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ORIGINAL ARTICLE

Picroside I and Picroside II from Tissue Cultures of *Picrorhiza kurroa*

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ABSTRACT

Background: *Picrorhiza kurroa* (PK) belongs to *Scrophulariaceae* family and is a representative endemic, medicinal herb, widely distributed throughout the higher altitudes of alpine Himalayas from west to east, between 3000 and 4500 m above mean sea level. **Objective:** The objective of the present study is to assess the production of picroside I and picroside II from tissue cultures of PK. **Materials and Methods:** Auxiliary shoot tips of PK were incubated in Murashige and Skoog medium supplemented with indole-3-butyric acid and kinetin phytohormones. The callus produced was collected at different time intervals and was processed for extraction of picroside I and picroside II followed by thin layer chromatography and high-performance liquid chromatography HPLC analysis. **Results:** The maximum growth index was found to be 5.109 ± 0.159 at 16-week-old callus culture. The estimation of picroside-I and picroside-II was carried out by (HPLC) analysis; quantity of secondary metabolite found to be 16.37 ± 0.0007 mg/g for PK-I and 6.34 ± 0.0012 mg/g for PK-II. **Conclusion:** This is the first attempt to produce the Picroside-I and II in large amount by the tissue culture technique. It can be observed that the method of callus culture can be used in production of secondary metabolites Picroside-I and II from PK

Key words: *Picrorhiza kurroa,* picroside I, picroside II, tissue cultures

SUMMARY

- *Picrorhiza kurroa* is a high value medicinal herb due to rich source of hepatoprotective metabolites,Picroside-I and Picroside-II.The medicinal importance of P. *kurroa* is due to its pharmacological properties like hepatoprotective, antioxidant (particularly in liver), antiallergic and antiasthamatic, anticancer activity particularly in liver and immunomodulatory. Shoot apices which were produced a good response was inoculated on selected medium i.e., on MS medium containing 2, 4 D (mg/l) + KN (1mg/l) for induction of callus. The initiation of callus was observed after 4weeks and it was light green and fragile Maximum growth was observed with 3% w/v of sucrose supplement. The callus culture was maintained and growth index was recorded after every subculture. The growth index was calculated from the obtained final dried weight divided by initial weight.

Plant-P.kurroa ⇨ Shootapex ⇨ Surface sterilisation of shoot

apex grows on M.S.Media ⇨ Multiple Shoot formation ⇨

These leaf disc,stemsegments grows on M.S.Media ⇨

Callus Initation ⇨ HPLC Analysis.

Abbreviations Used: PK-Picrorhizakurroa, IBA-Indole-3-butyricacid, KN-Kinetin, 2,4D-2,4Dichlorophenoxy acetic acid.

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INTRODUCTION

A number of plants are described in Ayurveda for the use in the treatment of liver disorders, and two species *Picrorhiza kurroa* (PK) Royle ex Benth and *Picrorhiza scrophulariiflora* Pennell of genus *Picrorhiza* are used extensively in traditional medicine.

PK is belonging to family *Scrophulariaceae*, is a representative endemic, medicinal herb, widely distributed throughout the higher altitudes of alpine Himalayas from west to east, between 3000 and 4500 m above mean sea level. The plant is commonly known as "kutki" or "katuka." PK, a well-known medicinal herb in Ayurvedic system of medicine, has been used traditionally in India and other Asian countries to treat disorders of liver and upper respiratory tract infections, reduce fever (pyrexia), dyspepsia, chronic diarrhea, scorpion sting, stomachic, cathartic, cholagogue, and anemia.[1,2] The root has bitter taste, cooling, cardiotonic, antipyretic, anthelmintic, laxative, and appetizer properties.[3] It is also useful in biliousness, bilious fever, urinary discharge, and asthma.[4] Several research groups have investigated the pharmacological actions of the species and reported that various activities exhibited by the plant such as hepatoprotective, antioxidant, antiallergic, antiasthma, anticancer, immunomodulatory, anti-inflammatory, hypolipidemic, immunostimulant, antileishmanial, anticholestatic, antiulcerogenic, antidiabetic, anthelmintic, antimalarial, cardioprotective, antiviral, and nitric oxide scavenging activities. It is the principle source of glycosides that is picroside-I, picroside-II, and kutkoside. Other identified active constituents are apocynin, drosin, and nine cucurbitacin glycosides.[5] The active constituents are obtained from shoots, roots, and rhizomes of the plant. PK is a high-value medicinal herb due to rich source of hepatoprotective metabolites, picroside-I and picroside-II and other metabolites such as picroside-III, picroside-IV, apocynin, androsin, catechol, and kutkoside. Other constituents are obtained from the dried root and rhizome.[6,7] The medicinal importance of PK is due to its pharmacological properties such as hepatoprotective,[8] antioxidant (particularly in liver),[9] antiallergic and antiasthmatic,[10] anticancer activity particularly in liver,[11] and immunomodulatory.[12] A

