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Evaluation of antioxidant and antiproliferative potential of endophytic fungus: *Aspergillus subramanianii*, isolated from *Gyrinops walla* Geartn, a medicinal plant

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Abstract
Natural products have played an important role in cancer therapy. Also, endophytic fungi produce secondary metabolites with a high potential for tumour suppression. The present study was designed to evaluate *in vitro* antiproliferative activity of an endophytic *Aspergillus subramanianii* (GenBank MT755962), isolated from *Gyrinops walla* in *Vero, RD* and *MCF-7* cells. The Crude ethyl acetate extract (CEAE) of endophytic *A. subramanianii* was assayed for antioxidant potential by DPPH and ABTS assays. The CEAE was subjected to brine shrimp lethality assay to determine the cytotoxicity. MTT assay and protein content were performed to evaluate antiproliferative potential against *Vero, RD* and *MCF-7* cells at different time intervals after exposure to the CEAE. Oxidative stress was evaluated by analysing GSH and NO levels in *RD* and *MCF-7* cells after 24 h exposure. DNA fragmentation and microscopic examination of cells stained with ethidium bromide/acridine orange (EB/AO) were used to visualize apoptosis. The mean IC_{50} values were 88.2±7.9 and 42.9±4.9 µg/ml for DPPH and ABTS assays respectively. The brine shrimp assay showed a mean LC_{50} of 1242.5±3.4 µg/ml. *RD* cells showed the highest antiproliferative activity followed by *MCF-7* cells over the time periods investigated. Both cancer cell lines showed a dose-dependent decrease in total protein content, GSH and NO levels. DNA fragmentation and EB/AO assays exhibited induced apoptosis after treatment with the CEAE. The CEAE has exhibited antiproliferative potential against both *RD* and *MCF-7* cancer cell lines compared to the normal cell line and has induced apoptosis via oxidative stress.

Keywords: *Aspergillus subramanianii*, RD, *Gyrinops walla*, antiproliferative, antioxidant, endophytic,

Introduction
Cancer has become the leading cause of death worldwide. It has become an emerging health problem affecting both developing and developed countries. According to the World Health Organization (WHO) reports published in 2014, 8.2 million patients died of cancer in 2012 and the annual number of cancer cases reported in 2012 was 14 million. The annual number of cancer cases is estimated to increase from 14 million in 2012 to 22 million in the next two decades [1].

Endophytes are defined as fungi and bacteria/actinomycetes that colonize the internal tissues of living plants without causing any immediate negative impacts [2]. In addition, a few endophytic archaea [3] and algae [4] have been reported. All of plant species host to endophytic microorganisms [5] which are found in macroalgae, mosses, ferns, conifers as well as angiosperms including grasses, palms, dicotyledonous shrubs and trees [6].

Endophytes were discovered in 1904, but scientists initially did not pay much attention to endophytes. The situation change dramatically in the decades to follow due to the detection of paclitaxel (PTX) in an endophytic fungus *Taxomyces andreanae*, isolated from *Taxus brevifolia*, the latter being the original source of this important anticancer drug [7]. Metabolites of endophytic microorganisms exhibiting a variety of biological activities against different diseases with a significant number of interesting natural bioactive compounds being reported during 1990-2000.

They have become a major interest area of research among the scientific community worldwide because of their pharmaceutical potential [6]. Natural products of endophytes have been identified as alkaloids, terpenoids, flavonoids, steroids etc. They have been recognized as antibiotics, anticancer agents, biological control and other bioactive compounds. A single endophyte may be able to produce several bioactive metabolites. As a result, the role of endophytes in the production of novel bioactive compounds for exploitation in medicine is receiving increased attention [5].

Occasionally, some endophytic compounds are the same as those produced by the respective host plants, suggesting that endophytes can serve as an alternative source of important plant secondary metabolites. Some examples are a taxol-producing endophytic fungus that was isolated from Taxus (*Taxomyces andreanae*), camptothecin-producing endophytic bacteria and fungi from several camptothecin-producing plants. This strategy would be an ideal alternative for the low-cost, rapid and efficient production of such compounds [8, 9].

Nevertheless, the current therapeutic methods and available drugs are not adequate to manage the conditions effectively, although several fungal-based agents were reported to possess strong antioxidants or anti-inflammatory activity *in vitro*; it does not guarantee their activity *in vivo* [10]. Identified antioxidant properties *in vitro* may be lost or reduced at the *in vivo* assessment stage due to less pharmacokinetic optimization. The frequency and severity of side effects at therapeutic doses of most anticancer drugs are well known. One of the strategies to overcome chemotherapy-induced toxicity is to use of alternate drugs or their analogues. Therefore, a continuous research effort to explore more sources of novel drugs and drug leads is an important task of the scientist involved in herbal medicine research around the world [11]. However, bioactivities of various endophytic *Aspergillus* spp. have been reported, but no published information on such properties is available on endophytic *Aspergillus subramanianii* fungus yet. Therefore, in the present study, we evaluated *in vitro* antioxidant, cytotoxicity and antiproliferative activity of an endophytic *Aspergillus subramanianii* (GenBank MT755962), isolated from *Gyrinops walla* (Thymelaeaceae), an endogenous plant to Sri Lanka that is generally known as 'Walla Patta" in Sinhala.

Materials and Methods

Chemicals

Folin-Ciocalteu′s phenol reagent1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethyl benzo thiazoline-6-sulfonic acid) (ABTS), Sodium Persulfate, cell culture and cytotoxicity chemicals were purchased from Sigma-Aldrich (P.O. box 14 508, St Louis, MO 63178 USA). Triton X-100, ascorbic acid, trolox and sulfanilamide were purchased from Fluka (FlukaChemie GmbH, CH-9471 Buchs). Tris-base, TES lysis buffer, Proteinase K and 100 bp DNA ladder were purchased from Promega (Promega Corporation, Madison, WI 53711-5399, USA). All chemicals used were of analytical grade. Fresh brine shrimp cysts were purchased from a fish aquarium (Thimbirigasyaya Aquarium, Colombo, Sri Lanka).

