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Antioxidant and anti-inflammatory potentials of the leaf extracts of *Calotropis procera* and *Enantia chlorantha*

Oluwasayo Esther Ogunjinmi [1,*], Peter Ifeoluwa Adegbola [2], Johnson Oladimeji Odedele [1], and Ganiyat Adeyinka Adedokun [1]

[1] *Department of Industrial Chemistry, Faculty of Natural Science, First Technical University, Ibadan, 200255, Nigeria*
[2] *Department of Biochemistry and Forensic Science, Faculty of Natural Science, First Technical University, Ibadan, 200255, Nigeria*

* *Corresponding author at: Department of Industrial Chemistry, Faculty of Natural Science, First Technical University, Ibadan, 200255, Nigeria.*
e-mail: oluwasayo.ogunjinmi@tech-u.edu.ng (O.E. Ogunjinmi).

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ABSTRACT

Inflammation and oxidative stress are involved in the aetiology of numerous human diseases. The two processes are interconnected such that one may appear before or after the other, but as soon as one of them appears, the other will. Consequently, targeting the two conditions may aid in the prevention or treatment of associated human diseases, and plants capable of performing the two functions together are of great advantage. This study was aimed at evaluating the antioxidant and anti-inflammatory potentials of methanol and aqueous extracts of *Calotropis procera* and *Enantia chlorantha*. The leaves of the two plants were extracted separately in water and methanol using a cold maceration method. Antioxidant activity was evaluated using the Lipid peroxidation (LPO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP), while the anti-inflammatory properties were evaluated using membrane stabilization and inhibition of protein denaturation assays. Qualitative phytochemical analysis was performed using standard methods. The results of the study showed that the aqueous extract of the two plants demonstrated better antioxidant activity than the methanol extracts. IC_{50} values of 61.60, 59.12, and 83.07 µg/mL were, respectively, for ascorbic acid, aqueous extracts of *E. chlorantha* and *C. procera*, while the methanol extracts of both plants recorded a value >150 µg/mL for DPPH. For LPO inhibition, the IC_{50} values were 191.79 µg/mL, >150 µg/mL for the aqueous, methanol extracts of *C. procera* and 228.25 µg/mL, 135.46 µg/mL for ascorbic acid and quercetin used as standards. The aqueous extract of *E. chlorantha* had a value of 161.95 µg/mL and the methanol extracts had a value >250 µg/mL. For the two anti-inflammatory methods used, the IC_{50} values for the plant were >250 µg/mL. Phytochemicals such as tannins, flavonoids, alkaloids, terpenoids, and phenols were identified in both plant samples. Overall, the results demonstrated the potential of the plant when used for the treatment of diseases related to inflammatory and oxidative stress.

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1. Introduction

The intricate interconnectivity between inflammation and oxidative stress emphasizes the importance of research on the pharmacological discovery of a potential modulator of these tightly linked pathophysiological processes. Via numerous studies, it is well established that many human diseases develop after dysregulation of the inflammatory and antioxidative defence mechanisms [1-3]. In addition, inflammatory injuries caused by the activation of proinflammatory cytokines and reactive oxygen species are the major trigger of this event. In the real sense, one of these two processes, *i.e.,* oxidative stress and inflammation may appear before or after the other, but as soon as one of them appears, the other is most likely to occur and eventually take part in the aetiology of different chronic diseases [4-8].

Inflammation, although a physiological process required for healing or fighting infection, can become chronic and the supposed physiological processes can go sore [6,9-12]. Similarly, free radicals known to function physiologically in the enhancement of signal transduction processes, the regulation of vascular tone, and against infectious attacks can become harmful [13-16]. Therefore, maintaining the balance and integrity of oxidative defense mechanisms is crucial in the prevention and treatment of diseases. Products of natural origin, such as plants, have been reported to play an frontier role in drug discovery, and many medicinal plants and compounds have been shown to be effective in regulation of the inflammatory process and provision of antioxidant function [17,18-22].

Calotropis procera belongs to the Asclepiadaecae family [23]. The plant is a perene Asian shrub common in adverse climate conditions and poor soils [24]. In Nigeria, it is known as tumfafia by Hausas, Kausu by Igbos, and bomubomu by Yorubas [25,26]. *C. procera* has found usefulness in the treatment of wound sores, skin diseases, diarrhoea/diarrhea, infections,

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ulcers [27], and cancer [28], as well as antioxidants and antimicrobials [29].

