ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Quantification of Phytochemicals in hairy root cultures of *Rubia cordifolia* Linn

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Manuscript Info

Manuscript History:

Received: 15 January 2015
Final Accepted: 22 February 2015
Published Online: March 2015

Key words:

alkaloids, flavonoids, hairy roots, phenolics, alizarin, *Rubia cordifolia* Linn.

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Abstract

Hairy roots were successfully induced using leaf and stem explant and were analyzed for the presence of Alizarin using RP-HPLC. The transgenic nature of hairy roots was confirmed by PCR. The growth curve study performed showed high biomass generation from the roots grown in MS media than that of ½MS and B5 media. The fresh and dry weight of hairy roots after four weeks of culturing found to be 86.92±2.5g and 4.74±0.14g respectively. These roots were further analysed for total phenolics, flavonoids and alkaloids using different solvent system**.** The Methanol extract of dried roots of B_5 media showed highest accumulation of Total Phenolic Content (139.719±0.856 mg GAE g^{-1}) and flavonoids (115.615±1.208 mg RE g^{-1}) while aqueous extract of dried roots from B_5 media showed highest (32.429±0.569 mg CE g^{-1}) accumulation of alkaloids. Alizarin content was estimated from the hairy roots grown on different media as well as field grown roots. Hairy roots grown on MS medium showed higest accumulation of alizarin which was obsreved to be 7.42 and 5.16 fold increase, in fresh and dry roots respectively, as compaired to field grown roots. These properties of hairy roots of *Rubia cordifolia* have opened up further avenues for the research.

INTRODUCTION

Rubia cordifolia Linn is a climbing perennial herb of family Rubiaceae, widely distributed in the hilly areas of India and also in the different parts of the world. It is known to accumulate useful phytochemicals especially anthraquinones. The presence of anthraquinones in plant species of Rubiaceae gave commercial importance and exhibited several activities such as antimicrobial (Sittie et al. 1999; Bringmann et al. 2008; Xiann et al. 2008), antifungal (Singh et al. 2006), anticancer (Zhang et al. 2007; Son et al. 2008; Patel et al. 2011), hypotensive, analgesic (Younos et al. 1990), antimalarial (Sittie et al. 1999), antileukemic and mutagenic properties, antioxidant (Joharapurkar et al. 2003; Rawal et al. 2008). The anthraquinones obtained are used to treat haematemesis, haematuria, inflammations, ulcers and skin diseases (Miyazawa and Kawata, 2006; Jaijesh et al. 2008; Gahindo et al. 2008). The hairy root cultures of *R. cordifolia* are known to accumulate higher amount of anthraquinones (Bulgakov et al. 2004), while callus is known to accumulate manjistin, purpurin (Mischenko et al. 1999); as well as rubiadin, quinizarin, lucidin and 1,8-dihydroxy-anthraquinone (Banyai et al. 2006).

In present study, an attempt was made to established hairy root system in *R. cordifolia* and the roots were further studied for total phenolic, flavonoid , alkaloid and alizarin content using RP-HPLC.

Materials and methods

Reagent and standards

Ascorbic acid, aluminium trichloride, ferric chloride, folin-ciocalteu reagent, sodium bicarbonate, 1,10-phenanthroline, tannic acid, rutin and colchicine were purchased from Sigma chemicals, USA. HPLC grade water, acetonitrile and alizarin standard were purchased from Merck, India.

Hairy root induction

Source of explants

The *in-vitro* shoot cultures of *R. cordifolia* were established from axillary bud explants on MS (Murashige and Skoog 1962) medium supplemented with 1.0 mg l^{-1} thiadiazuron (TDZ) as described earlier (Ghatge et al. 2011). The cultures were maintained under control conditions such as temperature 25+1^{O}C, relative humidity 70% and 16/8 hours photoperiod with light intensity (40 µmol m^{-2} s^{-1} photon flux density). One month old stem and leaf explants (~ 5 mm^2) were excised from *in-vitro* grown shoots and pre-cultured on MS basal medium for 2 days. The culture plates were incubated under control conditions as mentioned earlier and were used for the infection with *Agrobacterium rhizogenes.* Similarly, the *in-vitro* grown leaf explants of *Nicotiana tabacum* were pre-cultured on MS basal medium and used as control.

