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RESEARCH ARTICLE

ANTI-CANCER ACTIVITY OF *FLUGGEA LEUCOPYRUS* WILLD (KATUPILA) AGAINST HUMAN OVARIAN CARCINOMA AND CHARACTERIZATION OF ACTIVE COMPOUNDS

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ABSTRACT

Fluggea leucopyrus Willd has been used in the treatment for cancers in the traditional system of medicine in Sri Lanka. This study was aimed on evaluation of anti-cancer activity of F.leucopyrus against Human Ovarian carcinoma and identification of active compounds. Anti-cancer activity of ethyl acetate (EtoAc) extract of leaves and two compounds isolated from EtoAc extract were evaluated using MTS and hTERT assays on Human Ovarian carcinoma (A 2780). MTS assay showed a significant anti-proliferation activity giving IC_{50} (concentration of extract/compound that inhibits 50% of the activity) values of 36.35±0.17µg/mL, 12.36±1.0 µg/mL and 48.53±1.43 µg/mL for EtoAc extract and two compounds (compound-1 & compound-2) respectively. A rapid depletion of hTERT content in Human Ovarian cancer cells was observed for the compound-2 with the concentration range 50-200 µg/mL. The compounds-1 was identified as bergenin, a known compound, whereas the compound-2 was identified as a diastereoisomer of bergenin (at C-9 and C-14,) with the help of NMR and Mass spectral data, and with the comparison of reported data. Brine Shrimp Lethality assay for EtoAc extract showed LC_{50} (Lethal concentration) values of 2779.63 µg/mL, implying that no toxicity of plant extract towards brine shrimp. As conclusion, anti-cancer activity of leaves of F. leucopyrus against Human Ovarian cancers is confirmed in this study, and bergenin and its isomer were identified as the responsible compounds for the anti-cancer activity of this plant.

Key Words: Fluggea leucopyrus, anti-cancer activity, Bergenin, Human Ovarian carcinoma, MTS assay, hTERT assay.

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1.0 INTRODUCTION

Cancer is characterized as uncontrolled cell division caused by mutation of DNA of the cells and then invaded into other organs of the body making abnormalities of normal cell functions. Though conventional treatment methods include chemotherapy, radiotherapy and surgery has advanced, the mortality rate and the number patients suffered from the unwanted effects from chemotherapy and radiotherapy are not in significant reduction. Instead, the number of cancer cases reported worldwide is tremendously increasing due to various factors, mainly environmental and behavioural. According to WHO reports, cancers are known as the second leading cause for death globally and 9.6 million death reported in 2018, about 70% of deaths occur in low- and middle-income countries [1]. Therefore, finding cure for cancers using plant-based treatment has emerged as a promising alternative. At this endeavour, scientific investigation of medicinal plants which are known to have traditional uses in cancer treatment, is considered as prime important in order to prove their anti-cancer activity scientifically and to identify the active compounds.

Flueggea leucopyrus Willd (Phylanthreceae), locally known as "katupila", a plant as shrub, grown in many parts of the world including Sri Lanka specially in dry zones. The plant has been recognized as a potential candidate for discovering novel potent drugs and other pharmaceutical products due to its vast applications in traditional medicine, and recently it has been used in the treatment of cancers in complementary and alternative medicine in Sri Lanka [2], [3]. Cancer patients take decoctions prepared by boiling of leaves in water or as fresh or with traditional medicine recipes to improve the condition and cure the cancers. Among many studies reported on *F. leucopyrus* on its anti-cancer activities, chemical compositions [4] and anti-oxidant activities, the followings are highlighted; anti-oxidant and anti-proliferative activity of aqueous extract of leaves against human Hep-2 cells by Preethy Soyza *et al* [5], cytotoxic and apoptotic effect of the decoction of the aerial part of plant on Human Endometrial Carcinoma (AN3CA) Cells by Samarakoon *et al*.,[6] immunomodulatory activity of the plant and identification of bergenin as the active compound by Wijabandara *et al*., [7],[8] growth inhibitory activities against MCF-7 and MDA-MB-231 breast cancer cell lines and P-388 cell lines by Monkodkaew *et al*.[8],[9] etc. Though anti-cancer activity on various carcinoma has been recognized from *F. leucopyrus* by previous researchers, no studies against Human Ovarian carcinoma has been reported so far, and no responsible compounds for the anti-cancer activity have been identified from this plant to date. As Ovarian cancers are the fifth most common gynaecological malignancy [10],[11] and it can be diagnosed only later stage, it is important to find a cure through plant medicine. Therefore, aim of

this study was to evaluate anti-cancer activity of leaves of *F. leucopyrus* against Human Ovarian carcinoma and to characterize the responsible compounds present in the plant for its anti- cancer activity.

