Evaluation of the effect of two Extraction Methods on the Components of Essential oil of Trifolium pratense L. by GC-MS

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Abstract. In order to make full use of Trifolium pratense L. resources, GC-MS was used to evaluate the effects of two extraction methods on the volatile oil components of Trifolium pratense L. A total of 96 compounds were identified by GC-MS from the volatile oil of Trifolium pratense L. extracted by petroleum ether (PE) method and by steam distillation (SD) method. 67 and 42 compounds were identified respectively, with a total peak area of $2.14 \times 10^9$ and $1.83 \times 10^8$ respectively. The relative percentage of the volatile oil extracted from Trifolium pratense L. by PE method was higher than that of the others, which were Tetracontane (19.70%), Oleic acid (14.21%), 9,12-Octadecadienoic acid (12.51%) and 1 - (+) - Ascorbic acid 2,6-dihydroxadecanoate (12.13%). The relative percentage of the volatile oil distilled from Trifolium pratense L. by SD method were 13-docosenamide (28.52%), 7,9-di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione (3.34%) and L - (+) - ascorbic acid 2,6-dihexadecanoate (2.34%). There were rich compounds in the volatile oil of Trifolium pratense L. Compared with SD method, PE method has higher kinds of compounds and total peak area.

1. Introduction
The abnormal accumulation of melanin in the basal layer of the epidermis will lead to hyperpigmentation and lead to pigmentation diseases such as black spot and freckle, which will seriously affect people's physical and mental health [1]. Studies have shown that tyrosinase (Tyr) is the key enzyme of melanin synthesis in vivo, and its overexpression is the main cause of pigmentation. Therefore, inhibition of tyrosinase activity can block the biosynthesis reaction chain of melanin, reduce the production of melanin, and achieve the effect of prevention and treatment of pigmentation diseases. In recent years, many inhibitors have been proved to be able to effectively inhibit the activity of tyrosinase, but people prefer to use products from natural sources. Volatile oil from natural plant has aromatic and certain tyrosinase inhibitory activity, which has attracted the attention of researchers and consumers. Lemon essential oil and some of its volatile components were reported to exhibit good inhibitory effect on tyrosinase activity. Among the components of lemon essential oil, inhibitory rate on tyrosinase activity exerted by citral (2.16% m/m) and beta-pinene (14.30% m/m) was 76.64% and 61.82%, respectively, while that of d-limonene (61.41% m/m) was only 33.25%[2].Camphor (28.30%), 1, 8-Cineole (21.03%) and β-thujone (14.20%) were the major components in the leaf oil of Artemisia diffusa. The largest part of the leaf oil of Artemisia diffusa was formed by oxygenated monoterpenes (75.58%). Anti-tyrosinase activity of
Artemisia diffusa oil at 50% concentration (IC50) was 6.08 mg [3]. Xu Guang et al. [4] extracted the essential oil of Angelica dahurica by SD method. 64 compounds were identified by GC-MS. 39 of them were non terpenoids, 25 were terpenoids. The main components of the essential oil were Dodecanol (30.62%), 1-Pentaenol (16.15%), Basilene (2.9%), Terpinene (2.83%). The inhibitory activity of fresh Angelica dahurica volatile oil on tyrosinase in vitro was as high as (43.21 ± 1.96)%.

Trifolium pretense L. is a perennial herb of Trifolium Leguminosae. It is a traditional folk herb and widely used in curing skin trauma [5]. Trifolium pratense L. was reported to be rich in volatile oil [6]. In view of the fact that many kinds of plant volatile oils have tyrosinase inhibitory activity, the extraction and identification of the volatile oil from Trifolium pratense L. will lay a foundation for exploring whether it has tyrosinase inhibitory activity.

2. Materials and Apparatus

2.1. Plant materials

Trifolium pratense L. was purchased from Guizhou Province and identified as the whole dry plant of Trifolium pratense L. by Ouyang puyue, associate professor of Chinese Traditional Medicine Department, Guangdong food and Drug Vocational College. The certificate specimen is deposited in Guangdong food and Drug Vocational College.

