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Evaluation of anti-inflammatory Properties of Homoeopathic Gel preparations of calendula officinalis, arnica Montana, echinacea angustifolia and hypericum perforatum by Zymography- An *in vitro* study

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Abstract

Context: Inflammation is a physiological process in response to tissue damage resulting from microbial pathogen, infection, chemical irritation and/or wounding.

Aims: We investigated the anti-inflammatory effect of four homoeopathic medicaments Calendula officinalis, Arnica Montana, Echinacea angustifolia and Hypericum perforatum by Zymography a process of detection of enzymatic activity on gel electrophoresis.

Materials and Methods: An apparatus for gel electrophoresis was used. Sample preparation (10mg in 1ml DMSO) and buffer preparation (225µl of D/W + 25µl of stock solution) was made. The apparatus was assembled. Then casting of polyacrylamide gel was done and it was allowed to polymerize and then the gel electrophoresis was run at least for one and half hours and later removed and was incubated. After electrophoresis the gel was washed with zymogram renaturing buffer (ie 2.5% Triton X-100). Then it was stained with coomassie blue R-250 and later destained with an appropriate destaining solution.

Result: Coomassie blue staining of the gel revealed sites of proteolysis as translucent bands on a dark blue background. It showed a percentage of anti-inflammatory activity for Arnica Montana to be 85% for MMP-2 and 80% for MMP-9, for calendula officinalis 80% for MMP-2 and 75% for MMP-9, for echinacea angustifolia 75% for MMP-2 and 68% for MMP-9 and for hypericum perforatum 70% for MMP-2 and 60% for MMP-9.

Conclusion: Our findings confirmed the anti-inflammatory potential of all homoeopathic medicaments used.

Keywords: Homoeopathy, Zymography, Calendula officinalis, Arnica montana, Echinacea angustifolia, Hypericum perforatum, Gel electrophoresis

1. Introduction

Plants are utilized as therapeutic agents since times unmemorial in both organised and unorganised forms [1]. A herbal drug constitutes a major part in all traditional system of medicine [2]. Pharmacological activity of plants is often known as a result of millennia of trial and error but they have to be carefully investigated if we wish to develop a new drug that meet the criteria of modern treatment [3]. Homoeopathy has remained the most widespread and still a contentional mode of therapy. Homoeopathic medicines are prepared according to the methods endorsed in homoeopathic pharmacopoeias. However in the last few decades claims about the efficacy of homoeopathic medicines and their high dilution are being revisited using validated pharmacological assays [4]. Inflammatory reactions and haemorrhages are the main characteristics of tissue injury [5]. Inflammation also stimulates angiogenesis and tissue remodelling [6]. Healing of wounds weather from accidental injury or surgical intervention involves the activity of an intricate network of blood cells, tissue types, cytokines and growth factors [7]. Zymography is known as an electrophoretic technique commonly based on sodium dodecyl sulphate polyacrylamide gel electrophoresis which contains a substrate copolymerized within the polyacrylamide gel matrix for the detection of an enzymatic activity [8]. Also a study conducted by Kupai *et al* discussed about matrix metalloproteinase activity assays and importance of Zymography. These are zinc dependent endopeptidases capable of degrading extra cellular matrix including basement membrane. MMPs are associated with various physiological processes such as morphogenesis, angiogenesis, and tissue repair [9]. MMPs are secreted as inactive or latent pro-MMPs which undergo proteolytic activation by other MMPs or other proteases before they can degrade ECM proteins [10].

For the development of selective MMP inhibitor molecules reliable methods are necessary for target validation and lead development. Here major methods used for MMPs assay were discussed focussing on substrate zymography [9]. We investigated the anti-inflammatory efficacy of few homoeopathic medicines namely *Calendula officinalis*, *Arnica Montana*, *Echinacea angustifolia* and *Hypericum perforatum* by Zymography.

