FORMULATION, EVALUATION AND IN-VITRO ANTI-INFLAMMATORY ACTIVITY OF HERBAL SUSPENSION

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ABSTRACT:
Objective: The current study was aimed to
1) To formulate herbal suspension.
2) To evaluate herbal suspension.
3) To evaluate the in-vitro anti-inflammatory activity of herbal suspension of Panax ginseng.
Materials and Methods: Ethanolic extract of Panax ginseng was prepared in the laboratory by using Soxhlet extractor. Phytochemical screening was performed to determine the presence of phytoconstituents. Herbal suspension of Panax ginseng was formulated and evaluated and the in vitro anti-inflammatory activity of the suspension was assessed based on the ability of extract in suspension to inhibit protein denaturation.
Results: Preliminary phytochemical screening of ginseng extract revealed the presence of steroids, glycosides, triterpenoids, alkaloids, polysaccharides, amino acids, tannins and phenolic compounds. The prepared herbal suspension had shown good physical parameters and optimum stability and sedimentation rate. The maximum inhibition of protein denaturation was found to be 86.21% at 250µg/mL in the in-vitro anti-inflammatory activity of herbal suspension of Panax ginseng.
Conclusion: The in-vitro study revealed that the ginseng extract in the herbal suspension had shown significant anti-inflammatory activity. From this study we can also conclude that the prepared herbal suspension showed optimum parameters for physico chemical properties and stability.

Key words: Panax ginseng Meyer, Herbal Suspension, Formulation, Evaluation, Anti-inflammatory activity, Protein Denaturation, Extraction.

1. INTRODUCTION

Inflammation is a complex biological response of our body to harmful external stimuli such as microbial infections and chemical toxins [1]. It is closely associated with the release of pro-inflammatory mediators including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines such as interleukins (ILs) and tumour necrosis factor- α (TNF-α) [1-2]. The inflammatory response is characterized by several steps including coordinate activation of signalling pathways, complex changes in humoral and cellular components ,vascular permeability, expression of pro-inflammatory cytokines, chemokines and adhesion molecules in resident tissue cells, and infiltration of leukocytes mainly macrophages, neutrophils and dendritic cells and mediators of inflammation from the vascular system to remove the harmful stimuli and to initiate the healing process [1]. Inflammation not only results in tissue oedema, pain and damage, but also may contribute to chronic disease development, including obesity, diabetes, psoriasis, pharyngitis, arthritis, and cancer [2-3].

Panax ginseng Meyer, also known as Korean ginseng or Asian ginseng is a slow-growing perennial plant, belonging to the family Araliaceae [4]. *P. ginseng* root comprises 80 to 90% organic, approximately 10% inorganic substances, including many active constituents like ginsenosides or saponins, nitrogenous substances like alkaloids, carbohydrates, phytosterol, polyacetylene, fatty acids, amino acids, peptidoglycan, vitamins, essential oils, minerals and other phenolic compounds[4-5-6]. Ginseng contains 43 types of ginsenosides including protopanaxadiol-type ginsenosides, Rb1, Rb2, Rc, and Rd; protopanaxatriol-type ginsenosides, Re, Rf, and Rg1; and oleanane-type ginsenoside, Ro [6]. Ginseng has pharmacological properties such as antioxidant, anti-viral, anti-aging, anti-depressant, anti-fatigue, anti-obesity, and anti-carcinogenic and hepatoprotective effects. Newly discovered properties include antibacterial, antifungal, anti-inflammatory, activities [2-3-6]. Recent studies have further shown the anti-inflammatory effects of ginseng extracts and ginsenosides in cellular responses triggered by inducers like endotoxin, TNF-α, INF-γ and other stimuli [7].

Figure1. Roots of Panax ginseng.

The oral route of drug administration is the most important means of administrating drugs for systemic effects. Ayurvedic herbal formulations are also usually administered by oral route. In present study *Panax ginseng* will be selected for developing the herbal

suspension. Suspension is coarse dispersion of finely divided solid particles of drug dispersed in a continuous liquid medium, where the drug is not readily soluble. An aqueous suspension is a beneficial formulation system for administering an insoluble or poorly soluble drug to the body [8].

