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In vitro antioxidant properties and inhibitory effect of extracts and fractions of *Plectranthus glandulosus* leaves on copper sulfate (CuSO4)-induced oxidation in human low-density lipoprotein

Djamila Zouheira[1,2], Gabriel A Agbor[2], Randhir Singh[3], Sylviane Laure Poualeu Kamani[1], Anu Kajal[3], Shah Asma Farooq[3], Sylvie Lea Wansi Ngnokam[1]*

[1] Department of Animal Biology, Faculty of Sciences, University of Dschang-Cameroon

[2] Centre for Research on Medicinal Plants and Traditional Medicine (CRPMT), Institute of Medical Research and Medicinal Plants Studies (IMPM), Yaoundé-Cameroon

[3] Department of Pharmaceutical Sciences, Maharishi Markandeshwar University, Mullana, Ambala, India

ABSTRACT

Oxidation of LDL has been suggested to be an initial step in the development of atherosclerosis. This research work deals with the evaluation of antioxidant potential of *Plectranthus glandulosus* leaves extracts and fractions as well as its protective effects against human LDL oxidation. A preliminary quantitative phytochemical screening was carried out. Antioxidant potential was evaluated employing *in vitro* hydrogen peroxide, nitric oxide scavenging assays and TAOC test. The human LDL oxidation induced by $CuSO_4$ inhibition test was also performed. *Plectranthus glandulosus* leaves exhibited the presence of 18.3% of saponines, 25.6% of terpenoids and 36.2% of flavonoids. EAF exhibited highest hydrogen peroxide and nitric oxide scavenger activity (IC_{50} 13.63µg/ml and 24.59 µg/ml respectively). Ascorbic acid exhibited an IC_{50} value of 15.39µg/ml in hydrogen peroxide assay and 22.96 µg/ml in nitric oxide scavenging activity. EAF exhibited a higher TAOC (optical density 0.186±0.00) than that of ascorbic acid (0.162±0.001) at the concentration of 25µg/ml while at 200µg/ml N-BUTF exhibited a higher optical density (1.261±0.001) than that of ascorbic acid (1.065±0.001). EAF lengthened the lag time of the CD formation up to 150mins at the concentration 1mg/ml. TBARS formation inhibition at the concentration 0.5mg/ml, were no significant different between n-butanol (68.55%) ethyl acetate fraction (68.21%) and quercetin 67.69%). *Plectranthus glandulosus* is a promising prospect as an anti-atherosclerotic agent and needs a detailed study to establish the same.

Key words: *Plecthrantus glandulosus*; oxidation; LDL; atherosclerosis, antioxidant.

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***Address for Correspondence:** Wansi Ngnokam SL, Department of Animal Biology, Faculty of Sciences, University of Dschang-Cameroon

Abbreviation: LDL: low density lipoprotein; TAOC, total antioxidant capacity; CD, conjugated diene; TBARS, thiobarbituric acid reactive substances; EAF, ethyl acetate fraction; N-BUTF, n-butanol fraction; IC_{50}, concentration that produce half maximal inhibition.

INTRODUCTION

Oxidative stress is initiated by reactive oxygen species such as hydrogen peroxide (H_2O_2), peroxyl nitrite, as well as hydroxyl radical (.OH), superoxide anions (O2-), nitric oxide (NO.) which seeks stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidation[1]. These changes contribute to cancer, ageing, inflammation and cardiovascular disease such as atherosclerosis [2,1,3]. Atherosclerosis is a multifactorial complication leading to heart attack and stroke. Oxidation of low density lipoproteins (LDL) has been suggested to be an initial step in the development of atherosclerosis [4]. Oxidized LDL is taken up by macrophages at an enhanced rate via their scavenger receptors [5,6] leading to the formation of lipid-laden foam cells, the hallmark of the early atherosclerosis [5,7].

Antioxidants with free-radical scavenging activities could have great importance as prophylactic and therapeutic agents in diseases in which oxidants or free radicals are implicated [8,9]. The most common antioxidants currently in use include BHA- Butylated hydroxyl anisole, BHT Butylated hydroxyl toluene, propyl gallate, and tert-butyl-hydroquinone[10]. However, they have been suspected to be responsible for liver damage and carcinogenesis in laboratory animals [11]. Therefore, the researches on the development of the new, safe and effective antioxidants are nowadays vivaciously encouraged and botanicals might be the alternative solution.

Plecthrantus glandulosus Hook. F. (Lamiaceae) is one of natural products with culinary applications and health benefits. It is a climbing herbaceous plant widely distributed in West, Central and South of Africa [12]. It is used in Cameroon's traditional medicine to treat dermatitis, bellyache, venereal diseases, internal inflammation, lower abdominal and nerve ache. Known as Ava in Ewondo, the plant is used as condiment in the Ewondo tribe [12]. Earlier studies reported the antinociceptive and anti-inflammatory effects [13] as well as antioxidant and insecticidals activities [14,15,16]. As phytochemical constituents of *P. glandulosus*, one new methoxylated flavonoid derivative, plectranmicin and one new monoterpene derivative, plectranmicinol, together with seven known compounds including 5-hydroxy-3,7,2′,4′tetramethoxyflavone; 5,7-dihydroxy-3,2′,4′-trimethoxyflavone; 7-hydroxy-5,6,4′trimethoxyflavonen; 3-epi-betulinicacidn; 3O-β-D-glucopyranosylstigmasterol; β-sitosterol and 4-epi-fridelin were isolated from the whole plant [17].

The present study was undertaken to evaluate the *in vitro* antioxidant properties of the leaves extracts and fractions as well as their protective effects against human low density lipoprotein oxidation induced by copper sulfate ($CuSO_4$).

1. MATERIALS AND METHODS

1.1. Chemicals

Copper sulfate ($CuSO_4$), ethylenediaminetetra acetic acid (EDTA), trichloroacetic acid (TCA), thiobarbituric acid (TBA), solid potassium bromide (KBr), sodium chloride (NaCl), sulfuric acid, sodium phosphate, ammonium molybdate, sodium nitroprusside, dibasic sodium phospate (Na_2HPO4) (for PBS preparation), monobasic sodium phosphate (NaH_2PO4) (for PBS preparation), sulphanilamide, phosphoric acid (H_3PO_4), napthylethylenediamine dihydrochloride, hydrogen peroxide (H_2O_2), ascorbic acid, methanol, sulphuric acid, chloroform, ethanol, diethyl ether, n-butanol, were facilited by the Pharmacology Laboratory of the MM college of Pharmacy, Mullana, Ambala,India where different tests were conducted.

1.2. Plant Material

The fresh leaves of *P. glandulosus* were collected in Ngaoundere (Cameroon). The plant was identified at the National Herbarium of Cameroon, where the voucher specimen was deposited (41168-HCN). The fresh leaves collected from their natural environment were washed with distilled water three times, shade dried and powdered.

1.3. Extraction and fractionation of plant materials.

1.3.1. Preparation of aqueous extract

A powdered material (1200g) was macerated in 10 L of distilled water at room temperature for 48h.The filtered macerate was then dried in a ventilated drying oven (MANESTY-PETRIE) and the extract obtained was 133.84 g.

1.3.2. Preparation of ethanolic extract

Cold maceration was used to extrate 1200g of powder in 14 L of ethanol for 48 h. The mixture was filtered and the filtrate was concentrated in a rotary evaporator (BUCHI). The concentrated extract was dried using ventilated drying oven (MANESTY- PETRIE) to obtain 34.6g of dry powdered extract.

