



Ethosomal formulation composed of standardized *Orthosiphon stamineus* extract and sophorolipid inhibits angiogenesis of melanoma *in vivo*

Mansoureh Nazari Vishkaei ^a, Mohamed Khadeer Ahamed Basheer ^c, Amin Malik Shah Abdul Majid ^{a,b}

Abstract

Cancer disease involves the abnormal growth of cells that have the ability to invade other organs. Melanoma is one of the angiogenesis dependent cancer. *Orthosiphon stamineus* (EXOS) extract is a well-known anti-angiogenic herb in South Asia. Thin-film hydration method has been employed to develop ethosome, a flexible liposomal gel formulation composed of standard extract of O.S leaves (SLOS) using sophorolipid (SL) and investigated its physicochemical properties for topical drug delivery system. High Performance Liquid Chromatography (HPLC) determined $76.02.02 \pm 1.2\%$ entrapment of rosmarinic acid (RA) in ethosome vesicle. The average particle size of optimized vesicle was 189.9 nm with potential of -35.5 mV. SLOS at concentration of 1% demonstrated proper plastic flow, which could facilitate achieving effective and maximum skin permeation of EXOS. Permeation studies using albino mice revealed that the SLOS demonstrated higher penetrability through deeper layers of skin than the conventional hydroethanolic gel composed of EXOS. Cytotoxic test on normal endothelial cells confirmed the safety of SLOS formulation. *In vivo* melanoma tumor model study revealed that SLOS showed $97.07 \pm 0.6\%$ anti

-tumor activity during 16 days of study. These results revealed that SLOS vesicles can be considered for effective dermal therapeutic agent for the treatment of skin cancer.

Keywords: Ethosome; sophorolipid; *Orthosiphon stamineus*; Melanoma; Rheology; Topical drug delivery

Introduction

Melanoma is one the most aggressive types of skin cancer. Malignant melanoma has been reported as an angiogenic tumor type, unmistakably showing new vessel arrangement as an essential advance in infection movement from atypical melanocytes, through outspread development to the forceful vertical development stage (Vishkaei, Sultan, Basheer, & Majid, 2017)(Vishkaei, Sultan, Basheer, & Majid, 2017). *Orthosiphon stamineus Benth* is well known therapeutic herb in south Asia since long time ago for the treatment of bladder inflammation, eruptive fever, hepatitis, hypertension, and rheumatism (Hossain & Rahman, 2015; Vishkaei, Sultan, Khadeer, & Ahamed, 2017). It is abundant with flavonoids with recognized anti-oxidant, anti-inflammatory anti-tumor and anti-angiogenic properties (Shafaei et al., 2016). For the treatment of skin diseases, topical drug delivery is one of the desired types of administrations. Due to the selective permeable characteristics of outer layer of skin, which acts as

Significance | SLOS gel is a novel topical formulation and a promising treatment for skin cancer.

*Correspondence: **Amin Malik Shah Abdul Majid** at Department of Pharmacology, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Minden, 11800, Pulau Penang, Malaysia, Tel: +60124230842 Email: aminmalikshah@gmail.com

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Author Affiliation:

^a Department of Pharmacology, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Minden, 11800 Pulau Penang, Malaysia

^b ACRF Department of Cancer Biology and Therapeutics, the John Curtin School of Medical Research, Australian National University.

^c EMAN Biodiscoveries Sdn. Bhd., Suite 126, Level 1, EUREKA Complex, Universiti Sains Malaysia (USM) Campus, Minden - 11800, Pulau Penang, Malaysia

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a stringent barrier, in many conditions, permeation enhancers are required to maintain therapeutic quantities of medicine. Traditional liposomal techniques were identified to be efficient at topical drug delivery for the purpose of skin therapy (Bragagni, Scozzafava, Mastrolorenzo, Supuran, & Mura, 2015). However, the conventional liposomal technique has several major drawbacks, such as low drug entrapment, low drug-holding capability, low stability and inefficient drug delivery to the deeper level of the skin. In contrast, ethosomal particles through the phospholipid vesicular techniques that include high percentage of alcohol, were efficient at improving dermal and transdermal delivery of either lipophilic or hydrophilic compounds (Mbah, Builders, Agubata, & Attama, 2017). Ethosome vesicles are phospholipid-based elastic nano particles including high concentrations of ethanol (20–45%). Ethanol is identified as an effective permeation booster and has been integrated in the vesicles to make the elastic nano particles. It can mingle with the polar head group part of the lipid substances, causing the elimination of the melting point of lipid in the structure of the stratum corneum, consequently raising lipid fluidity, as well as permeability of cell membrane. The superior flexibility of vesicular membranes is attributed to the presence of ethanol in ethosome. This enables the vesicles to become more elastic and therefore can be easily absorbed into the skin layers by squeezing themselves through the pores of even smaller dimensions. Thereby, in transdermal drug delivery system with respect to the effective amount and depth of penetration, ethosomal technology is significantly more effective than either traditional liposomes or hydroalcoholic formulations (Malla et al., 2016). Some studies have also specified that ethosomal systems also own perfect stability of entrapment due to the fact that the existence of ethanol, that produces a negative surface charge, consequently hindering vesicle aggregation because of electrostatic repulsion. Ethosomal drug delivery system has proved safe and effective in human study, wherein 5-fluorouracil has shown promising result in penetration through human skin (Vishkaei, Sultan, Basheer, et al., 2017).

