

**Evaluation of the Anti-cancer Activity of most potent Ethanolic fraction of *Prunus avium* on EAC Cells in RPMI 1640**Lopamudra Roy<sup>1</sup>, Mounamukhar Bhattacharjee<sup>2</sup>

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Our present study aims to determine the most potent fraction of ethanolic extract of *Prunus avium* as well as to evaluate the anticancer activity of most potent fraction on EAC cells in long time incubation (by using RPMI 1640 media). Ethanolic extract of the fruit was prepared by using maceration (70% C<sub>2</sub>H<sub>5</sub>OH + 30% H<sub>2</sub>O) for 10 days followed by solvent evaporation by rotary vacuum evaporator. Most potent fraction was determined by fractionation by using Chloroform, n-butanol and ethyl acetate followed by qualitative and quantitative analysis. Most potent fraction was introduced into fresh collected and sufficiently diluted EAC cells to check the cell viability under microscope by using RPMI 1640 medium. Results of fractionation shows ethyl acetate fraction is probably most potential fraction due to presence highest amount of flavonoid and phenolic compounds than the other fractions. On addition of ethyl acetate fraction to fresh collected and diluted EAC cells, it shows IC<sub>50</sub> values 43.03 ± 0.54 µg/ml, 34.62 ± 0.21 µg/ml and 26.94 ± 0.22 µg/ml respectively on 2 hr, 4hr and 8 hrs of incubation by using RPMI 1640 medium. Results indicates that the ethyl acetate fraction of ethanolic extract of *Prunus avium* is the most potential fraction and it has good anti-cancer activity in long time incubation as well. Still future studies can be performed to isolate the bioactive principles.

**Keywords:** EAC Cells, RPMI 1640, *Prunus avium*, Anti-cancer activity.**INTRODUCTION**

*Prunus avium* is commonly known as 'sweet cherry' – which is a native of Europe and Western Asia and now the species is widely cultivated at the himachal and the western Himalayan region at high altitude <sup>(1)</sup>. Free radical scavenging activity of *Prunus avium* is already established <sup>(2)</sup> and these antioxidants play important role in the management of oxidative stress – which is a key factor to treat cancer. An antioxidant is the molecule that inhibits oxidation of other molecules by removal of free radical intermediates and as a result formation of oxidative stress is prevented. But all the antioxidants do not work by the same mechanism. Some of them work by free radical scavenging

assay (Example: Vitamin C, Vitamin E), by inhibition of free radical formation (Example: Flavonoids) and by cell damage repair <sup>(3)</sup>. Sweet cherries are thought to be alleviating the pain associated with gout and arthritis and composition of the fruit varies from one region to another depending upon the climatic condition <sup>(4,5)</sup>. Ethanolic extract of the plant already shows good amount of anti-microbial and radio protective activity <sup>(6)</sup> whereas in some cases it has been found as an activator of the human sperm samples <sup>(7)</sup>.

Conventional anticancer drug discovery and development have focused on the cytotoxic agents. The drug discovery paradigms selected agents that had significant cytostatic or cytotoxic

activity on tumor cell lines and caused regression of tumor. The anticancer agents were discovered mainly by serendipity or inhibiting metabolic pathways crucial to cell division. Their exact mechanisms of action were often a subject of retrospective investigation. For example, Farber et al. reported the use of folate analogues for the treatment of acute lympho-blastic leukemia (ALL) in 1948<sup>(8)</sup>, while its mechanism of action, inhibition of the dihydrofolate reductase, was reported by Osborn et al. in 1958<sup>(9,10)</sup>. Similarly, the nitrogen mustard, mustine, was used as a chemotherapeutic agent long before its mechanism of action was understood. Although this strategy has achieved significant success, the recent developments in molecular biology and an understanding of the pharmacology of cancer at a molecular level have challenged researchers to come up with target-based drugs.

RPMI-1640 is a medium containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate, for use in incubators using 5% CO<sub>2</sub> in air<sup>(11)</sup>. Additional sodium bicarbonate may be required for use in incubators containing higher percentages of CO<sub>2</sub>. These can be used for the purpose of long time incubation of the cell lines. Due to presence of high free radical scavenging activity of the ethanolic crude extract<sup>(2)</sup>, our aim is to find out the most potential fraction of the ethanolic extract of the fruit and to incorporate it into the freshly collected and diluted EAC cells to check its anticancer activity in terms of cell viability in long time (2, 4, 8 hrs) incubation by using RPMI 1640 media.

