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PHARMACOGNOSTIC AND PHYTOCHEMICAL INVESTIGATION, IN-VITRO ANTI-OXIDANT AND ANTI-INFLAMMATORY STUDIES OF METHANOLIC EXTRACT OF *CENTRATHERUM PUNCTATUM* CASS. LEAVES FORMULATED IN CREAM

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ABSTARCT

Centratherum punctatum Cass. also called lark daisy is a medicinal plant belonging to the family Asteraceae. It is a low maintenance perennial herb of 45-60cm in height grown in tropical areas. According to previous article leaves of this plant showed various medicinal properties such as anti-inflammatory, anti-microbial, anti-oxidant, and anti-proliferative activities. The present investigation is to formulate a cream containing methanolic extract of *Centratherum punctatum* leaves. The formulated cream was evaluated for various parameters and analysed for anti-oxidant and anti-inflammatory activities. Total phenolic content was estimated by FC [Folin-Ciocalteu] method. The methanolic extract of *Centratherum punctatum* showed good amount of phenolic content which might be responsible for its anti-oxidant and anti-inflammatory activities. Anti-oxidant properties were detected by FRAP [Ferric Reducing Anti-oxidant Power] assay and anti-inflammatory was investigated by protein denaturation method. The methanolic extract of *Centratherum punctatum* leaves when formulated to cream showed effective results and exhibits anti-oxidant and anti-inflammatory properties.

KEYWORDS: *Centratherum punctatum*, phenolic content, anti-inflammatory, anti-oxidant, FRAP, protein denaturation.

1. INTRODUCTION

Medicinal plants are known for their abundance of benefits and have also been used from ancient times as a cure for many things. Herbal preparations are less toxic and have fewer adverse effects than synthetic treatments. Hence, most preferred as it is safer. The study in these circumstances is mainly based on the revival of curiosity in medicine from naturally obtained sources with the idea that herbal medicaments will apparently show lesser side effects compared to synthetic medicaments.

Centratherum punctatum Cass. also called lark daisy is a medicinal plant belonging to the family Asteraceae. It is a low-maintenance bushy perennial herb of 45-60cm in height grown in tropical areas. It has stems with widely spread leaves with lavender blue-coloured flowers. The plant has a refreshing smell like that of pineapple.[1]

Inflammation and oxidative stress are linked to each other. Oxidative stress can cause chronic inflammation which in turn could initiate or mediate the most chronic diseases. One effect synergizes the effect of another, which is toxic to each other. Oxidative stress is implicated during inflammatory processes. The prolonged release of inflammatory mediators can provoke oxidative stress leading to chronic inflammatory diseases. Hence, they are correlated to one another.[2]

In case of our plant triterpene named squalene and palmitic acid esters such as hexadecenoic acid are responsible for anti-oxidant activity whereas flavones and flavanones are responsible for anti-inflammatory activity as well as anti-oxidant activity.[3]

Based on previous articles, it is well established that the *Centratherum punctatum* shows anti-inflammatory and anti-oxidant properties. Our effort is to incorporate those properties into herbal cream and evaluate them. This work includes formulating and evaluating herbal cream with anti-inflammatory and anti-oxidant properties.[4]

2. MATERIALS AND METHODS

2.1 Collection and Authentication of samples

Healthy and uninfected leaves of *Centratherum punctatum* were procured from the local areas of Udupi district, Karnataka and authenticated by Dayanand Karagi, Assistant horticulture officer in department of horticulture, Karwar, Karnataka, India.

2.2 Pharmacognostical evaluation

2.2.1 Organoleptic evaluation

Various organoleptic parameters of the plant leaves such as color, odor, shape, taste and size were studied.

2.2.2 Macroscopic evaluation

Macroscopic characters of *Centratherum punctatum* leaves were evaluated for the type of petiole, surface, leaf tip, base and venation.

2.2.3 Microscopic evaluation

In microscopic evaluation, studies were conducted on both grounds qualitatively and quantitively to understand the pharmacognostic characters of *Centratherum punctatum* leaves.

2.2.3.1 Qualitative microscopy

a. Transverse section of *Centratherum punctatum* leaves.

T.S of leaf was taken, stained and observed under the microscope.

b. Powder microscopy of *Centratherum punctatum* leaves.

The shade dried leaves were coarsely powdered and stained with few ml of phloroglucinol and conc. HCL, mounted with glycerin and observed under the microscope. The different powder microscopical characters were identified.

