Journal of Research in Pharmacy
Research Article
www.jrespharm.com

Formulation & Evaluation of ointment containing anthocyanin of *Garcinia indica*: A single arm open label study

Anita R. DESAI [1]*, Laxman S. VIJAPUR[1], Madhushree[1], Prakash NARABOLI[2]

[1] Department of Pharmaceutics. BVVS Hanagal Shri Kumareshwar College of Pharmacy, Bagalkote -587101, Karnataka, India.
[2] Department of Panchakarma. BVVS Ayurved Medical College and Hospital, Bagalkote-587101, Karnataka, India.
*Corresponding Author. E-mail: anitardesai@gmail.com. (A.D); Tel. 7259898837.

Received: 09November 2022 / Revised: 09 February 2023 / Accepted: 13 February 2023

ABSTRACT: The study was aimed to formulate and evaluate ointment containing anthocyanin extract to study of anti-fungal activity. Anthocyanin was extracted from the *Garcinia indica*.by maceration technique using 0.1% HCl(v/v) inwater asa solvent. Anthocyanin ointment were formulated using various excipients and different concentration of anthocyanin. Formulated ointment was evaluated for pH, spreadability, viscosity, extrudability, drug content, *in-vitro* anthocyanin release, and best formulation were evaluated for Clinical study. The pH of formulations was fairly constantabout6.6-6.8 with in the range of skin pH. Formulation F4 showed less viscous and showed good spreadability and F5 showed excellent extrudability compared to other ointment formulations. The formulations F5 showed better percentage anthocyanin content of 97.96±0.70 respectively. All formulations were evaluated for cumulative percentage anthocyanin release, were F5 formulation showed92% at the end of 6 hour. Anthocyanin extract and formulation F5 showed anti-fungal activity against fungi pathogen such as *Candida albicans* and *Candida krusei*. Formulation F5 showed positive results against *In-vivo* superficial dermatophytic infections and effect showed significant on all parameters.

KEYWORDS: Anthocyanin; *Garcinia indica*;Anti-fungal;Superficial dermatophytic infection study; Topical ointment.

1. INTRODUCTION

Anthocyanins are the most important water-soluble pigments in plants. They are liable for imparting splendid red, blue and purple shades to organic products, vegetables, blossoms and grains. In plants, they act as pollination and seed dispersal attractants, provide protection from UV radiation and have antiviral and antimicrobial properties in plants, because of there colorings and medicinal characteristics, anthocyanins have recently become one of the most promising components for the functional food industry[1]. Apart from fruits and vegetables, *Garcinia indica*extract especially from itsrind, are rich in anthocyanins[2].Anthocyanins are salts of flavylium or phenyl-2-benzopyrylium, which are phenolic chemicals that belong to the flavonoid group[3].Flavonoids and anthocyanin shows anti-fungal, anti-imflammatory, anti-microbial, anti-oxidant activity and anti-cancer activity. The stability of anthocyanin is dependent on pH, light, tempertare and its struture[4-9].

Kokam (*Garcinia indica choisy*) trees are found in humid tropical regions of Western Ghats of India. Most useful part of the plant is the fruit of Kokam. This fruit is of commercial importance owing to its enormous medicinal properties. Genus *Garcinia* of the Clusiaceae family includes around 200 species of trees or shrubs, of which *Garcinia indica*is the most common. In Ayurveda, Kokam is traditionally used for edema, rheumatism, delayed menstruation, constipation, bowel complaints, intestinal parasites, skin rashes and burns. This plant is also pharmacologically studied for free radical scavenging, anti-bacterial, anti-fungal, anti-cancer, anti-inflammatory, anti-obesity and anti-ulcer activities[10]. Kokum rind contains three important chemical constituents such as garcinol, hydroxycitric acid and anthocyanin pigment. Kokum is rich source of anthocyanin, it contains two major components such as cyanidin 3-glucoside and cyanidin 3-sambubioside [11].

Topical delivery is the application of a drug-containing formulation to the skin with the aim of limiting the drug's pharmacological or other effects to the skin's surface or deeper layers in order to treat cutaneous conditions like psoriasis or the cutaneous manifestations of more widespread illnesses like acne, because they deliver a treatment to a specific location, topical formulations are unquestionably among the most challenging

How to cite this article: Desai AR, Vijapur LS, Madhushree, Naraboli P. Formulation & Evaluation of ointment containing anthocyanin of *Garcinia indica*: A single arm open label study. J Res Pharm. 2023; 27(4): 1547-1559.

medications to develop. A topical formulation must interact with the skin environment after application in order to achieve optimal skin absorption, this interaction may impact the rate at which the component is released [12].The term "Topical delivery system" describes a technique for treating local ailments that involves applying the formulation to the skin, eyes, nose, and vagina. When a medicine is applied topically, it avoids the hepatic first pass metabolism, changes in gastric pH, and fluctuations in plasma levels that are typically experienced when a drug is delivered orally [13].
Topical delivery methods use a variety of pharmaceutical dosage form, including semesolids, liquid preparations, sprays, and powders. The most often used semisolid formulations for topical medication administration are ointments, gels and creams [14].

Fungi exist in two basic forms: yeasts and moulds. Yeasts are typically single, small, oval cells, whereas mould colonies consist of filamentous strands called hyphae [15]. Fungal infections can affect anyone, and they can appear on several part of the body. Different types of fungi can cause fungal infections. In some cases, fungi that aren't typically found on or inside your body can colonize it and cause an infection. In other cases, fungi that are normally present on or inside your body can multiply out of control and cause an infection. Fungal infections can be contagious. They can spread from one person to another. In some cases, you can also catch disease-causing fungi from infected animals or contaminated soil or surfaces. A fungal infection is also known as mycosis. Although most fungi are harmless to humans, some of them are capable of causing diseases under specific conditions [16].

