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Evaluation of Chemical, Antipsoriatic and Antiangiogenic Properties of Salt from Lonar Crater Lake Water

Prakash Bansode[1], Indumathi Somasundaram[2], Apurva Birajdar[2], Sanjay Mishra[3], Dhanashree Patil[3], Ahilya Waghmode[4] and Gajanan Rashinkar[1,✉]

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ABSTRACT

Lonar Crater lake was created by the impact of a massive meteor during the Pleistocene Epoch. Being a hypersaline and hyperalkaline soda lake, rich microbial diversity is reported earlier. Lonar lake water is used by local people and tribals against skin diseases. These observations prompted us to investigate the therapeutic potential of lake water against skin diseases. In this context, we have conducted pilot study to assess the antipsoriatic and antiangiogenic activity of the salt obtained from lake water using THP1 cell line by MTT assay and antiangiogenic activity by *in vivo* chicken chorioallantoic membrane (CAM) assay, as there is a close relation between psoriasis and angiogenesis. The results revealed that salt possess remarkable antipsoriatic and antiangiogenic activity.

KEYWORDS

Lonar crater, Psoriasis, Angiogenesis, THP1, Chorioallantoic membrane.

INTRODUCTION

Owing to the interesting physico-chemical and biological properties, study of soda lakes is the most captivating aspect of ecological science [1]. Lonar crater (latitude 19°58′, longitude 76°36′) is a near circular depression formed in Buldhana district, Maharashtra state, India during Pleistocene epoch by the impact of massive hypervelocity meteor that descended on the earth from space [2]. The crater has a diameter of around 1.88 km with a depth of 135 meters and is confined from all the sides by raised rims [3]. The crater encompasses worlds third largest natural saline lake formed due to the accumulation of water through rain, ground water seepage and the springs situated in the cliffs at the edge of crater [4]. The Lonar crater lake is the world's only hypersaline and hyperalkaline soda lake in basaltic rock. The lake water is highly alkaline with pH in the range of 9.5-10.5 owing to the presence of large concentration of salts [5]. One of the striking features of crater lakes is the remarkable colour of their waters [6]. A remarkable feature of Lonar lake water is the presence of massive algal blooms that result the lake to appear green. Fluctuations in physico-chemical parameters of brackish water with time play a major role in establishing the bio-distribution and dynamics of the quality of the water [7]. The lake depicts fascinating biodiver-

Author affiliations:

[1]Department of Chemistry, Shivaji University, Kolhapur-416004, India
[2]Department of Stem Cell and Regenerative Medicine, D.Y. Patil Educational Society, Kolhapur-400614, India
[3]Dr. Prabhakar Kore Basic Science Research Centre, KLE Academy of Higher Education and Research, Belagavi-590010, India
[4]Department of Botany, Rajarshi Chhatrapati Shahu College, Kolhapur-416003, India

✉To whom correspondence to be addressed:

Fax: +91 231 2692333
Tel: +91 231 2609169
E-mail: gsr_chem@unishivaji.ac.in

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sity with haloalkaliphilic microbial flora and fauna [8-10]. The fascinating features of Lonar crater lake has attracted attention of researchers from different domains of science for revealing various aspects of lake ecosystem. In addition, because of the presence of hypersaline conditions on Mars, studies of biodiversity in terrestrial saline environments like Lonar crater lake may have implications for the possibility of life on Mars [11]. An interesting feature of Lonar crater lake is myth in local and tribal people that showers from Lonar crater lake water (LCLW) helps to eradicate skin diseases. The lake finds citation in historical document Ain-i-Akbari indicating LCLW was used for making soaps in 16th century in India. These observations prompted us to investigate the therapeutic potential of LCLW salt against skin diseases.

