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Quantification of Gallic Acid, HPTLC, GC-MS Profiling and Anti-inflammatory Potential of Fermented Traditional Formulation "*Punarnavadyaristha*"

Research Article

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Abstract

Background: *Punarnavadyaristha* (PA), is a polyherbal fermented traditional formulation therapeutically known as anti-inflammatory in the classical text of Charak Samhita. However, there is no scientific evidence about its anti-inflammatory potential in the published literature. Objective: The present study was performed to validate the potential of PA against inflammation. Along with physicochemical parameters, the quantification of gallic acid and GC-MS profiling were performed to reveal the composition of PA. Materials and methods: GC-MS profiling of PA was performed to confirm the presence of bioactive compounds using the NIST library database. Quantitative HPTLC studies were performed for the quantification of gallic acid. The presence of reactive oxygen was assessed by DPPH assay.PA was assessed preclinically for acute oral toxicity and carrageenan (acute) and formalin (sub-acute) induced edema. Results and Discussion: The GC-MS profile of PA demonstrates the presence of 67 bioactive compounds. HPTLC studies confirm the presence of gallic acid in the formulation (4.2 %). It also shows free radical scavenging activity of 47% by DPPH assay. No acute toxicity was seen in rats up to 10.0 mL/kg dose of formulation.PA (in-house) formulation at the dose of (4 mL/kg) showed inhibition of formalin-induced paw edema by 74% and carrageenan-induced paw edema by 29%. Conclusion: The effectiveness of *Punarvadyaristha* on inflammatory disorder, safety, and antioxidant potential is authenticated by these studies. Standardization parameters also comply Ayurvedic Pharmacopoeia of India.

Keywords: Anti-inflammatory activity, Carrageenan, Formalin, *Punarnavadyarishtha*.

Introduction

Traditional formulations of Ayurveda are used in India for the prophylaxis and treatment of a wide range of unhealthy eating habits, which all contribute to an increase in inflammatory disorders, which further give rise to complicated symptoms. (1)

Accumulated Toxins and Inflammation

"*Ama*" is a word derived from the '*Am*' meaning Dhatu. The term "*Ama*" denotes a substance that remains undigested in the body. It refers to a substance that is unripe and would further go into biotransformation.(2) It is the outcome of weak digestion, which gives rise to the accumulation of toxins. *Ama* includes both endo and exotoxins, which may trigger an inflammatory response in the body. (3)

Allopathic drugs used for Inflammatory disorder

Many allopathic drugs are available in the market for the cure of inflammatory disorders, but limitations are renal and hepatic toxicity. (4)Ayurvedic literature has many herbal formulations that are very effective in managing renal and liver disorders and curing inflammatory disorders. So, here, safety and biocompatibility are preferred. (5)

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Classical Insight on Anti-inflammatory Formulations

Charaka Samhita, Sushruta Samhita, *Nigantus*, *Dravyagun*, etc., are classical texts of Ayurveda. (6) There are many polyherbal formulations praised in different Ayurvedic texts for *reducing inflammation*, out of which Punarnavadyaristha is recommended in Ayurveda for inflammatory, hepatic, and renal disorders. (7) *Punarnavadyaristha* is an ayurvedic fermentation alcoholic formulation prepared from *Shwet* and *Rakt Punarnava* (Boerhavia sp.) in association with other herbal ingredients. (8) *Punarnavadyaristha* is highly recommended in Ayurveda text for its potential on various metabolic disorders (9) So, in the present study, it is chosen for the exploration of anti-inflammatory potential.

Scientific evaluation of selected formulation *Punarvadyaristha*

Punarnavadyaristha is a polyherbal formulation the main active ingredient is Punarnava and therapeutic use may be attributed to the properties of *Punarnava*. According to Ayurveda, *Shweta* and *Rakt Punarnava* are potent and have the ability to balance the three doshas (Vat, Pitta, and Kapha). *Punarnava* are both

diuretic, purgative, anti-inflammatory (10), aphrodisiac, anti-anemic, and diaphoretic, and exhibit heart tonic and *rasayan* qualities. (11),Chemical analysis of *Boerhavia diffusa* (*Rakt Punarnava*) shows a broad range of phytochemicals, including rotenoids, flavonoids, xanthones, purine nucleosides, lignans, and steroids. (12)

Ancient formulation Arista (Hot decoction) was preferred for the present study due to the effective method of preparation including biomedical fermentation and prolonged shelf-life. This formulation is somewhat different from other *aristha* due to the absence of *Maduca longifolia* as Jaggery contains yeast spores that aid fermentation.(13) So, these ancient formulations are already standardized by the wisdom of our saints but we have to standardize them by using modern scientific parameters for worldwide acceptance.

