**Effect of African Walnut (Tetracarpidium conophorum) Seed Oil on 3-Methylcholanthrene-Induced Mammary Carcinogenesis and Expression of COX-2 and PPAR-γ in Female Wistar Rats**

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**ABSTRACT**

Breast cancer is a commonly diagnosed disease among women, and dietary lipids has been implicated in its incidence. This study investigated the effect of feeding African walnut seed oil (AWSO) on 3-methylcholanthrene (MCA) induced mammary cancer and expression of cyclooxygenase-2 (COX-2) and peroxisome proliferator activated receptor gamma (PPAR-γ) in female Wistar rats. AWSO was extracted with n-hexane in a Soxhlet apparatus and characterized by gas chromatography. Group A and B of 21 days old rats (15 each) were fed with diet containing 10% AWSO for 12 weeks. After 4 weeks of feeding, group A animals were administered MCA (250mg/kg) intraperitoneally. Another group (group C with 15 animals) was fed with diet containing no AWSO and administered MCA (250mg/kg) intraperitoneally after 4 weeks of feeding. Results revealed that animals fed with AWSO had lower tumor incidence (21.7%), tumor weight (1.22g) and tumor volume of 948mm$^3$ compare to the animals not fed with AWSO (87.4%, 9.41g, 6281mm$^3$ respectively. The expression of cyclooxygenase-2 was observed only in MCA treated animals and it was significantly less on AWSO fed group than on animals in group not fed with AWSO. The expression of PPAR-γ was significantly more on animals fed with AWSO than in group C (not fed with AWSO). Histological analysis of carcinogenesis was significantly (p < 0.05) more rapid on animals not fed with AWSO. Our results revealed that dietary AWSO reduce breast carcinogenesis induced by MCA.

**Keywords:** Tetracarpidium conophorum, 3-methylcholanthrene, cyclooxygenase-2, mammary cancer

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**INTRODUCTION**

*Tetracarpidium conophorum* has a long history as food plant and is grown by peasant farmers across West African rain forest. According to Ihemeji *et al.*, 2015, T. conophorum is widely distributed and consumed by the inhabitants of the Guinea Zone of West and Central Africa. (Nwanichi *et al.*, 2017). The tree bear capsules that are greenish in colour when young and greenish yellow when fully ripe (Chigioke *et al.*, 2015). The economic importance of the species lies in the edibility of its oil rich endospermous seed, which is consumed by diverse populations in Nigeria, Sierra Leone and the Lower Congo region (Kanu *et al.*, 2015). It grows along the African Coastline and it is thought to originate in South Western Nigeria (Wyk and Wink, 2017). In Nigeria, the *Tetracarpidium conophorum* plant flowers between November and early January and fruits between February and September with peak production in July. The immature fruits are usually green in color, but turn dark brown as they mature (Oluwole and Okusanya, 1993; Ojobor *et al.*, 2015; Uhunmwangho and Omorogie, 2017). They are plants having swollen, fleshy, sparsely branched stems and sometimes candelbroid in appearance. The fruit is a capsule 6-10cm long by 3-11cm wide containing sub-globular seeds 1-2.5cm long with a thin brown shell resembling the temperate walnut (Ojobor *et al.*, 2015; Nwachoko and Jack, 2015). Breast cancer is the most commonly diagnosed cancer in women and is the leading cause of cancer mortality in females around the world.

A strong positive correlation between fat intake and mortality from breast cancer has been shown (Wynder *et al.*, 1986; Ram and Geetanjali, 2018). It has been suggested that high dietary levels of unsaturated fatty acids enhance tumour development through increased synthesis of prostaglandins (Welsch *et al.*, 1992; Ayoola, 2011; Nwauzoma and Dappa 2013; Chigioke *et al.*, 2017). Cyclooxygenase (COX) that catalyzes the conversion of arachidonic acid to prostaglandins exists in two isoforms (COX-1 and COX-2). The constitutively expressed COX-1 is important for maintaining
Feeding the animals with diet containing *Tetracarpidium conophorum* oil: Female Wistar rats (21day old) were obtained from the animal house of the University of Medical Science, Ondo, and were housed in metal cages in a well-ventilated room and they were allowed access to water and ad libitum. The experimental diet comprised of chick pea (51.4%), wheat (15.0%), groundnut cake (10.0%), skim milk powder (6.0%), mineral mixture (2.16%), vitamin mix (0.2%) and *Tetracarpidium conophorum* oil (15.0%). Overall, 48 Wistar male rats were used. The remaining Animals were randomly divided into three major groups of 15 animals each. Group A animals were fed for 12weeks with diet containing *Tetracarpidium conophorum* oil (15%) and the animals injected with 3-methylicholanthrene (250mg/kg body weight) intraperitoneally injection after 4weeks of feeding. Group B were fed for 12weeks with diet containing *Tetracarpidium conophorum* oil (10%) only. Group C animals were fed for 12weeks with diet containing no *Tetracarpidium conophorum* oil, and were given 3-methylicholanthrene (250mg/kg body weight) intraperitoneally after 4weeks of feeding. The animals were palpated weekly to determine the time of appearance of tumors and body weight.

