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Anti-Tumour Potentials of Methanol Extracts of *Allium sativum* on Serum IL-10, and IFN-γ in 7,12 Dimethyl Benzene-(a)-Anthracene Breast Tumour-Induced Albino Rats

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Abstract

Breast cancer is an uncontrolled cell proliferation, leading to tumor formation, in this case tumor may grow in different breast areas, such as lobules, ducts, and connective tissue. The hallmark of cancer pathology is not the tumor itself, but the migration of transformed cells to different tissues. Presently, there is no known curative anti-cancer/tumor drugs. The aim of this research is to evaluate the effects of Garlic on serum level of IL-10 and IFN-γ in 7,12 Dimethyl Benzene-(a)-Anthracene Breast tumor-induced albino rats. Fresh garlic bulbs were identified/authenticated and subjected to Methanolic extractions. The total of 24 rats were grouped into 6 groups of 4 rats each. These groups are: The normal and positive controls, Acetylsalicylic acids, preventive, and treated group and synergistic group. All the groups were inducted with 65 mg/kg^{-1}b.w. of 7,12 Dimethylbenzene-(a)-anthracene with the exception of Group I, and observed for 14 days, before treatment with 100mg of ASA (III), and *Allium sativum (IV, V, VI)*. Rats were killed by surgical dislocation, 24hrs of the last treatment, the blood, were collected for ELISA evaluation and assessment. After the treatment with *Allium sativum* extracts and ASA in all the respective groups, the serum IL-10, was up-regulated while the IFN-γ was significantly down-regulated, when compared with the positive controls ($P \leq 0.05$) . The extract was found to also exhibit chemoprotective activities in group IV, where the level of IFN-γ and IL-10, remained absolutely within the normal range, when compared with group I, despite being induced with carcenogen. The research indicated that the ethanolic extracts of *A. sativum* may possess *an* anti-tumor, immunosuppressive and immunomodulatory effects, it may be use in th treatment of breast cancer patients.

Keywords: *Allium sativum*, IL-10, Cytokine, Breast Tumor, Immunomodulation

Introduction

Breast cancer is the most common tumor and the second cause of cancer-related death in women worldwide (GCI, 2018). It is a major global public health problem with estimated number of new breast cancer cases raised from about 641,000 cases in 1980 to 1.6 million cases in 2010 and 625,000 deaths in 2010 (Jemal *et al.,* 2011). In Nigeria, female breast cancer is recognized as major cause of morbidity and mortality with incidence rate ranging from 36.3 to 50.2/100,000 live birth (Jedy-Agba *et al.,* 2012).

Breast cancer an uncontrolled cell proliferation, leads to tumor formation and the development of a multifactorial disease. Breast cancer tumors may grow in different breast areas, such as lobules, ducts, and connective tissue, being ductal cancer the most common of all, however, the hallmark of cancer pathology is not the tumor itself, but the migration of transformed cells to different tissues of the tumor origin. (Nava-Castro *et al*., 2014).

There are numerous risk factors linked to breast cancer tumorigenesis. The principal is the gender, according to World Health Organization (WHO), breast cancer is the most frequent women malignancy in incidence and mortality

(GLOBOCAN, 2012). Pregnancy reduces breast cancer risk through breast cell maturation and diminishing estrogen exposure during gestation (Russo *et al.,* 2005). Breast cancer occur in women and rarely in men, symptoms include a lump in the breast, bloody discharge from the nipple and changes in the shape or texture of the nipple or breast (Sunders and Jassal, 2017). The fundamental issue in breast cancer control is prevention (primary and secondary), which depends on identification of the determinants of the disease, in terms of initiation and promotion. The possibility of using biomodulators of breast carcinogenesis, such as selective estrogen receptor modulators (SERMS), aromatase and cyclooxygenase inhibitors, and dietary factors is very promising (Powles, 2013). Cancer still remains associated with a very high mortality rates, which indicate existing difficulties of effective treatment. The role of herbal medicine in the treatment of many diseases was proven (Miraj, 2016). Medicinal plants constitute an effective source of both traditional and modern medicine. These plants have been shown to have genuine utility and about 80% of the rural population depends on them as primary health care (Akinyemi, 2000).

