

CHAPTER 5

Preservation of Fermented Locust Beans (*Parkia biglobosa*) Using Essential Oil from Lemon grass (*Cymbopogon citratus*)

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Abstract

Fermented locust beans (FLB), a highly nutritious condiment used to enrich foods in many parts of Nigeria has a very low shelf life of less than one week if unpreserved. Most of the methods used for preservation have presented limitations which were yet to be surmounted. The efficacy of preserving FLB using lemongrass essential oil (LCEO) was therefore investigated in this study. The essential oil was extracted from the leaves of *Cymbopogon citratus* and used to treat FLB at 2% (v/w). Untreated FLB served as the control. All samples were stored at 28 ± 2 °C and withdrawn periodically for organoleptic and microbiological analyses. Susceptibility of microbial isolates to LCEO and the effect of the treated FLB on the hepatic histology of rat were also investigated. Acceptance rating was high for the treated samples up to 33 days while ratings dropped from the 19th day in the untreated. A significant reduction of the bacterial load up to 70% due to treatment with LCEO was achieved. Thirteen bacteria were isolated with members of *Bacillus* species as the most dominant. All the isolates were susceptible to LCEO with diameter zones of inhibition ranging from 4.0 ± 1 mm to 57.7 ± 1.2 mm which compared favorably with the value (highest = 22.0 ± 1.7 mm) for many of the standard antibiotics used. Histomorphology showed normal hepatic architecture in rats fed with LCEO-treated FLB. Results showed that LCEO can serve as a natural and safe preservatives of FLB.

Key words: Fermented locust beans; Lemongrass essential oil; Preservation; Safety

5.1 Introduction

Seeds of *Parkia biglobosa* known as African locust beans (ALB) serve as source of nutritious condiment in at least 17 West African countries

including Nigeria (Ajayi *et al.*, 2015) as it comprises of protein (35%), lipid (29%) and carbohydrate (16%), and it is regarded as a good source of calcium (Zekeri and Usman, 2017), nutraceuticals and antioxidants (Kolapo *et al.*, 2007). The seeds are however inedible and must pass through several stages of processing in order to derive these nutritional benefits. The processes that lead to the production of edible fermented locust beans include a solid-state fermentation described in our previous study (Saliu *et al.*, 2019). This involves de-hulling locust bean seeds by boiling for about 12-24 hours or until the seeds become tender, followed by gentle pounding in a mortar or rubbing between the palms or trampling under foot with abrasive materials such as sand. The cotyledons that result after proper washing are again boiled for 3 to 4 hours, after which they are packed in large calabash and covered tightly with broad leaves such as those of banana plants, to allow spontaneous fermentation at room temperature for 2-3 days. Members of the genera *Staphylococcus*, *Bacillus* and some lactic acid bacteria were reported as the dominant organisms involved in the fermentation (Egbebi *et al.*, 2016; Zannou *et al.*, 2018; Saliu *et al.*, 2019).

The nutritional benefits derivable from fermented locust beans is often jeopardized due to the low shelf life of less than one week if unpreserved (Ojewumi *et al.*, 2016). Several methods including salting, drying, and refrigerating have been applied to increase the shelf life. Each of these have limitations that may discourage its use (Saliu *et al.*, 2019). A downstream processing involving drying and packaging as “bouillon cubes” was reported to improve the microbiological quality thereby increasing the shelf life from 5 days to over nine months (Ajayi *et al.*, 2015). However, the protein content of the bouillon was reduced due to the heat applied during processing and the use of binders in packaging.

Over the years, there has been a tendency to use natural preservatives such as essential oils, to forestall the negative effects that may arise from the use of synthetic chemicals in foods. Essential oils are valuable commodities in the fragrance and food industries (Hesham *et al.*, 2016). More than 250 types have been identified for the control of microbial and chemical spoilage of foods (William *et al.*, 2010). Furthermore, due to their richness in volatile compounds such as terpenes, aldehydes, ketones, alcohols, phenols, acids, ethers and esters, there is increased use of EOs as antioxidants, flavorings and colorants in the food industry. Essential oils are applied in the preservation of fish, cheese, meats, meat products, and fruits (William *et al.*, 2010, Govaris *et al.*, 2011; Braga, 2012; Laranjo *et al.*, 2015 and Campos *et al.*, 2016). The oils of lemongrass (*Cymbopogon citratus*) have been reported to possess antimicrobial, antifungal, antioxidant and antiseptic

activities which are essential in improving the shelf life and organoleptic property of foods (Mukarram *et al.*, 2021)

