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Trace elements study using x-ray fluorescence, charantin accumulation and squalene synthase gene expression in different *Momordica charantia* Linn fruit

Anwesh Roy[1], Anindita Chakraborty[2], Muthummal Sudarshan[3], Sayak Gangulic[4] and Sarmistha Sen Raychaudhuri[5*]

[1]Department of Biophysics, Molecular Biology and Bioinformatics, University of Calcutta, 92, APC Road, Kolkata- 700009, India. E-mail: anweshroy1990@gmail.com

[2]UGC-DAE CSR, Kolkata Centre Sector III, LB-8, Bidhan Nagar, Kolkata 700 098, India. E-mail: anindita.iuc@gmail.com

[3]UGC-DAE CSR, Kolkata Centre Sector III, LB-8, Bidhan Nagar, Kolkata 700 098, India. E-mail: sude@alpha.iuc.res.in

[4]Department of Biotechnology, St. Xavier's College (Autonomous), 30 Mother Teresa Sarani (Park Street), Kolkata – 700016, India. E-mail: sayakbif@yahoo.com

[5]Department of Biophysics, Molecular Biology and Bioinformatics, University of Calcutta, 92, APC Road, Kolkata- 700009, India. E-mail: sarmistha_rc@rediffmail.com

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Abstract

Momordica charantia is a medicinal plant comprising of antidiabetic properties. Along with this, it has been recognized as a good source of trace elements which are beneficial for health. So it is important to find the actual trace element content in different *M. charantia* fruits cultivated in different areas. We have found 12 elements varying within the fruit parts where the fruit pulp was found to be key reservoir of calcium, manganese, potassium and sulfur. Charantin, being one of the important antidiabetic compounds found in *M. charantia*, were estimated using HPLC and found to be accumulated at maximum in the Tamluk region. Squalene synthase is the rate limiting enzyme in phytosterol biosynthesis and therefore, we have characterized the *M. charantia* Squalene Synthase (McSQS) gene, went through an *in silico* study and tried to find the expression correlation with charantin content in *M. charantia* fruits, where we found that *McSQS* expression is highest in Tamluk region.

Keywords: *Momordica charantia, Charantin, Trace elements, EDXRF, Squalene synthase*

1. Introduction

Diabetes mellitus is a chronic metabolic disorder resulting from error of action and/or secretion of insulin which leads to a hyperglycemic condition in neonatal and/or adults (Njølstad *et al*., 2003). Diabetes is one of the rapidly increasing prevalent diseases and according to World Health Organization (2003) by 2030, the number of diabetic adults will become 370 million from177 million in 2000 (Ozougwu *et al*., 2013). In India, it may be set up to 79.4 million individuals, even greater than China and USA (Kaveeshwar and Cornwall, 2014). As antidiabetic therapies, antidiabetic drugs such as sulfonylurease, biguanides, guanides and also

* *Corresponding author: Sarmistha Sen Raychaudhuri, Department of Biophysics, Molecular Biology and Bioinformatics, University of Calcutta, 92, APC Road, Kolkata- 700009, India. E-mail: sarmistha_rc@rediffmail.com*

insulin are available but various plants showing hypoglycemic activities are reported to be effective against diabetes. Mostly the glycosides, alkaloids, terpenoids, flavonoids, etc. are the salient antidiabetic compounds found in different plants (Patel *et al.*, 2013).

Numerous studies have revealed that, *Momordica charantia*, a member of the Cucurbitaceae family, renders broad ranging health benefits such as anti-cancer, anti-viral, anti-inflammatory, analgesic, hypolipidemic and hypocholesterolemic effects (Tan *et al.*, 2016). Especially, *Momordica* fruits can reduce blood sugar level in diabetic rat as the fruit possesses phytochemicals such as momordicine, charantinetc, along with insulin-like peptides and few galactose-binding lectins (Patel *et al*., 2013). It was also demonstrated that aqueous solution of unripe *Momordica* fruits can fractionally stimulate insulin release from isolated beta cells in obese hypoglycemic mice (Grover and Yadav, 2004). Different parts of *M. charantia* such as leaf, vine, root, seed has anthelmintic, emmenagogue, antidiabetic, anti-pneumonial properties respectively, as well (Paul *et al*., 2010).

M. charantia L. is an important medicinal plant containing a large number of bioactive compounds. A wide array of recent studies has highlighted the hypoglycemic activity of *Momordica* (Bailey *et al*., 1985; Day *et al*., 1990; and Bortolotti *et al*., 2019). Its use as an alternative treatment of diabetes mellitus has been proposed since it does not have any major side effect unlike the current methods of diabetes treatment. Phytochemical screening of this plant revealed the presence of some chemical compounds possessing antidiabetic property. Among them charantin has drawn considerable attention from researchers (Jeevathayaparan *et al.,* 2017).

The mechanism of the antidiabetic activity of *Momordica*is being explored but has not been very well established. The most plausible mechanisms for hypoglycemic effects of extracts of *Momordica* may be due to some physiological, pharmacological and biochemical means (Akhtar *et al*., 2005; Raman and Lau, 1996; Ahmed *et al.*, 2004; and Hlaing and Kyaw, 2005). An interesting study proposes charantin to increase insulin sensitivity by causing reduced Protein Tyrosine Phosphatase (PTP) activity, which is a physiological antagonist in the insulin signaling pathway (Sharma *et al*., 1996). Extracts of *M. charantia* are also known to have cholesterol lowering effect (Joseph and Jini, 2013). Moreover, *M. charantia* has been shown to have an effective role in non insulin dependent diabetes mellitus (NIDDM) (Leung *et al*., 2009; and Bakare *et al*., 2010).

Although bitter melon is cultivated as a major cucurbit in almost every district of West Bengal, the cultivators remain mostly unaware about the actual content of the bioactive principles present in the crop. It is evident from the earlier studies that the fruits of *Momordica* possess extensive hypoglycemic principles (Paul *et al*., 2010). Taking this into account fruit pulp extracts have been used in the present investigation. Bitter melon was reported to increase the mass of â-cells and insulin production in the pancreas (Chao and Huang, 2003; and Shetty *et al*., 2005). An amelioration of about 30% in fasting blood glucose was observed when Streptozotocin induced diabetic rats have been provided with edible portion of bitter melon at 10% level in the diet (Shetty *et al.*, 2005). It has been observed in biochemical studies that bitter melon plays a role in the regulation of cell signaling pathways in pancreatic β cells, adipocytes and muscles. Ethyl acetate extracts of bitter melon have been found to lower plasma apoB-100 and apoB-48 in mice fed with high fat diet by activating peroxisome proliferator receptors (PPARs) α and γ (Chao and Huang, 2003; and Bao *et al*., 2013) and modulating the phosphorylation of insulin receptor and its downstream signaling pathway. In addition to that the momordicosides (Q, R, S and T) is known to stimulate GLUT4 (Glucose transporter type 4) translocation of the cell membrane and increases the activity of AMP-activated protein kinase (AMPK) in both L6 myotubes and 3T3-L1 adipocytes. This causes an enhancement in fatty acid oxidation and glucose disposal during glucose tolerance tests in both insulin-sensitive and insulin-insensitive mice (Tan *et al.*, 2008). The importance of *M. charantia*as a plant having antidiabetic activity is well established and our results would be useful for making polyherbal formulations or in further biotechnological applications Our study may advance the use of charantin in herbal medicine and may also lead to new drug discovery in future and intensive cultivation of these two elite populations may play a significant role in the improvement of commercial trade in West Bengal and also across India.