Psoriasis is a chronic inflammatory skin disease characterized by the loss of normal cellular homeostasis, enhanced epidermal proliferation, altered rates of differentiation with parakeratosis and inflammation [12]. The increased rates of cell proliferation and abnormal differentiation of keratinocytes is a consequence of the over expression of growth factors, their receptors, cytokines and angiogenic peptides [13,14]. Dithranol and acitretin are common drugs employed in antipsoriasis therapy [15]. Psoarisis has high correlation with angiogenesis. It is driven by several angiogenic factors with the change in the microvasculature during psoriasis. Pro-angiogenic mediators, such as tumor necrosis factor (TNF), vascular endothelial growth factor (VEGF), hypoxia inducible factor (HIF), IL-8 or angiopoietins and other angiogenic peptides are enriched in psoriatic skin [16]. Currently, wide variety of antiangiogenic drugs are available for treatment of psoriasis. Methotrexate is the most commonly used drug in systemic therapy for psoriasis that inhibits dihydrofolate reductase and therefore inhibits proliferation of immune tissue [17]. The immune suppressants like cyclosporine inhibit calcineurin whereas corticosteroids inhibit cytokine [18]. Other antiangiogenic drug used in psoriatic treatment are: Vitamin D3 analog [19], anti-TNF antibodies [20] and fumaric acid esters [21] are well established. Though several antiangiogenic drugs are well established in practice [22], many of them suffer from adverse drug reactions (ADRs) [23]. In addition, current drugs have several drawbacks in terms of efficacy, toxicity and undesirable side-effects and lead to poor patient compliance [24]. In view of these drawbacks, there remains a distinctive need to develop new agents for the more efficient treatment of psoriasis.

In view of myth regarding skin disease curing ability of LCLW and looking at need to develop more efficient antipsoriasis agents, we report herein our pilot study on the evaluation of therapeutic potential of salt obtained from LCLW (acronymed as LCLW salt) against psoriasis. Besides, vascular proliferation and angiogenesis being a key factor in psoriasis, we also report herein the antiangiogenesis effect of the LCLW salt.

EXPERIMENTAL

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and pen strep (a mixture of penicillin and streptomycin) were procured from Gibco Life Technologies (Auckland, New Zealand); MTT reagent, gentamycin (4 mg/mL) and amphotericin B (5 mg/mL) were purchased from Himedia Pvt. Ltd., Mumbai, India. Human monocytic leukemia (THP-1) cell line was procured from the National Center for Cell Science, Pune, India. X-ray photoelectron spectroscopy (XPS) analysis was performed in a Kratos AXIS Supra model instrument under 10^{-9} torr. The measurements were carried out with monochromatic Al Kα photons (1486.6 eV). The power of the X-ray source was kept constant at 600 W. The infrared spectrum was recorded on a Bruker ALPHA ECO-ATR spectrometer.

Isolation of LCLW salt: Water samples were collected from sampling site of Lonar crater (Kamaljamata temple) in 5 L air tight polythene container following APHA guidelines [25] and carried to the laboratory. The physical and chemical parameters were analyzed as per standard methods. Sampling was done in May 2015 at morning. The pH, temperature, hardness, alkalinity, turbidity and TDS were determined at the spot rest of the parameters. The pH of the LCLW was 8.72 and the temperature was 29 °C. The LCLW was evaporated at 100 °C to obtain desired LCLW salt. The salt concentration was approximately 2.5 %.

MTT cell proliferation assay: Human monocytic leukemia (THP-1) cells were counted by tryphan blue dye exclusion assay using a hemocytometer. The cells were seeded to 96 well plate at the density of 1×10^4 cells per well per 0.1 mL medium and were allowed to adhere by incubating for 24 h at 37 °C and 95 % humidity in CO_2 incubator (Eppendorf, New Brunswick, Galaxy 170R, Germany). The LCLW salt and methotrexate (1000, 500, 250, 125, 62.6 and 31.25 µg/mL) were seeded followed by incubation at 37 °C and 5 % CO_2. Cytotoxicity was evaluated at time intervals of 24, 48 and 72 h. After incubation, 20 µL of MTT solution (5 mg/mL in phosphate buffer saline) was added to each well and the plate was incubated in dark for 4 h at 37 °C. On completion of 4 h, supernatant was discarded and DMSO (100 µL) was added to each well to dissolve formazan crystals and absorbance was recorded at 492 nm filter using ELISA plate reader (Lisa Plus, India). The absorbance was calculated for percent cell viability filter using ELISA plate reader (Lisa Plus, India). The absorbance was calculated for percent cell viability [26,27].

