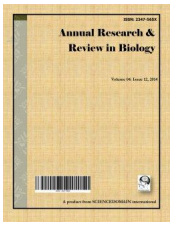

Annual Research & Review in Biology

25(5): 1-9, 2018; Article no.ARRB.40165
ISSN: 2347-565X, NLM ID: 101632869

In-vitro Antioxidant Effect of Thymoquinone

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Authors' contributions

The present study was the result of the collaboration of all authors. Author MB designed the study, performed the statistical analysis, proposed the protocol and wrote the preliminary version of the manuscript. Authors HK, WS, AK and AM managed the study analyses and managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2018/40165
Editor(s):
(1) Saleha Sadeeqa, Incharge Institute of Pharmacy, Lahore College for Women University, Pakistan.
(2) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) Ieong Xinfang, Malaysia.
(2) Bhaskar Sharma, Suresh Gyan Vihar University, India.
(3) Ahmad Eid, An-Najah National University, State of Palestine.
Complete Peer review History: http://www.sciencedomain.org/review-history/24089

Original Research Article

Received 27th January 2018
Accepted 4th April 2018
Published 10th April 2018

ABSTRACT

Aim: In recent years, natural products from medicinal plants have received considerable attention. Thymoquinone, the major compound from *Nigella sativa L*. Is the most used due to its several pharmacological properties. This study was undertaken to evaluate the antioxidant effect of thymoquinone which is very useful in controlling free radicals noxious species causing the induction and / or amplification of a number of pathologies.
Study Design: Spectrophotometric methods.
Place and Duration of Study: Biochemistry Department, Applied Biochemistry Laboratory, Nature and Life Sciences Faculty, Ferhat Abbas Setif 1 University, Setif, Algeria, between December 2016 and September 2017.
Methodology: *In vitro* antioxidant study was characterized by using free radicals scavenging methods with reducing power, lipid peroxidation and ß-carotene bleaching assays. All tests were realized by spectrophotometric methods.
Results: Our study showed that thymoquinone is a potent antioxidant. It is less effective as a scavenger of both DPPH and ABTS radicals with IC_{50} of 125.65 ± 0.76 and 332.5 ± 14.39 µg/ml,

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respectively; While it is a strong scavenger of hydroxyl radical with IC_{50} of 26.3 ± 0.59 µg/ml and very strong hydrogen peroxide scavenger with IC_{50} of 11.0 ± 0.57 µg/ml. As superoxide anion scavenger it has inhibition ability less than 50%. In lipid peroxidation, TQ had a very efficient activity it inhibits peroxidation of β- carotene by 73.58 ± 0.50%. Thus, in lipid peroxidation assay, TQ had also a significant activity with percent inhibition of 79.5 ± 2.12%.

Conclusion: Our results revealed that thymoquinone possesses a low antioxidant activity against DPPH, ABTS and superoxide anion radicals. Thus, it has a very low reducing power capacity, whereas it is a strong hydrogen peroxide, hydroxyl scavenger and lipid peroxidation inhibitor.

Keywords: Thymoquinone; antioxidant; scavenger; lipid peroxidation; reducing power.

1. INTRODUCTION

Oxidative stress can be defined as an imbalance in the oxidant-antioxidant balance which accompanied with over production of free radicals. These free radicals are molecules or molecular fragments which have one or more unpaired electrons in atomic or molecular orbital [1]. The unpaired electron(s) is characterized by high degree of reactivity. In living systems, the most important class of radical species is reactive oxygen species including superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) as free radicals and hydrogen peroxide (H_2O_2) as non-free radical [2]. Free radicals have a great capacity to damage all types of cellular constituents in the organism, which explains their involvement in the induction and / or amplification of several pathologies including cancer [3] cardiovascular disease [4] neural disorders [5] mild cognitive impairment [6] alcohol induced liver disease [7] aging [8] atherosclerosis [9] and arthritis and other inflammatory disorders [10,11]. The human body has a complex antioxidant system including enzymatic (superoxide dismutase, catalase, glutathione peroxidase and reductase,…) and non-enzymatic defenses (reduced glutathione, vitamine C, vitamine E,…) which reduced the free radicals noxious effects. Thymoquinone (TQ) is the major active monoterpene derived from *Nigella sativa*, it has been the subject of many recent studies due to its pharmacological properties such as anti-inflammatory [12] antidiabetic [13] neuroprotective [14] hepatoprotective [15] and anti-arthritic [16]. The aim behind our study is the evaluation of the antioxidant effect of TQ, which is very useful in controlling the noxious effect of free radicals causing the induction and / or amplification of several pathologies. *In vitro* studies are used to investigate the antioxidant effect of thymoquinone , such as scavenging of free radicals DPPH, ABTS, H_2O_2, $O_2^{\bullet-}$ and $OH^{\bullet}$ with the estimation of reducing power ability and lipid peroxidation inhibition activity. Ascorbic acid (Vitamin C) was used as standard due to its significant antioxidant activity.

