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Anti-inflammatory effects of orally and topically administered nanoformulations of *Malva parviflora* root extracts, and *Prunus persica* and *Cupressus sempervirens* exudates

Poloko Stephen Kheoane [a,*], Kingsley Chimaeze Mbara [c], Mosoatsi Lawrence Mputi [a], Ts'epo Arnold Lenkoe [a], Sebusiswe Magama [b], Mokonyana Mohale [a], Clemence Tarirai [c]

[a] Department of Pharmacy, National University of Lesotho, P.O. Box Roma 180, Lesotho
[b] Department of Biology, National University of Lesotho, Roma 180, Lesotho
[c] Department of Pharmaceutical Sciences, Tshwane University of Technology, Pretoria 0001, South Africa

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ABSTRACT

Background: Medicinal plants have been used traditionally as oral and topical herbs for treating inflammation and alleviating pain. Particularly in traditional Chinese medicine (TCM) practices, many plants from the genera *Malva, Prunus*, and *Cupressus* are used to treat various inflammation-related diseases. This study investigated *in vitro* and *in vivo* anti-inflammatory activity of the root extracts of *Malva parviflora*, the exudates of *Prunus persica, Cupressus sempervirens* and their chitosan nanoparticles and chitosan nanogels.

Methods: In vitro anti-inflammatory activities of *M. parviflora* root extracts, *P. persica* and *C. sempervirens* exudates were investigated using the protein denaturation assay method. A 1% bovine albumin reaction mixture in phosphate buffer and 80% (v/v) methanol was incubated with plant extracts or exudates at 37 °C and 70 °C. Cross-linked chitosan nanoparticles loaded with plant extracts or exudates were prepared by the gelation method. The entrapment efficiency of the plants in the chitosan nanoformulation was estimated using the phenolic content of plant materials. The nanoparticles-based nanogel was formulated by suspending nanoparticles in a gel base. Inflammation was induced in Wistar rats (230 – 270 g) by subcutaneous injection of 0.1 mL of 1% (w/v) carrageenan in the plantar tissue of the right hind paw of the rats. The rats (n = 48) were randomly divided into two experimental groups (A and B) of 24 rats each for oral and topical administration of nanoformulations, respectively. Each group (n = 24) was subdivided into 6 test group (n = 4), where test groups 1, 2, and 3 were treated with 500 mg/kg/BW each of *M. parviflora, C. sempervirens*, and *P. persica* nanoparticle/nanogel, either orally or topically, respectively. Test groups 4, 5, and 6, respectively served as positive control, placebo nanoparticles (*i.e.*, chitosan nanoparticles), and negative control, treated orally or topically with indomethacin (50 mg/kg/BW), chitosan nanoparticle/nanogel alone (500 mg/kg/BW/100 mg/kg/BW), and saline (3 mL).

Results: P. persica exudate had the highest TPC of 70.42 ± 0.53 µg of GAE/mg compared to *M. parviflora* root extract and *C. sempervirens* exudate with the 30.93 ± 1.65 µg of GAE/mg and 9.99 ± 0.65 µg of GAE/mg, respectively. *M. parviflora* root extracts had the highest *in vitro* protein denaturation (92.40%) compared to leaves and stem extracts. *P. persica* and *C. sempervirens* nanoparticles had the highest entrapment efficiencies (99.46% and 99.56%). *M. parviflora* root extract nanoparticles showed the greatest inhibition of oedema (90%) with oral administration, outperforming *P. persica* and *C. sempervirens* exudates nanoparticles (both 87%). *M. parviflora, P. persica and C. sempervirens* nanoparticles and placebos (chitosan) nanoparticles had better overall anti-inflammatory activity than indomethacin after 24 h. For topical applications, *C. sempervirens* exudate nanogel had the highest oedema inhibition, and placebo nanogel was more effective than the other plant nanogels and indomethacin after 24 h.

Conclusion: Formulating medicinal plants in nanoscale dosage forms provides an effective therapy against inflammation. This avenue could counteract the rapidly developing resistance of drugs to diseases and some side

* Corresponding author.
E-mail address: polokokheoane@yahoo.com (P.S. Kheoane).

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effects associated with the administration of multiple allopathic drugs. Further *in vivo* studies using steroidal and non-steroidal anti-inflammatory controls are recommended.

1. Introduction

Inflammation is a complex biological reaction of vascular tissues to damaging stimuli, which is associated with redness, swelling, heat sensation, and pain [1,2]. The release of chemical mediators from injured tissues and migrating cells accelerates inflammation. The movement of leukocytes from the venous systems to the damaged site and the release of cytokines are recognized as pivotal in the inflammatory reaction [3]. Cytokines are responsible for dilating blood capillaries and increasing permeability, thus increasing blood flow to the injured area [4].

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most used over-the-counter (OTC) drugs for inflammation treatment [5]. According to Kuchar et al. [6], these drugs inhibit cyclooxygenase enzymes (COX-1 and COX-2) which are required to convert arachidonic acid into thromboxanes, prostaglandins, and prostacyclins. The therapeutic benefits of NSAIDs stem from the absence of these eicosanoids [7]. More specifically, thromboxanes contribute to platelet adhesion, prostaglandins induce vasodilation, elevate the temperature set-point in the hypothalamus, and participate in anti-nociception [8,9]. Therefore, by inhibiting COX enzymes, NSAIDs effectively reduce swelling, fever, and pain. Furthermore, the long-term use of NSAIDs is associated with numerous side effects, and these include gastric ulceration, nausea, vomiting, and respiratory depression [10]. NSAIDs have many drug interactions which include interaction with anti-coagulants and some anti-epileptic drugs thereby prolonging their effects. For example, aspirin increases the effects of warfarin, phenytoin and valproic acid by displacing them from proteins they are bound to thus increasing the concentration of a free drug [11].

Several plant species have been traditionally used to treat inflammation and alleviate pain [12,13].

