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1st Cancer Update

Iridoid glycosides-Kutkin, Picroside I, and Kutkoside from *Picrorrhiza kurroa* Benth inhibits the invasion and migration of MCF-7 breast cancer cells through the down regulation of matrix metalloproteinases

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MMPs-1, 2, 9, 13;
Iridoid glycosides;
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Kutkin;
Picroside I;
Kutkoside

Abstract *Ethnopharmacological relevance:* Iridoid glycosides have been associated with decreased risks of cancer, such as hepatocarcinoma. Although *Picrorrhiza kurroa* has shown activity against hepatocarcinogenesis, its mechanism of action is poorly understood, further the anticancer activity of iridoid glycosides present in this plant has not been tested so far.
Aim of the study: Here, MCF-7 cell lines (Human breast cancer) were used to test whether *P. kurroa* extract (PE) and its isolated iridoid glycosides Picroside I (PS), Kutkoside (KS), and Kutkin (KT)

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exerts the anti-invasion activity via down-regulation of the expression of matrix metalloproteinases (MMPs). MMPs play an important role in solid tumor invasion and migration.

Materials and methods: The activity and expression of gelatinases (MMP-2 and MMP-9) and collagenases (MMP-1 and MMP-13), protein, and mRNA were detected by gelatin zymography, and RT-PCR. The migratory and invasive capacities of MCF-7 cell lines were measured by the wound scratch migration assay. The preliminary cytotoxicity testing was done by MTT assay and propidium iodide staining. Further the inhibition of inflammatory mediators was also done by quantification of nitrite inflammatory mediators.

Results: The study showed that PE and its isolated iridoids glycosides PS, KS, and KT exhibited considerable cytotoxic potential in a dose-dependent manner. Further PE, PS, KS, and KT inhibited MCF-7 cell invasion and migration, and decreased MMP-2, 9 and MMP-1, 13 activities. Furthermore, PS, KS, and KT reduced MMPs expression at protein and mRNA levels, and suppression of the inflammatory mediators was also exhibited.

Conclusions: Our results suggest that PS, KS, and KT may be the valuable anti-invasive drug candidates for cancer therapy by suppressing Collagenases and Gelatinases. PS, KS, and KT showed good results in comparison with PE. PS and KS exhibit almost comparable down regulation while KT exhibited maximum suppression of invasion, migration, and expression of MMPs.

1. Introduction

Based on the knowledge of cancer biology, it is clear that, once cancer cells have spread and formed secondary masses (metastases); cancers are largely incurable despite the progress in medicine. Two basic principles govern this problem: First, tumors are biologically heterogeneous and contain subpopulations of cancer cells with different properties, and stroma cells. Second, the metastatic process is a cascade of events that depend on both tumor cell properties and the microenvironment. A significant amount of evidence is accumulating that favors the notion that not only cancer cells, but also host cells may be useful therapeutic targets for the treatment of cancer patients (Joyce, 2005; Liotta and Kohn, 2001; De Wever and Mareel, 2002, 2003; Bhowmick et al., 2004; Desmoulière et al., 2004). Examples of key therapeutic targets include over expression of growth factor receptors and their signaling pathways (Bange et al., 2001; Colomer et al., 2001; Dollé et al., 2004), angiogenic factors (Dredge et al., 2003; Ziche et al., 2004), MMPs (matrix metalloproteinase's) (Egeblad and Werb, 2002; Yana and Seiki, 2002), and integrin receptors (Joyce, 2005; Guo and Giancotti, 2004; Xiao et al., 2004). Metastasis is a characteristic of highly malignant cancers with poor clinical outcome. Malignant tumor progression depends upon the capacity to invade, metastasize, and promote the angiogenic host response. One critical characteristic that metastasis cancer cells have acquired is the ability to dissolve basement membranes and the extracellular matrix (ECM). This degradative process is mediated largely by MMPs (Forrbes et al., 2003), which are a large family of at least 20 zinc-dependent neutral endopeptidases that together can degrade all known components of ECM (Parmo-Cabanas et al., 2006). Previous studies have shown the expression and activity of MMPs to be linked to an advanced stage of breast cancer, increased invasion of tumor cells, and building of metastatic formations (Duffy et al., 2000). Various identified MMPs-1, 2, 8, 9, 10, 11, 12, 13, 15, 19, 23, 24, 27, and 28 shows a stronger expression in breast cancer tissue compared to normal breast tissue and could thus seem to be associated with breast cancer development. Hence, all fourteen MMPs are candidates for future functional analyses and potential targets for new therapeutic agents for patients (Köhrmann et al., 2009). Two subclasses of MMPs viz. Gelatinases (MMP-2 and MMP-9) and Collagenases (MMP-1 and MMP-13) are associated with breast tumor progression. Invasion and metastasis are the most strenuous problems in the management of breast cancer. These events require diverse proteolytic enzymes, among which MMP-2 and MMP-9 play a significant role in degradation of type IV collagen, the major component of the basement membrane (Shah et al., 2009). Elevated levels of both MMP-2 and MMP-9 have been observed in blood from breast cancer patients and were repeatedly found to be associated with advanced stage, lymph node metastasis, and poor prognosis (Coskun et al., 2007; Farias et al., 2000; La Rocca et al., 2004; Leppa et al., 2004; Liu et al., 2006; Quaranta et al., 2007; Ranuncolo et al., 2003; Sheen-Chen et al., 2001; Talvensaari-Mattila and Turpeenniemi-Hujanen, 2005; Zucker et al., 1993, 1999). MMP-9 is abundantly expressed in various malignant tumors and is postulated to play a critical role in tumor invasion and angiogenesis (Chandler et al., 1997; Liabakk et al., 1996). Matrix metalloproteinase-1 (MMP-1) and G protein-coupled receptor, CXCR4 (Chemokine receptors), are elevated in these activated fibroblasts, in which they facilitate angiogenesis and matrix degradation, processes that are also vital to breast cancer metastasis (Eck et al., 2009). Some previous studies report also suggests a tumor-promoting role for MMP-1 in breast cancer (Decock et al., 2008). MMP-13 protein was detected in the cytoplasm of carcinoma cells and peritumoral fibroblasts. High level expression of MMP-13 protein in tumor cells was associated with more lymph node involvement and higher tumor grade. MMP-13 protein expressed by tumor cells correlates with the invasion and metastasis of breast carcinoma, and therefore, may serve as a poor prognostic marker for the patient (Zhang et al., 2008). Thus, the inhibition of MMPs activity is important for the prevention of cell invasion (Hidalgo and Eckhardt, 2001; Zhang et al., 2004).

