Revista Argentina de Clínica Psicológica
2020, Vol. XXIX, N°5, 1244-1253
DOI: 10.24205/03276716.2020.1119

Induction of IRG-1 Reduces Reactive Oxygen Species Production by Bioactive Compounds of Anise (*Pimpinella Anisum L.*) on LPS-Activated Macrophages

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Abstract

Plant extracts have become increasingly useful anti-inflammatory medicine and their contribution to primary healthcare in a significant population in the world. The research seeks to demonstrate the process of extracting plant's bioactive constituents from *Pimpinella Anisum L*. and to show the role played by plant's anise extract in Reactive Oxygen Species **(**ROS) production as reduced by expression of *IRG1* by activated macrophages.

The study outlines a new extraction method of *Pimpinella Anisum L*. at a low temperature (0 °C). The bioactive components of the anise extract were detected using GC/MS analysis. The macrophages (RAW264.7) were stimulated by 5 µg/ml of lipopolysaccharides (LPS) from E. coli for 8 hours. Then, post-treatment of the anise extract was performed on the macrophages by 3 separate varied concentrations of 100, 250 and 400 µg/ml of *Pimpinella Anisum L*. extract for 24 hours.

The aqueous plant extract showed high amounts of fifteen bioactive components bearing anti-inflammatory activities by GC/MS analysis. Further, the study provides a significant reduction in the cell viability were treated with the anise extract in comparison to the untreated LPS-activated macrophages. Moreover, mRNA levels and protein expressions of *IRG1* gene were increased significantly resulted in ROS production being inhibited in post-treated LPS-activated macrophages.

This is the first study reveals the impact of all chemical compositions in the *Pimpinella Anisum L.* extract's essential oil indicated to have the required properties of an anti-inflammation and antioxidant effects that might reduce pathological inflammatory responses as a valid pharmacological agent.

Keywords: Anise; *Pimpinella Anisum L*.; Essential oil; Phenolics; Antioxidant activity

Introduction

Inflammation is one of the body's self-protective phenomenon in which the body's cellular machinery provides a complex biological response against foreign particles. Numerous literature studies have also conclusively recommended several plant extracts with anti-inflammatory effect several model systems (Talhouk et al. 2007). This research used *Pimpinella Anisum L.* from which methanolic extract was obtained. *Pimpinella Anisum L*. is commonly found in Saudi Arabia where it is used by the local population to stimulate immunity when treating flue, fever, bronchitis, and diarrhea (Rebey et al. 2019).

The plant, which is classified under the Umbelliferon family, is among the oldest plants used mainly as a source of medicine. Besides Saudi Arabia, the plant is also found in Eastern Mediterranean Region, the Middle East, West Asia, Mexico, Spain, and Egypt (Anastasopoulou et al. 2020). The primary purpose of cultivating *P. Anisum* is to obtain its aniseeds from which 1.5–5per cent essential oil can be extracted. The fruits are also

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used as digestive, flavoring, carminative agents, as well as to relief gastrointestinal spasms (Sun et al. 2019). Anise is used in the food industry as an aromatic and flavoring agent for a range of foods including fish products, gums, and sweets (Özcan and Chalchat, 2006).

Cell culture investigations have shown how an array of free radicals and inflammatory cytokines produced by the central nervous system's resident immune cells and activated microglial cells and macrophages play a role in the antimicrobial activity (Rebey et al. 2019). As typical immune cells, the role macrophages play in inflammation is critical. Classically activated macrophages or M1 cells are also typically involved in macrophages and are activated by microbial products or by inflammatory cytokines such as lipopolysaccharides (Conforti et al. 2010) and are essential in the role of defensing an organism against inflammation (El Haliem and Mohamed, 2011).

Although several studies were carried out on anise (*P. Anisum*) extract, the effect of this plant's bioactive constituents on the *IRG1* expression that acts as a treatment for inflammation has not been comprehensive studied. Therefore, the objective of conducting this research was to evaluate the anti-inflammatory properties of the anise extracts' bioactive components on the production of ROS and the expression of *IRG1* by activated macrophages (Rodrigues et al. 2003).

