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Ch. Manogna
Department of Pharmaceutical Chemistry, Teja College of Pharmacy, Kodad, Nalgonda-508206, Telangana State, India.

Asish Bhaumik
Department of Pharmaceutical Chemistry, Teja College of Pharmacy, Kodad, Nalgonda-508206, Telangana State, India.

T Haritha
Department of Pharmaceutical Chemistry, Teja College of Pharmacy, Kodad, Nalgonda-508206, Telangana State, India.

SK Nasreen
Department of Pharmacology, Teja College of Pharmacy, Kodad, Nalgonda-508206, Telangana State, India.

M Sucharitha
Department of Pharmacology, Teja College of Pharmacy, Kodad, Nalgonda-508206, Telangana State, India.

M Uttara
Department of Pharmacology, Teja College of Pharmacy, Kodad, Nalgonda-508206, Telangana State, India.

Correspondence
AsishBhaumik, M. Pharm; PGDAIB; PGDCR & CDM
Asst. Professor
Department of Pharmaceutical Chemistry, Teja College of Pharmacy, Kodad, Nalgonda-508206, Telangana State, India.

Evaluation of cytotoxic activity of various extracts of sweet cherry (*Prunus avium*) against human colorectal adenocarcinoma HT-29 cell line

Ch. Manogna, Asish Bhaumik, T. Haritha, SK. Nasreen, M. Sucharitha, M. Uttara

Abstract
HT-29 is a human colorectal adenocarcinoma cell line with epithelial morphology. These cells are sensitive to the chemotherapeutic drugs 5-fluorouracil and oxaliplatin, which are standard treatment options for colorectal cancer. In addition to being a xenograft tumor model for colorectal cancer, the HT-29 cell line is also used as an *in-vitro* model to study absorption, transport, and secretion by intestinal cells. Under standard culture conditions, these cells grow as a nonpolarized, undifferentiated multilayer. Altering culture conditions or treating the cells with various inducers, however, results in a differentiated and polarized morphology, characterized by the redistribution of membrane antigens and development of an apical brush-border membrane. The main objective of the present research work is to isolate the bioactive molecules and evaluate the *in vitro* cytotoxic activity of methanolic and chloroform extracts of sweet cherry (MEC and CEC) of *Prunus avium.* The *in vitro* cytotoxic activity was carried out against human colorectal adenocarcinoma cell line HT-29 by SRB assay. The results obtained from the *in-vitro* studies performed by SRB assay by using human colorectal adenocarcinoma cell line HT 29 displayed that the various extracts of sweet cherry (MEC and CEC) possessed a very good anticancer activity. From the present studied it had been concluded that MEC and CEC, all were exhibiting the potential capability to kill the cancer cell when compared with standard drug 5-FU and the cell growth inhibition of MEC and CEC was found to be the highest 93.91% at 10 µg (IC_{50} = 2.5 µg/ml) and 94.25% % at 10 µg (IC_{50} = 2.1 µg/ml.

Keywords: HT-29, adenocarcinoma, xenograft, nonpolarized, cytotoxic activity, IC_{50} etc.

Introduction
Wonderfully delicious, cherry fruit is packed with full of health-benefiting nutrients and unique antioxidants. Cherries are native to Eastern Europe and Asia Minor regions. Botanically, the fruit is a “drupe” (stone fruit), belonging to the broad Rosaceae family of small tree fruits in the genus, Prunus. Some of the common “drupe” family fruits are plums, peaches, apricots etc. Although several species of cherries exist, two popular cultivars are wild or sweet-cherry, and sour or tart-cherry. While sweet cherries belong to the species of Prunus avium, tart variety belongs to that of Prunus cerasus. Cherries are drupe fruits with a central “stony-hard” seed surrounded by fleshy edible pulp measuring about 2 cm in diameter. Externally they covered by bright "shiny" red or purple, thin peel.

