Comparative Evaluation of *in vitro* Growth Characteristic and Secondary Metabolite Accumulation in Two Cultivars of *Withania coagulans*

-AUWC008 and AUWC025

Thamarai, R.S

(12PBT016)

Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women,

Coimbatore – 641 043

In Partial Fulfilment of the Requirement for the Degree of

Master of Science in Biotechnology

March, 2014

CERTIFICATE

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Signature of Supervisor

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THAMARAI. R.S

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*AUWC025 is equivalent to AUFWc025.

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INTRODUCTION

INTRODUCTION

In Indian systems of medicine, a large number of drugs of either herbal or mineral originhave been advocated for various types of diseases and other different unwanted conditions in humans (Brekhman and Dardimov, 1969). Ayurvedic medicines are largely based upon herbal and herbomineral preparations and have specific diagnostics and therapeutic principles (Patwardhan and Hopper, 1992). WHO estimated that 80% of the population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs. The developed nations are also looking for eco-friendly treatment of various diseases through plant based source (Kumar *et al*., 2011)

Medicinal plants play a vital role for the development of new drugs. Currently 80% of the world population depends on plant derived medicine for the first line of primary health care for human alleviation because it has no side effects (Mathur *et al.*, 2011).Medicinal plants are of great interest to the researchers in the field of biotechnology as most of the drug industries depend in part of plants for the production of pharmaceutical compounds (Chand *et al.,* 1997).

There are several plants used in Ayurvedic medicinal systems origin with potential therapeutic activity, which are widely used as Ayurvedic medicine (Puloke*t al*., 2011).The genus Withania is an important member of the family Solanaceae. Twenty three species of the Withania have been reported (Negi *et al*., 2006). *W. coagulans* is a small genus of shrub, which is distributed in the East of the Mediterranean region and extend to South Asia. In Punjab, the fruits of *W. coagulans* are used as the source of coagulating enzyme for clotting the milk which is called 'paneer'.*W.coagulans* commonly called Vegetable rennet,Paneer-Bandh, or Indian Cheese Maker and ishighly valued for its multiple medicinal properties (Mathur *et al.*, 2011).The dried seeds of *W.coagulans* Dunal of Solanaceae family, play a major role in indigenous system of medicine for the treatment of ulcers, dyspepsia, rheumatism, dropsy, *etc*. (Rasheed *et al*., 2012). *W.coagulans* (Stocks) Dunal is used to treat nervous exhaustion, disability, insomnia, wasting diseases, failure to thrive in children, impotence. Its fruits are used for liver complaints, asthma and biliousness. Antimicrobial, anti- inflammatory, antiumor, hepatoprotective, anti-hyperglycemic, cardiovascular, immuno-suppressive, free radical scavenging and central nervous system depressant activities of the plant have been reported (Mathur *et al.*, 2011).

These activities have been attributed to withanolides that are present in the plant (Rahman *et al.*, 2002).*W. coagulans* has received much attention in recent years due to the presence of a large number of steroidal alkaloids and lactones known as withanolides (Hemalatha *et al*., 2008). One of the most important withanolides is withaferin A, which is isolated from *Withania* extracts has anticancer properties (Yang *et al.*, 2007). Withanolides have also been reported to inhibit metastatis and quinine reductase activity (Leon and Kuttan, 2004). Some of them have been show to preferentially affect events in the cholinergic signal transduction cascade of the cortical and the basal forebrain, indicating their promise for the treatment of Alzheimer's disease (Kuboyama *et al*., 2005). Some studies have demonstrated that Withaferin-A has potent anti-inflammatory, anti-oxidant and antitumor properties (Yang *et al*., 2007; Bargagna *et al*., 2007). Several properties of Withaferin A have been reported: antiangiogenesis through NF- κB inhibition (Yokota *et al*., 2006); cytoskeletal architecture alteration by covalently binding annexin II (Falsey *et al*., 2006) and apoptosis induction through the protein kinase C pathway in leishmanial cells (Sen *et al*., 2007). Withanolides are naturally occurring steroids built on ergostane skeleton, in which C-22 and C-26 are appropriately oxidized to form a d-lactone ring. Withaferin A has antibacterial, anti-tumor, anti-inflammatory and immunomodulatory properties (Veeresham *et al*., 2006).

Plant tissue culture can be a potential source for important secondary metabolites such as pharmaceuticals and food additives. (Abouzid *et al*., 2010). Tissue cultures having different morphology analyzed for withanolide production showed that the inherent biosynthetic capability of the donor plant was retained in cultures and they produced withanolides invitro (Sharadha *et al.*, 2007).*In vitro* cultures is more advantageous over a single shoot formation for rapid clonal multiplication as well as for its conservation (Saritha and Naidu, 2007). Plant tissue culture techniques are extensively used for mass production of elite plants as well as to study the basic aspects of primary and secondary metabolism, morphogenesis and genetic engineering etc (Rajendra and D'Souza, 1999). Micropropagation is used to grow large numbers of plants, *in vitro*, in an aseptic and closely controlled environment that are genetically identical to a parent plant, as well as to one another. This will help in the mass multiplication of genetically superior species and also in selection and propagation of cultivars that are rich in withanolides. A micropropagation system through leaf explants culture has been developed for Withania coagulans (Jain *et al*., 2011). The root extract of this species has recently been accepted as

a dietary supplement in the United States. Harvesting roots is destructive for the plants and hence there is a growing interest in root culture as an alternative source for this important metabolite. Root cultures are typical examples that can be used for production of phytochemicals. Root cultures have been used as standard experimental system in studies of inorganic nutrition, nitrogen metabolism, plant growth regulation, and root development (Abouzid *et al.*,2010). The root cultures synthesized withanolides of which withaferin A was the major compound.

High Performance Liquid Chromatography gives both qualitative and quantitative measure of the withanolides present in a sample. Any novel secondary metabolites present can also be easily identified.

The main objectives of the present study are to establish protocol and analyse

- *In vitro* germination oftwo *Withania coagulans* cutivar AUFWc008 and AUFWc025.
- To identify the optimum concentration of growth hormones for shoot multiplication and root induction in both cultivar of *Withania coagulans.*
- To develop a HPTLC finger print for *in vitro* leaves of AUFWc008 and AUFWc025.
- To perform quantitative estimation of selected phytochemicals present in both cultivar *Withania coagulans.*

REVIEW OF LITERATURE

2.0 REVIEW OF LITERATURE

Ayurveda is the science of life and it aims at maintenance of health using holistic approach. Plants have a long therapeutic history over thousands of years and still considered to be promising source of medicine in the traditional health care system. The efficacy and safety of herbal medicine have turned the major pharmaceutical population towards medicinal plant's research (Hemalatha *et al*., 2008). They play a dominant role in the discovery of new therapeutics. They have always been a rich source of lead compounds e.g. morphine, cocaine, digitalis, quinine etc. Pharmacological screening of natural products has led to the discovery of a number of drugs. Different civilizations have developed their own indigenous system of medicines (Maurya*et al*., 2010). Medicinal plants as a functional group consist of the largest single group of plants with an approximate 30,000 species worldwide, covering over 5,000 genera and more than 1,000 families and sub-families.Around 8,000 species of plants are used by the people of India in primary health care for human, veterinary and agricultural applications (Singhal, 2005). A survey of the literature has shown that in various traditional systems of medicine *Withania coagulans* has been recommended for the treatment of various disorders (Maurya*et al.*, 2010).

In vitro micro propagation technology has sound and extensive potential for commercial rapid multiplication of plants because it is a quick method, allows round the year propagation of identical plants, and produces plants free from diseases (Kumar *et al*., 2007).

The literature related to the work **"Comparative evaluation of *invitro* growth characteristic and secondary metabolite accumulation in two cultivars of *Withania coagulans*"** was surveyed extensively and is presented in this chapter.

2.1*Withania coagulans*

2.2 Traditional medicinal properties of *Withania coagulans*

2.3 Secondary metabolites of *Withania coagulans*

2.4 *Invitro* studies on *Withania coagulans*

2.5 Analysis of withanolides

2.1 *Withania coagulans*

W. coagulans Dunal (synonym: Puneeria coagulans Stocks), commonly known asIndian rennet, Indian cheese maker, vegetable rennet (English), Panir ke phool, Panir band,Paneer bandh, Punir dodi (Hindi), Ning gu shui qie (Chinese), is a small genus of shrubs.It belongs to family Solanaceae (Maurya*et al*., 2010).

***Withania coagulans* plant**

2.1.1 Taxonomical classification

Kingdom: Plantae, Plants;

Subkingdom: Tracheobionta, Vascular plants;

Super division: Spermatophyta, Seeds plants;

Division: Angiosperms;

Class: Dicotyledons;

Order: Tubiflorae;

Family: Solanaceae;

Genus: *Withania;*

Species: *Withania coagulans*

(Maurya *et al.,* 2010).

2.1.2 Distribution

This shrub is common in East India, Nepal and Afghanistan, distributed in the East of the Mediterranean region and extend to South Asia (Maurya*et al*., 2010).The plant is native of the Asia-temperate (Western Asia: Afghanistan) andAsia-tropical (Indian Subcontinent: India, Nepal) regions. In India it occurs in drier parts of Punjab, Rajasthan, Simla, Kumaun and Garhwal (Gupta and Keshari ., 2013)

2.1.3 Morphological characterestics

Leaves are 1-2.2 cm., lanceolate-oblong, obtuse, entire, clothed with a persistent not easily detachable greyish tomentum, of a uniform colour on both sides, thick, more or less rugose, base acute, running down into an often obscure petiole; petiole 6 mm long. Flowers *of Withania coagulans* are dioecious, in axillary clusters; pedicles 0-6mm long, deflexed, slender. Fruits are 6-8 mm in diameter, globose, smooth, closely girt by the enlarged membranous calyx which is scurfy pubescent outside (Gupta and Keshari*et al*., 2013). The seeds of the plant are dark brown, ear-shaped and globrous and 2.5-3mm diameter. Berries are globose, red or brownish in colour, smooth and are enclosed in a leathery calyx (Naz *et al*., 2009).

2.2 Traditional medicinal properties of *Withania coagulans*

Withania coagulans (Paneer Doda) has been used since time immemorial in Indian Ayurvedic medicines (Mishra *et al.*, 2013). Different parts of this plant have been reported to possess a variety of biological activities(Gupta and Keshari, 2013). The fruit, leaves and root of *Withania coagulans* have been used as a treatment in many disorders (Mishra *et al*., 2013).

2.2.1 Fruits

The fruits of the plant are sweet and are reported to be sedative, emetic, alterative diuretic and it is gaining popularity as a antidiabetic (Hemalatha *et al*., 2008). In Northern India traditional healers use dry fruits of *Withania coagulans* for the treatment of diabetic patients (Gupta and Keshari, 2013). In Bombay, the berries have a reputation as blood purifiers. In Las Bella, the fruit is pounded and used as a cure for colicin. It also has antimicrobial, anthelmintic, antifungal, hepatoprotective, hypoglycemic, hypolipidemic,

cardiovascular, free radical scavenging, anti-inflammatory, antitumor, immunosuppressive, depressant property (Gupta *and* Keshari,2013).They are useful in chronic complaints of liver. The fruits are also used in dyspepsia, flatulent colic and other intestinal infections. They are employed for the treatment of asthma, biliousness and stranguary (Rahman *et al*., 2002). They are prescribed in infusion, either alone or conjoined with the leaves and twigs of *W.coagulans,* an excellent bitter tonic (Gupta and Keshari 2013).

The fruit is applied to wound. The round capsular fruit is used in the fresh state as an emetic and when dried it is used as a stomachic (Bakhthavar *et al.,* 2010). The volatile oil obtained from fruits of *W.coagulans* had antibacterial activity against *S. aureus* and *Vibrio cholera* and also found to have antihelminthic activity (Mishra *et al*., 2013).

***Withania coagulans* fruits**

The milk coagulating properties of the fruit is attributed to the pulp and husk of berries which possess an enzyme with coagulating activity. The aqueous fruit extract of *W. coagulans* has hypotensive, respiratory stimulant and smooth muscle relaxant activity in experimental animals. Oral administration of this extract (1.0 g/kg) was earlier reported to lower the blood sugar,serum cholesterol, serum LPO and hepatic LPO levels in normalas well as streptozotocin-induced diabetic rats (Maurya *et al*., 2010). The antimutagenic activity of *W. coagulans* fruit extracts was investigated on cyclophosphamide induced micronucleus formation in mouse bone marrow cells (Jain *et al*., 2012). The extract of *W. coagulans* fruit showed good antioxidant property in Fenton reaction model, the test drug(s) were compared with a low concentration of ascorbic acid (Mathur *et al*., 2011).

