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Abstract

Background: The discovery of the only animal pigment, bilirubin, in the plant *Strelitzia nicolai* has triggered a vast number of questions regarding bilirubin's formation and its role in the human body. Recent studies have confirmed that bilirubin at certain levels have many medical benefits. Various case studies have revealed that bilirubin is a potent antioxidant. Cervical cancer is one of South Africa's largest women's health crises. It is estimated that it affects one out of 41 South African women and kills approximately 8 women in the country every day. Thus, the aim of this study was to investigate if the aril extract of *Strelitzia nicolai* (Regel and Körn.) containing bilirubin possesses anti-cancer activity and to determine its effect on the induction of apoptosis.

Materials and methods: The DPPH activity was firstly used to determine the antioxidant effect of the extract. Thereafter, the cytotoxic effect was tested using the XTT assay. Apoptosis was confirmed and quantified using the Annexin V-PE kit and the morphology was studied using acridine orange and ethidium bromide. **Results:** The aril extract decreased cell viability by 52% and induced apoptosis in HeLa cells; as shown by the Annexin V-PE Apoptosis detection kit and morphological studies with acridine orange/ethidium bromide staining. **Conclusion:** The activity of the extract as a potent antioxidant was immensely enhanced as compared to the bilirubin standard. These results suggest that *S. nicolai* aril extract containing bilirubin works synergistically as opposed to bilirubin on its own. Furthermore, this extract might be a good candidate for the therapeutic intervention of cervical cancer.

Keywords: Bilirubin, *Strelitzia nicolai*, apoptosis, aril extract, antioxidant

Introduction

Bilirubin is an end product from the breakdown of haemoglobin molecules in red blood cells. First, microsomal haem oxygenase (HO) enzyme catalyses oxidation of haem to a green tetrapyrrolic bile pigment, biliverdin. Biliverdin is subsequently transformed to bilirubin by biliverdin reductase (McDonagh, 2001) and excreted from the body via the bile ducts of the liver, typically as the main component of bile. If bilirubin is not excreted it leads to toxicity of intracellular organelles and physiological processes. A build up of bilirubin leads to jaundice. Jaundice could result from three distinctive processes, i.e. (a) increased production of bilirubin; (b) decreased excretion by the liver and/or (c) bile duct obstruction. The deposition of bilirubin in connective tissue (skin, scleras, internal organs) result in a yellow colour, typical of jaundice. The deposition in parenchymal cells (in basal ganglia) is called kernicterus (intracellular accumulation pigments). This is a rare condition caused by increased levels of unconjugated bilirubin that is lipid-soluble and capable of crossing the blood-brain barrier. Other harmful effects of bilirubin toxicity include, the Gilbert syndrome, the Dubin-Johnson syndrome and the Crigler-Najjar syndrome (Lathe, 1972). *Strelitziaceae*, is a tropical monocotyledonous family generating colourful bracteate inflorescences with woody capsular fruits that contain vibrantly colored arillate seeds. Pirone, 2010 discovered bilirubin in the arils of the white bird of paradise tree (*S. nicolai*) and the bird of paradise (*S. reginae*), being present as the primary pigment, producing colour.

Even though bilirubin was assumed to be only a waste product for decades, recent advances has depicted that bilirubin serves as a potent antioxidant and anti-cancer agent (Temme et al., 2001). Thomas et al., 2008, found that the bilirubin pathway was a physiological cytoprotectant. Bilirubin, largely protects against lipid peroxidation. This antioxidant effect impacts cell survival, as cell death is more markedly augmented following depletion of bilirubin. When bilirubin acts as an antioxidant, it is oxidized to biliverdin, which is immediately reduced by biliverdin reductase to bilirubin. Llesuy and Tomaro, 1994, found that an increase in bilirubin could be a response to initial oxidative stress. They discovered that administering Co (II) to rats leads to oxidative stress, which precedes haem oxygenase induction.

The induction of this enzyme could be a mechanism, through the increase of bilirubin levels, to decrease the damage caused by oxidative stress.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) participate in processes, contributing to cancer progression but also play important roles in endogenous defenses, working to eliminate and controlling the spread of transformed cells. Due to recent increases in the number of cancer patients using antioxidant supplements, a greater understanding of both the damaging and protective actions of ROS in carcinogenesis is crucial to further advances in cancer treatment (Monks et al., 2004). Therefore, scientists consider a good antioxidant as also a good anticancer agent.

