Introduction

The genus *Hypericum* Linn. consists of nearly 288 species. Of which *H. perforatum* Linn., *H. maculatum* Crantz, *H. tomentosum* Linn., *H. bithynicum* Boiss., *H. glandulosum* Gilib. and *H. beliaricum* Linn. are reported to contain hypericin¹. *H. perforatum*, commonly known as St. John’s Wort (Hindi — Balsana, Bassant, Dendhu) is a potent medicinal plant and is one of the top selling herbs in USA. It is a herbaceous perennial plant of family *Hypericaceae* widely distributed in temperate regions of Europe, Asia, North Africa and USA. In India it grows in Himalayas at higher altitudes and in the hills of central parts of the country. The plant grows approximately one metre high with opposite and paired branches. The leaves are opposite and sessile, upto 2cm long, oblong and contain numerous translucent glandular dots, which are visible against light. The yellow flowers contain 5 petals with many stamens protruding. These flowers contain a group of reddish fluorescent dianthrone pigments with biological activity². It was also reported that the biosynthesis of hypericins is connected with morphogenesis and formation of dark red coloured oil glands on the leaves of the plant³. It has been known since Greek and Roman times, Dioscorides (1st century A.D) and Galen described its medicinal properties (2nd century A.D). It was widely used in the folk medicine in European countries as a soothing agent, an antiphlogistic in inflammation of bronchi and urogenital track, in haemorrhoid treatment, a healing agent in the treatment of traumas, burns, scabs and ulcers of various kinds and other local and general illness. Today this drug is little used for these traditional purposes but is largely used in the treatment of depression and AIDS.

Biosynthesis of Hypericin and Hyperforin

The biosynthesis of hypericin (Fig 1&2) involves a polyketide derivative through acetate pathway¹⁰⁷. The polyketide (1) with a series of intermediates transforms in to
Chemical composition

The plant is reported to contain following categories of chemical constituents:

<table>
<thead>
<tr>
<th>Category</th>
<th>Constituents</th>
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<tbody>
<tr>
<td>Dianthrone derivatives</td>
<td>Hypericin&lt;sup&gt;2,4&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Pseudohypericin&lt;sup&gt;5,6&lt;/sup&gt;</td>
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<td></td>
<td>Protohypericin&lt;sup&gt;7&lt;/sup&gt;</td>
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<tr>
<td>Phloroglucinol derivatives</td>
<td>Hyperforin&lt;sup&gt;8&lt;/sup&gt;</td>
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<td></td>
<td>Furohyperforin&lt;sup&gt;9&lt;/sup&gt;</td>
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<td></td>
<td>Adhyperforin&lt;sup&gt;10&lt;/sup&gt;</td>
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<tr>
<td>Xanthones&lt;sup&gt;11&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Essential oil</td>
<td>α-Pinene, β-Pinene, Limonene</td>
</tr>
<tr>
<td>Monoterpenes&lt;sup&gt;12&lt;/sup&gt;</td>
<td>Caryophyllene, Humulene</td>
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<tr>
<td>Sesquiterpenes&lt;sup&gt;13&lt;/sup&gt;</td>
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<tr>
<td>Flavonoids</td>
<td>Hyperoside&lt;sup&gt;14&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Quercetin&lt;sup&gt;16&lt;/sup&gt;</td>
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<td></td>
<td>Quercetrin&lt;sup&gt;17&lt;/sup&gt;</td>
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<td></td>
<td>Rutin&lt;sup&gt;18&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Biapigenin&lt;sup&gt;19&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Kaempferol&lt;sup&gt;19&lt;/sup&gt;</td>
</tr>
<tr>
<td>n-Alkanols&lt;sup&gt;20&lt;/sup&gt;</td>
<td>0.42% of total herb</td>
</tr>
<tr>
<td></td>
<td>1-Tetracosanol (9.7%), 1-Hexacosanol (27.4%), 1-Octacosanol (39.4%), 1-Triacontanol (23.4%)</td>
</tr>
</tbody>
</table>

Atrochrysone (2). Atrochrysone on dehydration converts into emodine anthrone (3). Emodine anthrone readily transforms into hypericin (4) via oxidative free radical chemistry.

