

Research Article



Finger Print Analysis of the Beta-Sito Sterol from Ethanolic Extract of *Benincasa hispida* Fruit by HPTLC Analysis

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ABSTRACT

The present study is deliberate on the isolation and purification of ethanolic extract of *Benincasa hispida* fruit by column chromatography and the presence of β -sitosterol in active fraction IV is to be analysed by HPTLC method. Ethanolic extract of *Benincasa hispida* fruit extract was subjected to column chromatography followed by TLC analysis. Similar fractions with the same R_f values were pooled. The pooled fractions were subjected to quantification of phenols, flavonoids, terpenoids and tannins. Fraction containing high amount of these secondary metabolites were subjected to HPTLC analysis using β -sitosterol, as a marker. The column chromatography analysis resulted in 84 fractions. When the fractions were subjected to TLC analysis R_f value were obtained for each fractions. According to the R_f value fractions were pooled into five fractions. Ethanolic extract of *Benincasa hispida* fraction IV showed the presence of different bands with different R_f values ranging from 0.04 to 0.83. Secondary metabolites in all fractions were determined. Total Phenols found in all fractions were range from 124.16 ± 4.07 to 137.7 ± 3.89 , total flavonoids range from 16.85 ± 0.75 to 37.95 ± 1.00 . Total tannins range from 6.3 ± 0.35 to 37.31 ± 0.36 and total terpenoids range from 4.25 ± 0.32 to 16.38 ± 0.30 . β -sitosterol was identified in fraction IV of ethanolic extract of *Benincasa hispida* fraction has many medicinal properties. β -sitosterol is type of phytosterol. So it can be used for the formulation of drugs for treatment of diseases like inflammatory, Hypercholesterolemia and cancer.

Keywords: *Benincasa hispida*, β -Sito sterol, column chromatography.

INTRODUCTION

B*enincasa hispida* belongs to cucurbitaceae family. These families have about 130 general and about 800 species. The plants of this family are collectively known as cucurbits.¹ Many ayurvedic researchers have turned their attention towards this family because this fruits of this family eaten by human in the form of salads or dessert or pickle. *Benincasa hispida* fruit was commonly recommended for management of peptic ulcer, hemorrhage from internal organs, asthma, cough, diabetes, epilepsy and other nervous disorders. It is an important ingredient of kushmanda lehyam, an Ayurvedic medicine widely used as rejuvenative agent (Rasayana) in treatment of epilepsy and other nervous disorders.²

In Ayurvedic system of medicine, *Benincasa hispida* fruit was used for treatment of schizophrenia and other psychologic disorders.³ The methanolic extract of fruit has been evaluated for it's anti-diarrheal potential against several experimental models of diarrhea induced rats.⁴ The methanolic extract of the fruit was reported to possess antiulcer, antihistaminic and antidepressant activities.⁵⁻⁶ Phytochemical review indicate the presence of triterpenes: alnusenol, multiflorenol, iso multiflorenol, flavones, isovitexin and sterols: Lupeol, lupeol acetate and beta-sitosterol.⁷

The preliminary phytochemical analysis and quantitative secondary metabolites was carried out on different extracts of *Benincasa hispida*.⁸ It was found that ethanolic

extract of *Benincasa hispida* has higher concentration of secondary metabolites. Thus ethanolic extract was taken for further studies.

The present study was focused to fractionate the ethanolic extract of *Benincasa hispida* by column chromatography and TLC method and fractions were subjected to quantitative estimation of phenols, flavonoids, tannins and terpenoids.

High concentration of secondary metabolites containing fraction was subjected to HPTLC analysis using Beta-sitosterol as standard. The secondary metabolites present in the active fractions have lots of pharmacological effects.

MATERIALS AND METHODS

Sample Preparation

Plant specimen for the proposed study was collected from medicinal plant vendor. Care was taken to select healthy fruits.

The fruit was authenticated by Dr. P. Jayaraman, Director of National Institute of Herbal science, Plant Anatomy Research Center, Chennai. The fruit specimen was used for the studies.

