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Original Article

Screening and Evaluation of Lectin and Anti-Cancer Activity from the Phloem Exudate/Sap of the Indian Dietary Ethnomedicinal Plants

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ABSTRACT

Objective: Lectins are extremely significant biomolecules to study several biological progressions. In this present investigation, we are screening the crude phloem exudate/ sap sample from different ethnomedicinal plants were evaluated for lectin and anticancer activity. **Methods:** The lectin activity of crude phloem exudate/sap samples were confirmed by haemgglutination assay and anticancer activity by using trypan blue, MTT and in-ovo CAM angiogenic assay. The tumor cell nuclei resulting in Giemsa stain, AO/EtBr stain, DNA Fragmentation and Caspase- 3 inhibitor assay. **Results:** Our experimental data show that the phloem exudate/sap sample S2 (*Musa acuminata*), sample S4 (*Euphorbia geniculate*) exerting the potent lectin activity, sample S5 exerting very low lectin activity against the trypsinized rabbit erythrocytes and decreases the cell viability in EAC cells *in-vitro*. Sample S2, S4 and S5 exerts significant cytotoxic effect against the various human cancer cell lines and regressed the neovasculature (development of new blood vessels) in the developing CAM embryos when compared to the other crude samples. The apoptotic inducing activity of crude phloem exudate/sap samples was revealed by DNA fragmentation assay, caspase-3 inhibitor assay and cellular morphology were studied by fluorescence staining methods. **Conclusion:** This study reports that some of the isolated crude phloem exudate/sap samples show potent lectin activity and anti-cancer activity in different human cancer cell lines. The further additional experiment needs to purify and characterize the bioactive lectin components from the potent sample which is responsible for pro-apoptotic, anti-angiogenic activity and mechanism involved.

Key words: lectin, haemagglutination, EAC, apoptosis, angiogenesis, VEGF.

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INTRODUCTION

Lectins are carbohydrate-binding proteins or glycoproteins, which are found ubiquitously in nature. 'Lectin' originates from the Latin word Legere, which means to select or to choose.[1] By binding to carbohydrates, lectins serve diverse biological functions. Lectins are present plants, animals, fungi, bacteria with the ability to interact with carbohydrates and agglutinate cells or precipitate glycoconjugates. They play important roles in defence against invasion of bacteria, virus or fungi.[2] They used in the field of blood typing diagnosing of microorganisms, mitogenic lymphocyte stimulation, acumen between normal and malignant cells. Lectins are classified based on their characteristics, binding domains and glycan structures. Interestingly, the majority of cancer biomarkers that are currently being used in the clinical settings are glycoproteins, which are structurally altered in their glycan moieties and aberrantly expressed.[3] Indeed, changes in glycosylation are believed to be the main feature in oncogenic transformation as glycans are known to be continuously involved in cancer evolving processes, such as cell signalling, angiogenesis, cell-matrix interactions, immune modulation, tumour cell dissociation and metastasis.[4]

Cancer is one of the most dreaded diseases of the 20th century and spreading further with a continuance and increasing incidence in the 21st century. Over 9 million new cancer cases are diagnosed and > 4.5 million people die of cancer P.A in the world. Due to adverse effects and complications, currently available chemotherapeutic agents have limitations in treatment. To overcome this problem, phytoconstituents from the plants exhibiting anti-cancer property is emerging as a tool in the prevention of tumour development.[5] Over more than 30% of cancer death was directly associated with dietary food habits. This is due to the changes in dietary and adaptation of a sedentary lifestyle and the burden of this disease are gradually increasing every day.[6] In the modem era, several researchers involved in order to develop therapeutics against cancer by using the medicinal plants. Lectins from plants are considered to be one of the major sources lectins which localized in various parts of plants.[7] However, numerous plant-based lectins have been well studied,

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but their physiological role of these lectins is poorly understood and it is argued that lectins which exhibit biological role need careful study in order to execute its molecular mechanism in the physiological conditions. Edible/Dietary lectins which are plant protein components with the ability to bind, selectively, free or conjugated saccharide in a reversible way by two or more binding sites, have been shown to induce lymphocyte proliferation or modulate several immune functions including agglutination, immunomodulation, anti-inflammatory, anti-cancer properties.

The current work exploits the use of edible/dietary medicinal plants available in the local region of Karnataka, India. Five plants, namely *Citrullus lanatus, Musa acuminata, Manilkara zapota, Euphorbia geniculate* and *Abelmoschus esculentus* were selected to screen for the presence of lectin and anticancer activity.

All the five plants were previously reported for their medicinal values against various pathological disorders, including, inflammation, diabetes, antibacterial etc.[8-15] but none of the above plants were screened for lectin and its anti-cancerous activity. The study aimed to exhibit the effectiveness of these plants against apoptosis and angiogenesis.

MATERIAL AND METHODS

Cells and reagents

K562 human leukaemia cells, MCF-7 human breast cancer cells, HeLa human cervical cancer cells, HT-29 human colon cancer cells and Ehrlich Ascites Carcinoma (EAC) cells were purchased from National Centre for Cell Sciences (NCCS) Pune, India. MEM, DMEM, Mecoys'5A, RPMI media, PBS, Supplements Fetal Bovine Serum (FBS), Trypsin and Antibiotics were purchased from Gibco, Invitrogen life technologies (Paisley UK). MTT, Caspase-3-Inhibitor, $rVEGF_{165}$ were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Acridine orange, Ethidium Bromide, Agarose, Trypan blue dye was purchased from SRL, India.

