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Molecular and Chemical Diagnosis of Curcumin Compound and Study its Antioxidant Activity in Two Varieties of Curcuma longa L. Plants

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Abstract: In this work, the Curcumin synthase 1 (*CURS1*) gene from Curcuma longa L., which plays an important role in the biosynthesis of the curcumin compound, was chosen. The objectives were to identify the genetic variations of *CURS1* in two turmeric plant varieties (NDH-98 and GNT-2) and their relationship with curcumin content. Using DNA sequencing technology, the results were analyzed, and the three-dimensional structure of the CURS1 protein was modeled in silico. The primer employed in this investigation amplified 900 bp fragments of the *CURS1* gene. A single nucleotide polymorphism (SNP) was identified in the first exon (g.850 C>G), which resulted in a change in the three-dimensional structure of the protein due to the substitution of the amino acid threonine with arginine. Haplotype and nucleotide diversity values were 1.00 and 0.00169, respectively. This investigation involved a relative measurement of the putative *CURS1* gene using DNA sequencing from the roots of GNT-2, compared to NDH-98, and an HPLC-assisted curcumin measurement for the two varieties. The amount of curcumin in the rhizomes of GNT-2 was 784.0 µg g^{-1}, while in NDH-98, it was 712.1 µg g^{-1}. The DPPH assay, which evaluates the free radical scavenging capacity of extracts, revealed that the methanolic fraction of Curcuma longa (NDH-98 and GNT-2) demonstrated a significant capacity to inhibit free radicals, with an IC50 value exceeding 125% of the total extract's capacity. Consequently, the methanolic extracts of NDH-98 and GNT-2 exhibited significant effects in the DPPH assay for scavenging free radicals, and regulating vitamin D levels.

Keywords: Antioxidant activity, *Curcuma longa, CURS1* gene, protein three dimensions, single nucleotide polymorphism.

Introduction

Curcuma longa L. (*C. longa*) is rhizome or substrate of a plant similar to ginger (Zingiberaceae). *C. longa* is a perennial herb that is usually known as turmeric, 60-90 cm tall, with short stems, large, oblong leaves, and rhizomes that are ovate, elliptical, or pear-shaped, occasionally branched, and brownish-yellow in color (Kocaadam & Şanlier, 2017).

Curcumin is among the most significant natural products sourced from the rhizomes of *C. longa* L., its production is impacted by rhizome decay and diseases of leaf spot produced by many different pathogens (Santhoshkumar & Yusuf, 2021). The major bioactive components of turmeric are called curcumin, demethoxycurcumin, and bisdemethoxycurcumin which are concentrations ranging from (60-80%), (15-30%) and (2-6%) respectively, these components are called polyphenolic curcuminoids (Wichitnithad *et al.,* 2009). *Curcuma longa* L. (Turmeric) has two varieties that are different in the content of curcumin: the normal percentages of GNT-2 are (4.6%) and NDH-98 (1.6%) (Ayer *et al.,* 2018).

Turmeric has long been known for its therapeutic effects on a variety of ailments. In Unani and Ayurvedic medicine, *C. longa* flowers are used externally to treat ulcers and inflammation, and internally to treat liver obstruction and jaundice. It is also used as an antiseptic for many other ailments, including hemorrhoids, bronchitis, asthma, colds, dental problems, digestive disorders, skin infections, blood purification, wounds, tumors, and liver diseases (Ayati *et al.,* 2019). Curcumin is detected in the rhizomes of the plant *C. longa* (Kita *et al.,* 2016), and the *C. zedoaria* (Lobo *et al*., 2009), as well as *C. amada* (Gilani *et al.,* 2015), *C. caesia* (Borah *et al.,* 2019) and *C. aromatica* (Lim *et al*., 2021). Curcuminoids have vital roles in hepatoprotective, antimutagenic, antidiabetic, anti-inflammatory and antimicrobial functions (Krup *et al.*, 2013). Recently, a class of bioregulators called "curcuminoids" has originated from the recognized curcumin.

