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# The Antioxidant and Antiproliferative Agents from the bark of Philippine *Alstonia scholaris*(L.) R. Br.(Apocynaceae)

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## ABSTRACT

*Alstonia scholaris* (L.) R. Br., locally known as "Dita", is a vital medicinal plant species under family Apocynaceae. The plant has been used for its medicinal properties for fevers, chronic diarrhea and dysentery. This study explored the antioxidant and cytotoxic potential of the extracts of *A. scholaris* bark. Volatile components of the bark of Philippine *A. scholaris*(AS) were isolated by solvent extraction techniques and analyzed by GC/MS and LC/MS-MS. Two solvent systems were used; a 50:50 solution of MeOH/DMSO and DCM. GC-EI-MS analyses of DCM extracts showed the presence of 2(4H)-benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl-,(R)- (peak area: 2.827%) and in MeOH:DMSO extracts β-amyrin (peak area: 37.10%), olean-12-en-3-yl acetate (peak area: 21.21%), lupenyl acetate (peak area: 16.61%) and lupeol (peak area: 13.93%). Energy dispersive X-ray spectroscopy exhibited that *A. scholaris* had the following elemental percentages (<ppm): K (2.128%), Ca (1.215%), Mg (0.997%), and carbohydrates, $C_6H_{10}O_5$ (94.947%). Since DCM extracts exhibited mostly fatty acids and methanolic extracts were found to be rich in triterpenes and alkaloids, MeOH:DMSO extracts were used in the bioassays. The free radical scavenging activity of AS was found to be highly efficient ($IC_{50}$ = 0.522 mg/mL). Crude extracts of AS gave the highest efficacy towards MCF-7 with a half maximal inhibitory concentration of 4.98 µg/mL, which was followed by HT-29, H69-PR, and THP-1 which gave $IC_{50}$ values of 5.18, 6.95, and 28.64 µg/mL, respectively. The free radical scavenging and antiproliferative actions on mutant cells can be correlated to the bioactive constituents characterized in **AS**.

**Conclusion** FQ and TFQ derivatives may unveil new antidiabesity and anticancer agents.

**Keywords**: *Alstonia scholaris*, gas chromatography-electron ionization-mass spectrometry, high performance liquid chromatography-mass spectrometry, energy dispersive X-ray spectroscopy, cytotoxicity assay.

## INTRODUCTION

Traditional medicine is still largely practiced in many parts of the Philippines as it has been proven to be the only form of accessible healthcare to dispersed populations. Among the most extensively used plants in traditional medicinal system in the country are the species of genus *Alstonia* R. Br. in the family Apocynaceae. *Astoniascholaris*(L.) R. Br., the most widely distributed species in the genus, is planted mainly for local use as sources of medicine and wood (Quisumbing, 1978). *A.*

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*scholaris* is known by various names, for example Black board tree, Devil's tree, kilky pine, milkwood pines, mill wood, Scholar's tree, White cheese wood and verbal in English (Khyade et al., 2014). Its species name "*scholaris*" was derived from the use of the word in making blackboards and wooden slates in schools around South East Asia to make blackboards (Arulmozhi et al., 2007b; Baliga, 2010, 2012).

Different organs of the *A. scholaris* are used in medicine for the treatment of malaria, jaundice, gastrointestinal troubles, cancer, etc. (Warrier et al., 1996; Khare, 2007). The most intensively used organ of *A. scholaris*, however, is the bark which was recently found to have antimicrobial properties. (Shetty et. al., 2014). The bark produces abundant white sap when cut which is found out to contain a toxic alkaloid, a form of defense for the plant (allelochemical) (Kaushik, 2011). In traditional medicinal system in the Philippines, the bark is regarded as a remedy for fevers, chronic diarrhea, dysentery (Quisumbing, 1978; Wiart 2006; Ragradio, et al., 2013). Early Spanish records reported *A. scholaris*bark alkaloid being used in hospitals as a quinine substitute (Pardo de Tavera, 1901). Decoction of *A. scholaris* bark is used as a febrifuge and tonic, as well as an emmenagogue, anticholeric and vulnerary (Pardo de Tavera, 1901; Kirtikar&Basu, 1918). About twenty-five grams of the bark is macerated in a bottle of muscatel or sherry to prepare tonic wine (Pardo de Tavera, 1901). For treating malaria, on the other hand, 5% decoction of the bark is prepared and is drink as tea (Pardo de Tavera, 1901; Kirtikar&Basu, 1918; Wiart 2006). Tincture of the bark is occasionally used as galactagogue (Pardo de Tavera, 1901) while the milky bark juice is applied to ulcers and rheumatic pains or mixed with oil to be used as dropsy (Pardo de Tavera, 1901).

Phytochemical investigations reported a wide range of chemical compounds in different organs of *A. scholaris*. These include iridoids, coumarins and flavonoids in leaves as well as terpenoids in barks and roots of *Alstonia scholaris*. Meanwhile, alkaloids, phlobatanins, simple phenolics, steroids, saponins and tannins have been identified from all organs of *A. scholaris* (Macabeo, et al., 2005; Cai, et al., 2007; Cai et al., 2008a; Cai et al., 2008b; Cai et al., 2010; Khyade and Vaikos, 2009; Khyade and Vaikos, 2010; Ragasa, et al., 2013; Ragasa et al., 2016). Among these class of compounds, alkaloids are the most important with over 70 distinct types reported in different organs of the plant, mainly in the leaves (Khyade et al., 2014).

This study is limited to the analyses of the constituents through gas chromatography mass spectroscopy, reversed phase liquid chromatography mass spectroscopy (ESI-QToF-MS/MS) in positive ion mode, and an energy dispersive X-ray spectrometer. Solvent systems used in this study were dichloromethane (DCM) and dimethylsulfoxide (DMSO)/methanol (MeOH). In this work, we established the antioxidant, and cytotoxic activities (H69PR, MCF-7, THP-1 and HT-29 immortalized cancer cell lines; HDFn normal human fibrobalsts) of crude **AS** MeOH:DMSOextracts.To the best of our knowledge, this is the first reported study using this methodology of these selected bioassays and chemical characterizations of *A. scholaris* bark.

## MATERIALS AND METHODS

### Sample collection and preparation

The bark of the *A. scholaris*, which was collected from Mariveles, Bataan, Philippineswas pulverized into a powdered biomass. The powder was used for various analytical techniques to characterize the aforementioned species. The bark of each species was ground using an osterizer followed by soaking ~3.0 grams of the powdered biomass in 20 mL of dichloromethane (DCM) for three hours. Extracts were filtered and then dried under nitrogen for one hour. The crude extract yielded approximately 0.4 grams. Extraction proceeded with a solution of 50:50 DMSO:MeOH according to a previously reported method (Harukaze et al., 1999).

