Title

Chemical Characterization of *Datura stramonium* and

It's Micropropagation

A dissertation II

Submitted by

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MSc. (Hons.) Biotechnology

For the award of degree in M.sc Biotechnology

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ABSTRACT

The extract of stem and leaves *Datura stramonium* were extracted in four different solvents: petroleum ether, methanol, chloroform and water. After the phytochemical testing, some extract had showed the presence of secondary metabolites. The extracts had also shown microbial activity over the four different bacterial strains. Methanolic extract had showed activity 50% on the *E. coli,* 75% on *bacillus subtilis,* 60% on *Salmonella typhium,* and 36.8% on *Bacillus cereus.* Spectrometric analysis had the highest amount (45.73µg/ml) of phenol is present in stem with methanol extract and lowest amount (1.09µg/ml) in stem with petroleum ether extract and leaf with water extract. It also showed that the highest amount (11.11µg/ml) of flavonoid is present in leaf and chloroform extract and lowest amount (0.39µg/ml) in leaf and water extract. In thin layer chromatography, scopolamine and hyoscine was observed in methanolic and chloroform extract of stem and leaves extract. From the HPLC results, the methanolic extract had higher amount of hyoscine (9.2 mg/g), Hyoscyamine (9.5 mg/g), atropine (2.5 mg/g) and scopolamine (2.0). The growth of callus was observed in MS media after 2-3 weeks from the beginning. The callus was grown in the combination of the three growth regulators that are 0.25mg/l BAP, 0.25mg/l kinetin and0.5mg/l NAA and the shootlet was seen after two weeks of callus formation.

ACKNOWLEDGEMENT

I take this opportunity to express my deep sense of gratitude to my esteemed supervisor Dr. Kuldip Chandra Verma who have helped enormously and always inspired me by their indispensable guidance and encouragement during the whole long project work. They have always supported and strengthened me in many direct and indirect ways. Their thoughtful and valuable reviews, constructive criticism and tireless review of the manuscript has immensely helped me to improve the work.

I also owe my thanks to Yogesh Sir, all of my family and friends for their constant help and support.

KULJEET KAUR

DECLARATION

I, Kuljeet Kaur, student of M.Sc. (Hons.) Biotechnology under department of biotechnology, Lovely Professional University, Punjab hereby declares that all the information furnished in this dissertation is based on my all intensive research and is genuine.

This dissertation to the best of my knowledge contains part of my work which has been submitted for the award of my degree**.**

Kuljeet Kaur

Regd. No. 11308720

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CERTIFICATE

I hereby declare that Kuljeet Kaur has worked on project entitled **"Chemical characterization of *Datura stramonium* and its micropropagation**" under my guidance at department of biotechnology, Lovely Professional University, Punjab.

To the best of my knowledge, the present work is the result of her original investigation and study. No part of the dissertation has ever been submitted for any other degree at any University. The dissertation is fit for submission and the partial fulfillment of the conditions for the award of degree.

Dr. Kuldip Chandra Verma

Project advisor

Content

LIST OF TABLES

LIST OF FIGURES

CHAPTER -1

INTRODUCTION

Datura stramonium is an annual plant which belongs to the solanaceae and is considered as the most important medicinal plant. *Datura stramonium*, also called as Jimson Weed, is that plant which is distributed in the most of the temperate regions of the world and is a rich source of many medicinal substances. It is a fouling smell plant, erect, freely branching herbs which forms bush up to 2-5feet tall. It is a most interesting plant with property of hallucinogenicity (**Drager**, 2002).

The whole plant is nacrotic and is very much helpful in treatment of asthma. Most of the parts of *Datura stramonium* contain toxic compounds which were sometimes used for causing delirious states and death. On the other hand it is also in the treatment of Parkinsonism and Haemorrhoids. It is a fascinatic topic while it has limited advantages economically, but the alkaloid content of the plant is very much in demand from past to the present. Alkaloids are large group of natural compound found as the secondary metabolite in plants. These alkaloids seems to occur in all tissues of the plant but is absent in the capsule and woody portion of the root and the stem. But in the largest diversity in constituents is found in the roots. The stem contains a lower number of tropane alkaloids and some amount is found in seeds, leaves and flowers. The alkaloids are the most numerous group of active herbal substances from which the tropane alkaloids have significant importance. They play a significant role in healing of some human organs such as eyes, nerve system, heart etc. The number of synthetic medicinal substances o the drug market has grown, from which 25% are herbal derived product. Tropane alkaloids are extracted from the cultivated plant materials. *In vitro* researches for the production of tropane alkaloids have led to the experience of some variables e.g. exogenic phytohormones, glucoses, micro and macro elements etc. It is shown that the production of the callus was mainly stemmed from the leaf explants rather than the peduncle explants and the best environment for leaf explants is MS culture comprising NAA hormone for the yield of tropane alkaloids (**Afsharypour *et al***, 1995).

The growing interest in secondary metabolites took the attention towards the secondary analysis. The chemical properties of these secondary metabolites like

dissociation constant, stereochemistry etc. are very much useful for chromatographic separation. It is often used as a nacrotic and local anesthetic drug in many societies (**Schulman and Bolton**, 1998).

The plants which are used as a medicine can be traced back about five million years. There are about 250,000-500,000 plant species present on earth from which only small amount of plants have shown medicinal properties. *Datura stramonium* is most important plant which is used as medicine from the past million years. Leaves and seeds of this plant are used to prepare many treatment recipes. Leaves are mixed with the mustard oil to treat skin disorders. Juice of flower petals are used in ear pains and seeds are used in cough, fever and asthma. The drug which is obtained from the plant is called as drug of natural or biological origin. Its extracts have shown a significant role in antimicrobial activity upon some human pathogens. Its secondary metabolites are helpful as antidiabetic and antiviral diseases. Its water extract have shown some insecticidal activities. The water extract of leaves also shown it activity upon lung, breast, head and neck cancer cell lines, its acetone and ether extract of seeds have shown activity upon laryngeal cancer cell lines, mammary and glioma cell lines. The ethanolic extracts have shown more antimicrobial activity than that of aqueous extract of *Datura stramonium (**Aqib et al.,* **2014**)*.

A wide range of medicinally important plant parts are used in different countries by collecting the plant extracts. The plant parts include root, stem, fruit and modified organs. Some plant extracts are collected in small quantity and is locally used only. Some plant extracts are collected in large quantity and used for marketing purposes as the raw material for herbal industries (**Mahesh *et al.,* 2008**).

The plants are rich in various secondary metabolites like tannins, tripenoids, flavonoid, alkaloids etc. These metabolites have been found to have antimicrobial activities and properties. Now a day, the modern allopathic medicines are known to have some adverse side-effects and also the microorganisms which developed, have become resistance to many antibiotics due to the continuous use of the antibiotics. It makes the treatment of disease very difficult. But the antibiotics are essential for curing bacterial infections *in vivo*. These infectious diseases have become the main reason for the death of many people every year, because bacteria divide rapidly and show mutagenic properties. The pathogenic bacteria develop antimicrobial resistance, thus many

antibiotics have become useless. The increased number of pathogen showed multiple drug resistance. So the health care association starts to investigate the antimicrobial activity of medicinal plants. A steroidal compound which was isolated from the *Datura metel* leaves had showed antifungal activity against some plant pathogenic fungi (**Ram *et al.*2013**).

fig.1 Plant of *Datura stramonium* (kaweahaoks.com)

Chemicals analysis of plant materal is very challanging and require some special proedures, due to its variability. So planner chromatography plays an important role in identification and quality control tests for herbal products. Traditionally, thin layer chromatography technique is used for the analysis of botanical or herbal raw material. The visualisation of the compound pattern i.e. compounds present in the herbal drug, is very important in the quality and stability of the product. TLC method provides fundamental data by visible pattern of bands. It is also used to demonstrate the cosistency of the given plant material. Therefore, TLC is aconvinient method for quality of the plant materal. Chromatography is used for the determination of alkaloids in the sample. Chemical properties like dissiciation constant, stereochemistry and spectroscopic data is very helpful in the establishment of chromatography.

HPLC is used to separate the mixture component and also used to identify and quantify that component. HPLC is also used in medical, research and manufacturing

purposes.Atropine, hyoscyamine and scopolamine are the medicinally important tropane alkaloids because it shows anticholinergic and central nervous system activities.

