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Effect of scopoletin on phagocytic activity of U937-derived human macrophages: Insights from transcriptomic analysis

Amgad I. Alkorashy[a], Ahmed S. Doghish[a,b,*], Ahmed I. Abulsoud[a,c], Mohamed G. Ewees[d,e], Tamer M. Abdelghany[d,e], Mostafa M. Elshafey[a], Walid F. Elkhatib[f,g,**]

[a] Department of Biochemistry, Faculty of Pharmacy (Boys), Al-Azhar University, Nasr City, Cairo 11651, Egypt
[b] Department of Biochemistry, Faculty of Pharmacy, Badr University in Cairo (BUC), Badr City, Cairo 11829, Egypt
[c] Department of Biochemistry and Biotechnology, Faculty of Pharmacy, Heliopolis University, Cairo 11785, Egypt
[d] Department of Pharmacology and Toxicology, Faculty of Pharmacy (Boys), Al-Azhar University, Nasr City, Cairo 11651, Egypt
[e] Davis Heart and Lung Research Institute, The Ohio State University, Columbus, OH 43210, USA
[f] Department of Microbiology and Immunology, Faculty of Pharmacy, Badr University in Cairo (BUC), Badr City, Cairo 11829, Egypt
[g] Microbiology and Immunology Department, Faculty of Pharmacy, Ain Shams University, African Union Organization St., Abbassia, Cairo 11566, Egypt

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ABSTRACT

Scopoletin is a botanical coumarin. Notably, scopoletin effect on phagocytic activity has not been addressed on transcriptomic level. Accordingly, this study investigated the effect of scopoletin on phagocytosis-linked gene transcription. Whole phagocytosis transcriptional profiling of stimulated U937-derived macrophages (SUDMs) in response to scopoletin as compared to non-treated SUDMs was studied. Regarding scopoletin effect on 92 phagocytosis-linked genes, 12 of them were significantly affected (p-value $<$ .05). Seven genes were down-regulated (*CDC42, FCGR1A/FCGR1C, ITGA9, ITGB3, PLCE1, RHOD & RND3*) and five were upregulated (*DIRAS3, ITGA1, PIK3CA, PIK3R3 & PLCD1*). Moreover, scopoletin enhanced phagocytic activity of SUDMs. The current results highlighted the potential use of scopoletin as immunity booster and as an adjuvant remedy in management of some autoimmune reactions. To the best of our knowledge, this is the first report that unravels the effect of scopoletin on phagocytosis via transcriptomic analysis.

1. Introduction

The use of natural products as immunomodulators has become in the center of research studies due to the role of immune modulation in immediate protection and treatment of different diseases. Several medicinal plants exert their anti-infective action not only by directly affecting the infectious agent, but also by enhancing natural and adaptive immune response of the host. Therefore, natural-remedies have become significant versatile means in the field of immunotherapy [1]. Phytoalexins are chemicals synthesized in plants and accumulate in response to invading pathogens as part of plant defense mechanisms [2–4]. They are chemically diverse and include different chemical entities including terpenoids, alkaloids, glycosteroids and polypropanoids (coumarins). Scopoletin (7-hydroxy-6-methoxycoumarin) is a member of coumarin family that includes warfarin, esculetin, herniarin, psoralen and imperatorin. Coumarins have been studied as a possible source of therapeutic agents with different pharmacological activities such as immunomodulation, anti-microbial, anti-viral, anti-cancer, anti-inflammatory and antioxidant [5]. Among the immunomodulatory effect, some Coumarins could activate macrophages to degrade extracellular albumin to smaller peptides that can be phagocytosed, resulting in more efficient resorption of edematous fluids in lymphoedema [6–11].