**GC-EI-MS Parameters**

Crude extracts from both solvents were analyzed by gas chromatography – mass spectrometry An Agilent GC MS 7890B with a HP-5MS (5% phenyl methyl siloxane) Ultra Inert column (30 m x 250 mm x 0.25 mm) with helium as carrier gas. The flow rate of the helium gas was set at 1.0587 mL/min, pressure was made to be at 9.4889 psi, with an average velocity of 37.862 cm/sec and hold time of 1.3206 min. The initial setpoint temperature was at 70□□C. The program was as follow: first ramp was set at 2 □C/min to 135□□C and held for 10 mins, second ramp had a rate of 4 $^{O}$C /min to 220 $^{O}$C and held for 10 mins, and finally, the last ramp had a rate of 3.5 $^{O}$C /min to 270□□C and held for 37 mins.

**LCMS Parameters**

LC-MS-MS analysis of the phytochemicals in the methanol - DMSO solvent extract was done using an Agilent Technologies 1200 Series HPLC with an RPC18 column and Bruker micrOTOF-Q II positive electrospray ionization (ESI) quadrupole time-of-flight (Q-TOF) MS/MS. Nitrogen gas was used as the nebulizer spray at an injection rate of 0.4 μL/s. The solvent system used was A: methanol with 5% formic acid, B: water with 5% formic acid. The flow rate was maintained at 0.4 mL/min and the solvent ratios were as follows; 0-3 mins (5% B), 4-15 mins (20% B), 16-35% (80% B) and 36 - 40 mins (5% B).

**Free radical scavenging activity**

The free radical scavenging activity of protonated methanolic extracts of leaves of **AS** were measured by a modified DPPH assay protocol (Viturroetal., 1999). Approximately ~0.3 mg of powdered biomass of each species was added to one mL of an extraction solvent made up of 7.0 mL MeOH, 2.95 mL $H_2O$ and 0.05 mL HCl. The biomass was soaked for 3 hours and was filtered to yield approximately ~50 mg of crude extract. Samples were then dried under nitrogen gas for 2 hours. 0.2 mM DPPH solution was prepared by diluting 3.94 mg of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) in 50 mL of methanol in a volumetric flask. A stock solution of **AS** was prepared at 4 mg of crude extract in one mL of methanol. The blank was made to be one mL of this solution in addition to 1 mL of methanol. One mL of the 4 mg/mL stock was added to a 10- mL test tube along with one mL of the 0.2 mM DPPH solution. A total of eight concentrations, from 2 to 0.0625 mg/mL, were made by serial dilution. Each sample absorbance was read at 515 nm (UV-VIS Shimadzu 2900) after samples were incubated at room temperature for 30 minutes. The $IC_{50}$ value of the sample, or the concentration of the sample which can inhibit 50% of the DPPH free radical, is determined by calculating the percent DPPH scavenging effect. This is calculated for by the following formula,DPPH scavenging effect (%) or Percent inhibition = $(A_o - A_1/ A_o) \times 100$

**Cell viability assay**

The bioactivity of the MeOH:DMSO extract from **AS**was tested on the following human cell lines (ATCC, Manassas, Virginia, U.S.A.): breast cancer (MCF-7), colon cancer (HT-29), small cell lung carcinoma (H69PR), human acute monocytic leukemia (THP-1); and a primary culture of normal human dermal neonatal fibroblast,(HDFn; ThermoFisher Scientific, Gibco®, USA). The cells were provided and maintained in the Cell and Tissue Culture Laboratory, Molecular Science Unit, Center for Natural Sciences and Environmental Research, De La Salle University. Culture conditions were performed in complete DMEM (Dulbecco's Modified Eagle Medium with 10% fetal bovine serum and 1X antibiotic-antimycotic; Thermo Fisher, Invitrogen, USA) and were incubated at 37°C with 95% humidity containing 5% $CO_2$.

The cytotoxic activities were assayed via PrestoBlue® (ThermoFisher Scientific, Molecular Probes®, Invitrogen, USA), a rezasurin-based viability assay. Viable cells are detected secondary to mitochondrial reductases which actively reduce blueresazurinto red resorufin with λ max at 570 nm. The relative amount of converted resorufin is directly proportional to the

metabolic activity of viable cells and can be spectrophotometrically measured at 570 nm.

Cultured cells at 90% confluence were harvested, counted and passaged in 96-well plates with viable final counts of 1.0 x $10^4$ cells/mL within each well. Adherent cells were allowed to form monolayers overnight, except for THP-1 which are floating cells. After overnight incubation, 100 µL of filter-sterilized **AS** extracts (200 µg/mL) were carefully subjected to two-fold serial dilutions in respective wells to final concentrations of 100, 50, 25, 12.5, 6.25, 3.12, 1.56,and 0.78 µg/mL, respectively. Further incubation for 72 hours was done after which 10 µL of PrestoBlue® were carefully added in each well. A minimum incubation of 1 hour was allowed for optimal reactions to take place. Wells with no **AS** extract served as negative untreated controls whilewells with Zeocin™ (ThermoFisher Scientific, Gibco®, USA), a DNA intercalating agent of the bleomycin/phleomycin family, served as positive cytotoxic control. Spectrophotometric measurements were done at 570 nm (BioTek ELx800,BioTek® Instruments, Inc., USA) and normalized to 600 nm (reference wavelength). The raw optical density (O.D.) readings were used to calculate percent cell viability through the equation below:

$$Cell Viability\ \% \left(\frac{(\text{O.D.570nm Treated Sample} - \text{O.D.570nm Blank})}{(\text{O.D.570nm Negative Control} - \text{O.D.570nm Blank})}\ \text{x}100\right)$$

Viability profiles were inferred via nonlinear regression including statistical analyses using GraphPad Prism 7.01 for Windows (GraphPad Software, Inc., USA). Half maximal inhibitory concentrations, ($IC_{50}$, the inhibitor concentration which promote 50% reduction in cell viability) were also derived from nonlinear regression data. All trials were performed in three replicates and are shown as mean ± SEM. The extra sum-of-squares F-test or Brown–Forsythe test was used to evaluate the differences in the best-fit parameters ($IC_{50}$) among data sets (treatments) and to determine the differences among dose-response curve fits according to the software's recommendation. One-way ANOVA ($p < 0.05$) was also conducted to determine significant differences among group variables, followed by the multiple comparison, Tukey's post hoc test ($p < 0.05$), to compare different pairs of data sets.

## RESULTS AND DISCUSSION

### Volatile Constituents of DCM Extracts

GC-MS analysis of dichloromethane **AS** extracts revealed eighteen volatile constituents. The compounds were listed in sequence of the corresponding retention time of the eluent through an HP-5MS column (Table 1 and Figure 1). Hard ionization of the crude extract revealed the presence of the major compounds; 2(4H)-benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl-,(R)- (peak area: 2.827%); ethanol, 2-[2-(2-butoxyethoxy)ethoxy]- (peak area: 0.539%); 5,9-dodecadien-2-one, 6,10-dimethyl-, (E,E)- (peak area: 0.369%); and bacchotricuneatin c (peak area: 0.329%) with retention times, 46.69, 43.57, 6.93 and 42.83 minutes respectively.

