

Original Research Article

In-vitro anti-inflammatory activities of 3-methoxy quercetin isolated from Nigerian mistletoe parasitic on *Garcinia kola* Heckel, Clusiaceae

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Abstract

Purpose: To evaluate the *in vitro* anti-oxidant and anti-inflammatory potential of the most potent and abundant metabolite, 3-methoxy quercetin (3-MQ), from extract fractions of mistletoe, *Loranthus micranthus* Linn (Loranthaceae) parasitic on *Kola acuminata* Schott & Endl, (Malvaceae), also known as *Garcinia kola* Heckel, (Clusiaceae).

Methods: Compounds isolated through a combination of chromatographic techniques were screened for *in vitro* antioxidant potential using the diphenyl picrazyl hydrazine (DPPH) radical-based model. Cell viability at 1–1000 μ M 3-MQ in 24h was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test. Lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate ($n = 5$) five (5) replicates.

Results: Ten (10) known compounds including 3-MQ (1) were isolated and characterized. 3-MQ exhibited highly significant ($p < 0.05$) antioxidant activity with 50 % inhibitory concentration (IC_{50}) of 15.0 μ M; concentrations $\leq 100 \mu$ M did not exert cytotoxic effect. 3-MQ, at 25 and 125 μ M concentrations, significantly ($p < 0.05$) inhibited the production of TNF- α by 82 and 100 %, respectively, compared to controls.

Conclusion: The results demonstrate the potent anti-inflammatory activity of 3-MQ and suggests its use as a potential alternative therapy for inflammation and related diseases.

Keywords: *Loranthus micranthus*, *Kola acuminata*, *Garcinia kola*, Anti-inflammatory, Cytotoxicity, Chemiluminescence, Antioxidant, TNF- α

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INTRODUCTION

Plant-derived natural products, including the quercetin-derivatives, are ubiquitous and undoubtedly form interesting scaffolds for the development of potent antioxidant-anti-inflammatory as well as possible antitumor congeners. This is illustrated by the fact that in

the flavonol group alone, over 230 new compounds were identified from 1986 to 1992 [1], while approximately 180 new structures were isolated in the subsequent years 2001-2003 [2]. The flavonol, quercetin is the most abundant specie in nature occurring as rhamnosylated or glucosylated derivative [3]. In food, quercetin occurs mainly as glycosides and usually, after ingestion, derivatives of quercetin are hydrolyzed

mostly in the gastrointestinal tract and then absorbed and metabolized [4-6]. Therefore, the content and form of all quercetin derivatives in food is significant for their bioavailability as the aglycone. Quercetin as a naturally occurring flavonoid protects cells against oxidative stress [7,8]. Several studies have shown that quercetin in its free or glycosylated form modulates certain physiological functions with potent anti-proliferative effects in numerous cell lines [9,10], pro-apoptotic effect in lung carcinoma cell lines [11], and inhibitory effect of bone metabolism [12].

It is well established that the pro-inflammatory cytokines, interleukin 1 (IL 1), interleukin 6 (IL 6), and tumor necrosis factor (TNF) are involved in the regulation of the immune response, hematopoiesis, and inflammation. These cytokines are actively involved in inflammatory responses [13]. Overproduction of reactive oxygen and nitrogen species (ROS and RNS, respectively) by phagocytes causes oxidative damage to membrane lipids, DNA, proteins and lipoproteins. These reactions have functional

consequences, which may be deleterious to cells and tissues. Thus, inhibition of ROS and RNS production is a popular target for the attenuation of many inflammatory diseases [14]. The reported anti-inflammatory mechanisms included reduction of the pro-inflammatory cytokines IL-6 and TNF- α , increasing anti-inflammatory IL-10 secretion, and reduction of cyclooxygenase-2 (COX-2) and nitric oxide synthase expressions [15]. Two excellent reviews of quercetin and derivatives with detailed emphasis on their structures and functions were published in 2008 and 2012 [16,17]. Our group investigated and reported the osteogenic properties of 3-MQ [18]. 3-MQ is a derivative of quercetin with an attachment of a methoxy group at position 3 of quercetin [18]. The present study aims to investigate the anti-inflammatory activities of this major constituent (3-MQ) of the Nigerian mistletoe used traditionally as in the management of inflammation and associated conditions. The structures of the studied compound, 3-MQ alongside nine (9) other co-isolated compounds as depicted in Figure 1.

