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2-(Benzhydryl sulfinyl)-N-sec-butylacetamide isolated from fig fruits as a potential immune response modulator

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ABSTRACT

The phagocytic activities of various immune cells can be enhanced by the use of medicinal preparations that can confer physiological homeostasis through the elimination of both pathogenic material and cells experiencing apoptosis. Nevertheless, studies on the achievement of this kind of reaction by the use of 2-(benzhydrylsulfinyl)-N-sec-butylacetamide as a novel derivative are seldom. Hence, we assess herein the impacts of this novel compound on macrophage cells (RAW 264.7) involved in phagocytic activities as well as on the growth of Gram-negative *Escherichia coli* (*E. coli*). A significant increase in both phagocytosis and bactericidal activities was observed, as indicated by the fact that *E. coli* was remarkably cleared in response to the treatment. In parallel, a highly significant increase ($p < 0.0001$) in the size of the cell, particularly the cytoplasm, was recorded. Additional analyses revealed that 2-(benzhydrylsulfinyl)-N-sec-butylacetamide derived from fig significantly modulates the levels of the inflammatory cytokines such as tumour necrosis factor α (TNF-α), Interleukin (IL) -2, IL-1ß, and IL-17 in the RAW 264.7 macrophage cell culture supernatants. Taken together, 2-(benzhydrylsulfinyl)-N-sec-butylacetamide could enhance the phagocytic activity of phagocytic cells through increasing the inflammatory mediators, reactive oxygen species (ROS), and cell size.

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1. Introduction

All over the history of science, extensive studies of plants have been conducted with the result of the identification of a large number of chemical compounds that altogether possess an overwhelming potential as therapies for a wide array of disease conditions (Alhazmi et al., 2021; Mohammed et al., 2023). An active immune system is the principal tool of the body to encounter viruses, bacteria, and other infectious microorganisms. Thus, it has been gradually recognized that the approach with the highest level of effectiveness can be achieved when the immune system is stimulated to encounter these pathogens (Wen et al., 2012). In health conditions, both potent protection and high nutritional value have been proven to be conferred by constituents extracted from various plants for medicinal uses (Bachrach, 2012; Esmaeil et al., 2017). A vast array of biological functions has been proven by a large number of studies to be provided by plant-derived organic compounds. Furthermore, evidence revealed more effective biological activities of raw materials extracted from various pants, including traditional herbs, in comparison to chemicals industrially synthesized, which is mainly attributed to a higher level of synergistic activities (Al-Salman et al., 2020; Mohammed et al., 2021). Examples may include compounds like flavonoids, alkaloids, lactones, terpenoids, glycosides, and polysaccharides that have been proven to possess the potential to modulate the immune responses (Al-Salman et al., 2020; Debnath

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et al., 2020). Such phytochemicals have the potential to be the main source of developing medicines with high levels of safety and effectiveness in providing the proper immune modulation necessary to combat different infections caused by various types of microorganisms. Nature-derived compounds commonly exert non-specific mechanisms for modulating immune responses. The identification and isolation of many plant-derived compounds with potential abilities to modulate immune reactions have been achieved overtime. Research has confirmed the medicinal value of many traditional remedies and paved the way to further analysis and future use in the field of pharmaceuticals (Alhazmi et al., 2021). Previous investigations on compounds extracted from mature black fig demonstrated positive impacts in terms of actions against microorganisms, oxidants, and inflammatory immune responses. The abundant availability of the fruits of this plant implies the possibility of the extraction of abundant and diverse plant derivatives that can show the potential to treat various diseases and make high economic value in the market for pharmaceuticals (Hajam & Saleem, 2022; Mahmoudi et al., 2016; Zhao et al., 2020). One example presented by Alsalman revealed that fig fruit extract was effectively used to treat infections caused by bacteria and other microorganisms (Al-Salman et al., 2020). Fig fruit extract contains a wide array of chemical compounds, including 2-benzhydrylsulfinyl)-N-hydroxyacetamide, that can be investigated for their potential as remedies to many physiological complications, including those caused by infections.

Catheter-associated urinary tract infections (CAUTIs) are commonly encountered nosocomial infections that result in critical illness and extended days in the hospital. It is remarkable that consequences such as higher rates of morbidity and mortality as well as elevated treatment expenses can be caused by a prolonged period of catheterization. CAUTIs are responsible for the majority of healthcare-related urinary tract infections (UTIs) (Al Rugaie et al., 2022; Tambyah & Maki, 2000), while a low percentage of such incidents (about 2–4%) might develop into bacteraemia, in which *Escherichia coli (E. coli)* is the most commonly recognized pathogen that is known to cause CAUTI (Tambyah & Maki, 2000). This bacterial species has the ability to make biofilm, from which it can start propagation (Al Rugaie et al., 2022). Bacterial isolates collected from CAUTIs patients show higher antibiotic resistance as compared to that of UTIs patients. Upon responding to infections, phagocytes show strong reactions to pathogens. This is clear from the mechanisms they use to regulate host defense and contribute to inflammation and homeostasis activities (Laskin et al., 2011). Phagocytes have strong capacities that enable them to take up and present bacterial antigens, as well as secrete cytokines. Additionally, they have the capacity to start and stop certain immunological responses (Wynn et al., 2013). Furthermore, these cells are proficient in identifying, handling, and eliminating xenobiotics and other foreign nanomaterials (Brandenberger et al., 2013). It is notable that encountering infectious pathogens, such as bacteria, largely depends on specific cells that are able to do phagocytosis. The majority of bacterial pathogens can be ingested and then destroyed by macrophages, but in parallel, cell death of these phagocytic cells can also be induced by certain bacterial species with higher virulence, e.g. *Listeria, Shigella*, and *Salmonella* (DeLeo, 2004). Nevertheless, recent research demonstrated that less virulent bacterial species, e.g. *E. coli*, are also capable of inducing cell death in phagocytes (Häcker et al., 2002). The innate arm of the immune system includes macrophages as one of its most important components that can professionally exert various activities against microorganisms. Furthermore, adaptive immune responses are shaped by macrophages when they phagocytose infectious microorganisms and present their antigens (Flannagan et al., 2015). Therefore, pathogens must avoid the killing processes created by macrophages in order to successfully start and maintain infections. Phagocytosis can be considered a good instance of a conical defense mechanism used to combat pathogenic microorganisms (Tauber, 2003). Within their cell walls, bacteria have certain constituents, e.g. lipoteichoic acid, which utilized to rearrange the actin cytoskeleton present in the macrophages. This process is mediated by toll-like receptors (TLRs) on phagocytes and leads to the formation of phagosomes that, as a result, internalize antigenic particles of the bacteria (Flannagan et al., 2012). New phagosomes do not immediately kill microbes since the nascent vacuoles represent the fluid phase that surrounds the phagocytic cell, whereas the membrane of the phagosome forms a direct derivative from the cellular membrane. Nevertheless, the nascent phagosome is biochemically remodelled in a rapid way after it acquires and loses certain processes, along with a significant decline in pH (Pitt et al., 1992). These events are collectively called 'phagosome maturation' which involves a number of tightly organized fission/fusion steps between the phagosome and the endo/lysosome. As a result, mature phagosomes are formed as

