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A concentrated phenolic compounds extract from *Khaya grandifoliola* CDC exhibits anti-oxidant and anti-TNFα activities

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Abstract
A concentrated phenolic compounds extract (KPE) from the plant *Khaya grandifoliola* was assessed *in vitro* for antioxidant and anti-inflammatory properties and its effect on erythrocytes Plasma Membrane Redox System (PMRS). Phenolic compounds quantity per g of dry matter (DM) were total phenols (57. 93±0.42 mg EGA) and flavonoids (2.84±0.04 mg EQ). Antiradicalar half scavenging concentrations were 6.25±0. 07 µg/mL and 11.65±0.92 µg/mL for DPPH° and HO° tests, respectively. Total Antioxidant Capacity was 76.82±0.35 mgEq AA/g DM and half inhibitory concentrations (IC_{50}) of 8.50±0.14 µg/mL, 8.7±0.14 µg/mL and 71.05±1.20 µg/mL found for inhibition of lipids and proteins oxidation and TNFα production in *Jurkat T* cells, respectively. KPE activated erythrocytes PMRS with an EC_{50} of 5.90±0.00 µg/mL. Conclusively, KPE may be suggested as potential ingredient of functional foods against health conditions involving oxidative stress and inflammation.

Keywords: *Khaya grandifoliola*, polyphenols, anti-oxidant, anti-TNFα, erythrocytes PMRS, *in vitro*

1. Introduction
Oxidative stress is involved in several pathologies such as rheumatoid arthritis, cancer, arteriosclerosis and the phenomena of ischemia/reperfusion [1-3]. Among sensitive cells to oxidative stress, erythrocytes whose antioxidant capacity depends on the activity of plasma membrane redox system (PMRS), are subjected to an excessive level of reactive oxygen species (ROS). When the activity of PMRS decreases, the antioxidant potential of blood plasma also decreases and, erythrocytes become weak and decrease in number throughout hemolysis. This situation could be used to design a useful method to evaluate antioxidant potential [4]. Low erythrocyte blood level is linked to various pathological statuses such as anemia and neurodegenerative diseases [5]. Moreover, ROS can contribute to extend inflammation process by inducing transcription factors such as nuclear factor kappa-B (NF-KB) [6]. When inflammation is abnormally regulated, pathologies such as type II diabetes, edema, cardiovascular diseases, obesity and tumors occur [7]. Also, NF-KB factor plays a crucial role in the inflammatory process for it induces the transcription of a range of genes that code for pro-inflammatory molecules including vasoactive amines, cytokines, chemokines and inducible enzymes [6, 7].
To solve these health problems associated with oxidative stress and inflammation, one of the treatment approaches is the use of medicinal plants, privileged sources of panoply of bioactive molecules such as polyphenols. In fact, polyphenols and particularly flavonoids are endowed with several beneficial biological activities including antioxidant and anti-inflammatory activities [8]. Bearing in mind the main interest of polyphenols, these biomolecules were extracted from *Khaya grandifoliola*, a medicinal plant used in Bamoun (A Cameroonian tribe) traditional medicine. This plant has been subject of several previous studies showing its antioxidant properties and high polyphenols content [9]. Recently, it has been proven that this plant extract is cytoprotective, inducer of nuclear translocation of Nuclear factor-E2-related factor-2 (Nrf2) [10] and protective of rat hepatocytes against the toxicity of paracetamol [11]. In this study, phenolic compounds from *K. grandifoliola* were extracted for the first time and their antioxidant and anti-inflammatory properties as well as ability to activate the erythrocytes PMRS determined.

2. Materials and Methods

2.1. Plant material: Stem bark of *K. grandifoliola* was collected in Foumban (Noun Division, West-Region, Cameroon). The botanical identification was done at the Cameroon National Herbarium, where voucher specimen is kept under the reference number 23434 YA. The plant sample was washed, rinsed with distilled water and dried at laboratory temperature, then ground to obtain the powder.