Lemongrass contains 1 to 2% essential oil on a dry basis and like other phytochemicals, its composition varies widely as a function of genetic diversity, habitat and agronomic treatment of the plant. Compounds such as hydrocarbon terpenes, alcohols, ketones, esters and aldehydes have constantly been reported. For instance, a GC-MS analyses of the LGEO reported by De-Silva *et al.* (2020), revealed the presence of geranial, neral, geraniol, geranyl acetate and Beta-caryophyllene as the main active principle of the oil. The geranial is responsible for the strong aroma, antimicrobial and anti-insecticidal activity of the oil, while neral confers the sweet odour, and antimicrobial activities. In addition, geraniol and geranyl acetate confer the antioxidant, anti-inflammatory and neuroprotective activities while Beta-caryophyllene is responsible for the distinctive gentle aroma and the anti-depressant activities of LGEO (Akhila, 2010).

In contrast to most synthetic preservatives, LGEO is considered “generally regarded as safe” for human consumption (FDA, 2022). It enhances the sensory quality and does not alter the physiochemical characteristics of foods (Hamad *et al.*, 2021). In addition, the sweet fragrance smell of LGEO favors its use as a natural food flavoring and may be of particular advantage in suppressing the rather unpleasant smell that characterize FLB in storage. These, and the reported antimicrobial properties of LGEO prompted this study which investigated its potential in extending the shelf life of FLB.

5.2 Materials and Methods

5.2.1 Collection of samples

Freshly produced fermented locust beans were purchased from a local producer in Ilorin into a sterile sample bottle, and moved immediately to the laboratory where treatment and analysis were done within 1 hour of collection. Lemongrass (*Cymbopogon citratus*) was obtained from a farm also in Ilorin. The lemongrass was authenticated at the Herbarium unit of the Department of Plant Biology, University of Ilorin, and assigned voucher number **UILH/001/800**.

5.2.2 Extraction of lemon grass essential oil (LGEO)

Pulverized fresh lemongrass (500 g) was hydro-distilled for 3 hours in a Clevenger-type apparatus, according to the British Pharmacopoeia specification (Usman *et al.*, 2010) and the oil collected into a sterile sample

tube, was weighed to determine the yield; and stored at 4 °C until required. Yield was calculated using the following formula:

$$\text{Yield of essential Oil} = \frac{\text{Weight of oil collected (g)}}{\text{Weight of leaves used (g)}} \times 100\%$$

5.2.3 Treatment of FLB samples with LGEO

The LGEO, 100 µL was thoroughly mixed with FLB (5 g) in each of 25 sterile bottles. Control was set up with untreated FLB in another set of 25 bottles. All samples were stored at 28±2 °C (room temperature), in laboratory cabinet. Samples were withdrawn in triplicates for analysis, daily for the first week, every other day for the next two weeks, two days interval for another seven sets of samples while the remaining were taken in three days intervals.

5.2.4 Organoleptic analysis of FLB

The colour, odour and texture of the samples were evaluated by a five-man panel on a five-point hedonic scale.

5.2.5 Microbiological analyses

The pour plate method was used to isolate total aerobic, enteric and lactic acid bacteria on Nutrient agar (NA), MacConkey agar (MA) and de Mann Rogosa Sharpe agar (MRS) respectively (Olayemi et al., 2017). Sabouraud dextrose agar (SDA) was used for the isolation of fungi (Olayemi et al., 2017). After growth, colonies were counted. Percentage reduction was obtained using the formular: $\frac{A-B}{A} \times \frac{100}{1}$

Where A was taken as the microbial count of isolate from untreated samples and B, the microbial count of isolate from treated samples. Isolates were identified based on their morphology, physiology and molecular characteristics.

5.2.6 Morphological and physiological characterization

Isolates on agar plates were observed for the color, shape, margin, surface texture and translucency of the colonies. Cell shapes, arrangements, Gram reaction and sporulation were obtained after the cells were stained appropriately and observed under the oil immersion lens of the light microscope. Motility was determined using the hanging-drop method. The tests done to obtain physiological characteristics of the isolates were catalase, oxidase, methyl red, Voges Proskauer, indole, citrate utilization,

starch hydrolysis, sugar fermentation, nitrate reduction and, growth in various concentrations of NaCl (Fawole and Oso, 2004).