Iridoids comprise part of a group of plant metabolites based on a monoterpene structure with a cyclopenta[*c*]pyranoid skeleton. They are present in various medicinal plants and are considered to be the active principles in some of them, especially in *Harpagophytum procumbens* and *Picrorhiza kurroa* (Ghisalberti, 1988). In the past few decades, several studies

have been performed not only to determine the pharmacological activity of this group of principles, but also in order to demonstrate that they are indeed the active principles of the medicinal plants that contain them. The most relevant effects demonstrated by the different iridoids include cardiovascular, anti-inflammatory, antitumoral, antiviral, and immunomodulator activities, along with antihepatotoxic and antispasmodic effects. They also exhibit purgative, choleretic, hypoglycemic, and hypolipidemic activities (Ghisalberti, 1988). The evidence for the anti-inflammatory activity of iridoids was also present in literature (Singh et al., 1993).

P. kurroa forms a major ingredient of many indigenous medical preparations especially useful for the treatment of diseases of liver, such as hepatitis (Mittal et al., 1978; Ansari et al., 1988), jaundice (Handa et al., 1986), as well as anaemia (Antarkar et al., 1988) and asthma (Langer et al., 1981). Previous studies report that *P. kurroa* shows anti-carcinogenic activity, anti-tumor activity (Joy et al., 2000) and also act on hepatocarcinogenesis induced by *N*-nitrosodiethylamine (Jose Jeena et al., 1999). However, there are no reports of its anticancer activity against breast cancer and further there is no report for the anticancer activity of iridoid glycosides viz. Kutkin, Picroside I, Kutkoside. The aim of this work is to study the cytotoxic activity of *P. kurroa* and the isolated iridoid glycosides viz. Kutkin, Picroside I, Kutkoside which are present in this plant against breast cancer (MCF-7) cell lines, down regulation of the MMPs, to check the migration and invasion of MCF-7 cancer cells and to find out the possible anti-metastatic mechanism.

2. Materials and methods

2.1. Chemicals

Cell lines were procured from National Center for Cell Science, Pune, India. DMEM (Dulbecco's Modified Eagle Medium), MEM (Minimum Essential Medium), RPMI (Roswell Park Memorial Institute), L-glutamine, Penicillin, and Streptomycin were procured from Himedia, Mumbai. FCS (Fetal Calf Serum) was purchased from Lonza, Belgium. The following chemicals were obtained from Sigma–Aldrich, USA: DMSO (Dimethyl sulfoxide), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Propidium iodide (PI). MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Merck, India, Paclitaxel (Dabur, India), Kutkin, Picroside I, Kutkoside (Rathee et al., manuscript no.; (FILE#9079), Production tracking number: LJLC 551607) were isolated in-house. All chemicals used were of analytical reagent grade or higher grade.

2.2. Instrumentation

ELISA plate reader (Bio Rad, USA), Polyvinylidene difluoride (PVDF) membrane filters (Millipore, Ireland), Rotary shaker (R 100/TW Luckham, England), Microscope Olympus 1X51 with Prog Res Software (Jenoptix, Japan), Gel Doc System (Bio Rad, USA), Gel assembly (BengloGenie, India). UV spectrophotometer (UV-2450 Shimadzu, Japan), Master cycler gradient (Eppendorf, Germany), Lyophilizer (Allied frost Lyophilizer, India), Deep freezer (Heto ultra freeze, UK).

2.3. Extraction and isolation

2.3.1. Preparation of extract

Methanolic extract (PE) of *P. kurroa* was prepared by the following method: Accurately weighed 50 gm of the powdered drug was extracted with methanol (100 mL × 4) under reflux on a water bath. The extract was filtered through a Whatman I filter paper, pooled, and concentrated under vacuum to dryness.

