Anti-inflammatory effect of Phytomarine R-L compound against lipopolysaccharide-induced pulmonary injury in BALB/c mice

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ABSTRACT

Background: This study deals with the induction of lipopolysaccharide-induced inflammation in animal model and treating this condition using phytomarine R-L compound (Bloomin’Age, Science of Living, Milan Italy) that is known to possess the anti-inflammatory properties and eventually determine their characteristics through the in-vivo experimentation.

Objective of the study: The present study was subjected to test the therapeutic anti-inflammatory/anti-oxidative and regenerative property of this phytomarine (SBF-LF) in an LPS-induced lung injury in mice models.

Methods: Mice received an intra-nasal administration of LPS at a concentration of 1 mg/ml weekly for four consecutive weeks followed by the oral administration of R-L compound at a concentration of 50 mg/kg body weight intermittently. Broncho-alveolar lavage fluids (BALF) as well as the lung tissues were periodically collected from
the control and experimental groups for histological examination. Likewise, the lung tissue homogenate obtained from induction and treatment groups was assayed for myeloperoxidase (MPO) assay, superoxide dismutase (SOD) activity and elastase assay.

**Results:** The results illustrated that LPS-induced mice group demonstrated an increased inflammatory cell infiltration causing an acute and persisting neutrophilic accumulation in the lung parenchyma with alveolar congestion. Furthermore, the influx of macrophages, lymphocytes as well as inflammatory mediators was observed during the BALF analysis. The treatment with R-L compound resulted in a significant decline in the permeation of inflammatory cells as observed by lung histology and BALF analysis.

**Conclusion:** Thus, the exploitation of R-L compound with the experimental groups along with other assay methods confirmed its robust anti-inflammatory and antioxidant properties and suggested epithelial regenerative capacity. Further analyzing the efficacy of the R-L compound might help understanding its potential as a therapeutic option in clinical settings.

**Keywords:** Lungs, BALB/c mice, Lipopolysaccharide, anti-inflammatory, R-L compound
INTRODUCTION

Inflammation is an adaptive response implicated in the aging process and underlying a broad spectrum of pathological as well as physiological processes affecting protective cellular mechanisms whenever tissue injury and infections occur. Acute inflammation mainly encompasses the migration of neutrophils from the vasculature into the inflammatory region that results in an interim matrix with the production of chemoattractant factors through platelets at the site of inflammation which inlay for a period of two to six weeks and gradually shifts to stage of chronic inflammation [1]. Our research work predominantly concentrates on the inflammatory condition in lungs causing asthma, chronic obstructive pulmonary disease involving the chronic bronchitis, as well as emphysema, pneumonia, and acute respiratory distress syndrome. Conscription of leukocytes at the site of inflammation mainly involves the functional response of a significant chemotactic action on cytokines [2]. Thus, the delivery of pathogens is known to be a potent method of inspecting the host’s inflammatory reaction [3]. Toxic substances such as LPS, an endotoxin from cell wall of gram-negative bacteria and present in tobacco smoke as well as in a range of environmental and occupational dusts exposure, can ultimately lead to acute and chronic pulmonary inflammation through the infiltration of neutrophils and monocytes into the alveoli.

Thus, LPS is a recognized trigger model for investigating the acute inflammatory response. This is associated to the release of pro-inflammatory cytokines encompassing the tumor necrosis factor alpha (TNF-α), interleukin-1β and interleukin-6. LPS-induced inflammation model in mice reiterates the facets of inflammatory cascade that are interconnected with the pulmonary inflammation and lung ailments in humans [4]. Sequentially, LPS provokes the immunological reactions by interrelating with the membrane receptor CD14 which is a 55 kDa glycosyl phosphatidylinositol connected membrane protein that stimulates the production of multiple cytokines while the protein kinases particularly Fgr, Lyn and Hck engage mainly in the signalling pathway of LPS. Within minutes of LPS stimulation, there is a copious proteins efflux which are tyrosine phosphorylated where the MAP kinases p42, p44 and p38 are enzymatically active and trigger the release of macrophages [5]. Concomitantly, the signalling mechanism of Type I integral protein involves the Toll like receptor 4 (TLR-4) engrosses the recruitment of intracellular adaptor proteins that transmit signals from the TIR domain, initiating the protein kinases as well as transcription factors that provoke the release of inflammatory mediators [6] whose involvement is known to be linked with acute and chronic inflammation in human. Therefore, using intranasal delivery for delivering substances in the bronchus mirrors a clinically compatible and interventional methodology [7, 8].

