Sonication Enhancement of Capsaicin Formation in Callus of Chili Pepper, *Capsicum annuum* L.

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Abstract—The current study investigates the induction of callus from leaf explants of chili pepper *Capsicum annuum* L. coupled with the isolation of capsaicin from alcoholic extracts. To determine which isolated alkaloid has a positive reaction, the Dragen Droff test is used. Alkaloid is identified using conventional diagnostic techniques, such as measuring the absorbance values of the isolated alkaloid with an ultraviolet spectrophotometer; the alkaloid is identified. The results show a complete identity among them, and with control. Thin layer chromatography data shows a 0.8 cm distance between one location from each tested sample with the same rate, which is 0.8 cm from the control's rate flow value. The chemical structure of studied samples is subsequently determined using nuclear magnetic resonance, which reveals similarities between the isolated alkaloid's structure and standard capsaicin. A quantitative analysis of the isolated alkaloids reveals variations in the amounts of generated explants relative to other explants. This study shows that fruits are the most effective source of alkaloids. It's interesting to note that the composition of the explant and the sonicated callus are identical. Since capsaicin's discovery, it has been used as a homeopathic remedy to treat burning pain using the concept of "treating like with like" or counterirritant, relieve minor pain associated with rheumatoid arthritis or muscle sprains and strains, and due to large consumption of this fruit recently, the current study is achieved to find out the structure and quantity.

***Index Terms*—Callus, Capsaicin, *Capsicum annuum* L., Dragendroff, Nuclear magnetic resonance, Sonication.**

I. Introduction

The Solanaceae family includes the genus Capsicum, which includes pepper and is known for its global distribution. Five domesticated species *Capsicum baccatum*, *Capsicum annuum*, *Capsicum pubescen*, *Capsicum frutescens*, and *Capsicum chinense* along with more than 31 different species it is a diverse genus. Spices, such as capsicum are widely used and prized for their distinct flavor and pungency. Pepper contains good amounts of provitamin A, carotenoids, Vitamins E and C, and phenolic compounds such as luteolin, quercetin, and capsaicinoids. All of these substances have antioxidant properties and are linked to various biological functions. Remarkably, individuals have used capsicum fruits in their diet to cure wounds, rheumatism, toothaches, coughs, and parasite infections. In addition, it has antibacterial, antiseptic, immune-modulatory, anticancer, and counterirritating properties (Batiha, et al., 2022). *C. annuum* has many angular twigs and reaches heights of 0.75–1.8 m in cultivated areas. This shrub is an annual. The leaves are simple, elliptical to lanceolate, and alternating in shape. The entire smooth margins are usually wrinkled. The tiny flowers are arranged in groups of two or three and have a diameter of 1.0–1.5 cm. The berries are smooth, glossy, and have many seeds; when ripe, they turn crimson. They lack sutures and might be long, cylindrical, ovoid, obtuse, or oblong. They lack sutures and might be long, cylindrical, ovoid, obtuse, or oblong with numerous smooth, rounds, discoid, golden seeds with an outward spine-aroma. It is a maximum length of 25 cm and a maximum width of 7 mm (Kraft, et al., 2014).

The five main chemicals found in the majority of Capsicum species are capsaicin (69%), dihydrocapsaicin (22%), nordihydrocapsaicin (7%), homocapsaicin (1%), and homo dihydro capsaicin (1%) (Perry, et al., 2007).

Because of their pungency, pepper fruits are noted for their intense, spicy flavor, which is attributed to secondary metabolites called capsaicinoids. The placental tissue and interlocular septum both generate a class of alkaloids. All peppers contain the two primary capsaicinoids, dihydrocapsaicin and capsaicin, in concentrations that can exceed 90% in the seeds, placenta, and pericarp of the fruit. Less than 20% of the total is made up of homodihydrocapsaicin, homocapsaicin, and nordihydrocapsaicin (Perucka and Oleszek, 2000; Giuffrida, et al., 2013). Even though capsaicin's pharmacological properties were first recognized in science in the middle of the 19th century, current research focuses on enhancing the compound's applications, isolation, and purification. Back then, capsaicin was said to be a substance with a variety of uses, such as a natural food addition and a host of health advantages (Papoiu and Yosipovitch, 2010).

