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*Proceeding Paper*

# Comparison of Health Benefiting Phytoconstituents of Australian Grown *Nigella sativa* Genotypes †

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**Abstract:** *Nigella sativa*, an annual herbaceous flowering plant of the Ranunculaceae family, is considered an important medicinal plant due to the presence of several bioactive compounds in its seeds, including both volatile and non-volatile compounds. The cultivation of numerous genotypes of *N. sativa* are witnessed in different parts of the world with varying compositions of such chemical compounds. Since the variation in composition determines the quality grade of the seeds, this study was carried out to explore the compositional variation of twelve different genotypes of *N. sativa* cultivated in Central Queensland, Australia. The results showed total phenolic content (TPC), FRAP and CUPRAC (antioxidants), and thymoquinone in the range of 291–529 mg GAE/100 g DW, 703–966 mg TE/100 g DW, 2533–3416 mg TE/100 g DW, and 219–349 mg/100 g DW, respectively. The highest value of TPC, thymoquinone, FRAP and CUPRAC was observed in genotype AVTKS#E, AVTKS#F, AVTKS#4 and AVTKS#D, respectively. The lowest value of TPC and FRAP was observed in genotype AVTKS#24 and the CUPRAC and thymoquinone was lowest in genotype AVTKS#23 and AVTKS#1, respectively. Monomeric anthocyanins were absent in the methanolic seed extracts of all nigella genotypes. There was a strong positive correlation among the TPC, CUPRAC and FRAP. However, despite thymoquinone being reported as a strong antioxidant in the literature, there was no significant correlation of thymoquinone with TPC or CUPRAC, and only a weak positive correlation with FRAP. Overall, the genotypes with comparatively higher value of thymoquinone, TPC and antioxidant capacity (both, FRAP and CUPRAC) showed particular potential for breeding programs.

**Keywords:** *Nigella sativa*; thymoquinone; antioxidants; total phenolics; seed extracts; health benefiting compounds

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## 1. Introduction

*Nigella sativa*, an annual herbaceous flowering plant of the Ranunculaceae family, is medicinally considered an important plant due to the presence of several valuable volatile and nonvolatile bioactive compounds in its seeds [1]. Some examples of volatile compounds are p-cymene, carvacrol, carvone, thymoquinone, thymol, thymohydroquinone, dithymoquinone, longifolene, $\alpha$-thujene, $\alpha$-pinene and sesquiterpene [1]. Besides volatile compounds, nonvolatile compounds in trace quantities have been reported in the ethanolic extracts obtained from *N. sativa* seeds, namely sterols and tocols, and two different types of alkaloids (isoquinoline alkaloids and indazole or pyrazole alkaloids) [2]. The isoquinoline includes nigellicimine and nigellicimine-N-oxide and the indazole includes nigellicine and nigellidine [3]. Although *N. sativa* seeds contain many valuable compounds, different factors such as genotype might affect the level of such compounds present in seeds and this variation in composition ultimately determines the quality grade of seeds.

There is limited information on the chemical compositional variation of the seeds and therapeutic value of *N. sativa* genotypes [4]. In particular, different genotypes of *N. sativa* are grown in different parts of Australia although there is a lack of data available in terms of thymoquinone composition, antioxidant capacities and TPC in them which would otherwise positively ascertain the therapeutic value. Therefore, the aim of this study was to investigate the compositional variation and therapeutic value of different genotypes of *N. sativa* cultivated in Central Queensland, Australia.

## 2. Materials and Methods

### *2.1. Seed Samples Production and Collection*

Twelve genotypes of *N. sativa* seeds were obtained from AgriVentis Technologies Pty Ltd. (https://www.agriventistechnologies.com.au) and sown on 1 May 2022, following a Randomized Complete Block Design (RCBD) with three replications at Central Queensland Innovation and Research Precinct (CQIRP) under same environmental and soil conditions. The details of genotypes have been illustrated in Table 1. The plants were harvested from the raised beds after the maturation stage was completed in mid October. The seeds from the harvested plants were used for the determination of variation of phytochemical composition and therapeutic values.

