J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 47(2): 497-507, 2023
Doi: 10.33483/jfpau.1212628

ORIGINAL ARTICLE / ÖZGÜN MAKALE

FORMULATION OF GEL CONTAINING *PHASEOLUS VULGARIS* L. EXTRACT AND TO EVALUATE ITS EFFICACY IN THE MANAGEMENT OF INFLAMMATION - A SINGLE ARM OPEN LABELLED CLINICAL STUDY

PHASEOLUS VULGARIS L. ÖZÜ İÇEREN JELİN FORMÜLASYONU VE İNFLAMASYON TEDAVİSİNDEKİ ETKİNLİĞİNİN DEĞERLENDİRİLMESİ - TEK KOL AÇIK ETİKETLİ KLİNİK ÇALIŞMA

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ABSTRACT

Objective: *The research works objective was to develop and test a gel that contained anthocyanin of Phaseolus vulgaris L. extract and to study it's anti-inflammatory activity.*
Material and Method: *By macerating Phaseolus vulgaris L. with 1% HCl (v/v) in methanol as the solvent, anthocyanin was extracted from the seed coat. Anthocyanin gels were produced utilizing a variety of polymers, including carbopol-940, sodium-CMC, chitosan, and other ingredients, including ethanol, lavender oil, propylene glycol, methyl paraben, and propyl paraben. All of the formulations of anthocyanin gel were studied for FTIR, pH, viscosity, spreadability, extrudability, drug content, and in-vitro drug release studies. The optimal formulation was then carried through a clinical investigation.*
Result and Discussion: *Formulations for anthocyanin gels can be made utilizing various gelling agents, such as carbopol-940, sodium-CMC, chitosan at different concentrations. Gel formulated using Chitosan (F3) showed better results of composition with decreased viscosity, enhanced*

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Submitted / Gönderilme : 01.12.2022
Accepted / Kabul : 15.03.2023
Published / Yayınlanma : 20.05.2023

extrudability, and a respectable amount of drug release. Formulation F3 by 2% of chitosan was best choice for anti-inflammatory activity in clinical study. Hence, Anthocyanin proved it's potential for inflammation that implies moderate to marked improvement with significant results in subjects.
Keywords: *Anthocyanin, anti-inflammatory, Phaseolus vulgaris L., topical gel*

ÖZ

Amaç: *Bu çalışmanın amacı, Phaseolus vulgaris L. ekstraktından antosiyanin içeren bir jel geliştirmek, test etmek ve bunun anti-enflamatuar aktivitesini incelemektir.*
Gereç ve Yöntem: *Phaseolus vulgaris L., çözücü olarak metanol içinde %1 HCl (v/v) ile maserasyonu yapılarak tohum kabuğundan antosiyanin ekstrakte edilmiştir. Antosiyanin jelleri, karbopol-940, sodyum-CMC, kitosan ve etanol, lavanta yağı, propilen glikol, metil paraben ve propil paraben gibi diğer bileşenler dahil olmak üzere çeşitli polimerler kullanılarak üretilmiştir. Antosiyanin jelin tüm formülasyonları FTIR, pH, viskozite, yayılabilirlik, ekstrüde edilebilirlik, ilaç içeriği ve in vitro ilaç salınım çalışmaları için incelenmiştir. Optimal formülasyon daha sonra klinik bir araştırma ile tespit edilmiştir.*
Sonuç ve Tartışma: *Antosiyanin jelleri için formülasyonlar, farklı konsantrasyonlarda karbopol-940, sodyum-CMC, kitosan gibi çeşitli jelleştirici ajanlar kullanılarak yapılabilmektedir. Kitosan (F3) kullanılarak formüle edilen jel, azalmış viskozite, gelişmiş ekstrüdasyon ve yeterli miktarda ilaç salınımı ile bileşimin daha iyi sonuçlar göstermiştir. Kitosan'ın % 2'si ile F3 formülasyonu, klinik çalışmada anti-enflamatuar aktivite için en iyi seçim olarak bulunmuştur. Bu nedenle, antosiyanin ile deneklerde orta ila belirgin iyileşme sağlayanmıştır ve antosiyaninin anti-enflamatuar potansiyeli olduğu kanıtlanmıştır.*
Anahtar Kelimeler: *Anti-enflamatuar, antosiyanin, Phaseolus vulgaris L., topikal jel*

