Potassium humate inhibits carrageenan induced paw oedema and a graft-vs-host reaction in rats

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Short title: Potassium humate inhibits inflammation.

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A**BSTRACT**

It has been shown in a previous study that brown coal derived potassium humate is safe and effective in suppressing contact hypersensitivity in rats. In this study the efficacy of potassium humate on other types of inflammation was determined. Preparative TLC followed by mass spectroscopy was used in an attempt to fingerprint the product.

The effects of potassium humate, at an oral dose of 60mg/kg bodyweight, on a delayed type hypersensitivity reaction, a carrageenan induced inflammation model and an allogeneic graft-versus-host reaction (GVHR) in rats were investigated. Paw oedema was used as a measure of inflammation.

It was found that potassium humate had no effect on the delayed-type hypersensitivity reaction but significantly inhibited the increase in paw volume of the carrageenan-induced oedema in rats which compared favourably with indomethacin treatment. Furthermore potassium humate inhibited the GVHR induced in normal and cyclophosphamide treated immune-incompetent rats.

The identification of a naturally occurring compound that is safe and effective in reducing different types of inflammation merits further evaluation in clinical trials.

Key words: Potassium humate; Graft-versus-host reaction; Anti-inflammatory; Carrageenan induced inflammation; Rats

Introduction

Humic substances are dark coloured and are a heterogeneous mixture of organic materials. Humic substances are widely spread in nature. They occur mainly in heavily degraded peat but also in all natural environments in which organic materials and microorganisms are, or have been present. It can be extracted from brown coal or peat (Hartenstein, 1981) or derived from bituminous coal (Bergh et al., 1997). Many of its properties are known, but its exact structure and function are still in question (Paciolla et al., 2002).

Humates have been used as folk remedies for the last 3000 years for a broad diversity of illnesses (Schepetkin et al., 2002). Mud baths, rich in humic and fulvic acids, were used to treat rheumatic conditions during the 19th century (Baatz, 1988; Kleinschmidt, 1988; Kovarik, 1988; Lent, 1988; Golbs et al., 1982). These patients experienced a subsidence of the pain, a relaxation of the tension in the back muscles, and were able to move more freely after treatment.

In a recent study it has been shown that potassium humate suppresses ear swelling in a contact hypersensitivity animal model, comparable to prednisilone (Van Rensburg et al., 2007). However, little is known of the possible mechanism of action of humate with reference to its anti-inflammatory properties. It has been found that oxihumate, a water-soluble humate obtained through a wet oxidation of bituminous coal (Bergh et al., 2002), decreases the expression of complement receptor 3 (CR3) by phorbol-12-myristate-13-

acetate (PMA) stimulated human neutrophils as well as the adhesion of these cells to a baby hamster kidney cell line expressing intracellular adhesion molecule-1 (ICAM-1) (Jooné and van Rensburg, 2004), possibly contributing to its anti-inflammatory effects.

The safety of brown coal derived potassium humate was studied by van Rensburg et al., 2007. This product, at 1000mg/kg body weight per day, had no effect on the safety parameters tested when administered to rats by gavage for one month nor did 500mg/kg body weight have any effect on pups after oral administration of the product to pregnant female rats on days 5 to 17 of pregnancy indicating the safety profile of this compound.

In the present study the anti-inflammatory properties of potassium humate have been determined using (i) a delayed type hypersensitivity reaction in rats immunized with sheep red blood cells (SRBC), (ii) a carrageenan-induced paw oedema model and (iii) an allogeneic graft-versus-host reaction (GVHR) in normal and cyclophosphamide treated immune incompetent rats.

Materials and methods

Materials and reagents

Zymate®, a potassium humate product, prepared from brown coal (leonardite) that was mined from a selected area, was supplied by Unique Health Trust (Milnerton, South

Africa). Indomethacin, cyclophosphamide, carrageenan and dexamethasone were obtained from Sigma Diagnostics (St Louis, MO, USA).

Thin layer and preparative thin layer chromatography

Analytical thin layer chromatography was performed using precoated aluminium backed Silica F-254 plates of 5 x 10 cm and 0.25 mm thickness (Macherey Nagel, Düren). The mobile phase was a mixture of acetonitrile:methanol:water:25% ammonium hydroxide in the ratio 17:6:6:6. The plates were run in standard closed TLC tanks in a mobile phase saturated atmosphere.