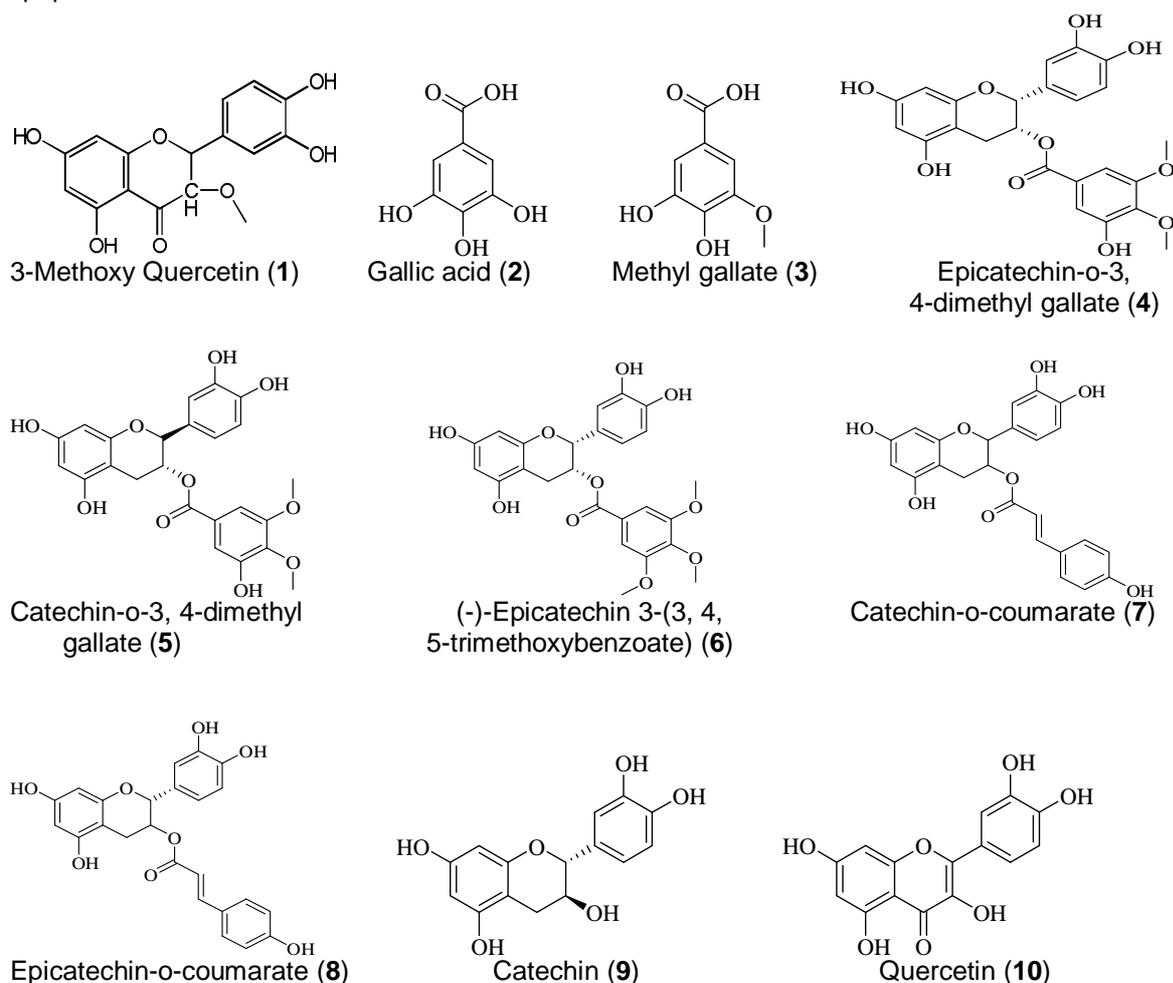


Figure 1: Structures of 3-MQ and other isolated compounds

EXPERIMENTAL

Collection of plant materials

Loranthus micranthus leaves parasitic on *Garcinia kola* were collected in October 2011, from different locations in Nsukka LGA, Enugu state Nigeria. The leaves were identified and certified by Mr AO Ozioko, a taxonomist of the Bioresources Development and Conservation Programme, Nsukka, Nigeria. A voucher specimen (no. BDC-1023-011) has been kept at the herbarium of the same centre for reference purposes.