Bacterial strain and culture condition

Agrobacterium rhizogenes strain NCIM 5140 procured from National Chemical Laboratory (NCL), Pune (India) and was established on YEB agar medium as described earlier by Patil et al. (2009). The culture plates were incubated in the tempearature controlled incubator at 27^{O}C, whereas the flasks of bacterial suspension cultures were maintained by growing on shaker at 100 rpm agitation speed. Optical density (O.D.) of the suspension culture was measured using double beam spectrophotometer (Shimadzu, Japan) at 600 nm. The density of the suspension culture was adjusted to 0.6 and used for transformation. The suspension culture was centrifuged at 4,000 rpm for 5 min and the pellet was re-suspended in 10 ml MS liquid medium and used for infection.

DNA Isolation and PCR confirmation:

Genomic DNA was extracted using CTAB method (Doyle and Doyle 1987) and transgenic nature of the hairy root was confirmed as per Teleke et al. (2011).

Growth kinetics

The hairy roots obtained from leaf explant were further used in investigation of the growth kinetic studies for four weeks. All the experiments were performed in triplicates using MS, ½ MS and B_5 media. The growth performance was recorded at the interval of 3 days till 27 days and dry weight was recorded after 27 days of culturing. After 27 days, the roots were harvested and were subjected to biochemical analysis of total phenolics, flavonoids, alkaloids and Alizarin content.

Sample Preparation:

The fresh and dry hairy roots of *R. cordifolia* were crushed in 50 ml of solvent and filtered through the muslin cloth. The extracts were centrifuged at 10,000 rpm for 10 minutes and supernatant was collected. The extracts were condensed on the rotary evaporator and stored at 4^{0}C. The assay protocol was followed as per Chaturvedi et al. (2011).

Quantitative determination of total phenolics content:

The total phenolics content from all the extracts was determined using the method of Singleton and Rossi (1965). The reaction mixture was prepared by adding 0.125 ml of 1% extract and 1.8 ml of Folin-Ciocalteu reagent. The assay mixture was allowed to stand for 5 min at room temperature. 1.2 ml of aqueous sodium carbonate was added to the mixture and the mixture was kept in dark for 90 min at room temperature. Absorbance was recorded at 765 nm using UV-VIS spectrophotometer. The total phenolics content was quantified comparing with standard curve of gallic acid and total content was expressed as mg of gallic acid equivalents per g of roots.

Quantitative determination of total flavonoids content:

The total flavonoids content from all the extracts was determined using the method proposed by Luximan-Ramma et al (2002). The reaction mixture was prepared by adding 1.5 ml of 1% extract and 1.5 ml of 2% methanolic aluminum chloride. The reaction mixture was incubated for 10 min at room temperature. The absorbance was measured at 368 nm. The total flavonoid content was quantified comparing with standard curve of rutin and total content was expressed as mg of rutin equivalents per g of root.

Quantitative determination of total alkaloid content:

The total alkaloid content from all the extracts was determined using the method of Singh et al. (2004). The reaction mixture was composed of 1% extract, 1 ml of 0.05M, 1-10 phenanthroline and 1 ml of 0.025 M ferric chloride. The reaction mixture was incubated in water bath at 70^{o}C for 30 min and absorbance was recorded at 510 nm. The total flavonoids content was quantified comparing with standard curve of colchicine and total content was expressed as mg of colchicine equivalents per gm of root.

Quantitative determination of Alizarin:

For quantification of Alizarin content the roots of field grown plant and the hairy roots were used. The plant material was refluxed with water:ethanol (75:25) for 18 hours. The samples were centrifuged at 10,000 rpm for 10 min and the supernatant was collected. These were evaporated on rotary vacuum evaporator and dried samples were eluted in ethanol and were filtered through 0.22 µm filter and used for HPLC analysis.

The quantitative analysis of Alizarin was performed by Waters HPLC system (model 2487) UV-Visible dual wavelength detector. The separation of Alizarin was conducted using a C18 column (Merck LiChrospher®), 5µm, 150×4.6 mm ID. The separation was carried out in isocratic mode using methanol and 10% aqueous acetic acid (60:40) as mobile phase with the flow rate of 1ml/min and the detector was set at 254 nm. The solvent used for separation was filtered through a nylon membrane (0.45µm×47mm) and degassed by sonication before use. The analysis of Alizarin was performed using some modification in method of Banyai et al. (2006).

