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Original Research

The Impact of *Tadbir* as a Pre-Application Treatment of Medicinal Plants on Their Chemical Profiles and Biological Activities in Traditional Persian Pharmacy

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Abstract

Based on basic traditional medicine practice, many plants undergo primary treatments to improve their pharmacological characteristics or to attenuate unwanted or unfavorable features of drugs before incorporating them into drug formulations. These treatments are called "*Tadbir*" in Traditional Persian Medicine (TPM). The purpose of these processes includes but is not limited to eliminating unnecessary compounds, excluding harmful properties such as toxicity and poignancy, and improving their overall natural properties and effectiveness. Here, the effect of vinegar and acetic acid treatment on three herbal specimens, including *Carum carvi* L. fruits (CC), *Trachyspermum ammi* (L.) Sprague fruits (TA), and *Nigella sativa* L. seeds (NS) were investigated. The treated and non-treated samples were subjected to essential oil and methanol extraction. Further, to assess the alterations in the essential oil constituents caused by *Tadbir*, samples were analyzed by Gas Chromatography/Mass Spectrometry (GC/MS) and HPTLC fingerprinting techniques. Total phenol and flavonoid content and DPPH free radical scavenging activity of methanol extracts were evaluated. As a result of the treatments, TA extracts showed significant rise in phenol and flavonoid contents. Total phenol content increased from 98.50±1.01 in non-treated increased to 181.20±0.27 mg GAE/g Ext. in the vinegar-treated TA fruit extract and total flavonoid showed a rise from 8.97±1.12 to 12.89±0.41 mg QE/g Ext. This may be the reason behind its lower IC_{50} values in DPPH free radical scavenging assay. Interestingly, *Tadbir* treatment of TA fruits with 4% acetic acid, lowered the IC_{50} value from 1019.42±75.65µg/mL in non-treated control to 274.2±17.22 µg/mL; while vinegar caused a lower degree of reduction in IC_{50} value (369.4±5.54 µg/mL) in DPPH free radical scavenging assay. However, CC fruit extracts, showed a decrease in phenolic content; while demonstrating an increase in flavonoids. It is also noteworthy that phenol and flavonoid contents were significantly enhanced in treated NS seed extracts. The results of all extracts were found significantly different ($p<0.05$) from each other and the non-treated control. The conclusive results of the present study may partly justify the pre-application of *Tadbir* treatments of medicinal plants in traditional pharmacy.

Keywords: *Trachyspermum ammi*; *Tadbir*; GC/MS; DPPH

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Introduction

Medicinal herbs have long been used to treat various ailments by human beings. These herbs are used either raw or in various types of pharmaceutical forms [1,2]. Extracts and essential oils from various medicinal plants are used in medicinal formulations and supplied by phytopharmaceutical manufacturing companies. In traditional Persian medicine practice, many plants, before entering formulations, undergo certain primary treatments, to modify their nature, properties, and/or chemical contents [3,4]. These types of treatments are mentioned in TPM resources in Iran under the title "*Tadbir*" [5]. The purpose of *Tadbir* of natural products is to include characteristic changes such as reducing toxicity, decreasing poignancy, removing wastes, and possibly altering the properties of the compounds or the initial nature of the drug [6]. The processes may include burning, soaking in water, vinegar, or milk, heating in oil or direct heat, boiling, and adding other compounds or medicinal plants. According to the claims made in standard literature sources of TPM, the products obtained from these processes can be used to treat certain types of diseases. In the case of many of these drug formulations that have been prepared by traditional text sources such as "*Qarabadin*", it has been stated that these drugs should not be given to the patients unless having undergone the *Tadbir* process [7-9]. Therefore, the present study seeks to investigate the role of the *Tadbir* process on chemical constituents and the biological efficacy of selected medicinal plant species. In the present study, the prescribed *Tadbir* process is to soak the medicine (plant fruits/seeds) separately in vinegar and acetic acid. Three herbal specimens, including, *Carum carvi* L. and *Trachyspermum ammi* (L.) Sprague fruits, and *Nigella sativa* L. seeds, were used in the present investigation. In the practice of TPM, these herbal seeds/fruits are soaked in vinegar before they are used in many formulations. So far, *C. carvi*, *T. ammi,* and *N. sativa* have exhibited many clinical and pharmacological effects such as analgesic, antimicrobial, anti-inflammatory, antibronchospasmic, antiparasitic, antioxidant, antihyperglycemic, lipid-lowering, antitumor and immunoregulating activities [10-14]. These plant species are used extensively in TPM as well as in folk remedies for the control and treatment of many diseases [15]. The present study was therefore undertaken to verify the effects of *Tadbir* process on the chemical constituents and antioxidant activity of each seed/fruit specimens, using chemical and instrumental methods of analysis. Finally, according to the results, the overall significance of *Tadbir* treatments and the changes that occurred, have been investigated.

Materials and Methods

Ethical considerations

The research proposal was approved by the Ethics Committee of Shiraz University of Medical Sciences, with code; IR.SUMS.REC.1395.S884 on December 14, 2016.

Plant specimens

Three plant specimens including *Carum carvi*, and *Trachyspermum ammi* fruits and *Nigella sativa* seeds were purchased from the licensed herbal medicine market and the voucher specimens were deposited at the herbarium of the Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences. The specimens were registered and identified by the plant taxonomist, and each was assigned a voucher number as *C. carvi* PM-903, *T. ammi* PM-901, and *N. sativa* PM-904. The seed and fruit specimens were stored in closed glass containers away from light, humidity, and heat until further treatment.

Chemicals and reagents

The quercetin standard and other chemicals and reagents procured from Merck Company, Darmstadt, Germany. Solvents used were of HPLC grade, manufactured by Samchun, Korea. The vinegar was purchased from an authorized local producer of natural grape vinegar (Sadeh-meimandi), in Meimand, a city in Fars province, Iran.

