International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 454-464 www.ijprajournal.com ISSN: 2249-7781

Capsicum chinenseJacq. Capsaicinmedicinal value and its HPLC analysis

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Date Of Submission: 01-04-2021 Date Of Acceptance: 14-04-2021

ABSTRACT: Medicinal plants which formsthe backbone of traditional medicine in the last few decades been the subject of very instance pharmacological studies. Acetone and acetonitrile solvent has the best ability to extract the Capsaicin from Capsicum chinense.Extract yield at optimum condition was then analyzed by High Performance Liquid Chromatography (HPLC) for quantifying the bioactive compounds. It is also suitable for the determination of drugs in biological and environmental samples. All the analyses were performed isocratically using degassed HPLC grade 50% acetonitrile (Merck) and 50% milli 'Q' water as a mobile phase. The chromatograms obtained for acetone and acetonitrile crude extracts of C.chinense showed major peaks, identified as capsaicin, which registered a difference of 1.199 min between the retention periods of the capsaicin (1.194 min) which corresponds to the standard Capsaicin. High-pressure liquid chromatography (HPLC) is considered the most reliable and rapid method [1] available for the identification and quantification of capsaicin and capsaicinoids. Gradient elution HPLC is a powerful technique required to separate samples that otherwise exhibit the general elution problem under isocratic conditions. The purpose of HPLC analysis of any drugs is to confirm the identity of a drug and provide quantitative results and also to monitor the progress of the therapy of a disease.

KEYWORDS: Capsaicin, Capsaicinoids, Gradient Elution, HPLC, Isocratic, Neuralgia

I. INTRODUCTION:

Capsicum chinense is an ever green shrub growing to 1.5m at a fast rate. Preferably it grows well in drained loamy soil. Capsicum chinense belongs to the family Solanaceae. It is native to Central America but now it has been cultivated worldwide. It has common name 'Habenaro Pepper' and other common names like Naga King Chilli, Yellow laternchilli, Scotch Bonnet Pepper etc., It is a tropical herb and known as World hottest Chilli. The fruit has extraordinary heat and flavouring. It has a lot of medicinal property like increasing perspiration, reduce pain sensitivity and stimulates the circulatory and digestive systems. The pungent fruit has tonic and antiseptic properties. It also irritates the tissues, increases blood supply to the area and reducing sensitivity to pain [2]. It is taken internally in the treatment of the cold stage of fevers, debility in convalescence or old age, varicose veins, asthma and digestive problems. Externally it is used in the treatment of sprains, unbroken chilblains, neuralgia, pleurisy etc.,

Fruits and vegetables have been recognized as natural source of various bioactive compounds[3, 4]. The pepper belongs to the genus Capsicum, which is comprised of more than 200 varieties. Capsicums are consumed worldwide and their importance has increased gradually to place them among the most consumed spice crops in the world (Bown, 2001). Capsicum has a significant role in traditional medicine and therapeutic applications are related to the capsaicinoid, phenolic compound and carotenoids content of peppers [5, 6]. Carotenoids are more than mere pigments and play an important role as antioxidants as well. Carotenoids protect cells and tissues from harmful reactive oxygen species (ROS), acting as scavengers of singlet molecular oxygen [7], peroxyl radicals [8] and reactive nitrogen species.

Phytochemicals are secondary metabolites of plants which are non-nutritive chemicals that have protective or disease prevention properties. Normally red fruits and vegetables predominantly contain phytonutrients such as lycopene, anthocyanins, which are also responsible for their colour. Orange and yellow fruits and vegetables contain antioxidants like beta-carotene, zeaxanthin, Vitamin C etc.,[9]. Hence, the biotechnology encompassing plant tissue culture and genetic engineering is becoming a functional tool of classical plant breeding to boost the crop improvement programs. In vitro plant regeneration

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 454-464 www.ijprajournal.com ISSN: 2249-7781

from cells, tissues and organ cultures, which is a fundamental process of Plant tissue culture through biotechnological approach in plant propagation, plant breeding and genetic improvement which serves the main purposes of micro-propagation of elite plants, selection of plants against biotic and abiotic stresses and the genetic transformation [10].

High performance liquid chromatography (HPLC) was introduced to pharmaceutical analysis. It has been developed for applicable analytical method providing rapid and versatile separation possibilities that meet the increasing requirements for purity testing of bulk pharmaceuticals and pharmaceutical products [11]. It provides a number of highly selective variants to resolve almost every type of separation problem. On the basis of this, HPLC and related techniques can be regarded as the most important analytical method in contemporary pharmaceutical analysis[12].

