Evaluation of polyherbal ayurvedic formulation ‘Peedantak Vati’ for anti-inflammatory and analgesic properties

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**ABSTRACT**

**Ethnopharmacological relevance:** Peedantak Vati (PV) is a polyherbal ayurvedic formulation, which is regularly prescribed by the ayurvedic practitioner for the inflammatory disorders and joints pain in India. It is composed of 23 different herbs and minerals, described in ayurvedic text for their anti-inflammatory and analgesic properties.

**Aim of the study:** To investigate anti-inflammatory and anti-nociceptive potential of ‘Peedantak Vati’ using in vitro and in vivo methods.

**Materials and methods:** In-vitro anti-inflammatory activity of PV was studied by estimating nitric oxide (NO) and LPS-induced pro-inflammatory cytokines IL-6 and TNF-α, using murine macrophage RAW264.7 and human monocyte THP-1 cell lines. PV's anti-inflammatory potential was studied in vivo using carrageenan-induced rat paw edema model. Similarly, anti-nociceptive property of PV was evaluated using hot plate, tail flick, formalin and writhing tests on CD-1 mice. Phytochemical profiling of hydro-alcoholic extract of PV was done using HPLC and HPTLC techniques to identify different marker compounds. These identified marker compounds were confirmed using LC-MS/MS analysis.

**Results:** In vitro results strongly suggest that, PV significantly (p < 0.001) inhibited NO release and LPS-stimulated pro-inflammatory cytokines IL-6 and TNF-α, in murine RAW264.7 and human THP-1 cells. Further, PV demonstrated significant (p < 0.05) anti-inflammatory activity at different time points after carrageenan injection with maximum effect at 2h (40.4 ± 5.2% at 400mg/kg). Similarly, PV significantly (p < 0.05) decreased nociceptive pain, studied using hot plate, tail flick, formalin and writhing tests. Moreover, HPLC and HPTLC methods were developed for the standardization of PV. Five marker phytocompounds viz. rutin, caffeic acid, colchicine, withaferin A and curcumin were identified and quantified by HPLC and HPTLC methods. The presence of these phytoconstituents was confirmed by LC-MS/MS analysis.

**Conclusion:** The findings of the study strongly suggest that, the polyherbal ayurvedic formulation ‘Peedantak Vati’ possesses remarkable anti-inflammatory and analgesic property, providing potent alternative for currently available allopathic medicines such as non steroidal anti-inflammatory drugs (NSAIDs).

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1. Introduction

Inflammation is one of the first protective responses of immune system to infection or cellular damages. This response can be against foreign pathogens, irritation as well as disorder or diseases like auto-immune or neurodegenerative diseases. It is a self-defense mechanism, characterized by redness, pain, swelling and a sensation of heat. The inflammatory responses play an important role in host survival although it can also lead to pathogenesis of many diseases such as cancer, rheumatoid arthritis, cardiovascular dysfunction (Grivennikov et al., 2010; Skeoch and Bruce, 2015; Libby, 2006) etc.

Inflammation is complex biological response of vascular tissue to harmful stimuli including pathogens, irritants, or damage cells, which leads to influx of neutrophils resulting in activation of macrophages (Schiott et al., 2013; MacMicking et al., 1997). This causes release of various inflammatory mediators such as NO, pro-inflammatory cytokines including TNF-α, interleukins (IL-6, IL-1β), prostaglandins (PGs) (MacMicking et al., 1997; Zelová and Hošek, 2013; Tanaka et al., 2014) etc. NO is a central inflammatory mediator which is produced by nitric oxide synthetase from L-arginine in response to inflammatory stimuli. Increased NO level is one of the well known causative agent of inflammatory disorders such as rheumatoid arthritis (RA) and ulcerative colitis (Wright et al., 2014). NO and PGs are widely known to act as secondary mediators of pro-inflammatory cytokines TNF-α and IL-6 (Skelly et al., 2013).

In most cases pain is associated with inflammation, regardless of cause behind inflammation. Now it is well understood and defined mechanism supported by scientific evidences that relief from pain is caused by alleviation of inflammation (Cooper et al., 2017). Most of anti-inflammatory drugs, particularly NSAIDs primarily act by inhibiting the production of PGs, thus interfering with inflammatory cascade (Ricciotti and FitzGerald, 2011). However, regular use of these medicine causes severe side effects including inflammation of gastrointestinal tract, renal failure and liver toxicity (Harirfroorosh et al., 2013).

Due to these severe side effects, in recent times interest in traditional medicine is rising. In this scenario, use of plant derived products to treat inflammation and related condition becomes viable and valid approach. It has been observed that, isolated molecule(s) from the plant fail to illicit desired therapeutic effect in comparison to whole plant extract (Kunnunmakkara et al., 2017). Moreover, according to the Indian traditional medicine system, a combination of substances such as polyherbal formulations is used to enhance the desired activity and eliminate unwanted side effects (Kono et al., 2015; Rasanoaivo et al., 2011; Zhou et al., 2016).