Standard solutions of different concentration of alizarin 10, 25, 50, 75 and 100µg/l were prepared in ethanol. The 20µl of each standard concentration was injected. The calibration graph was constructed by plotting area of alizarin versus concentration. The identified peak was compared with peak of standard and Alizarin was quantified in the sample.

Results and Discussion

Hairy root induction

The leaf explant of *Nicotina tabacum* when infected with *A. rhizogenes* showed cent percent response, where as the stem explant of *R. cordifolia*, showed 58.18% response with 4.56±0.92 roots per explant and leaf explant showed 38.26% response with 4.23±0.82 roots. (Table no 1) The initiation of hairy roots was observed within a week of *Agrobacterium* infection. (Fig. 1a) Similar result were also reported in allied species like *R. tinctorum* (Sato et al. 1991., Ercan et al. 1999), *R. nakai* (Park et al. 2009), *R. peregrine* (Lodhi and Charlwood 1996) and *R. cordifolia* (Shin and Kim 1996) using different explants like cotyledon and stem, with different strains of *A. rhizogenes*. (15834, R1000, 9365, 2628 etc). The Lee et al (2010) reported the influence of different strains of *A. rhizogenes* on hairy root induction and secondary metabolite production in *Rubia akane* Nakai, they found that R1601 is the good strain for hairy root induction amongst the 13333, 15834, R1000, R1200 with 85.6% response also they observed the highest biomass production with high content of alizarin and purpurin in same strain of *A. rhizogenes*.

The A. rhizogenes infects the plant and produce the hairy root. The strains of *A. rhizogenes* show the variation in hairy root induction, also it shows the difference in percent response to the different explant like stem, cotyledon and leaves. The different strains of *A. rhizogenes* also affects on biomass production and secondary metabolite production.

The transgenic nature of hairy roots was confirmed by PCR analysis using a set of specific primers which amplified 970bp and 498bp domains present on T-DNA region of the *A. rhizogenes* plasmid. (Patil et al. 2009). (Fig. 1b)

Table 1: Hairy root induction.

Explant used	No. of explant infected	% Response	Average no. of roots per explant
Stem	30	58.18±5.25	4.56±0.92
Leaf	30	38.26±7.83	4.23±0.82

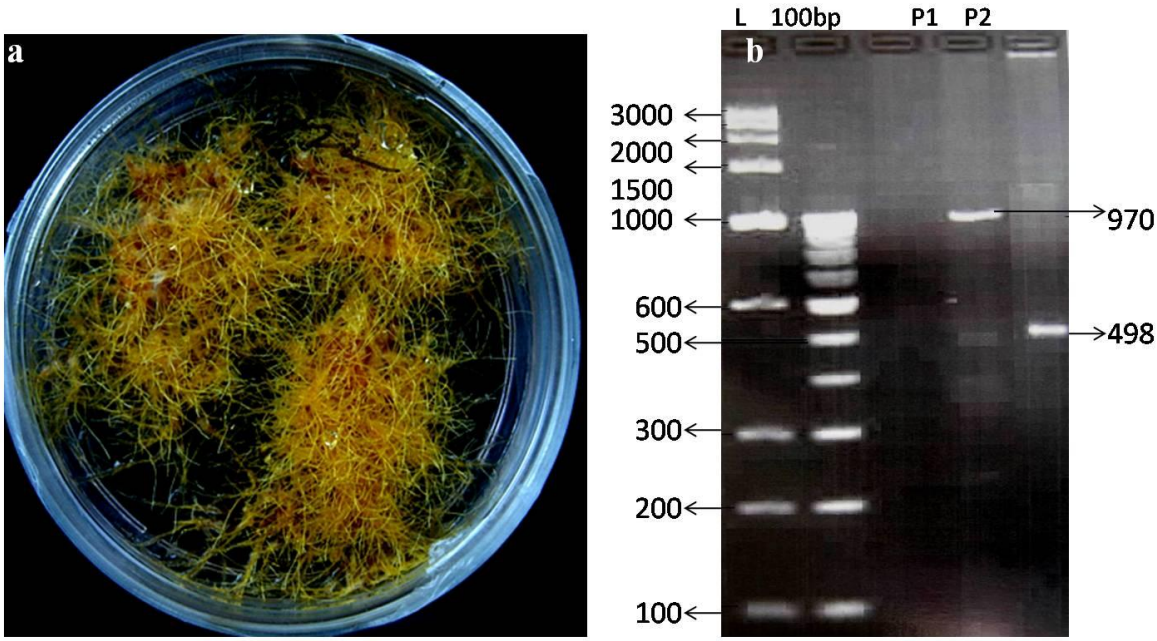

Figure 1- (a) A plate showing hairy roots of *Rubia cordifolia*. (b) PCR confirmation of hairy roots, Lane LMM and 100bp- Marker Ladder, Lane P1 and P2- Transformed hairy roots