2. MATERIELS AND METHODS

2.1 Materials

Thymoquinone, ascorbic acid (Vitamin C), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azino-bis 3-ethylbenzo-thiazoline -6-sulfonic acid (ABTS), hydrogen peroxide, sodium Salicylate, nitro blue Tetrazolium (NBT), potassium ferricyanide, trichloroacetic acid(TCA), ferric chloride and all reactive were purchased from Sigma Aldrich.

2.2 Methods

2.2.1 Effect of thymoquinone on free radicals scavenging

2.2.1.1 DPPH scavenging assay

The free-radical scavenging capacity of TQ was determined with DPPH which is the most basal antioxidant assay used [17]. DPPH is a stable free radical and does not dimerize as the most other free radicals because its spare electron is delocalized over the molecule [18]. It is measured as previously described by Sharma and Bhat [19] with slight modification briefly, different concentrations of TQ was prepared in methanol, the reaction was started by the addition of 200 µM DPPH solution in methanol. The reaction mixture was incubated in the dark at 30ºC for 30 min and the absorbance was measured at 517 nm using Spectronic Genesys 5 spectrophotometer. Ascorbic acid was used as standard. The percentage of antioxidant activity was determined according to the next equation:

$$\text{Inhibition (\%)} = [(A_C - A_S)/A_C] * 100$$

A_C is the control reaction absorbance and A_S is the TQ or ascorbic acid absorbance. The data are presented as mean ± SD.

2.2.1.2 ABTS scavenging assay

The ABTS radical scavenging activity is based on the estimation of ABTS radical cation formation. $ABTS^{*+}$ was generated by mixing 7 mM of ABTS solution with 2.45 mM of potassium sulfate then, the mixture was incubated at room temperature for 16 h. The solution was diluted to get an absorbance of 0.7±0.02 at 734 nm. 2 ml of ABTS solution were added to 0.3 ml of different concentrations of TQ, the mixture was incubated for 30 min, the absorbance was measured at 734 nm [20]. Ascorbic acid was used as standard. The percentage of antioxidant activity was estimated according to the next formula:

$$\text{Inhibition (\%)} = [(A_C - A_S)/A_C] * 100$$

A_C is the control reaction absorbance and A_S is the TQ or ascorbic acid absorbance.

2.2.2 Hydroxyl scavenging

The scavenging of $OH^{\bullet}$ was carried out according to the method of Smirnoff and Cumbes [21]. The Principe of this method is based on the production of $OH^{\bullet}$ in the reaction medium through the reaction of Fenton, then the OH product reacted with sodium salicylate to produce the hydroxyl salicylate complex. Briefly; 1.0 ml of $FeSO_4$ (1.5 mM), 0.7 mL of hydrogen peroxide (6 mM), 0.3 mL of sodium Salicylate (20 mM) and 1 ml of the various concentrations of TQ or ascorbic acid as standard. After incubation at 37° C for 1 h, the absorbance of the hydroxyl complex Salicylate was measured at 562 nm. The inhibition percentage of $OH^{\bullet}$ was calculated using the formula:

$$\text{Inhibition\%} = [1- (A_1-A_2) / A_0] * 100$$

A_0: Control absorbance (without TQ).
A_1: TQ or standard absorbance.
A_2: Absorbance without sodium salicylate.