2. Materials and methods

2.1 Preparation of aqueous suspension and plant extracts

Anise, *Pimpinella Anisum L.* (family, Apiaceae) were gathered from the southern area of Saudi Arabia, Abha. The plant species was identified by Dr. Alwahibi (Botany department, College of Science, King Saud University). A voucher specimen (#14427) of this plant has been deposited at the herbarium of the Botany Department, College of Science, KSU as a reference for the future. Seeds of anise, *Pimpinella Anisum L.* (family, Apiaceae) were blended and dissolved in water, followed by performance of extraction at a low temperature (0 °C/ 8 hours) which gave a compound of molecular weight 173.21 mg/ ml. The mixture was filtered and evaporated to dryness. The viscous powders obtained were then dissolved in the right volume to form a stock solution, was of a concentration of 50 mg/ml. The stock solution was stored safely at 4°C until used.

2.2 Gas chromatography/mass spectrometry (GC/MS) analysis

A multi-purpose sampler (Gerstel) was used to perform metabolite derivatization. Dried samples were mixed with 15µL pyridine, comprising of 20 mg/mL methoxyamine hydrochloride, for 60 minutes at 40°C. To the resulting solution, 15µL N-methyl-N-trimethylsilyl-triflouroacetamide (MSTFA) was added then the incubation of the samples at 40°C for 30minutes. An Agilent 5975C inert XL MSD coupled with an Agilent 7890A GC was used to perform GC-MS analysis. The GC-MS operated in Selected Ion Monitoring (SIM) mode (m/z 215.1, m/z 230.1, and m/z 259.1) and one sample was set to run within a total run time 24.3 min. Since data analysis was targeted, the use of Metabolite Detector was necessary to process all GC-MS chromatograms (Hiller et al. 2009).

2.3 Cell and cell Culture conditions

The American Type Culture was used to provide a mouse macrophage cell line, RAW264.7. The medium used for cultured flasks was Roswell Park Memorial Institute (RPMI-1640) which contained the modified Eagle's medium (DMEM) of high-glucose Dulbecco (SH30022.01, HyClone, USA) with 10% fetal bovine serum (FBS) as a supplement (FBS) (10099141C, Gibco, USA), 100 µg/mL streptomycin, and (SV30010, HyClone, USA) set at 37 °C packed in a humidified incubator with 5% carbon IV dioxide.

2.4 Cell stimulation and treatment

From the humidified incubator, seeding of the cells was further done at 1×10^6 cells/well or 1×10^5 cells/well in 25 and 96 well tissue culture plates respectively. The macrophages (RAW264.7) were stimulated by 5 µg/ml of lipopolysaccharides (LPS) from E. coli for 8 hours. The essential oil obtained from an aqueous solution extracted from anise was added to a culture media and the cells were then incubated in this media post-treatment and combined with 3 separate varied concentrations of 100, 250 and 400 µg/ml respectively for 24 hours and treated macrophages in culture media with only 400 µg/ml to check the aqueous extract's cytotoxicity on macrophages (El Haliem and Mohamed, 2011).

2.6 Cytotoxic activity assay

According to Talib and Mahasneh (2010), the cytotoxic effects of the anise "*Pimpinella Anisum L*". aqueous extract on macrophages were determined using MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

2.7 Measurement of LPS-induced Intracellular ROS

LPS-stimulated intracellular ROS was measured by detection of the 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) dye fluorescent intensity (Wan et al. 1993). The dye was also used to incubate the peritoneal macrophages (1×10^6 cell/ assay) under Invitrogen, 10 µg/ml for 24 hours. LSR II was used to immediately measure the fluorescence intensity (BD Biosciences).

2.8 RNA Quantification and Quantitative Real-time PCR

Qiagen RNeasy Mini Kit (Qiagen) was used to harvest the expected total RAW264.7 cells RNA, consistent with the manufacturer's protocols. Monitoring of RNA integrity and purity was done using and Agilent 2100 Bioanalyzer with RNA 6000 Nano assay kit and NanoDrop1 ND-1000 spectrophotometer. Technologies [9]. Quantitative real-time PCR (Q-PCR) analysis using the SYBR RT-PCR kit (Takara) and Light Cycler (Roche Applied Science). The performance of RT with Superscript® II (Life Technologies) and 200 ng poly(A)$^+$ RNA using oligo(dT) primers was done according to (Zur et al. 2003). The following primers were then used to perform PCR:

IRG1 (5-GGCTGTCCTACCTGTCCTCAC-3; 5-GGGCCTGTTTACGTACTGGAC-3′) and

GAPDH (5-TGAAGGTCGGTGTGAACG-GATTTGGC-3; 5-CATGTAGGCCATGAGGTCCACCAC-3).

