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ANTI-OXIDANT AND ANTI-CANCEROUS PROPERTIES OF *PYRUS PASHIA (BUCH. -HAM.EX D.DON) AGAINST* KB-3-1 CANCER CELL LINES *IN VITRO*

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ABSTRACT:

Background: Oral cancer is the fastest-growing cancer in men. Many medicinal approaches are tried step-wise for cancers. Homeopathy and Ayurveda are contributing much to cure cancers without side effects. Plants having antioxidant properties are now being utilized with different solvents to extract different chemical compounds to cure cancer. **Objective:** The current study aimed to examine the anti-cancer and antioxidant properties of acetone extract of *Pyrus pashia* (PP-AE) fruit against KB-3-1 cancer cell lines. **Materials and Methods:** The extract's total phenolic content was determined using a folin-ciocalteu reagent. The DPPH, ABTS, and H_2O_2 assay were used to measure the free radical scavenging activity of the PP-AE. The MTT assay was used to evaluate the cytotoxic efficacy of the PP-AE against KB-3-1 cancer cell lines at various concentrations. **Results:** Based on the observations, the total phenolic content was present in the PP-AE of fruit and all free radical scavenging assays have shown remarkable IC_{50} values. **Conclusion:** According to the current study, acetone extract of *Pyrus pashia* fruit significantly possesses antioxidant and anti-cancer properties against oral (KB-3-1) cancer cell lines. This effect may be used in the future to analyze particular chemical compounds in extract, capable of minimizing the growth of oral cancer cells.

Keywords: *Pyrus pashia,* KB-3-1, Antioxidant, MTT assay.

INTRODUCTION

Oral cancer is the cancer of mouth tissues including the tongue, gums, lips, floor and roof of the mouth, and tonsils. These are squamous cell carcinomas. [1]. It appears only when it is migrated to neck lymph nodes or other locations [2]. In men and women, it is the sixth most common cancer which contributes to 3 percent of all cancers [3]. In India, it is ranked in the top three cancers in the country [4]. More than 3 laths cases are being diagnosed every year which have oral squamous cell carcinoma (OSCC) [5]. The incidences of this cancer increase with age and some typical trends were found in the north and east regions [6]. Many approaches to finding its molecular basis exist, and miRNAs are used as new diagnostic and prognostic biomarkers [7]. Esophageal squamous cell carcinoma (ESCC) is a ferocious tumor and is a common histological subtype of Esophageal cancer (EC). It ranks 6th in mortality and 10th in morbidity worldwide. Radiotherapy, surgery, and chemotherapy are its current treatments. Mammalian targets of rapamycin (mTOR), Protein kinase B (AKT), and Phosphatidylinositol 3-kinase (PI3K) pathway are the survival pathways associated with malignant progression in these tumor cells. Many inhibitors are being tested that target different parts of this pathway. That particularly involves the downstream of signals between eukaryotic translation initiation factors(eIFs) and the progression of ESCC [8]. Chemotherapy is the best for metastasized cancer but its efficacy is limited and it also leads to many side effects for patients. So herbal medication is required for such types of cancers [9].

Herbal Plants

Plants such as *Swertia chirata, Allium sativum,* Zingiber officinale*, Asparagus recemosus,* Withania somnifera*, Catharnthus roseus, Tinosporia cardifolia, Mangifera indica, Ocimum santum, Lantana camera* and many more possess anti cancerous properties [10]. Medicines are prepared from the phytochemicals of plants and they are used in cancer therapeutics [11]. Saponins**,** flavonoids, and polyphenols have shown activities in arresting the cell cycle of cancerous cells, inducing apoptosis, suppressing cancer cell proliferation, and preventing oxidative stress [12].

***Pyrus pashia* Buch. -Ham. ex D. Don (The wild Himalayan pear)**

Taxonomic classification [13-14]

Scientific Name -	Pyrus pashia
Kingdom -	Plantae
Division -	Magnoliophyta
Class -	Magnoliopside
Order -	Rosales
Family -	Rosaceae
Subfamily -	Maloideae
Genus -	*Pyrus*
Species -	*P. pashia*

Vernacular names

Synonyms: *Malus pashia*, *Sorbus variolosa*, *Pyrus crenata*, *Pyrus kumaoni*, *Pyrus nepalensis* , *Pyrus variolosa* ,

Local Name: Hindi- Mahal Mol, Kainth, Panjabi - Kainth, Shegal, Nepali -Passi, Mayal, Kashmiri - Tangi, Kumaoni- Mehal Mol, Urdu- Batang

Geographical distribution

The Himalayan pear (Pyrus pashia Hamilton ex D. Don), is distributed around the Himalayas from Pakistan to Vietnam and from the northern regions

of India to the southern provinces of China. It is a small tree growing up to an altitude of 2000 m in the Himalayas on the western side. It is found in Himachal Pradesh, J&K, etc. In Himachal, it is widely distributed in Kangra, Hamirpur, Bilaspur, Mandi, Lower Kullu, and Una districts [15].