2.2.3 Hydrogen peroxide scavenging (H_2O_2) assay

The ability of TQ to scavenge hydrogen peroxide was estimated according to the method of Ruch et al. [22] with slight modification, H_2O_2 solution (120 mM) was prepared in phosphate buffer (50 mM, pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm. 850µL of different concentrations of TQ was prepared in phosphate buffer with 0.1% of tween 80 was added to 150 µl of hydrogen peroxide. The absorbance was determined at 230 nm after 10 min against a blank solution of phosphate buffer without H_2O_2. The hydrogen peroxide scavenging percentage was calculated according this formula:

$$\text{\% scavenged } (H_2O_2) = [(A_c \_ A_t)/ A_c] \text{ x100}$$

Where, A_c is the control absorbance and A_t is the test absorbance.

2.2.4 Scavenging of superoxide radical by alkaline DMSO Method

The scavenging of superoxide anion was estimated as described by Elizabeth and Rao [23]. Briefly, 0.1 ml of NBT (1 mg/ml dissolved in DMSO) and 0.3 ml of different concentrations of TQ or ascorbic acid as standard were added to 1 ml of alkaline DMSO which is prepared by addition of 1 ml DMSO to 5 mM NaOH. The absorbance was determined at 560 nm. Pure DMSO was used as blank instead of alkali DMSO. The inhibition % was calculated according to the following formula:

$$I\ \% = [(A_{\text{Control}} - A_{\text{sample}})/ A_{\text{Control}}]*100$$

2.2.5 Reducing power assay

The reducing power ability of TQ was evaluated as previously described by Oyaizu [24]. Briefly, 2.5 ml of various concentrations of TQ were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide (10 mg/ml). The reaction mixture was kept at 50ºC for 20 min, then 2.5 ml of 10% trichloroacetic acid were added. The mixture was centrifuged for 10 min at 3000 rpm and the supernatant (2.5 ml) was added to 2.5 ml distilled water and 0.5 ml of 0.1% of ferric chloride. The absorbance was measured at 700 nm, absorbance increasing explains reducing power ability increasing [25]. All the assays were carried out in three repetitions and the results are expressed as mean values ± SD. The TQ concentration which gives 0.5 of absorbance (EC_{50}) was calculated from the graph expressed the variation of absorbance in terms of TQ concentration. Ascorbic acid was used as standard.

2.2.6 Effect of thymoquinone on lipid peroxidation

2.2.6.1 β-carothene- Linoleic acid assay

This is a rapid assay to screen antioxidants, which is based on the oxidation of linoleic acid, which is an unsaturated fatty acid by Reactive

Oxygen Species produced by oxygenated water. The products formed will initiate the β-carotene oxidation, which will lead to discoloration [18]. The antioxidant activity of TQ was evaluated by the β -carotene linoleate model system as described previously by kabouche et al. [26] briefly, 0.5 mg β -carotene in 1 mL chloroform was emulsified with 25 µL linoleic acid and 200 mg tween- 40. Chloroform was eliminated at 40°C by rotavapor, 100 mL distilled water previously saturated with oxygen was slowly added to the residue and the solution was mixed to get a stable emulsion. 4 mL of this mixture is added into the test tubes which contains 200 µL of TQ or BHA as standard prepared in methanol at concentrations of 2 mg/mL. Immediately, zero time absorbance is determined at 470 nm. Then the tubes are kept for 120 min at 50ºC. Antioxidant activity was calculated as inhibition percentage (I%) relative to the control according to the following equation:

$$I\% = [1 – (As - As120)/ (Ac - Ac120)]*100$$

where As was initial absorbance, As120 was the sample final absorbance, Ac was negative control initial absorbance and Ac120 was the negative control final absorbance.

All assays were carried out in triplicate and the results presented as mean values ±SD.

2.2.6.2 Lipid peroxidation assay

The ability of TQ to inhibit the lipid peroxidation as total antioxidant activity was estimated as prescribed previously by Mitsuda et al. [27] with slight modification. Briefly, the emulsion of linoleic acid was prepared by mixing 0,028 g linoleic acid, 0,028 g tween- 40 and 10 ml of potassium phosphate buffer (0,04 M, pH 7.0). The reaction mixture contains 1ml of emulsion and 1ml of TQ or ascorbic acid as standard, The negative control contains 1ml of phosphate buffer was kept in dark at 40°C. At regular intervals during incubation, 20 µl of the mixture was diluted with 1ml of ethanol (75%), 20 µl of ammonium thiocyanate, and 20 µl of 20 mM of $FeCl_2$ in 3.5% HCL. The peroxide level was determined by reading the absorbance at 500 nm. These steps were repeated every 24 h for 6 days.

