Trends in Phytochemical Research (TPR)

Journal Homepage: https://sanad.iau.ir/journal/tpr

*Original Research Article*

# Exploration of metabolic variations, anti-cholinesterase, anti-heme biocrystallization, and anti-protein denaturation activities of ten *Capsicum* accessions under different stages of ripening

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**ABSTRACT**

Hydro-methanolic extracts of pericarps and seeds of 10 different *Capsicum* cultivars from three different species viz., *Capsicum chinense*, *C. annuum* and *C. frutescens* were assessed to determine metabolic variations and anti-cholinesterase, anti-malarial and anti-protein denaturation activities at four different stages of ripening, namely green unripe (GU), turning green-orange (TGO), orange ripe (OR) and red fully ripe (RFR) stages. Many of the ripening stages of the tested cultivars showed significant bioactivities in a dose-dependent manner. In addition, metabolites were identified using GC/MS. On the other hand, HPLC analysis revealed the highest capsaicin and dihydrocapsaicin content in the pericarp of GU, TGO and RFR stages of *C. chinense*. β-Carotene, capsanthin and lutein contents of *C. chinense* were also measured using HPTLC technique. The GU and RFR stages of *C. chinense* contained appropriate amounts of all three carotenoids. These results may be helpful in selecting the cultivars with the best attributes.

**ARTICLE HISTORY**

Received: 27 September 2023
Revised: 05 December 2023
Accepted: 01 March 2024
ePublished: 05 March 2024

**KEYWORDS**

Anti-cholinesterase
Anti-heme biocrystallization
*Capsicum* species
Cultivars
Ripening stages

## 1. Introduction

Plants containing various bioactive ingredients are gaining increasing attention due to their effectiveness in enhancing human health and nutrition (Idris et al., 2017; Unuofin et al., 2017). The proper identification of metabolites with bioactive properties is a fundamental step in any improvement program, such as selecting effective genotypes in plant breeding programs for various pharmacological and nutritional purposes (Chenet al., 2014; Ganie et al., 2015). Medicinal plants have long been revered for their profound therapeutic properties, offering a natural and holistic approach to healing various ailments. Packed with a myriad of bioactive compounds, these plants possess the ability to alleviate symptoms, boost immunity, and promote overall well-being (Agrawal and Jain, 2023; Singh et al., 2023). From traditional herbal remedies to modern pharmacological applications, the therapeutic potential of medicinal plants continues to be explored and harnessed by cultures worldwide. Whether it's the anti-inflammatory properties of turmeric (Choi et al., 2019), the calming effects of chamomile (Devi and Kumar, 2023), or the immune-boosting prowess of *Echinacea* (Chen and Yu, 2016), these botanical wonders serve as a testament to nature's profound healing capabilities. As we delve deeper into the realm of herbal medicine, we uncover a treasure trove of plant-based remedies that offer gentler alternatives to conventional treatments, empowering individuals to take control of their health in a sustainable and harmonious manner (Mohammad hosseini et al., 2019a; Mohammad hosseini et al., 2019b).

*Capsicum,* the genus known by different names such as chilli, chile pepper, bell pepper, hot pepper, sweet pepper etc., all over the world, belongs to the family Solanaceae. The family Solanaceae, encompassing diverse plants like tomatoes, potatoes, and bell peppers,

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contributes a wealth of culinary delights and medicinal treasures to our lives. *Capsicum* spp. are considered as one of the oldest, popular vegetables and spices cultivated mostly in the tropical and subtropical parts of the world. Chillies are appreciated not only because of their economic importance but also for their rich nutritional values. Chillies are abundantly produced in India and, because of their cheapest price, they are consumed across all over the country (Mehta, 2017). Several *Capsicum* sp. have been domesticated comprising mild and sweet to hot, strongly pungent, flavoured and aromatic ones. The sweet non-pungent peppers are consumed as vegetables, whereas pungent peppers are employed as hot spice. *Capsicum* plants are perennial shrubs consisting of about 27 species, among which 5 species, namely *Capsicum annuum* L., *C. chinense* Jacq., *C. pubescens* R., *C. baccatum* L. and *C. frutescens* L. are broadly cultivated. (Bosland and Votava, 2012).

The *Capsicum* fruit has been traditionally used as a vegetable, natural coloring agent and as a therapeutic medicine. A diverse group of bioactive phytochemical compounds consisting of phenolics, flavonoids, carotenoids, etc. are present in the fruit of *Capsicum* plants (Altemimi et al., 2017). Besides the vast economic importance of the peppers as vegetables and spices, the active principles present in the fruit of *Capsicum* account for its significant contribution in ethnopharmacological use (Palevitch and Craker, 2012). Dietary antioxidants protect us from cancer, diabetes, cardiovascular diseases, etc. Chilli peppers are rich in protective antioxidants like vitamin C, E, β-carotene, etc. (Villa-Rivera and Ochoa-Alejo, 2020). It has also been documented that *Capsicum* species exhibit varying degrees of antimicrobial properties against *Bacillus cereus, Bacillus subtilis, Clostridium sporogenes, Clostridium tetani, Streptococcus pyogenes* (Cichewicz and Thorpe, 1996) and on *Arcobacter butzleri, Arcobacter cryaerophilus, Arcobacter skirrowii, Campylobacter jejuni* and *Helicobacter pylori* (Dogan et al., 2018). Mature green fruits of *C. annuum* var. *acuminatum* showed α-amylase and α-glucosidase inhibitory activities while its fruits, at the premature green stage, inhibited acetylcholinesterase enzyme by the Ellman method (Loizzo et al., 2008). Aqueous extract of green *C. annuum* exhibited a protective effect against ethanol induced hepatotoxicity by acting as an antioxidant agent. It arrested ethanol induced apoptosis and brought down pro-inflammatory cytokine levels (Das et al., 2018). Chilli pepper is known to exhibit hypocholesteremic and hypolipidemic properties. It can be used in the intervention of cardiovascular diseases (Sanati et al., 2018). Furthermore, chilli pepper extracts are reported to have anti-inflammatory and anti-allergic properties (Jolayemi and Ojewole, 2013). Chilli fruits are used as stomachic remedies and represent preventive properties for diseases like rheumatism, arthritis, heart arrhythmias, bronchitis and chest colds with cough and headache (Pawar et al., 2011). Fruit extract of *C. annuum* and fine powder of *C. chinensis* were reported to have larvicidal activity against *Aedes albopictus* and *Culex quinquefasciatus* (Ombugadu et al., 2020).

Fruit pungency is the most desirable flavour trait found in chillies. Pungency is mainly because of the presence of capsaicinoids, which is a unique characteristic of chilli fruits. No other genus except *Capsicum* contains capsaicinoids. Piperine ($C_{17}H_{19}NO_3$), present in black pepper, has a similar composition as of capsaicin ($C_{18}H_{27}NO_3$), thereby giving a mildly pungent flavor. Capsaicinoids are amides naturally synthesized by condensation of vanillylamine and different-sized fatty acid chains in the presence of capsaicin synthase enzyme within the placenta of chilli fruits. Capsaicin is the most abundant capsaicinoid followed by dihydrocapsaicin. Other minor capsaicinoids are norcapsaicin, nordihydrocapsaicin, nornordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin (Maokom et al., 2014).

*Capsicum* fruits being one of the most common spices in the world and having a range of medicinal and nutritional value may be explored for their anti-cholinesterase activity for controlling neuro-degenerative diseases, anti-malarial activity to manage chloroquine resistant malaria and anti-protein denaturation activity for the intervention of arthritic pain.

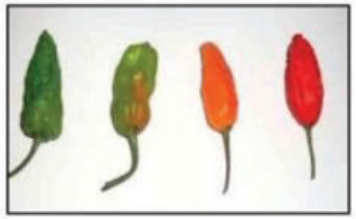
In the present study, we made an effort to understand *in vitro* anti-acetylcholinesterase, anti-malarial and anti-protein denaturation activity of different species and varieties (landraces) of sweet to hot *Capsicum* fruit pericarp as well as seeds along with placenta during their ripening stages from green unripe (GU) to turning green-orange (TGO) to orange ripe (OR) and finally to red fully ripe (RFR) stages. We also intended to identify the compounds present in the hydro-methanolic extracts that can be correlated to the above mentioned bioactivities from the metabolite profiles. Moreover, on the basis of their metabolite profiling using GC/MS based metabolomic and chemometric tools, we discriminated the tested cultivars (landraces) during their ripening stages.