The genus *Malva* (Malvaceae) comprises around 25–30 different species, distributed throughout the temperate, subtropical, and tropical regions of China, India, Central Asia, the Mediterranean, South America, America, Europe, and Africa [14]. Species of this genus contain secondary metabolites such as amino acid derivatives/proteins, phenolic compounds, terpenoids, coumarins, vitamins, fatty acids/sterols, leucoanthocyanidins, anthocyanidins, and anthocyanins [15,16]. In TCM, most species of the genus *Malva* are used for the treatment of inflammation, urinary, digestive, and respiratory disorders [17]. *Malva parviflora* L., commonly called Cheeseweed, has known anti-inflammatory properties. The infusion derived from its leaves has been historically used to treat swollen and inflamed purulent wounds [18]. Genus *Prunus* (Rosaceae) includes approximately 400–430 species of shrubs and trees, with widespread distribution spanning across Asian countries, Russia, Europe, North America, and African countries [19]. *Prunus* species are rich sources of different polyphenol compounds, including phenolic acids, flavonols, and anthocyanins [20]. A member of this genus, *P. mume* Sieb. Et Zucc.(improperly called *Fructus mume*) is one of the major components of the Chinese herbal formula Fructus Mume pill (FMP, Chinese: “Wu Mei Wan,”), which was approved for the treatment of gastrointestinal diseases in 2001 by the Chinese medical agency (SFDA) [21]. Additionally, in TCM, *Prunus persica* (L.) Batsch flowers have been historically used to treat blood stasis, constipation, and high blood pressure, and have also been used in cosmetology [22,23]. The genus *Cupressus* L. (Cupressaceae) comprises more than 20 species widely spread in the Mediterranean region, North America, and tropical Asia, including China [24]. Phytochemical screening on the genus *Cupressus* showed that they are rich in flavonoids, terpenes, phenols, and organic acids [25]. In TCM, various species of the genus *Cupressus* are used for treating conditions such as rheumatoid arthritis, pruritus, rheumatism, and pertussis [26]. The exudates of both genus *Prunus* and *Cupressus* have been shown to suppress the production of pro-inflammatory cytokines and upregulate their natural inhibitors [27,28]. This action is crucial in reducing inflammation and is more pronounced in the exudates due to their concentrated bioactive compounds [29], making them more effective in reducing inflammation compared to other plant parts. Although evidence of the direct use of *M. palviflora* and *C. sempervirens* in TCM has not been reported, the anti-inflammatory activities of their exudates [30–32], and the wide range of biological activities reported for other species of this genus, as well as for their constituents piqued our interest to study their potential use in modern Chinese medicine.

Nanoscale drug delivery systems (also called nanocarriers) deliver a drug precisely and safely to its target site at the right time and deliver a controlled release, thereby ensuring the maximum therapeutic effect [33]. Nanocarriers, because of their higher ratio of surface area to volume, show improved pharmacokinetics and biodistribution of therapeutic agents and thus minimize the toxicity of drugs [34]. They improve the solubility of hydrophobic compounds and render them suitable for parenteral administration. Furthermore, they increase the stability of a variety of therapeutic agents, like peptides and oligonucleotides, at the target site [34]. Polymeric nanoparticles, which are among the drug delivery systems, have gained recognition in recent research, owing to their biodegradability and biocompatibility with the human body [35,36].

The current conventional therapies for inflammation, including non-steroidal anti-inflammatory drugs, are taken three times per day, either orally or applied topically [10]. Oral administration is the most common and convenient route for delivering medications, but it is often linked to systemic side effects, particularly gastrointestinal issues, due to the widespread distribution of the drug throughout the body. In contrast, topical delivery aims to localize the action of the drug at the site of inflammation, which can help minimize systemic exposure and its associated side effects. Despite the significant benefits of topical drug delivery, there are still challenges, including variability in pharmacokinetic absorption and barriers to drug permeability. Owing to scientific evidence for the use of medicinal plants both as oral and topical formulations to treat inflammatory diseases [33], there is a need to compare these administration routes and enhance the efficacy and delivery of plant-based therapeutics through nano delivery systems. Therefore, this study investigated *in vitro* and *in vivo* anti-inflammatory activity of the root extracts of *Malva parviflora*, the exudates of *Prunus persica,* and *Cupressus sempervirens* and their chitosan nanoparticles and chitosan nanogels.

2. Materials and methods

2.1. Materials

2.1.1. Plant materials

M. parviflora (roots, stems, leaves) were sourced from Roma Valley, while *P. persica* and *C. sempervirens* exudates were sourced from the National University of Lesotho (NUL) Roma campus. The plants were identified by Mr Moretloa Polaki, a Botanist at the Department of Biology NUL, Roma campus. The *M. parviflora, P. persica* and *C. sempervirens* botanical specimens were assigned voucher specimen numbers RV 0011, RMC 0012 and RMC 0013, respectively, and were deposited at the National University of Lesotho Herbarium (ROML).

2.1.2. Reagents

Chitosan, sodium tripolyphosphate (TPP), hydroxypropyl

methylcellulose (HPMC), carrageenan, phosphate-buffered saline (PBS) at pH 7.4, bovine serum albumin, indomethacin, Folin-Ciocalteu reagent, and gallic acid were purchased from Merck (South Africa). All the reagents used were of analytical grade.

2.1.3. Animals

Wistar rats weighing (250 ± 20) g of either sex were donated by the Department of Biology, NUL. Wistar rats were selected because of their well-documented use in assessing anti-inflammatory activity and their physiological similarity to human inflammatory responses [58]. All experiments were performed in the morning in accordance with current guidelines for the care of laboratory animals and ethical guidelines.

2.2. Methods

2.2.1. Selection of plants

An extensive literature search was performed, and local knowledge was applied to identify medicinal plants traditionally used for treating inflammation. Literature search and indigenous knowledge led to the selection of *M. parviflora*. The *M. parviflora* plant was abundant locally and easily accessible as it was in season. Exudates from *P. persica* and *C. sempervirens* trees at the NUL Roma campus were used for the study.