The main aim of this research work was to develop an optimized topical ethosomal-based formulation of O.S extract with enhanced rate of penetration through deeper layers of skin and treat melanoma tumor model via topical application.

Materials and methods

Sophorolipid was given as a gift from Malaysian palm oil board. Orthosiphon stamineus standard extract was given as gift from natureceuticals.sdn.bhd.Pulau penang, Malaysia. Solvents of either HPLC or analytical grade were purchased from Merck. Rosmarinic Acid and other markers and rhodamine red from Sigma-Aldrich, USA, DMEM media Gibco/Life technology, UK and B16F10 cell line from ATCC, Rockville, MD, USA.

Animals

For permeation and tumor model studies healthy adult male Albino mice weighing 28–32 g were used. The study was approved by the

Animal Ethics Committee of Universiti Sains Malaysia, Penang, Malaysia [Protocol No: USM/IACUC/2017/ (109) (882)].

Preparation of ethosome dispersion

Ethosome formulation (SLOS) developed using thin film rehydration method with some modifications. Different ratio of phospholipids: O.S extract (EXOS) (2:1, 1:1 and 1:2) were suspended in 15 mL of 50% ethanol. The mixture was homogenised for 10 min at high-speed shearer/mixer. This mixture was agitated at 120 rpm in a shaking incubator for 3 hours at 40 °C. The final solution was concentrated using a rotary evaporator to prepare a thin film. Then, 40% ethanol added and covered it properly to avoid evaporation of ethanol and kept it in incubator shaker with 120 rpm for 1 hour. It was analysed for the entrapment percentage of different formulation using cold centrifuge method to select the best formulation for further studies (Rezaei-Sadabady, Eidi, Zarghami, & Barzegar, 2016).

Preparation of ethosome gel formulation:

The selected dispersion was chosen to make 1% and 2% gel utilizing acrypol. 1 and 2% W/V acrypol added gradually to the beaker contained selected dispersion of ethosome while stirring continuously. The pH of dispersion controlled to be in neutral pH using sodium hydroxide. After 1 hour collected the gel and kept in fridge (Ramadan, Goldie, & Anwar, 2017).

Characterization

Entrapment percentage

Entrapment efficiency of ethosomal formulation was assessed by defining the content of rosmarinic acid entrapped in the ethosomal vesicles using UV spectrophotometer at wavelength of 320 nm (Iizhar, Syed, Satar, & Ansari, 2016).

Particle sizes and zeta potential

The average particle size, PDI and zeta potential of selected formulation was assessed by a Zetasizer Nano ZS (Malvern Instruments Ltd, UK).

HPLC

HPLC analysis performed on RA as standard compound and SLOS using previously validated method ⁹. A RP-HPLC-diode-array detector at a 320 nm wavelength used to detect the marker compound (RA) and to ensure accuracy, precision, and sensitive detection. The assay conducted to quantify RA in SLOS compared with EXOS prepared in ethanol, using a gradient mobile phase of 0.1% formic acid in Acetonitrile at a flow rate of 1 mL/min in a reverse-phase acclaim polar advantage II C18 column (3 µm, 3 × 150 mm) with the separation time being 20 min (Shafaei, Ab Halim, Zakaria, & Ismail, 2017).

Transmission electron microscopy (TEM)

TEM study was done in order to verify the formation of SLOS. The samples were studied under CM12 TEM (Philips, Netherlands).

Zeta sizer and potential

The average particle size, PDI and zeta potential were determined by a Zetasizer Nano ZS (Malvern Instruments Ltd, UK).

Viscosity analysis of ethosome gel in different temperatures

MCR 302 Rheometer (Anton Paar, Malaysia) along with Parellel Plate PP25 (Diameter 25mm, Stainless steel) was used to study rheological analysis of SLOS. The temperature was controlled by Plate Peltier Temperature Controller P-PTD200. Software Anton Paar Rheocompass version 1.2. Prior to the rheological experiment SLOS gel was placed on the peltier plate in the way to cover the cone at the top of the plate. Then the SLOS gel was equilibrated at a controlled temperature dependent on the experimental condition for 3minutes before and after putting the cone over Peltier plate. After fixing the cone trimmed the gap by using this option in rheometer to obtain thin uniform film of SLOS gel under the cone. After that without disturbing the film between plate and cone removed the excess of gel from sides of the cone carefully.

Continuous flow ramp study

SLOS gels 1% and 2% were kept in the condition to increase shear rate from 1-200 S⁻¹ and the corresponding stress was analyzed at 4 °C, 25 °C and 32 °C.

Drug content

10ml of SLOS gel was dissolved in 1ml methanol and stirred for 30 min. Drug content was quantified using HPLC (Li et al., 2018).

Cell Viability study

Cytotoxicity of EXOS and SLOS were evaluated against endothelial cell line (EA.hy926) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described earlier. The experiment was repeated three times with four replicates for each concentration. The percentage of cell viability was calculated and presented as mean ± S.E.M.