5.2.7 Molecular characterization

QIAamp DNA Mini Kit (250) cat No. 51306 was used to extract genomic DNA of the isolates and the 16SrRNA genes were amplified using the 16SF (5'AGAGTTTGATCCTGGCTCAG 3') and 16SR (5'TACCTTGTTACGACTT 3') primers. The PCR product was purified from contaminants by electro-elution of the gel slice containing the excised desired fragments with Qiaquick gel extraction kit (Qiagen, USA). The elution was carried out in 300 µL of nuclease free water in order to enhance the purification of PCR products. Sequencing was performed using automated sequencer (ABI PRISM 310, Applied Biosystems, USA). Translated nucleotide sequences were analyzed for similarities using BLASTN tool (www.ncbi.nlm.nih.gov:80/BLASTN/). ClustalW Multiple Sequence Alignment was constructed using BioEdit v7.2.5 for Windows. A phylogenetic tree was constructed from the sequence alignment using the neighbor joining method while maximum composite likelihood method was used to compute evolutionary distance (Tamura et al., 2004) using the bioinformatics software Mega X (Kumar *et al.*, 2018).

5.2.8 Antimicrobial susceptibility test

The susceptibility of isolates to LGEO was determined using agar well diffusion method for the bacterial isolates and the poisoned plate technique (Balouiri *et al.*, 2016) for organisms isolated on SDA. Diameter zone of inhibition zones was measured in five planes and means used to represent antimicrobial activity. The diameter of fungal growth was also measured in five planes and antifungal activity calculated. Prior to use, LGEO was mixed with Tween 80 v/v to obtain 50% concentration. A further dilution v/v of the 50% diluent with Tween 80 was made to obtain 25% concentration. Tween 80 served as the negative control while the antibiotic discs used as positive control were: Ofloxacin 5µg (OFL), Amoxycillin/Clavulinate 30µg (AUG), Gentamycin 10µg (GEN) Cefpirome 10µg (CPR) Ceftriazone 30µg (CTR), Erythromycin 30µg (ERY) and Cloxacillin 5µg (CXC).

5.2.9 Histo-pathological assessment of LGEO using male Wistar rats

Ten healthy male Wistar rats, with an average weight of 150 g were acclimatized and fasted for 18 hours prior to treatment. The rats were randomly divided into 2 groups. One group was fed with the LGEO-treated

FLB and the other with the untreated FLB, for 21 days. The rats were sacrificed and liver tissues harvested for histological examination (Punithavathi *et al.*, 2011).

5.2.10 Statistical analysis

All data were measured in triplicates and ANOVA was used to obtain the means with standard deviations and test of significance ($p < 0.05$) using SPSS 20 for Windows

5.3 Results and Discussion

5.3.1 Yield of lemongrass leaf essential oil

An average yield of 0.48% (w/w) was obtained from eight consecutive extractions of fresh lemon grass that were used in the study (Table 1). This yield fell within the range obtained in previous studies (Weiss, 1997; Maiti *et al.*, 2006)

Table 1: Yield of Leaf essential oil of *Cymbopogon citratus*

Extraction Cycles	% Yield (w/w)
1	0.53
2	0.49
3	0.52
4	0.39
5	0.45
6	0.56
7	0.41
8	0.48

5.3.2 Effect of treatment with LGEO on the organoleptic properties of fermented locust bean samples

The organoleptic rating of the FLB samples is presented in Table 2. Using the fresh sample as the point of reference, the acceptance rate was slightly higher initially (0.29 average difference within the first 19 days) in the treated sample compared to the untreated. The gap widened (0.82 average difference for the rest of the storage period) as storage period increased. On the average, acceptability dropped lower than 3.0 in the untreated sample after 19 days of storage while the treated samples rating was high up to the 39th days. The sensory quality of the untreated sample in

this study was preserved beyond the commonly reported period of 3 to 5 days (Ajayi *et al.*, 2015; Ojewumi *et al.*, 2016) and this may be due to the aseptic condition observed during sample collection. The LGEO further extended the sensory quality by 20 days indicating a significant preservative activity. This is a remarkable improvement when compared with 4 days reported for another food sample 'Tofu' (Hamad *et al.*, 2019).

5.3.3 Effect of LGEO on the microbial population of FLB during storage

The microbial population of FLB reduced steadily over the period of storage up to 70% in samples treated with LGEO (Table 3). The population of enteric bacteria was generally low, laying credence to the hygiene observed in the collection of the FLB sample used. Contamination during production of FLB has been linked to the water quality (Ogunshe and Okereh, 2011) making it expedient to use potable water in washing the cotyledon prior to fermentation.

