert antioxidant activities, either by directly stimulating antioxidant response genes or by potentiating the bodies` own natural antioxidant defense mechanism. This study reported the high-performance liquid chromatography, invitro antioxidant activity, measurement of total phenolic and flavonoid contents of various solvent extracts of whole plant of Phyllanthus niruri in order to find possible treatment of disorders caused by oxidative stress such as neurodegenerative diseases. Results obtained revealed that, ethyl acetate fractions of P. niruri whole plants possess high flavonoid and polyphenolic compounds which ultimately leads to the potent antioxidant activity than other polar and nonpolar solvent fractions. Thus, this study suggests that, these fractions can be used as a potent source of natural antioxidant there by it can be used for the further investigation for the treatment of neurodegenerative diseases and other free radical induced pathological conditions.

Keywords: Phyllanthus niruri, HPTLC, ethyl acetate, flavonoid, polyphenol, antioxidant, neurodegenerative disease

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I. Introduction

Oxidative stress has been implicated in various pathological conditions including cardiovascular diseases, cancer and age-related disorders[1]. Medicinal plants containing variety of bioactive substances which are of considerable use against various diseases. The demand for natural food ingredients has resulted in extensive research on naturally occurring antioxidants which can neutralize highly reactive free radicals. In recent years, much attention has been devoted to natural antioxidant and their association with health benefits. The plant extracts or their metabolites have act as antioxidants in phytotherapeutic medicines to protect and treat various diseases for centuries related to oxidative stress and free radicals[2]. It produces various antioxidative compounds to counteract reactive oxygen species (ROS) in order to survive. ROS, which include free radicals such as superoxide anion radicals (O_2^-), hydroxyl radicals (OH) and non-free-radical species such as H_2O_2 and singlet oxygen (O_2), are various forms of activated oxygen. The antioxidant enzymes and free radical scavengers may provide a defensive mechanism against the deleterious actions of ROS. Free radicals generated in the body can be removed by body's own natural antioxidant defense e.g. glutathione, catalase etc. However, endogenous antioxidant defences are not completely efficient. Therefore, dietary antioxidants are essential to lower the overall effect of antioxidant stress due to excessive free radicals occurring in our system. Significant antioxidant properties have been recorded with phytochemicals that are essential for the decrease the incidence of many diseases. Therefore, the evaluation of antioxidant activity of various plant extracts is considered as an important step in the identification of their ability to scavenge the free radicals[3]. Phyllanthus niruri which belongs to the Euphorbiaceae family is also known as kidney stone crusher or seed-under-leaf and can be found in the tropical regions of Asia and America. P. niruri is a chief plant in the Ayurvedic tradition to treat pathological conditions of stomach, genitourinary system, liver, kidney and spleen. Recent studies have also revealed antidiabetic and antioxidant properties of this herbal extract both in vivo and in vitro[4]. The various extracts of the plant also proved to act as antiviral and antibacterial agent. Indigenously this plant is used for menstrual and other gynaecological problems. Many active phytochemicals such as flavonoids, alkaloids, terpenoids, lignin, polyphenols, tannins, coumarins and saponins have been documented from various parts of P. niruri. Extracts of this herb have been proven to have therapeutic effects in many preclinical studies[5]. Many crude plants found having antioxidant properties and among the compounds phenolic and flavonoid attracted as noteworthy choice for being used as antioxidants[6]. There are reports which indicate that P. niruri can block calcium oxalate crystals and stone formation in the kidney, ureter, and urinary bladder. A dual cholinesterase inhibitory-guided fractionation of P. niruri leaves afforded isocorilagin, a bioactive tannin possessing

good inhibitory activities against acetylcholinesterase (AChE) and butyryl cholinesterase (BChE) which are playing potential role in Alzheimer's disease pathogenesis[7].

The aim of the present study is to characterize the major chemical groups by high performance liquid chromatography (HPLC) of fractions from hydroalcoholic extract of whole plants of P. niruri (HAPN) and evaluate the different free radical scavenging activities including the 1,1-diphenyl picryl hydrazyl (DPPH), hydroxyl radical and their reducing power capacity of fractions from HAPN.

