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Food and medicinal plants from Nigeria with anti-*Helicobacter pylori* activities induce apoptosis in colon and gastric cancer cell lines

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ABSTRACT

Background: Food and medicinal plants are used traditionally in Nigeria to treat gastrointestinal (GI) disorders such as gastritis, peptic ulcer disease and GI-related cancers. We have previously reported that specific extracts of Nigerian food and medicinal plants inhibited the growth of *Helicobacter pylori,* a bacterium known to cause peptic ulcer disease, as well as gastric and colon cancer.

Objective: To determine the effects of three food plants, namely *Anogeissus leiocarpus* (DC.) Guill. & Perr. (African birch, Combretaceae), *Terminalia glaucescens* Planch ex Benth. (Nigerian chewing sticks, Combretaceae) and *Dillenia indica* L. (Elephant apple, Dilleniaceae) used in Nigeria for the treatment of GI disorders and cancer in six colon and gastric cancer cell lines, and two non-cancerous cell lines.

Methods: Cancer cells were grown in appropriate media and CellTiter-Glo® 2.0 and ApoTox-Glo™ Triplex assays were used to measure cell growth and apoptosis in SW480, SW620, HCT116, Caco2 cell lines, as well as AGS and NCI-N87 gastric cancer cells. Caspase-Glo® 3/7, and Caspase-Glo® 8 were used to determine caspase activities and apoptosis. Gene expression was measured using quantitative PCR.

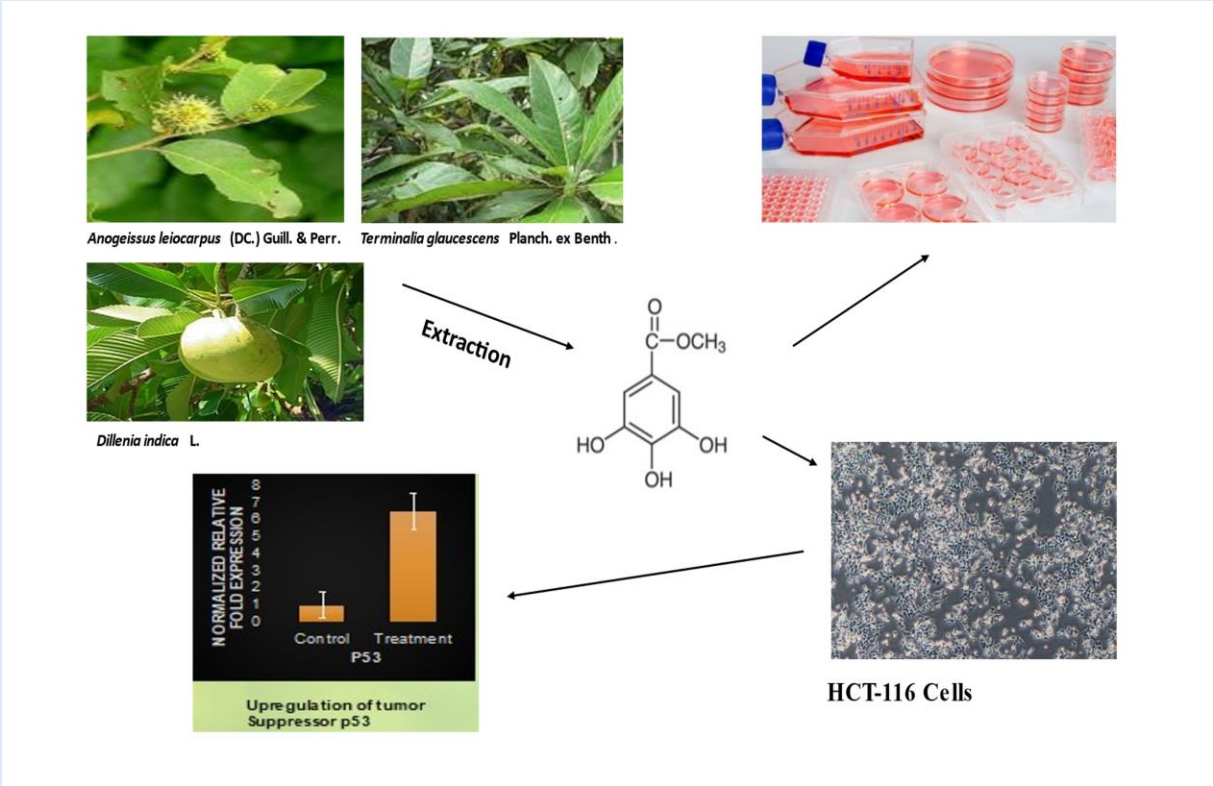
Results: The methanol extract of *A. leiocarpus* roots inhibited the growth of HCT-116, SW480 and SW620 colon cancer cells (IC_{50} of 15.8, 10.0, and 20.8 µg/mL, respectively), but weakly active in the AGS and NCI-N87 cells (IC_{50} 77.0 and >100

µg/mL respectively). The *T. glaucescens* extract was weakly active in HCT-116 cells (IC_{50} 64.9 µg/ml) and AGS cells (52.2 µg/ml). The *D. indica* extract was active in SW480 (IC_{50} 15.8 µg/mL), and weakly active in Caco2 (IC_{50} 35.2 µg/mL) and HCT-116 (IC_{50} 53.2 µg/mL). In HCT-116 cells, *A. leiocarpus* root extract and its aqueous partition increased caspase 8 activity, as well as mRNA expression of p53, while expression of Bcl-2 and HDAC1 mRNA was reduced. Bioassay guided fractionation of the aqueous partition led to the isolation and identification of the known compound methyl gallate.

Conclusions: These data suggest that the Nigerian food plants with anti-*Helicobacter pylori* activities increase the expression of the tumor suppressor p53 and inhibit HDAC1, thereby inducing apoptosis in HCT-116 colon cancer cells.