2.2.2. Preparation of plant extracts

M. parviflora roots, stems, and leaves were individually air-dried at ambient temperature for three weeks. The dried plant material was milled into a fine powder using a kitchen blender (Kenwood 2000, serial 00,045). About 10 g of powdered plant material was extracted in duplicate in 80 % methanol for 2 days with frequent agitation. Plant extracts were filtered through a Whatman No.1 filter paper. The filtrate was concentrated using a rotary evaporator (Model: ROVA-100, MRC Scientific Instruments) at 50 °C. The prepared concentrated extracts of *M. parviflora* were dried at 50 °C for 5 h in the oven (Model: HAS-T25, China), and the extracts were then stored in the fume hood for further drying for 24 h. The dried extracts were stored at 8 °C in air-tight screw-capped glass vials, until used. Dry *P. persica and C. sempervirens* exudates were collected directly from their tree sources and were ground into fine powder using a mortar and pestle. The grounded exudates were mixed in 2 % DMSO to prepare stock solutions and working solutions.

2.2.3. In vitro anti-inflammatory activity of plant extracts and exudates

The *in vitro* anti-inflammatory activity of plant extracts and exudates was determined using the protein denaturation method described by Gunathilake et al. [37] with modifications. Briefly, a 0.2 mL volume of 1 % bovine albumin was mixed with a 4.78 mL volume of phosphate-buffered saline (pH 6.4), and a 0.02 mL volume of plant extract or exudate in an Eppendorf tube. The plant mixture was agitated vigorously using a vortex mixer (Model: MX-S, BioBase), and the mixture was incubated in a water bath at 37 °C for 15 min. The reaction mixture was further heated at 70 °C for 5 min in the water bath and then cooled to room temperature. After cooling, the turbidity of the mixture was measured at 660 nm using a UV/VIS spectrometer (Model: OPTIZEN POP, Apex Scientific). The 80 % v/v methanol solution was prepared in phosphate buffer and used as the control.

The absorbance of the plant extracts, exudates, and controls was mathematically used to predict the percentage inhibition of protein denaturation. The percentage inhibition of protein denaturation of plant extracts and exudates was calculated using Eq. (1):

$$\%\ inhibition\ of\ protein\ denaturation = \frac{1 - A_2}{A_1} \times 100 \quad (1)$$

Where A_1 = absorbance of the control sample, and A_2 = absorbance of the test sample.

2.2.4. Determination of total phenolic content of plant extracts

The determination of the total phenolic content (TPC) of plant extracts and exudates was additionally performed to enable the characterization of the chitosan nanoparticles (*i.e.*, the entrapment efficiency). The TPC of plant extracts and exudates was determined using the Folin-Ciocalteu reagent method described by [38]. Gallic acid was used as a reference standard (2–100 μg/mL) for plotting the calibration curve. A volume of 0.5 mL of plant extract was mixed with a 1.5 mL volume of Folin-Ciocalteu reagent (diluted 1:10 with distilled water) and was neutralized with a 3 mL volume of sodium carbonate solution (7.5 %, w/v). The reaction mixture was kept in the dark at room temperature for 30 min with intermittent shaking for colour development. The absorbance of the resultant blue colour was measured by using a double-beam UV–Vis spectrophotometer at 765 nm wavelength. The TPCs were determined using linear regression equation obtained from the standard plot of gallic acid. The total phenolic content of samples was calculated as mean and expressed as mg of gallic acid equivalent (GAE)/g of dry extract.

2.2.5. Formulation and characterization of cross-linked chitosan nanoparticles

Chitosan nanoparticles were prepared by the ionic gelation method described by Yanat and Schroën [39]. The mechanism of this method involves crosslinking the protonated amino groups of chitosan and negatively charged groups of the polyanion such as sodium tripolyphosphate (TPP). A 500 mg of chitosan was dissolved in a 7.5 mL volume of 1 % acetic acid to produce chitosan solution. The TPP aqueous solution was prepared by mixing a 250 mg weight of TPP powder in a 10 mL volume of distilled water. A 2.5 mL volume of *M. parviflora* root extract or *P. persica and C. sempervirens* exudates (2.5 mg/mL) was mixed with a chitosan solution, then a 14 mL TPP solution was added drop-wise to the mixture while stirring vigorously using a magnetic stirrer. The mixture was stirred until it turned milky and was stored at 2 °C for 30 min. The mixture was sonicated on ice for 10 min at 20 % amplitude using the sonicator (Model: Q500, USA) to obtain a homogenous chitosan nanoparticle suspension. Each of the samples were prepared in three replicates.

2.2.5.1. Determination of particle size and zeta potential. The particle size and zeta potential (surface charge) of nanoparticles were measured using Zetasizer Nano ZS-90 (Malvern Instruments, Worcestershire, UK) [59]. All measurements were measured in triplicate at 25 °C and an angle of detection of 90 °C.

2.2.5.2. Determination of entrapment efficiency of chitosan nanoparticles. The entrapment efficiency of chitosan nanoparticles was determined according to the procedure described by Kheoane et al. [40]. The chitosan nanoparticles suspension was centrifuged (Model: CFGR-B18B, China) at 18,500 rpm at −4 °C for 30 min. The supernatant was collected and labelled 'first wash' while the pellet was resuspended in fresh PBS. The centrifugation of the resuspended pellet was repeated to obtain the 'second wash' of chitosan nanoparticles. The phenolic content of the washes was determined using the previously described Folin-Ciocalteu reagent procedure to estimate the amount of plant sample entrapped in nanoparticles. The entrapment efficiency was calculated using the following formula:

$$\text{Entrapment efficiency } (\%) = \frac{TPC - SPC}{TPC} \text{ X } 100 \quad (2)$$

where TPC = total phenolic content and SPC = surface phenolic content.

