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A comparative study of five *Mentha* species based on their protein and amino acid profiles

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Abstract:
The study aims to revise the classification of mint species belonging to the Lamiaceae family using chemotaxonomic properties. The dried leaf powder was used in chemical analyses. Traditional analytical methods (Biuret, Ninhydrin, Anthrone) were used in this chemical taxonomic study. Protein levels have been estimated using the biuret method. The studied plant species were divided into four groups. The first group included *M. longifolia* (with the highest protein level of 1.1513 mg\g, followed by *M. piperita* (0.8363 mg\g). The third group included *M.spicata* (0.52267 mg\g) while the fourth group included M. *aquatica* and *M. pulegium* (0.30333 - 0.2983 mg\g) protein, respectively. On the other hand, the amino acids determined by the ninhydrin method showed the presence of 2 groups of plant *M. aquatica* and *M. piperita* (2.6477 - 2.9243 mg/g, respectively) in whereas another group was occupied by *M. pulegium, M. longifolia,* and *M. spicata* plants (2.1773 - 2.1050 - 2.264 mg\g respectively). For carbohydrates, the results showed the presence of three groups. The first group is *M. piperita* (1.411 mg\g) and the second group, *M. spicata* and *M. longifolia* plant (0.90700 - 0.94167 mg\g respectively), the third group is *M.aquatica* and *M. pulegium* (0.76767 - 0.75367 mg\g).

Keywords: Chemotaxonomy, Flora of Libya, Lamiaceae, *Mentha*, Taxonomy.

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مقارنة خمس انواع من النعناع بناء على محتواها من البروتين والاحماض الامينية

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الملخص

هدفت هذه الدراسة إلى مراجعة تصنيف أنواع النعناع التابعة لهذه الفصيلة باستخدام الخصائص الكيميائية التصنيفية، وقد استُخدم مسحوق الأوراق المجففة في التحليلات الكيميائية. استُخدمت في هذه الدراسة التصنيفية الكيميائية طرق التحليل التقليدية (البيوريت، النهيدرين، الانثرون). وقد قُدِّرت مستويات البروتين باستخدام طريقة البيوريت. وقُسِّمت الأنواع النباتية المدروسة إلى أربع مجموعات. المجموعة الأولى احتوت على أعلى مستوى بروتين (1.1513 ملغم/جم) تتمثل في (مينثالونجي فوليا) تليها(مينثا ببريتا) (0.8363 ملغم/جم) والمجموعة الثالثة(مينثاسبيكاتا) احتوت على (0.52267 ملغم/جم) بينما احتوت المجموعة الرابعة على (مينثا أكواتيكاومينثابوليقيم) (0.30333 – 0.2983 ملغم/جم) بروتين على التوالي. من ناحية أخرى، أظهرت الأحماض الأمينية المحددة بطريقة ننهيدرين وجود مجموعتين (أكواتيكاوببريتا) من نبات النعناع – (2.6477 2.9243ملغم/جم على التوالي) في حين احتلت مجموعة أخرى النباتات (بوليقيم،لانجي فوليا، وسبيكاتا) (2.1773 – 2.1050 – 2.264 ملغم/جم على التوالي) . بالنسبة للكربوهيدرات، أظهرت النتائج وجود ثلاث مجموعات المجموعة الأولى(مينثاببيريتا) (1.411 ملغم/جم) والمجموعة الثانية (مينثاسبكاتاولانجي فوليا) النبات (0.90700 – 0.94167 ملغم/جم على التوالي) والمجموعة الثالثة (مينثأاكواتيكاوبوليقيم) (0.76767 – 0.75367ملغم/جم) . ونظرًا لأن بعض الأنواع مهددة بالانقراض، مثل النعناع الكواتيكا، المذكور في دليل النباتات الليبي، فإننا نوصي بالحث على اجراء المزيد من الدراسات الكيميائية التصنيفية لأنواع النعناع الموجود في ليبيا.

الكلمات المفتاحية: تصنيف كيميائي، فلورا ليبيا، الشفوية، النعناع، تصنيف

Introduction:

The Lamiaceae family, also referred to as Labiatae, boasts a vast distribution, encompassing approximately 236 genera and between 6900 to 7200 species (Qureshi *et al.*, 2024). Members of the

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Lamiaceae family are utilized for a variety of applications, with their predominant use being in the food industry due to their rich content of aromatic compounds. (Karageçili, and Gülçin, 2025). Sweet basil, spearmint, rosemary, sage, oregano, and garden thyme are merely a few examples of the species that are extensively employed in culinary production globally (Sharma, 2023).