Tadbir experimental procedure

To perform the *Tadbir* process, experiments were conducted in three groups consisting of *N. sativa* (NS) and *C. carvi* (CC), and *T. ammi* (TA) fruits, with a total of 27 samples. The dried seeds and fruits used in *Tadbir* groups (50 g each) were subjected to treatment by soaking separately in 500 mL of either traditional vinegar or 4% acetic acid for 72 hours and served as vinegar-treated and acetic-acid-treated (both as *Tadbir*-treated) samples before essential oil extraction. The NS group served as group 1 which consisted of a total of 3×50 g powdered seed as triplicate in each non-treated, vinegar-treated, and acetic acid-treated as subgroups. CC, with 3×50 g powdered fruit in each subgroup as non-treated, vinegar-treated, and acetic acid-treated was defined as group 2; while group 3 consisted of 3×50 g of powdered fruit specimens of TA as triplicate in each non-treated, vinegar-treated, and acetic acid-treated subgroup. All the treated and non-treated (control) seed and fruit samples of NS, CC, and TA have undergone simultaneous essential oil extraction.

Extraction of essential oil

After the treatment process was completed, seeds and fruits were removed from the solutions, dried, and grinded. To prepare essential oil, 50 g of each seed or fruit powder in 500 mL distilled water was subjected to hydrodistillation for 4 hours using a Clevenger-type

apparatus. One mL *n*-hexane was then added to the apparatus collector, and the resulted essential oil was collected in a screw-capped glass container dried over anhydrous sodium sulfate, and stored at 4 °C until used for analysis. All seed/fruit extractions were carried out in triplicate and thus, nine extraction processes were performed for nine seed or fruit samples each of NS, CC, and TA in triplicate (three for control, three for vinegar-treated, and three for acetic acid-treated groups). The percentage yields of essential oils were calculated and compared within each group. Finally, the results obtained from treated groups were compared to those of non-treated control, in terms of essential oil composition.

Gas Chromatography/Mass Spectrometry (GC/MS)

This method was used to identify various chemical constituents of essential oil samples. In this study, the Agilent GC instrument 7890 attached to a Mass spectrometer (MS) 5975C, was used. The capillary column used was HP-5MS (phenylmethyl siloxane 30 mm × 0.25 mm × 0.25 µm ID), Agilent Technologies Corporation. The initial column temperature was adjusted at 60 °C, while reached 220 °C at 5 °C/min and remained at this temperature for 10 minutes. The helium carrier gas was used at a flow rate of 1 mL/min. Mass spectra acquired at EI mode in a mass range of *m/z* 30-600. The voltage applied was 70 eV, and the interfacial temperature was 280 °C [16].

GC/MS analysis of essential oil

Each sample was diluted before injection into the GC/MS instrument by *n*-hexane. A volume of 1 µL of the essential oil solution was injected into the gas chromatograph in split mode with a split ratio of 1:50. The chemical components of essential oil after separation through the GC column appear in the form of individual peaks for each compound, which appears in the gas chromatogram, each with a specific retention time (RT). The compounds then enter the mass chamber and are detected, and the mass spectrum of each separated compound is obtained. Identification and quantification of essential oil components were initially performed by calculating KI (Kovats Index) values for each compound and then comparing these data with the information given in Wiley nl7, Adams book [17], NIST, and Pherobase mass spectral libraries [18,19].

Preparation of methanol extracts

Samples of three powdered seed/fruit specimens of NS, CC, and TA have undergone treatment *Tadbir* process individually before extraction with methanol. Triplicate samples of each treated and non-treated seed/fruit specimen were used for the methanol extraction. In this method, 15 g of each of the three powdered seeds was transferred separately into glass stoppered tubes and placed in three different groups, the group consisted of 3×15 g of each non-treated seed/fruit specimen or control (group 1), three powdered seed/fruit specimens soaked in traditional vinegar (group 2); while the group, consisted of 3×15 g of each seed/fruit specimens, soaked in 4% acetic acid solution (group 3) with nine samples in each group and totally 27 seed samples.

The content of each tube was shaken for 30 minutes and then filtered at room temperature. The residual crushed seeds/fruits that remained on filter paper were dried and stored at -4°C before methanol extraction. Each residual seed/fruit of treated and non-treated control groups was then placed in glass stoppered tubes and separately extracted with 150 mL methanol, by ultrasonic extraction at 30 °C for 15 min, using an ultra-sonicator. The content of each tube was filtered, and the filtrate was collected. The extract collected from each seed/fruit sample was concentrated in a rotary evaporator and freeze-dried. A total of 27 methanol extracts, were obtained from NS seeds, CC, and TA fruits. The dried extracts were carefully weighed, and the yields were calculated and finally stored at -20 °C until further analysis.

Determination of total phenolic content

The determination of total phenolic (TP) content was performed using the Folin-Ciocalteu method [20]. In this method, metal oxides are reduced by polyphenolic antioxidants, such as glycolic acid and catechins, creating a blue solution with an absorption maximum at 765 nm, and this color change is measured. The Folin-Ciocalteu reagent was diluted to a 1:10 ratio with distilled water and placed in the dark for further use. To prepare a sodium carbonate solution, 18.55 g of Sodium carbonate was dissolved in 250 mL of distilled water. The gallic acid solution in methanol was prepared at concentrations of 3.1, 6.25, 12.5, 25, 50, 100, and 200 µg/mL as a standard and the calibration curve was prepared. To obtain the calibration curve based on various gallic acid concentration, 0.5 mL of each gallic acid concentration was diluted with 2.5 mL of Folin-Ciocalteu reagent, and 2 mL solution of sodium carbonate (75 g/L) were added and kept at 20 °C in the dark for 30 minutes. The absorbance was recorded at 765 nm against methanol as blank. To measure the total phenol content of each methanol extract, 5 mg of the extract was dissolved in 20 mL methanol to obtain a concentration of 250 µg/mL. To measure the total phenol content of the extract, 0.5 mL solution of each extract was diluted with 2.5 mL of Folin-Ciocalteu and 2 mL of sodium carbonate (75 g/L) and kept in the dark for 30 min. The absorbance was measured against methanol, using a spectrophotometer. Distilled water

was used as blank. This test was performed in triplicate. The absorption-concentration curve was plotted using Excel software, and the line equation was generated. The content of phenol was calculated according to the following formula, and the content of phenols was expressed as mg Quercetin Equivalent (QAE)/g of Ext.

