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Stress response due to sodium azide treatment inside *Nigella sativa* L. plant and its effect on antioxidative property

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ABSTRACT

Nigella sativa is a potent medicinal plant having remarkable antioxidant property. More than hundred phytochemicals known for this plant, thymoquinone is one of the major active and strong antioxidative phyto-constituent found in it. Thymoquinone being a cyclicdione, reacts with sodium azide to synthesize its analogs α-azido ketones that are versatile with wide variety of reactions. The stress produced due to sodium azide, modulates the antioxidant system (antioxidative property) of the plant. The present study was aimed to observe the effect of sodium azide on antioxidative property and thymoquinone content within *Nigella sativa* plant. The results revealed the effect of sodium azide with strong positive correlation between percent yield and percent composition of thymoquinone in Ajmer Nigella 1(AN1) and UP Nigella 1 (UP1) in a concentration dependent manner. Minimum percent yield and percent composition of thymoquinone (analyzed by RP-HPLC) were observed (0.823 ± 0.035% for AN1 and 0.530 ± 0.020% for UP1) and (0.022 ± 0.002% for AN1 and 0.012 ± 0.002% for UP1), respectively at 100 μM concentration of sodium azide used. However, a strong negative correlation was observed with the decrease in thymoquinone content, with the increase in antioxidant property (−0.9369 and −0.9526) for AN1 and UP1, respectively. The findings clearly indicate the effect of sodium azide on thymoquinone content and their antioxidant property. The elevated antioxidant property might be due to the elicitation in production of other antioxidants or by the formation of analogs of thymoquinone that are more powerful antioxidants. Therefore, further research is also needed to further explore the interaction of sodium azide with thymoquinone that produce more potent antioxidant analogs and thus may be useful in various therapeutic applications.

1. Introduction

Nigella sativa is commonly known as black seed or black cumin, belonging to the family Ranunculaceae, abundantly grows in the region of west Asian and central European countries and Middle East countries (Dwita et al., 2019). For over 2000 years, it is conventionally used in the prevention and medication of various infirmities (El-Fatatry, 1975; El-Kadi and Kandil, 1986; Gali-Muhtasib et al., 2006). In middle East, *Nigella sativa* seed oil is used as a traditional herbal medication for various ailments of gastrointestinal treatments, lung diseases, cholesterolemia etc. (Khader et al., 2009; Shariq et al., 2015; Manju et al., 2015). Some recent studies revealed that rheumatoid arthritis can be cured by *Nigella sativa* seeds oil (Gheita and Kenawy, 2012). Phytocomponents derived from these seeds are commercially used in soaps, beauty products, cosmetics and oils etc. (Shomar, 2012). Various other *in vivo* and *in vitro* studies on *Nigella sativa* reveled its antiradical and antineoplastic competence (Thabrew et al., 2005). The antitumor activity was also found on human hepatoma cells and reveled its apoptotic potential (Thabrew et al., 2005). An *in vitro* study on breast cancer (MCF-7) cells showed that aqueous and methanolic extract of *Nigella sativa* reduced the cancer cell growth in a dose dependent manner (Farah and Begum, 2003). Similarly other study on, ethanolic and chloroform extracts of *Nigella sativa* effectually exterminated human epithelial cervical cancer cells by prompting apoptosis (Shafi et al., 2009). The HPLC analysis of *Nigella sativa* showed some potent active constituents that are identified as thymol thymoquinone, dithymoquinone, and thymohydroquinone (Ghosheh et al., 1999). Various other studies revealed that the pharmacological activity of *Nigella sativa* is mainly due to its oil constituent, that are rich in thymoquinone (IUPAC-2-methyl-5-isopropyl-1, 4-benzoquinone) and about 30–48% of the

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total content (Burits and Bucar, 2000; Ali and Blunden, 2003; Hajhashemi et al., 2004).

