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Phyto-chemical Screening & Evaluation of Antibacterial Activity of Polyherbal Formulation

Kruti Pandya*, Bhavna Solanki, Bijal Shah, Diksha Parekh, Hardik Soni, Kunal Maniar, Nilesh Gurav, Prateek Patel

Vasu Research Centre, A Division of Vasu Healthcare Pvt Ltd, Vadodara, India

Address for Correspondance: krutishah85@gmail.com

Abstract: Acnovin Capsule has a good amount of herbal ingredients that possess antimicrobial activity. They were screened to show their anti bacterial activity in three solvent extractions. They were also screened for the presence of pathogens, heavy metals and their Quality Control Parameters. All the ingredients have good activity on the skin. Few of the samples were also tested for their HPLC and HTLC charcaterization.

Keywords: Antimicrobial activity, Mc Farland standard turbidity, Heavy metal analysis, Microbial analysis, HPLC, HPTLC.

INTRODUCTION

The poly herbal formulation consists of ten important Ayurvedic ingredients that consist of Mahamajishthadi kwath, Panch neem churna, Sariva, Sonamukhi, Khadir twak, Haridra, Amla, Bibhitaki, Haritaki and Gandhak rasayana. The plant extracts were tested for their anti bacterial activity and were also screened for Phytochemical and other parameters.

The presence of some phytochemicals determines the antimicrobial properties of various plants. They give plants its colour, flavour, odour and are part of the defense system (disease resistance). Phytochemicals are bio actives, non nutrient plants compounds in fruits, vegetables, grains and other plants food that has been linked to reduce the risk of major degenerative disease[1]. Phytochemicals as plant derived chemicals, which are beneficial to human health and disease prevention[2]. It attracts beneficial and repel harmful organisms, serves as photoprotectant and respond to environmental changes. For examples, isoflavones, anthocyanins, and flavonoids do function as phytoalexins, a substance that assists a plant to resist pathogens[3].

- **Mahamajishtadi kwath**: it is used for all types of skin disorders, moreover it is also used in chronic gout, tumors, obesity and all types of filariasis. This is one of the best medicines for blood purification. It is also useful in syphilis. It is used in all types of boils, skin eruptions, ring worm, pruritis, itching and other major diseases related to skin.[4]
- **Amalaki**: **Photochemical:** Iron, calcium, silica, magnesium, B12, C, K. Fruit pulp contains moisture 81%, 5% proteins, fat1%, mineral matter 7%, fiber3.4%, carbohydrates 14%, calcium 0.05% and potassium 0.02%, iron 0.5mg/100g, nicotinic acid2mg/100g and vitamin C 600mg/100g. Fresh amla contains about 20 times more vitamin C than orange juice. Fruit is high in pectin & phyllemblin. Dry fruit contains tannins and 3-4 colloidal complexes. Other components are phyllembic acid, lipids, gallic acid,

emblicol, mucic acid, ellagic acid, and glucose. Seeds contain a fixed oil, phosphatides, and some essential oil with linolenic, linoleic, oleic, stearic, palmitic, myristic acids, and proteolytic and lipolytic enzymes. The plant as a whole promotes resistance towards illness and counteracts infection. It is a potent anti-inflammatory herb and is used in piles, gastritis, colitis etc. Emblica Officinalis strengthens the defense mechanisms against free radical damage induced during stress. The effect of Emblica Officinalis appeared to depend on the ability of target tissues to synthesize prostaglandins.[5-7]

- **Bibhitaki**: The principle constituents are Triterpenoids – cardiac glycoside saponins, bellericoside, bellericanin, Sterols – B-sitosterol, Tannin – gallic acid, ellagic acid. It also contains galloyl glucose and number of free sugars. The seeds contain protein and oxalic acid, while bark contains tannin and its oil contains palmitic, oleic and linoleic acids as major fatty acids. It has purgative, blood pressure depressant, antifungal, antihistaminic activity against viral hepatitis and vitiligo.
- **Haritaki:** Its paste with water is found to be anti-inflammatory, analgesic and having purifiying and healing capacityfor wounds. Its decoction is used for surgical dressing in healing the wounds. It has proven gastrokinetic effect i.e. it helps in moving the contents of stomach. So it can be used after surgeries and as adjuvant with other drugs that interfere with gastric motility as antihistaminics, atropine like drugs.
- **Panch neem churna**: 5 Different parts of the neem plant are used in this formulation (in equal quantities and they are – leaf, root, bark, flower and fruit). It cures all types of skin diseases, erysipelas, piles, deep & infected wounds, hepatitis as well as all the disorders caused by the vitiation of pitta, kapha and rakta. If a person continues to take it for one year, alongwith proper diet then it can cure any type of chronic skin disease as well as blood impurity disorders. It also acts as an immuno modulator.[8]
- **Acacia catechu (Khadir)**: It is used in skin diseases, leprosy, internally as well as externally. The person suffering from any type of skin disease should use this drug for internal use, bathing as well as for massaging purpose [9,10]
- **Sariva**: The drug consists of dried roots of *Hemidesmus indicus.* The chloroform and ethanol (95 %) extracts were reported to possess good anti fungal activity against *A.niger* and weak anti bacterial activity *E.coli*, *S.aureus* and *P.aeruginosa*.
- **Sonamukhi**: Senna is one of the most commonly used laxative drugs in the Eastern and Western countries for the treatment of constipation. Commercially available consists of the dried leaflets of Alexandria senna (*Cassia acutifolia* Delile) or Tinnevelly senna (C*assia angustifolia* Vahl) belonging to plant family Leguminosae [11, 12].
- **Haridra**: Rhizomes of haridra are commonly used for diverse medicinal purposes.. Haridra has a long tradition of use in the Ayurvedic systems of medicine, particularly as an anti-inflammatory agent and for the treatment of flatulence, jaundice, menstrual difficulties, hematuria, hemorrhage and colic. Many studies have demonstrated the antiallergic potential of Haridra. The active ingredient of Haridra effectively inhibits allergic symptoms such as airway constriction and airway hyper reactivity in animals exposed to allergens.
- **Gandhak Rasayan: Gandhaka Rasayana** is a compound preparation, being used by Ayurvedic physicians in clinical practice for thousands of years, in the treatment of skin diseases of bacterial and fungal origin