organelles that can degrade foreign particles through their strong killing capabilities (Fairn & Grinstein, 2012). The lysosomes that are newly formed in the phagocytes might be one of the major factors involved in the elimination of foreign antigens, a process that was shown to be dependent on the activation of the enzyme NADPH oxidase (NOX2) within the macrophage (Aberdein et al., 2013).

This enzyme mediates the production of superoxide anion (O_2–) which has high instability and triggers different reactions, leading to the production of noxious reactive oxygen species (ROS), including superoxide, peroxides, hydroxyl radical, a-oxygen, and singlet oxygen (Pospíšil et al., 2019). Once formed within the macrophage, ROS implement various functions that start with transforming signals among cells and end with the killing of the pathogen (Dupré-Crochet et al., 2013). Killing inside the phagocyte is a mechanism that has a high level of sophistication and involves complicated pathways to achieve microbiocidal activity (Aderem, 2003). Once the pathogen is bound to the receptors found on the surface of the phagocyte, particularly TLR2 and TLR4, a proinflammatory reaction is rapidly initiated with the involvement of NF-kB and other transcription factors as mediators (Schorey & Cooper, 2003). Activation of the phagocytic capabilities of macrophages is considered a high-potential approach to combating pathogens. Hanckock et al. reported a new methodology that includes immunomodulation activities that are directed by the host's natural mechanisms (Hancock et al., 2012). The aim behind such approaches is usually the initiation or enhancement of the white blood cells to phagocytose pathogenic particles while preventing tissue damage caused by inflammatory reactions (Cicchese et al., 2018). It has been reported that *S. aureus* has the ability to tolerate bactericidal effects mediated by neutrophils. This characteristic is outstanding and reflects the strong killing potential exerted by neutrophils. However, no thorough studies were made to address the interaction between phagocytes and *E. coli*. The present study was designed to assess the biological potential of 2-(benzhydrylsulfinyl)-N-sec-butylacetamide which has been isolated by our previous study (Ali et al., 2023) and found to be the main chemical compound found in fig fruits. We tested the effects of this compound, which is known to have antimicrobial properties, on the phagocytic activity of immune cells against Gram-negative *E. coli,* which is known to be pathogenic to humans. We assessed whether this compound has the ability to activate macrophages as bacterial uptake is increased, phagosomes are further acidified, and phagolysosome production is stimulated.

2. Materials and methods

2.1. Collection of fig fruits and isolation of the pure 2-(benzhydryl sulfinyl)-N-sec-butylacetamide

The plant, *Ficus carica L*. fresh fruits was collected in July 2018 from the center of agricultural research and applied natural sciences, university of Basrah, Annex number 0031150) in Al Kifl, in southeast Iraq, Basrah, Iraq. We followed the university's guidelines for collecting plants. The plant material was confirmed by Prof. Dr.Taha Y. M. Al-Edany at the Department of Plant Protection, College of Agriculture, University of Basrah, Basrah, Iraq, and a sample of the plant was saved in the same department with the voucher number of (BSR 2/1973). The isolation and identification processes have been handled by the authors and recently published (Ali et al., 2023) and the purity of this compound was 95%.

2.2. Isolation of bone marrow-derived macrophages

Using methods that were described previously, bone marrow-derived macrophages (BMDMs) were produced and isolated using the bone marrow tissues of C57/BL6 mice (7–8 weeks old, males) (Waheeb et al., 2020). Mice were housed at a temperature of 24 ± 2 °C and a humidity of 55 ± 10% on a 12:12 h light/dark cycle with a full supply of water and food. The experiments were approved by the Animal Use and Care Administrative Committee of the Division of Biotechnology, Department of Applied Sciences, University of Technology, Iraq (Approval ID-2018. ASBT53), and were in accordance with the Guidelines of the U.S. National Institutes of Health (NIH Publication No. 86–23, revised in 1996).

2.3. Cell proliferation assay

Cell proliferation of RAW 264.7 macrophage cells induced by 2-(benzhydrylsulfinyl)-N-sec-butylacetamide was assessed. The proliferation rate of cells that were not subjected to stimulation was regarded as baseline rate. The proliferation index was calculated, which represents the ratio of induced proliferation (cells treated with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide to baseline proliferation (non-exposed cells).

2.4. Measurement of cytokine levels

Measurement of the levels of interleukins and growth factors in cell culture supernatants following treatment with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide was conducted by utilizing ELISA kits (Quantikine ELISAR Mouse M-CSF (R&D, USA), Mouse IL-17, Mouse IL-2, Mouse IL-1β, and Mouse TNF-α (Bioscience, USA), according to the protocols described by the manufacturers.

2.5. Intracellular bacterial killing assays

E. coli strain was isolated from UTIs patients who attended the Medical City in Baghdad, Iraq. Standard biochemical methods were applied for the processing and identification at the Microbiology Laboratory, Division of Biotechnology, Department of Applied Sciences, University of Technology, Baghdad, Iraq. Bacteria were cultured in broth media to mid logarithmic phase then optical density was measured by using a spectrophotometer at 550 nm. Following centrifugation (3500 rpm; 15 min), the cells were washed three times with PBS (pH 7.2). BMDMs were either treated with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide (25 µg/ml) or left untreated. Infection of BMDMs with *E. coli* was achieved by applying an MOI of 1:100, with an incubation period of 90 min and a temperature of 37 °C. BMDMs were then treated with lysis buffer, and overnight cultures of serial dilutions of the lysates were made on Luria-Bertani (LB) agar plates to estimate the bacterial killing values (both total and extracellular), which were then utilized to calculate the values of the intracellular killing.