The biosynthesis of hyperforin (Fig 1 & 3) involves isoprenoid moieties, which are derived predominantly via the non-mevalonate pathway<sup>85</sup>. The acylphloroglucinol (4) moiety is generated via a polyketide type mechanism. This acylphloroglucinol is formed from isobutyryl-CoA (3) as a starter point, which in turn is formed from two units of pyruvate via α-acetolactate (1) and α-ketoisovalerate (2). The isobutyryl-CoA is subsequently lengthened by 3 malonyl CoA units and cyclization in to an intermediate, which would then lead to the acylchloroglucinol. Elaboration of hyperforin (5) from the unsubstituted acylchloroglucinol precursor is assumed to be with the participation of the geranyl pyrophosphate and three dimethylallylpyrophosphate units. While the sequence of remaining steps remain to be a large extent unidentified.

Pharmacological activities

Antidepressant activity — Hypericin in a standardized extract has shown a significant antidepressant activity by inhibiting the enzyme monoamino oxidase (MAO)<sup>21</sup>. The other mechanisms of antidepressant activity are, inhibition of dopamine β-hydroxylase<sup>22</sup> in vitro, inhibition of synaptic uptake of serotonin and dopamine<sup>23,24</sup>, inhibition of catechol-O-methyl transferase<sup>25</sup> in vitro, suppression of interlukin-6 in blood samples<sup>26 in vivo</sup>, modulation of expression of serotonin receptors<sup>27</sup>. The antidepressant activity of hyperforin is attributed to its inhibition of neuronal uptake of serotonin, norepinephrine and dopamine like many other antidepressants and also inhibits GABA and α-glutamate uptake<sup>28</sup>. The antidepressant activity of hypericum is not only limited to hypericin and hyperforin, xanthones of the plant are also reported to exhibit this property<sup>11</sup>.

Antiviral activity — Hypericin is a well-known photosensitizing agent used in the photodynamic therapy of cancer and viral infections. The photodynamic therapy involves the combination of photosensitizing agent and visible light at the absorption wavelength of the compound. Lavie et al<sup>29</sup> demonstrated the inhibitory effect of hypericin and pseudohypericin against vesicular stomatitis, influenza virus and herpes simplex virus types II and I. Inactivation of murine cytomegalovirus, sindbis virus and HIV-I on exposure to fluorescent light with hypericin treatment was reported first time by Hudson et al<sup>30</sup> and Lopez-Bazzocchi et al<sup>8</sup>. Both hypericin and pseudohypericin prevents the viral fusion by the generation of singlet oxygen up on illumination<sup>32</sup>. The antiviral activity against retroviruses like HIV involves combination of its photodynamic and lipophilic properties.
Fig. 1: Structures of Hypericin and Hyperforin

Fig. 2: Biosynthesis of Hypericin
Hypericin binds the cell membranes and crosslinks the viral proteins leading to its inability to retrieve the reverse transcriptase activity.

Anticancer activity —
Hypericins have been found active against human leukaemia, squamos cell carcinoma, nasopharyngial carcinoma, mouse mammary carcinoma and fibroblasts. Several studies have proven the significance of hypericin in photodynamic therapy of cancer both in vitro and in vivo. Hypericin seems to inhibit EGF (Epidermal Growth Factor) receptor and PTK (Protein Tyrosine Kinase) activity. A review on the mechanism of action of hypericin and its interaction with cellular components reveals that the photogenerated pH drop can achieve the potentiation of hypericin’s photodynamic activity. Hyperforin activates a mitochondria mediated apoptosis when added to MT-450 cells. In in vivo, hyperforin inhibited the growth of antologous MT-450 breast carcinoma in immuno-competent Wistar rats to a similar extent as that of Paclitaxel without any signs of acute toxicity.

The pharmacology of this plant also reveals a broad spectrum of activities.
like analgesic, anti-anxiety, anti-alcoholic, antispasmodic, antioxidant, calcium channel blocker, gene expression induction, wound healing, smooth muscle relaxant, sleep potentiation, anti-inflammatory, and anti-microbial.

**Production of Hypericin and Hyperforin through tissue cultures**

Over the last few years, St. John’s Wort producers have enjoyed a tremendous surge in interest and sales due to its potential in the treatment of depression and AIDS. Linde revealed the efficacy of St. John’s Wort when compared to the traditional antidepressants in his clinical studies. Apart from the efficacy in relieving depressive symptoms, St. John’s Wort popularity is rapidly expanding because of the botanical safety compared to traditional antidepressant medications.

