

ANTIOXIDANT, CYTOTOXICITY, AND STABILITY EVALUATION OF *GINKGO BILOBA* EXTRACT-BASED MICROEMULSIONS FOR ENHANCED THERAPEUTIC ACTIVITYMANISHA SINGH¹, SURYA PRATAP SINGH², RACHANA R^{1*}¹Department of Biotechnology, Jaypee Institute of Information Technology, A-10, Sector 62, Noida - 201 307, Uttar Pradesh, India.²Department of Biochemistry, Faculty of Science, Banaras Hindu University, Varanasi - 221 005, India. Email: Rachana.dr@gmail.com

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ABSTRACT

Objective: This study is aimed to evaluate the antioxidant (AO) potential, cytotoxicity, and stability of preformulated *Ginkgo biloba* standard extract microemulsion (GBME), to investigate if, it retains the therapeutic potential of EGB761 and remains safe and stable for a longer period.

Method and Results: GBME has shown enhanced AO (85.2±0.78%, IC₅₀=31.3±0.45 µg/ml) in comparison to EGB761 (74.1±0.51%, IC₅₀=49.4±0.05 µg/ml) using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay. Similarly, 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay has also shown that AO for GBME (94.6±0.04%, IC₅₀=11.4±1.03 µg/ml) was higher than EGB761 (78.6±1.20%, IC₅₀=34.6±0.81 µg/ml). Further, IC₅₀ value of antiradical unit of GBME was much lesser (ABTS=14.3±1.05 µg/ml and DPPH=17.03±1.8 µg/ml) in comparison to EGB761 (ABTS=34.1±1.62 µg/ml and DPPH=37.5±0.08 µg/ml). Equivalently, both, hydrogen peroxide scavenging activity, and nitric oxide activity were appreciably higher for GBME than the pure extract. The *in vitro* cytotoxicity assessment showed that GBME is quite safe (98.68±0.76% cell viability) in comparison to EGB761 (83.29±1.02%). Thereafter, these samples were tested for stability by evaluating their AO activity along with high-performance liquid chromatography analysis, for the major phytochemicals, after 1 year, and results suggested that AO of GBME remained stable while comparing with the freshly prepared GBME, whereas AO of EGB761 reduced significantly as compared to freshly taken EGB761 extract implying the degradation of phytochemicals supporting decrease in AO activity.

Conclusion: Therefore, the observed results suggest that GBME maintained AO and scavenging activity along with enhanced shelf life with no observed toxicity, which can be explored further for its potential therapeutic implications in various oxidative stress-induced central nervous system disorders.

Keywords: Phytoconstituents, Flavonoid, Nitric oxide, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay, radical scavenging activity, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay.

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INTRODUCTION

Medicinal plants are the most opulent resource of bioactive compounds that have a wide application in various diseases. Around 80% of the world population is dependent on herbal medicines for their therapeutic benefits but on the contrary, very limited numbers of phytochemicals are approved by governing and regulatory authority's worldwide [1,2]. Treating central nervous system (CNS) ailments has been a challenging task due to various unavoidable physiological limitations [3]. There are various biological barriers such as blood brain barrier and blood-cerebrospinal fluid barrier which obstruct the drug distribution toward the targeted sites of brain thereby, prohibiting the passive diffusion of several compounds into the CNS [4]. One of the important pathological mechanisms leading to the various CNS disorders is generation of reactive oxygen species (ROS) [5]. Although the molecular form of oxygen is an essential component for the survival of living forms, its other forms such as superoxide ion can cause internal cell damage due to their oxidative properties [6]. Similarly, many biological and physiological processes in human body also cause production of ROS and free radicals as residual products. Many of these species are neutral but are unstable as they carry unpaired electrons and to attain stability; they pair themselves with other odd or unpaired electrons [7]. When these species are released in excess, they eventually cause oxidative stress, leading to structural and functional damage to essential biomolecules of body [8]. Therefore, these free radicals are reported to start the chain reactions, finally causing the diseased conditions due to initiation of apoptotic and other changes [9,10]. Various medicinal and herbal plants are being used as remedies to overcome such irregularities. *Ginkgo biloba* is reported to be a potential tree for the

treatment and improvement of various neuronal disorders such as dementia, multiple sclerosis, Alzheimer's disease, tinnitus, and other cognitive impairments due to its polyvalent therapeutic actions [11]. It has been traditionally popular in China [12] and had also been extensively used as a nutraceutical and therapeutic drug compound in Europe and South Asian countries [13]. The standard extract of *G. biloba* (EGB761) is reported to have many important flavonoids such as quercetin-3-β-D-glucoside, quercitrin, rutin, quercetin, kaempferol, and isorhamnetin and terpene lactone components (ginkgolide A, B, C, bilobalide and *Ginkgo* toxin). It also includes 24% flavone glycosides and 6% terpenes (ginkgolides A, B, C, and bilobalide) [14,15].