Chick chorioallantoic membrane assay: The chick chorioallantoic membrane assay was performed by using zero hour black australorp fertilized eggs (N = 60 including YSM and CAM model) procured from Central Egg Hatching Centre, Kolhapur. The eggs were disinfected with 70 % ethanol and incubated in an incubator at 37 °C and 70 % relative humidity (monitored by hygrometer) until experiment. The LCLW salt concentration ranging from 100 µg/mL to 300 µg/mL was injected by window method on day 4 (Yolk sac membrane) and day 8 (chick chorioallantoic membrane) of incubation; window was closed with sterile surgical tape and was returned to the incubator for further development. Eggs were opened and the CAM was carefully dissected out of the eggs after 48 h of injection to assess angiogenesis, macroscopically. Untreated eggs were maintained as normal group, whereas 0.9 % saline injected eggs were used as control [28].

Macroscopic analysis: The eggs were broken off and antiangiogenic effect was assessed on all eggs after 48 h of injection. CAM was surgically removed from eggs in a bowl. Photographs of the developing CAM and its vasculature of both control

and treated eggs were obtained with a digital camera and exported to a computer for image analysis. The CAM area and the number of blood vessels were assessed.

Histology: The CAM was surgically removed and fixed in 10 % buffered formaldehyde for 10 h, dehydrated in graded alcohol, cleared in xylene and embedded in paraffin. Thick sections (5 µm) were cut in a plane parallel to the surface of the CAM and stained by haematoxylin-eosin which was observed under a light photomicroscope.

RESULTS AND DISCUSSION

Initially, the LCLW sample was collected following APHA guidelines. Various physico-chemical parameters of LCLW were studied and are summarized in Table-1. The results reveal high concentration values of most of studied physical and chemical properties.

TABLE-1
PHYSICO-CHEMICAL PARAMETERS OF LONAR CRATER LAKE WATER

Parameter	Result	Parameter	Result
pH	8.72	Alkalinity	6200 ppm
Temperature	27 °C	Salinity	7546 mg/L
Odour	Grouty	Turbidity	2.57 NTU
Colour	Dark green	CO_2	5.3 mg/L
Conductivity	21.6 µS/cm	Chlorides	4650 ppm
TDS	7561 ppm	Sulphate	180 ppm
Total hardness	640 ppm	–	–

LCLW was evaporated to get the desired LCLW salt. The X-ray photoelectron spectroscopy (XPS) analysis was performed to identify the composition of LCLW salt (Fig. 1). The XPS survey scan shows the presence of various salts such as phosphates (133 eV), sulphides (161.5 eV), sulphates (169 eV), chlorides (199 eV), carbonate (344 eV) and phosphate (349 eV) of calcium. Further, the survey spectrum of LCLW salt show the presence of salts of calcium, potassium, manganese, iron, cobalt, barium, nickel, copper, sodium and zinc. Moreover, the peaks for KCl and KBr appeared at 291 eV and 293 eV. Additionally, the peak at 780 eV appeared due to the presence carbonates of barium while peaks at 856 eV and 934 eV indicated the hydroxides of nickel and copper. The peak at 1071 eV appears for sodium due to auger electrons [29-33]. The LCLW salt was further subjected to FTIR analysis (Fig. 2). The FTIR spectrum indicated prominent peaks for sodium nitrate (1424 cm^{-1}), magnesium phosphate (1155 cm^{-1}), calcium carbonate (876 cm^{-1}), sodium carbonate (700 cm^{-1}) and calcium sulphate (639 cm^{-1}). After characterization, the LCLW salt was used for further studies without purification.

