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Emblica officinalis Gaertn. constituents and anti-proliferative activity against an ovarian cancer cell line

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Abstract

Emblica officinalis Gaertn. fruits contain structurally diverse phenolic phytochemicals (flavonols, phenolic acids, and hydrolysable tannins) that can exert beneficial effects on the human health. In this report, we describe: 1) methods to process and extract the chemicals from *Emblica officinalis* juice; 2) qualitative composition analysis of bioactive compounds in *Emblica officinalis* juice using HPLC-MS-MS techniques; and 3) anti-proliferative activity of the extract against SKOV-3 cells, a platinum resistant ovarian cancer cell-line. Fresh juice of *Emblica officinalis* fruits harvested locally in Jaunpur (Uttar Pradesh, India) grown under conventional farming conditions, were crushed with coffee grinder, filtered and concentrated to dry powder to achieve constant weights. HPLC-MS-MS analysis showed the presence of 15 compounds; phenolic acids, and hydrolysable tannins. Treatment with *Emblica officinalis* extract reduced proliferation of SKOV-3 ovarian cancer cells. In summary, *Emblica officinalis* fruit juice extract carries a rich profile of phenolic phytochemicals with ant-proliferative activity against a platinum resistant ovarian cancer cell-line.

Epithelial Ovarian Cancer (EOC) is the most lethal gynecologic malignancy faced by women. A highly asymptotic disease by nature, over 75% EOC cases present disseminated stage III/IV disease[4-7,12,14,15]. Five-year survival at stage-3 and 4- are less than 71% and 31%. Serous (68–71%), mucinous (3%), mixed (6%), endometrioid (7–11%) and clear cell (12–13%) are the key subtypes. One in every 75 women may face EOC risk[1]. Over 295,414 cases of EOC were identified in 2018 worldwide, accounting for 3.4% of all malignancies that women face. The Age Standardized Rate (ASR) of EOC was estimated to be 6.6 in 2018[5-7]. India alone contributes over 59,276 new EOC detections were expected by the year 2020. The annual EOC incidence in India is estimated to increase

to 371,000 a year by 2035 followed by 254,000 deaths[4,5].

Debunking plus platinum and paclitaxel-based chemotherapy generates nearly complete response initially, but EOC patients mostly perish due to recurrence and chemoresistance. Alternative lines of chemotherapies offer marginal benefits which are temporary in nature and beset overwhelmingly by the associated toxicities. EOC affects women of all ages but is most commonly diagnosed after menopause. The risk factors include menopause, age and family history of EOC and breast cancer[4]. Despite improved understanding of ovarian tumorigenesis and introduction of novel therapies, five-year survival rate of EOC patients remains dismal[4-7,12,14]. Identification of drive-like targets and their modulators are crucial to making significant improvement in the survival rates of women facing EOC. In addition, identification of dietary supplements that can control EOC proliferation may noticeable contribution to the survival rate of women at risk of EOC via their chemo-preventive actions.

In this report, we describe a method to process *Emblica officinalis* Gaertn. fruits, isolate chemical constituents, conduct analyses using HPLC-ESI-MS-MS techniques and demonstrate their effects on the proliferation of an ovarian cancer cell-line which is platinum resistant by nature. *Emblica officinalis* fruit has been the subject of numerous investigations relating to their health benefits[7-15]. The molecular mechanism and the effects of *Emblica officinalis* extract on the platinum resistant ovarian cancer is not known. We demonstrate that *Emblica officinalis* fruits are rich in phenolic acids, and hydrolysable tannins. Over 15 structurally diverse phenolic acids, and hydrolysable tannins were present in *Emblica officinalis* juice we analyzed. Using MTS assay, we demonstrate that phytochemicals present in *Emblica officinalis* juice can control proliferation of platinum resistant EOC in vitro. In summary, we demonstrate that *Emblica officinalis* fruits can control EOC proliferation *in vitro.*

Emblica officinalis samples :

Kanchan (NA4) popular *Emblica officinalis* variety are commonly cultivated and consumed in the Uttar Pradesh. They were grown in the normal soil conditions in Jaunpur, Uttar Pradesh, India. *Emblica officinalis* fruits were harvested, placed immediately in ice chest, and sent overnight in refrigeration to the Department of Botany, TDPG College, Jaunpur, Uttar Pradesh, India.

