Chemical analysis and protective effect of *Elaeis guineensis* kernel oil against calcium ion-induced mitochondrial membrane permeability transition

Anyasor, God'swill Nduka[*]; Onuoha, Ezinwanne Elizabeth and Gisarin Olusola

Department of Biochemistry, Benjamin S. Carson School of Medicine, Babcock University, Ilisan-Remo, Ogun State, Nigeria. P.M.B. 21244 Ikeja.

*corresponding author: anyasorg@babcock.edu.ng or anyasorgodswill@gmail.com

Abstract: This study evaluated the chemical property and effect of *Elaeis guineensis* kernel (EGK) oil on calcium ion-induced mitochondrial membrane permeability transition (MMPT) using standard *in vitro* methods. Results showed the EGK oil values for iodine (46.53±1.8 Wij's), saponification (246.33±1.2 mgKOH/g), acid (10.32±2.5%) and peroxide (3.03±0.4 mEq/kg). EGK oil (150 µg/ml) exhibited a maximal inhibitory (80.9±4.8%) effect on MMPT. Furthermore, EGK oil (100 µg/ml) maximally stabilized (96.72±5.2%) erythrocyte membrane against hypotonicity-induced hemolysis. In addition, EGK oil (250 µg/ml) inhibited heat-induced protein denaturation by 12.42±2.6% and mitochondria lipid peroxidation by 61.03±3.7%. This study showed that EGK oil could possess mitochondria protective agent(s).

1.0 INTRODUCTION

Mitochondria research has progressed in recent times as a result of the greater understanding of the central role played by the organelle in a number of disorders (Via et al., 2014; Payne and Patrick, 2015). Some disorders have been closely associated with the opening of mitochondria membrane permeability transition (MMPT) (Bernardi and Lisa, 2015) and mutations in the mitochondrial DNA (mtDNA) (Khan et al., 2015). MMPT has been implicated in intrinsic apoptotic pathway, formation of reactive oxygen species and derangement in the energy production of process (Treulen et al., 2016).

MMPT is a Ca^{2+}-mediated amplitude in the permeability of mitochondrial membranes that induces mitochondrial swelling and eventual lysis of the outer mitochondrial membrane (Wang et al., 2015). This phenomenon occurs after the opening of a putative mega channel, which consists of a voltage-gated anion channel, an adenine nucleotide translocator, cyclophilin D (a mitochondrial peptidylprolyl-cis, trans-isomerase), and other molecules (Desagher and Martinou, 2000; Carlos et al., 2015). Consequently, the proton motive force is dissipated and oxidative phosphorylation uncoupled resulting into adenosine triphosphate (ATP) depletion, bioenergetics failure and eventual cell death (Halestrap et al., 2004; Llorente-Folch et al., 2015).

A wide range of substances have been isolated or designed therapeutically to either activate or inhibit MMPT pore opening (Halestrap et al., 2004; Larmache et al., 2012; Anyasor et al., 2014). However, most of these compounds are synthetic, expensive, non-selective and toxic effects to normal cell, which would eventually progress into tissue degeneration. Hence, there is a need to investigate the potentials of organic materials particularly of plant origin to serve as protective agent against mitochondrial membrane damage.

Elaeis guineensis Jacq. commonly called African oil palm of the family of Arecaceae is the principal source of palm oil in the world (Obahiagbon, 2012). The reddish palm fruit bunch takes five to six months to mature and weighs about 40 - 50 kg (Obahiaghon, 2012). The fleshy fruit of *E. guineensis* yields palm oil from its mesocarp while palm kernel oil is extracted from the endosperm (Gervajio, 2005; Vaughan and Geissler, 2009).

Oil palm produces about 7,250 L oil per hectare per year which has made it a common cooking oil in most region of the world. In different countries, there is a high demand for palm and kernel oils by food and pharmaceutical industries which could be attributed to its reduced cost, oxidative stability of the refined products (Matthaus, 2007) and presence of high levels of natural antioxidants (Sundram et al., 2003; Tiku and Bullem, 2015).

