RESEARCH ARTICLE

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P53 Gene Expression and Nitric Oxide Levels after Artemisinin-Caffeine Treatment in Breast, Lungs and Liver of DMBA-Induced Tumorigenesis

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Abstract

Objective: With increasing incidence of cancers globally and limited resources in some affected countries, repurposing existing drugs for reducing tumorigenesis is highly important. Artemisinin and caffeine have potent anti-oxidative and anti-tumor properties but are therapies for other diseases. This study evaluated the biochemical and p53 gene modulatory effects of doses of artemisinin-caffeine combination on breast, lungs and liver tissues in rats induced with DMBA. **Methods:** After due ethical approval, 30 animals were treated with 40mg/kg single dose of 7,12-dimethylbenzene anthracene (DMBA) as a model for DNA damage and induction of carcinogenesis. Five animals each received normal saline (normal), low dose artemisinin (Art; 4mg/kg), low dose caffeine (Caff; 25mg/kg), low dose combination of caff + art (25+4mg/kg), high dose combination of caff + art (50+8mg/kg) or no treatment (DMBA). All treatment doses were orally administered daily for two weeks post DMBA treatment. Nitric oxide levels and p53 relative gene expression was carried out using primer-specific RT-PCR, GAPDH was used as loading control and amplicons were resolved by gel electrophoresis. **Results:** DMBA induced lesions in breast, liver, and lung tissues evident from histology analysis, compared to normal group. In all 3 tissues, caffeine (25mg/kg) and combination of caff + art (25+4mg/kg) significantly reduced p53 gene expression ($p < 0.05$), but there was significant increase in the group treated with low dose art (4mg/kg) and high dose caff + art, which were similar to DMBA group ($p<0.05$). In lungs, nitric oxide (NO) increased in all groups but not in caffeine, in the liver NO decreased with caffeine or its combination with art, compared to DMBA group. **Conclusions:** This study shows a dose-dependent synergistic anticancer effects of caffeine and artemisinin combination on p53 gene and nitric oxide regulation hence can mitigate tumor development.

Keywords: Cancer prevention- tumor suppressor gene- artemisinin- caffeine- DMBA

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Introduction

Cancer is a major risk factor for mortality worldwide. it is a malignant neoplasm arising from genetic mutations which could be hereditary, caused by environmental agents or errors in DNA replication (Bray et al., 2018). Assessment based on human developmental index on incidence and mortality of 36 cancer types in diverse ethnographical regions is approximately 18.1 and 9.6 million cases respectively and prevailing cancers in males and females include breast (female specific), lung, prostate (male specific), colon and liver (Ferlay et al., 2015; Kastenhuber and Lowe, 2017; Bray et al., 2018; GBD, 2020; Sung et al., 2021). In less developed and underdeveloped nations, mortality rates are on a steady rise compared to highly developed nations with increased incidence rates however less mortality records, and projections in death occurrence in underdeveloped regions may increase to 75% by 2030 (Siegel et al., 2019; Siegel et al., 2020). Several factors have been implicated in the development of cancers which includes frequent exposure of DNA to chemical carcinogens in the environment such as DMBA, which becomes metabolically activated via cytochrome p450 microsomal enzymes in the liver, forming DNA adducts that initiate carcinogenesis (Klaunig and Kamendulis, 2004; Klaunig et al., 2011; Jurel et al., 2014; Klaunig and Wang, 2018).

Tumor suppressor genes code for expression of tumor suppressor proteins such as TP53, APC, BRCA1/2, that inhibit proliferation and apoptosis-promoting proteins, as a response to DNA damage (Chow, 2010). TP53 gene, also known as the "custodian of the genome" due to its fundamental role in maintaining genomic stability in the G1 to S cell cycle checkpoint; mitigates damaged

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cells from proliferating (Ghosh et al., 2022). Signaling alterations in normal cell division induces several cellular responses, p53 becomes activated and dissociates from its de-regulator mouse double minute two (MDM2), and halts cell division after cells exposure to DNA-damaging agents (Shu et al., 2007; Niazi et al., 2018). Activated p53 transcript binds to DNA response elements of various target genes inducing their activation which function by isolating damaged cells for repair or mediating apoptosis (a programmed form of cell death) if repair mechanism is ineffective (Chow, 2010; Reisman et al., 2012). In healthy conditions, p53 expression is low and quickly undergoes proteasome and ubiquitin degradation through MDM2 (Haupt et al., 1997; Toledo and Wahl, 2006; Lukashchuk and Vousden, 2007).