Energy Dispersive X-Ray Emission Spectroscopy (EDXRF) is a very reliable technique to determine trace elemental content of biological sample as it is highly sensitive, non-destructive and multi-elemental technique (Johansson and Campbell, 1988). Different elements present in different medicinal plants have direct or indirect influence on the disease control and to cure and to prevent such diseases these herbs should be consumed in a regulated manner, and therefore EDXRF study to measure trace element concentration of medicinal plants can be conducted (Swain *et al*., 2012). Although bitter melon is cultivated as a major cucurbit in almost every district of West Bengal, the cultivators remain mostly unaware about the actual content of the bioactive principles

present in the crop. The present investigation has undertaken a rapid, cost effective, easily adaptable and well validated HPLC method for screening and identification of elite *M. charantia* populations available in our state. Out of the eight different accessions of two *M. charantia* varieties screened, two populations have been identified in the present study which contained substantially high amount of charantin in comparison to the other accessions. Intensive cultivation of these two elite populations may play a significant role in the improvement of commercial trade in West Bengal and also across India.

With this perspective we would try to analyze trace elemental profile of the collected fruits (preserved as lyophilized sample), root and stem from plants growing in these areas. β-sitosteroland stigmasterol, which are two components of charantin (Desai, 2015) are included among phytosterols and are synthesized via mevalonate-dependent isoprenoid pathway, in which squalene synthase acts as the key regulatory enzyme (Kim *et al*., 2011). Overexpression of sqaulene synthase can increase the phytosterol accumulation in plant system (Jin *et al*., 2005). Mi-Hyun Lee *et al.* (2004) showed that methyl jasmonate treatment can elicit *Panaz ginseng* squalene synthase expression in adventitious root.

2. Materials and methods

2.1. Sample collection

Samples in the form of fruits and seeds used for cultivation were collected from eight different locations of West Bengal, India namely Contai (Con), Tamluk (Tam), Kharagpore (Kgp), Bagnan (Bag), Behrampore (Beh), Duttapukur (Dut), Daspur (Das) and Basirhat (Bas). *M. charantia* var. *charantia* fruits and collected from the cultivators.

2.2. Lyophilazation

About 1 g of each fruits was freeze-dried using a Vertex Lyophilizer at -80°C for 48 h and homogenized by brittle fracture technique. These were then ground into powder by using mortar and pestle and preserved in vaccum.

2.3. Trace elements analysis using EDXRF

Pellets were made from freeze-dried samples using pelletizer. Three types of pellets were made from samples of eight different sites. Those are: (a.) fruit pulp (FP), (b.) seeds from fruit (SF) and (c.) seeds for cultivation (SC). The pellets were run for EDXRF analysis.

2.4. Charantin extraction

Charantin extraction was carried out according to Ahamad *et al*. (2015) with minor modifications. 100 mg of lyophilized fruit samples are suspended in 5 ml of 90% ethanol. The suspensions were sonicated for 1 hour in 50°C. Then the samples were centrifuged at 4000 rpm for 5 min and the supernatants were filtered through a 0.2 micron Acrodisc filter. Filtered solutions were evaporated at 60°C for 1-2 h. Then 1 ml methanol was added in each dried tube.

2.5. Charantin estimation by reverse-phase HPLC

The extracted samples dissolved in methanol were now run in reverse-phase HPLC C18 column. The mobile phase was methanol and water (methanol:water = 100:2 v / v). Stigmasterol (Sigma-Aldrich) was used as the standard. The retention time was found to be 8.3 min. Standards were also run with concentrations of 25, 50, 75, 100, 150 and 200 μg / ml.

2.6. Primer Designing

Primers were designed in order to characterize *M. charantia* Squalene Synthase gene by RT-PCR method followed by sequencing. The following sequence informations were taken into consideration collected from NCBI:

1. *Siraitiagrosvenorii* squalene synthase mRNA (KX231777.1)
2. *Gynostemmapentaphyllum* squalene synthase mRNA (FJ906799.1)
3. *Camellia oleifera* squalene synthase mRNA (JX914592.1)

4. *Diospyros kaki* cultivar Xiao Fang Shi squalene synthase (sqs) mRNA (FJ687954.1)
5. *Luffa acutangula*isolate La.SSsqualene synthase mRNA (KX548336.1)
6. *Trichosanthestruncata* isolate Tt.SS.01 squalene synthase mRNA (KX548327.1)

Primers were designed by performing multiple sequence alignment (ClustalW). The sequence of primers are given in the Table 1.

Table 1: Primers for *McSQS*

Name of Gene	Forward Primer Sequence	Reverse Primer Sequence
SQS1	5'ACCGTGACTGGCATTTTTC3'	5'GCTACGTAGTGGCAATATTCATC3'

2.7. Total RNA extraction and reverse transcription (RT) - PCR

M. charantia seeds were grown for 10 days in agar sucrose medium and 100 mg leaves were collected for total RNA extraction. Total RNA was extracted using PureLink RNA Mini kit [PureLinkRNA Mini kit, Ambion by Life Technologies, New Delhi, India]. After total RNA extraction, it is stored at -80°C. In order to check the purity and integrity of the total RNA extracted, spectrophotometric analysis[JASCO V-630 UV–Vis Spectrophotometer] is done at 260 nm and accordingly the RNA concentration is calculated from the optical density (O.D. / A260) obtained using the following formula:

RNA concentration (μg / mL) = $Absorbance_{260}$ * dilution factor

In our experiment, dilution factor is 300 as we have taken 1 μl RNA and 299 μl nuclease free water for dilution.

RT-PCR was carried out with gene-specific primers using Qiagen One Step RT-PCR kit. Each reaction mixture contained 2 μg total RNA. The RT-PCR conditions are given in Table 2. The RT-PCR product was run in 1.5% agarose gel electrophoresis stained with ethidium bromide visualized in gel documentation system (BioRad Molecular Imager, GelDoc XR, Milan, Italy). The remaining sample was sequenced partially sequenced by Sanger dideoxy method.

