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ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM *PICRORHIZA KURROA* AND ITS *IN-VITRO* ANTICANCER ACTIVITY

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ABSTRACT

Picrorhiza kurroa Royle ex Benth. from the Scrophulariaceae family is an important medicinal plant used in different formulations of herbal drugs that have hepatoprotective activity. Because of the presence of iridoid glycosides, such as picroside-I and picroside-II in underground part. According to the ethnopharmacological relevance Iridoid glycosides have been linked to decreased cancer risks, such as hepatocarcinoma. While *Picrorrhiza kurroa* has shown anti-hepatocarcinogenesis activity, its mechanism of action is poorly understood, the anticancer activity of iridoid glycosides present in this plant has not yet been tested so far. The anti-neoplastic activities of ethanolic extracts of *P. kurroa* rhizome and its isolated iridoid glycosides Picroside I and Picroside II were investigated in the present study. These compounds was identified by a spectrophotometric method. Furthermore, the cytotoxicity of the extracts was tested by Brine shrimp lethality bioassay, Trypan blue exclusion assay and MTT assay. Here, Hep G2 cell lines (Human hepatocellular carcinoma), Colo 205 (Human colon cancer), Normal cell line (L929) were used to test whether *P. Kurroa* extract and its isolated iridoid glycosides Picroside I and Picroside II exerts the anticancer activity. The study showed that *P. Kurroa* extract and its isolated iridoid glycosides Picroside I and Picroside II exhibited considerable cytotoxic potential in dose- dependent manner and were able to target

cells towards apoptosis. The study concludes that *P. kurroa* and its isolated iridoid glycosides like Picroside I and Picroside II have diverse therapeutic potential that may be useful for drug development or for its precursors.

Keywords: Cytotoxicity, Iridoid glycosides, *Picrorhiza kurroa* (*P. Kurroa*), Picroside-I (PI), Picroside-II (PII)

1. INTRODUCTION

In Present scenario, Cancer is one of the greatest killers worldwide and is spreading promptly **[1]**. Cancer describes a series of many diseases in which an uncontrollably dividing group of irregular body cells starts by violating the rules of normal cell **[2]**. Carcinogenesis is a multiple-step process consisting of initiation, promotion, and progression of uncontrolled cells. Initial step involves, damage to deoxyribonucleic acid (DNA) occurs. Then cells begin to proliferate and expand into abnormal cells during the promotion step. Finally during the progression step, further changes occur to these abnormal cells, leading to formation of malignant cells **[3]**. In 2012 around 14.1 million new cancer cases and 8.2 million deaths occurred worldwide based on GLOBOCAN estimates. The burden has moved over the years to less developed countries, which currently account for about 57 percent of cases and 65 percent of deaths from cancer worldwide **[4]**.

Colorectal cancer is the second most frequent cause of cancer mortality in the United States and the third most common cancer worldwide. Colon carcinogenesis is now a multi-stage cycle characterized by the accumulation of genetic modifications. Involving a number of tumor suppressor genes and oncogenes. Diet is considered one of the major factors that account for the incidence and mortality variation of cancer at these sites **[5]**. Approximately 1.2 million new cases and 608,000 deaths are reported each year. Many risk factors have been proposed in the development of colorectal cancer, such as advanced age, sedentary lifestyle and unhealthy eating habits **[6]**.

Hepatocellular carcinoma is the most common primary cancer of the liver. The disease has a poor five-year survival rate of less than 5 percent. In developing countries, hepatocellular carcinoma is a rare cancer **[7]**. According to data from GLOBOCAN 2012, The seventh most frequent reason of cancer associated death in India is liver cancer. In India, 70 percent – 80 percent of all HCCs are HBV-related; about 15 percent are HCV-related; and 5 percent are HBV-related. Alcohol alone represents approximately 8 percent of all HCCs **[8]**.

All cancers involve gene malfunction which controls the growth and

division of cells. Approximately 5 percent of all cancers are strongly hereditary in that an inherited genetic mutation produces a very high risk of developing one or more different cancer forms. Most cancers are not caused by inherited genes but by damage (mutation) to genes that occur during one's lifetime. Mutations can caused by internal factors such as hormones or nutrient metabolism within cells or by external factors such as tobacco, chemicals and sunlight **[9]**. Cell damage caused by free radicals appears to be a major contributor to aging and to degenerative disease of aging such as cancer, cardiovascular disease, cataracts, immune system decline and brain dysfunction. Epidemiological evidence consistently relates low antioxidant intake or low blood levels of antioxidants with increased chances of cancer risk. It has been stated antioxidant exert their protective effect by decreasing oxidative damage to DNA and by decreasing abnormal increases in cell division **[10]**.

Picrorhiza kurroa Royle ex Benth (Family: Plantaginaceae, formerly referred to as Scrophulareaceae) is a well-known medicinal herb popularly known as 'Kutki' or 'Kurro' and 'Indian Gentian,' growing at altitudes of 3000–5000m in the northwestern Himalayas. The plant is an everlasting shrub and historically well-known in the Indian Ayurvedic System to treat liver and respiratory disorders. *P. Kurroa* Pharmacological use of kurroa has potential as antioxidant, anti-inflammatory, hepatoprotective, anti-asthmatic, choleretic, anti-allergic and anti-cancer leading to effective aglycoside iridoid found in the plant. The 22 iridoid glycosides present in Picrorhiza genus. *P. Kurroa* has seven distinct iridoid glycosides, including kutkoside, kutkin, picroside V, mustaenosidic acid, pikuroside, boschnaloside and bartsioside. Iridoid glycosides of *P. Kurroa* are biosynthesized monoterpenes from isoprene, which generally appear as glycosides. Plants use these glycosides for their protection and defence against various microorganisms and insect infections. Such iridoid glycosides treat hepatic, infectious, bacterial, viral, mutagenic cancer and other infections effectively **[11]**.

2. MATERIALS AND METHODS

2.1. Chemicals

Chloroform were purchased from Fischer scientific lab,(India),Methanol were purchased from Rankem, (New Delhi), Ethanol, Ethyl acetate , Formic acid ,Glacial acetic acid, Toluene, Sodium chloride, Potassium hydrogen phosphate, Potassium chloride, Dimethyl sulphoxide (DMSO),5-Fluoro uracil were purchased from Research-lab Fine Chem. Industries (Mumbai), Trypan blue 0.4% solution were purchased from High Putity laboratory Chemicals, (Sarigam), Anemia salina leach

(Brine shrimp eggs) were purchased from central institute of fisheries education seven bungalows, (Andheri (W), Mumbai), Dried yeast was obtained in local market, MTT powder was obtained from Sigma-Aldrich, Silica (60-120), precoated with silica gel 60 F254 (20 × 20 cm, 0.2 mm thickness) were obtained from Merck Ltd (Mumbai, India). Acetonitrile, Orthophosphoric acid. The remaining chemicals and solvents used were of standard analytical grade and HPLC grade respectively.

2.2. Cell Line and Cell Culture.

Two cell lines (Human colon carcinoma (COLO 205) and Human hepatocellular carcinoma (HEP G2) were procured from National Centre for Cell Science, (Pune, India). The cell lines were grown as monolayer cultures in RPMI- 1640 media with 10% foetal calf serum (FCS) and 1% PSA (penicillin, streptomycin, and amphotericin) in a humidified atmosphere of 5% CO_2 at 37°C **[12]**.

2.3. Plant Material

Picrorhiza kurroa rhizomes were collected from Sava healthcare ltd. Pune in the month of JUNE 2019. The rhizomes are crushed into smaller sizes and kept in a tightly closed containers in dark places until subjected to the extraction process.

2.4. Preparation of herbal extract

For refluxing 500 gm of coarse powdered plant material (*Picrorhiza kurroa* rhizomes) was mixed with ethanol in a round bottom flask and refluxed for 2 hours, 4 hours & 6 hours separately at 80°C on water bath. For each cycle 500ml of ethanol was applied, and a total of three cycles were performed. Liquid extracts obtained were separated from the solid residue by vacuum filtration and the solvent was distilled off and liquid was evaporated to dryness at 40 ºC under reduced pressure (ethanol 337mbar), in a rotary evaporator (BÜchi, Switzerland)., the liquid extract was evaporated at 50-55ºC and yield of extract was recorded. The yield of the extract was calculated by using the following formula.

$$\text{Yield (\%)} = \frac{\text{The weight of the residue obtained}}{\text{The weight of the plant material taken}} \times 100$$

2.5. Isolation and characterization of marker compounds

2.6. Thin layer chromatography

Development of TLC profile for separation of phyto-constituents present in ethanol enriched extract. The TLC was performed on pre-coated silica plates (20 × 20 cm, 0.2 mm thickness) silica gel 60 F_{254}. The mobile phase were selected Ethyl acetate: Methanol: Glacial acetic acid: Formic acid (25:5:1:1, v/v/v/v) as mobile phase **[13]**. The sample application was

performed with the help of capillary. The linear ascending development was carried out in a solvent system (10 ml) of Ethyl acetate: Methanol: Glacial acetic Formic acid (25:5:1:1, v/v/v/v) in a twin trough glass chamber (20 ×10 cm) previously saturated with solvent for 25 min at room temperature (25–27°C) and relative humidity (40–45%). After development, TLC plates were dried with the help of hair dryer for 2 min. Visualization was done under UV at 254 nm. The TLC as shown in **Figure 1**.

