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In Vivo Anti-Inflammatory Assessment of a Topical Formulation Containing *Ehretia Cymosa* Extract Mediated-Silver Nanoparticles

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Silver nanoparticles (SNP) are the most preferred and most often used metallic nanoparticles in biomedical studies. However, there are only few studies on its application in topical drug delivery design.
Objectives: This study was carried out to design topical ointments containing n-hexane and methanol *Ehretia cymosa* leaf extracts mediated-silver nanoparticles for the treatment of inflammation.
Methods: Silver nitrate was reacted with n-hexane and methanol extracts of *Ehretia cymosa* leaf to synthesize SNP used in the formulation of an ointment. The SNP was characterized by visual observation, UV–visible spectroscopy, atomic absorption spectroscopy and FTIR spectroscopy. The physical characteristics of the ointment and spreadability were evaluated. Inflammation was inflicted by carrageenan-induced paw acute edema method in albino rats. The linear paw circumference was measured hourly after application of the ointment.
Results There was colour change as the synthesis progresses. The absorption peak of n-hexane SNP (N-SNP) and methanol SNP (M-SNP) was 450 nm and 430 nm respectively. The ointments were easy to administer with satisfactory spreadability but difficult to wash off. Ointments containing SNP had significantly higher activity ($p < 0.05$) than the crude extract and ointments containing M-SNP had significantly higher activity ($p < 0.05$) than ointments containing N-SNP.
Conclusion: The anti-inflammatory activity of ointment containing SNP synthesized with methanol extract is significantly higher compared to ointment formulations containing silver nanoparticle synthesized with n-hexane extract and the reference drug (diclofenac).
Keywords: *Ehretia cymosa extracts*, silver nanoparticle, anti-inflammatory activity, ointment formulation

INTRODUCTION

Metallic Nanoparticles (NPs) are nanosized metals with dimensions ranging from 1 – 100nm. They can be synthesized by different methods like sol-gel (Owens *et al.*, 2016), polyol (Dhand *et al.*, 2015), hydrothermal synthesis *(*Jaggessar and Yarlagadda, 2020), microemulsion (Malik *et al.*, 2012), and green synthesis method (Shah *et al.*, 2015; Dhuper *et al.*, 2012). Green synthesized metallic NPs are synthesized from microbes and plants extracts by reacting with inorganic metals like silver, gold, copper, zinc and palladium to form AgNPs (Odeniyi *et al.*, 2020; Masum *et al.*, 2019), AuNPs (Ahmed *et*

al., 2018), CuONPs (Rajesh et al 2018), ZnONPs (Hossain *et al.,* 2019) and PdNPs (Yaqoob *et al.,* 2020) respectively. The green synthesis method is the most widely reported and the most popular because it is biologically safe, stable, eco-friendly and cost-effective (Zhang *et al.,* 2020a). However, nanoparticles synthesized from plants extract are more popular and widely reported than those synthesized from microbes because plant extracts can be used in a simple manner to rapidly synthesize NPs (Ahmed *et al.,* 2017).

Silver NPs (SNPs) are the most preferred and most often used metallic NPs in biomedical because of its strong plasmon resonance thus making it to possess properties such as good conductivity, chemical stability and catalytic activity (Solati and Dorranian, 2015).

There are various reports of SNP possessing anti-inflammatory activity (Kedi *et al.,* 2020; Zhang *et al.,* 2020b; Sharma *et al.,* 2018), but there is little or no report of its application in topical drug delivery designs

So, this study was carried out to design topical ointments containing n-hexane and methanol

METHODOLOGY

Preparation of leaf extract

The leaves of *Ehretia cymosa* were collected in May 2019 from a local district in Ibadan, Oyo State, Nigeria. They were dried at room temperature for 14 days and milled into powder with a blender (Model 857 Williamette Industries, Kentucky USA). Powdered leaves (400g) was macerated separately in n-hexane and methanol for 72 hours with intermittent shaking.