2.3.2. Isolation of active constituents

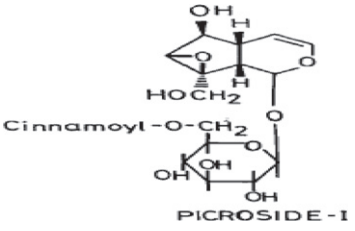
We isolated Kutkin (KS) (Scheme 1c), Picroside I (PS) (Scheme 1a) and Kutkoside (KS) (Scheme 1b) using column chromatography. The isolated compounds were characterized by recording melting point using DSC, UV, IR, and Mass spectra and identified by comparing with the reported data (Singh et al., 1993).

2.4. Cell culture

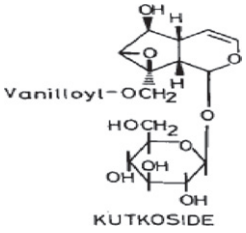
MCF-7 human melanoma breast cancer cells were grown in DMEM supplemented with 100 U/mL, Penicillin G, 100 μg/mL Streptomycin, 0.25 μg/mL, Amphotericin, and 10% heat inactivated fetal bovine serum. Cultures were maintained at 37 °C in a 5% CO_2, 95% air atmosphere.

2.5. Cytotoxicity assays

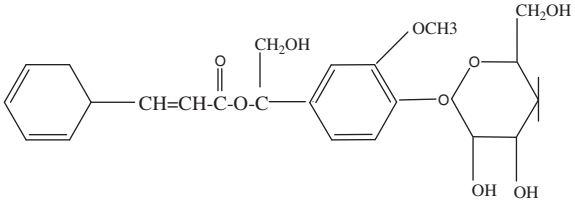
Cytotoxicity screening models provide important preliminary data to help in selecting plant extract with potential anticancer properties for future work. Two cytotoxicity assays were carried out on breast cancer cell lines.



(a): Picroside 1



(b): Kutkoside



(c): Kutkin

Scheme 1 (a–c) Structures of Picroside I, Kutkoside and Kutkin.

2.5.1. MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is taken up by the viable cells and reduced to formazan by the "Succinate-tetrazolium reductase" system that belongs to the mitochondrial respiratory chain functioning in metabolically active cells. Formazan formed, is a purple colored water-insoluble product that is largely impermeable to cell membranes, thus resulting in its accumulation within the healthy cells which is solubilized by adding DMSO. The optical density (OD) of purple colored solution developed was read using a conventional micro plate reader at 570 nm (maximum absorbance). The ability of cells to reduce MTT provides an indication of the mitochondrial integrity and activity, which, in turn, may be interpreted as a measure of viability and/or cell number (Tripathi and Mait, 2005). Exponentially growing cancer cells were harvested from 75 mm^2 flask (TPP, Switzerland) and a stock cell suspension was prepared. 5×10^4 cells/mL were seeded in a 96-well tissue culture plate with 200 μL of DMEM and incubated for 24 h. The dry extract and the isolated compounds (Kutkin, Picroside I and Kutkoside) were dissolved in a minimum amount of DMSO and were prepared in DMEM without phenol red. All the samples were first sterilized using PVDF membrane filters of 0.22 μm. Paclitaxel was used as a positive control and was used at 1 μM, 5 μM concentrations. Cells were treated with different concentrations, 50 μg/mL and 100 μg/mL of PE and 5 μM, 10 μM of isolated compounds (PS, KS, and KT) in 100 μL volume prepared in media without phenol red and with a positive control and were incubated for 48 h. The cells in the control group received no drug treatment. Each treatment was performed in triplicates. After the treatment, drug containing media was removed and washed twice with 100 μL of PBS (Phosphate buffer saline). To each well of the 96-well plate, 20 μL of MTT reagent (Stock: 5 mg/mL) was added and incubated for 4 h at 37 °C. Plates were shaken for 10 min and inverted with gentle tapping on tissue paper to remove the media. To solubilize formazan crystals in the wells, 100 μL of 100% DMSO was added to each well. Plates were placed on a Rotary shaker and agitated for 15–20 min. The optical density was measured by an Enzyme Linked Immunosorbent Assay (ELISA) plate reader at 570 nm with a reference wavelength of 630 nm. O.D of each well was read and expressed as percentage cell survival (absorbance of treated wells/absorbance of control wells × 100). Results were expressed as Mean ± S.E. O.D values (proportional to cell survival) was plotted against the extract concentrations. From the results, the active extract/s was considered to be significant which gave less than 50% survival at the exposure time of 48 h. The PE was further diluted in medium to produce 5 concentrations of 5, 25, 50, 75, and 100 μg/mL. 100 μL/well of each concentration was added to the plates in triplicate and incubated for further 48 h. Its cytotoxic effect was determined by MTT assay. The inhibitory concentration IC_{50} of the crude extract was determined as the drug concentration that decreased 50% of the O.D of the control (untreated) cells.

