Studying the possible biotransformation of Antihistaminic Loratadine using *Phaseolus vulgaris* L. cell suspension culture

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**ABSTRACT**

The aim of this research was to perform biotransformation for Loratadine drug to either Desloratadine or denovo secondary metabolites using cell suspension cultures of *Phaseolus vulgaris*, of the family Fabaceae. A solid medium was developed for the cultivation of *Phaseolus vulgaris* L. seeds and establishment of callifrom hypocotyls. The inoculation of the cell suspension cultures and biotransformation of Loratadine was monitored using the produced calli and TLC and HPLC techniques. Seeds of *P. vulgaris* L. were germinated on MS solid medium and the hypocotyls excised and thereafter cultured on MS medium supplemented with BA (0.5 mg/L) and 2,4-D (5 mg/L). The resulted calli were used to establish different sets of cell suspension cultures in variable amounts (2, 4 and 8/150 ml), respectively. Loratadine was added to each set with different concentrations (5, 10 and 15 mg/150 ml) and after 10 days of incubation on the shaker the samples were withdrawn for 4 consecutive days (11th to 14th day). A callus line of *P. vulgaris* was maintained for four years without change in growth rate. The cells reached their maximum bioavailability on the 23rd day (around 4 weeks). On adding Loratadine with variable concentrations (5, 10 and 15 mg/150 ml) to callus cells (2, 4 and 8 g/150 ml), some peaks evoked as traced by HPLC after 10 days of incubation but not the same results were obtained in all cases. Desloratadine peak was evoked in nearly all cases but with trace amounts together with other secondary new metabolites formed. This would be the first report of a biotransformation trial using *P. vulgaris* cell cultures which gave highly positive results. This would be the first report on *P. vulgaris* to be used in biotransformation of Loratadine.

**Key words:** *Phaseolus vulgaris*, Loratadine, Desloratadine, cell culture, biotransformation.

**INTRODUCTION**

*Phaseolus vulgaris* L. or common beans belonging to the family *Fabaceae* is a member of the subtribe *Phaseolinae*. They are dicotyledonary legumes, herbaceous, annual plants and popular as dry and green beans. They are of many varieties and widely distributed worldwide ([Source](http://www.users.globalnet.co.uk/~aair/allergy.htm)). *P. vulgaris* L. is rich in flavonoids; 2 groups of flavonoid glycosides exist as flavonols namely quercetin and kampeferol -3-O-glycosides and flavones namely apigenin and luteolin -7-O-glycosides and proanthocyanidins ([Onilagha and Islam, 2009](http://www.users.globalnet.co.uk/~aair/allergy.htm)). Furthermore, the seeds are consumed, worldwide as main source for proteins and fibers. Allergy is a disease of high prevalence since 1964 in school children ([Source](http://www.users.globalnet.co.uk/~aair/allergy.htm)). A fascinating new study by the Asthma and Allergy

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Foundation of America found that most patients are interested in finding a new prescription allergy medication because they are unsatisfied with the drug administered to them (http://www.vaxa.com/prescription-allergy-medication.cfm). The most commonly used treatments to relieve allergy symptoms are antihistaminics. They are classified into two (2) classes namely: mast cell stabilizers and histamine antagonist. This second class comprises of two (2) generations namely: the first generation (sedative antihistaminics): which became rarely used due to their side effects as they pass the Blood Brain Barrier (BBB) causing sedation which is considered a great drawback for its use, while the second generation includes drugs with limited ability to penetrate BBB and as such is free from side effects, especially sedation.

Both Loratadine and Desloratadine are drugs of choice to accomplish our study since they are more advantageous in allergy treatment than the other members of the second generation. They both have the longest elimination half-lives (11 and 26.8 h, respectively) and rarely eliminated unchanged, hence, the human body gets complete benefit from the drugs administered once daily to achieve patient compliance, least possible drug interactions and relieve different allergy symptoms by acting on H1 and H2 receptors (Estelle and Simons, 2003; Renwick, 1999; Babina et al., 2002).

Several attempts were reported concerning trials for biotransformation of Loratadine using animals, microorganisms, plant cell cultures and also chemically mediated transformations revealing the production of several secondary products including those mentioned in Table 1.

The main enzyme responsible for Loratadine metabolism giving its bioactive form is Desloratadine and this is made possible through CYP 450 3A4 by oxidation and hydrolysis; Desloratadine is long lasting depending on the concentration at steady state but however, considered of the longest half life time in comparison to other drugs of second generation antihistaminics (Estelle, 2003; Renwick, 1999; Babina et al., 2002).