Preparative thin layer chromatography was carried out on 20 x 20 cm glass plates with a 2 mm thick Silica Gel 60 F254 layer (Merck, Darmstadt). The same mobile phase as for the analytical TLC was used and the plates run under the same conditions as the analytical TLC. The tank was lined with a thick layer of filter paper to ensure saturation of the atmosphere.

Analyte zones were visualised by inspection under normal light as well as 254 and 360 nm UV light where several UV absorbing and fluorescing bands could be detected. These were marked and individually scraped from the preparative TLC plates and the removed silica extracted sequentially with 50 ml of methanol then water containing 0.25% ammonium hydroxide. These extracts were subjected to mass spectrometry by infusion of the extracts directly into a 4000 QTrap triple quadrupole mass spectrometer (Applied

Biosystems Sciex, Concordia, Canada) in both positive and negative ionisation mode in an attempt to determine the compounds identities. All masses were monitored between 70 and 1200 Dalton without any fragmentation.

Animals

Female Sprague Dawley and BD IX rats of 12 weeks old (weighing between 150g to 200g) were purchased from the National Health Laboratories Service (Rietfontein, South Africa). Rats were housed individually in cages in a temperature controlled room (22 °C) with a 12 hour day/night light cycle with ad libitum access to water and rat chow. Rats were allowed to acclimatise for at least one week before the study was initiated.

All animal experiments were carried out at the University of Pretoria's Biomedical Research Centre, Onderstepoort, South Africa, with the approval of the Animal Use and Care Committee of the University of Pretoria.

Potassium humate was administered by oral gavage at 60 mg/kg bodyweight, which was similar to that used by van Rensburg et al., 2007. Indomethacin and dexamethasone was administered at 10 and 30mg/kg bodyweight respectively, also by gavage, as described by Smit et al., 2000.

A delayed type hypersensitivity reaction in rats immunized with sheep red blood cells (SRBC)

A delayed type hypersensitivity reaction in rats immunized with sheep red blood cells was done according to a combination of the methods described by Sharma et al., (2004), Bani et al., (2005) and Manosroi et al., (2005). Thirty female Sprague Dawley rats were assigned to one of three groups; negative control group, positive control group and treatment group of ten rats each. Each rat was immunised on day one with an intraperitoneal injection of a SRBC suspension (1×10^8 SRBC in 0.5 ml phosphate buffered saline). The experimental group received potassium humate that was admonistered at 60mg/kg bodyweight once daily by oral gavage for 7 consecutive days, starting on day one. The control group received water once daily by oral gavage and the positive control group received dexamethasone (30mg/kg bodyweight) once daily by oral gavage on the sixth and seventh day after immunisation. On the seventh day after the sensitisation step the initial volume of the right hind paw of each rat was measured with a water displacement plethysmometer. Rats were then administered the different test compounds by oral gavage and then challenged by injecting a sheep erythrocyte suspension (1×10^8 sheep erythrocytes in 0.5ml PBS) subplanar into the right hind footpad of each rat. The volume of the right hind paw of each rat was measured 24 hours later with a plethysmometer. Paw oedema was expressed as the difference between the volumes of the initial paw compared to the paw measured 24 hours after the challenging step.

Carrageenan induced paw oedema

A carrageenan-induced paw oedema was executed according to methods described by Recio et al. (2000), Smit et al.(2000), Petersson et al. (2001) and Huber et al., (2002). Thirty female Sprague Dawley rats were assigned to one of three groups; control group, experimental group and positive control group of ten rats each. The experimental group received potassium humate (60mg/kg bodyweight) once daily by oral gavage for five consecutive days. On the fifth day of the experiment the initial right hind paw volume of each rat were measured with a water displacement plethysmometer, the control group received water by oral gavage, the experimental group received a final bolus of potassium humate (60mg/kg bodyweight) and the positive control group received indomethacin (10mg/kg bodyweight) by oral gavage. Carrageenan (50µl of a 2% solution in saline) was injected sub plantar into the right hind paw of each rat thirty minutes after administration of the test compounds. The paw volume was measured 60min, 120min, 180min, 240min and 300min after carrageenan administration with a plethysmometer. Paw oedema was expressed as the difference between the volumes of the initial paw measurement compared to the paw measured every hour after carrageenan administration.

Popliteal lymph node (PLN) assay

The effects of potassium humate on a graft-vs-host (GVHR) reaction were determined in normal and immune incompetent rats by using the PLN assay according to a modified method described by Skowron-Cendrzak et al. (1978) and Gutting et al. (2003).