1.3.3. Preparation of hydroethanolic extract

Hydroethanolic extract was prepared by maceration of powder (2400g) into ethanol /water (70:30) for 48h. The filtered macerate was concentrated using rotary evaporator (BUCHI) and dried out using ventilated drying oven (MANESTY- PETRIE) to obtain 263.09g of dry extract.

1.3.4. Fractionation

The hydro ethanolic crude extract (190g) was fractioned in hexane, ethyl acetate, n-butanol in increasing order of polarity. The hydro ethanolic extract was initially mixed with 500 ml of distilled water and 450 ml of hexane then transferred into a separating funnel. After shaking and decantation, the separation of two phases was obtained. The upper organic phase which represents the hexane fraction was transferred in to an erlenmeyer. The lower organic phase was extracted many times by adding fresh solvent (hexane) until a clear phase was obtained. The hexane fractions was then pooled and stored as such. The lower phase was mixed with subsequent solvents (ethyl acetate and then n-butanol) and they followed same treatment as hexane fraction. At the end of the fractionation the residue was called residual fraction. The four fractions were concentrated and dried.

1.4. Quantitative analysis of phytochemical constituents

1.4.1. Estimation of total flavonoids

P. glandulosus leaves (10 g) were extracted in triplicate with 80% aqueous methanol (100 ml) at room temperature. The whole solution was filtered using Whatman filter paper no. 42. The filtrate was transferred to a crucible and evaporated to dryness and weighed to a constant weight [18,19] (Ali *et al*., 2019; Boham *et al*.,1994).

1.4.2. Determination of total saponins

20 g of *P. glandulosus* leaves was taken in conical flask and 20% aqueous ethanol (100 ml) was added. The mixture was heated at about 55°C for 4 h over a hot water bath with continuous stirring. The mixture was filtered and the residue re-extracted with another 200 ml of 20% ethanol. The extracts were combined and reduced to 40 ml. The concentrate was transferred to a separating funnel and 20 ml of diethyl ether was added and shaken vigorously. Ether layer was discarded and aqueous layer was further purified. Concentrate was then fractionated with 60 ml n-butanol repeatedly for three times. Butanol fractions were combined and washed twice with 10 ml of 5% aqueous sodium chloride. Purified butanol fractions were evaporated to dryness in the oven to a constant weight. Saponin content was calculated as percentage[18,20] (Ali *et al.,*2019 ; Obdoni *et al*.,2001).

1.4.3. Dertermination of terpenoid

The extraction method of Ekwueme *et al.* (2015) [21] was used with some modifications. *P. glandulosus* leaves (50 g) were macerated with methanol and water (4:1) for 24 h at 37°C and filtered with Whatman filter paper. Filtrate was concentrated at 40 °C and concentrate was then acidified with 2M sulphuric acid and the mixture was then extracted with chloroform. Non-aqueous layer was separated and evaporated to dryness. Terpenoids content was calculated as percentage.

1.5. *In vitro* antioxidant tests

1.5.1. Hydrogen peroxide (H_2O_2) scavenging activity

The H_2O_2 scavenging activity was determined according to the method of Ruch *et al*. (1989) [22]. with some modifications. The mixture containing sample (1 ml; 25-400 µg/ml), phosphate buffer solution (PBS) (2.4 ml; 0.1 M, pH 7.4) and H_2O_2 solution (0.6 ml; 40 mM) was shaken vigorously and incubated at room temperature for 10 min. Absorbance of the reaction mixture was determined at 230 nm. Ascorbic

acid was used as positive control. The H_2O_2 scavenging activity was calculated as follows :

$$\% \text{ Inhibition} = 1 - \left(\frac{A_1 - A_2}{A_0}\right) \times 100$$

Where, A_0 is the absorbance of the control (water instead of sample), A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample only (phosphate buffer instead of H_2O_2 solution). The IC_{50} value represented the concentration of the compounds that caused 50% inhibition of H_2O_2.

1.5.2. Nitric oxide radical-scavenging activity

Nitric oxide assay was carried out following a slightly modified method of Lee *et al.* (2010) [23]. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitric ions that can be estimated by using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide (Marcocci *et al*.,1994). 5mM of Sodium nitroprusside in phosphate buffer saline (PBS) pH= 7.4 was mixed with 3.0 ml of different concentrations (25-400 µg/ml) of *P. glandulosus* leaves extracts and fractions. This reaction mixture was incubated for 150 min (25˚C). Further mixture of sulphanilamide (1%), H_3PO_4 (2%) and napthylethylenediamine dihydrochloride (0.1%) (Greiss reagent) was added to the above reaction mixture. Diazotization of nitrite with sulphanilamide and subsequent coupling with napthyl ethylenediamine generates a chromophore. The absorbance of the chromophore formed was noted at 546 nm. Ascorbic acid was used as the reference compound. Percentage inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{A_0 - A_t}{A_0} \times 100$$

Where A_o was the absorbance of the control (blank, without extract) and A_t was the absorbance in the presence of the test (extract). IC_{50} value represented the concentration of the compounds that caused 50% inhibition of H_2O_2.

1.5.3. Total antioxidant capacity (TAOC)

The basis for this assay was the reduction of Molybdenum VI to Molybdenum (V) and formation of a green phosphate/Molybdenum (V) complex in acidic conditions [24]. Different concentrations (25, 50, 100, 200 and 400 µg/ml) were prepared by dissolving the extracts/fractions in distilled water. 0.3 ml of the extract/fraction mixed with 3 ml of reagent. Reagent solution comprises of mixture of various reagent like sulfuric acid (0.6 M), sodium phosphate (28 mM) and ammonium molybdate (4 mM). After mixing both sample and reagent solution, mixture was incubated for 90 min (95˚C) after covering the test tubes with aluminium foil. Absorbance was recorded at 695 nm after cooling the solution at room temperature, against blank. Similar procedure was carried out with ascorbic acid which is used as standard. The total antioxidant activity was expressed as absorbance of the sample. A higher absorbance value would indicate a higher antioxidant activity[25].

1.5.4. *In vitro* CuSO4-induced LDL Oxidation inhibition study

1.5.4.1. Isolation of LDL

The LDL fraction was isolated from fresh plasma by single vertical discontinuous density gradient ultracentrifugation [26]. The density of the plasma was adjusted to 1.21 g/ml by the addition of solid potassium bromide (KBr) (0.365 g/ml). Centrifuge tubes were loaded by layering 1.5 ml of density-adjusted plasma under 3.5 ml of 0.154 mol/L NaCl, and centrifuged in a Beckman L7-55 ultracentrifuge at 40000 rpm at 10 °C for 150 min. The yellow LDL band, located in the upper middle portion of the tube, was collected into a syringe by puncturing the tube. The isolated LDL was dialyzed for 48h at 4°C against three changes of deoxygenated-PBS (0.01 mol/L Na_2HPO4, 0.16 mol/L NaCl, pH 7.4) containing 0.01% NaN3 and 0.01% EDTA. After isolation of total LDL, the protein content of LDL was measured [27].