2.5.2. Propidium iodide staining

PI binds to DNA by intercalating between the bases with little or no sequence preference. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20–30-fold. Propidium iodide is suitable for fluorescence microscopy, confocal laser scanning microscopy, flow cytometry, and fluorometry. PI is membrane impermeant and is generally excluded from viable cells. PI is commonly used for identifying dead cells in a population and as a counterstain in multicolor fluorescent techniques. (According to the manufacturer's protocol) Cells (5×10^5 cells/mL) were cultured at 37 °C for 3 days in medium that contained 10% FBS. After 24 h media was aspirated. Cells were treated with different concentrations, 50 μg/mL and 100 μg/mL of the crude extract and 5 μM and 10 μM of the isolated compounds in 100 μL volumes were prepared in media without phenol red and were incubated for 48 h. The cells in the control group received no drug treatment. At the end of the treatment, the drug containing media was removed. Cells were thoroughly washed 2–3 times with 3 mL of PBS to remove any trace amount of drug and fresh 10% DMEM was added. The cells were harvested, washed with PBS, fixed with 75% ethanol at 4 °C for 2 h, and treated with 0.25 mg/mL RNAse (Sigma) at 37 °C for 1 h. After washing, the cells were stained with 500 μg/mL PI at room temperature for 10 min. Digital images were taken under inverted fluorescence microscope, Olympus 1X51 with Prog Res Software from Jenoptix.

2.6. In vitro assays

2.6.1. Wound scratch migration assay

Cell migration is a complex phenomenon that requires the coordination of numerous cellular processes. The commonly used scratch wound healing assay of tissue-culture cell monolayers is by mechanically scratching the cell substrate with a sterile plastic pipette tip, a characteristically sized wound is created. Wound width is the measure of the distance covered by the cells at different concentration after a particular time point study. When wounded or scratched, the cell monolayer's respond to the disruption of cell–cell contacts and to growth factors at the wound margin by healing the wound through a combination of proliferation and migration. These processes reflect the behavior of individual cells as well as the properties of the cell sheet as a surrogate tissue. They have been used with multiple cell types and, as the monolayer heals the wound in a characteristic manner, used to study cell polarization, matrix remodeling, cell migration, and numerous other processes.

A cell migration assay was performed using a 24-well plate as previously described (Kim et al., 2003). 1×10^4 cells/mL were seeded in 24-well plates, prepared in DMEM, and incubated for 24 h. Cells were treated with different concentrations, 50 μg/mL and 100 μg/mL of the crude extract and 5 μM and 10 μM of the isolated compounds in 100 μL volume prepared in media without phenol red and with a positive control and were incubated for 48 h. The cells in the control group received no drug treatment. At the end of the treatment, a physical wound was given to all the plates in the presence of drug containing media with a sterile plastic micro tip in a linear fashion throughout the center of the plate. This media was removed and the cells were thoroughly washed 2–3 times with 2 mL of PBS to remove any trace amount of drug and the dislodged cells. One of the plates was immediately fixed in methanol. The rest of the plates were incubated with freshly prepared 10% DMEM for 24 h. After incubation, media was aspirated out; cells were washed twice with 2 mL of PBS, fixed with 75% ethanol at 4 °C for 2 h. After washing, the cells were stained with 500 μg/mL of dye at room temperature for 10 min. Digital images were obtained with an inverted fluorescence microscope.

2.6.2. Gelatin zymography assay

Zymography is an electrophoretic method based on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for measuring activity of proteolytic enzymes. The gel is impregnated with a protein substrate depending on the activity of the enzyme to be studied (e.g. type III gelatin) that is commonly chosen for studying gelatinases. Gelatin is degraded by the proteases resolved during the incubation period (conditioned media). Samples for Zymography are prepared in the standard SDS–PAGE treatment buffer but without boiling, and without a reducing agent (β-mercaptoethanol). Following electrophoresis, the SDS is removed from the gel (or Zymogram) by incubation in 2.5% Triton X-100. Staining the gel with Coomassie (Sigma) and destaining with 40% methanol, 10% acetic acid allows the bands of proteolytic activity to be detected as clear bands of lyses against a blue background. Within a certain range, the band intensity can be related linearly to the amount of protease loaded (Hauzenberger et al., 1999).

In 6-well plates, 3×10^4 cells/mL prepared in 10% DMEM were seeded and incubated for 24 h. Cells were treated with different concentrations, 50 μg/mL and 100 μg/mL of the crude extract and 5 μM and 10 μM of the isolated compounds in 100 μL volume were prepared in media without phenol red and with a positive control and were incubated for 48 h. The cells in the control group received no drug treatment. At the end of the treatment, the drug containing media was removed and the cells were thoroughly washed twice with 2 mL of PBS to remove any trace amount of drug. To the culture plate 2 mL of plain DMEM was added (without supplemented with 10% FBS) and incubated for 18–20 h. After incubation, the serum free media called as the conditioned media was centrifuged at 1400 rpm for 10 min to remove any cell debris. The cells in the culture were trypsinized, except that no extra fresh media was added to stop the trypsin activity instead the media collected from the culture plate itself was added. The conditioned media was concentrated by lyophilization and protein content estimated by Folin Lowry method, and stored at −80 °C until further use. The conditioned media was reconstituted using autoclaved water and to assess the gelatinase activity, samples (110 μL for 24 h and 150 μL for 48 h) were run on 10% (w/v) SDS–PAGE with certain modifications containing 2% (w/v) gelatin. Proteinase K (Sigma) served as a positive control. The gel apparatus was set up on a vertical mini gel assembly and the bottom was sealed with 0.8% agarose (HiMedia). Finally, the MMP gel was stained for 30 min with 0.25% (w/v) Coomassie blue in 10% acetic acid (v/v) and 50% methanol (v/v) and destained in 10% acetic acid (v/v) and 50% methanol (v/v). Gel documentation software was used and the images were taken.