HPLC is the prevailing separation techniques for non-volatile organics, drugs, metabolites or toxic residuals and element speciation using isocratic and gradient elution being still in progress. Assay by HPLC is one of the most common techniques used to measure the quality of the drug substances [13]. Gradient elution gave a short overall analysis with similar resolution of the critical pair compared to isocratic elution without sacrificing repeatability in retention time, peak area and peak height or linearity of the calibration curve [14]. HPLC is highly sensitive method for detection, identification and quantification of any chemical in particular samples using UV and visible absorbance [15].

II. MATERIALS AND METHODS:

Quantification Determination Of Phytochemical Screening Of Plant Materials

The plant materials such as callus, leaf, shoot, fruit, seeds, wild 1,wild 2 and wild 3 were collected, shade dried, grounded, sieved through 20-30 mesh to obtain a coarse powder and stored in an air-tight container until further processes. The Standard capsaicin was purchased from Sigma Aldrich chemicals co. St Louis, MO, USA. All solvents used for Capsaicinoid analysis were of HPLC grade from Himedia. The extraction and quantification of Capsaicinoids in polar aprotic solvent was performed according to Collins et al., with slight modifications [16]. The Chilli powder was mixed with acetone and acetonitrile solvents in the ratio of 1:10 (gram: millilitre). The mixture was placed in 120 ml glass bottle with Teflon lids. The bottles were capped and placed at 65°C in the Water bath for an hour and were swirled manually. The samples were removed from the water bath, cooled at room temperature and stored for further process. The supernatant was centrifuged at 10000 rpm and filtered through Whatman No.1 filter paper [17]. The filtrates were evaporated to dryness and crude obtained was stored at 5°C in refrigerator until further analysis was carried out [18].

THIN LAYER CHROMATOGRAPHY (TLC)

Qualitative analysis of the extracts was done by Thin layer chromatography. TLC method is providing a chromatographic plant extract fingerprint. TLC was carried out by using readymade pre-coated plates of size 10 cm × 15 cm × 0.2 cm, with aluminium backing (“Merck”, India) and coated with Silica Gel F_{254} were used. Starting line was marked at a distance of 1.0 cm from the bottom of the plate and the finishing line was marked at distance of 1.5 cm from the top of the Plate spots of standard and sample solutions in acetone and acetonitrile were applied with the help of micro-capillaries tubes[19].

Each spot contained 50 µl of the standard solution of strength 1 mg/ml (5 mg of standard Capsaicin, Sigma, USA applied effectively) and 10 µl of the sample solution from callus, shoot, leaves, fruit and seed extracts. Chromatogram development was done in vertical glass chambers saturated with following ratio (Petroleum ether: Chloroform: Acetonitrile – 4.5: 4.5: 1.5) was used as mobile phase. The chromatogram was developed in iodine chamber.

At the time, the plate was removed from the development chamber and the mobile phase was allowed to dry. After drying the plate was visualised under UV $_{254}$ nm and UV $_{366}$ nm and photographs taken. The developed plates sprayed with reagent of Vanillin-sulphuric acid reagent and kept in oven at 105°C till the colour of the spots appeared of both standard and sample were observed under a short and long wavelength of UV produced blue spots and normal light produced fluorescent spots for detection of UV quenching capsaicin in the sample and their comparison with the standards [20].

Rf was calculated as per the formula [21]

Rf = Distance travelled by the applied solute mixture front (cm) / Distance travelled by the solvent (mobile phase) front (cm).

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 454-464 www.ijprajournal.com ISSN: 2249-7781

High Performance Liquid Chromatography (HPLC):

The principle of HPLC is based on the distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase (packing material of the column). It is an extremely quick and efficient techniques to accurate result. All routine procedure for analytical HPLC was followed [22]. HPLC was carried out on a Shzimadzu instrument with a series D-7000 interface with HSM manager, L-7100 pump, L-7420 diode array detector, a rheodyne injector with a 25 µL injection loop. RP-18 column ("Merck", India) of dimension 250 x 4.6 mm with particle size of 5 mM was used.

The column was opened in the reverse phase mode. The data were recorded at a wavelength of 254 nm on a computer and the chromatograms and data reports were printed on HP Deskjet 200 printer (Hewlett Packard, India). All the solvents used were filtered through 0.22 or 0.45 µm pore size filters (Millipore Corporation, USA). The samples were filtered through 0.45 µm (Millipore filter) using a 5 ml disposable syringe (Millipore, Bedford, MA) into a sample vial. A Shimadzu (LC-10, Shimadzu, Japan) HPLC system equipped with LC- 10AS multisolvent delivery system, a SPD-10A UV-Vis detector at 280 nm and control of parameters with system controller unit (SCL- 10A) was used[23].