## MATERIALS & METHODS

**Reagents used:** Commonly used chemicals are ethanol, Chloroform, n-butane, RPMI-1640 (Sigma Aldrich) etc. All the other chemicals and reagents used were of high analytical grade.

**Preparation of Ethanolic Fruit Extract:** Ethanolic extract of the fruit was prepared by using maceration (70% C<sub>2</sub>H<sub>5</sub>OH + 30% H<sub>2</sub>O) for 10 days with intermediate shaking followed by filtration with Al<sub>2</sub>CO<sub>3</sub>. Solvent evaporation was done by rotary vacuum evaporator at 35°C followed by heating in water bath to get the ethanolic fruit extract<sup>(12)</sup>.

**Fractionation & Phyto-chemical screening:** Extracts were subjected to screen for various phyto-constituents such as Flavonoids, glycosides, alkaloids, amino acids, carbohydrates, tannins and phenols present in them by using

standard protocols<sup>(13,14,15)</sup>. Preliminary qualitative analysis was done to identify presence of secondary metabolites in the Chloroform, n-butanol and ethyl acetate fraction. The solvent free extract was taken and re-dissolved in the mixture of 15ml ethanol and 15 ml water and washed with petroleum ether by using separating funnel until a clear upper layer of petroleum ether was obtained. The concentrated solution of the lower layer was fractionated with 30 ml chloroform, ethyl acetate and n-butanol respectively. Then the resulting fractions were filtered, concentrated under reduce pressure and finally kept in freeze for further use. Isolation of different fraction from the hydro alcoholic extract was done by using separating funnel. Qualitative analysis was done to check the presence of various components like alkaloid, Flavonoids, tannin, glycoside etc and Quantitative analysis was done to explore amount of flavonoid compound and phenolic compounds equivalent to pyro-catechol and gallic acid present in Chloroform, n-butanol and ethyl acetate fraction.<sup>(16,17)</sup> The most potent fraction was used to check the anti-cancer activity on EAC cells.

### Collection & Maintenances of EAC Cells:

EAC cells were obtained from Pharmacology Research Lab., were maintained *in vivo* Swiss Albino mice by injecting 0.1 ml of tumor cell in the intra-peritoneal route. Transplantation of 2×10<sup>6</sup> cells per mouse was done on every 10<sup>th</sup> day and was drawn out from EAC tumor bearing mouse at the log phase (9<sup>th</sup> – 10<sup>th</sup> day of tumor cell inducing) of the tumor cells<sup>(18)</sup>.

**Preparation of Medium:** RPMI 1640 medium (Gibco, Grand Island, NY, USA) was supplemented with 10% fetal bovine serum (FBS) and was passed through 0.1 micron membrane filter for sterile purpose and ultimately maintained at 37 °C with 5% CO<sub>2</sub> concentration at CO<sub>2</sub> incubator<sup>(11)</sup>.

**In Vitro Cyto-toxicity of the most potent Ethyl-Acetate fraction over EAC Cells in long time incubation (2, 4, 8 hrs) by using RPMI 1640 medium:** Cyto-toxicity in case of long time incubation of ethyl acetate fraction of the extract was performed against EAC cells by using RPMI 1640 media. Freshly collected and diluted EAC (2×10<sup>6</sup>) were added with different concentration of the ethyl acetate fraction (10, 25, 50, 100 ml) along with sufficient volume of RPMI-1640 followed by incubation of 2, 4 and 8 hours respectively. Viability of the cells was determined by using Tryphan Blue exclusion method. Incubated fluid was taken in a WBC pipette and a

drop of the diluted cell suspension was placed on the Neubauer's counting chamber and the numbers of cells in the 64 small squares were counted under microscope. Percentage inhibition was calculated using the standard formulae<sup>(19)</sup>.

**Statistical Analysis:** Results were expressed as mean ± SEM & it was calculated by Graph Pad Prism version 7.00 software.