Peedantak Vati is one of the polyherbal ayurvedic formulation used by Indian population to treat pain and inflammatory disorders. The traditional name ‘Peedantak’ is composed of two words; ‘peeda’ (pain) and ‘antak’ (ending), altogether ‘pain ending’. The formulation is composed of 23 different plants and minerals as shown in Table 1. All of its components have detailed individually in the literature of Indian Traditional Medicine such as Charak Samhita (Trikamji, 2007), The Ayurvedic Pharmacopoeia of India et al. (2008), The Unani Pharmacopoeia of India (2007) and The Ayurvedic Formulary of India (2003) about its anti-inflammatory and pain relieving effects. Furthermore, all these plants viz. Commiphora wightii (Arn.) Bhand (Shishodia et al., 2008), Colchicum luteum Baker (Nair et al., 2012), Withania somnifera (L) Dunal (Gupta and Singh, 2014; Orrù et al., 2016), Strychnos nux vomica L. (Chen et al., 2012), Cyperus rotundus L. (Dang et al., 2011), Pluchea lanceolata (DC.) C.B. Clarke (Srivastava et al., 2014), Vitex negundo L. (Dharmasiri et al., 2003), Boerhavia diffusa L. (Bairwa and Jachak, 2015; Hiruma-Lima et al., 2000), Trigonella foenum-graecum L. (Bae et al., 2012), Opeculina turpethum (L.) Silva Manso (Aggarwal et al., 2011), Asparagus racemosus Willd. (Tiwari et al., 2017), Cissus quadrangularis L. (Bhujade et al., 2012), Curcuma longa L. (Lee et al., 2013), Zingerber officinalis Roscoe (Ojewole, 2006), Picrorhiza kurroa Royke ex Benth (Kumar et al., 2016), Trachypermum amni (L.) Sprague (Bairwa et al., 2012), Tinospora cordifolia (Willd) Miers (Patgiri et al., 2014), Dashmool (Grampurohit et al., 1992; Gupta et al., 1089) have been reported for the anti-inflammatory and/or analgesic potential and/or their beneficial effects on inflammatory disorders in peer reviewed journals.

Though PV formulation has been prescribed by ayurvedic practitioner for the treatment of inflammatory disorders, its scientific validation for anti-inflammatory and anti-nociceptive effect has never been explored so far. Keeping this in view, present study was designed to explore PV’s anti-inflammatory and anti-nociceptive effects using in vitro and in vivo methods. In addition, chemical fingerprinting of PV was done using different chromatographic techniques viz. HPLC, HPTLC and LC-MS/MS.

2. Materials and methods

2.1. Chemicals and reagents

Cell culture reagents viz. DMEM, RPMI, FBS, antibiotic/antimycotic and their supplements were purchased from Gibco, USA. Bacterial origin endotoxin LPS (O111:B4) Griess reagent, MTT, PMA, α-carrageenan, Indomethacin and standard compounds (> 95% by HPLC) such as berberine, rutin, caffeic acid, colchicine, cinnamic acid, quercetin, withaferin A, withanolide A and as curcumin were purchased from Sigma-Aldrich (St. Louis, MO, USA). ELISA reagents and kits (TNF-α, IL-6) were purchased from BD Biosciences. Tramadol hydrochloride (Tramacad, Cadila Pharmaceuticals) injection and sodium chloride injection I.P. 0.9% w/v (Infutec Healthcare Ltd) were procured from the local market. Analytical grade toluene, ethyl acetate, formic acid, chloroform, ethanol, diethyl ether, HPLC grade acetonitrile, methanol, and concentrated sulphuric acid, concentrated nitric acid were purchased from Merck India Ltd. Precoated TLC aluminum sheets silica gel 60F254 (10 × 10 cm, 0.2 mm thick) were obtained from E. Merck Ltd, India. All other chemicals and reagents were of the highest commercial grade.

2.2. Preparation of extract

About 20 g of PV formulation (Batch no. PTV203) was powdered and refluxed for 6 h at 88°C in 300 mL of 70% ethanol. The solution was filtered through Whatman filter paper no 41 to remove the particulate matter and filtrate was evaporated dry at 45°C under reduced pressure in rotary film evaporator. The final dried sample was weighed (3.12 g; extraction yield: 15.60% w/w) and stored under vacuum in the desiccators for further in vitro experiments and chromatographic method development.

The powdered form of PV was suspended in 0.25% Na-CMC, triturated to form uniform suspension and used for the in vivo experiments.

2.3. Cell culture for in vitro experiments

RAW264.7 and THP-1 cell lines were obtained from the National Centre For Cell Science (NCCS), India and cultured in Dulbecco's Modified Eagle's Medium (DMEM) and RPMI1640. Media was supplemented with 10% heat-inactivated fetal bovine serum (FBS), in presence of 100 U/mL concentrations of penicillin-streptomycin, 1 mM sodium pyruvate and 4 mM L-glutamine. Cells were grown at 37°C in a 5% CO2/air environment, following standard protocol.

2.3.1. MTT assay

MTT assay was performed according to protocol described earlier (Mosmann, 1983) with minor modifications. Briefly, cells were seeded in 96 well culture plates at density of 1 × 104/mL and treated with different concentrations of PV. After overnight treatment, 10 μL of MTT
### Table 1: Ingredients of Peedantak Vati.

