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Research Article

Effects of Tannin Extract from *Gongronema latifolium* Leaves on Lipoxygenase *Cucumeropsis manii* Seeds

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Lipoxygenase (EC 1.13.11.12) was partially purified from germinated seeds of *Cucumeropsis manii* to a purification fold of 47.14, enzyme activity recovery of 72.18%, and specific activity of 326.25 units/mg protein, using a three-step process of centrifugation, ammonium sulphate precipitation and gel filtration. Kinetic properties show maximum activity at pH 6.0 and at optimum temperature of 40°C. Inhibitory effects of the extract from *Gongronema latifolium* and two other known antioxidants: ascorbic acid and propyl gallate on lipoxygenase from *Cucumeropsis manii* were studied. Result shows presence of inhibition with IC50 of $4.2 \times 10^{-3} \pm 0.09 \times 10^{-3}$ g/L, $4.3 \times 10^{-2} \pm 0.11 \times 10^{-2}$ g/L and $7.9 \times 10^{-2} \pm 0.11 \times 10^{-2}$ g/L for the extract from ascorbic acid and propyl gallate, respectively. The extract when compared to the other antioxidants exhibits a competitive mechanism of inhibition. This tannin extract could be included during food processing as preservative against food deterioration that might be caused by oxidative enzymes such as lipoxygenase.

1. Introduction

Lipoxygenase (EC 1.13.11.12) (LOX) catalyzes the addition of molecular oxygen to the *cis*, *cis*-1, 4-pentadiene system in polyunsaturated fatty acids forming hydroperoxides and is widely distributed in plants [1], animals, and microorganisms [2]. The products of the degradation of polyunsaturated fatty acids and their derivatives can react with proteins, peptides, and aminoacids, which can result in off-flavour production and rancidity of oils thereby lowering nutritional values of oil-based foods [1].

Oleaginous seeds are known to contain LOX [3]. The seeds of *Cucumeropsis manii* are oil seeds [4], having mostly polyunsaturated fatty acids and are of high nutritive value and thus potential for human and livestock feed [5–7]. *Cucumeropsis manii* seed kernels are major soup ingredients and they are used as a thickner and flavour component of soups [4, 8]. Many fats- and oil-based foods including these soup thickeners are susceptible to deterioration as a result of enzymatic activities (of which lipoxygenase is one of them), thereby posing a storage problem [9]. Very few reports are available on lipoxygenases belonging to this family of Cucurbitaceae [10].

Owing to the uncertain future for chemical preservatives and public concern over residues in prepared products, there has been an increasing interest in the use of natural enzyme inhibitors as a nonchemical method for food preservation.

Gongronema latifolium is an herbaceous nonwoody plant from the family of Asclepiadaceae. It has milky or clear latex, widespread in the tropical, and subtropical regions especially in Africa and South America, with a moderate representation in Northern and South Eastern Asia [11]. In South Eastern and South Western Nigeria, *Gongronema latifolium* (whose leaves are bitter) is commonly called "utazi" and "arokeke", respectively, and is primarily used as spice and vegetables in traditional folk medicine [12]. Earlier reports on extract from this plant have focused mainly on their medicinal properties [13, 14], with little attempts at investigating their food preservative potentials. Sueeri [15] reported the use of bittering agents in brewing to produce the characteristic flavour, foam stability, and preservative properties in beer. Adenuga et al. [16] have used Nigerian bitter vegetables of *Gongronema latifolium*, *Vernonia amygdalina,* and *Garcia*

kola as substitutes for commercial hops in lager beer production. Apart from this, there is a dearth of information on the preservative potential of extracts from this plant with particular reference to its effect on some food-quality-related enzymes. It has been observed that the extracts of *Gongronema latifolium* contain phytochemical compounds including alkaloids, saponins, tannins (flavonoids), and glycosides [17]. Studies have shown that these phytochemicals found in *Gongronema latifolium* may influence cellular proteins with enzymic activities. Tannins have been shown to be strong inhibitors of oxidative enzymes present in foodstuffs [18]. Makkar and Singh [19] noted that extracts of tannin rich leaves of Oak (*Quercus incana*) inhibited various microbial enzyme activities of bovine rumen. Min et al. [20], while investigating the antibacterial activity of tannin extracts from perennial plants on mastitis pathogens, observed that the source and concentration are important factors that influence antimicrobial activity of tannins. Therefore, this report focuses on studies on the inhibitory effects of tannin extract from the leaves of *Gongronema latifolium* on lipoxygenase from *Cucumeropsis manii* seeds with a view to contribute to the knowledge that will assist in the processing of the seeds of *Cucumeropsis* and the leaves of *Gongronema latifolium* into value-added products.