The production of tropane alkaloids in the tissue culture depends upon the media composition. The effect of different factors like as source of the plant nutrition. Plant growth regulators and conditions for the production of the alkaloid tropane have been studied by using tissue cultures (**Iranbakhsh *et al.*,** 2007).

Micro propagation as a tool for the production of plant based medicines plays a tremendous role. The mode of regeneration by tissue culture is Callus-mediated organogenesis and regeneration through somatic embryogenesis. With the help of differential application of the growth factors and condition, the induction of the callus growth and subsequent differentiation and organogenesis are accomplished (**Jha and Pandey**, 2012).

Micropropagation is the alternative technique to clone the species of *Datura stramonium*. This plant contains alkaloids which have high medicinal value. The micropropagation of *Datura* species from leaf or anther is successful for the establishment of calli gives rise to the organogenesis (**Mahakant *et al.*, 2012**).

Plant tissue cultures are taking attention of the world, because the cells also synthesize specific compounds which are used as medicines. The plant tissue culture helped in producing those plants which are in high demand for the medicine and edible purposes (**Sheeba *et al.*, 2013**).

In response to wounding, infestation, or at graft, the callus forms naturally on plant (**Bottino**, 1981). The anthers and the pollen grains are cultured for the production of the haploid embryogenesis from the division of microspore and immature pollen grains (**Dodds & Roberts**, 1985). The *in vitro* embryo culture of *Datura stramonium* plant regeneration helped in the study of the enhancement of the callus induction and regeneration efficiency by adjusting the carbon source and concentration (**Amiri *et al.***, 2011).

Fig: 2. Fruits of *Datura stramonium*

It was found that *in vitro* cultivated plants can produce secondary metabolites in a control manner. It will also help to produce new secondary metabolites in future. For the callus production, leaf explants have been extensively used. Except leaf immature embryos are also a good source for instant somatic embryogenesis. Mature embryos are helpful in the callus production. **(Ashwini *et al., 2014*)**

CHAPTER-2

TERMINOLOGY

TLC	Thin Layer Chromatography
µg/ml	Micrograms per milliliter
µl	Micro litre
2, 4-D	2, 4- Dichlorophenoxyacetic acid
E. coli	Escherichia coli
FC	Folin - Ciocalteu reagent
g/L	Gram per Litre
Gms	Grams
HCl	Hydrochloric acid
M	Molar
mg/ml	Milligram per Milliliter
MHA	Muller Hinton Agar
Ml	Milliliter
Mm	Millimeter
mM	Millimolar
MS media	Murashige and Skoog media
NAA hormones	Naphthalene Acetic Acid
NaOH	Sodium Hydroxide

Nm	Nano meter
^{o}C	Degree Celsius
pH	Negative logarithm of Hydrogen ion
Psi	Pounds per Square Inch
Rpm	Rotation per Minute
UV-Vis	Ultraviolet- visible

CHAPTER-3

REVIEW OF LITERATURE

In vitro production of tropane alkaloids have led to the experience about some variables like exogenic phytohormones, micro and macro elements, glucose, vitamins and some other physical factors. For the production of the callus which was stemmed from leaf rather than the peduncle and for this production the best environment is the MS media containing NAA hormones in conc. of 0.5g/L (**Iranbakhsh and Riazi**, 2000). **Yoshimatsu *et al. (2010)* and Zhang *et al* (2010)** showed a positive connection between the root tissues and the tropane alkaloids production.

Table1. - Qualitative test of extract from root, stem, leaf, seed and fruit coat.

Plant parts	**Tannins**	**Saponin**	**Alkaloids**	**Iridoids**
Root	++	+	+	-
Stem	+	++	++	-
Leaf	+++	+++	+++	+
Seeds	+++	+++	+++	+
Fruit coat	+++	++	+	+

According to the Table1, the qualitative analysis of extracts from the root, stem, leaf, seed and fruits coat sample of *Datura metel Linn*, showed that the presence of phytochemical constituents such as alkaloids, tannins, saponins and iridoids. During the present investigation, it is found that leaf and seed contains more tannin, Saponin and alkaloids as compared to the other parts. Iridoids are absent in root and stem (**Jamdhade *et al***., 2010).

Table: 2. Phytochemical profiling of chloroform fraction, methanolic fraction and crude extract in *Datura stramonium. (*Ghias *et al., 2012)* 6

Chemical compound	Chloroform fraction	Methanolic fraction	Crude extract
Alkaloids	-	-	-
Terpenoids	+	+	+
+Flavonoids	-	+	+
Tannins	-	+	+
Saponins	+	-	+
Reducing sugar	+	+	+
Phenols	+	+	+

According to Ghias *et al. (*2012), the sample was extracted in methanol and after drying, the extract was suspended in methanol and chloroform (Table: 2.). Then the phytochemical profiling of methanolic and chloroform fraction and crude extract of leaves of *Datura stramonium* were done. It showed that the sample is having terpenoid, reducing sugars, phenols and other chemical compounds.

Datura metel. L. plants were propagated by collecting the nodal part from in vitro germinated seeds and field grown plants (**Mutukumar *et al***. 2004) from leaf discs of *Datura innoxia* callus was initiated with different capacities for alkaloids biosynthesis.

Iranbakhsh (2012) studied the effect of methanolic extract from root, stem and leaves of the *Datura stramonium* on the growth of some bacterial strains. He also studied the effects of *in vitro* cultivated callus extract of *Datura stramonium* on the same bacteria then that growth of the bacterial strains. He observed that the extract of plant and callus, have different activities over the bacterial strains. The *in vitro* cultivated

callus has more activity against the bacterial strains than the activity of the other extracts of the *Datura stramonium*.

According to **Jamdhade *et al***. (2010), the antimicrobial activity of aqueous extract of the root of four different species of *Datura*, which were screened against five human pathogenic bacteria. The *Datura innoxia* showed maximum antibacterial activity. While the remaining three extracts were less effective against the bacteria.

Antimicrobial activity of plants of Datura was carried out with the help of the well diffusion method (**Gachande *et al.* 2013**). Nutrients agar plates were prepared to which the inoculation of bacterial strains and fungal strains were done. The wells were prepared were about 5mm in diameter. In these wells about 0.5ml of the sample and the positive control was added. These plates were incubated at their optimum temperature for their bacteria and fungus growth. After the incubation zone of inhibition was observed (Table: 3).

Table: 3. Antimicrobial activity of *Datura stramonium* water extract (Ganchande, 2013)

Name of Organisms	Zone of Inhibition (mm)				
	Root part	Stem part	Leaf part	Negative control (water)	Positive control (streptomycin)
Bacillus subtilis	15 (50%)	15(50%)	31(96.7%)	-	30(100%)
E. coli	09(22.5%)	11(27.7%)	18(45%)	-	40(100%)
Staphylococcus aureus	9(75%)	11(91.6%)	10(83.3%)	-	12(100%)
Salmonella typhi	-	-	8(32%)	-	32(100%)

According to the studies of **Miraldi *et al*.** (2001) the level of tropane alkaloids in root was lower than the aerial part of the plant. Atropine is the predominant alkaloid in *Datura stramonium* in the generative period (**Oshawa *et al*.**, 1989). In contrast, scopolamine was predominant during all development stages of *Datura metel* plants (**Afsharypuor *et al*.**, 1995).

Using suspension cell culture, it was shown that the glucose presence in the media resulted in the high production of the alkaloid during the sixth week of culture. This was observed by **Majid and AmirJani** (1996). The biomass production of the root culture is more in the sucrose medium than in the monosaccharide medium but the high production of the alkaloids is most in the monosaccharide medium (**Schripsema** and **Verpoorte,** 1992).

The result obtained by **Hilton and Rhodes** in 1995, after considering the effect of different concentration of amino acid, the highest production of tropane alkaloid was happened with 10.3mM amino nitrate at third week. Small increase of the concentration of the amino nitrate dosage causes the decrease in the tropane alkaloids.

Amiri (2010) studied that the presence 2, 4-D is important for the callus growth from the leaf of *Datura stramonium*. But 2, 4-D failed to produce callus. Then they found that 1≥ mg/l kinetin with combination of the 2, 4-D for more callus initiation and proliferation from the explants. Then they conclude that the kinetin enhance the effect of 2, 4- D in callus formation for *Datura stramonium*.

