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# THE CALLUS INTECTION AND ISOLATION OF ALKALOID FROM DATURA METAL L

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**ABSTRACT:**

Datura metal L is an important medicinal plant containing the alkaloids like scopalamine(hyoscine),hyoscyamine(atropine) scutellaerin etc .The total alkaloids present in the callus were higher than the amount of alkaloids present in leaves .For the higher production of alkaloids the medium plays avital role.The addition of L-arginine were enhanced the alkaloid production The higher production of alkaloids like scopalamine (Hyoscine) and Hyoscyamine (Atropine) in sodium alignate were conformed by using FTIR – spectra and UV –visible spectra. The realase of alkaloids from the sodium alginate enttrapped cells was higher on 11$^{th}$ day of culture. In the present study indicated that the maximum amount of alkaloids were observered in callus (16.50mg/g d.wt) than leaves (12.37 mg/g d.wt) and seeds (11.5 mg/g dwt). The ms medium supplimented with different concentration of auxin (NAA) and cytokinin (BA) were enhanced maximum number of the shoot buds .Auxin rich medium promoted the rhizogenesis directly from callus.

**Keywords:** Datura metal L, Alkaloids,FTIR –spectra UV-spetra, sodium alignate.

**INTRODUCTION**

Medical plants deserve special mention because of their various pharmacological properties. Medicinal plants are still an important source of material for the pharmaceutical industries (Balantrin *et al*., 1985) comprising about 25% of prescribed drugs. As one of the biodiversity rich tropical countries, India has nearly 17,000 species of higher plants and 7500 of these plants have been reported to have medicinal value and about 400 plants are used in the regular preparation of ayurvedic, siddha and unani medicines. Medicinal plants are also important for their pharmaceutically valuable primary and secondary metabolites. Medicinal plants are of great interest in biotechnology. By the use of tissue culture various problems in plant biotechnology such as micro propagation, biosynthesis and biotransformation of biologically active compounds storage of plant cells and organs and genetic engineering of higher plants can be solved (Reinert and Balaji, 1977).

Medicinal plants are important for their pharmaceutically valuable secondary metabolites such as alkaloids, amino acids, antibiotics, various enzymes, steroids etc. they are also important for their production of coloring agent, perfumes, insecticides and vitamins. Collection of medicina plants on a mass scale from natural habitats is leading to the depletion of plantsresources. Organogenesis in a number of medicinally important plants has been reported in a number of plant species belonging to the family Solanaceae. Datura metal L has been selected for present study.( Alfermann, A.W., and Maike Petersen, (1995).

Datura metal L (Family-Solanacea is an annual herb, native of India, wild or naturalized throughout the tropics of both the hemispheres. It is valued in medicines as the leaves are of use for making cigarettes and fumigating powders for the relief of asthma. Ganja eaters often adulterated the ganja with Datura leaves. The juice of this plant is believed to be a cure of hydrophobia. The leaves are boiled and used as a poultice to relieve pain. The plant contains alkaloids such as hyoscyamine (atropine), scopolamine (hyoscine etc (Jaggi *et al*., 1989). It is a reputed drug for dog bites and respiratory alignments (Jain, 1994).

Recently it has been reported that the gene from Nicotiana tobacum L coding for pmt (Putrescine-N-methyltransferase has been inserted in to hairy roots of Datura metal under the contril of the constute camv 35s promater, in order to influence tropane alkaloid production (Moyano et al., 2003). Direct organogenesis in Datura metel (l) from nodal explants has been reported recently (Muthukumar et al., 2004). But the present study aims at the high frequency of shoot regeneration from the leaf explants of Datura metal L and to evaluate the potentiality of the plant tissues in the biosynthesis of alkaloids.

