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A Proposed Biosynthetic Pathway of Picrosides Linked through the Detection of Biochemical Intermediates in the Endangered Medicinal Herb *Picrorhiza kurroa*

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ABSTRACT:

Introduction – *Picrorhiza kurroa* Royle ex Benth is an important medicinal herb used in the preparation of several herbal drug formulations due to the presence of picroside-I (P-I) and picroside-II (P-II) along with other iridoid-glucosides derivatives.

Objective – The endangered status of *P. kurroa* coupled with lack of information on biosynthesis of P-I and P-II necessitate deciphering the biosynthetic pathway for picrosides.

Methods – LC with electrospray ionisation (ESI) and quadrupole time of flight combined with MS/MS was used to detect intermediates and assemble the picrosides biosynthetic pathway in *P. kurroa*.

Results – The presence of catalpol and aucubin, the major backbone structures of picrosides, along with intermediate metabolites boschnaloside, bartsioside and mussaenosidic acid, was confirmed in ESI negative mode with pseudomolecular ion peaks, that is, *m/z* 361, *m/z* 343, *m/z* 345, *m/z* 329 and *m/z* 375 ions and their fragmentation patterns.

Conclusion – The picrosides biosynthetic pathway is expected to provide a reliable platform towards understanding the molecular components (genes/enzymes) of P-I and P-II biosynthesis in *P. kurroa* for their eventual utilisation in various applications.

Supporting information can be found in the online version of this article.

Keywords: LC–MS; biosynthetic pathway; aucubin; bartsioside; picrosides; *Picrorhiza kurroa*

Introduction

Picrorhiza kurroa Royle ex Benth is a medicinal herb belonging to the family of Scrophulariaceae and mainly found in the Himalayan regions of India at altitudes of 3000–4300 m (Sood and Chauhan, 2010). It is widely used as a hepatoprotective (Saraswat *et al*., 1999) in various formulations such as Picroliv (Ansari *et al*., 1991), Katuki, Arogya, Livomap and Kutaki (Bhandari *et al*., 2009), and also possesses other pharmacological activities such as anti-carcinogenic (Joy *et al*., 2000), anti-oxidant (Rajkumar *et al*., 2011), immunomodulatory (Gupta *et al*., 2006), anti-allergic, anti-asthmatic (Dorsch *et al*., 1991), superoxide scavenging (Chander *et al*., 1992) and anti-diabetic (Joy and Kuttan, 1999) properties. *Picrorhiza kurroa* contains two major medicinal components, picroside-I (P-I) and picroside-II (P-II) along with kutkoside (Singh and Rastogi, 1972), picroside-III (P-III) (Weinges *et al*., 1972), picroside-IV (P-IV), verminoside, specioside (Li *et al*., 1998) and other iridoid-glucosides (Table 1) (Mondal *et al*., 2012). The increasing demand, limited cultivation and reckless collection from the wild have rendered *P. kurroa* a critically endangered plant species (Rai *et al*., 2000; Mehra *et al*., 2011; Sood and Chauhan, 2011).

Despite the several medicinal properties attributed to P-I and P-II, their biosynthetic pathway is unknown in *P. kurroa*. Picrosides are biosynthesised by the isoprenoid biosynthesis pathway through the precursor geranyl diphosphate (GPP) (Gahlan *et al*., 2012) and no information is available as of today on the formation of picrosides from GPP. The biosynthesis of P-I and P-II has been found to occur differentially in *P. kurroa*, the former in shoots and the latter in stolons or roots (Sood and Chauhan, 2010; Pandit *et al*., 2012). Whether both the mevalonate (MVA) and non-mevalonate (i.e. methylerythritol phosphate, MEP) pathways or either of them contribute to the formation of GPP, was undertaken through the expression analysis of pathway genes vis-à-vis differential P-I and P-II biosynthesis in different tissues/organs of *P. kurroa* (Pandit *et al*., 2012). Four genes (DXPS, ISPD, ISPE, MECPS) of the MEP pathway and one gene (PMK) of the MVA pathway had shown elevated levels of expression in shoots, thereby, correlating with the biosynthesis of P-I in *P. kurroa* and two genes of the MEP pathway showed higher expression in roots containing P-II. The expression analysis showed that both the MVA and MEP