2.7. Column Chromatography

Preliminary TLC experiment indicated the presence of Picroside I (A) and Picroside II (B) and were isolated with column chromatography. First, the 15 gm ethanol extract on silica gel (100-200 mesh) was subjected to column chromatography. The silica gel slurry was made from chloroform and transferred to column with vigorous shaking so that the silica gel settles in the column. The ethanol extract was then adsorbed to silica gel to form solid mass. Such solid masses then load on column-settled silica. The column was then eluted with 100% chloroform & then 3-4%v/v Methanol in Chloroform and Collected the all fractions of 100 ml each respectively. After observing the TLC fraction no. 1-2, 3-4, 5-6, 7-9, 10-12, 13-14, 15-17, 18-21, 22-23, 24-25, 26-28 were pooled together & concentrated. TLC was performed using mobile phase (Ethyl acetate: methanol: Glacial acetic formic acid 25:5:1:1, v/v/v/v) on pre-coated silica plate. The mixture fraction from the above column subjected to column chromatography on silica gel (230-400 mesh) by using gradient system with chloroform & methanol different ratio of solvent combination. Then column was eluted with first 100% chloroform & 5–7% v/v Methanol in chloroform, Collected the all fractions of 50 ml each respectively at a flow rate of 6-8 ml / min. TLC was performed using mobile phase (Ethyl acetate: methanol: Glacial acetic formic acid 25:5:1:1,v/v/v/v) on pre-coated silica plate. The TLC fraction no.1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 were pooled together & concentrated after observation shown in fig. 6.6. The isolated compounds were characterized by recording melting point (using DSC), NMR, IR, and mass spectra and identified by comparing with the reported data **[14]**. Further confirmation was obtained by co-TLC with standard Picroside I and Picroside II.

2.8. Cytotoxicity assays

Cytotoxicity screening models provide important preliminary data to help in selecting plant extract with potential anticancer properties for future work. Three cytotoxicity assays were carried out on Human colon carcinoma (COLO 205) and Human hepatocellular carcinoma (HEP G2).

2.8.1. Brine shrimp lethality assay

Principle

By this method, the bioactivity of natural product extracts, fractions and the pure compounds can be tested. In a simple zoological organism (Brine nauplii) the method uses in vivo lethality as a convenient monitor for screening and fractionation when discovering new bioactive natural products. The toxicity of brine is closely linked to 9 KB (human nasopharyngeal carcinoma) cytotoxicity. Thus, it is possible to detect and then monitor the fractionation of cytotoxic, as well as (in vivo murine leukaemia) active extracts using the Brine lethality bioassay.

Procedure

Samples of the isolated compound was prepared by dissolving 10mg of extract in 20ul of DMSO and making up to volume 10 ml with distilled water to obtain 1000µg/ml stock solution. From this stock, 100µl, 250µl, 500ul, 1000µl, and 2500µl were taken and up to 5 ml of distilled water volume was produced to obtain the final concentration of 20µg / ml, 50µg / ml, 100µg / ml, 200µg / ml and 500µg /ml. Three replicates were prepared for each dose level. Control vials were prepared by adding equal volumes of artificial sea water. **Hatching of Brine Shrimp eggs:** The 3.5 litters of artificial seawater was added to the egg hatching chamber and 1 capsule of brine shrimp eggs was added to the chamber's dark side, then covered on this side. The shrimp were allowed to hatch for two days and to mature as nauplii. During the hatching time constant oxygen supply was provided. The hatched shrimps are attracted to the light (phototaxis), and so nauplii free from egg shell was collected from the illuminated part of the tank. The nauplii was taken by a pipette from the fish tank and diluted in fresh clear sea water to improve visibility, and 10 nauplii were carefully taken by micropipette. The experiment for the bioassay was carried out according to the procedures described by **[15]**. Nauplii were collected along with water in a glass pipette, and 10 of those shrimps were transferred to each drug conc. vial containing 4.5 ml solution of brine (specific volume and suspension of the yeast) after they are counted in the stem of capillary against lighted background. In each experiment, 0.5 ml of isolated compound (P I & P II) were added to 4.5 ml of brine solution at various concentrations 20-500µg/ml respectively. In control vial added 4.5 ml of artificial sea water and 0.5 ml distilled water. The vial was kept on illumination. After 24 hr. survival of nauplii were counted, by 3X magnifying glass against dark background and the percentage lethality and LC50 values were determined by dose-response data were transformed into a straight line by means of a trendling fit linear regression

analysis (MS-Excel version-10); and the LC50 was derived from the best fit line obtained.

2.8.2. Trypan blue dye cell exclusion assay

Principle A living cell membrane has the ability to prevent the entry of certain dyes into the cell. Hence, the viable cells remain unstained and can be easily distinguished from the dead cells that take up the dye and appear blue under the light microscope.

Procedure:

Trypan blue: 100mg trypan blue dye was dissolved in 100ml PBS and stored at 4ºC.

Phosphate Buffer saline (PBS) preparation: 1. Sodium hydrogen phosphate:2.5 gm, 2. Disodium hydrogen phosphate:2,523 gm, 3. Sodium chloride: 8.2 gm, 4. Distilled water: 100 ml. **Sample preparation**: Sample of the isolated compound were prepared by dissolving 10mg of isolated compound (P I & P II) in 20µl of DMSO and volume make up to 10 ml with phosphate buffer saline (PBS), to get 1000µg/ml stock solution. From this stock 100 µl,1000 µl and 5000 µl were taken and volume was made up to 10 ml (in 10 ml vial capacity) with PBS solution to get the final drug conc.10µg/ml, 100µg/ml, and 500µg/ml. Three replicates were prepared for each dose level. Control vials were prepared by adding equal volume of 0.2% DMSO with PBS. **Standard drug solution preparation:** Samples of standard prepared by dissolving 10mg of 5- FU in 20µl of DMSO and volume make up to 10 ml with phosphate buffer saline (PBS-7.4 pH), to get 1000µg/ml stock solution. From this stock 20 µl/ml was took and volume was made up to10 ml (in 10 ml vial capacity) with PBS solution to get the final drug conc. 20µg/ml.

Procedure for cytotoxicity assay: [16]

In the stock cell suspension, cell count determined and COLO 205 & HEP G2 cells were found 2.3×10^{5}/0.1ml. From this stock, cell suspension was taken in micro wells of microtiter well plates. In the first well added only 0.1ml DMSO (0.1 % V/V with PBS-7.4 pH) and considered as control group. In the next 4 wells, 0.1 ml isolated compound (P I & P II) of concentration ranging from 10 ,20 ,40 and 100 µg/ml were added in respective micro wells considered as test groups. In the next 4 wells, concentration ranging from 10, 20, 40 and 100 µg/ml of 5-FU were added in respective micro wells of microtiter well plate. Considered as standard groups. Further, microtiter well plate was incubated at temperature 37ºC and 5% CO_2 incubator for period of 3 hours. After the incubation, in each microwell of microtiter well plate individually. 0.1 ml of trypan blue was added and mixed well.

Cell counting: The total numbers of dead and living cells in all the four corner squares of the Neuber's chambers were counted by using haemocytometer and the total no. of

viable cells was calculated by using following formula:

Total number of viable cells = A×B×10 ^{4}

Total dead cell count = C×B×10 ^{4}

Where,
A= Mean number of unstained cells (Viable cells).
B= Dilution factor of trypan blue dye (1:5).
C= Mean number of dead cells/ stained cells.
10^4=Conversion of 0.1 mm^3 to ml.

Percentage of cell viability for cytotoxicity was calculated using following formula:

% Viability = Viable cell count / Total cell count × 100

2.8.3. MTT Assay

Principle

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is a yellow coloured water soluble dye. Mitochondrial lactate dehydrogenase produced by metabolically active cells reduce MTT to water insoluble formazone crystals. When dissolved in appropriate solvent (DMSO) these formazane crystals exhibit purple colour. The intensity of purple colour is directly proportional to number of viable cells and be measured spectrophotometrically at 540nm.