Dissimilarities in agroclimatic conditions and factors of soil environments in turmeric varieties, also, affect the gene synthase expression of curcumin linked with curcumin production (Ayer *et al.,* 2020). Curcumin synthase 1 (*CURS1*), and they wo type III polyketide synthases (*PKSs*): curcumin synthase 2 (*CURS2*) as well as curcumin synthase 3 (*CURS3*), which have marginally different substrate specificity from *CURS1*, which are elements from the group of curcumin synthase (*CURS*) gene, and are contributing to the curcumin manufacturing pathway (Katsuyama *et al.,* 2009 a). The *CURS1* gene comprises two exons and one intron (Chakraborty *et al.,* 2021). Most polyketide plants are biologically synthesized through PKS, composed of simple keto synthase homodimers (Austin & Noel, 2003). Different studies suggested that CURS3 is expressed nearly in equal ways in the leaves of plants and rhizomes, but the relative expression of CURS1 and CURS2 was larger in the rhizomes than in the leaves. (Sandeep *et al.*, 2017). Antioxidants are an essential element of daily nutrition and help prevent cell oxidization through their role in decreasing free radicals (Godic *et al*., 2014). Antioxidants play a vital role in neutralizing free radicals and avoiding oxidative stress, which is related to the progression of numerous diseases such as cancer, diabetes, neurodegenerative diseases, and aging. Antioxidants accomplish this by scavenging reactive oxygen species (ROS), thus keeping cellular homeostasis and defensive in contradiction of harm to proteins, lipids, and DNA. (Ateya *et al*., 2023) In cooperation, endogenous and exogenous antioxidants contribute to this defensive consequence (Lawi *et al*., 2021; Al-Behadili *et al*., 2024). Free radicals are created continuously in cells of the human body within complex redox reactions, including different radicals, for example, superoxide (O2•-), hydroxyl (OH•), and singlet oxygen (1O2).

Such free radicals significantly impact the progression of various serious diseases, for example, neurodegenerative diseases cancer, diabetes, cardiovascular disease, atherosclerosis, and aging (Pham-Huy *et al.,* 2008). Antioxidants that forage free radicals and help prevent age-related degenerative diseases are available in exogenous and endogenous forms.

Therefore, antioxidants in food can reduce the risk of developing certain infections. Antioxidants have health benefits and can prevent or slow down the oxidation of food through the formation of free radicals when exposed to temperature, light, and air (Lim *et al.,* 2011; Akter *et al.,* 2022). Turmeric has demonstrated its pharmacological properties in numerous studies, including antioxidant, anti-inflammatory, immunomodulatory, antifungal, antiangiogenic, antimicrobial, analgesic, vasodilator, antidiabetic, and anti-Alzheimer (Ramadan et al., 2011; Akter et al., 2019). Therefore, this work aimed to understand the polymorphism of the curcumin synthase 1 (CURS1) gene curcumin content and antioxidant activity. This research focuses on the CURS1 gene from Curcuma longa L., which plays a vital role in curcumin biosynthesis. By analyzing genetic variations in two turmeric varieties (NDH-98 and GNT-2) and their association with curcumin content, this research aims to offer deeper insights into the molecular basis of curcumin production. Through a combination of DNA sequencing, in *silico* protein modeling, and curcumin quantification using HPLC, the study investigates the impact of single nucleotide polymorphisms (SNPs) on the CURS1 protein structure and their potential effect on curcumin yield and antioxidant capacity. This work sets the foundation for understanding genetic diversity in turmeric diversities and its implications for enhancing curcumin production and antioxidant activity, contributing to the broader applications of turmeric in health and agriculture.

Materials & Methods

This study was analyzed in the Laboratory of Genetic Engineering and Molecular Biology at the University of Misan, Iraq.

Genomic DNA extraction

The Fresh turmeric (*Curcuma longa* L.) rhizomes were collected from the markets of Maysan and Basrah governorates. The collected samples were cleaned, air-dried, and stored under controlled conditions to preserve their integrity before analysis. DNA was extracted from the original samples 70 to 90 mg of dehydrated rhizomes. For this purpose, the Genomic DNA Mini Kit (Plant) from Geneaid, Taiwan was modified according to the manufacturer's protocol (Liu *et al.,* 2022). The fragment (900 bp) of the *CURS1* gene in turmeric was amplified using primers F:5`-ATGGTGAAGAAGCGGTACCTG-3` and R:5`-GCTAATCAGTCAATCCAGATGG-3` (Santhoshkumar and Yusuf, 2021). The 50 µL volume used for PCR amplification contained 6 µL of genomic DNA, 25 µL of master mix, 4 µL of each primer, and 15 µL of free water. Initial denaturation at 95˚C for 15 min was followed by 35 cycles of denaturation at 95˚C for 20 s, annealing at 52˚C for 40 s, extension at 72˚C for a few minutes, and a final extension at 72˚C for 10 min. PCR products were identified by 1% agarose gel electrophoresis, ethidium bromide staining, and UV visualization. PCR products were sent to Macrogen, Inc., Korea for sequencing.

Bioinformatics analysis

BioEdit 7.0 software compared the *CURS1* gene sequencing data with GenBank accession number KM880189.1 (Hall, 1999). Genotypes

were found using the Geneious prime (version 2024.0.2) tool. Phyre2 V. 2.0 and EzMol V.1.22 software were used to predict the secondary and three-dimensional structures of the Curcumin synthase 1 protein (Kelley *et al.* 2015; Reynolds *et al.,* 2018; Faraj *et al.* 2023). diversity of Haplotype (Hd), and nucleotide diversity (π) were determined using DnaSP v5. 10 software (Rozas *et al.,* 2017; Hatem *et al.,* 2024).

