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PHARMACOLOGICAL OVERVIEW OF PROANTHOCYANIDINS FROM THE BARK OF *THESPESIA POPULNEA* (L.) AS AN ANTIOXIDANT AND CYTOTOXIC AGENT

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Keywords:

Thespesia populnea, Proanthocyanidins, Sephadex LH-20 chromatography, (epi)catechin, (epi)gallocatechin.

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ABSTRACT: The preliminary phytochemical analysis of the aqueous ethanolic extract of the bark of *Thespesia populnea* (L.) revealed the presence of alkaloids, unsaturated sterols, triterpenes, flavonoids and the absence of anthraquinones. The froth test indicated the presence of saponins. Most importantly this study revealed the presence of proanthocyanidins which has not been reported before. In the present study, ethyl acetate and aqueous soluble proanthocyanidin fractions (EASPA and AQSPA respectively) were extracted and purified by chromatography on Sephadex LH-20. Prussian blue test and acid catalyzed cleavage test revealed that proanthocyanidins have been successfully separated from non-proanthocyanidin phenolics. The yields of purified EASPA and AQSPA fractions were 0.081% and 0.72% (by weight) of the fresh bark. Acid catalyzed cleavage followed by TLC studies of both EASPA and AQSPA fractions showed the presence of two anthocyanidins, cyanidin and delphinidin, suggesting that they are composed of (epi)catechin and (epi)gallocatechin units with (epi)catechin being more abundant compared to the other. This was further confirmed by ^{13}C NMR spectroscopic studies. In addition, ^{13}C NMR spectroscopic studies revealed (epi)catechin to be the epi-isomer. The preliminary antioxidant activity of purified EASPA and AQSPA fractions was determined using the DPPH assay. The IC_{50} values of EASPA and AQSPA (0.0725 mg/mL and 0.0781 mg/mL respectively) were clearly lower than that of ascorbic acid (0.125 mg/mL), which is an established standard used in antioxidant studies. Thus, proanthocyanidin samples appear to possess better antioxidant capacity than ascorbic acid. In addition, EASPA and AQSPA fractions were shown to mediate cytotoxic activity against MCF 7 cells through SRB assay.

INTRODUCTION: *Thespesia populnea* (L.) is a medium sized, fast growing, evergreen tree belonging to the family Malvaceae and is naturalized in tropical countries. [1] In Sri Lanka, it is commonly known as Gan-suriya or Suriya. This is an important medicinal plant used in the traditional medical systems throughout the world.

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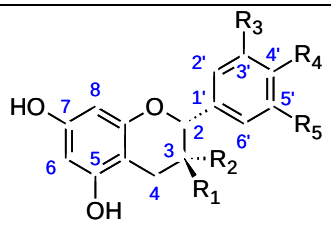
Almost all the parts of the tree are used to treat various diseases including diarrhea, skin and liver diseases, hemorrhoids and diabetes.[2] In addition, the bark of the tree is been used for the treatment of cancers in Sri Lanka. Although the phytochemistry of the bark has been exploited, the presence of an important secondary metabolite, proanthocyanidins has not been reported yet.

Proanthocyanidins belong to a class of polyphenolic compounds called flavonoids. Beside lignin, they are the most abundant class of naturally occurring phenolic compounds.[3] Phytochemical studies carried out on proanthocyanidins over the

last few decades have revealed that they occur widely in fruits, vegetables, nuts, seeds, flowers, and bark of a diverse range of plants. In nature, they occur as dimers, trimers, higher oligomers or polymers of monomeric flavan-3-ol units. The most commonly occurring monomeric flavan-3-ol units are (+)-catechin, (-)-epicatechin, (+)-gallocatechin, and (-)-epigallocatechin, while (+)-afzelechin and (-)-epiafzelechin occur to a lesser extent (**Fig. 1**).[4] The common proanthocyanidins consist of monomers that are linked through bonds between C4 and C8 or sometimes C4 to C6 bonds. These are called B-type proanthocyanidins. However, there can be an additional ether linkage formed between C2 and C7. These are called as A-type proanthocyanidins. [5] The structures of various proanthocyanidins vary depending upon the flavan-3-ol monomeric unit, the extent of polymerization, stereochemical differences and type of linkage. Thus, their natural occurrence is diverse.