The West Indian cherry, known as acerola (Malpighia emarginata) is native to West Indian islands and grown in Mexico, Texas regions in North America. Acerola belongs to tropical fruit-bearing shrub or small tree in the family Malpighiaceae and contains 2-3 tiny seeds. Acerola contains exceptionally high levels of vitamin-C and vitamin-A than North American and European cherries. Cherries are one of the very low calorie fruits. Nonetheless, they are rich source of phytonutrients, vitamins, and minerals. Both sweet as well as tart cherries are packed with numerous health benefiting compounds that are essential for wellness. Cherries are pigment rich fruits. These pigments, in fact, are polyphenolic flavonoid compounds known as anthocyanin glycosides. Anthocyanins are red, purple or blue pigments found in many fruits and vegetables, especially concentrated in their skin, known to have powerful anti-oxidant properties. Scientific studies have shown that anthocyanins in the cherries are found to act like anti-inflammatory drugs by blocking the actions of enzymes cyclooxygenase-1 and 2. Thus, consumption of cherries may offer potential health effects against chronic painful episodes

such as gout arthritis, fibromyalgia (painful muscle condition) and sports injuries. Research studies also suggest that anti-oxidant compounds in tart cherries can help the human body to fight against cancers, aging and neurological diseases, and pre-diabetes condition. Cherries compose of melatonin anti-oxidant. Melatonin can cross the blood-brain barrier easily and has soothing effects on the brain neurons, calming down nervous system irritability. It, thus, can help relieve neurosis, insomnia and headache problems [1-2].

Cherry taxonomy[3]

Cherries are members of the Rosaceae family, subfamily Prunoideae. They occupy the Cerasus subgenus within Prunus, being fairly distinct from their stone fruit relatives plums, apricots, peaches, and almonds. Prunus avium L. is the Sweet Cherry, and Prunus cerasus L. the Sour, Pie, or Tart Cherry.

Sweet Cherry Cultivars: There are less than 100 sweet cherry cultivars grown in the major production regions around the world today. 'Bing', 'Napoleon' (syn. 'Royal Ann'), 'Ranier', and 'Lambert' are the most important cultivars in North America. Pollinizers for 'Bing' are often 'Early Burlat', 'Black Tartarian', and 'Van'. There are a few self compatible cultivars such as 'Stella' and 'Lapins', but they are of poorer quality than 'Bing' and others that form the basis of the industry.

Sour Cherry: There is good evidence suggesting that P. cerasus, a tetraploid, arose from a natural cross between P. avium and P. fruticosa (Ground cherry). The geographic ranges of the two species overlap in northern Iran and Turkmenistan, which is the center of origin of sour cherry. From there, sour cherry followed a similar course to Europe as sweet cherry, and ultimately came to North America with English settlers. It is more tolerant of the humid, rainy eastern conditions, and therefore proliferated there more than sweets, where it is still cultivated today in greatest numbers. Low monetary returns make sour cherry a less attractive investment than sweet cherry. Thus, it has been planted in western states only to a limited extent. Michigan, the leading producer, grows sour cherries along the eastern shore of Lake Michigan, where the moderating influence of the lake on winter and spring temperatures is beneficial to production [4-5].

Botanical description

Plant: Sweet Cherry. Vigorous tree with strong apical control with an erect-pyramidal canopy shape, capable of reaching 50 ft. In cultivation, sweet cherries are maintained 12-15 ft in height. Leaves are relatively large (largest of cultivated Prunus), elliptic with mildly serrate margins, acute tips, petioled, and strongly veined.

Sour Cherry. Medium sized tree with a rounder, more spreading habit than the erect sweet cherry. Kept <15 ft in cultivation. Leaves elliptic with acute tips, mildly serrate margins, smaller than sweet cherry, with long petioles.

Flowers: Sweet Cherry. White, with long pedicels, borne in racemose clusters of 2-5 flowers on short spurs with multiple buds at tips; the distal bud is vegetative and continues spur growth. Spurs are long-lived, producing for 10-12 years. Ovary position. is perigynous with a distinct hypanthium, characteristic of stone fruits. Sour Cherry. Individual flowers are the same as for sweet cherry. Sour cherry inflorescence buds usually produce 2-4 flowers, with long pedicels, as in sweet cherry. However, many are borne laterally on 1-yr wood, not exclusively on spurs as in sweets. Spurs are shorter-lived on sour than sweet, gradually declining in productivity over 3-5 years. Sour cherries are the latest blooming of the stone fruits.

Pollination: Sweet Cherry. Pollination is absolutely essential for production, since sweet cherries are self-incompatible and need a high degree of fruit set (25-50%) for a commercial crop. In addition to self-incompatibility, there is a high degree of cross-incompatibility. Pollinizers are set every third tree in every third row, or a ratio of 8-9:1. Honey bees are the main pollinator.