At necropsy, mammary glands were exposed and tumors were excised. Tumor incidence, volume and weight were determined. Animals from each group were sacrificed at 4, 8, 12weeks, and the serum and tissues collected for enzymes and biochemical analysis. Portions of mammary tissue from no tumor bearing and tumor tissue were preserved in RNA later for gene expression studies. Another portion of tumor tissue was fixed in formalin (10%) for histopathological studies.

Fatty Acid Determination: Fatty Acids were determined according to the method of Mann and Caron (1995) as described by Uhunmwangho and Omorje (2017).

**COX-2 and PPAR-γ gene expression:** The liver samples were placed in triazole (a molecular grid RNA isolating reagent). The samples were homogenized and chloroform was added for homogenate gradient separation. This was followed by centrifugation at 15,000rpm for 15 minutes. After centrifugation, the upper phase (clear supernatant containing RNA) was aspirated into a new sterile eppendorf tube of 1.5ml. The clear supernatant was precipitated by adding isopropanol. This b was followed by centrifugation at 15,000rpm for 5 minutes. RNA pellet was air dried for 15 minutes and resuspended in nuclease free water (30 microliters). RNA samples were quantified and absorbance was checked using a spectrophotometer. RNA samples were optimized using PCR machine for 1 hour at 42°C. The samples were amplified and gel electrophoresis was carried out at 70 volts, 500 milli amperes for 10 minutes, the samples were placed in UV documentary for viewing the expression bands.
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**Statistical analysis**: The values were expressed as mean ± SE. Kruskal-wallis one-way analysis of variance (ANOVA) was used for COX-2 and PPAR-γ gene expression using Systat 7.0 software (Spss Inc., Chicago, USA). A difference with *P*<0.05 was considered statistically significant.

**RESULTS**

Table I summarizes the data on incidence, latency period and weight and volume of tumors in mammary gland. The incidence of tumors on *Tetracarpidium conophorum* seed oil (21.7%) was significantly (*P*<0.05) lower than animals that were fed with no *Tetracarpidium conophorum* seed oil but treated with MCA (87.4%). The tumor latency period was 4 weeks in MCA treated group without *Tetracarpidium conophorum* seed oil compared to 8 weeks in the oil treated group. The average size of tumor was generally larger in MCA treated group than in the animals treated with the seed oil. Similarly, average tumor volume was significantly (*P*<0.05) less in the seed oil treated groups than on MCA only group.

**Table 1**

| Effect of feeding *Tetracarpidium conophorum* seed oil on mammary carcinogenesis in MCA administered rats |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Animals fed with *T. conophorum* only | Animals fed with *T. conophorum* oil + MCA | Animals fed with MCA only |
| Tumor latency period | Symptoms not observed in these animals | 8 weeks | 4 weeks |
| Tumor incidence | 21.7% | 87.4% |
| Tumor weight (g)/ tumor bearing rat | 2.6 ± 1.45 | 9.4 ± 2.26 |
| Tumor volume (mm³)/ tumor bearing rat | 1774 ± 3.21 | 7342 ± 1.48 |

Values are mean ± SE; *P*<0.05 compared to *T. conophorum* oil group

**Expression of COX-2 and PPAR-γ**: The effect of dietary fat on expression of COX-2 and PPAR-γ was investigated in normal mammary gland, *T. conophorum* treated animal mammary gland and tumor tissue tumor bearing rats. The COX-2 was not expressed in normal mammary tissue but its expression was induced in response to MCA treatment (Fig. 1). In MCA treated rats, the expression of COX-2 was significantly greater in tumor bearing than in no tumor bearing and *T. conophorum* treated rats. Further, the expression of COX-2 was greater in tumor tissue than in other tissues studied. In carcinogen treated rats wherein no tumor appeared, the expression of PPAR-γ in both *T. conophorum* and positive control group was almost of the same magnitude as observed in their respective untreated counterparts (Fig. 2).

**Figure 1**

Effect of feeding *T. conophorum* oil on expression of COX-2 in mammary tissue of control and 3-MCA treated rats. Values are mean ± SE

**Figure 2**

Effect of feeding *T. conophorum* oil on expression of PPAR-γ in mammary tissue of control and 3-MCA treated rats. Values are mean ± SE,
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e 218 group administered with MCA but treated with Tetracarpidium conophor difference ( carcinogenesis. Fig 1.0 shows there was no significant but over expression of COX 2 gene results in excess production of prostaglandin E2 and increase in local estrogen biosynthesis by aromatase. Three major line of events that drives the process of mammary carcinogenesis: mutagenesis by creation of free radical involved in sustained prostaglandin biosynthesis; angiogenesis by stimulation of vascular endothelial growth factor by prostaglandin E2; and mitogenesis without natural apoptosis due to estrogen production by aromatase. Also, COX activity may also be linked to the metabolic activation and metabolism of 3-MCA and other polycyclic aromatic hydrocarbons through the cytochrome P-450 system (Shou et al., 1996).

