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Quality assessment of menthofuran rich essential oil of *Mentha piperita* (CIMAP-PATRA) stored at different temperatures and containers

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Abstract
The storage of the menthofuran rich essential oil of *Mentha piperita* (cv. CIMAP-Patra released by CSIR-CIMAP) is the major problem for its market sustainability. In the present study, the best storage conditions were optimized without degradation in menthofuran content and quality of essential oil. The quality of the essential oil was evaluated in two storage conditions (temperature: 25±2 °C and 4±0.5 °C), three types of storage containers (transparent glass, amber color glass, and aluminium bottles), and six storage periods (3, 6, 9, 12, 15, and 18 months). The results indicated that maximum degradation (52.45%) was reported in the oil sample which was stored in transparent vial at 25 °C. At 4 °C, degradation in menthofuran content was not pronounced irrespective of different containers, however, lowest degradation was reported in amber color bottle. Apart from the menthofuran, pulegone and isomenthone, minor fluctuations in other chemical constituents were recorded, but not in the proper pattern. The refractive index of the essential was not altered with storage condition and time, while optical density was decreased with the storage time. Results suggest that the storage of the menthofuran rich essential oil of *Mentha piperita* in amber color glass container at 4 °C is the best way to reduce the oxidation and degradation/conversion of menthofuran for a longer period of time.

Keywords: *Mentha piperita* (CIMAP-PATRA), essential oil, menthofuran, refractive index, optical rotation

1. Introduction
Essential oils produced from the aromatic plants consist pharmacological activities [1], which allow them to be considered as medicinal plants[2]. The essential oils of these aromatic plants have a wide application in the perfumery, food industry, meat processing, canning, liquors elaboration and confectionery [3]. The storage of these essential oils is the major concern for marketing. These oils hold high added value and should be handled carefully. There is a need to evaluate how the constituents and sensory quality attributes of essential oils vary during storage [4].
The interest on essential oils increases strongly worldwide, including those of *Mentha* genus. *Mentha*, an herb of the Lamiaceae family, has been long used in analgesic, astringent, carminative, expectorant and inflammatory disease-related pharmaceutical preparations [5]. The essential oil of each species has characteristic chemical signature e. g., menthol (75-80%) in *Mentha. arvensis*, carvone (60%) in *Mentha spicata* and linalool and linalyl acetate in *Mentha citrata*. In contrast, *Mentha piperita* known for its variable chemical constituents possess low and high menthofuran content in different varieties. Menthofuran emerged as a salient and valuable constituent in the essential oil sector, used for flavoring foods and drinks and herbal medicinal products [6]. The annual consumption of menthofuran is estimated to be about 150-200 MT[7]. The concentration of this vital chemical constituent in most of the Indian peppermint varieties CSIR- central Institute of Medicinal and Aromatic Plants, such as Kukrail, Pranjal, Tushar, and Madhuras, vary from 3-5%.
Pulegone and menthofuran are remarkable constituents of several mints (*Mentha*) species and, their 90 derived volatile oils, including peppermint (*Mentha piperita*), spearmint (*Mentha spicata*), European 91 pennyroyal (*Mentha pulegium* L.) and American pennyroyal (*Hedeo mapulegioides* L.). Pulegone is the 92 essential components of the volatile oils of European and American Pennyroyal where it comprises 85–97%, 93 (w/v) and about 30% (w/v) of the respective oil [8]. They have insecticidal and pesticidal activities. Hence, they are commercially

important. CSIR-CIMAP developed a new variety which is rich in menthofuran content. Though the condition for storage of synthetic Menthofuran has been explained by chemical industries (Sigma Aldrich), the storage of the menthofuran rich essential oil which is of plant origin i. e. natural product is a big problem due to the highly reactive nature of menthofuran under general conditions. Preservation of production is a very important problem to be solved by producers of these products for which sustainable technology is required for its long-term storage. Keeping in view above problems and prospects, experiments were conducted to optimize the temperature and container for storage of *Mentha piperita* (CIM-PATRA) essential oil for 18 months of storage.

2. Materials and Methods

2.1. Collection of plant material and oil extraction

The extraction of essential oil of the *Mentha piperita* (90 days old crop of CIMAP- Patra grown at experimental farm of CSIR-CIMAP) was performed through the hydro- distillation method. One liter of freshly distilled oil was dehydrated by anhydrous Na_2SO_4. Immediate after distillation, quality analysis of essential oil was analyzed by gas chromatography and remaining oil was transferred into three different types of vials i.e. (i) Transparent glass, (ii) amber color glass and (iii) aluminium, each of 50 ml capacity. Two vials of each were kept under (i) 25±2 ˚C and two under (ii) 4±0.5 ˚C. One bottle of each of the storage conditions used for chemical analysis and another one for recording physic-chemical properties. The total 6 were named as T1: Transparent glass bottle at 25±2 ˚C, T2: Amber color glass bottle at 25±2 ˚C, T3: Aluminium bottle at 25±2 ˚C, T4: Transparent glass bottle at 4±0.5 ˚C, T5: Amber color glass bottle at 4±0.5 ˚C, T6: Aluminium bottle at 4±0.5 ˚C. Changes in physico-chemical properties were recorded at three, six, nine, twelve, fifteen and eighteen months i.e. at three months intervals.