2. Materials and Methods

2.1. Materials. *Cucumeropsis manii* seeds and the leaves of *Gongronema latifolium* used for this study were procured from Orba Modern Market in Udenu Local Government Area of Enugu State, Nigeria and identified at the Botany Department of the University of Nigeria.

2.2. Methods. Linoleic acid (99% Sigma) used as a substrate was prepared in solubilized state as described [21]. Other analytical grade quality chemicals were purchased from local commercial sources and were freshly prepared unless otherwise stated.

2.3. Preparation of Plant Extract. This was carried out using the method of Kubicka and Troszunska [18]. The leaves of *Gongronema latifolium* were collected fresh, sun-dried and powdered. This was then subjected to extraction with 70% acetone at the ratio of 1 : 7 (w/v) in a shaking incubator for 30 min. The mixture was then centrifuged and the supernatant was collected. The pellets were extracted twice with the same solution. The resultant extract was evaporated under vacuum and analyzed for total phenolics according to the methods of Julkunen-Tiitto [22].

2.4. Isolation and Purification of Lipoxygenase. Four days germinated (in the dark) white melon seeds were used for this experiment. One hundred grams of ground seeds were used for the extraction of LOX in 0.05 M phosphate buffer, pH 7.5 as in the method of Buranasompob et al. [23]. The crude LOX obtained was partially purified using a modification of the method of Malekian et al. [9]. Solid ammonium sulphate was added to each sample to obtain 70% saturation. The sample was allowed to stand for 24 hr and then centrifuged at 9000 ×g for 10 min at 4°C. The precipitate was redissolved in 0.01 M phosphate buffer pH 7.5. This solution was introduced on a sephadex G-50 column previously equilibrated with 0.01 M phosphate buffer pH 7.5. 3 mL, fractions were collected after every 5 min interval, and the active fractions were pooled and designated as the partially purified enzyme.

2.5. Lipoxygenase Assay and Protein Determination. LOX activity was assayed according to the methods of Aurand et al. [21], with slight modifications. LOX activity was measured with a JENWAY 640 5 UV/VIS spectrophotometer (Beckman Instruments, Inc., Huston, TX, USA) at 234 nm at 30°C. The cuvette contained 2.9 mL of substrate solution and was placed in the sample compartment of the spectrophotometer. 0.1 mL of the enzyme solution was rapidly added, mixed, and the increase in absorbance (A) versus the blank was recorded for every 5 sec. One unit of LOX activity was defined as the change in absorbance of 0.001 U/min in 3 mL volume and 1-cm light path using linoleic acid as substrate at 234 nm. Protein was determined by the method of Lowry et al. [24].

2.6. Effect of Substrate Concentration. The kinetics for lipoxygenase reaction (Km and Vmax) were determined in duplicate using linoleic acid stock solution diluted with 0.01 M borate buffer pH 7.5 to attain different concentrations (0–3 mM). The reaction was followed in the spectrophotometer at 30°C. LOX activity at each substrate concentration was determined as described under the assay section.

2.7. Optimum pH and Temperature of LOX Activity. The following buffer systems were used at concentrations of 0.02 M: sodium acetate buffer (pH 5.0-6.0); sodium phosphate buffer (pH 7-8); Tris-Hcl, (pH 9.0-10.0). LOX activity at each pH was determined as described in assay method. Also, LOX activity was determined at 30, 40, 50, 60, and 70°C, respectively as described in the assay method. To minimize lag phase, the assay cocktail was preincubated in a circulating water bath to the required temperatures for 600 sec followed by the introduction of 0.1 mL of the enzyme solution. This was rapidly mixed and assayed as earlier described.

2.8. Lipoxygenase Inhibition Studies. The enzyme inhibitory effect was tested by adding different volumes of the solution (0–9.1 mg/mL) of the extract to the incubation mixture. The lipoxygenase activity was monitored after 5 min of incubation as an increase in the absorbance at 234 nm which reflects the formation of hydroperoxylinoleic acid in the presence and absence of the extract as in the LOX assay section. The extinction coefficient of $25\,mM^{-1}\,cm^{-1}$ was used for calculation of enzyme activity. This was also repeated with two known inhibitors of lipoxygenase: propyl gallate and ascorbic acid under the same condition. Each of the inhibitors was incubated in 50 mM phosphate buffer pH 7.5 with the enzyme before it was transferred to a cuvette containing the assay cocktail to start the reaction. The Lineweaver-Burk plot was applied to determine the kinetic mechanism of inhibition.