The current research trends in pharmaceutical industries exhibit the preference of phytochemicals over the chemically fabricated drugs due to their comparatively higher patient compliance, with less side effects [16]. Standardized extract of *G. biloba*, has higher content of potential compounds such as flavonoids, polyphenols, and terpenoids, but these compounds are not very stable and get degraded easily in gut and other tissue fluids and also have shorter shelf life [17]. There are various other factors which lead to their low bioavailability, bioefficacy, permeability, and poor stability [18]. Therefore, more effective and stable delivery system is required to enhance their therapeutic index. Microemulsions (ME) are a viable system to eliminate the degradation hydrolysis and oxidation of the phytochemicals [19]. Hence, this study is primarily focused on comparing the antioxidant (AO) along with stability and cytotoxicity assessment of previously formulated GBME with the pure EGB761 extract, to evaluate if the developed ME system could enhance the bioavailability, therapeutic index, stability with longer shelf life.

METHODS

The standardized EGB761 was obtained from the Ranbaxy Research Laboratory, India, as a gift sample, potassium persulfate ($K_2O_8S_2$), ascorbic acid (AA), phosphomolybdate reagent, gallic acid, sodium nitroprusside, Folin-Ciocalteu, aluminum chloride were procured from High Media Laboratories, Mumbai, Maharashtra, India, and all other chemicals used were of analytical grade.

Preparation of GBME

The EGB761 extract loaded oil in water (o/w) ME system was prepared by water titration method as published before [20]. Where, isopropyl myristate, tween 80, and ethanol were selected as oil, surfactant, and cosurfactant phases, respectively, along with water as aqueous phase. In addition, the stabilization of prepared ME formulation is equally essential and it is usually balanced by S_{mix} (surfactants and cosurfactants) components present in the colloidal system which equilibrates the interfacial tension between the oil droplets and aqueous phases [21], within the generally regarded as safe (GRAS) limits, thus all the excipients used in the formulation followed the criteria. The optimized formulation after approving their thermodynamic stability was further characterized by particle size, polydispersity index, zeta potential, transmission electron microscopy, Fourier transform infrared, and rheological studies analysis were accompanied by *in vitro* permeability kinetics [20].

In vitro studies – AO assays

Radical scavenging assays (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) [ABTS] and 2,2-diphenyl-1-picryl-hydrazyl-hydrate [DPPH])

The AO potential and antiradical unit (AU) activity of EGB761 and GBME were estimated by both ABTS and DPPH assays [22].

As discussed by Re *et al.* (1999) [23] in case of ABTS assay, which is based on the ability of test samples to scavenge the radical cations and inhibit the oxidation of ABTS. These radicals were produced in response to the reaction between ABTS (7 mM) solution in water with potassium persulfate ($K_2O_8S_2$) (2.45 mM). The reaction mixture (500 μ l of ABTS added with equal amount of $K_2O_8S_2$) was left for 16 hrs in the dark at 37°C and then diluted further with 27 ml of distilled water. Consequently, the DPPH assay which is widely used for analyzing AO activity of plant extracts specifically was also performed [24]. In this assay, reduction in the rate of chemical reaction absorption spectra was reflected by change of deep violet color in solution to pale yellow, after adding DPPH solution [25].

After this, in 20 μ l of test samples (EGB761 and GBME) (10-100 μ g/ml), 980 μ l of ABTS/DPPH reagent was mixed and stirred continuously (120 seconds) and incubated again for 30 minutes. Then absorbance was taken at 734 nm and 520 nm, respectively to estimate for scavenging effect of the same.

Furthermore, reduction in ABTS and DPPH free radicals due to the antiradical compounds (EGB761 and GBME) was estimated further at the above-mentioned wavelengths [26,27]. AA (10-50 μ g/ml) was used as a standard and scavenging effect of test samples (%) were calculated as [28]:

$$\text{Scavenging effect (\%)} = \frac{(A_{OC} - A_{OT})}{A_{OC}} \times 100$$

Where, A_{OC} and A_{OT} are the absorbance of control and test samples, respectively.