Materials and reagents for cell cytotoxicity and molecular biology studies

The following materials and reagents were procured and used for the study: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lipopolysaccharide, LPS (serotype 0128: B 12, L 4255; Sigma, St Louis, MO, USA), multi-well plate reader (Bio-Kinetic Reader-E312e®; Bio-Tech Instruments, Winooski, VT), ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA), enhanced luminol solution (Pierce Biotechnology Inc. Rockford, IL, USA), microplate luminometer (*Synergy® H1 Hybrid Multi-Mode*, BioTek Winooski, VT, USA), Gen 5 ® version 2.0 (BioTek Winooski, VT, USA), Phorbol 12-myristate 13-acetate (PMA), RPMI 1640 medium (Corning cellgro® RPMI; Mediatech Inc., Manassas, VA, USA), 2-mercaptoethanol (Gibco, Invitrogen, USA), PBS, DMSO. All materials and or reagents were used according to manufacturer's specifications and protocols.

Cell lines and culture medium

Laboratory stock of RAW264.7 mouse macrophage cell line (ATCC, MD, USA) was cultured in R-10 medium, consisting of RPMI 1640 medium (Corning cellgro® RPMI; Mediatech Inc., Manassas, VA, USA) supplemented with 10 % heat-FBS, 50 µM 2-mercaptoethanol (Gibco, Invitrogen, USA), 100 µg/mL penicillin, and 100 µg/mL streptomycin in a 5 % CO₂ humidified atmosphere at 37 °C.

Preparation of crude aqueous extract

Leaves of *Loranthus micranthus* parasitic on *Garcinia kola* were cleansed and dried under shade for 8 days. They were pulverized in mechanized laboratory grinder to fine powder. A total of 1.2 kg of the powdered plant materials was macerated repeatedly with distilled water (total volume; 12 L, 10 L; 7.5 L respectively). The

resulting aqueous extracts were lyophilized under vacuum affording dry powdered extracts which were weighed and their percent yield determined. The dry extracts were placed in amber-colored glass bottles and stored in a refrigerator (4 °C) until use. The yield obtained was 9.80 %.

Fractionation of crude extracts of mistletoe

Based on preliminary evaluated anti-inflammatory potential of different crude extracts (unpublished data), exactly 70 g of crude extract obtained from mistletoe parasitic on *Garcinia kola* was uniformly dispersed in 450 mL of distilled water and then carefully poured into a stoppered separatory funnel. Then, aliquots (500 mL) of analar grade hexane was poured into the funnel and vigorously agitated for 5 min to allow for equilibration. The funnel was mounted on a stand to allow for complete separation of the solvents into layers. The lower aqueous layer was run off and the upper hexane layer collected in a glass bottle. This process was repeated severally until the *n*-hexane no longer extract any further constituents from the extract dispersion. The hexane fraction was pooled and concentrated *in vacuo* (40 °C) to afford the dry hexane fraction. Then, solvents of increasing polarity in the order, chloroform<ethyl acetate<butanol<water were used accordingly as described above for hexane to afford corresponding fractions. The major fractions were screened for anti-inflammatory activities and active fractions subjected to column chromatography.

Isolation of 3-MQ (compound 1)

The isolation and characterization of compound 1 was as previously described [18,19].

Isolation of other compounds

Compounds **2-10** were isolated using a combination of various chromatographic techniques. Each time, the separation process was monitored by TLC and analytical HPLC. Specifically, compounds 2 and 10 were isolated from the *n*-butanol fraction while compounds 3-9 were from the ethyl acetate fraction. The spectra of all compounds were compared to existing literature data and were found to be unequivocally same.

Vacuum liquid chromatography (VLC)

Exactly 35.4 g of the ethyl acetate (EtOAc) fraction was fractionated on a silica gel VLC column (200-400 mesh, 20 x 6 cm) using

gradient elution of *n*-hexane-EtOAc (100:0, 90:10, 80:20....0:100 each fraction collected was 1.2 L), and of dichloromethane in methanol (90:10, 70:30, 60:40, 30:70....10:90, each volume 800 mL) to afford the 15 fractions (F1-F15). The fractions of interest, F6-F9 were subjected to further VLC sessions and later to sephadex LH20 column to afford sub-fractions F61, F62, F63, F67; F71, F72, F77; F81, F82, F83, F86 and F91, F92, F93, F97. The sub-fractions with substantial yields were either subjected to semi-preparative HPLC or sephadex column to obtain pure compounds **3-9**. This was repeated for the *n*-butanol fraction (22.7 g) to obtain compounds **2** and **10**. Each time, the separation and isolation were monitored closely by TLC and analytical HPLC.

