# Genetic transformation of *Datura stramonium* for the production of tropane alkaloids in hairy root cultures

**Submitted in partial fulfillment for the Degree of Masters of Applied Science (Biotechnology) in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa**

**Farnaaz Ally**

**Masters of Applied Sciences: Biotechnology**

**2022**

**PROMOTER/ SUPERVISOR** : **Prof B. Odhav**

**CO-PROMOTER/ CO-SUPERVISOR** : **Prof V. Mohanlall**

## REFERENCE DECLARATION

I, Ms F. Ally – 20907201 and Prof Bharti Odhav (full name of promoter/ supervisor) do hereby declare that in respect of the following dissertation:

Title: **Genetic transformation of *Datura stramonium* for the production of tropane alkaloids in hairy root cultures**

**1.** As far as we ascertain:

a) no other similar dissertation exists;

b) the only similar dissertation(s) that exist(s) is/are referenced in my dissertation as follows:

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**2.** All references as detailed in the dissertation are complete in terms of all personal communication engaged in and published works consulted.

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| **Signature of student** | **11/08/2021**<br>**Date** |
| **Signature of promoter/ supervisor** | **25/01/2022**<br>**Date** |
| **Signature of co-promoter/ co-supervisor** | **25/01/2022**<br>**Date** |

## AUTHORS DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof Bharti Odhav** and **Prof Viresh Mohanlall**.

**Student's Signature**

## PUBLICATIONS

F Ally and V Mohanlall. (2021). An overview of tropane alkaloids from *Datura stramonium* L. Journal of Pharmacognosy and Phytochemistry. 9(3): 05-13

F Ally and V Mohanlall. Anti-bacterial and antioxidant activity of *Datura stramonium* extracts. Journal of Pharmacognosy and Phytochemistry- Accepted, Pending Publication - Appendix 4

## CONFERENCE PRESENTATIONS

**Ally, F.,** Mohanlall, V and Odhav. B. (2014) Analysis of tropane alkaloids in field-grown plant cell and hairy root cultures of *Datura stramonium*. Royal Society of Chemistry- 2nd International Symposium on Natural Products, Lagoon Beach Hotel Cape Town, South Africa, September 2014. (Poster presentation)

**Ally, F.,** Mohanlall, V and Odhav. B. (2016) Analysis of tropane alkaloids in field-grown plant cell and hairy root cultures of *Datura stramonium*. South African Society of Microbiology Biennial Conference, Coastlands Hotel Umhlanga rocks, Durban South Africa, January 2016 (Oral Presentation).

**TABLE OF CONTENTS**

## ACKNOWLEDGEMENTS

I would like to thank and express my gratitude to the following:

- My parents, for all the hard work and dedication in ensuring I received the best education and pursued my dreams. May Almighty Allah bless them abundantly for all their sacrifices.
- My supervisor and co-supervisor, Prof B. Odhav and Prof V. Mohanlall, I express my heartfelt gratitude for all the support and effort in imparting knowledge, guiding and mentoring me over the years especially when I almost gave up. Prof Odhav, you have certainly been a mentor that has touched my life personally as well as academically and I will never forget your teachings. Prof Mohanlall, your patience and understanding and support in the last few months are priceless and I will forever be grateful for ensuring I complete.
- Research Technician Ms Evashni Rampersad, for her dedication in ensuring all instruments as well as chemicals, were available timeously.
- Mrs Devrani Naicker from the department of chemistry, Durban University of Technology, for sacrificing her time in teaching and guiding me along the path of my project and assisting me with analysis.
- Staff at the Department of Biotechnology especially Ms Priscilla Phillips, Mrs Sarla Juglal, Dr NP Mchunu, Prof Swalaha, Prof Mellem and Ms K Mellem.
- The National Research Foundation and Durban University of Technology for funding this project and providing me with a Scholarship.
- My friends, Kabange Kasumbwe, Fitsum Kassa and Melissa Naidoo for helping make each day a memorable one in the lab.

## ABSTRACT

*Datura stramonium* is well known for its tropane alkaloid content, specifically atropine and scopolamine. With the demand of tropane alkaloids in the pharmaceutical sector, as anti cholinergic agents, anesthetic or its use in transdermal patches, an alternate method to obtain higher yields is required. In addition, much interest has been developed around the plant for its potential biological profile due to its use in traditional medicine. This study investigated the potential production of tropane alkaloids in plant tissue cultures and the biological activity in crude extracts of *D. stramonium*.

Elicited hairy root cultures had displayed the highest atropine and scopolamine content as compared to *in vitro* leaf and callus culture extracts. The concentrations of atropine and scopolamine was 5.2 $\mu g.mL^{-1}$ and 5.01 $\mu g.mL^{-1}$ respectively. This was an increase of 1.57 and 1.21 fold respectively as compared to wild plant extracts. The biological profile of *D. stramonium* was investigated by determining its antimicrobial activity and anti-oxidant activity. The anti bacterial activity wsas investigated by the Kirby-Bauer disc diffusion assay. Root extracts displayed a broader spectrum of bactericidal activity (7 mm – 33 mm) as compared to leaf extracts. No fungicidal activity was displayed by both extracts. The anti oxidant activity was investigated using 2-diphenyl-1-picrylhydrazyl (DPPH) photometric assay. The activity displayed was dose dependant, i.e.; as the extract concentration increased, so did the anti oxidant activity. At a concentration of 1 $\mu g.mL^{-1}$, the radical scavenging capacity of root and leaf extracts was 64.4% and 31.7% respectively whereas, at the highest concentration of 1000 $\mu g.mL^{-1}$, the radical scavenging capacity of root extracts and leaf extracts was 98.4% and 45.8% respectively. The biosafety evaluation of leaf and root extract demonstrated a linear relationship between the concentration of extracts and percentage toxicity. As the concentration and exposure period of the extracts increased, the toxicity towards *A. salina* had also increased.

These results indicated the potential of plant, cell and tissue culture systems of *D. stramonium* to produce higher yields of atropine and scopolamine through optimization, and the potential of anti oxidant and anti bacterial components present in its leaf and root.

# 1. INTRODUCTION AND LITERATURE REVIEW

Many plants have been used as traditional medicine (Cock *et al*., 2019; Akinyemi *et al*., 2018). Literature in the past decade, highlight studies that confirm plants, serve as an infinite source of secondary metabolites that possess a wide range of biological activities such as antimicrobial, antioxidant, anti-inflammatory, antidiabetic, anticancer and anticholinergic (Santic *et al.*, 2017*;* Romulo *et al*., 2018).

*Datura stramonium* is an annual herbaceous shrub belonging to the *Solanaceae* family. According to phytochemical analysis, *D. stramonium* serves as a reservoir for tropane alkaloids especially Atropine, Scopolamine and Hyoscyamine (Castillo *et al*., 2019). Tropane alkaloids such as Atropine and Scopolamine are extensively used as anticholinergic agents in the clinical sector for the inhibition of the parasympathetic nervous system (Nisthala *et al*., 2016). Tropane alkaloid was initially derived by one-pot synthesis. Different disciplines and fields of research such as pharmacology and biochemistry, illustrated alternate methods for the chemical synthesis of tropane derivatives such as Ladenburg's method (Alsamarra, 2019).

However, this makes this makes the synthesis of tropane alkaloid difficult, expensive and time-consuming. The total yield of tropane alkaloids present in wild plant extracts, such as *Atropa belladonna,* is generally low. The availability and yield of tropane alkaloids in plants are dependent on the environment in which these plants are grown (Kohnen-Johannsen and Kayser, 2019). Plant cell and tissue culture technology is an alternate biotechnological approach for the in-house production of biologically active metabolites present in plants. The use of plant cell and tissue culture techniques are investigated and optimized to produce high yields of biopharmaceuticals (Anis and Ahmad, 2016).

Hairy roots are an independent genetically stable tissue culture system that has a high reproducibility rate and requires simple nutrients for growth (Ghatge and Desai, 2015). Lab trials of hairy root cultures have been scaled up to commercially produce biopharmaceuticals that are easily purified during downstream processing and further used

as an Active Pharmaceutical Ingredient (API) for the production of low toxic drugs with a broad application (Glenn *et al*., 2013; Fischer *et al*., 2015).

### 1.1 Scope of the Study

In research conducted by the WHO, it has been identified that a larger portion of the worlds population, ascertain to the use of traditional medicine as a primary health care (World Health Organization, 2018). This has led to researchers using plants as a source of identifying, isolating and characterizing compounds for pharmaceutical application. The phytochemical content in plants across the world varies due to their habitat and life cycle, thus resulting in the inconsistent yields of compounds (Liebelt *et al*., 2019). Hence, there has been much focus on plant cell cultures to serve as an incubator for the production of pharmacologically active ingredients for drug development.

Phytochemical studies on *D. stramonium* indicates the plant is a rich source of tropane alkaloids. Tropane alkaloids, Atropine and Scopolamine are high market value antispasmodic and anticholinergic agents (Ali *et al*., 2020). Mass cultivation of the plant is hindered due to land space and the production of tropane alkaloids is dependent on the environmental conditions and season (Chadha *et al*., 2020). To overcome these limitations, biotechnological approaches including the development of hairy root cultures have been employed to produce pharmaceutically valuable components.

Therefore the focus of this study was to develop and screen for the potential production of tropane alkaloids in hairy root cultures of *D. stramonium* and to investigate the potential pharmacological activity of *D. stramonium* roots and leaves.

The aim of this study was:

i. To assess and compare the production of tropane alkaloids, Atropine and Scopolamine in wild plants and tissue culture extracts of *D. stramonium*
ii. To determine the biological activity of crude *D. stramonium* leaf and root extracts.

This was achieved by completing the following objectives:

a) *In vitro* germination of *D. stramonium* seeds

b) Induction of callus cultures of *D. stramonium* on MS medium using leaves from *in vitro* explants
c) Establishing cell suspension cultures using callus cultures of *D. stramonium*
d) Induction of hairy roots on *D. stramonium* leaf explants by *A. rhizogenes mediated transformation*
e) Quantitative analysis of tropane alkaloids using High-Performance Liquid Chromatography
f) Assessing the antimicrobial and antioxidant potential of methonolic extract of *D. stramonium* using the Kirby Bauer Disc Diffusion and DPPH assay respectively.
g) Assessing the biosafety of the crude methonolic extracts of *D. stramonium* using the Brine Shrimp Assay.

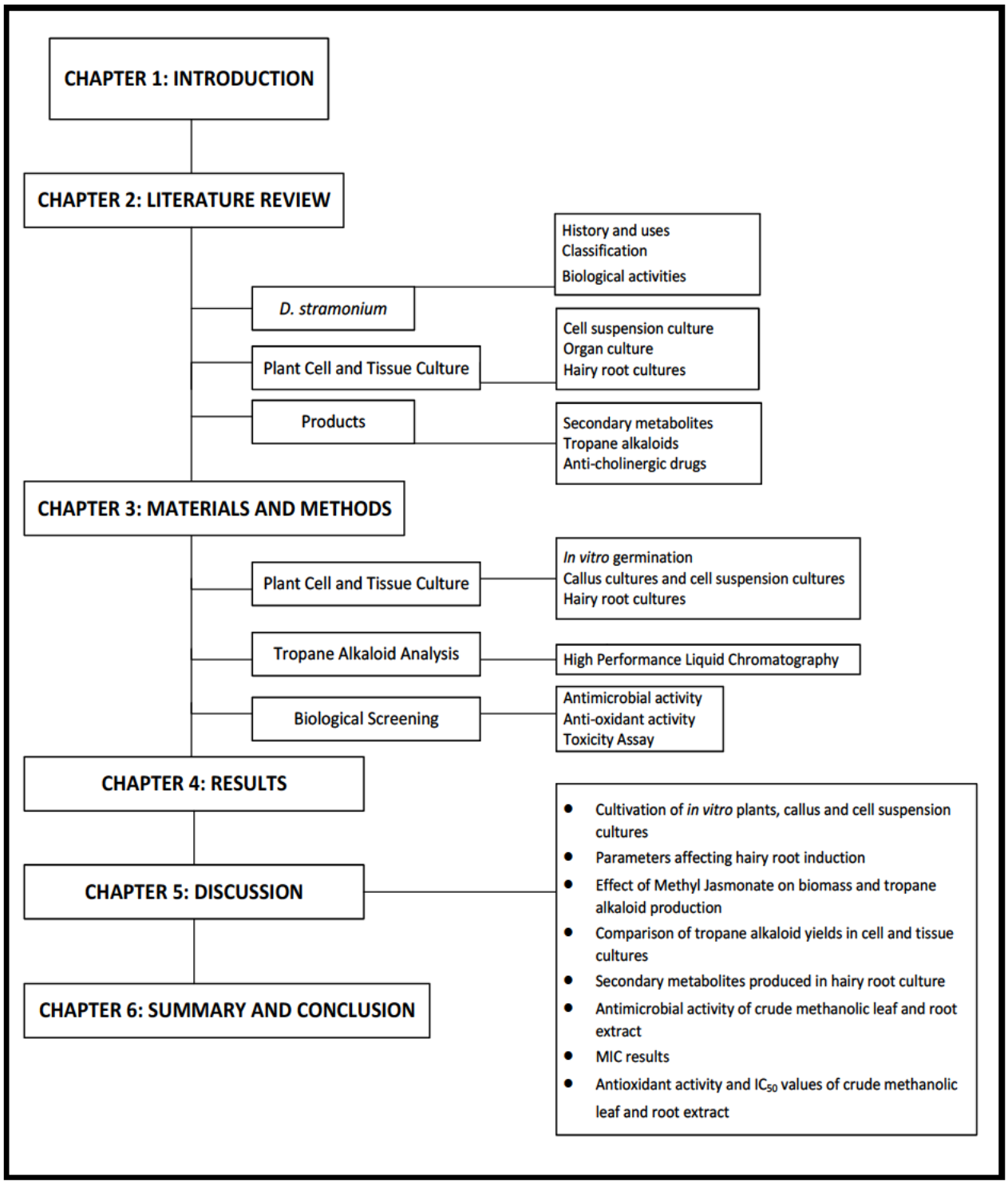


**Figure 1:** An overview of this dissertation divided into six chapters namely; Introduction, Literature review, Materials and Methods, Results, Discussion and Conclusion. Note that results are not shown in the flow diagram.

## 1.2 Medicinal Plants

For many years, plants has been used as a source of natural medicine around the world. The World Health Organisation (WHO), has recorded more than 80% of the worlds population utilizes traditional medicines, mainly plants (Jamshidi -Kia *et al*., 2018). Their application in traditional medicine and ethnomedicine has been extensively studied for industrial and pharmaceutical application to create biopharmaceuticals within an economic framework that will be used as an alternative to modern medical treatments (Tomlinson and Akerele, 1998). According to the 2001 statistics in Canada, plant-based medication had reached a total sales of 400 million U$ and have an increase of 15% annually (Uprety *et al*., 2012). Table 1 highlights plant-originated modern drugs and their application.

Over many years of trial and error, traditional healers have gained important knowledge on the use of plants. The plants employed for the treatment of various ailments were administered either in the form of teas, powders or a mixture of herbal formulation (Amber *et al.*2017). For example, plants within the *Gelsemiaceae* family has been employed by the Chinese for the curing of skin ulcers and headaches (Jin *et al*., 2014). The application and use of specific plants for ailments has been passed to generations through oral history and are now of attraction in Ayurveda and Homeopathic treatments, as described in Table 2 (Patwardhan and Vaidya, 2010). In the early 19$^{th}$ century, plant extracts and pastes which was traditionally used in many remedies were investigated for their potential *in vitro* activity. This had led to the isolation of bioactive compounds such as morphine from Opium poppy (Pandey *et al*., 2011; Azmir *et al*., 2013).

Analytical methods such as Preparative Column Chromatography have been optimized using multi-disciplinary biotechnological approaches for the isolation of active compounds from other natural resources. These isolated compounds eventually became the cornerstone of novel drug discovery (Balunas and Kinghorn, 2005). Although pharmaceutical companies employ molecular modelling and synthetic chemistry techniques in creating drugs, natural sources, in particular, medicinal plants still serve as an important source of novel drugs, drug leads and chemical entities. It has been reported that approximately 60% of modern drugs manufactured are off natural product origin (Nwonu *et al*., 2019). Natural products possess diverse structures with multiple stereocentres thus making them difficult to synthesize.

Hence, they are isolated from plants and employed as the starting point for the synthesis and production of novel drugs intending to have low toxicity and a diverse pharmaceutical application (Newman and Cragg, 2020).

**Table 1:** Examples of commonly used plant originated modern drugs (Yuan *et al.,* 2016*;* Mustafa *et al.,* 2017)

| **Drug** | **Therapeutic Use** | **Plant Source** |
|---|---|---|
| Acetyldigoxin | Cardiotonic | *Digitalis lanata* |
| Adoniside | Cardiotonic | *Adonis vernalis* |
| Atropine | Anticholinergic | *Atropa belladonna* |
| Cocaine | Local Anesthetic | *Erythroxylum coca* |
| Curcumin | Choleretic | *Curcuma longa* |
| Digitoxin | Cardiotonic | *Digitalis purpurea* |
| Ephedrine | Antihistamine | *Ephedra sinica* |
| Morphine | Pain reliever | *Papaver somniferum* |
| Quinine | Anti-malarial | *Cinchona ledgeriana* |
| Santonin | Ascaricide | *Artemisia maritima* |
| Taxol | Anti-cancerous | *Taxus brevifollis* |
| Vincristine | Anti-cancerous | *Catharanthus rosesus* |

**Table 2:** Common plants used in Ayurveda practices

| Plant Name | Ayurveda application | References |
|---|---|---|
| *Phyllanthus emblica* | Rasayana – disease prevention | Baliga *et al*., 2015 |
| *Withania somnifera* | Immunomodulatory | Chandran & Patwardhan, 2017 |
| *Semecarpus anacardium* | Antiarthritic | Shanuvas and Indhumathi, 2018 |
| *Aegle marmelos* | Antidiarrheal | Rakulini and Kalaichelvi, 2019 |
| *Santalum album* | Antiviral | Ravi Kumar *et al.,* 2019 |
| *Plumbago zeylanica* | Antitumour | Kapare *et al.,* 2020 |
| *Punica granatum* | Antidiarrheal | Yuliani and Mega, 2016 |
| *Ricinus communis* | Hepatoprotective | Kumar, 2017 |
| *Tinospora cordifolia* | Immunomodulatory | Salkar *et al*., 2015 |
| *Curcuma longa* | Antimicrobial | Maghimaa and Alharbi, 2020. |
| *Rubia cordifolia* | Antioxidant | Gupta and Gupta, 2017 |
| *Embelia ribes* | Antifertility | Choudhary *et al.,* 2021 |

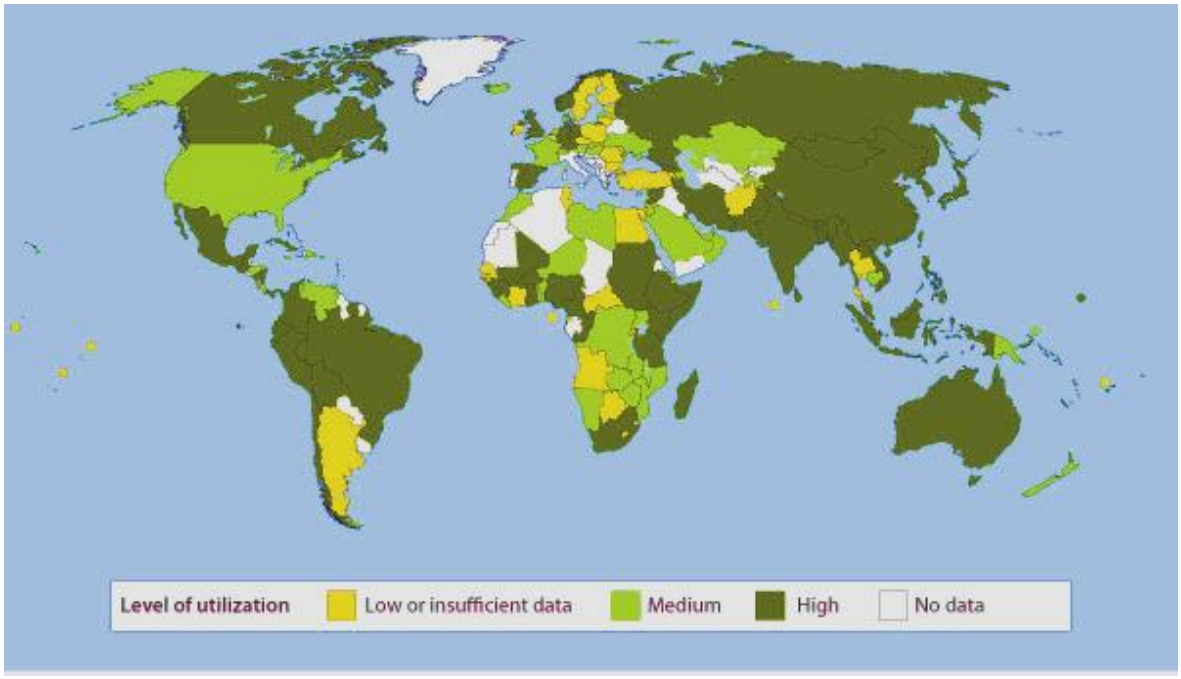


**Figure 2:** The utilization of plants as traditional and ethnomedicine across the world (Iwu, 2014).

### 1.3 Secondary metabolites

There is an assortment of organic compounds produced in plants, traditionally grouped and termed as secondary metabolites.These compounds play a vital role in the life cycle of plants, from acting as antibiotics and protecting the plant from pathogens to aiding plants in adapting to their environments (Yang *et al*., 2018). Plant secondary metabolites (PSM) are divided into three major groups depending on their biosynthetic origin. These groups are polyphenols, alkaloids, terpenes and steroids. Within the plant kingdom, not all secondary metabolites are widespread making them more specific to a particular genus and species, thus constituting the basis of chemotaxonomy and chemical ecology (Bourgaud *et al*., 2001; Misra and Srivastava, 2016). Secondary metabolites are of high value, as they have commercial and pharmacological applications (Table 3). Some applications of secondary metabolites are but not limited to their use for agrochemical synthesis, Flavour enhancers and biopesticides (Tiwari and Rana, 2015; Xu *et al*., 2016).

**Table 3:** Plant-derived compounds and their pharmaceutical value (Fabricant and Fransworth, 2001).

| **Product** | **Use** | ***Plant species*** | **Cost (US $/Kg)** |
|---|---|---|---|
| Ajmalicine | Antihypertensive | *Catharanthus roseus* | 37,000 |
| Artemisinin | Antimalarial | *Artemisia annua* | 400 |
| Berberine | Intestinal ailment | *C. japonica* | 3250 |
| Camptothecin | Antitumour | *Camptotheca acuminata* | 432,000 |
| Colchicine | Antitumour | *Colchium autumnale* | 35,000 |
| Diosgenin | Steroidal precursor | *Dioscorea deltoidea* | 1000 |
| Ellipticine | Antitumour | *Orchrosia elliptica* | 240,000 |
| Morphine | Sedative | *Papaver somniferum* | 340,000 |
| Vincristine | Antileukemic | *Catharanthus roseus* | 2,000,000 |
| Taxol | Anticancer | *Taxus brevifolia* | 600,000 |

### 1.4 *Datura stramonium*

The *Solanaceae* family contains plants that are a rich resource of tropane alkaloids. Research studies have indicated that *D. stramonium* contain sixty-four tropane alkaloids and derivatives of tropane alkaloids (Ally and Mohanlall, 2020). *D. stramonium*, commonly called Jimsonweed, Thorn Apple and Angels Trumpet is an annual herbaceous, dicotyledonous shrub belonging to the *Solanaceae* family. The plant originated in the territories of the Caspian Sea and spread to Europe in the first century. At present, the plant is found in most waste and dumpsites in Europe, Asia, America and Africa (Ally and Mohanlall, 2020).

The plant is described to grow at an approximate height of 2 meters with containing a toothed margin that is 5 - 18 cm broad and 10 - 20 cm long. Flowers of *D. stramonium* is described as axillary and resemble the shape of a trumpet thus giving its common name “Angel’s Trumpet”. A spiny capsule is a green fruit and upon drying splits open and releases black seeds. The flowers are usually cream, yellow or purple in colour (Figure 3) (Stace, 2010).

The plant is known to be a strong narcotic and poisonous. The ingestion of any part of the plant causes poisoning and may result in death. According to research based on *D.*

*stramonium* toxicity, the seeds and fruit are known to be the most toxic (Setshogo, 2015). There are no treatments available that can reduce the toxic effects of the seeds or fruit. There are many cases of Datura poisoning, the first recorded in the early 1990s in the United States of America. It was reported that many adolescents and young adults became ill and passed on from ingesting the leaves of *D. stramonium* (Adegoke and Alo, 2013). The symptoms of Jimsonweed poisoning are associated with dryness of the mouth and skin, severe thirst, dilation of the pupil, loss of eyesight, hallucination, palpitations, restlessness and loss of consciousness (Ozkaya *et al.*, 2015).

**Table 4**: Classification of Kingdom Plantae (Ally and Mohanlall, 2020)

| **Kingdom Plantae - Plants** |
|---|
| Subkingdom Tracheobionta – Vascular plants |
| Superdivision Spermatophyta – Seed plants |
| Division Magnoliophyta – Flowering plants |
| Class Magnoliopsida - Dicotyledons |
| Sub class Asteridae |
| Order Solanales |
| Family Solanaceae |
| Genus Datura |
| Species *Datura stramonium* |

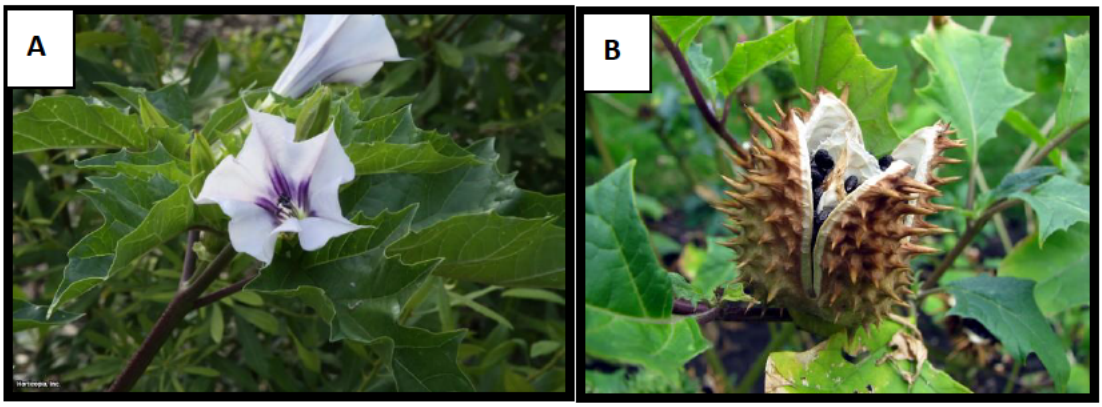


**Figure 3:** *D. stramonium* flower (A) and a spiny capsule containing seed (B).

### 1.4.1 Ethnopharmacological applications

Although many cases of *D. stramonium* intoxication and poisoning have been reported, ethnomedicinal and pharmacognostic research as per Gaire and Subed (2013) and Miraj, (2016) indicates that various parts of the plant were used independently or in a concoction for the treatment of different ailments. *D. stramonium* leaf infusions were also created to attain vapours which were used to relieve pain and inflammation associated with rheumatism and gout while the smoke of burnt leaves was used as a natural bronchodilator for the treatment of asthma and bronchitis. The fruit of *D. stramonium* which resembles a spiny capsule was processed with its contents to obtain a homogenised extract that was applied on the scalp for the treatment of dandruff and weak and brittle hair. This extract was also applied to skin abrasions and wounds to allow for quick healing. The leaves of *D. stramonium* were also used as a sedative for psychotic patients and the treatment of insomnia (Ally and Mohanlall, 2020). The liquid obtained from the flower of *D. stramonium* has been previously used in the treatment of an earache and was also noted for containing analgesic and antiasthmatic activities (Dwivedi *et al*., 2019).

### 1.4.2 Pharmacological activities

*D. stramonium* extracts were researched for their potential pharmacological profile (Rasila Devi *et al*., 2011; Sharma *et al*., 2014a). Ethanolic extracts of *D. stramonium* was investigated for use as larvicidal and mosquito repellent agents. At a concentration of 86.25 $mg.L^{-1}$, 16.07 $mg.L^{-}$ and 6.25 $mg.L^{-}$, the extracts exhibited larvicidal activity against *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasiciatus* respectively (Swathi *et al*., 2012).

*D. stramonium* leaf extracts, combined with extracts of other plants such as *Azadirachta indica* and *Coriandrum sativum,* were screened *in vivo* for their anti-inflammatory potential in albino rats. The combined ethanolic extracts were investigated using the carrageenan-induced rat paw edema method and the results of these plants exhibited anti-inflammatory activity comparable to the standard drug, Diclofenac (Sonika *et al*., 2010).

The antibacterial potential of *D. stramonium* extracts was investigated in conjunction with *W. somnifera* and *Terminalia arjuna.*The results indicated that these extracts in combination with crude ethanolic extracts of *D. stramonium* displayed antibacterial activity against *S. aureus*, *B. subtilus*, *E. coli*, *M. luteus* and *C. albicans* which were comparable to the standard drug, Ciprofloxacin (Sharma *et al*., 2009).

In 2017, a study by Baynesagne *et al.,* evaluated *D. stramonium* extracts against clinical pathogenic isolates of bacteria. The extracts, which was prepared using a four solvent system (acetone, ethanol, methanol and water), displated maximum antibacterial activity against *S. aureus* with a zone of inhibition of 18 mm. The minimum antibacterial activity was displayed against *E. coli* with a zone of inhibition of 8.2 mm (Baynesagne *et al*., 2017).

An investigation by Rajeshkanna *et al* (2016) on the ethyl acetate flower extracts of *D. stramonium* for its anticancer activity against human liver cancer cells (HepG2) using the MTT assay exhibited anticancer activity comparable to that of the standard, Gemcitabine with a $CTC_{50}$ value of 131.53 $\mu g.mL^{-1}$ (Rajeshkanna *et al.*, 2016).