Harle *et al*. (2004), Gamoun and Louhaichi (2024). A significant number of Lamiaceae species thrive in various ecosystems and exhibit considerable diversity with a global distribution. Most of these species are aromatic and contain a complex array of bioactive compounds that enhance their overall biological activity in both in vitro and in vivo settings (Pinto *et al*, 2021). The fragrant plant known as spearmint (*Mentha spicata* L.) can be used fresh or dried, as leaves or powder, as a herb for flavoring and seasoning, or as a traditional herbal tea. (Kanatt, *et al*., 2007). Additionally, because spearmint essential oil is used in pharmaceutical formulations, confections, cosmetics, and perfumes, it has commercial significance. A wide range of food items, including cheese and dough (an Iranian yogurt beverage), chocolate, drinks, jellies, syrups, sweets, ice creams, and chewing gum, have used both fresh and dried spearmint leaves as flavorings. (Okmen, *et al*., 2017). Mentha species are part of the Lamiaceae family and are extensively found across Europe, Asia, Africa, Australia, and North America (Lawrence, 2006; Mamadalieva *et al*., 2017). Plants from this genus can be located in numerous and varied environments (Lawrence, 2006; Brahmi *et al*., 2017). The genus Mentha can be divided into 42 species, 15 hybrids, and hundreds of subspecies, variations, and cultivars, according to recent research based on morphological, cytological, and genetic traits. Mint taxonomy is, in fact, extremely complicated, and consensus is not always reached. According to Tucker (2007), the genus *Mentha* is frequently separated into five sections: *Audibertia*, *Eriodontes*, *Mentha*, *Preslia*, and *Pulegium*. The majority of *Mentha* species are industrial crops that are grown for their essential oils, are perennial, and contain essential oils (Lawrence,2006). The science of chemotaxonomy is used for the classification of plants on the basis of their chemical constituents.

The concept of chemotaxonomy has been elaborated in the past century. According to DeCandolle, Plant taxonomy will be the most useful guide to man in his search for the future of plant taxonomy will mostly rely on chemical traits of plants, as well as new industrial and medicinal species. Both of these statements are

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relevant to current research on natural products. This study evaluated the morphological characteristics and DNA analysis of five species of the genus *Mentha* from various locations in the Al-Gabal al-Akhder region of northeastern Libya.

Materials and Methods:

Locations of Collection

The plant species employed in this study were gathered under various circumstances and from various locations. At the time of collecting, all of the materials were brand-new.

The fresh samples are stretched between newspaper sheets and family pressed inside a herbarium press, and were allowed to dry for species description.

After the description, a key is made for the five types, places of collection of photographs.

Determination of Total Protein by the Biuret Method Extraction

To 200g dry tissue of leaves, then placed in the oven, dried at 70 °C for at least 24 hours. 10ml of distilled water was added, the mixture was shaken for a couple of hours, and then centrifuged at 3500 rpm for 5 min. The supernatant was collected for the analysis of soluble proteins.

Reagent

The Biuret reagent was prepared by dissolving 3g of copper sulfate ($CuSO_4$ $5H_2O$) and 9g of sodium potassium tartrate in 500 mL of 1M sodium hydroxide. Five g of potassium iodide were added, and the reagent was made to 1 liter with 0.2 M sodium hydroxide.

Determination

Three ml of Biuret reagent were added to 2ml of the protein solution, and the mixture was warmed at 73˚C for 10 minutes, cooled, and the absorbance of the mixture was measured at 540nm using a spectrophotometer. A standard curve was prepared using different albumin concentrations. Dilution of the samples was required in some samples before treatment with the Biuret reagent. (Witman *et al.*, 1978).

Determination of Total Free Amino Acid using the Ninhydrin method

Amino acids are organic acids that contain one or more amino and carboxyl groups and therefore have acidic and basic properties. Most amino acids are crystalline and almost all are α-amino acids, in which the amino group is present on the α-carbon atom.

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Principle

The free-amino acids solution reacts with ninhydrin to create a blue color. The blue color is obtained from all amino acids that have a free α-NH2 group and therefore not from pralines. The intensity of the blue color obtained by the reaction with ninhydrin determines the amount of free amino acids present in the sample.

Reagents

(A)Distilled water and 80% ethanol (W/V).

(B) Ninhydrin reagent: 0.5g of ninhydrin and 0.05gof hydrindantin are dissolved in 12.5 ml of 60% methylcellulose solution. This was followed by the addition of, 4ml of 4M acetate buffer (pH 5.4), and the volume was brought up to 100 mL with methyl cello solution.