2.2. Quality analysis of essential oil

The constituents of fresh oil and oil from different treatments were analyzed using gas chromatography (Figure 1). The study of the oil from various treatments was also performed at 3 months interval and up to 18 months. The quality parameters were assessed through a gas chromatograph (Agilent-4890, fitted with Elite wax column (30 m×0. 25 mm×0. 25 m). The oven temperature was programmed from 40 ˚C (40-120 at 3˚C/) min with 9 minutes hold, 120-140˚C for 2 ˚C/min with 2- minute hold and 140-250 ˚C at 5˚C/min with 2 minutes final hold time). H_2 was used as the carrier gas at 5 psi constant column head pressure. Split ratio was 1:100, injection size 0.02 µl neat; injector and detector temperatures were maintained at 250 ˚C. Characterization of constituents was done on the basis of retention indices using index calculating software, co-injection with standards (Sigma), MS Library spectra (NIST/NIH Version 2.1 & Wiley-Registry of mass spectral data- 7th edition) and by comparing with mass spectral literature data.

2.3. Stability studies

Refractive index and optical rotation of fresh oil and oil from different treatments were evaluated with the help of the color chart, ATAGO Rx 7000 Refractometer and Horiba Sepa 300 high sensitive Polarimeter, respectively. To minimize the risk of oxidation during the sampling, inert gas (nitrogen) was blown every time and cap should be fitted.

2.4. Statistical analysis

The derived data of industrially influential chemical constituent Menthofuran were analyzed statistically by using the analysis of variance (ANOVA) technique for the completely randomized design of lab experimentation[9]. Differences among the treatments were considered significant at $P<0.05$. Hierarchical Cluster analysis (HCA) was applied to the treatment and also on the constituents to assess the dissimilarity between them.

3. Results

The picture of the essential oil stored in the different vials after 18 months of storage has been shown in Figure 2. Essential oil of *Mentha piperita* turns brown and loses its quality within few months in the presence of light. One of the considerable specifications in determination of consumer acceptance of a number of essential oil is color. It is visible from the picture that the color of the oil changed from pale yellow to brown at 25 °C. In transparent vial, darkness in the oil was more pronounced as compared to other two. At temperature 4 °C, slight variation in essential oil color was observed.

3.1. Chemical analysis

The gas chromatograph of the essential oil constituents is shown in the Figure 1. It is evident from the figure 1 that oil contained highest concentration of menthofuran (48.75%) followed by pulegone (30.12%), menthol (6.39%) and menthone (3.01%). The small amount of limonene, α-pinene, β-pinene1.8 cineole, β-myrcene, sabinene and isomenthone were also present in the oil. The menthofuran and pulegone contents were quite higher in of *Mentha piperita* (CIMAP-Patra) than reported in other varieties of the mints[10]. This is because the variety CIMAP- Patra is developed for the commercial extraction of the menthofuran from *Mentha piperita*.

The chemical composition of menthofuran and pulegone was significantly reduced during storage at two different temperatures (Table 1). The difference is more pronounced at higher temperature irrespective of vial types (Figure 2). Though, the essential oil was analyzed at two different storage temperatures (25±2 ˚C & 4±0.5 ˚C) which was stored at three different types of containers i.e. transparent glass vials, amber color glass vials and aluminium vials and chemical analysis was carried out at 3 months interval and last up to 18 months, only the variations in menthofuran content have been discussed in detail because this variety of *Mentha piperita* is commercially utilize for menthofuran extraction.

3.2. Effect of storage temperature, type of containers and storage period on the essential oil constituents

The Table 1 showed the percentage of essential oil constituents at day one (fresh oil) and after 18 months. It suggests that all the constituents of the oil of *Mentha piperita* were significantly reduced during storage. However, reduction in their concentration was less pronounced irrespective of different treatments. The finding of present investigation showed that in T1 (transparent glass bottle stored at 25 °C), pulegone concentration increased from 30.12% to 38.95%. A similar trend was observed for other constituent such as menthol (6.39% to 8.36%), menthone (3.01 to 3.57%) and α- pinene (from 0.84 to 0.88%) at the completion of the experiment (18 months) whereas, other compounds decreased in different proportions along with menthofuran (from 48.75% to 23.2%) which is the main compound of interest in the present experiment. In T2 (Amber color glass at 25 °C), only pulegone showed a slight increase during the experimentation period (30.0% to 30.85%). All other compounds exhibited a decomposing trend during and at the end of the experiment. In the T3 (Aluminium vial at 25 °C), the trend of pulegone evolution during 18 months of storage was from 28.63% to 31.54%. Other compounds which showed a similar trend in α- pinene (0.79% to 0.87%), β- pinene (0.92% - 1.01%), limonene (2.08% to 2.28%), 1.8 cineole (0.73% to 0.75%) and menthol (6.32% to 6.87%).