Exudate extraction

The fresh sap/phloem exudate from the different plants is collected by using a sterile needle as described for the other sap processing with some modifications.[16] The oozed out sap was mixed with ice-cold 10mM PBS (pH - 7.4) in the proportion of 1:10 (10%, v/v) and immediately kept in the magnetic stirrer at 4 °C to avoid sap coagulation. After homogenizing for 2-3 hr, the suspension was centrifuged at 12,000 rpm, 4 °C for 10 min to remove coagulated debris. The supernatant with the transparent golden brown extract of plant sap was subjected lyophilisation (Thermo Scientific PowerDry LL1500), used for the further assay. The samples were named as follows, *Citrullus lanatus* (*S1*), *Musa acuminata* (*S2*), *Manilkara zapota* (*S3*) *Euphorbia geniculate* (*S4*) and *Abelmoschus esculentus* (*S5*) and stored at -20 °C and used for the experiments.

Protein analysis

Protein concentration was determined by Lowry's method, using bovine serum albumin (BSA) as a reference standard with minor modification.[17] Briefly, 0.5 ml of crude latex samples were incubated with the Lowry reagent for 10 min at 37 °C. 0.5 ml of FC (Folin-Ciocalteu) reagent (1:1) was added to the reaction mixture and incubates the samples for 30 min at room temperature and absorbance was read spectrophotometrically.

Erythrocytes (RBCs) preparation

Fresh rabbit blood is collected in a sterile tube, containing an equal volume of the anticoagulant solution (Alsever's Solution) and centrifuged at 2500 rpm for 5 min. Collect the packed cells and repeat the above step until the supernatant becomes colourless and prepare the 2% of RBC suspensions by using the Alsever's solution.[18]

Haemagglutination assay

Haemagglutination (HA) tests were done as per the methods, as described earlier.[19] Briefly, 50 µl 2% trypsinized rabbit erythrocytes were incubated with crude samples (1 mg/ml) solution in a U-bottomed 96 well Microtiter plate, gently mixed and incubated at 37 °C for 1 h to visualize the agglutination and photograph. Agglutination titter is defined as the minimum amount of lectin required to form a positive agglutination referred to as 1titer value.

Effect of thermal and pH stability of crude phloem exudate/ sap samples

To determine the thermal stability, crude Phloem exudate/sap samples (50 µg) was incubated at various temperatures of (0 °C to 100 °C) for a period of 30 min and agglutination activity was carried as described above. For pH stability, crude Phloem exudate/sap samples were examined using the buffer, ranging from pH 2 to 12. The buffers used were: 50 mM glycine-HCl (pH 2 and 3), 50 mM Na-acetate- acetic acid (pH 4 and 5), 10 mM PBS (pH 6 and 7) and 50 mM Tris-HCl (pH 8-12). Crude Phloem exudate/sap samples (50 µg) was incubated with the equal volume of buffer for 60 min and the agglutination assay was performed as described earlier.[19]

Anticancer study

Animal and ethical statement

Swiss albino BALB/c female mice, aged 6-8 weeks, weighing 26 ± 1.5 g and New Zealand Swiss albino white Rabbit, weighing 1.5 ± 2 kg were housed under standard laboratory conditions and fed a diet of animal chow and water ad libitum throughout the experiment. The mice were maintained at the room temperature (22 ± 2 °C) with a good ventilation for a 12h day/night cycle. All the animal experimentations were approved by the Institutional Animal Ethics Committee (IAEC), (Approval No: BCP/IAEC/EXTP/06/2018) Bharathi College of Pharmacy, Bharathi Nagara, Mandya District, India. In accordance with the Committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines for laboratory animal facility.

In-vivo ehrlich ascites carcinoma (EAC) cell culture

The Ehrlich Ascites Carcinoma (EAC) cells were maintained by *in-vivo* passage in Swiss albino mice. EAC bearing mice were sacrificed and the tumour cells were withdrawn under aseptic conditions. A suspension of the withdraw tumour cells was made in 0.9 % sterile saline. Approximately (5×10^6 cells/ml) EAC cells were injected into the mice intraperitoneal region and growth was recorded from 1st day to14th every day. These cells grow in the mice peritoneum, forming an ascites tumour with massive abdominal swelling. The animals show a dramatic increase in body weight over the growth period and animals surrender to the tumour weight 12-14 days after transplantation.[20]

Preparation of EAC cells

The cells were isolated from the peritoneal cavity of EAC bearing mice after 10 days of transplantation. Two millilitres (ml) of saline was injected into the peritoneal cavity of mice and the peritoneal fluid containing the tumour cell was withdrawn and cells were washed with ammonium chloride to remove the RBCs, after ammonium chloride washing, again cells were washed with Phosphate buffer saline (PBS) and discard supernatant and repeat the above step twice. The obtained cells were dissolved in saline (1:5 ratio).

Trypan blue dye exclusion assay

Isolated phloem exudate/sap samples induce the cell viability in EAC cells was determined by trypan blue dye exclusion assay, as described previously.[21] Briefly, 5×10^4 cells were treated with or without crude

phloem exudate/sap samples (200 µg, 100 µg, 50 µg, 25 µg, 12.5 µg, 6.25 µg, 3.125 µg and 1.5625 µg) for 4h, stained with 0.25% trypan blue dye and counted under a microscope using a haemocytometer. The result was expressed as the percentage of viable cells in each treatment group were calculated and plot the graph.

Cell culture and maintenance

The variant human cancer cell lines, such as breast cancer (MCF-7), cervical cancer (HeLa) leukaemia (K562), colon cancer (HT-29), Chang liver cell (CCL-13) and Ehrlich Ascites Carcinoma cell (EAC) were procured from the National Centre for Cell Science (NCCS), Pune, India. The cells are maintained aseptically at 37˚C, in a mixture of MEM media for MCF-7, EAC cell lines, DMAEM media for HeLa cells, RPMI media for K562 cells and Mecoy's 5A Media for HT-29 cells with 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS) in a humidified atmosphere, containing 5% CO_2.

