DISCUSSION

Extracts of *Ginkgo biloba* L. leaves are widely available worldwide in herbal medicinal products, dietary supplements, botanicals, and complementary medicines. Several pharmacopoeias contain monographs for *Ginkgo* leaf, leaf extract, and finished products. *Ginkgo biloba* L. is one of the top-selling botanicals in the world. It is used mainly to improve cognitive functions[16]. Being a high value commodity, *Ginkgo* extracts may be subject of economically motivated adulteration.

The major contributors to the positive biological effects attributed to *Ginkgo* are the flavonol glycosides[21] and the terpene trilactones[18]. Negative markers of *Ginkgo* are also recognized and include mixture of ginkgolic acids in the leaves[108] *a*nd ginkotoxin in the seeds[41]. The first standardized extract of *Ginkgo biloba* prepared from the leaves was termed EGb 671®. The latter has been standardized to contain 6% total terpene trilactones and 24 % total flavonoids[14].

In Egypt the majority of *Ginkgo* products are marketed as dietary supplements, hence escape the strict control measures mandated for conventional medicines.[109]

The main objectives of the present work have been to assess the quality of *Ginkgo biloba* L. containing dietary supplements marketed in Egypt and to suggest improved method of analysis that couple both specificity and stability indicating properties.

The present study examined the following:

- Critical assessment of compendial methods for the analysis of *Ginkgo* extracts and products.
- Results of analyses of products containing *Ginkgo biloba* extract and marketed in Egypt according to USP monograph and recommended methods in this thesis.
- Importance of the study of extract before hydrolysis for the evaluation and stability testing of the examined *Ginkgo* extracts and products
- To emphasize the different methods used for counterfeiting of *Ginkgo* products.
- To suggest special recommendations to the Egyptian health authorities responsible for the registration and post-marketing surveillance of *Ginkgo* products.

1. Critical assessment of compendial methods for the analysis of *Ginkgo* extracts and products

Monographs for *Ginkgo* extracts are found in various pharmacopoeias, including the USP, Ph. Eur., BP, and USP compendium of dietary supplements. The USP monograph for *Ginkgo* extract specifies a flavonoid content of 22-27%, calculated as flavonol glycosides, terpene lactones content of 5.4-12% consisting of bilobalide ,ginkgolide A and ginkgolide B. As a parameter for quality/authenticity the USP method mandates to monitor a quercetin/kaemferol/isorhamnetin (Q/K/I) ratio of the hydrolysed extract based on their respective peak areas[20]. The kaempferol peak must be 0.8-1.2 times the size of the quercetin peak, and the peak for isorhamnetin must be not less than 0.1 times the size of quercetin peak. The USP microbial enumeration tests requirements are the absence of

salmonella species and *Escherichia coli*. The total aerobic bacterial count must not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g[107].

1.1. Estimation of the flavonol glycosides content

Recently El-Masry[110] suggested that current USP method for the assay of flavonol glycosides content in *Ginkgo* extracts is non-stability indicating. The method involves acid hydrolysis of flavonol glycosides to aglycones and back calculates the total flavonol glycoside content from the concentration of flavonol aglycones (Figures 10 and 11). This means that current pharmacopoeial methods are incapable of detecting adulteration of *Ginkgo* extracts with *Ginkgo* flavonol aglycones (quercetin and kaempferol) or glycosides yielding these aglycones upon hydrolysis (rutin). Although the Q/K/I ratio can detect inferior quality products containing *Ginkgo biloba* L. extracts, however it might fail to detect counterfeiting with aglycones. The BP and Ph Eur methods are essentially the same as USP method, both fail to detect counterfeiting with the flavonol glycosides and their hydrolytic products.

1.2. Estimation of the terpene trilactones content

Compedial methods for the estimation of terpene trilactones are very tedious. In addition, it involves the use of refractive index detector which suffers from low sensitivity[22]. Accordingly, analyses of tepene trilactones in eight products containing *Ginkgo biloba* L. extract were done in an accredited laboratory using validated HPLC-MS spectrometric methods.

1.3. Limits of ginkgolic acids

Compendial methods for the estimation of ginkgolic acids have a drawback that the entire extract is injected on the HPLC column with large volume (100µl) while the analytes constitute less than 0.0005%. This will lower the lifetime of expensive HPLC columns. Accordingly, analyses of ginkgolic acids in eight products containing *Ginkgo biloba* L. extract were done using a validated HPLC- MS method in an accredited laboratory.