Forty BD IX rats (recipients) were divided in four groups and weighed before and after the experiment. The rats were sensitised on day 0 of the study by an intraperitoneal injection of 1×10^6 leukocytes suspended in 0.5ml RPMI isolated from the spleens of Sprague Dawley rats (donors).

The first group received water by oral gavage daily from day 1 to day 13 of the study and 1 ml saline i.p. on day 3 of the study. The second group received water by oral gavage daily from day 1 to day 13 of the study and 1 ml cyclophosphamide (200mg/kg bodyweight) i.p. on day 3 of the study. The third group received potassium humate (60mg/kg bodyweight) by oral gavage daily from day 1 to day 13 of the study and 1 ml saline i.p. on day 3 of the study. The fourth group received potassium humate by oral gavage daily from day 1 to day 13 of the study and 1 ml cyclophosphamide (200mg/kg bodyweight) i.p. on day 3 of the study.

On day 7 of the study all of the BD IX rats received 5×10^6 viable mononuclear leukocytes suspended in 0.5ml RPMI, isolated from the spleens of Sprague Dawley rats by a sub-planar injection to the right hind footpad of the recipients. On day 13, six days after the injection the BD IX rats were terminated by euthanasia with CO_2 asphyxiation. The right and left PLN of each rat were removed and weighed. The PLN weight index was defined as the percentage increase of lymph node weight of experimental right PLN over control left PLN.

Statistical analysis

Data are expressed as means ± SEM. Statistical significance was calculated using one-way analysis of variance (ANOVA), followed by either Bonferroni test for pair-wise comparisons compared to control or Tukey's multiple comparison test, compared to the equivalent control.

Results and discussion

Humates have long been used as folk remedies for a broad diversity of illnesses, for virtually 3000 years (reviewed by Schepetkin et al., 2002) and were traditionally used in Asian herbal medicine to treat injuries, bone fractures, dislocations, diseases of the skin and diseases of the peripheral nervous system. Humates were also used by Greek physicians mainly as anti-inflammatory agents. Only a small number of scientific studies have been done to confirm the medicinal applications of humic acid. Shilajit, an exudate from the steep rocks of Afghanistan, which contains high levels of humic acid, reduced paw oedema of a carrageenan-induced inflammatory model when administered i.p. at 50mg/kg bodyweight (Schepetkin et al., 2002). A recent study has shown that brown coal derived potassium humate administered by gavage at a dosage of 60mg/kg bodyweight for 6 consecutive days, suppresses ear swelling in a contact hypersensitivity model, which was comparable to prednisilone (van Rensburg et al., 2007), confirming that potassium humate is absorbed and is physiologically available to elicit its effects at areas of inflammation.

Analytical and preparative TLC resulted in the same type of separation with three obvious pseudo-fronts forming on the plates at Rf 0.63, 0.92 and 0.98. A band of UV absorbing and long UV fluorescing compounds coincided with each of these pseudo-fronts. A typical TLC plate is shown in Fig. 1. The more concentrated the applied sample, the more of the sample remained at the application point. Severe streaking of UV absorbing compounds occurred between the pseudo-fronts. The strongly coloured compounds remained close to the origin with Rf values of 0.30 or less.

The compounds recovered from the preparative TLC plates displayed very different solubility properties. The compounds on the pseudo-fronts were easily extracted by methanol whereas the coloured compound near the origin could only be extracted with dilute ammonium hydroxide solution. The extracts which showed distinct colour differences were dried under a stream of nitrogen and resolublised in 50% methanol in 0.1% formic acid for mass spectral analysis.

The extracts of the zones with Rf of 0.47, 0.60 and 0.67 all showed that they were still complex mixtures of compounds with a high degree of similarity of the dominant ions detected by mass spectrometry using a triple quadrupole system. Interestingly most of the compounds (which numbered more than 15 in each extract) showed mass to charge ratios of less than 500 Dalton when using positive ionisation mode, implying mixtures of small molecules. A similar trend was seen using negative mode ionisation, however only four masses appeared to be from the same compounds. This means that there are more than

the 15 major compounds per PTLC band with the possibility of uncharged compounds adding further to the complexity. A typical mass spectrum of the PTLC band at Rf 0.63 is shown in Fig.2. These results confirm the complexity and supramolecular nature of humic acid (Baigorri et al., 2009).

In an attempt to investigate the effects of potassium humate on the delayed type hypersensitivity reaction, rats were immunized with SRBC. However, this product had no effect on the increase in foot volume of this model whereas dexamethasone treatment reduced the inflammation significantly (Fig.3). On the other hand both the potassium humate and indomethacin significantly decreased the carrageenan induced inflammation over the 300min period in rats from as early as 60min after the carrageenan injection (Fig.4). The results obtained with indomethacin are in agreement with that of Nantel et al. (1999).