Synthesis of silver nanoparticles

The SNP was synthesized by method described by Odeniyi *et al.,* 2020. Forty milliliters (40 mL) of extract was mixed with 100 mL freshly prepared 1 mM silver nitrate in 250 mL Erlenmeyer flasks. The mixture was kept at room temperature in a dark cupboard for 72 hours after which the mixture was centrifuged at 4000rpm for 15 minutes and the pellets obtained were dried.

Characterization of the synthesized silver nanoparticles

Visual observation

Color change was observed visually every 24 h as the reaction of the mixture of the plant extract and the AgNO3 proceeded for 72 h.

Ehretia cymosa leaf (a shrub, belonging to the family Boraginaceae – Fig. 1) extracts mediated-silver nanoparticles.

Figure 1*: Ehretia cymosa* leaf

UV–Visible spectroscopy

The absorption spectra of the reaction mixture at 24 h interval were measured at room temperature in the wavelength range of 300 -700nm using a UV-Visible Spectrophotometer (T90 + spectrophotometer, PG Instruments Ltd, Leicester, UK)

Atomic Absorption Spectroscopy (AAS)

Atomic Absorption Spectroscopy of the synthesized SNP was done by AAS Model 200 series Buck Scientific, USA to analyze the conversion of silver nitrate into silver nanoparticles at different reaction time intervals.

Fourier Transform Infrared (FTIR) spectroscopy

FTIR analysis of the synthesized SNP was carried out by the KBr method with PerkinElmer Spectrum spectrometer version 10.03.02 in the range of 400 - 4000 cm^{-1} at a resolution of 1- 4 cm^{-1}.

Scanning electron microscopy (SEM)

The size and morphology of the SNP was analyzed by SEM (JSM 6100, JOEL, Tokyo, Japan). A prepared film of the SNP was placed onto a carbon-

coated copper grid which was allowed to dry before measurement.

Ointment formulation

The ointment was formulated by triturating different concentrations of the crude extract and the SNP in a simple ointment BP base as shown in Table 1. Simple ointment was prepared by weighing 5 g each of wool fat, hard paraffin and cetostearyl alcohol into an evaporating dish then followed by the required quantity of white soft paraffin. The content of the evaporating dish was melted altogether at 70^{O}C with continuous stirring. The required concentration of the crude extract or SNP was then incorporated in the ointment base and transferred into a wide mouthed dispensing bottle where it was allowed to cool.

Table 1: Ointment formulation formula

Formulation code	Crude Extract (g)	SNP (g)	Wool fat (g)	Hard paraffin (g)	Cetostearyl alcohol (g)	White soft paraffin (g)
OB	-	-	5.0	5.0	5.0	85.0
NC1	1.0	-	5.0	5.0	5.0	84.0
NC2	2.0	-	5.0	5.0	5.0	83.0
NC3	3.0	-	5.0	5.0	5.0	82.0
NS1	-	1.0	5.0	5.0	5.0	84.0
NS2	-	2.0	5.0	5.0	5.0	83.0
NS3	-	3.0	5.0	5.0	5.0	82.0
MC1	1.0	-	5.0	5.0	5.0	84.0
MC2	2.0	-	5.0	5.0	5.0	83.0
MC3	3.0	-	5.0	5.0	5.0	82.0
MS1	-	1.0	5.0	5.0	5.0	84.0
MS2	-	2.0	5.0	5.0	5.0	83.0
MS3	-	3.0	5.0	5.0	5.0	82.0

OB = Simple ointment base, NC1, NC2 & NC3 = 1%, 2% and 3% n-hexane crude extract ointment respectively, NS1, NS2 & NS3 = 1%, 2% and 3% n-hexane-SNP respectively, MC1, MC2 & MC3 = 1%, 2% and 3% methanol crude extract ointment respectively, MS1, MS2 & MS3 = 1%, 2% and 3% methanol-SNP respectively.

