ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by Pharmascope Publications Journal Home Page: www.pharmascope.org/ijrps

Influence of macerating enzyme - Cellulase on the extraction of valuable compounds: Carotenoid and Camptothecin

Sujatha Natarajan, Sangilimuthu Alagar Yadav*, Sowmya Priya Manoharan

Department of Biotechnology, Karpagam Academy of Higher Education, Coimbatore-641 021, Tamil Nadu, India

Article History:

Received on: 06.01.2018
Revised on: 22.02.2018
Accepted on: 27.02.2018

Keywords:

Camptothecin
Carotenoid
Cellulase enzyme
Extraction
HPLC

ABSTRACT

Macerating enzymes breaks long chain compounds during maceration for extraction of industrially important phytomolecules. Cellulase is an important class of enzyme which helps in the extraction process of phytomolecules such as carotenoids, camptothecin from their natural sources as macerating enzyme. The extraction of phytoconstituents like carotenoids, camptothecin holds high commercial value. The uses of macerating enzymes help to the extraction process of phytomolecules and increase its yield. Quality of the product is also improved in terms of stability, texture and viscosity. Camptothecin is an important drug with potential anti-cancer activity. In this work, the effect of cellulase on the extraction of carotenoid from carrot, tomato and sweet potato have been studied. As a pioneer work, the production of camptothecin from endophytic fungi *Aspergillus niger* has been carried out using cellulase. The quantitative analysis of pharmaceutical important phytomolecules such as carotenoids and camptothecin were performed using UV-Visible spectrophotometer, HPLC with respective standard compounds. Carotenoid extraction was made from tomato, carrot and sweet potato with cellulose enzyme found 2.5±0.25µg/g, 2.2 ±0.18µg/g, 18± 1.75 µg/g respectively. Carotenoid extracted from carrot using enzyme yielded 1.47 times higher amount of carotenoid than that of without enzyme. Carotenoid extracted from tomato, showed the maximum difference of being 7.3 times higher with enzyme than without enzyme. The another industrially important phytomolecule camptothecin extraction was made from *Aspergillus niger* with cellulase enzyme yielded 0.5512 mg/g which is more than the camptothecin, extracted without enzyme (0.175 mg/g). Thus, it was observed that the use of cellulase enhanced the yield of both carotenoid and camptothecin from natural sources such as plants and fungi.

* Corresponding Author

Name: Sangilimuthu Alagar Yadav
Phone: +91-9047298492
Email: smuthu.al@gmail.com

ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v9i1.1200

Production and Hosted by

Pharmascope.org

INTRODUCTION

Cellulases is an important class of macerating enzymes which is widely produced by several microorganisms. Their immense potential in bioprocesses and products have recently been discovered. Their ability to convert cellulosic biomass enables them to act as biocatalysts. Thus they have wide spread industrial applications. Cellulase is produced by many microorganisms. Cellulolytic microorganisms use carbohydrates as energy source for growth as they are unable to use proteins or lipids as energy sources (Lynd *et al*., 2002). Among many microorganisms capable of

producing cellulase, fungi have immense ability to secrete large amounts of extracellular protein. Such fungal strains are most suited for production of extracellular cellulases. *Trichoderma* and *Aspergillus* are the most widely studied cellulase producers (Sukumaran *et al.,* 2005).

Cellulases have a plethora of potential applications in food biotechnology. Fruit and vegetable juice production requires improved methods of extraction, clarification and stabilization. The use of the macerating enzyme complex, Cellulase in the extraction and clarification of fruit and vegetable juices has shown a marked increase in the yield of juices (Minussi *et al.,* 2002 ; Carvalho *et al.,* 2008). Cellulases have also been used in the extraction of carotenoid for the production of food colouring agents (Cinar, 2005).

The colossal use of enzymes in the industrial world has called for a large demand of stable, highly active and specific enzymes. In 1995 it was estimated that the world sale of industrial enzymes would be 1.0 billion US dollars, while the world market for industrial enzymes are expected to be in the range between 1.7 and 2.0 billion US dollars by the year 2005 (Godfrey and West, 1996). According to a recent publication, the industrial enzymes have already reached a market of 1.6 billion US dollars. Interestingly, 60% of the total world supply of industrial enzymes are produced in Europe, and the remaining 40% from USA and Japan. Also, approximately 75% of the industrial enzymes are hydrolases and the second largest group are the carbohydrolases. (Demain, 2000).

