Research Article

Hesperidin Supplementation Improves *In Vitro* Nuclear and Cytoplasmic Maturation of Dromedary Camel Oocytes

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Abstract | *In vitro* maturation (IVM) of camel oocytes is a crucial component of assisted reproductive technologies (ARTs) that aim to enhance camel embryo production. The purpose of this study was to investigate the impact of hesperidin (HP), a potent antioxidant, on the maturation rate of she-camel oocytes. Different concentrations of hesperidin were used to identify the optimal dose for reducing oxidative stress, which negatively affects oocyte maturation. Good quality oocytes were matured in maturation medium supplemented with 0, 25, 50, or 75 uM hesperidin. Then, they were incubated in CO_2 incubator at 38.5 C, 5% CO_2 and 95% humidity for 36 hours. Results demonstrated that hesperidin significantly ($p < 0.05$) increased nuclear maturation rates in the 50 uM HP-treated group. In conclusion, hesperidin improved *in vitro* cytoplasmic maturation of she-camel oocytes in a concentration-dependent manner, as reflected by cumulus expansion and polar body formation. Further studies are needed to investigate subsequent fertilization and embryo developmental competence.

Keywords | Hesperidin, She-camel, Oocytes, Reproductive technologies, Maturation, Antioxidant

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INTRODUCTION

Camels have always played a valuable role in the desert as the only source of meat, milk and transportation under extreme dry conditions. However, research into improving commodity characteristics like milk or meat production has only recently begun to increase. The development of camel racing in the Middle East created higher value for racing animals, so the desire to improve reproductive efficiency in camels has become an area of focus for researchers (Skidmore, 2011).

IVM is a key process in *in vitro* embryo production (IVP), and optimizing it in any species could improve IVP success. Regulation of oocyte maturation influences not just the percentage of oocytes maturation, but also their fertilization and subsequent embryo development (Russo *et al.*, 2014).

Oocyte maturation is defined as a complex biological process that is controlled or influenced by many metabolic and endocrine processes, as well as external stressors. One such stressor category is oxidative stress, which is defined as an imbalance between the generation and accumulation

of reactive oxygen species (ROS) in cells and tissues and the system›s ability to detoxify or eliminate reactive species (Pizzino *et al*., 2017).

An elevated oxidative stress state promotes apoptosis, mitochondrial damage, telomere shortening, and bio macromolecule damage, all of which accelerate the aging process of the ovaries (Yang *et al*., 2012). Kandil *et al*. (2022) showed that, antioxidant supplementation alleviated oxidative stress while enhancing gene expression associated with oocyte development.

Various approaches have reported that supplementation of external antioxidants to the maturation medium is thought to be an essential defense factor against oxidative stress (Khattab *et al*., 2020) which significantly improved oocyte quality maturation and reduce oxidative stress (Khatun *et al*., 2024).

There have been many studies in other mammals (Kim *et al*., 2018; Park *et al*., 2022), showed that, hesperidin (HP) plays an important role in improving oocyte maturation by reducing reactive oxygen species (ROS) and increasing glutathione (GSH) levels that improved spindle organization, chromosomal alignment and cytoplasmic maturation, and increased blastocyst formation and embryo viability.

Although most studies on hesperidin have been conducted in monovulatory species such as mice and pigs, camels possess unique reproductive physiology, including follicular wave patterns that differ significantly from the single-ovulation cycles observed in monovulatory mammals (Skidmore, 2018). Moreover, dromedary camel oocytes contain high lipid contents, making them particularly vulnerable to oxidative damage during *in vitro* maturation and cryopreservation (Yassin *et al*., 2024). Therefore, it is reasonable to hypothesize that antioxidant supplementation, such as hesperidin, could provide similar or even greater protective benefits in dromedary camels, despite their distinct ovarian dynamics.

The current study investigated the effect of hesperidin, a potent antioxidant, on the maturation rate of she-camel oocytes.