$Cp = (Cg \times v) / M$

Cp: Phenolic content of the extract (mg/g).

Cg: The gallic acid concentration from the standard curve (mg/g).

V: The volume of extract tested.

M: Amount of dry extract (g).

Determination of total flavonoid content

The Dowd method was employed to measure the total flavonoid (TF) content of the extracts [2]. The calibration curve was initially plotted using quercetin as a standard. Different concentrations from 0.0-100 g/mL of quercetin in 5 mL methanol were prepared. Each concentration was mixed with 5 mL of aluminum chloride (2%) and incubated for 10 minutes at 25°C. The UV absorbance of each concentration was measured at 415 nm using a spectrophotometer, and the calibration curve was plotted. Then, triplicate samples of 5 mg of methanol extract were each dissolved in 20 mL of methanol, and further, a 3 mL portion was transferred into three test tubes and finally, 3 mL of a 2% solution of $AlCl_3$ was added to each and the mixture was incubated at 25 °C for 10 min. The absorbance was measured at 415 nm against methanol, spectrophotometrically. The values obtained for flavonoid content were expressed as mg quercetin equivalent/g of Ext (QE/g).

High-Performance Thin Layer Chromatography (HPTLC) Fingerprints

Considering that the process of *Tadbir* may affect the extracts' polar or non-polar compounds, each of the seeds/fruits (3 specimens of each of NS, CC, and TA), of which their freeze-dried methanol extracts were prepared, checked by the HPTLC technique using a standard procedure [22]. To prepare the chromatograms, a 1 mg/mL solution of each seed extract in methanol was prepared and the spots were loaded onto the chromateplates. HPTLC fingerprinting analysis was performed by selecting a suitable solvent using an HPTLC system (CAMAG, Basel, Switzerland). HPTLC screening of methanol extract was performed using toluene-ethyl acetate mixture (7:3) as the developing solvent. For thin-layer chromatography, 20×10cm silica gel 60 F_{254} plates (Merck) were used. Comparisons were made between the three samples including non-treated control, vinegar-treated, and acetic acid-treated groups of each seeds/fruit extract. The three seeds/fruit specimens of NS, CC, and TA comprising 9 samples of methanol extracts were applied onto the chromatogram using a CAMAG automatic TLC sampler 4. A 15 µL solution of each sample was loaded onto the chromatoplates along with a given distance of 1 cm. The mobile phase moving distance to the solvent front was adjusted to 80 mm. Following elution, the plates were removed from the tank, dried, and examined under visible and ultraviolet light at 254 and 366 nm. The plates were further sprayed with a freshly prepared anisaldehyde-sulfuric acid reagent and heated at 105 °C until the spots were visualized. To prepare the anisaldehyde-sulfuric acid reagent, 0.5 mL of anisaldehyde was dissolved in 10 mL of pure acetic acid to form a clear solution, and then methanol was added to reach a volume of 85 mL. Finally, 5 mL conc. Sulfuric acid was added, and the whole solution was vortexed until a clear solution was obtained.

DPPH free radical scavenging assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity of methanol extract was evaluated using a previously reported procedure [23]. Quercetin was used as the standard antioxidant compound. The calibration curve was plotted for different concentrations of quercetin (6.25-1600 µg/mL). Various concentrations of each extract (6.25-1600 µg/mL) were also prepared from a stock solution (3200 mg/mL) and used in the assay. Two hundred microliters of a 100 mM methanol solution of DPPH were mixed with 20 µL of each sample solution at different concentrations. The mixture was left in the dark for 30 minutes and the absorbance was recorded at 490 nm using a Stat Fax-2100 microplate reader (Awareness Technology, USA). A sample containing 20 µL of methanol and 200 µL of DPPH solution without extract, served as the control, while the blank contained an equal amount of extract in 20 µL of methanol. Analysis of each sample was performed in triplicate.

Statistical Analysis

Results were expressed as the mean ± SEM and analyzed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test using SPSS version 24.0 software. *P* values of <0.05 were regarded as statistically significance.

Results

Effect of Tadbir on Essential Oil Composition

The Tadbir process affected the seed and fruit essential oil yields of *N. sativa*, *C. carvi*, and *T. ammi*. Vinegar and acetic acid treatments significantly decreased the essential oil content of the seeds (Table 1).

The chemical components of the seeds' essential oils are presented in tables 2-4. The essential oil compo-

nents of the non-treated control, vinegar-treated, and acetic acid-treated seeds of NS exhibited the presence of 21, 24, and 24 compounds of different chemical classes, respectively. *o*-Cymene was found as the major chemical constituent among the essential oil components. The percentage of this compound declined from 43.43% in the non-treated control to 35.39%, and 32.13%, in the vinegar and acetic acid-treated samples, respectively (Table 2). The contribution of the monoterpene, α-thujene with 8.79% in a decreasing trend dropped to 3.5 and 2.7%, respectively by the action of vinegar and acetic acid. Terpinen-1-ol level was also significantly reduced from 7.77% in control to 0.88 and 0.99%, in vinegar and acetic acid-treated seed oils, respectively. Also, carvacrol with 6.32% in the control sample, decreased to 3.95 and 5.34%, with vinegar and acetic acid, respectively. While, the monoterpene thymoquinone with 6.26% in the control samples revealed a significant increase to 11.21 and 12.58%, with vinegar and acetic acid treatments respectively.

The sesquiterpene, longifolene, showed a contribution of 6.18% in the control sample; while increased to 6.64 and 6.87% by the action of vinegar and acetic acid, respectively.

Evaluation of the essential oil composition of NS seed declared the highest contribution by monoterpenes in the non-treated control (87.04%) as well as the vinegar and acetic acid-treated samples with 82.41 and 69.95%, respectively. Fatty acids were the second abundant group of compounds with 14.18%, in the acetic acid-treated NS essential oils components. Sesquiterpenes contribution in NS essential oil composition was found to be 7.36, 8.09, and 8.74%, for control, vinegar, and acetic acid-treated seeds (Table 2).

