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RESEARCH ARTICLE

ESTIMATION OF CAPSAICIN IN PHENYLALANINE AND VALINE-TREATED CELL SUSPENSION CULTURES IN *CAPSICUM ANNUUM* AND *CAPSICUM CHINENSE* USING HPLC

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Abstract

Capsaicin content was estimated in phenylalanine & valine (0.01, 0.02, 0.03 %) treated cell suspension cultures derived from zygotic embryo (non-fruit tissue) using HPLC in 10 *Capsicum* genotypes. Maximum amount of capsaicin (20.1±0.3µM/g) was observed in *C. chinense* var. Kotpar, while in *C. annuum* var. special bullet produced maximum amount of Capsaicin (18.2±0.3µM/g), whereas minimum amount of capsaicin was found in *C. annuum* var. Badiga-1 (4.5±0.7µM/g). Another observation was that capsaicin was not detected both in precursor and non-precursor treated cell suspension cultures of *C. annuum* var. california wonder & yolo wonder.

Introduction:-

Pungency of Chilli pepper (*Capsicum* species) fruit is due to a group of alkaloids known as capsaicinoids, which are expressed in the placenta. Among the 22 known capsaicinoids, Capsaicin (CAP) and Dihydrocapsaicin (DHC) together account for about 90% of pungency (Kawada *et.al.*, 1970) and the other capsaicinoids occur in smaller concentrations and are the "minor" capsaicinoids viz. Capsaicinoid nordihydrocapsaicin (NDHC), Homodihydrocapsaicin (HDHC), Homocapsaicin (HC) and Nonivamide (PAVA). Capsaicin has good export possibilities (Choudhury, 1967; Singh, 1996) because it is endowed with anticancer properties (Bosland, 1993; Surh, 1998; Ito *et.al.*, 2004; Oh *et.al.*, 2008; Yang *et.al.*, 2010). Pungency phenotyping in chilli pepper fruits is mandatory both for domestic and export purposes. However, the task of pungency phenotyping is cumbersome and time consuming, since it is carried out in dry fruits, after 4-5 months of commencement of cultivation and the trait is influenced by genotype, environment and their interactions.

Capsaicin is produced in fruits only. Industrial production of secondary metabolites through *in vitro* cultures was first reported by Pfizer Company during 1950 to 1960. *In vitro* production of secondary metabolites can be enhanced or regulated by precursor feed or growth hormones. However, small quantities of capsaicin were reported in non-fruit tissues like young leaves, shoots, nodal region, placental region and pericarp tissue by Umamaheswari & Lalitha, (2007). Varindra *et.al.*,(2003) reported that seedling derived callus showed low level of capsaicin content compared to pericarp derived callus culture in four varieties of *C. annuum.* Sudha & Ravishanker, (2003) reported enhanced level of capsaicin by precursor feed of salicylic acid and methyl jasmonate individually, with in combination there was no further enhancement of capsaicin production in suspension culture of *C. frutescence*. Johnson *et.al*, (1998) reported increased capsaicin production in *in vitro* seedling derived callus cultures of *C. frutescens* using various levels of P-fluoropheylalanine. Lindsey & Yeoman, (1984) reported accumulation of capsaicin in immobilized and precursor feed (phenylalanine & isocapric acid) in suspension cultures of *C. frutescens*

