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A Comparative Study of Hptlc Fingerprint Profile and Standardization of *Benincasa Hispida* (Thunb.) Cogn. Pulp and Seed

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ABSTRACT

Benincasa hispida belongs to the family Cucurbitaceae and has notable medicinal properties as it has anti-inflammatory, anti-asthmatic and antimicrobial properties. Methanolic extracts of B. hispida pulp and seed were obtained separately through reflux extraction to perform further experiments. Various experimental tests were carried out to understand the physicochemical, antimicrobial and fluorescence properties. HPTLC and phytochemical profiles were also obtained. Preliminary phytochemical analysis of the pulp showed the presence of alkaloids, flavonoids, saponins, steroids and triterpenes whereas incase of the seed, saponins and triterpenes were absent. Microscopy of the samples showed the presence of helical xylem vessels, stone cells and parenchyma cells in pulp; oil globules, and fibersin seed. The maximum antimicrobial activity of B. hispida pulp was seen at a sample concentration of 10µL where the activity was more than standard against Escherichia coli and Aspergillus niger and in case of the seed, maximum activity was seen at the same concentration as that of the pulp as there was an increase in activity in comparison to the standard against Aspergillus niger. The activity of the seed was equivalent to that of the standard against Escherichia coli. HPTLC profiling showed 5 bands each in 5 lanes and 7 bands each in 5 lanes of increasing concentration, in pulp and seed extracts respectively and a pattern unique to Benincasa hispida.

Keywords: *Benincasa hispida, Phytochemical, HPTLC profiling, Microbial activity, Physicochemical evaluation, Microscopical evaluation*

INTRODUCTION

Benincasa hispida (Thunb.) Cogn., commonly known as wax gourd or winter melon, is a tropical vine plant belonging to the family- Cucurbitaceae. *B. hispida* is a monoecious, stem-hairy, 5-angled, climbing or trailing herb with suborbicular stipuliform bract at the petiole root; simple,very hairy leaves on both surfaces, alternate, palmate or ovate blade in young plant, root cordate. When young, the fruits are 30-45 cm long, succulent, densely hairy, with a thick waxy deposit when ripe. Its texture is fine, yellowish white in colour, taste slightly acidic, smell slightly aromatic, nature course and odour is nonspecific[1]. It can grow to a length up to 80 cm and also have broad leaves and yellow flowers[2]. The Cucurbit species can grow in diverse climatic conditions, including arid deserts,tropical, subtropical, and temperate regions. It is a popular vegetable crop, especially among Asian communities both for nutritional and medicinal purposes[3]. Ash gourd can be grown successfully on practically all types of soils ranging from sandy to clayey; medium soils like loams with plenty of organic matter are most suitable[4].

The pharmacological studies revealed that various parts of these plants such as leaves, stems, flowers, fruits, seeds, roots etc. exhibit a plethora of pharmacological activity viz. hypolipidemic, antihyperglycemic, anticancer, antimicrobial, analgesic, anti-inflammatory, anti-stress and immunomodulatory activities[5]. In Ayurvedic medicine the seed is used in the treatment of coughs, fevers, excessive thirst and to expel tapeworms. The extract of seed is antiangionic, the bronchodilator and anti-ulcer effects reported in the methanolic extract of plant, n-triacontanol, lupeol and sitosterol are present[4]. Fruits were usually used as an aphrodisiac, cardio tonic, urinary calculi, laxative, diuretic, tonic, blood disease, psychosis, schizophrenia, epilepsy and other psychologic disorders, dyspepsia, fever, jaundice, menstrual amenorrhea, dysmenorrheal, menorrhagia and premenstrual syndrome[6, 7, 8].