2.2. Extraction of phenolic compounds [12]

From the powder obtained, 350g was extracted with 2L of hexane and shaken vigorously every two hours for 48 hours. The hexane lipid extract was eliminated and the residues of delipidated powder were dried at room temperature for 48 hours and weighed. The delipidated powder was then macerated in 2L of 70% methanol for 72 hours. The methanolic extract was recovered and the residue macerated a second time. After concentration and drying, the phenolic extract obtained was weighed and stored at 4 °C. The extraction yield was calculated as per the formula: yield = 100 x (Mass of extract)/ (Mass of bark powder)

2.3. Phytochemical screening and high performance liquid chromatography analysis of KPE extract

2.3.1 Phytochemical analysis

A 2% KPE solution was prepared in methanol. An adequate volume of this solution was used for the screening of polyphenols, flavonoids, tannins, anthraquinone, alkaloids, saponins, terpenes, tri-terpenes and sterols as previously described [13, 14].

2.3.2. High performance liquid chromatography (HPLC) analysis

The KPE was dissolved in dimethyl sulfoxide (DMSO) at 5 mg/ml and centrifuged (5000g, 25 °C, 10min) to remove insoluble material. After filtration, the supernatant was analyzed using a Dionex U3000 HPLC system (Thermofisher, Sunnyvale, CA) equipped with a temperature-controlled autosampler, column oven, and diode detector. 5 µL of supernatant was injected into the phenomenex EVO C18 100A, 2.6µ, 50 x 3 mm column (Torrance, CA) and the compounds were eluted by a gradient of 15% solvent A (10 mM formiat ammonium, 0.3 mM EDTA, pH 3.5) in 100% solvent B (methanol) for 20 min followed by re-equilibration of the column, using a flow rate of 0.5 mL/min at 25 °C.

2.4. Quantitative analysis of KPE

For these analyses, 1% KPE solution was prepared in methanol and used.

2.4.1 Assay of total polyphenols

Folin-Ciocalteux method described by (15) was used. In brief, 100 µL of extract solution, 0.2 mL of Folin-Ciocalteux's reagent v/v diluted to 10^{th} and 2 mLof distilled water were put in a test tube. After 3 minutes of incubation at room temperature, 1 mL of saturated sodium carbonate ($NaCO_3$) 20% solution was added. The whole was incubated at room temperature for 1 hour and the absorbance read at 765 nm. Gallic acid was used as a standard and the polyphenols content were expressed in mg gallic acid equivalent/g dry matter (mg EAG/g DM) using the calibration line.

2.4.2 Assay of flavonoids

In a test tube, 0.5 mL of extract solution and 0.5 mL of aluminum trichloride ($AlCl_3$) 2% were put and the mixture incubated for 30 min in the dark. Then, the absorbance of the yellow color read at 420 nm. Quercetin was used as standard and the flavonoids content determined from the calibration line and expressed in mg of quecretin equivalent/g of dry powder (mg EQ/g of DM).

2.4.3 Assay of flavonols [15]

One mL of 1% KPE solution, 1 mL of aluminum chloride ($AlCl_3$; 2% in ethanol); 1.5 mL of sodium acetate were successively added in a test tube and the mixture incubated (2.5 hours, 20 °C). Then, the absorbance of the developed yellow color was read at 440 nm. Quercetin was used as standard and the flavonol content determined from the calibration line and expressed as mg EQ/g DM.

2.5. Determination of the antioxidant properties of KPE

The antioxidant properties of KPE were determined using the 2,2-diphenyl-1-picryl hydrazyl (DPPH°) and hydroxyl (OH°) radicals scavenging, lipid and protein peroxidation inhibition, the Erythrocyte Plasma Membrane Redox System (EPMRS) assays and the total antioxidant capacity test. For these different analysis, the experiment was done in triplicate and the extract dissolved in DMSO and tested at final concentrations of 0.1; 1; 10 and 100µg/mL. Vitamin C was used as positive control and treated under the same conditions as the extract.

2.5.1 Free radical scavenging activities assay

2.5.1.1 DPPH° radical scavenging activity

The procedure used was that of (16). In brief, 3.1 mL of the methanolic solution of DPPH° 40 µg/mL, 50 µL of KPE extract were added in a test tube. In the negative control tube, the extract was replaced by 50 µL of DMSO. The mixtures were well homogenized and incubated in the dark for 30 minutes at room temperature and the absorbance read at 517 nm for scavenging percentage calculation as the following equation (E):

% scavenging = $(OD_{control}-OD_{test})/ OD_{control} \times 100$ (E), with:
$OD_{control}$: Optical Density of the negative control; OD_{test}: Optical Density of the sample