The aqueous extract and 3-b-hydroxy- 2, 3-dihydrowithanolide F of fruits of this plant has been shown to exert hepatoprotective activity against CCl4 induced hepatotoxicity in adult albino rats.Using the aqueous extract of *W. coagulans* fruits in experimental rats have a diuretic potential. The same hot aqueous extract of*W. coagulans* fruits has also increased the glucose utilizationin isolated rat hemidiaphragm cells (Jaiswal*et a*l., 2009). The aqueous extract of fruits of *W. coagulans* (1 g/kg; p.o.) showed 15% reduction in serum cholesterol level in Tritoninducedhyperlipidaemic rats in comparison with untreated animals. In rats with a high fat diet-induced hyperlipidaemia, the aqueous extract at the same dose administered for seven weeks showed a significantly reduced body weight, elevated serum cholesterol, triglycerides and lipoprotein levels. The animals treated with aqueous extract of fruits of *W. coagulans* and the reference drug Navaka guggulu showed less degenerative changes along with microvesicular fatty changes. The total extract of *W. coagulans* fruit has been reported tohave central nervous system (CNS) depressant activity inmice, rabbits and dogs. The extract was hypotensive inanimals and had respiratory stimulant and smooth musclerelaxant activity (Maurya *et al.*, 2010).

Recent investigation has shown that withanolides isolated from the aqueous extract of fruits possessed a good antihyperglycemic and antidyslipidemic activity (Prasad*et al*., 2010).Cardiovascular effects of withanolide isolated from *W. coagulans* fruits have been reported (Maurya *et al*., 2010).

The berries of the shrub are used for milk coagulation (Rahman *et al*., 2002). In some parts of the sub-continent, the berries are used as a blood purifier. In the Ormera Hills, the smoke is applied to aching teeth ‘to destroy the worm’. The twigs are chewed for cleaning teeth, and the smoke of the plant is inhaled for relief in toothache (Gupta *et al*., 2012). It is well known in the indigenous system of medicine for the treatment of ulcers, rheumatism, dropsy, consumption and sensile debility (Hemalatha *et al*., 2008). The hydro alcoholic extract of the berries of W. coagulans showed significant anti-inflammatory activity in carragenin induce rat paw oedemamodel (Rajurkar *et al*., 2001). The seeds are emmenagogue, diuretic; useful in lumbago, opthalmia; lessen the inflammation of piles (Gupta and Keshari, 2013)

2.2.2 Leaves

The leaves are used in Pakistan as vegetable, and as fodder for camel and sheep (Hemalatha *et al*., 2008). Honigberger says that the bitter leaves are given as febrifuge by the Luhanees (Gupta and Keshari*et al.*, 2013). Leaves of this plant are used as a coagulant and alterative (Gupta, 2012). They are also useful in lumbago, ophthalamia, and lessen the inflammation of piles (Hemalatha *et al*., 2008) They have been reported to inhibit the cell growth of various human cancer cell lines, including lung cancer (NCI-H460). Withaferin A from the leaves, are known to posses anti-cancer properties (Jayaprakasam *et al*., 2003) and also shows antiproliferative activity against head and neck squamous carcinoma, by reduced cell viability in cell lines *in vitro*. Also antibacterial properties have been demonstrated for isolated withanolides from ethanolic extract of the leaves (Khodaei *et al*., 2012).Antifungal properties have also been demonstrated in the withanolides isolated from ethanolic extract of the leaves (Maurya *et al*., 2010).

2.2.3 Roots

Roots of *Withania coagulans* have been used as a treatment in many disorders. It is used in treating burns and infectious wounds, arthritis, inflammation and rheumatism. The root is harvested in autumn and dried for later use. Antimicrobial, anti-inflammatory, hepatoprotective, anti-hyperglycemic, cardiovascular, immunosuppressive, free radical scavenging and central nervous system depressant activities of the plant have been reported (Mishra *et al*., 2013). In folk medicine, extracts of roots are used to treat gastroenteritis, vomiting, diarrhoea, dysentery, wounds, ulcers, toothache, coughs, sore throat, and inflamed gums.(Barad *et al.*, 2013)

The alcoholic, chloroform, and ethyl acetate extracts of *W. coagulans* roots can protect the morphine withdrawal syndrome in Albino mice. It shows the significant suppression in morphine induce withdrawal jump, induced by naloxone and decreases development of morphine dependence (Mohammad *et al*., 2012). The extract of *W. coagulans* roots exhibited hepato-protective activity against carbon tetrachloride (CCl4)-induced hepatotoxicity in adult albino rats of either sex due to the presence of 3-β-hydroxy-2, 3-dihydro-withanolide F (Jain *et al*., 2012).Treatment of root extract on induced skin cancer in mice exhibited significant decrease in the incidence and average number of skin lesions. In another study, the aqueous suspension of root powder inhibited

the mutagen induced lymphocyte proliferation and DTH reaction in rats. The root extract also enhanced total white blood cell count, inhibited delayed-type hypersensitivity reactions and enhanced phagocyte activity of macrophages (Barad *et al.*, 2013).

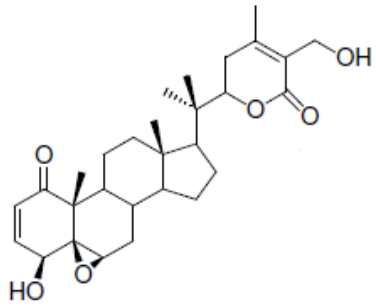
The root extract of this species has recently been accepted as a dietary supplement in the United States (Abouzid *et al.*, 2010).

2.3 Secondary metabolites of *Withania coagulans.*

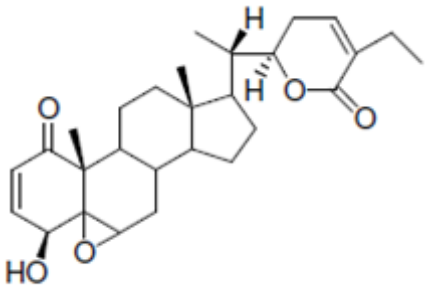
Secondary metabolites, such as alkaloids, terpenoids, flavonoids, and a host of glycosides, mediate the relation between plants and their environment (Kurz and Constabel, 2000). Preliminary Phytochemical study of 50% ethanolic extract of the root parts of *Withania coagulans* was found to contain carbohydrates, protein, some steroids, anthraquinone, flavonoids, tannin, phenolic compounds and triterpenoids.

W.coagulans berries contain the milk-coagulating enzyme, two esterases, free amino acids,fatty oil, an essential oil and alkaloids. The amino acids present are proline, hydroxyproline, valine, tyrosine,aspartic acid, glycine asparagin, cysteine and glutamic acid. Fourteen alkaloidal fractions have been isolatedfrom the alcoholic extract of the fruits. The seeds on petroleum ether extraction, give a yellow fatty oil and unsaponifiable matter. Fatty acid compositions are oleic, linoleic, palmitic, stearic and arachidonic acid. Theunsaponifiable matter consists of triacontain, three sterols including dihydrostigmasterol and β-sitosterol. Thedefatted meal from the seeds contains free sugar consisting of D-galactose and D-arabinose and traces ofmaltose. The leaves contain four steroidal lactones called Withanolides, viz Withaferin-A, 5, 20α(R)- dihydroxy-6α,7α-epoxy-1-oxo-(5α)-with a-2,24-dienolide and two minor withanolides, of which one is probably 5, 17-dihydroxy-1-oxo-6, 7-epoxy-22R-witha-2,24-dienolide the so called withanone (Gupta and Keshari, 2013).

Phytochemical investigation on plant *Withania coagulans* reported number of constituents. The withanolides, withacoagin, coagulan and withasomidienone have been isolated from plant along with other withanolides and withaferin (Hemalatha *et al.*, 2008).



Withaferin



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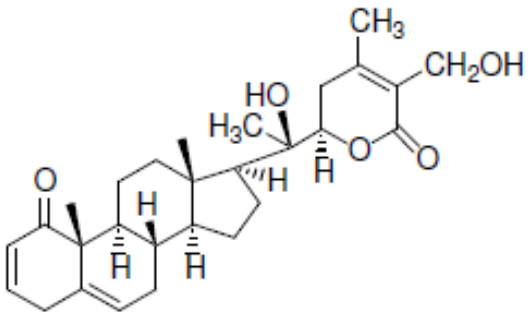

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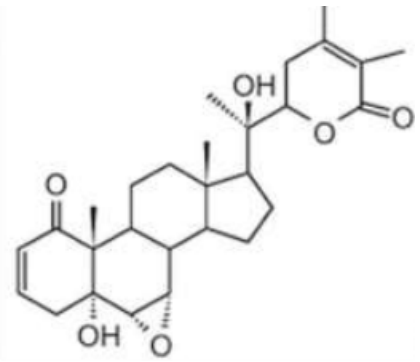


Withanolide

2.3.1 Specific activity of secondary metabolites

Withania coagulans is rich in steroidal lactones, which are known as **Withanolides**. Withanolides are naturally occurring polyhydroxy C28 steroidal lactones and several of them possess significant pharmacological activities. In the basic structure of all withanolides a six- or five-membered lactone or lactol ring is attached to an intact or rearranged ergostane skeleton (Maurya *et al.*, 2010)

A) Antimicrobial activity

Studies have shown that Withaferin A at a concentration of 10ml inhibited the growth of various gram-positive bacteria, acid fast bacilli, aerobic bacilli and pathogenic fungi. It was active against *Micrococcus pyogenes,aureus* and *Bacillus subtilis* glucose-6-phosphate-dehydrogenase (Gupta and Keshari, 2013).

Withanolides isolated from ethanolic extract of the whole plant showed antifungal properties (Mishra *et al*., 2013). Two withanolides (14,15β -epoxywithanolide I

[(20S,22R) 17β,20β-dihydroxy -14β, 15β-epoxy-1-oxo-witha-3,5,24-trienolide] and 17β-hy-droxywithanolide K (20S,22R) 14α,17β,20β-trihydroxy- 1-oxo-witha-2,5,24-trien-olide]) have been isolated from the whole plant of *W. coagulans*. The second compound was found to be active against a number of potentially pathogenic fungi. The antifungal activity of the crude extract, 17β-hydroxy withanoloied k and withanolide F were tested against nine highly pathogenic isolated fungi i.e. *Nigrospora oryzae*, *Aspergillus niger*, *Curvularia lanata*, *Pleuretus ostreatus*, *Stachybotrys atra*, *Allescheria boydii*, *Drechslera rostrata*, *Microsporum canis*, and *Epidermo-phyton floccosum*. These compounds also showed activity against gram positive (*S. aureus*) (Khodaei *et al.*, 2012).

B) Anticancer properties

Withaferin A is the most important of the withanolides isolated so far. Withaferin A has marked tumor inhibitory property when studied *in vitro* against cells derived from human carcinoma of nasopharynx (KB). It also acts as mitotic poison arresting the division of cultured human larynx carcinoma cells at metaphase. Withanolide A is well-known for its neuronal regenerating effect. It has good antibiotic and anti tumor activities. The studies also showed growth inhibitory and radio sensitizing effects *in vivo* on mouse Ehrlich ascites carcinoma. It also caused mitotic arrest in embryonal chicken fibroblast cells (Gupta and Keshari, 2013).

Phytochemical studies on Withaferin A have shown cytotoxity in vitro, against KB cell cultures derived of intra-muscular carcinoma. Withaferin A showed significant anticancer activity in animals cell cultures by decreasing the expression of the nuclear factor-kappa β and suppressing the intercellular tumor necrosis factor, therefore has the potential of apoptotic signaling in cancerous cell lines. Withaferin A showed inhibition growth and cytotoxic activity against human lung cancer cell lines (NCI-H460) (Choudhary *et al*., 2010).

C) Anti-inflammatory activity

Inflammation is a complex process occurring through a variety of mechanisms, leading to changes of local blood flow and the release of several mediators (Khodaei *et al*., 2012) Lalsare and Chutervedi, (2010) reported that various extracts of *Withania coagulance* fruits have anti- inflammatory activities. The aqueous extract of the plant have shown an anti-inflammatory activity in various rodent models (Mishra *et al*., 2013).