Whereas, amount of AUs (AU_{734} and AU_{520}) present in the tests compounds were estimated as [29]:

$$AU_{734} = (A_{R0} - A_{R1}) - (AR_{C0} - AR_{C1}) \quad (1)$$

$$AU_{520} = (A_{R0} - A_{R1}) - (AR_{C0} - AR_{C1}) \quad (2)$$

Where, A_{R0} is the absorbance recorded for the test samples at the onset of reaction (0 seconds), and A_{R1} is the readings of the same samples taken after incubation time (30 minutes) of the reaction. While AR_{C0} represents the absorbance shown by control sample at the beginning of reaction and AR_{C1} is the absorbance taken after 30 minutes of the same [30]. Now, since $AR_{C0} - AR_{C1}$ was always invariably equivalent to 0 hence, above equation can be reduced down to the following:

$$AU_{734} = (A_{R0} - A_{R1}) \quad (3)$$

$$AU_{520} = (A_{R0} - A_{R1}) \quad (4)$$

The IC_{50} values of the experimental models were also assessed to find out, the concentration of test samples that has inhibited 50% of the free radicals [31].

Hydrogen peroxide scavenging activity

Hydrogen peroxide is comparatively a very mild oxidant, present in phagosomes but due to its oxidizing behavior, it inactivates few enzymes such as human myeloperoxidase (MPO) and protein tyrosine phosphatases [32] and may alter many amino acids of protein structure by cleaving the peptidic bond, hence causing biocatalysis in cellular structures [33]. Therefore, to reduce down the excessive accumulation and toxic effects of H_2O_2 many AO phytocompounds are considered to be effective. Similarly, in this experiment, the capacity of *G. biloba* (EGB761) extract and its ME (GBME) were analyzed to scavenge the hydrogen peroxide by the method of Keser *et al.* (2012) [34]. In each aliquot (0.1 ml) of the experimental solutions (EGB761 and GBME) which were taken in different concentrations (10-100 μ g/ml), 50 mM phosphate buffer (pH 7.4) was added and final volume was made up to 0.4 ml. Further, to this 0.6 ml of hydrogen peroxide solution was added and incubated for 10 minutes; finally, absorbance was recorded at 230 nm [35]. The hydrogen peroxide scavenging activity of both was calculated and compared by AA which was used as a reference compound, by using following equation:

$$H_2O_2 \text{ activity (\%)} = \frac{(\text{Control absorbance} - \text{Sample absorbance})}{(\text{Control absorbance})} \times 100$$

Nitric oxide (NO) scavenging activity

NO is an essential biomolecule which controls and regulates various physiological mechanisms in human body such as transduction and propagation of neural signaling, regularizing the systemic circulation, antimicrobial and anticancerous activities but, when it reacts with free radicals, it develops peroxy nitrite anion (ONOO-) [36]. Therefore, we aimed to estimate the NO radical quenching ability of standard *Ginkgo biloba* (EGB761) along with its microemulsions (GBME), taking AA as a standard by spectrophotometric quantification. For this, 0.5 ml of 10 mM sodium nitroprusside (dissolved in PBS, pH 7.2) was added in 1 ml of test samples (EGB761 extract and GBME) taken at different concentrations (10-100 μ g/ml) and incubated at 25°C for 3 hrs. After this 1 ml of Griess reagent is added and the absorbance of purple colored chromophore coupled with naphthyl ethylenediamine dihydrochloride was recorded at 546 nm [37], this complex developed due to the diazotization of nitrite with sulphanilamide. Then, % of inhibition was calculated by below mentioned formula [38]:

$$\% \text{ inhibition} = \frac{I_0 - I_1}{I_0} \times 100$$

Where, I_0 = Absorbance of control and I_1 = Absorbance of test samples.

All the experiments were performed in triplicates, and the results are expressed as an average of all the independent determinations.

In vitro cytotoxicity studies: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay

The cytotoxicity of GBME was assessed on *Vero* cell line through the MTT colorimetric assay [39]. The cells were maintained in DMEM medium supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum in humidified atmosphere of 5% CO₂ at 37°C to get adhered. The cells were trypsinized and seeded in triplicates at the concentration of 5.0×10⁵ cells/ml in 96 well tissue culture plates. Thereafter, the cells were treated with different concentrations (10-100 µg/ml) of test samples (EGB761 and GBME) for 6 and 12 hrs individually, in each well. Subsequent to the incubation 20 µl of MTT solution (5 mg/ml) was added to the respective wells and incubated again for 4 hrs, the viable cells converted the yellow dye MTT to formazan crystals, which were dissolved in 200 µl of dimethyl sulfoxide and the optical density was determined by microplate reader at 570 nm [40].