2.5.1.2 Hydroxyl radical HO° scavenging activity

In a test tube, 50 µL of KPE extract, 0.7 mL of FeSO4 (3 mM), 1 mL of H_2O_2 (1 mM), 1 mL of distilled water and 0.4 mL of sodium salicylate (10 mM) were added according to (17). In the negative control tube, the extract was replaced by 50 µL of DMSO while the blank contained distilled water instead of sodium salicylate. Mixtures were incubated (1 hour, 37 °C) and absorbance read at 562 nm against blank. The different entrapment percentages were calculated using the above (E) formula

2.5.1.3. Phosphomolybdenum reducing assay (Total Antioxidant Capacity)

In each test tube, were successively added 50 µl of polyphenolic extracts, 1 ml of 0.6 M sulfuric acid, 1050 µl of 28 mM sodium phosphate and 1050 µl of molybdate of 4 mM ammonium. The mixtures were then incubated (90 minutes, 95 °C) and cooled. After incubation and cooling, absorbances were read at 695 nm. The Total Antioxidant Capacity (TAC) was estimated, as previously described by (16), in mg ascorbic acid equivalent per gram of extract (mg EAA/g of extract).

2.6. *In vitro* lipid and protein peroxidation inhibition assays

For lipid peroxidation inhibition assay, the procedure used by (10) was followed. In brief, lipid peroxidation was induced in rat liver homogenate with $FeCl_2$-H_2O_2. 50 µL of KPE extract was mixed with 1 mL of 10% (w/v) rat liver homogenate; then, 50 µL of H_2O_2 (0.5 mM) and $FeCl_2$ (0.5 mM) each, were added. The mixture was incubated (60 min, 37 °C) before the addition of 1 mL of trichloroacetic acid (15%, w/v) and thiobarbituric acid (0.67%) respectively. The resulted mixture was heated for 15 min at 100 °C in a water bath, followed by centrifugation (3000 g, 5 min, 4 °C). Finally, the supernatant was collected and the absorbances recorded at 532 nm.

As far as protein oxidation inhition assay is concerned, the method described by (19) was used. Quercetin was taken as positive control. In each test tube were added, 0.5 mL of 10% rat liver in 0.9 mL phosphate buffer (50 Mm, pH 7.4), plus 100 µLof KPE extract. The extract was replaced in blank and negative control by DMSO. Then, 250 µL of FeSO4 0.01 mM and vitamin C 0.1 mM were added except the blank in which it was replaced by 0.5 m¨L of distilled water. The mixture was incubated (30 min, 37 °C) and 1 mL of 2,4-dinitrophényl hydrazine (DNPH) 10 mM was added in positive and negative control whereas in blank 1 mL of HCl 2 N was added. All the tubes were incubated at room temperature and vortexed every 15 min. After incubation, 1 mL of 10% TCA was put in each tube. The mixtures were centrifuged (3000 trs/min, 10 min, 4° C) and the pellets washed three times with 2 mL of ethanol/ethyl acetate solution 50% v/v. The washed pellets were suspended in 1mL of urea 6M, incubated (37 °C, 10 min) and centrifuged (3000 trs/min, 5 min, 4° C). Absorbance of supernatant from the last suspension was read at 372 nm.

For both experiments, the result expressed as percentage of inhibition was determined with the above formula (E) for half inhibition concentration (IC_{50}) calculation.

2.7. Determination of the effects of KPE on the Erythrocyte Plasma Membrane Redox System (PMRS)

The procedure used was that of (4).

2.7.1 Sheep blood sample collection and erythrocytes isolation

After slaughtering of healthy sheep at the Yaoundé modern slaughterhouse, blood sample was collected in heparinized test tubes and transported to the laboratory in cooler containing ice pack sand and centrifuged (1800g, 10 minutes, 4 °C). The supernatant (Plasma) was discarded and the pellet (Erythrocytes) was washed twice with phosphate buffer salin pH 7.4 and conserved at 4 °C until use.

2.7.2 Erythrocytes treatment with KPE

To 300 µL volume of erythrocytes suspension, were added 2.4 mL de PBS containing 5 mM of glucose and 300 µL of KPE extract. The extract was replaced in control tube by 300 µL of PBS. The mixtures were incubated (37°C, 30 min), centrifuged (1800g, 10 minutes, 4°C) and pellets washed once with PBS. The resulting erythrocytes suspensions were collected and used for the PMRS activity assay.