Enantia chlorantha, on the other hand, belongs to the *Annonaceae* family. It grows in dense tropical forests and is popularly called Awogba in Yoruba [30]. The medicinal uses of the plant include antimalaria, antipyretic, antimicrobial, antioxidant, anti-inflammatory, and gastroprotection [31-33].

Although the potentials of plants as antioxidant and anti-inflammatory have been shown in different *in vitro* and *in vivo* studies [31,34,35], the present study was designed to evaluate the variation in the potential of plants when extracted with water and methanol and to deduce the likely mode of their anti-inflammatory potentials.

2. Experimental

2.1. Plant collection and drying

The leaves of *Calotropis procera* and *Enanthia chlorantha* were obtained from Masifa area, Ogbomosho, Nigeria, on 19 May 2023. The plant samples were identified and authenticated by a taxonomist and the samples were deposited in the herbarium with voucher numbers LHO 752 for *C. procera* and LHO 753 for *E. chlorantha*. Before air drying, the samples were removed from dust and sand by thoroughly washing them in running water and then spread on the laboratory work bench until a constant weight was reached. The air-dried samples were ground to powder in an electric blender ready for extraction [36].

2.2. Preparation of plant extracts

2.2.1. Preparation of methanol extract

The methanol extraction of the plants was done by a cold maceration method. One hundred grams of each pulverized sample was soaked in methanol in 1:10 for 72 hours with intermittent shaking. At the end of 72 hours, the samples were filtered separately with Whatman's No 1 filter paper and the filtrate was evaporated at 40 °C using a rotary evaporator. The crude extracts obtained were kept in a refrigerator (4 °C) until use [36].

2.2.2. Preparation of aqueous extract

Similarly, for the methanol extraction of the samples, one hundred grams each pulverized sample was soaked separately in distilled water in 1:10 for 72 hours. The macerated samples were kept in the refrigerator throughout the extraction process with intermittent shaking. After 72 hours duration, the samples were filtered separately with Whatman's No. 1 filter paper and the filtrate was subjected to freeze drying. Powdered samples obtained from freeze-drying were kept in a refrigerator (4 °C) until use [36].

2.3. In-vitro antioxidant analysis

2.3.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical inhibition

Eight milligrams of 1,1-diphenyl-2-picrylhydrazyl were dissolved in methanol (100 mL) to obtain a solution with a concentration of 80 µg/mL. For the radical inhibitory ability of the extracts and standard, 100 µL DPPH solution and 100 µL of different concentrations of the sample and standard were mixed in a 96-well microplate, incubated at room temperature for 30 min. The absorbance of the reaction was measured at the end of the 30 min incubation period at 517 nm using a microplate reader, absolute methanol was used as a control [37]. The percentage inhibition of DPPH was measured according to Equation 1.

$$\text{DPPH radical scavenging \%} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100 \quad (1)$$

where A_0 is the absorbance of the DPPH solution and A_1 is the absorbance of the sample.

2.3.2. Ferric reducing antioxidant power (FRAP)

The working solution of FRAP was prepared by mixing acetate buffer (300 mM, pH = 3.6), 2,4,6-tripyridyl-S-triazine (TPTZ) (40 mM dissolved with 40 mM HCl) and ferric chloride (20 mM in water) in a ratio 10:1:1. Microplate FRAP was performed according to previous reports with minor modifications [38]. Different concentration of already prepared plant extracts and standard (20 µL) was transferred to the 96-well microplate and then 280 µL of working FRAP solution which was immediately followed by missing. Thereafter, mixture was incubated at 37 °C in the dark for 30 minutes. The absorbance reading was recorded at A593 with a microplate reader.

2.3.3. Inhibition of lipid peroxidation

Reactive thiobarbituric acid species (TBARS) were used to estimate lipid peroxide formed in egg yolk homogenates serving as lipid-rich media [39]. Briefly, 50 µL of egg yolk (10% v/v) was added to 10 µL of the extract (different concentration). Distilled water was added to increase the volume to 100 µL. Followed by the addition of 5µL $FeSO_4$ with incubation at 37 °C for 30 min. After incubation, 150 µL of acetic acid and 150 µL of thiobarbituric acid (TBA) in sodium dodecyl sulfate (SDS) were sequentially added to the mixture, following vortex mixing and then heating at 95 °C for 1 hour. At the end of the heating period, 500 µL of butanol was added and the samples were centrifuged at 3000 rpm for 10 min. The absorbance of the upper layer (organic component) was read at 532 nm. The percentage inhibition was calculated according to Equation 2.