Methanolic seed extracts of *D. stramonium* were investigated for their potential antioxidant and anticancer activity. The antioxidant activity was conducted using the DPPH radical, superoxide radical, ABTS radical cation, $OH^-$ radical scavenging assays, Phoshomolybdenum reduction and $Fe^{3+}$ reducing power assays, whilst the anticancer activity was investigated using the MTT assay against MCF (breast cancer) cell lines. The antioxidant results demonstrated $IC_{50}$ values for DPPH radical, superoxide radical, ABTS radical cation, $OH^-$ radical scavenging assays of 35.26, 10.50, 49.36 $\mu g.mL^{-1}$ respectively. Cytotoxic activity for the MCF7 cell line was 66.84% at 500 $\mu g.mL^{-1}$ (Iqbal *et al*., 2017). Ethanolic leave extracts of *D. stramonium* were also investigated for their potential larvicidal and mosquito repellent activities. The results for the assay demonstrated potential larvicidal against *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* with $LD_{50}$ values of 86.25 $mg.L^{-1}$, 16.07 $mg.L^{-1}$ and 6.25 $mg.L^{-1}$ respectively. Mosquito repellency was displayed for durations of 2.7, 71.7 and 117.7 minutes against *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* respectively at higher concentrations (Swathi *et al*., 2012).

### 1.4.3 Phytochemistry

A total of sixty-four tropane alkaloids has been identified from *D. stramonium.* The major tropane alkaloids being Hyoscyamine, Atropine and Scopolamine, whilst the minor tropane alkaloid present is Tigloidin, Apoatropin and Aposcopolamine. Research conducted by Ryan *et al.,* (2015), has highlighted that the production of Atropine and Scopolamine occurs in different parts of the plants at different stages of its life cycle. In young stems and leaves, Hyoscyamine is always the predominant alkaloid (Ryan *et al.*, 2015). Table 5 illustrates examples of a few tropane alkaloids that have been utilized in the pharmaceutical industry. Although different organs of the plant produce tropane alkaloids, the overall presence of these phytochemicals, within the plant, is relatively low. In previous studies, research has shown the advantage and potential of plant cell and tissue culture systems to produce secondary metabolites in higher yields and continuously (Dias *et al*., 2016).

### 1.4.4 Biological activities of plant secondary metabolites

There is potential of new drug leads being discovered during the research of secondary metabolite research. Biological assays are conducted to investigate the potential use of phytochemicals identified as well as their potential role in the pharmaceutical industry. In this project, antimicrobial and antioxidant assays were conducted on crude plant extracts. These assays will be discussed as well as their importance.

#### 1.4.4.1 Antimicrobial activity

Over the last decade, infectious diseases have been the primary cause of millions of deaths around the world (Fenollar and Mediannikov, 2018). The development and recurrence of antimicrobial-resistant pathogenic bacteria occur as a result of bacterial DNA being of mutagenic nature and the constant transformation of bacterial cells which arises during plasmid exchange and uptake. The increased number of resistant pathogens to multiple antibiotics may develop immunity against all form of antimicrobial agents, hence being untreatable. Hence, there is always a constant search for novel antimicrobial agents that has a broad spectrum of activity at non-toxic dosages (Moloney, 2016). The diverse nature of phytochemicals within plants is responsible for their therapeutic application. Plants serve as an indigenous source of antimicrobial agents which has been one of many rationales in investigating the potential of plants as anti-microbial agents. Agar well, disc diffusion,

dilution and bioautographic methods are usually employed to investigate antimicrobial susceptibility. Many indigenous and exotic plants have been investigated for their potential antimicrobial activity (Mabona *et al*., 2013; Xiong *et al*., 2013; Akthar *et al*., 2014; Brusotti *et al*., 2014; Abdirahman and Batool, 2016).

#### 1.4.4.2 Antioxidant activity

Flavonoids, alkaloids, anthocyanins, dietary glutathione and carotenoids are secondary metabolites that are known for their free radical scavenging capacity (Ravimannan and Nisansala, 2017). These secondary metabolites are rich in antioxidant activities. Antioxidants function as peroxide decomposer, inhibitors of enzymes and singlet or triplet oxygen quenchers. Molecular oxygen is an example of an electron acceptor that reacts with free radicals and result in becoming radicals themselves. These free radicals are also known to react as reactive oxygen species which include hydrogen peroxide and hydroxyl radicals.

The presence of these free radicals in the human body causes oxidative damage to lipids, nucleic acids and proteins, which eventually causes inflammation, ageing, cancer and diabetes. Plant extracts are assessed for their antioxidant potential using various assays such as the DPPH and FRAP assays. The antioxidant potential and phenolic content of *Asparagus racemosus*, *Ocimum sanctum, Cassia fistula, Piper betel, Citrus aurantifola, Catharanthus roseus* and *Polyalthia longifolia*s were analyzed using the DPPH assay and the Folin-Ciocalteu method. The investigation had demonstrated antioxidant activity in alcoholic leaf extracts between 35% and 81%. The resulting antioxidant potential was due to the aggregation of phenolic compounds present in the extract and the presence of other phytochemicals such as ascorbic acid and tocopherol (Kaur and Mondal, 2014).

#### 1.4.4.3 The Biosafety of Plant Extracts

Research and historic practices have proved plants to possess pharmacological properties, plants may prove to be toxic if administered incorrectly (Ihegboro *et al*., 2020). One may expect plants to be safe as it has been traditionally used for many years, however not much of their toxic effects of commonly used plants are well documented in the literature.

The brine shrimp lethality assay is an example of a simple rapid, reliable, and inexpensive assay that can be used to test the safety of natural products. *Artemia salina* Leach,

commonly known as the Brine shrimp, is an invertebrate belonging to the *Artemiidae* family. It is known that a positive correlation exists between brine shrimp lethality and cytotoxicity (Dominguez-Martin *et al*, 2020).

### 1.3.5 Tropane alkaloids

Tropane alkaloids are bicyclic alkaloids that contain the tropane ring. Secondary metabolites containing the tropane ring are distributed in plants within the Proteaceae, Brassicaceae, Convolvulaceae, Rhizophoraceae, Euphorbiaceae, Solanaceae, and Erythroxylaceae family (Ally and Mohanlall, 2020). There are approximately 2000 structures that contain the tropane ring however the most common is Atropine and Scopolamine. Atropine, Scopolamine and their derivatives are commercially important as they are used as anticholinergic drugs (Courdavault *et al*., 2020).

The biosynthetic pathway of tropane and pyridine alkaloids, as indicated in Figure 4, shares a common polyamine metabolism in their early steps with a common precursor being Putrescine. The enzyme, Putrescine N-methyltransferase (PMT) is responsible for the removal of Putrescine from the polyamine pool as it catalyzes the N-methylation to form N-methylputrescine (MP). The tropane ring moiety of tropane alkaloids is derived as a result of Putrescine by MP synthesis. The first step of the tropane alkaloid biosynthesis occurs with PMT catalyzing the N-methylation of Putrescine. As indicated by Figure 4, Ornithine is the precursor of 1-methyl-pyrrolidinium cation, which is an intermediate for the biosynthesis of Nicotine and Hyoscyamine in plants. Nicotine is formed by combining 1-methyl-pyrrolidium cation with Nicotinic acid, whereas the condensation of the 1-methyl-pyrrolidium cation with acetate moiety forms Tropine. Tropine is then esterified with tropic acid to yield Hyoscyamine. The direct oxidation of Hyoscyamine results in the formation of Scopolamine. Hyoscyamine 6b-hydroxylase (H6H) is an oxoglutarate dependent dioxygenase that mediates the two-step reaction to generate Scopolamine (Lee *et al*., 2005).

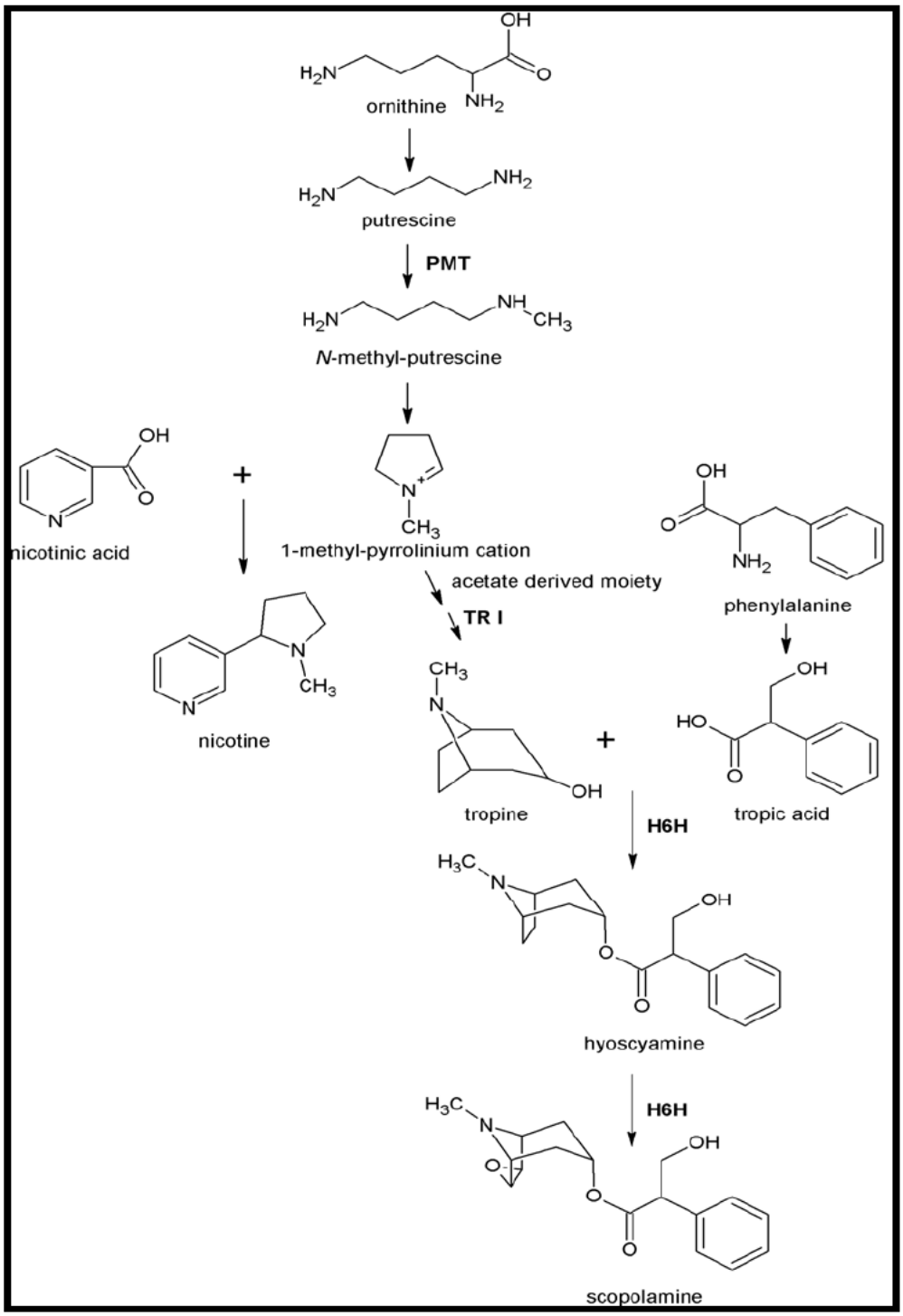


**Figure 4:** The biosynthetic pathway of tropane alkaloids sharing a common polyamine metabolism as pyridine alkaloids. The diagram highlights the importance of a precursor, Putrescine, and enzyme Putrescine N-methyltransferase (PMT), during the formation of Atropine and Scopolamine (Lee *et al.*, 2005)

### 1.3.5.1 Atropine

Atropine (Fig. 5), is one of the most common tropane alkaloids used in the pharmaceutical industry (Ally and Mohanlall, 2020). It is the racemic form of Hyoscyamine and is known to bind to muscarinic receptors which block the parasympathetic cholinergic neurons. The alkaloid acts on both peripheral and central muscarinic receptors. According to previous research, it was also found that Atropine inhibits the growth of enveloped viruses independent of the nucleic acid content . The antiviral activity of Atropine was investigated using the plaque reduction test and one-step growth experiments. It was found that Atropine was effective against the *Herpes Simplex, Influenza, Sindbis, Adenovirus* and *Japanese encephalitis* virus. Atropine was discovered to block the glycosylation of viral proteins of Herpes, hence the production of virions are inhibited. Furthermore, virions that were formed in the presence of Atropine were known to be non-infectious (Ally and Mohanlall, 2020).

The pharmaceutical application of Atropine is vast however low dosage is advised on treatment as it affects the cardiovascular system causing bradycardia. Atropine is used as an antidote to treat organophosphate poisoning as it increases acetylcholine release which inhibits cholinesterase (Vanova *et al*., 2018). It is also used to dilate pupil, decrease salivation and reduce gastrointestinal activity (Sayyed and Shah, 2014).

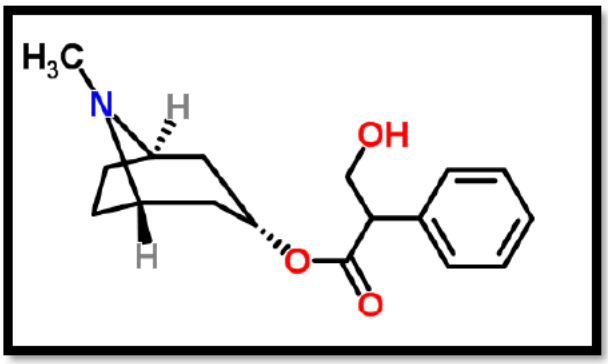


**Figure 5:** Chemical structure of the tropane alkaloid, Atropine

### 1.3.5.2 Scopolamine and Hyoscyamine

Scopolamine (Fig. 6A) is the 6, 7-β-epoxide of Hyoscyamine (Figure 6B) which is formed by the direct oxidation of Hyoscyamine without the intermediacy of a double bond. The hydroxylation of Hyoscyamine to 6β-Hydroxy-hyoscyamine is catalyzed by a 2-oxy-glutarate dependent dioxygenase, Hyoscyamine 6β-hydroxylase. The epoxidation of 6β-hydroxyhyoscyamine to Scopolamine is also catalyzed by hyoscyamine 6β-hydroxylase (Moradi *et al.*, 2020). Initially, Scopolamine was used in neuropsychopharmacology as an antidepressant and as a standard drug for the induction of age and dementia-related cognitive deficiency in healthy humans and animals (Furey *et al.*, 2010). However, the demand for Scopolamine in the pharmaceutical industry has grown due to its multiple applications and administrations.

Scopolamine is currently administered as a syrup or tablet for the treatment of nausea and vomiting. It has also been administered for the treatment of gastrointestinal spasms associated with irritable bowel syndrome (Qi *et al.*, 2019). The current hype of Scopolamine is associated with its use in creating a transdermal patch for treating motion sickness. This type of Scopolamine is known as the "transdermal scopolamine". In 1979, the first transdermal Scopolamine was manufactured by the company, Alza Corporation. Over the years the sale of transdermal Scopolamine has grown as it is the preferred choice for treating motion sickness (Pastore *et al.*, 2015). This is due to its easy administration, cost-effectiveness and availability. It has been envisaged that from 2016 to 2024, the expected Compound Annual Growth Rate (CAGR) growth rate for transdermal treatment between 4% and 6.7% (Gendelberg *et al.*, 2016; Mehrotra *et al.*, 2021).

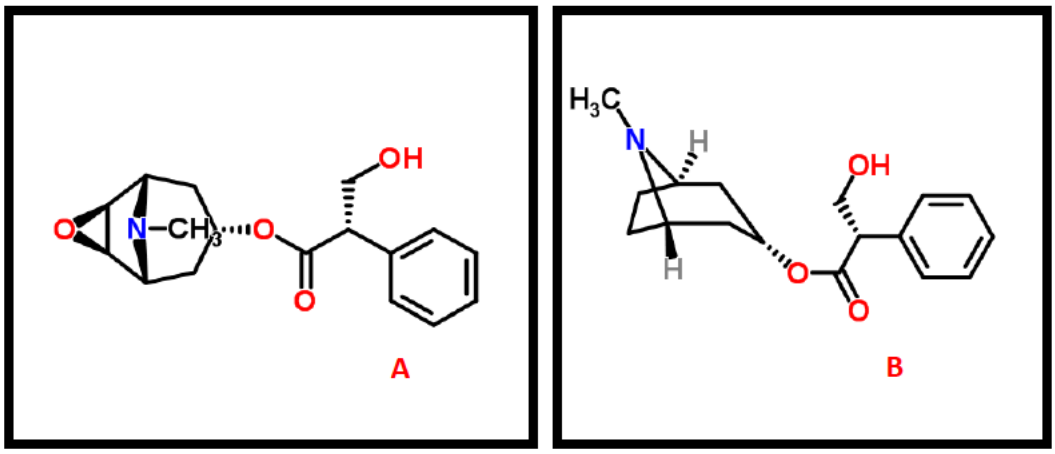


**Figure 6:** Chemical structure of Scopolamine (A) and Hyoscyamine (B)

**Table 5:** Pharmaceutical application of Tropane Alkaloids (Kukula-Koch and Widelski, 2017)

| Distribution | Examples | Pharmacological activities | Pharmaceutical applications |
|---|---|---|---|
| ***Ipomoea polpha* R.W. Johnson–giant sweet potato. Convovulvaceae, tuber** | Calystegines | Inhibitors of glycosidases | Calystegines are potent competitive inhibitors of the bovine, human and rat beta-glucosidase and alpha-glucosidase activities |
| ***Atropa belladonna* L.–belladonna/deadly nightshade, Solanaceae, leaf** | Atropine (racemic mixture), hyoscyamine, scopolamine, hyoscine | Parasympatholytic, mydriatic, spasmolytic | Preparations are mainly used against spasms and colic-like pains in gastrointestinal and biliary tract<br>Traditionally used as hallucinogenic, narcotic, anesthetic, and rheumatic pain relieving |
| ***Erythroxylon coca* Lam.–Erythroxylaceae, leaf** | Cocaine, hygrine, cinnamylcocaine, truxilline | Anesthetic, CNS stimulant, parasympathetic | Applications totally confined to ophthalmic, ear, nose and throat surgery |

## 1.5. Plant cell and tissue culture systems

Higher plants are an inexhaustible source of biochemicals that are of important value and is utilized in the pharmaceutical industry. The total yield of biochemicals vary in each species of plant. However, with the use of *in vitro* plant cells and tissue culture systems, the capability of plants to biosynthesize biochemicals can be exploited (Patel and Krishnamurthy, 2013). The increase in demand of plants for Ayurvedic use, increases the rate of depletion in a specific region, and lack of commercial planting resulting in endangerment to a species (Dawa *et al*., 2018; Lone *et al*., 2015).

There are many advantages of plant cell and tissue culture systems as compared to the field and whole plant cultivation such as:

- Plant cells are biosynthetically totipotent which means plant cells in culture retain their complete genetic information and therefore can produce a range of secondary metabolites that are found in the parent plant (Ramachandra Rao *et al*., 2002)
- Novel metabolites may be produced as the product profile may differ between the *in vitro* plant culture and the parent plant (Ramachandra Rao *et al*., 2002).
- Plant cell culture biotransformation systems can allow for the conversion of inexpensive precursors to novel or valuable compounds (Ramachandra Rao *et al*., 2002).
- Optimizing of cultural conditions allows for the higher accumulation of plant derived products in plant cell and tissue culture systems (Ramachandra Rao and Ravishankar, 1998)
- *In vitro* plant cultures are independent of climate and season, thus allowing for the plant derived product to be produced all year round (Ramachandra Rao *et al*., 2002).
- A definite advantage of plant cells for metabolite production is their ability to outweigh whole plant production systems by bypassing the long development times, variations in product yield and quality. It also eliminates contamination with fertilizers and pesticides. Good Manufacturing Practice (GMP) is easily implemented at all stages of metabolite production, *in vitro* (Hellwig *et al*., 2004*).*
- There is a more reliable secondary metabolite production which is simpler and predictable. In some cultures, the yield of secondary metabolite per gram fresh weight may be greater than the yield in wild plants (Ramachandra Rao *et al*., 2002)

- Phytochemicals can be isolated efficiently from cell cultures as opposed from complex whole plants (Efferth, 2019).
- Unwanted compounds which are present in the field-grown plants can be eliminated in cell cultures (Ramachandra Rao *et al*., 2002).

Plant and cell and tissue culture systems are divided into two categories viz., undifferentiated and differentiated cultures. The undifferentiated cultures refer to the callus and suspension cultures and differentiated cultures refer to the root, shoot and transformed hairy root culture. The type of culture to be employed depends on whether the same metabolites as the parent plant can be produced by the plant cell or tissue culture, the profile of the metabolite produced, the genetic stability and the growth profile of the plant cell or tissue culture (Mulabagal and Tsay, 2004). The key characteristics of the different plant cell and tissue culture systems are described in Table 6. The major advantage of hairy root cultures is that it produces compounds that are similar to roots of the parent plant, they are genetically stable and their growth is rapid as compared to untransformed roots and shoots.

**Table 6:** Characteristic of different plant and cell cultures (Yue *et al*., 2016)

| **Characteristics** | **Undifferentiated cell cultures** | **Organ cultures** | **Transformed tissues** |
|---|---|---|---|
| **Metabolite production** | Produces the same metabolite as the parent plant | Produces metabolite that requires cell differentiation | Produces root-derived metabolites |
| **Product profile** | Differ from the parent plant | Similar to the parent plant | Similar to the parent plant |
| **Genetic stability** | Low | High | High |
| **Growth Profile** | Rapid growth cycle | Slow growth cycle | Growth faster than untransformed |

### 1.5.1 Callus culture

Callus culture refers to undifferentiated tissues that occur around an injured or cut part of a plant. The cell masses formed are in friable form and are the basis of producing homogenous cell suspension cultures. The successful establishment of *in vitro* callus cultures is often achieved by a balance between chemical (growth medium) and the type of explants used. Murashige and Skoog (MS) media are common media used in plant tissue culture which was initially developed for the tissue culture of the tobacco plant (Dias *et al*.,2016). There is a high concentration of ammonia, nitrate, and potassium in MS media that contributes to the growth and morphogenesis of *in vitro* plant culture, thus making it the most popular used media in tissue culture. The culture medium required for the *in vitro* establishment of callus culture has four basic components: The essential elements (macronutrients, micronutrients and iron source), organic supplements (vitamins or amino acids), a fixed carbon source and Plant Growth Regulators (PGR). PGR's are critical media components that plays a vital tole in the development pathway of plant cells. (Dixon and Gonzales, 1994).

PGR is divided into five classes, mainly Auxins, Cytokinins, Gibberellins, Abscisic acid and Ethylene. Each PGR is responsible for a specific role in a plants lifecycle as described in Table 7. Cytokinins and auxins are two classes of plant growth regulators used in most tissue culture medium. The ratio of cytokinin to auxin present in the medium, determines the type of culture that will be established. Callus formation often occurs by an intermediate ratio of cytokinin and auxin.

**Table 7:** Plant growth regulators and their functions (Rademacher., 2015)

| PGR class | Function | Example |
|---|---|---|
| Auxin | Promote cell division and growth | Indole acetic acid (IAA)<br>2,4 Dichlorophenoxyacetic acid (2.4 D) |
| Cytokinin | Promote cell division | 6- Benzylaminopurine (BAP)<br>Kinetin |
| Gibberellins | Regulate growth and the influence of various developmental processes such as stem elongation, germination, dormancy, flowering, sex expression, enzyme induction and leaf and fruit senescence | Gibberellic acid |
| Abscisic acid | Inhibit cell division-commonly used to promote distinct developmental pathways such as somatic embryogenesis.<br>This a major aid in mediating the adaptation of the plant to stress. | Abscisic acid |
| Ethylene | Controls fruit ripening in climacteric fruits. Some cell cultures produce ethylene, which is built up, inhibits the growth and development of the culture. | Ethylene gas |

### 1.5.1 Plant cell suspension culture

Cell suspension cultures are initiated by agitating friable callus tissue in a liquid medium. They are composed of cells that are of different shape and size, therefore are known as a complex and heterogenic system. Cell suspension cultures are advantageous in the production of secondary metabolites as a result of their rapid growth cycle and enhanced nutrient uptake (Verpoorte *et al*., 2002). In cell suspension cultures, secondary metabolites are intracellularly based thus allowing the high concentration of cell masses to be produced before inducing secondary metabolites biosynthesis. There are many factors that influence the use of cell suspension cultures for the production of secondary metabolites (Yue *et al*., 2016). They are:

- Any cell of a plant can be multiplied to yield specific metabolites
- Secondary metabolites will be produced under controlled conditions, thus steered in the direction to meet supply and demand targets.
- Consistent product quality could be assured with the use of specific cell lines.

- Cell growth could be automatically controlled and metabolic processes could be regulated rationally, all contributing to the improvement of productivity and the reduction of labour and expenses.
- Production of substances in a chemically-controlled environment facilitates subsequent processing and product recovery steps.
- New routes of synthesis can be recovered from mutant cell lines which can lead to the production of novel products

On an industrial biotechnology application, plant cell suspension cultures can be achieved in bioreactors for industrial-scale production of secondary metabolites. Manipulation of chemical and environmental factors, PGR's and elicitors influences secondary metabolite production. Plant cell suspension culture requires suitable conditions such as agitation, light, aeration and other physical parameters to be maintained during incubation (Cardoso *et al*., 2019)

To achieve a homogenous suspension culture, various strategies have been adopted. For example, cell suspension cultures of *Podophyllum hexandrum* are difficult to maintain as the media turns brown and cells clump. This occurs as a result of a decrease in pH and resolved by adding pectinase which reduces clumping and polyvinyl pyrrolidone which prevents colour change in the media. In most callus and suspension cultures the desired compound is initially produced, however, sub-culturing and incubation over a long period reduce productivity. This occurs as a result of genetic variation due to a lack of nutrient. Hence, high yielding cell lines are selected for pilot studies (Chattopadhyay *et al*., 2001).

Although plant and cell tissue culture has potential, the industrial and commercialization of cell suspension cultures have been limited such as in the production of Shikonin from *Lithospermum erythrorhizon* and Berberine from *Coptis japonica* (Malik *et al*., 2016). Limited commercialization of the technology arises from economic feasibility due to biological and engineering factors. Plant cell cultures present a large cost on an industrial application due to their low product concentration and productivity (Davies and Deroles, 2014).

### 1.5.2. *Agrobacterium rhizogenes*-mediated transformation for the induction of hairy root cultures

*Agrobacterium rhizogenes* is a gram negative soil bacterium that causes hairy root disease in dicotyledonous plants. The hairy root disease occurs as a result of the Ri (root inducing) plasmid being transformed from the bacteria to the nuclear genome of the host plant. The Ri plasmid (Figure 7) is approximately 200 kb in size and contains two regions of T-DNA and Vir (Virulence) region, all of which contribute to hairy root disease (Rawat *et al*., 2019).

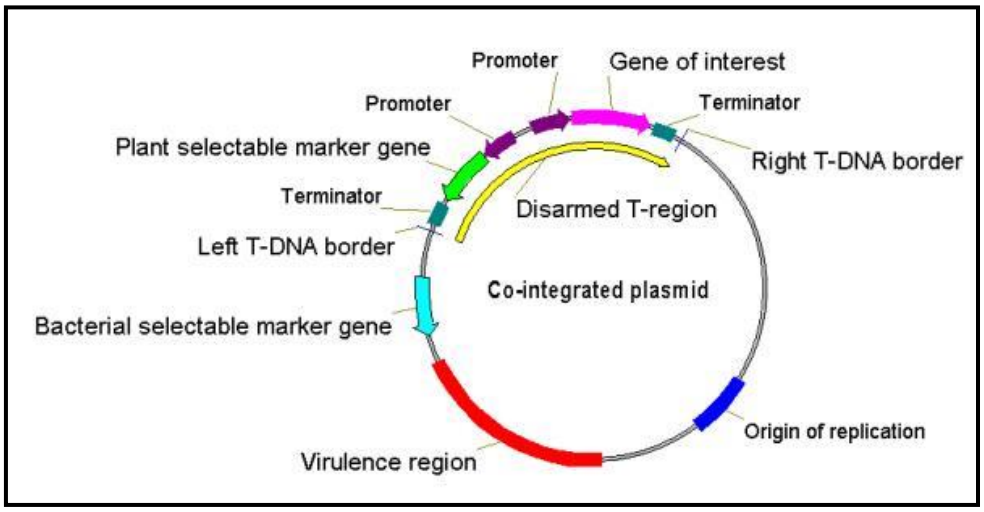


**Figure 7:** The Agrobacterium Root Inducing (Ri) Plasmid, highlighting key areas such as the virulence region, promoter and TDNA region (Rawat *et al*., 2019).

#### 1.5.2.1 Mechanism of interaction between *A. rhizogenes* and host plant

The mechanism of interaction between *A. rhizogenes* and a plant is initiated by the attachment of the bacterium to the surface of the plant cell. Wounded plants are susceptible to Agrobacterium infection. As demonstrated in Figure 8, at the site of infection, phenolic compounds such as acetosyringone are released. These phenolic compounds activate the *vir* region within the Ri plasmid. The *A. rhizogenes* plasmid consists of three genetic components that are essential for plant cell transformation. They are:

1) TDNA integrates into the plant genome and also termed mobile DNA.
2) The virulence (*vir*) area consisting of several *vir* genes which assist in the transfer of TDNA.
3) The “so-called” border sequence of 25 bp found in the chromosome of *A. rhizogenes*.

T-DNA consist of 24000 base pairs. These base pairs are encoded with enzymes responsible for producing opines and phytohormones. The integration of the TDNA into the plant genome results in the bacterium re-programming the plant cells and growth cycle, resulting in them being differentiated.