2.7.3 PMRS activity assay

A volume of 1.7mL PBS-glucose and 100 µL of 20 mM potassium ferrocyanide 20 mM were added to 200 µL previous erythrocytes suspension. In the blank tube, only 1.9 mL of PBS containing glucose and 0.1 mL of potassium ferrocyanide were added. All the tubes were incubated for 30 minutes at 37 °C and centrifuged (1800g, 10 minutes, 4 °C). To 200 µL of each collected supernatant were added respectively 1.2 mL of distilled water, 200 µL sodium acetate (0.3 M), 200 µL of citric acid (0.2 M), 100 µL of ferric chloride (3.3 mM) prepare in 0.1 N acetic acid and 100 µL disulfonic bathophenanthrolin acid (6.2 mM). The resulting mixtures were incubated for 10 minutes at room temperature and the absorbance of developed pink color were read against the blank at 535nm. The pourcentage of activation were calculated according to the following formula.

$$\% \text{ of activation of PMRS} = [(OD_{assay} - OD_{control}) / OD_{control}] \times 100$$

Where: $OD_{control}$: absorbance of the negative control tube OD_{assay}: absorbance of the test tube

2.8. Determination of the effect of KPE on tumor necrosis factor alpha (TNFα) production in *Jurkat T cells*

For the effect of KPE on TNFα production, Jurkat T cells (Cone E6-1, ATCC® TIB-152TM) were used. A suspension of $2x10^6$ cellules/mL between 11 and 12 passages were stimulated with a mixture of 50 ng/mL phorbol 12-myristate 13-acetate and 1 µg/mLionomycin (PI) in the presence of KPE extract. The methylene chloride/methanol (75/25; *v*/*v*) (F25) demonstrated anti-inflammatory fraction of *K. grandifoliola* (10) was used as standard. Cells were incubated for 4 hours at 37°C in the presence of 5% CO_2. After incubation, cells were centrifuged (600g, 10 minutes, 4 °C) and the supernatant was kept at -80 °C until use. TNF-α production was assayed in the supernatant using Human TNF-α ELISA MAX™ Deluxe kit (Biolegend, San Diego, CA, USA) according to the manufacturer protocol.

2.9. Data analysis

Data analysis was performed using GraphPad Prism 8.0.1 software. The results were expressed as mean±standard deviation and the different values were compared using the analysis of variance test "one-way ANOVA" followed by the multiple comparison test of Turkey with a p-value $p<0.05$.

Results

Extraction yields, Qualitative and quantitative phytochemical profile of KPE extract

Extraction yield of KPE was 19.07%. The qualitative screening revealed the presence of polyphenols, flavonoids, tannins and leuco-anthocyanins (Table 1). HPLC fingerprint of KPE showed several peaks with various retention times detected at 254 and 370nm, probably denoting the availability of aromatic compounds within the extract (Figure 1A and 1B), with a confirmation of pics observed by UV analysis (Figure 1C and 1D). The quantitative analysis showed that the total phenolic compounds, flavonoids and flavonols contents ranged from 0.44 to 57.93mgEGA/g of DM (Table 2).

Table 1: Phytochemical profile of KPE

Phytochemicals	TP	Fl	Ta	La	Fq	An	Al	TS	Sa	Su
KPE	+	+	+	+	+	-	-	-	-	-

Tp: Total polyphenols; Fl: Flavonoids, Ta: Tanins; La: Leuco-anthocyanes; Fq: Free quinones; An: Anthraquinones; Al: Alkaloids, TS: terpens and Sterols; Sa: Saponines; Su:Sugar; PEK: polyphenolic extract of *Khaya grandifoliola;* (+) = present; (-) = absent.