MTT assay

The cytotoxic effect of crude phloem exudate/sap samples was evaluated by using 3-(4, 5 dimethyl- 2-yl) -2, 5 diphenyl tetrazolium bromide (MTT) assay as described earlier.[22] In brief, 1 x 10^4 cells were seeded to 96 well plate and cells were exposed to crude phloem exudate/sap samples with a different concentration (200 µg - 1.562 µg). The cytotoxic effect of crude phloem exudate/sap samples was measured by adding 20 µl of (5 mg/ml) MTT solution to each well, incubating at the 37 ˚C for 4 h and reading the plate at 570 nm (Thermo Scientific Varioskan Flash Multimode Reader).

In-Ovo CAM angiogenesis assay

The recombinant VEGF165 ($rVEGF_{165}$) induced *in-ovo* CAM angiogenesis model was executed to study the anti-angiogenic effect of the crude Phloem exudate/sap samples. Fertilized eggs were procured from the local market of Malavalli, Karnataka, India. The eggs were grouped and incubated at 37 ºC in a humidified and the sterile atmosphere for 8 days (n = 5). The crude Phloem exudate/sap samples (10 µg/CAM) was treated on the growing CAM by making a small window on the eggshell as described earlier.[23,24] The windows were opened on the 12th day and inspected for changes in the microvessel density and photographed using a Nikon Digital camera.

Giemsa staining

Giemsa stain was performed to assess the apoptotic morphology as reported earlier. Briefly, Control and crude phloem exudate/sap samples treated EAC cells were collected and washed with phosphate buffered saline (20 mM, PBS) twice and smeared on a glass slide. Methanol and acetic acid (3:1) were used to fix the cells and stained by Giemsa solution (0.1%). The cells were observed under a light microscope and photographed using the CatCam 130 microscopic camera.[25,26]

Fluorescent analysis

AO/Et Br staining

Nuclear staining was performed according to the method as described previously.[27] Both control and crude phloem exudate/sap samples treated EAC cells were collected and washed with phosphate buffered saline (20 mM, PBS) twice and smeared on a glass slide. Methanol and acetic acid (3:1) were used to fix the cells and air dried. The cells were hydrated with PBS and stained with a mixture (1:1) of acridine orange/ethidium bromide (AO/EtBr) (4 µg/mL) solution in PBS for 5 to 10 min in the dark at the room temperature. The cells were immediately washed with PBS and viewed under a fluorescent microscope with the wavelength of 400nm-500nm and the cell morphological changes were observed under the fluorescent microscope (Carl Zeiss Axio Imager A2) and photographed.

DNA fragmentation assay

In-vitro genomic DNA from the EAC cells of control and treated with crude phloem exudate/sap samples were isolated using the phenol-chloroform-isoamyl alcohol method as described earlier with minor modifications.[28] And electrophoresed on 1.5% agarose gel. The DNA fragmentation was visualized under the Gel Doc system (Bio-Rad Universal Hood II) and documented.

Caspase – 3 inhibitor assay

Caspase-3 inhibitor assay was performed for evaluating the EAC induced caspase-3 dependent activity. EAC cells were neither or either pre-incubated with caspase-3 inhibitor Ac-DEVD-CHO (100 mM) for 1hr and treated with or without crude Sample treated for 4hrs. Cells were centrifuged and isolate the DNA and the fragmentation was visualised by 1.5% agarose gel and Documented.[29]

Statistical analysis

Values were expressed as a mean ± standard error (SEM). Statistical significance was evaluated by the one-way analysis of variance (ANOVA), followed by the use of Student's t-test by using the Graph Pad Prism 5.1.

RESULTS

Crude phloem exudate/sap samples show potent haemagglutinating activity in RBCs

Crude phloem exudate/sap samples were used to examine for the presence of lectin activity through the haemagglutination assay. The agglutination activity of crude phloem exudate/sap samples confirmations potent lectin activity against 2% trypsinized rabbit RBC's. The crude latex sample *S2* (*Musa acuminata*) exhibits high lectin activity of 4096HU/mg, sample *S4* (*Euphorbia geniculate*) exhibit moderate lectin activity of 128HU/mg and sample *S5* (*Abelmoschus esculentus*) exhibits low lectin activity of 16HU/mg titter value (Figures 1A and 1B and Table 1). Further, the stability of crude phloem exudate/sap samples was evaluated by different kinetic parameters such as temperature and pH. The results were found to be sample *S2*, were extremely thermostable even at the temperature of 70 °C, whereas at 80 °C, it lost 20% activity and it lost 40% activity at 100 °C, sample *S4* thermostable even at the temperature 50 °C, whereas at 80 °C, it lost 60% activity and it lost 70% activity at 100°C and sample *S5* thermostable at 20 °C, whereas at 80 °C, it lost 80% activity and the activity of agglutinating was completely (95%) inactivated at 100 °C (Figure 2A). Since the pH parameter of crude phloem exudate/sap sample *S2*, retained its visible agglutinating activity, even at a high pH of 9-10 and it was more stable at pH 4-8, But in the sample *S4* retained their agglutinating activity, even at the pH 6-8 and sample *S5* retained their agglutinating activity at pH 7 (Figure 2B). It is evident that sample *S2* are stable even at a high temperature and the pH variations. It is clearly evident from this observation that the lectin, responsible for the cell agglutinating activity is exerting the anti-tumour and antiangiogenic potential.

Table 1: Screening of edible/dietary lectins for heamagglutination activity (n=3).