P. vulgaris L. was reported to contain microsomal NADPH cytochrome P450 reductase (CPR) and NADPH Ferricytochrome b5 oxidoreductase. CPR is flavin adenine mono and dinucleotide linked (FMN and FAD), whereas B5R is FAD linked. The cytochromes are linked to the endoplasmic reticulum membrane and CPR exists on the outer membrane of the chloroplast (Brankova et al., 2007).

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Table 1: Loratadine biotransformation reactions performed by animal, micro-organisms, plant cell cultures lines and chemically.

<table>
<thead>
<tr>
<th>Culture type</th>
<th>Enzyme</th>
<th>Product</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human liver</td>
<td>Microsomal enzymes CYP3A4 with PMSF (Phenyl Methyl Sulfonyl Fluoride) and CYP2D6</td>
<td>R=OC(OH)CH₂; R=OC(OH)CH₂; R=H</td>
<td>(Robert et al., 1996)</td>
</tr>
<tr>
<td>Rat, monkey and rabbit</td>
<td>5 mg Ig polyclonal rabbit antibodies, CYP 3A1 inhibitor in rats and radiolabelled drug administered by Man, Monkey and Rat; C14*</td>
<td>R=H converted to R=OH hydroxyl desloratadine</td>
<td>(Robert et al., 1996)</td>
</tr>
<tr>
<td>Histamine receptor gene from the lungs of man cloned in Chinese Hamster cells</td>
<td>Histamine Receptor cloned sequence by PCR and mRNA Chlorpheniramine</td>
<td>R=H</td>
<td>(William et al., 2002)</td>
</tr>
<tr>
<td>Tracheal submucosal cells of Yorkshire white Male Swine</td>
<td>Add salt bridge and Ag, AgCl pellet electrodes, histamine (agonist), Acetyl choline (antagonist)</td>
<td>On adding agonist Desloratadine increases and on adding antagonist Desloratadine decreases</td>
<td>(Liu and Farley, 2005)</td>
</tr>
<tr>
<td>Female Rabbit and Guinea; Pig iris smooth muscle</td>
<td>In Rabbit; add Indomethacin, CO₂, O₂, Carbachol In GP; Loratadine added directly to iris smooth muscle to measure pupil mydriasis</td>
<td>In Rabbit; Desloratadine increases and replaces carbachol InGP; mydriasis occur</td>
<td>(Cordelus et al., 1999)</td>
</tr>
<tr>
<td>Man bronchial epithelial cells with explants</td>
<td>NO₂ gas</td>
<td>R=H</td>
<td>(Bayram et al., 1999)</td>
</tr>
<tr>
<td>Nicotiana tabacum (Petit Havana)</td>
<td>Kanamycin 50mgrams/L and Exogenous CYP 450 3A4</td>
<td>R=H</td>
<td>(Andreas and Matthias, 2009)</td>
</tr>
</tbody>
</table>
Therefore, according to the aforementioned postulations and literature on Loratadine drug and \textit{P. vulgaris} plant, we decided to establish cell culture of this plant and utilize it to incubate Loratadine to produce its biotransformation products. This would be the first report of a biotransformation trial using \textit{P. vulgaris} cell cultures.

**MATERIALS AND METHODS**

**Chemicals**

The solvents used were of the analar grade; Chloroform, Dichloromethane, Ammonia, Acetone and Dimethyl sulfoxide (DMSO). Murachige and Skoog medium (MS 1962), solid media components; MS salt (Duchefa haraleem Netherland), 2,4 – Dichlorophenoxy acetic acid (2,4-D)(Sigma London Chemical Company LTD Poole Dorset) and 6 Benzyl adenine (6BA)(Nice Chemicals Pvt. cochin, Roth company). Spraying reagents used were Dragendorff, Bismuth subnitrate, Distilled water, Glacial acetic acid and KI (Wagner, 1984).

**Equipments**

Seed inoculation was done under Laminar Air flow Cabinet Dalton 19 OAG. Media was cooked and prepared with Magnetic Stirrer, Labvolt Plaque Chauffante. Seeds for germination and calli for completing their growth were incubated in Hoffman Manufacturing INC. USA Incubator. Jars of liquid suspension media with callus cells and drug were put on Dalhan Labtech LTD.

