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In vitro production of secondary metabolite using *Atropa komarovii* Bline&Shal (Solanaceae) hairy root culture via *Agrobacterium rhizogenes* ATCC15834

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ABSTRACT

Background & Aim: A new sustainable tissue-based system is presented by plant hairy roots, preserving all of the several specialized types of cell with critical roles in allowing bioactive secondary molecules to be synthesized more consistently as usual. The system is also essential for studying the production of alkaloid in culture.

Experimental: The *Atropa komarovii* leaves were wounded and infected with soil gram-negative bacterium *Agrobacterium rhizogenes* ATCC15834. After three weeks, the transformation roots and control roots without infection, appeared, and for confirming that T-DNA Ri plasmid fragments were transformed and integrated to plant genome, the *rol*B gene region, was amplified using PCR. HPLC method was then used for assaying how two tropane alkaloids such as atropine (hyosciamine) and scopolamine (hyoscine) were produced in hairy roots,control roots, leaves and roots of plantlet.

Results: The data indicated that diagnostic 500bp *rol* B product amplification was exhibited to be present by all the transformed hairy roots. Scopolamine content in hairy roots was considerably greater than that in control roots but greatest (Hyoscyamine) atropine content was observed in control roots. Analysis of DW, FW and root length showed that fresh and dry root weight increased in hairy roots compared with that in non transformed root.

Recommended applications/industries: The present study demonstrated that secondary metabolite production using medicinal plants concerns many researchers worldwide today and hairy root culture is a useful method for producing tropane alkaloids in solanaceae.

1. Introduction

Pharmaceutical tropane alkaloids are most widely obtained from *Atropa komarovii* Bline&Shal in commerce, as compared to the other members of the Solanaceae. A novel means of obtaining greater amounts of pharmaceutical compounds of high value using medicinal plants like *Atropa komarovii* is provided by genetic engineering (Song and Walworth,

2013). While in vitro culture conventional roots often suffer from low growth rate as their main problem, hairy roots mediated with *Agrobacterium rhizogenes* grow fast, and are genetically and biosynthetically stable. Another advantage is the potential for successful large-scale culture (Sevon *et al*., 2002). As the hairy root disease causative agent (Akramian *et al*., 2008), when the soil-borne bacterium, *Agrobacterium rhizogenes* infects the host plant, transfer and integration of the T-DNA present between the TR and TL regions of the Ri-plasmid inside it into the nuclear genome can be observed (Hu *et al*., 2006). *Agrobacterium rhizogenes* can deliver a well-defined fraction of its own genome into the host cell in the form of a DNA molecule with only one strand in order to transform its host (Yelin *et al*., 2008). New opportunities are offered by hairy root cultures for producing phytochemicals of high value synthesized in roots (Zhao *et al*., 2012). It is advantageous in many ways to apply genetically modified hairy root cultures yielded through different biotechnological methods (Hank *et al*., 2003). The major characteristics of hairy roots include free culture condition, high growth rate under hormone, lateral branching, and plagiotropic, biosynthetic, and genetic stability (Nikravesh *et al*., 2010). The two alkaloids that are most frequently observed in the Solanaceae are scopolamine and hyoscyamine (atropine), and the poisonous, medicinal, and hallucinogenic properties of plants that contain them have made many of these plants quite popular (Samet*etal*., 2012). It is in the leaves and the main root that these alkaloids are most commonly accumulated, and hyoscyamine and scopolamine are the major compounds, respectively (Zarate *et al*., 2006). Hairy root cultures appear to promisean alternative for industrial scale production of tropane alkaloids (Marchev *et al*., 2012).

2. Materials and Methods

2.1. Material of the plant

The mature *Atropa komarovii* seeds were obtained in Gozlugurben on Ali-abad road, Golestan Province at 2500 m height. MS media were used for inoculating the sterilized seeds (Murashig and Skoog, 1962) supplemented with 30 g L^{-1} sucrose for one week in the darkness, and then they were maintained at 25^0C with a daily 16h light and 8h dark photoperiod. The five-week-old plantlets were 6 cm heighand 6 leaves.

2.2. Bacterial strain

In this research, leaf explants of *Atropa komarovii* cultivated with *Agrobacterium rhizogenes* ATCC 15834 were infected for yielding transformed (hairy) root cultures. The bacterium was maintained on LB solid medium as required. For twenty-four hours prior to infection, the bacterium was grown in the dark in 10 ml LB liquid medium containing 50 mg l^{-1}rifampicine at 28^0C on a rotary shaker at 100 rpm. A spectrophotometer was then used for measurement of the optical density at 600 nm (OD=0.7).