**Table 1.** Description of genotypes used for this study.

| Seed Lines | Genotypes (Description) |
|---|---|
| AVTKS#A | Konji-SV 3rd gen A.T. ADRA |
| AVTKS#4 | Kalonji-2 2016/17.B/DX W.B. Commercial Qty."066 (only) G.uselecy. Khan Academy |
| AVTKS#C | KALONJI 3. 2nd Gen;2016. "Oil seed = Kayman. 1007-phs-2017-TAZO |
| AVTKS#D | Konji -SV 4. #rd Gen AT Kevita III |
| AVTKS#E | KALONJI-2016 4th Gen in Oz/B/Stock 1007-phd-11Z-065. Riverdale-Hunter Valley |
| AVTKS#F | Nigella (M/S) KALONJI- This was selected for showing the best growth and the strongest under stress. Excellent yield |
| AVTKS#2 | Kalonji. AT. Commercial Qty. NA-6 |
| AVTKS#H | KALONJI-8 |
| AVTKS#1 | Kalonji. Bangladesh x Hunter Valley (D) |
| AVTKS#3 | Kalonji. Black Cumin. Al-Acc- E |
| AVTKS#23 | Konji-SV 3RD Gen. A.T. KEVITA III QLD (122) |
| AVTKS#24 | Konji-SV 3RD GEN. A.T. ADRA. 5TH AUST (122) |

### *2.2. Seed Extract Preparation*

The methanolic extraction protocol developed by Johnson et al. [5] was followed to prepare nigella seed sample extracts. Briefly, the dried nigella seeds were ground into uniform fine powdered form using a grinder. Then 2 g of powder was extracted twice with 90:10 MeOH:$H_2O$ (7 mL × 2) using vortex mixer and then end-over-end shaking at ambient temperature. The samples were then centrifuged and the supernatant was collected, combined, and brought final volume to 14 mL.

### *2.3. Total Phenolic Content (TPC) Analysis*

The method of Folin-Ciocalteu developed by Singleton and Rossi and modified by Johnson et al. [5] was used to estimate the TPC of the samples. Gallic acid solution with Milli-Q® water was used as a spectroscopy standard. The TPC of the samples was derived as a function of the equivalent absorbance of the standard solution of gallic acid in the 20 to 100 mg/L range. The linearity of the calibration curves of gallic acid standards was very good ($R^2 = 0.9964$), and the equation, $y = 0.0095x + 0.0064$, obtained from the calibration curves was used for the quantification of TPC in samples. The results were expressed as

milligrams of gallic acid equivalents (GAE) per 100 g of dry sample weight (mg GAE/100 g DW).

*2.4. Antioxidant Analysis*

The two methods, Ferric Reducing Antioxidant Capacity (FRAP) and Cupric Reducing Antioxidant Capacity (CUPRAC), were used for the antioxidant analysis in seeds following the protocal described previously by Johnson et al. [5]. For both methods, Trolox (6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid) with 100% ethanol was used as a spectroscopy standard. The FRAP values and CUPRAC values of the samples were derived as a function of the equivalent absorbance of the standard solution of Trolox between the ranges 10–150 mg/L and 50 to 500 mg/L, respectively.

The linearity of the calibration curves of Trolox standards for both, FRAP ($R^2$ = 0.9989), and CUPRAC ($R^2$ = 0.9985), was very good. The equations, y = 0.0056x + 0.0751 and y = 0.0014x + 0.1698, obtained from the calibration curves of Trolox standards for FRAP and CUPRAC, respectively were used for the quantification of total FRAP values and CUPRAC values in samples. All the results were illustrated as milligrams of Trolox equivalents (TE) per 100 g of the dry weight sample (mg TE/100 g DW).

*2.5. Total Monomeric Anthocyanin Analysis*

The total monomeric anthocyanin assay was carried out using a modification of the pH differential method described by Lee et al. [6].

*2.6. Quantification of Thymoquinone*

High-performance Liquid Chromatography (HPLC) was used for the determination of thymoquinone in *N. sativa* seeds extract. An Agilent 1100 HPLC system, comprising a G1313A autosampler, G1322A vacuum degasser, G1311A quaternary pump and G1365B multi-wavelength detector module was used. Thymoquinone was quantified following the protocol described by Mani et al. [7]. All quantitative analysis was performed with external standardization by measurement of peak areas of pure standards prepared in the range of 10–250 ppm with methanol. The linearity of the calibration curves of pure standards was very good ($R^2$ = 0.9997), and the equation, y = 30.645x +32.337 obtained from the calibration curves was used for the quantification of thymoquinone in samples.