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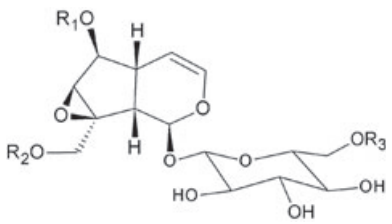
Table 1. Modifications in catalpol in the formation of picrosides and other related compounds



Catalpol

(R_1, R_2, R_3 = H)

Catalpol (R_1, R_2, R_3 = H)

Metabolite name	R_1	R_2	R_3
Picroside-I	H	H	(*E*)-cinnamoyl
Picroside-II	Vanilloyl	H	H
Picroside-III	H	H	(*E*)-Feruloyl
Picroside-IV	H	H	*p*-Coumaroyl
Picroside-V	(*Z*)-feruloyl	H	H
Verminoside	Caffeoyl	H	H
6-Feruloylcatalpol	(*E*)-feruloyl	H	H
Kutkoside	H	Vanilloyl	H
Veronicoside	Benzoyl	H	H
Specioside	*p*-Coumaroyl	H	H
Vanilloyl catalpol	H	H	Vanilloyl

pathways genes contribute to the formation of GPP in the biosynthesis of picrosides in *P. kurroa*.

All picrosides (P-I, P-II, P-III, P-IV, verminoside and specioside) are derivatives of catalpol (Fig. 1). Catalpol and its biosynthetic precursor aucubin (Damtoft, 1994; Ronsted *et al*., 2000) have been isolated from *P. scrophulariiflora* (Wang *et al*., 1993), however, neither have so far been detected in *P. kurroa*. Catalpol is found in many plant families, for example Scrophulariaceae, Lamiaceae and Bignoniaceae, and is biosynthesised from aucubin (Damtoft *et al*., 1993), which is further biosynthesised through 8-epi-deoxyloganic acid with a series of intermediates (Damtoft, 1983; Damtoft *et al*., 1993; Ronsted *et al*., 2000). 8-Epi-deoxyloganic acid is synthesised from 8-epi-iridodial and/or through an intermediate 8-epi-iridotrial or its cyclised form (Isabel and Kaplan, 2001) by cyclisation and oxidation.

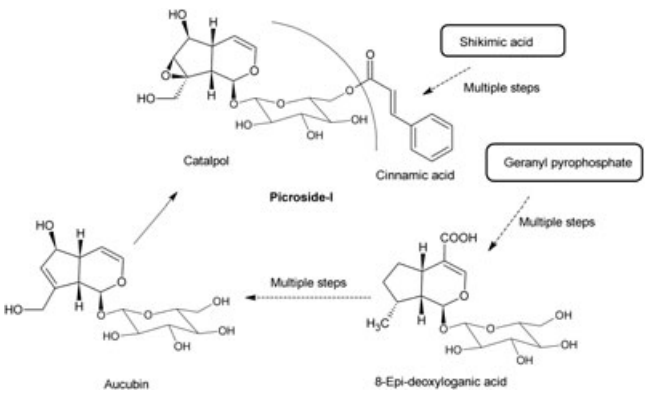


Figure 1. Catalpol as a building block for P-I and related compounds, showing its biosynthetic precursors in nature.

Therefore, there should be one more compound after cyclisation of 8-epi-iridotrial and before oxidation to 8-epi-deoxyloganic acid, which may be boschnaloside because there are two changes occurring in 8-epi-iridotrial to 8-epi-deoxyloganic acid, that is, oxidation and glucosylation. Feeding experiments with deuterium-labelled 8-epi-iridodial /trial prodiced no incorporation into aucubin in *Buddleja albiflora* (Scrophulariaceae), whereas feeding experiments in *Gardenia jasminoides* (Rubiaceae) formed tarennoside to geniposide. In *Scrophularia umbrosa* (Scrophulariaceae), 8-epi-iridodial/trial formed aucubin through geniposidic acid and bartsioside (Damtoft, 1994; Isabel and Kaplan, 2001). There are more intermediate compounds that lead to the formation of aucubin, that is, mussaenosidic acid and deoxygeniposidic acid (Damtoft *et al*., 1993, 1994; Ronsted *et al*., 2000). Thus, there have to be 14 steps after GPP leading to the formation of catalpol and one additional step in which picrosides are formed through the esterification of catalpol and aromatic acids which come from other biosynthetic pathways, that is, shikimic acid and phenylpropanoid pathways (Gahlan *et al*., 2012; http://www.genome.jp/kegg/pathway/map/map00940.html). The catalpol backbone is further modified by esterification of R_1, R_2 and R_3 groups, respectively, resulting in the formation of P-I, P-II and other metabolites in *P. kurroa*. The major aromatic acids modifying catalpol at respective positions are cinnamic acid, vanilic acid, *p*-coumaric acid, caffeic acid and ferulic acid. The current study propose a plausible biosynthetic pathway for picrosides biosynthesis in *P. kurroa* by using a bio-retrosynthetic approach, which includes assembling the biosynthetic pathway from earlier to later steps, that is from end-product to its precursor (Bachmann, 2010). We investigated the presence of different intermediates that were either not detected earlier in *P. kurroa* or their occurrence was reported but not arranged with respect to the biosynthesis of picrosides (online Supplementary Fig. 1). The LC/ESI–MS/MS method was used for the detection of intermediates to complete the biosynthetic pathway in *P. kurroa*.

