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*Research Article*

# Effect of African Walnut (*Tetracarpidium conophorum*) Seed Oil on 3-Methylcholanthrene-Induced Mammary Carcinogenesis and Expression of COX-2 and PPAR-ɣ 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-ɣ) 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 in group 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. (Nwanchi *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 Omoregie, 2017). They are plants having swollen, fleshy, sparsely branched stems and sometimes candlebroid 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

the homeostatic function, whereas COX-2 is upregulated in response to growth factors, tumour promoters and cytokines (Rita and Vinod, 2011). The overexpression of cyclooxygenase-2 is sufficient to induce mammary tumours in mice Peroxisome proliferators activated receptor gamma (PPAR-γ) is a ligand activated transcription factor and its activity is regulated by several natural ligands, including fatty acids and eicosanoids (Rita and Vinod, 2011). The PPAR-γ ligands promote differentiation and reduce growth rate of breast adenocarcinoma cell lines in vitro and promote regression of DMBA induced rat mammary tumours in vivo (Rita and Vinod, 2011). These studies suggest that COX-2 and PPAR-γ are the regulatory molecules in the development of mammary carcinogenesis.

African walnut oil has an important place in African dietary not only because of its characteristic flavor and pleasant aroma, but in terms of health benefits. It has been used for the treatment of various ailments including cancer, although there is no experimental evidence to support this contention (Ogunyinka *et al*., 2015; Ayeni and Nuhu, 2018). In the present study, we examined the effect of feeding African walnut oil on 3- methylcholanthrene induced rat mammary carcinogenesis. In order to provide further information on the mechanism by which dietary fats modulate mammary cancer development, we also examined the effect of this seed oil on the expression of COX-2 and PPAR-γ genes in rat mammary gland.

## MATERIALS AND METHODS

**The Study Location:** Department of Biochemistry Laboratory, University of Medical Sciences, Ondo City, Ondo State, Nigeria.

**Reagents/Chemicals:** All reagents used were of analytical grade. Methanol (Sigma Chemicals Co, London), Chloroform (Sigma Chemicals Co., London), Benzene (BDH Chemicals Ltd., Eng.), NaCl (BDH Chemicals Ltd., Eng.), Standard buffer tablets (BDH Chemicals Ltd., Eng.), Ethanol, 3-methylcholanthrene, Sulphuric Acid Aldrich Chemical Company, USA.

**Plant material (Sample collection):** Fresh *Tetracarpidium conophorum* fruits were obtained from farms in Ifetedo town near Ondo city, Ondo State, Nigeria. The fruits were authenticated by a Taxonomist of the Botany Department, University of Medical Sciences, Ondo. At each harvest, 40 fruits will be collected randomly from three regions of the plant as follows, apical region -10 fruits; middle region – 15 fruits; basal region -15 fruits. The collected fruits were cleaned with a moist soft cotton wool and then the seeds carefully separated from the fruits and dried at $65^0$C for 4 hrs. in an oven, crushed with a laboratory mortar and pestle and were kept in a well labeled air tight polythene bags or screw-capped bottles at $4^0$C for extraction.

**Extraction of oil from African Walnut:** The Soxhlet extraction method according to AOAC (1996) will be employed. The sample (5.0g) will be weighed into a weighed filter paper and folded neatly. This will be placed inside the pre-weighed thimble. The thimble with the sample will be inserted into the Soxhlet apparatus and extraction under reflux will be carried out with the n-hexane (40-60$^0$C boiling range) for 6 hours. At the end of extraction, the thimble will be dried in the oven for about 30minutes at $100^0$C to evaporate off the solvent and cool in a desiccator and later weighed and kept in the refrigerator.

**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-methylcholanthrene (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-methylcholanthrene (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 Manni and Caron (1995) as described by Uhunmwangho and Omoregie (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 $42^0$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.

**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 4weeks in MCA treated group without *Tetracarpidium conophorum* seed oil compared to 8weeks 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** I
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

**Table 2**
Major fatty acids composition (%) in mammary tissues of animals fed with and without seed oil diet

| **Fatty acids** | **Animals fed with diet containing seed oil (%)** | **Animals fed with diet without seed oil (%)** |
|---|---|---|
| g-Linolenic acid | 63.7 | 0.4 |
| Eicosadienoic Acid | 0.5 | - |
| Eicosatrienoic Acid | 2.7 | 0.4 |
| Docosahexaenoic Acid | 3.5 | - |
| Palmitic Acid | 1.1 | 0.7 |
| Heptadecanoic Acid | 38.1 | 0.5 |
| Elaidic Acid | 4.3 | 0.5 |
| Linolelaidic Acid | 12.8 | 30.7 |
| Oleic Acid | 0.8 | 0.1 |
| Arachidic Acid | 4.5 | 0.07 |
| Linolenic Acid | 1.76 | 0.01 |
| Arachidonic Acid | 0.07 | 0.01 |
| Palmitoleic acid | 4.2 | 12.5 |