2.2.3.2 Quantitative microscopy

Leaf surface constants such as stomatal number, stomatal index, vein islet and vein termination number were estimated using camera lucida which helped in confirming identity of *Centratherum punctatum* leaves.

2.3 Phytochemical evaluation of methanolic extract of *Centratherum punctatum*

2.3.1 Preparation of methanolic extract

2.5 gm of coarsely powdered leaves of *Centratherum punctatum* was placed in closed flask and 50ml of methanol was added and macerated for 48 hours. After 48 hours the macerated mixture was filtered.[5]

2.3.2 Qualitative Phytochemical evaluation of extract

The extract was screened for various bioactive phytoconstituents.[6]

2.3.3 Quantitative phytochemical analysis

Estimation of total phenolic content[7]

Phenolic compounds present in leaves of *Centratherum punctatum* might be responsible for anti-oxidant and anti-inflammatory activities. It was estimated by FC [Folin-Ciocalteu] method.

Standard stock solution was prepared by mixing 1000mg of gallic acid powder in 1 liter of distilled water. Slightly heated to dissolve.

Calibration standard solutions were prepared by pipetting different concentrations of standard solution from stock solution and added to 100ml volumetric flask and made up to volume using distilled water. Approximate concentrations of gallic acid in each of the calibration standard solutions are shown in table no.1.

Sample test solution was prepared by dissolving 1ml of methanolic extract of *Centratherum punctatum* leaves into 4ml of distilled water.

To 1 ml of different concentrations of standard solutions, 1 ml of test solution and 1 ml of blank, 15 ml of distilled water and 1 ml of Folin-Ciocalteu reagent was added. The contents of all the test tubes were mixed well and allowed the mixture to react for 6 minutes at room temperature. 3 ml of 20% sodium carbonate solution was added to each test tube and incubated for 1 hour in dark at room temperature. After 1 hour of incubation absorbance of all the reaction mixtures were measured at 765nm. The amount of total phenols was calculated from the calibration curve as a gallic acid equivalents by the following formula

$$T = (x \times v) \div m$$

Where, x=Concentration of gallic acid established from the calibration curve (mg/ml)

v=The volume of extract(ml)

m=The gram weight of plant extract

Table no.1: Preparation of calibration standard solutions.

Sl. No.	Volume of standard stock solution in ml	Approximate concentration gallic acid, mg/l
1	1.0	10
2	1.5	15
3	2.0	20
4	2.5	25
5	3.0	30
6	3.5	35
7	4.0	40
8	4.5	45
9	5.0	50

2.4 Formulation of cream containing methanolic extract of *Centratherum punctatum* leaves[8]

Table no.2: Composition of cream.

Sl. No.	Ingredients	Quantity
1	Methanolic extract	3ml
2	Stearic acid	1g
3	Cetyl alcohol	1g
4	Vitamin E	0.04ml
5	Castor oil	0.2ml
6	Triethanolamine	0.4ml
7	Honey	0.4ml
8	Perfume	2ml
9	Distilled water	Q. S

The various steps involved in preparation of cream are described below.

Oil phase was prepared by dissolving stearic acid, cetyl alcohol, vitamin E, castor oil and in required quantity at 70°C. Water phase such as triethanolamine, honey, distilled water of required quantity was dissolved separately at 70°C.

The water phase and methanolic extract of leaves of mentioned quantity were taken separately in a beaker and added gradually to beaker containing oil phase with continuous stirring. At this stage perfume (orange oil) was added and stirring was continued until homogenous, smooth, and even textured cream was formed.

The formulated cream was evaluated for various parameters. (viz. colour, odour, texture, homogeneity, type of smear, dye test, globular size, spreadability, irritancy, acid value, saponification value, pH etc.)[9]

2.5 Screening of anti-inflammatory activity by inhibition of protein denaturation method[10,13]

Anti-inflammatory activity of formulation was evaluated by inhibition of protein denaturation assay. In this assay egg albumin albumin was used as protein source. Ibuprofen gel was taken as positive control and distilled water was taken as negative control. Their absorbance was measured at 680nm.

Serial dilutions of different concentrations 200µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml and 1000 µg/ml of formulated cream and standard drug were prepared.