In the present study, COX-2 was undetectable in normal mammary tissue, the group fed with Tetracarpidium conophorum seed oil only, and its expression induced by 3-MCA treatment was significantly higher in tumor tissue as compared to Tetracarpidium conophorum seed oil treated mammary tissue. Cyclooxygenase-2 is an inflammation associated enzyme involved in the pathogenesis of carcinogenesis. Inhibition of COX-2 and blockade of prostaglandin cascade may lead to the reduction of carcinogenesis (Shiffs and Rigas, 1999; Alugoju et al., 2011) but over expression of COX-2 initiates and promotes carcinogenesis. Fig 1.0 shows there was no significant difference (p>0.05) between the group fed with Tetracarpidium conophorum seed oil only and the animals in group administered with MCA but treated with Tetracarpidium conophorum seed oil but there was a significant difference (p<0.05) in the expression of COX-2 between the groups fed with Tetracarpidium conophorum seed oil and the group which was induced with 3-Methylcholanthrene but was not treated with Tetracarpidium conophorum seed oil which implies that the use of Tetracarpidium conophorum seed oil has a high activity against COX-2 in groups fed with Tetracarpidium conophorum seed oil. This proves that Tetracarpidium conophorum seed oil extract was effective in suppressing the expression of COX-2 in groups that were fed with Tetracarpidium conophorum seed oil. This result is in agreement with Chinery et al., 1998 who conducted an experiment on cyclooxygenase-2 expression to colorectal cancer cells. We also explored the expression of PPAR-γ in 3-methylcholanthrene induced carcinogenesis in female Wistar rats, examining its correlation to breast carcinogenesis. In this study, there was no significant change in the expression of PPAR-γ in groups that were fed with Tetracarpidium conophorum seed oil extract but there was a significant difference in the expression of PPAR-γ between these groups and the group of animals administered with MCA only with the indication of that there was a reduction in the expression of PPAR-γ. This proves that our extract was effective in the expression of PPAR-γ which stimulates cell death in cancer cells. This result agrees with Shu-Fang et al., 2017 who conducted an experiment on PPAR-γ on epithelial ovarian cancer.

Earlier study showed that diets containing high levels of polyunsaturated fatty acids were effective in downregulating the development of carcinogenesis (Win, 2015). PUFAs like linolenic acid and docosahexaenoic acid were higher in group fed with the seed oil compared to the group which was not fed with the seed oil. The high concentration of administered walnut oil could be attributed to the high concentration of linolenic acids which in turn was incorporated into the cell membrane of the Wistar rat. This shows that our extract, Tetracarpidium conophorum seed oil contains PUFAs with potent anticancer properties which helps in curbing breast cancer as shown in Table 2.0. This result is in alliance with Campos-Perez and Martinez-Lopez, (2015) who conducted an
experiment on the role of polyunsaturated fatty acids in cancer prevention.

While vegetable oils contain large amount of linoleic acid known to have promotional role in carcinogenesis (Badawi et al., 1998) tetracarpidium conophorum seed oil contains GLA, which has been shown unequivocally to inhibit mammary carcinogenesis. In the present study, feeding Tetracarpidium conophorum seed oil started during mammary gland development period led to 66 per cent lower cancer incidence than in MCA fed rats. Feeding Tetracarpidium conophorum seed oil containing GLA during pubescent mammary gland development period lowers the population and proliferating activity of the terminal end buds cells (Ip et al., 1999) which are the target sites for development of adenocarcinomas in response to carcinogenic stimuli. In the present study, the feeding of Tetracarpidium conophorum seed oil started during the pubescent period of mammary gland development might have resulted in the decreased tumour incidence and progression to malignancy. The anticarcinogenic effect of GLA may be partly explained by its effect on the COX-2 at the level of mRNA as well as protein in cultured macrophage cell line (Cheng et al., 2004) it represses AP-1 mediated activation of COX-2 transcription in MCF-7 breast cancer cells (Degner et al., 2006). McCarty, (2000) hypothesised that activation of PPAR-γ may mediate a portion of the anticancer activity of conjugated LA. The treatment of colon cancer cells with GLA inhibits cell proliferation; increases expression of PPAR-γ and down regulates APC and c-myc proteins (Bozzo et al., 2007; Yausi et al., 2005). The higher tumour incidence and faster progression of MCA induced mammary carcinogenesis in rats fed with diet containing no Tetracarpidium conophorum seed oil compared to animals fed with the seed oil could be due, partly, to lack of high content of GLA acid in the diet. The promotion of mammary carcinogenesis in rats by n-6 polyunsaturated fatty acids is associated with enhanced expression of COX-2 (Badawi et al., 1998).

In conclusion, Tetracarpidium conophorum seed oil protects against MCA induced mammary carcinogenesis and the effect is mediated through decreased expression of COX-2 and increased expression of PPAR-γ. Further work is needed to understand the regulation of COX-2 and PPAR-γ and prostaglandin synthesis in response to dietary fat.

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REFERENCES

Nwaichi, E.O., Osuoha, J.O. & Monanu, M.O., (2017). ‘Nutraceutical potential of Tetracarpidium conophorum and
African Walnut seed ameliorates 3-Methylcholanthrene-Induced Mammary Carcinogenesis