## MATERIALS AND METHODS

### Nuterient medium

The culture medium consists of Murashige & skoog's medium (1962) containing organic, inorganic salts, vitamins with 2% sucrose and 0.8% Difco agar powder. The medium was supplemented with growth harmones such as alpha-Napthalene Acetic Acid (NAA), Indole Acetic Acid (IAA), 2,4-Dichlorophenoxy acetic acid (2,4-d), 6-Benzyl Amino Purine (BAP and kinetin (KIN) either alone or in combination at various concentrations. The Ph of the medium was adjusted to 5.8 + 0.1 with NaOH or Hcl before gelling with bacteriological agar. The gelled medium was dispersed into 150mm X 25 mm rim less culture tubes (20ml medium/tube) plugged with non absorbent cotton plugs and autoclaved at 1.06 kg/square cm pressure and 121 C for 15 min. for each treatment 7-14 replicate cultures were used and all experiments were repeated at least twice Double distilled water was used for the media. ( Baburaj,S.R., Damotharan and K.Santhaguru (1987).

### CULTURE CONDITION

The cultures were incubated in a growth room maintained at 25 +or-, after inoculation under cool white fluorescent light (ca.3000 lux, 14h/d) provided by Phillips fluorescent tubes (TL 40 W / 54,cool day – light, 6500k) With a relative humidity of 50-60 % (Bhalsing, S.R., and Maheswari, V.L., (1997).

**CULTURAL PROCEDURE**

The plant materials were excised and thoroughly washed in tap water and treatment with Teepol (detergent solution). The leaves were initially disinfected by rinsing in 90% ethanol for 15s followed by surface sterilization in an aqueous solution of 0.1% (W/v) Hg $Cl_2$ for 3-4min and 2-2 rinses in sterilized water. The leaf segments of 0.5 $cm^2$ cut and inoculated onto the sterilized Nutrient medium, one explants /tube, in a laminar airflow system.

The cultures were exposed to 14h/d illumination for callus induction and organogenesis after regeneration of shoot they were collected and cut into single node segment and sub cultured on medium supplemented with BAP or KIN. Subculture was repeated every 5 weeks for shoot production.( Asada, M. and M.L. Shuler, (1989).

**SUSPENSION CULTURE**

Transferring friable callus lumps to liquid medium of the composition 2,4-D(2mg/l) and BAP (0.5mg/l) at 100 rpm obtained suspension cultures.

After 5 days of transfer, large clumps of initial inoculums were removed by passing the suspension through stainless steel mesh of 60 micro m.

From this stock culture, 50ml of cell suspension was transferred to 250 ml conical flask and was maintained under continuous fluorescent light (3000 lux) at 25· C +or- 2 C on an orbital shaker (100-rpm).( Balantrin, M.F.,1985).

**ENTRAPMENT OF PLANT CELL**

Cell aggregates were adjusted to 2.5ml packed volume (500 mg FW), equal amount of culture medium (2.5ml) was added to the cell aggregates.

And the resulting suspension was mixed with 15 ml of 3% sodium alginate.

Alginate cell suspension was dropped under gravity through a1mm orifice into 500ml of 0.05M calcium chloride ($CaCl_2$) solution.

The alginate beads were collected and transferred to ErlenMeyer flask containing a medium supplemented with 2,4-D(2 mg/l), BAP (0.5 mg/l) and the different concentration of L-arginine (50-150 mg/l).

Alkaloids released from immobilized cells were collected at different time intervals and quantified through UV & FTIR spectrophotometrically.

**EXTRACTION OF ALKALOIDS:** 5mg of dried (callus/leaves/seeds/) sample was extracted by maceration with 95% ethnol of 1hr in aboiling water bath.

The combined extracts were filtered and the filtrate was evaporated to dryness. The dried residue was dissolved in 15ml hydrochloric acid and hydrolyzed for 2 hrs at 100 c in a water bath.( Cusido, R.M.,*et al*.,1999)

The hydrolysate was neutralized with 15ml of 1N sodium hydroxide. To this 10 ml of acetic acid was added and volume of 50 ml was made with distilled water.