Medicinal plants contain immunomodulatory and antioxidant properties, leading to anticancer activities. They have flexible immunomodulatory activity by invigorating both non-specific and specific immunity (Pandey and Madhuri, 2006). Certain phytochemicals from plants hold strong antioxidant capacity. The antioxidants may prevent and cure cancer and other diseases by protecting the cells from damage caused by free radicals. The body formulates a portion of the antioxidants it uses to counteract free radicals. These antioxidants are called endogenous antioxidants. However, the body relies on external (exogenous) sources, to acquire the rest of the antioxidants needed (Diplock et al., 1998).

Zucker et al., 2004, investigated a large number of subjects in the United States and with this they found that the odds ratio for colorectal cancer is reduced to 0.295 in men and 0.186 in women per 1 mg/dL increment in serum bilirubin levels. An additional study in Belgium by Temme et al., 2001, showed an inverse relationship between serum bilirubin levels and cancer mortality.

American Association for cancer research, 2013, found that a 7.02 incidence rate of lung cancer per 10,000 person-years for men with bilirubin levels of 0.68 mg/dL or less, compared with an incidence rate of 3.73 among men whose bilirubin levels were 1.12 mg/dL or more. This translates into a 51 percent increase in the risk of developing lung cancer in patients with low bilirubin (American Association for cancer research, 2013).

This newfound evidence gives insight, on the long perplexed reason as to why mammals evolved an energetically expensive and apparently unnecessary enzymatic step to converting the relatively innocuous biliverdin to the more toxic bilirubin.

Recently, our understanding of cancer has advanced in the realization that apoptosis and the genes that control it have a profound effect on the malignant phenotype. It is now clear that some oncogenic mutations disrupt apoptosis, leading to tumor initiation, progression or metastasis. Most cytotoxic anti-cancer agents induce apoptosis, increasing the possibility that defects in apoptotic programs contribute to treatment failure. The very same mutations that suppress apoptosis during tumor development also reduce treatment sensitivity. Apoptosis provides a conceptual framework to link cancer genetics with cancer therapy. This, together with the discovery of the only animal pigment in a plant (bilirubin) has lead us to the present study that investigates whether the aril extract that contains bilirubin; is a potential antioxidant and induces apoptosis in HeLa cell lines. This study also investigated the bilirubin standard that is available commercially, and its effects were compared to the aril extract. Many conventional drugs or their precursors are derived from plants. There are fundamental differences between administering a pure chemical and the same chemical in a plant matrix (Murthy et al., 2002).

Materials and methods

Collection of plant material

Fresh specimens (\pm 1 kg fresh weight) of *S. nicolai* (Voucher specimen: Dwarka and Baijnath 1 Ward Herbarium UDW) arils were collected from Kwa-Zulu Natal, South Africa during December 2013. The plants were identified using taxonomic keys by Professor H. Baijnath, School of Biological and conservation sciences (UKZN), South Africa.

Extraction of bilirubin from arils

Tissue (50 g) from the arils of the plant were ground by a mortar and pestle and extracted with 750 ml of chloroform for 72 hours and filtered through a Buchner funnel. The solution was re-extracted with 50 ml of chloroform, and the procedure repeated until the arils were colourless. This was then subsequently air-dried to form a powdery residue, which was dissolved in appropriate solvents, depending on the assay's prerequisites to give required concentrations for further analyses.

Preparative thin layer chromatography (TLC) to confirm the presence of bilirubin

Bilirubin was previously reported in the arils of *S. nicolai* and in order to confirm its presence, TLC was carried out with a commercial standard with a mobile phase of chloroform: methanol: acetic acid (97:2:1).

The aril extract of *S. nicolai* and bilirubin standard were analyzed on a Bruker Avance 500 MHz instrument. 10 mg of sample was dissolved in 0.5 ml CDCl₃ and the ¹H and ¹³C NMR spectra recorded and referenced to the deuterio chloroform signal at δ 7.24 relative the internal standard TMS. Chemical shifts were expressed in ppm (Jiang et al., 2004).