The analysis was carried out with the following conditions: column temperature 30°C, flow rate of mobile phase; 1 ml/min and data acquisition was made using Class LC-10 software. All analyses were performed isocratically using degassed HPLC grade 50% acetonitrile (Merck) and 50% milli 'Q' water as a mobile phase. The reverse-phase chromatographic column (Discovery C18 (250 x 4.6 mm, 5 mm), Supelco, and Bellefonte, PA, USA) was used for the detection of capsaicin and dihydrocapsaicin[24]. The flow rate was 1.0 ml/min. and the run was continued for 30 minutes for complete resolution and detection of all the Capsaicin.

The column was flushed injection by mobile phase for 10 min. to re-establish the initial conditions. Plotting of graphs and calculations of amounts of capsaicin in the tissues by the external standard method of quantification was done using routine protocols as described in the Instruction Manual for HSM Manager" as well as using the formula given by Scott [25],

$Cp(s) = ap(s) \times Cp(st)$

Where, Cp (s) is the concentration of solute in the mixture.

ap (s) is the area of the peak for the solute (p) in the sample chromatogram.

Cp (st) is the concentration of the standard in the reference solution.

The linearity of detector response for standards for Capsaicin was observed by injecting 25 µl from solutions of standards ranging in concentration from 0.625 µg-10 µg/ml [26] and plotting the standard curve Identification of Capsaicin in all tested samples was confirmed by retention time, co-chromatography with the standard and peak purity by wavelength [2].

III. RESULTS:

THIN LAYER CHROMATOGRAPHY (TLC)

TLC was performed on a readymade pre-coated Silica Gel F_{254} plates with aluminium packing. For the determination of alkaloids presence in the crude extracts, by comparing the samples with authentic sample Capsaicin (standard CAP, USA; strength of 1 mg/ml) was carried out.

For the chromatogram standardization, various concentration of mobile phases were screened viz, Petroleum ether: chloroform: acetonitrile (2.5:2.2:1.5), Petroleum ether: chloroform: acetonitrile (4.0:3.5:2.0), Petroleum ether: chloroform: acetonitrile (4.2: 4.2:1.0) and Petroleum ether: chloroform: acetonitrile (4.5:4.5:1.0) which produce different $R_f$values, 0.86, 0.75, 0.84, 0.84, 0.74 and 0.83 respectively. Among the different mobile phases Petroleum ether: chloroform: acetonitrile (4.5:4.5:1) produce $R_f$values of 0.54 used further screening of the extract.

Alkaloids were observed by the TLC plates in UV light at 254 nm (short wave length) and 366 nm (long wave length). After the chromatogram on TLC plate, the extracts showed CAP contents as dark black spot at 254 nm and fluorescent spot at 366 nm. The developed plates were dipped in Vanillin-Sulphuric acid reagent and kept in oven at 105°C till the colour of the spots appeared in both standard and sample were observed under short and long wave length of UV produced dark black spots and in normal light produced dark blue colour spots for detection of capsaicin in the sample and their comparison with the standards(Fig 1). The dark spots which are present in all the samples but in tract 2 (fruit) and tract 4 (seed) shows very dark spot which clearly shows the presence of the compound capsaicin.

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 454-464 www.ijprajournal.com ISSN: 2249-7781

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The quantification of alkaloids by HPLC has been carried out by isocratic analytical HPLC in RP-18 column with mobile phase. HPLC analysis can be modified depending on the level of quantification needed and produces a reliable result. All analyses were performed isocratically using degassed HPLC grade 50% acetonitrile (Merck) and 50% milli 'Q' water as a mobile phase [27]. The chromatograms obtained for acetone and acetonitrile crude extracts of C.chinense showed major peaks, identified as capsaicin, which registered a difference of 1.199 min (Plate a-i) between the retention periods of the capsaicin (1.194 min)(Plate f) which corresponds to the standard Capsaicin (Table 1; Fig.2 & Plate (a-i). In UV estimation the targeted capsaicin was observed at 280nm, with a prominent peak. The acetone and acetonitrile extract showed pungency level with 1,347,439 SHU and 1,266,250 SHU respectively [28].

IV. DISCUSSION

TLC AND HPLC ANALYSIS

The TLC profile of acetonitrile extract of Capsicum chinensefruit had capsaicin spot with fewer amounts of other compounds when compared to acetone. The study confirmed acetonitrile was the best solvent for the extraction of capsaicin from Capsicum chinensefruit [17, 29] reported that the content of capsaicin in Capsicum spices was found to be 0.73-3.7µg/gm dry weight. In the present TLC analysis, samples such as in vitro callus, leaf, shoot, fruit and seed were compared with the standard Capsaicin, and all the samples showed the presence of dark spot which is clearly noted the presence of capsaicin.

