ANTIOXIDANT SCREENING AND CYTOTOXICITY EFFECT OF TIGERNUT (CYPERUS ESCULENTUS) EXTRACTS ON SOME SELECTED CANCER-ORIGIN CELL LINES

Elom Seyram Achoribo 1, 2, Ming Thong Ong 1

1. Institute for Research in Molecular Medicine, University Sains Malaysia, Pulau Pinang, Malaysia

ARTICLE INFO

Article history:  
Received 12 September 2018  
Revised 25 November 2018  
Accepted 07 January 2019

Keywords:  
Antioxidant activity, cytotoxicity, phenolic compounds, flavonoids content, cancerous cell lines.

ABSTRACT

Cyperus esculentus is an underutilized crop that has been studied mainly for its nutritional. It contains compound such as Quercetin, beta-sitosterol that is known to have anti-cancer properties. Thus, this study sought to find its antioxidant ability and cytotoxic effect on few cancer cell lines. Qualitative tests were performed to determine the functional groups present in the extracts. DPPH and Aluminum Chloride assays, Folin and Ciocalteau Reagents were used for the antioxidant activity. Finally, MTT assay was used for cytotoxicity effect. Be extract gave the highest percentage inhibition of 48.52%. The IC50 value was 12.68 GAE mg/g. There was no significant difference in the relative percentage of cell viability obtained from both extracts, but rather at the different time of treatment. The extracts showed an anti-proliferative effect on MCF7 and MDA-MB-231 cell lines. The presence of flavonoids, phenol content and sterols might be involved in the cytotoxicity effect of the extracts on the cell lines. The cells were mostly affected at 48-hour post-treatment suggesting that the active compounds are depletable after 48h. Also, the extracts anti-proliferative activity is related to the characteristics of the cell lines investigated. The aqueous extracts of Tigernut are a good candidate for the formulation of anti-cancer treatments.

© EuroMediterranean Biomedical Journal 2019

1. Introduction

Cyperus esculentus is one of the underutilized and widely distributed plants in subtropical and tropical regions. It can be consume draw as a snack, processed into flour, oil or milk. It is used in food catering, crop industry and as a substitute in animal feed.

In Ghana, Tigernut is mainly used as a snack. Tigernut milk is one of the main products found in the market especially in Spain where it is commonly known as Horchata. In Africa, the same milk is prepared as porridge and beverage, especially in Nigeria.

Tigernut is found to assist in reducing the risk of colon cancer with its high content of dietary fiber, vitamins, minerals, and starch (1, 2). It is also known to be a good carrier of unsaturated-fatty-acid-rich oil (3) because of its fiber content. Epidemiological study on the influence of fiber on breast cancer has shown that the fiber content of Tigernut was a contributing factor in the reduction of the risk from getting breast cancer (4). Calcium, one of the minerals content of Tigernut has also been reported to have a reduced risk of polyps’ recurrence in the colon after earlier treatment in colon cancer patients (5).

The high content of oleic acid in Tigernut may help in the prevention of breast cancer (6). Oleic acid inhibits the overexpression of Her-2/neu, by interacting synergistically with anti-Her-2/neu immunotherapy thus aiding apoptosis in breast cancer cells. Moreover, oleic acid has also been found to be a modulator of tumor chemosensitivity in paclitaxel-based therapy (6).

Most works have been focused on the physiochemical, phytochemicals, mineral contents, organoleptic properties, nutritional values, and microbial load of Tigernut (7-10). Also, Tigernut has been used as nutritional- and texture- value adding an ingredient in food products like bread, cake, candies, drinks, yogurt. The effect of Tigernut on copulatory behavior, sperm parameters, testosterone level, anti-diabetic and anti-sickling
parameters were also explored (11–13). Few works, however, have been documented on the anticancer effect of Tigernut. Thus, this study sought to investigate the cytotoxicity effect of Tigernut milk and its aqueous-ethanolic extract on various cancer-origin cell lines. An examination of the phytochemical contents and antioxidant activities of the extracts was conducted to establish the possible relationship between these parameters and cytotoxicity effects found in cell-based assays.

2. Methods

Samples preparation

Cyperus esculentus Linn has been identified and registered with the University of Ghana herbarium, with registration number EL001. Two types (black and yellow) of the fresh nuts were collected, washed separately with distilled water and sun-dried. The dried nuts were milled to a powder, stored in sterilized bottles, labeled and kept at -20°C till further use.

One hundred grams of each type of the nuts were blended three times with 500 ml distilled water and sieved using filter bags of size approximately 9.5 x 7.0 cm to obtain the milk. The Tigernut milk was frozen at -80°C and freeze-dried to obtain the milk powders. The aqueous-ethanolic extract was prepared by macerating 100 g of the crude powder of each type of milled Tigernut with 70% Ethanol and the solution concentrated using a rotary evaporator. The concentrated solution was then frozen at -80°C and freeze-dried to obtain the aqueous-ethanol powder.

