TOTAL FLAVONOID CONTENT, MICROSCOPIC AND MICROBIAL EVALUATION OF SYRIAN GINKGO BILOBA PRODUCTS

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Ginkgo Biloba (GB) is a widely used finished herbal product. Due to its natural source there is no demand to evaluate its quality before marketing. Although GB products have shown promising potential with the efficacy, many of these products remain untested. The Current work aims to evaluate some quality parameter of GB finished products like total flavonoid content (TFC), microscopic, Loss On Drying (LOD) and microbial tests in comparison with GB crude plant. The TFC ranged from (22.86 to 78.74 mg/g). Microscopic findings revealed the presence of large amounts of starch grains in product A. Furthermore, most of the products were heavily contaminated with bacteria and fungi. As bacterial colony counts ranged from ($10^3$ to $7.5\times10^5$ CFU/g) and from ($1.7\times10^3$ to $0.75\times10^7$ CFU/g) for fungal colony counts. Most of the products exceeded the reference limits for LOD. Thus may pose a threat to consumer's health.

INTRODUCTION

The use of herbal products (HP) is continually expanding worldwide with many people now resorting to these products for treatment of various diseases. Yet, there is insufficient information about its safety, purity and effectiveness, which raise a great concern about the quality and safety of these products. Generally, it is believed that the risk associated with herbal drugs is very low, but several reports on serious effects indicate the need for development of safety profiles and stringent quality control systems for authentication and standardization of HP. Ginkgo folium (Ginkgoaceae) is a very important medicinal herb, as numerous studies reported GB ability to enhance peripheral circulation and slow the progression of Alzheimer’s disease. In fact, the chemical composition of GB is quite complicated, as flavonoids constitute are the largest groups of bioactive substances isolated from GB. Moreover, the content of flavonoids is considered as an important index for evaluating GB products quality. A variety of analytical methods can be used to quantify these compounds, however UV/Vis spectrophotometric determination became one of the most widely used methods for quantification of total flavonoids due to its simplicity and low cost of implementation. This approach becomes more critical due to the high cost or absence of reference substance needed for determination of individual flavonoids. On the other hand, the microscopic & microbial evaluation are essential steps in verifying HP purity, and safety. The current study aims to evaluate the total flavonoid content (TFC), Microscopic, Microbial and LOD testes of GB products, as they are the most important parameters for quality evaluation of HP. After all adhering GMP to all production stages is very important to obtain high quality products with no harmful effects.
MATERIAL AND METHODS

Chemical and Equipment

Automatic mill, Ethanol Solution 70%, aluminum chloride (10% w/v), potassium acetate (1M), distilled water, quercetin standard (Aldrich Chemical), Spectrophotometer (Shimadzu), chloral hydrate solution 60%, iodine solution 1%, nutrient and MacConkey agar medium (HiMedie-India), Sabouraud Dextrose Agar medium (SDA) (Accumix-India), Autoclave (Dampsterilsator Germany - Variokla), Incubator (Carbolite – UK), Micros Optical Microscope (Austria, Precisa-XB220), Switzerland. All solvents were of analytical grade.

Samples

Three different batches of three GB products (A, B and C) were purchased from local Syrian pharmacies. GB crude plant was kindly gifted from Syrian Pharmaceutical Industries (Homs, Syria). The crude plant was grinded into powder using the automatic mill. A and B products were capsules with dried leaves powders, while C products were capsules with dried leaves extract. The batches were coded 1, 2, 3 respectively.

Estimation of Total Flavonoid Content (TFC)

The total flavonoid content of GB samples were determined using the aluminum chloride assay. Fifty g of powdered samples was extracted by reflux using 70% ethanol as the solvent for 2 hrs. and repeated 3 times. The extracts then evaporated using a vacuum rotatory evaporator, and then dried in water bath on 50°C. Briefly, to 0.5 mL of sample, ethanolic solution of aluminum chloride (10% w/v), potassium acetate (0.1 mL of 1 M) and distilled water (4.3 mL) were added. After 15 min of incubation the mixture turns to pink whose absorbance was measured at 510 nm using a spectrophotometer. Obtained results were expressed in mg/g quercetin equivalents.

Microscopic Evaluation Method

Samples were tested by adding few drops of chloral hydrate solution 60% and the powders was studied using light microscope with 10 and 40 lenses. An iodine solution was used to detect the presence of starch grains in the studied samples. GB crude plant was used as comparative material.