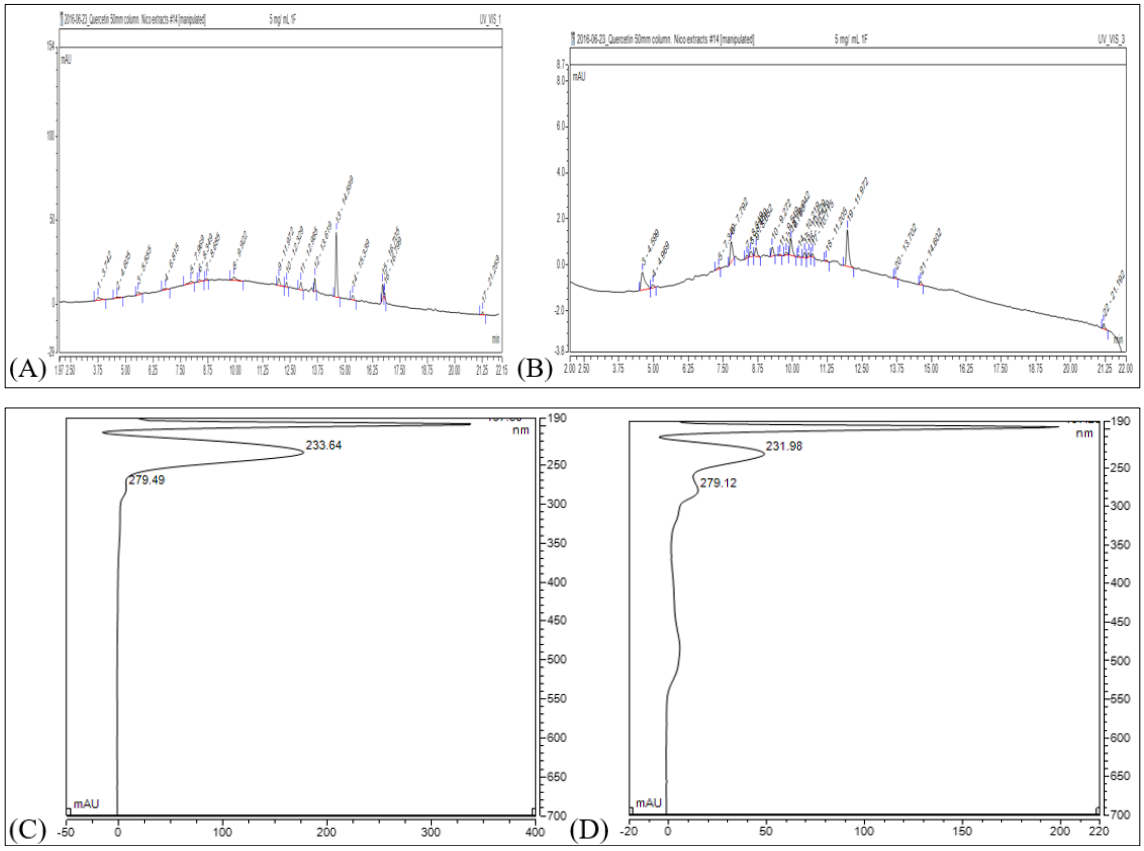

Fig 1: HPLC profile of KPE. (A): chromatogram at 254 nm; (B): chromatogram at 370 nm as function of retention time; (C): Absorption band in UV of the 13th pic at 254 nm; (D): Absorption band in UV of the 19th pic at 370 nm.

Table 2: Phenolic compounds content and TAC of KPE

Classes of compound	Total polyphenols (mgEGA/g DM)	Flavonoids (mgEQ/g DM)	Flavonols (mgEQ/g DM)
Content	57.93±0.42	2.84±0.04	0.44±0.02
TAC (mgEAA/g DM)	76.82±0.35		

KPE: *Khaya grandifoliola* Polyphénolic Extract; EGA: Equivalent of Gallic Acid; DM: Dried Material; EQ: Equivalent Quercetin; AA: Ascorbic Acid; TAC: Total Antioxidant Capacity

3. Antioxidant properties of the KPE extract

3.1 Antiradicalar property

The antiradical property was assessed by scavenging of the DPPH° and HO° radicals. KPE extract effectively trapped those two radicals in a concentration-dependent manner (Figure 2A and 2B) with respective IC_{50} value of 6.25±0.07µg extract/mL and 11.65±0.92µg extract/mL (Table 3). The corresponding activities for ascorbic acid (AA) were 5.00±0.00 µg extract/mL and 7.9±0.00µg extract/mL.