**Table 1.The chemical constituents of DCM extracts of AS.**

| | Compound | RT (min.) | RI[a] | % Peak Area | Functionality |
|---|---|---|---|---|---|
| *Alstonia scholaris*(AS) | | | | | |
| 1 | 5,9-Dodecadien-2-one, 6,10-dimethyl-, (E,E)- | 6.93 | 857 | 0.369% | unsaturated ketone |
| 2 | 2-Decanal, (Z)- | 30.62 | 1260 | 0.355% | aldehyde |
| 3 | Nonanoic acid | 31.78 | 1278 | 0.027% | carboxylic acid |
| 4 | Vanillin | 39.23 | 1392 | 0.010% | aromatic aldehyde |
| 5 | Bacchotricuneatin c | 42.83 | 1452 | 0.329% | clerodanediterpenoid |
| 6 | Ethanol, 2-[2-(2-butoxyethoxy)ethoxy]- | 43.57 | 1465 | 0.539% | diverse functional group |
| 7 | 2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)- | 46.69 | 1518 | 2.827% | diterpenoid |
| 8 | Dodecanoic acid (lauric acid) | 49.62 | 1569 | 0.096% | carboxylic acid |
| 9 | 1-Formyl-2,2,6-trimethyl-3-cis-(3-methylbut-2-enyl)-5-cyclohexene | 52.89 | 1627 | 0.058% | monocyclic sesquiterpene |
| 10 | Tetradecanoic acid (myristic acid) | 60.31 | 1765 | 0.132% | carboxylic acid |
| 11 | n-Hexadecanoic acid (palmitic acid) | 70.73 | 1976 | 0.095% | carboxylic acid |
| 12 | o-Anisic acid, 4-hydroxy-6-pentyl-, methyl ester, ester with 2-hydroxy-6-(2-oxoheptyl)-p-anisic acid | 87.43 | 2362 | 0.003% | aromatic carboxylic acid |

[a]Retention Index (HP-5ms column)

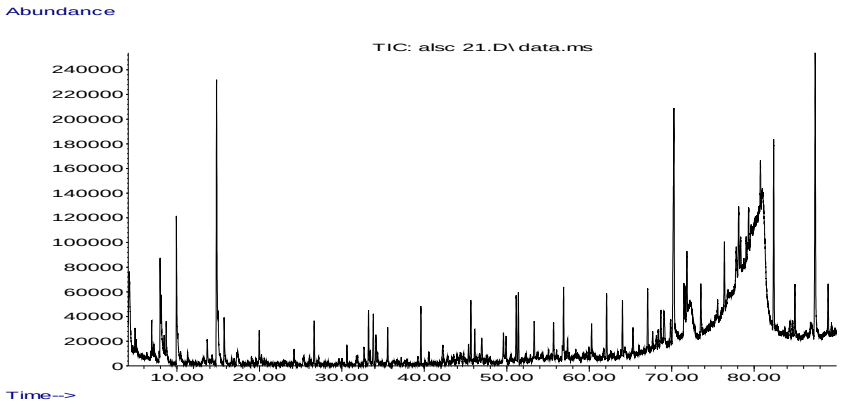



**Figure 1:Total ion GCMS chromatogram of DCM extract of AS with n-alkanes**

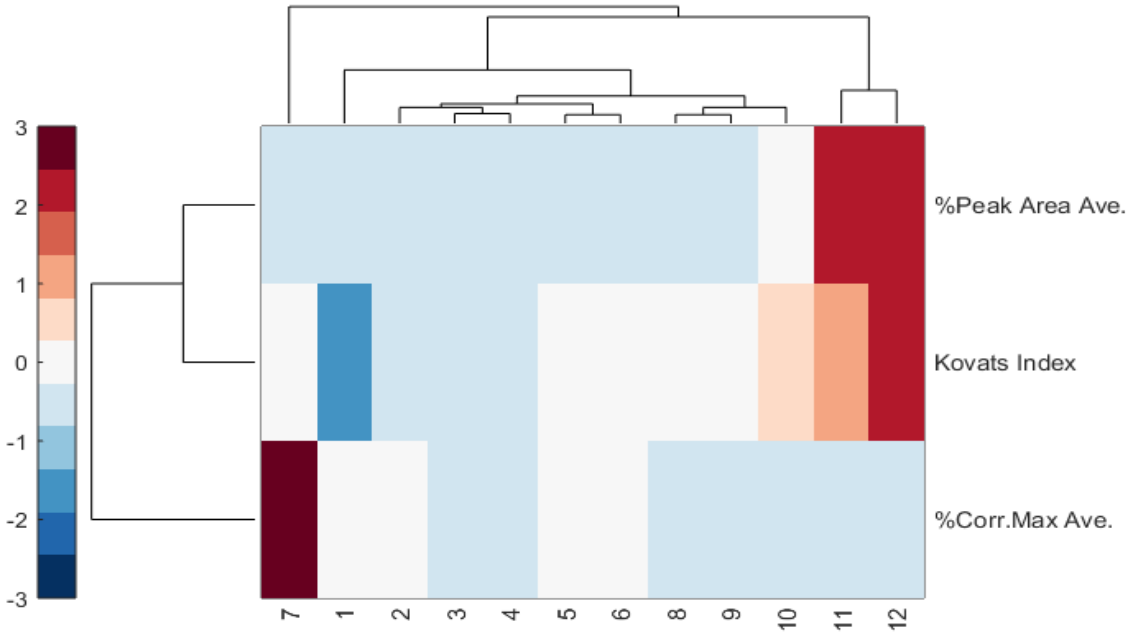



**Figure 2: Hierarchically clustered heat map of the compounds found in DCM extracts of AS gas chromatogram – mass spectrum.**

Hierarchical clustering, which involves calculating the distance matrices of data objects and then merging objects that are close to each other to form sub-clusters, has been utilized to interpret Gas Chromatography-Mass Spectrometry data with ease. In Figure 2, hierarchical clustering has been used and visualized through a heat map with dendrograms. This was generated through the clustergram function of MATLAB. Each column represents a compound in Table 1 and is labeled with its peak number. The rows represent the percent peak area, percent correlation maximum of AS, and the Kovats Index calculated in reference to 100 ppm alkane standards (Figures 2 and 4).

Figure 3 demonstrates the relation of 2-decenal, (Z)-, nonanoic acid, and vanillin in one cluster while n-hexadecanoic acid, and o-anisic acid, 4-hydroxy-6-pentyl-, methyl ester, ester with 2-hydroxy-6-(2-oxoheptyl)-p-anisic acid form another. The percent peak area of the two compounds in the later mentioned cluster is seen to be the highest and suggests the compounds' abundance in the AS extract. The clusters also suggested 2-decenal, (Z)-, nonanoic acid, and vanillin behave similarly and may have similar properties. A distinguishable compound, 2(4H)-benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)- is found separate from the other constituents. The heat map indicates benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)- has the highest percent correlation maximum, indicating that this compound has the highest abundance in AS.