Allium sativum commonly known as Garlic, the well-documented medicinal use of garlic throughout human civilization is one of the major reasons for the widespread consumption of garlic today, garlic is used to cure of variety of diseases including acute and chronic infections (Gilani and Rahman, 2015; Wargovich *et al*., 2016). One of the most important biological effects observed recently with garlic is prevention of cancer which is reported in various experimental models with mouse and rats (Milner, 2016; Wargovich *et al*., 2016).

Cytokines are low molecular weight regulatory proteins or glycoprotein that modulate the intensity and duration of immune response by stimulating or inhibiting the activation, proliferation, and/or differentiation of target cells (Smyth *et al.,* 2017). Different cytokines are known to have diverse role in breast cancer initiation and progression, the irregular cytokines levels can shift the immune responses from being beneficial to being harmful (Parkin *et al.,* 2016), so therefore, this present study is initiated to determine the preventive and therapeutic activities of Garlic on serum level of IL-10 and IFN-γ in 7,12 Dimethyl Benzene-(a)-Anthracene Breast tumor-induced albino rats. The findings from this study shall provide adequate evidence that garlic-containing substances can inhibits a variety of chemically induced mammary tumor in female Albino rats.

MATERIALS AND METHODS

Ethics statement

For procedures involving Wistar albino rats, the approval was sort and obtained from the UDUS's animal care and use committee. This study was conducted in accordance with the Helsinki guide for the care and use of laboratory animals.

Plant Collection and Identification

Fresh *Allium sativum* were purchased from Sokoto central market (Shagari Gate) Sokoto, Nigeria and was identified and authenticated at the herbarium Department of Pharmacognosy Usmanu Danfodiyo University where a voucher number was allocated (PCG/UDUS/AMAR/0001) and a voucher specimen was kept in the herbarium.

Preparation of Extract

Chemicals

All reagents used for the study were of an analytical grade. Ethanol used for extraction and phytochemical reagents were purchased from Science Technology CO., LTD. China.

Plant Extraction

The raw *Allium sativum* bulbs were peeled off to obtain garlic cloves, the cloves were crushed using kitchen manual blender. The extraction was obtained by soaking 876.133g in 1100.00ml of ethanol for 24-hrs at room temperature. The residue and the filtrate were obtained by filtering the soaked *Allium sativum* using a separating funnel. The residue was dried on cardboard paper and the filtrate was obtained as extract. The concentration of the extract was obtained by putting it in to a thermostat oven at 50-60^{o}C for some days. Following evaporation, 7.31g volume of extract was obtained.

Experimental Animals

A total of 45 female Wistar Albino rats weighing approximately 100-140g aged 8 - 10 weeks old, were purchased from the Veterinary Department of Ahmadu Bello University (ABU), Zaria. They were housed in well-ventilated cages under hygienic condition in animal house, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University Sokoto (UDUS), in an environment of ambient temperature (25 ± 5^{o}C), humidity (55 ± 5%) and the lighting period of about 12 hours daily. They were fed with standard commercial pelletized grower's feed (Vital® feed, Jos, Nigeria) obtained from Grand Cereal Soil Mills Limited, Jos, Nigeria. They were allowed free access to clean drinking water ad libitum throughout the experimental period. They were kept in cages to acclimatize for a period of two weeks before commencing the experiment. Cleaning of the animal cages was carried out on a daily and regular basis. The animals were maintained as described by Turner (2008). All the experimental protocols were incompliance with UDUS Faculty Animal Research Ethics Committee (FAREC).

Table 1 Experimental Design

Groups	Dose (mg/kg b.w)	Durations
Group I: NC	DH_2O only	28 days
Group II: PC	DMBA 65mg kg. bw	28 days
Group III: ASA	DMBA 65mg + ASA (100mg/kg/day)	14 +14 days
Group IV: Preventive	Extract (*A. sativum*) (500mg/kg/day) + DMBA 65mg	14 + 14 days
Group V	DMBA (65mg + Extract (1000 mg/kg/day).	14 + 14 days
Group VI	DMBA (65mg + Extract (1500 mg/kg/day).	14 +14 days

Key: n = number of rats, DH_2O = distilled water, mg/kg = milligram per kilogram, b. w = body weight, DMBA= Dimethylbenzene (a)anthracene

Induction of Mammary Tumor

A single dose of dimethylbenzene (a) anthracene (DMBA) 65 mg/kg body weight was injected once to the rat mammary glands subcutaneously. The rats were observed for the development of breast tumor after 14 days of induction.