Evaluation of ointment formulations

Physical characteristics of the formulations

The physical characteristics of the ointment formulations (color, texture, homogeneity, ease of application, and ease of removal) were determined by visual examination. The ointment texture and homogeneity was analyzed by rubbing a quantity of the ointment between the thumb and index finger.

Spreadability of formulation

Spreadability was determined according to established protocol (Djiobie Tchienou *et al.,* 2017). The ointment in excess was applied between two glass slides of 10 cm each and was compressed with a uniform weight of 100 g for 5 minutes to a uniform thickness between the slides. Excess ointment was wiped off then a 50 g weight was placed on the upper

slide. The time for the upper glass slide to move over the entire length of the lower glass slide was noted as the spreadability. The procedure was carried out in triplicate.

Carrageenan-induced rat paw edema

Inflammation was induced by carrageenan-induced rat paw acute edema method in albino rats weighing 180-200 g of either sex kept overnight prior to the experiment (Adeleye *et al.*, 2020). The study was carried out according to standard guidelines for the care and use of laboratory animals. The rats were divided into 14 groups (n=6). Group 1 received OB (negative control group), Group 2 – 4 received NC1, NC2 & NC3 respectively, Group 5 - 7 received NS1, NS2 & NS3 respectively, Group 8 - 10 received MC1, MC2 & MC3 respectively, Group 11 - 13 received MS1, MS2 & MS3 respectively while Group 14 received RD (the reference drug – diclofenac gel). Carrageenan (0.1ml, 1%w/v in normal saline) was injected into the sub-planar tissue of the right hind paw in all animal groups to induce inflammation. The linear paw circumference was measured using a thread and measuring ruler hourly after application of the formulation for 6 h (Gupta and Gupta, 2017).

The percentage (%) of inhibition of edema was calculated according to Equation 1 (Jaiswal and Sontakke, 2017)

$$\% \text{ inhibition} = \frac{To - Tt}{To} \times 100 \qquad \ldots\ldots\ldots 1$$

where, T_t is the paw size of rats receiving treatment at time t and T_o is the paw size of rats of control group at the same time.

Statistical analysis

Statistical differences were determined by GraphPad Prism 5 version 5.01 software using Student's *t*-test and one way ANOVA test. Data were considered statistically significant at $p < 0.05$

RESULTS AND DISCUSSION

Characterization of SNP

Visual observation

As the reaction of n-hexane extract with silver nitrate progresses, there was a gradual colour change from light green to brown after 72h while with the methanol extract the colour change was from brownish green to black. The change in colour indicates the formation of SNPs as a product of bioreduction of silver ions to silver atom.

UV–Visible spectroscopy

The UV-Vis Spectra of the synthesized n-hexane SNP (N-SNP) and Methanol SNP (M-SNP) is shown in Figures 2 and 3. N-SNP and M-SNP shows peak absorption spectra at 450 nm and 430 nm respectively. SNP spectra are sensitive to particle size – smaller particles have shorter and broad wavelength while bigger particles have higher and sharp wavelength (Das *et al.*, 2010). Therefore, M-SNP with a shorter and broad wavelength is smaller in size than N-SNP.