B. hispida is rich in phenolic compounds. Several other bioactive compounds present in it are isomultiflorenyl acetate, isovitexin, 1-sinapoylglucose, multiflorenol, 5-gluten-3-β-ylacetate, alnusenol, and benzylalcolcohol-O-α-l-arabinopyranosyl-(1-6)-β-d-glucopyranoside. *B. hispida* produces active phytochemicals such as triterpenes, proteins,vitamins, steroids, etc., showing importantgastro-protective, anti-oxidant and antipyretic effects[1]. Chemical analysis showed that the main sugars in the *Benincasa hispida* peels were galactose, glucose, xylose and sorbose[9]. *B. hispida* seeds contained high amounts of fatty acids 24.3%, saturated fatty acids represented 75.38% and unsaturated fatty acids (75.38%), it was apparent that linoleic and oleic are the principal fatty acid components in the seed's extracts[10]. The study emphasizes its high water content, making it a valuable source of hydration and its low-calorie nature, contributing to its dietary benefits. The aim of this paper is to study and analyze the microbiological, HPTLC,

pharmacognostic and phytochemical profile of *Benincasa hispida* (Thunb.) Cogn., to assess and understand its various applications and benefits.

MATERIALS AND METHODOLOGY

Collection of sample[11, 12]

In this study, samples of *Benincasa hispida* pulp and seed were collected from a local market at Bangalore. The robust mature fruit pulp and seed were targeted for our study, following a random sampling approach. The pulp and seeds were dried in the sunlight, later in the oven for 3-5 days and then ground to prepare a powder of the dried pulp and seed respectively. The powder was stored at room temperature for further analysis. The collected *B. hispida* was identified and authenticated by Plants of the World Online Herbarium.

Organoleptic evaluation[13, 14]

Organoleptic evaluation is a qualitative method based on the sample's morphological characteristics where it is evaluated based on sight, smell, taste, touch, and color. It is the conclusion drawn from studies that resulted from impressions on organs ofsenses.

Physicochemical evaluation

Determination of Total Ash[15]

Total ash is the amount of ash obtained after the sample has been incinerated and is devoid of carbon. It usually contains phosphates, carbonates, silicates and silica. An empty crucible was weighed after heating in the muffle furnace at 500 °C for 1 hour. 2 g of powdered sample was weighed and transferred into the dried and cooled empty crucible which was then placed in the muffle furnace with the temperature setting at 600 °C. After 4 hours, the crucible was removed from the furnace and cooled by placing in a desiccator for 30 min after which the following readings were noted.

Total Ash % =

where, A = Weight of sample (in grams)

B = Weight of crucible + contents after drying (in grams)

C = Weight of empty crucible (in grams)

Determination of Acid Insoluble Ash[15]

Total ash when treated with HCl reacts with materials to form soluble salts and insoluble residues consisting of mainly silica and acid insoluble ash. 25 mL of 2M HCl was added to the crucible containing the total ash obtained and covered with a watch glass. This setup was kept on a hot plate to boil gently for 5 min. The insoluble matter was filtered using a clean filter paper which was then rinsed repeatedly with hot water until it was neutral or free from acid. This filter paper containing the insoluble matter was then transferred into the original crucible. The crucible was dried on a hot plate and placed in the muffle furnace at a temperature setting of 500 °C. After 4 hours, the crucible was removed and cooled by placing it in a desiccator for 30 min after which the following readings were noted.

Acid Insoluble Ash % =

where, A = Weight of sample (in grams)

B = Weight of crucible + contents after drying (in grams)

C = Weight of empty crucible (in grams)

Determination of Alcohol Soluble Extract[16]

4 g of powdered plant material was weighed and macerated with 100 mL of 90% ethanol in a conical flask for 24 hours while shaking the contents frequently in the first 6 hours. Thereafter, the contents of the flask were rapidly filtered while taking precautions against loss of ethanol. An empty Petri plate was weighed and the contents obtained after filtration was poured into it and placed on the hot water bath till all the liquid components had evaporated. The petri plate was then dried by placing it in a hot air oven at a temperature setting of 105 °C, followed by placing it in a desiccator for 30 min. The following readings were noted.