INTRODUCTION

The immune system's biological response, inflammation, can be carried on by a variety of factors including pathogens, harmed cells, and toxic substances. The heart, pancreas, liver, kidney, lung, brain, digestive tract, and reproductive system may all experience acute or chronic inflammatory reactions, which may result in tissue damage or disease. Inflammatory cells are activated by both viral and non-infectious stimuli, as well as by cell injury, which also opens up inflammatory signalling pathways, most frequently the NF-B pathways. Organ-specific inflammatory responses, with an emphasis on the causes of inflammation, mechanisms of inflammatory response, and resolution of inflammation.

Redness, swelling, heat, pain, and loss of tissue function are signs of inflammation at the tissue level and are brought on by local immunological, vascular, and inflammatory cell reactions to infection or damage. Vascular permeability alterations, leukocyte recruitment and accumulation, and the release of inflammatory mediators are all significant microcirculatory events that take place throughout the inflammatory phase. A chemical signalling cascade that drives actions aimed at mending damaged tissues is started by the organism in response to tissue injury. These signals cause leukocytes to migrate to damaged areas from the overall circulation. The cytokines that are produced by these activated leukocytes cause inflammatory reactions [1].

Gel is a word used to describe semi-rigid systems in which the dispersing medium's ability to move is constrained by solvated macromolecules in the dispersed phase. Both "gel" and "jelly" can be traced back to the Latin word gelu, which meant "frost." Gel, which means "freeze" or "congeal," is derived from "gelatin." According to the USP, gels are semisolid systems made up of lattices of small, distinct particles or suspensions of large, liquid-pierced organic molecules or small, inorganic particles. Due to the presence of more covalent crosslinks, a higher density of physical bonds, or just less liquid, gels are typically thought to be more stiff than jellies. While some gel systems are transparent like water, and some others are turbid due to the ingredients' incomplete molecular dispersion or the possibility that they will form clumps that scatter light [2].

Phaseolus vulgaris L. belonging to the family Fabaceae or Leguminosae is one of the most widely used food crops and medicinal plants in the world and is well-known for its seed. *P. vulgaris* contains lysine, phenylalanine, and tyrosine, as well as carbs, protein, and other amino acids. In addition to

nutritional value, it contains bioactive substances such anthocyanin, phenolic acid, flavonoids, flavan-3-ol, condensed tannins [3].

MATERIAL AND METHOD

List of Chemicals

Anthocyanin (Black bean [*Phaseolus vulgaris* L.]), Methanol, Hydro Chloric acid Carbopol-940, Sodium Carboxymethyl Cellulose, Chitosan, Methyl paraben, Propyl paraben, Propylene glycol, Ethanol, Lavender oil. All ingredients used are analytical Grade.

Methods

Procedure for anthocyanin extraction: The seed coat powder of *Phaseolus vulgaris* L. taken in 1:10 ratio was soaked in 1% v/v solution of con. HCl in methanol, cover the beaker with aluminium foil and allow it stand in dark place for 24 hrs at room temperature. Later, muslin cloth was first used for filtering, then whatman filter paper. Anthocyanin was obtained by further filterate evaporation over a water bath at 40°C. The obtained dried anthocyanin was collected and preserved [4].

Identification Tests for Anthocyanin

IR Spectroscopy

FT-IR spectra of the drug sample that was obtained and the standard FT-IR spectra of the pure drug were compared using infrared spectroscopy [5].

Solubility Analysis

The drug's solubility in the intended dissolving medium was tested as aspect of the preformulation solubility analysis, which also involved choosing a suitable solvent to dissolve the drug and for anthocyanin release studies.

Initially we performed solubility by dissolving 0.5 g of Anthocyanin with 10 ml of methanol with continue stirring for 5-10 minutes. Then other solvents such as ethanol, phosphate buffer of pH 6.8 were used, as ethanol during formulation for dissolving Anthocyanin & phosphate buffer of pH 6.8 in drug release studies.