Abbreviations: ATCC, American Type Culture Collection; C5a, Complement component 5a; CD, Cluster of Differentiation; COPD, Chronic Obstructive Pulmonary Disease; DEPC, Diethylpyrocarbonate; ECACC, European Collection of Authenticated Cell Cultures; Fc, Fragment crystallizable; GvHD, Graft versus Host Disease; ICH, International Conference on Harmonization; MOI, Multiplicity Of Infection; NSCLC, Non-Small Cell Lung Cancer; OECD, Organization for Economic Co-operation and Development; PI3K, Phosphatidylinositde-3 Kinase; PLC, Phospholipases C; PRKC, Protein Kinase C; qHTS, quantitative High-Throughput Screening; RA, Rheumatoid Arthritis; Rho, Ras homolog; ROS, Reactive Oxygen Species; RSE, Relative Standard Error; SCLC, Small Cell Lung Cancer; SUDMs, Stimulated U937-Derived Macrophages

* Correspondence to: Department of Biochemistry, Faculty of Pharmacy (Boys), Al-Azhar University, Nasr City, Cairo 11651, Egypt.
** Correspondence to: Department of Microbiology & Immunology, Faculty of Pharmacy, Ain Shams University, African Union Organization St., Abbassia, Cairo 11566, Egypt.
E-mail addresses: ahmed_doghish@azhar.edu.eg (A.S. Doghish), walid-elkhatib@pharma.asu.edu.eg (W.F. Elkhatib).

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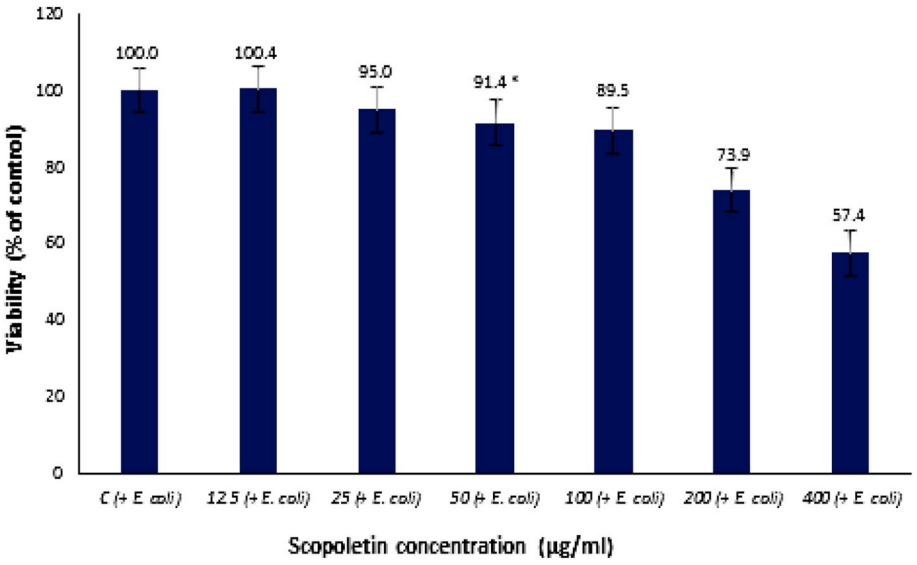

Fig. 1. Cytotoxicity of scopoletin on SUDMs, indicated as viability % vs. control (C) group. Cell viability was determined as described in Materials and Methods. Each column represents the % viability calculated from the mean absorbance of a triplicate, showing the standard error bars. *indicates the selected concentration for the main experiment; the highest concentration showing viability ≥90%.

Moreover, coumarin, 7-hydroxycoumarin and esculetin were found to stimulate the phagocytic activity of macrophages on the experimental model [12]. These findings open a new avenue of thinking for developing new coumarin-based immunity boosting, more specifically macrophage phagocytic enhancing drugs.

The mononuclear phagocytes are principal components of cellular defense against infections and composed of circulating monocytes and tissue macrophages [13]. Upon activation, macrophages engulf the invading organism; produce many cytokines and inflammatory mediators that finally lead to death of invading organism. Phagocytosis of infectious agent by macrophages stimulate the innate immune system response, which in turn coordinates the adaptive response by expressing high levels of major histocompatibility class II complex molecules to present antigens on their surface [14].