2.6. Phagosome/lysosome fusion assays

After isolation of BMDMs, they were suspended in RPMI and placed on 4-chambered slides (1×10^5 cells/ml) for 10 h. Then, 2-(benzhydrylsulfinyl)-N-sec-butylacetamide (25 µg/ml) was added for 1 h. Lyso-Tracker Red® at a concentration of 25 nM was added, and cells were incubated (37 °C for 60 min). They were later incubated with fluorescein isothiocyanate (FITC)-conjugated *E. coli* (MOI = 1:50) for 2 h. The introduction of Lyso-Tracker Red was achieved during the phase of infection. Cold, sterile phosphate buffered saline (PBS) was used for washing the cells for 5 times, followed by fixation with 4% paraformaldehyde (PFA) and examination with a fluorescence microscope (Olympus, Tokyo, Japan), where the unfused phagosomes that are carrying the FITC-labelled bacteria appeared in green, whereas lysosomes stained with Lyso-Tracker Red appeared in red. Since the two tagged fluorochromes were fused, the phagosomes and lysosomes showed yellow staining.

2.7. Analyzing phagocytosis of pHrodo E. coli bioparticles by BMDMs

After the isolation of BMDMs and their plating in a 4-well plate, they were subjected to pre-treatment with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide (25 µg/ml). Following the addition of 1 × PBS (pH 7.4) to the pHrodo-particles, they were vortexed, and buffer solution (50 µl) was added to them for 2 h. After fixation of cells and staining with DAPI, they were photographed at 400 × by means of a Zeiss confocal microscope, where blue dots indicate cell nuclei while red dots indicate the *E. coli*-pHrodo component within the phagocytes.

2.8. Assessment of the phagocytic activity

For their maintenance, BMDMs were kept (37 C°, 5% CO_2) in DMEM (Dulbecco's Modified Eagle Medium) medium, which contained 10% foetal bovine serum, penicillin (100 units/mL), and streptomycin (100 unit/mL). The measurement of the phagocytic activity was performed by following the method of Parra et al. (Parra et al., 2012). Briefly, seeding of cells (2×10^5) was performed in 6-well plates and then treated with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide (25 µg/ml, 24 h), followed by incubation with *Candida albicans* at a ratio of 1:5 for 60 min and stained with methylene blue (0.2%). Cells were observed and counted, where the blue cells were considered inactive and the transparent ones as active. Results were expressed as phagocytosis ratio (PR), which represents the percentage of phagocytic cells; phagocytosis index (PI), which reflects the number of phagocytized *C. albicans* per cell; and sterilizing rate (SR), which refers to the ratio of the sterilized *C. albicans* to the total engulfed *C. albicans*.

2.9. Measurement of cell size

Cells were first mounted on glass slides and then treated with 25 µg/ml of [2-(benzhydrylsulfinyl)-N-sec-butylacetamide)]. Cells were subjected to fixation by using methanol followed by staining by using Giemsa stain (Solarbio, Beijing, China). After being washed with PBS, observation and imaging of cells were achieved by utilizing the Carl Zeiss Axio Observer A1 Microscope (Oberkochen, Germany). The

sizes of cells and nuclei were calculated via the measurement of cell diameters in four fields that were selected on a random basis in each slide using ImageJ software. For the calculation of cytoplasmic size, nucleus size was subtracted from cell size.

2.10. Statistical analysis

Data were analyzed using GraphPad Prism (Mohammed et al., 2023). They were expressed as mean + SD. Data were considered statistically significant if the *p*-value was less than 0.05 at a 95% confidence interval (Ibrahim et al., 2021).

3. Results

3.1. 2-(Benzhydrylsulfinyl)-N-sec-butylacetamide modulated the proliferation of RAW 264.7 cells

The capability of 2-(benzhydrylsulfinyl)-N-sec-butylacetamide for modulating the proliferation of RAW 264.7 macrophage cells was investigated, as shown in (Figure 1). A follow-up for the ability of the cells to proliferate under treatment with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide was conducted for 24 h. Evidently, 2-(benzhydrylsulfinyl)-N-sec-butylacetamide reflected after 24 h of cell exposure a comparable ($p < 0.0001$) antiproliferative pattern vs. baseline untreated cells. The treatment for the next 24 h exerted an increasing trend compared to the values of baseline untreated cells. Macrophages exhibited a significant increase in their phagocytic activities with increases in cell number, particularly after being exposed to untreated cells. The ratio of exposed cells vs. unexposed cells showed a value of 0.578. In contrast, treatment with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide which has mitogenic effects on cells caused an increase in the proliferative capacity of the macrophages by 1.57 fold (Figure 1).

3.2. 2-(Benzhydrylsulfinyl)-N-sec-butylacetamide increases cytokines production

Levels of a number of proinflammatory cytokines (namely TNFα, IL-1β, IL-2 and IL-17) were assessed in culture media of macrophages after treatment with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide for 24 h. This analysis, together with the assessment of macrophage cell proliferation, was the tools used to evaluate the immunological potential of 2-(benzhydrylsulfinyl)-N-sec-butylacetamide. The results demonstrated effective stimulation of RAW 264.7 response, as reflected by levels of TNFα, IL-1β and IL-2, as well as Th17 cell response, as reflected by the IL-17 levels. The highest increase in cytokine levels in response to treatment was observed for TNFα and IL-1β. These are molecules with very strong inflammatory activities and are described as primary cytokines that mediate acute inflammatory reactions (Figure 2). This influence, along with the induction of the secretion of free radicals, provides clear evidence of the effectiveness of 2-(benzhydrylsulfinyl)-N-sec-butylacetamide in modulating immune responses. Furthermore, the activity of IL-6 in regulating immune responses involves the capability of inhibiting the secretion of TNFα, hence conferring negative feedback that could neutralize the acute inflammatory environment. The second cytokine found to be induced by the treatment was IL-1β (Figure 2). The production of this cytokine is performed by macrophages, monocytes, and a type of dendritic cells and it has an important role in the mediation of inflammatory reactions. Its involvement in a number of