2. MATERIALS AND METHODOLOGY

2.1. MATERIALS

2.1.1. Apparatus

Table 1. Name of Equipments

Sl. No.	Name of Equipment
1.	Soxhlet Extractor
2.	Round Bottom Flask
3.	Heating Mantle
4.	China Dish
5.	Digital Balance
6.	Beaker
7.	Measuring Cylinder
8.	Funnel
9.	Glass Rod
10.	Spatula
11.	Mortar and Pestle
12.	100 mesh size sieve
13.	Test tube
14.	Test tube holder
15.	Bunsen Burner
16.	Water bath
17.	Optical Microscope
18.	Stage micrometre
19.	Eye piece micrometre
20.	Glass slide
21.	Clarity test apparatus

Chemicals Used

Table 2.Name of Chemicals

Sl. No.	Name of Chemicals
1.	Panax ginseng extract
2.	Tween 80
3.	Sodium CMC
4.	Sodium Benzoate
5.	Sugar™ Free Gold
6.	Lemon Oil
7.	Distilled water

2.1. METHODOLOGY

2.2.1. PLANT MATERIAL

Collection and authentication: For this study, roots of plant *Panax ginseng* were collected and the sample was identified and authenticated by Indian Institute of Horticultural Research, Hessaraghatta Lake Post, Bangalore.

The roots of the plant were washed with tap water and dried in shade for 20 days prior to study.

2.1.2. EXTRACTION

The fresh, air-dried, powdered roots of *Panax ginseng* were extracted by repeatedly boiling and refluxing with ethanol until exhaustion using Soxhlet extractor[9].

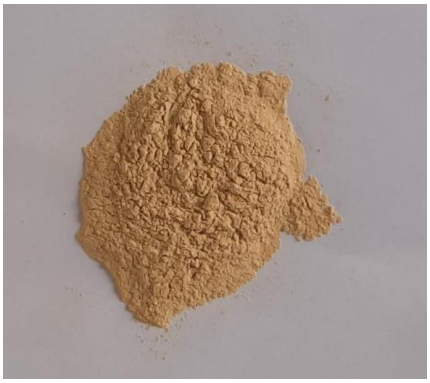

Figure2. Air dried and powdered Panax ginseng root extract.

2.1.3. PRELIMINARY PHYTOCHEMICAL ANALYSIS

A small portion of extract was subjected to qualitative chemical tests for identification of their active constituents. Tests for the presence of steroids, triterpenoids, glycosides, alkaloids, polysaccharides, amino acids, tannins and phenolic compounds were conducted as per the standard procedure.

I. Test for Steroid

a) Salkowski test: To 2ml of extract, 2ml chloroform and 2ml conc. H_2SO_4 was added and shaken well.

b) Liebermann-Burchard test: 2ml of extract was mixed with chloroform. 1-2ml acetic anhydride was added along with 2 drops of concentrated sulfuric acid added along the side of test tube.

c) Liebermann test: 3ml of extract was mixed with 3ml acetic anhydride, heated and cooled. A few drops conc. H_2SO_4 was added.

II. Test for Glycosides

A) Test for Cardiac Glycosides

a) Legal's Test (test for Cardenoloids): To the alcoholic extract 1ml pyridine and 1ml sodium nitroprusside was added.

b) Kellar-Killiani Test (Test for deoxysugars): To 2ml extract glacial acetic acid, 1 drop 5% $FeCl_3$ and conc.H_2SO_4 was added.

B) Test for Saponin Glycosides

a) Foam Test: The drug extract was shaken vigorously with water to obtain persistent stable foam.

b) A drop of Na_2CO_3 solution was added to 5ml of extract in a test tube. After vigorous shaking it was left to rest for 5 minutes.

C) Test for Anthraquinone Glycosides

a) Borntrager's Test: To 3ml extract dil.H_2SO_4 was added, boiled and filtered. To the cold filtrate equal volume of benzene or chloroform was added and shaken well. Organic solvent was separated. Ammonia was added.

D) Test for Coumarin Glycosides

a) The alcoholic extract was made alkaline.

III. Test for triterpenoid

a) Horizon test: 2ml of trichloroacetic acid was added to 1ml of extract.

b) Salkowski test: A few drops of conc. H_2SO_4 were added to extract solution, shaken well and allowed to stand.

IV. Test for Alkaloid

The alcoholic extract was evaporated separately and dil. HCl was added. It was shaken well and filtered. With the filtrate following tests were carried out

a) Dragendorff's test: To 2-3ml filtrate, few drops of Dragendorff's reagent were added.

b) Mayer's test: To 2-3ml filtrate Mayer's reagent was added.

c) Hager's test: To 2-3ml filtrate Hager's reagent was added.

d) Wagner's test: To 2-3 ml filtrate Wagner's reagent was added.

e) Tannic acid test: Test solution was treated with tannic acid.

f) Murexide test for purine alkaloids: To 3-4ml of test solution, 3-4 drops of con. H_2SO_4 was added. It was evaporated to dryness, cooled and 2 drops of NH_4OH was added.

