In-vitro establishment and long term maintenance of callus and regeneration of *Hypericum perforatum, a natural antidepressant*

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CERTIFICATE

This is to certify that the work entitled, "*In-vitro* establishment and long term maintenance of callus and regeneration of *Hypericum perforatum,* a natural antidepressant" submitted by Adit Yadav & Vikash Rana in partial fulfillment for the award of degree of Bachelors of Technology in Biotechnology of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Roma Sarkar 29.05.09

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Place: JUIT, Waknaghat.
Date:

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CHAPTER 1

ABSTRACT

St. John's Wort (*Hypericum perforatum*) is a perennial herb distributed in temperate and subtropical regions of the world. It proved to be a useful medicinal herb for its anti-depressant (for mild to moderate depression), anti-inflammatory, anti-viral properties.The key chemical components in the plant are hypericin and hyperforin.However,opinions differ as to the active constituent(s) attributable to anti-depressant properties.The present investigation aims at in-vitro establishment and long term maintenance of callus and regeneration of *Hypericum perforatum*,for eventual commercial exploitation of its phyto-pharmaceuticals

Successful induction of callus has been achieved in M.S. media.The studies showed that fully defined M.S.media in combination with benzyl adenine (0.1%) and 2,4-D (0.05%) was effective for both initiation and sustained growth of callus tissue. The process of subsequent subculturing of calli led to the complete regeneration of plant showing a rapid and vigorous growth.[Plants with vigrous leaf and root growth were obtained after they were subcultured into MS media with growth hormones, BA (0.5mg/lt.) and, kinetin (1mg/lt.).]

CHAPTER 2

OBJECTIVES

- **In-vitro establishment and long term maintenance of callus of *Hypericum perforatum,***
- **Regeneration of *Hypericum perforatum***

CHAPTER 3

LITERATURE REVIEW

Introduction

St. John's Wort (*Hypericum perforatum*) has been known for centuries, dating back to the ancient greek culture for its therapeutic values.The plant has been traditionally used for treating various kind of ailments like bacterial and viral infections as well as 'nervous conditions'. In the modern times there has been a renewed interest on this herb for its antidepressant properties. A great deal of scientific research has progressed focusing on the therapeutic aspects of the plant. St. John's wort is now one of the most commonly purchased herbal products in the USA. It is commonly prescribed for mild to moderate depression in country like Germany.

Distribution

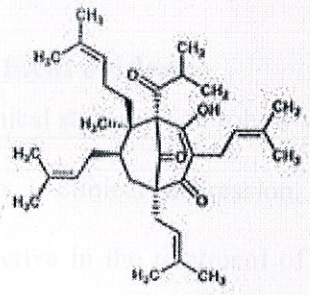
Hypericum perforatum grows naturally in temperate and subtropical regions of Europe, North America, Russia, India and China.In India it grows wild in the western Himalayas - at places in Jammu and Kashmir and Himachal Pradesh at altitudes above 1500 metres.

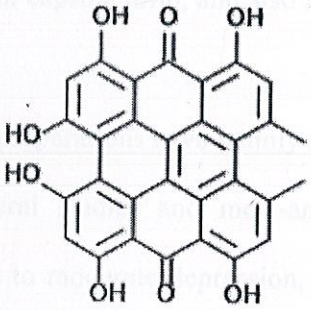
Plant description

St John's Wort is a perennial herb with rhizomatous roots and clusters of yellow flowers. Flowers bloom in late summer. Leaves exhibit prominent oil glands and the whole appear perforated when held up against the light.

Chemical composition:

It has different flavonoids (rutin, hyperoside, isoquercetin, quercitrin, quercetin, I3,II8-biapigenin, amentoflavone, astilbin, miquelianin), phenolic acids (chlorogenic acid, 3-O-coumaroylquinic acid),different naphtodianthrones (hypericin, pseudohypericin, protohypericin,protopseudohypericin), phloroglucinols (hyperforin and adhyperforin) and essential oils (composed mainly of sesquiterpenes). The naphthodianthrones hypericin and pseudohypericin along with the Phloroglucinol derivative hyperforin are thought to be the active components.[3],[4],[5]





Hypericin Hyperforin

Mechanism of Action

The apparent broad mechanism of action of St. John's Wort is not fully understood, yet biologically active constituents may include hyperforin and adhyperforin, hypericin and pseudohypericin, flavonoids, xanthones, oligomerics, procyanidines, and amino acids. Its

antidepressant activity may be mediated by serotonergic (5-HT), noradrenergic, and dopaminergic systems [1] as well as by means of γ-aminobutyric acid (GABA) and glutamate amino acid neurotransmitters. The action of St. John's Wort may be different from that of standard antidepressants[2].