Increasing demand for St. John’s Wort formulations, limited natural sources coupled with low content of therapeutically active constituents like hypericin and hyperforin, variation of these active constituents in wild plants and difficulty in synthesizing hypericins (because of its tautomeric forms) are the prime reasons to look for the alternative production of these medicinal agents. One of such alternatives is systematic cultivation under controlled conditions, which provides cultivation of the plant with moderate expenditure for labour and energy sources. In these circumstances plant tissue culture technique that is a bit expensive but allows the biotechnologists to attempt micropropagation, elicitation, permeabilization, immobilization and various other strategies to improve the production of active constituents compared to the intact plant. This technique has already been commercialized for the production of taxol, shikonin and rosmarinic acid.

**Callus formation and plant regeneration**

Santarean and Pretto reported callus formation and plant regeneration within few months, from leaf explants of the species. Li et al. reported callus and suspension cultures from the leaves and stems. Rani et al. established callus cultures of *H. perforatum* and reported the production of hypericin in callus is significantly less than that of intact plant. They also reported micropropagation from seeds. Baruah et al. reported *in vitro* regeneration by shoot tip culture. Regeneration potentiality was found to be constant through out the year in long-term cultures. The rooted plants could be successfully transferred to green house condition. Cordoso and Oliveira demonstrated plant regeneration from seeds and callus induction from nodal segments of *H. brasiliense Choisy*.

Usually secondary metabolites formed in plants serve as defense chemicals not only against predators, microbes and competing plants but also to protect the plant survival and growth in adverse conditions including the presence of toxic metals. In a way to see the environmental effects on the biosynthesis of hypericins, Briskin and Gawienowski reported that a higher light intensity and lower nitrogen resulted with an increased hypericin and pseudohypericin content. In contrast to this reduced levels of cellular capacity to produce hypericin in St. John’s Wort seedlings grown in Nickel supplemented medium was reported by Murch et al.

In order to eliminate the quality impairment in St John’s Wort products, Saxena et al. have reported six different culture systems for *in vitro* propagation of the plant to compare the biomass and secondary metabolite production with green houseplants. Of the six culture systems they found that growth in large vessel with gelled medium under forced ventilation (LFV system) was optimal for the production of biomass and secondary metabolites. Similarly Murch et al. reported the potential for acclimatization of *in vitro* grown St. John’s Wort plantlets to a nutrient film technique (NFT) hydroponic system in a controlled environment green house. The quantities of hypericins and hyperforin were found to be equal or more than that of green house grown plants.

**Cell suspension cultures of *H. perforatum***

In tissue culture techniques of medicinal plants, cell suspension cultures have wide range of application to study and optimize the production of important secondary metabolites using various strategies. Of the reports on *H. perforatum*, Bias et al. described an *in vitro* production of hypericins. They found that the cell aggregate size as an important requirement for large-scale production. Kirakosyan and Kaufman in their experiment to optimize the hypericin production in cell suspension cultures carried out the fractionation of
heterogeneous cell suspension cultures of the species by centrifugation through sucrose step gradient. The maximum hypericin content ca. 0.23 mg/g dry wt was reported from the population with the largest cells, further fractionation of the cells with the highest floating density revealed the highest percentage of hypericin ca. 0.90 mg/g dry wt. But the drawback of this technique is that the cultures are impossible to subculture to maintain their growth and production.

These reports reveal that the optimization of cell aggregates and their size are essential to enhance the production of hypericins in cell suspension cultures. In another study Gabriella Pasqua et al. reported the absence of hypericin in callus grown in dark and suspension cultures derived from it. Where as the callus that is used for shoot regeneration is grown under a photoperiod of 16 hours light has produced dark coloured globules and production of hypericin was observed. Based on this they conclude that the enzymes required for anthranol synthesis are missing in callus and suspension cultures. And on other hand cell differentiation with advanced stage of growth is required to obtain the biosynthesis of hypericins that in turn is related with the formation of dark coloured secretary globules.

**Chemical constituents of tissue cultured plants**

Initiation of shoot organ cultures from the germinated seedlings (after removal of rootlets) was reported by Zdunek and Allermann they found the accumulation of hypericin as a reddish fluorescent pigment in the leaves of differentiated plant from shoot organ cultures. The production of hypericin, pseudohypericin and flavonoids is reported by Kartnig et al. in *H. perforatum, H. maculatum, H. tomentosum, H. bithynicum, H. glandulosum* and *H. beliaricum*. The cell cultures of some strains of *H. perforatum* and *H. maculatum* produced high quantities of hypericin and pseudohypericin.