Experimental

Picrorhiza kurroa rhizome samples were collected from nursery-grown plants (Sairopa, Himachal Pradesh, 4500 m altitude, 31°38′–31°54′N and 77°20′–77°45′E) and a voucher sample has been submitted to the Himalayan Forest Research Institute (Shimla, HP, India) with the reference Herbarium Acc. No. 0670. Catalpol and aucubin standards were procured from Sigma-Aldrich (Bangalore, India). Methanol and acetonitrile solvents (HPLC grade; Merck, Mumbai, India) were used for method optimisation, extraction and sample preparation. Formic acid (99%, GR grade), ammonium acetate (GR grade) and acetic acid (GR grade) were procured from Merck. Milli Q Plus ultra-pure water was used in all the experiments (Millipore, Milford, MA, USA).

LC–MS/MS method for intermediates analysis

Plant rhizomes were ground to a fine powder in liquid nitrogen and 100 mg of sample was extracted with 80% methanol in a bath sonicator for 30 min, centrifuged at 12000 rpm and then filtered through a 0.22 μm polyvinylidene fluoride (PVDF) filter. Filtered extract was injected into a Waters 2695 separation module and Waters Micromass QTOF MS controlled by MassLynx 4.0. Separation was performed on a Waters Spherisorb® 5-μm ODS2 (250 mm × 4.6 mm) reverse phase column. The analytes were eluted at 1.0 mL/min flow rate with a gradient of 10 mM (*w/v*) aqueous ammonium acetate (pH 3.0) with formic acid as mobile phase A; 1:1 (*v/v*) mixture of acetonitrile and methanol as mobile phase B with linear gradient conditions; 0 min 20% B hold for 5 min; 20% to 30% B in next 5 min, hold for 15 min; 30% to 70% B in 15 min,

hold for 5 min; 20% B in next 5 min and equilibrated for 5 min. Micromass Q-TOF was operated in negative ionisation (NI) mode at 2731.0 capillary voltage, 30.0 sample cone voltage, 2.0 extraction cone voltage, 300 °C desolvation temperature, 100 °C source temperature, 7.0 collision energy and mass scan range of *m/z* 100 to 1000 for MS and for MS^2 it was *m/z* 60 to 600.

Detection, isolation and identification of catalpol and aucubin in *P. kurroa* samples

Grounded rhizomes were extracted in water using bath sonicator for 30 min, centrifuged at 12000 rpm and then filtered through 0.22-μm membrane filter for detection of catalpol and aucubin through LC-MS. The extract was separated by 0.1% acetic acid in water as mobile phase A and acetonitrile as mobile phase B eluted at 1.0 mL/min flow; 2% B from 0 to 15 min, 70% B in 0.5 min hold for 4.5 min, 98% in next 0.5 min equilibrated for next 4.5 min on Waters Spherisorb® 5 μm ODS2 (250 mm × 4.6 mm) reverse phase column at 35 °C. The column eluent was connected to the ESI source from 0 to 15 min, after which it was directed to waste untill it stopped in order to protect the source from a high concentration of other compounds. The Micromass Q-TOF was operated in negative mode at 3122.0 capillary voltage and 3.0 collision energy and other mass parameters were the same as used in previous studies. Catalpol and aucubin were isolated from the aqueous extract of rhizomes. The extract was subjected to preparative isolation by water (A) and acetonitrile (B). The isolation was performed through Waters Spherisorb® 5 μm ODS2 (250 mm × 20 mm) reverse phase column at 30 mL/min flow; 2% B from 0 to 10.5 min, 70% B in 0.2 min, hold for 3.3 min, 2% B in next 0.3 min and hold for 2.7 min for equilibration before next injection at ambient column temperature.

