ijlpr 2021; doi http://dx.doi.org/doi 10.22376/ijpbs/lpr.2021.11.4.L47-55

International Journal of Life science and Pharma Research

ISSN 2250-0480

Research Article

Botany

Phytochemical Study of Capsaicinoids Extracted From Indian Varieties of *Capsicum Annuum* and Quantification of Capsaicin By Chromatographic Methods

Ms Aditi khandekar[1], Ms Apeksha petare[1], Ms Yashashri kolalle[1], Ms Mayuri desai[1], Ms Sayali gaikwad[1], Ms Kajol chavan[1], Ms Nimisha vijayan[1] and Dr Kanchan chitnis[*2]

[1]*Students of Dept of Life Science, Ramnarain Ruia Autonomous College, L. N. Road, Matunga East, Mumbai, 400019*

[*2]*Corresponding Author, Assistant Professor, Dept of Life Science, Ramnarain Ruia Autonomous College, L. N. Road, Matunga East, Mumbai, 400019*

Abstract: The flavour and aroma of chillies is because of the presence of capsaicinoids. Among the capsaicinoids, 90% of the pungency is due to the presence of capsaicin (8-methyl-N-vanillyl-trans-6-nonenamide) and dihydrocapsaicin. From the perspective of the food and pharmaceutical industry, capsaicin being used as an active component, there is a need to find a quicker method of estimating capsaicin. Also it is imperative to determine and standardize a fingerprint of chilli extract of different varieties, to define a pure sample containing capsaicin by determining a marker peak for capsaicin, using HPTLC. Hence in this study capsaicin was estimated by HPLC, GC-MS and HPTLC methods. This study has successfully demonstrated a quick chromatographic separation of capsaicin by HPLC with Rt of just 2.9 min. GC-MS showed the capsaicin peak at Rt of 25.9 min. The fingerprint results of HPTLC indicated capsaicin at Rf 0.03 using Toluene: Ethyl Acetate (70:3) as mobile phase and Iodine vapours as detecting reagent. Besides, the Methanolic extracts of *Capsicum annuum* showed good antimicrobial activity with very negligible antioxidant properties. Byadgi chilli extract showed the most intense red brown colour with λmax at 500 nm and it thus justified its use as the most preferred one for contributing to colour in food. Keeping in mind the importance in the food industry, and monitoring of raw materials in the pharmaceutical industry, the methods of HPTLC and HPLC can be used to authenticate chilli powders available in the market and in pharmaceutical products for quick estimation of capsaicin.

Keywords: Capsaicin, Capsaicinoids, Capsicum, HPTLC, HPLC, GC-MS, antimicrobial, antioxidant.

***Corresponding Author**

dr kanchan chitnis , Assistant Professor, Dept of Life Science, Ramnarain Ruia Autonomous College, L. N. Road, Matunga East, Mumbai, 400019

Recieved On 01 April 2021
Revised On 12 May 2021
Accepted On 04 June 2021
Published On 05 July 2021

Funding This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

Citation Ms aditi khandekar , ms apeksha petare, ms yashashri kolalle, ms mayuri desai, ms sayali gaikwad, ms kajol chavan, ms nimisha vijayan and dr kanchan chitnis , Phytochemical Study of Capsaicinoids Extracted From Indian Varieties of Capsicum Annuum and Quantification of Capsaicin By Chromatographic Methods.(2021).Int. J. Life Sci. Pharma Res.11(4), 47-55 http://dx.doi.org/http://dx.doi.org/doi 10.22376/ijpbs/lpr.2021.11.4.L47-55