Shaker melting point was determined by Gallenkamp variable heater, while IR spectra were obtained from IR spectrometer 07122009 at Micro Analytical Center at Faculty of Science Cairo university, 1HNMR spectra obtained by JEOL apparatus SCC machine DELTA2NMR at National Center for Research and Main Laboratories for War Chemical, Mass spectra and apparatus equipped with electron spray ionization used to generate negative ions at Alazhar university and Main Laboratories for War Chemical.

HPLC peaks were obtained by Agilent technologies apparatus 1200 infinity series (Wilmington, DE, USA), ODS reversed phase column (C$_{18}$ 250×4.6mm i.d., 3 µm particle size) at National Organization for Drug Control and Research. TLC plates, silica gel 60F$_{254}$ and DC Keiselgel F$_{254}$ 20×20 cm were also used in the experiment. TLCs were monitored under UV lamp CN-6T VILBER LAURMAT, France and by spraying with Dragendorff reagent followed by heating in Memmert Oven. LC-MS apparatus used were TSQ Quantum Access MAX triple stage quadrupole mass spectrometer, Thermoscientific, New York, USA, equipped with an electrospray ionization (ESI) source. Xcalibur software version 2.2 was used to control the LC-MS/MS system; thereafter, the data was collected and analyzed in Helwan University.

**Substrate**

Loratadine and Desloratadine were taken from the National Organization for Drug Control and Research Standards Department and structures identified by IR, 1 H and 13C NMR spectral analyses and observed data were compared to reported data in the literature (Martindale 36$^{\text{th}}$ edition (1), British Pharmacopeia 2010 (2), British Pharmacopeia 2009 (2), United States Pharmacopeia 32, Clark’s 3$^{\text{rd}}$ edition (2). The substrate was dissolved in ethanol and then added to the liquid suspension media and methanol before HPLC analysis.

**Establishment of callus and cell suspension cultures**

**Preparation of Explants for Calli Induction**

\textit{P. vulgaris} seeds purchased from the Egyptian market were thoroughly washed with tap water and surface sterilized with 50% calcium hypochlorite (clorox) for 10 min followed by thorough rinsing with distilled water three consecutive times under sterile laminar air flow cabinet. The sterilized seeds were then incubated in incubator at 23 to 25°C for 7 to 10 days on sterilized cotton pads wetted with distilled water; thereafter, the resulted hypocotyls were excised and cut into pieces (1 to 2 cm) and inoculated on solid MS media.

**Formulation and preparation of culture media**

MS medium was prepared for calli formation by weighing 4.43 g/L of MS solid medium adding 3% sucrose in about 600 ml distilled water and supplemented with 2,4 D 5 mg/ml and BA 0.5 mg/ml and volume was completed to 1 L with distilled water; pH was adjusted from 5.7 to 5.8 using 0.1 N HCl or 0.1 N KOH before adding agar (0.8%w/v). After cooking on hot plate stirrer, 25 ml aliquots were dispensed into the culture bottles to be autodaved for sterilization at 120°C and 1.5 psi for 15 min. MS medium for cell suspension cultures were prepared by the same procedure but without adding agar and then divided into seven (7) jars each containing 150 ml.

**Callus induction**

The hypocotyls of \textit{P. vulgaris} were placed on MS solid media at temperature 25 ± 2°C 16 h / day photoperiod with light intensity 1000 Lux provided by cool white florescence lamp and then incubated for 3 to 4 weeks.
**Initiation of cell suspension culture**

Different weights of calli (2-4-8 g) were aseptically divided into suspension culture vessels containing 150 ml each of the culture media. The jars were shaken for 10 days (26.4°C and 55 rpm) before adding the substrate and thereafter completed for further 4 days after aseptic application of Loratadine. This procedure ensured homogenous interaction between calli cells and the drug to enable the author study the effect of the drug on the viability of the calli cells and the effect of the cells on the biotransformation of the drug under study.