2.6.3. Semiquantitative RT-PCR (mRNA analysis)

To check the mRNA expression of these proteases, cancer cells (2×10^6 cells/mL) were treated with PE and PS, KS and KT for 18 h. The total RNA was isolated using TRIzol RNA Extraction Kit in accordance with the manufacturer's instructions. Complementary DNA was synthesized from 1 μg of total RNA in a 20 μL reverse transcription reaction mixture. For semiquantitative PCR, aliquots of cDNA were amplified in a 25 μL PCR mixture as described previously (Zhang et al., 2004). The PCR products were size-fractionated and DNA bands were visualized by staining the gel with ethidium bromide. β-actin primers were used as normalization control. PCR was performed in the presence of 0.5 U Taq DNA polymerase (Takara, Japan) using primer sets that were specific to the following: human MMP-2 (5′-GTG CTG AAG GAC ACA CTA AAG AAG A-3′, 3′-GGA TGT TGA AAC TCT TCC TAC CGT T-5′); MMP-9 (5′-CAC TGT CCA CCC CTC AGA GC-3′, 3′-GGA ATA GCG GCT GTT CAC CG-5′); MMP-1 (5′-CCT TCT ACC CGG AAG TTG AG-3′, 5′-TCC GTG TAG CAC ATT CTG TC-3); MMP-13 (5′-GTG GTG TGG GAA GTA TCA TCA-3′, 5′-GCA TCT GGA GTA ACC GTA TTG-3′); β-actin (5′-TGT GAT GGT GGG AAT GGG TCA G-3′, 5′-TTT GAT GTC ACG CAC GAT TTC C-3′). The PCR products were separated on a 2% agarose gel containing ethidium bromide (0.5 μg/mL), visualized, and photographed using a gel documentation system.

3. Results

3.1. Cytotoxic effect of PE and iridoid glycosides PS, KS and KT

Initially, we measured the cytotoxicity of PE to MCF-7 cells by MTT and PI assays. Dose-dependent cytotoxic effect of PE against MCF-7 cells was shown in Fig. 1. IC_{50} value of the

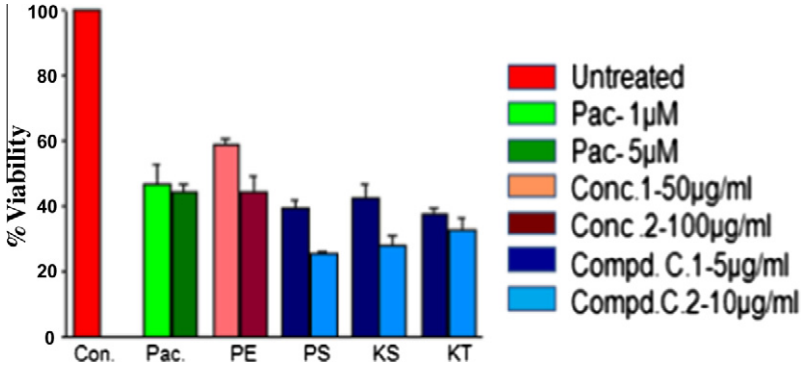


Figure 1 Cytotoxicity activity was evaluated by MTT assay. Cells were treated with 50 and 100 μg/mL of PE, 5 and 10 μM of PS, KS and KT each and positive control (Paclitaxel – 1 and 5 μM) for 48 h, thereafter cultures were evaluated as described in text. Percentage cell viability on MCF-7 human breast cancer cell line was calculated. Con. – control; Pac. – Paclitaxel; PE – *Picrorrhiza kurroa* extract; PS – Picroside I; KS – Kutkoside; KT – Kutkin.

Table 1 Cytotoxicity of methanolic extract of *Picrorrhiza kurroa* rhizomes on MCF-7 human cancer cell lines at various concentrations. IC50 for PE was found to be 61.86.

Concentration (μg/mL)	% Cell viability[a]
	MCF-7
5	84.40 ± 1.05
25	73.79 ± 0.41
50	61.86 ± 0.91
75	54.26 ± 0.75
100	41.80 ± 0.32

PE was found to be 61.86 (Table 1). PE among the 5 tested concentrations of 5, 25, 50, 75, and 100 μg/mL, did not significantly affect the cytotoxicity at the conc. 5 and 25 μg/mL, but at 75 and 100 μg/mL concentration it showed 95% cell viability. Thus, we treated cells with PE in the concentration of 50 μg/mL and 100 μg/mL during subsequent experiments. Investigation of the cytotoxicity by PI assay which is membrane impermeable and is generally excluded from viable cells. Results (Fig. 2) showed that the viable cells did not take the PI while the cells treated with PE extensively taken up the stain, which confirms its cytotoxic potential. Further on comparison of the cytotoxicity of the PS, KS, and KT isolated from *P. kurroa*