1. INTRODUCTION

Chillies belong to the family Solanaceae and genus *Capsicum* and they are used for their flavour and colour in the food industry. The flavour and aroma of chillies is because of the presence of capsaicinoids. They are a group of phenolic alkaloids. Among the capsaicinoids, 90% of the pungency is due to the presence of capsaicin (8-methyl-N-vanillyl-trans-6-nonenamide) and dihydrocapsaicin, with capsaicin making up to 71% of it. The commercial value of peppers is largely determined by their capsaicin content among many other parameters.[1] Capsaicin is also the active principle with pharmaceutical properties and has been used as a topical analgesic against arthritic pain and inflammation.[2,3] It is also protective against high cholesterol and obesity.[1] Sanatombi and Sharma[2] studied six different indigenous chilli cultivars grown in Manipur. They quantified capsaicin content by HPLC and Scoville Heat Unit (SHU). Pasupuleti *et al*[4] quantified capsaicin by GCMS and HPTLC. Cho and Kwon[5] in their study constructed a database of levels of capsaicin by HPLC in different foods consumed in Korea. According to Ryu *et al*[6], HPLC analysis of capsaicinoids is not practical for the mass analyses of samples. They suggested a colorimetric method instead by a selective chromogenic reaction with Gibbs reagent (2,6-dichloroquinone-4-chloroimide). By this method, a minimum of 1 µg/ml capsaicinoids could be detected. Moise *et al*[7] in their study extracted capsaicin from red hot pepper (*Capsicum annuum*) fruits of Romanian origin and separated it by TLC on silica gel plates using toluene-acetone-chloroform as mobile phase. Detection was carried out by exposure to iodine vapours. The spots of capsaicin were removed from the plate and the components were extracted by chloroform. The extracts were analysed by GC-MS. Antioxidant and antimicrobial activity of chilli have been studied[8,9] but the results vary greatly. Gandhi and Mashru[10] have detected adulterants in chilli powder with reference to Sudan I Dye as an adulterant. But standardization with respect to capsaicin content in chilli powder used as raw material has not been mentioned. From the perspective of the food and pharmaceutical industry, there is a need to find a method of estimating capsaicin, which will be quicker. Also to curb adulteration, it is imperative to find and standardize a fingerprint of chilli extract of different varieties and define a pure sample containing capsaicin. Thus the objective of this study was mainly to extract capsaicinoids from chilli peppers (*Capsicum annuum*) of Maharashtra origin (Lavangi Mirchi) and analyse capsaicin by High Performance Liquid Chromatography and Gas Chromatography Mass Spectrometry. Also it was an objective to determine antioxidant activity and antimicrobial activity of capsaicinoids from Lavangi Mirchi against Gram-negative and Gram-positive bacteria and fungi. Besides this, one more pertinent objective of the study was to perform fingerprinting by High Performance Thin Layer Chromatography, of few other varieties of chillies grown in different parts of India and to find a marker peak for capsaicin. The study also aimed at characterizing other varieties of chillies for their colour and their antioxidant activity.

2. MATERIALS AND METHODS

Different Indian varieties of chillies, bought from the local market, were used as samples. They were: Lavangi, Bor, Teja, Kashmiri, Pandi, Sankeshwari, Madras, Reshampatti and Byadgi.

2.1 Extraction of capsaicinoids from chilli peppers (Capsicum annuum)

Sample used was 10 g chilli pepper powder (variety Maharashtrian Lavangi Mirchi).

2.1.1 Extract 1 (Soxhlet extract)[11]

Ten grams of finely ground and sieved chilli powder was taken in a thimble of Whatman's paper, which was put in the Soxhlet apparatus. Methanol, 250 ml was taken in the round bottom flask and was used as solvent. The round bottom flask was then placed on the heating mantle. The extraction was performed for 18 hrs at a temperature of 60-65°C (methanol boiling point is 64.7°C). The apparatus was connected to a continuous supply of water. Final extract was filtered through Whatman's filter paper and was evaporated in the rotary evaporator at a temperature of 60°C.

2.1.2 Extract 2 (Cold extract)[11]

Ten grams of finely ground and sieved chilli powder was taken in a conical flask, 100ml of methanol was added to it and the sample was kept in an incubator shaker for 3 days. Then the sample was filtered through Whatman filter paper and was evaporated at room temperature. Both the extracts were stored in the refrigerator till further use.

2.2 Colour Analysis[11,12]

Finely ground and sieved chilli powder 1g of each of the varieties was taken in a conical flask, 20ml of methanol was added to it and the sample was kept in an incubator shaker for 24 hrs. Then the samples were filtered through Whatman filter paper and absorbance was recorded over wavelength range from 400nm to 700 nm.

