Leaf Extract and Active Fractions of *Dillenia pentagyna* Roxb. Reduce In Vitro Human Cancer Cell Migration Via NF-κB Pathway

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Abstract

**Background:** Different parts of *Dillenia pentagyna* have long been used in traditional medicines to cure several diseases including cancer. However, the mechanism(s) of anti-cancer effects are still unknown. We aimed to elucidate the anti-metastatic potential of ethanolic extracts of leaves of *D. pentagyna* (EELDP) and active fractions of it in highly metastatic human cancer cells. **Methods:** We screened different HPLC fractions of EELDP based on their anti-metastatic effect. We used TLC and ESI-MS for determining the presence of various phytochemicals in EELDP and fractions. We monitored in vitro anti-metastasis effect of EELDP (0-0.6 mg/ml) and active fractions (0-0.050 mg/ml) on various human cancer cells like A549, HeLa, and U2OS. **Results:** EELDP significantly reduced cell viability and cell migration in A549, HeLa, and U2OS cells. However, higher sensitivity was observed in A549 cells. We screened 2 active HPLC fractions F6 and F8 having anti-MMPs activity. EELDP and active fractions reduced metastasis via the NF-κB pathway, decreased the expression of Vimentin, N-cadherin, and increased the expression of Claudin-1. **Conclusion:** Significant reduction of metastasis by EELDP at a dose of 0.1 mg/ml or by active fractions at 0.050 mg/ml implicates that the active compound(s) present in crude or fractions are extremely potent to control highly metastatic cancer.

Keywords

*Dillenia pentagyna*, active fractions, cell migration, MMPs, EMT, NF-κB

Submitted June 16, 2022; revised September 5, 2022; accepted September 9, 2022

Introduction

Despite the development of drug design and pharmaceutical chemistry, medicinal plants are still indispensable for the majority of people worldwide to fight against several diseases.¹ Medicinal herb is a good source of traditional medicine as well as modern medicines, and phytochemicals isolated from it have been used as a template for synthetic drugs against several diseases including cancer.² *Dillenia pentagyna* has long been used in India from ancient times in the treatment of many disorders. As such no molecular mechanism is reported regarding this. In India, several tribal groups of people have been using crude extracts of many medicinal plants including *D. pentagyna* against various diseases including cancer.³ It would be worthwhile to use their knowledge for the treatment of several diseases. The people of Mizoram use methanol extract of *D. pentagyna* plant for gastric cancer. The stem bark extract of *D. pentagyna* is also reported to treat Dalton’s lymphoma.⁴ Leaf powder of this plant is used in the treatment of breast cancer in folk medicine.⁵ About 100 flowering species of the Dillenia genus are observed in the family Dilleniaceae and are available in native to tropical and subtropical regions of India, southern Asia, and Australia.⁶-⁸ Anticancer activities of other species of *Dillenia* are also reported.⁹-¹² But the detailed mechanism of action of the crude extract against various types of cancer is largely unknown. So, it is worthwhile to investigate the detailed

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mechanism of action of crude extracts and active fractions of *D. pentagyna* against various cancer cells for the further progress of folk medicines. In fact, cancer treatment nowadays is very costly and hence combating cancer using medicinal plant extract is becoming very popular. Our interest was to investigate the effect of ethanolic extract of leaf of *D. pentagyna* (EELDP) and its active fractions on cell proliferation, cell invasion, and expression of key markers of metastasis in human cancer cells.

In metastasis, the dissemination of cells from primary sites is the first step followed by invasion into the circulatory system, spreading throughout the whole body, and lodging the secondary target sites through extravasation of vascular walls. Matrix metalloproteinases (MMPs) are responsible for the degradation of the extracellular matrix to allow the cancer cells to migrate from one place to another. The MMPs activities depend upon the transcription of precursor pro-MMPs, conversion of active MMPs from pro-MMPs, and activity of tissue inhibitors of matrix metalloproteinases (TIMPs). In general, apoptotic properties are suppressed in highly proliferating cancer cells. Hence, the exploration of the reduction of metastasis properties in drug-resistant human cells like A549, HeLa, and U2OS could be a footing stone to understand the treatment strategy of cancer. Here, we present the anti-metastatic potential of EELDP on various human cancer cells like A549, HeLa, and U2OS could be a footing stone to understand the treatment strategy of cancer. Here, we present the anti-metastatic potential of EELDP on various human cancer cells such as A549, HeLa, and U2OS cells. Further, we have isolated a few HPLC fractions from crude and screened them depending on their anti-metastatic activity.

**Methods**

**Reagents**

Bovine serum albumin (BSA), trypan blue, and gelatin were purchased from Sigma-Aldrich, USA. DMEM, McCoy’s, fetal bovine serum (FBS), trypsin, anti-mitotic solution, and antibiotics were purchased from HiMedia India. NF-κB inhibitor BAY 11-7082 (B5556) was purchased from Sigma. Primary antibodies of MMP-2 (ab-37150), and β-actin (ab-151526) were purchased from Abcam, UK. Primary antibody MMP-9 (sc-21733), Alkaline phosphatase (AP) conjugated secondary antibody goat anti-mouse (sc-1004), and goat anti-rabbit (sc-1005) were obtained from Santa Cruz Biotechnology, Santa Cruz, USA. Primary antibodies for Claudin-1 (D5H1D) XP®, N-cadherin (D4R1H) XP®, Vimentin (D21H3) XP®, and NF-κB (D14E12) XP® were from Cell Signaling Technology (CST). Other reagents, biochemicals, and solvents like DMSO, methanol, acetonitrile etc were purchased locally.

**Plant Sample Identification**

In the year of 2012, a field trip was undertaken to the Similipal Biosphere Reserve (SBR), Mayurbhanj District, Odisha for the collection of this plant. With the help of Flora of Orissa, the plant was identified by a professional taxonomist of North Orissa University (see Acknowledgments) and the voucher specimen number NOU062/2012 was submitted. The information about this plant and collection details are shown in Table 1.