Loss on Drying Test (LOD)

It was conducted by taking 1 g of product A, B and GB crude plant, followed by heating in an oven at 105 °C for two hrs. The percentage of LOD is calculated by the equation:

\[
\text{Percentage of LOD} = \frac{\text{Weight of the material before drying} - \text{Weight of the material after drying}}{\text{Weight of the material after drying}} \times 100
\]

Microbial Evaluation Method

The samples were investigated for the presence of bacterial and fungal contamination by using the methods described in USP 41-NF36 with some modifications. All medium for microbial analysis was prepared according to manufacturer's guidelines. The culture tubes were prepared using distilled water, then it was diluted in a sufficient ratio to obtain an aqueous suspension that could be implanted and counted (10/100). The preservative efficacy was canceled by dilution to 100 ml. The spread plate method was used and 0.1 ml of each tube was implanted on the medium mentioned above then were incubated at 37 °C for 48 hrs., including the negative control for nutrient agar and MacConkey agar. SDA samples were incubated at 25 °C for 7 days. Results were reported as the counts of colony forming Units (CFU) per each gram or ml. Three samples of each tube were counted for CFU and the mean was calculated. At the end of incubation period, pathogenic bacterial isolates were preliminary characterized by colony morphology, Gram staining, and biochemical tests (catalase production).

RESULTS AND DISCUSSION

Estimation of Total Flavonoid Content (TFC)

The flavonoids content was calculated using the following linear equation based on the quercetin calibration curve: \[ Y = 0.0066 \times C + 0.1303, \text{R}^2 = 0.9978, \text{where} \ Y \text{is the absorbance and} C \text{is the flavonoids content in mg/g. The standard curve of quercetin clarified in Fig.1, as the results of TFC included in table 1.} \]
| Table 1: Total Flavonoid Content (TFC) in GB samples |
|-----------------|---------------|----------------|---------------|---------------|---------------|---------------|---------------|---------------|
| sample          | Crude plant   | A1  | A2  | A3  | B1  | B3  | C1  | C2  | C3  |
| TFC mg/g        | 23.48         | 58.89 | 78.74 | 43.43 | 27.98 | 26.01 | 22.86 | 24.43 | 23.28 |

The TFC in the studied GB samples ranged from 22.86 to 78.74 mg/g, as the lowest value was found in product C1 (22.86 mg/g), while the highest value was found in product A2 (78.74 mg/g). Moreover, TFC values vary between the batches of each product. It was observed that TFC values in all products were higher than TFC values in GB crude plant. This matches with the results of E. Pereira, as the TFC in commercial GB products in E. Pereira study were higher than TFC in the crude plant.11

Also, it was observed that the TFC in Capsules with GB dried leaves powders (products A and B) were higher than Capsules with GB dried extracts powders (product C). This also matches with the results of E. Pereira study as TFC values were higher in Capsules with GB dried leaves powders.11 On one hand, one of the extraction steps is the plant exposure to high temperatures thus, flavonoids chemical structure become unstable and cause decline in its content in the plant samples.11 On the other hand, the climatic and geographical conditions, environmental factors, soil as well as the age of the plant,13-15 in addition, the extraction and drying methods play a major role in the TFC variation in samples.16-17 Moreover, the different colors of GB leaves can affect TFC, as it was reported that the yellow leaves had the highest TFC, while the lowest content exhibited on leaves in green color. This indicated GB leaf in pure green color is not suitable for harvesting due to its low TFC. Furthermore, total flavonoids concentrations change rapidly in GB leaves in green-yellowish and yellow colors. Therefore determination of total flavonoids content in GB leaf could be used to decide the harvest time and estimate the quality of GB.4

Microscopic Evaluation
This test was conducted on products (A, B) and GB crude plant which is used as a comparative material. The results of microscopic tests were included in fig.2. The crude plant leaves powder was green-yellowish, while the products powders color ranged from green, yellow to green-yellowish. As the product A and B powders colors varied in their different batches. In fact, the leaves color may differ depending on season, harvesting, drying method and many other factors. The microscopic evaluation showed characteristic microscopic features of GB leaf lamina, which are fragment of upper and lower epidermal cells in surface view that is similar in shape, but lower epidermis is characterized by the presence of sunken anomocytic stomata.18 Also, xylem vessels with annular thicknesses appeared in all samples. Moreover, cluster crystal of calcium oxalate is one of the most important distinguishing elements of GB, where different sizes of these crystals were observed, and it reflected the leaf age and the chemical composition or quality of the leaf. The presence of particles with maximum sizes suggested that the leaves are of old age. Furthermore, starch grains were abundant particularly in product A2, it was ribbed with a central helium and presented in large amounts that impeded the microscopic detection to some extent, especially in product A3 (Fig.2). Starch grains were not observed in all batches of product B. In fact, some references reported that starch grains may be present in small amounts, but they do not present in these large quantities as it was observed in product A3. So,
the presence of this amounts of starch grains may be due to mixing GB powders with starch powders. On the other hand, aromatic cavities and secretory canals were also observed in frontal view, which are large and full of yellowish-brown content. Although some references do not classify it as a characteristic diagnostic component of GB leaves, but it was clearly observed in all samples. This findings are corresponding to GB comparative crude plant, references and matches with S. Ion study of GB microscopic features18-19.