*2.7. Statistical Analysis*

The experiments were performed on 72 samples (three replications in the field × 2 replications of laboratory analyses). Values were expressed as mean ± standard deviation (SD) (n = 6). Data was analysed by one-way ANOVA using IBM SPSS software. *p* values less than 0.05 were considered to be statistically significant. Pearson's correlation test was done to describe the relationship between the variables.

## 3. Results and Discussion

To our knowledge, this is the first study to provide information on health benefiting phytoconstituents—specifically TPC, CUPRAC, FRAP and thymoquinone—of a wide range of Australian grown nigella genotypes. These are presented in the following sections.

*3.1. Total Phenolic Content (TPC)*

The TPC of the twelve genotypes ranged between 291–529 mg GAE/100 g. The highest TPC was found to be present in genotype AVTKS#E (529 mg GAE/100 g), followed by AVTKS#4 and AVTKS#A with values of 492 and 477 mg GAE/100 g, respectively. The lowest value of TPC was observed in genotype AVTKS#24 (291 mg GAE/100 g), followed by AVTKS#23 which represented 294 mg GAE/100 g. The results obtained in this study are coherent with the values reported by some researchers. For example, Thippeswamy &

Naidu studied TPC in the methanolic seed extract of nigella sourced from India and observed an average value of 410 mg GAE/100 g [8]. However, a few researchers have reported both comparatively higher as well as lower values of TPC of some nigella seeds samples. For example, Haron et al. collected nigella seeds of Yaman, Iran and Malasya, prepared their methanolic extract and observed TPC in the range between 1619 to 3084 mg GAE/100 g [9]. Sen et al. on the other hand prepared methanolic seed extracts of nigella sourced from six different regions of Turkey and observed ≤ 292 mg GAE/100 g of TPC [10].

**Table 2.** Total Phenolics Content, Antioxidant Capacity (CUPRAC and FRAP) and Thymoquinone content in the Nigella seeds.

| Genotype (New) | Seed Lines | Total Phenolic (mg GAE/100 g DW) | FRAP (mg TE/100 g DW) | CUPRAC (mg TE/100 g DW) | Thymoquinone (mg/100 g DW) |
|---|---|---|---|---|---|
| 1 | AVTKS#A | 477 ± 36 $^{f,g,h}$ | 934 ± 45 $^{d,e}$ | 3188 ± 110 $^{b,c}$ | 311 ± 31 $^{e,f}$ |
| 2 | AVTKS#4 | 492 ± 37 $^{g,h}$ | 966 ± 45 $^{e}$ | 3411 ± 125 $^{c}$ | 288 ± 19 $^{d,e}$ |
| 3 | AVTKS#C | 380 ± 36 $^{b,c,d}$ | 866 ± 50 $^{c,d}$ | 3222 ± 148 $^{b,c}$ | 247 ± 25 $^{a,b,c,d}$ |
| 4 | AVTKS#D | 444 ± 38 $^{e,f,g}$ | 929 ± 35 $^{d,e}$ | 3416 ± 157 $^{c}$ | 281 ± 22 $^{c,d,e}$ |
| 5 | AVTKS#E | 529 ± 24 $^{h}$ | 873 ± 43 $^{c,d}$ | 3187 ± 78 $^{b,c}$ | 232 ± 8 $^{a,b}$ |
| 6 | AVTKS#F | 425 ± 28 $^{d,e,f}$ | 850 ± 37 $^{c,d}$ | 3081 ± 163 $^{b}$ | 349 ± 32 $^{f}$ |
| 7 | AVTKS#2 | 356 ± 18 $^{b}$ | 788 ± 32 $^{a,b,c}$ | 3200 ± 94 $^{b,c}$ | 238 ± 24 $^{a,b,c}$ |
| 8 | AVTKS#H | 363 ± 33 $^{b,c}$ | 821 ± 52 $^{b,c}$ | 3265 ± 167 $^{b,c}$ | 268 ± 25 $^{b,c,d,e}$ |
| 9 | AVTKS#1 | 418 ± 32 $^{c,d,e}$ | 822 ± 58 $^{b,c}$ | 3283 ± 212 $^{b,c}$ | 219 ± 22 $^{a}$ |
| 10 | AVTKS#3 | 375 ± 15 $^{b,c,d}$ | 838 ± 34 $^{b,c}$ | 3135 ± 91 $^{b}$ | 227 ± 23 $^{a,b}$ |
| 11 | AVTKS#23 | 294 ± 24 $^{a}$ | 763 ± 48 $^{a,b}$ | 2533 ± 107 $^{a}$ | 261 ± 26 $^{a,b,c,d}$ |
| 12 | AVTKS#24 | 291 ± 15 $^{(a)}$ | 703 ± 39 $^{(a)}$ | 2577 ± 62 $^{(a)}$ | 264 ± 26 $^{(a,b,c,d,e)}$ |