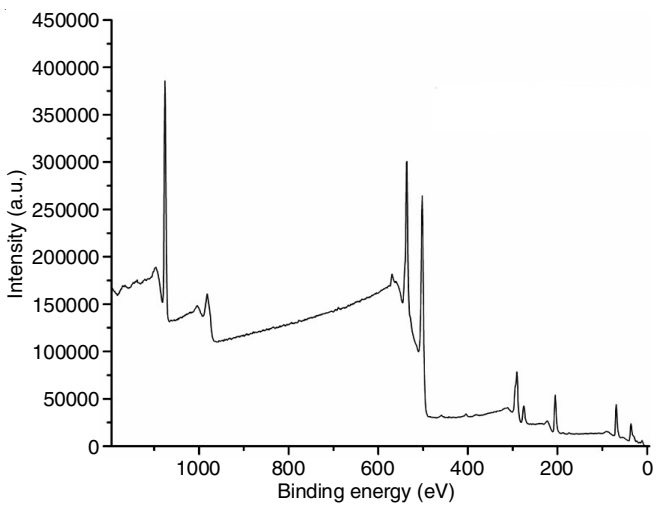


Fig. 1. XPS spectra of LCLW salt

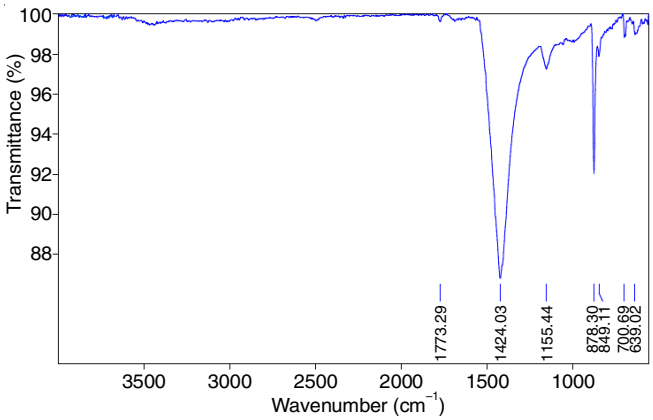


Fig. 2. FTIR spectra of LCLW salt

The antipsoriatic activity of LCLW salt was evaluated against human monocytic leukemia (THP-1) cells using MTT assay. The cells were seeded with different doses of LCLW salt and methotrexate and incubated at 37 °C. After 24, 48 and 72 h of incubation, cell viability was measured following general procedure. To focus the activity, the half maximal inhibitory concentration (IC_{50}) was calculated as the concentration required to inhibit the growth of THP-1 cells in culture by 50 % compared to the untreated cells. The IC_{50} values for LCLW salt and methotrexate are listed in Table-2. The results demonstrated inhibition of cell proliferation of THP-1 in a concentration dependent manner. At 24 h, LCLW salt was found to show no inhibitory effect on cell proliferation at concentrations greater than 1000 µg/mL but exhibited a concentration dependent cytotoxic effect with IC_{50} values 436.8 and 318.6 µg/mL at 48 and 72 h respectively.

TABLE-2
IC_{50} VALES OF LONAR CRATER LAKE WATER (LCLW) SALT AND METHOTREXATE

Time interval (h)	IC_{50} values (µg/mL)	
	LCLW salt	Methotrexate
24	< 1000	547.4
48	436.8	319.4
72	318.6	> 31.25

It is noteworthy to mention that IC_{50} value of methotrexate at 48 h is 319.4 µg/mL which is close to LCLW salt. These results indicate that LCLW salt exhibits remarkable antipsoriatic activity (Fig. 3). The results further revealed that LCLW salt is more selective to THP-1 cells than normal cells as there was low cytotoxic effect observed towards vero cell lines.