The estimate of curcumin by HPLC technology

The chemical profiling of curcumin in turmeric was performed using High-Performance Liquid Chromatography (HPLC). Rhizome samples were finely ground, and curcumin was extracted using a solvent mixture of methanol and water. The extracts were filtered and subjected to HPLC analysis using 100 µL of the sample injected into the SYKAMN HPLC system (Germany) equipped with a C18-ODS column (250 × 4.6 mm, 5 µm). The mobile phase consisted of a gradient mixture of acetonitrile and water with 0.1% formic acid, ensuring optimal separation (Ngamsuk *et al.,* 2019). The curcumin was detected on a wavelength of 425 nm, with the retention times of curcumin quantified against standard references.

Antioxidant capacity by DPPH assay

Dissolve 0.04 g of DPPH in 100 millilitres of methanol. To prepare the standard solution of Vitamin C and the samples, 0.5 g of Vitamin C was mixed with 100 ml of methanol and distilled water. The concentration of the standard solution was 5000 ppm, and additional concentrations (30, 60, 120, 250, and 500 ppm) were prepared from Vitamin C and samples using the dilution method. The mixture was shaken vigorously and left at room temperature for 30 minutes. Afterward, the absorbance was measured at 517 nm. The IC50 value of each sample (i.e., the sample concentration required to inhibit 50% of the DPPH free radicals) was calculated using a spectrophotometer (UV-VIS Shimadzu) and a logarithmic dose-inhibition curve. Lower absorbance of the reaction mixture indicates higher free radical activity. The following equation was used to calculate the percent DPPH scavenging impact:

$$\text{DPPH reduced (\%)} = \frac{\text{A0} - \text{A 1}}{\text{A0}} \times 100$$

The A0 was the absorbance of the blank, while the A1 was the absorbance in the existence of the test sample.

Statistical analysis

All statistical parameters were evaluated using SPSS 20.0 software. The relationship between genetic variation and curcumin accumulation was examined using Pearson's correlation coefficient. One-way ANOVA was used with a significant value of 0.05.

Results & Discussion

Genetic Variation

The results presented in this study of the *CURS1* gene showed that the total number of sequences (N) was 42, and the number of haplotypes (H) was 2 resulting in 1 genetic polymorphism (S). of haplotype and nucleotide diversities data were (1.00 and 0.00169 respectively), as shown in Table 1.

Table (1): The Genetic Diversity of Turmeric.

Gene	(N)	(H)	(S)	(Hd)	(π)
CURS1	23	2	1	1.00	0.00169

N: Number of Sequences; H: Haplotype; S: Number of polymorphic; Hd: Haplotype Diversity; π: Nucleotide Diversity

The data of Table 2 and Fig. 1 showed the analysis of nucleotides as well as protein in exon 1, in which they detected one SNP: cytosine (C) to guanine (G) at position 850 (g.850C>G). Thus, the amino acids altered to 293Thr>Arg.

Table (2): Potential type of amino acid change in the CURS1 protein of turmeric.

Gene	(N)	(H)	(S)	(Hd)	(π)
CURS1	23	2	1	1.00	0.00169

C: Cytosine, G: Guanine, Thr: Threonine, Arg: Arginine, aa: amino acid

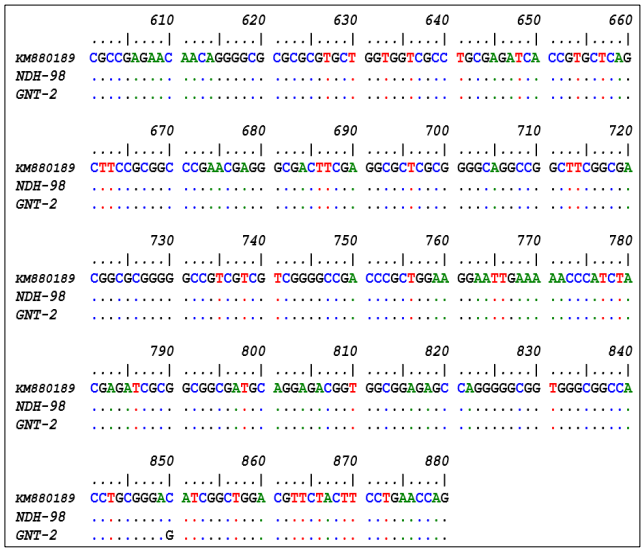

Fig. (1): CURS1 gene sequencing from GenBank (KM880189.1) with comparison to NDH-98 and GNT-2.