Procedure:

The cell line were cultured in DMEM medium which was supplemented with 10% heat inactivated fetal calf serum (FBS) and 1% Antibiotic – Antimycotic 100X solution. The cells were seeded at a density of approximately 5×10^3cells/well in a 96-well flat-bottom micro plate and maintained at 37ºC in 95% humidity and 5% CO_2 for overnight. Different concentration (160, 80, 40, 20, 10µg/mL) of samples was treated. The cells were incubated for another 24 hours. The cells in well were washed twice with phosphate buffer solution, and 20 µL of the MTT staining solution (5mg/ml in phosphate buffer solution) was added to each well and plate was incubated at 37ºC.After 4h, 100 µL of di- methyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals, and absorbance was recorded with a 570 nm using micro plate reader. The % inhibition or % cytotoxicity was calculated by using following formula:

Surviving cells (%) = Mean OD of test compound /Mean OD of Negative control ×100

Inhibiting cells (%) =100- Surviving cells

2.8.4. Statistical analysis

All analyses were carried out in triplicates. Data were presented as Mean ± SEM. Statistical analyses were performed

by one-way ANOVA. Statistical significance of the mean mortality at each concentration was analysed using one way analysis of variance (ANOVA) and compared using Dunnett multiple comparison test (Graph Pad Instat 7.04, USA). $p<0.05$ was taken as the criterion of statistical significance.

3. RESULTS:

3.1. Yield of extracts

The average % yield of extract of ethanol extract of rhizomes of *Picrorhiza kurroa* was found to be 71.42%

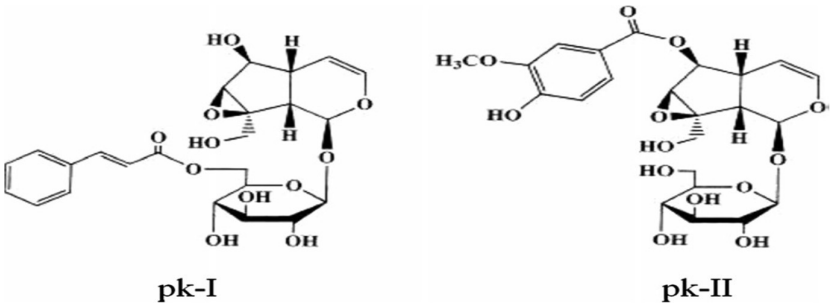
3.2. Isolation and Characterization of Picrosides

P. kurroa rhizomes are reported to be rich in irridoid glycosides and preliminary TLC fingerprint studies also showed the presence of Picroside I and Picroside II. Subsequently, we isolated these compounds. We first isolated the constituent Picroside I and then the Picroside II individually. In our experiment, we modified the earlier reported method to suit the sample and found it to be cost effective and the chemicals used were less toxic. We chose a less costly solvent such as methanol instead of ethanol, and chloroform was used instead of ethyl acetate as the yield was somewhat increased in the case of chloroform. We got a better resolution at 3-4% v/v of methanol for Picroside I and at 5–7% of methanol in chloroform for Picroside II. Several analytical methods, based on HPLC, Column chromatography, TLC, NMR, IR and LC-MS, have been reported for the determination of Picroside-I and Picroside-II in plant. The structural elucidation of isolated compounds was done by interpretation of IR, 1H & 13C NMR and Mass spectra. The two isolated compounds exhibited satisfactory IR, 1H & 13C NMR and Mass data.

Compound A (Picroside I)

UV absorption maxima: 290 nm. IR spectroscopic data (KBr) n cm_1: of Picroside I exhibited broad absorption peak at around 3365.17 $cm^{-1}$was assigned to the O-H stretching vibration of alcohol. C-H Bending vibration of aromatic compound were observed at 1523.49 cm^{-1}. The absorption peaks positioned at 2924.52$cm^{-1}$and 1452.14 cm^{-1} are assigned to the alkane C-H stretching and C-H bending respectively. C=C stretching vibrations of alkene were observed at 1637.27cm^{-1}. C-O stretching vibrations of ester were observed at 1257cm^{-1} . O-H Bending & C-O stretching vibrations of alcohol were observed at 1332.57 c m^{-1} and 1177 cm^{-1}. which confirmed all the possible functional groups in isolated compound. Molecular formula-$C_{24}H_{28}O_{11}$, Mass spectra: m=z picroside-I [m/z 515 (M + Na)+]. **[17]** Melting point: 128–130 ^{0}C (measured using DSC). Co-TLC with Picroside I standard developed in the solvent system of ethyl acetate: methanol: glacial acetic formic acid

25:5:1:1,v/v/v/v)gave only one band at RF 0.43 + 0.03. The characterization of isolated compounds was established ^{13}C-NMR spectral analysis and comparison with earlier reports **(Table 1)**. The isolated compound of Picroside I ^{1}H NMR spectrum revealed three types of proton signals: aromatic protons and hydroxy protons of the analyte and a solvent proton signal. The ^{1}H NMR spectrum showed hydroxyl protons resonating at δ 6.2 and in between 6.4 to 6.5 ppm two singlet also 7.3 to 7.5 ppm for aromatic proton and two singlet at7.6 ppm for aromatic proton another proton occurs at 7.8 ppm corresponding to aromatic ring compound A was identified as Picroside I (Scheme 1A) by comparing the melting point [18] and spectral properties with the reported data.



a) Picroside I **b) Picroside II**

Scheme 1 (a–b) Structures of Picroside I and Picroside II

Compound B (Picroside II)

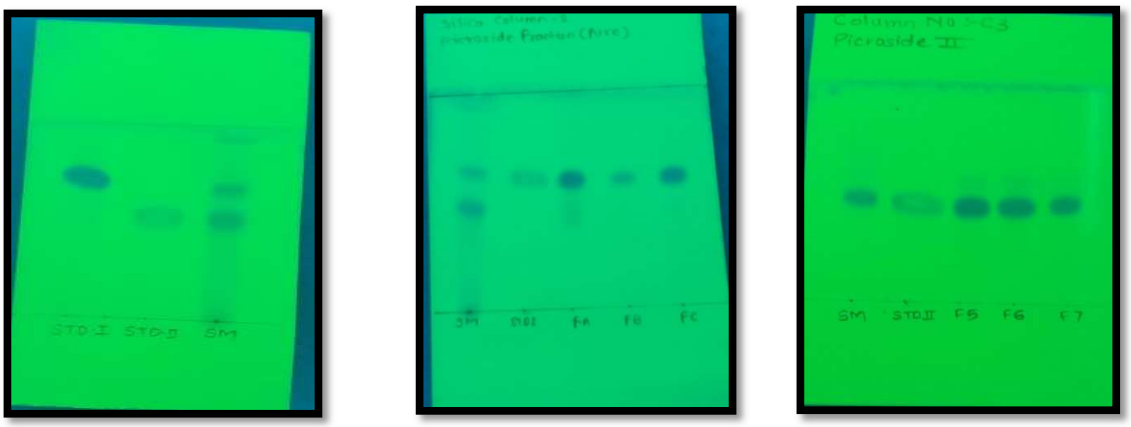
UV absorption maxima: 290 nm. IR spectroscopic data (KBr) n cm_1 of Picroside II exhibited broad absorption peak at around 3397.96 cm^{-1} was assigned to the O-H stretching vibration of alcohol. C=O stretching vibration of Carbonyl were observed at 1656.55 cm^{-1}. C=C stretching and C-H Bending vibrations of aromatic Ring were observed at 15.92.91 cm^{-1} and 1524.45 cm$^{-1.}$ The absorption peaks positioned at 1456 cm^{-1} are assigned to the Methyl Group of C-H Bending vibration. O-H Bending and C-O stretching vibrations of Alcohol were observed at 1282 cm^{-1}and 1077 cm^{-1}respectively. C-O stretching vibration of Alkyl Aryl

Ether were observed at 1256.4 cm^{-1}. C-O stretching vibration of ester were observed at 1159.97 cm^{-1}. The absorption peak positioned at 1106.94 cm^{-1} are assigned to the Aliphatic Ether C-O stretching. Which confirmed all the possible functional groups in isolated compound. Molecular formula- $C_{23}H_{28}O_{13}$,Mass spectra: m=z Picroside-II [m/z535 (M + Na)+].[17] Melting point: 172–173^{0}C (measured using DSC). Co-TLC with Picroside II standard developed in the solvent system ethyl acetate: methanol: glacial acetic formic acid 25:5:1:1,v/v/v/v)gave only one band at R_F

0.60 +0.03. The characterization of isolated compounds was established ^{13}C-NMR spectral analysis and comparison with earlier reports **(Table 1)**. The isolated compound of Picroside II ^{1}H NMR spectrum showed hydroxyl protons resonating at δ 6.3 and in between 6.7 to 6.8 ppm two singlet also 7 to 7.5 ppm for aromatic proton and another proton occurs at 7.8 ppm corresponding to aromatic ring. Alkene Proton appeared at 4.7 ppm as singlet. Methoxy Proton appeared at 3.71 ppm as singlet. Compound B was identified as Picroside II (Scheme B) by comparing the melting point and spectral properties with the reported data **[18]**.