During the last two decades, there is an extensive use of cellulases, hemicellulases and pectinases, especially in the textile, food, brewery, wine and in pulp and paper industries. Today, the world enzyme market accounts for approximately 20% of these enzymes, mostly from *Trichoderma* and *Aspergillus*. Currently, there are a variety of tailor-made enzyme preparations that are suitable for biotechnology being marketed by commercial enzyme producers. It is extensively used in the extraction of fruit and vegetable juices, fruit nectars, purees and olive oil. It has a pronounced use in improving the quality of bakery products, beer and wine. It is also used in animal feed technology. In recent times, these macerating enzymes have shown a substantially strong use in the extraction of valuable compounds. (Bhat, 2000).

The hydrolytic enzymes such as cellulases, hemicellulases and pectinases have a discernible property to penetrate the complex tissue matrix of polysaccharides and facilitate the removal of carotenoids due to their cell wall degrading ability (Bauernfeind, 1981). Enzyme assisted extraction has exhibited better results showing higher recovery, reduced solvent usage and lower energy consumption (Bunea *et al.,* 2009).

The need for Carotenoids especially for food, animal feed and pharmaceuticals appear to increase every year. Reports of Business Communication Company (BCC) shows that the current market value of commercially used carotenoids are nearly $1.2 billion in 2010, with a chance to grow to $1.4 billion in 2018 carotene, around $250 million in 2007, with a compound annual growth rate of 2.3 %. The market value has increased to just $261 million in 2010. This market is expected to grow to $ 334 million by 2018 at a compound annual growth rate of 3.1 % (Hudiyono, 2012).

Camptothecin (CPT) is a pilot compound used in anticancer treatment. CPT derivatives (CPTs) manifest anticancer activity both *in vitro* and *in vivo*. It has been shown to provide a strong anticancer activity against gastric carcinoma, hepatoma, leukemia, and tumors of the head and neck. The annual quantity extracted may reach 1000 kg camptothecin extracted and purified. Enhanced and cost effective extraction of camptothecin from natural sources is a promising strategy to increase their usage in pharmacological industries (Chu *et al.,* 2014).

Most enzymes of microbial origin are available commercially in purified and standardized form. However they are not economically viable for production purposes. An alternative is the use of crude enzyme enriched fractions obtained by fermentation using enzyme producing microorganism. In this study, crude extract of the cellulase enzyme was obtained using the fungal strain *Aspergillus niger*. The yield obtained from the extraction of carotenoids and camptothecin from sweet potato, carrot and tomato was compared by carrying out the extraction with cellulase enzyme and without cellulase enzyme. The novelty of this work arises from the fact that no previous work has been carried out to enhance the extraction of camptothecin using the cellulase enzyme. The objective of this study is to enhance the extraction of carotenoid from carrot, tomato, sweet potato and camptothecin from endophytic fungi *Aspergillus niger* using the cellulase enzyme.

MATERIALS AND METHODS

Production of cellulose

Cellulase producing *Aspergillus niger* was isolated from soil samples. Fermentation conditions for the production of cellulase were optimized. After the production it was characterised and purified for further study (Sujatha and Seethalakshmi, 2013).

Cellulase production depends upon several parameters including the composition of the fermentation medium. Optimization of the production media for abundance of the enzyme is an important step. Physico-chemical parameters such as substrate (Carboxy methyl cellulose) concentration, incubation period, pH, temperature, and supplemented substrate in submerged fermentation were standardized.

Extraction of carotenoids

Carrot, tomato and sweet potato were collected from market, koyembedu, Chennai. Cellulase produced from *Aspergillus niger* was used as mecerating enzyme. 1 g of carrot, tomato and sweet potato were homogenized with 50 ml sodium acetate buffer (pH 5), 0.3 ml of cellulase (0.054 UI) was added and the mixture was exposed to continuous stirring process with magnetic stirrer for 24 hrs at 37°C. After 24 hrs incubation, the combination of solvents with ratio of 1:1:1 was added to the mixture (petroleum ether: ethyl acetate: methanol) along with butylated hydroxytoluene (BHT) for extraction and separation. The mixture was taken for further liquid-liquid separation using separating funnel at room temperature with diethyl ether. The organic layer have collected for separation of carotenoid and dried over anhydrous sodium sulphate to get solid form. The extraction of carotenoid without enzyme was carried out using the same procedure. The samples were kept under nitrogen, at −20°C until further utilization. The carotenoid content was estimated spectrophotometrically, at 450 nm and HPLC. The total carotenoid content from the Carrot, tomato and sweet potato were calculated according to the formula (Pu *et al.,* 2013).