Nitric oxide (NO) is a free radical and signaling molecule that exerts a wide range of biological effects, including regulation of blood flow, neurotransmission, etc (Hirst et al., 2011). In cancer, it exerts both tumor promotion or inhibition depending on the concentration (Fukumura et al., 2006; Mocellin et al., 2001; Ridnour et al., 2007; Burke et al., 2013); NO at higher levels has anticancer properties mediated via pro-apoptotic effects on p53 by its accumulation which mediates growth arrest and apoptosis (Forrester et al., 1996; Ambs et al., 1997; Burke et al., 2013; Hu et al., 2020). NO is regulated to control cancer growth and this has been explored as a lone therapy or combination with anticancer compounds (Mocellin et al., 2001; Ridnour et al., 2007; Huerta, 2015; Hu et al., 2020). Studies have shown that artemisinin upregulates p53 pro-apoptotic and cell proliferative effects in a p53-dependent manner (Mondal and Chatterji, 2015). Further studies that elucidate p53 gene expression after artemisinin combination therapy in mitigating carcinogenesis is beneficial. Cancer chemotherapies are associated with toxicities and side effects that are highly detrimental to patient's health and the search for novel therapies continues to be vital in reducing cancer burden globally.

Natural compounds such as artemisinin has been found to be potential anti-cancer agents (Meshnick, 2002; Crespo-Ortiz and Wei, 2012). Artemisinin is isolated from Artemisia annua has been identified in retrospective studies to have anti-cancer properties via production of carbon-centered free radical in mitigating cancer cells and it is currently a popular anti-malarial drug (Dhingra et al., 1999; Meshnick, 2002; Nakase et al., 2008). Another natural compound; caffeine is a natural alkaloid occurring in over sixty plant parts however predominantly in roasted coffee bean and tea leaves (Barone and Roberts, 1996). It has been reported that caffeine has anti-tumor effects in vivo (Chung et al., 1998; Lou et al., 1999) and enhances cytotoxicity effect of anti-cancer treatment (Tomita and Tsuchiya, 1989; Wang et al., 2015). This study evaluated the synergistic effects of artemisinin and caffeine on p53 gene expression and nitric oxide levels in mitigating DMBA induced carcinogenesis in female Wistar rats.

Materials and Methods

Animals and Experiment process

Experimental animals used were thirty albino Wistar female rats weighing between 100-180 g of about six weeks, obtained from animal laboratory in Federal University of Abeokuta, Nigeria. All the Wistar rats were kept and nurtured at room temperature, and having access to standard food and clean water ad libitum in the Covenant University animal laboratory. Ethical approval was obtained from Covenant University Health Research Ethics Committee (CHREC/46/2020). Animals were allowed to acclimatize for two weeks post purchase and modified method of Lai and Singh (2006) using two doses of DMBA; 40mg/kg and subsequently 20mg/kg after a month of initial DMBA treatment respectively, was used to induce carcinogenesis. Animals were randomly grouped into six (n = 5); animals were given: vehicle (group 1 - normal control), 40mg then 20mg/kg DMBA (group 2 - negative control), DMBA + 25mg/kg Caffeine (group 3), DMBA + 4mg/kg Artemisinin (group 4), DMBA + 25mg/kg Caff + 4mg/kg Art (group 5) and DMBA + 50mg/kg Caff + 8mg/kg Art (group 6). Pure research grade artemisinin, caffeine and DMBA were purchased from Sigma-Aldrich® (United Kingdom). Artemisinin and caffeine stock solutions were prepared as 1mg/ml in water and DMBA was dissolved in olive oil. Drug solutions were administered orally once daily for two weeks. Animals were euthanized and tissues (liver, lungs and breast) were harvested in sterile tubes for hematoxylin and eosin staining for histology analysis and RNA extraction respectively.

