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Investigation of the anti-cancer and apoptotic properties of aqueous extract from fermented African locust bean seeds

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Abstract

Some fermented foods are reported to possess anti-cancer properties. Fermented African locust bean seeds is a condiment prepared from fermentation of *Parkia biglobosa*. It has been reportedly functional for various medicinal activities but not anti-cancer. The cytotoxic and apoptosis-inducing properties of the aqueous extract of the condiment were investigated in human cancer - hepatocellular (Hep-G2) and cervical (HeLa) and non-cancer cell lines. Cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and clonogenic cell survival assays. Apoptotic cell death and DNA fragmentation were also investigated. The results revealed cytotoxicity to both cell lines in a dose-dependent manner ($P < 0.05$) and selective activities between cancer and non-cancer cells. The IC_{50} values were 1.3 and 0.5 mg/mL for Hep-G2 and HeLa cells respectively. Furthermore, the extract induced apoptotic cell death in only Hep-G2 (73.03 ± 0.73) cells. The morphologic photomicrographs correlated well with other findings, indicating the cell-specific cytotoxicity of the condiment.

1. Introduction

Existing scientific reports reveal that many naturally fermented foods and beverages contain both functional and non-functional microorganisms (Tamang *et al*., 2016), with the ability to modulate specific target functions in the body leading to the well-being and health of the consumers. Some fermented foods and beverages have been reported to be useful in cancer prevention and treatment including *kefir* (Otes *et al*., 2003; Wang *et al*., 2009), sauerkraut a fermented vegetable in Germany (Kris-Etherton *et al*., 2002), *kimchi* (Park *et al*., 2014) and many others.

African locust bean (*Parkia biglobosa*) species represents one of the indigenous medicinal plants available across West and Central Africa. The fermented African locust beans (Figure 1) is a soup/stew condiment prepared by natural fermentation of *P. biglobosa* seeds. The condiment is well researched and reported to possess diverse nutritional and medicinal properties. This condiment can be eaten directly but it is mainly used as a spice in the preparation of various delicious meals. The fermented seeds are given various names depending on location, these include *dawadawa* in Ghana and northern Nigeria; *iru* in Benin Republic and southern Nigeria; and *soumbala* in Burkina Faso. Various extracts from the fermented seeds of *P. biglobosa* have been studied for their usefulness in the prevention, treatment, and management of various disease conditions (using various models) including *diabetes mellitus* (Odetola *et al*., 2006), hypertension (Ognatan *et al*., 2011), antioxidant and hypolipidaemic effect (Ayo-Lawal *et al*., 2014). There is however no study in the literature that has reported the cytotoxic activity of fermented *P. biglobosa* (FPB) seeds. The present study was done to investigate the cytotoxic and apoptotic effects of the aqueous extract of FPB seeds on HeLa and Hep-G2 cells.

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Figure 1. Fermented African locust bean seeds (A) prepared from raw *P. biglobosa* seeds (B)

2. Materials and methods

2.1 Preparation of the aqueous extract from FPB seeds

Indigenously prepared (Ayo-Lawal *et al.,* 2016) FPB seeds were obtained from a commercial producer and smoothened to a paste using a mortar and pestle in the Biochemistry laboratory of the Obafemi Awolowo University, Osun State, Nigeria. The aqueous extract was obtained by soaking the paste in distilled water for 48 hrs and the supernatant was collected. The residue was thereafter squeezed in a mesh filter cloth to collect all the filtrate which was then freeze-dried to obtain a dried marc.

2.2 Cell culture and growth

Hepatocellular (Hep-G2) and cervical (HeLa) cancer cell lines were obtained from the Department of Biotechnology, University of the Western Cape, South Africa. These cells were grown in complete Dulbecco's Modified Eagle Medium (DMEM Life Technologies, USA), supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin cocktail (100 U/mL penicillin and 100 μg/mL streptomycin, Lonza, USA) in a humidified incubator - Labotech, South Africa (5% CO_2 in air at 37°C).

2.3 Assessment of the cytotoxicity of the aqueous FPB seeds extract

Hep-G2 and HeLa cells were seeded in 96-well plates at 8×10^4/mL and 5×10^4/mL respectively and incubated for 24 hrs under standard conditions to attach. The cells were thereafter incubated with aqueous FPB seeds extract at various concentrations (0.5 – 2.5 mg/mL) for 24, 48 and 72 hrs respectively. Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, St. Louis, MO, USA) assay. Here, 10 μL of 5 mg/mL MTT (dissolved in phosphate buffered saline (PBS)) was added to each well of a 96-well plate containing treated cells, and then incubated at 37°C for 4 hrs. The formazan crystals formed were thereafter solubilized by adding 100 μL DMSO and absorbance read at 570 nm using the POLAR Star Omega plate reader (BMG Labtech, Germany).