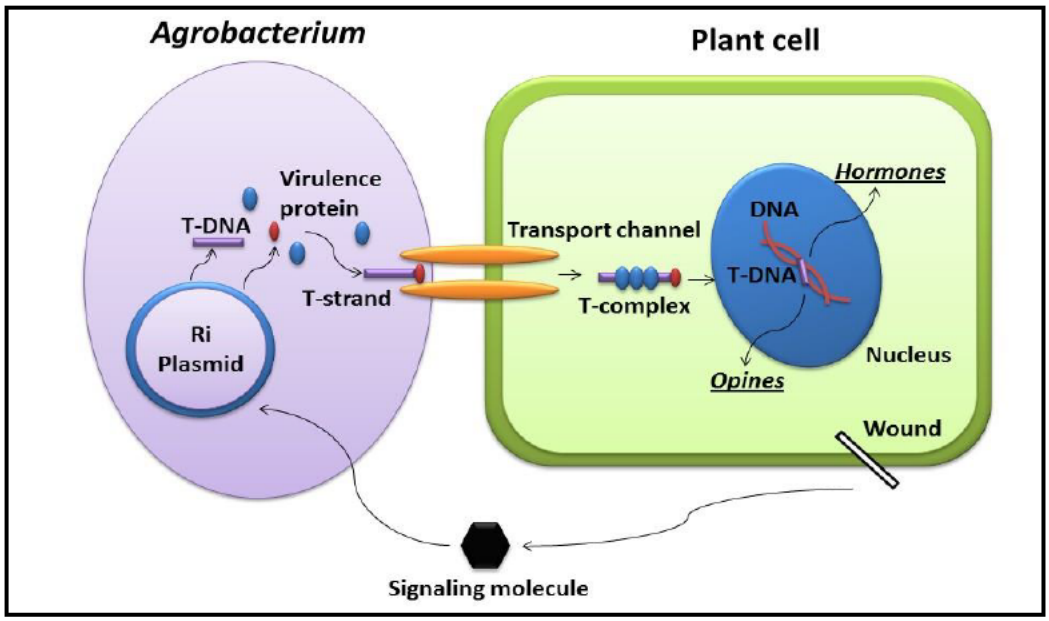


**Figure 8:** Infection of plant cell with *A. rhizogenes* indicating at the wounded site, the release of signalling molecules (phenolic compounds) that are responsible for activating the *vir* region within the Ri plasmid (Tzfira and Citovsky, 2006).


**Figure 9**: *A.belladonna* hairy root cultures. Sterile plantlet on MS ½ strength medium (A). Transformed roots emerged from wounded sites after infection with *A.rhizogenes* (B). Transformed roots growing after infection (C). Monoclone of hairy root (D) (Yang *et al*., 2011).

### 1.5.3. Characteristics of Hairy Roots

Hairy root cultures are also known as transformed roots. They are differentiated tissue of plants due to bacterial infection by *A.rhizogenes.* The hairy roots are characterized as fast-growing. Their average growth rate is approximately 0.1g to 2.0 g dry weight/litre/day. Hairy roots are morphologically described as lateral and highly branched and can grow in hormone-free media as described in Figure 9. Transformed roots offer many advantages stability compared to adventitious roots stability (Hakkinen and Oksman-Caldentey, 2018). Advantages are:

1. They offer genetic stability and can produce high content of secondary metabolites.
2. The productions of secondary metabolites in hairy roots are more stable compared to other types of plant cell culture.
3. Secondary metabolites produced in hairy roots are secreted in the growth medium.
4. They are new growing points and consequently lateral branches formed from hairy roots.
5. There is a definite growth pattern which is followed by the hairy roots, however, the production of metabolites is not growth-related.
6. Hairy roots are capable of synthesizing more than one metabolite thus making them economical for commercial processes.
7. Transformed root lines constantly produce metabolites over successive generations maintaining their genetic and biosynthetic.

The contribution of Plant Cell and Tissue culture systems, in attaining pharmaceutical valuable secondary metabolites are highlighted in Table 8.

**Table 8:** *In vitro* production of secondary metabolites in plant, callus and hairy root cultures.

| Plant Name | Active Ingredient | Culture Media and Plant Growth Regulator | Culture Type | Reference |
|---|---|---|---|---|
| ***Aconitum heterophyllum*** | Aconites | MS+2,4D+ Kinetin | Hairy root | Rawat *et al*., 2016 |
| ***Arachys hypogaea*** | Resveratol | G5 + 2,4-D + Kinetin | Hairy root | Kim *et al*., 2008 |
| ***Ammi majus*** | Triterpenoid | MS + 2,4-D + BA | Suspension cultures | Staniszewska *et al*., 2003 |
| ***Aspidosperma ramiflorum*** | Ramiflorin alkaloid | MS + 2-4,D + BAP + 30 g/l Sucrose | Callus | Oliveira *et al*., 2002 |
| ***Cinchona succirubra*** | Anthraquinone | MS + IAA + GA3 | Suspension | Jamwal *et al.*2018. |
| ***Coffea arabica*** | Caffeine | MS + 2,4-D + Kinetin | Callus | Ashebre, 2016. |
| ***Catharanthus roseus*** | Vincristine | MS + 2,4-D + GA3 | Suspension | Lee-Parsons and Royce, 2006 |
| ***Catharanthus roseus*** | Indole alkaloid | MS + 2,4-D + GA3 + Vanadium | Suspension | Zhu *et al*., 2015. |
| ***Eleutherococcus senticosus*** | Eleuthrosides | MS + 2,4-D | Suspension | Shohael *et al*., 2007 |
| ***Fabiana imbricata*** | Rutin | MS + NAA + 2,4-D | Callus and Suspenson | Schmeda-Hirschmann *et al*., 2004 |
| ***Hypericum perforatum*** | Hyperforin | MS + 2,4-D + Leusine | Multiple shoot | Karppinen *et al*., 2007 |
| ***Gymnema sylvestre*** | Gymnemic acid | MS + 2,4-D + IAA | Callus | Gopi and Vatsala, 2006 |
| ***Mentha arvensis*** | Terpenoid | MS + BA + NAA | Shoot | Phatak and Heble, 2002 |
| ***Momordica charantia*** | Flavonoid | MS + BAP + NAA | Callus | Agarwal and Kamal, 2007 |
| ***Ophiorrhiza rugosa var. decumbens*** | Camptothecin | MS + BA + Kinetin | Shoot | Vineesh *et al*., 2007 |
| ***Papaver somniferum*** | Alkaloids | MS + Kinetin | Callus | Almukhtar, A.S. 2018 |
| ***Rauolfia serpentina*** | Serpentine | MS + BAP + IAA | Callus | Salma *et al*., 2008 |

| | | | | |
|---|---|---|---|---|
| ***Silybum marianum*** | Silymarin | MS + IAA + Kinetin | Hairy root | Rahnama *et al*., 2008 |
| ***Stevia rebaudiana*** | Stevioside | MS + BA + NAA | Callus | Dheeranupattana *et al*., 2007 |
| ***Tinospora cordifolia*** | Berberin | MS + IAA + GA3 | Suspension | Rao *et al*., 2008 |
| ***Vitis vinifera*** | Anthocyanin | MS + BAP + NAA | Suspension | Qu *et al*., 2006 |
| ***Withania somnifera*** | Withanoloid A | MS + IAA + Kinetin | Hairy root | Murthy *et al*., 2008 |
| ***Zataria multiflora*** | Rosmarinic acid | MS + IAA + Kinetin | Callus | Françoise *et al*., 2007 |

### 1.6. Plant cell elicitation to produce secondary metabolites

Secondary metabolites are isolated from wild or cultivated plants as their chemical synthesis is difficult and infeasible (Brusotti *et al*., 2014). Although a plant cell and tissue culture system is an alternative method to produce pharmaceutical important secondary metabolite, there is limited success on an industrial scale. The limitation is due to the lack of understanding of the synthesis and regulation of secondary metabolites and the selection of high yielding stable cultures. There have been many techniques and technologies employed to enhance the production of secondary metabolites in plant cell cultures such as modifying of media, continuous and batch precursor feeding, plant cell immobilization and biotransformation (Davies and Deroles, 2014).

Elicitation is a process that induces or enhances the biosynthesis of metabolites to ensure survival, persistence, and competitiveness of plants an elicitor. An elicitor can be classified as biotic or abiotic depending on their source of origin of being endogenous or exogenous. The additional treatment of plant with an elicitor results in a range of defense mechanisms by the plant thus leading to the accumulation of plant defensive metabolites within intact plants or plant cultures (Gorelick and Bernstein, 2014).

The use of an elicitor in plant tissue culture has gained much popularity as it was hypothesized, researched, and proved that it has economic benefits for the pharmaceutical industries. There are various parameters which need to be considered prior to the addition of an elicitor to a culture. These parameters are but not limited to:

- The concentration of an elicitor,

- The duration of exposing the culture to the elicitor,
- The age of the culture,
- The cell line used,
- Plant Growth Hormones, the media and nutrient composition.

There have been reports of increased product accumulation in plant cells when modifying the above-mentioned parameters (Sánchez-Sampedro *et al*., 2005). Table 9 indicates the type of elicitors used in plant and cell culture and the enhanced secondary metabolite production after elicitation compared to that of a control.

**Table 9:** Elicitors used for the production of secondary metabolites by plant cell cultures (Ramirez-Estrada *et al*., 2016).

| Secondary metabolite | Plant | Elicitor | Culture Type |
|---|---|---|---|
| **Glycosylate triterpenes (Saponins)** | *Panax ginseng* | Methyl Jasmonate | Hairy root & cell suspension cultures |
| **Diterpine Alkaloids** | *Taxus chinensis & Taxus baccata* | Salicyclic Acid | Cell suspension cultures |
| **Alkaloid-Betalaine** | *Beta vulgaris* | Bacterial Extracts | Hairy root cultures |
| **Podophyllotoxin; 6-methoxypodophyllotoxin** | *Linun album* | Fungal Extracts | Hairy root cultures |
| **Ginsenosides** | *Panax ginseng* | Fungal Extracts | Cell suspension cultures |
| **Xanthones** | *Hypericum spp* | Salicyclic Acid | Hairy root & cell suspension cultures |
| **Phenolics & Flavanoids** | *Morinda citriflora* | Chitosan & Chitin | Adventitious root |
| **Tropane alkaloids** | *Catharantus roseus* | Methyl Jasmonate | Hairy root cultures |
| **Phenylpropanoids** | *Vitis vinifera* | Salicyclic Acid | Cell suspension cultures |
| **Tropanic Alkaloid** | *Catharantus roseus* | Salicyclic Acid | Hairy root cultures |
| **Rosmarinic acid** | *Lavandula vera* | Methyl Jasmonate | Cell suspension cultures |
| **Rutin** | *Fagopyrum tataricum* | Bacterial extract | Hairy root culture |
| **Diosgenin** | *Dioscorea zingiberensis* | Other cell wall fragments | Cell suspension culture |

# 2. MATERIALS AND METHODS

## 2.1 Acquisition and Preparation of Plant Material

Mature plants of *D. stramonium L* were collected locally in Durban, Kwa Zulu Natal. These plants were identified and verified by Botanist, Professor Himansu Baijnath. A voucher specimen (Baijnath sn.) was deposited in the Ward Herbarium, University of KwaZulu-Natal (Westville). The plant leaves, root and seed pods were washed to remove excess sand and dried as indicated in Figure 10. Healthy leaves and roots were dried and then mechanically ground to powder. All seeds were removed and inspected for insect debris, which was removed. The dried plant material was stored in an airtight container at room temperature until required.


**Figure 10:** *D. stramonium* plant being sorted and cleaned. Dried wild leaves (A) and Roots (B) before being ground for extraction and seed pods (C) from a dry spiny capsule of the plant.

## 2.2 Seed germination and shoot culture

All plant tissue culture procedures were conducted in a laminar flow cabinet (Scientific Engineering INC). The laminar flow cabinet was sterilized by UV light and disinfected by swabbing with 70% ethanol (Merck, South Africa) before use. All tissue culture instruments (forceps and scalpel) were autoclaved, dipped in 70 % ethanol and passed through a flame.

Mature seeds were obtained by immersing all seeds in water in a petri dish. Seeds that sank to the bottom of the petri dish were considered mature seeds and used for further investigations. Mature seeds were selected and surface sterilized for germination. The seeds were subjected to various surface sterilization regimes as described in Table 10. Each

sterilization regime was referenced as per literature with modifications. After each regime, seeds were rinsed thrice with sterile distilled water. Five seeds were placed on Murashige and Skoog hormone-free medium (Merck) and a replicate of three plates was prepared for each trial. All plates were incubated at room temperature under visible light. Post three weeks incubation, seeds were examined for germination and sterility. The sterilization trial that produced a moderate percentage of sterile and facilitated germination was selected as a treatment regime for seeds. These seeds were inoculated on MS medium without growth hormones for six weeks, to obtain germinated seedlings.

**Table 10:** The five different sterilization regime trials for seeds.

| Trial Number | Treatment regime | Exposure time (minutes) | Reference |
|---|---|---|---|
| **1** | 0.1% $Hg_2Cl_2$ | 5-7 | (Manzoor and Bhat., 2013) |
| **2** | 0.1% $Hg_2Cl_2$ and 1% hypochlorite | 3-5 | (Zayed *et al*., 2006) |
| **3** | 70% EtOH + 50% bleach | 2-3 | (El-Rahman *et al*., 2008) |
| **4** | 6% Sodium hypochlorite and Tween | 3-5 | (Al-Utbi *et al*., 2013) |
| **5** | 30% Sodium hypochlorite | 3-5 | (Naicker, 2012) |

Six weed old germinated seedlings were transferred onto MS media supplemented with 6-Benzyl Amino Purine (BAP) and 3-Indole Acetic Acid (IAA) at different ratios as described in Table 11 for optimum shoot development. The shoot development was used as explants to obtain further cell cultures for tissue culture systems required in the project.

**Table 11:** BAP and IAA ratios investigated to obtain optimum shoot medium

| **BAP mg.mL$^{-1}$** | **IAA mg.mL$^{-1}$** |
|---|---|
| 0.5 mg.mL$^{-1}$ | 1 mg.mL$^{-1}$ |
| 0.5 mg.mL$^{-1}$ | 0.5 mg.mL$^{-1}$ |

## 2.3 Cultivation of callus cultures

### 2.3.1 Induction of callus cultures

Leaf explants from shoot cultures were removed and with the aid of a scalpel. These leaves were cut into square discs. The square discs were placed on MS agar supplemented with two different hormone treatments i.e. 1 mg.mL$^{-1}$ BAP and 2, 4 Dichlorophenoxyacetic acid (2,4D) as described in Figure 11, and 1 mg.mL$^{-1}$ 2.4 D and 0.5 mg.mL$^{-1}$ BAP. These plates were incubated in the dark phase at 26˚C for 6 weeks. The callus cultures were maintained by subculturing at three-week intervals by transferring callus tissue onto fresh medium.

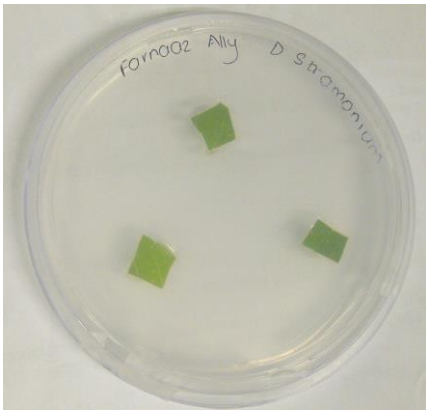

**Figure 11:** Leaves of *in vitro* cultures excised and placed on MS hormone supplemented medium before incubation

### 2.3.2 Microscopic analysis of callus

Initiated callus cultures from the explants in Petri dishes were placed under the dissecting microscope (Nikon SM2800) to view callusing of the explant and morphological parameters (colour and root-like structures) of induced callus at 100X magnification.

## 2.4 Cultivation of cell suspension culture

Cell suspension cultures were obtained by transferring 1 g of callus culture into 250 mL Erlenmeyer flasks containing 50 mL of MS liquid medium supplemented with 1 mg.mL$^{-1}$ BAP and 2, 4 D. The flasks were incubated at 26$^{o}$C at 150 rpm in the dark phase for one week. After a week of incubation, 50 mL of MS medium was added to the inoculum. The flasks were further incubated for 6 weeks. A sucrose feed of 30 g/L was prepared and fed to the

flask at a two-week interval. At the end of the cultivation period, the suspension cultures were centrifuged at 4000 rpm for 10 minutes to obtain the cell mass being produced. The total weight of the cells produced was obtained by the following equation:

$$(Mass\ of\ beaker + Cell\ mass) - Mass\ of\ beaker$$

## 2.5 Production of hairy root cultures

### 2.5.1 Cultivation of *A. rhizogenes*

*A. rhizogenes* (15834) was provided on Yeast extract-peptone (YEP) agar by the Council of Scientific and Industrial Research (CSIR), South Africa. Stock cultures of *A. rhizogenes* were prepared by streaking out a loopful of the culture onto YEP agar plates (Naicker *et al*., 2016). The strain was cultivated for 48 hours at 30°C and stored at 4°C in a refrigerator. The stock culture was used to prepare a cell suspension culture by streaking out a loopful of *A. rhizogenes* onto a YEP agar plate which was incubated for 24 hours at 30˚C. A loopful of this culture was then used to inoculate a 250 mL Erlenmeyer flask containing 50 mL of YEP liquid medium. The flasks were placed on a shaker and incubated for 24 hours at 30˚C. The optical density (OD) of the culture was determined spectrophotometrically at 600 nm and the culture was diluted with YEP medium to an $OD_{600nm}$ of 1, 0.5 and 0.2 for the transformation experiment.

### 2.5.2 Storage, Culture & Regeneration of *A. rhizogenes*

*A. rhizogenes* was cryopreserved by using the following protocol. A 24-hour liquid culture of *A. rhizogenes* was prepared as described in section 2.5.1. An aliquot of 1.5 mL of glycerol was added to 8.5 mL of the liquid culture. Aliquots of 1 mL of the prepared cells were then transferred to cryovials which were stored in an ultra-freezer at -80˚C. When required the cryovial was removed from the ultra-freezer, thawed and swabbed with 70 % ethanol. A loopful of the cells was used to inoculate a 250 mL Erlenmeyer flask containing 50 mL YEP liquid medium which was further incubated for 24 hours at 30˚C (Naicker, 2012).

### 2.5.3 Hairy root induction in *D. stramonium* using *A. rhizogenes*

*In vitro* grown shoot plants were used as a source of explants for the induction of hairy roots using *A. rhizogenes* (15834). The stems and leaves were inoculated with three different concentrations of the *A. rhizogenes* (15834) cultures ($OD_{600nm}$ of 0.2, 0.5 and 1). Leaf explants were inoculated by pricking and stem explants were cut. Explants inoculated with YEP medium served as the control. The infected and control explants were placed on MS hormone free medium and incubated for 4 weeks at 26˚C under standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}.m^{-2}$ and a 16 h photoperiod. The Agrobacterium transformation efficiency was determined by the following equation:

$$(Number\ of\ hairy\ roots \div Number\ of\ explants) \times 100$$

Induced hairy roots were excised followed by decontamination with 200 $mg.L^{-1}$ of Cefotaxime for five days. Confirmation of sterile hairy roots was done by placing hairy roots on YEP medium and incubating it at 30˚C for three days. Confirmed sterile roots, was then inoculated into an Erlenmeyer flask containing MS hormone free liquid medium following incubation at 26˚C under standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}.m^{-2}$ and a 16 h photoperiod with agitation at 150 rpm. The culture was maintained by sub-culturing onto fresh medium at four- week intervals. The culture sterility was checked at each subculturing interval to ensure a sterile culture was maintained throughout.

### 2.5.4 Elicitation of hairy root using Methyl Jasmonate

Elicitation of hairy roots was done according to Kang *et al*, 2004, with modifications. Two-week-old hairy root cultures were elicited using 1.0mM sterile filtered Methyl Jasmonate (Sigma-Aldrich, South Africa). Flasks were inoculated and incubated at 26˚C under standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}.m^{-2}$ and a 16 h photoperiod. Flask without MeJa served as a control. The measurement of root growth was achieved using the growth index (GI) formula. At 24, 48 and 72 hours, cultures were harvested and adventitious roots were separated from the medium. The roots were blotted dry and weighed as fresh weight (FW).

$$Growth\ Index = Harvested\ root\ dry\ weight\ (g) \div Inoculated\ root\ dry\ weight\ (g)$$

## 2.6 Extraction and analysis of tropane alkaloids

### 2.6.1 Extraction of tropane alkaloids from field roots and shoots and cell suspension cultures

Methanol (Merck, South Africa) was the solvent used for extraction. The preparation of the cultures remained the same however all extractions differed and were conducted as outlined below:

**Field roots**: Ground material was extracted with Methanol by agitation on a benchtop shaker at 150 rpm for 48 hours at room temperature.

**Shoot cultures**: Shoot cultures were removed from the jars, dried at 30$^{o}$C in an oven and crushed to a fine powder. Methanol was added to the powder and extraction was done on a platform shaker at 150 rpm for 48 hours at room temperature.

**Cell suspension culture**: To obtain cell mass, cultures were centrifuged at 4000 rpm for 10 minutes at 4$^{o}$C. The supernatant was retained as the extracellular component. The pellet, which represented the biomass and intracellular extract, was disrupted by sonication (Virsonic, Virtis) at 4 psi for 10 minutes. The vessel containing the biomass was kept on ice to avoid thermal damage to the extract. The cell mass and supernatant were agitated in methanol on a shaker at 150 rpm for 48 hours at room temperature.

**Hairy root culture:** Hairy roots were removed with the aid of sterile forceps and the media was retained as the extracellular component. The roots were dried, macerated and submerged in methanol for extraction at 150 rpm for 48 hours.

The preparation of intracellular extracts entailed filtration to separate powder or cells from the solvent. The solvent mix obtained was concentrated using a rotary evaporator with rotation at 60 rpm and a water bath set at 50$^{o}$C. The residue obtained was stored in 5 mL of 100% methanol, sealed and covered in foil to prevent evaporation and degradation by light.

### 2.6.2 Detection and quantification of tropane alkaloids by Liquid Chromatography

#### 2.6.2.1 HPLC system

The HPLC system consisted of a liquid chromatograph, a micro vacuum degasser (Shimadzu prominence DGO-20A3), an autosampler (prominence SIL20A), a diode array detector (Shimadzu prominence SPD-M20A) – 215 nm, a column oven (Prominence CTO-20A) and a controller. The Lab Solutions software was used to control the LC system for data processing.

#### 2.6.2.2 HPLC Method

Chromatographic separations were performed on a $C_{18}$ column. For the separations, a gradient mobile phase A [1% (v/v) formic acid in water] and mobile phase B [1% ( v/v) formic acid in methanol] was used. The gradient profile was set as follows: 0.00 min 10% B eluent, 10.00 min 90% B eluent, 17.00 min 90% B eluent, 17.10 min 10% B eluent, 23.00 min 10% B eluent. The flow rate was 0.2 mL/min and the column temperature was set at 50$^{o}$C. The injection volume was 2 µL for the extracts and standard mixtures.

#### 2.6.2.3 Standard solutions

Standard solutions of Atropine and Scopolamine were prepared for the calibration curve in the concentration range from $10^{-6}$ to $10^{-3}$ mg.mL$^{-1}$ in a solvent mixture [1 % ( v/v) formic acid in methanol: water =7:93]. The solutions were stored at 4$^{o}$C before use.

## 2.7 *In-vitro* biological activity of *D. stramonium* leaf and root extracts

### 2.7.1 Antimicrobial activity

The antimicrobial activity (antibacterial and antifungal) and the minimum inhibitory concentration of wild *D. stramonium* leaf and root extract were carried out using the agar disc diffusion method (Cos *et al*., 2006). Extracts were prepared as in section 2.6.1. All extracts were reconstituted in 100% Dimethyl sulfoxide (DMSO) before *in vitro* experiments.

#### 2.7.1.1 Antibacterial assay

The ten bacterial strains used were obtained from the stock collection in the Department of Biotechnology and Food Technology, Durban University of Technology. They were: *Escherichia coli* (ATCC 25922)*, Klebsiella pneumoniae* (ATCC 700603)*, Enterococcus faecalis* (ATCC 29212), *Bacillus cereus* (ATCC 10876)*, S. aureus* (ATCC 29213)*, Serratia marcescens* (DBT*_ZM)*, S. typhi* (DBT*_AF), *E. aerogenes* (DBT*_D), *P. vulgaris* (ATCC 6380) and *Providencia rettgerii* (DBT*_PR).

DBT*: Durban University of Technology Culture Collection reference strain based at the Department of Biotechnology and Food Science.

Cultures were plated out and verified. Stock cultures were stored in microbank vials (Davies Diagnostics, South Africa) using 50% glycerol. When required, the cultures were plated out on Mueller- Hinton Agar (Fluka, Biochemika) plates and grown in Mueller-Hinton Broth (Fluka, Biochemika) for 24 h at 37°C. Post incubation, the sterility of the cultures was checked by conducting Gram Stains. The microscopic view of cell morphology and Gram reaction of three test cultures is demonstrated in Figure 12. The concentrations of each bacterial suspension were adjusted to MacFarland standard of 0.5 absorbance which corresponded to $10^8$ CFU/mL.

A suspension (1 mL of $10^8$ CFU/mL) of the test bacteria was spread on MH Agar plates (Fluka, Biochemika). The filter disks were prepared by cutting 5 mm disks from Whatman No. 1 filter paper and they were dried in open sterile Petri dishes in a biological safety cabinet (Labotec Bioflow II, South Africa). These were inoculated with 20 µL of the sample at a concentration of 3 $mg.mL^{-1}$ and placed onto inoculated agar plates and incubated at 37°C

for 24 h. Each concentration was tested in triplicate. 100% DMSO (20 µL) was used as the negative control and ciprofloxacin 1 $mg.mL^{-1}$ (Fluka, Biochemika), was used as a positive control. The effect of the compound was determined by measuring the diameter of clearing around the disks in mm.

#### 2.7.1.2 Antifungal assay

Three yeast cultures, *Candida albicans* (DBT*_AB), *Candida utilis* (DBT*_AB) and *Saccharomyces cerevisiae* (DBT*_R) and four fungi, *Aspergillus flavus* (DBT*_AR*), Aspergillus niger* (DBT*_AE), *Fusarium oxysporum* (DBT*_AU), Penicillium sp. (DBT*_AC), were inoculated on Sabouraud Dextrose Agar (SDA) plates (Biolab, South Africa).

The yeast cultures were grown in Sabouraud Dextrose Broth for 24 h at 28 °C. The moulds were incubated at 28°C for 4 to 7 days in Sabouraud Dextrose Agar (SDA) until sporulation. The spores were collected in 10 mL sterile distilled water, counted in a Neubauer counting chamber and the concentration adjusted to $10^6$ spores/mL. Sterile distilled water containing the fungal spores ($10^6$ spores/mL) was poured over the SDA base plates (Biolab, South Africa). The filter disks were prepared by cutting 5 mm disks from Whatman No. 1 filter paper and they were dried in open sterile Petri dishes in a biological safety cabinet (Labtec Bioflow II, South Africa). These were inoculated with 20 µL of the sample at a concentration of 3 $mg.mL^{-1}$ and placed onto inoculated agar plates and incubated at 37 °C for 24 h. Each concentration was tested in triplicate. 100% DMSO (20 µL) was used as the negative control and whilst amphotericin B, 5 $\mu g.mL^{-1}$ (Fluka, Biochemika) was used as a positive control. The effect of the compound was determined by measuring the diameter of clearing around the disks in millimeters (mm).

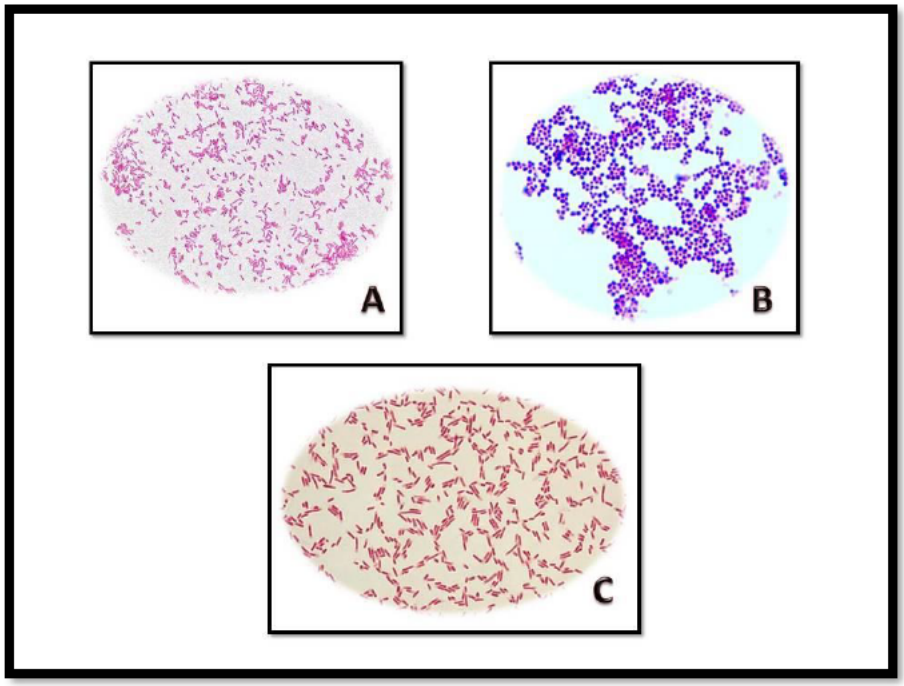


**Figure 12:** Gram stain of *E. coli* **(A),** *S .aureus* **(B)** and *S. marcescens* **(C)**

#### 2.7.1.3 Minimum inhibitory concentration

Where significant inhibitory effects were displayed against the test microorganism, this was further investigated by conducting a Minimum Inhibitory Concentration (MIC) investigation.