Extracts were labeled Bm (Black color Dry Tigernut milk); Sm (Small Roasted Dry Tigernut milk); Ye (Yellow colour Dry Tigernut milk); Be (Black colour Dry Tigernut 70% ethanolic extract); Se (Small Roasted Tigernut 70% ethanolic extract); Ye(Yellow colour Dry Tigernut 70% ethanolic extract). These were then stored at 4°C till used.

Phytochemical analysis

Qualitative analysis was run for the following compound groups: tannins, phlobatannins, saponin, flavonoids, phenolics, terpenoids/steroids, sterols, cardiac glycosides, anthraquinones, flavones, flavanones, and reducing sugars.

DPPH free radical scavenging assay

The antioxidative activity of the different Tigernut extracts was determined by the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay with some modifications as previously described (14). 100µLoF 1mg/ml extracts were each mixed with 3900 µL of 0.004% DPPH• in methanol solution. After 30 minutes of incubation at room temperature, the reduction in DPPH• free radical was measured at 517nm against Gallic Acid as standard, expressed in percentage inhibition and IC50 value.

Total phenolic content

Total phenol content was quantified using Folin-Ciocalteu Reagent (15) with some modifications. 50 µL portion of 1mg/ml samples, were each mixed with 3 mL of distilled water (dH2O) and 250 µL of a 1 in 10 diluted Folin-Ciocalteu phenol Reagent. The mixtures were then allowed to stand for 5 minutes, after which 750 µL of 20% Na2CO3 was added. This was thoroughly mixed, incubated for 30 minutes at room temperature and absorbance measured at 760 nm using a UV-VIS Spectrophotometer. Total phenolic content in each extract was then expressed as milligram Gallic equivalent per gram of spices sample (mg GAE/g).

Total flavonoid content

The flavonoid content was quantified using Aluminium Chloride Assay (16) with some modifications. An aliquot of 500 µL extract of 1mg/ml concentration was mixed with the following, 1500 µL of 99.9% ethanol, 100 µL of 1 M potassium acetate, 100 µL of 10% Aluminium Chloride and 3000 µL of distilled water. The resulting mixtures were then incubated for 30 minutes at room temperature and corresponding absorbance measured at 415nm. Flavonoid content of each extract was expressed as microgram Quercetin equivalent per gram (µgQE/g).

Cytotoxicity of the extracts on MCF7 and MDA-MB-231

The cytotoxic effect was measured using standard 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay after the cells have been treated with the ethanolic and milk extracts of Tigernut respectively for various time periods (24, 48 and 72 hours).

The assay was developed based on the method described by (17). Two thousand five hundred cells were seeded into each well of 96-well plates and cultured in the CO2 incubator at 37°C. After 24 hours, the cells were treated with various serial-diluted concentrations of Tigernut extracts and returned into the CO2 incubator at 37°C. The absorbance (OD) at 570 nm was read on a spectrophotometric plate reader (Multiskan spectrometer, Thermo Electron Co., Waltham, Massachusetts, USA). The proportion of surviving cells was calculated as: [(OD of the drug-treated sample - OD of blank)/(OD of control - OD of blank)] x100%.

All experimental data were derived from at least 3 independent experiments.

3. Results

Phytochemical Tests

The tests were conducted, and the results are summarized in Table 1.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Phenols</th>
<th>Flavonoids</th>
<th>Steroids</th>
<th>Saponins</th>
<th>Terpenoids</th>
<th>Sugars</th>
<th>Reducing Sugars</th>
<th>Alkaloids</th>
<th>Anthraquinones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bm</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sm</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ye</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Be</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Se</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Legend: + present; - not detected; Bm - Black color Tigernut milk; Sm - Small roasted Tigernut milk; Ye - Yellow color Tigernut milk; Be - Black color Tigernut ethanolic extract; Se - Small roasted Tigernut ethanolic extract.

Table 1 - Phytochemical contents of Ethanol and Aqueous extract of Tigernut

Out of the 10 phytochemicals investigated, 5 were found to be present in Ye, 4 in Be, and 3 in the other samples. All extracts contained flavonoids.
and phenolics however anthraquinones, reducing sugar, and phlobatannins were not detected in all the samples. Sterol was detected in all extracts apart from Be. Glycosides were present only in the Ye extract. Saponins were found in all ethanolic extracts and Bm. Terpenoids/steroids were detected in Be, Ym and Sm extracts. In general, the main phytochemical compounds present in both extracts are flavonoids, phenolics, and sterols. Saponins and tannins are specific to ethanol extract while terpenoids/steroids are to the aqueous extracts.