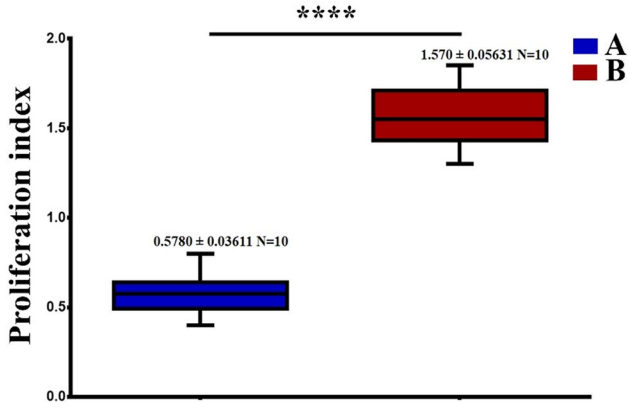

Figure 1. Proliferation of RAW 264.7 macrophages in response to stimulation with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide (50 µg/mL, 24 h). (A) Unstimulated macrophages. (B) 2-(Benzhydrylsulfinyl)-N-sec-butylacetamide treated macrophages. Results are expressed in terms of mean stimulation indexes ± SD from biological duplicates and technical triplicates. Significant differences are shown to be at the level of ****$p < 0.0001$.

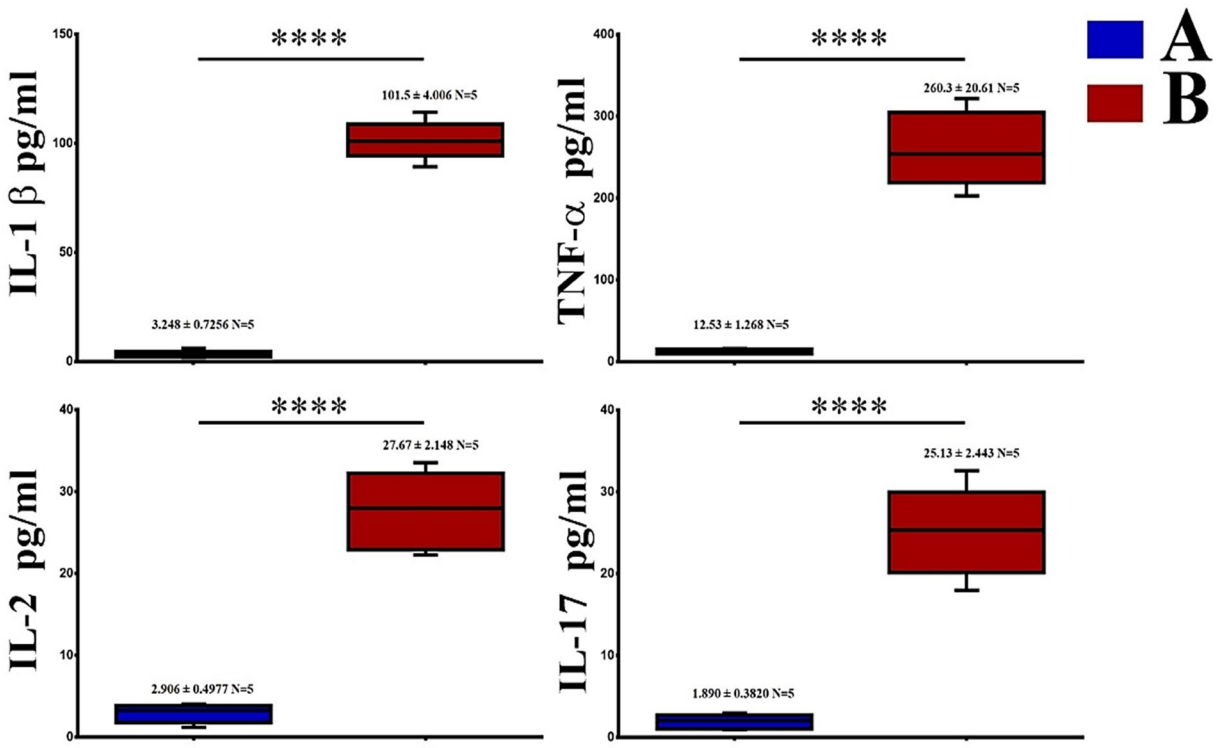

Figure 2. Effect of 2-(benzhydrylsulfinyl)-N-sec-butylacetamide on the cytokines production of RAW 264.7 macrophages. Control group of untreated macrophages (A). Levels of cytokines (pg/ml) in the culture media after treatment of macrophages with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide (25 µg/mL, 24 h) (B). The most significant elevation in the secretion of IL-17, IL-2, IL-1β, and TNF-α was recorded in treated cells in comparison to untreated cell culture. Results are expressed in terms of mean stimulation indexes ± SD from biological duplicates and technical triplicates. Significant differences are shown to be at the level of ****$p < 0.0001$.

other cellular events, such as proliferation, differentiation, and apoptosis, has been documented (Yaseen et al., 2023). Thus, the effective impact of 2-(benzhydrylsulfinyl)-N-sec-butylacetamide ($p < 0.001$) in inducing the release of IL-1 β in a time-dependent manner is a significant finding that suggests a role in the acceleration of cellular immunity. The present results related to the roles of 2-(benzhydrylsulfinyl)-N-sec-butylacetamide are in consistent with previous reports that showed that this compound could accelerate the release of TNF-α by RAW 264.7 cells *in vitro* (Lee et al., 2010). We also noticed the induction of IL-17 release ($p < 0.001$) following exposure of cells to 2-(benzhydrylsulfinyl)-N-sec-butylacetamide) for 24 h (Figure 2). This is an important observation, since interleukin-17 is well-known to be a pro-inflammatory cytokine that has a variety of impacts on innate immune cells. It was reported that IL-17 induced the secretion of granulocyte colony-stimulating factor (G-CSF) as well as the chemokines CXCL1 and CXCL2 (Paulovičová et al., 2022). Upon infection, IL-17 takes part in the reactions that are triggered to eliminate extracellular bacteria and fungi, which are achieved by the induction of antimicrobial peptides. A previous study recorded the anti-inflammatory impacts of Erigeron extract on A549 cells that are pre-stimulated with TNFα, IL-4, and IL-1β- (Sohn et al., 2009). The authors reported that inflammatory-related genes, including NOS1, NOS2A, IL-1 ß, IL-8, and CSF2, along with cell adhesion-related genes, e.g. SELE, MMP3, VCAM1, ICAM1, ITGA7 and ITGB2, experienced downregulation in TNF-alpha, IL-4 and IL-1ß-pretreated A549 cells exposed to Erigeron (Sohn et al., 2009). In general, interleukins are known to have important roles in the modulation of immune reactions and activities, including cell activation and proliferation, signalling, polarization, phagocytosis, and inflammation.