The role of scopoletin in phagocytosis remains unclear. Some studies support its ability to enhance phagocytic activity. For instance, scopoletin diminishes the intracellular survival of *Salmonella typhi* in a human macrophage cell line [15]. Moreover, a study performed on Noni fruit extract (containing scopoletin), proved the enhancing effect of Noni on the phagocytic activity of macrophages in cyclosporine A-induced immunocompromised mice [16]. On the other hand, scopoletin and scopolin could suppress the differentiation of osteoclastic murine macrophages by scavenging reactive oxygen species [17]. The mechanism of scopoletin suppressive effect on macrophages still requires to be elucidated on genetic level. Accordingly, the current study addressed the effect of scopoletin on transcription of 92 genes covering most of the phagocytosis pathway (including receptors and intracellular signaling mediators) in opsonized bacteria-stimulated U937-derived macrophages (SUDMs) using quantitative High-Throughput Screening (qHTS) which, to the best of our knowledge, may represent the first footprint of scopoletin as a potential immunomodulatory drug through transcriptomic and evidence-based research.

2. Materials and methods

2.1. Cell culture

Human U937 monocytic cell line (origin: Human Caucasian histiocytic lymphoma; lymphoblast, biological source: human lung, ECACC Cat. No.: 85011440, Passage no.: +3, purchased from Sigma-Aldrich, St. Louis, MO, USA) was maintained at 5×10^5 cells/ml cultures, in T-75 flasks (Corning, Corning, NY, USA), in RPMI 1640 medium, L-glutamine (2 mM), supplemented with 10% heat-inactivated FBS (Fetal Bovine Serum), 100 IU/ml penicillin G, and 100 mg/ml streptomycin (Gibco, Grand Island, NY, USA), and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Differentiation of monocytes into macrophages

Phorbol 12-myristate 13-acetate (PMA; from Sigma-Aldrich, St. Louis, MO, USA, Cat. No.: P1585), dissolved in dimethyl sulfoxide (DMSO) was used to activate U937 cell differentiation into macrophages, at a concentration of 100 ng/ml, and maintained for 4 days, as described previously [18,19]. The cells were observed to transform from suspended monocyte to attached macrophage due to increased expression of β2-integrins on cells' surface [20]. Scopoletin (Sigma-Aldrich, St. Louis, MO, USA, Cat. No.: S2500) was dissolved in DMSO, then diluted with medium to a final concentration of 0.1% as a stock solution [21].

2.3. Preparation of human serum-opsonized bacteria

A bacterial suspension of fixative-killed *E. coli* ATCC 7839 was provided by Department of Microbiology and Immunology, Faculty of Pharmacy, Al-Azhar University, in phosphate-buffered saline (PBS) with 1% BSA (Bovine Serum Albumin), with cell density of 1.5×10^8 cfu/ml. The killed bacterial cells were then opsonized by serum opsonins (antibodies and complements) via incubation with an equal volume of fresh human serum from a healthy 30-year old volunteer for 30 min at 37 °C [22]. The opsonized bacterial particles were pelleted by centrifugation at 8000 rpm for 5 min, and washed 3 times with sterile 1× PBS. Immediately before use (stimulation of macrophages), the bacterial cells were suspended in the same initial volume of sterile 1× PBS to maintain the initial cell density (1.5×10^8 cfu/ml).

2.4. Cytotoxicity assay

The cytotoxicity assay using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was performed as previously reported [23]. Attached macrophages were trypsinized and sub-cultured in fresh medium, into a 96-well plate at a count of 2×10^5 cells/ml and incubated for 3 h to re-attach to plastic surfaces. To the wells containing 100 µl of macrophage suspension (2×10^5 cells/ml), scopoletin was added in 2-fold escalating concentrations: 0 (control), 12.5, 25, 50, 100, 200, 400 µg/ml in triplicates, and incubated for 3 h. Macrophages were then stimulated for phagocytosis by adding the opsonized-bacteria at a multiplicity of infection of 20; 20 bacterial cfu for each macrophage, followed by 3-h incubation [15,21]. Afterwards, the medium in wells