**Incubation and extraction**

Seven sets of cell suspension cultures were established, each containing 150 ml liquid suspension medium and 4.3 g calli cells and left on shaker for 10 days and on the 10th day Loratadine authentic drug 10 ml =10 mg/ml -dissolved in the least possible amount of dimethyl sulfoxide DMSO was added and completed with ethanol to keep cells viable; this was added using aseptic technique and the drug passed through autoclaved bacterial micro-filter. The samples were collected on seven (7) successive days; each sample was dissolved in 10×3 ml of chloroform and thereafter sonicate to destroy the cells; every of the sample was added to the flask and Buckhner funnel and filter paper attached; it was first washed with distilled water, thereafter the sample was passed through filter paper and finally washed again with 10 ml chloroform. The content of the flask was taken and the lower organic chloroformic layer separated after shaking with chloroform. Round bottom flask was then used collect the organic layer after passing through anhydrous sodium sulfate to ensure absence of water droplets; the Round bottom flask was fixed to the rotavapour apparatus, while the organic solvent evaporated leaving behind the residue. The residue was dissolved in the least possible amount of chloroform solvent for TLC analysis. TLC plate of dimensions 5×10 cm was developed in pre-saturated flat bottom developing chamber with mobile phase acetone: ammonia: distilled water in ratio 18 ml:1.4 ml:0.6 ml and after mobile phase run, it was examined under UV lamp with short and long wave lengths of 254 and 365 nm; thereafter, HPLC was performed on all samples on Agilent Technologies 1200 infinity series (Wilmington, DE, USA), ODS reversed phase column (C18 250×4.6 mm i.d., 3 μm particle size) using mobile phase containing Buffer: Acetonitrile: Methanol with ratio 50:40:10 and flow rate 0.8 ml/min and the sample dissolved under test in HPLC grade methanol first and then in parts of the mobile phase (Bondili and Ramya, 2011).

**Cell viability test**

Sub-culturing line was maintained to get continuous cell line; 4 weeks old calli were sub-cultured on MS solid media 5 mg/L 2,4-D, 0.5 mg/L BAP and after 4 weeks well developed calli were obtained.

**RESULTS**

**Growth characteristics of calli and suspension culture of Phaseolus vulgaris**

MS solid media was established with auxins and cytokinins concentrations as reported from previous trials; 2,4-D 5 mg/ms/L and BAP 0.5 mg/ms/L which gave optimum calli formation after hypocotyls inoculation for 4 weeks giving healthy fast growing buff friable calli (Table 2). Sub-culturing for calli cells was achieved every 4 weeks otherwise calli cells becomes dark due to phenolic compounds accumulation. Fast growing calli cell lines were maintained for 4 years of successive work without change in rate of growth (Andreas and Matthias, 2009).

**Initiation of cell suspension culture**

Calli cells were incubated with variable amounts of liquid suspension media as single cells or cell aggregated of variable size or number and left on the shaker for 10 days in view of the fact that on the 10th day maximum increase in fresh weight of calli cells will be obtained; turbid cell suspension culture was also obtained.

**Detection of biotransformation product**

After incubation of Loratadine with P. vulgaris calli in liquid suspension media facing 90 rpm, 26°C and darkness on shaker, samples were withdrawn on seven (7) successive days and the evaporated samples analyzed using TLC and HPLC techniques. TLC was examined under UV lamp short wave length (254 nm) and large wave length (365 nm). Dragendorff reagent was used to spray TLCs to detect the presence of Loratadine and placed in an oven at temperature 150°C for 1 min; the presence of orange spots indicate Loratadine remnants since not all drug was completely biotransformed and even some of the biotransformed drug return by reversible reaction to give back Loratadine since the bond broken in Loratadine is highly unstable and easily hydrolysable.

Further investigations of the same extract was dissolved and filtered in HPLC grade methanol (Diluent 1) and then in part of the mobile phase buffer: Acetonitrile:Methanol 50:40:10 (Diluent 2) and HPLC technique applied to confirm the presence of many biotransformed compounds including Desloratadine with small amount in all samples with exception in the case of using 2 g calli cell samples. Thereafter, the samples of highest abundancy of
Table 2: Trials done with changing factors of calli cells amount, liquid suspension media and drug concentration to study the exact time of biotransformation occurrence.

<table>
<thead>
<tr>
<th>Liquid suspension media (ml)</th>
<th>Calli cells (g)</th>
<th>Authentic Loratadine (g)</th>
<th>Number of days on gyratory shaker</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>2</td>
<td>10</td>
<td>11-17 days</td>
</tr>
<tr>
<td>300</td>
<td>8</td>
<td>20</td>
<td>11-17 (leave the whole 300 ml on shaker for 10 days then on adding the drug divide it on 7 sterilized containers equally)</td>
</tr>
<tr>
<td>750</td>
<td>20</td>
<td>50</td>
<td>11-17 (leave the whole 750 ml on shaker for 10 days then on adding the drug divide it on 7 sterilized containers equally)</td>
</tr>
<tr>
<td>150</td>
<td>4</td>
<td>10</td>
<td>11-14 days</td>
</tr>
</tbody>
</table>

Desloratadine peak which were of 8 g calli cells, 10 and 15 mg Loratadine drug on 11th and 12th day were separated and LC/MS fragmentation done on them for verification of Desloratadine occurrence with its fragmentations.

