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Antiphotoaging effects of liposomal encapsulated superoxide dismutase extract of *Bacillus altitudinis* on ultraviolet-A-irradiated New Zealand rabbits' back skin

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ABSTRACT

Photoaging can occur due to sunlight exposure that emits ultraviolet-A rays. Therefore, this study aims to assess the antiphotoaging activity of superoxide dismutase (SOD) extract of *Bacillus altitudinis* liposomes on rabbit back skin. The liposomes were encapsulated using the film method. The antiphotoaging test was carried out on the back skin of New Zealand rabbits which were separated into four groups, namely normal, negative, and positive controls, as well as liposome *B. altitudinis*. They were then exposed to ultraviolet-A (UV-A) light for 2 weeks. The treatment was carried out by applying liposome extract of *B. altitudinis* to the back skin of rabbits once a day for 28 days. Observations were made to assess the increase in the percentage of collagen, elasticity, and moisture content. The dorsal skin of the test animals was observed histologically using van Gieson staining. The results showed that rabbits exposed to UV-A light for 2 weeks experienced a decrease in collagen, elasticity, and moisture contents. Liposomes of *B. altitudinis* can increase the collagen level elasticity and moisture content by 6.46%, 5.10%, and 26.90%, respectively. The histological staining revealed that *B. altitudinis* liposomes increased collagen levels by 36.62%. These results indicate the potential value of *B. altitudinis* liposomes as photoaging agents.

INTRODUCTION

The skin is the most prominent organ in the human body and accounts for approximately 16% of the total body mass. The epidermis and dermis are the two major components, where the epidermis is the outermost layer and is in direct contact with the external environment (Amaro-Ortiz *et al*., 2014). Consequently, it is often exposed to direct sunlight. Three forms of ultraviolet radiation reach the Earth, namely ultraviolet-A (UV-A) (315–400 nm), UV-B (280–315 nm), and UV-C (100–280 nm). Previous studies showed that sunlight contains 95% UV-A and 5% UV-B (Leiter *et al*., 2014). Through the stratospheric ozone, UV-C and UV-B can be removed from the Earth's surface (Rivas *et al*., 2020). Continuous exposure to UV-A rays can lead to short- and long-term health conditions, including erythema, photoaging, photo immunosuppression, and skin cancer (Rivas *et al*., 2020; Savoye *et al*., 2018).

Skin aging can be caused by a decline in the physiological activities of the organ. Extrinsic factors, such as UV radiation, stress, reactive oxygen species (ROS), and chemicals, can contribute to this process (Kang *et al*., 2020). UV-A rays can penetrate the dermis, where they cause DNA damage, oxidative stress, wrinkles, and inflammation (Tyrrell, 2004). It can also cause burns and accelerate photoaging on sensitive skin. Some signs of photoaging include wrinkling, wilting, laxity, sagging, patchy

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pigmentation, and dryness (Svobodova *et al*., 2006). Aging skin can progress to dermal atrophy and decreased fibroblast activity, thereby leading to decreased elasticity, increased roughness, and the formation of fine rhytids, as well as deeper and persistent folds (Sulistyoningrum *et al*., 2019). The inclusion of superoxide dismutase (SOD) is one strategy to prevent this photoaging (Hwang *et al*., 2019).

SOD is an antioxidant metalloenzyme that neutralizes free radicals caused by an increase in the level of ROS (Ighodaro and Akinloye, 2018). ROS level increases along with the duration of UV exposure (Thakur *et al*., 2017). To prevent cell damage, SOD has an action mechanism, which involves the conversion of superoxide anion (Islam *et al*., 2021) (Che *et al*., 2017). In the presence of the catalase enzyme, hydrogen peroxide (H_2O_2)in the mitochondria is converted to H2O and O2, and the glutathione peroxidase enzyme detoxifies H2O2 that diffuses into the cytosol (Hwang *et al*., 2019). Several studies revealed that SOD is present in various species of bacteria and fungi (Kang *et al*., 2020).