2.3 Antimicrobial activity of capsaicinoids[8,9]

Cold extract and Soxhlet extract of Lavangi chilli were tested against *Escherichia coli*, *Klebsiella pneumonia*e, *Candida albicans* and *Staphylococcus aureus* on Nutrient Agar, Muller Hinton Agar and Sabouraud Agar using Ampicillin as control (2.5 mg/ml). Sterile swab was dipped into individual culture suspension, and the surface of the agar was swabbed with it completely. The surface was allowed to dry for about 5 minutes before placing paper discs of 5 mm diameter, dipped in different concentrations of sample extracts, on the agar. Sample extracts concentrations prepared were 50, 75, 100, 150 and 200 mg/ml using sterile water as diluent. Plates were incubated at 37° C for 24 hrs and the zone of inhibition was measured.

2.4 Antioxidant activity of Capsaicinoids[13]

0.1mM solution of DPPH was prepared in methanol. (2,2-diphenyl-1-picrylhydrazyl) [0.002 g DPPH in 50 ml methanol stored in dark].1ml of the freshly prepared DPPH was added in 3ml of each of the sample extracts (1 g chilli powder extract in 20 ml methanol). After shaking the mixture vigorously, it was allowed to stand at room temperature for 30 minutes under dark conditions. 0.05M Ascorbic acid was used as reference. The Absorbance was measured at 517nm using a UV-VIS Spectrophotometer.

DPPH Scavenging effect (% inhibition) = (A_{std} / A_{sample}) x 100

Where, A_{std} = absorbance of standard and A_{sample} = absorbance of sample

2.5 High Performance Liquid Chromatography analysis[1]

The HPLC system used was Waters 1525 Binary HPLC Pump, 2707 Autosampler, column Waters Atlantis® T3 3 µm, 4.6 x 100 mm (silica based, reversed - phase C18). Detector was 2489 Dual Wavelength UV/Vis with Empower™ 2 Software. Column temperature was 40 °C. Mobile phase A was water, mobile phase B was methanol. Injection volume was 25 µl for sample Lavangi chilli extract in methanol (100 mg/ml) and standard Capsaicin in methanol (3mg/ml)

2.6 GC-MS analysis[14]

Lavangi chilli extract in methanol (100 mg/ml) was used as a sample using splitless injection mode and 260 °C as injection temperature. The column oven temperature was maintained at 40 °C. Instrument used was Shimadzu QP 2010 Ultra.

2.7 High Performance Thin Layer Chromatography analysis

Standard capsaicin of concentrations 50, 100, 200, 300, 400 and 500 ppm was prepared in methanol and 10 µl of each was spotted on HPTLC plate (Merck silica gel HPTLC plate 60 F 254). Absorbance in the form of Area Under the Curve was obtained for each standard spotted, which was proportional to concentration of capsaicin. Hence a standard curve using area under the curve was obtained. Overnight extracts of varieties of chilli (1g of sample in 20ml Methanol shaken for 24 hrs in incubator shaker and then filtered) were spotted on plate. Mobile phase used was Toluene: Ethyl Acetate (70:3).[11] For detection, Iodine vapours were used. Brown coloured bands of capsaicin were observed. The plate was analysed by using CAMAG TLC Scanner with winCATS software.

3. STATISTICAL ANALYSIS

Mean and Standard Deviation of readings in triplicates for zone of inhibition were calculated and presented.

4. RESULTS

4.1 Extraction of capsaicinoids from Lavangi chilli peppers

The total yield after Soxhlet extraction of 10 g of Lavangi chilli powder in 250 ml of methanol used as solvent, followed by evaporation done by Rotary Evaporator was found out to be 3.2989 g. The total yield after cold extraction of 10 g of Lavangi chilli powder in 100ml of methanol used as solvent, followed by evaporation done at room temperature was found out to be 1.8651g.

4.2 Colour analysis

Byadgi chilli extract showed the most intense red brown colour with λmax at 500 nm and it thus justified its use as the most preferred one for contributing to colour in food.