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hepatoprotective drug formulation, picroliv, has been prepared from the extracts of PK. Picroside-I is the major ingredient of picroliv therefore makes this compound a highly valued secondary metabolite of PK.[13,14] The PK plants have been recklessly collected from its natural habitat, thereby reducing its populations and putting it under the category of endangered plant species. In this regard, plant propagation through tissue culture has been evolved as a valuable method compared to conventional plant multiplication in the last few decades. Several studies based on micropropagation, synthetic seed production, and adventitious shoot regeneration of PK have been reported.[15,16] Therefore, the present study was carried out with the aim of production of from tissue culture of PK.

MATERIALS AND METHODS

Plant material

The PK plant was procured from the Himalayan Forest Research Institute, Manali, H. P, India and authenticated by a Taxonomist (Prof. V. S. Raju) of Kakatiya University, Warangal, Telangana.

Chemicals and compounds

Murashige and Skoog's (1962) (MS) media was purchased from HiMedia Pvt. Ltd., India. The chemicals acetonitrile and methanol high-performance liquid chromatography (HPLC) grade used for the analysis were purchased from Merck, India. Standards of picroside-I and picroside-II were purchased from Sigma-Aldrich, Mumbai, India.

Establishment of axenic cultures

Auxiliary shoot tips (0.5–1.5 cm long) excised from pot grown plants were washed in running tap water for 2 min and surface sterilized for 5–7 min with Bavistin (0.5%) (BASF, India, Ltd) and then washed with Tween 20 (10%) for 2–3 min. The excess surfactant solution was removed by washing with sterile distilled water. The shoot apices were immersed in ethyl alcohol (70%) for 30 sec and then immersed in sodium hypochlorite chloride (20%+Two Drops Tween20) (Merck, India) for 2–3 min followed by 4–5 times washings in sterile double distilled water. The disinfected shoot apices were cultured on MS medium supplemented with growth hormones (indole-3-butyric acid [IBA] and kinetin [KN]).

Preparation of media and incubation of cultures

MS media (1962) supplemented with concentrations of 2 mg/l IBA, 3 mg/l KN were prepared, pH adjusted to 5.8 using 0.1N HCl or 0.1N NaOH and solidified with agar–agar (Glaxo, Bacteriological Media) 0.8%w/v as a gelling agent. The media was autoclaved at 121°C and 15 lb in^{-2} pressure for 15–20 min. The cultures were incubated at 25 ± 2°C in plant tissue culture chamber with 70% relative humidity under 8 h dark/16 h photoperiod. The cultures were subcultured after every 4 weeks.

Establishment of callus cultures

Leaf discs and stem segments were excised from 4 to 5 weeks old *in vitro* growing plantlets of PK and cultured on callus induction medium for their dedifferentiation into callus cultures. The medium consisted of MS supplemented with 2, 4 dichlorophenoxyacetic acid (2, 4-D) (2 mg/l), KN (1 mg/l), sucrose 3% (w/v), and solidified with agar (0.8%) for their dedifferentiation into callus cultures. The medium was poured into 150 ml Erlenmeyer flasks (Borosil, India) by dispensing 40 ml media in each flask and inoculated with 4–5 explants in a flask. The cultures were incubated at 8 h dark/16 h photoperiod at 25 ± 2°C in a plant tissue culture chamber. Three to four weeks after inoculation, the explants were completely transformed into callus mass. The cultures were subcultured after every 15–20 days on callus induction media for 2 months to obtain good growth. The explants were observed for the development of callus and the callus was initiated after 6 weeks. The callus was subcultured in the same medium after every 4 weeks.[17]