Catechin: R_1=OH; R_2=H; R_3=OH; R_4=OH; R_5=H

Epicatechin: R_1=H; R_2=OH; R_3=OH; R_4=OH; R_5=H

Gallocatechin: R_1=OH; R_2=H; R_3=OH; R_4=OH; R_5=OH

Epigallocatechin: R_1=H; R_2=OH; R_3=OH; R_4=OH; R_5=OH

Afzelechin: R_1=OH; R_2=H; R_3=H; R_4=OH; R_5=H

Epiafzelechin: R_1=H; R_2=OH; R_3=H; R_4=OH; R_5=H

FIG.1: FLAVAN-3-OL MONOMERIC UNITS OF PROANTHOCYANIDINS

A significantly large number of biological and pharmacological properties have been reported for proanthocyanidins. Some of these are anticancer, anti-inflammatory, anti-aging and cardioprotective activities. [4] Most importantly they are reported to be good antioxidants. [6] Many life processes, by oxidative and reductive processes, constantly generate free radicals including reactive oxygen species (ROS). The antioxidant mechanisms in the body help to protect against the deleterious effects of ROS that are formed. However, under pathological conditions the balance between antioxidants and ROS can be impaired due to over production of ROS leading to ox idative stress.[7] This causes damages to DNA, lipids and proteins, leading to a number of adverse effects that contribute to diseases like cancer, diabetes, atherosclerosis, cardiovascular and neurodegenerative diseases.

Free radical scavengers or antioxidants protect cells against this oxidative damage. Therefore, the exploration of potent natural antioxidant compounds from plants has become an important area of research in natural product chemistry. In that context proanthocyanidins have gained significant interest in recent years as potential antioxidants. Today, nutritional supplements containing proanthocyanidin extracts from various plant sources are available, alone or in combination with other nutrients, as herbal extracts, capsules, or tablets.

The aim of the present study was to extract and purify proanthocyanidins of the bark of *Thespesia populnea* (L.) and evaluate their antioxidant and cytotoxic activities.

MATERIALS AND METHODS:

Chemicals and solvents:

All chemicals, reagents and solvents were of AR grade and purchased from Sigma-Aldrich, Germany. Water when used was distilled using GFL distillation apparatus.

Materials:

Column chromatography was performed using Sephadex LH-20 (25-100 µm, Bio-Science, Uppsala). Thin layer chromatography was performed on Cellulose F_{254} (0.2 mm, Merck) and developed with forestal solvent system (conc. HCl-glacial acetic acid-water, 3:30:10) and precoated Kieselgel 60 F_{254} plates (0.2 mm, Merck KGaA) using ethyl acetate: formic acid: dichloromethane: methanol (6.8:0.2:2.8:0.2) and ethyl acetate: formic acid: glacial acetic acid: distilled water (10:1.1:1.1:2.7) as solvent systems and visualized by spraying 2-aminoethyl diphenyl borinate (Natural Product Reagent – NPR).

Collection and preparation of plant material:
Fresh outer bark of *Thespesia populnea* (L.) was collected in the month of January (2014) from healthy trees situated within the premises of Meegoda Ayurveda Institute, Sri Lanka. The plant was botanically authenticated and a voucher specimen (M3 S08, 003) was deposited in the herbarium of the Department of Botany, University of Sri Jayewardenepura, Sri Lanka. The collected bark pieces of *Thespesia populnea* were washed with tap water. The bark pieces were then cut into small pieces and air-dried thoroughly under shade at room temperature for 2 weeks. The dried materials were stored in the refrigerator in polythene covers upon sealing.