$$\text{\% Inhibition of lipid peroxidation} = \left[\frac{(100 - \text{Asorbance of sample})}{\text{Absorbance of control}}\right] \times 100 \quad (2)$$

2.4. In-vitro anti-inflammatory assays

2.4.1. Erythrocyte membrane stabilization assay

Erythrocyte membrane stabilization of the extracts was measured as described by Sadique *et al.* [40] and Saket *et al.* [41]. Fresh whole human blood obtained early in the morning (10 mL) was centrifuged at 3000 rpm for 10 min and then washed in an equal volume of normal saline three times. The volume of the washed blood sample was measured and reconstituted (10% v/v) in normal saline.

For the membrane stabilization assay, the reaction mixture (2 mL) consisted of 1 mL of extract (different concentrations) and 1 mL of 10% RBCs suspension. In the control test tube, saline was added in place of the extracts, whereas diclofenac and indomethacin served as the standard drug. The reaction mixture was then incubated in a water bath at 56 °C for 30 min, cooled under running tap water, and then centrifuged at 2500 rpm for 5 min. The absorbance of the supernatants was read at 560 nm. All experiments were performed in triplicate for all test samples. Percentage of membrane stabilization was calculated using Equation 3.

$$\text{Protection \%} = \left[\frac{100 - \text{Absorbance of sample}}{\text{Absorbance of control}}\right] \times 100 \quad (3)$$

2.4.2. Inhibition of protein denaturation

The method described by Sakat *et al.* [41] and Mizushima and Kobayashi [42] was used to determine the inhibition of

Table 1. DPPH radical scavenging activity (%) of aqueous and methanol leaf extracts of *C. procera* and *E. chlorantha*.

Concentration, (µg/mL)	Percentage inhibition (mean ± SE) *				
	AECP	MECP	AEEC	MEEC	Ascorbic acid
10.00	16.98±2.31	14.65±1.71	24.77±0.63	13.44±0.59	1.68±0.33
20.00	26.88±0.31	19.92±0.24	34.69±0.32	17.22±0.58	39.37±0.32
50.00	46.13±2.17	20.86±0.78	56.73±0.91	18.57±0.99	64.79±0.08
100.00	60.01±0.31	22.42±0.23	65.63±0.48	21.07±1.76	72.53±0.26
150.00	67.31±0.20	23.03±0.35	80.83±0.74	22.62±0.81	83.40±0.03
IC_{50} (µg/mL)	83.07	>150.00	59.12	>150.00	61.06

* AECP: Aqueous extracts of *C. procera*, AEEC: Aqueous extracts of *E. chlorantha*, MECP: Methanol extracts of *C. procera*, MEEC: Methanol extracts of *E. chlorantha*.

Table 2. Ferric reducing antioxidant power of aqueous and methanol leaf extracts of *C. procera* and *E. chlorantha*.

Concentration, (µg/mL)	Percentage inhibition (mean ± SE) *				
	AECP	MECP	AEEC	MEEC	Ascorbic acid
10.00	41.06±1.68	12.90±1.71	51.73±0.04	10.25±0.09	57.22±1.41
20.00	53.25±0.17	13.32±0.24	55.12±0.21	11.69±1.23	68.67±0.80
50.00	56.82±0.07	14.82±0.78	68.04±0.48	11.88±1.27	114.22±0.15
100.00	63.75±0.67	15.92±0.23	76.29±0.47	13.25±1.03	133.82±0.50
150.00	71.20±1.01	17.20±0.35	108.82±0.79	15.29±1.33	197.65±1.77

* AECP: Aqueous extracts of *C. procera*, AEEC: Aqueous extracts of *E. chlorantha*, MECP: Methanol extracts of *C. procera*, MEEC: Methanol extracts of *E. chlorantha*.

Table 3. Phytochemical constituents of *Calotropis procra* and *Enantia chlorantha* leaves *.