Alcohol Soluble Extractive value % =

where, A = Weight of plant material (in grams)

B = Weight of petri plate + residue

C = Weight of empty petri plate

Determination of Water Soluble Extract[16]

4 g of powdered plant material was weighed and macerated with 100 mL of chloroform water in a conical flask for 24 hours while shaking the contents frequently in the first 6 hours. Thereafter, the contents of the flask were rapidly filtered. An empty petri plate was weighed and the contents obtained after filtration was poured into it and placed in the hot water bath till all the liquid components had evaporated. The petri plate was then dried by placing it in a hot air oven at a temperature setting of 105 °C, followed by placing it in a desiccator for 30 min. The following readings were noted.

Water Soluble Extractive value % =

where, A = Weight of plant material (in grams)

B = Weight of petri plate + residue

C = Weight of empty petri plate

Preliminary Phytochemical Evaluation[17, 18, 19]

Phytochemicals, also known as secondary metabolites, are biologically active compounds found in plants like alkaloids, flavonoids, saponins, steroids, triterpenes, phenols, tannins etc., These phytochemicals are found in various parts of the plant like leaves, bark, seeds, flowers and roots. The methanolic extracts were subjected to various chemical tests to detect the chemical constituents present in them. Different tests were performed for different constituents i.e., alkaloids (Dragendorff' s test, Mayer' s test), flavonoids (Shinoda test), saponins (Froth test), steroids (Liebermann-Burchard' s test, Salkowski test), triterpenes (Liebermann-Burchard' s test, Salkowski test) and phenols (Ferric chloride test).

HPTLC Studies[20]

Sample solutions were applied to the Silica gel 60 F254 (E. Merck) precoated TLC plates as sharp bands by means of Aspire automatic sample applicator. The spots were dried in a current of air. Chromatography was carried out in a glass chamber (Aspire). The mobile phase (Toluene: Ethyl acetate (8:2 v/v)) was poured into a twin trough glass chamber. The whole assembly was left to equilibrate and pre-saturate for 30 min. The plate was then developed until the solvent front had traveled at a distance of 80 mm above the base of the silica plate at 20 ºC and 50% relative humidity. The plate was visualized for detection by observing it under UV light (254 nm) and at long UV (366 nm). Then the derivatization was carried out with 10% H_2SO_4 solution. The densitometric scan was drawn using Just TLC software attached to Aspire HPTLC.

Microscopical Evaluation

Powder Microscopy[21]

It is a quality control method used for medicinal plants to study the specific microscopic characters using different staining reagents.

The powdered plant material was completely immersed in 10% HCl overnight. The sample was then filtered and rinsed with distilled water the next day. The soaked sample was completely drained of excess water, stained with safranin, and observed under Magnus MLX Plus microscope under 4X magnification followed by 10X, 40X, and 100X.

Powder Fluorescence[22]
Powdered samples, when treated with different chemical reagents, give characteristic colors when visualized under UV light. On adding 1-2 drops of nitrocellulose to the sample and observing it in the UV transilluminator, a change in color is noticed.

Fluorescence analysis[22]
The powdered sample of *B. hispida* pulp and seeds were treated with various chemicals like water, concentrated HNO_3, H_2SO_4, HCl, methanol, petroleum ether, hexane, chloroform, and ethanol. The powdered materials gave different colors with different chemicals when observed under UV light of short and long wavelengths.

Microbial Limit Test[23, 24]
Microbial testing is carried out for the detection of microorganisms in a product. We check for the amount of microbial activity in the sample chosen. The suitable media- Nutrient Agar, Potato Dextrose Agar and peptone water was prepared and sterilized. Serial dilution was performed by adding 10 g of powdered sample in 90 mL NaCl saline (10^{-1}). 9 mL of NaCl saline was poured in a series of 9 test tubes. 1 mL of sample from the first dilution was taken and mixed with the contents of the second test tube (10^{-2}). This process was continued for up to 4 dilutions. 0.1 or 1 mL of sample was taken from each dilution and poured into the respective petri plates and inoculated with the media by pour plate method. Incubate the bacterial plates with Nutrient Agar at 37 °C for 24-48 hours and fungal plates with the Potato Dextrose Agar at room temperature for 3-5 days. Observe and count for bacterial and fungal colonies with the help of a digital colony counter after the incubation period.