Laboratory Analysis

The present study was conducted at the Department of Immunology School of Medical Laboratory Sciences, Department of Pharmacological Sciences and Department of Pharmacognosy and Ethnomedicine Usman Danfodiyo University Sokoto. The following techniques were employed in the study.

Blood Sample Collection and Processing

A day (24 hours) after the last treatments blood samples was collected in sterile plain and EDTA bottles. The samples collected into a plain tube was allowed to clot at room temperature and later centrifuged at 3000g for 5 minutes. The clear unhaemolysed sera was transferred into labelled sterile serum bottles tightly caped and stored at -20◦C until.

Analytical Methods

Estimation of Serum Cytokines Concentrations

Serum IL-10 and IFN-γ Concentration were evaluated using Sandwich-ELISA Technique (using test kit procured from Sunlong Biotech. CO., LTD China). The procedure was carried out with strict adherence to the manufacturer's instructional manual.

Serum interleukin IL-10 and IFN-γ Serum concentration of IL-10 and IFN-γ were assessed by quantitative sandwich enzyme immunoassay technique (Sunlong Biotech. CO.,

LTD China). The procedure was carried out with strict adherence to the manufacturer's instructional manual.

Data Analysis and Presentation
Multiple comparisons of the mean value was carried out using one way analysis of variance (ANOVA) using SPSS software version 23.0 The results were presented using tables and figures.

Presentation of Results
Physical Properties of Ethanol Extract of *Allium sativum*
The physical properties of the ethanolic extract of *Allium sativum* is presented in Table 1. In this Table, 873 .133g of fresh *Allium sativum* bulb yielded 7.31 g extract of *Allium sativum* and the texture was semi-solid and light brown in color.

Initial and Final body Weight of Female Wistar Albino Rats
The initial and final body weight analysis of female Wistar albino rats is presented in Table 2, In this Table, six groups of 4 rats each shows the initial and final body weight of rat. The Table also shows the P and F – values.

Effect of Methanol Extract of *A. Sativum* on Serum IL-10 Concentration
The effect of ethanolic extract of *A. Sativum* on serum IL-10 concentration in (DMBA)-induced breast tumor/cancer in female wistar albino rats is presented in Table 4.8. In this Table, five groups of 5 rats each shows the doses of DMBA, extract and ASA (mg/kg B.W) with the concentration of serum IL-10 (pg/ml) respectively. In the Table, the F-value and the P- value is also presented.

Effect of Methanol Extract of *A. Sativum* on Serum INF-γ Concentration
Serum level INF-γ was significantly higher in positive control group (65 mg DMBA) when compared with negative control. However, INF-γ significantly decreases in DMBA+ 100 mg ASA, DMBA + 500 mg MEAS, DMBA + 1000 mg MEAS and DMBA + 1500 mg MEAS. However, there was a statistical difference ($P \leq 0.05$) in DMBA + 1000 mg MEAS and DMBA + 1500 mg MEAS when compared with DMBA + 100 mg ASA. Moreover, there was no statistical difference in the positive group, DMBA + 100 mg ASA, DMBA + 500 mg MEAS when compared with negative control.

Table 2: Physical Properties of Ethanol Extract of *A. sativum*

Plant	Extract	Yield	Texture	Color
Fresh *Allium sativum* bulb (873.133g)	Ethanol 1100.00ml	7.31g	Semi-solid	Light Brown

Key: g =gram, ml = milligram,

Table 3: Initial and Final Body Weight of Wistar Albino Rats

Groups	N	Initial body weight (Day 1) (g)	Final body weight (Day 29) (g)
1	4	125.80 ± 3.31	143.3 ± 3.49
2	4	125.00 ± 7.31	108.7 ± 6. 40 [a]
3	4	126.80 ± 11.86	144.4 ± 9.31[a]
4	4	128.80 ± 10.74	133.3 ± 2. 94 [a]
5	4	135.80 ± 9.55	156.9 ± 5.45 [b]
6	4	137.81 ± 7.55	159.2 ± 4.45 [b]
F value		0.230	0.918
P value		6.247	0.002

Values expressed as mean ± SEM, n= number of rats, g = gram. Values with superscript differ significantly at [a]$p < 0.05$ and [b]$p < 0.001$ using Turkey's HSD Post Hoc Multiple Comparison Test.