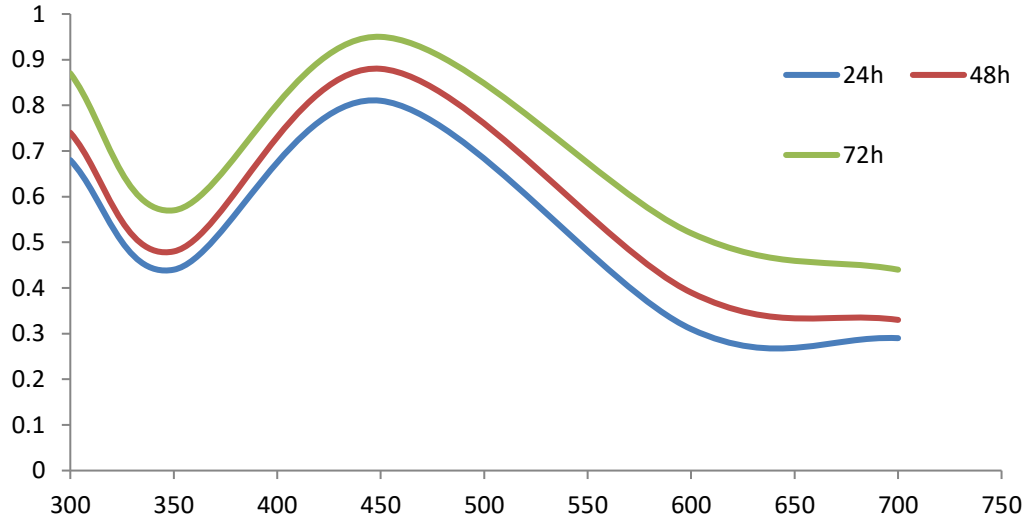

Figure 2: UV–vis spectra of N-SNP at different time interval

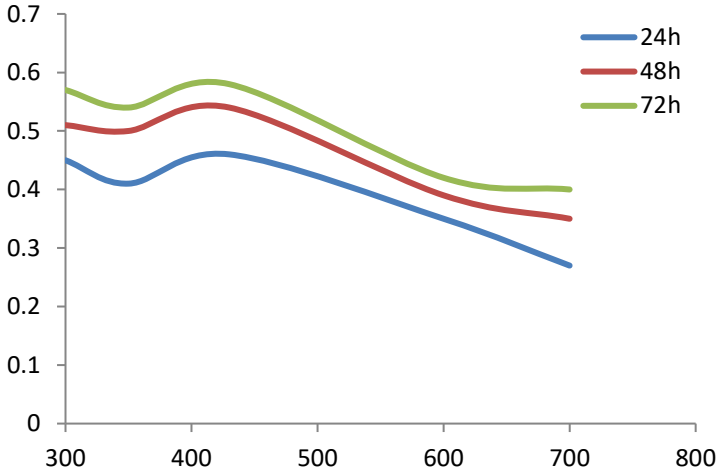

Figure 3: UV–vis spectra of M-SNP at different time interval

Atomic Absorption Spectroscopy (AAS)

Silver ion concentration in the reaction mixture over 72 h as analyzed by atomic absorption spectroscopy showed decrease in the concentration of silver ion affirming the conversion of silver ion to SNP. Methanol extract is more effective and efficient as a reducing and stabilizing/capping agent than n-hexane because of the fast decrease in the concentration of silver ion in the reaction mixture as shown in Fig. 4.