E. guineensis (EGK) oil contains fatty acids including lauric (45%), myristic (18%), palmitic (9%), oleic (15%) and linoleic (2%) acids. Its mineral content includes calcium (1.1 g/kg), phosphorus (3.4 g/kg), potassium (3.8 g/kg) and magnesium (1.8 g/kg). In addition, EGK oil contains amino acids which include histidine (2.5% protein), arginine (3.9% protein), lysine (3.5% protein), methionine (2.1% protein) and (tryptophan (2.8% protein) which perform many crucial and diverse functions in living systems

(Gervajio, 2005;Gunstone, 2013). Thus, this study was designed to evaluate the chemical analysis of EGK oil and investigate its effect on calcium ion-induced mitochondrial membrane permeability transition using *in vitro* models.

2.0 MATERIALS AND METHODS
2.1 *Elaeis guineensis* kernel oil and processing
One litre of *Elaeis guineensis* kernel (EGK) oil was purchased from a reputable local market at Warri, Delta state, Nigeria. Five hundred milliliters of n-hexane was added to 500 ml EGK oil to separate it further. The mixture (1:1 v/v) was filtered using the Whatman No.1 filter paper and subsequently concentrated using rotary evaporator (BuchiRotavapor RE; Switzerland) at 30°C. The concentrated EGK extract was immediately stored in an amber bottle at room temperature until further used.

2.2 Chemical analysis
2.2.1 Acid value: The acid value of EGK oil was determined by titrimetric method of Pearson (1970). A hot neutral alcohol (75 ml) was added to 5 g EGK oil with a few drops of 1% phenolphthalein indicator and mixed vigorously. Subsequently, the mixture was titrated with 0.1M NaOH solution with constant shaking until the pink colour remains permanent.

2.2.2 Saponification value: The saponification value of EGK oil was determined by the titrimetric method of Pearson (1981). An alcoholic KOH (25 ml) was added to 0.2 g EGK oil and the mixture was heated in boiling water for 1 h. Subsequently, 1ml 1% phenolphthalein was added to the mixture and titrated with 0.5N HCl.

2.2.3 Iodine value: The iodine value of EGK oil was carried out using titrimetric method of Pearson (1970). CCl_4 (10 ml) and 20 ml of Wij's solution was added to 0.2 g EGK oil and the mixture was kept in the dark for 30 min. Subsequently, 15 ml 10% KI and 100 ml distilled water was added to the mixture which was immediately titrated with 0.1M sodium thiosulphate using 1% starch as indicator.

2.2.4 Peroxide value: The peroxide value of EGK oil was determined by the Association of Official Analytical Chemist (AOAC) method (1984). One gram KI and 20 ml of glacial acetic acid and chloroform mixture was added to 0.2 g oil. The mixture was boiled for 30 s and transferred to 20 ml 5% iodide solution. The test tube was rinsed with 25 ml distilled water and subsequently titrated with 0.002N sodium thiosulphate solution using 1% starch as indicator.

2.3 Animals
Five male albino rats (Wistar strain) with an average weight of 150 ± 2.5 g were obtained from the Animal Facility, Babcock University. Animals were acclimatized for 14 days, maintained under standard condition of temperature and 12-hour dark/light cycle and were fed with water and commercial chow *ad libitum*. Animals were maintained and cared following the National Institute of Health (NIH) good laboratory animal care guidelines.

2.4 Procedure for the isolation of rat hepatic mitochondria
Rat hepatic mitochondria were isolated in accordance to the method described by Johnson and Lardy (Johnson and Lardy, 1967) with some modifications. Animals were anaesthetized using chloroform in an air-tight chamber and subsequently sacrificed to remove liver. The obtained liver was immediately weighed, homogenized and subjected to conventional differential centrifugation method to isolate the hepatic mitochondria in a buffer solution containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.4) and 1 mM EGTA. In the final wash solution, EGTA was omitted.

The protein content of mitochondrial fraction was carried out following the Folin-Ciocateau method (Lowry et al., 1951). Bovine serum albumin (BSA) was used as standard protein. The mitochondrial suspension was kept on ice and used fresh. All assays were carried out in an ice-cold medium in order to maintain the integrity of the mitochondria.