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Mill.cv.*annuum*. Lindsey, (1985) reported that 100-fold of capsaicin was produced in immobilizing the cells in reticulated polyurethane foam in suspension of *C. frutescens*, increases the levels of capsaicin by addition of precursor feed isocarpric acid. Holden *et.al.,* (1988) reported that tissue cultures produced low levels of capsaicin than fruits tissue in *Capsicum* species. Leete & Louden, (1968) reported that capsaicin is produced from phenylalanine & valine responsible for fatty acid moiety (precursor feed) in *C. annuum* species. Johnson, (1995) reported production of capsaicin which is produced through suspension culture was comparable to capsaicin standard. Capsaicin was extracted from callus as well as from the medium (Johnson & Ravishanker, 1998). Johnson *et.al.,* (1998) reported that activity profile of phenylalanine ammonia lyase and its relation to capsaicin formation in *p*-fluorophenylalanine-resistant cell cultures of *C. frutescens.* Putrescine treatment influenced the capsaicin production in cell suspension cultures of *C. frutescens* (Govindaswamy *et.al.,* 2003). Pandhair & Gosal, (2009) reported that increase capsaicin levels by precursor feed such as coumaric acid and a group of individual elicitors such as phycocyanin, salicylic acid, synapinic acid and curdlan. Prasad *et.al.,* (2006) reported that by the influence of 8-methyl-nonenoic acid and its possible regulatory role in capsaicin biosynthesis in *Capsicum spp*. The present paper reports estimation of capsaicin in phenylalanine & valine treated cell suspension cultures derived from zygotic embryos (non-fruit tissue) using HPLC in 10 *Capsicum* genotypes which are commercial cultivated in this region.

Materials and Methods:-

Seed of *Capsicum annuum* L. varieties Pragna & Teja obtained from ARS, Malyal, Warangal, Telangana State, India. *C. annuum* L varieties G-3, G-4, G-5, G-273 & LCA-335 obtained from Agricultural Research Station (ARS), LAM, Guntur, Andhra Pradesh, India. *C. annuum* L. variety Badiga-1 obtained from Seed Stores, Karnataka. *C. annuum* L. variety Special bullet obtained from S.K. Nursery & Seed Company, Parganas (North), West Bengal, India and *Capsicum chinense* Jacq. var. Kotpar obtained from Tezpur, Assam, India. *C. annuum* L. var. California Wonder & Yolo Wonder (positive controls) obtained from **POCHA AGRO SCIENCE (P) LTD, BHALASWA, NEW DELHI, INDIA.** Cultivated in local area and recently introduced were used in this study.

Isolation of Zygotic embryo:

Fresh seed of the above enlisted *Capsicum annuum* & *Capsicum chinense* were surface sterilized with 0.5% $HgCl_2$ for 5 minutes and washed thoroughly in sterile double distilled water. Mature zygotic embryo excised aseptically and cultured on MS medium (MS, 1962) consisting of 2, 4, Dichlorophenoxyacetic acid (2,4-D 2.0 mg/L) and Kinetin (Kn 1.0 mg/L). Pale green callus was initiated over the enter zygotic embryo and after 3 weeks-time the enter explant transformed in to friable callus

Callus induction:

Friable Callus (150 to 200 mg fresh weight) was sub- cultured for another 2 weeks -time period and subsequently maintained for one year

Preparation of cell suspension culture:

Friable callus (200 mg fresh weight) was transferred to 50 ml Erlenmeyer flask containing MS+ (2,4,D 2.0 mg/l) and (Kn 1.0 mg/l), with (or) without precursors- phenylalanine & valine (0.01, 0.02, 0.03 %) liquid medium. The flasks were placed on a orbital shaker with 90 RPM in dark at 27° C for 2 weeks. The friable callus dispersed into the liquid medium due to shaking and developed into a fine cell suspension culture. These cultures were maintained for 1 year.

Precursor's treatment in cell suspension cultures:

One week old cell suspension (25 ml) was centrifuged at 1000 RPM and the pellet was resuspended in 10 ml of fresh medium. Ten (10) µl of phenylalanine & valine (0.10 mg in 100 ml water), were added to 10 ml of fresh medium and incubated on an orbital shaker (90 RPM), under the same culture room condition as described above. The cells were harvested on 21 days (Third week) and used for estimation of capsaicin contents. The experiments were repeated thrice. A single value was recorded for Capsaicin content.

Quantification of capsaicin by HPLC

Equipment/Instruments:

HPLC:

Shimadzu SPD 10 A, Detector: LC- 10AD, Injector: Manual, Column: C18, Water Symmetry, 250 X 4.6mm, 5 µl, Flow rate: 1.0ml / min, Wavelength: 268nm, Software: N2000

Chemicals:
Acetonitrile, Methanol, Potassium Hydrogen Phosphate (monobasic) (Himedia, India), Chloroform, Toluene, Ethyl acetate, Diethylamine, Capsaicin (RM 21750-100-mg; Product code: 101208064; Mol. Wt.: 305.41 gm) (Sigma, India).