2.2.6. Formulation and characterization of nanogel

The formulation of nanogel was achieved by polymerization process whereby chitosan nanoparticles were entrapped in the hydroxylpropylmethyl cellulose polymeric gel base [41,42]. A 300 mg amount of hydroxylpropylmethylcellulose was mixed with a 20 mL volume of PBS pH 7.4 using a magnetic stirrer to form a nanogel base. The chitosan

nanoparticles were then added to the nanogel base and the mixture was homogenised by mixing with a magnetic stirrer. The uniformity of dose of the dosage form was achieved by controlling the viscosity of the gel base using PBS and magnetic stirring.

The initial measurement of the diameter of the inflamed areas (the right paw) and the follow-up measurements were taken to observe any changes in dimensions. The percentage inhibition of oedema was calculated using Eq. (3):

$$\%\ inhibition\ of\ oedema = \frac{Mean\ oedema\ increase\ in\ control\ sample - Mean\ oedema\ increase\ in\ test\ sample}{Mean\ oedema\ increase\ in\ control\ sample}\ x\ 100 \quad (3)$$

2.2.7. Animal studies

Wistar rats weighing 250 ± 20 g of either sex were donated by the Department of Biology, NUL. All the experiments were performed in the morning in accordance with current guidelines for the care of laboratory animals and ethical guidelines. Animals (n = 48) were randomly allocated to various treatment groups to ensure scientific reliability and placed in a clean polyacrylic cage. They were maintained under suitable laboratory conditions (temperature 25 ± 2 °C, relative humidity 50 ± 5 %, and exposure to 12-h light and dark cycle). Animals were allowed free access to the standard commercial chow and water *ad libitum* for the duration of the study. Dosing and outcome assessments were performed in a blinded manner to reduce bias. Each group included at least four animals to maintain statistical validity. Experiments were independently repeated to verify reproducibility. Differences between groups were analysed using appropriate statistical tests.

2.2.8. Ethical considerations

The research design and protocol were reviewed and approved by the Ministry of Health, Lesotho, and the Institutional Review Board on Research of the National University of Lesotho (ID150–2023 NUL/PHA/2023/5). Animals were treated with humane care in accordance with the principles outlined in the World Medical Association (WMA) statement on animal use in biomedical research (https://www.wma.net/policies-post/wma-statement-on-animal-use-in-biomedical-research/), as well as the European Union recommendations (Directive 2010/63/EU) for the protection of animals used for scientific purposes [43].

2.2.9. In vivo anti-inflammatory activity of plant extract and exudates

Oedema in the right hind paw of the rat was induced by an injection of 0.1 mL of 1 % (w/v) of carrageenan (Sigma-Aldrich; St Louis, USA) in 0.9 % normal saline subcutaneously in the plantar side of the right hind paw of the rat [44]. The paw diameter was measured before the carrageenan injection and hourly for 5 times, then after 24 and 48 h following treatment with plant nano-formulations. The rats (n = 48) were randomly divided into two experimental groups (A and B) of 24 rats each for oral and topical administration of nanoformulations, respectively). Each group consists of 6 test groups (n = 4) [63–65]. Test groups 1, 2, and 3 were treated with 500 mg/kg/BW each of *M. parviflora, C. sempervirens,* and *P. persica* nanoparticles/nanogel, either orally or topically, respectively. Rats in test groups 4 and 6 served as positive control and negative control and were orally or topically treated daily with 50 mg/kg/BW of indomethacin, and 3.0 mL of saline, respectively. Indomethacin was chosen as the standard positive control because it is a well-known NSAID with potent and well-documented effects in carrageenan-induced paw oedema models. It serves as a benchmark for evaluating the anti-inflammatory effectiveness of the plant-extract/exudate-loaded chitosan nanoparticles. Test group 5 served as placebo and was orally treated with 500 mg/kg/BW chitosan nanoparticles alone or topically treated with 100 mg of nanogel alone. The dose of the herbal nano-formulations was chosen in accordance with a previous study [45,46]. Animals were pretreated with formulations through gastric gavage or application directly to the paw 1 h before the administration of carrageenan.

2.2.10. Statistical analysis

Data obtained from this study were analyzed using Microsoft Excel 2016. The mean and standard deviation were calculated for paw diameter, and the percentage inhibition of oedema following administration of samples was calculated for all the animal groups. The means of the groups were compared using the analysis of variance (ANOVA) followed by a least significant difference (LSD) post hoc analysis. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Phenolic content

P. persica exudate had the highest TPC of 70.42 ± 0.53 μg of GAE/mg (Table 1) compared to *M. parviflora* root extract, leave extract, stems extract and *C. sempervirens* exudate with the 30.93 ± 1.65 μg of GAE/mg, 29.87 ± 1.15 μg of GAE/mg, 30.43 ± 1.55 μg of GAE/mg and 9.99 ± 0.65 μg of GAE/mg, respectively. The obtained TPC results on both *M. parviflora* root extract and *C. sempervirens* exudate contradict the results for protein denaturation. The TPC of the plant samples was expected to directly correlate with the protein denaturation or anti-inflammatory activity because phenols are natural antioxidants. However, the observed high protein denaturation of *M. parviflora* root extract and *C. sempervirens* exudate could therefore be attributed to other antioxidants (*e.g.*, ascorbic acid and vitamin E) contained in these plant samples.

3.2. In vitro anti-inflammatory activity of plant extract and exudates

The *M. parviflora* roots exhibited higher inhibition of protein denaturation of 92.40 ± 1.08 % (Table 1), which is in line with several scientific reports on the traditional use of this plant [47]. This observed protein denaturation directly translates to the anti-inflammatory activity of this plant. Methanolic extracts of *M. parviflora* roots could be formulated into conventional dosage forms as a potential homeopathic medicine to optimize their administration and also for improved patient

Table 1
Total phenolic contents and protein denaturation inhibition of plant extracts and exudates.