## 2. Experimental

### 2.1. Plant materials

The experimental samples were collected from different parts of West Bengal, India based on morphological variations at different stages of ripening from 2016 to 2018 only during the peak growing season, *i.e*, November to December. Ten different types of chilli fruits from three different *Capsicum* species, *C. chinense, C. annuum* and *C. frutescens* were collected (Fig. 1) during the peak winter season, *i.e.*, the months of November to December (Table 1). Fruits were sampled according to different ripening stages namely green unripe (GU), breaker/turning green-orange (TGO), orange ripe (OR) and red fully ripe (RFR) stages. In bird's eye chilli group of *Capsicum frutescens*, only the green black (GB) and black (BL) stages could be collected. In the clustered pendent downward chilli group of *Capsicum annuum*, the ripening stages were green unripe (GU), breaker/turning green-black (TGB) and black-orange ripe (BO).

The taxonomic identification of the studied chilli species and cultivars were done by Prof. Dr. Pinaki Acharya, Department of Horticulture, University of Calcutta, West Bengal, India. The voucher specimens of the different



1. *Capsicum chinense*
cv. Ghee smelling group (8– 9 cm. long)

2. *Capsicum frutescens*
cv. Bird's Eye chilli pepper group (4-5 cm. long)

3. *Capsicum frutescens*
cv. Tobasco group (1-2 cm. long)

4. *Capsicum annuum*
cv. clustered pendent downward
group (10 – 11 cm. long)

5. *Capsicum annuum*
cv. cherry group
(1-2 cm. in diameter)

6. *Capsicum annuum*
cv. pendent anaheim group
(8-10 cm. long)

7. *Capsicum annuum*
cv. small hot, pendent solitary
group (3-4 cm. long)

8. *Capsicum annuum* var. *glossum* of Shimlai Mirchi Group – Green,
Red and Yellow bell pepper (10-12 cm long)

9. *Capsicum annuum* of
long waxy group (15–20 cm. long)

**Fig. 1.** The morphological variations of the experimental samples displayed along with their ripening stages.

**Table 1**
List of *Capsicum* spp. studied along with their morphological characters.

| Sl. No. | Species / Cultivar. | Name of Cultivated Varieties / Landraces | Morphology, size of fruits | Collection spots, districts |
|---|---|---|---|---|
| 1 | *Capsicum chinense* Jacq. | Ghee (a kind of butter) smelling variety | Conical/inflated, pointed tipped, wrinkle surfaced, ridged, pericarp 8-9 cm. long | Barrackpore Station market, North 24 parganas Lat 22.759908 Long 88.370349 |
| 2 | *Capsicum frutescens* L. | Bird's eye chilli pepper group | Black pericarp, pointed, 4-5 cm. long | Contai market, East Midnapore Lat 21.781134 Long 87.7069292 |
| 3 | *Capsicum frutescens* L. | Tobasco group | Very small sized, pointed, 1-2 cm. long | Field cultivation of Baruipur, South 24 Parganas Lat 22.374367 Long 88.4328829 |
| 4 | *Capsicum annuum* L. | Clustered pendent downward, long black group | Pointed, slender, long, black, 10-12 cm. long | Vegetable market, Purulia Lat 22.8780863 Long 86.4994078 |
| 5 | *Capsicum annuum* L. | Cherry group | Roundish, smooth surfaced, 1-2 cm. in diameter | Field cultivation in Baruipur, South 24 Parganas Lat 22.374367 Long 88.4328829 |
| 6 | *Capsicum annuum* L. | Pendant Anaheim group | Long, slender, tips pointed, 8-10 cm. long | Vegetable market North Kolkata Lat 22.5671008 Long 88.2156787 |
| 7 | *Capsicum annuum* L. | Small hot pendent solitary group | Tips not pointed, smooth surface, short, 3-4 cm. long | Vegetable market of Jhargram village, West Midnapore Lat 22.454647 Long 86.9938421 |
| 8 | *Capsicum annuum* L | Large hot pendent solitary group | Tips not pointed, smooth surface, medium sized, 5-7 cm. long | Vegetable market, North Kolkata Lat 22.5671008 Long 88.2156787 |
| 9 | *Capsicum annuum* L. | Variety glossum Shimlai Mirchi group- green, red and yellow bell pepper | Pericarp fleshy, not pointed smooth surfaced, ridged, 10-12 cm. long | Lakshmikantapur station market, South 24 Parganas Lat 22.110001 Long 88.322828 |
| 10 | *Capsicum annuum* L. | Long waxy group | Very long sized, pericarp, smooth, thick, fleshy, not pointed, 15-20 cm. long | Field cultivation in Lauhati, North 24 Parganas Lat 22.608800 Long 88.519092 |

plant samples were deposited at the Calcutta University Herbarium (CUH), University of Calcutta, Kolkata, West Bengal, India.

## 2.2. Chemicals and reagents

Acetylcholinesterase enzyme from *Electrophorus electricus*, hematin porcine, chloroquine di phosphate, *N*- methyl-*N* (trimethylsilyl) trifluroacetamide, methoxyamine hydrochloride, fatty acid methyl esters (FAME) markers, vanillic acid, *O*-acetyl salicylic acid, benzene-1,2,4 triol, capsaicin, dihydrocapsaicin and adonitol were purchased from Sigma-Aldrich, St. Louis, USA. Hydrochloric acid, dimethyl sulphoxide, oleic acid, glacial acetic acid, pyridine, sodium dodecyl sulphate, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide, sodium bicarbonate and sodium acetate were procured from Merck Specialities Pvt. Ltd., Mumbai. Gallic acid, ferulic acid, 4-hydroxy benzoic acid, quinic acid, chlorogenic acid, caffeic acid, bovine serum albumin, acetyl thiocholine iodide and 5,5-dithiobis 2-nitro benzoic acid (Ellman's reagent) were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. 3,4-Dihydroxy benzoic acid was acquired from Hi Media Laboratories Pvt. Ltd., Mumbai. Galantamine hydrobromide, was acquired from Sun Pharmaceutical Industries, India. Ibuprofen was purchased from Abbott India Limited, Goa. All the organic solvents used were of analytical grade purchased from Merck Pvt. Ltd. For GC/MS analysis,

the solvents used were of HPLC grade purchased from Merck Pvt. Ltd.

### 2.3. Extraction of plant materials

Hydro-methanolic extracts of fruit pericarps and seeds with placenta were prepared by heating the liquid nitrogen crushed plant materials in 50% methanol at 70 °C, for 3 hours in a boiling water bath under constant stirring. The filtrate for each sample was then evaporated to dryness under reduced temperature and pressure. The crude extract of each sample was preserved at -20 °C for further analysis. The extracts were used to evaluate their anti-cholinesterase, anti-malarial and anti-arthritic activity. With the same solvent extracts, GC-MS, HPLC and HPTLC analyses were also performed under the optimal experimental conditions.

### 2.4. Assay for acetylcholinesterase inhibition

Acetylcholinesterase (AChE) inhibitory property was measured modifying the previously reported method of Debnath et al. (2021). In this relation, AChE from electric eel was used as enzyme source for AChE assay. The 5,5′-dithiobis (2-nitro benzoic acid) (DTNB) was used as color developer for the measurement of cholinesterase activity. A yellow anion (5-thio-2 nitro benzoate) was formed as a result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylcholine. Different concentrations of hydro-alcoholic extracts of pericarp and seed extract (10 µL) were added to 20 µL of AChE (19.93 unit/mL in phosphate buffer, pH 8) and 1 mL of buffer. The reaction was started by adding 10 µL of 5,5′-dithiobis (2 nitro benzoic acid, 0.5 mM) (DTNB) and 20 µL of acetylthiocholine iodide (0.6 mM) solution. The reaction mixture was incubated at 37 °C for 20 minutes. The optical density was measured spectrophotometrically at 412 nm immediately. The percentage inhibition of AChE activity of the plant extracts was calculated using the following formula:

Inhibition (%) = (A - S)/A × 100 (Eqn. 1)

Where A and S respectively represent the OD value for the control and the test sample. From triplicate test results, the data were represented as mean ± standard deviation (SD) and all the obtained results were analysed by Microsoft Excel, 2007. The value of $p < 0.05$ was considered as significant value of the data. Galantamine hydrobromide was used as positive control.

### 2.5. Assay for hematin polymerization inhibition

The potential of anti-malarial activity was evaluated using the protocol followed by Hussain et al. (2013) with slight modifications. Accordingly, 100 µL of plant sample dissolved in 10% DMSO was incubated with 100 µL of hematin (0.003 M, freshly prepared in 0.1 M NaOH), HCl (1 M) and oleic acid. The reaction volume was adjusted by sodium acetate buffer (0.5 M, pH 5). The samples were incubated for 4 hours with gradual shaking in an orbital shaker. After incubation, the samples were centrifuged at a speed of 14000 rpm, at 21 °C for 10 minutes. The hemozoin pellets were separated from the supernatant and re-suspended in 1 mL of NaOH (0.1 M) and incubated again for 1hour at room temperature. The hemozoin content was determined by measuring the absorbance of samples at 400 nm using UV-Visible spectrophotometer. The percentage inhibition of hematin polymerization inhibitory activity of the plant extracts was calculated following the formula:

Inhibition(%) = (AB - AS)/AB (Eqn. 2)

Here, AB represents the OD value of the blank, and AS denotes the OD value of the samples undergoing testing. The inhibition percentage of triplicate test results were represented as mean ± standard deviation (SD) and all these data were plotted by Microsoft Excel, 2007. The value of $p < 0.05$ was considered as significant value of the data. Chloroquine di phosphate was used as positive control.

