RESEARCH ARTICLE

IN VITRO ANTIOXIDANT ACTIVITIES OF ETHANOLIC EXTRACT AND TRITERPENOID FRACTION OF GARCINIA KOLA STEM BARK AND INHIBITION OF ARSENIC-INDUCED RAT LIVER AND KIDNEY MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITIONS.

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Abstract

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This study was designed to investigate the *in vitro* antioxidant activities and inhibitory effects of *G. kola* stem bark ethanolic extract (EEGK) and triterpenoid fraction (TFGK) on arsenic-induced liver and kidney mitochondrial membrane permeability transitions (MMPTs). DPPH (1-1-diphenyl 2-picryl hydrazyl), hydrogen peroxide scavenging (HPSA), hydroxyl radical scavenging (HRSA) and total antioxidant capacity (TAC) assays were performed to determine antioxidant activity *in vitro*. Rat liver and kidney mitochondrial fractions were isolated using differential centrifugation technique while MMPT assay was performed using spectrophotometric method. Results showed that EEGK had a significantly (P<0.05) high DPPH, HPSA, HRSA and TAC scavenging activity than TFGK. However, 30 μg/mL TFGK significantly (P<0.05) inhibited arsenic-induced liver and kidney MMPT by 92.59% and 88.89% when compared with EEGK (70.37% and 66.67%) and standard spermine (81.48% and 77.78%). Thus, data from this study indicated that ethanolic extract of *G. kola* stem bark exhibited higher antioxidant activity *in vitro* while TFGK substantially inhibited arsenic-induced liver and kidney mitochondrial membrane permeability transitions.

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Introduction:-

Sodium arsenic (NaAsO$_2$) is regarded as a high priority toxic contaminant due to its toxic effects in human (Bishayi, 2000). In the environment, inorganic arsenic exits as arsenate (pentavalent, As^{5+}) and arsenite (trivalent, As^{3+}) and are readily interconvertible in aquatic environment through redox and methylation reactions. Arsenite toxicity is known to be higher than arsenate under *in vitro* and *in vivo* conditions (Kulp et al., 2004; WHO, 2004). Arsenic contaminant in the environment has been through natural and man-made sources, including drinking water, agricultural pesticides, coal fired power plants, glassware manufacturing, wood preservatives, smelting and cosmetic industries (Kapaj et al., 2006; Flora et al., 2009).

Ingested arsenic is readily absorbed through the gastrointestinal tract, and distributed through blood circulation into liver, kidney, heart, lung, gastrointestinal tract and spleen leading to arsenicosis (Kitchin, 2001). On a molecular level, the mechanism of arsenic toxicity is through binding to protein free thiol groups (Tapio and Grosche, 2006). This leads to enhanced production of toxic oxygen radicals, inhibition of numerous cellular enzymes, DNA repair

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mechanisms interference, alteration of gene expression and oxidative stress (Shi et al., 2004; Jomova et al., 2011). Experimental findings have shown that mitochondrial reactive oxygen species are contributory to the toxicity induced by arsenic in flies, worms, mouse and human liver (Schmeisser et al., 2013; Chavan et al., 2017). In addition, hepatic damage and renal dysfunction have been reported as the most common complication associated with chronic arsenic exposure (Santra et al., 2000).

Mitochondria are known to serve as a target site for arsenic toxicity through the generation of toxic oxygen radicals and participation in the initiation of the apoptotic caspase-cascade. This process is associated with the formation of mitochondrial membrane permeability transition (MMPT) (Selvaraj et al., 2013). MMPT is a molecular events caused by opening of the inner mitochondrial membrane voltage-dependent anion channel known as the permeability transition pore under certain conditions including noxious agents, Ca^{2+} overload and oxidative stress. It is a sudden increase of inner mitochondrial permeability to solutes with a molecular mass up to 1.5 kDa and has been implicated in apoptotic or necrotic pathways leading to neurodegeneration (Bustamante et al., 2005; Rao et al., 2014).

The conventional treatments for arsenic-induced toxicity in patients are thiol containing chelating agents such as British Anti Lewisite (BAL; 2,3-dimercaprol), meso 2,3-dimercaptosuccinic acid (DMSA) and 2,3 dimercaptopropane-1-sulfonate (DMPS) (Flora et al., 2009; Chandranayagam et al., 2013). However, the use of these drugs is still limited due to accompanying adverse effects. In contrast to synthetic drugs, medicinal plants are usually less toxic with fewer side effects (Nasri and Shirzad, 2013).

