Chemo-Protective Effects of DL-Limonene Unaided and in-Blend with 6-Methyleneandrosta-1, 4-Diene-3, 17-Dione on N-Nitroso-N-Methylurea and Estrogen Sulfotransferase Breast Cancer-Induced Albino Female Rat

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ABSTRACT: Breast cancer is a serious prime hassle commonly diagnosed in women worldwide and it is currently the leading cause of cancer-related mortality. In N-nitroso-N-methylurea (NMU) and estrogen sulfotransferase (EST) breast cancer-induced female rat, the study evaluates the chemo-protective possibilities of DL-limonene (1:1) and its combination with 6-Methyleneandrosta-1, 4, Diene- 3, 17-Dione using standard techniques. Amongst others, data obtained reveals mild areas of clogged blood vessels and pulmonary inflammation revealed in the histopathology section. Interestingly, it's possible that DL-limonene alone at a concentration of 10% could be an effective breast cancer treatment. The findings also revealed that combining DL-limonene with 6-ADD at 5% and 12.5mg/kg could reduce the risk of toxicity associated with higher chemotherapeutic dosages in long-term treatment. Furthermore, at a modest dose, this combination may increase the use of aromatase inhibitors in premenopausal women. Despite the medicinal and therapeutic benefits of DL-limonene, it is best to take it in moderation due to its possible harmful effects on the blood vessels.

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Worldwide, breast cancer is a serious prime hassle commonly diagnosed in women. It is the second most common cause of cancer-related death in women after lung cancer (Siegel et al., 2020). Treatment for breast cancer can take a variety of forms, depending on the severity of the disease and the characteristics of the tumor (Moo et al., 2018). It is an extremely heterogeneous cancer type, with many molecular subtypes that are linked to different prognostic factors (Malhão et al., 2021). Immunohistochemistry and/or genetic analyses are commonly used to determine the molecular subtype, and it is classified based on the presence or absence of estrogen and progesterone receptors (ER and PR), as well as the eventual (over)expression of the oncogene human epidermal growth factor receptor 2 (HER-2) (Malhão et al., 2021). The use of natural plant products to ascertain innovative chemical entities is one of the most rapidly expanding topics of investigation (Stephane and Jules, 2020). Such plant products included Terpenes (Yazaki et al., 2017), diterpene, (Ravichandran et al., 2018; Klimek-Szczykutowicz et al., 2020). Non-clinical toxicological evaluations of natural and synthetic substances, often performed in rat and mice, are regarded vital for the development and validation of new medications, as well as to comprehend their application for human and pharmaceutical innovation (Houck and Kavlock 2008; Lima et al., 2012; Ferreira et al., 2015, 2019).

One of the most extensively utilized medicines for breast carcinoma is chemotherapy. However, because of the negative side effects and potential multidrug resistance that patients may develop, significant progress has been made in the quest for novel options, such as the use of plant-derived natural compounds. In light of this, the objective of this study was to investigate the chemo-protective effects of DL-limonene alone and in combination with 6-Methyleneandrosta-1, 4-diene-3, 17-dione on N-nitroso-N-methylurea (NMU) and estrogen.
MATERIALS AND METHODOLOGY

Chemical/Natural Compound: Nitrosomethyleneurea and Estradiol were purchased from Sigma-Aldrich chemical company China, 6-Methyleneandrosta-1, 4-diene-3, 17-dione was obtained from Sigma-Aldrich chemical company China, and DL-limonene was obtained from Bristol chemical company, Nigeria.

Experimental Animals and Study Design: Ninety (90) female albino rats at seven weeks of age were purchased from the Nigerian Institute of Medical Research (NIMR) Yaba, Lagos, Nigeria. They were examined for health status and housed and handled under ethical conditions approved by the Health Research Ethics Committee, College of Medicine, and University of Lagos, Nigeria. Rats were acclimatized for one week before being divided into eight groups and kept at 25 ± 3 °C and 60 ± 10% relative humidity with a 12-hour light/dark cycle. Animals were given a high-fat diet and water ad libitum.