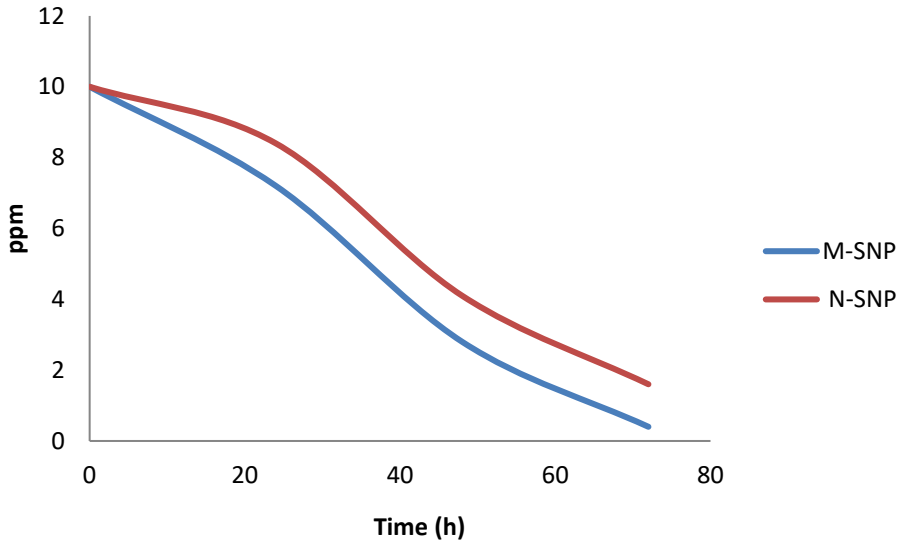

Figure 4: AAS analysis M-SNP and N-SNP at different time interval

Fourier Transform Infrared (FTIR) spectroscopy

FTIR spectroscopy was performed to identify the biomolecules involved in the synthesis of the SNP by comparing the absorption bands with standard values (Jyoti et al 2016). The FTIR spectra of M-SNP and N-SNP are shown in Fig. 5 and Fig. 6 respectively. The range of the peaks of M-SNP and N-SNP is 1038.94 cm^{-1} to 3356.37 cm^{-1} and 1335.40 cm^{-1} to 3341.28 cm^{-1} respectively. The absorbance bands between 3400-3300 cm^{-1} correspond to medium N-H stretching vibration indicating the presence of amine. The bands between 1650-1600 cm^{-1} correspond to medium C=C stretching vibration indicating the presence of conjugated alkene while the bands between 1390-1310 correspond to O-H bending indicating phenol. The functional groups in both M-SNP and N-SNP confirmed by FTIR spectroscopy are N-H, C=C and O-H. These functional groups are responsible for the synthesis, capping and stabilization of the SNP as reported in many articles (Karthik *et al.*, 2020; Balashanmugam and Kalaichelvan 2015).