Plant sample	Total phenolic content (μg GAE/mg)	Inhibition of protein denaturation (%)
M. parviflora (roots)	30.93 ± 1.65	92.40 ± 1.08
M. parviflora (leaves)	29.87 ± 1.15	91.37 ± 2.12
M. parviflora (stems)	30.43 ± 1.55	92.07 ± 1.80
P. persica exudate	70.42 ± 0.53	91.23 ± 1.63
C. sempervirens exudate	9.99 ± 0.65	91.57 ± 1.96

Total phenolic content: μg of GAE/mg of dry plant extract; Values = Mean ± SD, n = 4); GAE = Gallic acid equivalent.

compliance in the modern lifestyle. The increasing emergence of new disease burdens that pose inflammation to the human body and the side effects associated with the co-administration of multiple conventional drugs qualify novel dosage forms based on *M. parviflora*-based novel dosage forms as an alternative therapy. Similarly, both *P. persica and C. sempervirens* exudates just like *M. parviflora* showed high inhibition of protein denaturation of 91.23 ± 1.63 % and 91.57 ± 1.96 % respectively, suggesting their anti-inflammatory activities, which have not been previously reported in the literature.

3.3. Formulation and characterization of crossed-linked chitosan nanoparticles

3.3.1. Particle size and zeta potential

The particle size ranged from 620.4 ± 2.7 nm to 632.3 ± 2.0 nm while the zeta potential ranged from 25.9 ± 3.0 mV to 30.3 ± 0.5 mV (Table 2). This particle size falls within the nano-scale, thereby improving drug/or phytochemical loading in terms of the surface area available for loading of compounds. On the other hand, the surface charge of these particles is cationic and, therefore, creates some repulsive forces between nanoparticles. The repulsive forces between individual particles will enable their distribution in the aqueous medium and thus the uniformity of the dose of the dosage form [42].

3.3.2. Entrapment efficiency of nanoparticles

The entrapment efficiency of nanoparticles was estimated by quantifying the TPC of the nanoparticles washes following the centrifugal washing. Both *P. persica* and *C. sempervirens* exudates had the highest entrapment efficiency of 99.46 % and 99.56 % (Table 2) while *M. parviflora* entrapment efficiency was only 77.12 %. Chitosan with a high molecular weight is insoluble in either water or organic solvent, but only in a weakly acidic aqueous media [44]. Compounds from *M. parviflora* were extracted in 80 % v/v methanol implying that they were slightly more lipophilic in nature than being hydrophilic hence the observed lower entrapment efficiency compared to exudates. Both exudates on the other hand were waxy in nature and were only soluble in 2 % DMSO-PBS solution. The nature of the exudates and their overall composition could be favoured by the chitosan biopolymer and the crosslinking with TPP could have increased the total surface for binding.

3.4. In vivo anti-inflammatory activity of orally administered nanoparticles

The *in vivo* anti-inflammatory activities of *C. sempervirens* and *P. persica* exudates nanoparticles were higher than that of *M. parviflora* extract nanoparticles, the positive control indomethacin, as well as the placebo nanoparticles (Fig. 1). *C. sempervirens* exudate nanoparticles reduced paw swelling by 70.05 % at 1 hour after induction of inflammation, 97.67 % at 5 h, and 95.34 % after 24 h. *P. persica* exudate nanoparticles on the other hand, reduced the swelling by 62.60 % at 1 hour after induction to 97.93 % at 5 h and by 98.52 % after 24 h after inflammation induction. *C. sempervirens* offered the best anti-inflammation results at times promptly after administration indicating high efficacy as compared to other formulations. Interestingly, all the nano-formulations showed better inhibition of oedema than the positive control indomethacin which was administered as a free drug suggesting the anti-inflammatory properties of chitosan itself. The differences observed between *C. sempervirens* and *P. persica* exudates nanoparticles treatments were statistically significant ($p = 0.002$ and $p = 0.006$, respectively). Both *M. parviflora* root extract nanoparticles, and *C. sempervirens* and *P. persica* exudates nanoparticles exhibited high anti-inflammatory properties *in vivo*. However, further research focusing on the isolation, purification, and identification of specific compounds within these plant extracts and exudates may lead to the discovery of new lead molecules for the treatment and/or prevention of inflammation. Additionally, the observation that placebos demonstrated higher anti-inflammatory activity than the positive control, indomethacin, suggests the potential of chitosan nanoscale structures to serve as an anti-inflammation treatment on their own.

Table 2
Particle size and zeta potential of plant extract-loaded chitosan nanoparticles.

Nanoparticles	Particle size (nm)	Zeta potential (mV)	Entrapment efficiency (%)
M. parviflora NPs	620.4 ± 2.7	25.9 ± 3.0	77.12 ± 2.10
P. persica NPs	628.6 ± 1.8	29.0 ± 1.4	99.46 ± 4.11
C. sempervirens NPs	632.3 ± 2.0	30.3 ± 0.5	99.56 ± 3.43

Values = Mean ± SD, $n = 4$; NPs = Nanoparticles.

3.5. In vivo anti-inflammatory activity of topically administered nanogel

Topically administered samples of *C. sempervirens* exudate nanogel showed significant results ($p < 0.05$) compared to *M. parviflora* root extract nanogel and *P. persica* exudate nanogel over a 24-hour dosing period (Fig. 2). *C. sempervirens* exudate nanogel achieved an overall oedema reduction of 91.74 % after 24 h post-inflammation induction, whereas *M. parviflora* root extract nanogel and *P. persica* exudate nanogel inhibited oedema by 69.70 % and 69.49 %, respectively. The differences in anti-inflammatory activity of *C. sempervirens* formulations were statistically significant ($p = 0.01$). Surprisingly, the placebo produced better topical results than both *M. parviflora* root extract nanogel and *P. persica* exudate nanogel after 24 h following oedema induction. The observed effect could be attributed solely to the release kinetics of the plant-loaded nanoparticles, the physical interaction between phytochemicals in the plant material and the chitosan nanocarriers, and/or the route of administration itself.