**Leaf Extract Preparation by Percolation**

Fresh leaves were harvested during the flowering stage and completely dried at room temperature in the dark to avoid the loss of volatile oil and color. Completely dried condition was maintained for storing the materials to eliminate the growth of different types of microorganisms like bacteria, fungi, molds, and others. A fine powder was made by grounding the leaves using a mechanical grinder. The percolation method was followed up. Fifty grams of fine powder was soaked into 500 ml rectified spirit (Bengal Chemicals & Pharmaceutical Ltd, India) taken in a 1000 ml conical flask. The extraction was done at ambient temperature with repeated shaking (4 × 5 minutes) for a day. Whatman® 41 papers were used to filter the decoctions and the filtrate was concentrated under low pressure using a rotary evaporator (Sonax, India) at 40°C, yielding 7.09%. The dry sample was stored at −20°C temperature until further use. For experimental purposes, a stock of 20 mg/ml was made by dissolving the dry residue in autoclaved water.

**TLC and HPLC of the Leaf Extract**

TLC was performed in pre-coated glass sheets (silica gel 60 F254, Merck, Germany) for the separation of compounds present in the EELDP. 1 mg/ml solution of crude extract was prepared in ethanol and 5 µl aliquots were placed on TLC plate(s) in triplicate. Then the TLC plates were developed with the solvent combinations Butanol: Dichloromethane: Formic acid (8:1:1, v/v), Ethyl acetate: Butanol: Acetic acid (8:1:1, v/v), and Acetone: Dichloromethane: Formic acid (2:7:1, v/v) at room temperature. Then the plates were dried in an oven for 5 minutes at 90°C. A better separation was observed in the last solvent combination. The spots were visualized by using one set of chromatograms by spraying with alcoholic 5% sulfuric acid in ethanol followed by heating for 5 minutes at 100°C. Compounds were detected by comparing the spot colors and RF values with the following literature data. Then the leaf extract was run in an HPLC to know about the complexity of the mixture. The dried leaves were dissolved in 20% acetonitrile and filtered through a CHROMAFIL® Xtra H-PTFI filter (pore size 0.45 µm, filter 13 mm, MACHEREY-NAGEL, Germany) before injecting the sample into the HPLC. Then HPLC analysis was done in Shimadzu, LC-20AT system (model DGU 20A3, Japan). Here, the reverse-phase HPLC column (Sunfire TM prep C18 column, Waters [10 mm × 250 mm, 5 µm]) was used. Solvent A (H₂O with 0.1% trifluoroacetic acid [TFA,
HPLC Analysis of Leaf Extract to Obtain Different Fractions

Based on the preliminary HPLC run, we again performed HPLC to collect different fractions from EELDP in the same solvent system, solvent A = 0.1% TFA in water (HPLC grade) and solvent B = 0.1% TFA in acetonitrile (HPLC grade). The plant sample was dissolved in ethanol and filtered through Agilent Captiva Econo filter Nylon (pore size-0.2 µm, filter 13 mm) before sample injection in the instrument. HPLC analysis was performed in Breeze™ 2 HPLC System, Waters. Here also the C18 reverse phase preparative column (PHENOMENEX, Gemini-NX 5 µ C18 110 A, AX, size-250X 21.2 mm) was used. The gradient program is given in Table 3. The run time was 60 minutes. 650 µl of each sample was injected and a flow rate of 4.0 ml/min was used at ambient temperature. Spectra were recorded from 200 to 500 nm with a resolution of 1.2 nm.

Preparation of Dry Fractions

After collecting the different fractions (fraction1-fraction 10 or F1-F10) from HPLC, the organic solvent of each fraction was removed by using a rotary evaporator (rotary water bath-EYELA SB-1200 and rotary condenser-EYELA N-1200 A) and the remaining water was completely dried out by using lyophilizer (ScanVac, Labogene). The dried fractions are stored in glass vials.

Cell Culture

A549 (human non-small cell lung cancer cell line), HeLa (human cervical cancer cell line), and L-132 (normal embryonic lung cells) were purchased from National Centre for Cell Sciences, Pune, India. U2OS (human osteosarcoma-HTB-96) was obtained from ATCC. A549, HeLa, and L-132 cells were allowed to grow in the DMEM medium and U2OS cells were grown in McCoy’s 5A medium. Both DMEM and McCoy’s medium were supplemented with 10% FBS (fetal bovine serum), 1% penicillin, neomycin, and streptomycin (complete medium). The cells were kept in a humidified incubator (5% CO2) at 37°C temperature. Mycoplasma testing was done and showed negative results.

Cell Viability and Cell Morphology Study After Treatment With Crude Extract and Active Fractions

Cell viability study was performed by trypan blue assay after 24 hours of treatment of EELDP (0-0.6 mg/ml) and active fraction 6 (F6) and fraction 8 (F8) (0-100 µg/ml) on A549 and L-132 cell lines as per our earlier reports and others. The number of viable cells (%) was counted after treatment with EELDP and active fractions for 24 hours and represented...
in a scatter plot with a linear fit. The IC$_{50}$ value (where 50% of cells remain viable) was calculated from this graph. The cellular morphology of A549 and L-132 cell lines were studied after 24 hours of treatment with EELDP (0-0.6 mg/ml) and photographed under a light microscope (Carl Zeiss, Germany).

**In Vitro Scratch Wound Healing Assay After Treatment With Crude Extract and Active Fractions**

To study the wound healing assay, around $1 \times 10^5$ cells of A549, HeLa, and U2OS were seeded in a 35 mm culture plate and kept overnight in an incubator. In each culture plate, a similar single scratch was made by the tip after the attachment of cells. After 24 hours of treatment of EELDP (0-0.6 mg/ml) with and without NF-$\kappa$B inhibitor BAY 11-7082 (0-10 µM), cells were washed gently by PBS and photographed under the light microscope (Carl Zeiss, Germany) immediately (0 hours) and after 24 hours of treatment. We also treated the A549 cells with fractions F6 and F8 (0-50 µg/ml) for 24 hours after the scratch and performed the experiment in the same way as mentioned above.