Ex vivo rat aortic ring assay

The rat aortic ring assay was carried out with slight modification (Ng, Salhimi, Majid, & Chan, 2010). Briefly, aortic rings (1 mm thickness) taken from thoracic aortas of 12-14 weeks old male Sprague Dawley rats were seeded individually in 48-wells plate in 300 µl serum free M199 media containing 3 mg ml⁻¹ fibrinogen and 5 mg ml⁻¹ aprotinin. 50 NIH U ml⁻¹ thrombin in 0.15 M NaCl were added in each well. After 90 min incubation at 37°C, equivalent concentrations of SLOS and EXOS dissolved in 0.3 ml M 199 medium supplemented with 20% HIFBS, 0.1% ε-aminocaproic acid, 1% L-Glutamine, 2.5 µg/ml amphotericin B, and 60 µg/ml gentamicin, were added to each well. Suramin and 1% ethanol were used as positive and negative controls, respectively. On day four, the medium was replaced with a fresh one containing the compound. On day five, aortic rings were photographed at 4x magnification using an inverted light microscope (Olympus). The angiogenic response was determined by measuring the distance of blood vessels outgrowth from the primary tissue explants using the same instrument with the aid of Leica Quin software package. The results are presented as mean percent inhibition to the negative control ± S.E.M, (n = 3).

Ex vivo skin Permeation study

In this study, a Franz diffusion apparatus was used with minor modifications to obtain an efficient diffusion surface area of about 2

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cm². In this experiment, 2 × 2 cm area of mouse abdominal skin was shaved until all fur was removed. Then the cleaned and shaved skin explants were excised from abdominal regions of albino mice. The skin explant was mounted as a barrier between the donor compartment and the receptor compartment of the Franz diffusion apparatus. Test sample (SLOS or EXOS) was applied on the mounted skin surface in the donor compartment and covered with parafilm to avoid evaporation of ethanol. The receptor section of the diffusion cell was filled with phosphate buffer pH 7.4. The entire setup was installed on a magnetic stirrer, so that the solution in the receptor section was continuously stirred by means of magnetic beads at 50 rpm. The temperature was maintained at 32 ± 0.5°C to mimic skin body temperature. At specific time intervals for 24 h, samples were taken and the quantity of rosmarinic acid content was estimated spectrophotometrically at 320 nm, as explained earlier. In the receptor section an equivalent quantity of fresh phosphate buffer pH 7 was replaced at each time interval. The analysis was done in triplicate using different skin explants from 3 animals for each test sample for each time point (n=3). Average percentage ± S.E.M of drug permeated through the skin over time was plotted.

In vivo melanoma tumor model in albino mice model

B16F10 melanoma cells were injected subcutaneously into the right flank of each mouse (4–6 week old, 20–22g) C57BL/6 mice.

The animals were divided to 3 groups of 6 animals each. Group one was treated with empty ethosome topical gel as negative control. Group two and three were applied 1000mg/kg of SLOS and EXOS respectively at equivalent concentration once per day. Various parameters i.e., tumor size and body weight were identified every 3 days and tumor volume was measured using the following formula;

$$\text{Tumor volume (mm)}^3 = (L \times W \times D) / 2$$

While L, W and D stand for length, width and depth of tumor in mm respectively.

The anti-melanoma effect of ethosomal formulation of *O. stamineus* was analyzed using following equation;

$$\text{Anti-melanoma efficacy} = \% \Delta T / \Delta V$$

$\Delta T = T - \Delta 0$ and $\Delta C = C - \Delta 0$ (T, $\Delta 0$ and C is equal to average of tumor volume at t₀, in different days and in various treated and untreated groups.

All the animals were euthanized after 4 weeks when the tumor in untreated group exceeded to more than 1000mm³. Eventually the survived animals were euthanized using CO₂, then cervical dislocation was done. The images of animals were taken by camera Canon and the tumor were collected and preserved in 4% paraformaldehyde. The tumors were sent to Gribbles pathology laboratories (Penang, Malaysia) to do haematoxylin/EXOS in staining in order to observe them under microscope to visualize the apoptosis/necrosis caused by the treatment after finishing the period of the study.

Statistical analysis

Data are expressed as the mean ± S.E.M. Statistical analysis was performed using ANOVA test with graph prizm software. The level of probability at 0.05 was used as the level of significance.

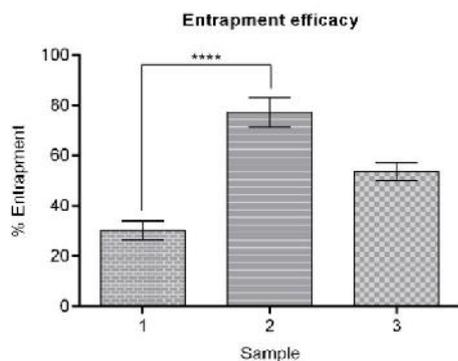


Figure 1 | Entrapment percentage of SLOS. Ethosomes were formulated by mixing O.S ethanol-water extract with sophorolipid with different ratios (1-3).