The antifungal comprise a large and diverse group of drugs used to treat fungal infections. Antifungal drugs can take orally, apply them topically, or administer them intravenously through an IV drip. Antifungal medications usually work either by killing the fungal cell or stopping them from growing and multiplying. Parts of the cell that the antifungal drugs target include the fungal cell membrane and the fungal cell wall. These are both protective parts of the cell that can cause the cell to leak and die when damaged [17].

The major objective of this study is to extract anthocyanin and prepare a herbal topical formulation that contains anthocyanin concentrate. The present study aimed to formulate herbal ointment with anthocyanin extract to study thepotential of prepared formulation on the superficial dermatophytic fungal infections in the patients.

2. RESULTS

2.1. Confirmatory test of Anthocyanin

Test	Observation	Inference
a) UV-Visible spectrophotometer	λ max observed at 516 nm	Anthocyanin Confirmed
b) 1ml *Garcinia indica* extract mixed with 2ml 2M NaOH	Color changes to green color	Anthocyanin Confirmed
c) 1ml *Garcinia indica* extract boiled 5min with 2ml 2m HCl	Extract remains stable purple color	Anthocyanin Confirmed

Table 1: Anthocyanin confirmatory test

2.2. Homogeneity:

All the prepared ointment formulations were visually analyzed they were found to be clear, there was no aggregation found and free from presence of particles.

2.3. Centrifugation:

There were no observable sediment in centrifuge tests and the ointments kept their uniformity.

2.4. Evaluation of formulation

2.4.1. pH determination, Percentage Anthocyanin content, Extrudability, Viscosity, Spreadability and *In-vitro* anthocyanin release study.

Formulation Code	pH (Mean ± SD)	% Anthocyanin Content (Mean ± SD)	Extrudability in percentage (Mean ± SD)	Viscosity in cps (Mean ± SD)	Spreadability in gm.cm/sec (Mean ± SD)
F1	6.7± 0.05	96.33 ± 2.13	90.2 ± 0.2	39850 ± 126	5.13 ± 0.26
F2	6.8± 0.32	93.5 ± 1.86	94.3 ± 4.88	32013 ± 287	5.34 ± 0.27
F3	6.6± 0.05	96.63 ± 2.11	93.7 ± 2.27	36019 ± 293	5.33 ± 0.29
F4	6.6± 0.11	95.7 ± 1.56	94.73 ± 1.15	29050 ± 369	5.24 ± 0.37
F5	6.8± 0.3	97.96 ± 0.70	95.86 ± 2.05	24498 ± 214	5.39 ± 0.19

Table 2a: pH, percentage anthocyanin content, extrudability, viscosity, spreadability(n=3).

Time (hours)	Percentage Anthocyanin Release (%)					
	Formulation Code					
	Anthocyanin	F1	F2	F3	F4	F5
0.5	19.2±0.05	16±0.11	17.3±0.11	18.4±0.15	17.9±0.13	18.6±0.15
1	35.9±1.03	28.2±0.32	27.3±0.22	28.4±0.25	26±0.11	28.32±0.30
2	43.1±1.25	33.4±1.56	35.7±1.32	36.86±0.9	37.4±1.22	37.1±1.18
3	57±0.98	41.7±1.13	44.2±1.10	47.1±1.21	46.6±1.19	47.1±1.21
4	66.7±1.36	53.8±0.65	54.5±0.78	65.5±1.26	57.3±0.98	65.8±1.28
5	85.6±0.67	71.2±0.12	74.4±0.32	76.5±0.66	76.5±0.66	84.4±0.62
6	95.2±0.56	87.3±0.95	86.8±0.92	91±1.41	88.4±0.98	92±0.39

Table 2b: *In-vitro* Release tudies of Anthocyanin (n=3).

2.5. Anti-fungal Activity

Sl.no	Organisms	Zone of Inhibition (mm)						
		F1	F2	F3	F4	F5	F6 Aanthocyanin Extract	Standard (Fluconazole)
01	***Candida albicans***	R	R	R	R	11 mm	12 mm	38mm
02	***Candida krusei***	R	R	R	R	10mm	15mm	39mm

R-Resistance

Table 3: value of zone of inhibition by disc diffusion method

(A) **(B)**

Figure 1: Zone of Inhibition of Anthocyanin extract and Ointment formulations v/s standard (Fluconazole) A) *Candida albicans*. B) *Candida Krusei.*