Similar anti-inflammatory effects were obtained for potassium humate in an experimentally induced GVHR in which case the potassium humate inhibited the inflammatory reactions in both the normal and immune incompetent groups (Fig. 5).

Pro-inflammatory cytokines involved in GVHRs contribute to the pathological damage of target organs (Antin and Ferrara, 1992). These cytokines activate cytotoxic T lymphocytes, resulting in the amplification of local tissue injury and further promotion of inflammation, which ultimately leads to target tissue destruction in transplant recipients. It has been shown that humic acid markedly reduced lipolysaccharide-induced adhesion

molecules; ICAM-1, VCAM-1 and E-selectin expressed by cultured human umbilical vein endothelial cells at a dose of 100µg/ml (Gau et al. 2000). The inhibition of these adhesion molecules might provide an explanation to one of the possible mechanisms in which potassium humate inhibits these inflammatory reactions.

An interesting *in vitro* finding has recently been published by Van Rensburg and Naude, (2009), indicating that potassium humate inhibits both the alternative and classical pathways of complement activation as well as the release of the inflammatory cytokines, TNF-α, IL-1β and IL-6. A fungal metabolite i.e. K76 monocarboxylic acid, which has been described as an inhibitor of both the alternative and classical complement pathways, inhibited leukocyte accumulation in the subcutaneous air pouch of rats in a zymosan induced reaction (Satoshi and Tsurufuji, 1985), whereas anti TNF-α therapy has been successfully used as treatment of severe acute rejection of intestinal transplantations (Pascher et al., 2005). This could perhaps explain some of the effects seen in this study. However further investigation into the mechanism by which potassium humate inhibits inflammation, needs to be done.

Interestingly, potassium humate also inhibited the loss of bodyweight of the cyclophosphamide treated rats (Fig 6), indicating that potassium humate may be of use in the treatment of immune compromised patients suffering from weight loss. It is worth mentioning that Botes et al. (2002) reported that HIV infected individuals, treated with 2, 4, 6 and 8g oxihumate per day for 2 weeks, showed no signs of toxicity and even gained weight compared to the placebo groups.

In summary it was found that potassium humate, given by gavage, had no effect on the delayed type hypersensitivity reaction but reduces the paw volume of carrageenan-induced oedema in rats similar to indomethacin as well as the GVH reaction induced in normal and cyclophosphamide treated immune incompetent rats.

Although this study was done on female Sprague Dawley rats, as suggested by Huber et al., (2002) the possibility of the effects of physiological functions and the influence thereof on the effects of the product needs to be taken into consideration. Preclinical studies using both male and female animals will be considered in the planning of future experiments.

The identification of a naturally occurring compound that is safe and effective in reducing different types of inflammation similar to known anti-inflammatory drugs merits further evaluation in the treatment of patients suffering from inflammatory conditions.

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LEGENDS TO FIGURES

Fig. 1. Thin layer chromatography of potassium humate dissolved in water at concentrations of 1.0% (lane 1) and 0.5 % (lane 2) on silica using acetonitrile:water:ammonium hydroxide [15:8:2] as mobile phase and visualized under UV light.

Fig. 2. A positive ionisation mode mass spectrum of the methanolic extract of the band with Rf 0.63 in the PTLC separation of Zymate. Note the large number of dominant masses despite not fragmenting the ions.

Fig. 3. The effects of potassium humate (60mg/kg bodyweight) and dexamethasone (30mg/kg bodyweight) on a delayed type hypersensitivity reaction in rats immunized with sheep red blood cells (SRBC). Data are expressed as percentage weight changes of the rat paw volumes expressed as means ± SEM. Statistical significance was calculated using ANOVA, followed by Tukey's multiple comparison tests, compared to the untreated control group.

***$P < 0.0001$.

Fig. 4. The effects of potassium humate (at 60mg/kg bodyweight treated orally) and indomethacin (10mg/kg bodyweight) on a carrageenan induced inflammation in rats. Data are expressed as percentage weight changes of the rat paw volumes expressed as

means ± SEM. Statistical significance was calculated using ANOVA, followed by Tukey's multiple comparison tests, compared to the untreated control group.
***P < 0.0001.