2.5 Determination of mitochondrial membrane permeability transition
Mitochondrial membrane permeability transition (MMPT) was determined using spectrophotometric method as described by Lapidus and Sokolove (1993). The isolated hepatic mitochondria fraction (0.4 mg/ml) and 0.8 μM rotenone were pre-incubated for 3.5 min followed by the addition of 5 mM sodium succinate. To determine the effect of Ca^{2+} as a triggering agent; mitochondria were incubated in the presence of 0.8 μM rotenone for 3 min. This was followed by the addition of 3 μM Ca^{2+} and 30 sec later sodium succinate was added. In order to determine the effect of 1mM spermine (a natural polyamine used as standard MMPT inhibitor) or EGK oil extract, the mitochondria were pre-incubated in the presence of rotenone and spermine or EGK oil extract (50, 150 and 300 μg/ml) for 3 min. This was followed by the addition of Ca^{2+} and 30 sec later sodium succinate was added. MMPT was quantified as change in absorbance at 540 nm wavelength at a time interval of 30 sec for 12 min using double beam UV/Visible spectrophotometer (T80 model, PG instrument).

The percentage inhibition of MMPT opening was calculated as:

$$\frac{\Delta TA^{12} - \Delta V^{12}}{\Delta TA^{12}}$$

Where ΔV^{12} = change in absorbance for EGK oil extract at 12 min.

ΔTA^{12} = change in absorbance for triggering agent at 12 min.

2.6 Hypotonicity-induced membrane haemolysis assay

The effect of EGK oil extract on hypotonicity-induced haemolysis of red blood cell membrane was carried out according to the method by Vangalapati and Chippanda (2011) with some modifications. The assay mixture which contained 1 ml of phosphate buffer (pH 7.4), 2 ml of hypo saline (0.36%), 0.5 ml of erythrocyte suspension (10% v/v) with 0.5 ml of EGK oil extract or standard drug (diclofenac sodium) of varying concentrations (100, 250, 500 and 1000 μg/ml) in a final volume of 4 ml were incubated at $37^{o}C$ for 30 min and centrifuged after cooling under running tap water for 5 min at 3000 rpm. The control contained 2.5 ml of distilled water but did not contain test sample or standard drug. The hemoglobin content in the suspension was estimated using spectrophotometric method at 560 nm.

The percentage inhibition of haemolysis or membrane stabilization was calculated using the following equation:

$$\frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

2.7 Anti-denaturation of protein assay

The effect of EGK oil on anti-denaturation of protein assay was carried out using the method of Mizushima and Kobayashi (1968) with some modifications. A stock solution of 1 mg/ml EGK oil extract was prepared using dimethyl sulphoxide (DMSO) as a solvent. From this stock solution, four different concentrations of 100, 250, 500 and 1000 μg/ml were prepared. One milliliter of each extract was transferred to test tubes using a micro pipette. One milliliter of 5% w/v bovine serum albumin (BSA) was added to all the above test tubes. The control consists of 1 ml 5% w/v BSA solution with 1 ml DMSO. The standard consists of 1 ml of diclofenac sodium in distilled water with 1 ml 5% w/v BSA solution. The test tubes were incubated at 27°C for 15 min and subsequently heated in a water bath at 70°C for 10 min. The test tubes were later cooled for 10 min. The absorbance was determined by using double beam UV/Visible spectrophotometer (T80 model, PG instrument) at 660 nm. The percentage inhibition of heat-induced denaturation of protein was determined using the following formula:

$$\frac{Absorbance\ of\ control - Absorbance\ of\ test}{Absorbance\ of\ control} \times 100$$

2.8 Inhibition of mitochondria lipid peroxidation assay

The inhibition of hepatic mitochondria lipid peroxidation assay was carried out following the method described by Ohkawa et al (1979). The reaction mixture contained 0.1 ml of rat hepatic mitochondria in mannitol-sucrose-hepes (MSH) buffer pH 7.4, 0.1 ml EDTA, 0.1 ml $FeSO_4$, 0.1 ml H_2O_2, and 0.5 ml varying concentrations of EGK oil (0.25, 0.5 and 1.0 mg/ml) and made up to 1 ml. The reaction mixture was incubated for 1 h at 37°C. After the incubation, 0.5 ml of the reaction mixture was treated with 0.2 ml sodium dodecyl sulfate, 1.5 ml thiobarbituric acid and 1.5 ml acetic acid. The total volume was made up to 4 ml by distilled water and kept in a water bath at 95 - 100°C for 1 h. Two milliliters reaction mixture was subsequently mixed with 3 ml n-butanol (BDH Chemicals Ltd, England) and centrifuged at 3000 rpm for 10 min and absorbance was measured at 535 nm using double beam UV/Visible spectrophotometer (T80 model, PG instrument). The test tubes without Fenton's reaction mixture and EGK oil served as blank, while those without EGK oil alone were taken as control.