2.6. Single arm open label superficial dermatophytic clinical study.

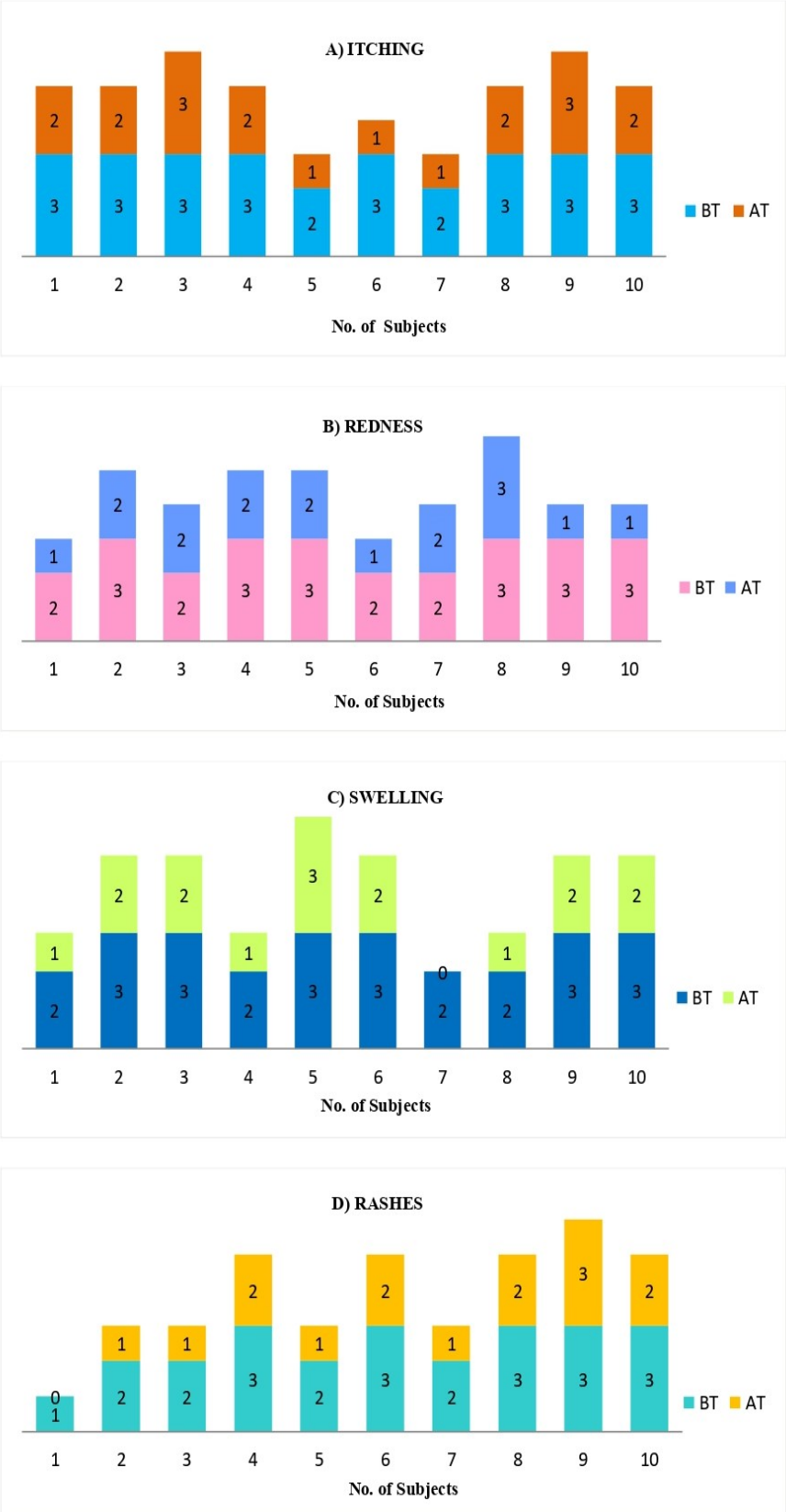

Figure 2: Effect of Anthocyanin ointment on subjects with following parameters before treatment (BT) and after treatment (AT); (A) Itching (B) Redness (C) Swelling (D) Rashes.

2.6.1. Distribution of subjects according to different grades of clinical study after treatment.

Gradings	Parameters			
	Itching	Redness	Swelling	Rashes
Grade 0	0%	0%	10%	10%
Grade 1	30%	40%	30%	40%
Grade 2	50%	50%	50%	40%
Grade 3	20%	10%	10%	10%

Table 4: Distribution of subjects according to different grades of Itching, Redness, Swelling and Rashes after treatment.

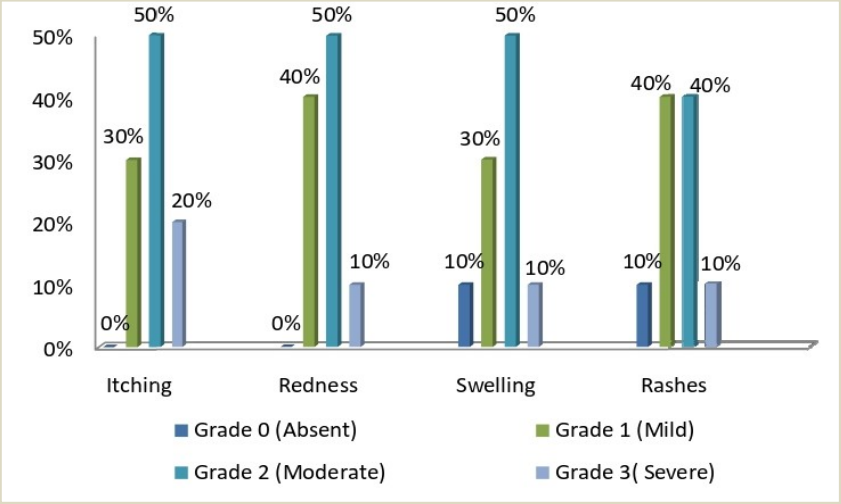

Figure 3: Distribution of subjects according to different grades of Itching, Redness, Swelling and Rashes after treatment.

2.6.2. Pie chart represented before treatment and after treatment of clinical study

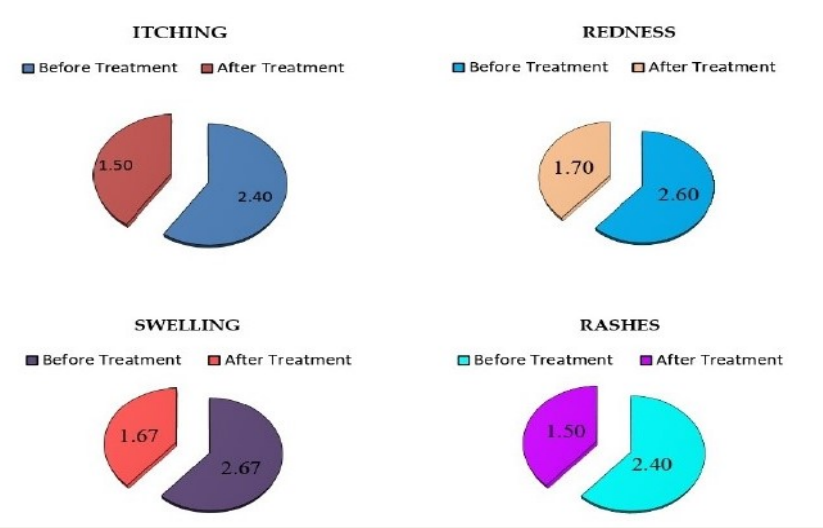

Figure 4: Effect of anthocyanin ointment on itching, redness, swelling, rashes before & after treatment. n=10, p=0.0007, p=0.0039, p<0.0001, p< 0.0001.