Measurement of free radical scavenging activity using the DPPH assay

The scavenging activity (antioxidant capacity) of the plant phytochemicals on the stable radical, DPPH, was evaluated according to the method by Murthy et al., 2002 with some modifications. A volume of 150 µl of methanolic solution of the compound at different concentrations (500, 250, 125, 62.5, 31.3 and 15.6 µg/ml) were mixed with 2.85 ml of a methanolic solution of DPPH (0.1 mM). An equal amount of MeOH and DPPH without sample served as a control. After 30 min of reaction at room temperature in the dark, the absorbance was measured at 517 nm against methanol as a blank using a UV spectrophotometer. The percentage free radical scavenging activity was calculated according to the following equation: % Scavenging activity = $(A_c - A_s) / A_c \times 100$. Where A_c = Absorbance of control and A_s = Absorbance of sample

Cell culture

The HeLa (human cervical cancer cell line) was donated by Ms Joubert from the department of infectious diseases, Nelson Mandela School of Medicine, University of KwaZulu Natal. Cells were grown at 37°C in a humidified incubator under 5% CO₂ in DMEM supplemented with foetal calf serum (10 %) and antibiotics (penicillin: 10 000 U/ml, streptomycin sulphate: 10 000 U/ml). Culture medium was replaced every two days. After confluency, the cells were trypsinized and subcultured.

Cell viability (XTT assay)

The XTT cytotoxicity assay was conducted according to Jiang et al., 2004 with minor modifications. The assay was carried out in flat-bottomed microtitre plates (96 well) (Cellstar, Greiner, Germany). Cells (50 µl; $\pm 1.2 \times 10^3$) were added into each well and aril extract (50 µl) in a twofold dilution factor (1000 µg/ml – 7.8 µg/ml) and media (50 µl) were added to the respective wells. The same procedure was implemented for the bilirubin standard purchased from Sigma Chemicals. In the control wells, cells (50 µl) and Taxol® (250 µg/ml) were added respectively. The plates were incubated (37°C) in a humidified incubator (5 % CO₂ atmosphere) for 24 hrs, 48hrs and 72hrs. Thereafter XTT (20 µl) reagent was added and the plates further incubated (4 h; 37°C) in a humidified incubator (5 % CO₂ atmosphere). The absorbance was read (578 nm) on an ELISA plate reader (Digital Analogue Systems, Italy) and the percentage viability determined using the formula:

% Cell viability = Absorbance of treated cells / Absorbance of untreated cells × 100

Confirmation and quantification of apoptosis

The Annexin V-PE Apoptosis detection kit (BD Biosciences) was used as per manufacturer's protocol. The HeLa cells were seeded in plates (24 well) and left to adhere overnight. They were then treated with the aril extract of *Strelitzia nicolai* (250 µg/ml), bilirubin standard (7.8 µg/ml) and Taxol® (250 µg/ml). After 24 hours, the cells were trypsinized, washed twice with PBS and resuspended in binding buffer (1 X at 50 000 cells/ml). FITC Annexin V (5 µl) and propidium iodide (PI) (5 µl) were added, vortexed and incubated (15 min) in the absence of light. Thereafter, binding buffer (400 µl) was added to each tube and the results analysed by flow cytometry (BD Accuri C6).

Assessment of morphology in apoptosis

The membrane changes of apoptosis were observed using acridine orange and ethidium bromide. A stock solution (100 X) was made up by adding ethidium bromide (50 mg) and acridine orange (15 mg). This was dissolved in ethanol (1 ml; 95 %) and distilled water (49 ml). This was then mixed well and divided into aliquots (1 ml). The working stock solution (1 X) was made up of an aliquot (1 ml) of the 100 X stock solution and diluted with PBS (99 ml).

A sterile cover slip was inserted into the 24 well plates using sterile forceps. The HeLa cells were seeded in plates and left to adhere overnight. They were then treated with the aril extract of *Strelitzia nicolai* (250 µg/ml), bilirubin standard (7.8 µg/ml) and Taxol (250 µg/ml). After 24 hours, the media was removed and 1ml of acridine orange-ethidium bromide solution was added to each well. Thereafter, the cover slips were removed and mounted on a slide and viewed

using a fluorescence microscope set up to excite for fluorescein (i.e., with a 495nm primary filter and a 515 nm secondary filter). Cells were viewed under the 10X objective where viable normal cells stained bright green with intact structured nuclei and viable apoptotic cells stained green with highly condensed or fragmented nuclei. Non-viable normal cells were identified as those with chromatin stained bright orange and with an organized structure whilst non-viable with apoptotic nuclei were observed to have highly condensed and fragmented chromatin.

Data analysis

Data was analyzed using the Graphpad prism 5 for individual comparisons and data was expressed as the mean \pm standard deviation. P values less than 0.05 were considered to be statistically significant.