Table 2: Effect of treatment with leaf essential oil of *Cymbopogon citratus* on the organoleptic properties of fermented locust bean samples

Storage period	Untreated				Treated			
	Odour	Colour	Texture	A	Odour	Colour	Texture	A
0	4.6±0.55 ^a	4.6±0.55 ^a	5±0	4.7	4.6±0.55 ^a	4.6±0.55 ^a	5±0	4.7
1	4.2±0.45 ^{ab}	4.8±0.45 ^a	4.6±0.55 ^a	4.5	4.8±0.45 ^a	4.8±0.45 ^a	4.8±0.45 ^a	4.8
2	4.2±0.45 ^{ab}	4±0.71 ^{bc}	4.4±0.55 ^a	4.2	4±0.71 ^a	4.6±0.55 ^a	4.6±0.55 ^a	4.4
3	3.4±0.55 ^{bcd}	3.8±0.84 ^{bc}	4±0.71 ^{ab}	3.7	3.8±0.84 ^{ab}	4±0.71 ^{abc}	4±0.71 ^{ab}	3.9
4	4.6±0.55 ^a	4.4±0.55 ^{ab}	3.4±0.55 ^{cd}	4.1	4.4±0.55 ^a	4.4±0.89 ^{ab}	4.4±0.89 ^a	4.4
5	4.4±0.55 ^{ab}	4.2±0.45 ^{bc}	3.4±0.55 ^{cd}	4.0	4.2±0.45 ^a	4.2±0.45 ^{ab}	4.2±0.45 ^{ab}	4.2
6	3.4±0.55 ^{bcd}	4.4±0.55 ^{ab}	3.6±0.55 ^{bcd}	3.8	4.4±0.55 ^{ab}	4.2±0.45 ^{ab}	4.2±0.45 ^{ab}	4.3
7	3.2±0.45 ^{cde}	4.2±0.45 ^{abc}	4.2±0.45 ^{ab}	3.9	4.2±0.45 ^a	4.2±0.45 ^{ab}	4.2±0.45 ^{ab}	4.2
9	3.2±0.45 ^{cde}	4.2±0.45 ^{bc}	3.6±0.55 ^{bcd}	3.7	4.2±0.45 ^{ab}	4.6±0.55 ^a	4.6±0.55 ^b	4.5
11	3.2±0.45 ^{cde}	3.4±0.55 ^{cd}	3.8±0.84 ^{bc}	3.5	3.4±0.55 ^{ab}	4.2±0.45 ^{ab}	4.2±0.45 ^{ab}	3.9
13	3.2±0.45 ^{cde}	3.6±0.55 ^{bcd}	3.8±0.84 ^{bc}	3.5	3.6±0.55 ^{ab}	3.8±0.84 ^{bc}	3.8±0.84 ^b	3.7
15	3.6±0.55 ^{bcd}	3.8±0.84 ^{bc}	3.8±0.84 ^{bc}	3.7	3.8±0.84 ^{ab}	3.8±0.45 ^{bc}	3.8±0.45 ^b	3.8
17	2.4±0.55 ^{fgh}	3.4±0.55 ^{cd}	3.4±0.55 ^{cd}	3.0	3.4±0.55 ^{ab}	4.2±0.84 ^{abc}	4.2±0.84 ^{ab}	3.9
19	2.6±0.89 ^{fg}	3.4±0.55 ^{cd}	3.2±0.45 ^{cd}	3.1	3.4±0.55 ^{ab}	3.8±0.45 ^{ab}	3.8±0.45 ^b	3.7
21	2.4±0.55 ^{fgh}	3.2±0.45 ^{cde}	3±0.71 ^d	2.9	3.2±0.45 ^{bc}	3.2±0.45 ^{cd}	3.2±0.45 ^{bc}	3.2
24	2.2±0.45 ^{fgh}	2.2±0.45 ^{fg}	2.6±0.55 ^{ef}	2.3	3.2±0.45 ^{bc}	3.2±1.1 ^{cd}	3.2±1.1 ^{bc}	3.2
27	2.2±0.45 ^{fgh}	2.4±0.55 ^{ef}	2.2±0.45 ^{fg}	2.3	2.4±0.55 ^{cde}	3.4±1.14 ^{bcd}	3.4±1.14 ^{bc}	3.1
30	2.2±0.45 ^{fgh}	2.2±0.84 ^{fg}	2.8±0.45 ^{de}	2.4	3.2±0.84 ^{bc}	3.2±0.45 ^{cd}	3.2±0.45 ^{bc}	3.2
33	2.2±0.45 ^{fgh}	2.6±0.55 ^{def}	2.2±0.45 ^{fg}	2.3	2.6±0.55 ^{cde}	3.4±0.55 ^{bcd}	3.4±0.55 ^{bc}	3.1
36	2.2±0.45 ^{fgh}	2.2±0.45 ^{fg}	1.6±0.89 ^{hi}	2.0	2.2±0.45 ^{ef}	3.4±0.55 ^{bcd}	3.4±0.55 ^{bc}	3.0
39	2±0.71 ^{gh}	2.6±0.55 ^{def}	1.2±0.45 ⁱ	1.9	2.6±0.45 ^{cde}	3.2±0.45 ^{cd}	3.2±0.45 ^{bc}	3.0
42	1.8±0.45 ^{gh}	2.2±0.45 ^{fg}	1.2±0.45 ⁱ	1.7	2.6±0.55 ^{cde}	2.6±0.55 ^{de}	2.6±0.55 ^d	2.6
46	1.6±0.55 ^{ghi}	2.2±0.45 ^{fg}	1.2±0.45 ⁱ	1.7	2.2±0.45 ^{ef}	2.8±0.84 ^{cde}	2.8±0.84 ^{cd}	2.6
50	1.4±0.55 ^{hi}	1.4±0.55 ^g	1.2±0.45 ⁱ	1.3	2.2±0.45 ^{ef}	2.8±0.45 ^{cde}	2.8±0.45 ^{cd}	2.6
54	1±0 ⁱ	2.2±0.45 ^{fg}	2.2±0.45 ^{fg}	1.8	1.4±0.55 ^f	2.8±0.45 ^{cde}	2.8±0.45 ^a	2.3
58	1±0 ⁱ	2.2±0.45 ^{fg}	1.2±0.45 ⁱ	1.5	2.2±0.45 ^{ef}	1.8±0.84 ^e	1.8±0.84 ^e	2.0