Among the fresh seed samples, capsaicin recovery using acetonitrile and ethanol was 27% greater than recovery using acetone ($p < 0.05$,) Fresh sample extractions with ethanol and acetonitrile were also 23–29% greater than extractions with those solvents on freeze-dried and oven-dried seeds ($p < 0.05$). Significantly more capsaicin (39%) was recovered from oven-dried seeds than freeze-dried seeds using acetone ($p < 0.05$), yet statistically similar amounts were extracted from fresh and oven-dried preparations [30].

The method of measuring the pungent property of chilli (Scoville test) is now used widely across the world, but is done by High Performance Liquid Chromatography (HPLC) [31]. The results of HPLC quantitative analysis of the capsaicinoid fraction showed a slight increase of these compounds content in red fruit of Capsicum[32].

Retention time for the constituents is 8.4 min for nordihydrocapsaicin, 9.3 min for capsaicin, 14.3 min for dihydrocapsaicin, and 17.7 min for homodihydrocapsaicin. These peaks have been verified using an HPLC–mass spectrometry (LC–MS) analysis conducted at McCormick and Co. (Hunt Valley, Md.).

In our protocol, capsaicinoids were extracted using acetonitrile instead of 95% ethanol saturated with sodium acetate, as required in the ASTA (1985) procedure.

The HPLC analyses allowed identification and determination of capsaicin and dihydrocapsaicin which were the main capsaicinoids in the different chilli and pepper samples. RP-HPLC-PDA method was used for analysis of major components of capsaicinoids (capsaicin and dihydrocapsaicin) after sample preparation using MSE and SPE methods[33, 34]. In this discussion, it is very clear that HPLC analysis is a very useful method to analysis the purity and quality of the compound Capsaicin. In the present study, HPLC analysis, capsaicin was detected at a flow rate between 1.194 min^{-1} to 1.199 min^{-1} and using the mobile phase is an isocratic mode that is, without altering the solvent pumping throughout the course of HPLC run.

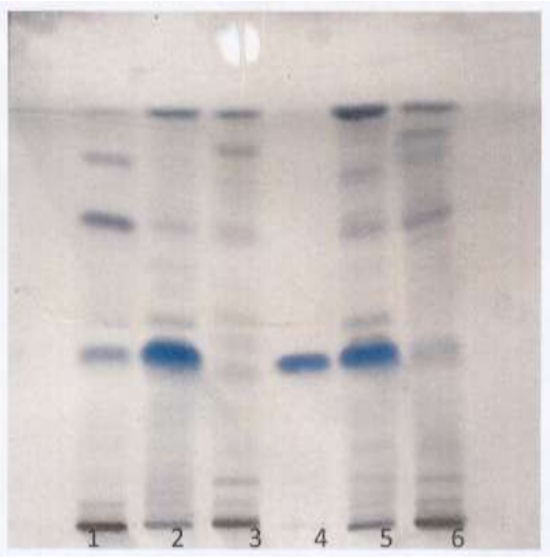
V. CONCLUSION

The study revealed that Capsicum chinensehas a great source of potential bioactive compound and it seems important to identify and characterize the compounds isolated for their use in the food, pharmaceutical, medicinal and therapeutic industries. The most abundant of these components are capsaicin (trans-8 methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin (8 methyl-N-vanillylnonanamide), which are responsible for about 90% of the spiciness [35]. The chromatography analysis are necessary for characterizing the plant profile in the conservation parts.The alkaloids are one of the most divorcees groups of secondary metabolites found in living organisms and have an array of structure type, biosynthetic pathway, and pharmacological activities [36]. There is no doubt that Capsicum chinense is being one of the hottest chilli in the world, has enormous potential for commercialization. The finding that Naga King Chilli is a result of gene introgression from C.

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 454-464 www.ijprajournal.com ISSN: 2249-7781

frutescensinto C. chinensefavour the prospect of doing wide hybridization among the Capsicum species for development of elite genotypes[37].

FIG.1 TLC PHOTO DOCUMENTATION OF CAPSICUM CHINENSE PLANT EXTRACTS SUCH AS CALLUS, LEAF, SHOOT, FRUIT AND SEED.



After derivatizationVanillin-sulphuric acid

Track-1. 15 µl (Callus), Track-2. 15 µl (Fruit), Track-3. 15 µl (leaf), Track-4. 15 µl (marker), Track-5. 15 µl (Seed),Track-6. 15 µl (Shoot)

TABLE 1. QUANTIFICATION OF CAPSAICIN CONTENT FROM DIFFERENT PARTS OF CAPSICUM CHINENSE BY HPLC PROFILE

Plant Sample	**Plant part**	**Capsaicin Concentration (mg/g)**
Wild 1	Whole fruit	2.27
Wild 2	**Whole fruit**	**2.66**
Wild 3	Whole fruit	2.23
Callus	**Callus**	**2.49**
Leaf	Leaf	2.28
Shoot	Shoot	1.61
Fruit	**Fruit**	**2.86**
Seed	**Seed**	**2.70**