Table 1: Purification profile for LOX from *Cucumeropsis manii* seeds.

Procedure	Total protein (mg)	Total activity (units)	Specific activity units/mg protein	Activity yield (%)	Purification fold
Crude extract	26,120.00	180,800.00	6.92	100.00	1.0
70% $(NH_4)_2SO_4$ fractionation	5,000.00	138,000.00	27.60	76.33	4.0
Sephadex G-50 chromatography	400.00	130,500.00	326.25	72.18	47.14

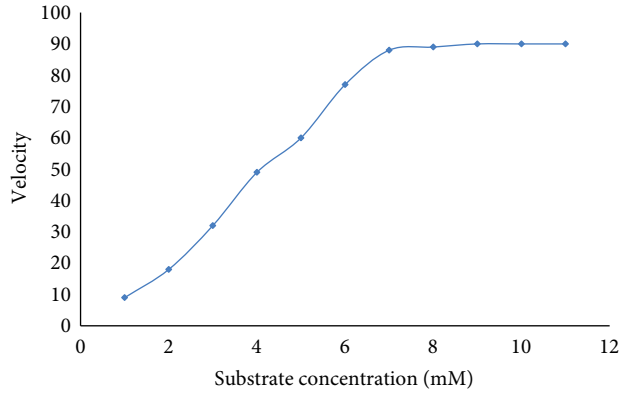

Figure 1: Michaelis-Menten plot showing the effect of substrate concentration on LOX.

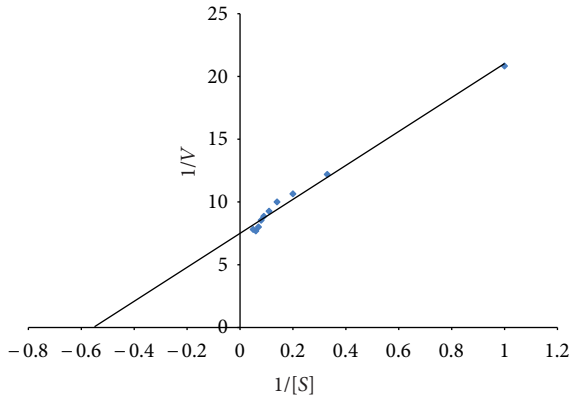

Figure 2: Lineweaver-Burk plot for the determination of Km and Vmax.

2.9. Statistical Analysis. Each experiment was performed in triplicate. Results are expressed as the mean ± S.D. Statistical analysis was performed using SPSS package and statistical significance is expressed as, $P < 0.05$. For kinetic studies, each experiment was performed in duplicate and result expressed as the mean.

3. Results

3.1. Enzyme Purification. Lipoxygenase was purified about 47.14-fold with protein recovery of about 72.18% and LOX-specific activity of 326.25 units/mg protein. The purification profile is shown in Table 1.

3.2. Effect of Substrate Concentration on LOX Activity. The linoleic acid hydroperoxidation rate followed Michaelis-Menten kinetic equation (Figure 1). Figure 2 shows the Lineweaver-Burk plot for the determination of Km and Vmax. A plot of the reciprocal of hydroperoxidation activity rates ($1/V$) versus the reciprocal of linoleic acid concentrations ($1/[S]$) ranging from 0.0 to 3 mM was constructed. Vmax was calculated from the intercept to be 1.44 ± 0.02 units/mg/min, while Km value for linoleic acid for the partially purified LOX extract was 1.61 ± 0.019 mM (by extrapolating the Lineweaver-Burk plot to the hypothetical point where $1/V = 0$).

3.3. pH and Temperature Optimum. Figure 3(a) shows the activity profile for white melon seed lipoxygenase across the pH range of 3–10, used in this study. The optimum pH was at 6.0. Maximum lipoxygenase activity was observed at 40°C (Figure 3(b)).