MATERIALS AND METHODS

Plant material

Samples of *C. maxima* (Burm.) Merr growing in Egypt used in this study were collected from Shehab Mazhar Botanical Garden (Albarageel, Giza). The authentication of the plant was kindly confirmed by Agriculture engineer Mrs Therese Labib, senior botanist, El-Orman botanical garden, Giza, Egypt. *C. maxima* (Burm.) Merrill peels were dried and voucher specimens, (No. 30.3.16.1) are kept in the Herbarium collection of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

Isolation and identification of hesperidin

According to Lahmer *et al*. (2015), hesperidin was isolated from dried peels of *C. maxima* using the following procedure. Briefly, 20 g of dried and powdered peels were defatted in a Soxhlet extractor for 4 h using 150 mL of n-hexane at 40–60 °C. The defatted powder was then extracted with methanol in a Soxhlet apparatus for 2 h until exhaustion. The methanolic extract was evaporated under vacuum to a syrupy consistency. The resulting extract was mixed with 6% acetic acid in water (50 mL), the precipitate was filtered using Buchner funnel to give crude hesperidin, washed with 6% acetic acid, and dried at 60 °C to yield 190 mg of yellowish-white powder (0.95% w/w).

The obtained compound was soluble in methanol and produced a dark purple color under UV light, which turned yellow after exposure to ammonia vapor. The identity of the isolated flavonoid was confirmed as hesperidin by ^{1}HNMR and ^{13}CNMR spectroscopy, consistent with previously published data (Lahmer *et al*., 2015). Detailed spectral data are provided in the Supplementary Information (Supplementary Figures S1, S2, S3; Supplementary Table S1) and the purity of isolated hesperidin was compared with standard hesperidin that purchased from Simga-Aldrich company (H5254-25G) (Supplementary Figure S4).

Biological study

This work was conducted at Department of Artificial Insemination and Embryo Transfer, Animal Reproduction Research Institute (ARRI), Al-Haram, Giza, Egypt during the period from December 2024 to May 2025.

The chemicals used in this study were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA).

Forty eight ovaries were collected from apparently normal reproductive organs of slaughtered she-camel of unknown age and breeding history at El-basatin slaughterhouse. Ovaries were extracted within 2 h of slaughter. The ovaries were brought to the lab and kept at 25-30°C in sterile normal saline (0.9% NaCl) in a thermos container supplemented with 100 uM streptomycin (Masoud *et al*., 2023). The ovaries were washed 3 times in pre-warmed saline (37°C) and extra tissues were removed and maintained in warm normal saline at 37°C.

Oocytes were aspirated from follicles (3-8 mm in diameter) using a device consisting of a 20-gauge needle connected

to a 10-mL syringe (Abdoon, 2001). Collected oocytes were transferred to phosphate buffer saline (m-PBS) to which bovine serum albumin (4 mg/ml) was added and examined under a stereomicroscope (Iwasaki *et al*., 2018).

Cumulus–Oocyte Complexes (COCs) were classified according to their physical traits according to Assidi and Sirard (2013). The retrieved COCs were classified into 4 grades based on their morphological appearance:

- Grade A (good), COCs with six layers of dense compact cumulus cells investment and evenly granular homogenous ooplasm.
- Grade B (fair), similar to grade A, but with 2-4 layers cumulus cells.
- Grade C (poor), partially or completely denuded oocytes.
- Grade D (very poor), characterized by highly scattered cumulus cells and dark irregular ooplasm.

Ovaries were collected on five different days (replicates), and in each replicate approximately 20 COCs per treatment were cultured. Thus, each treatment group included a total of 100 oocytes across five replicates. Oocytes collected on each day were randomly allocated across all treatment groups to minimize variability due to collection day or donor animal.