To 2ml of different concentrations of standard and test solutions 2.8ml of phosphate buffer saline and 0.2ml of egg albumin was added. All the contents of test tubes were mixed well and incubated at 37°C for 20 minutes. After incubation the reaction mixtures were heated at 70°C for 5 minutes and cooled to room temperature. The absorbance of cooled reaction mixtures were measured at 680nm and % inhibition was calculated using the formula mentioned below.

$$\%\ Inhibition\ = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} * 100$$

2.6 Screening of anti-oxidant activity by FRAP [Ferric Reducing Anti-oxidant Power] assay[11]

For evaluation of anti-oxidant activity Ferric Reducing Anti-oxidant Power [FRAP] assay was selected where ferrous sulphate [FeSo4] was considered as standard. Serial dilutions of different concentrations 0.02g/ml, 0.04g/ml, 0.06g/ml, 0.08g/ml, and 0.10g/ml of formulated cream and standard drug were prepared. To 1ml of different concentrations of standard and test solutions 2.5 ml of phosphate buffer saline and 2.5ml of 1% potassium ferricyanide solution was added and incubated at 50°C for around 20 minutes. Once incubation time is over 2.5 ml of 10% TCA [tri- chloro- acetic acid] was added to each sample and test tubes were centrifuged at 3000rpm for 10 minutes. From these centrifuged samples 2.5 ml of supernatant was collected in separate test tubes. To those new test tubes having 2.5 ml of

supernatant, 2.5 ml of deionised water and 2.5 ml of 5% ferric chloride was added. Absorbance was measured at 700nm.

$$\%Reduction\ ability = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} * 100$$

3. RESULTS AND DISCUSSIONS

3.1 Pharmacognostical evaluation

3.1.1 Organoleptic evaluation

Organoleptic properties of the leaves showed that *Centratherum punctatum* leaves are oval in shape and green in colour having aromatic pineapple like odour and bitter in taste. It's average length and breadth was found to be 5.3 and 2.6cm.

3.1.2 Macroscopic evaluation

It is a petiolate leaf having double serrate margin and attenuate base including dichotomous veins with the pubescent surface and acute tip.

3.1.3 Microscopic evaluation

3.1.3.1 Qualitative microscopy

a. T. S of *Centratherum punctatum* leaves

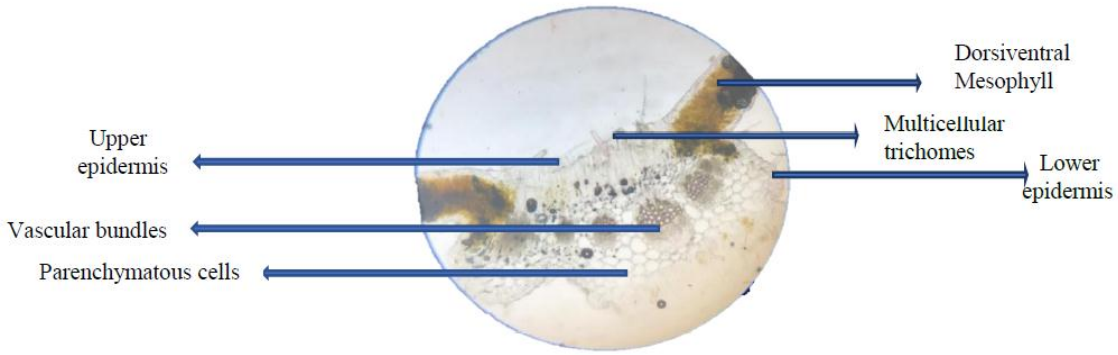

Fig no.1 T.S of *Centratherum punctatum* leaves.

b. Powder microscopy of *Centratherum punctatum* leaves.