Except these, all other compounds exhibited lower content in different proportions. In T4 (Transparent glass vial at 4 °C), only pulegone (29.87% to 30.85%) and menthol (6.60% to 6.65%) showed an enhancement whereas all other 9 compounds showed degradation during the experimental period. In T5 (Amber color glass vial at 4 °C), pulegone increased from 29.64 to 30.37% and other chemical constituents which showed the same trend, were β- pinene (0.97% to 0.98) limonene (2.18% to 2.21%), 1.8 cineole (0.73% to 0.86%) and menthone (3.00% to 3.08%) all other chemical constituents showed decreasing trend. In T6 (Aluminium bottle at 4 °C), most of the compounds like α - pinene (0.72% to 0.85%), β- pinene (0.87% to 0.98%), sabinene (0.41% to 0.46%), β- myrcene (0.500% to 0.51%), limonene (2.05% to 2.20%), 1.8 cineole (0.70% to 0.74%), Menthone (2.06% to 3.12%) pulegone (29.70% to 31.86%) and Menthol (6.68% to 6.80%) showed an increasing trend except Menthofuran and isomenthone. It clearly indicates that the quantity of Pulegone always showed an increasing trend, whereas, menthofuran and isomenthone showed decreasing trend in all the treatments irrespective of temperature, container type and storage period.

3.3. Effect of storage temperature, type of containers and storage period on the menthofuran content

Figure 3 showed the variations in the mentofuran content in different treatments. It was about 49.0% in fresh oil and reduced gradually under ambient temperature stored in transparent glass vials (T1) and there was a decrease near exponential of 2.66%, 9.83%, 19.87%, 30.12%, 37,5%, and 52.45% after three, six, nine, twelve, fifteen, and eighteen months, respectively. The decrease in menthofuran content was found minimum (30.3%) in amber color glass bottle which was closely followed by aluminium bottle (30.0%) in ambient temperature. The decrease in menthofuran content in the amber color bottle was 1.24%, 1.65%, 7.02%, 18.59%, 27.06% and 37.39% after three, six, nine, twelve, fifteen, and eighteen months respectively whereas in aluminium bottle it was 2.69%, 4.34%, 6.21%, 19.25%, 28.15% and 37.88% after three, six, nine, twelve, fifteen, and eighteen mon`ths respectively. The data also revealed that statistically significant decrease or considerable changes in menthofuran content started after 6^{th} month in transparent vial whereas in amber color bottle and in aluminium bottle significant decrease was reported after 9 months of storage. There was no significant difference in the menthofuran content in amber color bottle and aluminium bottle, when oil was stored in the refrigerator however in transparent bottle significant decrease in menthofuran was reported after 15 months of storage. The difference was about 3.06%, 3.88%, 4.29%, 5.52%, 8.17%, and 12.47% in transparent glass bottle in amber color bottle it was 0%, 0.61%, 1.22%, 1.22%, 1.63%, and 3.27%, whereas in aluminium bottle it was 0.61%, 1.64%, 1.64%, 4.32%, 7.40%, and 8.43% after three, six, nine, twelve, fifteen, and eighteen months of storage respectively.

3.4. Physico-chemical properties of Essential oil as affected by different treatment

The variation in the refractive index (n) of the essential oil in different treatments is given in the Figure 4. Insignificant change in the refractive index of the essential oil was observed with the time (0.00140 to 0.00202) however, it was observed that refractive index was increased from 1^{st} day of observation till 18^{th} month of storage but increment was negligible. Opposite scenario was observed for the optical rotation $[\alpha]^{25}_{D}$ of essential oil. It decreased considerably with an enhancing period of storage and reached the lowest in transparent glass bottle stored at 25 °C followed by aluminium bottles and amber color glass bottles irrespective of storage temperatures (Figure 5).

3.5. Hierarchical Cluster analysis (HCA)

The data set obtained from oil constituents and quality was employed to perform cluster analysis, applying the average paired distance algorithm. Results were reported in the form of the dendrogram, shown in Figure 6. On the basis of the connecting distances two distinctive clusters were defined. The oil quality data after eighteen months was taken in triplicates. C is defined as the control. The groups of T1, T2 and T3 were taken for transparent, amber and aluminium container at 25 °C storage conditions while T4, T5 and T6 were taken for transparent, amber and aluminium container at 4 °C storage conditions. HCA proved highly selective in grouping the oil samples according to their quality. Control is clustered in one group and have close proximity with T4, T5, and T6. This suggests that oil stored at 4 °C posses same quality irrespective of different containers.