Previous studies also reported that *Bacillus altitudinis* has SOD activity against Alternaria alternata (Sun *et al*., 2021). Another study showed that *B. altitudinis* exhibited similar activity of 85.09% ± 1.24% (unpublished data). Free radicals are one of the leading causes of skin aging, and SOD has a powerful ability to remove them (Shanbhag *et al*., 2019). SOD is expected to be utilized as cosmetic raw material with antiphotoaging activity. SOD treatment is expected to reduce the signs of skin aging, such as decreased collagen, elasticity, and skin moisture (Zhou *et al*., 2020).

The use of liposomes as a delivery mechanism for lipid-based active compounds has been developed. The increasing utilization of these molecules is due to their biodegradability, biocompatibility, and low toxicity. They can also transport both active water-soluble and fat-soluble active substances. Due to their structure, chemical composition, and size, liposomes can be used as carriers of active substances in various applications (Hou *et al*., 2022).

MATERIALS AND METHODS

Materials

Phosphate buffer solution, ethanol P.A., phospholipids (Phospholipon 90G), cholesterol (Sigma), and sterile distilled water were acquired from Sigma/Aldrich, St. Louis, MO. Furthermore, six male New Zealand rabbits, aged 7–8 months, were procured from the Pharmacology Laboratory of Setia Budi University, Surakarta, Indonesia. Formalin and van Gieson's staining were purchased from Oxoid, Hampshire, UK. Nutrient agar (NA), brain heart infusion (BHI), and phosphate buffer saline (PBS) were obtained from Merck®, Darmstadt, Germany.

Extraction of the enzyme SOD *B. altitudinis*

A total of 1–2 doses of *Bacillus altitudinis* bacteria suspension grown on NA media was taken and placed into 10 ml of BHI media, followed by incubation at 37°C for 24 hours. SOD enzyme extraction was carried out by inoculating 2% of the suspension into 300 ml BHI and then it was incubated at 37°C for 24 hours. The results of bacterial incubation were harvested by centrifugation at 5,000 rpm and 4°C for 15 minutes with three repetitions. The pellets obtained were mixed with PBS buffer. The cells were lysed using mechanical methods, namely sonication at an amplitude of 50 for 5 minutes with a pause every 1 minute under cold temperatures. The suspension was centrifuged again at 5,000 rpm for 15 minutes at a cold temperature. The supernatant obtained was then placed in a microtube and stored at 4°C as a SOD extract (Guo *et al*., 2015) (Hartmann and Asch, 2019).

Liposome encapsulation SOD extract of *B. altitudinis*

Liposomes were produced by combining 50 µM/ml phosphatidylcholine and 25 µM/ml cholesterol dissolved in 5 ml of ethanol in a 300 ml round-bottom flask until it became homogenous. The solvent was evaporated under a pressure at 50°Cin a rotary evaporator until a thin lipid film was formed on the surface of the flask. The hydration of the thin film was carried out using 5 ml of *B. altitudinis* SOD enzyme extract at 0.5 mg/ml concentration in phosphate buffer solution (PBS, PH = 7.8) with a rotary evaporator for 15 minutes at 37°C. At 4°C, the liposome dispersion was sustained for an hour, and a sonicator was used for 8 minutes to produce equally sized products (Karn *et al*., 2013; Picon *et al*., 1995; Porfire *et al*., 2009). Liposome SOD was characterized and evaluated by calculating the encapsulation efficiency and measuring the particles. The encapsulation efficiency test was carried out by calculating the absorption of the solution, followed by measurement with a UV–Vis spectrophotometer at the maximum wavelength. Subsequently, the concentration was calculated using the calibration curve equation and the free drug levels (FD). The experiment was carried out three times, and the adsorption efficiency was calculated using the formula:

$$\%\ EE = \frac{TD\text{-}FD}{TD} \times 100\%$$

Description:

TD = total compounds contained in the formula

FD = the number of compounds detected in the supernatant (not adsorbed)

The liposome particle size test was carried out using a PSA (particle size analyzer) in the technology laboratory of Yogyakarta State University, Indonesia.