Running Title: Inhibitory Effects of Nigerian food plants on GIT Cancers

Keywords: African birch, AGS, Apoptosis, *Anogeissus leiocarpus, Dillenia indica*, Elephant apple*, Terminalia glaucescens*, Nigerian chewing sticks, HCT-116, SW480, SW620



INTRODUCTION

The bacterium *Helicobacter pylori* (*Hp*) is associated with gastritis ,peptic ulcer disease (PUD), and over time may lead to the development of mucosa-associated lymphoid tissue gastric lymphomas and gastrointestinal tract (GIT) cancers [1-4]. Treatment of PUD and prevention of *Hp*-induced GIT depends on the eradication of *Hp.* In Nigeria and the United States, colorectal cancers (CRC) cause significant morbidity and mortality, with > 50% of CRC patients dying due to the disease. In patients with metastatic CRC, only 10% will survive for 5 years or longer [5]. When detected early, surgical resection in

combination with adjuvant radiation and chemotherapy may be curative. Treatment with adjuvant chemotherapy has been shown to reduce the relapse rate by 40% in patients with stage III colorectal cancer [6]. Globally, gastric cancer (GC) also remains a problem and metastatic GC remains difficult to cure, with poor outcomes and a < 10% five-year survival rate [7-9]. One review of gastric cancer in Nigeria reported an 80-85% seroprevalence of *Hp* infections in healthy individuals, and most gastric cancers were associated with *Hp* infections [10-12]. New treatment options are needed for both gastric and colon cancers, including nutritional and chemopreventative agents.

Despite the widespread use of use of Western medicines for the treatment of GI disorders and cancers, food and medicinal plant extracts are still used by ~70% of Nigerians as treatment [13, 14]. Previously, we have reported the anti-*Hp* activities of food and medicinal plant extracts from countries around the world including in Nigeria, Thailand, and the USA [15-26]. In Nigeria, plants with inhibitory effects against *Hp* included *Abelmoschus esculentus* L. Moench (okra) [25], *Allium ascalonicum* L. (shallots) [19], *Myristica fragrans* Houtt. (nutmeg) [26] and *Theobroma cacao* L. (cocoa) [20], *Anogeissus leiocarpus* and *Terminalia glaucescens* (Nigerian chewing sticks) [21]. *Anogeissus leiocarpus* (DC.) Guill. & Perr. (African birch) of the family Combretaceae is found mainly in Africa, South Asia and the Arabian Peninsula [27]. It is used locally in the treatment of many diseases including diabetic ulcers, pulmonary disorder, and malaria; as well as infectious diseases such as infected wounds and ulcers, tuberculosis, and pneumonia. The plant's root is known for its antibacterial effect in gum infection, and as such it is used as chewing stick [28-30]. *Terminalia glaucescens* Planch. ex Benth., is a large tree belonging to the family Combretaceae and is found in the Tropics. The plant's parts such as leaves, stem and root are used traditionally in various countries for the treatment of oral infections, dysentery, skin infections, as well as diabetes and cancer. Phytochemical constituents including flavonoids, cyclic triterpenes, tannins isolated from trees of this genus are known to possess antimicrobial, anti-cancer and hepatoprotective properties [31-33]. *Dillenia indica* L. (Elephant's apple, family Dilleniaceae), a species of Dillenia found in the Tropics including Nigeria and tropical Asia has been reportedly used in traditional medicine. The fruit juice is used as a cardiotonic and for the relief of fever, abdominal pain, and cough while the mixed juice of the leaves, fruit and bark, is used for treatment of diarrhea and cancer [34-35].

In this work, we tested specific Nigerian food and medicinal plant extracts with significant anti-*Hp* activities, namely *A. leiocarpus*, *T. glaucescens* and *Dillenia indica* L. (Elephant apple, Dilleniaceae) to determine their effects on colon and gastric cancer cell lines.

MATERIALS AND METHODS

Plant Collection and Extract Preparation: The food and medicinal plants *Anogeissus leiocarpus*, *Terminalia glaucescens* and *Dillenia indica* were collected and identified previously as we have described [21]. The Department of Botany at the University of Ibadan identified the Elephant apple (*Dillenia indica*) and a voucher specimen (UIH-22427) was deposited in the herbarium. Dried and pulverized plant samples were extracted to exhaustion in methanol at room temperature, and the extracts were filtered, combined, and dried in vacuo. Dried extracts were refrigerated at 4^{o}C until further use. Fifty grams (50 g) of the methanol extract were dissolved in 95:5 (water: methanol), defatted, and partitioned using organic solvent and water. Each partition was air-dried and stored at 4^{o}C. Methanol (MeOH), dichloromethane (DCM), ethyl acetate (EtOAc) and water (Aq) partitions were dissolved in DMSO (0.02% final concentration) for concentrations ranging from 5 - 100 µg/mL. The water partition of

methanol root extract of *A. leiocarpus* showed the most significant activity in the cell lines and was further followed up using bioassay guided fractionation.

Isolation and identification of the bioactive compounds from Anogeissus leiocarpus: The water partition of *A. leiocarpus* was separated on a Sephadex LH-20 column (60 x 1000 mL) using methanol: water to obtain fractions F1 – F6 as we have described [21]. Fractions 5 and 6 were the most active and were pooled as well as separated using reverse phase C18 silica gel, resulting in tested in SW480 and HCT116 cells. Fraction F5b (10% methanol fraction, 1.79 g) was further separated by loading 1.70 g of fraction unto HILIC (Bulk HILIC flash, 50 µm, Agela Technologies, USA) column (10 x 250 mm) and eluted with methanol: water starting from 100% methanol to yield F5bi - x. Sub-fraction F5bvi (43 mg, 50% Methanol HILIC fraction) being the most active was solubilized in water and ethanol. The water-soluble portion was extracted with four aliquots (4 x 200 µL) of ethyl acetate, combined and air-dried. The dried compound was crystallized and recrystallized from ethyl acetate with dichloromethane. A white crystalline compound (5 mg) was obtained and determined to be the known compound methyl gallate using NMR and X-ray crystallography [36].