Table 2: RT-PCR Condition

Stage	Temperature	Time	
Initial Denaturation	95°C	2 min	
Denaturation	94°C	1 min	40 cycles
Primer Annealing	53.5°C	1 min	
Strand Synthesis	72°C	1.5 min	
Final Extension	72°C	10 min	

2. 8. Relative mRNA expression analysis by reverse transcription RT-PCR

The mRNA were extracted from all the collected samples by the above mentioned method and the RT-PCR was carried out in the same way as mentioned earlier. The RT-PCR products were run in 1.5% agarose gel electrophoresis stained with ethidium bromide visualized in BioRad gel documentation system. Relative mRNA expression profile was analyzed densitometrically from the band intensity of the gels using Image J software. The expression profile was compared against endogenous control (β-actin).

2.9. In Silico Studies

Computational methods exploit the sequence signatures of disorder to predict whether a protein is disordered, given its amino acid sequence. We have used IUPred2A, a web interface that allows to identify disordered protein regions using IUPred2 and disordered binding regions. Hydropathy plots (Kyte and Doolittle) allowed

the visualization of hydrophobicity over the length of a peptide sequence and are useful in determining the hydrophobic interior portions of globular proteins as well as determining membrane spanning regions of membrane bound proteins. We have used Phyre2 Server (http: / / www.sbg.bio.ic.ac.uk / ~phyre2) to perform homology modeling to precisely predict the structure which is used for homology or comparative modeling of protein three-dimensional structures. Phyre2 analyzed secondary structure, domains with their algorithm and predicted the *in silico* model for the partial McSQS protein sequence. They took several templates, aligned the query sequence with those PDB sequences and we came up with the best model taking the factors such as alignment coverage, confidence, percentage identity into account. PROCHECK server checked the stereo chemical quality of a protein structure, producing a number of PostScript plots analyzing its overall and residue-by-residue geometry.

3. Results

3.1. Sample collection

Samples are collected from the from the eight different cultivation areas across the West Bengal, India; where *M. charantia* are cultivated frequently throughout the year. The images of the samples are given in the Figure 1.

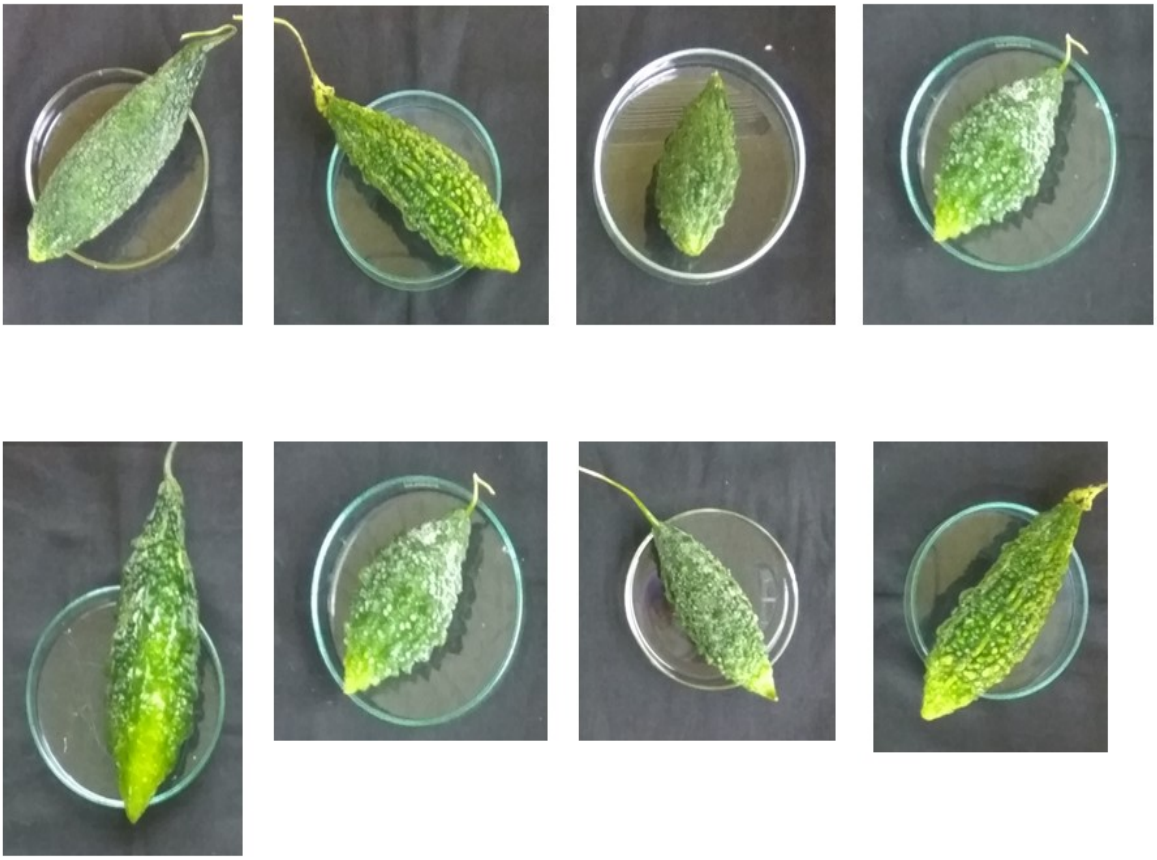

Figure 1: Fruits of different collection site a. Contai (Con); b. Tamluk (Tam); c. Basirhat (Bas); d. Bagnan (Bag); e. Behrampore (Beh), f. Duttapukur (Dut), g. Daspur (Das), h. Kharagpore (Kgp)

Trace Elemental Variations: The fruits and seeds collected from the farmers were subjected to EDXRF analysis. A comparative data sets were obtained and arranged as Tables 3-14. The data showed variation in 12 elements across different cultivation areas and different fruit parts (SC: Seed for cultivation; SF: Seed within the fruit, FP: Fruit pulp) (Figure 2).