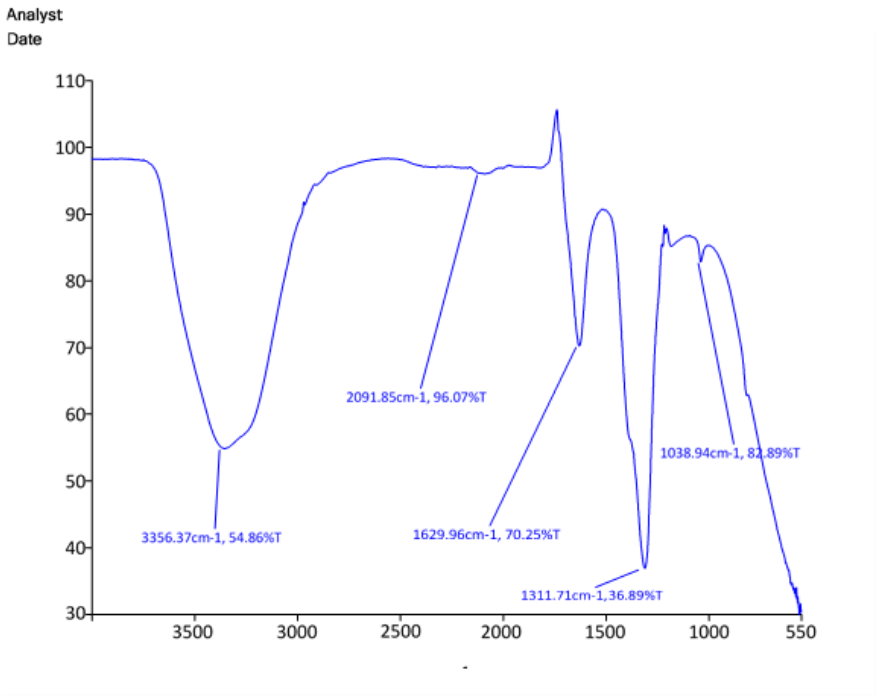

Figure 5: ***FT-IR of M-SNP***

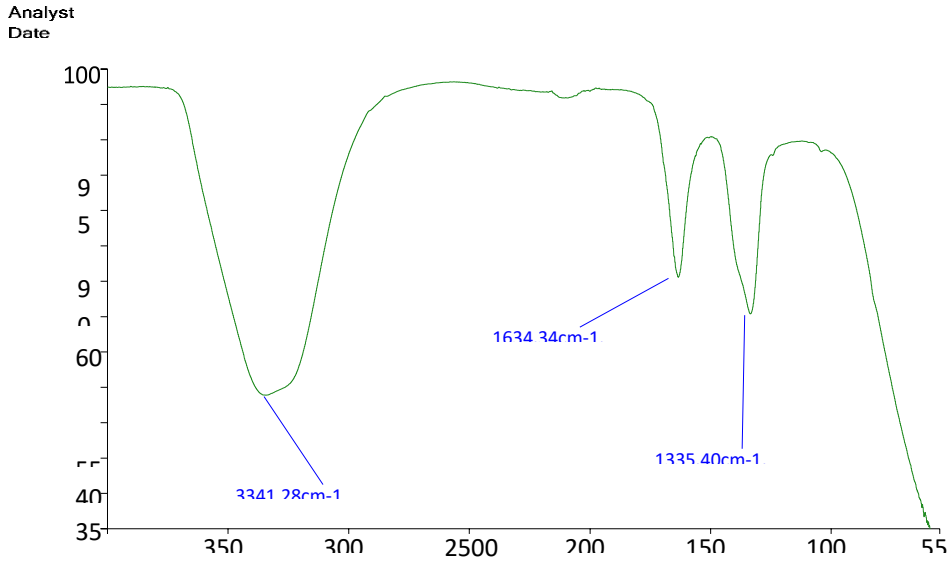

Figure 6: FT-IR of N-SNP

Scanning Electron Microscopy

SEM evaluation was performed to study the size and morphology of the biosynthesized SNP. The SEM image of the M-SNP in Fig. 7 reveals nano-sized irregularly shaped aggregated particles.

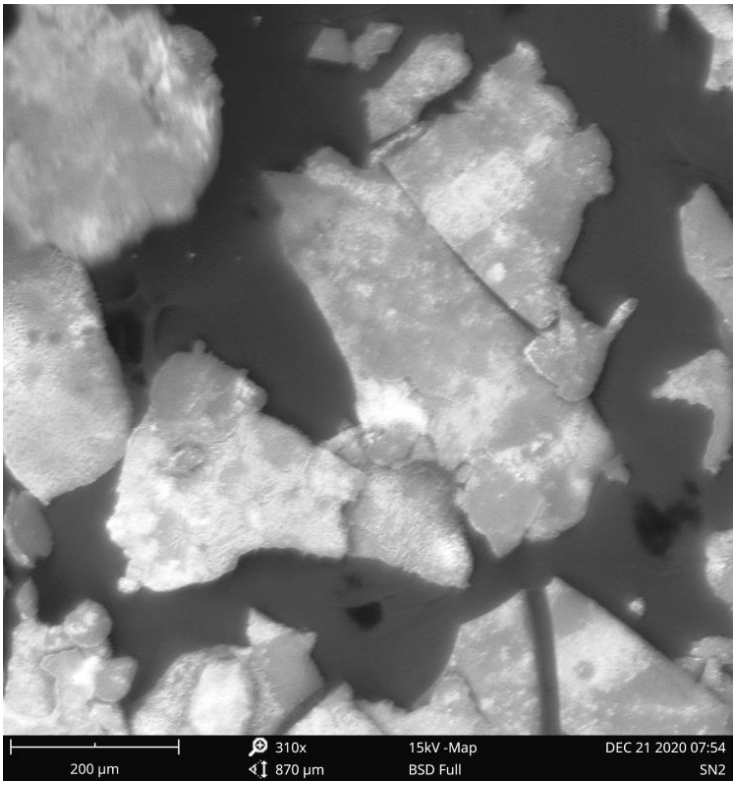

Figure 7: SEM image of M-SNP

Evaluation of ointment formulations

Physical characteristics of the formulations

The formulations containing n-hexane crude extract and Methanol crude extract were light green and light brownish in colour respectively while N-SNP and M-SNP were slightly brown and slightly black in colour respectively. The simple ointment base was colourless. All the formulations were soft and smooth in texture, and homogenous in appearance. They were easy to administer but difficult to wash off due to the presence of oleaginous ointment base.