### 2.6. Assay for protein denaturation inhibition

*In vitro* protein denaturation assay was performed by the method of Mizushima and Kobayashi (1968) along with Sakat et al. (2010) with slight modifications. In this regard, the reaction consisted of a mixture with 200 µL of the test solutions and 200 µL of aqueous bovine serum albumin (BSA, 5%). pH of the reaction mixture was adjusted using glacial acetic acid. The reaction tubes were first incubated at 37 °C for 20 min and thereafter were heated at 70 °C for 10 min. After cooling, the relevant turbidity was measured at 660 nm while using sample along with distilled water devoid of BSA, as a blank. The percentage inhibition of protein denaturation was calculated using the formula:

Inhibition(%) = (A - B)/A (Eqn. 3)

In this context, A stands for the absorbance of the control set, while B represents the absorbance of the tested sample. The triplicates of inhibition(%) for each dilution were represented as mean ± standard deviation (SD) and all the obtained data were evaluated by Microsoft Excel, 2007. The value of $p < 0.05$ was considered as significant value of the data. Ibuprofen was used as positive control.

### 2.7. Chemical profiling of the herbal extracts using GC/MS analysis

Agilent 7890 A GC [software driver version 4.01 (054)] interfaced with 5795 C inert MSD with Triple Axis Detector was employed for GC-MS analysis. HP-5 MS capillary column [Agilent J&W; GC Columns (USA)] of dimensions 30 m × 0.25 mm × 0.25 µm was used in the analytical arrangement. The method of Kind et al. (2009) was followed with a few modification by Das et al. (2016). The oven temperature program for analysis was set as oven ramp 60 °C (initial 1 min hold), to 325 °C with an increasing rate of 10 °C per min. The oven temperature was held for 10 min before cooling down producing a total run time of 37.5 min. The injection temperature was set at 250 °C, the MSD transfer line at 29 °C and the ion source at 230 °C. Helium was used as the carrier gas at a flow rate of 0.723 mL/min (carrier linear velocity of 31.141 cm/s). Adonitol was added to the dried crude extract and was followed by derivatization using methoxyamine hydrochloride

and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide. FAME markers prepared in chloroform was added to the sample before injection. Derivatized samples were injected via splitless mode on to the column. A solvent delay of 5.90 minute was allowed to check overloading of sample in the column. Chromatographic peaks and mass spectra were recorded. Automated mass spectral deconvolution and identification system (AMDIS) was used to deconvolute and identify metabolites from chromatographic peaks. The fragmentation patterns of the mass spectra as well as retention times (RT) of samples were compared with entries of mass spectra, RT in Agilent GC-MS Metabolomics RTL Library (2008) (Agilent Technologies, USA). Many metabolites were further confirmed by chromatographic data with that of authentic metabolite samples. Normalization of peak areas of different metabolites were done by dividing the peak area by dry weight of crude extract followed by peak area of adonitol as the internal standard. The relative response ratios obtained by this process was further used for data interpretation.

### 2.8. HPLC analysis

The HPLC analyses were carried out on an Agilent 1260 infinity series HPLC system equipped with a quarternary pump and a diode array detector. The chromatographic conditions include Waters Symmetry $C_{18}$ column [dimension: 150 mm × 4.6 (internal diameter) and 5 µm particle size]. The UV detection wavelength for both capsaicin and dihydrocapsaicin was set at 280 nm (Usman et al., 2014). Mobile phase consisted of a binary mixture of water and methanol which was run in a linear gradient modifying the method reported by Waite and Aubin (2008). Crude dry samples were extracted using methanol (HPLC grade) and centrifuged. Supernatant was then filtered using a 0.2 µm membrane filter and 20 µL of the sample with a range of concentration from 10 to 50 mg/mL was subsequently injected manually into the system at ambient temperature. The linear gradient elution was applied using 100% water (solvent A) and 100% methanol (solvent B) at a constant flow rate of 1.50 mL/min for a total run time of 20 minutes. The gradients were as follows: 40% B, increased to 85% B over 8 min, increased to 99% B over 5 min and then returned to initial ratio (40% B) over 7 min.
Different concentrations ranging between 0.01 to 15 mg/mL of capsaicin and 0.01 to 3 mg/mL of dihydrocapsaicin were prepared and run into the system. The peak areas of the standards were plotted into a calibration curve to find out unknown concentration of capsaicin and dihydrocapsaicin in the samples. The content of capsaicin and dihydrocapsaicin (µg/mg) of the extracts were calculated from the calibration curve. Then, 20 µL of an eluent containing water and methanol in a ratio of 9:1 was injected after every sample set to avoid measurable carryovers. Injection of both standards were made at the beginning of each work module.

### 2.9. HPTLC analysis

High performance-thin layer chromatography was carried out on pre-coated silica gel 60$F_{254}$ plates of 0.25 mm thickness and the operating software used was WinCATS. Acetone extracts of only *Capsicum chinense* fruit pericarp were analysed for lutein, β-carotene and capsanthin following Hernandez et al. (2012) and Das et al. (2017).

### 2.10. Statistical analysis

Each experiment was performed in triplicates and percentage inhibition was calculated using the formula mentioned as Eqn. 1, Eqn. 2 and Eqn. 3. Regression equations were prepared from the concentrations of the extracts and percentage inhibition of enzyme activity using Microsoft Excel, 2007. $IC_{50}$ values as a measure of concentration of sample required to inhibit the enzyme activity by 50% were calculated from the regression equations.

### 2.11. Multivariate data analysis of polar metabolites

Multivariate analysis such as PLS-DA (partial least squares-discriminant analysis) of different varieties of *C. annuum*, *C. chinense* and *C. frutescens* were carried out with the help of Metaboanalyst 4.0, for metabolomic data analysis and interpretation.

## 3. Results and Discussion

### 3.1. AChE inhibition

***Capsicum chinense*:** All the pericarp and seed extracts of four ripening stages (GU, TGO, OR and RFR) of the ghee smelling group inhibited the AChE enzyme in a dose-dependent manner. Both pericarp and seed of the TGO stage showed the highest activity with $IC_{50}$ value of 1.593 ± 0.049 and 1.497 ± 0.037 mg/mL, respectively.
***Capsicum frutescens*:** 50% hydro-methanolic extracts of all the pericarp and seed of erect upward bird's eye chilli group showed inhibition against AChE in a dose-dependent manner. All the extracts of the upward tobacco group except seed of TGO stage inhibited the enzyme. Among these two landraces of *C. frutescens*, the highest activity with the lowest $IC_{50}$ value (0.789 ± 0.048 mg/mL) was observed in the pericarp of BL stage of erect upward bird's eye chilli group followed by the pericarp of GB stage of the same ($IC_{50}$ = 0.833 ± 0.041 mg/mL).
***Capsicum annuum***: Pericarp and seed extracts of all the ripening stages of clustered pendent downward group inhibited the AChE enzyme in a dose-dependent manner. Both pericarp and seed of OR stage of the cherry group did not show any activity. Pericarp of OR stage and seed of RFR stage of the pendent Anaheim group did not inhibit AChE enzyme. Extracts of all the stages of large hot pendent group except the seed of RFR stage showed AChE inhibitory activity. Only the pericarp and seed of GU stage and pericarp of TGO stage of small hot pendent solitary group showed inhibition against AChE enzyme. The seed extract of GU and RFR stage of the long waxy group did not show any inhibition. None of the seed extracts from the green, yellow and red bell pepper inhibited AChE. However, seed extract of the GU stage of the large hot pendent group and pericarp

extract of BO stage of clustered pendent downward group showed the highest inhibition of all the samples tested.

In case of pericarps, the BO ripening stage of the clustered pendent downward group of *Capsicum annuum* showed the highest activity with an $IC_{50}$ value of 0.671 ± 0.007 mg/mL followed by BL and GB stage of erect upward bird's eye chilli group of *Capsicum frutescens* with $IC_{50}$ values of 0.789 ± 0.05 and 0.833 ± 0.036 mg/mL. In case of seeds, the highest inhibition was observed in the GU stage of large hot pendant group followed by the GU stage of clustered pendent group of *Capsicum annuum* with $IC_{50}$ values of 0.627 ± 0.029 and 0.845 ± 0.041 mg/mL. The $IC_{50}$ values of all the tested samples are depicted in the Fig. 2(A).