Equipment

Cells were incubated at 37°C in a humidified CO_2 incubator (SHEL LAB/Sheldon Manufacturing Inc. Cornelius, OR 97113, USA). Olympus (1X70-S1F2) inverted fluorescence microscope (Olympus Optical Co. Ltd. Japan) was used to observe cells. Photographs of cells were taken using a Nikon D700 camera (Nikon D700, Japan). Absorbance was measured using Shimadzu UV 1601 UV visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The electrophoresis was performed in a gel electrophoresis unit (Cleaver scientific ltd, UK). DNA bands were observed using a gel documentation system (Major Science, USA). The gel was photographed using a UVI pro gel documentation system (UVItec UK).

Sources of fungus

The authors used an endophytic fungus isolated from the medicinal plant *Gyrinops walla*. The fungus was identified as *Aspergillus subramanianii* by internal transcribed spacer (ITS) and β-tubulin gene sequencing; then BLASTn search (GenBank MT754563 and MT755962).

Cell culture

Kidney epithelial cells extracted from an African green monkey (*Vero*) and human Rhodomuscularsarcoma (*RD*) cell lines were procured from the Medical Research Institute, Sri Lanka. Human breast adenocarcinoma (*MCF-7*) cell line was obtained from Human Genetics Unit, University of Colombo, Sri Lanka. Cell lines were cultured in minimum essential medium (MEM) supplemented with heat-inactivated fetal bovine serum (FBS), 7.7% sodium bicarbonate, 3% glutamine and an antibiotic solution (penicillin/streptomycin cocktail).

Extraction of fungal metabolites

Isolated *A. subramanianii* fungus was inoculated onto 25 plates of potato dextrose agar (PDA) and incubated at room temperature for three weeks. Agar medium with fungal mycelia was then cut into small pieces and macerated with 150 ml of ethyl acetate. The contents were filtered and the above step was repeated twice. The filtrates were pooled and rotary evaporated at 40 °C. Finally, the dried crude ethyl acetate extract (CEAE) of *A. subramanianii* was obtained as a sticky paste and was stored at -20 °C until used [12].

The DPPH assay

The DPPH assay was carried out as described previously [13] using 96-well micro titrate plates. The CEAE was dissolved in analytical grade methanol (ME). Equal volumes of the DPPH (0.2 mM) and the fungal extract (50-100 µg/ml) were mixed and incubated under dark conditions at room temperature for 30 min. Absorbance was then obtained at 517 nm. The free radical scavenging activity was calculated as the following equation.

% I=(absorbance of control-absorbance of sample)/ absorbance of control x 100%.

The control contained equal volumes of DPPH solution and methanol. Ascorbic acid (1-10 µg/ml) was used as the positive control. The concentration of sample required to scavenge 50% of the DPPH (IC_{50}) was calculated using the linear segment of the curve obtained with the percentage of free radical scavenging activity (%I) against concentration.

The ABTS assay

The ABTS assay was carried out as described previously [14] using 96-well micro titrate plates. Freshly prepared ABTS

(7 mM) and $Na_2S_2O_8$ (4.9 mM) were mixed up in a 1:1 ratio (v:v) and incubated under dark conditions for 12-16 h. The optical density of this mixture was then adjusted to 0.7 at 735 nm by adding analytical-grade ethanol. The CEAE was dissolved in ME. Equal volumes of the above solution and the fungal extract (20-80 µg/ml) were mixed and incubated under dark conditions at room temperature for 6 min and the absorbance was obtained at 735 nm. The control contained equal volumes of ABTS solution and methanol. Trolox (1-10 µg/ml) was used as the positive control. The free radical scavenging activity and the EC_{50} were calculated as described earlier.

The brine shrimp lethality assay

The Brine shrimp lethality assay was carried out as described previously [15]. About 0.2 g of the cysts were added to artificial seawater (3.8%). Constant aeration and a 100 W light source were supplied for 48 h. Active nauplii free from egg shells were collected and the nauplii were fed with dry yeast before using for the assay. The CEAE were dissolved in artificial seawater and the concentration gradients of the CEAE (1000-1500 µg/ml) were made. Thirty nauplii were then transferred to each concentration and kept at room temperature for 24 h. The nauplii were subjected to continuous aeration and exposed to yellow light. The death percentages of nauplii were determined as (total nauplii-alive nauplii) / total nauplii x 100%. The positive control was a concentration series of $K_2Cr_2O_7$ (1-10 µg/ml). Then concentration of sample required to kill 50% of the nauplii (LC_{50}) was calculated using the linear segment of the curve obtained with the percentage of death against concentration.

The MTT assay

The MTT cell viability assay was carried out as described previously [16]. The cells (2×10^5 cells/well) were seeded in 96-well plates and incubated overnight with 300 µl of the medium. Monolayers with a 70% confluent were treated with different concentrations of the CEAE and incubated for 24, 48 and 72 h separately at 37 °C in a Carbon Dioxide (CO_2) incubator. The spent medium was then carefully removed and 100 µl of MTT (5 mg/ml) was added. Cells were incubated at 37 °C in an incubator for 3-4 h under dark conditions. The supernatant was then carefully removed without disturbing the crystals and 100 µl of 0.05% (v/v) acidic isopropanol was added to each well. The absorbance of the resulting solutions was measured at 570 nm. Percentage of cell viability = {absorbance of the sample /mean absorbance of the negative control) x 100%. The concentration of sample required for 50% of cell death (EC_{50}) was calculated using the curve obtained with the percentage of cell viability against the concentration. The positive control contained Cycloheximide (5 mM, 20 µl) and the negative control contained only growth medium was simultaneously carried out with all experiments.