Spreadability of formulations

Spreadability as shown in Fig. 8 is a parameter which denotes the ability, ease of application and extent an ointment would spread on the skin after application (Oladimeji *et al.*, 2015). All formulations had satisfactory spreadability.

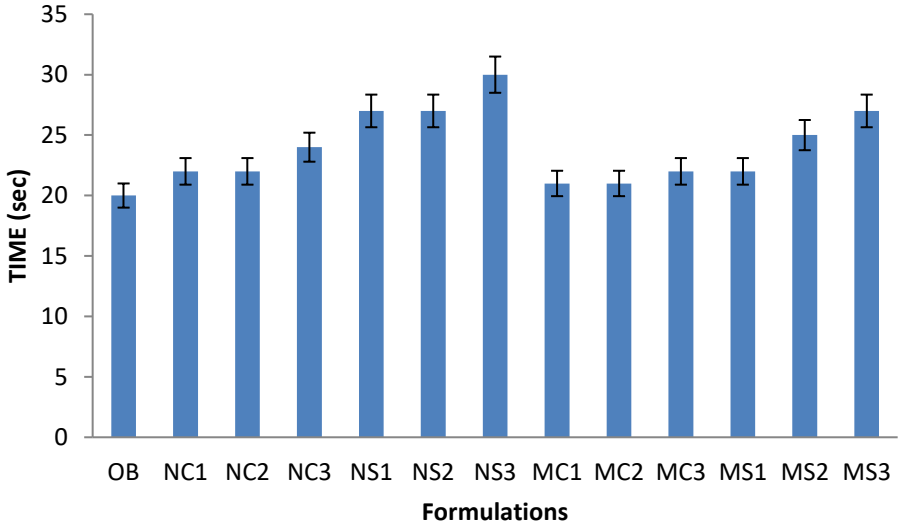

Figure 8: Spreadability of formulations

Anti-inflammatory activity of formulations

Inflammation was induced by injecting carrageenan into the sub-planar tissue of the right hind paw of albino rats. Carrageenan induces inflammation by the activation and release of biochemical and pro-inflammatory mediators which include histamine, bradykinin and prostaglandin into a damaged tissue (Gupta *et al*, 2015). Inflammation is a response to tissue damage characterized by pain, swelling, redness, heat and pain (Pashmforosh *et al.*, 2018). The experimental data compares percentage inhibition of inflammation of the formulations every hour over a six-hour period after administration of the formulations. As shown in Fig. 9, there was significant difference in the anti-inflammatory activity of the formulations containing SNP when compared with formulations containing the crude extract with SNP formulations having higher activity ($p < 0.05$). There was also significant difference in the anti-inflammatory activity of the formulations containing methanol (crude extract and SNP) compared with n-hexane (crude extract and SNP) with methanol formulations having higher activity ($p < 0.05$). This can be correlated to the peak absorption spectra of both N-SNP and M-SNP (Fig.1 and Fig. 2). The shorter and broader wavelength of M-SNP indicates that it has smaller particle size which facilitates rapid penetration into the rat paw to elicit anti-inflammatory activity (Krishnan and Mitragotri 2020). The ointments were able to penetrate the rat paw to reduce the inflammation by suppression of pro-inflammatory mediators. The ointment base formulation (OB) containing no active ingredient (negative control group) had little or no activity on inflammation. At 6 h of treatment, 11.2% inhibition of inflammation was achieved which could have been as a result of the rat's natural body regulatory function. The reference drug – diclofenac had limited extent of activity compared to all the methanol crude and SNP formulations expect formulations MC1 and MC2. However, it had a comparative activity with NS3, MC3 and higher activity than all the n-hexane crude and SNP formulations expect NS3 formulation. It was also observed that anti-inflammatory activity increased with increase in concentration.