Figure 5: The effects of potassium humate (at 60mg/kg bodyweight treated orally) on the PLN assay in normal and cyclophosphamide (cyclophos.) (200mg/kg bodyweight) treated, immune compromised rats. Data are expressed as percentage weight change of the PLN calculated as means ± SEM. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.
**P < 0.001, ***P < 0.0001.

Figure 6: The effects of potassium humate (at 60mg/kg bodyweight treated orally) on the change in body weights of normal and cyclophosphamide (cylcophos.) (200 mg/kg bodyweight) treated, immune compromised rats. Data are expressed as percentage weight change calculated as means ± SEM. Statistical significance was calculated using ANOVA, followed by the Bonferroni test for pair-wise comparisons compared to control.
*P < 0.05

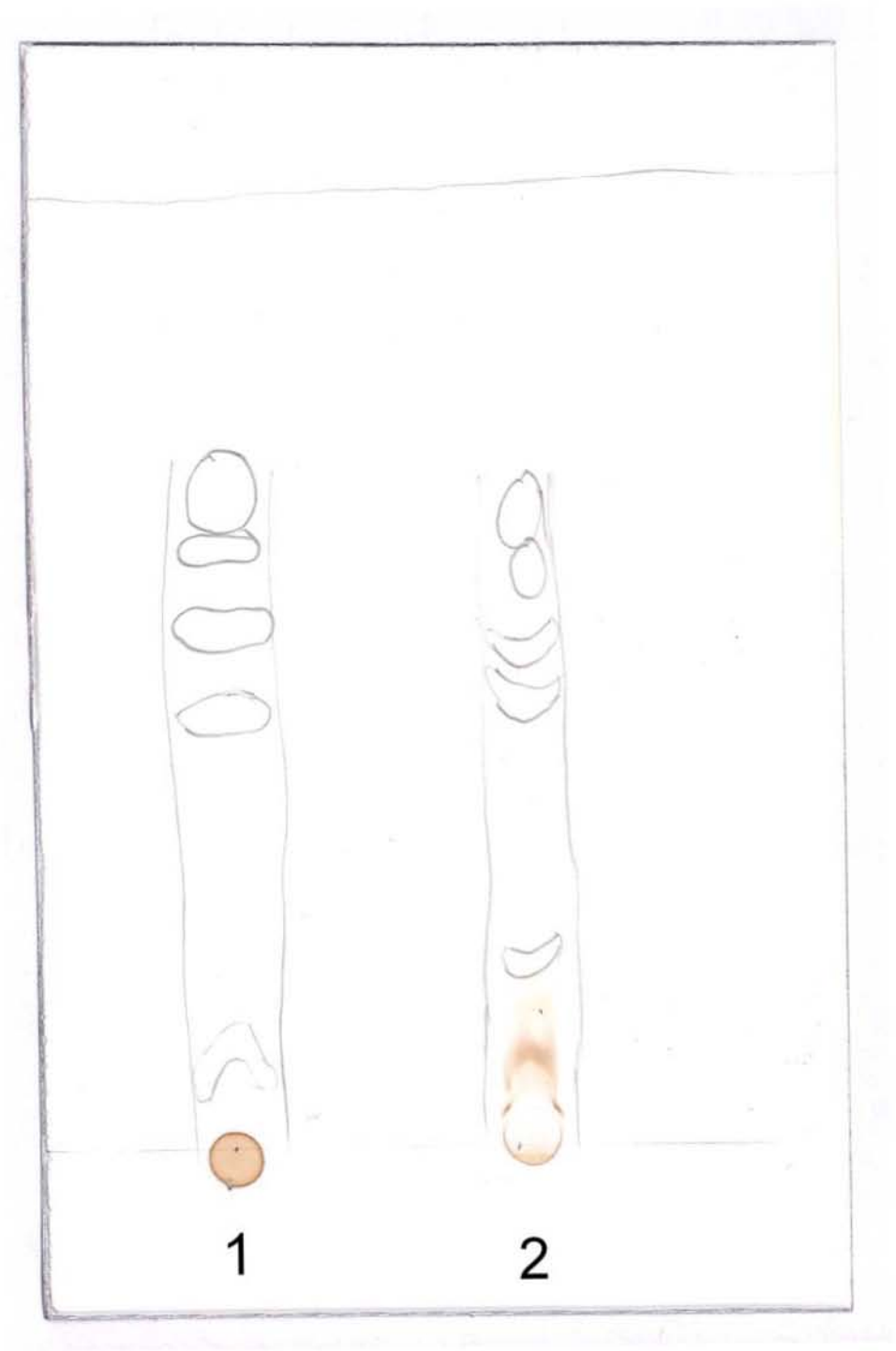
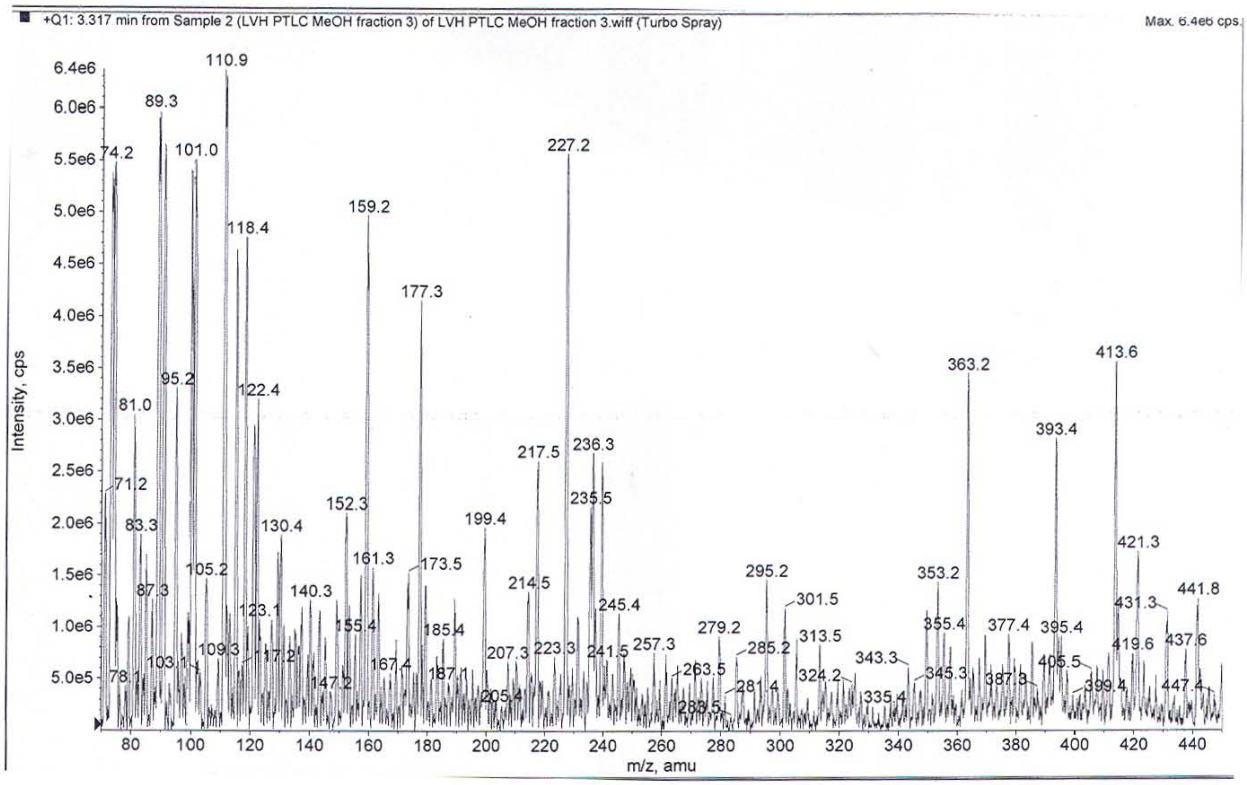