Use as anti-depressant

Today,St John's Wort is most widely known as a herbal remedy for depression in the USA and Germany. In some countries, such as Germany, it is commonly prescribed for mild depression, especially in children and adolescents.[6]. It is also found effective as a natural remedy for treating seasonal affective disorder or SAD (a form of depression that occurs during the winter because of lack of sunlight).

Standardized products of the plant are generally available over the counter in the USA. Extracts are usually sold in tablet or capsule form, and also in teabags and tinctures.

Clinical evidence

Clinical studies of St John's Wort preparations have mainly focused on the efficacy of the herb in clinical depression. Several studies and meta-analyses have found it to be effective in the treatment of mild to moderate depression, with fewer side effects than many conventional antidepressants.[7]

Evidence for efficacy

A comprehensive meta-study indicated that extracts of *Hypericum perforatum* may be significantly more efficacious than placebo for the treatment of mild to moderate

depression.[8] This study, which covered the results from 23 smaller, earlier studies, is most often cited by manufacturers and other supporters of St John's wort.

An updated review of the above study based on new inclusion of data revealed that *Hypericum* preparations were significantly superior to placebo, and similarly effective as standard antidepressants. [9]

A further meta-analysis confirmed that *Hypericum* was more efficacious than placebo and as efficacious as tricyclic antidepressants, with little side effects. [10]

Other medical uses

Alcoholism: It is hypothesized that because of SJW's ability to relieve depressive symptoms, people tend to lose out on alcohol addiction.[11]

Hyperforin, a major constituent, has also been found to have antibacterial properties; in ultrapurified form a concentration of 0.1 mg/ml kills methicillin-resistant Staphylococcus aureus.[12]

Since hyperforin can stimulate the release of the neurotransmitter norepinephrine, it is presumed that the drug might alleviate the symptoms of attention-deficit hyperactivity disorder (ADHD). However, a randomized controlled trial of St. John's wort found no significant difference between the herbal extract and placebo in the management of ADHD symptoms over eight weeks.[13,14].

Of all the the medicinal properties of St.John's Wort, the herb has been valued most for its efficacy in curing depression and other psychiatric disoders, in many parts of the world. Though the plant is indigenous to India, still, its potential remains to be explored; so far there is no suitable product based on this plant. Of late, there has been a growing

research interest on this plant [18] in India. Keeping in view of its therapeutical potential, we intended to carry out the present investigation with a primary objective for in-vitro culture of the plants for the purpose of conservation as well as for commercial exploitation of its active components which are medicinally useful.

CHAPTER 4

MATERIALS AND METHODS

Materials

Plants stocks collected from NBPGR,regional station,Shimla had been used for the present study. These plants were planted in the JUIT experimental garden and are being maintained there.The leaves,stems and roots were used for further experiments as explants.

Methodology- I

Preparation of culture media:

Stocks were made for the preparation of MS media. The various stocks of MS media were mixed together and then sucrose (30 gm./lt.) was added to it. Growth hormones BA (0.1%), 2,4-D (0.05%) were then added. The pH was adjusted to 5.5-5.6. Agar (8 gm)

was finally added to the mixture. The distilled water was then added to adjust the final volume to 1 litre. Media was then heated till boiling and poured into flasks. then, flasks were autoclaved at 121 °C and 15 psi.

Preparation of explants for inoculation and incubation

Explant, mainly consisting of leaves and stem and also some portions of root were taken and thoroughly washed under tap water for 15 minutes, which was followed by washing with detergent Labolline.The treated plant materials were then subjected to sterlisation using Bavistin(0.5 gm/100ml) for 60 seconds and Mercuric chloride(0.1 gm/100ml)for 45 seconds, followed by repeated washing with autoclaved water.Inoculation of the explant was done into autoclaved flasks containing M.S. media in the Laminar Air Flow.The inoculated flasks were then incubated in the culture room at 25 ±2°C under fluorescent light.Callus was induced using M.S. media supplemented with different growth hormones,viz. benzyl adenine and 2,4-D.

Methodology- II

Plants with vigrous leaf and root growth were obtained after continuous subculturing of calli in advanced stages of development.These were then subcultured into MS media with growth hormones, BA (0.5mg/lt.) and, kinetin(1mg/lt.).