No	Phytochemicals	*C. procera*	*E. chlorantha*
1	Tannins	++	+
2	Glycosides	+	+
3	Resins	-	+
4	Saponins	++	++
5	Phlobotannins	+	+
6	Flavonoids	++	+
7	Steroids	+	-
8	Phenols	+	++
9	Reducing Sugar	-	-
10	Alkaloids	++	++
11	Terpenoids	+	+

* '+' = Present; '++' = Strongly present; '-' = Absent.

protein denaturation. The reaction mixture consisted of extracts (different concentrations) and 1% aqueous solution of bovine albumin fraction and was incubated at 37 °C for 20 min. After incubation, the mixture was heated to 51 ° C for 20 min and then cooled before measuring the turbidity spectrophotometrically at 660 nm. Inhibition of protein denaturation was calculated using Equation 4.

$$\text{Percentage inhibition} = \frac{(\text{Abs. of control} - \text{Abs. of sample/standard})}{\text{Abs.of control}} \times 100 \quad (4)$$

2.5. Phytochemical screening

The qualitative analysis of the phytoconstituents of the two plant samples was carried out using standard methods described by Olasunkanmi *et al.* [36].

2.6. Statistical analysis

All analyses were performed in triplicate, and the average value was calculated.

3. Results and discussion

3.1. In vitro antioxidant activity

The aqueous and methanol extracts of the samples were evaluated for their radical scavenging properties by comparing them with the appropriate standard (Tables 1 and 2). Authors support the use of more than a single evaluation method for the determination of antioxidant efficacy of plant samples [41]. Therefore, in this study, the antioxidant properties of the two plant samples were evaluated using the DPPH and FRAP methods.

3.1.1. DPPH radical inhibition

The change in the colour of the DPPH radical from purple to yellow is the behaviour of a potent antioxidant sample when interacting with DPPH radicals. The colour change indicates transfer of electrons from the antioxidant to the radical to become a stable compound that is monitored by measuring the decrease in absorbance [43-45]. In this study, the DPPH radical scavenging activity demonstrated by the two plant samples is given in Table 1 and the ranking of their potency was performed utilizing with the IC_{50} values. The extracts demonstrated concentration-dependent inhibition of the radicals with the aqueous extracts of both plants, demonstrating the best inhibition of DPPH radicals relative to the methanol extracts. The aqueous extracts of *E. chlorantha* and *C. procera* have IC_{50} values of 59.12 and 83.07 µg/mL, respectively, while the values for the methanol extracts were respectively > 150 µg/mL. A similar study has shown a higher antioxidant activity of aqueous extracts compared to extracts of polar organic solvents such as ethanol [46], while Namadina *et al.* [34] reported a lower activity than that of the present study. Significant antioxidant activity is related to the presence of plant flavonoids and other polyphenols [47] and the phytochemical analysis of the plant samples (Table 3) shows the presence of phytochemicals associated with antioxidant activities. Namadina *et al.* [34] reported similar phytochemicals in the methanol extract of *C. procera.*

3.1.2. Ferric reducing antioxidant power (FRAP) and Lipid Peroxidation

Similar to the DPPH radical inhibition, the reducing power of the aqueous extracts was demonstrated in a concentration-dependent manner, while the methanol extracts demonstrated a much lower reducing antioxidant power (Table 2). By demonstrating reducing antioxidant power, the plant samples have the potential to inhibit metal-induced chain reaction, decomposition of peroxides, and prevention of continued hydrogen abstraction [48]. In the literature, it is evidenced that the formation of highly reactive oxidative species such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals can be catalysed by free iron through the Haber-Weiss

Table 4. Percentage inhibition (mean±SE) of lipid peroxidation, protein denaturation, and membrane stabilization by aqueous and methanol leaf extracts of *C. procera*.

Sample	Percentage inhibition of lipid peroxidation (LPO) (µg/mL)					IC_{50} (µg/mL)
	50	100	150	200	250	
Aqueous extract	23.46±1.43	28.86±0.41	45.81±0.85	55.49±0.55	57.12±0.16	191.79
Methanol extract	6.11±0.37	10.50±0.50	13.24±0.57	27.19±0.19	32.59±1.82	> 150
Ascorbic acid	12.88±0.38	19.86±0.38	31.87±0.94	45.47±0.19	54.60±0.59	228.25
Quercetin	29.61±0.16	40.46±1.86	56.10±0.17	66.29±0.47	74.30±0.56	135.46
	Percentage inhibition of protein denaturation					
Aqueous extract	13.39±0.40	22.25±1.60	25.56±1.07	26.54±1.48	40.47±3.31	>250
Methanol extract	1.99±1.16	13.34±0.50	23.95±3.73	32.60±0.88	32.71±4.36	>250
Diclofenac	20.07±1.36	24.81±0.23	33.61±0.87	46.14±0.60	53.88±1.01	230.37
Indomethacin	10.83±1.16	10.74±1.45	62.79±1.36	70.69±0.23	71.29±0.55	163.10
	Percentage membrane stabilization					
Aqueous extract	1.20±0.17	8.89±0.88	13.39±1.24	15.04±1.48	21.08±0.17	>250
Methanol extract	18.16±0.33	17.47±1.01	24.92±0.17	31.64±0.84	43.12±0.38	>250
Diclofenac	8.15±1.48	13.16±0.97	23.30±0.97	38.06±0.41	47.58±1.27	>250
Indomethacin	28.08±3.12	32.18±0.57	42.68±0.38	59.61±0.32	78.10±0.73	157.36