Phytochemical screening:
Dried bark pieces (500 g) were refluxed with 1250 mL of 80% aqueous ethanol for 1 hour. The resulting extract was cooled to room temperature and filtered. This extract was subjected to preliminary phytochemical screening to detect the presence of secondary metabolites such as alkaloids, sterols, triterpenes, flavonoids, proanthocyanidins and anthraquinones according to previously published methods.[8]

Confirmatory test for flavonoids:
The presence of flavonoids in the bark extract was confirmed by carrying out thin layer chromatographic studies on precoated Kieselgel 60 F_{254} plates using Natural Product Reagent (NPR) as the visualizing agent. The developed spots were observed under UV light before and after spraying the reagent at 254 nm and 365 nm respectively.

Test for saponins:
The bark pieces (5 g) were ground and subjected to froth test to detect the presence of saponins.

Extraction of proanthocyanidins:
The extraction of proanthocyanidins from the bark of *Thespesia populnea* (L.) was carried out according to a previously published method with minor modifications. [9] The bark pieces (400 g) were refluxed with 1400 mL of 70% aqueous acetone containing 0.1% ascorbic acid for a period of 2 hours and filtered. The filtrate was saturated with sodium chloride. The aqueous layer was removed and the remaining acetone layer was washed with the aqueous phase of sodium chloride saturated 70% aqueous acetone containing 0.1% ascorbic acid. The acetone was evaporated under *vacuum* at 40˚C. The resulting crude proanthocyanidin residue was dissolved in an equal volume of distilled water and defatted with petroleum ether (40-60˚C). Then the aqueous layer was extracted with ethyl acetate (400 mL x 3). The combined ethyl acetate extracts were dried over anhydrous sodium sulphate and filtered. The solvent was removed under *vacuum* at 40 ˚C to produce the crude ethyl acetate soluble proanthocyanidin (EASPA) fraction as a light maroon colour powder (0.54 g). The aqueous fraction was freeze dried to yield the crude aqueous soluble proanthocyanidin (AQSPA) fraction as a pale maroon colour powder (4.10 g).

Purification of proanthocyanidins:
Both crude EASPA and AQSPA fractions were purified by chromatography on Sephadex LH-20, according to published methods.[10] The Sephadex LH-20 column was equilibrated with 95% aqueous ethanol and the EASPA fraction (0.50 g) was applied to the column after dissolving in 95% aqueous ethanol. The non-proanthocyanidin phenolics were eluted with the same solvent system (3000 mL). Proanthocyanidins were eluted with 70% aqueous acetone (1000 mL). Collected fractions were examined with Prussian blue and acid catalyzed cleavage tests. Fractions that gave a positive result for both the tests were combined. The acetone was removed under *vacuum* at 40 ºC and the resulting aqueous residue was freeze dried to yield 0.30 g of purified EASPA as a very light maroon colour powder.

Crude AQSPA fraction (0.50 g) was dissolved in 30% aqueous methanol containing 0.1% ascorbic acid and applied to the Sephadex LH-20 column, which was previously equilibrated with the same solvent. The non-proanthocyanidin phenolics were eluted first with 30% aqueous methanol containing 0.1% ascorbic acid (1000 mL) and then with 30% methanol (1500 mL). As before, the proanthocyanidins were eluted with 70% aqueous acetone (1000 mL). In this case also the collected fractions were examined with Prussian blue and acid catalyzed cleavage tests. The fractions that gave positive results to both tests were combined.

The acetone was removed under *vacuum* at 40 ˚C and the resulting aqueous residue was freeze dried to yield 0.35 g of purified AQSPA as a very pale maroon colour powder.

The yield percentages for EASPA and AQSPA fractions were 0.081% and 0.72% respectively by weight of the fresh bark.

Determination of monomeric composition of proanthocyanidins:

Acid catalyzed cleavage studies:

Purified EASPA (0.010 g) and AQSPA (0.010 g) were refluxed with 25 mL of 2 M HCl and the anthocyanidin fractions were extracted using amyl alcohol. The resulting amyl alcohol solutions were co-chromatographed alongside anthocyanidin standards on cellulose TLC plates using forestal as the solvent system.[11]

^{13}C NMR spectroscopic studies:

Purified EASPA and AQSPA were analyzed by ^{I3}C NMR spectroscopy in deuterated methanol (BrukerAvance III 400 spectrometer at 100 MHz). The observed chemical shifts are as follows.