Confirmatory Test for Anthocyanin

- Using methanol as a blank, a UV-Visible Spectrophotometer was used to confrim the presence of anthocyanin in the extract. A spectra in the 200-800 nm UV-visible range was recorded.
- 2M HCl was dissolved in 1 ml of *Phaseolus vulgaris* L. extract, and the mixture was heated for 5 minutes at 100°C. The observation that the extract maintains its stable colour indicates that anthocyanin is present.
- When 1 ml of *Phaseolus vulgaris* L. extract is mixed with 2M NaOH, the extract's colour changes to show that anthocyanin is present [6,7].

Preparation of Anthocyanin Gel

The formulation of different gels, using concentrated *Phaseolus vulgaris L.* as gelling agents, Carbopol-940, Sodium CMC, and Chitosan were utilized along with extract (Anthocyanin) as the active ingredient as mentioned in Table 1 [8].

Table 1. Composition of gel formulations with different polymers such as Carbopol- 940, Sodium Carboxymethyl Cellulose and Chitosan

Weight taken in g (For 100g)	F_1	F_2	F_3
Extract	1	1	1
Carbopol-940	2	-	-
Sodium CMC	-	3	-
Chitosan	-	-	2

Table 1 (*continue*). Composition of gel formulations with different polymers such as Carbopol-940, Sodium Carboxymethyl Cellulose and Chitosan

Methyl paraben	0.2	0.2	0.2
Propyl paraben	0.02	0.02	0.02
Propylene glycol	5	5	5
Ethanol	5	5	5
Lavender oil	q.s	q.s	q.s
Water upto	100	100	100

Preparation of Carbopol-940/Sodium Carboxymethyl Cellulose/ Chitosan

Weigh accurately gelling agents, mix it with water/ glacial acetic acid and keep it aside for 24h for complete swelling. Methyl and propyl parabens (used as preservatives) were dissolved in half of the formulation water and heated further to 40°C. Gelling agents were then added and stirred with the mixture for 30 minutes at 1200 rpm using a magnetic stirrer. Anthocyanin extract in a predetermined quantity was weighed and thoroughly combined with propylene glycol and ethanol. A few drops of lavender oil were then added to the gel after this mixture had been gradually added and well combined into a gel [9-11].

By observing the consistency of gel water containing 1% glacial acetic acid was used, the weight of ingredients was taken in grams, for liquids volume was converted in mass during formulation.

Characterization of Anthocyanin Gels

pH Determination

The pH of the extract was measured using a digital pH metre after extraction for 48 hours, one week, two weeks, one month, and three months. Three repetitions of 1g of extract in 10 ml of distilled water were carried out [12].

Homogeneity

All prepared gels were placed in containers and inspected visually to determine their homogeneity. They were examined to check for aggregates and appearance [13].

Centrifuge Test

After 48 hours of preparation, formulations were placed into tubes that were 10 cm long and 1 cm wide, and they were centrifuged for 60 minutes at 2000 rpm using a centrifugal equipment (PR-24) At 5, 15, 30, and 60 minutes, the formulation's stability and sedimentation were determined [14].

Temperature Change Test

Tubes containing the formulation were placed at temperatures of 2-8°C, 25°C, and 40–45°C, and their appearance quality was checked 48 hours, 1 week, 2 weeks, 1 month, and 3 months afterwards. This was done to check the formulation's stability in various seasons and temperature conditions [15].

Evaluation of Anthocyanin Gels

Viscosity

A DV-E Brookfield viscometer was used to measure the formed gel's viscosity. Using spindle no. 64, the gels were rotated at 50 rpm, and the appropriate dial reading was recorded [16].

Spreadability

Utilizing a modified apparatus made of a wooden block with a pulley at one end, spreadability was evaluated. By using this method, spreadability was characterized based on the gels' properties of slip and drag. On this ground slide, an excess of the gel (approximately 2 g) under investigation was applied. The gel was then placed in a sandwich between this glass slide and another glass slide with a hook and a fixed ground slide dimension. For five minutes, a weight of 20 g was placed on the slide’s

top to push out air and create a consistent gel film between the slides. The edges of the gel were scraped clean of extra. Next, a 20 gramme pull was applied to the top plate. With the use of a thread fastened to the hook, record the amount of time (in seconds) needed for the top slide to travel 6.5 cm. Better spreadability is indicated by a shorter interval [17].

Extrudability

A clamp was used to stop any rollback and a closed collapsible tube containing about 20 gm of gel was squeezed firmly at the crimped end. The gel was extruded after the cap was removed. The extruded gel's volume was collected and weighed [18,19].

