A Novel Drug Carrier System Using Clinoptilolite For Ginkgo Biloba Leaf Extract

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ABSTRACT

The use of natural and synthetic zeolites for pharmacological applications in possible encapsulation and/or adsorption of different ions and molecules in their open frame-work, and the subsequent release has emerged as a promising field during last years. In this study, the possible use of clinoptilolite-rich mineral as a novel drug carrier for the active constituents present in Ginkgo biloba leaf extract solutions was investigated.

In the first place, the extraction conditions for the compounds of interest (terpene lactones and flavonoid aglycones) within the leaf material were optimized. The extraction processes were performed with 80% ethanol aqueous solution due to its being non-toxic in nature and widely-used solvent for the preservation of the antioxidant activity possessed by the extract. For the quantification of the extracted species, High Performance Liquid Chromatography (HPLC) analysis was conducted. The quantification of terpene lactones was achieved with Evaporative Light Scattering Detection (ELSD), whereas flavonoid glycosides were quantified by Ultraviolet (UV) detection after being converted to their aglycone forms via acidic hydrolysis.

In the adsorption studies, clinoptilolite having a particle size in the range 25-106μm was experimented. The flavonoid aglycone constituents were found to be selectively adsorbed on the clinoptilolite surface, whereas terpene lactones were determined to be adsorbed on the clinoptilolite surface in negligible amount. Therefore, the study concentrated on the adsorption of flavonoid constituents on the surface of the zeolitic material.

Finally, the antioxidant activity determinations of the extract solutions were performed by the Trolox Equivalent Antioxidant Capacity (TEAC) assay. The antioxidant activity measurements performed for the Ginkgo leaf extract and commercial tablet extract solutions both showed decreasing antioxidant activities via adsorption. The decrease in antioxidant activity was related to the adsorption of phenolic constituents on the clinoptilolite surface.

ÖZ

Doğal ve sentetik zeolitlerin, kapsül şeklinde kaplanmasının ve/veya değişik iyon ve moleküllerin açık iskelet yapılarına adsorplanmasının, ve sonraki salınımlarının farmakolojik uygulamalarda kullanımı son yıllarda umut verici bir alan oluşturmuştur. Bu çalışmada, klinoptilolitçe zengin doğal zeolitin, Ginkgo biloba yaprağı ekstrakt çözeltilerindeki aktif maddeler için yeni bir ilaç taşıyıcı olarak kullanımı incelenmiştir.

Öncelikle, yaprak malzemesinde mevcut istenen bileşenler (terpen laktonlar ve flavonoid glikozitler) için ekstraksiyon koşulları optimize edilmiştir. Ekstraksiyon işlemlerinde, zehirsiz yapısı ve ekstraktın antioksidan özelliğini koruması açısından en çok kullanılan çözgen olması nedeniyle, 80%'lik ethanol sulu çözeltisi kullanılmıştır. Ekstrakte edilen türlerin miktar tayini için Yüksek Performanslı Sıvı Kromatografisi (HPLC) kullanılmıştır. Terpen laktonların miktar tayini Buharlaştırmalı, Işık Saçtırmalı Dedektör (ELSD) ile belirlenirken, flavonoid glikozitlerin miktarı asidik hidrolizle aglikon formlarına dönüştürüldükten sonra Ultraviole (UV) dedeksiyonuyla belirlenmiştir.

Adsorpsiyon çalışmalarında, 25-106μm aralığında tanecik boyutuna sahip klinoptilolit denenmiştir. Flavonoid aglikon bileşenleri seçimli olarak klinoptilolit yüzeyine adsorplanırken, terpen laktonların klinoptilolit yüzeyine ihmal edilebilir derecede az miktarda adsorplandığı bulunmuştur. Bu nedenle, çalışma flavonoid bileşenlerin zeolitik malzemenin yüzeyine adsorplanması üzerine yoğunlaştırılmıştır.

Son olarak , ekstrakt çözeltilerin antioksidan aktivite tayinleri Trolox Eşdeğer Antioksidan Kapasite (TEAC) yöntemiyle belirlenmiştir. Ginkgo yaprağı ekstraktesi ve ticari tablet ekstraktesi çözeltileri için yapılan antioksidan aktivite ölçümleri, adsorpsiyonla antioksidan aktivitenin düştüğünü göstermiştir. Antioksidan aktivitedeki düşme, fenolik bileşenlerin klinoptilolit yüzeyine adsorplanmasıyla ilişkilendirilmiştir.

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CHAPTER 1

INTRODUCTION

In recent years there have been a renaissance of interest in natural and herbal remedies worldwide, partly because of the realization that modern medicine is not capable of providing a "cure-all" solution against human diseases and that the presence of unwanted side-effects is almost unavoidable. Unlike modern drugs that invariably comprise a single active species, herbal extracts and/or prescriptions contain multiple active constituents. Interestingly, natural compounds contained in these "herbal cocktails" can act in a synergistic manner within the human body, and can provide unique therapeutic properties with minimal or no undesirable side-effects (Huie, 2002).

A key factor in the widespread acceptance of natural or alternative therapies by the international community involves the "modernization" of herbal medicine. In other words, the standardization and quality control of herbal materials by use of modern science and technology is critical. At present, however, quality-related problems (lack of consistency, safety, and efficacy) seem to be overshadowing the potential genuine health benefits of various herbal products, and a major cause of these problems seems to be related to the lack of simple and reliable analytical techniques and methodologies for the chemical analysis of herbal materials (Huie, 2002). Therefore, it is necessary to determine the chemical constituents responsible for medicinal activity in the herb extracts and to standardize methods to ensure reliability and reproducibility of pharmacological and clinical assays (Wang et al.,2003).

Sample preparation is the crucial first step in the analysis of herbs, because it is necessary to extract the desired chemical components from the herbal materials for further separation and characterization. Thus, the development of "modern" sample-preparation techniques with significant advantages over conventional methods (e.g. reduction in organic solvent consumption and in sample degradation, elimination of additional sample clean-up and concentration steps before chromatographic analysis, improvement in extraction efficiency, selectivity, and/or kinetics, ease of automation, etc.) for the extraction and analysis of medicinal plants is likely to play an important role in the overall effort of ensuring and providing high-quality herbal products to consumers worldwide (Huie, 2002).

Among those herbal medicines stated above, Ginkgo biloba extract (GBE) has been one of the most widely consumed phytopharmaceutical. There are many applications that demonstrate the efficacy of GBE for scavenging free radicals, treatment of disturbances of circulation disorders, cerebrovascular insufficiency, dementia, peripheral vascular disease such as arterial occlusive diseases, and age-dependent damages.

Natural zeolite (NZ) and their modified forms have been evaluated as a gastric antacid, anti-diarrheic, anti-hyperglicemyc, hipocholosterolemic and as a matrix for the release of ions and organic molecules. These products are considered as active principles, therefore, it is important from the pharmaceutical point of view, to determine if the co-administration with conventional drugs is possible (Farias et al., 2003).

Considering the purported therapeutic efficacies of Ginkgo biloba leaf extract and the possible use of clinoptilolite as a drug/drug carrier, the clinoptilolite-rich mineral was experimented to be used as a novel drug carrier for *Ginkgo biloba* L. Extract (GBE) in this study. Also, the contribution of flavonoids to the antioxidant activity of Ginkgo biloba leaf extract was investigated while comparing its activity with commercial antioxidants and reference compounds.

Throughout this manuscript, chapters investigate the subjects under study. Firstly, definition and pharmacological action of Ginkgo biloba L. is stated in Chapter-2. Then, the chemical analysis of the active constituents within the extracts obtained from Ginkgo leaves are presented in Chapter-3. The following chapter discusses the general characteristics of zeolites and more specifically, clinoptilolite as the adsorbent used in the study. Chapter-5 explains the mechanism of adsorption from solution and the key parameters affecting this process. The next chapter represents the experimental section of the study. Finally, the results and their corresponding discussions are stated in Chapter-7.

CHAPTER 2

GINKGO BILOBA L. EXTRACT

2.1 Definition

The Ginkgo L. (synonyms Pterophyllus salisburiensis, Salisburia macrophylla, maiden hair tree) is a tree of the family Ginkgoaceae, dating back 200 million years. Individual trees have been to live for hundreds of years reaching heights of 30-40 m and diameters of 4 m. The resistance of this "living fossil" to mutagenic insults as well as to modern day environmental toxins has sparked an interest in the biologic and pharmacologic potential of the antioxidant and free-radical scavenger abilities of components of Ginkgo leaf extracts (Ponto et al., 2003). Figure-2.1 shows the photographs of fresh Ginkgo biloba leaves and a commercial, standardized Ginkgo biloba leaf extract tablets.

Figure 2.1: Photograph of Ginkgo biloba leaves and a commercial product of Ginkgo biloba leaf extract from GNC.

French and German companies have approved a standardized form of G. Biloba leaf extract (EGb 761, developed by Beaufour-Ipsen Pharma, Paris, France) and Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany) that contains 24% flavonoid

glycosides, 6% terpene lactones and less than 5 ppm ginkgolic acid, the component that has allergenic properties (Smith et al.,2004).

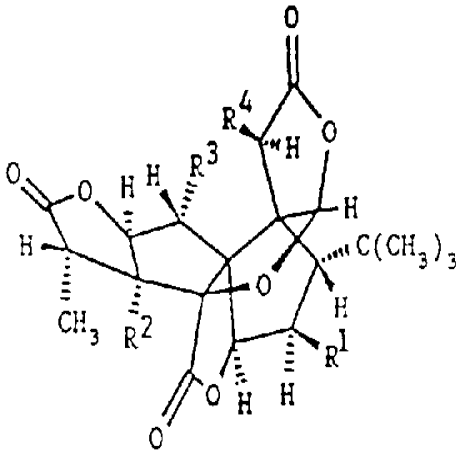
Depending on the country of origin, time of harvest, etc., the percentages of individual constituents found in G. biloba can vary considerably; therefore it is highly desirable to have a standardized preparation with a known constituent makeup for the purposes of clinical trials, drug regulation, human consumption and for purposes of research and reproducibility. Van Beek and Lelyveld (1992) noted that the concentration of lactone terpenoids was the lowest during spring and gradually increased until reaching maximum during late summer or early autumn.

2.2 Pharmacological Action

The two major fractions of the extract, the terpenes and the flavonoids, having separate properties as shown in Figure-2.2 and Table-2.1, are responsible for giving this extract its unique polyvalent pharmacological action. The terpene lactones are represented by the ginkgolides A, B, C, J and M and bilobalide.

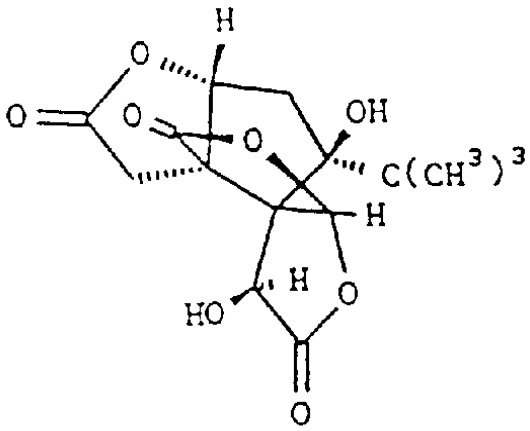
The ginkgolides are platelet-activating factor (PAF) antagonists, able to reduce platelet aggregation, thrombin activity and fibrinolysis. Therefore, they have the potential to improve blood circulation and lower blood pressure. The increase in blood circulation allows increased delivery of oxygen and glucose to the brain following an ischemic event (Smith et al.,2004). The effects of the extract on bleeding and clotting, as well as its other actions on the cardiovascular system, could have both beneficial and detrimental effects on function of the central nervous system, for example, through differential consequences for development of occlusive or hemorrhagic strokes (Gold et al.,2002). Clinical tests on ginkgolides A and B were conducted and reported by different research groups, and ginkgolide B is now believed the most active compound among these terpenoids (Lang et al.,1999).

Bilobalide, a sesquiterpene trilactone constituent of G. biloba leaf extracts, can reduce cerebral edema produced by triethyltin, decrease cortical infarct volume in certain stroke models, and reduce damage from cerebral ischemia. It has been reported that bilobalide is unstable under neutral or basic conditions (Lang et al.,1999). Figure-2.2 presents the main terpene lactone groups present in Ginkgo biloba leaf extracts.



R1	R2	R3	R4	Ginkgolide
H	OH	H	OH	A
H	OH	OH	OH	B
OH	OH	OH	OH	C
OH	H	OH	OH	M
OH	OH	H	OH	J

Ginkgolides



Bilobalide

Figure 2.2: Main terpene lactone groups present in Ginkgo biloba leaf extracts.

Free radicals have been implicated in a variety of illnesses such as arthritis, atherosclerosis, cardiovascular disorders, as well as neurodegenerative diseases. They play a role in inflammation and possibly in the multi-stage process of carcinogenesis. Free radicals can oxidise lipoproteins in the blood resulting in atherosclerosis and are also able to deaminate DNA and alter the structure of amino acids in proteins via oxidation and nitrosation. Aging and the illnesses associated with it are thought to result from oxidative stress in the body (Zhao et al.,2000).

Phenolic constituents (flavonoid glycosides) of the extract can inhibit the activity of superoxide dismutase and monoamine oxidase, two widespread enzymes that

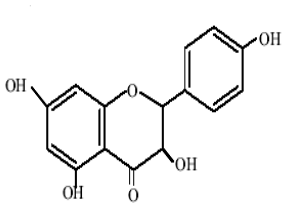
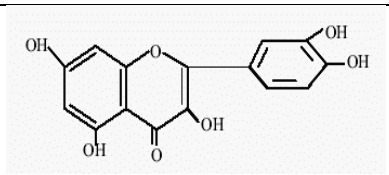
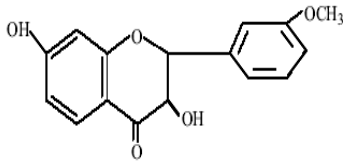
contribute to the production of free radicals in the brain and body. These antioxidant substances can scavenge free radicals that might injure neurons and thereby retard age-related changes in brain and other functions. (Gold et al.,2002). In recent years it has been identified that oxidative stress (free radicals) is a major contributing factor to the development of age-related (senile) cataracts and that the normal antioxidant protective mechanisms in the lens have been found to be significantly compromised in cases where senile cataracts occur. Strong evidence indicates that oxidative stress from ultra-violet light (sunlight) and radiation exposure induces free radical damage to the lens that contributes to senile cataracts development. The lens of the eye has been shown to be devoid of the antioxidant enzymes, superoxide dismutase (SOD), catalase and glutathione peroxidase, and is thus completely dependent upon nutritional antioxidants including Vitamin E, Vitamin C, selenium, carotenes and Ginkgo biloba L. extract for their antioxidant defenses (Meschino, 2002).

The antioxidant effect of the flavonoid fraction may be achieved by either direct attenuation of reactive oxygen species (ROS), chelating pro-oxidant transitional metal ions, expression of antioxidant proteins such as superoxide dismutase (SOD), and increase in antioxidant metabolites such as glutathione. The flavonoid fraction of the extract appears to be more effective against hydroxyl radicals than the terpene fraction. The chemical structure of the flavonoids in general is comprised of an aromatic ring and a double bond. Therefore, the flavonoids react preferentially with, and directly scavenge, the hydroxyl radicals. Additionally, the phenolic hydroxyl groups on the flavonoids may be able to chelate pro-oxidant transition heavy metal ions (e.g., Fe^{2+}), consequently inhibiting the formation of new hydroxyl radicals (Smith et al.,2004).

Antioxidant activity of phenolic compounds was correlated to their chemical structures. Structure-activity relationship of some phenolic compounds (e.g. flavonoids, phenolic acids, tannins) has been studied (Rice-Evans et al., 1996; Lien et al., 1999; Son and Lewis, 2002). In general, free radical scavenging and antioxidant activity of phenolics (e.g. flavonoids, phenolic acids) mainly depends on the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules, and is also affected by other factors, such as glycosylation of aglycones, other H-donating groups (-NH, -SH), etc. For example, flavonol aglycones such as quercetin, myricetin, and kaempferol, containing multiple hydroxyl groups, had higher antioxidant activity than their glycosides such as rutin, myricitrin, astragalin. The glycosylation of flavonoids reduced their activity. There were many reports on antioxidant components,

generally focusing on flavonoids and phenolic acids (Cai et al., 2004). Table 2.1 tabulates the main flavonoid structures present in Ginkgo biloba leaf extracts.

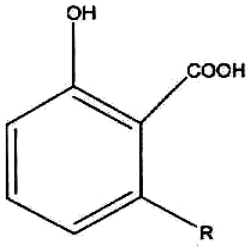
Table 2.1: Main flavonoid structures present in Ginkgo biloba leaf extract solutions (Singh, 2002).

Flavonoid	Structure	Molecular formula	Molecular weight(g/mol)
Kaempferol		$C_{15}H_{10}O_7$	338.3
Quercetin		$C_{15}H_{10}O_7$	302.25
Isorhamnetin		$C_{16}H_{12}O_7$	317

The diterpenes and sesquiterpenes, rather than the flavonoids or biflavonoids, appear to be the main component of the ginkgo extract that is responsible for the protection of brain tissue against brain injury due to reduced oxygen or blood flow. Although the flavonoids and biflavonoids found in ginkgo extract do have antioxidative properties in vitro, they are not able to pass through the blood-brain barrier in sufficient quantity to reach an effective concentration to reproduce these effects within the brain. Therefore, the diterpenes and sesquiterpenes are probably responsible for and clinical effects. In particular, ginkgolide B and bilobalide may provide the neuroprotective and anti-apoptotic effects attributed to ginkgo extracts (Gold et al.,2002).

However, some alkylphenols (ginkgolic acids, cardanols and cardols) (Figure 2.3) have been identified as potentially hazardous constituents in Ginkgo extracts. Indeed, they are considered as the compounds responsible of the allergic skin reactions frequently reported after contact with Ginkgo fruits. These compounds, besides strong

allergenic properties, possess possibly mutagenic and carcinogenic activity and do not contribute to the therapeutic action of Ginkgo extracts. Accordingly, a requirement for a maximum concentration of ginkgolic acids in G. biloba extracts has been included in the draft monographs of US and European pharmacopoeias by establishing a limit value of 5 ppm (Fuzzati et al.,2003). Figure 2.3 illustrates the structures of ginkgolic acids.



$GA_{15:1}$ R= $(CH_2)_7$-CH=CH-$(CH_2)_5CH_3$

$GA_{13:0}$ R= $(CH_2)_{12}CH_3$

$GA_{17:1}$ R= $(CH_2)_9$-CH=CH-$(CH_2)_5CH_3$

$GA_{15:0}$ R= $(CH_2)_{14}CH_3$

Figure 2.3: The structures of ginkgolic acids.

In summary, GBE is currently marketed for enhancing brain function using the following language:

- Promotion and maintenance of mental sharpness, concentration, and focus

Restorative effects on the brain- specifically memory function and the ability to concentrate. Enhances nootropic activities-agents known to enhance mental clarity, memory and alertness

- Enhances blood circulation and oxygen supply to the brain and extremities. Increases the amount of oxygen in the blood supply to the brain
- Promotes the elasticity and strengthening of blood vessels and capillary walls

Increases vascular tone by supporting healthy circulation in the arteries

- Increases the uptake of glucose across the blood-brain barrier
- Powerful antioxidant that protects brain cells and the cardiovascular system

Potent antioxidant that traps, neutralizes and eliminates free radicals before they damage healthy cells. Maintains healthy levels of platelet aggregating factor (PAF) (Ponto et al.,2003)

CHAPTER 3

CHEMICAL ANALYSIS OF GINKGO BILOBA LEAF EXTRACT

Ginkgo leaf extracts typically contain dozens of organic compounds. Besides trace amounts of ginkgolides and bilobalide, there are at least 12 phenolic compounds, ~30 flavonoid compounds, and variable amounts of tannins, anacardic acids, chlorophyll, and lipids. All of the reported separation methods are based on the small polarity differences between ginkgolides and the major "impurities", which are usually hydroxyl group-bearing or oxygen-containing compounds. Therefore, the reported separation processes would require many hours utilizing a silica gel column, more than one solid-phase extraction column, or repeated extractions with large amounts of organic solvents (Lang et al.,1999).

3.1 Extraction of Active Constituents

3.1.1 Definition

Interest in herbal remedies has led to rapid improvements in the extraction techniques to isolate the active principles from herbs. A herbal extract is defined as the compound and/or compound mixtures obtained from fresh or dried plants, or parts of plants: leaves, flowers, seeds, roots and barks, by different extraction procedures. Development of a more efficient extraction method to obtain maximum yield of the active components is the basis of the extraction of herbal remedies.

Table 3.1 briefly summarizes the paths for the extraction of active constituents within the Ginkgo biloba L. extracts:

Table 3.1: Extraction methods previously experimented for Ginkgo biloba leaves and products

Author & Date	**Extracted Sample**	**Extraction Method**
Yu et al., (2003)	Ginkgo biloba L.	- 10 g, shaken in certain amount of 70% ethanol at 150 rpm - Filtered through 0.2 μm nylon membrane - The filtrate(Extract A) was concentrated by rotary evaporator - The concentrate was finally dissolved in 100 ml methanol as crude extract
Yoon et al, (1997)	Ginkgo biloba L.	- 1 g dried&pulverized sample, extract with 100 ml methanol for 24 h - Filter the solution - Concentrate with rotary evaporator - Dissolve in 5 ml methanol
Ganzera et al., (2001)	Ginkgo extract (commercial)	- Extract 500 mg sample 3 times with 3 ml methanol by sonication for 10 min. - After centrifugation at 3000 rpm for 10 min, collect the supernatants in 10 ml flask and make up the volume with methanol
Chin et al., (2000)	Ginkgo extract (commercial)	- Grind 4-5 tablets and add 20 ml of 80% methanol solution to dissolve the sample - Shake for 30 min and leave the sample till two phases are separated - Remove 10 ml of the upper layer and treat with 10 ml 5.5% HCl solution - Reflux the solution in 85^0C water bath for 30 min - Filter the solution for HPLC analysis
Chiu et al., (2002)	Ginkgo biloba L.	- Place 20 g powder in 30×100 mm thimble and extract with 200 ml of 95% ethanol for 24 h. - Hydrolyze 20 ml solution of with 10 ml of 25% HCl for 120 min at 80^0C.

Table 3.1: Extraction methods previously experimented for Ginkgo biloba leaves and products (continued).

Author & Date	Extracted Sample	Extraction Method
Sloley et al., (2003)	Ginkgo extract (commercial)	- Dissolve the samples in 25% aqueous HCl:methanol(10:70 v/v) at a concentration of 4 mg/ml. - Clarify the samples by centrifugation - Heat 200 µl of this solution at 100^0C for 25 min and filter the sample for HPLC analysis
Hostettman et al, (1995)	Ginkgo biloba L.	- Reflux 600-800 mg ground leaf material in a 50-ml round bottom flask fitted with a reflux condenser for 15 min in 5 ml methanol-water(90:10) - Filter the solution - Extract the leaf materal a second time with 5 ml of methanol:water(90:10) - Filter and combine the collected extracts - Evaporate to dryness under a stream of N_2 - Reconstitute the residue with 1 ml methanol - Filter for HPLC analysis
Hostettman et al, (1995)	Ginkgo extract (commercial)	- Place 400 mg of extract into 50 ml volumetric flask along with 40 ml of methanol:water(90:10) - Sonicate the sample for 20 min at 60^0C until completely dissolved - Filter the solution for HPLC analysis
Xu et al., (2001)	Ginkgo biloba L.	- Extract dried leaves(1 kg) three times with 4 l of 70% ethanol for 3 h at 65^0C - Concentrate the combined extracts by rotary evaporator at 50^0C, then dilute to 4 l. - Filter the extract solution - Extract with 4 l heptane to remove ginkgolic acid and ginkgol.

3.1.2 Factors Affecting Extraction

Numerous cases affect the extent to which the extraction could be performed best. The most important of all those cases are the solvent type and composition, ratio of the quantity of the material to be extracted to the volume of the solvent and the particle size of the extracted material. Figure-3.1 presents two extract solutions obtained from Ginkgo biloba leaf powder and commercial Ginkgo biloba leaf extract tablet powder.

Figure 3.1: Extracts of Ginkgo leaf powder and the commercial GNC tablets obtained by solvent extraction.

3.1.2.1 Solvent Type and Composition

Solvent selection for the extraction process mainly depend on the polarity of the desired constituent to be extracted. By adjusting the polarity of the solvent, one can easily extract the target component.

Chiu et al.(2002) indicated in their study that Ginkgo's lactones and flavonoids have less affinitive solubility in water than in ethanol. Therefore, it is also quite important to optimize the water content of the extracting solvent system.

3.1.2.2 Solid-to-liquid Ratio

In the extraction process, it is quite important to optimize the solid-to-liquid ratio. When the solvent amount is high, the resultant extract won't be concentrated, which means the detection of the terpene constituents by ELSD is quite difficult. However, the concetrated extract solution when injected to the HPLC column causes the column and the injection port line clogged. On the contrary, when the solvent amount is small, then the contact of the extracted particle with the solvent wouldn't be achieved, so the extraction process would be ineffective.

3.1.2.2 Particle Size of the Extracted Material

The extraction efficiency is strongly affected by the particle size of the extracted material. Chiu et al.(2002) investigated the extraction yields of lactones and flavonoids for two sizes of Ginkgo leaves powder (larger than and smaller than 105 μm). According to their experimental data, a particle size smaller than 105 μm had the best extraction efficiency of terpene lactones and flavonoids. This finding corresponded that the extraction rate increased with a decrease of particle size owing to a smaller intra-particle diffusion resistance for a smaller particle size.

3.2 Separation and Detection of the Active Constituents

The separation of compounds can be achieved rapidly and routinely with chromatography. There are a multitude of chromatographic techniques but they all have in common separation of compounds through the use of variations in mobile and stationary phase. Liquid chromatography (LC) is primarily used for the separation of high-molecular weight, involatile or/and polar compounds in three modes, i.e.normal phase, reverse-phase and gel-permeation chromatography. Reverse-phase chromatography is the most widely used separation mode. The term reversed-phase liquid chromatography derived from the fact that the mobile phase is more polar than the stationary phase. A vast range of applications has been reported using this technique, especially in the area of pharmaceutical drug analysis and other biologically important compounds.

3.2.1 Terpene Lactones

Many methods have been tried to analyze ginkgolides and bilobalide. In the method using initial high-performance liquid chromatography (HPLC) with ultraviolet (UV), many trace impurities interfere because of the poor UV characteristics (200-220 nm) of terpenes and their low concentrations in GBE and phytopharmaceuticals. Furthermore, other compounds such as flavonoids which are present in higher concentrations and have stronger UV absorption can interfere with determination of ginkgolides. Therefore, determination of ginkgolides in GBE leaves is problematic (Amanlou et al.,2000).

Several investigators have applied refractive index as an alternative detection method. Although this method is more suitable and has been used with considerable success, sensitivity and baseline stability still remain a problem. Other techniques including gas chromatography-flame ionization detection after silylation, HPLC-mass spectrometry and NMR have been proposed and are used. However, these three methods are not suitable for routine determination in terms of cost, time or complex sample preparation (Tang et al., 2003).

Evaporative light scattering detection (ELSD) is a mass and non-selective detector that responds to the number and size of nonvolatile particles. Therefore, it is applied to the analysis of poor UV absorption and nonvolatile compounds. In 1995, Componovo et al. reported the coupling of HPLC with ELSD in order to detect ginkgolides and BB. Results indicate that it is one of the most suitable methods for routine determinations of such compounds (Tang et al., 2003). Figure-3.2 illustrates a typical HPLC-ELSD chromatogram of a standard mixture of pure compounds present in Ginkgo biloba leaf extract solutions.

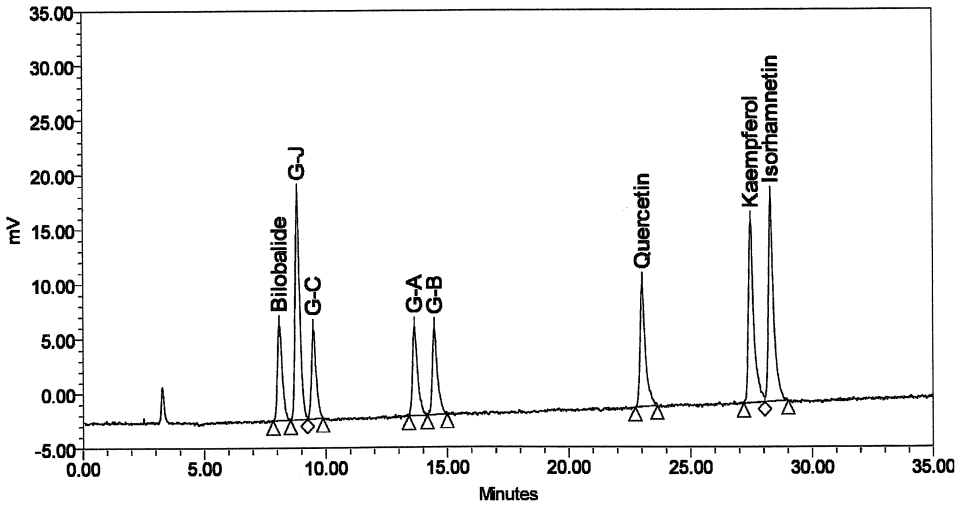
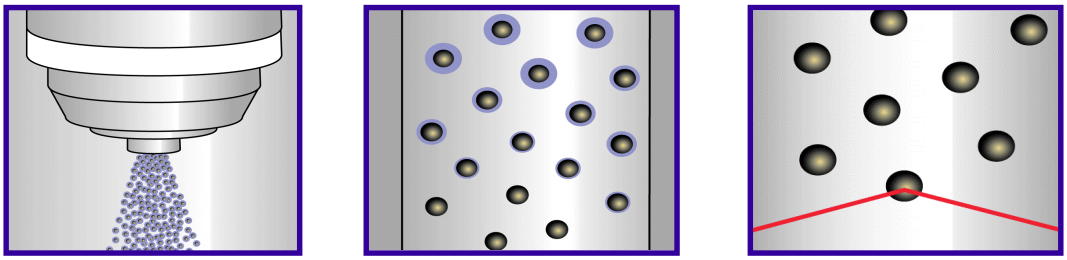


Figure 3.2: A typical HPLC–ELSD chromatogram of standard mixture with 200 ng of ginkgolide A, B, C, bilobalide, quercetin, kaempferol and isorhamnetin and 350 ng of ginkgolide J on column (Li et al.,2002).