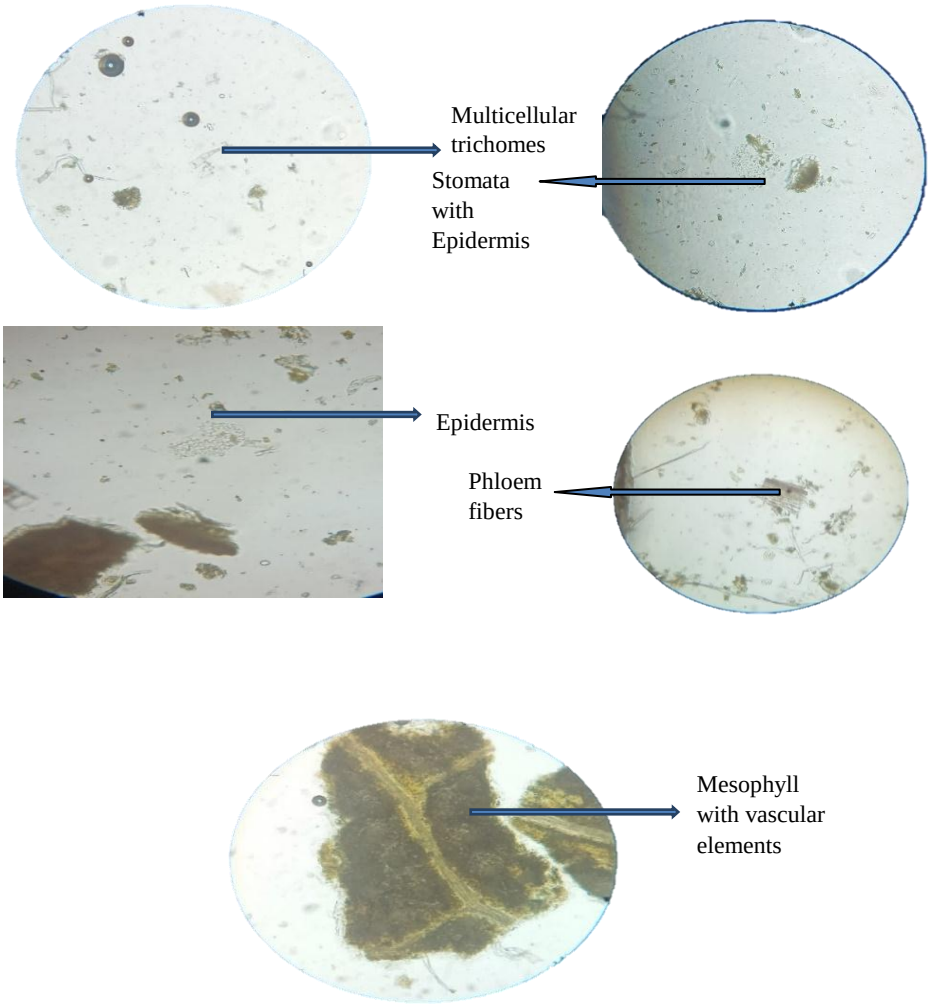

Fig no.2 Powder microscopy of *Centratherum punctatum* leaves.

3.3.1.2 Quantitative microscopy

Average stomatal number and stomatal index of upper epidermis of leaf was estimated to be 60.975 and 8.2 respectively. Average vein islet and vein termination number of leaf was estimated to be 93.52 and 23.21 respectively.

From pharmacognostical evaluation of *Centratherum punctatum* leaves it was concluded that leaves are of light green colour with refreshing smell like that of pineapple. It is a petiolate leaf having double serrate margin and attenuate base including dichotomous veins with the pubescent surface and acute tip. The T.S of the leaf showed upper and lower epidermis appeared as wavy in structure. The cells of outer layer are thin walled and consist of multicellular trichomes. Further vascular bundles, dorsiventral mesophyll and

parenchymatous cells were observed. Through Powder microscopy mesophyll, trichomes, stomata with epidermis phloem fibres and epidermis were viewed.

3.2 Phytochemical evaluation of methanolic extract of *Centratherum punctatum* leaves.

3.2.1 Qualitative phytochemical evaluation of extract

Table no.3 Results of phytochemical analysis.

Sl. No.	Phytoconstituents	Test	Methanolic extract
1	Carbohydrates	Molisch's Test	_
2	Proteins	Biuret test	_
3	Fats and oils	Spot test	_
4	Alkaloids	Mayer's test	+
		Wagner's Test	+
5	Resins	Acetone water test	_
6	Tannins	Ferric chloride test	++
		Gelatin test	+++
7	Cardiac glycosides	Baljet test	_
8	Anthraquinones	Bontrager's test	_
9	Saponins	Foam test (soap test)	_
10	Coumarins	Alkali test	_
11	Flavonoids	Shinoda test	+

