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Original Article

In vitro antiproliferative studies of extracts of the marine molluscs: Tympanatonus fuscatus Var radula (linnaeus) and Pachymelania aurita (muller)

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Abstract: This study aimed to investigate the antimitotic and antiproliferation activities of crude acetone-methanol and aqueous extracts of two marine molluscs commonly found in the Niger Delta region of Nigeria; *T.fuscatus* and *P.aurita*, against human cancerous cell lines (DU145, Hep-2, and HCC1395) cell lines *in vitro*. The antimitotic activity of the extracts was evaluated using *Allium cepa* root meristematic cells. Antiproliferative activity of the plant extracts against the cancerous cell lines was compared with normal cell line (VeroE6). Doxorubicin was used as a positive control. Gene expression studies using qPCR for the proapoptotic genes, CASP3, CASP8 and P53 were also carried out. The alcohol extract of *T.fuscatus* (TFAC) exhibited the most promising activity against all the cancer cell lines tested (DU145 IC_{50} = 96.48 ± 1.36 µg/ml, HCC 1395 IC_{50} = 61.44 ± 2.45 µg/ml, Hep2 IC_{50} = 0.52 ± 0.36 µg/ml) and also had the highest selectivity index of 4.94, 7.78 and 921.97 for DU145, HCC 1395 and Hep-2 cells respectively. Furthermore, TFAC was the only extract that significantly upregulated the expression of caspase 3, caspase 8 and P53. Thus, these findings suggest potential exploitation of TFAC as an anticancer agent.

Keywords: Tympanatonus fuscatus, Pachymelania aurita, allium cepa, Hep2, DU145, HCC1395, Vero E6, antimitotic, antiproliferative, CASP3, P53

Introduction

Cell division is a normal and routine physiological process that occurs in tissues. A balance between proliferation and programmed cell death (apoptosis) is generally maintained by tightly regulated processes under normal physiological conditions. Certain mutations in DNA caused by agents (chemicals and radiation) and disruptions in the programmed and tightly regulated processes lead to cancer. Carcinogenesis is a process by which normal cells are transformed into cancer cells. It is characterized by a progression of changes at both, cellular and genetic level, that reprogram a cell to undergo uncontrolled division, thus forming a mass or tumour that can spread to other distant locations in the body via metastases [1]. Cancer is one of the leading causes of death in the developed world [2-4]. Many of the chemotherapeutic drugs used for treating cancer actually work by inhibiting cell division (antimitotic activity) and by selectively targeting fast-dividing cells (antiproliferative activity). However, unfavorable side effects and resistance on many anticancer agents have become serious problems [5]. Thus, there is a growing need to develop safe and more effective therapeutic agents for cancer treatment especially from nature-based compounds or dietetic products [6].

Marine organisms have been shown to possess bioactive compounds with anticancer activity. Sponges, coelenterates and microorganisms are the principal sources of these therapeutic compounds. Algae, echinoderms, tunicates, molluscs, bryozoans are also prime sources of

anticancer drugs [7]. *Tympanatonus fuscatus* Var Radula and *Pachymelania aurita* are two common marine mollusc species found in the Niger-delta region of Nigeria, where they are primarily used as a source of protein and although they are used in a number of traditional medicines, no study has been carried out to evaluate their antiproliferative activity. In view of the bioactive compounds with anticancer activity that have been discovered in marine molluscs, this study aims at evaluating the antiproliferative and thus anticancer activity of extracts from both *Tympanatonus fuscatus* Var Radula and *Pachymelania aurita*.

Materials and methods

Reagents and chemicals

Doxorubicin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reagent, dimethyl sulfoxide (DMSO), Agarose, Glutamine, penicillin, streptomycin and trypsin were obtained from Sigma Aldrich. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco, Thermofisher. FIREScript RT Complete Oligo-(dT) cDNA synthesis kit and HOT FIREPol® EvaGreen® (no ROX) qPCR mix kit were obtained from Solis BioDyne, Estonia. 5 min DNA RNA extraction Kit was obtained from BioFactories, Monrovia, CA, USA. 100 bp ladder was obtained from New England Biolabs, MA, USA.