Group AO – NMU and Estradiol induced only: Group BO – Feed and Water: Group CO — NMU and Estradiol induced rat treated with DL limonene (5%): Group DO — NMU and Estradiol induced rat treated with DL limonene (10%): Group EO – NMU and Estradiol induced rat treated with 6-ADD (12.5mg/kg): Group FO – NMU and Estradiol induced rat treated with 6-ADD (25mg/kg): Group GO— NMU and Estradiol induced rat treated with DL-limonene (5%) and 6-ADD (12.5mg/kg): Group HO — NMU and Estradiol induced rat treated with DL-limonene (10%) and 6-ADD (25mg/kg)

Preparation of Nmu and Beta-Estradiol: The carcinogen NMU as indicated by (Minari and Okeke, 2018) was dissolved shortly before administration in phosphate/citrate-buffer-ed saline at pH 4.2 (1 part buffer to 14 parts saline). Beta-estradiol was dissolved in 1 mL ethanol and to a volume of 20 mL of olive oil.

Acute Toxicity Test: Acute toxicity was carried out following the protocol of Chinedu et al., (2013).

Induction of Cancer in Animals: Cancer was induced using a modified protocol of Sajjadi and Bathaie (2016). NMU was administered through intraperitoneal injection. The experimental groups received 100mg/kg/body weight of NMU (four times) once per week for the first four weeks and Beta estradiol was administered twice per week. Rats were carefully checked daily, body weight was taken weekly, and the weight of the tumour was taken twice a week.

Drug Schedules

DL-Limonene Alone: The powdered food was supplemented with DL-limonene to achieve a final limonene concentration of 5% to 10%. The diet (prepared weekly) was placed in a glass jar until required. Rats were fed a fresh diet daily for 12 weeks.

6-Methyl-Indrorostenedione Alone: 0.1 mg/ml of 6-ADD was dissolved in 2 mL of DMSO and 20 mL of saline. This was administered daily at a dose of 12.5 and 25 mg/kg (10 mg, 0.1 ml, and 0.2 ml per rat) subcutaneously for 12 weeks.

Combination Treatment: Rats were given a 5% and 10% (optimal) dose of dl-limonene in their diet, and a dose of 12.5 mg/kg and 25 mg/kg of 6-ADD was given subcutaneously for 12 weeks.

Sample Collection and Preparation: After the experimental period, all the animals were sacrificed at the end of 12 weeks by cervical dislocation. Organs were harvested and fixed in formalin for histopathology. Blood was collected by orbital venous plexus bleeding in EDTA bottles for hematological parameters and in plain bottles kept in a slanting position to induce separation of serum from whole blood and centrifuged (5000 rpm for 20 minutes) in plain sterile bottles for biochemical and anti-oxidant evaluation (parasuraman et al., 2010).

Liver Function Test: Blood was collected in sterile vial without anticoagulant for serum separation. Sera samples were analysed for liver function test, biochemical parameters viz. total protein determined using the Biuret method as reported by Gornall et al. (1949). Plasma Bilirubin, which was used for bilirubin determination, was determined by the method described by Tietz et al. (1994). Plasma Aspartate Aminotransferase (AST) activity was determined following the principle described by Reitman and Frankel (1957). Plasma Alanine Aminotransferase (ALT) activity was determined following the principle described by Reitman and Frankel (1957) and the determination of nitrite level was assayed using the method of Palmer et al., (1987).

Kidney Function Test: Blood was collected sterile vial without anticoagulant for serum separation. Sera samples were analysed for Blood Ureal Nitrogen (BUN) using the methods methods of Veniamin and...
Vakirtzi–Lemonias (1970) and Creatinine was determined by the methods of Tietz et al. (1994).