The alcoholic extract and total alkaloids showed a significant anti-inflammatory effect in acute inflammation induced with egg albumin. 3-β-Hydroxy-2, 3-dihydrowithanolide F exhibited a significant anti-inflammatory activity at 10 mg/kg in sub acute models of inflammation such as granuloma formation and formalin-induced arthritis in rats (Maurya *et al*., 2010)**.**

D) Cardiovascular effects

A new withanolide, with a unique chemical structure similar to the aglycones of the cardiac glycosides (mol. wt. 488 6, m. p. 260-261 degrees), was isolated from the fruits of *W. coagulans*, and was screened for cardiovascular effects. At a dose of 5 mg/kg body weight, the withanolide produced a moderate fall of blood pressure in dogs (34 ± 2.1, mm Hg) which was blocked by atropine and not by mepyramine or propranolol. In rabbit Langendorff preparation and ECG studies, it produced myocardial depressant effects; but in perfused frog's heart, it produced mild positive inotropic and chronotropic effects. Extracted coagulin L from *W. coagulans* fruits also showed significant drop of a fasting blood glucose profile and improved the glucose tolerance in mice (Maurya *et al* ., 2010).

E) Hepatoprotective activity

Protective effect of 3β-hydroxy-2,3-dihydro withanolide F isolated from *Withania coagulans* was tested against CCl4- induced hepatotoxicity, and the compound was found to possess marked protective effect. A comparison of the protective properties showed that it is more active than hydrocortisone on a weight basis (Gupta and Keshari, 2013). Coagulin L from *W. coagulans* fruits was reported to have antihyperglycemic activity in rats (Maurya *et al*., 2010).

The aqueous extract of fruits of this plant has been shown to exert hepatoprotective activity. Since the steroidal compounds (glucocorticoids) having anti-inflammatory properties are used in some hepatic disorders, 3-b-hydroxy-2,3 dihydrowithanolide F has been screened for its hepatoprotective effect. It has shown hepatoprotective activity against CCl4-induced hepatotoxicity in adult albino rats of either sex (150–200 g) at 10 mg/kg (Maurya *et al*., 2010).

F) ImmunosuppressiveEffects

Withaferin A and withanolide E were reported to have specific immunosuppressive effects on human B and T lymphocytes as well as on mice thymocytes. A known withanolide, coagulin-H, was evaluated for its effect on various cellular functions related to immune responses including lymphocyte proliferation, interleukin-2 (IL-2) cytokine expression. These results were compared with prednisolone. Coagulin H also significantly inhibited IL-2 production by 80%. Docking studies predicted that coagulin H bound to the receptor binding site of IL-2 more effectively than prednisolone. Based on the computational and the experimental results, coagulin H was identified as a potential immunosuppressive candidate (Rahman *et al*., 2002).

G) Antihyperlipidemic activity

The aqueous extract of *W.coagulans* fruits in high fat diet induced hyperlipidemic rats, significantly reduced elevated serum cholesterol, triglycerides, lipoprotein and the LPO levels. The hypolipidemic effect of *W.coagulans* fruits were found to be comparable with ayurvedic product containing Commiphora mukkul (Hemalatha *et al.,* 2008). The coagulanolide isolated from fruits of Withania coagulans has antidyslipidemic effect on mice (Maurya *et al.,* 2010).

Treatment with coagulanolidealong with four knownwithanolides 1-3 and 5 isolated from *Withania coagulans* fruits,showed significant inhibition on postprandial rise in hyperglycemia post-sucrose load in normoglycemic ratsas well as streptozotocin-induced diabetic rats. The hydroalcoholic extract of *Withania coagulans* dried fruits was effective and comparable to atorvastatin in controlling the high cholesterol diet-induced hyperlipidemia in rats. The compound also caused significant fall in fasting blood glucose profile and improved the glucose tolerance of db/db mice (Gupta., 2012)

H) Wound healing activity

The hydroalcoholic fraction of the methanolic extract of *Withania coagulans* was administered in the form of 10% w/w ointment topically and at a dose of 500 mg/kg body weight orally to streptozotocin-induced diabetic rats. The hydroalcoholic fraction in both the forms, i.e., topical (10% w/w ointment) and oral (500 mg/kg body weight, p.o.)

showed a significant increase in the rate of wound contraction compared to diabetic controls (Gupta, 2012).

2.4 *In vitro*studies on *Withania coagulans*

The *in vitro* shoot cultures could provide an alternative to field grown plants harvested for the production of therapeutically valuable compounds (Jain*et al.,* 2011). Production of secondary metabolites in tissue cultures is usually higher when plant cells are organized into tissues/organs (Abouzid *et al*., 2010). Tissue cultures having different morphology analyzed for withanolide production showed that, the inherent biosynthetic capability of the donor plant was retained in cultures and they produced withanolides *in vitro*. *In vitro* culture is more advantageous over a single shoot formation for rapid clonal multiplication as well as for its conservation (Valizadeh *et al.,* 2009).

Natural propagation of *W. coagulans* is through seeds, is very slow and unreliable, also poor due to environmental constrains. The plant species is threatened in its habitat and has been declared critically endangered (Jain *et al*., 2011). Micropropagation has proved to be more advantages over conventional methods of vegetative propagation

In vitro mass-multiplication and conservation strategies have several benefits and they are :

- Medicinal plants can be quickly cultivated.
- Continuous supply of plant materials from elite germplasm lines, which can make significant contributions to the exploitation of therapeutic properties of the plant species.
- Eliminate the need forharvest from the wild.
- The in vitro-propagated medicinal plants furnish a ready source of uniform, sterile, and compatible plant material for mass-multiplication and germplasm conservation of rare, endangered, and threatened medicinal plants.
- The multiplication rate is greatly increased.

Thus, these could provide alternatives to field plant harvesting for the production of therapeutically valuable compounds. Recently there is an increased interest in using in vitro techniques for medicinal plant regeneration using different kinds of explants because

these are faster for ex situ multiplication and use small amounts of tissues, and may succeed when other methods fail (Rathore *et al*., 2013).

The study ofValizadeh and Valizadeh, (2011)highlights a complete micropropagation protocol for *W.coagulans* through adventitious shoot multiplication. *In vitro* response of *W. coagulans was* varying depending on explants. The comparison of callusing potential of different explants shown that leaf explants is best one, as its callusing capacity was 100%, but no shoots could be regenerated from this source of callus and callus were turned brown after three sub cultures. Valizadeh and Valizadeh, (2009).

Shoot bud differentiation, elongation of microshoots and induction of roots of *Withania coagulans* varied in different PGRs combinations and concentrations. Plant hormones are necessary for shooting, elongation and rooting (Debnath, 2008). The effects of auxins and cytokinins on shoot multiplication of various medicinal plants were reported by Rout *et al.,* (2000). Adventitious shoot regeneration of *W. coagulans* was reported earlier by Jain *et al*. (2011). They showed that multiple adventitious shoot bud differentiation occurred on medium fortified with 2.3 μM kinetin (Kn) and higher levels of BA (22.2 μM). Valizadeh and Valizadeh, (2011)investigation showed that the highest number of shoots was observed in MS medium supplemented with 2.0 mgl-1 BA and 0.5 mgl-1 IBA.

2.4.1Seed germination

Seeds of *W. coagulans* showed a good ratio of germination (50%) on full strength MS agar media after treatment with conc. H2SO4 for 2 min. The roots of the sterile plantlets showed slowed growth when cultured in MS liquid media without growth hormones. The growth hormone IBA (0.25 mgL-1) was added to MS media to promote growth of the *in vitro* cultured roots. The growth was in the form of compact mass from which lateral roots were protruding (Abouzid *et al*., 2009)

A recent report of Rathore *et al.,* (2013) states that exactly 95.5 ± 0.34% explants responded within 8–10 days (d) and produced multiple shoot buds (4.1 ± 0.10 shoots of 2.95 ± 0.15 cm length) on 0.8% agar-gelled Murashige and Skoog's (MS) basal medium supplemented with 8.88 μM 6-benzylaminopurine (BAP), 0.57 μM indole-3-acetic acid (IAA), and additives (100 mg L−1 L-ascorbic acid, 25 mg L−1 each citric acid, adenine sulphate and L-arginine). The shoots in cultures were multiplied by repeated transfer on

MS medium with 4.44 μM BAP, 0.57 μM IAA, and additives. Maximum number (19.1 ± 0.28) of healthy (6.15 ± 0.25 cm) and viable shoots differentiated on this medium.

2.4.2 Callus induction

Valizadeh *et al*., (2009) have reported that leaf and internode explants of *Withania coagulans* Dunal were used to evaluate the effect of growth regulators on the *in vitro* callus induction and plant regeneration. In cultures with leaf explants highest callus growth in terms of dry weight (76±5.34 mg) was reported in MS medium fortified with 2 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} Kin. Best callusing rates (42%) and dry weight (86±3.68 mg) occurred in the presence of 4 mg L^{-1} 2, 4-D with combination of 0.25 mg L^{-1} BA from internodal explants. Shoot induction was obtained just from callus induced from internode explants on MS medium containing BA (2 mg L^{-1}) with IBA (0.5 mg L^{-1}), but the yield of shoot regeneration was unsatisfactory (18-33%). Shoot bud proliferation occurred through both adventitious and de novo routes depending on the hormonal regime of the culture medium.

The work of Jain *et al., (*2011) reported that green compact nodular organogenic callus developed on Murashige and Skoog (MS) medium supplemented with 2.3 lM kinetin (Kn) and lower levels of 6–benzyladenine (BA) (13.3 lM) while multiple adventitious shoot bud differentiation occurred on medium fortified with 2.3 lM kinetin (Kin) and higher levels of BA (22.2 lM). Shoot buds were transferred to proliferation medium containing 2.2 lM BA, 2.3 lM Kin, and 3.9 lM phloroglucinol (PG) for further growth and development of shoot system

2.5 Analysis of withanolides

Extraction is an important step in the itinerary of phytochemical processing for the discovery of bioactive constituents from plant materials. Selection of a suitable extraction technique is also important for the standardization of herbal products as it is utilized in the removal of desirable soluble constituents, leaving out those not required with the aid of the solvents. Further, selection of suitable extraction process and optimization of various parameters are critical for up scaling purposes i.e. from bench scale to pilot plant level.

Rajasekar *et al.,* (2011) reported that for preparation of methanol and aqueous extracts from root, stem and leaves conventional methods like sonication, refluxation, percolation and counter current extractions are used.

The study by Dhanani *et al*., (2013) study extraction was done with water and water-alcohol as solvent using UASE and MASE since in most of the herbal preparations water or water-alcohol is used as a solvent. Conventional extraction using refluxing was also carried out for the comparison of extract yield and phytochemical qualities of the extract.

The plant is increasingly become popular in ayurvedic medicines due to the demand of its phytochemicals. HPTLC method has been used for the determination of phytochemicals in *Withania coagulans* (Shetty *et al.,* 2012). Withaferin A was estimated in herbal extract and polyherbal formulations by high performance thin layer chromatography (HPTLC). As there is no official HPTLC protocol for quantitation of the phytochemicals, an attempt was made to quantify withaferine A in herbal extract and polyherbal formulations produced from *W. somnifera.* Precoated silica gel G (aluminium backed) plates were used as stationary phase and toluene:ethyl acetate: formic acid (50:15: 5) was used as mobile phase. Detection and quantification were performed by densitometry at k 213 nm. The linear range was 11g - 3lg. This HPTLC method was found to be reproducible, accurate, and precise (Mahadevan *et al.,* 2003).

The HPTLC method for routine quality control of present species can be carried out using this method for different extracts of plant parts and serve in qualitative, quantitative and was appropriate for standardization of the drug. The HPTLC fingerprint is also suitable for rapid and simple authentication and comparison of subtle differences among samples of identical plant resource. HPTLC is more efficient, faster method and the results are more reliable and reproducible. In combination with digital scanning profiling, HPTLC also provides accurate and precise *R*f values and quantitative analysis of sample by in situ scanning densitometry aided by formation of easily detected derivatives by post-chromatographic chemical reactions as required, as well as a record of the separation in the form of a chromatogram with fractions represented as peaks with defined parameters including absorbance (intensity), *R*f, height and area . Furthermore, the feature of a pictorial fluorescence image of HPTLC coupled with a digital scanning profile is more and

more attractive to herbal analysts for constructing a herbal chromatographic fingerprint by means of HPTLC (Kamboj *et al.,* 2011).