4.3 Antimicrobial activity of Capsaicinoids

Table 1: Soxhlet extracts of Lavangi chilli at different concentration and corresponding zone of inhibitions in mm

Soxhlet extracts	*Escherichia coli*		*Klebsiella pneumoniae*		*Staphylococcus aureus*		*Candida albicans*	
Sample extract conc mg/mL	NA	MH	NA	MH	NA	MH	Sabouraud Agar	MH
200	-	6.16+0.29	10.5+0.5	6+0.5	8+0.5	18.16+0.29	7.67+0.58	8+1
150	7.83+1.04	6.5+0.5	7+1	-	10.33+0.57	11.5+0.5	-	6.33+0.57
100	8.83+0.76	6.67+0.58	7.5+0.5	6.5+0.5	8.33+0.76	8.67+0.58	7+0.5	5.67+0.58
75	-	-	7.67+0.58	6.5+0.5	8.16+0.76	10.5+0.5	6.33+0.58	6.5+0.87
50	-	8.33+0.58	7.83+0.29	6.16+1.25	9+0.86	8+1	-	7.83+0.29

Mean and Std Dev readings are given; n=3 (NA= Nutrient Agar, MH=Mueller Hinton Agar)

Table 2: *Cold extracts of Lavangi chilli at different concentration and corresponding Zone of inhibitions in mm*

Cold extract	*Escherichia coli*		*Klebsiella pneumoniae*		*Staphylococcus aureus*		*Candida albicans*	
Sample extract conc mg/ml	NA	MH	NA	MH	NA	MH	Sabouraud Agar	MH
200	12±1	5.67±0.47	10±0.82	7.67±0.47	15±1.63	6.83±0.23	10.5±0.41	6.16±1.02
150	-	6±0.5	7.66±1.52	-	12.16±0.76	-	7.66±0.58	-
100	-	5.33±0.57	-	7±1	11.16±1.75	7±1	5.83±0.29	5.5±0.5
75	8±0.5	6.5±0.5	8±1	6.33±0.57	-	6.16±0.76	-	6.5±0.5
50	7.83±0.28	5.16±0.28	8±0.5	8.33±0.57	-	10.16±0.28	-	6.33±0.84

Mean and Std Dev readings are given ; n=3 (NA= Nutrient Agar, MH=Mueller Hinton Agar)

According to Tables 1 and 2 and Figures 1, 2, 3, and 4, the cold chilli extract at 200mg/ml displayed antibacterial activity against *Escherichia coli and Klebsiella pneumoniae* in Nutrient Agar. It did not show activity at lower concentrations. Soxhlet extraction failed to show any appreciable activity against *E coli* but at 200mg/ml displayed antibacterial activity against *Klebsiella pneumoniae* in Nutrient Agar. The cold and Soxhlet extracts, both showed maximum antibacterial activity against *Staphylococcus aureus* in Nutrient Agar as well as Mueller Hinton Agar. Highest zone of inhibition of 18 mm diameter indicated prominent antibacterial activity against *Staphylococcus aureus.* The cold and Soxhlet chilli extracts showed minimum antifungal activity against *Candida albicans.*

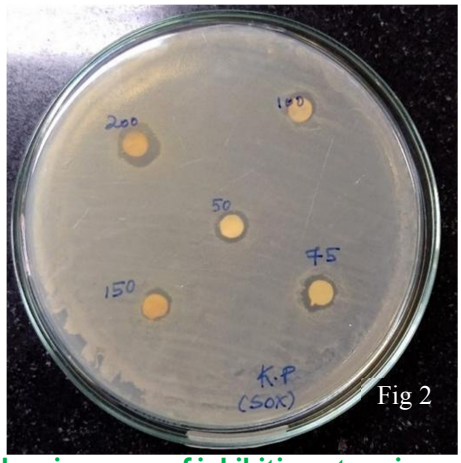


Fig 1: NA plate showing zone of inhibition at various concentrations of chilli extract (Soxhlet) against Staphylococcus aureus.

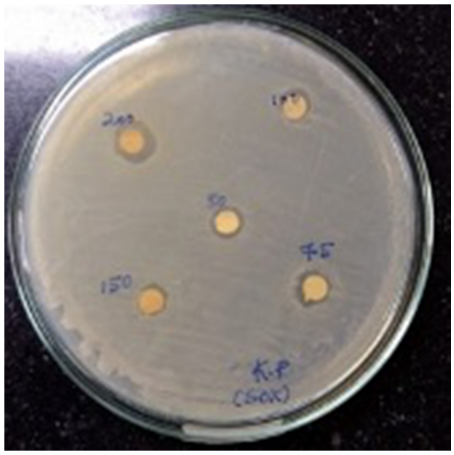


Fig 2: NA plate showing zone of inhibition at various concentrations of lavangi chilli extract (Soxhlet) against Klebsiella pneumoniae.