Oocytes with AandB COCs grade were washed three times in TCM-199 *. Groups of 10–15 oocytes were placed in 100 µL droplets of TCM-199 medium supplemented with Earle's salts, 10 uM FSH, 10% FCS, 50 uM sodium pyruvate, 2.6 mg/ml sodium bicarbonate, and 50 uM gentamicin. The medium was supplemented with either 0 , 25 uM, 50 uM or 75 uM hesperidin and then oocytes were incubated for 36 h at 38°C under 5% of CO2 in air with 95% humidity (Khattab *et al*., 2020).

The expansion of cumulus cells and the extrusion of the first polar body were used to assess cytoplasmic maturation (Khattab *et al*., 2020).

Cumulus expansion was evaluated morphologically under a stereomicroscope and classified into two categories: (i) Total expansion, representing expansion of cumulus cell mass to at least 2 times its original diameter away from zona pellicida (Z.P.); and (ii) High expansion, representing expansion, representing expansion of cumulus cell mass to at least 3 times its original diameter away from Z.P. characterized by the dispersion of all cumulus cell layers and clear visibility of the zona pellucida (Chauhan *et al*., 1999).

Statistical analysis

Percentages were calculated per replicate (day) and analyzed using one-way ANOVA, with day (n=5) as the unit of replication. Tukey's post-hoc test was applied for multiple comparisons. Data are presented as mean ± SEM, and significance was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Data presented in Table 1 demonstrate that supplementation of the maturation medium with 25 and 50 uM hesperidin significantly increased oocyte maturation rates, as evidenced by cumulus–oocyte complexes (COCs) expansion (Figure 1) and polar body extrusion (Figure 2). At 50 uM, the maturation rate was highest, with total expansion (85% vs. 69% control), high expansion (50% vs. 33% control), and polar body extrusion (50% vs. 32% control). These findings indicate that hesperidin significantly enhanced nuclear and cytoplasmic maturation in camel oocytes ($p \leq 0.05$).

In the High expansion parameter, the control group (33%) and the 25 uM group (43%) were not significantly different, despite numerical differences, whereas both were significantly lower than the 50 uM group (50%).

Similar improvements in oocyte competence with hesperidin were previously reported in porcine and murine oocytes (Kim *et al*., 2019; Kandil *et al*., 2022).

Table 1: Effect of antioxidants on cytoplasmic maturation rate of Dromedary camel.

Groups	No. of oocytes (N)	Total expansion (mean ± SEM)	High expansion (mean ± SEM)	Polar body (mean ± SEM)
Control	100	69±4.65 a	33±4.73 ab	32±4.69 ab
25 uM	100	81±3.94 b	43±4.98 bc	41±4.94 bc
50 uM	100	85±3.56 b	50±5.03 c	50±5.03 c
75 uM	100	61±4.90 a	26±4.41 a	23±4.23 a

Different superscripts within the same column indicate significant differences at $P \leq 0.05$ (Tukey's post-hoc test). Groups sharing at least one superscript letter are not significantly different from each other.

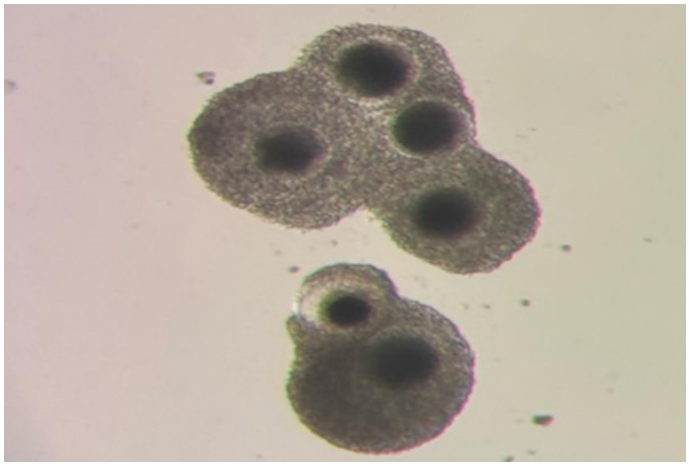

Figure 1: Oocytes after incubated 36 h and expansion of (COCs) clearly defined.

