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Studies on the Use of In vitro Synthesized Red Dye of Arnebia hispidissima L. in Textile Industries

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The shikonin-derivatives produced in the cultures of A. hispidissima were tested for dyeing cotton and woolen fibers. The maximum extraction of red dye (shikonin/alkannin) was extracted in chloroform. The dye was further separated in 5-6 fractions with help of thin layer chromatography (TCL) and column chromatographic (CC) methods. The dye was tested to obtain different color shades on cotton cloths and woolen fabrics with different types of mordants. Herbal mordants like, Prosopis galls extracts and fruit extracts of two species of Terminalia were also used. Different color shades were produced by the mordants. Color fastness tests were also done to check the fastness of the shades and were found positive. The dye was also used to manufacture various cosmetic products.

Keyword: Shikonin/alkannin, Arnebia hispidissima, Red Dye, Mordants.

INTRODUCTION: The discovery of Indigo, the most important Indian natural dye is as old as textile making itself. History reveals that Chinese have recorded the use of dyestuff even before 2600 BC.[1] But after that most of the natural dyes were replaced by synthetic dyes. Natural dyes are now-a-days again in demand not only in textile industry but in cosmetics, leather, food and pharmaceuticals also. Since the last decade, there has been a significant revival of interest in the application of natural dyes on textile materials all over the world, possible because of increasing awareness of environment, ecology and pollution control.[2]

The interest in the use of natural dyes has been growing rapidly due to the result of stringent environmental standards imposed by many countries in response to toxic and allergic reactions associated with synthetic dyes.[1] The synthetic dyestuffs produce hazardous by-products, some of which possess carcinogenic intermediates and hence a ban has been imposed by Germany and some other European countries on the use of benzidine dye in textile garments exported into their countries.[3]

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The rich biodiversity of our country has provided us a plenty of raw materials yet a sustainable linkages must be developed between the cultivation, collection and their use. The roots of various species of the family Boraginaceae are source of naturally occurring hydroxynaphthoquinones and especially isohexenylnaphthazarins, commonly known as alkannin and shikonin. It has antibiotic properties against all types of microorganisms. The naphthoquinones - alkannin derivatives and shikonin (red dye) are produced by number of plants including *Alkanna tinctoria, Macrotomia euchroma, Onosma echiodes, Arnebia* species and *Lithospermum erythrorhizon*.[4] The roots of *A. hispidissima* contain a red purple dye[5] which is used by the tribals for coloring their clothes. The roots are used as an alkanet. This dye is also locally used in textile industry, calico-printing, paints etc. Because of its intense red color it is also used as an important dyestuff for fabrics and cosmetics.[6]

A. hispidissima is known to accumulate shikonin in its roots but it fails to provide sufficient raw material for commercial production. The shade of color, a plant produces will vary according to season at which the plant is picked, how it was grown, soil conditions, etc.[7] This entails the essentiality for developing rapid and efficient *in vitro* propagation methods as well as genetic improvement of *A. hispidissima* for meeting the ever-increasing demand of high-valued shikonin. The production of the red pigment shikonin in culture has been reviewed and discussed in many texts.[8,9,10,11,12,13,14,15] The present investigation deals with the extraction of natural dyes from the cells, callus and root cultures of *A. hispidissima* and to test the *in vitro* produced dye in dyeing of textile fibres.

Materials and Methods

The explants of *Arnebia hispidissima* were collected from the selected sites in Rajasthan, India, during the month of November to March. The nodal segments (2.0-3.0 cm in length) harvested from the field grown plants were used as explants. Explants were surface sterilized under aseptic condition in Laminar air flow hood with 0.1% $HgCl_2$ (w/v) for 3-5 min followed by 4-5 times washing with autoclaved water.

The shikonin/alkannin dye/pigment were synthesized by *in vitro* methods like, callus culture, cell suspension culture and root cultures on different media like MS[16] (Murashige and Skoog), WP[17] (Woody Plants), B_5[18], White's[19] and M-9.[20,21] Effects of auxins and cytokinins on pigment production were studied. The cultures were kept under 20 to 35°C temperature and 10 to 35 µmol m^{-2} s^{-1} Spectral Flux Photons (SFP) for maximum production.