2.3. Co- and hairy root induction

The induction of hairy roots was performed by placing cut axenic leaf explants from three week- old micropropagated shoots. Infections, were carried out by wounding the leaves with scalpel and immersing them into the bacterial suspension for 15 min, and the leaves with no bacterial treatment that served as control were gently wounded. The infected and control leaves were co-cultivated for 48h on MS medium with no antibiotics and phytohormones. The explants were then transferred into the same medium that contained the antibiotic cefotaxime (500 mg l-1) for elimination of bacteria. Next, they were incubated at 26±20C under a photoperiod composed of 16 hours of light and 8 hours of dark, and the uninfected (control) leaves were cultured while the same conditions held. After 25 days, the hairy roots were induced. After transferred into MS basal medium that was hormone-free and contained 30 gL^{-1} sucrose, they were supplemented with cefotaxime 500 mgL^{-1}. Then, every three weeks, cefotaxime concentration was divided by two form 500 mgL^{-1} to 250 mgL^{-1}.

2.4. Fresh (FW) and dry weight (DW) determination

After three weeks, the hairy root and control root were induced, then we evolved them from the medium, rinsed them with distilled water, and weighted (FW) them after removal of the water with filter paper. When the fresh roots were dried at 60^0 C in an electric oven, dry weight (DW) was measured. This was triplicated during four consecutive weeks on each sample.

2.5. Length of the roots

For comparison between hairy root and control root lengths, they were measured during five weeks. In the first week, no significant difference was observed between the treatments, but there were significant differences between the hairy roots and control ones from the second to the fifth week.

2.6. DNA analysis and PCR analysis

Based on Doyle and Doyle's (1980) CTAB method, the genomic DNA that was used on PCR as template was isolated from roots that were excised from leaves of the infected and uninfected types. For PCR analysis for detection of *rol* B gene in hairy roots, the extracted DNA was employed. For the sake of *rol*B gene region amplification we used forward 5´ATGGATCCCAAATTGCTATTCCCCAGGA 3´ primer and then reverse 5´TTAGGCTTCTTTCATTCGGTTTACTGCCGC-3´ primer based on Martins *et al.* (2003). PCR reaction was performed in 20 µl that contained 14.7 ml of H_2O, 20 µl of PCR buffer, 0.5 µl of dNTP, genomic DNA, 50 ng, Taq DNA polymerase, 1 u, 10 pmoles primers, and $MgCl_2$ 2 mM. For detecting the presence of *rol* B gene, we denatured the template at 94^0C for a minute, annealing at 57^0C and extract at 72 ^{0}C for 1.0 min, followed by a 10-min extension of 72 ^{0} C with a Thermocycler. For separation of the DNA fragments, we ran the amplified product on 0.8% (w/v) agarose gel at 100 V.

2.7. Alkaloid extraction

The arial parts such as a the transformed roots, control roots,leaves and roots of *Atropa komarovii* were finely powdered in a mill, and hexane was used for percolating 0.8 gr of each sample. Removal of the solvent under lowered pressure and temperature was followed by re-extraction of the residue with methanol. The solvents were evaporated under the reduced pressure. The extract was directly analyzed by HPLC (Hashimoto *et al*., 1993).

2.8. HPLC analysis

The alkaloids underwent HPLC analyses based on Vasdekis *et al*. (2008) with slight modification. Hyoscyamine and scopolamine were analyzed with high-performance liquid chromatography. There was an ultraviolet-visible detector and a C_8 reverse phase column in the HPLC system, with the mobile phase being acetonitrile, $H_3PO_4$50 Mm (pH 2.9) 15:85 (v/v). The flow speed was1 ml per minute. The detection wavelength and injection volume were 254 nm and 20 µl each time. After prepared in methanol, the standard samples of scopolamine and hyoscyamine were diluted into 10, 20, 40 and 60 ppm and were obtained through serial dilution of the stock solution with methanol. The peak area of the standard alkaloid samples against their concentrations was plotted for construction of the calibration graphs. Linear calibration graphs with good correlation were obtained for standard solutions.