Table 5. Percentage inhibition (mean±SE) of lipid peroxidation, protein denaturation, and membrane stabilization by aqueous and methanol leaf extracts of *E. chlorantha*.

Sample	Percentage inhibition of lipid peroxidation (LPO) (µg/mL)					IC_{50} (µg/mL)
	50	100	150	200	250	
Aqueous extract	25.98±0.35	31.94±0.69	53.49±0.14	60.15±1.43	65.60±0.37	161.95
Methanol extract	2.37±0.14	6.62±0.50	12.09±0.19	24.24±0.25	26.47±0.40	> 250
Ascorbic acid	12.88±0.38	19.86±0.38	31.87±0.94	45.47±0.19	54.60±0.59	228.25
Quercetin	29.61±0.16	40.46±1.86	56.10±0.17	66.29±0.47	74.30±0.56	135.46
	Percentage inhibition of protein denaturation					
Aqueous extract	13.17±0.12	17.22±0.15	25.22±0.34	34.48±0.16	39.93±0.38	>250
Methanol extract	5.30±2.64	13.63 ±1.45	16.29±0.62	31.45±0.24	32.58±0.34	>250
Diclofenac	20.07±1.36	24.81±0.23	33.61±0.87	46.14±0.60	53.88±1.01	230.37
Indomethacin	10.83±1.16	10.74±1.45	62.79±1.36	70.69±0.23	71.29±0.55	163.10
	Percentage membrane stabilization					
Aqueous extract	4.50±1.11	13.45±0.71	28.43±0.21	30.14±0.30	35.38±0.99	>250
Methanol extract	1.91±0.93	7.99±3.67	20.96±0.51	25.21±0.62	36.14±3.27	>250
Diclofenac	8.15±1.48	13.16±0.97	23.30±0.97	38.06±0.41	47.58±1.27	>250
Indomethacin	28.08±3.12	32.18±0.57	42.68±0.38	59.61±0.32	78.10±0.73	157.36

and Fenton reactions [49,50]. Transition metals are highly reactive and are a major lipid oxidation pro-oxidant [51], thus perpetuating the progression of a chain reaction with consequent membrane damage and initiation of disease. In this study, the antioxidant activity demonstrated by plant extracts is a testament to their ability to inhibit the propagation of radical chain reactions. However, to further establish these potentials, plant samples were subjected to inhibition of the lipid peroxidation assay and the result is presented in Table 4. Iron-stimulated lipid peroxidation of phospholipids in egg yolk is characterized by an electron transfer reaction, and inhibition of the process may serve as an indicator of the protective ability of a plant extract. The subsequent peroxidation of lipids can cause tissue damage that is consequential to general well-being. In this study, the water-extracted components of the two plants were able to inhibit the lipid peroxidation process, while lower activity was observed for the methanol extract. The efficacy of the aqueous extracts was slightly higher than that of the ascorbic acid that was used as the standard. Previous studies have shown that the plant extracted in aqueous medium has a strong inhibitory potential against lipid peroxidation [52].