**In Vitro Cell Migration Assay**

We checked the migratory property of A549, HeLa, and U2OS cell lines by using a Thin Cert™ multi-well plate (pore size 8 µm) after treatment of EELDP following our previous report. In brief, around $6 \times 10^5$ numbers of A549, HeLa, and U2OS cells were seeded in a 60 mm plate and allowed to grow for 17 hours. The treatment of EELDP (0-0.6 mg/ml) was then done in a serum-free medium for 24 hours at 37°C in a CO$_2$ incubator. Cells were gently washed twice with PBS and an equal number of cells (around $2 \times 10^5$ cells) from each control and treated plate were counted and seeded in serum-free medium in the upper chamber of the Thin Cert™ multi-well plate. The lower chamber of the plate contained a 2 ml medium with 10% serum. Cells were kept in an incubator for 17 hours for migration from the upper chamber to the lower chamber through the membrane. After that incubation period, the medium from the lower chamber was discarded and the cells were dislodged using trypsin, and the total cells that migrated from the upper chamber to the lower chamber were counted by hemocytometer. The experiment was repeated 3 times for each kind of cell line.

**Gelatin-Zymography After Treatment With Crude Extract and Screening of the Active Fractions Having Anti-MMPs Activity**

Gelatin zymography of A549, HeLa, and U2OS was done after EELDP (0-0.6 mg/ml) treatment to study the activity of MMPs as per our earlier report. Further A549 cells were treated with 10 fractionated parts (F1-F10) (0-50 µg/ml) to screen out the active fractions which show anti-MMPs activity in A549 cells.

**Real-Time PCR for MMP-2 and MMP-9**

The total RNA was extracted from A549 cells with and without treatment of EELDP (0, 0.3, and 0.6 mg/ml) using TRIZol Reagent (Invitrogen; life technologies) and then cDNA was prepared using random hexamer as per our earlier report. The MMP-2 (F primer: CGCTCAGATCCGTTGGTAG; R primer TGTCACGTGGCGTCACAGT) and MMP-9 (F prime: CCCCTGAGACCTGAGAACC and R prime: CCGAAGTGAACCATAACC) transcripts were monitored using SYBR green (Invitrogen) in Step OnePlus™ Real-Time PCR System (Applied Biosystem; Life Technologies).

**Western Blot After Treatment With Crude Extract and Active Fractions**

Western blot was done for the expression of various marker proteins in A549, HeLa, and U2OS after 24 hours of treatment of EELDP or by fractionated parts (F6 and F8) following our earlier reference. We checked MMP-2, MMP-9, NF-$\kappa$B, Vimentin, N-cadherin, Claudin-1 proteins, and $\beta$-actin (internal control) in all 3 cell lines after 24 hours of treatment of EELDP. Further, we also prepared the cell lysate from A549 cells treated with EELDP (0-0.3 mg/ml) in the presence and absence of NF-$\kappa$B inhibitor BAY 11-7082 (0-10 µM) for 24 hours to check the expression of MMP-2, MMP-9, NF-$\kappa$B, and $\beta$-actin. The secreted MMP-2 and MMP-9 in the culture after 24 hours of EELDP treatment were also detected by western blot (denoted as a medium). Further, we also checked the expression of MMP-2, MMP-9, NF-$\kappa$B, and $\beta$-actin from cell lysate of A549 after 24 hours of treatment of active fractions F6 and F8 (0-50 µg/ml) using DMSO as a negative control. The concentrations of the antibodies used are as follows: MMP-2 (Abcam-37150, 1:5000) and MMP-9 (sc-21733, 1:1000), NF-$\kappa$B (D14E12, 1:1500), Claudin-1 (D5H1D, 1:1500), N-cadherin (D4R1H, 1:1500), Vimentin (D21H3, 1:1500), $\beta$-actin (ab151526, 1:10000), and the HRP conjugated secondary antibodies goat anti-mouse (sc-2066, 1:5000), goat anti-rabbit (sc-2005, 1:7000) and goat anti-rabbit (sc-2007, 1:7000). We have used the same blot for multiple protein expressions in western blot. Either we stripped the blot using a different secondary antibody or we cut the blot for the expression of multiple proteins.

**TLC of the Active Fractions**

TLC of active fractions F6, F8, and the crude EELDP was performed in different solvent systems to check the presence...
of some secondary metabolites like betulinic acid (BA), β-sitosterol (BS), β-sitosterol glucoside (BSG), gallic acid (GA), and quercetin (Q). Different solvent systems were used as the mobile phase. Based on the best separation, solvent system chloroform, methanol, acetic acid, and water in combination were selected in the ratio of 7:3:1:1. Solvent was poured into the TLC development chamber. A blotting paper was placed inside the development chamber for proper saturation. TLC plates (MERC, TLC Silica gel 60 F254, dimension 7 cm × 6 cm) were pre-heated for activation and used for TLC analysis. An origin line was drawn with a pencil at 0.5 cm from the bottom to spot the sample. A solvent front line of approximately 0.3 cm was drawn at the other end of TLC. Samples (F6, F8, and EELDP dissolved in ethanol) and standards were spotted on the same TLC plate at a certain distance. The sequence was from left to right β-Sitosterol (BS), betulinic acid (BA), β-sitosterol glucoside (BSG), quercetin (Q), gallic acid (GA), fraction 6 (F6), fraction 8 (F8), and crude extract (EELDP). After spotting TLC, it was dried and placed in the development chamber. Prepared TLC was allowed undisturbed to develop in the TLC chamber until the mobile phase was not reached up to the solvent front. After the complete development of TLC, it was taken out and dried.

TLC spots were visualized under UV (254 nm), iodine chamber, and with the help of ρ-Anisaldehyde spraying reagent. Spots present on TLC were marked and analyzed by comparing the retention factor value of sample compounds with the standard compounds.