(epi)catechin ^{13}C NMR (100 MHz, CD_3OD) δ: 36 (C4), 75.6 (C2), 71.7 (C3), 97 (C6), 105.6 (C8), 113.8 (C5′), 114.7 (C2′), 117.9 (C6′), 131.3 (C1′), 145 (C3′, C4′), 155.2 (C5, C7, C8a) (epi)gallocatechin ^{13}C NMR (100 MHz, CD_3OD) δ: 105.3 (C2′, C6′), 131.3 (C4′), 145 (C3′, C5′)

Antioxidant activity:

The free radical scavenging capability of purified EASPA and AQSPA fractions were determined using DPPH assay according to the method published by W. Brand-Williams *et al*. (1994) with some modifications. [12] Proanthocyanidin sample stock solution (2.0 mg/mL) was diluted to final concentrations of 1.0, 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/mL in methanol. The DPPH solution was prepared by dissolving 19.60 mg of DPPH in 500 mL of methanol. Sample solutions at various concentrations (1.5 mL) were allowed to react with freshly prepared DPPH solution (1.5 mL). The reaction mixtures were allowed to stand at room temperature for 30 minutes and then the discolouration was measured at 517 nm using the UV-Visible spectrophotometer (Perkin Elmer Lambda 35 double beam scanning spectrophotometer). All the measurements were taken in triplicate. The DPPH solution mixed with methanol (1.5 mL) in the absence of samples/standard was used as the control and methanol was used as the blank. Ascorbic acid was used as the standard. The DPPH radical scavenging activity of the proanthocyanidin samples was calculated according to the following equation and the IC_{50} values were determined.

$$\text{DPPH scavenging effect (\%)} = [(A_\circ - A_1) / A_\circ] \times 100$$

Where $A_\circ$ is the absorbance of the control and A_1 is the absorbance in the presence of the test compound.

Cytotoxic activity:

The cytotoxic activity of purified EASPA and AQSPA fractions were determined using Sulphorhodamine B (SRB) assay according to the method published by Mitry *et al*. (2000). [13] The MCF 7 cells were harvested by trypsinization and were seeded in 96-well cell culture plates. For 24 hours, these cell cultures were maintained in the DMEM (Dulbecco's Modified Eagle Medium), after which they were exposed to different sample concentrations of 5, 10, 25, 50, 100, 200, 400 and 600 µg/mL purified EASPA and AQSPA fractions. These plates were then incubated for 24 and 48 hours. The morphological changes of the MCF 7 cells were observed under the light microscope after 24 and 48 hours.

The SRB assay was then performed to determine the cell survival after exposure to different concentrations of purified EASPA and AQSPA fractions. A volume of 50 µL of ice-cold 50% trichloroacetic acid solution was gently layered on top of the medium in each well of the 96-well plate, overlaying the cells. This was then incubated at 4ºC for 60 minutes. Using tap water the wells were then rinsed for 5 times and the cells were stained with 0.4% SRB solution (100 µL/well) at room temperature for 15 minutes. The excess SRB solution was poured off and using 1% acetic acid, the wells were rinsed for 5 times in order to remove the unbound dye, and left to air dry. Unbuffered tris-base solution [tris(hydroxymethyl) aminomethane] of 10 mmol/L (200 µL/well) was

added to solubilize the bound SRB dye, after which the plates were placed on a shaker at room temperature for 1 hour. Then, the plates were read at OD 540 nm, using a microplate reader (EL_x800 Universal Micro Plate Reader, BIO-TEK INSTRUMENTS, USA) and the results were expressed as a percentage of control values using the following equation and the IC_{50} values were determined.

$$\text{Percentage of control} = [(A_t - A_b)/(A_c - A_b)] \times 100$$

Where A_t is the absorbance of the test compound, A_b is the absorbance of the blank and A_c is the absorbance of the control.