Procedure

1) Different concentration gradients of a standard solution of glycine were prepared and treated with ninhydrin reagents and read in a manner similar to that followed in the case of the test. The readings obtained were used to plot the standard curve by plotting the optical density at 590nm against glycine concentration.

2)3.6 grams of plant tissue were taken and were extracted with 80 % ethanol (80ml of ethanol with 20ml of distilled water) at a reflux temperature of 70 to 80degrees Celsius in a Soxhlet machine for one hour. The extract was cooled to room temperature.

3) The extract was filtration by centrifuging at 4000rpm after cooling. The supernatant is collected and brought to a final volume of 50ml.

4) A quantity (1ml) was placed in a test-tube, to which was added 2ml of 0.5 % ninhydrin Reagent, and the mixture was stirred well and heated in a water bath (70˚C-80˚C) for 10 minutes.

5) The solution was allowed to cool to room temperature for color development. This was done to a known volume with distilled water.

6) The optical density value of the colored solution was then measured at a wavelength of 590 nm in a Spectrophotometer against a blank (ethanol).

Estimation of Total Carbohydrates by Anthrone Method:

Reagent Anthrone

This solution was created by dissolving 0.1 grams of Anthrone in 100 ml of H2SO4, which was prepared by mixing 500 ml of conic acid with 200 ml of water. The reagent was allowed to sit for 30 to 40 minutes, with occasional stirring to achieve clarity. The production of Anthrone from benzene and light petroleum is viable

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with certain commercial samples. The reagent is prepared fresh daily and utilized within a 12-hour period.

Estimation of Carbohydrates

Plant extracts. The leaves were thoroughly extracted using 70% ethanol, and the resulting extracts were evaporated to dryness under vacuum, then rinsed with hot water and rinsed with aluminum hydroxide. Take 0.6 grams of the dry tissue and add hydrochloric acid, that is made up 0.2 mg of Hcl add to 100 ml of distilled water, put it in a water bath for 2 - 3 hours, keep samples until it cools, then add a little of sodium carbonite, wait for the reaction to complete and the vaporstoevaporate, then centrifuge at 3500 rpm for 10 minutes, then take 0.5 ml from each finished samples up to 1 ml after then add the Anthrone reagent, 4 ml to each test tube, and then putitin a water bath for 10 minutes, take absorbance at 630 UV spectrophotometer (Yemm, and Willis, 1954).

A standard curve was prepared using different glucose concentrations. Dilution of the samples was required in some samples before treatment with the Anthrone reagent.

Results

Proteins Determination

Significant differences were observed between the protein values analyzed statistically from the standard curve obtained using different concentrations of egg albumin (Fig. 1) (Table 1), until the five species could be separated in the four groups as confirmed by the statistical method used (standard deviation).

1-The first group with the highest protein value is *M. longifolia*.

2-Followed by *M. piperita,* which occupied the second position (second group)**.**

3- The third group, *M. spicata*.

4-The last position with the lowest value was represented by *M. aquatica and M. pulegium* (fourth group)**.**

Values are expressed as means ± SD; each sample group consisted of three replicates. Mean values within the same column that do not share the same superscript letter (a, b, c, d) were statistically significantly different from each other with $p<0.05$.

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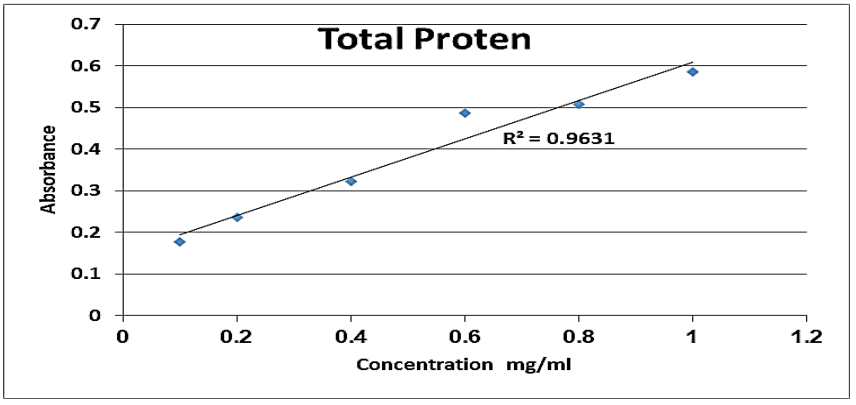

Fig (1) Standard Curve for the Estimation of Proteins (Conc.mg/ml against Absorbance nm)

