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To develop and evaluate the *in-vitro* anti oxidant and anti-inflammatory activity of polyherbal Gel

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ABSTRACT

Herbal medicines are used for their safety, efficacy, cultural acceptability and lesser side effects. The chemical constituents present in plants are a part of the physiological functions of living system and hence they are believed to have better compatibility with the human body. These drugs are made from renewable resources of raw materials by eco-friendly processes and will bring economic prosperity. An herb is a plant or plant part used for its scent, flavor, or therapeutic properties. They are sold as tablets, capsules, powders, teas, extracts, and fresh or dried plants. People use herbal medicines to try to maintain or improve their health. Products made from botanicals, or plants, that are used to treat diseases or to maintain health are called herbal products, botanical products, or phytomedicines. A product made from plants and used solely for internal use is called an herbal supplement.

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INTRODUCTION

Herbal treatments are employed because they are safe, effective, culturally acceptable, and have fewer negative effects. Because plant chemical ingredients are part of the physiological operations of living systems, they are thought to be more compatible with the human body [1]. These pharmaceuticals will create economic prosperity because they are made from renewable raw ingredients using eco-friendly procedures [2]. Herbs are plants or plant parts that are utilised for their smell, flavour, or therapeutic effects. Dietary supplements include herbal medicines [3]. They are available in the form of tablets, capsules, powders, teas, extracts, and fresh or dried plants. Herbal remedies are used by people in an attempt to preserve or enhance their health [4]. Herbal products, botanical products, or phytomedicines are products made from botanical or plants that are used to treat illnesses or maintain health [5]. An herbal supplement is a plant-derived substance that is only used internally. Medicinal plants, commonly referred to as medicinal herbs, have been identified and utilised in traditional therapeutic practises since prehistoric times. Plants generate hundreds of chemical substances for a number of purposes, including resistance and protection from insects, fungi, illnesses, as well as herbivorous mammals. Whether in modern or traditional medicine, medicinal plants are used to preserve health, to treat a specific illness, or both. In 2002, the Food and Agriculture Organisation estimated that over 50,000 medicinal plants were utilised globally. In 2016, the

Royal Botanic Gardens, Kew estimated that 17,810 plant species have a therapeutic application, out of around 30,000 plants for which usage of any type is documented. Plants have been the foundation of various traditional medicinal systems around the world, and they continue to supply humans with novel treatments. The World Health Organisation (WHO) describes traditional medicine (including herbal medications) as therapeutic practises that existed before the establishment and spread of modern medicine, frequently for hundreds of years, and are still in use today. Herbal medicine is a combination of the therapeutic experience of a generation of indigenous medicine practitioners. Traditional remedies include medicinal herbs, minerals, organic substances, and so on. Herbal medications have been in use in Indian, Chinese, Syrian, Roman, Egyptian, Greek texts for thousands of years as per recorded evidences.

MATERIALS AND METHODS

Materials

Curcuma longa, Zingiber officinale, Ocimum tenuiflorum, ethanol, ammonium chloride, silica gel-G, dihydrogen phosphate, sodium chloride, iodine, hexane, ethyl acetate, Carbopol, triethanolamine, benzoic acid, sodium lauryl sulphate, glycerin, dragondroff's reagent, fehling's reagent, ferric chloride, sodium acetate, hydrogen peroxide, ascorbic acid, phosphate buffer, diclofenac sodium, fresh hen's egg albumin, methylparaben eucalyptus oil.

Plant materials:

Rhizomes of Curcuma longa, rhizomes of Zingiber officinale and leaves of Ocimum tenuiflorum was collected from Gudur, Nellore district, Andhra Pradesh, India. The plant specimen was verified to be of the correct species by Dr.K. Vishnu Vardhan, a botanist from the Department of Botany, S.K.R. Government junior college, Gudur.

Principle of soxhlation:

The components are extracted utilising the condensed vapours of the solvent by the Soxhlet extractor. The sample powder comes into touch with the condensed vapours, and the soluble part of the powder is combined with the solvent.

Preparation of extract:

The Soxhlet extraction process was used to prepare aqueous-ethanolic (30:70) extract of polyherbal formulation for 4 extracts (1:1:1). The collected plant materials were shade dried until the moisture was removed from the plant materials. After drying the plant materials was grinded to a coarse powder and stored in a sealed container for further experiments. The polyherbal formulation of (1:1:1) were taken 10 g of tulsi, turmeric and ginger powders. After preparing thimble pour ethanol onto the thimble and macerate for 2 hours. After maceration starts the soxhlation apparatus.