Then aglygone complex was complexed with 5ml of 0.05% aqueous methyl orange in acetate buffer (pH: 4.7) for the calorimetric estimations.

The methyl orange agygone complex was extracted with extracted with 50 ml of choloroform totally 100 ml of extracted with 50ml of chloroform totally 100ml of extract was transferred in to separating funnel and allowed it for 24 hrs. After separation alkaloid solution was collected and quantified through UV &FTIR spectrophotometrically. (Pooja Bhatnagar *et al*.2004 ).

Table 1: **Effect of different concentrations and combinations of NAA and BA on shoot bud formation from leaf explants of Datura metal L cultured on MS medium.**

| GROWTH REGULATORS | | EXTEND OF CALLUS GROWTH | NUMBER OF SHOOT BUDS/EXPLANT |
|---|---|---|---|
| **NAA** | **BA** | | |
| 0.5 | 0.5 | +++++ | 2.71 |
| | 1.0 | ++++ | 5.65 |
| | 2.0 | +++ | 7.84 |
| | 3.0 | ++ | 12.71 |
| | 4.0 | ++ | 17.56 |
| | 6.0 | ++ | 9.81 |
| 1.0 | 0.5 | ++++ | - |

| | | | |
|---|---|---|---|
| | 1.0 | ++++ | - |
| | 2.0 | ++ | 1.75 |
| | 3.0 | ++ | 5.85 |
| | 4.0 | ++ | 7.71 |
| 1.5 | 0.5 | +++ | - |
| | 1.0 | +++ | - |
| | 2.0 | ++ | - |
| | 3.0 | ++ | 4.14 |
| | 4.0 | ++ | 3.87 |

Values are mean of 7 replicates each containing one explants.

Relative values of callus formation: ++=low; +++++ =High

**Table 2: Showing the alkaloid content of seeds leaves and callus.**

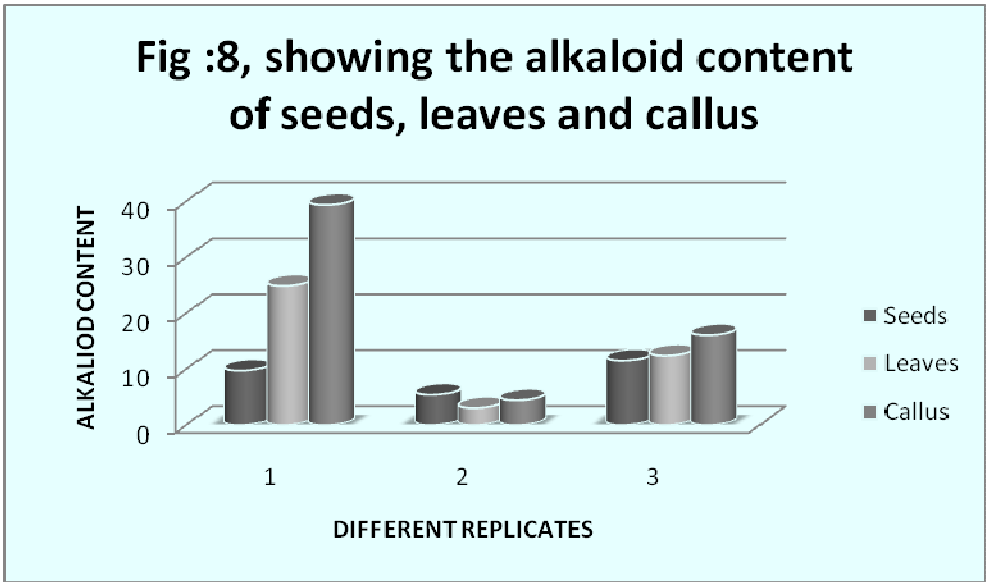
| SOURCE | FRESH WEIGHT(g) | DRY WEIGHT(g) | ALKALOID CONTENT (mg/gdwt/100ml) |
|---|---|---|---|
| Seeds | 9.70±0.70 | 5.39±0.35 | 11.50±0.56 |
| Leaves | 24.87±0.52 | 3.01±0.41 | 12.37±0.42 |
| Callus | 39.35±0.37 | 4.418±0.38 | 16.05±0.65 |