Stability testing of GBME and EGB761 extract

The stability of test samples was evaluated by the method reported by Basheer *et al.*, 2013 [41]. In this assay, the stability of EGB761 and GBME in various concentrations (10-100 µg/ml) was tested, in terms of their AO activity (ABTS and DPPH radical scavenging) after storing them to room temperature (37°C) for 1 year and then comparing it with the AO of the fresh samples. The optimized and final formulation encapsulating the EGB761 extract was analyzed and quantified by using reversed phase, high-performance liquid chromatography (HPLC) (Waters) method [42] to evaluate the stability of important AO components (Quercetin, Isorhamnetine, and Kaempferol) after storing them for 1 year. The chromatographic analysis was done on C₁₈ column with detection at 370 nm, and temperature was fixed at 35°C. A stock solution (10 mg/ml, with respect to extract present) of GBME was prepared in mobile phase with subsequent dilutions (2-10 mg/ml). The isocratic mobile phase for HPLC was consisted of methanol and phosphoric acid (0.3%) in equivalent ratio (50:50), which was degassed by passing through a 0.45 µm Millipore filter and sonicated for 10 minutes and pumped at a flow rate of 1 ml/minute [43,44].

RESULT AND DISCUSSIONS

Preparation of GBME

The GBME formulation was developed earlier [20] (particle size of 259.8±6.3 nm, PDI score of 0.186±0.092 and zeta potential as -9.87±2.23 mv) using isopropyl myristate, tween 80, and ethanol as, oil, surfactant, and cosurfactant phases, respectively. Surfactant and cosurfactant components are very important in ME formulation as they reduce down the interfacial tension between the 2 phases (oil and water) [45]. In the present study, tween 80 which is a nonionic, hydrophilic surfactant, composed of same polar heads but different lipophilic tails are reported to have highest solubilization capacity, lowest toxicity and irritability properties, aiding in enhancing the therapeutic efficiency of GBME along with short chain alkanol (ethanol), which is known for improving the ME formulation by affecting the interfacial energy, when it interacts with surfactant monolayers [46].

Radical scavenging assays (ABTS and DPPH)

The measurement of AO activity through ABTS assay for EGB761 extract and GBME showed the higher AO activity of GBME (89.2±0.78%) in comparison to EGB761 (74.1±0.51%) at the highest concentration (100 µg/ml) (Fig. 1a). Consequently, the IC₅₀ value of GBME was also noted significantly lesser (29.6±0.03 µg/ml) than the EGB761 (47.1±0.32 µg/ml) (Fig. 1a). Similarly, in case of DPPH estimation, AO activity of GBME (94.6±0.04%) was again recorded to be higher than EGB761 (77.6±1.20%), and IC₅₀ value for GBME was estimated to be distinctly lower (12.8±0.55 µg/ml) than EGB761 (31.1±0.81 µg/ml) hence, reflecting more effective activity of GBME at lower concentrations too (Fig. 1b). Their IC₅₀ value during ABTS assay gives an insight that GBME with all its components has equally comparable AO as the standard (AA) alone. Initially, it was observed that AO activity of GBME was recorded lower (17.6±1.6%)

as compared to EGB761 (22.61±0.82%) at 10 µg/ml, as suggested by Koga *et al.*, 2011 [47] this phenomenon may arise due to some of the phytochemicals getting partitioned into the oil phase and it took some time by them to partition out from the oil phase into the external medium. Later it was observed that, with the increase in concentrations, GBME exhibited fairly better activity than the extract. As, DPPH is highly sensitive toward even the weakest AOs and can very well analyze both hydrophilic and lipophilic components thus, in DPPH assay GBME, at its lowest concentration of 10 µg/ml also recorded higher (46.4±1.6%) AO activity than extract (33.17±1.8%) as represented in Fig. 1a and b. The reason for enhanced activity may be contributed by the components of the ME including extract as well as surfactant cosurfactants of the carrier system (ABTS=35.4±0.27% and DPPH=43.2±1.0%).

Antiradical action of pure extract (EGB761), ME without extract and GBME in existence of ABTS as a source of radical enhancer (Table 1) was also compared with standard (AA). The IC₅₀ values of all the test samples (AA, EGB761 and GBME) were calculated and it was being observed that EGB761 showed the highest IC₅₀ value (34.6±0.81 µg/ml), whereas IC₅₀ value of GBME (12.8±0.55 µg/ml) (Table 2) was slightly higher than the standard (AA=11.2±0.26 µg/ml), (p<0.5) suggesting the enhanced scavenging activity even at lower concentration.