Cell lines

The permanent human Laryngeal carcinoma (Hep2), Human prostate carcinoma (DU145), Human breast carcinoma (HCC1395) and Kidney epithelial cells from African Green monkey (Vero E6) were obtained from American Type Culture Collection, (ATCC; Rockville, MD, USA). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 µg/mL penicillin and streptomycin and were cultured or maintained in an incubator at 37°C under a humidified atmosphere of air containing 5% CO_2.

Sample collection and authentication

Live *Tympanotonus fuscatus var radula* and *Pachymelania aurita* were purchased from the Oron Beach Market, Oron, Akwa Ibom State, Nigeria (GPS coordinates: 4°49'37.6''N 8°14'04.4''E) in August. Molluscs were transported alive to the laboratory, where they were washed thoroughly to remove mud and then deshelled to collect both the flesh and hemolymph. Samples were authenticated morphologically, before deshelling, by a zoologist in the Department of Zoology, Obafemi Awolowo University, Ile-Ife, Nigeria.

Extract preparation

The alcohol extracts of *T.fuscatus* and *P.aurita* were prepared using the method described [8]. 200 g of mollusc flesh in its hemolymph was macerated using a blender and extracted twice with 1 L acetone for each cycle. Each cycle of extraction with acetone was carried out overnight at room temperature with constant stirring using a magnetic stirrer and the homogenate was filtered using a muslin cloth. After acetone extraction, the biomass residue of the sample was subjected to two cycles of extraction using a total of 1.500 L of methanol. The Acetone and methanol fractions were combined and concentrated by evaporation using a rotary evaporator at 40°C then stored at 4°C.

The aqueous extract was prepared by homogenizing 500 g of mollusc flesh in its hemolymph with 2 L of Phosphate buffered saline (PBS, pH 7.2 with 0.1 M PMSF) using a blender. The homogenate was left to extract for 48 hrs at 4°C after which it was centrifuged at 10,000 g using a cold centrifuge and then freeze dried. The freeze dried powder was stored at 4°C, until needed.

Antimitotic assay

Evaluation of the antimitotic activity of the extracts of *P.aurita* and *T.fuscatus* was carried out using the *Allium cepa* assay [9]. *A.cepa* bulbs were grown in distilled water at room temperature for 48 hrs, after which the bulbs were treated with different concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1 and 3% v/v of extract in distilled water) of the crude extracts of *T.fuscatus* and *P.aurita* for 24 hrs. Bulbs grown in 0.25 M sodium benzoate were used as positive control while bulbs grown in distilled water were used as negative control. Root tips from each bulb was harvested, fixed in Carnoy's fixative (1:3 acetic acid: ethanol v/v) for 24 hrs. The root tips were then washed thoroughly with distilled

water and hydrolysed with 18% HCl at room temperature for 5 mins. After hydrolysis, the roots were washed and the Meristematic layer cut and placed on slides. One drop of aceto-orcein was dropped on the root tips and left for 2 mins, after which the root tips were crushed using a metal rod and another drop of aceto-orcein added and left for another 2 mins. The cover slips were then carefully lowered on to the slides to avoid air bubbles and the slides thus prepared were observed using an Olympus Light Microscope, with a camera attachment, at 40× magnification to count cells and observe for chromosomal aberrations. Five slides were observed for each concentration and photomicrographs were made. The Mitotic index was determined using the calculation:

$$\text{Mitotic index (MI)} = \frac{\text{Number of cells in mitosis}}{\text{Total number of cells}}$$

Cytotoxicity assay

The cytotoxicity of the extracts of both *Tympanotonus fuscatus var radula* and *Pachymelania aurita* were assessed against VeroE6 cells by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) as the chromophore [10]. Briefly, following three to four cycles of subculturing, the Vero E6 cells were seeded into 96-well plates at 5000 cells/well and left to incubate for 24 hrs, followed by treatment with crude extracts of *T.fuscatus* and *P.aurita* at concentrations starting from 100 µg/ml and making a 3-fold serial dilution. Well A served as no drug control. Cells used as positive controls were treated with doxorubicin. The cells were left to incubate for 48 hrs after which 10 µL of freshly prepared 5 mg/ml MTT stock solution was added to each well and left to incubate for 2 hrs. After incubation, the media was discarded and 50 µL of DMSO was added to each well. Each plate was analysed by reading the absorbance at 570 nm. % Cell survival was calculated using the formula:

$$\text{Survival\%} = 100 \times \frac{\text{Absorbance of treated cells} - \text{Absorbance of culture medium}}{\text{Absorbance of untreated cells} - \text{Absorbance of culture medium}}$$