Semi-preparative HPLC

The mobile phase was either HPLC grade methanol with nanopure water and with or without 0.1 % trifluoro acetic acid (TFA) in gradient manner and reverse-phased. The maximum quantity of fraction injectable was 3 mg in 1ml of methanol. The flow rate was set at 5 ml/min in the system described thus; pump was Merck Hitachi L-7100; detector was also Merck Hitachi UV detector L-7400; column was Knauer, Berlin, Germany (300 x 8 mm; internal Diameter), prepacked with Eurosphere, 100-10 C18, with integrated pre-column.

Analytical high performance liquid chromatography (HPLC)

Analytical HPLC was used to probe the distribution of constituents in the extracts and fractions and match them with existing compound libraries (plants and marine). This served to estimate the purity of the isolated compound(s) and guide in the choice of isolation techniques. The reverse phase gradient flow of solvent system was methanol and nanopure water in an equilibration pH of 2.0 and run time remained 35 min or 60 min depending on outcome. The injection volume from an auto sampler remained 20 μ L with peaks detected by an array of UV-Vis photodiode detectors usually at 235, 254, 280 and 340 nm wavelength. The complete description of the entire system was as follows: Pump-Dionex P580A LPG; detector-Dionex Photodiode Array Detector UVD 340S; Column thermostat-STH 585; Auto sampler- ASI-100T, HPLC programme used-Chromeleon (v.6.3). The instrumentation comprises a pump, the detector, injector, separation column and solvent reservoir. The column (125 x 2 mm,

internal diameter) was pre-filled with Eurosphere-100 C18 of 5 μ m pore size. The system had an integrated pre-column.

Antioxidant assay of the isolated compounds

All the compounds were assessed for antioxidant potential using DPPH in methanol and at a wavelength of 517 nm.

Cell cytotoxicity assay

The effect of 3-MQ on the viability of RAW264.7 mouse macrophages was determined using a modification of the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) cytotoxicity assay originally described by Mosman [20]. To modify the original MTT assay technique, the volumes of reagents and temperature of incubation were reduced appropriately.

Assessment of inhibition of LPS-induced release of nitric oxide by macrophages

Nitric oxide production and release by RAW264.7 cells was measured indirectly by determining nitrite accumulation in culture supernatant using the Griess reaction.

Determination of effect of 3-MQ on the production of TNF- α

The effect of 3-MQ on LPS-induced release of tumour necrosis factor (TNF- α) by macrophages was determined according to established protocols [21].

Chemiluminescence determination of ROS and RNS scavenging by 3-MQ

Chemiluminescence of RAW264.7 macrophages was evaluated in Hank's balanced salt solution (HBSS; pH 7.4) by a modification of the enhanced-luminol microplate assay [21].

Statistical analysis

Data are expressed as mean \pm SEM. The data obtained in experiments with multiple treatments were subjected to one-way ANOVA followed by Newman-Keuls post-hoc multiple comparison test using Prism 3.0 version software. Where necessary, Student's 't' test was used to study statistical significance ($p < 0.05$) in experiments with only two treatments.

RESULTS

Antioxidant activity of the compounds

Out of the ten compounds tested (Figure 1), compound 1 (3-MQ) exhibited highly significant ($p < 0.05$) antioxidant activity compared to the controls (Table 1). This further informed its choice for further detailed anti-inflammatory research work.

Table 1: Radical scavenging activity of isolated compounds (DPPH method)

Compound	Radical Inhibition (%)	IC ₅₀ (μM)
1	89.9 ± 1.00	15.00
2	69.0 ± 1.00	35.00
3	72.5 ± 2.50	29.00
4	70.0 ± 2.70	25.00
5	65.0 ± 3.90	38.50
6	60.0 ± 3.70	39.70
7	61.0 ± 4.20	40.00
8	66.0 ± 5.60	36.80
9	70.0 ± 4.60	28.20
10	72.0 ± 4.10	24.00
Ascorbic acid	94.8 ± 4.70	7.50
Chlorogenic acid	98.9 ± 1.20	4.50

Compound 1 (3-MQ) exhibited the highest radical scavenging activity

Effect of 3-MQ on cell viability

The effect of 3-MQ on the viability of RAW 264.7 cells is shown in Figure 2. The compound, beyond a concentration of 100 μg/mL caused a sharp reduction in the number of viable cells. However, the high concentration of compound used in the study suggests that it is relatively safe.