2.2. Reagents and Apparatus

The reagents used in this study included: Petroleum ether (boiling range from 60°C to 90°C) was analytical pure. N-hexane was chromatographic pure. The apparatus used in this study included: A11 crusher (Aika (Guangzhou) Instrument and Equipment Co., Ltd.); BSA2202S electronic balance (sai duolis scientific instrument (Beijing) Co., Ltd.); RE-5205 rotary evaporator (Shanghai Yarong Biochemical Instrument Gactory); GCMS-QP2010 Ultra Gas Chromatography-mass Spectrometer (Shimadzu Institute, Japan).

3. Method

3.1. Extraction of volatile oil

3.1.1. PE method. Weigh 50g of Trifolium pratense L. powder, add 500g of petroleum ether (boiling range 60-90°C), after ultrasonic treatment for 6h (Power: 800W, temperature: 40°C), soak for 20h, filter and collect the extraction liquid, remove the petroleum ether by rotary evaporation at 35°C, and obtain the volatile oil. Take proper amount of n-hexane to dissolve and dilute, and then use it for GC-MS detection.

3.1.2. SD method. Weigh 50g of Trifolium pratense L. powder, add 1500g of distilled water, soak for 2h, put it in the rotating evaporator, keep it slightly boiling for 6h, collect the fraction, add anhydrous sodium sulfate to dry, and obtain the volatile oil. Take appropriate amount of volatile oil and dilute it with n-hexane for GC-MS detection.

3.2. GC-MS Conditions

3.2.1. Gas chromatography conditions. The GC system used chromatographic separation was achieved by using a fused capillary column Rts-5MS, length (30m×0.25mm×0.25m). The initial column temperature was 100°C for 5min, and it was raised to 200°C at 6°C/min for 10min. Then it was raised to 250°C at 2°C/min for 10min. The carrier gas was high purity helium with a flow rate of 0.8 mL/min. The injection temperature is 250°C, the interface temperature is 280°C, and the desorption time is 2min. There is no split injection. The total time of GC is 66.67min.
3.2.2. Mass spectrometry conditions. Electron bombardment ion (EI) source is used as ionization mode. The monitoring method is full scanning. The scanning range is 35-500. The ionization energy is 70eV. The ion source temperature is 230°C.

3.3. Qualitative analysis

Qualitative analysis was carried out by searching NIST 11 standard library, selecting compounds with a matching degree of more than 80%, and combining the results of literature reports for qualitative analysis. The relative percentage content of each compound in total volatile components was calculated by peak area normalization method.

4. Result

It can be seen from table 1 that 67 and 42 compounds were identified by GC-MS from the volatile oil extracted from Trifolium pratense L. by PE method and SD method, with total peak areas of $2.14 \times 10^9$ and $1.83 \times 10^8$, respectively. The compounds with higher relative percentage in the volatile oil extracted from Trifolium pratense L. by PE method were Tetracontane (19.70%), Oleic acid (14.21%), 9,12-Octadecadienoic acid (12.51%), 1-(-)-Ascorbic acid 2,6-dihydroxadecanoate (12.13%), Octadecanoic acid (4.96%), 13-Docosanamide (4.59%), Ovirane, Hexadecyl (4.35%) and Phytol (4.08%) and they accounted for 76.53% of the volatile components. The compounds with relatively high content in the volatile oil extracted from Trifolium pratense L. by the SD method were 13-Docosenamide (28.52%), 7,9-Di-tert-butyl-1-oxaspiro (4, 5) deca-6,9-diene-2,8-dione (3.34%), 1-(-)- Ascorbic acid 2,6-dioxoacid 2,6-dihydroxadecanoate (2.34%), Phenol, 2,4-bis (1,1-dimethylhydroxyl) (2.10%), 9-Octadecenamide (2.03%), Dodecane, 4, 6-Dimethyl (1.98%) and 1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester (1.86%) and they accounted for 42.17% of the volatile components.