Values are means of five determinations (± SD). ^{abcdeghi} Means with different superscript in a column are significantly different (p<0.05). A= Acceptance value

Table 3: Microbial Count of bacteria isolated from fermented locust beans samples

Storage Period (days)	Colony Count (CFU/g)								
	NA (10 ⁶)			MA (10 ⁶)			SDA (10 ⁷)		
	Untreated	Treated	% Redu	Untreated	Treated	% Redu	Untreated	Treated	% Redu
0	91±0 ^t	83±1 ^{bc}	9	0±0 ^c	0±0 ^c	NA	61±2 ^{mn}	59±2 ^{mn}	3
1	83±1 ^u	71±1 ^{ghi}	14	0±0 ^c	0±0 ^c	NA	56±2 ^o	36±2 ^{mno}	36
2	101±1 ^{rs}	86±1 ^{ab}	15	0±0 ^c	0±0 ^c	NA	52±1 ^p	43±1 ^p	17
3	121±0 ^p	87±2 ^g	28	0±0 ^c	0±0 ^c	NA	48±1 ^m	51±0 ^{mno}	-6
4	103±2 ^r	69±2 ^{hijk}	33	53±1 ^b	35±1 ^b	34	63±1 ^m	42±1 ^m	33
5	134±0.5 ⁿ	83±0 ^{bc}	38	0±0 ^c	0±0 ^c	NA	67±0 ^l	46±1 ^{lm}	31
6	111±2 ^q	79±2 ^{de}	29	0±0 ^c	0±0 ^c	NA	59±2 ^m	43±2 ^{nm}	27
7	127±1 ^o	77±0 ^{ef}	39	0±0 ^c	0±0 ^c	NA	63±0 ^k	37±2 ⁿ	41
9	99±0 ^s	67±2 ^{ijk}	32	0±0 ^c	0±0 ^c	NA	93±2 ^k	41±0 ⁿ	56
11	132±1 ⁿ	73±1 ^{fgh}	45	81±1 ^a	47±2 ^a	42	97±0 ^{ij}	55±0 ^{ijk}	43
13	148±0 ^m	73±2 ^{fgh}	51	0±0 ^c	0±0 ^c	NA	94±0 ^{ij}	57±0 ^{ijk}	39
15	163±1 ^k	71±1 ^{ghi}	56	0±0 ^c	0±0 ^c	NA	96±1 ⁱ	62±1 ^{ef}	35
17	122±0 ^p	69±2 ^{hijk}	43	0±0 ^c	0±0 ^c	NA	98±1 ^h	53±1 ^l	46
19	164±1 ^j	66±0 ^{kl}	60	0±0 ^c	0±0 ^c	NA	101±0.5 ^g	43±0 ^{gh}	57
21	162±1 ^k	65±1 ^{klm}	60	0±0 ^c	0±0 ^c	NA	109±2 ^f	47±0 ^{ef}	57
24	167±0 ^m	73±1 ^{fgh}	56	0±0 ^c	0±0 ^c	NA	115±1 ^g	62±1 ^{fg}	46
27	156±1 ^k	69.3±1 ^{hij}	56	0±0 ^c	0±0 ^c	NA	113±0 ^e	57±0 ^{fg}	50
30	178±1 ^l	62±1 ^m	65	0±0 ^c	0±0 ^c	NA	121±1 ^{ef}	53±0 ^{ef}	56
33	185±0 ^g	63±2 ^{lm}	66	0±0 ^c	0±0 ^c	NA	119±0 ^{ef}	49±0 ^{ef}	59
36	181±0 ^k	67±0 ^{ijk}	63	0±0 ^c	0±0 ^c	NA	123±0 ^e	53±2 ^{ef}	57
39	192±1 ^f	69±2 ^{hij}	64	0±0 ^c	0±0 ^c	NA	127±1 ^{ed}	61±1 ^{ed}	52
42	204.±2 ^e	73±2 ^{fgh}	64	0±0 ^c	0±0 ^c	NA	133±2 ^b	71±2 ^c	47
46	226±2 ^d	77±2 ^{ef}	66	0±0 ^c	0±0 ^c	NA	139±2 ^a	83±0 ^{cb}	40
50	236±1 ^c	79±0 ^{cd}	67	0±0 ^c	0±0 ^c	NA	128±1 ^c	87±2 ^b	32
54	252±1 ^a	82±2 ^{bc}	68	0±0 ^c	0±0 ^c	NA	135±2 ^b	91±2 ^a	33
58	247±2 ^b	74±1 ^{fg}	70	0±0 ^c	0±0 ^c	NA	141±2 ^a	64±1 ^a	55