2.9. Statistical analysis

Each piece of data was the mean obtained from three replicates. SPSS V22.0 was used for performing the statistical analyses. We utilized t-test and the univariate procedure at statistical significance of $P < 0.05$.

3. Results and discussion

3.1. Hairy root induction

For three weeks after infection of *Agrobacterium rhizogenes* ATCC 15834, the hairy roots were induced. In the control leaves, the roots were induced after a month. Most of the leaves treated with *A. rhizogenes* were induced. Apart from some other species of *Atropa*, nothing has been reported so far on transformation in *Atropa komarovii*. According to the findings obtained from infection of five to six young *Atropa komarovii* leaves by *A. rhizogenes* strain ATCC15834, the plantlet has high sensitivity to this strain due to transformed roots possibly emerging from 70 percent of the leaves. Visible roots were formed after 10-12 days of infection where bacteria had been inoculated on leaf discs. Hairy root formation occurred on the infected leave midribs, and roots were formed in control explants. Very rapid growth of transformed roots with plenty of lateral branching on negative geotropism and hormone-free MS basal medium was also observed. We excised the hairy roots and the control roots from the necrotic explant tissues and subculture three weeks later on fresh agar solidified medium that contained 500 mgl^{-1}cefotaxime in the dark (Fig.1).

Researchers have made use of various parameters, such as plant tissue age, bacterial suspension density, and bacterial strain, for plant infection via *A.rhizogenes* (Bensaddek *et al*., 2008). Another important factor for establishment of hairy roots is the source of explants

that must be true to type of the plant and should not have any genotypic variation (Khatodia *et al*., 2013).

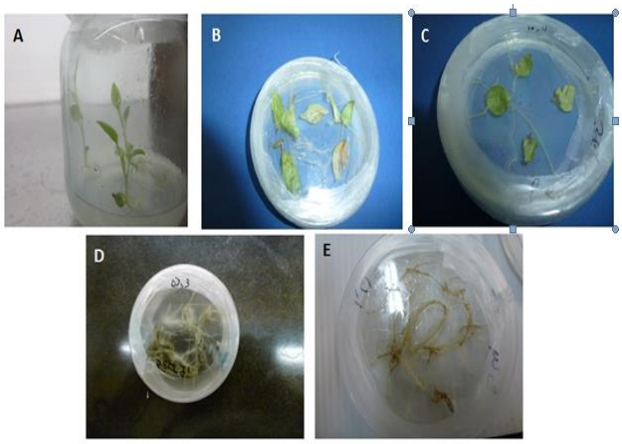

Fig 1. Establishment of hairy root of Atropa komarovii .A: plantlet of Atropa komarovii in MS medium; B: Transformed roots emerged from wounded site with *Agrobacterium rhizogenes*, 21 days after infection; C: Roots emerged from leaves segments free of bacteria; D: Hairy roots culture in MS medium without plant growth regulator; E: Non transformed roots in MS medium without plant regulator.

3.2. PCR analysis for rol B gene

The presence of *rol*B gene in the genomic DNA of the putative transgenic and non- transgenic (control) regenerated hairy roots was confirmed by PCR using specific primers yielded fragments of 500bp. The hairy root transgenic DNA played the role of a template. There was no amplification in the control hairy roots (negative control) with the primers. It was exhibited by all of the transformed hairy roots that diagnostic 500bp *rol*B product amplification was present (Fig. 2). The results indicate that the *A. rhizogenes* that was applied in the research impressively induced hairy roots on explants proper for use for hairy root mass production in *Atropa komarovii*. We utilized *A. rhizogenes* ATCC 15834, which is one of the most commonly used strains for hairy root induction, and its agropine type includes two separate T-DNA regions, namely, TR-DNA and TL-DNA (Sevon *et al*., 2002). There are lots of plants for which *rol*B alone sufficiently induces rooting and exists in every Ri plasmid, with an identity of about 60% between strains and *rol* genes (Britton *et al*., 2007), functioning as cell growth and differentiation modulators in *A.rhizogenes* (Widorento *et al*., 2012). According to earlier research on *rol*B gene transformed plant culture, secondary metabolism is activated in most cultures but not all of them (Bulgakove *et al*., 2002). It should be noted, however, that the above studies have been restricted to specific plant species. There are several reports of the impact of *rol*B gene on hairy root induction and secondary metabolite production stimulation. An example is the production of tropane alkaloid in the hairy root cultures of *Atropa belladonna* (Bulkagov *et al*., 2002).