Sl.No.	Plant Name	Family	Titre Value (HU/mg)
01	*Citrullus lanatus*	*Cucurbitaceae*	0
02	*Musa acuminata*	*Musaceae*	4096
03	*Manilkara zapota*	*Sapotaceae*	0
04	*Euphorbia geniculate*	*Euphorbiaceae*	128
05	*Abelmoschus esculentus*	*Malvaceae*	16

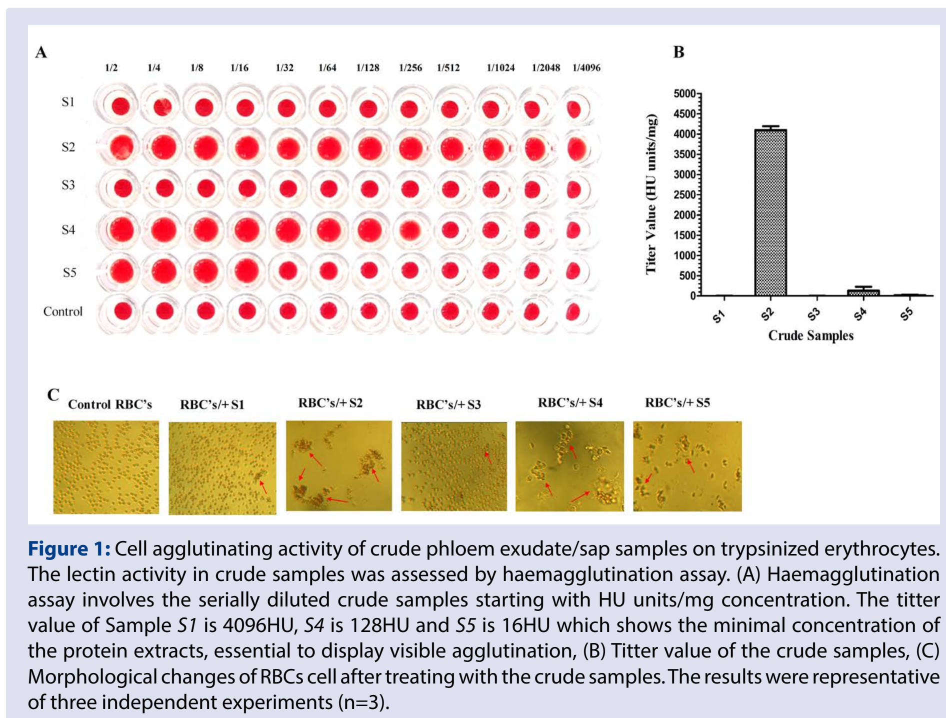

Figure 1: Cell agglutinating activity of crude phloem exudate/sap samples on trypsinized erythrocytes. The lectin activity in crude samples was assessed by haemagglutination assay. (A) Haemagglutination assay involves the serially diluted crude samples starting with HU units/mg concentration. The titter value of Sample *S1* is 4096HU, *S4* is 128HU and *S5* is 16HU which shows the minimal concentration of the protein extracts, essential to display visible agglutination, (B) Titter value of the crude samples, (C) Morphological changes of RBCs cell after treating with the crude samples. The results were representative of three independent experiments (n=3).

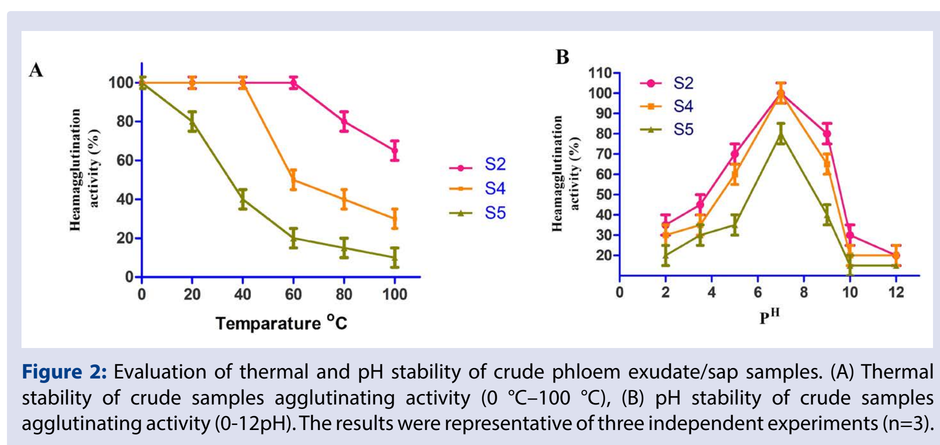

Figure 2: Evaluation of thermal and pH stability of crude phloem exudate/sap samples. (A) Thermal stability of crude samples agglutinating activity (0 °C–100 °C), (B) pH stability of crude samples agglutinating activity (0-12pH). The results were representative of three independent experiments (n=3).

Crude phloem exudate/sap samples decrease EAC cell viability *in-vitro*

To determine cell viability of crude phloem exudate/sap samples in EAC cells and cells were incubated with different concentration of crude phloem exudate/sap samples (200 µg, 100 µg, 50 µg, 25 µg, 12.5 µg, 6.25 µg and 3.125 µg) for 4 hrs, stained With 0.25% trypan blue dye and counted under a microscope using a haemocytometer. Significant 100% of viability in control cells, 95% viability was observed in Sample *S1*, 15% of viability in Sample *S2*, 90% of viability in sample *S3*, 35% of viability in sample *S4* and 75% of viability in sample *S5* at the concentration of 200µg after 4 hrs of incubation (Figure 3).

Crude phloem exudate/sap samples inhibit cancer cell proliferation

Cytotoxicity activity of crude phloem exudate/sap samples was evaluated on the various human cancer cell lines of various origins and EAC of the mouse origin, human colon cancer (HT-29), breast cancer (MCF-7), cervical cancer (HeLa) and leukaemia (K562) cells. The obtained IC_{50} value against each of the cell lines is assessed by MTT assay (Table 2). It is apparent from the results that all the six cancer cell lines are sensitive to the sample *S2* and sample *S4* and sample *S2* which have inhibited the proliferative effectiveness in EAC, HeLa and MCF-7 cell lines when compared to the other cell lines and sample *S4* which have inhibited the proliferative effectiveness in EAC, HeLa and HT-29 cell lines when compared to the other cell lines. In contrast, sample *S2* and *S4* did not exhibit any changes in the viability of the normal Chang liver cell line (CCL-13), at a higher concentration of 1000 µg/ml and IC_{50} were found to be 835.75 µg in sample *S2* and 840.5 µg in sample *S4* (Data not have shown). The graph indicates the prominent anti-proliferative activity of crude phloem exudate/sap samples (Figures 4A-4C).