Over the last decades, the prominence of natural compounds such as the plants and marine species naturally containing many active chemicals have been studied for their potentially health benefits, either preventative or therapeutic. To refrain from the utilization of classic medicine, the prominence of functional foods along with the phytomedicine might sustain the ministrations of the prevailing medical conditions. Functional foods are not like dietary supplements and are rather traceable through natural compounds that can be taken through normal diet. The
promising features of functional food can be employed against viral infections, heart disease, respiratory disease, inflammation and cancer. Moreover, the plant-based components of functional foods can furnish both taste as well as food aroma that can be employed as expectorant, antitussive, antimicrobial and irritation soothers in pulmonary medicine [9]. For instance, flavonoids display a broad array of pharmacological activities dealing with the modulation as well as progression of several pulmonary disease conditions [10-13]. Some, albeit still extremely limited, experimental studies have investigated the therapeutic potential of marine extracts in LPS-induced lung injury [14, 15].

Rhodiola rosea, which is mostly found in Europe and Southeast Asia, has a rich polyphenol content (41.4 ± 3.41%) [16] and its salidroside moieties are known to possess various pharmacological activities, such as antioxidant, anti-inflammation, anti-apoptotic, anti-depressant, anti-aging, neuroprotective, cardioprotective effects while alleviating cigarette-smoking experimental COPD [17]. Another interesting compound which attracted our research interest was a lipoprotein fraction obtained without chemicals from T. trachurus fish from the Galician coast of the Atlantic Ocean. Lipofishin, i.e., a subclass under the proteolipids, which forms a new group of lipoproteins extracted from the muscle of Trachurus fish have been demonstrated to possess anti-inflammatory properties [18]. After years of research, in 2021, we devised a phytomarine compound (Bloomin’Age, Science of Living, Milan Italy) by accurately mixing of high-salidroside specific bioactive fractions (SBF) isolated from the Rhodiola rosea and the lipoprotein extracted from the muscle of Trachurus trachurus fish (Rhodiola rosea plant extract and Lipoproteins from Trachurus sp). Our previous results showed the significant anti-inflammatory, antioxidant, potential of R-L compound [19].

Thus, the aim of the present study was to test the therapeutic anti-inflammatory/anti-oxidative and regenerative property of this phytomarine (SBF-LF) in an LPS-induced lung injury in mice model.

METHODS

Reagents: LPS, phosphate buffered saline (PBS), sodium phosphate buffer, Ethylene diamine tetra-acetic acid (EDTA), Sodium hydroxide (NaOH), Concentrated sulphuric acid (H₂SO₄), Glycerin, Giemsa stain, ethanol, methanol, saline, DNase free water and R-L compound, 10% formaldehyde, Xylene, Tris, Ethylenediaminetetra-acetic acid disodium salt dihydrate (Na₂EDTA-2H₂O), pyrogallol, ammonium chloride, potassium bicarbonate, 3, 3′, 5, 5′-Tetramethylbenzidine (TMB), dimethyl sulphoxide (DMSO). Rhodiola SBF and LF-Trachurus sp (R-L compound) were a kind gift from ReGenera R&D International for Aging Intervention, Italy.

Animals: Healthy female BALB/c mice (Mus musculus) aged 8 to 12 weeks old weighing about 20-25 grams approximately were housed in animal house, Chettinad Hospital and Research Institute (CHRI) with the approval number: IAEC 4/ Proposal 29/ A. Lr: 11/ Dt: 20.12.18. They were placed in plastic cages with adsorbent bedding matter with 12h light/dark cycle under standard conditions. Further, ad-libitum feeding of water and food pellet was provided during the in vivo experimentation.