Gene expression analysis

From liver, lung and breast tissues, total RNA was extracted using TRIzol™ reagent (Invitrogen, USA) based on manufacturer's instruction. Reverse transcriptase polymerase chain reaction was performed using Easy Script® One-Step cDNA super mix (Trans-Gene®, Strasbourg, France). The cycling condition used for p53 PCR is as follows: 45ºC for 30mins, 94ºC for 30secs, 57.3ºC for 30secs, 72ºC for 30secs and 72ºC for 7mins and steps 2-4 were repeated for 40 cycles (Alsalman et al., 2019) and GAPDH was used as loading control. Primer sequence used for PCR are as shown Table 1.

Nitric Oxide assay

From the tissue homogenate, 100µl of sample was diluted with 150µl of distilled water, 250µl of sodium hydroxide (NaOH) was added and incubated at room temperature for 5minutes. Then, 13µl of zinc sulphate was added and centrifuged at 4,000 rpm for 30 minutes. From the supernatant, 400µl was added to 400µl of Griess reagent. Absorbance was read at 540nm within 30minutes of incubation. Nitrite production was used as indirect measure of nitric oxide following the method of Yucel et al., (2012) calculated as:

$$\text{Nitrite} = [OD_{sample} - OD_{Blank} / \text{slope}] \times n\ (\mu M)$$

where OD = absorbance, slope = slope of standard

curve, n = dilution factor

Statistical analysis

All results are represented as mean ± standard error of mean, and analysed using GraphPad prism statistical software (version 8.4.3) and Excel. Intensities of the gel bands were quantified and means were compared using one-way ANOVA followed by Turkey's test. Statistically significant values are considered at $p < 0.05$.

Results

p53 gene expression

The results of other antioxidant parameters have been previously reported (Dokunmu et al., 2021). The histological and molecular mechanism of tumor prevention in breast, liver and lungs of DMBA-treated rats was determined by checking modulative effects of artemisinin and caffeine as combined or single oral doses on p53 gene expression relative to GapDH in all groups. Figure 1A-D shows the photomicrographs of the liver architecture after treatment in the different groups. Combination of caff+art (25 mg+4 mg/kg) synergistically showed the most positive modulatory effects with no lesion or tissue damage compared to DMBA and Art groups. The relative fold change compared to GapDH are shown in Figures 2 – 4, caff+art (25mg+4mg/kg) reduced p53 expression to 0.69 in breast (Figure 2a-b), 0.84 in liver (Figure 3a-b) and 0.69 in lungs (Figure 4a-b) tissues, respectively This was similar to levels in normal group (0.88, 0.32 and 0.48, respectively) and significantly lower than the corresponding DMBA group with p53 expression levels of 2.75, 5.35, 2.84 in breast, liver and lungs respectively, $p < 0.05$.

Nitric oxide levels

Nitric oxide level was determined in the liver and lung tissues only. Figure 5 shows the nitric oxide levels across the 6 treatment groups. Mean NO levels in the liver was slightly lower in artemisinin (54.4 µM), caffeine-artemisinin [25+4 mg/kg] (40.5 µM) and caffeine-artemisinin [50+8 mg/kg] (38.5 µM) groups,