The conditions under which PCR was performed are detailed in (Basel-Vanagaite et al. 2006).

2.9 Statistical analysis

The graphs of the results were computed using Origin Pro software and Graph Pad Prism, then used the SAS version 9.4 software was used to follow up for the groups considered significantly different by Waller-Duncan Post Hoc analysis using (Cary, NC).

3. Results

3.1 Chemical Constituents of 50 mg/ml of an aqueous extract of anise "*Pimpinella Anisum L.*" essential oil (%) of 15 compounds

The Chemical Constituents contained in the essential oil anise "*Pimpinella Anisum L.* aqueous extract by supercritical extraction involving the use of CO_2 identified in anise oil by GC/MS after separation (Table 1). Fifteen compounds contained in the anise oil of the *Pimpinella Anisum L.* extract were identified and quantified and found to represent 99.82% of anise oil. The anise oil's trans-anethole was found to contain phenylpropanoid as the most abundant component in the anise oil, 91.21% of the entire anise oil extract. Other compounds include a phenylpropanoid γ-himachalene (1.2%); Estragole, Limonene, and O-isoeugenol comprising of 0.9%, 0.8%, and 0.71%, respectively. Others include chemotypes such as cis-anethole (0.6pc), α-pinene (0.63pc), 3-Octanol (0.4pc), α-phellandrene (0.18pc), and p-cymene (0.16pc), linalool (0.5%) and p-anisaldehyde, while Sabinene (0.2%) and α-zingiberene in the anise oil.

Table 1. **The chemicals constituents of anise "*Pimpinella Anisum L.*" essential oil (%) of 15 compounds**

Compound	%MS	Activity
1. α-pinene	0.63	Anti-inflammation, Antioxidation (Dhami et al., 2019)
2. linalool	0.5	Anti-inflammation (Kim et al., 2019)
3. α-phellandrene	0.18	Anti-inflammation, Antioxidation (Kumar et al., 2019)
4. p-cymene	0.16	Anti-inflammation, Antioxidation (Thapa et al., 2020)
5. Limonene	0.8	Anti-inflammation, Antioxidation (De Souza et al., 2019), Anti-cancer (Li et al., 2016)
6. o-isoeugenol	0.71	Anti-inflammation, Antioxidation (Sun et al., 2019)
7. α-zingiberene	0.2	Anti-inflammation, Antioxidation (Sen et al., 2019)
8. γ-himachalene	1.2	Anti-inflammation, Antioxidation (Yin et al., 2019)
9. Estragole	0.9	Anti-inflammation, Antioxidation (Hegazi et al., 2019)
10. Methyl chavicol	0.62	Anti-inflammation, Antioxidation (Ahmed et al.,2019)
11. Cis-anethole	0.7	Anti-inflammation, Antioxidation (Luís et al., 2019)
12. p-anisaldehyde	0.5	Anti-inflammation, Antioxidation (Amodeo et al., 2019)
13. 3-Octanol	0.4	Anti-inflammation, Antioxidation (Alade et al., 2019)
14. Sabinene	0.2	Anti-inflammation (Ryu et al., 2019)
15. Trans-anethole	91.21	Anti-inflammation, Antioxidation (Kim et al., 2017; Chen et al., 2020)
Total identified compounds (15)	99.9	The whole extract has an anti-inflammation and antioxidation effects

3.2 Effects of an aqueous extract of anise "*Pimpinella Anisum L.*" on the Cell Viability and cytotoxic effects of RAW264.7 Macrophages

The cell viability and cytotoxic effects of an aqueous extract of anise "*Pimpinella Anisum L*. on macrophages were determined using two methods, the direct counting of living and dead cells using a haemocytometer with an inverted microscope and by MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Using the direct counting method, values of percentage cell viability of the murine RAW264.7 macrophages at different concentrations of the extracts after 24 hours incubation are shown in (Figure 1). It was noted that the results of the cell viability showed a very high significant (***P < 0.001) increase in the dead cell numbers in macrophages stimulated with LPS but untreated with the anise extract (positive control) compared to the LPS- stimulated macrophages and treated with the anise extract used (100, 250, 400 µg/ml) and compared to the untreated macrophages (negative control). LPS-activated RAW264.7 macrophages that treated with the anise extract for the concentrations (100, 250) were showed a significant (**P<0.01) decrease gradually in the cell viability.