LPS-induced lung inflammation: For induction of acute phase of inflammation in lungs, 1 mg of lipopolysaccharide was dissolved in 1ml of sterile saline. Then, each mouse was administered 10μl of LPS (1 mg/ml dose concentration) intra-nasally (IN) to the nares of the mice through micropipette by lightly anaesthetizing with isoflurane and was given once in every week for four consecutive weeks [20]. Treatment
of R-L compound was subjected to oral administration with a specific concentration 50 mg/kg body weight was predetermined for the drug treatment. Thus, the R-L compound was given orally to the BALB/c mice at a specific dose concentration of 15 mg/ml (i.e., each mice received 1.5 mg for approximately ~30g body weight) at regular time intervals (1st, 3rd, 5th, 7th day of week 5) according to protocol [21].

**Experimental groups:** Mice were randomized and divided into three groups. Group 1 (n=6) received oral administration of phosphate buffered saline (PBS) which was allotted as negative control. Group 2 (n=6) received LPS (1 mg/ml) which was administered intra-nasally once a day in a week for 4 consecutive weeks and they were sacrificed at the end of four weeks which was allotted as induction/positive control group. Group 3 (n=6) received a selected oral administration of 50µl of R-L compound from week 5 continuously at regular intervals after LPS induction to evaluate its effect on the biochemical parameters (Fig.1).

Figure 1: Experimental Design of the study

**Tissue Collection and Processing:** After instillation of deep anesthesia, the animals were euthanized, and the lungs were collected appropriately from the experimental groups. Mice lungs were obtained for light microscopy without induction or perfusion with fixatives to avoid any translocation of leukocytes within the lung parenchyma or vessels. A portion of lung was used for histology by fixing it in 10% formaldehyde and the remaining portion of lung was stored at -20°C till homogenization and processed for further experimental studies.

**Histology assessment of lung tissue:** Lung tissues were fixed and embedded in paraffin. Tissue sections were cut at 4–5 μm from paraffin embedded lung tissues and mounted on glass slides for hematoxylin and eosin stain and examined by an optical microscope [22].

**Analysis of BALF:** The experimental protocol of BALF collection involves euthanizing the mouse through inhalation of carbon-di-oxide gas and taking them back to the laminar airflow chamber. Then, mice were moistened with ethanol and positioned in supine
position by pinning the legs with needles on Styrofoam boards. Dissection of the mice was performed, the organs were exposed, and the fluid was accurately collected about ten times in an Eppendorf tube and stored in ice. The processing of the BALF involved centrifuging the fluids for 10 minutes at 4°C and resuspending the cell pellet in 200 μl of PBS. This was followed by centrifuging the fluids for 10 minutes at 4°C and resuspending the cell pellet in 100 to 200 μl of ACK lysis buffer and placing it on ice for 10 minutes to lyse the RBCs. 1 ml of PBS was added to stop the lysis. Finally, the fluids were centrifuged again at 800G for 10 minutes at 4°C and the cell pellet was resuspended in PBS and used for the experimental procedures and assay methods [23].

**Estimation of BALF by Giemsa stain:** Bronchoalveolar lavage fluid was centrifuged at 1500 revolutions per minute for 10 minutes and red blood cells were extracted by hypotonic lysis. After a second centrifugation the pellet was dissolved in 500 µl saline. For differential cell count analysis, the pulmonary fluids were diluted with saline to retrieve 1 x 10⁵ cells/100 µl of saline. Polymorphonuclear cells and macrophages were identified by morphological examination of smears and stained with Wright-Giemsa solution.

**Elastase assay:** The elastase assay was carried out for the lung tissues. Elastase stock has a concentration of 3.33 mg/ml which is to be dissolved in sterile H₂O and the substrate (N-succinyl-ala-ala-ala-p-nitroanilide) in 1.6mM of buffer. 50 μl of tissue supernatant were combined with the elastase enzyme followed by incubation for 15 minutes. Then, substrate was added to the above reaction mixture and absorbance was read at 402nm.

**Myeloperoxidase (MPO) assay:** The MPO assay was carried out for the lung tissues. The peroxidase activity with 3, 3’, 5, 5’-Tetramethylbenzidine (TMB) was measured suitably in the spectrophotometer. Lung tissue supernatant was combined with 0.75 mM hydrogen peroxide (H₂O₂) and TMB solution and the plate was incubated at 37°C for 5 min. The reaction was stopped by adding 2M sulphuric acid (H₂SO₄) and absorption was measured at 450 nm to estimate MPO activity [24].

