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GC-MS analysis and inhibitory effect of *Kigelia africana* Leaf extract and fractions on 5-lipoxygenase

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Abstract
The study investigated the inhibitory role of kigelia africana leaf extracts and fractions on 5-Lipoxygenase oxidative enzyme. GC-MS analysis was carried out on the ethyl acette fraction. Fresh leaves of *Kigelia africana* was extracted with 70% (v/v) ethanol and fractionated with n-hexane, ethyl acetate and butanol. Plant extract and fractions were estimated for total phenol content and flavonoid content using gallic acid and quercetin as standard respectively. Anti-lipoxygenase activity was determined from the extract and fractions at 0.1 mg/ml to 0.5 mg/ml. Fraction which exhibited the most favourable inhibitory potential was subjected to GC-MS fingerprinting to identify the phytoconstituents. The highest total phenolic and total flavonoid content was obtained from the ethyl acetate fraction with values of 22.55±0.55 gallic acid equivalent and 4.63±0.19 quercetin equivalent respectively. Lipoxygenase inhibitory activity of the plant extract and fractions showed that the crude extract, aqueous and ethyl acetate fractions exhibited their highest inhibitory potential at 0.5 mg/ml while n-butanol and n-hexane showed their highest inhibitory effect at 0.2 mg/ml and 0.4 mg/ml respectively. GC-MS analysis of the ethyl acetate fraction revealed the presence of several constituents with anti-inflammatory properties. *Kigelia africana* leaf extract and fractions could serve as a non-selective redox inhibition of 5-lipoxygenase.

Keywords: inflammation; lipoxygenases; kigelia africana; antioxidant; leukotrienes

1. Introduction

Lipoxygenases are a group of highly efficient oxidative enzymes having a non-heme iron atom in their active site and are involved in the metabolism of poly-unsaturated fatty acids (PUFA) [1]. These enzymes are involved in the regulation of inflammatory responses by generation of pro-inflammatory and anti-inflammatory mediators known as leukotrienes and lipoxins respectively. They catalyse the addition of oxygen into poly-unsaturated fatty acids (PUFAs) such as arachidonic acid and linoleic acid [2]. Studies revealed that their mechanism of action involves the activation of nuclear factor kB [3, 4], a major transcription factor implicated in signal transduction and pathogenesis of inflammatory diseases such as asthma, arthritis, inflammatory bowel diseases (IBD) and chronic obstructive pulmonary diseases (COPD) [5-7]. This subsequently indicates the relationship between lipid signaling and transcription factor activation. As such, several data have suggested that inhibition of lipoxygenase activity and leukotriene biosynthesis can be a valuable approach for treatment of such inflammatory diseases [8]. The recognition of the function of PUFAs, especially arachidonic acid metabolites in numerous inflammatory diseases has inadvertently led to a significant drug discovery effort around arachidonic acid metabolizing enzymes. Unfortunately, progress in this research area has been hindered due to lack of specificity of the developed inhibitors and sparse information on arachidonic acid metabolising enzymes and their functions. Hence, a more thorough research has been encouraged to study the activity of arachidonic acid metabolizing enzymes, especially the lipoxygenases and development of more selective inhibitors [1]. Further research revealed that 5- lipoxygenase inhibitors can act as non-selective antioxidants by reducing the active-site ferric iron thereby interrupting the catalytic cycle [9]. Examples of these non-selective redox-inhibitors include secondary metabolites like flavonoids and coumarins. Also, compounds that possess high radical scavenging activity or metal chelating activity are capable of inhibiting 5-lipoxygenases [9-11].

Kigelia africana (Lam.) Benth, commonly referred to as sausage tree because of its big and extended fruits is native to African and extensively cultivated in Southern, Central and West Africa [12, 13]. It belongs to the family Bignoniaceae and the fruits, stem, leaves and roots have been found to possess several secondary metabolites and phytochemicals [14]. As a result of

this, Kigelia africana plant has been said to possess so many medicinal properties. Most importantly, *K. africana* are known to be a rich source of flavonoids when compared with other medicinal plants [15, 16]. However, the role of this plant on the lipoxygenase enzyme has not been investigated. Considering the many medicinal purposes for which this plant is used, there is therefore need to investigate its potential to inhibit this inflammatory oxidative enzyme, lipoxygenase.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and drugs

5-Lipoxygenase enzyme, linoleic acid, gallic acid, quercetin, Follin Ciocalteu's reagent and ascorbic acid were obtained from Sigma Fine Chemicals Limited, Uppsala, Sweden and British Drug House (BDH) Chemicals Limited, London. All other reagents used in the study were of analytical grade and were obtained from reputable sources.

2.1.2 Plant collection, authentication and preparation

Fresh leaves of *Kigelia africana* (Lam.) Benth were collected from Ayegunle, Ekiti State, Nigeria. Leaves were identified and authenticated at IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife. *Kigelia africana* leaves were air-dried and pulverized with impact mill. Fine powder was soaked in 10 L 70 % (v/v) ethanol for 72 hr with regular mixing. The extract was sieved using a cheese cloth and filter paper (Whatman No. 1). Filtrate obtained was dried under controlled temperature with a rotary evaporator.