By using hairy root culture of *Datura stramonium* also shows the effect of the concentration of the calcium on the production of the tropane alkaloid. The production of tropane alkaloids with the decrease in the calcium content (**Pinol *et al***. 1999).

CHAPTER-4

SCOPE OF THE STUDY

Datura stramonium is used to make medicines for curing violent activities of the brain or the nervous system. It is also used to cure other nervous disorders. It also unwinds the bronchial tube muscles, food pipe and urinary tract. It also helps in curing Parkinson's disorder. But if the preparation of the plant medicine is not proper then it leads to highly toxic. *Datura stramonium* contains alkaloids, tannins which are used to produce medicines like analgesic and antiasthamatic actions. Alkaloids and tannins contain methylated nitrogen compound.

So to produce any medicine from *Datura stramonium*, proper study of constitutes should be given priority. Because the lack of knowledge about the constituents may give rise to a toxic result. Many tests are used to determine the constituents of this plant. The scope of study is to identify the overall activity of the *Datura stramonium* on various types of microbes. Study is also related to the total phytochemical contents in the extract of different plant parts in different solvent by using spectrophotometer.

To study the Micropropagational properties of *Datura stramonium* is also very important to grow this plant *in vitro*. *In vitro* cultivation of this plant is used to increase the desired content in the plant as present in the wild plant. It will also increase the economical value of this plant for the medicinal purpose.

CHAPTER-5

OBJECTIVE

- The phytochemical testing and total phenolic and flavonoid content in *Datura stramonium.*
- To determine the antimicrobial activity of *Datura stramonium.*
- Thin layer chromatography and HPLC of *Datura stramonium.*
- Micropropagation of *Datura stramonium.*

CHAPTER-6

MATERIAL AND METHOD

6.1. Collection of *Datura stramonium* plant leaves. Plant leaves and stems were collected from village Khalwara, V.P.O. Phagwara. Then leaves and stems were allowed to air dried at room temperature in shade. Then the dries leaves and stems were crushed by electric grinder to make it in the powdered form. The powder was stored in plastic bags for further use.

6.2. Extraction of *Datura stramonium* material from powdered leaves. The powdered leaves and stems were weighed in four parts, 10 gms. each. The extraction was done by using different solvents, 100ml methanol, 100ml petroleum ether, 100ml water and 100ml chloroform. Each powder was dissolved in these different solvents for about 3 hours in rotary shaker at 60 rpm. After 3 hours, the mixture was centrifuged at 5000 rpm for 25 minutes. Then the supernatant was filtered by using Whattman's filter paper 1. The solvent was evaporated by using hot plate. The crude sample was made 1mg/ ml in concentration and stored in container at 4^{o}C for further use.

(a)

(b)

Fig.2. Solvent evaporation (a) *Datura stramonium* stems extract, (b) *Datura stramonium* leaf extract

6.3. Testing for phytochemical constitution of *Datura stramonium*

6.3.1. Test for alkaloids (Meyer's reagent test). 100µl of each sample extract was taken in a test tubes and 2 ml 2% HCl was added in each test tube. Heated to boiling and then filtered the sample using whattman's filter paper. 100 µl of Meyer's reagent is added in the filtrate and mixed it properly. Then, observed for the turbidity of the solution.

6.3.2. Test for reducing sugar. 100 µl of each sample extract was taken in a test tubes and 1 ml of distilled water in the test tubes. Tubes were then allowed to heat Then 100 µl of Fehling's solution (Fehling's solution A and Fehling solution B) in the hot sample mixture and mixed it by shaking. Then, observed for the colour change from green to brick red.

6.3.3. Test for saponin. 100µl of each sample extract was mixed with 1 ml of distilled water and shook it well. Then, observed for the foam formation in the test tube.

6.3.4. Test for Phenolic compound (Ferric chloride test). 100 µl of each sample extract was taken in the test tubes and 5 ml distilled water was added to it. Then the diluted sample was filtered. Then to the filtrate 5% Ferric chloride was added. Then, observed for the colour change in the test tube.

6.3.5. Test for tannins. 100µl of each sample extract was taken in the test tubes. 1 ml of distilled water was added to it. 50 µl of Ferric chloride was added in the diluted sample and mixed properly by shaking. Then, observed for the change in the colour of the sample.

6.3.9 Test for flavonoid. 100µl of each extract was taken in the test tubes. The extract was then mixed with diluted NaOH and mixed well. After mixing 200 µl of hydrochloric acid was added. Then, observed for the colour change in the solution.

6.4. Antibacterial of *Datura stramonium*.

6.4.1. Preparation of media.

For the antibacterial and antifungal activity Mueller Hinton Agar (MHA) was used. 6.84gms. of MHA was dissolved in 180 ml of distilled water and autoclaved at 15 psi for 15 minutes. Under aseptic conditions, media was poured in petriplates and allowed to solidify. After solidification each plate was divided into two parts by using marker. One half of the plate was marked as control and other was marked as extract.

6.4.2. Test for Antibacterial using disc diffusion.

For the test of anti bacterial activity, two strains of bacteria were used. One was Bacillus subtilis and other was Escherichia coli and Salmonella typhium.

For disc diffusion, discs were prepared by dipping them in 25µl of the extract and left overnight. The positive or standard control for this test, Gentamycin was used (2mg/2ml).

6.5. Total phytochemical determination.

6.5.1. Total Phenolic contents. Total phenolic contents were determined by using Gallic acid as standard. The Gallic acid was prepared in 1mg/ml conc. Then different concentration from 0.01 to 0.10 mg/ml was prepared from the stock in different test tubes. Then 5ml distilled water was added to each test tube. FC reagent was prepared in

1:1 about 30 ml and then 2ml of FC reagent was added to each test tube. Then 2ml of 20% of sodium bicarbonate, prepared in 0.1 sodiumhyroxide, was added to each test tube and mixed well. These tubes were incubated at 37^{o}C for half an hour. After incubation the absorbance was taken at 765nm in UV-Vis spectrophotometer.

For the extract absorbance, 2µl of extract was added in 998ul distilled water. Then 5ml of distilled water was added to it. Then 1:1 FC reagent was added to the extract solution and then 20% sodium bicarbonate was added to it. Then the extract was incubated at 37^{o}C for half an hour. Then absorbance was taken at 765nm in UV-Vis spectrophotometer.

6.5.2. Total flavonoid contents. Total flavonoid contents were determined by using Quercetin as standard. Quercetin was prepared as 1mg/ml in methanol from which 0.01 to 0.10 concentrations were prepared in different test tubes. Then 1.5ml methanol was added to each test tube. After addition of methanol, 10% aluminum chloride was added to each test tube and mixed well. Then 0.1ml of 1M potassium acetate solution was added to each test tube and then 2.8ml of distilled water was added to each test tube. Then the tubes were incubated at 30^{o}C for 60 minutes. After incubation the absorbance of the standard was taken at 415nm in UV-Vis spectrophotometer.

0.5ml of each extract was taken in test tubes into which 1.5ml of methanol was added. Then 0.1ml of 10% Aluminum chloride was added and mixed well. Then 0.1ml of 1M potassium acetate was added to the test tubes. Then 2.8ml of dist. water was added and the test tubes were incubated at 30^{o}C for 60 minutes. After 60 minutes absorbance was taken at 415 nm.

6.6. Thin layer Chromatography: For thin layer chromatography, Silica Gel G60 was used. Slurry of silica gel G60 was prepared by mixing it in the distilled water. The slurry was then spread over the TLC plate and allowed it to air dry. After air drying, the plates were activated by keeping them in hot air oven at 70-100^{o}C for one hour. In the mean time, solvent system was prepared i.e. toluene, ethyl acetate and formic acid (36:12:5). After the activation of plates, spotting of sample was done above 1cm from the base, by using capillary tube. Then these spotted plates were kept in the TLC chamber. The solvent was then allowed to move on the plate. The plates were taken out when the solvent reached close to the other end of the plate. The plates were seen under UV-transilluminator. The movement of the solvent and the different was noted.

6.7. High Performance Liquid Chromatography: High performance liquid chromatography is an improved form of the simple chromatography the solvent is forced to through the column under high pressure upto 400 atmosphere rather than solvent being drip under the effect of gravity. For HPLC, crude extract of *Datura stramonium* was diluted by adding methanol in the different sample extract for the HLPC analysis. After performing TLC only those samples were selected which showed more bands after visualizing under UV- transilluminator. Selected samples were packed in eppendorf tubes and sent to Central Drug Research Institute, Lucknow, for HPLC analysis.