Experiments were performed in triplicates and the half-maximal cytotoxic concentration (IC_{50}) was calculated from log dose-response curves using the Graph Pad Prism software for Windows. Cell viability (percentage of control) (Badmus *et al.,* 2015) was calculated as:

(Average absorbance of treated cells/Average absorbance of control cells) x 100

2.4 Investigation of cell morphology

Morphological changes in HeLa and Hep-G2 cells were observed after treatment with the aqueous FPB extract for 24 hrs. Briefly, HeLa (5×10^3/well) and Hep-G2 (8×10^3/well) cells were seeded into a 24-well plate (in triplicates) and incubated for 24 hrs to attach. Thereafter, HeLa and Hep-G2 cells were treated with the previously determined IC_{50} concentrations of 0.5 and 1.3 mg/mL respectively for 24 hrs, and washed once with PBS, cells morphologies were viewed under the ZEISS Primo Vert inverted microscope (ZEISS, Germany) and images captured at x200 magnification.

2.5 Assessment of cell survival - the clonogenic assay

Reproductive cell death was assessed in HeLa and Hep-G2 cells following treatment with the aqueous of FPB extract for 24 hrs using a previously reported protocol (Badmus *et al.,* 2015). Briefly, HeLa (3.28 x 10^5) and Hep-G2 (5.25 x 10^5) cells in 5 mL were seeded in 60 mm dishes and incubated for 24 hrs. Thereafter, the cells were treated with the same concentration of the extract as above, for 24 hrs while the untreated cells were used as control. Treated and untreated cells were trypsinized and further seeded at 1 x 10^3 into a 35-mm dish and cultured again depending on proliferation rate (HeLa for 5 days; Hep-G2 for 7 days). During the culturing stage of the experiment, media was changed daily, and cells were thereafter fixed with 750 μL of the mixture of methanol: acetic acid (3:1) for 15 mins at room temperature. The cells were then stained with crystal violet for 10 mins at room temperature and photomicrographs of the air-dried dishes were captured using the ZEISS Primo Vert inverted microscope. Experiments were performed in triplicates and the total number of colonies on each dish was counted using a colony counter (TT 201 Techmel and Techmel, USA).

2.6 Flow cytometric analysis of apoptosis

The flow cytometer based APOPercentage^{TM} assay was utilized to quantify apoptosis in treated and untreated cells (Meyer *et al.,* 2008). Briefly, HeLa and Hep-G2 cells were seeded at a density of 1 x 10^5 into 24-well plates and allowed to attach for 24 hrs under standard incubation conditions. Thereafter, three

different extract concentrations representing values < IC_{50}, IC_{50}, and > IC_{50} were used to treat Hep-G2 (0.8, 1.3, 2.0 mg/mL) and HeLa (0.3, 0.6, 0.9 mg/mL) cells. Positive control cells were treated with 6% DMSO for 24 hrs followed by staining with the APOPercentage[TM] dye dissolved in PBS (1:160) and analysis using the BD Accuri[TM] C6 flow cytometer (BD Biosciences, Belgium).

2.7 DNA fragmentation analysis of apoptosis

Qualitative analysis and confirmation of apoptotic induction in Hep-G2 and HeLa cells were done by investigating DNA fragmentation following treatment with the extract for 24 hrs in 1.5% agarose gel electrophoresis. Briefly, HeLa (100,000/well) and Hep-G2 (100,000/well) cells in 3 mL complete DMEM medium were incubated under standard conditions for 24 hrs and thereafter separately treated with 0.6 mg/mL and 1.3 mg/mL extract concentrations respectively, being the respective IC_{50} values. The control cells were on the other hand not exposed to any extract treatment (negative control). Cells were prepared as previously described by Bossú (1999) and the DNA marker (Fermentas Fast Ruler[TM]) was used as a reference. The electrophoresis analysis was run in standard TBE buffer (voltage 90 for 30 mins) and used for the migration of molecules in the samples based on size and electrical charge. Fragmented DNA movements were visualized by placing the gel on an ultraviolet transilluminator and photographing. Bands were compared with those of the DNA marker.