Antioxidant activity was presented as inhibition percentage (I %) relative to the control using the following equation:

$$I\% = [(Ac - At)/ Ac] *100$$

Where, A_c is the control absorbance and A_t is the test absorbance.

All assays were carried out in three repetitions and the results presented as mean values ±SD.

2.3 Statistic Analysis

All tests were performed in triplicate. Results are expressed as mean ± SD. All statistical analyses were carried out by Graphpad prism 5. Linear regression analysis was used to calculate the 50% inhibitory concentration (IC_{50}) values and Student's test to compare them. The *P* value < 0.05 was considered statistically significant.

3. RESULTS

3.1 Effect of Thymoquinone on Free Radicals Scavenging

3.1.1 DPPH and ABTS assays

Both DPPH and ABTS radicals are reduced in the presence of such hydrogen or electron-donating antioxidants. Our results indicated that the IC_{50} value of TQ was 125.65 ± 0.76 µg/ml (Fig. 1) and 332.5 ± 14.39 µg/ml (Fig 2), in DPPH and ABTS radical-scavenging assays, respectively. These values showed that TQ had significantly low antioxidant potential than ascorbic acid by 79 and 22 times, respectively (Table 1).

3.1.2 Hydroxyl, hydrogen peroxide and superoxide anion scavenger

In hydroxyl radical ($OH^{\bullet}$) svavinger assay, our findings indicated that TQ is an efficient hydroxyl radical scavenger with IC_{50} value of 26.3 ± 0.59 µg/ml. Despite its ability as $OH^{\bullet}$ scavenger was 5 times less efficient as compared with ascorbic acid (Fig 3 and Table 2); While the results of H_2O_2 radical scavenging assay showed that TQ is more effective than ascorbic acid with IC_{50} value of 11.0 ± 0.57 and 17.5 ± 0.56 (µg/ml), respectively (Fig. 4 and Table 2).

The $O_2^{\bullet-}$ radical is the most dangerous free radicals in humans and also the source of hydroxyl radical ($OH^{\bullet}$). Our results illustrated that TQ had very low superoxide anion scavenging activity, less than 50 %. Its best inhibition was 48.55 ± 0.68 % which is obtained at a high concentration equal to 1 mg/ml (Figs. 5 & 6).