Extraction of *Picroside-I and Picroside-II*

The callus was collected at time intervals of 4, 8, 12, 16, 20, and 24 weeks. It was dried, weighed, made into small pieces, and refluxed twice with methanol (70% v/v) for 20 min. The supernatant liquid was separated and evaporated at room temperature. The dried extract was dissolved in methanol and carried out the thin layer chromatography (TLC) and HPLC analysis to find the active constituents present in extract.

Determination of *Picroside-I and Picroside-II*

Thin layer chromatography analysis

The methanolic extract was analyzed by using precoated silica gel G plates of 250 μm layer, UV 254 (Merck) by cochromatography with authentic samples of obtained from Sigma Aldrich, USA. The TLC analysis was carried out by trial and error method and detected the spot using Klenzaids Bioclean Devices, UV chamber.[18]

Preparation of standard solutions for high-performance liquid chromatography analysis

Stock solution of picroside-I and II (1.0 mg/ml) was prepared in methanol and diluted to a series of appropriate concentrations (15.625, 31.25, 62. 5, 125, 250, 500, and 1000 μg/ml) of picroside-I and II with the same solvent. An aliquot (20 μl) of the diluted prepared standard solutions was injected into the HPLC for the construction of six-point calibration curve.

Preparation of sample solutions

The dried extract (100 mg) was dissolved in methanol. The methanolic extracts were filtered and dried under reduced pressure at 50 ± 5°C. The dried filtrate (10 mg) was redissolved in mobile phase (1 ml), filtered through 0.45 μm membrane, and an aliquot (20 μl) of the filtrate was injected into HPLC for analysis.

High Performance Liquid Chromatogram analysis

HPLC analysis was performed on a Shimadzu Ultra-Fast Liquid Chromatography (UFLC) system equipped with binary gradient pump, LC-20AD UV/Visible detector. The chromatographic resolution was achieved on a Luna C-18 (2) column (250 × 4.6 mm, 5 μm particle size) from phenomenex. The mobile phase used for the analysis was water: acetonitrile (75:25) in isocratic elution with a flow rate of 1 ml/min at 270 nm detection wavelength.[19]

RESULTS

Establishment of multiple shoots

Modified method of Sood and Chauhan[17] was used for shoot culture development of PK, and proliferation into multiple shoots was observed within five to 6 days of inoculation. These were maintained at 8 h dark/16 h photoperiod and 25 ± 2°C in a plant tissue culture chamber followed by subculture after every 4 weeks of shoot proliferation. Finally, it formed multiple shoots in 2 months with good growth as shown in Figure 1.

Callus initiation and maintenance

Shoot apices which were produced a good response were inoculated on selected medium, i.e., on MS medium containing

2, 4 D (mg/l) + KN (1 mg/l) for induction of callus. The initiation of callus was observed after 4 weeks and it was light green and fragile [Figure 2]. Maximum growth was observed with 3% w/v of sucrose supplement. The callus culture was maintained and growth index was recorded after every subculture. The growth index was calculated from the obtained final dried weight divided by initial weight and was tabulated in Table 1.

Thin layer chromatography and HPLC analysis of *Picroside-I and Picroside-II*

To identify the production of picroside I and II in callus cultures, qualitative analysis with TLC was done and the Rf values of Picroside-I and II was found to be 0.44 and 0.38, respectively, in chloroform: methanol: formic acid (7:2:1) mobile phase.

The HPLC analysis gave the same retention time as the standard for a presumptive Picroside-I and Picroside-II as shown in chromatogram [Figure 3a and b], i.e., 10.14 and 5.6 min for Picroside-I and Picroside-II, respectively. The kinetic pattern for production of Picroside-I and Picroside-II was shown in Table 1 and Figure 4. The measurements from three independent cultures were made at each time point. The maximum value was observed as 16.37 ± 0.0007 mg/g for Picroside-I and 6.34 ± 0.0012 mg/g for Picroside-II after 16 weeks.