2.8 Statistical analysis

The GraphPad Prism software version 5.0 for Windows (GraphPad Software, Inc., California) was used for estimating IC_{50} via the linear regression equation and for plotting the graphs. Data obtained was analysed and expressed as Mean±Standard Error of Mean and one-way analysis of variance (ANOVA) followed by Dunnet multiple comparison post-hoc test was used to determine statistical significance (p<0.05) across groups.

3. Results

3.1 Cytotoxicity of aqueous FPB seeds extract to cancer cells

The aqueous extract of FPB seeds was found to be significantly (P<0.05) cytotoxic to both Hep-G2 (Figure 2) and HeLa (Figure 3) cells in a concentration-dependent manner. The IC_{50} concentrations at 24 hrs were approximately 1.3 mg/mL and 0.6 mg/mL for Hep-G2 and HeLa cells respectively, suggesting that HeLa cells were more susceptible to the cytotoxic activity of the extract. Furthermore, the cytotoxic effects of the extract to cells was found to increase with increased

incubation time as evidenced by the progressive decrease in IC_{50} values with time (Table 1).
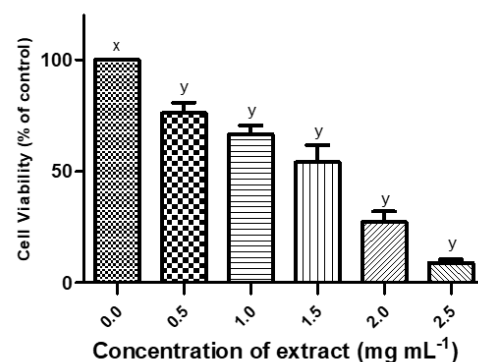

Figure 2. Cytotoxicity of aqueous fermented *P. biglobosa* seeds on Hep-G2.
*y is significantly different from x (P<0.05) in each case.
Hep-G2 cells were treated with increasing concentrations of fermented *P. biglobosa* extract for 24 hrs; cell cytotoxicity was assessed using MTT assay
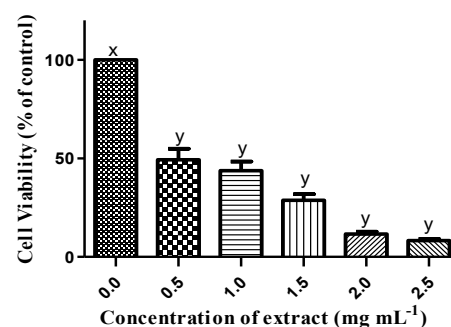

Figure 3. Cytotoxicity of aqueous fermented *P. biglobosa* seeds on HeLa cells. *
y is significantly different from x (P<0.05) in each case.
HeLa cells were treated with increasing concentrations of fermented *P. biglobosa* extract for 24 hrs; cell cytotoxicity was assessed using MTT assay

Table 1. Incubation time versus half maximal inhibitory concentrations (IC_{50}) (mg/mL) of aqueous fermented *P. biglobosa* extract in different cell lines

Incubation Time (hrs)	Hep-G2 (Human Hepatocellular Cells)	HeLa (Human Cervical Cells)
24	1.3	0.6
48	1.2	0.4
72	0.8	0.3

3.2 Morphological alterations in Hep-G2 and HeLa cells following treatment with aqueous FPB extract

The Hep-G2 and HeLa cells not treated with the aqueous extract of FPB maintained their original structure and conformation with increased proliferation, reaching up to 90% confluence at the end of the experiment and remaining attached to the bottom of the plates. In contrast, the extract-treated Hep-G2 and HeLa cells had less cell volume (seeding density was equal in both treated and untreated cells prior to treatment). Cells

in the treated dishes appeared to have lost cell-cell and cell-plate contacts and remained floated in the medium, with perturbed morphology. Dead cells were also captured in both extract-treated dishes (Figure 4).
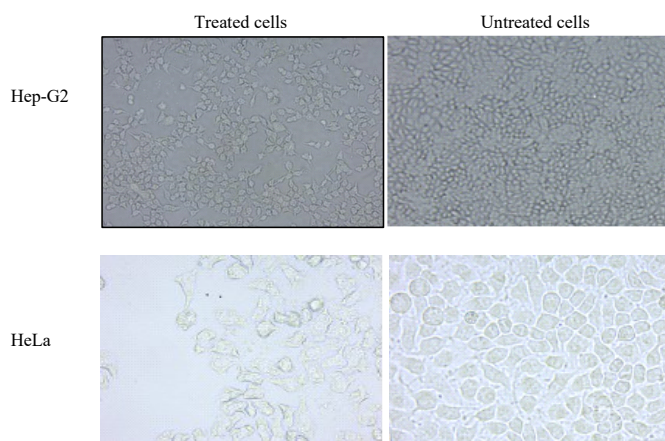