Anthocyanin Content

By accurately dissolving 2 g of gel in 100 milliliters of pH 6.8 phosphate buffer, the drug content of the gel formulation was determined. To fully solubilize the drug, the volumetric flask holding the gel solution was shaken for two hours. With the aid of filter paper, the solution was purified. Utilizing a UV-visible spectrophotometer (UV Shimadzu, Japan) with a maximum 536 nm reading and phosphate buffer as a blank, drug absorbance was measured using the method with appropriate dilution [20].

In-vitro Drug Release Studies

Using a modified diffusion testing apparatus, the *in-vitro* drug release study of anthocyanin from the prepared formulations was investigated. As a diffusion medium, freshly made phosphate buffer (pH 6.8) was employed. Gelatin sheet was used as a semi-permeable membrane that had been pre-soaked in the diffusion medium over the previous night. It was then attached to one end of a specially made glass cylinder with an inner diameter of 3.4 cm that was open on both sides. A glass cylinder known as the donor chamber was gently pipetted with 2 gm of the gel formulation within donar chamber. The cylinder was suspended in a 50 ml diffusion medium-filled beaker (acceptor chamber) so that the membrane just touched the surface. Using a magnetic stirrer, the acceptor chamber was kept at a temperature of 37 ± 2ºC. while being rotated at a rate of 50 rpm. At hourly intervals, 4 ml of the sample were taken out and replaced with an equal volume of diffusion media. The aliquots were examined using a UV spectrophotometer at 536 nm [21].

Clinical Studies

A Single Arm Open Label Clinical Study

Anti-inflammatory activity study for formulated Anthocyanins rich extract gel was done in prospective observational study over time period of 1 month at outpatient of BVVS Ayurved medical college & Hospital, Bagalkot. Prior approval from Institutional Ethics Committee (Reference. No: BVVS/IEC/AMVB-2020-21/767) on Human Subject Research was obtained and documented. The 10 subjects who was having Osteoarthritis of age 25-70 years were included of either sex in the study after obtaining the written consent from the subjects were treated. The data was extracted according to predefined study criteria. The subjects were instructed for the procedure of application of gel thrice a day. On each visit of study centre the containers of medication containing Anthocyanin gel was given for treatment. Subjects analysis of inflammation was made by the investigator for parameters like pain, swelling, tenderness, pain during flexion, pain during extension, crepitus. Each of these parameters was graded as absent (0), mild (1), moderate (2), severe (3) and the four scores added together to give clinical score. The data for present study was collected from subjects case report and progress chart. The obtained final results were statistically processed using the student paired 't' test [22].

RESULT AND DISCUSSION

Phaseolus vulgaris L. anthocyanin herbal gel formulations were developed and evaluated for their ability to reduce inflammation. Gel was prepared through dispersion process using a variety of polymers, including chitosan, carbopol-940, and sodium-CMC. They evaluated the pH, viscosity, spreadability, extrudability, drug content, and *in-vitro* drug release tests of the extracted drug and prepared formulation. The formulation was subjected for a clinical investigation.

Identification Tests for Anthocyanin

FTIR Studies

By using an IR spectrophotometer, compatibility studies were carried out. The peaks found in the spectra of each sample correlate to the peaks found in the spectrum of anthocyanin. The physical mixture of Anthocyanin and polymers indicates that the drug was compatible with formulation components, hence their is no interaction between them.

Solubility Analysis

The anthocyanin extract was completely soluble in methanol and other organic solvents, it was only partially soluble in water.

Confirmatory Test for Anthocyanin:

- A UV-visible spectrophotometer was used to analyse a *Phaseolus vulgaris* L. extract, and an absorbance at 536 nm confirmed the presence of anthocyanin.
- *Phaseolus vulgaris* L. extract was mixed with 2M HCl, the colour of the mixture remains stable red colour, which confirms the presence of Anthocyanin.
- *Phaseolus vulgaris* L. extract was mixed with 2M NaOH, the colour of extract red turns to stable brown colour, which confirms the presence of Anthocyanin.

Development of UV Spectroscopic method: The anthocyanin extract λ_{max} was found to be 536 nm and result of obtained peak is represented in Figure 1.