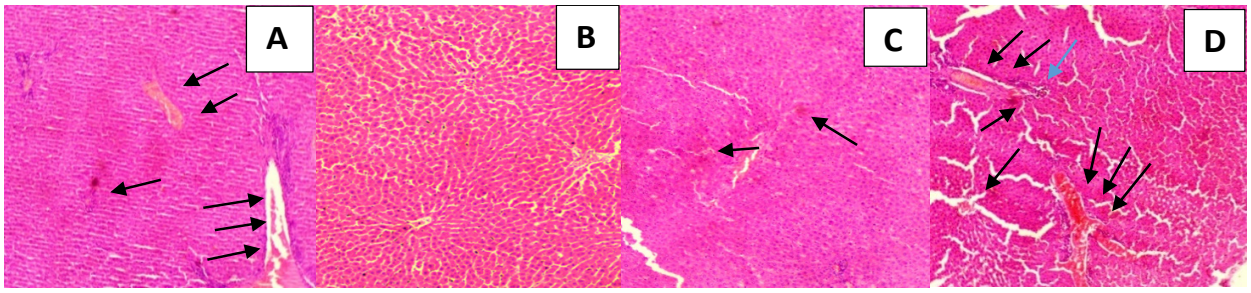

Figure 1. Photomicrograph of the Liver after Treatment with Artemisinin and Caffeine. A: DMBA Negative control group – histologic section of liver showing general structure, Central vein (CV), portal vein (PV) and the basophilic portion with nucleus and the acidophilic cytoplasm of the acinar cells. Vascular congestion of blood vessel seen; B: Normal control group histologic section of liver showing general structure, Central vein (CV), portal vein (PV) and the basophilic portion with nucleus and the acidophilic cytoplasm of the acinar cells. No abnormalities are seen; C: Caffeine (25mg/kg) showing histologic section of liver showing general structure, Central vein (CV), portal vein (PV) and the basophilic portion with nucleus and the acidophilic cytoplasm of the acinar cells. No abnormalities are seen; D: Histologic section of liver showing general structure, Central vein (CV), portal vein (PV) and the basophilic portion with nucleus and the acidophilic cytoplasm of the acinar cells. Vascular congested blood vessel is seen.

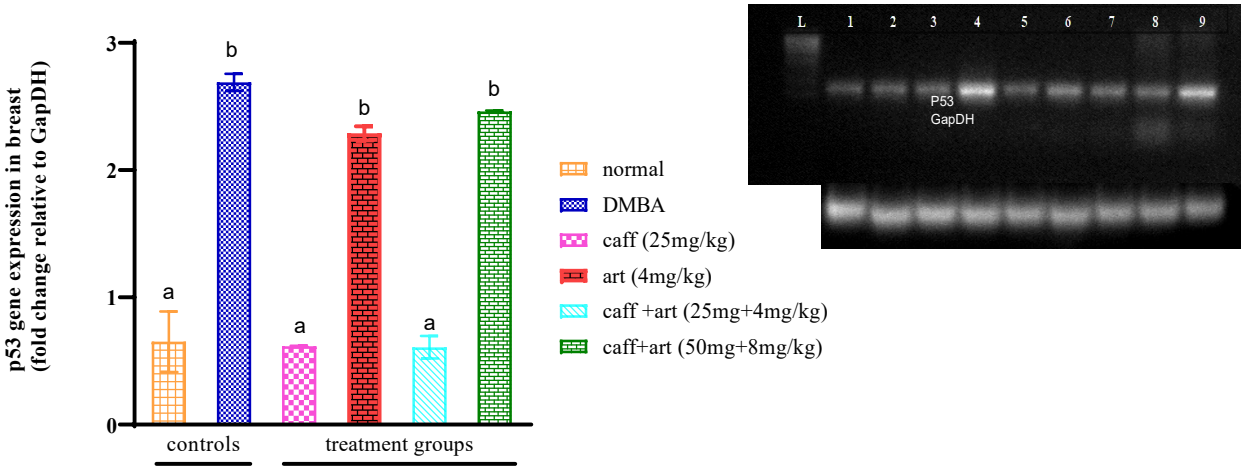

Figure 2. Relative Fold Change in p53 Gene Expression in Breast Tissue. Gel image shows caffeine [lane 3] and caffeine-artemisinin (25+4mg/kg) [lane 5] significantly reduced p53 expression similar to normal [lanes 1-2], compared to DMBA [lanes 4,6,9] and higher dose combination [7] or artemisinin alone [8].