Fig 3: NA plate showing zone of inhibition at various concentrations of chilli extract (cold) against Staphylococcus aureus.

Fig 4: MH plate showing zone of inhibition at various concentrations of chilli extract (Soxhlet) against Staphylococcus aureus.

4.4 Antioxidant activity of Capsaicinoids

Table 3: *Antioxidant activity of chillies*

Sr.no	Samples	Absorbance at 517nm	Scavenging activity in %
1	Ascorbic Acid(standard)	0.03	100.00
2	Bor	0.88	3.40
3	Teja	0.62	4.84
4	Pandi	0.6	5.00
5	Sankeshwari	0.93	3.22
6	Madras	0.73	4.11
7	Reshampatti	0.82	3.66
8	Kashmiri	1.02	2.94
9	Byadgi	1.05	2.86
10	Lavangi	0.64	4.68

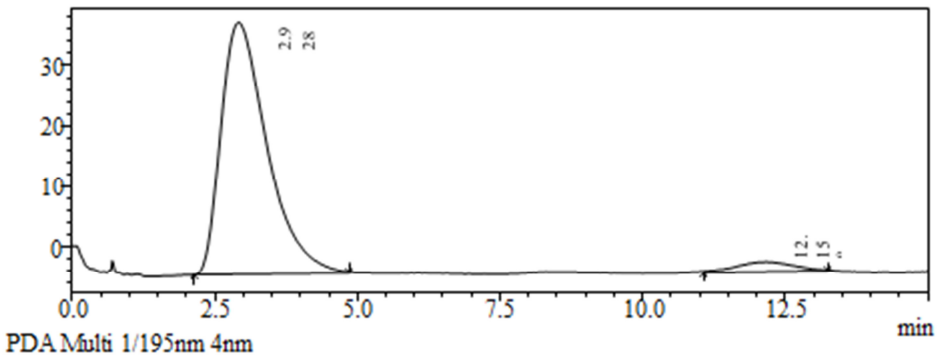
According to Table 3, the antioxidant property was found to be highest in the Pandi variety of chilli, followed by Teja and Lavangi. Lowest was found to be in Byadgi. Overall these extracts demonstrated a very low antioxidant activity relative to the reference standard of ascorbic acid.

4.5 High Performance Liquid Chromatography analysis of chilli extract

Lavangi cold extract sample: 100mg/ml. (PDA 195nm 4nm)

Table 4: *Peak height and area under curve*

Peak	Rt (min)	Area	Height	Area %	Height %
1	2.928	2318822	41704	96.010	96.495
2	12.158	96357	1515	3.990	3.505
Total	-	2415179	43219	100.000	100.000



Standard Capsaicin: 3mg/ml (PDA 195nm 4nm)

Fig 5: Chromatographic peak of sample

Table 5: Peak height and area under curve					
Peak	Rt (min)	Area	Height	Area %	Height %
1	2.876	1362013	24488	100.000	100.000