Evaluation of the volatile compounds of TA fruit essential oils revealed the presence of 5, 4, and 4 compounds in control, vinegar-treated, and acetic acid-treated groups, respectively. The vinegar and acetic acid treatment reduced the number of essential oil components to 4 (Table 3). Thymol was the most abundant component in TA fruit essential oil samples obtained from control, vinegar-treated, and acetic acid-treated groups with 55.88, 63.62, and 68.09%, respectively. The proportions of the monoterpenes and thymol were increased as a result of *Tadbir* treatments with vinegar and acetic acid respectively. This compound has been reported earlier, as the major essential oil constituent of TA fruits [24]. *o*-Cymene content in the control (23.57%) was reduced in a decreasing trend to 20.22% and 17.41% with vinegar and acetic acid treatment, respectively. γ-Terpinene showed 18.31% contribution in the non-treated control samples which decreased to 15.32% and 13.20%, in vinegar and acetic acid-treated samples respectively (Table 3). The proportion of the monoterpene, β-pinene was found to be 1.62% in the essential oil of non-treated TA fruits; while dropped to 0.94 and 0.86%, by *Tadbir* operations with vinegar and acetic acid respectively. Also, the monoterpene β-myrcene percentage was observed to be 0.61 in the non-treated control, which was eliminated due to the *Tadbir* processes. As given in table 3, monoterpenes were found to be the main constituent of TA essential oil (99.06-99.9%).

Careful examination of the essential oil composition of non-treated CC fruits showed the presence of 19 compounds from different chemical groups, which were reduced to 17 on vinegar and acetic acid treatments. The essential oil content of CC fruits exhibited the presence of the aromatic compound, cuminaldehyde with a contribution rate of 33.50% in the control sample, which increased to 35.14 and 34.75 %, by vinegar and acetic acid treatment, respectively (Table 4).

The percentage of γ-terpinene in the control (27.67%), increased to 30.61% by vinegar treatment; while slightly reduced to 27.07% by the action of acetic acid. *P*-Cymene content (14.71%) decreased to 13.22 and 14.02%, by the action of vinegar and acetic acid respectively. Also, *p*-Cymene-α-ol, as a member of the monoterpene group, increased from 6.57% in the control to 7.08 and 7.61%, by the influence of vinegar and acetic acid, respectively. Monoterpene components showed the highest contribution (96.32%) in acetic acid-treated CC fruit essential oil while it was found to be 95.80 and 96.23 % in control and vinegar-treated samples respectively. The monoterpene hydrocarbon,1-isopropylidene-3-n-butyl-2-cyclobutene content of CC fruit essential oil was 3.51% in the control which showed a slight increase to 3.76% by vinegar treatment; while decreased to 3.31% by acetic acid treatment (Table 4).

HPTLC fingerprinting of seeds/fruits methanolic extracts

HPTLC fingerprinting of methanol extracts of the treated and non-treated seeds/fruits demonstrated minor variations in the main compounds present in vinegar and acetic acid-treated methanol seed and fruit extracts (Figure 1-3). Minor differences were observed between the treated and non-treated samples when the chromatograms were sprayed with reagent and evaluated under UV light at 366 nm.

Effect of Tadbir on total phenol and flavonoid content

To monitor the quantitative changes that occurred in the major non-volatile constituents of the seeds, total phenol (TP) and total flavonoid (TF) contents of the treated and non-treated seeds were measured and the results were then compared. Determination of TP in CC methanolic fruit extract revealed the average total phenolic content in the non-treated control samples

(119.21±1.68 mg GAE/g of Ext.), which dropped to 89.27±5.62 and 108.2±3.77 mg GAE/g of Ext. respectively by vinegar and 4% acetic acid treatment (Table 5). The TF content of the methanol extract of non-treated CC fruits was found to be 28.43± 0.35 mg QE/g extract; while that of vinegar-treated fruits decreased to 24.69 ± 0.32 mg QE/g Ext. Treatment of fruits with acetic acid, increased the TF content of CC methanolic fruit extract to 34.38 ± 0.20 mg QE/g Ext. (Table 5). As shown in table 5, the TP content of the methanolic fruit extract of TA was found to be 98.50±1.01 mg GAE/g Ext. Vinegar treatment of the fruits increased the phenolic content to 181.20±0.27 mg GAE/g Ext.; while the acetic acid-treated fruits showed a lower extent of increase in TP content (129.56±2.49 mg GAE/g Ext). Based on the results obtained in this group, it can be inferred that *Tadbir* with both vinegar and acetic acid has led to a significant increase in the phenolic content of the fruits.

Total flavonoid content in the non-treated methanol fruit extract of TA was found to be 8.97±1.12 mg QE/g Ext., while those of the vinegar- and acetic acid-treated fruits were increased to 12.89±0.41 mg QE/g Ext. and 9.92±0.33 mg QE/g Ext., respectively (Table 5). In other words, vinegar, increased the flavonoid content of the fruits to a higher extent, compared to acetic acid (Table 5).

Measurement of the TP content of methanol extracts of NS seeds is presented in table 5. The average content of TP in the control group was found to be 24.38±1.95 mg GAE/g Ext. Vinegar and acetic acid treatment increased the TP content to 45.74±1.13 and 43.15±1.39 mg GAE/g Ext. respectively. As it is evident from the results given in table 5, *Tadbir* with vinegar showed a greater rate of increase in phenolic content of the seeds (>1.8 fold) compared to acetic acid.

The results also declared that non-treated NS seed extract contains 2.58±.06 mg QE/g Ext. of flavonoids. Vinegar treatment increased the amount of TF to 3.86±1.44 mg QE/g Ext.; whereas acetic acid declined the TF content of the seeds nominally to 2.08±.0.24 mg QE/g (Table 5). As a result, the treatment of NS seeds with vinegar declared an increase in the flavonoid content, but a decrease was observed in acetic acid-treated seeds (Table 5).

Tadbir process and alterations in free radical scavenging activity

This study revealed an increase in DPPH free radical scavenging properties of the methanolic extracts of treated CC fruits, both through vinegar and acetic acid (Table 6). Treatment with vinegar lowered the IC_{50} value from 373.8±1.23 µg/mL in non-treated control to 304.24±6.48 µg/mL, while this decrease was found to be moderate in the acetic acid-treated group (333.13±4.75) compared to control (373.8±1.23).