Table 1. Primer Sequence for Selected Genes

Primer	Sequence	Direction
TP53 A	5-AACAATGGCCCAGTCTAATGGGA-3	Forward
TP53 B	5-ACAGATGTTGCCTGATGTCTGGGA-3	Reverse
GAPDH-A	5-AGTGCCAGCCTCGTCTCATA-3	Forward
GAPDH-B	5-GATGGTGATGGGTTTCCCGT-3	Reverse

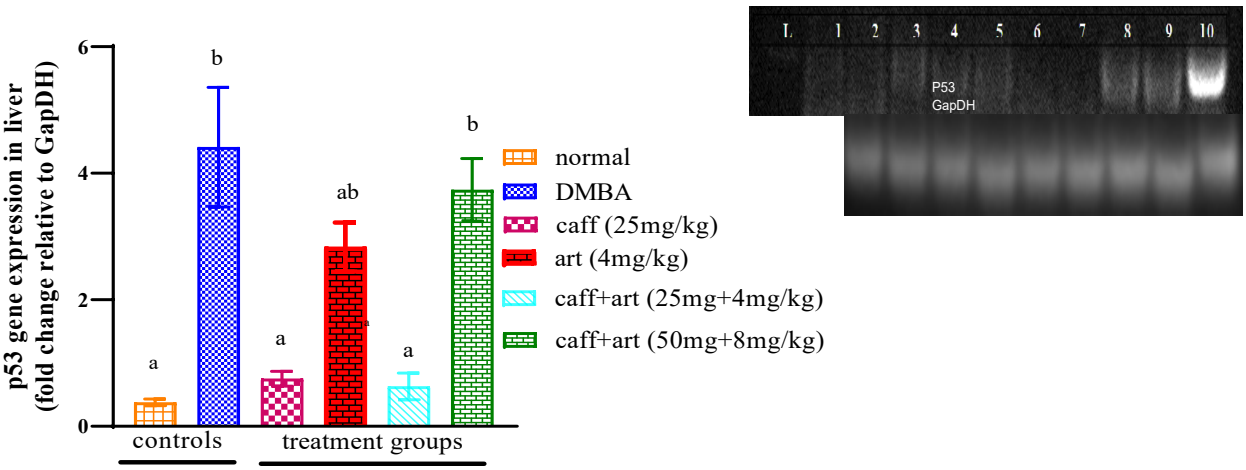

Figure 3. Relative Fold Change in p53 Gene Expression in Liver Tissue. Gel image shows caffeine-artemisinin (25+4mg/kg) significantly reduced p53 expression [gel lanes 1-4,6-7] than higher doses [lane 5,8], or DMBA group [gel lanes 9,10]

respectively compared to other groups. In the lungs, nitric oxide levels was 2-2.5 times significantly higher than levels in the liver ($p < 0.05$). Paradoxically NO levels reduced slightly in caffeine [25mg/kg] (110 µM) but higher in artemisinin group (190 µM).

Discussion

As cancer incidence increases globally (Sung et al., 2021), efforts towards reducing the incidence of cancers are important, however there are few preventive strategies for mitigating against tumorigenesis or reducing the growth of existing tumors. While new therapies are being developed, the duration from development to deployment as well as affordability still remains a major challenge. Repurposing existing drugs for the management or prevention of cancers has been in existence for long; whereby existing drugs for other diseases are adapted in cancer treatment for their cytotoxicity effects or combined to enhance anti-cancer drug effects (Li et al., 2016; Berns et al., 2020; Schein, 2021). Several genes have been implicated in the development of cancer malignancies, but p53 gene is one of the tumor suppressor genes that respond to endogenous or exogenous distress signals