MATERIALS & METHODS

Phytochemical Analysis:

Phytochemical evaluation were carried out for the different materials used in the poly herbal formulation which are as given below. [13-15]

1. pH and LOD of all the ingredients

The results are as tabulated in **Table 1.**

Sr No	Name of the Ingredient	Physio Chemical Parameters	
		pH (1 % w/v solution)	Loss on Drying
1	Mahamajishtadi kwath	5.00 ± 5.33	5.58 ± 0.46
2	Panch neem churna	4.61 ± 0.34	4.30 ± 0.34
3	Sariva	4.51 ± 0.19	2.88 ± 0.45
4	Sonamukhi	5.40 ± 0.36	2.88 ± 0.45
5	Khadir twak	3.39 ± 0.36	2.43 ± 0.47
6	Haridra	4.20 ± 0.43	2.75± 0.47
7	Amla	2.95 + 0.45	4.87 ± 0.46
8	Bibhitaki	3.92 ± 0.36	3.79± 0.36
9	Haritaki	-	-
10	Gandhak rasayana	-	-

Table 1 Physico Chemical Parameters

2. The **Water and Alcohol Soluble Extractive** are as below.

The results are as tabulated in **Table 2 .**

Sr No	Name of the Ingredient	Physio Chemical Parameters	
		Water soluble (% w/w)	Alcohol Soluble (% w/w)
1	Mahamajishtadi kwath	89.68 ± 0.84	-
2	Panch neem churna	41.6 ± 0.84	-
3	Sariva	-	78.48 ± 1.06
4	Sonamukhi	84.48 ± 0.84	-
5	Khadir twak	85.12± 0.84	-
6	Haridra	-	-
7	Amla	88.48 ± 0.84	86.00 ± 0.84
8	Bibhitaki	74.96 ± 1.06	78.48 ± 1.06
9	Haritaki	66.16 ± 0.93	68.64 ± 0.95
10	Gandhak rasayana	-	-

Table 2 Water and Alcohol Soluble Extractive

3. The **Ash Values and the different assays** are as tabulated below

The results are as tabulated in **Table 3**

Sr No	Name of the Ingredient	Physio Chemical Parameters		
		Ash Value	Various Assays	
			Assays	Result
1	Mahamajishtadi kwath	4.15 ± 0.34	-	-
2	Panch neem churna	7.65 ± 0.36	-	
3	Sariva	-	Tannin Saponin	10.58 ± 0.36
4	Sonamukhi	-	Sennoside	14.5 ± 0.651
5	Khadir twak	-	Tannin	14.40 ± 0.62
6	Haridra	6.8 ± 0.461	Curcumin Volatile Oil	15.49 ± 0.75 7.2 ± 0.38
7	Amla	4.1 + 0.38	Tannin	29.30 ± 0.73
8	Bibhitaki	6.5 ± 0.487	Tannin	53.31 ± 0.42
9	Haritaki	4.17 + 0.36	-	-
10	Gandhak rasayana	-	-	-

Table 3 The Ash Values and the different assays

4. Quality Control paramters of the final product.

The results are as tabulated in **Table 4.**

Sr No	Test	Specification	Result
1	Moisture(by KF)	NMT 3.2 %	1.05 %
2	Bulk density	0.600 to 0.900 g/ml	0.675 gm/ml
3	Disintegration time	NMT 30 mins	08 min 23 sec
4	Wt. Variation	± 7.5%	Complies
5	Ph 2% Soln. Con.	3.00 to 5.00	4.21
6	Dissolution test	NLT 50%	83.57 %
7	Assay of Tannin	NLT 5.0%	9.87 %
8	Identification (By TLC Test)	Complies	Complies

Table 4 Quality Control Parameters of the poly herbal formulation ; NLT: Not Less Than; NMT: Not More Than

5. Microbial Analysis

Microbial analysis was carried out as per procedure of Indian pharmacopoeia 2007 and WHO Guidelines. It included the test of Total Bacterial Count, Total Fungal Count, and presence of pathogens like *Escherichia coli, Salmonella ebony, Pseudomonas aeruginosa,* and *Staphylococcus aureus*. Pure culture of *Escherichia coli* (NCIM: 2065; ATCC: 8739), *Salmonella ebony* (NCIM: 2257 NCTC: 6017), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6358) were obtained from NCIM Pune. The media used for the microbial limit test were of HiMedia Pvt. Ltd.[16, 17]