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 454-464 www.ijprajournal.com ISSN: 2249-7781

FIG 2. QUANTIFICATION OF CAPSAICIN CONTENT FROM DIFFERENT PARTS OF CAPSICUM CHINENSE BY HPLC PROFILE

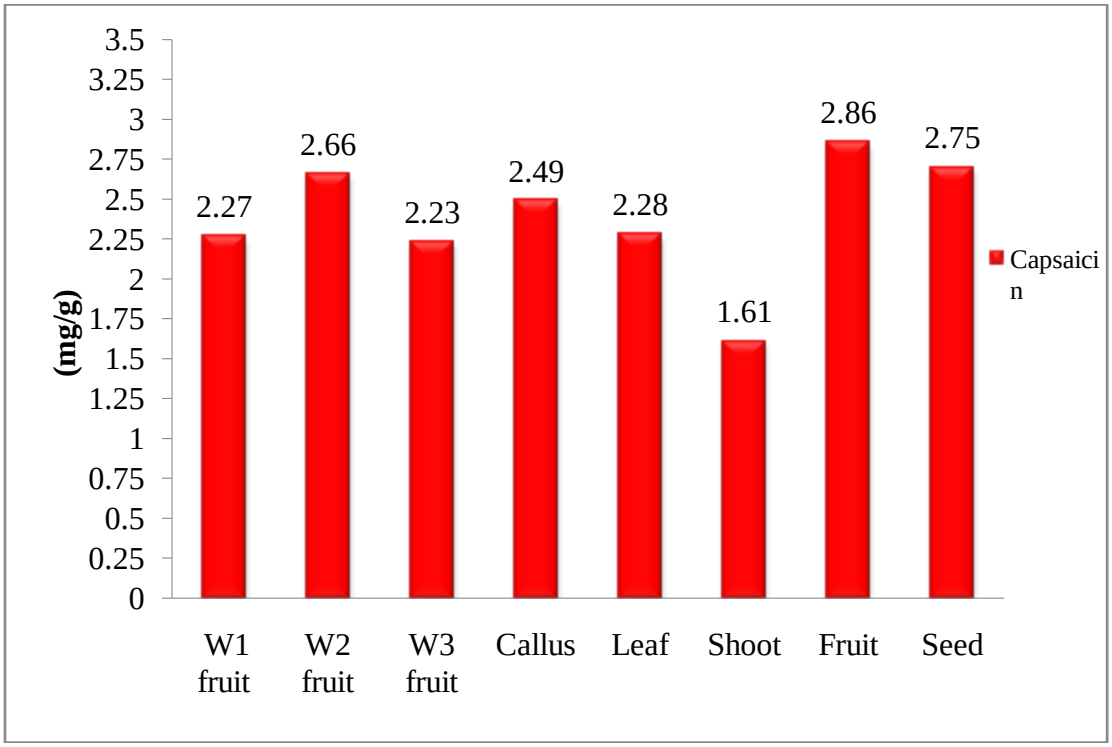
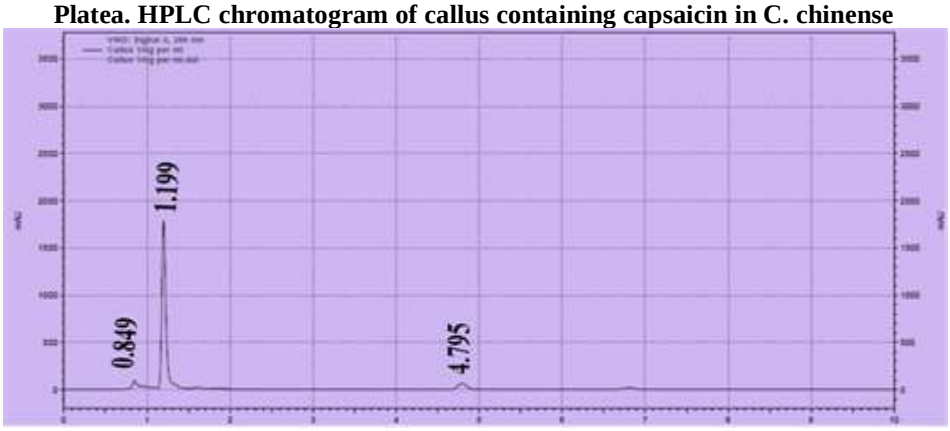


PLATE (a-i). HPLC CHROMATOGRAM OF DIFFERENT EXTRACTS OF CAPSICUM CHINENSE SHOWINGCAPSAICIN.