3. DISCUSSION

The current study aimed to formulate antifungal ointment from anthocyanin extract of *Garcinia indica* by maceration method using aqueous containing 0.1% HCl. Ointment were formulated by fusion method and tested for various evaluation criteria such as pH, viscosity, extrudability, spreadability, *in-vitro* release and antifungal activity.

3.1. Formulation studies.

Formulated ointment prepared by using different anthocyanin concentrations along with various excipients as mentioned in the table no 5. The main purpose of adding excipients is to increses the bulk of the formulation along with impartning desired properties. Eucalyptus oil was used as penetration enhancer because fungi have lipophilic cell membrane whic helps in penetration of anthocyanin in fungal cells and also it may increases the partition coefficient, diffusion coefficient, drug solubility in fungal cells. Cetyl alchol used as emulsifying agent to treat itchy skin and minor skin irritations. White soft paraffin used as ointment base to treating dry skin conditions such as eczema, ichthyosis, pruritus etc. Propylene glycol used as co-solvent. The types and usage rates of excipients decided from refering the literature.

3.2. Confirmation test for anthocyanin.

The maximum absorbance of the anthocyanin extract was at 516 nm in UV-Visible spectrophotometer. The addition of 2ml 2M NaOH to the 1 ml of aqueous extract of *Garcinia indica*, the initial red colour turn to green colour reported in table 1 due to the formation of a quinoidal base that confirms the presence of anthocyanin[18]. 1ml of *Garcinia indica* extract mixed with 2ml 2M HCl and heated for 5 minutes at 100°C. Extract remains in magenta (purple) color, due to the dominant form of anthocyanin shown in table 1.

3.3. Homogeneity.

Formulated ointment was investigated for the presence of a particle, improper mixing aggregation and another residue. So prepared ointments were filled in a transparent container and visually inspected. All the formulations were found to be homogeneous and there was no aggregate formation of particles.

3.4. Centrifugation.

The ointments homogeneity and sedimentation were investigated using a centrifuge test at 10,000 revolutions per minute (RPM). Additionally, upon inspection, there was no sediment, and the ointments maintained their homogeneity.

3.5. Evaluation Parameter

3.5.1. pH

To determine the pH of the various anthocyanin ointment formulations were conducted, and the results are reported in table 2a. All ointment formulations had pH values between 6.6 to 6.8, Formulaion F1 (6.7±0.05), F2(6.8±0.32), F3(6.6± 0.05), F4(6.6± 0.11), F5(6.8± 0.3), which is within the range of pH that the skin hence ointments were considered non-irritants and can be used for topical applicationl[19].

3.5.2. Extrudability.

Extrudability testing is necessary to assess how easily product may be removed from their packaging and applied. The extrudability of the various anthocyanin ointment compositions was tested. Data shown in the table 2a. Formulation F1 showed (90.2±0.2), F2(94.3±4.88), F3(93.7±2.27), F4(94.73±1.15), F5(95.86±2.05). The extrudability values shows that the ointment has good extrudability. Among these formulations, the formulation F5 showed better extrudability than other formulations[20].

3.5.3. Percentage anthocyanin content:

Studies on the percentage anthocyanin content of the various anthocyanin ointment formulation were conducted and data shown in the table 2a. All ointment formulations showed good percentage drug content as formulation F1 showed (96.33±2.13%), F2(93.5±1.86%), F3(93.7±2.27%), F4(94.73±1.15%) F5(95.86±2.05%). Among these formulations, the formulation F5 showed better percentage drug content than other formulations[21].

3.5.4. Viscosity.

Viscosity tests were performed on various anthocyanin ointment formulations. Ointment formulations majorly depend upon its viscosity. If the oinment has a higher viscosity, the drug is released slowly, but if the ointment has a lower viscosity, the drug diffuses quickly into the diffusion medium. The various anthocyanin ointment formulations were subjected to viscosity studies. Formulation F1 showed (39850±126), F2(32013±287), F3(36019±293), F4(29050±369), F5(24498±214). All the ointment formulations showed optimum viscosity and the ability to remain in the site of application for prolonged time. data shown in the table 2a[22].

3.5.5. Spreadability.

Spreadability tests were conducted on the various anthocyanin ointment formulations. All the ointment formulations showed optimum spreadability. Formulation F1 showed(5.13±0.26), F2(5.34±0.27), F3(5.33±0.29), F4(5.24±0.37), F5(5.39±0.19). The spreadability data show that a modest amount of shear made the ointment easily spreadable. Low viscosity ointment exhibits superior spreading characteristics as data represented in the table 2a[23,24].

3.5.6. *In-vitro*anthocyanin release study.

The various anthocyanins ointment formulations were subjected to *in-vitro* anthocyanin release studies and data is shown in the table 2b. The percentage anthocyanin release between every time intervals were fairly constant up to 6 hours. All the ointment formulations showed good percentage anthocyanin release. Among these formulations, the F5 (92%) formulation shows better anthcyanin release than F1(87.3%) F2(86.5%), F3(91%), F4(88.4) formulation. Viscosity is an important physical property that affects the rate of drug release. In general, an increase in viscosity decreases the rate of drug release. In our study, formulation with less viscosity such as F5 showed better rate of anthocyanin release than formulation with more viscosity such as F1,F2,F3,F4.