Antiphotoaging activity of SOD extract of *B. altitudinis* liposomes on the back skin of New Zealand rabbits

Animal acclimatization

Six male New Zealand rabbits, weighing 2–4 kg and aged 7–8 months, were procured from the Pharmacology Laboratory of Setia Budi University, Surakarta, Indonesia. A cage was set up for the test animals and they were acclimatized for 1 week (Lee *et al*., 2014). Standard laboratory settings of ambient temperature (25°C ± 2°C), relative humidity (55 ± 5%), and a 12-hour light /12-hour dark cycle were applied to the rabbit cage. They were fed a regular pellet diet with unrestricted access to water. All animal tests were carried out in line with the guidelines given by the Health Research Committee of Dr. Moewardi General Hospital, Indonesia (Decision Number: 213/II/HREC/2021) (Chen *et al*., 2019).

Animal model: UV-A irradiation

A total of six rabbits were used in this study, and their back was shaved. The skin was cut into four sections, forming a

circle with a diameter of 2 cm (Manosroi *et al*., 2012). The exposed back skin of the rabbit was treated using an *Exoterra® Daylight Basking Spot* lamp containing UV-A rays at a 30 cm distance with 63.69 J cm^{-2}/hour for 6 hours per day in 2 weeks (Amaro-Ortiz *et al*., 2014; Chen *et al*., 2019). A skin analyzer was used to assess the wrinkle formation based on the collagen, moisture, and elasticity percentages.

Liposome treatment on back skin of rabbits

The rabbit's back was shaved to expose the skin in four sections: part I: normal control, without UV-A radiation, part II: negative control (liposomes without *B. altitudinis* extract), part III: positive control (collagen liposomes), and part IV: *B. altitudinis* liposome extract. Liposomes with and without *B. altitudinis* extract, as well as collagen liposomes, were smeared onto the backs of UV-A-induced rabbits once a day for 28 days (Duraivel *et al*., 2014; Suksaeree *et al*., 2018). Wrinkle parameters were then analyzed after the treatment. Furthermore, the percentage of collagen, moisture, and elasticity was measured using the skin analyzer. The device was linked to a computer, where the *Skin Analyzer EH 900 U driver* CD has been loaded. The skin was analyzed and photographed with a camera handset, after which the photographs and data obtained were input into a computer for electronic microscopy analysis. The skin images and analysis results were displayed on the monitor (Lee *et al*., 2014).

Histological staining

After 28 days of treatment, rabbit skin tissue was harvested without the animals' suffering. They were placed in a closed container with cotton containing ether at a lethal dose (403 ppm) (Boatman *et al*., 2014) and left until the animals died. For each treatment, tissue with a 2 × 2 cm diameter and a 0.5 cm thickness was extracted. It was then treated with 10% formalin in a volume 10 times the amount of tissue removed. The test animals were then burned and the ashes were properly buried. The extracted skin sample was placed in a 10% neutral buffered formalin solution for 24 hours. After fixation with a buffered formalin solution for 8–48 hours, the biopsied/operated-on tissue slices were deparaffinized with xylene, followed by van Gieson's staining. The sample was then embedded in paraffin, sliced to a thickness of 1–20 µm, stained with van Gieson, and viewed under a microscope. Observations were made to identify the collagen formed as a fiber in rabbit skin tissue. In a picture by van Gieson containing picric acid and collagen, fuchsin acid often appears red (Chen *et al*., 2019).

Data analysis

The results of antiphotoaging activity on rabbit back skin using a skin analyzer with parameters of collagen percentage, moisture content, and elasticity percentage were analyzed using paired *T*-Test and ANOVA statistical methods before and after the treatment.

RESULTS AND DISCUSSION

Liposome encapsulation of *B. altitudinis* extract

Phosphatidylcholine (*Phospholipon 90 G*) and cholesterol were evaporated with a rotary evaporator to produce liposomes using a modified thin-layer hydration approach. The thin layer formed on the flask's wall was then hydrated until a liposome layer was formed using a phosphate buffer solution containing SOD extract. Furthermore, the liposome layer obtained had a slightly whitish color, as shown in Figure 1.

Liposome encapsulation results from SOD of *B. altitudinis* extract yielded 5 ml of liposomes. The film method was appropriate for the production of large liposomes, which can encapsulate SOD. During the production process, the SOD enzyme was able to sustain its activity. Low- or high-molecular-weight compounds can be absorbed by lipomers as active molecules (Porfire *et al*., 2009).