The cytotoxic effect is shown in (Figure 1). The cell viability increased in a dose-dependent manner of the anise extract on RAW264.7 cells. There was no significant cytotoxicity due to the anise extract on RAW 246.7 at the higher concentration used (400 µg/ml), when macrophages were treated with anise extract at the final concentration used (400 µg/ml) without stimulation.

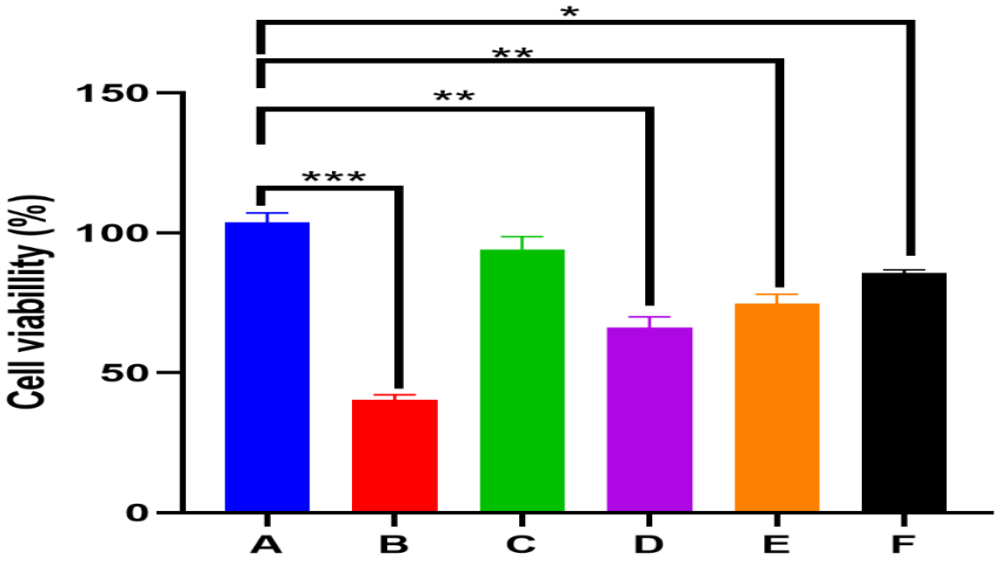

Figure 1. **Cell viability changes in RAW264.7 cells by MTT. (A) Control = 0 LPS, (B) LPS = 5 µg/ml, (C) 400 µg/ml of anise extract, (D) LPS + 100 µg/ml of anise extract, (E) LPS + 250 µg/ml of anise extract, (F) LPS + 400 µg/ml of anise extract. Results are presented as the mean ± SD (n=3). Cell viability values with different superscript letters indicating that they are significantly different with reference to Waller-Dunkan test (at *P<0.05, **P < 0.05, ***P < 0.01).**

3.3 Anise "*Pimpinella Anisum L.*" extract reduces LPS-induced Reactive Oxygen Species (ROS) Production in Murine Macrophage RAW264.7 cells

To investigate LPS-induced ROS in macrophage RAW264.7 cells, detection of fluorescent oxidative product of DCF-DA was used to examine cell oxygen bursts. As shown in (Figure 2), after 8 h LPS (5 µg/ml) stimulation of macrophage cells rapidly induced significant ROS production. The results revealed that the anise extract significantly (***P<0.001) much higher reduced the production of ROS in the LPS-activated RAW264.7 macrophages were treated with the anise extract for both concentrations (250 and 400 µg/ml), compared to the macrophages in the positive control setup (exposed to LPS), although the rate reduction of ROS generation was a significant (**P < 0.01) in the low dose anise (100 µg/ml) setup. Conversely, there was no significant difference in the values recoded in the macrophages treated with anise extract alone at final the concentration of (400 µg/ml), compared to those in the negative control (untreated cells). These changes in the recorded values were significant at ***P < 0.001; **P < 0.01; *P<0.05 (Figure 2).