3.6. Anti-fungal studies.

The topical preparations were evaluated against particular fungal pathogen strains. The agar well disc diffusion method was used for the antifungal activity. Standard strains of *Candida albicans* and *Candida krusei* were used and a flucanazole was used as a control or for comparison. The zones of growth inhibition for each different anthocyanin concentration containing ointment formulation and also anthocyanin extract demonstrated that it was effective against *Candida albicans* and *Candida krusei* under investigation. As shown in table 3. Anthocyanin extract and formulation F5 containing anthocyanin concentrate (2.5%) was found to be most effective against *C. albicans and C. krusei* respectively and it is represented in figure 1.

3.7. Single arm open label superficial dermatophytic clinical study.

The superficial dermatophytic infection is one of the most common dermatologic diseases. Some of these infections are extremely resistance to therapy. Traditionally, the aqueous extracts are used orally and topically to cure skin diseases. In our study we have used anthocyanin concentrate of 2.5% as an anti-fungal agent in the form of ointment for the single arm open label superficial dermatophytic infection clinical study and anthocyanin of *Garcinia indica* hasanti-fungal properties was confirmed by *in-vitro* testing prior to the clinical study. As the study was for targeted medical condition with experimental therapy of topical anthocyanin for the management of dermal fungal infection, hence single arm open label clinical study design was chosen and control group were excluded.

Cyanidine-3-glycoside and Cyanidine-3-sambubioside are two major components found in the anthocyanin of *Garcinia indica*. Flavonoids which are potent antioxidants which contributes in the reduction of swelling. The delphinidin-3,5-glucoside is another major component present in the anthocyanin that contains anti-oxidant properties which help to prevent the redness. Chemical structure of anthocyanin is an important in determining their potential role and anthocyanin are described by an electron deficiency and also recognized as free radicals therefore anthocyanin considered to be used for preventing itching and rashes and to cure dermatitis or other mild fungus infections. Anthocyanin can reduce the expression of cyclooxygenase 2 (COX-2) through an NF-κB and Mitogen-activated protein kinase (MAPK) pathways dependence.[25]

Ointment containing anthocyanin (2.5%), was applied twice a day on the infection part/area for 1 month on 10 subjects including males and females. After 1 month from time of application clinical parameters associated with the superficial dermatophytic infection by fungus were rechecked and statistical analyzed results as shown in figure 4 indicated there is substantial reduction in infections and statistically significant in superficial dermatophytic infections caused by fungus.

3.7.1. Itching

The effects of anthocyanin on the itching of superficial dermatophytic infection in subjects, the values are expressed as mean n=10. Eight subjects were having Grade III itching before treatment in that 5 subjects showed moderate itching, 1 subject showed mild itching and 2 subjects doesn't showed any improvement after treatment as shown in figure 2. Two subjects were having Grade II itching before treatment in that 2 subjects showed mild itching after treatment. The statistical analysis revealed that the percentage relief of itching after treatment was 67.85% which implies moderate improvement and it showed statistically extremely significant in itching.

3.7.2. Redness

The effect of anthocyanin on the redness of superficial dermatophytic infection in subjects, the values are expressed as mean n=10. Six subjects were having Grade III redness before treatment in that 3 subjects showed moderate redness, 2 subjects showed mild redness and 1 subject doesn't showed any improvement after treatment shown in figure 2. Four subjects were having Grade II redness before treatment in that 2 subjects showed mild redness and 2 subjects doesn't showed any improvement after treatment. The statistical analysis revealed that the percentage relief of redness after treatment was 65.38% which implies moderate improvement and it showed statistically very significant in redness.

3.7.3. Swelling

The effect of anthocyanin on the Swelling of superficial dermatophytic infection in subjects, the values are expressed as mean n=10. Six subjects were having Grade III swelling before treatment in that 5 subjects showed moderate swelling and 1 subject showed mild swelling after treatment shown in figure 2. Four subjects were having Grade II swelling before treatment in that 3 subjects showed moderate swelling and 1 subject doesn't showed any improvement after treatment. The statistical analysis revealed that the percentage relief of swelling after treatment was 62.54% which implies moderate improvement and it showed statistically extremely significant in swelling.

3.7.4. Rashes

The effect of anthocyanin on the rashes of superficial dermatophytic infection in subjects, the values are expressed as mean n=10. Five subjects were having Grade III rashes before treatment in that 4 subjects showed moderate rashes and 1 subject showed mild rashes after treatment shown in figure 2. Four subjects were having Grade II rashes before treatment in that 4 subjects showed mild rashes and 1 subject doesn't showed any improvement after treatment. The statistical analysis revealed that the percentage relief of rashes after treatment was 62.5% which implies moderate improvement and it showed statistically extremely significant in rashes.

By the above statistical analysis, we can understand that treatment had positive effect and results showed extremely significant in itching, swelling and rashes and very significant in redness parameters.

4. CONCLUSION

Antifungal ointment formulations can be prepared using different concentration of anthocyanin extract. Among the used different anthocyanin concentrations, formulation F5 contains higher concentration of anthocyanin(2.5%) showed better results compared to other formulations. anthocyanin extract and formulation F5 showed anti-fungal activity against fungi pathogens such as *Candida albicans* and *Candida krusei.* Our research and results indicated that there is potential and phenomenal protective role of anthocyanin ointment F5 and statistical analysis results showed extremely significant in itching, swelling and rashes and very significant in redness parameters and it showed substantial reduction in the superficial dermatophytic fungal infections.