The hydration phase of the encapsulation procedure optimized the absorption of the active substance. SOD encapsulation was also performed at 50°C with a certain pressure. The method used in this study is in line with that of Karn *et al*. (2013), where the process was performed at 450°C and 10 megapascals. The results of the measurement test using PSA showed that the liposome SOD extract of *B. altitudinis* met the standard nanoparticle size, namely 90–150 nm. The particle size obtained was included in the nanocategory. The smaller the particle size is, the easier the active compound is distributed to target cells or tissues (Naibaho *et al*., 2019). Measurement of encapsulation efficiency of liposome SOD extract of *B. altitudinis* showed a value of 80%. This indicates that the method can be used to adsorb the test compound to ensure that a high content of the compound is obtained during the encapsulation process (Filipczak *et al*., 2020).

Previous studies revealed that human recombinant Cu/Zn- SOD (rh-Cu/Zn SOD) can mitigate thermal harm caused by local burns. After 24 hours of treatment, human recombinant Cu/Zn-SOD gel encapsulated in liposomes can minimize the size of skin lesions caused by thermal injury. Rh-Cu/Zn SOD gel encapsulated in liposomes can also diminish skin swelling after 72 hours of treatment (Vorauer-Uhl *et al*., 2001). Therefore, this study aims to determine the antiphotoaging activity of *B. altitudinis* liposomes on rabbit back skin.

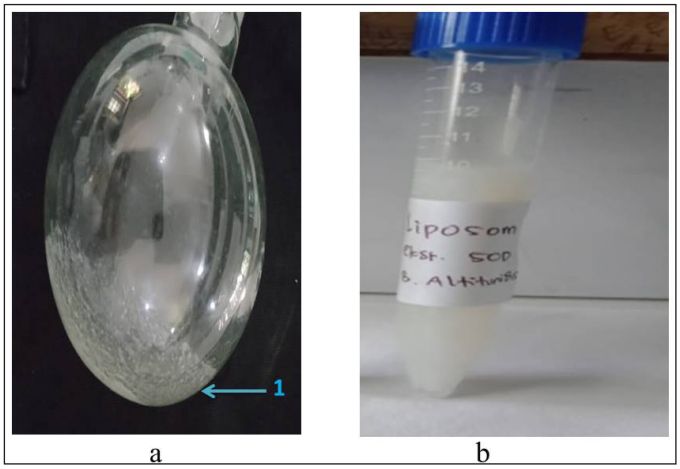

Figure 1. Liposome encapsulated SOD extract of *B. altitudinis*, a: formation of the liposome layer, b: liposome encapsulation of SOD of *B. altitudinis*, 1: liposome layer.

UV-A irradiation of the rabbit's back skin

Rabbit's back skin was exposed to UV-A for 2 weeks, which reduced the percentage of collagen, elasticity, and moisture in the skin, as shown in Table 1.

The results of this study reveal that exposure to UV-A rays can decrease the percentage of collagen, elasticity, and moisture. This indicates that the rays can alter the biological mechanism of the skin, specifically accelerating aging. This finding is consistent with a previous study that UV-A can harm collagen fibers (Svobodova *et al*., 2006). Matrix metalloproteinases (MMPs) with elastase activity were expressed at higher levels in the dermis after repeated exposure. These increased levels affect the tissue's elastic fibers and can decrease skin elasticity (Imokawa and Ishida, 2015).

Exposure to UV-A rays can lead to several adverse effects, including degenerative aging, inflammation, and cancer. Sunlight carries 90%–95% of the environment's UV-A because it can penetrate the ozone layer (D'Orazio *et al*., 2013). Furthermore, UV-A has a longer wavelength compared to UV-B and UV-C, thereby allowing it to penetrate deeper into the dermis. This ray is also effective at creating ROS that can indirectly harm DNA through photosynthesis (Svobodova *et al*., 2006).

UV exposure accounts for 50% of skin damage, where it induces the generation of ROS, including superoxide anion radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen (O_2). ROS destroys collagen by increasing the level of cytokines and lowering the synthesis of the transcription factor, which decreases collagen production (Zhuang *et al*., 2009). Changes in the mechanical characteristics of the stratum corneum and significant macroscopic skin damage, including chapping and cracking, inflammation, infection, scarring, and aberrant desquamation, are caused by UV exposure (Biniek *et al*., 2012) revealing that UV can significantly reduce intercellular strength, strain, and cohesiveness.