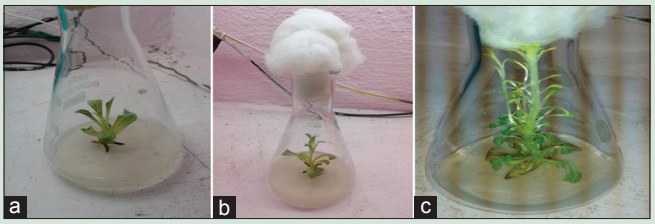

Figure 1: (a) Shoot Apex, (b) Shoot proliferation, (c) Multiple Shoot Formation

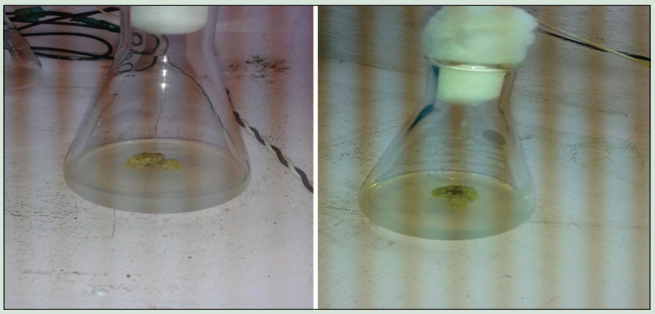

Figure 2: Callus initiation and maintenance

DISCUSSION

A good response was observed on shoot apices inoculated on selected medium. MS medium containing 2, 4 D (2 mg/l) + KN (1 mg/l) with addition of 3% w/v of sucrose found to be more suitable for the induction of callus from shoot apices. In this, medium callus was initiated and observed after 4 weeks. The callus was light green and fragile. Maximum growth was observed when supplemented with 3% w/v of sucrose. However, callus growth as unaffected in MS supplemented with 1%, 2%, and 4% of w/v of sucrose; this is in agreement with previously reported methods.[18,20]

Growth efficiency of callus is measured as described by Loyola-Vargas *et al*. (2006) that at a given sampling time, both fresh and dry weights are measurements of complete biomasses of the tissues. No reference to the actual growth capacity is taken into consideration. Growth index is a relative estimation of such capacity as it correlates the

Table 1: Growth index of callus and Production Kinetics of Picroside I and II

Age weeks	Growth index Mean±SD	Picroside I (%w/w)	Picroside II (%w/w)
4	1.923±0.108	00.84±0.0001	0.393±0.001
8	2.378±0.109	3.38±0.0008	0.43±0.001
12	2.804±0.081	5.15±0.0015	4.83±0.0006
16	5.109±0.159	16.37±0.0007	6.34±0.0012
20	3.566±0.107	9.91±0.0004	4.54±0.0012
24	1.437±0.150	2.06±0.0008	3.61±0.0012