Results are presented and compared using the IC_{50} concentration values. The IC_{50} values were calculated using Graphpad Prism software (version 7) by constructing a scatterplot of the mean responses to different concentration of extracts (Survival %) against the logarithm of concentration. A sigmoid curve was fitted. The equation used was: dose-response; log (inhibitor) vs. response-3 parameters: Y = Bottom + (Top-Bottom)/(1+10^(X-LogIC_{50})). The selectivity index (SI) was also calculated from the IC_{50} ratio of normal epithelial (veroE6) and cancerous cells.

Antiproliferation assay

Evaluation of antiproliferative activity of the extracts and peptide fractions of *T.fuscatus* and *P.aurita* was carried out with cultured Hep2, HCC1395 and DU145 cells using the MTT Cell Proliferation Assay previously described.

Gene expression studies

The effect of the extracts on the expression of CASP3, CASP8 and P53 genes was evaluated using a Real Time PCR (qPCR) method [10]. 80% confluent Hep2 cells, in T75 flasks, were treated with crude extracts of *T.fuscatus* and *P.aurita* at concentrations equivalent to the calculated IC_{50}. Negative control cells were exposed to fresh growth media. The cells were incubated for 48 hrs after which they were detached using trypsin and total RNA extraction was carried out, using a commercial extraction kit (BioFactories, 5 min DNA), according to the manufacturer's instructions. The purity and concentration of the extracted RNA were determined using a Nanodrop spectrophotometer (Thermofischer). For generation of cDNA, 50 ng/µl concentration of each RNA sample was prepared, 10 µl of which was reverse transcribed using a FIREScript RT Complete Oligo-(dT) cDNA synthesis kit (Solis Biodyne, Tartu, Estonia) according to the instructions provided by the kit manufacturer. qRTPCR of the samples was performed in a LightCycler instrument (Roche Applied Science, USA) using a HOT FIREPol® EvaGreen® (no ROX) qPCR mix kit (Solis BioDyne, Estonia). The amplification reactions were in a total reaction volume of 20 µL containing the PCR mix, 5 µL of cDNA, and gene specific primers (10 pmol for each). Primers were designed using the NCBI primer design tool [11]. The sequences of the primers (Inqaba Biotec, South Africa) used in this experiment were as follows: Caspase 3 forward: 5'-CGGCGCTCTGGTTTTCGTTA-3', Caspase 3 Reverse: 5'-CAGAGTCCATTGATTCGCTTCC-3', Caspase 8-forward: 5'-GGAACTTCAGACACCAGGCA-3',