Two main bioactive iridoids (Picroside-I and Picroside-II) from *Picrorhiza kurroa* Royle ex Benth were successfully isolated and purified with prep-RP-HPLC using binary gradient Orthophosphoric acid and acetonitrile. The gradient elution program gave better peak shape and required resolution with a short run time. The fraction collector was set as time based from time 4.205 min for P-II and 8.42 min for P-I. The purity of fractions were analyzed by HPLC and found to be highly pure and identified by retention time with reference standard. The purity of Picroside I & Picroside II is 96.42% and 89% resp.

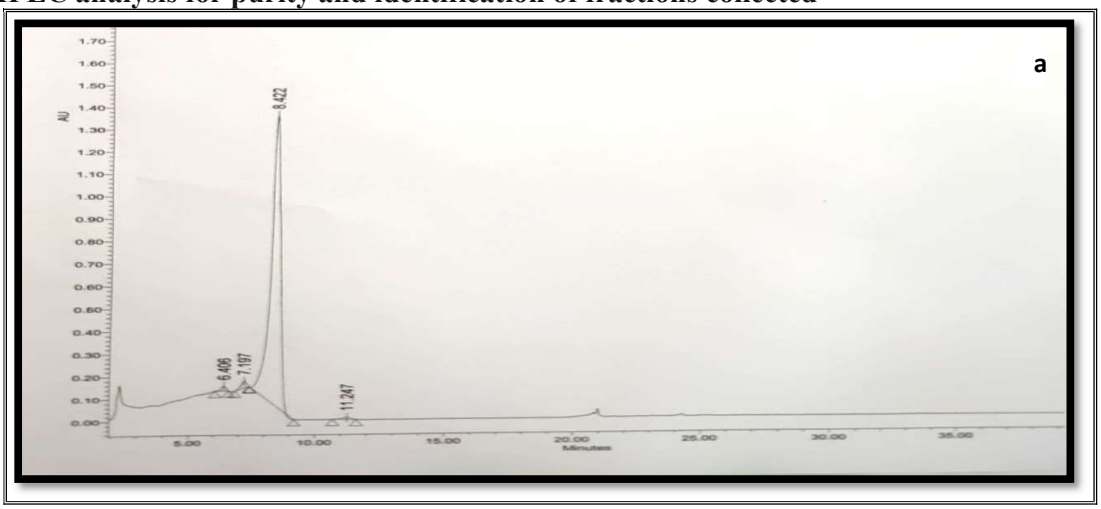
TLC Fingerprint and Co-Chromatography



a) Standard PI Standard PII Ethanolic ext **b) Ethanolic ext Standard PI PI/F-A PI/F-B PI/F-C** **c) Ethanolic ext Standard PII F5 F6 F7**

Figure 1: TLC fingerprint profile of *P. kurroa* Rhizomes under UV 254nm (a) Ethanolic extract of *Picrorrhiza kurroa* rhizome b) Picroside I c) Picroside II

HPLC analysis for purity and identification of fractions collected



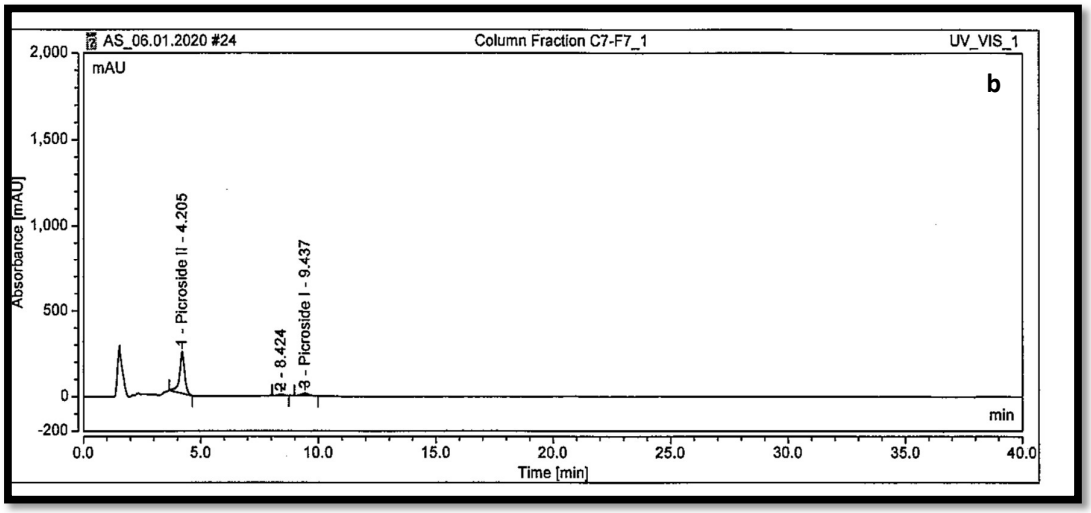


Figure 3: a) HPLC chromatogram of fraction collected from prep-RPHPLC between 8.422 min for PI
b) HPLC chromatogram of fraction collected from prep-RP-HPLC between 4.205 min for PII

Table 1: ^{13}C NMR data (500 MHz DMSO-D6 and 125 MHz) δ ppm spectral data for picroside-I and picroside-II

Position no	Interpreted value ^{13}C NMR P- I	Position no	Interpreted value ^{13}C NMR P- II
1	93.95	1	93.69
3	140.38	3	141.03
4	102.69	4	101.56
5	37.72	5	35.37
6	78.30	6	80.32
7	62.74	7	59.91
8	64.71	8	65.48
9	42.01	9	41.82
10	62.74	10	61.56
1’	98.39	1’	98.31
2’	74.51	2’	73.46
3’	76.08	3’	79.98
4’	70.15	4’	70.39
5’	76.08	5’	78.08
6’	64.71	6’	61.56
1’’	134.25	1’’	122.91
2”	130.24	2”	114.18

3”	128.69	3”	147.43
4”	134.25	4”	151.78
5”	128.69	5”	114.61
6”	130.24	6”	123.92
CO	166.92	CO	166.47
α	145.22	*α*	55.07
β	117.27	COCH3	-

3.3. Brine Shrimp Lethality bioassay

Table 2: The mean % mortality after 24 hours (Mean ± SEM) on treatment of absolute ethanol extract of Rhizomes of *Picrorhiza kurroa* & Bioactive compounds (Pk I & Pk II and 5-flurouracil on brine shrimp lethality bioassay.

Treatments	Dose µg/ml	Mean % Mortality after 24 hrs. (Mean ± SEM)	LC 50 (µg/ml)
Standard group: 5-Flurouracil	20	33.33 ± 3.33	158.76 µg/ml
	50	43.33 ± 3.33	
	100	53.33 ± 3.33	
	200	76.66 ± 8.819	
	500	83.33 ± 6.667	
Test group: Absolute ethanol extract of rhizomes of *Picrorhiza kurroa*	20	13.33 ± 3.33	288.83 µg/ml
	50	30.00 ± 5.774	
	100	36.66 ± 6.667	
	200	46.66 ± 3.33	
	500	70.00 ± 5.774	
Standard group: 5-Fluorouracil	5	13.33± 3.33	62.53 µg/ml
	25	33.33 ± 3.33	
	50	43.33 ± 3.33	
	75	56.66 ± 8.819	
	100	73.33 ± 3.33	
Test group (Picroside I)	5	6.66 ± 3.33	85.54 µg/ml
	25	23.33± 3.33	
	50	30±5.774	
	75	40.00 ± 5.774	
	100	60.00 ± 5.774	
Test group (Picroside II)	5	10.00 ± 5.774	73.32 µg/ml
	25	26.66 ± 6.667	
	50	33.33 ±8.819	
	75	50.00 ±5.774	
	100	66.66 ± 8.819	

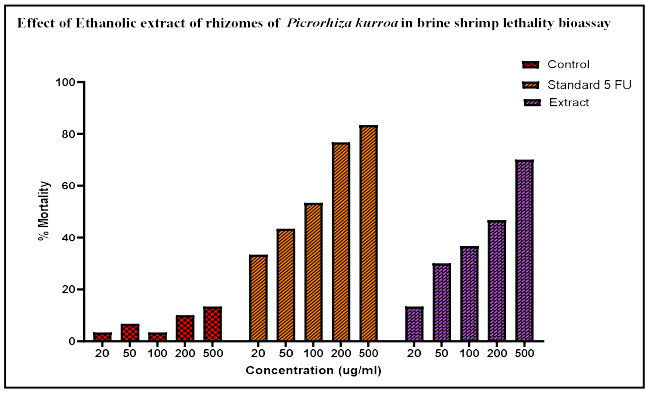


Figure 2: Effect of absolute ethanol extract of rhizomes of *Picrorhiza kurroa* brine shrimp lethality bioassay