Secondary structure prediction

Analysis of the chain secondary structure by Phyre2 predicted α-helix, β-strand, and a random coil of the protein chain (Fig 2 and 3). The α-helix in the predicted secondary structure of the CURS1 protein was 32% and 34% in NDH-98 and GNT-2, respectively. This was followed by random coil (8, 10%) and β-strand (14, 13%) (Table 3). The CURS1 protein showed a predominance of helices and β-strands, highlighting that the CURS protein has a larger transmembrane position and stronger bonding.

Table (3): Predicted structure of secondary CURS1 proteins by Phyre2

Cultivars	Parameters		
	α-Helix	β-strand	Random coil
NDH-98	32%	14%	8%
GNT-2	34%	13%	10%

Three-dimensional protein structure

Modeling homology of the automated homology protein modeling tool EzMol was used to create three-dimensional protein structures. The 3D fig was used to determine the amino acid change belongs the CURS1 protein. One single-nucleotide polymorphism (SNP) was detected in the protein. The changes in the structure of threonine to arginine, indicate changes in the structure of the three-dimensional protein (Fig. 4 A, B).

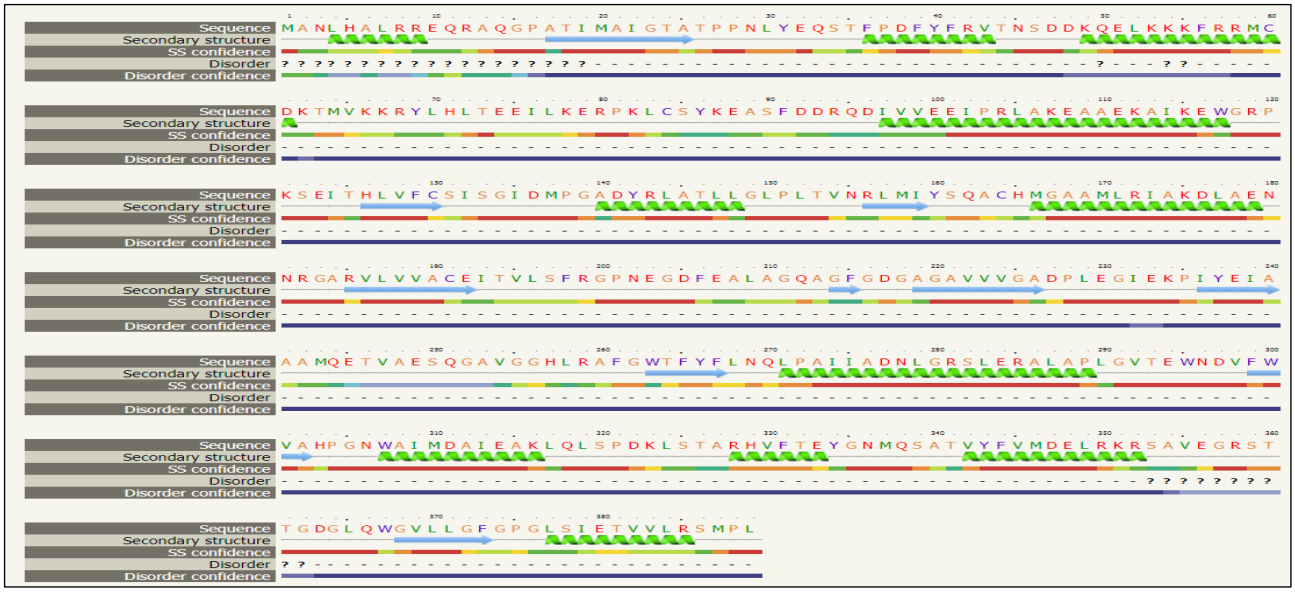

Fig. (2): Analysis of CURS1's secondary structure in NDH-98

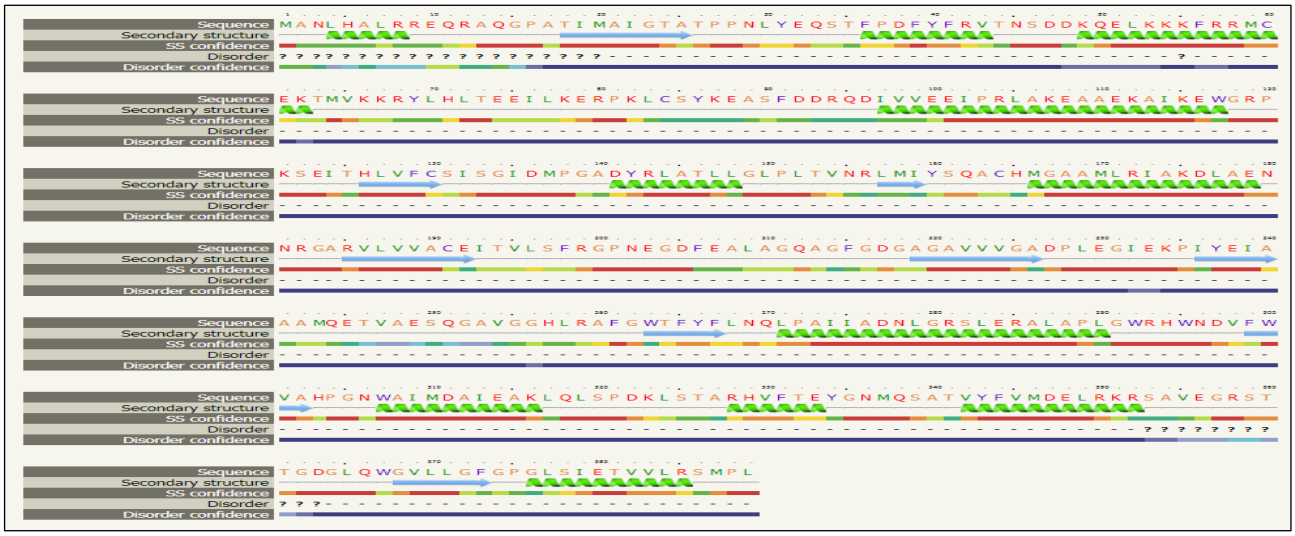

Fig. (3): Analysis of CURS1's secondary structure in GNT-2.

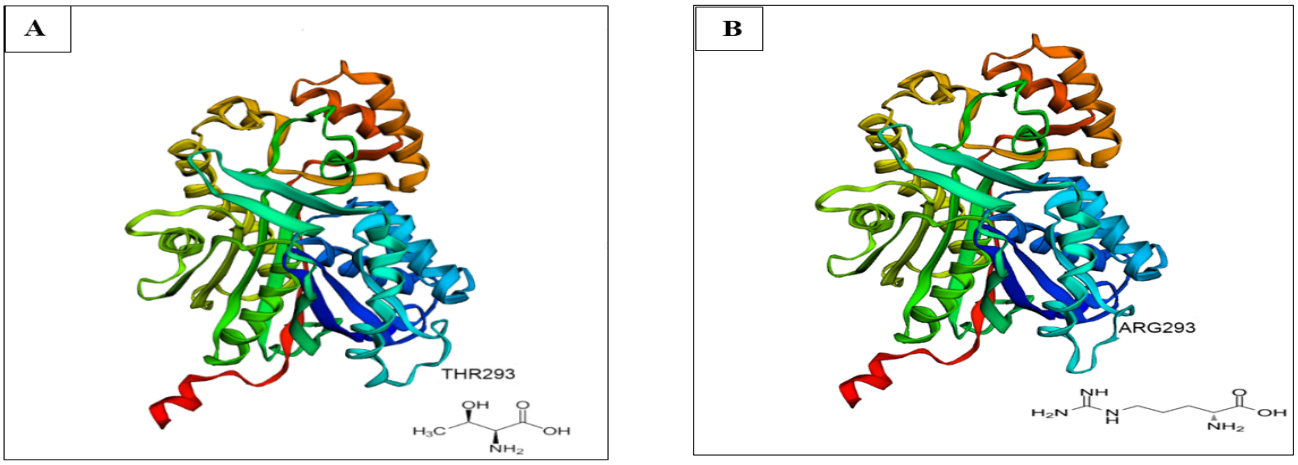

Fig. (4): Model 3D structure of CURS1 protein in Turmeric by Phyre2 software. A: NDH-98, B: GNT-2

Determination of the curcumin content

The HPLC determined that the curcumin content was increased in GNT-2 rhizomes compared to NDH-98 rhizomes. The content of curcumin was 784.0 µg g^{-1} and 712.1 µg g^{-1}, respectively (Table 4 and Fig 5).

Table (4): Determination of the curcumin content in the plant *Curcuma longa* L. (NDH-98, GNT-2) Using HPLC technology

Gene	Mutation	Type of plant	Curcumin µg g^{-1}	*p*. value
CURS1	293Thr>Arg	GNT-2	784.0*	0.04
		NDH-98	712.1	