### 3.2. Anti-heme biocrystallization assay

Anti-heme biocrystallization assay was carried out for all the *Capsicum* samples and percentage inhibition was noted at 3 mg/mL for the pericarp and seed extracts of all the ripening stages.

***Capsicum chinense:*** Both pericarp and seed of all the ripening stages of ghee smelling group of this species exhibited anti-heme biocrystallization activity. The highest activity was shown by the pericarp of TGO stage with 91.25 ± 0.68% inhibition followed by seeds of GU stage with inhibition(%) of 89.93 ± 0.36%.

***Capsicum frutescens*:** Both pericarps and seeds of BL and GB stage of erect bird's eye chilli group showed remarkable activities with 99.25 ± 0.29% (pericarp BL), 99.14 ± 0.37% (seed BL), 97.53 ± 0.11% (pericarp GB) and 90.10 ± 0.34% (seed GB) respectively to inhibit the biocrystallization of hematin. Except the seed of TGO stage (30.18 ± 0.58%), seed (48.0 ± 0.66%) and pericarp (46.91 ± 0.69%) of OR stage and seed of RFR stage (44.54 ± 0.66%) of upward tobasco group all the other stages of extractions showed more than 50% inhibition. Pericarp of RFR stage followed by seeds of GU stage of ripening showed higher activities such as 90.32 ± 0.27% and 73.78 ± 0.5%, respectively.

***Capsicum annuum*:** All the ripening stages of pericarps and seeds of pendent anaheim, large hot pendent, small hot pendent solitary, cherry, clustered pendent downward, long waxy and glossum bell pepper groups showed anti-heam biocrystallization activity more than 50%, except the seeds of OR stage of long waxy group. The pericarp of TGO stage of pendent anaheim group, seed of TGO stage of large hot pendent group, seeds of OR and RFR stages of cherry group, pericarp of TGB stage of clustered pendent downward group, seeds of TGO stage of long waxy group and the pericarp of red stage of glossum bell pepper showed 98.94 ± 0.31%, 97.12 ± 0.20%, 99.0 ± 0.27% and 99.18 ± 0.33%, 99.14 ± 0.43%, 99.79 ± 0.11%, 99.75 ± 0.16% inhibition of polymerization of hematin protein which exhibited strong anti-plasmodial or anti-malarial activity. Out of all these values, the highest inhibition was obtained in the seed extract of TGO stage of long waxy group of chilli, *i.e.*, 99.79 ± 0.11%. The anti-heme biocrystallization activity of all the tested samples are represented in the Fig. 2(B).

### 3.3. Anti-protein denaturation assay

Pericarp and seed extracts of different ripening stages of all the samples were subjected to anti-protein denaturation assay at a concentration of 292.68 mg/mL. Among all the species and cultivars, the highest inhibition was exhibited by the pericarp of BL stage of erect bird's eye chilli group with 99.23 ± 0.16%.

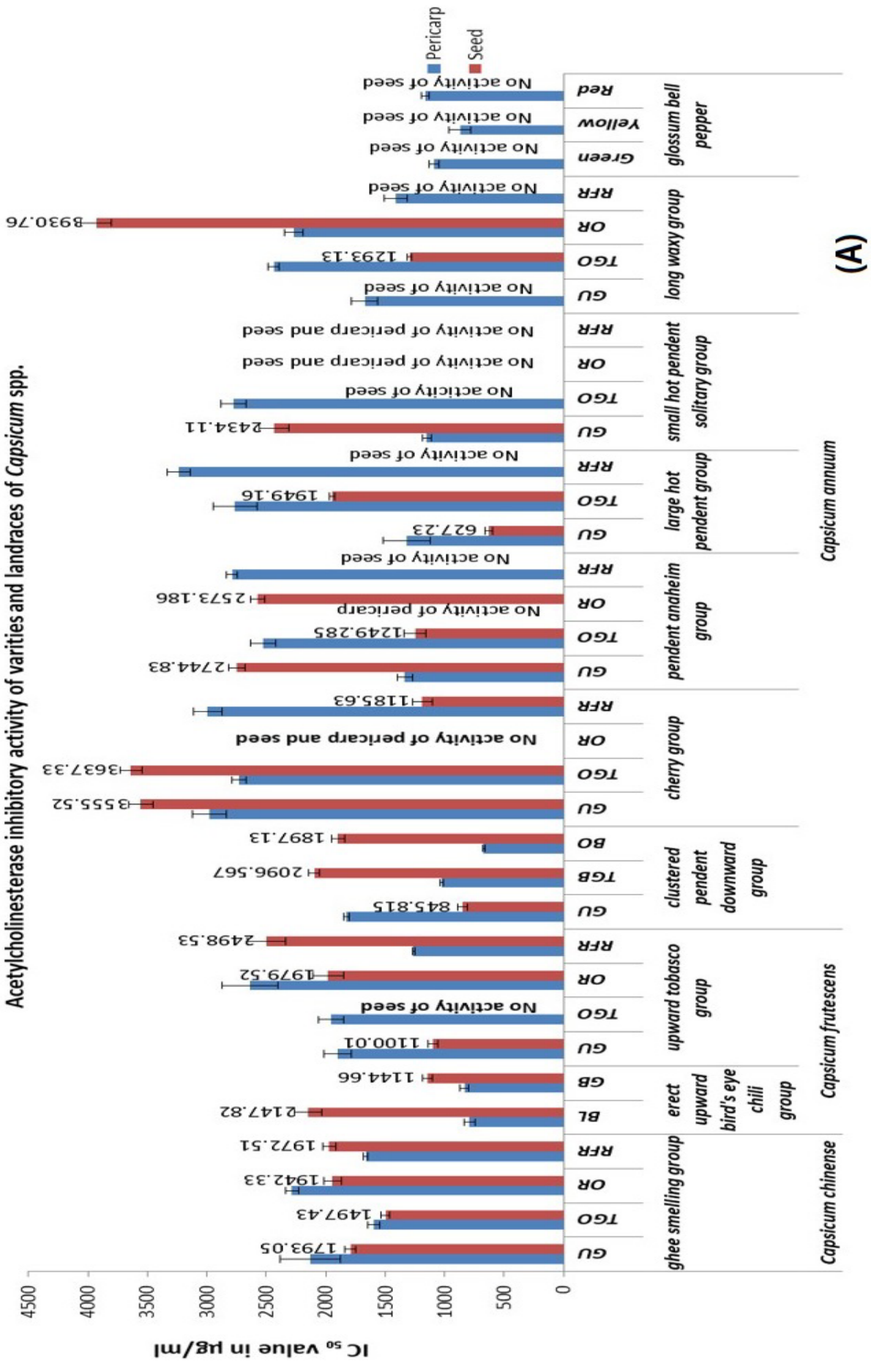
***Capsicum chinense*:** In pericarp, only the GU stage showed anti-protein denaturation activity with 98.27 ± 0.18% and all the other stages showed below 50% inhibitory activity which were considered as no activity. On the contrary, the seeds of all the four stages showed inhibition with the highest activity was shown by the seeds of OR stage with 90.0 ± 0.24% inhibition.