2. Results of analyses of products containing *Ginkgo biloba* L. extract and marketed in Egypt according to USP monograph and recommended methods described in this thesis

In the present thesis, two commercial extracts and ten products containing *Ginkgo biloba* L. extract were assayed for their flavonol glycosides content. Eight products were assayed for terpene trilactones and ginkgolic acids. Microbial count analyses for some products were carried out. Accelerated stability testing was carried out for the complying products (A, B, C, D, and E).

The percentage of flavonol glycosides and the Q/K/I ratio in extracts after hydrolysis were determined as essential pharmacopoeial requirements. In addition, the following are the suggested modifications.

- Percentage of two available primary glycosides (rutin and quercetrin) in extracts was determined before hydrolysis as a useful indicator of stability which is circumvented when samples are hydrolyzed to back calculate flavonol glycoside content.

-Percentage of quercetin before and after hydrolysis in *Ginkgo biloba* L. extracts was determined as a tool to identify the quercetin adulterated products.

- The ratio of aglycones (quercetin and kaempferol) to glycosides (quercetrin) before hydrolysis was determined as an indicator for hydrolysis; increase in the ratio indicates hydrolysis

- HPLC fingerprints of *Ginkgo biloba* L. containing products were carried out to detect inferior, hydrolyzed, and adulterated products.

- HPTLC fingerprints of *Ginkgo biloba* L. containing products and *Ginkgo biloba* L. extracts were performed as a quick confirmatory tool for adulteration.

Preparations containing extracts of *Ginkgo biloba* L. leaves which were analysed by HPLC were found to contain variable amounts of flavonoid compounds and terpene trilactones.

Results of analyses of rutin and quecetrin content in products A and B which complied with USP requirements showed a range of (4.4 - 5%) and (5.8 - 6.5 %) respectively as shown in Table 7. HPLC fingerprints of products A and B were found to be the same as the standardized *Ginkgo biloba* L. extract before hydrolysis as shown in Figure 16. Percentage of quercetin before hydrolysis in products A and B was very small (0.09%) as shown in Table 6 and thus the ratio of their aglycones to glycosides before hydrolysis was also very small suggesting no hydrolysis (Table 8). Percentage of terpene trilactones in products A and B complied with USP requirements, suggesting that these two products are of a good quality (Table 9). It is worth mentioning that products A and B are registered as medicines according to their product inserts.

Analysis of rutin and quercetrin content in products C, D, E, and F before hydrolysis revealed that they contained high percentage of rutin ranged from (17.4-25%) and low percentage of quercetrin ranged from (0.99-1.54%) which suggest an inferior quality products adulterated with rutin (Table 7). This was confirmed by the HPLC fingerprints of the four products before hydrolysis which showed only one prominent peak corresponding to rutin (Figure 17). The percentage of quercetin aglycone before hydrolysis in products C, D, E, and F was about 5 times higher than that of products A and B which passed all compendial requirements (Table 6). The ratio of their aglycones to glycosides before hydrolysis was high compared to products A and B suggesting hydrolysis and/or counterfeiting (Table 8). Products C, D, and E failed the USP requirements for terpene trilactones (Table 9).

It was interesting to note that the first batch of product G had high concentration of quercetin (1.09%). This indicates either adulteration with quercetin aglycone or hydrolysis. The percentage of quercetin before hydrolysis was 12 times higher than that of products A

and B (Table 6) and thus the ratio of aglycones to glycosides was high (1.48) suggesting hydrolysis or counterfeiting with quercetin (Table 8). However, HPLC fingerprint of the second batch (G*) before hydrolysis showed one prominent peak due to rutin (Figure 18) suggesting adulteration with rutin (Table 7). This indicates that some manufacturers use different sources for their raw material of *Ginkgo biloba* L. extracts according to availability. The first batch (G) failed the USP requirements for terpene trilactones content (Table 9).

Product I was found to be adulterated with unknown flavonol glycoside containing kaempferol aglycone. It is reflected in the 6 times increase in the ratio of kaempferol to quercetin (1/6.13/0.31). HPLC fingerprint of product I was not the same like *Ginkgo biloba* L. standardized extract before hydrolysis (Figure 18). The percentage of rutin and quercetrin in product I was very small compared with the two USP complied products (A and B) suggesting inferior quality product (Table 7). Surprisingly it complied with USP requirements for terpene trilactones content (Table 9).