Figure 4. Morphological alterations in Hep-G2 and HeLa Cells following 24 hrs treatment with aqueous FPB extract. Morphological alteration in Hep-G2 and HeLa cells following treatment with aqueous extract of fermented African locust bean seeds. Hep-G2 and HeLa cells were treated with the aqueous extract for 24 hrs. Cells were washed, morphology viewed and the images were captured by ZEISS Primo Vert inverted microscope at x200 magnification

3.3 Clonogenic effects of the aqueous fermented Parkia biglobosa seeds extract on Hep-G2 and HeLa cells

There was a significant reduction in the number of colonies formed in both Hep-G2 and HeLa cells treated with the aqueous FPB extract compared with the untreated cells (Figure 5). The untreated dish containing Hep-G2 cells had 556.66 ± 24 colonies, while the treated Hep-G2 cells had only 71.33 ± 4 colonies. Similarly, the number of colonies in the untreated HeLa dish was 832.66 ± 23, while the treated cells had only 115.33 ± 6 colonies (Figure 5).
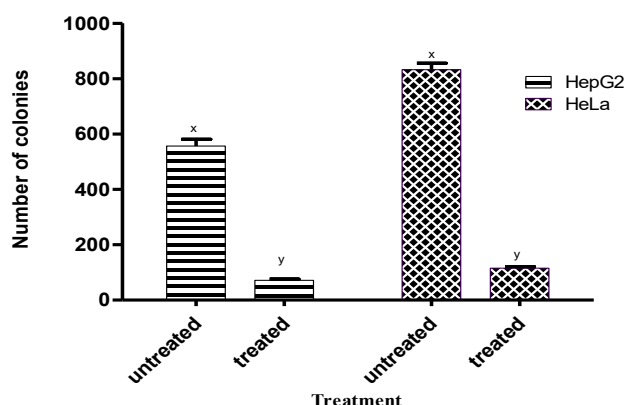

Figure 5. Clonogenic effects of aqueous fermented *P. biglobosa* seeds extract on Hep-G2 and HeLa Cells.
Following treatment of HeLa and Hep-G2 cells with aqueous FPB extract, cells were fixed and processed. Cell colonies were counted using a colony counter (TT 201 Techmel and Techmel, USA).

3.4 Induction of apoptosis in Hep-G2 and HeLa cells following treatment with the aqueous extract of FPB seeds

In this study, the aqueous extract of FPB seeds was found to induce concentration-dependent apoptosis in Hep-G2 (Figure 6) and not in HeLa cells (Figure 7). Three concentrations representing $< IC_{50}$, IC_{50} and $> IC_{50}$ were used in each case, and experiments were performed in triplicates. The average numbers of apoptotic cells measured in the wells treated with the IC_{50} extract concentrations were 73.03 ± 0.73 and 35.10 ± 3.28 for the Hep-G2 and HeLa cells respectively.
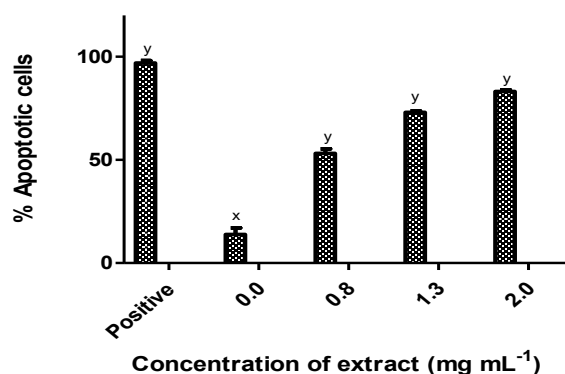