Materials and Methods

Gel preparations of *Calendula officinalis*, *Arnica montana*, *Echinacea angustifoila* and *Hypericum perforatum* were used. The objectives of this study was to evaluate the *in vitro* anti-inflammatory properties of the preparations by Zymography.

The following solutions were used for the procedure

	Resolving gel	Stacking gel
Acrylamide	3.3ml	1.7ml
Resolving gel buffer	1.25ml	1.12ml
SDS (10%)	100 μ l	100 μ l
APS (1.5)	500 μ l	500 μ l
Gelatin	1ml	
Water	3.8ml	6.5ml
TEMED	10 μ l	10 μ l
	10ml	10ml

Sample Preparation

10mg in 1ml DMSO

1. Add 50 μ l of tonsils sample + 50 μ l compound and then

incubate for 1 hour

2. From prepared / preincubated sample take 20 μ l of 2 X non reducing buffer + 20 μ l sample
3. Final loading: 30 μ l into the wells

Buffer Preparation

225 μ l of D/W + 25 μ l of stock solution to make it 250 μ l

Procedure

Arrange the apparatus

1. Essential component of the apparatus

- 1 Tank
- 1 CEU (central electrode unit) with gasket
- 1 set of connecting cords
- Two clamps
- Two set of plastic spaces
- Two acrylic combs
- Set of flat & stiff glass plates (notched & rectangular)

2. Assembling the apparatus (Fig 1)

- Slide the CEU into the tank, fasten in place by tightening screw on the back side
- On the CEU arrange the sandwich consist of the notched plate, spacers & rectangular plate on it
- Seal the sides by placing the spacers
- Insert the clamps along the edges of the plate & turn the screw clockwise to clamp the sandwich to the CEU.
- Seal the bottom of the sandwich with the 3-5 ml of molten agar/ agarose



Fig 1: Apparatus for gel Electrophoresis

Casting Polyacrylamide slab gel

1. Resolving gel buffer was poured up to the 3/4th mark on the glass slab.
2. It was allowed to set, once the mixture was set it was checked in the side arm flask of the beaker in which the mixture was prepared. Water was poured & lid was placed.
3. It was allowed for 30 mins after which the water tank was removed & the resolving gel was checked by tilting the apparatus side wards for the polymerization.
4. Preparation of stacking gel was done once the resolving gel was polymerized, stacking gel was poured on top of the resolving gel completely or the remaining 1/4th. A comb of appropriate size was placed.
5. The gel was allowed to polymerize for 30 mins
6. Once the mixture was set, remove the comb was removed & the wells created were marked.
7. The wells were washed with D/W twice.
8. The water from the wells was removed
9. 225 μ l of D/W + 25 μ l of stock solution ie reservoir

buffer was added on the upper tank & lower tank

10. The samples which were previously incubated for 1 hour were now loaded
11. The electrodes were connected (Black to Black & Red to Red)
12. Then the gel electrophoresis was run
13. In case current did not flow through the apparatus, the connections for the apparatus were checked weather been fitted with polarity sensing circuit which prevented the passage of current in the reverse direction
14. It was run for at least one & half hour & the samples reached 3/4th of the gel glass slab.

Dismantling the sandwich & removal of polyacrylamide gel

1. When the dye front was about 5mm from the bottom the electrophoresis is complete
2. At the end of the run the power supply was switched off, the electrical rods disconnected & the lid removed
3. The buffer from both cathodic & anodic tank was

- siphoned out & the buffer was discarded
4. The tank was tilted backwards by placing a buffer under one side
5. The clamps were loosened by turning the screw anti clockwise & removed & rinsed with water
6. The gel sandwich was lifted & placed horizontally on a sheet of paper. The gel sandwich was inserted open/ spatula between the plates & prised apart
7. The orientation of the gel were marked & carefully peeled off from the plate
8. The gel was transferred into the triton X-100 & kept for 1 hour incubation
9. It was kept in incubation buffer over night

After electrophoresis, the gel was put in a plastic dish & washed with zymogram renaturing buffer (ie 2.5% Triton X-100) for 1 hour to remove SDS from the gel & allow proteins to denature. Then it was stained with coomassie blue R-250 for 1 hour. Gel was destained with an appropriate coomassie blue R-250 destaining solution for about 2 hours.