Platea. HPLC chromatogram of callus containing capsaicin in C. chinense



International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 454-464 www.ijprajournal.com ISSN: 2249-7781

Plate b. HPLC chromatogram of in vitro shoot containing capsaicin in C. chinense

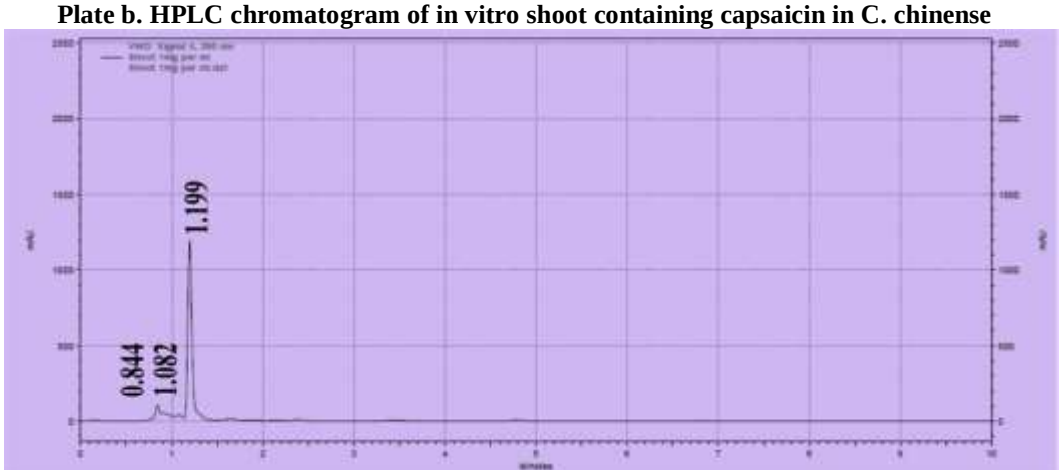


Plate c. HPLC chromatogram of in vitro leaf containing capsaicin in C. chinense

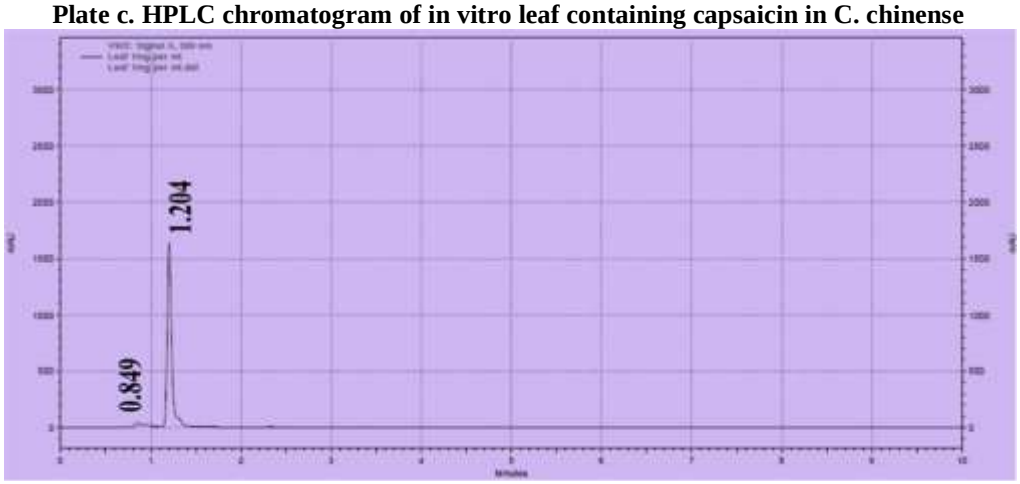
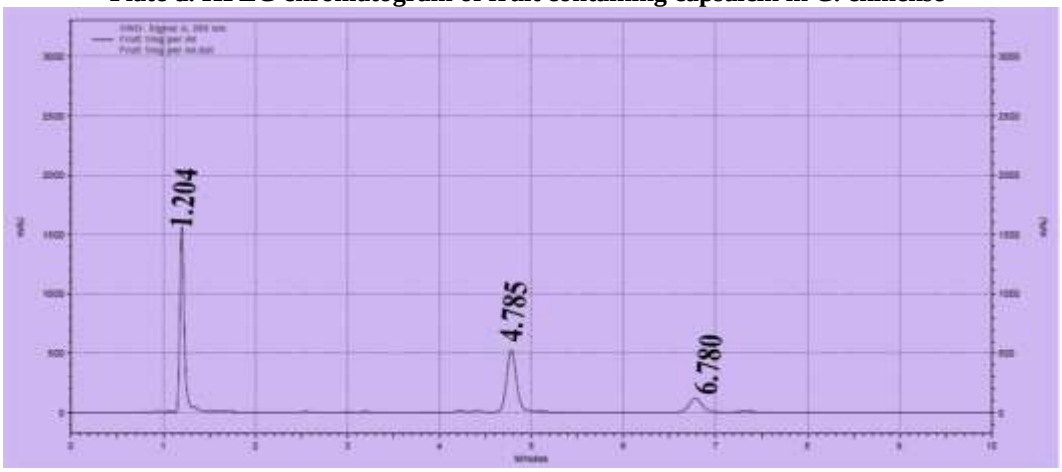