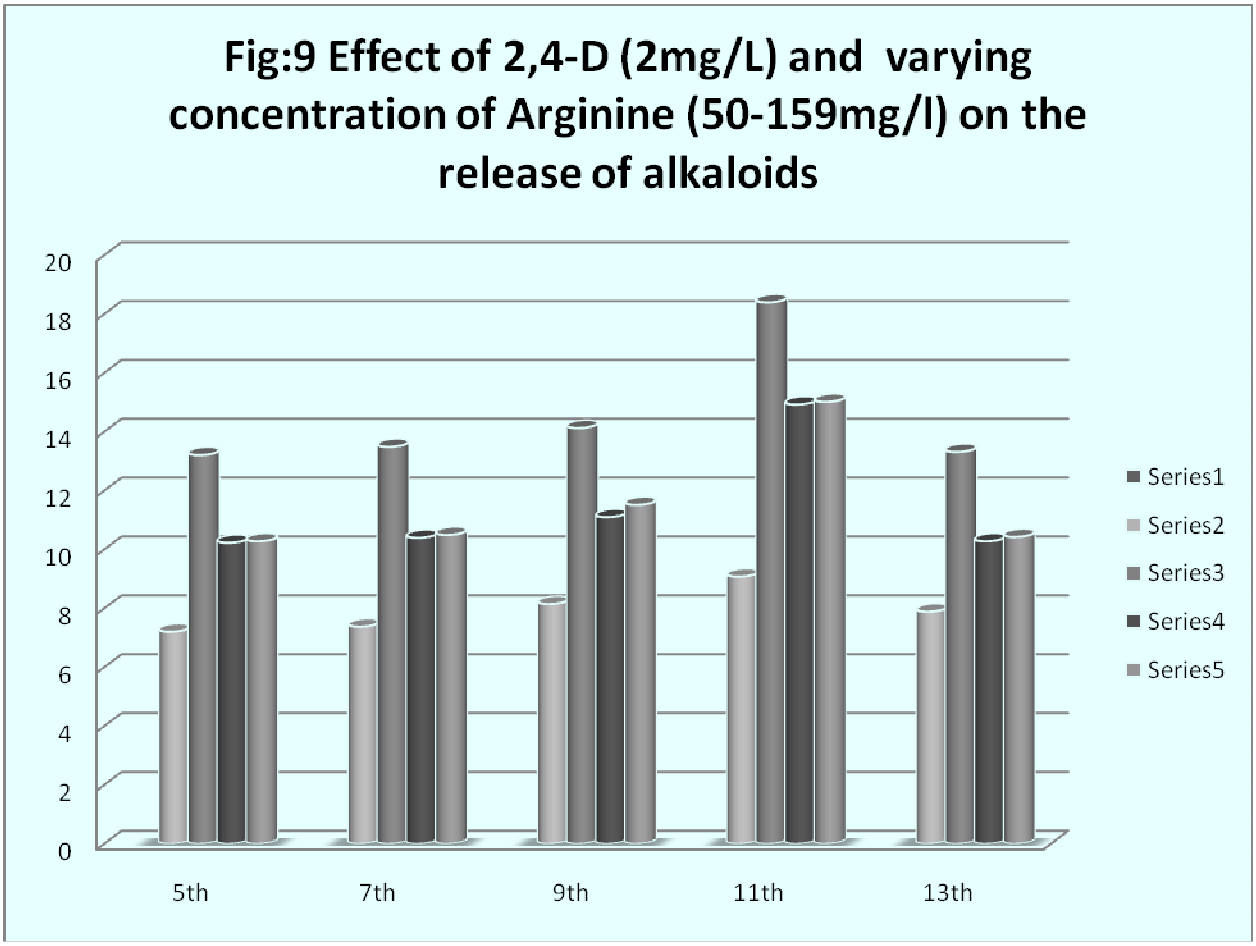
The data (mean value ± standard deviation) were calculated from the measurement of 7 replicates.