According to the analysis of the results of the common components of the volatile oil, there were 13 kinds of the same components extracted from Trifolium pratense L. by PE method and the SD method, but the relative percentage content of each component was quite different. The proportion of common components of essential oil extracted from Trifolium pratense L. by PE method were Tetracontane (19.70%), 13- Docosenamide (28.52%), 7,9-Di-tert-butyl-1-oxaspiro (4, 5) deca-6,9-diene-2,8-dione (3.34%), 1-(-)- Ascorbic acid 2,6-dioxoacid 2,6-dihydroxadecanoate (2.34%), Phenol, 2,4-bis (1,1-dimethylhydroxyl) (2.10%), 9-Octadecenamide (2.03%), Dodecane, 4, 6-Dimethyl (1.98%) and 1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester (1.86%) and they accounted for 42.17% of the volatile components.

According to the analysis of the results of the specific components of the volatile oil, there are 54 specific components in the volatile oil extracted from Trifolium pratense L. by PE method, accounting for 57.96% of the volatile components. Among them, Oleic acid (14.21%), 9, 12-Octadecadienoic acid (12.51%), Octadecanoic acid (4.96%), Ovirane, hexadecyl (4.35%) and Phytol (4.08%) were the most abundant compounds. There are 29 specific components in the volatile oil extracted from Trifolium pratense L. by SD method, with a relative percentage of 28.52% and a peak area of $8.79 \times 10^7$. The relative percentage of the total volatile oil extracted from Trifolium pratense L. by PE method was 4.59% and the peak area was $1.03 \times 10^8$.

According to the analysis of the results of the specific components of the volatile oil, there are 54 specific components in the volatile oil extracted from Trifolium pratense L. by PE method, accounting for 57.96% of the volatile components. Among them, Oleic acid (14.21%), 9, 12-Octadecadienoic acid (12.51%), Octadecanoic acid (4.96%), Ovirane, hexadecyl (4.35%) and Phytol (4.08%) were the most abundant compounds. There are 29 specific components in the volatile oil extracted from Trifolium pratense L. by SD method, accounting for 14.90% of the volatile components. Among them, 1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester (1.86%), 1, 2-Benzenedicarboxylic acid, butyl 8-m ethylonyl ester (1.41%), Phynol, 2, 2'- methylenebis [6-(1, 1-dimethyl) - 4-methyl (1.38%) and Heptadecane, 8-methyl (1.11%) were the most abundant compounds.

5. Discussion

96 compounds were identified by GC-MS from the volatile oil of Trifolium pratense L. extracted by PE method and distilled by SD method. 67 and 42 compounds were identified respectively. The total peak areas were $2.14 \times 10^9$ and $1.83 \times 10^8$, respectively. The results showed that there were rich compounds in the volatile oil of Trifolium pratense L., and the compounds and the total peak area of the volatile oil extracted by PE method were higher than that by SD method. The volatile oil of Trifolium pratens
e L. has been reported to have significant antibacterial activity against Melissococcus pluton and Bacillus subtilis [7]. The highest content of volatile compounds extracted from Trifolium pratense L. were $\beta$-myrcene (4.55%), $\alpha$-cymene (3.59%), Limonene (0.86%) and Tetrahydroionone (1.56%), and they were highlighted due to their biological activity. The essential oil showed good radical-capturing capacity and also had lipid peroxidation effects. [8]. In view of the reported antibacterial and antioxidant effects of the essential oil of Trifolium pratense L., its inhibitory effect on tyrosinase and other medicinal value are worth further study.