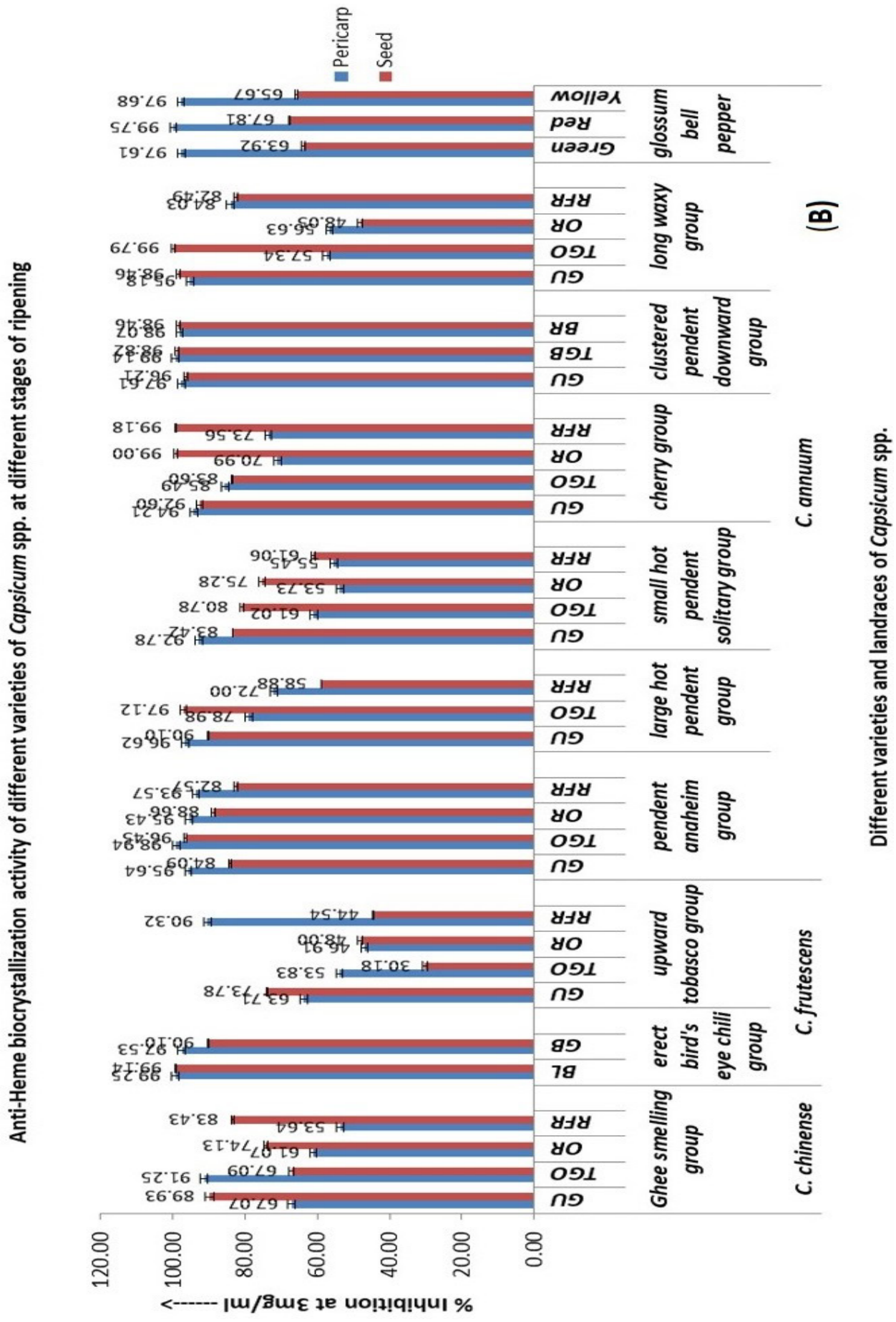
***Capsicum frutescens*:** The pericarps of BL stage of erect upward bird's eye chilli group exhibited notable anti-protein denaturation activity, *i.e.*, 99.23 ± 0.16%. The seeds of GB stage of this particular cultivar showed only 51.05 ± 0.34% inhibition. In case of upward tobacco group, only the pericarp of the OR stage showed its activity against denaturation of protein with inhibition of 77.82 ± 0.00%.

***Capsicum annuum***: Out of the four ripening stages, only the GU stage of the pericarp of pendent Anaheim group showed 67.84 ± 0.21% inhibition, whereas, all the stages except the OR stage of the seeds showed inhibition with the highest inhibition was measured at 88.10 ± 0.27% in the GU stage. According to our findings, no activity was detected in the large hot pendent group, neither in the pericarp nor in the seeds. Only the seeds of RFR stage of small hot pendent solitary group showed mild inhibition of protein denaturation (59.84 ± 0.25%) activity. Only the seeds of GU stage of cherry group showed inhibition with 65.49 ± 0.20%. Seeds of both GU and RFR stages of clustered pendent downward group demonstrated high activity with 91.06 ± 0.17% and 82.25 ± 0.30%, respectively. Only the RFR stage of seeds of long waxy group displayed mild inhibition towards protein denaturation activity with 54.48 ± 0.12%. The pericarps of green, red and yellow glossum bell pepper and seeds of green and yellow glossum bell pepper exhibited anti-protein denaturation property with 58.57 ± 0.23%, 82.13 ± 0.21%, 79.97 ± 0.31%, 67.05 ± 0.13% and 52.11 ± 0.21%, respectively. In this regard, the percentage inhibition of all the tested samples at the mentioned concentration is shown in Fig. 2(C).

### 3.4. Estimation of Capsaicin and dihydrocapsaicin content

Capsaicin and dihydrocapsaicin content were measured for each sample using HPLC-DAD. Accordingly, the highest capsaicin content (657.41 ± 112.95 µg/mg) was noted in the seeds of TGO stage followed by pericarp of GU (565.62 ± 206.69 µg/mg), TGO (524.24 ± 295.42 µg/mg) and RFR stage (357.21 ± 226.42 µg/mg) of ghee smelling group of *Capsicum chinense*. RFR and OR stage of seed as well as GU stage of pericarp of the upward tobacco group contained the second highest range of capsaicin content of 268.12 ± 10.46, 260.84 ± 6.96 and 257.51 ± 25.24 µg/mg, correspondingly.


Acetylcholinesterase inhibitory activity of varities and landraces of Capsicum spp.
IC50 value in µg/ml
Pericarp
Seed
4500
4000
3500
3000
2500
2000
1500
1000
500
0
No activity of seed
No activity of seed
No activity of seed
8930.76
1293.13
No activity of seed
No activity of pericarp and seed
No activity of pericarp and seed
No activity of seed
2434.11
No activity of seed
1949.16
627.23
No activity of seed
2573.186
No activity of pericarp
1249.285
2744.83
1185.63
No activity of pericarp and seed
3637.33
3555.52
1897.13
2096.567
845.815
2498.53
1979.52
No activity of seed
1100.01
1144.66
2147.82
1972.51
1942.33
1497.43
1793.05
GU
TGO
OR
RFR
BL
GB
GU
TGO
OR
RFR
GU
TGO
OR
RFR
BO
TGB
GU
TGO
OR
RFR
GU
TGO
OR
RFR
GU
TGO
OR
RFR
Red
Yellow
Green
long waxy group
small hot pendent solitary group
large hot pendent group
pendent anaheim group
cherry group
clustered pendent downward group
upward tobasco group
erect upward bird's eye chili group
glossum bell pepper
Capsicum chinense
Capsicum frutescens
Capsicum annuum
(A)
Names of varieties and landraces of Capsicum spp.


Anti-Heme biocrystallization activity of different varieties of Capsicum spp. at different stages of ripening
Pericarp
Seed
% Inhibition at 3mg/ml ------>
120.00
100.00
80.00
60.00
40.00
20.00
0.00
C. chinense
C. frutescens
C. annuum
(B)
Ghee smelling group
erect bird's eye chilli group
upward tobasco group
pendent anaheim group
large hot pendent group
small hot pendent solitary group
cherry group
clustered pendent downward group
long waxy group
glossum bell pepper
GU
TGO
OR
RFR
GB
BL
BR
TGB
Green
Red
Yellow
Different varieties and landraces of Capsicum spp.