Garcinia kola Heckel is a dicotyledonous plant belonging to the family of Clusiaceae. It is a perennial crop that is distributed throughout West and Central Africa (Iwu et al, 2009; Farombi and Owoeye, 2011). *G. kola* is a medium sized evergreen tree that is about 15 - 17 m tall with a fairly narrow crown. The leaves are 6 - 14 cm long and 2 - 6 cm across. The small flowers are covered with short red hairs (Iwu, 1993). The fruit is a drupe of 5 - 10 cm in diameter and weighs 30 - 50 g. It is usually smooth and contains a yellowish red pulp. The fruit changes color during maturation from green to orange, and each fruit contains 1 - 4 seeds (Juliana et al., 2006).

G. kola has been referred to as a "wonder plant" because every part of it has important medicinal properties (Farombi and Owoeye, 2011). It is commonly called "bitter cola" or "male kola" due to the reported aphrodisiac property. *G. kola* stem bark is used in folklore remedies as a purgative and the latex is externally applied to fresh wounds to prevent sepsis. A decoction of *G. kola* stem bark is used as therapy for the treatment of dysmenorrhea, fever, inflammation and burns (Adesina et al, 1995).

G. kola stem bark contains a complex mixture of phenolic compounds such as biflavonoids, xanthines and benzophenone, kolanone, kolaflavanone and garcinia flavanone (Iwu, 1993). Data obtained from our laboratory through gas chromatography-mass spectrometry analysis showed the presence of fatty acids, oxalic acid, erucic acid and13-docosenoic acid in the ethanolic extract of *G. kola* stem bark while its triterpenoid fraction contained 3,4-dimethyl-2,5-dihydrofuran, 9,15-octadecadienoic acid, oleic acid, 9,12-octadecadienal, trans-farnesol and brassidic acid previously known to elicit hepatoprotective and nephroprotective properties. Therefore, this study was designed to investigate the antioxidant activities and inhibitory effects of ethanolic extract and triterpenoid fraction of *G. kola* stem bark on arsenic-induced liver and kidney MMPTs. This was carried out with the rationale to proffer scientific explanation to the ameliorative effects of *G. kola* stem bark fraction against metal-induced tissue damage.

Materials and Methods:-
Plant material collection and Identification:-
Stem barks of *G. kola* were collected from an Ilishan farm in Ogun State and authenticated at the Herbarium of the Forestry Research Institute of Nigeria, Jericho, Ibadan, Oyo State with voucher number FHI/110398.

Plant processing and extraction:-
Stem barks of *G. kola* were thoroughly washed to remove debris, chopped and oven-dried at 40°C. Dried stem barks were pulverized, using a mechanical blender and 200 g was soaked with 1.6 L 70% ethanol and mixed intermittently for 48 h at room temperature. The suspension was filtered using Whatman No. 1 filter paper and concentrated using a rotary evaporator (Buchi Rotavapor RE, Switzerland) at 40°C and stored in a refrigerator until further use. Furthermore, triterpenoid fraction *of G. kola* stem bark was prepared according to the method described by Pramod et al. (2006). Pulverized *G. kola* stem bark (250 g) was soaked with 95% ethanol and the suspension was mixed

intermittently for 7 days at room temperature. Subsequently, the extract was filtered using Whatman No.1 filter paper. The filtrate obtained was concentrated using a rotary evaporator (Buchi Rotavapor RE, Switzerland) at 40°C to obtain an ethanolic extract. The obtained ethanolic extract was further partitioned between ethyl acetate and water. Ethyl acetate fraction was further partitioned using n-hexane to isolate triterpenoids. Ethanolic extract and triterpenoid fraction of *G. kola* stem bark were stored in a refrigerator until further use.

Animals:-
Five male albino rats (Wistar strain) weighing between 150 - 200 g were purchased from an inbred colony at Babcock University Animal Facility. Animals were allowed to acclimatize in aerated cages under a natural light condition at room temperature and fed with commercial pelleted diet and water *ad libitum* for two weeks. All animal experiments and protocols conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals (2011). Ethical clearance with certificate number BUHREC227/16 was obtained from the Babcock University Health Research Ethics Committee.