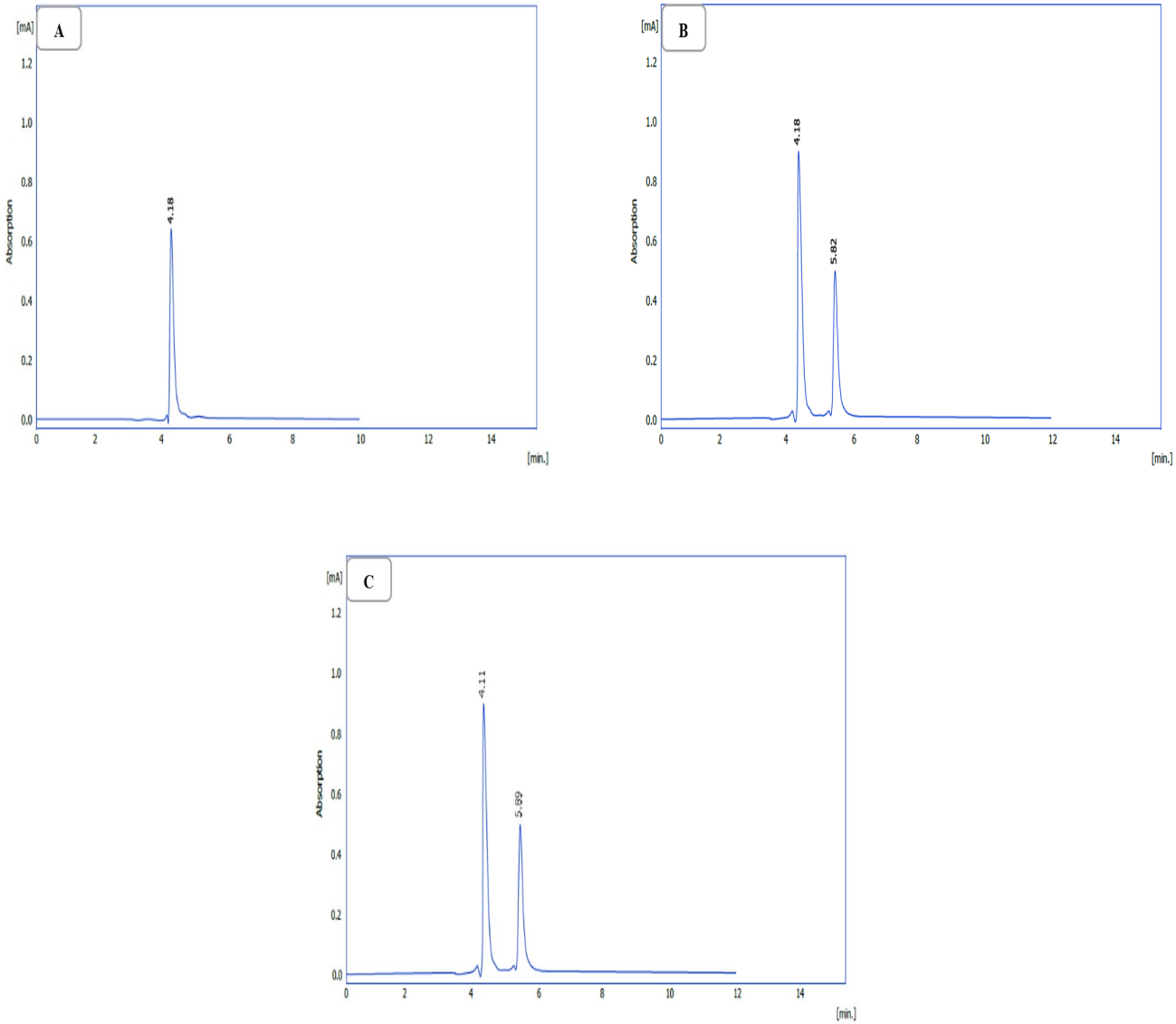

Fig. (5): Chromatographic separation of curcumin (A) standard (B) NDH-98 (C) GNT-2.

Anti-Oxidant Study of NDH-98, GNT-2 and Vit. C methanolic Extracts Using DPPH Assays shown in Fig 6, the percentage of inhibition of free radicals by different concentrations (30, 60, 120, 250, and 500 µg g^{-1}) of turmeric methanol extract can be determined. of NDH-98 and GNT-2 methanol extracts at different concentrations showed higher free radical inhibition than the Vit C control used for the DPPH free radical scavenging assay. The inhibition of GNT-2 by turmeric methanol extract at different concentrations (500, 250, 120, 60, and 30 µg g^{-1}) was 94.9%, 82.08%, 66.97%, 52.11%, and 32.61%, respectively, while the inhibition of NDH-98 was 92.66%, 79.80%, 63.58%, 49.11%, and 29.08%, respectively.