Hairy root growth kinetics

Among the different types of growth media used for the hairy root cultures of *R. cordifolia*, MS medium was found most suitable in comparison to ½ MS and B5 medium. (Fig. 2) Growth kinetics of hairy roots revealed significant increase in the fresh and dry biomass with increase in the incubation period from 3 to 27 days. At the end of the 27 days, almost 9 and 11-fold increase in fresh and dry biomass has been observed, respectively (Table 1). Similarly, the increase in hairy root biomass has been reported in the hairy roots of *R. tinctorium* (Ercan et al. 1999) and *R. arken* (Park et al. 2009).

Table 2: Growth pattern of *Rubia cordifolia* hairy roots in MS medium

Harvest in days (from the day of inoculation)	Fresh weight (g)	Dry weight (g)
3	9.92 ± 0.04	0.426 ± 0.02
6	12.7 ± 0.56	0.526 ± 0.02
9	13.2 ± 0.44	0.66 ± 0.02
12	17.84 ± 1.14	0.86 ± 0.05
15	38.0 ± 3.8	1.6 ± 0.06
18	37.2 ± 2.14	1.98 ± 0.12
21	58.42 ± 4.0	2.0 ± 0.66
24	65.3 ± 3.4	3.52 ± 0.1
27	86.92 ± 2.5	4.74 ± 0.14

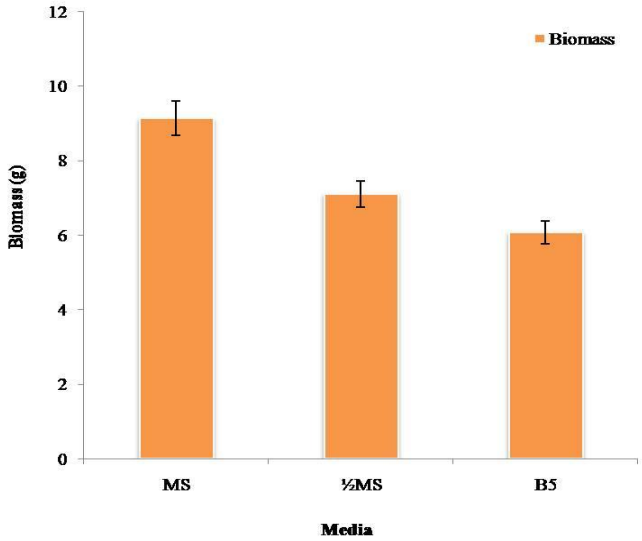

Figure 2- Total biomass recovered after 27 days of culturing on different media

Total Phenolic content

Dried roots of field grown plant showed highest phenolic content in methanol extract (41.021±0.813 mg GAE g^{-1}), and the lowest content was observed in aqueous extract of fresh roots (8.929±0.948 mg GAE g^{-1}). In dried hairy roots, highest phenolic contents was recorded in the methanol extract of hairy roots grown on B_5 medium (139.719±0.856 mg GAE g^{-1}), where as the lowest content was observed in aqueous extract of hairy roots grown on MS medium (56.210±0.687 mg GAE g^{-1}). In the fresh hairy roots, the highest phenolic content was observed in ethanol extract of hairy roots grown on MS medium (75.719±1.893 mg GAE g^{-1}) and lowest content in aqueous extract grown on ½ MS medium (15.719±0.694 mg GAE g^{-1}) (Table 3).

Dried hairy roots harvested from B_5 medium contained comparatively higher phenolic compounds than those grown on MS, ½ MS media and roots of field grown plant. Fresh hairy roots on MS medium exhibited higher phenolic compound compared to those grown on B_5 and ½ MS media and roots of field grown plants. The highest phenolics were recorded in methanol dried roots extract and ethanol fresh root extracts respectively. Methanol was the best solvent for extraction of phenolic compounds for different extracts prepared from dried roots, while, ethanol was found to be the best solvent for extraction of phenolic compounds for different extracts prepared from fresh roots.