Figure 1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Max. 6.4e6 cps.
Intensity, cps
m/z, amu

Figure 2

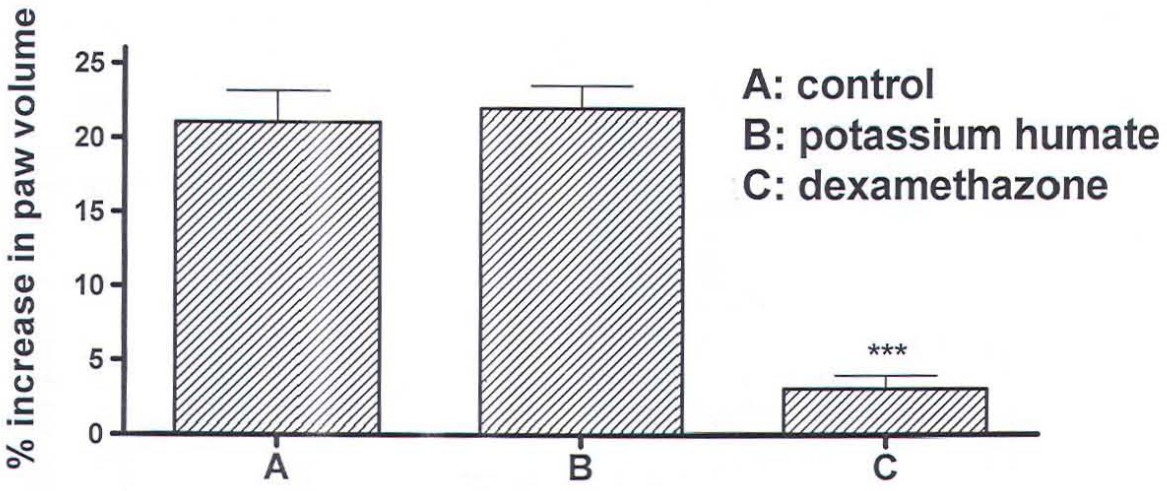
% increase in paw volume
25
20
15
10
5
0
A
B
C
***
A: control
B: potassium humate
C: dexamethazone