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Common name</th>
<th>English name</th>
<th>Scientific name</th>
<th>Part(s) used</th>
<th>Quantity (mg)</th>
<th>Voucher specimen number</th>
<th>Reference(s) for traditional use (anti-inflammatory &amp;/or pain relief)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Suranjan meethi</td>
<td>Colchicicum</td>
<td>Colchicum luteum Baker</td>
<td>Rhizomes</td>
<td>36</td>
<td>QC1/RM/12-17/3182</td>
<td>UPI, P-II, Vol-III</td>
</tr>
<tr>
<td>7</td>
<td>Nirgundi</td>
<td>Five leaved Chaste Tree</td>
<td>Vitex negundo L.</td>
<td>Leaves &amp; roots</td>
<td>20 &amp; 71.6</td>
<td>NISCAIR/RHMD/CONSULT/2017/3034-61-45</td>
<td>API, P-I, Vol-I, p95</td>
</tr>
<tr>
<td>10</td>
<td>Nishodh</td>
<td>Indian Jalap</td>
<td>Operculina turpethum (L.) Silva Manso</td>
<td>Roots</td>
<td>20</td>
<td>QCD1/RM/11-17/3038</td>
<td>API, P-I, Vol-III, p21</td>
</tr>
<tr>
<td>16</td>
<td>Ajvayan</td>
<td>Thyme</td>
<td>Thymuspermum ammi (L.) Sprague</td>
<td>Fruits</td>
<td>3.5</td>
<td>NISCAIR/RHMD/CONSULT/2017/3034-31-5</td>
<td>API, P-I, Vol-I, p129</td>
</tr>
<tr>
<td>17</td>
<td>Giloy</td>
<td>Indian Tinospora</td>
<td>Tinospora cordifolia (Willd.) Miers.</td>
<td>Stems</td>
<td>1.8</td>
<td>NISCAIR/RHMD/CONSULT/2017/3034-61-19</td>
<td>API, P-I, Vol-I, p41</td>
</tr>
<tr>
<td>18</td>
<td>Dashmool</td>
<td>(Composition of 10 different roots)</td>
<td></td>
<td>Roots</td>
<td>35.7</td>
<td>QCD1/FP/10-17/1719</td>
<td>API, P-I, Vol-I, p10, 47, 73, 74</td>
</tr>
<tr>
<td>19</td>
<td>Shilajeet</td>
<td>Mineral Pitch</td>
<td>Mineral Pitch</td>
<td>Exudates</td>
<td>35</td>
<td>QCD1/SF/06-17/853</td>
<td>Charak Samhita</td>
</tr>
<tr>
<td>20</td>
<td>Godanti Bhasma</td>
<td>–</td>
<td>Processed gypsum</td>
<td>–</td>
<td>20</td>
<td>QCD/FP/02-16/393</td>
<td>API, P-I, Vol-VII, p11</td>
</tr>
<tr>
<td>21</td>
<td>Mukta shukti Bh.</td>
<td>–</td>
<td>Processed pearl oyster</td>
<td>–</td>
<td>20</td>
<td>QCD/FP/02-16/032</td>
<td>Charak Samhita</td>
</tr>
<tr>
<td>22</td>
<td>Yograj Guggulu</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>20</td>
<td>QCD/FP/12-17/2002</td>
<td>API, P-II, Vol-I, p11</td>
</tr>
<tr>
<td>23</td>
<td>Praval Pihti</td>
<td>–</td>
<td>Processed red coral powder</td>
<td>–</td>
<td>3.5</td>
<td>QCD/FP/10-15/073</td>
<td>API, Vol-I, p19</td>
</tr>
</tbody>
</table>

(Ingredients 1–16 in the form of fine powders; Ingredients 17–19 in the form of aqueous extracts.)

* API: The Ayurvedic Pharmacopoeia of India, UPI: The Unani Pharmacopoeia of India, AFI: The Ayurvedic Formulary of India.
stock solution (5 mg/mL) was added to 100 μL media in each well and incubated in CO2 incubator for 3–4 h until formazan crystals were observed. Water insoluble formazan crystals were solubilized in acidic isopropanol (0.1 N HCl) and absorbance was taken using Envision microplate reader (Perkin Elmer) at 590 nm.

2.3.2. Nitric oxide assay

RAW264.7 cells were seeded in 24 well culture plates at the density of 2 × 10^5 cells/well. Cells were treated with vehicle or PV (15.62, 31.25, 62.5, 125 and 250 μg/mL) for 60 h. Cells were incubated with LPS (500 ng/mL) and incubated for an additional 24 h at 37°C in a CO2 incubator. NO release in the culture media was determined using modified Griess reagent, following manufacturer’s protocol. Equal volume of modified Griess reagent solution was added to cell supernatant and absorbance was recorded at 540 nm using Envision Microplate reader (Perkin Elmer). Modulation of NO production was measured (nitrate content) using the standard curve of sodium nitrite.

2.3.3. Cytokines level measurement

For cytokines level modulation studies, human monocyte THP-1 cells (5 × 10^5 cells/well) were seeded in 24 well culture plates and treated with 25 ng/mL LPS. For experiment, PV was added to the wells at final conc. of 50, 100, 150, 200 and 250 μg/mL. After treating cells for an hour, LPS was added at final concentration 500 ng/mL except in control wells. Cell supernatants were collected after 4 and 24h to measure different cytokines such as TNF-α and IL-6 using ELISA kits (Equikine, France). ELISA assay was performed according to manufacturer’s protocol and absorbance was recorded at 450 nm using Envision microplate reader (Perkin Elmer).

2.4. Experimental animals

Male Wistar rats (160–180 g) and CD-1 mice (20–22 g) were procured from Liveon Biolabs Pvt. Ltd (India) and Hylasco Biotechnology Pvt. Ltd, India respectively. All the animals were placed in optimal controlled environment with relative humidity of 60–70% with 12:12 h light and dark cycle in a registered animal house (1964/PO/Rc/S/17/ CPCSEA). The animals were maintained on standard pellet diet (Golden Feed, India) and sterile filtered water ad libitum. All the animal experiments were approved by the Institutional Animal Ethical Committee and were performed in accordance with the guidelines of the Committee.

2.4.1. Dose selection for in-vivo experiments

The animal equivalent doses of PV powder for rat and mouse studies were calculated based on body surface area. The recommended dose of the PV for the human is 1–2 tablets twice a day. The weight of each tablet is 500 mg. According to this, total humane dose is 1500 mg/60 kg/day (25 mg/kg/day). Animal equivalent doses (mg/kg) for rat and mouse were calculated by multiplying human equivalent dose (mg/kg) by factor 6.2 and 12.3 respectively (Nair and Jacob, 2016). According to this, therapeutic equivalent doses (TEDs) for rat and mouse were found to be approx. 200 and 400 mg/kg respectively. However, two different doses for rat and mouse studies were selected i.e. TED and HD (High dose), 200 and 400 mg/kg for rat and 400 and 600 mg/kg for mouse.