In recent years, numerous techniques for obtaining capsaicinoids from spicy peppers have been devised,

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including organic solvent extraction, microwave-assisted extraction, supercritical fluid extraction, and ultrasound-assisted extraction (Marincas, et al., 2018; Teng, Zhang and Devahastin, 2019; Fabela, et al., 2019; Wang, et al., 2021). The first stage in designing an extraction process is selecting a good solvent producing a large amount of the targeted substance. The most abundance used solvents for removing capsaicinoids include water, acetonitrile, methanol, and ethanol (Barbero, et al., 2008). To achieve high extraction productivity, a number of additional relevant variables are also considered during the solvent selection process. These include sample quantity, temperature, extraction duration, solvent volume, and the operations' repeatability and reproducibility. This research investigated the induction of callus from leaf explant of *C. annuum* L. coupled with the isolation of capsaicin from alcoholic extracts of fruit and callus of the chili pepper plant.

II. Materials and Methods

A. Plant Material and Processing of Explants

Healthy and uniform seeds of *C. annuum* were obtained from local markets, first washed then sterilized using 6% sodium hypochlorite solution (NaOCl) (Jadid, et al., 2023) for 5 times followed by rinsing them using sterilized distal water twice for 2.0 min then dried using sterilized filter paper. Sterile five seeds were transferred into flasks containing 25 mL of agar-solidified Murashige and Skoog Free of plant growth regulators (PGRs) MSO medium free of PGRs. Samples were kept in a tissue culture room at 23°C under 2000 lux light at (16/8) regime.

B. Establishment of Callus

Two-month-old sterile seedlings were used to establish callus, 0.5 cm^2 leaf segments, 0.5 cm stem segments, 0.5 cm leaf petiole, and steam apex were cultured on 50 mL agar solidified MSO media enhanced with varying amounts of PGRs (Table I). Induced calli were subcultured every 4 weeks.

C. Exposure of Callus to Ultrasonic Wave

One gram of callus was exposed to the ultrasound wave, which was carried out at 47.6 kHz on the digital ultrasonic cleaner (Barasonic 221, Germany) for 30 min. Ultrasound treatments were performed in a water bath at a constant temperature of 25°C (El-Sattar and Tawfik, 2023).

D. Callus, and Fruits Alcoholic Extraction Preparation

Fifty grams of fruit and callus samples were dried in an oven at 40°C for 24 h. The samples were then crushed in a mortar with 100 mL of 70% methanol added. After 45 min of centrifugation at 4°C and 13.000 g, the supernatant was removed. Next, 100 mL of methanol was added and allowed to sit for 24 h. Finally, it was heated at 50°C and allowed to cool before another 45-min centrifugation at 4°C and 13148× *g* was performed (Lucia, et al., 2020).

E. Isolation of Capsaicin Alkaloid

The supernatant from the last step was transferred into rotary evaporator at 50°C to precipitate. To dissolve the precipitate 20 mL of 2 N HCL was added. This mixture undergoes filtration using filter paper and 20 mL of Ethyl acetate) (CH_3-COO-CH_2-CH_3). The mixture was kept in a separating funnel and shacked manually. Then the ethyl acetate was neglected and ammonia solution was gradually added to reach pH 9. After passing the mixture through filter paper, 50 mL of chloroform were added to the filtrated product, which was then put in a separating funnel and manually Shacked 3 times. Then chloroform layer appeared in a bottle and discarded the remaining. The bottle was then placed in a 50°C water bath to evaporate the chloroform. The remaining product was weighed, and samples were kept at 5°C in a dark place (Berlinck and Kossuga, 2007).