### Table 1. Volatile compositions extracted from Trifolium pratense L. by PE method and SD method

| No. | Retention \( t_R/min \) | Compound name | Percentage of compound in total area /% | Peak area/10\(^7\) |
|-----|-------------------------|---------------|---------------------------------------|-------------------|
|     |                         |               | PE method | SD method | PE method | SD method |
| 1   | 9.073                   | 1-Octanol, 2-butyl | 0.38 | - | 0.84 | - |
| 2   | 9.416                   | Dodecane, 4,6-dimethyl | 0.04 | 1.98 | 0.09 | 0.61 |
| 3   | 10.191                  | Nonadecane | 0.04 | 0.17 | 0.09 | 0.05 |
| 4   | 10.253                  | 1-Decanol, 2-hexyl | 0.03 | 0.58 | 0.06 | 0.18 |
| 5   | 10.515                  | Cyclohexane, 1-ethyl-2-propyl | - | 0.23 | - | 0.07 |
| 6   | 12.938                  | Heptadecane | 0.04 | 0.57 | 0.08 | 0.18 |
| 7   | 12.973                  | Tetradecane | - | 0.21 | - | 0.06 |
| 8   | 13.174                  | 2-methyltetrasosane | - | 0.50 | - | 0.15 |
| 9   | 13.988                  | Alloaromadendrene | 0.40 | - | 0.90 | - |
| 10  | 14.12                   | Pentadecane, 2,6,10,14-tetramethyl | - | 0.33 | - | 0.10 |
| 11  | 14.163                  | Cyclooctadecane | 0.04 | - | 0.09 | - |
| 12  | 14.198                  | 2-methylloctacosane | - | 0.34 | - | 0.11 |
| 13  | 14.42                   | Nonane, 3-methyl-5-propyl | 0.03 | - | 0.06 | - |
| 14  | 14.502                  | Aromandendrene | 0.06 | - | 0.13 | - |
| 15  | 15.174                  | Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylenehexyl) | 0.06 | - | 0.13 | - |
| 16  | 15.331                  | 1-Cycloheptene, 1,4-dimethyl-3-(2-methyl-1-propene-1-yl)-4-vinyl | 0.12 | - | 0.27 | - |
| 17  | 15.455                  | Phenol, 2,4-bis(1,1-dimethylethyl) | 0.05 | 2.10 | 0.10 | 0.65 |
| 18  | 15.98                   | 2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl | 0.06 | - | 0.13 | - |
| 19  | 16                      | Hexadecane | - | 0.62 | - | 0.19 |
| 20  | 16.796                  | Epiglobulol | 0.10 | - | 0.22 | - |
| 21  | 16.888                  | Octadecane | - | 0.22 | - | 0.07 |
| 22  | 17.1                    | 1H-Cycloprop[e]azulene-7-ol, decahydro-1,1,7-trimethyl-4-methylene | 0.03 | 0.22 | 0.06 | 0.07 |
| 23  | 17.23                   | Caryophyllene oxide | 0.15 | - | 0.34 | - |
| 24  | 17.305                  | (-)-Globulol | 0.63 | 1.49 | 1.41 | 0.46 |
| 25  | 17.445                  | Decane, 3,8-dimethyl | - | 0.16 | - | 0.05 |
| 26  | 17.488                  | Viridiflorol | 0.16 | - | 0.37 | - |
| 27  | 17.509                  | Heptadecane, 2,6,10,15-tetramethyl | - | 0.46 | - | 0.14 |
| No. | Compound Description                                      | Retention Time (min) | Area (%), Peak Width (s) | Area (%), Retention Time (min) |
|-----|-----------------------------------------------------------|----------------------|--------------------------|--------------------------------|
| 28  | 2-Naphthalenemethanol, 2,3,4,4a,5,6,7,8-octahydro-alpha, | 17.724               | 0.05 - 0.12              | -                              |
|     | alpha,4a,8-tetramethyl                                    |                      |                          |                                |
| 29  | Tetradecane, 4-methyl                                     | 17.923               | - 0.34 - 0.11            | -                              |
| 30  | Hentriacontane                                            | 18.466               | - 0.71 - 0.22            | -                              |
| 31  | Selina-6-en-4-ol                                          | 18.773               | 0.06 - 0.14              | -                              |
| 32  | Dodecane, 2,6,10-trimethyl                                | 19.172               | - 0.90 - 0.28            | -                              |
| 33  | Octadecane, 1-chloro                                      | 19.477               | 0.07 - 0.15              | -                              |
| 34  | Squalane                                                  | 19.845               | - 0.28 - 0.09            | -                              |
| 35  | 2-Hexyldecyl acetate                                      | 20.008               | 0.03 - 0.06              | -                              |