Ishiguro et al. reported a new xanthone named paxanthonin and demethyl paxanthonin together with the known compounds padiaxonanthone and tripteroide from callus tissues of *H. perforatum* flowers. Schmidt et al. reported xanthone 6-hydroxylase from cell cultures of *H. androsaemum* which is capable of hydrolysing 1,3,5 and 1,3,7-trihydroxy xanthone at 6th position. They also reported prenylated xanthone aglycones and their glycosides from cell cultures of the same plant. Two new xanthone glycosides, patuloside A and B have been isolated by Ishiguro et al. from cell suspension cultures of *H. patulum Thunb.* and their structures were elucidated by spectral studies.

Ishiguro et al. demonstrated that cell suspension cultures derived from the callus of *H. perforatum* flowers are capable of producing a new phloroglucinol derivative named as paglucinol. Yazaki and Okuda reported the production of procyanidins in *H. erectum Thunb.* callus and multiple shoot cultures. This is the first case of tannin production in tissue cultures of this genus. Guedes et al. reported the variation in essential oil components in *in vitro* shoots of *H. androsaemum* developed from excised apical buds when compared to the *in vivo* cultivated plants.

**Elicitation**

Elicitors are the agents of biotic or abiotic origin that can stimulate the defense mechanism of the plant cells to induce the excessive production of secondary metabolites. Plant tissue cultures are the best tools to have the biochemical manipulations with these elicitors to enhance the production of active constituents. Enhancement of production of hypericins and hyperforin was observed when tissue cultures of *H. perforatum* were subjected to elicitation. Kirakosyan et al. reported that mannan at 0.1 mg/ml concentrations stimulated the production of hypericin and pseudohypericin 2 and 4 times, respectively when compared to control shoot cultures. The compound β-1,3-glucan stimulated pseudohypericin production about 2.5 times when compared to control shoot cultures. In a similar study on shoot cultures, they reported that cork pieces enhanced the production of pseudohypericin by three-fold (0.4 mg/g dry wt). The stimulating effect of cork tissue was attributed to its insoluble components either by “massage effect” or “flotation effect”. Walker et al. reported that jasmonic acid improved the production of pseudohypericin by three-fold (0.4 mg/g dry wt). The stimulating effect of cork tissue was attributed to its insoluble components either by “massage effect” or “flotation effect”. Walker et al. reported that jasmonic acid improved the growth of biomass and production of hypericin by two times that of control cell suspension cultures. They also demonstrated the difference in growth and hypericin production of cells under light and dark conditions. Increased levels of hypericin and hyperforin by abiotic elicitors, methyl jasmonate and salicylic acid in meristem cultures and increased...
levels of hypericin by biotic elicitor *Colletotrichum gloeosporioides* was reported by Sirvent and Gibson\(^8\) in greenhouse grown plant.

Understanding the regulation of biosynthesis, optimization of culture conditions, selection of cell lines and use application of yield improvement strategies are essential to use the tissue cultures to produce hypericins and hyperforin on large scale.

**Extraction**

As the compound hypericin is highly photosensitive, the extraction process for St. John’s Wort must be carried out under dark conditions. Wagner and Bladt\(^2\) extracted the plant material with different solvents and the yields were analyzed for each kind of solvent, its concentration and extraction temperature. They found that optimal yields were obtained with 80% methanol at 80°C. Water and ethanol as (40:60), (20 : 80) for the extraction of dried flowers\(^1\) and dried *hyperici herba*\(^8\), respectively. Chatterjee *et al*\(^8\) described hydroalcoholic extraction using methanol and ethanol, it was concluded that both the solvents produce similar levels of hypericin but widely differing amounts of hyperforin. They also reported an extraction technique using \(\text{CO}_2\) by which 35% hyperforin was extracted but the amount of hypericin was undetectable.

The most commonly followed method involves the use of methanolic extraction in water bath shaker or ultrasonic bath. This basic method was followed for the dried flowering tops\(^8\), leaves\(^4\), plant material\(^8\), mixed capsule powder\(^8\) and St. John’s Wort powder\(^8\). The Soxhlet extraction with methanol was described for the plant material\(^6,8,98\) and commercially available formulations of St. John’s Wort\(^9\). Reported extraction methods with combination of solvents includes acetone, ethanol and methanol (1:1:1) for freeze dried cells\(^9\), ethanol and acetone (3:2) for capsule powder\(^9\), methanol and acetone (1:1) for air dried blossoms by Soxhlet extraction\(^9\). Michael\(^9\) carried out a similar extraction with methanol and 100mM triethylamine acetate for commercial St. John’s Wort powder and the extract was directly injected in to HPLC system for analysis. Hansen *et al*\(^9\) used 80% d-methanol (\(\text{CD}_3\text{OD}\)) in D\(_2\)O for extraction followed by HPLC-High Field NMR and Mass spectrometry for the structure elucidation of naphthodianthrones, flavonoids and other constituents of *H. perforatum* crude extract. Sirvent *et al*\(^8\) and Sirvent and Gibson\(^9\) reported an extraction method from fresh plant material for the analysis of hypericins involves the removal of chlorophyll with chloroform in the absence of light followed by extraction of the remaining plant material with acetone. The dried stem and reproductive materials were extracted directly with acetone.