TABLE I
Media used for Callus Induction using MS media that is Enhanced with different PGR (Benzyl adenine, Naphthalene Acetic Acid, and 2,4-Dichlorophenoxy Acetic Acid), from Leaf Explant of Chili Pepper, Seedlings of *Capsicum Annuum* L.

Media	Plant growth regulator combinations		
MSO*		Control	
MS	BA 1 mg/0.1+0.2 mg/l NAA	BA 1 mg/0.3+0.2 mg/l NAA	BA 1 mg/0.5+0.2 mg/l NAA
	BA 1 mg/0.1+0.4 mg/l NAA	BA 1 mg/0.3+0.4 mg/l NAA	BA 1 mg/0.5+0.4 mg/l NAA
	BA 1 mg/0.1+0.8 mg/l NAA	BA 1 mg/0.3+0.8 mg/l NAA	BA 1 mg/0.5+0.8 mg/l NAA
MSO		Control	
MS	BA 1 mg/0.1+0.2 mg/l IAA	BA 1 mg/0.3+0.2 mg/l IAA	BA 1 mg/0.5+0.2 mg/l IAA
	BA 1 mg/0.1+0.4 mg/l IAA	BA 1 mg/0.3+0.4 mg/l IAA	BA 1 mg/0.5+0.4 mg/l IAA
	BA 1 mg/0.1+0.8 mg/l IAA	BA 1 mg/0.3+0.8 mg/l IAA	BA 1 mg/0.5+0.8 mg/l IAA
MSO		Control	
MS	BA 1 mg/0.1+0.2 mg/l IBA	BA 1 mg/0.3+0.2 mg/l IBA	BA 1 mg/0.5+0.2 mg/l IBA
	BA 1 mg/0.1+0.4 mg/l IBA	BA 1 mg/0.3+0.4 mg/l IBA	BA 1 mg/0.5+0.4 mg/l IBA
	BA 1 mg/0.1+0.8 mg/l IBA	BA 1 mg/0.3+0.8 mg/l IBA	BA 1 mg/0.5+0.8 mg/l IBA
MSO		Control	
MS	BA 1 mg/0.1+0.2 mg/l 2,4-D	BA 1 mg/0.3+0.2 mg/l 2,4-D	BA 1 mg/0.5+0.2 mg/l 2,4-D
	BA 1 mg/0.1+0.4 mg/l 2,4-D	BA 1 mg/0.3+0.4 mg/l 2,4-D	BA 1 mg/0.5+0.4 mg/l-1 2,4-D
	BA 1 mg/0.1+0.8 mg/l 2,4-D	BA 1 mg/0.3+0.8 mg/l 2,4-D	BA 1 mg/0.5+0.8 mg/l 2,4-D

MSO*: Free from plant growth regulators.

F. Characterization of Isolated Capsaicin Using DragenDroff

Identification protocols were utilized to detect the presence of alkaloids in extracts to ensure that the isolated compound is alkaloid and its type.

To identify the type of alkaloids and make sure it was extracted in a good quality the DragenDroff method was followed:

The preparation of the Dragendroff solution was combining 2 g of bismuth nitrate Bi $(NO_3)_3.5H_2O$ with 25 mL of glacial acetic acid (solution 1), followed by 40 g of potassium iodide in 100 mL of distilled water (solution 2). Next, 10 mL of the first solution and 10 mL of the second, 20 mL of glacial acetic acid, and 100 mL of distilled water were combined to make the DragenDroff solution (Modified from Narasimhan and Shanta, 2003).