Method:-

Callus cell suspension cultures (500 mg fresh weight) of both precursors treated and non- treated were oven-dried at 35 to 40° C for 4 to 6 hours, grinded using mortar and pestle. Samples were processed within 1 day of grinding. Dried cell suspension cultures and acetonitrile (1:10- gram: ml) was transferred to 60 ml glass bottles with teflon lid. Glass bottles were incubated at 60° C in a water bath for 2h. The glass bottles were swirled manually every hour. The glass bottles were removed from the water bath and cooled to room temperature. The extract separated into precipitate (debris) which settled in the bottom and the liquid supernatant on the top . Supernatant (1.5 ml,) was filtered using Whatman No 1 filter paper and stored in 1.5 ml glass vial , capped and stored at 5° C until further use. A 10-µl aliquot of the samples were used for HPLC analysis (Fig. 1 & 2).

Procedure:-

Samples were injected into injector port of Shimadzu- SPD 10 A. Polar mobile phase Methanol guided the sample into the column and the steady flow rate of pump was adjusted to 1.0ml/min. The HPLC was used to determine retention time, height of peak and area of peak.

Result and Discussion:-

Capsaicin expression in cell suspension cultures :
Capsaicin was estimated in one year old cell suspension cultures using HPLC technique in 12 chilli pepper genotypes viz. *C. annuum* varieties G-3, G-4, G-5, G-273, LCA-335 (G & LCA-series), Pragna & Teja (W-series), Badiga-1, Special bullet and *C. chinense* var. Kotpar, *C. annuum* L. California wonder & Yolo wonder.

A detectable amount of capsaicin was not observed in non-precursor treated cell suspension culture of all 12 chilli peppers. However, a quantifiable amount of capsaicin was observed in precursor treated cell suspension cultures of only 10 chilli pepper genotypes (Figs. 3 & 4). Maximum amount of capsaicin (20.1±0.3µM/g) was observed in *C. chinense* var. Kotpar. While in *C. annuum* variety Special bullet maximum amount of Capsaicin (18.2±0.3µM/g) was observed. In *C. annuum* varieties Teja (14.3±0.2µM/g), G-5 (6.5±0.8µM/g), G-273 (5.8±0.9µM/g) G-4 (5.6±0.7µM/g), G-3 (4.9±0.1µM/g), LCA-335 (4.5±0.7µM/g), Pragna (4.5±0.1µM/g) was observed, minimum amount of capsaicin was found in *C. annuum* var. Badiga-1 (2.5±0.7µM/g). An interesting observation was that capsaicin was not detected both in precursor and non-precursor treated cell suspension cultures of *C. annuum* varieties California wonder & Yolo wonder.

In this study, capsaicin was estimated in phenylalanine and valine (0.01, 0.02, 0.03%) grown cell suspension cultures of 12 commercial chilli pepper genotypes viz. *C. annuum* varieties G-3, G-4, G-5, G-273, LCA-335 (G & LCA-series), Pragna & Teja (W-series), Badiga-1, Special bullet, California wonder, Yolo wonder and *C. chinense* var. Kotpar using HPLC.

Capsaicin was detected in 10 genotypes in phenylalanine and valine (0.01, 0.02, and 0.03%) based cell suspension cultures. Maximum amount of capsaicin (20.1±0.3µM/g) was observed in *C.chinense* var. Kotpar, while in *C. annuum* variety Special bullet produced maximum amount of Capsaicin (18.2±0.3µM/g) and minimum amount of capsaicin was found in *C.annuum* in Badiga-1 (4.5±0.7µM/g). Similar, observation was reported by (Bosland & Baral, 2007, Sanatombi *et.al.*, 2010, Prasad *et.al.*, 2013). Capsaicin was detected in 10 commercial chilli pepper genotypes, while it was not detected in 2 chilli pepper genotypes viz. California Wonder & Yolo Wonder.