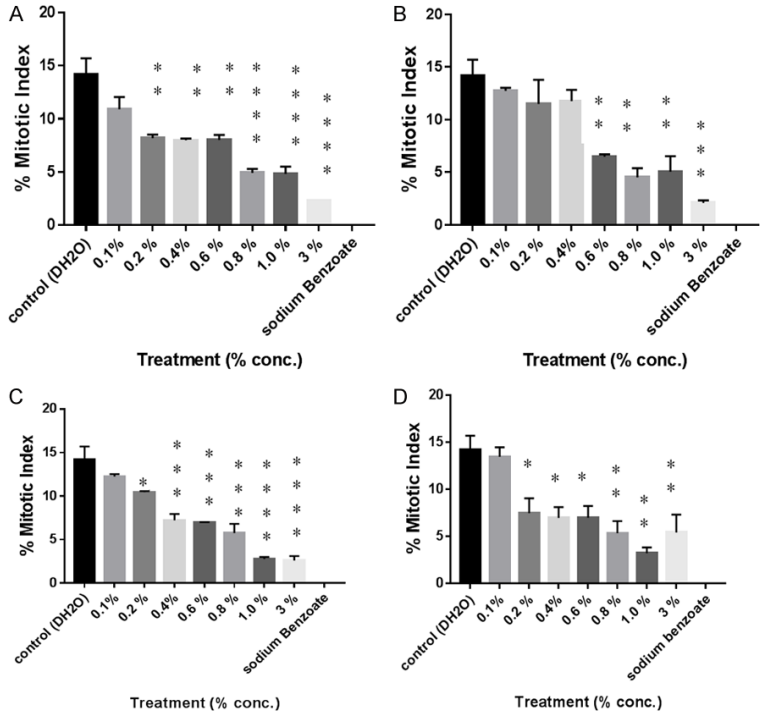

Figure 1. Antimitotic activity as indicated by the mitotic index (% MI) of *Allium cepa* roots treated with PAAQ (A), PAAC (B), TFAQ (C) and TFAC (D). Data is presented as Mean ± SEM. *indicates P < 0.05, **indicates P < 0.01, ***indicates P ≤ 0.001, and ****indicates P ≤ 0.0001 compared to the control.

Caspase 8-reverse: 5'-CCTCCGCCAGAAAGGTACAG-3', P53 forward: 5'-TGACACGCTTCCCTGGATTG-3', P53 reverse: 5'-GTTTTCAGGAAGTAGTTTCCATAGG-3', GAPDH-forward: 5'-GGCCACTAGGCGCTCAC-3', GAPDH reverse: 5'-GGCGCCCAATACGACCAAAT-3'.

The following conditions were used for the PCR amplification reactions: an initial incubation step at 95°C for 12 min, followed by 50 amplification cycles, each one consisting of a denaturation step at 95°C for 15 s, an annealing step at 58°C for 30 s and an extension step at 72°C for 1 min. Following this reaction, a melting curve analysis was performed in order to verify the specificity of the amplicon. The LightCycler software supplied by the instrument manufacturer (Roche Applied Science) was used for processing and analyzing the data. The relative expression of genes was quantitated following the standard 2-Δct calculation using the housekeeping gene, GAPDH, for normalization, where Ct denotes the crossing threshold value calculated.

Results

Antimitotic activity

Actively dividing cells were observed in root cells in the negative control group (distilled water) with a mitotic index of 14.17%. No dividing cell was observed on exposure of *A.cepa* root tip cells to 0.25 M sodium benzoate (positive control). Hence, the mitotic index for the sodium benzoate-treated *A.cepa* cells was 0.0%. The extracts of *P.aurita* and *T.fuscatus* showed antimitotic activity as indicted by a decrease in mitotic index (%) in a dose dependent manner (**Figure 1**). Antimitotic activity was found to be significant at concentrations ≥ 0.2% of PAAQ (8.18 ± 0.33; $P < 0.01$), TFAQ (10.44 ± 0.14; $P < 0.05$) and TFAC (7.47 ± 1.58; $P < 0.01$) while antimitotic activity was found to be significant at concentrations ≥ 0.6% of PAAC (6.48 ± 0.23; $P < 0.01$) when compared with the control mitotic index (14.17 ± 1.52).

Chromosomal abnormalities

No chromosomal abnormalities or mitotic aberrations were observed in the root tips of negative control group. The cells in root tips showed normal mitotic phases including prophase, metaphase, anaphase and telophase (**Figure 2A-E**). However, chromosomal abnormalities were observed in cells of *A.cepa* roots treated with the extracts. The most common chromosomal abnormalities observed were anaphasic bridges. Other abnormalities observed in extract treated root tip cells include formation of binucleolar cells, c-metaphase, micronuclei formation and vagrant chromosomes (**Figure 2F-J**).

Cytotoxicity assay

Treatment of VeroE6 cells with the standard drug doxorubicin (positive control) at a concen-