Microbial Activity
Antibacterial activity[25]
The in-vitro antibacterial activity test was conducted against two different bacteria- *Escherichia coli* and *Bacillus cereus*, as these bacteria multiply rapidly and show results quickly. The sample was prepared by adding 40 mg of plant extract in 10 mL DMSO solution. The antibacterial activity was determined by a disc method where gentamycin was used as standard and distilled water as control. 1.5 mL of sample was added at different concentrations of 10, 20, 30 mg in the respective vials and sterilized discs were allowed to soak for an hour. Nutrient Agar media was poured into petri plates and allowed to solidify followed by inoculation of bacteria by spread plate method. The discs were removed from vials, completely drained of excess liquid and placed on the agar gently. Further, the petri plates were incubated at 37 °C for 24 hours. The zones of inhibition around each disk were then measured.

Antifungal activity[25]
The in-vitro antifungal activity test was conducted against *Aspergillus niger*. The sample was prepared by adding 40 mg of plant extract in 10 mL DMSO solution. The antifungal activity was determined by disc method where Fluconazole was used as standard and distilled water as control. 1.5 mL of sample was added at different concentrations of 10, 20, 30 µL in the respective vials and sterilized discs were allowed to soak for an hour. Potato Dextrose Agar media was poured into petri plates and allowed to solidify followed by inoculation of bacteria by spread plate method. The discs were removed from vials, completely drained of excess liquid and placed on the agar gently. Further, the petri plates were incubated at room temperature for 48 hours. The zones of inhibition around each disk were then measured

RESULTS

Organoleptic evaluation

Table 1. Organoleptic evaluation of Benincasa hispida pulp and seed

Sl. No	Parameter	Observation	
		Pulp	Seed
1	Color	White	White/ yellow
2	Shape	Round/ oval	Oval, drop shaped
3	Texture	Slimy, slippery, wet	Slimy, slippery, wet
4	Taste	Mild taste like cucumber	Tasteless
5	Odour	Strong bitter odor	Mild bitter odor

Physicochemical evaluation

Table 2. Physicochemical evaluation of Benincasa hispida pulp and seed

Sample	Total ash (%)	Acid insoluble ash (%)		Water soluble extract value (%)		Alcohol soluble extract value (%)		
	OV	LM	OV	LM	OV	LM	OV	LM
Pulp	12.19%	<12%	1.754%	<1%	25.21%	<24%	10.84%	<10%
Seed	5.07%	NA	4.161%	NA	20.08%	NA	11.75%	NA

OV: Obtained value LM: Limit

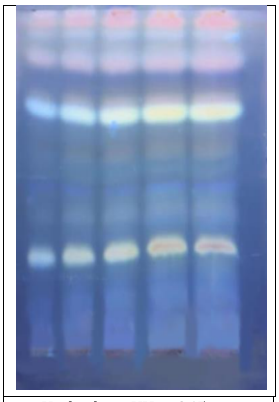
Preliminary phytochemical evaluation

Table 3.Preliminary phytochemical evaluation of Benincasa hispida pulp and seed

Sl. No	Test	Result	
		Pulp	Seed
1	**Alkaloids** a. Dragendorff's test b. Mayer's test	 Positive Positive	 Positive Positive
2	**Flavonoids-** Shinoda test	Positive	Positive
3	**Saponins**- Froth test	Positive	Negative
4	**Steroids** a. Liebermann-Burchard's Test b. Salkowski test	 Positive Positive	 Negative Negative
5	**Triterpenes** a. Liebermann-Burchard's Test b. Salkowski test	 Positive Negative	 Positive Negative
6	**Phenols-** $FeCl_3$ test	Negative	Negative
7	**Tannins**	Negative	Negative