Table (1) Levels of total protein in samples of *Mentha* Species (Mean ±SD)

Samples	Mean ±SD
M. aquatica	**0.2983 ±0.0711 D**
M. piperita	**0.8363 ±0.0280 B**
M. pulegium	**0.30333 ±0.00681 D**
M. longifolia	**1.1513 ±0.0405 A**
M. spicata	**0.52267 ±0.01201 C**
Standerd	**0.38617 ±0.00242 D**

Total Amino Acids Determination:

Regarding the amino acid values calculated from the standard curve (Fig. 2), the statistical analysis showed the presence of two groups (Table 2) instead of the four groups observed for the protein values. However, the positions occupied by the different species in terms of their amino acid content are modified in the case of amino acids compared to those reported for the proteins in the previous section (4.2.1.) *M. piperita* had the highest amino acid content, followed by *M. aquatica* with the second highest values, while the third position was taken by *M. pulegium*, and the fourth position was occupied by *M. longifolia* and *M. spicata*.

It is also noted that the difference obtained by the statistical analysis for the five species was not highly significant, which cannot be a decisive factor to determine whether these five species should be placed in a single group:

Accordingly, the two groups are:

1- The first group is occupied by *M. aquatica* and *M. piperita* with the highest amino acid contents.

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2- The second group consists of *M. pulegium*, *M. longifolia*, and *M. spicata*.

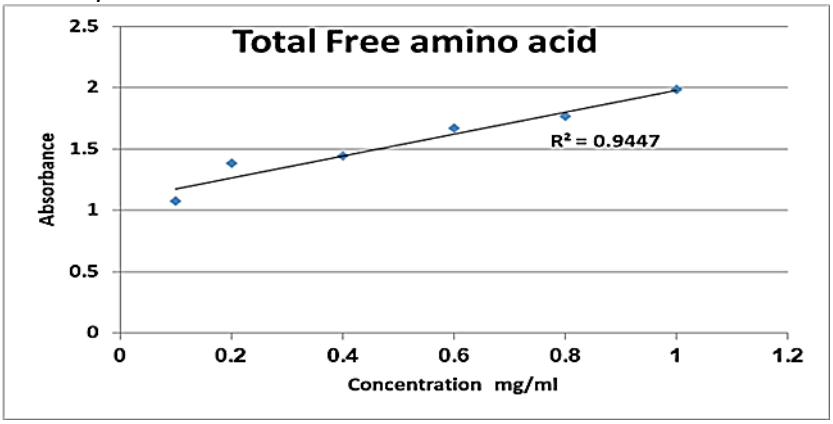

Fig (2) Standard Curve for the Estimation of Amino Acids (Conc.mg/ml against Absorbance nm)

Table (2) Levels of Total amino acids in samples of *Mentha* Species (Mean ±SD)

Samples	Mean ±SD
M. aquatica	**2.6477 ±0.0576 A**
M. piperita	**2.9243 ±0.0325 A**
M. pulegium	**2.264 ±0.236 B**
M. longifolia	**2.1050 ±0.0688 B**
M. spicata	**2.1773 ±0.0506 B**
Standerd	**1.5469 ±0.0448 C**

Values are expressed as means ± SD; each sample group consisted of three replicates. Mean values within the same column that do not share the same superscript letters (a, b, c, d) were statistically significantly different from each other with $p<0.05$.

Estimation of total carbohydrates:

Regarding the carbohydrate values calculated from the standard curve (Fig. 3), the statistical analysis of the findings demonstrated that the five species' carbohydrate contents varied, and statistical analysis revealed the existence of three groups (Table 3). M. piperita had the highest carbohydrate content, followed by M. spicata and M. longifolia, while the third position was occupied by M. aquatica and M. pulegium.

It was also observed that the difference obtained by the statistical analysis for the five species was not highly significant, which cannot

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be a decisive factor to determine if these five species should be placed in a single group.