The total carotenoid content from the Carrot, tomato and sweet potato were calculated according to the formula (Hurst *et al.,* 1997).

$$X \text{ (mg carotenoid)} = (A \times V \times 1000)/ (2500 \times l \times 100)$$

Where:

A = the sample absorbance at 450 nm

V = sample volume (ml)

2500 = molar absorption coefficient for carotenoid (E1%)

l = 1 cm – the length of spectrophotometer curve

Extraction of Camptothecin from endophytic fungi

Fungal Endophyte *Aspergillus spp*. was obtained from Life Teck Research Centre for the present study. For the production of Camptothecin, spore suspensions of the culture were inoculated in Erlenmeyer flask (500 ml) with SDA broth (200ml) enriched by 1% peptone and yeast extract. The cultures were incubated in a rotary shaker (220 rpm) at 28^{o}C for, 96 hours (Neagu *et al.,* 2014).

Extraction of mycelia

After four days of incubation the fully grown mycelia were harvested. The mycelia was separated from the broth by filtration and the mycelia were thoroughly washed with sterile distilled water and then homogenized. The resulting homogenate were extracted with equal volume of chloroform: methanol (4:4 v/v) solvent mixture. The extraction was carried out with cellulase enzyme and without cellulase enzyme separately. The residue was obtained after stripping off the solvent. The crude camptothecin obtained was used for further analysis (Pu *et al.,* 2013).

Quantification analysis of camptothecin using HPLC

The extraction of camptothecin from *Aspergillus niger* was carried out using cellulase enzyme. The standard camptothecin was purchased from Sigma Aldrich Inc, minimum 95% HPLC powder, empirical formula- $C_{20}H_{16}N_2O_4$ and formula weight- 348.35. Camptothecin standard solutions within the range from 1 - 100 µg/ml concentrations were prepared for HPLC analysis. The camptothecin standard was prepared by dissolving in a solution of DMSO and absolute HPLC grade methanol in a ratio of 5: 50 (v/v). The standard sample solutions for HPLC were filtered using 0.2 µ syringe filter before injection. The analysis of extracts was done in High Performance Liquid Chromatographic system (HPLC) equipped with LC8A pump, SPD-M 10 A p photo array detector in combination with class LC 10 A software (Shimadzu). The presence of camptothecin in the samples was detected by comparing with the retention time of the standard sample. The area of the standard was compared with area of the sample and the amount of camptothecin in the extracts was calculated. Conditions of the HPLC were liquisorb RP-18,25cm X 10µm; gradient elution 0.04 $CHCl_3$: CH_3OH 4:1 upto 1:1, 10 min., flow rate 3.5ml/min., UV detection 254nm (Wiedenfeld *et al.,* 1997).

RESULTS AND DISCUSSION

Optimization of cellulose production and carotenoid extraction

The optimum incubation period, pH, temperature, nitrogen source and substrate concentration for fermentative production of the enzyme, the selected strain was cultivated with varying parameters of incubation period range of 48, 72, 96, 120

and 144 hrs, pH range of 4.5, 5, 5.5, 6 and 6.5, temperature range of 30°C, 35°C ,40°C, 45°C and 50°C, nitrogen source (ammonium nitrate) at concentrations of 0.5%, 1%, 1.5%, 2% and 2.5% and substrate concentrations of 2%, 4%, 6%, 8% and 10% and monitored continuously (Hudiyono *et al.,* 2012). The optimum parameter recorded during the submerged fermentation was observed the highest yield of cellulose at temperature (40°C), pH (6.0), substrate concentration (8%), incubation time (120 hrs) and nitrogen source (1%). The carotenoid extraction from carrot using cellulose as macerating enzyme found to be 2.5±0.25µg/g and 1.7 ±0.15µg/g was observed in extraction without macerating enzyme (cellulase). The carotenoid content isolated from tomato with cellulase enzyme was found to be 2.2±0.18µg/g and lowest yield was observed without mecerating enzyme for extraction (0.3±0.02µg/g). The same way carotenoid extracted from sweet potato with enzyme and without enzyme treatment yielded 18± 1.75 µg/g and 15±0.78 µg/g respectively. The figure.1 shows the yield of carotenoid content from carrot, potato and sweet potato by extraction using macerating enzyme cellulase and absence of cellulase enzyme. Among the selected natural plant sources the sweet potato contains more carotenoid than the carrot and potato. Carotenoids are a group of natural pigments with colours varying from yellow to red. They are produced by higher plants, some bacteria and algae. They are found abundantly in nature (Bauernfeind, 1981). As naturally coloured pigments, carotenoids can be used as food colourants. Artificial or synthetically prepared colour rendering compounds are widely used as food colorants. Natural pigments such as these can easily replace them as safe food colourants. However, the production of large quantities of these pigments is restricted due to the usage of toxic chemicals such as petroleum, benzene etc. (Bunea *et al.,* 2009). Hence, a valid alternative to the extraction of carotenoids using toxic chemicals, can be obtained through enzyme assisted carotenoid extraction. Thus carotenoids can safely and adequately be used as food colourants. This will immensely increase their market value and availability, putting these naturally coloured compounds to good use. The naturally occurring carotenoid were extracted using macerating enzyme from tomatoes (Hurst *et al.,* 1997). Cellulase and pectinase enzymes mediated extraction to enhanced yield of carotenoids from orange peels, sweet potato, carrots (Cinar 2005) and red capsicum (Nath *et al.,* 2016) Cellulases and hemicellusases to improve the extraction of carotenoids from *Physalis allekengi* (Bunea *et al.,* 2009).