Materials and Methods

Procurement and Authentication of Herbs

All herbs were procured from the nursery and additives were purchased from the local market in Moradabad and authenticated in the Department of Botany, IFTM University by Dr. Ashok Kumar, Taxonomist, and voucher specimens of herbs were kept there in the herbarium for future reference.

Formulation of *Punarnavadyaristha* (PA)

Punarnavadyaristha is a fermented polyherbal formulation, made with the ingredients listed in Table 1.

Table 1: Formulation composition of *Punarvadyaristha* (Each 100 mL contains)

S. No.	Ingredients	Botanical name	Herbs with anti-inflammatory potential (References)	Quantity
1	Sveta Punarnava	*Boerhavia verticillata* Poiret(Rt.)	-	2.34 g
2	Rakta Punarnava	*Boerhavia diffusa* L (Rt.)	(14)	2.34 g
3	Bala (Kharainti)	*Sida cordifolia* Linn(Rt.)	(15)	2.34 g
4	Atibala (kanghi or thuthi)	*Abutilon indicum* (L.)(Rt.)	(16)	2.34 g
5	Patha (velvet leaf)	*Cissampelos pareira* L. (Rt.)	(17)	2.34 g
6	Vasa (Vasaka)	*Adhatoda vasica* Nees(Rt.)	(18)	2.34 g
7	Guduci (Giloy)	*Tinospora cordifolia* (Willd.) Miers(St.)	(19)	2.34 g
8	Citraka (citrak)	*Plumbago zeylanica* L.(Rt.)	(20)	2.34 g
9	Nidigdhika (Kantakari)	*Solanum xanthocarpum* L.(Pl.)	(21)	2.34 g
10	Jala for decoction	Water	-	Q. S
11	Guda	Jaggery	-	156.25 kg
12	Madhu	Honey	-	11.52 g
13	Hema (Naag kesher)	*Mesua ferrea*		0.39 g
14	Tvak (Dalchini)	*Cinnamomum zeylanicum* Blume(St.Bk.)	(16)	0.39 g
15	Ela (Elaychi)	*Elettaria cardamomum* (L.)(Sd)	(22)	0.39 g
16	Marica	*Piper nigrum* L. (Fr)	(23)	0.39 g
17	Ambu (Hrivera)	*Coleus vettiveroides* K.C. Jacob(Rt)	-	0.39 g
18	Patra (Tejapatra)	*Cinnamomum tamala* Nees. Laurus nobilis (Lf)	(24)	0.39 g

Note: The decoction herbs are in the same quantity ie 2.34g while prakshepa (additives are in the same quantity 0.39mg) St=stem, Bk=Bark, Rt=Roots, Pl=Whole plant, Lf=Leaf

Method of Preparation

All the raw materials (herbs, sugar honey, etc. were taken in as described in Ayurvedic Pharmacopoeia of India (API). The ingredients numbered 1 to 9 (decoction ingredients) were washed, dried, and individually powdered then sieve number 44 was used to obtain coarse powder.

Additives or Prakshepa dravas ingredients numbered 10 to 18 were clean, dry, and powdered individually, and sieve number 85 was used to obtain fine powder. A sufficient amount of water was added to the decoction ingredients, soaked overnight, heated, reduced to half volume, and filtered by muslin cloth to obtain decoction. (25) After that, a variety of components were added to the decoction, given time to dissolve, and then filtered through muslin fabric., then the filtrate was moved to a sanitized container.

Madhu (honey) and Jaggery (fermentation initiator) were added to finely powdered Prakshepa Dravyas (These are merely restricted to pharmaceutical processing and can be viewed as aids of contemporary pharmaceutical research. In addition to its synergistic effects, Prakshepa Dravya also plays a pharmaceutical role. (26)

The container's mouth was sealed. The fermentation process was maintained in the container, and it was regularly monitored for indications that the fermentation was finished. The fermented material was passed through a neat and clean muslin cloth, further packed in air-tight containers, and kept for maturation. (27)

Description

PA is a dark brown, transparent liquid with a sharp flavor. It has no foam either.