3.2.2 Quantitative phytochemical evaluation of extract

3.2.2.1 Estimation of total phenolic content

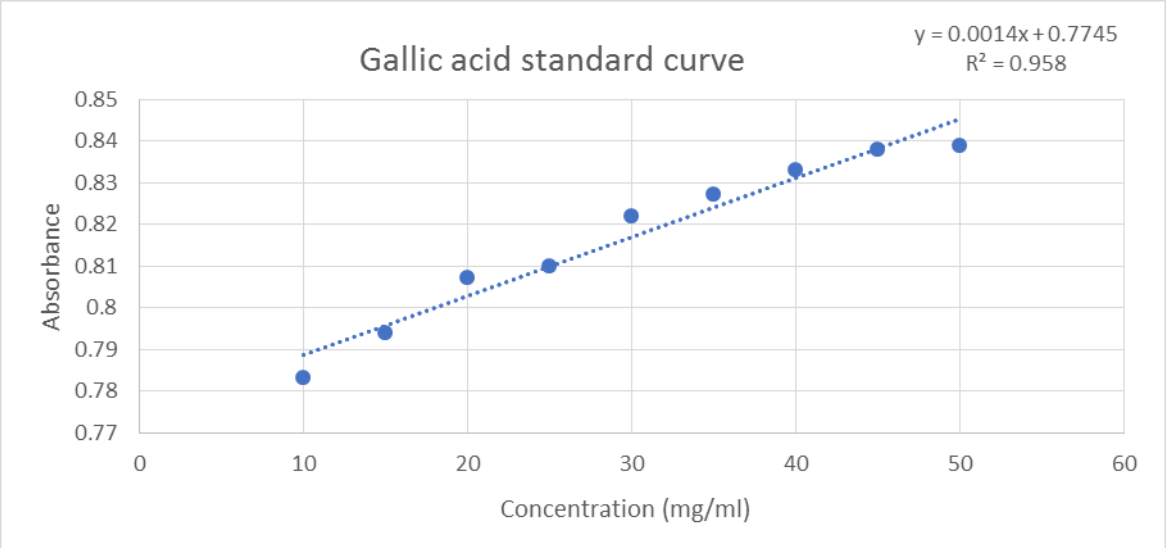

Fig no.3 Calibration curve of gallic acid.

$y = mx + c$

x =27.5

Total phenolic content=550 gallic acid equivalents/100g of dry extract.

Tests for phytochemicals such as alkaloids, tannins and flavonoids showed positive results during phytochemical analysis of methanolic extract. Total phenolic content which was estimated using FC method showed higher amount of phenolic content which might be the possible reason for exhibiting anti-inflammatory and anti-oxidant activity.

3.3 Evaluation of formulated cream

The formulated cream was evaluated for various parameters.

- **Organoleptic evaluation**

The formulated cream consisted of light green color with aromatic odor having smooth and creamy texture.

- **Homogeneity**

The homogeneity of formulation was tested and was found to be good by appearance and touch.

- **After feel**

Emollient, no residue was left after application of cream.

Fig no.4 Cream containing methanolic extract of *Centratherum punctatum* leaves.

- **Type of smear**

Non-greasy.

- **Dye test**

The amaranth solution was mixed with the cream. A drop of cream was placed on microscope slide. Covered it with a cover slip and examined it under microscope. The dispersed globules

appeared white, the ground appeared pinkish red, which indicates the cream as o/w type of cream.

- **Globular size**

The average globular size was found to be 26.38µ.

- **Spreadability Test**[12]

Excess cream was placed between the two glass slides and 240 g weight was placed on the glass slide for 5 min to compress the cream to a uniform thickness. The time in seconds required to separate the two slides was taken as a measure of spreadability.

S* = m * l/t

m – weight tied on upper slide[240g]

l* – Average length of glass slide [0.5]

t – time in s

Average spreadability was found to be 14.71gm.cm/sec.

- **Irritancy test**

The cream was applied to the specified area of hand and checked for the irritancy. No irritancy, erythema and oedema were found.

- **Acid value**

Take 2g of substance dissolved in accurately weighed, in 10ml mixture of equal volume of alcohol and solvent ether, the flask was connected to reflux condenser and slowly heated, until sample was dissolved completely, to this 1 ml of phenolphthalein added and titrated with 0.1N of NaOH until faintly pink colour appears after shaking for 30 seconds.

Acid value = n*5.61/w

Where, n= the number of ml of NaOH required

w=the weight of substance

Acid value=16.83

- **Saponification value**

Introduce about 2g of substance refluxed with 25ml of 0.5 N alcoholic KOH for 30 minutes, to this 1ml of phenolphthalein added and titrated immediately with 0.5 N HCL.