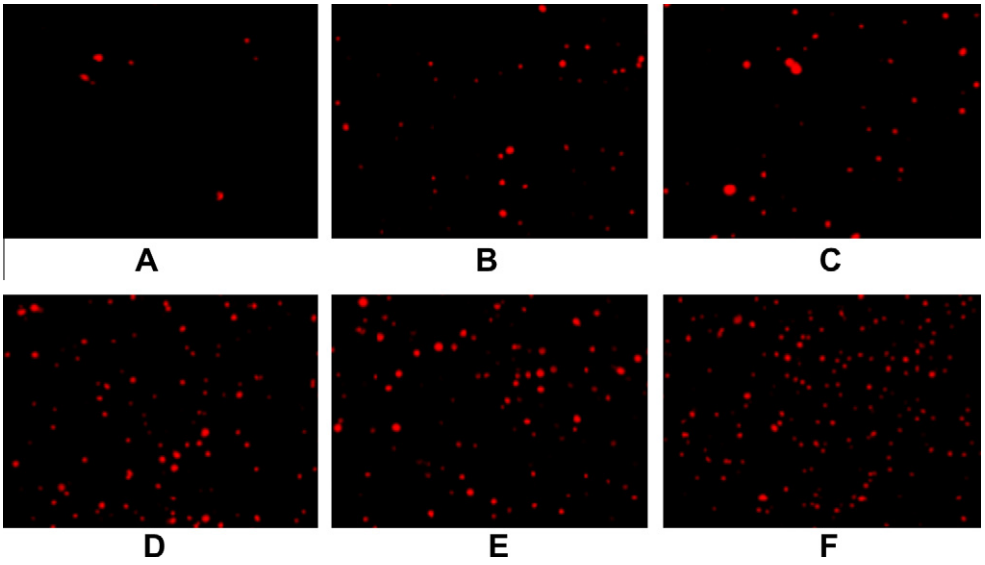


Figure 2 Effect of PE and PS, KS and KT on MCF-7 (breast cancer) cell line. Treated cell were incubated for 48 h and cell death was evaluated by Propidium iodide staining. (A) Control; (B) PE conc. 1 (50 μg/ml); (C) PE conc. 2 (100 μg/ml); (D) Picroside I (PS) (5 μM); (E) Kutkoside (KS) (5 μM); (F) Kutkin (KT) (5 μM).

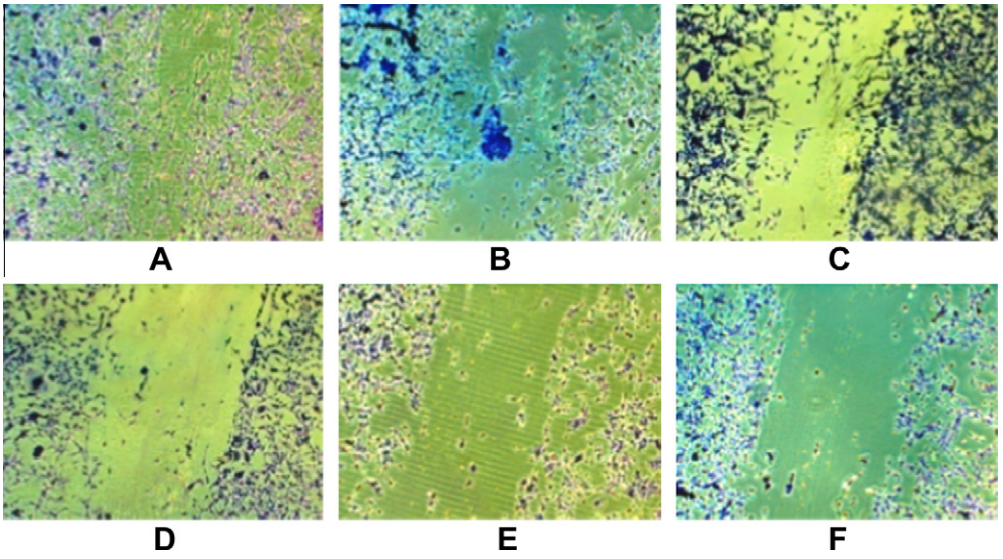


Figure 3 Effect of PE and PS, KS and KT on MCF-7 (breast cancer) cell line. Treated cell were incubated for 48 h and cell migration was evaluated by wound scratch migration assay. (A) Control; (B) PE conc. 1 (50 μg/ml); (C) PE conc. 2 (100 μg/ml); (D) Picroside I (5 μM); (E) Kutkoside (5 μM); (F) Kutkin (5 μM).

Table 2 Comparision of cytotoxicity of *Picrorrhiza kurroa* extract and its isolated compounds on MCF-7 human cancer cell line (breast cancer).

Sample	Concentration	% Cell viability[a] MCF-7
Paclitaxel	(1 μM)	46.63 ± 2.84
	(5 μM)	43.96 ± 2.49
Picrorrhiza kurroa Rhizomes	(50 μg/mL)	61.86 ± 0.92
	(100 μg/mL)	41.80 ± 0.32
Picroside I	(5 μM)	39.21 ± 2.48
	(10 μM)	25.53 ± 0.74
Kutkoside	(5 μM)	42.60 ± 3.78
	(10 μM)	28.23 ± 2.97
Kutkin	(5 μM)	37.85 ± 1.65
	(10 μM)	25.98 ± 3.25

[a] Mean ± S.D. (n = 3).