The Evaporative Light Scattering Detector (ELSD) is designed for use with High Performance Liquid Chromatography (HPLC) systems to analyze any compound that has sufficiently lower volatility than the mobile phase. Some of its application areas include the analysis of carbohydrates, pharmaceuticals, lipids, triglycerides, underivitized fatty and amino acids, polymers, surfactants, nutraceuticals, and combinatorial libraries (ALLTECH ELSD 2000 Manual, 2003).

The unique detection principle of evaporative light scattering detectors involves nebulization of the column effluent to form an aerosol, followed by solvent evaporation in a heated drift tube, and the detection of the remaining non-volatile solute particles in the light scattering cell. Figure-3.3 schematically presents the basic operating principles of Evaporative Light Scattering Detection (ELSD).



(a) (b) (c)

Figure 3.3: Three basic operating principles of ELSD, (a) nebulization, (b) evaporation, (c) detection.

In ELSD, the nebulizer-gas flowrate and drift tube temperature are the major instrumental parameters affecting the signal response. The nebulizer gas flowrate determines the size of the droplets formed during nebulization. In general, large droplets are formed at low gas flowrates, which results in spikes and noisy signals. On the other hand, increasing the gas flowrate results in a marked decrease of signal response owing to the reason that smaller droplets scatter less light. With respect to the drift tube temperature, solvent evaporation is not completed at low temperature and the detector response is decreased at high temperature.

3.2.2 Flavonoid Glycosides

The total amount of flavonoid glycosides is not easy to estimate due to their great variety and the difficulty to get the pure standards. Fortunately, by acidic hydrolysis, even the great variety of flavonoid glycosides can be reduced to three major aglycones: isorhamnetin, kaempferol and quercetin (Yu et al.,2003). Then, the quantification of the total flavonoid glycosides can be achieved in terms of the amount of these three aglycone structures which are all commercially available and easy to analyze by RP-HPLC. High performance liquid chromatography with ultra-violet detection (HPLC-UV) approach (350-370 nm) has been used for the determination of the total flavonoids in the dried leaves, standardized extracts ($\geq 24\%$) and finished drugs (van Beek, 2002).

3.2.3 Ginkgolic Acids

Thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) were initially used for qualitative analysis of the ginkgolic acids. Verotta and Peterlongo (1993) reported the scale-up separation and identification by supercritical fluid extraction (SFE) and capillary gas chromatography-mass spectrometry (GC-MS) as trimethylsilyl derivatives, but no recovery data or extraction efficiencies were given. Recently, liquid chromatography-electrospray ionization (ESI-MS) has been successfully applied to analyze ginkgolic acids (He et al.,2002).

Since all ginkgolic acids share the same polar groups, they elute together in HPLC chromatograms. Therefore, a reliable method should be adopted in order to resolve the peaks of ginkgolic acids. He et al.(2002) developed a reversed-phase argentation HPLC system with the mobile phase system consisting of methanol-5% acetic acid (90:10). They found that the addition of 0.03 mol/l silver ion as complexation agent to the eluent

showed a sharp separation and a considerably increased selectivity between ginkgolic acids as shown in Figure-3.4.

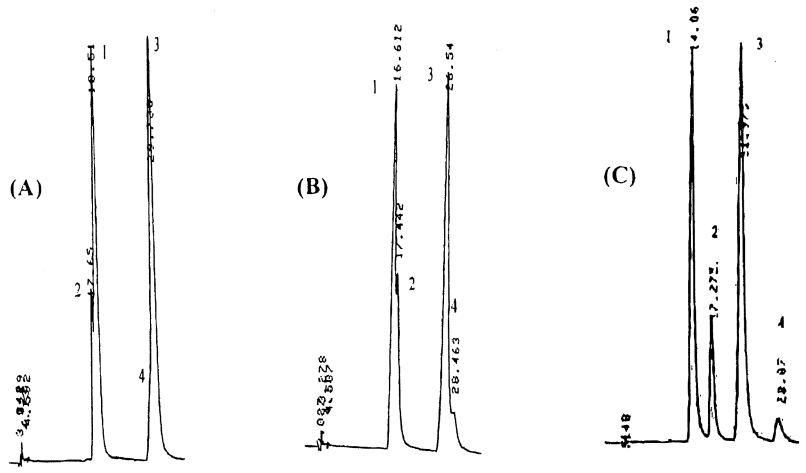


Figure 3.4: HPLC separation of ginkgolic acids with and without the addition of silver ion. Mobile phase: (A) methanol-5% aqueous acetic acid(90:10); (B) A + 0.02 mol/l [Ag^+]; (C) A + 0.03 mol/l [Ag^+]. Peaks: 1=$GA_{15:1}$; 2=$GA_{13:0}$; 3=$GA_{17:1}$; 4=$GA_{15:0}$ (He et al.,2002).

On the other hand, due to the existence of ginkgolic acids in very small amounts within Ginkgo biloba leaf extract solutions, the ginkgolic acid concentration should be increased by removing chlorophyll and other co-extracted impurities. Therefore, the extraction step plays a crucial role in further detection of ginkgolic acids.

CHAPTER 4

ZEOLITES

4.1 Definition

Only a few of the existing natural zeolites in the world are found in sufficient quantity and purity as required by industry. Within this group, silica-rich heulandite (clinoptilolite) and mordenite are the most important and play a significant industrial role. Important uses of zeolite minerals include water softening, gas and petroleum processing, mining, sewage treatment, paper products, among others (Englert et al.,2004).

Synthetic zeolites are manufactured on a large scale for industrial use, but natural zeolites have not yet found extensive application as commercial molecular sieves, even though a few, particularly, clinoptilolite, are abundant in volcanogenic sedimentary rocks. Of the more than 40 natural zeolite species known today, clinoptilolite is the most abundant in soils and sediments.

4.2 Crystal Structure of the Zeolites

Zeolites are porous, crystalline, hydrated aluminosilicates of alkali and alkaline earth cations that possess a three-dimensional structure. The zeolite framework consists of an assemblage of SiO_4 and AlO_4 tetrahedra joined together in various regular arrangements through shared oxygen atoms to form an open crystal structure containing pores of molecular dimensions into which guest molecules can penetrate. The negative charge created by the substitution of an AlO_4 tetrahedron for a SiO_4 tetrahedron is balanced by exchangeable cations (e.g., Na^+, K^+, Ca^{2+}, Mg^{2+}), which are located in large structural channels and cavities throughout the structure. These cations play a very important role in determining the adsorption and gas-separation properties of zeolites. These properties depend heavily on the size, charge density, and distribution of cations in the porous structure (Armenta et al., 2001). Figure 4.1 shows the tetrahedron primary building unit of zeolite framework.

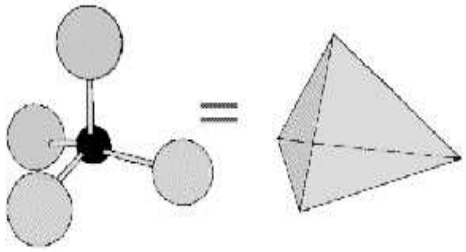


Figure 4.1: Tetrahedron primary building unit

4.3 Clinoptilolite-rich Natural Zeolite

Natural zeolites possess many desirable ion exchange, molecular sieving and catalytic properties, which make them valuable minerals. Certain natural zeolites such as mordenite and clinoptilolite have made headway in these areas because of the ready availability, low prices and high Si/Al ratio (Kurama et al., 2002).

The framework of clinoptilolite is formed by two parallel channels of 10-member rings (channel A) and eight-member rings (channel B) connected to a third channel C of eight-member rings. The approximate channel sizes (A^o) are: A, 4.4 ×7.2; B, 4.1×4.7; C, 4.0 × 5.5. Figure-4.2 illustrates the channel and crystal structure of clinoptilolite.

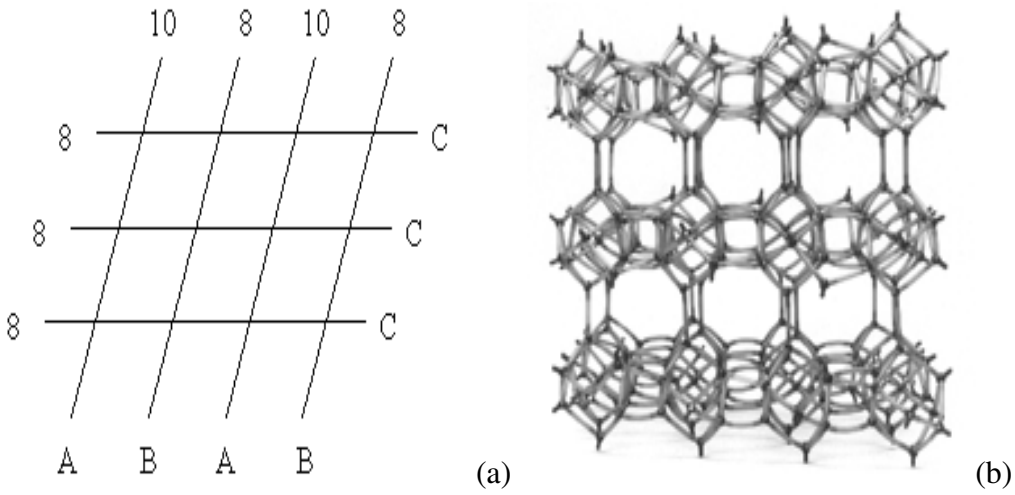


Figure 4.2: Channel (a) and crystal structure (b) of clinoptilolite

Small hydrated cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) can easily enter the channels of clinoptilolite and compete for the major exchangable-cation sites, designated as M(1), M(2), M(3), and M(4). The major cations are located and distributed as follows: M(1) is located in channel A, where Na > Ca; M(2) is located in channel B, where Ca > Na; M(3) is located in channel C, where there is only K; and M(4) is located in channel A, where there is only Mg (Armenta et al.,2001). Figure-4.3 shows the main components in the clinoptilolite structure.

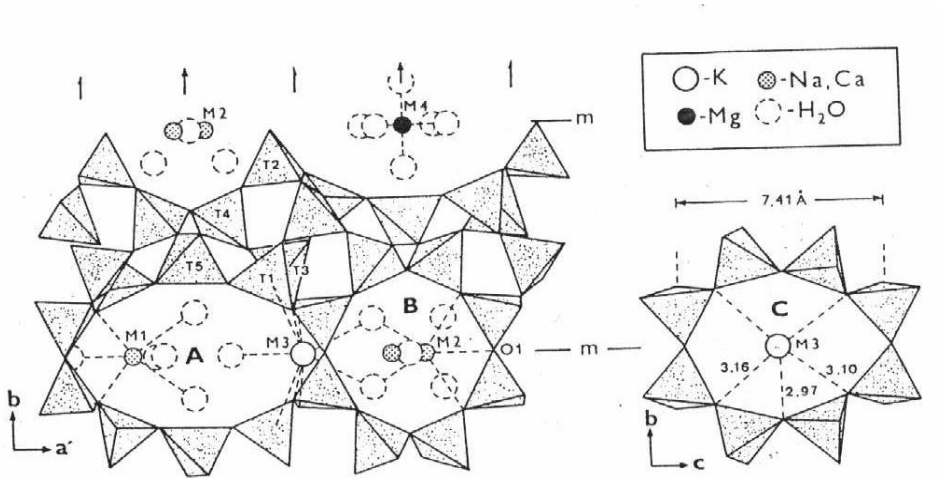


Figure 4.3: Main components in the clinoptilolite structure

4.4 Biomedical Applications of Clinoptilolite

The use of the natural zeolites in animal health and nutrition, and also their long-term chemical and biological stability has been studied in the literature. Pavelic et al.(2001) reported that clinoptilolite treatment of mice and dogs suffering from a variety of tumor types led to improvement in the overall health status, prolongation of life-span, and decrease in tumor size.

In particular, several works on biomedical applications of natural clinoptilolite have been published during the past five years. Considering that many biochemical processes are closely related to ion-exchange, adsorption and catalysis, it is expected that natural zeolites could make a significant contribution to the pharmaceutical industry and medicine in the near future (Farias et al., 2003).

Besides, due to its amphoteric character, clinoptilolite tends to neutralize the aqueous medium acting either as proton acceptor or as a proton donor. For example, if

an appropriate dosage of clinoptilolite is able to increase the stomach pH, and to decrease the intestine pH as it goes through the gastrointestinal tract, it may modify the absorption of certain drugs at both organs (Rivera et al.,2000).

Rivera et al.(2003) studied the adsorption of the model drugs -metronidazole, sulfamethoxazole and aspirin- on the purified natural clinoptilolite from Tasajeras deposit (Cuba) (37-90μm), in order to investigate the possibility of using this mineral as carrier in slow-release dosage forms. Since these molecules were larger than the clinoptilolite channels and it was impossible for the drugs to enter them, they adopted the 8- and 10-ring window models to represent the interaction with the external surface of that zeolite. They found that the presence of drugs on the zeolite does not produce structural changes, and results in a strong decrease of the specific surface area.

In order to accept the in-vivo administration of zeolitic material, the material should be made in compliance with the requirements of NIRB 1152 as stated in Table 4.1.

Table 4.1: Chemical requirements for the NZ (Fuentes et al.,1997).

Elements	**Requirement(%)**	**Toxic elements**	**Requirement(%)**
SiO_2	64.00-66.00		
Al_2O_3	10.00-12.00	F	<10 ppm
Fe_2O_3	1.50-2.20	Pb	<10 ppm
FeO	0.20-0.5		
MgO	0.30-0.90	As	<3 ppm
CaO	2.50-6.00	Cd	<2 ppm
Na_2O	1.00-2.00		
K_2O	1.00-2.00	Hg	<5 ppm
P_2O_5	0.05-0.07		
H_2O	4.00-7.00		

In addition to those requirements stated in Table 4.1, the zeolitic phase of the natural zeolite must be in the range of 75-80% (Fuentes et al.,1997). Therefore, the enrichment of the zeolitic phase by an appropriate method is to be considered (e.g. fluidized bed technique).

CHAPTER 5

ADSORPTION FROM SOLUTION

5.1 Definition

Adsorption occurs whenever a solid surface is exposed to a gas or liquid. It is defined as the enrichment of material or increase in the density of the fluid in the vicinity of an interface. Under certain conditions, there is an appreciable enhancement in the concentration of a particular component and the overall effect is then dependent on the extent of the interfacial area. For this reason, all industrial adsorbents have large specific surface areas (generally well in excess of 100 m^2 g^{-1}) and are therefore highly porous or composed of very fine particles (Rouquerol et al., 1999).

Adsorption separation methods may be a better way to increase the active ingredient contents within the extract solution because the problems in the adsorption separation process, such as gradient elution and longer separation period, could be resolved by selecting appropriate adsorbents and designing better operating conditions.

5.2 Factors Affecting Adsorption

In the case of adsorption with zeolite mineral, the difference in the adsorptive behaviour is fundamentally related to the polarity of the molecules, and the nature of the zeolitic material. Other key parameters are the initial solution concentration and the solid loading.

5.2.1 Polarity of the Solvent

Hydrophobic interaction and hydrogen bonding are known to be the major mechanisms in physical adsorption. In an aqueous solution, hydrogen bonding between solutes and adsorbents become less important for adsorption but the hydrophobic interaction plays a dominant role in adsorption due to the strong hydrogen bonding characteristics of water. It has been reported that non-polar adsorbents were effective for adsorbing non-polar solvent (Yoon et al.,1997).

5.2.2 Nature of the Zeolitic Material

The key to an adsorption separation process is to use an adsorbent with a high selectivity, which may be finely designed by modification of its surface chemistry and its pore structure.

In adsorption studies with zeolite mineral, washing of the zeolitic material directly affects the adsorption process. The removal of some secondary phases, from NZ to washed-NZ, as thin layers attached on the zeolitic surface facilitates, to some extent, a more effective interaction between the drug and the zeolitic surface (Farias et al.,2003). Also, the adsorption of non-polar molecules would be favored in the case of increased Si/Al ratios of the zeolitic material.

5.2.3 Solid-to-liquid Ratio

The amount of adsorbent should be well-designed relative to the amount of the solution, in which the adsorption process is carried out. The adsorption medium should be concentrated with the adsorbent, so that the maximum total contact surface could be achieved. On the other hand, excessive amount of adsorbent would cause some of the surface of the adsorbent not to interact with the solution. Generally, the optimum solid-to-liquid ratio is to be selected in order to reach the best adsorption performance.

5.3 Previous Studies on the Adsorption of Ginkgo biloba L. Extract

There exist a few studies regarding the adsorption of Ginkgo biloba leaf extract constituents in literature. The most important ones among those studies were listed in the Table 5.1.

Table 5.1: Previous studies on the adsorption of Ginkgo leaf extract constituents.

Author and Date	Adsorbed constituents	Adsorbent
Yu et al.,(2003)	Flavonoid glycosides	Hydrophobic and anion-exchange membranes
Xu et al.,(2000)	Flavonoid glycosides and terpene lactones	Copolymer of methacrylate and divinylbenzene(MA-DVB)
Xu et al.,(2000)	Flavonoid glycosides and terpene lactones	PVA-gelatin beads
Yoon et al.,(1997)	Flavonoid glycosides	Polycarboxyl ester resin(XAD-7)

Yu et al.(2003) investigated the adsorption of flavonoid glycosides on the surface of adsorptive membranes including commercial hydrophobic and anion-exchange membranes. Prior to the adsorption process, they prepared the crude *Ginkgo biloba* L. extracts using ethanol aqueous solution. They found that hydrophobic and anion-exchange membranes exhibited close adsorption performance.

Xu et al.(2000) synthesized macroporous polymethacrylate beads via suspension polymerization of methacrylate (MA) and divinylbenzene (DVB) as crosslinker. Thus, they obtained a kind of adsorbent with high selectivity for ginkgo flavonol glycosides and terpene lactones. They concluded that the adsorbed amount of terpene lactones increased with increasing MA content, whereas the adsorbed amount of both flavonol glycosides and terpene lactones increased with decreasing content of DVB moiety in the adsorbent.

In another study, Xu et al.(2000) synthesized spherical adsorbents derived from the glutaraldehyde cross-linked hybrid of gelatin and polyvinyl alcohol (PVA) and investigated their adsorption selectivity for ginkgo flavonol glycosides and terpene lactones in detail. They found that an adsorbent with an appropriate gelatin content could selectively adsorb flavonol glycosides with a high adsorption capacity based mainly on hydrogen bonding interactions, while showing only a fairly low adsorption capacity for terpene lactones.

Besides, Yoon et al.(1997) developed a simple purification method in which flavonoid compounds were selectively adsorbed onto a polycarboxyl ester resin (XAD-7) from methanol extract of ginkgo leaves. They concluded their study that the pH did not affect the adsorption of flavonoids and the solution polarity played a key role in the selective adsorption.

CHAPTER 6

EXPERIMENTAL

Experiments will be conducted according to the diagram shown in Figure 6.1.

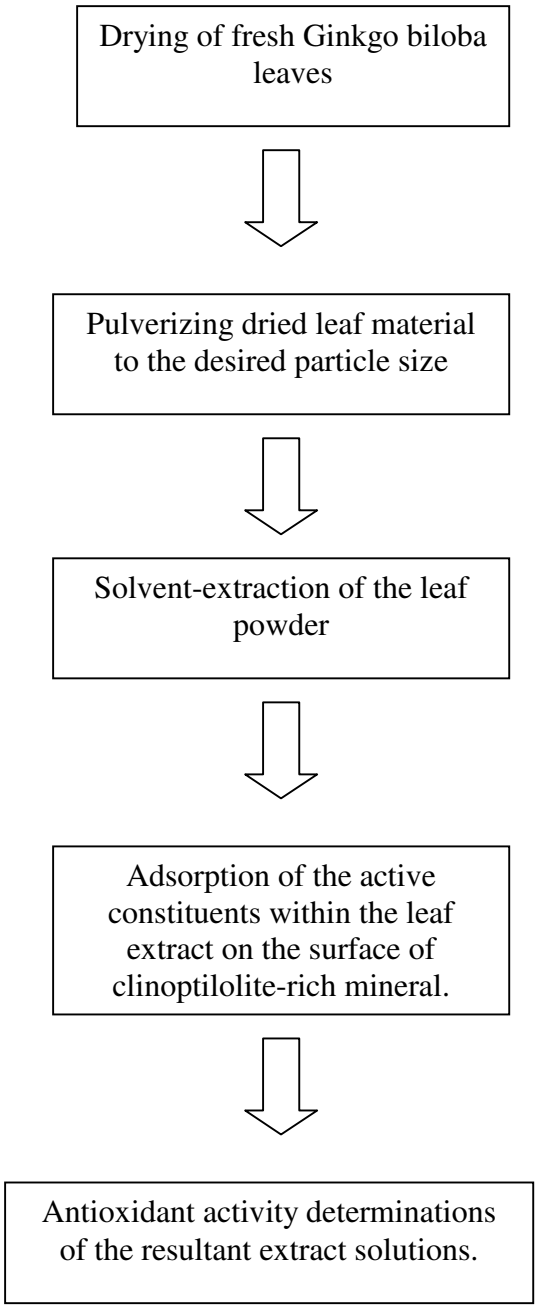


Figure 6.1: Flow-chart of the experimental study.

6.1 Materials

6.1.1 Standard Compounds

Quercetin, Kaempferol, Isorhamnetin and Ginkgolide C were purchased from Fluka, Ginkgolide A, Ginkgolide B and Bilobalide were from Sigma. Internal standard Morin was from Sigma. Mixture of ginkgolic acids standard was supplied from Indena Chemical Laboratories (Milan, Italy).

6.1.2 Reagents

Folin Ciocalteu's phenol reagent, ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) and α-Tocopherol were obtained from Sigma, sodium carbonate was from Fluka, Trolox was from Aldrich, L(+)Ascorbic acid and hydrochloric acid (37% purity) were from Merck Co. (Hohenbrunn, Germany).

6.1.3 Solvents

Methanol, acetone and trifluoroacetic acid (TFA) were obtained from Merck Co., ethanol was from DOP. All solvents were HPLC grade.

6.1.4 Samples

6.1.4.1 Ginkgo biloba Leaves and Tablets

The fresh Ginkgo leaves were collected from a male Ginkgo tree in the botanical garden of Ege University at the end of August, 2003. Commercial Ginkgo biloba L. tablets were supplied from General Nutrition Centers Inc. (GNC).

6.1.4.2 Zeolitic Material

Clinoptilolite-rich mineral was collected from Gördes (Manisa) deposit in the western part of Turkey (Enli Madencilik A.Ş.). The characterization study of the zeolitic material is presented in Appendix-A.

Since natural zeolite is of sedimentary origin, most of the grains have attached on the surface small amounts of non-zeolitic phases that still remain after the purification of the raw material. These phases might modify the interaction between the drugs and the zeolitic surface (Farias et al., 2003). For this reason, the clinoptilolite samples were

washed via shaking the samples in volumetric flasks filled with deionized water in a constant-temperature water bath kept at 100^0C. The water was replaced for several times until the spent water was almost clear. Then the clinoptilolite samples were dried in an oven by raising the temperature up to 350^0C in increments and left at that temperature for 16 hr. The sample was further dried in an oven under vacuum at 150^0C for 8 hours.

6.2 Methods

6.2.1 Extraction Studies

Mantle et al.(2000) investigated various combinations of solvent type and extraction techniques to determine the optimum conditions for plant tissue extraction and found that highest levels of antioxidant activity were consistently obtained using 80% ethanol:20% water. Owing to this reason, the crude Ginkgo biloba L. extracts were prepared using 80% ethanol in this study. Also, ethanol was chosen because of its nontoxicity for dietary purpose, and a high-percentage ethanol solution was employed for the expectation of better extraction performance.

Besides, because of the poor stability of ginkgo terpene lactone in alkali medium, the experiments were carried out in the media with a pH value of 2-6.5.

6.2.1.1. Preparation of the dry leaf material for extraction

The fresh Ginkgo leaves were collected from the same area (botanical garden of Ege University) and the same period of day (end of August, 2003, from 9 a.m. to 11:30 a.m.), because it can exist some differences between the quantity and chemical composition of the active constituents extracted from different geographical areas, different growing stage and period of day. August was chosen for the collection of the leaf material, since the concentration of lactone terpenoids was the lowest during spring and gradually increased until reaching maximum late summer or early autumn (van Beek et al., 2001).

Prior to drying stage, leaves were washed with distilled and deionized water, respectively. Then, the leaves were oven dried at 37^0C for 3 consequtive days. Drying at room temperatures higher than 55^0C should be avoided, since it can lead to inactivation of phenolics or could decrease their extractability in solvents and affect the quantification. The dried leaves, having a relative humidity value of ≤ 4 % (Sartorius,

MA100) were pulverized in a blender to particle sizes of 106-250µm. Homogenization by blender was performed full speed at room temperature and separated by 1 minute time intervals to prevent possible frictional heating of the sample. The leaf powder was mixed thoroughly and stored in amber glass bottles at about 20^0C till further use.

6.2.1.2 Extraction Kinetics

Extracted samples were prepared by weighing 2.43 g powdered commercial Ginkgo leaf extract from GNC and dissolving the samples in 10 ml, 80% aqueous ethanol solutions by subjecting the extract solutions in a constant temperature water bath kept at 29^0C with a revolution speed of 168 rpm. Then, for every hour 5 ml of the aliquot samples from the upper layer of the extract solutions were subjected to hydrolysis with 5.5% ethanolic solution in a constant temperature water bath kept at 85^0C for 30 minutes. The hydrolyzed samples were filtered over 0.45µm syringe filter and stored at $+4^0$C until HPLC analysis.

For many analytical methods using HPLC as the end step, the samples cannot be directly injected into the instrument. Sample preparation is therefore needed to isolate the analytes of interest, to pre-concentrate them in order to lower detection levels and also to protect the analytical column from substances which may potentially damage the bed of packing material (Lough et al., 1996). Solid-phase extraction (SPE) is one of the most common forms of sample preparation. With SPE a liquid sample is injected into the top of a plastic syringe shape column containing a small amount (often 100-500 mg) of a selective adsorbent as shown in Figure 6.6. The properties of the adsorbents are similar to HPLC columns and so the same principles apply for the retention and desorption of analytes.

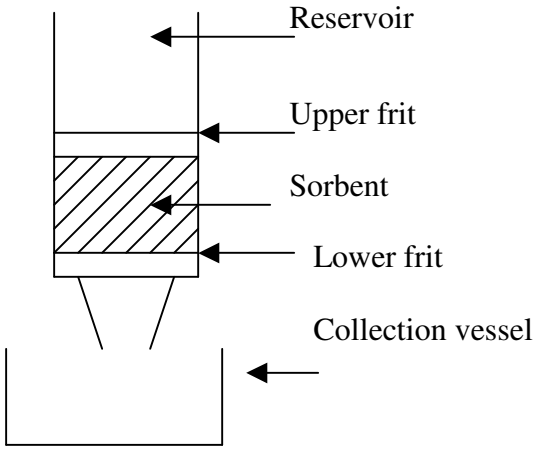


Figure 6.2: A typical solid phase extraction cartridge

The SPE cartridges normally require pre-conditioning before use, typically a wash with 1 cm^3 of first methanol, and then deionized water. Therefore, in the time of analysis, the samples were brought to room temperature and solid-phase extraction (SPE) cartridges (AccuBond, reversed-phase, non-polar, octadecyl silane packing material) were used to remove the non-polar matrix compounds from the extract solution before injection to HPLC.

6.2.1.3 Extraction with Different Solvent Systems

In order to extract the desired constituents within the Ginkgo biloba leaf and extracts, several extraction paths were stated in literature. To avoid too many polar impurities, in almost all approaches water is an important constituent of the solvent initially used for the extraction of ginkgolides from Ginkgo leaves. Normally an organic solvent like methanol or acetone is added to improve the rate of extraction because Ginkgolide A and especially Ginkgolide B are poorly soluble 100% water at room temperature (van Beek, 2002).

Camponovo et al.(2000) compared the efficiency of methanol, methanol-water (1:1) and refluxing water and reported that all three solvents extracted the terpene trilactones equally well. However, methanol:water (1:1) was the preferred choice because it gave the most clean extract. Also they reported that the addition of a small percentage acid, e.g., 1% acetic acid, would reduce the amount of co-extracted chlorophyll and would diminish decomposition of the rather labile bilobalide during

extraction. The extraction studies performed were tabulated in tables from Table 6.1 to Table 6.8.

Table 6.1: Extraction of Ginkgo leaf powder with MeOH.

1) 12.5 g Ginkgo leaf powder (106-250µm) was sonicated 3 times during 10 min with each time 75 ml MeOH (centrifuge if necessary). 2) The collected extract was centrifuged at 3000 rpm during 10 min 3) Supernatants were combined in a 250 ml volumetric flask 4) MeOH was added in order to make up the volume to 250 ml and the extract solution was filtered over 0.45µm syringe filter and stored at+4^{0}C until analysis.

Table 6.2: Extraction of Ginkgo leaf powder with acetone derived from methanolic extract.

1) 40 ml methanolic leaf extract prepared as stated in Table-6.1 was evaporated under reduced pressure at 45^{0}C. 2) The constituents were redissolved in 40 ml acetone. 3) The resultant extract solution was stored at +4^{0}C until further analysis

Table 6.3: Extraction of Ginkgo leaf powder and commercial GNC tablets with acetone.