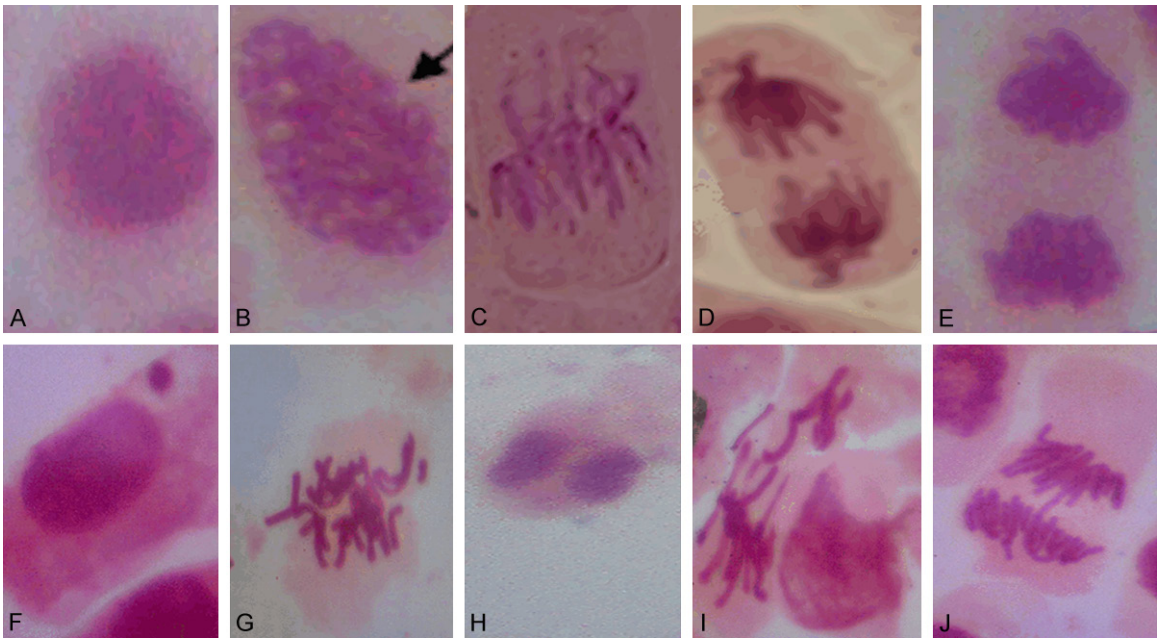

Figure 2. Normal mitotic phases in the control *allium cepa* cells. Phases shown: interphase (A), prophase (B), metaphase (C), anaphase (D), and telophase (E). chromosomal aberrations observed in Allium cepa cells after treatment with varying concentrations of the aqueous and alcohol extract of *P.aurita* and *T.fuscatus* including Micro-nucleus (F), C-metaphase (G), Bi-Nucleated Cell (H), vagrant chromosome (I) and anaphasic bridges (J).

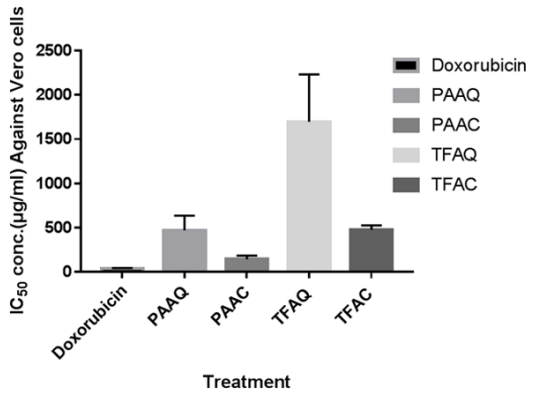

Figure 3. IC_{50} concentrations of the various extracts of *P.aurita* and *T.fuscatus* against the normal (non-cancerous) cells; VeroE6. Doxorubicin was used as standard anticancer drug. Data is presented as Mean ± SEM.

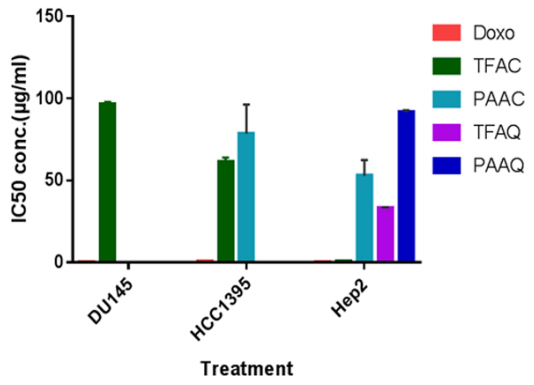

Figure 4. IC_{50} concentrations of the various extracts of *P.aurita* and *T.fuscatus* against cancer cells (DU145, HCC1395 and Hep2). Doxorubicin was used as standard anticancer drug. Data is presented as Mean ± SEM.

tration of 100 µg/ml, resulted in inhibition of cell growth by 98.76% (IC_{50} = 0.98 ± 0.01 µg/ml). The alcohol extract of *P.aurita* (PAAC) exhibited the highest level of cytotoxicity to VeroE6 cells, resulting in a 25% inhibition in cell survival at 100 µg/ml of the extract (IC_{50} = 143 ± 42.73 µg/ml) (**Figure 3**). All other extracts exhibited only slight cytotoxicity to VeroE6 cells. Treatment of Vero E6 cells with PAAQ, TFAQ and TFAC at a concentration of 100 µg/ml led to inhibition of cell survival by 9.98%, 1.11% and 10.4% respectively.