**Superoxide dismutase (SOD) activity:** SOD activity in the cell extract was estimated by measuring its ability to inhibit the auto-oxidation of pyrogallol according to the method of Markland [25]. 1ml of assay mixture containing 0.05M sodium phosphate buffer (pH8.0), 0.01M EDTA and 0.27mM pyrogallol (Solution of pyrogallol was made in100mM HCl). The absorbance was measured for 5min at 420nm. The enzyme activity was expressed as U/mg protein, where1U is the amount of enzyme required to bring about 50% inhibition of the auto-oxidation of pyrogallol.

**Statistical Analysis:** Data was obtained from independent experiments performed in triplicate manner and were presented as mean ± SEM. The unpaired student t test was used to determine significant differences between two groups of data. The p values of <0.05, <0.01 and <0.001 were considered as statistically significant and are indicated by asterisks (*, ** and ***) respectively. All the data was analyzed with Graph Pad Prism 5.0 software.

**RESULTS**

Animal disease activity index of the mice was analyzed by interpreting the change in the body weight, input of water, and food consumption. Thus, changes in body
mass were assessed during the in vivo experiment. Initially, in LPSfed mice, there was a gradual loss of body weight and water and food intake when compared to normal mice, whereas, in LPS + R-L compound fed mice there was a marginal gain in the body mass, water and food consumption (Fig. 2-a, 2-b and 2-c). During animal experiments, the veterinary pulse oximeter was used to measure the saturated peripheral oxygen (SpO₂) level in all groups at regular time intervals during LPS induction as well as under R-L compound treatment. From graphical representation, Fig. 2-d depicts a gradual SpO₂ decline in the LPS induced mice in contrast to control mice, whereas a slight increment in the LPS + R-L compound treatment to the lung injury mice model.

Figure 2: Effect of body mass changes in mice (2-a) was observed during the LPS induction as well as during R-L treatment; (2-b) Water and food consumption was observed during LPS induction; (2-c) Water and food consumption was monitored during R-L treatment; Pulse oximeter reading (2-d) during LPS induction and R-L treatment.

Histology assessment of lung tissues: Control mice showed the normal histological structure of the lung sections stained with hematoxylin and eosin. Microscopically, the control tissue section was seen with its lung parenchyma exposed to normoxic condition with alveolar septa and pulmonary capillaries which are found to be preserved (Fig. 3-a). In LPS-induced mice group, the lung tissue sections showed alveolar congestion and enlargement, hemorrhage, infiltration, or aggregation of inflammatory cells such as macrophages, neutrophils and even lymphocytes in airspaces, engorgement of the blood vessel walls and
thickening of the alveolar walls as the significant signs of the progression of the disease condition (Fig. 3-b). Thus, the treatment of inflammatory lung changes with the drug (R-L compound) were found to impede the inflammatory cell infiltration in the lung parenchyma as well as in the alveolar and bronchial walls suggesting the recovery of lung tissue to the normoxic condition (Fig. 3-c).

Figure 3: Effect of R-L on lung inflammation. Lung sections were obtained from control mice (a) group; LPS treated mice (b) group, LPS + R-L compound mice group (c). After hematoxylin and eosin staining, histological examination of lung tissue sections was observed under light microscopy at 20X magnifications.

BALF smear stained using Giemsa stain: The processed BALF was smeared in a glass slide and fixed with methanol followed by Giemsa stain. The smeared glass slide was observed with a compound microscope at 10X magnification. Therefore, in LPS treated group, the BALF cytological analysis revealed that three distinct types of alveolitis were observed namely the lymphocytes, neutrophils, and eosinophils. Especially, in Fig. 4-b, the presence of alveolar casts of amphophilic, amorphous material in honeycomb pattern of cells were observed, the infiltration of inflammatory cells in BALF and in the lung, parenchyma was observed following exposure to LPS in mice. Alveolar macrophages are found which is associated with the acute phase of inflammation respectively. Then, in Fig. 4-c the R-L compound treated group was observed with a minimal level of inflammatory cells accumulation in comparison with LPS treated group.