G. Thin Layer Chromatography (TLC)

Using a micropipette (0.5–10 μL), samples were manually loaded, 10 mm above the lower border of the plates, with up to 2 μL of the crude extract (corresponding to 4–8 mg of dry tissue). One consistent hit was used to deliver the entire sample volume, with adjacent samples placed 5 mm apart. In every instance, the application spot's diameter was <2 mm. After loading, warm air was used for 30 s to dry the application areas. A solvent mixture consisting of 95:5 ethanols to chloroform was used for the separation process (Wagner, Bladt and Zgainski, 1984). To ensure that the chamber environment was completely saturated, solvent mixes were made right before usage and introduced to the chromatography tank fifteen minutes before development.

H. Ultraviolet (UV) Spectrophotometer

For the UV technique analysis of capsaicin, a (AGILENT CARY 100/300 series Uv-Vis, Malaysia) twin beam UV/visible spectrophotometer with a 1.0 cm^3 matched quartz cell was used. Samples were dissolved in chloroform and applied to quartz cells. Standard capsaicin obtained from Chungwoo Food Republic of Korea. The analytical-grade, easily accessible chloroform was used to dilute the capsaicin (Baravkar, et al., 2023).

I. Determination of Chemical Structure by nuclear magnetic resonance (NMR)

The samples were dissolved in 500 μL of chloroform for the NMR analysis. Using an NMR spectrometry (Shimadzu 60MHz, Japan) coupled to a 5 mm probe at 295 K, NMR data gathering was carried out. To record 1 H NMR, a typical pulse program called zg30 was utilized. On customized graph paper that was improved by an NMR device, dates were recorded (Bora, et al., 2021).

J. Statistical Analysis

One-way analysis of variance was used to evaluate all the data using the GraphPad prism9 statistical package, version 9.3.1 software. The results were expressed as means ± Standard Division at 0.01(Gupta, et al., 2019).

III. Results and Discussion

A. Plant Material and Explant Preparation

Leaf segments grown on MS medium with various amounts of PGRs added as supplements showed that the MS media supplemented with 0.4, 0.8 mg/L of NAA and its combinations with 0.3, 0.5 mg/L of BA had the greatest percentage (100%) of callus induction (Table II).

B. Identification of Potential Capsaicin Derived FROM Alcoholic Extraction

The orange color and sticky texture of capsaicin that was separated from fruit and callus adhered to the walls of the isolating vessels.

C. Diagnosis of Capsaicin Derived from Leaf Callus and Fruit

The results of detecting the alkaloids isolated from different plant parts of *C. annuum* and the callus derived from them confirmed the effectiveness of this test, as it was turned orange when the solution was added (Fig. 1).

D. Rate Flow

Data showed that the flow distance rates for all spots separated from the alkaloids isolated from different

TABLE II
Induction of callus from *Capsicum annuum* leaf segments on solidified MS medium supplemented with various concentrations of auxins (Naphthalene Acetic Acid, Indole Acetic Acid, Indole Butyric Acid, 2,4-Dichlorophenoxy acetic acid) and Cytokinin (Benzyl Adenine)

Auxins (mg/L)/ Cytokinin (mg/L)		Percentage of induction (%)								Number of callus-producing leaf segments							
		NAA				IAA				IBA				2,4-D			
		0.0	0.2	0.4	0.8	0.0	0.2	0.4	0.8	0.0	0.2	0.4	0.8	0.0	0.2	0.4	0.8
BA	0.0	0	2	3	6	0	4	5	5	0	3	4	6	0	4	6	6
		0	33	50	100	0	67	83	83	0	50	67	100	0	67	100	100
	0.1	1	4	4	5	3	5	5	5	2	3	5	6	3	5	6	6
		17	67	67	83	50	83	83	83	33	50	83	100	50	83	100	100
	0.3	3	5	6	6	6	6	6	6	6	6	6	6	6	6	6	6
		50	83	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	0.5	4	4	6	6	6	6	6	6	6	6	6	6	6	6	6	6
		67	67	100	100	100	100	100	100	100	100	100	100	100	100	100	100

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explants of *C. annuum* (Chili pepper) and the callus derived from them were identical to each other and with the average flow distance of the standard capsaicin solution (Control) (Table III and Fig. 2), as the average flow distance for each of the spots that appeared was (0.8 cm). The spot flow rate is the same as the standard solution of capsaicin alkaloid.