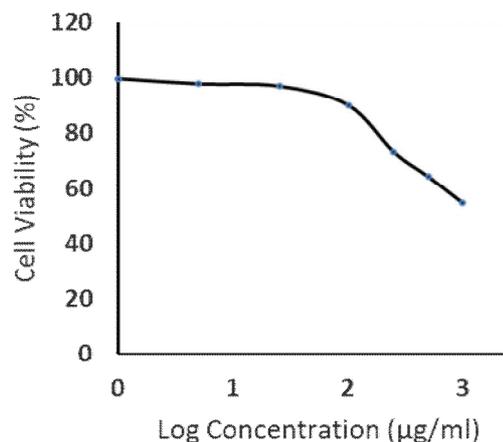


Figure 2: Effect of 3-MQ on viability of RAW 264.7 cells

Effect of 3-MQ on nitrite production by RAW 264.7 cells

3-MQ significantly caused a dose-dependent reduction in the amount nitrite produced by the RAW 264.7 cells compared with both controls. 3-MQ caused a graded inhibitory response on the production of nitric oxide by the RAW 264.7 cells (Figure 3). This suggests the potential anti-inflammatory activity of the compound.

Inhibitory effect of 3-MQ on tumour necrotic factor (TNF-α) production

The compound, 3-MQ caused a dose-dependent graded inhibitory response on the production of TNF-α by the RAW 264.7 cells (Figure 4). This suggests significant potential anti-tumor and anti-inflammatory activity of the compound.

