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IDENTIFICATION OF LUPEOL DERIVED FROM IN VITRO CALLUS CULTURE OF HEMIDESMUS INDICUS (L.) R.BR. (ANANTAMOOL)

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ABSTRACT

Hemidesmus indicus (L.) R. Br. is very useful herb in pharmacology. It is used a lot due to its medicinal properties. There are many drug preparations perform by using this plant. Many phytochemicals are present in root, leave and stem of Hemidesmus indicus (L.) R. Br. An efficient medium for callus formation was developed in MS and B5 media supplemented with 2, 4-D, NAA, BA and kinetin. The maximum percentage of callus induction was observed in MS medium containing 1.0 mg/l NAA and 2.0 mg/l KN for in vitro biosynthesis of antioxidants such as lupeol, from Hemidesmus indicus (L.) R.Br. (Anantamool) cultures. Used of identification method is Thin layer chromatography (TLC). In result of Thin layer chromatography analysis, Rf value (Retardation factor) of standard and that of sample extracts lies almost in same range which confirms that extracts contain is Lupeol.

01. INTRODUCTION

Hemidesmus indicus (L.) R.Br. is known as Anantamool. It is placed in Asclepiadaceae family and it is a creeper hedge plant. Hemidesmus indicus (L.) R.Br. is recognized as Sugandi in early years. It's high medicinal value known from 1000 years. Hemidesmus indicus (L.) R.Br contains various phytochemicals which are used in allopathic and unani medicinal system. Plant roots and leaves are used in Ayurvadic medicinal system. Literature survey and also from our studies revealed that Hemidesmus indicus (L.) R.Br. contains lupeol octocosonoate, β-sitosterol, α and β amines, tetracyclic triterpene alcohols, small amount of resin acids, alkaloids, flavonoids tannins, saponins, glycosides, phenols and ketones in its roots (Subramanian and Nair 1968). Hemidesmin and Emidin (Mandal et al. 1991) are

present in the stem. Tannins are present in leaves (Alam et al. 1998). Due to its use in treating different diseases, It has gained a lot of importance in medicinal industry.

Lupeol is found in Hemidesmus indicus (L.) R.Br. plant. Lupeol is a pharmacological active pentacyclic triterpenoid. It has several potential medicinal properties. Currently Lupeol is used for much major medicine. Lupeol is used as content of medicines given in i.e. anticancer disease. It is high demand in domestic and international market. It is antidyslipidemic, ant mutagenic effect, antioxidant, antiinflamaentry. Chromatography is used for Product isolation, Product identification and component detection. Component detection is passed form stationary phase through flow of mobile phase. Sample components are separated based on different migration rates.

Thin layer chromatography (TLC) was introduced in 1938 by Izmailov and Sheraiber when they used this technique to separate components in plant extracts on 2mm thick plates.

02. MATERIAL AND METHOD

2.1 Collection of plant Material

Sterile leaf and root explants (young and old) excised from micro propagated shoot culture were used for the experiment. Transversely as explants for callus induction, long shoots and roots segment which are well expanded 1.5 cm to 2 cm terminal inter-nodal segment under the mortal end.

2.1.1. Preparation of callus

The root/leaves segments were excised from the micro propagated plant for callus induction. Callus was initiated on MS medium fortified with varied concentrations and combination of different auxins like, 2-4 Dichloro acetic acids (2-4D), napthalene acetic acid (NAA) and cytokine like, kinetin (Kn) in 250 ml culture flasks. The pH is adjusted with in acceptance criteria (5.8 pH) previously accumulating agar and sterilization in autoclave as per procedure (Temperature limit 121 ^{o}C, 108 k Pa for 20 minutes).Media are incubated at 25 ±1°C below 16 hours photoperiod. Callus induction of culture is incubated in as same condition for shoot culture/ in total darkness. For callus sub culturing, tiny piece are transferred in solid medium. Suitable selective MS medium and hormones are used for callus induction by explants. Subculture is performed in fifteen days for same medium. All developed callus are compared. Biomass of callus is determined on frequently approximate 56 days. Production of

phytochemicals was assessed. Fresh weight of harvest callus was determined. Callus for 60°C for one day and callus weight were recorded respectively.