Botanical description

Pyrus pashia is a small to medium shrub tree height up to 10-15 m. It is grown along roadsides, in scrub forests, streams, and wastelands in the western Himalayas. Plants possess spiny branches and at younger stages, they have tomentose leaves. Buds are ovoid. Deciduous stipules. Petioles are1.5 to 3 cm long and glabrescent. 7-13 flowers arranged in corymbose inflorescence. Flowers are 2-5 cm in diameter. White petals, obovate. Stamens are 15-20 shorter than petals. Ovary with 3-5 locules. Its flowers are also edible and have medicinal utility. Fresh flowers are cooked into 'raita' and young leaves are cooked as 'kachru'. Shoot tips, leaves, and flowers are also cooked as a vegetable. Plants possess antiproliferative, hepatotoxic, and anti-inflammatory properties. Besides it is used for diarrhea and hair loss and as a tea beverage by the Monapo community at Twang. Fruits of *Pyrus pashia* are used for dehydration, headaches, fever, gastrointestinal disorders, hysteria, and epilepsy. Edible flowers are also used in many communities for cardiovascular diseases and cancer [16-18].

Picture 1&2 *Pyrus pashia plant and fruits (Photo at the backyard of Nav Vibhor PSSS Sulgwan Hamirpur HP)*

KB-3-1 (Oral) cancer cell Lines

There are various cancer lines for oral cancers. Such as CAL 27 (Caucasian, Tongue Squamous Cell Carcinoma), HSC-3(Japanese, Tongue Squamous Cell Carcinoma), UM -SCC-1 (Caucasian Tongue Squamous Cell Carcinoma), FaDu (Caucasian, Hypopharynx Squamous Cell Carcinoma), KB 3-1 (Human Mouth Cancer Cell Carcinoma), (SCC-4 (African American, Tongue Squamous cell Carcinoma [19]. KB 3-1 cancer cell lines were first time established in an old woman in 1975 during her biopsy of cervical carcinoma exhibiting multidrug resistance (MDR). These cells show resistance against anthracyclines, taxanes, vinca alkaloids, and other broad-spectrum chemotherapeutic agents. This resistance is maintained by membrane transporter p-glycoprotein which expels drugs from intracellular fluid. The cell lines are thus used to study drug efflux mechanisms and molecular signalling pathways [20-21]. Colchicine has been investigated in the management of these cancer cell lines. It has induced apoptosis, disrupting the microtubular dynamics that leads to the stoppage of cell division, and antimetastatic properties [22-23]. Cisplatin is the most widely used chaemotherauptic drug

against such cancers but having side effects.[24]. Rapamycin was isolated from soil samples having bacteria *streptomyces hygroscopicus*. It selectively targets mTORC1 that have inhibitory effects on inflammatory responses, and the formation of colonies, and promotes cellular death and autophagy in oral cancer cells. Rapamycin was treated against Ca9-22 gingival squamous carcinoma *in vitro* and Rapamycin-Cisplatin combination treatment was explored [25-26].

MATERIALS AND METHODS

Plant material

The fruits *of Pyrus pashia* were collected from the backyard of Nav Vibhor public senior secondary school Sulgwan Hamirpur, Himachal Pradesh, India. Plants and fruits were identified and authenticated by the Department of Agriculture, Abhilashi Group of Institutions, Mandi, HP.

The preparation of *Pyrus pashia* fruit acetone extract (PP-AE):

The fruits were washed and after the removal of dirt, they were shade-dried in an open area. The dried fruits along with seeds were then powdered by using a mortar and pestle. The powder is stored between 4 to 8 °C until needed. Each 50 g of powdered plants was extracted using 500 ml acetone through cold maceration for seven days and exhausted. In each instance, the combined extract was evaporated at 30-40 °C under reduced pressure until it was scorched [27].