Quantitative phytochemical analysis of G. kola stem bark extract/fraction:-
Total phenolic content was determined according to the method described by Singleton et al. (1999). Total flavonoid content was by Aluminum chloride method as described by Ordonez et al. (2006). Tannin content was determined according to the modified vanillin-HCl methanol method as described by Noha (2011). Saponin content was determined according to the method described by Okwu and Josiah (2006). Alkaloid content was determined using the method described by Onyilagba and Islam (2011).

Determination of G. kola stem bark extract/fraction antioxidant activity using in vitro methods:-
DPPH radical scavenging activity was determined using the method described by Mensor et al. (2001). Hydrogen peroxide scavenging activity was measured according to the method described by Ruch et al. (1989). Hydroxyl radical scavenging activity of *G. kola* stem bark extract/fraction was measured according to the method of Halliwell et al. (1987). Total antioxidant capacity of the extract/fraction was determined using phosphomolybdate method as described by Priesto et al. (1999).

Liver and kidney Mitochondrial fractions isolation:-
Mitochondrial fractions from the liver and kidneys of rats were isolated using conventional differential centrifugation technique as described by Hogeboom et al. (1948). Rat liver and kidney mitochondrial fractions were isolated in a buffer solution containing 210 mM mannitol, 70 mM sucrose, 5 mM 2-(4-[2-hydroxyethyl] piperazin-1-yl) ethanesulfonic acid (HEPES) at pH 7.4 and 1 mM ethylene glycol tetraacetic acid (EGTA). Mitochondrial protein content was determined by Folin-Ciocateu method using bovine serum albumin as standard protein as described by Lowry et al. (1951).

Assessment of mitochondrial membrane permeability transition:-
Mitochondrial membrane permeability transition was assessed according to the method of Lapidus and Sokolove (1993). Change in absorbance of mitochondria was monitored at 540 nm using a double beam UV-visible spectrophotometer (T80 model, PG instrument UK). Mitochondrial fraction (0.4 mg/mL) was suspended in a medium containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES-potassium hydroxide (pH 7.4), 0.8 μM rotenone and 5 mM succinate. MMPT was induced using sodium arsenite while spermine, a polyamine served as an inhibitor of MMPT.

Statistical Analysis:-
Statistical analysis was carried out with the aid of SPSS for Windows: SPSS Inc., Chicago, standard version 17.0 to determine difference between means using independent sample t-test. Data were reported as mean ± standard error of mean. Data were considered significant at $P<0.05$. Fifty percent inhibitory concentration (IC_{50}) for test fractions was calculated using GraphPad Prism® 7.00. Graphical presentations were plotted using Microsoft Excel 2013 version; Microsoft Office Suite 2013.

Results:-
Table 1 shows that the percentage yields of *G. kola* stem bark ethanolic extract and triterpenoid fraction were 32.45% and 20.17% respectively. EEGK had significantly ($P<0.05$) elevated total phenol (0.52 ± 0.01 GAE mg/g) and flavonoid (0.34 ± 0.04 QE mg/g) contents when compared with TFGK phenol (0.46 ± 0.02 GAE mg/g) and

flavonoid (0.26 ± 0.01 QE mg/g) contents. In addition, tannin, saponin and alkaloid contents in pulverized *G. kola* stem bark were 0.06 ± 0.01%, 3.33 ± 0.40% and 1.73 ± 0.02% respectively.

Data in Figure 1 showed that 20 - 100 μg/mL standard ascorbic acid, EEGK and TFGK significantly scavenged (P<0.05) DPPH radical in a concentration dependent manner. The minimum and maximum DPPH scavenging activity for standard ascorbic acid were 18 ± 1.07% and 67.56 ± 1.49% at 20 and 100 μg/mL respectively, while EEGK had 15.28 ± 1.94% and 61.93 ± 0.97%, and TFGK had 12.41 ± 0.84% and 41.02 ± 1.93% at 20 and 100 μg/ml, respectively. Furthermore, Table 2 showed that EEGK (IC_{50} = 79.14 ± 0.02 μg/mL) had a higher DPPH scavenging activity than TFGK (IC_{50}= 102.1 ± 0.01 μg/mL).

Data in Figure 2 showed that 20 - 100 μg/mL standard ascorbic acid, EEGK and TFGK significantly (P<0.05) scavenged hydrogen peroxide in a concentration dependent manner. The minimum and maximum hydrogen peroxide scavenging activity for standard ascorbic acid were 45.78 ± 0.20% and 91.11 ± 1.50% at 20 and 100 μg/mL respectively while EEGK had 16.56 ± 0.50% and 55.90 ± 1.6%, and TFGK 13.89 ± 1.7% and 37.95 ± 0.50% at 20 and 100 μg/mL respectively. Furthermore, Table 2 showed that EEGK (IC_{50} = 94.03 ± 1.02 μg/mL) had a higher hydrogen peroxide scavenging activity than TFGK (IC_{50} = 188.70 ± 1.25 μg/ml).