Cancer cell growth and treatment: The AGS and NCI-N87 gastric cancer and SW480, SW 620, HCT116 and Caco-2 colon cancer cells, normal comparison control cells, rat L6 muscle cells, and hFOB human osteoblasts were grown and maintained according to American Type Cell Culture guidelines. The AGS cells were grown and maintained as we have previously described [37] [Mahady *et al*., 2019]. The SW620 (CCL-227) cells were cultured in L-15 (30-2008) + 10% FBS + 1% P/S incubated in 100% air at 37°C. The HCT 116 colon cancer cells were cultured. [38].

CellTiter-Glo$^{2.0}$ cell proliferation assay: The cell viability assay CellTiter-Glo$^{2.0}$ was performed as described by the manufacturer and as we have previously described [38]. Cells were seeded in opaque-walled 96-well plates in triplicate at 2.5 x 10^4 cells in 100 µL/well and left overnight to attach. For treatment, fresh media was added to each well, then MeOH extracts, or DCM, EtOAc or Aq partitions at concentrations of 5-100 µg/mL. 5-Fluorouracil (positive control) and negative control (0.02% DMSO) wells were also prepared. SW620 cells were incubated in 100% air at 37^{o}C for 72 hours. At the end of the incubation period, 100 µL of CellTiter-Glo$^{2.0}$ Reagent (Promega Corporation, Madison, WI, USA) was added to each well and luminescence was determined using a Synergy HT Plate reader (Biotek, Winooski, VT) and Gen5 1.11 software. The median inhibitory concentration (IC_{50}) was calculated after 72 hours of treatment using GraphPad Prism 9.0 as described prior. [38].

Caspase activities: The activities of caspase 3/7 and 8 were measured in cultured colon and gastric cancer cells after treatment with the Aq partition of *A. leiocarpus* at the IC_{50} concentrations. The details of the Caspase-Glo® 3/7 and Caspase-Glo®8 assays (Promega Corporation, Madison, WI, USA) were as we have previously described [38]. Luminescence was measured on a Synergy HT Plate reader (Biotek, Winooski, VT, USA) using the Gen5 1.11 software. The ApoToxGlo™ Triplex Assay was performed as we have previously described [38]. Briefly, HCT-116 cells were seeded in 96 well plates and then treated with the *A. leiocarpus* aqueous partition at the IC_{50} concentration or vehicle controls. After incubation, the assay reagents were added and luminescence was determined on a Synergy HT Plate reader (Biotek, Winooski, VT) and Gen5 1.11 software to detect caspase activation.

RNA extraction and qPCR: Total RNA was isolated from cells treated with *A. leiocarpus*-Aq partition at the IC_{50} concentration using Trizol (ThermoFisher Scientific,

Waltham, MA, USA) using the methods that have been previously described [38]. Quantitative polymerase chain reaction (qPCR) was performed using a Power SYBR Green RNA- to-C_T 1- step kit (Applied Biosystems, Foster City, CA, USA) as described on a Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using primer sequences described below [38]. PCR cycling conditions were as we have previously described [38]: 48°C for 30 min, 95°C for 10 min, followed by 50 cycles of 95°C for 15 sec. and 60°C for 1 min. Quantitation of gene expression was performed using the $^{\Delta\Delta}$CT calculation.

Table 1: Sequences of primer used in real-time PCR analysis

Gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
Bcl-2[1]	CGCATCAGGAAGGCTAGAGT	AGCTTCCAGACATTCGGAGA
Bax[1]	TGCCAGCAAACTGGTGCTCA	GCACTCCCGCCACAAAGATG
β-actin[2]	TGACGTGGACATCCGCAAAG	CTGGAAGGTGGACAGCGAGG
HDAC1[3]	CTACTACGACGGGGATGTTGG	GAGTCATGCGGATTCGGTGAG
HDAC3	CTACTACGACGGGGATGTTGG	GAGTCATGCGGATTCGGTGAG
SIRT1	TGCTGGCCTAATAGAGTGGCA	CTCAGCGCCATGGAAAATGT
p53	AAGTCTGTGACTTGCACGTACTCC	GTCATGTGCTGTGACTGCTTGRTAG

[1]BCl2 and BAX primer: https://www.ncbi.nlm.nih.gov/pubmed/23347444
[2]B actin primer: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0117058
[3]HDAC1 primer: https://www.ncbi.nlm.nih.gov/pubmed/25500546

Statistics: The statistics were analyzed using GraphPad/Prism version 9.0 (GraphPad Software, Inc. La Jolla, CA, USA). One-way ANOVA with Tukey's multiple comparison tests were used to analyze the PCR data, with $p<0.05$ being statistically significant. A $p > 0.05$ was considered statistically significant.

RESULTS

Median inhibitory concentrations of plant extracts:

Treatment with *A. leiocarpus* root (ALR), *A. leiocarpus* stembark (ALS), *T. glaucescens* and *Dillenia indica* leaf (DIL), *D. indica* stembark (DIS) extracts, partitions and fractions showed that the methanol extracts of ALR and ALS (Table 2), and ALR-Aq were the most active, against all cell lines tested (Table 3), IC_{50} of 14.6-76 µg/ml. ALR-Aq treatments reduced the viability of HCT-116 cells (IC_{50} of 14.6 µg/ml), while the methanol extract of *D. indica* stembark was active in SW480 (IC_{50} 15.8 µg/ml), Caco2 (IC_{50} 35.23 µg/ml) and HCT-116 (IC_{50} 53.2 µg/ml) (Table 2). Only the ALR methanol extract was active against the resistant SW620 colon cancer cell line (Table 2). Since chemotherapy may have deleterious effects on muscle and bone, we tested the extracts in the normal cell lines: L6 rat muscle cells and hFOB human osteoblasts. None of the extracts tested reduced the viability of these normal cells in concentrations >100 µg/ml, indicating there were no effects on normal muscle or bone cells (data not shown).