Thymoquinone was pioneerly quarantined from *Nigella sativa* excerpts in 1963 (El- Dakhakhny, 1963). Subsequently, various studies based on antioxidative property and its significant therapeutic properties have been reported (Dajani et al., 2016; Manju et al. 2016). Various other investigations were implemented to elucidate the anti-cancerous property of thymoquinone, which revealed discriminatory cytotoxic effect on various cancer cells lines of human (Gali-Muhtasib et al., 2004). Apart from thymoquinone anti-tumor activities and cell death, it was found to impede with other tumorigenic progressions, such as metastasis incursion and angiogenesis (Khan et al., 2015; Peng et al., 2013). Moreover to conventional chemo or radio therapy, thymoquinone can demolish cancerous cells, by the modification of mechanisms for resistance (Zhang et al., 2016; Velho-Pereira et al., 2011). Various *in vitro* and *in vivo* antitumor activity of thymoquinone had shown a prominent apoptotic effect (Majdalawieh et al., 2017), but its poor efficiency (Effenberger et al., 2010) and deprived bio-availability (Elmowafy et al., 2016; Ganea et al., 2010) is the prime drawback for its consideration as significant remedial phyto-compound. Another major downside, which limits its usage as a therapeutic agent is its lower solubility and higher cytotoxic dosage. In the direction to improve this snag several types of nano-carrier/nanoencapulated thymoquinone have been prepared (Alam et al., 2012; Singh et al., 2013). Recent studies showed that nanoencapulated thymoquinone (polyethylene glycol coated and Poly-N-isopropylacrylamide nanoparticles) have potent effect than the synthesized nanothymoquinone because of the smaller size (Verma et al., 2013). Based on the previous findings and drawbacks in the therapeutic applications of thymoquinone, the present study was aimed to synthesize structural analogs of thymoquinone within *Nigella sativa* plant by the treatment of sodium azide (Moore et al., 1970), which might be more potent antioxidants. On the other hand the treatment of sodium azide may cause stress and can elicit other antioxidants. The effect of sodium azide on two tomato cultivars under stress conditions was already reported by (Abdulrazaq and Ammar, 2015), where it showed significant effect on various morpho-physiological parameters. In another study, the effect of sodium azide was studied on *Capsicum annuum,* where it showed significant effect on various morpho-physiological and biochemical parameters (Dahot et al., 2012). With consideration to such studies, sodium azide was used in the present study, where it was hypothized that it might improve the antioxidative property of the plant due to stress induced resulting in the synthesis of structural analogs of thymoquinone, which might be more potent antioxidants. However, further research is needed to fully explore the interaction of sodium azide with thymoquinone to produce analogs, which are more potent antioxidants and thus useful for therapeutic applications. The therapeutic phytochemicals thus obtained would be significant in pharmaceutical and neutraceutical industry.

2. Materials and methods

2.1. Plant material

Nigella sativa seeds were obtained from National Research Centre on Seed Spices, Tabiji, Ajmer, Rajasthan, India. It was designated as Ajmer Nigella 1 (AN1). The other seeds were collected from the local seed market of Lucknow, Uttar Pradesh and designated as (UP 1). Seeds of AN1 and UP1 were thoroughly rinsed with distilled water. Afterwards, it was sowed in pots containing Vermiculite and supplemented with 0 μM (control), 5 μM, 10 μM, 20 μM, 50 μM, 100 μM and 200 μM concentrations of sodium azide. The plants after ten weeks of germination *i.e.,* at the budding stage were used for present study. *Nigella sativa* leaves (5 g) were homogenized in 10 ml 100% methanol (HPLC grade) to conduct RP-HPLC (Reverse phase - High Performance Liquid Chromatography) analysis (Iqbal et al., 2018). The 1 mg of each extracts AN1 and UP1 were dissolved with 1 ml methanol to prepare 1 mg/ml of stock solution and filtered (Whatman No. 1 filter paper) and stored at −20 °C for further studies.

2.2. Physiological parameters

2.2.1. Spectrum scanning

Spectrum scanning was done using spectrophotometer (Shimadzu UV- 1800) for absorbance of pure thymoquinone. Maximum absorbance was obtained using the procedure of Belete and Dagne (2014) with some alterations.