Table. 3: Total phenolics content in *Rubia cordifolia* hairy roots

Media	Solvent	mg GAE/g fresh wt	mg GAE/g dry wt
Field grown	Water	8.929 ±0.948	26.084 ±0.776
	Ethanol	13.282 ±0.958	34.716 ±0.525
	Methanol	12.573 ±1.051	41.021 ±0.813
MS	Water	32.210 ±0.909	56.210 ±0.687
	Ethanol	75.719 ±1.893	67.929 ±0.977
	Methanol	40.912 ±1.170	109.473 ±1.297
½ MS	Water	15.719 ±0.694	81.263 ±0.859
	Ethanol	66.807 ±0.977	64.280 ±1.461
	Methanol	23.719 ±1.789	85.894 ±0.957

B5	Water	38.175 ±0.775	68.070 ±0.882
	Ethanol	58.877 ±1.378	131.298 ± 1.144
	Methanol	50.035 ±1.764	139.719 ± 0.856

Total Flavonoid Content (TFC)

The highest flavonoid content was observed in methanol extract of dried roots (30.045±1.336 mg RE g^{-1}), and lowest content was observed in methanol extract of fresh roots (10.186±0.674 mg RE g^{-1}).

In dried hairy roots, highest flavonoid contents were recorded in the methanol extract of hairy roots grown on B_5 medium (115.615±1.208 mg RE g^{-1}),where as the lowest content was recorded in ethanol extract of hairy roots grown on ½ MS medium (44.541±1.178 mg RE g^{-1}) (Table 4).

The flavonoid content was highest in methanol extract of fresh hairy roots grown on B_5 medium (42.237±0.940 mg RE g^{-1}) which was lowest in hairy roots grown on MS medium (15.167±0.922 mg RE g^{-1}) (Table 4).

Results represented here indicated that the dried roots possess the highest flavonoids content as compared to the fresh roots. From the various media studied, the hairy roots grown on B_5 medium, in both, fresh as well as dry hairy roots; showed highest content of flavonoids.

Table. 4: Total Flavonoids content in *Rubia cordifolia* hairy roots

Media	**Solvent**	**mg RE/g fresh wt**	**mg RE/g dry wt**
Field grown	Water	12.220 ±0.730	24.665 ±0.478
	Ethanol	13.505 ±0.947	21.954 ±1.112
	Methanol	10.186 ±0.674	30.044 ±1.336
MS	Water	19.127 ±0.773	79.060 ±1.129
	Ethanol	28.926 ±1.386	63.199 ±0.811
	Methanol	15.167 ±0.922	112.483 ±1.275
½ MS	Water	22.393 ±1.078	77.606 ±1.663
	Ethanol	36.979 ±0.660	44.541 ±1.178
	Methanol	25.078 ±1.289	95.369 ±1.170
B5	Water	23.534 ±1.312	83.892 ±1.162
	Ethanol	36.979 ±0.887	70.246 ±0.911
	Methanol	42.237 ±0.940	115.615 ±1.208

Total Alkaloid content.

The alkaloids analysis of roots revealed that the highest content was found in dried roots compared to fresh roots. Amongst all the media studied, B_5 medium for dried hairy roots and MS medium for fresh hairy roots were found to yield highest alkaloid contents. Among the various solvents used for extraction of alkaloids, aqueous for dried roots, where as methanol for fresh roots, were found to be the best solvent for alkaloids.

The alkaloid analysis showed the highest level both in roots from field plants (12.293±0.756 mg CE g^{-1}) and hairy roots in aqueous extract, which reduced considerably in ethanol extract of fresh roots (3.574±0.464 mg CE g^{-1}).

The highest alkaloid content was recorded in the aqueous extract of hairy roots grown on B_5 medium (32.429±0.569 mg CE g^{-1}), where as the lowest content was recorded in ethanolic extract of hairy roots grown on MS medium (18.566±0.884 mg CE g^{-1}) (Table 5).

The methanol extract of fresh hairy roots grown on MS medium showed alkaloid content of 23.269±0.507 mg CE g^{-1}; where as the lowest content was observed in ethanol extract of hairy roots grown on MS medium (5.923±0.534 mg CE g^{-1}) (Table 5).

