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Metabolite Profiling of *In vitro* Established Cultures of Picrorhiza Kurroa Royle ex Benth in Different Growth Regime (s)

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ABSTRACT

Picrorhiza Kurroa Royle ex Benth, a Scrophulariaceae member, is an endangered species according to Red Data Book and highly demanded medicinal herb due to established hepatoprotective function. Several monoterpenoid glycosides are present which are known as picroside I, picroside II, picroside III, more recently picroside IV and V are being identified specially for hepatomodulation in model laboratory animals and human.The plant has been over consumed for its valuable terpene glycosides. Biotechnological approaches through in vitro establishment of the important germplasm of Picrorhiza Kurroa Royle ex Benth at germplasm evaluation facility from suitable stage of its growth and development of leaf explants which in turn shall allow for the defined conditions for in vitro establishment under micro environment to look upon the production of picrosides in vitro. Present research work is an attempt toward quantification of Picroside(s) under regimes of various kind of phytoregulator(s) selected on the basis of growth behavior of callus. Callus cultures were established in vitro using leaves as an explant. TDZ (0.75 mg/L) + IBA (0.75 mg/L) was found better for callusing while BAP (0.50 mg/L) + Kinetin (0.75 mg/L) was found the best for shoot proliferation. Results of present study revealed that it might be possible that both mevalonate and non mevalonate pathways are involved in Picroside(s) biogenesis. GA_3 seems to down regulate the biosynthesis of picrosides as GA_3 also follows the same biosynthetic pathways as reported for picrosides. Electrophoresis of protein reveals bands like 84, 67, 58, 37 and 25 kDa which were observed in all samples taken at different days interval while other bands were seen in 12 and 18 days samples harvested from shoot tissue obtained from callus were subjected into suspension cultures, which implies that some proteins are produced under the increased concentration of GA_3 in different suspension cultures at laboratory scale. Although the exact mechanism of picroside(s) metabolism requires to be explored in detail, however, our results are indicative of the increased amount of GA_3 showed prominent effect on picroside(s) metabolic pathways.

Key words: *In vitro propagation, Picrorhiza kurroa Royle ex Benth, Picroside (s).*

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INTRODUCTION

The term medicinal plant consists of different types of plants used in herbal remedies and a few of these plants have curative properties. In the developing countries, more than 3.3 billion people utilize medicinal plants on a regular basis which proves that medicinal plants are the "backbone" of traditional medicine[1]. Around the world 34 hotspots are recognized, out of which two major hotspots *viz.* the Eastern Himalayas and the Western Ghats are located in India[2]. Out of total medicinal plant species known globally, India has 20% plant species of medicinal value and which are in use while the world average stands at 12.5% [3]. But according to Hamilton[4], India has about 44% of flora, which is used medicinally. Due to increasing global demand of medicinal plants for production of herbal medicines that lead to overexploitation of most of the medicinal plants in India. The industrial usages of medicinal plants are infinite. These ranges from their utilization as traditional medicines, herbal drinks, and health promoting foods including nutraceuticals to galenicals, phytopharmaceuticals, industrially produced synthetic analogs of phytochemicals for their use as pharmaceuticals etc[5].