The *Benincasa hispida* fruit (pumpkin) skin was peeled off and seeds were removed. The fruit plup were taken and cut into pieces, dried and then ground to powder. The dried powder was extracted with ethanol using soxhlet apparatus. Then the ethanolic extract of *Benincasa*



hispid fruit was subjected into the column chromatography.

Silica Gel Column Chromatography

The ethanolic extract was subjected to column chromatography using different solvent systems. (n - Hexane to ethanol) Silica gel (100-200 mesh) was used as a stationary phase. Column chromatography was done by using a glass column.

The dimension of the column was 46×2cm. The column was packed with silica gel by wet packing method where in a padding of cotton was placed at the bottom of the column. Slurry was made with the required amount of stationary phase with silica gel (100-200 mesh) in the solvent of lowest polarity (n-hexane) and poured into the column to form a bed of silica.

The extract was made into mixture (1:3 ratio) then poured onto the top of silica gel column, a layer of cotton was covered again and allowed to percolate. The column was then eluted gradiently with solvents of increasing polarity. The fractions were collected and concentrated using rotary evaporator. All the concentrated fractions were subjected to Thin Layer Chromatography (TLC).

Separation of Compounds by TLC using different Solvent System

All fractions were tested by TLC analysis. The TLC pre-coated silica gel (Merk-60 F254, 0.25mm thick) plate were developed using a CAMAG twin trough glass tank which was pre-saturated with the mobile phase for 1 hour and each plate was developed to a height of about 10cm. The composition of mobile phase was optimized by using different mobile solvents of varying polarity.

After development, plates were removed and dried and spots were visualized using 0.5% (w/v) vanillin solution prepared in 4% (w/v) HCl.⁹ Compounds were detected as violet color spots. Then the plates were visualized at visible light, UV-254 nm and 365 nm.¹⁰ Fractions with the similar R_f values were pooled and the organic solvent was removed by rotary evaporator. The fractions were then used for further analysis.

Estimation of Total Phenols

Total phenolic content was estimated using the Folin-Ciocalteu method of Yu.¹¹ Fractions (100µL) was mixed thoroughly with 2 ml of 2% Na₂CO₃. After 2 minutes 100µl of Folin-Ciocalteu reagent was added to the mixture. The resulting mixture was allowed to stand at room temperature for 30 min and the absorbance was measured at 743 nm against the blank.

A calibration curve was established using various concentration of gallic acid. The values were expressed in mg/g of sample.

Estimation of Total Flavonoids

The determination of flavonoids was performed according to the colorimetric assay of Chang.¹² To 1ml of

fractions, 3 ml of methanol, 0.2ml of 1 M potassium acetate, 0.2ml of 10% aluminium chloride and 5.6ml of distilled water was added and left at room temperature for 30 minutes. Absorbance of the mixture was read at 415 nm using UV spectrophotometer. Calibration curve was prepared using quercetin, as standard.

Estimation of Tannins

The estimation of tannin was determined by the method of Peri and Pompei¹³. 1ml of sample fractions was taken. The volume was made upto 1ml with distilled water and 1ml of water serves as the blank.

To this 0.5ml of folin phenol reagent (1:2) followed by the 5ml of 35% sodium carbonate was added and kept at room temperature for 5 minutes. Blue color was formed was read at 640nm. A standard graph (gallic acid-1mg/ml) was plotted, from which the tannin content of the fractions was determined. The total tannin content was expressed in mg/g of extract.

Estimation of Terpenoids

Terpenoids was estimated according to the method of Ing-Luen.¹⁴ 0.2ml of fraction was evaporated by keeping it in boiling water bath and to the residue 0.3ml of vanillin/glacial acetic acid (W/V), 1ml of perchloric acid was added and incubated at 60 °C for 45min.

Tubes were cooled in ice and to the mixture 5ml of glacial acetic acid was added and the color intensity was absorbed at 548nm using spectrophotometer. Vanillin/glacial acetic acid, perchloric acid and solvent alone served as blank. Ursolic acid was used as a standard and expressed as mg/g extract.

