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Anti-tumour and Anti-inflammatory Effects of Fractions of *Annona muricata* leaf-extract on dimethylbenzanthracene-induced mammary tumours in Wistar rats

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Abstract

The major challenge concerning tumours and cancers is that they currently do not have a cure. The side effects from the available treatment options have made research gravitate towards phytotherapy. *Annona muricata* is one of the highly recommended plants due to its usage albeit in crude form in treating several diseases especially, tumours and cancer inclusive. However, the fraction of its extracts responsible for these effects, is still unknown. This study was designed to investigate the anti-tumour and anti-inflammatory effects of Hexane, ethyl-acetate, butanol and aqueous fractions of *A. muricata* leaves in dimethylbenzanthracene-induced (DMBA) mammary tumours. Forty-two female Wistar rats were divided into 7 groups of 6 animals each. Group 1 was Control and were on basal diet; Groups 2 to 7 were tumour-induced with 0.05mg/g DMBA subcutaneously with soy oil as vehicle. Group 2 was untreated; Group 3 was treated with doxorubicin (0.0125mg/g); Groups 4 to 7 were treated orally with the four fractions respectively at 12.5mg/g for 14 days. Data obtained were subjected to Analysis of Variance using Bonferroni post-hoc test. $P<0.05$ was taken as statistically significant. *Annona muricata* fractions reduced the TNF-α and CRP concentrations which were elevated in response to tumour induction, with the butanol fraction having the highest reduction potential, with a value of 0.02 ± 0.08 and 0.15 ± 0.04 respectively. However, these reductions were not statistically significant across all treatment groups. This study shows that all four fractions of *A. muricata* are potent inhibitors of inflammation, particularly in DMBA-induced mammary tumours. However, the butanol fraction proves to be the most potent in altering the process of inflammation via the TNF-α and the CRP signalling pathways.

Key words: Tumour, Cancer, *Annona muricata*, dimethylbenzanthracene, doxorubicin.

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Introduction

A tumour or neoplasm is an abnormal mass of tissue that is formed when cells grow and divide uncontrollably. These cells undergo more mitotic division and less programmed cell death (apoptosis) (National Cancer Institute, 2015; Yvette, 2019). It is known that a tumour can develop in almost any cell of the body and the process through which it is formed is called tumorigenesis. When tumours develop to the extent which they gain potential for metastasis, cancer is formed. Cancer is an autoimmune disease in which tumour cells become malignant and gain potential for metastasis (Smetana Jr, K., Lacina, L., Szabo, P., Dvorankova, B., Broz, P. & Sedo, A., 2016).

There is presently no cure for cancer, but there are some treatments that have been adopted over the years but with accompanying side effects like fatigue, anemia, bruising, bleeding, infections, hair loss (Rady, I., Bloch, M. B., Chamcheu, R. N., Mbeumi, S. B., Anwar, M. R., Mohammed, H., Babatunde, A. S., Kuiate, J., Noubissi, F. K., El Sayed, K. A., Whitfield, G. K. & Chamcheu, J. C., 2018; Laksmitawati, D. R., Prasanti, A. P., Larasinta, N., Syauta, G. A., Hilda, R., Ramadaniati, H. U., Widyastuti, A., Karami, N., Afni, M., Rihibiha, D. D., Kusuma, H. S. W., Wahyu, W., 2018). This is why the attention of researchers has recently shifted to the use of alternative therapy, particularly herbal therapy. *Annona muricata* (*A.* muricata) is one of the herbal plants that have been recognized for the treatment of several infections and diseases. *A. muricata* (also called Graviola) belongs to the Annonacae family (Rady et al., 2018). Its healing and anti-inflammatory and anti-tumour potentials have particularly been recognized in liver infections, kidney infections, malaria and wound healing processes (Laksmitawati et al., 2016; Rady et al., 2018). However, there remains a question of which fraction of *A. muricata* is responsible for its anti-inflammatory and anti-tumour potentials. This study was therefore designed to investigate the anti-tumour and anti-inflammatory effects of fractions of A*nnona muricata* leaf-extract in Wistar rats.

2. METHODOLOGY

Experimental Design

Forty-two female Wistar rats weighing about 100-120g, at 8weeks of age were used. They were housed under standard 12:12h light/dark cycle. The animals were divided into seven groups of six animals each.