Sour Cherry: Sour cherries are self-fertile, and require no pollinizers.

Sweet Cherry: A drupe; ½" to 1 1/4″, round or heart-shaped, glabrous, with long pedicel attached. The pit is generally smooth, and encloses a single seed. The skin color is generally deep red or purple (often referred to as "black"), yellow, or rarely white. Yellow fruit often have a red cheek. The flesh color varies from white to dark red. Fruit is borne on short spurs that arise from older wood. Sweet cherries require only about 2-3 months for fruit development. Thinning is unnecessary.

Fig A: Cherry-bloom-sweet-cherry-tree and Fig B: Cherry-bloom-sore-cherry-tree

Fig D: Cherry-ripe-sour-cherries and Fig E: Cherry-ripe-sweet-cherries

Pharmacological actions [6]

Cherries are a nutritional powerhouse fruit with so many incredible health benefits. One cup of raw cherries has 87 calories, 22 grams of carbohydrates, 1 gram of protein and 3 grams of fiber. Enjoy them now while they are at their peak because their season is way too short. Read on for some of the great health benefits of eating cherries.

Ten Great Health Benefits of Eating Cherries

* Cherries, known as a "super-fruit", are packed with antioxidants called anthocyanins which aid in the reduction of heart disease and cancer.

- Cherries are one of the few food sources that contain melatonin, an antioxidant that helps regulate heart rhythms and the body's sleep cycles.
- Cherries are an excellent source of beta carotene (vitamin A). In fact they contain 19 times more beta carotene than blueberries and strawberries.
- Cherries are rich in vitamins C, E, potassium, magnesium, iron, folate and fiber.
- Cherries are referred to as "brain food", aiding in brain health and in the prevention of memory loss.
- Because cherries contain anthocyanins, they can reduce inflammation and symptoms of arthritis and gout.
- Eating cherries reduces the risk of diabetes.
- Cherries are a good source of fiber which is important for digestive health.
- Cherries are a great snack or dessert choice important for weight-maintenance.
- Because of their powerful anti-inflammatory benefits, cherries are said to reduce pain and joint soreness for runners and athletes after workouts.

Materials and Methods

Drugs and chemicals: The standard drug 5-flurouracil purchased from Local Retail Pharmacy Shop and solvents and other chemicals used for the extraction and phytochemical screening were provided by Institutional Store and were of LR and AR grade.

Cell culture: The cell culture human colorectal adenocarcinoma HT-29 cell line was provided by National Centre for Cell Science (NCCS), Puneand was grown in Eagles Minimum Essential Medium (EMEM) which contained 10% fetal bovine serum (FBS). All cells were maintained at 37°C, 100% relative humidity, 5% CO2, 95% air and the culture medium was changed twice a week.

Apparatus: Round bottom flask, water condenser, heating mantle, motor and pestle.

Methodology for the extraction [7]: Weigh 20 g of sweet cherry fruits paste (ripen can be mashed to prepare a paste) into a 250 ml round-bottomed flask. Add 50 ml of methanol and 60 ml of dichloromethane. Heat the mixture under reflux for 5 min on stem-bath with frequent shaking. Filter the mixture under suction and transfer the filtrate to a separating funnel. Wash this mixture containing bioactive compounds with three portions of 150 ml each with sodium chloride solution. Dry the organic layer over anhydrous magnesium sulfate. Filter and evaporate most of the solvent in vacuum without heating and obtained methanolic extract of sweet cherry (MEC) of *Prunus avium*. Same procedure was followed for the preparation ofchloroform extract of sweet cherry (CEC) of *Prunus avium.*

Phytochemical screening: Preliminary Phytochemical screening of ethanolic and ethyl acetoacetate extracts of sweet cherry of *Prunus avium* had shown the presence of various bioactive compounds such as carbohydrates, amino acids and peptides, phytosterols, carotenoids, andpolyphenols etc [8-10].

Evaluation of *in vitro* cytotoxic activity by SRB assay against human colorectal adenocarcinoma HT29 cell line

Principle: Sulforhodamine B (SRB) is a bright pink aminoxanthine dye with two sulfonic acid group. Under mild acidic conditions SRB dye binds to basic amino acid residues in trichloro acetic acid (TCA) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of visible at least two order of magnitude [11, 12].