Cell line culture

The oral cancer cell lines (KB-3-1) were arranged from the National Centre for Cell Science (NCCS) in Pune, India. The cells (10000 cells/well) were grown in DMEM medium supplemented with 10% FBS and 1% antibiotic solution for 24 hours in a 96-well plate. The cells were kept in a humidified incubator with 5% CO_2 at 37°C in 95% air [28].

Chemicals

Folin-Ciocalteu reagent, DPPH, Na_2CO_3, Methanol, Gallic Acid, Ascorbic Acid, Ethylenediaminetetraacetic acid (EDTA), H_2O_2. The reagents and all chemicals used were of analytical grade.

Total Phenol Content Estimation:

Principle:

The Foiln-Ciocalteu phenol reagent is a mixture of phospho-tungstic acid and hetero-poly phosphomolybdic, where the molybdenum and tungsten are in 6+ states. When specific reducing agents are used to reduce the material, the molybdenum blue and tungsten blue, are produced.

Procedure:

The phenolic compounds were determined by the Folin-Ciocalteu reagent. The test sample dilutions were combined with 50µl of diluted folin ciocalteu reagent and 40 µl of aqueous Na2CO3 (1.0 M). According to the setup table, reaction mixtures were prepared, and allowed to stand for 15 minutes, and then absorbance was measured at 760 nm with the double-beam JASCO V-630 spectrophotometer. Gallic acid was prepared as a standard curve in a 50:50 v/v water mixture: methanol at concentrations ranging from 25 µg/mL to 300 µg/ml [29].

Hydroxyl Free Radical Scavenging Assay:

Principle:

A kit of H_2O_2 assay for measuring hydrogen peroxide includes cell culture, supernate, urine, serum, plasma, and other biological fluids. This kit's

objective is to quantify low-level H_2O_2 concentrations in biological matrices. A color reagent reacts with xylenol orange dye in an acidic solution with sorbitol and ammonium iron sulphate to produce a mixture of purple colour which is directly proportional to the amount of H_2O_2 in the sample.

Procedure:

Following were added in the order to the 96-well plate: 10µl of plant extract (concentration as specified in the excel sheet), 24µl of phosphate buffer (50 mM, pH 7.4), 10µl of ascorbic acid (-SD Fine- F13A/0413/1106/62) and 24.14 mg of deoxyribose (SRL-84384), 88µl FeCl3 (Fischer Scientific-Cat no.-23585) (10mg/ml), 28 µl H2O2 (Neurochem Laboratories-HP6520) (6%), water up to 33 ml, and 10µl of plant extract (Concentration as per mentioned in excel sheet). Gallic acid (SRL-Cat no.-5995-86-8) with a concentration as indicated in the Excel sheet was standard. After incubation, 10% TCA (Fischer Scientific-Cat no. 28444) and 50µl of 1% TBA (HiMedia-Cat no. RM1594) were added to each well. A chromogen in pink was created. Then the absorbance was measured at a wavelength of 540 nm. [30-31].

Calculation:

Scavenging activity was calculated by the following formula...

$$\frac{A(control)-A(sample)}{A(control)} x\,100$$

A (Sample): Absorbance of the extracts/standard.

A (control): Absorbance of the control and

ABTS Radical Scavenging Activity

Principle

Either manganese dioxide or potassium persulfate oxidizes ABTS. ABTS cation radicle absorbs radiation of a wavelength of 743 nm and gives blue blue-green colour upon losing an electron by the Nitrogen atom of ABTS. When Trolox I is added which is a hydrogen-donating antioxidant, the colour is lost as the Nitrogen atom quenches the hydrogen, and absorbance is decreased at 743 nm [32-33].