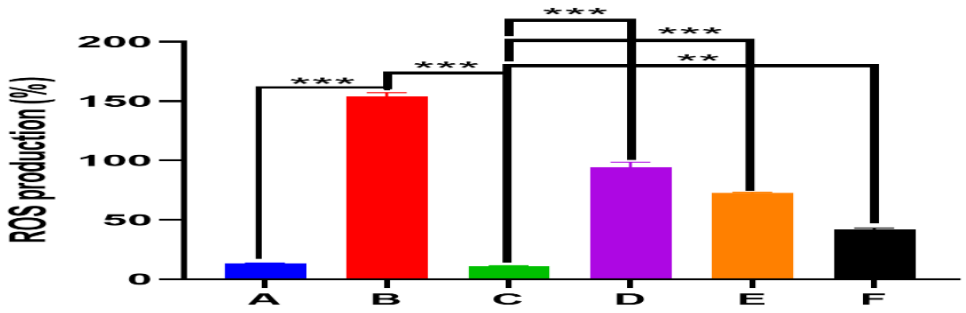

Figure 2. **Anise "*Pimpinella Anisum L*" aqueous extract's inhibitory effect on ROS production in the case of LPS stimulated macrophages. (A) Control = 0 LPS, (B) LPS = 5 µg/ml, (C) 400 µg/ml of anise extract, (D) LPS + 100 µg/ml of anise extract, (E) LPS + 250 µg/ml of anise extract, (F) LPS + 400 µg/ml of anise extract. Results are expressed as the mean ± SD (n=3). ROS Production values with varying superscript letters are significantly different based on the Waller-Dunkan test (at *P<0.05, **P < 0.01, ***P < 0.001).**

3.4 Anise "*Pimpinella Anisum L.*" extract upregulates *IRG1* expression in LPS-activated RAW264.7 macrophages

So as to investigate how the anise extract bioactive compounds affect *IRG1* expression over dose after exposure to a pro-inflammatory stimulus, we conducted an analysis of the different levels it expresses itself based on the following aspects, (Figure 3), where (A) a negative control with untreated macrophages, (B) a positive control in which macrophages exposed to LPS, (C) treatment of macrophages with only 400 ug/ml of anise extract, (D, E, F) LPS infected RAW264.7 macrophages and later treated with (100, 250 and 400 ug/ml) of anise extract under the inflammation conditions indicated, respectively.

The results were obtained by RT-PCR indicated that the expression of *IRG1* is determined by either anise extract dose or LPS infection. LPS-activated RAW264.7 macrophages and treated with (100 ug/ml) of anise extract resulted in *IRG1* being significantly (**P < 0.01) in comparison to the positive, negative controls and treatment of macrophages with only 400 ug/ml of anise extract. Moreover, the expression levels of *IRG1* in LPS-activated RAW264.7 macrophages treated with concentrations of (250 and 400 µg/ml) of anise extract showed a significant (***P < 0.001) induction.