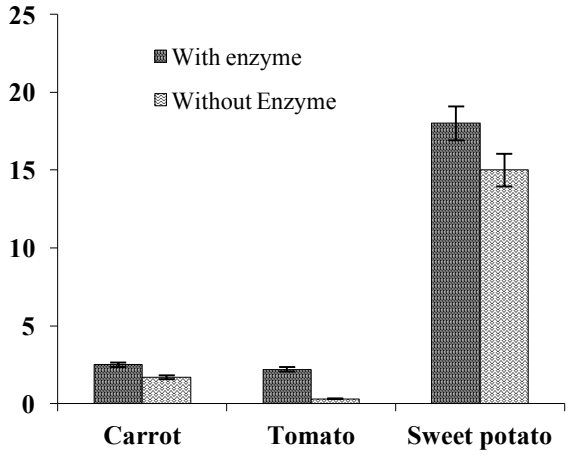


Figure 1: Yield of carotenoid with and without macerating enzyme (cellulase)

In the carotenoid extracted from carrot, tomato and sweet potato, the extraction carried out with enzyme was found to yield better results in all the three cases. It was also observed that sweet potato yielded a greater amount of carotenoid than carrot and tomato. Earlier report stated that, β-carotene extraction using enzyme yielded was high than without using of the enzyme for extraction. The same method used for the extraction of such valuable phytomolecules lycopene, plant pigments.

The α-amylase, β-amylase, cellulase and hemicellusases are also used as macerating enzyme for the extraction of industrially important molecules.

Extraction of Camptothecin from *Aspergillus niger*

Camptothecin (CPT) is an alkaloid originally isolated from *Camptotheca acuminate* and it has strong antitumor potential. The enzyme maceration methods are predominantly used nowadays for the extraction of industrially important molecules to increase the yield. However the enzyme assisted extraction of camptothecin has not been carried out previously. CPT constitute an important class of anticancer drugs with a wide spectrum of activities in many solid tumors, including lymphoma, gastric cancer and colorectal cancer (Hurst *et al.,* 1997). The camptothecin extracted was characterised using HPLC. The chromatograms of camptothecin standard, camptothecin extracted with enzyme and camptothecin extracted without enzyme were obtained. The extraction yield of captothecin from *A.niger* by using the macerating enzyme cellulase showed higher yield (0.5512mg/g) compared to the absence of cellulase enzyme in extraction process (Figure 2,3). The commercially available camptothecin was used as standard in HPLC analysis and found the retention time at 10.321

mins, the nearer range of retention time was observed for camptothecin extracted from *A. niger* with cellulase enzyme (10.209 mins) and without cellulase enzyme (10.841 mins) treatment of extraction. Singh *et al*., (2010) reported that camptothecin from *Nothapodytes nimmoniana* by HPLC. This is the first report on enzyme assisted extraction of camptothecin with improves yield.