Chemicals :

HPLC grade methanol, acetonitrile, formic acid, and water were obtained from Fisher Scientific (Fair Lawn, NJ, USA). All compounds were identified using LC-MS fragmentation.

Extraction of phenolic acids and flavonols:

The refrigerated fruits samples were crushed and grounded in a coffee grinder into paste form, filtered using Cora cloth and dried in oven at 45°C. For stability purpose small amount of sodium benzoate was added before drying and shipped to Rutgers University and Rochester Medical school USA for chemical and anticancer properties the juice extract was

dissolved in 1 mL of mobile phase (water) and filtered using a PVDF syringe filter. The filtered extract was analyzed by LC-MS-MS.

Analysis of Phenolic acids and Tannins :

Phenolic acids and tannins were analyzed in Dionex UltiMate® 3000 LC system. A Gemini® 150 x 4.6 mm C18 110 Å, 5 µm LC column was used, and phenolic acid and tannins were detected at 280 nm. All solvent systems and elution gradients as below. At a flow rate of 1mL min^{-1}, Solvent-A mobile phase (water+0.5% formic acid) and Solvent B (Acetonitrile+0.5% formic acid) the following gradient was used: 0 min, 100% A (solvent- A water+0.5% formic acid); 10 min 20% A; 20 min, 40% A; 40 min, 0 % A; held at 0% A for 15 min. Column equilibration was carried out by flowing 100% Solvent A for 5 min before and after each injection. Effluent from the column was introduced into the triple-quadrupole mass spectrometer under APCI ion source.

MS Spectrometry :

An Applied Biosystems API 3000TM triple-quad LC-MS/MS mass spectrometer coupled with the Dionex UltiMate® 3000 LC system was used in LC/MS-MS analysis. MS data was obtained under atmospheric pressure chemical ionization (APCI) in negative and positive ion detection mode, with following parameters: Curtain gas: 12 psi, Nebulizer gas: 7 psi, Nebulizer current: -2.0 mA, Entrance potential: -10 V, Focusing potential: -300 V, Declustering potential: -60 V, Collision energy: -50 V, Collision cell exit potential: -5.0 V, Source temperature: 500 ºC. Identifications of all 15 compounds were made by comparing retention times, UV spectral patterns, and APCI-MS-MS fragmentation patterns.

Cell viability assay :

SKOV-3 cells were seeded (5000/ well) in triplicate wells in a round bottom 96 well cell culture plates (Nest Biotechnology, catalog number: 701001) in 100uL FBS)+Strep supplemented DMEM medium (GIBCO, catalog number: 11965-092) and allowed to adhere overnight. Media was replaced with Water or *Emblica officinalis* dry powder dissolved in 100uL medium was added to well. The cells were incubated with vehicle/*Emblica officinalis* extract solution for 48 hours. Media was replaced with MTS (Promega, Celltiter 96 Aqueous one solution,1:10 catalog number G3580). The cells were incubated for 2-3 hours. The optical density (OD) of the wells was recorded using BioRad iMark microplate reader at 490nM wavelength. The %age viability of *Emblica officinalis* treated cells was calculated relative to water treated cells normalized to 100%, each datapoint subtracted with average O.D values of 3 cell free blank wells containing RPMI media and MTS dye.

Statistical analysis :

The viability of the SKOV-3 cells in the treatment versus vehicle treated cells was compared by two tailed unpaired t –test using GraphPrism Pad 8th edition.