1.5.4.2. Continuous monitoring of formation of conjugated dienes in LDL

After isolation, LDL was adjusted to 150 µg/mL of LDL protein with 10 mM PBS, pH = 7.4. 1ml of aliquots extracts/fractions at various concentration (0.25, 0.5 and 1mg/ml) were added to 1ml of LDL solution (150µg/ml). The oxidative modification of LDL was initiated by addition of 0.1ml of freshly prepared 10 µM CuSO4 solution at 37°C [28]. Then the sample containing LDL and copper sulfate without extract/fractions (negative control sample) and samples containing copper sulfate and extracts/fractions (test samples) were prepared. Quercetin was used as the positive control sample and prepared in the same manner as the test samples. The appearance of conjugated diene was monitored at 234 nanometers once every 10 min for a period of 3 h and after 24 h at 37 °C in a UV/Visible spectrophotometer. The oxidation-time curve (lag time) was plotted.

1.5.4.3. Assay of the formation of thiobarbituric acid reactive substance (TBARS)

Lipid peroxidation end product, TBARS, was measured as per the modified method of Buege and Aust (1978) [29]. After the oxidation process with $CuSO_4$, 0.1ml of EDTA (2mM) was added to the sample mixtures to stop oxidation reaction and the samples (in triplicates) were subjected to quantification of LDL oxidation products. To the samples mixtures, 1ml of trichloroacetic acid (TCA 15%) and 1ml of thiobarbituric acid (TBA 0.67%) in 0.05N NaOH was added and incubated at 90°C for 20 min. After cooling down at room temperature, the samples were centrifuged at 3000rpm for 15 min and the absorbances of the aliquots were recorded in a spectrophotometer at 532 nm. The percentage inhibition of TBARS formation was calculated using the following equation:

$$\text{Inhibition (\%)} = (A0 - AS) \times 100/A0$$

Where A0 is the absorbance of the control and AS is the absorbance of the reaction mixture containing extracts, fractions or quercetin. IC_{50} value (concentration of the sample to produce half maximal inhibition) of the most potent sample was calculated from the graph of the inhibition of TBARS.

1. Stastistical Analysis

The statistical analysis was carried out using SPSS 16.0 for window in which One-Way ANOVA was used and $P < 0.05$ were set as significant. IC50 was calculated using GraphPad PRISM, version 5.00 for windows (GraphPad software).

2. RESULTS

2.1. Quantitative phytochemical contents of *Plectranthus glandulosus* leaves

Quantitative phytochemical screening of *P. glandulosus* leaves revealed the presence of saponins, flavonoids, and terpenoids. However, the plant leaves is highly concentrated in flavonoids (36.2%), compared to terpenoids (25.6%) and saponins (18.3%) (Table 1).

Table 1: percentage of phytochemical compound present in *Plectranthus glandulosus* leaves

Phytochemical compound	Saponins	Flavonoids	Terpenoids
Proportion in *P glandulosus* leave	**18.3%**	**36.2%**	**25.6%**

2.2. *In vitro* antioxidant activities

2.2.1. Hydrogen peroxide scavenging activity

Hydro ethanolic extract is a better scavenger of hydrogen peroxide than aqueous and ethanolic extracts. The scavenging activity induced with the hydro ethanolic extract varied significantly ($F_{(4, 10)}$ =4871; P<0.001) from 57.35% (at 25 µg/ml) to 91.78% (at 400 µg/ml) (Table 2) while among fraction ethyl acetate exhibited the most potent significant ($F_{(4, 10)}$ =4035; P<0.001) hydrogen peroxide scavenging activity ranging from 63.72% (at 25 µg/ml) to 89.58 (at 400 µg/ml) (Table 3) . The positive control ascorbic exhibited a significant scavenging activitiy ranging from 63.72 to 93.25% when applied at 25 µg/ml and 400 µg/ml respectively. The inhibition concentration (IC_{50}) value was 18.33µg/ml for hydro ethanolic extract at correlation coefficient value (r) of 0.9756. The IC_{50} value of the ethyle acetate fraction was found to be 13.63 µg/ml at the correlation (r) of r=0.9976 13.63 µg/ml at the correlation (r) of r=0.9976 and 15.39 µg/ml for ascorbic acid at correlation coefficient value (r) of 0.9876.

Table 2: Percentage of inhibition and inhibition concentration values of hydrogen peroxide scavenging activity of *P. glandulosus* leaves extracts and ascorbic acid

Plant extracts	Conc (µg/ml)	Percentage of inhibition	IC_{50} (µg/ml)	r
Aqueous extract	25	6.86 ± 0. 21	43.92	0.9482
	50	57.35 ± 0. 00		
	100	69.11 ± 0. 36		
	200	70.82 ± 0. 21		
	400	84.55 ± 0.36		
	$F_{(4, 10)}$	37900***		
Ethanolic extract	25	11.64 ± 0. 21	43.63	0.9492
	50	60.17 ± 0. 21		
	100	69.11 ± 0. 36		
	200	73.28 ± 0. 21		
	400	87.13 ± 0.36		
	$F_{(4, 10)}$	30850***		
Hydro ethanolic extract	25	57.35 ± 0.36	18.33	0.9756
	50	66.54 ± 0.36		
	100	69.11 ± 0.36		
	200	83.45 ± 0.36		
	400	91.78 ± 0.21		
	$F_{(4, 10)}$	4871***		
Ascorbic acid	25	63.72 ± 0.21	15.39	0.9876
	50	68.38 ± 0.36		
	100	84.80 ± 0.21		
	200	88.23 ± 0.00		
	400	93.25 ± 0.21		
	$F_{(4, 10)}$	9251***		

Each value represents Mean of inhibition ± SD. ***p<0.001: significant difference within the percentages of inhibition of each sample; r: correlation coefficient, F: mean square between-group/mean square within-group.

Table 3: Percentage of inhibition and their inhibition concentration values of hydrogen peroxide scavenging activity of *P. glandulosus* leaves fractions and ascorbic acid

Plant extracts	Conc (µg/ml)	Percentage of inhibition	IC_{50} (µg/ml)	r
Hexane fraction	25	36.40 ± 0.00	29.72	0.9803
	50	66.66 ± 0.21		
	100	68.13 ± 0.56		
	200	81.86 ± 0.42		
	400	88.97 ± 0.37		
	$F_{(4, 10)}$	9068***		
Ethyl acetate fraction	25	63.72 ± 0.21	13.63	0.9976
	50	75.73 ± 0.36		
	100	82.11 ± 0.20		
	200	87.50 ± 0.37		
	400	89.58 ± 0.21		
	$F_{(4, 10)}$	4035***		
n-butanol fraction	25	55.27 ±0.20	16.24	0.9787
	50	63.84±0.21		
	100	66.54 ±0.36		
	200	75.73 ±0.36		
	400	84.56 ±0.37		
	$F_{(4, 10)}$	3874***		
Residual fraction	25	19.48 ± 0.36	35.54	0.9756
	50	64.09 ± 0.21		
	100	71.81 ± 0.21		
	200	73.28 ± 0.21		
	400	82.35 ± 0.00		
	$F_{(4, 10)}$	34000***		
Ascorbic acid	25	63.72 ± 0.21	15.39	0.9876
	50	68.38 ± 0.36		
	100	84.80 ± 0.21		
	200	88.23 ± 0.00		
	400	93.25 ± 0.21		
	$F_{(4, 10)}$	9251***		

Each value represents Mean of inhibition ± SD.***p<0.001: significant difference within the percentages of inhibition of each sample; r: correlation coefficient, F: mean square between-group/mean square within-group.