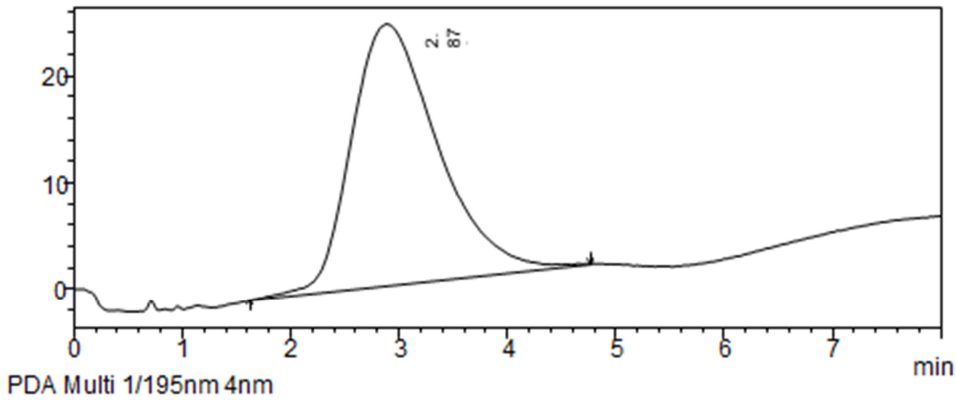


Fig 6: Chromatographic peak of standard

After calculating from AUC (Table 4 and 5, Figure 5 and 6), the sample had 5.11 mg/ml of capsaicin per 100 mg/ml extract. This means 10gm of Lavangi chilli, which gave 3.2989 g extract containing 168.574 mg/ml. ie.1685.73 mg/ml per 100 g material= 1.685%.

4.6 GC-MS analysis of chilli extract

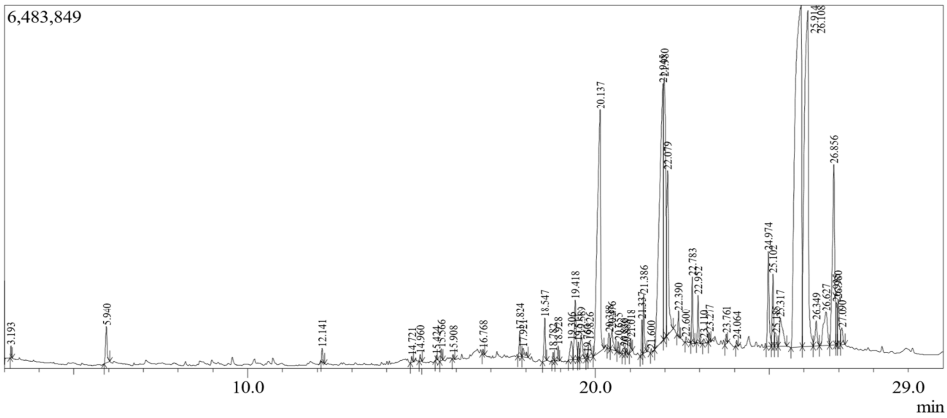


Fig 7: Chromatogram of sample: 100mg/ml

The total number of compounds detected in GC-MS analysis were 53 (Fig7). Amongst these 53 compounds, following 7 compounds had the highest peak as well as area percentage.

Table 6: Seven major compounds detected in Lavangi chilli cold extract by GC-MS				
No.	R.T	Area	Area %	Name
1	20.137	27622282	9.34 %	n-hexadecanoic acid
2	21.945	36624311	12.348%	9,12-Octadecadienoic acid (Z, Z)-
3	21.980	18161146	6.14%	9-Octadecenoic acid (Z)-, hexadecyl ester
4	22.079	11677962	3.95%	n-Octyl-acetamide
5	25.914	80017116	27.06%	Capsaicin
6	26.108	47864997	16.18%	Dihydrocapsaicin
7	26.856	15608768	5.28%	1-Propyl 9,12-octadecadienoate

Capsaicin (Rt 25.9) and dihydrocapsaicin (Rt 26.1) (Table 6) which are the major capsaicinoids, responsible for the pungency of chilli peppers were present in the sample and they occupied the highest area percentage in the chromatogram corresponding to the concentration of these capsaicinoids as compared to other compounds present in the sample.

3.7. HPTLC analysis

Table 7: Rf and Area under curve for standard capsaicin			
No	Std Capsaicin conc ppm	Rf	AUC
1	50	0.05	732.3
2	100	0.05	1783.2
3	200	0.03	1603.4
4	300	0.05	2952.4
5	400	0.05	4146.8
6	500	0.05	4698.8