Picrorhiza kurroa Royle ex. Benth(family *Scrophulariaceae*) is a herbal medicinal plant found in the Western Himalayan region, between the elevation of 3000-5000 m [6,7,8]. It is valued as a hepato-protective, immunomodulator, anti- periodic, anti-inflammatory, stomachic, anthelmintic, carminative, expectorant, cardio-tonic, laxative etc[9,10,11,12].This herbal medicine mainly effective against liver disorders. The crude extract of the plant shows good results in liver damage caused by carbon tetrachloride, paracetamol, galactosamine, and alcohol. Aqueous rhizome extracts of *Picrorhiza kurroa* Royle ex. Benth has demonstrated hepatoprotective and antioxidant properties on CCl4 induced liver toxicity in albino rats[13]. According to Ayurveda, the plant has utility as laxative, liver-stimulant, and appetite, and febrifuge. *Picrorhiza kurroa* is also beneficial in bronchial asthma and epidemic jaundice. It is also used to ease stomachaches and is believed to promote appetite. The herb is also effective in 'Kapha' disorders, billows fever, urinary discharge, hiccup, blood troubles, burning sensations and leukoderma[14]. *Picrorhiza kurroa* has antioxidant and anti-neoplastic activities[15] and an iridoid glycoside isolated from its roots is an effective immmuno modulator mostly enhances macrophage capacity amid contaminations[16]. Recently rhizome extracts of *Picrorhiza kurroa* have likewise appeared to have antagonistic to malarial action[17,18]. The cultivation of majority of medicinal plants is difficult as they either do not produce seeds or seeds are too little and do not germinate in soils and as a result exploitation from natural habitat is unavoidable. However, the *Picrorhiza kurroa* propagates vegetatively through stolons, which at first emerge as a young bud, develop to a full grown stolon and then eventually into a rhizome with independent shoots and roots[19]. This plant yields a crystalline product called ''kutkin,'' in its root, which is usually a mixture of two major C9 iridoid glycosides, i.e., picroside-I (6-Otrans-cinnamoyl catalpol) and kutkoside (10-Ovaniloylcatalpol)[20]. Demand for medicines obtained from medicinal plants is increasing day by day and warrants use of plant tissue culture strategy for mass propagation of the plants.

Picroside-I and Picroside-II are iridoid derivatives of monoterpenes[21]. The precursors of monoterpenes i.e., geranyl diphosphate (GPP) are formed by the condensation of isopentenyl pyrophosphate (IPP) and dimethyl allyl pyrophosphate (DMAPP)[22]. At the molecular level, biosynthesis and accumulation of terpenoids are controlled by the structural and regulatory genes in different plant species[23]. The methylerythritol 4-phosphate (MEP) and mevalonate (MVA) biosynthetic pathways resulting in the biosynthesis of DMAPP (dimethylallylpyrophosphate) and IPP (isopentenyl pyrophosphate), from which GPP has formed that act as the building blocks for the biosynthesis of Picroside -I and Picroside-II.

Plant secondary metabolites are used in the pharmaceuticals, food additives, flavors, and other industrial materials either as part of a final product or as a raw material[24]. Different strategies like treating with elicitors and invoking biotic and abiotic stresses have been used in both plant cell culture and intact plants to boost the production of secondary metabolites[25]. Elicitors refer to chemicals that can initiate physiological and morphological responses and secondary metabolism in plants. Elicitation causes a variety of defensive reactions and the accumulations of secondary metabolites[26]. Gibberellic acid (GA_3), a phytohormone, is also well known as an effective elicitor for the production of secondary metabolites. The rate of germinated seeds was reduced to 48% in the presence of NaCl and increased to 76% after seeds priming with 5 mM GA3. The activity of superoxide dismutase, guaiacol peroxidase and ascorbate peroxidase in primed and non-primed seeds increased in the presence of NaCl and after priming of seeds with 5 mM GA3. Main purpose of this study is to analyse the picroside II content in the different regime of growth and development and Total protein profiling from *in vitro* differentiated leaf in the different regime of growth and development.

MATERIAL AND METHODS

Plant material:

The plants of *Picrorhiza kurroa* Royle ex Benth were collected from Chamoli, North West Himalayan Region of Uttarakhand in the month/year of July-2015 by earlier workers in Bioprospecting and Metabolomics Laboratory. These plants were established in Plant Germplasm Evaluation Facility (250C, 65.0% RH) at College of Basic Sciences and Humanities, G.B.P.U.A. &T., Pantnagar-263145 in the same duration which was then subsequently established *in vitro* and maintained during the present investigation.

Callus and Shoot induction:

The explants (leaves) were cultured on MS medium supplemented with IBA, and TDZ in different combinations to initiate callus. After callus induction, fully grown friable callus was cut into 2-3cm pieces and transferred to the shoot medium having different combination on BAP and kinetin. Within 3-4 weeks shoots induction occurs.