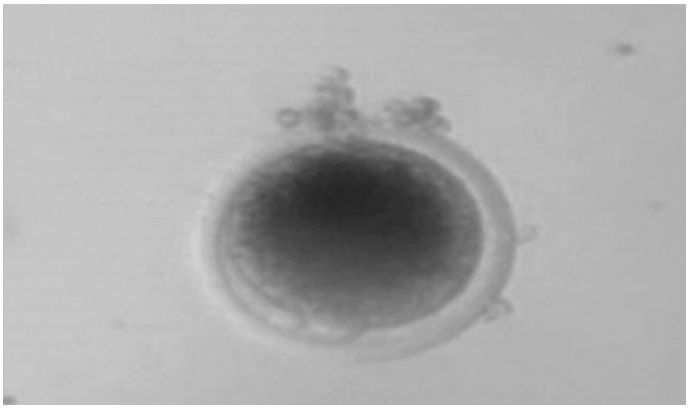

Figure 2: Oocytes after denuded the first polar body appeared (nuclear maturation).

Hesperidin had a dose-wise effect. On one hand, low doses caused enhancement of expansion and maturation, on the other hand, high dose caused retardation of the aforementioned parameters.

The observed improvements may be related to the known antioxidant and anti-inflammatory activities of hesperidin, which have been reported in other species to reduce reactive oxygen species (ROS) accumulation and increase intracellular glutathione (GSH) (Kim *et al*., 2018; Park *et al*., 2022). However, since ROS and GSH levels were not directly measured in the present study, these potential mechanisms remain speculative and warrant further biochemical investigation in camels. Antioxidant supplementation during *in vitro* maturation has been shown to enhance mitochondrial activity and distribution, improving fertilization rates (Kandil *et al*., 2022). In porcine oocytes, hesperidin supplementation increased glutathione (GSH) levels and improved both nuclear and cytoplasmic maturation (Kim *et al*., 2019). Moreover, hesperidin upregulates PCNA and FSH-R expression, genes critical for folliculogenesis and reproductive outcomes (Shoorei *et al*., 2023).

In contrast, 75 uM hesperidin exerted toxic effects, significantly reducing maturation rates (Table 1). Total expansion declined to 61%, high expansion to 26%, and polar body extrusion to 23%. Morphologically, oocytes at this concentration exhibited poor cumulus expansion and defective polar body formation, reflecting impaired nuclear maturation. These results suggest that supra-optimal doses disrupt redox balance and induce cytotoxicity, consistent with prior reports of antioxidant over-supplementation impairing gamete quality (Rodríguez-Varela and Labarta, 2020; Budani and Tiboni, 2020).

The first polar body is considered a reliable indicator of oocyte meiotic competence and quality. In this study, polar body extrusion peaked at 50 uM (50%) and was lowest at 75 uM (23%). High-quality oocytes are typically associated with intact, smooth, non-fragmented polar bodies, which predict developmental potential (Yang *et al*., 2022).

Collectively, these findings highlight that hesperidin exerts a dose-dependent effect on camel oocyte maturation. Intermediate concentrations (25–50 uM) promoted optimal COC expansion and nuclear maturation, while excessive doses (≥75 uM) impaired oocyte quality. This aligns with the broader principle that adequate antioxidant supplementation enhances gamete competence, but that excess levels can disrupt cellular balance and induce apoptosis (Gualtieri *et al*., 2021). Future investigations should assess fertilization success, embryo viability, and long-term reproductive performance of hesperidin-treated camel oocytes.

CONCLUSION

This study demonstrated that supplementation of *in vitro* maturation (IVM) medium with hesperidin improved cytoplasmic maturation of dromedary camel oocytes in a concentration-dependent manner. The optimal effect was observed at 50 uM, which significantly enhanced cumulus cell expansion and first polar body extrusion compared to the control group. Conversely, a higher concentration (75 uM) exerted toxic effects, resulting in reduced maturation rates. These findings highlight the potential of hesperidin as an effective antioxidant supplement for improving camel oocyte IVM protocols, although further studies are warranted to assess its impact on subsequent fertilization and embryo development.