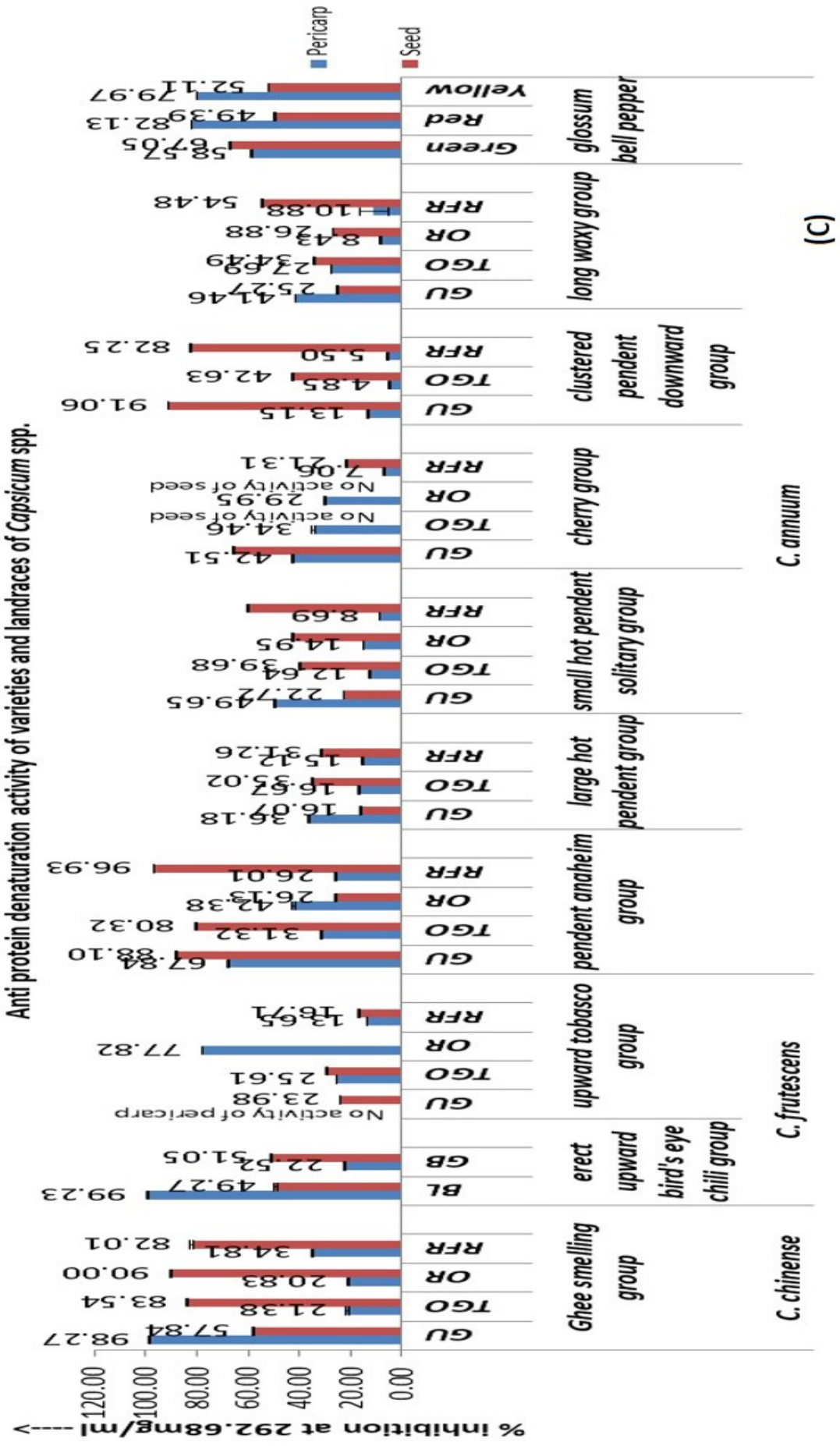



**Fig. 2. (A)** Acetylcholinesterase inhibitory activity; **(B)** Anti-hemebiocrystallization activity and **(C)** Anti-protein denaturation activity of different varieties and landraces of *Capsicum* sp. at different stages of ripening.

Dihydrocapsaicin was found to be excessively higher in ghee smelling group of *Capsicum chinense* than in any other groups tested. Pericarp of GU (192.27 ± 15.67 µg/mg), TGO (204.33 ± 122.60 µg/mg) and OR stage (173.92 ± 75.89 µg/mg) and seeds of TGO (121.35 ± 16.65 µg/mg) and OR stage (155.41 ± 5.65 µg/mg) of ghee smelling group contain high amount of dihydrocapsaicin. The ghee smelling group is a very hot variety of chilli that belongs to the same species as that of Bhutjolokia or "ghostpepper" whose scoville heat units (SHUs) are more than 1 million. Bosland and Baral (2007) reported that there may have been a genetic introgression from *C. frutescens* to bhutjolokia. Our study reveals that the ghee smelling variety, a probable relative of bhutjolokia of *C. chinense* has capsaicin and dihydrocapsaicin content highest among all the groups analyzed. The excessive hotness and pungency may be due to the high amount of these capsaicinoids present in the ghee smelling group. The full profile of capsaicin and dihydrocapsaicin content of all the samples at different ripening stages is presented in Table 2.

### 3.5. HPTLC analysis of carotenoids from *C. chinense*

The ghee smelling variety of the *C. chinense* group of chilli peppers studied here is not widely consumed by the people of West Bengal. We tried to find out the quantity of carotenoids present in the pericarp of the fruit at different stages of ripening. The amount of β-carotene, capsanthin and lutein present in each stage of ripening of ghee smelling variety of *C. chinense* reveals that the GU (β-carotene: 255.97 ± 3.52; lutein: 46.86 ± 10.97 µg/mg) and the RFR stages (β-carotene: 167.57 ± 40.13; capsanthin: 921.22 ± 311.25; lutein: 30.05 ± 13.52 µg/mg) contain good amounts of the carotenoids. The carotenoid content of all the ripening stages of the pericarp of ghee smelling variety is calculated and depicted in Table 3.

### 3.6. GC-MS based metabolomics and chemometric analyses

GC-MS based metabolite profiling enabled identification of a total of 118 metabolites (Supplemtary material 1 and Supplementary material 2) with the presence of several sugars and polyols, organic acids, amino acids, fatty acids, phenols and other metabolites from the pericarp and seeds of different varieties and landraces of *C. annuum, C. frutescens* and *C. chinense* at different ripening stages. PLS-DA (Partial least squares-discriminant analyses) segregated each ripening stage on the basis of their metabolite profiles. The PLS-DA 2D scores plot of both pericarp and seed (Fig. 3) show distinct separation of the ripening stages.

Among the 118 metabolites studied, some of the compounds were found to have significant correlation to anti-cholinesterase, anti-heme biocrystallization and anti-protein denaturation activities. The correlated compounds were subjected to three corresponding assays which revealed that a number of phenols have inhibitory activities. Those compounds were compared to the standard marketed drugs. The phenolic compounds identified in the experimental *Capsicum* species and landraces showing bioactivity are presented in Table 4.

Out of several methodologies, one approach to treat neurodegenerative disorders like Alzheimer's disease, insomnia, memory loss, Parkinsonism and others, is to inhibit the enzyme acetylcholinesterase which breaks down the neurotransmitter like acetycholine, needed for neurotransmission and ultimately causes impairment in cholinergic transmission. So, in this research, we inspected the compounds found in the specific ripening stages of a very common spice crop, *Capsicum*. From the Table 4, it is evident that there are several compounds which have $IC_{50}$ values less than that of known standard drugs / inhibitors, *in vitro*. Ilkay et al. (2007) revealed that they have subjected gallic acid, chlorogenic acid and caffeic acid to AChE inhibition at a concentration of 1 mg/mL. In this study, only gallic acid showed approximately 15% inhibition to AChE while the other two compounds did not show inhibition at 1 mg/mL. Furthermore, we have successfully found out the $IC_{50}$ values of all the three compounds, where we see that gallic acid has an $IC_{50}$ value (32.92 ± 2.16 µM) close to the standard inhibitor galantamine hydrobromide (22.38 ± 0.44 µM). Though the other two compounds chlorogenic acid (219.18 ± 9.84 µM) and caffeic acid (687.35 ± 5.39 µM) had inhibited the AChE enzyme, their $IC_{50}$ values are much higher than that of the standard drug.