Table 1: Composition of Media used in vitro propagation of Picrorhiza kurroa Royle ex Benth

Explant	MS Media + Phytoregulators	
	Callus induction/proliferation	**Shoot induction/ proliferation**
Leaf	a)IBA 0.50 mg/L +TDZ 0.75 mg/L b)IBA 0.75 mg/L +TDZ 0.50 mg/L c) IBA 0.75 mg/L +TDZ 0.25 mg/L d) IBA 0.50 mg/L +TDZ 0.75 mg/L	a)BA 0.25 mg/L + Kinetin 0.75 mg/L b)BA 0.50 mg/L + Kinetin 0.75 mg/L c)BA 0.75 mg/L + Kinetin 0.75 mg/L d)BA 1.00 mg/L + Kinetin 0.75 mg/L

Suspension culture:

The 21 days plantlets from shoot cultures of *P. kurroa* were grown in jam bottle having 50 ml of liquid growth medium. Different concentrations of hormonal elicitor i.e. GA_3 (0ppm, 0.5ppm, 1.0ppm, 1.5ppm) were added. The suspended cells were harvested at 0, 6, 12 and 18 days from the culture medium by filtration and used for protein extraction and HPLC analysis.

Determination of Picroside-II in control and treated samples in *in vitro* liquid cultures of *Picrorhiza kurroa* Royle ex Benth:

Extract preparation for HPLC:

Picrosides were extracted essentially as described by Kawoosa[29]. The frozen samples (100 mg) were ground to fine powder in liquid nitrogen followed by addition of 1ml of 80% HPLC grade methanol.. Extracts were pooled, centrifuged at 10,000g for 20 min. and the supernatant was used for picroside-II estimation. Samples were filtered through the 0.22µm filter before injecting[17]. The chromatographic separation was carried out on an Agilent controller HPLC system using reverse phase non polar C-18 column eluted in an isocratic mode with a mixture of 0.05% tri fluoro acetic acid and methanol: acetonitrile

(1:1) in 70:30 respectively. The column elutes were monitored using PDA (Photodiode Array) detector. Isocratic elution was carried out at a flow rate of 1.0 ml/ min with an injection volume of 20 µl. Picrosides were monitored at 264 nm. The mobile phase was also filtered through 0.45 µm membrane using filtration assembly connected with a vacuum pump. The mobile phase was degassed properly before applying it on HPLC to avoid air bubble. Analysis was repeated for three replicates each and the means and standard deviations were calculated.

Protein isolation:

For extracting the soluble proteins from the leaf, 25 ml of extraction buffer (10% trichloroacetic acid (TCA) in chilled acetone with 0.07% β-mercaptoethanol) were added to one gram of finely powdered leaf, followed by 1 minute of vortex agitation. Homogenate obtained was kept at -20^{o}C for 2-3 hours, and then it is centrifuged at 12000xg at 4^{o}C for 15 minutes. Obtained pellet was resuspended in 80% acetone containing 0.07% β-mercaptoethanol and 2 mM EDTA and kept at 20°C overnight[27]. After centrifugation pellet was again resuspended in 100% acetone containing 0.07% β-mercaptoethanol, 2mM EDTA and then centrifuged at 12000xg, at 4°C for 20 minutes and then the pellet is vacuum dried. The lyophilized powder obtained from vacuum dried pellet was resuspended in 0.5-1.0 ml rehydration buffer (7M Urea, 2M Thiourea, 2% CHAPS, 25mM DTT added fresh) and kept at 25°C for 4 hours. After incubation insoluble precipitates were removed by centrifugation at 12,000xg for 30 minutes at 4°C. Bradford protein assay was used for the estimation of protein concentration in the finally obtained supernatant[28].

SDS-PAGE of the total soluble protein

Total soluble proteins extracted at different time intervals (0, 6, 12, 18 days) were subjected to electrophoresis on 5% stacking gel and 12% separating gel. A medium range protein marker (14.3-97.4 kDa) is used to determine the molecular weight of the unknown proteins.