II. PROCEDURE

A. Plant material and preparation of plant extract

Fresh whole plant of P.niruri (PN) were collected from farm fields around Erode and Namakkal district, Tamilnadu, India and authenticated by pharmacognosy division of SKM siddha and ayurvedha company(India) pvt limited. After authentication, the fresh healthy whole plant P.niruri was dried properly in shade for 3 weeks, segregated, pulverized by a mechanical grinder and passed through a 40-mesh sieve. The powdered plant materials were stored in an airtight container and used for further studies. The powdered drug was defatted with n-Hexane using Soxhlet apparatus. The extraction was performed using maceration with 70% ethanol (1:5 w/v) for 7 days with occasional shaking to obtain hydroalcoholic extract of PN (HAPN). The liquid extract was evaporated using a rotary evaporator at 40°C and until a thick extract was formed. The residue was then weighed and stored in a sealed container. The residue was suspended in water and partitioned successively with petroleum ether, ethyl acetate and n-butanol (a total of two aliquots of 100 ml each) [8]. The percentage yield of successive solvent extractive values for Phyllanthus niruri was calculated. The residue was stored in airtight container for further studies. The final residue thus obtained from various fractions were then subjected to HPTLC analysis and assessment of antioxidant activity.

B. Phytochemical screening

Phytochemical screening of the crude extract and fractions was carried out in accordance with standard test procedures described by Evans[9].

C. Determination of phenolic content

The total phenol content was determined by adding 0.5 ml of the aqueous extract to 2.5ml, 10% Folin–Ciocalteau's reagent (v/v) and 2.0 ml of 7.0% sodium carbonate. The reaction mixture was incubated at 45oC for 40 min, and the absorbance was measured at 765 nm in the spectrophotometer. The mean of three readings was used and the total phenol content was expressed as milligrams of gallic acid equivalents/g extract[10].

D. Determination of Flavonoid content

Total flavonoid content was measured by the aluminium chloride colorimetric assay [11]. An aliquot (1 ml) of extracts or standard solutions of quercetin (20, 40, 60, 80 and 100 µg/ml) was added to a 10 ml volumetric flask containing 4 ml of distilled water. To the flask, 0.30 ml of 5% $NaNO_2$ was added and after 5 min, 0.3 ml of 10% $AlCl_3$ was added. After 5 min, 2 ml of 1M NaOH was added and the volume was made up to 10 ml with distilled water. The solution was mixed and absorbance was measured against the blank at 510 nm. The total flavonoid content was expressed as mg quercetin equivalents (QE*)*

TABLE 1: PERCENTAGE YIELD OF EXTRACT OBTAINED BY MACERATION

S.No	Extracts	Colour	Yield % (W/W)
1.	Hexane	Dark Green	22.30
2.	Hydroalcoholic	Dark brown	40.12
Fractions from hydroalcoholic extract			
a.	Petroleum ether	Brown	20.65
b.	Ethyl acetate	Light Brown	32.70
c.	n-butanol	Light Brown	14.15
d.	Water	Brown	15.95

E. HPTLC analysis for fractions

Test solutions 10µl and 5 µl of standard solution was loaded as 6mm band length positioned 10 mm from the bottom of the plate, using an automated TLC applicator Camag Linomat 5 instrument with inert gas flow providing delivery from the syringe at a speed of 150nl/s. Each TLC plate was developed to a height of about 80 mm with a mobile phase of toluene: ethyl acetate: formic acid: methanol (3:6:1.6:0.4 v/v/v/v) under laboratory conditions (60oC). The plate was kept in Twin Trough Chamber 10x10cm and captured the images at white light, UV 254nm. Finally, the plate was fixed in a CAMAG TLC Scanner 3 was used to densitometrically to quantify the bands using WIN CATS software (Version 4 X). The scanner operating parameters were: (Mode: absorption / remission; Slit dimension; 6.00 x 0.45 mm; scanning rate: 20 mm/s and monochromator band width: 20 nm at an optimized wavelength 254, 366 nm and in visible range) The peak table, peak display and peak densitogram were noted. The obtained results were compared with standard quercetin, rutin and gallic acid.