Table 4 : Effect of Methanol extract of *A. sativum* on Serum IL-10 Concentration in *DMBA*-induced Breast Cancer in Female Wistar Albino Rats

Groups	No	Dose (mg/kg b.w)	IL-10 (pg/ml)
1	4	Non DMBA	20.02 ± 0.41
2	4	65 mg DMBA / Single dose	12.62 ± 0.66 [c]
3	4	65 mg DMBA+100 mg ASA /14 days	15.4 ± 0.29 [b, c]
4	4	65 mg DMBA+500 mg MEAS/14 days	17.83 ± 0.22 [a, c]
5	4	65 mg DMBA +1000 mg MEAS/14 days	16.04 ± 0.60 [c,]
6	4	65 mg DMBA +150 0 mg MEAS /14 days	18.05 ± 0.63 [a, b,c]
P- value			0.000
F- value			3.624

Values expressed as mean ± SEM, n= number of rats, mg/kg = milligram per kilogram, b.w = body weight, DMBA = Dimethylbenzene (a) anthracene, ASA = acetylsalicylic acid, pg/ml = pictogram per mil. Values with superscript differ significantly at $^{a}p \leq 0.05$ and $^{b}p \leq 0.01$ and $^{c}p \leq 0.001$using Turkey's HSD Post Hoc Multiple Comparison Test.

Table 5 : Effect of Methanol Extract of *A. Sativum on* Serum IFN- Concentration in DMBA-Induced Breast Cancer in Female Wistar

Groups	No	Dose (mg/kg b.w)	IFN-γ (pg/ml)
1	4	Non DMBA	60.56±0.91
2	4	65 mg DMBA / Single dose	72.41±2.35
3	4	65 mg DMBA+100 mg ASA /14 days	72.64±2.53
4	4	65 mg DMBA+500 mg MEAS/14 days	65.60±2.70
5	4	65 mg DMBA +1000 mg MEAS/ 14 days	54.59±7.02 [b, c]
6	4	65 mg DMBA +1500 mg MEAS /14 days	53.50±3.67 [b, c]
P-value			0.004
F- value			5.14

Data in the table above are expressed as Mean ± SEM, $^{b}P \leq 0.05$ when compare with 65 mg DMBA /Single dose, $^{c}P \leq 0.05$ when compare with 65 mg DMBA + 100 AA/10 days, IL-6 = Interleukin 6, DMBA = 7,12 dimethylbenzyl (α) anthracene, MEAS = Methanolic extract of Allium sativum, ASA = Acetyl salisalic acid . Values differ significantly at $P \leq 0.05$. (One-way ANOVA) by instant grap pad prism VI followed by Turkey Post-Hoc Test.

Discussion

In this study, Mammary gland tumors were induced by a single dose of 65 mg/kg B.W of DMBA diluted in Dimethyl sulfoxide (DMSO). The first assessment 7 days after DMBA administration revealed at least two or more rats developed at least 1 mammary tumor (80%), at 14th day all rats developed one or two tumor at the mammary glands (100%).. This finding agrees with previous studies by Avula *et al.,* (2014). Twenty young virgin Sprague-Dawley female rats, aged 47 days, received 20 mg of 7,12dimethylbenzene-(a) anthracene (DMBA) intragastrically by gavage. Eight weeks after DMBA injection, 16 rats presented at least 1 breast tumor (80%). After 13 weeks, all of them (100%) developed breast carcinomas that were confirmed by histopathological analysis. In another study, carcinomas of the breast were produced in 72 female Sprague-Dawley rats bred

in the laboratory. At the ages of 50, 53, and 56 days, the rats received an i.v. injection of DMBA, 2 mg, in a fat emulsion. The result revealed Breast tissue was fixed in formalin and processed for light microscopic studies (Tariq *et al.,* 2010). This provides adequate proof that DMBA can practically lead to the initiation of tumor/cancer. Except that of group four which were first treated with extract (*A. sativum*) prior to DMBA induction of the same dose and duration with all groups however, in this group (IV), only few rats shows a sign of tumor initiation this is due to the preventive effect of phytochemical constituent present in *A. sativum* this finding is in agreement with a previous study by Moayad *et al.* (2006) designed to investigate the pre amd post chemoprotection of garlic on 7, 12-dimethylbenzene (a) anthracene (DMBA) mammary cancer in female albino rats. The result of their findings indicate that garlic got both pre and post effect on DMBA infused rats (Moayad *et al*., 2006).