Crude phloem exudate/sap samples inhibits in-ovo CAM angiogenesis

The efficacy of the crude phloem exudate/sap of all the plant samples in inhibiting angiogenesis was studied in $rVEGF_{165}$ induced CAM *in-ovo* angiogenesis model. The results of the *in-ovo* CAM assay revealed that none of the crude phloem exudate/sap samples was able to inhibit the blood vessel formation in the growing CAM of the chick embryo except sample *S2*, *S4* and *S5*. A clear avascular zone around the implanted sample *S2*, *S4* and *S5* containing rVEGF165 were evident. There was a decrease of ~ 80% of the growing blood vessel in the sample *S2*, 65%

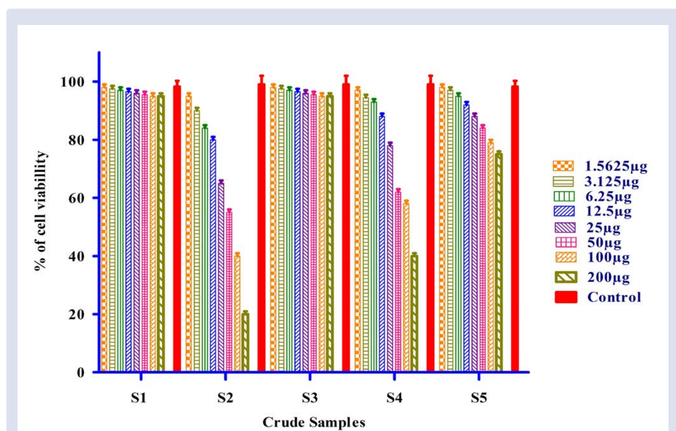

Figure 3: To evaluate *in-vitro* effect of crude phloem exudate/sap samples on EAC cell viability: (A) EAC cells were incubated with different crude phloem exudate/ sap extract of the plants such as *Citrullus lanatus* (*S1*), *Musa acuminata* (*S2*), *Manilkara zapota* (*S3*) *Euphorbia geniculate* (*S4*) and *Abelmoschus esculentus* (*S5*) and at different concentration i.e. 200 µg, 100µg, 50 µg, 25 µg, 12.5 µg 6.25 µg, 3.125µg and 1.5625 µg for 4hrs at 37°C. Percentage of viable cells were determined by tryphan blue dye exclusion method.

Table 2: IC_{50} value of the sample *S2* and *S4* against the various human cancer cell lines.

Cell Line Name	IC_{50} Value Sample S2	IC_{50} Value Sample S4
HeLa	40.25 µg ± 1.0	46.75 µg ± 1.2
HT-29	62.40 µg ± 1.5	48.25 µg ± 1.5
K562	73.75 µg ± 1.5	76.75 µg ± 1.3
MCF-7	42.50 µg ± 1.2	63.50 µg ± 1.0

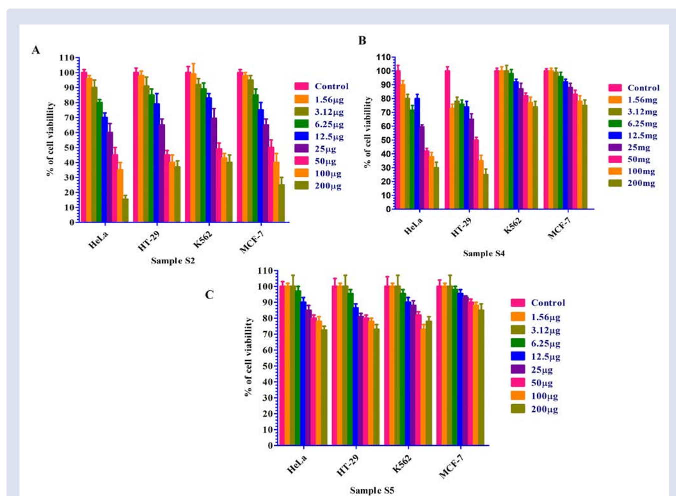

Figure 4: To evaluate the *in-vitro* cytotoxic effect of crude phloem exudate/sap samples on different human cancer cell line with different concentration (200µg to 1.562µg). (A-C) *In-vitro* cytotoxic potential of crude samples on cancer cells.

inhibition in the sample *S4* and 35% inhibition in the sample *S5* treated CAM (Figures 5A-5F).

Crude phloem exudate/sap samples induces apoptosis of EAC cells *in-vitro*

To evaluate the isolated crude phloem exudate/sap samples induced cell death of EAC cells *in-vitro*. EAC cells were treated with crude phloem exudate/sap samples were subjected to apoptosis analysis through Giemsa staining and the result shows the apoptotic morphological characteristics such as apoptotic body's cells, plasma membrane rupture, chromatin condensation, membrane blebbing and the irregular shape of cells in the treated cells but not in the control cells (Figure 6A). Further, fluorescent apoptotic morphology was assessed by Ao/EtBr dual staining method. Acridine orange is a vibrant dye and will stain both live and dead cells. Ethidium bromide will stain only the cells that have lost their membrane integrity. Live cells will look uniformly green. Early apoptotic cells will stain green and contain bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells will also incorporate ethidium bromide and therefore stain orange, but, in contrast to necrotic cells, the late apoptotic cells will show condensed and often fragmented nuclei. Necrotic cells stain orange but have a nuclear morphology resembling that of viable cells, with no condensed chromatin observed in treated EAC cells but not in control EAC cells (Figure 6B). Sample (*S2*) and sample (*S4*) exhibits potent apoptotic activity in EAC cells.