3.4. Lipoxygenase Inhibitory Effects. LOX activity was monitored as an increase in the absorbance at 234 nm, which reflects the formation of hydroperoxylinoleic acid. The highest inhibitory effect was obtained for crude extract of *G. latifolium* ($IC_{50} = 4.2 \times 10^{-3} \pm 0.09 \times 10^{-3}$ g/L). As far as the two other known inhibitors were compared, a higher inhibition of LOX is caused by ascorbic acid ($IC_{50} = 4.3 \times 10^{-2} \pm 0.11 \times 10^{-2}$ g/L) than propyl gallate ($8.0 \times 10^{-2} \pm 0.09 \times 10^{-2}$ g/L) (Table 2). The type of inhibition was deduced from Lineweaver-Burk double reciprocal plots and both the *Gongronema latifolium* leaf extract and ascorbic acid exhibited competitive pattern of inhibition while that of n-propyl gallate is noncompetitive (Figures 4(a), 4(b), and 4(c)).

4. Discussion

The result recorded here suggests that the purification procedure used here is suitable for purification of LOX from *Cucumeropsis manii* to the extent of our purification. Ammonium sulphate is the most frequently used method at the first stage of enzyme purification and gave a good separation of the protein. The enzyme recovery is quiet low especially when compared with results obtained by a number of researchers [2, 25], this might be as a result of differences in enzyme extraction method and the assay conditions [26]. A Km value of 1.61 ± 0.019 mM obtained in this experiment suggests low affinity of this LOX from *Cucumeropsis manii* for linoleic acid especially when compared with those observed in literature 0.131 ± 0.019 mM for barley lipoxygenase [27],

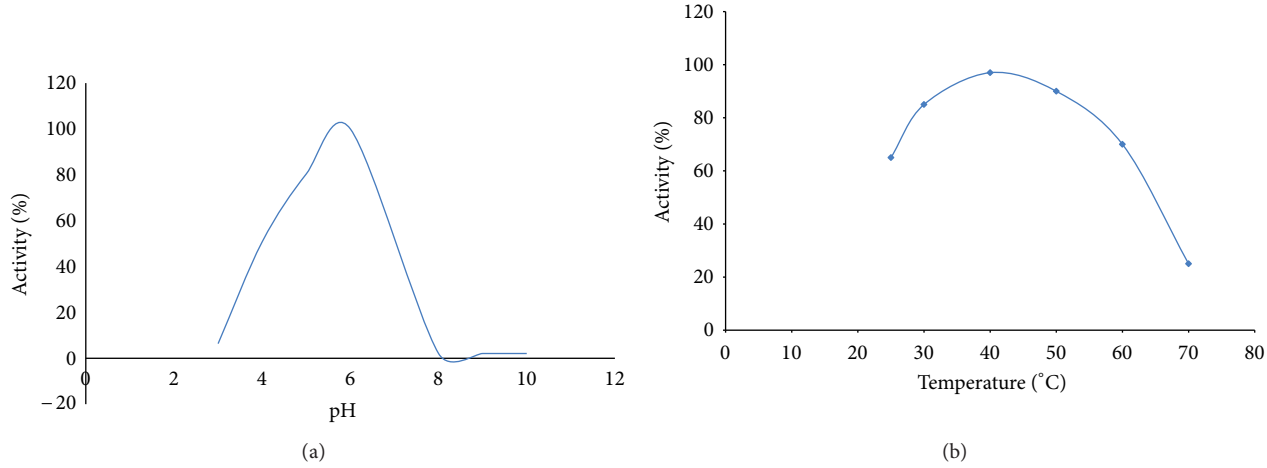

FIGURE 3: (a) pH profile for the activity of lipoxygenase from *Cucumeropsis manii* seeds. (b) Optimum temperature for LOX activity from *C. manii*.