Table 2: Median inhibitory concentrations (IC_{50}) of methanol extracts of *Anogeissus leiocarpus, Dillenia indica* and *Terminalia glaucescens* in colon and gastric cancer cells

Plant/part	Extract	Cell lines and IC_{50} (µg/mL)					
		SW480	**SW620**	**Caco2**	**HCT116**	**AGS**	**NCI-N87**
Anogeissus leiocarpus, stembark	MeOH Ext	21.82	ND	>100	48.31	ND	ND
Anogeissus leiocarpus, root	MeOH Ext	15.8	10.0	>100	20.8	77.13	>100
Dillenia indica, leaves	MeOH Ext	>100	ND	>100	>100	ND	ND
Dillenia indica, stembark	MeOH Ext	15.8	ND	35.23	53.29	>100	>100
Terminalia glaucescens	MeOH Ext	>100	ND	>100	64.94	52.25	>100
5FU		3.42	13.0	14.79	0.98	18.64	0.09

Note: Cell viability was determined using the CellTiter-Glo2.0 Reagent (Promega Corporation, Madison, WI, USA). The IC_{50} was calculated using log (inhibitor) vs. normalized response analysis with GraphPad Prism 9.0. (GraphPad Software, Inc. La Jolla, CA, USA). ALS - *Anogeissus leiocarpus* stem bark; ALR - *Anogeissus leiocarpus* root; DIL - *Dillenia indica* leaf; DIS - *Dillenia indica* stem bark; TG – *Terminalia glaucescens*; MeOH – Methanol extract; 5FU – 5–Fluorouracil; ND – Not Determined.

Since the ALRAq partition (water soluble) was the most active in HCT-116 cells, it was used for further assay follow-up and bioassay-guided fractionation on a Sephadex column (Table 3). Fractions 5 and 6 were the most active against HCT-116 colon cancer cells (Table 4).

Table 3. IC_{50} of the *Anogeissus leiocarpus* root methanol extract, partitions and fraction

Cell line	***Activities of Anogeissus leiocarpus*** **root (ALR) extract and partitions (IC_{50} µg/mL) compared with 5-fluoruracil**						
	MeOH Extract	**DCM partition**	**EtOAC partition**	**DCM partition**	**Aqueous partition (ALRAq)**	**5FU**	Doxorubicin
SW480	15.8	15.14	58.51	>100	15.0	3.42	ND
HCT116	20.8	16.48	45.82	>100	14.6	0.98	ND
Caco2	>100	53.08	63.83	>100	37	14.2	ND
AGS	77.13	85.76	>100	>100	60	18.64	0.079
NCI-N87	>100	>100	>100	>100	76	0.097	0.048

Note: MeOH – Methanol extract; EtOAc – Ethyl acetate partition; DCM – Dichloromethane partition; Aq – Aqueous partition; 5FU – 5–Flourouracil

Table 4. Median inhibitory concentrations of the aqueous partition from the *Anogeissus leiocarpus* root (ALRAq) methanol extract

Cell line	IC_{50} (µg/mL) activities of the fractions from the *Anogeissus leiocarpus* root Aqueous (ALRAq) partition					
	F1-100% H2O	**F2-20% MeOH**	**F3-40% MeOH**	**F4-60% MeOH**	**F5-80% MeOH**	**F6-100% MeOH**
SW480	>100	>100	45.64	26.27	46.72	46.84
HCT116	>100	69.48	36.89	19.26	15.41	14.27
Caco2	>100	>100	>100	91.11	>100	>100
AGS	>100	>100	>100	87.54	87.87	>100
NCI-N87	>100	>100	>100	95.57	12.39	60.15

Note: The *A. leiocarpus* aqueous partition was loaded unto Sephadex LH-20 column (60 x 1000 mL) and eluted with a stepwise gradient of water: methanol to obtain fractions F1 – F6. Fractions were air-dried and screened for activity in cancer cell lines.

ALRAq activated caspase 8, reduced viability and increased cytotoxicity in HCT-116 and SW480 colon cancer cells:

Increased caspase 8 activity in HCT-116 cells was observed at 2 hrs. after treatment (Figure 1), while SW480 showed elevated caspase 8 levels at 18 hrs. post-treatment with ALR-Aq at the IC_{50} concentration (15.0 µg/mL). No effects were observed on the activation of caspase 3/7 in the same cells.