There is a close relationship between psoriasis and angiogenesis as the treatment of antiangiogenic agents may be the promising way in treating psoriasis. Physiological angiogenesis

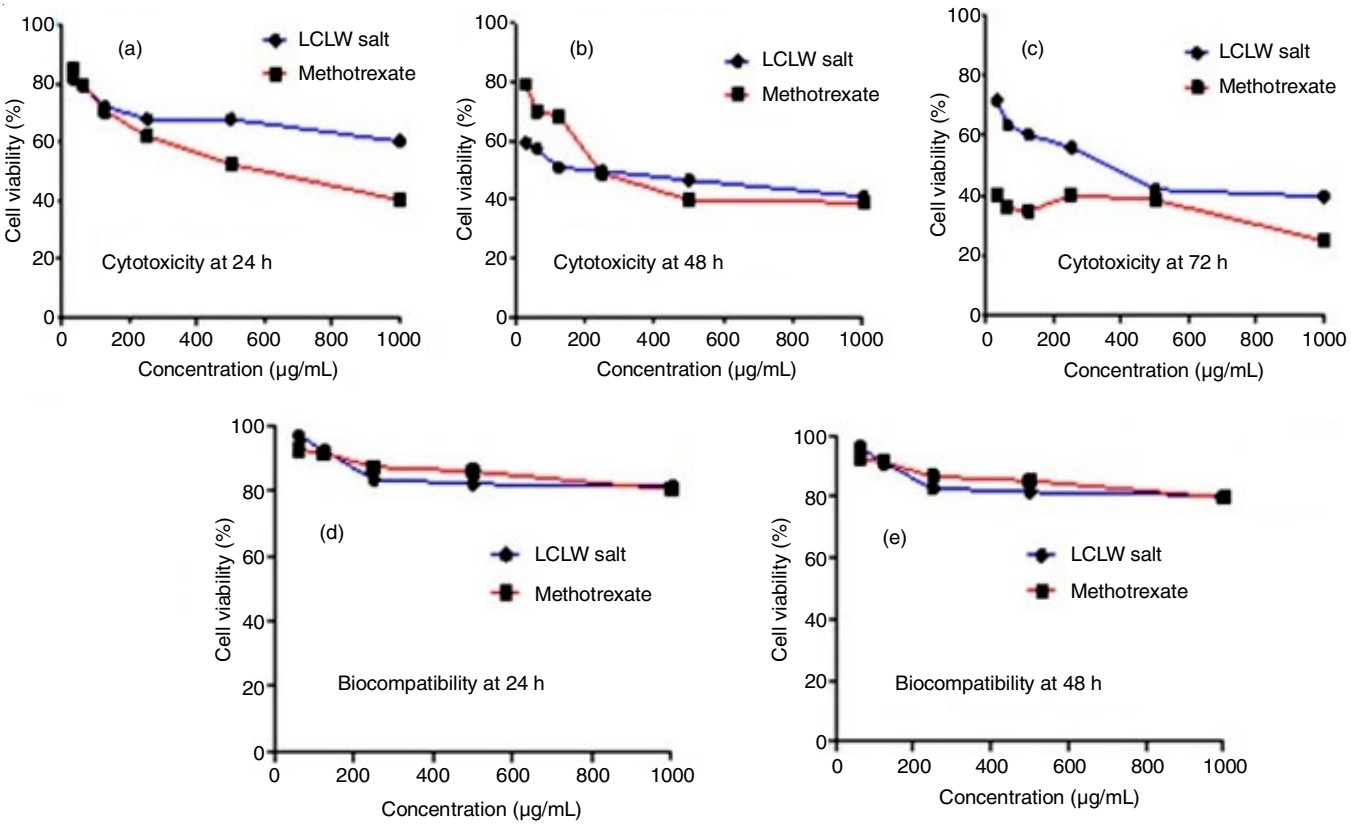
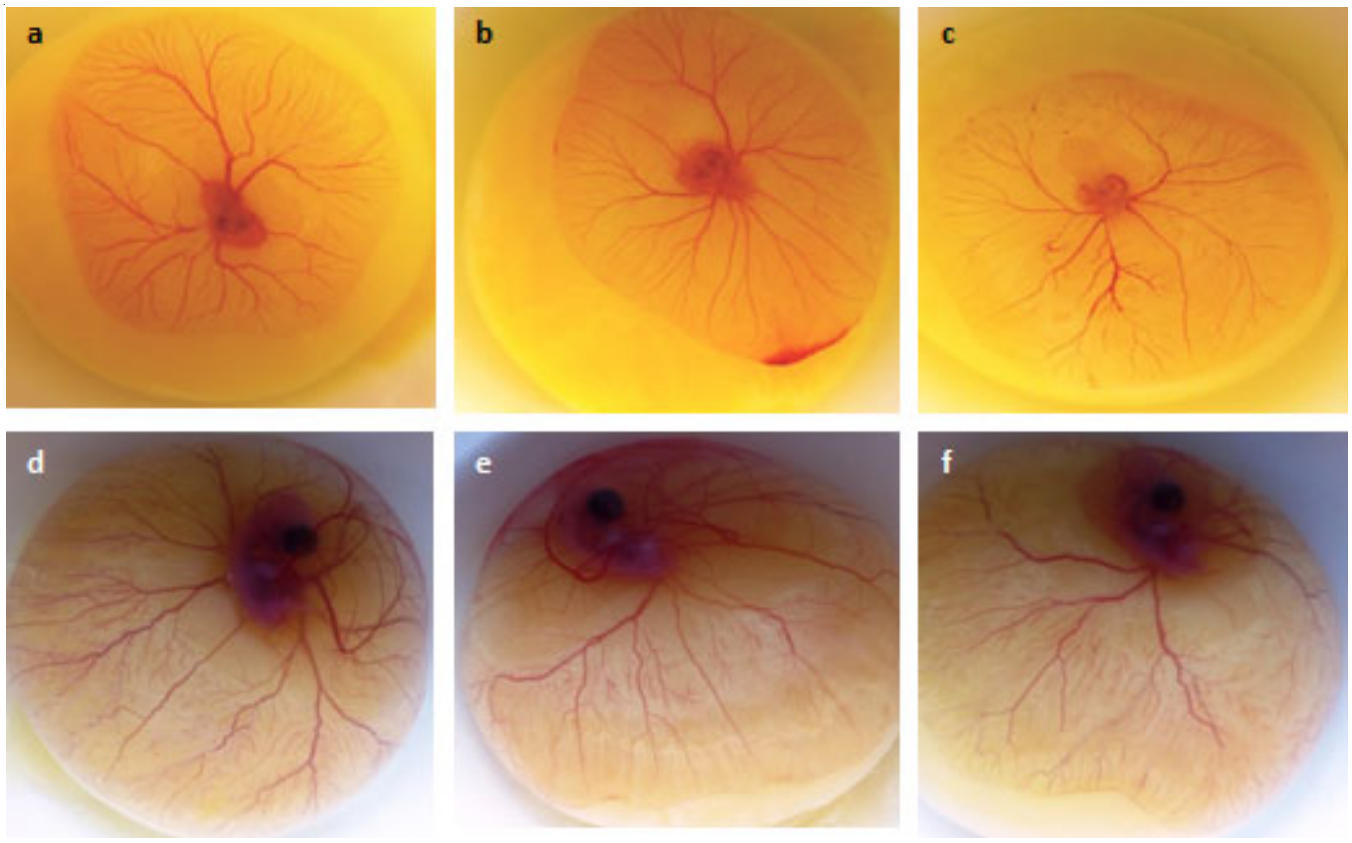