Percentage inhibition of lipid peroxidation was calculated as:

$$\frac{Absorbance\ of\ control - Absorbance\ of\ test}{Absorbance\ of\ control} \times 100$$

2.9 Statistical analysis

Statistical analysis was carried out using SPSS analytical software for windows; SPSS Inc., Chicago, standard version 17.0 to determine differences between the mean of the test samples using student t-test analysis and One Way Analysis of Variance (ANOVA). The differences with probability values of $P < 0.05$ was considered significant. Microsoft Office Excel version 2010 for Windows was used to determine the percentage inhibition and graphical representation of results. All analysis were performed in triplicate and data reported as mean ± standard error of mean.

3.0 RESULTS

Table 1 showed results of the chemical analysis of EGK oil values as follows: iodine (46.53 ± 1.8 Wij's), saponification (246.33 ± 1.2 mgKOH/g), acid (10.32 ± 2.5%) and peroxide (3.03 ± 0.4 mEq/kg).

Table 1: Chemical analysis of *E. guineensis* kernel oil

PARAMETER	AMOUNTS
Acid value	10.32 ± 2.5 %
Saponification value	246.33 ± 1.2 mgKOH/g
Iodine value	46.53 ± 1.8 Wij's
Peroxide value	3.03 ± 0.4 mEq/kg

Figure 1 showed that the change in absorbance of mitochondria fraction incubated with Ca^{2+} at 12 min had a large amplitude of MMPT (-0.216 ± 0.45) when compared to mitochondria fraction incubated in the absence of Ca^{2+} (-0.005 ± 0.03). This was reversed when mitochondria fraction was

incubated with Ca^{2+} and spermine (-0.011 ± 0.12) at 12 min.

Figure 2 showed that EGK oil extract (50 - 300 µg/ml) significantly (P<0.05) inhibited Ca^{2+}-induced hepatic MMPT with maximal (80.9 ± 4.8%) and minimal (62.96 ± 7.2%) inhibitions at 150 and 300 µg/ml respectively.

Figure 3 showed that EGK oil stabilized erythrocyte membrane against hypotonicity-induced haemolysis in a reversed concentration dependent manner with maximal (96.72 ± 5.2%) and minimal (86.48 ± 4.9%) inhibitions at 100 and 1000 µg/ml respectively while Diclofenac sodium (DS) stabilized erythrocyte membrane against haemolysis in a concentration dependent manner with maximal (98.92 ± 4.7%) and minimal (92.69 ±

4.5%) inhibitions at 1000 and 100 µg/ml respectively. Furthermore, Figure 3 indicated that EGK oil inhibited heat-induced protein denaturation in a reverse concentration dependent manner with the highest (12.42 ± 2.6%) and lowest (4.28 ± 0.5%) inhibitions at 250 and 1000 µg/ml respectively while DS inhibited heat-induced protein denaturation by 97.89 ± 3.9% and 97.92 ± 4.5% at 100 and 1000 µg/ml respectively.