Crude phloem exudate/sap samples treatment induces caspase-3 mediated apoptosis

The evaluation of apoptotic pathway was further carried out by determining the DNA fragmentation assay. EAC cells treated with crude phloem exudate /sap sample *S2* and *S4* showed distinctive DNA fragmentation which then leads to the formation of DNA laddering that had higher apoptotic cells while the DNA of *S1*, *S3* and *S5* sample cells did not show any fragmentation (Figure 7A). Caspases have been shown to play a vital role in apoptosis induced by several deleterious and physiologic provocations. Inhibition of caspases can delay apoptosis, associating a potential role in drug screening efforts. Further investigation on the molecular insights, underlying apoptotic downstream signalling pathway revealed that the activation of caspase-3 protease in crude phloem exudate/sap samples treatment induced EAC cell death. In order to check the involvement of caspase-3 upstream activator of endonuclease, the cells were treated with or without caspase-3- inhibitor (Ac-DEVD-CHO), prior to the treatment with crude samples. The results clearly suggest that the DNA fragmentation is due to increased endonuclease activity but the Ac-DEVD-CHO, a specific inhibitor of caspase-3 enzyme inhibited the DNA fragmentation (Figure 7B). DNA Degradation and caspase-3 inhibitor assays showed that the sample *S2* and *S4* induce Caspase-3 mediated apoptosis in EAC cells. The above data is clear the confirmation of the induction of pro-apoptotic activity of crude phloem exudate/sap samples.

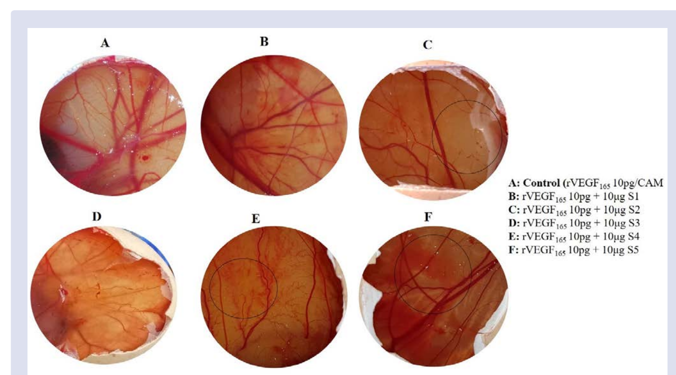

Figure 5: The anti-angiogenic activity of isolated crude phloem exudate/sap samples in *in-ovo* angiogenic CAM model. (A) *In-ovo* CAM photographs showing the crude phloem exudate/sap samples inhibit the blood vessel formation in the growing CAM of the chick embryo except for sample *S1* and *S3*. A clear avascular zone around the implanted samples containing rVEGF165 was evident.

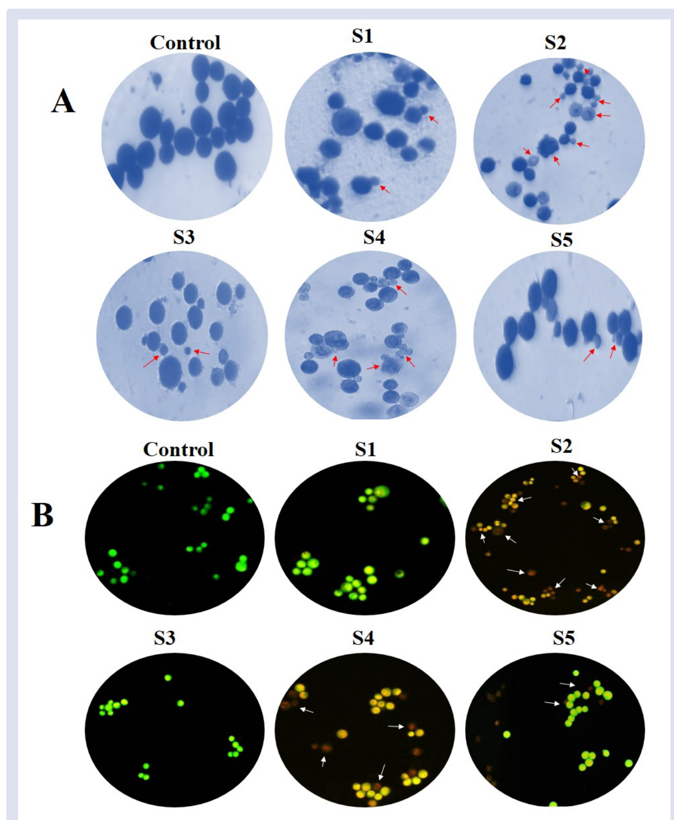

Figure 6: *In-vitro* effect of crude phloem exudate/sap samples on induction of EAC cell apoptosis. Pro-apoptotic effect of crude samples were analysed by nuclear staining technique after treatment, (A-B) Treated and untreated EAC cells were fixed in methanol/acetic acid solution (3:1) and stained with Giemsa and AO /Et Br to visualize apoptotic features such as plasma membrane degradation, membrane blebbing or apoptotic bodies upon treatment when compared to control cell morphology and the results were documented. Arrow marks in the image indicate the apoptotic morphological changes of cells (Early, late apoptotic and Necrotic cells). Sample (*S2*) and Sample (*S4*) exhibits potent pro-apoptotic activity when compared to other samples.