TABLE 3: TOTAL PHENOLIC AND FLAVONOID CONTENT

S.No	Fraction	Hydroalcoholic extract	Petroleum ether	Ethyl acetate	n-Butanol	Aqueous
1.	Total phenolic content (mgGAE/g) (±SEM)	70.36± 0.52	20.09± 0.01	86.48 ± 0.28	69.13 ± 0.02	36.48± 0.28
2.	Total flavonoid content mg QE/g	58.18 ±1.07	10.78 ±0.56	73.12 ± 1.07	52.65 ± 0.23	43.52± 0.58

TABLE 2: PHYTOCHEMICAL SCREENING

S.No.	Test	Hexane	Ethanol 70%	Ethyl acetate	n-butanol	Water
1.	Alkaloids	-	+	+	+	-
2.	Corbohydrates	-	+	+	+	-
3.	Glycosides	-	-	+	-	+
4.	Terpenoids	-	-	-	-	-
5.	Proteins	-	+	+	+	+
6.	Amino acids	-	+	+	+	+
7.	Steroids	+	-	-	+	-
8.	Flavonoids	-	+	+	+	+
9.	Phenols	-	-	+	-	-
10.	Tannins	-	-	+	-	-
11.	Quinones	-	-	-	-	-
12	Anthraquinones	-	-	-	-	-
13	Saponins	+	-	-	-	-

TABLE 4: HPTLC PEAK TABLE FOR HAPN

Peak	**Start R_f**	**Start Height**	**Max R_f**	**Max height**	**Max %**	**End R_f**	**End height**	**Area**	**Area %**	**Assigned substance**
1	0.04	0.4	0.05	17. 3	1.95	0.07	0.5	246.4	0.55	unknown
2	0.12	3.5	0.16	31.2	7.565	0.18	24.0	919.5	8.53	rutin
3	0.23	39.5	0.26	62.2	7.00	0.33	0.20	17360.7	5.27	unknown
4	0.41	36.3	0.49	130.4	14.68	0.58	0.1	8069.1	18.02	unknown
5	0.60	0.2	0.70	173.8	19.55	0.74	135.0	9714.4	21.70	unknown
6	0.74	135.1	0.77	151.9	17.10	0.81	51.4	26261.8	13.99	Gallic acid
7	0.82	51.6	0.87	197.1	22.18	0.91	115.8	19486.9	21.19	Quercetin

F. DPPH radical scavenging activity

The free radical scavenging activity of the fractions was measured in vitro by 2,20- diphenyl-1-picrylhydrazyl (DPPH) assay according to the method described earlier[12]. A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extract in methanol containing 0.02–0.1 mg of the extract. The reaction mixture left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid was used as positive control. The ability to scavenge DPPH radical was calculated by the following equation:

DPPH radical scavenging activity (%) = [(Abs $_{Control}$ - Abs$_{Sample}$)]/ (Abs$_{Control}$)] x 100

where Abs $_{Control}$ is the absorbance of DPPH radical + methanol; Abs $_{Sample}$ is the absorbance of DPPH radical + sample extract /standard.

G. Hydroxyl Radical Scavenging activity

Hydroxyl radical scavenging activity of different fractions was evaluated by the method as previously described[13]. Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell. The model used is ascorbic acid-iron- EDTA model of $HO^{\cdot}$ generating system. This is a totally aqueous system in which ascorbic acid, iron and EDTA conspire with each other to generate hydroxyl radicals. The reaction mixture (1.0 ml) consist of 100 µl of 2-deoxy-Dribose (28 mM in 20 mM KH_2PO_4 -KOH buffer, pH 7.4), 500 µl of the extract, 200 µl EDTA (1.04 mM) and 200 µM $FeCl_3$ (1:1 v/v), 100 µl of H_2O_2 (1.0 mM) and 100 µl ascorbic acid (1.0 mM) which is incubated at 37ºC for 1 h. 1.0 ml of thiobarbituric acid (1%) and 1.0 ml of trichloroacetic acid (2.8%) are added and incubated at 100ºC for 20 min. Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants.1.0 ml extract is mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (30 mM) and incubated at 50°C for 20 min. Thereafter, 2.5 ml of trichloroacetic acid (600 mM) is added to the reaction mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) is mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (6 mM) and absorbance is measured at 532 nm against an appropriate blank solution. Ascorbic acid was used as positive control.