2.2. Preparation of extracts

2.2.1 Purification of Solvents

2.2.1.1. Ethanol

Weight of previously dried magnesium turning 5.0 gram and iodine 0.5 gram and placed in 50 ml to 75 ml absolute alcohol. Will heat the liquid mixture until it turns into a methanol, 900ml alcohol was added. The liquid mixture is refluxed for ½ hour. Methanol was received from the distillation and will use it now.

2.2.1.2. Distilled

Water

BY powered drug material of aqueous extraction.

2.2.2. Preparation of Extracts

2.2.2.1. Ethanol extract

Ethanol extraction was keeping in oven for 50^0 C and crammed in soxhlet kit and the extraction liquid mixture until it turns into a ethanol. The liquid extract was filtered when in hot condition and extracts was distilled in vacuum under pressure in sequence to remove the completely. For dried into desiccators. Take weight and interpreted, extracts % yield in expression of air dried powdered crude material.

2.2.2.2. Aqueous extract

Ethanol extract extraction was kept in oven for 50 °C and crammed in soxhlet kit and the extraction liquid mixture until it turns into a methanol. The liquid extract was filtered when in hot condition and extracts was distilled in vacuum under pressure in sequence to remove the completely. For dried into desiccators. Take weight and interpreted, extracts % yield in expression of air dried powdered crude material.

2.3 Thin layer Chromatography (TLC)

2.3.1. Preparation of stock solution: Take Approximately 10 mg of lupeol and dissolved in 5 ml respective methanol solvents. The Flaks is sonicated for 10 minutes with respective and was used for spotting on TLC plates.

2.3.2. Preparation of standard: Take 50 µ/ml of stock solution in volumetric flask and added in 10 ml methanol.

2.3.3. Preparation of sample: 1 g of callus extracts were weighed and dissolved in 25 ml Methanol: chloroform (1:1). The Flask is solicited for 15 minutes. After sanitation flask are rotted in 50 rpm and stands in 01 day for room temperature. The solution is filtered with what- man paper. Finally flittered with 0.45 µm membrane.

2.3.4. Preparation of mobile phase

The mobile phase is used for Aqueous and Ethanol phase.

For Aqueous Phase-: Hexane: Dichloromethane: Methanol (2:3:5)

For Ethanol-: Ethyl acetate: Methanol (4:6).

2.3.5. Preparation of plates: Slurry of silica- gel and distilled water was prepared in a mortar through constant triturating with pestle. The slurry was spread evenly on clean grease free glass plates. The plates were dried in air and thereafter heated in oven at 110ºC for about 30 minutes to activate them.

2.3.6. Application of samples on TLC plate: Sample was applied on the plate with the help of a capillary tube at a distance of about0.5 cm from the developing solution. The solvent from the plate was removed by air-drying and position of the spot was marked.

2.3.7. Saturation of TLC chamber: The inner wall of the chamber was lined with filter paper on three sides, the solvent system was poured up to a height of about 1 cm from the base, grease was applied on the rim of the chamber and it was covered with glass plate. The chamber was allowed to stand for approximate 30 minutes and by that time the filter paper inside the chamber was completely drenched by the solvent system, making the chamber completely and evenly saturated with solvent system.

2.3.8 Development of TLC plates: The solvent vapor is saturated in TLC chamber and plate was positioned at vertically. The Mobile phase had move approximate 80 percentage to spotting line, take the plate out of the developing chamber.

2.3.9. Detection of TLC plates: The spots are representing various compound .Following detection method steps are given below.

i. The Plate was visualized at UV-254 nm and UV-365nm.

ii. By spraying reagent on TLC plate and observed.