2.2.2. Nitric oxide scavenging activities

Tested with the aqueous, ethanolic and hydro ethanolic extract, a moderate nitric oxide scavenging activity was observed by the hydro ethanolic extract which varying significantly ($F_{(4, 10)}$ =3268; P<0.001) from 22.58% (at 25 µg/ml) to 51.10% (at 400 µg/ml) (Table 4). Among fraction the most potent fraction ethyl acetate exhibited a moderate nitric oxide scavenging activity ranging significantly ($F_{(4, 10)}$ =3148; P<0.001) from 29.75% (at 25 µg/ml) to 56.81% (at 400 µg/ml) (Table 5). The positive control (ascorbic acid) exhibited a high nitric oxide scavenging activity varying significantly ($F_{(4, 10)}$ = 614.971; P<0.001) from 34.77% (at 25 µg/ml) to 59.14% (at 400 µg/ml). The calculated IC_{50} of the hydro ethanolic extract was found to be 27.42 µg/ml at the correlation (r) 0.9805. Ethyl acetate fraction exhibited an IC_{50} of 24.59 µg/ml at the correlation (r) 0.9838. The positive control (ascorbic acid) exhibited an IC_{50} value of 22.96 µg/ml (r=0.9612).

Table 4: Percentage of inhibition and inhibition concentration values of nitric oxide scavenging activity of *P. glandulosus* leaves extracts and ascorbic acid as

Plant extracts	Conc (µg/ml)	Percentage of inhibition	IC_{50} (µg/ml)	r
Aqueous extract	25	18.10 ± 0.31	32.41	0.9772
	50	23.65 ± 0.53		
	100	26.34 ± 0.92		
	200	33.33 ± 0.53		
	400	38.71 ± 0.54		
	$F_{(4, 10)}$	539.836***		
Ethanolic extract	25	15.95 ± 0.31	38.49	0.9796
	50	23.83 ± 0.30		
	100	29.93 ± 0.31		
	200	31.72 ± 0.54		
	400	40.86 ± 0.00		
	$F_{(4, 10)}$	2228***		
Hydro ethanolic extract	25	22.58 ± 0.00	27.42	0.9805
	50	25.27 ± 0.00		
	100	36.20 ± 0.62		
	200	40.86 ± 0.00		
	400	51.10 ± 0.00		
	$F_{(4, 10)}$	3268***		
Ascorbic acid	25	34.77 ± 0.31	22.96	0.9612
	50	38.17 ± 0.54		
	100	42.65 ± 0.82		
	200	47.84 ± 0.92		
	400	59.14 ± 0.54		
	$F_{(4, 10)}$	614.971***		

Each value represents Mean of inhibition ± SD. ***p<0.001: significant difference within percentages of inhibition of each sample; r: correlation coefficient, F: mean square between-group/mean square within-group.

Table 5: Percentage of inhibition and inhibition concentration values of nitric oxide scavenging activity of *P. glandulosus* leaves fractions and ascorbic acid as

Plant extracts	Conc (µg/ml)	Percentage of inhibition	IC_{50} (µg/ml)	r
Hexane fraction	25	19.17 ± 0.30	33.19	0.9706
	50	22.04 ± 0.00		
	100	26.70 ± 0.31		
	200	35.30 ± 0.30		
	400	39.25 ± 0.00		
	$F_{(4, 10)}$	3873***		
Ethyl acetate fraction	25	29.75 ± 0.31	24.59	0.9838
	50	38.17 ± 0.54		
	100	45.16 ± 0.00		
	200	46.77 ± 0.00		
	400	56.81 ± 0.31		
	$F_{(4, 10)}$	3148***		
n-butanol fraction	25	21.50 ±0.53	31.61	0.9888
	50	26.52 ±0.31		
	100	36.02 ±0.00		
	200	39.78 ±0.00		
	400	45.70 ±0.00		
	$F_{(4, 10)}$	3779***		
Residual fraction	25	11.11 ± 0.31	57.66	0.9501
	50	16.13 ± 0.54		
	100	24.01 ± 0.30		
	200	25.80 ± 0.53		
	400	34.05 ± 0.31		
	$F_{(4, 10)}$	1371***		
Ascorbic acid	25	34.77 ±0.31	22.96	0.9612
	50	38.17±0.54		
	100	42.65 ±0.82		
	200	47.84±0.92		
	400	59.14 ±0.54		
	$F_{(4, 10)}$	614.971***		

Each value represents Mean of inhibition ± SD. ***p<0.001: significant difference within the percentages of inhibition of each sample; r: correlation coefficient, F: mean square between-group/mean square within-group.

2.2.3. Total antioxidant capacity (TAOC)

Generally, the total antioxidant capacity of the extracts and fractions as well as ascorbic acid increased with the increasing concentration. Hydro ethanolic extract showed a total antioxidant capacity varying from 0.098A (at 25 µg/ml) to 0.949A (at 400 µg/ml) better than ethanolic and aqueous extract (Table 6). Among fraction ethyl acetate showed the most potent total antioxidant capacity ranging from 0.186A (at 25 µg/ml) to 1.026 A (at 400 µg/ml). Ascorbic acid exhibited a higher total antioxidant capacity varying significantly from 0.162A (at 25 µg/ml) to 2.298 A (at 400 µg/ml) (Table 7).

Table 6: Absorbance of the total antioxidant capacity (TOAC) of *P glandulosus* leaves extracts and ascorbic acid at the wavelength 760nm.

	Absorbance (wavelength = 760nm)			
Concentration (µg/ml)	**Aqueous extract**	**Ethanolic extract**	**Hydroethanolic extract**	**Ascorbic acid**
25	0.083 ±0.000	0.055 ±0.000	0.098 ±0.000	0.162±0.001
50	0.086 ±0.001	0. 087 ±0.001	0.191±0.000	0.388±0.001
100	0.118 ±0.000	0. 153 ±0.01	0.2910±0.001	0.8513 ±0.000
200	0.197 ±0.000	0.234 ±0.000	0.663 ±0.000	1.065 ±0.001
400	0.366 ±0.001	0. 500±0.000	0.949 ±0.000	2.298 ±0.001
$F_{(4,10)}$	70740***	3655***	337600***	2403000***

Analyses were done in triplicates. Each value represents Mean of absorbances ±SD. ***p<0.001: significant difference whitin sample absorbances; r: correlation coefficient, F: mean square between-group/mean square within-group.

Table 7: Absorbance of the total antioxidant capacity (TOAC) of *P glandulosus* leaves fractions and ascorbic acid at the wavelength 760nm

	Absorbance (wavelength = 760nm)				
Concentration(µg /ml)	**Hexane fraction**	**Ethyl acetate fraction**	**N-butanol fraction**	**Residual fraction**	**Ascorbic acid**
25	0.091±0.000	0.186±0.000	0.095±0.000	0.0870±0.001	0.162±0.001
50	0.184±0.000	0.285±0.001	0.195±0.000	0.182±0.001	0.388±0.001
100	0.291±0.000	0.850±0.001	0.785±0.001	0.452±0.001	0.851±0.000
200	0.741±0.000	1.001±0.002	1.261±0.001	0.897± 0.001	1.065±0.001
400	1.026±0.002	2.289±0.001	2.010±0.000	1.001±0.001	2.298±0.001
$F_{(4,10)}$	343300***	1026000***	1888000***	306200***	2403000***

Analyses were done in triplicates. Each value represents Mean of absorbances ±SD.***p<0.001: significant difference whitin sample absorbances; r: correlation coefficient, F: mean square between-group/mean square within-group.