**Loss on Drying (LOD) Results**

The LOD results is included in Table 2. It was observed that LOD average values were higher in crude plant (17.4%) than the finished products. As product A had the lowest LOD average value of (11.33 %). Moreover, all average values of the samples exceeded the permissible limits for LOD which indicates a high moisture content. As the preserving and drying methods can affect LOD results20.

**Microbial Evaluation**

The results of Total Aerobic Count (TAC) on Nutrient Agar are included in Table 3 and The results of Total yeasts and molds count (TYMC) on SDA are included in Table 4. Results indicated that 75% of the samples were bacterial contaminated. Furthermore, it was observed that product A2 and A3 were within the acceptable limits10, while all batches of product B exceeded the permissible limits. This results matches with the results of Ratajczak study21. There was no bacterial growth on all batches of Product C. The absence of bacterial growth in all batches of product C may be due to the fact that this product is a dry extract, and extraction is a stringent way of treatment thus kill all types of living cells, including spores22.

![Fig. 2 : The microscopic results of Ginkgo Biloba samples](image-url)
### Table 2: The results of Loss On Drying test (LOD)

<table>
<thead>
<tr>
<th>A %</th>
<th>B %</th>
<th>Ginkgo leaf %</th>
<th>Reference limits (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.33</td>
<td>13.46</td>
<td>17.4</td>
<td>NMT 11%</td>
</tr>
</tbody>
</table>

### Table 3: The results of Total Aerobic Count (TAC) on Nutrient

<table>
<thead>
<tr>
<th>Product</th>
<th>Type</th>
<th>Batch No.1</th>
<th>Batch No.2</th>
<th>Batch No.3</th>
<th>Reference limits (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Dried leaves Powder</td>
<td>$1.5 \times 10^6$</td>
<td>$1.9 \times 10^3$</td>
<td>$1.7 \times 10^3$</td>
<td>$^{3}$NMT $10^5$</td>
</tr>
<tr>
<td>B</td>
<td>Dried leaves Powder</td>
<td>$1.3 \times 10^6$</td>
<td>$1.7 \times 10^6$</td>
<td>$1.6 \times 10^6$</td>
<td>NMT $10^5$</td>
</tr>
<tr>
<td>C</td>
<td>Dried leaves extract</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>NMT $10^6$</td>
</tr>
<tr>
<td>Crude plant</td>
<td>Dried leaves Powder</td>
<td>$0.7 \times 10^7$</td>
<td>$0.75 \times 10^7$</td>
<td>$0.71 \times 10^7$</td>
<td>NMT $10^5$</td>
</tr>
</tbody>
</table>

NMT: Not more than

1According to USP41-NF36 (21), 2Colony forming units per gram, 3Not more than. TYMC: Total yeasts & molds count.

### Table 4: The results of Total yeasts and molds count (TYMC) on SDA

<table>
<thead>
<tr>
<th>Product</th>
<th>Type</th>
<th>Batch No.1</th>
<th>Batch No.2</th>
<th>Batch No.3</th>
<th>Reference limits (10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Dried leaves Powder</td>
<td>$1.2 \times 10^3$</td>
<td>$3.0 \times 10^3$</td>
<td>$10^3$</td>
<td>NMT $10^5$</td>
</tr>
<tr>
<td>B</td>
<td>Dried leaves Powder</td>
<td>$1.0 \times 10^4$</td>
<td>$1.1 \times 10^4$</td>
<td>$1.05 \times 10^4$</td>
<td>NMT $10^5$</td>
</tr>
<tr>
<td>C</td>
<td>Dried leaves extract</td>
<td>$1.1 \times 10^3$</td>
<td>$1.7 \times 10^3$</td>
<td>$1.3 \times 10^3$</td>
<td>NMT $10^5$</td>
</tr>
<tr>
<td>Crude plant</td>
<td>Dried leaves Powder</td>
<td>$6.5 \times 10^5$</td>
<td>$7.5 \times 10^5$</td>
<td>$6.0 \times 10^5$</td>
<td>NMT $10^5$</td>
</tr>
</tbody>
</table>