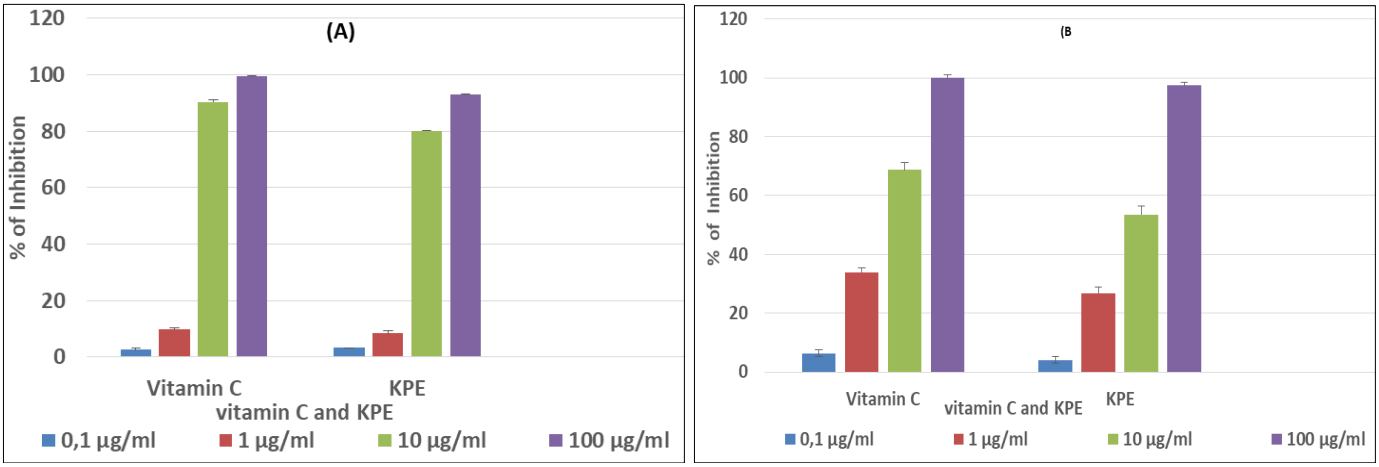

Fig 2: Radical scavenging activity of KPE (A): DPPH° and (B): HO°

Table 3: IC_{50} and EC_{50} values (µg/mL) of KPE activities

	Tested extract and Standards			
Assays	**Ascorbic Acid**	**Quercetin**	**F25**	**KPE**
DPPH radical scavenging	5.00±0.00			6.25±0.07
HO° radical scavenging y	7.90±0.00			11.65±0.92[a]
Inhibition of lipid peroxidation	8.70±0.14			8.50±0.14
Inhibition of protein oxidation		9.40±0.14		8.40±0.14
Activation of the redox system		4.60±0.00		5.90±0.00
TNFα inhibition			32.10±1.13	71.05±1.20[a]

KPE: *Khaya grandifoliola* Polyphenolic Extract; F25: Proven anti-inflammatory fraction of *K. grandifoliola*; EC_{50}: Efficient Concentration 50; IC_{50}: Inhibitory Concentration 50;[a]: significantly different from standard at $p < 0.05$.

3.2 Lipids and proteins oxidation inhibitory property

KPE exhibited good inhibitory activity of membrane lipid peroxidation and proteins oxidation in a concentration-dependent manner (Figure 2C and 2D). This observation was also justified by low values of half inhibitory concentration (IC_{50}) (Table 3) not significantly ($p<0.05$) different from that of the standard ascorbic acid and quercetin.