Table 3: Effect of 2, 4-D (2 mg/l), BAP (2mg/l) and varying concentration of arginine (50-150 mg) on the release of alkaloids from alginate-entrapped cells of Datura metal L.

| MS+Growth regulators +L – arginine | | | ALKALOID RELEASED (mg/g d wt/100ml) DAYS | | | | |
|---|---|---|---|---|---|---|---|
| 2,4-D (mg/l) | BAP (mg/l) | L-arginine | $5^{th}$ | $7^{th}$ | $9^{th}$ | $11^{th}$ | $13^{th}$ |
| 2.0 | 0.5 | - | 7.21±0.46 | 7.38±0.38 | 8.15±0.38 | 9.08±0.43 | 7.89±0.49 |
| 2.0 | 0.5 | 50 | 13.21±0.43 | 13.49±0.58 | 14.13±0.16 | 18.40±0.39 | 13.32±0.29 |
| 2.0 | 0.5 | 100 | 10.23±0.64 | 10.40±0.64 | 11.10±0.23 | 14.93±0.47 | 10.28±0.55 |
| 2.0 | 0.5 | 150 | 10.28±0.27 | 10.50±0.47 | 11.52±0.18 | 15.02±0.39 | 10.43±0.54 |

The data (mean value ± standard deviation) were calculated from the measurement of 7 replicates.



**RESULTS AND DISCUSSIONS**

**CALLUS ESTABLISEMENT**

Leaf explants of Datura metal L (fig:1) were routinely cultured on MS medium supplemented with auxins (NAA and cytokinins (BA) in varying concentrations either individually or in combinations. The proliferation of callus started from the cut ends of the explants after 2 or 3 weeks (Fig:2). Callus formation was high when the medium was supplemented with both auxin and cytokinin. Differential requirement of these two groups of growth regulators was observed. In general, the required auxin concentration for callusing was higher than that of cytokinins. This was true for all combinations of growth regulators tried in this study. Maximum callus growth was obtained after 20 days of culture and this contributed about 95% of growth was obtained after 20 days of culture studies (Dodds and Roberts.1985). Browning of callus tissue may be due to the production polyphenolic compounds produced by the cultured cells. In the present study browning was avoided by aub culturing every 5 weeks.( Curtis, W.R.,*et al.*,1995) (Choi, H.J.,B.Y.Tao and M.R.Okos,(1995)( Decendit, A and J.M.Merillon(1996).

Fig: 1Datura Metal L Plant.