E. UV Spectrophotometer

The results of estimating the absorption degree by photometric spectrometry confirmed that the alkaloid isolated from fruit and callus derived from different explants of *C. annuum* (Chili pepper) have the same degree of absorption at the highest wavelength λ max (Fig. 3), as the degree of absorption reached 280 nm, which is similar to the degree of absorption at the highest wavelength of the capsaicin alkaloid. These results confirm again that the alkaloid isolated is a capsaicin compound.

TABLE III
AVERAGE VALUES OF FLOW DISTANCE OF THE SPOTS SEPARATED FROM FRUIT AND DIFFERENT CALLUS TYPES OF *CAPSICUM ANNUUM* (CHILI PEPPER), PLANTS

Isolated alkaloid source	Average flow distance (cm) ± SD
Standard capsaicin (control)	0.8±0.002
Fruit	0.8±0.003
Stem calli	0.8±0.005
Leaf petiole calli	0.8±0.008
Leaf calli	0.8±0.009
Stem apex calli	0.8±0.009

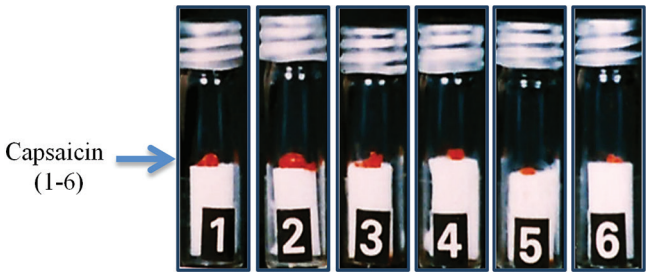

Fig. 1. Quantitative determination of capsaicin isolated from different explants of *Capsicum annuum* (Chili pepper) and the derived callus. (1) Standard capsaicin (Control) (arrowed). (2) Capsaicin isolated from fruit. (3) Capsaicin isolated from stem callus. (4) Capsaicin isolated from leaf petiole callus. (5) Capsaicin isolated from leaf callus. (6) Capsaicin isolated from stem apex callus.

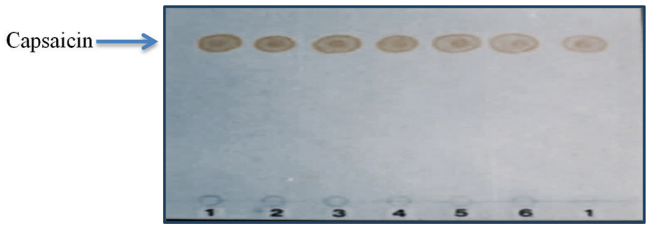

Fig. 2. Detection of capsaicin obtained from fruit and callus derived from different explants of *C. annuum* (Chili pepper) plants using thin layer chromatography technique. (1) Standard capsaicin (Control) (arrowed). (2) Capsaicin isolated from fruit. (3) Capsaicin isolated from stem callus. (4) Capsaicin isolated from leaf petiole callus. (5)Capsaicin isolated from leaf callus. (6) Capsaicin isolated from stem apex callus.

F. Quantitative Estimation of Capsaicin

The results related to the quantitative estimation of the capsaicin alkaloid isolated from fruit and callus derived from different explants of *C. annuum* (Chili pepper) showed a difference in the content of these tissues of callus (Table IV). Data showed that leaf callus was the type of callus that contained the most capsaicin, as its concentration reached 0.64 mg/10 g dry weight, superior to its concentration in fruit and other types of callus, more than that sonication treatment obviously rise the capsaicin quantity in the callus (Table V).