HPTLC of *Withania somnifera* methanolic extract was performed on Si 60 F_{254} (20s cm × 20 cm) plates with toluene:ethyl acetate:formic acid (5:5:1), as mobile phase. It showed about 96.0 and 96.7%, average recovery of withaferin-A and withanolide-A respectively. The calibration curves were linear for both in the range of 200–3,200 ng. The technique has been applied, for the first time, for the estimation of withaferin-A and withanolide-A in different parts of the two morphotypes of *Withania somnifera*. The method is simple, precise, specific, sensitive and accurate and can be used for routine analysis as well as for quality control of raw materials and herbal formulations (Sharma *et al.,* 2007).

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

The various materials and experimental procedures employed in the study **"Comparative evaluation of *in vitro* growth characteristic and secondary metabolite accumulation in two cultivars of *Withania coagulans*"** are described under the following headings

3.1 Plant material

3.2 Chemicals

3.3 Plant tissue culture media

3.4 Media preparation and sterilization

3.5 *Invitro* propagation of *Withania coagulans* seeds

3.5.1 Germination of *Withania coagulans* seeds

(AUF Wc 008 and AUF Wc 025).

3.5.2 Multiple shoots induction

3.6 Adventitious root induction

3.7Analysis of secondary metabolites in leaf samples

3.7.1 Extraction of secondary metabolites

3.7.2 Quantitativeestimation of selected photochemicals

3.7.3 High performance thin layer liquid chromatography analysis of secondary metabolites

3.1 Plant materials

Surface sterilized seeds of *Withania coagulans* were germinated *in vitro* and seedlings were maintained on MS basal medium with regular sub culturing. The seeds were obtained from Banaras Hindu University, Varanasi. Leaves excised from two months old aseptic plantlets maintained *invitro* were used as explants.

3.2 Chemicals and equipments

Chemicals used for this study were purchased from HiMedia unless otherwise mentioned. Elix – 3water was used for the entire work. *W.coagulans* leaves and *roots* were extracted individually using solvents and the extracted samples were analyzed for the presence of secondary metabolites. The solvents used were Chloroform (Rankem), Ethyl acetate (Rankem), HPLC grade Methanol (Rankem), Formic acid (Qualigen). Standard withanolides for HPTLC were purchased from (Chromodex). HPTLC was performed on precoated Silica gel aluminum60 F254 plates (E.MERCK, Germany) in a Semiautomatic CAMAG Linomat5 device. Quantification was carried out using Spectrophotometer andcolorimeter (ELICO CL63).

3.3 Plant tissue culture media

Full strength and half strength of MS media (Murashige and Skoog, 1962) were used for all the plant tissue culture experiments. The various growth regulators supplemented to standard medium for the study is indicated in the **table 3.1.** The composition of stock solution is given in **Appendix 1.**

Table 3.1 Media used for the study

S.No	Treatments	Hormone supplementation mg/ml	
A. Multiple shoot induction (mg/ml)			
		KIN	**BAP**
1.	T0	0	0
2.	T1	0.5	0
3.	T2	0	0.2

4.	T4	0	0.5
5.	T5	0.5	0.5
6.	T6	1.0	0.5
7.	T7	0.25	0
8.	T8	1.0	0.25
B. Adventitious root induction (mg/ml)			
9.	R0	IBA	IAA
10.	R1	0.25	0
11.	R2	0.5	0
12.	R3	0.5	0.25
13.	R4	1.0	1.0
14.	R5	4.0	1.0
C.Suspension medium for roots			
15**.**	S	½ MS	3% sucrose

3.4 Media preparation and sterilization

The macronutrients, micronutrients, vitamins, potassium iodide, Fe EDTA and myo-inositol were taken from the stock solutions according to the requirement of the culture medium. Sucrose (30 g/l) was added and mixed well. The growth regulators at required concentrations were added. The pH of the media was adjusted to 5.6-5.8 using 0.1N NaOH or 0.1N HCl, agar at a concentration of 8gm/ml was added to the media as a gelling agent and steamed to melt the agar. It was then dispensed in clean culture bottles (30 ml per bottle) and autoclaved at 15 lbs pressure at 121ºC for 20 minutes.

3.5 *In vitro* propagation of *Withania coagulans*

3.5.1 Germination of *Withania coagulans* seeds (AUFWc008, AUFWc025):

The seeds were washed in tap water for about 10 minutes to remove the dust particles. After repeated washes, the seeds were washed twice in distilled water and soaked overnight for imbibition. The water was decanted and the seeds were rinsed in 70% (v/v) ethyl alcohol for 3 minutes, followed by a wash in sterile distilled water. The seeds were placed in 0.1 % mercuric chloride under sterile conditions for 30 seconds with occasional swirling. The seeds were finally washed thrice with sterile distilled water and then inoculated in ½ MS / MS solid basal medium supplemented with 2% sucrose and incubated either in Dark or Light until the seeds germinated. After the appearance of the first leaf, the bottles were placed at 16 hour photoperiod to provide healthy growth conditions. All the *in vitro* experiments were carried out in sterile conditions and in a laminar air flow chamber following method of Valizadeh, J and Valizadeh, M (2009). The growth index was calculated using formula

$$\text{Germination (\%)} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds inoculated}} \times 100$$

3.5.2Multiple shoots induction

The shoot tips from the germinated seedlings were used as the source of explants for multiple shoot induction. The shoot tips were carefully excised and inoculated on MS basal medium supplemented with various concentrations of BAP and Kin (Table 3.1) T0-T8 under sterile conditions. The explants were cultured at a 16 hour photoperiod and a constant temperature of 22°C was maintained throughout the culture period. After a period of 30 days, the number of multiple shoots was counted. The explants were subcultured after a period of 30 days and total number of shoots was counted.

3.6 Adventitious root induction

The healthy leaf explants from the plantlets maintained in MS basal medium were inoculated on MS basal medium supplemented with various concentrations of IBA and IAA (Table 3.1) R0-R5 under sterile conditions. The explants were cultured under a photoperiod of 16 hours at a constant temperature of 22°C for a period of 30 days, and the number of roots was counted.

3.7 Analysis of leaf and root extracts.

A various leaf and root samples were collected from a period of one month. The wet weight and dry weight of each collected sample were noted. The dried samples were then subjected to the extraction and analysis of the secondary metabolites.

3.7.1 Extraction of secondary metabolites

One gram of *in vitro* leaf androot powder of *Withania coagulans* was taken and the evaluation of secondary metabolites was carried out as indicated in the following flow chart following method of Gupta and Rana , 2007.

Leaf + 1ml ammonia

↓

Incubate at room temperature for 20 mins

↓

Add 50 ml methanol + sonicate for 20 mins.

↓ **Keep in shaker 2 hrs at 150-200 rpm**

The extract was filtered off using Whatmann No: 1 filter paper

↓

The residue were allowed to interact with another 50ml of methanol for overnight

↓

The same procedure was followed till the completion of fourthextraction

↓

Methanol fraction

↓ **Flash evaporater/ water bath at 40°C to evaporate methanol**

Dissolved in HPLC grade methanol

↓

Quantification for HPTLC

3.7.2 Quantitative estimation of selected phytochemical:

The methanolic leaf extract was used to estimate the phytochemicals following the procedures given in the previous studies. Phytochemical estimation given below

S.No	Phytochemical	Methanolic leaf extract (ml)	Reference	Appendix
1.	Flavanoids	0.1	Cameron *et al* ., (1943)	2
2.	Saponins	0.2	Buccou *et al*.,(1977)	3
3.	Alkaloids	0.5	Muthumani *et al.,* (2010)	4
4.	Phenols	0.5	Kumbhare *et al*., (2012)	5
5.	Steroids	0.5	Wall *et al.,* (1952)	6

3.7.3 High Performance Thin Layer chromatographic analysis of secondary metabolites

The High Performance Thin Layer Chromatography analysis was carried out on 20cm x 10cm precoated silica gel aluminum plate 60F254(E.MERCK, Germany). The plates were washed with methanol. The methanolic extract of samples were applied to the plates as 8mm bands, under a stream of nitrogen, by means of a CAMAG (Switzerland) Linomat V semiautomatic sample applicator fitted with a 100µl Hamilton HPTLC syringe. Linear ascending development to a distance of 8cm was carried out on 20x10cm twin trough chamber saturated with 11ml of the mobile phase, Toluene: Ethyl Acetate: Formic acid (5: 5: 1). The optimized chamber saturation time for mobile phase was 30min at room temperature (25ºC±2). TLC plates were dried in a current of air with the help of an air dryer. The banding patterns were visualized in 254nm, 366nm and white light and the Rf values were calculated. Densitometric scanning was performed with Camag TLC scanner III in the reflectance –absorbance mode at 540nm after spraying with Anisaldehyde Sulphuric acid (85mL methanol: 10mL glacial acetic acid: 5mL sulphuric acid: 0.5mL anisaldehyde) and analysed by Win CATS software (1.3.0 Camag) (Jirge*et al*, 2011).

RESULTS AND DISCUSSION

4.0 RESULTS AND DISCUSSION

The present study entitled **"Comparative evaluation of *in vitro* growth characteristic and secondary metabolite accumulation in two cultivars of *Withania coagulans*".** The results obtained during the course of the study are presented and discussed under the following headings

4.1*In vitro* germination of seeds

4.2 Micropropagation

4.1.3 Multiple shoots induction in varying concentrations of BAP and KIN.

4.3 Root induction

4.3.1 Effect of IBA and IAA combination on root induction

4.4 Phytochemical analysis

4.5 Chromatographic analysis

4.1*In vitro* germination of seeds

The natural populations of *W. coagulans* are not sustainable in that they cannot reproduce fast enough in the wild to keep up with the exploitationrate, hence the rate of regeneration and exploitation are not balanced (Rathore *et al.,* 2013). So *in vitro* germination of *Withana cogulans* serves as an alternative to natural propagation.

The seeds of *Withania coagulans* of cultivars AUFWc008 and AUFWc025 were inoculated after surface sterilization. Sterilization protocol followed is similar to that used by Valizadeh and Valizadeh, (2009). Seeds were thoroughly washed in running tap water for 30 minutesand were rinsed in 70% ethyl alcohol for 30 seconds followed by 0.1% mercuric chloride for 30 seconds. Seed were then washed under sterile conditions with sterile distilled water and imbibed over night in sterile distilled water. These seeds were theninoculated in half strength MS basal medium for germination studies.

The cultures were transferred to 16-hour photoperiod after the occurrence of the first leaf. Non-etiolated, healthy germinated seedlings, were observed. In this study it was found that 1/2MS basal medium supplemented with 2% sucrose and culturing in the dark

at 22°C resulted in 80% germination for AUFWc 008 variety and 67% germination for AUFWc025 variety within a period of 20 days and found to be best for the germination. And the germination percentage was higher in the dark when compared to the light. Rate of germination was also higher in 22°C than in room temperature.

Abouzid *et al*., (2010) showed 50% seed germination on full strength MS agar media after treatment with conc. H2SO4 for 2 min. It shows treated seeds with conc. H2SO4 and germinated aseptically on 3% sucrose-agar MS medium.

Kumar *et al* ., 2011in his studies inoculated seeds in glass containers with 50ml of half-strength MS medium for germination. The leaf explants were derived from 30 days old seedlings grown *in vitro.*

4.2 Micropopagation

The *in vitro* elongated shoots of *Withania coagulans* AUFWc008 and AUFWc025 variety were excised and transferred on to MS media fortified with various concentrations of BAP and Kin. MS medium without growth regulators served as control

Plate 4.1 and 4.2 Shows shoot multiplication of AUFWc008 and AUFWc025 under influence of growth harmones BAP and KIN.