RESULTS AND DISCUSSION

***In vitro* callus and shoot culture establishment:**

0.75 TDZ mg /L + 0.75 IBA mg/ L were used for callus induction from leaf explants. While Sood[30] reported that IBA (0.5 mg/L) + 2, 4-D (2.0 mg/L) was most effective for callus induction (56.3%) in *P. kurroa* leaf derived callus and 38.3% in case of stem explants. Calli were transferred to shoot induction medium. Best result was observed in 0.5 BAP mg/ L + 0.75 Kinetin mg /L. Shoots were regularly sub cultured and maintained on same medium composition. Helena[31] reported maximum shoot induction frequency in 1.0 mg /L BA + 0.75 mg /L Kinetin in leaf derived callus and 1.0 mg/ L BA + 1.0 mg /L Kinetin in stem derived callus respectively.

Estimation of picroside II through HPLC:

Elicitors treated and untreated cultures were studied for picrosides content estimation. According to the R^2 value (0.9851) of the calibration curve, the linearity of the calibration curve was acceptable. The equation of the calibration curve was as Y=122776X, where Y and X are peak area of picroside II and concentration of picroside II in the samples respectively. It was observed that picroside content was affected in all different days treatment as compared to control cultures when treated with Hormonal elicitor (GA_3). Decreased picroside II concentration indicates that GA3 downregulates the biosynthesis of secondary metabolite picroside in Picrorhiza kurroa.

Total soluble protein profiling by SDS-PAGE:

Total protein profiling at tissue level provides an insight to understand the possible role of different proteins in specific tissues in the biogenesis pathway. Hence, protein profiling is done to find out the protein which is expressed during the biosynthesis of secondary metabolites through MVA and MEP pathway. Total protein profiling is done by SDS-PAGE. Total protein was isolated from shoots *in vitro* suspension cultures, according to the procedure followed by Sanjeeta[32]. The isolated protein was subjected to electrophoresis on

12% polyacrylamide gels. A number of bands were seen in the gel, some are clearly visible and other was not clear. In the 0-day sample, five bands were clearly seen having the molecular weight (84, 62, 48, 37, 25 kDa). In 6th day samples, five bands were seen having the molecular weight (86, 67, 58, 35, 25 kDa). In 12th day samples, seven bands were seen having the molecular weight (81, 67, 52, 43, 37, 30, 24 kDa). In 18th day samples, seven bands can be seen clearly having the molecular weight (85, 78, 67, 58, 46, 40, 25 kDa). Some protein bands like 84, 67, 58, 37, 25 kDa bands were seen in all samples taken at different day's interval while other bands were seen in 12th and 18th days samples, which implies that some proteins are produced under the increased concentration of GA3.

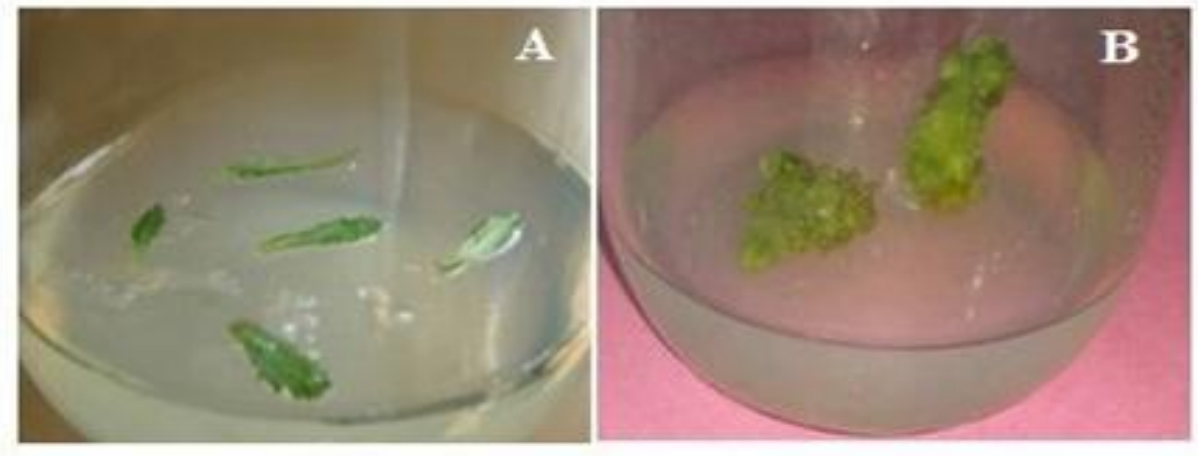

Fig. 1: Callus induction from in vitro maintained plant leaf of Picrorhiza kurroa Royal ex Benth (A) Leaf explants, (B) callus after 2 weeks of culture