G. Determination of Chemical Structure by NMR

The results of determining the chemical structure of the alkaloid isolated from *C. annuum* (Chili pepper) fruits and callus derived from different plant parts confirmed that they have the same chemical structure of the standard capsaicin alkaloid (Fig. 4a-c). This confirms conclusively that the isolated alkaloid is the same as the capsaicin alkaloid.

Capsaicin is an alkaloid that is a member of the class Capsaicinoids. Its scientific name is [(E)-N-[(4-hydroxy-3-methoxyphenyl) methyl]-8-methylnon-6-enamide] and its

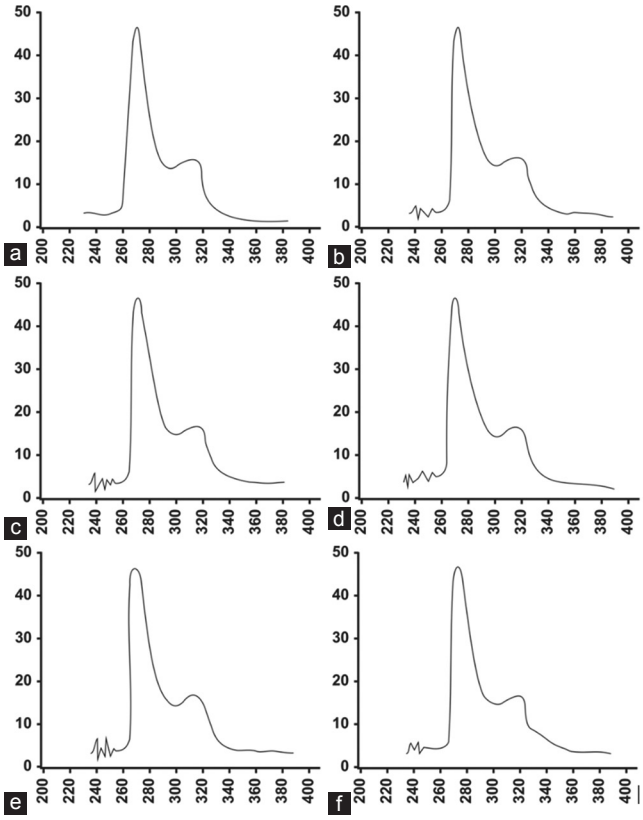

Fig. 3. Absorption Degree of alkaloid isolated from fruit and callus derived from different explants of *C. annuum* (Chili pepper) using UV spectrophotometer. (a) Standard capsaicin (Control). (b) Capsaicin isolated from the fruit. (c) Capsaicin isolated from stem callus. (d) Capsaicin isolated from leaf petiole callus. (e) Capsaicin isolated from leaf callus. (f) Capsaicin isolated from stem apex callus.