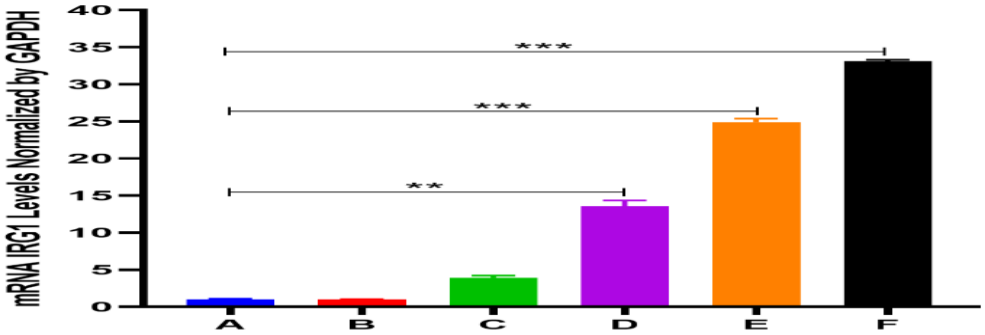

Figure 3. **Expression of *IRG1* in murine (RAW264.7) macrophages. *IRG1* mRNA expression was analyzed by RT-PCR assay. *GAPDH* expression were identified as controls for loading and infection of the sample, respectively. (A) control samples (unexposed and untreated macrophages), (B) LPS infected RAW264.7 macrophages under pro-inflammatory conditions, where RAW264.7 mock infection of macrophages with LPS at the final concentration (5 µg/ml) for 8 h, (C) macrophages were treated with only 400 µg/ml of anise extract, (D) macrophages were treated with LPS (5 µg/ml) + 100 µg/ml of anise extract, (E) macrophages were treated with LPS (5 µg/ml) + 250 µg/ml of anise extract, (F) macrophages were treated LPS (5 µg/ml) + 400 µg/ml of anise extract. Data in the bar graphs are means ± SE and significantly different based on the Waller-Dunkan test (at *P<0.05, **P < 0.01; ***P < 0.001). All tests were conducted at least triplet samples independently.**

4. Discussion

The given research project revealed the empirical findings on the anise's invariable qualities with a particular focus on the plant's anti-inflammation characteristics and its phytochemical components (Patra et al. 2020; Rebey et al. 2019).

The research identified that anise, plant-related extracts held at specific temperature (0 ℃) for 8 hours, as well as plant's internal oils incorporate a range of active biological elements that can be widely used in the food and/or feeding industries, medical and pharmaceutical fields, and cosmetic areas (Yu et al. 2020). Nevertheless, plants with aromatic qualities as well as their extracts have to comply with standards and be thoroughly supervised on their structure to provide valid data for this research project. In vitro tests that involve normalized extracts must be performed before actual in vivo experiments to approve the extracts' functional value (Bhatt et al. 2020; Patra et al. 2020). As a result, adequate alternative methodologies for improving shelf life and functional qualities of the animal products can be designed, meeting the consumer's topical needs in natural, healthy and top-quality food. The plant can also be utilized as an antiviral drug for treating main types of influenza (A and B). In addition, the plant has other useful molecules that can be used for achieving antiviral outcomes, which have been documented in the literature (Yu et al. 2020). Aside from antiviral qualities, anise is found useful in securing other effects, among which are antioxidant, antifungal, antimicrobial, anti-inflammatory and anthelmintic effects. Other reports indicate that anise can also provide insecticidal, secretolytic, antinociceptive, gastroprotective, as well as sedative, estrogenic, expectorant and spasmolytic outcomes (Bhatt et al. 2020; Patra et al. 2020; Yu et al. 2020).

Plants in habitats in the world have been locally used as medicine since ancient times, and origin of current pharmaceutical drugs. Evaluation of a wide range of pharmacological characteristics of these plants may be as a result of the new natural substances found in them that are capable of treating particular diseases. Moreover, there is special importance in the identification of the medicinal plants' active principles in the role of new drug introduction process (Orav et al. 2008).

It has been used widely as a traditional medicine in Saudi Arabia. As a result, several studies were conducted on the plant's essential oil and extracts to identify its pharmacological properties and chemical compounds as an anti-inflammation and as antioxidant treatment (Bhatt et al. 2020; Iannarelli et al. 2018). With regard to recent studies conducted on the compositions of essential oil considering several locations, the findings showed that γ-himachalene and trans-anethole are usually the major components (Ullah et al, 2014) with high efficacy to remedy the inflammation and oxidation reactions (Mahdavi et al. 2018).

The anise extract has varied bioactive compounds depending on the species and provenance of the plant, the extraction method as well as the solvent type. So, the results in this research showed considerable anti-inflammation and antioxidant activities coupled with protective effect to counter inflammation conditions (Anastasopoulou et al. 2020; Rodrigues et al. 2003). Also, while our extraction method involved performance at low temperature (0℃) and water was used as solvent, as these conditions ensured the bioactive components are protected and large amounts of these compounds were produced as aqueous anise extract (Bettaieb et al. 2018; Patra et al. 2020).

In this context, several studies on gene expression profiling of human microglial cells and macrophages have identified immunoresponsive gene 1 (Irg1) to be among the genes that are most highly up regulated under pro-inflammatory conditions, for example bacterial infections (Tavallali et al. 2017). The studies have conclusively demonstrated that increased levels of *IRG1* expression in chicken spleen macrophages have also been detected following an infection with Salmonella enterica (Rebey et al. 2020). Moreover, research findings have shown that *IRG1* can increase one's susceptibility to Marek disease virus (Kara et al. 2020).