Antiphotoaging of liposome encapsulating SOD extract of *B. altitudinis*

The percentage of collagen in the back skin of rabbits is one of the antiphotoaging factors, and it was determined using the *Skin Analyzer EH 900 U*. The resulting area under the curve (AUC) values are presented in Table 2.

Exposure to UV-A reduced the elasticity of rabbit's back skin, while treatment with *B. altitudinis* liposome extract increased this parameter. The percentage improvement in skin elasticity of rabbit's back skin after bending is presented in Table 3.

Treatment of the back skin of rabbits with liposome extract of *B. altitudinis* after UV-A exposure can enhance skin moisture content by more than 25%, as shown in Table 4.

The treatment with liposome extract of *B. altitudinis* can significantly enhance the percentage of collagen, elasticity, and moisture compared to the negative control, as shown in Tables 2–4.The improvement is associated with the SOD extract content in liposomes, which reduced ROS levels, thereby allowing the skin to produce more collagen and elastin. These findings are in line with those of Thakur *et al*., 2017, that SOD obtained from the thermophilic bacterium *Bacillus licheniformis* SPB-13 has strong antioxidant activity against ROS. The addition of chemical

Table 1. Decrease collagen, elasticity, and moisture content percentage before and after UV A exposure.

Group	Before UV-A exposure	After UV-A exposure	Decreased (%)
Collagen	61.0 ± 1.83	55.0 ± 1.33	9.80
Elasticity	62.0 ± 2.05	55.7 ± 1.25	10.20
Moisture	12.2 ± 2.20	7.3 ± 0.95	40.20

Table 2. AUC value and collagen percentage gain level.

Group	AUC value	Collagen percentage gain level (%)
Negative control	1,552.9 ± 14.2 ^{bcd}	0
Normal control	1,707.2 ± 22.7 ^{abd}	9.94
Positive control	1,777.0 ± 41.8 ^{acd}	14.43
Liposome of *B. altitudinis*	1,653.3 ± 22.7 ^{abc}	6.46

Description: a = significantly different from negative control, b = significantly different from positive control, c = significantly different from normal control, d = significantly different from *B. altitudinis* group.

Table 3. AUC value and elasticity percentage gain level.

Group	AUC value	Elasticity percentage gain level (%)
Negative control	1,598.2 ± 15.4 ^{bcd}	0
Normal control	1,703.5 ± 22.3^{a}	6.59
Positive control	1,729.1 ± 26.6 ^{acd}	8.19
Liposome of *B. altitudinis*	1,679.7 ± 30.9 ^{ab}	5.10

Description: a = significantly different from negative control, b = significantly different from positive control, c = significantly different from normal control, d = significantly different from *B. altitudinis* group.

Table 4. AUC value and moisture content gain levels.

Group	AUC value	Moisture content gain (%)
Negative control	230.9 ± 15.4 [d]	0
Normal control	347.0 ± 18.5 [abcd]	50.29
Positive control	335.8 ± 28.7 [ad]	45.45
Liposome of *B. altitudinis*	293.7 ± 29.9 [abc]	26.90

Description: a = significantly different from negative control, b = significantly different from positive control, c = significantly different from normal control, d = significantly different from *B. altitudinis* group.

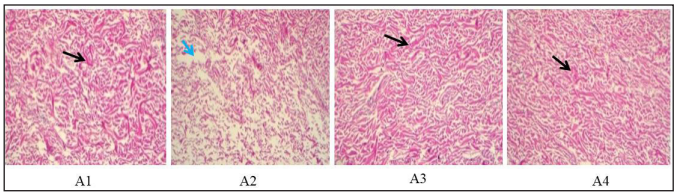

Figure 2. A: (histology) connective tissue of the rabbit's back skin, A1: normal group, A2: negative control group, A3: positive control group, and A4: liposome SOD extract of *B. altitudinis* group, showed collagen density, showed collagen looseness.

Table 5. Collagen density percentage based on the histological observation.