Results and discussion

For identification and characterisation of intermediates in the picrosides biosynthetic pathway, 80% methanolic extract was injected directly into the mass spectrometer so that intermediates were detected without any degradation. As all the intermediates are glucosidic compounds, which are highly polar and may become ionised in negative mode and unstable in nature, ESI negative ionisation (NI) mode was used at low collision energy, optimum source and desolvation temperature in order to obtain high molecular ion peaks. Desired pseudomolecular ion peaks, that is, *m/z* 361, *m/z* 343, *m/z* 345, *m/z* 167, *m/z* 329, *m/z* 375, *m/z* 511, *m/z* 491 and *m/z* 147 ions, were obtained in ESI negative mode (online Supplementary Fig. 2a,b), which correspond to catalpol, boschnaloside, aucubin, vanilic acid, bartsioside, mussaenosidic acid, P-I, P-II and cinnamic acid, respectively, on the basis of their theoretical mass in NI. Lower ion counts were detected for deoxygeniposidic acid, geniposidic acid and 8-epi-deoxyloganic acid, which may be due to system noise or due to their minute quantity in the extract.

All masses were analysed by HPLC coupled with a MS (online Supplementary Fig. 3) and subjected to mass fragment analysis (MS^2). The peaks at *m/z* 345 and *m/z* 361 that corresponded to aucubin and catalpol, respectively, could not be resolved in the present method and also gave very low pseudomolecular ion peaks, therefore, a different method was developed to detect these compounds through LC–MS (online Supplementary Fig. 4), but again the ion count was low. Finally, the catalpol and aucubin were isolated through preparative HPLC (online Supplementary Fig. 5), the fractions were concentrated and then subjected to MS^2 scan to confirm their presence in the extract.

Boschnaloside, bartsioside, mussaenosidic acid, P-I, P-II, catalpol and aucubin showed pseudomolecular ion peaks at

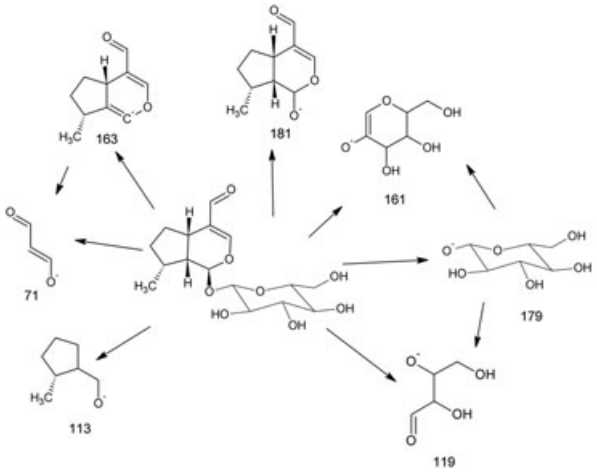


Figure 2. Plausible fragmentation pathway of boschnaloside.

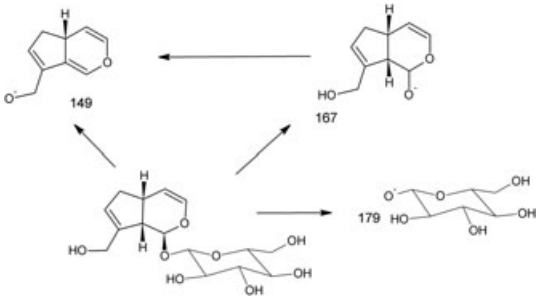


Figure 3. Plausible fragmentation pathway of bartsioside.