Values are means of three determinations (± SD). ^{a-u} Means with different superscript in each column are significantly different (p<0.05).

No organism was isolated on MRS, hence it is excluded from the table. NA = not applicable; % Redu = % reduction

Table 4: Morphological and biochemical characteristics of bacteria isolated from fermented locust beans

Isolates	Colonial Morphology				Cellular Morphology				Biochemical Characteristics																				TSI			Sugar Fermentation		
	Shape	Colour	Elevation	Edge	Optical Characteristics	Gram's reaction	Cell shape	Cell arrangement	Spore	Spore location	Spore size	Spore shape	Bulged/infant cell	Motility	5% NaCl	6.5% NaCl	Nitrate reduction	Catalase	Oxidase	Indole	Urease	Citrate	Methyl red	Voges Proskauer	Starch hydrolysis	TSI			Sugar Fermentation					
																										Surface	Butt	Gas	Glucose	Mannitol	Lactose			
A	I	Cr	F	L	TL	+	Co	S	-	NA	NA	NA	NA	-	+	+	-	+	+	-	+	+	-	+	+	+	-	-	A	-	-			
B	C	Cr	F	E	Op	+	Co	Cl	-	NA	NA	NA	NA	-	+	+	+	+	-	-	+	+	+	+	+	+	+	-	A	AG	+			
C	C	W	F	E	TP	+	Co	Cl	-	NA	NA	NA	NA	-	+	-	+	+	-	-	+	+	+	+	-	+	-	-	A	A	+			
D	I	Cr	Ra	L	TL	+	R	Ch	+	Tr	Sm	O	In	+	+	-	+	+	+	-	-	-	+	-	+	+	+	-	A	A	-			
E	Rh	Cr	F	E	TL	+	R	C	+	NA	NA	NA	NA	-	+	+	+	+	+	-	+	-	-	+	+	-	+	-	A	A	+			
F	C	Cr	Ra	E	TL	-	R	S	-	M	Sm	O	In	-	+	+	+	+	-	-	+	+	-	-	+	+	-	+	A	A	-			
G	C	Cr	Ra	E	TL	+	Co	Cl	+	NA	NA	NA	NA	-	+	+	+	+	+	-	+	+	-	-	+	+	+	-	A	-	A			
H	C	W	R	E	TL	-	R	S	-	NA	NA	NA	NA	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	AG	A	-			
I	I	P	F	U	TP	-	R	Ch	-	NA	NA	NA	NA	-	+	+	+	+	-	-	-	+	+	-	+	-	-	AG	AG	-				
J	I	Cr	Ra	L	TL	+	R	S	+	Tr	Sm	O	In	-	+	-	+	+	-	-	-	-	+	+	+	+	+	-	A	A	+			
K	I	Cr	Ra	L	TL	+	R	S	+	Tr	Sm	O	In	-	+	-	+	+	-	-	-	-	+	+	+	+	+	-	A	A	-			
L	I	Cr	Ra	L	TL	+	R	S	+	Tr	Sm	O	In	-	+	-	+	+	+	-	+	-	+	+	+	+	+	-	A	A	+			
M	I	Cr	Ra	L	TL	+	R	S	+	Tr	Sm	O	In	-	+	-	+	+	+	+	-	-	+	+	+	+	+	-	A	A	+			