2.5. Evaluation of in-vivo anti-inflammatory and anti-nociceptive activities

2.5.1. Carrageenan induced paw edema

Carrageenan induced paw edema test was performed according to the modified methods described earlier (Winter et al., 1962; Sakat et al., 2014). Wistar rats were divided into different groups (eight animals per group) based on basal paw volume (0 h) measured using 37140 plethysmometer (Ugo Basile, Italy). Inflammation was induced by the subcutaneous injection of λ-carrageenan (0.1 mL of 1% solution in normal saline) into the planter side of the left hind paw. The paw was marked with ink at the level of lateral malleolus and volume was measured up to the mark at 1, 2, 3, 4 and 5 h after injection of carrageenan for all the animals. Further, animals were treated orally with vehicle or PV at 200 and 400 mg/kg or Indomethacin at 10 mg/kg, 1 h before carrageenan challenge. Paw edema was calculated by subtracting 0 h (basal) paw volume from the respective paw volumes at 1, 2, 3, 4, and 5 h. The anti-inflammatory activity (%) was calculated for each animal using the following formula: Mean paw edema of control animals (mL) – Paw edema of each test animals (mL)/Mean paw edema of control animals (mL) × 100.

2.5.2. Hot plate test

The hot plate test was performed to measure response latencies according to previously described method (Arrau et al., 2011; Eddy and Leimbach, 1953) with minor modifications. Albino mice were initially screened for basal latency time into different groups (eight animals each). Test groups were treated orally with PV 400 and 600 mg/kg, while the control group received 0.25% Na-CMC. TMD (Tromadol hydrochloride Inj.) was used as a standard drug and administered intraperitoneally at the dose of 40 mg/kg. After 1 h of drug treatment, all the animals were placed into the perspex cylinder of the hot plate (Ugo Saile, Italy) maintained at 55.0 ± 0.5°C and time to discomfort reaction (licking paws or jumping) was recorded as response latency. The latency time was recorded at 30, 60, 90, 120 and 180 min after the administration of test and standard drugs. A cut-off point of 20 s was considered to avoid any possible accidental paw damage. The percentage of maximum possible effect (% MPE) was calculated as ([(LT1 - LT2)/((LT1 - LT0)] × 100). LT0 and LT1 were the latencies time before and after drug administration, and LT2 was the cut off time.

2.5.3. Tail flick test

Tail flick test as an acute model of pain was used to assess anti-nociceptive effect of the drugs by measuring the latency of response. Tail flick test was performed as described earlier (Keyhanfar et al., 2013; Meymandi et al., 2006) with minor modifications using a plantar test device (7370 plantar test; Ugo Basile, Italy) at high-intensity infrared radiation (infrared intensity of 99) with cut off time of 5 s. The animals were divided into four different groups containing eight animals each with mean basal latency time 2.0 ± 0.2 s. All the groups of animals were treated orally with vehicle or PV 400 and 600 mg/kg or TMD at 40 mg/kg (i.p.). The latency time was recorded at 30, 60, 90 and 120 min after the administration of test and standard drugs. The percentage of maximum possible effect (% MPE) was calculated by the formula mentioned above.

2.5.4. Formalin test

To assess chemical-induced acute and tonic pain, method of Hunskaar and Hole (1987) was followed with minor modifications. Eight animals in each group were treated with vehicle or PV (400 and 600 mg/kg, p.o.) or INDO (10 mg/kg, p.o.) 1 h before 1% formalin injection (20 μL) in normal saline (v/v). Immediately after the formalin injection in dorsal hind paw, the time of pain reactions were recorded that the animals remained licking or biting the paw during the first phase (0–5 min) and the second phase (20–30 min) of the test. Percent anti-nociceptive pain activity in each phase was calculated by the formula: (Mean Control-Test) X100/Mean Control

2.5.5. Acetic induced writhing test

The modified method (Torri et al., 2007) of writhing test was used to assess visceral pain. Animals were divided into four groups with 8
animals in each. One hour after the treatment of 0.25% Na-CMC or PV (400 and 600 mg/kg; p.o.) or INDO (10 mg/kg; p.o.), all the animals were received intra-peritoneal injections of 0.6% acetic acid (0.1 mL/10 g), for induction of writhing. A writh is indicated by abdominal constriction and full extension of the hind limbs. After 10 min of the acetic acid injection, the numbers of writhing were registered for 20 min for each session. Percent anti-nociceptive pain activity was calculated by the formula mentioned in Section 2.5.4.

2.6. Statistical analysis

The data are expressed as mean ± standard error of mean (SEM) for each group. Statistical analysis was done using GraphPad Prism version 7.0 software. A one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test was used to calculate statistical difference for in-vitro and in-vivo tests. Two-way ANOVA followed by Newman-Keuls multiple comparison test was used to calculate statistical difference for carrageenan induced paw edema test. A value of \( p < 0.05 \) was considered as significant.

2.7. Phytochemical analysis

Physicochemical analysis of PV was performed qualitatively and quantitatively using colorimetric analyses. The phytoconstituents present in the formulation were analyzed quantitatively based on their class of molecules. Alkaloids were determined using 1,10-phenantroline in ethanol and the results were expressed as colchicine equivalents (Singh et al., 2004). Folin–Ciocalteau reagent in an alkaline medium was used to assess phenols and the results were expressed as the gallic acid equivalents (Singh et al., 2002). Flavonoid content was measured and expressed as the quercetin equivalent (Zhishen et al., 1999). Total saponin content was determined using Vanillin-Sulphuric acid colorimetric reaction with some modifications (Makkar et al., 2007) and the values were expressed as diosgenin equivalents.