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TABLE 5: HPTLC PEAK TABLE FOR ETHYLACETATE FRACTION OF HAPN

Peak	Start R_f	Start Height	Max R_f	Max height	Max %	End R_f	End height	Area	Area %	Assigned substance
1	0.11	0.2	0.17	57.3	9.01	0.19	50.5	2213.3	7.50	Rutin
2	0.20	50.7	0.22	60.6	9.52	0.29	5.1	2651.9	8.99	Unknown
3	0.35	12.2	0.38	34.0	5.35	0.39	17.6	619.8	2.10	Unknown
4	0.48	10.9	0.51	21.2	3.32	0.56	4.1	812.6	2.75	Unknown
5	0.63	2.7	0.76	234.3	36.82	0.80	40.6	13063.4	44.27	Gallic acid
6	0.80	40.9	0.84	229.0	35.98	0.93	0.2	10145.8	34.38	Unknown

TABLE 6: HPTLC PEAK TABLE FOR AQUEOUS FRACTION OF HAPN

Peak	Start R_f	Start Height	Max R_f	Max height	Max %	End R_f	End height	Area	Area %	Assigned substance
1	0.03	0.4	0.04	11.8	1.15	0.55	0.0	90.3	0.16	Unknown
2	0.17	18.0	0.24	74.8	7.30	0.30	1.1	3748.8	6.46	Unknown
3	0.30	1.1	0.31	13.3	1.30	0.32	1.8	70.7	0.12	Unknown
4	0.33	4.0	0.36	33.6	3.27	0.38	28.0	855.1	1.47	Unknown
5	0.42	35.2	0.44	47.0	4.59	0.45	44.2	908.0	1.56	Unknown
6	0.45	44.3	0.49	74.0	7.21	0.57	1.3	3733.2	6.43	Unknown
7	0.59	0.0	0.76	379.1	36.96	0.80	180.3	266686.7	45.98	Gallic acid
8	0.80	181.1	0.84	392.0	38.22	0.93	3.0	21945.6	37.81	Quercetin

TABLE 7: HPTLC PEAK TABLE FOR AQUEOUS FRACTION OF HAPN

Peak	Start R_f	Start Height	Max R_f	Max height	Max %	End R_f	End height	Area	Area %	Assigned substance
1	0.06	0.4	0.14	218.2	16.59	0.22	0.5	12213.4	20.43	Unknown
2	0.22	0.2	0.25	15.2	1.15	0.27	0.5	294.6	0.49	Unknown
3	0.31	3.2	0.38	54.6	4.15	0.38	51.9	2020.9	3.38	Unknown
4	0.43	68.3	0.48	104.0	7.91	0.57	1.9	6136.1	10.27	Unknown
5	0.58	0.2	0.71	317.7	24.16	0.72	314.6	13518.4	22.62	Unknown
6	0.72	315.2	0.76	357.5	27.19	0.81	63.9	15838.6	26.50	Gallic acid
7	0.81	63.9	0.84	247.8	18.85	0.92	0.7	9745.5	16.31	Quercetin

TABLE 8: PERCENTAGE INHIBITION OF HAPN AND ITS FRACTION ON DPPH RADICAL SCAVENGING ACTIVITY

Concentration	Standard	Hydroalcoholic	Pet ether	Ethyl acetate	n-Butanol	Aqueous
20	85.82	84.92	53.07	95.41	91.20	88.28
40	87.04	85.70	55.14	96.12	91.86	90.22
60	87.89	90.02	57.29	96.49	94.50	91.08
80	92.26	93.64	66.30	96.84	95.94	93.76
100	94.95	93.80	66.89	97.03	96.51	94.53

TABLE 9: PERCENTAGE INHIBITION OF HAPN AND ITS FRACTION ON HYDROXYL RADICAL SCAVENGING ACTIVITY

Concentration	Standard	Hydroalcoholic	Pet ether	Ethyl acetate	n-Butanol	Aqueous
20	74.82	64.51	15.3	72.96	73.38	62.51
40	77.04	64.97	18.56	76.29	74.43	62.82
60	77.89	69.12	18.73	78.90	78.35	69.20
80	81.20	71.56	24.14	84.67	84.47	72.05
100	83.67	71.76	37.03	87.45	85.62	73.61

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TABLE 10: PERCENTAGE INHIBITION OF HAPN AND ITS FRACTION ON REDUCING POWER

Concentration	Standard	hydroalcoholic	Pet ether	Ethyl acetate	n-Butanol	Aqueous
20	0.207	0.080	0.012	0.198	0.178	0.110
40	0.210	0.085	0.028	0.214	0.210	0.118
60	0.329	0.135	0.044	0.344	0.301	0.144
80	0.385	0.170	0.051	0.402	0.398	0.207
100	0.486	0.210	0.051	0.490	0.420	0.263