Key: I=irregular, C=circular, Cr=cream, F= flat, L=lobate, TL= translucent, Co= cocci, R=rods, S=single, Cl= cluster, ND=Not determined, A=acid, AG= Acid and gas, NR=No reaction, OP=opaque, TP= transparent, E=entire, Ra=raised, Rh=rhizoid, Ch=chain, U= undulate, P=pink, W= white, TSI = Triple Sugar Iron Agar

5.3.4 Organisms isolated from fermented locust beans

Thirteen bacteria were isolated and characterized (Table 4). Out of these, nine were identified by the 16SrRNA sequencing (Table5) based on significant susceptibility to LGEO. The most dominant isolate belonged to the genus *Bacillus*. Others were *Staphylococcus*, *Enterobacter*, *Micrococcus* and *Yersinia*. Lactic acid bacteria were not isolated in this study as against previous reports of their isolation and involvement in the production of FLB (Adeoye *et al.*, 2018). *Bacillus* and *Staphylococcus* are the most commonly reported fermenting organisms in the production of fermented locust beans (Odunfa, 1981; Ajayi, 2014) and their isolation in this study is confirmatory.

A polyphyletic relationship of the isolates was revealed in analyzing the evolutionary distance and phylogenetic relationship (Figure 1). The isolates clustered together, with the exception of *Enterobacter hormoaechi* MJ_1_27F and *E. xiangfangenesis* M5S2B6 which form a clade independently. All *Bacillus* species identified were found to be from the same roots, truly identifying their generic relationship while *Staphylococcus hyicus* NCTC944 remained separated from the *Bacillus* root confirming the generic variation as expected in the two genera.

5.3.5 Antibacterial activities of LGEO and some antibiotics on bacteria isolated from FLB

At 25% v/v of LGEO, all the isolates were inhibited with diameter zone of inhibition ranging from 4.0 ± 1 mm to 44.3 ± 0.6 mm, except *E. xiangfangenesis* M5S2B6. None of the isolates was inhibited by Tween 80 which was used for dilution and as negative control. At a higher concentration (50%) *E. xiangfangenesis* M5S2B6 was also inhibited with 10.3 ± 1.2 mm inhibition zone. The inhibitory activities of the essential oil compared favorably with those of the antibiotics used as positive control (Table 6). Results indicate that LGEO has antibacterial activity and may be useful in controlling microbial growth that could lead to spoilage of FLB. A similar report on the antibacterial activities of LGEO however indicated higher activities against Gram negative bacteria compared to the Gram positives (Naik *et al.*, 2010).

Table 5: Molecular characteristics of bacteria isolated from fermented locust beans

Isolates	Organism	Probable Identity	Accession Number	Number of bases
D	<i>Staphylococcus hyicus</i> NCTC944	91.50%	LR134264	2675237
F	<i>Bacillus licheniformis</i> TS6	95.80%	MN013952.1	877
G	<i>Enterobacter hormoaechi</i> MJ_1_27F	93.53%	MF768986.1	1385
H	<i>Bacillus</i> sp.	97.26%	LC482469.1	1153
I	<i>E. xiangfangenesis</i> M5S2B6	91.00%	MG928407.1	1444
L	<i>Bacillus subtilis</i> SRR21	93.86%	KY401500.1	996
M	<i>Bacillus siamensis</i> HQB720	95.02%	kT758584.1	1043
N	<i>Bacillus licheniformis</i> PS4	83.15%	JH411561.1	942
O	<i>Bacillus tequilensis</i> JS56	94.23%	MK110355.1	1040

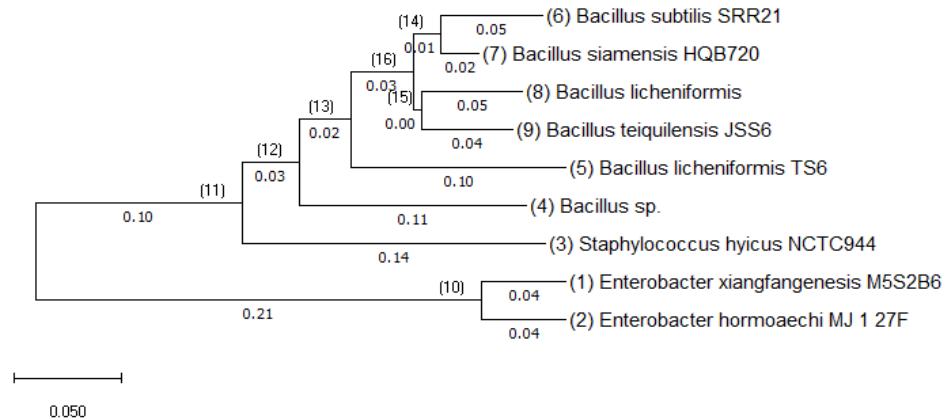


Figure 1: Evolutionary relationships of taxa of bacteria isolated from fermented locust beans samples