3.3 Analysis for scopolamine and hyoscyamine

The hyoscyamine and scopolamine content of the transformed roots, untransformed roots (control), roots and leaves of the plantlet were compared. To obtain the tropane alkaloid values, we cultured the hairy and untransformed roots in MS solid medium, and we subcultured them every two weeks, and for the root and leaves of the plantlet, we used the plantlet in MS solid medium and then investigated their scopolamine and hyoscyamine contents. The content of hyoscyamine was higher in the control root than in the other treatment (Fig. 3), and the content of scopolamine washigher in the hairy root than in the other treatments (Fig. 4).

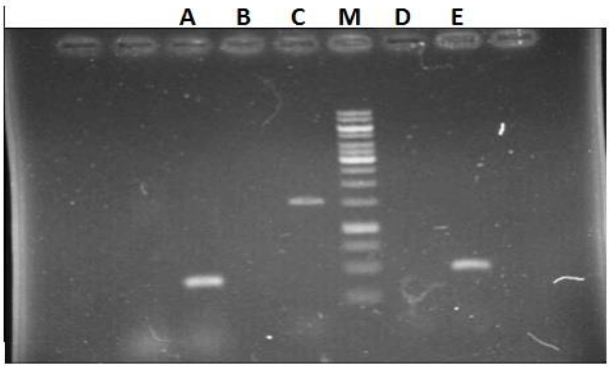

Fig 2. PCR analysis of hairy root culture of *Atropkomarovii* transformed *Agrobacterium rhizogenes* ATCC 15834. lane M –Marker (1kbp): lane A - genomic DNA of hairy root culture showing amplified fragment of *rol*B (500bp): lane B- genomic DNA from normal root culture (negative control): lane C- PCR(positive control): lane D - PCR (negative control): lane E- genomic DNA of hairy root culture showing amplified fragment of *rol*B (500bp).

The HPLC profile of the hairy root cultures is represented in Figure 9. The retention time of the scopolamine contents of the hairy root culture was compared with those of the standard scopolamine and hyoscyamine (Sigma). In the HPLC profile, the RT

value showed similar peaks for both the standard and the extract of hairy root culture. The results concerning the main tropane alkaloids (scopolamine and hyosciamine) from *Atropakomarovii* plant tissue with hairy roots show a significantly greater scopolamine amount than the hyoscyamine amount in hairy roots, as opposed to the case in the other treatments (Fig. 3). Oppositely, the maximum amount of hyoscyamine was observed here in the control roots (Fig. 4).

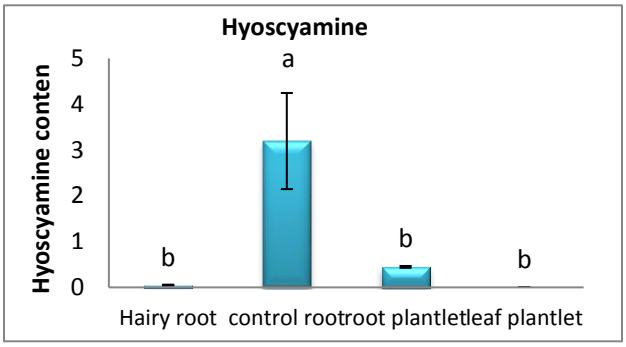

Fig 3. Analyses for hyoscyamine contents in hairy root,control roots, root and leaf plantlet of *Atropakomarovii.*

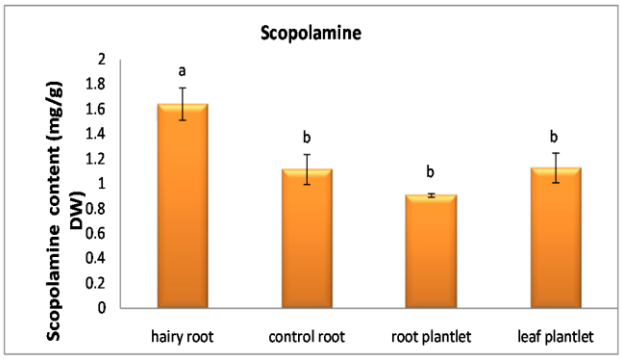

Fig 4. Analyses for scopolamine contents in hairy root,control roots, root and leaf plantlet of *Atropakomarovii.*