2.2.2. RP-HPLC analysis

UFLC (Make - Shimadzu corporation Kyoto, Japan; Model - LC-20AD/SPD-20A) was used to perform RP-HPLC investigation. Lab Solution Lite software (Shimadzu Corporation Kyoto, Japan) was used for data attainment. Genuine standards of pure thymoquinone (certified purity 99.0%) was obtained from Sigma-Aldrich and methanol (HPLC-grade Rankem, RFCL Limited) were used for the study.

2.2.2.1. Instrumental conditions. RP-HPLC analysis was executed as per the procedure of Hadad et al. (2013) and Iqbal et al. (2018), with some modifications. An isocratic system was used with a flow rate of 1.0 ml/min for the analysis. The movable phase was methanol: water (70:30 v/v), maximum absorbance was at 254 nm, 9.059 min of retention time and all determinations were performed at ambient temperature (25–30 °C). Before use, the mobile phase, sample/solvent were filtered through syringe filters (0.22 μm Millipore) and degassed by using bath sonicator.

Standard stock solutions of thymoquinone was prepared with a concentration of 1 mg/ml using methanol as solvent with taking care of it's stability (light and heat). To plot calibration curve, stock solution of pure thymoquinone (1 mg/ml) was diluted with concentrations of 0.0312–1.0 μg/ml, using mobile phase as solvent. For analysis, 20 μl of each unknown sample and standard was evaluated and chromatographed (run in triplicate). For validation of peak of thymoquinone and to refute the insignificant deviation in retention time, each sample (AN1 and UP1) was introduced together with standard solution of pure thymoquinone. Furthermore, standard thymoquinone (concentrations ranging from 0.0312 to 1.0 μg/ml) was injected at an increasing concentrations to ratify the peak and retention time. To attain the calibration curve, peak areas were evaluated alongside the subsequent concentrations (Fig. 1).

2.3. Total antioxidant activity

Using the standard procedure (minor alterations) of Cacig et al. (2006); Iqbal et al. (2017) the total antioxidant activity was elucidated. Plant leaves (1 g) were homogenized using distilled water (4 ml) and incubated (4 °C) for 24h. The incubated extract was filtered (Whatman No. 1 filter paper) and stockpiled (4 °C) for future study. The spectrophotometric analysis was done using UV-VIS-1800, Schimadzu, Japan spectrophotometer at 535 nm. The reaction mixture consisted of 0.18 ml $KMnO_4$ (0.01M); 0.42 ml H_2SO_4 (2M); distilled water (2.3 ml) and sample (0.1 ml) prepared in 3.0 ml glass cuvette. Standard solution of ascorbic acid (HiMedia-TC094) of concentration 0.01 mM/ml was used as standard. The readings were taken in triplicate to evaluate the mean activity (Fig. 2). Using the formula of Cacig et al. (2006); Iqbal et al. (2017) the antioxidant activity was calculated.

3. Statistical analysis

Results are expressed as mean ± standard deviation. Two way ANOVA was applied between percent yield, percent composition of thymoquinone and total antioxidant activity by GraphPad Prism software followed by Bonferroni posttests. Correlation analysis was made using Microsoft excel amongst AN1 and UP1 samples with

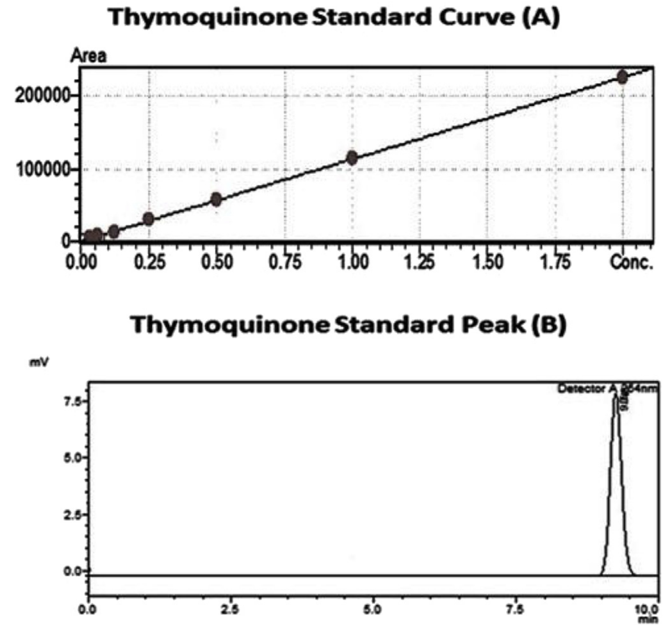

Fig. 1. Pure thymoquinone (99.0%) standard curve (figure A) with mean coefficient of determination (R^2) = 0.999 and % RSD was 7.848 using concentration range (0.0312–1.0 µg/ml). Pure thymoquinone standard peak (figure B) with time of 9.059 min.