It is worth mentioning that, *Tadbir* treatment with both vinegar and acetic acid has led to a significant reduction in IC_{50} values and hence increased anti-DPPH properties of methanol extract of TA fruits. The antioxidant effects of methanolic fruit extracts of TA on DPPH free radicals were evaluated and quercetin was used as the standard antioxidant (Table 6). The IC_{50} value for the non-treated fruit extracts of TA was recorded as 1019.42±75.65µg/mL. Interestingly, acetic acid treatment lowered the IC_{50} value to 274.2±17.22 µg/mL, while vinegar treatment caused a lower degree of reduction in IC_{50} value (369.4±5.54 µg/mL). The effects of methanol extracts of non-treated control, vinegar- and acetic acid-treated NS seeds on DPPH free radical inhibition were also investigated (Table 6). Analysis of the results revealed inefficient anti-DPPH characteristics of the non-treated control (IC_{50} = 424.53±30.35 µg/mL), but the IC_{50} values of vinegar and acetic acid-treated extracts, unexpectedly increased to 662.87±23.49 and 1320.97±169.52 µg/

Table 1. Essential oil yield of treated (*Tadbir*) and non-treated *T. ammi* and *C. carvi* fruits and *N. sativa* seeds

Specimens	Weight (g)	Essential oil (mL)	Yield (%)
T. ammi (non-treated control)	50	2.9±0.1	5.8±0.3
T. ammi – vinegar	50	1.7±0.2	3.4±0.1
T. ammi – acetic acid (4%)	50	2.6±0.3	5.2±0.2
C. carvi (non-treated control)	50	2.7±0.1	5.4±0.1
C. carvi – vinegar	50	2.3±0.2	4.6±0.4
C. carvi – acetic acid (4%)	50	1.9±0.1	3.8±0.2
N. sativa (non-treated control)	50	1.5±0.2	3.0±0.3
N. sativa – vinegar	50	1.1±0.2	2.2±0.1
N. sativa – acetic acid (4%)	50	0.75±0.03	1.5±0.1

Table 2. Comparison of treated and non-treated *N. sativa* seed essential oil compositions

No.	Components		NS1[a]		NS2[b]		NS3[c]	
		RI[Rep*]	**RI[Exp**]**	**Area %**	**RI[Exp]**	**Area %**	**RI[Exp]**	**Area %**
1	α-Thujene	927	927	**8.79**	927	**3.5**	927	2.74
2	α-Pinene	936	935	1.89	935	0.78	935	0.06
3	Sabinene	973	974	1.13	-	-	-	-
4	β-Pinene	977	979	2.21	980	1.06	979	0.90
5	α-Terpinene	1017	1018	0.6	1060		1018	0.07
6	*o*-Cymene	1032	1027	**43.43**	1026	**35.39**	1026	32.13
7	*dl*-Limonene	1029	1030	1.83	1030	1.30	1030	1.08
8	γ-Terpinene	1059	1059	2.93	1060	4.81	1059	2.36
9	α-Terpinolene	1086	1088	-	1090	0.37	-	-
10	Unknown	-	1098	1.26	-	-	-	-
11	Terpinen-1-ol	1119	1121	**7.77**	1121	**0.88**	1121	0.99
12	Unknown	-	1167	0.58	-	-	-	-
13	4-Terpineol	1177	1179	1.42	1179	4.05	1180	4.22
14	Unknown	-	1206	1.63	-	-	-	-
15	Propanal, 2-methyl-3-phenyl	1245	-	-	1242	1.22	1242	0.90
16	Thymoquinone	1249	1253	**6.96**	1253	**11.21**	1253	12.58
17	Borneol acetate	1291	1288	0.44	-	-	1288	0.44
18	Thymol	1290	1291	1.32	1292	**13.99**	1292	6.32
19	Unknown	-	-	-	1295	0.56	-	-
20	Carvacrol	1299	1302	**6.32**	1301	**3.95**	1301	4.53
21	2,4-decadienal	1319	-	-	1317	3.50	-	-
22	α-Longipinene	1351	1355	1.18	1355	1.65	1355	1.47
23	Longifolene	1416	1411	**6.18**	1411	**6.84**	1411	6.87
24	α-Gurjunene	1409	-	-	-	-	1688	0.40
25	Tetradecanoic acid	1768	-	-	-	-	1759	0.38
26	Ent-pimara-8(14),15-diene	1954	-	-	1953	0.55	1953	0.33
27	Hexadecanoic acid	1984	-	-	1980	0.74	1982	7.05
28	1,13-Tetradecadiene	1385	-	-	1381	0.42	-	-
29	Unknown	-	1984	0.58	1984	1.06	1984	1.95
30	Unknown	-	2106	1.21	2106	1.57	2106	4.26
31	Linoleic acid	2130	-	-	2130	0.43	2134	5.04
32	9-Octadecenoic acid	2122	-	-	-	-	2138	1.71
Total			**99.66**			**99.83**		**92.87**
Monoterpenes			**87.04**			**82.41**		**69.95**
Sesquiterpenes			**7.36**			**8.09**		**8.74**
Fatty acids			-			**1.17**		**14.18**
Fatty Aldehydes			-			**3.30**		**-**

[a]Non-treated control (NS1), [b]Vinegar-treated (NS2) and [c]4% acetic acid-treated (NS3)
*RI= Retention Index, Rep= Reported, Exp=Experimental

mL, respectively (Table 6).