2(4H)-Benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl-,(R)- also known as dihydroactinidiolide is naturally found in certain woods and teas which contribute to its 'woody aroma' (Yannai., 2004). Benzofuran derivatives, which were found to be the principal constituents of *Leucaenaleucocephala* (Lam.)

de Wit, a plant species common to tropical and subtropical regions: were able to induce strong hypoglycemic and hypolipidemic properties (Chowtivannakul et. al. 2016).

**Volatile Constituents of MeOH:DMSO extracts of AS**

Crude extract of the more polar solvent was used to target the isolation of phenolic compounds, alkaloids and larger 104.73 and 98.80 mins respectively.

terpenoids. *Alstonia scholaris* was found to have ten constituents (Table 2 and Figure 3, 4) in total; 2 alkaloids, 6 terpenoids and 2 triterpenoid acetate derivatives. The major constituents were identified to be; β-amyrin (peak area: 37.10%) olean-12-en-3-yl acetate (peak area: 21.21%), lupenyl acetate (peak area: 16.61%) and lupeol (peak area: 13.93%) with retention times 97.60, 104.24,

**Table 2. Constituents of MeOH:DMSO extracts of AS**

| | Compound | RT(min.) | RI[a] | % Peak Area | Functionality |
|---|---|---|---|---|---|
| 1 | Vincoridine | 77.74 | 2727 | 0.83% | alkaloid |
| 2 | Aspidodasycarpine, N-methyl- | 85.32 | 2986 | 0.65% | alkaloid |
| 3 | Campesterol | 93.96 | 3230 | 2.25% | triterpenoid |
| 4 | β-Sitosterol | 96.66 | 3331 | 2.70% | triterpenoid |
| 5 | β-Amyrin | 97.60 | 1452 | 37.10% | triterpenoid |
| 6 | Urse-12-en-3-one | 97.99 | 1465 | 3.21% | triterpenoid |
| 7 | Lup-20(29)-en-one | 98.24 | 1518 | 2.39% | triterpenoid |
| 8 | Lupeol | 98.80 | 1569 | 13.93% | triterpenoid |
| 9 | Olean-12-en-3-yl acetate | 104.24 | 1627 | 21.21% | triterpene acetate |
| 10 | Lup-20(29)-en-3-ol, acetate, (3β)- | 104.73 | 1765 | 16.61% | triterpene acetate |

[a]Retention Index (HP-5ms column)

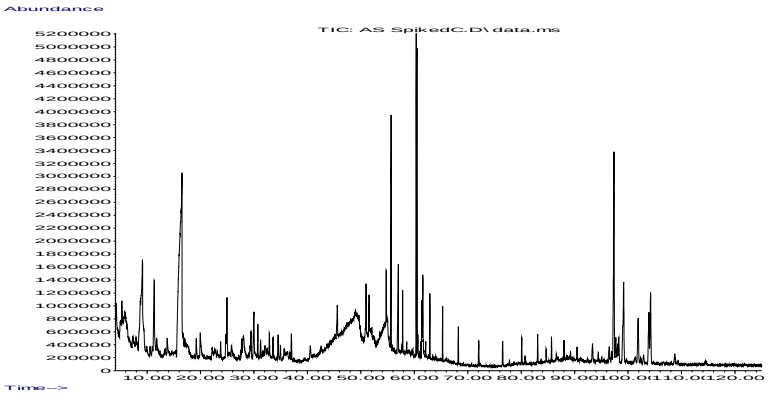



**Figure 3:Total ion GCMS chromatogram of MeOH/DMSO extract of AS with n-alkanes**

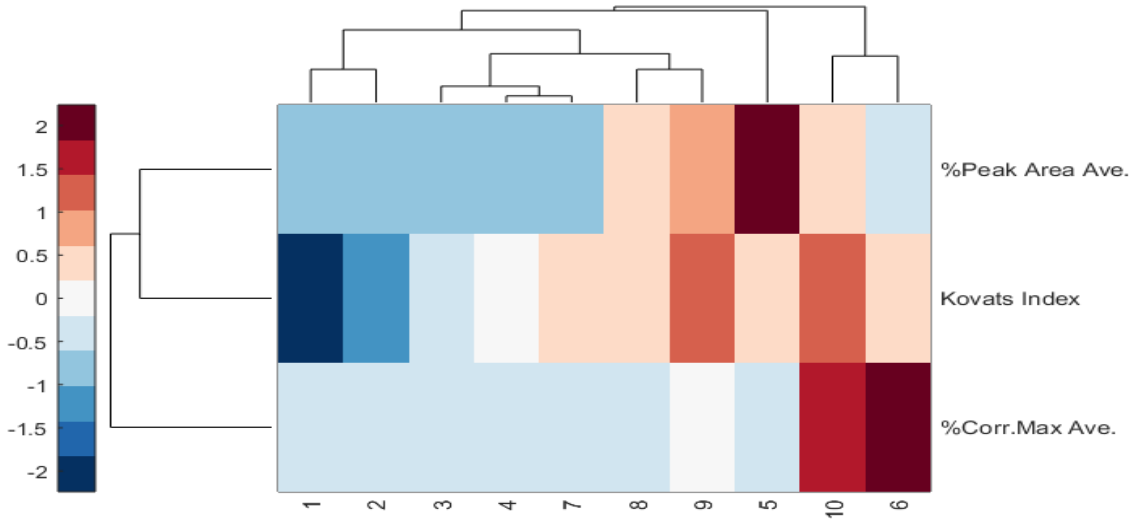



**Figure 4: Hierarchically clustered heat map of the compounds found in MeOH:DMSO 50:50 extracts from AS gas chromatogram – mass spectrum.**

**LC-ESI-Q-ToF-MS/MS analysis of AS**

Resultant fragmentation patterns and ion abundances indicated the presence of aspidodasycarpine, N-methyl- (384 m/z), an alkaloid and lupeol, a triterpenoid (426 m/z). GC-EI-MS and LC-ESI-MS analyses exhibited the presence of the aforementioned constituents in MeOH:DMSO extracts of **AS**, as seen in Figure 5 - 7.

Methanolic**AS**extracts (40 ppm) were analyzed by LCMS targeting masses 385 m/z and 427 m/z. The following fragments were formed when MRM analysis was carried out with a collision energy (CE) of 30 eV for aspidodasycarpine, N-methyl-: 385 m/z $[M+H]^+$, 367 m/z $[M\text{-}OH]^+$, 310 m/z $[M+H\text{-}CH_2\text{-}COOCH_3]^+$. Selected fragments formed from lupeol were 427.22 $[M+H]^+$, 409.21 $[M\text{-}OH]^+$ and 384.18 $[M\text{-}C_3H_9]^+$.