Determination of protein content

The protein content in the cell lysates of *RD* and *MCF-7* cells obtained after treatment with the CEAE was determined as described previously [17]. The cell lysate was prepared by treating the cells with 1 ml of 0.1% (v/v) Triton X-100. The contents were then sonicated for 20 sec and cell lysates were obtained by centrifuging the content at 4000 rpm for 5 min. A mixture (solution A) was freshly prepared by mixing the 2% Na_2CO_3 (w/v), 1% (w/v) $CuSO_4$ and 2% (w/v) sodium potassium tartrate at a ratio of 100:1:1 (v:v:v). NaOH (100 µl; 2M) and the cell lysate (100 µl) were mixed and the mixture was incubated in a water bath at 100 °C for 10 min. A volume of 100 µl of solution A was added to each well and aliquots of 50 µl of Folin reagent were added after 10 min. The mixture was incubated for 30 min at room temperature under dark conditions and the absorbance was measured at 550 nm. The protein content was determined by using a calibration curve constructed with bovine serum albumin (BSA). Negative controls were untreated cell lysate. The cell lysate was replaced with deionized water for the blank.

Determination of reduced glutathione level (GSH)

The GSH levels in the cell lysates of *RD* and *MCF-7* cells after treatment with CEAE were determined as described previously [18]. Aliquots of 200 µl of cell lysates were treated with 100 µl of 2.5% (w/v) Sulfosalicylic acid and centrifuged at 6000 rpm for 10 min. Aliquots of 200µl of PBS and 250 µl of 5% 5, 5'-dithiobis-2-nitrobenzoic acid were added to 250 µl of supernatant and the absorbance of the reaction mixtures was measured immediately at 412 nm. The GSH level of each cell line after treatments was determined using a calibration curve constructed with glutathione. The negative controls consisted with untreated cell lysates and all reagents. Blank wells contained all reagents without the cell lysates replaced with PBS.

Determination of nitric oxide

The spent medium of *RD* and *MCF-7* cells obtained after treatment with the CEAE was used to determine the nitric oxide production level in cells by Griess-Nitrate assay according to [18]. Equal volumes of growth medium and Griess reagent were incubated at room temperature for 10 min. Absorbance was then measured at 540 nm. The nitric oxide production levels of each cell type after treatment were determined by using a calibration curve constructed with $NaNO_2$. The negative control was the growth medium of untreated cells and the Griess reagent. The Griess reagent with PBS was used as the blank.

Determination of Morphological Changes of Treated Cells

The morphological changes of *Vero*, *RD* and *MCF-7* cells after treatment with the CEAE were observed under the inverted fluorescence microscope and photographs of cells were taken using a Nikon D700 camera.

Ethidium Bromide and Acridine Orange (EB/AO) Staining

The EB/AO staining for the *RD* and *MCF-7* cells was carried out to determine the induction of apoptosis by the CEAE as described previously [19]. The cells were treated with the CEAE for 24 h. The growth medium was replaced with 20 µl of PBS. Aliquots of 2 µl of the EB/AO mixture consisting of Ethidium Bromide and Acridine Orange (100 mg/ml) in PBS were then added. The cells were observed immediately under the inverted fluorescence microscope with UV light (254 nm) and photographs of cells were taken. The changes in morphology were compared with the positive and negative controls.

DNA fragmentation assay

The DNA fragmentation assay for *RD* and *MCF-7* cells after treatment with the CEAE (15, 30, 60 µg/ml) was determined as described previously [20]. After 24 h of exposure, the cells were trypsinized with 750 µl of Trypsin-EDTA solution and incubated at 37 °C for 10 min. The contents were centrifuged at 2000 rpm at 4 °C for 5 min and the cell pellet was carefully obtained. Aliquots of 20 µl of TES lysis buffer and 10 µl of RNase were mixed thoroughly with cell pellets and incubated at 37 °C for 4 h. A volume of 10 µl of Proteinase K was then added and incubated at 50 °C for 18 h. Then 5 µl of 6X DNA loading buffer with loading dye was mixed with 5 µl of DNA sample and loaded to a well of 2% (w/v) agarose gel. Also, 2 µl of 100 bp DNA ladder was loaded into another well. The electrophoresis was carried out for nearly 2 h under 45 V and DNA bands were observed and photographed using a gel documentation system after staining with ethidium bromide (0.5 µg/ml). Negative controls and positive controls were DNA samples obtained from untreated cells and cells treated with Cycloheximide (50 µg/mL) respectively.

Statistical analysis

All the results of the experiments were expressed as the mean±standard deviation (Mean±SD). The measurements were performed in triplicate and the values shown are representative of at least three independent experiments. The linear/nonlinear regression analysis was applied using Microsoft Offices Excel 2013 to determine the EC_{50} or IC_{50} values and the calibration curves. The R^2>0.99 was considered as linear for the calibration curves. Significant differences in each test result were statistically analyzed using paired sample T-test with $p < 0.05$ significance using SPSS version 16.

Results

Extraction of Fungal Metabolites

The yield obtained from the pooled ethyl acetate extracts (CEAE) of *A. subramanianii* endophytic fungus was 252 mg.

The DPPH assay

The scavenging activity was increased with concentrations of the CEAE and the standard. The concentration of the CEAE of *A. subramanianii* required to scavenge 50% of the DPPH free radicals (Mean IC_{50}) was 88.2±7.9 µg/ml. the CEAE exhibited intermediate antioxidant activity with DPPH free radical scavenging assay. The IC_{50} value of the CEAE was higher than that of the ascorbic acid (4.1±0.3 µg/ml), indicating that the required concentration of all tested samples to scavenge 50% of DPPH radical is higher than that needed for the standard.