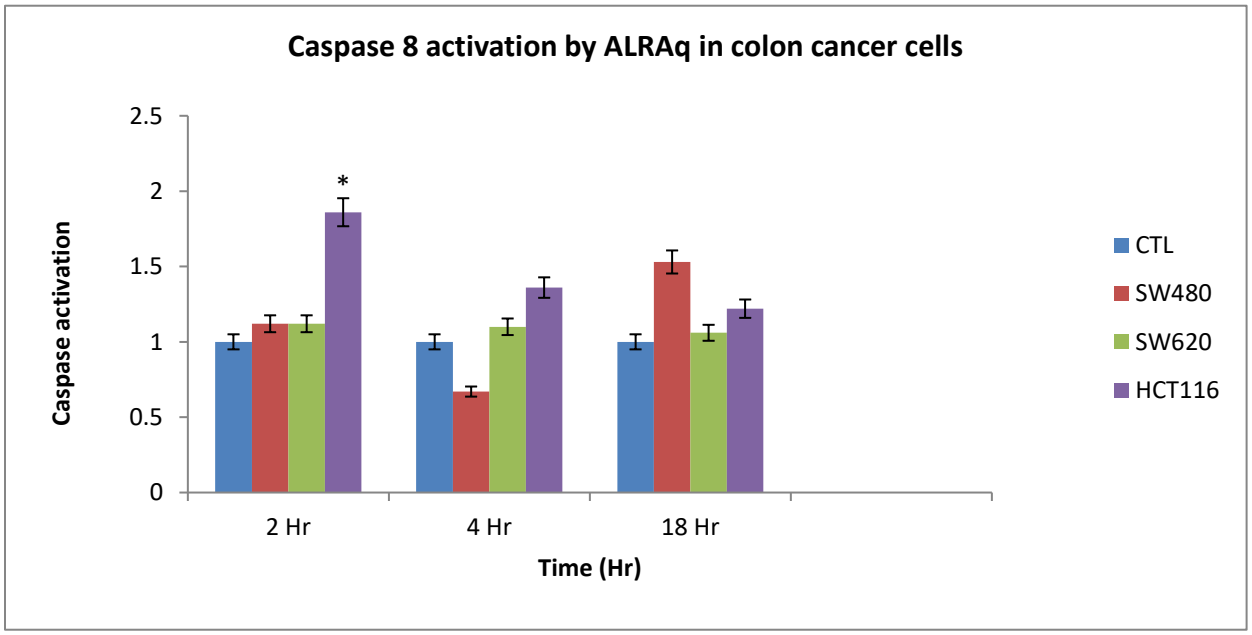


Figure 1: Caspase 8 was activated in SW480, SW620 and HCT116 cells treated with 15 – 17 µg/mL of ALR-Aq. The fluorescent Caspase-Glo® 8 assay was used to measure caspase 8 induction. The induction of caspase 8 activity in HCT-116 cells by ALR-Aq was statistically significant *$p<0.05$ at two hours.

SW480 cells treated with ALRAq at the IC_{50} concentration, showed an increase in cytotoxicity as measured by the ApoToxGlo assay that corresponded with a reduction in cell viability and caspase 8 activation over 72 hours (Figure 2). Cell viability was reduced at 18 hours and cytotoxicity increased between 12-24 hours corresponding with the increase in caspase 8 activity.

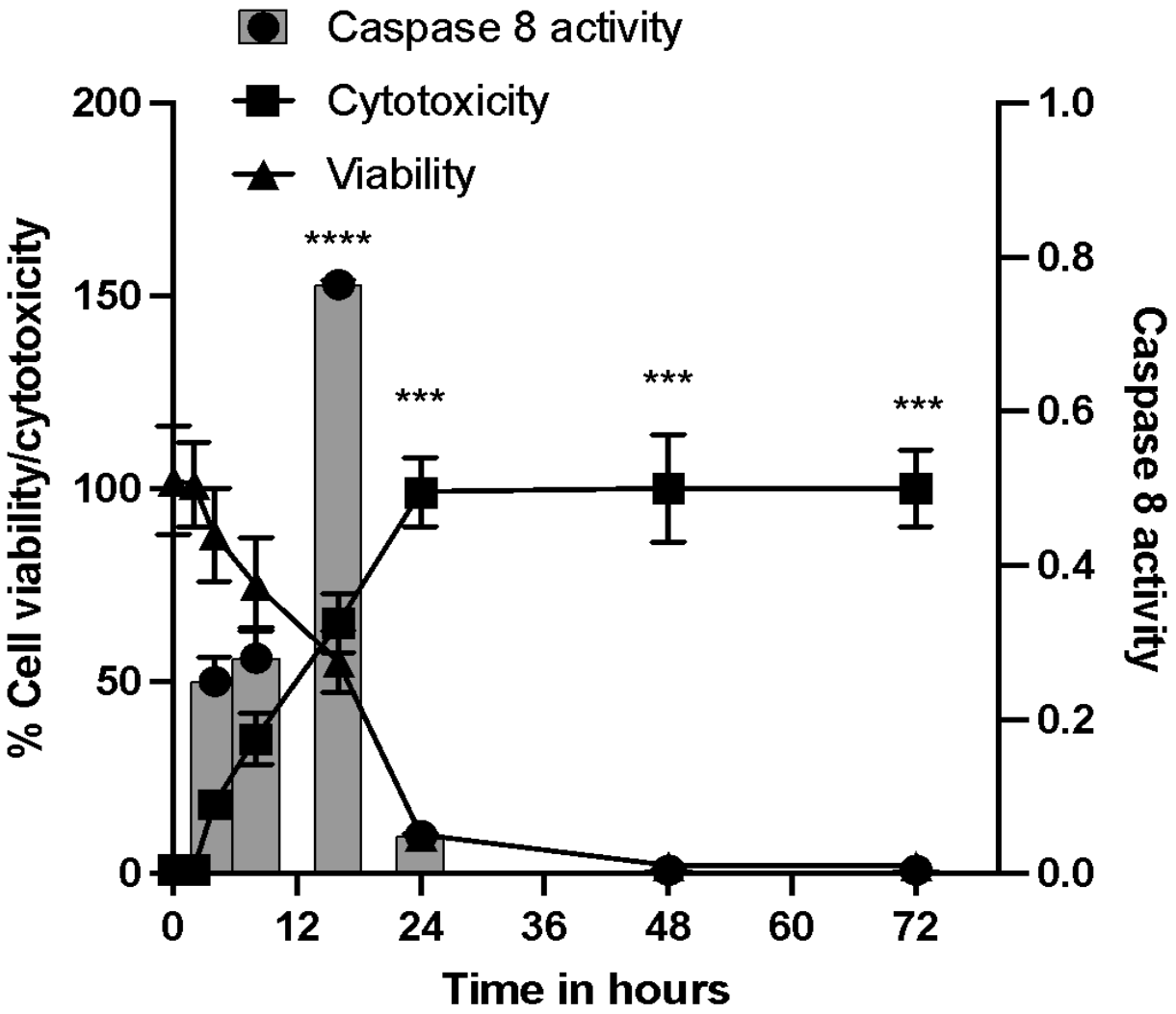


Figure 2: Results from the ApoTox-Glo™ triplex assay in SW480 cells over time. Caspase 8 was activated in SW480 cells after treatment for 2-18 hours with ALR-Aq. Cytotoxicity, and cell viability were also concomitantly determined. SW480 cell viability was reduced at 18 hours and cytotoxicity increased between 12-24 hours, corresponding with increased caspase 8 activity. ****p<0.0001; ***p<0.001.