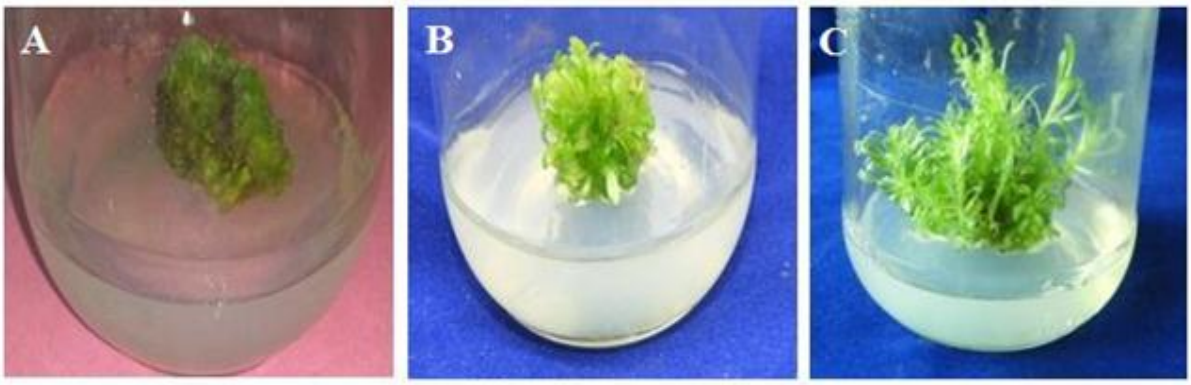

Fig. 2: Shoot induction from callus of Picrorhiza kurroa Royal ex Benth in MS medium supplemented with BAP 0.50 mg/L + Kinetin 0.75 mg/L (A) Callus (B) After 2 weeks of culture (C) After 4 weeks of culture

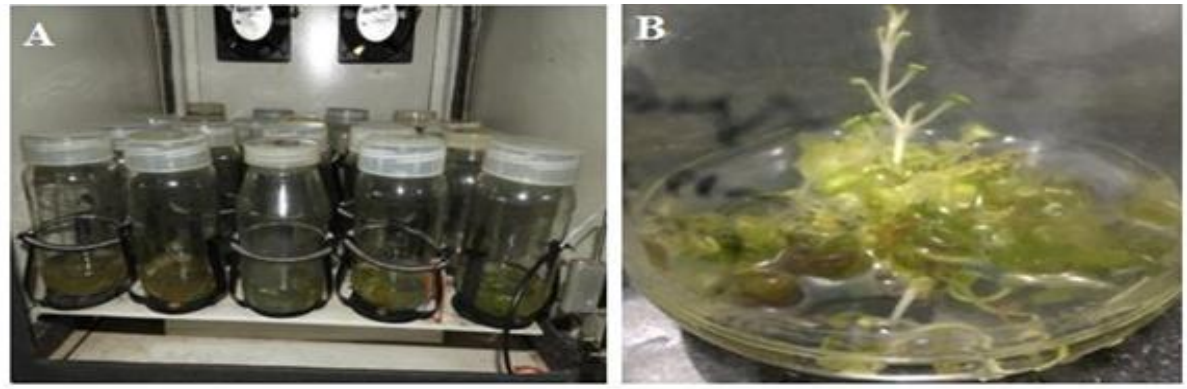

Fig. 3: (A) In vitro shoot cultures in MS liquid medium in orbital shaker (B) Transferred shoots in MS-Liquid medium at 12 days