Saponification value=(b-a) *28.05/w

The volume in ml of titrant consumed in titration of sample=a

The volume in ml of titrant consumed in titration of blank=b

The weight of substance in gm =w

Saponification value= 126.22

- **pH**

pH of the cream was found to be 7.47

Formulated cream containing methanolic extract of *Centratherum punctatum* leaves was evaluated for various parameters. The formulated cream was smooth in texture and homogenous with no irritancy to skin and showed pH 7.47 which is suitable for skin.

3.4 Screening of anti-inflammatory activity by inhibition of protein denaturation method

Table no.4: % Inhibition of Ibuprofen gel and sample at different concentrations.

Sl. No.	Concentration, µg/ml	% Inhibition of Ibuprofen	% Inhibition of sample
1	200	0.94	19.53
2	400	4.10	22.47
3	600	7.26	25.58
4	800	11.43	28.30
5	1000	13.37	29.30

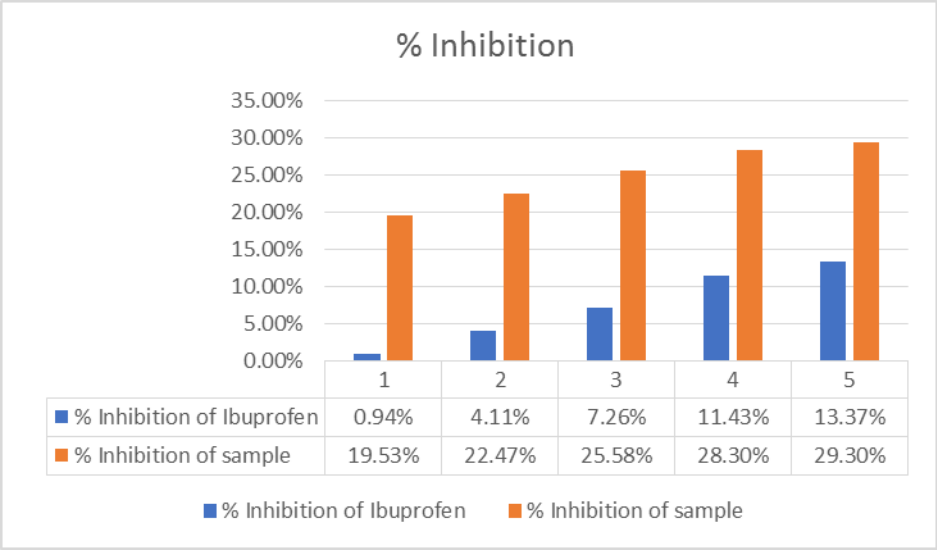

Fig.no.5 Column chart indicating % Inhibition of Ibuprofen and sample.

Anti-inflammatory activity of cream containing methanolic extract of leaves was investigated using inhibition of protein denaturation method and results indicated that as the concentration increases percentage inhibition also increases. Ibuprofen gel was taken as positive control which also showed increase in percentage inhibition with increase in concentration.

Comparatively, the formulated cream showed higher percentage inhibition with respect to standard drug having similar concentration which also indicates that sample showed higher anti-inflammatory activity comparing to standard with similar concentrations.

3.5 Screening of anti-antioxidant activity by FRAP [Ferric Reducing Anti-oxidant Power] assay

Table no.5 % Reduction ability of standard and sample at different concentrations.

Sl. No.	Concentration, g/ml	% Reduction ability of standard	% Reduction ability of sample
1	0.02	8.81	41.52
2	0.04	9.32	42.37
3	0.06	9.49	45.08
4	0.08	12.54	54.23
5	0.10	16.44	61.18