ALR-Aq alters gene expression associated with apoptosis: ALR-Aq treatments reduced Bcl-2 mRNA expression in SW480, SW620 and HCT-116 cells, while Bax mRNA expression was not significantly altered, and therefore, treatment of HCT-116, SW480 and SW620 with ALR-Aq increased the Bax/Bcl-2 ratio increasing apoptosis in these cell lines (Figures 3A-I).

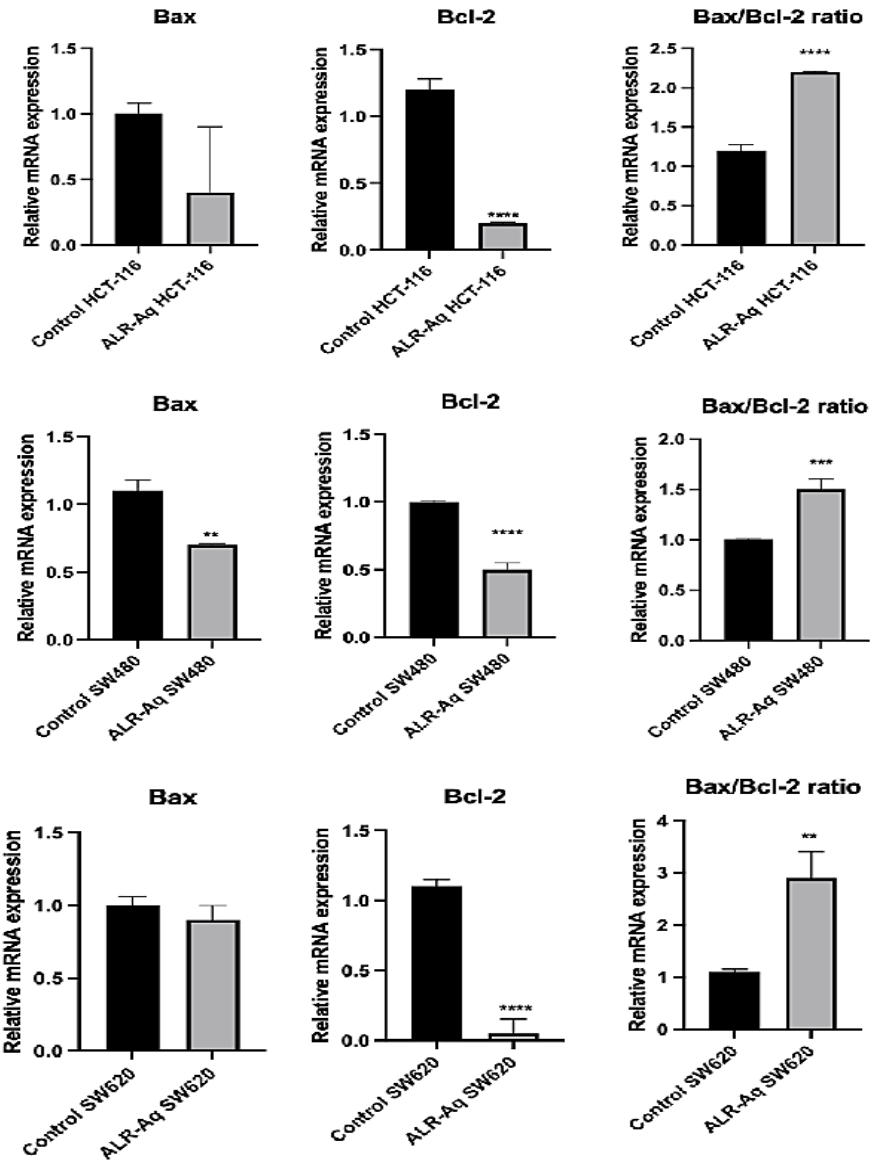


Figure 3 A-I: ALR-Aq treatments significantly Bcl-2 mRNA expression, thereby increasing the Bax/Bcl-2 ratio and inducing apoptosis. Apoptotic gene expression in was measured after treatment with 15 µg/ml of ALR-Aq for 2 hrs. **A-C**: Treatment of HCT-116 cells with ALR-Aq significantly downregulated Bcl-2 mRNA expression and significantly increased the Bax/Bcl-2 ratio increasing apoptosis. **D-F**: SW480 cells treated with ALR-Aq exhibited a significant decreased in Bcl-2 mRNA expression and increased Bax/Bcl-2 ratio, indicating apoptosis. **G-I**: SW620 cells treated with ALR-Aq showed a significantly reduced Bcl-2 mRNA expression and a significant increase in the Bax/Bcl-2 ratio indicting apoptosis. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

ALRAq increased p53 mRNA expression, and reduced HDAC1 and SIRT1 expression: ALR-Aq treatment (IC_{50} concentration) significantly ($p < 0.05$) increased p53 mRNA expression in HCT-116 ($p<0.05$) and SW480 cells ($p < 0.01$). No effects in SW620 cells were observed. In HCT-116 cells, ALRAq treatment also significantly reduced the expression of HDAC1 ($p < 0.01$) and SIRT1 ($p < 0.01$). In the SW620 cell line, a significant reduction in SIRT1 mRNA expression was observed ($p < 0.05$) (Figure 4).