Physicochemical studies

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Punarnavadyaristha's physicochemical analyses were carried out using the protocol outlined in the Indian Ayurvedic Pharmacopoeia. (28)

Phytochemical Screening

Punarnavadyaristha underwent phytochemical screening to determine whether secondary metabolites such as alkaloids, phenolics, flavonoids, and glycosides were present. It is very important for polyherbal formulation in which secondary metabolite dominates in presence.

GC – MS profile of In- house formulation of *Punarnavadyarista* for identification of volatile constituents

GC/MS is a highly favored analytical technique in metabolomic research. Using an Agilent 7890A GC, a thorough GCMS analysis of Punarnavadyaristha was performed by previously published procedures. Chromatographic peak origins are useful as indications, particularly for interpreting biotransformation pathways. Retention indices (RI) were determined for components using the C8–20 standard (~ 40 mg/L each, in hexanes). Peaks are characterized by the NIST14 database and reported literature, and were identified in terms of quantification using peak area and abundance.

HPTLC Analysis of *Punarvadyaristha* for analyzing Gallic acid content

Silica gel F254 plates (E. Merk) were used of dimension 20x10 cm. (29) The calibration mode was multi-level, the statistics mode was CV, and the evaluation mode was peak height and area. The sample applicator was CAMAG linomat 5. The Standard solution of gallic acid was prepared by dissolving 100 mg in 100 mL methanol. While the PA 20µl sample was placed on a TLC plate. The solvent system Toluene: Ethyl acetate: Formic acid in the ratio 4.5:5.5:0.5 was used as the mobile phase. UV light at 366nm was used for spot detection. The dosage speed was 150nl/s and the dosage volume were 0.2ul. For PA application position was 73.1mm, and the applied volume was 5.0 microlitres development chamber was a Twin trough chamber 20x10 cm. The detector was a CAMAG TLC Scanner. The plates were scanned at 290nm. The software used for analysis was WINCATS. Methanol and Gallic acid were procured from CDH, New Delhi.

Method Validation

To validate the process of densitometric analysis of gallic acid in the PA calibration curve of gallic acid was obtained. ICH guidelines were followed to determine LOD and LOQ and recovery of gallic acid.

In vitro Antioxidant activity

The 1,1-diphenyl-2-picryl hydrazyl (DPPH) technique was used to examine PA's ability to scavenge free radicals. [30] A stock solution was prepared by dissolving 25 milligrams of DPPH (available from CDH chemicals, New Delhi) in 100 millilitres of methanol. The DPPH stock solution was filtered with methanol to create a manageable mixture that had an absorbance of roughly 0.950 at 517 nm. In a test tube, 100 µL of Punarnavadyaristha and 3 mL of DPPH solutions were combined. Three milliliters of DPPH-containing solution in 100 µL of methanol was the standard used. After that, the tubes were kept completely dark for thirty minutes. Therefore, the absorbance was calculated at 517 nm. The antioxidant % was calculated using the following formula.Antioxidant activity percentage = [(Ac−As) ÷Ac] × 100; where: Ac—Control reaction absorbance; As—Testing specimen absorbance

In Vivo Acute Toxicity Studies Pre-Clinical Studies of PA on Inflammation

Animals

Wistar albino rats weighing between 100 and 120 grams were kept in the IFTM University's Animal House in Moradabad, Uttar Pradesh, India. All animals were kept in clean, sanitary polypropylene cages that had 12 hours of light and dark cycles and a consistent temperature of 24± 1°C. The animals had unrestricted access to drinking water and were given a regular pellet (Hindustan Lever Ltd., India). The Institutional Animal Ethical Committee (IAEC; reference number IAEC/ 2021/33) approved the CPCSEA criteria, and all experiment methods and procedures were properly approved. The NIH publication no. 82-23, updated 1985) Principles of Laboratory Animal Care criteria were properly followed. (30)

Chemicals and drugs

Carrageenan (Sigma-Aldrich, St Louis, MO, USA), Formaldehyde (Merck Limited, Mumbai, India), and Indomethacin(31) (Acichem Laboratories, Mumbai, India) were used for the study.