***Emblica officinalis* fruits are rich in polyphenolics and tannins:** Qualitative composition analysis of bioactive compounds in *Emblica officinalis* juice using HPLC-ESI-MS-MS techniques showed the presence of

Caffeic acid, Quinic acid, Mucic acid-2-O-gallate, Mucic acid-gallate, Gallic acid, Trimethyl-O-ellagic acid, Ellagic acid-O-hexoside, Chlorogenic acid, Ethyl brevifolinacarboxylate, Brevifolibcarboxylic acid, Brevifolin, Galloylglucose, Genestic acid-o-hexoside, Benzoic acid +Catechin, Methyl gallate and Dimethyl-o-ellagic acid in varying compositions. The LC-MS profiles and retention times (rt) of each of these constituents are shown in the Figure-1 and Table-1. Benzoic acid and catechin showed higher area percentage but that should be otherwise (59-87%) at rt of 22.672 minutes (Figure-1 and Table-1) since 0.5% of sodium benzoate was added to juice before drying process as stabilizer. Mucic acid-gallate, Ellagic acid-O-hexoside, Brevifolin and Dimethyl-o-ellagic acid showed least quantitative presence in our analysis (Figure-1 and Table-1).

***Emblica officinalis* fruit extracts inhibited the viability of SKOV-3 ovarian cancer cells :** Viability of SKOV-3 cell lines before and after *Emblica officinalis* crude extract treatment was determined by the CellTiter 96® AQueous One Solution Assay (Promega Corp, Madison, WI) following the manufacturer's recommendations. This colorimetric assay measures relative ability of mitochondria to reduce a substrate [MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] into a soluble blue colored formazan product quantified by measuring the absorbance at 490 nm. The resulting OD is directly proportional to the number of living cells**.** As shown in the Figure-2 crude *Emblica officinalis* extract significantly reduced the viability of SKOV-3 cells ($p=0.0005$, $R^2=0.96$) within 48 hours of treatment.

Emblica officinalis is a member of the Phyllanthaceae family whose consumption in the Indian diet has continued to increase due to cultural awareness and anecdotal observations that consumption of *Emblica officinalis* fruits can confer health benefits[8-14]. Perceived health benefits of foods rich in phenolic phytochemicals in fruits, vegetables, whole grain cereal, red wine, green tea inspire their increased consumption. Indeed, consumptions of phenolic phytochemicals have been associated with reduced risk of certain types of cancers, cardiovascular, and neurodegenerative diseases[1-,24]. *Emblica officinalis* fruits currently ranks amongst top 5 super fruits. Over 90% of the world's *Emblica officinalis* fruits are grown in India and Nepal. The color, size, and shape of *Emblica officinalis* fruits vary with the cultivar type, and the differences among the phenolic compound profiles in different *Emblica officinalis* cultivars arises from sun light exposure, soil make up, temperature, irrigation, influences of abiotic conditions and climatic factors. As a result, considerable diversity of quantity and composition of phenolic phytochemicals compounds can arise in the cultivars owing to these factors.

Health benefits of *Emblica officinalis* have been described *in vitro*, *in vivo* and including human subjects[6,8-11,13,16-,24]. Anticancer activity of *Emblica officinalis* has been described[1,5]. A randomized, double blind, placebo controlled, multicenter clinical trial assessed the efficacy and safety of *Emblica officinalis* extract in patients with dyslipidemia[17,18]. A randomized, double-blind, placebo-controlled clinical trial showed that oral consumption of *Emblica officinalis* fruit extract improved skin conditions in healthy