3.3. 2-(Benzhydrylsulfinyl)-N-sec-butylacetamide augmented bacterial intracellular killing

The number of *E. coli* cells phagocytosized by BMDMs was studied following the infection, both with and without treatment with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide, with the aim of assessing and comparing the processes of phagocytosis and intracellular killings. Cells subjected to pre-treatment with

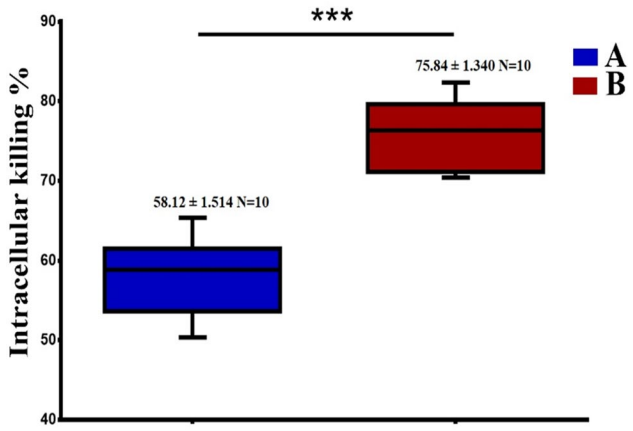

Figure 3. The increases of the intracellular killing activity of BMDNs against *E. coli* after treatment with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide. Results are expressed in terms of mean ± SD. (A) Control BMDMs. (B) BMDMs following treatment with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide. Significant differences are shown to be at the level of ***$p < 0.001$.

2-(benzhydrylsulfinyl)-N-sec-butylacetamide demonstrated a clear elevation in their capability of intracellularly killing the phagocytosed bacteria (Figure 3). The bacterial killing activity of phagocytes was shown to be mediated by the activation of NOX2, which results in the production of superoxide anion (SOA) within the membranes of phagosomes (Nauseef, 2019; Winterbourn, 2020). The ability of BMDMs to produce SOA was measured for the purpose of evaluating NOX2 and ROS activities in these cells. When compared to the control group, phagocytes treated with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide) demonstrated a marked elevation in the activities of NOX2 and ROS, accompanied by remarkable production of SOA in response to encountering *E. coli* (Figure 4). Hence, treated cells are believed to trigger increased NOX2 activation as a response to elevated ROS production, which is known to be a main pathway of enhancing the clearance of bacteria (Liu et al., 2011). Other cell death mechanisms, including destruction of DNA and RNA as well as cell wall damage, were out of the scope of the present work, despite their reported roles in this context (Akhavan et al., 2012). An earlier work demonstrated that gold nanoparticles (GNPs) and graphene oxide fakes (GOFs) exerted significant enhancement of phagocytes to digest Gram-positive -negative bacterial species that infect humans, namely *S. aureus* and *E. coli*. The combination of these two compounds showed a significant induction of bacterial killing via the mechanisms of phagosome maturation and ROS production, an effect that was mediated by NOX2 pathway (Al Rugaie et al., 2021).

The maturation of phagocytes occurs following their activity of ingesting bacteria and can be indicated by the decline in pH of the phagosomes along with the decline in the fusion between the phgosomes and the lysosomes (Lee et al., 2020). We observed a significant decline in cells pre-treated with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide as well as a decline in the ingestion of *E. coli* in comparison to that in the untreated cells (Figure 5). Cells that experienced loading with Lyso-Tracker Red-loaded showed selective labeling of the late endosomes/lysosomes. This permitted the maturation events of *E. coli*-FITC-ingesting phagosomes to be examined via the assessment of colonization with Lyso-Tracker Red. Such co-localization events were noticeable after 30 min in BMDMs that were subjected to pre-treatment with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide. Simultaneously, the majority of *E. coli*-FITC entities exerted lower a magnitude of co-localization with Lyso-Tracker Red in the untreated cells (Figure 4). Our results imply that pre-treatment of cells with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide caused an enhancement of phagosome maturation following the ingestion of *E. coli*.

3.4. 2-(Benzhydrylsulfinyl)-N-sec-butylacetamide stimulated phagocytosis

The activity of BMDMs to phagocytose bacteria was assessed via the observation of the uptake of tagged *E. coli*, which were unable to emit fluorescence until their transfer into the lysosome (low pH) (Figure 5). Phagocytosis of the pHrodo *E. coli* bioparticles was compared following BMDMs pre-treatment with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide. The untreated cells showed lower phagocytotic activity as compared to the pre-treated cells.

