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Anti-mitotic and anti-proliferation potentials of aqueous and methanolic extracts of fermented *Citrullus vulgaris* (*ogiri-egusi*) seeds

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Abstract

There is a growing scientific evidence of the health-enhancing benefits of fermented food and beverages, especially in cancer prevention and treatment. *Ogiri-egusi* is an indigenously fermented condiment consumed in some West African countries, and prepared mainly from natural fermentation of melon (*Citrullus vulgaris*) seeds. The condiment has been previously reported to possess anti-oxidative and anti hyperlipidemic properties. In this study, different concentrations of *ogiri-egusi* extracts were exposed to actively dividing cells from *Allium cepa* meristematic roots and *Saccharomyces cerevisiae* for the antimitotic and antiproliferative investigations, respectively. There was a statistically significant reduction in mitotic activities of the treated *A. cepa* meristematic cells compared to the untreated cells. The mitotic index (MI) of the untreated cells was 28.38 ± 4.58 . At a concentration of 1.0 mg/ml, the extracts induced a reduction in the MI to 6.02 ± 2.74 and 4.94 ± 0.61 in the aqueous and methanolic extracts-treated cells, respectively. Some chromosomal aberrations were also observed. In addition, proliferation in yeast cells was significantly inhibited at all the concentrations of the aqueous extract examined as well as at the highest concentration of the methanolic extract. These results provide useful insights to the cytotoxic and genotoxic effects of fermented *Citrullus vulgaris*.

Keywords: Fermented *Citrullus vulgaris*, *Allium cepa*, antiproliferation, cytotoxicity, genotoxicity

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Introduction

Cancer is the second leading cause of death globally, and was responsible for an estimated 9.6 million deaths in 2018 (WHO, 2018). Among other characteristics, incessant proliferation is an important part of cancer development and progression. The cancer cells therefore develop systems

to survive beyond their normal life span and proliferate abnormally in a process involving mitosis (Feitelson et al., 2015). Mitosis is an important and elaborate process in actively proliferating cells, resulting in the division of duplicated sets of chromosomes and producing two genetically identical daughter cells (Chan et

al., 2012). The vulnerability of cancer cells when they undergo division serves as a critical intervention point in chemotherapy. The strategy involves a prolonged arrest of cells in mitosis, culminating in mitotic cell death (Chan et al., 2012). Among plant species, *Allium cepa* assay has been used to evaluate disturbances in the mitotic cycle and chromosome aberrations (Khanna and Sharma, 2013). The test has been used for monitoring the genotoxic, cytotoxic and mutagenic nature of different test chemicals.

Fermented foods and beverages have been an integral part of the human diet since time immemorial, with unproven potential health-enhancing properties. The traditional beliefs regarding the medicinal importance of some fermented foods are now scientifically verified. Medicinal

importance varies from one fermented product to another and includes quicker digestion of protein and carbohydrates, removal of natural toxins in food, improved mental health and lipid profile, anti-diabetic, etc (Marshall and Mejia, 2011; Kwak et al., 2012; Park et al., 2014; Selhub et al., 2014). In addition, some fermented foods have been reportedly beneficial in cancer prevention and treatment (Park, 1995; Hur et al., 2000; Kris-Etherton et al., 2002; Mueller and Voigt, 2011).

Ogiri egusi (Figure 1) is a condiment prepared naturally by fermentation of melon (*Citrullus vulgaris*) seeds. It is consumed in many West African countries (Odunfa, 1981; Achi, 2005), and is locally known as *ogiri-egusi* in many parts of Nigeria.