H.Reducing power antioxidant activity

The reducing power was determined as described as earlier[14]. Reducing power is associated with antioxidant activity and may serve as an important indicator of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants.1.0 ml extract is mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (30 mM) and incubated at 50°C for 20 min. Thereafter, 2.5 ml of trichloroacetic acid (600 mM) is added to the reaction mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) is mixed with 2.5 ml of distilled water and 0.5 ml of FeCl3 (6 mM) and absorbance is measured at 700 nm. Ascorbic acid was used as positive control.

III. Results and Discussion

The amount of materials that can be extracted from a plant depends on the vigour of the extraction procedure and the possibility also exists of sample-to-sample variation in the extracted material. The crude HAPN yield of all plant parts varied from 40.12% W/W dry raw materials. The percentage yield of various fractions from HAPN, were summarized in Table 1. This result is in line with a work conducted earlier[15], which performed Soxhlet extraction using organic and aqueous solvents to obtain various crude extracts from Phyllanthus niruri L. The authors reported that the yield was high when using water and polar solvents (ethanol and methanol), however the yield was significantly decreased with non-polar solvents (hexane and petroleum ether). Preliminary phytochemical screening showed the presence of flavonoids, terpenes, coumarins, lignans, tannins, carbohydrates and alkaloids. However, steroid and terpenoids are not identified. The results are summarized in Table 2. As previous study[16] reported that, from thin layer chromatography (TLC) and HPLC analysis, P. niruri L aerial extracts showed a positive result for the presence of phenols, flavonoids, tannins, saponin, steroids, and lipids. The results from the phytochemical tests differ from other studies due to at least three possible factors such as geographical location, extraction procedure and sensitivity of the techniques. Presence of hydroxyl group in the plant phenolic compounds plays a vital role for antioxidant property with free radical neutralizing activity[17]. This is due to their redox properties, which play an important role in adsorbing and neutralising free radicals, quenching singlet or triplet oxygen and decomposing peroxides. Total phenolic content of various fraction of HAPN were determined using Folin-Ciocalteu reagent. Total phenolic content of the sample was calculated based on the standard curve for gallic acid and the results were expressed as mg of gallic acid equivalent (GAE)/g of dried extractives and was tabulated in Table 3. The phenolic content of ethyl acetate fraction from HAPN is higher in amount than petroleum ether fraction. Various clinical studies confirmed the prolonged use of polyphenol enriched diet can prevent many chronic diseases including AD[18]. Plants with higher biologically active polyphenols act as neuroprotective agents which shows significant protection against neural injuries and degeneration. As a subclass of this family, flavonoids act as a potent antioxidant, anti-inflammatory and signalling pathways modulatory agents. Flavonoids are considered as well-known antioxidants due to their radical scavenging, metal ion chelating and lipid peroxidation inhibiting activities. AD occur mainly due to oxidative stress. Flavonoids may prevent or slower the progression of AD that are act by inhibiting the enzymes such as β-secretases, cyclin dependent kinase and glycogen synthase which are playing key role in phosphorylation of tau proteins. Flavonoids takes part in neural rehabilitation and lost cognitive performance recovery which are considered as potent anti-AD effects[19,20]. In our study total flavonoids content was determined by aluminium chloride colorimetric method. Flavonoid content of the samples was calculated based on the standard curve for quercetin and the results were expressed as mg of quercetin equivalent (QE)/g of dried extractives and was tabulated in Table 3. The flavonoid content of ethyl acetate fraction from HAPN was estimated as 73.12± 1.07mg of QE/g of dried extract which is very high in comparison with the flavonoid content of other fractions.

The various fraction of HAPN having the potentiality to scavenge the free radical contains flavonoids which was analysed by using HPTLC. The peak table, peak display and peak densitogram were noted. Thus, the presence of flavonoids and phenolic compound in the various fraction of HAPN was confirmed by HPTLC analysis and were shown in Table 4-7 and Figure 1&2. It was observed that the aqueous fraction showed the presence of quercetin, ethyl acetate fraction contained rutin, n-Butanol fraction contains quercetin and all the fractions contained gallic

acid. They were confirmed from the chromatogram after derivatization as in Figure 3. Calixto, et al., reported that P. niruri contained flavonoids quercetin, quercitrin, isoquercitrin, astragalin, rutin and physetinglucoside21. Mediani, et al22., reported that it contained a high amount of rutin, followed by gallic acid, quercetin.