2.3. Inhibition of human low density lipoprotein oxidation induced by $CuSO_4$

2.3.1. Continuous monitoring of formation of conjugated dienes in LDL and kinetics of CuSO4 induced LDL oxidation

A dose-dependent decrease of conjugated dienes formation and the increase in lag time period of LDL oxidation was observed in samples containing aqueous, ethanolic, hydroethanolic extract and quercetin at the concentration of 0.25, 0.5 and 1mg/ml. At the concentration 1mg/ml, hydroethanolic extract lengthened the lag time of conjugated diene (CD) formation the most (110 min) (Figure 1c). At the same concentration, ethyl acetate fraction exhibited the best lag time period (150 min) followed by n-butanol fraction (130mins) (Figure 1c). The absorbance of conjugated diene formed in quercetin sample at 1mg/ml increased insignificantly from 0.134 to 0.155 between 0 to 24hours at this concentration. At the concentration of 0.25mg/ml ethyl acetate fraction and n-butanol were found to lengthened the lag time of conjugated diene (CD) formation up to 60mins and 70mins respectively better than that of hexane and residual fraction (30 min and 20 min respectively) (Figure 2 a).

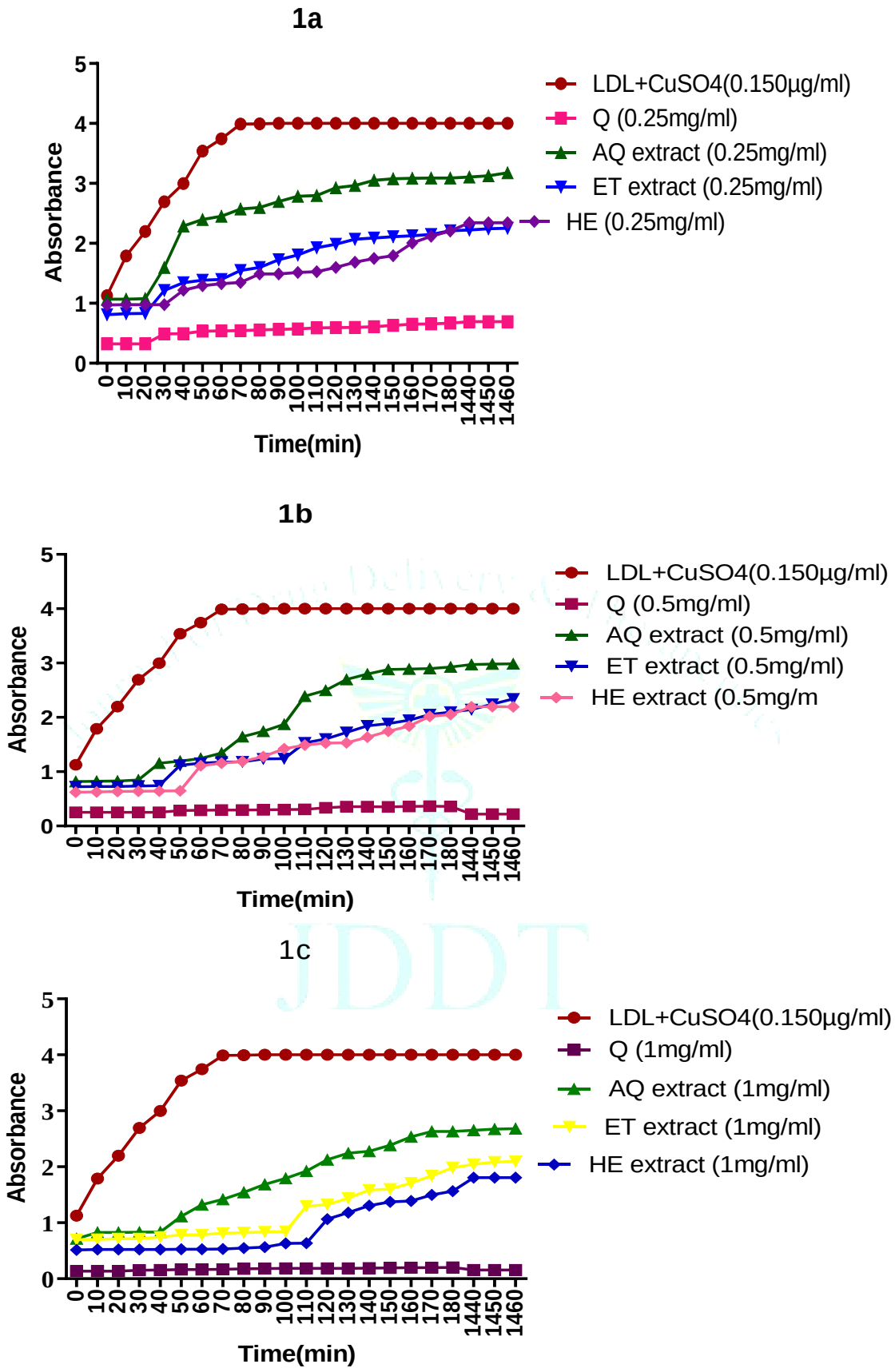

Figure 1: Effect of *Plectranthus glandulosus* leaves extracts on oxidized LDL (ox-LDL) human molecules. The increased lag time of conjugated diene (CD) formation along with decreased absorbance at 234nm indicates the extent of ox-LDL inhibition by the respective extracts against the CuSO4 (0.150µg/ml) induced LDL oxidation. All the extracts and quercetin were used at 0.25mg/ml (Figure 1a), 0.5mg/ml (Figure 1b) and 1mg/ml (Figure 1c).

$LDL+CuSO_4$= native low density lipoprotein Copper Sulfate; Q = Quercetine; AQ extract = Aqueous extract; ET extract = Ethanolic extract; HE = Hydro ethanolic extract.