The values are reported as means ± SD of 6 replicate analysis (n = 3 × 2). Values followed by identical superscript letters along the column are statistically similar.

### *3.2. Antioxidant Capacity and Monomeric Anthocyanins*

FRAP values of different genotypes were observed in the range between 703–966 mg TE/100 g. The highest value was found in genotype AVTKS#4 (966 mg TE/100 g), followed by AVTKS#A and AVTKS#D representing 934 and 929 mg TE/100 g, respectively. The lowest value was in genotype AVTKS#24 (703 mg TE/100 g), followed by AVTKS#23 and AVTKS#2, which represented 763 and 788 mg TE/100 g, respectively. The FRAP values obtained in this study matches with the values reported by Mani et al. [11]. They reported the FRAP values of methanolic seed extracts of nine different nigella genotypes in the range between 532–805 mg TE/100 g while studying nine different genotypes of nigella in Australia. However, some researchers have reported lower values of FRAP. For example, Kamiloglu et al. reported 182 mg TE/100 g in 80% methanolic seed extract of nigella from Turkey [12].

Furthermore, CUPRAC values ranged between 2533–3416 mg TE/100 g DW. The highest value was found in genotype AVTKS#D (3416 mg TXE/100 g), followed by AVTKS#4, AVTKS#1, AVTKS#H and AVTKS#C, AVTKS#2, AVTKS#A and AVTKS#E, which represented 3411, 3283, 3265, 3222, 3200, 3188 and 3187 mg TE/100 g, respectively. The lowest value was found in genotype AVTKS#23 (2533 mg TE/100 g), followed by AVTKS#24 which represented 2577 mg TE/100 g. The CUPRAC values of nigella seed extracts have been reported by few researchers. For example,Kamiloglu et al. and Toma et al. reported 2260 and 355 mgTE/100 g, respectively which showed that there is a variation in the CUPRAC value of nigella obtained from different origin and sources [12,13].

The monomeric anthocyanin content was also studied in the methanolic seed extract of nigella genotypes. However, negative results were observed, which indicated the absence of anthocyanins in the nigella samples. Mehmood et al. also did not find any anthocyanins in the methanolic or aqueous seed extracts of nigella from Pakistan origin [14].

Ishtiaq et al. furthermore studied the aqueous seed extracts including seven separate organic solvents seed extracts (methanol, ethanol, chloroform, diethyl ether (DEE), n-hexane, acetone, butanol) in Pakistan, but they also did not observe any sign of anthocyanin and leucoanthocyanins in the samples studied [15].

### *3.3. Thymoquinone Content*

The highest contributed compound was observed to be a thymoquinone in all the HPLC chromatograms obtained from the analysis of methanolic seed extracts. The thymoquinone concentration ranged between 219–349 mg/100 g DW. The highest concentration of thymoquinone was found to be present in genotype AVTKS#F (349 mg/100 g DW), followed by AVTKS#A, which represented 311 mg/100 g DW. The lowest concentration of thymoquinone was observed in AVTKS#1 (219 mg/100 g DW), followed by AVTKS#3, AVTKS#E, AVTKS#2, AVTKS#C, AVTKS#23 and AVTKS#24 representing 227, 232, 238, 247, 261 and 264 mg/100 g DW, respectively. A higher value of thymoquinone has been reported by a few authors compared to values obtained in our study. For example, Foudah et al. investigated the thymoquinone concentration in the methanolic extract of nigella seeds obtained from 6 different countries (Saudi Arabia, Egypt, Jordan, Palestine, Syria and India) and recorded the values in the range between 651–1076 mg/100 g [16]. On the contrary, lower values of thymoquinone content compared to our study have also been reported by some authors. For instance, Herlina et al. investigated the methanolic seed extracts of nigella obtained from India and Kwait and reported thymoquinone in the range between 10–29 mg/100 g [17].