The oil constituents of all treatments were employed to perform cluster analysis, applying the average paired distance algorithm. Results were reported in the form of the dendrogram shown in Figure 7. On the basis of the connecting distances two distinctive clusters were defined.

4. Discussion

Overall, the vials kept at ambient temperature reported degradation in menthofuran content after six months of storage, whereas, degradation was very minor in all vials which were kept in refrigeration. It is attributed to photo degradation of the oil. Light plays an important role in essential oil quality degradation and temperature plays a more crucial role in quality of essential oil [11]. Essential oil stability is crucially influenced by ambient temperature in several respects [12]. Also increase in the temperature accelerates chemical reactions and volatility in the oil constituent. It is also corroborated with the susceptibility of essential oils to auto oxidation at different storage temperatures [13, 14]. Dominant alteration in essential oil from cardamom, clove bud, lavender, pine and rosemary were revealed in decreasing amount of terpenic hydrocarbons such as β-caryophylline, β-myrcene, β-pinene, sabinene or γ-terpinene and an overall rise of p-cymene upon temperature increases [15, 16].

Menthofuran being a monoterpene, exhibited considerable decomposition upon exposure to temperature. Similar observations on terpenic conversion reactions upon heating have been reported both for isolated compounds[11,14,17] as well as for essential oils [18]. Moreover, essential oil components are easily transformed by oxidation, cyclization or dehydration reactions owing to their structural relationship within the same chemical group [14]. Apart from menthofuran, pulegone and isomenthone, all chemical constituents showed minor or major fluctuations in all the said treatments.

Temperature plays a crucial role during storage of chemicals. It was reported that if *Ocimum basilicum* L. was dried at 45 °C, there was a significant increase in constituents like trans-berbamotene, linalool and 1,8- cineole etc., whereas the amount of methyl chavicol and eugenol were decreased at this temperature [19]. Leaves of *Cymbopogon winterianus* Jowitt. were dried at different temperatures and best results were obtained at 60 °C without affecting the chromatographic profile of essential oil [20]. Same results were also reported by some other workers [21-23] on *Ocimum basilicum* L., *Filipendula ulmaria*, and *Juniperus Phoenicia* L. respectively. These results indicated that essential oil composition is affected by the drying and storage temperature. Packaging plays a vital role in quality control during storage [24]. Dry okra kept in polythene package provide the best storage condition, which indicated that packaging material and storage times significantly affected the quality parameters of stored products [25]. Precaution should be taken concerning the effect of atmosphere, moisture heat and light may be taken into consideration so that the active constituents of stored material remain stable for a more extended period. It has been proved by the experiments that the chemicals which are contained in the plants are altered i.e. they can be enhanced or decreased due to storage periods. However, it has been observed that storage can increase or decrease the activity of some compounds in the stored products.

Refractive index was not significantly altered during storage which suggests that the container type and temperature have no effect on the refractive index of the essential oil. It indicates that the components were neither degraded nor polymerized and remained as monoterpenoid or sesquiterpenoid and their derivatives. These results are similar to the other reports on changes in refractive index of the essential oils [26, 27]. However, it might be possible that the inter-conversion of the chemical constituent of the oil may alter the optical density of the oil. For example, methofuran decrease with the storage time while pulegone content was increased considerably (30.11% to 38.95%) with the increase in the storage period. Similar trend leading to an increase in menthol content was noticed (6.39% to 8.36%). The optical activities of pulegone and menthol are ±23.5 and ±50.1 $[\alpha]^{25}_{D}$ where, as for menthofuran is ±100$[\alpha]^{25}_{D}$.

The first group contained pulegone, menthol and menthofuran and α pinene suggesting that these constituents may be inter-converted during the storage period. It is reported that menthofuran has been identified as common precursor for the secondary transformation of menthane lactones in Mentha species[28] and lead to clustering in the one group. The rest other constituents are clustered in the second cluster indicating that they may either inter converted during the storage or follow the same degradation pattern in treatments.