1) 12.5 g Ginkgo leaf powder (106-250µm) and 12.5 g powdered GNC tablets were sonicated 3 times during 10 min with each time 75 ml acetone (centrifuge if necessary), separately. 2) The collected extracts were centrifuged at 3000 rpm during 10 min 3) The supernatants were collected in a 250 ml volumetric flask 4) Acetone was added in order to make up the volume to 250 ml and the extract solutions were filtered over 0.45µm syringe filter. 5) Both extracts were stored at +4^{0}C until further analysis.

Table 6.4: Extraction of commercial GNC tablets with methanol.

1) 4 to 5 commercial GNC tablets (3.2405 g) were ground and 20 ml 80% aqueous methanol solution was added to dissolve the sample.
2) After shaking for 30 min., the mixture was placed aside until the two phases were separated.
3) 10 ml of the upper layer was removed and treated with 10 ml, 5.5% HCl solution.
4) The solution was refluxed in 85^0C water bath for 30 min.
5) Finally, the solution was filtered over 0.45µm syringe filter for further analysis.

Table 6.5: Extraction of commercial GNC tablets with ethanol.

1) 12.15 g ground commercial GNC tablets were dissolved in 50 ml 80% aqueous ethanol solution was added to dissolve the sample.
2) After shaking for 30 min., the mixture was placed aside until the two phases were separated.
3) 30 ml of the upper layer was removed and treated with 30 ml, 5.5% HCl solution.
4) The solution was refluxed in 85^0C water bath for 30 min.
5) Finally, the solution was filtered over 0.45µm syringe filter for further analysis.

Table 6.6: Extraction of Ginkgo leaf powder with ethanol.

1) 10 g Ginkgo leaf powder (106-250µm) was put in 100 ml absolute ethanol.
2) After shaking for 6 hours., the mixture was placed aside until the two phases were separated.
3) 30 ml of the upper layer was removed and treated with 30 ml, 5.5% HCl solution.
4) The solution was refluxed in 85^0C water bath for 30 min.
5) Finally, the solution was filtered over 0.45µm syringe filter for further analysis.

Table 6.7: Extraction of Ginkgo leaf powder with ethanol.

1) 10 g Ginkgo leaf powder (106-250μm) was put in 100 ml, 80% ethanol solution.
2) After shaking for 6 hours, the mixture was placed aside until the two phases were separated.
3) 30 ml of the upper layer was removed and treated with 30 ml, 5.5% HCl solution.
4) The solution was refluxed in 85^0C water bath for 30 min.
5) Finally, the solution was filtered over 0.45μm syringe filter for analysis.

Table 6.8: Extraction of GNC tablets with ethanol.

1) 30 g Ginkgo leaf powder (106-250μm) was put in 100 ml, 80% ethanol solution.
2) After shaking for 6 hours, the mixture was placed aside until the two phases were separated.
3) 30 ml of the upper layer was removed and treated with 30 ml, 5.5% HCl solution.
4) The solution was refluxed in 85^0C water bath for 30 min.
5) Finally, the solution was filtered over 0.45μm syringe filter for further analysis.

6.2.2 Adsorption Studies

The adsorption studies were performed as the procedures stated in the tables below, from Table 6.9 to Table 6.15.

Table 6.9: Adsorption of the methanolic leaf extract.

1) 35 ml methanolic leaf extract, prepared as stated in Table 6.1, was put into a flask filled with 5 g clinoptilolite conditioned with 10 ml methanol
2) Adsorption process was conducted for 24 h in thermoshake at 150 rpm at T_{room}.

Table 6.10: Adsorption of the acetone leaf extract derived from the methanolic extract.

1) 40 ml of the acetone leaf extract, which was reduced from methanolic extract and prepared as stated in Table 6.2, was put into a flask filled with 5 g clinoptilolite conditioned with 10 ml acetone. 2) Adsorption process was performed for 24 h in thermoshake at 150 rpm at T_{room}.

Table 6.11: Adsorption of the methanolic leaf extract.

1) 25 ml of the methanolic leaf extract (as tabulated in Table 6.1) was put into a flask filled with 10 g clinoptilolite. 2) Adsorption process was performed for 24 h in constant temperature water bath set at 29^0C with the revolution speed of 150 rpm.

Table 6.12: Adsorption of the acetone leaf extract derived from the methanolic extract.

1) 25 ml of the acetone leaf extract, reduced from 30 ml methanolic extract (as tabulated in Table 6.2), was put into a flask filled with 10 g clinoptilolite. 2) Adsorption was carried out for 24 h in constant temperature water bath set at 29^0C with the revolution speed of 150 rpm.

Table 6.13: Adsorption of the acetone leaf extract.

1) 35 & 25 ml acetone leaf extracts (extraction procedure in Table 6.3) were put into separate flasks filled with 5 g clinoptilolite conditioned with 10 ml acetone and 10 g clinoptilolite without conditioning with acetone, respectively. 2) Adsorption was carried out for 24 h in constant temperature water bath set at 22^0C with the revolution speed of 168 rpm.

Table 6.14: Adsorption of the ethanolic extract of commercial GNC tablets.

1) 20 ml of the ethanolic GNC tablet extracts (extraction procedure in Table 6.5), were subjected to adsorption with 5 and 10 g zeolite for 24 h by shaking the solutions in a water bath kept at 29^0C with a revolution speed of 168 rpm. 2) The resultant solutions were filtered over 0.45μm syringe filter and kept at +40C until further analysis.

Table 6.15: Adsorption of the ethanolic leaf extract.

1) 20 ml of the ethanolic leaf extracts (extraction procedure in Table 6.6) were subjected to adsorption with 0.5, 2, 5 and 10 g zeolite for 24 h by shaking the solutions in a water bath kept at 29^0C with a revolution speed of 168 rpm. 2) The resultant solutions were filtered over 0.45µm syringe filter and kept at +40C until further analysis.

6.2.3 Quantification With HPLC

6.2.3.1 Optimization of the Chromatographic Conditions

For the separation and detection of the active constituents within the methanolic extract of Ginkgo biloba L., LiChrospher RP-18 column having dimensions of 3×250 mm and a packing particle size of 5µm was used. The column temperature was set at 30^0C and the injection volume was 20µl. According to the mild operating parameters of ELSD and the applicable conditions, methanol-water system was selected as eluent. Therefore, a gradient elution system was adopted with the mobile phases; A: water (containing 5%methanol & 0.05% Trifluoroacetic acid, TFA) and B: methanol (containing 0.05% TFA). The rate of mobile phase flow was 1 ml/min. The gradient conditions were stated as shown in Table 6.16.

Table 6.16: Gradient conditions for HPLC detection (Fitzloff et al.,2002).

Final time (min)	A (%)	B (%)
0	75	25
35	25	75
36	10	90
42	10	90
43	75	25
50	75	25

For the UV- detection for the flavonoid compounds, the wavelength was set at 350 nm.

In ELSD, the nebulizer-gas flow rate and drift tube temperature are the major instrumental parameters affecting the signal response. In general, large droplets are formed at low gas flow rate, which results in spikes and noisy signals. On the other hand, increasing the gas flow rate results in a marked decrease of signal response. With respect to the drift tube temperature, solvent evaporation is not completed at low temperature and the detector response is decreased at high temperature. Due to these restrictions, the operating conditions for ELSD were optimized as shown in Table 6.17.

Table 6.17: Optimized ELSD conditions

Drift tube temperature	82.6^{0}C
Nitrogen flow rate	2.3 l/min
Gain	16
Impactor Position	Off

The experimental set-up for which all of those optimization steps were followed is shown in Figure 6.3.

Figure 6.3: Experimental set-up for the chromatographic study.

6.2.3.2 Calibration of Standard Compounds For HPLC Analysis

6.2.3.2.1 Calibration Graphs of Flavonoid Aglycones

For the determination of the calibration graphs of the standard reference aglycones, the procedure stated by Chin et al.(2000) was adopted. According to their assay, quercetin, kaempferol, isorhamnetin, and morin (internal standard) were measured precisely and were dissolved separately in 80% methanol solution.

Quercetin solution was further diluted to 500, 110, 53, and 26 μg/ml; kaempferol solution to 470, 100, 50, and 26μg/ml; isorhamnetin solution to 270, 54, 27, and 13 μg/ml. Internal standard (440 μg/ml) was spiked into each dilution, which was then analyzed by HPLC. The standards were filtered through a 0.45-μm membrane filter before 20 μl-injection. The flavonoid compounds, quercetin, kaempferol and isorhamnetin and the internal standard, morin, yielded retention times as 17.0, 22.4, 21.3 and 14.5 min, respectively, at a wavelength of 350 nm.

The standard curves were obtained by plotting the peak area ratios (Y-axis) of standard compounds to the internal standard versus the concentrations of standard compound (X-axis). According to these standard curves, the linear regressions (Y =mx + b) and correlation coefficients were computed.

When each aglycone content in the chromatogram of the hydrolyzed extract solution was determined using the aglycone calibration curves, the total flavonoid glycoside content was evaluated using the formula (Yu et al.,2003):

$$\sum \text{(amount of each aglycone)} \times 2.51 = \text{total amount of flavonoid glycosides}$$

where total flavone glycoside values were calculated by multiplying the sum of the quercetin, kaempferol and isorhamnetin concentrations by the suggested conversion factor of 2.51.

6.2.3.2.2 Calibration Graphs of Terpene Lactones

Ginkgolide A (GA), Ginkgolide B (GB), Ginkgolide C (GC) and Bilobalide (BB) stock solutions were prepared with the concentration of 10 mg/ml in 80% aqueous methanol solution, and the resultant solutions were kept in a freezer until analysis.

In the case of the analysis, GA (100, 250, 500, 800 μg/ml), GB (75, 200, 400, 600 μg/ml), GC (75, 200, 400, 600 μg/ml) and BB (100, 250, 500, 800 μg/ml) standard solutions were prepared by the dilution of the stock standard solution with 80% aqueous methanol solution.

According to the quantitative principles of ELSD, detector response is given by the relation $y = ax^b$, where y is the peak area, x is the sample concentration, and a and b are numerical coefficients. Therefore, a plot of peak area versus sample concentration is not linear, but in log-log scale, relationship between peak area and sample concentration is linear. Calibration was performed with four different standard solutions of external standards.

6.2.3.2.3 Calibration Graph for the Ginkgolic Acid Standard Reference Mixture

Ginkgolic acid standard reference mixture stock solution was prepared with the concentration of 2 mg/ml in ethanol, and the resultant solution was filtered through a 0.45-μm membrane filter and kept in a freezer until analysis.

In order to prepare working standard solution for HPLC analysis, the stock solution was further diluted to 800, 600, 400, 200 and 100 μg/ml. The ginkgolic acid standard reference mixture yielded retention time as 1.5 min at a wavelength of 310 nm with mobile phase B only, containing 0.03 mol/l silver nitrate. The flow rate of the mobile phase was 1 ml/min.

6.2.4 Total Phenolic Content and Antioxidant Activity Determinations of Ginkgo Biloba Leaf Extracts

6.2.4.1 Measurement of Total Phenolics and Tannins Using Folin-Ciocalteu Method

A number of studies showed that antioxidant activity of plant extracts is correlated with total phenolics rather than with individual compound (Gao et al.,2000). Due to this fact, Folin-Ciocalteu method was adopted to quantify total phenol content of the extract solutions.

The method for total phenol is useful in order to know the efficiency of extraction of phenolics in solvents. Figure 6.4 illustrates the schematic diagram of the method.

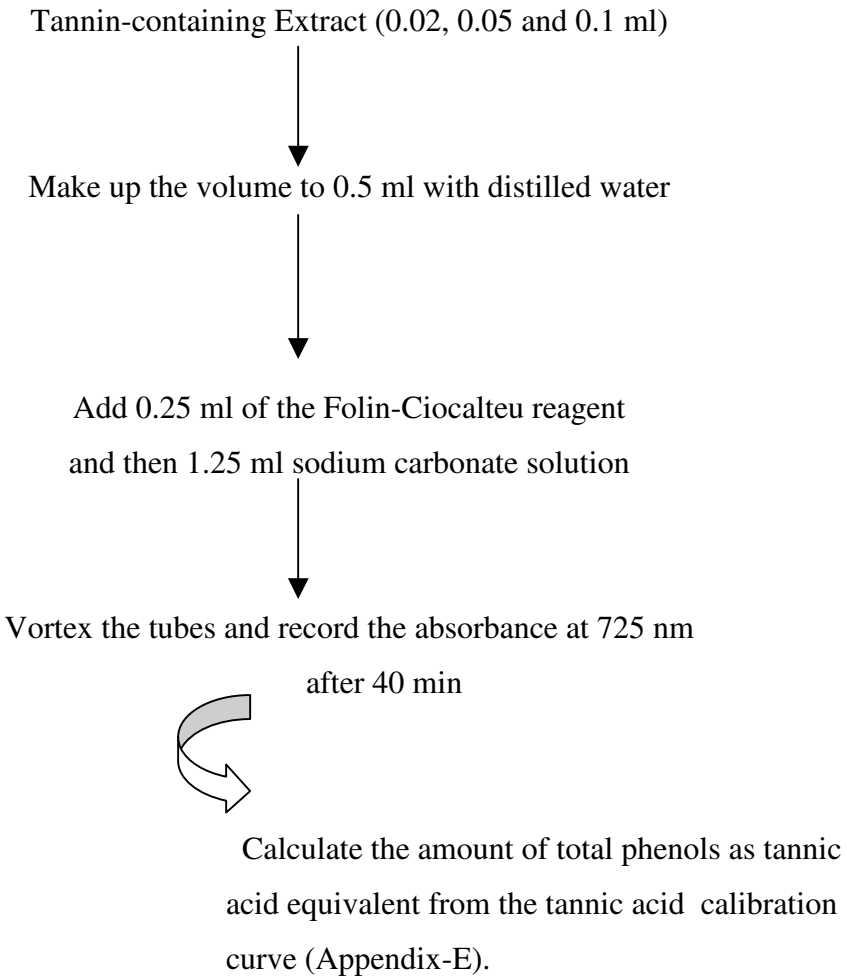


Figure 6.4: Determination of the amount of total phenolics by Folin-Ciocalteu method

6.2.4.2 Measurement of Total Antioxidant Activity

According to "ABTS Radical Cation Decolorization Assay" as stated by Re et al(1999), ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. Because ABTS and potassium persulfate react stoichiometrically at a ratio of 1:0.5, this will result in incomplete oxidation of the ABTS.

The radical was stable in this form for more than two days when stored in the dark room at room temperature.

For the study of phenolic compounds and food extracts, the $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.7 ($\pm$ 0.02) at 734 nm and equilibrated at

30^0C. Stock solutions of phenolics in ethanol were diluted such that, after introduction of a 10µl aliquot of each dilution into the assay, they produced between 20%-80% inhibition of the blank absorbance. After addition of 1.0 ml of diluted $ABTS^{\cdot+}$ solution ($A_{734\ nm}$ = 0.700 ± 0.02) to 10 µl of antioxidant compounds or Trolox standards (final concentration 0-15 µM) in ethanol, the absorbance reading was taken at 30^0C exactly 1 min after initial mixing and up to 6 min.

The percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of concentration of antioxidants and of Trolox for the standard reference data.

Figure 6.5 illustrates the detailed reaction mechanism for the TEAC assay.

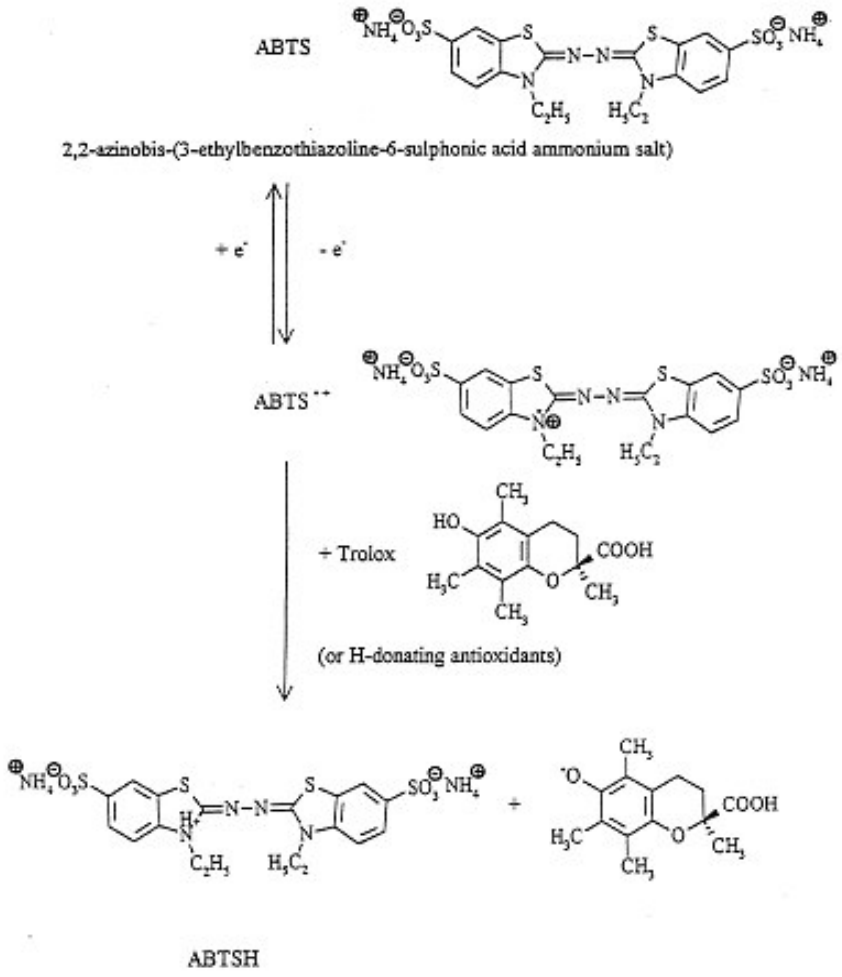


Figure 6.5: ABTS Radical Cation Decolorization Assay (Re et al., 1999).

CHAPTER 7

RESULTS AND DISCUSSION

7.1 Extraction Studies

7.1.1 Extraction Kinetics

Time needed to extract all of the active constituents by the solvent extraction was one of the crucial points in the extraction process. Therefore, a set of experiments was performed in order to establish the inter-relationship between contact time and extracted phenolics concentration. The extraction kinetics study performed for the commercial GNC tablet showed that the extraction of the active constituents with 80% aqueous ethanol completed at the end of 6th hour as shown in Figure-7.1. Further extraction resulted in no significant change in concentration of flavonoid constituents. Therefore, contact time of 6 hr was considered enough for an efficient extraction process.

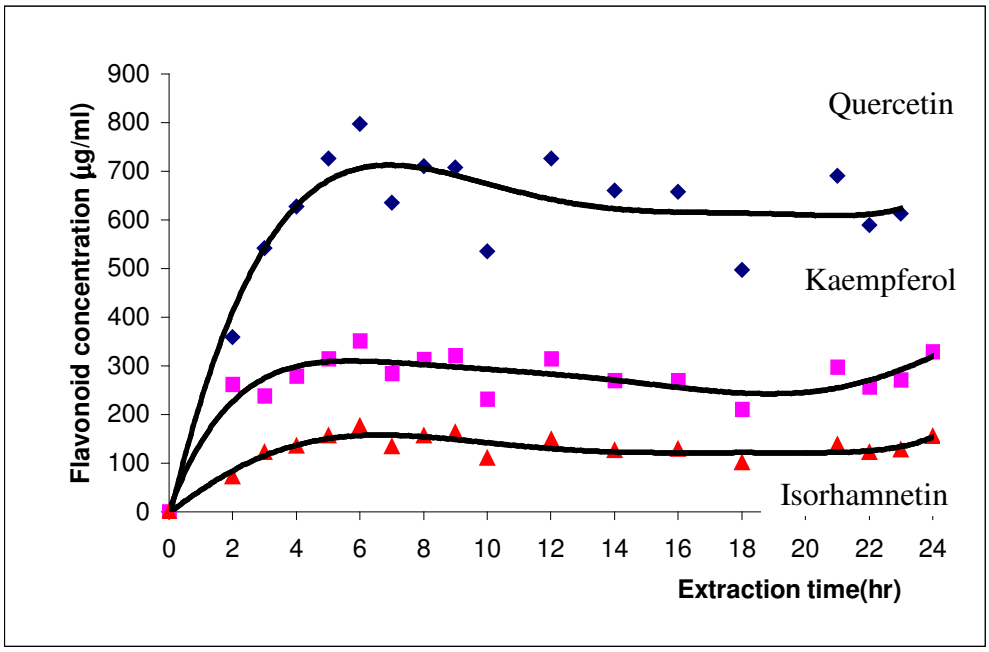


Figure 7.1: Solvent extraction kinetics results for the three flavonoids: quercetin, kaempferol and isorhamnetin for commercial GNC tablet extract in 80% ethanol solution.

Since extraction processes were conducted in constant temperature water bath, the processes were considered to be isothermal. Since isothermal assumption was adopted, the solution for the uptake curve is:

$$\frac{m_t}{m_\infty} = 1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left(\frac{-n^2\pi^2 D_c t}{r_c^{\,2}}\right) \qquad (7.1)$$

In the long time region, where the fractional uptakes are greater than 70%, the uptake curve approaches the asymptotic form as:

$$\frac{m_t}{m_\infty} = 1 - \frac{6}{\pi^2}\exp\left[-\frac{\pi^2 D_c t}{r_c^{\,2}}\right] \qquad (7.2)$$

for which a plot of ln $(1- m_t/m_\infty)$ versus t is linear with slope $-\pi^2 D_c/r_c^{2.}$

A simplified expression for the initial region of the uptake curve where the fractional uptakes are smaller than 30%, may be obtained by neglecting the higher terms as:

$$\frac{m_t}{m_\infty} \approx \frac{6}{\sqrt{\pi}}\sqrt{\frac{D_c t}{r_c^{\,2}}} \qquad (7.3)$$

for which a plot of m_t/m_∞ vs $\sqrt{t}$ is linear with the slope $\left(\frac{6}{\sqrt{\pi}}\right)\sqrt{\left(\frac{D_c}{r_c^{\,2}}\right)}$ (Ruthven, 1992).

Table 7.1 tabulates the experimental uptake data for the three aglycone constituents (quercetin, kaempferol and isorhamnetin).

Table 7.1: Uptake data via extraction of the three aglycone constituents.

	Quercetin	**Kaempferol**	**Isorhamnetin**
Time (hr)	$\mathbf{m_t / m_\infty}$	$\mathbf{m_t / m_\infty}$	$\mathbf{m_t / m_\infty}$
0	0	0	0
3	0.72	0.72	0.79
4	0.84	0.85	0.87
5	0.97	0.96	1.00

Since the experimental data fit the long time region, the resultant effective diffusivity value for the aglycone constituents can be tabulated as in Table 7.2.

Table 7.2: Effective diffusivities of the three aglycone constituents.

Aglycone	**$D_c \times 10^{10}$ (m^2/s)**
Quercetin	1.76
Kaempferol	1.8
Isorhamnetin	2.0

The effective diffusivity values calculated for the aglycone constituents were found to be quite close to each other due to the fact that the constituents have similar molecular structures.

7.1.2 Extraction with Different Solvent Systems

Type and composition of the extracting solvent determined the extent to which the active constituents could be extracted. In the extraction studies, three different solvents(methanol, acetone and ethanol) were used to see the effect of solvent on extraction efficiency. In the literature the most clean extracts from Ginkgo biloba leaf and commercial Ginkgo tablets were obtained using methanol as an extraction solvent. Figure 7.2 shows a fingerprint chromatogram of methanolic Ginkgo biloba leaf extract.

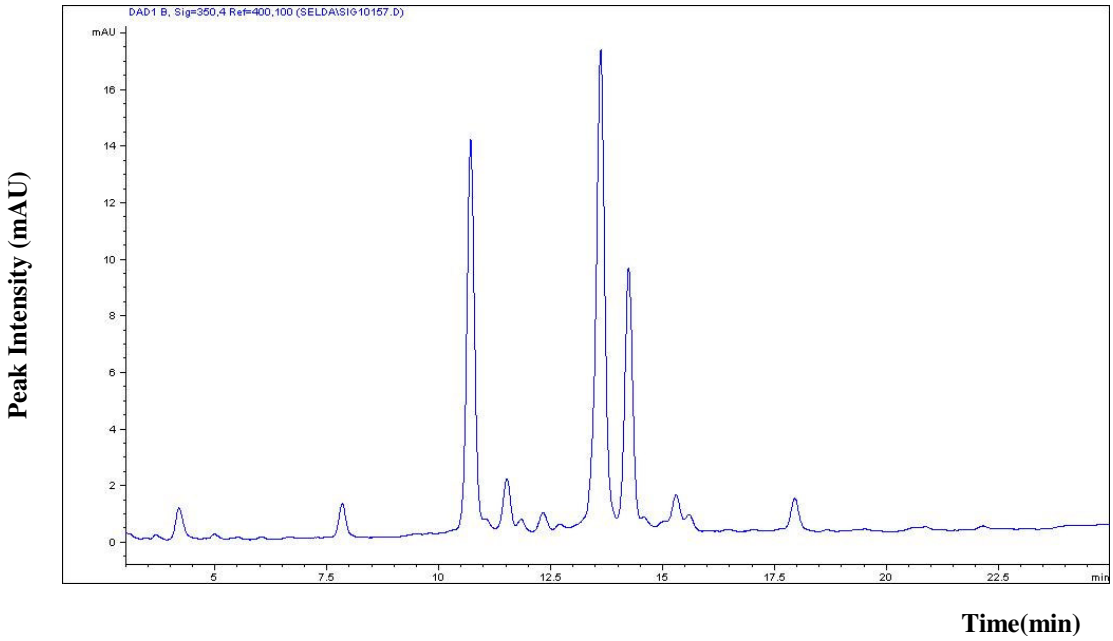


Figure 7.2: Methanolic Ginkgo biloba leaf extract (extraction procedure in Table 6.1).

But due to the toxicity of methanol as an extracting solvent for pharmaceuticals, leaf powder was extracted using methanol followed by complete evaporation of this solvent. Finally, the constituents were redissolved in acetone, one of the widely used solvents for GBE. Since the eluotropic strength, i.e., polarity, of acetone was much smaller than methanol, acetone could not recover all of the dried residue from the inner surface of the glass chamber of the rotary evaporator as illustrated in Figure 7.3.

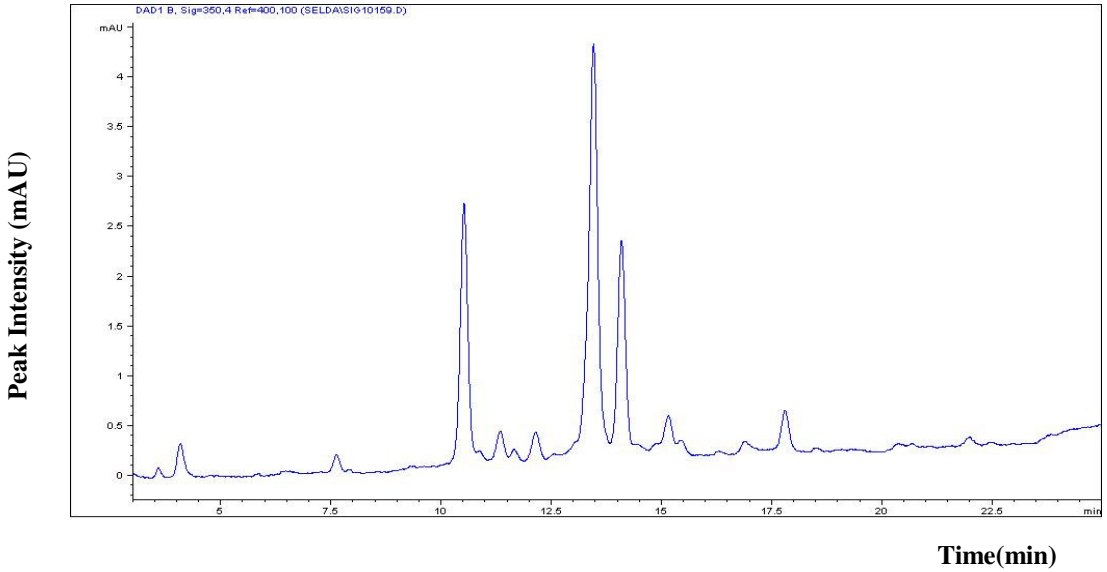


Figure 7.3: Ginkgo biloba leaf extract obtained by evaporating the methanolic extract under reduced pressure at 45^0C and redissolving the constituents in acetone (extraction procedure in Table 6.2).

When pure acetone was used for the extraction of Ginkgo leaves, the peaks were not resolved so clear as was in the case for methanol. Also, the peak areas were much smaller when compared with methanol extract as shown in Figure 7.4.

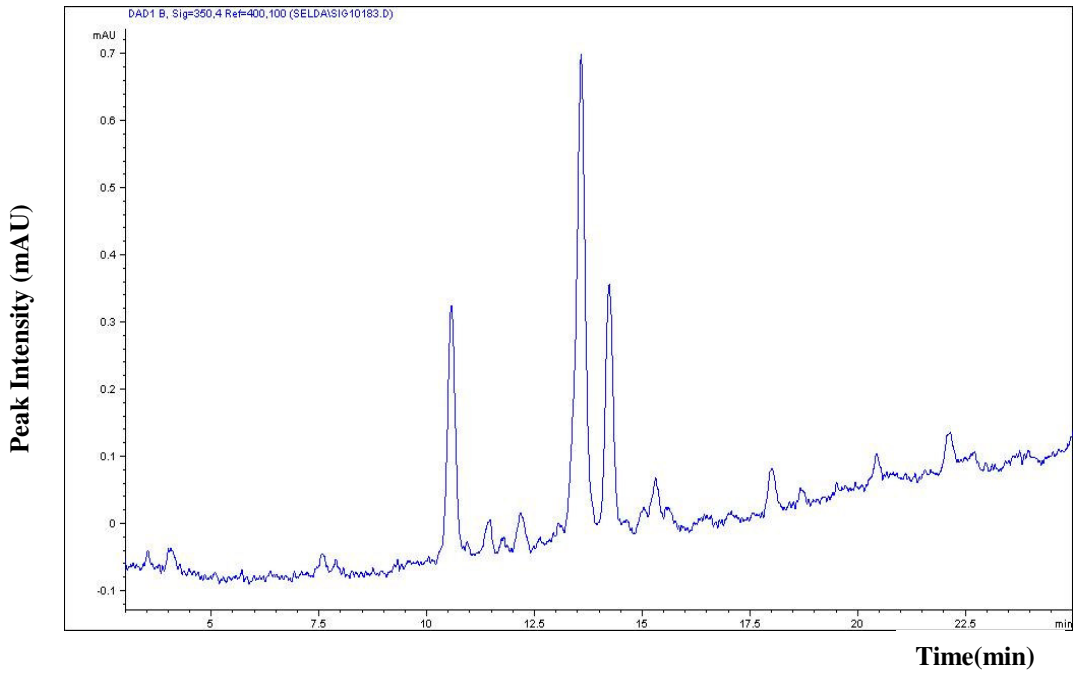


Figure 7.4: Ginkgo biloba leaf extract prepared with acetone as solvent (extraction procedure in Table 6.3).