**RESULTS**

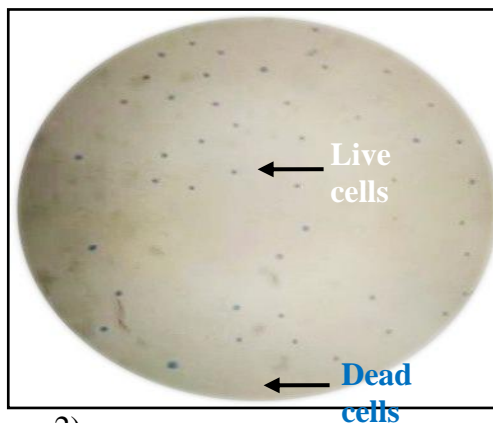
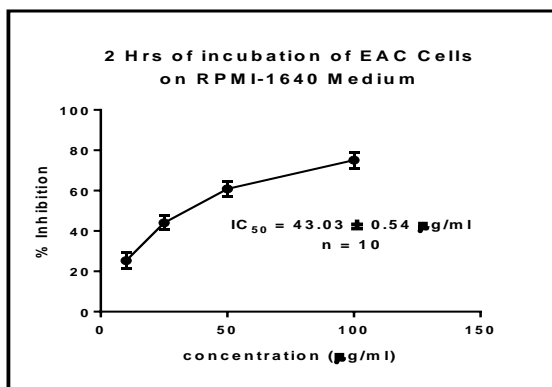
**Results of Qualitative and Quantitative Analysis of Fractionation:** Results obtained on qualitative analysis of different fraction have been described in the following table (Figure 1):

Secondary Metabolites	Fraction of Ethyl Acetate	Fraction of Chloroform	Fraction of n-butanol
Alkaloid	+	+	+
Flavonoid	+	+	+
Tannin	--	+	+
Fat & Fixed oil	--	--	--
Saponine	--	--	--
Glycoside	+	+	+
Carbohydrate	+	+	+

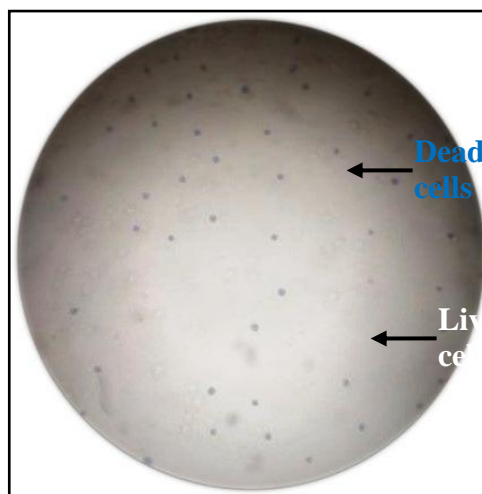
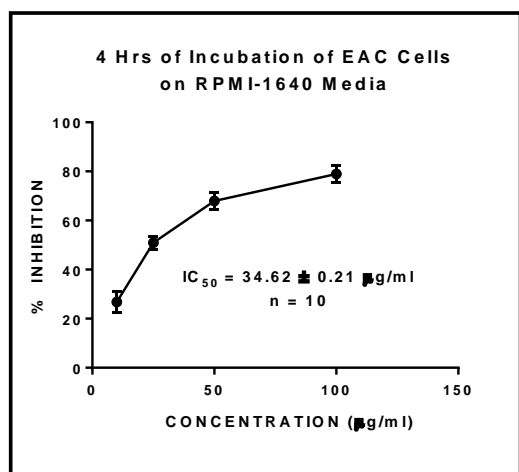
(Figure: 1) (+ = Present, - = Absent)

Total amount of Pyrocatechol, gallic acid and Flavonoids (quercetin) compounds of chloroform, ethyl acetate, n-butanol fraction of ethanolic extract of *Prunus avium* were  $16.23 \pm 0.39$ ,  $43.26 \pm 0.42$ ,  $23.83 \pm 0.94$ ;  $4.50 \pm 0.47$ ,  $9.95 \pm 0.27$ ,  $5.94 \pm 0.36 \mu\text{g}$  and  $0.17 \pm 0.09$ ,  $1.35 \pm 0.18$ ,  $0.78 \pm 0.07 \mu\text{g}$  equivalent of extract respectively. From this result it was concluded that ethyl acetate fraction is probably the most potential fraction due to presence highest amount of Flavonoids (Quercetin) and phenolic compounds (Pyrocatechol and Gallic acid) than the other fraction.