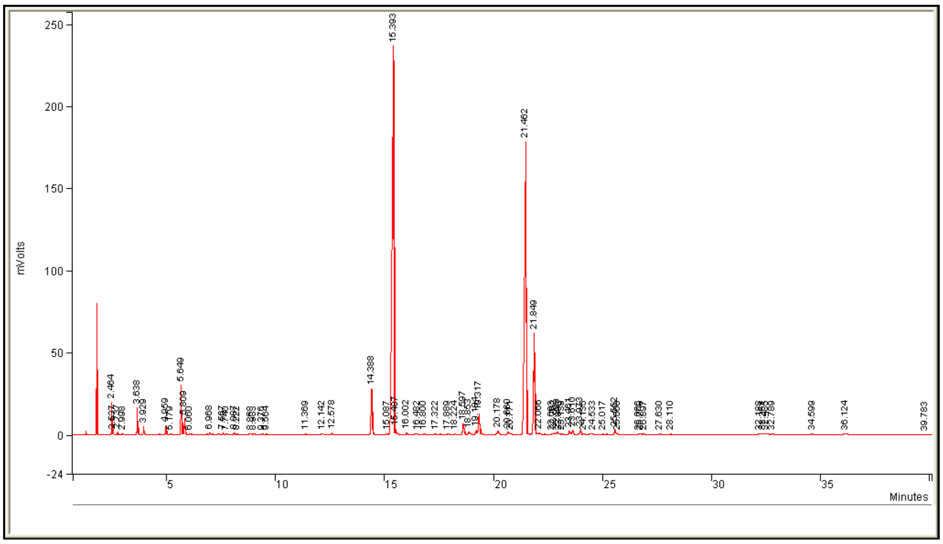

Fig 1: Chromatogram of the essential oil after distillation

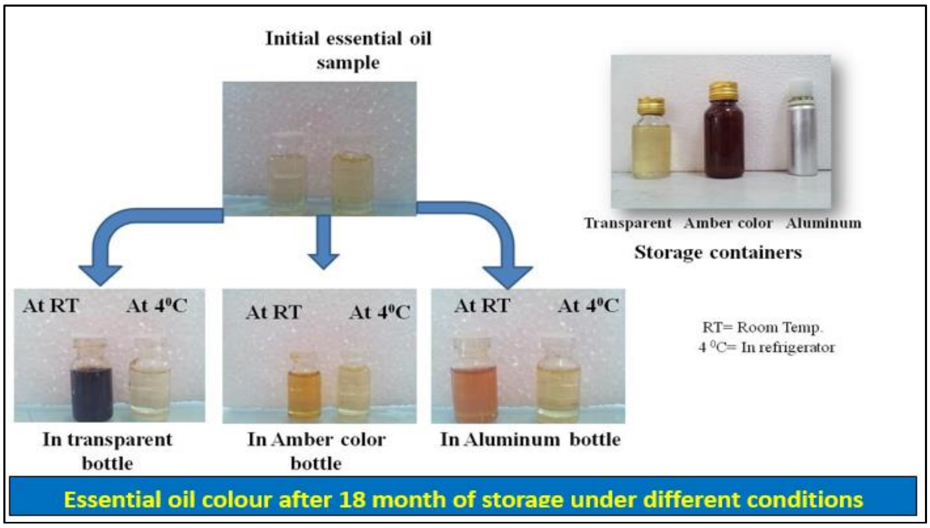

Fig 2: Effect of the storage temperature, container, and storage time on the essential oil colour

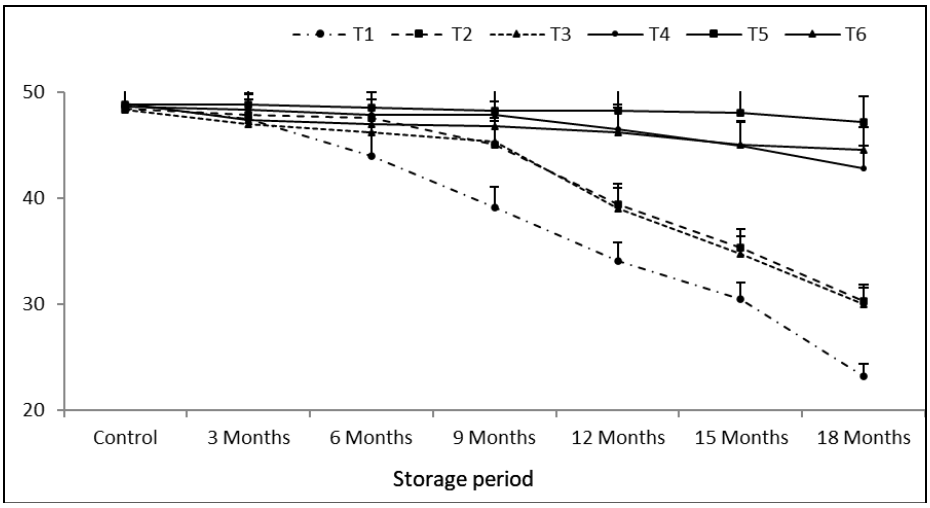

Fig 3: Variations in Menthofuran concentration in different treatments Where T1= Room temperature + Transparent bottle, T2 = Room temperature + Amber colour bottle, T3= Room temperature + Aluminium bottle, T4 = 4 °C +Transparent bottle, T5 = 4 °C + Amber colour bottle, T6 = 4 °C + Aluminium bottle