After completion of soxhlation process keeps the reflex condenser for recollecting the solvent used in the soxhlation.

Evaluation of polyherbal powders

Detection of total ash:

In an ignited and weighted silica crucible, polyherbal powder (1:1:1) 2grms was placed. It was burnt at a low temperature until it was after being rid of carbon, it was cooled and weighed. The percentage of ash was estimated using the air-dried.

Detection of water-soluble ash:

Total ash was added to 25ml of water and heated for 5 min then filtered through ash-free filter paper (Whatman filter paper) the water insoluble substance was weighed after the filter paper was rinsed in hot water and burned in the silica crucible. The water soluble ash was determined by removing the water-insoluble materials from total ash.

Detection of extractive values:

Alcoholic soluble extractive:

Sample(5g) was weighed and macerated in 100ml of alcohol in a closed flask for 24hrs. to prevent alcohol loss, the contents of the flask were rapidly filtered, then 25ml of the filtrate was evaporated until it was dry at 105^{o}c in a tarred shallow dish and then weighed. Using the air-dried powder, the proportion of alcohol-soluble extractive was calculated.

Moisture content:

To determine moisture content, a 5g pre-drying weight sample was placed in the oven. The pre-drying weight was compared to the post-drying weight to assess how much moisture was removed. The moisture content was calculated as a percentage.

Qualitative phytochemical screening of ethanolic extract

The 4 Ethanolic extract of poly herbal formulation were screened for their chemical constituents. A little amount of dried extract was utilised to determine the alkaloids, carbohydrates, and other constituents, phenols. The following procedures are used to isolate flavonoids, steroids, as well as triterpenoids. The total flavonoid content of the ethanolic extract of CZO. was estimated by reported method.

Aluminum chloride method used for flavonoid content determination [6, 7].

In vitro anti-oxidant activity

Hydrogen peroxide scavenging activity

Hydrogen peroxide (H2O2) belongs to a non-radical reactive species that is produced in the body by various oxidising enzymes such as SOD. It is the least reactive ROS yet has the greatest ability to cross biological membranes.

1 ml of sample (1 mg/ml) was mixed with 3 ml of methanol, 0.2 ml of 10% aluminium chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water and remains at room temperature for 30 min.

The absorbance of the reaction mixture was measured at 420nm with UV-Visible spectrophotometer [8].

The content was determined from extrapolation of calibration curve which was made by preparing gallic acid solution (50-150 μg/ml) in distilled water. Calibration curve for gallic acid was obtained by plotting absorbance on Y-axis and their corresponding concentration on X-axis. The concentration of flavonoids was expressed in terms of μg/ml.

In Vitro Anti-Inflammatory Activity

Protein Denaturation Method:

Protein denaturation method was used for the estimation of anti-inflammatory activity

In Vitro

In a beaker mix 0.2 ml of egg albumin (collected from fresh hen's egg), 2.8 mL of phosphate buffer saline (PBS pH-6.4) as well as 2mL of diclofenac sodium at various concentrations (10, 20, 30, 40, 50 g/ml). As a control, prepare the same volume of double-distilled water (Figures 1 and 2).

Incubate the mixtures at 370°C for 15 minutes in a BOD incubator, then heat at 700°C for 5 minutes, chill the solution, and measure the absorbance at 660nm. Assume the vehicle is empty (Figures 3, 4 and 5).

HRBC Membrane Stabilization Method

HRBC method was used for the estimation of anti-inflammatory activity INVITRO. Blood was collected from healthy volunteers and was mixed with equal volume of sterilized Alsevers solution. This blood solution was centrifuged at 3 000 rpm and the packed cells were separated. The packed cells were washed with isosaline solution and a 10% v/v suspension was made with isosaline. This HRBC suspension was used for the estimation of anti-inflammatory property. Different concentrations of extract, reference sample and control were separately mixed with 1mL of phosphate buffer, 2 mL of hypo saline and 0.5 mL of HRBC suspension. All the assay mixtures were incubated at 37 °C for 30 minutes and centrifuged at 3 000 rpm. The supernatant liquid was decanted and the hemoglobin content was estimated by a spectrophotometer at 560 nm [9].