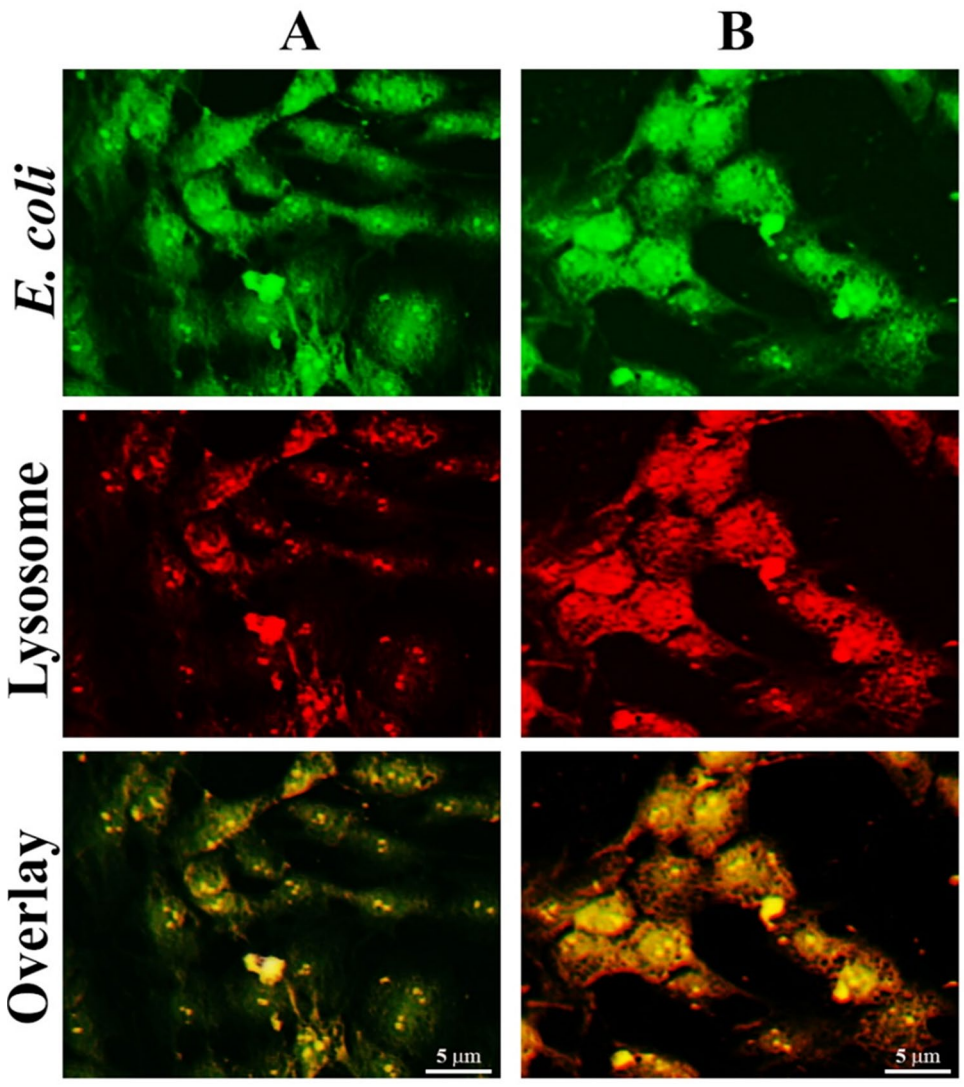

Figure 4. The enhanced co-localization of lysosomes and fluorescein isothiocyanate (FITC)-conjugated *E. coli* in response to treatment with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide. Co-localization is shown in yellow, while lysosomes stained with Lyso-Tracker Red are shown in red. (A) control cells. (B) 2-(benzhydrylsulfinyl)-N-sec-butylacetamide treated cells. Scale bar, 5 µm.

The present results demonstrate the ability of 2-(benzhydrylsulfinyl)-N-sec-butylacetamide to enhance the ability of the treated cells to phagocytose and kill the bacteria. Phagocytosis is a major mechanism adopted by immune cells to recognize and present antigens, leading to the elimination of invading pathogens, tumor cells, and apoptotic cells from organs (Das et al., 2014). It is possible to augment the impact of this mechanism through the use of certain compounds found in a number of edible fungal species, including polysaccharides and lectins (de Melo et al., 2016; Ni et al., 2013). Nevertheless, due to the scarcity of research attempting to address the impacts of fig on phagocytic activity, we designed this study to test how pre-treatment with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide could influence the ability of macrophages to phagocytose pathogenic bacteria. We also aimed at testing the mechanisms that could possibly involve in this process. The ability of RAW 264.7 macrophages to phagocytose different targets, including beads, bacteria, and *Candida albicans,* was first assessed. The results revealed that the ratio of cells that were able to phagocytose beads was significantly higher than that in the untreated group ($p < 0.001$) (Figure 6). Similar behavior was noticed with regard to *E. coli*, as shown in Figure ($p < 0.0001$). The results collectively imply that pre-treatment with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide caused an increase in the macrophage's ability to phagocytosis. Notably, pre-treatment with 2-(benzhydrylsulfinyl)-

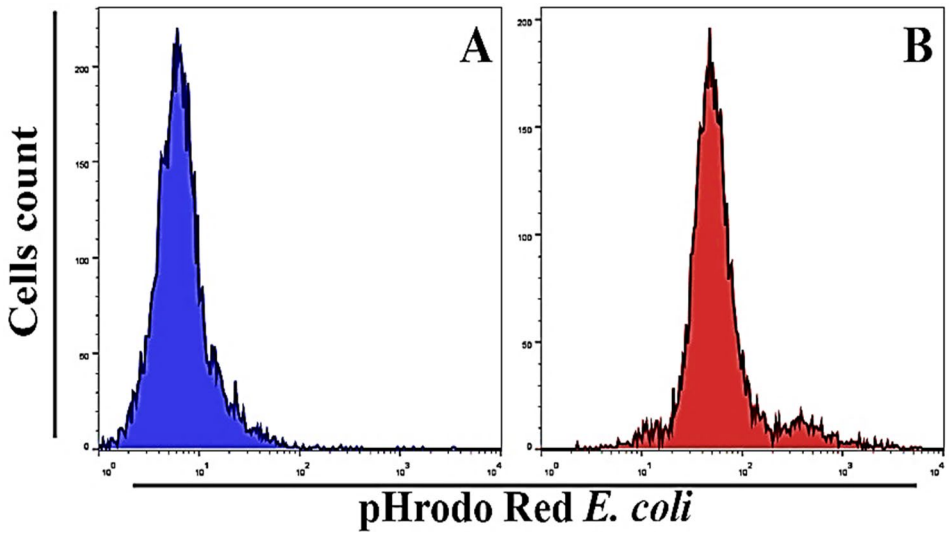

Figure 5. Improvement of the phagocytic activity of pHrodo *E. coli* bioparticles after treatment with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide. (A) BMDMs pre-treated with pHrodo *E. coli* bioparticles. (B) BMDMs following pre-treatment with 10 µg/ml of 2-(benzhydrylsulfinyl)-N-sec-butylacetamide and then with pHrodo *E. coli* bioparticles.