Data in Figure 3 showed that 20 - 100 μg/mL standard ascorbic acid, EEGK and TFGK significantly (P<0.05) scavenged hydroxyl radical in a concentration dependent manner. The minimum and maximum hydroxyl radical scavenging activity for standard ascorbic acid were 18.22 ± 0.34% and 75.82 ± 2.50% at 20 and 100 μg/mL respectively while EEGK had 17.59 ± 1.95% and 70.16 ± 2.80%, and TFGK 17.49 ± 2.90% and 68.51 ± 1.50% at 20 and 100 μg/mL respectively. Furthermore, Table 2 showed that EEGK (IC_{50} = 70.08 ± 0.11 μg/mL) had a higher hydroxyl radical scavenging activity than TFGK (IC_{50} = 74.22 ± 0.14 μg/mL). In addition, data in Figure 4 showed that EEGK (1.11±0.01 mg ascorbic acid equivalent/g) had a significantly (P<0.05) higher TFGK (1.07±0.01 mg ascorbic acid equivalent/g).

Table 3 shows that 10 – 30 μg/mL EEGK and TFGK inhibited arsenic-induced MMPT in a concentration dependent manner. Furthermore, 30 μg/mL TFGK (Δ540 nm = -0.02±0.00 and -0.02±0.01) significantly (P<0.05) inhibited arsenic-induced liver and kidney MMPT than EEGK (Δ540 nm = -0.08±0.01 and -0.06±0.01) respectively. In addition, 10 - 30 μg/mL TFGK inhibited arsenic-induced liver MMPT by 77.78 to 92.59% and arsenic-induced kidney MMPT by 61.11 to 88.89% respectively, while 10 - 30 μg/mL EEGK inhibited arsenic-induced liver MMPT by 44.44 to 70.37% and arsenic-induced kidney MMPT by 50.00 to 66.67% respectively. Spermine also inhibited arsenite-induced liver (Δ540 nm = -0.05±0.02) and kidney (Δ540 nm = -0.04±0.01) MMPT by 81.48% and 77.78% respectively.

Table 1:- Quantitative phytochemical analysis of *G. kola* stem bark.

Test parameters	EEGK	TFGK
Total Phenols (GAE mg/g extract)	0.52±0.01[*]	0.46±0.02
Flavonoids (QE mg/g extract)	0.34±0.04[*]	0.26±0.01
	Pulverized *G. kola* stem	
Tannin content (%)	0.06 ± 0.01	
Saponin content (%)	3.33 ± 0.40	
Alkaloid content (%)	1.73 ± 0.02	
Percentage yield	32.45	20.17

GAE: - Gallic acid Equivalent   QE: - Quercetin Equivalent; * indicates significantly elevated when compared with TFGK at P<0.05; Values were expressed as mean ± standard error of the mean (SEM) (n=3). EEGK: - Ethanolic extract of *G. kola* stem bark; TFGK: - Triterpenoid fraction of *G. kola* stem bark

Table 2:- Fifty percent inhibitory concentration (IC_{50}) values for ascorbic acid, EEGK and TFGK in different antioxidant models.

Test samples	IC_{50} (μg/mL)		
	DPPH	HPSA	HRSA
Ascorbic acid	67.96 ± 0.01	26.08 ± 0.07	58.84 ± 0.05
EEGK	79.14 ± 0.02[*]	94.03 ± 1.02[*]	70.08 ± 0.11[*]
TFGK	102.10 ± 0.01	188.70 ± 1.25	74.22 ± 0.14