V. Test for Polysaccharides

a) Iodine test: 3ml test solution was mixed with a few drops dilute iodine solution.

b) Tannic acid test for starch: With 20% tannic acid solution the test solution gives precipitate.

VI. Test for Amino Acids

a) Ninhydrin Test (General test): 3ml of test solution was heated with 3 drops of 5% ninhydrin solution in boiling water bath for 10 minutes.

b) Test for tyrosine: 3ml of test solution was heated with 3 drops of Millions reagent.

c) Test for cysteine: To 5ml test solution 40% NaOH and 10% Lead acetate solution was added. The solution was then boiled.

VII. Test for Tannins and Phenolic compounds

To 2-3ml of alcoholic extract the following reagents were added:

a) Ferric chloride test: 5% $FeCl_3$

b) Lead acetate test: Lead acetate

c) Gelatin solution

d) Bromine water

e) Acetic acid solution[10-11]

2.1.4. FORMULATION OF SUSPENSION

The composition of formulation for preparing three different 100 ml of suspensions of *Ginseng* was selected. The 100 mesh size fine particles of the drug were properly mixed by triturating. After that the drug was added to water and different additives such as Tween-80, sodium carboxymethyl cellulose (CMC), sweetening agent, flavoring agent, and sodium benzoate used for its better stability during shelf life of formulation. [8]

Figure 3. Size reduction of powdered ginseng extract for the formulation of suspension.

Table 3.Composition of herbal suspension

SL. No.	Name of Ingredient	Quantity Taken		
		F1	F2	F3
1.	Panax ginseng	1g	1g	1g
2.	Tween 80	0.1w/v	0.1w/v	0.1w/v
3.	Sodium CMC	0.5%	0.7%	1.0%
4.	Sodium Benzoate	1g	1g	1g
5.	Sugar™ Free Gold	0.1g	0.1g	0.1g
6.	Lemon Oil	1ml	1ml	1ml
7.	Purified Water q. s	100ml	100ml	100ml

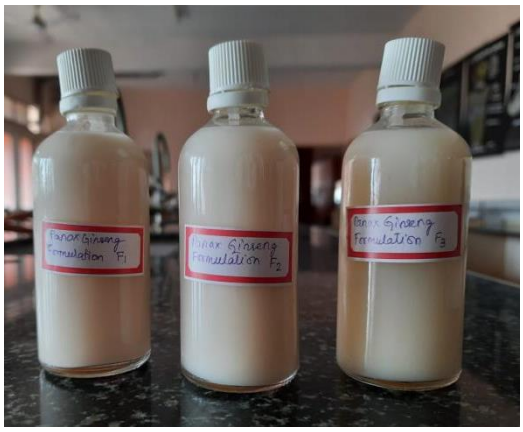

Figure 4. Formulation of F1, F2, F3 form of herbal suspension.

2.1.5. IN-VITRO ANTI-INFLAMMATORY ACTIVITY

Inhibition of protein denaturation:

The denaturation of proteins is one of the sources of inflammation. Hence, protein denaturation can be applied as in vitro screening model for anti-inflammatory compounds. The protein denaturation assay was conducted by taking 0.25ml of different concentrations of Herbal extract formulation F3 (12.5, 25, 50, 100 and 250 µg/ml) with 2.25ml of 1% bovine serum albumin (BSA). The pH of the reaction mixture was adjusted to 6.3 using 1 N HCl and vortexed and then incubated at 37°C for 20 min. The mixture was further incubated at 57°C for 20 min and thereafter allowed to cool at room temperature. Then 2.5 ml of phosphate buffered saline (pH 6.3) was added to each tube and the measurement of turbidity was conducted at 660 nm. Distilled water was added for control. The percentage of inhibition of protein denaturation was calculated using the below given formula.

Percentage of Inhibition = [(Abs of Control – Abs of Sample) ÷ (Abs of Control) × 100][12]

2.1.6. EVALUATION

Herbal suspension was evaluated for the following parameters using suitable methods. Particle size, Appearance, Clarity, Rate of sedimentation, Stability Studies.

3. RESULTS AND DISCUSSION

3.1. Preliminary phytochemical screening:

The phytochemical analysis is important to evaluate the possible medicinal utilities of a plant and also to determine the active principles responsible for the known biological activities exhibited by the plants. These secondary metabolites contribute crucially towards the biological activities of medicinal plants such as hypoglycemic, antidiabetic, antioxidant, antimicrobial, anti-inflammatory, anticarcinogenic, antimalarial, anticholinergic, antileprosy activities etc. The results of phytochemical analysis of the ginseng extract revealed the presence of various phytoconstituents as shown in the table below.