Plate d. HPLC chromatogram of fruit containing capsaicin in C. chinense



International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 454-464 www.ijprajournal.com ISSN: 2249-7781

Platee. HPLC chromatogram of seed containing capsaicin in C. chinense

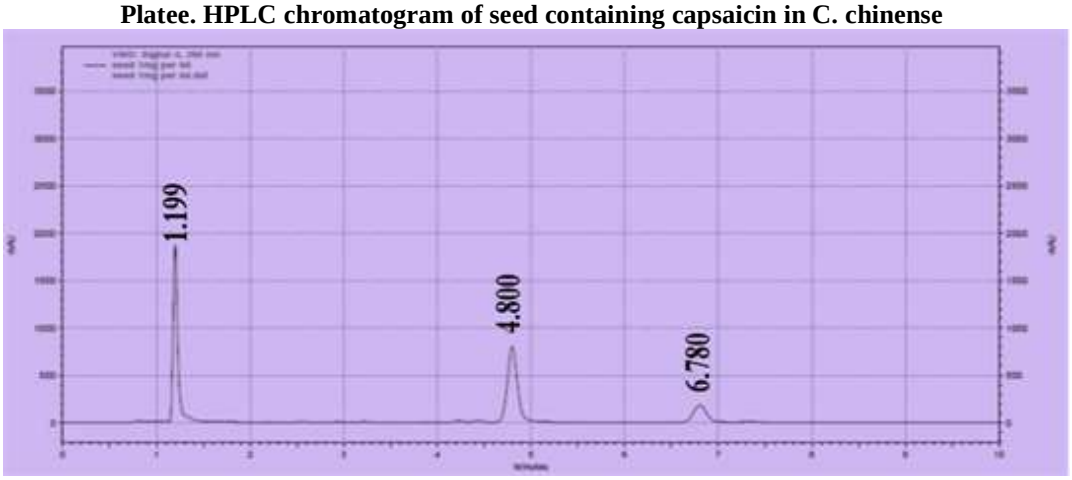
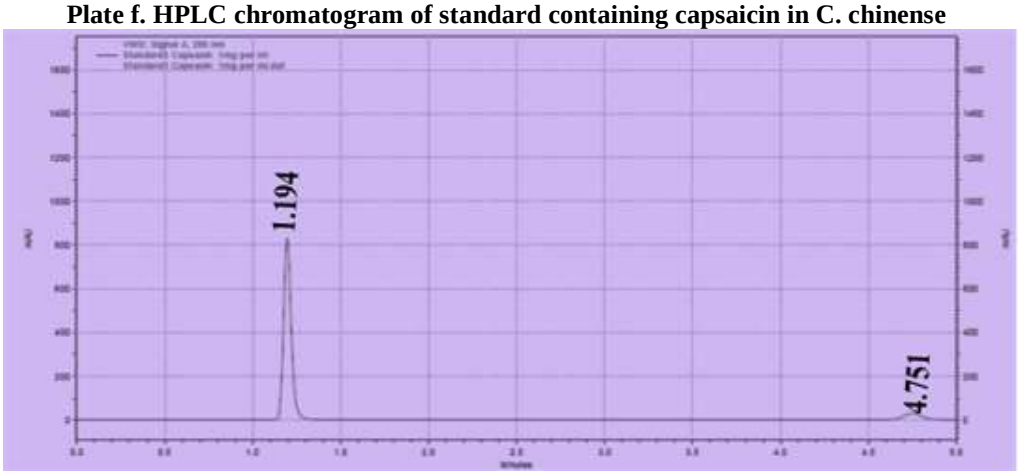
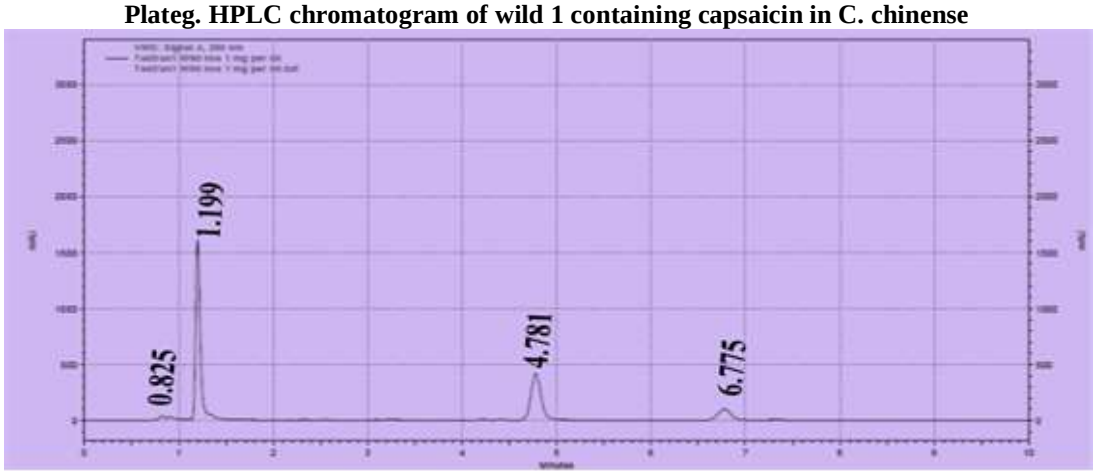