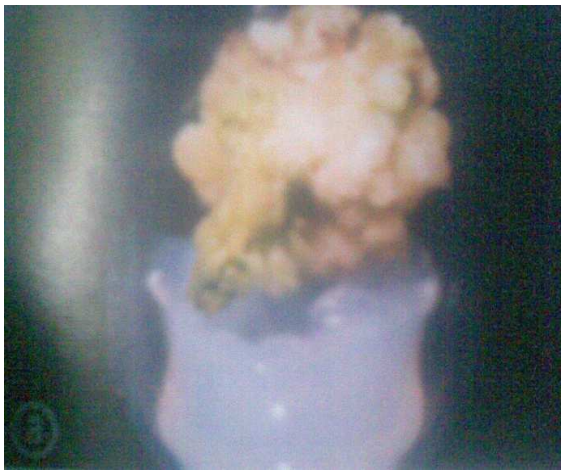


Fig-2: Leaf callus

**REGENERATION OF SHOOTS**

The explants were maintained in the same medium and the results were tabulated (Table:1). In the present study auxin-rich (1 mg/l NAA + 0.5 mg/BA) MS medium favored root initiation directly from callus (Fig:6). Small greenish nodule emerged on the surface of the callus after 3-week culture, when the medium was supplemented with the 0.5 mg/l NAA and 1mg/l BA (Fig: 3). These nodules later grew into shoots (Fig: 4). The shoot buds were formed in MS medium with various combinations of NAA and BA (Fig-1) (Palazon, J.*et al*.,1999) Direct shoot formation from leaf explants was observed in medium containing 0.5 mg/l NAA and 5.0mg/l BA with little callus (Fig:5). The optimum concentration of NAA and BA for shoot bud initiation was found to be 0.5 mg/l and 4.0mg/l respectively (Table-1). Shoot buds were also observed in cultures supplemented with only BA. But the addition of low concentration of auxin along with cytokinin had an added advantage in intiation of mare number of shoot buds (Table-1). The higher requirement of cytokinin for shoot proliferation in callus cultures has been reported by many workers (Flick et al, 1983; Baburaj et al., 1994) (Dodds, J.H and L.H.Roberts(1985) Dornenberg, H. and D.Knorr,(1995).. Incorporation of a balanced ratio of growth supplements could therefore bring out cellular differentiation leading to organ formation in callus tissue.

**Fig: 3) Regeneration of shoot bud from Callus**

Fig:4)Callus with enlarged shoot

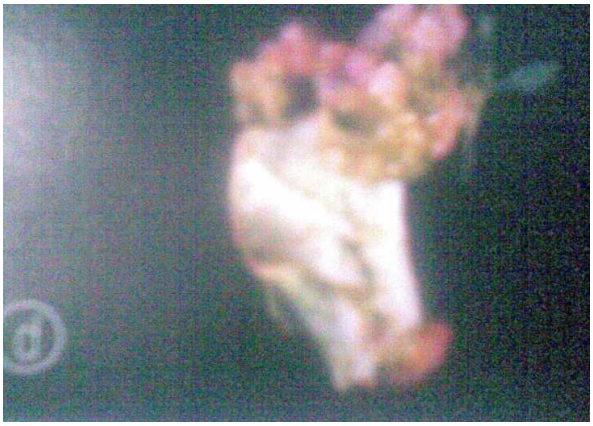


Fig: 5)Direct regeneration of shoot from leaf explants

Fig:6)Callus with root

Fig:7)Sodium alginate entrapped cells

## ALKALOID EXTRACTION

The alkaloid contents of dried leaves, seeds and callus were quantified from chloroform extracts. The callus cultures were maintained with various concentrations of growth regulators. The addition of2, 4-D (2 mg/l) with 0.5 mg/l BAP is suitable for callus growth and alkaloid present in leaves (12.37 mg/g d wt) and (11.5 mg/g d wt) (table: 2).

Similarly the alkaloids released from the sodium alginate entrapped cells were quantified (Plate-1f). Here the sodium alginate entrapped cells were suspended in MS liquid medium supplemented with 2, 4-D (2 mg/l) and BAP (0.5 mg/l) and also with different concentration of L-arginine (Table: 3). It has been observed that the addition of L-arginine (50mg/l) along with 2, 4-D (2 mg/l) and BAP (0.5 mg/l) enhanced the higher release of alkaloid. Similar results were obtained in Solanum laciniatum Where highest solasodine content (0.4277 mg/g dwt) was recorded in the cultures growing on medium containing L-arginine (100mg/l), when compared to the alkaloid content of calli maintained without the addition of L arginine (Pooja Bhatnagar et al., 2004). In the present study the release of alkaloids from the sodium alginate entrapped cells increased from first day up to $11^{th}$ day of culture. Decline in the release of alkaloids was observed from $12^{th}$ day onwards (fig-2). Identical results were observed in the production of anthocyanin in cell suspension cultures of Vitis vinfera (Decendit and Merillon 1996). Here the release of alkaloid was maximum (18.40 mg/g dwt) on $11^{th}$ day when the medium was added with 2,4-D (2 mg/l), BAP (0.5 mg/l) and 50 mg/l L-arginine. These results were further confirmed with UV-visible spectra and FTIR spectra studies as discussed in the following passages.( Moreno, P.R.H. *et al.*,1995).