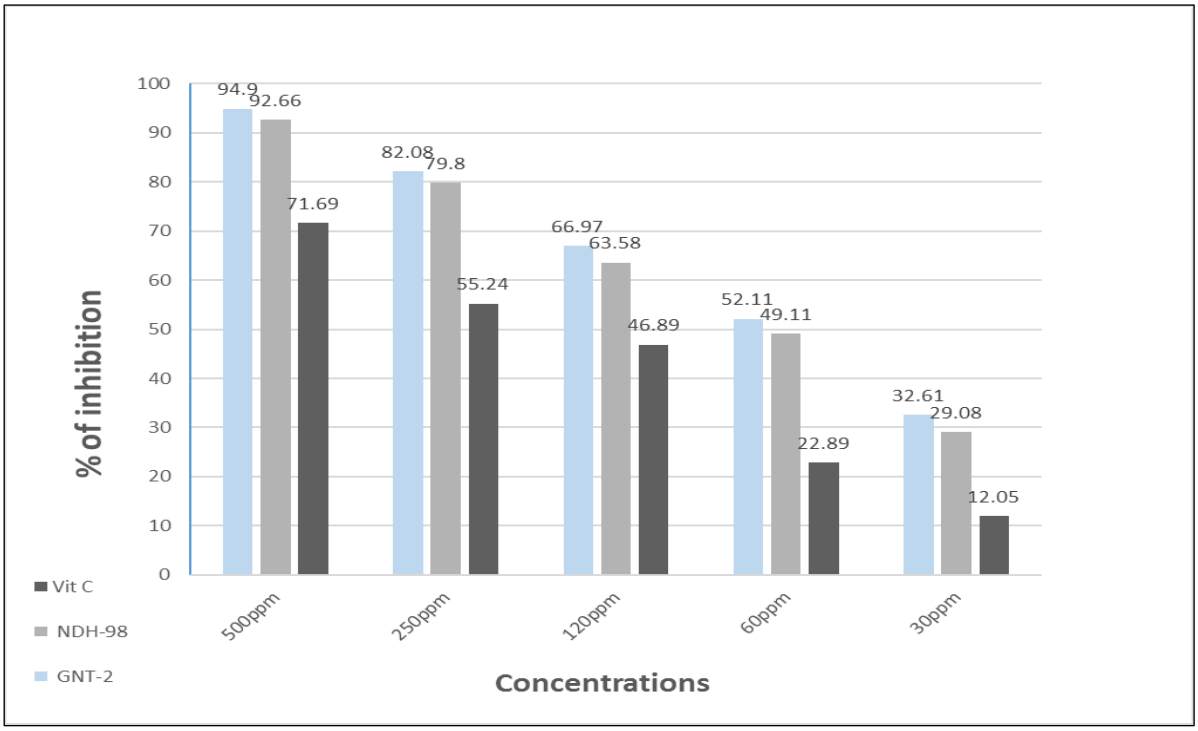

Fig. (6): The percentage of inhibition of free radicals' graph of NDH-98, GNT-2, and Vit. C using DPPH assay.

Discussion

There are few studies on the polymorphism of the *CURS1* gene in turmeric. The main region analyzed is intron 1 (Kita *et al.,* 2016). Identifying different gene variants and revealing the relationship between these polymorphisms and the curcumin content trait is of great significance for improving this trait in plants (Lan *et al.,* 2018). The cloned CURS1 putative sequence showed higher homology with the CURS sequence in the database by ORF determination to determine the protein characteristics (Koo *et al.,* 2013). The results showed that there was a gene mutation in this gene (*CURS1*), which changed the encoded amino acids due to the change in the nitrogenous base sequence, resulting in partial or complete differences in the amino acid sequence. This may cause complete or partial variations in the structure of the enzyme responsible for the synthesis of the active constituents (Santhoshkumar & Yusuf, 2020).

Different studies have shown that curcumin biosynthesis in turmeric is crucially influenced by three different genes including CURS (*CURS1*, *CURS2*, and *CURS3*). Three isoforms of the curcumin synthase gene play an important role in synthesizing the many

vital curcuminoids (Krup *et al*., 2013). The curcumin production varied between the two Curcuma species, and the ratio of 293Thr>Arg was detected by expressing the *CURS1* gene. The recent results suggested that GNT-2 can act as a possible alternative to NDH-98 in the separation of natural curcumin (Sandeep *et al.,* 2017). The results presented here, are consistent with those of Ayer *et al.* (2018). NDH-98 had a lower curcumin content, while GNT-2 had a variety with a higher curcumin content. Although there is little data on the relationship between *CURS1* polymorphisms and the trait of curcumin content, it has been correlated positively with curcumin biosynthesis and physicochemical features in turmeric (Rafi *et al*., 2015; Santhoshkumar & Yusuf, 2020). This proves the gene can be categorized as a sign of these multiple traits (Jyotirmayee & Mahalik, 2022).