**ESI-MS of Active Fractions**

Fraction 6 (F6) and fraction 8 (F8) were selected among all fractions which were collected from the HPLC technique. Samples (F6 and F8) were diluted 100 times in a mixture of 1:1 0.1% formic acid LCMS grade water: LCMS grade EtOH. Blank was used the same except for the sample (fraction 6 and fraction 8). Blank and samples were run in ESI (+) ve mode in Waters Xevo-G2-XS-Qtof instrument. A direct infusion was carried out at 5 µl/min. The acquisition was made for 1 minute. Source voltage, source temperature, and desolvation temperature were 3 kV, 100°C, and 250°C respectively. The instrument was calibrated with sodium formamide (CH$_3$NNaO) and then the lock mass of leucine enkcephalin was checked. The mass spectrum was obtained for each sample and analyzed with an elemental composition program in Mass Lynx software.

**Statistical Analysis**

The significance value between control and treated samples was calculated by Dunnett's test using one-way ANOVA using IBM SPSS Statistics 21. Here “*” defines the significance value 0.01 < P ≤ 0.05, “**” defines the significance value 0.001 < P ≤ 0.01 and “***” defines the significance value P ≤ 0.001. Every experiment was repeated 3 times and then the mean with the standard deviation (mean ± SD) was calculated.

**Results**

**TLC and HPLC Studies of the EELDP**

The chromatograms of TLC of EELDP are given in Figure S1. TLC was performed in different solvent compositions. Among different types of solvent compositions of mobile phases, the suitable one was found to be acetone: dichloromethane: formic acid (2:7:1, v/v). Six spots were visualized (Retention factor Rf 0.5, 0.52, 0.54, 0.7, 0.89, and 0.98) after spraying with 5% sulfuric acid in ethanol. When the spots of the plate were exposed and developed under UV 254 nm, several dark and light bands in a green background were observed which indicated the presence of various organic compounds. Two extra spots of Rf values 0.1 and 0.93 were observed when the same slides were developed under UV 360 nm exposure. The multi-colored spots such as pink, yellowish gray, purple, green, etc indicated the presence of various polar and nonpolar compounds. The presence of flavonoids (pink), saponins (yellowish gray), terpenoids (purple), and steroids (dark green) are confirmed. The results obtained from this TLC study suggested the presence of various groups of phytochemicals or bioactive compounds in our leaf extract which might be responsible for the activity.

Based on UV absorbance spectra and a comparison of the chromatographic retention times, a preliminary reversed-phase high-performance liquid chromatographic (HPLC) technique was performed to identify some of the peaks/compounds present in the crude extract of EELDP. It is evident from the HPLC profiles shown in Figure S2 that our leaf extract is a mixture of various compounds which need to be separated well.

**HPLC of EELDP to Obtain Different Fractions**

We further performed the HPLC following the protocol mentioned in the materials and methods section to collect different fractions. Here, in a preparative HPLC instrument, the same solvent was used but the gradient was different for collecting the fractions.

At first in the analytical HPLC instrument, we checked whether some distinct peaks were obtained or not by setting the gradient in this solvent system mentioned in the materials and methods section. We found that some peaks were obtained at the specified gradient of the mobile phase. Then we transferred into a preparative HPLC instrument to collect the fractionated parts that were found in the analytical instrument (UFLC SHIMADZU). The run

\[ \text{value} \ 0.001 < P \leq 0.01 \text{ and “***” defines the significance value } P \leq 0.001. \]
time was 60 minutes in a preparative HPLC instrument. The representative spectrum is shown in Figure 1. Three wavelengths 220, 254, and 280 nm were set in a single run.

![Figure 1](image1.png)

**Figure 1.** HPLC profile of EELDP in gradient run in the solvent system 0.1% TFA in water and solvent system 0.1% TFA in acetonitrile. The X-axis denotes the retention time (minutes) of compounds eliminated in solvent and the Y-axis denotes the intensity (AU) of them.

We collected 10 fractions denoted as F1 (retention time 3.886 minutes), F2 (retention time 5.507 minutes), F3 (retention time 6.682 minutes), F4 (retention time 18.224-19.78 minutes), F5 (retention time 21.687 minutes), F6 (retention time 23.260-25.903 minutes), F7 (retention time 26.747 minutes), F8 (retention time 34.840 minutes), F9 (retention time 46.445-47.137 minutes), and F10 (retention time 48.532-50.975 minutes).

**EELDP and the Active Fractions Reduce MMPs Activity**

The activity of MMP-2 and MMP-9 after treatment of different concentrations of EELDP in A549, HeLa, and U2OS cell lines were reduced significantly \( (P \leq 0.01) \) as shown in Figures 2A, 2C, and 2E respectively. By using densitometry analysis, we calculated the % of reduction of activity of both MMP-2 and MMP-9 in treated samples compared to control in each cell line. The graphical representation of MMP-2 and MMP-9 of A549, HeLa, and U2OS are shown in Figures 2B, 2D, and 2F respectively. EELDP treatment...
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reduced MMP-2 activity in A549 cells by 24% and 82% at 0.1 and 0.3 mg/ml respectively. The same doses of EELDP reduced MMP-9 activity by 33% and 90% respectively in A549 cells. We also performed the Coomassie blue gel staining for a set of experiments showing equal loading in each dose in each cell line (shown in Figure S3).

We screened all the collected fractions (F1-F10) having anti-metastatic activity as detected by gelatin zymography in the A549 cell line (data not shown). We observed that F6 and F8 fractions had higher anti-MMPs activity, so we performed different experiments using these 2 fractions—F6 and F8. The data is shown in Figure 3A and 3B respectively.