Results

TLC profile of compounds in the arils of *S.nicolai*

The TLC profile revealed two major compounds (B and C) present in the aril extract. The R_f value of compound B matched that of the bilirubin standard (0.93 in chloroform: methanol: acetic acid (97: 2: 1)). This indicated the presence of bilirubin in the aril extract of *S. nicolai*.

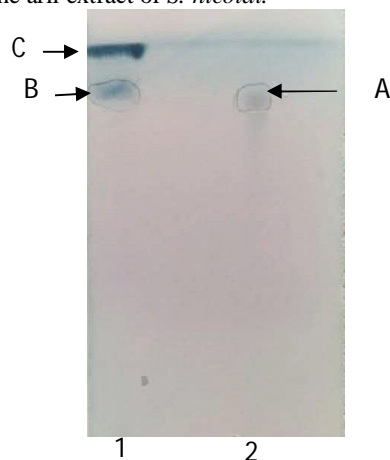


Figure 1: TLC of the chloroform extract of *S. nicolai* arils using chloroform: methanol: acetic acid (97: 2: 1) as the mobile phase and developed with *p*-anisaldehyde spray reagent. Spot 1 is the aril extract and spot 2 is the bilirubin standard.

NMR

The ^1H NMR spectrum of the aril extract showed similar resonances to that of the bilirubin standard, confirming its presence as one of the major compounds in the aril extract. In essence, the methyl, methylene and ABX coupled protons of the olefinic groups could be identified.

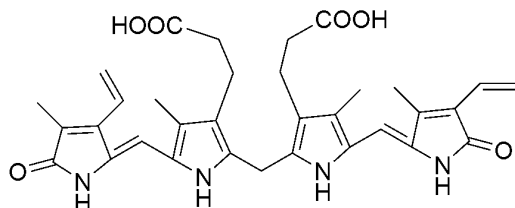


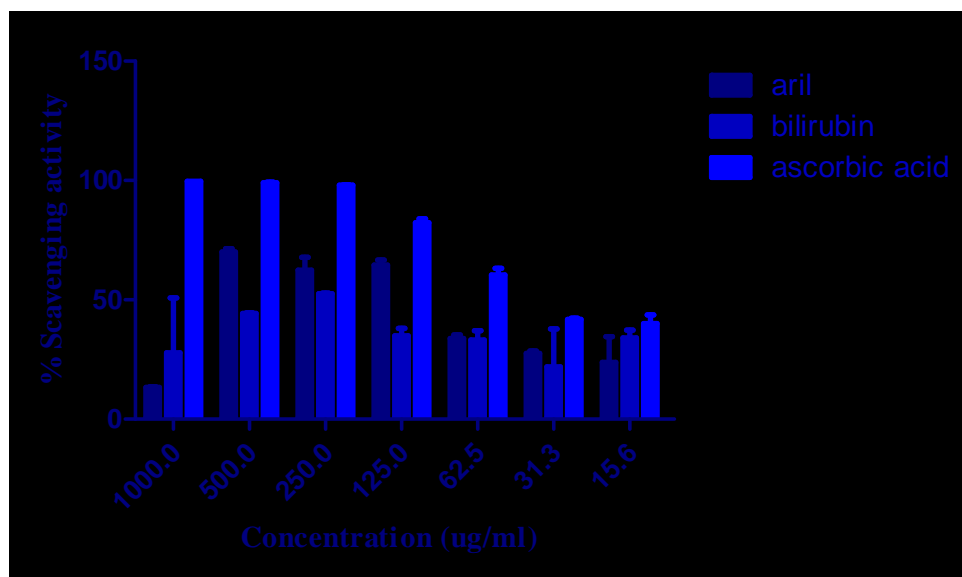
Figure 2: Bilirubin-IX α demonstrating intramolecular H-bonding

Table 1: ¹H NMR data of aril extract and bilirubin standard (values are given in ppm)