**Principle of the assay**

The lowest concentration at which an antimicrobial agent inhibits microbial growth is termed as minimum inhibitory concentration and is determined by a dilution method outlined by (Grela *et al.,* 2018). In the dilution tests, microorganisms are tested for their growth competence in a series of microplate wells containing broth as well as dilutions of the microbial agent.

In determining the MIC values, a growth indicator such as p-iodine tetrazolium violet (INT) is used. The INT reaction is based on the transfer of electrons from NADH, a product of the threonine dehydrogenase (TDH) catalysed reaction, to form the tetrazolium dye (INT). TDH from bacteria catalyses the oxidation of the threonine which is NADH dependent to form 2-amino-3-ketobutyrate and NADH. During microbial growth, an electron is transferred from NADH to INT violet resulting in formazan dye, a purple colour (Mohanlall and Odhav, 2013).

The chemical reaction is illustrated in Figure 13 which describes the reaction pathway of threonine dehydrogenase and INT coupling reagent during the colorimetric assay.

**MIC assay**

A 100 µL of MH Broth was added to each well on a 96 microtiter well plate. A 100 µL of each of the compound at a concentration of 6 $mg.mL^{-1}$ was added in well 2 and serially diluted to the 9$^{th}$ well, from which a 100 µL was then discarded resulting in each well have a consistent volume of culture media and drug. In each of the ten wells, 10 µL of culture standardised according to McFarland's standard was added to the wells. Well 10 and 12 was used as a negative control and contained the culture and broth only whilst Well 1 contained the positive control and Well 13 the sterility control containing media only. The plates were incubated at 37°C for 16 hours. Post incubation, 50 µL of 0.02 $mg.mL^{-1}$ INT was added to each well and further incubated for 30, 60 and 90 minutes. Results were qualitatively analysed after each incubation period by visualising the colour changes on the plate.

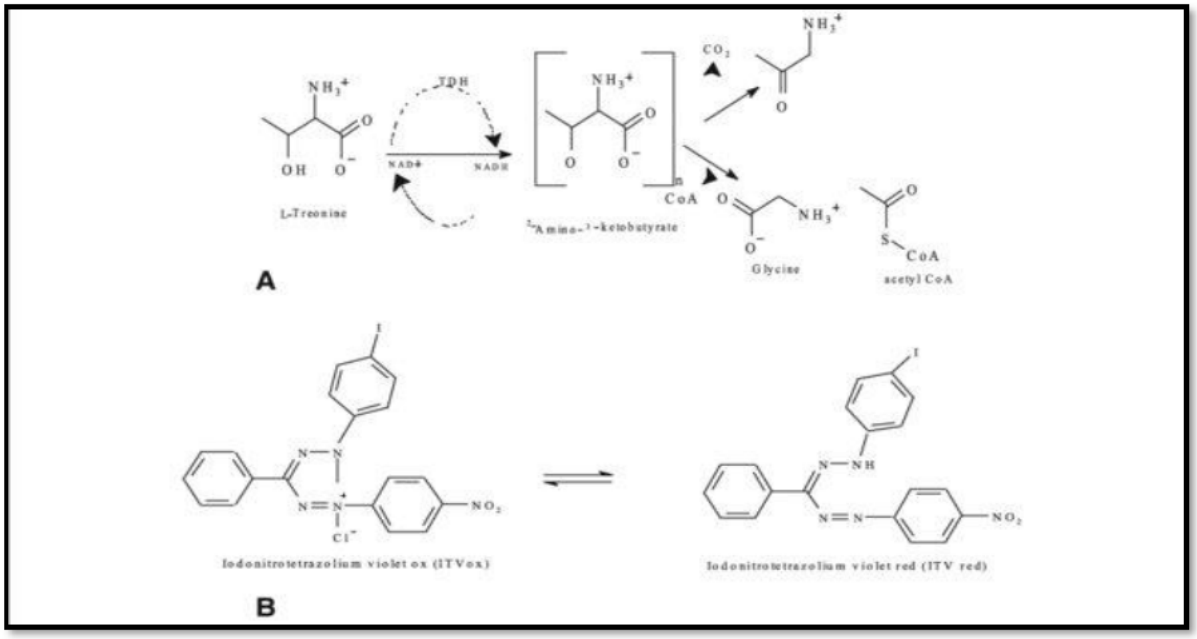


**Figure 13**: Reaction pathway of threonine dehydrogenase (A) and INT coupling reagent used for the MIC colorimetric assay (B) (Mohanlall and Odhav, 2013).

### 2.7.2 Antioxidant activity

The antioxidative properties of the *D. stramonium* leaf and root extracts were tested using the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) photometric assay described by (Choi *et al.*,

2002). DPPH is a free radical which forms a stable molecule on accepting an electron or hydrogen donor as indicated in Figure 14.

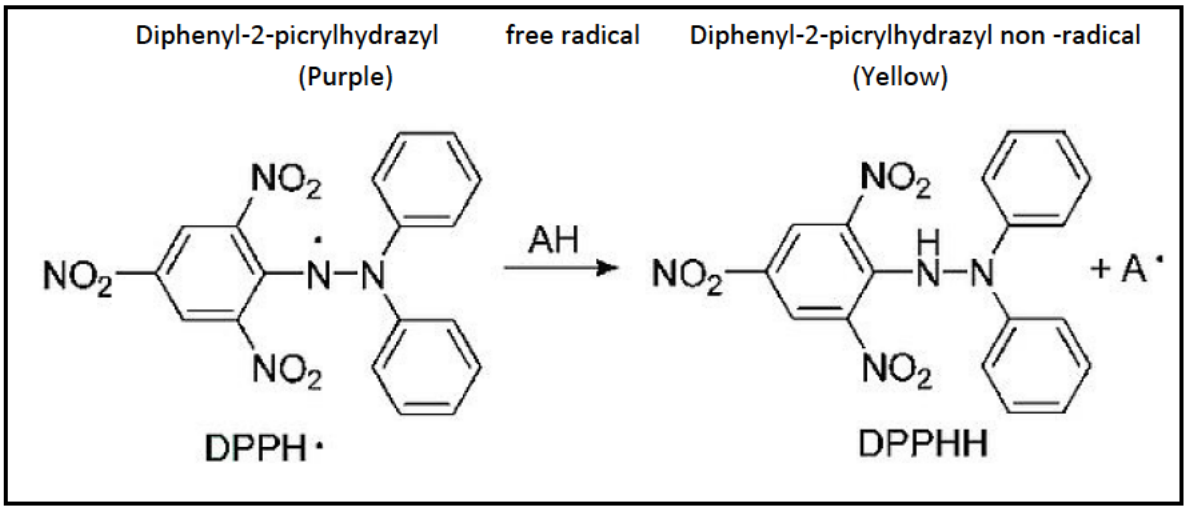


**Figure 14:** Reaction of DPPH with antioxidants which results in a yellow change in colour (Teixeira *et al.*, 2013).

#### 2.7.2.1 Preparation of the compounds

Stock solutions (2000 μg.mL$^{-1}$) of the extracts were prepared in methanol and diluted to final concentrations of 1000, 500, 250, 100, 50, 10 and 1 μg.mL$^{-1}$ in methanol. Rutin found in the buckwheat plant, *Fagopyrum esculentum* was used as a comparative standard and positive control.

#### 2.7.2.2 DPPH photometric Assay

1 mL of a 0.3 mM DPPH in methanol was added to 2.5 mL of sample solution of different concentrations and was allowed to react at room temperature for 30 min. 1 mL methanol plus the 2.5 mL of test solution was used as a blank and 1mL of DPPH solution and 2.5 mL methanol was used as a negative control. The positive control was 1 mL of DPPH solution plus 2.5 mL of 1 mM Rutin. Each test was carried out in triplicate and results are expressed as the mean ± standard deviation (Choi *et al.*, 2002). The absorbance values were measured using a Varian Cary 1E UV-Visible spectrophotometer at 518 nm and the average absorbance values were converted into the percentage antioxidant activity, using the following equation:

$$\textbf{Scavenging capacity (\%)} = \frac{\text{(Absorbance of sample - Absorbance of blank)}}{\text{Absorbance of negative contol}} \text{ X } 100$$

### 2.7.3 Brine Shrimp (*Artemia salina)* Lethality Assay

The brine shrimp lethality assay is an alternate technique used to assess the toxicity of plant extracts.

#### 2.7.3.1 Sample preparation

10, 100, 500 and 1000 µg.mL$^{-1}$ of the extracts were prepared in 100% DMSO. To eliminate any toxic effect due to DMSO, 100% DMSO was used as a control. The test involved hatching the shrimp and then exposing the larvae to *D. stramonium* leaf and root extracts.

#### 2.7.3.2 Hatching the shrimp

25 mg of Class C *Artemia salina* eggs were purchased from Natures Petland, Durban, South Africa. These were added to artificial saltwater and kept at room temperature. The pH was adjusted to 9.0 using 1 M sodium carbonate (Sigma-Aldrich) to avoid a risk of death to the *Artemia* larvae by a decrease of pH during incubation. They were incubated in a hatching chamber at room temperature. After 24 h, 15 mL of yeast solution was added to the chamber for every litre of saltwater to feed the larvae. 48 h after the eggs were incubated; the larvae were extracted by picking up the moving larvae and visibly counting them in a counting chamber.

#### 2.7.3.3 Bioassay

Using a six-well microtiter plate, ten shrimps were transferred into wells containing 5 mL of sterile seawater which was supplemented with a drop of yeast solution of 3 mg.mL$^{-1}$. A 100 µL dose of each compound was added to the well. 100% DMSO was used as a negative control. Post 2, 4, 6, and 24 h of incubation, percentage death at each dose and controls was determined. The number of shrimp death was substituted into the formula below to obtain the percentage of death caused by the compound at respective doses (Meyer *et al*., 1982).

$$\% \text{ Death} = \left[ \frac{\text{Number of shrimps dead}}{10} \right] \text{X} \quad 100$$

# 3. RESULTS

## 3.1 Plant Material

All plant material obtained was washed and dried before using. Although phytochemicals can be extracted from fresh plant material, the dried powdered material was preferred due to the traditional use of the plant and the period between harvesting plants and conducting experiments on them. For the current research, only leaves and roots were selected as Atropine and Scopolamine are predominantly found in these parts of the plant. Table 12, indicates the dried plant material obtained and percentage yield extracted from plant material.

**Table 12:** Percentage yield of dry weight obtained from fresh plant material

| Plant Organ | Initial material weight (g) | Dry weight (g) | % Yield of dry weight |
|---|---|---|---|
| Leaves | 180 | 74 | 41 |
| Roots | 180 | 89 | 49 |

## 3.2 Seed germination and shoot culture

The maximum seed germination and sterility of *in* vitro grown plants of *D. stramonium* was determined by conducting 5 different sterilization regimes. On completion of the different sterilization regimes, the number of germinated seeds and sterile seeds differed. The seeds exposed to 0.1% mercuric chloride resulted in 100% sterility however only 11% of the seeds had germinated. Seeds exposed to mercuric chloride in conjunction with hypochlorite resulted in 100% sterility with no germination. Seeds exposed to 70% ethanol in conjunction with 50% bleach resulted in 40% sterility and 20% of the seeds germinated. Seeds exposed to 6% sodium hypochlorite with tween resulted in 5% sterility and 60% of the seeds germinated (Fig. 15). Figure 17(a) graphically demonstrates the comparison of different sterilizing regimes on seeds of *D.stramonium*

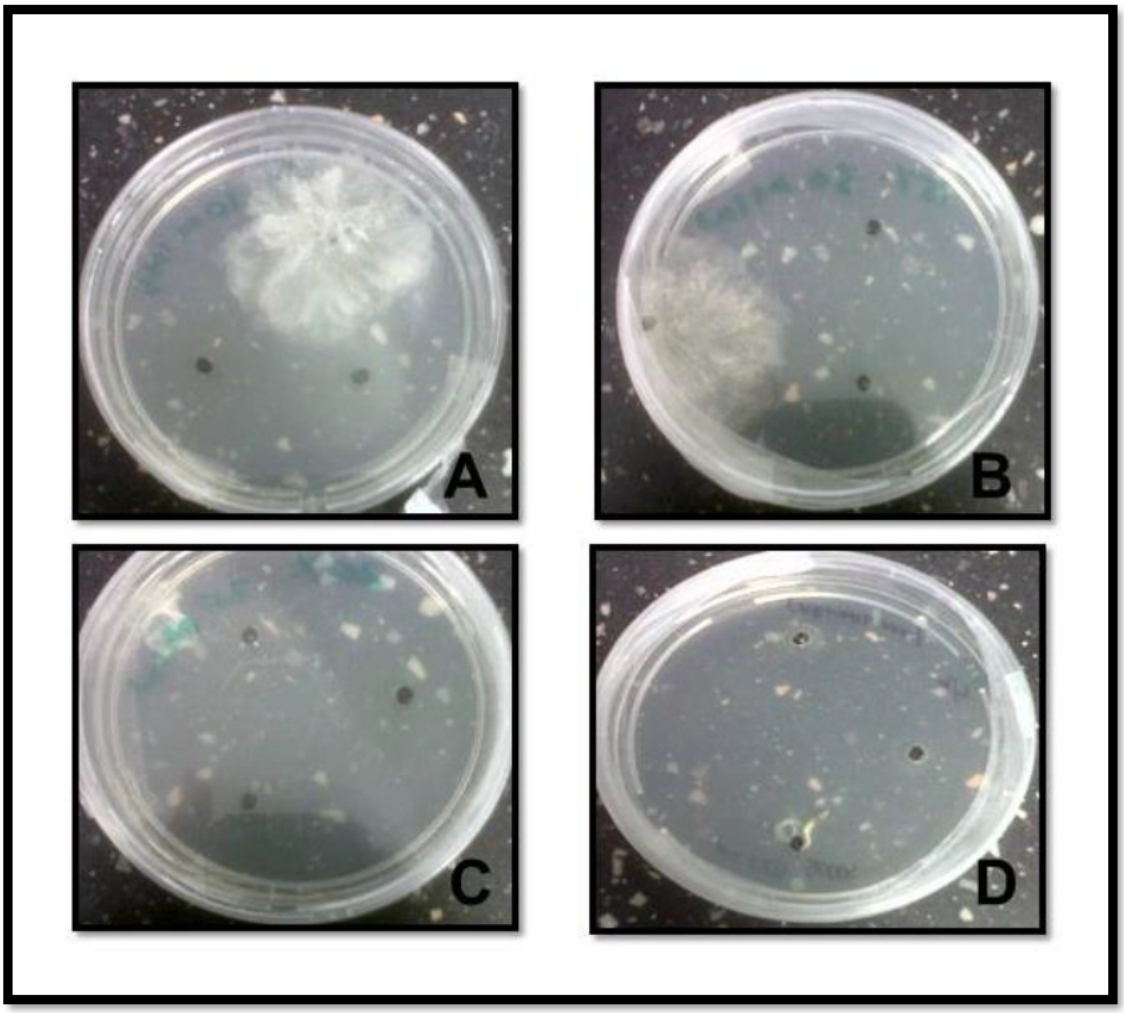


**Figure 15:** Fungal contamination on seeds post-treatment with 70% Ethanol and 50% bleach (A), and 6% Sodium hypochlorite and Tween (B). Sterile seeds produced by 0.1% mercuric chloride (C) following 10% germination (D)

Seeds exposed to 30% sodium hypochlorite resulted in 100% of the seeds being sterile with 40% of the seeds have germinated (Figure 17B). Hence, exposure to 30% sodium hypochlorite was used as a sterilisation protocol for *D. stramonium* seeds and germination was noticed after three weeks.

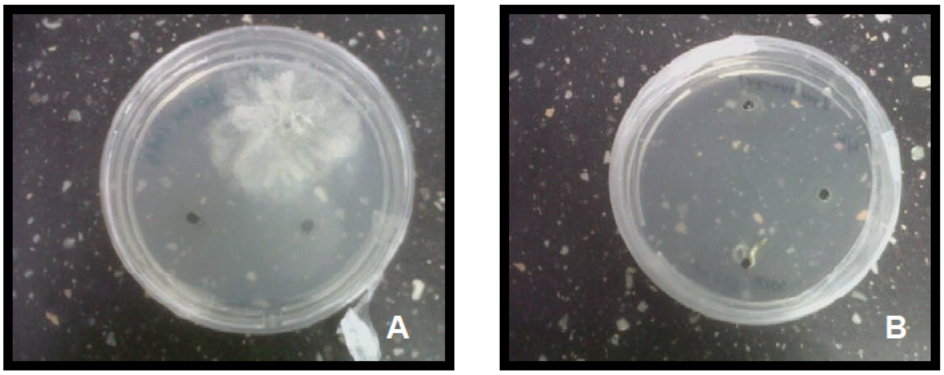


**Figure 16:** Seeds contaminated after being sterilised with 70% ethanol and bleach (A). Sterile seeds obtained after being sterilised with 30% sodium hypochlorite (B).

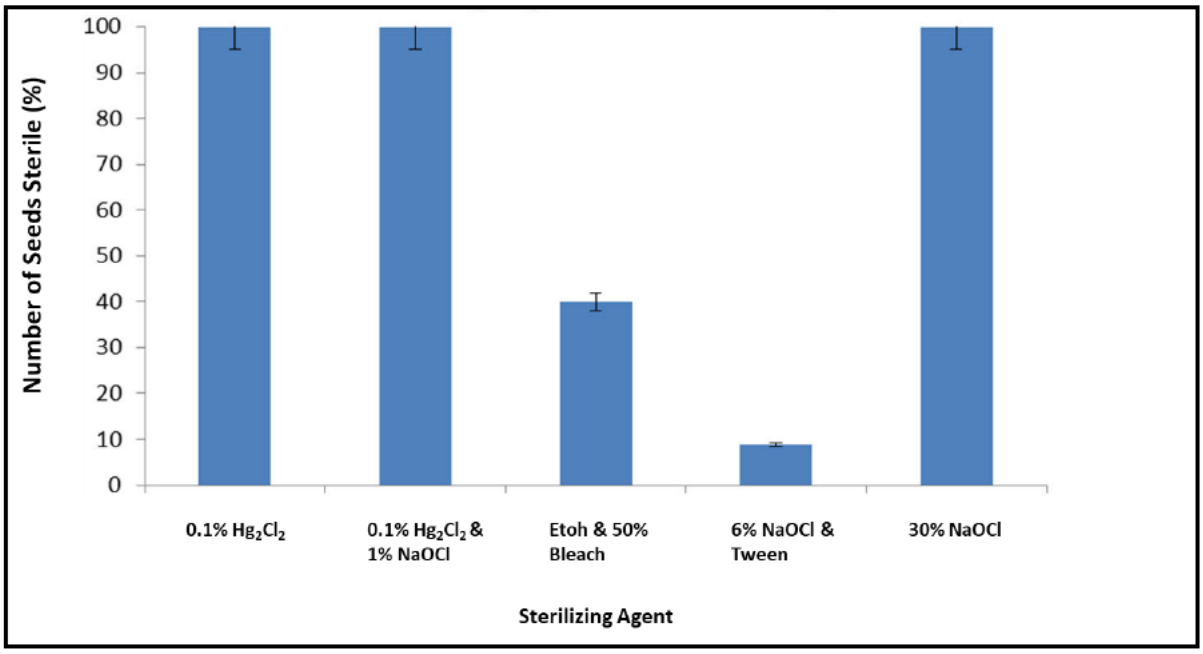


**Figure 17 (a):** Sterilization efficacy of various sterilizing agents on *D. stramonium* seeds. 100% sterility was demonstrated by 0.1% $Hg_2Cl_2$ ,0.1% $Hg_2Cl_2$ and 1% Sodium hypochlorite and 30% Sodium Hypochlorite. Six percent Sodium hypochlorite and Tween was least effective as a sterilizing agent on the seeds (n=3, SD ±0.5).

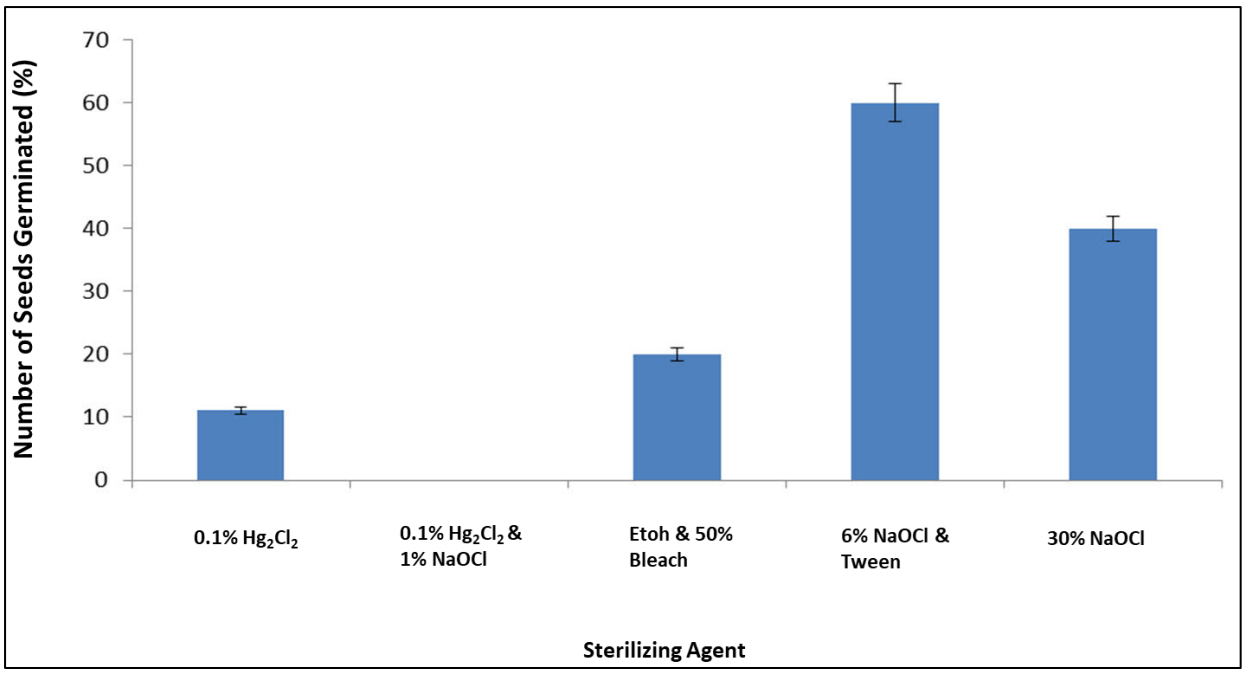


**Figure 17 (b):** Effect of sterilizing agents on seed germination. The most number of seeds was germinated by 6% Sodium hypochlorite and Tween (60%), followed by 30% Sodium hypochlorite (40%), 70% Ethanol and 50% Bleach (20%), and 0.1% Mercuric chloride (11%). No seeds germinated post sterilization with0.1% Mercuric chloride and Sodium hypochlorite (n=3, SD ±0.8).

Germinated seedlings (Fig. 18A) were transferred to MS medium containing BAP and IAA for shoot development as indicated in Figures 18B and 18C. Seedlings on MS medium supplemented with 1 $mg.mL^{-1}$ IAA and 0.5 $mg.mL^{-1}$ BAP resulted in proper shoot and root development as indicated in Figure 19A and 19B, whereas seedlings inoculated on MS medium supplemented with 0.5 $mg.mL^{-1}$ and 0.5 $mg.mL^{-1}$ resulted in slow growth of the plant, callus formation and no root development as indicated by Figures 19C and 19D.

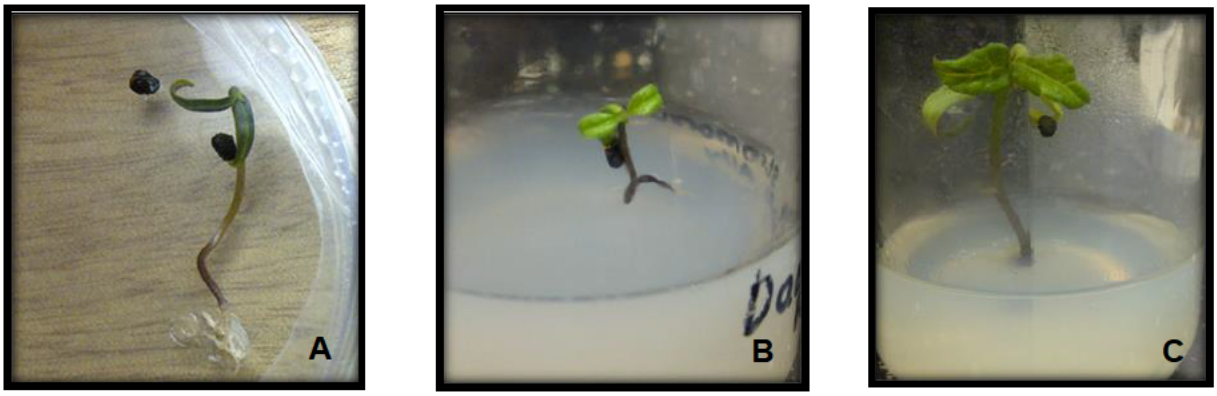


**Figure 18:** Germinated seedlings of *D. stramonium* on hormone free MS medium (A). Shoot cultures of *D. stramonium* MS medium supplemented with 1 $mg.mL^{-1}$ BAP and 0.5 $mg.mL^{-1}$ $^{1}$ IAA after six weeks (B) and after seven weeks (C).

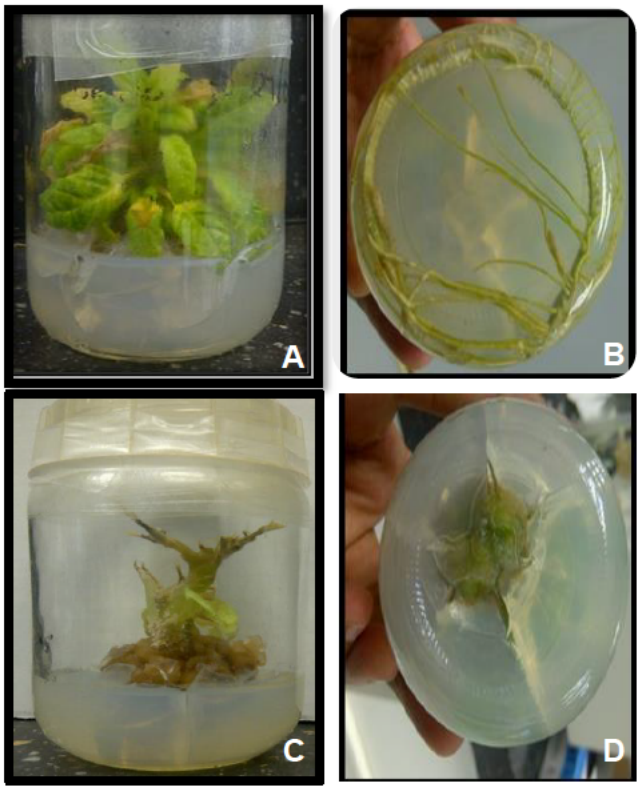


**Figure 19:** Seventeen week old *in vitro* cultures of *D. stramonium* on MS medium supplemented with 1 $mg.mL^{-1}$ IAA and 0.5 $mg.mL^{-1}$ BAP (A and B) and 0.5 $mg.mL^{-1}$ IAA and 0.5 $mg.mL^{-1}$ BAP (C and D).

### 3.3 Callus cultures

After six weeks of incubation, callus was observed on the surface and cut-ends of the leaf explants (Fig. 20A). Leaf explants inoculated on MS medium containing Kinetin and 2,4 D produced callus that was hard, dry and brown (Fig. 20C), whereas leaf explants inoculated on MS medium supplemented with BAP and 2,4D produced callus that was white and crystal-like in appearance (Figure 20B). Sub-cultured callus tissue grew successfully on medium and remained friable. These friable calluses were used as inoculum for the initiation of suspension cultures.

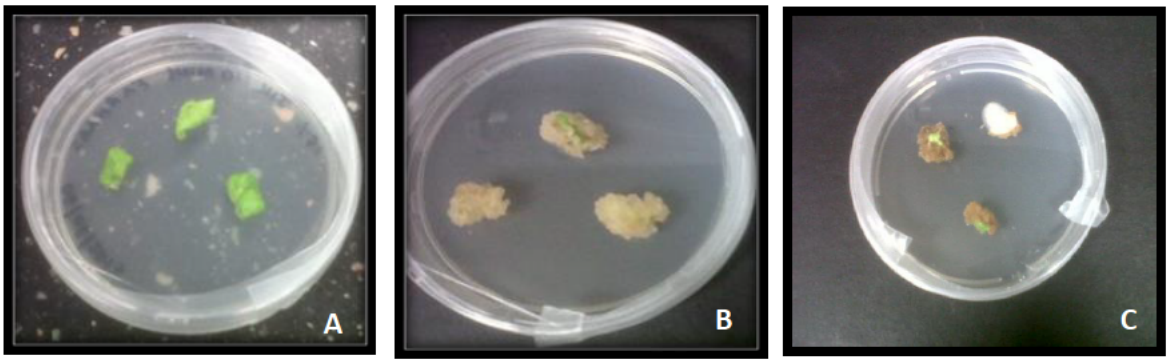


**Figure 20**: Leaf explants viewed two weeks after incubation (A), friable callus on MS Medium supplemented with 2,4D and BAP after six weeks of incubation (B) and callus formed on MS medium supplemented with Kinetin and 2,4 D (C).

### 3.4 Cell suspension cultures

Friable callus tissue was transferred into MS liquid medium supplemented with BAP and 2,4D (Fig. 21). The callus tissue had dispersed into small aggregates and actively grew. Cell suspension cultures were scaled up to 350 mL. The initial culture was cream in colour and at the end of 6-week cultivation; the culture was caramel brown in colour with approximate biomass of 48.7 g produced per flask.