| 36  | Decane, 1-iodo                                            | 20.256               | - 0.71 - 0.22            | -                              |
| 37  | Tetradecanoic acid                                        | 20.564               | 0.08 - 0.19              | -                              |
| 38  | Heptadecane, 2-methyl                                     | 20.682               | - 0.46 - 0.14            | -                              |
| 39  | Heptadecane, 3-methyl                                     | 20.822               | - 0.25 - 0.08            | -                              |
| 40  | Sulfurous acid, hexyl octyl ester                         | 20.945               | - 0.15 - 0.05            | -                              |
| 41  | 2,4-Diphenyl-4-methyl-1-pentene                           | 21.125               | 0.15 - 0.34              | -                              |
| 42  | Pentadecanal                                              | 21.634               | 0.02 - 0.05              | -                              |
| 43  | Hexadecane, 1-iodo                                        | 22.014               | - 0.43 - 0.13            | -                              |
| 44  | 2-Pentadecanone, 6,10,14-trimethyl                        | 22.062               | 0.47 - 1.06              | -                              |
| 45  | 1,2-Benzene dicarboxylic acid, bis(2-methylpropyl) ester  | 22.401               | - 1.86 - 0.57            | -                              |
| 46  | 3,7,11,15-Tetramethyl-2-hexadecen-1-ol                    | 22.45                | 0.14 - 0.31              | -                              |
| 47  | Cyclopentane, decyl                                       | 22.52                | 0.03 - 0.08              | -                              |
| 48  | Heptadecane, 8-methyl                                     | 22.94                | - 1.11 - 0.34            | -                              |
| 49  | Pentadecanoic acid, ethyl ester                           | 23.068               | 0.04 - 0.08              | -                              |
|     | 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione | 23.333               | 0.04 3.34 0.09 1.03     | -                              |
| 50  | Hexadecanoic acid, methyl ester                           | 23.769               | 0.25 0.66 0.56 0.20      | -                              |
|     | Tetratetracontane                                         | 24.015               | - 0.16 - 0.05            | -                              |
|     | Cyclopentadecanone, 2-hydroxy 1-Hexadecen-3-ol, 3,5,11,15- | 24.053               | 0.10 - 0.21              | -                              |
|     | tetracemethyl                                             | 24.305               | 0.05 - 0.11              | -                              |
|     | Dibutyl phthalate                                         | 24.457               | 1.75 - 3.92              | -                              |
|     | 1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester   | 24.477               | - 1.41 - 0.44            | -                              |
| 57  | 1-(+)-Ascorbic acid 2,6-dihexadecanoate                   | 24.61                | 12.13 2.34 27.15 0.72    | -                              |
| 58  | 2-Cyclohexylnonadecane                                    | 24.965               | - 0.13 - 0.04            | -                              |
| 59  | Hexadecanoic acid, ethyl ester                            | 25.478               | 0.67 - 1.50              | -                              |
| 60  | 5,5-Diethylheptadecane                                    | 25.749               | 0.03 - 0.08              | -                              |
| 61  | 4-Oxazolcarboxylic acid, 4,5-dihydro-2-phenyl-, 1-methylethyl ester | 26.933               | 0.29 - 0.65              | -                              |
| 62  | Heptadecanoic acid                                        | 27.562               | 0.16 - 0.37              | -                              |
| No. | Retention Time | Compound Description                                           | DL (ppm) | DL (ppm) | DL (ppm) | DL (ppm) |
|-----|----------------|-------------------------------------------------------------|----------|----------|----------|----------|
| 63  | 28.637         | 9,12-Octadecadienoic acid (Z,Z)-methyl ester               | 0.10     | -        | 0.22     | -        |