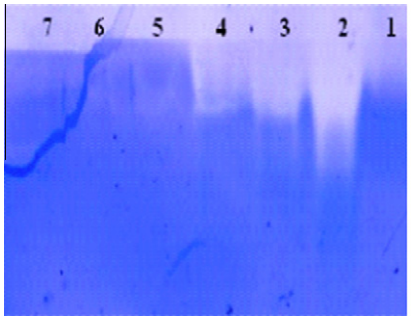
on MCF-7 cells with that of the PE, results showed that the pure compounds exhibits significant cytotoxicity as compared to the PE. Among PS, KS, and KT, the compound KT showed higher cytotoxicity in both of the cytotoxicity assays, while the compounds PS and KS exhibit almost equivalent activity (Table 1, Fig. 1). The data showed that the activity of PS, KS, and KT was found to be comparable to that of positive control paclitaxel on MCF-7 cells. On the basis of these results *P. kurroa* and its iridoid glycosides were selected for further studies. To investigate whether PE and iridoid glycosides PS, KS, and KT inhibits tumor invasion and migration, wound migration, gelatin zymography, and RT-PCR assays were performed in PE and PS, KS, and KT-treated MCF-7 cells. The invasiveness of serum-induced MCF-7 cells was significantly increased, compared to untreated cells, as determined with the wound scratch migration invasion assay (data not shown). Treatment with 50 and 100 μg/mL of PE reduced the invasiveness of cells induced by serum in a dose dependant manner, while compounds PS, KS, and KT in 5 μM and 10 μM significantly blocked tumor invasion. Furthermore the compound KT showed to inhibit the invasion and migration of MCF-7 cells more considerably than KS and PS. In addition, PS, KS, and KT suppressed the migration of MCF-7 cells across the wounded space in a time and dose dependant manner (Fig. 3). These results suggest that the PE and compounds PS, KS, and KT may be used for suppressing MCF-7 cell metastasis and migration (see Table 2).

3.2. Effect of PE and iridoid glycosides PS, KS and KT on the activity of MMPs

Invasion of cancer cells in the extracellular matrix requires MMPs activity. To determine whether PE and isolated compounds directly inhibits MMPs activity, we performed the zymographic analysis and it revealed that MMPs proteins were constitutively expressed in untreated MCF-7 cells. Zymographic evaluation was performed on supernatants of the MCF-7 cultures treated with 50 μg/mL and 100 μg/mL PE and 5 μM conc. of the compounds PS, KS, and KT. Conditioned medium of the MCF-7 cells contained an activity corresponding to gelatinases (MMP-2, -9) and collagenases (MMP-1, -13) (Fig. 4). Less difference in MMPs gelatinase activity was observed after treatment with PE compared to the solvent-treated cells. We further examined whether isolated compounds PS, KS, and KT could suppress MMPs expression and the invasion of breast cancer cells. In contrast, MMPs release was reduced in the supernatants of cells treated with PS, KS, and KT and interestingly the isolated compounds exhibit the comparable down-regulation of MMPs expression. Our results from zymogram gels indicated that PS, KS, and KT potentially blocked MMPs expression in human breast cancer MCF-7 cells at a concentration of 5 μM. These results suggested that PS, KS, and KT could play a crucial role in the down regulation of MMPs expression. (Fig. 4A and B)

3.3. Effects of PE and iridoid glycosides PS, KS and KT on the activities and expressions of MMPs

Because MMP-2, MMP-9, MMP-1, and MMP-13 have been indicated to be associated with cancer invasion, we, therefore, investigated whether these molecules were involved in PE and isolated compounds PS, KS. and KT mediated invasion down-regulation. We confirmed the inhibition of PE and isolated compounds PS, KS, and KT on MMPs activity by RT-PCR (m-RNA analysis). Next, we measured the expression levels of MMPs to examine whether reduced MMPs activities were due to changes in their protein and gene levels or in the levels of other regulatory molecules involved in the activation process. We found that PS, KS, and KT significantly inhibited gelatinases MMP-1,-9 and collagenases MMP-1, -13 activity in MCF-7cells, while the PE showed less inhibition of MMPs expression as compared with the isolated compounds. PS, KS, and KT and suppressed the expression of MMP protein



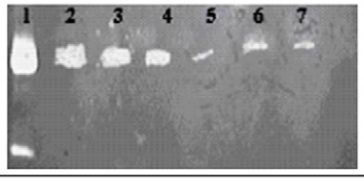


Fig: 4 B. 1: Proteinase K; 2: Control; 3: P. kurroa Ext. conc.1 (50 μg/ml); 4: P. kurroa Ext. conc.2 (100 μg/ml); 5: Picroside I (5 μM); 6: Kutkoside (5 μM); 7: Kutkin (5 μM). After 48 h treatment

Figure 4 Effect of PE and PS, KS, and KT on MCF-7 (breast cancer) cell line. Treated cell were incubated for 72 h and Gelatinase activity was evaluated by Gelatin Zymography. (1) Control; (2) Proteinase K; (3) PE conc. 1 (50 μg/ml); (4) PE conc. 2 (100 μg/ml); (5) Picroside I (5 μM); (6) Kutkoside (5 μM); (7) Kutkin (5 μM).