Fractionation of *A. muricata*

The ethanol extract of A. *muricata* was first obtained by the technique of Yang, H., Liu, N. & Lee, S., 2016. *A. muricata*. leaves were collected from Ilishan-Remo, Ogun State, Nigeria. The taxonomic identity was verified by a botanist. The *A. muricata* leaves were collected and dried in 40°c oven for two days and the dry leaves were macerated into pieces of approximately 1cm×1cm in size. The macerated leaves were then blended till a fine powder was achieved. The powder was carefully sieved till it was fine in texture, the end yield was weighed and found to be 1835.26g. This was then mixed in a jar with 95% ethanol at ratio 1:8 (55g of the *A. muricata* powder and 440ml of 95% ethanol). The process was done for 20 jars, resulting in 1100g of *A. muricata* powder and 8800ml of 95% ethanol. The mixture was left for 24hours then dried in the oven at 40°c until a thick paste was formed (crude extract).

Successive solvent extraction:

The various fractions of *A. muricata* were extracted and analyzed, using successive solvent extraction (Agrawal, M., Agrawal, Y., Itankar, P., Patil, A., Vyas, J. & Kelkar, A., 2012) to obtain the heaxane, ethyl acetate, butanol and aqueous fractions.

ANIMAL GROUPING AND TREATMENT

This study was done with the random distribution of 42rats into seven (7) groups of six (6) rats each, which is seen in Table 1 below:

Tumour induction and blood sample collection

All animals were on basal diet for 7days, after which all animals in group 2-7 were induced with mammary tumours by administering 0.05mg/g of dimethylbenzanthracene (DMBA) subcutaneously using soy oil as vehicle. This was done according to the method of Barros et al., 2004. Animals were monitored continuously for 12 weeks while on basal diet (Food + water). At week 13, doxorubicin was administered intraperitoneally to group 3 animals daily, at a

dose of 0.0125mg/g for two consecutive weeks while the animals of groups 4-7 (test groups) were treated with the four fractions of *A. muricata* respectively via oral administration at a dose of 12.5mg/g for two consecutive weeks. On the last day of treatment, animals were placed on a 12hour fast, after which blood samples were collected retro-orbitally, using capillary tubes. The blood of each animal was collected into heparinized tubes, centrifuged with a cold centrifuge for 15 minutes and serum was collected for TNF-α and CRP ELISA assay.

TABLE 1. Animal grouping and treatment

GROUP	GROUP NAME	TREATMENT
1	Control	Food + Water
2	Negative control	Food + Water + Dimethylbenzanthracene (0.05mg/g)
3	Reference group	Food + water + Dimethylbenzanthracene (0.05mg/g) + Doxorubicin (0.0125mg/g)
4	Hexane group	Food + Water + Dimethylbenzanthracene (0.05mg/g) + Hexane fraction of *A. muricata* (12.5mg/g)
5	Ethyl-acetate group	Food + Water + Dimethylbenzanthracene (0.05mg/g) + Ethyl-acetate fraction of *A. muricata* (12.5mg/g)
6	Butanol group	Food + Water + Dimethylbenzanthracene (0.05mg/g) + Butanol fraction of *A. muricata* (12.5mg/g)
7	Aqueous group	Food + water + Dimethylbenzanthracene (0.05mg/g) + Aqueous fraction of *A. muricata* (12.5mg/g)