Table 3: Variation in barium (Ba)

	SC	SF	FP
Bas	72.65 ± 2.7	70.89 ± 2.48	75.81 ± 5.22
Bag	87.75 ± 10.2	70.25 ± 3.83	78.23 ± 8.6
Con	68.04 ± 1.85	67.25 ± 1.32	83.83 ± 4.89
Tam	80.16 ± 6.91	71.19 ± 3.53	72.58 ± 2.66
Beh	70.57 ± 3.62	58.74 ± 4.8	67.76 ± 0.37
Das	72.98 ± 4.63	71.25 ± 2.45	77.25 ± 1.32
Dut	68.91 ± 0.9	63.53 ± 4.06	89.39 ± 11.32
Kgp	67.37 ± 1.2	65.07 ± 3.75	67.71 ± 0.12

Table 4: Variation in calcium (Ca)

	SC	SF	FP
Bas	2115.7 ± 24.72	2178.33 ± 30.59	4443.08 ± 79.27
Bag	2013.23 ± 30.91	2638.97 ± 32.09	4583.28 ± 101.31
Con	2034.45 ± 35.57	2994.06 ± 61.03	4722.44 ± 31.5
Tam	2035.8 ± 57.24	3746.56 ± 67. 09	4191.01 ± 154.38
Beh	1856.77 ± 41.53	3098.45 ± 49.91	5781.95 ± 131.56
Das	2001.33 ± 33.42	4349.77 ± 93.55	4503.3 ± 77.31
Dut	2087.87 ± 51.41	3982.77 ± 667.71	4342.55 ± 98.12
Kgp	1987.69 ± 67.07	4086.43 ± 60.42	4498.59 ± 122.73

Table 5: Variation in copper (Cu)

	SC	SF	FP
Bas	91.39±4.01	51.88±1.58	33.49±2.73
Bag	65.23±0.95	51.55±3.82	30.39±0.708
Con	70.38±1.95	64.64±0.48	30.38±2.37
Tam	65.26±4.66	61.53±1.61	20.68±0.58
Beh	62.85±3.06	59.74±2.41	47.92±1.73
Das	81.23±4.84	51.66±0.42	29.06±1.007
Dut	85.29±3.19	49.06±1.99	34.2±1.45
Kgp	69.37±2.89	58.07±1.55	29.03±0.903

Table 6: Variation in chromium (Cr)

	SC	SF	FP
Bas	2.83 ± 0.04	2.47 ± 0.15	2.26 ± 0.16
Bag	2.86 ± 0.09	2.61 ± 0.58	1.58 ± 0.48
Con	3.48 ± 0.16	2.41 ± 0.14	1.89 ± 0.206
Tam	2.54 ± 0.06	2.23 ± 0.26	2.03 ± 0.45
Beh	2.47 ± 0.09	2.35 ± 0.26	1.9 ± 0.306
Das	2.85 ± 0.27	2.29 ± 0.48	2.07 ± 0.95
Dut	2.49 ± 0.1	2.06 ± 0.32	1.69 ± 0.202
Kgp	2.69 ± 0.12	1.48 ± 0.43	0.87 ± 0.53

Table 7: Variation in iron (Fe)

	SC	SF	FP
Bas	107.4 ± 5.22	81.63 ± 1.66	137.97 ± 12.57
Bag	135.98 ± 5.38	92.62 ± 3.67	109.66 ± 8.89
Con	108.3 ± 2.86	97.61 ± 1.78	111.42 ± 3.705
Tam	118.6 ± 5.82	106.79 ± 6.23	113.9 ± 2.97
Beh	107.33 ± 5.63	98.45 ± 8.63	112.89 ± 2.46
Das	154.15 ± 6.69	105.89 ± 8.89	108.82 ± 8.4
Dut	194.07 ± 10.4	84.48 ± 28.46	85.73 ± 12.56
Kgp	108.46 ± 5.49	101.63 ± 10.02	109.21 ± 10.22

Table 8: Variation in manganese (Mn)

	SC	SF	FP
Bas	18.22 ± 0.58	37.5 ± 0.84	65.79 ± 1.16
Bag	43.64 ± 1.27	52.85 ± 1.92	63.13 ± 2.59
Con	29.19 ± 3.01	46.09 ± 0.33	78.66 ± 3.04
Tam	34.07 ± 1.43	78.96 ± 6.29	155.61 ± 6.48
Beh	25.37 ± 1.52	59.74 ± 3.31	109.66 ± 2.28
Das	27.69 ± 2.07	40.43 ± 2.26	46.3 ± 2.22
Dut	38.04 ± 1.59	45.25 ± 1.18	45.38 ± 0.88
Kgp	28.62 ± 1.48	54.21 ± 0.97	66.83 ± 0.441

Table 9: Variation in nickel (Ni)

	SC	SF	FP
Bas	4.67 ± 0.38	6.83 ± 0.46	5.54 ± 0.85
Bag	8.77 ± 0.45	17.11 ± 0.99	11.1 ± 0.84
Con	10.57 ± 0.46	17.55 ± 0.52	6.4 ± 1.09
Tam	9.95 ± 1.51	14.99 ± 0.56	7.12 ± 0.092
Beh	6.22 ± 0.71	12.67 ± 0.75	3.13 ± 0.99
Das	8.64 ± 0.62	14.89 ± 0.81	9.83 ± 0.605
Dut	2.52 ± 0.12	2.73 ± 0.45	1.21 ± 0.64
Kgp	8.27 ± 0.61	8.47 ± 1.04	2.45 ± 0.47

Table 10: Variation in potassium (K)

	SC	SF	FP
Bas	6105.91 ± 282.74	6755.04 ± 246.41	25732.55 ± 1084.97
Bag	6713.75 ± 100.31	10256.63 ± 150.16	26797.28 ± 951.94
Con	4908.04 ± 207.15	13807.81 ± 622.42	27062.32 ± 367.32
Tam	5328.34 ± 16.67	20016.1 ± 541.64	33685.52 ± 869.5
Beh	5183.63 ± 97.32	20234.53 ± 743.8	37002.83 ± 1463.28
Das	5042.76 ± 157.33	20034.17 ± 730.7	36230.88 ± 1234.7
Dut	6006.88 ± 125.63	18976.31 ± 531.65	34047.21± 535.77
Kgp	5432.19 ± 129.06	22341.86 ± 644.07	34683.98 ± 532.99

Table 11: Variation in rubidium (Rb)

	SC	SF	FP
Bas	13.39±1.04	6.53±1.29	44.67 ±1.3
Bag	13.94±1.45	6.1±0.96	71.04 ±1.83
Con	15.15±0.42	15.83±0.55	48.65 ±2.63
Tam	12.08±1.81	7.7±1.19	18.88 ±1.26
Beh	11.75±0.97	6.61±1.53	62.24 ±1.3
Das	10.25±1.07	7.49±0.67	60.34 ±2.41
Dut	14.92±0.96	13.7±1.77	35.96 ±0.95
Kgp	12.65±0.52	9.37±2.63	76.39 ±1.44

Table 12: Variation in strontium (Sr)