Table. 5: Total alkaloids content in *Rubia cordifolia* hairy roots

Media	Solvent	mg CE/g fresh wt	mg CE/g dry wt
Field grown	Water	3.934 ±0.250	12.293 ±0.756
	Ethanol	3.574 ±0.464	9.673 ±0.282
	Methanol	4.556 ±0.319	11.022 ±0.234
MS	Water	8.141 ±0.549	29.104 ±0.877
	Ethanol	5.923 ±0.534	18.566 ±0.884
	Methanol	23.269 ±0.507	23.057 ±0.537
½ MS	Water	7.637 ±0.596	27.625 ±0.373
	Ethanol	7.805 ±1.161	21.265 ±0.305
	Methanol	12.564 ±1.069	22.877 ±0.951
B5	Water	9.563 ±0.421	32.429 ±0.569
	Ethanol	8.040 ±0.690	28.521 ±1.050
	Methanol	23.101 ±0.433	20.615 ±0.445

Determination of Alizarin content

The RP-HPLC chromatogram showed presence of alizarin in the sample at retention time (RT) 25.27 min (Fig. 3a) which has been confirmed with the standard of the alizarin (RT = 25.33, Fig. 3b). The results on alizarin content showed maximum accumulation in dry matter in comparison to fresh matter of hairy roots grown on different types of growth media and normal roots of field grown plants (Fig. 5); however the content was found to be significantly higher in dry matter of hairy roots than normal roots. Among the different types of growth media used for alizarin accumulation, the hairy roots grown on MS basal liquid medium showed significantly higher accumulation (2.15, 1.5, 7.42-fold) in fresh and (2.04, 1.46, and 5.16-fold) dry hairy roots than the roots grown on half MS and B5 medium and normal roots of field grown plants, respectively (Fig. 5). Besides, the hairy roots grown on the growth media (MS, 1/2MS and B5) revealed significantly higher content of alizarin leached out in MS and B5 medium than ½ MS (Fig. 6). The accumulation of alizarin has been correlated with the increase in dry biomass of the hairy roots on different types of growth media (data not shown). The results of the present investigation showed comparatively superior accumulation of alizarin in the hairy roots of *R. cordifolia* in comparison to the alizarin accumulation reported from hairy roots of *R. akane* (Park et al. 2009; Lee et al. 2010) and *R. tinctorum* (Banyai et al. 2006). Further research on enhancement of alizarin should be undertaken using different types of elicitors and precursors to scale up the alizarin production from hairy roots of *R. cordifolia*.

Hairy roots have several advantages such as their fast growth, simple nutrient requirements, reproducible growth in liquid cultures, they are easy to maintain and there is no threat to the environment (Patil et al. 2009). Hairy roots exist as a genetically stable tissue culture system and the genetic instability problems are rarely reported as observed in callus and suspension cultures. Further the metabolism in hairy roots is similar qualitatively and quantitatively to non transformed plant materials. These advantages make hairy roots an ideal source to produce and

harvest numerous medicinal drugs as has been reported earlier in various plants (Liu et al. 2006; Yogananth and Basu 2009). The presence of desirable phytochemicals in hairy roots of *R. cordifolia* has opened up the avenues of genetic engineering to boost the contents of these metabolites.