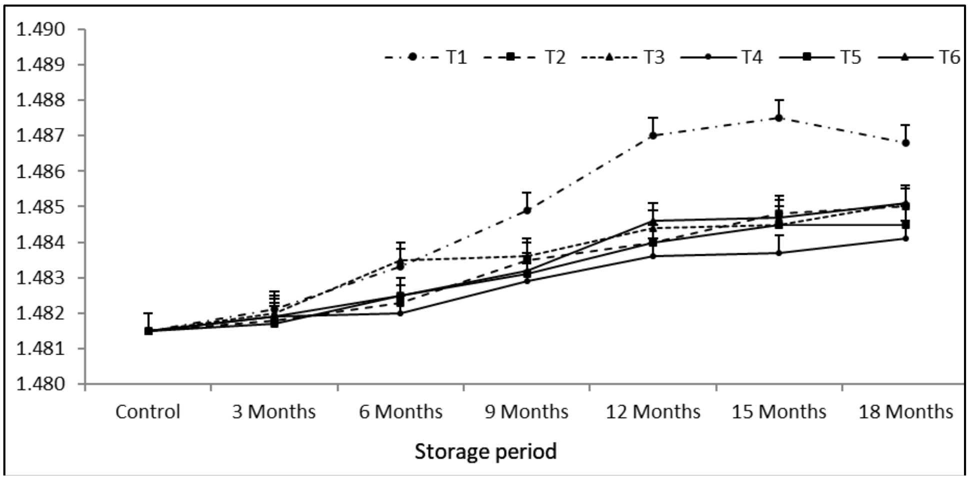

Fig 4: Variations in refractive index of essential oil in different treatments Where T1= Room temperature + Transparent bottle, T2 = Room temperature + Amber colour bottle, T3= Room temperature + Aluminium bottle, T4 = 4 °C +Transparent bottle, T5 = 4 °C + Amber colour bottle, T6 = 4 °C + Aluminium bottle

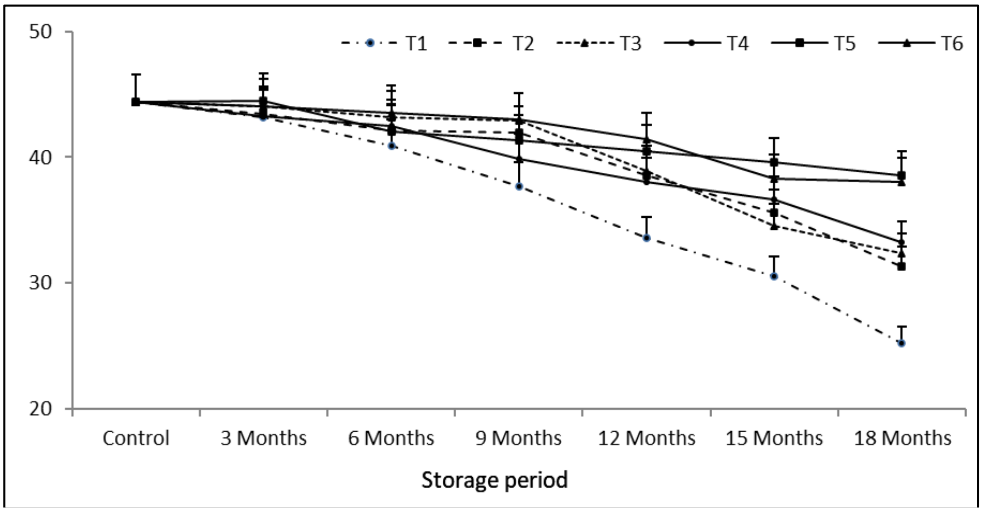

Fig 5: Variations in optical rotation of essential oil in different treatments Where T1= Room temperature + Transparent bottle, T2 = Room temperature + Amber colour bottle, T3= Room temperature + Aluminium bottle, T4 = 4 °C +Transparent bottle, T5 = 4 °C + Amber colour bottle, T6 = 4 °C + Aluminium bottle