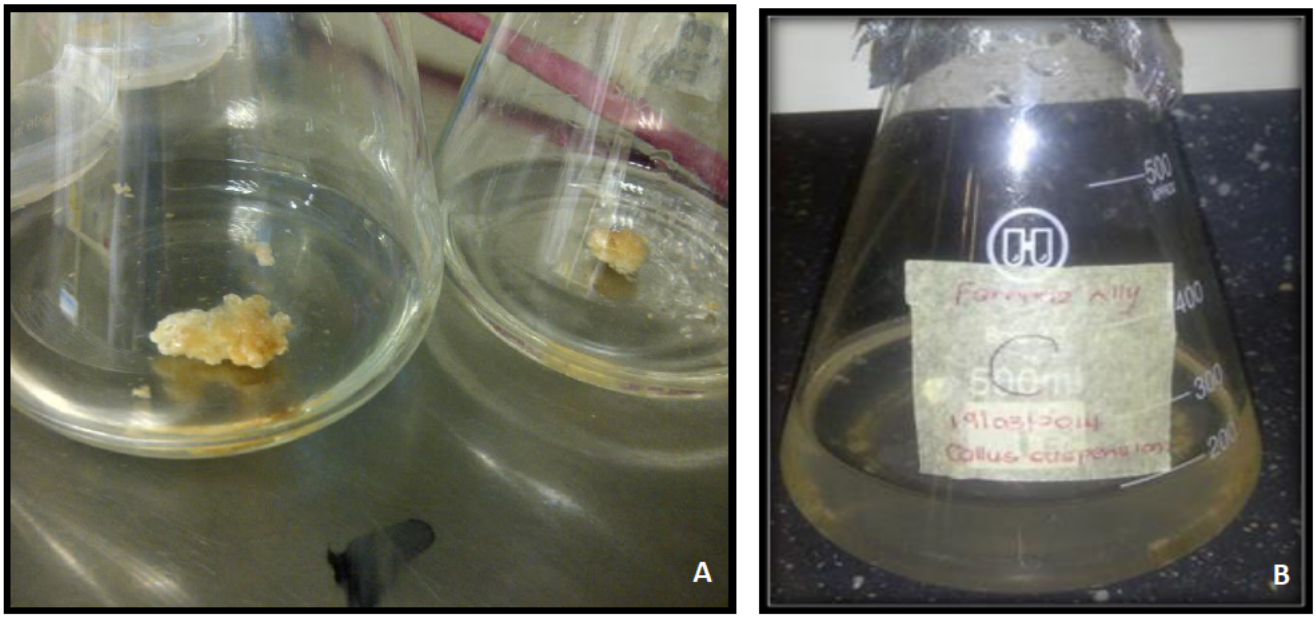


**Figure 21:** Day 1 of cell suspension cultures-friable callus in MS medium **(A).** Cell suspension culture after a week incubation with friable callus attached to sides of the flasks **(B).**

### 3.5 Hairy root cultures

*A. rhizogenes* (15834) was used to transform *D. stramonium* leaf and stem explants obtained from shoot cultures. As summarized in Table 13, there were no hairy roots formed on wounded regions of leaf explants hence, at an $OD_{600nm}$ of 1, 0.5 and 0.2, leaves were not susceptible to *A. rhizogenes* transformation. At an $OD_{600nm}$ of 0.5, stem explants were susceptible to *A. rhizogenes* infection as hairy roots emerged from wounded sites after two weeks of incubation as indicated in Figure 22B. Sixty percent of stem explants were successfully transformed. A higher concentration of *A. rhizogenes* killed the stem explants as they appeared brown and no hairy roots emerged. Highly branched hairy roots were transferred onto MS hormone free medium containing 200 $mg.L^{-1}$ Cefotaxime to inhibit the growth of Agrobacterium and provide sterile hairy roots as indicated in Figure 22C. Sterile hairy roots were transferred onto MS hormone free liquid medium. At the end of nine weeks of incubation, 36.4 g of biomass was produced in three culture flasks. This equated to 0.24 g of hairy root per ml of culture media.

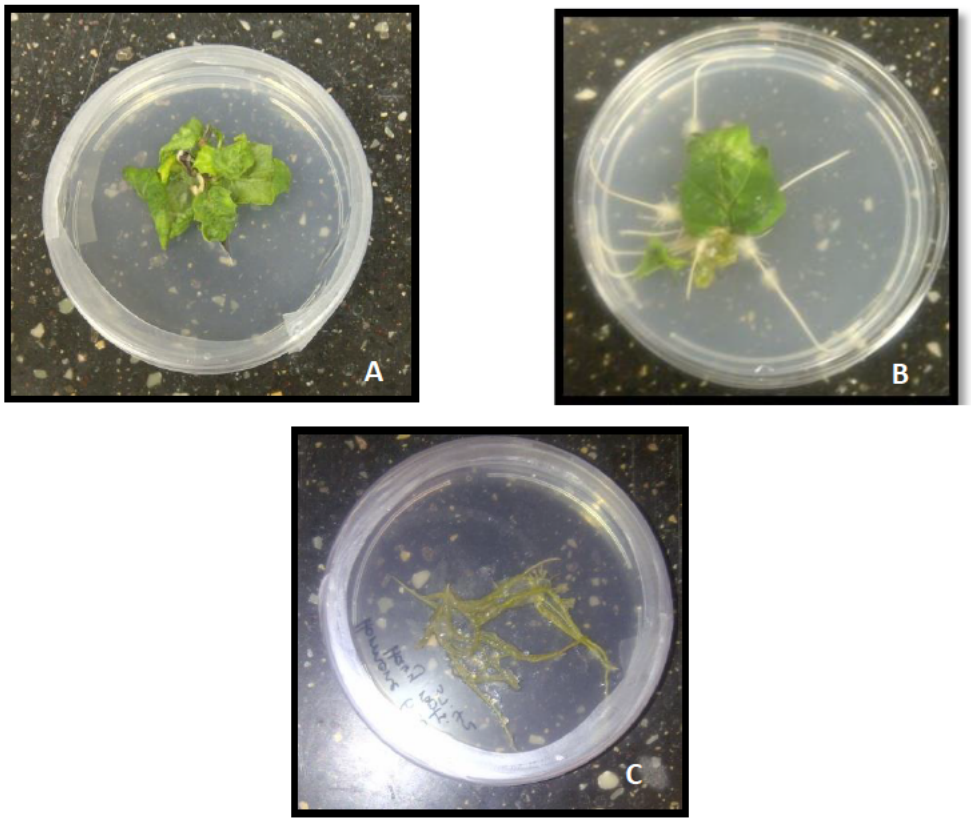


**Figure 22:** Wounded explants on Hormone free medium **(A).** Successful hairy root transformation on stem explants **(B)** and sterile hairy root on MS hormone free medium containing Cefotaxime **(C).**

**Table 13:** Concentration of *A. rhizogenes* used to transform *D. stramonium* and the transformation efficiency of leaf and stem explants

| Explant | OD / concentration of bacteria | No. of transformed plants* | Transformation efficiency (%)* |
|---|---|---|---|
| **Leaf** | Control | 0 | 0 |
| | 0.2 | 0 | 0 |
| | 0.5 | 0 | 0 |
| | 1 | 0 | 0 |
| **Stem** | Control | 0 | 0 |
| | 0.2 | 3 | 10±0.3* |
| | 0.5 | 6 | 60±1.67* |
| | 1 | 0 | 0 |

* Data represented as mean ± standard deviation, Number of samples (n=3)

### 3.6 Effect of Elicitor on hairy root biomass production and alkaloid content

Methyl Jasmonate (MeJA) was added in hairy root cultures and monitored for 72 hours. The initial colour of the roots was white and the texture was soft. After an incubation period of 24 hours, there was a change in the morphological characteristics of the hairy roots (Fig. 23). The hairy roots culture containing MeJA was more "string-like" and darker in colour compared to the control containing no MeJA.

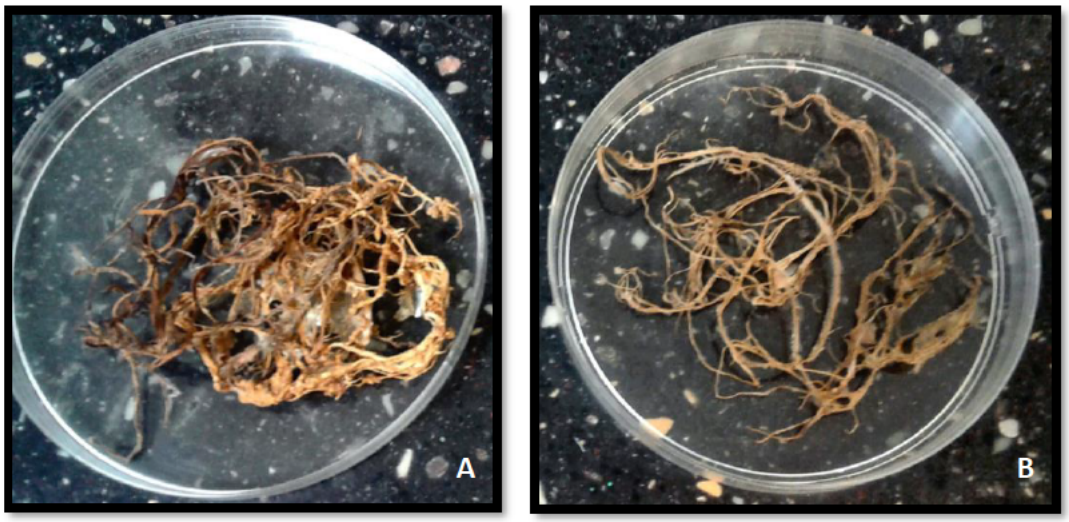


**Figure 23:** Elicited hairy roots (A) and Non-elicited hairy roots (B) after an incubation period of 24 hours.

After an incubation period of 24 hours, hairy roots began to turn brown. Although there was a change in the morphological characteristics of the hairy roots, the final wet weight mass achieved was 42.1 g as opposed to the control final weight of 35.6 g. Figure 24 indicates the biomass growth over 72 hours. The growth index of the elicited hairy root, over three days is shown in Table 14.

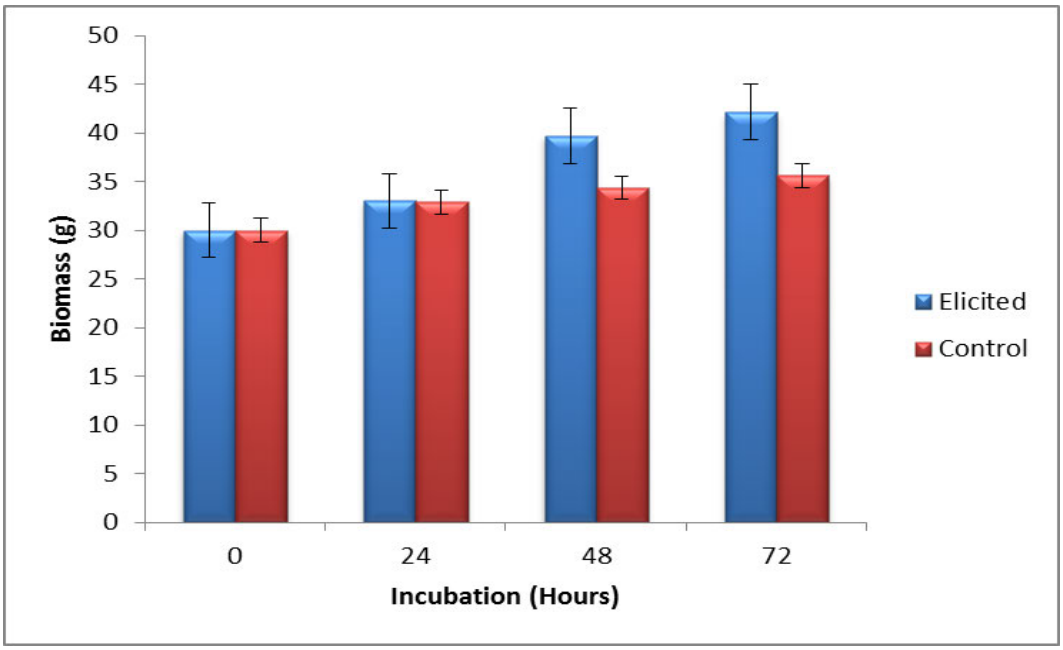


**Figure 24:** Biomass produced during elicitation over 72 hours.

**Table 14:** Biomass and Growth index of hairy root cultures

| Incubation Time (Hours) | Elicited cultures (MeJa +) | | Control Culture (MeJa-) | |
|---|---|---|---|---|
| | **Biomass (g)** | **Growth Index** | **Biomass (g)** | **Growth Index** |
| **0** | 30 | 1 | 30 | 1 |
| **24** | 33±0.4 | 1.18 | 32.9±0.2 | 1.01 |
| **48** | 39.7±0.4 | 1.29 | 34.4±0.3 | 1.07 |
| **72** | 42.1±0.1 | 1.4 | 35.6±0.5 | 1.18 |

## 3.7 Detection of tropane alkaloids in field root, plant cell and tissue culture extracts by High Performance Liquid Chromatography.

HPLC analysis had indicated that the Atropine and Scopolamine standards eluted at retention times (Rt) of 4.201 - 4.927 and 3.439 – 3.504 minutes respectively (Fig. 27 and 28). The field leave extract showed a peak at 4.241 minutes indicating the presence of Atropine and the absence of Scopolamine. The wild root extract showed peaks between 3.494 - 4.939 minutes. The *in vitro* intracellular leave extract showed peaks between 3.501 - 4.968 minutes. The *in vitro* extracellular callus extract showed peaks between 3.481 - 4.918 minutes. The intracellular hairy root extract showed peaks between 3.504 - 4.926 minutes. The extracellular hairy root extract showed peaks between 3.481 - 4.914 minutes. Elicited cultures with MeJa showed peaks between 3.480 - 4.927 minutes for the intracellular extract and 3.475 - 4.910 minutes for the extracellular extract.

The Atropine and Scopolamine content in the various extracts was calculated using the standard curve of Atropine and Scopolamine (Fig. 25 and 26). With reference to the analyses, it was evident that the highest amount of Atropine and Scopolamine was detected in the intracellular extract of elicited hairy root cultures at concentrations of 5.2 µg.mL$^{-1}$ and 5.01 µg.mL$^{-1}$ respectively.

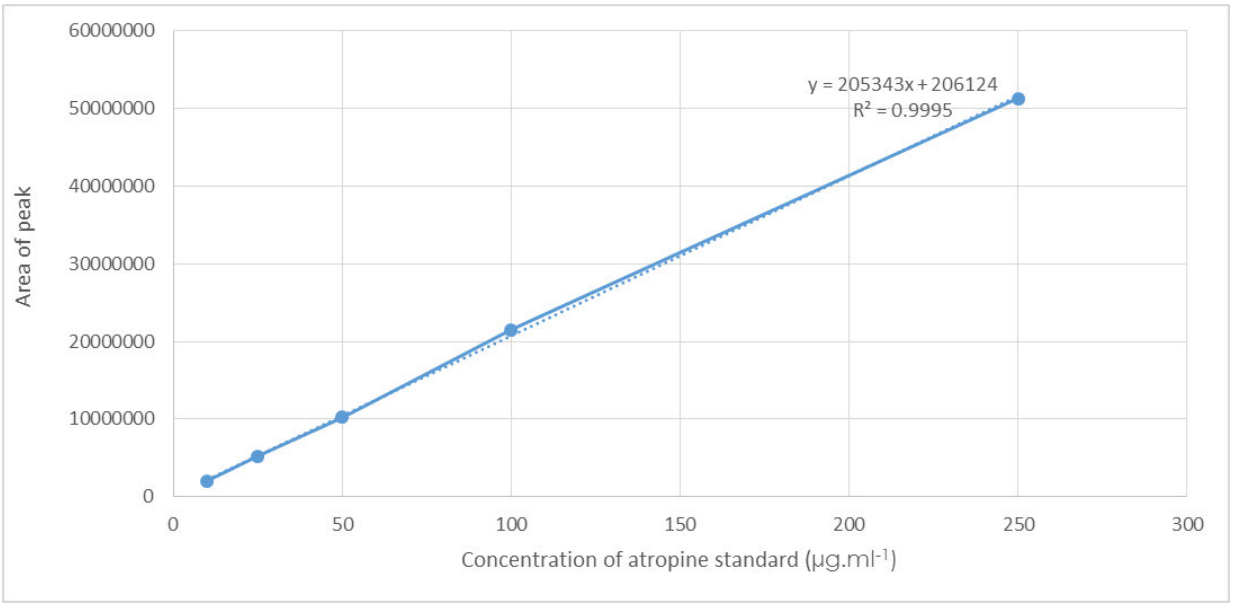


**Figure 25:** Standard curve of Atropine with an $R^2$ value of 0.9995.

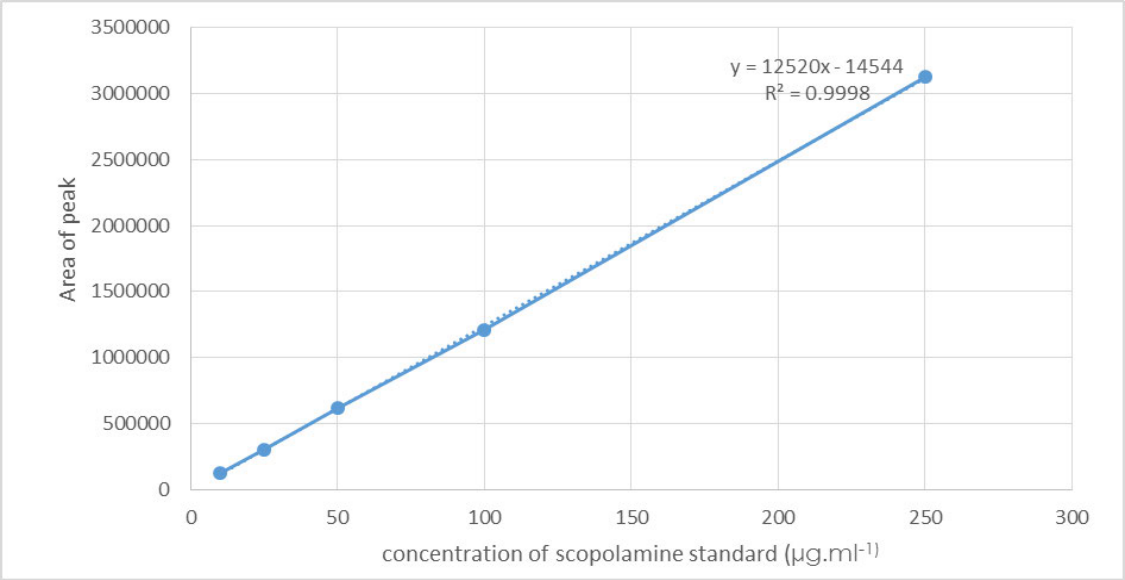


**Figure 26:** Standard curve of Scopolamine with an $R^2$ value of 0.9998.

The highest Atropine and Scopolamine concentration was produced in MeJa supplement hairy roots at 5.2 µg.mL$^{-1}$ and 5.01 µg.mL$^{-1}$ respectively. Non-supplemented hairy root cultures produced Atropine at 3.37 µg.mL$^{-1}$ and Scopolamine at 4.26 µg.mL$^{-1}$. Wild root extract producing Atropine at 3.32 µg.mL$^{-1}$ and Scopolamine at 4.11 µg.mL$^{-1}$. Extracts of *in vitro* shoot cultures showed no detection of Atropine and Scopolamine, whereas wild leave extracts produced Atropine at 2.18 µg.mL$^{-1}$. The Atropine and Scopolamine concentration of field, plant cell and tissue culture of extracts is summarized in Tables 15 and 16. A graphical comparison of Atropine and Scopolamine content in each extract is demonstrated in Figure 29.

**Table 15:** Concentration and yield of Atropine from *D. stramonium* field, plant cell and tissue culture of extracts.

| Plant material | [k]Atropine (µg.mL$^{-1}$) | Yield of Atropine in extract (µg.g$^{-1}$) | Increase in Atropine production as compared to field roots |
|---|---|---|---|
| **Wild leaves** | 2.18±0.03 | 0.436 | |
| **Wild roots** | 3.32±0.20 | 0.664 | |
| ***In vitro* shoot cultures** | - | - | |
| **Callus culture (IC)** | 0.78±0.10 | 0.156 | |
| **Callus culture (EC)** | 0.75±0.06 | 0.003 | |
| **Hairy root culture (IC)** | 3.37±0.07 | 0.36 | 1.01 |
| **Hairy root culture (EC)** | 0.32±0.03 | 0.674 | |
| **Elicited culture (IC)** | 5.23±0.03 | 0.54 | 1.57 |
| **Elicited culture (EC)** | 0.37±0.05 | 0.14 | |

IC (intracellular extract), EC (extracellular extract). Fold increase represents the increase in Atropine concentration present in hairy root cultures compared to *D. stramonium* field roots.
*Mean data represented (n=3)

**Table 16:** Concentration and yield of Scopolamine from *D. stramonium* field, plant cell and tissue culture extracts.

| Plant material | *Scopolamine (µg.ml$^{-1}$) | Yield of Scopolamine in extract (µg.g$^{-1}$) | Increase in Scopolamine production as compared to field roots |
|---|---|---|---|
| **Wild leaves** | - | - | |
| **Wild roots** | 4.11±0.08 | 0.822 | |
| ***In vitro* shoot cultures** | - | - | |
| **Callus culture (IC)** | 3.17±0.03 | 0.634 | |
| **Callus culture (EC)** | 1.29±0.02 | 0.01 | |
| **Hairy root culture (IC)** | 4.26±004 | 0.85 | 1.03 |
| **Hairy root culture (EC)** | 0.12±0.04 | 0.24 | |
| **Elicited culture (IC)** | 5.01±0.02 | 1.002 | 1.21 |
| **Elicited culture (EC)** | 0.76±0.04 | 0.038 | |

IC (intracellular extract), EC (extracellular extract). Fold increase represents the increase in Scopolamine concentration present in hairy root cultures compared to *D. stramonium* field roots.
*Mean data represented (n=3).

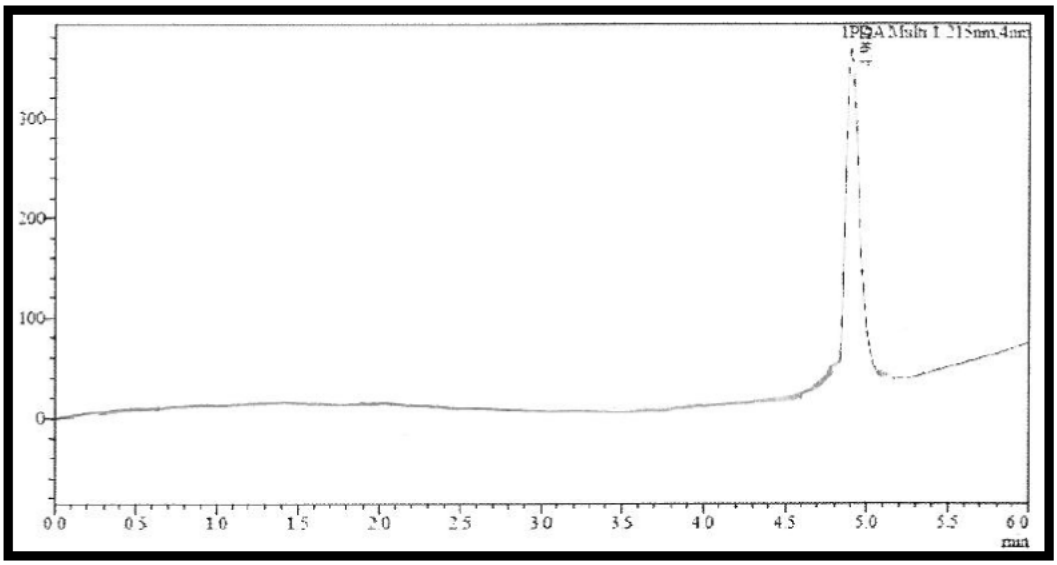


**Figure 27:** Chromatogram of Atropine standard eluting at 4.90 minutes.

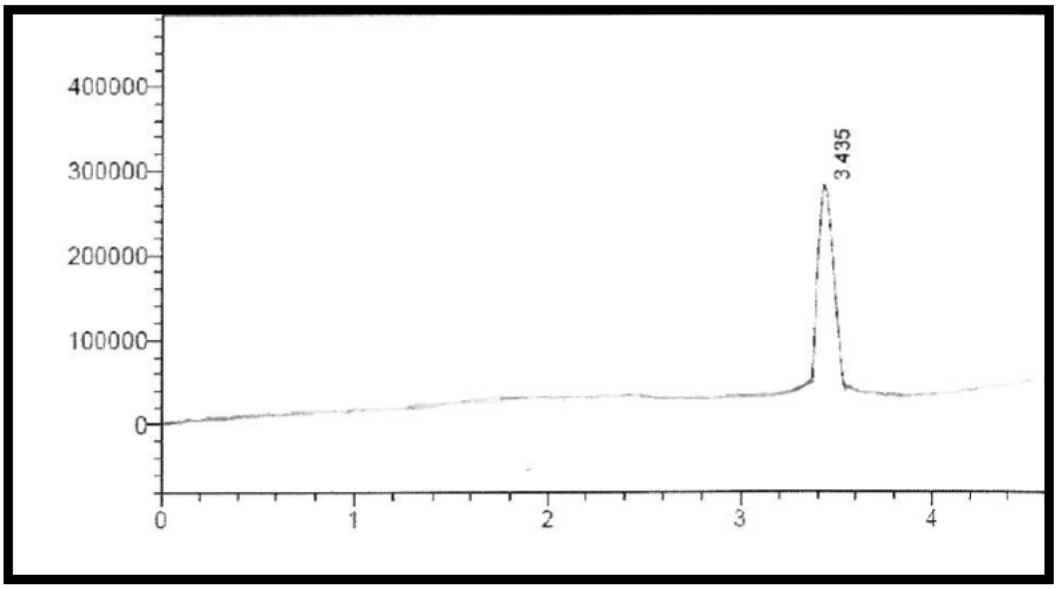


**Figure 28:** Chromatogram of Scopolamine standard eluting at 3.435 minutes.

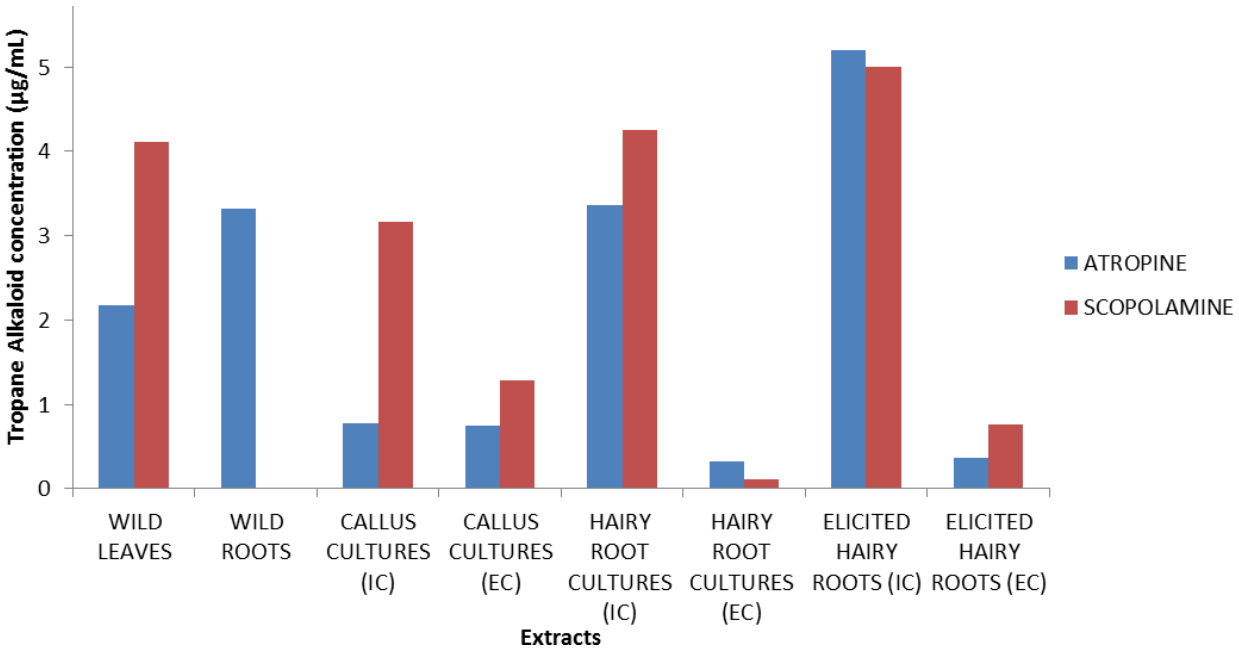


**Figure 29:** Comparison of Atropine and Scopolamine present in the various extracts with the highest Atropine and Scopolamine content present in elicited intracellular hairy root cultures. The lowest Atropine and Scopolamine content was present in the extracellular hairy root cultures. No Scopolamine was detected in the wild root extract.

## 3.8 *In vitro* biological activity of *D. stramonium* leaf and root extract

### 3.8.1 Antimicrobial activity

The leaf and root extracts of *D. stramonium* were screened for their potential antimicrobial activity against bacteria and fungi using the Kirby-Bauer disc diffusion assay. The results of antimicrobial screens are indicated in Table 17. Amongst the two extracts, a good bactericidal effect was displayed by root extracts against *S. marcescens, E. coli*, *S. aureus*, *K. pneumoniae*, *S. typhi*, *E. aerogenes*, *B. cereus* and *P. rettgeri*. Zones of inhibition ranged between 7 mm - 33 mm. There was no bactericidal effect displayed against *P. vulgaris* and *E. faecalis*. However, only the leaf extract exhibited bactericidal activity against *E. faecalis* with a zone of inhibition of 7 mm. There was no antifungal activity displayed by both extracts.