Plate 4.1:Shoot multiplication of AUFWc008 under influence of growth harmones BAP and KIN

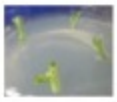
Treatments	0 day	5 days	10 days	15 days	20 days
Control					
T1					
T2					
T3					
T4					
T5					
T6					
T7					

Plate 4.2: Shoot multiplication of AUFWc025under influence of growth harmones BAP and KIN

Treatments	0 day	5 days	10 days	15 days	20 days
Control					
T1					
T2					
T3					
T4					
T5					
T6					
T7					

4.2.3 Multiple shoots induction in varying concentrations of BAP and KIN:

From the **table4.1** and **4.2**it is clear that increasing concentrations of BAP and KIN showed an increasing effect on multiplication of shoots. The effect followed an elevated pattern as the days progressed, maximum shoot multiplication occurred in MS basal media supplemented with 4.44 µM/L BAP and 2.32/L µM/L KIN within 20 days for both cultivars of *Withania coagulans.* An increasing effect was observed till 30 days from period of culture and no significant increase was observed after 30 days. But, BAP or KIN alone shows only less number of shoot multiplication. Combination of both growth regulators in MS basal medium supplemented with 4.44 µM/L BAP and 2.32/L µM/L KINshowed high number of shoot multiplication (5.66±0.52) and (5.33±1.30) for AUFWc008 and AUFWc025 variety respectively. Compared to AUFWc025 cultivar maximum shoot multiplication was observed in AUFWc008 cultivar of *Withania coagulans*. It proves that 4.44 µM/L BAP and 2.32/L µM/L KINas the best media combination best for shoot multiplication of AUFWc 008 cultivar of *Withania coagulans.*

Our results were supported by Jain *et al*., 2011 reported that various concentrations and combinations of plant growth regulators like 6–benzyladenine and kinetin were added in the medium to optimize growth and differentiation. It shows that the combination of BAP and KIN was the most important factor for shoot regeneration from leaf explants of *W. coagulans.* It showed that green compact nodular organogenic callus developed on Murashige and Skoog (MS) medium supplemented with 2.3 µM kinetin (Kn) and lower levels of 6–benzyladenine (BA) (13.3 µM) while multiple adventitious shoot bud differentiation occurred on medium fortified with 2.3µM kinetin (Kn) and higher levels of BA (22.2 µM).

In the study of Rathore *et al*., 2013 the best combination for shoot multiplication was found to be 1.11 µM BAP and 0.57 µM IAA which not only resulted in production of higher shoot numbers (19.1 ± 0.28) but also produced significantly lengthy (6.15 ± 0.25 cm) and viable microshoots. Among different concentrations and combinations, BAP and KIN tested for their efficacy in promoting the rate of shoot multiplication rate, BAP at lower (1.11 µM) concentration along with 0.57 µM IAA, was found to be significantly better than KIN at similar concentrations and its combination with BAP.

Table 4.1: Influence of varying concentrations of BAP and KIN on multiple shoot induction in AUFWc008

S.No	BAP µM/L	KIN µM/L	MEAN NO. OF SHOOTS			
			After 5days	After 10days	After 15days	After 20Days
T0	0	0	0	0	0	0
T1	2.22	0	1.55±0.24	2.55±0.3	3±0.57	3±0.5
T2	0	1.11	2±0.23	2.55±0.24	2.66±0.22	2.88±0.30
T3	0	1.16	2.11±0.38	2.77±0.36	2.77± 0.22	3±0.23
T4	2.22	2.32	2.22±0.66	2.77±0.22	2.88±0.44	3.66±0.33
T5	4.44	2.32	2.88±0.42	3.66±0.23	5.55±0.41	5.66±0.52
T6	1.11	0	1.44±0.37	1.88±0.35	2±0.28	2±0.28
T7	4.44	1.16	1.44±0.50	3.22±0.40	3.44±0.47	3.55±0.41

*Each treatment contained 3 replicates and each experiment was repeated thrice

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	28.15203	1	28.15203	7.016534	0.01103	4.051749
Within Groups	184.5631	46	4.012242			
Total	212.7152	47				

The significance level ofnumber of shoot multiplication on 20th day were carried out using one one way ANNOVA by excel. **P-value** obtained is **0.01103** when the control values compared to other treatments values it showed difference significance. Among the treatments T4 showed the high significant difference. T1 and T3 showed the par difference.

Fig 4.1: Multiple shoots induction in varying concentrations of BAP and KIN for AUFWc008

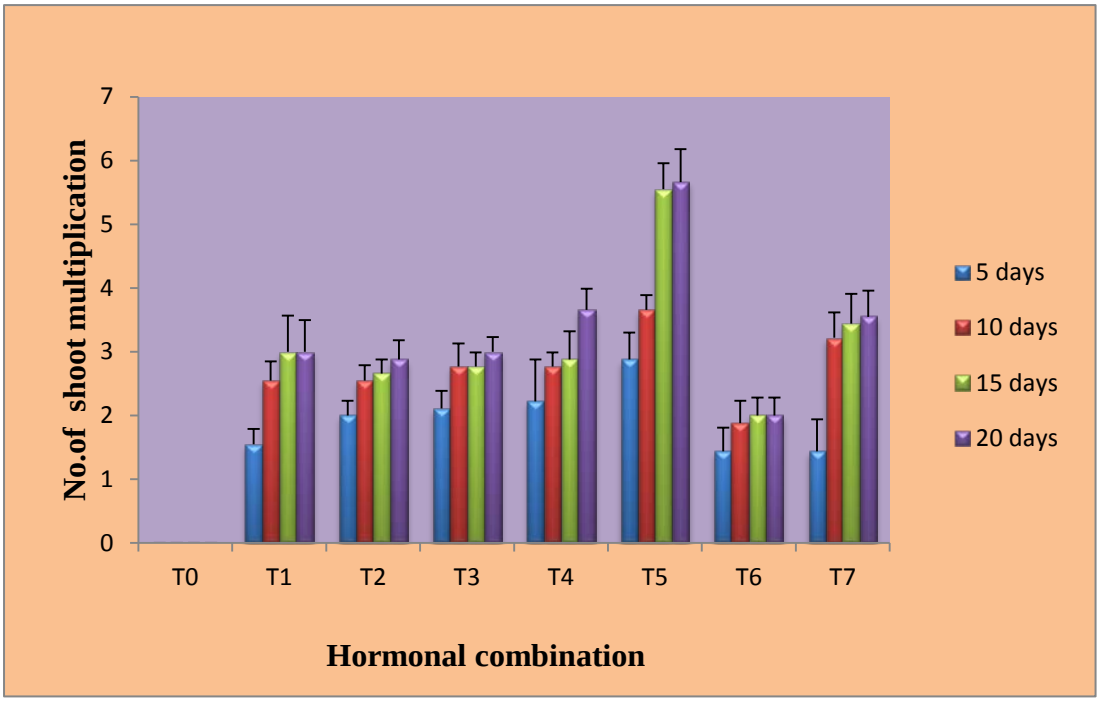


Table 4.2: Influence of varying concentrations of BAP and KIN on multiple shoot induction in AUFWc025

S.No	BAP µM/L	KIN µM/L	MEAN NUMBER OF SHOOTS			
			After 5days	After 10days	After 15days	After 20Days
T0	0	0	0	0	0	0
T1	2.22	0	1.88±0.35	1.88±0.42	2.33±0.52	2.66±0.52
T2	0	1.11	1±.0.23	1.66±0.28	2.55±0.37	2.66±0.33
T3	0	1.16	2.33±0.23	2.77±0.32	2.88±0.35	3±0.28
T4	2.22	2.32	1.77±0.22	1.88±0.26	2.22±0.43	2.66±0.40
T5	4.44	2.32	3.11±0.56	4±0.40	5.0 ± 0.84	5.33±1.30
T6	1.11	0	1.44±0.33	1.55±0.37	2.22±0.64	2.44±0.62
T7	4.44	1.16	1.88±0.45	2.88±0.30	3±0.33	3.11±0.30

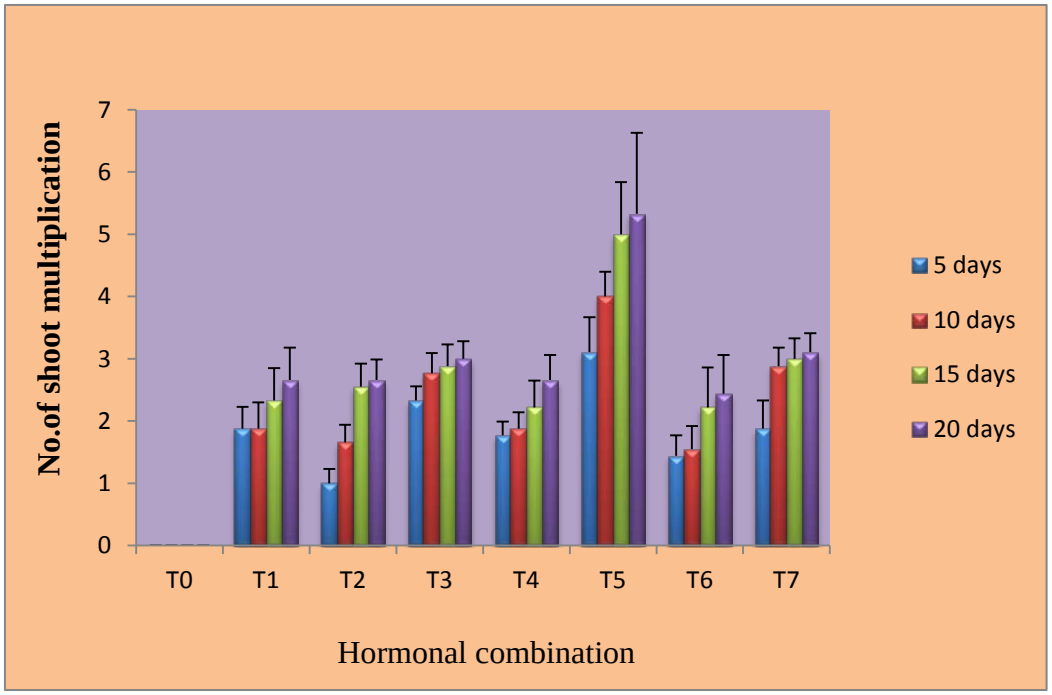
*Each treatment contained 3 replicates and each experiment was repeated thrice

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	37.471	1	37.471	8.718714	0.004946	4.051749
Within Groups	197.6973	46	4.297767			
Total	235.1683	47				

For AUFWc0025 the significance level of No of shoot multiplication on 20th day were carried out using one one way ANNOVA by excel. **P-value** obtained is **0.01103**.When the control values compared to other treatments values it showed difference significance. Among the treatments T4 showed the high significant difference. T1, T2 and T3 were on par.

Fig 4.2: Multiple shoots induction in varying concentrations of BAP and KIN for AUFWc025:



4.3 Root induction:

4.3.1 Effect of IBA and IAA combination on root induction:

Plants are the good source of secondary metabolites, especially in plants producing pharmaceutical compounds in their roots. The roots are generally considered to be enriched with bioactive withanolides and used in polyherbal formulations (Asthana *et al*., 1989).

Harvesting roots is destructive for the plants and hence there is a growing interest in root culture as an alternative source for this important metabolite (Abouzid *et al*., 2010). Root cultures are typical examples that can be used for production of phytochemicals. Root cultures have been used as standard experimental system in studies of inorganic

nutrition, nitrogen metabolism, plant growth regulation, and root development (Loyola, 1995).

Induction of rooting is affected by several intrinsic and extrinsic factors (Martin, 2002).Important developmental processes, such as root-hair formation, primary root growth and lateral root formation, are particularly sensitive to changes in the internal and external concentration of nutrients. The responses of root architecture to nutrients can be modified by plant growth regulators, such as auxins, cytokinins and ethylene, suggesting that the nutritional control of root development may be mediated by changes in hormone synthesis, transport or sensitivity. Recent information points to the existence of nutrient specific signal transduction pathways that interpret the external and internal concentrations of nutrients to modify root development(Bucio *et al*., 2003).

The both varieties of *Withania coagulans* maintained in MS basal medium for atleast 2 generations and fresh leaves were selected for the induction of adventitious roots. The work of Gray (1991) suggests that a half-strength of MS salts promotes root induction in *in vitro* condition. The leaf explants were cut into pieces from the AUFWc008 and AUFWc025 varieties of *Withania coagulans* and inoculated on MS medium supplemented with different concentrations of IBA and IAA, **plate 4.3 and 4.4** Shows the adventitious root induction of *Withania coagulans* (AUFWc008, AUFWc025) in various combination of IBA and IAA.

Table 4.3 and 4.4 shows the influence of various combination of auxin on root induction of AUF Wc 008, AUF Wc 025 varieties of *Withania coagulans* respectively.