Figure 1: *Citrullus vulgaris* (melon) seeds (A) were used to prepare *Ogiri-egusi* (B) uncontrolled solid state fermentation.

by

It is an important condiment useful as a nutritious, functional, non-meat protein supplement in soups and sauces (Mortarjemi, 2002; Achi, 2005). Furthermore, *ogiri-egusi* is rich in antioxidants (Ayo-Lawal et al., 2015) and has a number of medicinal properties; for instance, the oil extracted from the condiment after fermentation has been reportedly useful for medicinal purposes by traditional healers (Ajayi and Ajayi, 2009). Supplementation of feed with *ogiri-egusi* also significantly increases the plasma levels of HDL-C and reduces the LDL-C cholesterol in tyloxapol induced hyperlipidaemic rats (Ayo-Lawal et al., 2015). This study aimed to investigate the potential effects of *ogiri-egusi* extracts on actively proliferating cells. The possible

genotoxic, anti-mitotic and anti-proliferation activities were examined using the *A. cepa* and yeast cell proliferation models.

Materials and Methods

Aqueous and methanolic extraction from fermented Citrullus vulgaris seeds

Indigenously prepared fermented *C. vulgaris* seeds (FCVs) were purchased from a local industry and were manually smoothened using a mortar and pestle in the laboratory to form a smooth, uniform paste. The aqueous extract of FCVs was obtained by soaking the paste in distilled water (1:5 w/v) for 48 h with constant stirring. The supernatant was collected and the residue squeezed in a mesh cloth to

obtain all the extract. The extract was freeze-dried (CHRIST BETA 1-8 LD Plus, Germany) to obtain a dried marc. To obtain the methanolic extract, a known weight of the original paste was extracted with methanol (1:5 w/v) in the Soxhlet apparatus for 18 h. The methanol solution was subsequently concentrated in a rotary evaporator (Buchi RII, Switzerland) set at 40°C.

Anti-mitotic evaluation of the extracts of fermented C. vulgaris seeds

Allium cepa assay was used to investigate the anti-mitotic activity of the extracts as previously described (Gadano et al., 2002; Solange and Haywood, 2012). Dried *A. cepa* bulbs were sprouted in distilled water for 48 h. Sprouted bulbs with uniform roots were grouped and transferred to varying concentrations (1.0, 2.5, 5.0, 10.0 and 20.0 mg/mL) of methanolic and aqueous extracts of FCVs for 24 h. Sprouts in distilled water and 0.25 M sodium benzoate were used as untreated and positive control, respectively. After 24 h, the roots were harvested and fixed in 1:3 acetic acid: alcohol. The slides were prepared according to squash technique described by Laosebikan and Olorode (1972). The slides were viewed under a light microscope (Olympus, model CKX31). The photomicrographs were taken with AMSCOPE MT microscope camera version 3.001 attached to a light microscope. Experiments were performed in triplicates.

Mitotic index was expressed as:

$$\text{Mitotic index} = \left(\frac{\text{number of dividing cells}}{\text{total number of cells}} \right) \times 100 \%$$

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Anti-proliferation evaluation of extracts of fermented C. vulgaris seeds

The assay protocol followed was as previously described by Periyanyagam et

al. (2013). Briefly, a pure yeast (*Saccharomyces cerevisiae*) cell inoculum containing 25.4×10^4 cells (average) was prepared in sterilized potato dextrose broth (PDB) referred to as yeast inoculum. The assay mixture consisted of 0.5 mL yeast inoculum in 2.5 mL PDB, and 1.0 mL of various concentrations (1.0, 2.5, 5.0, 10.0 and 20.0 mg/mL) of FCVs aqueous and methanolic extracts (Periyanyagam et al., 2013). Untreated control contained distilled water with no extract, while the positive control contained 1.25 mg/mL methotrexate. These were incubated at 37°C for 24 h. Then, 100 µL of mixture was pipetted and thoroughly mixed with equal volume of 0.1% methylene blue dye. Cell viability was counted using a hemocytometer. Percentage cell viability was calculated as follows:

$$\text{Viable} = (\text{average number of viable cell in one square} \times 2 \times \text{dilution factor} \times 10^4)$$

$$\text{Percentage of cell survival} = \left(\frac{\text{total viable cells}}{\text{total number of cells}} \right) \times 100 \%$$

Results

Effects of extracts of fermented C. vulgaris on mitotic activity in A. cepa meristematic root cells

The data obtained in this study revealed that the aqueous (Table 1) and methanolic (Table 2) extracts of FCVs at the various concentrations negatively affected cell division in treated bulbs unlike the untreated cells. Again, the reduction was directly proportional to the extract concentrations in both cases. The cell division was completely halted at higher concentrations (10 and 20 mg/ml) of the two extracts as no dividing cell was observed. Actively dividing cells in all the stages of cell division were observed in cells grown in water (negative control) while sodium benzoate (positive control) showed a total inhibition of mitosis in *A. cepa* meristematic root cells.