6.6. *In vitro* Micropropagation of the *Datura stramonium.*

6.6.1. Media preparation. 150 ml Murashige and Skoog media (MS media) was prepared by adding 666.1 mg MS basal salt containing vitamins in 250 ml distilled water. The media was then supplemented with 30 gm/l sucrose. Then pH of the solution was adjusted up to 5.7 by adding acid or base to it. After adjusting pH, 8% agar was added to the MS media solution and growth regulators (BAP, Kinetin and NAA) were added to the media. Then the media was autoclaved at 15 psi for 15 minutes. The autoclaved media was added to two phyta jars, 75 ml in each and allowed to solidify. This was done inside the laminar air flow.

6.6.2. Explants sterilization: Some **s**tems were cut about 2-3 cm having one node at least. The stems were washed in running tap for about 25-45 minutes. Then the stems were washed with detergent. Then these stems were washed with sterile distilled water inside the laminar air flow. Then the surface was sterilized by using 0.1% Mercuric chloride for about half minute. Then again these stems were sterilized by using sterile distilled water.

6.6.3. *In vitro* establishment of seed. The sterilized stems were inoculated on the culture medium. The inoculated media was kept in the photoperiod for about 16 hours. After the photoperiod the inoculated media was kept at 25^{o}C.

CHAPTER-7

RESULT AND DISCUSSION

7.1. Weight of crude extract: After concentrating the extract, we got the crude extract having different weights. The weight of the sample is given in Table: 4. From the table 4, we observe that the leaf with water extract has more weight (1.2885gm) than the other and the stem with petroleum ether has low weight than the other extracts (0.0641gm).

Table: 4. Weight of crude extract of *Datura stramonium*.

Sample name	Weight of crude extract (gms)
Stem and petroleum ether extract (S1)	0.0641
Stem and methanol extract (S2)	0.8142
Stem and chloroform extract (S3)	0.1784
Stem and water extract (S4)	0.7349
Leaf and petroleum ether extract (S5)	0.3544
Leaf and methanol extract (S6)	0.9285
Leaf and chloroform extract (S7)	0.2732
Leaf and water extract (S8)	1.2885

7.2. Phytochemical testing:

7.2.1. Test for Alkaloids (Meyer's reagent test). After adding Meyer's reagent some of the extract solution got turbid. This turbidity indicated the presence of alkaloid in the plant (Table: 5).

7.2.2. Test for reducing sugar. After adding Fehling's solution, the colour of the diluted sample changes from green to brick red which indicated the presence of reducing sugar (Table: 5).

7.2.3. Test for saponin. After shaking the sample and water solution for 15 minutes, foam formed in some of the solution. This indicated the presence of saponin in the plant extracts (Table: 5).

7.2.4. Test for Phenolic compounds (Ferric chloride test). In filtrates, when 5% ferric chloride was added, the colour of the some filtrates changed to dark green which indicated the presence of the presence of the Phenolic compound (Table: 5).

7.2.5 Test for tannins. When the ferric chloride solution was added in extract solution, the colour of some extract solution changed to dark green which indicated the presence of the catecholic tannins(Table: 5).

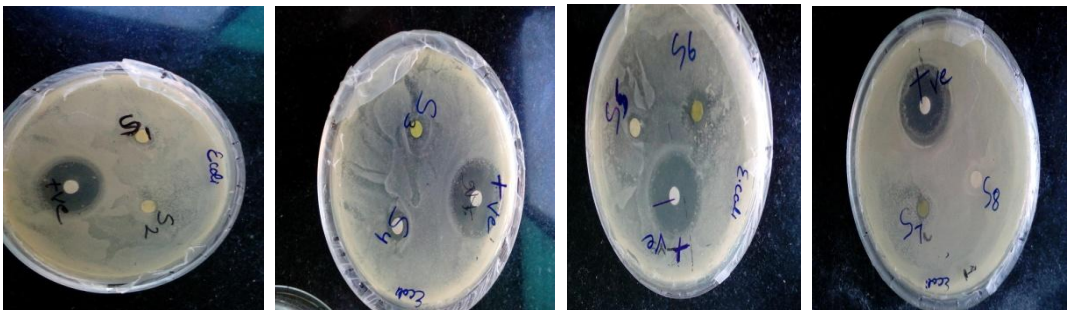
7.2.6. Test for flavonoid. After adding HCl the yellow scales turned colorless in some extracts. This colour change indicated the presence of flavonoid in the extract (Table: 5).

Table.5. Phytochemical testing of extract of *Datura stramonium*.

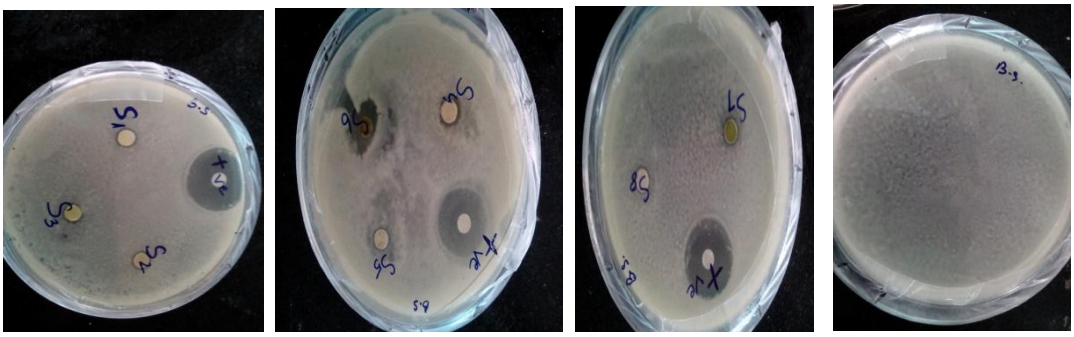
Sample	Alkaloid	Reducing sugar	Saponin	Phenolic compound	Tannins	Flavonoid
Stem and petroleum ether extract	-	-	-	-	-	-
Stem and methanol extract	+	+	+	+	+	+
Stem and chloroform extract	-	-	-	-	-	-
Stem and water extract	+	+	+	+	+	+
Leaf and petroleum ether extract	-	+	-	-	-	+

Leaf and methanol extract	+	+	+	+	+	+
Leaf and chloroform extract	+	+	-	_	-	+
Leaf and water extract	-	+	+	+	+	-

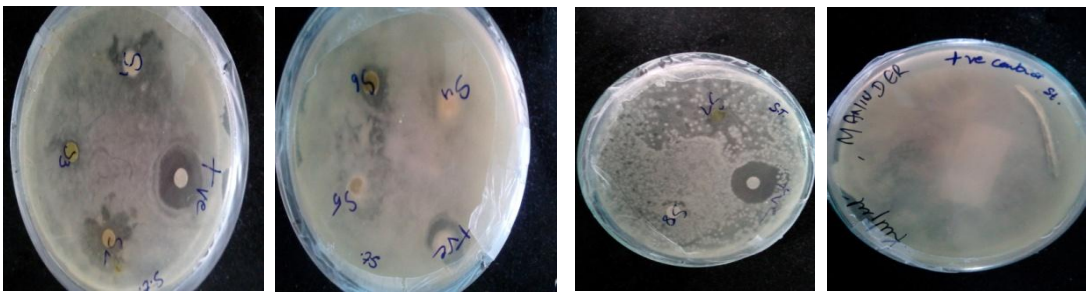
7.3. Antimicrobial activity of extract of *Datura stramonium*. Medicinal plants are the rich source of antimicrobial active agents. Plants are also used as medicines in many parts over the world and also as the powerful drugs source. The result of antimicrobial activity against different microorganisms by stem and leaves of *Datura stramonium* is shown in Fig: 3.