Table 4.Results of Phytochemical screening of *Panax ginseng* extract

Test Performed	Name of the test	Result
A. Steroids	1) Salkowski test	+ve
	2) Liebermann-Burchard test	+ve
	3) Liebermann test	+ve
B. Glycosides	1) Legal's test	+ve
	2) Keller-Killiani test	+ve
	3) Borntrager's test	-ve
	4) Foam test	+ve
	5) Na2CO3 solution	+ve
	6) Alkalinisation of extract	-ve
C. Triterpenoids	1) Horizon test	+ve
	2) Salkowski test	+ve
D. Alkaloids	1) Dragendorff's test	+ve
	2) Mayer's test	+ve
	3) Hager's test	+ve

	4) Wagner's test	+ve
	5) Tannic acid test	+ve
	6) Murexide test	-ve
E. Polysaccharides	1) Iodine test	+ve
	2) Tannic acid test	+ve
F. Amino Acids	1) Ninhydrin test	+ve
	2) Test for tyrosine	+ve
	3) Test for cysteine	-ve
G. Tannins and Phenolic compounds	1) 5% FeCl3 solution	-ve
	2) Lead acetate solution	+ve
	3) Gelatin solution	+ve
	4) Bromine water	+ve
	5) Acetic acid	+ve

+ve indicated the presence and –ve indicated the absence of respective class of compound. Preliminary phytochemical screening revealed the presence of steroids, glycosides, triterpenoids, alkaloids, polysaccharides, amino acids, tannins and phenolic compounds in the ginseng extract.

3.2. EVALUATION OF HERBAL SUSPENSION

Appearance:

The three different formulations F1, F2 and F3 of herbal suspension were evaluated for their physical appearance[8]. The results of the evaluation are shown in the table given below.

Table 5. Results of Physical test for herbal suspension

Sl. No.	Parameters	F1	F2	F3
1.	Nature	Liquid	Liquid	Liquid
2.	Colour	Cream/ Barely yellow	Cream/ Barely yellow	Cream/ Barely yellow
3.	Odour	Pleasant	Pleasant	Pleasant
4.	Texture	Suspension Free from gritty texture	Suspension Free from gritty texture	Suspension Free from gritty texture
5.	Appearance	Smooth Elegant	Smooth Elegant	Smooth Elegant

Clarity: The three different formulations F1, F2, F3 of herbal suspension were evaluated for clarity using clarity test apparatus. The results showed that the suspensions were found to have no foreign particles when held against light and dark backgrounds.

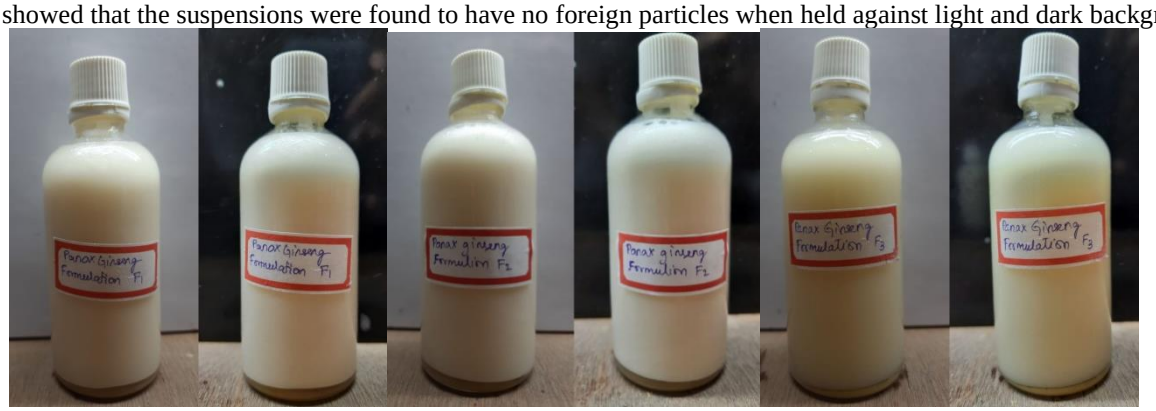

Figure5. Evaluation of clarity for F1, F2 and F3 forms of herbal suspension.

Particle size analysis: The range of particle size distribution of the F3 herbal suspension was approximately found to be from 3-10µm by optical microscopic method. In the case of F1 and F2 formulations, the particle size slightly change over a period of two week, but after one month due to formation of clumpy mass particle size measurement was not possible[13].