In this study we have detected capsaicin in phenylalanine and valine grown cell suspension culture derived from zygotic embryo (non-fruit tissue). It is well known that phenylalanine and valine are precursor for capsaicin biosynthesis (Prasad *et. al.*, 2006). Another interesting observation was that both in precursor & non precursor grown cell suspension cultures of *Capsicum annuum varieties* california wonder and yolo wonder capsaicin was not detected. This could be due to 15 bp deletion in non-pungent chilli pepper variety Yolo wonder (Rodriguez *et.al*, 2012).

In this study we report detection of capsaicin in phenylalanine and valine grown cell suspension cultures of 10 commercial chilli derived from zygotic embryo (non-fruit tissue) which can be maintained in laboratory conditions and can be grown in bioreactor.

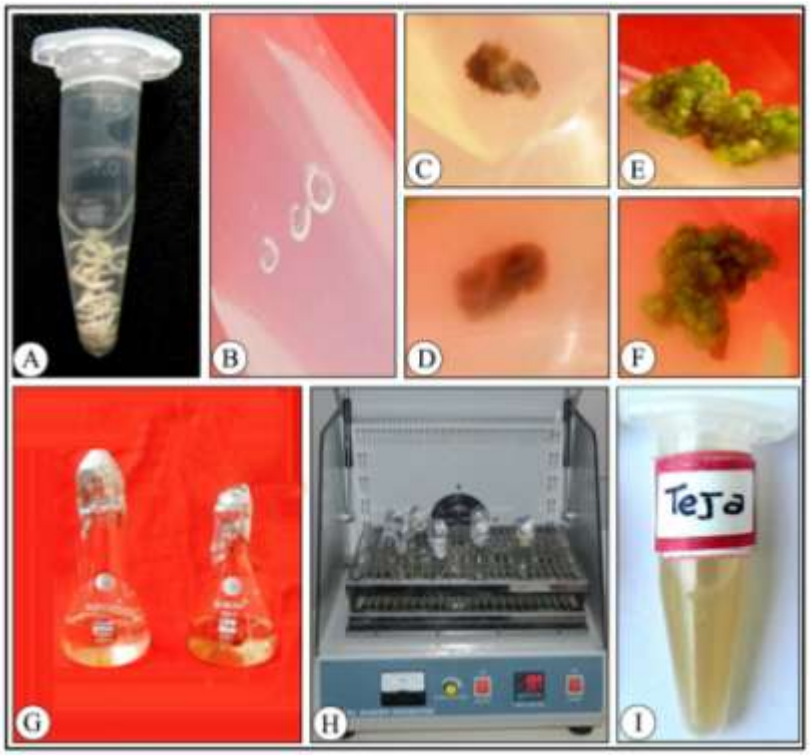

Fig: 1:- Callus induction in Zygotic Embryos of *C. annuum* L. var. Teja cultured on MS + 2, 4-D (1.5 mg/L) after 4 weeks of culture. A & B: Zygotic embryos, C & D: Callus (3 and 4 weeks old respectively), E & F: Sub-cultured callus, G: Cell suspension culture, H: Orbital shaker equipment & I: Sample extraction in acetonitrile.