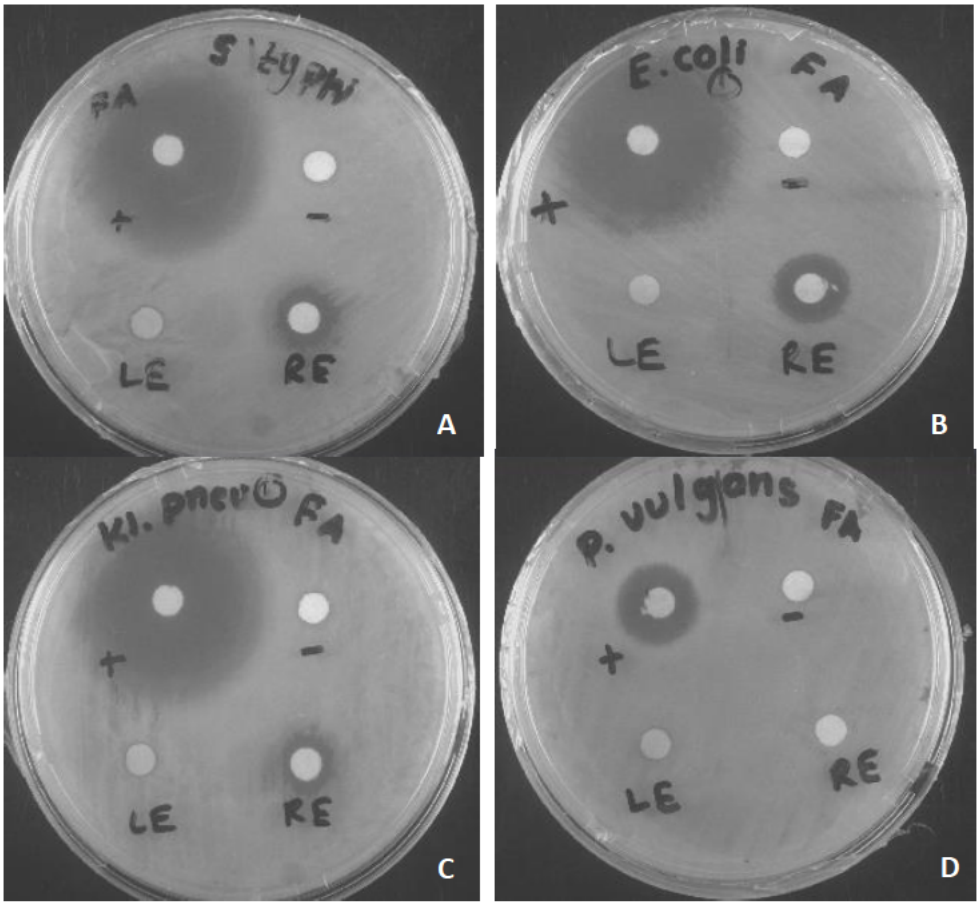


**Figure 30:** Antimicrobial screening of *D. stramonium* leaf and root extracts against *S. typhi* (A), *E. coli* (B), *K. pneumoniae* (C) and *P. vulgaris* (D), with root extracts displaying greater potential as an antibacterial agent.

**Table 17:** Summary of antibacterial activity of *D. stramonium* root extracts.

| Bacterial Strain | Root Extracts (mm)*[a] | Positive control (mm)**[b] |
|---|---|---|
| ***S. marcescens*** | 10 | 35±0.01 |
| ***E.coli*** | 15 | 35±0.01 |
| ***S.aureus*** | 11 | 37±0.01 |
| ***K.pneumoniae*** | 10 | 35±0.1 |
| ***S. typhi*** | 10 | 35±0.03 |
| ***E.aerogenes*** | 14 | 27±0.03 |
| ***B.cereus*** | 7 | 38±0.05 |
| ***P.rettgeri*** | 31 | 40±0.01 |
| ***P.vulgaris*** | n/a | 15±0.01 |
| ***E.faecalis*** | n/a | 20±0.01 |

* Data represented as mean ± standard deviation, Number of sample (n=3), n/a = no activity, [a] ($3mg.mL^{-1}$), **Positive control = Ciprofloxacin, [b] ($1mg.mL^{-1}$), Negative control= DMSO.

#### 3.8.1.1 Minimum Inhibitory Concentration

The MIC values were calculated using the 96 well plate dilution method. For all microorganisms, a standard antibiotic, Ciprofloxacin, was included as a positive control. Since all compounds were solubilized in methanol and made up to a final concentration of 3 $mg.mL^{-1}$, the same quantity of methanol to water was included as a negative control (solvent). Table 18, summarizes the results obtained during the minimum inhibitory concentration assay. It was evident that *E. coli*, *S. marcesens* and *K. pneumoniae* were inhibited at low concentrations of the crude root extract (0.375 $mg.mL^{-1}$). *B. cereus*, *P. rettgeri*, *S. aureus*, *S. typhimurium* and *E. aerogenes* were inhibited at higher concentrations of the crude root extracts (1.5 $mg.mL^{-1}$).

**Table 18:** Minimum Inhibitory concentration of *D. stramonium* root extracts

| Bacterial culture | Time (min) | Crude extract ($mg.mL^{-1}$) |
|---|---|---|
| ***B. cereus*** | 30 | 3 |
| | 60 | 1.5 |
| | 90 | 1.5 |
| ***P. rettgeri*** | 30 | NI |
| | 60 | 3 |
| | 90 | 3 |
| ***S. marcescens*** | 30 | 1.5 |
| | 60 | 0.1875 |
| | 90 | 0.1875 |
| ***E. coli*** | 30 | 0.75 |
| | 60 | 0.75 |
| | 90 | 0.375 |
| ***S. aureus*** | 30 | 1.5 |
| | 60 | 1.5 |
| | 90 | 1.5 |
| ***K. pneumoniae*** | 30 | 1.5 |
| | 60 | 0.375 |
| | 90 | 0.375 |
| ***S. typhimurium*** | 30 | NI |
| | 60 | 3 |
| | 90 | 1.5 |
| ***E. aerogenes*** | 30 | NI |
| | 60 | 3 |
| | 90 | 1.5 |

*NI represents no inhibition of the microorganism against the crude root extract of *D. stramonium*. All results obtained were compared to the positive control Ciprofloxacin.

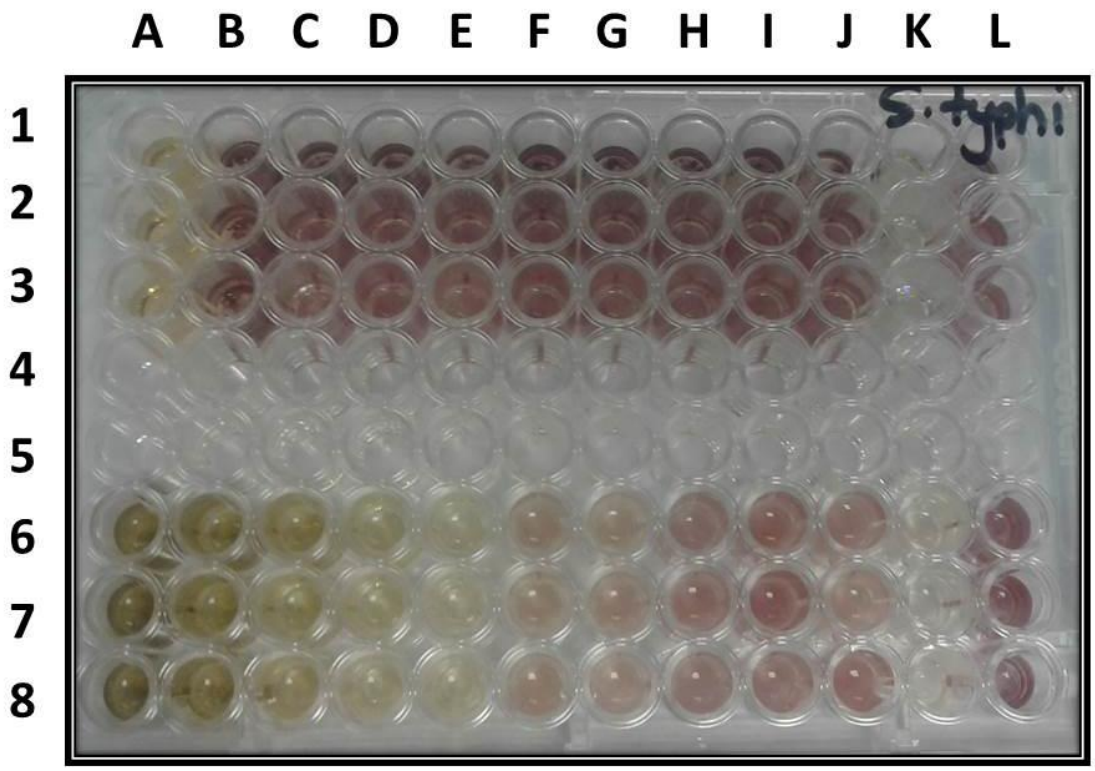


**Figure 31:** A 96 well plate post-30-minute incubation, indicating the minimum inhibitory concentration of crude leaf and root extracts against *S. typhimurium*. The antibacterial potential of crude root extract and leaf extract is displayed in triplicates in rows 1-3 and 6-8 respectively. Column A - crude extracts. Column B to J - crude extract at 6 $mg.mL^{-1}$ two-fold diluted. Column K - positive control containing bacterial culture and positive control ciprofloxacin. Column L - negative control containing culture only.

### 3.8.2 Antioxidant activity

The DPPH photometric assay was conducted to investigate the free radical scavenging potential of *D. stramonium* leaf and root extracts. The results obtained are shown in Figure 32 and presented in Table 19. Both leaf and root extracts displayed antioxidant activity however their percentage activity varied in each extract. The therapeutic effect of medicinal plants is related to their antioxidant potential. At the lowest concentration of 1 $\mu g.mL^{-1}$, the scavenging capacity of root extracts and leaf extracts was 64.4% and 31.7% respectively whereas at the highest concentration of 1000 $\mu g.mL^{-1}$ root extracts and leaf extracts was 98.4% and 45.8% respectively.

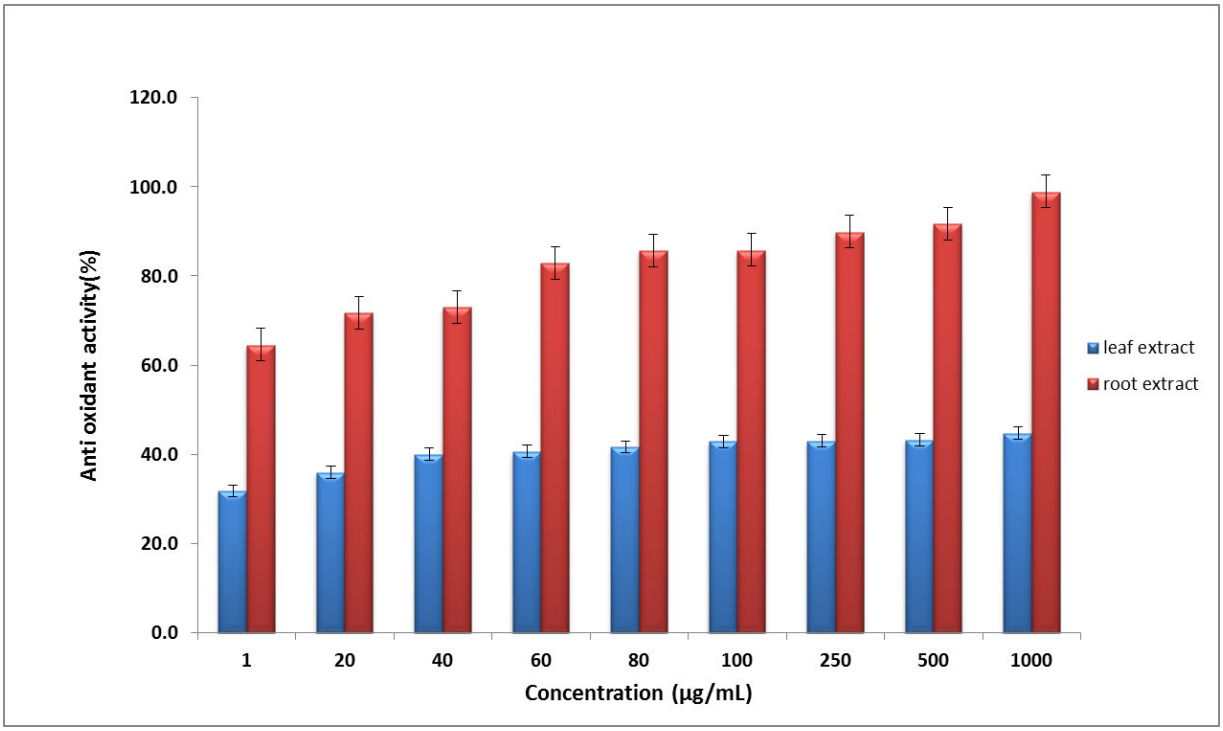


**Figure 32:** Comparative analysis of antioxidant activity obtained from root and leaf extracts of *D.stramonium*, with more potential displayed by root extracts.

**Table 19:** Antioxidant activity of *D. stramonium* leaf and root extracts.

| | **Antioxidant activity (%)** | | |
|---|---|---|---|
| **Concentration µg.mL$^{-1}$** | **Leaf Extract** | **Root Extract** | **Positive control 1mM Rutin** |
| **1000** | 44.8 ± 0.4 | 98.9 ± 0.5 | 100 |
| **500** | 43.2 ± 0.4 | 91.7 ± 0.9 | 100 |
| **250** | 43.0 ± 0.5 | 89.9 ± 0.7 | 100 |
| **100** | 42.9 ± 1.6 | 85.8 ± 0.4 | 100 |
| **80** | 41.6 ± 1.3 | 85.7 ± 0.8 | 100 |
| **60** | 40.6 ± 1.4 | 83.0 ± 1.0 | 100 |
| **40** | 40.0 ± 1.2 | 73.0 ± 0.4 | 100 |
| **20** | 35.9 ± 1.0 | 71.7 ± 0.3 | 100 |
| **1** | 31.8 ± 0.8 | 64.5 ± 0.3 | 100 |

* Data represented as mean ± standard deviation, Number of samples (n=3)

### 3.8.3 Brine Shrimp (*Artemia salina)* Lethality assay

The brine shrimp lethality assay is a preliminary test that is used to investigate the potential threat of the plant extracts at high concentrations. From the results obtained it was evident that leaf and root extract was potent against *Artemia salina* at low concentrations of 10 $\mu g.mL^{-1}$ within 4 and 6 hours of exposure. There is a directly proportional relationship displayed by the extract concentration and exposure time of the extract against *Artemia salina*. The higher the concentration of the extract and the longer the exposure period, the more toxic it is. These results can be viewed in Table 20 below.

**Table 20:** Percentage toxicity displayed by *D. stramonium* leaf and root extracts against *Artemia salina*

| **Concentration ($\mu g.mL^{-1}$)** | **Time (Hours)** | ***Leaf extract (Methanolic)** | ***Root extract (Methanolic)** |
|---|---|---|---|
| **10** | 2 | 0 | 0 |
| | 4 | 10 | 0 |
| | 6 | 80 | 7 ± 0.8 |
| | 24 | 100 | 97 ± 0.1 |
| **100** | 2 | 0 | 0 |
| | 4 | 20 | 0 |
| | 6 | 67± 0.3 | 10 |
| | 24 | 100 | 100 |
| **500** | 2 | 0 | 20 |
| | 4 | 30 | 23 |
| | 6 | 60 | 43 |
| | 24 | 100 | 100 |
| **1000** | 2 | 0 | 20 |
| | 4 | 44 ± 0.6 | 27± 0.6 |
| | 6 | 100 | 70 |
| | 24 | 100 | 100 |

* Data represented as mean ± standard deviation, Number of samples (n=3)

# 4. DISCUSSION

There are many genera of plants within the Solanaceae family, that contain tropane alkaloids. Some studies have been of much interest regarding tropane alkaloid content in the genus *Datura*. In the latter part of the 1990s, much focus was drawn to *Datura innoxia* and *Datura metel.* However, recent studies over the years have identified 64 tropane alkaloids and their derivatives in *D. stramonium*.

The popularity of tropane alkaloids in the pharmaceutical industry has grown due to their application in transdermal patches and anticholinergic drugs. Anticholinergic drugs are responsible for inhibiting acetylcholine which is responsible for involuntary muscle movement in the lungs, gastrointestinal tract, urinary tract and of those responsible for symptoms associated with Parkinson's disease (Sheu *et al*., 2019). Amongst many neurological disorders, the number of individuals affected with Parkinsons is the fasting growning. The Global Burden of Disease study, has highlighted an increase of 118% of the Worlds Population internationally to be affected with Parkinsons, specific for the period 1990-2015. This equates to approximately 6.2 million individual (Dorsey and Bloem, 2021).

The most common method of synthesizing tropane alkaloids is by the "Robinsons one-pot synthesis" with modified parameters to attain specific yields (Alsamarra, 2019). There are also methods for semi-synthetic and synthetic tropane derivatives. These methods are complex and time-consuming, with yields and purity varying. On a large scale industrial synthesis of drugs, there is often an increase in waste material such as solvents and effluents that are linked to health and safety concerns. The control of this waste often becomes an additional cost and loss to the business. Therefore the availability of these tropane and tropane derivative cores from natural sources can prove to alleviate cost and safety concerns.

Early research conducted, plant cell and tissue culture techniques were employed for the production of tropane alkaloids and their derivatives (Jouhikainen *et al*., 1999). Plants within the *Solanaceae* family, such as *Atropa belladonna* and *Brugmansia candida* have been employed for establishing cell and root cultures, thus allowing for the production of these

alkaloids on an industrial scale using various biotechnological approach (Pitta-Alvarez and Giulietti, 1995). This project focused on analyzing and comparing the yields of Atropine and Scopolamine present in extracts obtained from wild plants and tissue culture systems of *D. stramonium.*

The initiation of plant cell and tissue culture systems requires a source of sterile explants. To obtain a sterile explant, seeds and organs from mature plants need to be decontaminated before being used.

One of the major problems which arise in producing axenic seedlings is the presence of microbes inside the seeds which may be released upon germination. The choice and concentration of a seed sterilizing agent, as well as its exposure time, plays a role in obtaining sterile seeds. In this study, prior to the genetic transformation of *D. stramonium* using *A. rhizogenes,* a source of sterile plants was required. The seeds obtained from the wild plant were subjected to five sterilization treatment regimens as indicated in Table 10. Treatment of seeds with 30% Sodium hypochlorite resulted in 100% sterility as well as 40% germination. This protocol was employed for subsequent experiments. Although treatment with Mercury chloride resulted in 100% sterility, there was no germination of those seeds.

Sodium hypochlorite is one of the most employed surface sterilization agents in plant tissue culture (Barampuram *et al*., 2014). Eapen *et al*. (2015) utilized 30% sodium hypochlorite as a surface sterilizing agent in producing *in-vitro* cultures of *Arabidopsis thaliana, Pisum sativum* and *Zea mays L*. It was reported that this sterilization regime had produced sterile seedlings with no embryo damage. A primary requirement for commencing *in vitro* cultures from seeds, is disinfection. The concentration and sterilization period of disinfectant used, has a direct effect on the germination of seedlings and its sterility (Yasemin *et al*., 2018).

The growth of *in-vitro* cultured plants is dependent on environmental conditions such as temperature, light and chemical needs such as the choice of growth medium and supplements. Plant growth regulators are important supplements in the selection of media used. They play a vital role in the developmental pathway of plant cells. In this project,

maintaining the *in vitro* cultures of *D. stramonium* was dependent on the ratio of cytokinin and auxin used to supplement MS media. As indicated in Table 11, the choice of cytokinin and auxin utilized was IAA and 6-BAP respectively. The ratio between the cytokinin and auxin used determines the type of culture that will be established or regenerated. It was observed that an equal ratio of 6- BAP (0.5 $mg.mL^{-1}$): IAA (0.5 $mg.mL^{-1}$) resulted in the formation of callus and no root development occurred (Fig. 19 C and D). A higher auxin concentration of 1 $mg.mL^{-1}$ supported root and shoot formation as indicated in Figure 19 A and B. A study conducted by Ngomuo *et al*, (2013)., highlighted that in MS media, a higher concentration of 6-BAP and lower concentration of IAA enhances fresh weight and better root formation in *Musa* var. "Yangambi".

Callus cultures were derived using *in vitro* leaf explants. Leaf explants were subjected to two hormonal treatments, i.e., MS medium supplemented with 2,4 D and 6-BAP and MS medium supplemented with 2,4 D and Kinetin. As per the results obtained, callus formation in the presence of 2,4 D and Kinetin occurred at the edge of the leaf explants. The callus was dry, and could not be used as an inoculum for cell suspension cultures. It has been reported that brown callus formed under the influence of Kinetin is a result of triterpenes produced within the callus cells (Stojakowska and Kisiel, 1999). Callus formation in the presence of 2,4 D and BAP was cream and friable. 2,4 D and BAP belong to a PGR class called Auxin and Cytokinin respectively, and each PGR class is responsible for cell growth and division. The synergistic effect of a higher 2,4 D concentration to a lower BAP concentration was demonstrated to have the fastest callogenic response from leaf, root and internodal explants (Sen et. al, 2014).

Cell suspension cultures were initiated from the friable callus formed under the influence of 2,4 D and BAP using liquid MS medium supplemented with sucrose as required for callus induction. The suspended callus tissues were initially off-white and cream. The cells were dispersed in aggregates. Suspension cultures were sub-cultured and maintained every two weeks. This resulted in new aggregates being formed. Maintaining suspension cultures for continuous callogenesis was reported for many species such as *Gramineae* and *Passiflora alata* Curtis (Vasil and Vasil, 2012; Pacheco *et al*., 2012). After a six-week interval, approximately 48.7 g of callus culture was produced per 350ml flask. This was a yield of 0.14

g of callus produced per ml of media. The morphology of the callus was caramel brown. The browning of callus in suspension cultures could be due to intrinsic nutrient limitation or the accumulation of gases during callogenesis. The overcoming of the browning as per literature would have been the scheduled low dosing of auxin. This would have also increased the yield of callus produced per ml of media (Hussain *et al*., 2012).

The transformation of *D. stramonium* to produce hairy roots was dependent on the choice of explants used. The current research investigated the transformation of stem and leaf explants of *in vitro* shoot cultures. Higher transformation efficiency was demonstrated by stem explants, as compared to young leaves (Fig. 22). The concentration (optical density) and age of *A. rhizogenes* had influenced the transformation and survival of the transformed material. The successful transformation was obtained by infecting explants with *A. rhizogenes* (15834) at an $OD_{600nm}$ of 0.5 for incubation at 26˚C for four weeks. The wounding and infection period demonstrated in this project is much longer as compared to genetic transformation of *S. anacardium L*, that had a 61% transformation efficacy with an exposure period of 5 minutes to *A.rhizogenes* (15834) incubated for 20 days (Panda *et al*.,2017). Many factors affect Agrobacterium transformation. These include the type, age and tissue of the plant, the concentration and age of the Agrobacterium culture, the type of explant used for infection and media components (Nourozi *et al*., 2016).

Transformed explants were decontaminated using Cefotaxime as the presence of bacteria for further culturing would have resulted in a slow growth rate and deterioration of induced culture eventually leading to culture loss (Naicker *et al*.,2016). Sterile hairy root cultures was subcultured in a fed batch system of hormone free MS liquid medium, resulted in a biomass yield of 0.24 $g.mL^{-1}$ over 9 weeks. This yield is much lower as compared to a study on the biomass obtained in hairy root cultures of 3 Datura species grown in hormone free MS media supplement with sucrose at 20 $g.L^{-1}$ (Harfi *et al*.,2015). The increase yield of hairy root biomass is dependant on optimization of different intrinsic and extrinsic parameters, such as media composition with salt strengths, type and concentration of carbon sources, vitamin supplementation, sucrose concentration, agitation and light (Verma *et al*.,2015).The growth and yield of hairy root biomass is also dependant on media such as high salt media know as LS or MS, favours the growth of hairy root in dicotyledonous plants whereas low salt media

favours excessive bacterial manipulation resulting in frequent subculturing of hairy root cultures (Santos *et al*., 2016).

The elicitation of hairy root cultures using Methyl Jasmonate, resulted in 42.1g of hairy root biomass over a period of 72 hours. This was an increase in the growth index of 1.4 as the initial weight of the hairy root culture was 35.6g. Hairy roots exposed to MeJA was filamentous like, containing fine branches. However after two weeks of incubation, there was a morphological change in the roots. Hairy roots became brown in colour, which could be associated with lack of nutrients in the medium. In addition to the change in morphological appearance, the increase in biomass was much slower as compared to the rate highlighted in studies associated with elicitation (Shakeran *et al*., 2015; Gantait and Mukherjee, 2021). The methodology for bulking of hairy root biomass involved adding fresh medium at a four week interval. However, in previous research, as opposed to a fedbacth system, hairy roots post elicitation was always subcultured into new medium or supplemented with additional sources of macronutrients and micronutrients at a set interval (Sharifi *et al*. 2014).

Extracts of wild plant and *in vitro* cultures were quantified for Atropine and Scopolamine using HPLC. Scopolamine was absent in the leaf extracts of wild plants and *in vitro* cultures as compared to Atropine which was only absent in *in vitro* leave extracts. Callus and cell suspension cultures produced negligible amounts of Atropine at 0.78 $\mu g.mL^{-1}$. Scopolamine was produced at a concentration of 3.17 $\mu g.mL^{-1}$. It is reported that the biosynthesis of Hyoscyamine and Scopolamine is reduced after a series of subcultures (Dhoot and Henshaw, 1977). According to Oliveira *et al*. (2002), *Aspidosperma ramiflorum* produced calli which were yellow and friable however the yield of tropane alkaloid was six times less compared to mature plants (Oliveira *et al*., 2002). The analysis of Atropine and Scopolamine in wild and *in vitro* cultures of *D. stramonium* indicated elicited hairy root cultures exceeded the concentration of Atropine and Scopolamine in wild and hormone free hairy roots. A higher concentration of Atropine and Scopolamine was found in elicited roots at a concentration of 5.2 $\mu g.mL^{-1}$ and 5.01 $\mu g.mL^{-1}$ respectively. This was a 42% increase as compared to the concentration of Atropine and Scopolamine found in wild roots.

According to Afsharypur *et al*. (1995), Scopolamine and Atropine content in *Datura metel* increased gradually with growth development. It was reported that the highest amount of Scopolamine was accumulated in the roots after 16 weeks of incubation, supporting the fact that the incubation period plays a role in metabolite production. The variation of Atropine and Scopolamine content differs between plant parts and developmental stages. Atropine is known to be more prevalent in the vegetative period in roots as compared to scopolamine. The analysis concluded that roots accumulate a higher amount of Atropine whilst aerial parts of the plant accumulate a higher amount of Scopolamine (Kohnen- Johannsen and Kayer, 2019). This contradicts the results obtained in this research project as higher concentrations of Atropine and Scopolamine were found in hairy root cultures as opposed to aerial parts of the plant. According to Jakabova *et al*. (2012), Scopolamine and Hyoscyamine are predominant alkaloids in the Datura genus and occur in all organs of the plants. This study further concluded that Atropine was abundantly present in *D. stramonium* and the lowest in *D. innoxia*.

Secondary plant metabolite production is limited in disorganized plant tissue cultures as compared to the whole plant. This has also been reported for the production of Scopolamine in undifferentiated *in vitro* cultures of plants within the Solanaceae family. The low or no production of Scopolamine in callus cultures could be a result of auxin present in the medium. A study focused on the alkaloid production in *A. belladonna* plant and root culture indicated that the key enzymes in the tropane alkaloid biosynthetic pathway, such as PMT, is inhibited in callus cultures (Rothe *et al*., 2003).

The *in-vitro* synthesis of tropane alkaloids is often triggered by environmental factors thus the production of secondary metabolites is affected by cultural conditions. Selecting a good hairy root line is important. Plants naturally producing tropane alkaloids generate high producing cell lines such as *D. stramonium, A. belladonna* and *H. niger* (Jaremicz *et al*., 2014). Although the biosynthesis of secondary metabolites is genetically controlled in hairy roots, there are nutritional and environmental factors that influence biomass yield, growth rate and secondary metabolites produced. These factors include the presence of a carbon and nitrogen source in the medium, exogenous growth hormone, light intensity, and

temperature. The maintenance of optimum culture conditions results in a high density of root culture.

Hairy root cultures produce a stable number of phytochemicals however the exudation into the culture medium is poor and the accumulation of phytochemicals in hairy roots is limited due to feedback inhibition. Research has shown that media manipulations have aided in the release or overproduction of phytochemicals in hairy root cultures (Nielsen *et al*.,2019). Treatment of cultures with 5 mM of $H_2O_2$ induced the release of tropane alkaloids from hairy root cultures of *Atropa belladonna.* Common methods for the recovery of secondary metabolites in culture medium simultaneously requires elicitation, membrane permeability and *in* situ product removal (Cai *et* al., 2012).

Hairy root growth and alkaloid accumulation are also affected by the nitrate concentration in the medium. This has been demonstrated in a study focused on the tropane alkaloid production in *Atropa belladonna*. An increase in the nitrate concentration to 95 mM per 100 ml of media had qualitatively and quantitatively displayed changes in the fresh weight of hairy roots and the yield of alkaloids in *A. belladonna*. Thus, the concentration of nitrate in the medium has a direct influence on the total alkaloid content, Scopolamine and Atropine ratio in a cell line (Chashmia *et al*.,2 010).

In order for the enhanced production of secondary metabolites, various methods have been applied. Elicitation is an alternate method of enhancing secondary metabolite production in plant cultures. Elicitors such as Salicylic acid and Methyl Jasmonate are capable of inducing physiological and biochemical changes within a culture. For example, the use of MeJA, Salicylic acid and Acetylsalicylic acid are common elicitors that have previously been used in experiments to enhance tropane alkaloid accumulation in *Solanaceae* species (Kheradmand *et al*., 2017; Sharma and Agrihotri, 2021). Elicitors are also responsible for the stimulation of phytoalexins. Phytoalexins are responsible for stimulating plant defense, resulting in secondary metabolite production and its extracellular release into the medium.