For the extraction of hypericin and hyperforin from cell cultures of *Hypericum* spp., fresh or freeze-dried cells are subjected to methanolic extractions with slight modifications using sonication or shaker\(^7,77,79,98\). In *in vitro* studies, Yanyan *et al*\(^99\) demonstrated an extraction with cold acetonitrile for the determination of hyperforin in human plasma. In the quantitation of hypericin in biological fluids, Liebes *et al*\(^100\) reported an extraction procedure with ethyl acetate for the determination of hypericin in HIV infected plasma samples. Bauer *et al*\(^103\) in their work for the determination of hyperforin, hypericin and pseudohypericin in human plasma hypericin was extracted from plasma samples with phosphate buffer (pH 4.0) and acetonitrile mixture (2:3). In the same study, hyperforin was extracted with a mixture containing n-hexane: ethyl acetate (90:10) at room temperature.

In modern methods, Barnes\(^102\) reported a computer controlled, counter current, solvent extraction system using methanol/water solvent in which 99% of the methanol is recovered. Similarly Yanyan and Ang\(^103\) reported a small-scale supercritical fluid extraction (SFE) method for the selective extraction of phloroglucinols from St. John’s Wort leaf/flower mixtures using supercritical carbon dioxide and optimized the conditions. Using this method hyperforin and adhyperforin were extracted.

**Analysis**

There are many reports for the identification and quantification of active constituents viz., hypericin, hyperforin and their analogs. TLC and HPLC analysis used by many researchers reported in the literature are summarized.

**Thin Layer Chromatography**

Wagner and Bladt\(^104\) described TLC detection of hypericin from methanolic extracts and commercial trade samples of *H. perforatum* on TLC
plates. The solvent system consists of ethylacetate-formic acid-glacialacetic acid-water (100:11:11:26). The hypericins were seen as prominent red-violet fluorescent zones in UV-365nm after treatment with NP/PEG (Natural Products-Poly Ethylene Glycol) reagent. The hypericins were also identified in visible region as green-brown after chemical treatment with 10% pyridine in ethanol. Mulinacci et al 81 described TLC-densitometry for the qualitative analysis of hypericin using CAMAG TLC system. The mobile phase used was toluene-ethylacetate-formic acid (50:40:10). Densitometric evaluation of the spots was performed by using CAMAG TLC Scanner II, detection by fluorescence is under an excitation wavelength of 313 nm. Kartnig and Gobel 86 explained TLC-densitometry for the determination of hypericins using TLC aluminum sheets coated with silica gel 60 and were developed in unsaturated tanks. The mobile phase used was toluene-ethylformiate-formic acid (5:4:1) and the detection was made with UV light at 360nm. Adam et al 85 described a TLC method for detection of hyperforin using a mixture of hexane and ethyl acetate (9:1 v/v) as mobile phase. Spraying a mixture of anisaldehyde-sulphuric acid-acetic acid (1:2:100 v/v) did the detection. Hyperforin afforded a blue spot with Rf value of 0.42.

**High Performance Liquid Chromatography**

The pharmaceutical significance of St. John’s Wort by the active constituents has led to their evaluation by various isocratic and gradient types of HPLC methods in the dried plant material, flowering tops, tissue cultures, commercial formulations and in vitro animal studies.

**Dry plant Material**

There are few reports of HPLC for the estimation of active constituents from dried powdered plant material. Ganzera et al 106 reported a HPLC method for the determination of active constituents which consists of a Phenomenex Synergi-NAX RP 80 A column (150 × 4.6mm, 4 µm). The mobile phase consisted of 10 µm ammonium acetate buffer adjusted to pH 5.0 with glacial acetic acid (A) and a 9:1 mixture of acetonitrile and methanol (B) with gradient elution. The detection wavelength was 270nm. Sirvent and Gibson 87 reported a rapid isocratic HPLC analysis of hypericins using Diazem-phenyl (Metachem, 5 µm, 250 × 4.6 mm) column, and acetonitrile-methanol-water-phosphoric acid (48:40:10:2) as mobile phase. Primary detection of hypericins was done at 590nm with secondary detection at 254nm.