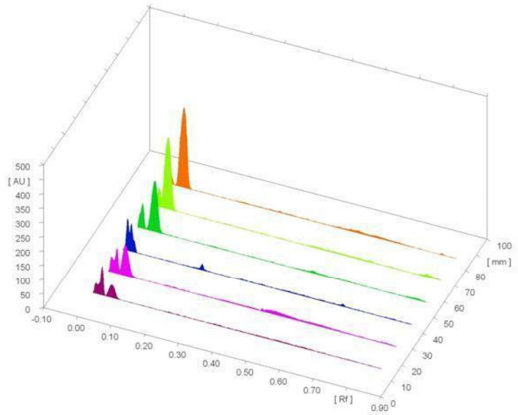


Fig 8: Peak heights of standard capsaicin in 3D

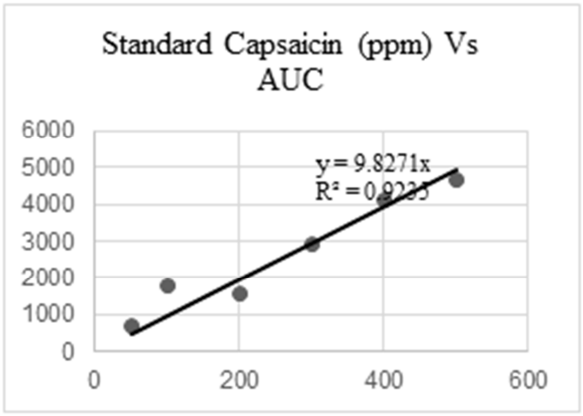


Fig 9: Standard capsaicin concentration Vs AUC

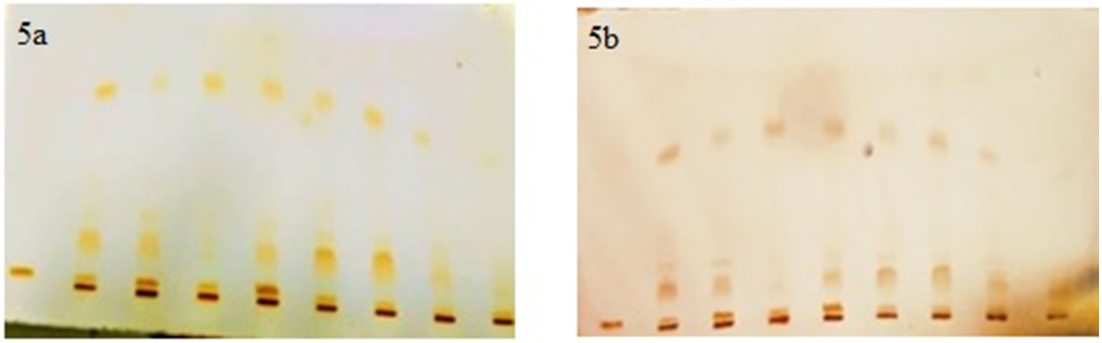


Fig 10: HPTLC fingerprint of varieties of chillies (5a) without developing with Iodine solution (5b) after developing with Iodine solution

Table 8: Rf and Area under curve for capsaicin from varieties of chillies				
No	Sample of chilli	Rf	AUC	Concentration of capsaicin calculated from std equation (ppm)
	Std 300 ppm	0.03	2952.4	300.43
1	Bor	0.03	552.6	56.23
2	Teja	0.03	1699.6	172.95
3	Pandi	0.04	530.5	53.93
4	Sankeshwari	0.04	2657.4	270.42
5	Madras	0.03	288.4	29.35
6	Reshampatti	0.03	122.3	12.46
7	Kashmiri	--	--	--
8	Byadgi	0.03	111.2	11.32

The fingerprint results of HPTLC (Tables 7 and 8, Figures 8, 9 and 10) can be used to authenticate the chilli powders and extracts used in the food and pharmaceutical industry. Rf 0.03 can be used as marker peak for capsaicin with the conditions given in this study. Table 8 also showed that Sankeshwari had a higher amount of capsaicin amongst other varieties of chillies.