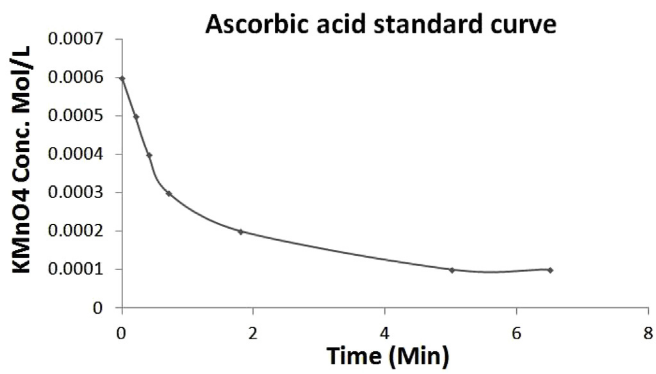

Fig. 2. Oxidative reaction of $KMnO_4$ with ascorbic acid (standard) 0.01 (mM/ml). The time until the standard (ascorbic acid) induces a decrease of the permanganate concentration up to one half was noted to be 0.66 min.

consideration to percent composition of thymoquinone and total antioxidant activity.

4. Result and discussion

The treatment in plant by sodium azide, results in stress conditions and as a consequence, over production or deprived production of phytochemicals were synthesized by the plant. With consideration to this process the present study was conducted. The salt stress (sodium azide) was given to the experimental plant *Nigella sativa*, and as a consequence, biochemical alterations were occurred. Sodium azide reacts with thymoquinone (phytochemical of interest) to form its analogs (Faiz et al., 2015). In the analysis, it was observed that the percent yield of extract decreases with the increase in sodium azide concentration (1.173 ± 0.064% for AN1 and 0.750 ± 0.050% for UP1) at control (0 µM) of sodium azide while (0.823 ± 0.035% for AN1 and 0.530 ± 0.020% for UP1) at 100 µM of sodium azide. However, further increasing concentration (200 µM of sodium azide), has no effect on the yield of the extract. This is in agreement with the previous report of Dahot et al. (2012), where the higher concentration of sodium azide inhibited the germination rate in *Capsicum annum* resulting in decreased morpho-physiological and biochemical parameters at 1% and 2% of sodium azide treatment. These findings indicate the interaction of sodium azide with phytochemicals, resulting in decreased yield of extract (Fig. 3). In a study, the phytochemical exploration of *Ocimum sanctum* (aqueous extract) was carried out for percent yield determination; suggest that the percent yield was associated with the chemical constituent of the plant extract (Sawant and Godghate (2013); Fahad Al-Qurainy 2009; Sawant and Godghate (2013); Rajesh et al., 2013). Hence, percent yield might be pondered as a significant aspect for phyto-extracts with consideration to it's bioactive constituents. However, in the present study when two way ANOVA was applied, a significant variation was observed (p value ≤ 0.05) between various concentrations of sodium azide treatment. It was found to be the p value of 0.003 for different concentrations treatment. However, no significant variation was observed between the two samples *i.e.,* AN1 and UP1 (p value ≥ 0.05).