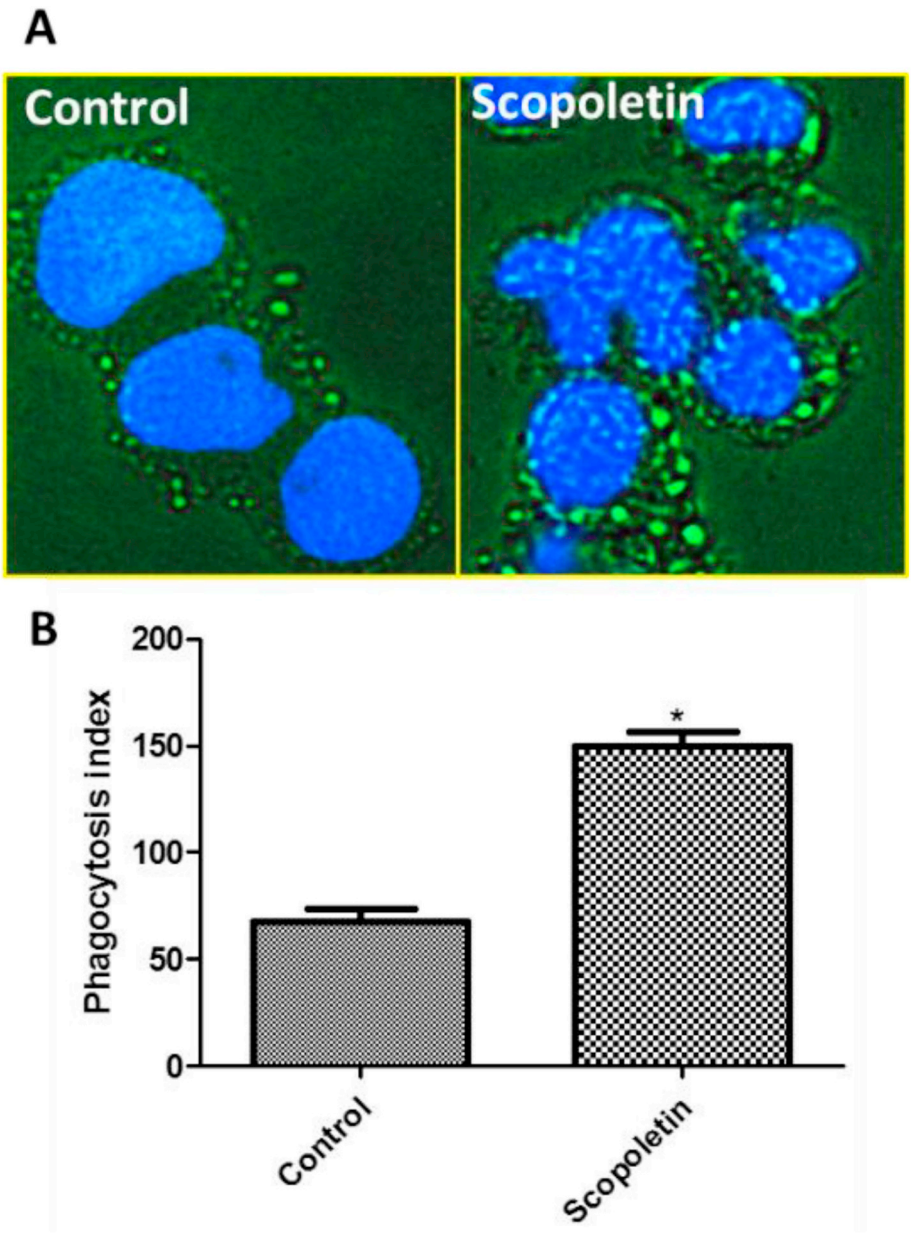

Fig. 2. Assessment of Phagocytosis index of macrophages. A: representative photos from control and Scopoletin treated cells using phase-contrast and fluorescence merged imaging mode. B. calculated Phagocytosis index according to the equation mentioned in methods. Values were obtained from at least 5 photos from each group. Data are represented as mean ± SE. * statistically significant from control at $P < 0.0008$ using unpaired *t*-test as test for significance.

was discarded and MTT reagent was added at a concentration of 50 μg/ml into each well. After incubation at 37 °C, the developed formazan blue crystals were dissolved in acid isopropyl alcohol. Optical absorbance at 570 and 630 nm, and Δ absorbance were determined with BioTek™ Epoch™ microplate reader (Fisher Scientific, Pittsburgh, PA, USA), through which % viability was calculated (Control group represented 100% viability).