female subjects[15]. *Emblica officinalis* extract was shown to attenuate attenuates age-related renal dysfunction by oxidative stress[16]. *Emblica officinalis* was found effective in preventing high fructose diet-induced insulin resistance and atherogenic dyslipidemia profile in ovariectomized female albino rats[8]. *Emblica officinalis* was found as a modulator of Alzheimer's disease risk factors and associated physiological changes[16]. *Emblica officinalis* extract was shown to promote procollagen production and inhibits matrix metalloproteinase-1 in human skin fibroblasts[6]. *Emblica officinalis* was shown to enhance mitochondrial spare respiratory capacity by increasing mitochondrial biogenesis and antioxidant systems in a murine skeletal muscle cell-line[20]. *Emblica officinalis* was shown to prevent fructose-induced hepatic steatosis in ovariectomized rats[9]. *Emblica officinalis* extract was found to inhibit lipopolysaccharide-induced procoagulant and pro-inflammatory factors in cultured vascular endothelial cells[13].

Fruits analyzed in this study showed presence and distribution of Caffeic acid, Quinic acid, Mucic acid-2-O-gallate, Mucic acid-gallate, Gallic acid, Trimethyl-O-ellagic acid, Ellagic acid-O-hexoside, Chlorogenic acid, Ethylbrevifolinacarboxylate, Brevifolibcarboxylic acid, Brevifolin, Galloylglucose, Genestic acid-o-hexoside, Catechin, Methyl gallate and Dimethyl-o-ellagic acids and catechin being the highest in the quantity. Similar constituent patterns have been described elsewhere as well[23,24]. *Emblica officinalis* extracts showed statistically significant inhibition of SKOV-3 cell-lines, an ascites derived platinum resistant ovarian cancer cell-line (Figure-2). Which constituent of *Emblica officinalis* is primarily responsible for anti-viability effects against SKOV-3 cell-lines is unclear. Our future studies aim to purify and isolate each of the constituents in singular homogeneity to test and validate the anti-viability effects against platinum resistant ovarian cancer cell-lines.

Ovarian cancer remains an unconquered lethal disease to women[4-7,12,14,15]. Current repertoire of therapies combining surgical debulking with chemotherapies have failed so far to rescue every EOC patient from the risks of morbidities and impending mortalities despite recent advances in ovarian tumorigenesis and introduction of novel therapies. More than 60% women diagnosed with EOC disease perish within 5 years of diagnoses due to lack of therapies in recurrent and resistant settings[4-7]. Women in developing countries like India are often malnourished and receive discriminatory lesser health care. Prevailing poverty, environmental pollution, lack of education, lack of dedicated health care facilities and providers for women combined with and discriminatory and suppressive culture practices predispose women in India considerably to the increased dangers of cancers and deaths. It is anticipated that *Emblica officinalis* can significantly alter the outcomes of the women in India due to underlying therapeutic benefits. Multi-layers of benefits exhibited of *Emblica officinalis* summarized above combined with the anticancer activity exhibited in our experiment (Figure-2) shows the potential to reduce the disproportionate burden of ovarian cancer diseases that women in India face due to poor health care infrastructure, lack of education and overly repressive societal and environmental factors. Future studies will interrogate the mechanism of molecular actions in ovarian cancer models in vitro and potentially *in vivo*.