Procedure: The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0x10^5 cells/ml using medium containing 10% new born sheep serum. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately) 10,000 cells was added. After 24 hrs, when a partial monolayer was formed, the supernatant was flicked off, washed once and 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml of different concentration of extracts (MEC and CEC) of cherry fruits were added to the cell in microtitre plate. The plates were incubated at 37^0c for 72 hrs in 5% CO_2 incubator, microscopic examination was carried out and observations were recorded every 24 hrs. After 72 hrs, 25µl of 50% TCA was added to wells gently such that it forms a thin layer over the test extracts to form overall concentrations 10%. The plates were incubated at 4^0c for 1 hr. The plates were flicked and washed five times with tap water to remove traces of medium sample and serum and were then air dried. The air dried plates were stained with 100 µl SRB and kept for 30 mints at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then air dried. 100 µl of 10 mM Tris base was then added to the wells to solubilise the dye [13]. The plates were shaken vigorously for 5 mints. The absorbance was measured using micro plate reader at a 540 nm. The % growth inhibition was calculated by the following formula:

% cell growth inhibition = 100-{(At-Ab/Ac-Ab)}x 100

At = Absorbance value of test compound

Ab = Absorbance value of blank

Ac = Absorbance value of control

Results and Discussion

Phytochemical screening

Preliminary Phytochemical screening of ethanolic and ethyl acetoacetate extracts of sweet cherry of *Prunus avium* had shown the presence of various bioactive compounds such as carbohydrates, amino acids and peptides, phytosterols, carotenoids, andpolyphenols etc.

Biological screening

The results for cell growth inhibition by the extracts such as MEC and CEC against HT 29 cell lines for various concentrations is shown in table 1 and 2. As the concentration increases there is an increase in the cell growth inhibition and it was found that MEC with the highest 93.91% growth inhibition at 10 µg (IC_{50} = 2.5 µg/ml) and CEC with the 94.25% growth inhibition at 10 µg (IC_{50} = 2.1 µg/ml). In the USNCI screening program a compound is generally considered to have *in vitro* cytotoxic activity, if the IC_{50} value following incubation between 48 hrs and 72 hrs is less than 4 µg/ml or 10 µM.In the present study IC_{50}values below 4 µg/ml were displayed by the various extracts of sweet cherry of *Prunus avium*. The IC_{50} value of standard drug 5-FU was found to be 1.3 µg/ml with 96.62 % growth inhibition at concentration 75 µg/ml.

IC_{50} Determination [14]

IC_{50} is the acronym for "half maximal inhibitory concentration". IC_{50} value indicates the concentration needed to inhibit a biological or biochemical function by half (e.g.

inhibition of enzymes, affinity to cell receptors). Amongst others, determination of IC_{50} is commonly calculated via linear interpolation: The activity of an enzyme is determined after exposure to a series of inhibitor concentrations. IC_{50} is calculated by the following formula:

IC_{50}= (50% - Low Inh%) / (High Inh% - Low Inh%) x (HighConc-Low Conc) + Low Conc.

Low Inh% / High Inh%: % inhibition directly below / above 50% inhibition

Low Conc / High Conc: Corresponding concentrations of test compound.

Table 1: For percentage (%) of cell growth inhibition of methanolic extract of cherry fruits (MEC) on HT29 Cell lines by SRB Assay

Serial no.	Concentration of the Extracts	Absorbance of extracts	Inhibition of cell growth (%)
1	10 µg/ml	0.018	93.91
2	20 µg/ml	0.034	88.51
3	30 µg/ml	0.055	81.41
4	40 µg/ml	0.068	77.02
5	50 µg/ml	0.086	70.94
6	75 µg/ml (5-FU)	0.010	96.62
7	Control	0.296	0