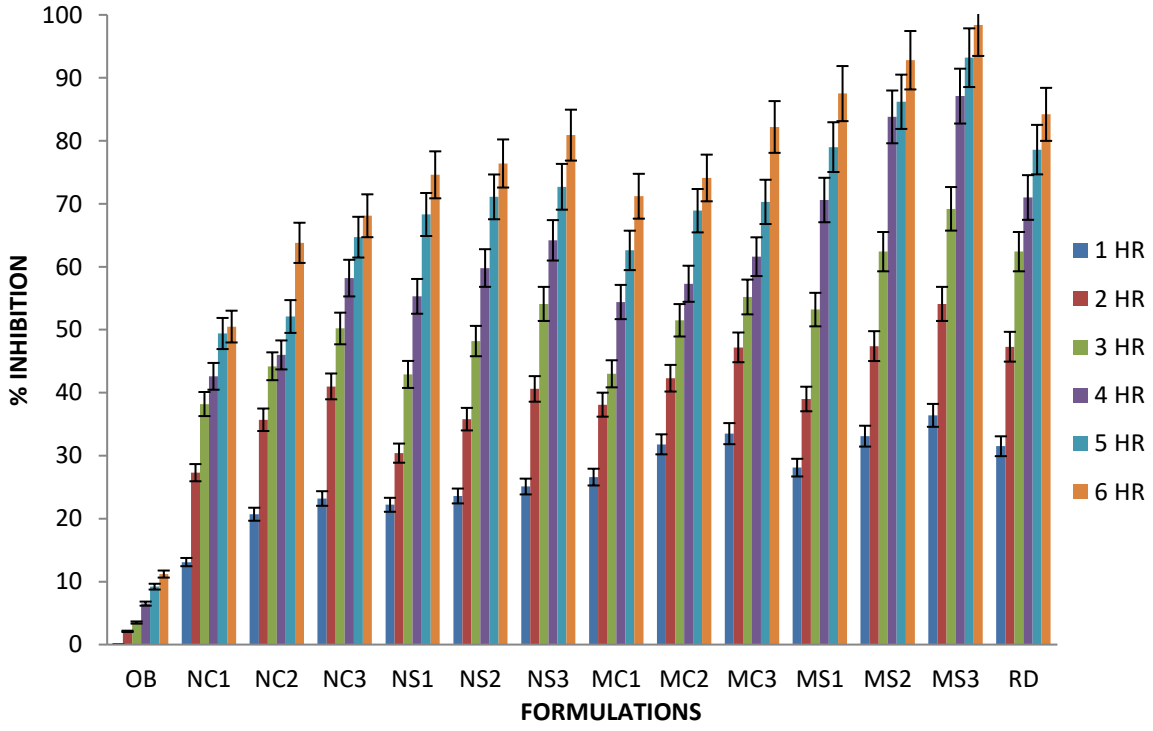

Figure 9: percent Inhibition of formulations

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CONCLUSION

The study was carried out to assess the *in vivo* anti-inflammatory activity of ointment containing silver nanoparticle synthesized with n-hexane and methanol extract of *Ehretia cymosa* leaf. All the formulations were soft and smooth in texture, and homogenous in appearance with satisfactory spreadability. The anti-inflammatory activity of ointment formulations containing silver nanoparticle synthesized with

methanol extract is significantly higher compared to ointment formulations containing silver nanoparticle synthesized with n-hexane extract and the reference drug (diclofenac).

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Conflict of Interest: None declared

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