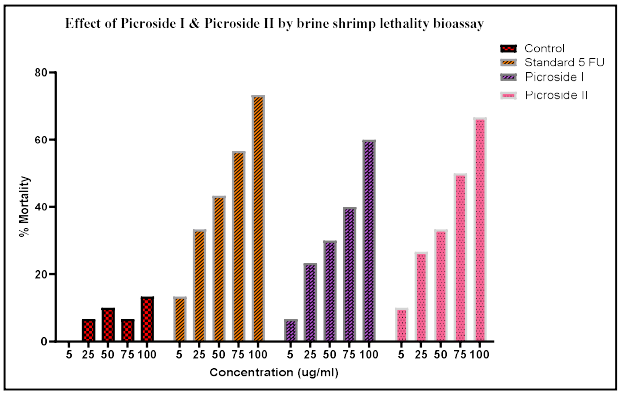


Figure 3: Effect of isolated bioactive compounds Picroside I &Picroside II from *Picrorhiza kurroa* brine shrimp lethality bioassay

3.4. Trypan blue dye cell exclusion assay

All the values are expressed Mean ± SEM and n=4, *P < 0.05, ****P<0.0001 using one way ANOVA coupled with "Dunnett t test", criterion for significance. ****P<0.0001 is considered as significant when standard group compared with control group and #### indicate test groups compared with control group in **Table 3**.

Table 3: In-vitro cytotoxic effect of Picroside I & Picroside II from rhizomes of *Picrorhiza kurroa* on COLO205cell line & HepG2 by trypan blue dye cell exclusion

Treatments	Dose µg/ml	% Mean cell viability (Mean ± SEM)	
		Cell lines	
		COLO 205	Hep G2
Control (DMSO)	0.2 % v/v	94.30 ± 0.5532	93.28 ± 0.6205
Standard group: 5 -Fluorouracil	10	56.85 ± 0.2650****	57.40 ± 0.03844****
	20	53.85 ± 0.3634****	52.04 ± 0.2983****
	40	50.66 ± 0.3402 ****	47.09 ± 0.4350 ****
	80	44.51± 0.4128****	41.32 ± 0.4328****
	160	33.42 ± 1.073 ****	37.63 ± 1.014 ****
Test group 1 (Picroside I)	10	90.91 ± 0.4070 ####	89.88 ± 0.2724####
	20	87.98 ± 0.3751####	85.97± 0.2709####
	40	81.28 ± 0.5588####	79.52 ± 0.7240 ####
	80	78.27 ± 0.4824 ####	73.74 ± 0.2849 ####
	160	71.60 ± 0.2689 ####	70.55 ± 0.4383####
Test group 2 (Picroside II)	10	88.83 ± 0.2030####	87.62 ±0.2397 ####
	20	83.89 ± 0.2583####	82.07 ± 0.5871 ####
	40	78.17 ± 0.0290####	75.66 ± 0.4446####
	80	71.65 ± 0.2651####	70.63 ± 0.3564####
	160	69.34 ± 0.1027 ####	68.70 ±0.2890####

Table 4: In-vitro cytotoxic effect of absolute ethanol extract of rhizomes of *Picrorhiza kurroa* on COLO205cell line & HepG2 by trypan blue dye cell exclusion

Treatments	Dose µg/ml	% Mean cell viability (Mean ± SEM)	
		Cell lines	
		COLO 205	Hep G2
Control (DMSO)	0.2 % v/v	92.57 ± 0.7965	92.85 ± 0.2633
Standard 5 - Fluorouracil	10	55.52 ± 0.6447 ****	52.78 ± 0.3415****
	100	44.11 ± 0.7255 ****	46.93 ± 0.3828 ****
	500	21.74 ± 0.3554****	19.26 ± 0.5507****
P.Kurroa Extract	10	71.90 ± 0.3374 ####	69.99 ± 0.2267 ####
	100	57.57 ± 0.4241####	60.78 ± 1.199 ####
	500	27.45 ± 0.6437 ####	24.26 ± 0.7085 ####

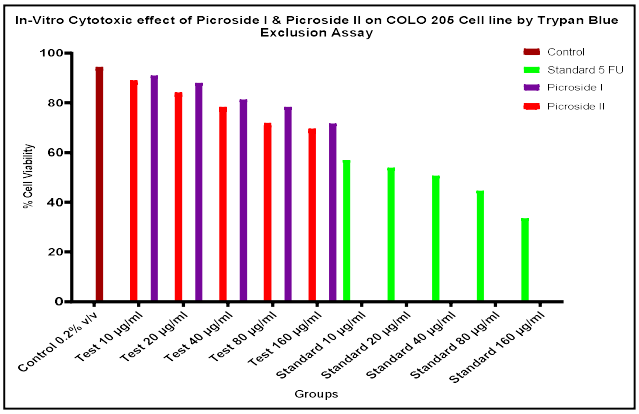


Figure 4 (a): Effect of Picroside I & Picroside II on COLO 205 cell line by Trypan blue dye cell exclusion assay

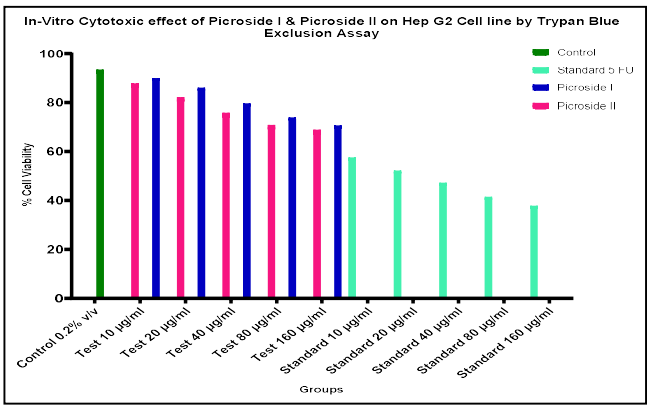


Figure 4. (b): Effect of Picroside I & Picroside II on Hep G2 cell line by Trypan blue dye cell exclusion assay

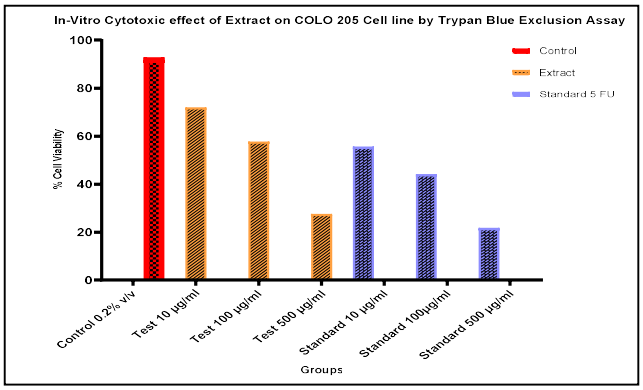


Figure 5 (a): Effect of ethanolic extract of rhizomes of *Picrorhiza Kurroa* on COLO 205 cell line by Trypan blue dye cell exclusion assay

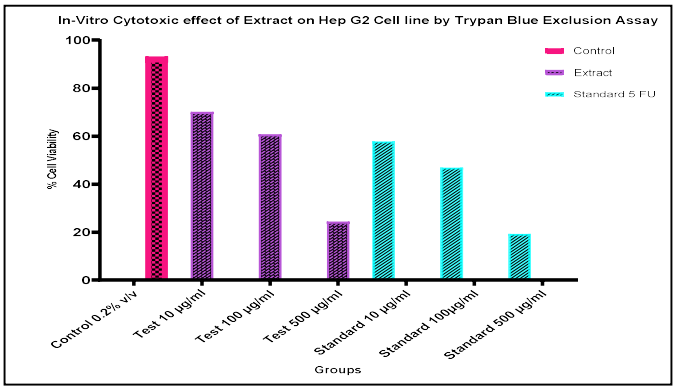


Figure 5(b): Effect of ethanolic extract of rhizomes of *Picrorhiza Kurroa* on Hep G2 cell line by Trypan blue dye cell exclusion assay

3.5. MTT assay:

Table 5: In-vitro cytotoxic effect of Picroside I & Picroside II from *Picrorhiza Kurroa* on COLO 205 & Hep G2 cell line by MTT assay method