| 64  | 28.848         | 9,12,15-Octadecatrienoic acid, methyl ester                | 0.14     | -        | 0.31     | -        |
| 65  | 29.59          | Phytol                                                     | 4.08     | -        | 9.14     | -        |
| 66  | 30.553         | 9,12-Octadecadienoic acid                                  | 12.51    | -        | 28.02    | -        |
| 67  | 30.855         | Oleic Acid                                                 | 14.21    | -        | 31.83    | -        |
| 68  | 31.478         | 9,12-Octadecadien-1-ol                                     | 0.30     | -        | 0.68     | -        |
| 69  | 31.813         | Octadecanoic acid                                          | 4.96     | -        | 11.10    | -        |
| 70  | 32.356         | Hexadecanide                                               | 0.05     | -        | 0.11     | -        |
| 71  | 33.141         | Octadecanoic acid, ethyl ester                             | 0.11     | -        | 0.24     | -        |
| 72  | 35.699         | Octanamide, N,N-dimethyl                                   | -        | 0.53     | -        | 0.16     |
| 73  | 37.004         | Hexadecanoic acid, tert-butylidimethylsilyl ester          | 0.27     | -        | 0.61     | -        |
| 74  | 39.274         | Cyclooctane, tetradeyl                                     | 0.25     | -        | 0.55     | -        |
| 75  | 39.924         | 4,8,12,16-Tetramethylheptadecan-4-oxide                    | 0.28     | -        | 0.63     | -        |
| 76  | 40.412         | 9-Octadecenamide                                           | 0.14     | 2.03     | 0.31     | 0.62     |
| 77  | 41.006         | Eicosenoic acid                                            | 1.29     | -        | 2.89     | -        |
| 78  | 41.886         | Phytol, acetate                                            | 0.28     | -        | 0.63     | -        |
| 79  | 42.417         | Phenol, 2,2'-methylenetris[6-(1,1-dimethylethyl)-4-methyl]  | -        | 1.38     | -        | 0.43     |
| 80  | 42.694         | Tetracosane                                                | -        | 0.20     | -        | 0.06     |
| 81  | 43.012         | Octacosyl acetate                                          | -        | 0.24     | -        | 0.08     |
| 82  | 44.419         | 9,12-Octadecadienoic acid, tert-butylidimethylsilyl ester  | 0.22     | -        | 0.50     | -        |
| 83  | 47.351         | Decanoic acid, 10-(2-hexylcyclopropyl)                     | 0.23     | -        | 0.53     | -        |
| 84  | 47.776         | Hexatriacontane                                            | 1.01     | -        | 2.26     | -        |
| 85  | 47.836         | Diisooctyl phthalate                                       | -        | 0.45     | -        | 0.14     |
| 86  | 49.553         | Docosanoic acid                                            | 0.59     | -        | 1.31     | -        |
| 87  | 50.071         | Tetrapentacontane                                          | -        | 0.13     | -        | 0.04     |
| 88  | 52.134         | Octadecanal                                                | 0.42     | -        | 0.94     | -        |
| 89  | 53.676         | 6a,12a-Dihydro-6H-(1,3)dioxol[5,6]benzofuro[3,2-c]chromen-3-ol | 1.34     | -        | 2.99     | -        |
| 90  | 55.086         | 7-Methoxy-3-(p-methoxyphenyl)-4H-chromen-4-one             | 1.12     | -        | 2.50     | -        |
| 91  | 56.012         | Hexadecanol                                                | 0.36     | -        | 0.80     | -        |
| 92  | 57.262         | 13-Docosanamide                                            | 4.59     | 28.52    | 10.29    | 8.79     |
| 93  | 60.083         | Oxirane, hexadeacyl                                        | 4.35     | -        | 9.73     | -        |
| 94  | 63.235         | Tetracontane                                               | 19.70    | 0.38     | 44.13    | 0.12     |
| 95  | 63.769         | 1-Heptacosanol                                             | 2.35     | -        | 5.26     | -        |
| 96  | 65.087         | Oxirane, heptadecyl                                        | 1.39     | -        | 3.11     | -        |
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