2.3.10. Optimization of solvent system: All extract were analyzed by TLC. These fractions constituted of mainly non volatile mixtures of compounds. The Visualization observation was taken inside the TLC plate in UV lamp and spraying reagent on plate.

03. RESULTS AND DISCUSSION

3.1. Standardize the medium and growth regulators for callus culture from different explants

Sterile leaf and root explants (young and old) excised from micro propagated shoot culture were used for the experiment. Transversely as explants for callus induction, long shoots and roots segment which are well expanded 1.5 cm to 2 cm terminal inter-nodal segment under the terminal end. Callus induction frequency was determined for different media by pooling the values over different accessions. of the different media tested, callus induction was observed in every medium.

3.2 Effect of auxin and cytokinin on induction and growth of callus

The small, compact and slow growing callus was preliminary observed in explants cultured in MS medium supplemented with various concentration and combination of 2-4D, NAA and Kn. The explants enlarged and callus developed on the cut surface after 7-10 days while the some explants developed friable callus which covered entire surface of the explants within 28 days. The callus was green at the beginning and turned dark gray after 3-4 weeks. The callus established on several media could be divided into two types, compact and friable callus (Table number 01).

Callus induction was also observed in MS media containing and combination of auxin alone i.e. 2, 4 D and Naphthalene acetic acid within 10-12 days of incubation the root explants depending upon the concentration induces callus.

There is a number of differences in % of callus formation and average fresh weight of callus. Callus induction was different in different hormone concentrations. The maximum % of callus induction is observed 88% in MS medium C-7 containing 1.0 mg/l Naphthalene acetic acid with maximum callus growth in terms of fresh weight is 946.48 ± 6.6, which results is found in soft friable callus (table number 1). However the maximum dried weight is observed 125.37 ± 0.281 in MS supplemented with 1.0 mg/l Naphthalene acetic acid and 0.5 mg/l KN.

Callus color is showed light cream to dark green. Only light green and pale yellow callus are produced phytochemicals. Hence the callus produced by C7 was used selected for further study.

Other auxin supplemented media has low moisture content of callus than it. Callus has pale yellowish green color. Callus with green in color more compact and hard granular which grown on medium supplemented with 2, 4, D.Better callus induction and proliferation observed in callus from root culture on MS medium supplemented with 2, 4, D (1.0 mg/l).On twelve day after inculcation 75% proliferation was recorded up to 40 day with 4 days interval. Callus response was found to high at 24 day compared to shorter duration.

The results indicated that auxins are very important in callus induction and different type of auxins had various effects. In callus induction of anantamool Naphthalene acetic acid is superior to 2,4,D. Dried weight of callus is affected by cytokines in terms of auxins.

Table No.01. Effect of different growth regulators for callus Formation of Hemidesmus indicus (L.) R.Br. (Anantamool).

S.NO.	Name of Medium	Medium + Growth hormones mg/l	% of callus induction X±SD	Fresh wt. of callus (mg) X±SD	Dry wt.of callus (mg) X±SD	Texture of callus
1	C1	MS + 1.0 2,4D	60.12±5.21	344.25±10.5	74.24±0.22	Compact
2	C2	MS + 2.0 2,4D	75.26±3.64	430.32±10.0	89.53±0.25	Compact
3	C3	MS + 1.0 KN	67.53±4.18	345.43±11.0	83.19±0.32	Friable
4	C4	MS + 2.0 KN	79.42±5.83	455.83±8.5	87.67±0.36	Friable
5	C5	MS + 0.5 KN	57.35±4.37	545.32±4.9	87.23±0.32	Friable
6	C6	MS + 0.5 NAA	55.24±6.19	601.73±5.3	97.79±0.42	Friable
7	C7	MS + 1.0 NAA	88.38±5.48	946 .48± 6.6	101.14±0.28	Friable
8	C8	MS + 2.0 NAA	78.62±6.51	843.63±8.9	89.56±0.33	Friable
9	C9	MS +1.0 NAA +1.0 mg/l KN	85.17±3.92	856.39±9.7	125.37±0.281	Friable
10	C10	MS + 2.0 NAA + 2.0 mg/l KN	62.34±4.43	940.28± 00	102.24±0.242	Friable

3.4 Thin layer Chromatography (TLC)

Thin layer Chromatography (TLC) method was develop for identification of Lupeol present in Anantamool roots/ callus. The analysis of sample can be completed within 2 days. The Rf value for each substance is the distance it has moved divided by the distance the solvent front

has moved. Usually, the center of each spot is the point taken for measurement. The results of ethanol and aqueous extracts medium are shows in table number 02.