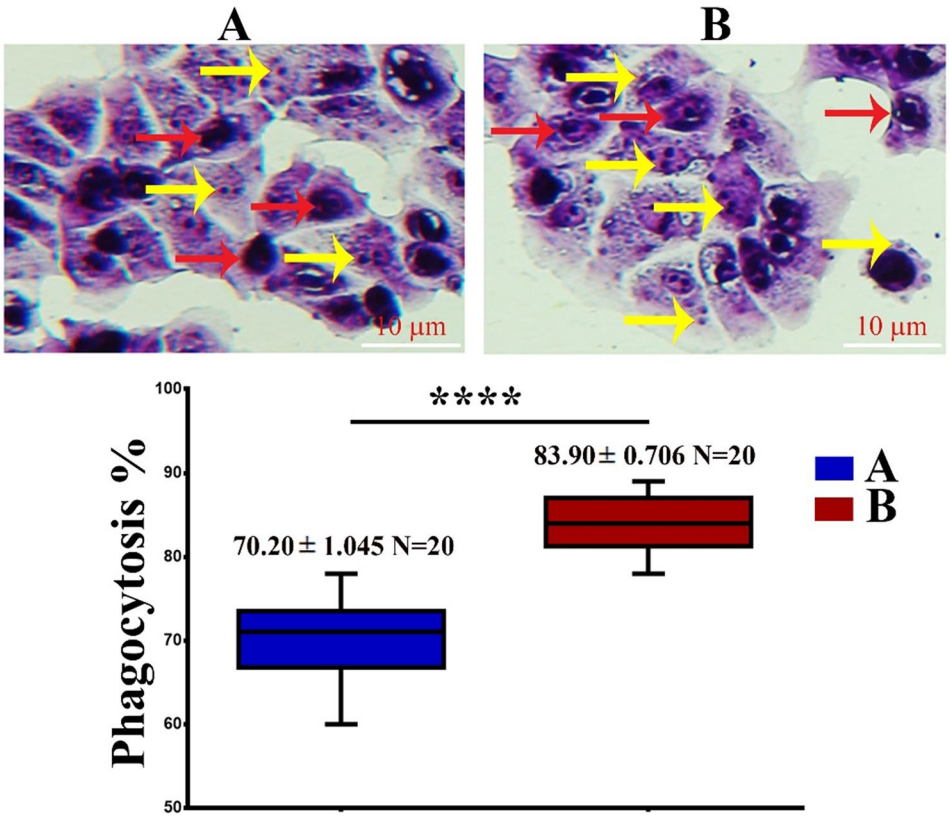

Figure 6. Phagocytosis of *Candida albicans* by RAW 264.7 macrophages after treatment with [2-(benzhydrylsulfinyl)-N-sec-butylacetamide)] for 24 h. Macrophages treated with 25 µg/mL (B). Control group of untreated macrophages (A). Methylene blue was used to stain the macrophages, followed by imaging. Red arrows: Nuclei; Yellow arrows: phagocytized *Candida albicans*. GraphPad Prism was employed to analyze the results. Significant differences are shown at the level of $p < 0.0001$ (****). Scale bar, 10 µm.

N-sec-butylacetamide in the case of *C. albicans* resulted in marked enhancement of both PR and SR. This suggests that the macrophage's capability of killing microorganisms was also enhanced.

3.5. Effect of 2-(benzhydrylsulfinyl)-N-sec-butylacetamide in macrophages size

The capability of granulocytes to phagocytose particles is proportionally correlated to the initial cell

size (Evans, 1989). Thus, the measurement of RAW 264.7 cell size was performed with the aim of exploring the mechanism of the enhancement of phagocytosis using 2-(benzhydrylsulfinyl)-N-sec-butylacetamide. The size was assessed in cells dyed with Giemsa. In addition, the staining outcome demonstrated the occurrence of enlarged macrophages that were obviously stretching out pseudopods as a result of exposure to 2-(benzhydrylsulfinyl)-N-sec-butylacetamide (Figure 7). Statistical analysis revealed that both cell and cytoplasm sizes experienced a significant increase following treatment with 2-(benzhydrylsulfinyl)-N-sec-butylacetamide ($p < 0.0001$) (Figure 7). Together, these findings reveal that 2-(benzhydrylsulfinyl)-N-sec-butylacetamide was able to induce enlargement in the macrophage size. In agreement with these findings, the increase in both cell and cytoplasm sizes was shown to take place upon macrophage activation (Kim et al., 2004). Therefore, it is not logical to postulate that the increased capability of cells to conduct phagocytosis can be attributed to the larger initial cell size and cytoskeletal rearrangement as a result of exposure to 2-(benzhydrylsulfinyl)-N-sec-butylacetamide.

In principle, phagocytosis refers to a complex process in which the phagocytes engulf, ingest, and eliminate the pathogens, apoptotic cells, as foreign large particles of size that are larger than 0.5 µm. Phagocytosis is performed by specific phagocytes that include macrophages, neutrophils, dendritic cells, etc. This process is essential for all aspects of the homeostatic reactions of body tissues and is involved in a variety of immunological events, including inflammation. During phagocytosis, several mechanisms are activated where ROS and nitrogen species are generated. This gives importance to the way in which phagocytosis is modulated by different mechanisms of immune system modulation. Previous reports demonstrated the activities by which Echinacea stimulates phagocytosis performed by granulocytes (Borchers et al., 2000) and PBMCs (Rininger et al., 2000). A later study described the stimulation of macrophages to digest pathogens by crude polysaccharides extracted from Echinacea in a mouse model (Yang et al., 2018), Our current data shows the effects of direct RAW cell exposure to 2-(benzhydrylsulfinyl)-N-sec-butylacetamide are consistent with those previous reports.