Antiproliferative activity

Even at the highest concentration tested (100 µg/ml), cell survival rates of 100% were recorded for extracts of *P.aurita* and the Aqueous extract of *T.fuscatus* after 48 hrs of treatment

Table 1. Selectivity Index of the various extracts of *P.aurita* and *T.fuscatus* against cancer cells tested, indicating their margin of safety

Treatment	Selectivity Index		
	DU145	HCC 1395	Hep2
Doxorubicin	4.08	1.96	3.77
TFAC	4.94	7.78	921.97
PAAC	-	1.82	2.71
TFAQ	-	-	51.36
PAAQ	-	-	5.147

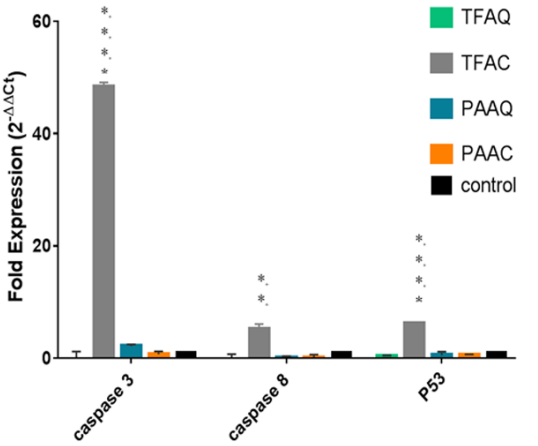

Figure 5. Fold expression change levels of apoptosis-related genes in Hep2 cells after 48 h of exposure to the IC_{50} concentrations of TFAC, PAAC, PAAQ and PAAC against Hep2 cells. Data presented as Mean ± SEM. **indicates $P < 0.01$ and ****indicates $P \leq 0.0001$ compared to the control.

against DU145 cells (**Figure 4**). However, the alcohol extract of *T.fuscatus* (TFAC) exhibited antiproliferative activity against DU145 cells (IC_{50} = 96.48 ± 1.36).

The alcohol extracts of *P.aurita* (PAAC) and *T.fuscatus* (TFAC) alone exhibited antiproliferative activity against HCC1395 cells after 48 hrs of treatment (**Figure 4**). At a concentration of 100 µg/ml, treatment with PAAC resulted in a 63.64% inhibition of cell survival (calculated IC_{50} = 78.41 ± 17.89), while treatment with TFAC resulted in 81.21% inhibition (calculated IC_{50} = 61.44 ± 2.45).

All extracts exhibited antiproliferative activity against Hep2 cells in a dose dependent manner after 48 hrs of treatment (**Figure 4**). The highest activity among all the tested extracts was shown by TFAC with an IC_{50} of 0.52 ± 0.36, while the aqueous extract of *P.aurita* (PAAQ) was the least active (with an IC_{50} of 91.52 ± 1.35).

Selectivity index

Selectivity of the cytotoxic activity of the extracts was determined by comparing the antiproliferative activity IC_{50} of each extract against each cancerous cell with that of the normal mammalian cell line VeroE6 (**Table 1**). A selectivity index greater than 3 was considered as highly selective. TFAC appears to be both the most potent and most selective against all cells tested, with selectivity indexes above 3 in all cases (DU145 IC_{50} = 96.48 ± 1.36 µg/ml, SI 4.94; HCC 1395 IC_{50} = 61.44 ± 2.45 µg/ml, SI 7.78; Hep2 IC_{50} = 0.52 ± 0.36 µg/ml, SI 921.97).

Gene expression of CASP3, CASP8 and P53

Due to the fact that all extracts exhibited antiproliferative activity against Hep2 cells, this cell line alone was used in the gene expression studies. The data show that the expression levels of p53 and caspase-3 and -8 were significantly increased by 6.1 fold, 48.3 fold and 5.1 fold respectively (**Figure 5**), after treatment with the alcohol extract of *T.fuscatus* (TFAC). This suggests that TFAC induced apoptosis by upregulating pro-apoptotic genes. The other extracts (PAAC, TFAQ and PAAQ) did not elicit a significant upregulation (nor Down-regulation) of these apoptotic genes.