Proton group	<i>S. nicolai</i> aril extract	Multiplicity	Bilirubin standard
2-CH ₃	1.63	Singlet	1.29
7-CH ₃	2.08	Singlet	2.00
13-CH ₃	2.08	Singlet	2.06
17-CH ₃	2.83	Singlet	2.12
8 & 12- CH ₂ CH ₂ CO ₂ H	2.32	Triplet	2.87
8 & 12- CH ₂ CH ₂ CO ₂ H	2.08	Triplet	1.57
10-CH ₂	4.16	Singlet	4.00
18-CH=CH ₂ *	~5.30 (centroid)*	ABX system	5.36
18-CH=CH ₂ *	~6.22 (centroid)*	ABX system	6.23
18-CH=CH ₂ *	~6.6 (centroid)*	ABX system	6.59
3-CH=CH ₂ *	~5.63 (centroid)*	ABX system	5.61
3-CH=CH ₂ *	~5.66 (centroid)*	ABX system	5.64
3-CH=CH ₂ *	~6.57 (centroid)*	ABX system	6.67
5-H	6.24	Singlet	6.20
15-H	6.15	Singlet	6.15
21-H	10.25	Broad singlet	10.56
22-H	10.83	Broad singlet	10.81
23-H	10.94	Broad singlet	10.70
24-H	9.33	Broad singlet	9.31
COOH	12	Broad singlet	13.73

Anti-oxidant activity

The DPPH radical scavenging activity revealed that the aril extract had a greater percentage radical scavenging activity than the bilirubin standard. The highest activity was considered at a concentration of 500 µg/ml generating a scavenging activity of 70.9%. Bilirubin produced the highest radical scavenging activity at 250 µg/ml with 52.6%. These results were analyzed using graphpad prism 5. The anova one way test confirmed that the aril extract and bilirubin standard at each different concentration when compared to each other and then to the positive control ascorbic acid showed no significant difference. This validates that the treatments act in a similar manner to the positive control. The Tukey's multiple comparison test validated that aril vs Bilirubin were not significant. The aril vs ascorbic acid was significantly different (*) and bilirubin vs ascorbic acid revealed that they were to a great extent significantly different (**).

**Figure 3:** Antioxidant activity of the aril extract of *S. nicolai*, bilirubin and positive control ascorbic acid.

XTT assay

The aim of the cell viability assay was to determine which of the concentration of the aril extract and bilirubin standard produced the most toxic effect on the HeLa cell line. The effect was observed for 24, 48 and 72 hours. These results were also compared to that of Taxol® at 250 µg/ml (previously determined). The optimal dose and period were used in imminent methodologies to confirm apoptosis.

The concentrations and percentage cell viability when analysed using Graphpad prism 5 via the One Way Anova test and Kruskal-wallis test revealed that the only significant difference shown between the different concentration of the arils and bilirubin at the different time period was within the 24 hour period.

Apoptotic cell death was seen after 24 hours (Figure 3) in the aril extract (52%) and bilirubin standard (20%). This was observed in the aril extract at a concentration of 250 µg/ml and the bilirubin standard at 7.8 µg/ml. Therefore these two concentrations were used to further confirm of apoptosis.

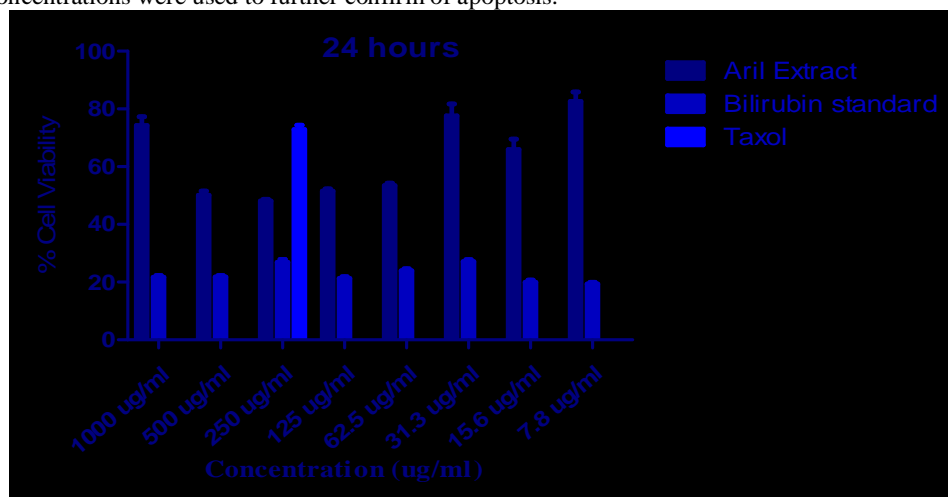


Figure 4: Effect of the aril extract of *S. nicolai*, bilirubin and positive control (Taxol®) on HeLa cells after 24 hours.