Dose selection and treatment

In the classical texts, a 15-ml dose of aristha has been mentioned per day for an adult human of 70 kg. Considering the dose in humans and the extract yield (≈10%), the formulation dose in rats (as per body surface area about man) was approximately 1 mL /kg. Therefore, graded doses of Punarnavadyaristha were tested to find an optimal efficacy against formalin and carrageenan-induced paw edema. Using an orogastric tube, the arista was given once daily in a volume of 1 ml/100 g of animal body weight. So the dose for rats with average weight of 200-350g is 2mL(low dose)and 4mL(high dose) and For every experimental paradigm under study, specifics on dosage, administration timing, and therapy length have been included.(32)

Anti-inflammatory activity

Punarvadyaristha formulation's anti-inflammatory activity was evaluated in Formalin and Carrageenan-induced inflammation. (32)

Formalin-induced paw edema

There were four groups of animals (n = 6). Every rat was given a sub-planter injection on the first and third day of the experiment, containing 0.1 ml of 3% v/ v formalin in its hind paw. The control or first group received 0.5% CMC and group II received

Indomethacin (10 mg/kg). The first treatment dose was administered 60 minutes before the formalin injection and was continued for seven days.

The remaining groups III and IV were treated with (2 and 4mL/kg, PA, i.p.), whereas the Paw thickness was assessed using a plethysmometer by the procedure at 0 and 3 hours and seven days after treatment. The calculation of the percent inhibition in paw volume between the treated and control groups follows.

All calculation was done by using a Graph pad. All data are shown in ± Sd and are significant to control. Percent inhibition = (A − B/A) x100

Where, A and B are the mean paw volume of the treated and control groups, respectively, at

3h or on the seventh day. (33)

Carrageen-induced paw edema

From among the animals, four groups of six animals each were chosen at random (Morris 2003). Group I received normal saline (10 ml/kg), group II received indomethacin (10 mg/kg), and groups III and IV received therapy (2 and 4 mL/kg PA, *i.p.*). Each rat was given a subcutaneous injection of carrageenan (1%, 0.05ml) in the sub-plantar tissue of its right hind paw thirty minutes after the previously mentioned intraperitoneal dose. The inflammatory response to the carrageenan injection was measured using a plethysmometer at 1, 2, 3, and 24 hours later. The following method was used to determine the proportion of inhibition (anti-inflammatory activity) of edema: comparing the average foot swelling of the drug-treated animal and the standard to that of the control. All calculation was done by using a Graph pad. All data are shown in ± Sd and are significant to control. Percent inhibition = A-B/A × 100; Where A represents the edema volume of control and B is the paw edema of the tested group.(34)

Results

The standardization parameters and biological evaluation outcomes are discussed here.

Physicochemical studies

Various physicochemical properties of Punarnavadyaristha were evaluated according to the procedure mentioned in Ayurvedic Pharmacopoeia of India. As described in Table 2.

Table 2: Physicochemical evaluation of in-house formulation

Parameters	In-house Punarnavadyarista	Parameters in API
Total solid Contents	12.33%	NLT 11.49%
Specific Gravity	1.04	1.02-1.3
Alcohol Content	9.43%	5-10%
Reducing Sugar content	6.12%	NLT 5.8%w/v
pH	3.80 ± 0.07	4.1
Test for Methanol (methyl salicylate test)	Methanol was absent in the formulation	Absent

#NLT=Not less than

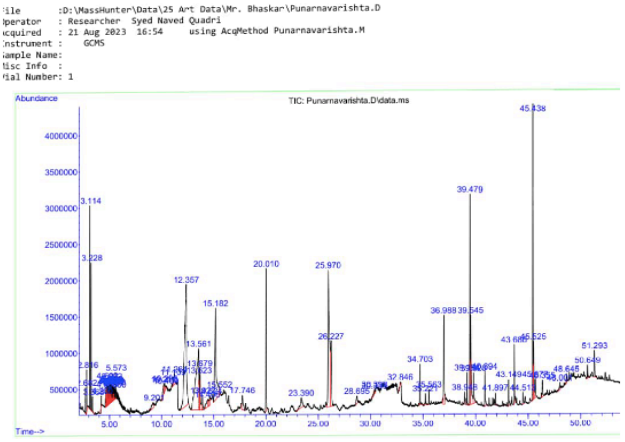
Phytochemical Screening

PA is a polyherbal formulation so it shows the presence of many secondary metabolites in evaluation, Phenolics dominate in presence. As shown in table 3.