5. MATERIALS AND METHODS

5.1. Materials

White soft paraffin obtained from Loba chemie Mumbai. Genuine chemical Co in mumbai provided propylene glycol. Cetyl alcohol obtained from Thermo fisher scientific Mumbai, Eucalyptus oil obtained from SDFCL Mumbai. All in gradients used are analytical grade.

5.2. Methods

5.2.1. Extraction of Anthocyanin from *Garcinia indica*.

By soaking kokum rind powder in acidifier water that contains 0.1% hydrochloric acid in a beaker. The beaker lid was covered with aluminium foil and it was left at room temperature for 24 hours in a dark place. Filtration was done using muslin cloth after 24 hours. The filtrate was then dried by evaporation in a hot air oven at 40°C. The anthocyanin extract was then collected and stored at 4°C as shown in figure 5.[26]

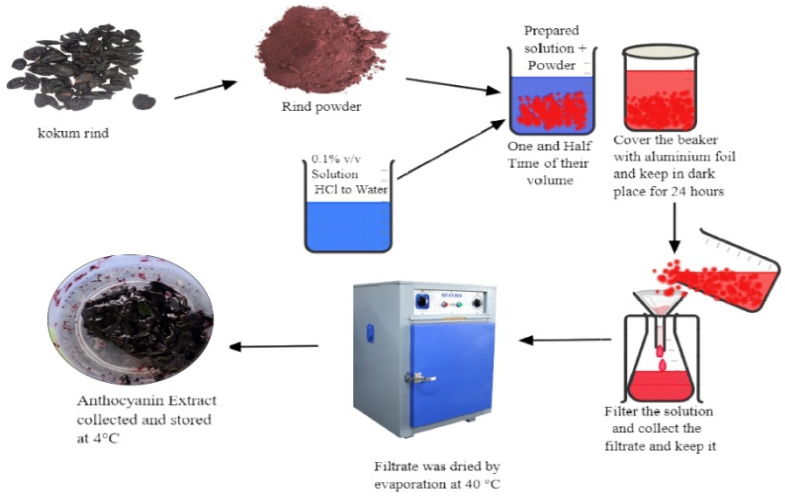

Figure 5: Schematic diagram for extraction of anthocyanin.

5.2.2. Ointment preparation method

Formulations containing anthocyanin extract were prepared by fusion method as shown in table 5. In this method, the constituents of the base were placed together in a melting pan allowed to melt together at specific temperature. After melting, the ingredients were stirred gently then cooled. Then anthocyanin extract was added respectively in the base. Then eucalyptus oil was added as a penetration enhancer and mixed properly by using ointment slab[27].

Formulation For 100 gm	Weight taken (gm)				
	F1	F2	F3	F4	F5
Anthocyanin concentration	0.5	1	1.5	2	2.5
Eucalyptus oil	8	8	8	8	8
Cetyl alcohol	10	10	10	10	10
White soft paraffin	76.5	76	75.5	75	74.5
Propylene glycol	5	5	5	5	5

Table 5: Composition of anthocyanin ointment formulation.

5.3. Confirmatory test for anthocyanin

- Using aqueous as a blank, the presence of anthocyanin in the extract was confirmed using a UV-Visible spectrophotometer. A spectrum was taken in the UV- Visible range that is 200 nm – 800 nm[28]
- One ml of the *Garcinia indica* extract mixed with 2 ml of 2M NaOH and change in the color was observed[18].
- One ml of the *Garcinia indica* extract was mixed with 2ml 2M HCl and heated for 5min at 100˚C.Stability in the color observed[29].

5.4. Homogeneity

Visual inspection was used to check the homogeneity of every developed ointment. They were examined for the absence of lumps[19].

5.5. Centrifugation

Prepared formulations were separately centrifuged in a tube of 10 cm long and 1 cm width for 10 minutes with 10,000 rpm and then studied for sedimentation and ointment stability[20].

5.6. Evaluation of Formulation

5.6.1. pH determination

The pH of various formulation was determined by using digital pH meter. The 0.5gm of the weighed formulation was dispersed in 50ml of distilled water and the pH meter was calibrated with standard buffers solution before measurement and every time the measuring was repeated 3 times and the mean was calculated [19].

5.6.2. Extrudability

About 5 gm of the ointment formulation was filled in a clean, lacquered aluminum collapsible tube on crimped end of the tube then clamp was applied to avoid any rollback, and the cap was removed and ointment was extruded. The extrudability was then determined by measuring the amount of ointment extruded through the tip. The extruded ointment was collected and weighed and the percentage of ointment extruded was calculated and grades were allotted[16]. Calculated by using following formula[20].

$$\% \text{ Extrudability} = \frac{\text{Amount of ointment extruded from the tube}}{\text{Total amount of ointment filled in the tube}} \times 100$$

(>90% Extrudability: Excellent)
(>80% Extrudability: Good)
(>70% Extrudability: Fair)

5.6.3. Percentage anthocyanin content

Content of anthocyanin in the formulation was determined by diluting 10mg of anthocyanin equivalent to ointment in 100 ml of 6.8 pH phosphate buffer. Absorbance was recorded by using UV-Visible spectrophotometer at 516 nm using phosphate buffer as blank. Then anthocyanin content was calculated from the linear regression equation obtained from the calibration data and average of three determination was noted[21].

5.7.4. Viscosity

The viscosity of the prepared ointment formulations was determined using Brookfield viscometer using spindle no.64. The viscosity was measured in cps at 10 rpm at each speed, the reading was recorded. The viscosity determination of samples was repeated tree times [22].