On the other hand, for the same amount of commercial Ginkgo extract powder, the extract yielded higher recovery rates (i.e., higher peak areas) in acetone as shown in Figure 7.5. This may be the result of the fact that the extraction of Ginkgo standardized extracts is less of a problem than Ginkgo leaves because matrix effects and diffusion do not play a role (van Beek et al.,2002).

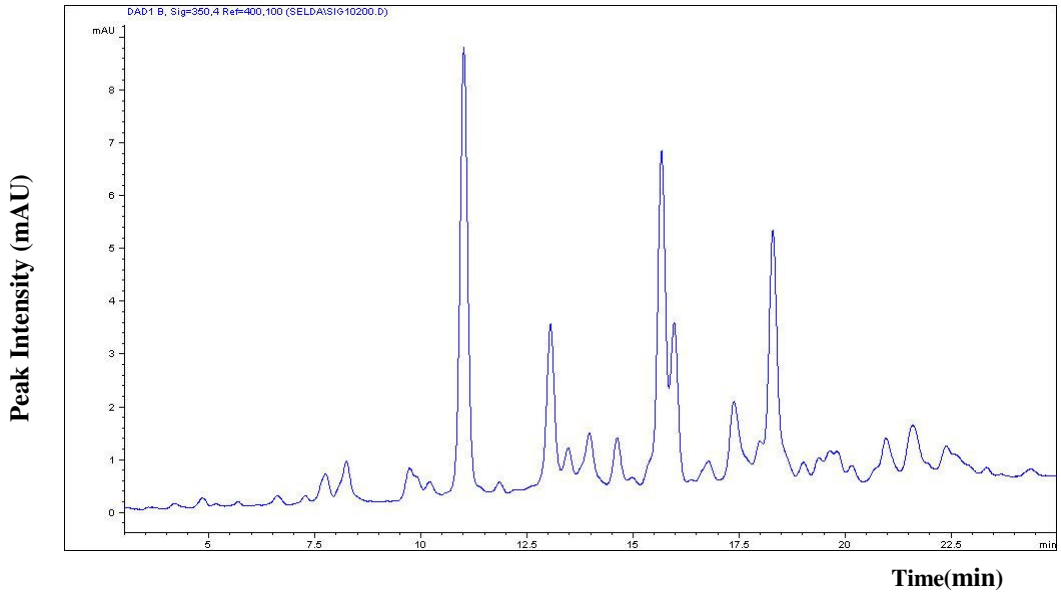


Figure 7.5: Commercial Ginkgo biloba leaf extract (GNC) prepared with 100% acetone as the extraction solvent (extraction procedure in Table 6.3).

Due to its non-toxicity and safety for the extraction processes for herbal medicines, ethanol was selected as the extracting solvent in this study. From the UV absorbance reading at 350 nm, the total flavonoid glycosides could not be obtained due to the great variety of the flavonoid compounds as seen in Figure 7.6a. However, with the hydrolysis step, the flavonoid constituents were converted to their aglycone forms, which could be easily detected and quantified from the corresponding chromatogram as illustrated in Figure 7.6b.

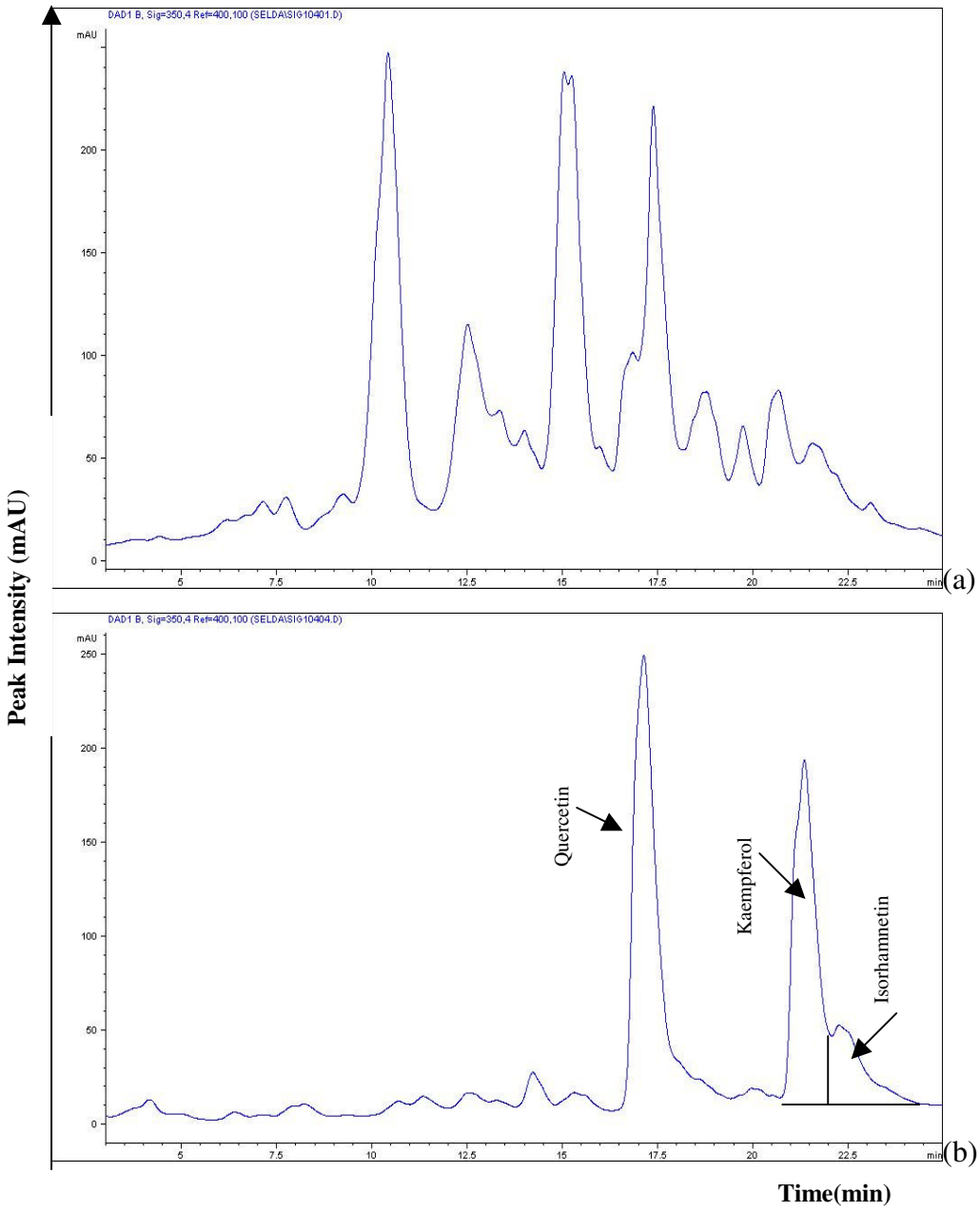


Figure 7.6: Commercial Ginkgo biloba leaf extract prepared with 80% aqueous ethanol solution as solvent (extraction procedure in Table 6.5) before (a) and after (b) hydrolysis with 5.5% HCl solution at 85^0C, without solid-phase extraction (SPE).

Crude leaf extracts contain significant amounts of more apolar compounds like ginkgolic acids, biflavones and chlorophyll. Not only do these apolar, matrix constituents cause the unresolved peaks of the analytes of interest, but also cause the clogging of the HPLC column. Owing to this reason, the extract solutions were subjected to solid-phase extraction (SPE) for sample clean-up and the peaks were better resolved as seen in Figure 7.7.

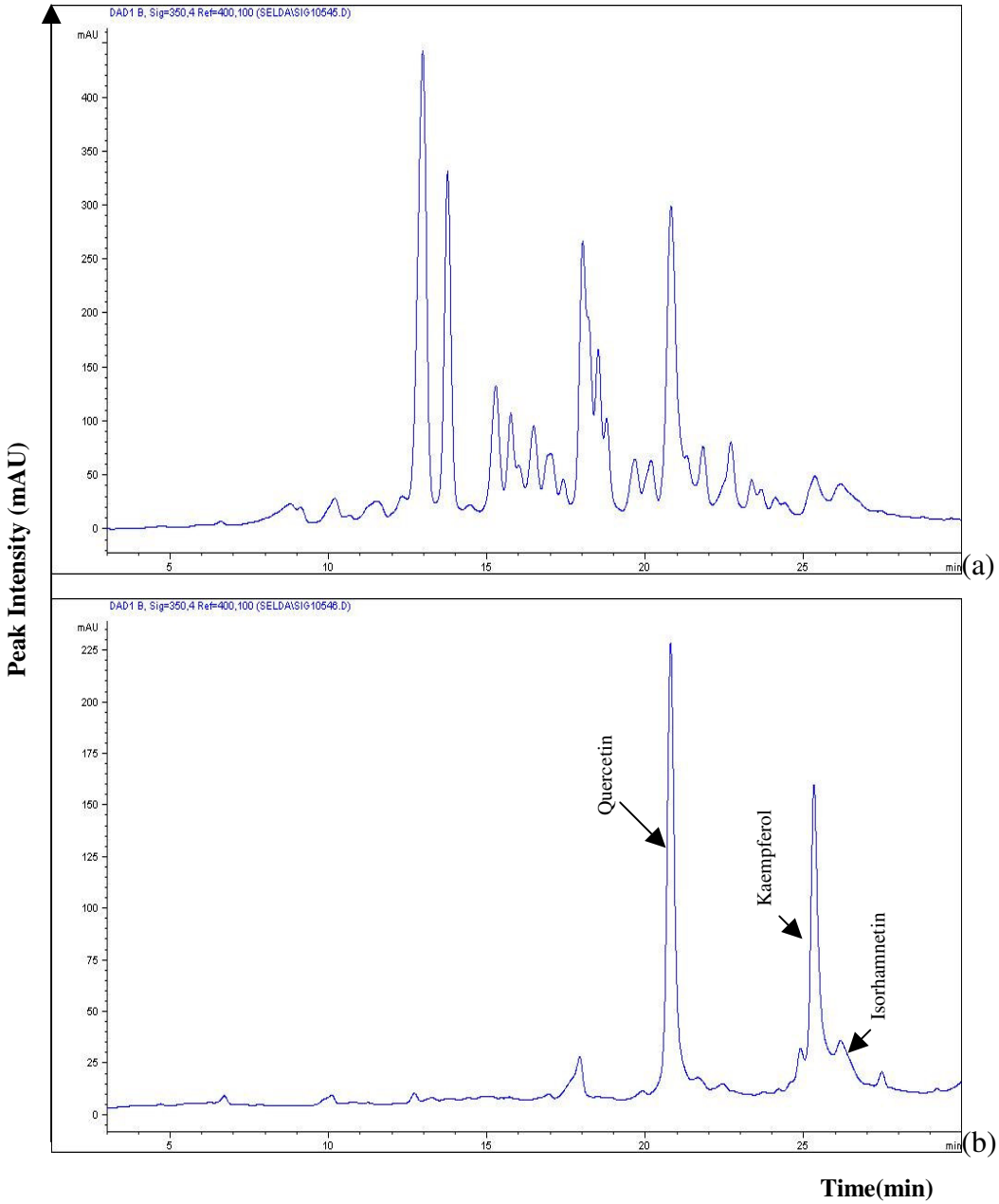


Figure 7.7: Commercial Ginkgo biloba leaf extract prepared with 80% aqueous ethanol solution as solvent (extraction procedure in Table 6.8) before hydrolysis (a) and after hydrolysis (b), with SPE.

Although the flavonoid glycosides were very polar in nature, their polarity decreased when converted to their aglycone forms. As a result, injection of the hydrolyzed extract solution through non-polar solid-phase extraction (SPE) cartridges resulted in the binding of noticable amount of those aglycones to the packing material and the decrease in the peak areas of the corresponding aglycones.

The adjustment of the polarity of the extracting medium played a crucial role in the determination of the extraction efficiency of the active constituents since there are considerable differences in the polarity between the individual terpene trilactones with G-B being the most apolar and G-C being the most polar (van Beek.,2002). Therefore, the water content of the organic solvents should be selected in order to reach the best extracting solvent. For example, when the leaf powder was extracted with 100% ethanol as its HPLC chromatogram was shown in Figure 7.8, instead of 80% ethanol as shown in Figure 7.9, the peak resolution was not so good when compared with the former case. Also, the peak intensities were much smaller.

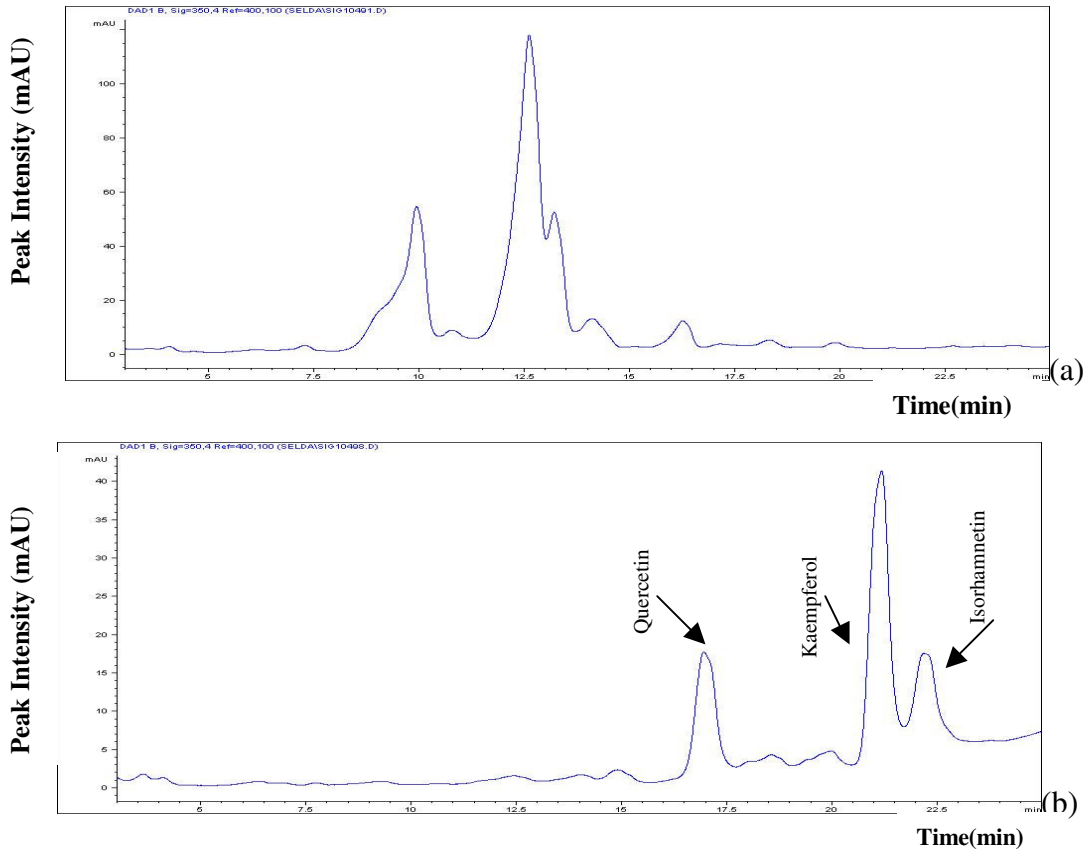


Figure 7.8: Ginkgo biloba leaf extract prepared with absolute (100%) ethanol (extraction procedure in Table 6.6), before hydrolysis (a) and after hydrolysis with 5.5% HCl solution for 30 min at 85^0C (b).

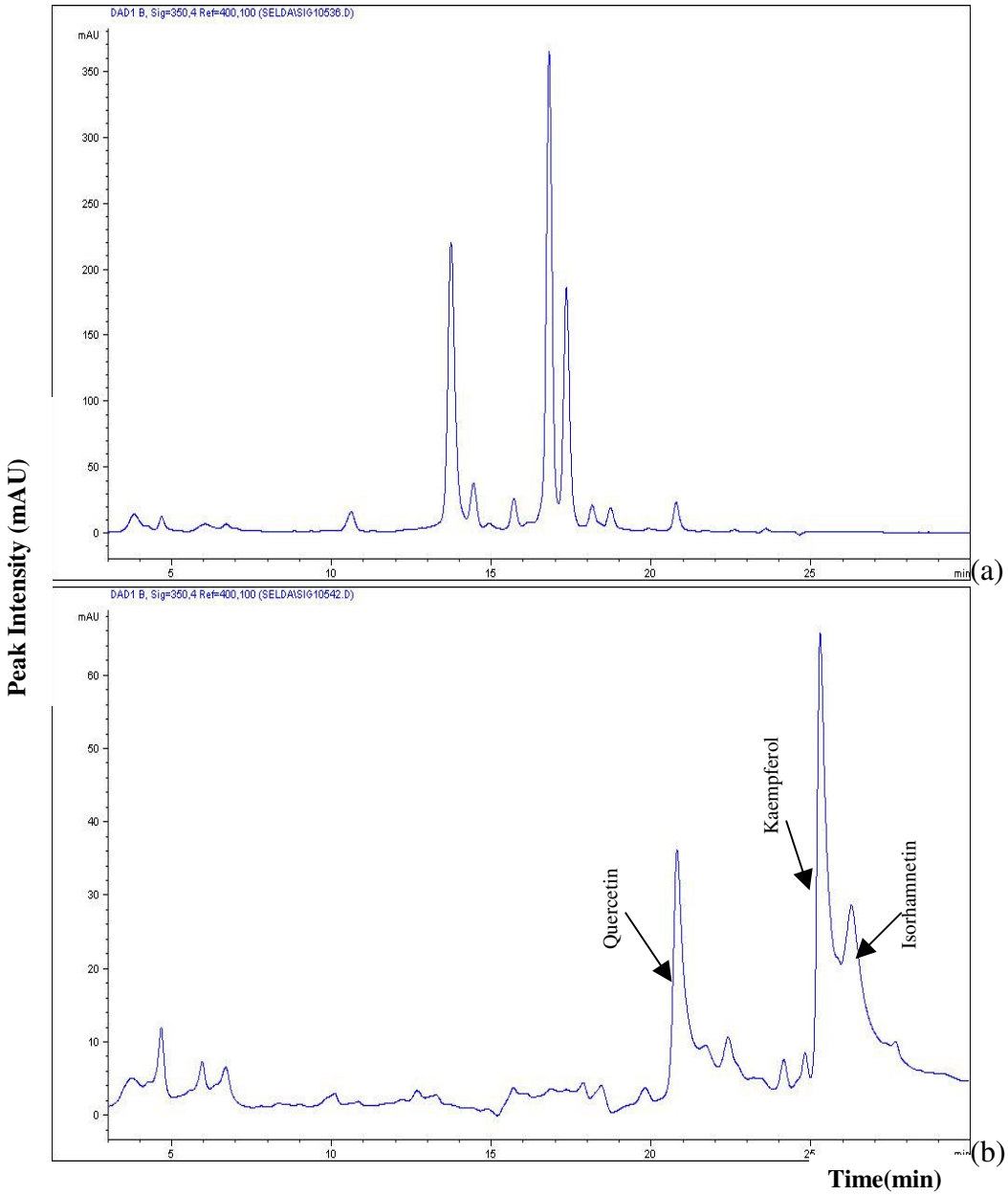


Figure 7.9: Ginkgo biloba leaf extract prepared with 80% ethanol (extraction procedure in Table 6.7), before hydrolysis (a) and after hydrolysis with 5.5% HCl solution for 30 min at 85^0C (b).

Also, when the aglycone peaks of the Ginkgo leaf powder extracts and the commercial tablet powder extracts were compared, it was seen that the quercetin content was much higher than the other two aglycones for the commercial tablet extract, whereas for the leaf extract the quercetin content was smaller than kaempferol content. This was probably due to the enrichment of the glycoside derivative of quercetin in the commercial extracts because of its being the most effective antioxidant constituent within the extract.

The quantification of terpene lactones was more problematic when compared with the flavonoids contituents. Not only were terpenes extracted in quite small quantities, but also the hydrolysis of the extract solution caused the decrease in the amount of terpenes while converting them to some extent to their hydrolysis products, the hydroxyl acids. This caused the detection of these compounds much more difficult by Evaporative Light Scattering Detection (ELSD). Therefore, all the quantification of terpenes present in GBE solutions were performed before hydrolyzing the samples. In Figure 7.20, one can see the ginkgolides in Ginkgo biloba leaf extract solution after SPE.

7.2 Adsorption Studies

7.2.1 Adsorption of Flavonoid Glycosides

Different extraction systems were experimented in order to determine the best adsorption efficiency. Since the interaction between the extracted species and the clinoptilolite-rich mineral was basically controlled by the chemical nature of the two contacting surfaces, the difference in the polarities of the interacting groups played a dominant role.

Methanol was selected as the extracting solvent for Ginkgo biloba L. powder (106-250μm), and the adsorption process was monitored for 5 and 10 g. zeolite. Due to lack of commercially available reference substances and the complex separation conditions, the normal procedure for the quantitative analysis of flavonol glycosides in Ginkgo leaves and extracts is an acidic hydrolysis followed by HPLC of the resulting aglycones (van Beek, 2002). As a result, fingerprint chromatograms were investigated in the first place to get an overall picture on how the adsorption process influenced the corresponding peak areas. When the fingerprint chromatograms in Figure 7.10, for before and after adsorption cases, were examined, no considerable decrease in the peak areas was observed. This was mainly the result of the fact that methanol was itself very polar in nature, hence the target compounds would not preferentially adsorb on the clinoptilolite surface, due to its polarity competing with the polarity of clinoptilolite.

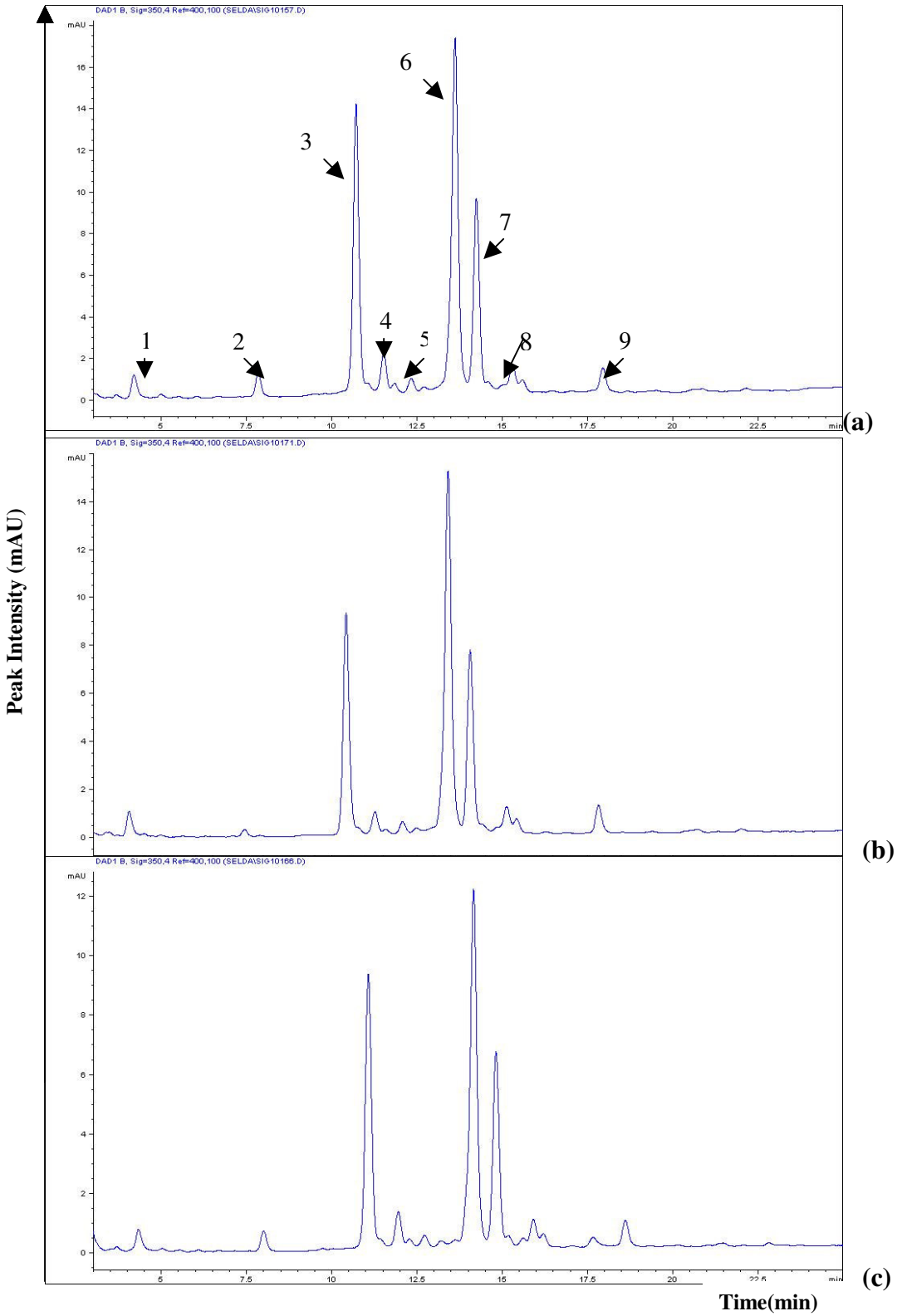


Figure 7.10: Methanolic leaf extract (extraction procedure in Table 6.1) (a), after adsorption with 5 g zeolite (b), after adsorption with 10 g zeolite (c).

Table 7.3 presents the decrease in the selected peak areas of the fingerprint chromatogram due to adsorption with clinoptilolite.

Table 7.3: Peak areas of the marked peaks on the methanolic leaf extract fingerprint chromatograms.

Peak #	1	2	3	4	5	6	7	8	9
Untreated	14.10	13.71	159.47	18.18	7.62	225.47	104.42	9.51	15.37
5 g zeolite adsorbed	12.61	8.60	114.48	13.26	6.15	204.30	88.90	7.86	14.53
10 g zeolite adorbed	9.39	-[a]	110.10	10.55	5.40	166.94	77.10	6.68	11.51

[a] Not detected.

The results tabulated in Table 7.3 were also illustrated in Figure 7.11 as shown below:

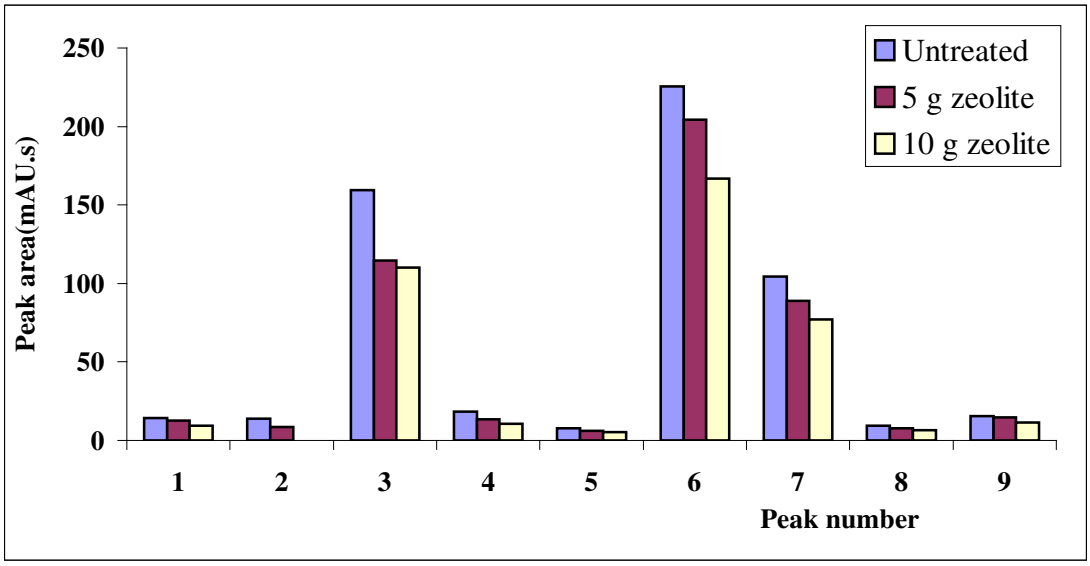


Figure 7.11: Decrease in the selected peak areas of Ginkgo leaf extract due to adsorption with 5 and 10 g zeolite in 20 ml extract solution.

In order to investigate the effect of polarity on adsorption, the methanolic extract was evaporated under reduced pressure and the extracted constituents were redissolved in another solvent with lower polarity(i.e. eluotropic strength, listed in Appendix-H), acetone. In this case, the fingerprint chromatograms shown in Figure 7.12 presented a reasonable decrease in the peak area due to the preferential adsorption of the extracted species on the surface of clinoptilolite.