**DPPH free radical scavenging assay**

The percentage inhibition (I %) of the various samples was calculated using the following formula:

\[ I \% = \frac{Abs(\text{blank}) - Abs(\text{sample})}{Abs(\text{blank})} \times 100 \]

Be extract has been identified to possess the highest percentage inhibition at 48.52%. The IC50 of the extract calculated from the equation \( y = 4.901x - 12.258 \) obtained from Figure 1 gave a concentration of 12.68 GAE mg/g. The DPPH scavenging activity was also depicted in figure 1.

**Total Phenolic (TPC), and the Total Flavonoid (TFC) contents of Tigernut extracts**

Figure 2 and 3 depict the total phenol and flavonoids contents of both ethanol and aqueous extracts. In Figure 2, all extracts have shown a level of phenol content with Be giving the highest value. Both solvents had an affinity for phenolic compounds while the aqueous extracts presented more flavonoids content (Figure 3). The black and roasted Tigernut contain more phenolic compounds than the Yellow Tigernut. Also, the aqueous extracts contained more flavonoids compared to the ethanol extracts. Likewise, Sm and Bm’s flavonoid contents are higher than Ym.

**MTT assay**

Different types of cells lines were first screened and found to be more susceptible to the treatment of aqueous extracts compared to the ethanol extracts (data not shown). Also, the extracts were more toxic to the breast cancer-origin cell lines. Thus, the results below showed the effect of the aqueous extracts of Tigernut on breast cancer-origin cell lines (MCF7 and MDA-MB-231) at lower concentrations (0-25µg/ml).

A sharp decrease in the percentage of cell viability at extract concentration of 6.25µg/ml was observed, and the percentage of viability stayed almost constant with increasing concentration. MCF7 and MDA-MB-231 cells were affected at different incubation time points. MDA-MB-231 was affected most at 24hours post-treatment (Figure: 4) while MCF7 was affected at 48-hours post-treatment (Figure 5).
At 72-hours post-treatment, the extracts showed little effect on the cell viability (Figure 6).

At 24h post-treatment, both Ym and Bm extracts cause cytotoxicity to MDA-MB-231 with a cell death percentage of approximately 40% which remained constant with increasing concentrations (Figure 4). Compared to MDA-MB-231, MCF7 cell lines were mostly affected by Ym after 48h post-treatment with 52% and 40% cell death at 3.125 µg/ml and 12.5 µg/ml respectively. Only a decrease of about 20 to 28% was observed in the percentage of the cell viability at 72-hours post-treatment for both cell lines (Figures 5 and 6).

4. Discussion

The Sterols are found in the aqueous extracts. In literature, sterols had been shown to possess anti-cancer effects against ovary cancer (18), estrogen-dependent human breast cancer (19), lung cancer (20) and stomach cancer (21). Also, according to Mercian and colleagues (22), phytosterol may inhibit cell growth, cell metastasis, cell invasion activities and stimulate apoptosis in cancer-origin cell lines. It is, therefore, possible that the decrease in cell viability seen in MCF7 might be attributed to the presence of sterols. Example of one of these sterols that affect MCF7 breast cancer is beta-sitosterol (23).

Flavonoids were found to be present in all extracts. These are well-known antioxidants (24) especially quercetin which is found to reduce breast cancer risk in a cohort study (25). They are also known to have anti-angiogenic, anti-inflammatory(26, 27, 28), apoptotic, anti-allergic effects, cytostatic, estrogenic and anti-estrogenic effects. According to Primiano and colleagues (29), flavonoids might slow down cell proliferation because of their binding to the estrogen receptor. Our results have indicated an anti-proliferative activity of the extract on most of the cell lines investigated.

In MCF7, an estrogen receptor positive cell line, a significant decrease in cell viability was detected between 6.25 µg/ml and 25 µg/ml of extract concentrations, and the viability was maintained at that level thereafter independent of the increase in the concentration of the extract. Alternatively, flavonoids can affect cancer cells by triggering the process of apoptosis (30) and are also potent inhibitors of mitogen signaling processes via affecting various kinase activities (31, 32, 33).