Table 2: Effect of Hormonal elicitor treatment on picroside accumulation *In vitro* shoot culture at different time intervals

S. No.	Concentration of elicitor(GA3)	Production of picroside mg L^{-1} (W/V)			
		0^{th} Day	6^{th} Day	12^{th} Day	18^{th} Day
1	0 ppm	161.75±1.25	51.49±3.27	26.37±0.86	29.26±0.65
2	0.5 ppm	112.99±0.054	34.04±1.43	24.14±0.41	24.73±0.15
3	1.0 ppm	105.88±7.31	29.63±1.06	19.45±0.48	22.32±1.25
4	1.5 ppm	190.82±5.04	24.83±1.59	18.87±0.37	20.67±0.02

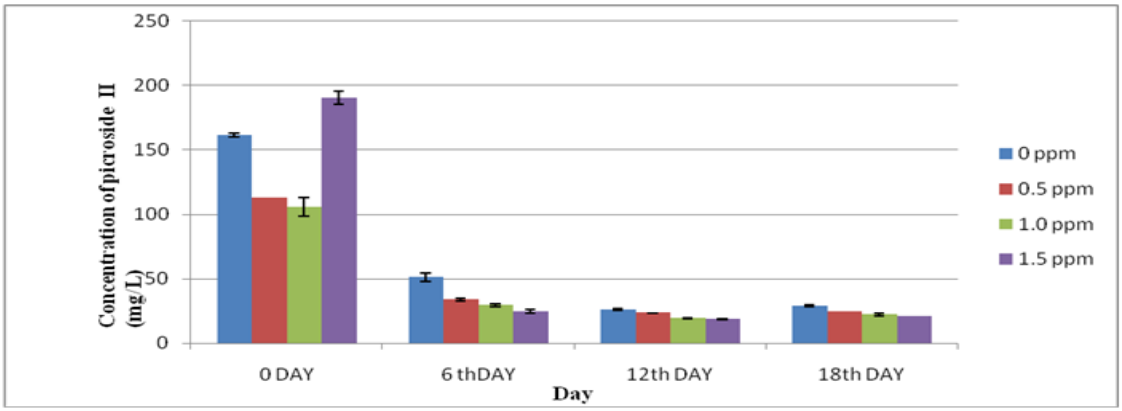

Fig. 4: Effect of Hormonal elicitor GA3 treatment on picroside-II accumulation in vitro shoot cultures in MS liquid medium at different time intervals