NOVELTY STATEMENT

While the beneficial effects of antioxidants on oocyte maturation have been reported in other species, the impact of hesperidin on dromedary camel oocyte IVM has not been investigated before. This study is the first to demonstrate a concentration-dependent effect of hesperidin, identifying 50 uM as the optimal dose to improve cytoplasmic maturation of she-camel oocytes.

AUTHOR'S CONTRIBUTION

Dalia E. Ali: conceptualization, methodology, compound isolation and analysis, investigation, writing—original draft, and reviewing and editing. M. A. Al-Tammar, A. Montaser, A. O. Hegab, A. M. F. ElRuby: Oocyte collection, experimental work, data analysis, prepare first manuscript draft. All authors have read and agreed to the published version of the manuscript

Generative AI and AI-assisted technology statement

The authors declare that no Genrative AI was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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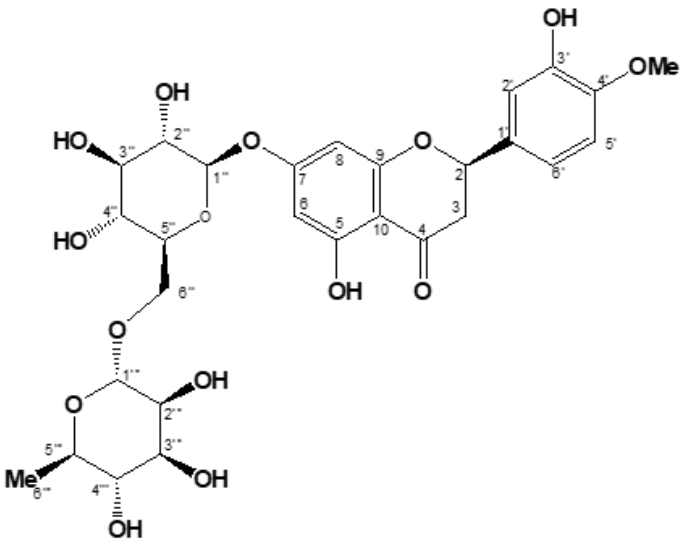
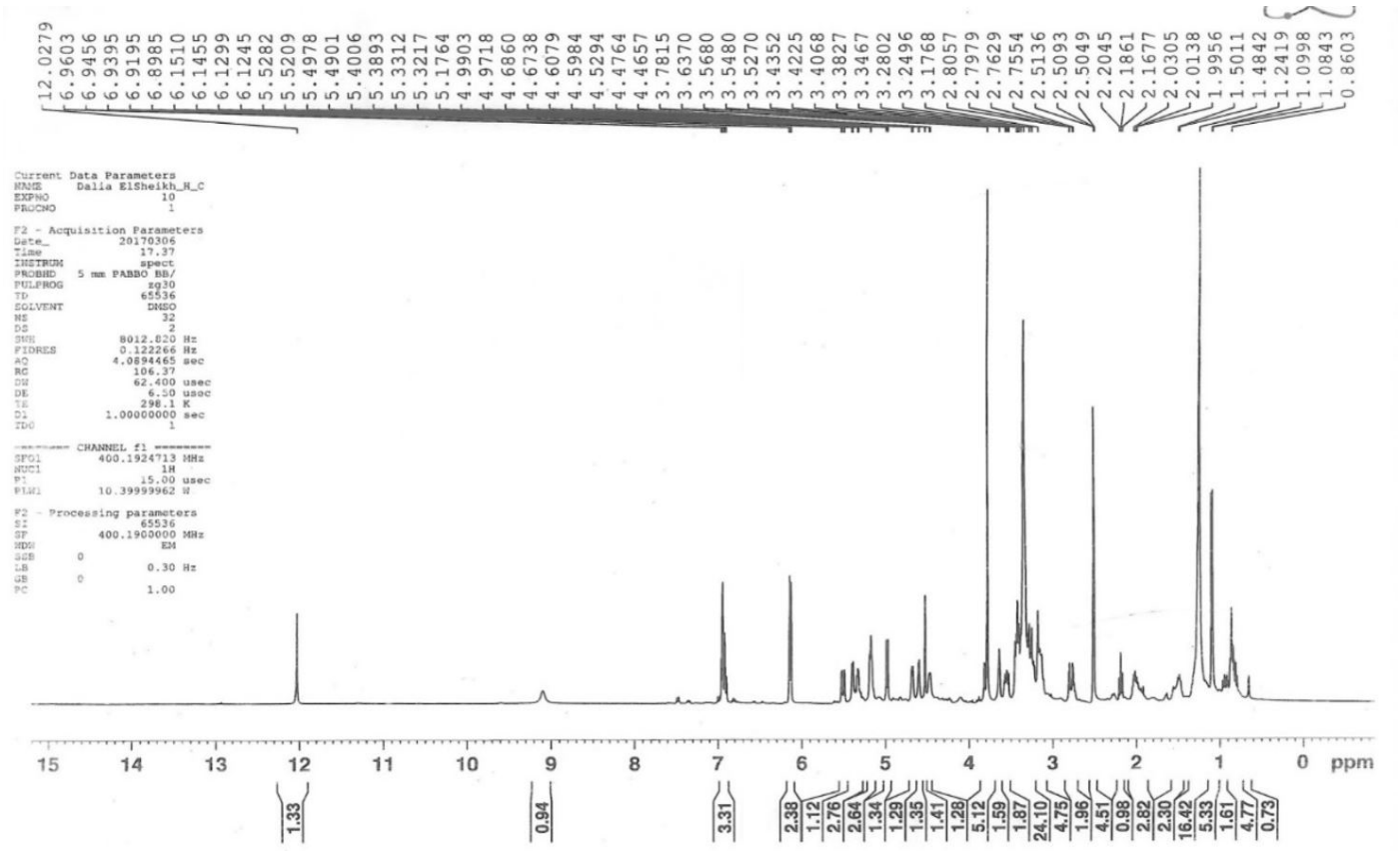
Supplementary Table S1: NMR spectral data of the isolated compound.