Many studies have focused on the biological activities of phenolics which are potent antioxidants and free radical scavengers. The antioxidant activity of phenolics is mainly due to their redox properties, which allows them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. The interest in phenolic compounds derived from vegetables and their roles in nutrition are therefore increasing. Phenolic compounds are also known to play an important role in stabilizing lipids against peroxidation and inhibiting various types of oxidizing enzymes. The differences in the flavonoid structures and their substitutions influence the phenoxyl radical stability, thereby affecting the antioxidant properties of the flavonoid 23. Enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), etc and non-enzymatic antioxidants such as carotenoids, ascorbic acid, phenolic compounds, flavonoids, etc act by one or more mechanisms like reducing activity, free radical scavenging, potential complexing of pro-oxidant metals and quenching of singlet oxygen. It is possible to reduce the risks of chronic diseases and prevent the disease progression by either enhancing the body's natural antioxidant defense or supplementing with proven antioxidants.

When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color (although there would be expected to be a residual pale-yellow color from the picryl group still present). The degree of color change is proportional to the concentration and potency of the antioxidants. A huge decrease in the absorbance indicates significant free radical scavenging activity of the extract under test24. The results were expressed as ascorbic acid (AA) equivalents (AAE/g) and shown in Table 8. Our findings indicate that 20μg/mL of the fractions were found to be in the order ethyl acetate > n-butanol > aqueous > HAPN > pet ether (97.03%) and ascorbic acid conferred 96.82% on DPPH radicals. The logic behind this behaviour may be explained by the presence of phytoconstituents that are capable of donating hydrogen or electron to a free radical to scavenge the potential damage by them. These results suggest that fractions have high radical scavenging activity. Radical scavenging activities are very important to prevent the deleterious role of free radicals in AD.

Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell. Table 9 showed the hydroxyl radical scavenging activity of various fraction of HAPN and compared with ascorbic acid. It was observed that ethyl acetate and n-butanol fraction had higher activity compared to ascorbic acid. The molecular mechanisms underlying the pathogenesis of cell death in neurodegenerative or amyloid diseases, could be the direct production of hydrogen peroxide during formation of the abnormal protein aggregates A(beta) and alpha-synuclein by a metal-dependent mechanism in which addition of Fe (II), by Fenton's reaction. Hydrogen peroxide subsequently converted to hydroxyl radicals. 25,26. From the above finding our HAPN fractions can be used to nullify the deleterious effects of hydroxyl radicals in various disease processes.

The reducing capacity of a compound is the potent indicator of its antioxidant activity. The reducing properties are generally associated with the presence of reductones, which have been shown to exhibit antioxidant action by breaking the free radical chain by donating a hydrogen atom and may have great relevance in the prevention and treatment of diseases associated with oxidants or free radicals27.All fractions of our study showed reducing activity compared to ascorbic acid which was a reference antioxidant and was summarized in Table 10. The ethyl acetate fraction showed increased reducing activity with the increasing concentration of the extract compared to other fractions and standard drug.

As our results, indicating that the ethyl acetate fractions showed greater antioxidant activity due to the presence of higher amount of phenolic and flavonoid contents. The previous studies 28,29 suggested that methanol extract had a better TPC and DPPH activity when compared to water extract based on their polarity. We believed that the greater antioxidant property of ethyl acetate fractions might be by less polarity. Plant antioxidant activity, however, also depends on other non-polyphenolic compound such as vitamins, minerals and carotenoids. These non-polyphenolic compounds may exert synergistic effect with TPC and TFC, which could further enhance the antioxidants activities. Further studies are needed to identify which phenolic compounds are responsible for the antioxidant activity of the species and assess the way in which the phenolic substances contribute to this activity.

IV. CONCLUSION

In conclusion, the present study provides the evidence that the HAPN and fractions obtained from HAPN contains flavonoid and phenolic contents. They show potential antioxidant and free radical scavenging activity. These *In-vitro* assays demonstrate that this ethyl acetate fraction is an important source of natural antioxidant and can be used for preventive and therapeutic purpose for diseases caused by oxidative stress and free radicals.

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