5.7.5. Spreadability

Spreadability was determined by the apparatus which consists of a glass plateblock, which was provided by a pulley at one end. By this method spreadability was measured on the basis of slip and drag characteristics of ointments. About 0.5gm of sample was placed on this ground slide. The ointment was then sandwichedbetween this slide and another glass slide having the dimension of fixed ground slideandprovidedwiththehook. A 20 gm weighted was placed on the top of two slides for 5 minutes to expel air and to provide a uniform film of the ointment between the slides. Excess of the ointment was scrapped off from the edges. The top plate was then subjected to pull of 20 gm. With the help of string attached to the hook and the time (in seconds) required by the top slide to cover a distance of 7.5 cm be noted. A shorter interval indicates better spreadability. Spreadability was calculated using the following formula[23,24].

$$S = M \times L/T$$

Where, S=Spreadability
M=Weight in the pan (tied to the upper slide in gm)
L=Length moved by the glass slide(7.5cm),
T=Time(in sec.)

2.6. *In-vitro* Anthocyanin release study

The *in-vitro* releases of Anthocyanin were studied using a modified diffusion testing apparatus. The freshly prepared phosphate buffer (pH 6.8) was used as a diffusion medium. Dialysis membrane grade15k were placed on top of the receptor chambers of the cells following the addition of the release media. Then the donor chambers were mounted on the membranes and clamped tightly. 10 mg of drug equivalent to ointment loaded into the donor chambers and 250 µl of the release medium were added to the top of the sample. The stirring speed of the modified diffusion cells was set at 100 rpm. About 4 ml of sample was withdrawn at a time interval of 1 hour and replaced with an equal volume of fresh diffusion medium. The aliquots were analyzed at 516 nm using UV spectrophotometer [30].

2.7. Anti-fungal activity study

The customized agar well disc diffusion method was used for the antifungal activity. Standard strains of *Candida albicans* and *Candida krusei* were used and a Fluconazole was used as a control or for comparison. Sabouraud agar media were used for fungi. After agar solidified, 50µl (0.05ml) of the different fungal cultures were spread onto plates using a sterile spreader. Plates were punch with 6mm diameter wells and filled with 25µl (0.025ml) plant extract and 0.1mg ointment formulations simultaneously, Fluconazole (100µg/ml). The fungal plates were incubated at room temperature. The diameter of the zone of inhibition was measured in millimeters after 120 hr [31,32].

2.8. Single arm open label clinical study

Superficial dermatophytic study was done for formulated anthocyanin concentrate ointment was done in prospective observational study over time period of 1 month at outpatient of BVVS Ayurved Medical College & Hospital. After obtained the approval from Institutional ethics committee on human subject research of BVVS Ayurved [BVVS/IEC/AMVB-2020-2/768] was obtained and documented**.** The 10 subjects, who had superficial dermatophytic infections of age 18-65 years were included either sex in the study after obtaining the written consent form from the subject and were treated. The data were extracted according to predefined study criteria. The subjects were instructed for the procedure of application of ointment twice a day. Subjects' analysis of infection was recorded, and parameter like swelling, redness, rashes and itching by the subjects. Each of these parameters were graded as absent (Grade 0), mild (Grade 1), moderate (Grade 2), severe (Grade 3) and given a corresponding score of 0-3, the four scores added together to give clinical score as shown in table 4. The data for present study as shown in figure 3 were collected from subject's case report and progress chart. The obtained final data were statistically processed using the paired student 't' test[33].

Acknowledgement: I wish to express my sincere gratitude to Dr. Mahantesh Salimath. Principal of BVVS Ayurved Medical College and Hospital, Bagalkote. For providing necessary facilities to carry out the clinical study.

Author contribution: Concept – A.D., L.V.; Design – A.D., L.V., P.N.; Supervision – A.D., L.V., P.N.; Resources – A.D., L.V., M; Materials – M.; Data Collection and/or Processing – M, P.N,. A.D; Analysis and/or Interpretation – A.D., L.V., P.N.; Literature Search – A.D., M.; Writing – A.D., L.V., M; Critical Reviews – A.D., L.V., P.N.

Conflict of interest statement: The authors declared no conflict of interest

REFERENCES

[1] Khoo HE, Azlan A, Tang ST, Lim SM. Anthocyanidins and anthocyanins: Colored pigments as food, pharmaceutical ingredients, and the potential health benefits. Food Nutr Res. 2017;61(1):01-21.[Cross Ref]
[2] Padhye S, Ahmad A, Oswal N, Sarkar FH. Emerging role of Garcinol, the antioxidant chalcone from *Garcinia indicachoisy* and its synthetic analogs. J Hematol Oncol. 2009;2(1):1-3.[Cross Ref]
[3] Paz R, Tamara G, Nalda R, Elena S, Jorge C and Carmen S. Encapsulation of phenols and anthocyanin's from pomegranates (*punica granatum L.*) by spray drying. Int J Food Sci Tech. 2010;45(7):1386-1394. [Cross Ref]
[4] Schaefer HM, Rentzsch M, Breuer M. Anthocyanins reduce fungal growth in fruits. Nat Prod Commun. 2008;3(8): 1267-1272. [Cross Ref]
[5] Pojer E, Mattivi F, Johnson D, Stockley CS. The case for anthocyanin consumption to promote human health: review. Compr Rev Food Sci Food Saf. 2013;12(5):483-508.[Cross Ref]
[6] Parle M, Dhamija I. Golden benefits of drinking kokam-cola. Int Res J Pharm. 2013;4(5):5-9.[Cross Ref]
[7] Jagtap P, Bhise K, Prakya V. A phytopharmacological review on *Garcinia indica*. Int J Herb Med. 2015;3(4):02-7.[Cross Ref]
[8] Bontempo P, De Masi L, Carafa V, Rigano D, Scisciola L, Iside C, Grassi R, Molinari AM, Aversano R, Nebbioso A, Carputo D. Anticancer activities of anthocyanin extract from genotyped *Solanum tuberosum* L."Vitelotte". J Funct Foods. 2015;19:584-593.[Cross Ref]