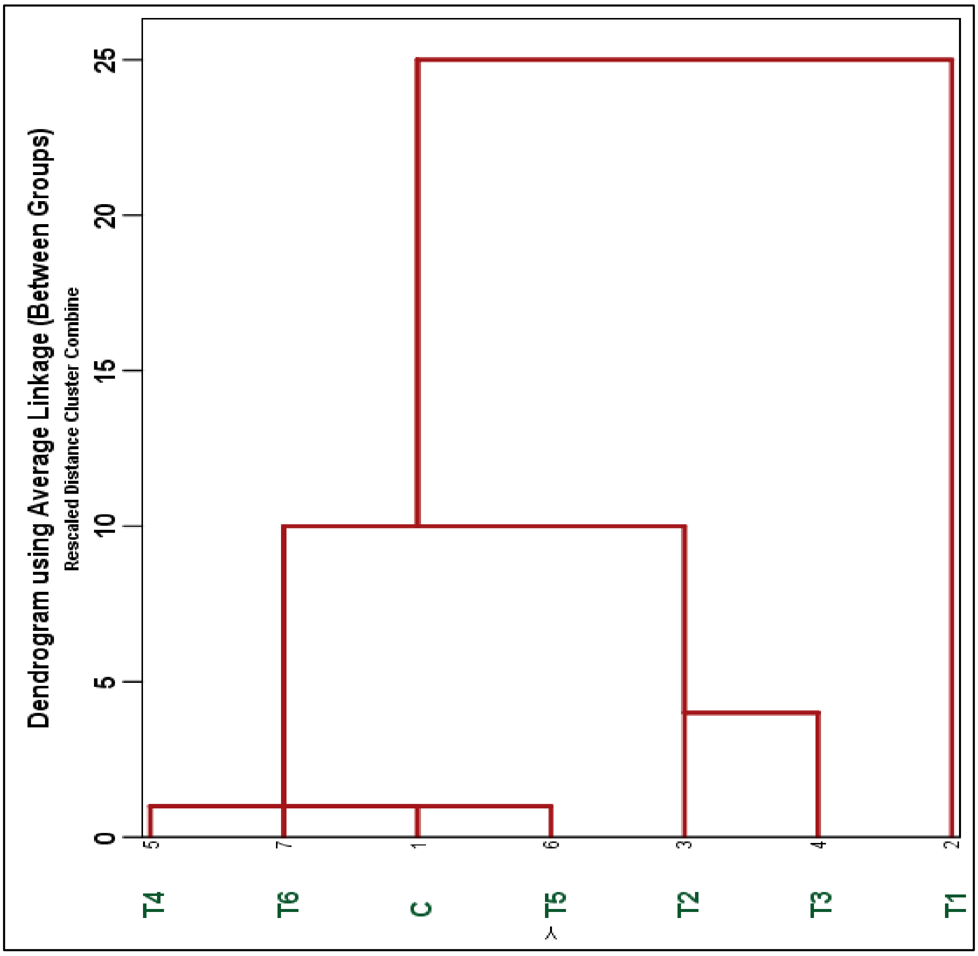

Fig 6: Hierarchical cluster analyses of different treatments

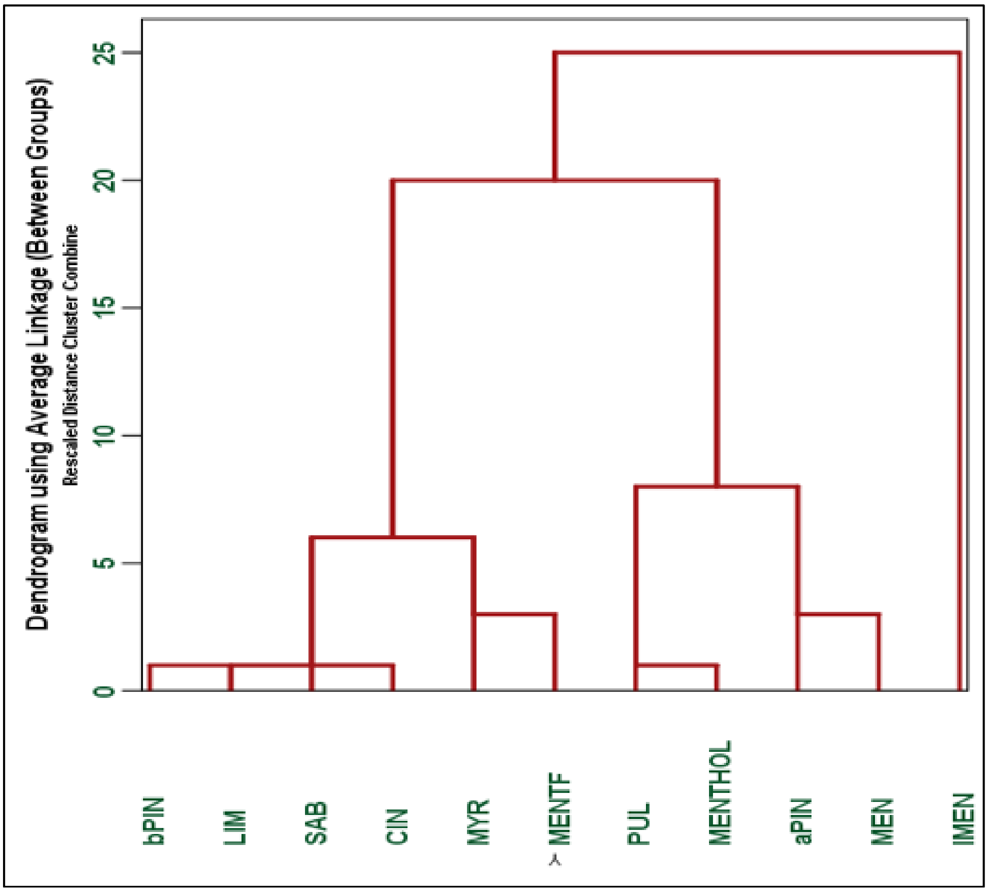

Fig 7: Hierarchical cluster analyses of oil constituents

Table 1: The constituents of the essential oils stored in different vials and at different temperatures