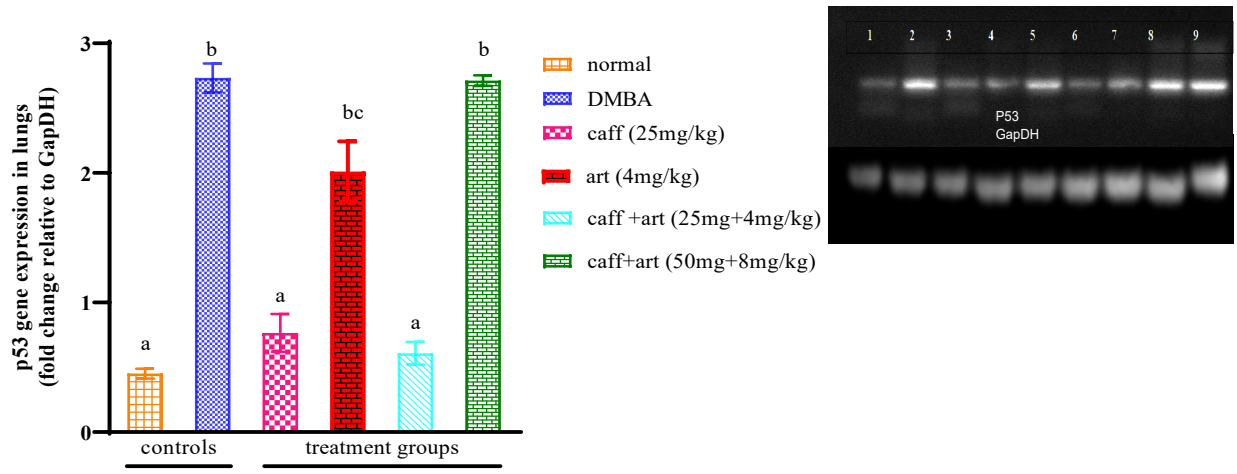

Figure 4. Relative Fold Change in p53 Gene Expression in Lung Tissue. Gel image shows caffeine alone [gel lane 6] and caffeine-artemisinin (25+4mg/kg) [3,4] similar to normal [1], significantly reduced p53 expression than higher combination doses [5], or DMBA [2,8-9].

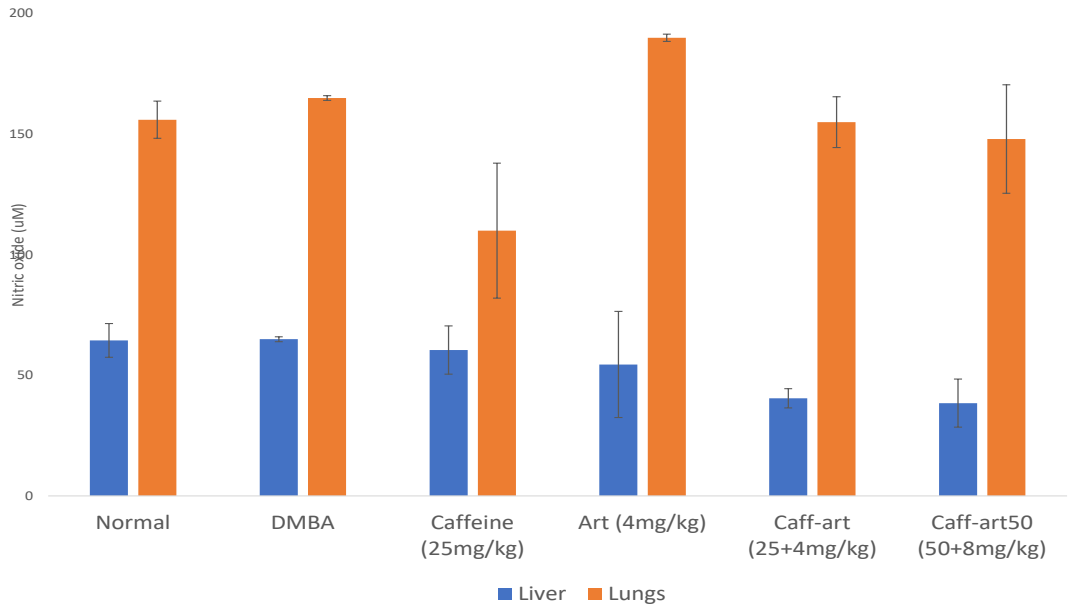

Figure 5. Nitric Oxide Levels in Liver and Lungs Following Treatment with Caffeine, Artemisinin or caffeine+artemisinin Combinations

that disrupts the stability and homeostasis of the genome. After sensing DNA damage, p53 is activated whereby it induces a number of cellular responses such as halting the cell division at the G1/S phase checkpoint of the cell cycle to promote either the elimination (programmed form of cell death) or repair of damaged cells (damaged genome is safely isolated) ultimately reducing their risk of propagating mutations (Surget et al., 2014; Kastenhuber and Lowe, 2017).