Results

Coomassie blue staining of the gel revealed sites of proteolysis as translucent bands on a dark blue background. The background stains blue with coomassie stain where the gelatin is degraded, white bands appear indicating the presence of gelatinases. The upper bands are gelatinases B (MMP9) which runs at about 95 KBand the lower bands are gelatinases A (MMP-2) (Fig 2). The results have shown the percentage of anti-inflammatory for Arnica Montana to be 85% for MMP-2 and 80% for MMP-9, 80% for MMP-2 and 75% for MMP-9 with calendula officinalis, 75% for MMP-2 and 68% for MMP-9 with echinacea angustifolia and 70% for MMP-2 and 60% for MMP-9 with hypericum perforatum. The results are tabulated in Table 1

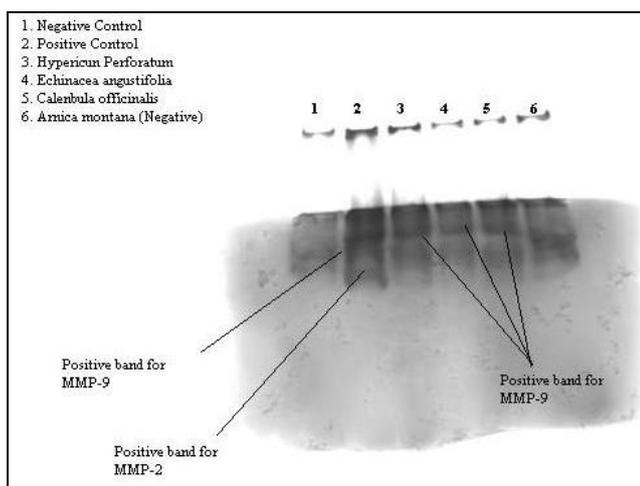


Fig 2: Bands showing anti- inflammatory activity after electrophoresis

Table 1: Percentage of anti- inflammatory activity

Drug Name	Percentage of activity	
	MMP-2	MMP-9
Arnica Montana	85	80
Calendula Officinalis	80	75
Echinacea angustifolia	75	68
Hypericum perforatum	70	60
Positive control	100	95
Negative control	Nil	Nil

Discussion

Inflammation is a physiological process in response to tissue damage resulting from microbial pathogens [11]. The functioning of immune system is finely balanced by the activities of pro inflammatory and anti-inflammatory mediators or cytokines [12]. Also inflammation activates a variety of inflammatory cells which induce and activate oxidant generating enzymes [13]. Hypericum perforatum is a well-known medicinal plant that has been in use for a decade [14]. In this study Hypericum perforatum showed 70% of anti-inflammatory activity against MMP-2 and 60% activity against MMP-9. Anti-Inflammatory activity has been found pointing to the possible relevant role of hypericin and related compounds in determining the anti-inflammatory activity of hypericum preparations [15]. Previous studies have shown the Hypericum perforatum extract inhibited LPS induced PGE2 and nitric oxide production and attributed part of the activity of a highly active fraction of the extract to a group of four compounds including pseudohypericin, amentoflavone, quercetin, and chlorogenic acid. Also in addition to PGE2 and nitric oxide TNFα and IL-1 were also measured and it was found that the four compounds inhibited both [16]. Researchers also reported protein kinase C inhibitory activity with both hypericin and pseudohypericin. The protein kinase C inhibition may contribute to the anti-inflammatory effects associated with hypericum as hypericin has been found to inhibit the release of archidonic acid and leukotriene B4 [17]. It is stated that Hypericum perforatum extract contain many different classes of constituents including flavonoids and biflavonoids, phlorglucinols, naphodianthrones, caffiec acid derivatives and other unidentified compounds [18]. Echinacea contains at least six chemical constituents with pharmacologic activity: polysaccharides, flavonoids, chicoric acid glycosides, essential oils, polyacetylenes, and alkylamides [19]. Anti-inflammatory activity of Echinacea extracts have been attributed to direct inhibition of Hyaluronidase. Echinacea-derived alkamides have immunomodulatory and anti-inflammatory activity [20]. Also it was presented in a study that showed inhibition of PGE2 by Echinacea species which may be one process of contributing to the anti-inflammatory capability [21]. In the present study Echinacea showed anti-inflammatory activity to be 75% against MMP-2 and 68% against MMP-9. Alkylamides have become a major focus for researchers studying Echinacea & studies with this class of compounds to a vast repertoire of immunomodulatory activities including anti-inflammatory properties. Also it was indicated in experiments that alkylamides are consistent inhibitors of PGE2 production. Also regardless several of extracts of Echinacea containing a variety of alkylamides, ketones, caffiec acid derivatives at low concentration were able to inhibit PGE2 significantly. Also it was found that the anti-inflammatory properties in Echinacea was not simply due to one constituent but several acting in a synergistic or additive manner [21]. In the field of homoeopathy arnica Montana preparations have been used with very good levels of tolerance for acute traumata such as strains, contusions and bruises. According to recent research helenalin in arnica Montana inhibits NF-Kappa B in the micromolar region a transcription factor that plays a substantial role in the inflammatory process [22]. Systemic inflammation is involved in the pathogenesis of many diseases. LPS is a prototypical endotoxin which can directly activate