Rate of Sedimentation: The herbal suspensions were evaluated for sedimentation ratio and F3 form of herbal suspension shows sedimentation ratio of 1.04 after 270 minutes which is better than F1 and F2 form of suspension which showed sedimentation ratio of 1.13 and 1.07 respectively after 270 minutes[14].

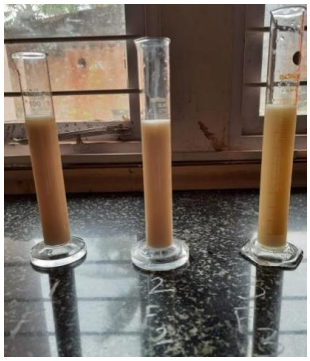

Figure 6. Rate of sedimentation of F1, F2, and F3 forms of herbal suspensions.

Table 6.Results of Rate of Sedimentation of Herbal suspensions

Sr. No.	Time (min)	Ultimate Height (Hu) (ml)	F1		F2		F3	
			Final Height (Ho) (ml)	Sedimentation Ratio (Hu/Ho)	Final Height (Ho) (ml)	Sedimentation Ratio (Hu/Ho)	Final Height (Ho) (ml)	Sedimentation Ratio (Hu/Ho)
1.	30	100	99	1.01	99	1.01	99	1.01
2.	60	100	97	1.03	98	1.02	98	1.02
3.	90	100	96	1.04	97	1.03	98	1.02
4.	120	100	95	1.05	96	1.04	98	1.02
5.	150	100	94	1.06	96	1.04	97	1.03
6.	180	100	92	1.08	95	1.05	97	1.03
7.	210	100	91	1.09	95	1.05	97	1.03
8.	240	100	90	1.11	94	1.06	97	1.03
9.	270	100	88	1.13	93	1.07	96	1.04

Stability study: The results reveal that in F3 formulation, no changes were noticed in all the tested parameters and turbidity/homogeneity of the suspension after 1 month of observation. There was no significant change observed in physicochemical and organoleptic behaviour in F3 formulation. However, F1 and F2 formulations did not show good dispersible pattern, homogeneity and formation of clumpy mass was observed.

The herbal suspensions F1, F2, F3 were prepared by adding different concentrations such as 0.5%, 0.7% and 1.0% of sodium CMC. Sodium CMC improves viscosity and stability of suspension. These formulations were evaluated for various quality parameters to determine their stability and acceptability. When the concentration of suspending agent increases in the suspensions an appreciable increase in viscosity was found. The data obtained from these evaluations revealed that F3 formulation of suspension was found to have good dispersible property with the sedimentation rate and stability studies. This indicated that F3 formulation of herbal suspension was found to be the most optimum and acceptable amongst the three prepared formulations. Hence, F3 formulation of herbal suspension was used for the in-vitro anti-inflammatory activity evaluation[8-13].

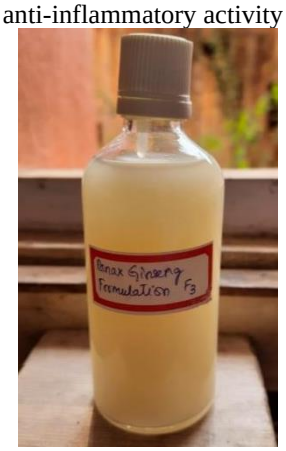

Figure7. F3 Formulation of herbal suspension.

3.3. INVITRO ANTI INFLAMMATORY ACTIVITY:

Absorption at wavelength 660nm.

Table 7. Results of Effect of Herbal Plant Extract Formulation F3 on Inhibition of Denaturation of Protein

Sl. No.	Conc. (µg/ml)	Abs of Herbal suspension formulation	% Inhibition of Herbal suspension formulation
1	12.5	0.664	32.66
2	25	0.597	39.45
3	50	0.505	48.78
4	100	0.418	57.61
5	250	0.136	86.21
6	Control	0.986	

Denaturation of proteins is a well- documented source of inflammation. As a part of the research on the mechanism of the anti-inflammatory activity, ability of extract in the formulation to inhibit protein denaturation was studied. It was successful in inhibiting heat induced albumin denaturation at different concentrations as shown in Table 5. Maximum inhibition, 86.21% was observed at 250µg/ml.

4. CONCLUSION

Traditional herbs are a considerable source of therapeutic agents. Ginseng contains a complex mixture of chemical constituents that have multiple and varied physiological effects on the human body. It is generally touted for its anti-inflammatory and antioxidant and effects.

The results of this research indicate that the extract of *Panax ginseng* in the herbal suspension shows significant anti-inflammatory activity.

From this study we can also conclude that the prepared herbal suspension showed optimum parameters for physicochemical properties and stability.

5. ACKNOWLEDGEMENT

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