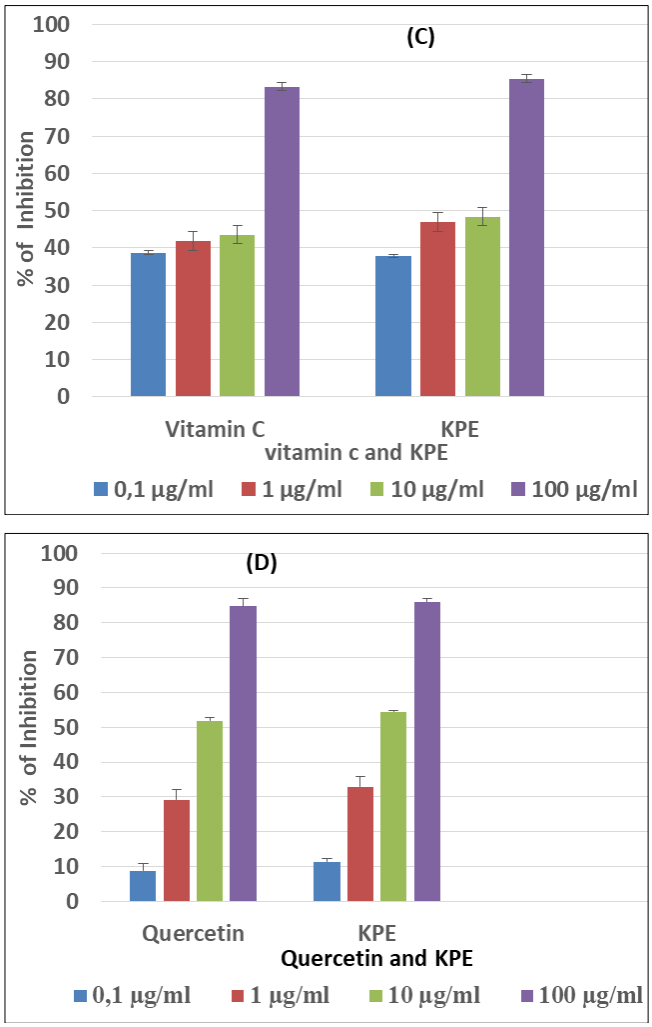

Fig 2: Inhibitory effect of KPE (C): lipid peroxidation; (D): protein oxidation.

3.3 Total antioxidant capacity

The total antioxidant capacity was assessed by the phosphomolybden method. KPE strongly reduced the phosphomolybdic complex as demonstrated by its relatively high obtained value (Table 2).

3.5 Activation of the plasma membrane redox system

KPE effectively activated the plasma membrane redox system of sheep red blood cells in a concentration-dependent manner as shown in the Figure 3. KPE activated the PMRS with an EC_{50} comparable to that of (Table 2).

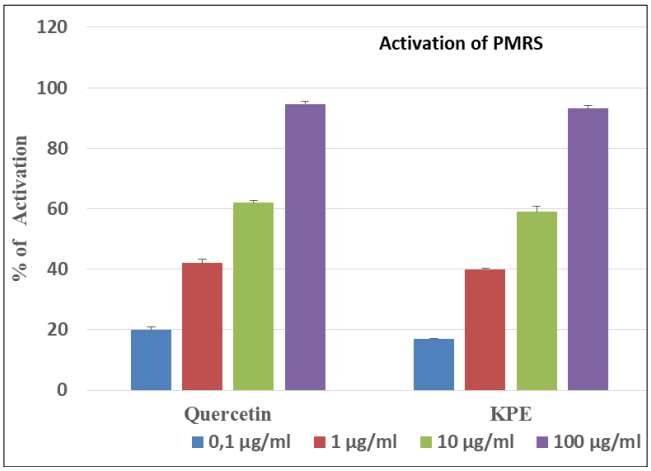

Fig 3: Activation effect of KPE on the erythrocyte PMRS.

4. Anti-TNFα activity

KPE was found to inhibit TNFα production in Jurkat T cells in a concentration dependent manner (Figure 4) with an IC_{50} less pronounced than that of F25 (Table 2).

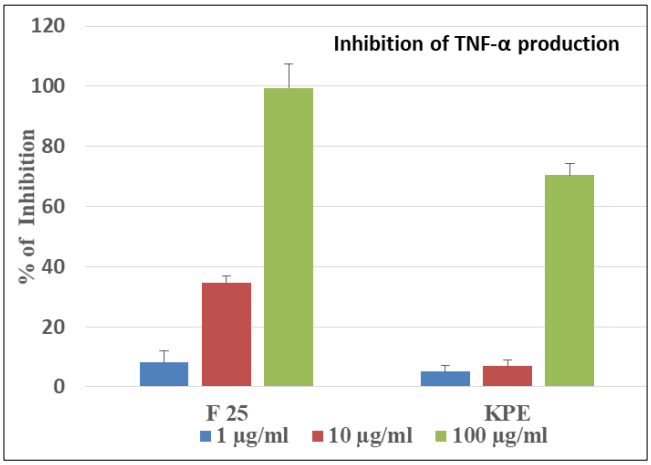

Fig 4: Inhibitory **e**ffect of KPE on the TNF-α production in Jurkat T cells. KPE: *Khaya grandifoliola* polyphnolic extract; F25: proven anti-inflammatory fraction from *K. grandifoliola.*

5. Discussion

Oxidative stress caused by an excess of reactive oxygen species (ROS) lead to several pathologies [20, 21]. ROS are critical mediators of oxidative stress pathways and play a significant role in the onset and progression of inflammatory responses [22]. These mediators can activate transcription factors such as nuclear factor kappa-B (NF-KB) which induce the transcription of several genes that code for pro-inflammatory molecules such as Tumor Necrosis Factor α