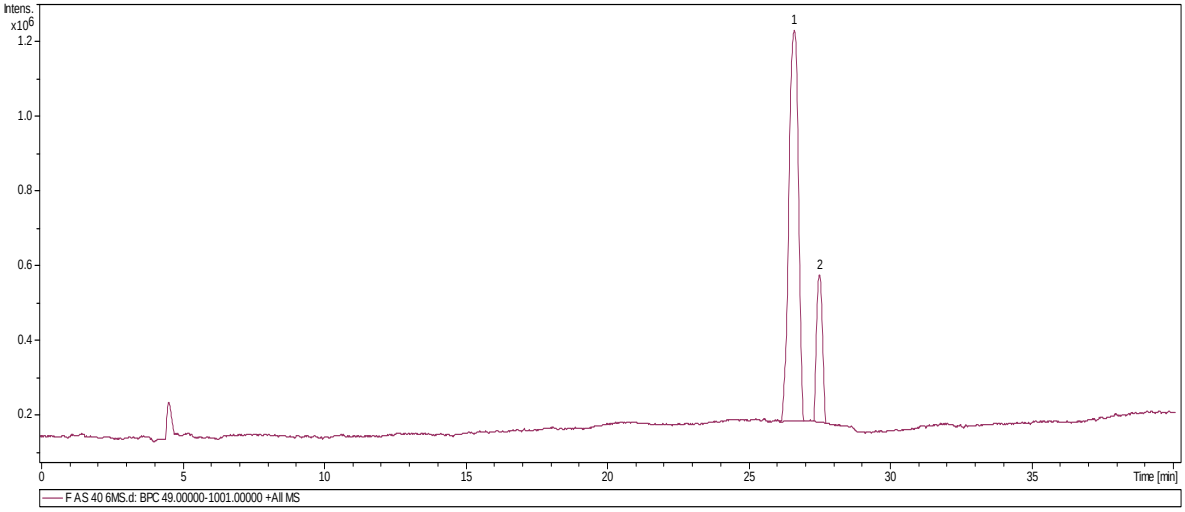



**Figure 5: Total ion chromatogram of a 40 ppm *Alstonia scholaris*extract in positive ion mode**

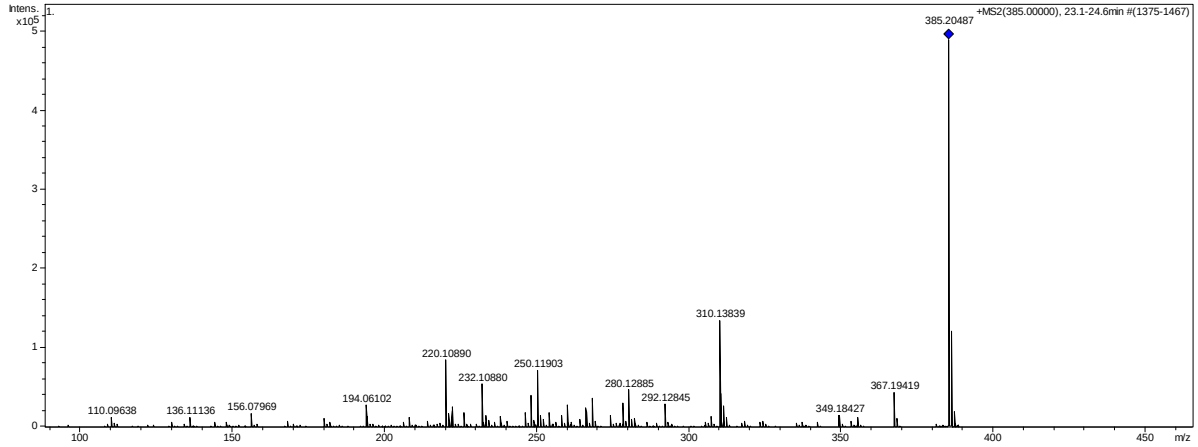



**Figure 6: Targeted (MRM) positive ion mode ESI-MS/MS mass spectrum of aspidodasycarpine, N-methyl-**

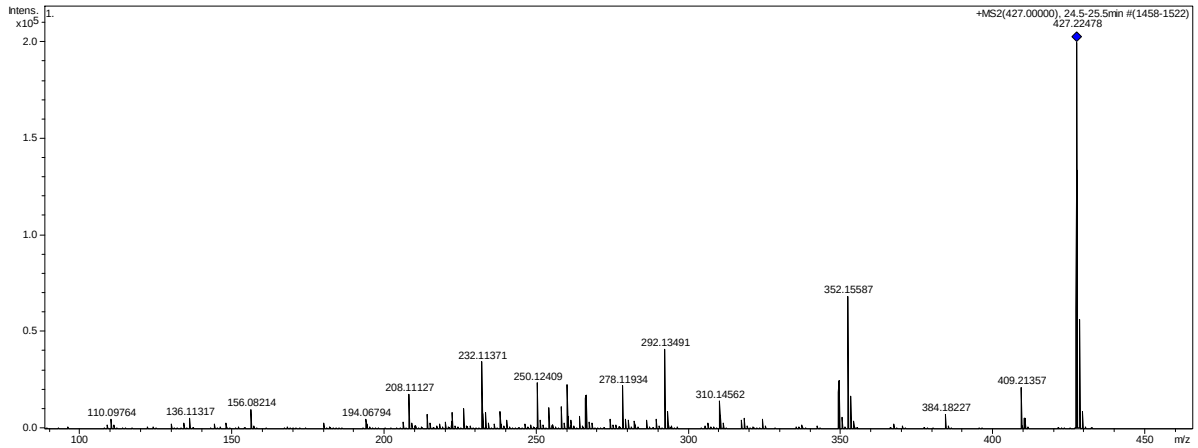



**Figure 7: Targeted (MRM) positive ion mode ESI-MS/MS mass spectrum of lupeol**

**EDX analysis of AS**

The chemical compositions of the adsorbents (Figure 8) were found to have an elemental percentage (<ppm) of K (2.128%), Ca (1.215%), Mg (0.997%), and carbohydrates, $C_6H_{10}O_5$ (94.947%). An increase in the consumption of potassium rich foods has been well established to normalize blood pressure and cardiovascular dynamics, alleviate bone and kidney disease, and prevent strokes (Sebastian et al., 2006).

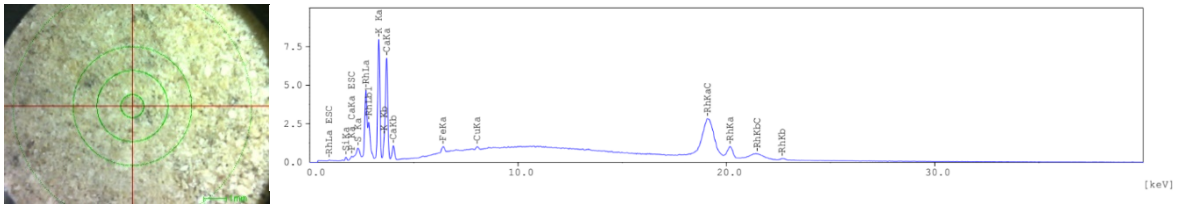



**Figure 8. EDX image and X-ray energy spectrum of AS bark**

**Antioxidant activity of AS**

The free-radical scavenging activity of **AS** exhibited high antioxidant activity as seen in Figure 9 ($IC_{50}$: 0.9497 mg/mL). The prevention of antioxidant effects of phytosterol such as □-sitosterol, stigmasterol, and campesterol, from lipid peroxidation was investigated and evaluated with 2,2,5,7,8-pentamethyl-6-chromanol. This study found that the phytosterol, stigmasterol mechanistically acts as an antioxidant, a moderate free radical scavenger, and can stabilize membranes (Yoshida et al., 2003).