(a)



(b)



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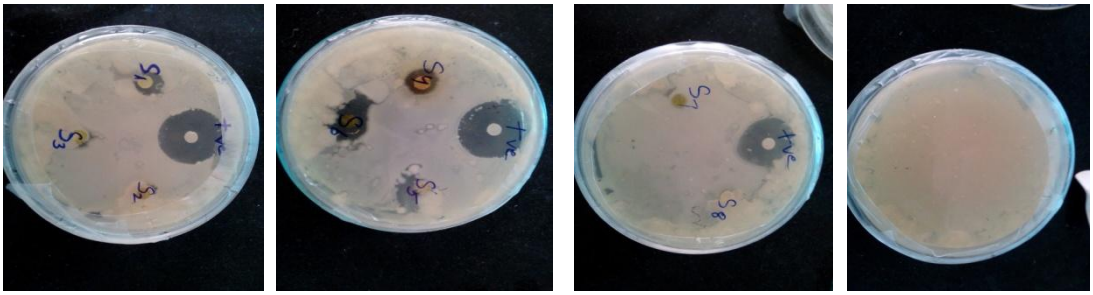


(d)

Fig: 4. Antimicrobial activity of *Datura stramonium* (a) Inhibition over *E. coli*, (b) Inhibition over *Bacillus subtilis*, (c) Inhibition over *Salmonella typhium*, (d) Inhibition over *Bacillus cereus.*

Zone of inhibition value is shown in Table: 5. Table: 6 show that the leaf with methanolic extract is highly active over the given bacterial samples. It showed activity 50% on the *E. coli,* 75% on *bacillus subtilis,* 60% on *Salmonella typhium*, and 36.8% on *Bacillus cereus.*

Table: 6. Antibacterial activity for *Datura stramonium*.

Sample name	**Activity over *E. coli***	**Activity over *Bacillus subtilis***	**Activity over *Salmonella typhium***	**Activity over *Bacillus cereus***
Gentamycin	100%	100%	100%	100%
Stem and petroleum ether extract (S1)	41.6%	33.3%	0%	31.5%
Stem and methanol extract(S2)	0%	33.3%	50%	0%
Stem and chloroform extract(S3)	16.6%	33.3%	30%	0%
Stem and water extract (S4)	50%	41.6%	0%	31.5%
Leaf and petroleum ether extract(S5)	0%	33.3%	0%	0%
Leaf and methanol extract(S6)	50%	75%	60%	36.8%
Leaf and chloroform extract(S7)	33.3%	25%	50%	0%

Leaf and water extract(S8)	0%	29.1%	0%	0%

Methanolic extract of leaves showed activity 50% on the *E. coli*, 75% on *bacillus subtilis*, 60% on *Salmonella typhium*, and 36.8% on *Bacillus cereus.* It is very much close to the **Ganchande (2013)** antimicrobial activity.

7.4. Total phytochemical test:

7.4.1. Total Phenolic content test. Total phenolic content in different polarity solvent such as petroleum ether, methanol, chloroform and water, in different plant parts are shown in Table: 7. The result showed that the highest amount (45.73µg/ml) of phenol is present in stem with methanol extract and lowest amount (1.09µg/ml) in stem with petroleum ether extract and leaf with water extract.

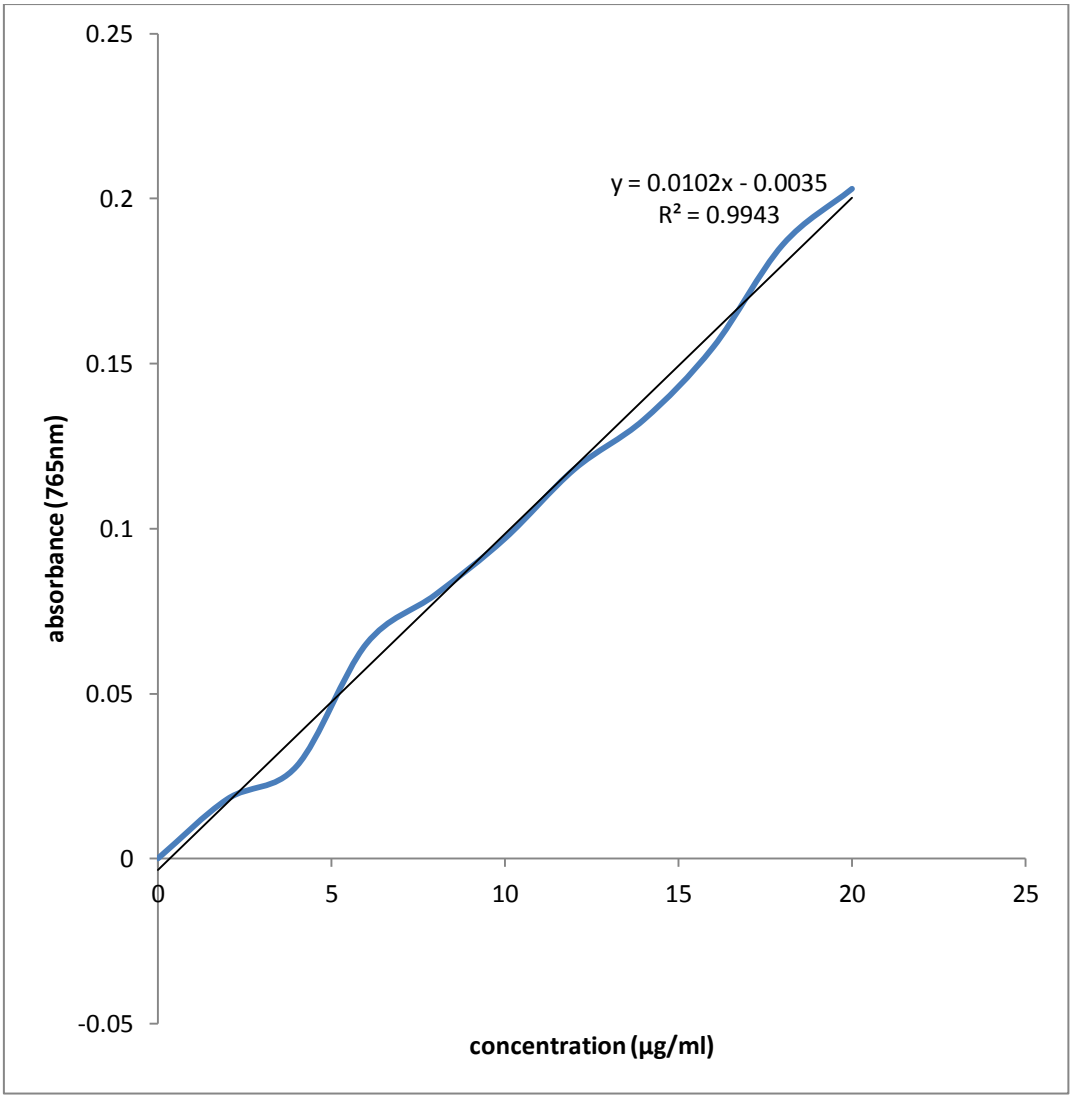


Fig: 5. Gallic acid standard curve

Table: 7. Total phenolic content present in *Datura stramonium*.

Sample name	**Total phenolic content (conc.) µg/ml**
Stem with petroleum ether extract (S1)	1.09
Stem with methanol extract (S2)	45.73
Stem with chloroform extract (S3)	3.29
Stem with water extract (S4)	14.63
Leaf with petroleum ether extract (S5)	4.39
Leaf with methanol extract (S6)	11.34

Leaf with chloroform extract (S7)	17.56
Leaf with water extract (S8)	1.09

- Above value is the mean of 3 replicates

7.4.2. Total flavonoid content. Total flavonoid content in different polarity solvent such as petroleum ether, methanol, chloroform and water, in different plant parts are shown in Table: 8. Result showed that the highest amount (11.11µg/ml) of flavonoid is present in leaf and chloroform extract and lowest amount (0.39µg/ml) in leaf and water extract.