Figure 3. (A) Gelatine zymography and equal loading after 24 hours of treatment of F6 (50 µg/ml) in A549 cells. From left to right lane denotes marker, control, DMSO, and F6 (50 µg/ml). (B) Gelatine zymography and equal loading after 24 hours of treatment of F8 (50 µg/ml) in A549 cells. From left to right lane denotes DMSO, control, and F8 (50 µg/ml). In each picture, the upper gel for zymography and the lower gel corresponds to equal loading. (C) Bar diagram of activity of MMPs obtained from 3 independent gelatine zymography after 24 hours of treatment of F6 (0-50 µg/ml) in A549 cells. (D) Bar diagram of activity of MMPs obtained from 3 independent gelatine zymography after 24 hours of treatment of F8 (0-50 µg/ml) in A549 cells. In both cases, the activity of control was taken as 100%, accordingly the activity of treated sample was calculated. Each bar represents the mean with the standard deviation of activity of MMPs from 3 independent experiments. Here * denotes $0.01 < P \leq 0.05$. 

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These 2 fractions F6 and F8 are termed active fractions. About 20% and 76% reduction of activity of MMP-2 and MMP-9 was observed with 50 µg/ml of F6 treatment. On the other hand, the reduction of MMP-2 activity was almost 46% whereas the reduction of MMP-9 was around 89% by the 50 µg/ml of F8 shown in Figure 3C and 3D respectively.

Cell Viability and Cell Morphology Study by EELDP and Active Fractions

We have used 3 different cell lines such as A549, HeLa, and U2OS for cell viability assay in control and after treatment. A dose-dependent decrease in cell viability was observed in all cell types (data not shown) for EELDP. However, a greater effect was found in A549 cells as shown in Figure 4A. The IC_{50} value is 0.35 mg/ml for A549 cells. Notably, no such significant decrease in cell viability was observed in the L-132 cell line after EELDP treatment shown in Figure 4B.

Cell viability was also checked in the A549 cell line after 24 hours of treatment of F6 and F8 (0-100 µg/ml) as shown in Figure 4C. DMSO (0.5%) was used as a negative control. More than 80% of cells remain viable at the highest dose (100 µg/ml) for both 2 fractions F6 and F8.

We compared the cell morphology of A549 and L132 cells after treatment with EELDP. EELDP treatment makes damage in to the cells and hence the A549 cells look round, wrinkled, and reduced in size, unlike the untreated control cells which show normal healthy shapes like pebble shapes showing cell-cell adhesion. Notably, no significant effect was observed in normal cells L-132. The typical pictures of the cell morphology of A549 and L-132 are shown in Figure S4. This data implicates that the EELDP selectively kills cancer cells keeping the normal cells least affected.

In Vitro Scratch Wound Healing Assay by EELDP and Active Fractions

We made a similar scratch in each petri plate of control and treated in all 3 cell types A549, HeLa, and U2OS, and photographed immediately (0 hours) and after 24 hours of EELDP (0-0.6 mg/ml) treatment. The % of total wound closure of control and treated cells of each cell line was measured and plotted accordingly. The graphical data is shown in Figure 5. EELDP treatment reduced the wound-closing property of 3 cancer cell lines compared to the untreated control. The images of a scratch wound assay for A549, HeLa, and U2OS cells are shown in Figures S5A to S5C respectively. The mean value of % total would heal after 24 hours of treatment obtained from the different independent sets of experiments are shown in the bar diagrams Figure 5A (for A549), Figure 5B (for HeLa), and Figure 5C.
De et al. The wound healing for each cell line after each treatment for 24 hours was compared with respect to 0 hours. It is evident from the figure that EELDP showed a higher effect in A549 compared to the other 2 cell types. The % of wound closing is around 16%, 5%, and 2% at a dose of 0.1, 0.3, and 0.6 mg/ml in A549 respectively.

We further checked the in vitro scratch wound healing assay after 24 hours of treatment with F6 and F8 (0-50 µg/ml) in A549 cells. Here, we performed this experiment at even lower doses (10 and 30 µg/ml). 0.25% DMSO was used as a negative control as usual. The image of the scratch wound for A549 cells by F6 and F8 are shown in Figures S5D and S5E respectively. The bar diagram of the wound healing assay of F6 and F8 are shown here in Figure 5D. From the above diagram, it was found that with increasing the concentration of fractions, wound closing of metastasis cells was inhibited gradually. Among the 2 fractions, the inhibition effects of F8 were comparatively higher than F6.

EELDP Reduces In Vitro Cell Migration

The in vitro cell migration assay of cancer cells after treatment of EELDP was measured by this experiment. EELDP (0-0.6 mg/ml) treatment for 24 hours significantly reduced the migration of A549, HeLa, and U2OS cells. But the inhibition effect was comparatively higher in A549 cells than that of the other 2 cell types HeLa and U2OS. The graphical representation of % cell migration of all 3 cell types A549, HeLa, and U2OS at different doses of EELDP (0-0.6 mg/ml) after 24 hours of treatment is shown in Figure 6. By taking

Figure 5. The graphical representation of % of total wound closure in control as well as treated samples in all 3 cell types A549 (A), HeLa (B), and U2OS (C). (D) represents a bar diagram of in vitro wound healing assay in A549 cells after 24 hours of treatment of F6 and F8 at concentrations 0 to 50 µg/ml. Here 0.25% DMSO was used as the negative control. % of total wound closure was calculated after 24 hours of treatment. Each bar represents the mean with standard deviation from 3 independent experiments. Here “***” defines $0.001 < P \leq 0.01$ and “****” defines $P \leq 0.001$. 

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the number of cells migrated through the membrane in the control set after 24 hours of treatment as 100%, cell migration at different doses of EELDP was calculated as about 62% and 22% at doses 0.1 and 0.3 mg/ml respectively whereas the number was nil at higher dose 0.6 mg/ml in A549 cells. Similarly, % of cell migration for the other 2 cell types was also calculated and plotted as shown in Figure 6.