Plate f. HPLC chromatogram of standard containing capsaicin in C. chinense



Plateg. HPLC chromatogram of wild 1 containing capsaicin in C. chinense



International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 2 Mar-Apr 2021, pp: 454-464 www.ijprajournal.com ISSN: 2249-7781

Plate h. HPLC chromatogram of wild 2 containing capsaicin in C. chinense

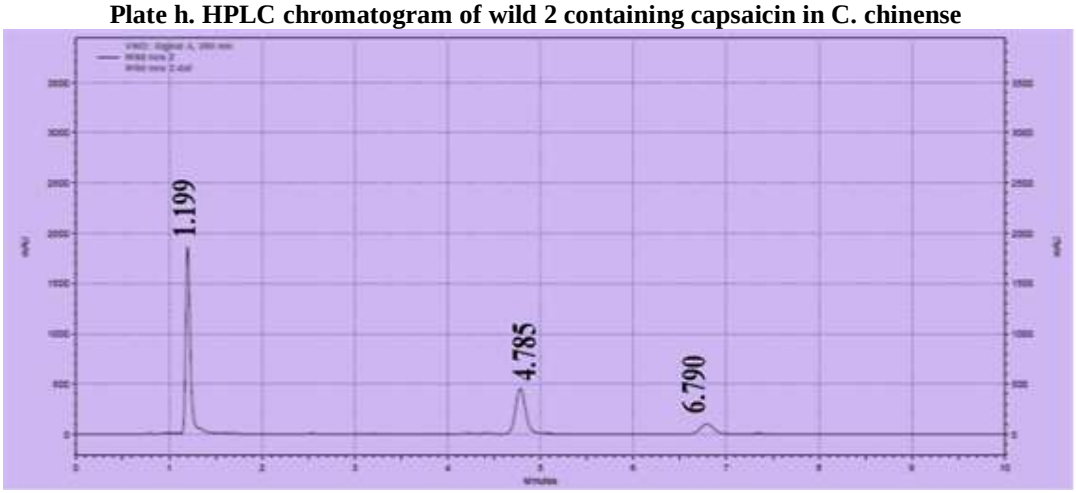
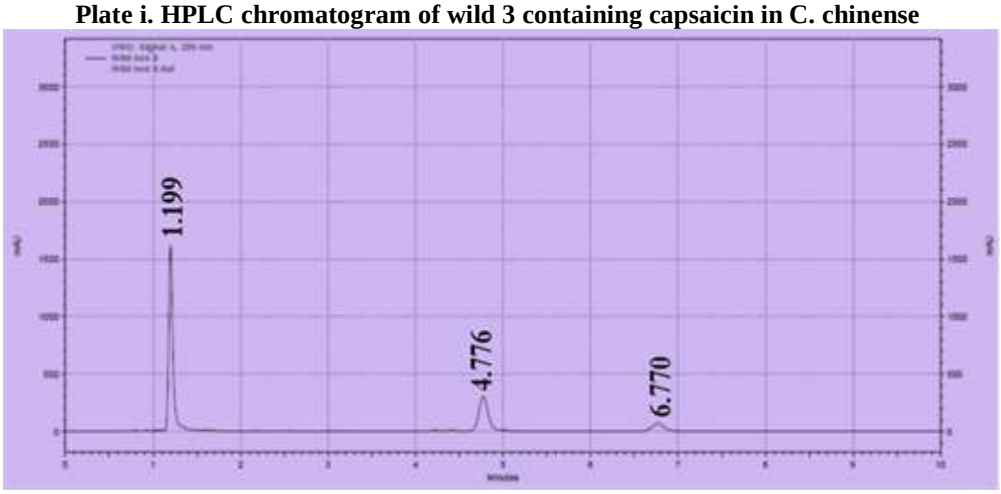


Plate i. HPLC chromatogram of wild 3 containing capsaicin in C. chinense



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