4. Discussion

Plant materials have been used by man from time immemorial to successfully cure different ailments associated with inflammation. Studies have shown the anti-inflammatory activities and therapeutic benefits of members of the genus *Malva, Prunus*, and *Cupressus* in TCM [17,22,23,26]. However, the traditional claims of these plants as anti-inflammatory agents have not been substantially evaluated using modern scientific techniques.

The anti-inflammatory property of the plants was assessed by protein (albumin) denaturation bioassays. Tissue protein denaturation, which predisposes tissues to injury is a characteristic feature of inflammatory responses [48]. The *M. parviflora* parts (roots), *P. persica,* and *C. sempervirens* exudate exhibited higher inhibition of protein denaturation of 92.40 %, 91.23 %, and 91.57 % respectively (Table 1), suggesting that these plants could probably contain metabolites that preserve protein structures in tissues against inflammatory mediators such as heat, chemicals, strong acids or bases. Denaturation of tissue proteins is implicated in several inflammatory disease conditions such as rheumatoid arthritis, serum sickness, glomerulonephritis, and systemic lupus [49]. Therefore, the inhibition of protein denaturation by extracts from various parts of these medicinal plants can be used to indicate their anti-inflammatory potential.

P. persica exudate had the highest TPC of 70.42 ± 0.53 μg of GAE/mg (Table 2) compared to *M. parviflora* root extract and *C. sempervirens* exudate with the 30.93 ± 1.65 μg of GAE/mg and 9.99 ± 0.65 μg of GAE/mg, respectively. The obtained TPC results on both *M. parviflora* root extract and *C. sempervirens* exudate contradict the results for protein denaturation. The TPC of the plant samples was expected to directly correlate with the protein denaturation or anti-inflammatory activity because phenols are natural antioxidants. However, the observed high protein denaturation of *M. parviflora* root extract and *C. sempervirens* exudate could therefore be attributed to other bioactive compounds

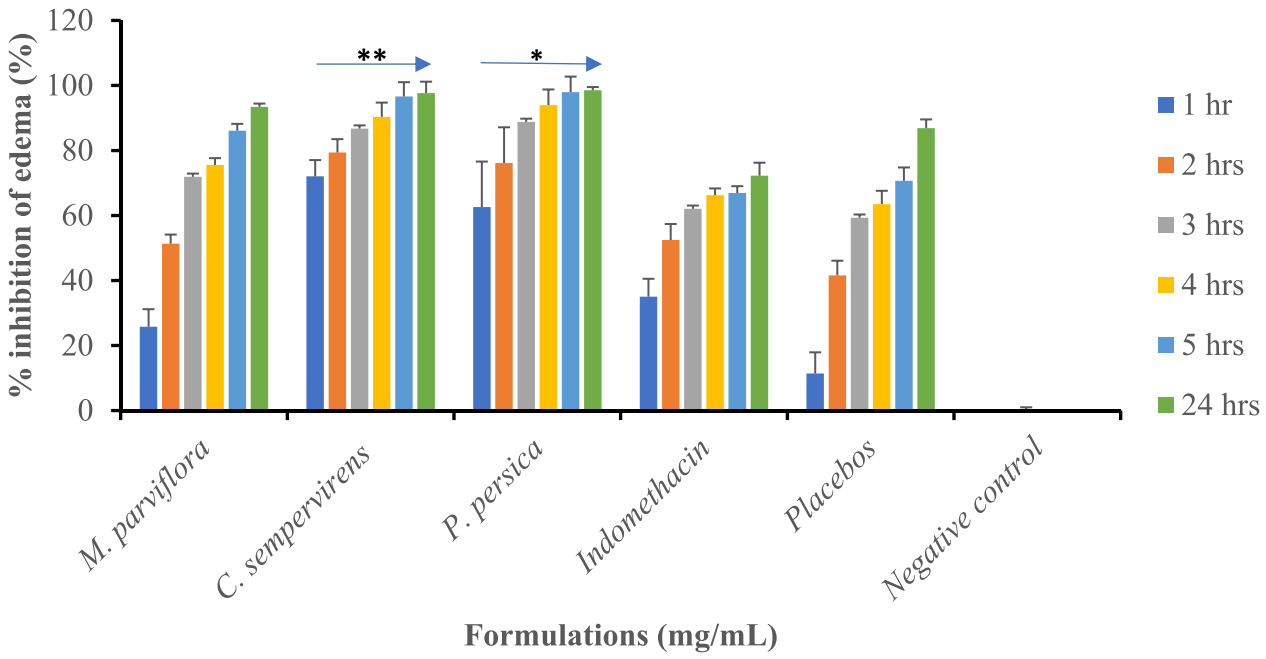

Fig. 1. Inhibition of rat paw oedema following oral treatment with plant extracts and exudates.
** = Statistically significant values ($p = 0.002$); * = statistically significant ($p = 006$); (Values = Mean ± SD, $n = 4$).