Figure 6. Induction of apoptosis in Hep-G2 cells following treatment with aqueous FPB seeds. Positive well contained 6% DMSO.
*y is significantly different from negative control (x) at P<0.05.
Investigation of apoptotic induction by APOPercentage™ assay. Hep-G2 cells were treated for 24 hrs with three increasing concentrations of aqueous extract of fermented *P. biglobosa* seeds. The cells were stained with the APOPercentage™ dye and evaluated using a flow cytometer. Each bar chat represents the means±SEM of three independent experiments.
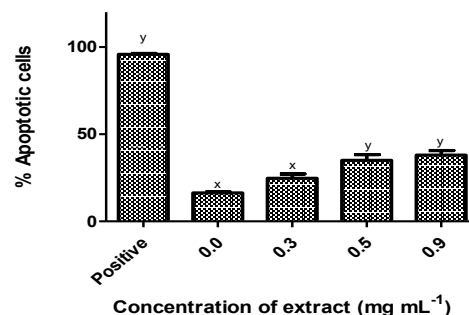

Figure 7. Apoptotic induction in HeLa cells following treatment with increasing concentrations of aqueous FPB seeds extract. Positive well contained 6% DMSO.
*x is not significantly different from negative control (0.0) while y is significantly different at P<0.05.
Investigation of apoptotic induction by APOPercentage™ assay. HeLa cells were treated for 24 hrs with three increasing concentrations of aqueous extract of fermented *P. biglobosa* seeds. The cells were stained with the APOPercentage™ dye and evaluated using a flow cytometer. Each bar chat represents the means±SEM of three independent experiments.

3.5 Induction of DNA fragmentation in Hep-G2 and HeLa cells following treatment with the aqueous extract of FPB seeds

The electrophoretic wells (in both gels) loaded with samples from the untreated cells clearly indicated the absence of DNA fragments; neither smears nor bands. On the other hand, a band corresponding to about 250 base pair or less on the DNA marker was seen for the wells loaded with treated Hep-G2 cells (Figure 8A). A weaker band was seen in the wells loaded with treated HeLa cells, indicative of some DNA fragments of similar size (Figure 8B) which is suggestive of apoptotic induction in both cells.
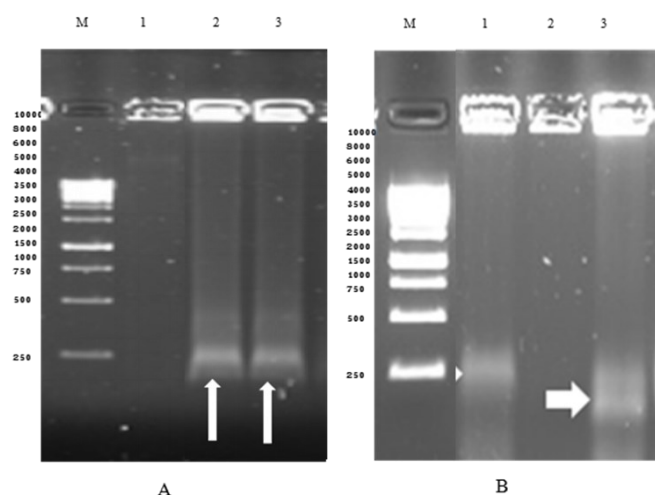

Figure 8. Qualitative analysis of DNA fragmentation by agarose gel electrophoresis in treated and untreated Hep G2 (A) and HeLa (B) cells. Hep-G2 (M – DNA marker, 1 – Untreated sample (control), 2,3, - Aqueous FPB extract-treated sample. HeLa (M – DNA marker, 2 – Untreated sample (control), 1,3, - Aqueous FPB extract-treated sample

4. Discussion

Fermented indigenous foods and associated microbiota have been associated with various health benefits for many years. These effects could be achieved via various mechanisms including the activation of the immune system, mitigation of carcinogenicity, and induction of apoptosis (Ghosh *et al.,* 2019) among others. Cytotoxic compounds which selectively initiate apoptosis in cancer cells and hence slow down proliferation are considered potential candidates for cancer therapy (Xiao *et al.,* 2007). In this study, the aqueous extract of the seeds of fermented *P. biglobosa,* (which is the readily-edible state of the condiment) was found to have significant cytotoxic effects on both Hep-G2 and HeLa cells, with the latter being more susceptible. Microscopic examination showed distortions in the morphology of Hep-G2 and HeLa cells which correlated with the observed cytotoxic effects. In addition, the clonogenic assay demonstrated that the extract exerted a lasting cytotoxic effect on the Hep-G2 and HeLa cells, thus inhibiting the ability of the cells to reproduce and form colonies compared with the untreated cells.