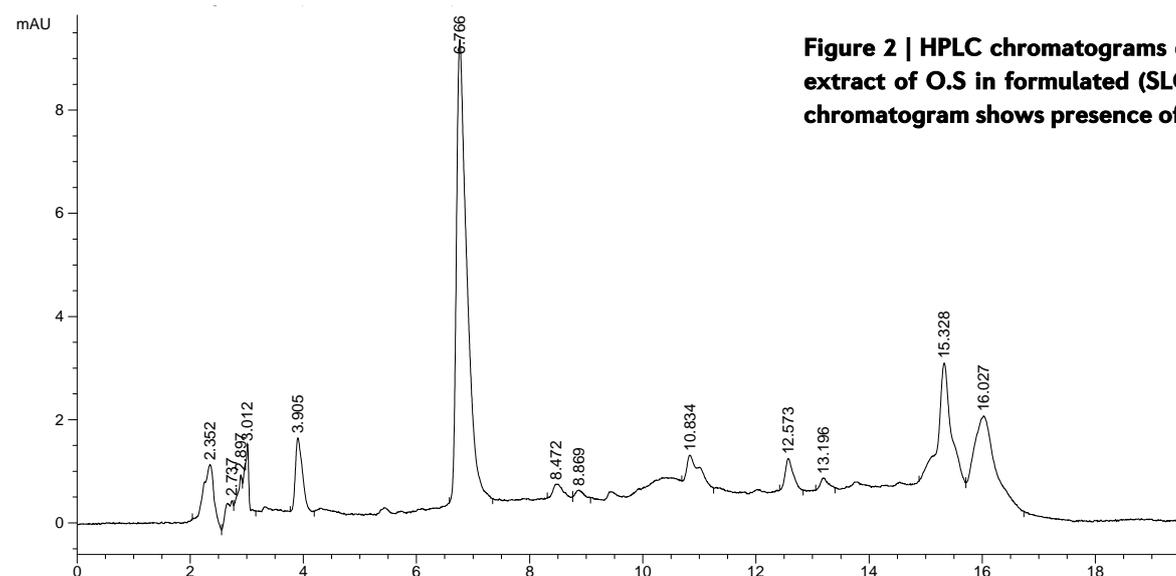


Figure 2 | HPLC chromatograms of crude extract of O.S in formulated (SLOS). The chromatogram shows presence of RA.

Results and discussion

Entrapment analysis

UV spectroscopy analysis revealed that the entrapment efficiency was less in formulation-1 composed of ratio 1:2 of sophorolipid: extract was 30.38±3.78, n=3. After that formulation-2 which is composed of ratio 2:1 of extract: sophorolipid. Figure 1 shows the percentage of RA entrapped in formulations. However, the formulation-2 which is composed of sophorolipid: extract at a 1:1 ratio, showed significantly ($P<0.0001$) highest entrapment efficiency with 77.23±5.9. Based on the entrapment results, formulation-2 was chosen for the further physico-chemical and pharmacological characteristic studies.

HPLC standardization of O.S-ethosome formulation and crude extract

HPLC analysis of rosmarinic acid (RA) (Figure2) enabled the determination of the extent of active compound entrapment in the SLOS compared with EXOS. These results confirmed entrapment analysis using UV. It showed 76.02±1.2% RA entrapped in ethosomal selected formulation.

TEM

The SLOS formulation was dissolved in water and studied under TEM. Figure 3 shows the presence of the predominantly round-shaped particles of the SLOS. However, few flat depressions on the surface of most of the particles suggest the flexible nature of the ethosomal particles.

Zeta sizer and potential

Zeta sizer has shown SLOS with the diameter of 189.9 nm with PDI of 0.404 (Figure 4 B). The zeta potential showed -35.5mv (Figure 4 A).

Rheological study

Continuous flow ramp study

The 1% SLOS gel showed a suitable plastic flow behavior with yield stress of approximately 60.099±4.20 Pa at low concentration of polymer at 4 °C. Figure 5 shows the changes in yield stress by increasing the temperature as well as changing the concentration of acrypol. Viscosity of samples was decreased by increasing the temperature while increasing the shear rate. Comparatively, viscosity of 2% gel in all the conditions was recorded to be more than the 1% SLOS gel.

The SLOS gel, irrespective of acrypol concentration demonstrated good spreadibility and was free of agglomerates. The rheological properties of SLOS gels was analysed to select proper acrypol percentage and application. For dermal and transdermal formulations, the proper SLOS gel must have plastic flow (Jain, Patel, Madan, & Lin, 2016). Results of viscosity analysis demonstrated that 1% SLOS gel proved to be more pronounced than the formulation composed of 2% gel, with respect to the transdermal penetration.

Effect of SLOS on Cell viability

Cytotoxicity effect of SLOS formulation on normal cell line (EA.hy926) showed that SLOS did not affect the viability of the cells. The test was conducted utilizing colorimetric measurement of cell

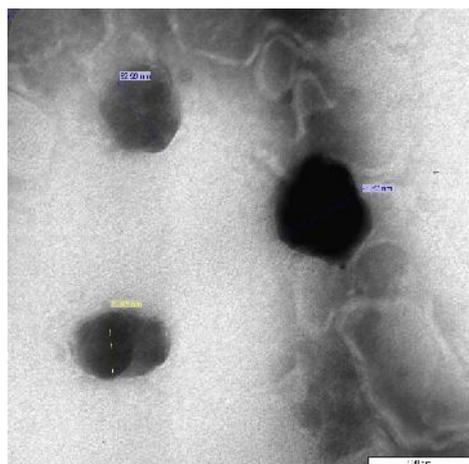


Figure 3 | The presence of the predominantly round-shaped particles of the SLOS.