**Spectra results**

**UV-Visible spectra:**

The UV-visible spectra are recorded to identify the electrode transitions taking place in substances containing σ-bonds, π-bonds, and lone pair of electrons, chromophores and aromatic rings. The UV- visible spectra of

chloroform extract of seed and callus were recorded, and are presented in figures (3a, 3b) respectively. Occurrence of peaks at 234-606 nm reveals the presents of scopolamine and hyoscyamine reported to be present in the Datura metal L (Jaggi et al., 1989). By making a direct comparison of the spectra of callus. And seed, we can conclude that these two extracts have similar alkaloid compound. The absorption spectrum of the purified alkaloid shows a distinct peak at 663 nm in callus. However it was not observed in the extract of seeds. It indicates the formation of the alkaloid exhibiting the yellow color only in callus. Other alkaloids may be formed in both cases (seed and callus)( Muthuvel, M.,et al.,2005) Suzuki,T.,et al.,1987) (Zehra, M.,*et al*.,1999*).*

**FTIR SPECTRA:**

IR-spectra of compounds reveal the nature of chromophore, ring system, functional groups, isotopic effects, hydrogen bonding, etc. the finger point region is characteristic of the compounds. The IR-spectrum of the chloroform extract of seeds, leaves and callus were recorded (fig- 4, 5, 6). Occurrence of peaks at 405-3432 $cm^{-1}$ reveals the presence of scopolamine (hyoscine) and hyocyamine (atropine) reported to be present in Datura metal L (Jaggi, et al., 1989). The spectrum shows a strong peak at 3000-4000 $cm^{-1}$, this is due to the vibration mode of OH groups present in the alkaloid. Since the stretching (symmetric & anti-symmetric) the OH- group occurs in this region. We observed that particular peak in seeds at (3019- 3432 $cm^{-1}$), in leaves at (2090-3415 $cm^{-1}$), and callus at (3019-3431 $cm^{-1}$) indicate the presence of OH- groups. Likewise the presence of ester group in general is 1660-1750 $cm^{-1}$, it shows peak in seeds at (1638-1713 $cm^{-1}$), in leaves at (1642-1713 $cm^{-1}$) and in callus at (1714 $cm^{-1}$) indicates the presence of ester group in the alkaloid. Normally the range of 1200-1450 $cm^{-1}$ is the finger print region revealing the presence of more number of benzene rings. The finger print region is observed in seeds at (1218-1394 $cm^{-1}$), in leaves at (1289-1409 $cm^{-1}$) and in callus at (1216-1402 $cm^{-1}$ ). Generally the methyl group occurs at (2000-3000 $cm^{-1}$), that region is observed in seeds at (2924-2981 $cm^{-1}$), in leaves at (2090 $cm^{-1}$), and in callus at (2404-2977 $cm^{-1}$). Presence of phenyl group normally in the range between (400-1500 $cm^{-1}$) in seeds it is observed at

(405-1046 $cm^{-1}$), in leaf at (403-769 $cm^{-1}$) and in callus at (459-1080 $cm^{-1}$). By making a direct comparison of the spectra of seed, leaf and callus, we can conclude that all the three extract have extract have similar alkaloid compounds. In the case of leaves and callus have some distinct peak reveals that the excess amount of that particular compound. Comparison of intensity of three seeds and leaves are higher, it implies that both have some extra compound other than alkaloid. (Flick, C.E., D.A.Evans and W.R.Sharp(1983) Engvild, K.C. (1973). Ganapathi, G., and F. Kargi,1990 and Tabata, M., et al .,1976).

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