RESULTS AND DISCUSSION: Preliminary phytochemical screening of the 80% aqueous ethanolic extract of the bark of *Thespesia populnea* revealed the presence of alkaloids, unsaturated sterols, triterpenes, flavonoids and the absence of anthraquinones. In addition, the froth test indicated the presence of saponins. These results were in accordance with published reports.[14] However, there are no reports that claim the presence of proanthocyanidins in *Thespesia populnea* bark. The present study for the first time revealed the presence of proanthocyanidins in the bark extract.
Both flavonoids and proanthocyanidins are based on the same basic heterocyaclic ring skeleton. The acid catalyzed cleavage test converts proanthocyanidins to coloured anthocyanidins. During the flavonoid test, they are reduced into flavones and flavylium salts in the presence of magnesium turnings to produce a colour change.

Since proanthocyanidins are present in the extract, under acidic conditions of the flavonoid test, the proanthocyanidins can produce a false-positive result. Therefore, the presence of flavonoids in the bark extract was confirmed by thin layer chromatographic studies using NPR as the visualizing agent. All flavonoids cause fluorescence quenching at UV-254 nm. The orange and yellow colour spots observed under UV-364 nm confirmed the presence of flavonoids in the bark extract, in addition to proanthocyanidins. [15]

Proanthocyanidins have been extracted from different plant sources. According to published reports aqueous acetone has been frequently used as the extracting solvent of which 70% aqueous acetone has been reported as the most efficient solvent. [16] Thus, in the present study the extraction of proanthocyanidins from the bark of *Thespesia populnea* was carried out according to a previously published method with minor modifications [9] using 70% aqueous acetone containing 0.1% ascorbic acid. Ascorbic acid was added to the solvent to prevent the oxidation of extracting proanthocyanidins.

Crude proanthocyanidins in the acetone/water extract were partitioned into ethyl acetate and water to yield EASPA and AQSPA fractions respectively. Removal of the solvent in ethyl acetate fraction under *vacuum* yielded crude EASPA (0.54 g). The proanthocyanidin fraction in water upon freeze drying yielded crude AQSPA (4.10 g). According to published reports, the EASPA fraction contains mostly low molecular weight proanthocyanidins while AQSPA fraction contains mostly high molecular weight proanthocyanidins.[10]

Both crude EASPA and AQSPA fractions were purified by chromatography on Sephadex LH-20. All the fractions eluting from the LH-20 column were analyzed for the presence of phenolics by the Prussian blue test and the proanthocyanidins by the acid catalyzed cleavage test to anthocyanidins. Positive results for both the tests were obtained only for the fractions eluting with acetone/water (7:3). This confirmed the presence of proanthocyanidins in those fractions and an effective separation from non-proanthocyanidin phenolics. These fractions upon freeze drying yielded the purified EASPA as a very light maroon colour powder and purified AQSPA as a very pale maroon colour powder in 0.081% and 0.72% respectively, by weight of the fresh bark. Taking into account the moisture in the bark (78.18%), the proanthocyanidin content of the fractions were 0.37% & 3.30% respectively. When considering both fractions together, total proanthocyanidin yield was 3.67% on dry weight basis.

Proanthocyanidins are compounds that yield anthocyanidin pigments upon acid catalyzed cleavage reactions. [17] The oligomers or polymers of (epi)catechin produce cyanidin, whereas those of (epi)gallocatechin produce delphinidin. The rare

mono substituted (epi)afzelechin based oligomers or polymers yield pelargonidin.[18] Acid catalyzed cleavage followed by thin layer chromatography studies of the purified EASPA and AQSPA fractions alongside anthocyanidin standards showed the presence of two anthocyanidins, cyanidin and delphinidin. The spot for cyanidin was much more intense than that of delphinidin suggesting that cyanidin is more abundant compared to the other. This study revealed that both EASPA and AQSPA are composed of (epi) catechin and (epi)gallocatechin monomeric units with (epi)catechin being more abundant.