HPTLC Analysis

HPTLC studies were carried out following Harborne and Wagner.¹⁵ CAMAG HPTLC system equipped with Linomat 5 applicator, CAMAG scanner was used. 100 µl of the fraction was evaporated to dryness and residues were extracted with 3 ml chloroform.

1 mg of the marker compound β-sitosterol was dissolved in 3 ml of chloroform separately. 5 and 10 µl of test extract fraction and 5 µl of the marker were applied on aluminium plate pre-coated with silica gel 60 F₂₅₄ of 0.2 mm thickness using CAMAG Linomat 5 applicator. The plate was developed in developing chamber using Toluene: Ethyl acetate: Formic acid (9.0: 1.0: 0.1) as mobile phase.

The developed plates were visualized in visualizing chamber and scanned using CAMAG scanner 4 at uv 254, 366 and 620nm.

The plate was dipped in Vanillin-Sulphuric acid, and heated at 105 °C till the spots appeared. R_f values and densitometric scan were recorded. Post-derivatisation and a spectral scan were performed between 500 to 700 nm to obtain superimposable spectra of the β-sitosterol marker and the same in test extract.



RESULTS AND DISCUSSION

Column Chromatography

By silica gel chromatography column totally 84 fractions were obtained and was subjected to TLC analysis. Based on the R_f values fractions were pooled into five fractions.

Fractions were dried using rotary evaporator. Yield of the ethanolic extract of *Benincasa hispida* fruit fractions was shown in Table 1.

The yield of different fractions of ethanolic extract of *Benincasa hispida* fruit was with a descending order ethanol>ethylacetate>n-hexane.

Total Phenols, Flavonoids, Tannins and Terpenoid Contents

Table 1 summarizes the results of total Phenols, flavonoids, tannins and terpenoid contents in ethanolic different fraction of *Benincasa hispida* fruit. The phenolic content of the different fractions of *Benincasa hispida* was expressed as mg/g of gallic acid equivalents. Total phenols found in all the fractions were range from 124.16 ± 4.07 to 137.7 ± 3.89 mg/g. The flavonoids present in all the fractions were range from 16.85 ± 0.75 to 37.95 ± 1.00 mg/g. The tannin content was range from 6.3 ± 0.35 to 37.31 ± 0.36 mg/g. Terpenoids concentration in all the fractions was range from 4.25 ± 0.32 to 16.38 ± 0.30 mg/g.

Table 1: Total Phenol, Flavonoid, Tannin and Terpenoid contents in fractions of Ethanolic extract of *Benincasa hispida* fruit

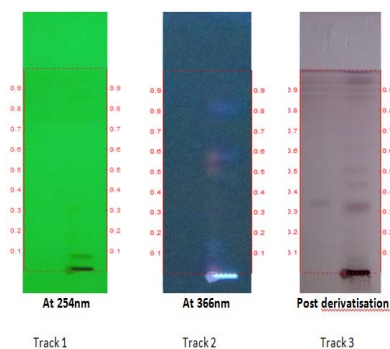
Contents	Fraction I Hex:EA (50:50)	Fraction II EA (100)	Fraction III EA:EOH (50:50)	Fraction IV EOH (100)	Fraction V EOH:EOH (50:50)
Percentage of Yield (%)	1.96	4.54	4.0	9.09	7.30
Phenol (mg/g)	124.16 ± 4.07	127.56 ± 3.52	132.7 ± 4.29	137.7 ± 3.89	134.93 ± 4.51
Flavonoid (mg/g)	16.85 ± 0.75	18.58 ± 0.27	27.2 ± 1.4	37.95 ± 1.00	28.41 ± 1.24
Terpenoids (mg/g)	4.25 ± 0.32	9.48 ± 0.27	4.30 ± 0.32	16.38 ± 0.30	12.5 ± 0.27
Tannin (mg/g)	6.3 ± 0.35	31.76 ± 1.26	19.31 ± 0.24	37.31 ± 0.36	28.31 ± 0.26

Values are expressed as Mean \pm SD (n=6); Legends: Hex-Hexane, EA-Ethyl acetate, EOH-Ethanol.