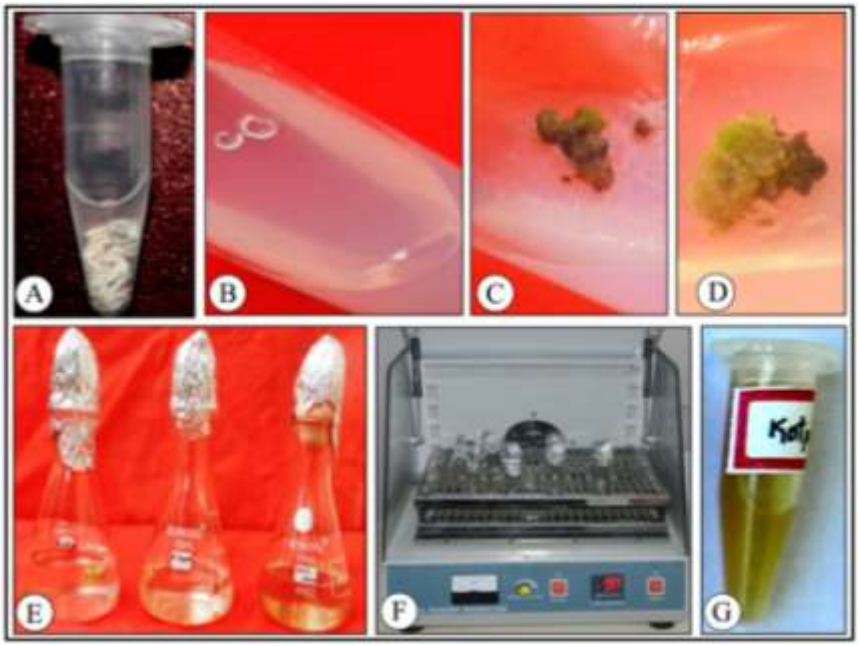

Fig 2:- Callus induction in Zygotic Embryos of *C. chinense* Jacq. var. Kotpar cultured on MS + 2, 4 -D (1.5 mg/L) after 4 weeks of culture. A & B: Zygotic embryos, C: Callus (3 and 4 weeks old respectively), D: Sub-cultured callus, E: Cell suspension culture, F: Orbital shaker equipment, G: Sample extraction in acetonitrile.