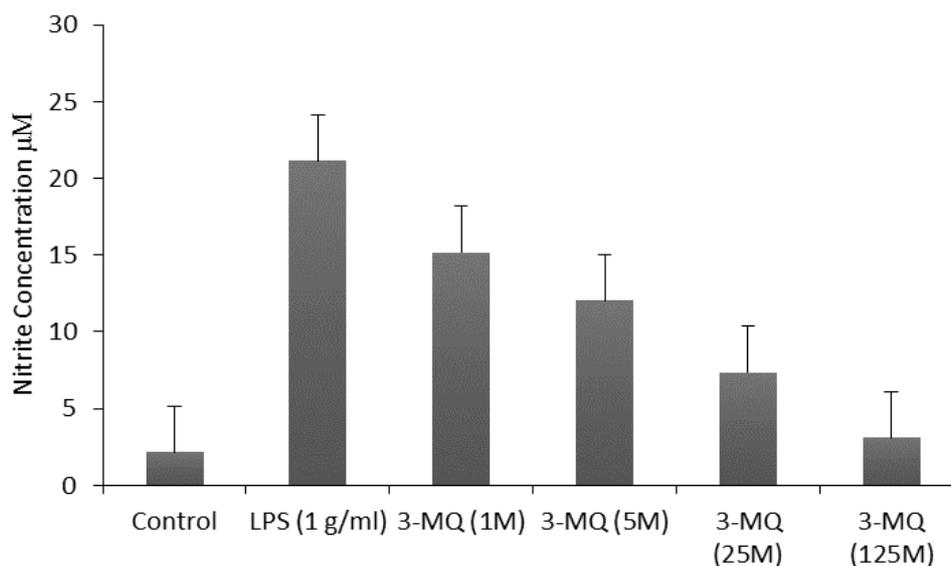


Figure 3: Effect of 3-MQ on nitrite production by RAW 264.7 cells

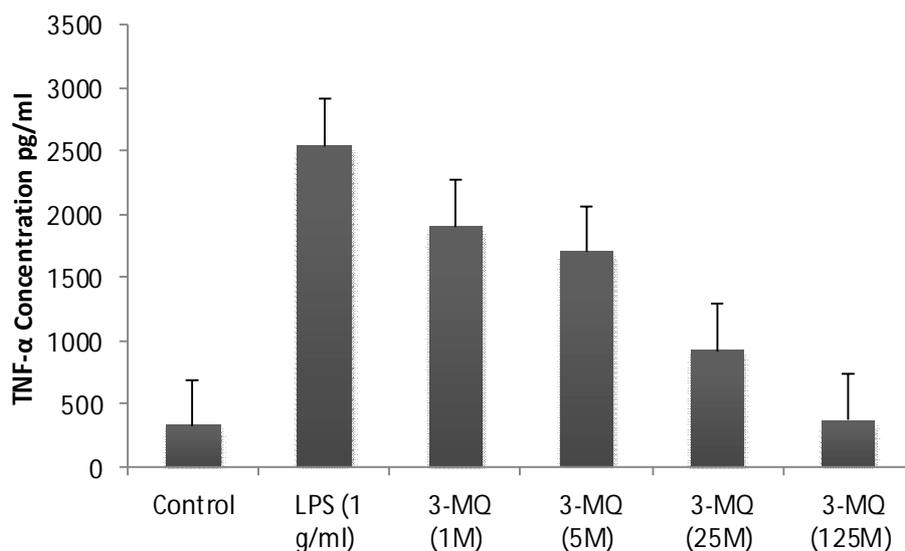


Figure 4: Effect of 3-MQ on the production of TNF- α

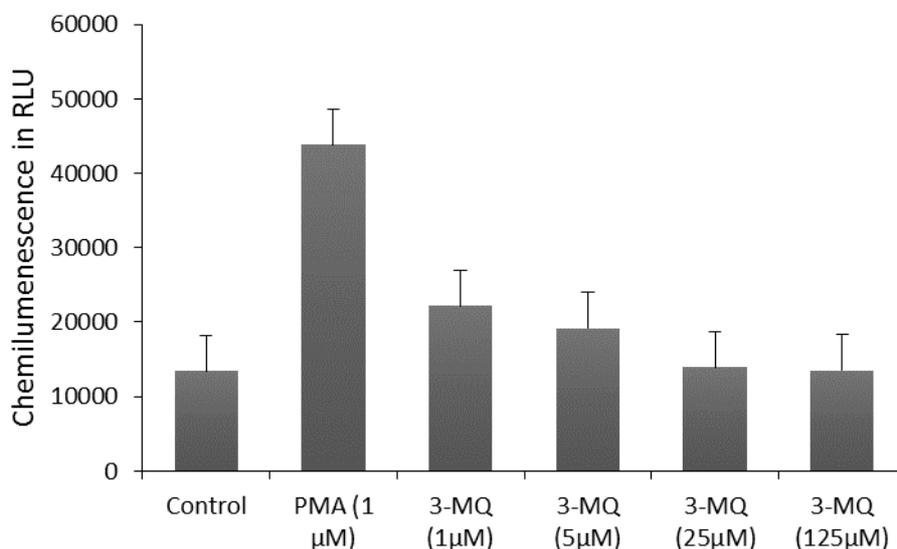


Figure 5: Effect of 3-MQ on chemiluminescence of PMA-activated RAW264.7 macrophages cells

Effect of 3-MQ on chemiluminescence of PMA activated RAW264.7 macrophages cells

The compound, 3-MQ significantly inhibited chemiluminescence of PMA activated RAW 264.7 macrophage cells in a dose-dependent manner (Figure 5). This suggests that the compound has potent anti-inflammatory activity.

DISCUSSION

The present study shows that the Eastern Nigeria mistletoe extract is rich in flavonoids and related polyphenols. These compounds showed moderate to highly significant antioxidant activities with 3-MQ as the most potent (Table 1). It is well known that inflammation is significantly correlated with oxidative stress and hence

compounds with potent radical scavenging activity are probable candidates for anti-inflammatory or antitumor screening. Coupled with the presence of gallic acid and its derivatives in high concentration, mistletoe extracts has justification for its many ethnomedicinal uses as anti-inflammatory, anti-rheumatic, antimicrobial and antihypertensive agent [18]. In response, an attempt was made to study the cytotoxic effects and the anti-inflammatory properties of 3-MQ, obtained in very high amount from the extract.