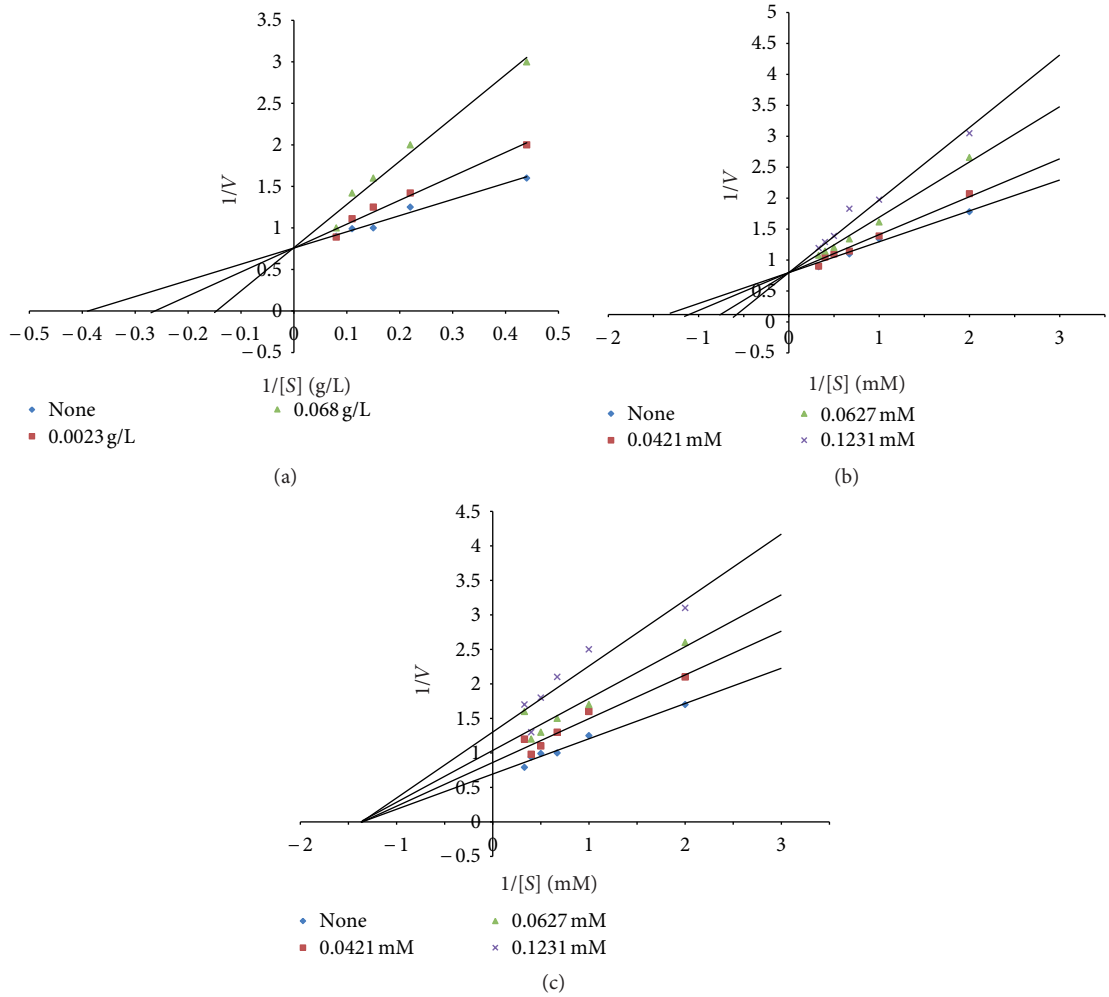

FIGURE 4: Inhibition studies on *Cucumeropsis manii* seed LOX. (a) shows a competitive inhibition with the crude extract. (b) shows a competitive inhibition with ascorbic acid. (c) Noncompetitive inhibition with propyl gallate.

Table 2: Results of lipoxygenase effects of crude *Gongronema latifolium* and two other known antioxidants.

	Final concentration (g/L)	Activity of lipoxygenase (K_{cat})	%Inhibition	IC_{50} (g/L)
Crude extract	2.3×10^{-3}	0.312 ± 0.41	68.80 ± 0.35	$4.2 \times 10^{-3} \pm 0.09 \times 10^{-3}$
	6.8×10^{-3}	0.289 ± 0.11	71.10 ± 0.20	
	9.1×10^{-3}	0.250 ± 0.23	75.00 ± 0.19	
Control	—	1.000 ± 0.22	—	
Ascorbic acid	7.4×10^{-3}	0.952 ± 0.12	95.2 ± 0.20	$4.3 \times 10^{-2} \pm 0.11 \times 10^{-2}$
	1.1×10^{-2}	0.800 ± 0.16	80.0 ± 0.23	
	2.17×10^{-2}	0.571 ± 0.20	57.1 ± 0.22	
Control	—	1.000 ± 0.29	—	
Propyl gallate	8.93×10^{-2}	0.769 ± 0.09	76.9 ± 0.11	$8.0 \times 10^{-2} \pm 0.09 \times 10^{-2}$
	1.33×10^{-2}	0.667 ± 0.11	66.7 ± 0.19	
	2.61×10^{-2}	0.476 ± 0.85	47.6 ± 0.09	
Control	—	1.000 ± 0.89	—	