Treatments	Dilutions µg/ml	% Inhibition			
		Cell lines			
		Colo 205	IC50 µg/ml	Hep G2	IC50 µg/ml
Test group 1: Picroside I	10	7.83	278.25 µg/ml	3.06	284.39 µg/ml
	20	12.32		6.38	
	40	17.68		15.48	
	80	21.06		21.1	
	160	29.07		26.43	
Test group 2: Picroside II	10	11.9	258.95 µg/ml	9.2	248.12 µg/ml
	20	16.7		14.5	
	40	23.8		21.13	
	80	26.77		24	
	160	30.56		32.01	
Standard group: 5-Fluorouracil	10	12.57	202.74 µg/ml	15.21	200.52 µg/ml
	20	19.23		20.05	
	40	25.85		27.78	
	80	29.85		29.75	
	160	37.76		38.51	

Table 6: In-vitro cytotoxic effect of ethanolic extract of rhizomes of *Picrorhiza Kurroa* on COLO 205 & Hep G2 cell line by MTT assay method

Treatments	Dilutions µg/ml	% Inhibition			
		Cell lines			
		Colo 205	IC50 µg/ml	Hep G2	IC50 µg/ml
Test group: *P.kurroa* Extract	10	24.8	331.78	30.32	322.04
	100	44.6		48.1	
	500	62.9		62.42	
Standard group: 5-Fluorouracil	10	28.12	319.67	29.46	307.34
	100	46.54		47.89	
	500	63.67		64.77	

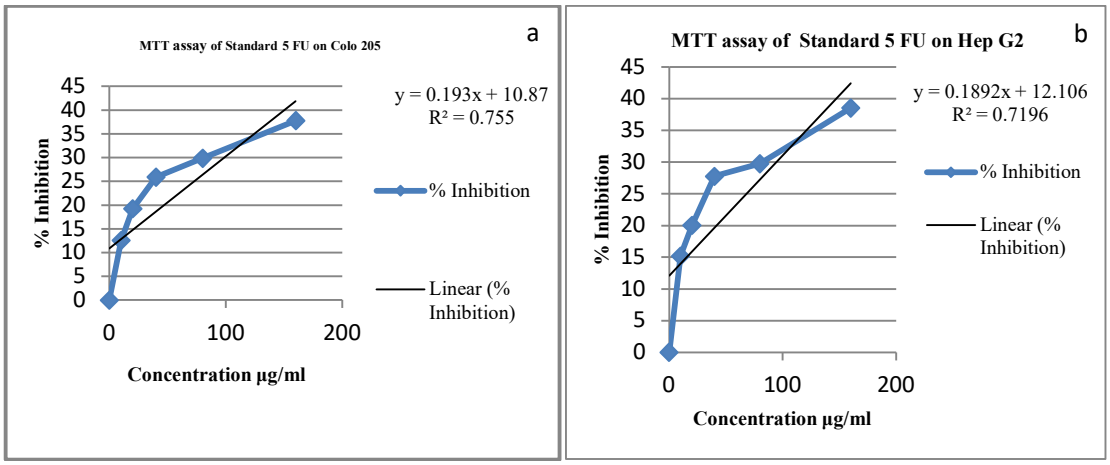


Figure 6: Calculation of IC_{50} by linear regression analysis of standard 5 fluorouracil at different concentrations of on both cell line by using MTT assay on a) Colo 205 and b) Hep G2 cell line

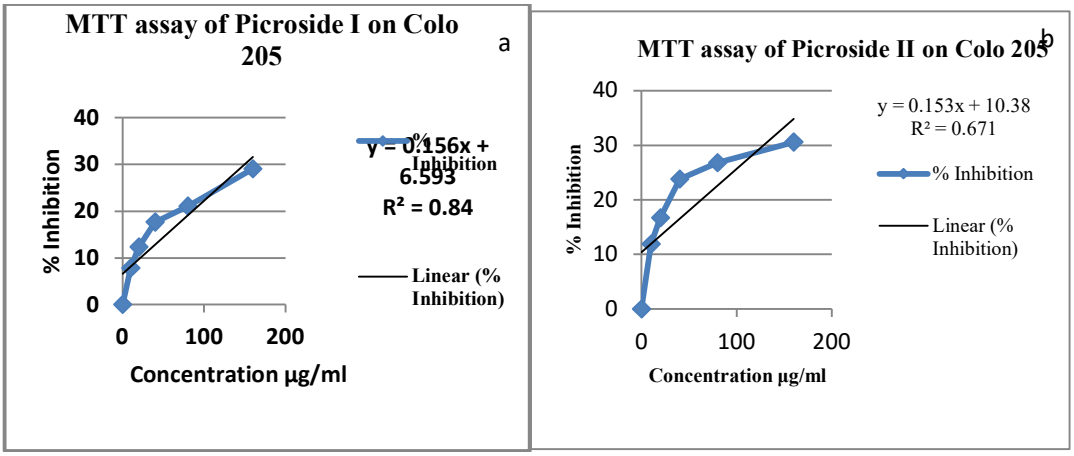


Figure 7: Calculation of IC_{50} by linear regression analysis of picrosides from *P. Kurroa* rhizomes on COLO 205 cell line by using MTT assay a) Picroside I and b) Picroside II

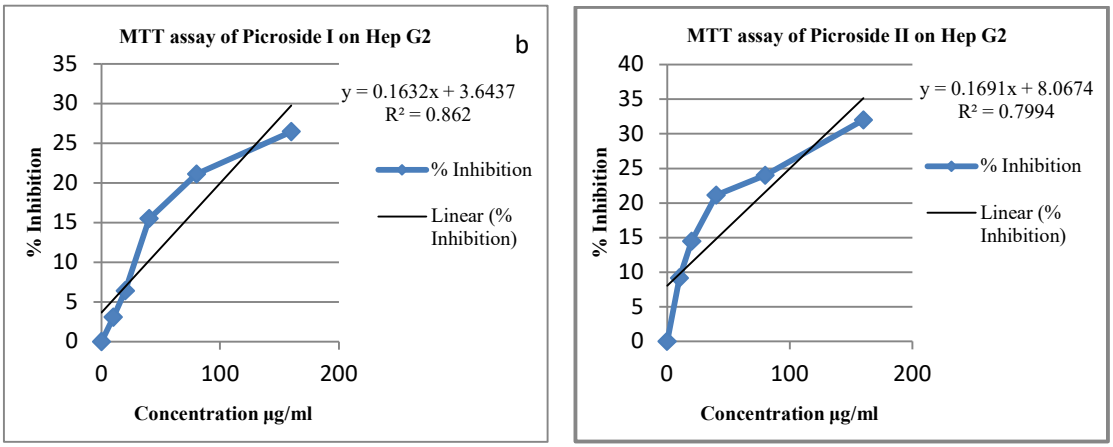


Figure 8: Calculation of IC_{50} by linear regression analysis of picrosides from *P. Kurroa* rhizomes on Hep G2 cell line by using MTT assay a) Picroside I and b) Picroside II

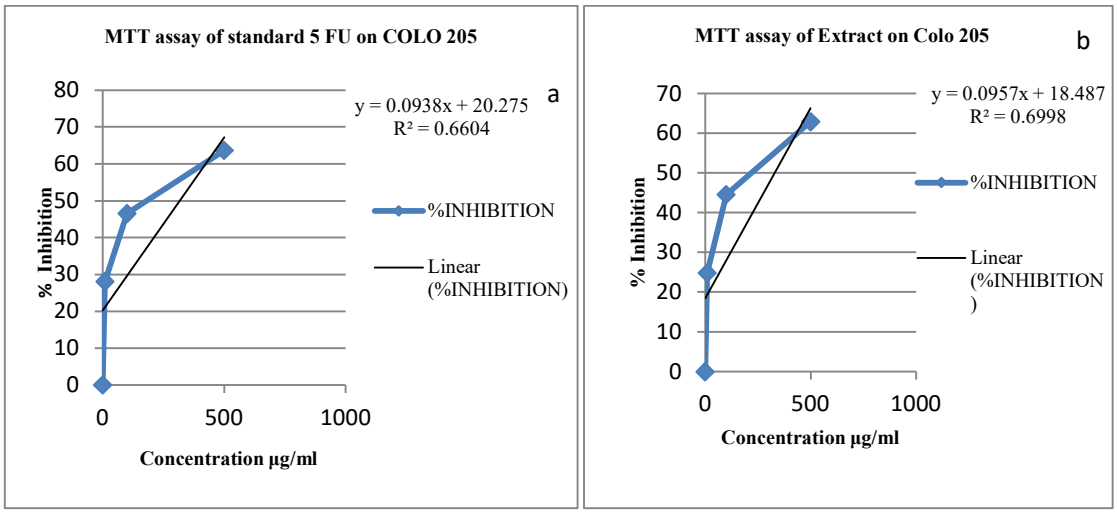


Figure 9: a) Calculation of IC_{50} by linear regression analysis of standard 5 fluorouracil at different concentrations on Colo 205 by using MTT assay b) Calculation of IC_{50} by linear regression analysis of *P. Kurroa* extract on Colo 205 cell line by using MTT assay