* indicates significantly reduced when compared with TFGK at P<0.05

DPPH: - 1-1- diphenyl 2-picryl hydrazyl radical scavenging activity
HRSA: - Hydroxyl radical scavenging assay
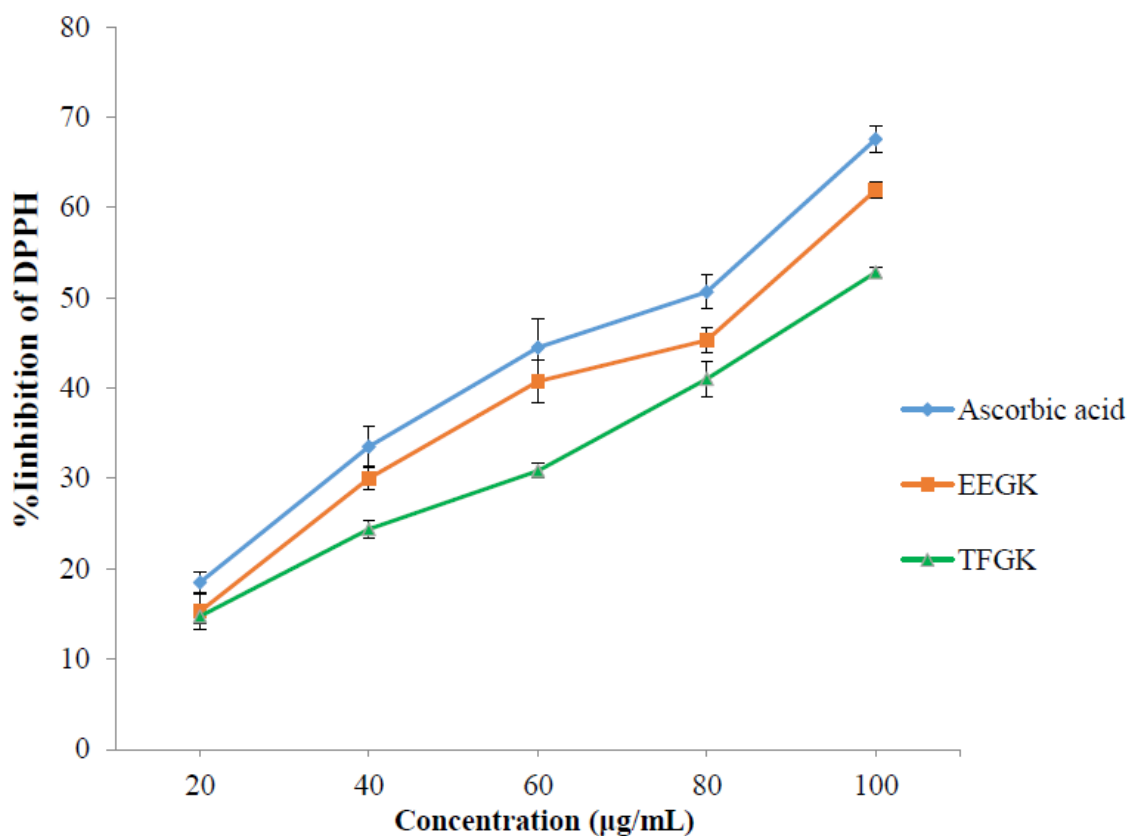HPSA: - Hydrogen peroxide scavenging activity
EEGK: - Ethanolic extract of *G. kola* stem bark
TFGK: - Triterpenoid fraction of *G. kola* stem bark

Table 3:- Inhibitory effects of varying concentrations of ethanolic extract of *G. kola* stem bark (EEGK) and triterpenoid fraction of *G. kola* stem bark (TFGK) on sodium arsenic-induced liver and kidney MMPT energized by succinate for 12 min at Δ540 nm.

Parameters	Inhibition of arsenite-Induced liver MMPT		Inhibition of arsenite-induced kidney MMPT	
	Absorbance at Δ540 nm	%	Absorbance at Δ540 nm	%
5 µg/mL Arsenic	-0.27 ± 0.01^e	-	$-0.18\pm0.02^\delta$	-
10 µg/mL Spermine	-0.05 ± 0.02^{bc}	81.48	$-0.04\pm0.01^\beta$	77.78
10 µg/mL EEGK	-0.15 ± 0.02^d	44.44	$-0.09\pm0.01^\gamma$	50.00
20 µg/mL EEGK	-0.09 ± 0.02^c	66.67	$-0.08\pm0.01^{\beta\gamma}$	55.56
30 µg/mL EEGK	-0.08 ± 0.01^c	70.37	$-0.06\pm0.01^\beta$	66.67
10 µg/mL TFGK	-0.06 ± 0.02^c	77.78	$-0.07\pm0.02^{\beta\gamma}$	61.11
20 µg/mL TFGK	-0.04 ± 0.01^{bc}	85.19	$-0.04\pm0.01^\beta$	77.78
30 µg/mL TFGK	-0.02 ± 0.00^a	92.59	$-0.02\pm0.01^\alpha$	88.89