The percentage hemolysis was estimated by assuming the hemolysis produced in the control as 100%.

Herbal gel was made using a mechanical stirrer and the gelling chemical Carbopol 934 in a 1% w/w concentration with deionized water.

The skin pH (6.8-7) was then maintained by adding tri-ethanolamine dropwise with continuous stirring.

Various concentrations 100mg, 250mg, 500mg, 750mg, 1000mg polyherbal ethanolic extract of (1:1:1) has been added to the gel and swirled for a sufficient amount of time to ensure homogenous mixing of the extract in the gel base.

These formulas were kept in a cool, dry place. The formulation was tested for the following criteria. The formulation fo Polyherbal gel according to the [Table 1].

Evaluation studies for gel:

Organoleptic evaluation

Colour and appearance were recorded as physical parameters.

Viscosity

A Brookfield viscometer with a spindle number was used to determine the viscosity of the gel.

Spreadability:

The equipment, which consists of a wooden block with a pulley at one end, was used to determine spreadability. Spreadability was determined using this method based on the slip and drag characteristics of gels. On the ground slide, an excess of gel (approximately 2 g) was placed under investigation. The gel was then Utilising a hook and a glass slide of the same size as the fixed ground slide, they were positioned between one another. A 1 kg weight was set on top of the two slides for 5 minutes in order to let air out and create a uniform gel coating between the slides. The borders were scraped clean of extra gel. The top plate was then pulled with an 80-gram weight using a thread that was attached to the hook, and the amount of time (in seconds) that it took for the top slide to move 7.5 cm was noted. The spreadability improves with decreasing interval length.

The following formula was used to determine

spreadability,

$S=M\times L/T$

Where

S = Spreadability; M = (Tied to the upper slide) Pan weight; L = The length that the glass slide moved; T = The amount of time (in seconds) needed to separate the top slide from the bottom slide [10].

Extrudability:

The gel compositions were placed in conventional capped collapsible aluminium tubes and crimped shut. The weights of the tubes were taken down [11]. Between two glass slides, the tubes were secured. When 500 g were positioned over the slides, the cover was taken off. The amount of gel that was extruded was measured and gathered. Calculated extruded gel percentages include >90% excellent, >80% good, and >70% fair (extrudability >80%).

Homogeneity:

All created gels were placed in containers and visually inspected for uniformity [12]. They were examined for the existence of aggregates and their appearance.

Inhibition of Protein Denaturation Reaction mixtures were incubated in a water bath at 37○C 2○C for 15-20 minutes before being heated to 70○C and maintained for 5 minutes [13].

The reaction mixture was then allowed to cool for 15 minutes at room temperature. A colorimeter was used to measure the absorbance 206 of the reaction mixture before and after denaturation at 660 nm for each concentration (10 g/ml, 20 g/ml, 40 g/ml, 60 g/ml, 80 g/ml, and 100 g/ml).

Each test was repeated three times, and the mean absorbance was recorded each time. Using the following formula, the percentage of protein inhibition was calculated in relation to the control.

RESULT AND DISCUSSION

Physical appearance and % yield of extracts:

The Curcuma longa, Zingiber officinale, Ocimum tenuiflorum extracts were prepared using ethanol.

The prepared extract was coded as CZO. The curcuma longa extract was prepared using ethanol, The prepared extract was coded as cl.

The zingiber officinale extract was prepared using ethanol, the prepared extract was coded as zo.

The Ocimum tenuiflorum extract was prepared using ethanol, the prepared extract was coded as not (Tables 1, 2 and 3).