Accordingly, the three groups are:

1- The first group is occupied by *M. piperita*
2- The second group consists of *M. spicata* and *M. longifolia*
3- The third group represented *M. aquatica* and *M. pulegium*

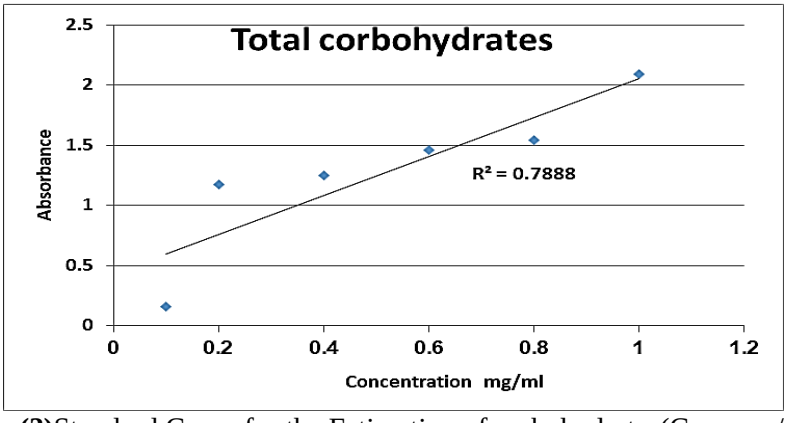

Fig (3) Standerd Curve for the Estimation of carbohydrates(Conc.mg/ml aginst Absorbance nm)

Table (3) Levels of Total carbohydrate in samples of *Mentha* Species (Mean ±SD)

Samples	Mean ±SD
M. aquatica	**0.76767 ±0.01501 B**
M. piperita	**1.411 ±0.00643 A**
M. pulegium	**0.75367 ±0.00643 B**
M. longifolia	**0.94167 ±0.00814 AB**
M. spicata	**0.90700 ±0.00721 AB**
Standerd	**1.2730 ±0.0422 AB**

Values are expressed as means ± SD; each sample group consisted of three replicates. Mean values within the same column that do not share the same superscript letter (a, b, c, d) were statistically significantly different from each other with $p<0.05$.

Discussion:

Wright *et al*. (1963), Greenhouse (1982), and Urich, K. (2013) demonstrated the use of proteins to distinguish species from

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other taxa, suggesting that protein biochemistry and function are better than morphological differences, differences between organisms and species.

This made it possible to coordinate the economic characteristics studied with the distribution map. It was also found to have an advantage over the multi-protein system used for serological comparison. Because it may be possible, for example, to establish the homology of large storage globulins in all angiosperms. In addition, Akubugwo *et al*. (2007), reported the differences in the protein content of *Solanumnigricum Virgincum*, *Telferia occidentalis*, and *Buchholzicorica*.

The application of protein determination in chemical taxonomy was endorsed by Turki (1999). Tedesco *et al*. (1997) made a similar suggestion, using soluble proteins from semi-woody stem cuttings in a chemical taxonomy and arguing that they were superior to pollen and seeds since they were available sooner during the study period. They also used the same protein pattern to identify species. However, in our case, the multi-protein system provided results that could be used to classify the five species into three groups.

It is well-known that amino acids are the building blocks of proteins and, at the same time, represent the final products of their degradation.

The fact that the amino acids of *M. aquatica* and *M. piperit a* (group 1) are more numerous than those of *M. longifolia with* high protein content can be explained by the presence of more amino acids in the first due to of either a higher rate of protein degradation or a greater assimilation of amino acids into proteins by the latter, thus leaving more free soluble amino acids.

The differences observed in the total amino acids, in this study, could be explained and confirmed by the information from the work previously reported by Mino, Y.*et al*. (1993), who obtained results working on two varieties of *Daturastromonium*, which supports the proposal that these plants should be considered two species of a single species and not two separate species. Variations in protein content are also attributed to variations in the quantity and quality of their amino acids, as they are accepted as more accurate than morphological characteristics in the expression of genetic variations (Hawkes, 1968; cited from Greenhous *et al*., (1982).

The slight difference in carbohydrate content between the species can be attributed to the fact that they are medicinal plants.

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Conclusion:

Our comparative analysis, focusing on the protein, amino acid, and carbohydrate content across the five *Mentha* species represented in Libya, revealed significant quantitative variability among them. This nutritional heterogeneity underscores the dual importance of the *Mentha* genus as both a source of significant therapeutic properties and an untapped potential nutritional resource for local development. Based on these findings and established knowledge, its cultivation should be given greater attention. We recommend focusing cultivation efforts not only to protect endangered species, such as water mint (*Mentha aquatica*), but also to prioritize the propagation of species with superior nutritional profiles to maximize local health benefits. This approach is further necessitated by the observation that some valuable species are currently not found in the locations mentioned in the Libyan flora.

To fully understand and utilize the medicinal and nutritional potential of this genus, further in-depth taxonomic, physiological, and phytochemical studies should be conducted on the five *Mentha* species found in Libya. Additionally, further taxonomic studies of medicinal plants are essential to expand the verified sources of herbal medicines in the region.

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