Figure 4 showed that EGK oil inhibited ROS-induced lipid peroxidation in rat hepatic mitochondrial fraction in an inverse concentration dependent manner. This results showed that EGK oil inhibited hepatic mitochondria lipid peroxidation maximally by 61.03 ± 3.7% and minimally by 25.52 ± 4.8% at 0.25 and 1.0 mg/ml respectively.
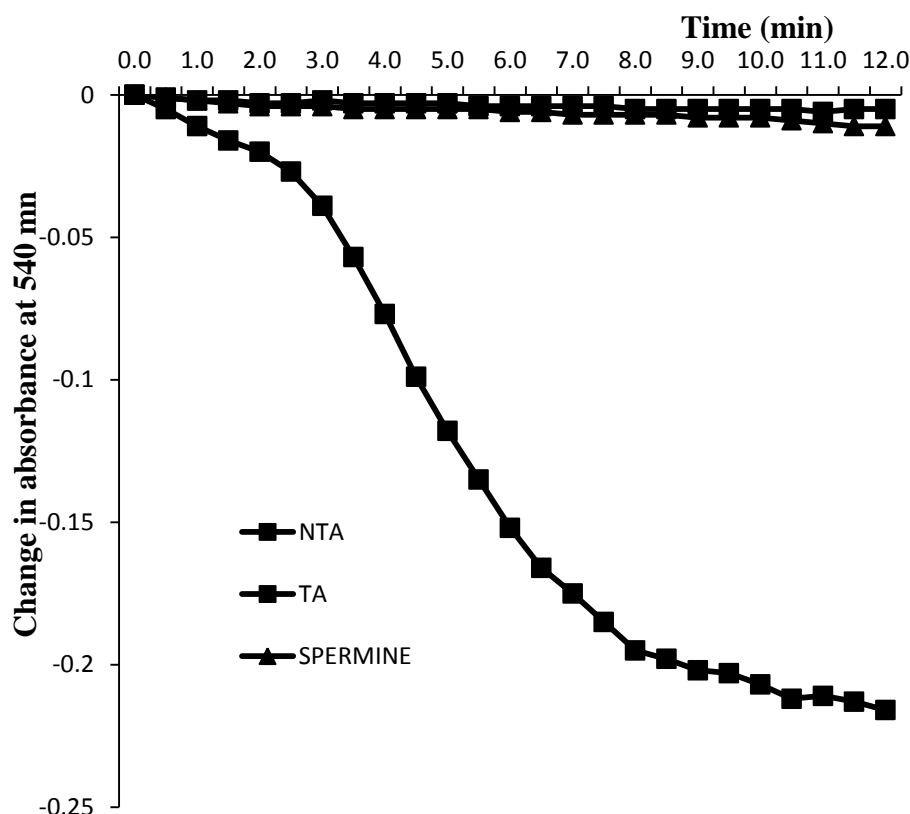