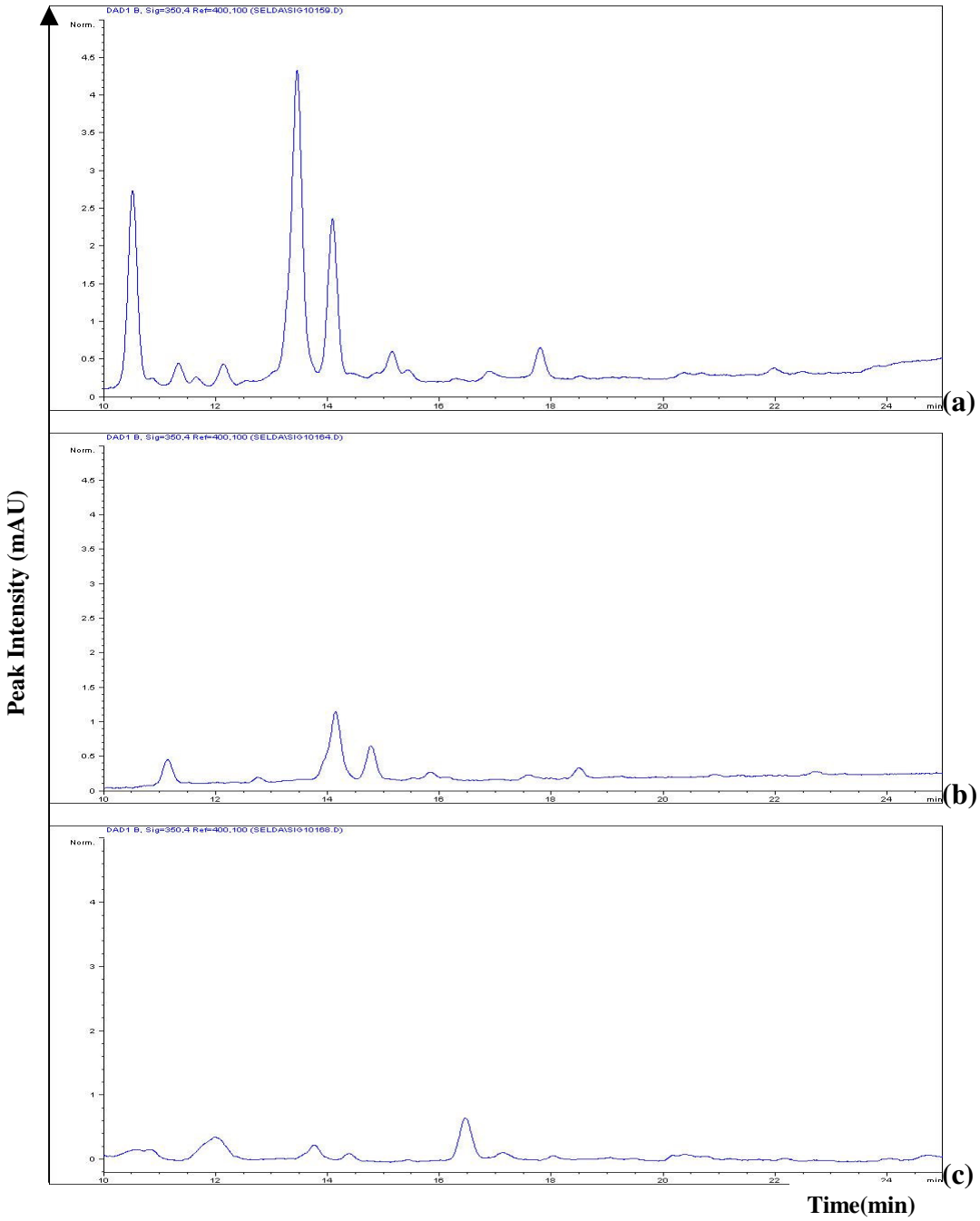


Figure 7.12: Ginkgo biloba leaf extract obtained by evaporating the methanolic extract under reduced pressure at 45^0C (extraction procedure in Table 6.2) (a), after adsorption with 5 g zeolite (b), after adsorption with 10 g zeolite (c).

This was also proved with the adsorption within the acetone extract of commercial Ginkgo biloba tablets powder. The fingerprint chromatograms illustrated in Figure 7.13, showed more noticable decrease for the corresponding peak areas.

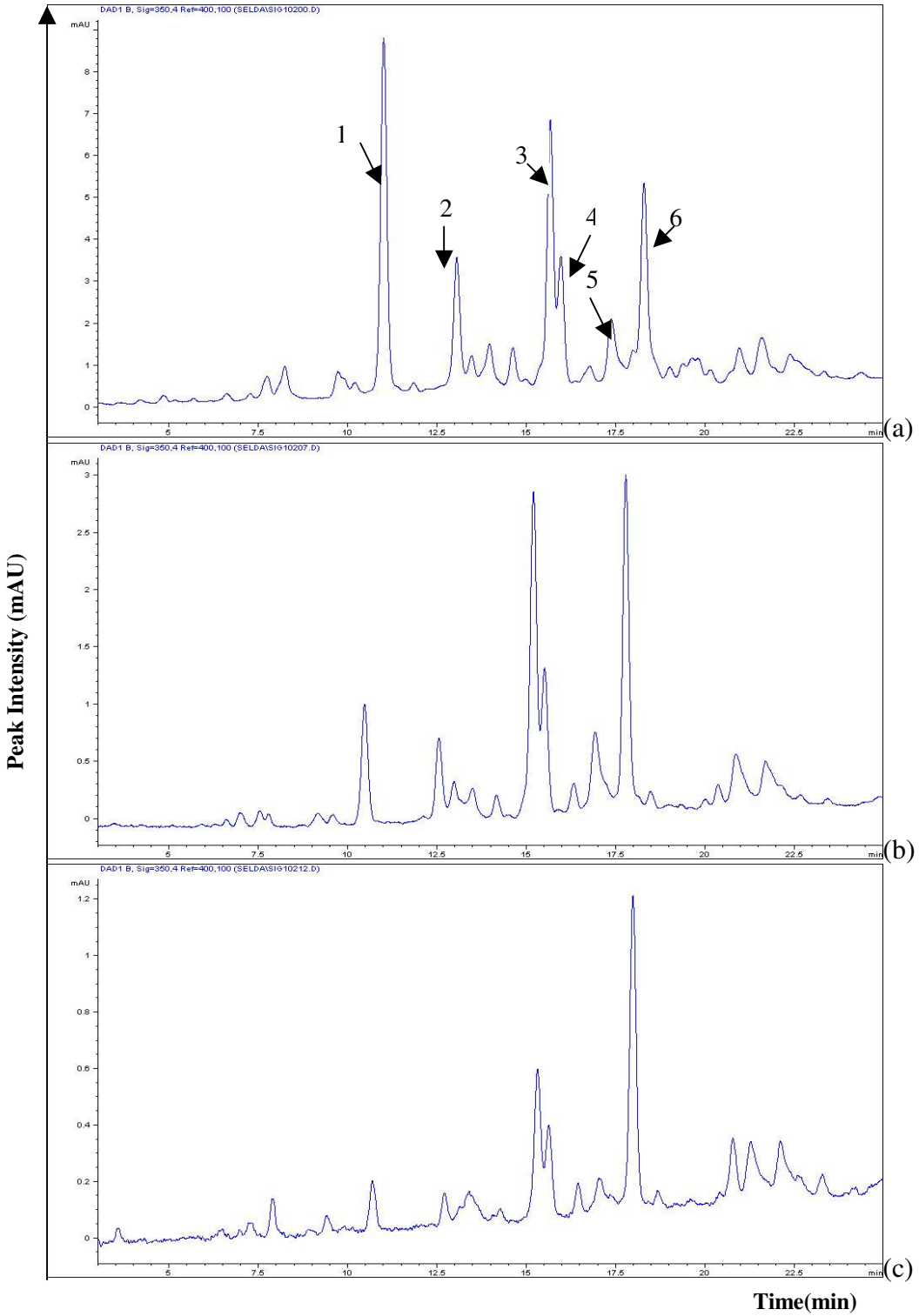


Figure 7.13: Commercial Ginkgo biloba leaf extract (GNC) prepared with acetone as solvent (extraction procedure in Table 6.3) (a), after adsorption with 5 g zeolite (b), after adsorption with 10 g zeolite (c).

The adsorption results of commercial Ginkgo biloba leaf extract (GNC) prepared with acetone, are presented as decrease in the peak areas for the selected peaks in the fingerprint chromatograms in Table 7.4.

Table 7.4: Peak areas of the marked peaks on the fingerprint chromatograms of acetone extract of GNC tablets.

Peak #	**1**	**2**	**3**	**4**	**5**	**6**
Untreated	106.97	35.39	85.40	38.78	21.84	70.18
5 g zeolite adsorbed	13.68	7.96	39.51	16.95	11.37	33.54
10 g zeolite adsorbed	-[a]	-[a]	7.40	3.98	-[a]	13.84

[a] Not detected.

The results presented in Table 7.4 were also figured out in Figure 7.14.

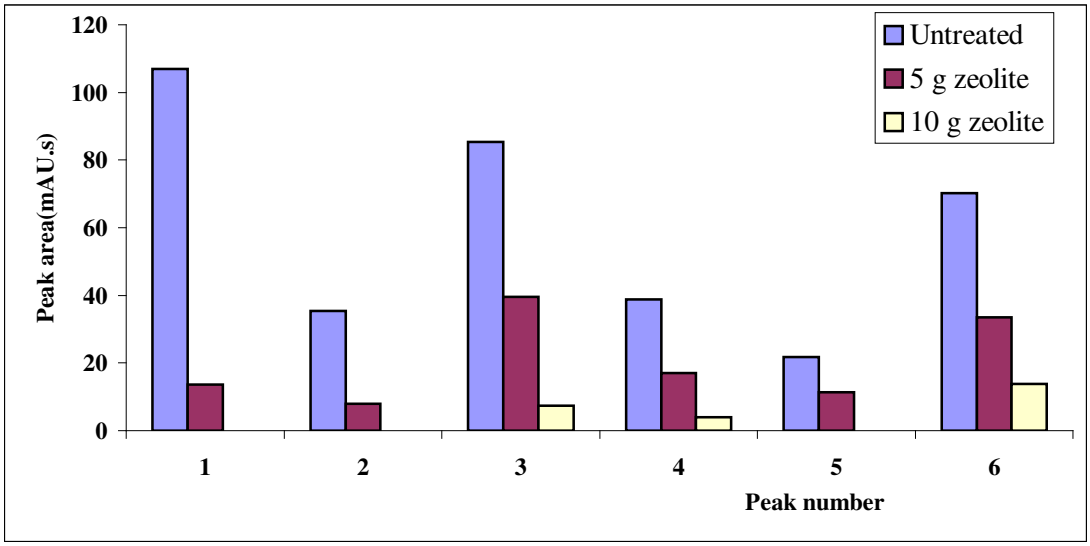


Figure 7.14: Decrease in the selected peak areas of GNC extract due to adsorption with 5 and 10 g zeolite in 20 ml extract solution.

In order to make sure that the decrease in the peak areas were mainly caused as a result of adsorption on zeolite surface, not on the inner surface of the flask made of silica-glass, control experiments were performed for which no clinoptilolite was added to the extract solution. At the end of the experiments, no change in the fingerprint chromatogram for the control experiment was detected.

Although, there was an evidence that some of the extracted constituents were adsorbed on the zeolite surface, the key point was to detect exactly the type of the species that adsorbed on the adsorbent surface and to quantify the exact adsorbed amount. For this reason, hydrolyzed ethanolic extract of commercial Ginkgo biloba L. tablets powder was subjected to adsorption with 5 and 10 g. zeolite, respectively. The

decrease in the peak areas of the three flavonoid aglycones -quercetin, kaempferol and isorhamnetin- were illustrated in Figure 7.15 and measured with respect to the peak area of the internal standard, morin.

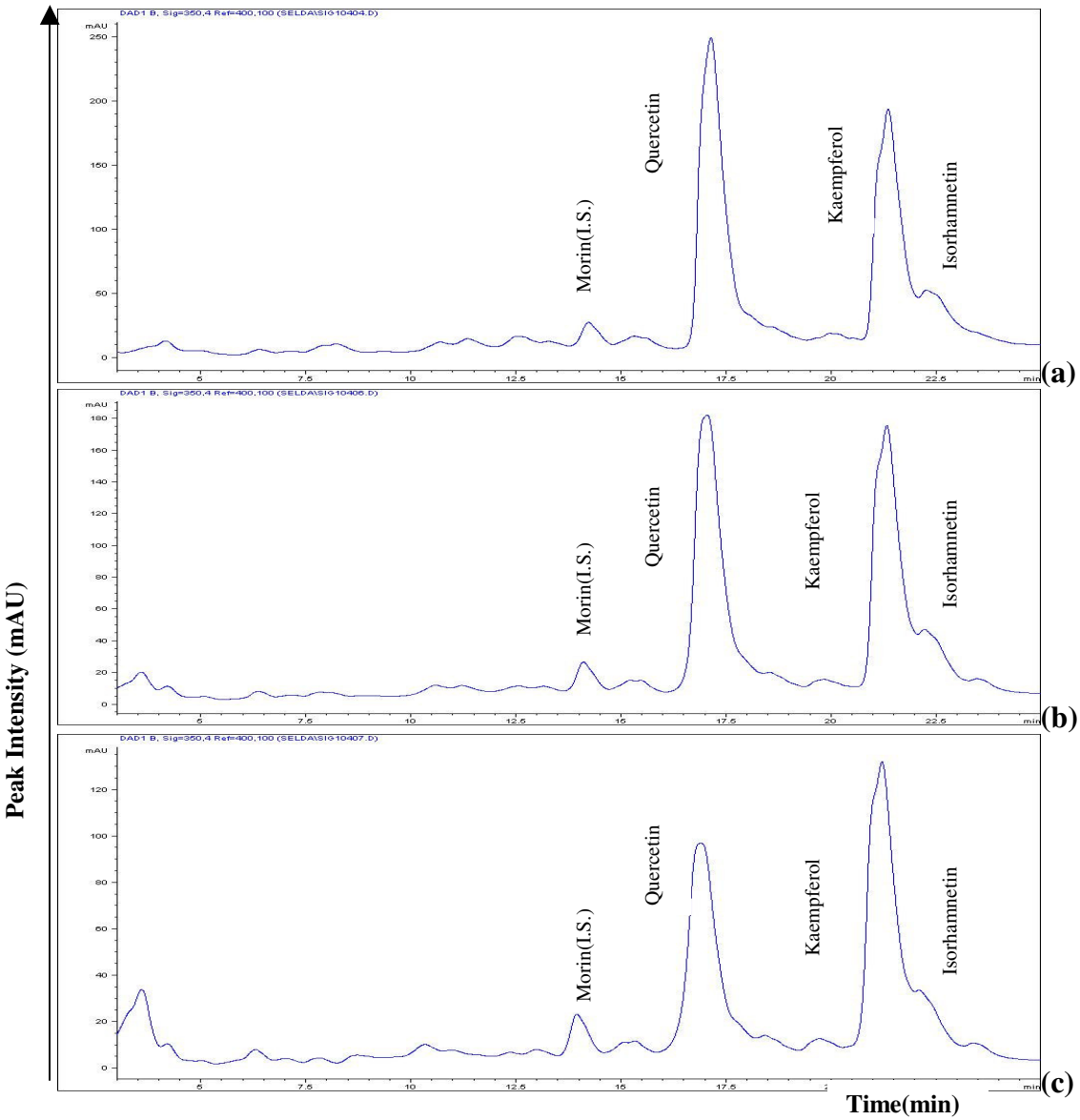


Figure 7.15: Commercial Ginkgo biloba leaf extract prepared with 80% aqueous ethanol solution (extraction procedure in Table 6.5) (a), after adsorption with 5 g zeolite (b), after adsorption with 10 g zeolite (c).

The adsorption results for the GNC tablet extract solution illustrated in Figure 7.15 were also tabulated in Table 7.5.

Table 7.5: Adsorption results for the flavonoid aglycones present in the GNC tablet extract (corresponding chromatogram presented in Figure 7.11)

	Quercetin conc.[a] (µg/ml)	**Kaempferol conc.[a] (µg/ml)**	**Isorhamnetin conc.[a] (µg/ml)**	**Total flavonoid glycosides conc. (µg/ml)[b]**
Before adsorption	824.72±3.1	382.02±5.8	68.18±2.6	3200.04±3.8
After ads. with 5 g zeolite	633.41±11.2	345.72±9.5	58.31±2.6	2603.96±7.7
After ads. with 10 g zeolite	337.31±18	286.07±7.4	39.34±0.3	1663.43±8.6

[a] Mean and RSD values obtained from triplicate injections

[b] $\sum$ (amount of each aglycone) × 2.51 = total amount of flavonoid glycosides

As the results are also interpreted in Figure 7.16, clinoptilolite-rich mineral selectively adsorbs quercetin rather than the other aglycone constituents.

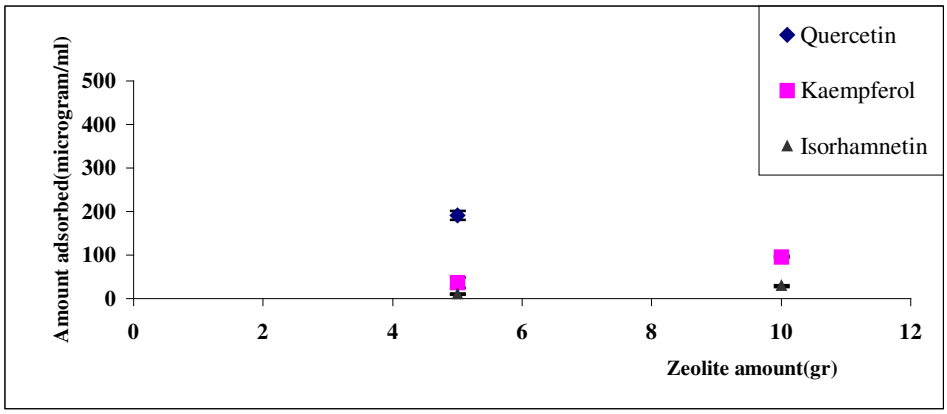


Figure 7.16: Adsorption results for the flavonoid aglycones present in 20 ml of commercial Ginkgo extract for 5 and 10 g zeolite loading.

In order to validate this interpretation, four different solid-to-liquid ratios (0.5 g, 2 g, 5 g and 10 g zeolite) were experimented for Ginkgo biloba L. powder, extracted with 100% ethanol. 0.5 g zeolite could not adsorb considerable amount of aglycone constituents, whereas with the increasing solid loading to the adsorption medium, the adsorbed quantities increased sharply as illustrated in Table 7.6. The peak areas were adopted for the calculations, again, by constructing the corresponding peak area of the internal standard morin as shown in Figure 7.17.

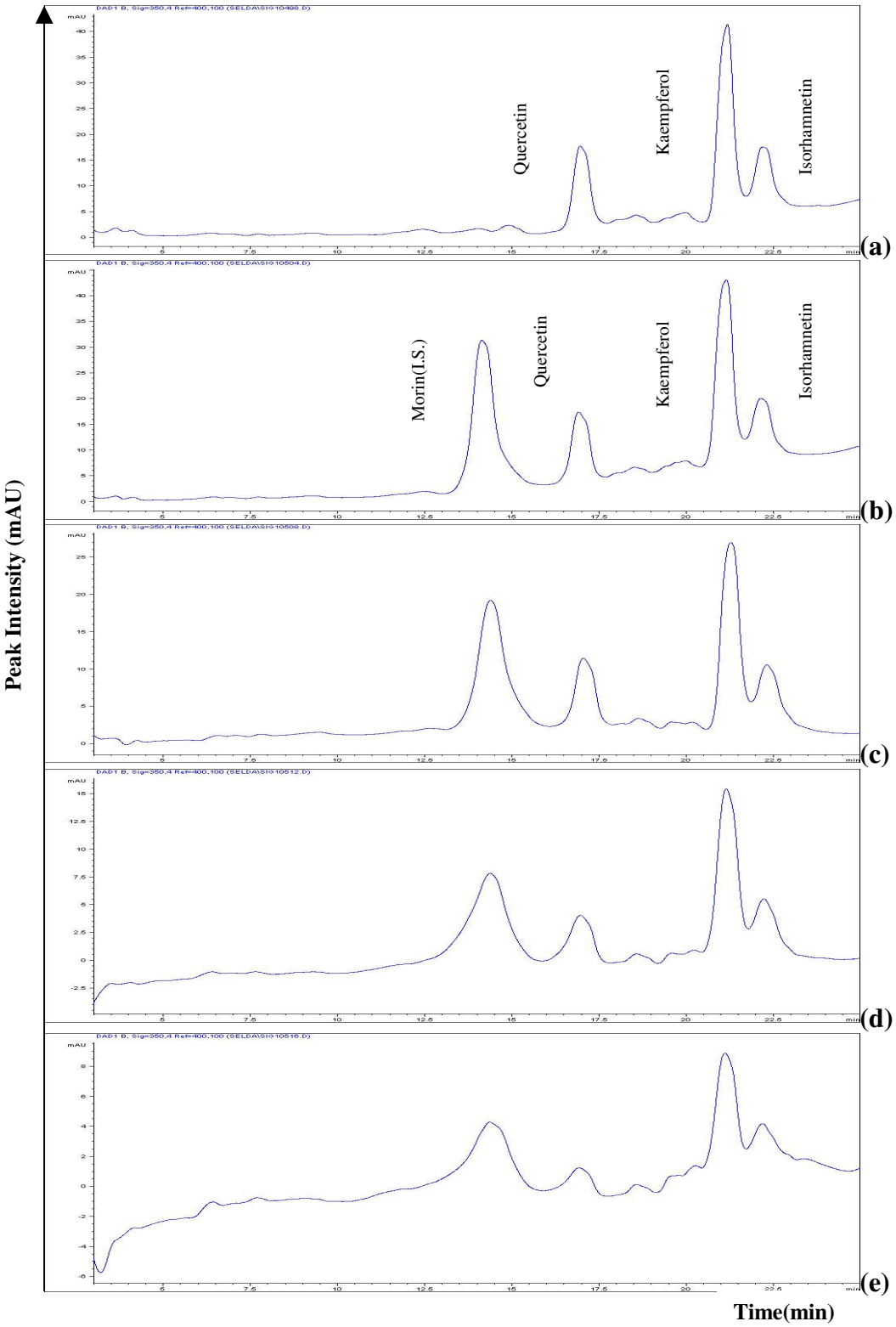


Figure 7.17: Ginkgo biloba leaf extract prepared with absolute (100%) ethanol and hydrolyzed with 5.5% HCl solution for 30 min at 85^0C (a) (extraction procedure in Table 6.6), after adsorption with 0.5 g zeolite (b), 2 g zeolite (c), 5 g zeolite (d) and 10 g zeolite (d).

The adsorption results for the Ginkgo biloba leaf extract illustrated in Figure 7.17 were also tabulated in Table 7.6.

Table 7.6: Adsorption results for the aglycones in ginkgo leaf extract.

	Quercetin conc.[a] (µg/ml)	**Kaempferol conc.[a] (µg/ml)**	**Isorhamnetin conc.[a] (µg/ml)**	**Totalflavonoid glycosides conc. (µg/ml)[b]**
Before adsorption	45.54	62.28	21.29	324.07
After ads. with 0.5 g zeolite	42.93	61.02	21.28	314.33
After ads. with 2 g zeolite	31.06	45.38	17.63	236.11
After ads. with 5 g zeolite	20.02	32.07	11.09	158.56
After ads. with 10 g zeolite	7.41	15.98	4.65	70.39

[a] Mean values obtained from triplicate injections

[b] $\sum$ (amount of each aglycone) × 2.51 = total amount of flavonoid glycosides

As stated before, initial concentration of quercetin within the extract obtained from Ginkgo leaf powder was found to be less than the two aglycones, kaempferol and isorhamnetin when compared with the commercial Ginkgo tablet extract as tabulated in Table 7.5. These adsorption results were also interpreted in Figure 7.18.

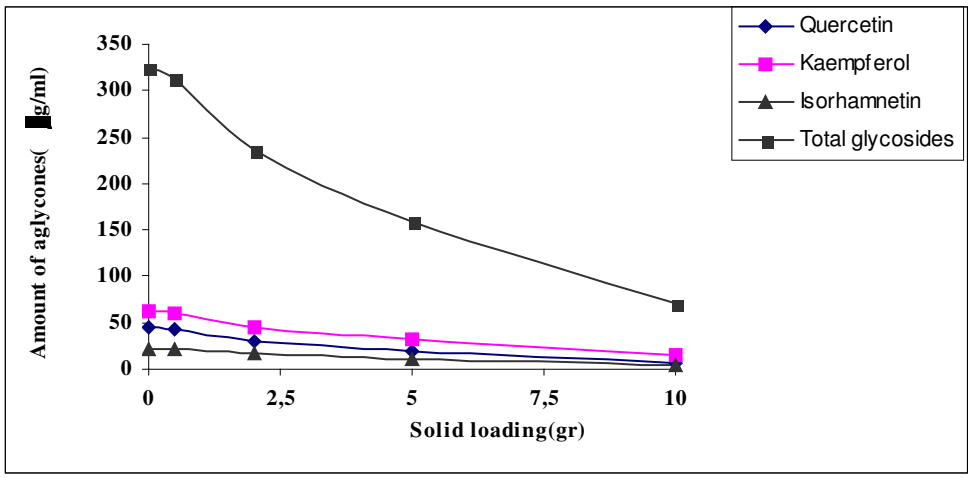


Figure 7.18: Adsorption results for the three aglycone constituents and the total flavonoid content for different solid loadings.

In order to investigate the effect of initial solution concentration on adsorption of flavonoid aglycone constituents, three different concentrations were experimented: 75, 100 and 125 g dry leaf material/l 80% ethanol solution. The zeolite amount added to each extract solution was constant as 10 g. The percent adsorbed amounts increased with increasing initial solution concentration as presented in Table 7.7. For 75 g/l initial solution concentration, the percent decrease in the amount of quercetin, kaempferol and isorhamnetin were calculated as 29.96%, 16.10% and 31.17%, respectively. For 100 g/l initial solution concentration, percent increase in the adsorbed amounts were 37.43%, 29.63% and 42.41%, respectively. And for 125 g/l initial solution concentration, these values were evaluated as 58.61%, 31.92% and 46.69%, respectively. Finally, the percent increase in the adsorbed amount of total flavonoid glycosides for 75 g/l, 100 g/l, and 125 g/l were calculated as 23.50%, 34.31% and 42.96%, respectively.

Table 7.7: Amounts of each aglycone constituent before and after adsorption with 10 g zeolite.

Initial solution concentration(g/l)		**75 g/l**	**100 g/l**	**125 g/l**
Quercetin conc.[a] (mg/l)	**Before ads.**	107.15	126.53	148.76
	After ads.	75.05	79.17	61.57
Kaempferol conc.[a] (mg/l)	**Before ads.**	180.54	218.59	249.66
	After ads.	151.48	153.82	169.98
Isorhamnetin conc.[a] (mg/l)	**Before ads.**	83.81	77.64	115.42
	After ads.	57.69	44.72	61.53
Total amount of flavonoid glycosides (mg/l)	**Before ads.**	932.48	1061.13	1289.71
	After ads.	713.37	697.03	735.62

[a] Mean values obtained from triplicate injections

[b] $\sum$ (amount of each aglycone) × 2.51 = total amount of flavonoid glycosides

The adsorption results tabulated in Table 7.7 are also illustrated in Figure 7.19 as shown below:

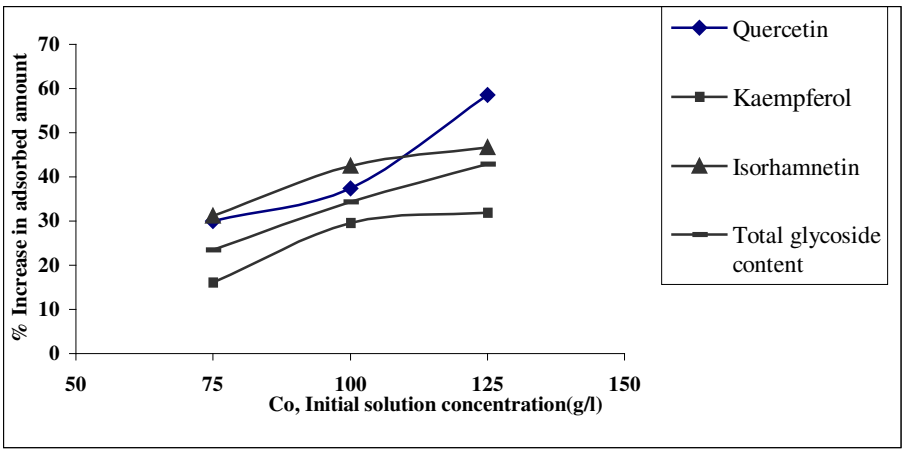


Figure 7.19: % Increase in the adsorbed values for the three aglycone constituents and the total flavonoid glycosides due to varying initial solution concentrations.

Since the cyclo- or benzene structures in the molecular configuration of flavonoid glycosides are hydrophobic, and the -OH residuals in the phenolic groups possibly form anions (Yu et al.,2003), it would be certainly very difficult to adsorb flavonol glycosides based on the usually mentioned surface physical adsorption mechanism. Therefore, the adsorption mechanism of flavonoid aglycones could rather be explained by hydrogen-bonding interactions with the zeolitic material, since flavonoid aglycones are effective hydrogen bonding donors (Xu et al.,2000).

7.2.2 Adsorption of Terpene Lactones

The terpene constituents were detected by ELSD within the commercial Ginkgo tablet extract with the retention times 4.3, 5.4, 8.8 and 9.4 min for Bilobalide, Ginkgolide C, Ginkgolide A and Ginkgolide B, respectively, as shown in Figure 7.20.