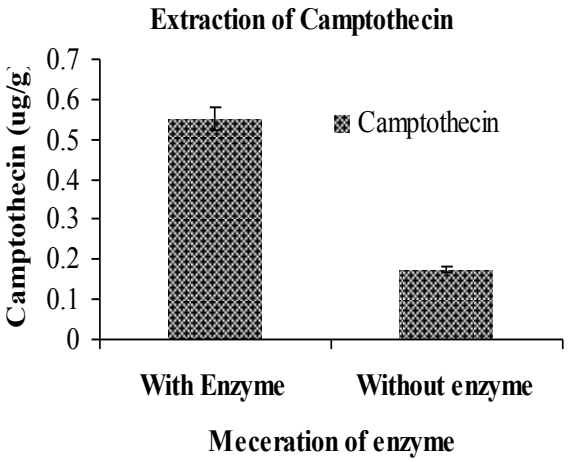
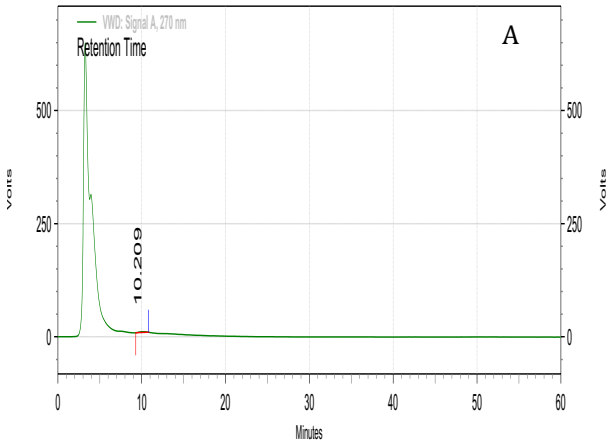
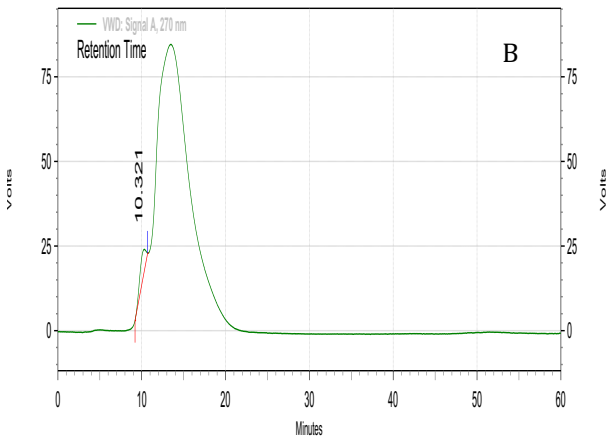


Figure 2: Yield of camptothecin with and without macerating enzyme (cellulase)





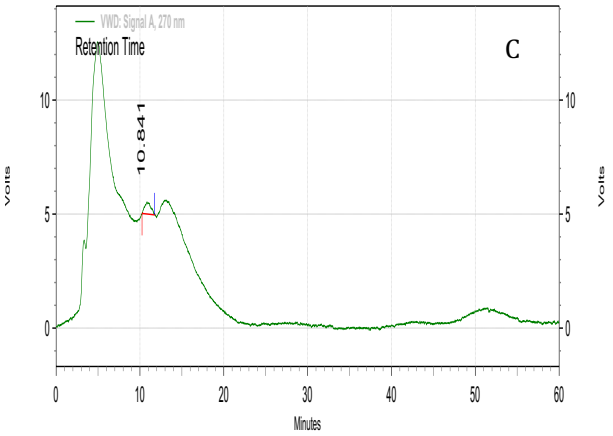


Figure 3: Quantification of camptothecin yield with and without cellulase enzyme treatment compared to the commercially available camptothecin. A. chromatogram of camptothecin standard; B. chromatogram of camptothecin with enzyme treatment; C. chromatogram of camptothecin without enzyme treatment

CONCLUSION

Macerating enzyme cellulase was obtained from *Aspergillus niger*. It was used to enhance the extraction of carotenoid and camptothecin. Carotenoid was extracted from carrot, tomato and sweet potato. Camptothecin was extracted from endophytic fungi *Aspergillus niger*. The results obtained in this study clearly shows that the amount of carotenoid yielded high in sweet potato compared to the carrot and tomato with macerating enzyme cellulase. And also, the anti-cancer camptothecin extracted from *Aspergillus niger* using cellulase as macerating enzyme yielded high compared to the without enzyme treatment of extraction. This is the first report of camptothecin extraction using macerating enzyme cellulase. Beside the enhancement in the yield of the released camptothecin, the use of crude enzymes prevents the use of high quantities of organic solvents and served as a cost-effective method of extraction. The macerating enzyme treatment method for the extraction of industrially important phytomolecules is a cheapest and cost effective method.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the authorities of Karpagam Academy of Higher Education for financial assistance and necessary facilities to carry out this research work.