2.0 MATERIALS AND METHODS

2.1 Material

The fresh leaves of *F. leucopyrus* were collected from Tangalle, Southern province of Sri Lanka. All the solvents and chemicals were purchased from Sigma-Aldrich through local agents. Human Telomerase Reverse Transcriptase (hTERT) assay kit was purchased from Life Sciences Advanced Technologies, 96® Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega) and Human Ovarian carcinoma cell line (A 2780-Adhesive type) (ECACC) were used for anti-cancer assays. Bio-safety cabinet class-II, Carbon dioxide incubator (Heal Force HF 240), ELISA plate reader and Light Microscope (Leica) were used to carry out bioassay experiments. Haemocytometer was used for cell counting after staining.

2.2 Extraction of plant material

After authentication of plant material with the help of Taxonomist at the Department of Botany, University of Ruhuna, Sri Lanka, plant material was washed, air dried, grounded into fine powder and subjected to the extraction. An aqueous extract of plant material was prepared by subjecting to Soxhlet extraction at 100^0C (4 hrs) followed by freeze drying. Ethyl acetate extract (EtoAc) was prepared using maceration with methanol (3 days), filtration and then concentration followed by solvent-solvent partitioning with EtoAc. The excess EtoAc was distilled off under reduced pressure and extracts were stored at freezer in a close container to be used in the experiments.

2.3 Cell line and maintaining cell culture

Cell line of Human Ovarian carcinoma (A2780) was cultured in 75cm^2 cell culture flask with RPMI 1640 medium (Sigma). Cell culture was maintained in carbon dioxide incubator (5% CO_2, 37^0 C and 100% humidity) at the Department of Chemistry, University of Ruhuna. A2780 cells were grown in RPMI 1640, 0.01mg/mL bovine insulin, penicillin and streptomycin at viable cells 4x104/mL concentration and it was incubated in 5 % CO_2, 37^0C, and 100 % humidity. Cultured medium was removed and the cell layer was washed with PBS volume equivalent to the half the culture medium. It was repeated if the cells are adhered strongly. Trypsin/EDTA (0.25 w/v) was pipetted onto the washed monolayer using 1 mL per 25 cm^2 of surface area and the flask was rotated to cover monolayer with trypsin and excess trypsin was decanted. The flask was returned

to the carbon dioxide incubator and left for 10 minutes at 5 % CO_2, 37 ^{0}C, and 100 % humidity. Cell counting was performed and making the cell density of 4 x 10^4 viable cells/mL and cells were transferred to culture flask containing pre-warmed RPMI 1640 with 0.01 mg/mL bovine insulin, penicillin and streptomycin [12]. Cell culture media was used as negative control and the cyclophosphamide was used as the positive control.

2.4 MTS Cell Proliferation assay with crude extracts and isolated compounds

The viable and dead Human Ovarian cancer cells were identified via staining with the Trypan blue 0.4 % solution. The required viable cell concentrations were prepared for subculture for MTS cell proliferation assay. Human Ovarian carcinoma cells were seeded at their optimal cell density of 1×10^4 of viable cells/wells into a 96-well microtiter plate (Sterilin) and incubated overnight (at 5 % CO_2, 37 ^{0}C, 100 % humidity for 24 hours) to allow cell attachment. The stock solutions with concentration of 200 µg/mL plant extracts were prepared by dissolving 0.0050 g of each dry solid sample of plant extracts (aqueous and EtoAc) in 25 mL of 0.4 % Dimethyl sulphoxide (DMSO) in Phosphate-buffered saline (PBS). The cells were exposed into various concentrations 1, 10, 50, 100, and 200 µg/mL of plants extracts and each treatment was made as duplicate and then incubated at 5 % CO_2, 37 °C, 100 % humidity for 24 hours. After incubation, 20 µL of the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reagent in combination with phenazine methosulfate was added to the wells and the cells were further incubated for 3 hours. After adding 20 µL of 10% Sodium Dodecyl Sulphate (SDS) to the above wells, absorbance was measured at 490 nm using ELISA plate reader. Same procedure was followed with the isolated compounds from EtoAc extract. IC_{50} (the concentration of plant extract where the response is reduced to half) was calculated using the corrected graph and mean values were compared by One-way ANOVA with Duncan's post hoc comparisons at $P < 0.05$ [13].