2.5. Assessment of phagocytosis index

The phagocytosis index was calculated as described previously [24]. Cells were cultured in 6-well plate on glass coverslips to sub-confluence. Cells were removed from the CO_2 incubator, and treated with scopoletin (the highest concentration showing viability not less than 90%), and incubated for 3 h. Macrophages were then stimulated for phagocytosis by adding the opsonized-bacteria at a multiplicity of infection of 20; 20 bacterial CFU for each macrophage, followed by 3-h incubation in CO_2 incubator at 37 °C. After fixation, cells nuclei were counterstained with 1 μM DAPI (4′, 6-Diamidino-2-phenylindole, dihydrochloride) for 5 min at 37 °C. Coverslips were mounted on slides and cells were visualized using Leica epifluorescent microscope using merged phase-contrast and DAPI blue fluorescence channels. Digital images of randomly selected fields were captured and the number of engulfed cells in more than 200 macrophages was counted. The phagocytic index was calculated according to the following formula: phagocytic index = (total number of engulfed cells/total number of counted macrophages) × (number of macrophages containing engulfed cells/total number of counted macrophages) × 100.

2.6. Stimulation and treatment of macrophages

Macrophages, in the T-75 flasks at cell density 2×10^5 cells/ml, were treated with scopoletin (50 μg/ml), and stimulated with opsonized-bacteria, in the same manner described in the previous section;

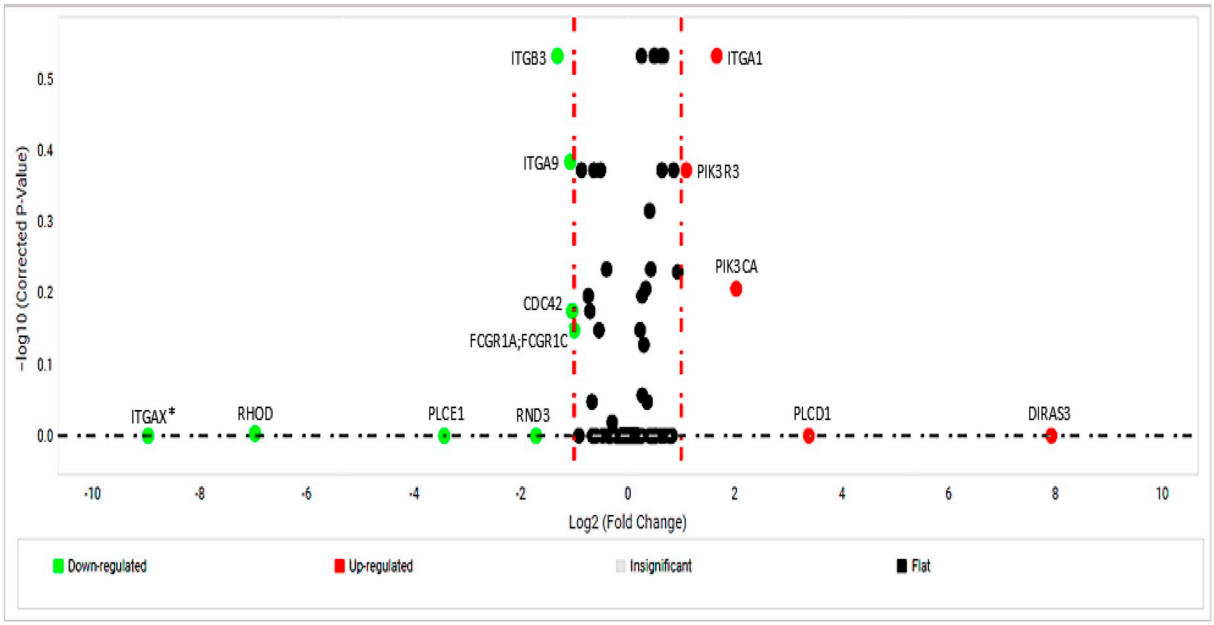

Fig. 3. Volcano plot of $\log_2$ (fold change = RQ) vs. $-\log_{10}$ (corrected *p*-value) for all target genes in the phagocytosis qPCR array, in response to scopoletin. *ITGA3* and *PRKCB* were used as endogenous controls/reference genes. Genes with $0.500 \geq RQ \geq 2.000$; $-1.000 \geq \log_2$ (fold change) ≥ 1.000 were considered significantly down- or up-regulated respectively. Results with p-value ≤ 0.05; corrected p-value ≤ 1; $-\log_{10}$ (corrected p-value) ≥ 0 were considered statistically significant. * denotes the statistically unreliable RQ values for genes whose ΔC_T RSE values are greater than 30%.