The ABTS assay

The mean IC_{50} / required concentration to scavenge 50% of the $ABTS^+$ free radicals (42.9±4.8 µg/ml) of the CEAE of *A. subramanianii* is significantly higher (p<0.05) than that of the positive control, Trolox (2.1±0.15 µg/ml). The results indicate that the antioxidant capacity of the CEAE was lower than the positive control.

The brine shrimp lethality assay

In vivo cytotoxic effect was assayed by brine shrimp lethality assay showed that the CEAE of *A. subramanianii* possessed a cytotoxic effect with a Mean LC_{50} value of 1242.5±3.4 µg/ml which was greater than that of $K_2Cr_2O_7$ standard (12.4±1.2 µg/ml), indicating that the CEAE is nontoxic as previously reported [21].

The MTT assay

The viability of *Vero, RD* and *MCF-7* cells after 24, 48 and 72 h treatment with the CEAE of *A. subramanianii* were examined by the MTT assay (Table 1). A dose-dependent decrease in cell viability was observed with the concentration and the exposure time for all cell lines. The EC_{50} values obtained for the *Vero* cells were significantly higher (p<0.05) than that of the *RD* and *MCF-7* cells demonstrating a less responsive to antiproliferative activity to healthy cells.

Determination of protein content

The protein content of the cell lysates of *RD* and *MCF-7* was determined after 24 h exposure to the CEAE and calculated relative to the negative controls (Figure 1). The total protein content was decreased with the concentration of the CEAE of *A. subramanianii* in both cancer cell lines tested. The mean percentage of protein content of the treated *RD* cells to that of the negative control was only 28% at the lowest experimental concentration (10 µg/ml). However rapid decrease in protein synthesis was observed in *MCF-7* cells at concentrations higher than 40 µg/ml. The total protein content of the negative controls of *RD* and *MCF-7* cell lysates was 1867.2±14.1 and 893.7±8.7 µg/ml, and the positive control treated with Cycloheximide was 489.3±9.5 and 375.3±9.2 µg/ml respectively.

Determination of reduced glutathi1one level

The reduced glutathione level of the cell lysates of *RD* and *MCF-7* was determined after 24 h exposure to the CEAE and calculated relative to the negative controls (Figure 2). The GSH levels were also decreased with the concentration of the CEAE of *A. subramanianii* in both cancer cell lines. The percentage GSH level of the positive control treated with Cycloheximide for *RD* and *MCF-7* cells were 6.1±0.5 and 5.9±0.3 µg/ml; the negative controls of *RD* and *MCF-7* cell lysates was 10.6±0.3 and 13.5±1.3 µg/ml respectively.

Determination of nitric oxide production

The nitric oxide production of the cell supernatants of *RD* and *MCF-7* was determined after 24 h exposure to the CEAE and calculated relative to the negative controls (Figure 3). The results indicated that there was a decrease in NO level in tested two cancer cell lines with the concentrations of the CEAE of *A. subramanianii*. At the lowest experimental concentration, the mean percentage of NO level in treated *RD* and *MCF-7* cells was 55% and 27% of the negative control respectively. The GSH of the negative controls of *RD* and *MCF-7* cell lysates was 0.077±0.006 and 0.82±0.07 µg/ml, and the positive control treated with Cycloheximide was 0.48±0.07 and 1.24±0.15 µg/ml respectively.

Correlation between NO% and GSH% was studied using the data obtained at each concentration for both cancer cell lines to investigate any association between NO levels on GSH levels (Figure 4). The Pearson correlations values obtained for *RD* and *MCF 7* cells were 0.9815 and 0.9984 respectively.

Morphological changes

The untreated *Vero* and *RD* cells were observed as spindle shape/elongated form while *MCF-7* cells were observed as star-shaped cell clusters. The adherent cells of all three cell types with prominent morphological features were lost after treatment with the CEAE of *A. subramanianii*. The treated cells became oval or irregular in shape with highly condensed contents in the culture medium and showed a dose-dependent increase in cell death with increasing concentrations of the CEAE. Similar morphological changes were observed with both cancer cell types treated with Cycloheximide (positive control), (Figure 5).

Ethidium Bromide and Acridine Orange (EB/AO) Staining

The nuclei of untreated live cells of *RD* and *MCF-7* appeared in bright green colour. Staining of the same cell lines with EB/AO after treatment of the CEAE of *A. subramanianii* showed characteristic features of apoptosis; cytoplasmic blebbing, light orange nuclei, nuclear fragmentation and chromatin condensation (Figure 6).

DNA fragmentation assay

A smear-like DNA fragmentation pattern was observed by the treated *RD* and *MCF-7* cell lines with the CEAE of *A. subramanianii* fungus even at lower concentrations (15 µg/ml) (Figure 7). Only a heavy DNA band was observed in negative control. The positive control also reflected a smear-like DNA fragmentation pattern.

Discussion

The DPPH assay offers a convenient and accurate method for titrating the oxidizable groups of natural products and is widely used to investigate the *in vitro* antioxidant ability of natural products [22]. Based on the IC_{50} value of the sample, classified their antioxidant potential into three groups; 10-50 µg/ml-strong, 50-100 µg/ml-intermediate, >100 µg/ml-weak [23]. The CEAE of *A. subramanianii* exhibited intermediate antioxidant activity with DPPH free radical scavenging assay in this experiment. Both ABTS and DPPH assays show low capacity towards radical scavenging compared to the positive controls. However, previous studies have discussed the strong antioxidant potential of different crude extracts of *Aspergillus* spp. [12, 24, 25].