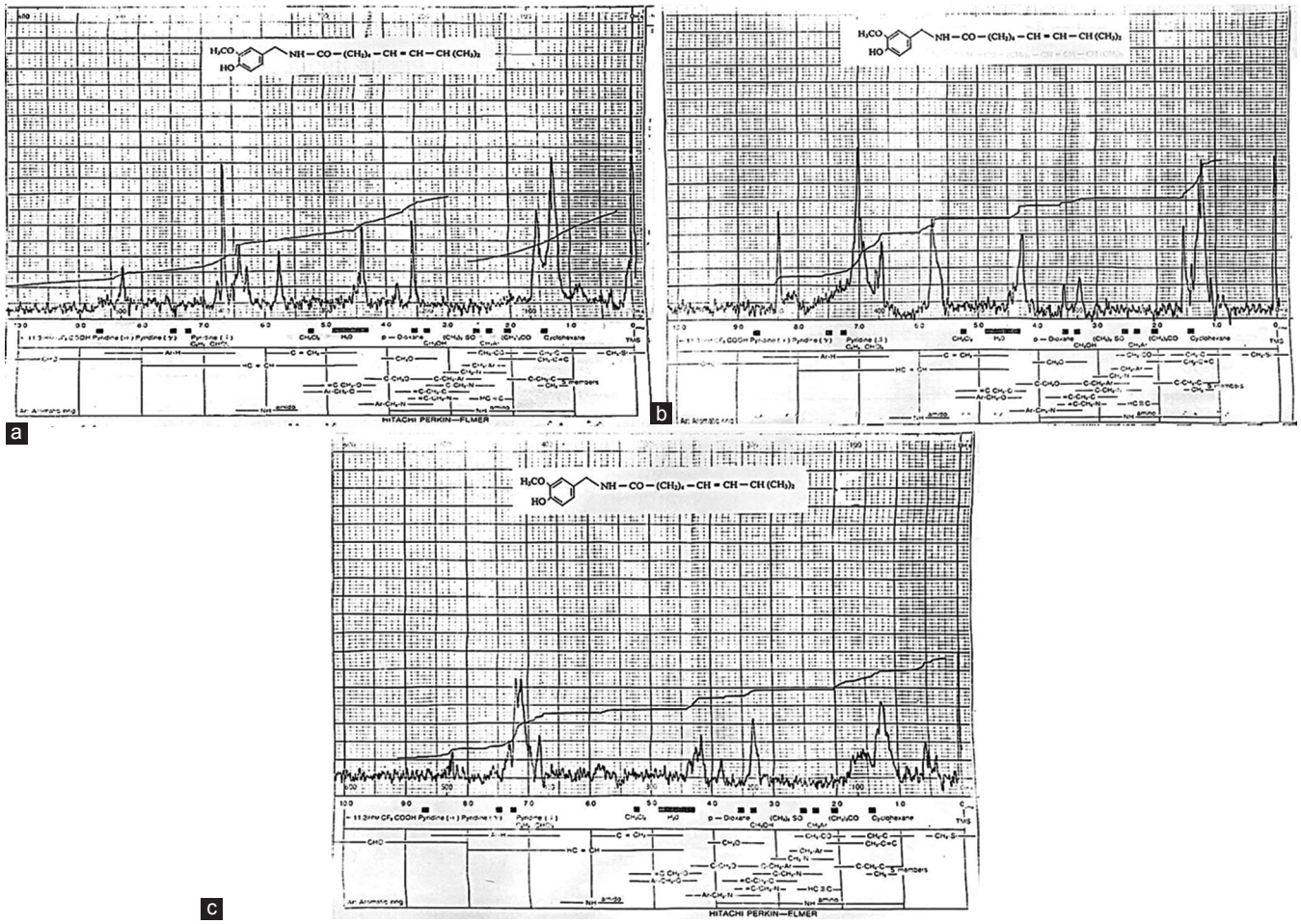

Fig. 4. Chemical composition of the capsaicin extracted from fruit and callus from several *C. annuum* (Chili pepper) explants through NMR technique. (a) Standard capsaicin (Control). (b) Capsaicin isolated from fruit. (c) Capsaicin isolated from different callus tissue.

TABLE IV
Quantitative Estimation of Capsaicin Obtained from Fruit and Different Callus Types of C. Annuum (Chili Pepper)

Isolated capsaicin source	Weight mg/10 g dry weight
Fruit	5.0±0.002
Stem calli	0.48±0.004
Leaf petiole calli	0.36±0.005
Leaf calli	0.64±0.007
Stem apex calli	0.26±0.002

TABLE V
Quantitative Estimation of Capsaicin Obtained from Sonicated Callus of *C. Annuum* (Chili Pepper)

Source of capsaicin	Weight mg/10 g dry weight
Steam calli (cont.)	1.5±0.008
	0.48±0.004
Leaf petiole calli (cont.)	0.9±0.002
	0.36±0.005
Leaf calli (cont.)	1.9±0.011
	0.64±0.007
Steam apex calli (cont.)	0.7±0.001
	0.26±0.002

chemical formula is $C_{18}H_{27}NO_3$. Its weight in molecules is 305.4 g/mol (Mol, et al., 2024).