Table 6: Antimicrobial activities of LGEO and some antibiotics on bacteria isolated from fermented locust beans samples

Isolate	Diameter zone of inhibition around bacterial colony (mm)									
	EO_25µL	EO_50µL	OFL	AUG	GEN	CTR	ERY	CXC	CPR	TWN 80
<i>S. hyicus</i> NCTC944	4.7±0.6 ^a	41.0±1 ^c	17.3±1.2 ^c	21.2±0.8 ^d	20.3±1.2 ^d	16.3±1.2 ^c	18.8±1.8 ^c	11.2±1 ^b	0	0
<i>B. licheniformis</i> TS6	4.0±1 ^a	57.7±1.2 ^c	31.0±1.0 ^d	0	18.0±1 ^c	12.0±1 ^b	0	0	0	0
<i>E. hormoaechi</i> MJ_1_27F	43.0±0 ^c	47.5±0.5 ^c	30.0±1 ^b	0	12.67±0.6 ^a	10.3±0.6 ^a	0	0	0	0
<i>Bacillus</i> sp.	18.6±3.8 ^a	25±1 ^b	24.7±0.6 ^b	0	22.0±1.7 ^b	0	0	0	21.3±0.6 ^b	0
<i>E. xiangfangensis</i> MSS2B6	0	10.3±1.2 ^a	18.0±1 ^b	0	0	0	0	0	21.7±0.6 ^b	0
<i>B. subtilis</i> SRR21	35.3±0.6 ^a	36.±2.9 ^a	0	0	0	0	0	0	0	0
<i>B. siamensis</i> HQB720	43.3±0.6 ^a	43.0±0 ^a	0	0	0	0	0	0	0	0
<i>B. licheniformis</i> PS4	44.3±0.6 ^a	44.7±0.6 ^a	0	0	0	0	0	0	0	0
<i>B. tequilensis</i> JSS6	43.0±1 ^a	45.3±0.6 ^a	0	0	0	0	0	0	0	0

5.3.6 Effects of LGEO on the histomorphology of the liver of male Wistar rats

The liver architecture of the male Wistar rats fed LGEO-treated FLB samples did not differ significantly with those given the untreated sample. The liver tissue had a preserved lobular architecture although an insignificant inflammation was observed, no features of acute or chronic damage was observed (Plates 1a and 1b). The hepato-protective ability of LGEO has been demonstrated (Uchida *et al.*, 2017; Amujal *et al.*, 2018). However, mild periportal hepatitis was reported in Wistar rats exposed to 500 mg/Kg of the essential oil of *Phoenix dactylifera* although, no pathological change occurred in rats treated with lower doses (Oluyele *et al.*, 2022). This brings to fore, the importance of dosage in the use of essential oils as food preservatives and underscores inclusion of toxicity tests in this study.

5.4 Conclusion

In this study, the leaf essential oil of *Cymbopogon citratus* achieved up to 70% reduction of the microbial load of Fermented Locust Beans and inhibited the bacteria isolates most of which were *Bacillus*, *Staphylococcus* and *Enterobacter* species that could cause post-production spoilage. In addition, the shelf quality in terms of consumers' acceptance was improved up to 39 days of storage with no significant histopathological effect. The leaf essential oil of *Cymbopogon citratus* is therefore recommended as preservatives of Fermented Locust Beans. A study of its effect on the nutritional quality of the FLB will provide more baseline information to support this recommendation.

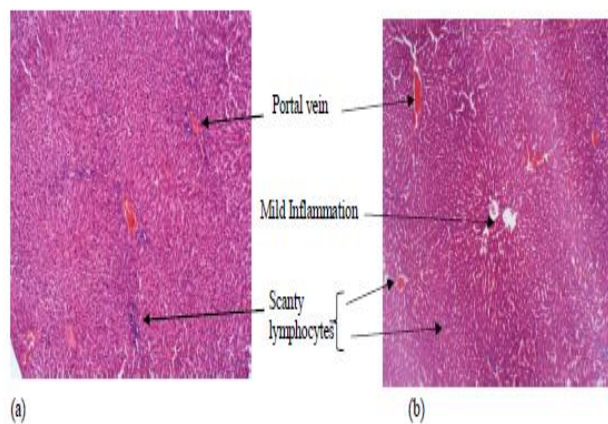


Plate 1: Photomicrograph of the histomorphology of the liver of male Wistar rat (a) fed with untreated FLB; (b) fed with FLB treated with LGEO (Magnification = $\times 400$)

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