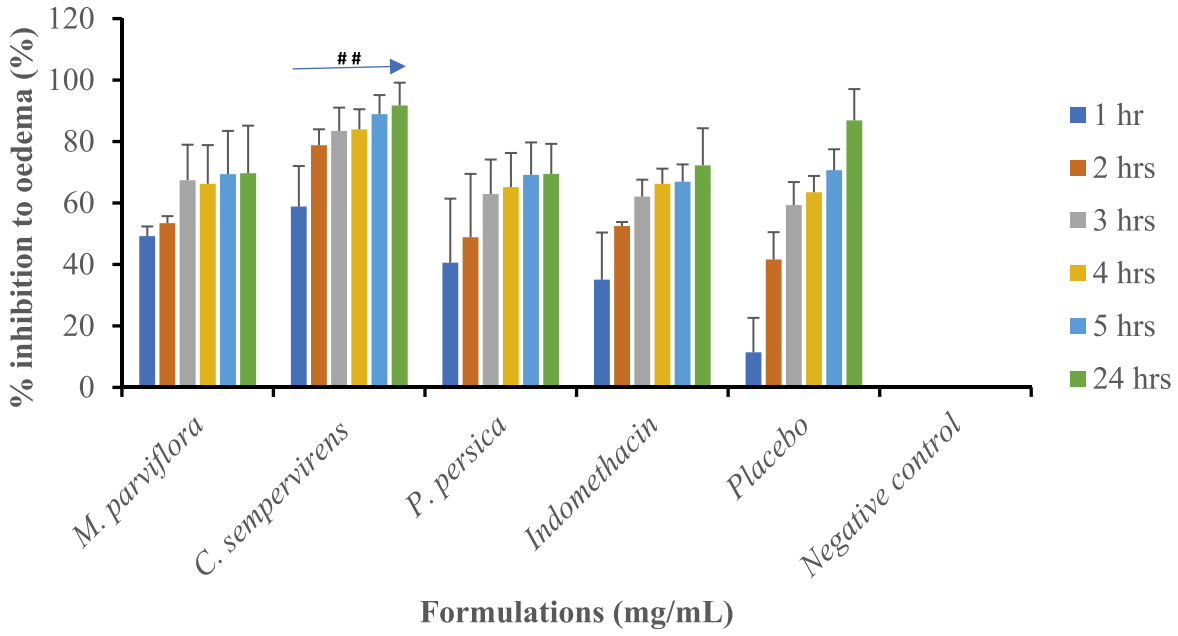

Fig. 2. Inhibition of rat paw oedema following topical treatment with plant extracts and exudates.
= Statistically significant values ($p = 0.01$); (Values = Mean ± SD, $n = 4$).

contained in these plant samples, as the anti-inflammatory efficacy of a plant extract is not solely dependent on its phenolic content. Compounds such as alkaloids, flavonoids, tannins, and glycosides can also play crucial roles in inhibiting protein denaturation and thereby increasing anti-inflammatory effects. The study on *Barringtonia racemosa* L. showed that even with low phenolic content, the extracts exhibited significant anti-inflammatory activity [48], suggesting the involvement of other compounds that could act on different molecular targets involved in the inflammatory process, such as inflammatory transcription factors, cytokines, and enzymes, including cyclooxygenase and lipoxygenase [50].

Moreover, apart from inhibition of protein denaturation, other mechanisms such as the stabilization of cell membranes, modulating immune cell activity, and the scavenging of free radicals, also play crucial roles in the anti-inflammatory effects [50]. Previous study has reported that the methanolic extract of *Melipona beecheii* honey interacted with cell membranes, to prevent damage and stabilize the cell membrane structure regardless of the phenolic content [51]. *Quercus* robur extracts have previously been shown to inhibit macrophage polarization toward M1-phenotype, thereby potentially shifting them towards a more anti-inflammatory M2-phenotype [52]. These alternative

mechanisms highlight the complexity of plant-based anti-inflammatory activity and the potential for multiple pathways to contribute to the overall effect.

Chitosan gained popularity in biomedical and/or pharmaceutical applications because of its versatile properties, such as availability, affordability, biocompatibility, antibacterial activity, anti-inflammatory activity and biodegradability [60]. Chitosan nanoparticles are synthesized through the ionic gelation process, whereby the cationic ammonium groups from chitosan interact with the anionic phosphate groups provided by tripolyphosphate (TPP). The number of unneutralized ammonium groups present in the chitosan matrix determines the surface charge of these nanoparticles. Given that the number of positive charge sites on chitosan exceeds that of the negative charges from TPP, the resultant surface charge of chitosan nanoparticles is cationic. This interaction between the positive charges of chitosan and the negative charges of TPP promotes proper crosslinking of chitosan nanoparticles, hence improved encapsulation of the plant extracts and exudates. The high encapsulation efficiency of plant extracts and exudates was achieved by optimizing the chitosan-TPP mass ratio (*e.g.* 2:1) and adjusting the pH to 4.6. Furthermore, the use of methanol-extracted plant extract and non-polar plant exudates minimized degradation and improved interaction with chitosan matrix. The particle size and zeta potential of plant extract-loaded chitosan nanoparticles were evaluated by zeta sizer analysis, as shown in Table 2. The particle size ranged from 620.4 ± 2.7 nm to 632.3 ± 2.0 nm, while the zeta potential ranged from 25.9 ± 3.0 mV to 30.3 ± 0.5 mV (Table 2). This indicates that the particle size falls within the upper limit of nano-scale range (ie., 1000 nm). Although the synthesized nanoparticles had sizes ranging from 620.4 ± 2.7 nm to 632.3 ± 2.0 nm, which are slightly above the preferred nano-range (usually < 200 nm), they still showed significant anti-inflammatory activity. This indicates that despite their larger size, the nanoparticles maintained beneficial surface properties, such as a positive zeta potential (25.9–30.3 mV), which may have improved their interaction with inflamed tissues and their retention at the inflammation site. However, larger particles might also influence the rate of cellular uptake and diffusion through biological barriers, especially for topical delivery. Future research will focus on adjusting synthesis parameters, such as chitosan concentration, TPP ratio, pH, and stirring speed, to reduce particle sizes below 300 nm, which is suitable for improved tissue penetration and systemic distribution, without sacrificing entrapment efficiency or stability [61,62]. The higher the positive or negative values of zeta potential, the stronger the surface electrical charge becomes, which reflects the magnitude of surface electrical charges on nanoparticles (NPs), influencing their electrostatic interactions and, consequently, their physical stability. In general, zeta potential values > +30 mV are indicative of high stability, while values near +20 mV suggest only short-term stability. In contrast, zeta potential values < +5 mV are associated with low stability, leading to rapid aggregation of nanoparticles [53].