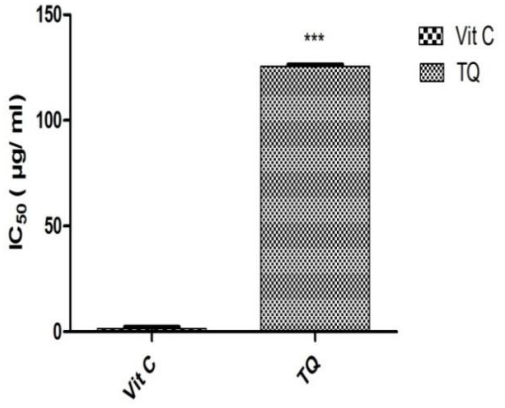

Fig. 1. DPPH radical scavenging effect of TQ and ascorbic acid

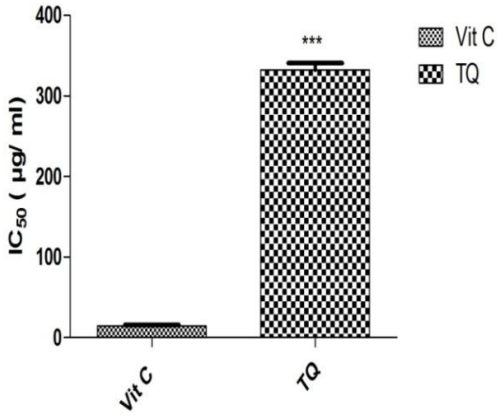

Fig. 2. ABTS radical scavenging effect of TQ and ascorbic acid

*Values were presented as the mean ± SD of triplicate. ***: p ≤ 0.001.*

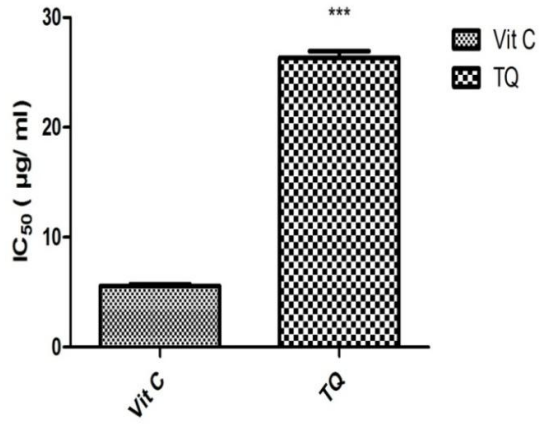

Fig. 3. $OH^{\bullet}$ radical scavenging effect of TQ and standard

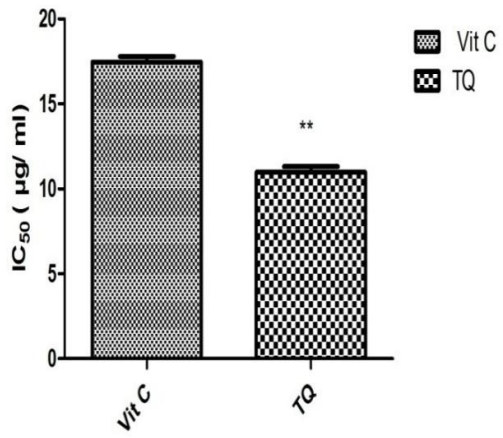

Fig. 4. H_2O_2 radical scavenging effect of TQ and standard

*Values were presented as the mean ± SD of triplicate. **: P ≤ 0.01, ***: p ≤ 0.001*

Table 1. IC_{50} of TQ and ascorbic acid against DPPH and ABTS radicals

Samples	IC_{50} for DPPH radical (µg/ml)	IC_{50} for ABTS radical (µg/ml)
Thymoquinone	125.65 ± 0.76 ***	332.5 ±14.39***
Ascorbic Acid	01.59 ± 0.78	14.96 ± 1.681

Values were presented as the mean ± SD of triplicate.
****: p ≤ 0.001.*

Table 2. IC_{50} of TQ and ascorbic acid against reactive oxygen species ($OH^{\bullet}$, H_2O_2 and $O_2^{\bullet -}$)

Samples	IC_{50} for $OH^{\bullet}$(µg/ml)	IC_{50} for H_2O_2 (µg/ml)	IC_{50} for $O_2^{\bullet -}$ (µg/ml)
Thymoquinone	26.3 ± 0.59 ***	11.0 ± 0.57 **	Inhibition less than 50%
Ascorbic Acid	5.57 ± 0.18	17.5 ± 0.56	54.27 ±11.23

Values were presented as the mean ± SD of triplicate.
***: P ≤ 0.01, ***: p ≤ 0.001.*

3.2 Reducing Power

In the reducing power assay, the existence of antioxidant causes the conversion of the ferric ions to ferrous ions. This reaction causes color change of the reaction mixture from yellow to bluish green. The bluish green color intensity is directly proportional to the reducing power capacity of the extract. Both TQ and ascorbic acid have shown reducing power activity in a dose dependent manner (Figs. 7 & 8) with EC_{50} value 713.25 ± 7.13 and 6.55 ± 0.09 µg/ml, respectively (Table 3). TQ had significantly less reducing power ability than ascorbic acid by 109 times.

3.3 Effect of Thymoquinone on Lipid Peroxidation

The aim behind these assays was to estimate the capacity of TQ to inhibit the lipid peroxidation caused by free radicals. We used linoleic acid as a source of hydroxyl-peroxide after its oxidation in the emulsion. Our results indicated that TQ inhibited the peroxidation of β-carotene significantly by 73.58 ± 0.50%. Its activity was less than BHA which was 97.2 ±1.28% (Fig. 9). Thus, in lipid peroxidation assay, TQ had also a significant activity with 79.5 ± 2.12% in the last day of the assay (Fig. 10).