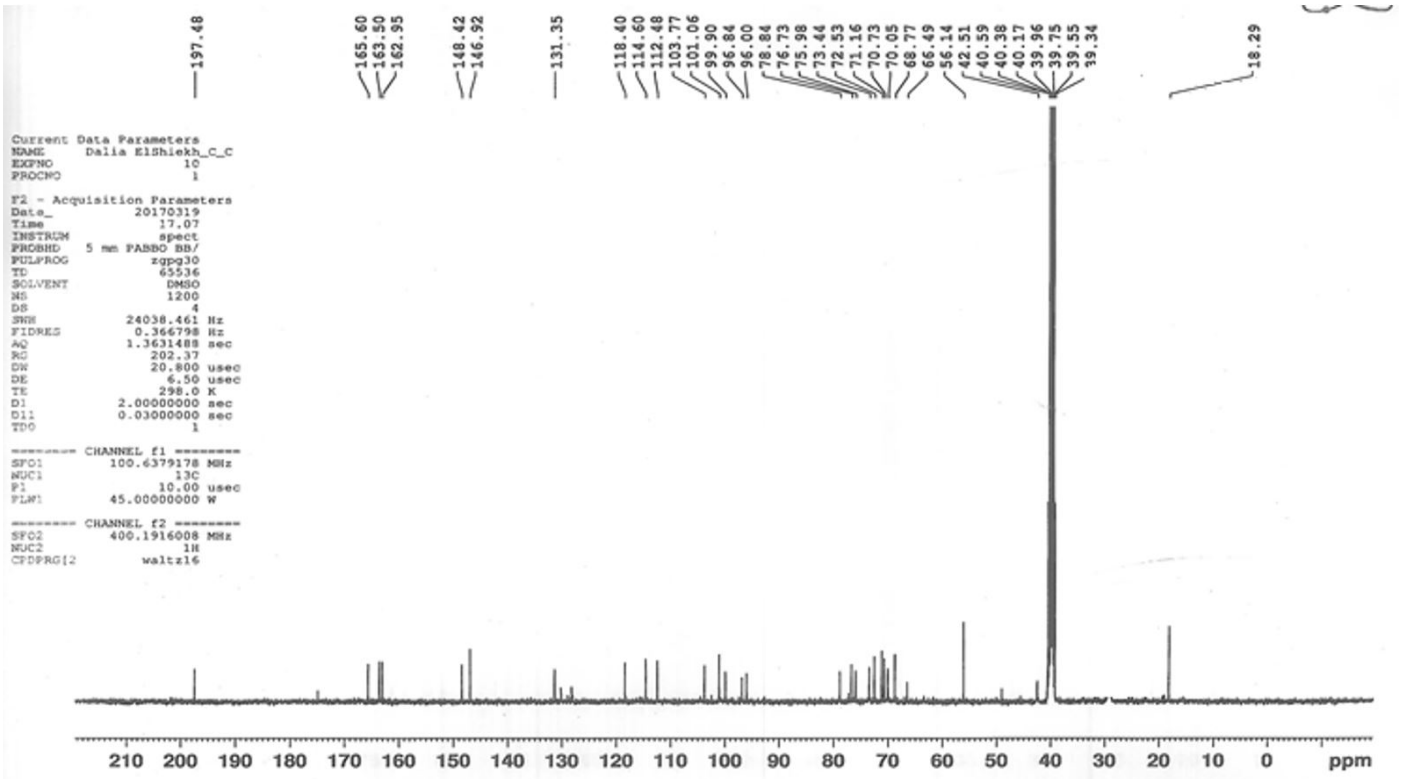
Carbon No.	^{1}HNMR (DMSO-d_6, 400MHz)	^{13}CNMR (DMSO-d_6, 100MHz)
2	5.5(1H, dd, *J*=12 Hz, 3Hz)	78.84
3	3.24(1H, dd, *J*= 16, 8Hz, H-3a) 2.79(1H, dd, *J*=17, 3Hz, H-3b)	42.51
4		197.48
5	12.03 (1H, s, 5- OH)	163.5
6	6.13(1H, d, *J*=2 Hz, H-6)	96.84
7		165.6
8	6.15(1H, d, *J*=2 Hz)	96
9		162.9
10		103.77
1`		131.35
2`	6.94(1H, d, *J*=2 Hz, H2`)	114.6
3`	9.18 (1H, s, 3` – OH)	146.92
4`		148.42
5`	6.93 (1H, d, *J*=8 Hz, H-5`)	112.48
6`	6.96 (1H, dd, *J*=2 and 8 Hz, H-6`)	118.4
1``	4.98(1H, d, *J*=8 Hz)	99.9
2``	3.2-3.2(m, sugar protons)	72.53
3``		75.98
4``		71.16
5``		76.73
6``		66.49
1```	4.74(1H, br s)	101.06
2```	3.2-3.2(m, sugar protons)	68.77
3```		73.44
4```		70.73
5```	2.2(1H, d, *J*=6 Hz)	70.05
6```	1.08(3H, d, *J*=6 Hz)	18.29
OCH_3	3.78(3H, s, 4`-OCH_3)	56.14