2.5 Human Telomerase Reverse Transcriptase (hTERT) assay

Human Ovarian carcinoma cells were seeded at their optimal cell density of 1×10^4 viable cells into a 96-well microtiter plate (Sterilin) and were incubated overnight (at 5 % CO_2, 37 ^{0}C, 100 % humidity for 24 hours). The cell culture superannuates were obtained by centrifugation for 15 minutes at 3000 rpm and used for hTERT assay. Kit standard (Life Sciences Advanced Technologies) and testing extracts were added per well according to the instruction in the commercial kit and then it was covered with adhesive strip and incubated at 37 °C for 2 hours. The wells were decanted without washing as instructed. Biotin-antibody (1x) 100µL was added to each well. It was incubated for 1 hour at 37 °C. Each well decanted and completed with three repeated

washing. The plate was inverted and blotted with clean blotting paper for addition of HRP-avidin (1x) (each well, 100µL) and another 1hour incubation was continued at 37^0C. The washing process was repeated for five times and TMB Substrate (3,3′,5,5′-Tetramethylbenzidine, each 90µL) was added to each well and incubated for 15 minutes at 37°C in dark place. Stop solution (50µL) was added to each well via mixing. The optical density of each well was determined within 5 minutes using ELISA reader at 450 nm. A standard curve was done by plotting the mean absorbance for each standard on the x-axis against the hTERT concentration on the y-axis. The hTERT content of each sample was obtained according to the standard curve and the dose response graphs of hTERT content vs plant extract concentration were plotted [14],[15].

2.6 Brine shrimps micro-well cytotoxicity assay

Brine shrimp bioassay is a rapid general, bench top bioassay used in preliminary assessment of toxicity of bioactive compounds and extracts. An extract or a compound is considered as cytotoxic when the LC_{50}'s $\leq$ 30 µg/mL [16]. Freshly hatched Artemia salina (n=10) was added to diluted series of aqueous and EtoAc extracts of 10, 20, 50, 100, 250, 500, 750 & 1000 µg/mL-1 in artificial sea water in 96-well microwell plates as triplets. After incubation for 24 hours, the number of live nauplii was counted and LC_{50} (lethal concentration) was calculated using SPSS statistical package.

2.7 Analysis and purification of crude extracts

As EtoAc extract was found to be potent extract in both anti-proliferation activity and inhibition of human telomerase activity, it was purified using flash column chromatography (60 g of silica, 2g of plant extracts) using gradient elusion with hexane as the first eluent and then increasing polarity by mixing EtoAc at different ratio with hexane. Repeated columns were employed to obtain the compounds in high purity stage based on the TLC results.

2.8 Structure elucidation of active compounds

Out of four compounds isolated the compound-1 and compound-2 showed the highest activity against cell proliferation in MTS assay and inhibition of human telomerase in hTERT assay, those two compounds were subjected to set of spectroscopic measurements; NMR (^{1}HNMR, H-H COSY ^{13}CNMR and DEPT135-^{13}CNMR spectra) and Mass spectra (EI-MS) in order to elucidate the structures. All the spectroscopic studies were carried out at Kanagawa University, Hiratsuka, Japan.

2.9 Statistical analyses

Microsoft excel 2010 was employed to produce dose-response curves and SPSS 17 for statistical comparison of IC_{50}. One-way ANOVA with Duncan's post hoc comparisons at $P < 0.05$ was used for statistical comparisons. All data are presented as a mean value with its standard deviation indicated (Mean ± SD).

3.0 RESULTS

3.1 Results of purification of crude extracts using column chromatography

Four compounds were isolated in pure stage from EtoAc extract in the repeated flash column chromatography and all the compounds were solid at the room temperature (Table-1).

Table -1: Compounds isolated from EtoAc extract

Compound	Amount /mg	Physical appearance
1	179.50	Yellowish white solid
2	52.50	White solid
3	9.20	Brown solid
4	3.30	Black solid

3.2 Results of MTS assay

The inhibition of proliferation of cancer cells that was measured in MTS assays are given in the graph of 1a, 1b and 1c for EtoAc crude extract, isolated compounds-1 and compound-2 respectively. The corresponding IC_{50} values for MTS assay are given in the table-2.