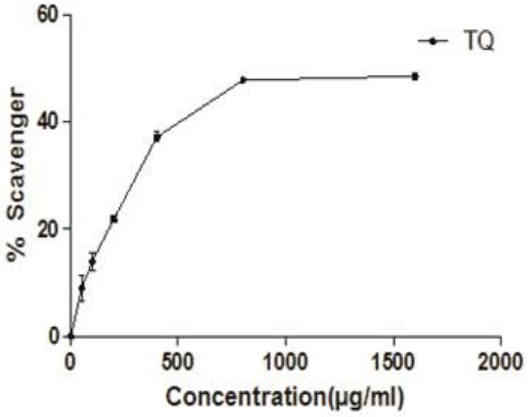

Fig. 5. $O_2^{\bullet-}$ radical scavenging effect of TQ

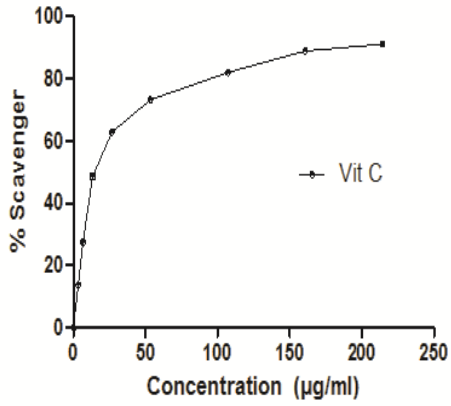

Fig. 6. $O_2^{\bullet-}$ radical scavenging effect of ascorbic acid (Vit C)

Values were presented as the mean ± SD of triplicate

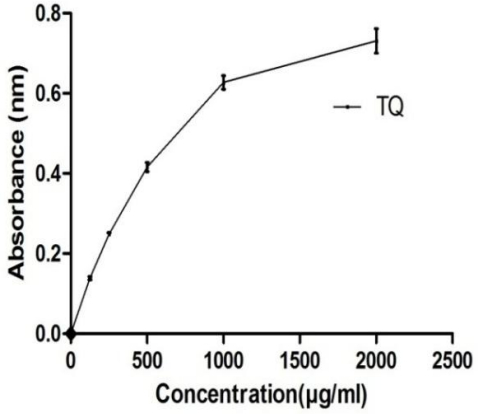

Fig. 7. Reducing power of TQ

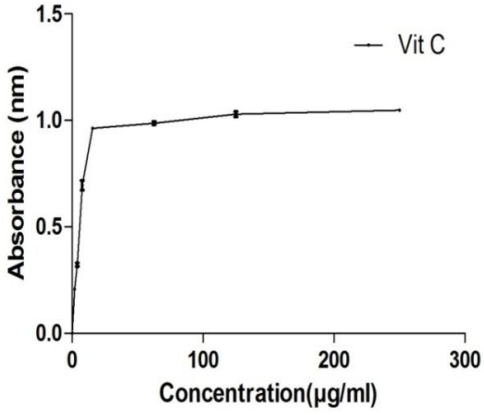

Fig. 8. Reducing power of ascorbic acid (c.vit)

Values were presented as the mean ± SD

Table 3. Reducing power activity of TQ and standard

Samples	EC_{50} reducing power (µg/ml)
Thymoquinone	713.25 ± 7.13***
Ascorbic acid	6.55 ± 0.09