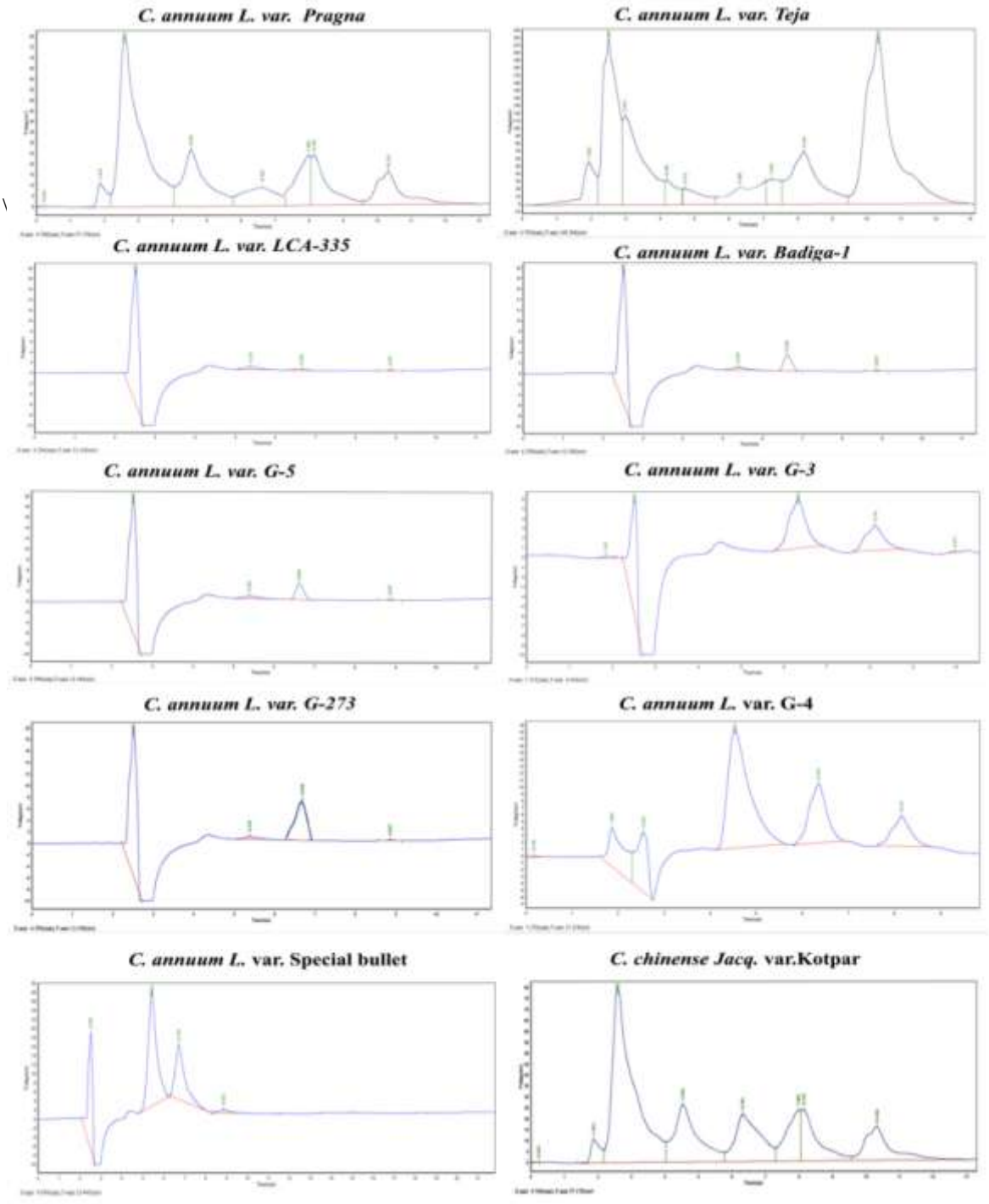

Fig: 3. Estimation of capsaicin content in in vitro zygotic embryo/callus cell suspension cultures of capsicum species using HPLC.

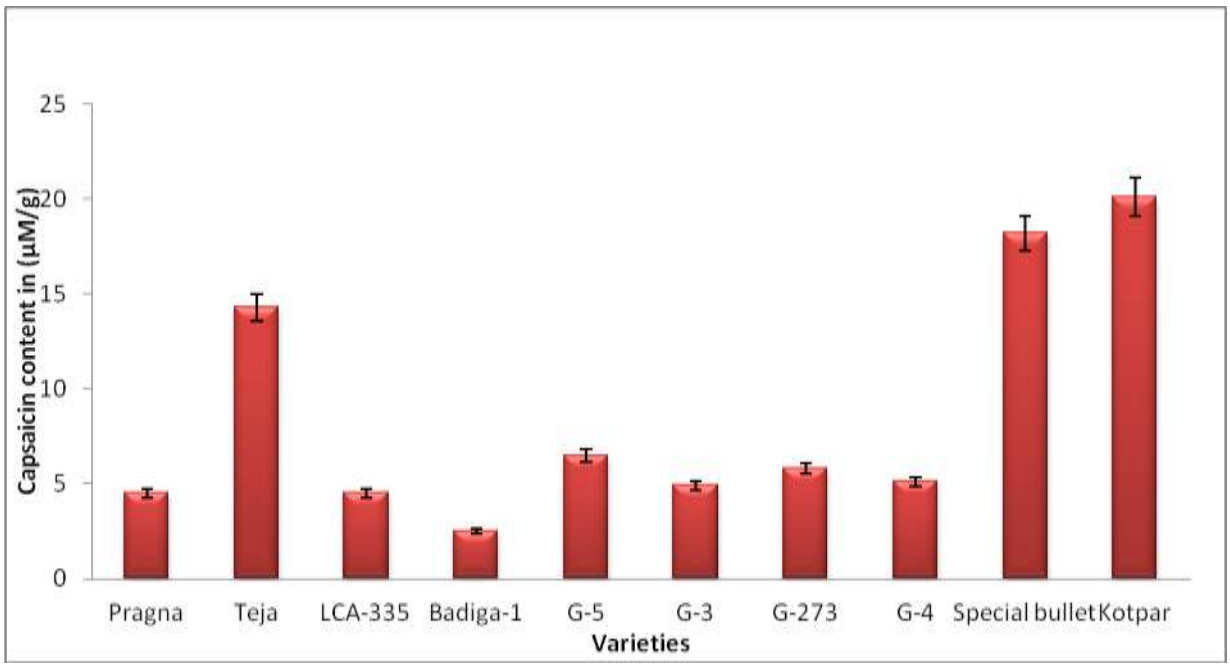

Fig: 4:- Capsaicin content (M/g) extracted from in vitro zygotic embryo/ cell suspension cultures of capsicum species using HPLC.

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