**Flowering Tops**

In view of major contributions of heterodianthrones and phloroglucinols from flowers, HPLC methods were established for the extractions exclusively made from air-dried blossoms. Poutaraud et al 10 reported a HPLC method for the quantification of naphthodianthrones and phloroglucinols using short linear gradient system. The analysis was carried out at 40°C on a Nucleosil – 100 end capped RP 18 column using three solvents A-acetonitrile, B-water: 85% phosphoric acid (99.7:0.3, v/v) and C-methanol with a linear gradient programme. The detection of hyperforins is at 270 nm and hypericins at 590 nm including their protoforms. Broilis et al 85 (1998) described a HPLC method for the identification and quantification of active constituents using 201 TP 5/4 RP-18 column 250 × 4.5mm i.D. 5 µm, 300 °C, protected with an Alltech direct-connect universal column prefilter of 2 µm porosity. The separation followed a linear gradient programme with eluents A-water: 85%, phosphoric acid (99.7:0.3 v/v), B-acetonitrile, and C-methanol. The active constituents were detected at 270nm. Piperopoulos et al 85 reported the determination of naphthodianthrones by LC-electrospray mass spectrometry, using a Lichrosorb RP 18 column (125 × 4mm i.d., 5 µm) protected with a Lichrospher 100 RP 18 guard column (4 × 4mm i.d., 5 µm). The solvent system was methanol-acetonitrile (5:4) (solvent A) and 0.1M aqueous triethylammonium acetate (solvent B) in a gradient programme. The detection was in the visible range at 590nm. A HPLC-DAD quantitative analysis of hypericin described by Mulinacci et al 81 consists of 4.6 × 250mm, 5 µm Lichrosorb RP 18 column maintained at 26°C. Three mobile phase components consists of water adjusted to pH 3.2 by addition of H3PO4(component A), methanol (component B) and CH3CN (component C) were used to prepare the multistep linear solvent gradient programme. Hypericin and pseudohypericin were easily identified and quantified at 590nm.

**Tissue Cultures**

To study the accumulation of phytoconstituents in tissue culture experiments, Tolonen et al 83 described
a HPLC method for determination of naphthodianthrones and phloroglucinols from cell culture extracts. The compounds were eluted in Waters Xterra RP18, 2.1 \times 50\text{mm} column with 3.5 \mu m particle size at 35°C. The elution was done with a gradient programme using aqueous 20\text{mM ammonium acetate (A) and acetonitrile (B).} All the naphthodianthrones and phloroglucinols were well separated and detected by tandem mass spectrometry. Kirakosyan et al\textsuperscript{77} reported a HPLC method for the analysis of hypericins in shoot cultures using Cosmosol 5C 18-MS C 4.6 \times 250 \text{mm column.} The mobile phase consisted of A-0.5% TFA (Trifluoro Acetic acid) in water, B-70% MeCN - 29.5% methanol - 0.5% TFA in a gradient-time programme. The peaks were observed on the chromatogram by UV absorption at 588 nm. Walker et al\textsuperscript{79} in a HPLC-MS method for the analysis of hypericin in cell suspension cultures, used a 5\mu m, C18 column of 25 \times 4.6 \text{cm} size. The solvent system used was A-70% solution of 1% ammonium phosphate (\textit{pH} 7 adjusted with NaOH) and 30% acetonitrile, B-acetonitrile, 30% water with a gradient programme. The hypericin was detected in visible absorbance at 590 nm by a PDA (Photo Diode Array) detector. Kartnig et al\textsuperscript{1} described a HPLC for the determination of hypericins in cell cultures of various \textit{Hypericum} species and their chemotypes. The analysis was carried out with a column of Lichro Cart RP 18 Supersphere 250 \times 4\text{mm} with RP 8 precolumn. Eluent used was acetonitrile-methanol-water-phosphoric acid (55:20:24:1). The UV detection was done at 254 nm. Dias et al\textsuperscript{98} reported a HPLC method for the analysis of the phenolic fractions from \textit{in vivo} and \textit{in vitro} biomass of \textit{Hypericum} spp. The analysis was carried out using a Lichro Cart RP 18 end capped Supersphere column (150 \times 4\text{mm ID}, 4\mu l) with a precolumn of the same material. Mobile phase consisted of water-formic acid (95:5) as eluent A and methanol as eluent B with a gradient programme. The detection of hypericin was done at 590nm.