Different letters and symbols across columns denotes significantly higher inhibition of MMPT at $P<0.05$

Figure 1:- DPPH radical scavenging activities of ethanolic extract of *G. kola* stem bark (EEGK) and triterpenoid fraction of *G. kola* stem bark (TFGK)
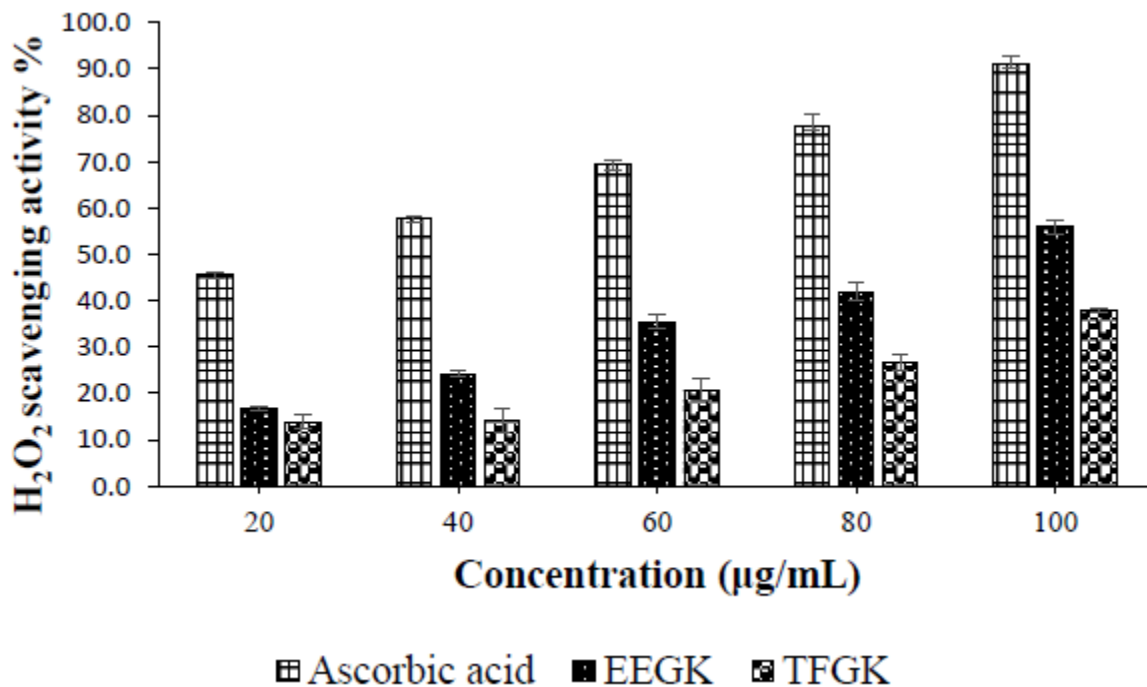
Figure 2:- Hydrogen peroxide scavenging activities of ethanolic extract of *G. kola* stem bark (EEGK) and triterpenoid fraction of *G. kola* stem bark (TFGK)
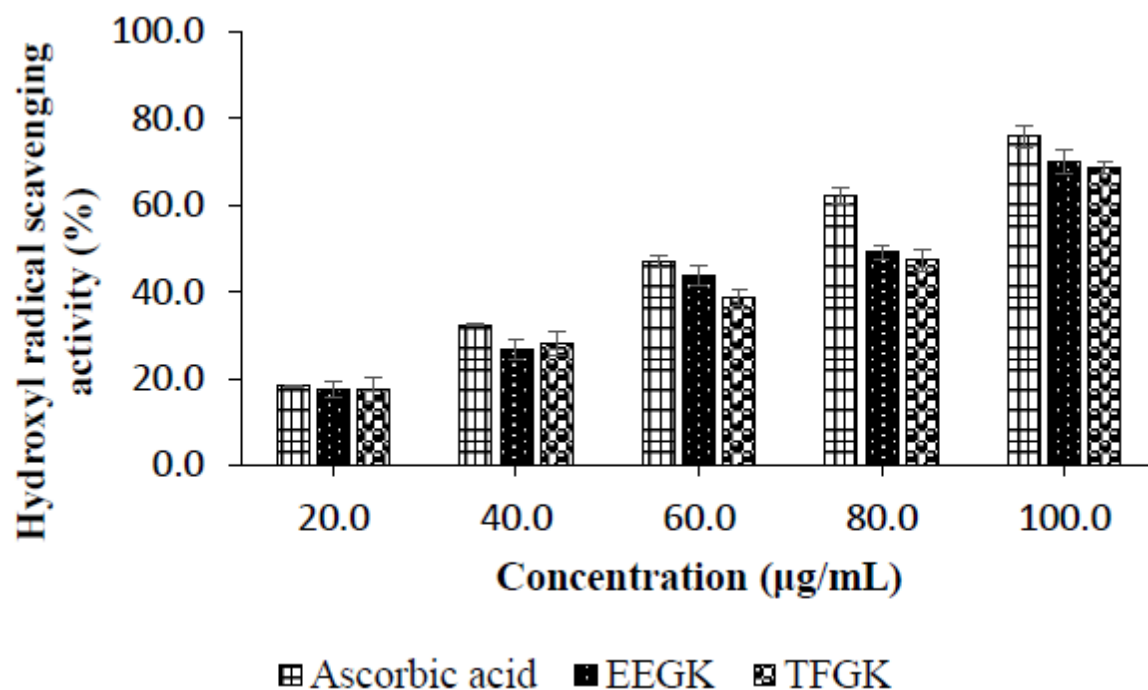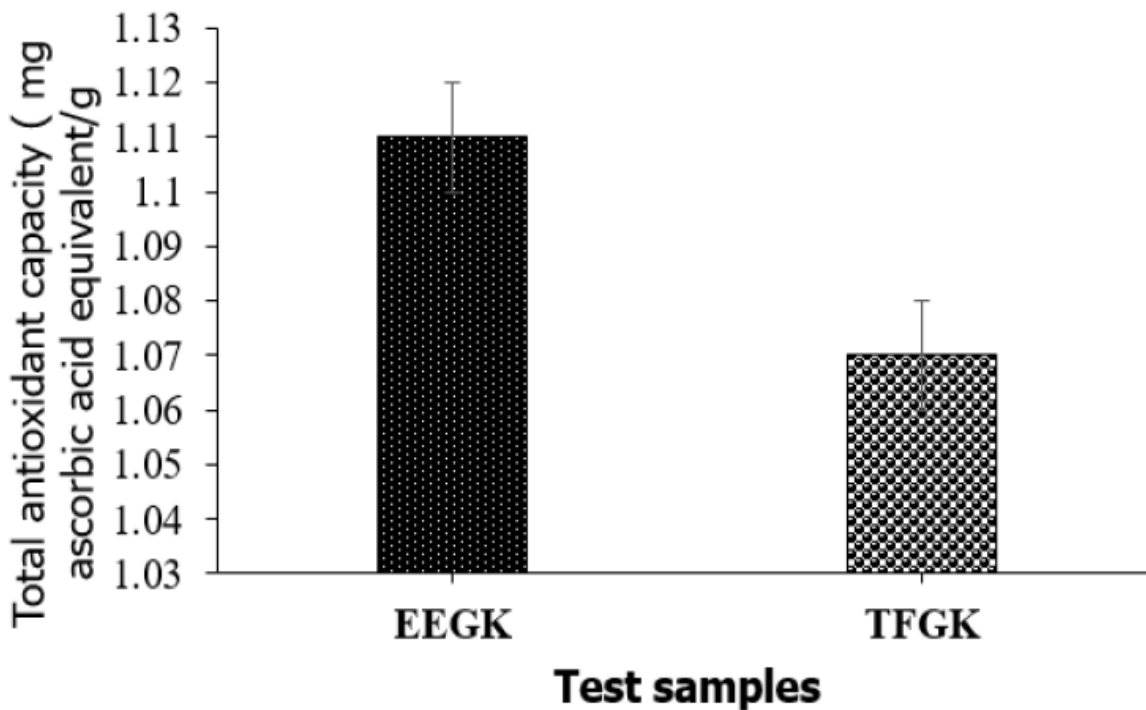