Discussion

As described in TPM sources, it has been recommended to process NS seeds with grape vinegar before usage as it may cause bronchospasm in patients with hot temperaments [4]. However, the overall biological effectiveness of NS seeds has been attributed to their thymoquinone content [25]. In the present study, the thymoquinone content of the seeds was increased owing to *Tadbir* treatment with vinegar and acetic acid. Reduction of *o*-cymene level in NS seed essential oil following the *Tadbir* process may lead to attenuation of some undesirable side effects already reported for this compound [26]. To verify the impact of *Tadbir* on the chemical components of TA, particular attention should go to the significant increase in thymol content, which occurred by the action of the *Tadbir* process. Thymol, the major compound in TA and various other plant essential oils has demonstrated numerous *in vitro* and *in vivo* pharmacological activities [27,28]. TA has traditionally been used to treat infection, arthritis, colic, diarrhea, and gastrointestinal problems. TA essential oil is a potent inhibitor of bacterial, fungal, and parasitic pathogens *in vitro* which can potentially contribute to the treatment of all types of vaginal infection including trichomoniasis [29].

The rise in γ-terpinene content of CC fruit essential oil caused by the *Tadbir* process, deserves mention, as this compound has demonstrated diverse pharmacological properties [30]. Meanwhile, the antioxidant properties of γ-terpinene have been attributed to its remarkable steric influence on the active sites in the DPPH molecule [31]. Cuminaldehyde is an oxygenated monoterpene and a component of essential oils of CC fruits, which has demonstrated antioxidant, antibacterial, antifungal, anticancer, anti-inflammatory antiplatelet, antidiabetic, antimalarial, and attenuating symptoms of Parkinson's disease [32].

GC/MS investigations of the fruit essential oils revealed significant changes in the types and contents of their chemical components, resulting from the acidic environment of the *Tadbir* process. Acid treatment can lead to hydrolysis and subsequent molecular changes or breakdown of some terpenic or other major essential oil components due to the oxidation and mild hydrolytic reactions that may cause migration of specific double bonds when they undergo vinegar and acetic acid treatments. [33,34].

Based on the changes that occurred in the essential oil composition resulting from the *Tadbir* process, it can be inferred that mild transformation reactions might have taken place in the chemical components of essential oil, by *Tadbir* processes. Therefore, these alterations in the composition of essential oil seem to be vital in the practice of TPM, aiming either at the optimization of pharmacological properties or attenuating particular undesirable characteristics of herbal drugs [35,36].

Despite the presence of several spots on the thin-layer chromatogram at 254 nm due to UV absorption, no clear distinction could be observed between the compound profiles of treated and non-treated CC methanolic fruit extracts. In vinegar and acetic acid-treated samples, a spot just above the base spot with slightly lower polarity showed an increased absorption intensity than the non-treatment control. *Tadbir* treatment with vinegar affected the thin-layer profile of TA methanolic fruit extract, which demonstrated the absence of polar compounds and the presence of compounds of lower polarity with UV absorption at 366 nm. Following treatment with 4% acetic acid, NS methanolic seed extracts showed a distinct spot-on TLC after spraying with anisaldehyde-sulfuric acid reagent, visualized at 366 nm, not detected in the vinegar-treated and non-treated control samples.This study clearly revealed an increase in DPPH free radical scavenging properties of the methanol extracts of *Tadbir*-treated CC fruits. Vinegar treatment of CC fruits led to a higher decrease in the phenol content, compared to the acetic acid. In the case of CC fruit extracts, no clear correlation could be observed between the increased anti-DPPH activities and TP and TF contents of vinegar and acetic acid-treated methanolic fruit extracts. Therefore, the increase observed in anti-DPPH free radical activity could be mainly due to the changes that have occurred on other major components of CC fruit extract, such as mono- and sesquit-

Table 3. Composition of treated and non-treated *T. ammi* fruit essential oils

No.	Components	TA1[a]			TA2[b]		TA3[c]	
		RI^{Rep*}	**RI^{Exp**}**	**Area%**	**RI^{Exp}**	**Area%**	**RI^{Exp}**	**Area%**
1	β-Pinene	977	980	1.62	980	0.94	980	**0.86**
2	β-Myrcene	991	992	0.61	-	-	-	-
3	o-Cymene	1032	1032	**23.57**	1032	**20.22**	1032	17.41
4	γ-Terpinene	1059	1066	**18.31**	1066	**15.32**	1065	13.20
5	Thymol	1290	1307	**55.88**	1310	**63.62**	1312	68.09
	Total			**99.99**		**99.06**		**99.46**

aNon-treated control (TA1), bVinegar-treated (TA2) and c4% acetic acid-treated (TA3)
RI= Retention Index, *Rep= Reported, **Exp=Experimental

Table 4. Composition of treated and non-treated *C. carvi* fruit essential oils

No.	Components	CC1[a]			CC2[b]		CC3[c]	
		RI^{Rep}	**RI^{Exp}**	**Area%**	**RI^{Exp}**	**Area%**	**RI^{Exp}**	**Area%**
1	α-Thujene	927	928	0.58	928	0.37	-	-
2	α-Pinene	936	936	1.55	936	1.31	936	**0.85**
3	Sabinene	973	976	1.85	976	0.65	975	**0.49**
4	β-Pinene	977	981	2.44	981	2.16	980	**1.61**
5	β-Myrcene	991	992	1.00	992	1.02	992	**0.72**
6	p-Cymene	1033	1033	**14.71**	1033	**13.22**	1030	14.02
7	dl-Limonene	1029	1036	3.36	-	-	1034	**4.79**
8	β-Ocimene	1050	1049	0.36	1049	0.4	-	-
9	γ-Terpinene	1059	1070	**27.47**	1071	**30.61**	1067	27.05
10	α-Terpinolene	1086	1092	0.36	1093	0.46	1091	0.38
11	4-Terpineol	1177	1181	0.62	1181	0.91	1180	0.85
12	Carveol	1197	1198	1.05	1198	1.25	1197	1.28
13	Cuminaldehyde	1239	1257	**33.50**	1258	**35.14**	1254	34.75
14	Unknown	-	1271	0.6	-	-	1270	0.36
15	1-Isopropylidene-3-n-butyl-2-cyclobutene	1297	1292	3.51	1293	3.76	1291	3.31
16	p-Cymen-α-ol	1287	1300	**6.57**	1301	**7.08**	1298	7.61
17	p-Mentha-1,4-dien-7-ol	1333	1332	0.39	1332	0.36	1331	**0.39**
18	α,α-4-Trimethyl benzyl ester	1428	1425	0.99	1426	1.29	1425	**1.53**
	Total			**99.91**		**99.99**		**99.97**
	Monoterpenes			**95.80**		**96.23**		**96.32**
	Hydrocarbons			**3.51**		**3.76**		**3.31**

aNon-treated control (CC1), bVinegar-treated (CC2) and c4% acetic acid-treated (CC3)
RI= Retention Index, *Rep= Reported, **Exp=Experimental