Plate 4.3 The adventitious root induction of AUFWc008 in various combination of IBA and IAA

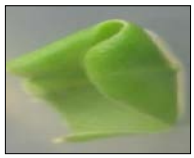
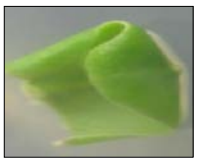
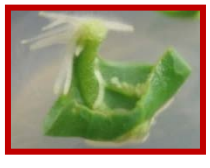
Treatments	0 day	10 day	20 days	30days
Control				
T1				
T2				
T3				
T4				
T5				

Plate 4.3 The adventitious root induction of AUFWc025 in various combination of IBA and IAA

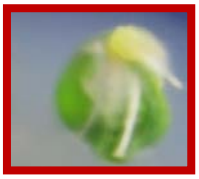
Treatments	0 day	10 days	20 days	30 days
Control				
T1				
T2				
T3				
T4				
T5				

Table 4.3: Influence of varying concentrations of combination of auxin on root induction in AUFWc008 leaf explants

S.NO	IBA µM/L	IAA µM/L	NUMBER OF ROOTS INDUCED	
			After 20days	After 30Days
T0	0	0	0	0
T1	1.23	0	0	4.22 ±0.46
T2	2.46	0	1.56±0.38	6.44±0.60
T3	2.46	1.42	2.22±0.28	7.11±0.79
T4	4.29	5.71	2.33±0.29	11.44±0.76
T5	19.70	5.71	6.67±0.44	21.78±0.79

*Each treatment contained 3 replicates and each experiment was repeated thrice

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	223.1538	1	223.1538	8.398586	0.006529	4.130018
Within Groups	903.3937	34	26.5704			
Total	1126.547	35				

The significance level of No of root induction on 30th day were carried out using one one way ANNOVA by excel. **P-value** obtained is 0.006529. When the control values compared to other treatments values it showed difference significance. Among the treatments T5 showed the high significant difference.

Fig 4.3: Effect of IBA and IAA combination on root induction of AUF Wc 008

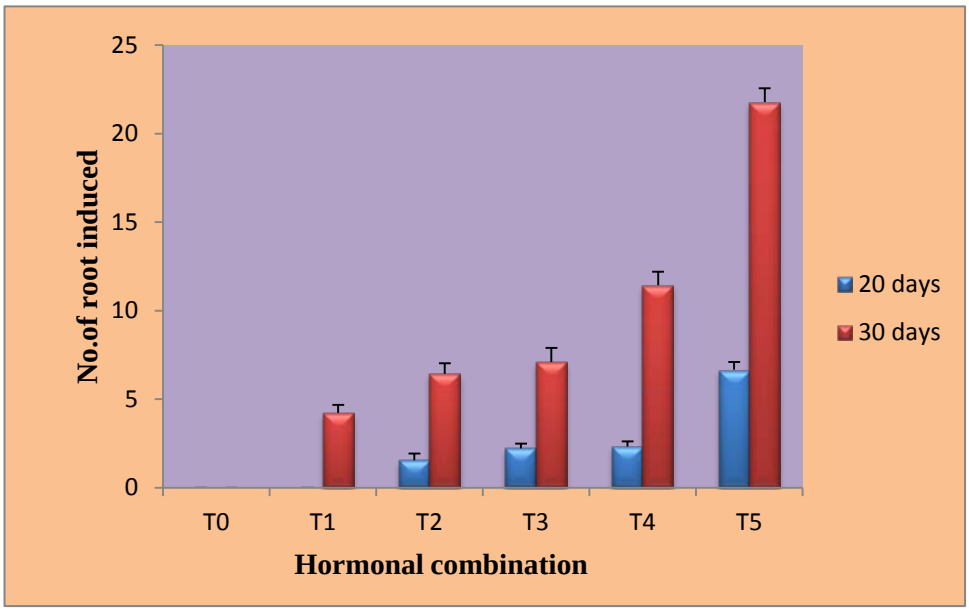


Table 4.4: Influence of varying concentrations of combination of auxin on root induction in AUFWc025 leaf explants

S.NO	IBA µM/L	IAA µM/L	NUMBER OF ROOTS INDUCED	
			After 20Days	After 30Days
T0	0	0	0	0
T1	1.23	0	0	7.66±0.0.37
T2	2.46	0	1.00±0.23	8.22±0.36
T3	2.46	1.42	2.22±0.28	10.22±0.64
T4	4.29	5.71	2.11±0.26	8.77±0.59
T5	19.70	5.71	9.55±0.44	18.44±0.58

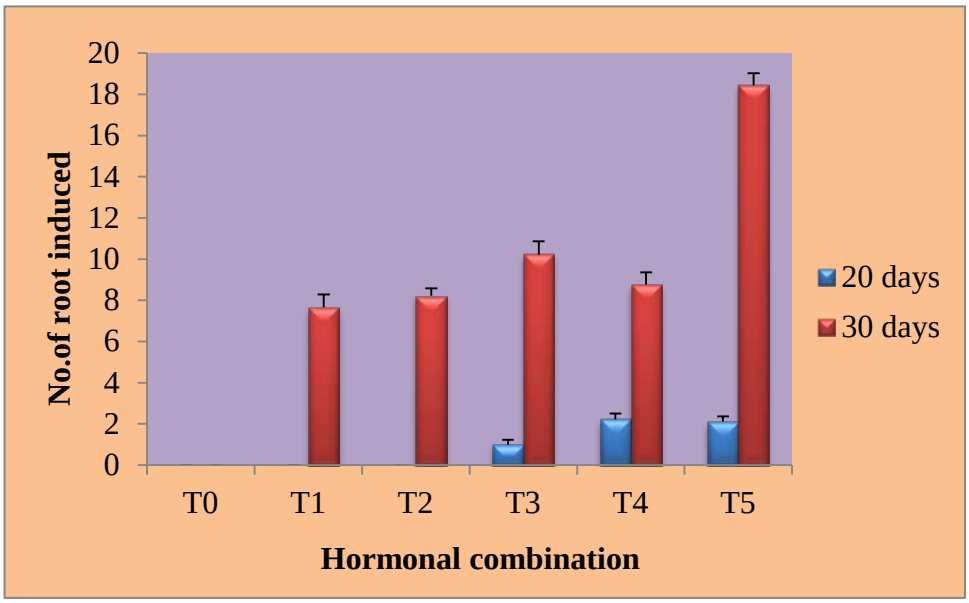
*Each treatment contained 3 replicates and each experiment was repeated thrice

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	261.0917	1	261.0917	15.33587	0.000412	4.130018
Within Groups	578.8468	34	17.02491			
Total	839.9386	35				

The significance level of No of root induction on 30th day were carried out using one one way ANNOVA by excel. **P-value** obtained is 0.000412 When the control values compared to other treatments values it showed difference significance. Among the treatments T5 showed the high significant difference.

Fig 4.4: Effect of IBA and IAA combination on root induction of AUF Wc 025



From the **table 4.3** and **4.4** results revealed that IBA and IAA induced adventitious roots from leaf explants after 12-15 days of cultures. It was found that there was no root induction upto 10th day. Different combinations and concentrations of 1AA and IBA showed various responses for adventitious root induction. An increasing effect of root induction was found on increasing concentration of IBA for both varieties of *Withania coagulans*. The result showed that as the days progressed the root induction was increasing.

Maximum root induction were observed in MS media supplemented with 19.7 µM/L IBA and 5.71 µM/L IAA (21.78±0.79) and (18.44±0.58) root induction was observed in AUF Wc 008, AUF Wc 025 varieties of *Withania coagulans* respectively. Comparing to all combinations MS basal media supplemented with 1.23 µM/L IBA alone for AUF Wc 008 showed minimum root induction (4.22 ±0.46). For AUF Wc 025 MS basal media supplemented with 2.46 IBA µM/L showed minimum root induction (7.66±0.62). It shows that higher concentration of IBA plays an important role in root induction.

Mavridou, 1993 proved the concentration of IBA and way of its treatment also influences root induction. It was observed that a number of roots were induced on increasing medium concentration of IBA at 22.85 µM/L /L.

The studies of kumar *et al*., 2011 shows the similar results that optimum rooting efficiency (64.0±0.22) as well as the best root number were achieved on MS medium fortified with IBA 24.66 µM/L.

Our studies was supported Valizadeh and valizadeh, 2009 reported that shoot explants induced roots in half strength MS medium containing IBA (9.85 µM/L). Medium with 9.85 µM/Lproduced highest number of roots (23 roots).

Rathore *et al*., 2013 indicated that half-strength MS medium amended with 200 mg/LAC without any PGR supplementation failed to promote rooting. Rooting occurred *in vitro* on half-strength MS medium containing 200 mg L−1 AC and root inducing auxins. Exactly 67.3 ± 0.01% root induction in condition *in vitro* on half-strength of MS salts containing 200 mg L^{-1} AC and 29.52 µM IBA within 13–15 days was found. The effect of 29.52 µM IBA on induction, thickness, strength, numbers (4.9 ± 0.23), and length of roots (2.8 ± 0.10) was significantly higher as compared to other concentrations of IBA, NOA, and their combinations. On medium with NOA and lower (less than 29.52 µM) concentrations of IBA, roots showed delayed and poor response. On higher concentration of IBA, percentage rooting remained high; however, the number and length of roots reduced along with swelling of shoot base. Effect of IBA was found significant in inducing rooting as compared to NOA.

4.4 Phytochemical analysis

The term quantitative analysis refers to the establishing and proving the identity of a substance. The active ingredients, after isolation, can be incorporated into the modern medicine system for the development of newer formulation for therapeutic ailments (Salwaan *et al*., 2012). Several specific reactions operating temporally and spatially are responsible for the production of secondary metabolites in medicinal plants. Changes of the environmental factors or placing a plant into tissue culture may also produce a new, and sometimes unexpected, secondary metabolic profile.

The plant extracts were subjected to preliminary phytochemical screening for the detection of various plant constituents present. The phytochemistry of *Withania* species has been studied extensively by several workers and several groups of chemical such as steroidal lactones, alkaloids, flavonoids, tannin etc. have been identified, extracted, characterized and isolated (Jain *et al.,* 2012). Different phytochemistry studies have been done on *W. coagulans* and various compounds have been isolated from the plant. The phytochemical investigations on *W. coagulans* up to 2011 reported a number of phytoconstituents (Khodaei *et al*., 2012).

The quantitative analysis of the phytochemicals in AUF Wc 008 and AUF Wc 025 variety of *Withania coagulans* showed that there is a variation in the phytochemicals. **(Table 4.5)** From the analysis of AUF Wc 008 and AUF Wc 025 variety of *Withania coagulans* the results revealed that flavanoid content of the both samples were higher when compared to steroid, alkaloid, phenol and saponin accumulation. Among the five phytoconstituent studied the saponin accumulation in AUF Wc 025 was very least. Steroid and phenol accumulation in both varieties of *Withania coagulans* was higher than the saponin and alkaloid content.AUF Wc 025 variety showed lower accumulation of alkaloids, phenol, saponin and flavanoid when compared toAUF Wc 008 variety except in steroids which showed higher accumulation in AUF 025 varieties.

Flavonoids:

Flavonoids comprise the most common group of plant polyphenols and provide much of the flavor and color to fruits and vegetables. It has been recognized that flavonoids show antioxidant activity (Pourmorad *et al*., 2006). More than 5000 different flavonoids have been described. Interest in the possible health benefits of flavonoids has increased owing to their potent antioxidant and free-radical scavenging activities observed *in vitro.* The six major subclasses of flavonoids include the flavones (e.g., apigenin, luteolin), flavonols (e.g., quercetin, myricetin), flavanones (e.g., naringenin, hesperidin), catechins or flavanols (e.g., epicatechin, gallocatechin), anthocyanidins (e.g., cyanidin, pelargonidin), and isoflavones (e.g., genistein, daidzein). Most of the flavonoids present in plants are attached to sugars (glycosides), although occasionally they are found as aglycones (Ross *et al*., 2002).

The flavonoid compositions of the samples were found to 18.715±1.225 mg/ml (AUF Wc008) and 16.565±0.305mg/ml (AUF Wc 025) respectively.

Alkaloid:

Alkaloids are a diverse group of low-molecular-weight, nitrogen-containing compounds

found in about 20% of plant species. Many of the alkaloids for which structures have been described function in the defense of plants against herbivores and pathogens. The potent biological activity of some alkaloids has also led to their exploitation as pharmaceuticals, stimulants, narcotics, and poisons. Other important alkaloids of plant origin include caffeine, nicotine, cocaine, and the synthetic *O*,*O*-acetylated morphine derivative heroin (Facchini *et al*., 2001).