Extraction of shikonin-derivatives (red dye)

The cells/callus/root tissues of *Arnebia hispidissima* were air dried/oven dried at 35 to 40°C for 24 hours. These were weighed encrusted and powered. Extractions were done in Acetone, Benzene, carbon-tetrachloride, Chloroform, Ethanol, Ether and Toluene. Chloroform was finally selected and used as solvent for extraction of the red dye complex as this was found to be most effective. Extraction was done 3 to 4 times at room temperature (25 to 30°C). The combined chloroform extracts were filtered through Whatman paper (Whatman International Ltd, Maidstone, England). The filtrate was washed with water, and the chloroform layer containing pigments like substances was dried and evaporated *in vaccuo*. The chloroform soluble red pigments were separated by the following methods:

1. Preparative thin layer chromatography (TLC) on pre-coated Silica coated Aluminum sheets (Silica Gel 60,F.254) with concentrating zone (Merck, Darmstadt). The dye concentrated in chloroform was applied with Micro-pipettes (Rochester Scientific Company, Inc., Rochester, New, York, USA). The pigments were separated using $CHCl_3$ (in a glass chamber) as solvent. Five of the six pigments isolated were further analyzed.

2. Column chromatographic Method, the pigments extracted from known quantity of plant

tissues were extracted and dried. The $CHCl_3$ extract was reduced to 5-10 ml. These were loaded on Silica Gel (60-120 mess) Column (75 cm long and 3 cm diameter). The pigments were separated by Column Chromatography using chloroform as solvent. The chloroform fractions separated in the silica gel column were collected in flasks. The individual fractions collected were dried in vaccuo and quantified.

The extracted dye was used for dyeing of cotton cloths and woolen fabrics at optimized dyeing conditions like, dye extraction time 60 min, material-to-liquor ratio 1:20, temperature 60^o C and dyeing time 50 min, using combination of mordants. Mordanting is a pre-dyeing process that makes the fibre receptive to dye. Mordant is a chemical that when 'cooked' with fibres attached it to the fibre molecules. A dye molecule attaches itself to the mordant. Herbal dyes require mordant which are metallic salts of aluminium, iron, chromium, copper and others, for ensuring the reasonable fastness of the color to sunlight and also washing.

Bleached plain weave fabric obtained from the market was used for the study. Analytical reagents (AR) grade Stannous chloride, Potassium Alum Sulphate, Ferrous Sulphate, Copper Sulphate, and Potassium Dichromate were used as mordants. *Prosopis* galls and fruit extracts of *Terminalia chebula* (Harar), and *T. bellerica* (Bahera) were used as natural mordants in this study. Depending upon the mordant used, the dye obtained on textiles from the cultures extract may give different shades.

Results and Discussions

Herbal dyes are best with natural fibres such as cotton, linen, wool, silk, jute, ramie and sisal.[7] These dyes are classified[22] on the basis of their chemical structure; Flavones (yellow and brown), 90% of all yellow dyes are flavnoids. The fastness of these yellow dyes is greatly affected by the mordant and the photosensitivity of the chromophores; Iso-quinoline (yellow), the only basic dyestuff known from nature; Cromene (orange yellow); Napthoquinones (Brown and purple grey), although an array of napthoquinones occur in nature, only a few are important as dyes; and Anthraquinones (red), over 95% of known natural red dyes fall into this category. Anthraquinone dyes surpass all other classes of dyes in their fastness properties.
Tissue culture technology has been applied extensively for production of red pigments.[13,23,24,15]

Fig. 1.

Fig. 1A: Dried callus of *A. hispidissima* contains red dye.
Fig. 1B: Extracted dye from the cells, callus and *in vitro* produced roots of *A. hispidissima*.
Fig. 1C: Separated fractions of dye obtained from column chromatographic methods.
Fig. 1D: Different color shades on cloth and wool samples obtained from different mordants with *A. hispidissima* dye.
Fig. 1E: Cosmetic products like moisturizer, nail polish, facial creams, shoe polish, candles and soap made with help of red dye.