	SC	SF	FP
Bas	8.23 ± 0.88	6.52 ± 0.03	9.49 ± 0.65
Bag	7.32 ± 0.68	6.98 ± 0.4	11.55 ± 0.46
Con	6.62 ± 0.13	7.65 ± 0.8	13.19 ± 0.5
Tam	7.9 ± 0.71	6.55 ± 0.06	8.63 ± 1.1
Beh	6.6 ± 0.58	5.79 ± 0.43	12.24 ± 0.4
Das	7.31 ± 0.54	7.69 ± 0.58	9.3 ± 1.32
Dut	7.17 ± 0.64	11.98 ± 0.07	21.81 ± 0.87
Kgp	6.51 ± 0.71	6.91 ± 0.12	12.29 ± 0.22

Table 13: Variation in sulfur (S)

	SC	SF	FP
Bas	34602.88 ± 295.7	36692.71 ± 909.138	17477.97 ± 525.98
Bag	32064.34 ± 198.85	39209.29 ± 1123.24	16588.14 ± 411.83
Con	34282.85 ± 892.35	36199.19 ± 1503.73	14464.94 ± 19.16
Tam	31645.43 ± 319.73	35771.97 ± 917.93	15477.14 ± 401.57
Beh	29708.06 ± 211.55	40314.75 ± 1254.77	19583.57 ± 797.34
Das	33736.26 ± 373.38	23651.04 ± 605.32	18144.41 ± 302.25
Dut	31546.86 ± 553.6	21398.07 ± 475.8	19244.52 ± 228.52
Kgp	31027.34 ± 542.76	14659.63 ± 290.9	12052.67 ± 234.84

Table 14: Variation in zinc (Zn)

	SC	SF	FP
Bas	91.39 ± 4.01	51.88 ± 1.58	33.49 ± 2.73
Bag	65.23 ± 0.95	51.55 ± 3.82	30.39 ± 0.708
Con	70.38 ± 1.95	64.64 ± 0.48	30.38 ± 2.37
Tam	65.26 ± 4.66	61.53 ± 1.61	20.68 ± 0.58
Beh	62.85 ± 3.06	59.74 ± 2.41	47.92 ± 1.73
Das	81.23 ± 4.84	51.66 ± 0.42	29.06 ± 1.007
Dut	85.29 ± 3.19	49.06 ± 1.99	34.2 ± 1.45
Kgp	69.37 ± 2.89	58.07 ± 1.55	29.03 ± 0.903

Figure 2: Graphical representation of variations in trace elemental content in different parts of fruits across the different aras- (a) Barium, (b) Calcium, (c) Copper, (d) Cromium, (e) Iron, (f) Manganese, (g) Nickel, (h) Potassium, (i) Rubidium, (j) Strontium, (k) Sulfur, (l) Zinc

Figure 2 (Cont.)

Charantin estimation: Charantin was estimated by RP-HPLC and found to be accumulated at highest level in the fruit pulp of Tamluk and lowest in Duttapukur. Variation in charantin content was observed in fruit pulp from different areas. No charantin was found in seeds. The data is given in Table 15 and the charantin peak is given in the Figures 3 and 4.The peak at 8.33 corresponds to the stigmasterol- component of charantin.

Table 15: Charantin content across different cultivation area

Area	Charantin in mg/g fresh weight
Bas	2.372333
Bag	2.293
Con	2.0462
Tam	2.147967
Beh	1.380733
Das	1.445233
Dut	2.0946
Kgp	2.057167

SQS characterizaion and expression: In order to characterize the SQS gene, the RT-PCR was carried out and a 211 bp fragment was obtained shown in the Figures 5 and 6. The fragment was sequenced and submitted in the NCBI (GenBank: MH778311.1). The McSQS was found be expressed at the highest rate in the Tamluk fruits and lowest in the Duttapukur fruits, similar to the charantin content (Figure 7).