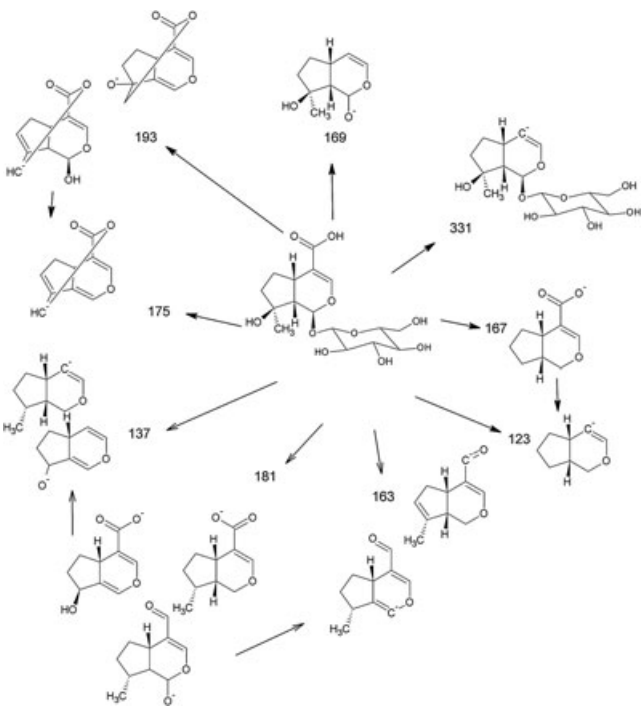


Figure 4. Plausible fragmentation pathway of mussaenosidic acid.

m/z 343, *m/z* 329, *m/z* 375, *m/z* 511, *m/z* 491, *m/z* 361 and *m/z* 345, respectively in NI (online Supplementary Figs 6–12). All masses were subjected to collision induced dissociation (CID) and gave characteristic loss of a *m/z* 162 ion of a glucose molecule either from the pseudomolecular ion peak or from their daughter ions, which confirmed them as glucosidic compounds. During LC – MS analysis, deprotonated molecular mass corresponding to boschnaloside was observed at a retention time of 3.2 min (online Supplementary Fig. 3), that is, *m/z* 343. The MS^2 spectra of ion *m/z* 343 gave characteristic daughter ions at *m/z* 181 $[M-H-162]^-$, which was the aglycone part of boschnaloside, and at *m/z* 179 of glucose $[M-H]^-$ (online Supplementary Fig. 13). The dehydration of glucose from the ion at *m/z* 343 resulted in a daughter ion at *m/z* 161 $[glu-H-H_2O]^-$ and, at the same time, dehydration of aglycone of boschnaloside $[M-H-162-H_2O]^-$ gave a signal at *m/z* 163 along with two other peaks at *m/z* 71 and *m/z* 113 (Fig. 2). The deprotonated ion at *m/z* 329 corresponding to bartsioside was observed at a retention time of 39.4 min (online Supplementary Fig. 3). The MS^2 spectra of the ion at *m/z* 329 confirmed it to be a glucosidic compound due to the presence of an *m/z* 179 glucose deprotonated ion (online Supplementary Fig. 14). Further, the presence of daughter ions at *m/z* 167 representing the aglycone part of bartsioside $[M-H-162]^-$ and its dehydrated ion *m/z* 149 $[M-H-162-H_2O]^-$ pointed towards bartsioside (Fig. 3).

The deprotonated pseudomolecular ion corresponding to mussaenosidic acid was observed at a retention time of 14.9 min (online Supplementary Fig. 3), i.e., *m/z* 375, which was a deprotonated $[M-H]^-$ peak. Further, the *m/z* 331 ion $[M-H-CO_2]^-$ gave a characteristic fragment that showed the presence of the carboxylic acid group and *m/z* 169 $[M-H-CO_2-162]^-$, which produced the decarboxylated aglycone part of mussaenosidic acid also present in the MS^2 spectra (online Supplementary Fig. 15). The MS^2 spectra of *m/z* 375 also gave daughter ions at *m/z* 193 of dehydrated cyclised aglycone moiety $[M-H-162-H_2O-H_2]^-$, *m/z* 169 that corresponded to $[M-H-162-CO_2]^-$, *m/z* 175 possibly from dehydration of *m/z* 193 $[M-H-162-2H_2O-H_2]^-$, and *m/z* 181 due to $[M-H-179\text{-}OH+H_2]^-$ as a characteristic of 3′-OH group and other companion ions (Fig. 4). P-I and P-II deprotonated ions, that is, *m/z* 491 and *m/z* 511, were observed at retention times of 20.7 min and 15.3 min respectively (online Supplementary Fig. 3).