The monomeric composition was further confirmed by ^{13}C NMR spectroscopic studies. The signals of the ^{13}C NMR spectrum of the purified EASPA fraction were assigned using those reported in the literature for other proanthocyanidins. [19, 20] The spectrum shows characteristic ^{13}C signals consistent with proanthocyanidins composed of (epi)catechin and (epi)gallocatechin units, with (epi)catechin being more abundant. All the signals relevant to (epi)catechin were observed in the spectrum. The aromatic carbons of the A-ring appeared in the range 160-90 ppm localized towards the two ends. The signal due to C-8 of the extension unit falls between 110-105 ppm. Comparison of the C-8 chemical shift of the extension unit with that published suggests the probable interflavanoid linkage to be B-type where the flavanyl units are singly linked between C-4 and C-8. The aromatic carbons of the B-ring appear in a narrow range between 150-110 ppm. The rest of the signals in the spectrum starting from 90 ppm are due to the aliphatic carbons of the C-ring. The region between 90–70 ppm is sensitive to the stereochemistry of the C-ring. The signal at 75.6 ppm of the C-2 extension unit is consistent with 2,3-*cis* stereochemistry.

Thus, the monomeric unit of procyanidin can be specifically identified as epicatechin. The presence of (epi)gallocatechin was also confirmed by ^{13}C NMR spectroscopic studies. The broad peak at 145 ppm is assigned to C3’ and C4’ of (epi)catechin. This signal overlaps with the sharp and high signal assigned to C3′ and C5′ of (epi)gallocatechin. This is a characteristic signal that shows the presence of (epi)gallocatechin. The signals which appeared at 131.3 ppm due to C4′ and 105.3 ppm due to C2′ and C6′ also confirmed the presence of (epi)gallocatechin. Due to the low abundance of (epi)gallocatechin in EASPA all the signals relevant to (epi)gallocatechin were not observed in the ^{13}C NMR spectrum of EASPA and thus was not possible to deduce the stereochemistry of this monomeric unit. The ^{13}C NMR spectrum of AQSPA was similar to that of EASPA. It also clearly showed the presence of both epicatechin and (epi)gallocatechin while epicatechin being more abundant.

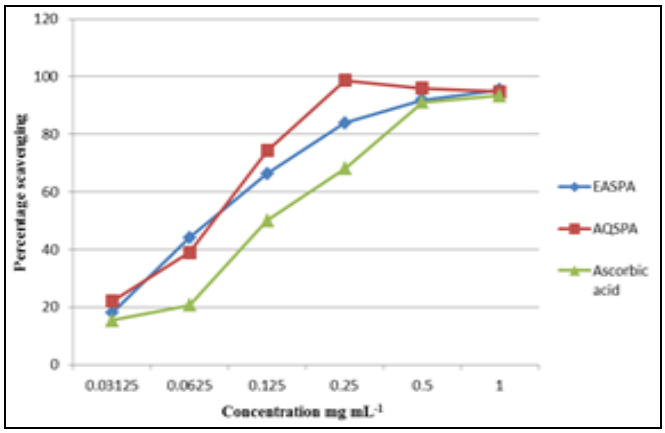


FIG.2: DPPH RADICAL SCAVENGING ACTIVITY OF EASPA, AQSPA AND ASCORBIC ACID

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical, which is commonly used for the evaluation of antioxidant activity of plant extracts. The DPPH solution undergoes a colour change from purple to yellow when it is mixed with a substance that is capable of donating an electron or hydrogen atom. The quantitative measurement of the colour change is obtained by measuring the absorbance at 517 nm. [21] Based on values obtained, the percentage of scavenging can be calculated. The percentage scavenging versus sample concentration for EASPA, AQSPA and ascorbic acid are given in **Fig. 2**. As evident from **Fig.2**, the IC_{50} values for purified EASPA and AQSPA are 0.0725 mg/mL and 0.0781 mg/mL respectively. The value for ascorbic acid is 0.125 mg/mL.

The IC_{50} values for proanthocyanidin samples are similar and these values are clearly lower than that of ascorbic acid, which is an established standard used in antioxidant studies. Thus, proanthocyanidin samples appear to possess better antioxidant potential than the standard ascorbic acid.