2.8. HPLC analysis

The hydro-alcoholic extract of PV (HA-PV) was diluted in 50% methanol (1 mg/mL) and subjected to high performance liquid chromatography (HPLC) analysis. Waters binary HPLC system (Waters Corporation, Milford, MA, USA), equipped with a column oven, autosampler (Waters 2707) and photo diode array (PDA) detector (Waters 998) was used for analyses. A reversed phase C18 analytical column of 4.60 × 250 mm and 5 μm particle size (Sunfire, Waters, USA) was
utilized and its temperature maintained at 30°C.

A clear separation was achieved by using a mobile phase consisting of 0.1% formic acid (A) and acetonitrile (B). The following gradient program was used: 5% B (0 min, flow rate 1 mL/min), 10% B (4 min, flow rate 0.9 mL/min), 23% B (5 min, flow rate 1.1 mL/min), 28% B (14 min, flow rate 1 mL/min), 35% B (20 min, flow rate 1 mL/min), 43% B (25 min, flow rate 0.9 mL/min), 45% B (35 min, flow rate 0.9 mL/min) 5% B (39 min, flow rate 1 mL/min) and 5% B (40 min, flow rate 1 mL/min). After filtration through 0.45µm PTFE membrane filters, 20μL of HA-PV was injected. Different concentrations of standard solution were prepared to make standard curve. Analysis of standard mixture and test samplesolution was done insix setsto ensure repeatability of the method. An analytical methodology of marker compound quantification in HA extracts of PV was validated in accordance with ICH guidelines. The limits of detection (LOD) and quantitation (LOQ) were estimated as the minimum concentration of marker compound is able to produce signal-to-noise ratios (S/N) of 3 and 10, respectively. To assess the linearity of the method, marker solutions (1.0mg/mL, triplicate) was added in extract solution (1.0mg/mL) at concentrations of zero, 20.0, 40.0, 60.0, 80.0, 100.0µg/mL. Analytical curves were obtained by ratio of marker peak areas at 270nm. The precision and accuracy experiments were performed triplicate. The precision were expressed as residual standard deviation (RSD%) and accuracy was measured as percent deviation from nominal concentration.

2.9. LC-MS/MS analysis

LC-MS/MS analysis was performed to confirm the phytocomstituents, which were identified and quantified by HPLC. The HA-PV was diluted with 50% methanol (1 mg/mL) and subjected to LC-MS/MS analysis using Waters Xevo TQS micro with UPLC H class instrument. A reversed phase C18 analytical column of 2.1 × 100 mm and 1.8 µm particle size (HSS T3, C18 Column from Waters, USA) was used at 30°C. HPLC method was used as guidance point for LCMS/MS analysis. Binary
gradient of water and acetonitrile were used as mobile phase with constant flow rate of 0.4 mL/min. Gradient elution was programmed as follows: 5% B (0 min), 10% B (1 min), 23% B (1.5 min), 28% B (3.8 min), 35% B (6.5 min), 43% B (8 min), 45% B (9.5 min), 95% B (12 min), 5% B (20 min).

2.10. HPTLC analysis

Standardization of HA-PV (2 mg/mL) was performed using HPTLC system (CAMAG, Switzerland) equipped with a sample applicator (ATS4), development chamber (ADC2). Simple and precise HPTLC methods were developed, optimized and validated using a precoated TLC plate (60 F254, Merck, Germany) with mobile phase as chloroform: methanol: water: formic acid in the ratio of 7:0.65:0.1:0.2 (v/v). The TLC chamber was saturated with mobile phase for 20 min at room temperature. Detection and quantification was achieved at 218 nm using integrated TLC scanner and analyzed by WinCats software. Analytical performance of the proposed HPTLC method was validated according to the ICH guidelines with respect to the linearity, detection and quantitation limits.

2.11. ICP-MS analysis

Presence of heavy metals was determined following standard protocol (citation). Briefly, 2 gm of PV was digested in 10 mL of concentrated nitric acid and placed in hot oven at 60°C. After complete digestion ultra pure elemental water was added to make complete 50 mL solution. Internal standard of known concentrations were added to the sample and the solution was subjected to heavy metal analysis by direct injection using instrument in helium gas mode (Agilent Technology model 7800). For data processing software provided with the instrument Mass hunter was used and results were calculated in PPM and experiments were performed in triplicates and RSD% values were calculated.

3. Results

3.1. Effect of PV on NO release

Cytotoxic level of PV was determined by standard MTT assay. The study results demonstrated that, PV was found to be safe for cells at ≤250 µg/mL concentration (data not shown). Hence, all the in vitro assays were performed below cytotoxic concentration of PV. The effect of PV on NO release was studied using RAW264.7 cells (Fig. 1A) and THP-1 (Fig. 1B) cells. Cells treated with LPS (500 ng/mL) led to significant increase (p < 0.001) in NO production in LPS control of RAW264.7 and THP-1 cells as compared to respective normal control (NC). However cells treated with PV at 62.5 (p < 0.05), 125 (p < 0.001) and 250 (p < 0.0001) µg/mL showed significant decrease NO production by RAW264.7 cells. However, THP-1 cells treated with PV at 100 (p < 0.05), 150 (p < 0.01), 200 (p < 0.001) and 250 (p < 0.001) µg/mL showed significant decrease NO production.

