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COMPARATIVE STUDY OF CAPSAICIN FROM *IN VITRO* CULTIVATED AND NATURALLY CULTIVATED *CAPSICUM* FRUITS EXTRACTS

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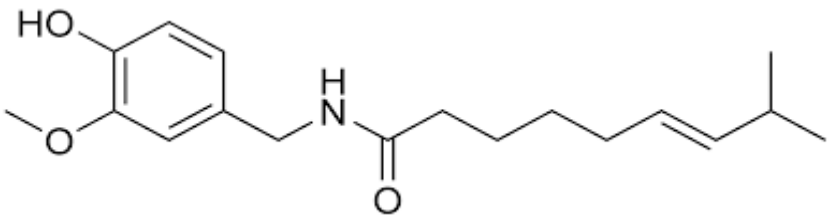
ABSTRACT
Capsaicin is a unique alkaloid found in primarily in fruit of *Capsicum* species. The present work deals with comparative analysis of capsaicin from *in vitro* cultivated and naturally cultivated *capsicum* fruits extracts. For callus induction young leaves and placental region from the fruit pods were used as explants. They were cultured on Murashige and Skoog (MS) medium supplemented with various combinations of 2, 4-D, kinetin and BAP growth hormones. Murashige and Skoog medium with 3mg/l 2, 4-D and 1mg/l kinetin showed significant callus induction and proliferation of callus as explants for single cell suspension culture. The fruit extract, callus extract and depleted medium of single cell suspension culture was taken for the estimation of capsaicin by spectrometric method. The single cell suspension culture made from placental callus showed more content of capsaicin as compare to others.

KEY WORDS: Capsaicin, *Capsicum* species, 2, 4-D, kinetin, MS medium

INTRODUCTION

Capsaicin is the major component of the capsaicinoids in capsicum species and is what provides its spicy flavor. The pungency, a commercially important attribute of peppers, is due to the presence of six chemically related compounds viz. capsaicin, dihydrocapsaicin, norcapsaicin, nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin; which constitute the "capsaicinoids" group1 (Perucka and Materska 2001). Topical application of capsaicin has long been clinically used to treat persistent pain, such as osteoarthritic pain, post-herpetic neuralgia of the trigeminal nerve, migraine prophylaxis, diabetic neuropathy, HIV-associated distal sensory neuropathy, and intractable pain in cancer patients (Lee Y *et al*, 2000). Generally extracted directly from fruit, high demand has driven the use of established methods to increase production through extraction and characterization.

Plant cell and tissue culture technology is being used for the large scale production of specific secondary metabolites which can be used as food additives, nutraceuticals, and pharmaceuticals. (Zhong *et al*, 2001). Over time these methods have improved.



Chemical structure of Capsaicin
(8-Methyl-N-vanillyl-trans-6-nonenamide) Goci.E *et al*,(2014)

Capsaicin is present in large quantities in placental tissue, which holds the seeds. The present work depicts comparative analysis of capsaicin from *in vitro* cultivated and naturally cultivated *capsicum* fruits extracts and efficient extraction, identification and quantification of capsaicin from *Capsicum sp*. in different samples by TLC and UV spectrophotometric analysis.

MATERIALS AND METHODS

Collection of plant material:

Capsicum fruits were collected from market for direct extraction of Capsaicin. For *in vitro* germination the viable seeds of *Capsicum annuum* L. were surface sterilized by 0.1% mercuric chloride ($HgCl_2$) under sterile conditions with gentle agitation for 7 min. The seeds were then rinsed thoroughly with double distilled water. The sterilized seeds were germinated aseptically on Murashige and Skoog's (1962) basal medium in bottles. The seeds were incubated at 25±2ºC in growth rooms with continuous light (16 hours light/dark conditions). For preparation of explants seven day old seedlings were used. Young leaves and placental nodal region were used as explants for culturing.

Composition of medium:

The culture medium used for the present work was Murashige and Skoog (MS) basal medium. The MS medium containing different supplements was used to study callus induction and proliferation maintaining pH 5.5
The combinations of plant growth regulators studied were as follows:

LCulture e	2,4-D (mg/l)	Kinetin (mg/l)	BAP (mg/l)
a *In Vitro* germinated plantlet	--	--	--
f Leaf Callus	2	--	--
Placental tissue callus	3	1	--
C Single cell suspension from a placental callus l l	0.5	--	--

Callus Initiation (from *In Vitro* germinated seeds):

For callus initiation process, leaf segments (each 1cm in length) were taken. These segments were inoculated on 30 ml MS medium supplemented with, 2mg/1 2,4-D and 3% sucrose. Then allowed to solidify by adding 1% agar (callusing medium). The calli were maintained by regular sub culturing at 4-week intervals with fresh callusing medium. Callus cultures were incubated at 28˚C with photoperiod 16h.