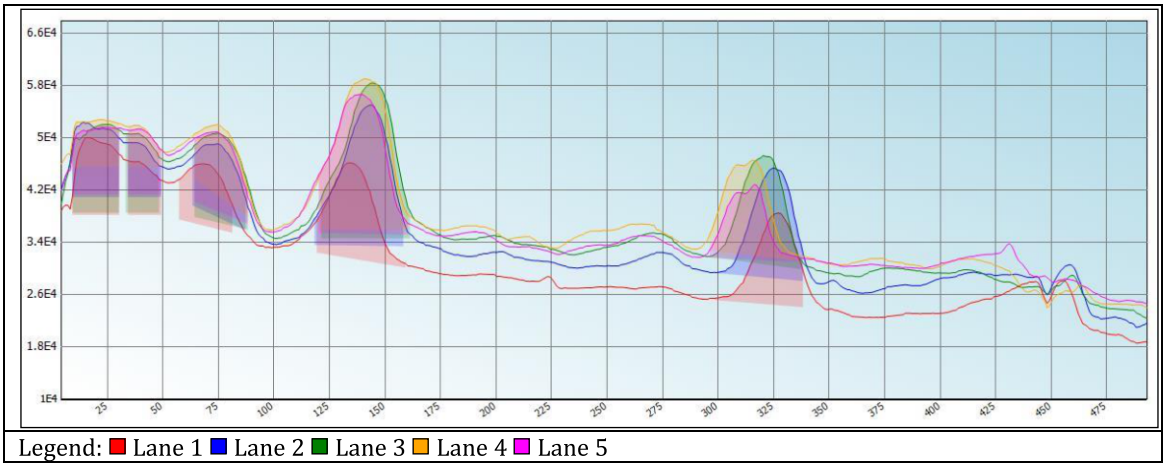
HPTLC studies

Fig 1. HPTLC studies of Benincasa hispida pulp



Under long UV at 365 nm (without filter)

Analysis



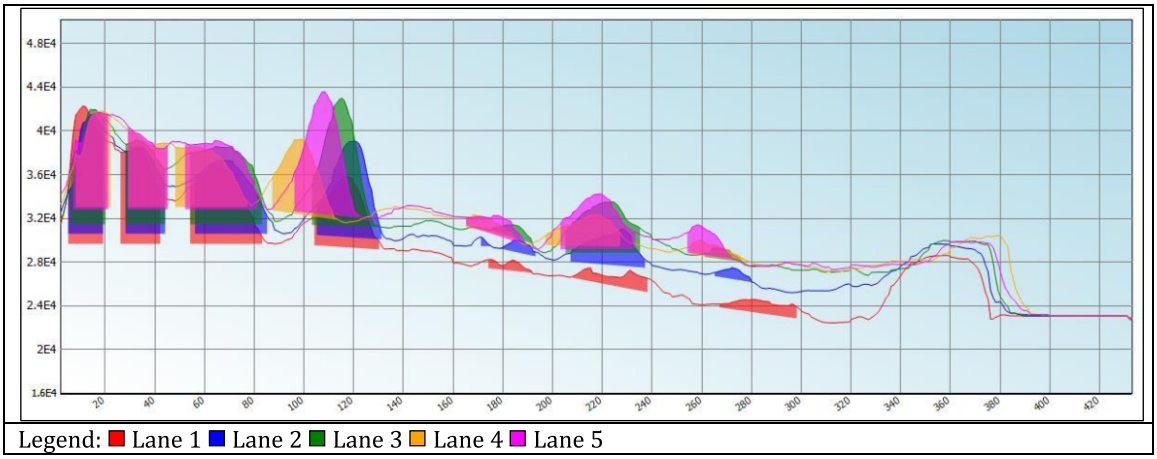
Lanes

ID	Width	Bands	Volume
1	62	5	704.29
2	62	5	899.82
3	67	5	1156.6
4	74	5	1052.25
5	91	5	1278.59

Fig 2. HPTLC studies of Benincasa hispida seed

Under long UV 365 nm (without filter)

Analysis



Microscopical evaluation

Table 4. Powder Microscopy

ID	Width	Bands	Volume
1	75	7	456.96
2	65	7	406.29
3	66	7	476.02
4	59	7	418.41
5	74	7	403.68