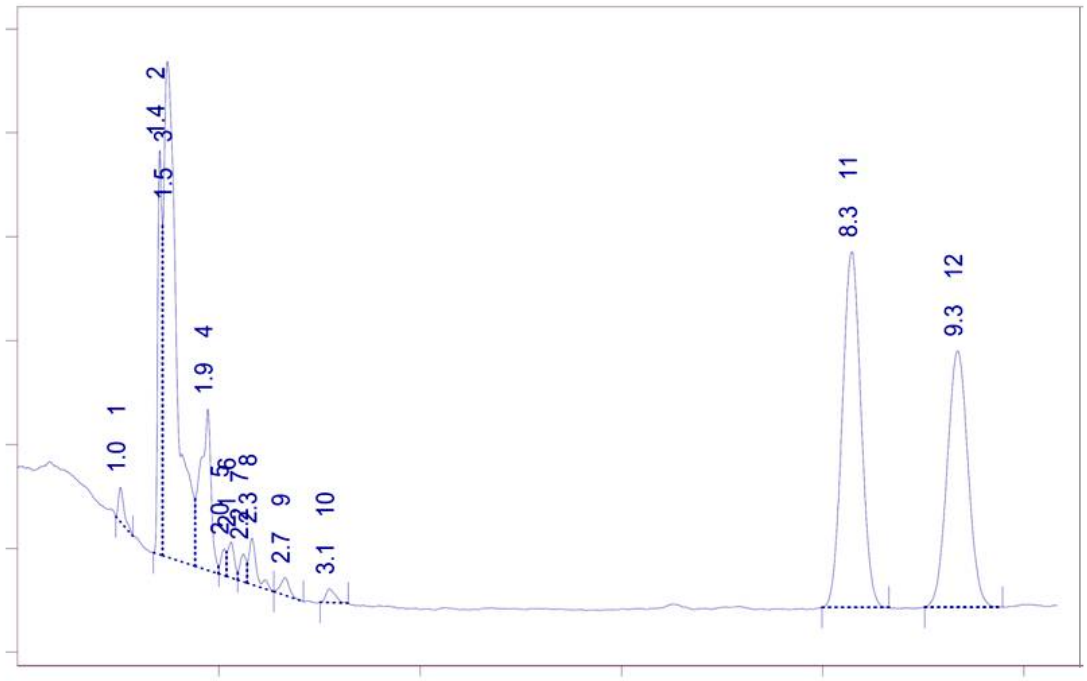

Figure 3: RP-HPLC chromatogram for charantin

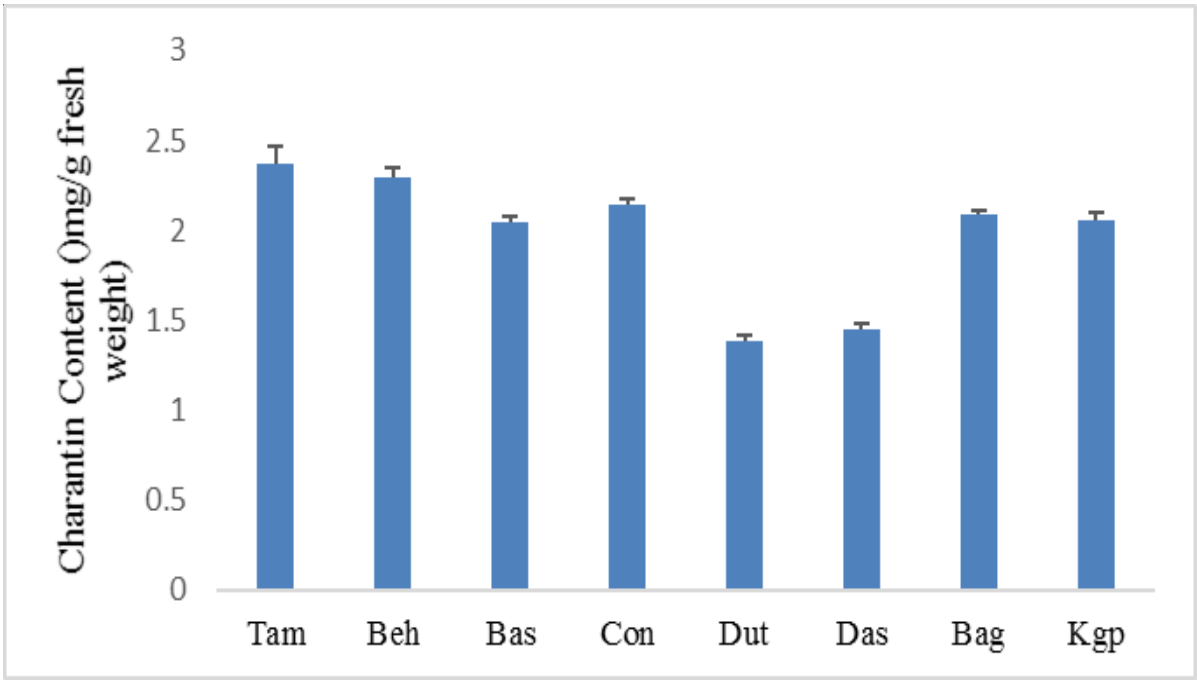

Figure 4: Charantin variation across different cultivation area

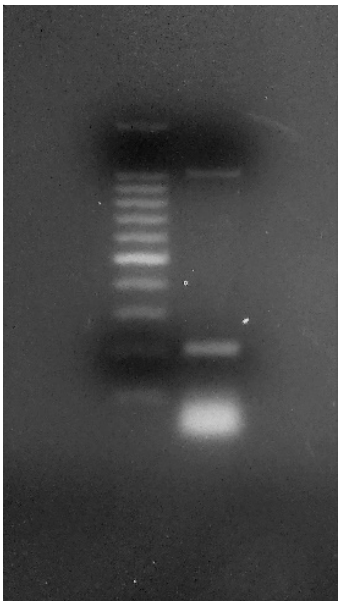

Figure 5: McSQS fragment for partial sequencing

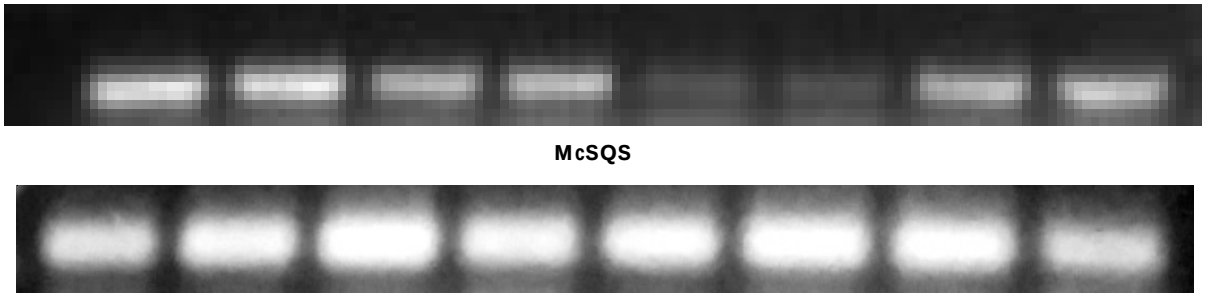

Figure 6: Agarose gel electrophoresis of (a) McSQS and (b) β-actin

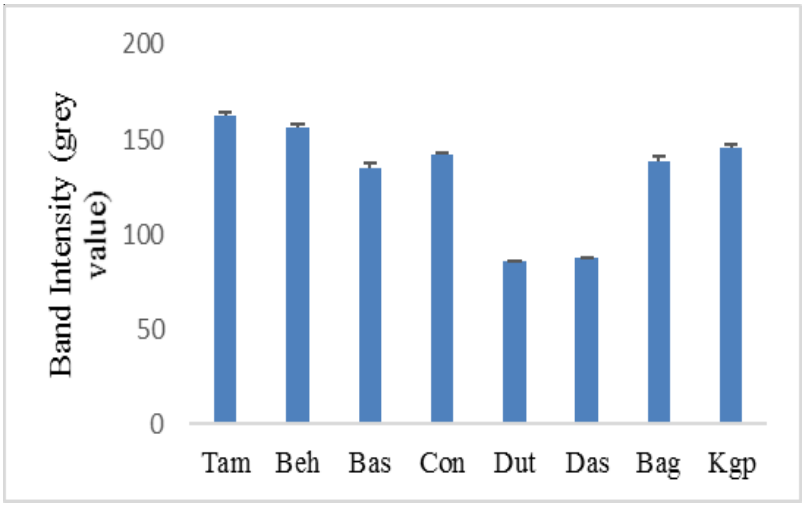

Figure 7: Expression of McSQS gene in the fruit pulp across the different areas

In Silico studies: The IUPred2A data showed that there is no disordered structure present in the McSQS sequence as the score is significantly below the threshold value 0.5 (Figure 8). The hydropathy plot found no membrane spanning region in the protein (Figure 9). Phyre2 server performed alignments and 99 alignment data where only 20 alignment results were able to be modeled by the Phyre2. Taking the parameters into account, the best result was obtained that aligned with the template fold familyterpenoid synthase, squalene synthase, superfamilyterpenoid synthase (d1ezfa). 69 (97% of the residue) have been modelled with 99.7% confidence (Figures 10 and 11). PROCHECK determined the quality of the structure as it found 0% residue in disallowed region (Figures 12a and b).