together with a non-treated control in triplicates. SUDMs were then harvested/pelleted and stored at −80 °C till RNA extraction.

2.7. RNA extraction

Total RNA extraction and RT-qPCR reactions were carried out according to kits manufacturers' instructions. Sterile and filtered DEPC (diethylpyrocarbonate)-treated water was used in all RNA protocols. TRIzol® Reagent, Ambion™ DNase I, RNase-free (Thermo Fisher Scientific Inc., Waltham, MA, USA) and RNeasy Mini Kit (Qiagen, Germantown, MD, USA) were used for RNA extraction and purification. Total RNA of each sample was reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.8. Array gene expression and RT-qPCR

To identify potential targets associated with phagocytosis affected by scopoletin vs. control, TaqMan™ Array Human Phagocytosis of Microbes (Cat. No.: 4414178) was used. A 96-well plate contained 92 assays of phagocytosis-associated genes and 4 assays of reference genes (18S, GAPDH, HPRT1, GUSB) as endogenous controls (Supplementary material Table 1). Gene names are in accordance with the approved symbols from the HUGO Gene Nomenclature Committee (HGNC) database.

All assays were run in triplicates; 3 plates for scopoletin-treated SUDM cultures versus 3 plates for non-treated control cultures ($n = 3$). To ensure qPCR results integrity, instruments calibration and cDNA quality were checked. The cDNA concentrations were homogeneous among samples. The cDNA concentration and purity were determined by NanoDrop 2000c (Thermo Fisher Scientific Inc., Waltham, MA, USA). The cDNA concentration means ± standard error values were 861.3 ± 41.19 and 873.7 ± 38.48 for control and treated samples, respectively. The cDNA absorbance (A260/A280 values) ranged from 1.81 to 1.84 for the 6 samples. In each well of the 96-well plate, a total volume of 20 µl; comprising 10 µl of TaqMan™ Universal Master Mix II, no UNG (Uracil-N glycosylase) (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 10 µl of cDNA diluted in DNase-free water, was included. The concentration of cDNA samples was 80 ng in 10 µl for each 20 µl PCR reaction/well. PCR array plates were run using ViiA™ 7 Real-Time PCR System and TaqMan Gene Expression Assays.

2.9. Data analysis

Cytotoxicity was analyzed using GraphPad Prism 5 and % viability values were expressed as means ± standard errors. Relative gene expression data were generated and analyzed by QuantStudio™ Real-Time PCR Software V1.2 (Applied Biosystems, by Thermo Fisher Scientific Inc.). Thermo Fisher Cloud software was used for the statistical analysis, using the RQ (Relative Quantification) method for different groups. Differences of p-value < 0.05 were considered significant, taking into consideration the Benjamini-Hochberg false discovery rate for p-values. Reference/endogenous control genes were selected using GeNorm score method for reference gene validation and normalization [25,26], by Thermo Fisher Cloud software. Based on the lowest observed scores (lowest variation), *ITGA3* and *PRKCB* were selected as endogenous controls in the current study. All reactions were performed in triplicates and ΔC_T measurements with a relative standard error (RSE) of more than 30% were considered statistically unreliable [27].

3. Results

3.1. Effect of scopoletin on phagocytosis index

To test the effect of scopoletin (used concentration: 50 µg/ml; the highest concentration showing viability not less than 90% (Fig. 1) on the phagocytic function of macrophages, phase-contrast imaging counterstained with fluorescent nuclear DAPI stain was used and at least 10 images were taken from either scopoletin treated or non-treated cells. Interestingly, scopoletin treatment markedly increased phagocytosis index compared to untreated macrophages (Fig. 2).