Fig. 3. Cytotoxicity of LCLW salt at 24, 48 and 72 h incubation on THP-1 Cells (a-c) and biocompatibility against vero cells at 24 h and 48 h (d-e)



(a & d) Control, (b & e) 200 µg/mL, (c & f) 300 µg/mL (on 6th and 8th day of incubation resp.)

Fig. 4. Effect of LCLW salt on yolk sac model (a-c) and CAM (d-f)

is induced only transiently during processes such as wound healing, pregnancy or in the corpus luteum formation during female reproductive cycle. However, pathological angiogenesis occurs under conditions such as tumor growth, retinopathy and chronic inflammation, as observed during rheumatoid arthritis or psoriasis [34,35]. This created inquisitiveness to study the antipsoriatic and antiangiogenic activity of LCLW salt. The chicken chorioallantoic membrane (CAM) assay was undertaken to study antiangiogenic effect of LCLW salt similar to other antiangiogenic drugs reported [36]. After treating the CAM with LCLW salt, the vascular network presented several macroscopic changes as compared to the control group. Antiangiogenic effect was seen at both 200 and 300 µg/mL, with significant result obtained at 300 µg/mL (Fig. 4). Inhibition of blood vessel formation and branching pattern was evident at 48 h of treatment. Hemorrhagic areas were observed between modified capillaries. Dilated, irregular vessels with stasis coupled with short capillaries as compared to control were significantly observed at 300 µg/mL. Sterile 1X PBS which is used as vehicle solution (negative control), did not show any antiangiogenic effect. There was apparent reduction in the number of tertiary vessels in LCLW salt treated CAM as compared to control (Table-3, Fig. 5). However, unlike 200 µg/mL, there were apparent differences in number of primary and secondary vessels seen at 300 µg/mL.

Evaluation for microscopic specimens confirmed the macroscopic observations. CAM treated with control, appeared well vascularized where the blood vessels were well formed with distinct capillary plexus beneath the ectoderm. However, the CAM treated with LCLW salt was hemorrhagic and extra-

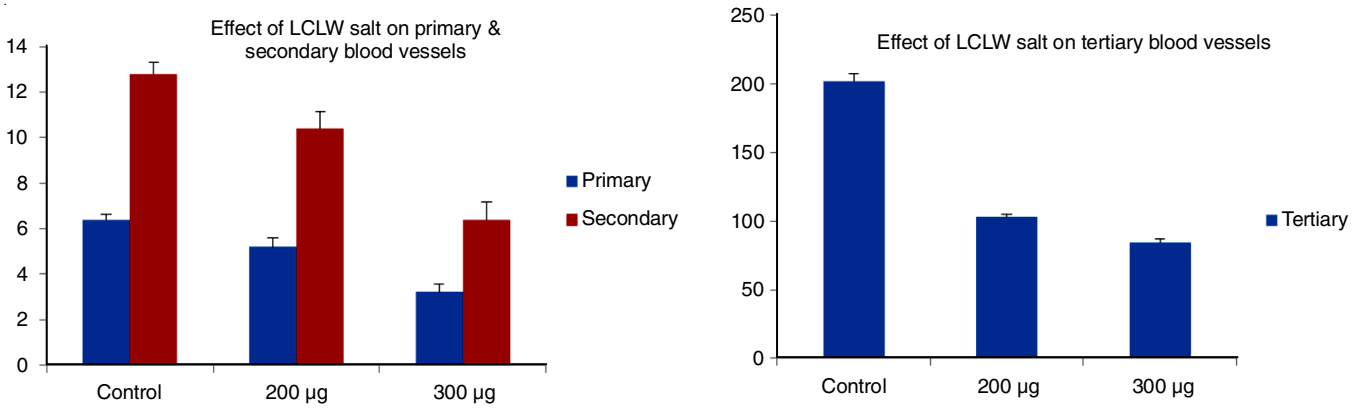
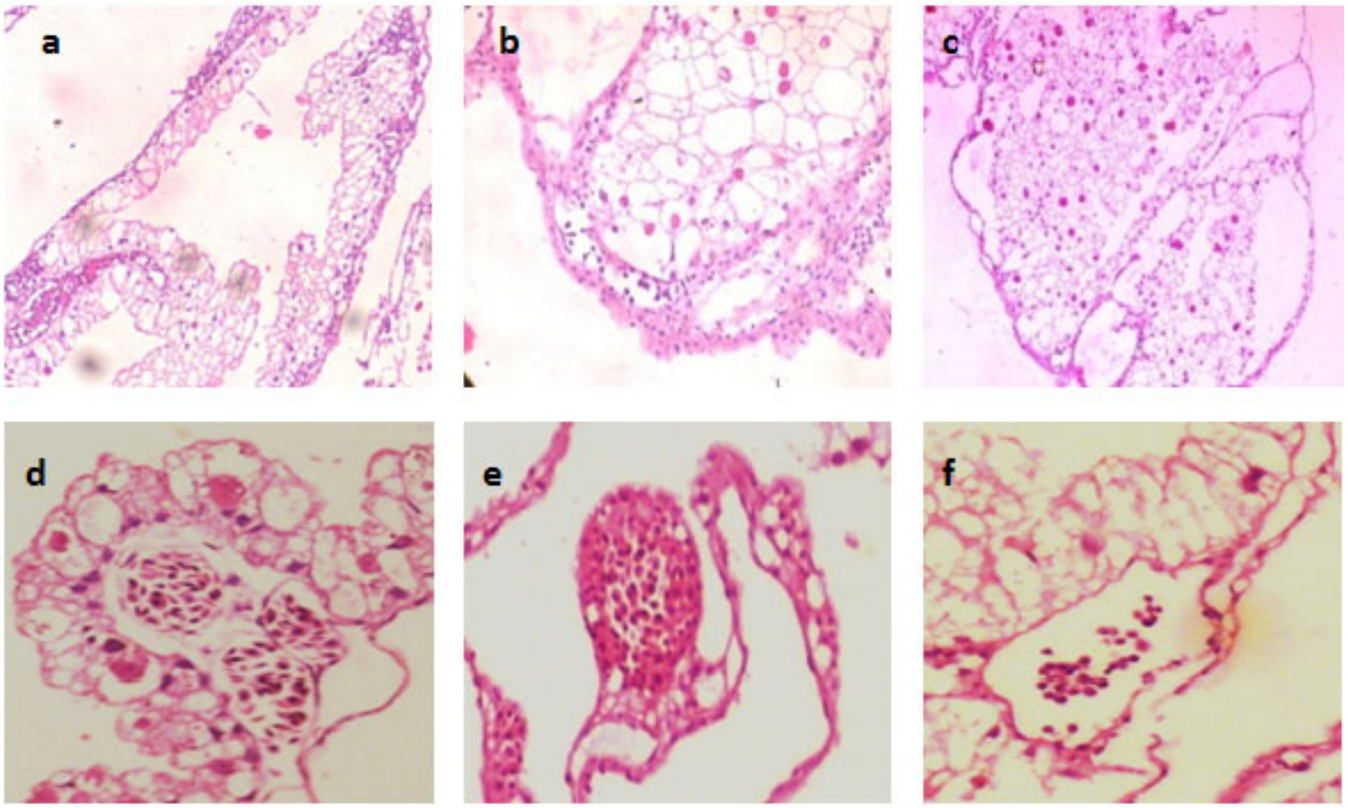