**EELDP Reduces MMP-2 and MMP-9 Transcripts**

We measured the expression of both MMP-2 and MMP-9 in RNA level in the A549 cell line after 24 hours of treatment of EELDP (0-0.6 mg/ml) by real-time PCR using SYBR green master mix as described in the materials and methods section. Expression of MMP-2 and MMP-9 was reduced by about 75% and more than 80% respectively after treatment with 0.3 mg/ml EELDP in comparison to the control as shown in Figure 7. However, at a higher dose of 0.6 mg/ml the reduction was even more for both the MMPs.

**EELDP Reduces MMP-2, -9 Via NF-κB Pathway**

We measured the expression of both MMP-2 and MMP-9 in protein levels inside the cells (mentioned as “lysate”) as well as secreted in culture medium (mentioned as “medium”) in Figure 8. The cell lysate was prepared using the protocol mentioned in the materials and methods section. The concentrated medium, which was used in gelatine zymography, was used here as a sample in the western blot technique to check the secreted MMPs, if any. Figure 8A and 8D represent the MMPs present in the medium as well as in lysate for A549, and HeLa cell lines respectively, and Figure 8G represents the MMPs in cell lysate for U2OS cell lines after 24 hours of treatment of EELDP. The corresponding graphical presentations are placed accordingly below each set of the blot. Figure 8B and 8C denote the % of the expression of MMP-2, -9 in medium and whole cell lysate respectively in A549 cells, whereas Figure 8E and F denote the same in HeLa cells. Figure 8H is the graphical presentation of the % of the expression of the same proteins in the whole cell lysate of U2OS cells. It is clear from the figures that the expression of MMPs was reduced in both lysate and medium. The reduction of secretion of MMPs in the medium is due to less amount of expression inside the cell. Due to less secretion of MMPs, activity was also getting down which was observed in our zymography results. Our RT-PCR data (Figure 7) also confirms our western blot data. We also checked the expression of NF-κB, one of the transcription factors of these 2 proteins MMP-2 and MMP-9, in 3 cell lines A549 (Figure 8A), HeLa (Figure 8D), and U2OS (Figure 8G) after 24 hours of treatment of EELDP (0-0.6 mg/ml). This data implicates that EELDP reduces the NF-κB expression dose-dependently in all 3 types.

To understand whether the alteration of the EMT pathway by EELDP treatment through the NF-κB pathway or not, the NF-κB inhibitor BAY 11-7082 (0-10 µM) was used in combination with crude extract of EELDP (0-0.3 mg/ml) for 24 hours and checked the in vitro wound healing assay and the expression of MMP-2, MMP-9, NF-κB, and internal control β-actin in A549 cells shown in Figure 9. The image of the scratch wound for A549 cells at 0 and 24 hours is shown in Figure S6. The total wound closure (%) after 24 hours of treatment was compared to that at 0 hours of treatment in

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**Figure 6.** In vitro cell migration of 3 different cells A549, HeLa, and U2OS cells after 24 hours of treatment of EELDP (0-0.6 mg/ml). Each bar shows the mean with the standard deviation of the percentage of cell migration at different doses of EELDP obtained from 3 independent experiments. Here “**” defines $0.001 < P \leq 0.01$ and “***” defines $P \leq 0.001$.

**Figure 7.** Expression of MMP-2 and MMP-9 in mRNA level by RT-PCR in A549 cells after 24 hours of treatment of EELDP (0-0.6 mg/ml). Each bar shows the mean with standard deviation obtained from 3 independent experiments. Here “***” defines $P \leq 0.001$. 

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A549 cells as shown in Figure 9A. The expression of MMP-2, MMP-9, NF-κB, and internal control β-actin in A549 cells treated with crude extract with and without BAY 11-7082 is shown in Figure 9B. It was found that combined treatment prevented the wound-closing property more than a single treatment. Furthermore, combined treatment reduced expressions of MMP-2, MMP-9, and NF-κB more compared with a single treatment of EELDP or BAY 11-7082 separately. So, EELDP reduces cell migration via reducing MMPs expression through the NF-κB pathway.

### Active HPLC Fractions of EELDP Reduce MMP-2, -9 Via NF-κB Pathway

The expression of MMP-2, MMP-9, and one of their transcription factor NF-κB in whole cell lysate after 24 hours of treatment of the HPLC fractions F6 and F8 (0-50 µg/ml) in A549 cells was detected by the western blot technique. Here, 0.25% DMSO, which is present at a 50 µg/ml concentration of each fraction, was used as a negative control. Both the fractions F6 and F8 are capable of reducing the expression of MMP-2 and MMP-9 significantly (0.01 < P ≤ 0.05) at 50 µg/ml compared to the control and the reduction of MMP-9 is higher than that of MMP-2 which corroborates our zymography data. This reduction of MMPs is due to the reduction of their transcription factor NF-κB which is clearly shown in Figure 10A for F6 and Figure 10B for F8. So, our data implicate that F6 and F8 reduce MMP-2, -9 expression via the NF-κB pathway.

### EELDP and Active HPLC Fractions Alter the EMT Marker in Favor of Reducing Metastasis

A higher effect of EELDP was found in A549 cells compared with other cells such as HeLa and U2OS, as evident from the studies mentioned above. Hence, we checked the expression of N-cadherin, Vimentin (mesenchymal genes), and Claudin-1 (epithelial genes) in A549 cells treated with
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EELDP (0-0.6 mg/ml) for 24 hours. EELDP reduced the expression of mesenchymal proteins, that is, N-cadherin and Vimentin whereas increased the expression of epithelial proteins like Claudin-1 as shown in Figure 11. This data implicates that EELDP prevents cancer cell progression by inhibiting the EMT (epithelial-mesenchymal transition) pathway via altering marker proteins.

We have also checked the expression of EMT markers like N-cadherin, Vimentin (mesenchymal genes), and Claudin-1 (epithelial gene) in A549 cells after 24 hours of treatment of F6 and F8 (0-50 µg/ml) shown in Figure 12A and 12B respectively. From the following figures, it is found that the 2 active fractions from EELDP (F6 and F8) are capable to reduce the expression of mesenchymal proteins N-cadherin and Vimentin whereas increasing the expression of epithelial proteins like Claudin-1 in A549 cells. This data implies that these 2 fractions can prevent cancer cell progression to some extent via altering EMT (epithelial-mesenchymal transition) marker proteins.