Table 1: Cell count and mitotic indices in *A. cepa* meristematic root cells exposed to various concentrations of aqueous extract of fermented *C. vulgaris* seeds

Treatment	Prophase	Metaphase	Anaphase	Telophase	Mitotic Index (Mean \pm SEM)
0.0	1825	75	100	115	28.38 \pm 4.58*
2.5	215	40	20	25	5.93 \pm 2.17*
5.0	75	0	5	5	2.67 \pm 1.28*
10.0	0	0	0	0	0
20.0	0	0	0	0	0
Sodium Benzoate	0	0	0	0	0

Notes: *p < 0.05 statistical difference between FCVs extracts, negative and positive controls 0.25M Sodium benzoate = positive control

Table 2: Cell count and mitotic indices in *A. cepa* meristematic root tip cells exposed to various concentrations of methanolic extract of fermented *C. vulgaris* seeds

Treatment	Prophase	Metaphase	Anaphase	Telophase	Mitotic Index (Mean \pm SEM)
0.0	1825	75	100	115	28.38 \pm 4.58*
2.5	170	45	5	90	3.93 \pm 0.65*
5.0	35	25	0	0	1.12 \pm 0.32*
10.0	0	0	0	0	0
20.0	0	0	0	0	0
Sodium Benzoate	0	0	0	0	0

Additionally, all normal stages of cell division were observed in untreated and low concentrations-treated cells even though there was reduction in mitotic indices in the latter (Figure 2). Chromosome aberrations were observed in the bulbs treated with 5 mg/ml aqueous extract. The chromosomes were observed

to be more contracted and some cells were more elongated than observed in untreated cells. Laggards were also noted. At 20 mg/ml, the cells were more elongated and distorted with no dividing cells in aqueous and methanolic extracts, respectively, resulting in the death of the cells (Figure 3).

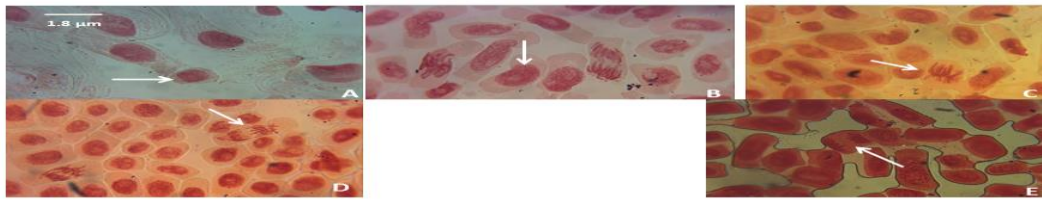


Figure 2: Normal stages of cell division in untreated actively dividing *A. cepa* meristematic root
 A. Interphase (arrowed)
 B. Prophase (arrowed)
 C. Metaphase (arrowed)
 D. Anaphase (arrowed)
 E. Telophase (arrowed)

Figure 2: Normal stages of cell division in untreated and low-concentration treated *A. cepa* cells