**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).

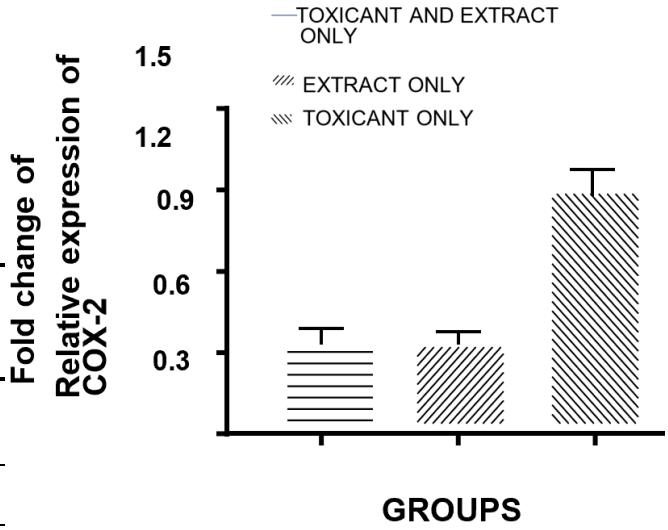


**Figure 1**
Effect of feeding *T. conophorum* oil on expression of COX-2 in mammary tissue of control and 3-MCA treated rats. Values are mean ± SE

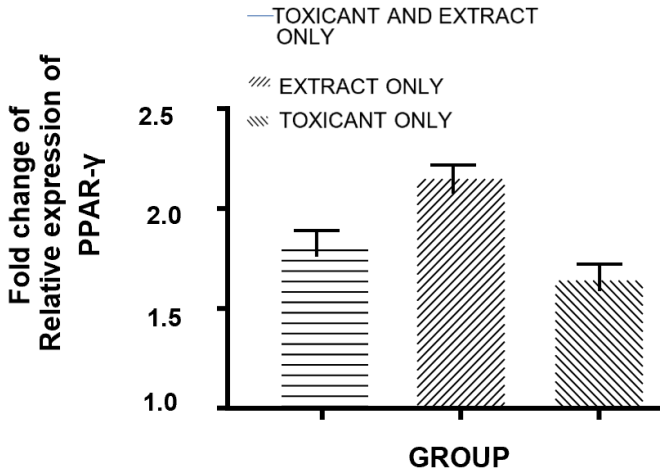


**Figure 2**
Effect of feeding *T. conophorum* oil on expression of **PPAR-γ** in mammary tissue of control and 3-MCA treated rats. Values are mean ± SE,

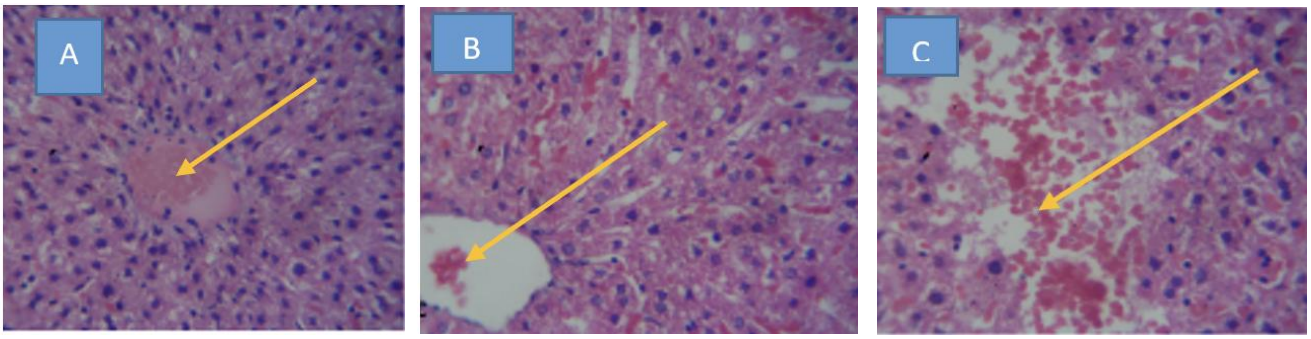


**Plate 1**

Histopathological section of mammary tumors tissue: *T. conophorum* treated tissue and normal tissues Histology of liver in control and treated animals. H & E staining; Magnification = X400. Hemorrhaging central vein (black arrows) with fat droplets.

*A = Methylcholanthrene + walnut oil, B = Walnut oil only (positive control), C = Methylcholanthrene only (negative control)*

*Plate a and b showed normal architecture of mammary tissues while plate c shows marked pathologic hemorrhaging into surrounding tissues along with hepatocytic degeneration.*

## DISCUSSION

We examined the effect of feeding *Tetracarpidium conophorum* oil on 3-MCA induced mammary carcinogenesis and the expression of COX-2 and PPAR-γ genes. Fewer tumor incidence, smaller tumor size and greater tumor latency period on *Tetracarpidium conophorum* seed oil treated group than on the MCA only group, which is are suggestive of protection conferred by *Tetracarpidium conophorum* seed oil in mammary gland carcinogenesis. The biological role of *Tetracarpidium conophorum* seed oil in mammary carcinogenesis may be explained by their ability to regulate the pathway of prostaglandin synthesis. Mammary carcinogenesis is triggered by inappropriate induction and upregulation of COX-2. It was observed (Harris *et al.*, 1999) that the 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 poly unsaturated 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) t*etracarpidium 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 stimulus. 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; Yasui *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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