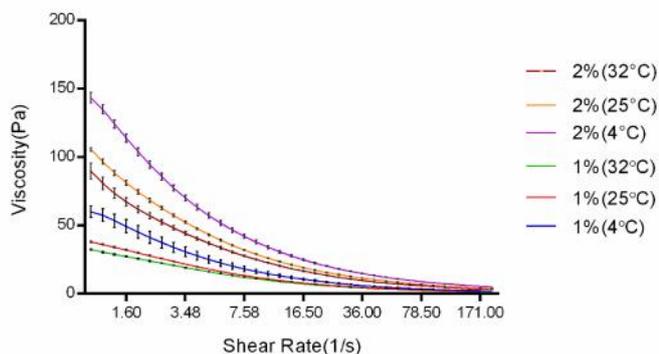


Figure 5 | The effect of shear rate on viscosity of 1% and 2% gel of SLOS in 4 °C, 25 °C and 32 °C (n=3).

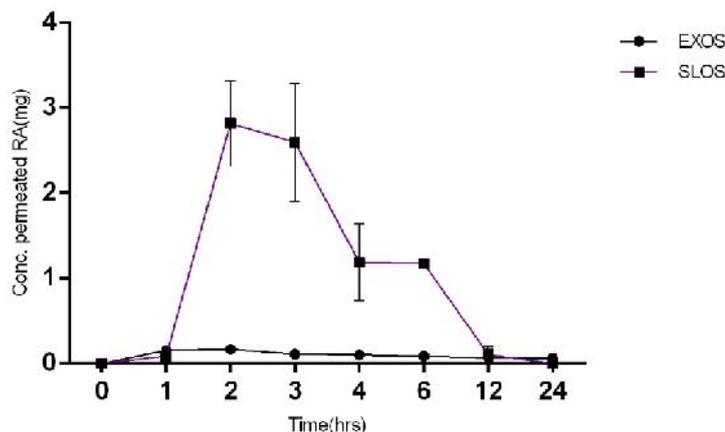
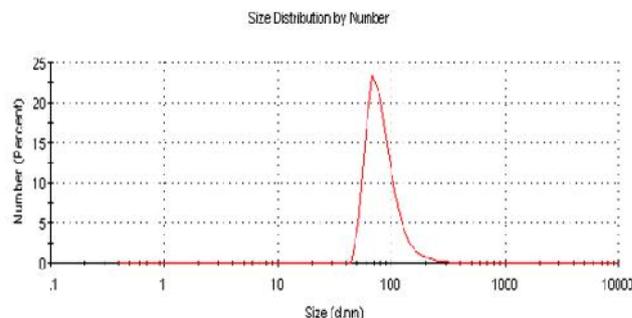
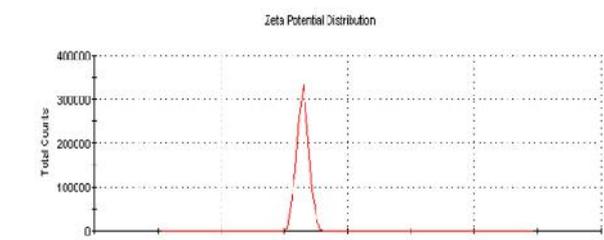


Figure 4 | (A) Zeta potential (B) Zeta sizer results of SLOS sample after dissolving in water.

viability after 48 hours treatment with SLOS compared to EXOS crude extract at concentrations 100 and 50 µg/mL. Figure 6 demonstrates the percentage of EA.hy926 cell viability after treatments.

Ex vivo Permeation study

In this study, 1g of the test product was applied onto the isolated skin mounted on Franz diffusion cell. After 24 hours, the transdiffused solution was collected and the marker compound of EXOS was quantified using HPLC. The results of the test showed that the concentration of RA found in the transdiffused solution after application of the hydroethanolic gel of EXOS and the SLOS gel was 0.11±0.009 µg/ml and 0.23±0.01 µg/ml, Figure : Permeation studies. Permeation of EXOS and SLOS gel in 24 hours of topical application mice skin. The data is presented as mean ± SEM, n=3.

respectively (Figure 4.B). This result indicates the SLOS has shown 2 times more permeability than that of EXOS. So based on enhance permeation efficacy of formulation it has the potential to be used for topical application *in vivo* study.

Ex vivo rat aortic ring assay

The anti-angiogenic potential of SLOS compared with EXOS was investigated in the rat aortic model. Figure 7 shows the microvessels outgrowth from the untreated aortic rings. On the contrary, aortic rings treated with SLOS exhibited sopped outgrowth at concentration of 100 µg/ml (Figure 7B). The anti-angiogenic effect on explants of rat aorta showed a significant dose dependent relationship (P < 0.05). At 50 µg/ml, SLOS and EXOS inhibited vascularisation by 58±5.08 and 34.61±1.8% respectively. Doubling the dose of SLOS and EXOS to 100 µg/ml led to a complete inhibition of angiogenesis by 100% for SLOS but 74.44±5.20% inhibition by EXOS. Suramin, which was used as a positive control showed 100% inhibition of microvessels outgrowth at 100 µg/ml (Figure 7F).

In this study, angiogenesis assays was employed to evaluate the antiangiogenic activity of SLOS compared with EXOS. The results showed that SLOS perturbed the new blood vessels formation in rat aortic ring explants 25.56% more than EXOS at high concentration of 100 µg/ml (Figure 7).