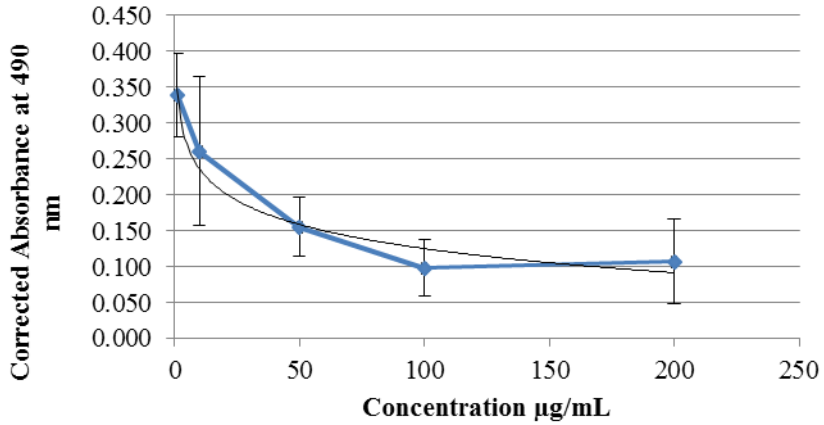

Figure 1a : Proliferation of Human Ovarian cancer cells in response to various concentrations of EtoAc extract of leaves

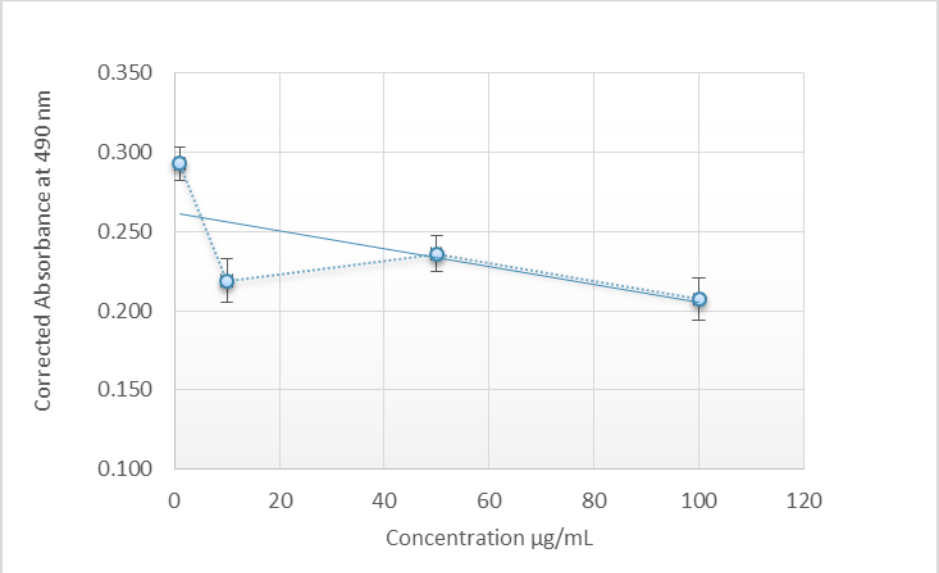

Figure 1b : Proliferation of Human Ovarian cancer cells in response to various concentrations of compound-1