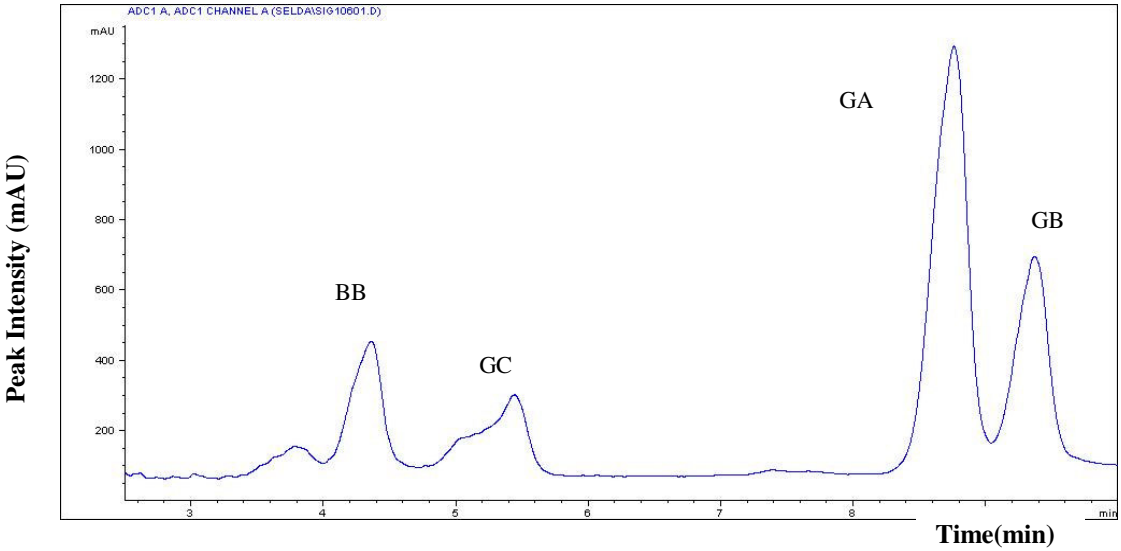


Figure 7.20: ELSD chromatogram of ethanolic (80%) extract of commercial Ginkgo tablet extract, showing the four terpenoid constituents: Bilobalide (BB), Ginkgolide C (GC), Ginkgolide A (GA) and Ginkgolide B (GB).

The adsorption studies were conducted by subjecting the extract solutions to adsorption with 0.5, 2, 5 and 10 g clinoptilolite-rich mineral. Resultant chromatogram peak areas for each terpene constituent were represented in terms of concentration (μg/ml) by inferring the corresponding standard curves of terpenoids (Appendix-B). Adsorption results are presented in Table 7.8 where results are tabulated as mean +/- 1 RSD values.

Table 7.8: Adsorption results for the terpenoids in commercial Ginkgo tablet extract.

	Bilobalide conc.[a] (μg/ml)	**Ginkgolide C conc.[a] (μg/ml)**	**Ginkgolide A conc.[a] (μg/ml)**	**Ginkgolide B conc. (μg/ml)[b]**
Before adsorption	172.2±3.5	126.2±1.6	600.9±36	412.3±22.3
After ads. with 0.5 g zeolite	188.4±6.3	128.7±5.1	726.8±11.6	456.3±11.8
After ads. with 2 g zeolite	181.5±4.5	127.7±6.1	693.9±1.2	449.6±3
After ads. with 5 g zeolite	177.92± 3	128.1±1.7	690.7±32.2	446.65±15.6
After ads. with 10 g zeolite	181.3±5.6	126.1±5.1	680.5±16.1	410.1±30.7

[a] Mean values obtained from 5 replicate injections.

The adsorption results for the terpene lactone constituents are also illustrated in Figure 7.21.

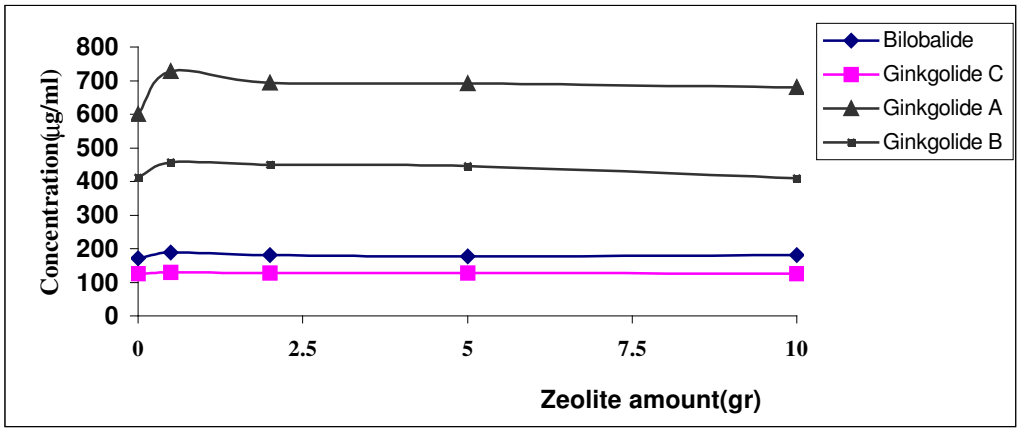


Figure 7.21: Adsorption results for the four terpenoid constituents: Bilobalide (BB), Ginkgolide C (GC), Ginkgolide A (GA) and Ginkgolide B (GB) for different amounts of zeolite loading.

As Table 7.8 and Figure 7.21 both indicate, the adsorption separation of terpene lactones on the clinoptilolite surface could not be achieved. Despite small differences between the five cases, the adsorption results were accepted nearly the same due to possible sensitivity differences while injecting the samples. The attempt to adsorb the terpenoids on the clinoptilolite surface can be attributed to the fact that ginkgolides are highly cage-like molecules and it is difficult for them to change their confirmation prior to being adsorbed on the surface of the adsorbent (Xu et al.,2000).

The cage-like structure of ginkgolides are maintained by C_4-O-C_{12} oxygen bridge and the bridge leads to the formation of an oxygen plane which is responsible for the specific interaction with protein microcosmic planar surface. The approximate planar electron atmosphere of ginkgolides is the base of its being adsorbed on the polar surface with the same scale microcosmic plane of the adsorbent as ginkgolides (Corey et al.,1988). The presence of these oxygen atoms yield an overall negative charge to the terpenoid structure, which in turn prevents these molecules from adsorption on the clinoptilolite surface, which is negatively-charged in nature. This negative charge results from the substitutions within the lattice of Al^{+3} for Si^{+4}, the broken bonds at the Si-O-Si (siloxane group) of clinoptilolite particularly generated at the particle surface during grinding and the lattice imperfections (Ersoy et al.,2002).

Although, there exist –H atoms (except Ginkgolide C) within the terpene lactone structures, electrostatic forces might overcome the hydrogen bonding interactions, so that adsorption could not be achieved for the terpene lactones.

7.2.3 Adsorption of Ginkgolic Acids

The adsorption experiments were performed for 100 ml of 80% ethanolic extract solutions of both Ginkgo leaf powder (10g) and the commercial product (GNC) (30 g). The amount of clinoptilolite were 0.5, 2, 5 and 10 g for 20 ml extract solutions. Unfortunately, due to HPLC results, no considerable change in the peak areas was observed. This could be explained by the weak affinity between the adsorbent surface and the ginkgolic acids due to the hydrophilic character of clinoptilolite and its modified forms (Si/Al ratio ≈ 5, 3) and the non-polar character of the ginkgolic acids. Therefore, modification of the zeolitic material by increasing its Si/Al ratio could lead to different results, since under such conditions, the adsorption of non-polar molecules would be favored (Farias et al., 2003).

7.3 HPLC Analysis Results

7.3.1 Optimization of ELSD Parameters

By setting the nebulizer gas flowrate as 2.3 l/min and drift tube temperature as 82.6^0C, the HPLC chromatogram for terpene lactones and the flavonoid aglycones present in the commercial Ginkgo biloba tablet extract were detected as shown in Figure 7.22. The terpene lactones (Ginkgolide A, Ginkgolide B, Ginkgolide C and Bilobalide) had retention times as 8.7, 9.4, 5.7 and 4.5 min, respectively, under ELSD. Also, the flavonoid constituents, quercetin, kaempferol and quercetin were detected at 18.2, 22 and 23 min, respectively, with ELSD.

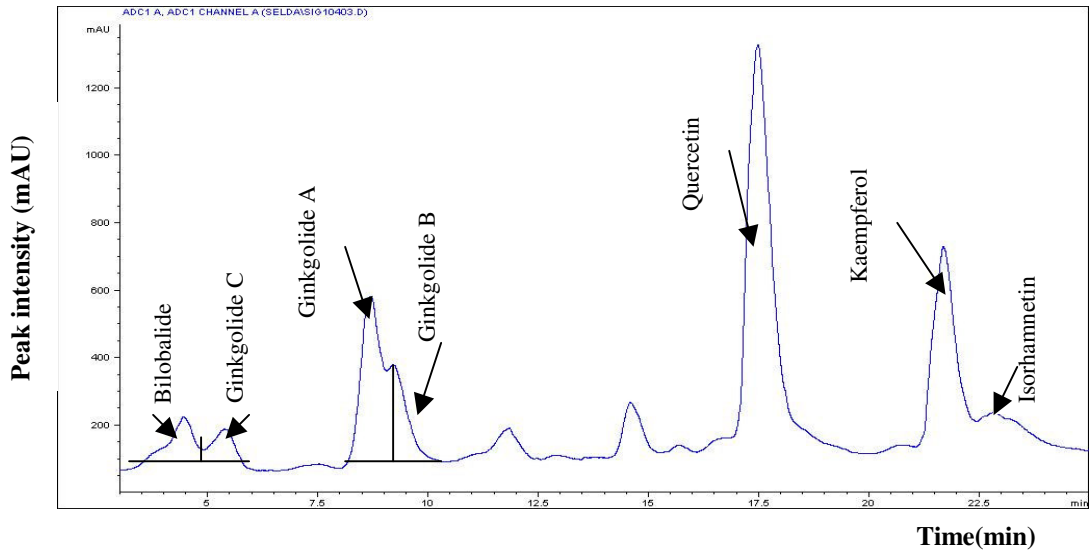


Figure 7.22: HPLC-ELSD chromatogram of the commercial Ginkgo biloba leaf extract.

However, detection of terpene lactones (GA, GB, GC and bilobalide) within the Ginkgo leaf extract by ELSD could not be achieved. This might be due to the complex plant matrix shadowing the presence of these compounds. A more selective extraction procedure for terpenes may overcome this problem.

The resultant ELSD chromatogram for terpene and flavonoid constituents within Ginkgo extract solutions was consistent with the one presented by Li et al (2002)., as in Figure 3.2.

7.3.2 Calibration Results for Flavonoid Glycosides

Linear ranges, correlation coefficients and regression equations for each aglycone according to the internal standard method are presented in Table 7.9.

Table 7.9: Calibration data for flavonoid aglycones including linear ranges, correlation coefficients (R^2) and regression equations

Aglycone	Linear Range (µg/ml)	Linear Range (µg/ml)	Regression Equation[a]
Quercetin	26-500	0.9959	Y=0.0114 X
Kaempferol	25-470	0.9944	Y = 0.0193 X
Isorhamnetin	54-500	0.9815	Y = 0.0031 X

[a] Y reflects the peak area, X the amount of compound in µg/ml; the detector response is linear.

The calibration graphs for individual standard reference aglycone compounds (for duplicate injections of each dilution) were obtained as shown in Appendix-C.

7.3.3 Calibration Results for Terpene Lactones

Linear ranges, correlation coefficients and regression equations for each terpene are given in Table 7.10.

Table 7.10: Calibration data for terpene lactones including linear ranges, correlation coefficients (R^2) and regression equations.

Terpene	Linear Range (μg/ml)	Correlation coefficient (R^2)	Regression Equation[a]
Ginkgolide A	100-800	0.9984	Y = 1.533X + 0.106
Ginkgolide B	75-600	0.9983	Y = 1.3685X + 0.488
Ginkgolide C	75-600	0.9942	Y = 1.4915X+ 0.087
Bilobalide	100-800	0.9975	Y = 1.4667X + 0.201

[a] Y reflects the log value of peak area, X the log value of the amount of compound in μg/ml; the detector response is linear.

The calibration graphs for individual standard reference terpene lactone compounds (for duplicate injections of each dilution) were obtained as shown in Appendix-B.

7.3.4 Calibration Result for the Standard Reference Mixture of Ginkgolic Acids

Optimized HPLC conditions were not effective for the detection of ginkgolic acids. The mobile phase was adopted as methanol, containing 0.05% TFA. By the addition of 0.03 mol/l silver nitrate ions (electronic acceptor) to the eluent, complexation of isolated double bonds (containing π electrons) of ginkgolic acid structure thus favoring the partition of ginkgolic acids toward the mobile phase (He et al.,2002) was aimed. Unfortunately, the resolution of ginkgolic acids could not be achieved. This was due to the fact that, the ethanolic extract cannot be analyzed directly for the detection of ginkgolic acids without removing the coextracted, interfering compounds having much stronger UV absorption near the detection wavelength and higher concentration than ginkgolic acids.

Therefore, the corresponding ginkgolic acid containing chromatograms for the Ginkgo leaf extract solution and the commercial product extract were presented in Figure 7.23. Also, the calibration graph for the standard reference mixture of ginkgolic acids (for four replicate injections of each dilution) was shown in Appendix D.

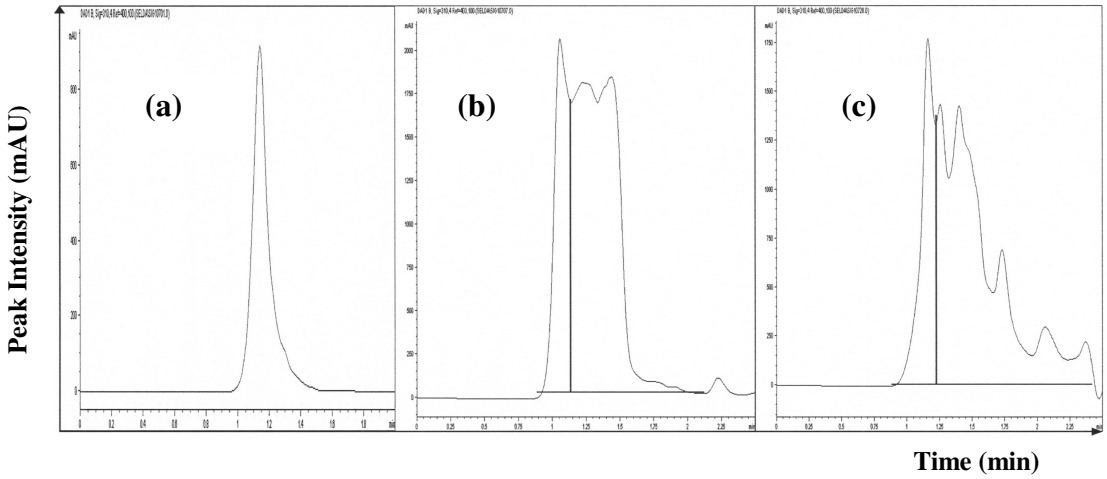


Figure 7.23: Ginkgolic acids standard mixture peak (a), and the corresponding Ginkgolic acid containing peaks for Ginkgolic acids commercial Ginkgo tablet extract (30 g extracted in 100 ml, 80%ethanol soln.) (b) and Ginkgo leaf extract (10 g extracted in 100 ml, 80% ethanol soln (c).

As a result, a more selective extraction procedure for ginkgolic acids should be adopted in order to achieve the peak resolution for individual ginkgolic acid constituents. The peak areas of ginkgolic acid containing peaks for Ginkgo biloba leaf extract solution and the commercial leaf extract solution were evaluated as 4651.58 mAu.s/ g dry material and 1815.4 mAu.s/g dry material, respectively. The difference in the peak areas might be correlated to the additional ginkgolic acid constituents within leaf extract solutios.

7.4 Phenol Content and Antioxidant Activity Determinations

7.4.1 Phenol Content Determinations

In order to determine the total phenolic content of different extract solutions, acetone and ethanol were used as extracting solvents in varying ratios with water. Figure 7.24 presents a clear comparison of various acetone/water and ethanol/water systems.

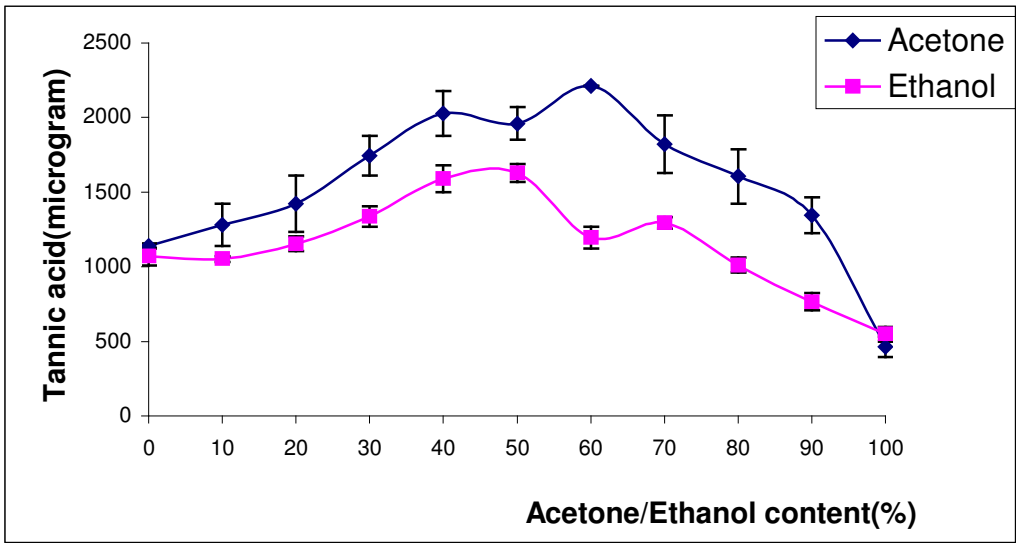


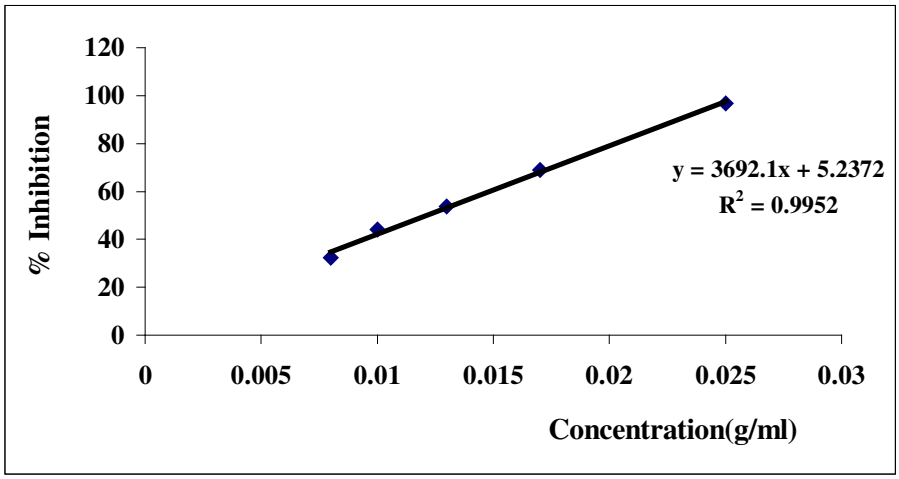
Figure 7.24: Total phenolic content of different extraction systems, taken as the average values of triplicate measurements.

The above figure also validates the fact that 60% acetone extraction yielded the highest quantity of phenolics and had the best pharmacological efficacy as stated by most of the pharmacopeias.

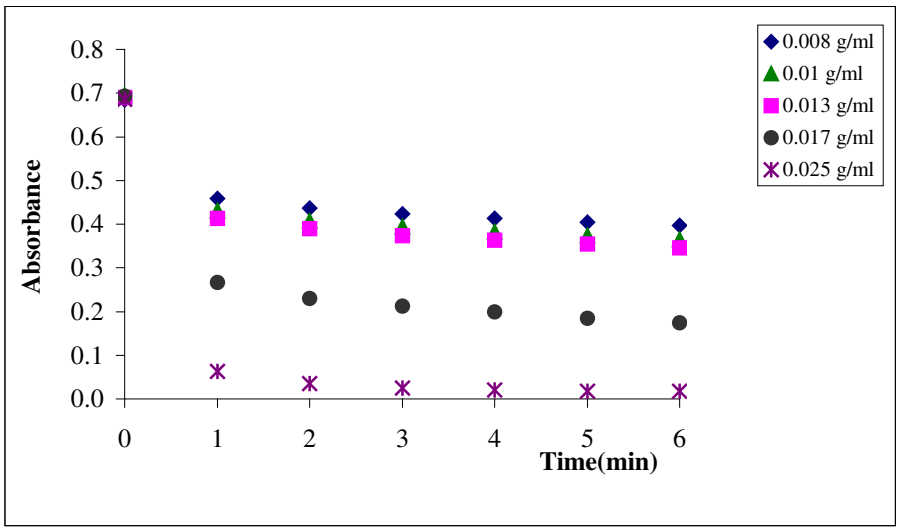
When the phenolic content of the extract solutions prepared with ethanol were compared, it was seen that 50% ethanol solution yielded the highest recovery of the phenolic constituents. However, since the efficiency of the extraction process considers both the extraction of terpene lactones and flavonoid gycosides, 80% ethanol solution was selected as the extraction solvent for the process.

7.4.1 Antioxidant Activity Determinations

The antioxidant activity measurements of the extract solutions were performed according to the TEAC (Trolox Equivalent Antioxidant Capacity) assay reported by Re et al (1999). The inhibition of the ABTS free radical by different extract solutions as a function of time were evaluated as follows:

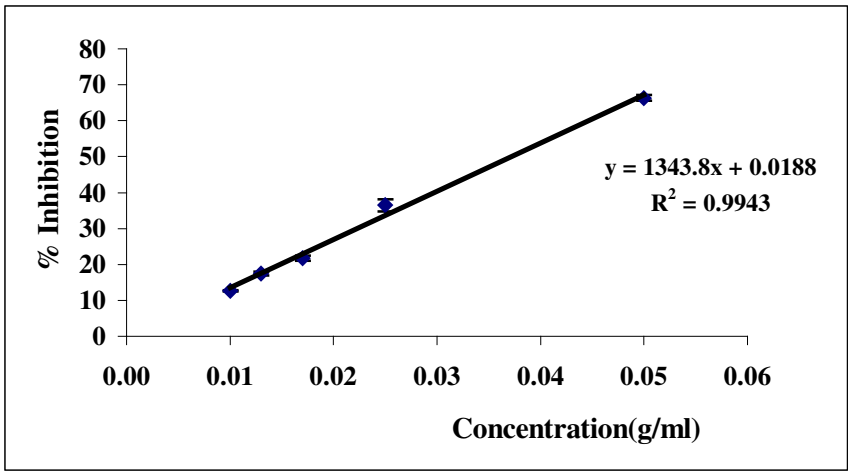


(a)

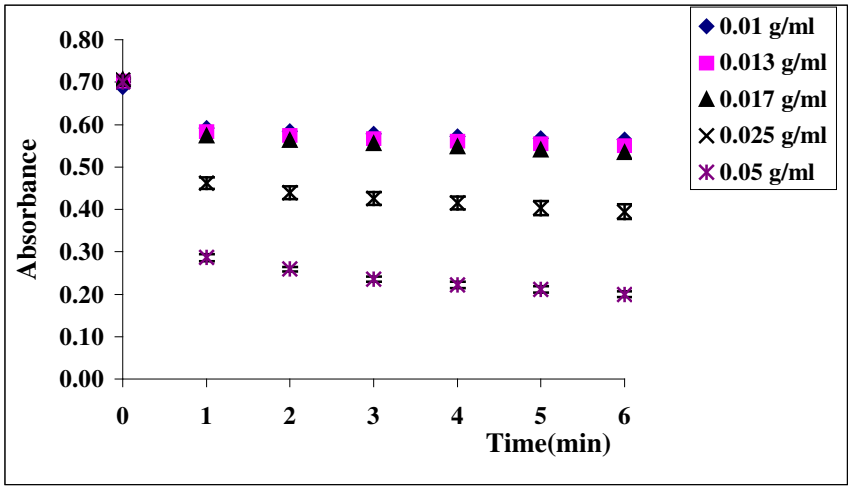


(b)

Figure 7.25: Concentration-response curve for the absorbance at 734 nm for $ABTS^{\cdot+}$ as a function of concentration of ethanolic Ginkgo leaf extract solution (a) and its time dependency (b).

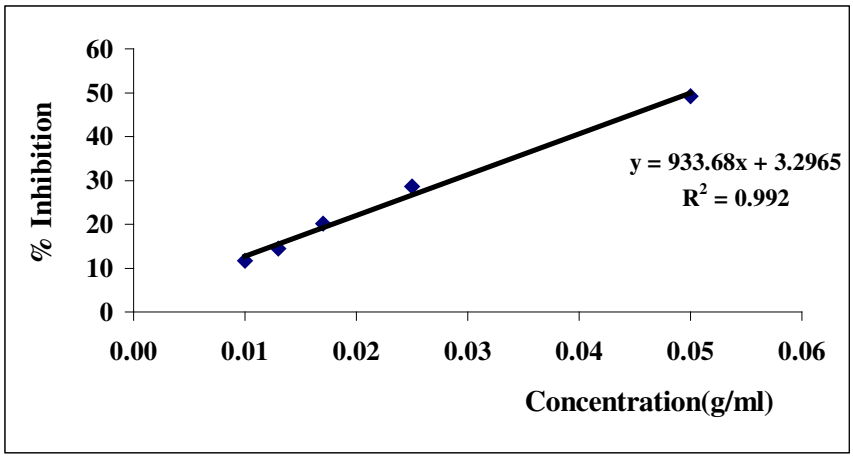


(a)

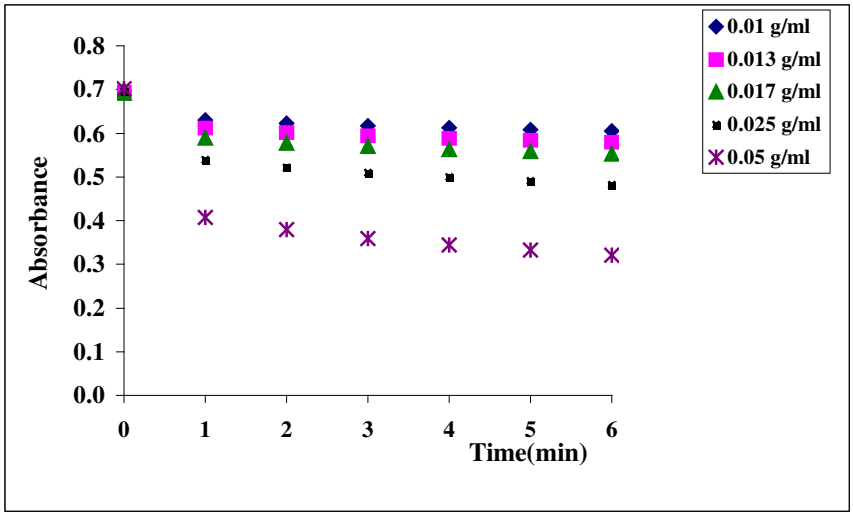


(b)

Figure 7.26: Concentration-response curve for the absorbance at 734 nm for $ABTS^{\cdot+}$ as a function of concentration of ethanolic Ginkgo leaf extract solution after treatment with 5 g zeolite (a) and its time dependency (b).



(a)



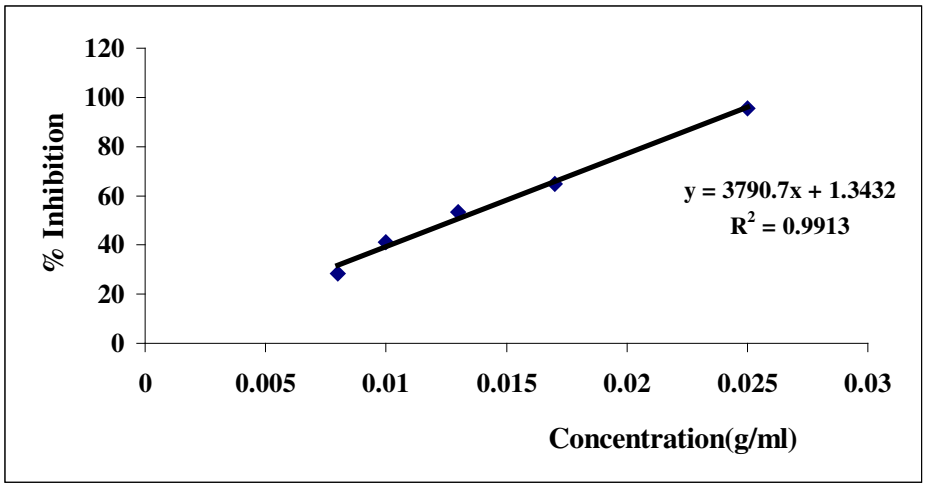
(b)

Figure 7.27: Concentration-response curve for the absorbance at 734 nm for $ABTS^{\cdot+}$ as a function of concentration of ethanolic Ginkgo leaf extract solution after treatment with 10 g zeolite (a) and its time dependency (b).

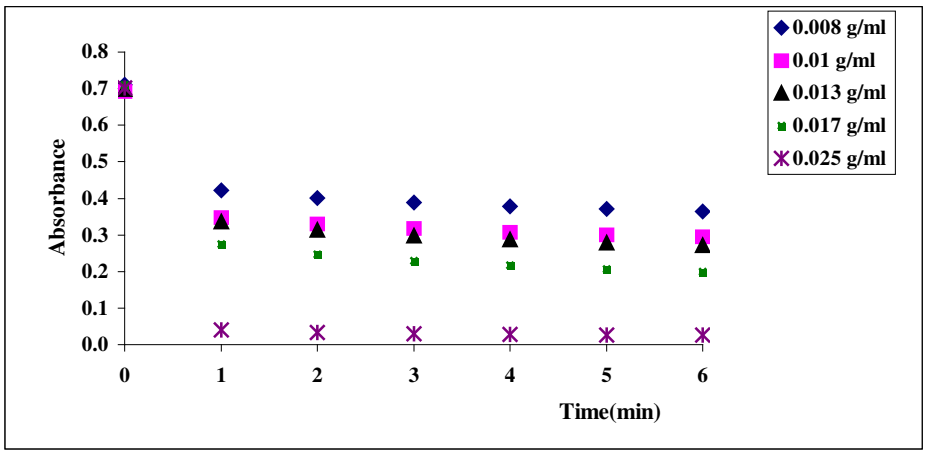
The more rapidly the absorbance decreases, the more potent the antioxidant activity of the extract solution. Since the absorbance decreased more sharply for the leaf extract solution when compared with the extract solutions after adsorption with clinoptilolite-rich mineral, the antioxidant activity of initial extract solution was the greatest. The smaller decrease in the absorbance values due to adsorption demonstrated the decrease in the total antioxidant activity.