**Determination of Enzymatic Antioxidants:** This was assessed in Red Blood Cells (RBC). The reduced glutathione (GSH) content of liver tissue as non-protein sulphhydryls was estimated according to the method described by Sedlak and Lindsay (1968). Superoxide Dismutase activity (SOD) was determined as described by Sun and Zigma (1978). Catalase activity was determined according to Sinha et al., (1972). Malondialdehyde (MDA), an index of lipid peroxidation, was determined using the method of Bueg and Aust (1978). The assay of glutathione peroxidase (GPx) was determined by the method of Rotruck et al. (1973).

**Hematology:** Red Blood Cells (RBC). White blood cell (WBC), Haemoglobin (HGB), packed cell volume (PCV) platelets (PLT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), granulocyte (GRAN%) and lymphocyte (LYMHP) was estimated using an automated blood analyser.

**Histological Determination:** For microscopic evaluation, liver, kidney, lungs, and ovary tissues were fixed in a fixative (10% formal saline) and embedded in paraffin, sectioned at 4 to 5 m, and subsequently stained with haematoxylin and eosin. Sections were studied under a light microscope at 40 and 100 magnifications. Slides of all the treated groups were studied and photographed. All specimens were compared histologically with a reference (Ito, 1986).

For the evaluation of experimental mammary tissues, the tissues were processed, sectioned at 3 microns and were stained using the conventional haematoxylin and eosin. The tissue slides were interpreted according to the groups.

**Statistical Analysis:** Statistical analysis of data was assessed by one way analysis of variance (ANOVA using SPSS (16.0) followed by post hoc test least significant difference (LSD). The Data were expressed as the Mean ± S.D. of 10 animals per group (n=10/group). Values were considered statistically significant at p<0.05.

**RESULTS AND DISCUSSION**

Figure 1. Shows the body mass of the rat treated with NMU and Estradiol. It was observed that treatment with NMU and Estradiol caused a 43% decrease in body weight gain of rodents when compared with control B. In contrast, supplementation with DL-limonene at both 5 and 10% showed 40.5% and 43% decline in weight compared to control B and no statistical difference when compared with control A.

**Fig 1:** The effect of 5% and 10% DL-limonene, 12.5 and 25 mg/kg 6-ADD only and in combination on the body mass of induced NMU and EST. Treated rats. Rat were treated daily for 12 weeks and body weight taken weekly. AO (Negative control) – Induced rats only, BO (Positive control) – Control experiment, CO --- Induced rat treated with DL limonene (5%), DO - Induced rat treated with DL limonene (10%), EO --- Induced rat treated with 6-ADD (12.5mg/kg), FO -Induced rat treated with 6-ADD (25mg/kg), GO-- Induced rat treated with DL-limonene (5%) and 6-ADD (12.5mg/kg), Group HO ---- Induced rat treated with DL-limonene (10%) plus 6-ADD given s.c. Error bars represent s.c.m.(25mg/kg).

Moreover, supplementation with 6-ADD only at 12.5mg/kg and 25mg/kg showed 24% and 30% decrease in weight gain compared to control B and showed a statistical increase in weight gain when compared with NMU and EST. induced rat.
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Plate 1: Histological sections of kidney tissue of experimental rat. (HE-100X):
(1A) Kidney tissue showing congested blood vessels
(1B) Sections showing no abnormalities
(1C) Sections showing mild vascular congestion
(1D) Sections showing mild vascular congestion
(1E) Sections showing mild vascular congestion
(1F) Sections showing no abnormality
(1G) Sections showing mild vascular congestion
(1H) Sections showing normal study.

Plate 2: Histological sections of liver tissues of experimental rat.
(2A) Sections showing severe vascular congestion with edema
(2B) Sections showing no abnormalities
(2C) Sections showing congested blood vessels
(2D) Sections showing mild vascular congestion
(2E) Sections showing vascular congestion
(2F) Sections showing vascular congestion
(2G) Sections showing vascular congestion
(2H) Sections showing severe vascular congestion with Edema.