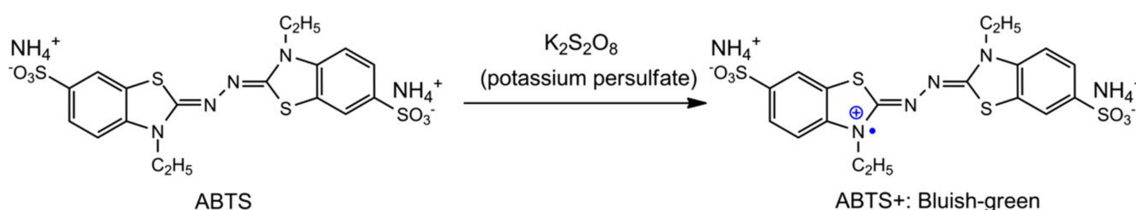


Fig.1 Oxidation of ABTS

Procedure

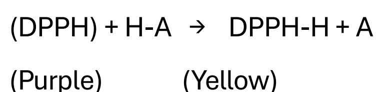
ABTS (SRL-Chem-Cat no.-28042) radicles were prepared by mixing APS (2.45 mM) and ABTS (7mM) solution, which was diluted 100X to prepare ABTS free radical reagent. Added 10µl of different stock of the standard (Ascorbic Acid -SD Fine-F13A/0413/1106/62, Concentration as per mentioned in excel sheet) and samples (As per mention in excel sheet) to the 200µl of ABTS free radical reagent in 96 well plate and incubated at RT for 10 min in dark. After incubation measure the absorbance of the decolorization at 750nm using a microplate reader (iMark, BioRad). Results were presented concerning negative control. IC-50 was

calculated using Software Graph Pad Prism 6 [33-37].

DPPH Scavenging Assay:

Principle:

1,1-diphenyl-2-picrylhydrazyl (DPPH) is deep violet due to its picryl group. The colour of the molecule disappears when combined with a material that can donate a hydrogen atom, turning it into a pale yellow colour. The powder has a red colour and is stable [38].



Procedure:

0.1 ml of 0.1 mM DPPH solution was mixed with 5µl of a distinct stock of the test compound in a 96-well plate. The reaction was set up in triplicate, and blanks with 0.2 ml of DMSO/methanol. 5 µl of a compound at various concentrations were made in duplicate. The plate was rested for thirty minutes in the dark. After the incubation, the decolorization was measured at 495 nm. with the help of a microplate reader (iMark, BioRad). A reaction mixture having 20µl of deionized water was made control. In comparison to the control, the scavenging activity was expressed as "% inhibition". Utilizing Software Graph Pad Prism 6, IC-50 was computed [39-40].

Calculations

DPPH Scavenging activity = $((Abs_{Control} - Abs_{Sample})/Abs_{Control}) \times 100$

In vitro Cytotoxicity Evaluation of acetone extract of PP fruits on KB-3-1 cells

Principle:

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, which is known as the MTT reagent, is a mono-tetrazolium salt made up of three aromatic rings. Two of which are phenyl moieties and one of which is thiazolyl, encircling a positively charged quaternary tetrazole core ring with four nitrogen atoms. Upon reduction of MTT, the core tetrazole ring is disrupted and formazan, a violet-blue water-insoluble dye, is formed. The MTT reagent is reduced to formazan by metabolically active cells and can cross both the cell membrane and the inner membrane of the mitochondria in viable cells, most likely because of its positive charge and lipophilic structure [41].

Procedure:

In cell culture MTT Solution (a final concentration of 250µg/ml) was added and incubated for 2 h. After incubation, the culture supernatant was removed and the cell layer matrix was dissolved in 100 µl Dimethyl Sulfoxide (DMSO) and then read in an Elisa plate reader (iMark, Biorad, USA) at 540 nm and 660 nm. IC-50 was calculated by using the software Graph Pad Prism -6. Images were captured under an inverted microscope (Olympus ek2) by using a Camera (AmScope digital camera 10 MP Aptima CMOS) [41- 43].

RESULTS

Total Phenol Content Estimation

Table 1: Total phenolic content

Sample Code	Phenolic Content (µg GAL Equivalence/mg)
PP-AE	140.1333

Folin ciocalteu reagent was used to determine total phenolic content. Results obtained from the experimental work that the acetone extract of *Pyrus*

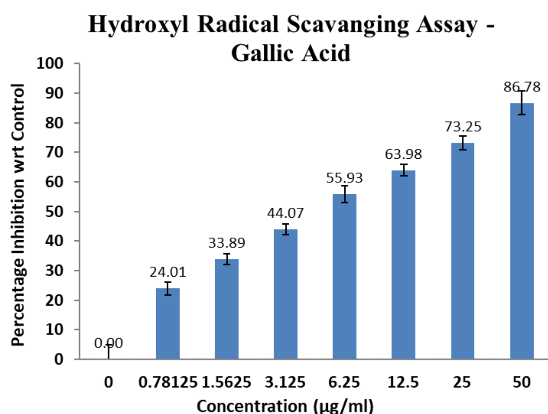
pashia (PP) fruits possesses a content of 140.13 µg GAL Equivalance/mg.