The results are as tabulated in **Table 5.**

Name of the Sample	TBC	TFC	*E.coli*	*S.aureus*	*P.aeruginosa*	*Salmonella spp*
Mahamajishtadi kwath	25 x 10^2 cfu/g	Absent	Absent	Absent	Absent	Absent
Panch neem churna	16 x 10^2 cfu/g	Absent	Absent	Absent	Absent	Absent
Sariva	12 x 10^2 cfu/g	Absent	Absent	Absent	Absent	Absent
Sonamukhi	26 x 10^2 cfu/g	Absent	Absent	Absent	Absent	Absent
Khadir twak	45 x 10^2 cfu/g	Absent	Absent	Absent	Absent	Absent
Haridra	16 x 10^2 cfu/g	Absent	Absent	Absent	Absent	Absent
Amla	32 x 10^2 cfu/g	Absent	Absent	Absent	Absent	Absent
Bibhitaki	16 x 10^2 cfu/g	Absent	Absent	Absent	Absent	Absent
Haritaki	26 x 10^2 cfu/g	Absent	Absent	Absent	Absent	Absent
Gandhak rasayana	38 x 10^2 cfu/g	Absent	Absent	Absent	Absent	Absent

Table 5 Microbial Analysis Report

6. Heavy Metal Analysis

Accurately weigh 2 g of the sample in a kjeldahl flask. An acid mixture of HNO_3:$HClO_4$ (4:1) was added in the flask and heated continuously till the solution becomes colorless. The sample was then transferred to a 25 ml volumetric flask and volume was made up with distilled water. A reagent blank was synchronously prepared accordingly to the above procedure. The standard of Lead (Pb), Cadmium (Cd), Arsenic (As) and Mercury (Hg) were prepared as per the protocol in the manual and calibration curve developed for each of them. The sample were analyzed for the presence of Pb, Cd, As, and Hg using atomic absorbance spectrophotometer (AAS) 6300 (by SHIMADZU) [18]

The results are as tabulated in **Table 6.**

Name of the Sample	Lead (10 ppm)	Cadmium (0.3 ppm)	Arsenic (10 ppm)	Mercury (1 ppm)
Mahamajishtadi kwath	ND	0.0660	0.1100	ND
Panch neem churna	1.6260	0.0810	0.4350	ND
Sariva	0.0000	0.1420	ND	0.0670
Sonamukhi	ND	0.0238	ND	ND
Khadir twak	3.1850	0.1210	0.1720	ND
Haridra	4.1830	0.1260	0.0850	ND
Amla	2.0059	0.2290	ND	ND
Bibhitaki	2.3610	0.0520	0.5830	ND
Haritaki	0.5200	ND	ND	ND
Gandhak rasayana	0.3990	0.0242	0.0400	ND

Table 6 Heavy Metal Analysis Report; ND: Not Detected

7. Bioassay

Test Organism: All the clinical strains were procured from NCIM, Pune that included four gram negative organisms and 1 gram positive organisms as stated below.

E.coli (NCIM NCIM: 2065; ATCC: 8739), *P.aeruginosa* (ATCC 9027, NCIM 2200), *Salmonella* (NCIM 2257, NCTC 6017), *K.pneumoniae and S.aureus* (ATCC 6358, NCIM 2079),

They were immediately sub-cultured by inoculating a loopful in Nutrient Broth (Hi Media, M002) and then incubated at 35-37°C for 18-24 hours. They were then streaked onto Nutrient agar (Hi Media, MM 012) plates and the plates were inverted and incubated at 35-37°C for 18-24 hours. They were then stored at 4°C till use.

Extraction of active principle

7.1 Aqueous Extraction: 10 g of the extract was weighed accurately and dissolved in 100 ml of distilled water taken in a 250 ml round flat bottomed flask. This was then kept on a soxhlet apparatus and refluxed for 3 hours, after which it was allowed to cool down to room temperature and filtered using a Whatman Filter paper no 1. The filtrate was then collected and dried to dryness first on a water bath and then in an oven. After drying the residue was scraped out and different aliquots were dissolved in 5 ml sterile water and were stored at 4°C till used for further analysis.

7.2 Methanolic Extraction: 10 g of the extract was weighed accurately and dissolved in 100 ml of methanol solution taken in a 250 ml round flat bottomed flask. This was then kept on a soxhlet apparatus and refluxed for 3 hours, after which it was allowed to cool down to room temperature and filtered using a Whatman Filter paper no 1. The filtrate was then collected and dried to dryness first on a water bath and then in an oven. After drying the residue was scraped out and different aliquots were dissolved in 5 ml sterile water and were stored at 4°C till used for further analysis.