Figure 3

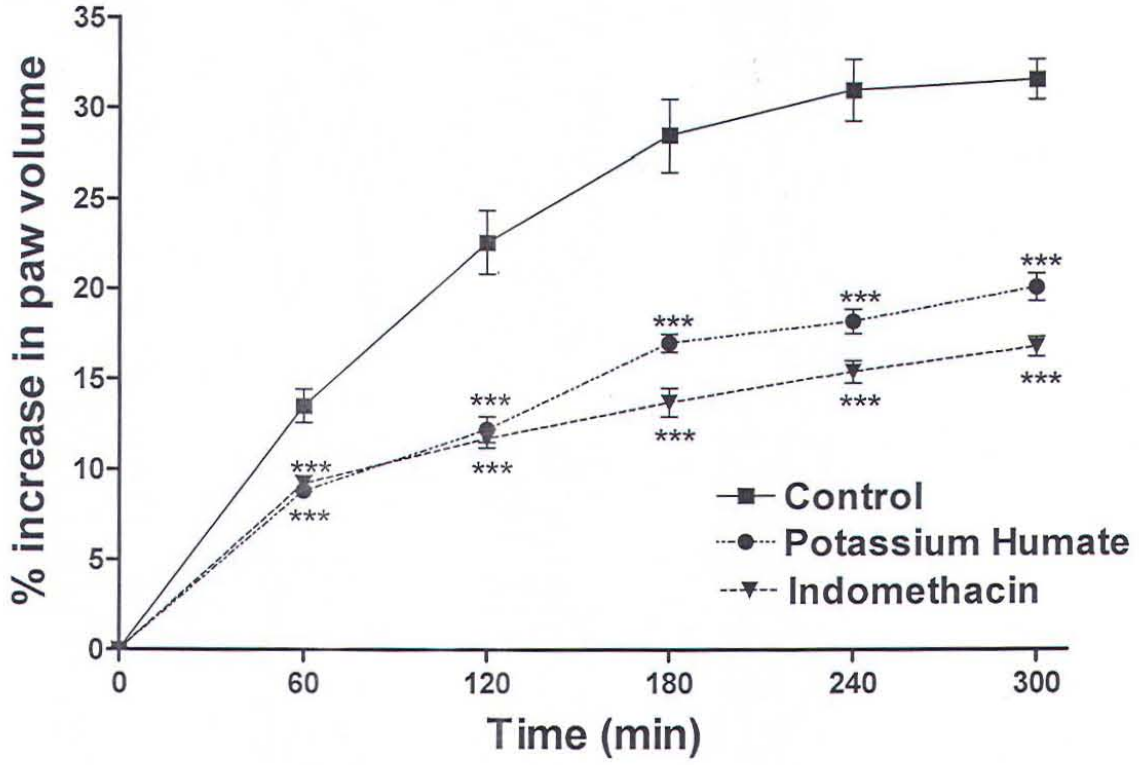
% increase in paw volume
35
30
25
20
15
10
5
0
0
60
120
180
240
300
Time (min)
Control
Potassium Humate
Indomethacin
***