Table 4.1.Powder microscopy ofBenincasa hispida pulp

Fig 1: Helical xylem vessels

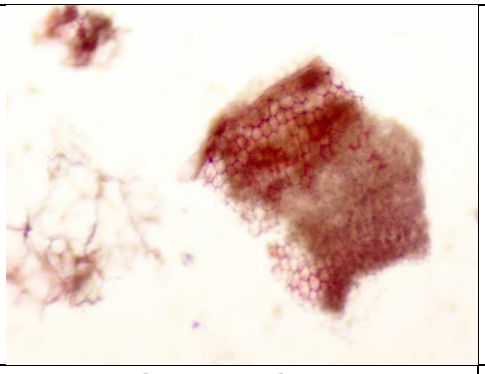


Fig 2: Epidermis and Parenchyma

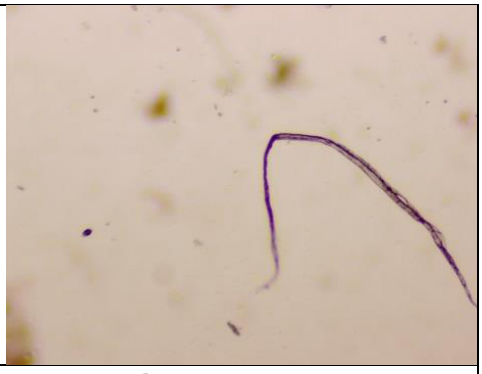


Fig 3: Trichome

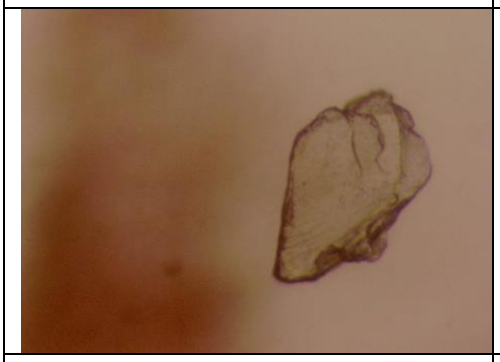


Fig 4: Stone cells

Fig 5: Thick walled cell

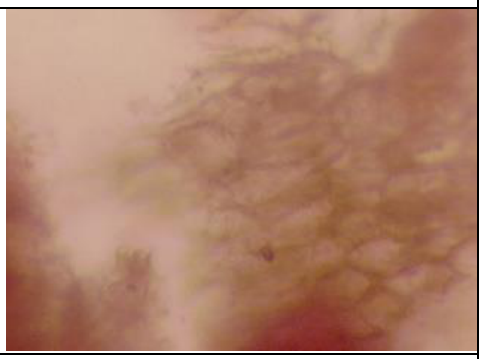


Fig 6: Stomata

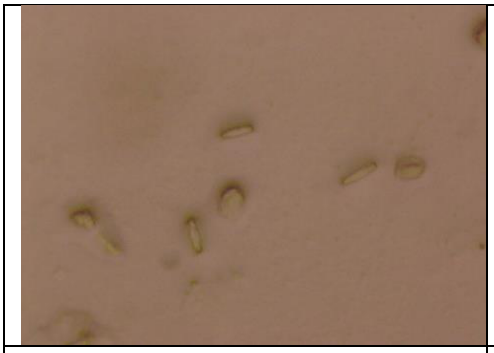


Fig 7: Parenchyma cells and oil globules

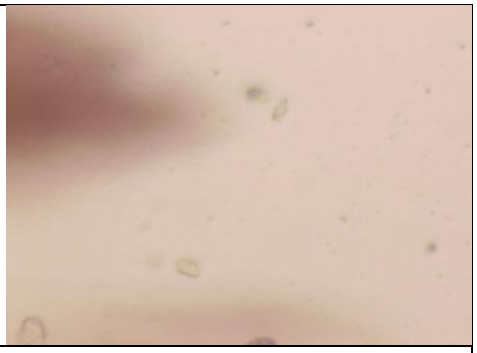


Fig 8: Oil globules

Table 4.2. Powder microscopy of Benincasa hispida seed

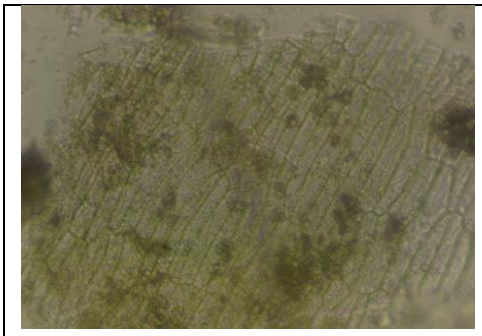


Fig 1: Epidermis

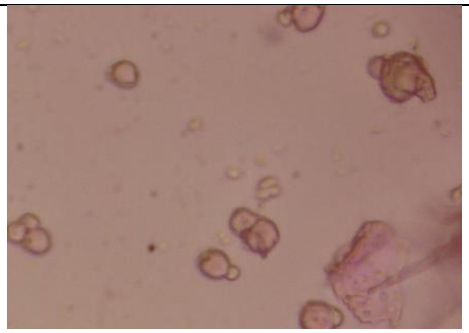


Fig 2: Oil globules

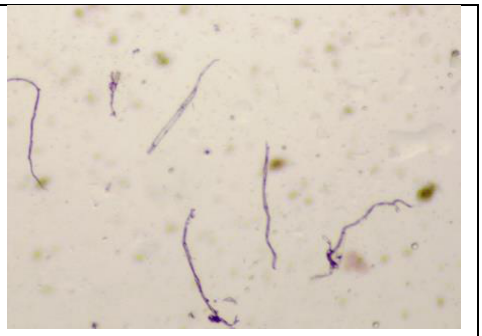


Fig 3: Fibers

Fluorescence analysis

Table 5.Fluorescence analysis of Benincasa hispida pulp and seed

Sl. No	Reagents	Color(Pulp)	Color(Seed)
1	No reagent (raw sample)	Blue	Blue
2	Water	Blue	Blue
3	Conc. HNO_3	Brownish yellow	Brownish-yellow
4	Conc. H_2SO_4	Blue	Blue
5	Dil. HCl	Light blue	Light blue
6	Methanol	Bright blue	Bright blue
7	Petroleum ether	Bright blue	Bright blue