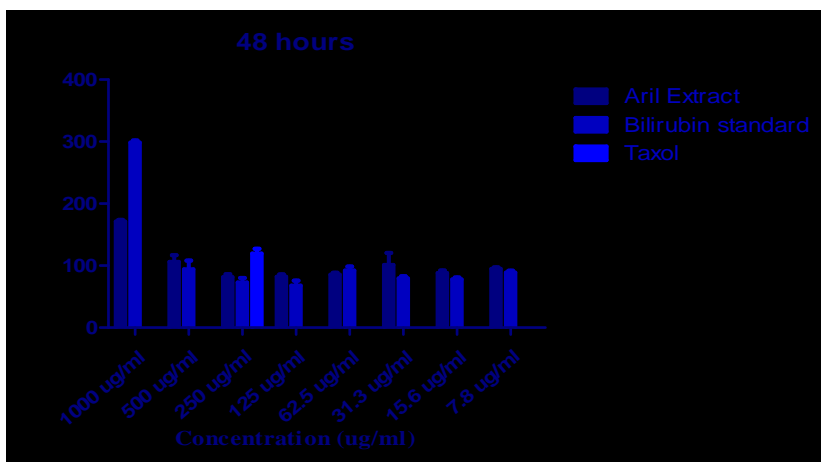


Figure 5: Effect of the aril extract of *S. nicolai*, bilirubin and positive control (Taxol®) on HeLa cells after 48 hours.

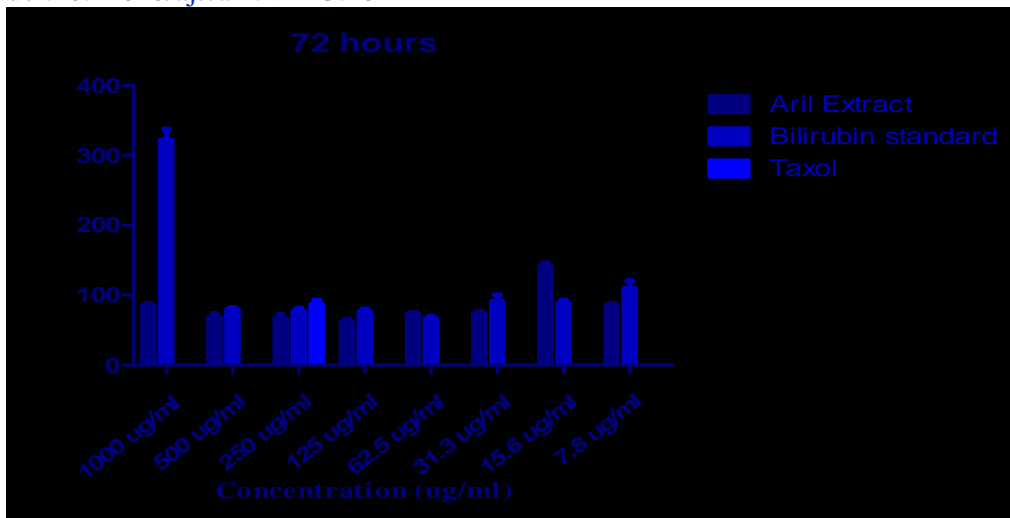
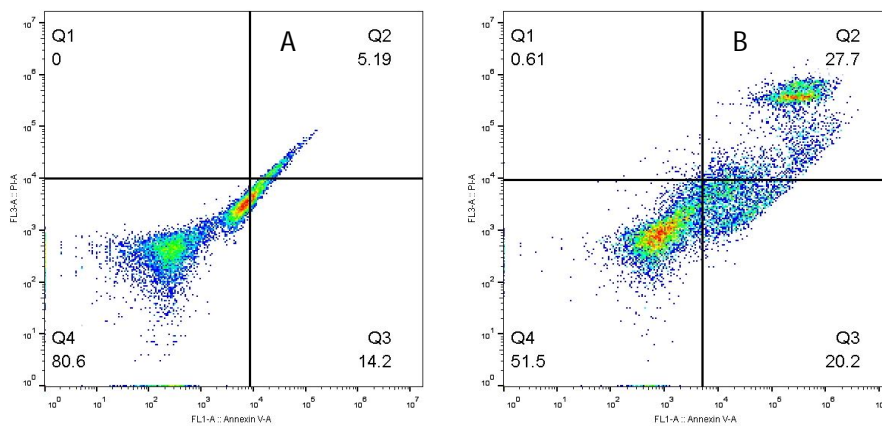


Figure 6: Effect of the aril extract of *S. nicolai*, bilirubin and positive control (Taxol®) on HeLa cells after 72 hours.