3.2. Effect of PV on IL-6 and TNF-α level

To determine the effect of PV on pro-inflammatory cytokine production, differentiated THP-1 macrophage cells were pretreated with PV extracts at different concentrations (50, 100, 150, 200, 250 µg/mL) and challenged with LPS after 1 h. Fig. 2 displays significant rise (p < 0.001) in IL-6 level as compared to NC. Treatment of PV at 200 and 250 µg/mL led to significant (p < 0.01) decrease in IL-6 level measured at 4 h. However, PV showed remarkable decrease in IL-6 production at 50 (p < 0.01) and 100–250 µg/mL (p < 0.0001) estimated at 24 h time point. The anti-inflammatory effect of PV was displayed in a conc.-dependent manner.

Similarly, cytokine TNF-α level in LPS treated cells was prominently (p < 0.001) increased in LPS control as compared to NC (Fig. 3). THP-1 cells treated with PV exhibited substantial decrease in TNF-α level at 4 h (200 and 250 µg/mL; p < 0.01). Similarly, treatment of PV at 100 (p < 0.01), 150 (p < 0.01), 200 (p < 0.0001) and 250 µg/mL (p < 0.0001) showed remarkable decrease in TNF-α level measured at 24 h and the effect observed was conc.-dependent.
The anti-inflammatory effect of PV was assessed by the carrageenan induced paw edema model (Fig. 4). The intraplantar injection of carrageenan in vehicle control rats led to a time dependent increase in paw volume that was maximal at 4 h followed by marginal decrease at 5 h. Animals treated orally with PV at 200 mg/kg displayed significant (p < 0.05) decrease in paw edema at 3 h, whereas high dose 400 mg/kg showed significant decrease in paw volume at 2 (p < 0.01) and 3 h (p < 0.05). The effect of 200 mg/kg dose was not significantly (p > 0.05) different as compared to high dose 400 mg/kg. Further, high dose of PV demonstrated maximum anti-inflammatory activity (data not shown) as 40.4 ± 5.2% at 2 h, followed by gradual decrease up to 5 h. The standard drug INDO at 10 mg/kg exhibited prominent (p < 0.0001) anti-inflammatory activity (> 50%) at all time points except 1 h (44.9 ± 6.5%).

### 3.4. Anti-nociceptive activity

#### 3.4.1. Hot plate test

The PV was evaluated for anti-nociceptive pain effect using hot plate test is shown in Fig. 5. Control animals showed no significant difference (p > 0.05) in the latency time at different time points as compared to basal readouts (0 min). PV at 400 mg/kg exhibited significant (p < 0.05) increase in latency time at 30 and 90 min as compared to basal latency and high dose 600 mg/kg showed considerable increase in latency time at 90 (p < 0.01) and 120 min (p < 0.05) as compared to basal latency. The % MPE (data not shown) shown by the high dose of PV was found to be maximal (> 50%) at 90 and 120 min. The standard opioid analgesic TMD at 40 mg/kg; treated intra-peritoneally displayed noticeable (p < 0.05) increase in latency at 90, 120 and 180 min. The anti-nociceptive pain activity shown by the

### Table 2

Quantification of marker compounds in HA-PV by HPLC.

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<tr>
<th>S. no</th>
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<th>Name</th>
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<th>% content</th>
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<tr>
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### Table 3

HPLC method validation.

<table>
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<tr>
<th>S. no</th>
<th>Name of compound</th>
<th>LOD (ppm)</th>
<th>LOQ (ppm)</th>
</tr>
</thead>
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<td>1.0 ppm</td>
</tr>
<tr>
<td>2</td>
<td>Caffeic Acid</td>
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<td>1.0 ppm</td>
</tr>
<tr>
<td>3</td>
<td>Withaferine A</td>
<td>0.5 ppm</td>
<td>1.0 ppm</td>
</tr>
<tr>
<td>4</td>
<td>Colchicine</td>
<td>0.5 ppm</td>
<td>1.0 ppm</td>
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<tr>
<td>5</td>
<td>Curcumin</td>
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</table>

#### 3.3. In vivo anti-inflammatory effect of PV

The anti-inflammatory effect of PV was assessed by the carrageenan induced paw edema model (Fig. 4). The intraplantar injection of carrageenan in vehicle control rats led to a time dependent increase in paw volume that was maximal at 4 h followed by marginal decrease at 5 h.
TMD was found to be > 50% at all time points. Importantly, the % MPE of PV of high dose was found to be similar to that of TMD at 90 min (data not shown).

3.4.2. Tail flick test

Anti-nociceptive pain effect of PV was studied using the predominantly spinally mediated tail flick latency test (Fig. 6). The data suggest, PV at 400 mg/kg exhibited noticeable (p < 0.01) increase in latency only at 90 min as compared to basal latency. However, PV at 600 mg/kg showed significant anti-nociceptive property by increase in latency time at 60 (p < 0.01), 90 (p < 0.0001) and 180 min (p < 0.01) with MPE (data not shown) as 30 ± 3.8% at 90 min. However, TMD displayed gradual increase in % MPE of anti-nociceptive pain potential till 90 min. The results of the study clearly indicate the PV’s considerable anti-nociceptive pain activity possibly mediated by spinal mechanism.

Fig. 10: A) LC-MS/MS chromatogram of marker compounds in HA-PV (Full scan) B) LC-MS/MS chromatogram of Rutin C) LC-MS/MS chromatogram of Caffeic acid D) LC-MS/MS chromatogram of Colchicines E) LC-MS/MS chromatogram of Withaferin A F) LC-MS/MS chromatogram of Curcumin.
3.4.3. Formalin test

This method elucidates central and peripheral nervous system activities. Formalin-induced pain is biphasic in which first phase involves direct stimulation of sensory nerve fibers representing neuropathic pain and second phase involves inflammatory pain mediated by prostaglandins, serotonin, histamine, bradykinin, and cytokines such as IL-1β, IL-6, TNF-α, eicosanoids and NO. In the present study, PV tested at 400 and 600 mg/kg showed no significant (p > 0.05) activity in the early phase (Fig. 7A), similar to that of INDO. However, in late phase PV showed remarkable (p < 0.0001) anti-nociceptive pain activity at 400 (76.4 ± 3.3%) and 600 mg/kg (90.6 ± 3.1%) (data not shown) by decreasing paw licking time (Fig. 7B). The standard drug INDO at 10 mg/kg displayed significant (p < 0.0001) anti-nociceptive pain activity (81.1 ± 4.2%) by lowering paw licking time.