### *3.4. Correlation between Different Variables*

Table 3 shows the correlation range (r = 0.681–0.808, $p < 0.01$) between TPC, CUPRAC and FRAP, demonstrating a strong positive linear correlation among the TPC, CUPRAC and FRAP. Our result is analogous to the reports of many authors who have reported positive correlation between TPC, FRAP and CUPRAC, although to varying level [18,19]. This supports the hypothesis that the majority of the antioxidant activity in nigella can be attributed to phenolic compounds.

Furthermore, there is very limited information available in the literature to understand the relationship of thymoquinone with TPC and antioxidant capacity. The table below confirms for the first time with a large sample size (n = 72) that there was no significant correlation of thymoquinone with TPC and CUPRAC. However, it showed a weak positive linear correlation ($r^{-} = 0.272$, * $p < 0.05$) with FRAP. This is notable as thymoquinone has previously been reported to have a strong antioxidant potential by many researchers including Cobourne-Duval, et al. [20]. However, in our work, it did not show any strong correlations with TPC, CUPRAC and FRAP. It is worth noting that Gupta et al. and Hossen et al. did report a strong positive correlation of thymoquinone with antioxidant capacity while using DPPH method [21,22]. Consequently, further research is required in this area.

**Table 3.** Pearson linear correlation analysis between various variables. The number of samples measured for each variable was 72.

| Variables | TPC | FRAP | CUPRAC | Thymoquinone |
|---|---|---|---|---|
| TPC | - | 0.808 ** | 0.681 ** | 0.15(NS) |
| FRAP | - | - | 0.764 ** | 0.272 * |
| CUPRAC | - | - | | 0.01(NS) |
| Thymoquinone | - | - | | - |

NS—not significant ($p > 0.05$), * $p < 0.05$, ** $p < 0.01$.

### *3.5. Factors Responsible for Variation in Chemical Composition*

Many factors, including genetic and evolution, ontogenic, agriculture practice and environmental factors (biotic and abiotic factors) have been reported to be responsible for variation in different metabolites of plants [23]. For example, Saxena et al. collected 23 genotypes of nigella seeds from the different parts of India, grew in similar condition, harvested the seeds, extracted oil using haxane and soxhlet appartus and observed significant variation in TPC (129–212 μg GAE/mL) in oils [24].

Study of the variation in nigella plant is very important. For example, investigation of the phytoconstituents variation in nigella genotypes and identification of the best quality of genotype in terms of valuable phytoconstituents such as thymoquinone and using it directly for the commercial production might ascertain the potential nutritional and therapeutic value and add substantial market value. Furthermore, the study of variation also adds better opportunity of selection to breeders in plant improvement programme such as better genotypes can be used in hybridization programme to obtain the new genetic resource for important econmic trait.

## 4. Conclusions

The important health benefiting components, TPC, antioxidant capacity (CUPRAC and FRAP) and thymoquinone, were systematically evaluated in the seeds of a wide range of Australian grown nigella genotypes for the first time. The result of this study showed the genotypes AVTKS#4 and AVTKS#D with comparatively higher value of antioxidant capacity (both FRAP and CUPRAC), while the genotypes AVTKS#F and AVTKS#E, with highest value of thymoquinone and total phenolic, respectively. Therefore, these genotypes showed potential for use in breeding programmes in terms of their thymoquinone content, total phenolics and antioxidant capacity. The present study also observed strong positive linear correlation between the TPC, CUPRAC and FRAP, but thymoquinone did not show any significatn correlation with TPC and CUPRAC and only showed a weak positive correlation with FRAP.

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