Figure 5 Relative mRNA expression of gelatinases MMPs shows a down regulation in collagen degrading enzymes upon treatment. (1) Control; (2) PE conc. 1 (50 μg/ml); (3) PE conc. 2 (100 μg/ml); (4) Picroside I (5 μM); (5) Kutkoside (5 μM); (6) Kutkin (5 μM).

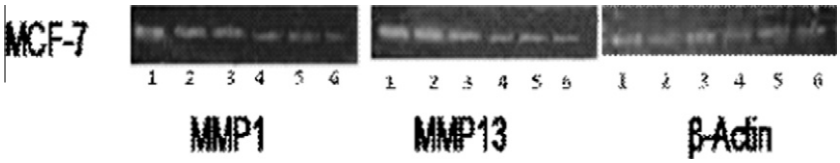


Figure 6 Relative mRNA expression of collagenases MMPs shows a down regulation in collagen degrading enzymes upon treatment. (1) Control; (2) PE conc. 1 (50 μg/ml); (3) PE conc. 2 (100 μg/ml); (4) Picroside I (5 μM); (5) Kutkoside (5 μM); (6) Kutkin (5 μM).

and mRNA levels (Figs. 5 and 6) which forms a specific complex with the MMPs and thus inhibits the activation of MMPs. Additionally, PS, KS, and KT suppressed MMPs transcriptional activity in a dose dependent manner. This indicated that isolated compounds PS, KS, and KT have the tendency to inhibit the metastasis of cancer.

4. Discussion

Invasion and metastasis are fundamental properties of malignant cancer cells. In multiple steps of these processes, degradation of environmental barriers such as ECM and basement membrane is the initial step, and a number of proteolytic enzymes participate in degrading these barriers (Woessner, 1991). Among these enzymes, MMPs play major roles. Especially, MMP-2 (gelatinase A) and -9 (gelatinase B) degrades type IV collagen constituting the major structural component of the basement membrane and ECM, and they are believed to be crucial in the invasion of malignant tumors (Aimes and Quigley, 1995; Kleiner and Stetler-Stevenson, 1993). The activity of MMP-2 and -9 is often found to be elevated in tumor tissues and malignant cells (Dredge et al., 2003). MMP-2 and -9 have been implicated as playing an important role in cancer invasion and metastasis (Levy et al., 1991; Librach et al., 1991). Invasion is a characteristic feature of carcinoma, which frequently shows early invasion into blood vessels as well as tumor metastasis, which occurs subsequently (Pasco et al., 2005). Thus, the discovery of novel natural products and their components to block cancer invasion and migration are the goals of cancer researchers. Anticancer properties have been associated with the components of various natural products including green tea polyphenols, resveratrol, limonene, and organosulfur compounds from garlic (Kaegi, 1998). Especially, baicalein and baicalin inhibited migration of the human vascular endothelial cells in a dose-dependent manner (Liu et al., 2003). Green tea polyphenols and one of its constituents (epigallocatechin gallate) caused a strong inhibition of the gelatinolytic activity of MMP-9 and the secretion of MMP-2 (Demeule et al., 2000; Annabi et al., 2002).

In the present study, the methanolic extract of *P. kurroa* and its isolated compounds KT, PS, and KS were analyzed the effect on MMP-2, -9, and MMP-1, -13 activities. PE and isolated compounds KT, PS, and KS showed a significant inhibitory activity on both classes of MMPs, and the IC50 for PE from the cytotoxicity was found to be 61.86. Among the compounds KT showed a significant inhibitory activity on MMPs, at a concentration of 5 μM. In addition, PE has good cytotoxic activity on MCF-7 cells, PE significantly inhibited the invasion of MCF-7 cells at the concentration of 100 μg/mL. In conclusion, these results suggested that PE has anti-invasive and anti-metalloproteinase activity, and PS, KS, and KT are the major players of the inhibitory potential. The results may serve as a leader compound with the development of anti-tumor drugs.

In conclusion, our data strongly imply that PE and PS, KS, and KT inhibit tumor invasion and migration. Firstly, by inhibiting the migration of MCF-7 cells. Secondly, by suppressing both classes of MMPs at proteins and mRNA levels and considerable down regulation of both the subclasses of MMPs was exhibited by all of the PS, KS, and KT These studies provide important information about the antiinvasive and antimetastatic nature of PS, KS, and KT prospecting these candidates for developing into potential anticancer therapeutics and also have potential avenues for targeting the spread of cancer. We here report that PE and its isolated iridoids compounds PS, KS, and KT inhibited invasion- or metastasis-associated protease activities. The inhibition was also related to the suppression of transcriptional level; however, its mechanism of action still remains elusive. In addition, all three isolated compounds inhibit degradation and cellular invasion of ECM and basement membrane. This study provides an additional activity of the antimetastatic potential beyond antitumor activity mediated by PE and PS, KS and KT.

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