8	Hexane	Bright blue	Bright blue
9	Chloroform	Bright blue	Bright blue
10	Ethanol	Bright blue	Bright blue
11	Nitrocellulose	Bright blue	Bright blue

Microbial limit test

Table 6. Microbial limit test of Benincasa hispida pulp and seed

Sl. No	Dilution	Pulp		Seed	
		No. of colonies	CFU	No. of colonies	CFU
1	control	No growth	0	No growth	0
2	10^{-1}	30	$30*10^{-1}$	37	$37*10^{-1}$
3	10^{-2}	22	$22*10^{-2}$	33	$33*10^{-2}$
4	10^{-3}	16	$16*10^{-3}$	27	$27*10^{-3}$
5	10^{-4}	14	$14*10^{-4}$	9	$9*10^{-4}$

CFU: Colony Forming Units

Microbial activity

Antibacterial activity

Table 7. Antibacterial activity of Benincasa hispida pulp andagainst Bacillus cereus and E. colirespectively

Sl. No	Concentration of antibiotic	Zone of inhibition (cm)			
		Pulp		Seed	
		Against *Bacillus cereus*	Against *E.coli*	Against *Bacillus cereus*	Against *E.coli*
1	Standard- Gentamycin	2.5 cm	1.5 cm	2.8 cm	1.1 cm
2	10 µL	1.0 cm	2.0 cm	1.6 cm	1.1 cm
3	20 µL	0.9 cm	1.0 cm	1.2 cm	1.0 cm
4	30 µL	1.0 cm	1.3 cm	1.0 cm	0.9 cm

Antifungal activity

Table 8. Antifungal activity of Benincasa hispida pulpand seed against Aspergillus niger