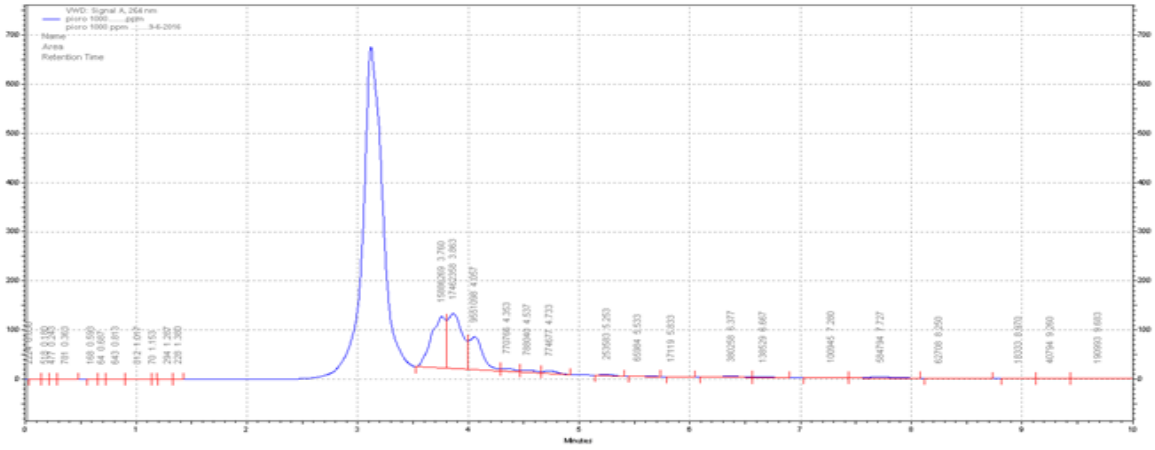

Fig. 5: Standard compound Picrodise II: Chromatogram of 1000 ppm

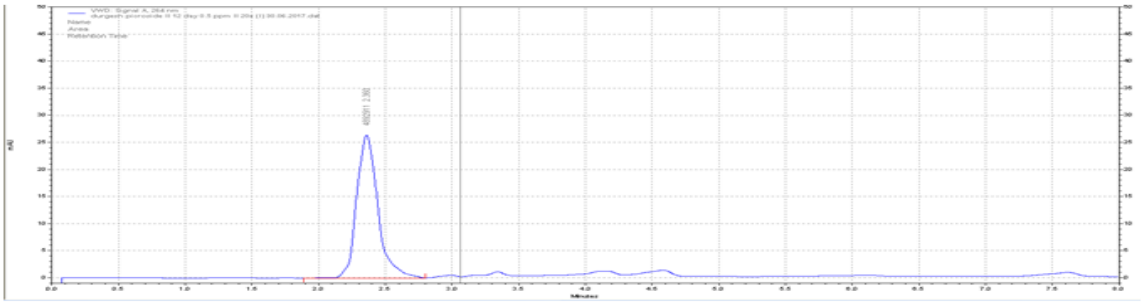

Fig. 6: Methanolic extract of Picrorhiza kurroa showing peak of Picroside II at 12 day 1.5 ppm treated sample

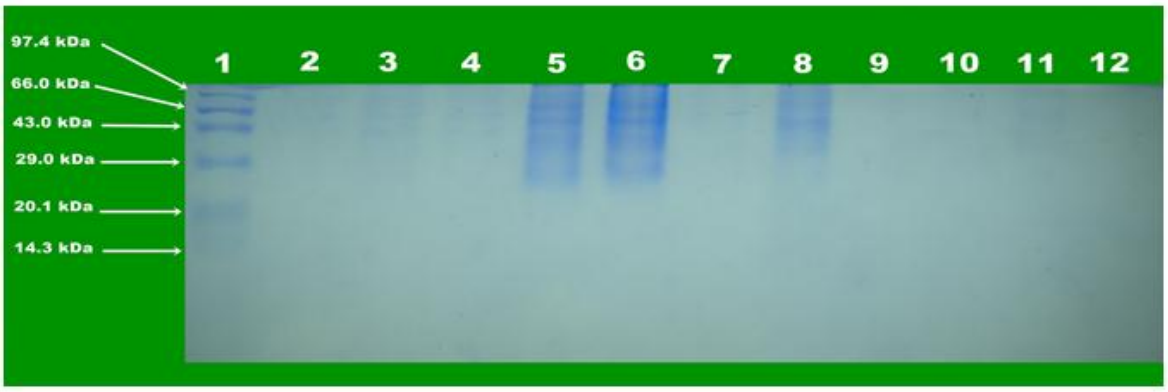

Fig. 7: SDS-PAGE of soluble protein extracted from 18th-day samples 1) Protein marker, (2, 3 and 4) 0 ppm sample, (5, 6 and 7) 0.5 ppm sample, (8, 9 and 10) 1.0 ppm sample, (11, 12) 1.5 ppm sample

CONCLUSION

Findings are indicative for the fact that *Picrorhiza kurroa* Royle ex Benth may be propagated through tissue culture methods in order to maintain its bioactive constituents. It also enables the mass propagation of this herb from a minimal of plant material to safe guard biodiversity related issues for its conservation in various niches. Large quantities of biomass required for extraction of active constituents can be made available throughout the year through suspension cultures without causing further threat to this important species. On the basis of elicitation response of GA_3, it might be proposed that both mevalonate and non-mevalonate pathways are involved in the metabolism of Picroside II as higher GA_3 concentration down regulates the metabolism of Picroside II. However, proposed hypothesis necessitates to be established through various other experimentations such as tissue specific over expression and knock out studies of rate limiting gene(s) to draw any tangible conclusion.

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