Figure 4: Microscopic images of bronchoalveolar lavage fluid (BALF) stained with Giemsa. Control group 4(a), LPS treated mice group 4(b); LPS + R-L compound treatment group 4(c) were observed at 10X magnification. BAL cells depict eosinophils, neutrophils, macrophages, and lymphocytes in LPS challenged mice.
**Colorimetric Assay Methods**

**I. Elastase Assay:** The enzymatic activities of elastase in control and treatment groups are summarized in Fig. 5-a. The elastase activity significantly increased in the LPS treated group because the alveoli lost their elasticity and secreted the enzymes which are known to be at higher levels in comparison with LPS + R-L treated mice group which exhibit a substantial decrease in their enzymatic reaction.

**II. Myeloperoxidase Assay:** In myeloperoxidase assay, the absorbance is inversely proportional to the level of MPO enzyme. The enzymatic activities of myeloperoxidase in control and treatment groups are summarized in Fig. 5-b. The tissue lysate depicts an increased level of MPO activity in the LPS treated mice group due to enhanced infiltration of enzymatic levels of myeloperoxidase from the azurophilic granules from the neutrophils due to necrosis caused by inflammation. In contrast with the LPS + R-L treated mice group showing a subsequent decrease in MPO enzyme reaction.

**III. Superoxide dismutase (SOD) Assay:** During LPS induction, the free radical accumulation will be more which is counteracted by the scavenging activity of the SOD enzyme. The specific activities of SOD in control and treatment groups are summarized in Fig. 5-c. The SOD tissue lysate was known to significantly increase in the LPS treated in contrast to LPS + R-L treated mice group, showing a significant decrease in SOD appropriately.

![Figure 5](image-url)

**Figure 5:** Effect of R-L compound on different assay methods in LPS induced mice. The assay methods such as (5-a) Elastase assay; (5-b) Myeloperoxidase Assay; (5-c) Superoxide dismutase Assay were performed from the lung tissue homogenate extracted from different experimental grouping of mice. Data are represented as the mean ± SD.
DISCUSSION

Generally, infections, as well as their correlated inflammatory mechanisms, lead to a rapid infiltration of neutrophils at the site of inflammation through the peripheral blood [26]. Thus, the neutrophils are necessitated in healthy lungs where they play a significant role as constituent of innate immunity shielding against infection. However, they could also cause substantial damage together with macrophages.

Treatment with salidroside has also been shown to alleviate the pro-inflammatory cytokines, chemokines as well as nitric oxide via NF-κB signaling pathway during a variety of in vitro and in vivo inflammatory conditions such as ethanol-induced acute gastric ulcer, murine endotoxemia, murine asthma and on RAW264.7 and J774.1 cell tests [30 - 35]. In the above experimental settings and others, salidroside has acted by setting down the nuclear factor kappa- Initiation of DNA-binding as well as mitogen-activated protein kinases signal or extracellular signal-related kinase transduction pathway production [36] is related with inflammation.

In this study, we evaluated the biological activity of R-L compound after 4-weeks induction of LPS lung damage model in mice in order to test the potential therapeutic intervention, during the acute phase, with the R-L compound which was orally administrated during the fifth week (i.e., Day 35). After the treatment with R-L compound, the lung tissue sections showed significantly beneficial effects in comparison with the control. Thus, R-L compound proved to significantly affect the acute inflammatory burst while also preventing its progression with minimal damage. Concomitantly, the enhanced MPO concentration observed in untreated mice, in RL-treated mice decreased in parallel with an overt reduction in the inflammatory cells’ infiltration into the lungs, in agreement with Guan et al. [31]. A great deal of lung injury research studies has been focused on the early phase acute events leading to the destruction of and cytokines at the inflammatory site, as observed in the BALF and in the lung parenchyma of mice after exposure to LPS [27, 28]. Indeed, after 3 days of LPS at 0.5 mg/kg lung injury induction, a remarkable neutrophil infiltration was found [28]. Rhodiola rosea L. extract contains p-tyrosol, salidroside, rosavin, pyridine, rhodiosin and rhodionin which over the past two decades, has been shown to exhibit an anti-inflammatory and anti-oxidative property [29]. epithelial cells in the alveoli and the endothelial cells in the capillary walls, infiltration of inflammation cells and edema of alveoli [7]. This sequence of events at a different degree and duration has also been advocated as one of the multifactorial detrimental factors involved in COPD and smoking-related COPD as we also previously highlighted in a book chapter [37]. No matter the severity and the stage of exotoxin/endotoxin-induced lung injury, tissue and BALF inflammatory changes and alveolar-capillary barrier disruption are common superimposing features. In this respect, it was of interest to note that R-L treated mice could significantly, albeit partially, improve their falling SpO₂ from ≤65% to 80%, as accurately measured by pulse oximeter.