Placental tissue callus:

The pod of Capsicum fruit was surface sterilized 0.1% mercuric chloride ($HgCl_2$) under sterile conditions with gentle agitation for 7 min. Then was rinsed thoroughly with double distilled water. Cut longitudinally with the sterile blade to expose the placental to cut along the inner pericarp and made into small bits of 1 cm length. These segments were then inoculated on 30 ml MS medium supplemented with 3% sucrose, 3mg/1 2,4-D and 1 mg/1 kinetin and solidified with 1% agar (callusing medium).

Extraction of capsaicin:

1. From direct fruit by Soxhlet extractor:

In extraction process, the shade dried Capsicum fruit were powdered in electric grinder. 10 g of the powder was extracted with 100 ml 95% ethanol in the Soxhlet apparatus for three hours from the start of reflux. Temperature was maintained at 60-70^0 C

2. From leaf callus and placental tissue callus:

Each grown callus from leaf sample and placental tissue was taken for extraction. Weight callus taken into the sterile mortar and pestle with the 95% ethanol and crush. The final product was filtered through the Whatmann filter paper number 1, the filtrate was used as sample for further analysis.

Single cell suspension from placental callus:

The cell suspension culture were prepared by transferring placental callus to MS medium (without Agar) containing 2, 4-D 0.5mg/l and sucrose 1% in 250ml of sterile Erlenmeyer flask. The obtained cultures were then placed on Rotary shaker at agitation speed of 100rpm. After 12 days of shaking, the original crude suspension was subcultured and separated cell sample from subculture for further estimation of Capsaicin.

Quanlitative estimation of capsaicin:

Thin Layer Chromatography was used to identify the presence of capsaicin in above four samples, on TLC silica gel 60 glass plate (Merck). The standard capsaicin at concentration of 1mg/ml was spotted as a reference on the TLC plate.

Sample of 10µl of the four extracts were spotted onto the plates and allowed to dry for 15 min. Petroleum ether: Chloroform: Acetonitrile in the ratio of 40: 45: 15 was used as the mobile phase. The chromatogram was developed in iodine chamber and viewed under the UV light. The standard capsaicin Rf value was calculated and compared with the extracts.

Quantification by UV spectrophotometer:

The simple linear regression curve was plotted by using standard capsaicin purchased from Sigma Chemical. A stock solution of one milligram capsaicin per milliliter of ethanol was dissolved and different concentrations were prepared from the stock solution. The optical density was recorded at 280 nm and linear regression equation was generated. The capsaicin concentrations in samples were calculated using capsaicin linear regression equation and it was expressed as microgram of capsaicin per millilitre.

Confirmation of Capsaicin:

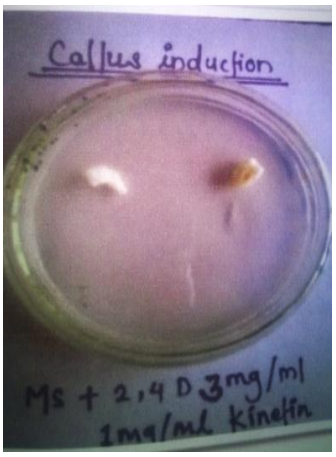
a) Mayer's Test:
The Mayer's Test was performed to determine presence of alkaloid. To a few drops of the Mayer's reagent, 2 mg of prepared extract was added. Formation of white or pale yellow precipitate indicates the presence of alkaloids.

b) Total phenolic contents of hot peppers were analyzed using the Folin- Ciocalteu reagent method of Sadasivam and Manickam. One ml of 10^{-1} dilution Folin- Ciocalteu reagent (Fisher) and 2ml of 7.5% (w/v) Na_2CO_3 were added to 0.1ml of solvent extract. After vortexing for 10 seconds, the mixture was incubated at 45ºC in water bath for 15 min. The absorbance was measured with spectrophotometer at 650nm. The concentration of total phenolic contents was determined as milligram of gallic acid equivalent by using an equation that was obtained from standard Gallic acid graph, four samples were analyzed.

RESULT AND DISCUSSION

Plant tissue culture is promising approach for large scale production of phytochemical and has several advantages over whole plant production. Callus initiation involves three major selections of explants, suitable medium and culture conditions. The callus induction was observed after 30 days from leaf sample and 46 days from placental tissue of fruit.