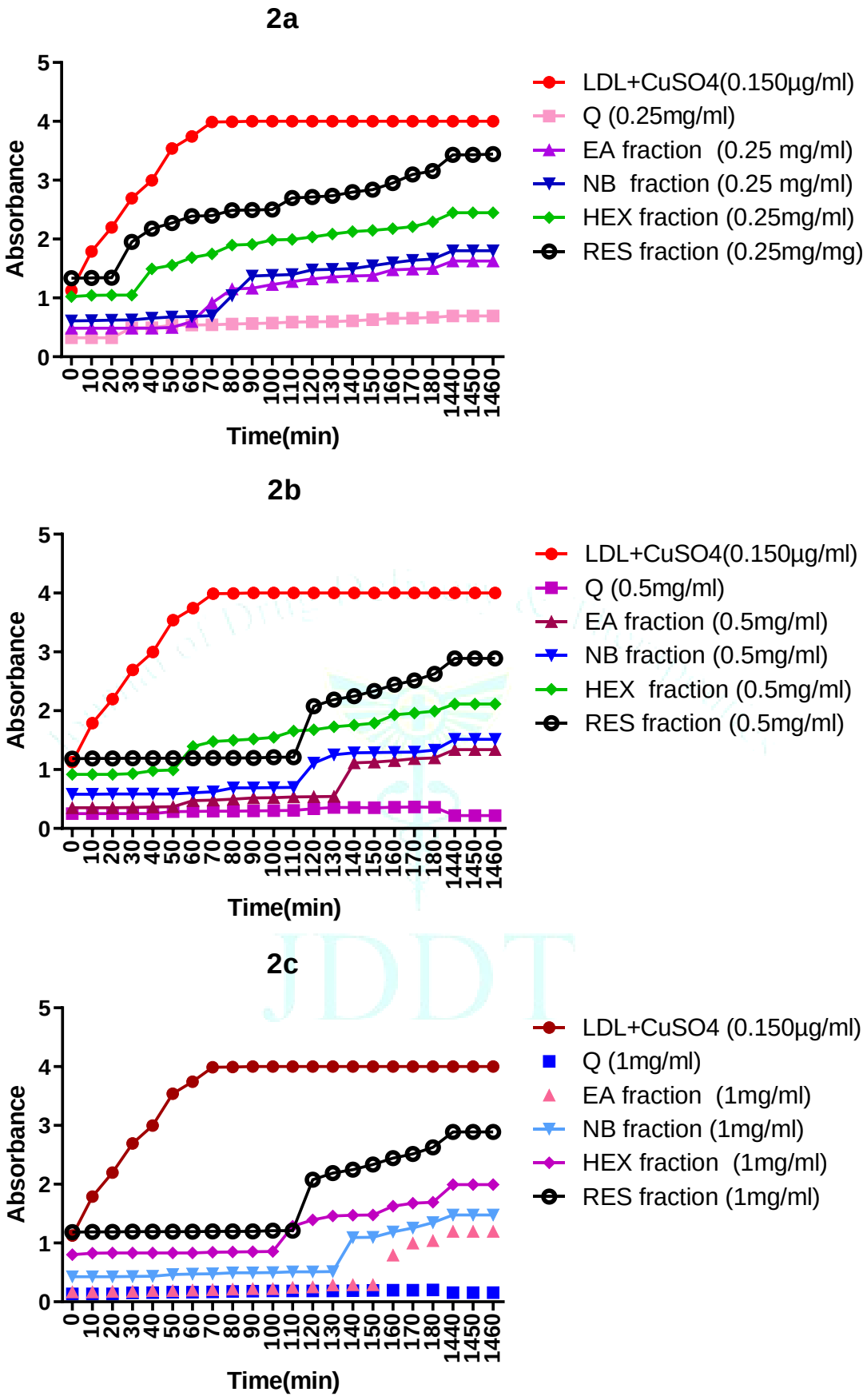

Figure 2: Effect of *Plectranthus glandulosus* leaves fractions on oxidized LDL (ox-LDL) human molecule. The increased lag time of conjugated diene(CD)formation along with decreased absorbance at 234nm indicates the extent of ox-LDL inhibition by the respective fractions against the CuSO4 (0.150µg/ml) induced LDL oxidation. All the fractions and quercetin were used at 0.25mg/ml (Figure 2a), 0.5mg/ml (Figure 2b) and 1mg/ml (Figure 2c).

LDL+$CuSO_4$ = native low density lipoprotein Copper Sulfate; Q = Quercetine; EA fraction = Ethyle acetate fraction;

NB fraction = N-butanol fraction; HEX fraction = Hexane fraction; RES fraction = Residual fraction.

2.3.2. Formation of Thiobarbituric Acid Reactant Substances (TBARS)

Hydroethanolic extract most highly inhibited the formation of TBARS than ethanolic and aqueous extract (Table 8) at the concentration of 0.25 and 0.5mg/ml, there were no significant difference between hydro ethanolic extract and quercetine percentage of inhibition (56.75% and 66.67% respectively for hydro ethanolic extract; 60.34% and 67.69% respectively for quercetine respectively). Ethyl acetate and n-butanol fractions also most highly inhibited the formation of TBARS than hexane and residual fractions (Table 8). No significant difference was observed betwen n-butanol and ethyl acetate fraction as well as quercetine at the concentration of 0.25 and 0.5 mg/ml (61.71% and 68.55% for n-butanol fraction; 56.75 and 68.21% for ethyle acetqte fraction; 60.34% and 67.69% for quercetine respectively). Quercetin exhibited an IC_{50} of 0.9003 mg/ml at correlation coefficient value (r) of 0.0006) while ethyle acetate fraction exhibited 0.9902mg/ml at correlation coefficient value (r) of 0.027).

Table 8: IC50 values and Correlation coefficient (r) of different extracts and fractions in the formation of LDL peroxidation end products (TBARS)

Plant extracts	Conc (mg/ml)	Percentage of inhibition	IC_{50} (mg/ml)	r
Aqueous extract	0.25 0.5 1	29.56 ± 1.09*** 47.36 ± 1.25*** 50.10 ± 0.81***	1.158	0.0271
Ethanolic extract	0.25 0.50 1	45.96 ± 2.41*** 52.30 ± 1.00*** 61.20 ± 1.08***	1.184	0.1088
Hydroethanolic extract	0.25 0.5 1	56.73 ±1.25 66.66 ±1.05 81.56 ± 1.37***	1.089	0.0717
Hexane fraction	0.25 0.50 1	57.94 ± 1.02* 68.20 ± 1.02 75.04 ± 1.80***	1.275	0.1240
Ethyl acetate fraction	0.25 0.5 1	56.75 ± 0.78*** 68.20 ± 0.51 88.37 ± 0.59***	0.9902	0.0277
N-butanol fraction	0.25 0.50 1	61.70 ± 1.06 68.54 ± 0.78 78.80 ± 0.78***	1.233	0.1269
Residual fraction	0.25 0.5 1	42.73 ± 0.30*** 50.59 ± 1.06*** 62.22 ± 1.80***	1.076	0.0652
Quercetine	0.25 0.50 1	60.34± 0.29 67.69 ± 0.51 96.41 ± 0.88	0.9003	0.0006

Each value represents Mean of inhibition ± SD. **p < 0.05; ***p < 0.001* significant difference compared to quercetin, r: correlation coefficient

3. DISCUSSION

Production of cardiac reactive oxygen species has been associated with atherosclerosis development [30,31]. Free radicals and the oxidation of low density lipoprotein (LDL) are the preliminary steps in the apparition of this disease. It is of paramount importance to search for agents capable with antioxidant capacity with a view to combating atherosclerosis. The present study was designed to evaluate the hydrogen peroxide, nitric oxide scavenging activity, total antioxidant capacity and inhibitory effects of extracts and fractions of *P glandulosus* on copper sulfate (CuSO4)-induced oxidation in human low density lipoprotein by *in vitro* method.

Phytochemical screening on *P glandulosus* leaves confirmed the presence of saponins (18,3%), flavonoids (36,2%) and terpenoids (25,6%). All phenolic compounds including flavonoids have been studied mainly for their properties against oxidative damage by scavenging dangerous free radicals like super oxide anion, hydrogen peroxide, hydroxyl radical and nitric oxide generated during normal metabolic processes which can lead to inflammation, allergie, bacterial infection, cancer, tumor, viral infection, atherosclerosis [2,32,33].Terpenoids are known to have antimicrobial, antiviral, anti- inflammatory, antittumor activities and protective effects on the cardiovascular system [34]. Tsopmejio *et al* (2019) [17] isolated one new methoxylated flavonoid derivative, plectranmicin and one new monoterpene derivative, plectranmicinol, together with seven known compounds from the whole plant of *P glandulosus.* Saponins have been associated with hypoglycemic activity, accelerating metabolism of cholesterol in the liver,

antifungal, antimicrobial, veridical and anti-inflammatory activities [35]. These results confirmed that *P glandulosus* has pharmacologically active components which can act against many diseases and specially atherosclerosis.