Plate 3: Histological sections of lungs tissues, (HE-100X).
(3A) Congested blood vessels
(3B) Normal blood vessels
(3C) Moderate pulmonary Inflammation
(3D) Moderate pulmonary inflammation
(3E) No abnormalities seen
(3F) No abnormalities seen
(3G) section showing mild vascular congestion
(3H) mild Congested blood vessels.

Plate 4: Histological section of ovary tissue. (HE-100X).
(4A) Normal ovary section
(4B) Normal ovary section
(3C) Normal ovary section
(4D) Normal ovary section
(3E) No abnormalities seen
(4F) No abnormalities seen
(4G) No abnormalities seen
(4H) No abnormalities seen..
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In addition, supplementation with the combination of DL-limonene and 6-ADD at 5% and 12.5mg/kg showed 30% decrease in body weight gain compared to control B and 13% increase in weight gain when compared with NMU and EST. induced rat. Furthermore, supplementation with the combination of DL-limonene and 6-ADD at 10% and 25mg/kg caused a critical decline in body weight gain compared with control B and NMU/EST. induced rat.

Figure 2 depicts the effects of DL-limonene, 6-ADD, and a combination of both at various concentrations on the tumor growth of NMU and estradiol-induced mice. The findings revealed that NMU and estradiol significantly enhanced mammary gland weight. Surprisingly, supplementation with DL-limonene at a concentration of 5% downgraded the tumor growth in the NMU-induced rat, but treatment with DL-limonene (at a concentration of 10% only) ameliorated the alteration of tumor growth.

Furthermore, while there was no sign of tumor growth in the 6-ADD alone administered (at 25 mg/kg), there was evidence of tumor growth and a substantial increase in tumor volume in the 6-ADD only administered (at 12.5 mg/kg). Furthermore, combining DL-limonene with 6-ADD (at 5 and 2.5 mg/kg) resulted in complete reversion of tumor growth in NMU and estradiol-induced rats. In the experimental groups, the effects of DL-limonene, 6-ADD, and their combinations on hematological parameters are shown in Table 1. According to the findings, the rats in group FO had a statistically significant decrease in WBC, whereas all other blood profiles indicated an increase in mean values, but were statistically insignificant (P > 0.05). A s shown in Table 2, the metabolic profile of the treatment groups did not show any significant alterations. The effects of DL-limonene, 6-ADD, and their combination on liver and kidney function in serum are shown in Figures 3 and 4. Among the experimental groups, studies indicated no significant variations in the liver of the experimental rats. While in the kidney, studies revealed a significant increase in urea in group CO and statistically insignificant variations in creatinine.

Plates 1 to 5 show histopatological changes in vital organs and breast tissue of control and experimental rats. The renal histology of groups AO, CO, DO, and EO indicated mildly congested blood vessels in plate 1, whereas no abnormalities were observed in groups BO, FO, and HO. Plate 2 depicts the liver's histological alterations.

From the findings, Group A had severe vascular congestion with edema, and Group HO had severe vascular congestion, whereas the other experimental groups had mildly clogged blood vessels in some areas. Plate 3 shows Lung histology revealed occluded blood vessels in groups AO, GO, and HO and mild pulmonary inflammation in groups CO and DO, while EO and FO revealed normal tissues.

Plate 4 depicts the results of ovarian histopathology. Studies indicated no abnormalities in the ovarian tissue of the experimental animals. Plate 5 depicts the findings of breast tissue histopathology. Investigation revealed ductal hyperplasia, adenosis, and secretion in group AO and normal terminal epithelial buds (TEB) and stroma in group DO.

Notwithstanding, ductal hyperplasia, adenosis, and normal stroma were also seen in CO and GO, but in EO, fibro-myxoid stroma was discovered, as well as sections exhibiting a lesion made up of proliferating breast ducts and normal stroma. Furthermore, FO and HO revealed severe desmoplasia indicating invasive carcinoma.