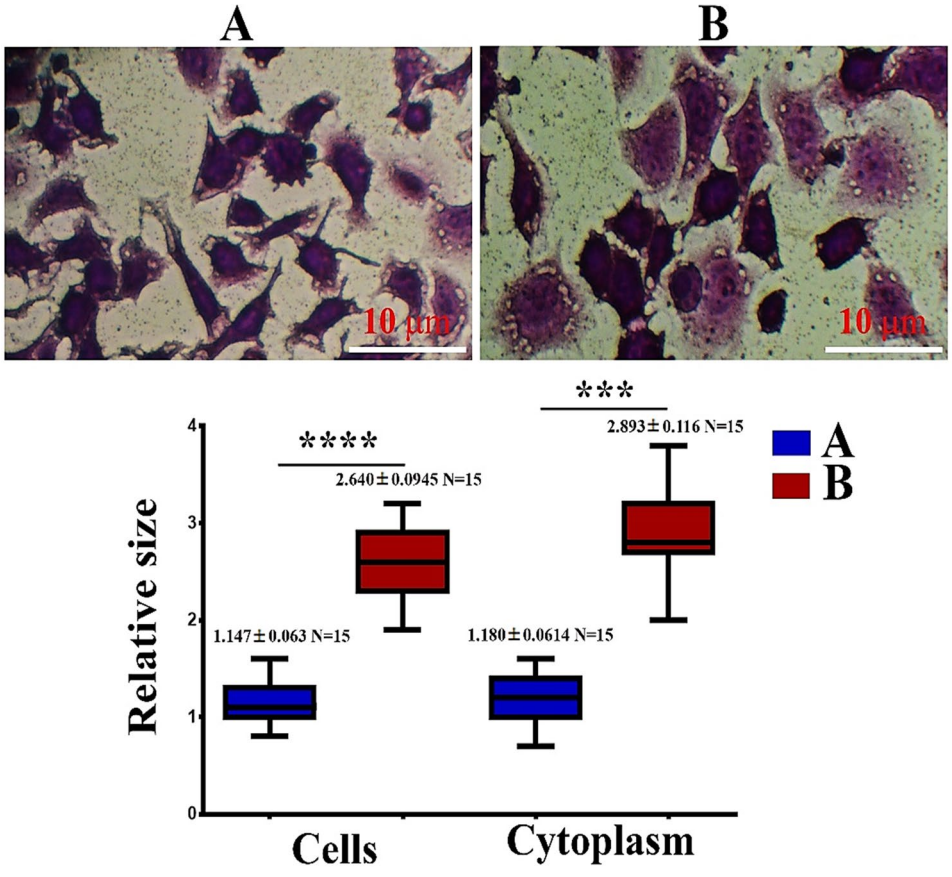

Figure 7. The impact of [2-(benzhydrylsulfinyl)-N-sec-butylacetamide)] on macrophage size. (A) Untreated macrophages. (B) Macrophages exposed to 2-(benzhydrylsulfinyl)-N-sec-butylacetamide. Staining of macrophages was achieved using Giemsa stain. GraphPad Prism was employed to analyze the data. Significant differences are shown at the levels of $p < 0.001$ (***) and $p < 0.0001$ (****). Scale bar, 10 µm.

It is of high importance to note that 2-(benzhydryl sulfinyl)-N-sec-butylacetamide is among the bioactive chemicals that are found more scarcely in other plants and fruits in comparison to their occurrence in figures. 2-(Benzhydryl sulfinyl)-N-sec-butylacetamide has in its composition a carboxyl group as well as free sulfur atoms, rendering it of higher biological activity as compared to chemical compounds by virtue of the synergistic action of these components. It is suggested that the higher content of sulfur compounds is the fundamental reason behind the efficiency of this compound as a therapy. Its electronic coupling takes place within the plasmalemma and leads to several effects in which cells are injured, demolished, and removed. It also has four strong bonds, i.e. C=C, S=O, C=O, and N–H, which occur as stretching bonds. In earlier work, we provided evidence that 2-(benzhydryl sulfinyl)-N-sec-butylacetamide has the characteristic of polarity and occurs in the aqueous extract of the plant. These findings are consistent with a recent report on the capability of this compound, together with trastuzumab, to increase phagocytic activities by the interaction with FcY receptors. This mode of action can be utilized in treatment protocols for ovarian cancer (Ali et al., 2023).

The effects found in our study against *E. coli* can be attributed to the occurrence of active compounds in the plant extract that are capable of generating and stimulating important complement factors, e.g. C3b and C3bi, both of which are able to bind to these bacteria. Later, bound *E. coli* is exposed to the recognition mechanisms of phagocyte receptors, e.g. CR1 (CD35) and CR3 (CD11b). In the same manner, another candidate pathway through which several components in plant extracts can be active is by inducing the Fcc receptors to be overexpressed on the phagocytic cell, thereby facilitating the interaction between the opsonized *E. coli* and the receptors. When Fcc receptors like FccRIII (CD16) and FccRII (CD32) are expressed, the phagocytic activity of phagocytes is augmented (Butcher et al., 2001). Such molecules can be activated through the secretion of a number of molecules, e.g. ROS, which are able to penetrate the mitochondrion and then cause macrophages to be activated to exert their inflammatory functions.

4. Conclusions

The present work highlights the impacts and mechanisms by which 2-(benzhydryl sulfinyl)-N-sec-butylacetamide exerts its influence on the process of phagocytosis. We show that this compound can be of high value as an agent used actively in immune modulation therapies. Furthermore, the study confers reference information in relation to the effectiveness of bioactive compounds in modulating phagocytosis. We also further established the contribution of 2-(benzhydrylsulfinyl)-N-sec-butylacetamide to the killing activity provided by macrophages against *E. coli*. Thus, we can conclude that 2-(benzhydrylsulfinyl)-N-sec-butylacetamide is essential for increasing the antibacterial mechanisms to work properly and has potent antibacterial characteristics that would be of benefit when applied in various areas of medicine and biochemistry.

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Author contributions

Conceptualization, Eman T. Ali, Hussein N. K. AL-Salman, Majid S. Jabir, Ghassan M. Sulaiman; methodology, Eman T. Ali, Hussein N. K. AL-Salman, Majid S. Jabir; validation, Majid S. Jabir, Ghassan M. Sulaiman, Hamdoon A. Mohammed; investigation, Majid S. Jabir, Ghassan M. Sulaiman, Hamdoon A. Mohammed, Mosleh M. Abomughaid; resources, Eman T. Ali, Hussein N. K. AL-Salman; data curation, Majid S. Jabir, Ghassan M. Sulaiman, Hamdoon A. Mohammed; writing—original draft preparation, Eman T. Ali, Hussein N. K. AL-Salman, Majid S. Jabir; writing—review and editing, Eman T. Ali, Hussein N. K. AL-Salman, Majid S. Jabir, Ghassan M. Sulaiman, Hamdoon A. Mohammed, Mosleh M. Abomughaid; visualization, Hussein N. K. AL-Salman, Majid S. Jabir, Ghassan M. Sulaiman; supervision, Majid S. Jabir, Ghassan M. Sulaiman, Hamdoon A. Mohammed; project administration, Ghassan M. Sulaiman, Hamdoon A. Mohammed. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All the data and results supporting the conclusions of this research are available in the article.

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