*Values were presented as the mean ± SD of triplicate. ***: p ≤ 0.001*

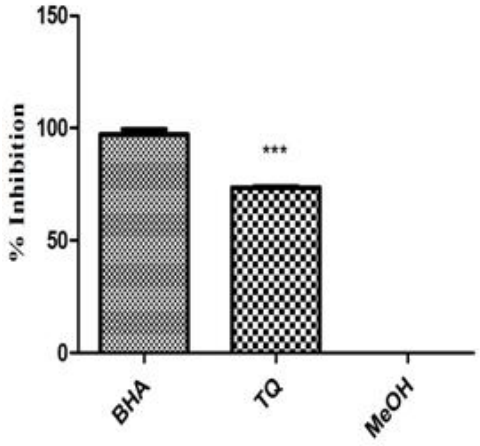

Fig. 9. Effect of TQ and BHA on β-carotene/linoleic acid assay

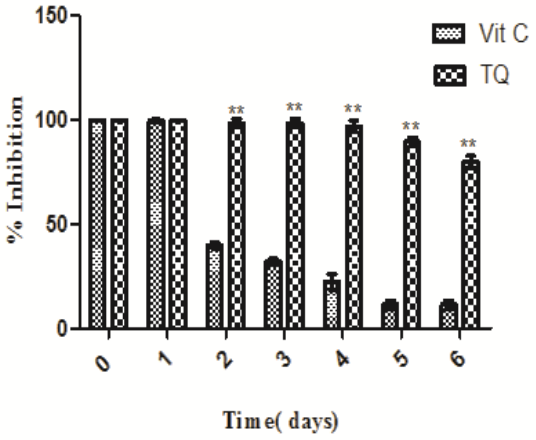

Fig. 10. Effect of TQ and ascorbic acid on lipid peroxidation

*Values were presented as the mean ± SD, ***: p ≤ 0.001, **: P ≤ 0.01*

4. DISCUSSION

In the present study, we evaluated the *in vitro* antioxidant capacity of TQ. In literature, there are few studies which are interested in the *in vitro* antioxidant activity of TQ.

Our results showed that TQ had significantly low antioxidant potential against DPPH and ABTS radicals than ascorbic acid, this is due to its low capacity to react as hydrogen or electron-donating antioxidants, these results confirmed the previous studies [28,29] where they had shown that TQ possesses a relative antioxidant activity as donor of proton or electron, while its reduced form (thymohydroquinone) exerts a highly radical-scavenging capacity.

However, the antioxidant capacity of TQ against reactive oxygen species including hydroxyl radical and hydrogen peroxide showed a significant activity, the scavenging ability of TQ against hydrogen peroxide radical showed that TQ is more effective than ascorbic acid. It is also a strong hydroxyl radical scavenger. These findings confirmed the previous study [28]. $OH^{\bullet}$ is the most potent reactive oxygen species in the biological system that is responsible to the oxidation of polyunsaturated fatty acid of cell membrane phospholipids causing lipid peroxidation and cell damage [30] Our findings in hydroxyl radical scavenger capacity are very important and confirmed our previous suggestion [15] where we evaluate the hepato-curative effect of TQ which is explained by its antioxidant effect and anti lipid peroxidation which protect the hepatocytes damages. But, in the superoxide anion scavenging assay, TQ had significantly very low activity; it is less than 50%. Whereas, in the previous studies [31,32] TQ was strongly inhibited superoxide anion production by NADPH oxidase in human polynuclear neutrophils stimulated with fMLF and it was a significant inhibitor of superoxide anion generation by xanthine- xanthine oxidase assay, the IC_{50} was in nanomolar range, respectively. These findings indicated that TQ is an efficient inhibitor of superoxide anion production in enzymatic system, but it is less effective in a chemical system as scavenger.

The results of reducing power ability of TQ indicated that it had a very low activity as a reducer agent of Fe^{+3} iron ions to Fe^{+2}. In literature, there is no study that dealt with the reducing power of TQ stated above; While the effect of TQ on lipid peroxidation free radical mediated process, indicated that TQ inhibited

significantly the lipid peroxidation, in both β-carotene/ linoleic acid and lipid peroxidation assays, these results could be explain by its significant effect as reactive oxygen species scavenger such as hydroxyl radical which is the most free radical which induced the lipid peroxidation [30] these results can give some information to the antioxidant capacity of thymoquinone [33]. Our findings indicate that the antioxidant property of TQ is mainly exerted through the scavenging ability of free radicals specifically reactive oxygen species and not due to reduced iron ions [29]. Our study could be implicated in future research as a primary vision regarding thymoquinone controlling free radicals noxious species causing the induction and / or amplification of several pathologies.

5. CONCLUSION

Our results revealed that TQ possesses a relative antioxidant activity against DPPH, ABTS and superoxide anion radicals. It has a very low reducing power capacity While Thymoquinone is a significant hydroxyl radical and hydrogen peroxide scavenger, thus it is a strong inhibitor of lipid peroxidation. These results could be very interested to promote the investigation of thymoquinone as *in vivo* antioxidant agent, therefore could be the object of recent research as treatment of several pathologies induced and/ or amplified by increasing in reactive oxygen species level.

ACKNOWLEDGEMENTS

The authors acknowledge the University of Setif 1. Research was performed in the Laboratory of Applied Biochemistry, Faculty of Nature and Life Sciences, University of Setif 1, Setif 19000, Algeria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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