We can conclude that alkaloids seem to be distributed in *Atropa komarovii* as specified by the tissue. According to a significant number of studies in the field, in some *Atropa* species, the tropane alkaloid synthesis site is in the roots and is then transported to the aerial organs (Ashtiania and Sefidkonb, 2011). According to the above explanation, the control roots had high amounts of hyoscyamine (atropine), so it could be concluded that the content of hyoscyamine was high in aerial parts. The observation that several biosynthetic genes are overexpressed in plants controlling gene expression in the target bioengineering pathway constitutes a promising, practical strategy to change the accumulation of particular secondary metabolic products (Liu *et al.*, 2010). According to Liu's reports, enhanced hydroxylase activity was shown by the engineered *A. belladonna* hairy roots, which produced scopolamine concentration five times more than the control roots. The research introduced two rate-limiting enzyme genes into *A.belladonna*, which included putrescine*N*-methyltransferas and hyoscyamine 6β-hydroxylase, and *h6h* and *pmt* genes were integrated into the genomic DNA of transgenic plants, and this enzyme, is in charge of transforming hyoscyamine to scopolamine in two stages, in hairy roots which is higher than in non transformed roots so it can be said that hairy roots have more scopolamine and higher DW and FW than control roots

3.4. Analysis for fresh weight and dry weight

In the first and second weeks, there is no difference between the hairy root and control root in fresh weight, but significant change was seen in the third and fourth weeks when fresh weight was greater in the hairy than in the control roots (Fig. 5). Dry weight exhibited a significant difference between the two treatments from the first week, and this difference was observed during four weeks. In the first week, no significant difference was observed between the treatments, but the difference between hairy and control roots was significant from the second to the fifth week (Fig. 6).

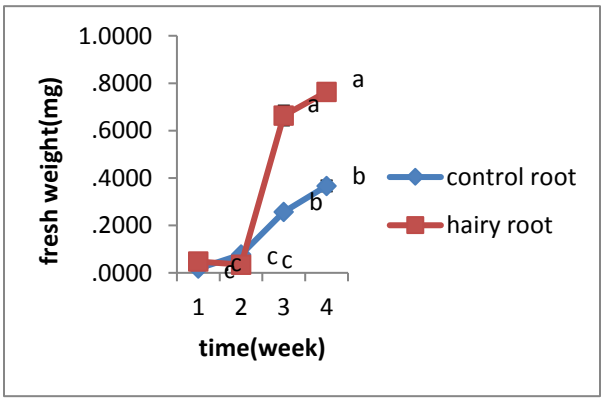

Fig 5. Changes of fresh weight in hairy root and control during four weeks in *Atropa komarovii*. Different letters on bars refers to significant difference (P<0.05).

3.5. Lengths of the roots

In the first week, no significant difference was observed between the treatments, but there was a significant difference between the hairy roots and control roots from the second to the fifth week (Fig. 7).

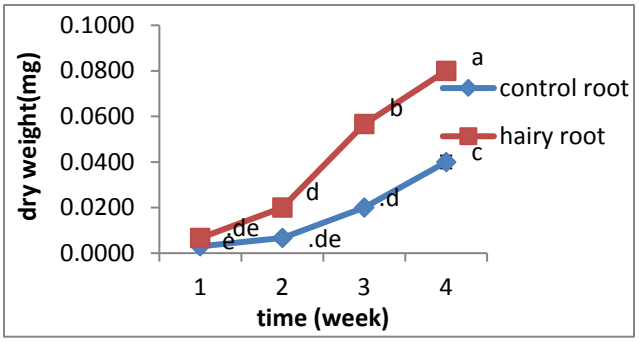

Fig 6. Changes of dry weight in hairy root and control during four weeks in *Atropa komarovii*.
Different letters on bars refers to significant difference (P<0.05).

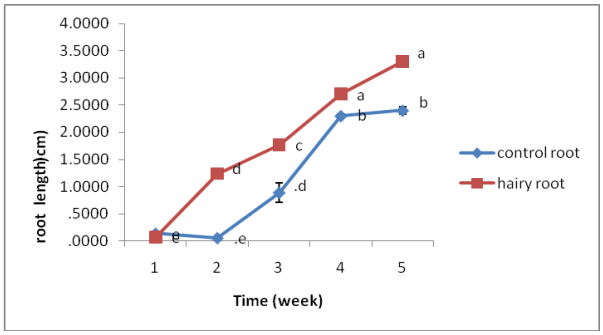

Fig 7. Time course of hairy roots and control roots length in *Atropa komarovii* .
Different letters on bars refers to significant difference (P<0.05).