7.3 Ethyl acetate Extraction: 10 g of the extract was weighed accurately and dissolved in 100 ml of ethyl acetate solution taken in a 250 ml round flat bottomed flask. This was then kept on a soxhlet apparatus and refluxed for 3 hours, after which it was allowed to cool down to room temperature and filtered using a Whatman Filter paper no 1. The filtrate was then collected and dried to dryness first on a water bath and then in an oven. After drying the residue was scraped out and different aliquots were dissolved in 5 ml sterile water and were stored at 4°C till used for further analysis.

- **Preparation of Mc Farland standard Turbidity Standards**

Mc Farland standards were prepared by adding specific volumes of 1% sulphuric acid and 1.174% barium chloride. Mac Farland 0.5 standard were used in this study, which contains 99.5ml of 1% sulphuric acid and 0.5 ml of 1.174% barium chloride. Solution is dispensed into tubes comparable to those used for inoculum preparation, which were sealed tightly and stored in dark at room temperature. The Mc Farland 0.5 standard provides turbidity comparable to a bacterial suspension containing $1.5x10^8$ cfu/ml (NCCLS 1993)

- **In Vitro Anti Bacterial Study:**

The modified agar-well diffusion method of Cappuccino and Sherman (1999) was employed to study the antibacterial activity of the plant extracts. 3.7% of Muller Hinton Agar was mixed with hot distilled water and autoclaved at 15 lb pressure for 15 minutes. After autoclaving, it was allowed to cool to 45° C-50° C. Then the medium was poured into sterilized Petri dishes with a uniform depth of approximately 4 mm. The agar medium was allowed to solidify and then stored at 4°C till used for further analysis.

- **Disc Diffusion (Kirby–Bauer) Method**

The disc diffusion test was done for each isolate on Mueller-Hinton agar. The turbidity of the broth was adjusted according to 0.5 Mc Farland standards by adding sterile saline. A sterile cotton swab was saturated by dipping into standardized bacterial culture. Lawn culture of the test strain was prepared by swabbing to give a uniform inoculum to the entire surface. The plates were allowed to dry, after which wells were bored in the middle of the well with the help of a cork borer and 0.1 ml of the sample was loaded into the well. The plates were first incubated at 25° C for 30 minutes and then shifted to 37°C for 18 – 24 hours. After incubation the plates were examined and zone of inhibition were measured. All the tests were carried out in triplicates and their mean value was calculated.

To screen the anti bacterial activity against the tested organisms a standard was used which was antibiotic amoxycillin (5 mg/ml) which showed a good zone of inhibition against the tested organism.

The results are as tabulated in **Table 7.**

Sr No	Name of the Organism	Aqueous Extraction	Methanolic Extraction	Ethyl acetate Extraction
1	*E.coli*	8 ± 0.57	7 ± 0.37	6 ± 0.37
2	*P.aeruginosa*	21 ± 0.81	25 ± 0.73	20 ± 0.73
3	*Salmonella*	9 ± 1.58	7 ± 0.37	20 ± 0.73
4	*S.aureus*	25 ± 0.81	31 ± 0.84	22 ± 0.73
5	*K.pneumoniae*	5 ± 0.37	7 ± 0.37	20 ± 0.73

Table 7 The zone of inhibition exhibited by the poly herbal formulation; Sample Concentration (500 mg/ml); n = 3

8.0 HPLC (High Performance liquid chromatography)

It is a chromatographic technique that is used to separate a mixture of compounds. HPLC typically utilizes different types of stationary phases, a pump that moves the mobile phase(s) and analyte through the column, and a detector that provides a characteristic retention time for the analyte. The pump provides the higher pressure required to propel the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. This allows for a better separation on columns of shorter length when compared to ordinary column chromatography. The figures/graphs are as depicted in **Figures 1 to 6**

Chromatographic Conditions

Mobile Phase: Water: acetonitrile: glacial acetic acid

9 1 0.2

Detection: 272 nm

Pressure: 121 kgf/cm^2

Temperature: Room Temperature

Flow Rate: 1 ml/min

Column: luna C 18 250 x 4.6 mm (5 µ)