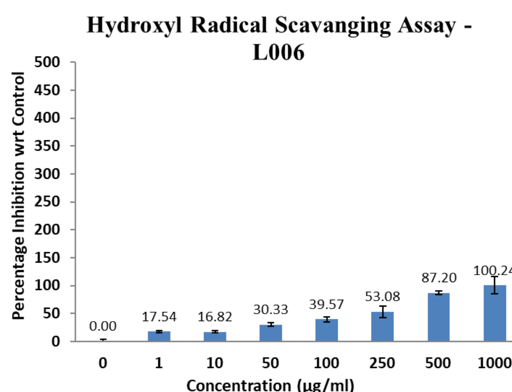
Hydroxyl Radical Scavenging Assay

Based on the results obtained from the study, Hydroxyl Free Radical Scavenging Activity was observed (IC_{50}=129.8 ± 0.12) in comparison to standard Gallic acid (IC_{50} = 4.522 ± 0.026 µg/ml).

Table 2: Antioxidant properties of PP-AE using H_2O_2 assay compared to Gallic acid.

Sample Code	IC_{50} Value (µg/ml)
Gallic Acid	4.522 ± 0.026
PP-AE(L006)	129.8 ± 0.12





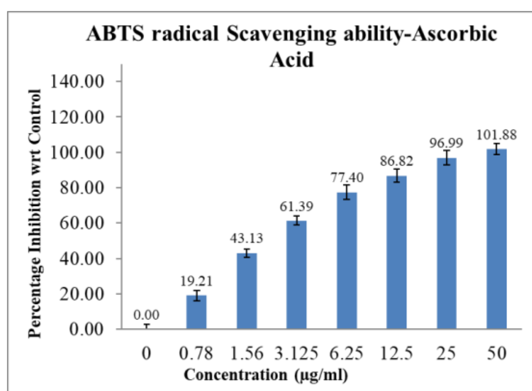
Graph 1&2: Comparative account of Antioxidant property of PP using H_2O_2 assay compared to Gallic acid

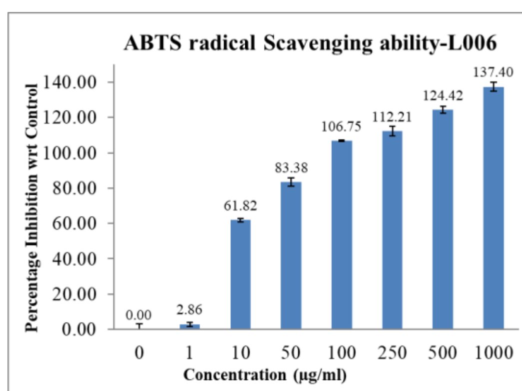
ABTS Radical Scavenging Assay

Table 3: Antioxidant property of PP-AE using ABTS assay compared to Ascorbic acid.

Sample code	IC50 value (µg/ml)
Ascorbic Acid	2.168 ± 0.049
PP-AE	7.55 ± 0.14

Antioxidant property (ABTS) was observed dose-dependent manner in PP acetone extract (IC_{50}= 7.55 ± 0.14 µg/ml) as compared to the standard ascorbic Acid (IC_{50} = 2.168 ± 0.049 µg/ml). 7.55 µg of PP-AE was found equivalent to 2.168 µg of the standard Ascorbic acid.





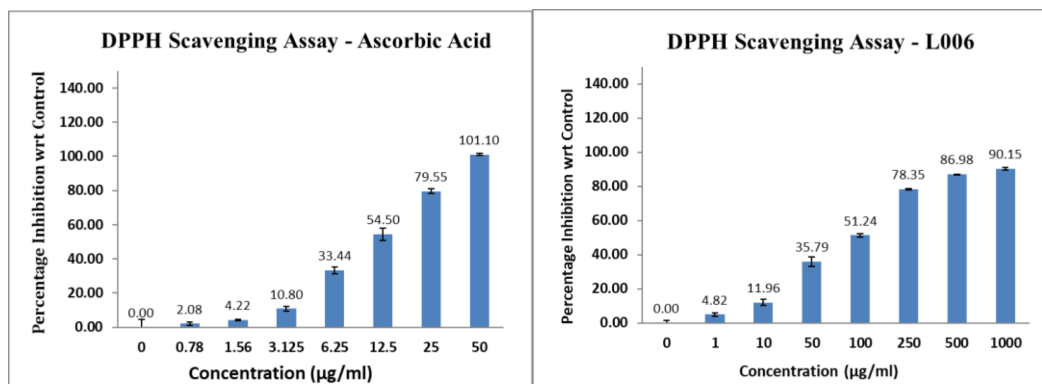
Graph 3: Antioxidant property of PP-AE comparative to ascorbic acid using ABTS assay

DPPH Scavenging Assay

DPPH scavenging activity was observed in PA-EA as compared to standard ascorbic acid (IC_{50}= 85.75 ± 0.030µg/ml).