5. DISCUSSION

Our results consolidated the findings of Liljana *et.al*[12] that the Soxhlet extraction gives better yield. Byadgi chilli extract showed the most intense red brown colour with λmax at 500nm and it thus justified its use as the most preferred one for contributing to colour in food. Due to the good optical properties obtained for this oleoresin, it could have a high commercial value in order to be used as a colouring and flavouring agent in various products. Sankeshwari chilli has a higher amount of capsaicin and hence it justified its use for pungency in food preparations along with Byadgi which is used for colour. The antimicrobial results obtained in this study are similar to Riquelme and Matiacevich[8] and Molina-Torres *et al.*[9] The cold and Soxhlet extracts, both showed maximum antibacterial activity against *Staphylococcus aureus* in Nutrient Agar as well as Mueller Hinton Agar. According to Nasser *et* al[15] for antimicrobial assay MHA should be used and not NA as NA gives multiple errors and MHA is best suited for such studies. Present study could not confirm this as the results obtained were not conclusive. The values obtained by DPPH method could not be compared with other reported results, because different methods have been used, and also there are different ways of expressing the results.[16] Oleoresins contain compounds showing antioxidant activities due to their redox properties that allow them to act as reducing agents and metal chelators.[17] Maksimova *et al*[18] reported that the antioxidant activity of ethanolic oleoresins from hot peppers depends on the concentration of capsaicin. Results in this study could not confirm that. The retention times in HPLC in this study match with those given by Barbero *et al*.[19] They developed a method using 0.1% acetic acid in water and 0.1% acetic acid in methanol which helped in the separation of capsaicinoids in less than 3 min. Perkin Elmer lab has also reported retention times as 2.3 and 2.85 for capsaicin and dihydrocapsaicin respectively using water and methanol as solvent A and B.[1] Whereas, Kajiya *et al*[20] reported Rt of 9.1 min for capsaicin and 12.5 min for dihydrocapsaicin, using 1% acetic acid in water (A) and acetonitrile (B) as mobile phases. The results in this study showed much faster separation and lower Rt of 2.9 min for capsaicin. The method of GC-MS presented in this study can be used to separate capsaicin at Rt 25.9 and dihydrocapsaicin at Rt 26.1. A simple and efficient method for extraction and quantification of capsaicin using high performance thin layer chromatographic (HPTLC) was developed and validated by Das *et al*[21] using Chloroform: Methanol: Acetic acid: Hexane (2.85: 0.15: 0.15: 1, v/v/v) as mobile phase and densitometric analysis in absorbance mode at 282 nm. They obtained peaks of capsaicin at Rf value of 0.78. The fingerprint results of HPTLC in this study indicated capsaicin at Rf 0.03 using Toluene: Ethyl Acetate (70:3) as mobile phase and Iodine vapours as detecting reagent. Besides the detection of adulterants in the chilli powder by methods already mentioned in the literature[10], this HPTLC method can be used to detect and estimate amount of capsaicin in the chilli powder used as raw material, as a means to standardize and authenticate it to be used in the food preparations.

6. CONCLUSION

A powdered mixture of Sankeshwari and Byadgi variety of chillies for pungency and color respectively are already known to be used in food preparations. Present study justified the same, from colour analysis and HPTLC analysis of capsaicin in the chilli varieties. The Methanolic extracts of *Capsicum annuum* showed good antimicrobial but negligible antioxidant properties. This study has successfully demonstrated a quick chromatographic separation of capsaicin by HPLC with Rt of just 2.9 min. GC-MS showed the capsaicin peak at Rt of 25.9 min. The fingerprint results of HPTLC indicated capsaicin at Rf 0.03 using Toluene: Ethyl Acetate (70:3) as mobile phase and Iodine vapours as detecting reagent. Keeping in mind the importance in the food industry, and monitoring of raw materials in the pharmaceutical industry, the methods of HPTLC and HPLC can be used to authenticate chilli powders available in the market and for quick estimation of capsaicin respectively.

7. ACKNOWLEDGEMENTS

The authors acknowledge P.S. Ramanathan Laboratory and Herbal Research Laboratory of Ramnarain Ruia Autonomous College, for the assistance in HPTLC, HPLC and GC-MS analyses.

8. AUTHORS CONTRIBUTION STATEMENT

Ms Aditi, Ms Apeksha, Ms Yashashri, Ms Mayuri, Ms Sayali, Ms Kajol, Ms Nimisha were all involved in the collection of sample, analyses, data compilation, preparation of graphs, under the guidance and inputs from Dr Kanchan Chitnis. Dr Kanchan Chitnis designed the manuscript and the final manuscript was prepared by incorporating inputs from all the authors.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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