Figure 1: Soxhlet extraction of Polyherbal compound

Figure 2: Recovery of solvent

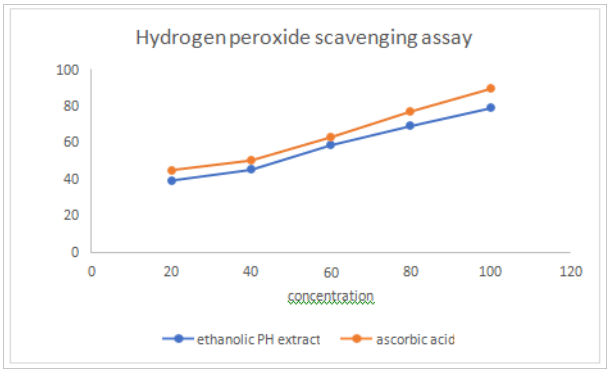

Figure 3: CZO ethanolic extracts' hydroxyl radical scavenging properties

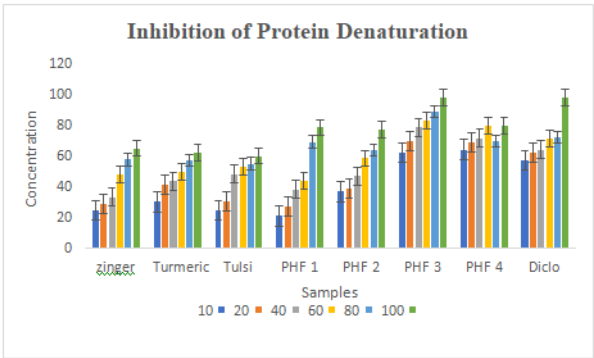

Figure 4: Inhibition of Protein Denaturation

Table 1: Formulation of Polyherbal Gel

S.No	Ingredients	Quantity
1.	Carbopol	1g
2.	Polyherbal extract	1g
3.	Methyl paraben	0.02g
4.	Distilled water	100ml
5.	Triethanolamine	Qs
6.	Eucalyptus oil	qs

Table 2: Percentage Yield of Extracts

S.no	Extract	%Yield	Physical appearance of the extract		Odour	Taste
			colour	Consistency		
1	CZO	10.5%	Dark greenish brown	Oily viscous	Characteristic	Sour
2	Curcuma longa	5.05%	Dark yellowish brown	Light viscous	Characteristic	Pleasantly bitter
3	Zingiber officinale	4.57%	Brownish yellow	Oily viscous	Characteristic	Pungent
4	Ocimum tenuiflorum	4.91%	Dark green	Viscous	Characteristic	mint

Table 3: Determination of Total Ash

S.NO	INGREDIENTS	% VALUE
1	CZO	9.1 %
2	Curcumin longa	16.6 %
3	Zingiber officinale	7.6%
4	Ocimum tenuiflorum	3.1%

Table 4: Water-soluble ash

S.NO	INGREDIENTS	% VALUE
1	CZO	2.99 %
2	Curcumin longa	3.93 %
3	Zingiber officinale	3.366%
4	Ocimum tenuiflorum	1.7 %

Table 5: Moisture content

S.NO	Ingredients	Moisture content	Alcohol Soluble Extractive value
1	CZO	30.75%	22.71 %
2	Curcumin longa	3.14 %	0.4 %
3	Zingiber officinale	8.13%	8.55%
4	Ocimum tenuiflorum	81.02 %	59.2%

Table 6: Phytochemical Tests

Chemical constituents	Tests	Czo	Curcuma longa	Zingiber officinale	Ocimum tenuiflorum
Alkaloids	Dragendroff's test	+	+	+	+
	Mayer's test	-	+	-	+
	Wagner's test	-	-	+	+
	Hager's test	+	+	+	+
Carbohydrates	Molisch's test	+	+	+	+
	Fehling's test	+	+	+	+
	Benedict's test	-	+	-	+
Amino acids & proteins	Biuret test	+	-	+	+
	Ninhydrin test	-	-	-	-
Flavonoids	Lead acetate test	-	+	-	-
	Shinoda test	+	+	+	+
	Alkaline test	+	+	+	+
Phenols	FeCl3	-	+	+	-
Tannins	Bromine water test	+	+	+	+
	Gelatin test	+	+	+	+
Steroids & triterpenoids	Salkowski test	-	-	-	-
	Libermann – buchard test	-	-	+	+
Saponins	Froth test	-	+	-	+
	Foam test	+	-	+	-

Table 7: Total Flavonoid Content

EXTRACT	TOTAL FLAVONOID CONTENTS
CZO	71.42
Curcuma longa	51.86
Zingiber officinale	62.81
Ocimum tenuiflorum	76.89

Table 8: TLC

S.NO.	Sample	Mobile Phase	Reagents For Detection	Colour Of Spot	No. Of Spots	Rf Value
1	CZO	Hexane: Ethyl acetate	Iodine fumes	Green and yellow	3	0.87 0.6 0.52