Antioxidant activity of *A. scholaris* bark (trunk) has been studied within *in vitro* study models. There is no report on *in vivo* antioxidant potential for this species. Thus far, the reported *in vitro* radical scavenging and antioxidant activities have used crude alcoholic extracts of stem bark, leaves, flower and fruit of *A. scholaris* (Arulmozhi et al., 2007a; Ravi Shankar et al., 2008; Kumar et al., 2010; Arulmozhi et al., 2010, James et al., 2011). Observed radical scavenging and antioxidant potentials of the leaves in *A. scholaris* were ascribed to its phenolic and flavonoid content while that of flower and fruit extracts to their flavonoid content (Ravi Shankar et al., 2008; Kumar et al., 2010).

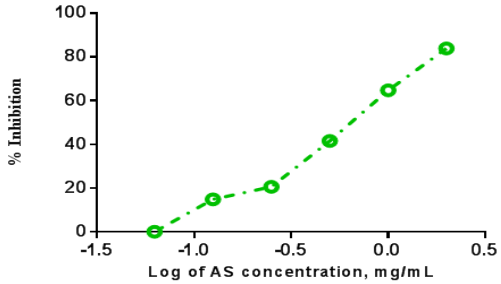
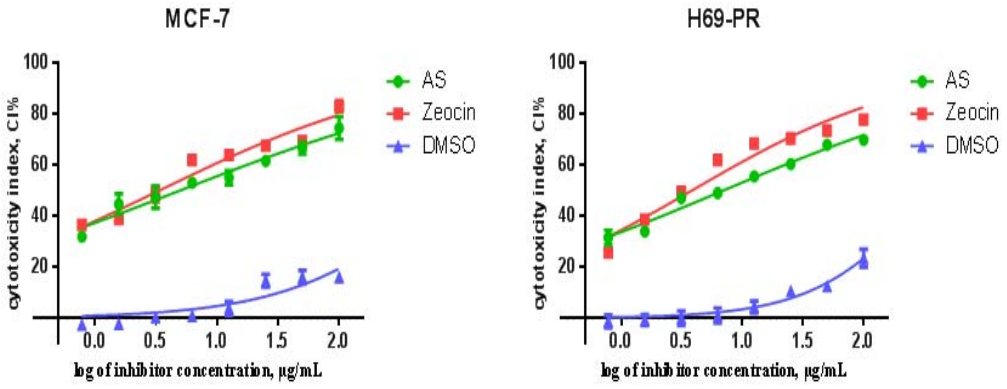



**Figure 9. Free radical scavenging analyses of AS**

**Cytotoxicity assays**

The cytotoxic activities of the **AS**MeOH:DMSOcrude extract was investigated on MCF-7, H69PR, HT-29, THP-1 and HDFn human cell lines. Analyses on wild type HDFn resulted in $IC_{□□}$ values exceeding 100 □g/mL. Illustrations of the respective cell viability indices (per cent cell viability) as a function of the logarithmic values of the concentrations used are presented in Figures 7 and 8. Most of the charts exhibited the characteristic sigmoidal inhibitory dose-response curve. Graphs comparing the cytotoxic profiles of **AS** and Zeocinpresenting the viability of each targeted cell line are found in Figure 7. The results of each individual inhibitor on all the cell lines are shown in Figure 7. $IC_{50}$ values of the isolates and the positive control are synopsized in Table 3.



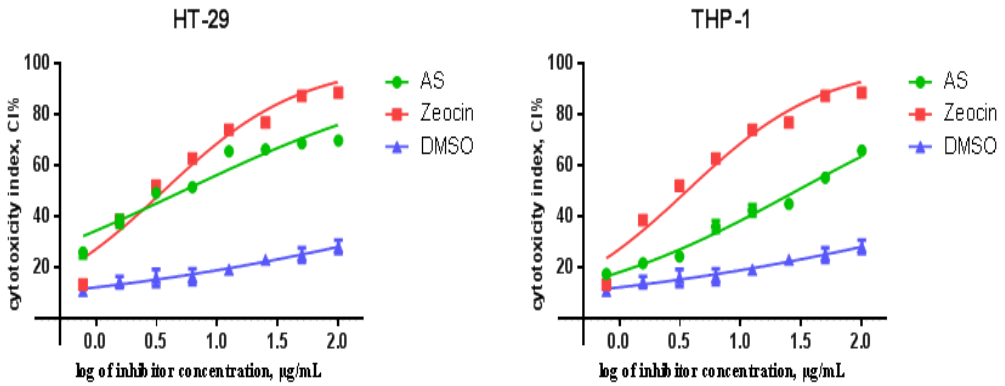



**Figure 10.**Dose-response curves displayed the cytotoxic activities of AS, Zeocin, and DMSO on the cell viability of MCF-7, H69-PR, HT-29, THP1, and HDFn: Each plot showed the effect of AS and controls against each cell line. Data are presented as mean ± SEM. GraphPad Prism 7.01 was used to perform extra sum-of-squares F-test to (A) evaluate the significance of the best-fit-parameter (half maximal inhibitory concentration) among dissimilar treatments, and to (B) determine the differences among the dose-response curve fits. MCF-7 F(Dfn, DFd) = (A) (2, 48) = 3960, $p < 0.0001$ and (B) F (2, 21) = 40.82, $p < 0.0001$; H69-PR (A) F (2, 48) = 5340, $p < 0.0001$ and (B) F (2, 21) = 30.95, $p < 0.0001$; HT-29 (A) F (2, 46) = 9512, $p < 0.0001$ and (B) F (2, 21) = 24.48, $p < 0.0001$; THP-1 (A) F (2, 48) = 3657, $p < 0.0001$and (B) F (2, 21) = 10.93, $p = 0.0006$; HDFn (A) F (2, 48) = 1850, $p < 0.0001$and (B) F (2, 21) = 26.35, $p < 0.0001$.

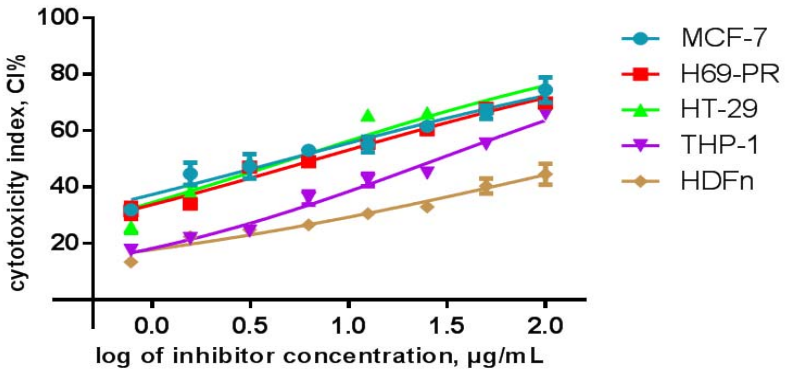



**Figure 11.**Dose-response curves indicating the cytotoxic activities of AS on the cell viability of MCF-7, H69-PR, HT-29, THP-1, and HDFn. Each plot specifies the effect of AS against each cell line. Data are shown as mean ± SEM. GraphPad Prism 7.01 was used to perform extra sum-of-squares F-test to (A) evaluate the significance of the best-fit-parameter (half maximal inhibitory concentration) among different treatments, and to (B) determine the differences among the dose-response curve fits. The results are: AS, (A) F (4, 80) = 722.0, $p < 0.0001$ (B) F (4, 35) = 4.893, $p = 0.0031$.