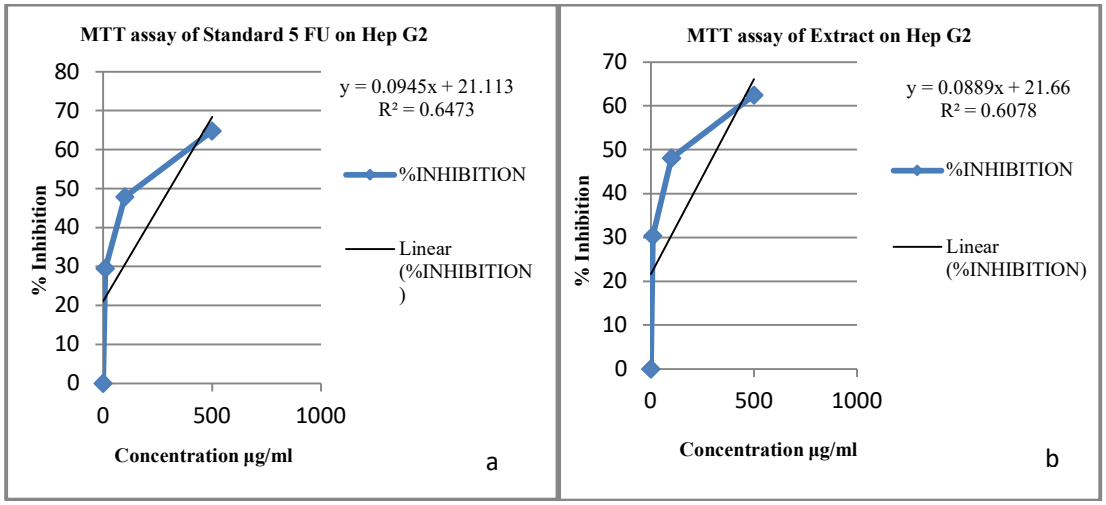


Figure 10 a) Calculation of IC_{50} by linear regression analysis of standard 5 fluorouracil at different concentrations on Hep G2 by using MTT assay b) Calculation of IC_{50} by linear regression analysis of *P. Kurroa* extract on Hep G2 cell line by using MTT assay

Table 7: In-vitro cytotoxic effect of Picroside I, Picroside II & absolute ethanol extract of rhizomes of *Picrorhiza kurroa* on L-929 cell line by MTT assay

Treatments	Dose µg/ml	% Mean cell viability (Mean ± SEM)
		L-929 Normal cell line
Standard 5 Fluorouracil	10	98.62 ± 0.2969
	20	96.90 ± 0.3230
	40	95.25 ± 0.06566
	80	94.73 ± 0.3040
	160	89.07 ± 0.4551
Picroside I	10	96.92 ± 0.1833
	20	96.33 ± 0.03333
	40	89.53 ± 0.08819
	80	83.20 ± 0.05774
	160	75.23 ± 0.1764
Picroside II	10	96.90 ± 0.3512
	20	95.27 ± 0.08819
	40	94.39 ± 0.1498
	80	89.11 ± 04271
	160	86.43 ± 0.2404
P.kurroa Extract	10	95.33 ± 01453
	20	94.20 ± 0.05774
	40	93.34 ± 0.1835
	80	93.43 ± 0.1914
	160	85.13 ± 0.03333

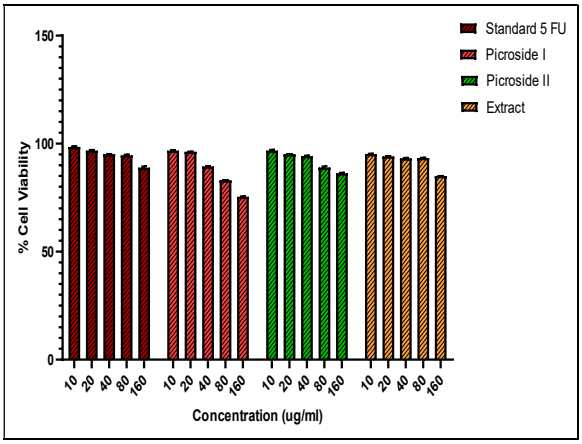


Figure 11: Cytotoxic effect of Picroside I & Picroside II & absolute ethanol extract of rhizomes of *Picrorhiza Kurroa* on L-929 cell line by MTT assay

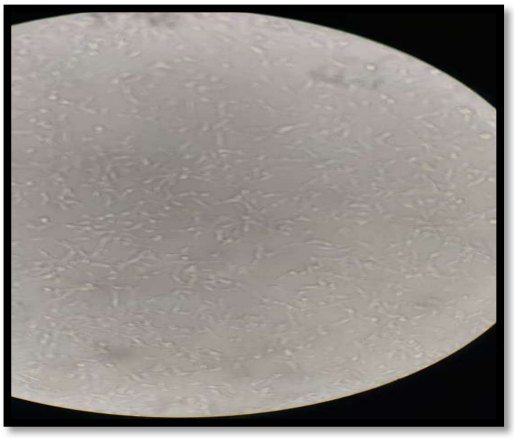


Figure 12: Identification of viability Normal cell line by MTT assay

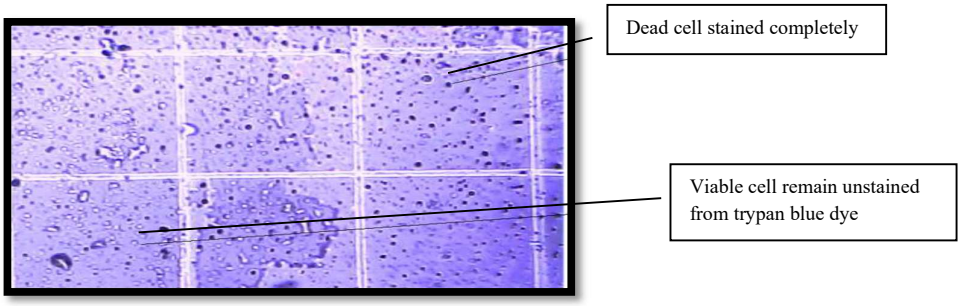


Figure 13: Identification of viable and dead cell in Trypan blue dye cell exclusion assay: Unstained cells observed in figure indicated viable cells, while completely blue stained cells counted as dead cells

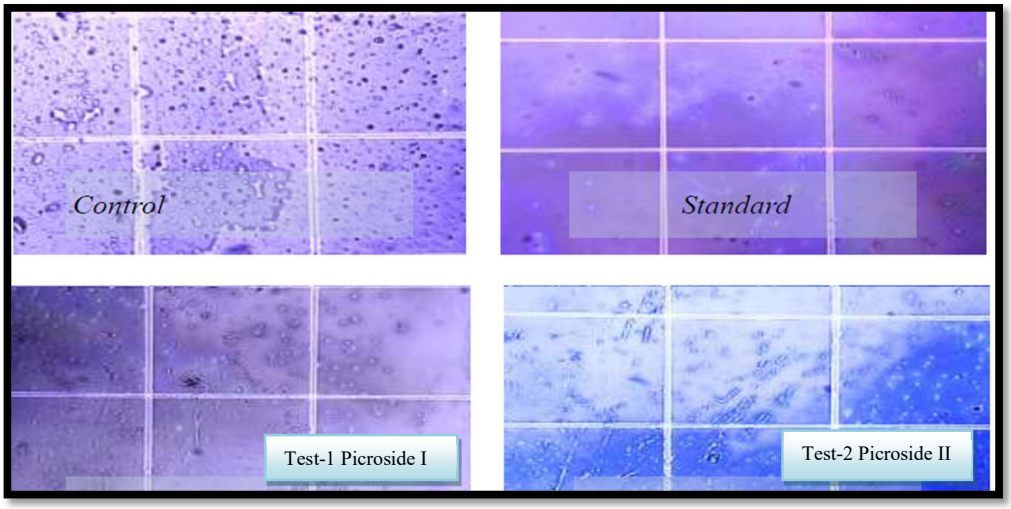


Figure 14: Effect of Picroside I & Picroside II extract on Hep G2 cell line by trypan blue dye cell exclusion assay: Control group showed number of viable cells, while in standard group lesser viable cells observed as compared to all other groups. Test-1 group of Picroside I showed less dead cells while test-2 Picroside II group showed more no. of dead cells as compared with Picroside I

4. DISCUSSION:

Preliminary phytochemical investigation of ethanolic extract of *P. kurroa* indicate presence of alkaloids, flavonoids, glycosides, protein, resin, saponin, sterol and **[19]**. Recently it has reported that the active principles of Kutkin (picroside I and picroside II) are also present in aerial part of the plant. Till date, only roots and rhizomes were investigated for their activity **[20]**. Therefore, *P. kurroa* was selected for the evaluation of anticancer activity based on its reported phytoconstituents on human cancer cell lines by in-vitro models in present research work. *P. kurroa* extract have antioxidant potential that help protect cells from the oxidative stress and oxygen free radicals **[21]**. The previous research study, has been reported that; the identified the chemical constituents of 70% hydro alcoholic fraction of *Picrorhiza Kurroa* by LC–ESI–MS/MS which showed the presence of iridoid glycosides such as picroside I, picroside II, picroside III, picroside IV, kutkoside, pikuroside and flavonoids like apocynin and vanillic acid. *P. kurroa* exhibited DPPH radical scavenging and metal chelating activities **[22]**.