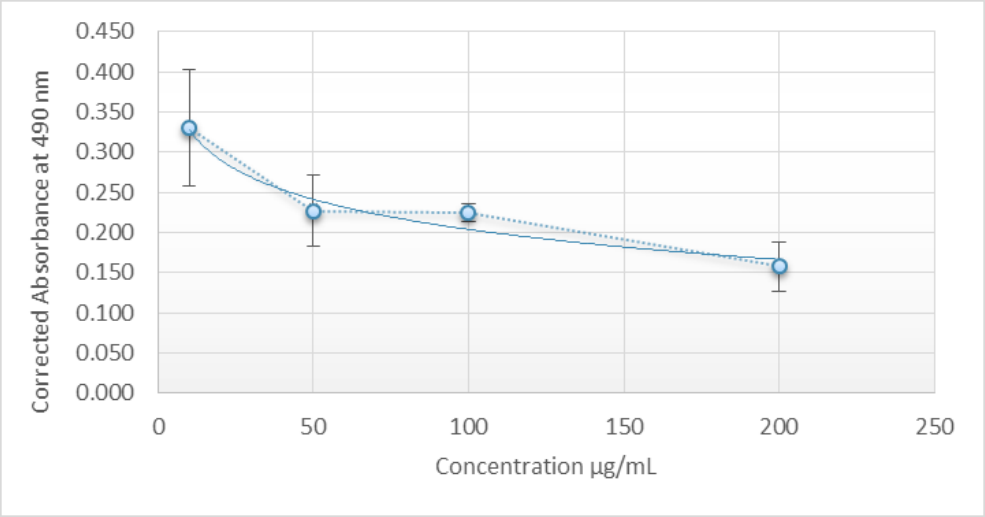

Figure 1c : Proliferation of Human Ovarian cancer cells in response to various concentrations of compound-2

Table -2: IC_{50} values in MTS assay

Extract / Compounds	**IC_{50} µg/mL**
EtoAc extract	36.35±0.17
Compound-1	12.36±0.41
Compound-2	48.53±1.43

3.3 Results of hTERT Assay

In hTERT assay, a rapid depletion of hTERT content in Human Ovarian cancer cells was observed in the range of 50-200 µg/mL with the compound-2 (Figure 2).

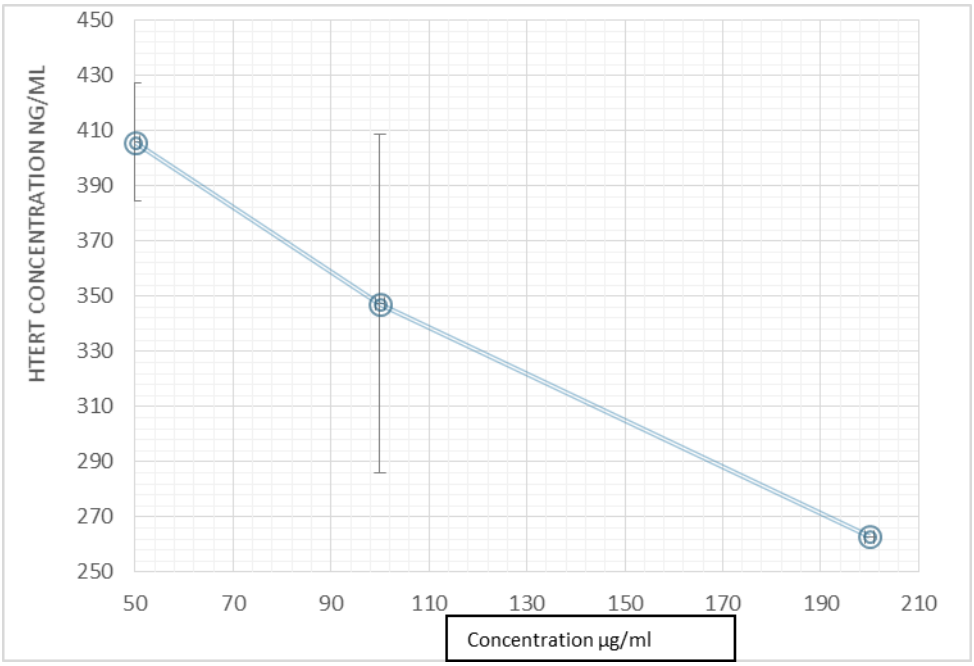

Figure 2: Variation of hTERT content in Human Ovarian cancer cells in response to various concentrations of compound-2

3.4 Results of Brine Shrimp Lethality assay

The results of Brine shrimp lethality assays showed the LC_{50} (Lethal concentration) value of 2779.63 µgmL^{-1} for ethyl acetate extracts indicating no toxicity towards brine shrimp.

3.5 Results of spectroscopic analysis

The spectral data of ^{1}HNMR,^{13}CNMR, COSY and Mass of two compounds are given below. After analysing spectroscopic data, and with the comparison of literature data, the compound-1 was identified as bergenin (Figure 3) which is a known anti-oxidant compound and the compounds -2 was characterized as a diastereoisomer of bergenin at C-9 & C-14 (Figure 4).