2.1.3 Partitioning into fractions

Ethanol extract was partitioned successively with solvents of different polarities to obtain the hexane, ethyl acetate and butanol fractions using a separating funnel. Respective fractions obtained were dried under controlled temperature with the aid of a rotary evaporator. Aqueous fraction was also obtained. All the fractions were weighed and kept inside a desiccator for *in vitro* assays.

2.2 Method

2.2.1 Determination of lipoxygenase (LOX) inhibitory concentration

Inhibition of lipoxygenase was determined spectrophotometrically according to the procedure of Lyckander and Malterud [17] with minor modifications. The principle of inhibition is based on the oxidation of linoleic acid which is catalyzed by lipoxygenase. The reaction forms a product with a conjugated double bond that can be measured spectrophotometrically at 234 nm.

Lipoxygenase solution (100 µL) prepared in 0.2 M boric acid buffer was mixed with 25 µL *K. africana* crude extract and solution of the various fractions. The various mixtures were incubated at 25 ^{o}C for 3 mins after which 250 µL linoleic acid (250 µM) was added to initiate the reaction. Rate of product formation (Ve) was observed at 234 nm for 3 min and the absorbance was taken. Methanol (1 %) served as negative control which is the activity of the enzyme without sample (extract or fraction) while quercetin was the standard drug used as positive control. Lipoxygenase enzyme inhibitory concentration was determined from the percentage inhibition.

$$\text{Percentage Inhibition} = \frac{(\text{Ve of negative control - Ve of sample})}{\text{Ve of negative control}} \text{ X } 100$$

Ve of control = enzyme activity without the fraction
Ve of sample = activity of enzyme with the fraction.

One unit of lipoxygenase activity is defined as one micromole of hydroperoxide formed per minute at 25 ^{o}C.

2.2.2. Determination of total Phenol and flavonoid contents

Kigelia africana crude extract and fractions were evaluated for their total phenol and flavonoid contents according to the method of Singleton and Rossi [18] and Miliauskas *et al.* [19] respectively.

2.2.3 Gas chromatography-mass spectroscopic (GC-MS) analysis

Agilent USA hyperherated to a mass spectrophotometer (5975 C) having a triple axis detector and equipped with a ten microliter auto injector syringe was used while Helium gas served as the carrier. An aliquot of 1 µl of ethyl acetate solution was injected into the column with the injector temperature at 250 ºC. GC oven temperature started at 110 ºC and holding for 2min and it was raised to 200 ºC at the rate of 10 ºC/min, without holding. Holding was allowed at 280 ºC for 9 min with program rate of 5 ºC/min. The injector and detector temperatures were set at 250 ºC and 280 ºC respectively. Ion source temperature was maintained at 200 ºC. The mass spectrum of compounds in samples was obtained by electron ionization at 70 eV and the detector was operated in scan mode from 45-450 amu (atomic mass units). A scan interval of 0.5 seconds and fragments from 45 to 450 Da was maintained. The total running time was 36 minutes. Total elution time of 47.5 mins was recorded. Analyzed sample was then compared using the mass spectra obtained with National Institute of Standards and Technology Library standard mass spectra II (NIST II).

2.3 Statistical analysis

All measurements were done in triplicate and results were expressed in terms of Mean ± SEM using Graphpad Prism 5.0 Statistical software. One-way analysis of variance (ANOVA) was conducted and p value less than 0.05 was considered as significantly different.

3. Result and Discussion

Table 1: Total phenolic and total flavonoid content of the ethanol extract and fractions of *Kigelia Africana*

Extract/Fraction	TFC (mg QUE/g)	TPC (mg GAE/g)
Ethanol extract	0.67 ± 0.27	7.84 ± 0.42
n-Hexane	0.44 ± 0.14	5.73 ± 0.00
Ethyl acetate	4.63 ± 0.19	22.55 ± 0.55
n-Butanol	0.51 ± 0.34	18.77 ± 0.55
Aqueous	0.81 ± 0.07	12.67 ± 0.72

Values expressed per g of plant extract and are means of triplicate determination ± standard deviation [Mean ± SEM (n = 3)]

QUE: Quercetin Equivalent. GAE: Gallic Acid Equivalent.
TFC: Total Flavonoid Content.
TPC: Total Phenolic compound.