A rapid and efficient method for high frequency direct plant regeneration without intervening callus formation from nodal segment has been

developed which allows the availability of plants all around the year. Same time cells, callus and root cultures were also established to synthesize the pigments in laboratory conditions. So that dependency on the uncertain natural production could be avoided.[14] It will help to design strategies for bridging the gap between ever-increasing demand and supply of raw products necessary for obtaining shikonin for cosmetic, dyeing, food, medicinal, and pharmaceutical industry.

The callus, suspension, root culture of *A. hispidissima* synthesized and produced shikonin-derivatives in culture. The media composition affected the pigment synthesis. Maximum dye was produced on the M-9 medium. The callus cultures producing alkannins was oven dried (Fig. 1A), and the dye was extracted (Fig. 1B) and quantified. On this medium 42 mg (per g dry weight of tissues) dye was produced. Agar-gelled, as well as in suspension culture the final concentration of synthesized dye/pigment was found almost equal. The quantitative (relative) yields of alkannins produced by the tissue cultures and separated by TLC and column chromatography (Fig. 1C).

Table 1: Effect of different media on the production of red dye by the cultures of *Arnebia hispidissima*.

S.No.	Media used	Production of red dye (mg/g dry weight)
1.	MS	40
2.	WP	32
3.	B5	30
4.	White's	31
5.	M-9	42
6.	MG-5	35

Dyeing of cotton, woolen and synthetic cloths is possible with herbal dyes. Since this is a plant-derived product, shikonin-derivatives of *A. hispidissima* can also used coloring agents for edible products/food additives. Such applications have been reviewed by several researchers.[25,26,27]

It has been reported that the non-polar hydrocarbons of the protein fibres are considered to be chiefly responsible for the incorporation of the non-polar dye in the structure of wool.[28] The dye extracted from *A. nobilis* exhibits good affinity for wool and cotton fabrics.[29] The dyeing mechanism corresponds well to partition mechanism, confirming that this naphthoquinonoid based dye is absorbed by wool and cotton as a disperse dye.

The different types of shades appeared on white cotton cloth and wool fabrics are shown in Table 2, with various types of modrants. The fruit extracts of *T. chebula* and *T. bellerica* were also produced shades from light brown to dark brown (Fig. 1D). Good wash and light fastness were achieved. Light fastness and wash fastness was found for all the shades. But in some shade it is not very stable. This is due to the reason that superficial dye on the surface is removed more in lighter shade than in dark one.[29]

The yields of shikonin-derivatives were 32, 30, 31, and 35 mg per g dry weight of tissues on WP, B_5, White's and MG-5 media respectively. On MS medium however, the dye production was 40 mg per g dry weight of tissues. The highest yield of the dye was (42 mg per g dry weight) was synthesized on M-9 medium. The effects of media on dye production in culture are shown in Table 1.

The dye complex extracted from the cultures was used to manufacture various cosmetic products. The cosmetic products like moisturizer, nail polish, facial creams, shoe polish, candles and soap etc. (Fig. 1E) could be prepared. The dyes produced by the cultures of *A. hispidissima* are soluble in several types of edible oils/ghee and milk. These are also soluble in terpentine oils/varnishes.

Table: 2. Effect of different mordants on the appearance of shades on the cloths and wool with the dye extracted from the cultures of *A. hispidissima*.

S. No.	Mordant used	Final color/shade of cloth or wool
0.	Control	White
1.	Unmordanted	Light cream
2.	Stannous chloride	Pale to organgish color
3.	Potassium Alum Sulphate	Light blue
4.	Ferrous Sulphate	Light cream color with green tone
5.	Copper Sulphate	Ash color
6.	Potassium Dichromate	Ash color with blue tone
7.	*Prosopis* galls	Ash color with green tone
8.	*Terminalia chebula* (Harar) fruit extract	Light brown (cloth as well as wool)
9.	*Terminalia bellerica* (Bahera) fruit extract	Brown (cloth as well as wool)

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