From the *in vitro* antioxidant tests results, it appeared that *P glandulosus* leaves extracts and fractions effectively reduced the generation of hydrogen peroxide. Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly and once inside the cell, it can probably react with Fe^{2+} and possibly Cu^{2}+ ions to form hydroxyl radicals and this may be the origin of many of its toxic effects [1,36]. Hydro ethanolic extract was the better scavenger among extracts. Among fractions, the better scavenger activity was observed with ethyl acetate fraction.

Nitric oxide is an unstable free radical involved in many biological processes which are associated with several diseases. It reacts with oxygen to produce stable product nitrate and nitrite through intermediates and high concentration of nitric oxide can be toxic and inhibition of over production is an important goal [37,38]. Hydro ethanolic extract was the most active extract and ethyle acetate the most active fraction and showed a moderate nitric oxide scavenging activity which is not negligible compared to that of the standard ascorbic acid at the concentration of 400µg/ml.

The total antioxidant capacity (TAOC) of samples with higher electron donating activity can terminate the radical chain and turn free radicals into more stable products [39,40]. Starting to concentration 100µg/ml to 400µg/ml each sample acted differently from the other no doubt due to differents concentrations and extraction solvent. Also their differents components can act by synergistic, antagonistic or additive effects and produce new physiological properties [41].

The unsaturated portions of lipids especially the double bonds of fatty acids present in lipid molecules are most vulnerable to oxidative stress by free radicals and ions that lead to altered lipid structures resulting in the proatherosclerotic breakdown products [41,42]. High level of iron and copper ions (Cu^{2+}) was indicated in the arterial walls of the atherosclerotic individuals by epidemiologic studies and thus, these redox-active metal ions have been implicated in playing a very important role in oxidizing the native LDL molecule both *in vivo* and *in vitro* [41,43]. The modification of the polyunsaturated fatty acids presents in the LDL molecule and their molecular rearrangement by iron and copper ions are responsible in the formation of conjugated dienes (CD) [41]. *P glandulosus* leaves extracts (aqueous, ethanolic, hydrothanolic extract) and fractions (hexane, ethyle acetate, n-butanol and residual fraction) increased the lag time of the conjugated diene (CD) formation compared to the negative control sample (LDL+0.150µg/ml of $CuSO_4$) which proved the evidence of oxidation by a gradual increase in absorbance which is proportional to the formation of conjugated diene (CDs). For CD formation, there has been a consensus that increase in lag time indicates the inhibition of LDL oxidation by the antioxidant compounds [41,44]. There was a gradual decrease in absorbance with quercetin after 24 hours indicating the decreased oxidation of native LDL compounds preventing LDL oxidation and ox-LDL-mediated atherogenesis. The mechanism responsible for the inhibition of LDL oxidation mihgh be attribute to flavonoids which enable them to bind to the LDL molecule, subsequently offering protection against oxidation through their radical-scavenging capacity [45].

During the course of LDL oxidation, the lag phase is followed by rapid oxidation (propagation phase) when lipid peroxides are formed. Then comes the breakdown of the double bonds (decomposition phase), and aldehydes, especially malondialdehydes (MDA), are formed [41]. different extracts and fractions of *P glandulosus* leaves inhibit the formation of TBARS. This inhibitory effect might be attributed to the antioxidants capacity of extracts and fractions. Antioxidants may act as electron donors to the free radicals (here, Cu^{2+}) to make them stable molecules, thus interfering the oxidation of LDL molecules [41].

CONCLUSION

Results from this present study demonstrated the antioxidant potential of *P glandulosus* leaves. Ethyle acetate fraction exhibit remarkable antioxidant activities and possess the more potent capacity for preventing LDL oxidation induced by copper sulfate. *P. glandulosus* might constitute a promising prospect as an anti-atherosclerotic agent and needs a detailed study to establish the same.

Conflict of interest statement

The authors declare there is no conflict of interest.

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Authors' contributions

Sylvie Lea Wansi Ngnokam: supervision conceptualization, design, drafted manuscript **Djamila Zouheira:** design, performed experiments, data analysis, drafted manuscript **Gabriel Agbor Agbor:** supervision conceptualization, design **Randhir Singh:** supervision, design, drafted manuscript **Sylviane Laure Poualeu Kamani:** design**,** drafted manuscript Anu **Kajal**: performed experiments **Shah Asma**: performed experiments.

REFERENCES

1. Hazra B, Biswas S and Mandal N. Antioxidant and free radical scavenging activity of *Spondias pinnata.* BMC Complementary and Alternative Medicine, 2008; 8:63:1-10.
2. Suja S, M**o**hanasundari L. Antioxidant and free radical scavenging activity of the mixture of ethanolic extracts of *alpinia speciosa* and *alpinia calcarata* rhizome. International Journal of Pharmacy and Pharmaceutical Sciences. International Journal of Pharmacy and Pharmaceutical Sciences, 2016; 8(8):164-170.
3. Braca A, Sortino C, Politi M, Morelli I, Mendez J. Antioxidant activity of flavonoids from *Licania licaniaeflora*. Journal of Ethnopharmacology, 2002; 79:379-81.
4. Naidu K A and Thippeswamy N B. Inhibition of human low density lipoprotein oxidation by active principles from spices. Molecular and Cellular Biochemistry, 2002; 229:19–23.
5. Aviram M, Dornfeld L, Rosenblat M, Volkova N, Kaplan M, Coleman R, Hayek T, Presser D, and Fuhrman B.

Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E–deficient mice. The American Journal of Clinical Nutritrion, 2000; 71:1062–76.
6. Parthasarathy S, Printz DJ, Boyd D, et al. Macrophage oxidation of low-density lipoprotein generates a modified form recognized by the scavenger receptor. Arteriosclerosis, 1986; 6:505–10.
7. Berliner JA, Navab M, Fogelman AM, *et al*. Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. Circulation, 1995; 91:2488–96.
8. Vikas B, Akhil B S, Remani P, K Sujathan Free Radical Scavenging Properties of *Annona squamosa.* Asian Pacific Journal of Cancer Prevention, 2017; 18(10):2725-2731.
9. Marnett LJ. Oxiradicals and DNA damage. Carcinogenisis, 2000; 21:361-70.
10. Gulcin I, Beydemir S, Ahmet AH, Mahfuz E, Emin BM. In vitro antioxidant properties of morphine. Pharmacological Research. 2001; 49:59–66.
11. Biswas M, Haldar P K, and Ghosh A K. Antioxidant and free-radical-scavenging effects of fruits of Dregea volubilis. Journal of Natural Science, Biology and Medicine. 2010; 1(1):29–34.
12. Pele J, Berre S. Aliment d'origine végétal au Cameroun. ORSTOM, 1986; Fond 554 documentaire No 14525 Cpte B.
13. Fongang ALM, Nguemfo EL, Zangueu CB, Ngando HL, Belle PEK, Dongmo AB. Antinociceptive and Anti-inflammatory Effects of the Aqueous Leaves Extract of *Plectranthus glandulosus*. Hook. F. (Lamiaceae) in Mice and Rats. Pharmacologia, 2016; 7(1):60-66.
14. Danga Y.S.P, Nukenine E.N, Younoussa L and Esimone C.O. phytochemicals and larvicidal activity of *Plectranthus glandulosus* (lamiaceae) leaf extracts against *Anopheles gambiae*, *Aedes aegypti* and *Culex quinquefasciatus* (diptera: culicidae). International Journal of Pure and Applied Zoology, 2014; 2(2):160-170. 567.
15. Danga SPY, Nukenine EN, Younoussa L, Adler C, and Esimone CO. Efficacy of *Plectranthus glandulosus* (lamiaceae) and *Callistemon rigidus* (myrtaceae) leaf extract fractions to *Callosobruchus maculatus* (coleoptera: bruchidae) Journal of Insect Science 2015; 15(1):1-6
16. 16. Goudoum A, Ngamo TLS, Ngassoum MB, and Mbofung C M. Antioxidant activities of essential oils of *Clausena anisata* (Rutaceae) and *Plectranthus glandulosus* (Labiateae), plants used against stored grain insects in North Cameroon. International Journal of Biological and Chemical. Sciences, 2009; 3(3):567-577. 563.
17. Tsopmejio J P, Momeni J, Tsopjio F N, Tedjon V S, Mkounga P, Shehla N, Iqbal C M., Nkengfack A E. Bioactive secondary metabolites from *Plectranthus glandulosus* Hook. (Lamiaceae). Phytochemistry Letters, 2019; 30:133–137
18. Ali AM, Saeed AA M and Fdhel T A. Phytochemical analysis and antimicrobial screening of selected Yemeni folk medicinal plants. Journal of Medicinal Plants Studies, 2019; 7(5):108-114.
19. Boham B A, Kocipai-Abyazan R. Flavonoids and condensed tannins from leaves of Hawaiian *vaccinium vaticulatum* and *V. calycinium*. Pacific Science. 1994; 48:458-463.
20. Obdoni B O, Ochuko P O. Phytochemical studies and comparative efficacy of the crude extracts of some Homostatic plants in Edo and Delta States of Nigeria. Global Journal of Pure Applied Science. 2001; 8b:203-208.
21. Ekwueme F N, Nwodo O F C, Joshua P E, Nkwocha C, Eluka P E. Qualitative and Quantitative Phytochemical Screening of the Aqueous Leaf Extract of *Senna mimosoides*: Its Effect in in vivo Leukocyte mobilization induced by inflammatory stimulus. International Journal of Current Microbiology and Applied, 2015; 4(5):1176-1188.
22. Ruch R J, Cheng S, Klaunig J E. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis. 1989; 10(6):1003-1008.
23. Lee S H, Lee J B, Lee K W, Jeon Y J. Antioxidant properties of tidal pool microalgae, *Halochlorococcum porphyrae* and *Oltamannsiellopsis unicellularis* from Jeju Island, Korea. Algae, 2010; 25:45-56.
24. Prieto P, Pineda M and AguilarM. Spectrophotometric Quantitation of Antioxidant Capacity through the Formation of Aphosphomolybdenum Complex: Specific Application to the Determination of Vitamin E. Analytical Biochemistry, 1999; 269:337–341.
25. Li C. and Wang M H Antioxidant Activity of Peach Blossom Extracts. Journal of Korean Society for Applied Biological Chemistry., 2011; 54(1):46-53.
26. Roghini R, Vijayalakshmi K. Free Radical Scavenging Activity of Ethanolic Extract of Citrus paradisi and Naringin -An *in vitro* Study. International Journal of Pharmacognosy and Phytochemical Research, 2018; 10(1):11-16.
27. Bradford M M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochememistry, 1979; 72:248-54.
28. Ahmadvand H, Ani M, and Moshtaghie A A. Inhibitory effect of *Allium cepa* extract on ldl oxidation induced by $cuso_4$ in vitro compared with *allium sativum* and *allium ascalonicom.* Iranian journal of pharmacology & therapeutic, 2011; 10:61-65.
29. Buege J A and Aust S D. "Microsomal lipid peroxidation. Methods in Enzymology, 1978; 52: 302–310.
30. Agbor AG, Akinfiresoye L, Sortino J, Johnson R, Vinson JA. Piper species protect cardiac, hepatic and renal antioxidant status of atherogenic diet fed hamsters. Food Chemistry, 2012; 134:1354–1359.
31. Heymes C, Bendall JK, Ratajczak P, Cave AC, Samuel JL, Hasenfuss G, *et al.* Increased myocardial NADPH oxidase activity in human heart failure. Journal of the American College of Cardiology, 2003; 41(12):2164–71.
32. Battisti V, Maders LD, Bagatini MD, Santos KF, Spanevello RM, Maldonado PA, Brulé A.O, Araújo Mdo C, Schetinger MR, Morsch VM. Measurement of oxidative stress and antioxidant status in acute lymphoblastic leukemia patients. Clinical Biochemestry, 2008; 41: 511-518.
33. Balasundram N, Sundram K, Sammar S. Phenolic compound in plants and agri-industrial by products antioxidant activity occurrence and potential uses. Food. Chemestry, 2006; 99:191-203.
34. Alves-Sylva JM, Zuzarte M, Marques C, Salgueiro L, Girao H. Protectives effects of Terpenes on the Cardiovascular System: Current Advances and Futures Perspectives. Current Medicinal Chemistry. 2016; 40:4559-4600.

35. Sapna D, Desai D G, Harmeet K. Saponins and their Biological 610 Activities. Pharma Times, 2009; 41(3):13-16.
36. Miller MJ, Sadowska-Krowicka H, Chotinaruemol S, Kakkis JL, Clark DA: Amelioration of chronic ileitis by nitric oxide synthase inhibition. Journal of Pharmacology and Experimental Therapeutics, 1993; 264(1):11-16.
37. Roghini R, Vijayalakshmi K. Free Radical Scavenging Activity of Ethanolic Extract of Citrus paradisi and Naringin -An *in vitro* Study. International Journal of Pharmacognosy and Phytochemical Research, 2018; 10(1):11-16.
38. Menaga D, Ralakumar S, Ayyasamy PM. Free radical scavenging activity of methanolic extract of *Pleurotus florida* mushroom. International Journal of Pharmacy and Pharmaceutical Sciences, 2013; 5(4):601-606.
39. Nedamani E R, Mahoonak A S, Ghorbani M, Kashaninejad M. Evaluation of antioxidant interactions in combined extracts of green tea (*Camellia sinensis*), rosemary (*Rosmarinus officinalis*) and oak fruit (*Quercus branti*). Journal of Food Sciences and Technology, 2015; 52(7):4565–4571.
40. Pan M, Jiang T, Pan J. Antioxidant activities of rapeseed protein hydrolysates. Food and Bioprocess Technology, 2011; 4:1144–1152.
41. Rahman M A, Abdullah N, and Aminudin N. Inhibitory Effect on *in Vitro* LDL Oxidation and HMG Co-A Reductase Activity of the Liquid-Liquid Partitioned Fractions of *Hericium erinaceus* (Bull.) Persoon (Lion's Mane Mushroom). BioMed Research International, 2014; 2014:1-9.
42. Morgan J and Leake D S, "Oxidation of low density lipoprotein by iron or copper at acidic pH. Journal of Lipid Research, 1995; 36(12):2504–2512.
43. Lynch S M and Frei B "Mechanisms of copper- and iron-dependent oxidative modification of human low density lipoprotein. Journal of Lipid Research, 1993; 34(10):1745–1753.
44. Yoshida H and Kisugi R. "Mechanisms of LDL oxidation. *Clinica Chimica Acta*,2010; 411(23-24):1875-1882.
45. Fuhrman B, Buch S, Jacob V, Belinky P A, Coleman R, Hayek T and Aviram M. Licorice extract and its major polyphenol glabridin protect low-density lipoprotein against lipid peroxidation: in vitro and ex vivo studies in humans and in atherosclerotic apolipoprotein E-deficient mice. The American Journal of Clinical Nutrition, 1997; 66:267-275.