Figure 1:Plant stocks of St.John's Wort being maintained in the JUIT experimental garden

Figure 2: Inoculated Explants

CHAPTER 5

RESULTS AND DISCUSSION

Callus initiation and development

The callus initiation was achieved in M.S. media supplemented with 2,4-D (0.05%) and Benzyl adenine (0.1%). It was observed that callus growth was initiated after a period of ten days,after inoculation was carried out. Full fledged callus growth which included the appearance of leaves occurred after a duration of 2 weeks.

Initially, it was observed that roots and leaves that were used in the inoculation in most cases did not yield any callus initiation. It was assumed that the concentration of auxin (2,4-D) in the media (0.05%) in case of leaves and root was not sufficient enough to induce callus growth.Thus, the concentrations of auxin (2,4-D) and cytokinin (Benzyl Adenine) were enhanced to 0.1% and 0.2% respectively, which led to initiation of callus growth in leaves and root explants as well.

Thus,it may be concluded that M.S.media in combination with 0.1% of 2,4-D and 0.2% of Benzyl adenine is optimum for callus initiation, for stems, leaves and roots.

Regeneration of *Hypericum perforatum*

The process of continuous subculturing of calli, yielded vigorous leaf and root growth in 15 flasks. Plants with vigrous leaf and root growth were obtained after they were subcultured into MS media with growth hormones, BA (0.5mg/lt.) and, kinetin (1mg/lt.). The regeneration of the complete plant grown about 2 inches long was achieved in 75 days from the time of inoculation.