In order to examine the effect of the adsorbent amount on the antioxidant activity of the extract solutions, Figure 7.26 and Figure 7.27 were compared for 0.025 g/ml and 0.05 g/ml solution concentrations, since 0.01 g/ml, 0.013 g/ml and 0.017 g/ml solution concentrations didn't yield comparable decrease for both absorbance vs. time plots. Regarding that the initial absorbance values were the same, the absorbance values

recorded at the end of 6 min for 0.025 g/ml solutions as $A_{t=6\ min}$=0.394 and $A_{t=6\ min}$=0.483 for 5 and 10 g zeolite additions, respectively. Also for 0.05 g/ml solutions, these values were recorded as $A_{t=6\ min}$=0.394 and $A_{t=6\ min}$=0.483 for 5 and 10 g zeolite additions, respectively. So, the extract solution after treatment with 5 g zeolite yielded a steeper decrease in its absorbance value, indicating that it possesses higher antioxidant activity.

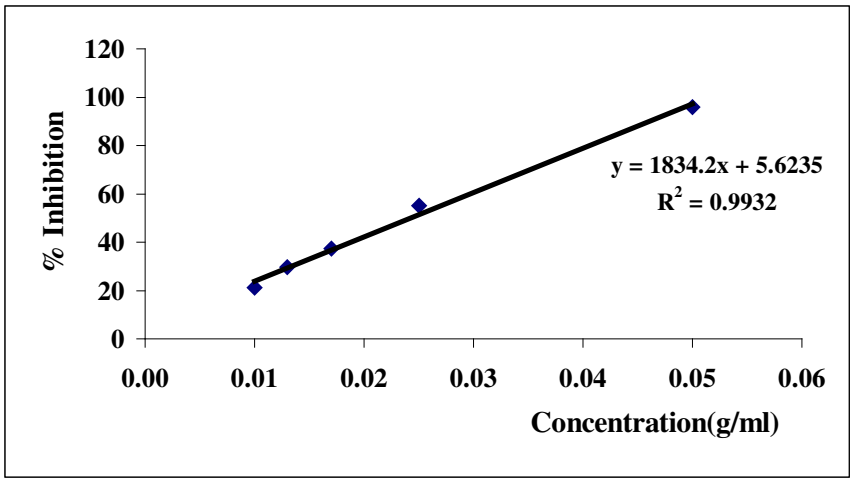


(a)

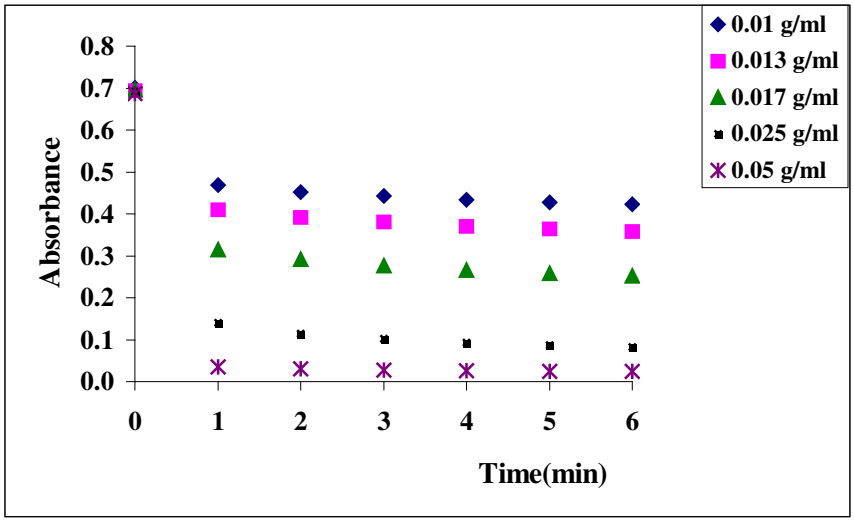


(b)

Figure 7.28: Concentration-response curve for the absorbance at 734 nm for $ABTS^{\cdot+}$ as a function of concentration of ethanolic GNC tablet extract solution (a) and its time dependency (b).

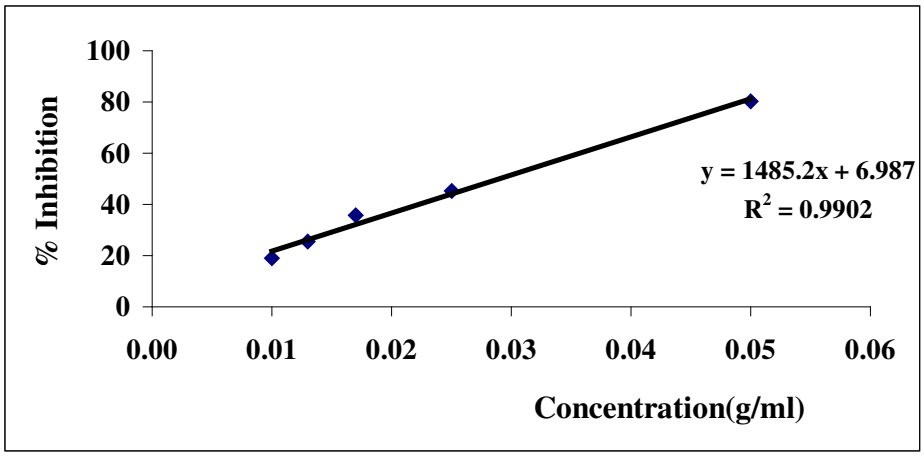


(a)

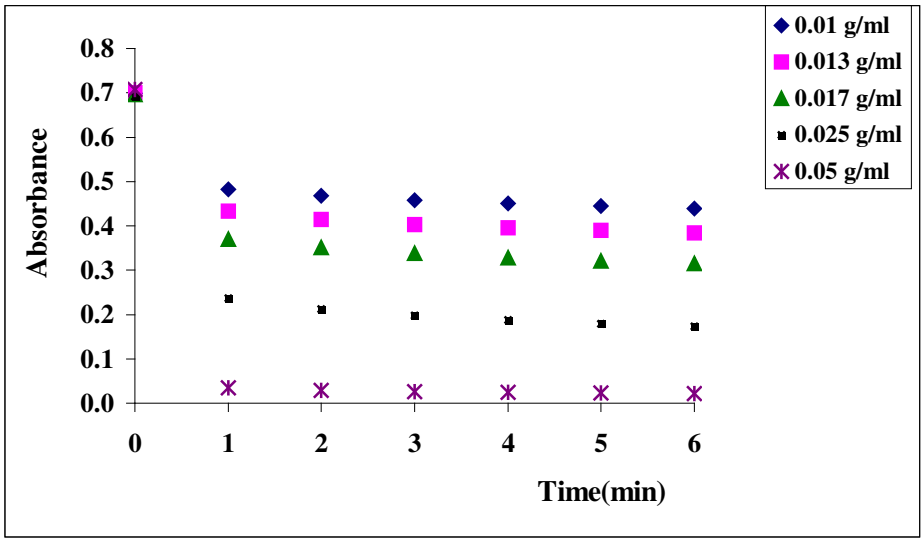


(b)

Figure 7.29: Concentration-response curve for the absorbance at 734 nm for $ABTS^{\cdot+}$ as a function of concentration of ethanolic GNC tablet extract solution after treatment with 5 g zeolite (a) and its time dependency (b).



(a)



(b)

Figure 7.30: Concentration-response curve for the absorbance at 734 nm for $ABTS^{\cdot+}$ as a function of concentration of ethanolic GNC tablet extract solution after treatment with 10 g zeolite (a) and its time dependency (b).

Also for the commercial GNC tablet extract solution, the absorbance decreased more sharply when compared with the extract solutions after adsorption with clinoptilolite-rich mineral, demonstrating that the antioxidant activity of initial extract solution was the greatest.

In order to examine the effect of the adsorbent amount on the antioxidant activity of the extract solutions, Figure 7.29 and Figure 7.30 were compared for 0.025 g/ml and 0.017 g/ml solution concentrations, since these concentration values showed more clear absorbance decrease curves when compared to the other concentrations. Regarding that the initial absorbance values were the same, the absorbance values recorded at the end of 6 min for 0.025 g/ml solutions as $A_{t=6\ min}$=0.083 and $A_{t=6\ min}$=0.173 for 5 and 10 g zeolite additions, respectively. Also for 0.017 g/ml solutions, these values were

recorded as $A_{t=6\ min}$=0.251 and $A_{t=6\ min}$=0.315 for 5 and 10 g zeolite additions, respectively. So, the extract solution after treatment with 5 g zeolite yielded a steeper decrease in its absorbance value, indicating that it possesses higher antioxidant activity.

The concentration of antioxidant giving the same percentage inhibition of absorbance of the radical cation, $ABTS^+$ at 734 nm as 1 mM (0.25 g/l) Trolox is calculated in terms of TEAC by dividing the gradient of the plot of the percentage inhibition of absorbance vs. concentration plot for the antioxidant in question is divided by the gradient of the plot for Trolox (Appendix-F). The antioxidant activities of standard antioxidants kaempferol, quercetin and propyl gallate (PG) were measured as 0.76 TEAC, 1.25 TEAC and 2.45 TEAC, respectively (Appendix-G). This results were consistent with the TEAC values stated for kaempferol, quercetin and PG as 1.45 TEAC, 2.76 TEAC (Koleva et al.,2001) and 2.08 TEAC (Altıok, 2003), respectively.

However, in complex mixtures, additive, synergistic, or inhibitory effects among the components have been observed. To evaluate the contribution of each single sample component, knowledge of its molecular structure is required. Unfortunately, in the search of novel active compounds in complex matrixes, this is not always possible (Koleva et al.,2001). Therefore, the total antioxidant activity of plant extracts is rather expressed in terms of μmol of Trolox per 100 gram of dry weight (DW) (Cai et al.,2004). Then, the antioxidant activities of the ethanolic (80%) Ginkgo biloba leaf extract and commercial Ginkgo leaf extract solutions were calculated as 225.5 mmol Trolox/ 100g dry leaf powder and 77.17 mmol Trolox/100g dry extract powder, respectively. Hence, our Ginkgo leaf extract solution showed nearly three times the antioxidant activity possessed by the commercial Ginkgo leaf extract.

When the antioxidant activity of the extract solutions were measured before and after adsorption with clinoptilolite-rich mineral, the total antioxidant activity of the extract solutions decreased due to adsorption of the phenolic constituents on the surface of clinoptilolite. The TEAC values for the corresponding extract solutions and the change in their antioxidant activities due to adsorption were presented in Table 7.11.

Besides, due to its ability to host pharmaceuticals, the adsorbed species on the clinoptilolite surface were assumed not to loose their antioxidant activity after adsorption. Dashevsky and his co-workers (1995), investigated the process of adsorption-desorption of quercetin on the medicinal adsorbent “Dniper”, a carbonic adsorbent with a high specific surface area and high adsorption capacity, in order to

create drug forms with controlled release. They concluded their study as on the surface of the adsorbent, quercetin did not loose its antioxidant activity.

The antioxidant activity determinations using TEAC assay were performed for the unhydrolyzed extract solutions regarding the possibility of degradation of especially phenolic constituents under harsh hydrolysis conditions (85^0C). However, Cai et al.(2004) reported that flavonol aglycones, such as quercetin, myricetin, and kaempferol, containing multiple hydroxyl groups, had higher antioxidant activity than their glycosides, such as rutin, myricetin, astragalin.

Table 7.11: TEAC values of the extract solutions, before and after adsorption.

	Before adsorption	**Treated with 5 g zeolite**	**Treated with 10 g zeolite**
Ginkgo leaf extract solution	225.5 TEAC[a,b]	82.1 TEAC[a,b]	57.03 TEAC[a,b]
Commercial (GNC) ginkgo leaf extract solution	77.17 TEAC[a,b]	37.35 TEAC[a,b]	30.24 TEAC[a,b]

[a]TEAC, Trolox equivalent antioxidant activity, expressed as mmol Trolox per 100 gram dry weight (DW).
[b] All values were mean of triplicates.

These results suggest that there is a strong relationship between the total phenolic content and the antioxidant activity expressed as TEAC. Hence, the antioxidant activity in Ginkgo biloba leaf extract solutions is largely due to the presence of phenolic constituents.

CONCLUSION

The possible use of Turkish clinoptilolite-rich natural zeolite as a novel drug carrier for Ginkgo biloba L. extract was investigated throughout this study.

The Ginkgo leaves were extracted by solvent extraction with various solvents and, the extraction and adsorption efficiencies were investigated according to the data collected from High Performance Liquid Chromatography (HPLC) analysis. The results were compared with the results obtained from the extracts of commercial Ginkgo tablets.

Extraction efficiencies were found to be highly affected by the solvent type and composition. Even a slight change in the polarity of the extracting solvent system highly affected the extent to which the compounds of interest were extracted through the extraction medium. In literature, several extracting solvents were experimented (methanol, acetone,ethanol and water) in order to decide on the best solvent system for the extraction of the target compounds, terpene lactones and flavonoid glycosides. Due to the lack of commercially available flavonoid glycoside standard compounds, fingerprint chromatograms were investigated in the first place in order to get a rough estimate of the amount of the extracted species. The exact quantification of the extracted flavonoid glycoside constituents was achieved via acidic hydrolysis of these compounds to their aglycone forms.

Although methanol yielded the cleanest extract and good resolved peaks in the HPLC chromatograms, 80% ethanol solution was adopted for the extraction processes due to its non-toxic nature for the use in dietary purposes and wide application in antioxidant activity determinations for the herbal extract solutions.

Since the molecular size of the extracted species were much greater than the dimensional size of the clinoptilolite channels, only surface adsorption phenomena was expected to occur. Adsorption studies showed that flavonoid aglycone constituents were selectively adsorbed on the surface of the zeolitic material. The adsorption efficiencies for these constituents were investigated for different extraction solvents. The polarity of the extracting solvent determined the degree of adsorption of aglycones. Solvents with lower polarity, e.g. acetone, showed higher adsorption rates when compared to other solvents due to its polarity competing with the polarity of the adsorbent.

The terpene lactone constituents were found not to adsorb on the surface of clinoptilolite-rich mineral. This result can be attributed to the fact that these compounds

possess highly non-polar surface structure. As a result, the physical adsorption of these constituents on the surface of hydrophilic clinoptilolite could not be achieved.

The extraction and adsorption studies of ginkgolic acid constituents could not be achieved in this study. This is due to the fact that a more selective extraction process should be adopted for ginkgolic acids, whereas for the extraction of Ginkgo biloba leaves a single-step solvent extraction was adopted.

Antioxidant activity determinations were also performed for the leaf extract solutions before and after adsorption via Trolox Equivalent Antioxidant Activity (TEAC) assay. The decrease in the total antioxidant activity values due to adsorption demonstrated that the phenolic constituents i.e. flavonoid compounds were adsorbed on the surface of the zeolitic material.

Finally, the use of clinoptilolite as a novel drug carrier for the flavonoid aglycone constituents should be developed regarding the release studies of those compounds as a future perspective of this study.

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APPENDIX-A

CHARACTERIZATION OF THE ZEOLITIC PHASE

A1- Particle Size Distribution

The sample was homogenized and sieved into a fine particle size (Fritsch, Pulverisette) varying within the size range determined by the particle size analyzer (Micromeritics, Sedigraph 5100) as shown in Figure A.1. Chemical composition of the zeolitic material under investigation is briefly summarized in Table A.1.

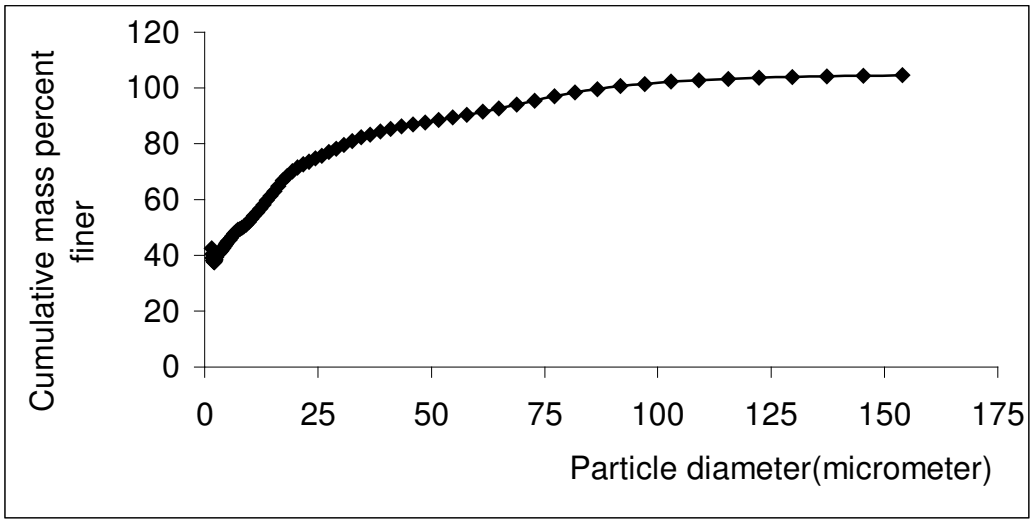


Figure A.1: Particle size distribution of the natural zeolite used in the study.

A2- SEM Studies

Scanning electron microscopy (SEM, Philips XL 30S FEG) was used for photomicrographs as well as to analyze the zeolite composition (Energy Dispersion X-ray, EDX). The sample was initially placed in a vacuum chamber for coating with a thin layer (few nanometers) of gold (Au). To obtain a more representative chemical composition of the sample bulk, the analysis was done on 10 different grains of each sample, and the element contents were averaged as tabulated in Table A.1.

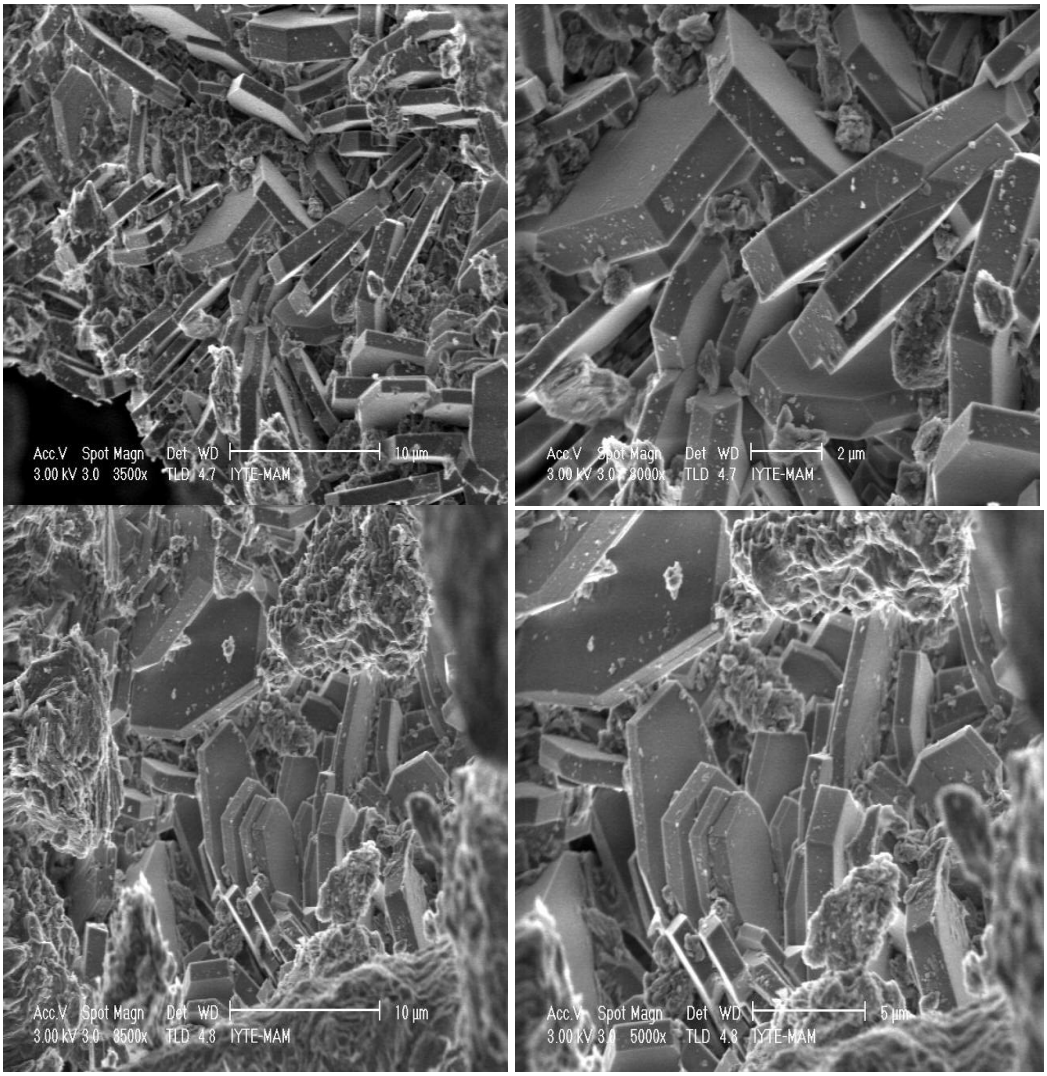


Figure A.2: SEM micrographs of the clinoptilolite-rich mineral used in the study.

Table A.1: Chemical composition (weight%) of washed natural zeolite obtained from EDX analysis.

Elements	Weight %[a]	Relative Standard Deviation (RSD)[b]
SiO_2	70.01	± 1.91
Al_2O_3	15.58	± 0.91
Fe_2O_3	3.34	± 1.93
MgO	2.49	± 0.30
CaO	2.30	± 0.6
Na_2O	1.51	± 0.34
K_2O	3.68	± 0.58
P_2O_5	0.98	± 0.44

[a] Mean values of 10 different grain particles

[b] Relative standard deviation, V= $\frac{s}{\overline{X}}$

where s = standard deviation

$\overline{X}$ = mean of sample (Zar, 1999).

A3- Thermal Analysis

Thermal gravimetric analysis (TGA) of the zeolitic material was conducted using Labsys^{TM} SETARAM type of instrument. Approximately 10 mg sample was heated at a heating rate of 10^0C/min under nitrogen purge stream with the pressure 1.5 bar up to 1000^0C. The total amount of water lost from the sample up to 1000^0C was determined as approximately 10% as illustrated in Figure A.3.

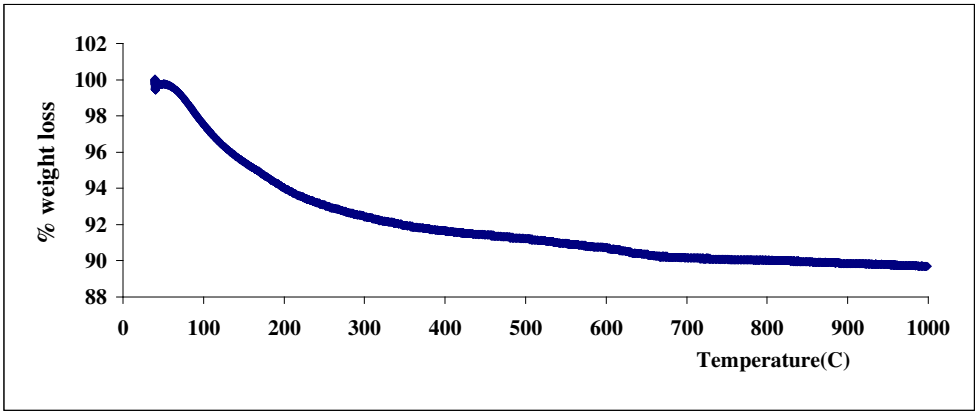


Figure A.3: Thermal Gravimetric Analysis (TGA) curve of the zeolitic material under examination.

As Derivative Thermal Gravimetric Analysis (DTGA) curve presented in Figure-A.4 illustrates, the clinoptilolite used in this study dehydrated in two steps; one up to 200 ^{0}C, and the second one between 200 and 800 ^{0}C, yielding average weight losses as 6% and 4%, respectively.

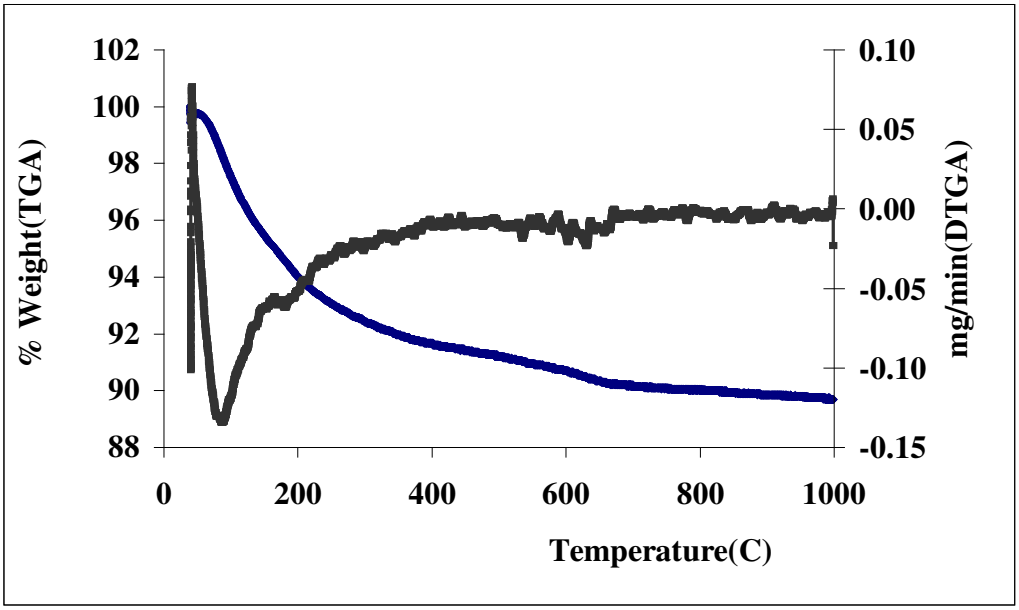


Figure A.4: DTGA curve of the original clinoptilolite sample used in the study.

A4- N_2 Adsorption Studies

A4-1 Assessment of Surface Area

A4-1.1 Brunauer-Emmett-Teller (BET) Method

In the original work of Brunauer, Emmett and Teller (1938) it was found that Type II nitrogen isotherms on various adsorbents gave linear BET plots over the approximate range p/p^0 = 0.05-0.35 (Rouquerol et al., 1999).

The BET equation is conveniently expressed in the linear form:

$$\frac{p/p^0}{V(1-p/p^0)} = \frac{1}{V_m \times C} + \frac{C-1}{V_m \times C} \times (p/p^0) \qquad \text{(A4.1)}$$

Thus, the BET plot of $(p/p^0)/[V(1-p/p^0)]$ versus p/p^0 should be a straight line with slope s = (C-1)/ n_mC and intercept i=1/n_mC. By solving the these two simultaneous equations, one can reach:

$$V_m = 1 / (s + i) \quad \text{(A4.2)}$$

and

$$C = (s / i) + 1 \quad \text{(A4.3)}$$

Then, the surface area (A) can be calculated as:

$$A = V_m \times L \times a_m \quad \text{(A4.4)}$$

where

L = Avogadro's number

A_m = surface area occupied by one molecule adsorptive (Sing et al., 1985).

A4-1.2 Langmuir Model

The Type-I isotherm is assumed to conform to the Langmuir model. Langmuir model can be expressed as:

$$\theta = \frac{V}{V_m} = \frac{b \times p}{1 + b \times p} \quad \text{(A4.5)}$$

where

p = pressure

b = adsorption coefficient

V = volume adsorbed at p

V_m = monolayer capacity

At low p, $(1 + b \times p) \sim 1$, then $\theta = b \times p$ (Henry's Law)

And at high p, $(1 + b \times p) \sim b \times p$, then $\theta \rightarrow 1$.

In the linear form:

$$\frac{p}{V} = \frac{p}{V_m} + \frac{1}{V_m \times b} \quad \text{(A4.6)}$$

Plot of p/ V vs. p gives slope= 1/ V_m and intercept = 1 / V_mb in the range $0.01 \leq p / p^o \leq 0.1$ providing the best correlation coefficient.

Then, the surface area (A) can be calculated as stated in Equation A4.4.

A4-2 CALCULATION OF SURFACE AREA

The specific surface area was evaluated at 77.45 K by nitrogen gas adsorption methods, using Micromeritics ASAP 2010 model static volumetric adsorption instrument. The samples were dried in an oven at 200^0C for 3 hours prior to degassing and degas conditions were adjusted as 350^0C and 24 hours.

In order to make comparison, BET and Langmuir surface areas and parameters of each model for the clinoptilolite sample are summarized in Table A.2.

Table A.2: BET and Langmuir Surface Areas with Model Parameters for the clinoptilolite-rich mineral.

BET Model			**Langmuir Model**		
Surface Area (m^2/g)	**C**	**Vm (cc/g STP)**	**Surface Area (m^2/g)**	**b**	**Vm (cc/g STP)**
34.90	-73.5	8.02	50.23	0.35	10.98

The physisorption isotherm of the clinoptilolite-rich mineral is Type-IV as seen in Figure A.5, therefore the surface area obtained by the BET model seems to fit the adsorption process and present the external surface area better than does the Langmuir model.