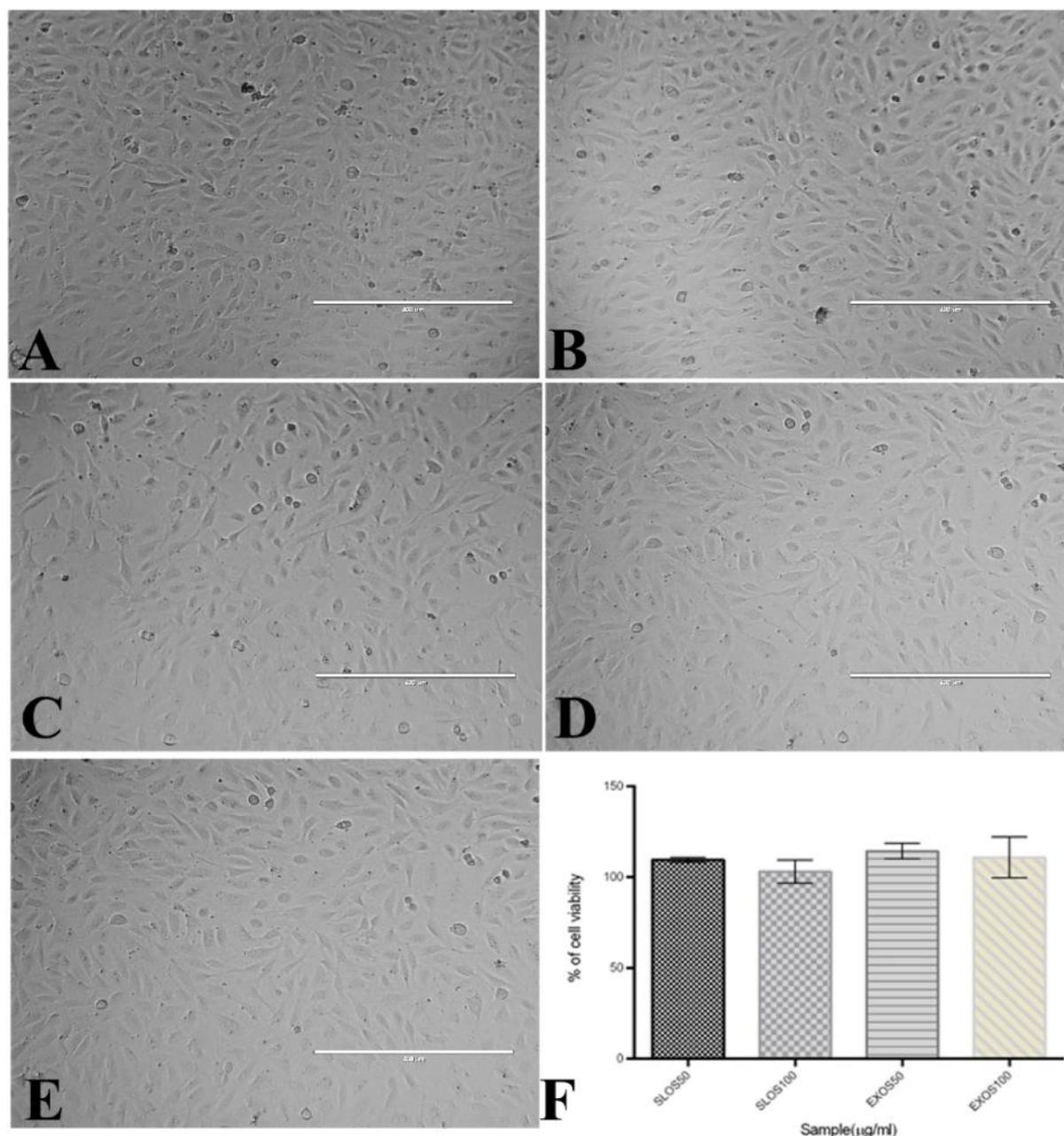


Figure 6 | (A, B) EA.hy926 cell line treated with SLOS at concentration 50 and 100 µg/mL.(C,D) treated with EXOS equivalent concentration of 50 and 100 µg/mL. (E) negative control. Percentage of EA.hy926 cell line viability after treatment. SLOS and EXOS refer to sophorolipid ethosomal formulation and crude extract of O.S, respectively. Statistical analysis was conducted by one-way ANOVA.

***In vivo* anti-melanoma tumor model**

The *in vivo* antitumor effect of topical SLOS evaluated compared with EXOS by inducing the B16F10 cells subcutaneously in albino mice. After 6 days the treatment based on dosage forms of SLOS and EXOS were started (Figure 8). Figure 8 demonstrated enhanced antitumor effect of SLOS compared with EXOS gel in 16 days of treatment (P<0.05).

The volume of tumors in all the groups decreased gradually by increasing the dosage form (Figure 8). The highest dose of SLOS showed the highest inhibition effect after 16 days of treatment. Evaluation of *in vivo* melanoma study revealed dose and time dependent antitumor activity (%ΔT/ΔC) in SLOS, EXOS and negative control groups respectively.

Antitumor analysis showed non-formulated topical EXOS gel has shown 58.95±5.7% tumor inhibition after applying equivalent concentration of 1000 mg/kg of treatment. SLOS of same dosage form revealed 97.07317±0.6% tumor inhibition. The current results revealed that high concentration of SLOS could significantly suppress the growth of the melanoma tumor compared with non-formulated gel of O.S extract and negative control.