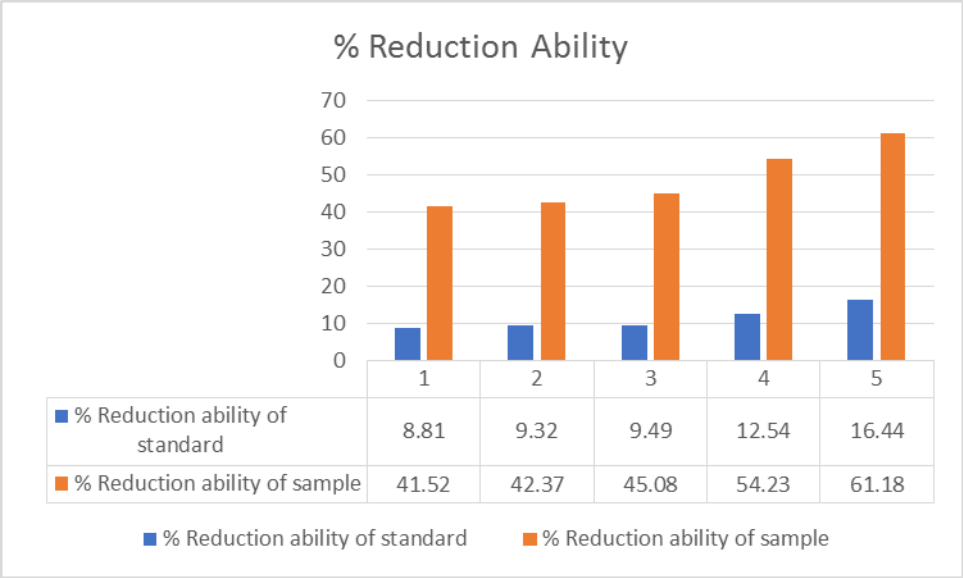

Fig.no.6 Column chart indicating % Reduction ability of standard and sample.

For evaluation of anti-oxidant activity FRAP assay was selected. Ferrous sulphate was considered as standard. This assay was selected because it can provide a quick, sensitive and easy way to measure the anti-oxidant capacity of our formulation as it can give anti-oxidant capacity as low as 0.2 Mm Fe2 + equivalents.

Results of FRAP assay concluded that higher the concentration greater the anti-oxidant activity. A blue chromophore was formed after adding ferric chloride which indicated that ferric ions are reduced to ferrous ions through anti-oxidants present in our cream containing methanolic extract of leaves.

The main findings of this investigation are that methanolic extract of *Centratherum punctatum* leaves when formulated into a cream exhibits anti-oxidant and anti-inflammatory activity and carry potent medicinal properties.

4. SUMMARY AND CONCLUSION

Leaves of *Centratherum punctatum* was collected from local areas of Udupi and they were tested for its various pharmacognostical properties which helped us to identify the sample. Phytochemical evaluation revealed that different bioactive phytoconstituents such as alkaloids, tannins and flavonoids were present.

From the previous articles it was cleared that these leaves had various pharmacological activities including anti-inflammatory and anti-oxidant activity. It is also well established that inflammation and oxidative stress are linked to each other i.e., one synergizes the action of another. Hence effort was made to formulate a cream containing methanolic extract of *Centratherum punctatum* leaves which can be applied topically for the treatment of inflammation and oxidative stress.

Total phenolic content was estimated by FC [Folin-ciocalteu] method. The methanolic extract of *Centratherum punctatum* showed good amount of phenolic content which might be responsible for its anti-inflammatory and anti-oxidant activities. In-vitro assays were employed to investigate the anti-inflammatory and anti-oxidant properties of formulated cream. Anti-inflammatory activity was investigated by inhibition of protein denaturation method. For evaluation of anti-oxidant activity Ferric Reducing Anti-oxidant Power [FRAP] assay was selected. Results of anti-inflammatory assay indicated that as the concentration increases percentage inhibition also increases. Ibuprofen gel was taken as positive control which also showed increase in percentage inhibition with increase in concentration. Comparatively, cream containing methanolic extract of leaves showed higher percentage inhibition with respect to standard drug having similar concentration. Results of FRAP assay indicated that higher the concentration greater the anti-oxidant activity. A blue chromophore was formed after adding ferric chloride which indicated that ferric ions are reduced ferrous ions through anti-oxidants present in our cream containing methanolic extract of leaves.

In-vitro assays which was carried out to investigate anti-inflammatory and anti-oxidant activities showed good results and indicated that anti-inflammatory and anti-oxidant activity

of formulated cream containing methanolic extract of *Centratherum punctatum* leaves is dependent on concentration. As the concentration increases their activity also increases.

Hence, the study summarizes that methanolic extract of *Centratherum punctatum* leaves when formulated into a cream exhibits anti-inflammatory and anti-oxidant activity and it can be applied topically for the treatment of inflammation and oxidative stress.

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