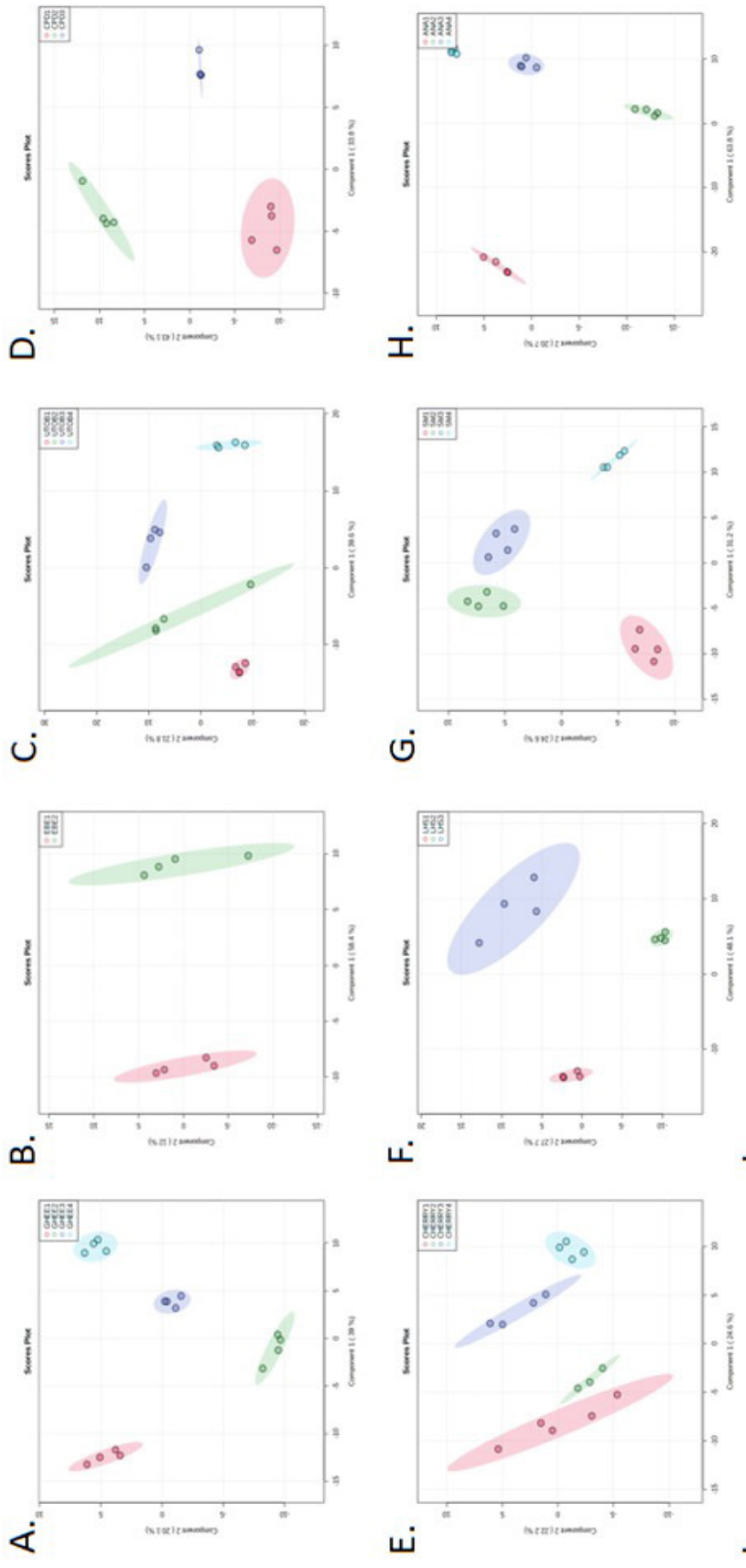
The *Plasmodium* parasite utilizes the host haemoglobin as its source of nutrition. As the haemoglobin degrades, it renders large amount of toxic heme in the host cell. The parasite converts heme monomers to insoluble cyclic dimers of hemozoin to detoxify the environment. The spectrophotometric assays to screen alternative and novel plant based anti-malarials employ hemozoin, the malarial pigment as the drug target. Our study revealed four phenolic compounds that inhibit the heme biocrystallization, namely gallic acid, chlorogenic acid, vanillic acid, and benzene 1,2,4-triol for the first time. Sharifi-Rad et al. (2022) studied the chemical composition of different *Artemisia* spp. and reported the presence of caffeic acid, chlorogenic acid, vanillic acid and different derivatives of benzene. *Artemisia* is a popular genus known for its pharmacological importance, especially anti-malarial and anti-insecticidal properties. Aldulaimi et al. (2017) tested gallic acid analogues to validate anti-malarial action of phenolic acids and revealed that the conjugates of gallic acid showed moderate activity. A group of researchers (Fordjour et al., 2020) working at the University of Ghana, reported that gallic acid was found to have highest potential for anti-malarial action, while chlorogenic acid responded moderately to *in vitro* anti-malarial drug test. Based on this study, it can be said that chlorogenic acid ($IC_{50}$ = 0.33 ± 0.001 µM) has very close rather better anti-heme biocrystallization activity than the standard drug, chloroquine diphosphate ($IC_{50}$ = 0.65 ± 0.045 µM) (Table 4). This study reveals two more compounds that can act as potential anti-malarial agent by inhibition of heme biocrystallization, namely vanillic acid ($IC_{50}$ = 270.55 ± 4.82 µM) and 1,2,4-benzene triol ($IC_{50}$ = 420.66 ± 2.02 µM) which are newly reported by our research group.

**Table 2**
Capsaicin and dihydrocapsaicin content of different *Capsicum* spp. fruits at different stages of ripening.

| *Capsicum* species | Variety | Ripening stages | Capsaicin content (µg/mg) (Mean ± SD) | | Dihydrocapsaicin content (µg/mg) (Mean ± SD) | |
|---|---|---|---|---|---|---|
| | | | Pericarp | Seed | Pericarp | Seed |
| *C. chinense* | Ghee smelling group | GU | 565.62±206.69 | 199.45±136.51 | 192.27±15.67 | 72.49±18.78 |
| | | TGO | 524.24±295.42 | 657.41±112.95 | 204.33±122.60 | 121.35±16.65 |
| | | OR | 141.11±61.18 | 184.23±50.88 | 173.92±75.89 | 155.41±5.65 |
| | | RFR | 357.21±226.42 | 118.69±7.64 | 78.86±24.39 | 22.76±1.32 |
| *C. frutescens* | Erect upward bird's eye chilli group | GB | 2.84±0.53 | 23.04±1.37 | 7.92±0.26 | 11.70±0.41 |
| | | BL | 28.64±1.83 | 5.52±2.17 | 13.73±0.37 | 17.47±0.22 |
| | Upward tobacco group | GU | 257.51±25.24 | 191.43±16.04 | 53.41±7.75 | 33.68±1.69 |
| | | TGO | 32.44±10.29 | 3.69±1.62 | 21.85±2.63 | 10.05±0.15 |
| | | OR | 97.03±1.97 | 260.84±6.96 | 27.21±1.62 | 44.77±0.69 |
| | | RFR | 95.92±6.89 | 268.12±10.46 | 29.11±9.28 | 45.32±2.06 |
| *C. annuum* | Clustered pendent downward group | GU | 10.05±4.26 | 106.08±25.05 | 17.02±0.80 | 37.84±3.98 |
| | | TGB | 12.79±12.51 | 15.98±14.63 | 18.63±2.03 | 17.09±2.69 |
| | | RFR | 9.37±5.75 | 38.14±4.30 | 21.90±1.15 | 23.79±0.88 |
| | Cherry group | GU | 20.42±3.19 | 23.34±3.02 | 13.43±0.53 | 23.03±1.19 |
| | | TGO | 11.71±1.30 | 5.07±2.82 | 9.98±0.61 | 18.06±2.37 |
| | | OR | 28.64±2.99 | 44.14±10.87 | 24.30±0.78 | 23.24±2.05 |
| | | RFR | 5.82±0.81 | 22.82±0.23 | 10.08±0.45 | 41.85±1.50 |
| | Large hot pendent group | GU | 17.28±1.42 | 16.01±2.72 | 12.30±0.65 | 12.09±7.54 |
| | | TGO | 5.12±1.56 | 36.24±1.22 | 9.57±0.84 | 11.04±0.21 |
| | | RFR | 11.97±0.78 | 12.88±4.73 | 9.75±0.51 | 8.13±0.87 |
| | Small hot pendent group | GU | 27.09±2.09 | 23.79±7.53 | 16.63±0.11 | 26.09±2.59 |
| | | TGO | 21.29±2.10 | 40.62±13.53 | 17.31±0.92 | 33.14±4.42 |
| | | OR | 10.28±1.10 | 51.25±9.36 | 9.63±0.58 | 36.11±2.21 |
| | | RFR | 3.58±0.86 | 31.29±0.99 | 8.55±0.64 | 14.33±0.71 |
| | Anaheim group | GU | 6.90±1.13 | 18.51±0.05 | 3.63±0.50 | 13.97±0.23 |
| | | TGO | 5.82±0.13 | 5.32±0.88 | 4.10±0.18 | 2.91±0.73 |
| | | OR | 7.61±0.04 | 10.87±1.38 | 4.29±0.05 | 3.75±097 |
| | | RFR | 8.37±0.11 | 86.50±0.57 | 4.60±0.01 | 67.10±3.13 |
| | Long waxy sweet group | GU | Not detected | Not detected | Not detected | Not detected |
| | | TGO | | | | |
| | | OR | | | | |
| | | RFR | | | | |
| | Bell peppers | Green | Not detected | Not detected | Not detected | Not detected |
| | | Yellow | | | | |
| | | Red | | | | |

**Table 3**
Carotenoid content of Ghee smelling variety of *C. chinense*.

| *C. chinense* | Content in µg / mg (Mean ± SD) of plant sample | | |
|---|---|---|---|
| Ripening stages | β-Carotene | Capsanthin | Lutein |
| GU | 255.97±43.52 | Not detected | 46.86±10.97 |
| TGO | 47.11±5.25 | 135.21±13.00 | 7.19±7.32 |
| OR | 21.30±1.90 | 136.35±49.82 | 2.10±1.64 |
| RFR | 167.57±40.13 | 921.22±311.25 | 30.05±13.52 |



PLS-DA 2D scores plot of pericarp of A. Ghee smelling group, B. Upward erect bird's eye group, C. Upward tobasco, D. Clustered pendent downward group, E. Cherry group, F. Large hot pendent group, G. Small hot pendent group, H. Anaheim group, I. Long waxy group, J. Glossum bell pepper.