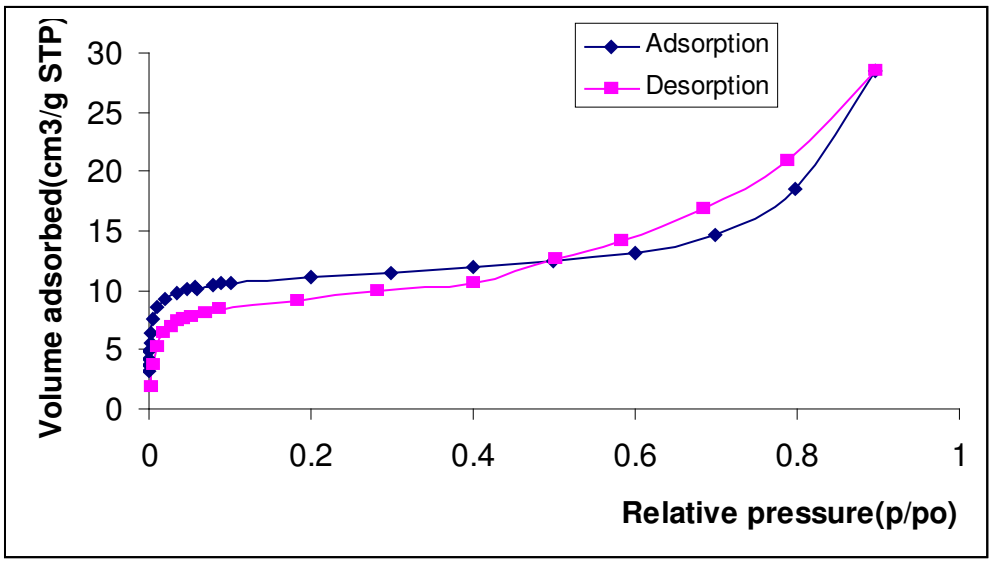
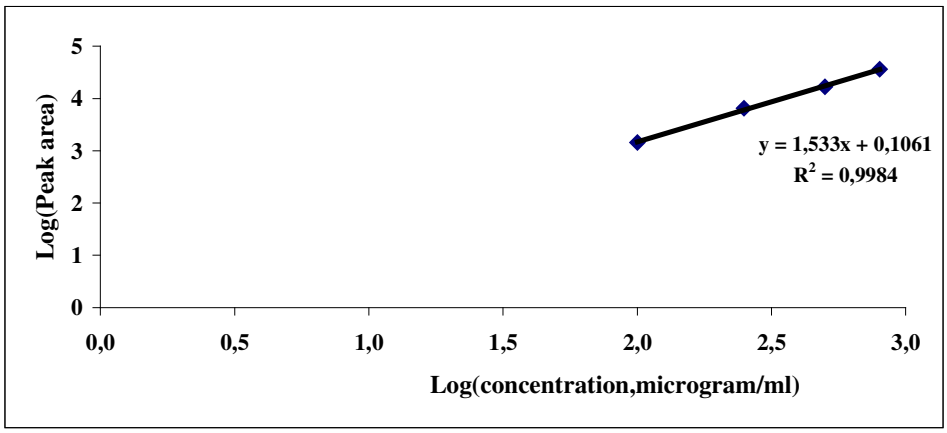


Figure A.5: Adsorption-desorption behaviour of the clinoptilolite used in the study.

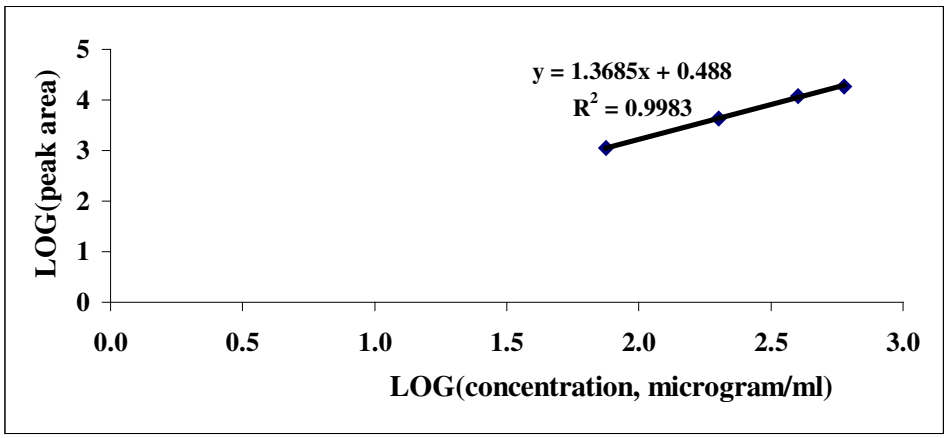
APPENDIX-B

CALIBRATION CURVES FOR TERPENE LACTONES

Figure B.1 illustrates the calibration curves for terpene lactone compounds (Ginkgolide A, Ginkgolide B, Ginkgolide C and Bilobalide) in Ginkgo biloba L. extracts.



(a)



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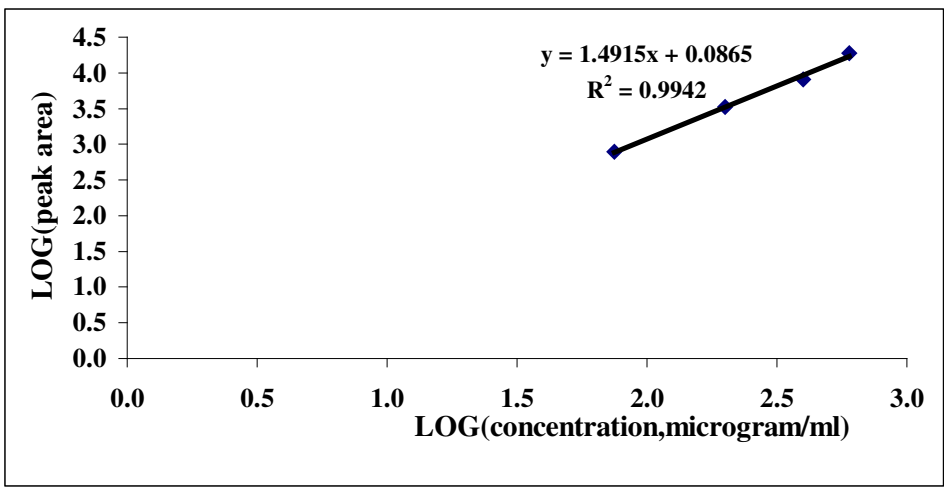
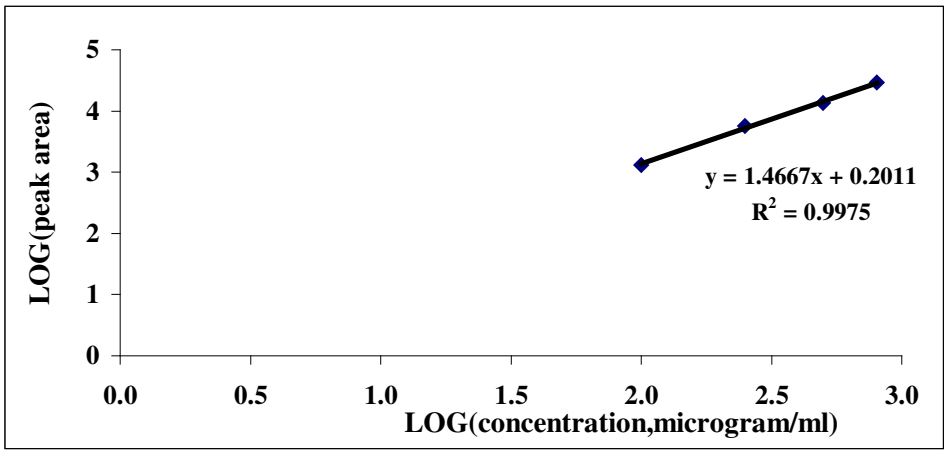


(c)



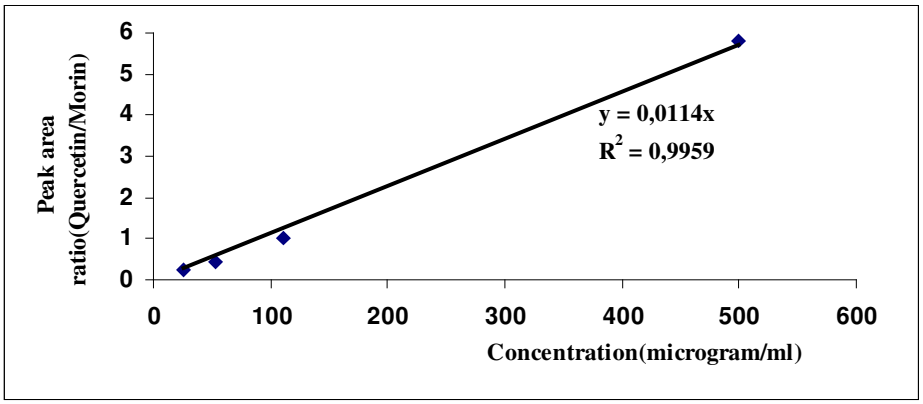
(d)

Figure B.1: Calibration curves for Terpene Lactone Compounds; Ginkgolide A (a), Ginkgolide B (b), Ginkgolide C (c) and Bilobalide (d) in Ginkgo biloba L. Extract.

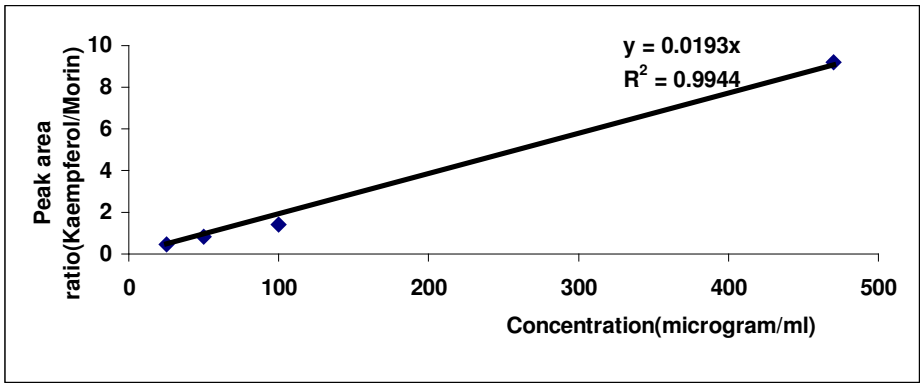
APPENDIX-C

CALIBRATION CURVES FOR FLAVONOID GLYCOSIDES

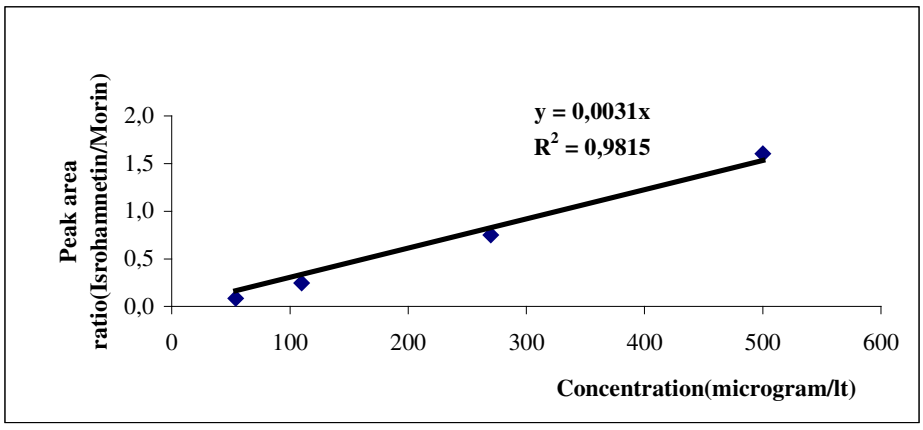
Figure C.1 shows the calibration curves for flavonoid glycoside compounds (quercetin, kaempferol and isorhamnetin in Ginkgo biloba leaf extract.



(a)



(b)



(c)

Figure C.1: Calibration curves for flavonoid glycoside compounds; Quercetin (a), Kaempferol (b) and Isorhamnetin (c) in Ginkgo Biloba Leaf Extract.

APPENDIX-D

CALIBRATION CURVE FOR THE GINKGOLIC ACID STANDARD REFERENCE MIXTURE

Figure D.1 illustrates the standard calibration curve for the ginkgolic acid standard reference mixture.

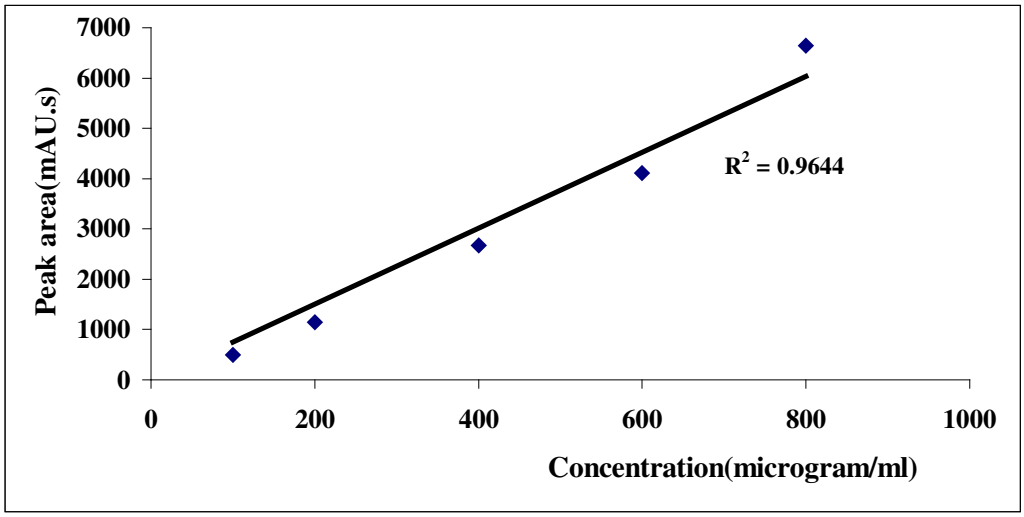


Figure D.1: Standard calibration curve for the ginkgolic acid standard reference mixture.

APPENDIX-E

TANNIC ACID CALIBRATION CURVE

Figure E.1 shows the tannic acid calibration curve for the Folin-Ciocalteu reagent method.

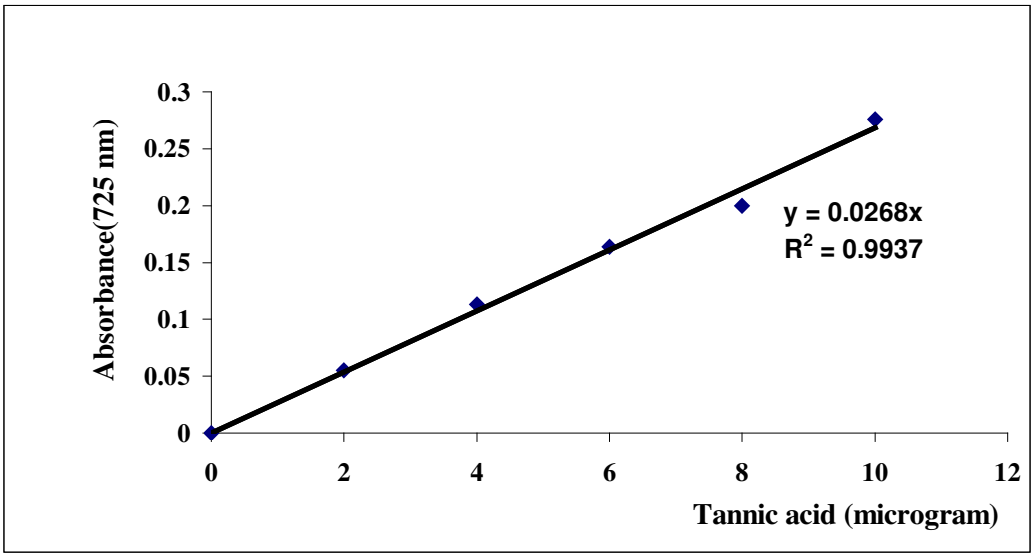


Figure E.1: Tannic acid calibration graph for Folin-Ciocalteu Method.

APPENDIX-F

TROLOX STANDARD CURVE OBTAINED FROM ABTS$^{\cdot+}$ ASSAY

Figure F.1 illustrates the concentration-response curve for the absorbance at 734 nm for ABTS$^{\cdot+}$ as a function of concentration of standard solution of Trolox.

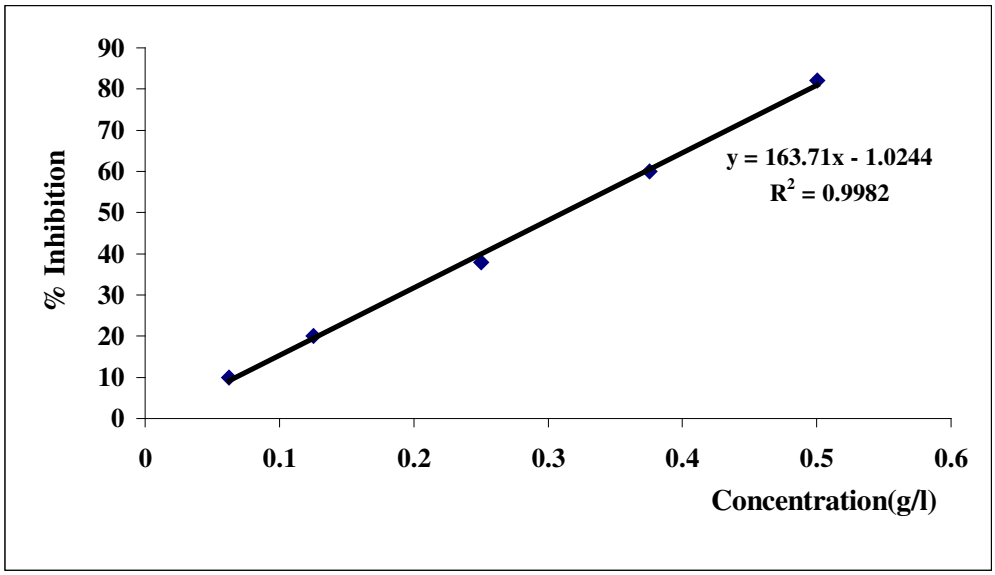
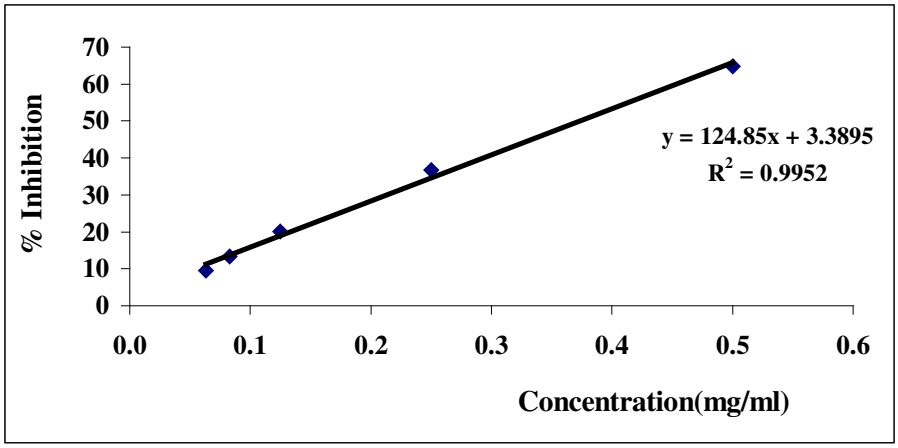


Figure F.1: Concentration-response curve for the absorbance at 734 nm for ABTS$^{\cdot+}$ as a function of concentration of standard solution of Trolox (Altıok, 2003).

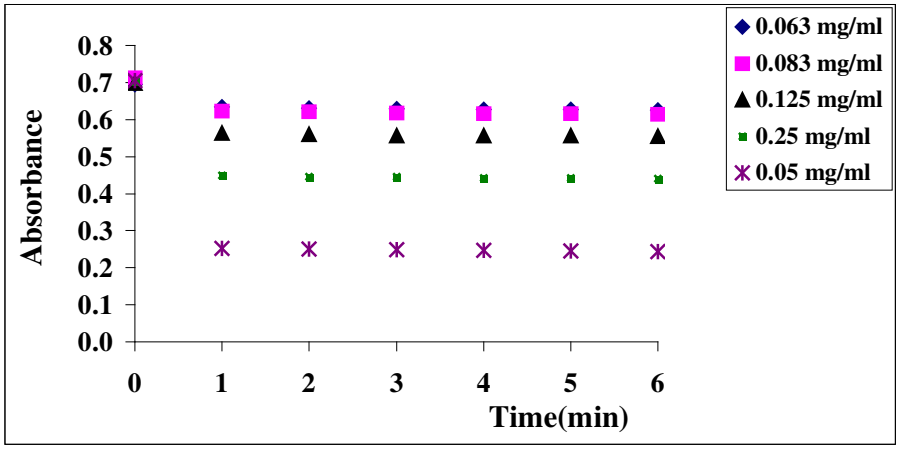
APPENDIX-G

% INHIBITION PLOTS OF KAEMPFEROL, QUERCETIN AND PROPYL GALLATE (PG) OBTAINED FROM ABTS$^{\cdot+}$ ASSAY

Figure G.1 illustrates the concentration-response curve for the absorbance at 734 nm for ABTS radical cation as a function of concentration of kaempferol and its time dependency.



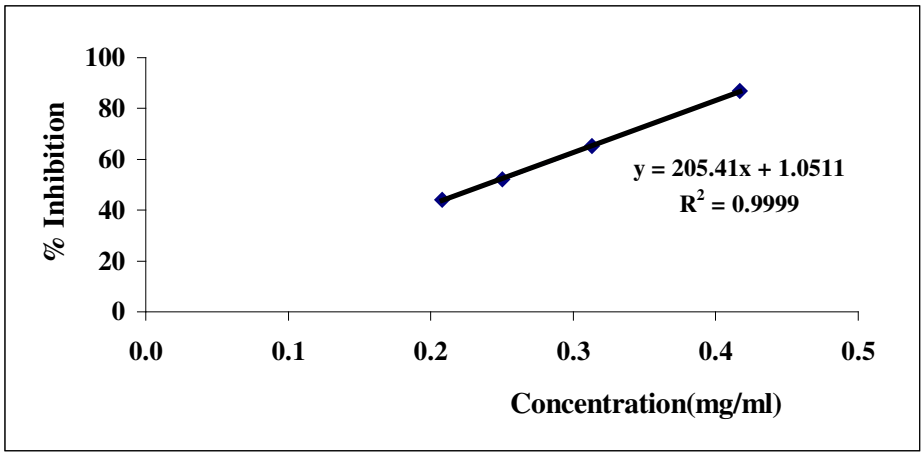
(a)



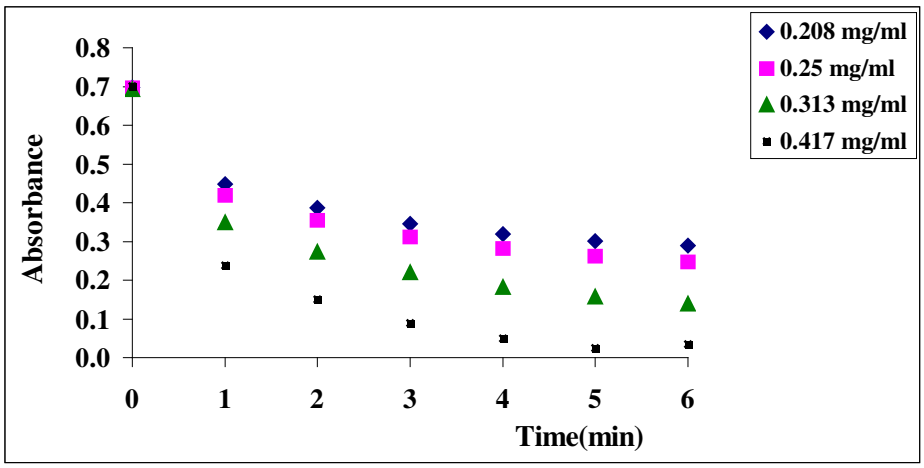
(b)

Figure G.1: Concentration-response curve for the absorbance at 734 nm for ABTS$^{\cdot+}$ as a function of concentration of kaempferol (a) and its time dependency (b).

Also, Figure G.2 presents the concentration-response curve for the absorbance at 734 nm for ABTS radical cation as a function of concentration of quercetin and its time dependency.



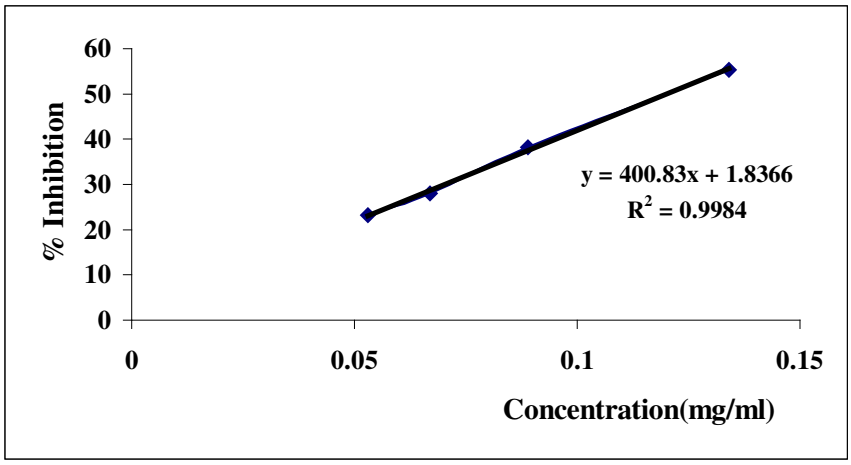
(a)



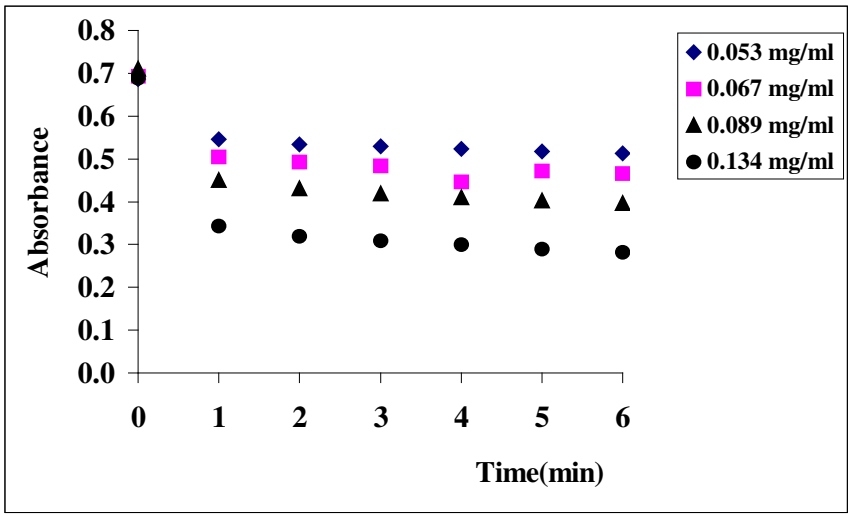
(b)

Figure G.2: Concentration-response curve for the absorbance at 734 nm for $ABTS^{\cdot+}$ as a function of concentration of quercetin (a) and its time dependency (b).

Besides, Figure G.3 presents the concentration-response curve for the absorbance at 734 nm for ABTS radical cation as a function of concentration of synthetic antioxidant, propyl gallate (PG) and its time dependency.



(a)



(b)

Figure G.3: Concentration-response curve for the absorbance at 734 nm for $ABTS^{\cdot+}$ as a function of concentration of synthetic antioxidant, propyl gallate (PG) (a) and its time dependency (b).

APPENDIX-H

ELUOTROPIC STRENGTH OF SOLVENTS ON VARIOUS SORBENTS

Table H.1 tabulates the eluotropic strength of solvents on various sorbents.

Table H.1: Eluotropic Strength of Solvents on Various Sorbents

Solvent	$\varepsilon^0(Al_2O_3)$[a]	$\varepsilon^0(SiOH)$[a]	$\varepsilon^0(C_{18})$[a]	P`
Pentane	δ0.00	δ0.00	-	0.0
Hexane	0.00-0.01	0.00-0.01	-	0.1
Iso-octane	0.01	0.01	-	0.1
Cyclohexane	0.04	0.03	-	0.2
Carbon tetrachloride	0.17-0.18	0.11	-	1.6
1-Chlorobutane	0.26-0.30	0.20	-	1.0
Xylene	0.26	-	-	2.5
Toluene	0.20-0.30	0.22	-	2.4
Chlorobenzene	0.30-0.31	0.23	-	2.7
Benzene	0.32	0.25	-	-
Ethyl ether	0.38	0.38-0.43	-	2.8
Dichloromethane	0.36-0.42	0.32-0.32	-	3.1
Chloroform	0.36-0.40	0.26	-	4.1
1,2-Dichloroethane	0.44-0.49	-	-	3.5
Methyl ethyl ketone	0.51	-	-	5.7
Acetone	0.56-0.58	0.47-0.53	8.8	5.1
Dioxane	0.56-0.61	0.49-0.51	11.7	4.8
1-Pentanol	0.61	-	-	-
Tetrahydrofuran	0.45-0.62	0.53	3.7	4.0
Methyl t-butyl ether	0.3-0.62	0.48	-	2.5
Ethyl acetate	0.58-0.62	0.38-0.48	-	4.4
Dimethyl sulfoxide	0.62-0.75	-	-	7.2
Diethylamine	0.63	-	-	-
Acetonitrile	0.52-0.65	0.50-0.52	3.1	5.8
1-Butanol	0.70	-	-	3.9
Pyridine	0.71	-	-	5.3
2-Methoxyethanol	0.74	-	-	5.5
n-Propyl alcohol	0.78-0.82	-	10.1	4.0
Isopropyl alcohol	0.78-0.82	0.60	8.3	3.9
Ethanol	0.88	-	3.1	-
Methanol	0.95	0.70-0.73	δ1.0	5.1
Ethylene glycol	1.11	-	-	-
Dimethyl formamide	-	-	7.6	6.4
Water	-	-	-	10.2

[a] Values represented as ranges indicate multiple sources

The "δ" symbol means that these are defined values