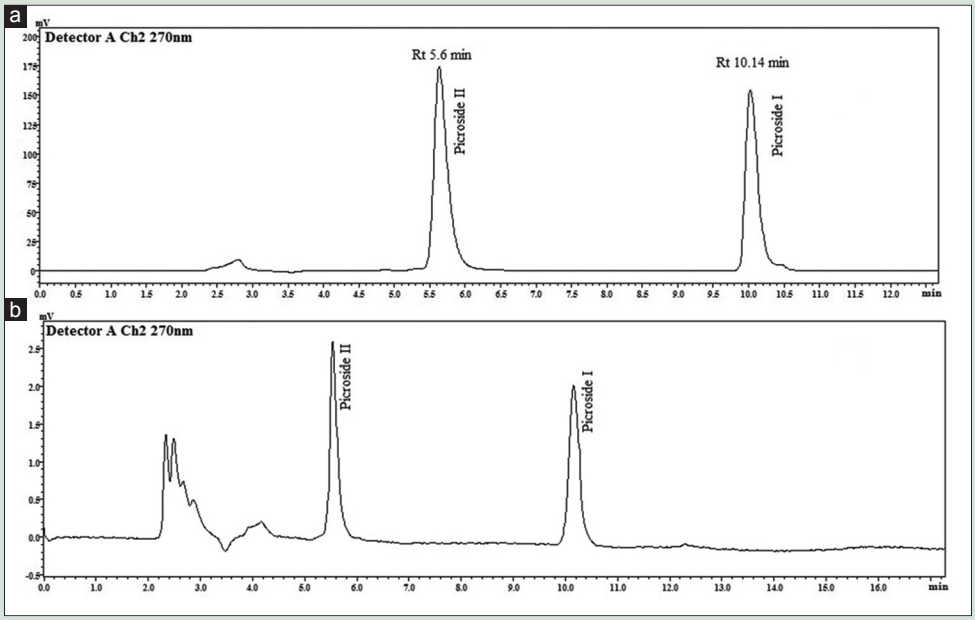

Figure 3: (a) Standard chromatogram of Picroside I and Picroside II, (b) Chromatogram showing *Picroside-I and Picroside-II* in extracts of callus culture

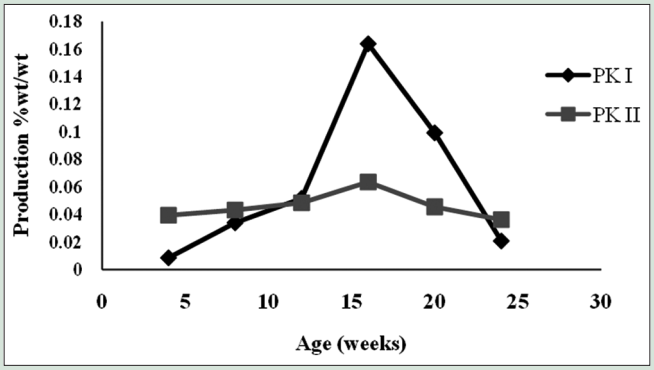

Figure 4: Production kinetics of *Picroside-I and Picroside-II*

biomass data at the sampling time to that of the initial condition. It is calculated as the ratio of the accumulated and the initial biomass. The accumulated biomass corresponds to the difference between the final and the initial masses.[21] In the present study, the callus cultures were maintained and growth index was calculated after every subculture. The maximum growth index was found 5.109 ± 0.159 at 16-week-old callus culture.

The production of picroside-I and picroside-II from callus cultures was confirmed by TLC and HPLC analyses. TLC analysis was carried out using solvent system (chloroform: methanol: formic acid) gave one spot when derivatized with UV light each for the Picroside-I and II with two different R_f value. Their R_f values were same as with that of standard samples.

Under optimum conditions, callus culture was analyzed by using RP-HPLC at 270 nm. Two compounds (Picroside-I and Picroside-II) were identified from the callus extract by matching their retention times against those of the standards. Peak assignment was confirmed by injection of standards. These measurements taken together provide strong evidence for quantifying the production of Picroside-I and Picroside-II from callus culture. The maximum growth production in callus culture was observed to be 16.37 mg/g for Picroside-I and 6.34 mg/g for Picroside-II after 16 weeks. Hence, there is an age correlation between production of secondary metabolites and growth curve. Several studies reveal that maximum production of secondary products is achieved during the active growing stage of the cells.[22,23]

So far, there are no reports on the tissue cultures of PK on the production of Picroside-I and Picroside-II. This is the first report to produce the Picroside-I and Picroside-II in large amount by the tissue cultures of PK. As described by Lima *et al.*, natural resources have been extensively exploited for medicinal purposes and a great number of plants have been employed in folk medicine although the active principles are found in very low quantity in isolated substances concentrations.[24] In this case, plant cell culture was the most efficient technique used to obtain higher amounts of secondary metabolites, i.e., Picroside-I and Picroside-II.

CONCLUSION

This is the first attempt to produce the Picroside-I and II in large amount by the tissue culture technique. It can be observed that the method of callus culture can be used in production of secondary metabolites Picroside-I and II from *P.kurroa*.

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Conflicts of interest

There are no conflicts of interest.

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