The optimal way to assess a compounds ability to scavenge free radicals is to quantify its activity of free radical scavenging by using the stable free radical called DPPH (Mensor *et al.,* 2001). This free radical can be dissolved in ethanol, resulting in a purple color, and is constant at room temperature. This color turns without color in the occurrence of antioxidants. This method is simple, reliable, and rapid (Lee *et al.,* 2003). This study showed that curcumin has a powerful and superior antioxidant effect compared to vitamin D. Curcumin has a positive impact on life and can be used to reduce or prevent the production of harmful oxidation products in the body. Our results are consistent with those of Asouri *et al.* (2013) who found the main reason for the biological effects of curcumin and showed strong antioxidant activity.

The results presented in this study agree with those of Alafiatayo *et al.* (2014) who depend on a comparison between the antioxidant activity of different turmeric varieties and indicate that the plant *C. longa* with the highest scavenging activity, with little effect by the plants *C. amada* and *C. xanthorriza*. In addition, the results of our study are consistent with the study of Nahak and Sahu (2011). They examined the activity of antioxidants of ethanol extracts of the plants *C. aromatica, C. longa, C. amada*, and *C. zedoaria*. They found that *C. longa* had higher antioxidant activity. The differences between the antioxidant activities of different turmeric varieties in this work and others may be caused by genetic variation Variations in the chemical structure of turmeric, and variations inside the geographical regions where turmeric is grown, including soil type and climate.

Conclusion

The CURS1 protein of turmeric was modeled by using bioinformatics methods. In multiple sequence alignments, higher homology was found between the CURS1 proteins in NDH-98 and GNT-2. According to secondary structure studies, the CURS1 protein is dominated by alpha helices, followed by beta strands and random coils. Phyre2 was used to check the tertiary structure predictions. After validation, the model was uploaded to the PMDB server. One of the most difficult aspects of reviewing data from NMR or X-ray crystallography methods is predicting the 3D model of the protein used in computer studies. Therefore, using computer analysis to detect protein structure is among the most powerful tools for exploring the structure and functions of different proteins. Finally, it helps to study the molecular differences expressed between different turmeric species to elucidate the precise gene regulatory mechanisms that control the accumulation of curcumin in these species.

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Contributions of authors

W.A.A: Samples collection, analysis part of data; write part of the manuscript.

F.A.H: Validation, editing, and writing review.

T.S.A: Suggest a title, and analyze the other part of the data.

S.H.F: Analyze and write another part of the data.

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Conflicts of interest

The authors declared that there is no conflict of interest.

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التشخيص الجزيئي والكيميائي لمركب الكركمين ودراسة فعاليته المضادة للأكسدة في صنفين من نبات الكركم

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المستخلص: في هذا الدراسة تم اختيار جين (*CURS1*) Curcumin synthase 1 من نبات الكركم الذي يلعب دورًا مهمًا في تخليق مركب الكركمين. كان الهدف هو تحديد تعدد أشكال النيوكليوتيد المفرد (SNPs) لجين *CURS1* في نوعين من نباتات الكركم (NDH–98 و GNT–2) والعلاقة بمحتوى الكركمين، باستخدام تقانة تتابع القواعد النيتروجينية، وتم تحليل النتائج ورسم البنية ثلاثية الأبعاد لبروتين *CURS1* بواسطة برامج المعلوماتية الحيوية. ضخمت قطعة بطول 900 زوج قاعدي من جين *CURS1*. تم تحديد تعدد أشكال النيوكليوتيد المفرد (g.850 C>G) في الاكسون الأول، أدت هذه الطفرة إلى تغيير في البنية ثلاثية الأبعاد للبروتين نتيجة لتغيير الحامض الأميني من الثريونين إلى الأرجينين. كان تنوع النمط الفردي وتنوع النيوكليوتيدات 1.00 و0.00169 على التوالي. كانت كمية الكركمين في جذور 784.0 GNT–2 ميكروجرام / جرام، بينما كانت في 712.1 NDH–98 ميكروجرام / جرام، على التوالي التي تم قياسها باستخدام تقانة HPLC. أظهر اختبار DPPH، الذي يحسب قدرة إزالة الجذور الحرة للمستخلصات، أن الجزء الميثانولي من كركم (NDH–98 و GNT–2) لديه قدرة كبيرة على تثبيط الجذور الحرة، مع قيمة IC50 التي تزيد عن 125٪ من إجمالي قدرة المستخلص على تثبيط الجذور الحرة. ونتيجة لذلك، كان لمستخلصات NDH–98 و GNT–2 ذات الطبيعة الميثانولية تأثيرًا كبيرًا على اختبار DPPH لإزالة الجذور الحرة، وكان هذا مصحوبًا بالتحكم في مستوى فيتامين د.

الكلمات المفتاحية: النشاط المضاد للأكسدة، الكركم، جين *CURS1*، التركيب ثلاثي الابعاد للبروتين، تعدد أشكال النيوكليوتيدات المنفردة.