GC – MS profile of In- house formulation of Punarnavadyarista

The GC – MS profile of Punarnavadyarista shows the presence of bioactive compounds. It contains many herbs and additives which go through fermentation to produce final outcome. The following chemical compounds are revealed in this formulation. As shown in Fig.1 and Table:4

Fig 1: GC-MS profile of Punarvadyaristha showing peaks and area occupied by chemical constituents



The compounds identified by GC-MS profiling according to mass/charge ratio were identified by NIST database as documented in Table 4.

Table 3: Phytochemical screening of Punarvadyaristha

S.No.	Experiment	Test	Inference	Punarnavadyarista
1	Test for Alkaloids	Mayers's Test	Cream colour precipitate	+
		Wagner's Test	Reddish orange precipitate	+
		Hager's Test	Yellow precipitate	+
		Dragendorff's Test	Reddish orange precipitate	+
2	Test for Glycosides	Baljet Test	Brick red color	+
3	Test for Tannins	Ferric chloride Test	Blue colour	+
4	Test for Saponins	Foam Test	Persistent froth	+
5	Test for Flavonoids	Lead Acetate	Yellow ppt	+
		H2SO4 Test	Yellow colour	+
		Alkaline Reagent Test	Yellow colour	+

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6	Test for Terpenoids	Libbermann burchard's Test	Blue green colour	+
7	Test for Carbohydrate	Selivanoff's Test	Cherry red colour	+
		Benedict Test	Brown colour	+
		Fehling Test	Brick red precipitate	+
		Molisch's test	Violet ring appears	+
8	Test for Phenolics	Lead Acetate Test	Precipitate obtained	++
		FeCl3 Test	Black colour obtained	+
9	Test for Protein	Million Test	Red colour	+
#(+) indicates presence, while(–) indicates absence				

Table 4: The GC MS analysis of Punarnavadyarista shows the major presence of the following compounds

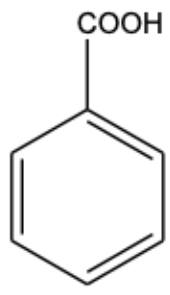
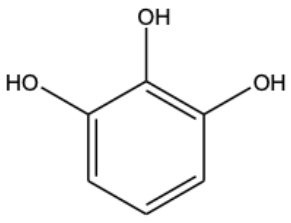
S. No.	Peak	Area	Cas NO	Compound
1	2.682	0.14	000109-52-4	Pentanoic acid
2	2.816	0.39	997005-05-6	1-Hydroxybut-3-en-2-one
3	3.114	3	024347-58-8	2,3-Butanediol
4	3.228	1.80	000513-85-9	Butane-2,3-diol 2,3-Butanediol
5	3.368	0.33	022696-02-2	1-Deutero-cyclobutano
6	4.200	0.04	000098-00-0	2-Furanmethanol
7	4.655	0.34	997012-98-7	1-(ethylthio)-1-propene
8	4.692	0.65	997012-98-7	1-(ethylthio)-1-propene
9	4.757	0.61	997012-98-7	1-(ethylthio)-1-propene
10	4.828	0.72	006975-85-5	2-Butanone, 4-methoxy-Propane
11	4.901	0.23	006975-85-5	2-Butanone
12	4.974	0.69	997040-27-8	Lactamide
13	5.023	0.74	053818-14-7	1,2-Propanediol diformate Ethane
14	5.092	0.34	053818-14-7	1,2-Propanediol diformate
15	5.132	0.45	053818-14-7	1,2-Propanediol diformate
16	5.219	0.14	010597-89-4	N acetyl muramic acid
17	5.248	0.11	000540-67-0	Aloxiprin
18	5.300	0.4	019900-84-6	Methoxymethyl isothiocyanate
19	5.357	0.22	019900-84-6	Methoxymethyl isothiocyanate
20	5.410	0.45	019900-84-6	Methoxymethyl isothiocyanate Ethane
21	5.500	0.16	997229-07-0	Cysteine, N-isopropyloxycarbonyl
22	5.573	0.5	997229-07-0	L-Cysteine, N-isopropyloxycarbonyl
23	9.201	0.25	997059-92-8	N-methyl-N-(methyl-d3)aminoheptane
24	10.212	0.45	997032-92-2	2,3-Dimethylpenta-2,4-dienoic
25	10.298	0.33	001746-81-2	N'-(4-chlorophenyl)-N-methoxy-N-methyl-
26	11.109	0.32	000111-48-8	Ethanol
27	11.264	0.57	000075-35-4	Ethene
28	12.357	10.69	028564-83-2	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-
29	13.561	13.82	000065-85-0	Benzoic acid
30	13.623	0.79	001070-34-4	Ethyl hydrogen succinate
31	13.679	1.47	000099-86-5	1,3-Cyclohexadiene,1-methyl-4-methylethyl)-
32	13.922	0.6	004362-24-7	1,3-Dioxolane, 4-methylene
33	14.406	0.66	02319-57-5	1,2,3,4-Butanetetrol
34	14.624	0.53	004775-93-3	Dithiodilactic acid
35	15.182	7.6	00067-47-0	5-Hydroxymethyl-2-furaldehyde
36	15.552	0.11	000288-47-1	Thiazole
37	17.746	0.94	997211-72-3	cyclobutene,3,4-bis(dimethoxymethyl)
38	20.010	3.60	000088-04-0	Chloroxylenol
39	23.390	0.94	001689-79-8	Thiophene
40	25.970	11.81	063785-74-0	methyl ester
41	26.227	2.74	063785-74-0	methyl ester
42	28.695	0.51	007557-00-8	Quinazoline
43	30.339	0.16	997098-02-3	3-Deoxy-d-mannoic lactone Sorbitol
44	30.392	0.1	74644-45-4	1,3-Methylene-d-arabitol Sorbitol
45	32.846	0.01	997102-41-6	Polygalitol
46	34.703	0.98	000504-96-1	Neophytadiene