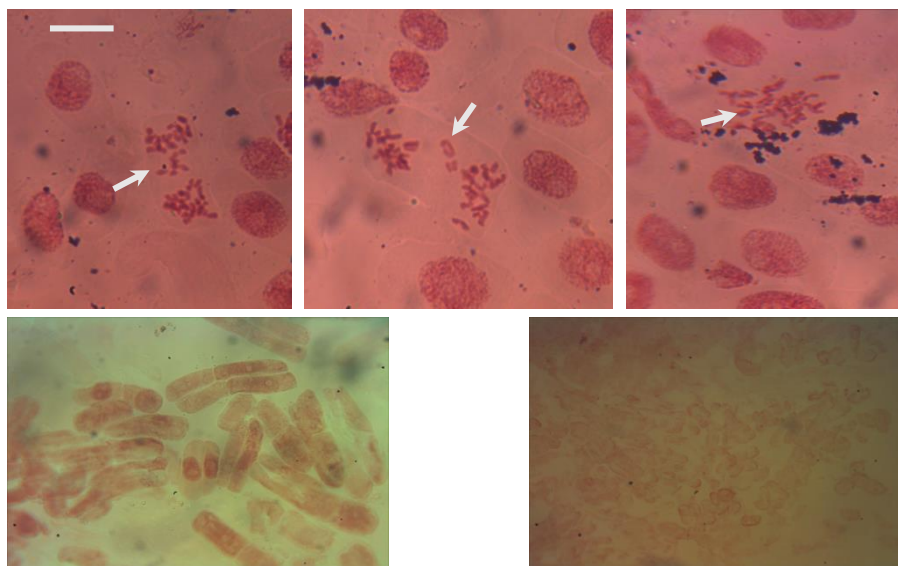


Figure 3: Chromosomal aberrations in *A. cepa* root cells exposed to aqueous and methanolic FCVs extracts

Anti-proliferative activity of FCVs extracts

There was statistically significant reduction in the rate of proliferation of cultured *S. cerevisiae* cells exposed to FCVs extracts for 24 h as concentration of the extract increased compared to the untreated

control. Proliferation of yeast cells was significantly inhibited at all the concentrations of aqueous extract (Figure 4) examined. However, activity of the methanolic extract was better described as oscillatory; the significant activity observed at the first two concentrations dropped at the next two and then picked up again at the highest concentration (Figure 5).

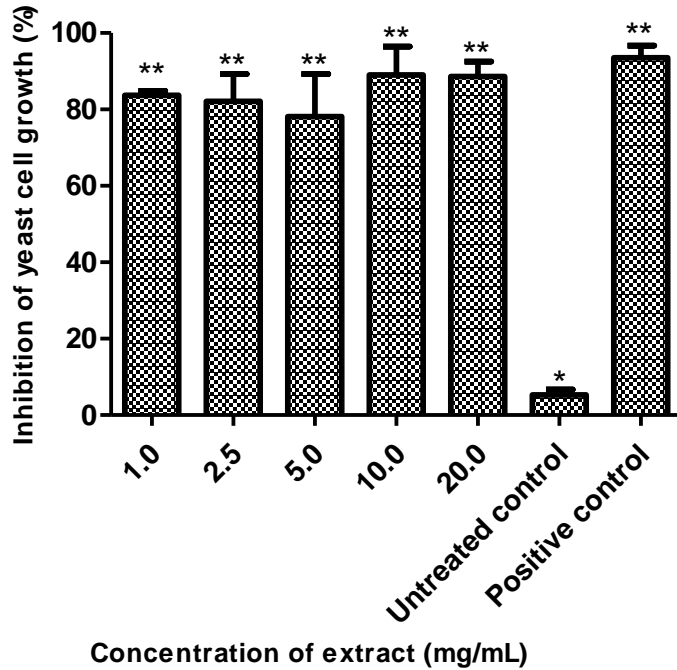


Figure 4: Yeast cells proliferation under the influence of aqueous extract of FCVs. Values represent Mean \pm SEM of three independent readings. **is significantly different from the untreated control (*) ($p < 0.05$).