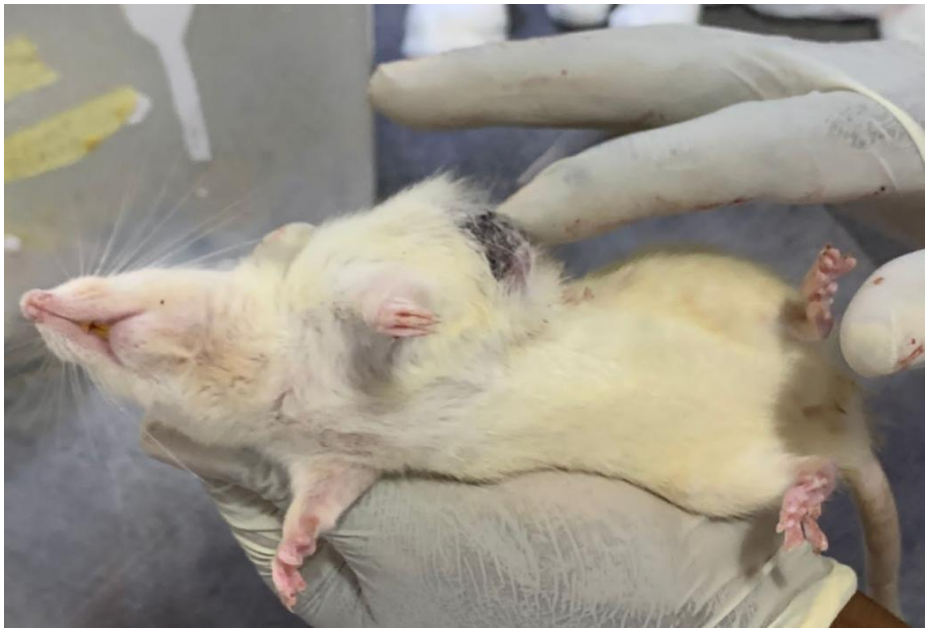

FIGURE 1 Animal with fully developed mammary tumour at week 12

Tissue sample collection

At the end of week 12, animals were sacrificed by cervical dislocation under diethyl ether anesthesia, after an overnight fast. The tumours were carefully removed from rats and kept on a glass plate in ice jackets.

3. STATISTICAL ANALYSIS

Results were presented as mean ± standard error of mean (SEM) and and the significance of means were tested using one-way analysis of variance (one-way ANOVA), as well as Bonferroni post hoc test, using the Graph Pad Prism software. A p-value < 0.05 was regarded as statistically significant.

4. RESULT

TNF-α ASSAY RESULT

The result obtained showed that when compared with control, TNF-α levels reduced insignificantly across all groups except the hexane group where it increased insignificantly. When compared with the negative control group, TNF-α levels increased insignificantly in the hexane and aqueous groups, while it reduced insignificantly in reference, ethyl-acetate and butanol groups. In comparison with the reference group, TNF-α levels increased insignificantly in test groups except the butanol group where it reduced insignificantly (See figure 2).

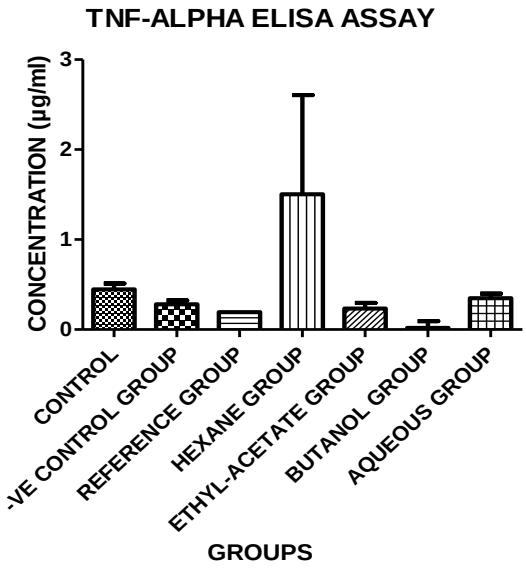

Figure 2 Graph of serum activity of TNF-α levels across all groups (Data are expressed as mean ± SEM)

CRP ASSAY RESULT

The result obtained showed that when compared with the control group, CRP levels increased insignificantly in the negative control, hexane and ethyl-acetate groups while it reduced insignificantly in the reference, butanol and aqueous groups. When compared with the negative control group, CRP levels increased insignificantly in the hexane and ethyl-acetate groups while it reduced insignificantly in the reference, butanol and aqueous groups. In comparison with the reference group, CRP levels increased insignificantly in all test groups except butanol group (See figure 3).

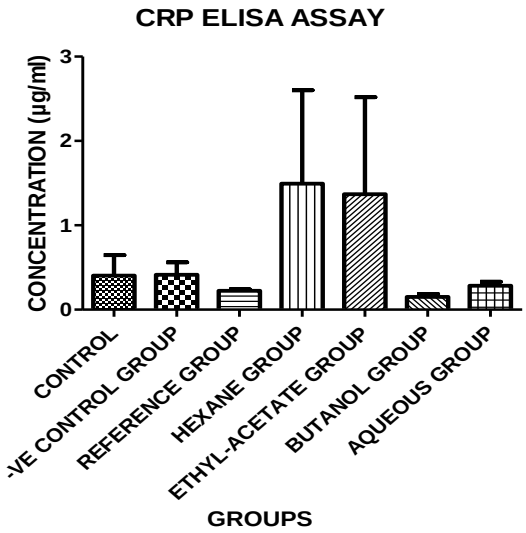

Figure 3 Graph of serum activity of CRP levels across all groups (Data are expressed as mean ± SEM)