Figure 3:Calli in initial stages of growth

Figure 4: Calli in advanced stages of growth

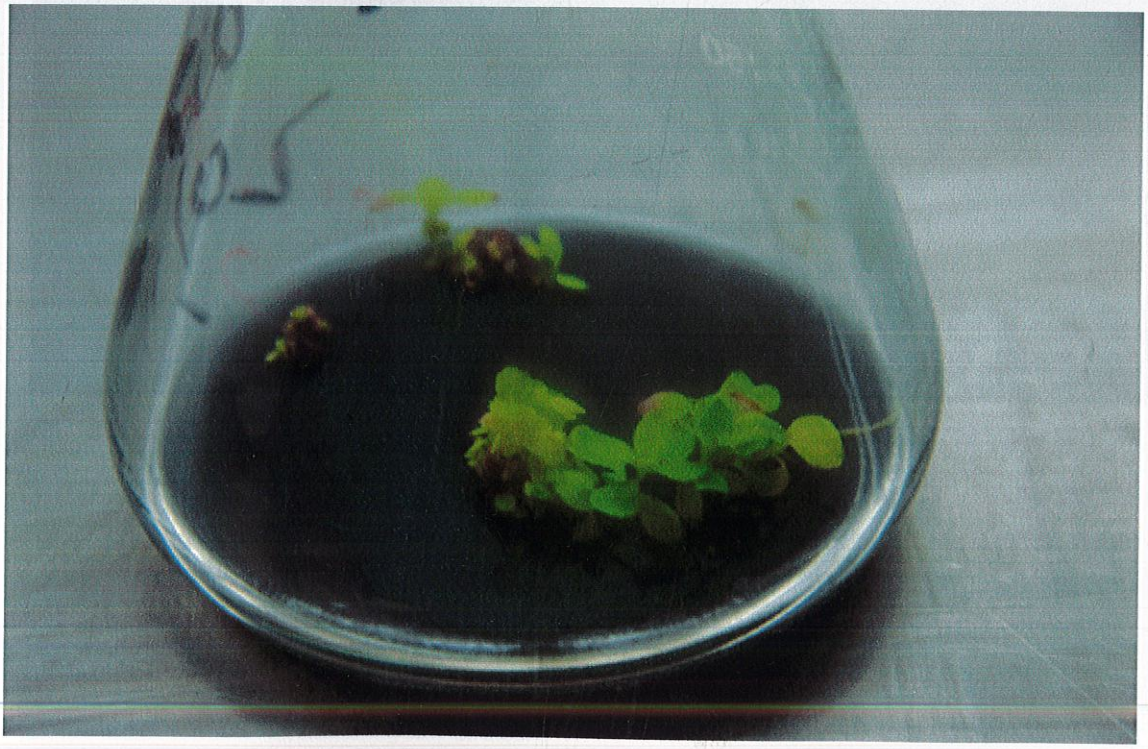


Figure 5 : Flasks showing vigorous vegetative growth after subculturing

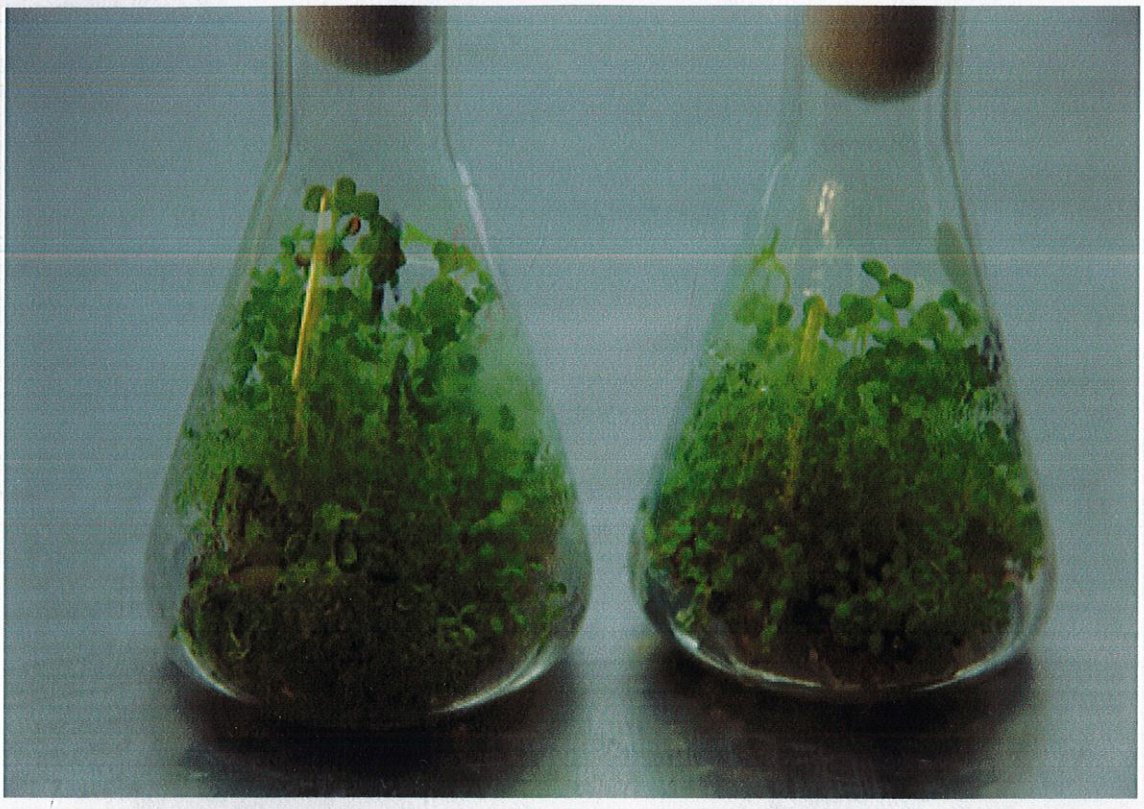


Figure 6: Flasks with leaf and root growth after the plantlets were subcultured in growth hormones, BA (0.5mg/lt.) and, kinetin(1mg/lt.).

Figure 7: Flask exhibiting root growth

Figure 8: Flasks collectively showing extensive root and leaf growth

CONCLUSION

The present study standardised a protocol, and callus was successfully induced which finally led to a rapid regeneration of complete plantlets of Hypericum perforatum, showing extensively vigorous vegetative stage, attributable to the high ploidy (2n=64) level of the species. It has been reported that the regenerated plants of *Hypericum perforatum* contain the secondary metabolites at a significant level [19]. Thus, such a vigorous growth in *in-vitro* culture, appears to be promising for commercial exploitation of its phytochemicals.

The *in-vitro* culture for *Hypericum perforatum* has several advantages: (a) It offers to be an effective means for conservation of the species. In India,the plants thrive only in a very restricted eco-climatic zones, and is extremely sensitive to any deviation from its optimum growing conditions. The species is considered threatened now at the wake of impending global rise of temperature. (b) In-vitro culture holds great potential as sustainable sources of the phytochemicals even during the period of dormancy of the plants during the winter months(December to February in Himachal Pradesh) in their natural habitat. (c) The application of tissue culture technique opens up prospect for screening of elite strains adaptable to grow in a wider eco-geographical climate, and also with an enhanced level of the phyto-pharmaceuticals by exploitation of somaclonal variation.

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