Additionally, high levels of *IRG1* expression has also been shown in the initial events preceding implantation in a woman's uterus (Hashem et al. 2020), the specific phase whereby the inflammatory cytokine secretion level is high by the uterus' endometrial cells along with the secretion of the immune system cells that are mobilized to the implantation site (Karimzadeh et al. 2012).

Remarkably, several studies suggest that *IRG1* bring about endotoxin tolerance through the increase of A20 expression in the immune system's macrophages by producing ROS (Li et al. 2013), and based on this premise, the present study has found out that there are bioactive compounds' anti-inflammation effects in the aqueous anise extract on ROS production and *IRG1* expression. As the results indicated, macrophages exposed to LPS

5 µg/ml followed by post-treated with anise extract of different concentrations (100, 250 and 400 µg/ml) indicated that an induction in the expression of *IRG1* expression resulted in ROS production being inhibited. As such, our results propose that ROS production was significantly reduced after the macrophages were posttreatment with different anise extract concentrations, an indication of dose-dependent inhibition of ROS production that is induced by LPS at 100, 250 and 400 µg/ml concentrations, respectively.

In order ascertain that the bioactive compounds contained in anise extract influence *IRG1* expression levels decrease and reduction in ROS production, MTT and the cell viability assays were successively performed and results obtained was that all the exposed LPS macrophages and post-treated with anise extract had viability data exceeding 50% and greater than 95% in the case of all post-treated macrophages unexposed to LPS, giving comparable results between negative and positive controls. These findings posit that the concentrations of anise extract at which the tests were conducted are not cytotoxic to the RAW 264.7 macrophages (Figure. 1). The study limitations associated with infecting lipopolysaccharides (LPS) in terms of empirical animal tests. Eventually, more experiments are needed to make sure that the RAW264.7 achieved the inflammatory phase.

Conclusion

In the context of the present study, we managed to conclusively demonstrate how bioactive compounds in the anise extract could induce *IRG1* expression hence decreasing ROS production in LPS-activated macrophages, which resulted anti-oxidation and anti-inflammation effects. These results, therefore, provide a link between *IRG1* induction and ROS reduction as well as the effect of the bioactive compounds following LPS infection. Because of the broad spectrum of *P. Anisum* 's pharmacological effects, and that no clinical studies have been performed so far on the plant's therapeutic effect and on the mRNA expression of *IRG1* expression as antioxidant and anti-inflammation, this research recommends that the tested extraction method used in this research to conduct more clinical trials to evaluate the plant's beneficial effects in mice and human models.

Abbreviations

GC/MS: gas chromatography/mass spectrometry analysis
DCF-DA: 2',7'-dichlorodihydrofluorescein diacetate
P. Anisum: *Pimpinella Anisum L.*
RAW264.7: murine macrophage cell line
***IRG1*:** immune-responsive gene 1
ROS: Reactive Oxygen Species
LPS: lipopolysaccharide
E. coli: Escherichia coli
M1 macrophages: macrophages produce nitric oxide (NO) or reactive oxygen intermediates (ROI)
MTT: Abbreviation for the dye compound 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor.
mRNA: Messenger RNA
RT-PCR: Real-time polymerase chain reaction
µg/mL: microgram/ millileter
µl: microlitter

Declarations

Ethics approval and consent to participate

Not Applicable

Consent for publication

Not Applicable

Availability of data and materials

All relevant data are within the paper

Competing of interest

The authors declare that they have no competing interests.

Funding

This research was funded and approved by the Deanship of Scientific Research at Princess Nourah Bint Abdulrahman University through the Fast-track Research Funding Program.

Acknowledgements

Not Applicable

Authors' contributions

'AH'. Formal analysis Performed experimental works, Investigation, Methodology, Resources, review, and editing; 'ARW'. Conceptualization, Formal analysis, Investigation, Methodology, review, and editing; 'AQ'. Performed experimental works, Data curation, Methodology, Resources, review, and editing; 'AI'. Methodology, Resources, Funding acquisition, review, and editing; 'AN'. Conceptualization, Formal analysis, Methodology, Software; 'AA'. Resources, Methodology, Software; 'ASW'. Project administration, Formal analysis, Investigation, wrote the original draft and submitted the paper as a corresponding author. All authors read and approved the final manuscript.

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