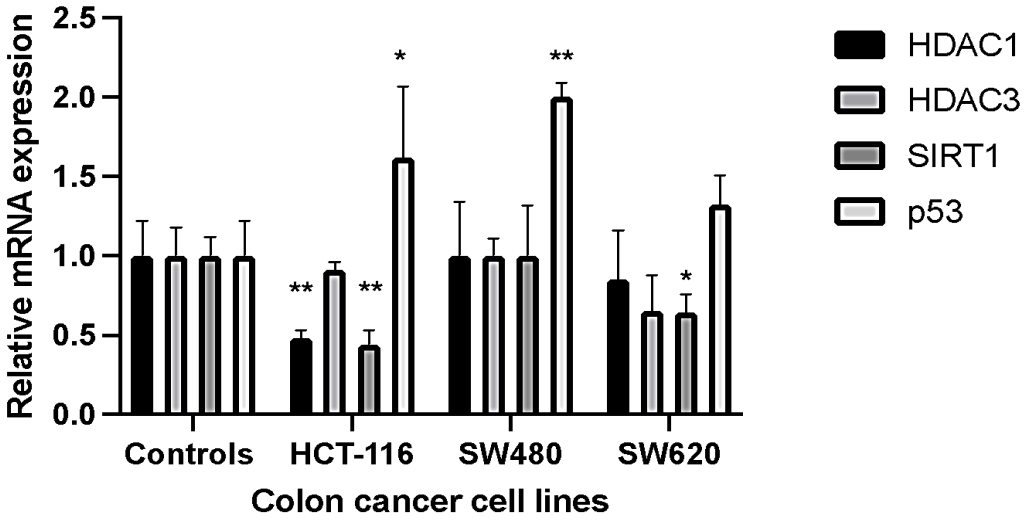


Figure 4. Treatment of HCT-116, SW480 and SW620 cells with ALR-Aq at the IC50 concentration for 2 hrs. alters gene expression of p53 and HDACs. Trizol was used to extract total RNA from cells that had been treated with aqueous partition of *A. leiocarpus* at IC_{50} concentration for 2 hours. Data showed that gene expression of both HDAC1 and SIRT1 HCT-116 cells was significantly reduced ($p<0.01$), while SIRT1 expression was significantly reduced in SW620 cells ($p<0.05$). In HCT-116 and SW480 cells, p53 gene expression was significantly upregulated ($p<0.05$) and ($p<0.01$), respectively. *$p<0.05$; **$p<0.01$.

DISCUSSION

Nigeria is a highly diverse country, and they commonly use many foods as medicines. . It has been estimated that ~70% of the population use food and medicinal plants as part of their primary healthcare [13]. Unfortunately, many of the medicinal and food plants used traditionally to treat GI disorders and cancers have little supporting experimental evidence [39-40]. Previously, we described the anti-*Helicobacter pylori* (*Hp*) activities of food and medicinal plant extracts from Nigeria that included *A. leiocarpus* and *T. glaucescens* [21]. In this work, we report that plant extracts with anti-*Hp* activities, namely *T. glaucescens*, *D. indica* and *A. leiocarpus* reduced cell growth and increased apoptosis in HCT-116, SW480 and SW620 cells, but were only weakly active in AGS and NCI-N87 gastric cancer cells. Previous investigations have shown that extracts of elephant apple (*D. indica*), an edible fruit that is used in Indian cooking and is also a favorite of elephants, reduced the proliferation of U937, HL60 and K562 leukemia cells, and in breast and granulosa cancer cells [38, 41]. Saowakhon et al., [42] tested extracts of Thai medicinal and food plants, including *D. indica,* and showed that this plant extract reduced the growth of many different cancer cell lines in an *in vitro* study. However, *D. indica* had not been reported to have cytotoxic effects on gastric and colon cancer cells.

Here, we have also demonstrated that extracts of *Anogeissus leiocarpus*—a tall, deciduous tree endemic to Africa that produces an edible water-soluble gum—used in drinks and chewing gum reduced the viability of cultured gastric and colon cancer cells. In Nigerian traditional medicine, the bark, leaves and roots of this tree are used for the treatment of common cold, gastrointestinal ailments, and cancer, while the twigs are used as chewing sticks for oral hygiene [43-44]. Previous studies have reported that extracts of *A. leiocarpus* have anti-cancer effects and were cytotoxic in Ehrlich ascites

carcinoma [44], induced apoptosis in COV and MCF-cells [38], had antiproliferative effects in HepG2 hepatocarcinoma [45], and reduced angiogenesis in a mouse xenograph model of colon cancer [46]. However, prior to this work there were no reports of the effects of this plant on the colon cancer cells lines SW480 and SW620 or in gastric cancer cell lines, and few mechanisms of action or active compounds have been reported. In this work, MeOH extracts of *A. leiocarpus* roots and stembark reduced the viability of AGS, HCT-116, SW480 and SW620 cells with IC_{50}s ranging from 15.8-77.13 µg/ml. Interestingly, none of the *A. leiocarpus* extracts or partitions were active against Caco2 cells (IC_{50} > 100 µg/ml). The *T. glaucescens* MeOH extract was weaky effective against HCT-116 and AGS cells, (IC_{50}s 64.94 and 52.25 µg/ml, respectively). The *D. indica* MeOH leaf extract was not active in the tested cells, while a MeOH extract of *D. indica* stembark was active in SW480, Caco2 and HCT-116 cells with IC_{50}s of 15.8, 35.23 and 53.29 µg/ml, respectively.

Further testing of the active *A. leiocarpus* MeOH extract led to the identification of water-soluble partition (ALR-Aq). The partition (ALR-Aq) had an IC_{50} of 15.0 µg/ml in SW480 and 14.6 µg/ml in HCT-116 cells. ALR-Aq contained one compound, and using column chromatography, semi-prep HPLC and x-ray crystallography, this compound was identified as the known compound methyl gallate [37]. Methyl gallate is a commonly known bioactive compound present in many fruits and vegetables. Methyl gallate is known for having many biological effects which includes reduction of cancer cell proliferation as well as induction of apoptosis in various cancer cell lines [47-49].