Potential health benefits of many fermented foods have been attributed to the biologically active peptides, vitamins, and other compounds produced by the bacteria responsible for the fermentation process (Sanlier *et al.,* 2010). In this study, the observed cytotoxic activities of FPB could have been caused by the bioactive peptides in the extract. Our previous unpublished data showed that the most abundant prevailing organisms in the FPB extract used in this study were the *Bacillus* spp, (especially *B. subtilis*). This finding is in agreement with previous studies by Odunfa (1981) who reported that members of the *Bacillus* spp. and *Staphylococcus* spp. as the prevailing organisms involved in the production of FPB. In addition, Odunfa and Oyewole (1986), found that all *Bacillus* species isolated from FPB were proteolytic and only 97.3% were lipolytic. Since protein is the major component of most fermented legumes such as *P. biglobosa*, *Bacillus* species serves to break down the protein into peptides and amino acids (Tamang and Nikkuni, 1996; Isu and Njoku, 1997). In addition, the fermentation of legume-based food substances is known to increase the content of phenolic compounds in the seeds which increases their nutritional value.

Apoptosis remains the major cell death pathway for removing unwanted and harmful cells in a non-disruptive manner. Abnormal evasion from apoptosis is typical of cancer cells, so the ability of a compound or an extract to induce apoptosis in cancer cells could be a promising lead to the anti-cancer potential of such a compound (Frankfurt and Krishnan, 2003). Here, the induction of apoptosis in HeLa and Hep-G2 cells was quantified following treatment with the aqueous extract of FPB extract for 24 hrs using the flow cytometer-based APOPercentage[TM] assay. The dye was only taken up by apoptotic-committed cells, recognized by phosphatidyl serine externalization (Meyer *et al.,* 2008) compared to DMSO which is also an apoptosis-inducing agent (Banič *et al.,* 2011). Significant activity was obtained in the Hep-G2 cells, unlike in the HeLa cells. This could suggest that different pathways might be involved in the cytotoxic activity of the extract in the two cancer cells investigated.

The detection of DNA fragmentation is currently one of the most frequently used techniques for highlighting apoptotic cells in tissues. One of the biochemical hallmarks of apoptosis is the degradation of DNA by endogenous dehydrogenases, which cut the internucleosomal regions into DNA fragments of ~200

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base pairs (Arends, *et al.,* 1990; Compton, 1992). The electrophoretic gel obtained in this study attested to the presence of DNA fragments corresponding to about 200 base pairs, indicative of apoptosis in both Hep-G2 and HeLa cells.

Two plausible indications emanated from the findings from this study; either HeLa cells could have been killed before the analysis, hence the dye could not bind to dead cells, or the assay is more applicable to cells that undergo apoptosis via the intrinsic pathway. Hence, if the observed cell death occurred via the extrinsic pathway, then the APOPercentage[TM] assay could not have demonstrated such cell death.

The anti-cancer activities of some fermented indigenous foods and associated microbiota have been well reported (Watanabe, 2013; Kwak *et al.,* 2014; Park *et al.,* 2014); and the induction of apoptotic pathways was suggested to be one of the ways by which fermented foods exert their anti-cancer effects. In this study, the aqueous extract from fermented African locust beans was found to significantly induce apoptosis in Hep-G2 cells. In another report, *Kefir*, a drink produced by milk fermentation and its cell free extract, was reported to exert its anti-carcinogenic effects via the induction of apoptotic mechanisms (Pẽnas *et al.,* 2017), as well as anti-proliferative effect (Ghoneum and Gimzewski, 2014) on cancer cells. *Kefir* was also reported to be cytotoxic and induced apoptosis in HT-29 and Caco-2 colorectal cancer cells (Khoury *et al.,* 2014; Maalouf *et al.,* 2011).

5. Conclusion

The results obtained from this study further corroborate available information on the nutritional and medicinal values of fermented *P. biglobosa* seeds. Furthermore, the results indicate that the aqueous extract of fermented *P. biglobosa* seeds was significantly cytotoxic to both human hepatocellular and cervical cancer cells and induced cell death in the cells via apoptosis. This was the first attempt to investigate the effects of any extract from this well-known Nigerian food condiment on cancer cells. The results obtained provide a promising lead for further investigation, isolation, characterisation and the potential development of an anti-cancer nutraceutical from the condiment.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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