[9] Vijapur VP, Vijapur LS, Desai AR, Madhushree, Mudalagi SV, Daddenavar AP, Hatti LM, Kustagi V. Formulation, characterization and *in-vitro* evaluation of herbal sunscreen cream containing anthocyanin. Der Pharm chem. 2022;14(6): 1-6.[Cross Ref]
[10] Swami SB, Thakor NJ, Patil SC. Kokum (*Garcinia indica*) and its many functional components as related to the human health: a review. J food Res Technol. 2014;2(4):130-142.[Cross Ref]
[11] Zahid Z, Rezgui M, Nisar S, Azeem MW. Phytochemistry and medicinal uses of underutilized tree *Garcinia indica:* A detailed review. Int J Chem Biol Sci. 2016;10 :40-45.[Cross Ref]
[12] Singh Malik D, Mital N, Kaur G. Topical drug delivery systems: a patent review. Expert Opin Ther Pat. 2016; 26(2): 213-228.[Cross Ref]
[13] Tadwee IK, Gore S, Giradkar P. Advances in topical drug delivery system: A review. Int J Pharm Res Sci. 2012; 1(1):14-23.[Cross Ref]
[14] Verma A, Singh S, Kaur R, Jain UK. Topical gels as drug delivery systems: A review. Int J Pharm Sci Rev Res. 2013;23(2):374-382.[Cross Ref]
[15] Gary Garber. An Overview of Fungal Infections. Drugs.2001; 61(1): 01-12.[Cross Ref]
[16] Healthline:https://www.healthline.com/health/fungal-infection. (Accessed on: 26 June 2022).
[17] Medicinal news today: https://www.medicalnewstoday.com/articles/antifungal#types. (Accessed on 22 June 2022).
[18] Pallavi R, Elakkiya S, Tennety SS, Devi PS. Anthocyanin analysis and its anticancer property from sugarcane (*Saccharum officinarum* L.) peel. Int J Res Pharm Chem. 2012;2(2):338-345.[Cross Ref]
[19] Shaikh SC, Sanap D, Bhusari DV, Jain S, Kochar PP, Memon FS. Fabrication and evaluation of herbal ointment formulations of *Moringa Olifera* for topical delivery. Univers J Pharm Res. 2018; 3(4): 37-41.[Cross Ref]
[20] Muthukumar S, Parvin NI, Shobana S, Vimala N, Vinesha R, Sundaraganapathy R. Formulation and evaluation of novel herbal ointment for the treatment of fungal infection. Res J Pharm Technol. 2021; 14(3):1459-1464.[Cross Ref]
[21] Avish DM, SR. Lahoti. Formulation and evaluation of ointment containing sunflower wax. Asian J Pharm Clin Res. 2019; 12(8):115-120.[Cross Ref]
[22] Asija R, Dhaker PC, Nema N. Formulation & evaluation of voriconazole ointment for topical delivery. J Drug Discov Ther. 2015; 26(3):07-14.[Cross Ref]
[23] Navitha R, Rasheed SH, Manjunath SY. Formulation and evaluation of herbal ointment containing *Cajanus Cajan* extract. Int J Res Phytochem Pharmacol Sci. 2020; 1(2):52-57.[Cross Ref]
[24] Khan Y, Jan SU, Gul R. Formulation and evaluation of mefenamic acid ointment using penetration enhancers. Int J Curr Pharm Res. 2020; 12(6):114-118. [Cross Ref]
[25] Desai AR, Vijapur LS, Terdal S, Madhushree. Formulation and evaluation of gel containing *Punica Granatum*L. Extract for anti-inflammatory and anti-microbial activity. RGUHS J Pharm Sci. 2022;12(3):1-10.[Cross Ref]
[26] Nayak CA, Srinivas P, Rastogi NK. Characterisation of anthocyanins from *Garcinia Indica Choisy*. Food chem. 2010; 118(3): 719-724.[Cross Ref]
[27] Ghodke Amol D, Jain Shirish P, Bhusari Dipak V, Bochare Umesh J, Kothari Chetana R, Kochar Pooja P. Formulation and evaluation of anti-inflammatory & analgesic herbal ointment of *Punica granatum* peel. Word J Pharm Pharm Sci. 2019; 8(8):1307-1317.[Cross Ref]
[28] Gallik S. Determination of the anthocyanin concentration in table wines and fruit juices using visible light spectrophotometry. Cell Biol. 2012;2:1-2.[Cross Ref]
[29] Rakkimuthu R, Palmurugan S, Shanmugapriya A. Effect of temperature, light, pH on the stability of anthocyanin pigments in cocculus hirsutus fruits. Int J Multidiscip Res Mod Educ. 2016; 2(2):91-96.[Cross Ref]
[30] Bao Q, Newman B, Wang Y, Choi S, Burgess DJ. *In vitro* and *ex vivo* correlation of drug release from ophthalmic ointments. J Control Release. 2018;276: 93-101.[Cross Ref]
[31] Nagendra K, Kusum R, Ramachandran HD. Anti-microbial, antifungal and anti oxidant activity of ethanolic extracts of *Garcinia Indica* fruit. Int J Current Microbiol Appl Sci. 2014; 3(9):800-804.[Cross Ref]
[32] Varalakshmi KN, Sangeetha CG, Shabeena AN, Sunitha SR, Vapika J. Antimicrobial and cytotoxic effects of *Garcinia indica* fruit rind extract. Am Euras J Agric Environ Sci. 2010;7(6):652-656.[Cross Ref]
[33] Marty FM, Ostrosky-Zeichner L, Cornely OA, Mullane KM, Perfect JR, Thompson III G. Isavuconazole treatment for mucormycosis: a single-arm open-label trial and case-control analysis. Lancet infect dis. 2016; 16(7):82837. [Cross Ref]