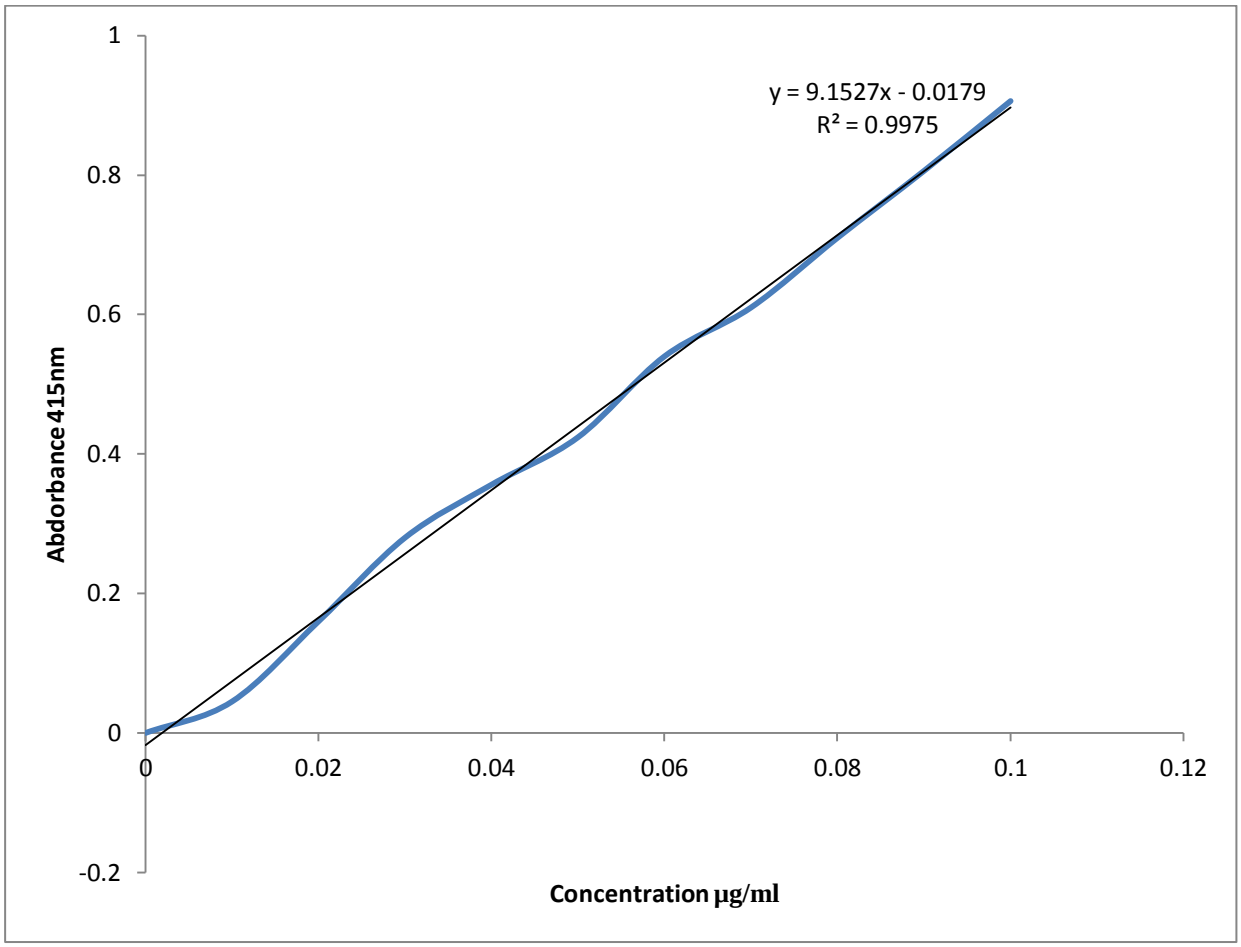


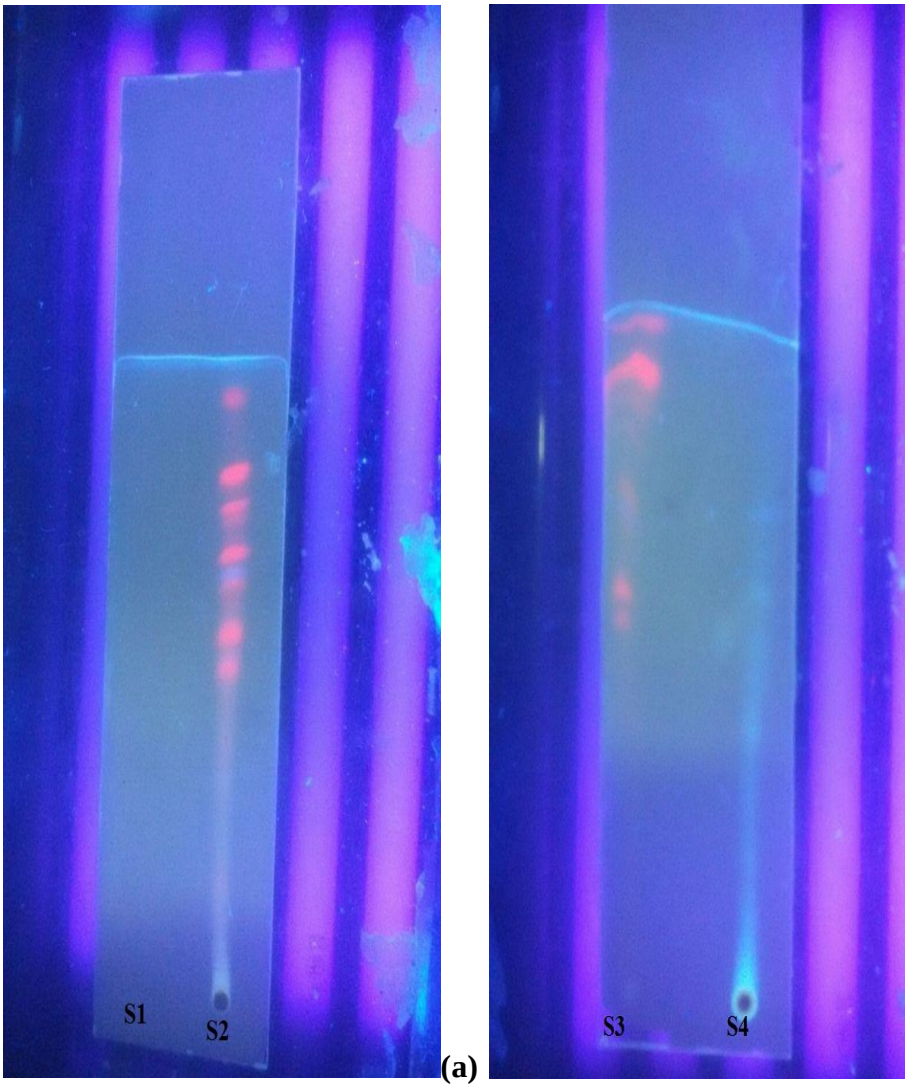
Fig: 6. Quanicitine standard curve

Table: 8. Total flavonoid content present in *Datura stramonium*

Sample name	Total flavonoid content (conc.) µg/ml
Stem with petroleum ether extract (S1)	2.18
Stem with methanol extract (S2)	3.17
Stem with chloroform extract (S3)	9.92
Stem with water extract (S4)	0.44
Leaf with petroleum ether extract (S5)	9.43
Leaf with methanol extract (S6)	4.16
Leaf with chloroform extract (S7)	11.11
Leaf with water extract (S8)	0.39

- Above value is the mean of 3 replicates

7.5. Thin Layer Chromatography: TLC was done for the testing of the phenolic contents in the extracts. It was observed that the phenolic compound is present in the methanolic and chloroform extract of stems and leaves.



(a) (b)

Fig: 7. TLC plate visualization under UV- transilluminator. (a) TLC of Stem with Petroleum Ether Extract (S1) and Stem with methanol extract S2, (b) TLC of Stem with Chloroform Extract (S3) and Stem with Water Extract (S4).

When the plate was seen under the transilluminator, in these samples bans at different positions were observed, which showed the presence of different phenolic compound in the sample. The movement of the solvent and sample and its value of retention factor are given in Table: 9

Table: 9. **Retention factor of *Datura stramonium.***

Sample name	Retention factor value	Phytochemical
Stem with petroleum ether extract (S1)	0	-
Stem with methanol extract (S2)	0.52, 0.53, 0.57, 0.66, **0.68**, 0.70, 0.76, 0.84	Scopolamine
Stem with chloroform extract (S3)	0.54, 0.6, 0.91, 0.98	Unknown
Stem with water extract (S4)	0.38, **0.68**	Scopolamine
Leaf with petroleum ether extract (S5)	0.61, 0.77, 0.85	Unknown
Leaf with methanol extract (S6)	0.07, 0.14, 0.24, 0.29, 0.4, **0.47**, 0.51, 0.61, 0.78, 0.83, 0.96, 0.97	Hyoscine, unknown
Leaf with chloroform extract (S7)	0.36**, 0.47**, 0.54, **0.68**, 0.80, 0.91, 0.98	Hyoscine, Scopolamine
Leaf with water extract (S8)	0	-

7.7: HPLC of Methanolic and Chloroform Extract of *Datura stramonium* Leaves and Stem.