Rf value (Retradation factor) of standard and that of sample extracts lies almost in same range which confirms that extracts contain is Lupeol.

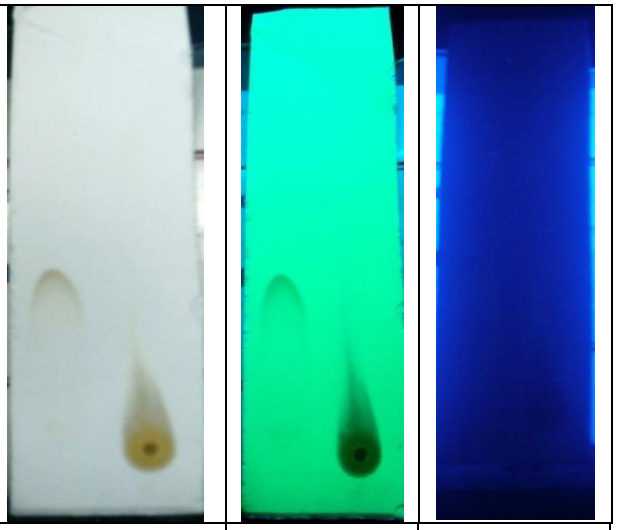
Detection and Calculation of Rf Value

The Rf Value of the spot was calculated using the formula –

Rf = Distance travelled by the solute/ Distance travelled by the solvent

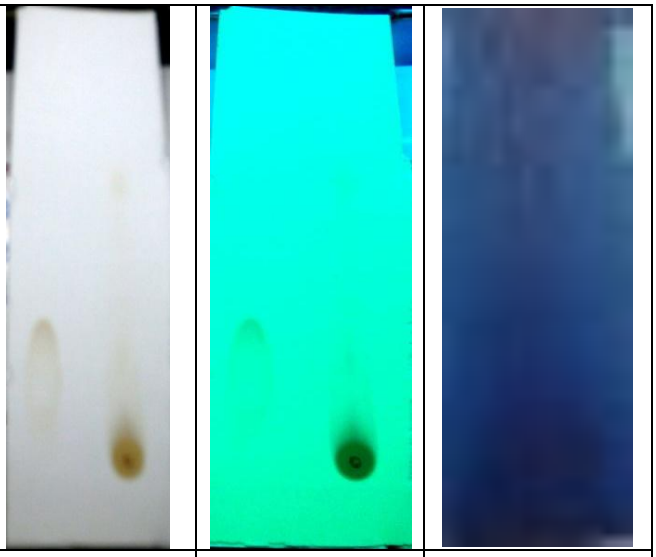
Table number 02. Rf value of Aqueous and Ethanol extract.

S.no	Extracts	Solvent system	Rf Value
1	Ethanol extract	Ethyl acetate: Methanol (4:6)	0.78 and 0.71
2	Aqueous extracts	Hexane:Dichloromethane:Methanol (2:3:5)	0.75 and 0.51



Normal light | **UV light** | **Long UV**

Figure-Aqueous extract for TLC.



Normal light	UV light	Long UV

Figure-Ethanol extract for TLC.

4.0 CONCLUSIONS

The highest percentage of callus induction was observed in MS medium (C7) containing 1.0 mg/l NAA with highest callus growth in terms of fresh weight which resulted in soft friable callus. Rf value (Retardation factor) of standard and that of sample extracts lies almost in same range which confirms that extracts contain is Lupeol.

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