p53 is one of the highly mutated genes in human cancers, the mutant p53's structures and functions are altered from being a proto-oncogene to an oncogene (Ghosh et al., 2022). Mutant p53 interacts with transcriptional factors or alters pathways such as SMAD/TGF-β signalling pathway, leading to uncontrolled cell proliferation and metastasis (Miah et al., 2019). Mutations, overexpression or non-functional alterations occur in p53 gene in over 50% cancers, and are associated with different cancers (Perri et al., 2016). Howbeit, functional p53 expression is fundamental to its remarkable anti-tumour characteristics (Chow, 2010; Niazi et al., 2018; Shu et al., 2007). On the other hand, nitric oxide is signaling molecule that plays a dual role as a pro-apoptotic and tumor promotion molecule by modulating accumulation of p53 which mediates growth arrest (Forrester et al., 1996; Ambs et al., 1997; Burke et al., 2013; Hu et al., 2020). Our study reports the combined anti-tumor effects of artemisinin and caffeine on breast, liver and lungs tissues via modulation of p53 and nitric oxide pathways. Their lone anti-cancer effects are well documented however the synergistic and biochemical mechanisms behind their combined activities are unclear and at what doses at these occurs.

From this study, results on breast, liver and lung p53 gene expression revealed a marked reduced expression of p53 mRNA post treatment in groups treated with caffeine (25 mg/kg) or caffeine + artemisinin (25+4mg/kg), and a paradoxical marked upregulation of p53 mRNA in groups treated with artemisinin alone (4 mg/kg) or higher dose of caffeine + artemisinin (50+8 mg/kg), which was similar to negative control group. In the DMBA negative control group, which were not treated with artemisinin or caffeine, p53 gene was upregulated (about 4-5 folds) compared to low dose caffeine + artemisinin, caffeine alone or normal control, in all tissues. Possible mechanism for the protective effects of artemisinin is that artemisinin selectively kills pre-cancerous cells, by reacting with iron to form free radicals that generate ROS and oxidative stress that kill cells. However, it is puzzling that artemisinin alone did not show significant protective effects on DMBA carcinogenesis in the tissues. In our study, tumor development was inhibited, other studies reported action on developed tumors; which have high intracellular iron concentration (Meshnick, 2002; Hamacher-Brady et al., 2011; Li et al., 2016), which is important for the anticancer activity of artemisinin.

Other mechanisms of anticancer activity of artemisinin and its derivatives include induction of apoptosis (Singh and Lai, 2004; Mercer et al., 2007; Slezakova and Ruda-Kucerova, 2017), inhibition of angiogenesis (Verma et al, 2017), arrest of cell cycle at G0/G1 (Tran et al, 2014) and ferroptosis; a specific form of programmed cell death caused by the iron-dependent accumulation of lipid reactive oxygen species and depletion of plasma membrane polyunsaturated fatty acids in cell lines or tissues (Lin et al., 2016). Caffeine is a major component in some common drinks which includes coffee and tea and several studies have revealed that caffeine contributes to the chemo-preventive effect of caffeinated tea (He et al., 2003). Several natural bioactive compounds possess anticarcinogenic effects in vivo and in vitro e.g. caffeine (Tomita and Tsuchiya, 1989; Lou et al., 1999; Wang et al., 2015). At low dosage (16.5–450 μM), Caffeine

induced apoptotic effect in JB6 Cl41 cells (He et al., 2003; She et al., 2001) synergistic chemotherapeutic effect with cisplatin, inducing apoptosis in lung cancer cell lines - HTB182 and CRL5985 (Wang et al., 2015) and in animal models (Chung et al., 1998; Higuchi et al., 2018; Lou et al., 1999; VanderPloeg et al., 1991). Caffeine augments p53 apoptotic activity by aiding it phosphorylation at the serine amino acid terminal which in turn increases its stability, concentration and transcriptional activity to its target gene such as BAX and caspases (Wang et al., 2015).