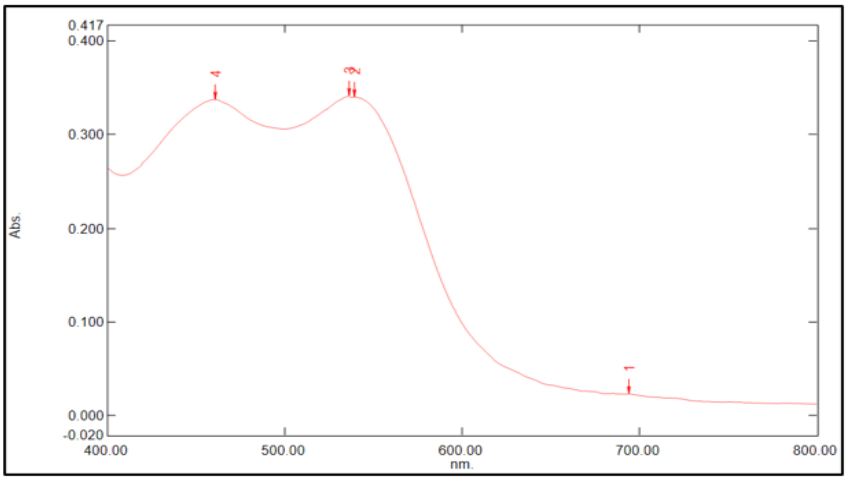

Figure 1. UV-Visible λ_{max} of Anthocyanin

Characterization of Anthocyanin Gels

pH Determination

The pH range of the all-gel formulations, which correlates to the normal pH range of the skin, was between 6.3-6.7.

Homogeneity

There were no aggregates formed and all the formulations were found to be homogenous.

Centrifuge Test

The gels maintained their uniformity and there was no observable sediment.

Temperature Change Test

There was no appearance change observed.

Table 2. Evaluation of Anthocyanin gels

Formulation Code	Parameters (n=3)			
	Viscosity in Cps ± S. D	**Spreadability in gm.cm/sec ± S. D**	**Extrudability in % ± S. D**	**Anthocyanin Content in % ± S. D**
F_1	11889.3 ± 9.01	26 ± 0.05	80.2 % ± 0.10	81.8 % ± 0.0005
F_2	11761.3 ± 9.01	40 ± 0.25	79.2 % ± 0.15	88.1 % ± 0.0005
F_3	11660 ± 10	43 ± 0.05	90.6 % ± 0.05	94.3 % ± 0.0005

The viscosity of F_3 was lower than F_1 and F_2, viscosity is inversely proportional to rate of drug release as viscosity increases drug releases decreases. Considering the results of other evaluation parameter of F_3 which was having lower viscosity, better spreadability, excellent extrudability, good Anthocyanin content, highest drug release, hence F3 was considered for clinical evaluation.

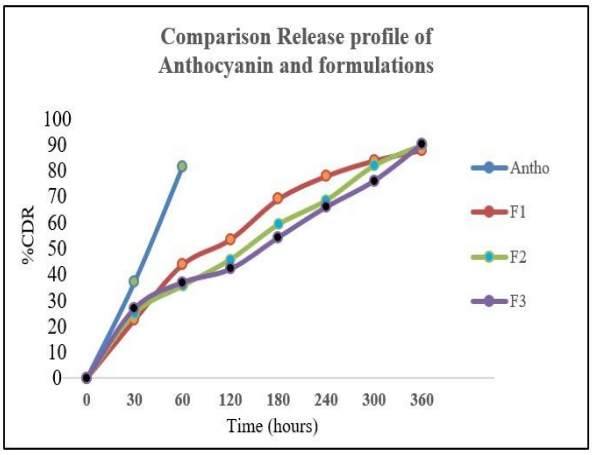

Figure 2. Comparison of cumulative drug release of pure drug and all formulations at the end of 6 hours

Evaluation Studies

Viscosity

All of the gel formulations exhibited good viscosity and the ability to adhere to the application site for an extended period of time. Among these formulations, gels made with sodium carboxy methyl cellulose and chitosan were less viscous than made with carbopol-940.

Spreadability

A small amount of shear was needed to spread the gel. Compared to gels made with carbopol-940 and sodium carboxy methyl cellulose, chitosan-made gels were easier to spread because they had reduced viscosity.