Prior to RP-HPLC analysis, standard thymoquinone spectrum scanning was performed in order to determine its maximum absorbance. The maximum absorbance of standard thymoquinone (purity 99.0%) was observed at 254 nm (Fig. 4). The outcome was in virtuous agreement with the study of Belete and Dagne (2014). The RP-HPLC analysis for percent composition of thymoquinone was estimated and it was found that maximum percent was at control (0 µM) concentration of sodium azide (0.081 ± 0.007% for AN1 and 0.048 ± 0.007% for UP1), minimum thymoquinone composition was estimated at 100 µM concentration of sodium azide (0.022 ± 0.002% for AN1 and 0.012 ± 0.002% for UP1) (Fig. 5). However, further increasing concentration (200 µM of sodium azide), has no effect. Furthermore, when two way ANOVA was applied, a significant variation was observed (p value ≤ 0.05) between the different concentration of sodium azide treatment with both samples *i.e.,* AN1 and UP1 (p value 0.002 and 0.001), respectively.

The correlation between percent yield and percent composition of thymoquinone for AN1 and UP1 was found to be strongly positive as 0.8837 and 0.9574, respectively. A strong positive correlation between the percent yield and percent composition indicates strong effect of sodium azide on the plant, thereby, altering the desired phytochemical to a greater extent. The reduction in thymoquinone content with respect to percent yield indicates the alteration of thymoquinone structure. It might be a possibility of alteration in thymoquinone to its analogs form, as we know when thymoquinone reacts with sodium azide, it gets converted to 3-amino-G-azido-2-methyl-5-isopropyl-1,4-benzoquinone (Moore and Shelden, 1969) (Faiz et al., 2015) (Fig. 6). The conformational changes in the structure results the formation of new compounds. Thus, in the present study there is a strong possibility of the alteration of thymoquinone to its analogs. Fieser and Hartwell (1935) reported the synthesis of azidoquinone in ethanolic solution by 2-azido-l,4-napthoquinone treatment with the corresponding 2-chloroquinone with azide ion. The main reaction was Cl^{-1} or Br^{-1} substitution with quinones in a nucleophilic displacement reaction of azide ion with halogen. The azidoquinones are made predominantly in elevated yields of more than 85% and generally precipitated with virtuous purity (purification). The 2-azido-5-amino-l,4-benzoquinones were obtained by disproportionation (thermal) of the analogous di-azido-hydroquinones. It was observed that all of the above mentioned compounds were thermally decomposes at their respective melting points, however are quite stable for a long period at ambient temperature kept in dark condition (Moore et al., 1969). These previous findings are the strong base of our hypothesis to conduct the present study. However this cannot be acclaimed as a yardstick to synthesize thymquinone analogs within the plant as various other factors are also plays a vital role in a phytochemical biosynthesis.

The antioxidant activity revealed that maximum activity was found at 100 µM concentration of sodium azide (4.460 ± 0.487 and 3.070 ± 0.177 mM equivalent ascorbic acid/g tissue for AN1 for UP1),