(TNF-α). TNF-α is strongly implicated in several diseases associated with inflammation. Antioxidants and anti-inflammatories play an important role in the body's defense system. Antioxidants act through various mechanisms such as free radical scavenging, reduction of oxidative compounds, chelation of transition metals or biomolecules protection [23]. It is therefore important to use several methods to assess the antioxidant potential of a plant extract [24]. Polyphenolic compounds have demonstrated several biological properties, which result in the modulation of the homeostasis of key cellular processes, such as the metabolism, the energy balance, the redox equilibrium, the cell signaling, the inflammatory response, and the control of oxidative stress [25]. In this study, a concentrated phenolic compounds extract (KPE) was prepared from *K. grandifoliola with an extraction yield of 19.07%.* Antioxidant as well as anti-TNFα activities of this extract were assessed after phytochemicals screening. The total polyphenols content of KPE is higher (57.93±0.42 mgEGA/g of DM) compare to that of *Mentha longifolia* leaf extract 23.52 ± 0.14 mgEGA/g [26]. Hence, it has an important part of flanonoids (2.84±0.04 mgEQ/g of DM). The qualitative colorimetric analysis was complemented by analytical HPLC (Figure 1) performed at two wavelengths (254 and 370 nm) that correspond to the absorption zones of flavonoids [27]. UV analysis of the two major peaks (Peak 13 and 19) at those wavelengths showed they have an absorption band, in flavonoids absorption zones, between 231.98 and 279.49 nm, respectively. Free radicals scavenging activity of KPE was assessed through DPPH° and HO° tests. DPPH° is the synthetic radical commonly used to assess the antiradical activity of plant extracts. It is a free radical that can be stabilized by an antioxidant atom of hydrogen. In this study, KPE effectively scavenged DPPH° radical in a concentration-dependent manner with an IC_{50} comparable to the one of Ascorbic Acid (AA). Since DPPH° free radical is not in biological systems, we have tested the capacity of KPE to interact with the hydroxyl radical HO°. This radical could be formed in biological cells and it is one of the most biological reacting and dangerous radicals. It's especially the initiator of the macromolecules damage (3). Therefore, the scavenging of the HO° radical can be an effective means of defense against several diseases associated with oxidative stress. This radical generated *in vitro* by the $FeSO4/H_2O_2$ system, was effectively scavenged by KPE and the activity (IC_{50}: 11.65±0.92 µg extract/mL) was higher than that obtained with the methanolic extract of Zingiber officinale (IC_{50}: 22.36 µg/ml) [28]. Moreover, the total antioxidant capacity (TAC) of KPE was assessed and found to be 76.82±0,35mgEAA/g of DM. Phenolic compounds concentrated in KPE express a TAC not too different of that of *Mentha longifolia* leaf extract [26]. All those antioxidant properties of KPE could limit exposure of erythrocytes to excess ROS which could decrease the PMRS activity and consequently the concentration of extracellular ascorbic acid and the antioxidant blood potential. Erythrocytes PMRS plays an essential role in maintaining the level of plasma ascorbic acid, the primary antioxidant and the main reduced equivalent of this system [4]. KPE as well as quercetin stimulated erythrocyte PMRS. These activities were similar to those exhibited on erythrocyte PMRS by curcumin extracted from *curcuma longa* [5]. Polyphenols as anti-inflammatory agents, act through protection of proteins from denaturation, regulation of pro-inflammatory mediators and transcription factors involved in the inflammatory process [29]. Inflammation is the first response to tissue injury, but it must be controlled immediately. The NF-KB factor plays a very important role in this process as it induces the transcription of a wide range of genes that code for pro-inflammatory mediators such as TNF-α [22]. KPE effectively inhibited the production of TNF-α but less than the methylene chloride/methanol fractions of *K. grandifoliola* (F25) which was three times as active. This anti-inflammatory activity may be related to the presence of flavonoids which act either by inhibiting the nuclear translocation of NF-KB or by inhibiting transcription or translation [30].

Conclusion

KPE is a concentrate of phenolic compounds from *Khaya grandifoliola* stem bark. Phenolic compounds are secondary plant metabolites with remarkable health-promoting properties. KPE presented a rich phenolics profile and ensured a good antioxidant and anti-inflammatory properties inhibiting lipids and proteins oxidation and the production of TNF-α *in vitro*. The extract also activated erythrocyte PMRS and therefore might contribute to maintain the level of plasma antioxidant status. All those health benefits of KPE suggest the extract as potential ingredient of functional foods to combat health conditions involving oxidative stress and inflammation.

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