macrophages^[23] and induce production of inflammatory cytokines such as IL-1, IL-6, TNF α and inflammatory mediators including nitric oxide and prostaglandin E2^[24]. In a study it was shown arnica Montana significantly reduced the release of IL-1, IL-6 and TNF α from the LPS stimulated RAW 264.7. Also arnica Montana extracts and its active constituents are reported to inhibit the binding between NF- κ B and DNA and inhibit NF dependent gene expression²⁵. Phosphorylation and degeneration of I κ B, NF- κ B's inhibitory subunit, stimulates NF- κ B. NF- κ B activation by T cells, B cells and epithelial cells is inhibited by helenalin which in turn blocks κ B-driven gene expression^[26]. In this study arnica montana demonstrated the highest anti-inflammatory activity with 85% for MMP-2 and 80% for MMP-9. Calendula extracts heals wounds as well as internal and external ulcers. It is an antiseptic and in addition improves blood flow to the affected area^[27]. In the present study Calendula officinalis showed a good anti-inflammatory activity with 80% against MMP-2 and 75% against MMP-9. In a study conducted by Preethi *et al* showed that the extract of Calendula officinalis flowers showed significant anti-inflammatory activity in both acute & chronic models of the study. Cytokines are the key molecules that can inhibit or propagate inflammation by activating or deactivating the genes involved in cellular process. Also acute inflammation causes the release of INF- γ into the circulation & mediates host inflammatory responses. The treatment with Calendula officinalis extract lowered the INF- γ level. Also the expression of cyclooxygenase 2 a key enzyme involved in inflammatory process was found to be inhibited by the treatment with the extract. So the Calendula officinalis extract may be exerting its anti-inflammatory activity through modulating the activity of proinflammatory cytokines as well as by inhibiting the expression of cox-2^[28]. Studies using cultures of human and murine fibroblasts demonstrated that extracts of Calendula officinalis stimulate fibroblast migration and proliferation in a PI3K-dependent manner. Calendula officinalis is also reported to enhance angiogenesis *in vivo*^[29].

Conclusion

Several journal reviews cite a high prevalence of complementary and alternative medicine use by surgical patients. The current study confirmed the anti-inflammatory potential of medicaments used. Use of various herbs and traditional medicines is safe as well as economical. In this study all medicines used exhibited a good anti-inflammatory activity with Arnica Montana showing the highest activity. However further research is needed to determine the main compounds responsible for this activity.

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