Since the bark of *Thespesia populnea* is being used for the treatment of cancers in the traditional medical system in Sri Lanka, and proanthocyanidins have been reported to mediate anticancer effects, it was interesting to find out whether the extracted proanthocyanidins from the bark of *Thespesia populnea* also possess anticancer activity. Thus, the cytotoxic activity of purified EASPA and AQSPA fractions in MCF 7 cells was evaluated using the SRB assay.

The results of these assays are shown in **Fig. 3** and **4**. The SRB assay is a widely used method for the evaluation of cytotoxic activity. SRB is an aminoxanthin dye, which has the ability to bind to basic amino acid residues in proteins on surfaces of viable cells under mild acidic conditions and undergo lysis under basic conditions.

The dye that is released is responsible for the colour and absorbance. The cytotoxic effect leads to a reduction of viable cancer cells and results in reduced absorbance.[22] The IC_{50} values of purified EASPA and AQSPA fractions after 24 hours were 266.8 µg/mL and 186.1 µg/mL respectively. After 48 hours the IC_{50} values were 150.0 µg/mL and 150.8 µg/mL respectively. As evident from the **Fig.3** and **4**, both EASPA and AQSPA fractions showed a dose-dependent inhibition of cancer cell survival. This was confirmed by the observation of the MCF 7 cells under the light microscope which showed a dose-dependent reduction of the MCF 7 cells. Thus, the purified proanthocyanidin samples also mediate cytotoxic activity against MCF 7 cells.

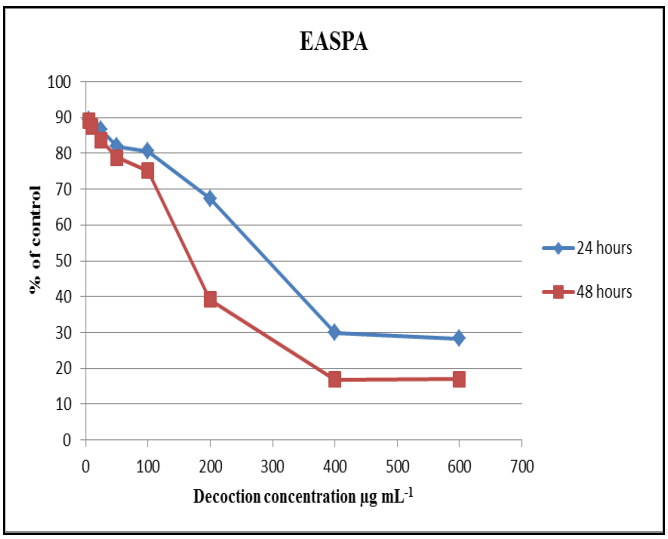


FIG.3: CYTOTOXIC ACTIVITY OF PURIFIED EASPA

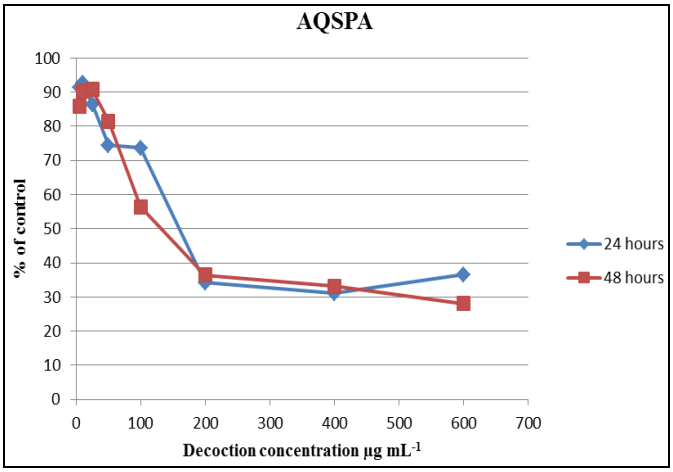


FIG.4: CYTOTOXIC ACTIVITY OF PURIFIED AQSPA

CONCLUSION: The presence of proanthocyanidins in the bark of *Thespesia populnea* is reported for the first time in the present study. Proanthocyanidins have been successfully extracted and purified from non-proanthocyanidin phenolics. They consist of epicatechin and (epi) gallocatechin monomeric units and possess antioxidant and cytotoxic activities.

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