Tissue culturing is a useful method for producing natural phytochemicals on an industrial and laboratory scale (Gammoudi, et al., 2019). Fruits from the *C. annuum* plant, also known as chili pepper, have been utilized as natural colorants, flavorings, food veggies, and medicinal remedies since ancient times. These days, a huge range of sweet and spicy peppers are consumed in many different ways all over the world. It's interesting to note that *C. annuum*, which has a large variety, is the most significant chili pepper in terms of commerce worldwide (Hernández-Pérez, et al., 2020).

The low yields of target compounds have frequently hindered the use of plant cell cultures for biochemical synthesis. When it comes to *in vitro* chili pepper cultures, capsaicin levels have been artificially raised by the following methods nutritional limitation, adding pre-cursors and intermediate salts to the culturing medium (Nisha, 2024), or immobilization of cells (Yaacob, et al., 2022). All of these methods, nevertheless, have not been able to raise capsaicin quantities to levels seen in fruits bearing chili peppers.

Especially in the interlocular septum of fruits and the placenta's capsaicinoid-secreting structures, capsicum species produce and store capsaicin (Nisha, 2024). It's unclear if these structures are necessary for *in vitro* cultures to produce enough capsaicin. However, recent research has demonstrated

that immobilized placental tissues have a higher capability for producing capsaicin than immobilized cells, suggesting that competence in biosynthesis and capsaicin accumulation requires a particular state of organization and differentiation (Poornima, et al., 2024).

It is not unexpected to find competing effects on the accumulation of capsaicin in chili peppers because capsaicin is a consequence of a metabolic process that shares pre-cursors and intermediates with other biosynthetic routes (e.g., proteins, lignins, anthocyanins, coumarins, flavonoids, etc.) (Elshafie, et al., 2023).

Sonication serves as a substitute stressor for cells or tissues, and clinical medicine makes extensive use of ultrasound. The short wavelength and high frequency of sonication provide excellent directional properties. However, sonication can reflect off the surfaces of tissues and exhibit a limited variety of diffractive phenomena. The greater the number of living cells in the samples, the higher the total metabolic activity; Sonication is a form of alternate stress and an elastic mechanical wave in a medium. This type of energy-driven ultrasonic vibration can produce a variety of microscopic mechanical effects, including the mechanical transfer of materials, the heating effect, and the cavitation effect (Liu, et al., 2003).

Ultrasound may alter a cell's metabolism by causing an increase in enzyme activity. Furthermore, ultrasound has the potential to boost mass transfer, including the absorption of nutritional components, and to promote the selectivity and permeability of cell membranes and cell walls. It is well known that ultrasound makes it easier for molecules to flow through membranes. The function of Ca^{+2}-ATPase, H^{+}-ATPase, and other ion channels in the plasma membrane may be impacted by ultrasonic stimulation. These channels are critical for cell development. (Fontana, et al., 2021).

IV. Conclusion

The study focused on the induction of callus using the chili pepper explants, and 0.8 NAA mg/L of supplement added to MS medium only was suitable for the induction of callus from all explants. Isolation of capsaicin alkaloid was obtained from alcoholic extracts of fruit and leaf callus, The Dragendroff test showed a positive reaction and the UV spectrophotometer showed a complete identity of isolated capsaicin with control. TLC and NMR techniques also confirmed the similarity between isolated capsaicin and slandered capsaicin. The findings of this study showed that the production of capsaicin in chili pepper callus cultures and fruits differs, sonication leads to an increase in the quantity of capsaicin compared with un-sonicated tissues. Never forget that the variation in the rates of synthesis and breakdown affects the total amounts of a secondary byproduct in cell cultures. The pace at which capsaicin degrades within *in vitro* cultures has to be investigated further.

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