47	35.221	0.26	997109-72-0	4-(2,2,6-Trimethylcyclohexyl)-2-butanone
48	35.563	0.34	0504-96-1	Neophytadiene
49	36.988	2.42	000057-10-3	n-Hexadecanoic acid
50	38.948	0.21	000112-63-0	9,12-Octadecadienoic acid, methyl ester
51	39.194	0.62	000150-86-7	2-Hexadecen
52	39.479	5.42	002420-56-6	10(E),12(Z)-Conjugated linoleic acid
53	39.545	3.24	000060-33-3	Linoleyl methyl ketone
54	39.820	0.89	000060-33-3	Octadecanoic acid
55	40.894	0.92	000072-48-0	Alizarin
56	41.897	0.24	000970-06-9	1,7-Diphenylnaphthalene
57	43.149	0.62	002345-28-0	2-Pentadecanone
58	43.686	1.17	023470-00-0	Hexadecanoic acid
59	44.513	0.26	000481-74-3	9,10-Anthracenedione
60	45.438	6.58	003443-82-1	9,12-Octadecadienoic acid
61	45.525	1.79	002277-28-3	9,12-Octadecadienoic acid
62	45.677	0.50	000478-94-4	6-Methyl-9,10-didehydroergoline-8-carboxamide
63	46.355	0.48	997805-60-6	14.alpha.-Cheilanth-12-enic Methyl Ester
64	48.001	0.28	997453-21-7	1-(3-Chlorophenyl)-5-phenyl-1,2,3-triazol-4-amine
65	48.645	0.19	997453-21-7	1-(3-Chlorophenyl)-5-phenyl-1,2,3-triazol-4-amine
66	50.649	0.34	997381-78-0	(4-Chlorophenyl)[(2R,6S)-2,6-dimethylpiperidin-1-yl]methanimine
67	51.293	0.82	000083-47-6	Gamma sitosterol

Note: The compounds shows dominance in quantity are Benzoic acid, 9,12-Octadecadienoic acid, Linoleyl methyl ketone, 10(E),12(Z)-Conjugated linoleic acid, methyl ester and Chloroxylenol

In this investigation, a spectrum of structural and biologically related endogenous species including methyl esters (35), fatty acids, Benzoic acid, 2,3 Butanediol, and Ethanol, were used as examples to illustrate the multi-origination and multipeak phenomena of GC/MS. Our findings revealed that the peaks of fatty acids, alcohol, and Benzoic acid (as a plant metabolite arose from phenylalanine in the shikimate pathway) (36). Dietary Benzoic acid is also known for its anti-inflammatory potential.(37)