**Table 3.Cytotoxic activities ($IC_{50}$) values of the AS crude extract and Zeocin against MCF-7, H69-PR, MCF-7, HT-29, THP-1, and HDFn.**

| Sample | $IC_{50}$* (µg/mL) | | | | |
|---|---|---|---|---|---|
| | **MCF-7** | **H69-PR** | **HT-29** | **THP-1** | **HDFn** |
| **AS** | 4.98 | 6.95 | 5.18 | 28.64 | >100 |
| **Zeocin** | 3.37 | 3.83 | 3.78 | 3.59 | 4.08 |

*$IC_{50}$ values were extrapolated from dose-response curves calculated from nonlinear regression analysis using GraphPad Prism 7.01. The unpaired two-tailed t-test was the treatment employed for each cell line to evaluate the significant variances among the data groups. The statistical data were as follows: MCF-7, t (14) = 0.5917, p = 0.5635; H69-PR, t (14) = 0/7661, p = 0.4564; HT-29, t (14) = 0.6196, p = 0.5455; THP-1, t (14) = 2.112, p = 0.0531; HDFn, t (14) = 3.723, p = 0.0023.

Crude extracts of **AS** gave the highest efficacy towards MCF-7 with a half maximal inhibitory concentration of 4.98 µg/mL, which was followed by HT-29, H69PR and THP-1 which gave $IC_{50}$ values of 5.18, 6.95 and 28.64 µg/mL, respectively. Significant differences, based on Tukey's post hoc multiple comparison, was found between almost all cell lines ($p < 0.05$) except for the paired treatment of MCF-7 vs. HT-29 which was found to be not significantly different ($p > 0.05$). The data subsets of all the cancer cell lines and Zeocin exhibited that the paired treatments were statistically similar ($p > 0.05$).

No significant differences, as found in the unpaired t-test between the paired treatments of **AS** vs. Zeocin ($p > 0.05$) in MCF-7 trials. In fact, there was not a significant difference in the scores for mean values of AS (M = 54.32, SD = 4.73) and mean values for Zeocin (M = 58.69, SD = 5.67) conditions; t (14) = 0.5917, p = 0.5635. These results exhibited that the antiproliferative action of both **AS** and Zeocin were comparable.

Trials using H69-PR showed that the **AS** cytotoxic activity was comparable to Zeocin displayed no significant differences between the two inhibitors ($p > 0.05$) as found through an independent – samples t-test comparison. Results for the unpaired t-test exhibited that the mean values of **AS** (M = 51.89, SD = 5.05) and mean values for Zeocin (M = 58.22, SD = 6.54) conditions; t (14) = 0.7661, p = 0.4564 were comparable.

Post hoc analysis obtained that there were no significant differences between the paired treatments ($p > 0.05$) of HT-29 of **AS** and Zeocin. The HT-29 mean values of AS (M = 54.42, SD = 5.70) and for Zeocin (M = 60.38, SD = 7.75) conditions; t(14) = 5494, p = 0.5914. The antiproliferative action can be inferred from the $IC_{50}$ values of HT-29 trials with **AS**, t (14) = 0.6196, p = 0.5455.

The global or shared results of THP-1 with inhibitor trials rejected the null hypothesis and correlated that both data subsets are unique ($p < 0.05$). Significant differences were seen between the two inhibitors ($p < 0.05$) and the results for the unpaired t-test disclosed the values of **AS** (M = 38.54, SD = 6.00) and for Zeocin (M = 61.67, SD = 9.17) conditions; t (14) = 2.112, p = 0.0531.

**AS** was established not to be cytotoxic to the normal human cell control**,** HDFn, with an $IC_{50}$ value substantially surpassing concentrations of 100 µg/mL. Multiple comparisons of **AS** and immortalized cell lines examinations confirmed that there were significant differences and the null hypothesis (one curve for all data sets) was rejected (log $IC_{50}$ the same for all data sets) as verified by the extra sum-of-squares F-test of the best-fit-parameter (half maximal inhibitory concentration) among different treatments, F (4, 80) = 722.0, $p < 0.0001$, and to the differences among the dose-response curve fits F (4,

35) = 4.893, p = 0.0031. Similarly, as pertains to their respective $IC_{50}$ results, trials with each aberrant cell line treated with Zeocin were found to not be significantly disparate ($p > 0.05$). Zeocin a known cytotoxic agent gave $IC_{50}$ values of 3.37, 3.83, 3.78, 3.59, and 4.08 µg/mL for MCF-7, H69-PR, HT-29, THP-1, and HDFn, respectively and multiple comparisons showed no significant difference among the trials ($p > 0.05$).

Assessment of the effectiveness of **AS** on the four immortalized cancer cell lines revealed that the integrity of H69PR, MCF7, and HT-29 consistently decreased in all the trials and the action of cytotoxicity was analogous to the positive control Zeocin. For the most part, trials observed in the cancer cell lines indicated low half maximal inhibitory concentrations (< 7 µg/mL) which were acquired for all of the trials. MCF-7 was highly responsive to **AS**; while the least responsive to **AS** was THP-1. Wild type HDFn trials incubated with **AS** were substantiated to be non-cytotoxic and gave half maximal inhibitory concentrations of greater than 100 µg/mL; whereas Zeocin treated normal cells reached their half maximal inhibitory concentrations at less than 5.00 µg/mL.

The observed bioactivity could be instigated by the alkaloids vincoridine and aspidodasycarpine, N-methyl-, since other similar alkaloids specifically, *O*-acetylmacralstonine, villalstonine and macrocarpamine, were found to possess pronounced activity against a human normal cell line (breast fibroblasts) and other human cancer cell lines, StMl1 1a (melanoma), Caki-2 (renal cell carcinoma), MCF-7 (breast adenocarcinoma), and LS174T (colon adenocarcinoma) with $IC_{50}$ values ranging 2 - 10 µM (Keawprdub et al., 1997). Phytosterols and triterpenes, such as campesterol, β-sitosterol, and lupeol were previously reported to exhibit strong antileukemic cell proliferation in two sub-fractions ($IC_{50}$ = 2.80 and 32.89 µg/mL) (Suttiarporn et al., 2015).