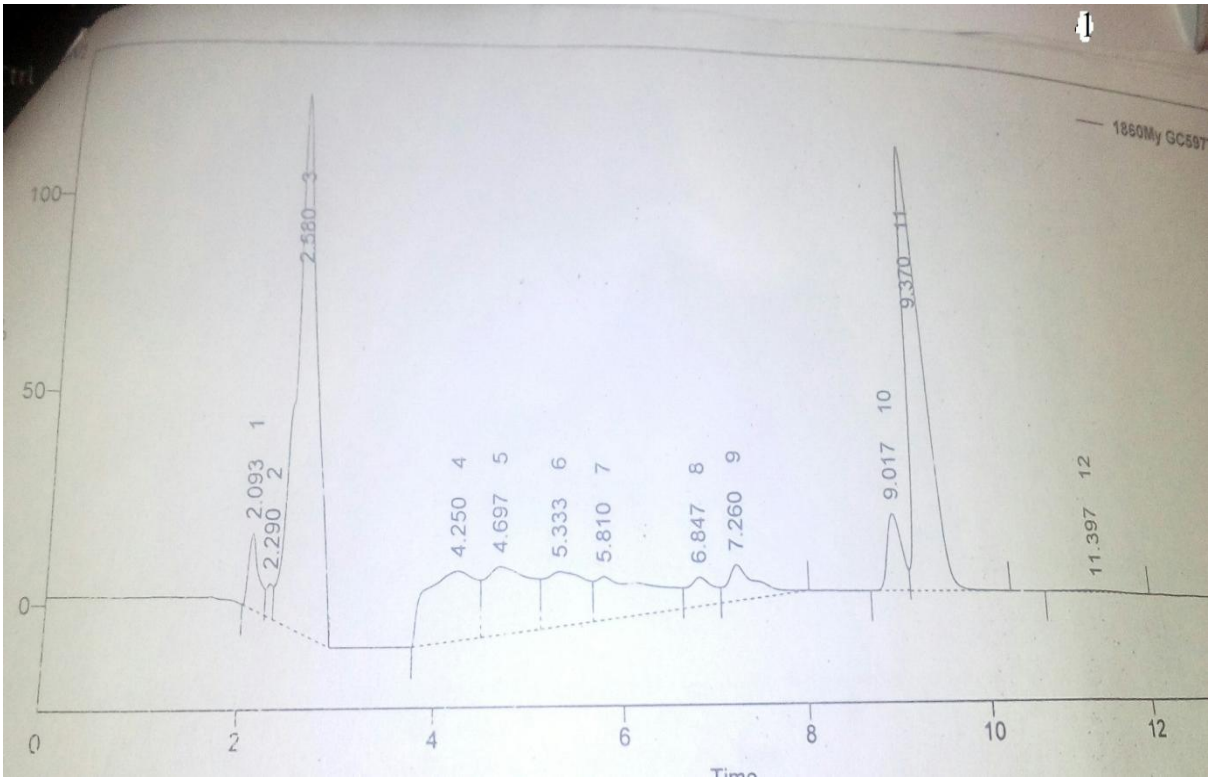


Fig: 8. HPLC of methanolic extract of *Datura stramonium* leaves.

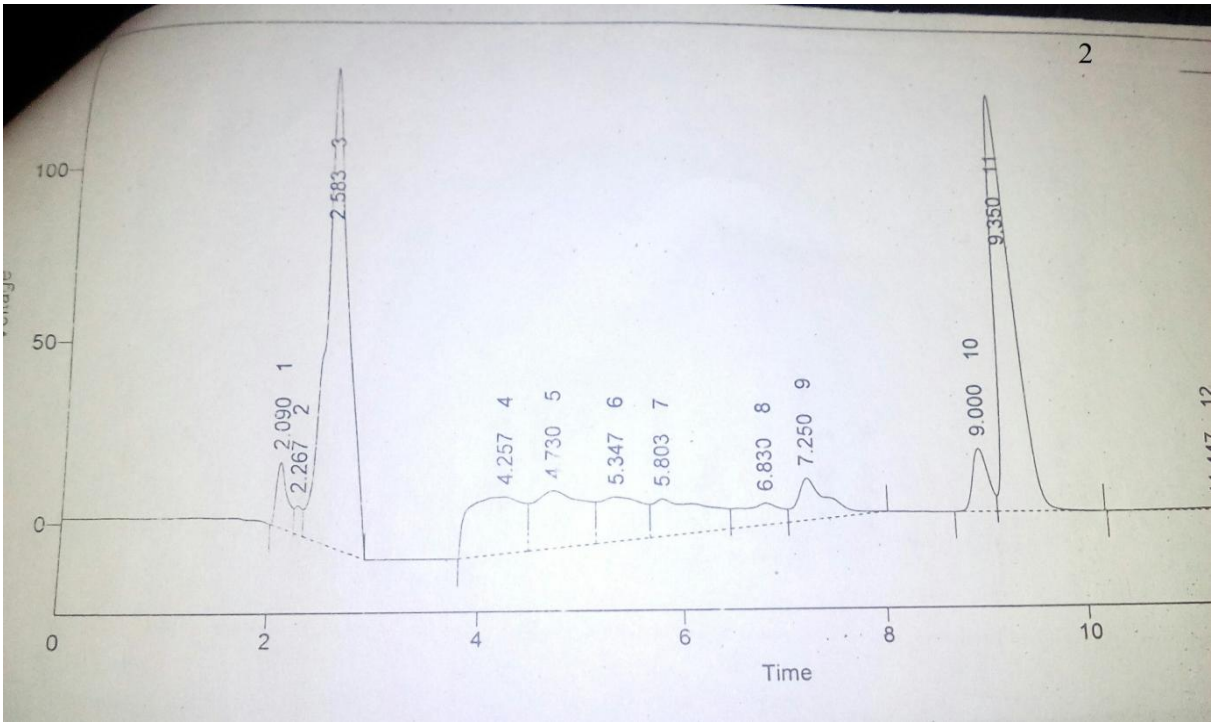


Fig:9. HPLC of methanolic extract of *Datura stramonium* stem.

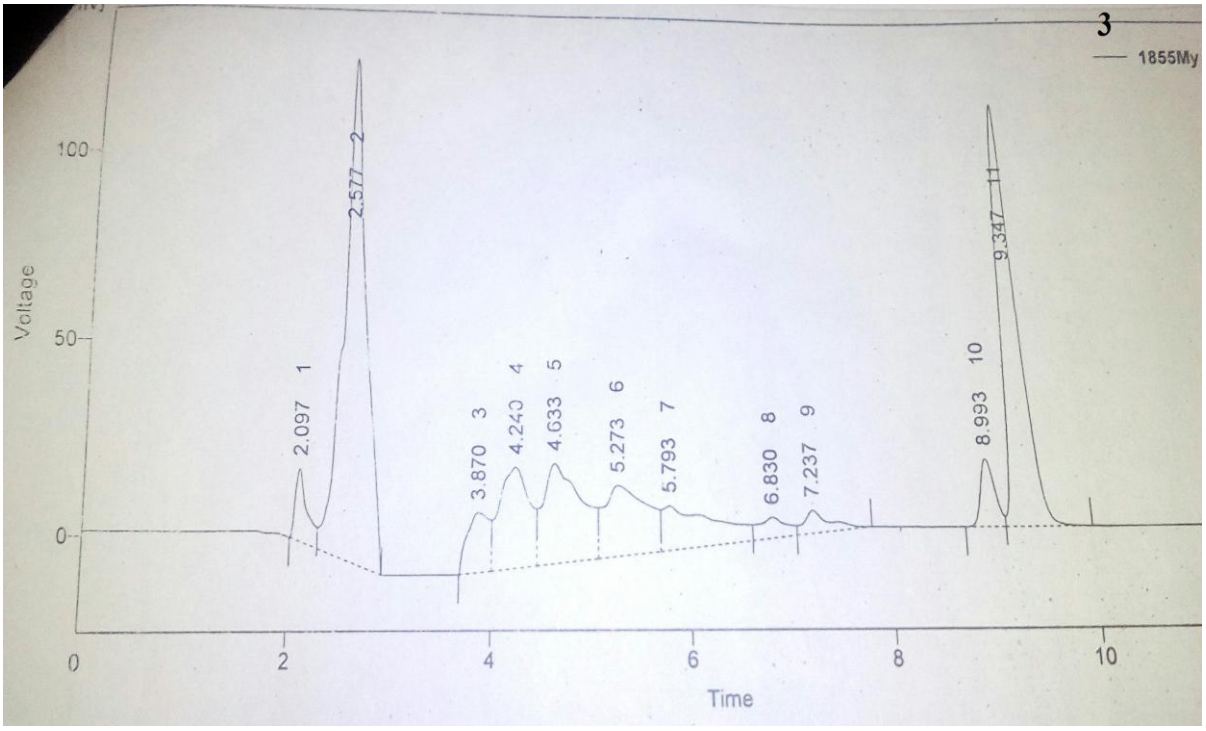


Fig: 10. HPLC of chloroform extract of *Datura stramonium* leaves.