Two major compounds which are identified by HPTLC and GC-MS in PA

Figure 2: Benzoic acid	Figure 3: Gallic acid
	

HPTLC Analysis of Punarvadyaristha

The current work is the first report on the quantification of Gallic acid in PA. The HPTLC densitometric chromatogram of gallic acid in PA is presented in fig 4. The peaks resolving at Rf 0.41 in PA were found superimposable with the bands obtained from gallic acid as a standard marker. (38) HPTLC analysis is shown in Table 5,6

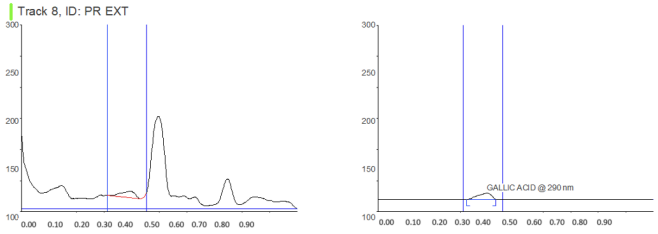
Table 5: Quantification of Gallic acid in Punarnavadyaristha

Track	Volume in Vial	Rf	Area	Amount in µg
1	0.2 µl	0.41	320.45	200
2	0.4 µl	0.41	581.26	400
3	0.8 µl	0.41	1174.85	600
4	1.2 µl	0.41	1642.11	800
5	1.6 µl	0.41	2074.44	1000
6	2.0 µl	0.41	2287.33	1200
7	5µL	0.41	417.39	211.30µg

Table 6: Validation of HPTLC Method

Validation Parameters	Outcomes
Linearity range (ng spot$^{-1)}$	100-1000
Correlation coefficient (R^2)	0.98101
Regression Equation	Y=178.5+2.261*X
Limit of detection (ng spot$^{-1)}$	13.296
Limit of quantification (ng spot$^{-1)}$	40.29
Accuray study (% recovery average of 3 replicates± SEM)	98.55±0.34
Quantity of gallic acid in PA	4.2% w/v

Figure 4: HPTLC of Punarnavadyaristha shows the presence of gallic acid at 290 nm at R*f* 4.1



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The HPTLC method was validated for Accuracy, Robustness, LOD, LOQ, and linearity ICH guidelines, 2003(39).

In vitro Antioxidant activity

In vitro Antioxidant activity of PA was performed by spectrophotometric analysis: As shown in Table 7

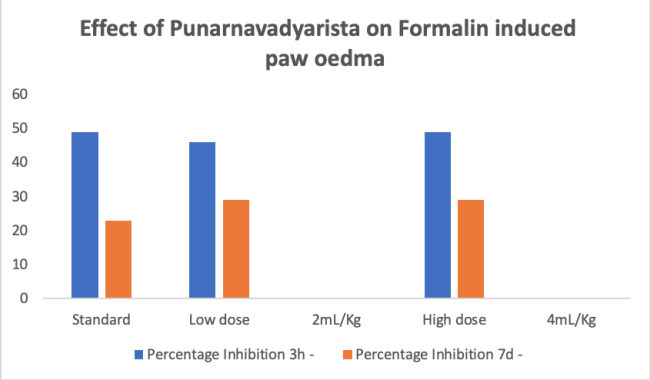
Table 7: Antioxidant potential of PA

Evaluation	Absorbance at 517 nm	% of Antioxidant
Control	1.087	-
PA	0.568	47

In vivo Acute Toxicity Studies Pre-Clinical Studies of PA on Inflammation

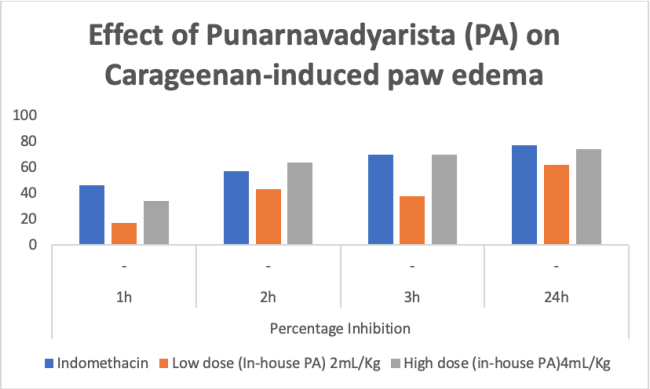
No signs of Acute toxicity were observed on prescribing PA up to 10mL/Kg. Punarnavadyarista did not produce any mortality in Wistar albino rats during a 72-h period at a 10-mL/kg oral dose. Rats didn't show any signs of toxicity, such as seizures, ataxia,diarrhoea, or increased diuresis. (40). Animals on prophylaxis of PA when administered with Formalin produce the following inflammatory response as shown in Figure 5.