Table 2: Lipoxygenase inhibitory activity of ethanol extract and fractions of kigelia africana leaf

Concentrations (mg/ml)	Ethanol Extract	Aqueous Fraction	N-Butanol Fraction	Ethyl-acetate Fraction	N-Hexane Fraction	Quercetin
0.1	82.69 ± 2.83	73.34 ± 0.27	75.36 ± 2.69	59.44 ± 0.69	81.27 ± 1.11	93.02 ± 0.17
0.2	80.61 ± 1.67	84.41 ± 1.41	89.03 ± 0.63	72.25 ± 3.75	79.39 ± 1.21	94.85 ± 0.27
0.3	83.71 ± 2.31	79.12 ± 3.97	77.18 ± 3.15	85.72 ± 1.00	76.27 ± 1.53	92.63 ± 2.82
0.4	87.63 ± 0.32	82.71 ± 0.63	82.39 ± 0.45	82.12 ± 0.62	87.69 ± 0.41	97.90 ± 0.46
0.5	91.71 ± 0.50	85.81 ± 0.12	84.83 ± 0.69	91.66 ± 0.86	86.16 ± 0.78	96.68 ± 0.67

Values expressed are means of triplicate determination ± standard deviation [Mean ± SEM (n = 3)]

Table 3: Prominent compounds obtained from ethyl acetate fraction of kigelia africana using GCMS analysis

Peak no	Retention Time	Peak Area (%)	Name of Compound
1	10.955	2.28	Phenol
1	10.955	2.28	Phosphonic acid, (p-hydroxyphenyl)
2	14.340	7.02	Phenol, 2-methoxy-
3	17.734	1.93	Ethanone, 1-(3-methylphenyl)-
4	37.341	13.49	Cyclopentane, 1,2-dimethyl-1-(1-methylethenyl)-
5	37.843	7.84	3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol
6	38.967	47.58	Hexadecanoic acid, ethyl ester
7	39.752	19.85	Phytol

Peaks corresponding to NIST (II) Library with the respective retention time

The processes of redox regulation of NF-κB and other transcription factors are highly intricate and are proposed to work by varying the level of IKKβ activity [20]. Reactive oxygen species (ROS) are reported to play an active role in both innate and immune responses and as such can affect several steps involved in these processes. However, unravelling the processes of signal transduction by ROS has remained the subject of intense study. NF-κB alteration by free radicals has reportedly led to oxidative stress. The consequence of this as reported by Khatami [21] is chronic inflammation, autoimmune diseases and cancer.

Phytotherapy has been described as the treatment of diseases or several other metabolic syndromes with medicinal plants which are beneficial to human body. Studies have reported the surprising effects that nutrients from medicinal plants play in the management of several human diseases [13]. One of the plants reckoned to play such significant role is *Kigelia africana* and several bioactive compounds such as polyphenols and tannins have been confirmed to be present in the plant [15]. Studies have also shown that flavonoids, phenols, polyphenols, tannins and coumarins are able to inhibit the activities of some inflammatory mediators and enzyme [23, 24]. From the study, the total phenol content (TPC) in K. africana crude extract and fractions (determined as gallic acid equivalent (GAE)) showed that ethyl acetate fraction has the highest phenolic content (22.55 ± 0.55 mg GAE/g) with the phenolic contents order of EAF > AqF > BF > EE > HF (Table 1). Likewise, highest total flavonoid content of the plant was obtained from the same fraction (4.63 ± 0.19 mg QUE/g), while the least content was observed in n-hexane fraction in the order of EAF > AqF > EE > BF > HF. These results are comparable with those obtained by other authors which revealed that K. africana is flavonoid-rich when compared with other medicinal plants [15]. Furthermore, free radicals have been implicated in oxidative stress leading to incidence of several pathological diseases. The involvement of polyphenols like flavonoids have been implicated in the suppression of inflammation linked disorders due to their phenolic group which helps to stabilize highly reactive oxygen radical species thereby acting as redox inhibitors [16]. *In vitro* lipoxygenase inhibition has been reported to represents a useful standard for the selection of plants with anti-inflammatory potentials [25]. Lipoxygenases are key enzymes implicated in diseases mediated by inflammation and free radical activities leading to several diseases like neurodegenerative diseases, AIDS, cardiovascular diseases, cerebral ischemia and certain immunological dysfunctions [15]. Its inhibition could be a way of finding new compounds with anti-inflammatory ability [26]. Inhibitory effect of *Kigelia africana* fractions and extract on the enzyme was evaluated at different concentrations (Table 2). The plant showed a remarkable inhibitory effect on lipoxygenase enzyme at all the concentrations when compared with the standard drug, quercetin. However, at the highest concentration of 0.5 mg/ml, ethanol extract and ethyl acetate fraction exhibited higher inhibitory effect (91.71 ± 0.50 and 91.66 ± 0.86 respectively). This could have been a consequence of high concentration of polyphenolic compounds as indicated by the high concentrations of flavonoid and phenols in the ethyl acetate fractions of the plant. Due to its high volatile behaviour, ethyl acetate has been observed to extract more active phyto constituents than other constituents. GC-MS analysis of the ethyl acetate fraction of *k. africana* revealed the presence of several phyto constituents (Table 3) including hexadecanoic acid and phytol which upregulate antioxidant activities and is linked to the prevention of inflammation, restoration of cellular dysfunction and protection against DNA damage and apoptosis [27]. Hence, the inhibitory effect of ethyl acetate fraction of *K. africana* may be associated with the presence of numerous health benefiting phytoconstituents

4. Conclusion

Plants with high polyphenolic contents like kigelia africana may serve as an effective inhibitor of lipoxygenase enzymes. However, further work is needed to identify the active anti-lipoxygenase agent present in the plant.

5. Conflict of interest

Conflict of interest declared none.

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