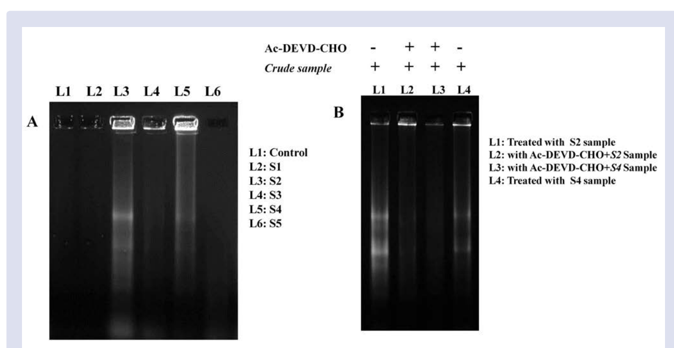

Figure 7: *In-vitro* pro-apoptotic effect of crude phloem exudate/sap samples of EAC cells were analysed by Caspase-3 inhibitor and DNA fragmentation Assay. DNA was isolated from cells treated with and without crude phloem exudate/sap samples and resolved on 1.5% agarose gel for DNA degradation. (A): Crude samples induces genomic DNA fragmentation. Lane 1: Control DNA, Lane 2:*S1*, Lane 3:*S2*, Lane 4:*S3*, Lane 5: *S4*, Lane 6:*S5* (B) Effect of caspase-3 inhibitor on crude samples induced DNA fragmentation. Lane 1: *S2* treated sample, Lane 2: with Ac-DEVD-CHO+*S2* Sample, Lane 3: with Ac-DEVD-CHO+*S4* Sample and Lane 4: *S4* treated sample.

DISCUSSION

Medicinal herbs and plants continue to play a significant role in drug discovery and development, predominantly in cancer research. The overwhelming contribution of natural products to the increase of the chemotherapeutic arsenal is evidenced by the fact that 50% of all the anticancer drugs approved worldwide between 1940 and 2006 were either natural products or natural products derived many of these novel anticancer agents have a history of using. In early 1991, Liener reported that lectin from soybean exhibited an effective anti-tumour activity which inhibited the growth of a transplanted tumour in rats.[30,31] Since then consequent years witnessed an explosive growth in the use of lectins in biomedical and cancer research. Apart from the potent cytotoxicity towards cancer cells, plant lectins are also indicated for the reduction of treatment associated side-effects as adjuvant agents during chemotherapy and radiotherapy in Europe for several years.[32] The presence of lectin has been detected in a large number of plant species seen worldwide, but very few lectins have been separated in pure form and these attempts were mainly done in global flora. So, the present investigation demonstrates the wide occurrence of lectins in locally available plants. For many years, it was alleged that lectins are a toxic substance which may harmful to the physiological system. According to Dr. Peter J. D'Adamo "eat right for your blood type", which explains the significant effect of lectin in the diet. Lectins present in the sap/phloem exudates which plays an important role in the plant defence system as well.[33] The plants that showed haemagglutination come under different families. There are many reports about the presence of lectins in the medicinal plant. This suggests that the lectins were not confined to a particular family. Some of the plants included in this study are used as food material while others are utilized in the indigenous system of medicine. Based on the early reports, in this present illumination, we screened the 05 edible/dietary plant phloem exudates/sap for lectin activity by using rabbit RBCs. Out of the 05 plants screened, 3 plants showed the presence of haemagglutinating activity against the rabbit RBCs tested, which are *Musa acuminata*, *Euphorbia geniculate* and *Abelmoschus esculentus*. The remaining 2 plants don't show the haemagglutinating activity against the rabbit RBCs tested, which are *citrus lanatus* and *Manilkara zapota*. Phloem exudate/sap from the *Musa acuminata* exhibit high lectin activity against all the kind of RBCs tested and it belongs to *Musaceae* family, Moderate lectin activity *in Euphorbia geniculate*, it belongs to *Euphorbiaceae* family and low lectin activity in *Abelmoschus esculentus*, it belongs to *Malvaceae* family (Figures 1A-1C and Figures 2A-2B). The majority of the lectins from *Musaceae* family were mannose-specific lectin which is reported previously.[34] Edible/Dietary lectins from the plant origin spread an overhaul due to its promising anti-cancer tools in the field of cancer biology. Lectins were used as diagnosing marker and massacre agents in cancer treatment.[35]

Here, we report the anti-cancer activity for the first time of the crude phloem exudates/ sap samples with a potent lectin activity, real as a striking approach for cancer treatment without damaging the host. As evident from our results, crude phloem exudate/sap sample *S2, S4* and *S5* decrease cell viability in EAC cells *in-vitro* when compared to the other samples in tryphan blue assay (Figure 3). Further, we evaluate the cytotoxic effect of crude phloem exudates/sap sample from different plants was assessed by MTT assay by using different human cancer cell lines *in-vitro.* sample *S2, S4* and sample *S5* kills cancer cells at a lower concentration and exhibits the potent cytotoxic effect in EAC, HeLa, HT-29 and MCF-7 cell line when compared to the other cell lines (Figures 4A-C and Table 2). This may due to the presence of soluble bioactive peptide /protein molecules and other small molecules which may responsible for the cytotoxic potential of cancer cells.

The growth of a tumour and metastasis are dependent on the formation of new blood vessels. Most elegant investigation of the correlation between the onset of angiogenesis and tumour growth was carried out by Folkman *et al*.[36] The tumour growth and metastasis are well-connected with several angiogenic growth factors, nutrients, oxygen supply etc.[37,38] Normal angiogenesis heavily depends on the vascular endothelial growth factor (VEGF) and its isoforms. VEGF plays a major role in developing the microvessel density in the pre-existing blood vessel and contributes to the tumour development. This finding suggests that microvessel density is a valuable prognostic factor.[39] Our study reveals that the crude phloem exudate/sap samples from the *S2*, *S4* and *S5* plants have expressively reduced the blood vessels in non-tumoral $rVEGF_{165}$ induced CAM assay and it helps to prove the *S2*, *S4* and *S5* samples are capable to bock the newly forming blood vessel and it's an anti-angiogenic activity. This clearly shows that angiogenesis is closely linked to the microvessel density of the tissue and clinical assertiveness of a tumour (Figures 5A-5F).