The cytotoxicity profile for 3-MQ revealed no significant cytotoxic effects at concentrations of 0, 5, 25 100 μ g/mL. However, toxicity was observed at concentrations of 250, 500 and 1000 μ g/mL. The observed toxicity on the RAW 264.7 cells was dose-dependent. Higher concentrations

of 3-MQ (>1000 µg/mL) were toxic to the cells. In contrast, Yen-Chou *et al* [22] reported a concentration-dependent inhibitory effects of quercetin and its pentaacetate derivative, on LPD-induced NO production in without obvious cytotoxic effect on the RAW 264.7 cells.

Expectedly, the methoxy group at position 3 of quercetin moiety which enhances lipophilicity of the molecule (increased cellular permeation by the compound) could probably be responsible for this observed toxicity at much higher doses. It is therefore, reasonable, to use optimal doses of 3-MQ that will elicit the required biological activity without precipitating toxicity. Although nitric oxide exerts anti-inflammatory effect under normal physiological conditions, it is considered as a pro-inflammatory mediator that induces inflammation due to excessive production in abnormal conditions. Lipopolysaccharide (LPS)-challenged RAW 264.7 cells, which have not been pre-treated with 3-MQ, resulted in excessive production of nitric oxide (NO).

There was a dose-dependent reduction in the production and release of NO for the cells pre-treated with 3-MQ. Specifically, when compared to the positive control group (group without LPS), there was inhibition of NO release at concentrations of 25 and 125 µM of 3-MQ. At 125 µM, 3-MQ almost restored the concentration of NO under normal physiological conditions (94.88 % reduction). This finding corroborate an earlier report that flavonoids and their glycosides, such as apigenin, wogonin, luteolin, tectorigenin, and quercetin inhibited NO production in LPS-activated RAW 264.7 cells as measured by nitrite formation at 10-100 µM [23]. This finding supported an earlier report by Luo *et al* [24] who found that quercetin and hyperoside exhibited concentration-dependent enzyme inhibitory effects on NO production in activated cells. Although further mechanistic studies are required to understand the possible pathway of action, it is plausible to propose a mechanism for this observed activity, possibly due to reduction of induced nitric oxide synthase (iNOS) enzyme expression since nitric oxide (NO) produced by inducible iNOS is one of the inflammatory mediators.

There was excessive production of TNF-α by the lipopolysaccharide-activated RAW 264.7 cells. TNF-α is a pro-inflammatory cytokine, and hence its presence in an abnormal concentration signals inflammation. It was observed that 3-MQ pre-treated RAW 264.7 cells suppressed the production of the pro-inflammatory agent; TNF-α. This inhibition was observed to be dose-dependent, with maximum inhibition occurring at

the 125 µM concentration of 3-MQ. This inhibitory activity is comparable to the positive control group (group without LPS). Therefore, 3-MQ was able to restore the normal production of TNF-α by the RAW 264.7 cells when activated by the LPS. A previous study reported several anti-inflammatory mechanisms to include reduction of the pro-inflammatory cytokines IL-6 and TNF-α, increasing anti-inflammatory IL-10 secretion, as well as reduction of cyclooxygenase-2 (COX-2) and nitric oxide synthase expression [15].

Regarding the anti-inflammatory property, ROS and RNS release by activated RAW264.7 cells was significantly inhibited by 3-MQ. $p < 0.05$ Inflammation processes trigger off reactions between macrophages and microorganisms leading to the excess production of mediators such as reactive oxygen species (ROS) and nitrogen species (RNS), nitric oxide (NO), as well as various cytokines. Oxidants and nitrogenous compounds generated by macrophages to destroy phagocytized pathogens are also involved in tissues injury associated with inflammatory process [25]. Numerous studies have found out that phenolic acids and flavonoids, which are biologically active molecules, act as scavengers of free radicals and inhibitors of nitric oxide and inflammatory cytokines production by macrophages and/or neutrophils. 3-MQ, a flavonoid, exerts anti-inflammatory properties by inhibiting chemiluminescence reactions and scavenging ROS and RNS.

CONCLUSION

The observed reduction in the pro-inflammation agents (NO and TNF-α) and the radical scavenging activity against ROS and RNS in LPS-stimulated RAW 264.7 cells in a dose-dependent manner is an indication of the anti-inflammatory activity of 3-methoxy quercetin. The cytotoxicity of higher doses of 3-MQ needs to be further investigated. In addition, mechanistic studies on this compound would be helpful in view of its *in vitro* anti-inflammatory activity observed in this study.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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