**Formulations**

Owing to the huge demand for hypericin and hyperforin as antidepressants and in the treatment of AIDS, number of St. John’s Wort formulations are coming in the market. In order to ascertain the quality of these products the most sensitive and reproducible methods of HPLC were reported.

Catharina et al\textsuperscript{86} described a HPLC method for the determination of hypericin and pseudohypericin derivatives in dietary supplements and functional foods. The constituents were eluted in a reverse phase column, Phenomenex Luna 3\mu m, C\textsubscript{18} (ODS-2) 4.6 \times 150 \text{mm using acetonitrile-triethylammonium acetate buffer (4:1) as solvent system.} Benzo[k]fluoroanthene was used as internal standard. The components were detected at 290nm and 590nm. Confirmation analysis was carried out with LC/electrospray ionization (ESI)/MS analysis.

Liu et al\textsuperscript{92} established a RP-HPLC method with PDA detection and LC-electrospray ionization - MS confirmation of major active constituents in St. John’s Wort dietary supplements. The analysis was carried out on a Lichrospher end-capped RP-C\textsubscript{18} 3\mu m, 250 \times 4\text{mm column with a linear gradient programme.} The mobile phase consists of A-0.5% TFA in Water, B-0.5% TFA in mixture of methanol-acetonitrile (13:7) in linear gradient programme. Absorption was measured with PDA detector at full spectrum (200-790 nm). Li and Fitzloff\textsuperscript{87} established an RP-HPLC method with PDA detection for the determination of major constituents in St. John’s Wort dietary supplements. The analysis was carried out at 20°C on a Waters YMC OD3-Aq\textsuperscript{18} RP-18 column, which was protected by a Waters delta-PAK RP-18 guard column. The mobile phases consisted of water (containing 20% methanol and 0.5% TFA) and acetonitrile (containing 10% methanol and 0.5% TFA) and the analysis followed a linear gradient programme. The UV absorption for hypericin and hyperforin along with other constituents were studied at 270nm and 590 nm. Michael\textsuperscript{94} described HPLC analysis of hypericin with PDA and MS detection. The HPLC column used for system 1 was C\textsubscript{8} Waters, 150 \times 2.1 \text{mm with 0.3 ml/min flow rate and for system 2 was C\textsubscript{4} Waters 150 \times 3.0 \text{mm with 0.4ml/min flow rate.} The chromatographic eluents were A-10mM triethylamine acetate (\textit{pH} 7.0), B-methanol, C-acetonitrile in a linear gradient programme. The detection of hypericin was done at 588 nm. Hansen et al\textsuperscript{95} in a HPLC method on-line coupled to High-Field NMR and mass spectrometry for structure elucidation of naphthodianthrones, flavonoids and other constituents of \textit{H. perforatum} used Knauer column 120 \times 4 \text{mm i.d. packed with Apex-1 ODS, 5\mu m column packing material. The mobile phase was made up of A-acetonitrile and
0.1% acetic acid (5:95 v/v), B-acetonitrile and 2mM ammonium acetate (98.5 v/v) in gradient conditions. The detection of components was done at 254nm. Denke et al.\(^\text{88}\) used Hypersil ODS 5µm, 250 × 4.6 mm column for the analysis of hypericin in their studies on biochemical activities of extracts. The mobile phase consisted of mixture of methanol/ethyl acetate/buffer, pH 2.1 (800/195/205/v/v/v) with isocratic elution. Hypericins were detected at 590nm. In another report\(^\text{89}\) for the analysis of constituents they used Macherey – Nagel EC 125/4.6 Nucleosil 100-5 C\(_{18}\) AB column. The eluents were A - 50 mmol/L sodium phosphate (pH 2.3), B-50 mmol/L sodium phosphate, (pH 2.3), 40% methanol and 49% acetonitrile in gradient-time programme. The detection of constituents was done at 254nm using photodiode array detector. Reyes and Koda\(^\text{9}\) described a HPLC method for the simultaneous determination of hypericins and stabilized hyperforin in commercial St. John’s Wort preparations using C\(_{18}\) column. Isoocratic elution was done with a mobile phase consisting of acetonitrile and 0.3% v/v phosphoric acid (90:10 v/v), with simultaneous fluorescence (315/590 nm, excitation/emission) and UV (273 nm) detection.