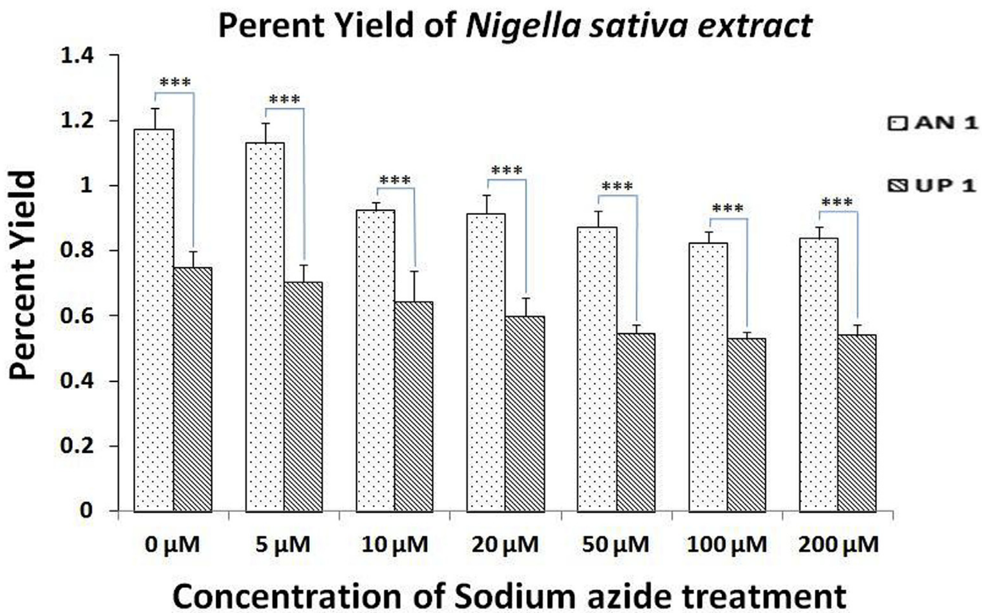

Fig. 3. Graphical representation of percent yield of *Nigella sativa* extract. Data represented as mean ± SD with two way ANOVA. *P value* (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$) were considered as statistically significant.

respectively. While, minimum activity was observed at 0 µM (control) concentration of sodium azide (1.330 ± 0.112 and 1.163 ± 0.041 mM equivalent ascorbic acid/gram tissue for AN1 for UP1) respectively. However, further increasing concentration (200 µM of sodium azide), deprived the antioxidant property, clearly indicating the stress response of the sodium azide treatment on plant (Fig. 7). The previous finding revealed that sodium azide increasing doses have no effect on *Helianthus annuus* germination, due to higher doses of sodium

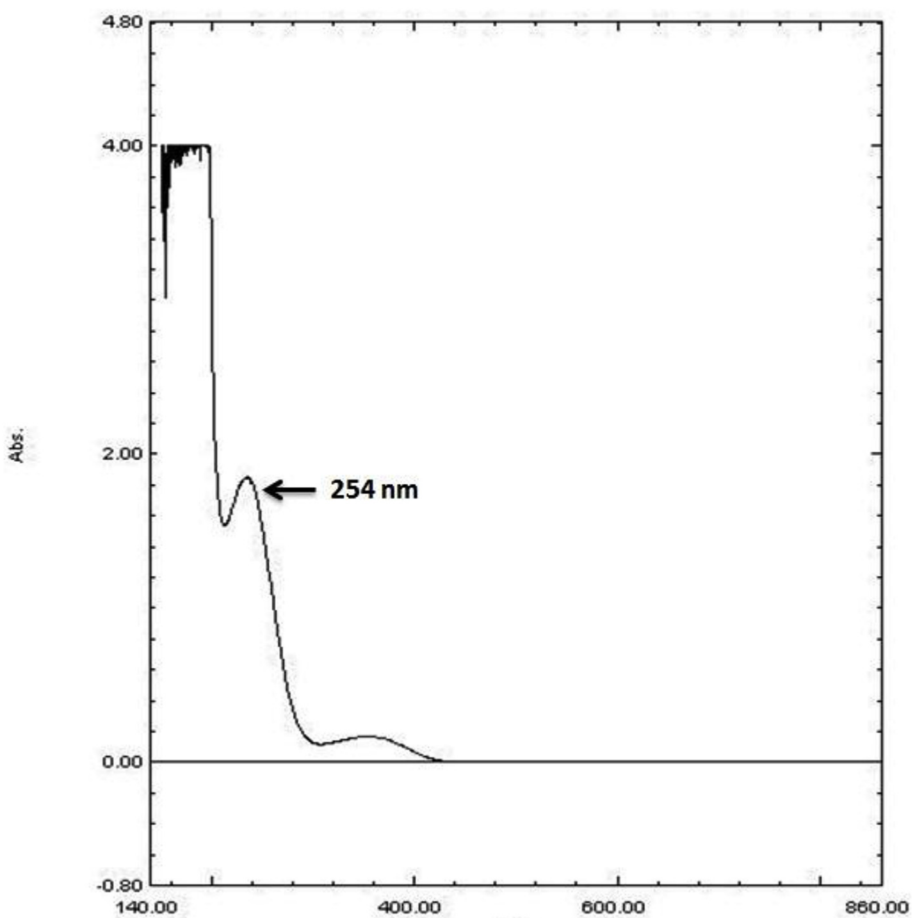

Fig. 4. Spectrum scan of thymoquinone showing maximum absorbance at 254 nm. Spectrum scanning was done using spectrophotometer (Shimadzu UV- 1800).