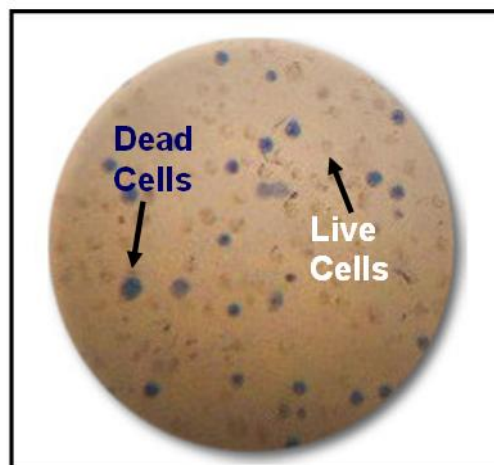
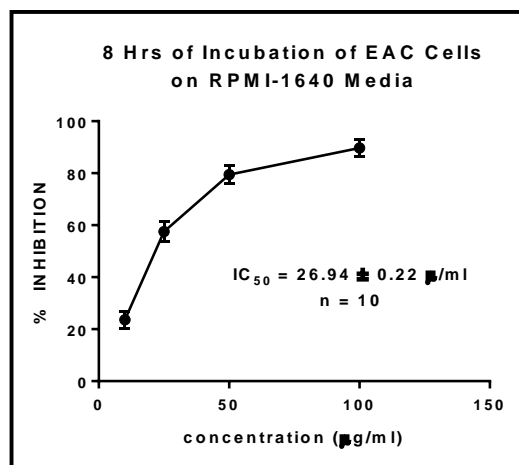
**In Vitro Cyto-toxicity of the most potent Ethyl-Acetate fraction over EAC Cells on RPMI 1640 medium:** Number of viable and non-viable cells found under microscope was counted on 2, 5 and 8 hours (n=10) for each concentration and Percentage Inhibition values were calculated by using standard formulae. In X-axis Concentration ( $\mu\text{g/ml}$ ) was plotted and in Y-axis value of Percentage Inhibition was plotted (Figure: 2, Figure: 3, Figure: 4).  $\text{IC}_{50}$  values found for 2 hrs, 4 hrs and 8 hrs were  $43.03 \pm 0.54 \mu\text{g/ml}$ ,  $34.62 \pm 0.21 \mu\text{g/ml}$  and  $26.94 \pm 0.22 \mu\text{g/ml}$  respectively.



(Figure: 2)



(Figure: 3)



(Figure: 4)

## DISCUSSION & CONCLUSION

The most abundant ROS represented in living inflammatory cells, is super-oxide, as well as hydrogen peroxide and highly toxic hydroxyl radicals and all of them are highly reactive to the living cells. Natural systems, like reduced glutathione, vitamins, and free fatty acids are considered an essential pool of antioxidants.<sup>(20)</sup> Oxidative stress is the situation where the production of oxidants exceeds the capacity to neutralize them & leads to damage of cell membranes, lipids, nucleic acids, proteins, and constituents of the extra-cellular matrix, such as proteo-glycans and collagens<sup>(21)</sup> and this generation of oxidative stress leads to the cancer. There are different therapeutic approaches can be used to decrease the oxidative stress – which includes scavenging of free radicals, inhibition of free radical producing enzymes, enhancing the antioxidant system or targeting the signaling

routes involved in the inflammatory cascade. Amongst the intracellular ROS generated, super-oxide plays a pivotal role in inflammation<sup>(22)</sup>. Various plant-derived extracts were used as an important source of anti-oxidant activity and fruit of '*Prunus avium*' and its fractions also shows the same.

Cancer cells exhibit greater ROS stress than normal cells do, partly due to oncogenic stimulation, increased metabolic activity and mitochondrial malfunction. In one side, at low ROS level, it facilitates cancer cell survival since cell-cycle progression driven by growth factors and receptor tyrosine kinases (RTK) require ROS for activation<sup>(23)</sup> and chronic inflammation, a major mediator of cancer, is regulated by ROS. On the other side, a high level of ROS can suppress tumor growth through the sustained activation of cell-cycle inhibitor<sup>(24, 25)</sup> and induction of cell death as well as senescence by

damaging macromolecules. In fact, most of the phytomolecules having anticancer activity kill cancer cells by augmenting ROS stress.<sup>(26, 27)</sup>

Due to presence highest amount of Flavonoids (Quercetin) and phenolic compounds (Pyrocatechol and Gallic acid) in the Ethyl-acetate fraction, it was probably the most potent fraction having highest free radical scavenging activity and this was responsible for the viability of the cells found under microscope. More the concentration of the ethyl acetate fraction of the extract was used, changes in the cell viability was observed under microscope and it also had been found in IC<sub>50</sub> value. Due to presence of RPMI 1640 medium, long time incubation was performed and IC<sub>50</sub> value for 2 hours, 4 hour and

8 hours found were  $43.03 \pm 0.54 \mu\text{g/ml}$ ,  $34.62 \pm 0.21 \mu\text{g/ml}$  and  $26.94 \pm 0.22 \mu\text{g/ml}$  respectively. Minimum IC<sub>50</sub> value indicates the maximum scavenging as well as anti-cancer effectivity of the ethyl-acetate fraction. Taken together, the study has established that Ethyl-acetate fraction is one of the most potent fractions of the ethanolic crude extract and it shows good anti-cancer activity as well. However more in vivo experiments and single molecule isolation is necessary for the ultimate conclusion.

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