Anticancer potentials of stem bark, root bark and leaves of *A. scholaris* was linked to the alkaloid fraction and isolated alkaloids from the plant are available in several *in vivo* and *in vitro* study reports (Kamarajan et al.,1991; Keawpradub et al.,1997; Saraswathi et al., 1998; Keawpradub et al., 1999a; Jagetia and Baliga, 2006; Sharma et al., 2010; Ahmad et al., 2016; Wang et al., 2017). The observed anticancer/tumor effect of *A. scholaris* was also believed to be associated to the cytotoxic and anti-proliferative effects (Surya Surendren et al., 2012). The alkaloid echitamine chloride isolated from *A. scholaris* was detailed to exhibit anticancer effect on fibrosarcoma cells and against fibrosarcoma in rats (Kamarajan et al., 1995; Saraswathi et al., 1998). In a separate study, Saraswathi et al. (1999) demonstrated that echitamine chloride also affected mitochondrial and cellular respiration of S-180 cells which lead to the reduction of cellular energy pool resulting to loss of viability.

Several indole alkaloids, the bisindoles, from the root bark methanol extract of *A. scholaris* studied against two human lung cancer cell lines, MOR-P (adenocarcinoma) and COR-L23 (large cell carcinoma) showed pronounced cytotoxic activity on both cell lines (Keawpradub et al., 1997; Keawpradub et al., 1999b). Further testing of bisibdoles: O-acetylmacralstonine, villalstonine and macrocarpamine against a normal human cell line (breast fibroblasts) and several human cancer cell lines such as StMl11a (melanoma), Caki-2 (renal cell carcinoma), MCF7 (breast adenocarcinoma) and LS174T (colon adenocarcinoma), also exhibited promising activities (Keawpradub et al., 1999a).

In addition to echitamine chloride and indole alkaloids, the anticancerouschemomodulatory activity of hydroalcholic extract prepared from the stem bark of *A. scholaris* was also studied in combination with berberine hydrochloride in Ehrlich ascites carcinoma-bearing mice at different doses (Jagetia and Baliga, 2004). Results of this study has shown that these combinations have greater antitumor effects with toxic side effects observed at high

concentration of berberine hydrochloride. This study also found that the efficacy of combining hydroalcholic*A. scholaris* extract with berberine hydrochloride were most effective when given during the early stages of carcinoma.

Cytotoxic effect of different fractions of *A. scholaris* (according to seasonal variations) were evaluated on the HeLa cells with samples collected during monsoon, summer and winter seasons (Jagetia and Baliga, 2005). In this study, the dose dependent cell killing effect of *A. scholaris*was observed to be more prominent from samples collected during summer. Moreover, the cytotoxic effect was greatest for residue followed by the whole extract and chloroform fraction. However, least cytotoxic activity was observed in the steroidal fraction.

The stipulated active cytotoxic limits of natural products are 20 µg/mL or less for crude extracts. (Geran et al., 1972) The findings in this work disclosed that crude dichloromethane extracts from *A. scholaris* can be candidates for chemotherapeutic drugs or used as a corollary for medical protocols in dealing with the management of human colorectal cancer, human breast adenocarcinoma, and human small cell lung carcinoma.

## CONCLUSION

The results of this work show that **AS** bark was found to be an effective free radical scavenger and that the MeOH:DMSOcrude extracts of **AS** gave the highest efficacy towards MCF-7 which was followed by HT-29, H69-PR, and THP-1. Post hoc multiple comparisons of MCF-7 vs. HT-29 were deduced to be not significantly different ($p > 0.05$). The antiproliferative action of **AS** on H69PR, MCF-7, and HT-29 was comparable to the positive control Zeocin. Normal HDFn trials incubated with **AS** were substantiated to be non-cytotoxic and gave half maximal inhibitory concentrations of greater than 100 µg/mL. The results of this work related that crude MEOH:DMSO extracts from *A. scholaris* could possibly be candidates for chemotherapeutic regimens or as a supplementation in the management of human colorectal cancer, human breast adenocarcinoma, human acute monocyticleukemia,and human small cell lung carcinoma.

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# العوامل المضادة للأكسدة والمضادة للتكاثر من لحاء الفلبينية *Alstonia scholaris* (L.) R. Br. (أبوسيناسي)

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**4-قسم الأحياء جامعة دي لاسال،الفلبين**

**ملخص**

*Alstonia scholaris* (L.) R. Br.، المعروفة محليا باسم "Dita"، هو نوع من النباتات الطبية الحيوية تحت عائلة أبوسيناسي. وقد استخدم النبات لخصائصه الطبية للحمى والإسهال المزمن والدوسنتاريا. استكشفت هذه الدراسة الإمكانات المضادة للأكسدة والسامة للخلايا من مقتطفات من لحاء *العلماء A.* تم عزل المكونات المتطايرة لحاء الفلبينية *A. scholaris* (AS) بواسطة تقنيات استخراج المذيبات وتحليلها من قبل GC/ MS و LC/ MS− MS. واستخدم نظامان للمذيبات؛ حل 50:50 من MeOH/ DMSO وDCM. وأظهرت تحليلات MS−EI−GC من مقتطفات DCM وجود 2 (H4)−(R)−(منطقة الذروة: 2.827%) وفي MeOH: DMSO مقتطفات α−amyrin (منطقةالذروة: 37.10٪)، yl−3−en−12−olean خلات (منطقةالذروة: 21.21٪)،خلاتلوبينيل (منطقةالذروة: 16.61٪) ولوبيول (منطقةالذروة: 13.93٪). وأظهرالتحليلالطيفيللأشعة السينية المشتت للطاقة أن *ألف scholaris* كان لديه النسب المئوية الأساسية التالية (<ppm): K(2.128%)، كاليفورنيا (1.215%)، Mg(0.997%)، والكربوهيدرات، $C_6H_{10}O_5$ (94.947%). منذ مقتطفات DCM عرضت في الغالب الأحماض الدهنية ومقتطفات الميثانول وجدت لتكون غنية في triterpenes وقلويدات، تم استخدام مقتطفات DMSO:MeOH في التطبيقات الحيوية. تم العثور على نشاط الكسح الراديكالية الحرة من AS لتكون فعالة للغاية ($IC_{50}$ = 0.522 ملغ / مل). أعطى المستخلصات الخام من AS أعلى فعالية نحو 7−MCF مع تركيز مثبط ة نصف القصوى من (4.98 ميكروغرام/غرام/مل)، الذي تبعه 29−HT، 69H−PR، و1−THP الذي أعطى قيم $IC_{50}$ من 5.18، 6.95، و 28.64 −ز/مل، على التوالي. يمكن ربط الكسح الراديكالية الحرة والإجراءات المضادة للتكاثر على الخلايا المتحولة إلى المكونات النشطة بيولوجيا التي تتميز في AS.خاتمة: قد تكشف مشتقات FQ و TFQ عن عوامل جديدة مضادة للسرطان.

**الكلمات الدالة:** *Paederiafoetida* Linn، دراسة كيميائية نباتية، نشاط مضاد للأكسدة، نشاط مضاد للميكروبات، نشاط مسكن، نشاط مضاد للديدان.

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