**TLC of the Active Fractions and Crude Extracts**

Further, we performed TLC of active fractions F6, F8 including crude extract EELDP using some standard secondary metabolites like betulinic acid (BA), β-sitosterol (BS), β-sitosterol glucoside (BSG), gallic acid (GA), and quercetin (Q) using the solvent system (chloroform:methanol:acetic acid:water = 7:3:1:1) based on the best separation of the compounds to check their presence. It had already been reported that betulinic acid and β-sitosterol are present in the leaves of most Dillenia species. The sequence of standard compounds and samples from left to right were β-Sitosterol (BS), betulinic acid (BA), β-sitosterol glucoside (BSG), quercetin (Q), gallic acid (GA), fraction 6 (F6), fraction 8 (F8), and crude extract (EELDP). After development under UV (254 nm) chamber, I2 chamber, and spraying with p-Anisaldehyde, the following spots were visualized given in Figure 13A (under UV), Figure 13B (under I2 chamber), and Figure 13C (spraying with p-Anisaldehyde). From the figures, it was found that the active fractions (F6 and F8) did not contain the above standard compounds that we had run in TLC. The other compounds may be present in the fraction which couldn’t be resolved in the solvent we used, but show biological activities. Our crude extract EELDP was a mixture of various compounds like flavonoids, terpenoids, saponins, steroids, and so many phytochemicals as evident in Figure S1. Here in Figure 13C, the rightmost row of EELDP was also showing a light yellowish-gray spot after p-Anisaldehyde spraying indicating the presence of other compounds in the crude.
ESI-MS of Active Fractions F6 and F8

Electrospray ionization mass spectrometry (ESI-MS) is a technique where the ionization of macromolecules was done by applying high voltage to a liquid to form an aerosol. After the ionization of molecules, spectra were obtained depending on their molecular mass. Here we ran our 2 fractions F6 and F8 with respect to the blank (1:1 0.1% formic acid LCMS grade water: LCMS grade EtOH) in which they were dissolved. The mass spectrum is shown in Figure 14. From lower to upper, the spectrum denotes blank, DMSO, fraction 6 (F6), and fraction 8 (F8) respectively. It is clear from the figure that each fraction (F6 and F8) contains multiple compounds with different molecular weights. So, the results that we got from the above experiments are the combined effect of these molecules together. Samples (F6 and F8) contained different peaks from the blank and DMSO. We only considered those peaks that are not present in the blank and DMSO. The spectrum of F6 and F8 showed that these 2 fractions have compounds of the same molecular weight. Maybe they are isomers and their retention time is different in HPLC. So, we got the different fractions in HPLC. We require further analysis to distinguish each molecule separately and find out the structure of those molecules that will show a positive result.

Discussion

Crude EELDP (0-0.6mg/ml) treatment reduces wound healing, cell migration, and activity of MMP-2, -9 via the NF-κB pathway in various human cancer cells. Moreover,
such treatment alters a few EMT makers like Vimentin, N-cadherin, and Claudin-1 in favor of reducing epithelial to mesenchymal transition and thereby reducing metastasis potential. EELDP treatment reduces the expression of MMP-2, -9 in a dose-dependent manner in all 3 cell types. Hence, reduced activity of MMP-2, -9 under EELDP treatment is due to transcriptional down-regulation of the genes. The degradation of the extracellular matrix by MMPs is the key event for the migration of cancer cells. Reduction of cell migration by suppression of MMPs activity is well documented. Phosphorylation of NF-κB leads to transcriptional up-regulation of MMP-2, -9. In our experimental condition, EELDP without or with NF-κB inhibitor Bay 11-7082 reduces NF-κB expression. This data implicates that inhibition of cell migration through reduced expression of MMP-2, -9 after EELDP treatment is via the NF-κB pathway. Other than the regulation of MMPs, NF-κB promotes angiogenesis, activates anti-apoptotic genes, and thereby makes the cancer cells resistant to several treatment modalities. We also observed that EELDP induces apoptosis (data not shown here). Hence, our observation shows that EELDP can target multiple signaling pathways via suppressing NF-κB expression. It is to be noted that we did not check the expression of MMPs other than MMP-2, -9, so their role in cell migration cannot be ruled out. Moreover, the role of other known modulators of

**Figure 11.** Expression of some EMT marker proteins after 24 hours of treatment of EELDP (0-0.6 mg/ml) in A549 cells. The lowermost panel is β-actin used as the internal control. Here C-control, 0.1-0.1 mg/ml, 0.3-0.3 mg/ml, and 0.6-0.6 mg/ml concentration of EELDP.

**Figure 12.** Expression of some EMT marker proteins after 24 hours of treatment of F6 (A) and F8 (B) (0-50 µg/ml) in A549 cells. In each set, the upper is for Vimentin, the middle is for N-Cadherin, and the lower denotes Claudin-1. The lowest panel of each blot represents β-actin used as the internal control. DMSO was used as the negative control.
MMPs like AP1/AP2, SP1 etc is not explored here.32-34 EELDP reduces cell migration in several human cancer cells like A549, HeLa, and U2OS. Hence, EELDP’s anti-metastatic effect is not cell-specific but generalized. The whole study of metastasis inhibition by EELDP and its active fractions is shown in Figure 15.