Quantification of apoptosis

A difference in the number of viable cells in the untreated control and cells that were treated with the aril extract and bilirubin standard is evident. The aril extract and bilirubin standard produced more apoptotic cells than the positive control Taxol®. The aril extract facilitated twice the production of apoptotic bodies compared to the bilirubin standard on its own.



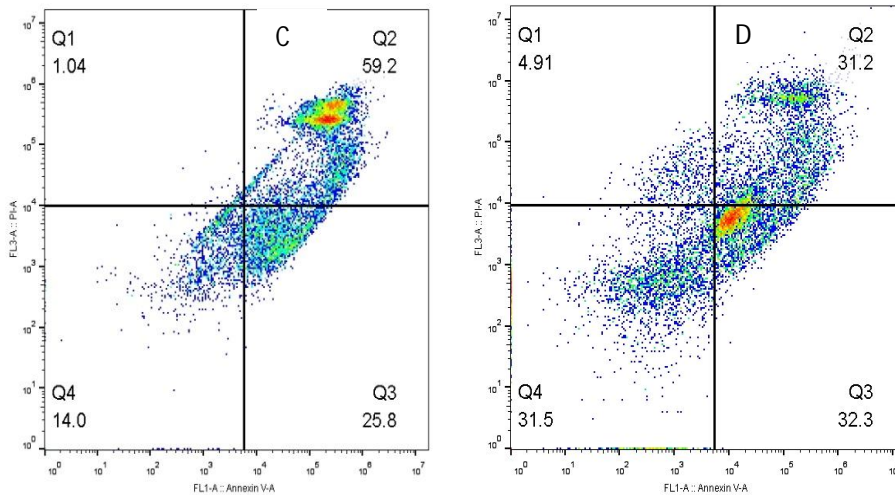


Figure 7: Flow cytometry analysis demonstrating the externalization of phosphatidylserine in HeLa cells treated for 24 hours with (A) untreated control cells (B) positive control Taxol® (250 µg/ml); (C) *S. nicolai* aril extract (250 µg/ml); and (D) bilirubin standard (7.8 µg/ml). Annexin V- PE Apoptosis Detection assay results are presented in a scatter plot form, denoting the percentage of cells that are necrotic (upper left), viable (lower left), apoptotic (lower right) and late apoptotic (upper right).

Morphological study of apoptosis

Acridine orange and ethidium bromide double staining (Figure 8. A1) shows viable normal cells that are attached and contained intact membranes. Figure 8B shows cells that were treated with Taxol®. There are only a few cells, as most of the dead cells were removed along with the media. B1 shows some cells that were still viable and attached. B2 shows non-viable normal cells and B3 shows a viable apoptotic cell. Figure 8C exemplifies cells treated with the aril extract where C1 demonstrates viable apoptotic cells which show membrane blebbing, C2 is evidence of apoptosis as this shows nuclear material from surrounding cells and C3 demonstrates non viable normal cells. Figure 8D is HeLa cells treated with bilirubin, where D1 indicates non-viable normal cells, D2 shows normal viable attached cells and D3 illustrates viable apoptotic cells starting to blebb with an uneven membrane.