Figure 1(a): Callus Induction of Leaf explants



(b): Callus Induction of placental explants

Comparative Study on the extraction of Capsaicinoids from *Capsicum* spp. were studied by Amruthraj *et al*. (2014). Capsaicin present in direct fruit was extracted by using Soxhlet extractor and for calli of leaf and placental tissue; Capsaicin was extracted by maceration method using 95% ethanol. In single cell culture process, after overnight (12 days) shaking, the original crude suspension was then sub cultured and separated the cell sample. The capsaicin extracted from the four samples resolved on TLC plate was viewed under the UV light at 308 nm. The capsaicin bands were visible in the iodine vapor and under UV illuminator at 302 nm. The standard capsaicin Rf value 0.078 was corresponded to the spot observed in the all extracts. After comparing with standard the four samples revealed presence of capsaicin.

The quantitative estimation of capsaicin in obtained four sample extracts was calculated using the following equation (Y = 0.0182X + 0.0171, R = 0.9981) for the UV spectrophotometer estimation (Figure 2a and b) and it was expressed as milligram of capsaicin per millilitre.

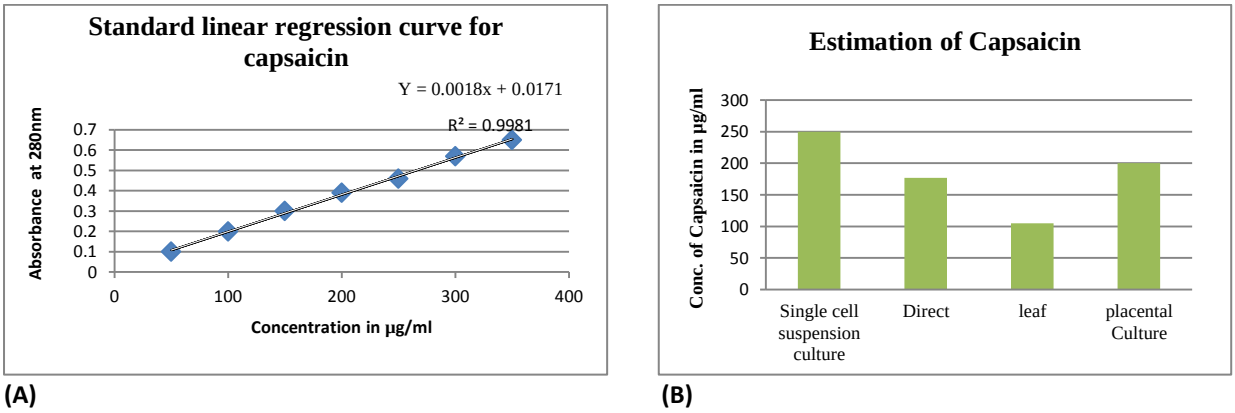


Figure 2:a. Standard linear regression curve for capsaicin by UV spectrophotometer, b. Spectroscopic analysis of four samples revealing concentration of total capsaicin content.

All these extraction four samples showed creamish pale yellow precipitate, indicating presence of alkaloids according to Mayer's test as studied by Kishore *et al*, (2017).
Arnnok, (2000) stated that the phenolics are complex organic substances, which contain more than one phenolic group. Polyphenolics can be divided into many different subcategories, such as flavonoids and non-flavonoid components.

The total phenolic contents of given four samples of *Capsicum sp*. were investigated by Folin-Ciocalteu method using gallic acid equivance shown in (Table 1). The results showed that a gradual increase of phenolics concentration was observed from Leaf Callus, Direct Fruit, and Placental Culture to Single Cell Suspension Culture. Among the obtained samples, Single Cell Suspension Culture had highest amount of total phenolics i.e., 140.5±0.55 and the lowest amount i.e., 64.0±0.76 from Leaf Callus.

Table 1. Total phenolic contents of four samples of *Capsicum sp*.

Capsicum sp. samples	Phenolic contents (mg/100g)
Single Cell Suspension Culture	140.5±0.55
Direct Fruit	77.6±0.82
Leaf Callus	64.0±0.76
Placental Culture	88.1±0.14

Further study on Capsaicinoid production via cell or tissue culture can be augmented be addition of biosynthetic pathway precursors and intermediaries as phenylalanine, ferulic acid and vanillyl amine showing encouraging results (Johnson, 1996).

CONCLUSION

Capsaicin is a unique alkaloid found in primarily in fruit of *Capsicum* species, providing its spicy flavor. The present study clearly reported the efficient phytochemical extraction, separation, identification and quantification of capsaicin from *Capsicum* spp in different samples by TLC and UV spectrophotometric for the purification of capsaicin. Capsaicin was extracted in different ways, directly from fruit, *in vitro* grown callus of leaf and placental tissue and from single cell suspension culture. The TLC profile with retention factor 0.078 corresponding to standard capsaicin was observed in all extracts. From figure 2b, we can conclude that single cell suspension culture developed from placental tissue showed more content of capsaicin as compare to others with maximum amount of phenolics recorded i.e.,

140.5±0.55 than the direct fruit extract, hence the above study depicts, single cell suspension culture made from placental callus showed more content of capsaicin as compare to others.

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