Replication	Normal control (%)	Negative control (%)	Positive control (%)	Liposome of *B. altitudinis* (%)
1	21.82	15.83	38.21	36.47
2	24.45	18.52	38.43	36.55
3	24.87	19.17	41.46	36.83
Average	23.71	17.84	39.37	36.62
SD	±1.65	±1.77	±1.82	±0.19

stressors such as H_2O_2 and menadione induced oxidative stress. The addition of SOD at a concentration of 30 g/ml was able to boost cell viability by 65%. This indicates that SOD can neutralize free radicals, protect cells from harm, and extend their longevity. Hwang *et al*. (2019) revealed that it protects cells against apoptosis triggered by superoxide by converting the compound to O_2 and peroxide. Armaini and Imelda (2021) showed that SOD can be increased by giving Scenedesmus dimorphus polysaccharides (SDP) at a dose of 0.8 mg/g BW. The administration of SDP can stimulate collagen synthesis, repair skin tissue, increase catalase activity, and reduce malondialdehyde better compared to vitamin E. Furthermore, SDP shows benefits as an antioxidant and antiaging agent.

Another study revealed that plant extracts fermented with *Lactobacillus buchneri* (PELB) bacteria can reduce UV-induced antiaging action. PELB can stimulate the expression of skin moisture activator genes and it can increase the level of type I collagen (Kang *et al*., 2020). SOD can inhibit receptor activation and the expression of the AP-1 and MMP genes by increasing collagen levels. It can also suppress the activation of cytokine receptors by ROS due to UV-A exposure. Lee *et al*. (2014) stated that SOD is a very powerful intracellular antioxidant and the first line of defense in neutralizing free radicals by catalyzing the superoxide anion into hydrogen peroxide (H_2O_2) and O_2, thereby reducing the effects of ultraviolet radiation. Active SOD can minimize the production of excessive ROS and significantly reduce DNA and RNA damage that leads to cell death. It can also inactivate the AP-1 formation pathway that regulates MMP expression, thereby preventing collagen degradation (Treiber *et al*., 2012).

Histological staining on the back-skin tissue of rabbits

At the end of the observation (28th day), histological tests were carried out on the rabbit skin using the van Giesen method. Furthermore, van Gieson's staining is commonly used for identifying collagen fibers. On the histological structure of the rabbit's back skin, an increased quantity of red colors indicates an increased collagen density, as shown in Figure 2.

The findings of the study using the *Optilab* tool are displayed as a bright red photograph of the connective tissue area, and the fraction area is entered into the *Image J* application to calculate the percentage of collagen density, as shown in Table 5.

Histological examinations on the skin of the negative group revealed that they have the lowest density of collagen, followed by the normal group, *B. altitudinis* liposomes, and the positive control. In this study, liposomes of *B. altitudinis* can increase the density of collagen by up to 36.62%. This indicates that they can serve as UV-protective agents for the skin. Liposomes of *B. altitudinis* can improve the histological structure of collagen compared to the normal controls, which shows that they have the potential to be used as an anti-aging agent. The average SOD activity of *B. altitudinis* liposomes was 85.09% ± 1.24% (our unpublished data).

Based on a previous study, SOD can maintain oxidation-reduction homeostasis by delaying aging and increasing collagen density. Furthermore, increased collagen stimulated collagen formation by activating the adenosine monophosphate-activated protein kinase and Nrf2/HO-1 pathways in aged mice. Administering SOD can cause skin thickening, as observed in this study, where animals treated with the compound showed thicker skin compared to others. Previous studies revealed that young mice have thicker skin compared to aged mice (Lee *et al*., 2021).

CONCLUSION

The results showed that there was a decrease in the percentages of collagen, elasticity, and moisture after the back of a rabbit was exposed to UV-A. *In vivo* treatment with liposome-encapsulated SOD extract of *B. altitudinis* showed antiphotoaging activity by significantly improving the test parameters.

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AUTHORS' CONTRIBUTIONS

AI and RMR designed the study and wrote the manuscript for publication. NN conducted the laboratory experiments and assessed the data. RMR and SH edited the outline and completed the manuscript.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

ETHICAL APPROVALS

The research protocol for the animal study was approved by the Health Research Committee of Dr. Moewardi General Hospital, Indonesia (Decision Number: 213/II/HREC/2021).

DATA AVAILABILITY

All generated and analyzed data are contained in this article.

PUBLISHER'S NOTE

Regarding jurisdictional claims in published institutional affiliation, this publication is impartial.

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