The Brine shrimp lethality assays have been used to determine *in vivo* cytotoxicity of natural products. The results can be used as a guide to determine the possible therapeutic indications, mainly cytotoxic and antiproliferative properties [21]. The present study shows that the CEAE of endophytic *A. subramanianii* does not exert cytotoxicity (non-toxic LC_{50} > 1000 µg/ml) towards the brine shrimps. However, a previous study reports that crude ethyl acetate extract of endophytic *A. tamari* has cytotoxicity against brine shrimps with LC_{50} of 38.483 µg/ml [26].

MTT is converted to a purple formazan product by cleavage of the tetrazolium ring by mitochondrial enzymes of viable cells. The cell membrane is impermeable to formazan products and accumulates in viable cells which can be measured at 570 nm [19]. The present study shows that the CEAE of endophytic *A. subramanianii* exerts less cytotoxicity against healthy *Vero* cells after 24, 48 or 72 h of treatment compared to the two cancer cell types investigated. Furthermore, *RD* cells demonstrated higher (p<0.05) antiproliferative activity (EC_{50} < 30 µg/ml) after 48 and 72 h of treatment compared to *MCF-7* and *Vero* cells revealing its selective cytotoxicity towards *RD* cells (Table 1). However, a recent review on anticancer compounds from plant-derived endophytic fungi reports that, secondary metabolites belong to *Aspergillus* spp. isolated from different plant species (*Torreya grandis bark, Ipomoea batata. Avicennia marina, Tabebuia argentea leaves, Sequoia sempervirens inner bark* and *Hyptis suaveolen*) are effective against *MCF-7* cells on anticancer activity [27]. The present study also provides evidence for the presence of secondary metabolite/s with anticancer potential in the CEAE of endophytic *A. subramanianii* isolated from *G. walla.*

The protein content in the cell lysates of *RD* and *MCF-7* cells after 24 h exposure to the CEAE of endophytic *A. subramanianii* which reflects the cell viability, also provides evidence that *RD* cells exhibits has higher cytotoxicity than *MCF-7* (Figure 1). Small molecules originating from natural products inhibit protein synthesis and target a specific step in protein synthesis in cancer cells [18]. Therefore, the current results suggest that the CEAE of endophytic *A. subramanianii* may induce an inhibitory mechanism of protein synthesis in cancer cells, causing cell death or as a results of cell death induced by other mechanisms, terminate the protein synthesis. However, further studies are needed to confirm the mechanisms. Nevertheless, several types of inhibitors of protein synthesis in cancer cells have already been identified from natural products, some of which have high therapeutic potential [28].

Induction of apoptosis in cancer cells tested by the CEAE of endophytic *A. subramanianii* was observed in the presence of AO/EB staining. The AO nucleus penetrates both living and dead cells, appearing green colour. EB is taken up only by cells with damaged cell membranes. Therefore, live cells are uniformly green and apoptotic cells are stained orange or exhibit orange fragments, nuclear fragmentation, the presence of apoptotic bodies, and blebbing [29].

The light microscopic photographs following the treatment with the CEAE of endophytic *A. subramanianii* indicate prominent features of apoptosis. Furthermore, fragmented DNA suggests that the CEAE of endophytic *A. subramanianii* may exert its cytotoxicity via the induction of the apoptotic pathway.

Exposure to reactive oxygen and nitrogen species (RONS) can induce cell death through apoptosis in various cell types. Glutathione (GSH) is the most rapidly acting and abundant substance in human cells that detoxifies RONS and regulates the redox status of the cell. The depletion of intracellular GSH can be recognized as an early indicator of apoptosis [30]. Also, the level of GSH is an important determinant of the activity of anticancer agents [31]. The levels of GSH were decreased along with the concentration of the CEAE in both *RD* and *MCF-7* cells (Figure 2). The results suggest that the depletion of GSH by the fungal extract may be associated with the inhibition of GSH synthesis or the direct conjugation of GSH with the compounds present. Accumulation of RONS in the cells causes a redox imbalance in cancer cells which promotes oxidative stress induced DNA damage preceding the induction of apoptosis [32].

Nitric oxide plays a dual role in cell death by either activating apoptotic pathways or shutting them down [33]. Pro or antitumor effects depend on the concentration and at

high concentrations NO induces apoptosis [34]. It has been found that depletion of the liver GSH increases the oxidative stress and decreases NO synthesis in rats [35]. Furthermore, another study describes that GSH depletion inhibits the early signaling events leading to inducible nitric oxide synthase (iNOS) expression [35]. Taken together with NO and GSH levels as represented in Figures 2,3 and 4, we can suggest that, depletion of GSH leads to a decrease in NO with increasing concentration of the fungal extract indicating inhibition of NO synthesis. However, the present study shows that NO levels in two tested cancer cell lines (*RD* and *MCF-7*) decreased gradually with the concentration of the fungal extract and does not indicate a pro and antitumor activity of NO as described previously [34]. Instead, oxidative stress induced as a result of concentration dependent intracellular GSH depletion with the fungal extract predispose *RD* and *MCF-7* to apoptosis [37].

Conclutions

The present study provides evidence for the selective antiproliferative potential of the crude ethyl acetate extract (CEAE) of endophytic *Aspergillus subramanianii* fugues isolated from the *Gyrinops walla* plant against *RD* and *MCF-7* cancer cell lines compared to healthy *Vero* cell line. The CEAE stimulates oxidative stress by reducing GSH levels in both *RD* and *MCF-7* cancer cells. Also, it breaks genomic DNA and shows characteristic microscopic features of apoptosis. Also, the brine shrimp results suggest that the CEAE have no cytotoxicity towards the normal healthy cells.