Sl. No	Concentration of antibiotic	Zone of inhibition (cm)	
		Pulp	Seed
1	Standard- Fluconazole	1.1 cm	1.1 cm
2	10 µL	2.6 cm	1.8 cm
3	20 µL	1.6 cm	2.2 cm
4	30 µL	1.9 cm	1.2 cm

DISCUSSION

The plant under study *Benincasa hispida* was identified and authenticated as per the study requirements. The organoleptic and physicochemical studies conform with the limits mentioned in API. The preliminary phytochemical studies of methanolic extract of *B. hispida* pulp and seed showed varied results in pulp except phenol and tannins, all other tested group of chemicals viz., alkaloids, flavonoids, saponins, steroids, and triterpenes were present, whereas in the methanolic extract of seed of *B. hispida*, only alkaloids, triterpenes and flavonoids showed positive result, and remaining four groups of compounds such as saponins, steroids, phenols and tannins were absent. Further, it is interesting to note that both phenols and tannins were absent in pulp and seed of *B. hispida*. As far as HPTLC fingerprinting profile is concerned, in the pulp, 5 bands were observed in all 5 lanes of 2 µL, 4 µL, 6 µL, 8 µL and 10 µL at Rf values 0.37, 0.72, 0.85, 0.92 and 0.96 and in the HPTLC fingerprinting profile of the seed, 7 bands in total were observed in 5 lanes of 2 µL, 4 µL, 6 µL, 8 µL and 10 µL at Rf values 0.39, 0.5, 0.6, 0.77, 0.86, 0.93, and 0.963.

The pulp and seed of *B. hispida* exhibited various normal and fluorescent colors when they were treated with water, different types of acids, andvarious other solvent systems. In the microbial limit test, the colony-forming unit (CFU) both in bacteria and fungus, ranges were well within the limits as mentioned in API. Having a large group of chemicals, pulp and seed of *B. hispida* exhibited moderate to significant antimicrobial activity. The activity of pulp of *B. hispida* is very less in 10 µL against *Bacillus cereus*, whereas it is 25% more than standard against *E. coli* in the same concentration. At the dose of 10µL extract of seed of *B. hispida*, 50% activity of the standard was seen against *Bacillus cereus*, whereas it is almost same with that of standard against *E. coli* in 20µL and 30µL of the extract. Additionally, in all three concentrations of 10 µL, 20 µL and 30 µL of the seed and pulp extract, the activity was very high and significant against *Aspergillus niger*, notably more than that of the standard Fluconazole. The antimicrobial activity of *B. hispida* may be attributed to the group of chemicals present in plant parts under study. The HPTLC fingerprint profile of methanolic extract of this plantshowed 5 bands each in 5 lanes and 7 bands each in 5 lanes of increasing concentration, in pulp and seed extracts respectively, and it can be used asquality standard method for identification and authentication.

CONCLUSION

From the above study, it can be concluded that pharmacognostic and phytochemical evaluation will possibly help as a valuable resource for the identification, authentication, and preparation of the monograph of *Benincasa hispida*. The present work was embraced with a perspective of setting down a benchmark that could be valuable in recognizing the authenticity of therapeutically important medicinal plants. Microscopical studies have demonstrated the presence of helical xylem vessels, trichome, stone cells, thick walled cells, stomata, parenchyma cells and oil globules in pulp of *B. hispida*, whereas in that of *B. hispida* seed, only epidermis, oil globules and fibers were observed.

The phytochemical investigation showed the presence of alkaloids, flavonoids, saponins, steroids, and triterpenesin *B. hispida* pulp, whereas in that of*B. hispida*seed, only alkaloids, triterpenes and flavonoids showed positive result.Thus, obtained results can be utilized for the quality control of the crude drug/ drugs.

This type of study helps standardize the drugs and can be used to differentiate closely related or allied species. The HPTLC results provide standard fingerprints that can be used as a reference result for the identification and QC of the drug. This can also be used as a good tool in preparing the monograph. The various physicochemical parameters were established which are also important in analyzing adulteration and mishandling of the crude drug. Further spectral studies and in vivo studies are required to know their exact chemical composition and therapeutic efficacy.

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