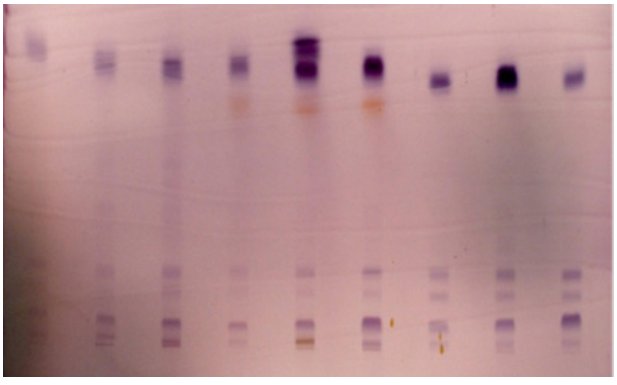

Figure 1. HPTLC Chromatogram of methanol extract in the visible wavelength sprayed with the Anisaldehyde/Sulphuric acid reagent; S1: non-treated control of methanolic fruit extract of *C. carvi;* S2: vinegar-treated methanolic fruit extract of *C. carvi*; S3: acetic acid-treated methanolic fruit extract of *C. carvi*; S4: non-treated methanolic fruit extract of *T. ammi*; S5: vinegar-treated methanolic fruit extract of *T. ammi*; S6: acetic acid-treated methanolic fruit extract of, *T. ammi*; S7: non-treated control, methanolic seed extract of *N. sativa*; S8: vinegar-treated methanolic seed extract of *N. sativa*; S9: acetic acid-treated methanolic seed extract of *N. sativa*.

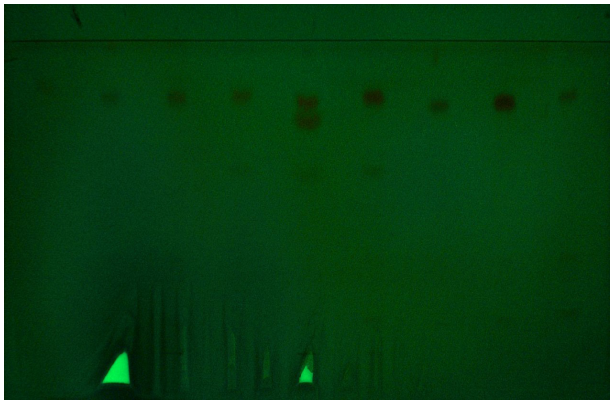

Figure 2. HPTLC Chromatogram of methanolic extracts at 254 nm sprayed with the Anisaldehyde/Sulphuric acid reagent; S1: non-treated (control) fruit extract of *C. carvi*; S2: vinegar-treated fruit extract of *C. carvi*; S3: acetic acid-treated fruit extract of *C. carvi*; S4: non-treated (control) fruit extract of *T. ammi*; S5: vinegar-treated fruit extract of *T. ammi*; S6: acetic acid-treated fruit extract of, *T. ammi*; S7: non-treated (control) seed extract of *N. sativa*; S8: vinegar-treated seed extract of *N. sativa*; S9: acetic acid-treated seed extract of *N. sativa*.

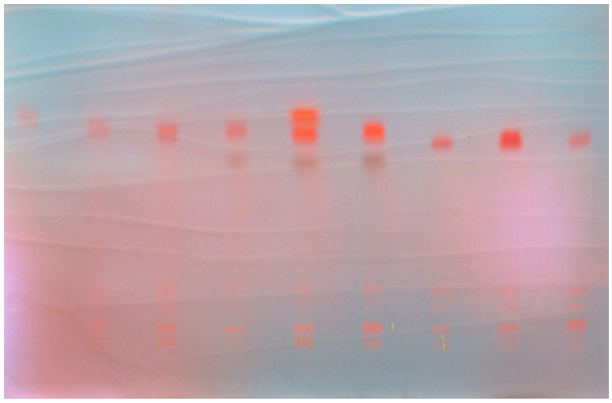

Figure 3. HPTLC Chromatogram of methanolic extracts at 366 nm sprayed with the Anisaldehyde/Sulphuric acid reagent; S1: non-treated (control) fruit extract of *C. carvi*; S2: vinegar-treated fruit extract of *C. carvi*; S3: acetic acid-treated fruit extract of *C. carvi*; S4: non-treated (control) fruit extract of *T. ammi*; S5: vinegar-treated fruit extract of *T. ammi*; S6: acetic acid-treated fruit extract of, *T. ammi*; S7: non-treated (control) seed extract of *N. sativa*; S8: vinegar-treated seed extract of *N. sativa*; S9: acetic acid-treated seed extract of *N. sativa*.

Table 5. Total phenol and flavonoids of methanolic fruit/seed extracts of *C. carvi* (CCM), *T. ammi* (TAM), and *N. sativa* (NSM)

Extract	Yield (%)	Phenol content (mg GAE/g Ext)	Flavonoid content (mg QE/g Ext)
CCM1[a]	2.4	119.21±1.68	28.43±0.35
CCM2[b]	2.4	89.27±5.62	24.69±0.32
CCM3[c]	1.6	108.2±3.77	34.38±0.20
TAM1[a]	2.8	98.50±1.01	8.97±1.12
TAM2[b]	1.8	181.20±0.27	12.89±0.41
TAM3[c]	2.6	129.56±2.49	9.92±0.33
NSM1[a]	4.3	24.38±1.95	2.58±0.06
NSM2[b]	5.8	45.74±1.13	3.86±1.44
NSM3[c]	8.2	43.15±1.39	2.08±0.24

[a] Non-treated Samples (CCM1, TAM1, NSM1), [b] vinegar-treated samples (CCM2, TAM2, NSM2), [c] 4% acetic acid-treated samples (CCM3, TAM3, NSM3).

erpenoid glucosides, lignans, and alkaloids in addition to isoflavonoids, flavonoid glycosides, and other phenolic compounds that have been reported earlier from caraway fruit [37-39]. Results of antioxidant screening revealed that the *Tadbir* process was ineffective in improving the DPPH free radical scavenging effect of NS seed extract. These findings may be attributed in part to the low flavonoid content observed in the control and treated NS methanolic seed extracts.