Figure 1: The inductive effect of calcium ion on MMPT formation and inhibitory effect of spermine energized by sodium succinate was measured spectrophotometrically at Δ540 nm for 12 min. TA indicates triggering agent; NTA indicates no triggering agent
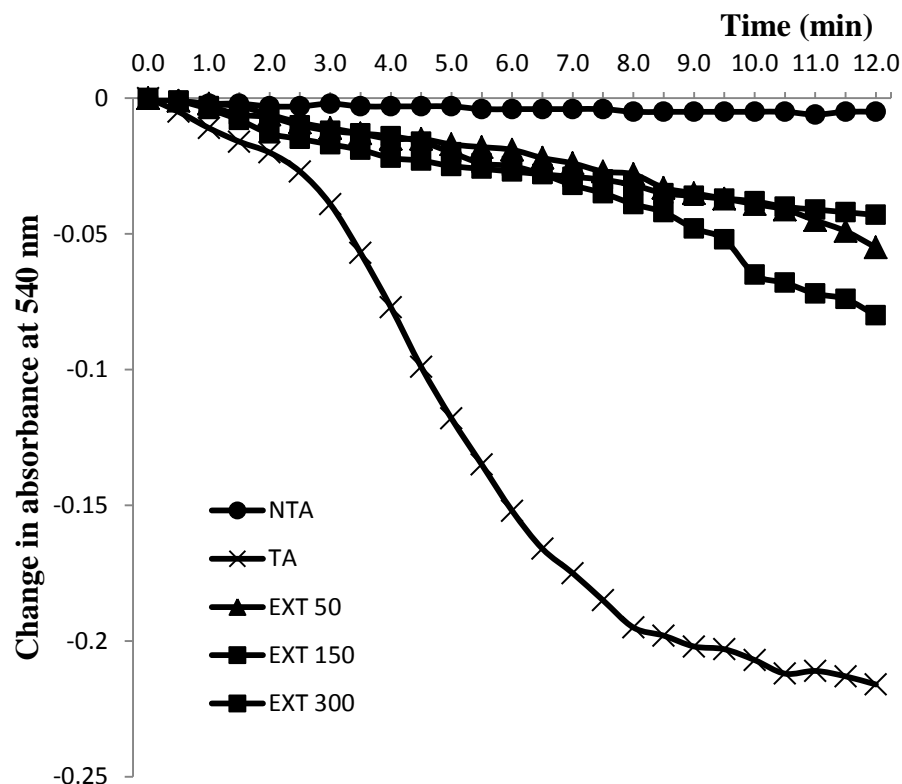
Figure 2: Inhibitory effects of 50, 150 and 300 μg/ml *E. guineensis* kernel oil extract on Ca^{2+}-induced hepatic MMPT formation as measured spectrophotometrically at Δ540 nm for 12 min. TA indicates triggering agent; NTA indicates no triggering agent
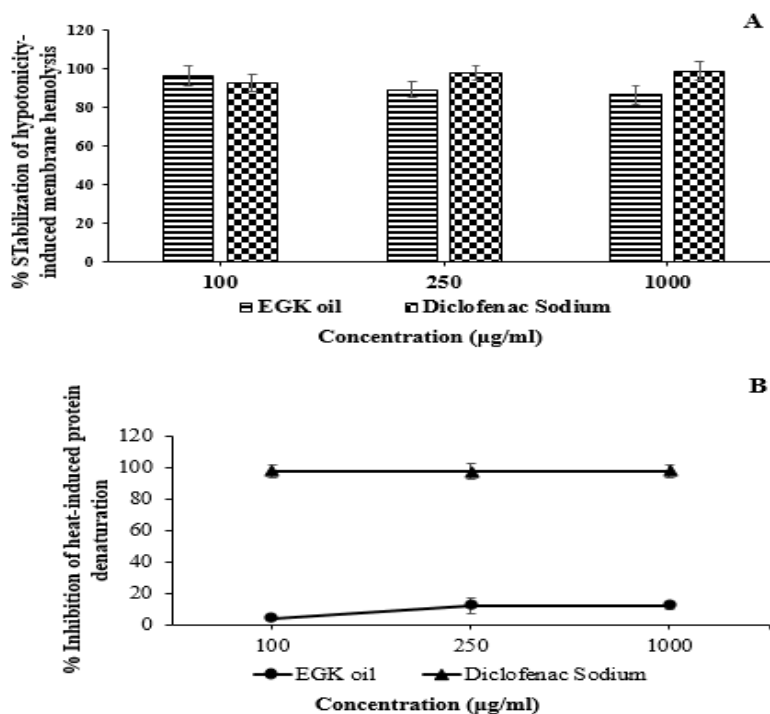
Figure 3: A: Percentage stabilization of hypotonicity-induced hemolysis of erythrocyte membrane by 100, 250 and 1000 μg/ml *E. guineesis* kernel (EGK) oil extract and standard Diclofenac sodium. **B :** Percentage inhibition of heat-induced protein denaturation by 100, 250 and 1000 μg/ml EGK oil extract and Diclofenac sodium
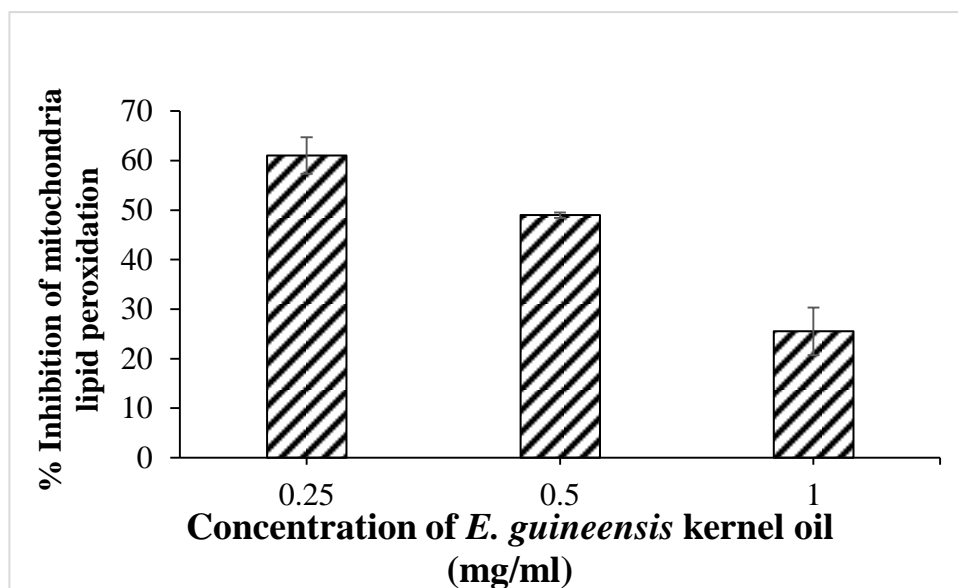
Figure 4: Percentage inhibition of mitochondria lipid peroxidation by 0.25, 0.5 and 1 mg/ml *E. guineesis* kernel oil