In this study, there was up-regulations of serum IL-10 concentrations across the treatment group ($p \leq 0.05$). The findings suggest that there is consistent activity of MEAS on secretion of IL-10 across all groups. IL-10 displays both immunosuppressive and immunostimulating activities (Pooja, *et al.,* 2012). IL-10 blocks the production of pro inflammatory cytokines such as IFN-γ, TNF-α, IL-6, IL-12 and Th2 derived cytokine like IL-5, but induced IgE synthesis of IL-4 (Pooja *et al.,* 2012). As IL-10 in turn strongly inhibits APC derived IL-12 production (Xiao *et al.,* 2015). Some cytokines such as IL-12 antagonistically opposed the functions of IL-10 (Xiao *et al.,* 2015). Our findings agree with the study of Li *et al. (*2009) which reported that aqueous extract of MILE up regulates IL-10 production. However, study by Luo *et al.* (2014) disagree with our findings, they reported that aqueous MILE down regulate IL-10 production. Glycoside in MILE has been proven to possess portent immunostimulating effect by significantly increasing IL-10 expression in mice (Li *et al.,* 2009).

From our findings, it's practically proven that ethanolic extract of *A. sativum* possesses a potent both preventive, therapeutics as well as immunomodulating effects on IL-10.

IFN-γ, or type II interferon, is a cytokine that is critical for innate and adaptive immunity against viral bacterial and protozoal infections. IFN-γ is an important activator of macrophages and inducer of Class II major histocompatibility complex (MHC) molecule expression. Aberrant IFN-γ expression is associated with a number of auto inflammatory and autoimmune diseases (Alhassan *et al.*, 2021). The importance of IFN-γ in the immune system stems in part from its ability to inhibit viral replication directly, and most importantly from its immunostimulatory and immunomodulatory effects. IFN-γ is produced predominantly by natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response, and by CD4 Th1 and CD8 cytotoxic T lymphocyte (CTL) effector T cells once antigen-specific immunity develops (Smyth *et al.,* 2017). IFN-γ is secreted by T helper cells (Th1 cells), cytotoxic T cells (TC cells), macrophages, mucosal epithelial cells and NK cells. IFN-γ is Type II interferon serologically distinct from Type I interferons by its acid-labile, while the type I variants are acid-stable (Miller *et al.,* 2017). IFN-γ has antiviral, immunoregulatory, and anti-tumor properties. It alters transcription in up to 30 genes producing a variety of physiological and cellular responses (Miller *et al.,* 2017). NK cells and CD8+ cytotoxic T cells also produce IFN-γ. IFN-γ suppresses osteoclast formation by rapidly degrading the RANK adaptor protein TRAF6 in the RANK-RANKL signaling pathway, which otherwise stimulates the production of NF-κB (Gerger *et al.,* 2010).

In our present study, MEAS, have been proven to possess potent immunostimulating effect by significantly decreasing IFN-γ production in Albino rat. The level of serum IFN-γ was drastically reduced after MEAS and ASA treatments across the group, the higher doses of MEAS been more effective, when compared with the standard treatment of ASA ($p \leq 0.005$). The observed downregulations in the level of IFN-γ may slow the onset of breast cancer in Albino rat, this could also lead to increasing receptor sensitivity and repairing of mammary cells (Mumm *et al.*, 2013). Our findings suggest

that increase concentration of MEAS over a prolonged period will lead to a significant level of cell repair.

Saponins stimulates lymphocytes (Cho *et al.,* 2002). The study findings show that there is significant decrease in IFN-γ concentration which corresponds with increase dose of MEAS across the treatment group ($p \leq 0.05$). Since there is a general decrease in IFN-γ secretion across the group, these findings suggest stimulative activity of MEAS on immune cells.

Conclusion

The present study it can be concluded that the ethanolic extract A*llium sativum* practically shows *an* anti-tumour and immunomodulatory effects in tumor/cancer inducted rats. The extract was able to Upregulate serum IL-10 while significantly downregulating serum IFN-γ. Therefore, *Allium sativum* may be use or serve as a better preventive and therapeutic options in the management of Breast cancer patients.

Recommendations

Considering the findings of this study, In view of the potentials effects of ethanolic extract of *A. sativum*, it's recommended that there is a need to carry out clinical trials to see if the same result will be replicated in human. Therefore, further and more research studies should initiate.

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