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The potential of naturally occurring bioactive compounds limonene have been demonstrated to possess anticancer agent. The D-isomer of limonene, D-limonene, occurs more commonly in nature compared to the L-isomer and can be extracted from citrus fruit peels (Zhou et al., 2021). D-limonene is a major component of citrus essential oil (Bozkurt et al., 2017) and is widely used as a flavoring agent in food, as well as cosmetics, medical products, personal care items, and cleaning products (Ravichandran et al., 2018). While L-limonene is mainly found in abundance in pine needles (Erasto and Viljoen, 2008). The efficacy of D-limonene has been reported to induce inhibition of the cell growth-regulatory proteins like Ras (Chaudhary et al., 2012), induces activation of caspase-3 and caspase-9, PARP cleavage, and Bax supermolecule, induces necroptosis through the mitochondrial pathway and through the suppression of the PI3K/AKT pathway (Jia et al., 2013). Within the in vivo models, the effectiveness of D-limonene has been reported to exhibit varied effects on many hallmarks of melanoma (Chaudhary et al., 2012; Chidambaram et al., 2012). Due to increasing attention of limonene in our industrial and pharmaceutical sectors, administrative effects and potentials of racemic mixture of limonene (DL) at 1:1 was evaluated. Four deaths were noticed (two at Group FO and two at HO). However, elevated mortality was also observed in the vehicle group, which can be attributed to the modified dosage of 400 mg/kg/bwt of NMU instead of the 50 mg/kg/bwt used in previous research. This is in corroboration with Minari and Okeke's prior report (2018). Studies consider body weight loss to be quite important from a toxicological standpoint if it changes by at least

<table>
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<th>Parameters</th>
<th>AONMUT-EST</th>
<th>BO(CONT)</th>
<th>CO</th>
<th>DO</th>
<th>EO</th>
<th>FO</th>
<th>CO/EO</th>
<th>DO/FO</th>
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<td>WBC (10^9 µL^-1)</td>
<td>7.56 ± 3.09</td>
<td>5.92 ± 7.34</td>
<td>13.84 ± 3.88</td>
<td>15.04 ± 11.52</td>
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<td>RBC (10^6 µL^-1)</td>
<td>5.892 ± 0.64</td>
<td>6.892 ± 6.496</td>
<td>6.976 ± 6.906</td>
<td>6.434 ± 6.906</td>
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<tr>
<td>HGB (g -dl)</td>
<td>12.46 ± 0.94</td>
<td>14.06 ± 14.72</td>
<td>13.16 ± 13.36</td>
<td>13.88 ± 14.24</td>
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<tr>
<td>PVC (%)</td>
<td>43.02 ± 4.46</td>
<td>40.62 ± 32.96</td>
<td>43.42 ± 45.64</td>
<td>42.68 ± 42.68</td>
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<td>PLT (10^9 µL^-1)</td>
<td>541.4 ± 580.4</td>
<td>618.6 ± 658.4</td>
<td>608 ± 806.2</td>
<td>622 ± 622</td>
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<td>MCV (10^-15)</td>
<td>73.4 ± 2.02</td>
<td>62.26 ± 63.36</td>
<td>70.96 ± 62.6</td>
<td>66.38 ± 70.62</td>
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<td>MCH (pg)</td>
<td>21.22 ± 1.66</td>
<td>18.22 ± 18.7</td>
<td>29.54 ± 19.18</td>
<td>20.58 ± 23.28</td>
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<td>MCHC(g/dL)</td>
<td>29.08 ± 1.6</td>
<td>29.34 ± 30.3</td>
<td>40.9 ± 30.72</td>
<td>31 ± 33.46</td>
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<td>GRAN%</td>
<td>28.94 ± 9.98</td>
<td>30.98 ± 21.8</td>
<td>32.12 ± 20.3</td>
<td>22.4 ± 22.4</td>
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<td>LYMHP (%)</td>
<td>35.34 ± 22.64</td>
<td>15.26 ± 32.9</td>
<td>58.44 ± 32.45</td>
<td>67.8 ± 63.24</td>
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10%. (Lu et al. 2014; Morais et al. 2016; Silva et al. 2016). A decrease in body mass in groups CO and DO in the second and fourth weeks could be due to food aversion. A significant drop in weight in groups AO and B could be due to NMU and EST toxicity, while a dip in HO could be owing to the combination therapy of DL-limonene (10%) with 6-ADD at a 25mg/kg dose.