4.0 DISCUSSION

From this present study, the chemical analysis of the EGK oil showed elevated values when compared to the findings from the study reported by Akinyeye et al. (2011). The iodine value indicated that unsaturated fatty acids were present in the oil while the saponification value denoted large number of ester bonds. This suggested that EGK oil may contain intact fat molecules. Previous study had implicated saturated fatty acids in the generation of cytotoxic reactive oxygen species (2016). Reactive oxygen species (ROS) including superoxide anion and hydroxyl radicals have been shown to provoke mitochondria-mediated cell death through induction of mitochondrial membrane permeability transition (2015).

The acid value of EGK oil in this present study indicated that the oil was suitable for general use. Previous study had shown that the acid value of vegetable oil is an indication of the extent of triglyceride de-esterification within the oil by lipases, heat or light and could be used to ascertain the general condition or edibility of the oil (Demian, 1990). In addition, the peroxide value of EGK oil was lower than the standard peroxide value (10 mEq/kg). This suggested that the EGK oil was stable. Previous study had shown that the peroxide value in vegetable oil determines the extent of fatty acid oxidation as a measure of oil stability (Dermian, 1990).

Furthermore, EGK oil inhibited Ca^{2+}-induced mitochondrial membrane permeability transition pore (MMPT) pore opening in normal rat liver. This indicated that EGK oil could possess the capacity to protect the mitochondria against agents that elicit mitochondria-mediated intrinsic apoptotic pathway perhaps by stabilizing the inner mitochondrial membrane. Previous study by Lapidus and Sokolove (1993) showed that spermine inhibited Ca^{2+}-induced hepatic mitochondrial membrane permeability transition in normal rat by stabilizing the mitochondria inner membrane.

Further investigation showed that EGK oil stabilized erythrocyte membrane against hypotonicity-induced haemolysis. This suggests that EGK oil contains membrane stabilizing compounds. Previous studies have shown that plant-based substances could confer protection against lysis of bio-membranes (Oyedapo et al., 2004; Anyasor et al., 2015). EGK oil also inhibited heat-induced protein denaturation. This indicated that EGK oil may possess the capacity to protect membrane protein from heat-induced denaturation. Previous study had suggested that the anti-denaturation of protein could have resulted from compounds that have the capacity to prevent molecular alterations in the electrostatic, hydrogen, hydrophobic and disulphide bonds in a protein's structure (William, 2009).

More so, the anti-lipid peroxidation study showed that EGK oil protected mitochondrial membrane against ROS-induced lipid peroxidation *in vitro*. This also indicated that EGK oil possesses mitochondrial membrane protective property perhaps through stabilization of membrane lipids. Previous study had shown that peroxidation of mitochondria membrane is critical for the initiation of apoptotic cascade that might eventually lead to cell death (Zhong and Yin, 2015; Mahmoud et al., 2015). The disrupted mitochondrial membrane integrity had been shown to release deleterious

factors including precursors of highly reactive oxygen species (Subhashini and Edgar, 2013). Previous study had also shown that compounds which can protect against ROS-induced lipid peroxidation are useful in preventing cell death and tissue damage (Ajith, 2010).

4.1 CONCLUSIONS

This study showed that EGK oil extract possesses mitochondrial protective compound(s) and could also explain its long-standing use in traditional medicine as therapy against inflammatory diseases. Further investigation is recommended to identify the mitochondrial membrane protective agent(s) that could be harnessed for pharmaceutical or nutraceutical purposes.

4.2 ACKNOWLEDGEMENTS

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