Figure 5:



The inflammatory effect on animals on the administration of Carrageenan on 5th day is shown in Figure 6 below:

Figure 6: Effect of PA on Carrageenan-induced paw edema



The effect of PA is combating odema in formalin and carrageenan induced model is significant as compared to standard drug indomethacin in both cases.

Discussion

The In-house (Punarnavadyarista was prepared according to the Ayurvedic Formulary of India) and it was evaluated for its physicochemical parameters,

The percentage of self-generated alcohol in PA that was produced during preparation is limited to 5–10%. Punarnavadyaristha was made per Volume 2 of the Ayurvedic Formulary of India, and its preclinical effects against induced inflammation, on the Wistar albino rats were examined.(27)

Efficacy in acute and sub-acute inflammation was evaluated and it was found effective in combating both formalin-induced and carrageenan-induced inflammation. Thus, it verifies the traditional claim. It shows 211.30ng of Gallic acid in a 5 µL sample. The total amount of Gallic acid present in the 100 mL sample is approximately 4.2 mg (4.2%w/v). Gallic acid, a polyphenol found in various plants, has been studied using pharmacology to understand its potential therapeutic effects and mechanisms of action. It is Gallic acid interacts with proteins involved in inflammation, such as NF-κB, COX-2, and TNF-α, to reduce inflammation. (41) Gallic acid interacts with antioxidant enzymes, such as SOD, CAT, and GPx, to scavenge free radicals and reduce oxidative stress. Gallic acid interacts with proteins involved in cancer, such as p53, Bcl-2, and VEGF, to induce apoptosis, inhibit tumor growth, and suppress angiogenesis. (42)

(43) GC -MS studies show the presence of 67 bioactive compounds in PA.(44) The presence of Benzoic acid in GC-MS also signifies poly phenolic compounds in formulation, which may arise from shikimic acid pathway. The presence of various secondary metabolites confirms its efficacy in the reduction of ama (toxins in Ayurveda) and enhanced metabolism (45). The short-chain fatty acids found in GC-MS profiling also have similarities with products of microbial metabolism in the human body which aids in the digestion and reduction of toxins (46) So, this may be attributed to the digestion of inflammatory toxins (Ama) and its activity in inflammatory disorder.

The Sarangdhar Samhita, an Ayurvedic text, emphasized the use of polyherbalism to increase therapeutic efficacy. To provide the intended therapeutic effects, individual plants' active phytochemical ingredients are insufficient. A higher therapeutic impact and decreased toxicity can be obtained by combining various herbs in a certain ratio. Moreover, polyherbal compositions promote synergy. (47)

Poly-herbal formulation PA is evaluated by GC-MS and it shows the presence bioactive compounds 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-, Benzoic acid, Chloroxylenol, methyl ester 10(E),12(Z)-Conjugated linoleic acid, Linoleyl methyl ketone in significant amount. (48)

According to a GC-MS study, while many chemicals are maintained during fermentation, some vanish. The parent molecules that either vanished or were preserved throughout fermentation may be responsible for the presence of novel phytochemical substances in the later phases of fermentation. It implies

that intrinsic microorganisms act as a mediator in the biotransformation of phytochemicals. Because of the presence of phytochemicals such as phenolics, flavonoids, tannins, and phytosterols—of which bacteria are also a part—these medications have antioxidant properties.(49)

Conclusion

The in-house formulation of Punarnavadyarista is evaluated for its anti-inflammatory activity, and it showed significant anti-inflammatory activity as compared to the standard drug Indomethacin. In an exploration of mechanistic study, antioxidant potential may be responsible for its anti-inflammatory effects. Its composition shows that 67 metabolites of secondary metabolism are incorporated and HPTLC analysis with Gallic acid shows its presence of 4.2mg/100 ml. Gallic acid has various mechanisms of anti-inflammatory potential so its presence in Punarvadyaristha may be responsible for its potency. So, in the future Punarvadyaristha made by the traditional method, contain active bioactives may be used to cure inflammatory disorders.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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