Lipoxygenase activity was assayed as change in absorbance at 234 nm after 5 min of incubation in the presence and absence of the extract, propyl gallate, and ascorbic acid, respectively. The equation $C = A \cdot V \cdot /\epsilon \cdot l \cdot v.$ was applied in the calculation of lipoxygenase activity where A is the value of absorbance increase, V is the vol of incubation mixture, ϵ is the extinction coefficient for linoleic acid (25×10^{-3} mol · L · cm^{-1}), l is the length of the cuvette (1 cm), and v is the volume of enzyme (0.1 mL). Experiments were conducted in triplicates and $P < 0.05$.

0.67 μM for durum wheat lipoxygenase [28]. Low affinity is an indication that lipoxygenase requires the use of high substrate concentration for its assay. The optimum pH of 6.0 obtained in this report is within the range of pH optimum for plant lipoxygenases (5.5–7.5) [25]. An optimum temperature of 40°C suggests that LOX from *C. manii* might not be suitable for operation at very high temperatures, but it is in agreement with the result of Koksel et al. [27], who determined maximum linoleic acid hydroperoxidation activities at 40°C. Though temperatures of 25–30°C were used in the past for studying LOX activity by a number of researchers.

In this work, the LOX inhibitory properties were tested on partially purified LOX from *Cucumeropsis manii*. Food spoilage caused by oxidative rancidity involves a reaction between the lipid and molecular oxygen. The reaction takes place at the double bonds of unsaturated fatty acids and can be accelerated by singlet oxygen, free radicals, metal ions (iron, cooper, and cobalt), light, radiation, and enzymes containing a transition metal prosthetic group such as lipoxygenases [29]. LOX specifically oxygenates polyunsaturated fatty acids and/or their esters and acylglycerols containing the *cis*, *cis*-1, 4 pentadiene double-bond system located between carbons 6–10 counting from the methyl terminus [30]. Consequently it causes off-flavour and off-odour in food. There is little published information on the inhibition of LOX in *Cucumeropsis manii*, especially in regard to storage characteristics of its value-added products. Lipoxygenases are sensitive to antioxidants and the most common mechanism of action may consist in either inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy or lipidperoxy radicals formed in the course of enzymic peroxidation or via chelation of its nonheme bound iron or by reduction of its ferric form, suggesting a competitive kind of inhibition [28]. However, noncompetitive or mixed competitive/noncompetitive inhibition of LOX has been reported [26]. The inhibitory properties of the extract from *Gongronema latifolium*, ascorbic acid, and n-propyl gallate were compared using linoleic acid as substrate. The strongest lipoxygenase inhibition was obtained for the crude extract ($IC_{50} = 4.2 \times 10^{-3} \pm 0.09 \times 10^{-3}$ g/L). Although ascorbic acid showed the stronger LOX inhibition effect ($IC_{50} = 4.3 \times 10^{-2} \pm 0.11 \times 10^{-2}$ g/L), than propyl gallate ($8.0 \times 10^{-2} \pm 0.09 \times 10^{-2}$ g/L), the difference between the inhibitory effects of the crude extract and ascorbic acid is not significant $P < 0$. Baraniak and Krzepilko [26] observed that phenolic compounds which are endogenous inhibitors in plant sources could act mostly as scavengers of free radicals rather than as inhibitors of lipoxygenase generating free radicals released from fatty acid oxidation. Kubo et al. [31] have observed that anacardic acid competitively inhibited various prooxidant enzymes involved in the production of the reactive oxygen species and acts by chelating divalent metal ions such as Fe^{2+} or Cu^{2+} from the prosthetic group of enzymes. Baysal and Demirdoven [29] have observed the presence of Fe^{2+} in LOX, though Trop et al. [32] had earlier suggested that lipoxygenase is the only oxidative enzyme which is not inhibited by those substances which bind to the prosthetic group of enzymes. These controversies are subject of further research.

5. Conclusion

Preservation technologies aimed at inactivating enzymes with deteriorative actions could include the use of enzyme inhibitors such as the tannin extract from the leaves of *Gongronema latifolium*. This work suggests that *Cucumeropsis-manii*-based food products could be protected against LOX using tannin extract from this plant instead of heating which could destroy other important nutrients inherent in them or using chemical preservatives which will raise acceptability questions by consumers.

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