Fig. 5. Effect of LCLW Salt on primary, secondary and tertiary blood vessels



(a & d) Control, (b & e) 200 µg/mL, (c & f) 300 µg/mL (on 6th and 8th day of incubation resp.)

Fig. 6. Antiangiogenic effect of LCLW salt on chick CAM

TABLE-3
AVERAGE NUMBER OF BLOOD VESSELS OF NORMAL AND LONAR CRATER LAKE WATER (LCLW) SALT TREATED CHICKEN CHORIOALLANTOIC MEMBRANE (CAM)

Experimental group	Number of blood vessels		
	Primary	Secondary	Tertiary
Control	6.4 ± 0.24	12.8 ± 0.49	202.2 ± 5.65
200 µg	5.2 ± 0.37	10.4 ± 0.75	103 ± 2.19
300 µg	3.2 ± 0.37	6.4 ± 0.75	84.6 ± 2.52

vasations of RBCs in the mesenchyme tissue of CAM are seen. Unlike the vehicle control, the LCLW salt treated CAM showed no distinct and well differentiated germ layers as shown in Fig. 6.

Conclusion

In conclusion, the present study reveals remarkable anti-psoriatic and antiangiogenic activity of salt obtained from Lonar crater lake water. It is believed that after further intense investigations, LCLW salt could emerge as an ideal source of therapeutics in treating profused angiogenesis as well as an ideal source towards psoriasis treatment. Further in-depth study in this regard, would create a leap towards formulating a novel composition with LCLW salt in treating psoriasis successfully in the nearby future.

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