Cell viability

Small cell culture aggregates on MS medium supplemented with 5 mg/ms/L 2, 4-D and 0.5 mg/ms/L BAP indicate noticeable growth within 4 to 6 weeks which later proliferate to well-developed calli cells.

DISCUSSION

Application of biotransformation technique for production of secondary metabolites using plant tissue culture catalyzed the transformation of readily available inexpensive precursors to more valuable final products by means of plant enzymes. Plant cultures retained an ability to transform exogenous substrates into products of interest including aromatic, steroid, alkaloid, coumarin, terpenoid, lignin and other molecular species. It is not necessary for the compounds to be natural intermediates in plant metabolism. Plant bioconversion systems may be used alone to produce novel chemicals or in combination with organic synthesis. Multistep processes catalyzed by cell or organ culture often generate intermediary metabolites which help to establish biosynthetic pathways (Krishna, 2002; Dekker, 2004; Maria et al., 2005; Colin et al., 2006; Kintzios, 2008, 2009; Franssen and Walton, 1999).

_P. vulgaris_, the plant of choice to apply biotransformation study of Loratadine contain microsomal NADPH cytochrome P450 reductase(CPR) and NADPH ferricytochrome _b_5 oxidoreductase. CPR is Flavin adenine mono and dinucleotide linked (FAD and FMN), while BSR is FAD linked. NaCl or salinity increases the activity of reductases. This study was carried out on beans and leaves which indicate that the enzymes are highly prevalent in the plant parts (Brankova et al., 2007).

Plant tissue culture and the growth of plant cells outside an intact plant is a technique essential in many areas of plant sciences. It relies on maintaining plant cells in aseptic conditions on a suitable nutrient medium. The culture can be sustained as a mass of undifferentiated cells for an extended period of time or regenerated into whole plants (Murashige and Skoog, 1962; Schenk and Hildebrandt, 1972; White, 1963; George et al., 1987). Thus, we inoculated the explants in a suitable medium under aseptic technique and from this callus initiated the suspension cell culture medium composed of 33 ml liquid MS, 2 mg/L and 2, 4-D. We thereafter took the friable callus with the sterile forceps and then applied it to the liquid medium and placed it on a shaker at 90 rpm and 26°C. There was lack of photoperiod to break down the cell aggregates into smaller clumps of cells to maintain a uniform distribution of cells and cell clumps in the medium that provide gas exchange. This plot shows plant culture growth profile. The lag phase: it was subcultured from it in fresh medium for cells to regain ability to divide. The Exponential phase: rapid cell division depends on the cells and nutrition and as such this
stage varies in duration but is usually short and only gives rise from 3 to 4 generations. The Linear and Progressive deceleration: rate of cell division declines. Finally, the Stationary phase: this is the phase where cell division ceases (Abo and Hildebrandt, 1971, 1973).

Sub-culturing line was maintained to get continuous cell line; 4 weeks old calli were sub-cultured on MS solid media 5 mg/L 2, 4 D, 0.5 mg/L BAP and after 4 weeks well developed calli were obtained. After the trials were done, we concluded that Desloratadine obtained in the following conditions: 4 to 8 g calli/150 ml liquid suspension media with authentic Loratadine 5 or 10 or 15 mg/ms added; 26°C, 90 rpm and in darkness for 10 days; Desloratadine appears on the 11th to 14th day but maximum result was obtained in 8 g calli/150 ml liquid suspension media with authentic Loratadine 10 or 15 mg/ms added on 11th and 12th day. These results were done on the extracellular samples and measures drug concentration in liquid suspension media; compartmentalization and Loratadine biotransformation intracellularly still requires further investigation.

**Conclusion**

We were able to achieve fast growing calli cells of *P. vulgaris* for consecutive 4 years without change in rate of growth and cell suspension culture of high viability was maintained for 10 days on shaker; 90 RPM, 26°C, and previous reported papers showed *Phaseolus* has metabolizing enzymes which could metabolize Loratadine. After the study, resulted biotransformation using TLC and HPLC proved occurrence of biotransformation of Loratadine to secondary metabolites including Desloratadine in small amount. The study of the BAV and pharmacological action of the other secondary metabolites formed were compared to Loratadine and Desloratadine. The study of the biotransformed secondary metabolites intracellularly– the compartmentalization of the biotransformed drugs still need to be further investigated. Study of the calli cells produced from hypocotyls of *P. vulgaris* is however a promising system for biotransformation of other drugs.

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