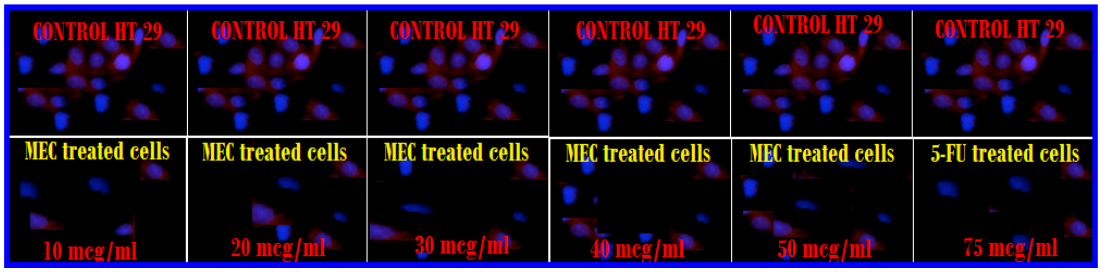

Fig 1: Percentage (%) of cell growth inhibition by MEC on human colorectal adenocarcinoma cell line HT 29

Table 2: For percentage (%) of cell growth inhibition of chloroform extract of cherry fruits (CEC) on HT29 Cell lines by SRB Assay

Serial no.	Concentration of the Extracts	Absorbance of extracts	Inhibition of cell growth (%)
1	10 µg/ml	0.017	94.25
2	20 µg/ml	0.033	88.85
3	30 µg/ml	0.054	81.75
4	40 µg/ml	0.066	77.70
5	50 µg/ml	0.079	73.31
6	75 µg/ml (5-FU)	0.010	96.62
7	Control	0.296	0

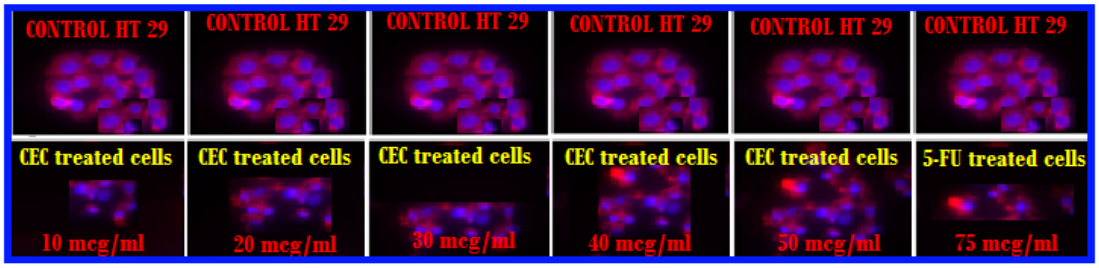

Fig 2: Percentage (%) of cell growth inhibition by CEC on human colorectal adenocarcinoma cell line HT 29.

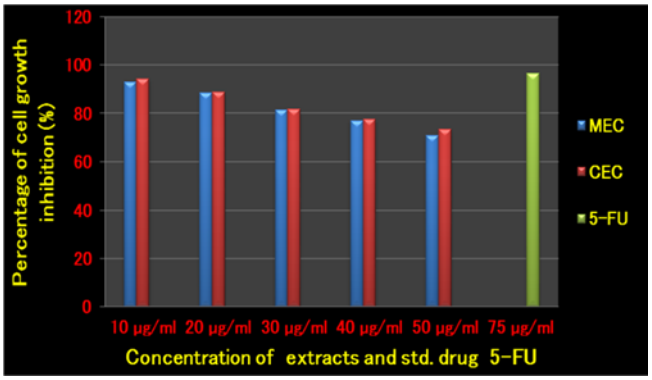

Fig 3: Percentage (%) of cell growth inhibition by MEC and CEC on HT29 cell line.

Conclusion

The results obtained from the present studies displayed that the Preliminary Phytochemical screening of methanolic and chloroform extracts of sweet cherry of *Prunus avium* had shown the presence of various bioactive compounds such as carbohydrates, amino acids and peptides, phytosterols, carotenoids, and polyphenols etc and the results obtained from the *in-vitro* studies performed by SRB assay by using human colorectal adenocarcinoma cell line HT 29. HT 29 cell lines displayed that the various extracts of sweet cherry (MEC and CEC) possessed a very good anticancer activity. From the present studied it had been concluded that MEC and CEC, all were exhibiting the potential capability to kill the cancer cell when compared with standard drug 5-FU and the cell growth inhibition of MEC and CEC was found to be the highest 93.91% at 10 µg (IC_{50} = 2.5 µg/ml) and 94.25% % at 10 µg (IC_{50} = 2.1 µg/ml).

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