Treatment	Storage period	α pinene	β pinene	sabinene	β myrcene	Limonene	1,8 cineole	Menthone	Menthofuran	Isomenthone	Pulegone	Menthol
C	1 day	0.84±0.03a	0.97±0.04ab	0.42±0.01ab	0.59±0.02a	2.19±0.09a	0.74±0.03b	3.01±0.13b	48.75±2.18a	0.23±0.01a	30.12±1.34b	6.39±0.28b
T1	18 months	0.88±0.03a	0.57±0.02c	0.27±0.01c	0.25±0.01d	1.53±0.06b	0.55±0.02c	3.57±0.15a	23.20±1.03d	0.23±0.01a	38.95±1.74a	8.36±0.37a
T2	18 months	0.39±0.01b	0.49±0.02c	0.21±0.00d	0.46±0.02c	1.22±0.05c	0.45±0.02d	2.53±0.11c	30.30±1.35c	0.0±0.00c	30.84±1.37b	6.32±0.28b
T3	18 months	0.87±0.03a	1.01±0.04a	0.40±0.01b	0.49±0.02bc	2.28±0.10a	0.75±0.03b	3.17±0.14ab	30.00±1.34c	0.19±0.01b	31.54±1.41b	6.86±0.30b
T4	18 months	0.77±0.03a	0.86±0.03b	0.39±0.01b	0.55±0.02ab	2.06±0.09a	0.71±0.03b	2.97±0.13b	42.80±1.91b	0.17±0.01b	30.85±1.37b	6.65±0.29b
T5	18 months	0.82±0.03a	0.98±0.04a	0.42±0.01ab	0.58±0.02a	2.21±0.09a	0.86±0.03a	3.07±0.13b	47.20±2.11ab	0.22±0.01a	30.37±1.35b	6.33±0.28b
T6	18 months	0.85±0.03a	0.97±0.04ab	0.46±0.02a	0.51±0.02bc	2.20±0.09a	0.74±0.03b	3.12±0.13b	44.50±1.99ab	0.18±0.01b	31.86±1.42b	6.80±0.30b

C= control (content after distillation), T1= Transparent glass stored at 25 °C, T2= Amber glass stored at 25 °C, T3= Aluminium bottle stored at 25 °C , T4= Transparent glass stored at 4 °C, T5= Amber glass stored at 4 °C, T6= Aluminium bottle stored at 4 °C

Table 2: Menthofuran content in different storage temperature, containers and period compares with control (content after distillation) of CIMAP- Patra

Storage condition	Storage at 25 °C			Storage at 4 °C		
Storage Period	**Transparent glass bottle (T1)**	**Amber colour glass bottle (T2)**	**Aluminium Bottle (T3)**	**Transparent glass bottle (T4)**	**Amber colour glass bottle (T5)**	**Aluminium Bottle (T6)**
Control	48.8±2.44a	48.4±2.42a	48.3±2.41a	48.6±2.43a	48.8±2.44a	48.9±2.44a
3 Months	47.5±2.37a	47.8±2.39a	47.0±2.35a	48.3±2.41a	48.8±2.44a	47.4±2.37a
6 Months	44.0±2.20ab	47.6±2.38a	46.2±2.31a	47.8±2.39a	48.5±2.42a	47.0±2.35a
9 Months	39.1±1.95b	45.0±2.25ab	45.3±2.26a	47.8±2.39a	48.2±2.41a	46.8±2.34a
12 Months	34.1±21.70bc	39.4±1.97bc	39.0±1.95b	46.5±2.32a	48.2±2.41a	46.2±2.31a
15 Months	30.5±1.52c	35.3±1.76cd	34.7±1.73bc	44.9±2.24a	48.0±2.40a	45.0±2.25a
18 Months	23.2±1.16d	30.3±1.51d	30.0±1.50c	42.8±2.14a	47.2±2.36a	44.5±2.22a

5. Conclusion

The present study concludes that, temperature plays crucial role in maintaining quality of menthofuran rich essential oil of CIMAP-Patra and the same can be maintained up to eighteen months or more if kept in air tight amber color bottles under refrigeration (±4 °C). The quality can be maintained only up to six months if kept at room temperature. Enhancement in the pulegone content was noticed with enhancing period of storage. Minor fluctuations in all other chemical constituents were also noticed under different storage treatments; however, there was no definite trend. The changes occurred in refractive index and optical rotation also supports the results of quality changes. Therefore, it is always better to use fresh oil of *Mentha piperita* (CIMAP-Patra) or as soon as possible after distillation. However, findings of present investigation suggest that the oil of this variety can be stored safely beyond 18 months under refrigerator temperature (±4 °C). The safe period of storage under refrigeration may be 15 months if stored in transparent bottle and 18 months if stored in amber color glass bottles or aluminium bottles. Further, work on safe storage period by using other types of containers like plastic bottles should also be under taken to reduce cost of storage.

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