Histopathological Examination

Anti-melanoma effect of SLOS compared to EXOS gel was evaluated by histological analysis of stained tumor cross sections. Figure 10 demonstrated the histology of tumors excised from animals treated with empty ethosome as negative control which revealed minimum necrosis compared to other groups with plenty of intra tumor blood

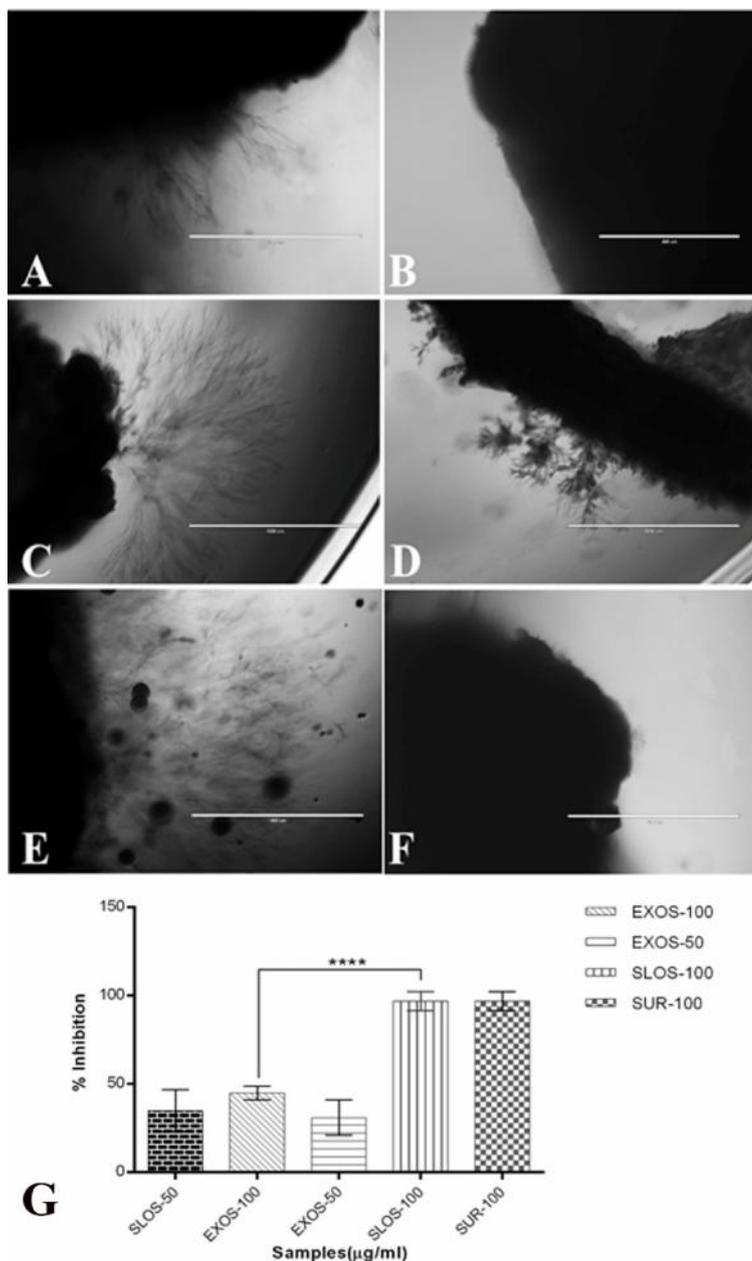


Figure 7 | (A, B) EA.hy926 cell line treated with SLOS at concentration 50 and 100 µg/mL.(C,D) treated with EXOS equivalent concentration of 50 and 100 µg/mL. (E) negative control. Percentage of EA.hy926 cell line viability after treatment. SLOS and EXOS refer to sophorolipid ethosomal formulation and crude extract of O.S, respectively. Statistical analysis was conducted by one-way ANOVA.



Figure 8 | Anti-melanoma effect of SLOS formulation in B16F10 tumor implanted in mice. (1,2) are negative control at day 0 of treatment. (3,4) are treated group with formulation at day 0 of treatment



Figure 9 | Anti-melanoma effect of SL-OS formulation in B16F10 tumor implanted in mice. (1) is the animal treated with EXOS, (2) is negative control after 16 days. (3,4) are treated group with SLOS after 16 days treatment.