The entrapment efficiency of nanoparticles was estimated by quantifying the TPC of the nanoparticles washes following the centrifugal washing. Table 2 presents the data from samples encapsulated into chitosan nanoparticles. Both *P. persica* and *C. sempervirens* exudates nanoparticles had the highest entrapment efficiency of 99.46 % and 99.56 % (Table 2), while *M. parviflora* root extract nanoparticle entrapment efficiency was only 77.12 %. Chitosan with a high molecular weight is insoluble in either water or organic solvent, but only in a weakly acidic aqueous medium [44]. Compounds from *M. parviflora* were extracted in 80 % v/v methanol, implying that they were slightly more lipophilic in nature than being hydrophilic, hence the observed lower entrapment efficiency as compared to exudates. Both exudates on the other hand were waxy in nature and were only soluble in 2 % DMSO-PBS solution. The nature of the exudates and their overall composition could be favored by the chitosan biopolymer and the crosslinking with TPP could have increased the total surface for binding.

In this study, the efficacy of *M. parviflora, P. persica*, and *C. sempervirens* as anti-inflammatory agents in the carrageenan-induced paw edema model was evaluated. The assessment of the *in vivo* anti-inflammatory activity of the selected medicinal plants using a 500 mg/kg dose in the carrageenan-induced rat paw-edema model showed significantly high anti-inflammatory activity during the period from the 1st - 24 h. This suggests that extracts of both *M. parviflora* and *P. persica*, and *C. sempervirens* plant exudates could abrogate carrageenan-induced inflammatory response that inhibits histamine, serotonin, bradykinin, PMN leukocyte infiltration, and the prostaglandin generation *in vivo* [54]. Interestingly, the anti-inflammatory activity of all the nano-formulations increased up to 24 h and showed better inhibition of oedema than the positive control indomethacin which was administered as a free drug. Further research focusing on the isolation, purification, and identification of specific compounds within these exudates may lead to the discovery of new lead molecules for the treatment and/or prevention of inflammation. Additionally, the observation that placebos demonstrated higher anti-inflammatory activity than the positive control, indomethacin, suggests the potential of nanoscale structures to serve as a treatment on their own.

Topically administered samples of the selected medicinal plants using 100 mg of the nanogels showed significant anti-inflammatory activity against carrageenan-induced oedema paw in rats over a 24-hour dosing period (Fig. 2). Surprisingly, while *C. sempervirens* showed the highest anti-inflammatory activity, the placebo produced better topical results than both *M. parviflora* and *P. persica* after 24 h following oedema induction. The observed effect could be attributed to the release kinetics of the plant-loaded nanoparticles, the physical interaction between phytochemicals in the plant material and the chitosan nanocarriers, and/or the route of administration itself. Moreover, chitosan nanoformulations may exert their anti-inflammatory effects through mechanisms beyond protein denaturation and edema inhibition. Previous studies have shown that these formulations can modulate the expression and release of key pro-inflammatory and anti-inflammatory cytokines, such as IL-6 and IL-10, thereby influencing the overall inflammatory response [55]. Furthermore, chitosan nanoformulations target various pathophysiological mechanisms of inflammation, including proinflammatory mediators, transcription factors, and enzymes such as COX (both COX-1 and COX-2), which play essential roles in arachidonic acid metabolism and prostaglandin synthesis, leading to decreased inflammation [56,57].

5. Limitations

This study used *in vitro* and *in vivo* models of inflammation to investigate the possible therapeutic effects of phytochemical nano-formulations of *M. parviflora, P. persica,* and *C. sempervirens*. However, it also suffers from potential limitations. From the findings of this study, it could be suggested that the improved anti-inflammatory activities of the nano-formulations of *M. parviflora, P. persica,* and *C. sempervirens* could be due to their phytochemical composition. Moreover, biosafety of the prepared plants-nanoparticles is another area of concern. Although medicinal plants and nano-delivery systems are generally considered to be safe, they are often associated with potentially harmful effects. Therefore, there is a need for the toxicological evaluation of these herbal-based nano-formulations and to determine the phytoconstituent (s) responsible for their anti-inflammatory effects and the underlying molecular mechanisms for their beneficial effects. A limitation to the generalizability of the study is that it did not consider gender or sex issues.

6. Conclusion

Chitosan-based polymeric nanoparticles possess inherent anti-inflammatory activity and enhance the anti-inflammatory effects of *M. parviflora, P. persica* and *C. sempervirens*. Therefore, herbal-based polymeric nanoparticles could offer an alternative sustained-release

treatment for inflammation, which could be safer due to the biodegradable nature of the chitosan polymer. Formulating medicinal plants in nanoscale dosage forms could also counteract the rapidly developing resistance of drugs to diseases and some side effects associated with the administration of multiple allopathic drugs. Although indomethacin was used as the standard positive control in this study, future *in vivo* studies could include other anti-inflammatory agents like diclofenac (a rapid-acting NSAID), dexamethasone (a corticosteroid), or celecoxib (a selective COX-2 NSAID). This would allow for exploring different anti-inflammatory mechanisms and assessing the comparative effectiveness of the plant-loaded nanoparticles.

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CRediT authorship contribution statement

Poloko Stephen Kheoane: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kingsley Chimaeze Mbara:** Writing – review & editing, Validation, Project administration, Data curation. **Mosoatsi Lawrence Mputi:** Investigation, Methodology, Writing – original draft, Resources. **Ts'epo Arnold Lenkoe:** Writing – original draft, Resources, Methodology, Investigation. **Sebusiswe Magama:** Supervision, Resources, Methodology. **Mokonyana Mohale:** Methodology, Investigation. **Clemence Tarirai:** Writing – review & editing, Validation, Supervision.

Declaration of competing interest

The authors hereby declare that they have no conflicts of interest.

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