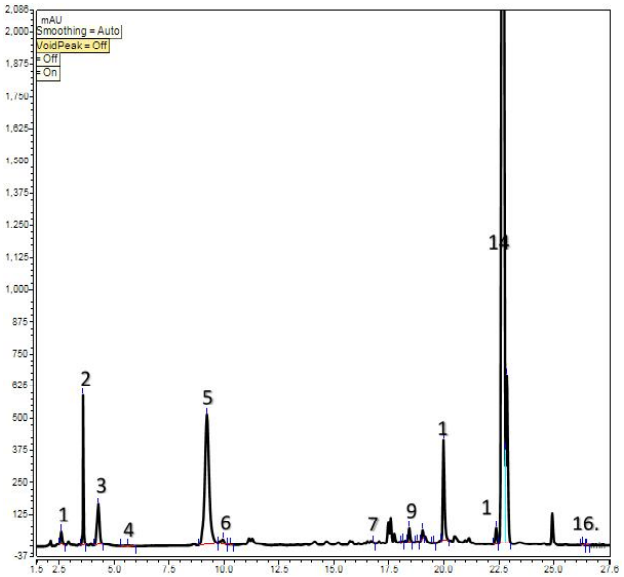

Figure-1. LC-Ms profile of *Emblica officinalis* extract. Phenolic acids and tannins were analyzed in Dionex UltiMate® 3000 LC system. A Gemini® 150 x 4.6 mm C18 110 Å, 5 µm LC column was used and phenolic acid and tannins were detected at 280 nm. All solvent systems and elution gradients as shown in materials and method sections. MS Spectrometry was conducted by an Applied Biosystems API 3000TM triple-quad LC-MS/ MS mass spectrometer coupled with the Dionex UltiMate® 3000 LC system was used in LC/MS-MS analysis. MS data was obtained under the conditions described in the Materials and Methods section.

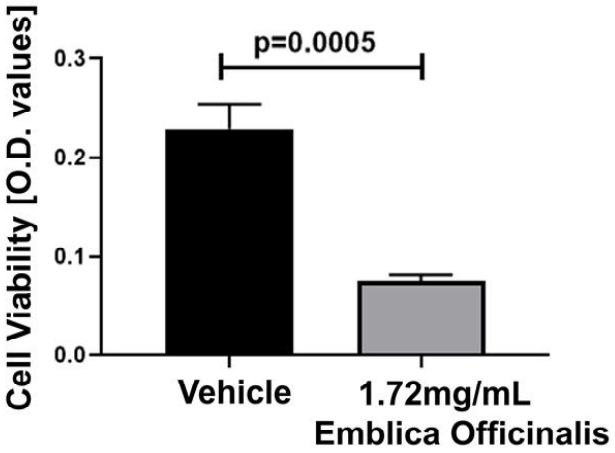

Figure-2. *Emblica officinalis* extract reduced the viability of SKOV-3 ovarian cancer cells. SKOV-3 cells (5000/well) were treated with vehicle or *Emblica officinalis* extract for 48 hours at the indicated concentration. The viability of the cells was determined by measuring the optical density of treated or untreated cells using MTS assay following the methods and materials shown in Materials and Methods Section. The optical density values in treated group was lower than vehicle group (t-test, two tailed, p=0.0005, R^2=0.96). The statistical analysis was performed using Graphprism Ed-8.

Table-1. Retention times (RT) and area percentage of identified phenolic and tannins in concentrated juice of *Emblica officinalis* fruits. Details of instrumentation, methods of analyses and calculations are provided in the Materials and Method sections

Peak no.	Compound name	Retention time in min	Area in %
1	Caffeic acid	2.595	0.63
2	Quinic acid	3.588	2.19
3	Mucic acid-2-O-gallate	4.283	2.49
4	Mucic acid-gallate	5.627	0.04
5	Gallic acid	9.221	13.1
6	Trimethyl-O-ellagic acid	9.941	0.26
7	Ellagic acid-O-hexoside	18.122	0.05
8	Chlorogenic acid	18.423	0.54
9	Ethyl brevifolinacarboxylate	18.787	0.04
10	Brevifolibcarboxylic acid	19.137	0.29
11	Brevifolin	19.537	0.01
12	Galloylglucose	19.978	4.18
13	Genestic acid-o-hexoside	22.376	0.62
14	Benzoic acid +Catechin	22.672	59.87
15	Methyl gallate	26.291	0.06
16	Dimethyl-o-ellagic acid	26.513	0.01

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Author's contributions:

VP identified the variety, collected, processed and extracted the *Emblica officinalis* juice and preserved it in refrigerator for analysis. AC performed the LC-MS analysis under supervision of lead researcher and coordinated cell culture studies. Both VP and AC reviewed the data. VP and AC wrote the manuscript. Both authors approved the final version of the manuscript.

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