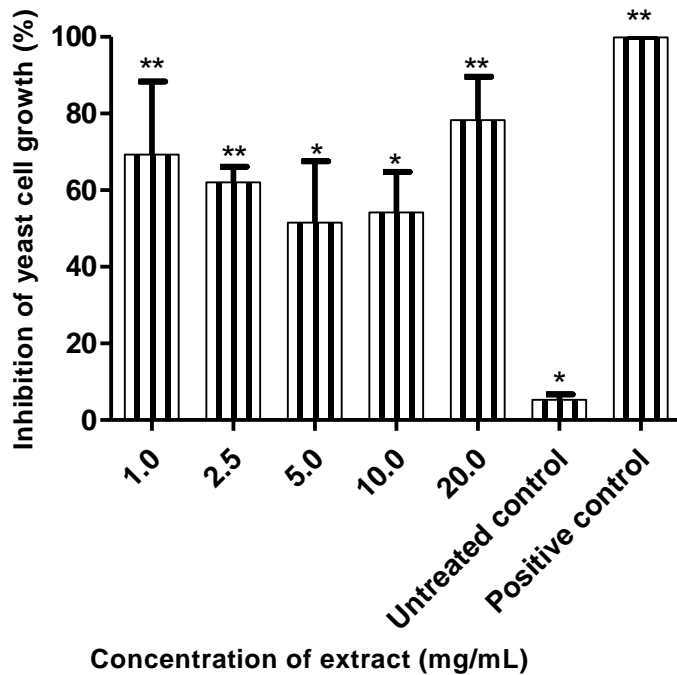


Figure 5: Yeast cells proliferation under the influence of methanolic extract of FCVs. Values represent Mean \pm SEM of three independent readings. ** is significantly different from the untreated control (*), while * is not significantly different from the untreated control ($p < 0.05$).

Discussion

The *A. cepa* assay is an ideal bio-indicator useful in the identification of the alterations

occurring over a cell cycle because the samples are exposed to cells in constant mitotic division (Bagatini et al., 2009).

Furthermore, the reliability of the test system to evaluate the genotoxic potential of medicinal plants has been established (Knoll et al., 2006; Solange and Haywood, 2012) because the test uses a model that is adequately sensitive to detect numerous substances that cause chromosomal alterations.

This study demonstrates that aqueous and methanolic extracts of fermented *C. vulgaris* seeds inhibited cell division and hence growth in the actively dividing roots of *A. cepa* cells. This was indicated by the reduction in mitotic index, which was long established as an important criterion or indicator of adequate growth and proliferation of tissues (Walker, 1954; Gadano et al., 2002). Furthermore, the aqueous extract was genotoxic to the chromosomes leading to the induction of some aberrations. Higher concentrations of the extract induced aberrations in the chromosomes leading to cell death. The genotoxic potential of various extracts from plants have been previously reported and established using this model (Khanna and Sharma, 2013; Periyannagam et al., 2013; Akinpelu et al., 2019). Induction of aberrations in dividing chromosomes as observed in this study has important biological consequences as it results from wide arrays of DNA changes resulting from cellular exposure to genotoxic agents (Obe et al., 2002). Put together, these results suggest that treatment with the aqueous and methanolic extracts of FCVs impair the integrity of the mitotic materials. A prominent feature of cancer is constant proliferation of cells; anti-mitotic agents are used in cancer management to inhibit abnormal proliferation of transformed cells.

Cell-based assays are important fundamental validation steps and are generally important in the identification of cytotoxic compounds (Barberis et al., 2005). Cancer therapy, involving cytotoxic drugs, kills cells that have a high basal level of proliferation and regeneration (Feitelson et al., 2015). Yeast cells-based assays have been described as excellent model for the cytotoxic screening of natural products due to the genetic malleability and a high degree of relatedness between yeast

cellular (cell cycle machinery) processes and those of human cells (Barberis et al., 2005; Qaddouri et al., 2011).

In this study, aqueous extract of FCVs significantly inhibited proliferation in yeast cells at all the concentrations examined compared with control. The methanolic extract showed statistically significant activity compared with control at the lowest (first and second) as well as the highest concentrations. While the highest concentration showed the most potent activity. The results suggested that the extracts of *ogiri-egusi* possess antiproliferation activity in yeast cells.

Conclusion

The results of this study revealed that the aqueous and methanolic extracts from *ogiri-egusi* possessed cytotoxic and genotoxic potentials in the two models adopted in this study. These valuable findings require further investigations for more potent nutraceuticals from the condiments. Extract concentrations used in this study are not in any way comparable to the quantity of the condiment used for cooking. Studies are ongoing to investigate the potential activities of these extracts on cancer cell lines and possible apoptotic activity.

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