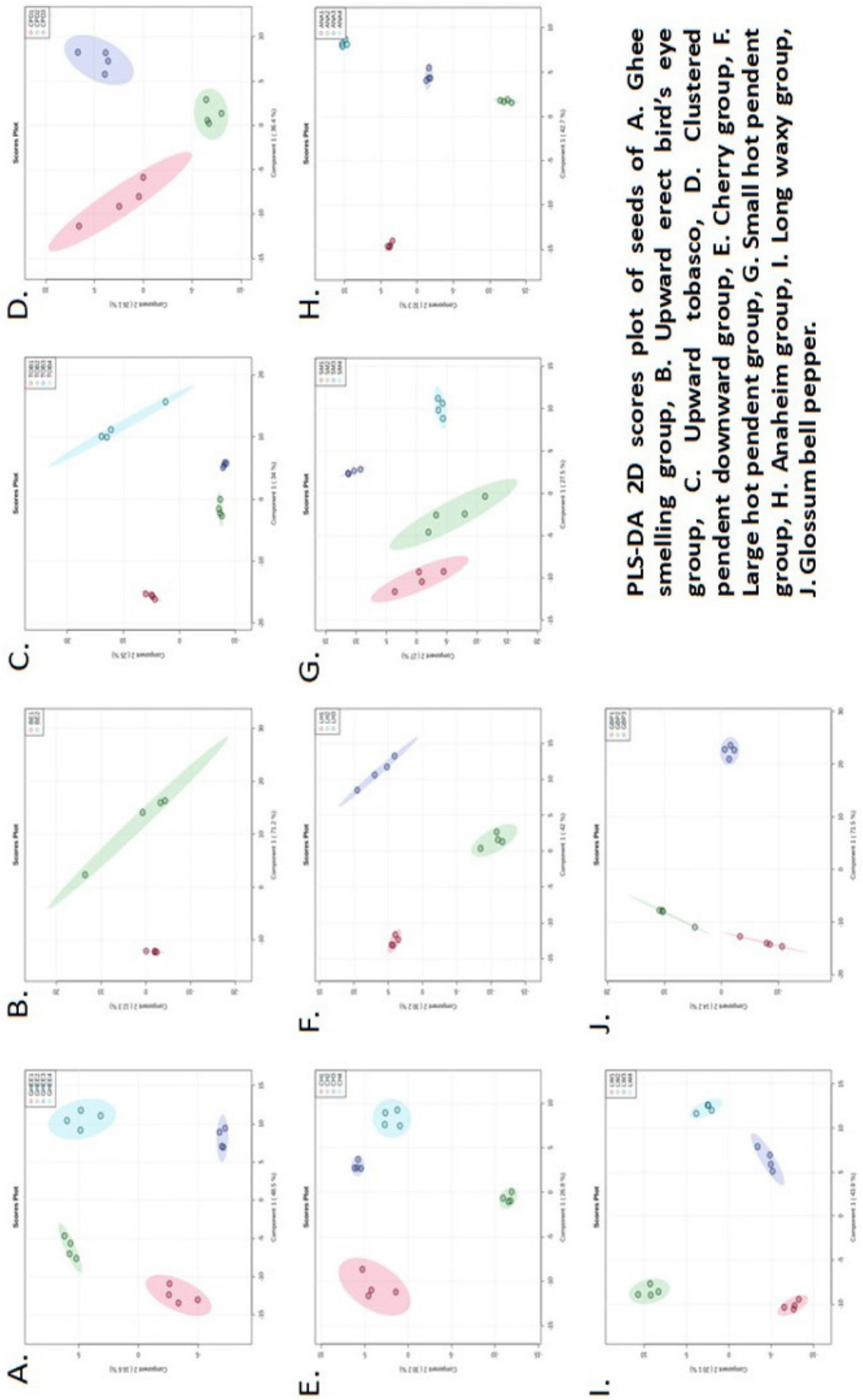



**Fig. 3.** PLS-DA 2D scores plot of pericarps and seeds of different varieties of *Capsicum* sp. showing segregation of different ripening stages.

**Table 4**
$IC_{50}$ values of correlated compounds and comparison with standard drugs.

| | Names of compounds | $IC_{50}$ (µM±SD) |
|---|---|---|
| Compounds correlated to AChE inhibitory assay | Galantamine hydrobromide (Standard drug) | 22.38±0.44 |
| | Gallic acid | 32.92±2.16 |
| | 1,2,4-Benzene triol | 32.79±0.93 |
| | Chlorogenic acid | 219.18±9.84 |
| | Caffeic acid | 687.35±5.39 |
| Compounds correlated to anti-heme biocrystallization assay | Chloroquine di phosphate (Standard Drug) | 0.65±0.045 |
| | Chlorogenic acid | 0.33±0.001 |
| | Vanillic acid | 270.55±4.82 |
| | Benzene1,2,4-triol | 420.66±2.02 |
| | Gallic acid | 565.35±16.18 |
| Compounds correlated to anti-protein denaturation assay | Ibuprofen (Standard Drug) | 96.95±0.17 |
| | O-Acetylsalicylic acid | 4.50±0.15 |
| | 4-Hydroxybenzoic acid | 7.63±0.06 |
| | Quinic acid | 5.56±0.19 |
| | Capsaicin | 7.25±0.08 |
| | 3,4-Dihydroxybenzoic acid | 14.83±0.31 |
| | Vanillic acid | 15.64±0.26 |

The denaturation of proteins as one of the causes of inflammation is well-documented. Production of auto-antigens in certain rheumatic diseases may be due to *in vivo* denaturation of proteins. A number of anti-inflammatory drugs are known to inhibit the denaturation of proteins. Ali et al. (2012) concluded that presence of phenolic compounds increases the thermal stability of proteins because phenolic interactions change secondary structure of proteins. In the present study, a number of phenolic compounds identified from the metabolic profile that had correlation with anti-protein denaturation activity, were tested for the same assay individually. *O*-acetyl salicylic acid ($IC_{50}$ = 4.50 ± 0.15 mM), 4-hydroxy benzoic acid ($IC_{50}$ = 7.63 ± 0.06 mM), 3,4-dihydroxy benzoic acid (protocatechuic acid) ($IC_{50}$ = 14.83 ± 0.31 mM), quinic acid ($IC_{50}$ = 5.56 ± 0.19 mM) and vanillic acid ($IC_{50}$ = 15.64 ± 0.26 mM) significantly inhibited protein denaturation *in vitro*. Capsaicin is a US-FDA approved neuropeptide-active agent used in the treatment of pain related to arthritis and musculoskeletal pain. It has the capacity of depleting supply of substance P to the nerves. Substance P is a neuropeptide that acts as a chemical mediator from peripheral to the central nervous system. Depletion of this neuropeptide prevents transmission of impulses to the brain and renders joints insensitive to the pain feeling. The function of substance P is involved in the pathogenesis of various diseases including but not limited to cancer, diabetes, rheumatoid arthritis, myocarditis, heart failure, epilepsy, migraine, thrombosis, pruritus, depression, and anxiety (Graefe and Mohiuddin, 2022). Capsaicin is the pungent ingredient of chilli peppers and is approved as a topical treatment of neuropathic pain. The analgesia lasts for several months after a single treatment. Capsaicin selectively activates TRPV1, a $Ca^{2+}$-permeable cationic ion channel that is enriched in the terminals of certain nociceptors (Chung and Campbell, 2016). Therefore, capsaicin is also tested for the same anti-protein denaturation assay and the *in vitro* 50% inhibitory concentration was obtained which was significantly lower ($IC_{50}$ = 7.25 mM ± 0.08) than the standard drug Ibuprofen ($IC_{50}$ = 96.95 ± 0.17 mM) tested.

## 4. Concluding remarks

This study has provided valuable insights into the anti-cholinesterase, anti-heam biocrystallization and anti-arthritic potentialities of both non-pungent sweet (long waxy group and green, yellow and red glossum bell pepper) as well as pungent hot *Capsicum* [*Capsicum chinense* (ghee smelling group), *C. frutescence* (erect upward bird's eye chilli group, upward tobacco group) and *Capsicum annuum* (clustered pendent downward group, cherry group, pendent Anaheim group, large hot pendent group, small hot pendent solitary group)]

fruits with special emphasis on their ripening stages. Since they are consumed all over the world at different ripening stages, they can be generally considered safe. The selection of proper ripening stages may serve as potential leads to drug targets for neurodegenerative diseases, malaria and arthritis. The phenolic compounds which were correlated and further assayed in this work may be substantiated through *in vivo* models.

Cultivation and consumption of *Capsicum* fruits should be encouraged more. Apart from the biological activities shown by the plant samples, this study provides supplementary information on chemotaxonomic significance and baseline data for the preference of suitable genotypes in plant breeding based on nutritional and pharmacological importance. The study may be beneficial in selecting the cultivars with the best attributes.

## Author contribution statement

Conceptualization and literature search were performed by Mamita Debnath and Jhelam Chatterjee. The first draft of the manuscript was prepared by Susmita Das. Susmita Das critically analyzed and gave suggestions to finalize the manuscript. All authors read and approved the final manuscript.

## Conflict of interest

The authors declare that they donot have any conflicts of interest.

## Acknowledgements

GC-MS based work was supported by DST-FIST Program, Govt. of India [Grant Number:S R/FST/LS1-459/2010], conducted in the Central Instrumentation Facility, Department of Botany, University of Calcutta, West Bengal, India.

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