Figure 3:- Hydroxyl radical scavenging activities of ethanolic extract of *G. kola* stem bark (EEGK) and triterpenoid fraction of *G. kola* stem bark (TFGK)

Figure 4:- Total antioxidant capacity (TAC) of activities of ethanolic extract of *G. kola* stem bark (EEGK) and triterpenoid fraction of *G. kola* stem bark (TFGK).

Discussion:-

In this present study, appreciable quantities of flavonoids, phenols, tannins, saponins and alkaloids were detected in EEGK and TFGK. This suggested that *G. kola* stem bark could possess bioactive compounds. The presence of these phytochemicals might explain the rationale for the ethnomedical use of *G. kola* stem bark as therapy against metal-induced liver and kidney toxicities. Previous study had shown that flavonoids possess the capacity to scavenge reactive chemicals, including hydrogen peroxide, hydroxyl radicals, lipid peroxyl radicals and superoxide anions (Buhian, 2016). These chemical radicals are unstable molecules with the propensity to abstract electrons from tissue macromolecules thereby eliciting deleterious effects on biochemical systems (Tentscher, 2013). Furthermore, phenols, flavonoids, tannins, and saponins present in plant materials had been reported to elicit hepatoprotective, nephroprotective, anti-inflammatory and antioxidant activities (Osadeba, 2012). These notable biological effects might be due to the presence of functional moieties which confers protection to the body system against the insurgence of deleterious reactive chemical species (Araujo et al., 2015).

Data from this study showed that the EEGK contained higher total phenol and flavonoid contents than TFGK. This suggested that EEGK could possess higher antioxidant and anti-inflammatory activities than TFGK. Previous study had reported that the solvent ethanol has a higher extractive capacity to penetrate the cell wall of plant materials to isolate mostly polar phyto-compounds, including phenols and some flavonoids than non-polar solvents (Sharmin et al., 2016). Furthermore, to gain some insight as to the antioxidant potentials of EEGK and TFGK, selected *in vitro* antioxidant models were adopted. The study showed that EEGK consistently exhibited a higher radical scavenging activity than TFGK for DPPH, hydrogen peroxide, hydroxyl radical and total antioxidant capacity assays. This indicated that EEGK possesses higher antioxidant activity than TFGK. This effect could be attributed to the presence of phenolic and flavonoid compounds and the obtained data seems to be in agreement with higher total phenol and flavonoid contents in EEGK than TFGK. Previous study had reported that plant extracts with antioxidant activity have the capacity to readily release proton(s) (H^+) to neutralize the unstable chemical species. This is also corroborated by the reports that ascorbic acid exerts antioxidant action by donating protons to neutralize free radicals (Shalaby and Shanab, 2013).

Further study showed the capacities of EEGK and TFGK to reverse arsenic-induced liver and kidney mitochondrial membrane permeability transitions (MMPTs). Arsenic induced MMPT has been implicated as a molecular marker

for arsenic tissue toxicity (Selvaraj et al., 2013). Data in this present study showed that the different concentrations of EEGK and TFGK inhibited arsenic-induced liver and kidney MMPTs in a concentration-dependent manner. This suggested that the EEGK and TFGK contained mitochondrial protective compounds against arsenic-induced liver and kidney toxicities. Previous study had reported that sodium arsenic inhibited complex I and II leading to the disruption of mitochondrial electron transport chain, altering the bioenergetics status with the formation of mitochondrial reactive oxygen species (ROS) inducing MMPT with subsequent activation of apoptotic pathway (Hosseini et al., 2013). However, phenol and flavonoid antioxidants present in plant extracts have been previously shown to act as scavengers of mitochondrial ROS and inhibitors of MMPT (Anyasor et al., 2014; Katerina and Gonzola, 2016). Percentage inhibitions of arsenic-induced liver and kidney MMPTs by TFGK were found to be higher when compared to EEGK and natural MMPT inhibitor, spermine. This indicated that TFGK possesses a higher ameliorative effect against arsenic-induced mitochondrial membrane perturbation than EEGK. This observation could account for one of the possible molecular events associated with the previously reported hepatoprotective and nephroprotective properties of *G. kola* stem bark against arsenic-induced tissue damages. More so, the inhibition of arsenite-induced liver and kidney MMPTs by TFGK might be through metal chelation than antioxidant activity or a combination of both properties.

Conclusion:-
It is therefore recommended that the triterpenoid fraction of *G. kola* stem bark be further explored as a potential source for drug(s) to be used as therapy against arsenic elicited tissue toxicity. In addition, data from this study has provided some scientific insight to explain the ethnomedical use of *G. kola* stem bark as therapy for liver and kidney dysfunctions.

Conflict of interest:-
Authors declare that there are no known conflicts of interest of interest with regards to the writing and financing of this research work.

Acknowledgements:-

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