Detector: UV Visible

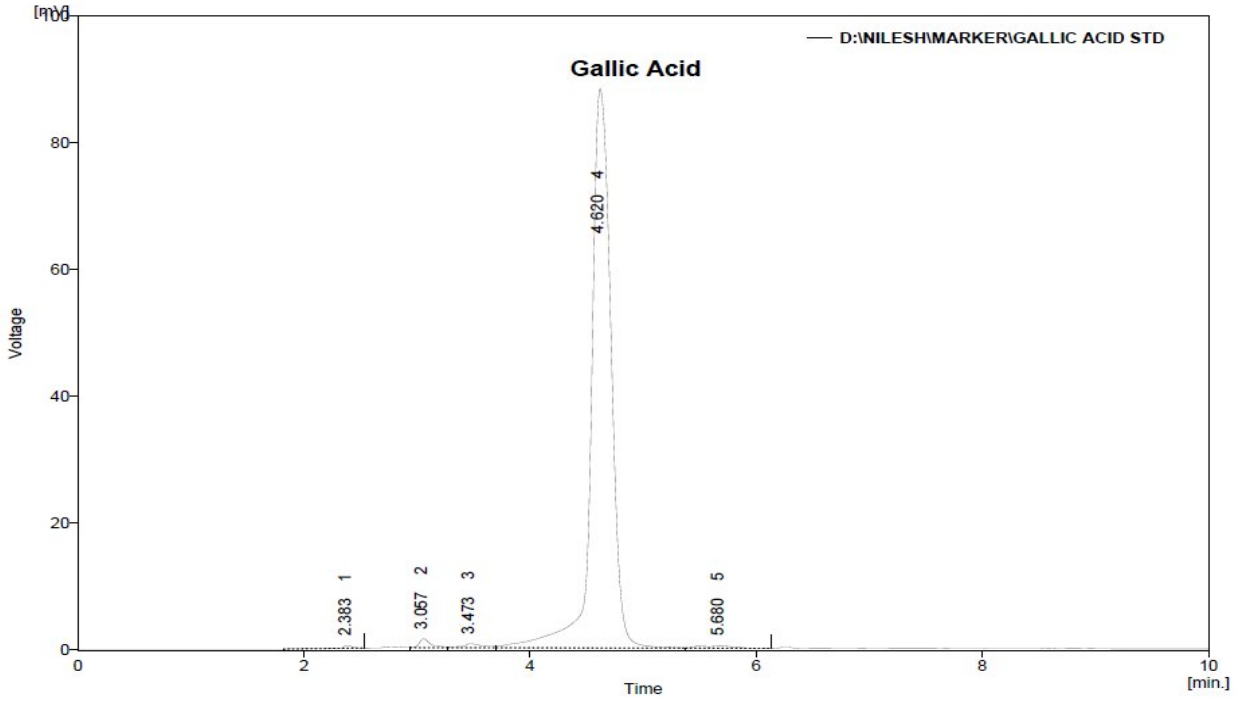

Figure 1 Reference Standard Gallic acid

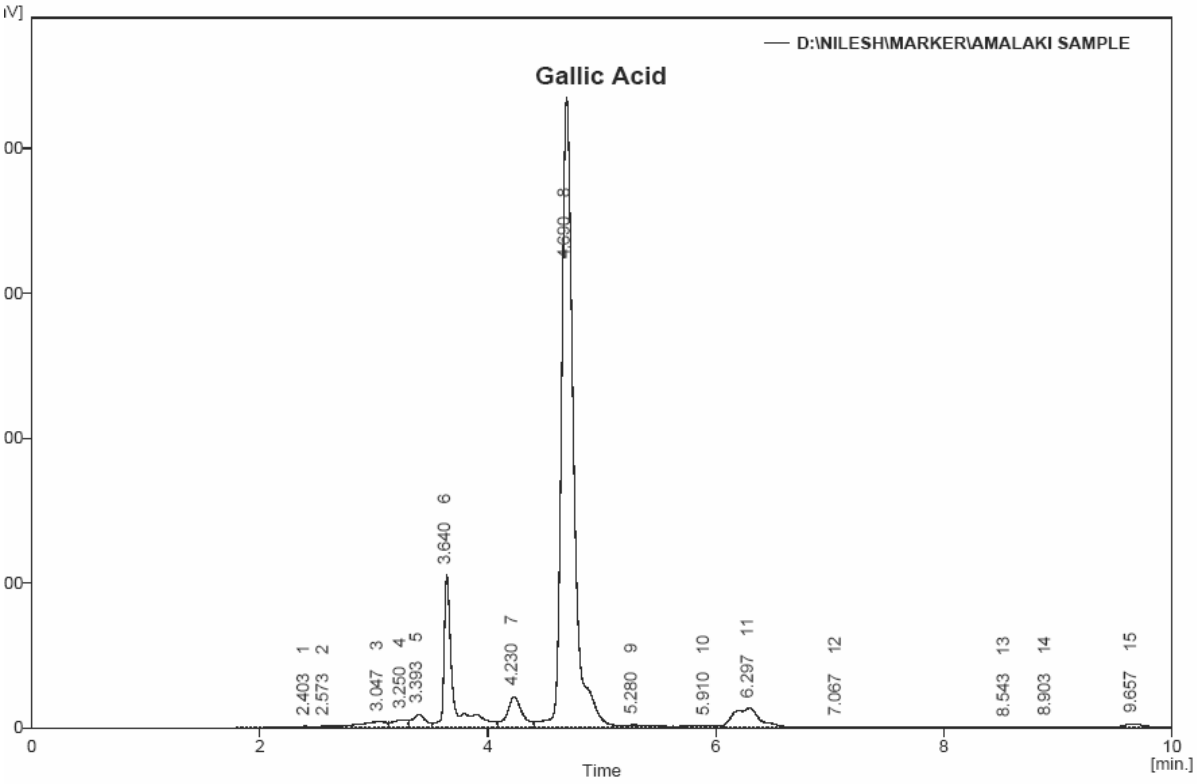

Figure 2 Amalaki

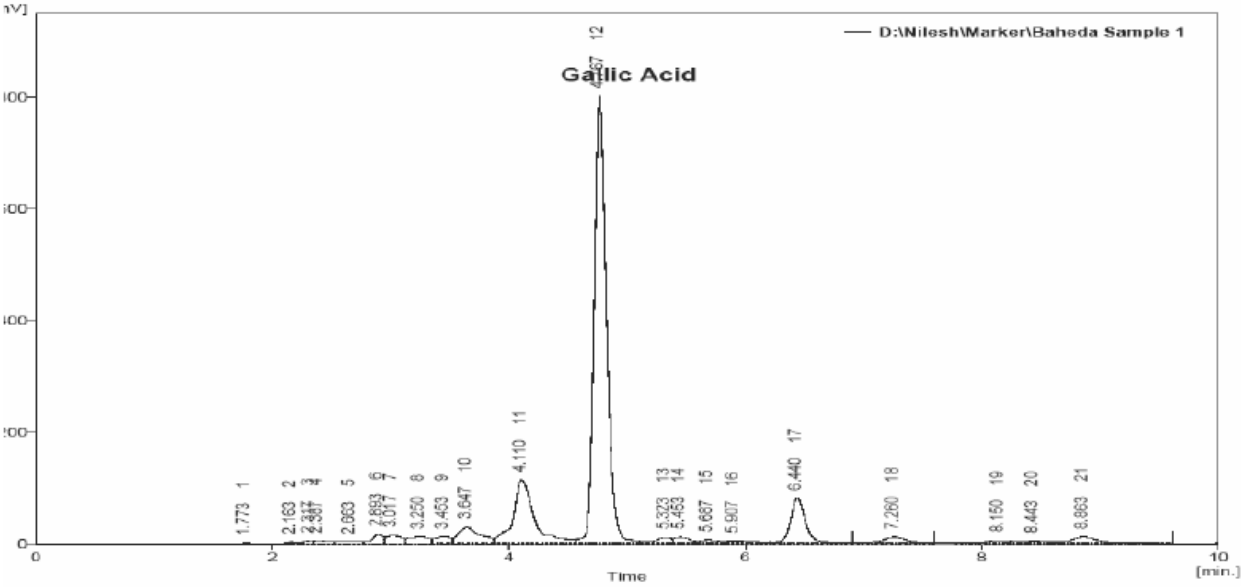

Figure 3 Bibhitaki

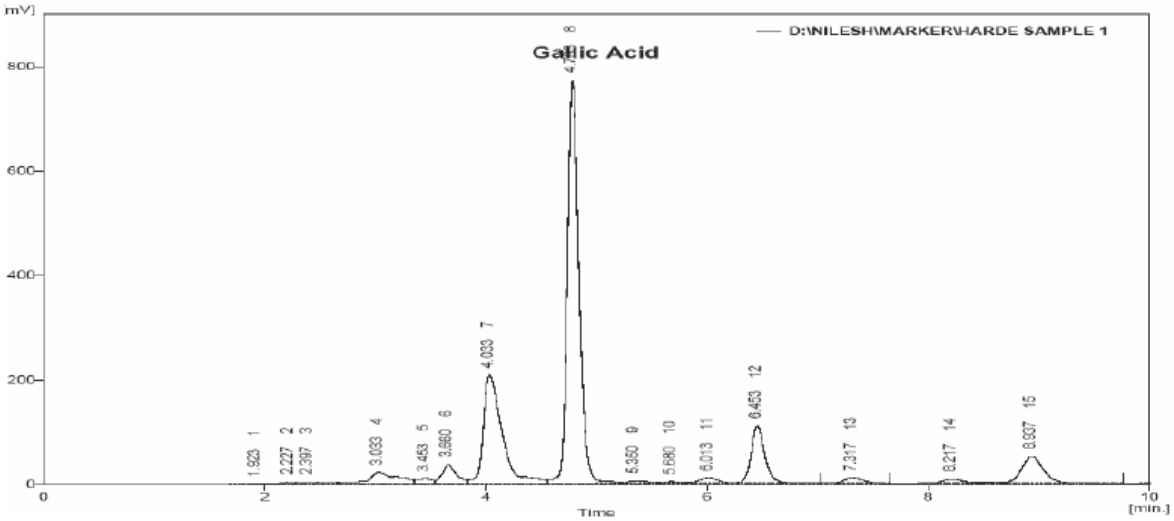

Figure 4 Haritaki

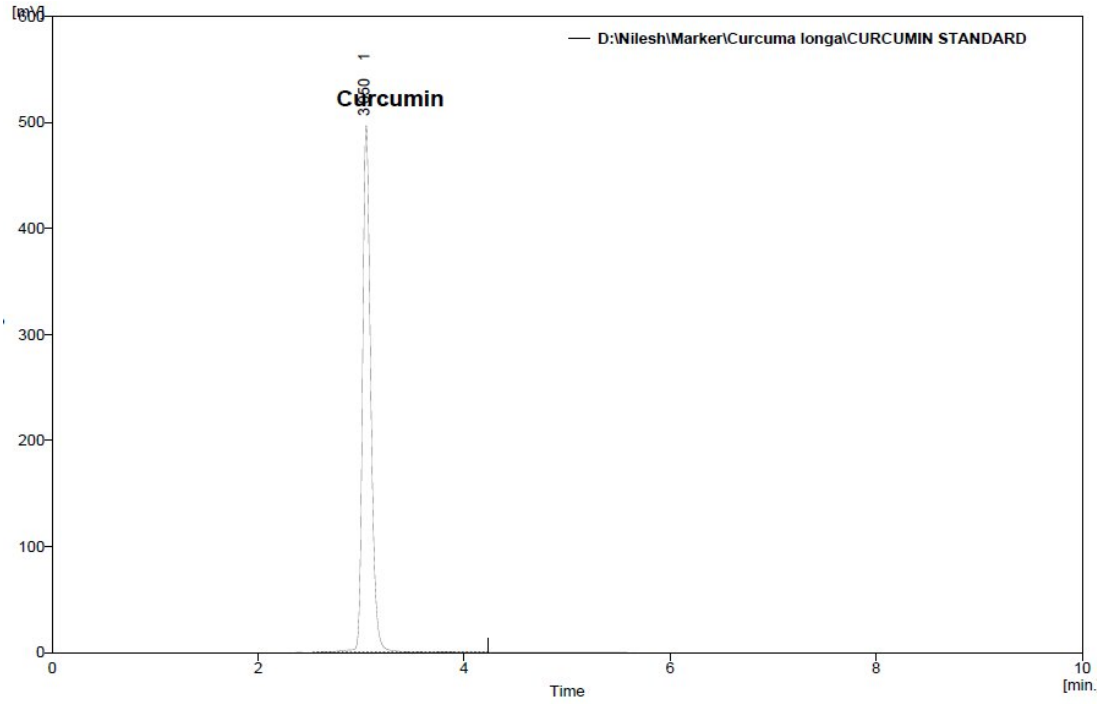

Figure 5 Reference Standard - Curcumin

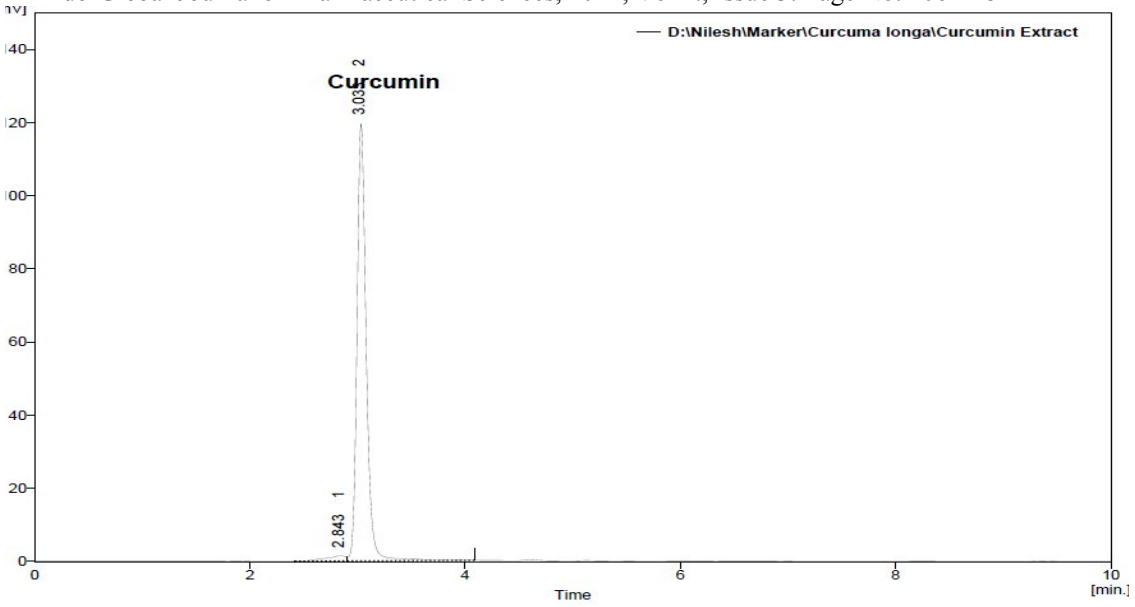

Figure 6 Curcumin Extract (Haridra)

9.0 HPTLC (High Performance thin layer chromatography)

High performance thin layer chromatography (HPTLC) is used for the quality assessment for the evaluation of botanical materials. It allows for the analysis of a broad number of compounds present. It is a liquid chromatography which involves the separation of the compounds on the basis of their polarity.

Procedure: Apply 6 µl of Test solution on a precoated silica gel 60 F_{254} TLC plate (E.Merck) of uniform thickness of 0.2 mm. Develop the plate in the solvent system to a distance of 8 cm. Spray the plate with anisaldehyde – sulphuric acid reagent. Heat the plate at 100 – 105°C until the colour develops. Scan the plate densitometrically at 540 nm. Record the peak area under curve and plot the calibration curve.