Figure 4

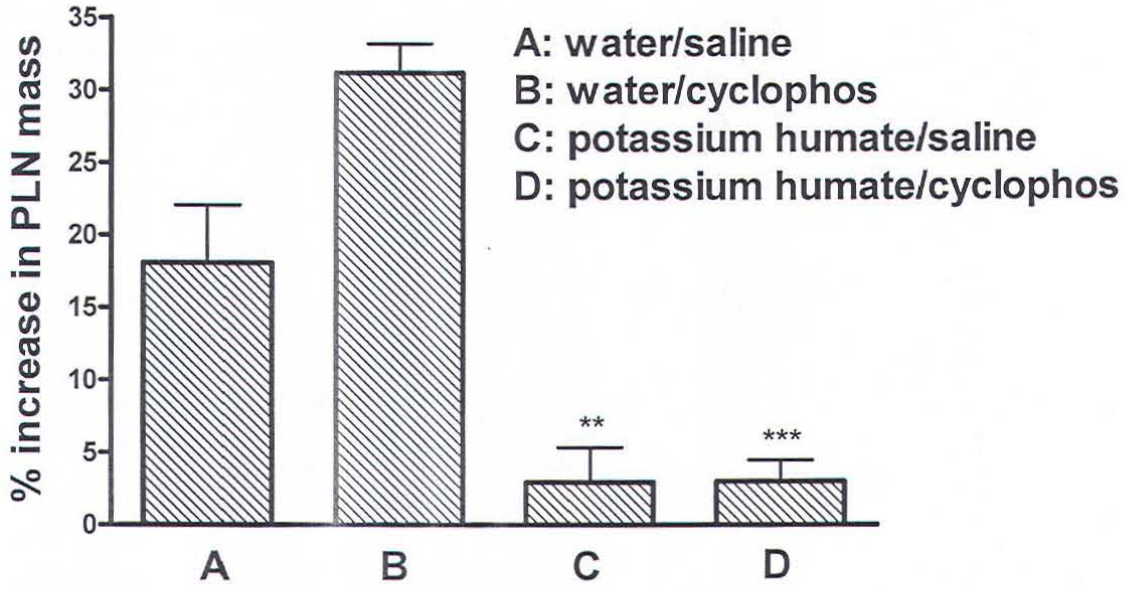
% increase in PLN mass
35
30
25
20
15
10
5
0
A: water/saline
B: water/cyclophos
C: potassium humate/saline
D: potassium humate/cyclophos
**
***
A
B
C
D

Figure 5

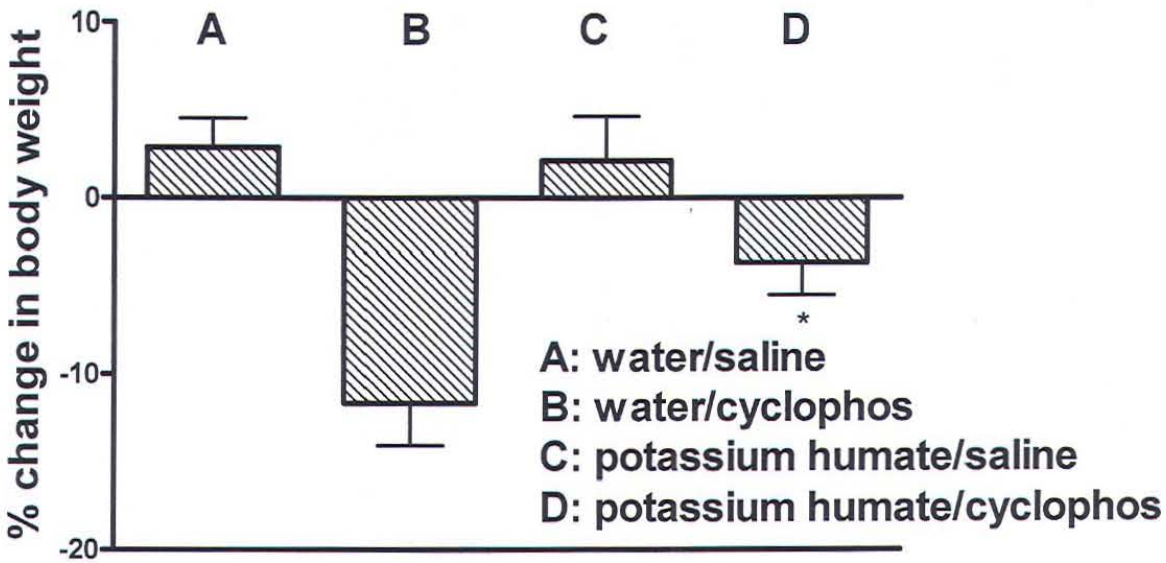
% change in body weight
10
0
-10
-20
A
B
C
D
*
A: water/saline
B: water/cyclophos
C: potassium humate/saline
D: potassium humate/cyclophos

Figure 6