Discussion

A tumour or neoplasm is an abnormal mass of tissue that is formed when cells grow and divide uncontrollably (National Cancer Institute, 2015). These cells undergo more mitotic division and less programmed cell death, that is apoptosis (National Cancer

Institute, 2015; Yvette, 2019). Presently, because there is no cure for tumours and cancers, attention of researchers have shifted to the use of alternative therapy, particularly herbal therapy. *A. muricata* is one of the herbal plants that has shown great promise in the treatment of tumours and cancers in its crude form. There remains the question of which fraction(s) of *A. muricata* is responsible for its anti-inflammatory and anti-tumour potentials. This study was therefore designed to investigate the anti-tumour and anti-inflammatory effects of fractions of A*nnona muricata* leaf-extract in Wistar rats.

TNF-α (Tumour necrosis factor-alpha) is a serum mediator of inbuilt immunity, capable of hemorrhagic necrosis in tumours. It is produced as a precursor of the membrane-bound polypeptide processed by proteolysis. When cells become cancerous, the tissues are infiltrated with monocytes, T-cells and other cells capable of producing TNF-α, including peripheral cells capable of producing soluble TNF-α receptors (Chadwick, W., Magnus, T., Martin, B., Keselman, A., Mattson, M. P. & Maudsley, S., 2008). Therefore, removal of soluble TNF-α receptors may lead to local enhancement of TNF-α activity which may help enhance tumour cell death without associated systemic toxicities (Steven, F. J., Thomas, E. I., Stephen, M. P., Santosh, K., Francesco, M. M., Anton, R. E. & Amir, J., 2018). This means that in tumorigenesis, there is an increase in blood concentration of TNF-α, serving as an anti-tumour agent, except it is not completely reliable as exogenous TNF-α might cause apoptosis in malignant cells (Anne, M., Celine, C., Thierry, L., Nathalie, A., Nicolas, M. & Bruno, S., 2019). The result obtained from this research shows that when compared with the control group, TNF-α levels reduced across all groups except the hexane group where the level was elevated. When compared with the negative control group, TNF-α levels increased in the hexane and aqueous groups, while it reduced in the reference, ethyl-acetate and butanol groups. In comparison with the reference group, TNF-α level was elevated in all test groups except the butanol group. This postulates that the butanol fraction of *A. muricata* is more effective in inhibiting the expression of TNF-α and consequently, reversing tumorigenesis in DMBA-induced mammary tumours in Wistar rats.

C-reactive protein (CRP) is a pentametric, acute-phase reactant from the pentraxin family. It is synthesized by the liver, primarily in response to IL-6 action on the gene responsible for the transcription of CRP during the acute phase of an inflammatory/infectious process. Its level rises in response to inflammation. CRP has both pro-inflammatory and anti-inflammatory properties, with its levels rising and falling rapidly with the onset and removal of the inflammatory stimulus respectively (Nehring, S. M., Goyal, A., Bansal, P. & Patel, B. C., 2021). The result of this study shows that when compared with the control group, CRP level was elevated in the negative control, hexane and ethyl-acetate groups while it reduced in the reference, butanol and aqueous groups. In comparison with the negative control group, serum level of CRP was elevated in the hexane and ethyl-acetate groups while it reduced in the reference, butanol and aqueous groups. This suggests that the reference drug, doxorubicin, as well as the butanol and aqueous fractions of *A. muricata* are effective in reversing the process of tumorigenesis, hence the reduction in CRP levels. Serum level of CRP was elevated in all test groups except the butanol group, when compared with the reference group. This result corresponds with the finding that serum level of CRP increases in response to inflammation (Nehring et al., 2021) and it postulates that the four fractions of *A. muricata* are potential inhibitors of CRP expression in DMBA-induced mammary tumours in Wistar rats.

6. CONCLUSION

The result obtained from this research suggests that the reference drug, doxorubicin is more effective in reducing serum levels of TNF-α and CRP when compared with the hexane, ethyl-acetate and aqueous fractions of *A. muricata* but the butanol fraction of *A. muricata* is the most effective. Therefore, it can be said that

the butanol fraction of *A. muricata* is a potent anti-inflammatory treatment in DMBA-induced mammary tumours in Wistar rats.

In conclusion, this study shows that all four fractions of *A. muricata* are potent inhibitors of inflammation, particularly in DMBA-induced mammary tumours. However, the butanol fraction proves to be potentially most effective in altering the process of inflammation via the TNF-α and the CRP signaling pathways.

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