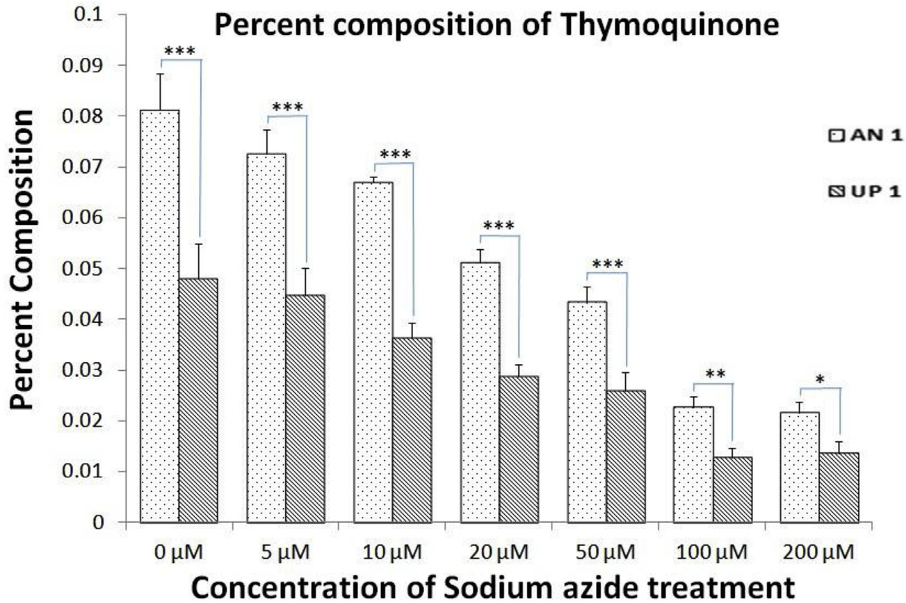

Fig. 5. Graphical representation of percent thymoquinone in *Nigella sativa* extract. Data represented as mean ± SD with two way ANOVA. *P value* (*$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$) were considered as statistically significant.

azide disrupt the physiological activities and cause cell death (Mostafa, 2011). Although various morphological and physiological effect of sodium azide on plants were studied but none on its antioxidant effect was established, so it's a pioneer report of such effect on a plant by sodium azide. However, when two way ANOVA was applied, a significant variation was observed (p value ≤ 0.05) between the different concentrations of sodium azide treatment and was found to be the p value 0.022 for different concentrations treatment. Although, no significant variation was observed between the two samples *i.e.,* AN1 and UP1.

Strong negative correlation was found between total antioxidant activity and the percent composition of thymoquinone *viz.* −0.9369 and −0.9526 for AN1 and UP1, respectively. The findings revealed that, at higher concentration (100 µM) of sodium azide treatment, thymoquinone content decreases with the increase of total antioxidant level. There are two possible factors responsible for this, first there may be an increasing production of other antioxidants due to stress conditions, that results in increased antioxidant activity. The second possibility is there may be some conformational change in thymoquinone structure, due to the rearrangement reaction after sodium azide treatment, resulting in analogs formation. These analogs are the more potent antioxidant, thus enhancing the antioxidative property of the *Nigella sativa* plant.

A recently published report, suggest that analogs of thymoquinone were synthesized by treatment of sodium azide and form the reduced product, by the interaction with the aldehydes generates the Schiff bases. These analogs have exhibited better anti-proliferative activity, tremendous chemo-sensitizing activity against *in vitro* pancreatic cancer and also in amalgamation with gemcitabine (Yusufi et al., 2013). Another study also revealed that thymoquinone minimized the sodium arsenate induced neurotoxicity in animal rat model. The findings exposed the antioxidant mechanism of thymoquinone that ameliorate the oxidative stress in the nervous system of the rat after exposure of sodium arsenate (Kassab and El-Hennamy, 2017). The antioxidant property of thymoquinone is well known in various previous studies, but its analogs are still yet to be explored, that might be more powerful antioxidative agents for future pharmacological use.