In HCT-116 colon cancer cells, ALR-Aq/methyl gallate reduced cell proliferation and intracellular ATP production (data not shown) indicating the possible involvement of mitochondrial apoptosis in its mechanism of action. Since the *A. leiocarpus* extract and the water partition (ALR-Aq) induced apoptosis, we further measured caspase activation and gene expression associated with apoptosis to determine potential mechanisms of action.

There are two apoptotic pathways in mammalian cells, the mitochondrial (intrinsic) and death receptor (extrinsic) pathways. The mitochondrial pathway is mediated through the Bcl-2 proteins activities while the death receptor pathway is mediated by receptor of the tumor necrosis factor [50-52]. Each of these two pathways requires the activation of caspases (cysteine-aspartic acid protease) for cellular apoptosis [50-52]. Caspase-3/7 is the effector caspase for mitochondrial apoptosis, and caspase 8 is the initiator caspase for death receptor apoptosis [50-52]. Here we show that ALR-Aq treatment of HCT-116 and SW480 cells did not induce caspase 3/7 activation (data not shown), but instead activated caspase 8, suggesting the involvement of extrinsic apoptosis in both cell lines.

Caspase activation increased the expression and activity of Bax, a pro-apoptotic protein, as well as reduced Bcl-2 (anti-apoptosis) protein activity and expression [51-52]. Thus, we measured Bax and Bcl-2 gene expression and the Bax/Bcl-2 ratio in treated SW480, HCT-116 and SW620 cells, using qPCR. The B-cell lymphoma 2 (Bcl-2) proteins regulate mitochondrial apoptosis and the Bax to Bcl-2 ratio determines cellular apoptosis [51-52]. When Bcl-2>Bax, apoptosis is reduced, but when Bax is greater than Bcl-2, apoptosis is induced [51]. ALR-Aq/methyl gallate treatment of SW480, HCT-116 and SW620 cells withthe IC_{50} concentration reduced the expression of the anti-apoptotic protein Bcl-2 mRNA

and increased the Bax/Bcl-2 ratio, thereby suggesting induction of the intrinsic apoptotic pathway was also occurring in all three cell lines.

The induction or activation of tumor suppressor protein p53 or TP53 is essential for the management of stimuli including oxidative stress, DNA damage and oncogenic events, that mediate the cell cycle, apoptosis, and autophagy [53-55]. ALR-Aq/methyl gallate treatment of HCT-116 and SW480 cells significantly increased p53 mRNA expression above controls, indicating that p53 may be involved in the activity of ALR-Aq. Interestingly, modification and dysregulation of p53 occurs in >50% all human cancers [56].

We further measured the HDAC expression, since the expression p53 can be altered by epigenetic modifications, including acetylation via histone acetyltransferases and deacetylation via histone deacetylases (HDACs) [57-59]. HDACs modify histones and non-histone proteins; and thereby change gene expression [60-62]. HDACs are classified into four classes: class I (HDAC1, 2, 3 and 8), class II, class III (SIRT1-7) and class IV [63-65]. Sirtuin 1 (SIRT1) is a nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase known to mediate many signaling pathways involved in aging, cell cycle, metabolism, and apoptosis [66]. Inhibition of SIRT1 reduced cancer cell growth and induced apoptosis [66]. Several HDACs and SIRTs have been reported to deacetylate p53, including HDAC 1, 3 as well as SIRT 1 and 3 [67-69]. HDAC and SIRT inhibition reduced p53 deacetylation, and thereby improved the stability and function of p53 as a tumor suppressor [68, 70-71]. Our data show that ALR-Aq, containing the one compound methyl gallate, inhibited the expression of both SIRT1 and HDAC1 mRNA in HCT-116 cells, suggesting that ALR-Aq/methyl gallate acts as an HDAC inhibitor. In support of our results, previous investigators have reported that HDAC inhibitors are reported to activate p53 signaling, reduce Bcl-2 gene expression, raise the Bax/Bcl-2 ratio and induce apoptosis [72-73]. Thus, the data presented in this work suggest that ALR-Aq/methyl gallate may act on the epigenome to inhibit HDACs and SIRTs, which increases the expression of p53 tumor suppressor in HCT-116 cells, leading to a reduction in Bcl-2 gene expression, and inducing apoptosis.

CONCLUSION

In this work we demonstrate that three Nigerian food plant extracts and partitions inhibited the cell growth of HCT-116, SW480 and CaCo2. However, all extracts were weakly active in gastric cancer cells AGS and NCI-N86, and not active in the SW620 resistant colon cancer line. In the colon cancer cell lines SW480 and HCT-116 cells, the aqueous partition, ALR-Aq/methyl gallate, induced apoptosis, increased caspase 8 activity, decreased the expression of Bcl-2 mRNA, and raised the Bax/Bcl-2 ratio; as well as increased p53 expression and inhibited HDAC expression in HCT-116 cells. Methyl gallate was identified as the only compound in ALR-Aq and appears to be the active compound. Interestingly, the primary activity of these food plant extracts resided in the aqueous partitions, thus keeping with their traditional use.

Abbreviations: ALR-Aq: *Anogeissus leiocarpus* root-aqueous, Aq: Aqueous, ATCC: American Type Culture Collection, ATP: Adenosine triphosphate, Bax: Bcl-2 associated X protein, Bcl-2: B-cell lymphoma 2, DCM: Dichloromethane, DIS-Aq: *Dillenia indica* stem- aqueous, DMSO: Dimethyl sulfoxide, EtOAc: Ethylacetate, FBS:

Fetal bovine serum, HDAC: histone deacetylase, hFOB: human osteoblasts, IC: Inhibitory concentration, ME/MeOH: Methanol, mRNA: messenger Ribonucleic acid, NAD, Nicotinamide adenosine dinucleotide, NMR: Nuclear magnetic resonance, P/S: Penicillin/Streptomycin, RNA: Ribonucleic acid, RTPCR/qPCR: Real-time polymerase chain reaction (also known as quantitative polymerase chain reaction), UIH: University of Ibadan herbarium, SIRT: Sirtuin.

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