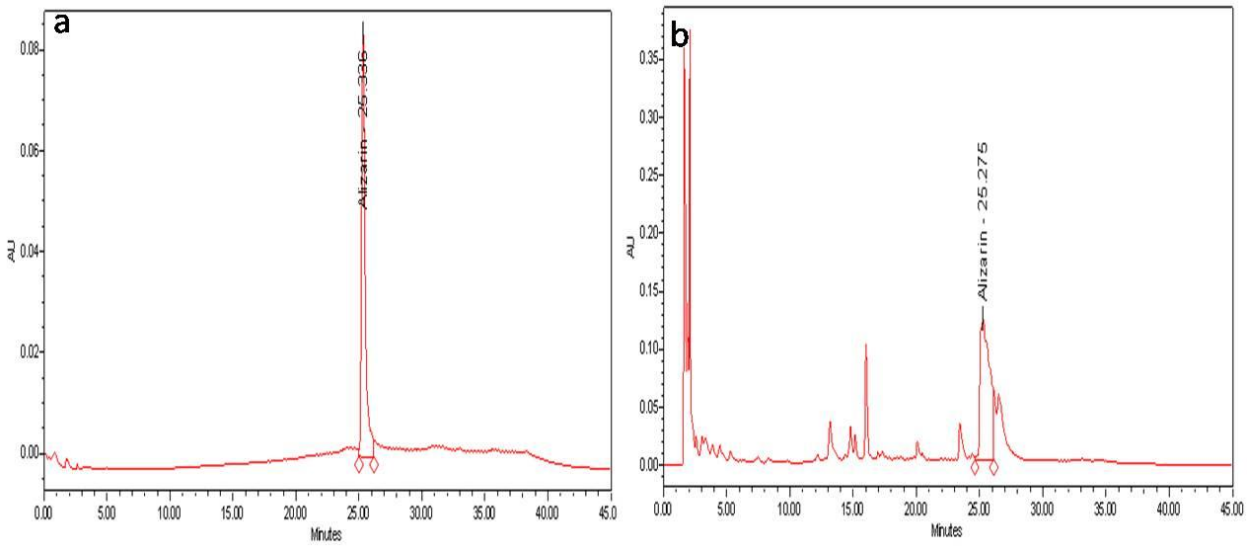

Figure 3- a) Chromatograph of Alizarin standard, b) Chromatograph of Alizarin sample

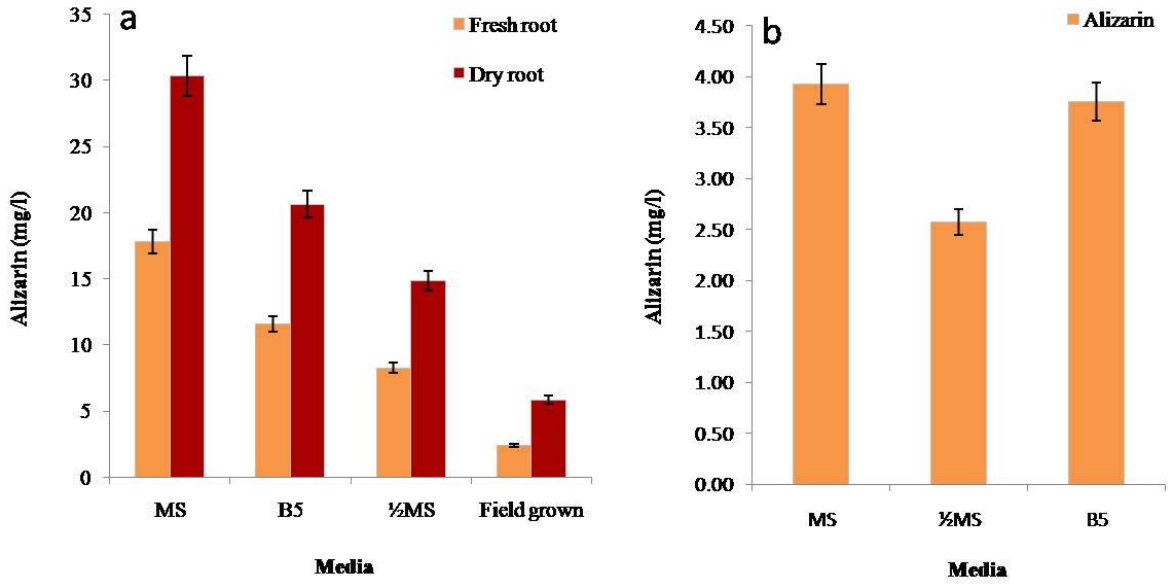

Figure 4- a) Alizarin content in field grown roots and hairy roots grown on different culture media, b) Alizarin content liberated in different culture media.

Conclusion.

This research showed an efficient method of inducing hairy roots in Rubia cordifolia using A. rhizogenes. Hairy root induction in *Rubia* induce significant increase in accumulation of economically important Phytochemicals. We observed 5.16 fold increase of alizarin content in dried hairy roots as compared to dried field grown roots. We also observed a significant increase in the levels of phenolics, flavonoids and alkaloids, in the hairy roots. It has been proved that hairy root accumulate higher amount of metabolites having commercial importance hence efforts need to

be made to develop methods for commercial production of hairy roots from which high yields of secondary metabolite can be obtained.

Acknowledgements:

Authors are thankful to Dr V A Bapat for his guidance and Dr U U Shedbalkar for her assistance and higher authority of university for providing necessary facilities.

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