Extrudability

The extrudability values show that the gels have good extrudability. The gel made with chitosan among the formulations had excellent extrudability to the gels made with carbopol-940 and sodium carboxy methyl cellulose.

Anthocyanin Content

All gel formulations exhibited better drug content percentages ranging from 80 to 95 percent. The formulation F3 showed good percentage drug release than other formulations.

In-vitro Drug Release Studies

All gel formulations exhibited good drug release percentages. Gels made with chitosan performed better in terms of release among these formulations than those made with carbopol-940 and sodium carboxy methyl cellulose. The rate of drug release often decreases as viscosity increases.

Clinical Studies

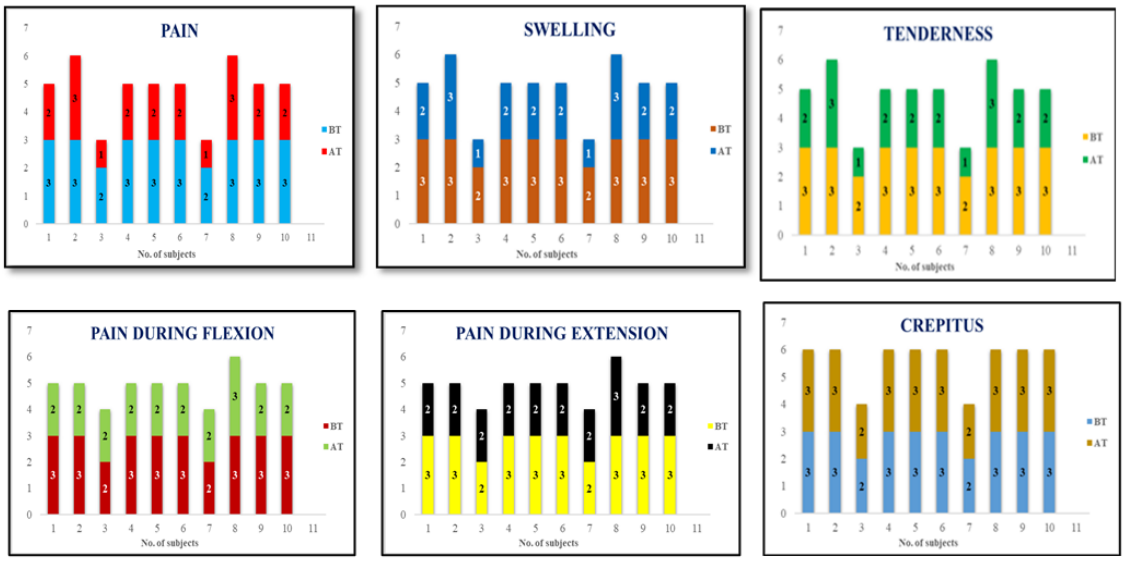

Figure 3. Effect of anthocyanin gel on subjects before treatment (BT) after treatment (AT)