Compound-1: Yellowish white powder, MF; $C_{14}H_{16}O_9$, 1H NMR (DMSO-d6 400 MHz): δ 7.0 (s, 1H, H-4), 4.95 (d, 1H, H-9), 3.95 (t, 2H, 1H from H-16 & H-14), 3.80 (dd, 1H, H-13), 3.72 (s, 3H, H-15), 3.55 (m, 2H, 1H from H-16 & H-11), 3.38 (dd, 1H, H-12) and 3.15 (t, 1H). ^{13}C NMR (DMSO-d6, 400 MHz): δ 151.43 (C-2), 148.56 (C-5), 142.0 (C-7), 120.11 (C-6), 118.44 (C-3), 116.12 (C-8), 114.17 (C-4), 109.98 (C-11), 82.09 (C-14), 74.12 (C-13), 72.57 (C-9), 71.16 (C-12), 61.00 (C-16), 60.42 (C-15). IUPAC: (2R,3S,4S,4aR,10bS)-3,4,8,10-Tetrahydroxy-2-(hydroxymethyl)-9-methoxy-3,4,4a,10b-tetrahydropyrano[3,2-c] isochromen-6(2H)-one.

Compound-2: White solid, MF; $C_{14}H_{16}O_9$, ^{1}H NMR (methanol-d4, 400 MHz): δ 7.0 (s, 1H, H-4), 4.95 (d, 1H, H-9), 4.0 (dd, 2H, 1H from H-16 & H-14), 3.85 (dd, 1H, H-13), 3.75 (s, 3H, H-15), 3.65 (m, 2H, 1H from H-16 & H-11), 3.42 (dd, 1H, H-12). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 163.33 (C-2), 150.92 (C-5), 148.03 (C-7), 140.55 (C-6), 118.02 (C-3), 115.90 (C-8), 109.42 (C-4), 81.73 (C-11), 79.65 (C-14), 73.65 (C-13), 72.06 (C-9), 70.66 (C-12), 61.06 (C-16), 59.77 (C-15). IUPAC: (2*R*,3*S*,4*S*,4a*S*,10b*R*)-3,4,8,10-tetrahydroxy-2-(hydroxymethyl)-9-methoxy-3,4,4a,10b-tetrahydropyrano[3,2-*c*] isochromen-6(2*H*)-one.

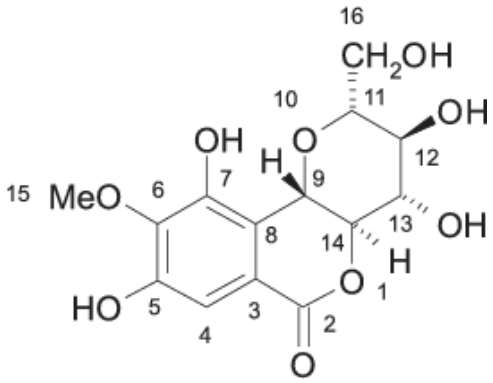

Figure 3: Compound-1: Bergenin

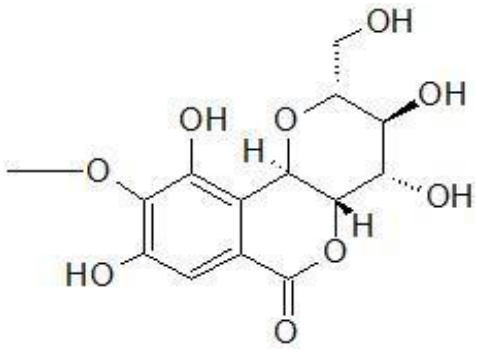

Figure 4: Compound-2: Isomer of Bergenin

4.0 DISCUSSION

Among gynaecological malignancies, Ovarian cancer is the eighth most common occurring cancers in women and about 300,000 of new cases has been reported in 2018 globally [17]. Ovarian cancer is neither common nor rare but that has an overall cure rate of less than 40% across all stages. More than 90% of Ovarian cancers are considered to be arisen from epithelial cells that covers the Ovarian surface or, more frequently, line subserosal cysts [18],19]. Therefore, development of treatment modalities based ethnopharmacology has been emerged as a global interest.