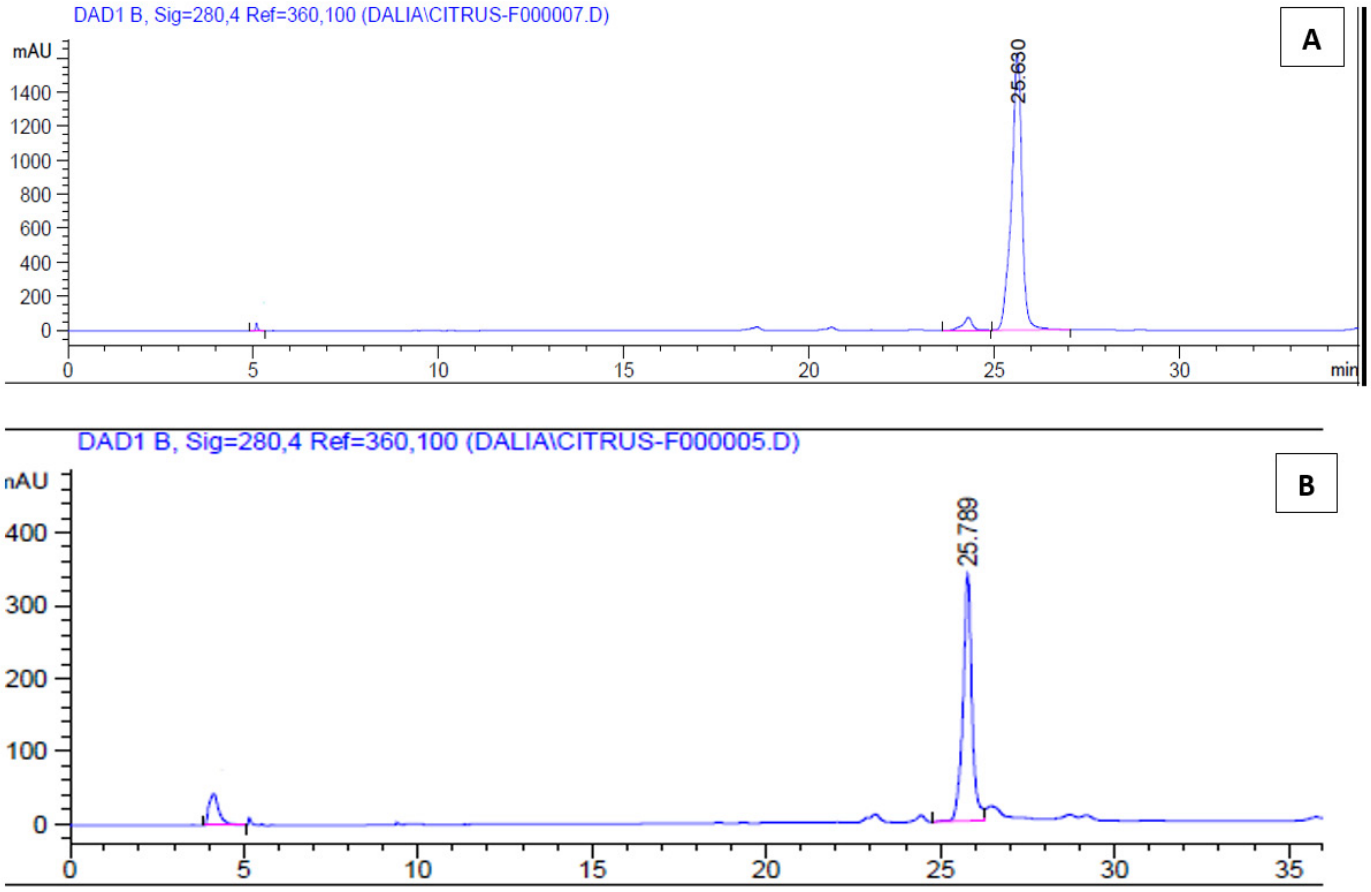
Supplementary Figure S1: Chemical structure of hesperidin.



Supplementary Figure S2: ^{1}H-NMR spectrum (in DMSO-d_6) of the isolated compound.



Supplementary Figure S3: ^{13} C- NMR spectrum (in DMSO-d_6) of the isolated compound.



Supplementary Figure S4: HPLC chromatogram of isolated hesperidin (A) and standard hesperidin (B).