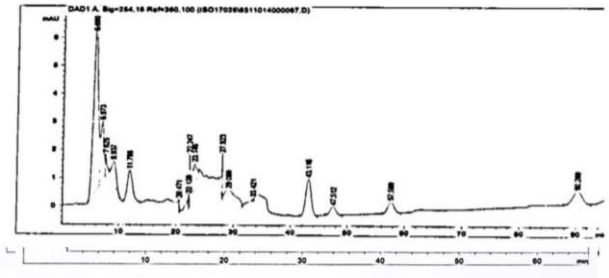

Fig 8. Scopolamine and hyoscyamine standard HPLC chromatogram.

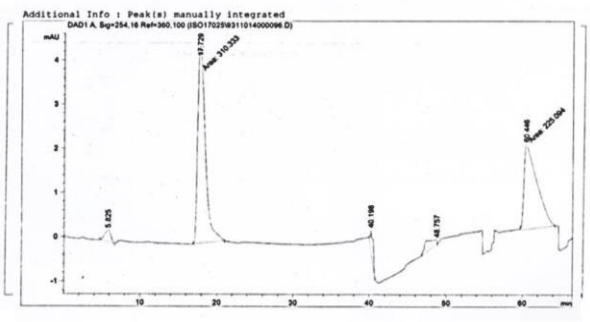

Fig 9. Scopolamine and hyoscyamine HPLC chromatogram of *Atropakomarovii* hairy roots culture.

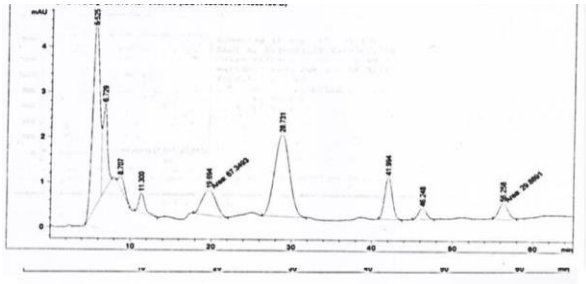

Fig 10. Scopolamine and hyoscyamine HPLC of *Atropakomarovii* control roots culture.

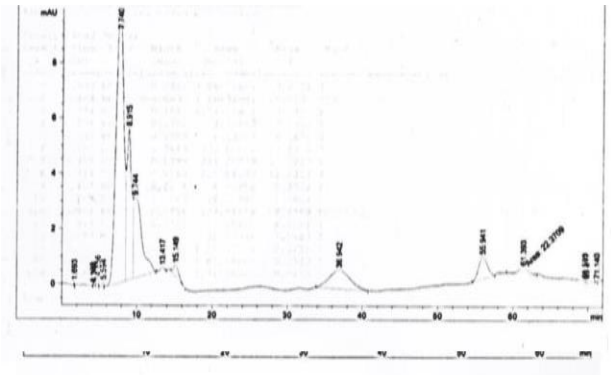

Fig 11. Scopolamnine and hyoscyamine HPLC of *Atropakomarovii* leaves culture.

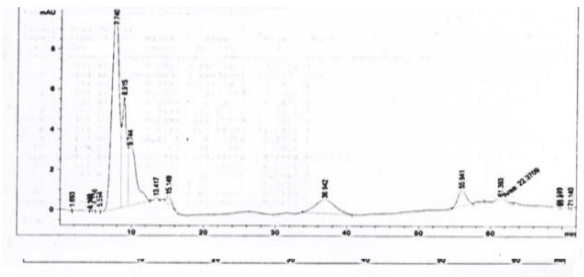

Fig 12. Scopolamine and hyoscyamine HPLC of *Atropakomarovii* root culture.

4. Conclusion

Scopolamine is the important compound in *Atropa komarovii*. The present study was designed to increase scopolamine production in hairy roots. Developing hairy root cultures mediated with *Agrobacterium rhizogenes* constitutes a significant prospect for high-value secondary metabolite production in commerce. In the future, we can improve productivity for instance by developing the biosynthetic pathway through overexpressing genes that codify enzymes in the metabolic pathway for increasing useful secondary metabolites in plant medicine.

5. Acknowledgements

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