Figures and Tables

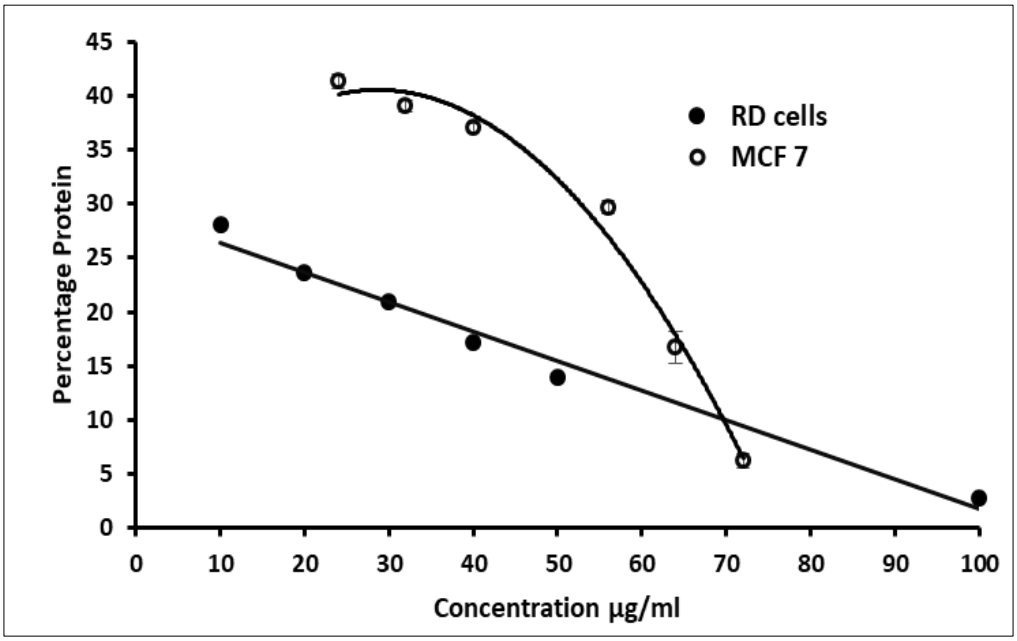

Fig 1: The percentage protein content of *RD* and *MCF-7* cells after 24 h treatment with the CEAE of *A. subramanianii* calculated relative to the respective negative controls

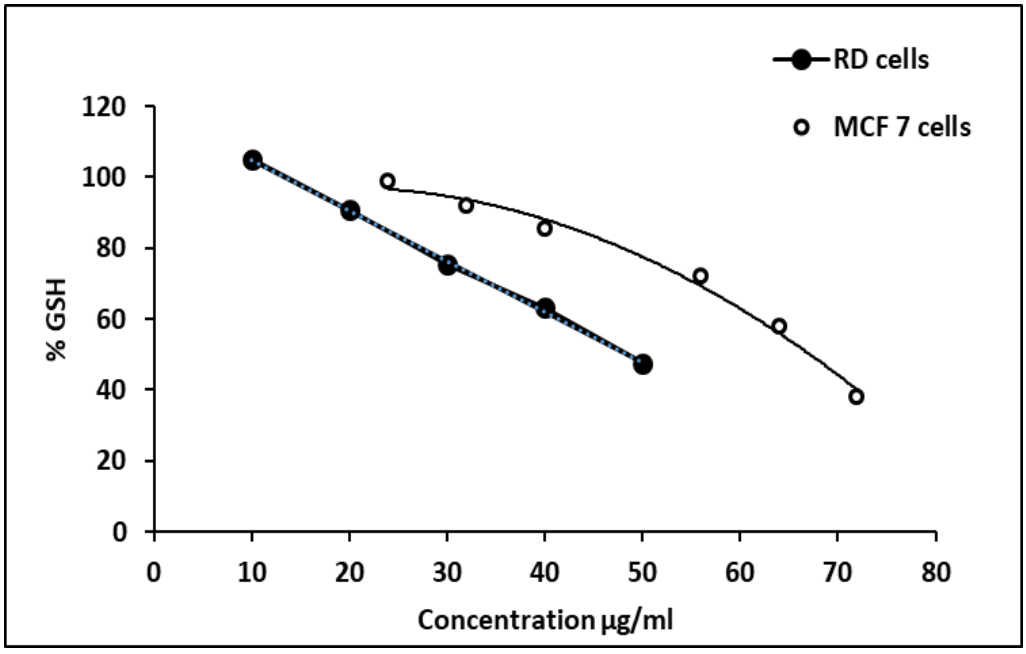

Fig 2: The reduced glutathione level (GSH) of *RD* and *MCF-7* cells after 24 h treatment with the CEAE *of A. subramanianii*, calculated relative to the respective negative controls

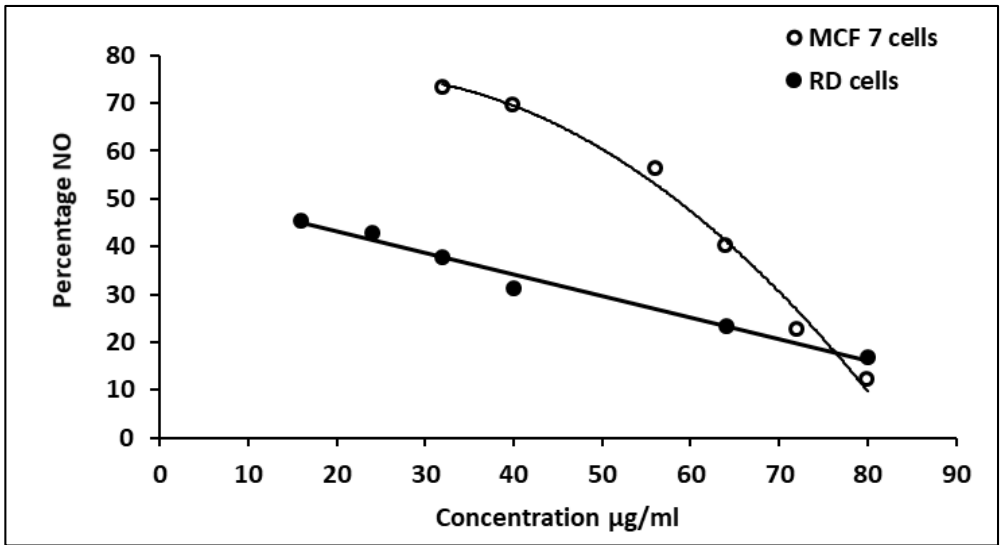

Fig 3: The Nitric Oxide level (NO) of *RD* and *MCF-7* cell lines after 24 h treatment with the CEAE of *A. subramanianii*, calculated relative to the respective negative controls