Table 9: The ethanol extracts' capacity to scavenge hydroxyl radicals of CZO

Sample	Concentration	Absorbance (Mean ± SEM)	Percentage inhibition (Mean ± SEM)	IC50
	20 μg/ml	0.187±0.001	39.29±0.294	
	40 μg/ml	0.176±0.003	45.10±0.779	
Ethanol extract	60 μg/ml	0.162±0.001	58.58±0.372	52.20 μg/ml
	80 μg/ml	0.122±0.006	68.95±1.532	
	100 μg/ml	0.092±0.005	78.73±3.351	
Ascorbic acid	20 μg/ml	0.142±0.002	44.93±0.450	51.41 μg/ml
	40 μg/ml	0.117±0.001	50.23±0.226	
	60 μg/ml	0.106±0.003	62.86±0.756	
	80 μg/ml	0.090±0.004	76.95±1.001	
	100 μg/ml	0.081±0.002	89.33±0.388	

Table 10: Percent inhibition of Protein denaturation

	10	20	40	60	80	100
ZINGER	24.83±1.91	29.12±0.38	33.15±0.38	48.13±0.87	57.71±0.21	65.13±1.28
TURMERIC	30.24±0.30	41.31±0.19	43.53±0.17	49.7±0.17	57.26±0.21	62.54±0.25
TULSI	24.8±0.36	30.39±0.30	48.49±0.24	52.86±0.25	55.23±0.182	60.12±1.78
PHF-1	21.19±0.33	27.12±0.97	38.14±0.78	43.74±0.38	69.26±0.26	78.72±0.29
PHF-2	37.12±0.28	39.16±0.86	47.14±0.96	58.62±0.46	64.20±0.178	77.16±0.76
PHF-3	62.519±0.50	69.89±8.87	78.98±0.65	83.12±0.54	89.16±0.65	98.12±0.78
PHF-4	64.47±0.86	69.26±0.91	71.90±0.31	79.90±0.32	69.88±0.76	79.898±0.145
DICLO	57.14±0.64	62.12±0.721	64.15±1.32	71.287±0.33	72.16±0.24	98.16±1.78

Table 11: HRBC Membrane Stabilization

	20	40	60	80	100
PHF-1	42.15±0.17	51.27±0.18	61.11±0.176	68.12±0.74	68.54±0.89
PHF-2	34.18±0.72	39.76±0.14	40.10±0.72	51.82±0.14	66.74±0.14
PHF-3	48.25±0.19	58.16±0.82	61.11±0.14	66.52±0.79	82.74±0.95
PHF-4	41.16±0.19	47.82±0.17	54.18±0.26	65.19±0.99	79.16±1.24
DICLO	51.25±2.15	62.25±0.19	74.12±0.98	78.52±0.89	84.12±1.02

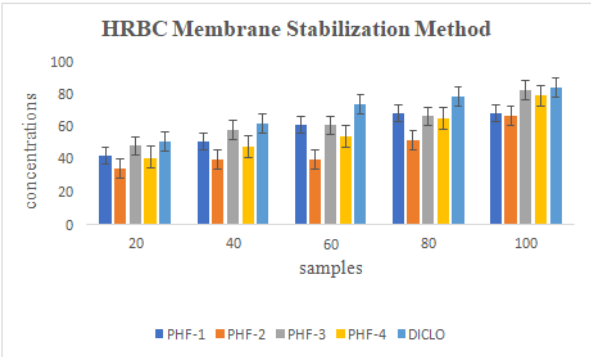

Figure 5: Method for HRBC Membrane Stabilisation

Physicochemical Parameters

Determination of Alcohol Soluble Extractive value:

The alcohol-soluble components of the polyherbal formulation (such as alkaloids, proteins, amino acids, and carbohydrates) were determined using pharmacopoeial procedures and in-house standards; the findings are shown in Table (Tables 4, 5 and 6).

Evaluation of Polyherbal Powder:

Qualitative phytochemical test for CZO extract

Several assays were used to conduct qualitative phytochemical screening, and the results demonstrated that ethanol extract of CZO. powder contains phe-

Table 12: Characteristics of gel formulation made with Carbapol

Formulati code	Conc. (%)	pH*	Viscosity* (poise)	Spread abil- ity*gcm/se	Drug con- tent* (%)	Extrudabili	Nature of gel
F1	0.5	7.56	0.381	31.13	99.3	Good	Pale green, smooth, homogenous, translucent
F2	1	7.48	0.382	44.03	99.6	Good	Pale green, smooth, homogenous, translucent
F3	1.5	7.67	0.388	55.29	98.3	Excellent	green, smooth, homogenous, translucent
F4	2	6.88	0.367	66.20	104	Good	Dark Green, smooth, homogenous, translucent

nols, flavonoids and absence of alkaloids, carbohydrates and steroids & triterpenoids (Tables 7, 8 and 9).