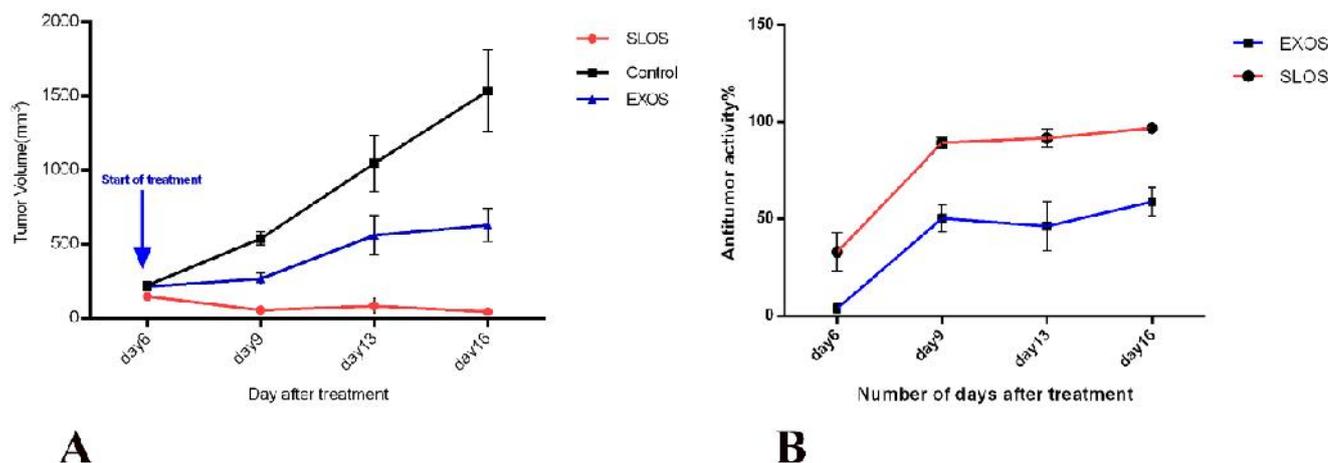


Figure 10 | Subcutaneous melanoma tumor in albino mice. (Control) Untreated animals, owing the largest size of tumor, (EXOS) animals after topical treatment with crude extract gel at concentration 1000mg/kg body weight. (SLOS) Animals after topical treatment with ethosomal gel at equivalent concentration. (A)Tumor dimensions were analysed every three days and values are demonstrated as mean melanoma tumor size(mm³) ±S.E.M. (B) shows the antitumor percentage of 1000 mg/kg body weight of SLOS compared with equivalent concentration of EXOS 16 days treatment in animals. All the studies are demonstrated as mean ±S.E.M.

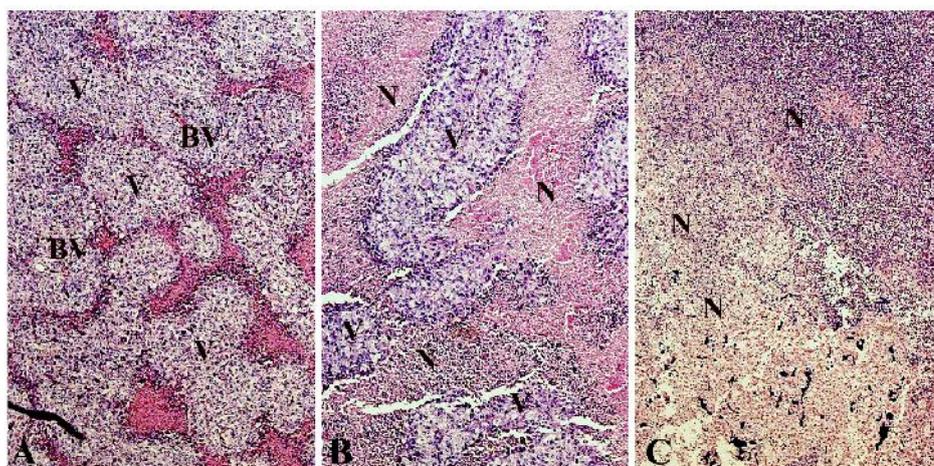


Figure 11 | Cross sections of melanoma tumor tissues stained with hematoxylin/eosin. The tumor cross sections were evaluated for signs of necrosis. (A) The viable melanoma cells and lower extent of necrosis compared to all treated groups, blood vessel and melanin distribution in negative group. (B) The tumor treated with equivalent concentrations at 1000mg/kg of EXOS and (C) 1000 mg/kg of SLOS. V stands for live tumor cells, N sands for necrosis, BV stands for blood vessels. All the images have been taken at 20x magnification.

vessels supply. In the animals treated with SLOS at 1000, 2000 and 3000 mg/kg, the tumors showed increasing trend of necrosis by increasing the dosage of treatment. The cross sections of the tumors in different groups demonstrated disorganized cells, necrosis and reduction of number of blood vessels. Tumor sections in control group demonstrated intact melanoma solid tumor tissues with just small necrotic section and vast area of intra-tumor blood vessels (**Error! Reference source not found.**). Furthermore, the lung cross section of negative group demonstrated large metastatic tumor cells (**Error! Reference source not found.**) distribution of melanin in metastasized lung which is more than all treated groups of animal tumors.

Conclusions

The current study reports the development of a novel sophorolipid ethosomal based gel formulation of O.S extract with high drug-
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entrapment capability, effective viscoelasticity, remarkable stability and efficient penetrability. In this study, a gel formulation of ethosome composed of *Orthosiphon stamineus* extract was optimized with 77.23±5.9 % entrapment. Flow ramp test study revealed that 1% gel demonstrated strong potential with optimum viscosity for topical gel formulation. Cytotoxic test on normal cell line confirmed the safety of SLOS *in vitro*. Physicochemical analysis confirmed the presence of the active compounds in SLOS. Vesicles were developed with smooth surface, which could help in holding up of the drug, prevented the drug-leakage, and thus enhanced permeation efficacy compared with conventional hydroethanolic gel composed of the EXOS. In vivo melanoma tumor model in mice also confirmed the enhanced effect of SLOS compared with EXOS. Altogether, it can be concluded that as SLOS gel is a novel topical formulation that can be a promising treatment methodology for skin cancer.

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