This study of *F. leucopyrus* on its anti-cancer activity against Human Ovarian Carcinoma has given promising results, and the results showed that it can effectively inhibit the proliferation of cancer cells and inhibit Human Telomerase Reverse Transcriptase enzyme which is directly responsible with telomerase activation of cancer cells [17]. MTS assay has been recognized as one of the most widely used methods for the quantification of cell proliferation. As cell proliferation is a prominent indicator to understand the survival or death of the cells, monitoring the progressing of carcinoma is a reliable measure. In this assay, NAD(P)H-dependent dehydrogenase enzymes in viable cells cause the reduction of MTS tetrazolium compound to form coloured formazan product

which is soluble in cell culture media. This causes a significant increase in colour intensity, which could be easily quantified by measuring the absorbance at 490-500 nm [20]. In this study, the concentration dependent inhibition of Human Ovarian cancer cell proliferation was observed for ethyl acetate extract of leaves of *F.leucopyrus* and the isolated compounds-1 and compound-2. A rapid inhibition of the proliferation of Human Ovarian cancer cells occurred in the concentration range from 1-50 µg/mL of EtoAC crude and a significant reduction of absorbance in 50 µg/mL - 100 µg/mL concentration (Figure 1a) which indicates a prompt inhibition of proliferation of Human Ovarian cancer cells. The compound-1 isolated from the EtoAc extract has shown rapid inhibition of cell proliferation in the range 1-10 µg/mL and slow inhibition in 10-100 µg/mL (Figure 1b). The compound-2 also showed significant inhibition of the cell proliferation in the range of 10 µg/mL -100 µg/mL (figure 1c). The IC_{50} (the concentration of an inhibitor which the response is reduced by half) values were calculated and the mean values were compared by One-way ANOVA with Duncan's post hoc comparisons at $P < 0.05$, and given in the table-2, in which the crude EtoAc extract (36.35±0.17 µg/mL) and the pure compounds (12.36±0.4µg/mL for compound-1 and 48.53±1.43 µg/mL for compound- 2) have shown signification inhibition effect against cell proliferation of Human Ovarian carcinoma.

Telomerase is an enzyme which maintain the length of telomerase and it has two components: a core reverse transcriptase protein (hTERT) and an RNA (hTR). The expression of hTERT is thought to have important predictive significance in different forms of human malignancies [21], [22]. Telomerase activation allows a cell to continue division and attain immortality, and the inhibition of telomerase activity results the retardation of cell proliferation, which has made a potential new target for cancer treatment [23]. Therefore, hTERT assay measures how concentration of hTERT content in carcinoma cells changes with the concentration of targeted drugs. The results of hTERT assay in this study showed that the compound-2 is a potent inhibitor of Telomerase activity and consequently rapid depletion of hTERT content occurred in the range of 50-200 µg/mL (Figure 2). Brine shrimp lethality assay is important to trace any toxicity of plant compounds towards normal cells and study proves that *F.leucopyrus* is not toxic giving the LC_{50} values 2779.63 μgmL^{-1} for EtoAc extracts of *F.leucopyrus*.

As ethyl acetate extract was found to be the most active against proliferation of Human Ovarian carcinoma, the purification of the crude extract using column chromatography was yielded four compounds. All the compounds were subjected to MTS and hTERT assays and out of four, only two compounds showed higher activity in both assays as noted above. Therefore, these two compounds were characterized by means of spectroscopy (^{1}HNMR,^{13}CNMR, COSY, Mass) and with the comparison of literature data [24]. The compound -1 was identified as bergenin (Figure

3), which is a known polyphenolic compound and potent anti-oxidant, and it has been isolated from few plants including *F. leucoyrus* by previous researchers [7]. Bergenin has been known to possess a wide range of pharmacological activities, such as antifungal activity, anti-inflammatory activity, anti-hepatotoxic, antiulcerogenic, anti-HIV, antiarrhythmic, and neuroprotective properties [7],[25],[26]. The other active compound, compound-2, was characterized as the diastereoisomer of bergenin at C-9, C-14 positions (Figure 4) in its HNMR spectrum. This compound was first identified in this study as a potent anti-cancer compound and also this is the first study in which the active compounds have been isolated and characterized against cancer from the plant *F. leucopyrus*.

5.0 CONCLUSION

The study reveals that the leaves of F. *leucopyrus* possesses potent anti-cancer activity against Human Ovarian carcinoma. The ethyl acetate extract of the leaves was the most active extract and anti-cancer compounds present in the leaves were bergenin and its distereoisomer. These two compounds showed higher anti-proliferative effects towards Human Ovarian cancer cells as well as potent inhibition of human telomerase enzyme which is known to play active role in cancer cell mobilization. This is the first study reported on characterization of active compounds against cancer from the herb *F. leucopyrus*. The isomer of bergenin at C-9 & C-14 is the novel anti-cancer compound identified in the study.

6.0 ACKNOWLEDGEMENT

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7.0 CONFLICT OF INTEREST

The authors declare co conflict of interest

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