Nitric oxide is a short-lived free radical that plays an important role as a signaling molecule in the body. Nitric oxide radical (●NO) is produced from L-arginine by many cell types by endothelial nitric oxide synthase in the vasculature (Davis et al., 2001; Thomas, 2015). NO is considered a vasculo-protective molecule and its regulation has been reported as a potential cancer therapy (Forrester et al., 1996; Ambs et al., 1997; Burke et al., 2013; Hu et al., 2020). Nitric oxide mediates endothelium-dependent vaso-relaxation by its action on guanylate cyclase in the vascular smooth muscle cells, and also displays antiproliferative properties and inhibits platelet and leukocyte adhesion to vascular endothelium (Davis et al., 2001; Thomas, 2015). Nitric oxide levels in the liver were significantly lower than levels in the lungs, we could not measure nitric oxide levels in breast tissues. The levels in the liver were lower in artemisinin, caffeine-artemisinin (25+4 mg/kg) and caffeine-artemisinin (50+8mg/kg) groups compared to other groups. In the lungs, paradoxically, nitric oxide levels reduced significantly in caffeine (25 mg/kg) group only, endothelial NO• is an effector of caffeine on smooth muscle targets, and NO• availability can alter caffeine activity (Chang et al., 2016).

High levels of NOS (nitric oxide synthase) expression and NO cause cytostatic or cytotoxic effects in tumor cells, however lower levels promote tumor growth by increasing angiogenesis via upregulation of vascular endothelial growth factor (Forrester et al., 1996; Ambs et al., 1997; Xu et al., 2002; Burke et al., 2013; Hu et al., 2020). Our results suggest tissue specific anti-oxidant activity on nitric oxide levels (Adelani et al., 2021), and variability of levels in the tissues, higher levels of nitric oxide were detected in the artemisinin alone group compared to caffeine group but a combination of caffeine and artemisinin showed a slightly reduced level hence indicating a synergistic effect of the combination of both compounds. Studies have reported that accumulation of p53 through a negative feed-back mechanism results in down-regulation of inducible NOS (Ambs et al., 1998); which could explain much lower NO levels in the liver of groups treated with higher doses of caffeine+artemisinin combinations which had higher levels of p53 expression.

In cancer, nitric oxide has been shown to inhibit or promote tumorigenesis depending on the levels (Xu et al., 2002), and site of action where it can promote aggression such as in lung cancer (Colakogullari et al., 2006; Choudhari et al., 2013; Luanpitpong and Chanvorachote, 2015). Reactive nitrogen species radicals like nitric oxide (•NO) and nitrogen dioxide (•NO2-) can react with superoxide to generate highly reactive molecule ONOO-, triggering a cascade of harmful events (Davis et al., 2001; Thomas, 2015). Therefore, its chemical environment, i.e. presence of •O2-, determines whether •NO exerts protective or harmful effects. Other observations from this study showed hepatocyte lesion, congested hepato-portal vascular system associated with infiltrations of leucocytes and pre-localized necrosis of hepatic cells in DMBA and high dose caffeine-artemisinin (50+8 mg/kg) group, this finding is similar to other studies (Ali et al., 2013; Dakrory et al., 2015). The induced hepatic damage also resulted in elevated liver function enzymes previously reported (Dokunmu et al., 2021), by causing leakage of liver enzymes into blood circulation (Dakrory et al., 2015). Renal inflammation was also observed suggesting reno-toxicity. The overall results showed that caffeine or plus artemisinin was able to prevent the initiation process of DMBA's carcinogenesis by minimizing the damages caused by the carcinogen.

We conclude that animals treated with low dose caffeine alone at (25 mg/kg) or combined treatment of artemisinin-caffeine (4 mg/kg+25 mg/kg) showed similar histoarchitectural result, and revealed that nitric oxide and p53 pathways were modulated by caffeine-artemisinin treatment in a synergistic manner. Conceivably, based on these mechanisms, consumption of caffeine and artemisinin beverages can benefit cancer patients with a potency to delay tumorigenesis and this should be tested for further studies.

Author Contribution Statement

TMD led the design and supervision of the research, TMD and NAI wrote the manuscript, SCO, OUA, DOE, IBA participated in the conduct and analysis, all authors read and approve the final manuscript. The data for this study is contained in the manuscript.

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