3.4.4. Writhing test

The anti-nociceptive pain effect of PV against visceral pain was assessed by the acetic acid induced writhing test. Normal control animals showed maximum writhing response 72.5 ± 11.8 induced by intraperitoneal injection of 0.6% acetic acid in normal saline (Fig. 8). Orally administered PV at 400 and 600 mg/kg showed remarkable (p < 0.0001) and dose-related anti-nociceptive activity as 81.0 ± 4.0 (writhes: 13.8 ± 2.9) and 90.7 ± 4.6% (writhes: 6.8 ± 3.4) respectively. The standard NSAID drug, INDO also produced considerable (p < 0.0001) decrease in number of writhes (23.8 ± 4.4) with 67.2 ± 6.2% anti-nociceptive pain activity (data not shown).

3.5. Chemical analyses

HA-PV was prepared for all the downstream analyses. HPLC and HPTLC analytical techniques were utilized for chemical profiling, identification and quantification of marker compounds in PV. Identified compounds were confirmed by LC-MS/MS analysis.

3.5.1. Phytochemical analysis

The colorimetric analyses of PV indicated the presence of phenols, saponins, flavonoids and alkaloids. Quantitative analyses revealed the presence of alkaloids (560 ± 4.51 µg of colchicine equivalent/mg of extract), phenolic (2.73 ± 0.28 µg of gallic acid equivalent/mg of extract), flavonoids (0.20 ± 0.05 µg of quercetin equivalent/mg of extract) and saponins (268 ± 5.78 µg of dioxygenin equivalent/mg of extract).

3.5.2. Identification and quantification of marker compounds by HPLC

Phytochemical profiling of HA-PV was performed using HPLC technique. A binary gradient method for HPLC was developed and optimized. HA-PV was analyzed along with mixture of standard marker compounds. Altogether five different compounds viz. rutin, caffeic acid, colchicine, withaferin A and curcumin were identified using newly developed method. Each compound was identified and confirmed using its retention time and UV profile in photodiode array (PDA) detector under similar conditions (Fig. 9A-B). These marker compounds were quantified in HA-PV and represented in Table 2. The HPLC method validation ensures reliability of results and reducing batch to batch variations. The retention time for different markers was validated with slope, linearity and accuracy (S1, Table A). The method was considered linear between concentrations of 20–100 µg/mL for Colchicine, Withaferin A, Curcumin, caffeic acid. The mean regression equation was curcumin was \( y = 16,364 \times -86,483 \), whereas for rutin it was \( y = 29,039 \times -879,282 \). The LOD and LOQ for identified marker compounds were obtained (Table 3).

3.5.3. Confirmation of marker compounds by LC-MS/MS

LC-MS/MS analysis was carried out to confirm marker compounds, which were identified and quantified by HPLC. Total ion chromatogram (TIC) both in +ve and -ve mode were obtained (Fig. 10A) Ionization pattern of major peaks were compared to the library and prediction was subjected to validation. Fragmentation pattern of predicted standards and HA-PV peaks were compared with standards to confirm the identity. Different marker compounds (Table 4) including caffeic acid, colchicine and curcumin were confirmed for their presence in HA-PV by HA-PV by LC-MS/MS (Fig. 10A-F).

3.5.4. Chromatographic profiling by HPTLC

HPTLC profile with optimized mobile phase chloroform: methanol: water: formic acid in the ration of 7.0:0.65:0.1:0.2 gave sharp peaks of different components present in HA-PV (Fig. 11A). Comparing with reference standards, two well defined peaks with Rf value of 23 and 28
Fig. 11. A) Profile of hydro-alcoholic extract of Peedantak Vati (HA-PV) by HPTLC at $\lambda = 210$ nm. B) Densitogram of reference marker Colchicine by HPTLC at $\lambda = 210$ nm. C) Densitogram of reference marker Withaferin A by HPTLC at $\lambda = 210$ nm.
were identified as colchicines and withaferin A, respectively. The identity of the Colchicine and withaferin A band in sample chromatogram was confirmed by comparing chromatogram (Fig. 11: B, C) and Rf values of reference standards (Table 5). The percent area was represented by area normalization. The calibration plots were linear within the concentration range of 40–200 and 80–240 ng/spot for colchicine and withaferin, respectively (S1, Fig. A). The correlation coefficient intercept and slope were 0.990835, + 8638, 206.01 and 0.9984, + 5.099, 91.044 for colchicines and withaferin A, respectively. Quantitative evaluation of identified standards colchicine and withaferin were found 0.0295% and 0.049% per 100µg PV respectively (Table 5). The LOD for colchicine and withaferin A was 40 and 80 ng/spot and LOQ was 120 and 240 ng/spot, respectively (Table 6).

### 3.5.5. Heavy metal analysis by ICP-MS

Plants are known for heavy metal absorbance and accumulation. Therefore, AYUSH (Anonymous, 2003) had set standard limits (citation) for different heavy metal in herbal origin products. Important heavy metal content was measured in PV and found to be below safe standard limit (Table 7). PPM content of Arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb) were 0.788, 0.186, 0.687 and 0.476 respectively with low RSD% value in experiments performed in triplicates.