1According to USP41-NF36 (21), 2Colony forming units per gram, 3Not more than. TYMC: Total yeasts & molds count
Crude plant was more contaminated than finished herbal products (A, B) due to treatment processes which reduce microbial load. This is corresponding to the results of Santos study\textsuperscript{23}. In fact, microbial tests often reveal the presence of bacilli type bacteria and its presence is associated with food borne diseases, moreover, this type of bacilli is dangerous because of its ability to form internal resistant spore which could live on products in a dormant state for a long time and couldn't be easily removed. No growth was observed on McConkey medium of any of the studied samples which may indicate the sample were free of negative gram bacteria, and that corresponds to A. Okunlola study\textsuperscript{24}. The positive Catalase test indicates the presence of Staphylococcus aureus colonies in product A. Moreover, all samples were heavily contaminated with fungi, and mostly of Aspergillus Flavus, which is considered a great hazard to health due to Aspergillus toxic spores which may be formed, as in a study of Tournas\textsuperscript{25}. As large number of fungi colonies indicates continuous spoilage of the product with negative effects on its quality and pharmacological properties\textsuperscript{26}. Storage in poorly ventilated place, for example, may lead to high temperature and increased moisture, moreover dried plant materials absorb moisture easily, which leads to the increase in microbial load of products\textsuperscript{27}.

The LOD results showed a very strong correlation with products microbial load using the Pearson, as Pearson coefficient was 0.9675 for correlation between LOD and bacterial growth, while it was 0.9360 for correlation between LOD and fungal growth, which indicated that bacterial and fungal growth increases as moisture content of products increases.

**Conclusion**

The use of herbal products has increased rapidly with no applying of quality control in the same acceleration of products spread. In this research, the TFC, microscopic, LOD and microbial tests of Syrian GB products were conducted. Flavonoids are key candidate compounds for evaluating the quality of GB products. The results confirmed the presence of GB leaf which is stated on the product label. Large amounts of starch grains were observed in some products. Moreover, most of products were highly contaminated with bacteria and fungi. Consequently, these products may affect patients health badly as well as affect product stability. However, finished products were generally less contaminated than crude plant. The elevated levels of contamination indicate inadequate storage procedures, failure to follow good hygienic practices and failure to adherence to GMP. The WHO recommends to store fresh medicinal plants at low temperatures, and therefore farmers should be trained for GMP, planting practices, Good agriculture and Good collection practice (GACP) and appropriate storage. All countries should make efforts to build consumers trust in herbal products by ensuring their safety and following GMP regardless of where it is manufactured and purchased.

**Acknowledgments**

The authors are thankful to The Pharmacy Institute, Al-Baath University for using laboratory equipment.

**REFERENCES**


محتوى الفلالفونويد الكلي، التقييم المجهرى والميكروبي لمنتجات الجنكوبيلوبا السورية

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يعد الجنكوبيلوبا من المستحضرات العشبية واسعة الاستخدام، وكونه من مصدر طبيعي، فلا يُطلب تقييم جودة مستحضراته قبل التسويق. وعلى الرغم من أن مستحضرات الجنكوبيلوبا قد أثبتت فعالية واعدة فإن العديد منها لم تتم مراقبة جودته. تهدف هذه الدراسة إلى تقييم بعض معالجات مستحضرات الجنكوبيلوبا وذلك من ناحية تحدي المحتوى للفلافونويدات والاختبارات المجهرية والفق بالتجريف والنقاوة المكروبيولوجية بالمقارنة مع النبات الخام. تراوحت قيم المحتوى الكلي للفلافونويدات من 22.86 إلى 78.77 مجم/جم.

أظهرت النتائج وجود كميات كبيرة من حبيبات النشأة في المستحضر A، وعلاوة على ذلك كانت أغلب المستحضرات ذات حمل ميكروبي عالي، حيث تراوح التعداد الكلي للجراثيم من CFU/g من 10٠ إلى 10٠٠ CFU/g، ومن 7.5 × 10٠ إلى 7.5 × 10٠ CFU/g، وتم للفطور.

تجاوزت معظم المستحضرات الحدود الدستورية المسموحة لاختبار الفقد بالتجريف، إن هذه المعطيات قد تهدد صحة المستهلك.