Table 4: Antioxidant properties of PP-AE using DPPH assay compared to Ascorbic acid.

Sample code	IC_{50} value (µg/ml)
Ascorbic Acid	10.35 ± 0.028
PP-AE (L006)	85.75 ± 0.030



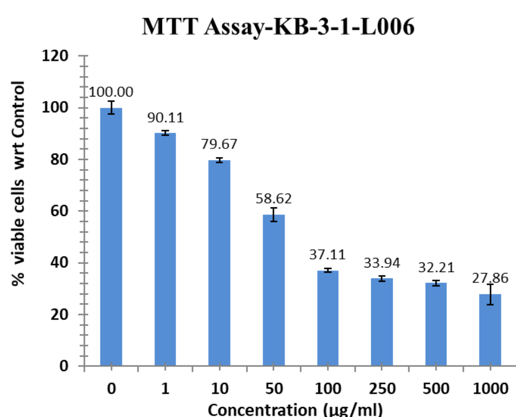
Graphs 4 & 5: Comparative account of Antioxidant properties of PP-AE and Ascorbic acid using DPPH

***In -Vitro* cytotoxicity evaluation of *Pyrus Pashia* (Fruit) acetone extract on KB-3-1 cell lines**

Based on the results obtained from the MTT assay, it was observed that when the Hep G2 and KB-3-1 cell lines were exposed to different concentrations of the sample, no cytotoxic activity was observed in PP-AE (not converged) against liver cancer cells and (IC_{50}= 91.63 ± 0.13 µg/ml) against oral cancer cells. The IC_{50} is the concentration of an inhibitor/sample/ formulation at which the viable cells are reduced by half.

Table 5: Cytotoxic activity of PP-AE extract against KB-3-1cells

Sample code	Cell line	IC_{50} value (µg/ml)
PP-AE(L006)	KB-3-1	91.63 ± 0.13



Graphs 6: KB-3-1cell lines exposed to different concentrations of PP acetone extract

DISCUSSION

Cancer is the deformation of cellular mechanisms. It damages DNA and causes the death of normal cells. Uncontrolled growth explains extra lipid metabolism and starvation of normal cells [44]. Chemotherapy is effective with side effects, so many countries are now switching over to medicinal plants that have some secondary metabolites [45]. Antioxidants like phenols, carotenoids, and terpenes are effective against free radicals that cause cancers [46-48]. The ethanolic fruit extract of this plant was studied through the Enzyme-linked immune sorbent assay (ELISA) method for the quantization of protein levels of cytokinin (IL-10, IL-6, and TNF-alpha) in lipopolysaccharide-stimulated RAW macrophages. Free and bound phenolic contents of fruit extract are rich in epicatechin, catechin, chlorogenic acid, and arbutin that inhibited pro-inflammatory cytokinin (interleukin 6 and tumor necrosis factor-alpha) expression and 5-Lipooxygenase, Cyclooxygenase -2 activities. Oral intake of the extract also showed an anti-inflammatory

effect in paw tissues by reducing cellular infiltration and decreasing pro-inflammatory cytokinin [49-51]. In the current study, the acetone extract of *Pyrus pashia* fruit is active in all assays like DPPH IC_{50}= 85.75 ± 0.030 μg/ml, hydroxyl radical scavenging assay IC_{50}= 129.8 ± 0.12 and ABTS scavenging assay IC_{50}= 7.55 ± 0.14. The presence of its phenol content of 140.1333 μg/mg makes plant extract effective in treating cancer cells. This study confirms the remarkable IC_{50} value for cytotoxicity by MTT assay against oral cancer cells to 91.63 ± 0.13 μg/ml.

CONCLUSION

The result of the current study suggests that the acetone fruit extract of the *Pyrus pashia* has remarkable antioxidant and anti-cancerous efficacy against KB-3-1 cells, which might be due to its free radical scavenging activities and due to its phenol contents. Thus, it is concluded that the study of this extract and its phytochemicals can surely be an alternative treatment for oral cancers.

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