The literature has shown that edible/dietary plant lectins can act as an auspicious agent to induce polyclonal activation of lymphocytes[40] and they kill cancer cells through apoptosis, *in-vitro*.[41-45] Apoptosis is a significant way to maintain cellular homeostasis between cell division and cell death. The fragmentation of DNA/chromosomes as an important biochemical process occurs during apoptosis. One of the failures of tumour cells is to initiate apoptosis, following the DNA damage. The failure in the apoptosis leads to malignancy, an important hallmark of cancer.[46] The primary approaches in the cancer treatment are reinstalling the apoptotic machinery within cancer cells or the induction of apoptosis in neoplastic cells which could be an effective measure in the controlling of cancer. So, the induction of apoptosis in cancer cells is one of the useful strategies for anticancer drug development with minimal or no side effects. In this admiration, many studies were performed for screening of apoptosis-inducing lectin compounds from phloem exudate/sap sample from the medicinal plants. In the present study, we found that *S2*, *S4* and *S5* samples showed significant anti-proliferative and induction of apoptosis in EAC cells *in-vitro*. The *S2*, *S4* and *S5* sample treated cells exhibit the morphological and biochemical changes that characterize the apoptosis. Cells and nuclear shrinkage, chromatin condensation, the formation of apoptotic bodies and phagocytosis by neighbouring cells characterize the main morphological changes of the apoptosis process.[47] Giemsa stain, AO/EtBr staining study very clearly showed that cell and nuclear shrinkage, apoptotic body formation in the treated cell when compared to untreated cells (Figures 6A-6B). Cleavage of chromosomal DNA into oligonucleosomal size fragments is a biochemical hallmark of apoptosis.[48] Caspases have been shown to play a vital role in apoptosis induced by several deleterious and physiologic provocations. Inhibition of caspases can delay apoptosis, associating a potential role in drug screening efforts. Caspase-3 inhibitor can be used to inhibit caspase-3 activity and to study events downstream of caspase-3 activation. Further Caspase-3 inhibitor assay and DNA fragmentation study reveal the apoptosis-inducing activity of crude exudate samples (Figures 7A-7B). Similarly, a large number of natural foodstuffs have proven the anti-cancer property of inducing apoptosis pathways in transformed cells during carcinogenesis process.[49] As it is non-toxic, it is possible to take up further studies. Hence, purification and characterisation of the lectin component(s) and investigation of the molecular mechanism associated with anti-angiogenic and apoptotic inducing pathways are in progress.

CONCLUSION

In conclusion, our study clearly demonstrates that the isolated crude phloem exudate/sap samples strongly inhibit agglutination in rabbit erythrocytes and is shows the potent cytotoxic effect in the different human cancer cell lines. Crude phloem exudate/sap samples induce cell viability and apoptosis in EAC cells *in-vitro* and the formation of new blood vessels in *In-ovo* CAM model. Induction of apoptosis and inhibition of anti-angiogenic properties of isolated crude phloem exudate/saps samples is surely given us a ray of positivity in the field if new anti-cancer drug discovery. However, we have to carry out a further additional experiment to purify and characterize the bioactive lectin components from the potent sample which is responsible for pro-apoptotic, anti-angiogenic activity and mechanism involved.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

ABBREVIATIONS

EAC: Ehrlich Ascites Carcinoma; **HeLa:** Human Cervical Cancer Cell Line; **MCF-7:** Michigan Cancer Foundation-7 (Human Breast Cancer Cell line); **HT-29:** Human Colorectal Adenocarcinoma Cell Line; **K-562:** Leukaemia Cancer Cell Line; **CCL-13:** Chang Liver Cell Line (Normal); **MTT:** (3-(4,5-Dimethylthiazol-2-yl) -2,5-Diphenyltetrazolium Bromide); **AO/Et Br:** Acridine orange/Ethidium bromide; **RBCs:** Red Blood cells; PBS: Phosphate Buffer Saline;**CAM:** Chorio Allantoic Membrane; **rVEGF165:** Recombinant Vascular Endothelial Growth Factor 165; **HA:** Haemagglutination; ***S1*:** *Citrullus lanatus;* ***S2*:** *Musa Acuminata*; ***S3*:** *Manilkara zapota;* ***S4*:** *Euphorbia geniculate;* ***S5*:** *Abelmoschus esculentus.*

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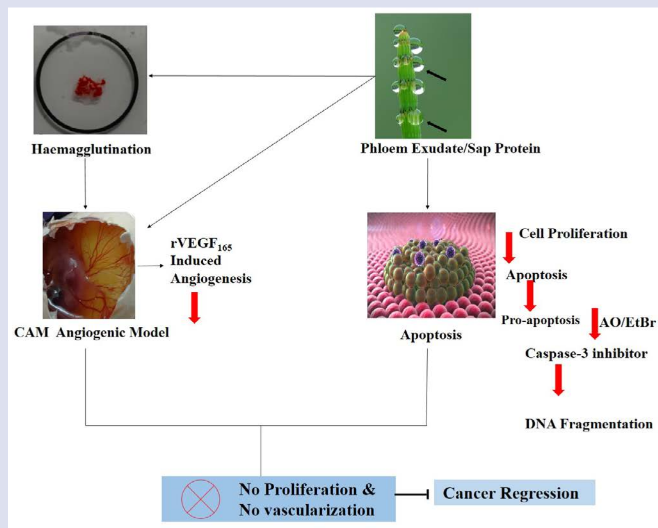
GRAPHICAL ABSTRACT



SUMMARY

- Crude phloem exudate/sap samples show strong haemagglutination activity.
- Crude phloem exudate/sap samples exhibit potent anticancer activity against the various human cancer cell lines in *in-vitro*.
- Crude phloem exudate/sap samples significantly decrease the cell viability and apoptosis in EAC cells *in-vitro*.
- Crude phloem exudate/sap samples reduce the newly formed microvessel (blood vessels) in rVEGF$_{165}$ induced in-ovo CAM angiogenic model.
- Interestingly Crude phloem exudate/sap samples did not show any toxicity in normal Chang liver cell line.

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