**In vitro Animal Studies**

HPLC method of analysis was even successful in estimating the concentration of active constituents in *in vitro* studies (human plasma). Yanyan et al.\(^\text{99}\) in a HPLC method reported the determination of hyperforin in human plasma using Luna C\(_{18}\)-150 × 4.6 mm, 3µm particle size column. The mobile phase consists of methanol-acetonitrile (3:2 v/v) mixed with water (92:8). The hyperforin was detected at 287 nm with Waters model 996 PDA detector.

Leibes et al.\(^\text{100}\) reported a HPLC method for the quantification of hypericin in biological fluids. The analysis was carried out on a reverse phase, phenyl 4µm, and 0.45×10cm radial pack analytical column. The mobile phase consisted of Solution A-70% solution of 0.1% ammonium phosphate (pH 7 with NaOH) and 30% acetonitrile, Solution B-70% acetonitrile and 30% water, in a linear gradient programme. The detection of hypericin in visible range was done at 590nm. Chi and Franklin\(^\text{106}\) reported a HPLC method for the measurement of hypericin in plasma using 5µm particle size mixed mode (C\(_{18}\)/CN) analytical column and a model 975 UV detector set to the optimal wavelength of 272nm using end point detection. The mobile phase consisted of 81% acetonitrile in water adjusted to pH 4.5 (with 1M H\(_3\)PO\(_4\)). Bauer et al.\(^\text{101}\) reported a HPLC method with fluorescence and UV detection for the determination of hypericin, pseudohypericin and hyperforin in human plasma. Hypericin and pseudohypericin were separated at 60°C in a Lichrospher RP select B column (5µm, 250 × 4.6mm i.d.) with a guard column and quantification by fluorescence detection at 315/590nm. For mobile phase preparation 6.1ml of concentrated phosphoric acid in 950ml distilled water, pH adjusted to 4.0 by NaOH (30% in water) made up to volume 1000ml with distilled water. A 300ml of this aqueous solution was combined with 450ml methanol and 250ml tetrahydrofuran to constitute the mobile phase. For hyperforin, samples were analyzed at 50°C on a Phenomenex Luna C\(_{18}\) column (5µm, 250 × 4.6mm i.d.) with a guard column and quantified by UV detection at 273nm. The mobile phase consisted of acetonitrile, 0.01M sodium hydrogen phosphate buffer (pH 2.4) (90:10):

**Conclusion**

The review describes chemical profile, pharmacology and analytical methods of *H. perforatum* with special reference to hypericin and hyperforin. Analysis by TLC and a number of HPLC methods were reported for the determination of hypericin and hyperforin from plant material, tissue cultures, commercial products and *in vitro* (plasma) studies have been summarized for further applications. Limited natural sources, variation of amounts of active constituents in wild plants, efficacy and safety of natural phytoconstituents led to their alternative production by *in vitro* methods. Hence, tissue culture methods have been described for the production of these valuable secondary metabolites.

To meet the increasing demand for novel compounds, reports on micropropagation and elicitation of key constituents by tissue culture methods could offer a possible solution. To further strengthen these approaches there is a need to attempt transformed root cultures, permeabilization and immobilization in cell cultures. Molecular studies and metabolic engineering of hypericin, hyperforin and their analogues may contribute significantly to enhance their production through tissue cultures.
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St. John’s Wort may be toxic during pregnancy and lactation in rats

In recent years, concerns about St. John’s Wort (Hypericum perforatum Linn.) use during pregnancy and breastfeeding have emerged. The scientists from Italy investigated the toxicity of this herb in Wistar rats, administered prenatally and during breastfeeding (from 2 weeks before mating to 21 days after delivery). Two doses of the extract were chosen, 100 mg/kg per day, which, based on surface area, is comparable to the dose administered to humans, and 1000 mg/kg per day. A microscopical analysis of livers, kidneys, hearts, lungs, brains, and small bowels was performed. A severe damage was observed in the livers and kidneys of animals euthanized postnatally on days 0 and 21. The lesions were more severe with the higher dose and in animals that were breastfed for 21 days; however, an important renal and hepatic damage was evident also with the dose of 100 mg/kg per day. In addition, similar serious hepatic and renal lesions were evident also in animals that were exposed to hypericum only during breastfeeding. The results indicated that appropriate histological studies should be performed in other animal species to better evaluate the safety of hypericum extracts taken during pregnancy and breastfeeding [Gregoretti B, Stebel M, Candussio L, Crivellato E, Bartoli F and Decorti G, Toxicity of Hypericum perforatum (St. John’s Wort) administered during pregnancy and lactation in rats, Toxicol Appl Pharmacol, 2004, 200 (3), 201-205].