Product J was found to be hydrolysed or adulterated with free aglycones (quercetin, kaempferol, and isorhamnetin). The ratio of aglycones to glycosides before hydrolysis was very high (8.94) suggesting that the product was hydrolysed (Table 8). This was confirmed from HPLC fingerprint of product J prior to hydrolysis which was completely different from HPLC fingerprint of the USP standardized *Ginkgo* extract. HPLC fingerprint of product J showed two prominent peaks due to quercetin and kaempferol and minor peaks due to flavonol glycosides (Figure 18). The percentage of quercetin aglycone before hydrolysis was very high (2.98%) compared to that of USP complied products A and B (0.09%) as shown in Table 6.

Product H contained in addition to *Ginkgo biloba* L. extract trihydroxyethyl rutin (troxerutin®). The latter upon hydrolysis yields quercetin. Thus USP method of assay is not suitable for the estimation of flavonol glycosides content of this product (Figure 18). In addition product H contains only 14 mg of Ginkgo extract, however, studies confirmed that the recommended dosage of an oral standardized extract is 120-240 mg daily.

HPLC fingerprint analyses of the examined commercial *Ginkgo biloba* L. extracts 1 and 2 suggest that they are adulterated with rutin. HPLC fingerprints of both extracts failed to show the recognizable peaks corresponding to the USP standardized *Ginkgo* extract (Fiqure15).

HPTLC analysis of the products containing *Ginkgo biloba* L.extract demonstrated that Products found to be adulterated with rutin showed much stonger quenching in the rutin zone than the other products. Products A and B demonstrated all zones typical for USP standardized extract of *Ginkgo biloba* L.. Inferior quality products demonstrated faint zones (Figure 12 and 13).

Microbial enumeration tests and determination of limits of ginkgolic acids were carried out as two important safety parameters required by the USP monograph of *Ginkgo biloba* L. products and extracts. Microbial enumeration test of the pharmaceutical products was carried out following the procedures described in USP 36. All samples were found to meet the requirements of the tests for absence of *Salmonella* species and *Escherichia*

coli. The total aerobic microbial count and the total combined molds and yeasts count did not exceed the required limit.

It is worth mentioning that five products out of eight failed the USP requirements for the limit of ginkgolic acids and showed extremely high limits ranged from 29 to 1528 ppm compared to the acceptable compendial requirements (5 ppm). These results confirm the use of inferior quality *Ginkgo biloba* L. extracts in these products which did not take into considerations the required steps to remove ginkgolic acids.[22]

Our results were similar to those obtained in other studies of *Ginkgo* dietary supplements in different countries which also failed to contain active ingredients in amounts declared by their producers [20,28,91,99].

3. Importance of the study of extract before hydrolysis for the evaluation and stability testing of the examined *Ginkgo* extracts and products

HPLC chromatographic fingerprints of *Ginkgo* extracts or their marketed products before hydrolysis clearly identified complying extracts (products A and B) as shown in Figure 15 and counterfeit or hydrolysed products as shown in Figure16 and 17. It is clear from the obtained results of analyses of products and extracts that the target for counterfeiting is the flavonol glycosides or their aglycones (Figure16 and 17).

The results obtained in this thesis suggest that HPLC fingerprints analysis of *Ginkgo* extracts containing products before hydrolysis, determination of quercetin content before and after hydrolysis, and ratio of *Ginkgo* aglycones to glycosides are assets for identification of hydrolysed products.

4. Different methods used for counterfeiting of *Ginkgo* products

Ginkgo extracts may be adulterated with pure flavonol aglycones, pure flavonol glycosides (eg. rutin, hyperoside, quercetrin) or other plant materials containing flavonols and flavonol glycosides. Sources of flavonols and flavonol glycosides are much less expensive than terpene trilactones, so it is far more likely that if adulteration occurs, it would be with some form of flavonol and adulteration candidates are probably quercetin and rutin.

5. Special recommendations to the Egyptian health authorities responsible for the registration and post-marketing surveillance of *Ginkgo* products are made

The method of assay of *Ginkgo* flavonol glycosides must be stability indicating method since it is well known that flavonol glycosides are more readily absorbed than their aglycone counterparts are. Accordingly, intact flavonol glycosides are reported to be responsible for the biological activity. So it is very important to evaluate the extracts qualitatively besides the numeric specifications.[28,85].

Researchers in the research and development (R&D) department should judge whether the compendial method is stability indicating or not, before carrying out stability testing and derivation of shelf-life of products. It is needless to say the health authorities examining registration files should ensure that stability testing of the products was made

by stability indicating method, otherwise stability testing and derivation of shelf-life would be meaningless.

Manufacturers must obtain their *Ginkgo* extract from reliable suppliers who apply GMP especially that *Ginkgo biloba* L. contains up to 35 flavonol glycosides and the composition of final extract depends on the method of extraction adopted.