Estimation of Total Flavonoids

Using the regression equation y = 0.0043x + 0.0104, R2 = 0.9968, the amount of total flavonoid content was calculated from the gallic acid calibration curve. The overall flavonoid content of the ethanolic extract of CZO was 71.42, of curcuma longa was 51.86, of zingiber officinale was 62.81, of Ocimum tenuiflorum was 76.86, gallons of gallic acid equivalents per kilogramme of dry material. Within these portions, Ocimum tenuiflorum contains high flavonoid content as compared to curcuma longa and zingiber officinale (Tables 10, 11 and 12).

The thin layer chromatography was used to isolate the non-volatile mixtures. The PHE was used in TLC to estimate the RF values for individual components. The PHE consists of three mixtures so, the TLC results in three spots. The RF values of the three spots are(0.87, 0.6,0.52).

In-vitro antioxidant Activity

Hydroxyl radical scavenging activity of extracts:

The values are expressed as the MeanSEM (n=3). The breakdown of deoxyribose by the Fe+3-ascorbic acid-EDTA-H2O2 system was greatly reduced by CZO and ascorbic acid at concentration ranges of 20-100 g/ml in a dose dependent manner, demonstrating Poly herbal extract's considerable hydroxyl radical scavenging action.

The antioxidant activity of the ethanol extract was significant (IC50=52.20 g/ml) compared to standard (IC50=51.41 μg/ml).

At 100 μg/ml, the percentage inhibition. Value was 78.73% for ethanol extract, while ascorbic acid possesses 79.33% scavenging activity at same concentration.

Protein denaturation technique for in vitro anti-inflammatory action

The table as well as image demonstrate how zinger, turmeric, tulsi, and PHF 1–4 ethanolic extracts affect the prevention of protein denaturation. All samples were at various concentrations (between 20 and 100 g). offered significant defence against protein denaturation. Comparing PHF-3 to different formulations and individual ethanolic extracts, the highest % inhibition was found at 100g/ml. It has sizable activity comparable to that of diclofenac sodium standard. The majority of researchers have noted that one underlying cause of rheumatoid arthritis is protein denaturation. In some rheumatic illnesses, the production of autoantibodies may be triggered by the denaturation of proteins.

The work is motivated by the need for effective natural anti-inflammatory agents with fewer side effects to replace chemical therapies. Table and image depict the impact of PHFs 1 through 4 on the stabilisation of the HRBC membrane. Highest level of stabilisation was observed in PHF -3 (82.74% at

100μg/ml) as compared to other formulations. It possesses significant activity equivalent to that of diclofenac sodium. It is possible that the substantial anti-inflammatory efficacy is related to the presents of secondary metabolites such as flavanoids, terpenoids, phenols.

Polyherbal evaluations

Carbopol 934, various amounts of ethanolic extract of PHE, methylparaben, distilled water, and triethanolamine were used to make the polyherbal gel. The appearance, viscosity, spreadability, pH, and homogeneity of the prepared gels were tested, and the findings are reported in Table. All gel compositions are pale green in colour, transparent, and have a smooth application feel. All of these formulas demonstrated optimal viscosity. All developed formulations had pH values ranging from 6 to 7, which is regarded as appropriate to avoid skin irritation when applied to the skin. When made and after one month, all formulations are homogenous and free of gritty particles.

Conclusion

Using the study as a foundation, the findings revealed that the polyherbal gels made from the Polyherbal extracts (Ginger, Turmeric and Tulasi) When compared to normal Diclofenac gel, it demonstrated strong anti-inflammatory effect. Because phytochemical studies revealed the presence of glycosides, carbohydrates, flavonoids, steroids, and resin in the ethanolic extracts, these compounds may restrict the synthesis of prostaglandins and bradykinins, antagonise their action, as well as exert their function. When compared to individual extracts, the polyherbal gels demonstrated a synergistic effect that may be effective for the treatment of local inflammation.

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Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

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