Fewer studies have also been reported on the anticarcinogenic activity of *P.Kurroa* rhizomes extract in cell lines in vitro. Initial antioxidant and anti-neoplastic activity of methanolic and aqueous extract of *P. kurroa* is reported in human breast carcinoma (MDA-MB-435S), human hepatocellular carcinoma (Hep3B), human breast cancer (MCF-7) and human prostate cancer (PC-3) **[23]**. Picrosides have shown different anticancer activities like free radical scavenging activity, metal ion chelator, detoxifying activity, cell cycle regulation and apoptotic induction. Detailed mechanism for anti-cancer potential of picrosides is yet to be elucidated in different cells as it acts on different molecular target **[22]**.

In the present study, the ethanolic extract of *P.kurroa* and its isolated iridoid glycosides viz. Picroside I and Picroside II were found significantly cytotoxic effect in Brine shrimp lethality bioassay. Cytotoxicity activity experiment was carried out on the polar fractions of *Cochlospermum tinctorium* using the Brine shrimp lethality bioassay method by Akpemi Audu Musa, in 2012, which reported that LC_{50} values of the extracts were determined by linear regression analysis method. This research findings were supported to present study **[24]**. In our study, observed that LC_{50} value of ethanolic extract from rhizomes of *P.kurroa* was **288.83 µg/ml** in Brine shrimp bioassay and it would act as cytotoxic agent. According to Clarkson's index, observed LC50 value (**288.83 µg/ml)** classified in medium toxic class, based on the LC50 value determined from Brine shrimp

lethality bioassay extract that are cytotoxic and effective anticancer agent. In the other hand, studies have showed that the isolated compounds Picroside I and Picroside II of *P.kurroa* exhibited LC_{50} values of **85.54 µg/ml** and **73.32 µg/ml** respectively. According to Clarkson's index, observed LC_{50} value (**85.54 µg/ml and 73.32 µg/ml)** respectively classified in highly toxic class **[25]**. According to Clarkson's index, observed LC_{50} value of ethanolic extract of the Rhizomes of *P.kurroa* classified in medium toxic class as compared to its isolated compounds was highly toxic. The Picroside II was Slightly toxic as compared to Picroside I according to Meyer's toxicity index for BSLA **[26]**. To correlate the cytotoxicity of the *P.kurroa* rhizome extract and its isolated iridoid glycosides like Picroside I and Picroside II to human cancer cells (Hep G2 and COLO 205) by trypan blue exclusion assay and MTT assay.In vitro method Trypan blue dye cell exclusion assay based on cell membrane integrity and enables visual dysfunction of viable and non-viable cells, since it stains the ones with damaged membranes, generally preceding death. Percentage mortality of cancer cell lines was showed that at dose level 500µg/ml of ethanolic extract of the Rhizomes of *P.kurroa is* Viz COLO 205 (72.55%) and HepG2 (75.74%) when compared with control (7.43% and 7.15 %) respectively. On cell lines Hep G-2 showed more percentage of cytotoxicity activity at dose **500µg/ml** while on COLO 205 showed less percentage cytotoxicity activity at dose 500µg/ml as compared to 10µg/ml and 100µg/ml dose levels.

In different test dilutions of Picroside I and Picroside II, at dose level **160µg/ml** showed that maximum number of percentage mortality of cancer cell lines Viz. COLO 205 (28.4% and 30.66%) respectively and HepG2 (29.45 % and 31.3 %) when compared to the control group 5.7% and 6.72% respectively. Thus Picroside I and Picroside II exhibited considerable cytotoxic potential in a dose-dependent manner. The percentage mortality of ethanolic extract *P.kurroa* on cancer cell lines showed that higher percentage mortality at higher dose as compared to its isolated compound (Picroside I and Picroside II).It was observed that on both cell lines Picroside I was produced less Percentage mortality as compared to Picroside II. The percentage mortality of ethanolic extract *P.kurroa* on cancer cell lines showed that higher Percentage mortality at higher dose **500µg/ml** as compared to its isolated compound showed higher percentage mortality lower dose at **160µg/ml.** Results showed that the pure compounds exhibits significant cytotoxicity as compared to the extract *P.kurroa.*

In study, the cytotoxic activity of ethanol extract of rhizomes of *P.kurroa* and its isolated compounds Picroside I and Picroside II were also determined by using MTT assay. In this study, ethanolic extract of *P.kurroa* and its isolated compounds Picroside I and Picroside II showed concentration dependant cytotoxicity on two cell lines (COLO 205 and Hep G2). The ethanolic extract of *P.kurroa* on COLO205 & HepG2 cell line was found that at the dose level **500 µg/ml** maximum % inhibition were observed i.e. 62.9% and 62.42 % resp. While the IC_{50} was found by using linear regression analysis IC_{50} value for standard on COLO205 and HepG2 cell line was 319.67 µg/ml and 307.34 µg/ml respectively and for test on COLO205 and HepG2 cell line was 331.78 µg/ml and 322.04 µg/ml respectively. The maximal inhibitory concentration of ethanolic extract *P.kurroa* on cancer cell line Hep G2 showed a significant inhibitory effect as compared with COLO205.

In different test dilutions of Picroside I and Picroside II, at dose level 160µg/ml have shown maximum % inhibition on cancer cell lines were observed Viz COLO 205 (29.07 % and 30.56% respectively) and HepG2 (i.e.26.43% and 32.01% respectively). While the IC_{50} was found by using linear regression analysis IC_{50} value for standard on COLO205(202.74 µg/ml) and HepG2 (200.52µg/ml) and for test on COLO205(278.25 µg/ml and 258.55 µg/ml respectively) and HepG2 (284.39 µg/ml and 248.12 µg/ml respectively). The maximal inhibitory concentration of Picroside II on cancer cell lines exhibited the significant Percentage inhibitory concentration as compared to Picroside I. The inhibitory concentration of ethanolic extract of *P.kurroa* it produces maximum inhibitory action at higher dose level as compared to it's isolated compounds i.e. Picroside I & Picroside II observes that even at low dose level it produces inhibitory effect . Among the compounds Picroside II Showed a significant inhibitory activity on both cell lines at a concentration **160µg/ml**. In addition, ethanolic extract of *P.kurroa* good cytotoxicity on both cell lines at the concentration **500 µg/ml.**In the present study, the cell viability of the **Normal cell line-L-929** was also assessed by using MTT assay. In different test dilutions of ethanolic extract of *P.kurroa* and its isolated compounds Picroside I &Picroside II, at dose level 160µg/ml showed that maximum percentage viable normal cell lines were observed Viz. extract of *P.kurroa (*85.13 %), Picroside I (75.23%) and Picroside II (86.43%).On the Normal cell line effect of ethanolic extract of *P.kurroa* and its isolated compounds Picroside I & Picroside II exhibited slight toxic effect as compared to cancer cell line. *P.kurroa* and its isolated

compounds Picroside I & Picroside II less toxicity to normal tissues has been suggested as possible candidates for their capability to improve the efficacy of anticancer drugs. These results suggested that ethanolic extract of *P.kurroa* bear anti-neoplastic activity that may have prospective clinical use as precursor for preventive medicine. Further studies on the isolation of the constituent compounds are in prospect.

5. CONCLUSION:

Ethanolic extract of rhizomes of *Picrorhiza Kurroa* Royle ex Benth showed significant cytotoxic activity on invertebrates (Brine shrimp), animal cancer cell line and human cancer cell lines in MTT assay method and trypan blue dye cell exclusion assay. *P.Kurroa* extract, Picroside I and Picroside II are the major players of the inhibitory potential. antimetastatic nature of *P.Kurroa* extract, Picroside I and Picroside II prospecting these candidates for developing into potential anticancer therapeutics and also have potential avenues for targeting the spread of cancer.Further studies are warranted to decipher the probable mechanism by which absolute ethanol extract of rhizomes of plant *Picrorhiza Kurroa* Royle ex Benth exert anticancer effect.

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