The extracts have shown some level of free scavenging activity (Figure 1) which might be due to the presence of the phytochemical mentioned above (Table 1). The aqueous extracts had more affinity for flavonoids content (Figure 3). The extractability of a component depends generally on the extraction medium polarity and the ratio of solute to solvent. Therefore, the difference observed in the concentration of the flavonoids content might be due to the high polarity index of the aqueous solution (PI=9). Also, phenolic compounds are often extracted in higher amounts in more polar solvents, therefore, the presence of polyphenols in both extracts (Figure 2). Further, the solubility of polyphenols mainly depends on the presence and position of hydroxyl groups, the molecular size and the length of constituent hydrocarbon chains. Thus, the ability of the water extracts (Ym, Bm, Sm) to have almost the same total phenol content might be attributed to a similarity in the structural composition of the phenols present in the different species of Tigernut used. The properties and characteristics of the phenols present in each might be considered as well.

Bivariate correlation analysis showed that Ym has a strong negative correlation of 0.999 between the free radical scavenging activity index (DPPH) and the total phenolic content index (TPC), p = 0.026. This means that DPPH activity increases with decrease content of TPC with Ym extract. Consequently, it can be said that polyphenols content does not play much role in the free scavenging activity observed. No correlation was observed within the rest of the groups. The linear regression analysis also showed that TPC, TFC, and DPPH values are typically negatively related (negative beta coefficient).
Below are the equations obtained:

\[ y = -0.468x + 24.347, r^2 = 0.999 \text{ for Ym;} \]
\[ y = -0.351x + 25.09, r^2 = 0.964 \text{ for Sm;} \]
\[ y = 0.11x + 8.56, r^2 = 0.934 \text{ for Bm;} \]
\[ y = -2.75x + 32.16, r^2 = 0.564 \text{ for Ye;} \]
\[ y = -0.29x + 124.85, r^2 = 0.729 \text{ for Be;} \]
\[ y = 0.26x + 25.66, r^2 = 0.912 \text{ for Se.} \]

The MTT assay showed a relative percentage of cell viability between 80% and 49%. The ability of the extracts to inhibit the cells proliferation was mostly affected by the incubation period. An effect seen at 24h showed an immediate effect of the extract to initiate the expression or inhibition of genes involved in cell growth. As time increases, the extracts might be more available to the cells by undergoing some modifications either by oxidation and/or polymerization that render them more cytotoxic to the cells. For example, flavonoids that are present in all extracts can interact with protein through their phenolic nucleus. This interaction can either develop a strong dispersion or electrostatic interactions with non-polar amino acid residues followed by the simultaneous release of water. As a result, there is scavenging of reactive oxygen species, autoxidation, and/or enzymatic reactions. It may also be that the extracts have already initiated the reaction but instead of the first order product causing the observed effect, the second order reactions did.

The difference in the cytotoxicity effect within each category of cell lines might be due to the characteristic of each cell lines. For instance, MCF7 is affected most at 48h which may be due to its being an estrogen receptor positive (ER+) and p53 wild-type cell line while MDA-MB231 an estrogen receptor negative (ER-) and p53 mutant cell line, was affected at 24h. p53 is a gene that mostly affects cell proliferation and its expression halts the cell cycle and favors apoptosis. p53 is also a redox-sensitive protein, so any redox imbalance that might be induced by the extracts might affect the function and/or structure of the mutant p53 protein thus promoting cell death. In addition, ER+ cell liaises directly with cyclin D1 which initiates the cell cycle.

Moreover, the increase in concentration (dose) of the extracts did not further augment the killing effect on the cells but rather keep them at an almost constant level. Thus, the extracts have an antiproliferative effect on the cell lines, suggesting their effect on the cell cycles. Flavonoids, tannins, saponins, and sterols are seen to affect cell proliferation of the studied cell lines (34, 35, 36). In addition, they are known to affect MAPK/ERK and PI3K/AKT signaling pathways involved in cell survival, and progression (37, 38). Furthermore, these compounds activate proteins involved in apoptosis such as CASP3, CASP9 and inhibit BCL2 (37, 39).

Therefore, the extracts are suspected to provoke the antiproliferative effect via the inhibition of the mentioned cell growth-specific pathways.

4. Conclusions

The present work has shown that the aqueous extracts of Tigernut could be used in the management of breast as an adjuvant treatment due to its anti-proliferative effect. The presence of flavonoids and sterols might be involved in the cytostatic effect observed. The percentage of cell death accrued seems not to be much affected by the increase in concentration. The extracts effect on the cell lines reduced at 72-hours post-treatment, suggesting that, the active compounds are depleatable after 48h. Also, the difference in the time of incubation showed that the extract effect on the cell lines depends on the cells characteristic.

Acknowledgements

The work was supported by Universiti Sains Malaysia. Elom Seyram Achoribo is recipient of The World Academy of Sciences-Universiti Sains Malaysia (TWAS-USM) fellowship.

References

12. Monaco CC Uwakwe AA. Proximate composition and in-vitro anti sickling property of Nigerian Cyperus esculentus (Tigernut sedge); Trees for Life Journal 2009; 4:2