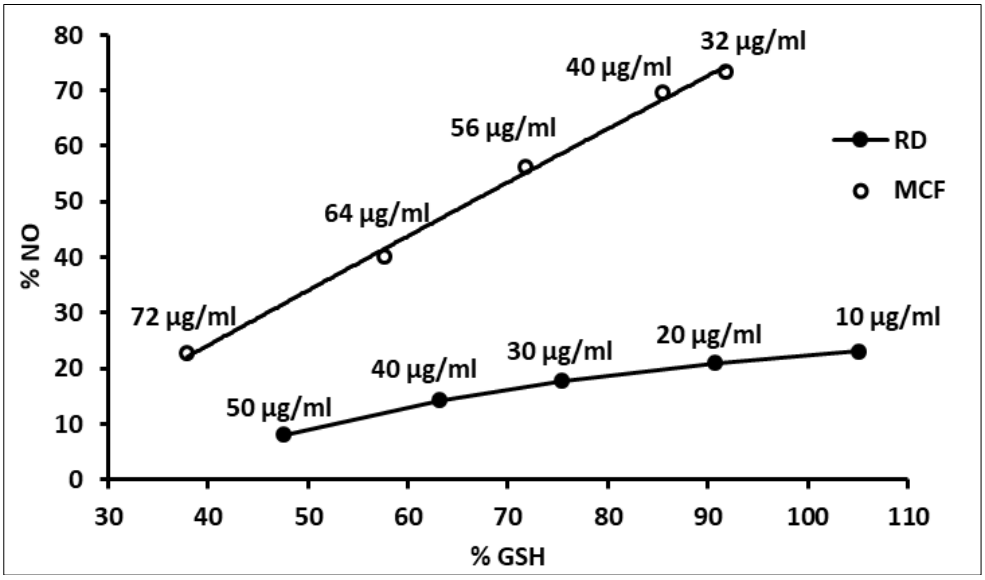

Fig 4: Relationship between percentage NO and percentage GSH levels obtained at each concentration after exposure to the CEAE of *A. subramanianii*. Respective concentrations treated with *A. subramanianii* are shown on the lines

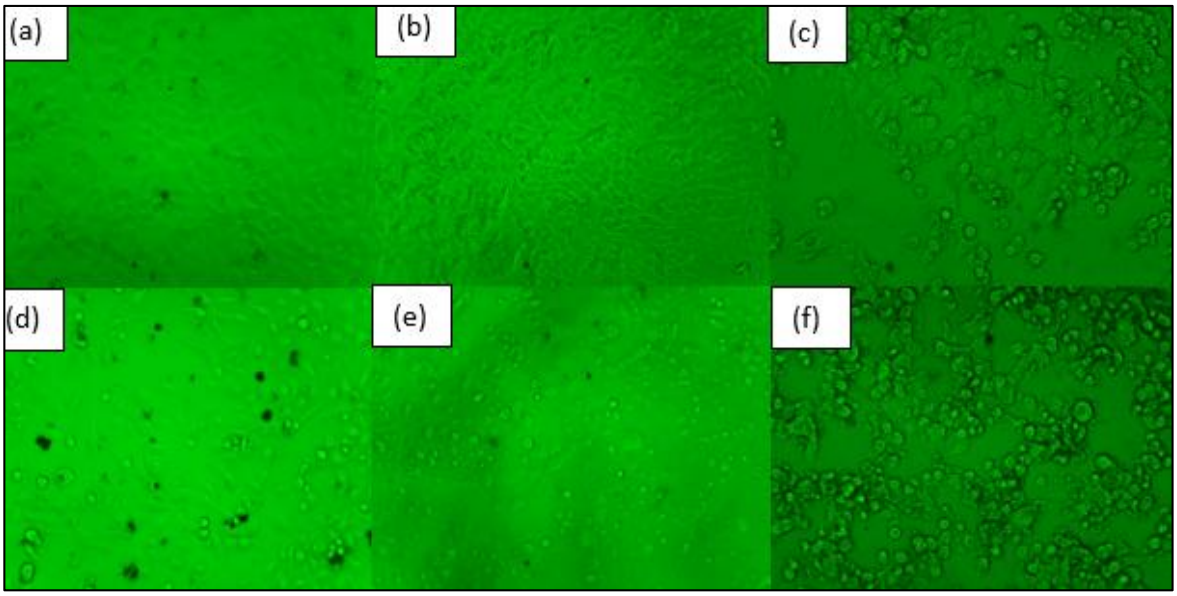

Fig 5: Light microscopic images of untreated cells of (a) *Vero*, (b) *RD* and (c) *MCF-7* and treated with the crude ethyl acetate extract of *A. subramanianii* fungus for 24 h at their respective EC_{50} of (d) *Vero*, (e) *RD* and (f) *MCF-7*