The estimation of alkaloid showed that the samples varied in their alkaloid content 3.495± 0.155 mg/ml (AUF Wc 008) and (AUF Wc 025) 3.1± 0.08 mg/ml

Saponin

Saponins are high-molecular-weight glycosides, consisting of a sugar unit(s) linked to a triterpene or a steroid aglycone. Many saponins have detergent properties. The saponins can be divided into three major classes according to the structure of genin: Triterpene glycosides, steroid glycosides and steroid alkaloid glycosides (Madland *et al*., 2013). Saponins occur constitutively in a great many plant species, in both wild plants and cultivated crops. Saponins are generally known as non-volatile, surface active compounds that are widely distributed in nature, occurring primarily in the plant kingdom (Nihal *et al*., 2008).

The saponin content in the *Withania* leaf samples ranged from 2.07±0.013 mg/ml in AUF Wc 008 to of 3.24±0.02 mg/ml in AUF Wc 025.

Phenol:

Phenol is a monosubstituted aromatic hydrocarbon. In its pure state, it exists as a colorless or white solid. This pure compound is mixed with water and commercially sold as a liquid product. Phenol is produced through both natural and anthropogenic processes.

It is naturally occurring in some foods, in human and animal wastes, and in decomposing organic material, and it is produced endogenously in the gut from the metabolism of aromatic amino acids. The primary oxidative metabolites include hydroquinone and catechol, which are also substrates for conjugation. Secondary products of hydroquinone or catechol metabolism, including benzoquinone and trihydroxybenzene, can also be formed. Once absorbed, phenol is widely distributed in the body, although the levels in the lung, liver and kidney are often reported as being higher than in other tissues (Haber *et al*., 2006).

The estimation of phenol revealed that the presence of phenol content ranges from 6.11±0 mg/ml (AUF Wc 008) to 4.95±0 mg/ml (AUF Wc 025).

Steroid:

The major chemical constituents of the Withania genus, the withanolides, are a group of naturally occurring C28-steroidal lactone triterpenoids built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring (Mirjalili *et al*., 2009). The constituents of Withania roots are the steroidal alkaloids and steroidal lactones. They belong to a class of constituents called the withanolides with the main active chemical constituent Withaferin A, a phytosteroid (Sharma *et al*., 2011).

The steroid content of the root samples ranged from 9.05±0 mg/ml in AUF Wc 008 to 11.47±0.22 mg/ml in AUF Wc 025.

Table 4.5: Quantitative analysis of phytochemicals

(Expressed in mg/g dried leaf)

S.No	Sample	Flavonoid	Alkaloid	Phenol	Saponin	Steroid
1.	AUFWc 008	18.715±1.22	3.495± 0.15	6.11±0	3.24±0.02	9.05±0
2.	AUFWc 025	16.565±0.30	3.1± 0.08	4.95±0	2.07±0.01	11.47±0.22

4.5 Chromatographic analysis

The chemical composition of a medicinal plant may vary substantially with the developmental stage of the plants. Therefore, investigations on ontogenetic variation of secondary metabolites from different classes have received considerable interest from plant scientists over several decades (Praveen *et al.,* 2010).

Among the modern Analytical tools HPTLC is a powerful analytical method equally suitable for qualitative and quantitative analytical tasks. HPTLC is playing an important role in today analytical world, not in competition to HPLC but as a complementary method (Andola *etal.,* 2010).

This method is simple, precise, specific, sensitive and accurate and can be used for routine analysis as well as for quality control of raw materials and herbal formulations (Sharma *et al*., 2007). HPTLC offers many advantages over other chromatographic techniques such as unsurpassed flexibility (esp. stationary and mobile phase), choice of detection, user friendly, rapid and cost effective. Thus, HPTLC is most widely used at industrial level for routine analysis of herbal medicines (Jirge*et al*., 2011).

In this study HPTLC analysis was done to estimate the quantity of withanolide A and withaferin A present in both varieties of *Withania coagulans* (AUF Wc 008 and AUF Wc 025). The leaf extracts were spotted on HPTLC plates and the mobile phase used in this study was Toluene: Ethyl acetate: Formic acid. In the work of Sharma *et al*., 2007 various compositions of mobile phases were tested to get better resolution of withaferin-A and withanolide-A. He proved that the resolution of withaferin-A and withanolide- A, with symmetrical and reproducible peaks, was achieved by using mobile phase consisting of toluene:ethyl acetate:formic acid (5:5:1). So this mobile phase was in our study. Anisaldehyde and Conc. Sulphuric acid was used as derivatizing agent (85mL methanol: 10mL glacial acetic acid: 5mL sulphuric acid: 0.5mL anisaldehyde).

Standard withanolide A solution (0.1mg/ml) was also spotted in varying concentrations of 2µl, 6µl, 10µl, 12µl in order to quantitatively estimate the amount of Withanolide A present in all the leaf samples. The standard and methanolic extracts of samples were applied to the plates as 6mm bands under a stream of nitrogen, at the rate of 150 nl/s by means of a CAMAG (Switzerland) Linomat V semiautomatic sample applicator fitted with a 100µl Hamilton HPTLC syringe. Linear ascending development to

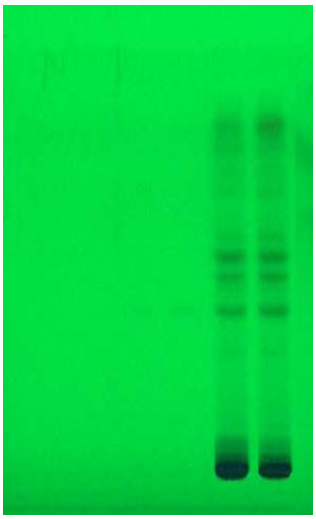
a distance of 8cm was carried out on 20x10cm twin trough chamber saturated for 10mins at room temperature (25ºC±2) with 20ml of the mobile phase, the plates were dried in a current of air with the help of an air dryer. The banding patterns were visualized at 254nm and 366nm respectively. **Plate 4.5** shows HPTLC plate at 254nm and **plate4.6** shows HPTLC plate at 366nm for withanolide A and Withaferin A

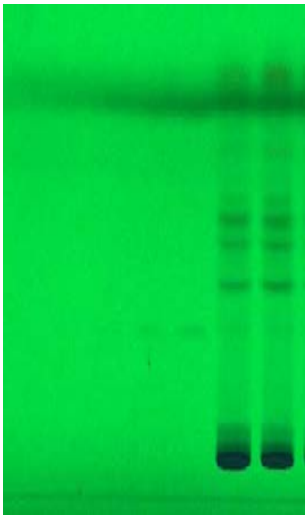
The *in vitro* sample of *Withania coagulans* 008 *and* 025was found to have larger number of spots indicating an increased number of phytochemicals. In the work of Gupta *et al.*, 1996 observed maximum contents of withanolide-A and withaferin-A in leaves.

The both varieties of *Withania coagulans*almost showed similar banding patterns for withanolide A and withaferin A. The developed plates were then subjected to Densitometric scanning with Camag TLC scanner III using Savitsky-Golay 7 filter in the reflectance –absorbance mode at 234nm at the speed of 200mm/s, the D2 and W lamp was chosen to scan the plates and the slit dimensions were set at 4.00 x 0.30 mm, Micro. All the tracts were scanned and the peak was displayed. The withanolide A peak was viewed as a separate spot with Rf values around 0.43-0.47 and for withaferin A peak was viewed as a separate spot with Rf values around 0.30-0.37 as obtained in the standardization of mobile phase. The peak analysis revealed the increase in concentration of Withanolide A and Withaferin A along the increase in volume **Fig 4.6**and **4.7**respectively.

The amount of withanolide A in each of the samples were quantified from the peak areas in comparison with the peak area of the standards using the peak area as an evaluation mode and at multilevel calibration modes and a linear regression graph was obtained using CAMAG software.

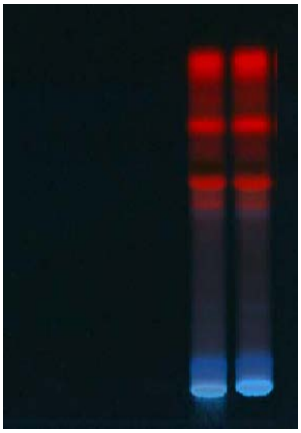
Plate 4.5: HPTLCplate at 254nm for Withanolide A and Withaferin A

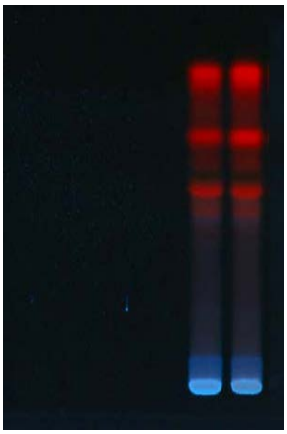




Withanolide A 1 2 Withaferin A 1 2

Plate 4.6: HPTLC plate at 366nm for Withanolide A and Withaferin A.



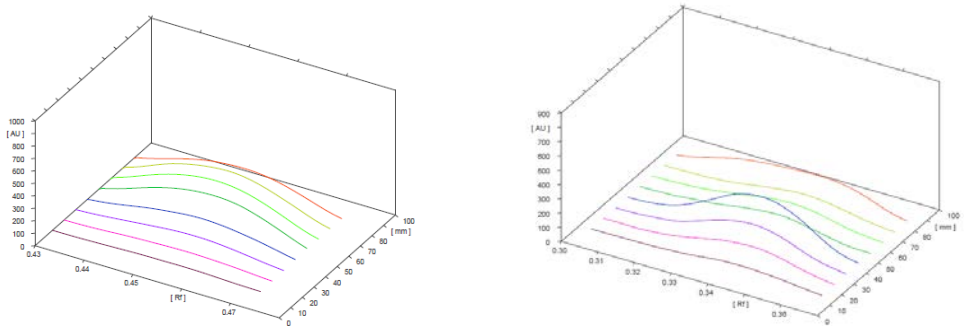


Withanolide A 1 2 Withaferin A 1 2

Lane 1- AUFWC008 (leaf)

Lane 2- AUFWC025 (leaf)

Fig 4.5: Scan of all tracks at 234nm for standard withanolide A and withaferin A at varying concentration



Withanolide A **Withaferin A**

Fig 4.6:Linear regression graph for standard Withanolide A

S.No	STANDARD		PEAK AREA
	Volume (µl)	Concentration	
1.	2	200ng	249.3
2.	4	400ng	550.9
3.	8	600ng	1069.7
4.	16	800ng	1870.1

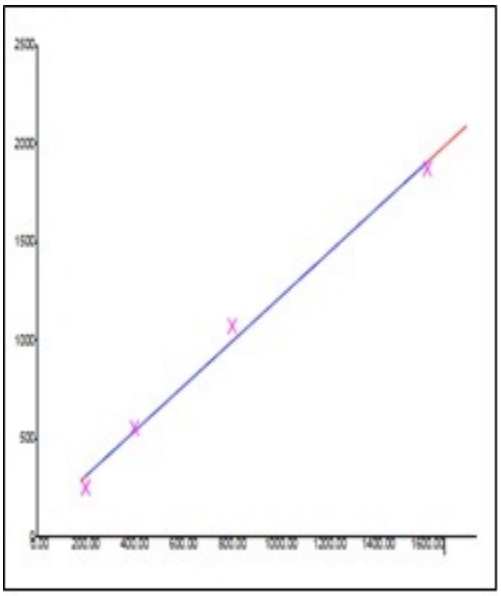


Fig 4.7:Linear regression graph for standard Withaferin A

S.No	STANDARD		PEAK AREA
	Volume (µl)	Concentration	
1.	2	200ng	782.2
2.	4	400ng	1800.6
3.	8	600ng	2824.2
4.	16	800ng	5051.0