Table 2: HPTLC shows max R_f and area occupied by fraction IV of *Benincasa hispida* at 254nm, 366nm and 620nm

Peak	Start Position	Start Height	Max Position	Max Height	Max%	End Position	End Height	Area	Area %
HPTLC shows max R_f and area occupied by fraction IV of <i>Benincasa hispida</i> at 254nm (10 μl plant extract fraction)									
1	0.02 R_f	60.3AU	0.04 R_f	375.6AU	50.78%	0.08 R_f	0.2AU	6876.2 AU	48.15%
2	0.08 R_f	0.4AU	0.11 R_f	212.7 AU	28.76%	0.14 R_f	0.4AU	3520.9 AU	24.66%
3	0.16 R_f	2.4AU	0.21 R_f	18.4 AU	2.48%	0.22 R_f	17.9AU	489.0 AU	3.42%
4	0.22 R_f	17.6AU	0.24 R_f	21.3 AU	2.88%	0.27 R_f	0.1 AU	428.4 AU	3.00%
5	0.28 R_f	0.2AU	0.31 R_f	11.2 AU	1.51%	0.33 R_f	4.7 AU	259.0 AU	1.81%
6	0.34 R_f	4.9AU	0.38 R_f	37.7 AU	5.10%	0.41 R_f	8.6 AU	997.7 AU	6.99%
7	0.42 R_f	8.3AU	0.43 R_f	12.0 AU	1.62%	0.46 R_f	1.9 AU	202.1 AU	1.42%
8	0.85 R_f	0.3AU	0.89 R_f	50.8 AU	6.87%	0.96 R_f	1.2 AU	1506.7 AU	10.55%
HPTLC shows max R_f and area occupied by fraction IV of <i>Benincasa hispida</i> at 366nm (10 μl plant extract fraction)									
1	0.02 R_f	93.1AU	0.04 R_f	333.6AU	78.23%	0.11 R_f	0.0AU	6856.7AU	68.94%
2	0.16 R_f	2.4AU	0.20 R_f	40.4 AU	9.47%	0.26 R_f	5.1AU	1609.0AU	16.18%
3	0.60 R_f	4.2AU	0.62 R_f	14.2AU	3.34%	0.65 R_f	5.7AU	332.7 AU	3.35%
4	0.65 R_f	5.8AU	0.69 R_f	38.2 AU	8.96%	0.77 R_f	1.4 AU	1147.3 AU	11.54%
HPTLC shows max R_f and area occupied by fraction IV of <i>Benincasa hispida</i> at 620nm (plant extract fraction 10μl)									
1	0.02 R_f	23.2AU	0.04 R_f	290.0AU	53.50%	0.07 R_f	0.1AU	4777.2 AU	38.03%
2	0.09 R_f	1.2AU	0.11 R_f	47.5AU	8.76%	0.14 R_f	0.5AU	664.9 AU	5.30%
3	0.32 R_f	3.0AU	0.41 R_f	125.2AU	23.11%	0.47 R_f	0.2AU	4979.1 AU	39.68%
4	0.48 R_f	0.2AU	0.53 R_f	48.9 AU	9.02%	0.57 R_f	4.3 AU	1323.9 AU	10.55%
5	0.58 R_f	4.4AU	0.61 R_f	20.2 AU	3.73%	0.66 R_f	2.2 AU	548.8 AU	4.37%
6	0.78 R_f	0.0AU	0.83 R_f	10.2 AU	1.88%	0.85 R_f	7.5 AU	259.4 AU	2.07%
HPTLC shows max R_f and area occupied by fraction IV of <i>Benincasa hispida</i> at 620nm (β-sitosterol standard(5μl))									
1	0.36 R_f	5.9AU	0.42 R_f	40.4AU	100.0%	0.47 R_f	5.4AU	1317.2 AU	100.0%





Legends: A-Fraction IV at 254nm, B- Fraction IV at 366nm, C- Fraction IV and Beta sitosterol in post derivatisation at 620nm.