A research study indicated biotic elicitors such as bacterial cultures (*B. cereus*) were shown to increase the fresh and dry weight of hairy roots (Guru *et al*., 2021). Further investigations had indicated that biotic elicitors produce indole acetic acid which increases the growth rate of roots and improves the water and inorganic substance uptake. The presence of the biotic elicitor triggers the production and accumulation of secondary metabolites in transformed root cultures which are later released at a low scale in the medium as an antibacterial component (Shakeran *et al*., 2015). Although elicitation has proved to enhance the production of secondary metabolites, the overall yield of metabolites is dependent on various parameters. These parameters include the type of cell line, the growth phase of the culture, the media and nutrient composition, the type and concentration of the elicitor and its total exposure period pre-harvest.

For the pharmaceutical industry, the production of secondary metabolites is crucial, as this serves as a hub of value-added products for novel chemical entities used to combat microbial infections and degenerative disease. Although plants are readily available, their biological activities, usage and toxicity are dependent on their phytochemical composition. Although *D.* stramonium is well known for the production of tropane alkaloids, research has shown the potential of the plant to produce a range of phytochemicals that have potential as antimicrobial and antioxidant agents (Jain *et al*., 2015). Methanolic root and leaf extracts of *D. stramonium* were screened for their antimicrobial, antioxidant and toxicity potential using the disc diffusion, DPPH and Brine shrimp lethality assay respectively.

As indicated in Figure 26, root extracts displayed excellent antibacterial activity which was comparable to the positive control, Ciprofloxacin. In literature, most plant extracts exhibit antibacterial activity against Gram-positive microorganisms compared to Gram-negative bacteria. However, *D. stramonium* root extracts were potent against Gram-negative bacteria only. The antibacterial activity displayed by the root extracts is similar to the antibacterial activity displayed by hydroalcoholic and methanolic extracts of *Datura fastuosa* (Al-Snafi, 2017).

In a study conducted by Altameme *et al* (2015), the alkaloid content in leaf extracts of *D. stramonium* was assessed for potential antibacterial activity. It was found that leaf extract was potent against *E. coli*, *P. mirabilis* and *S. aureus*. However, this contradicts the results obtained in this project as root extracts displayed a broader spectrum of antibacterial activity as compared to leaf extracts.

The antibacterial potential of alkaloid extracts from D. stramonium leaves against *E. coli*, *P. mirabilis*, *S. aureus*, *P. aerogenosa* and *K. pneumonia* was assessed. The research highlighted minimum antibacterial activity of the alkaloid extract with zones of inhibition between 0.8 mm-2.1 mm (Altameme et al.,2015). The minimal antibacterial activity displayed in alkaloid extract and no anti bacterial activity displayed by leaf extract in the project herein, could be due to the method of extraction used. This is justified due to previously conducted in vitro studies; that highlighted antibacterial potential of methanolic and hexane extract against E. coli, S. aureus and B. subtilis ( Pranay et al., 2010).This is also validated due to crude extracts obtained using solvent based system chloroform and acetone, had displayed antibacterial activity between 8.2mm and 18.1mm(Baynesagne et al.,2017). The phytochemical analysis of these extracts indicated the presence of multiple classes of secondary metabolites such as also tannins, cardiac glycosides, steroids and flavonoids. The presence of multiple phytochemicals within an extract, enhances the antibacterial characteristic (Wink, 2015).

There was no antifungal activity exhibited by both root and leave extracts. This contradicts investigations conducted by Sharma *et al*. (2014b), who indicated the potential of ethanolic *D. stramonium* extracts as antifungal agents (Sharma *et al*., 2014b). The antimicrobial activities of Datura extracts are solely due to the phytochemical composition of various plant organelles. For example, although plants within the Datura genus are known for their tropane alkaloid production, palmitic acid constitutes 50 % of the phytochemical composition in roots together with phenolic compounds and tannins being present (Al-Snafi, 2017). Hence the antibacterial activity displayed could be as a result of the total phytochemicals in *D. stramonium* root extracts working synergistically. If these phytochemicals had to be isolated and re-evaluated for their antimicrobial potential, the bioactivity may increase or decrease.

Reactive oxygen is an example of an oxidant that damages protein, DNA and enzymes in our bodies, thus causing an imbalance in redox homeostasis. Antioxidants are molecules that are capable of inhibiting oxidants either by reducing or radical scavenging capacity. The antioxidant activity of *D. stramonium* leaf and root extract was evaluated using nine different concentrations. As indicated in Figure 32, both root and leaf extract displayed antioxidant activity. At a concentration of 1 $\mu g.mL^{-1}$, the scavenging capacity of root extracts and leaf extracts was 64.4% and 31.7% respectively whereas, at the highest concentration of 1000 $\mu g.mL^{-1}$, root extracts and leaf extracts were 98.4% and 45.8% respectively. However, a greater antioxidant potential was displayed by root extracts. A direct relationship between the concentration and antioxidant activity was demonstrated. In *D. meta,* leaf extracts were investigated using the DPPH assay. The antioxidant potential was concentration-dependent and a positive correlation was determined between the phenolic and flavonoid content of the leaf extract and the antioxidant activity (Roy *et al*., 2016).

Although *D. metal* is a different species compared to *D. stramonium*, they belong to the same family, Solanaceae, where plants within this family have similar phytochemical profiles which include tropane alkaloids, glycosides, flavonoids and phenols (Sangeetha *et al*, 2014). A study by Kasote *et al.* (2015) supports the antioxidant property herein of the *D. stramonium* extracts however, extraction solvents can be further investigated and optimized to facilitate maximum extraction of antioxidants.

Traditional medicinal plants have displayed a range of bioactivity, their biosafety is likely to differ between species as well as their therapeutic effect when used in a concoction with other plants. Many plants contain phytochemicals that have genotoxic and mutagenic characteristics hence the administration of plant extracts as crude drugs are dose-dependent on ensuring no further harm to the patient. Therefore, alongside biological activities, toxicity studies must be conducted. There are many assays used to assess the toxicity of plant extracts and plant derived compounds, against different biological models, for example, *in vivo* assays on laboratory animals. However, recent studies employed efforts for alternative biological assays that include species of *Artemia salina*, *Artemia franciscana*, *Artemia urmiana* and *Thamnocephalus platyurus*. These toxicity tests are considered a useful tool for preliminary assessment of toxicity (Carballo *et al*., 2002).

As indicated in the results, the lowest concentration of 10 µg.mL$^{-1}$ for both root and leaf extracts, had indicated high toxicity of 97% and 100% respectively. This can be related to the fact that crude plant extracts contain a diverse community of phytochemicals, some of which may display high toxicity while some which are not toxic, hence displaying a synergistic effect of the phytochemicals present in the crude extracts. In conclusion of their experiments, it was found that different concentrations of *D. stramonium* extracts display a variance of cytotoxicity against human cultured lymphocytes, however, at all concentrations, the cell proliferation of human cultured lymphocytes is inhibited. It was also noted that at 50 µg. mL$^{-1}$ and 125 µg.mL$^{-1}$ *D. stramonium* extracts caused DNA fragmentation on cultured human lymphocytes (Akal *et al*., 2014).

The toxicity of *D. stramonium* against *Artemia salina* can be compared and validated against those cases reported after the ingestion of *D. stramonium*. The ingestion of *D. stramonium* by humans and animals causes acute anticholinergic poisoning which often leads to death. Pharmaceutical studies estimated a lethal dosage of Atropine and Scopolamine at 10 mg. mL$^{-1}$ and 4 mg.mL$^{-1}$ respectively. With these dosages, 60 minutes post-ingestion, symptoms such as blurred vision; coma, seizures and hyperthermia can occur and persist for 48 hours (Spina *et al*., 2007). The antimicrobial and antioxidant screening of *D. stramonium* leave and root extracts indicates that there are valuable phytochemicals that can be isolated and evaluated independently for their potential use as pharmaceuticals.

# 5. SUMMARY AND CONCLUSION

*D. stramonium* is one of many plants within the *Solanaceae* family that is capable of producing tropane alkaloids and has been widely used in folk and traditional medicine. Of all the tropane alkaloids produced, Atropine and Scopolamine are alkaloids of pharmaceutical demand as they exhibit anticholinergic activity and have an annual global revenue of approximately $131 million annually. However, to meet the supply and demand of the pharmaceutical industry, plants cannot be used as the only source of Atropine and Scopolamine, as their growth is seasonal and there will be an inconsistent yield of metabolites produced. Hence, this study was focused on screening the potential of *D. stramonium* hairy roots in producing Atropine and Scopolamine. This study also evaluated wild plant leaf and root extracts for potential biological activities.

The study was initiated by establishing an *in vitro* plant system. This was established by surface sterilizing wild seeds with 30% sodium hypochlorite followed by germination on a hormone-free MS medium. Germinated seedlings were then transferred to MS media supplemented with IAA and BAP, followed by a 16-hour photoperiod incubation. Leaf explants obtained from these seedlings were further used to create callus cultures using hormones 2,4 D and BAP and hairy root cultures using *A. rhizogenes*. A further trial, preliminary investigating the use of Methyl Jasmonate as an inducer to increase tropane alkaloid production, demonstrated a marginal increase in Atropine and Scopolamine content in hairy root cultures. The environmental optimization of hairy root cultures post elicitation, such as pH, nutrients and incubation period may result in an increase in the biomass weight. Further to this, the optimization of intracellular tropane alkaloids extraction by trialling different solvent system may provide a greater recovery of endogenous atropine and scopoloamine from leafs, and roots. Thus overall increasing the yield of atropine, scopolamine and hyoscyamine in tissue culture systems of *D.stramonium*.

Although research has demonstrated the plants within the *Datura* family are rich sources of tropane alkaloids, this project served as a base in investigating the antibacterial and antioxidant potential of the plant to also broaden its value in the pharmacological industry.

It was demonstrated that the leaf extract was more potent in displaying biological activities as compared to the root extracts.

It has been concluded that plant and cell tissue culture systems of *D. stramonium* are capable of producing valuable pharmaceutical alkaloids and their natural extracts possess biological activity. Plant cell and tissue cultures offer the advantage of controlling the production of secondary metabolites within plants. By combining techniques of fermentation technology, biochemistry and molecular biology, the production of important pharmaceutical metabolites can be exploited using plant tissue culture and be site-specific in enhancing production using elicitation and plant growth regulators. These metabolites may be exploited and used as an Active Pharmaceutical Ingredient for the synthesis of novel broad-spectrum drugs which will be cheap and effective at low dosage.

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# APPENDICES

## APPENDIX 1: Antioxidant activity of *D. stramonium* root extracts

Table 22: Antioxidant activity of *D. stramonium* root extract samples

| Concentration (µg/mL) | Antioxidant Activity Sample 1 | Antioxidant Activity Sample 2 | Antioxidant Activity Sample 3 | Average antioxidant activity | Std Deviation |
|---|---|---|---|---|---|
| **1** | 64.8 | 64.3 | 64.5 | 64.5 | ±0.25 |
| **20** | 71.8 | 71.9 | 71.4 | 71.7 | ±0.25 |
| **40** | 72.7 | 73.5 | 72.9 | 73.0 | ±0.41 |
| **60** | 81.8 | 83.7 | 83.3 | 83.0 | ±0.99 |
| **80** | 86.6 | 85.2 | 85.2 | 85.7 | ±0.82 |
| **100** | 85.4 | 86.0 | 86.1 | 85.8 | ±0.37 |
| **250** | 89.6 | 90.7 | 89.3 | 89.9 | ±0.74 |
| **500** | 91.5 | 90.9 | 92.7 | 91.7 | ±0.89 |
| **1000** | 98.8 | 98.5 | 99.5 | 98.9 | ±0.49 |

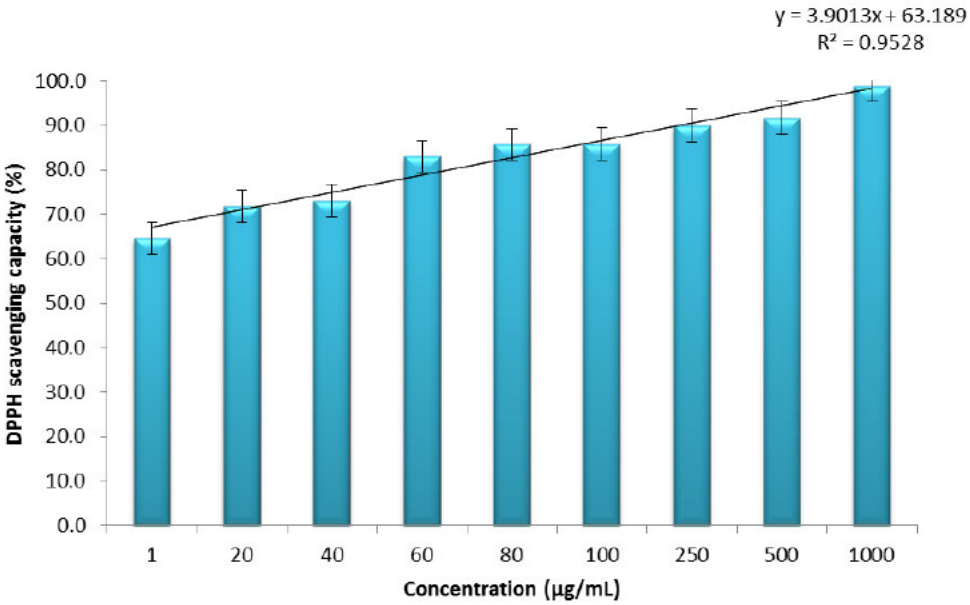


**Figure 33:** Graphical representation of the average antioxidant activity by *D. stramonium* root extracts

## APPENDIX 2: Antioxidant activity of *D. stramonium* leaf extract

**Table 23:** Antioxidant activity of *D. stramonium* leaf extract samples

| Concentration (µg/mL) | Antioxidant Activity Sample 1 | Antioxidant Activity Sample 2 | Antioxidant Activity Sample 3 | Average antioxidant activity | Std Deviation |
|---|---|---|---|---|---|
| 1 | 30.9 | 32.0 | 32.4 | 31.8 | ±0.8 |
| 20 | 35.2 | 35.5 | 37.0 | 35.9 | ±1.0 |
| 40 | 38.7 | 41.2 | 40.1 | 40.0 | ±1.2 |
| 60 | 39.0 | 41.3 | 41.5 | 40.6 | ±1.4 |
| 80 | 40.6 | 41.1 | 43.2 | 41.6 | ±1.3 |
| 100 | 41.2 | 43.0 | 44.4 | 42.9 | ±1.6 |
| 250 | 42.4 | 43.2 | 43.3 | 43.0 | ±0.5 |
| 500 | 43.2 | 43.6 | 42.9 | 43.2 | ±0.4 |
| 1000 | 44.9 | 44.4 | 45.2 | 44.8 | ±0.4 |

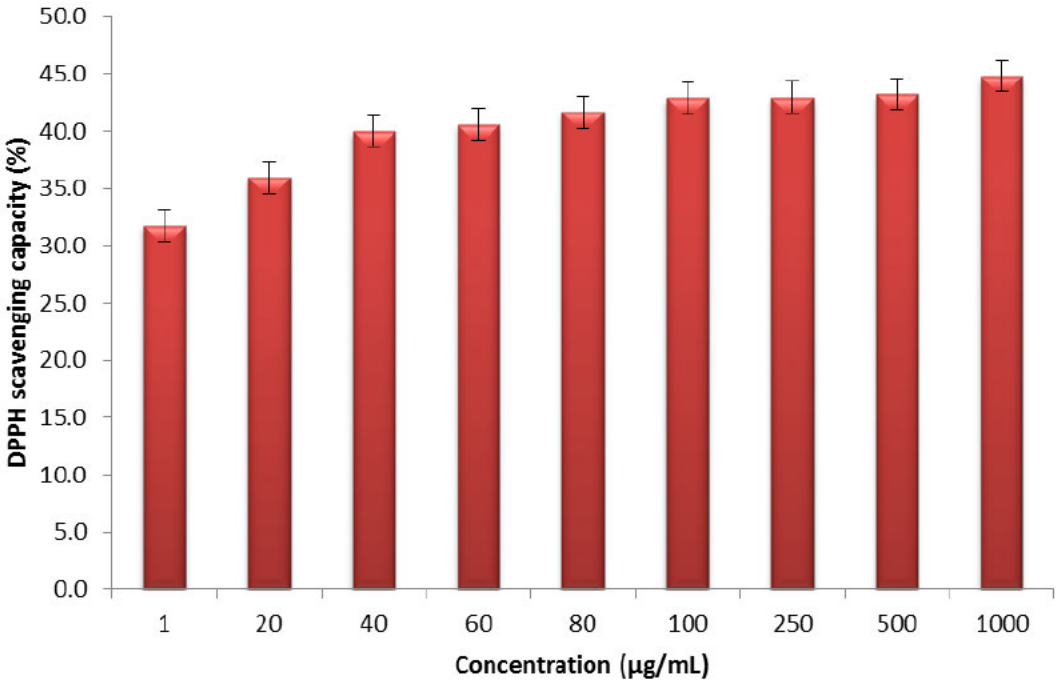


**Figure 34:** Graphical representation of the average antioxidant activity displayed by *D. stramonium* leaf extracts.

Appendix 3

Journal of Pharmacognosy and Phytochemistry 2020; 9(3): 05-13

Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(3): 05-13
Received: 04-03-2020
Accepted: 07-04-2020

**F Ally**
Department of biotechnology and food technology, Faculty of applied sciences, Durban University of Technology, P.O Box 1334, Durban, 4000, South Africa.

**V Mohanlall**
Department of biotechnology and food technology, Faculty of applied sciences, Durban University of Technology, P.O Box 1334, Durban, 4000, South Africa.

**Corresponding Author:**
**F Ally**
Department of biotechnology and food technology, Faculty of applied sciences, Durban University of Technology, P.O Box 1334, Durban, 4000, South Africa.

# An overview of tropane alkaloids from *Datura stramonium* L.

**F Ally and V Mohanlall**

**Abstract**
*Datura stramonium* L., a wild-growing plant of the Solanaceae family, is widely distributed throughout the world. It contains a variety of tropane alkaloids such as atropine, hyoscyamine, and scopolamine In Ayurvedic medicine, *D. stramonium* has been used for curing various human ailments, including ulcers, wounds, inflammation, rheumatism and gout, sciatica, bruises and swellings, fever, asthma and toothache. A few previous studies have reported on the pharmacological effects of *D. stramonium*; however, complete information regarding the phytochemistry remains unclear. This comprehensive review includes information on botany and phytochemistry of the major tropane alkaloids produced by *D. stramonium*.

**Keywords:** *Datura stramonium,* tropane alkaloids, atropine, hyoscyamine, scopolamine

## Introduction
*Datura stramonium,* also known as Jimson Weed, Locoweed, Angel's Trumpet, Thorn Apple, Devil's Trumpet is a hallucinogenic plant found in urban and rural areas. *Datura stramonium* is an annual herbaceous shrub belonging to the Solanaceae family. The plant originated in the territories of the Caspian Sea and spread to Europe in the first century. At present, the plant is found in most waste and dumpsites in Europe, Asia, America and South Africa (Weaver and Warwick, 1984) [44]. However, in other parts of the world, such as Germany and France, *D. stramonium* is cultivated (Moore, 1972) [24]. It is a wild growing flowering plant and was investigated as a local source for tropane alkaloids which contain a methylated nitrogen atom ($N\text{-}CH_3$) and include the anti-cholinergic drugs atropine and scopolamine.
Morphologically, the plant is described to have leaves with a toothed margin that are approximately 5-18 cm broad and 10-20 cm long. Flowers of *D. stramonium* can be described as axillary and resemble the shape of a trumpet thus giving its common name "Angel's trumpet". A spiny capsule is the fruit which is green and upon drying split opens and releases black seeds. The flowers are usually cream, yellow or purple in colour. The plant usually grows to a height of two meters (Stace, 2010) [38].
The plant is known to be a strong narcotic and poisonous. The ingestion of any part of the plant causes poisoning and may result in death. According to research based on *D. stramonium* toxicity, the seeds and fruit are known to be the most toxic. There are no treatments available that can reduce the toxic effects of the seeds or fruit. There are many cases of Datura poisoning, the first recorded in the early 1990's in the United States of America. It was reported that many adolescents and young adults became ill and passed on from ingesting the leaves of *D. stramonium* (Adegoke and Alo, 2013) [1]. The symptoms of Jimsonweed poisoning are associated with dryness of the mouth and skin, severe thirst, dilation of the pupil, loss of eyesight, hallucination, palpitations, restlessness and loss of consciousness.

## Taxonomy and description
*D. stramonium* is an annual plant. The stem is herbaceous, branched and glabrous or only lightly hairy. By cultivation the plant reaches a height of about one meter (Nadkarni and Nadkarni, 1996 [25]; Jarald and Edwin, 2007 [17]). The branching stems are spreading, leafy, stout, erect, Smooth and pale yellowish green in color, branching repeatedly in a forked manner. Leaves are hairy, big, simple dentate, oval glabrous, apposite veins of leaves are pale black, stalked, 4-6-inch-long, ovate and pale green. The upper surface is dark and grayish-green, generally smooth, the under surface paler, and when dried, minutely wrinkled (Figure 1a). *D. stramonium* bears funnel shaped, white or purple coloured flowers, with 5 stamens and superior ovary (Figure 1c). The average length of flower is about 3 inches. The calyx is long, tubular and swollen base surrounded by five sharp teeth. Corolla is funnel shaped. Stem stalk is pale blue or greenish white. Seeds are black, kidney shape and flat [Gary *et al.*, 2005 [11];

Gupta, 2008) [12]. Fruits are as large as walnuts and full of thorns (hence the English name "thorn apple") (Figure 1b). The plant is strong narcotic but has a peculiar action on the human which renders it very valuable as medicines. The whole plant is poisonous and the seeds are the most active; neither drying nor boiling destroys the poisonous properties.

The genus name Datura is derived from "dhatura", the Bengali name for the plant, while the epithet stramonium combines the Greek word "strychnos" for nightshade, and "makinos" meaning mad, referring to the narcotic properties of the species. Until relatively recently it had been customary to distinguish between the white-flowered *D. stramonium* and the purple-flowered *D. tatula*. However, chemotaxonomic studies have confirmed that these are both forms of *D. stramonium* (Haegi, 1976 [14]; Hadkins *et al.*, 1997 [13]). Variants of *D. stramonium* have been described with 2n = 12, 25, 26, 36 or 48 chromosomes. Morphological variants include var. *tatula* which has purple flowers and sub-equal spines on the capsule; var. *stramonium*, with white flowers and shorter spines on the lower part of the fruit (Table 1).

**Table 1:** Classification of Kingdom Plantae

| | |
|---|---|
| Domain | Eukaryota |
| Kingdom | Plantae |
| Phylum | Spermatophyta |
| Subphylum | Angiospermae |
| Class | Dicotyledonae |
| Order | Solanales |
| Family | Solanaceae |
| Genus | Datura |

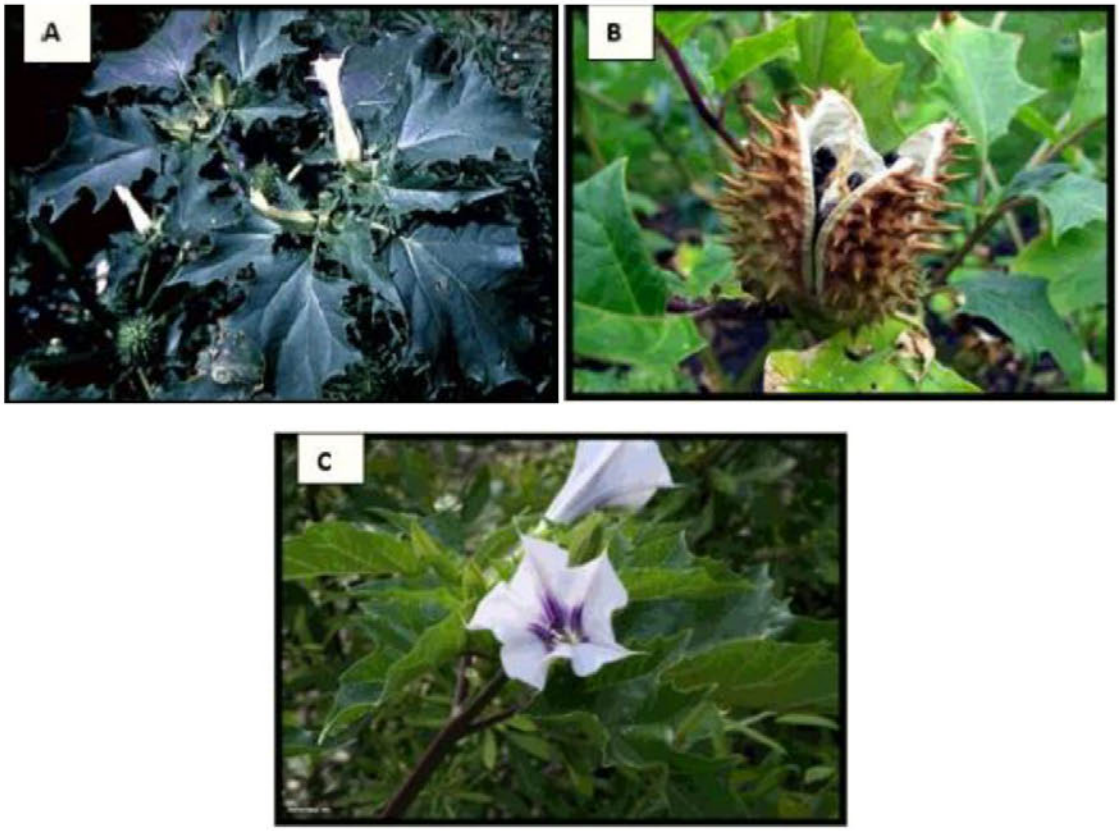


**Fig 1:** *Datura stramonium*. A: Datura plant (leaves and flowers); B: *D. stramonium* fruit; c: *D. stramonium* flower

**Distribution**

*Datura stramonium* originated in the tropical regions of Central and South America and has become a cosmopolitan weed in the warm regions of North, Central and South America, Europe, Asia, Africa and New Zealand. It is now found throughout almost all the USA except for the north-west and northern Great Plains. It was recorded in Virginia, USA, by 1676, where its seed was used as a narcotic by British soldiers. *Datura stramonium* is found on most soil types but prefers rich soils. *Datura stramonium* is a common weed of gardens, waste places, and farmyards. In recent years, the species has started to appear as a weed of cultivated ground, particularly in soybean, bean and maize fields in southern Ontario and Quebec. It prefers open communities, and it most commonly occurs in association with annual. Biennial or short-lived perennial weeds. At present it grows in waste places in Europe, Asia, America and South Africa. *D. stramonium* is cultivated in Germany, France, Hungary, South America and throughout the world (Jarald, 2007) [17].

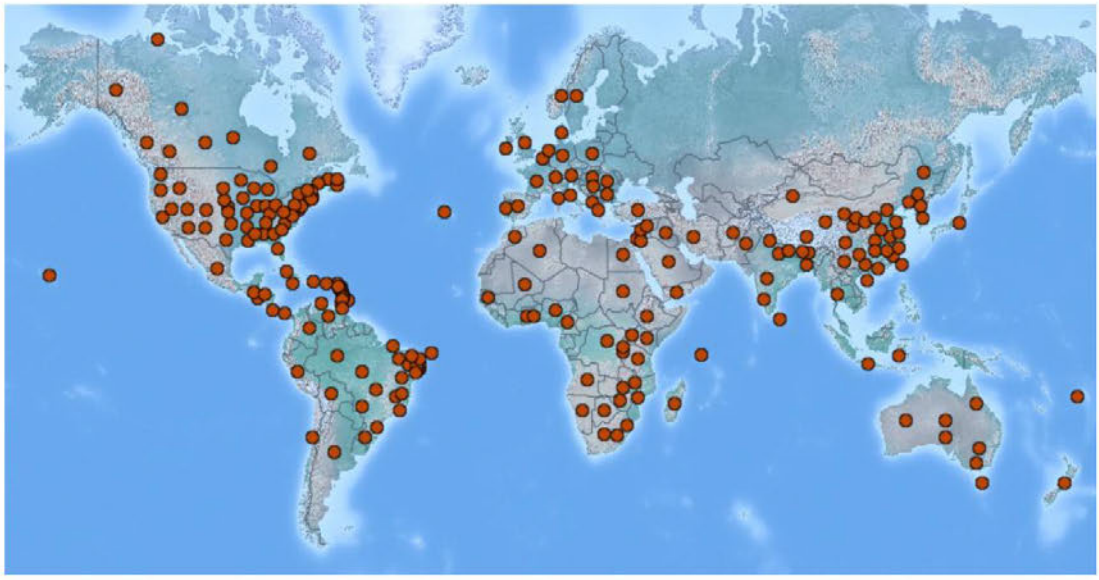

**Fig 2:** Present worldwide distribution (red) of *Datura stramonium*

**Review procedure**

Information presented in this paper was collected from different sources found online. Electronic databases such as Science Direct, ISI Web of Science, Google Scholar, Scopus and MEDLINE were used for data mining. The reviewed online sources include scientific studies published in articles, theses, books, book chapters, journals and abstracts. Potential literature sources were found by searching for the terms and/or phrases like taxonomic hierarchy, traditional medicine, folk medicine, folkloric uses, indigenous medicine, ethno medicine, ethno botany, economic uses, horticultural uses, cultural uses, phytochemistry, active compounds, biological activities and pharmacological properties of *D. stramonium*.