3.2. In-vitro anti-inflammatory studies

3.2.1. Membrane stabilization assay

Both membrane stabilization and inhibition of protein denaturation assays are in vitro approaches to assess the anti-inflammatory potential of test compounds [53], plant compounds modulate the cellular activities of inflammatory-related cells by stabilizing their membranes to prevent cell degranulation [54,55] and consequently prevent lysosomal enzyme impairment. In this study, the methanol extract of *C. procera* demonstrated a membrane stabilization capacity comparable to that of diclofenac but lower than that of indomethacin. As clearly observed in Tables 4 and 5, the methanol extracts of *C. procera* and *E. chlorentha* were better membrane stabilizers than their respective aqueous extracts, contradicting the previous submission of Fadahunsi *et al.* [53] who showed better efficacy of the aqueous extracts than of the ethanol extract. As observed in the literature, various plant compounds such as flavonoids, saponins, and tannins, have the potential to contribute to the maintenance of membrane integrity [53-55] and because these phytochemical classes of compounds were identified in the two plant samples forms the basis to suspect them as contributors to the observed activity. Furthermore, both aqueous and methanol extracts of *C. procera* and *E. chlorentha* showed lower anti-inflammatory effects than standard diclofenac and indomethacin by exhibiting lower inhibition of protein denaturation.

Protein denaturation is one of the leading causes of injury [56] and inhibition of the process is significant in the anti-rheumatoid effect of non-steroidal anti-inflammatory drugs (NSAIDs). Furthermore, the denaturation of proteins *in vivo* may be the cause of autoantigen production in certain arthritic conditions [57]. Therefore, a substance capable of preventing protein denaturation could be a potential anti-inflammatory candidate. In this study, although both plant samples demonstrated lower effects than the standards used, notwithstanding, their potential cannot be overemphasized.

In general, observations from this study showed that the plants contain important constituents that could have contributed to the observed activity. Flavonoids such as 3-O-rutinosides of quercetin, kaempferol, and isorhamnetin, in addition to the flavonoid 5-hydroxy-3,7-dimethoxyflavone-4'-O-β-glucopyranoside [58] previously isolated from the leaves of *C. procera*, are important compounds with diverse pharmacological efficacies.

4. Conclusions

This study presented the results of two important plants with the potential to exhibit antioxidant and anti-inflammatory activities. Aqueous extracts demonstrated better efficacy than the methanol extract. From this study, it is clear that the extraction solvent had a significant influence on the efficacy of plant extracts. Overall, the results demonstrated the potential of the plant when used for the treatment of inflammatory and oxidative stress-related diseases. Future studies would characterize the active principle(s) in the plant samples for drug development.

Disclosure statement

Conflict of interest: The authors declare that they have no conflict of interest.
Ethical approval: All ethical guidelines have been adhered.

CRediT authorship contribution statement

Conceptualization: Oluwasayo Esther Ogunjinmi, Peter Ifeoluwa Adegbola; Methodology: Oluwasayo Esther Ogunjinmi, Peter Ifeoluwa Adegbola; Software: Peter Ifeoluwa Adegbola; Validation: Oluwasayo Esther Ogunjinmi, Peter Ifeoluwa Adegbola; Formal Analysis: Oluwasayo Esther Ogunjinmi, Peter Ifeoluwa Adegbola, Johnson Oladimeji Odedele, Ganiyat Adedokun; Investigation: Oluwasayo Esther Ogunjinmi, Peter Ifeoluwa Adegbola, Johnson Oladimeji Odedele, Ganiyat Adedokun; Resources: Johnson Oladimeji Odedele, Ganiyat Adedokun; Data Curation: Oluwasayo Esther Ogunjinmi, Peter Ifeoluwa Adegbola, Johnson Oladimeji Odedele, Ganiyat Adedokun; Writing - Original Draft: Oluwasayo Esther Ogunjinmi, Peter Ifeoluwa Adegbola; Writing - Review and Editing: Oluwasayo Esther Ogunjinmi, Peter Ifeoluwa Adegbola; Visualization: Oluwasayo Esther Ogunjinmi, Peter Ifeoluwa Adegbola; Funding acquisition: Johnson Oladimeji Odedele, Ganiyat Adedokun; Supervision: Oluwasayo Esther Ogunjinmi, Peter Ifeoluwa Adegbola; Project Administration: Oluwasayo Esther Ogunjinmi, Peter Ifeoluwa Adegbola.

ORCID and Email

Oluwasayo Esther Ogunjinmi
oluwasayo.ogunjinmi@tech-u.edu.ng
https://orcid.org/0000-0003-3294-3137
Peter Ifeoluwa Adegbola
peter.adegbola@tech-u.edu.ng
https://orcid.org/0000-0002-4008-1728
Johnson Oladimeji Odedele
johnson.odedele808@gmail.com
https://orcid.org/0009-0009-0090-2972
Ganiyat Adeyinka Adedokun
ganiyat.adedokun@tech-u.edu.ng
https://orcid.org/0009-0009-0050-5758

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