Results of the DPPH free radical assay indicated that the *Tadbir* process significantly reduced the IC_{50} values of methanolic fruit extracts of TA and thus increased the DPPH free radical scavenging activity of the treated samples. The *Tadbir* processes with vinegar and acetic acid have therefore led to overall improved DPPH scavenging properties of the extracts. The improvement of antioxidant activity of these extracts may be attributed to the changes that have occurred in the molecular structures of their chemical constituents.

As compared to vinegar treatment, *Tadbir* with acetic acid showed a superior DPPH free radical scavenging effect of TA methanolic fruit extracts (Table 9). However, the presence of alkaloids, steroids, glycosides, saponins, flavonoids, and essential oil compounds like thymol, *γ*-terpinene and *o*-cymene in TA fruits, can lead to its therapeutic effects via their synergistic actions [40,41].

Both phenol and flavonoid contents have increased exclusively in vinegar and acetic acid-treated TA methanolic fruit extracts. This might be the reason behind the decrease in IC_{50} values and the manifestation of antioxidant activity by this extract. This, however, has not happened in the case of CC, as the content of phenol has declined, but the flavonoid content has increased by *Tadbir* treatment. The changes that occurred were reversed in NS methanolic seed extracts, as the phenolic content was increased; while the flavonoid content was decreased as a result of *Tadbir* treatment.

The results of the present study revealed that the *Tadbir* process caused an increase in the total phenol and flavonoid contents of the tested fruit and seed extracts. Numerous studies have documented the positive relationship between phenol and flavonoid contents and the antioxidant activity of plant extracts [42-47]. As evidenced by another study, flavonoid, coumarin, and polyphenolic compounds increased in vinegar and heat-treated *Bunium persicum* fruit extracts. A major objective of this process was to decrease medication side effects while increasing its effectiveness.

Therefore, the decrease in IC_{50} values and thus improvement in the antioxidant activity of the extract may be attributed to the increase in total phenol and flavonoids of the methanolic fruits and seed extracts following *Tadbir* treatment. However, the simultaneous contribution of other classes of chemical constituents of the extracts cannot be ignored.

In traditional pharmaceutical practice, many herbs or natural medicines undergo an initial treatment before prescription. Numerous species of medicinal plants have been considered as having hot and dry temperaments traditionally. The CC and TA fruits and NS seeds used in this study contain essential oils that

may partly contribute to their warm and dry nature. Therefore, the process of *Tadbir* using vinegar or acetic acid, causes physicochemical changes in the bioactive content of plant materials, concerning the TPM approach, and can modify the overall nature as well as other important characteristics of traditional herbal drugs. Looking into the individual profile of essential oils and components of methanol extracts, under the experimental conditions of this study, the *Tadbir* process led to variations in the chemical constituents of the fruits and seeds that affected the rate of DPPH scavenging of the extracts. The formation of analogous compounds such as enantiomeric or quasi-enantiomeric forms, as a consequence of the acidic environment of the *Tadbir* process may affect the nature and the degree of efficacy of these drugs. Since the process of *Tadbir* is conducted using various kinds of traditional methods, the numerous physicochemical changes that may occur, require individual investigations. These traditional approaches can also lead to the preparation of new herbal formulations and merit further attention.

Considering the differences observed in the performance of vinegar and acetic acid, understanding the chemical and biological transformations that may occur during *Tadbir* with these materials is of substantial practical importance. The present investigation revealed that, despite a remarkable increase in total phenol content by vinegar treatment, 4% acetic acid-treated TA fruit extract showed higher free radical scavenging properties. Also, an increase was detected in the thymoquinone content of the essential oil; while a decline in its terpinene-1-ol, *o*-cymene, and α-thujene contents. This evidence demonstrates that vinegar and acetic acid may interact differently with plant components. Therefore, further research on various medications is needed to understand the purposeful analogy behind the *Tadbir* process with these materials and the changes in traditional formulations' physicochemical behavior.

Table 6. Antioxidant effects of methanolic fruit and seed extracts of *C. carvi*, *T. ammi*, and *N. sativa* against DPPH free radical

Extract	*C. carvi* IC_{50} (µg/mL)	*T. ammi* IC_{50} (µg/mL)	*N. sativa* IC_{50} (µg/mL)
Non-treated (control)	373.8±1.23	1019.42±75.65	424.53±30.35
Vinegar-treated	304.24±6.48	369.4±5.54	662.87±23.49
Acetic acid-treated	333.13±4.75	274.2±17.22	1320.97±169.52
Quercetin	79.86±1.54	83.25±1.60	81.42±2.14

Conclusion

The process of *Tadbir* has been purposefully designed to accomplish specific goals in traditional pharmaceutical practice. This study investigated the influence of *Tadbir* treatment on the chemical components of three fruits/seeds routinely used for their tremendous medicinal and pharmacological benefits. Based on the results of this study, *Tadbir* makes noticeable changes in the molecular structure and volatile and non-volatile chemical components of traditional drugs that can be traced accurately by chemical and instrumental analysis methods, as performed in the present study. These changes may occur in the physicochemical and pharmacological properties of treated drugs. It should be acknowledged that these reactions are carried out gently to modulate or attenuate some, but not all, of the adverse effects of specific undesirable or unwanted components, which result in reducing potential toxicity or adding some new properties to the drug formulations. Therefore, *Tadbir* may be considered a smart or meaningful process frequently used in TPM. Since *Tadbir* is being performed using various types of treatments, it can be expected that the dimensions of changes caused would be distinctive in different plant products and hence encourage further research to measure its performance in preparing traditional drugs and phytopharmaceutical formulations.

Conflict of Interests

The authors declare no conflict of interest.

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