Figure 1: TLC photo documentation of fraction IV of plant extract and marker compound β -sitosterol

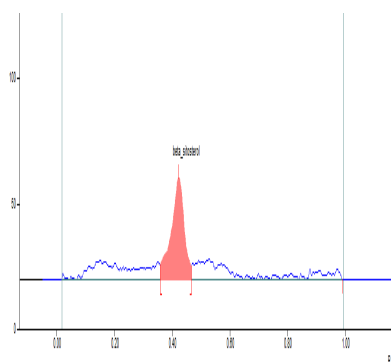


Figure 4: HPTLC finger print of ethanolic fraction of standard β -sitosterol at 620nm (β -sitosterol standard(5 μ l))

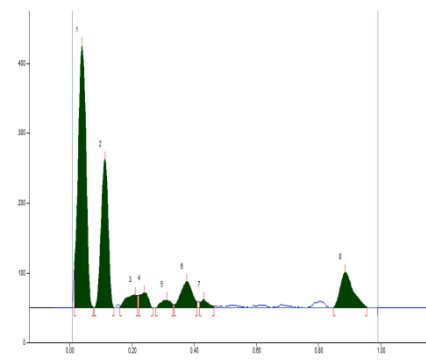


Figure 2: HPTLC finger print of fraction IV of *Benincasa hispida* fruit at 254nm (10 μ l plant extract fraction)

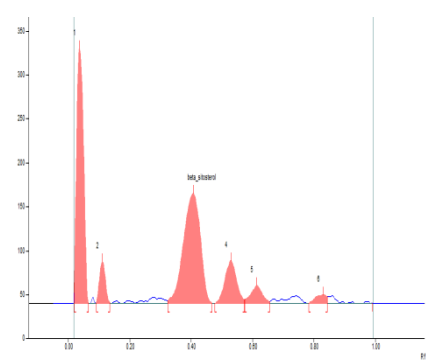


Figure 5: HPTLC finger print of ethanolic fraction IV of *Benincasa hispida* fruit at 620nm (plant extract fraction 10 μ l)

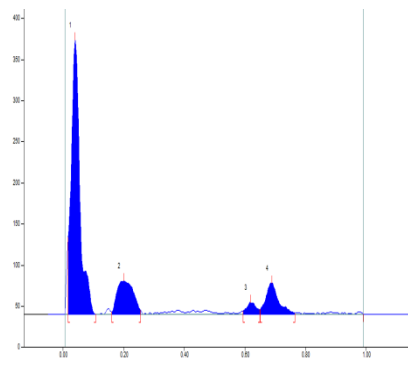


Figure 3: HPTLC finger print of ethanolic fraction of *Benincasa hispida* fruit at 366nm (10 μ l plant extract fraction)

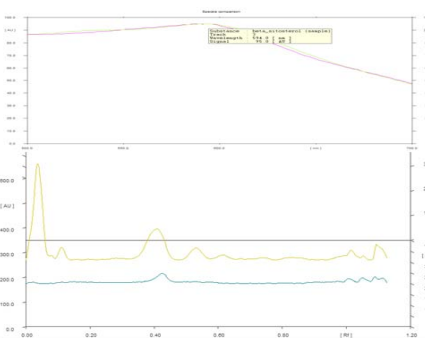


Figure 6: Spectral scan at 500nm to 700nm and 3-D chromatogram at 620nm

HPTLC Analysis

The TLC plate was visualized under UV light at 254nm and 366nm. A Photograph of a TLC plate after chromatography of β -sitosterol standard and ethanolic extract of *Benincasa hispida* fruit fraction IV shown in Figure 1. The Chromatograms shown in Figure 1 indicate that all the sample constituents were clearly separated without any tailing and diffuseness. Figure 2 shows HPTLC fingerprint of ethanolic fraction IV of *Benincasa hispida* fruit at 254nm.

Table 2 reveals HPTLC max R_f and area occupied by ethanolic fraction IV of *Benincasa hispida* fruit at 254nm. 10 μ l fraction of ethanolic extract of *Benincasa hispida* was used for process.