The P-I pseudomolecular $[M-H]^-$ ion, that is, *m/z* 491 gave daughter ions in MS^2 spectra (online Supplementary Fig. 16) at *m/z* 199 $[M-H-292]^-$ by the loss of cinnamic acid and glucose moiety, and at *m/z* 147 and *m/z* 103 that showed the presence of cinnamic acid. A daughter ion at *m/z* 169 $[M-H-292-CH_2OH]^-$ was due to the loss of the CH_2OH group from *m/z* 199 ion (onloine Supplementary Fig. 17). The MS^2 ion at *m/z* 511 (online Supplementary Fig. 18), which corresponds to P-II, gave daughter ions at *m/z* 167 and *m/z* 123 for vanilic acid, and at *m/z* 349 due to aglycone moiety $[M-H-162]^-$ (online Supplementary Fig. 19).

The presence of catalpol and aucubin was confirmed by a different LC–MS method, and their pseudomolecular ions were observed at retention times of 5.8 min and 10.8 min respectively (online Supplementary Fig. 20). The MS^2 of *m/z* 361 ion (online Supplementary Fig. 21), corresponding to catalpol, gave fragments at *m/z* 199, which was the aglycone moiety of catalpol $[M-H-162]^-$, *m/z* 181 due to $[M-H-162-H_2O]^-$, *m/z* 169 due to $[M-H-162-H_2O-CH_2OH]^-$ and *m/z* 151 due to $[M-H-162-2H_2O-CH_2OH]^-$ (online Supplementary Fig. 22). The MS^2 of *m/z* 345 ion (online Supplementary Fig. 23), which represented aucubin, gave daughter ions at *m/z* 183, representing the aglycone moiety of aucubin $[M-H-162]^-$, *m/z* 179 and *m/z* 161 from the glucose, *m/z* 165 that represented the dehydrated aglycone of aucubin $[M-H-162-H_2O]^-$, *m/z* 147 possibly due to dehydration of *m/z* 165 ion and again on dehydration *m/z* 119 (online Supplementary Fig. 24).

The catalpol and aucubin along with three other metabolites, that is, boschnaloside, bartsioside and mussaenosidic acid, suggest that the pathway leading to the formation of picrosides in *P. kurroa* (Fig. 5) is similar to that previously shown in related plants. Thus, glucosylation of iridotrial gives boschnaloside, which undergoes hydroxylation and oxidation to form mussaenosidic acid. Further transformation can take place by decarboxylation and dehydration to bartsioside. Bartsioside can be changed to aucubin by hydroxylation and the aucubin further converted to catalpol by epoxidation. Finally, catalpol can be converted to picrosides and other related compounds by esterification of different free hydroxyl groups of catalpol.

This study has reported a complete biosynthetic pathway for picrosides and related compounds in *Picrorhiza kurroa* for the first time. The picrosides biosynthetic pathway can be expected to help in understanding the biology and molecular basis of

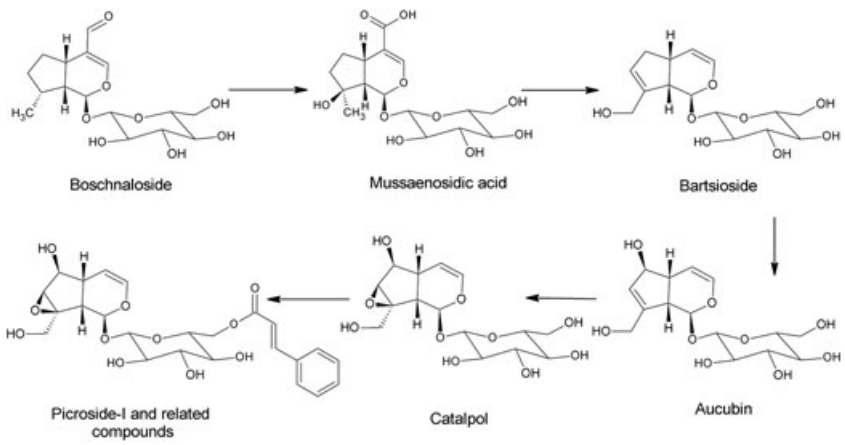


Figure 5. Plausible biosynthetic pathway of P-I and related compounds in *P. kurroa*.

their biosynthesis with respect to genes/enzymes for their eventual utilisation in controlled production of picrosides and other related metabolites in *P. kurroa*.

SUPPORTING INFORMATION

Supporting information can be found in the online version of this article.

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