**Ethno medicinal uses**

Flower petals from *D. stramonium* are crushed and the extracted juices are used for the treatment of ear infections. The seeds are used as purgative and for the treatment of cough and fever. A small quantity of seeds are used for asthma and tonsil problems (Savithramma *et al.*, 2007) [32]. Seeds are usually smoked due to its narcotic action (Khan *et al.*, 2013) [18]. External injuries, wounds, bleedings and pain is treated with leaf paste and extract (Njoroge, 2012) [26]. Fruit oil is used for to relieve body pain (Vijendra and Kumar, 2010) [41]. Leaf or whole plant is anti-inflammatory and antispasmodic (Dwivedi *et al.*, 2008) [9]. The extract of leaves is also used for baldness and the juice of the fruit is applied to scalp for falling hairs and as an antidandruff (Khan and Khatoon, 2008) [19]. Dried leaves and seeds are used as anticholinergic and sedative (Wazir *et al.*, 2004) [43]. The leaves of *D. stramonium* L. are used for the relief of headache and vapours of leaf infusion is used to relive the pain of rheumatism and gout (Biswas *et al.*, 2011) [6]. The smoke from the burning leaf is inhaled for the relief of asthma and bronchitis (Savithramma *et al.*, 2007) [32]. It is also applied to smooth painful wounds and sores. Seeds and leaves of *D. stramonium* were used to sedate hysterical and psychotic patients, also to treat insomnia (Khanra *et al.*, 2015) [20]. It is also used to relax the smooth muscles of the bronchial tube and asthmatic bronchial spasm. It is also used in the treatment of parkinsonism and hemorrhoids (Ivancheva *et al.*, 2006) [16]. Scopolamine is also found in the plant, which makes it a potent cholinergic-blocking hallucinogen that has been used to calm schizoid patients. Its leaves, containing hyoscyamine and atropine, can be used as an immensely powerful mind-altering drug. The seeds of Datura are analgesic, anthelmintic and anti-inflammatory and as such, they are used in the treatment of stomach and intestinal pain that results from worm infestation, toothache, and fever from inflammation (Ivancheva *et al.*, 2006) [16]. *Datura stramonium* plants are frequently used as antiparasitic and repellents to insect infestation (Das *et al.*, 2012) [8].

**Pharmacology**

*D. stramonium* extracts were researched for its potential pharmacological profile (Rasila Devi *et al.*, 2011 [29], Sharma *et al.*, 2014a) [36], Swathi *et al.* (2012) [39] investigated the potential of *D. stramonium* ethanolic extracts as larvicidal and mosquito repellent agents. At a concentration of 86.25 mg/L, 16.07 mg/L and 6.25mg/L, the extracts exhibited larvicidal activity against *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasiciatus* respectively.

*D. stramonium* leaf extracts, combined with extracts of other plants such as *Azadirachta indica* and *Coriandrum sativum*, were screened in vivo for their anti-inflammatory potential in albino rats. The combined ethanolic extracts were investigated using the carrageenan-induced rat paw edema method and results of these plants exhibited anti-inflammatory activity which comparable to the standard drug, diclofenac sodium. However, this anti-inflammatory activity, the synergistic effect of combined plant extracts and further studies pertaining to crude *D. stramonium* extracts is still to be conducted (Sonika *et al.*, 2010) [37].

Combined methanolic *D. stramonium* and *D. innoxia* extract, displayed antibacterial activity against Gram-positive bacteria in a dose-dependent manner. This antibacterial potential was further investigated in another study in conjunction with *W. somnifera* and *Terminalia Arjuna*. Results indicated that these extracts in combination with crude ethanolic extracts of *D. stramonium* displayed antibacterial activity against *S. aureus*, *B. subtilus*, *E.coli*, *M. luteus* and *Candida albicans* which were comparable to the standard drug ciprofloxacin (Sharma

*et al.*, 2009) [35]. *D. stramonium* extracts using four solvent systems (water, acetone, ethanol and methanol) was assessed for its potential antibacterial activity against clinical pathogenic isolates. The maximum antibacterial was displayed by chloroform extract against *S. aureus* with a zone of inhibition of 18 mm, whilst the least antibacterial activity was displayed by the acetone extract against *E.coli* with a zone of inhibition of 8.2 mm (Baynesagne *et al.*, 2017) [3].

A study in 2016, investigated ethyl acetate flower extracts of *D. stramonium* for its anti-cancer activity against human liver cancer cells (HepG2) using the MTT assay. The extract exhibited anticancer activity comparable to that of the standard Gemcitabine with a CTC50 value of 131.53 µg/ml (Rajeshkanna *et al.*, 2016). Methanolic seed extracts of *D. stramonium* was also investigated for their potential antioxidant and anticancer activity. The antioxidant activity was conducted using the DPPH˙ radical, superoxide radical, $ABTS^{\cdot}$+ radical cation, $OH^{\cdot}$ radical scavenging assays, Phophomolybdenum reduction and Fe3+ reducing power assays, whilst the anti-cancer activity was investigated using the MTT assay against MCF (breast cancer) cell lines. The antioxidant results demonstrated $IC_{50}$ values for DPPH radical, superoxide radical, ABTS+ radical cation, $OH^{\cdot}$ radical scavenging assays of 35.26, 10.50, 49.36 µg/mL respectively. Cytotoxic activity for MCF7 cell line was 66.84% at 500 µg/mL (Iqbal *et al.*, 2017).

Ethanolic leaf extracts of *D. stramonium* was also investigated for its potential larvicidal and mosquito repellent activities. The results for the assay demonstrated potential larvicidal against *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* with $LD_{50}$ values of 86.25 mg/L, 16.07 mg/L and 6.25 mg/L respectively. Mosquito repellency was displayed for durations of 2.7, 71.7 and 117.7 minutes against *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasiciatus* respectively at higher concentrations (Swathi *et al*., 2012) [39].

**Phytochemistry**

Amongst the many phytochemicals present in *D. stramonium*, minor and major tropane alkaloids have been predominantly present in the plant. Major alkaloids include Atropine, Scopolamine, Hyoscyamine, Apo scopolamine and 7-hydroxyhyoscyamine. Studies conducted on the distribution of Atropine and Scopolamine revealed that the production of Atropine and Scopolamine occurs in different parts of the plants at different stages of its life cycle. In young stems and leaves, hyoscyamine is always the predominant alkaloid. There has been a total of 64 tropane alkaloids which has been identified in *D. stramonium*, all of which has been included in many pharmacopoeias due to its anticholinergic potential (Ryan *et al.*, 2015). Two new tropane alkaloids, 3-phenylacetoxy-6, 7-epoxynortropane and 7-hydroxyapoatropine were tentatively identified. The alkaloids scopoline, 3-(hydroxyacetoxy) tropane, 3-hydroxy-6-(2-methylbutyryloxy) tropane, 3a-tigloyloxy-6-hydroxytropane, 3,7-dihydroxy-6-tigloyloxytropane, 3-tigloyloxy-6-propionyloxytropane, 3-phenylacetoxy-6,7-epoxytropane, 3-phenylacetoxy-6-hydroxytropane, aponorscopolamine, 3a,6a-ditigloyloxytropane and 7-hydroxyhyoscyamine are reported for the first time for this species (Berkov *et al.*, 2006) [4].

The main components of essential oil were sterols and their derivatives, and the major constituents of *Datura stramonium* essential oil are sterols and their derivatives and 5α-Ergosta-7,22-dien-3β-ol (16.53%), 3-Hydroxycholestan-5-yl acetate (14.97%), and 26,26-Dimethyl-5, 24(28)-ergostadien-3β-ol (10.39%) (Wang and you, 2012). The seeds also contained higher concentration of phytate, tannin and oxalate than the seed coat. In seed coat calcium, iron, potassium, sodium and phosphorus were higher than the seeds (Oseni *et al.*, 2011). Polar extractives contained saponins, steroids, alkaloids, and glycosides (Shagal *et al.*, 2012) [34]. The primary biologically active substances in *D. stramonium* are the alkaloids atropine and scopolamine (Ivancheva *et al.*, 2006) [16].

**Tropane alkaloids**

Tropane alkaloids are bicyclic alkaloids that contain the tropane ring. There are secondary metabolites distributed in plants within the Proteaceae, Brassicaceae, Convolvulaceae, Rhizophoraceae, Euphorbiaceae, Solanaceae, and Erythroxylaceae family. There are approximately 2000 structures that contain the tropane rings however the most common are Atropine and scopolamine. Atropine, Scopolamine and their derivatives are commercially important as they are used as anticholinergic drugs.

The different classes of Tropane alkaloids; cocaine, scopolamine/hyoscyamine and the calystegines share a common precursor biosynthetic route beginning with the amino acids L-ornithine and L-arginine (Figure 3). In plants, ornithine and arginine are derived from glutamate, an amino acid which is directly connected to the nitrogen assimilation. Ammonia is incorporated into Glutamate via the glutamine synthetase-glutamate synthase pathway. Glutamate is the precursor in several polyamine pathways. In order to form putrescine from the amino acids ornithine or arginine, ornithine is decarboxylated by ornithine decarboxylase and arginine undergoes a three-step reaction, including decarboxylation, hydrolysis of the imine functionality of guanidine and hydrolysis of urea which is catalyzed by the enzymes arginine decarboxylase; agmatine deiminase and N-carbamoylputrescine amidase, respectively.

The activities of arginine decarboxylase and ornithine decarboxylase were suppressed in Datura plants by using the specific irreversible inhibitors DL-difluoromethylarginine and DL-difluoromethylornithine, respectively in order to probe the nature of these two routes to putrescine biosynthesis. These experiments indicated that the two routes do not act independently from each other and that the arginine decarboxylase exhibited a higher activity than the ornithine decarboxylase (Robins *et al.*, 1991). Putrescine (Tetramethylenediamine) is an intermediate in several metabolic pathways. It can be formed to spermidine by a spermidine synthase catalyzed reaction using decarboxylated S-adenosyl methionine and putrescine as substrates. Putrescine can also be methylated to N-methylputrescine by the enzyme putrescine N-methyltransferase (Biastoff *et al.* 2009) using S-adenosyl methionine. The next step in TA biosynthesis is the oxidative deamination of N-methylputrescine to 4-methylaminobutanal which is catalyzed by N-methylputrescine oxidase (Mizusaki *et al.*, 1972) [23]. This diamine oxidase requires copper as a cofactor. N-methylpyrrolinium, a central intermediate, is formed by spontaneous cyclization of N-methylputrescine. Chemically, this reaction is an intramolecular Schiff base formation. N-methylpyrrolinium cation is a branchpoint in Tropane alkaloid and nicotine biosynthesis (Courdavault. 2010) [7].

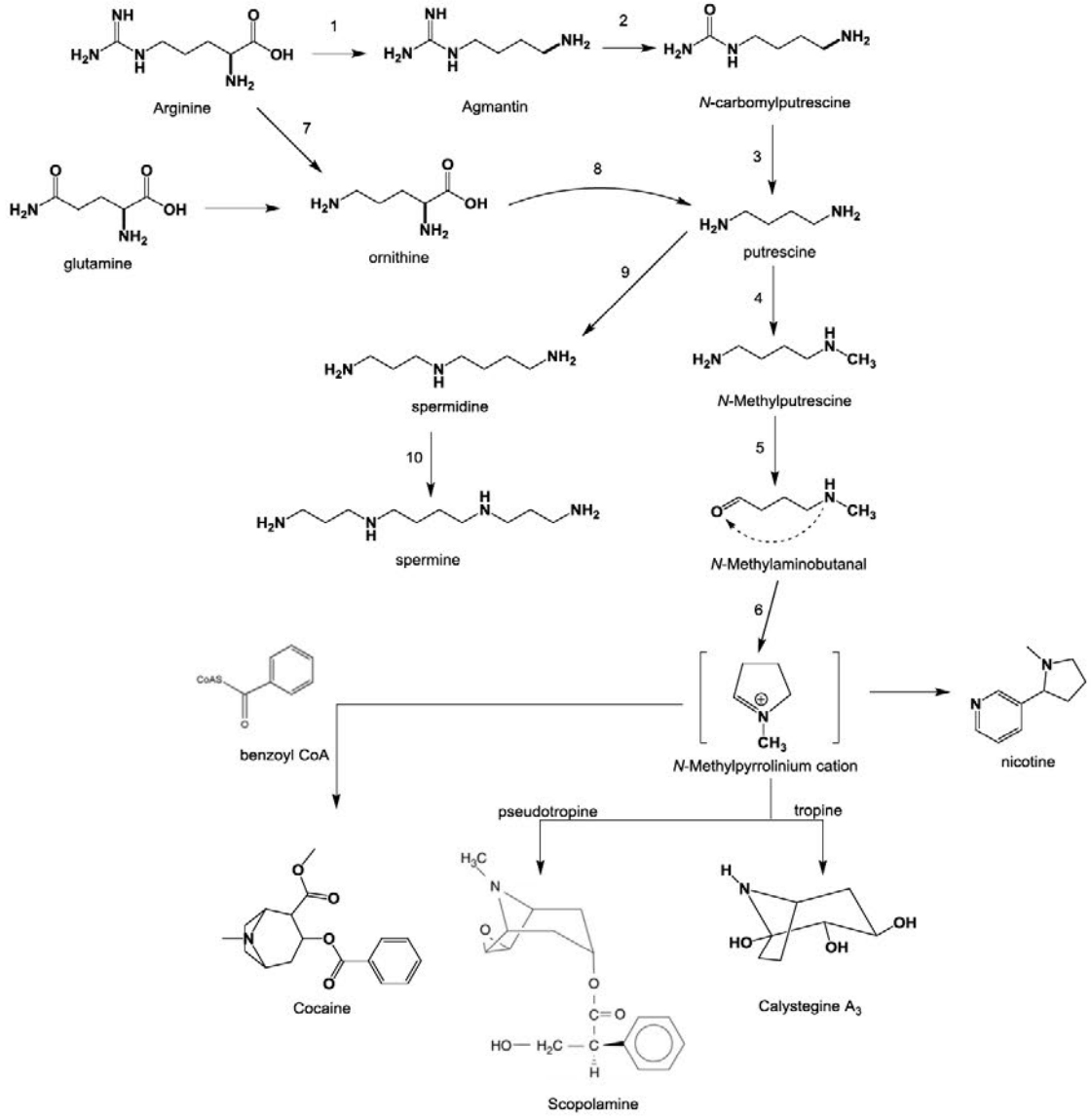


**Fig 3:** Early stages of Tropane Alkaloid biosynthesis; 1 = arginine decarboxylase; 2 = agmatine deiminase; 3 = N-carbamoylputrescine amidase; 4 = putrescine N-methyltransferase; 5 = N-methylputrescine oxidase; 6 = spontaneous cyclization; 7 = arginase; 8 = ornithine decarboxylase; 8 = spermidine synthase; 9 = spermine synthase

**Atropine**

Atropine is one of the most common tropane alkaloids used in the pharmaceutical industry (Fig. 4). It is the racemic form of Hyoscyamine and is known to bind to muscarinic receptors which block the parasympathetic cholinergic neurons. The alkaloid, acts on both peripheral and central muscarinic receptors. According to previous research, it was found that Atropine inhibits the growth of enveloped viruses independent of the nucleic acid content. The anti-viral activity of Atropine was investigated using the plaque reduction test and one step growth experiments. It was found that Atropine was effective against the Herpes simplex virus, Influenza, Sindbis, Adenovirus and Japanese encephalitis virus (Willoughby *et al*., 2005) [45]. Atropine was discovered to block the glycosylation of viral proteins of Herpes; hence the production of virions are inhibited. Furthermore, virions which were formed in the presence of Atropine were known to be non-infectious.

The pharmaceutical application of Atropine is vast however lose dosage is advised on treatment as it affects the cardiovascular system causing bradycardia. Atropine is used as an antidote to treat organophosphate poisoning as it increases acetylcholine release which inhibits cholinesterase. It is also used to dilate pupil, decrease salivation and reduce gastrointestinal activity (Sayyed and Shah, 2014) [33].

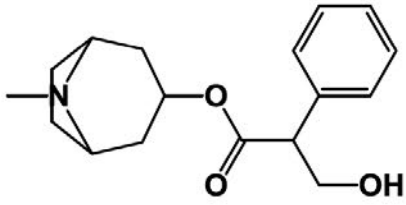

**Fig 4:** Chemical structure of Atropine - 1α H, 5α H-Tropan-3-α ol (±)-tropate (ester), sulfate monohydrate

**Biosynthesis of Atropine**

The initial stages of Atropine biosynthesis is identical to that that of all tropane alkaloids. Arginine and Ornithine metabolism leads to the formation of Putrescine, Putrescine is methylated to form *N*-Methyl putrescine by the enzyme *N*-putrescine Methyltransferase. *N*-Methyl putrescine is converted to N-methylpyrrolinium by spontaneous cyclization. N-methylpyrrolinium serves as the branch point for tropane synthesis. The biosynthesis of Atropine starting from L-Phenylalanine first undergoes a transamination forming Phenyl pyruvic acid which is then reduced to Phenyl-lactic Acid. Coenzyme A then couples Phenyl-lactic acid with Tropine forming Littorine, which then undergoes a radical rearrangement initiated with a $P_{450}$ enzyme forming hyoscyamine aldehyde. A dehydrogenase then reduces the aldehyde to a primary alcohol making Hyoscyamine, which upon racemization forms atropine.

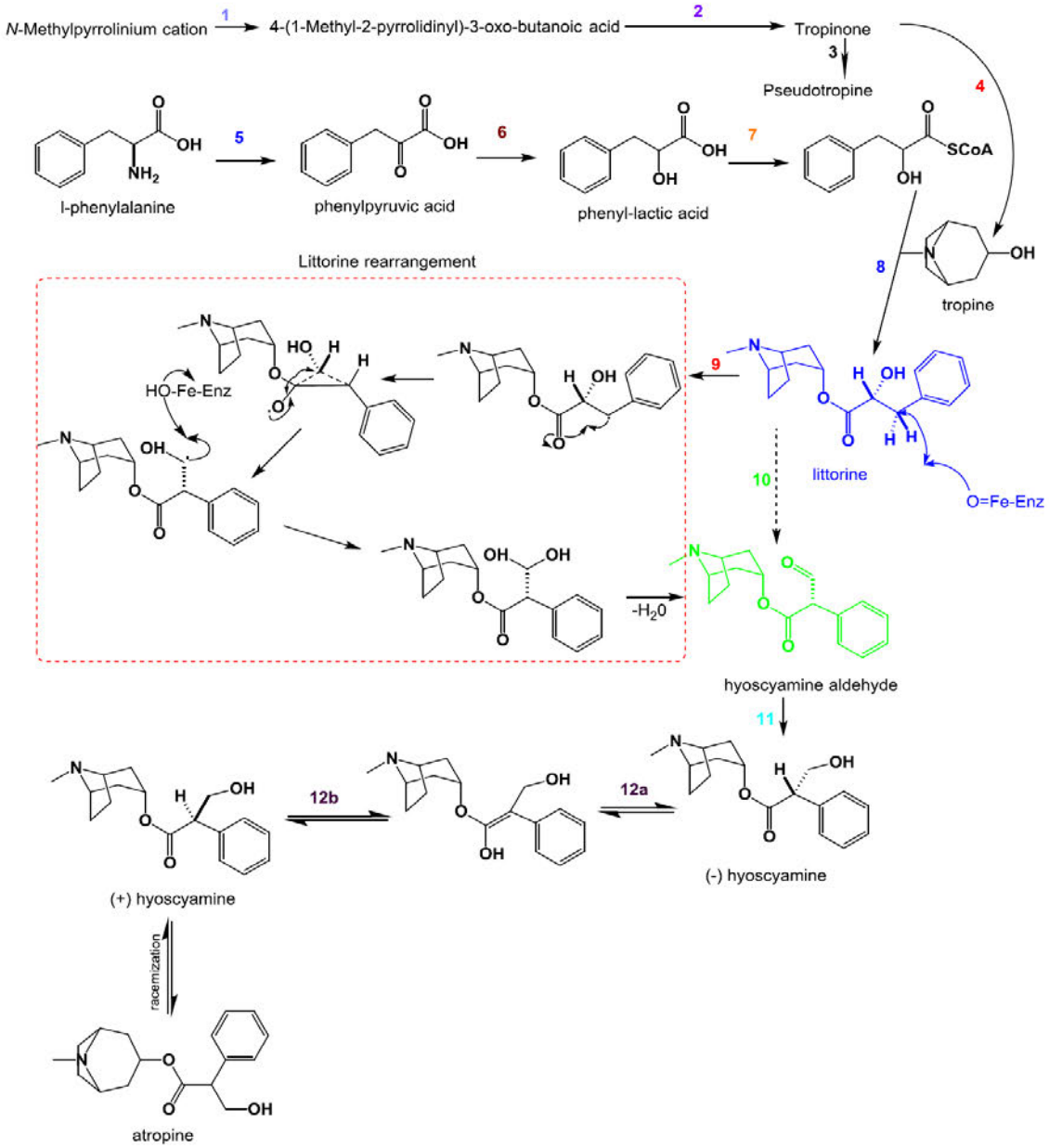


**Fig 5:** Atropine biosynthesis, starting with the L-phenylalanine; 1 = polyketide synthase; 2 = cytochrome P450 enzyme; 3,4 = tropinone reductase I/II; 5 = L-phenylalanine deaminase; 6 = D-phenyl lactate dehydrogenase; 7 = phenyl lactate CoA-transferase 8 = littorine synthase; 9 and 10 = cytochrome P450 littorine mutase/monooxygenase; 11 = unidentified alcohol dehydrogenase; 12a and 12b = hyoscyamine 6β-hydroxylase

**Scopolamine and Hyoscyamine**

Initially, Scopolamine was used in neuropsychopharmacology as an antidepressant and as a standard drug for the induction of age and dementia-related cognitive deficiency in healthy humans and animals (Furey *et al.*, 2010) [10]. However, the demand for Scopolamine in the pharmaceutical industry has

grown due to its multiple applications and administrations. Scopolamine is currently administered as a syrup or tablet for the treatment of nausea and vomiting. It has also been administered for the treatment of gastrointestinal spasms associated with irritable bowel syndrome and has also been used for eye inflammation. The current hype of Scopolamine is associated with its use in creating a transdermal patch for treating motion sickness. This type of Scopolamine is known as the "the transdermal scopolamine". In 1979, the very first transdermal Scopolamine was manufactured by a company Alza Corporation. Over the years its sale has grown as transdermal Scopolamine is the preferred choice for treating motion sickness. This is due to its easy administration, cost-effectiveness and availability. It has been envisaged that in the period of 2016 -2024, the expected CAGR growth rate for Scopolamine will be 6.7%.

Hyoscyamine is an antagonist of muscarinic acetylcholine receptors. It blocks the action of acetylcholine at parasympathetic sites in sweat glands, salivary glands, stomach secretions, heart muscle, sinoatrial node, smooth muscle in the gastrointestinal tract, and the central nervous system. It increases cardiac output and heart rate, lowers blood pressure, and dries secretions. Hyoscyamine is used to provide symptomatic relief to various gastrointestinal disorders including spasms, peptic ulcers, irritable bowel syndrome, pancreatitis, colic, and cystitis. It has also been used to control some of the symptoms of Parkinson's disease.

**Biosynthesis of Scopolamine and Hyoscyamine**

The biosynthesis of scopolamine begins with the decarboxylation of l-ornithine to putrescine by ornithine decarboxylase. Putrescine is methylated to N-methylputrescine by putrescine N-Methyltransferase (Ziegler and Facchini, 2008) [46]. Putrescine oxidase that specifically recognizes methylated putrescine catalyzes the deamination of this compound to 4-methylaminobutanal which then undergoes a spontaneous ring formation to N-methylpyrrolium cation. In the next step, the pyrrolium cation condenses with acetoacetic acid yielding hygrine. No enzymatic activity could be demonstrated that catalyzes this reaction. Hygrine further rearranges to tropinone. Subsequently, tropinone reductase I converts tropinone into tropine which condenses with phenylalanine-derived phenylactate to littorine. A cytochrome P450 classified as Cyp80F1 (Li *et al.*, 2006) [22] oxidizes and rearranges littorine to hyoscyamine aldehyde.

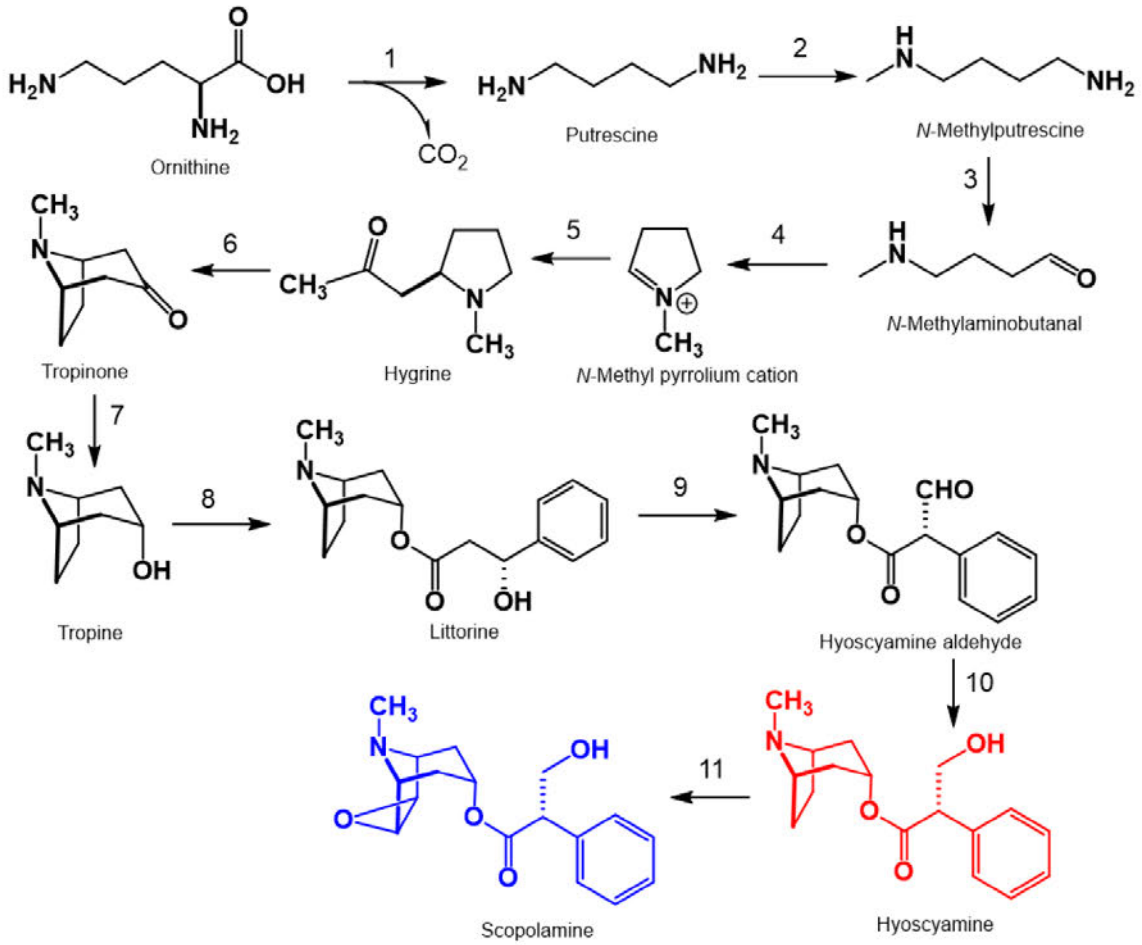


**Fig 6:** Scopolamine and Hyoscyamine biosynthesis, starting with Ornithine. 1 = ornithine decarboxylase; 2 = putrescine N-Methyltransferase; ; 3 = N-Methylputrescine oxidase; 4 = spontaneous cyclization; 5 = unknown enzyme; 6 = spontaneous cyclization; 7 = tropinone reductase I; 8 = littorine synthase; 9 = littorine mutase/ monooxygenase (CYP80F1) 3 = PYKS = polyketide synthase; CYP82M3 = cytochrome P450 enzyme TR-I/II = tropinone reductase I/II; littorine synthase not known); littorine mutase/ monooxygenase (CYP80F1); 10 = 11 = unidentified alcohol dehydrogenase; 11 = hyoscyamine 6β-hydroxylase (H6H)

Scopolamine (Fig. 7A) is the 6, 7-β-epoxide of hyoscyamine (Fig. 7B) which is formed from hyoscyamine by means of 6β-hyroxyhyoscyamine. The hydroxylation of hyoscyamine to 6β-Hydroxy-hyoscyamine is catalyzed by a 2-oxy-glutarate dependent dioxygenase, Hyoscyamine 6β-hydroxylase. The epoxidation of 6β-hyroxyhyoscyamine to Scopolamine is also catalyzed by hyoscyamine 6β-hydroxylase.

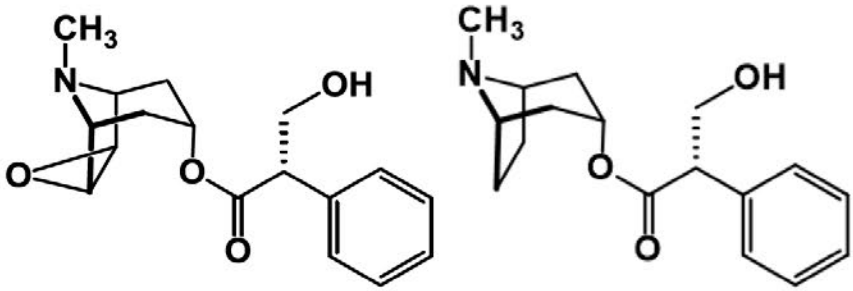


**Fig 7:** Chemical structure of Scopolamine [9-methyl-9-oxido-3-oxa-9-azoniatricyclononan-7-yl 3-hydroxy-2-phenylpropanoate] - (A) and Hyoscyamine [(1S, 5R)-8-methyl-8-azabicyclo [3.2.1] octan-3-yl] (2S)-3-hydroxy-2-phenylpropanoate] - (B)

## Conclusion

The phytochemistry of *D. stramonium* has been documented in this in depth review. In view of its multiple pharmaceutical applications, more pharmaceutical screening and quantitative structure-activity relationship and molecular docking studies need to be conducted. The metabolic pathways and metabolomic production of the major tropane alkaloids presented in this review would be helpful in promoting research with emphasis on the manipulation of metabolic pathways for the enhanced production of the secondary metabolites. This review will also provide a new platform for the research and development of new secondary metabolites for medical application and agroindustry.

## Acknowledgments

The National Research Foundation of South Africa and the Durban University of Technology are acknowledged for financial and infrastructural support.

## Conflict of Interest

No conflict of interest exists amongst authors.

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