### 4. Discussion

The aim of the present study was to establish scientific evidences of the polyherbal ayurvedic formulation Peedantak Vati’s biological activities. Keeping this in view, present study was designed to evaluate PV’s anti-inflammatory and anti-nociceptive pain potential using different in-vitro and in-vivo models.

NO level was evaluated using LPS-stimulated murine macrophage cells RAW264.7 and human THP-1. Macrophages produce inflammatory mediators such as NO and other free radicals, in addition to numerous cytokines such as TNF-α, IL-1β, and IL-6 (Yang et al., 2012) during inflammation. Significant inhibition of NO production was observed in a concentration-dependent manner, which suggests remarkable anti-inflammatory activity.

Pro-inflammatory cytokines TNF-α and IL-6 are biomarkers of the inflammation, and their role in diseases such as RA, inflammatory bowel disease, sarcoidosis, psoriasis (Glaudemans et al., 2010; Sanchez-Munoz and Dominguez-Lopez, 2008), systemic lupus erythematosus and Crohn’s disease (Gabay, 2006) has been reported. The level of IL-6 and TNF-α in LPS-stimulated human THP-1 cells was measured using ELISA at different time points. The results clearly demonstrate that, the treatment of PV causes significant decrease in IL-6 and TNF-α levels in a conc. dependent manner. Also, it was observed that, IL-6 level was robustly inhibited by PV in comparison to TNF-α, which could be due to modulation of NF-kB signaling pathways.

Further in vivo studies were performed to confirm the anti-inflammatory effects of PV in rat inflammation models. Paw edema model is widely used as a simple and reliable model to assess anti-inflammatory activity of various agents (Ashok et al., 2010; Fernandes et al., 2010). It is a biphasic event, During the early phase of inflammation (0–2 h) mediators like histamine, 5-hydroxytryptamine and Bradykinin play important role, while during the late accelerating phase (post 2 h) there is elevated production of PGs, and production of COX-2 (Di Rosa et al., 1971; Sakat et al., 2014). PV treatment at low dose was found to be active only in the late phase. However, at the high dose of PV displayed considerable inhibition in both early and late phase of inflammation induced by carrageenan by inhibiting the release of different inflammatory mediators disturbing cyclooxygenase pathway.

PV was evaluated by hot plate and tail flick tests to assess its anti-nociceptive effect via supraspinal and spinal mechanisms respectively (South and Smith, 1998). The oral treatment of PV demonstrated a dose dependent, significant anti-nociceptive pain activity in hot plate test, suggesting its activity is mediated by supraspinal mechanism. With tail flick test, PV displayed remarkable anti-nociceptive potential via spinal mechanism particularly at high dose. Similar to reference drug TMD showed considerable reduction in tail flick latency time and confirm its analgesic potential. The results suggest that the anti-nociceptive activity of PV Altogether involves both supraspinal and spinal mechanism.

The acetic acid induced writhing reflex is a model of visceral pain, which is simple and commonly used method for the screening of peripherally acting analgesic drugs (Abdollahi et al., 2003; Ezeja et al., 2011; Golshani et al., 2004). The data of the writhing test emphasized the significant peripheral anti-nociceptive activity of PV by reducing writhing response. As standard NSAID drug INDO at 10 mg/kg also exhibited prominent anti-nociceptive activity, known to relieve the pain response peripherally by inhibiting production of prostaglandins, thromboxane by acting on cyclooxygenase enzymes.

The formalin test discriminates pain into early and late phases. It is useful not only for assessing the analgesic substances but also for elucidating the mechanism of analgesia (Shibata et al., 1989). The first phase involves neuropathic pain and second phase involves inflammatory pain mediated by prostaglandin and cytokines such as IL-1β, IL-6, TNF-α, eicosanoids, and NO (Chichorro et al., 2004; Hunskar and Hole, 1987). Although, PV displayed marginal activity in the first phase at high dose, it displayed prominent activity in the second phase at both dose levels.

### Table 5

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### Table 6

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<th>Intercept</th>
<th>% RSD of slope</th>
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<th>LOQ (ng/spot)</th>
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<td>Colchicine</td>
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### Table 7

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* AYUSH guidelines.
Standardization of herbal formulations is essential in order to assess the quality of drugs, based on the concentration of their active principles (Chawla et al., 2013). We developed HPLC, HPTLC and LC-MS/MS methods for standardization of PV. The different markers compounds were identified and quantified by chromatographic techniques. Method was validated for HPLC and HPLC. This will help in maintaining consistency of the product. LC-MS/MS analysis confirmed presence of standard marker compounds, identified by HPLC and HPTLC, which substantiate the robustness of newly developed method and analysis. This method of analysis was found suitable for simultaneous identification and quantification of these five phytoconstituents from different class of phyto-compounds such as flavonoids (rutin), phenols (curcumin) and phenolic acids (caffeic acid), alkaloids (colchicine) and terpenoids (withaferin A) in a single run. Furthermore, these identified compounds can be used for quality control, consistency and accuracy of the PV formulation.

5. Conclusion

Based on the results shown in the present study, it can be concluded that polyherbal ayurvedic formulation ‘Peedantak Vati’ possesses significant anti-inflammatory and analgesic properties, providing potent and promising herbal alternative to currently available NSAIDs. Our studies also show that PV’s anti-inflammatory activities could be targeting arachidonic acid cascade and modulating pro-inflammatory cytokines.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors are thankful to Patanjali Research Foundation Trust and Dr. Sumnitro Nig. Divya Pharmacy, Patanjali Ayurved Ltd., Haridwar, India for providing the Peedantak Vati formulation to carry out the project.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2019.01.028.

References