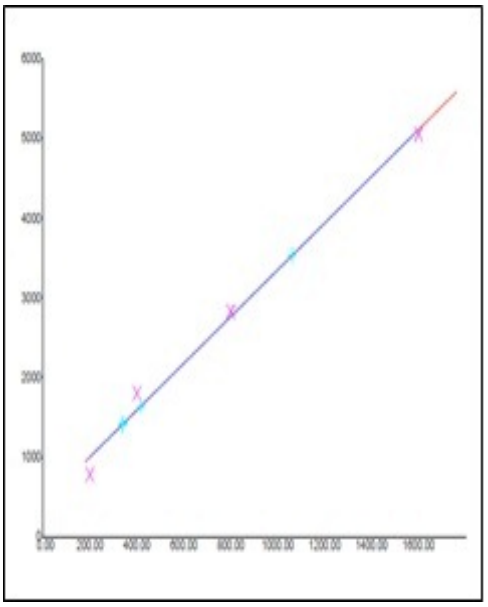


Table: 4.6 Amount of Withanolide A in the leaf samples

S.No	SAMPLE	Rf	PEAK AREA	WITHANOLIDE A (mg/g)
1.	AUF Wc 008	0.47	3779.9	0.16
2.	AUF Wc 025	0.47	2964.9	0.20

Table 4.7: Amount of Withaferin A in the leaf samples

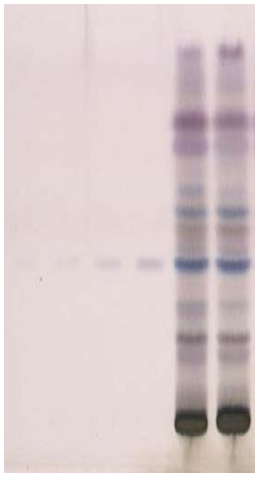
S.No	**SAMPLE**	**Rf**	**PEAK AREA**	**WITHAFERIN A (mg/g)**
1.	AUF Wc 008	0.47	1639.1	0.20
2.	AUF Wc 025	0.47	1405.9	0.16

The amount of withanolide A and withaferin A varied in the both varieties of *Withania coagulans.* The amount of withanolide A and withaferin A in each leaf samples is presented in the tables **4.6** and **4.7** respectively. The withanolide A concentration in two leaves samples ranged from **0.12 mg/g** (AUF Wc 025) to **0.16 mg/g** (AUF Wc 008). The withaferin A concentration in two leaves samples ranged from **0.16 mg/g** (AUF Wc 025) to **0.20 mg/g** (AUF Ws 008). The concentrations of various secondary plant products are strongly dependent on the growing conditions (Kannan *et al*., 2012). The results revealed that more amount of secondary metabolites are present in AUF Wc 008 when compared to AUF Wc 025 variety of *Withania coagulans.*

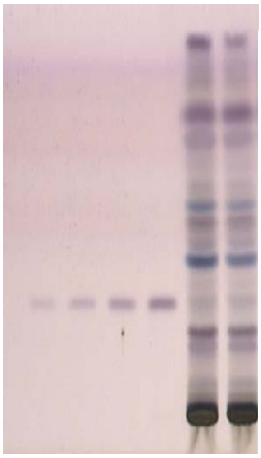
The plates were then derivitized by dipping in a solution of 10% H_2SO_4 and then drying in an oven at 120^oC for about 10mins. The derivitized plates were viewed in a visualiser under white light. **Plate 4.7** Shows withanolide and withaferin standard was visible as clear spots with different Rf values in white light.

After derivitization, several spots were observed and the results were similar to the ones observed under 366nm. HPTLC results revealed that AUF Wc 008 contains more concentration of withanolide A and withaferin A than AUF Wc 025 variety of *Withania coagulans*. The enrichment of these metabolites in the leaves may be attributed to environmental factors, genotypes, morphotypes or cultivation practices (Sharma *et al*., 2007).

Plate 4.7: Derivitized HPTLCplate under white light



Withanolide 1 2



Withaferin 1 2

Solvent system-: Toluene: Ethyl acetate:

Formic acid: (5:5:1)

Developing agent – Anisaldehyde sulphuric acid

Lane 1 – *invitro*AUFWc008 leaves

Lane 2 – *invitro*AUFWc025 leaves

SUMMARY ANDCONCLUSION

5.0 SUMMARY AND CONCLUSION

The results of study entitled **"Comparative evaluation of *in vitro* growth characteristics and secondary metabolite accumulation in two cultivars of *Withania coagulans*"** are summarized as follows.

- AUFWc008 and AUFWc025 cultivars of *W.coagulans* were taken for the study. Germination of the two varieties was attempted.MS basal medium supplemented with 2% sucrose and cultured in the dark at 22°C resulted in 80% germination for AUFWc008 variety and 67% germination for AUFWc025 variety in a period of 20 days and found to be best for the germination.
- Germination percentage was found to be higher in the dark at 22°C.
- Among two cultivars of *Withania coagulans* AUFWc008 showed better germination than AUFWc025.
- Shoot multiplication was attempted for mass multiplication *in vitro* in 7 different combinations of BAP and KIN supplemented MS media were analysed to study the influence of growth regulators on shoot multiplication. MS media supplemented with 4.44µM/L BAP and 2.32µM/L KIN (T5) was found to be best media combination for shoot multiplication in AUFWc008.
- As roots are the part that is therapeutically active, root induction invitro from leaf explants was studied for mass culture. Among the 5 different combinations of IAA and IBA tested, MS media supplemented with 19.70µM/L IAA and 5.71µM/L IBA (T5) and 3% sucrose was found to be the best medium for adventitious root induction in both cultivars of *Withania coagulans*.
- The quantitative phytochemical estimation was done to find which cultivar of *Withania coagulans* shows higher accumulation of secondary metabolites. AUFWc008 was found to contain high amount of flavonoids (18.71±1.22), alkaloids (3.49± 0.15), phenol (6.11±0), and saponins (3.24±0.02).exceptsteroid content (9.05±0). Steroid content(11.47±0.22)was found high in AUFWc025.
- The HPTLC fingerprint of AUF Wc 008 and AUF Wc 025 leaves showed similar banding patterns. But the concentration of major withanolides varied in both cultivars of *Withania coagulans.*

- AUFWc008 cultivar of *Withania coagulans* showed higher accumulation of withaferin A and withanolide A compared AUFWc025.

To conclude the study, among the two cultivars analysed for germination, in vitro shoot multiplication, adventitious root induction and accumulation of secondary metabolites in vitro, it was found that, AUFWc008 cultivar of *Withania coagulans perfomed better than* AUFWc025. In vitro multiplication method presented in this report would be helpful in large-scale restoration programs of *W. coagulans* through mass-scale multiplication as natural adaptation of this important medicinal plant is very poor. The present study may be useful to supplement the information with regard to its standardization and identification and in carrying out further research and its use in Ayurvedic system of medicine.

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6.0 BIBLIOGRAPHY

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APPENDICES

APPENDIX-I

COMPOSITION OF MS MEDIUM

Ingredients	Composition (mg/ L)	Stock Solution (W/V) (g)
MS Macro I (10 X)		**1000ml**
NH_4NO_3	1650	16.5
KNO_3	1900	19
$MgSO_4.7H_2O$	370.6	3.7
KH2PO4	170	1.7
100 ml		
MS Macro II (10 X)		**1000 ml**
$CaCl_2.2H_2O$	439.8	4.398
100 ml		
Fe-Na EDTA (1000 X)		**100 ml**
Fe-Na EDTA	36.7	36.7
1 ml		
Micro Nutrients (1000 X)		**100 ml**
NaMoO4.7H2O	0.25	0.025
CuSO4.5H20	0.025	0.0025
CoCl2.2H2O	0.025	0.0025
$MnSO_4.4\ H_2O$	13.2	1.32
$ZnSO_4.4H_2O$	8.6	0.86
H3BO3	6.2	0.62
1 ml		
KI (1000X)	0.83	100ml
Myo-Inositol (100 X)		**100 ml**
Myoinositol	100	1
10 ml		
MS Vitamins (1000 X)		**100 ml**
Nicotinic Acid	0.5	0.05
Pyridoxine HCl	0.5	0.05
Thiamine HCl	0.1	0.01
Glycine	2	0.2
1 ml		

APPENDIX 2

ESTIMATION OF FLAVANOIDS (Cameron *et al*., 1943)

PRINCIPLE

A Portion of plant was weighed and carried out in two steps, firstly MeOH: H20 (9:1) and then MeOH: H_2O (1:1) solvent added to make liquid slurry and mixture left to 12hrs .Filtration to separate the extract from plant material was / carried out rapidly for using glass wool or cotton wool plug in neck of filter funnel two extracts were combined and evaporated 1/13 original volume or most of MeOH had been removed. Resultant aqueous extract was cleared if low polarity contaminants such as Fats, Terpenes, Chloroform and Xanthophylls' by extraction with hexane and or chloroform. This was repeated several times and extract combined. The solvent extracted aqueous layer containing bulk of Flavonoids was concentrated.

MATERIALS

• **Vanillin reagent** - 1%in 70%H2SO4

• **Catechin standard** - 110 ug/ml

PROCEDURE

1. Aliquot of extract was pipetted into test tube and evaporated to dryness.
2. Then added 4ml of Vanillin reagent.
3. A standard was also treated in the same manner.
4. Then equal amount of distilled water was added.
5. Kept in boiling water bath for 15 minutes.
6. Took the readings at 360 nm.
7. Calculated the amount of flavonoids present in the sample.

CALCULATION

Express the amount in mg / g or 100 g sample.

APPENDIX –3

ESTIMATION OF SAPONIN

(Baccou *et al.*, 1977)

MATERIALS

• **Standard:** 0.1 g Diosgenin dissolved in 10 ml of HPLC grade methanol.

• **Reagent A:** 0.5 ml Anisaldehyde in 99.5 ml Ethyl Acetate.

• **Reagent B:** 50ml con.H2SO4 in 50 ml Ethyl Acetate.

PROCEDURE

1. Pipetted out 0.2, 0.4, 0.6, 0.8 and 1.0 ml of Standard into a series of test tubes and 0.3ml of the sample extract into another test tube.

2. Make up the volume to1 ml in all the test tubes. A tube with 1 ml of ethyl acetate serves as the blank.

3. 0.5ml of Reagent A was added to all the tubes.

4. Equal amount of Reagent B was then added.

5. Kept in boiling water bath maintained at 60^{0}C for 20 minutes.

6. Cooled to room temperature and the absorbance was measured at 430 nm.

CALCULATION

Express the amount in mg / g or 100 g sample.

APPENDIX 4

ESTIMATION OF ALKALOIDS

(Muthumani *et al.*, 2010)

MATERIALS

- **Standard:** 10mg of pure caffeine and dissolve in 25ml of 20% acetic acid A.R., dilute as aliquot a further 10 times with 20% acetic acid. This solution contains 40mcg/ml
- **20% Acetic acid.**
- **Chloroform**
- **Anhydrous Na_2SO_4**

PROCEDURE

- Into four suitable separators were pipetted 0,1,2 and 3 of 40mcg/ml standard solution
- The volume of each was made up to 5ml with 20% acetic acid
- To each separator 5 ml of acetate buffer and 1ml of methyl orange were added.
- After shaking for 10 sec.
- 5 ml of chloroform was added.
- The separators were stopped and shaken for 3 min.
- After standing for a few minutes chloroform layers were withdrawn into dry test tubes, dried with small amount of anhydrous Na_2SO_4
- Absorbance read on a spectrophotometer at 420nm using 10mm cells.
- From the reading standard curves was constructed.

CALCULATION

Express the amount in mg / g or 100 g sample.

APPENDIX –5

ESTIMATION OF PHENOL

(Kumbhare *et al.*, 2012)

MATERIALS

- **Standard:** 1ml of Gallic acid dissolved in 100ml of distilled water
- **Folin-Ciocalteau**
- **Sodium carbonate**

PROCEDURE

1. Pipetted out 0.5, 1, 1.5, 2, and 2.5 ml of Standards into a series of test tubes and 0.5ml of the sample extract into another test tube.
2. 1.5ml of Folin-Ciocalteau reagent was added to all tubes.
3. Allowed tubes to stand in room temperature for 5 minutes.
4. Added 4.0ml of 20% sodium carbonate to each tubes.
5. Make up the volume to10 ml in all the test tubes.
6. All tubes were incubated at room temperature for 30 minutes.
7. Absorbance was measured at 765 nm.

CALCULATION

Express the amount in mg / g or 100 g sample.

APPENDIX –6

ESTIMATION OF STEROIDS

(Wall *et al.*, 1952)

MATERIALS

- **Libermann Burchard Reagent :**(Acetic Anhydrate and Sulfuric acid) 0.5ml Sulfuric acid dissolved 10ml of a Acetic Anhydrates and kept in ice.
- **Standard:** 10mg Cholesterol dissolved in 10ml of Chloroform

PROCEDURE

1. Pipetted out 0.5, 1, 1.5, 2, and 2.5 ml of Standards into a series of test tubes and 0.2ml of the sample extract into another test tube.
2. Added 2ml of Libermann Burchard reagent to all the tubes.
3. Made up equal volume in all tubes with Chloroform.
4. Covered with carbon paper and Incubated at room temperature in dark for 30 minutes. A green colour was developed.
5. Read the absorbance at 640 nm.

CALCULATION

Express the amount in mg / g or 100 g sample.