The results are as tabulated in **Table 8 & Figure 7.**

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.03	1.0	0.06	18.8	1.46	0.07	0.1	287.1	0.62
2	0.14	0.2	0.15	14.7	1.14	0.17	0.2	190.7	0.41
3	0.20	0.2	0.28	363.7	28.30	0.32	34.1	13491.7	29.00
4	0.32	34.1	0.35	59.5	4.63	0.36	36.8	1658.6	3.56
5	0.36	36.8	0.38	85.2	6.63	0.41	54.7	2865.5	6.16
6	0.41	54.7	0.46	154.6	12.03	0.51	52.9	7942.2	17.07
7	0.51	52.9	0.53	96.9	7.54	0.55	0.6	2025.3	4.35
8	0.55	1.7	0.60	81.0	6.30	0.61	74.3	2550.2	5.48
9	0.61	74.3	0.64	120.4	9.37	0.65	98.5	3322.6	7.14
10	0.65	98.5	0.67	182.6	14.21	0.79	9.3	8684.4	18.67
11	0.86	0.6	0.91	107.9	8.40	0.97	0.0	3508.2	7.54

Table 8 Densitometry Results

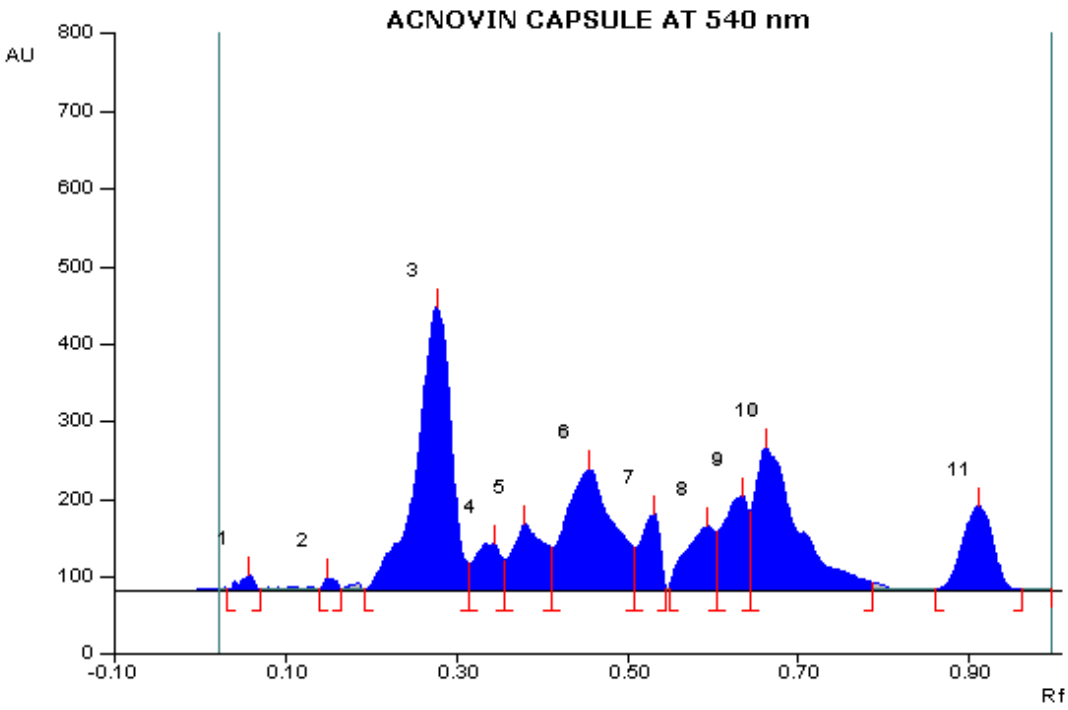

Figure 7 Chromatogram of the final poly herbal formulation

DISCUSSION & CONCLUSION

The final product was made keeping in mind the activity of the ingredients in regard to the skin. All the raw materials used have good effect on the skin. The sample is tested for all the possible parameters and is known to have good anti microbial activity as well.

The phytoconstituents principally responsible for Sonamukhi's characteristic action are two anthraquinone glycosides namely; sennoside A and sennoside B.

Sennoside A and B together are responsible for upto 40 – 60% activity of crude senna. Senna also contains small quantities of other anthraquinones such as sennosides C and D, rhein 8-glucoside, rhein-8- diglucoside, aloe-emodin, 8-glucoside, anthrone diglucoside and rhein. Additionally senna contains napthalene glycosides (tinnevellin glycoside and 6-hydroxy musizin glycoside), flavonoid (kaempferol), phytosterols, myricyl alcohol, salicylic acid, chrysophenic acid, and mucilage, resin and calcium oxalate[25, 26]

- The root bark of **Sariva** showed antioxidant activity. The ethanolic extract was reported to be effective chemoprotective agent and prevented oxidative stress and tumour in skin. On the **Skin** it is cooling, sweet and bitter flavours and affinity for rakta dhatu clears inflammation from the skin; used in eczema, psoriasis, urticaria, acne rosacea and acne from aggravated bhranjaka pitta. It 'cleans' the blood, reduces lymphatic swellings, stops itching and reduces suppuration[27-29]

Haridra is useful both internally as well as externally. It is known for its antiallergic potential. It also have been cited for anti-inflammatory, anti-bacterial, and antioxidant effects

Amalaki is used in eczema and psoriasis**.** For **immunity system** the seed's infusion is a tonic and is used in general debility. It is known to improve immunity of the body and helps to control infection.

Bibhitaki primarily supports the healthy formation of three bodily tissues-nutrients plasma (rasa dhatu), muscle (mamsa dhatu) and bone (asthi dhatu). It has been used both internally as well as externally.

- In Ayurveda Haritaki is the best for 'Srotoshodhana' or purifying the channels of body. It is also useful in skin disorders with discharges like allergies, urticaria and other erythematous disorders.

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