3.2. Effects of scopoletin on phagocytosis-associated genes expression

Array gene expression relative quantifications are well-explained (Fig. 3, and Supplementary material Table 1). Of 92 target genes

associated with phagocytosis, 12 were significantly affected by scopoletin; 7 genes were downregulated: *CDC42* (RQ = 0.488), *FCGR1A* and its pseudogene *FCGR1C* (RQ = 0.500), *ITGA9* (RQ = 0.476), *ITGB3* (RQ = 0.403), *PLCE1* (RQ = 0.093), *RHOD* (RQ = 0.008), and *RND3* (RQ =0.305). On the other hand, 5 genes were upregulated: *DIRAS3* (RQ = 242.537), *ITGA1* (RQ = 3.177), *PIK3CA* (RQ = 3.177), *PIK3R3* (RQ = 4.087), *PLCD1* (RQ = 10.474) with different significance levels. A gene was considered downregulated when it showed a minimum decrease of 50% on mRNA (transcription) level in comparison to control, equivalent to a cutoff of 0.5 fold change, while considered upregulated if showed a minimum increase of 100%; 2.0-fold change cutoff.

4. Discussion

Coumarins class of chemicals is involved in plant defense/immunity mechanisms, among other phytoalexins. Some functional assays have proved the stimulating effects of different coumarins on the phagocytic capabilities of different phagocytes [15,28,29]. To the best of our knowledge, no molecular assays were performed on the transcriptional level to depict the underlying mechanisms of scopoletin action on gene level. Comparing to the previous reports, the current study is unique in shedding light on genomic evidence-based medicine for the potential applications of scopoletin through transcriptomic analysis. Accordingly, qHTS assay was conducted on scopoletin to unveil its effects on phagocytosis signaling pathways. The main classes of phaogocytosis-allied mediators/effectors are: Fc receptors, Complements & Complement receptors, Fibronectin, Vitronectin & Integrin receptors, Lectin (mannose-like) receptors, Scavenger receptors, Toll-Like receptors, Phosphatidylinositde-3 Kinase, Phospholipases C, Protein Kinases C, Rho family GTPases, and Actins; all represented in the qPCR array used [30–35]. The current study covers a detailed elicitation of the scopoletin effects on the affected phagocytosis-mediated genes in light of their different functions.

The affected member of Fc receptors family is *FCGR1A* (CD64) (downregulated; RQ = 0.500). The Fc receptors bind to microbial particles opsonized with antibodies and induce their internalization, followed by downstream processes, like production of reactive oxygen species (ROS) and pro-inflammatory cytokines [30,33]. *FCGR1A* encodes a high-affinity Fc-gamma receptor (CD64) which binds to the Fc fragment of IgG-opsonized particles. Being downregulated, CD64's contribution to the phagocytic process seems to be limited by scopoletin, but on the other hand, its contribution towards aggravating autoimmune inflammatory responses will also decrease [36,37], as it's been proved to have a role in pathogenesis of many autoimmune inflammatory conditions/diseases including Rheumatoid Arthritis (RA) and other arthritic models [38–40]. These findings suggest that scopoletin may possess a balanced immunmodulatory activity. Interestingly, *Erycibe obtusifolia*, containing scopoletin, has anti-arthritic effect through reducing angiogenesis in synovium in an induced-arthritis model [41]. Another study reported scopoletin as a suppressor of differentiation of osteoclastic macrophages by scavenging ROS [17]. Scopoletin could be a good candidate to be tested against other autoimmune inflammatory diseases/conditions associated with increased expression of CD64 including Erythema Nodosum Leprosum, familial Mediterranean fever, Crohn's disease, chronic obstructive pulmonary disease (COPD), Kawasaki disease, Alopecia Areata, immune thrombocytopenic purpura and systemic lupus erythematosus (SLE). CD64 was also reported to be upregulated in acute graft versus host disease (GvHD) [42].