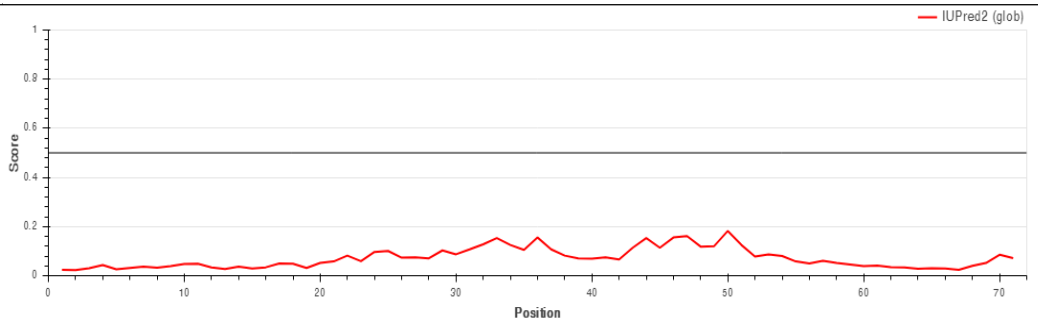

Figure 8: IUPred2A data for intrinsically disordered region of Mc Squlaene Synthase (McSQS)

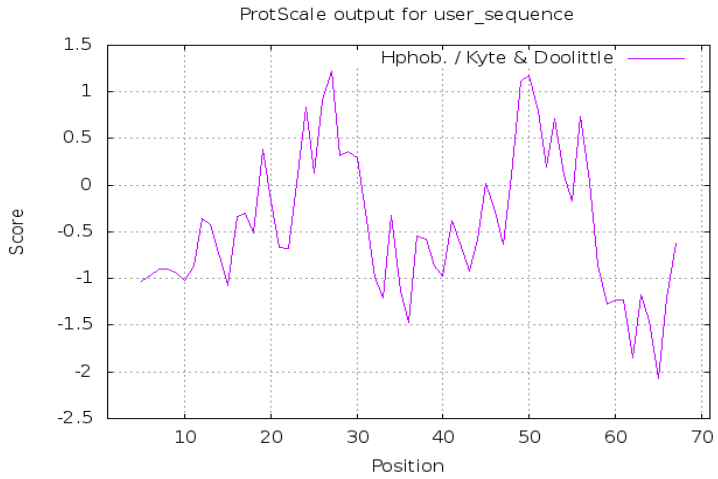

Figure 9: Hydropathy plot of Mc Squlaene Synthase (McSQS). The values above zero indicates the presence of hydrophobicity

Figure 10: Domain analysis of Mc Squlaene Synthase (McSQS) by Phyre2. The red color in the SS confidence row indicates confidence to be high (9) where purple indicates low (0). In this figure 72% was found to be in alpha helix where 0% was beta sheet

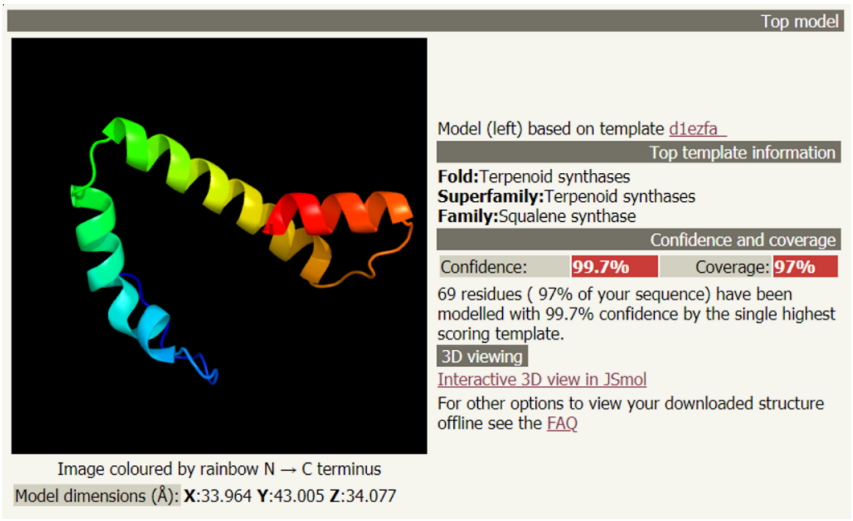

Figure 11: The 3D model of Mc Squlaene Synthase (McSQS) using Phyre2A server

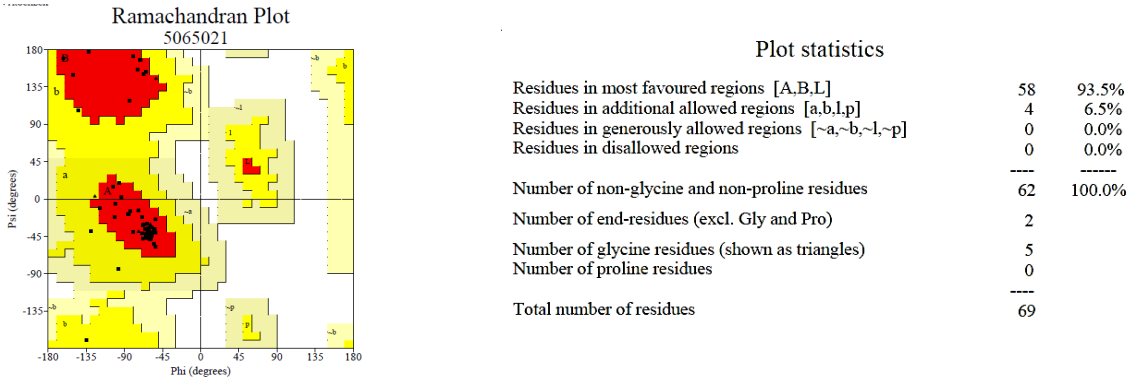

Figure 12: a: Ramachandran Plot and b: Plot Statistics of the Mc Squlaene Synthase (McSQS) using PROCHECK indicating the allowed and restricted region of the predicted structure