According to the results displayed in groups EO and FO of Figure 2, the use of 6-ADD alone in young female rats depends on dose and way of administration. Thus, at 12.5mg/kg, there was no evidence of mammary tumour, while at 25mg/kg, 6-ADD elevates breast tumour. This is in accordance with the results stated in a prior report by Kubatka et al. (2008). Also, more Findings among the other groups in Figure 2 revealed that limonene at 10% (Group DO), irrespective of its sub-types, is a potent cancer prevention and control agent. At 5%, DL-limonene is sufficient enough to cause tumor

**Fig 3:** Effects of DL-limonene, 6-ADD and Combination on liver marker enzyme in serum

3A: ASTL-Aspartat Aminotransferase; 3B ALTL—Alanine Aminotransferase; 3C S-TP2- SerumTotalprotein; 3D ALP2S- Alkaline Phosphatas; 3E BILT3-Total Bilirubin; 3F BILD2-Bilirubi

**Fig 4:** Effects of DL-limonene, 6-ADD and Combination on kidney function in serum

Haematological and biochemical profiles were gauged due to their relevance in risk assessment and ability to identify early and subtle xenobiotic toxicity as well as changes in medication response. (Ramaiah 2007; Morais et al. 2016; Ferreira et al. 2016, 2019). From the study, there were no significant alterations in the blood and metabolic profiles of the experimental animal groups. This finding is consistent with previous findings that showed that certain terpenes are either non-toxic or have minimal toxicity (Hariri et al. 2011; Costa et al. 2012).
regression. The most significant finding was that suboptimal doses of an aromatase inhibitor, 6-ADD, could be used in combination with a suboptimal dose of DL-limonene to ameliorate tumour alteration in rats, similar to that obtained with maximally effective doses of either DL-limonene at 10% or 6-ADD (12.5) at suboptimal doses separately. Due to the liver is the primary organ responsible for biotransformation of compounds and, in collaboration with the kidneys, processes vital and endogenous molecules, including plant-derived hepatotoxic components, exposure to xenobiotics, such as drugs, carcinogens, and secondary metabolites, can result in liver injuries (Ivarado-Rico and Castro 2010; Ferreira et al., 2016; Shalan et al., 2017; Ferreira et al., 2019). Most histological sections of the organs showed severely congested blood vessels with edema, mildly congested blood vessels, and mild pulmonary inflammation, which could be due to the carcinogenic effects of NMU and EST induced in the rats, or the biotransformation of DL-limonene and 6-ADD, as previously mentioned. Ductal hyperplasia is a sort of early precursor lesion that functions as a pathway in the development of breast cancer and increases the chance of invasive carcinoma (sin et al., 2010).The carcinogenic effects of NMU and EST provided in the experimental rat resulted in the establishment of ductal hyperplasia and fibro-myxoid stroma among the Groups in plate 5. Invasive ductal carcinoma was found in the FO and HO groups, possibly as a result of the action of 6-ADD administered at 25mg alone and the combination of DL-limonene and 6-ADD at high concentrations (10 and 25mg).

**Conclusion:** The current study suggested that DL-limonene's potential in the treatment of breast cancer can be reached at moderate dosages, either alone or in combination therapy, thereby reducing the risk of toxicity that could occur if greater chemotherapy levels were employed in chronic treatment. Furthermore, at a modest dose, DL-limonene combination may increase premenopausal women's use of aromatase inhibitors. More research is needed to uncover other natural chemicals that can be used in conjunction with 6-ADD to improve its efficacy in premenopausal women.

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