There are 8 spots (Figure 2). R_f values were as follows 0.04, 0.11, 0.21, 0.24, 0.31, 0.38, 0.43 and 0.89 were found to be more predominant and the maximum percentage was 50.78%, 28.76%, 2.48%, 2.88%, 1.51%, 5.10%, 1.62% and 6.67% respectively.

Table 2 and Figure 3 shows HPTLC fingerprint of ethanolic fraction IV of *Benincasa hispida* fruit at 366nm and HPTLC shows max R_f and area occupied by ethanolic fraction IV of *Benincasa hispida* fruit at 366nm. There are 4 spots

with R_f values as follows 0.04, 0.20, 0.62 and 0.69 and its maximum percentage is 78.23%, 9.47%, 3.34% and 8.96%.

Table 2 and Figure 5 denotes HPTLC fingerprint of β -sitosterol at 620nm and max R_f and area occupied by ethanolic fraction IV of *Benincasa hispida* fruit at 620nm. In that 5 μ l β -sitosterol marker was read at 620nm. It was showed in figure 4. R_f value was 0.42. Maximum percentage was 100%.

Table 2 and Figure 5 showed, 10 μ l plant extract at 620nm has different peaks with different R_f values 0.04, 0.11, 0.41, 0.53, 0.61 and 0.83 with maximum percentage of 53.50%, 8.76%, 23.11%, 9.02%, 3.73% and 1.88%. β -sitosterol also observed.

β -sitosterol in fraction IV was proved by chromatogram. The peak corresponding to β -sitosterol from the sample solution had same retention factor (R_f 0.41) as that of the retention factor from the β -sitosterol standard (R_f 0.42).

Figure 6 showed 3D chromatogram of plant extract and standard β -sitosterol and spectral scan at 500 to 700nm of plant extract and β -sitosterol. HPTLC fingerprint of the test fraction has been performed. It shows that presence of β -sitosterol in the fraction IV of ethanolic extract of *Benincasa hispida* fruit extract. It has been confirmed by photo documentation, densitometric scan and super

impossible spectra at 594 nm after derivatisation with vanillin-sulphuric acid.

Polyphenolic compounds such as the total phenolic and total flavonoids were implied in various biological activities like antioxidant, antidiabetic, anticarcinogenic, antimicrobial, antiallergic, antimutagenic and anti-inflammatory activities. It has better antioxidant potential due to the presence of phenols and flavonoids in *Benincasa hispida* fruit extracts.⁸ Phytochemical such as terpenoids and tannins have anti-inflammatory effects¹⁶⁻¹⁷ and also decrease blood sugar level.¹⁸ HPTLC fingerprinting of particular plant species will not only help in the identification and quality control of a particular species but also provide basic information which is useful for the isolation, purification, characterization and identification of (marker) chemical compounds of the species. Thus the present study has provided sufficient information about therapeutic efficacy of the drug and also in the identification, standardization and quality control of *Benincasa hispida*. β -sitosterol possess medicinal value like induced apoptosis in the leukemia cells¹⁹ and also prevents hepatotoxicity and it containing cyclopentano perhydro phenanthrene ring so it competes for cholesterol absorption in the intestine and in turn reduces the level of cholesterol in the blood.²⁰

The present HPTLC analysis reveals the presence of β -sitosterol in active fraction of ethanolic extract of *Benincasa hispida*. Hence ethanolic extract of *Benincasa hispida* fruit fraction IV may be used as therapeutic drug for the treatment of diseases like liver diseases, inflammatory, neurological diseases and lipid abnormalities.

CONCLUSION

The result obtained from HPTLC analysis shows the presence of β -sitosterol in fraction IV of *Benincasa hispida* fruit are implying their importance to human health. These investigations play an important role in standardization of particular drug and ensure the therapeutic efficacy. So it can be concluded that the fruit of *Benincasa hispida* fraction IV can be used as a good source for the isolation of β -sitosterol and this drug can be act as anticancer and antihyperlipidemic drug.

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