Our prior studies had shown the upregulating property of R-L compound on SIRT-1 and Klotho-1, among others [19]. Although in the present research we did not measure SIRT-1, this seems to support the finding that in a mouse model of asthma, lung SIRT1 expression decreased [38]. Authors suggested that elevating lung SIRT1 levels may be a new strategy for asthma [38] and this is likely to play a role also in tight junctions’ integrity as was more recently demonstrated [39]. Klotho-1 a further vitagenes enhanced by R-L compound in our vitro work seems to be in line with the very recent report that its overexpression protects the peritoneal membrane [40].
CONCLUSIONS

Our current SARS-COVID-19 pandemic has identified that counteracting and possibly inhibiting the inflammatory cascade represents the most significant target of pharmaceutics in curbing the lung inflammation at the early acute phase. The relevant findings observed with respect to the R-L compound which helped sparing the inflammation-induced alveolar tissues degradation with a possible remodeling of the alveolar air sacs associated to reduction of elastase was a very noteworthy finding. These latter biochemical and histological effect were notably mirrored by a functional counterpart, i.e., a partial but significant variation of pulse oximeter readings. Indeed, this clinically applicable parameter clearly differentiate among normal control mice against mice induced with LPS as well as the R-L treated mice. These data might hold some interest in the present scenario of the SARS-COVID-19 pandemic which brought respiratory distress and drastic fluctuation in saturated oxygen levels of COVID-19 positive patients, as well in re-infected vaccinated patients, with severe supervening infections. Finally, the potential adjuvant intervention using phytomarine R-L compounds under appropriate dosing and formulation may yield a potential application in clinical protocols treating from asthma and COPD in future while being devoid of adverse effects.

Limitations of the Study: Although, more in vivo studies are warranted to unveil deeper imaging and molecular mechanisms in the pathological conditions of the pulmonary inflammation, the present pilot study shows the potentialities of R-L compound which might be beneficial to the human health strategies dealing with the respiratory disease conditions.

List of Abbreviations Used: AHR: Airway hyperresponsiveness, BALF: Bronchoalveolar Lavage Fluid, COPD: Chronic obstructive pulmonary disease, CCL-2: Chemokines (chemokines c-c motif ligand), CRP: C-reactive protein, CXCL: Chemokine C-X-C motif ligand, DMSO: Dimethyl sulphoxide, H$_2$SO$_4$: Sulphuric acid, IFN-γ: Interferon gamma, IL-8: Interleukin-8, IN: Intra-nasal, LAR: Lymphocyte antigen receptors, LPS: Lipopolysaccharide, MIP: Macrophage inflammatory proteins, MPO: Myeloperoxidase, NaOH: Sodium hydroxide, NF-B: Nuclear transcription factor-B, Na$_2$EDTA: Ethylenediaminetetraacetic acid disodium salt dihydrate, PBS: Phosphate buffered saline, ROS: Reactive oxygen species, STATs - Signal transducers and activators of transcription, SOD: Superoxide dismutase, SBF: Specific bioactive fractions, SPB: Sodium phosphate buffer, SpO$_2$: Saturated peripheral oxygen, SARS-COVID: Severe Acute Respiratory Syndrome – Coronavirus, TNF-α :Tumor necrosis factor alpha, TLR-4: Toll like receptor 4, TGF-β: Transforming growth factor beta, NF-κB: Transcription factor nuclear factor, TLR: Toll like receptor, TMB: 3, 3', 5, 5'-Tetramethylbenzidine, κBa Inhibitory protein.

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Availability of Data and Materials: Readers can access the data upon request to Corresponding author.
**Ethics Approval:** The Animal Ethics Committee, Chettinad University approved the experimental methodologies under the approval number: IAEC 4/ Proposal 29/ A. Lr: 11/ Dt: 20.12.18 and the experimentation were carried out under the supervision of the Animal Welfare Committee, CPCSEA.

**Conflicts of Interest:** Each author declares that he or she has no commercial associations (e.g., consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

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