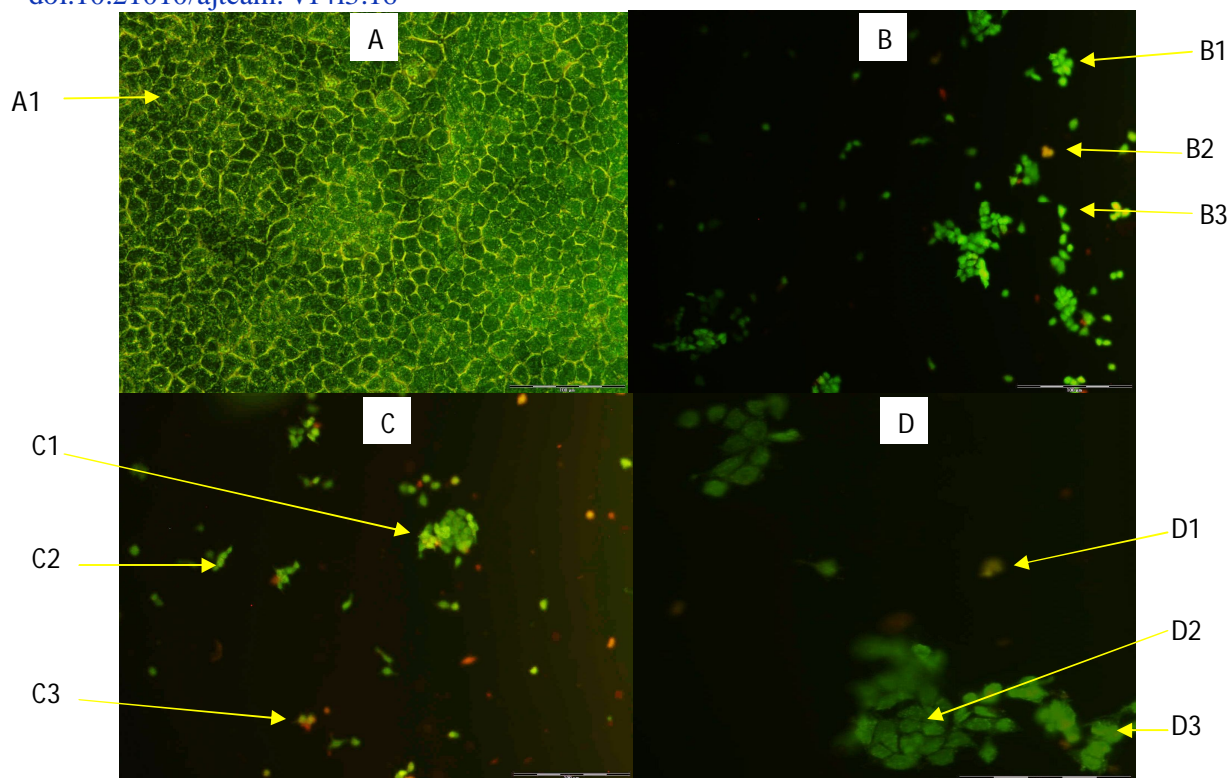


Figure 8: Fluorescent microscopic observation of cell membranes of HeLa cells treated with (A) untreated control cells (B) Taxol® (250 µg/ml); (C) *S. nicolai* aril extract (250 µg/ml); and (D) bilirubin standard (7.8 µg/ml). All observation was made at 10X magnification.

Discussion

The disadvantage of conventional chemotherapy is the severe side effects. A successful anticancer drug should kill or debilitate cancer cells without causing extreme damage to normal cells (Sheehan and Hrapchak, 1987). Natural compounds isolated from medicinal plants, with potential anticancer properties, have been of increasing interest. Thus, the focus of this study was to screen the aril extracts of *S. nicolai* that contains bilirubin as well as to screen a bilirubin standard on its own, to determine which would act as a prominent inducer of apoptosis.

The antioxidant results of this study revealed that the aril extract from *S. nicolai* produced a higher radical scavenging activity than the bilirubin standard. This is an initial indication of the strength of these extracts as an anticancer agent.

The XTT assay revealed the cytotoxicity of the HeLa cells that were treated with both the aril extracts and bilirubin showed a toxic effect after 24 hours, after which the cells disintegrated.

This study further investigated the type of cell death. The Annexin-V FITC detection kit was intended to detect specific changes in the cell surface membrane which are signature events of early apoptosis. The aril extract showed only a small number (14%) of cells that were viable, 25.8% were underwent early apoptosis and 59.2% experienced late apoptosis. In cells that were treated with bilirubin, 31.5% were still viable, whereas 32.3% were going through early apoptosis and 31.2% late apoptosis.

Morphological features play a primary role in the description of cell death. Often cell death includes both apoptosis and necrosis. The acridine orange–ethidium bromide stains, further confirmed apoptosis with characteristic cell detachment and membrane blebbing. This double staining technique showed that aril extract produced a larger number of detached smaller more rounded cells than the positive control Taxol® and the bilirubin standard, thus showing effects of late apoptosis.

Conclusion

The results indicated that *S. nicolai* possesses potential chemo preventive and therapeutic properties. The aril extract containing bilirubin showed enhanced activity compared to bilirubin on its own. These treatments indicate that the anti-cancer effects are related to two straight forward processes: inhibition of cell proliferation and initiation of apoptosis on HeLa cells *in vitro*. Whilst this study was an *in vitro* investigation, *in vivo* experiments can provide further insight into the use of this extract for the treatment of cancer.

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