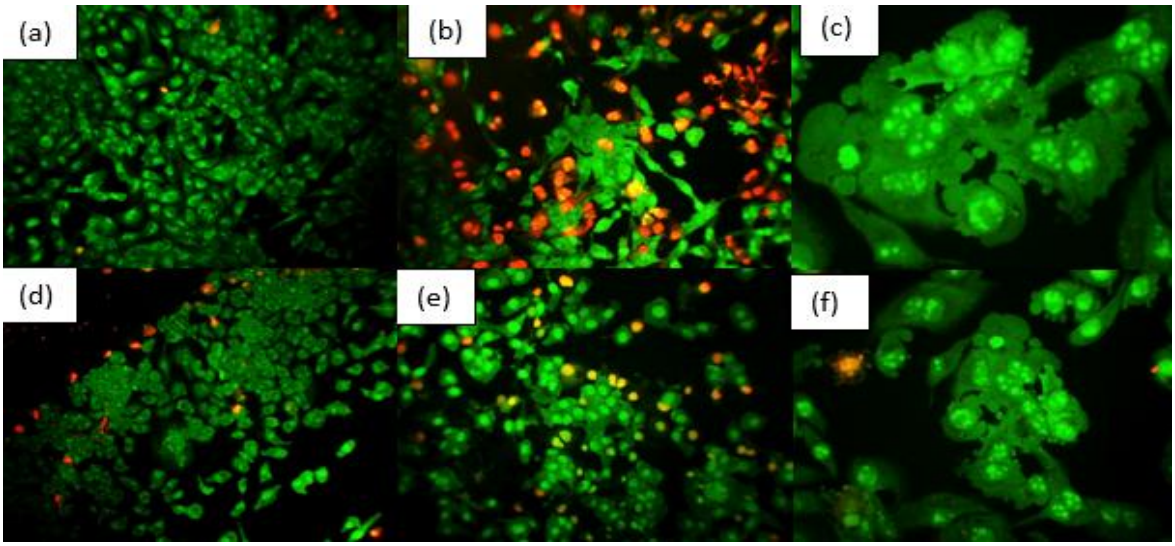

Fig 6: Apoptotic morphology detection by Acridine orange/ethidium bromide (AO/EB) fluorescent staining of (a) *RD*-negative control (b) *RD*-treated with the crude ethyl acetate extract/CEAE of *A. subramanianii* fungus for 24 h (40 µg/ml) (c) *RD*-cytoplasmic blebbing (d) *MCF-7*-negative control (e) *MCF-7*-treated with the CEAE for 24 h (60 µg/ml) (f) *MCF-7*-cytoplasmic blebbing

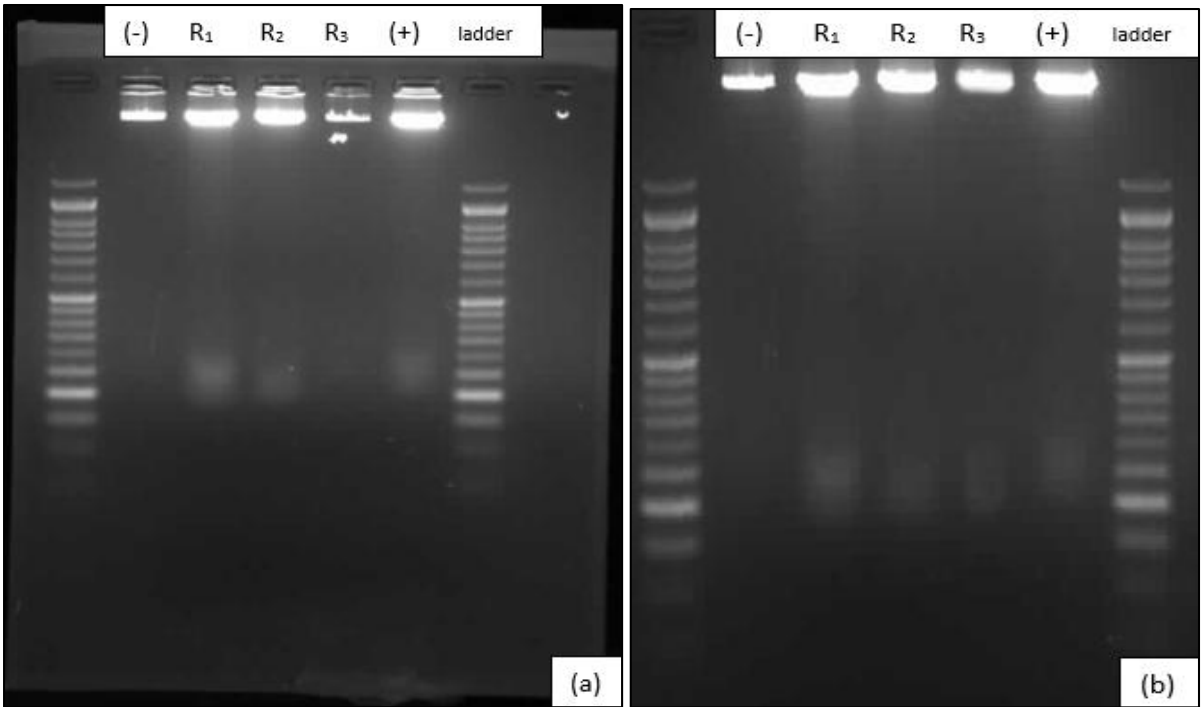

Fig 7: Agarose gel electrophoresis shows DNA fragmentation indicating induction of apoptosis by the CEAE of *A. subramanianii* in (a) *RD* and (b) *MCF-7* cells

Table 1: Mean EC_{50} values for MTT assay against *Vero*, *RD* and *MCF-7* cell lines after 24, 48 and 72 h treatment with the CEAE of *A. subramanianii* (N=9)

Cell line	EC_{50} value (µg/ml), 24 h	EC_{50} value (µg/ml), 48 h	EC_{50} value (µg/ml), 72 h
Vero (healthy)	90.7 ± 2.3	81.6 ± 1.0	67.7 ± 2.4
RD	40.7± 0.4	22.5 ± 0.8	19.9 ± 0.3
MCF-7	64.3 ± 0.8	61.9 ± 0.6	53.9 ± 0.7

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Ethical Approval

Not applicable

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