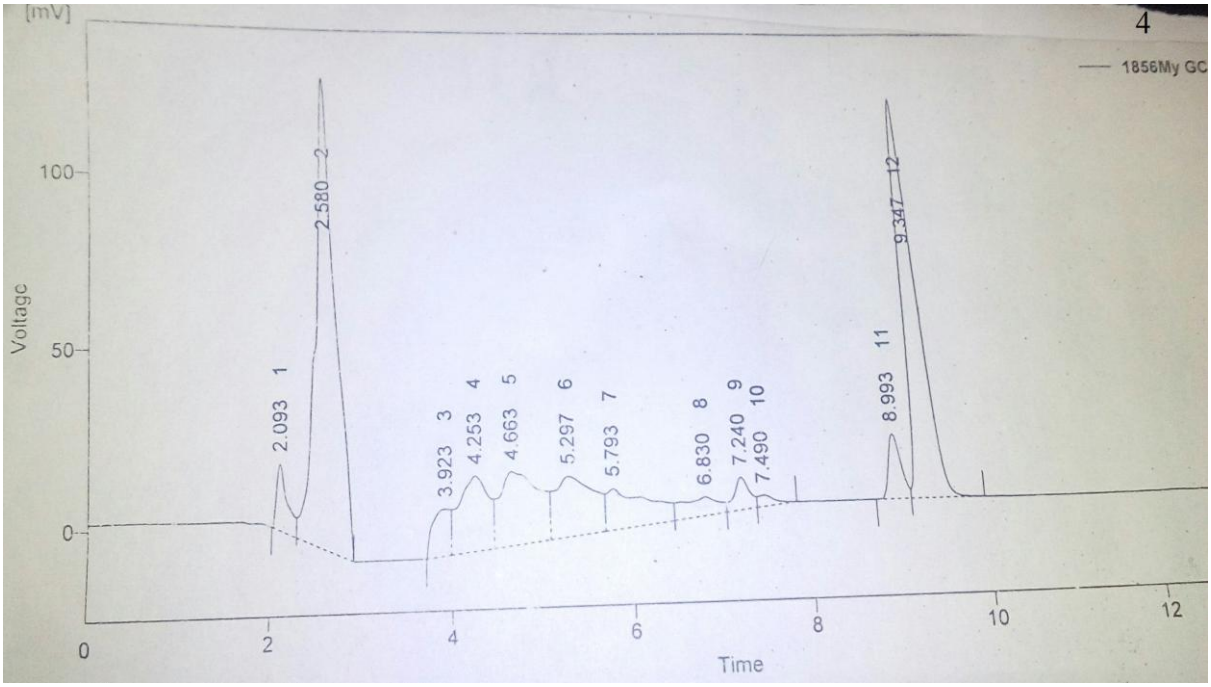


Fig: 11. HPLC of chloroform extract of *Datura stramonium* stem.

Table: 10. HPLC Analysis of *Datura stramonium* plant extracts.

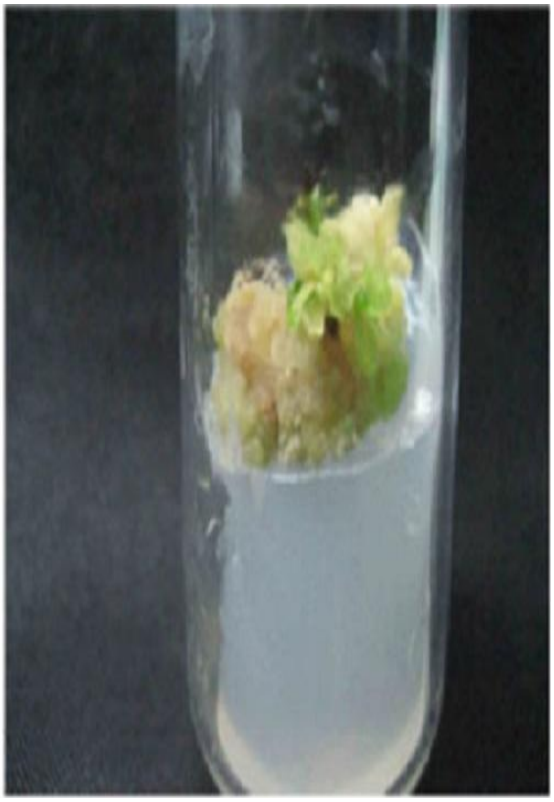
Sample Name	Hyoscine (mg/g)	Hyoscyamine (mg/g)	Atropine (mg/g)	Scopolamine (mg/g)
Methanolic extract of *Datura stramonium* leaves.	9.2	9.5	2.5	2.0
Methanolic extract of *Datura stramonium* stem	9.0	8.7	2.0	1.8
Chloroform extract of *Datura stramonium* leaves.	8.5	8.8	2.2	1.6
Chloroform extract of *Datura stramonium* stem.	7.8	8.0	1.8	1.4

The HPLC data of four extract of *Datura stramonium* had shown that the methanolic extract of leaves contain higher amount of hyoscine (9.2 mg/g), hyoscyamine (9.5 mg/g), atropine (2.5mg/g) and scopolamine (2.0). The lowest amount of hyoscine (7.8 mg/g), hyoscyamine (8.0mg/g), atropine (1.8 mg/g) and scopolamine (1.4 mg/g) was observed in *Datura stramonium* stem with chloroform extract.

7.8. *In vitro* micropropagation of *Datura stramonium*. The growth of callus was observed after 2-3 weeks from the beginning. The callus was grown in the combination of the three growth regulators that are 0.25mg/l BAP, 0.25mg/l kinetin and0.5mg/l NAA.

Fig: 12. Inoculation of nodal explants of *Datura stramonium* in MS media

(a)



(b)

Fig: 13. (a)Callus growth of *Datura stramonium* after 3rd week of inoculation.

(b) Shoots Initiation in callus of *Datura stramonium*.

The callus formed was then sub cultured to the fresh sterilized media taken in a cultured tube for the further growth of the plant. The shootlet was then observed after two weeks of the subculture.

CHAPTER-8

CONCLUSION

The extract of leaves of *Datura stramonium* was collected by using different solvent system that was petroleum ether, methanol, chloroform and water. Two parts of *Datura stramonium* was used one is stem and other is leaves. For the phytochemical testing, These tests give the positive result. After the phytochemical testing, total phenolic and flavonoid content was analyzed by using spectrophotometer. It gave the result that stem with methanol extract have more phenolic content and lower in stem and leaf with water extract, the flavonoid compound is present more in leaf with chloroform extract.

The microbial analysis gave result that the methanolic extract of leaves have more antimicrobial activity against many human pathogenic bacteria. Microbial activity was absent in some extract but according to some literature, those extracts should have some microbial activity. The main reason would be the location difference. By taking the sample of Punjab region comparison can be done in future. So, the effect of regional difference can be studied.

Then thin layer chromatography was done by using toluene, ethyl acetate and formic acid. This test showed that the methanol and chloroform extract of stem as well as leaf has more no. of phenolic content than the other. Then HPLC was carried out from Central Drug Research Institute, Lucknow. From the HPLC data hyoscine and hyocyamine is present abundantly in methanol extract of leaf and stem. In future, the unknown Retention factor values are shown by thin layer chromatography can be analyzed and the unknown peaks which were coming in the HPLC graph can be analyzed, so that the unknown phytochemicals can be identified and analyzed.

Micropropagation was also done by using Murashige and Skoog media containing vitamins, sucrose but without agar. For the micropropagation, we use nodal explants of this plant which showed callus after 3rd week of inoculation and shoot initiation was observed after two weeks of callus formation.

CHAPTER-9

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