5. Conclusion

A concentration-dependent increase in antioxidative property with the decreases in thymoquinone content was observed at higher concentrations of sodium azide treatment. The decrease in antioxidative property could be due to the increasing production of other antioxidants because of stress conditions or maybe some conformational change in thymoquinone structure after rearrangement reaction with sodium azide treatment, resulting in analogs formation. These analogs are the more potent antioxidant, thus enhancing the antioxidative property of the *Nigella sativa* plant. It was further observed that AN1 verity of *Nigella sativa* produced higher percent of thymoquinone

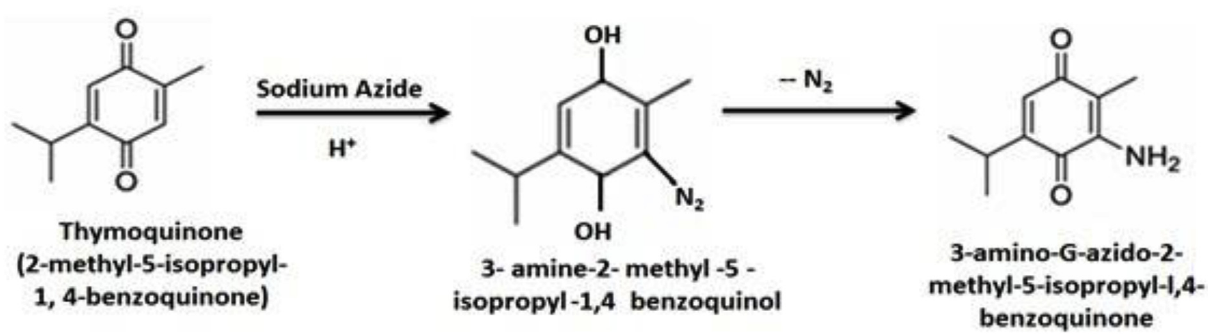

Fig. 6. Rearrangement reaction of thymoquinone with sodium azide. It undergoes intramolecular oxidation-reduction reaction to yield aminoquinones.

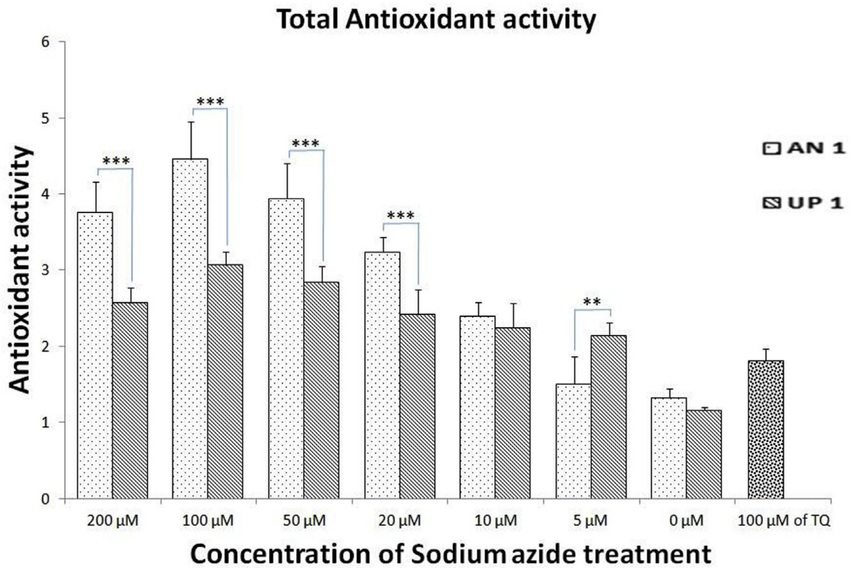

Fig. 7. Graphical representation of total antioxidant activity of *Nigella sativa* extracts with reference of pure thymoquinone 100 µM (as standard for comparison). Data represented as mean ± SD with two way ANOVA. *P value* (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001) were considered as statistically significant.

content and antioxidant activity than UP1. Further, more investigations are strongly recommended to recognize and screen out such produced analogs of thymoquinone, that might be an excellent source of antioxidant and can be useful in the pharmaceutical and nutraceutical industry. It would be a novel approach for herbal therapeutic application.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101171.

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