Statistical Analysis: for Paired "t" Test

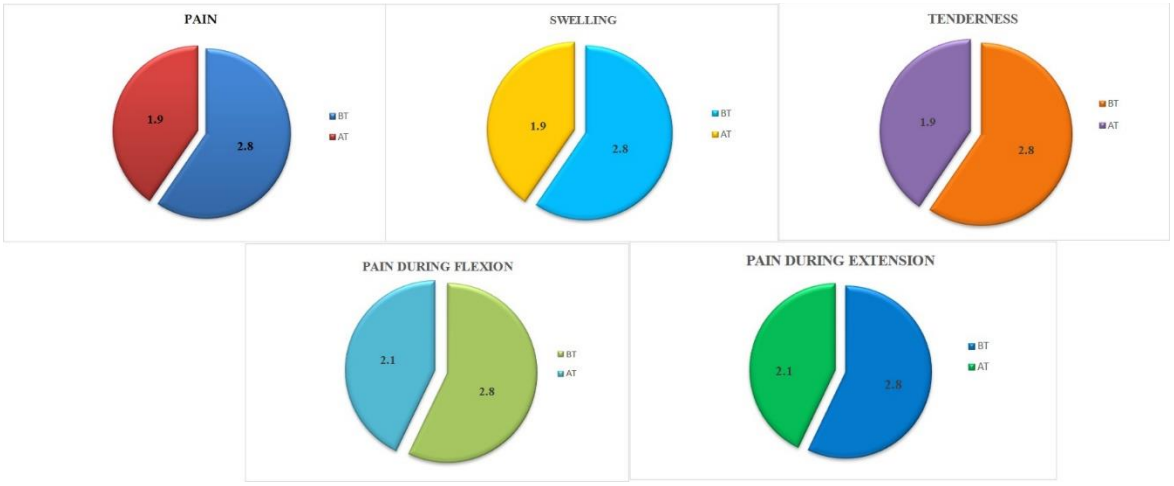

Figure 4. Improvement of parameters within subjects

In the present investigation, extraction of Anthocyanin from *Phaseolus vulgaris* L. for the formulation of topical gel and it's potential for anti-inflammatory activity was evaluated. Anthocyanin-containing topical gel has been shown to have anti-inflammation properties. The formulated gel of 2% concentration using chitosan polymer was applied topically to the subjects in the age-group of 25-70 years for 32 days in BVVS Ayurved Medical College & Hospital Bagalkot.

The subjects diagnosed for Osteoarthritis were included in the study, after obtaining the written consent, the subjects were treated. Subjects were instructed for the procedure of application of gel thrice a day. Further distribution of subjects were made based on the grades of pain, swelling, tenderness, pain during flexion, pain during extension, crepitus for indication of improvement with statistical analysis.

Pain

The effect of Anthocyanin gel on the subjects with pain according to the progress of administered formulation as; the mean score which was 2.80 before the treatment was reduced to 1.90 after the treatment with percentage relief of 67.85% which implies moderate improvement in subjects. This difference is considered to be extremely statistically significant.

Swelling

The effect of Anthocyanin gel on the subjects with swelling according to the progress of administered formulation as; the mean score which was 2.80 before the treatment was reduced to 1.90 after the treatment with percentage relief of 67.85% which implies moderate improvement in subjects. This difference is considered to be extremely statistically significant.

Tenderness

The effect of Anthocyanin gel on the subjects with tenderness according to the progress of administered formulation as; the mean score which was 2.80 before the treatment was reduced to 1.90 after the treatment with percentage relief of 67.85% which implies moderate improvement in subjects. This difference is considered to be extremely statistically significant.

Pain During Flexion

The effect of Anthocyanin gel on the subjects with pain during flexion according to the progress of administered formulation as; the mean score which was 2.80 before the treatment was reduced to 2.10 after the treatment with percentage relief of 75% which implies moderate improvement in subjects. This difference is considered to be extremely statistically significant.

Pain During Extension

The effect of Anthocyanin gel on the subjects with pain during extension according to the progress of administered formulation as; the mean score which was 2.80 before the treatment was reduced to 2.10 after the treatment with percentage relief of 75% which implies moderate improvement in subjects. This difference is considered to be extremely statistically significant.

Crepitus

The effect of Anthocyanin gel on the subjects with crepitus according to the progress of administered formulation as; the mean score which was 2.7 before the treatment remained 2.7 after the treatment which implies same improvement in subjects.

The data for present study were collected from subjects case report and progress chart. All the above results indicated the difference was statistically significant.

ACKNOWLEDGMENTS

I wish to express my deep sincere gratitude to Dr. Mahantesh Salimath, Principal and Dr.Prakash V Naraboli, Professor, Department of Panchakarma of BVVS Ayurved Medical College and Hospital, Bagalkot for their guidance and co-operation to carry out my clinical study.

AUTHOR CONTRIBUTIONS

Concept: A.D., L.V.; Design: A.D., L.V., M.S.; Control: A.D., L.V., M.S.; Sources: A.D., L.V., S.P.S.; Materials: S.P.S.; Data Collection and/or Processing: A.D., M.S., S.P.S.; Analysis and/or Interpretation: A.D., L.V., M.S.; Literature Review: A.D., S.P.S.; Manuscript Writing: A.D., L.V., S.P.S.; Critical Review: A.D., L.V., M.S.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

Reference no: BVVS/IEC/AMVB-2020-21/767 from Institutional Ethics Committee (IEC for research on human subjects) of BVVS Ayurved Medical College & Hospita, Bagalkot. On August 23rd 2021.

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