Our ethanolic crude extract is having phytochemicals like flavonoids, terpenoids, steroids, and saponins as per our TLC data. Our data is corroborated by several reports which show the presence of these phytochemicals in the plant *D. pentagyna*.3,35 It is always worthwhile to fractionate the crude and isolate active ingredients. We are proceeding to single compound isolation. So far, we have isolated some fractions from the crude and screened 2 very useful fractions (F6 and F8). These 2 fractions show the significant anti-metastatic property at a dose as low as 30 to 50 µg/ml. Here lies the significance of the natural composition of phytochemicals which become more effective than a single compound because of synergism among phytochemicals present in the active fraction.36,37 Chemotherapy using a single drug is not preferred because of its side effects and drug-related toxicities. Instead, the combined therapy is coming up and showed a better effect than the single mode of treatment due to synergism. Many reports also show the synergistic effect when a chemotherapeutic drug is combined with crude extracts or natural products.38,39 In this way, one can reduce chemotherapeutic drug-related toxicities and side effects. Here also we observed synergistic

Figure 13. TLC chromatogram of standard compounds along with samples (F6, F8, and EELDP) developed under different chambers. (A) TLC developed in UV chamber at 254 nm, (B) under iodine chamber, and (C) after ρ-Anisaldehyde spray.

Figure 14. ESI-MS spectra of DMSO, fraction 6 (F6), and fraction 8 (F8) correspond to the blank (1:1 0.1% formic acid LCMS grade water: LCMS grade EtOH) in which they were dissolved. From the lower spectrum to the upper one is for blank, DMSO, F6, and F8 respectively. The molecular mass/charge (m/z) is along the X-axis where the Y-axis denotes % of the relative abundance of each molecule.

Figure 15. Pictorial diagram showing the inhibition of in vitro metastasis by EELDP and active fractions (F6 and F8) treatment. “Up arrow” and “down arrow” represents the up-regulation and down-regulation of the genes respectively.
reduction of expression of MMP-2, -9, and NF-κB after combined treatment with crude EELDP and Bay 11-7082. This data implicates that judiciously chosen protein inhibitors can be combined with our crude extracts or active fractions for better outcomes.

Despite the wide use of this crude extract of this plant by a large number of people in India, detailed study regarding the anti-cancer activity of various parts of D. pentagyna is very few.4,5,8 As such there is no report on the anti-metastatic effect of crude extracts of D. pentagyna. So, this manuscript is going to be the first report that describes the anti-metastatic potential of EELDP and its active HPLC fractions. The key point of this study is that about 62% reduction of cell migration was obtained at a very low concentration (0.1 mg/ml) of crude extract. Out of these 2 active fractions, F8 is having very potent ingredients to reduce metastasis because of the significant reduction of expression of MMPs, NF-κB, and alteration of N-cadherin, Vimentin, Claudin-1 are observed at a concentration of 30 to 50 µg/ml. So, the active phytochemicals present in the EELDP and the active fractions F6/F8 are very potent to inhibit in vitro migration or cancer progression because purified compounds would give the same anti-metastatic effects in manyfold lower concentrations of crude or fractions. Our ESI-MS data implicates that each active fraction is having multiple compounds, so it is hard to assume the responsible compound and needs further study. The plant is known to be safe with many traditional uses to cure diseases. Ripen fruits and flowers are known to be edible. Leaves are commonly eaten by elephants.40 The authors studied the ethanolic extract showed cytotoxicity for human cancer cells but non-toxic for normal human cells. Thus, the present study is no doubt the footing stone for further studies. Our ESI-MS data implicates that each active fraction is having multiple compounds, so it is hard to assume the responsible compound and needs further study. The plant is known to be safe with many traditional uses to cure diseases. Ripen fruits and flowers are known to be edible. Leaves are commonly eaten by elephants.40 The authors studied the ethanolic extract showed cytotoxicity for human cancer cells but non-toxic for normal human cells. Thus, the present study is no doubt the footing stone for further studies.

Conclusions

EELDP shows the anti-cancer effect by decreasing metastasis potential via the NF-κB pathway at a concentration as low as 0.1 mg/ml of crude extract in several human cancer cells. Active fractions isolated from this crude extract also have a significant anti-metastatic effect at a concentration of 30 to 50 µg/ml. So, the active ingredient(s) present in this EELDP or in F6/F8 fractions is very much potent to kill aggressive human cancer cells like A549, HeLa, and U2OS.

Acknowledgments

DD thanks the DST for DST-INSPIRE fellowship (DST/INSPIRE Fellowship/2014/37), PC thanks ICMR for her ICMR-SRF fellowship (BMS/FW/CMB/2015-24000/JUN-2016/05/WB-GOVIT). UG thanks DST and DBT for the infrastructural facility under projects DST-FIST (SR/FST/LSI-623/2014) and BT/PR 4809/BRB/10/1028/2012 respectively. UG also thanks UGC-SAP (DRS-II) program for infrastructural facilities. UG thanks Bose Institute, Kolkata for providing infrastructural facilities for HPLC and ESI-MS. UG thanks CSIR-IICB, Kolkata for providing us TLC facility. SKP thanks the authorities of North Orissa University for providing the necessary facilities for the collection, identification, and extraction of plant materials. Special thanks go to Dr. Anil K. Biswal and Dr. A. K. Bastia, (N.O.U) for identifying the plant. We also thank Dr. Laxmipriya Padhi, Yugal K. Mohanta, Subhrakanta Jena (North Orissa University), for the collection of plant samples and expert technical help during the extraction of plant materials.

Author Contributions

UG: Investigation, supervision, planning of the experiments, and drafted the manuscript; DD: all the experimental work and analyzed the data except real-time PCR; PC: real-time PCR, SKP: identification, collection of the plant sample, and prepared the leaf extract and also performed the preliminary TLC and HPLC experiment. All authors read and approved the final manuscript.

Availability of Data and Materials

All the information related to our work is given main manuscript and supplementary file.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was supported by the DST [Grant Numbers DST/INSPIRE Fellowship/2014/37, DST-FIST (SR/FST/LSI-623/2014)]; ICMR [Grant Number BMS/FW/CMB/2015-24000/JUN-2016/05/WB-GOVIT]; UGC [Grant Number UGC-SAP (DRS-II)]; and DBT [Grant Number BT/PR 4809/BRB/10/1028/2012].

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Supplemental Material

Supplemental material for this article is available online.

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