CONFLICT OF INTERESTS

There is no conflict of interests.

REFERENCES

Bauernfeind, J.C. 1981. Carotenoids as food colourants and vitamin A precursors: technological and nutritional applications. *Natural food colours, cap I,* New York: Academic Press

Bhat. M.K.2000. Cellulases and related enzymes in biotechnology. *Biotechnology Advances*. 18:355–383.

Bunea. A., Lujerdean. A., Pintea. A., Andrei. S., Socaciu. C. 2009. Using Cellulases and Hemicellulases to Improve Better Extraction of Carotenoids from the Sepals of PhysalisAlkekengi L. *Bulletin UASVM Animal Science and Biotechnologies*, 66: 1-2.

Carvalho .L., Castro. I ., Silva. C. 2008. A study of retention of sugars in the process of clarification of pineapple juice (*Anana scosmosus*, L. Merril) by micro- and ultrafilteration*. Journal of Food Engineering*. 8(4): 447-454.

Chu Chu., Jialin Xu., Dongping Cheng., Xingnuo Li., Shengqiang Tong., Jizong Yan., Qingyong. Li.2014.Anti-Proliferative and Apoptosis-Inducing Effects of Camptothecin20(s)-O-(2-pyrazolyl-1)acetic Ester in Human Breast Tumor MCF-7 Cells. *Molecules*. 19: 4941-4955.

Cinar. I. 2005. Effects of cellulase and pectinase concentrations on the colour yield of enzyme extracted plant carotenoids. *Process Biochem.* 40: 945-949.

Demain. A. L.2000.Microbialbiotechnology. *Trends Biotechnology*. 18: 26–31.

Godfrey.T.,West.S.1996. Introduction to industrial enzymology. In: Godfrey T, West S, *Industrial enzymology*. 2: 1–8.

Hudiyono. S., Septian.A. 2012. Optimization Carotenoids Isolation of The Wastw Crude Palm Oil Using α-Amylase, β- Amylase and Cellulase. IOSR *Journal of Applied Chemistry.* 2(2): 07-12.

Hurst. P.I., Nielsen. J., Sullivan. P.A., Shepherd. M.G.1997. Purification and Properties of a Cellulase from *Aspergillus niger*. *Biochem. J.* 165: 33-41.

Lynd. L R., Weimer. P J., Van Zyl .W. H .,Pretorius .I. S. 2002. Microbial cellulase utilization: Fundamentals and biotechnology. *Microbiol Mol Biol Rev*. 66: 506-577.

Minussi R.C., Pastore G. M., Duran N. 2002. Potential applications of laccase in food industry. *Trends in Food Science and Technology.* 13: 205-216.

Nath. P., Kaur. C., Rudra. S.G., Varghese. E. 2016. Enzyme-Assisted Extraction of Carotenoid-Rich Extract from Red Capsicum (*Capsicum annuum*), *Springer Nature*. 5(2): 193–204.

Neagu. D., Leopold. L.F., Thonart. P., Destain. J., Socaciu. C. 2014. EnzymeAssisted Extraction of Carotenoids and Phenolc Derivatives from Tomatoes, *Bulletin UASVM 10 Animal Science and Biotechnologies*. 71(1): 20- 26.

Pu. X ., Qu. X ., Chen. F., Bao. J., Zhang. G ., Luo. Y. 2013. Camptothecin-producing endophytic fungus Trichoderma atroviride LY357: isolation, identification, and fermentation conditions optimization for camptothecin production. *Appl Microbiol Biotechnol.* 97: 9365–9375.

Singh. I., Kumaravadivel. N., Gnanam. R ., Vellaikumar. S. 2010. RP-HPLC analysis for camptothecin content in Nothapodytes nimmoniana, an endangered medicinal plant. *Journal of Medicinal Plants Research*. 4(3): 255-259.

Sujatha. N., Seethalakshmi.I.2013. Production and optimization of Cellulase produced by *Aspergillus* species isolated from soil. *Indian Journal of Applied Microbiology.* 16(2): 85-91.

Sukumaran .R., Singhania. R ., Pandey. A. 2005. Microbial cellulases- Production, applications and challenges. *Journal of Scientific & Industrial Research*. 64: 832-844.

Wiedenfeld. H., Furmanowa. M., Roeder. E., Guzewskaand. J ., Gustowski. W. 1997. Camptothecin and 10-hydroxycamptothecin in callus and plantlets of *Camptotheca acuminate. Plant Cell Tissue and Organ Culture* .49: 213- 218.