4. Discussion

In the present investigation, we have found significant variations among 12 different elements in the fruit parts across the different cultivation areas. Earlier studies have revelaed that in spite of being a non essential element, Ba were accumulated in plants like *Lactuca sativa* in the range of 0.13-29.2% and induced phytotoxicity (Lamb *et al.*, 2013). Calcium is an important trace element for plants and required for structural roles in the cell wall and membranes, as a counter-cation for inorganic and organic anions in the vacuole, and as an intracellular messenger in the cytosol (Clarkson *et al.*, 1993).Calcium is acquired from the soil solution by the root system and translocated to the shoot via the xylem. The Ca flux to the xylem is high, and a rate of 40 nmol Ca h^{-1} g^{-1} f. wt root is not unreasonable in an actively growing plant (White and Martin, 2003). Cucurbits such as *Trichosanthes dioica* is rich in Ca (Roy and Chakrabarti, 2003) and we also found high accumulation of Ca in all the areas, especially in fruits from Behrampore. Copper (Cu) is an essential redox-active transition metal that is involved in many physiological processes in plants because it can exist in multiple oxidation states *in vivo*. Under physiological conditions Cu exists as Cu2+ and Cu+. Cu acts as a structural element in regulatory proteins and participates in photosynthetic electron transport, mitochondrial respiration, oxidative stress responses, cell wall metabolism and hormone signaling (Clarkson *et al.*, 1993; and Raven *et al.*, 1999). As an essential metal, copper is required for adequate growth, cardiovascular integrity, lung elasticity, neovascularization, neuroendocrine function, and iron metabolism (Harris, 1997) suggesting copper to be beneficial for health. In our experiments Cu was found to be accumulated differentially in different places. Fe

is essential for plant growth and the ability of plants to respond to Fe availability ultimately affects human nutrition, both in terms of crop yield and the Fe concentration of edible tissues (Morrissey *et al.*, 2009). Iron is an essential micronutrient for almost all living organisms because of it plays critical role in metabolic processes such as DNA synthesis, respiration, and photosynthesis. Further, many metabolic pathways are activated by iron, and it is a prosthetic group constituent of many enzymes (Rout and Sunita, 2015). In our study Fe is found to variably accumulated in different area indicating differential availablity in fruits. Manganese is an essential element for normal plant growth, and most soils contain sufficient of it in an available form to supply the needs of all vegetation and Mn usually acts as an activator of enzymes and is often able to be replaced by other metal ions, involved in activating enzyme-catalysed reactions including phosphorylations, decarboxylations, reductions and hydrolysis reactions and therefore affects processes such as respiration, amino acid synthesis, lignin biosynthesis and the level of hormones in plants (Burnell, 1988). We have found a variable accumulation in Mn content across the different cultivating areas reflecting the difference in Mn content in different fruit parts as well as different areas. The element Ni is considered as an essential plant micronutrient because it acts as an activator of the enzyme urease (Polacco *et al*., 1977).Ni may have a key participation in plant antioxidant metabolism, especially in stressful situations (Fabiano *et al.*, 2015) and in our studies Ni has been accumulated differentially in fruit parts across the areas which may be induced due to the toxic elements accumulation in the plant. Potassium (K) is one of the vital elements required forplant growth and physiology. Potassium is not only a constituent of the plant structural formation butit also has a regulatory function in several biochemical processes related to protein synthesis,carbohydrate metabolism, and enzyme activation. Several physiological processes depend on K,such as stomatal regulation and photosynthesis (Mirza *et al*., 2018). *M. charantia* is rich in potassium (Upadhyay *et al*., 2015) and in our study, we have found different K content in different fruits, as well as fruit parts. The alkali metal ion Rb, despite its higher molecular weight, has several similarities to K. More than 80% of the variability in tissue concentrations of Rbwas accounted for, when K saturation and pH of rhizosphere soils were introduced (Nyholm and Tyler, 2000). Sulfur (S) is a crucial nutrient for plant growth and Sulfur not only plays structural, catalytic and regulatory functions but also acts as a major cellular redox buffer to protect the cells from oxidative stress. The supply of S to plants is essential for vegetative growth and allows production of seeds with high quality (Boldrin *et al*., 2016). We have found accumulation of S in seeds greater than fruit pulp, probably explaining the yeild of better seeds and the depostion of S is variable too. Zinc is essential for the growth in animals, human beings, and plants it is vital to the crop nutrition as required in various enzymatic reactions, metabolic processes, and oxidationreduction reactions. In addition, Zn is also essential for many enzymes which are needed for nitrogen metabolism, energy transfer and protein synthesis (Babar *et al.*, 2013). Zn is found to be varying within the different fruit parts and areas in our present study.

Charantin is a cucurbitane-type in *M. charantia* exhibiting antidiabetic property. It is a 1:1 mixture of two compounds, i.e., sitosteryl glucoside and stigmasteryl glucoside. Oral or intravenous administration of charantin has produced hypoglycemic effect (Paul *et al.*, 2010). Nivitabishekam *et al*. (2008) showed that orally administered powdered methanol extract of bitter melon containing 0.51% charantin reduces serum glucose level significantly in adult male stz-diabetic rats and dried powder of bitter melon containing 0.04%- 0.05% charantin, having a dose of 2000mg / day in a human treatment group causes significant reduction in fructosamine (Fuangchan et al, 2011). Charantin has been frequently detected and quantified using HPTLC (Ahamad *et al*., 2015) and HPLC (Tan *et al,* 2016). Charantin was found to be variable across the different cultivars of different areas. Maximum charantin content was found in Tamluk and Duttapukur showed the least charantin content. The SQS expression also showed the similar pattern which may reflect the true image of the charantin content in different *M. charantia* fruits.

In our *in silico* studies we have found data supporting the inferences regarding McSQS protein structure. IUPred2A is one of the most widely tools for the prediction of disordered regions of a protein (Erdos *et al*., 2020). The IUPred2A data in our experiment also confirms the absence of intrinsically disordered structure in our sequence which increases the quality of the predicted translated protein from the partial cDNA sequence obtained from the Sanger sequencing. Therefore we moved towards further studies. Though no membrane spanning region was found in our partial sequence of the protein, squalene synthase from Ginseng sp was most likely to be present in the plasma membrane (Ding *et al.*, 2015). Figure 9 which shows few values above zero predicting hydrophobicity may reflect the membrane bound properties of McSQS but may require further investigation as well. Kelly and Sternberg (2009) stated that Phyre server is a potent and reliable computational

tool for predicting structure of a protein. The domain analysis shows 72% to be helix in our structure(Figure 10). The 3D model structure of GgSQS1 showed that the protein motif 1 and 2 are dull of alpha helices (Aminfar *et al*., 2018). The built model by us using the Phyre2A (Figure 11) represents a predicted structure which has 97% identity with the template of the server. This entire *in silico* study is therefore likely to enhance our theoretical knowledge about the McSQS protein and induces us to explore further as far as protein research is concerned.

5. Conclusion

In this article, we tried to find the variation in deposition of different trace elements in the different parts of *M. charantia* plants and found the fruit pulps more reach in significant micronutrients. This data might influence the cultivation of bitter melon in the respective area, which is beneficial for the farmers of the state, locally as well as globally. The Charantin content variation may also be an aspect regarding this. Here we've also tried to find the micronutrient and charantin enriched zones in our state which can be a guiding force for the agricultural development in upcoming times. The data of characterization and in silico study of SQS gene has the prominent potential to encourage further studies as far as overexpression, cloning, transgenic plants are concerned. As a whole, our findings are probably one step forward towards phytoremediation to fight diabetes as well as plant science research.

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