Regarding integrin receptors, 3 integrin genes were affected significantly including *ITGA9*, *ITGB3* (downregulated), and *ITGA1* (upregulated). Integrin receptors bind microbial particles opsonized non-specifically with fibronectin and vitronectin, secreted by the macrophage itself and other cellular sources [34]. Integrins are well-known for their role in cell invasion and migration, thus they may have a critical role in metastasis and cancer progression [43]. Moreover, *ITGA9* was proved to have a critical role in the pathogenesis of RA as well [44–46]; indicating potential application of scopoletin in treatment of RA. *ITGB3* is upregulated in pelvic organ prolapse disorder in women [47]; thus scopoletin can be considered as a potential remedy for this disorder by targeting *ITGB3*.

ITGA1, being upregulated by scopoletin, can be used to enhance non-specific binding of foreign bodies to macrophage surface as the first step of phagocytosis. The downregulation of salivary glands' *ITGA1* in Sjögren's syndrome, due to defective androgen secretion, leads to salivary glands' malfunctioning [48]. In view of that, scopoletin can be further tested for its capability of alleviating symptoms of Sjögren's syndrome by upregulating *ITGA1*'s expression. In the light of the qHTS being capable of unraveling potential beneficial as well as toxicological effects, upregulating *ITGA1* (CD49a) may induce T-cell-mediated hepatitis [49]. However, scopoletin was reported to have a hepatoprotective activity through antioxidant mechanisms [50], necessitating further toxicological studies to confirm or negate its overall effect.

Concerning intracellular signaling mediators in phagocytosis, phosphatidylinositde-3 kinase (PI3K) is essential in recruiting signaling molecules. PI3K is recruited to TLRs and its activation is involved in NF-KappaB-mediated cytokine production [32,35]. PI3K is composed of a 110 kDa catalytic subunit (including domain α; *PIK3CA*) and an 85 kDa regulatory subunit (including domain 3; *PIK3R3*) [51,52]. Upregulating both subunits is favorable in terms of phagocytic process augmentation.

The second class of signaling mediators is Phospholipase C isozymes; one was downregulated (*PLCE1*) and the other was upregulated (*PLCD1*) in response to scopoletin. Phospholipases C (PLC) is essential in accumulating intracellular calcium and activating Protein Kinase C (PRKC) family members. PLC mediate microbe-induced pro-inflammatory signaling in macrophages past activating PRKC [13,32,34,35]. Furthermore, *PLCE1* is down-regulated in cystic fibrosis [53]; thus, scopoletin may be studied for possible role in cystic fibrosis. Interestingly, *PLCE1* and *PLCD1* were downregulated and upregulated nearly by the same fold-change (> 10) indicating that both of them are regulated through the same mechanism.

The third class of affected signaling mediators by scopoletin is Rho family GTPases; *CDC42*, *RHOD*, *RND3* were downregulated and *DIRAS3* was upregulated. Rho GTPases are the points of intersection for most of the signaling pathways regulating the whole phagocytic process. They are the main controllers of actin cytoskeleton remodeling and endosome dynamics [13,32,34,35].

RHOD depletion can improve the formation of favorable actin structures/rearrangements as stress fibers and edge ruffles with improved dynamics. This affects endocytosis, cell migration and proliferation [54]. In line with that, RHOD was found to be overexpressed in multiple myeloma [55]. In view of that, downregulating *RHOD* by scopoletin, by *125* fold, may play a role in the underlying mechanism by which scopoletin enhances phagocytosis. While *RND3* downregulation by scopoletin also appears to be in favor of phagocytosis boosting by the same mechanism; as *RND3* negatively regulates the organization of the cytoskeleton causing loss of adhesion [56]. *DIRAS3* was extremely upregulated by *242* fold in response to scopoletin. The encoded protein plays a role in autophagy by regulating the autophagosome initiation complex in cancer cells [57,58]. As such, scopoletin may be studied as a potential anti-cancer agent.

5. Conclusion

Scopoletin may have several pharmacological effects that extend from enhancement of phagocytosis and immunomodulatory effects to prevention and treatment of cancer progression and metastasis. It might be considered in management of other diseases such as some autoimmune disorder, GvHD, pelvic organ prolapse, Sjögren's syndrome, and cystic fibrosis. Scopoletin should be widely investigated through various experimental animal models to validate these findings.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2020.03.022.

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