Discussion

Cancer is abnormal or unregulated cell growth occurring due to uncontrolled cell division (mitosis) thereby forming tumour masses. Hence, any compound that can slow down or impede cell division in rapidly growing cells has potential as an anticancer therapeutic. The meristematic region in the roots of the *Allium cepa* plant undergoes repeated divisions which have been noted to be similar to the rate of cancer cell division in humans. The *Allium cepa* assay method is widely used for evaluating the anti-mitotic activity of compounds and therefore their antiproliferative potential [2, 12-14]. The decrease in the mitotic index in the roots tip of *Allium cepa* after treatment with the aqueous and alcohol extracts of *T.fuscatus* and *P.aurita* suggests these extracts have the ability to inhibit actively dividing cells.

Furthermore, any agent capable of causing a disturbance in the cell cycle would cause an increase in genomic instability and apoptosis. The chromosome aberrations observed upon treatment with the extracts, which included bridges, C-mitosis and vagrant chromosomes, are an indication of the effect of the extracts in either inhibiting DNA synthesis, spindle formation, inhibition of prophase initiation or blocking G2-phase in the cell cycle preventing the cell from entering mitosis [4, 12, 15]. Micro-nuclei are a result of the development of the isolated chromosome due to an unequal distribution of genetic material [16]. Cells bearing micronuclei stages were observed at interphase and prophase. The most frequent abnormality observed was anaphasic bridges, which may be caused by stickiness of the chromosomes. This stickiness may restrict their free movement and thus their separation becomes incomplete, hence, they remain connected by bridges [15, 17].

The selectivity index (SI) is a ratio that measures the window of safety between cytotoxicity to normal cells and antiproliferative activity against cancer cells [18]. The higher the SI ratio, the more effective and safe a drug would be during *in vivo* treatment. A high selectivity index generally indicates that a compound has reasonable selectivity for binding to the cancer cells over mammalian cells *in vitro.* The alcohol extract of *T.fuscatus* (TFAC) appears to be both the most potent and most selective against the cancer cells tested. TFAC inhibited the growth of all cancer cells tested (and with the lowest IC_{50}) and it had the highest selectivity index for all cancer cells tested. This indicates its potential as an anticancer drug.

Apoptosis is a genetically controlled mechanism of cell death that is essential for the elimination of old or damaged cells during normal development and for the maintenance of tissue homeostasis. Evasion of apoptosis is considered to be one of the hallmarks of human cancers. In this study, the significant upregulation of the P53, CASP3, and CASP8 genes by the alcohol extract of *T.fuscatus* is an indication that it induces apoptosis in the cancer cells (Hep2) via P53 and caspase dependent pathways, while the antiproliferative activity of its aqueous extract and the extracts from *P.aurita* are a result of other mechanisms independent of the P53 and caspase apoptotic pathways as these extracts did not lead to a significant upregulation of the CASP3, CASP8 and P53 genes.

Apoptosis is executed by caspases and several upstream regulatory factors, such as P53, which direct their proteolytic activity [19, 20]. Once activated, p53 activates expression of downstream genes leading to either cell cycle arrest (to allow repair and survival of the cell) or it initiates apoptosis to discard the damaged cell through multiple mechanisms, including transactivation of specific target genes, downregulation of a distinct set of genes, and transcription-independent mechanisms [21, 22]. Several lines of evidence suggest that p53 death signals lead to caspase activation [19, 20, 23, 24]. Activation of caspases ensures that the cellular components are degraded in a controlled manner, with minimal effect to surrounding tissue [25].

Conclusion

The extracts from *P.aurita* and *T.fuscatus* have been demonstrated to possess antimitotic activity and antiproliferative activity. However the alcohol extract of T.fuscatus, TFAC, exhibited the most promising and most selective cytotoxic activity. The mechanism of action of the antiproliferative activity of TFAC can be linked to its upregulation of the pro-apoptotic genes; p53 and caspase-3 and 8.

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Disclosure of conflict of interest

None.

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