Hydrogen peroxide scavenging activity

Hydrogen peroxide as mentioned above is known to be a weak oxidizing agent, and when it gets reacted with Fe²⁺ or Cu²⁺ complexes inside the cells, it forms highly reactive hydroxyl radicals which lead to its detrimental effects [46]. Hydrogen peroxide scavenging ability of both the test samples (EGB761 and GBME) was found to be dose dependent as it increases with the increase in concentrations of test samples (Fig. 2). IC₅₀ value for GBME and EGB761 were found to be 18.4±1.06 µg/ml and 42.8±0.45 µg/ml, respectively, in comparison to

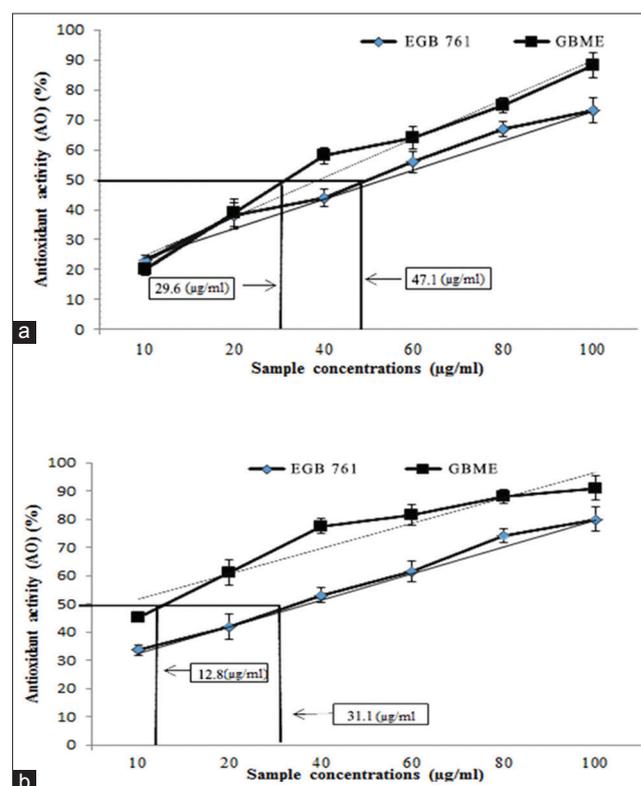


Fig. 1: (a) Antioxidant (AO) activity (%) against 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) of EGB761 and GBME with IC₅₀ values. (b) AO activity (%) against 2,2-diphenyl-1-picryl-hydrazyl-hydrate assay of EGB761 and GBME with IC₅₀ values. EGB761: Purified standard extract of *Ginkgo biloba*, GBME: EGB761 loaded microemulsion

Table 1: AU in ABTS and DPPH assay against EGB761 and GBME at various concentrations (10-100 µg/ml)

Sample conc. (µg/ml)	AU - ABTS method		AU - DPPH method	
	EGB761	GBME	EGB761	GBME
10	0.141±0.02	0.296±0.05	0.221±0.02	0.395±0.006
20	0.238±0.01	0.254±0.01	0.356±0.004	0.570±0.09
40	0.318±0.07	0.392±0.14	0.552±0.04	0.583±0.15
60	0.546±0.09	0.550±0.07	0.619±0.06	0.834±0.11
80	0.567±0.11	0.605±0.2	0.675±0.13	0.846±0.08
100	0.612±0.17	0.668±0.03	0.684±0.01	0.945±0.07

AU: Antiradical unit, ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), DPPH: 2,2-diphenyl-1-picryl-hydrazyl-hydrate, EGB761: Extract of *Gingko biloba*, GBME: EGB761 loaded microemulsion

Table 2: IC₅₀ value of EGB761 and GBME against DPPH and ABTS assay

AU	IC ₅₀ value of EGB761 (µg/ml)		IC ₅₀ value of GBME (µg/ml)	
	DPPH assay	ABTS assay	DPPH assay	ABTS assay
	31.1±0.81	47.10±0.32	12.8±0.55	29.6±0.03

AU: Antiradical unit, ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), DPPH: 2,2-diphenyl-1-picryl-hydrazyl-hydrate, EGB761: Extract of *Gingko biloba*, GBME: EGB761 loaded microemulsion

the standard (AA) which was 12.5±0.83, reflecting the fact that GBME has almost equivalent quenching ability as that of, standard and better than the pure extract. This improved characteristic of GBME can be attributed to the combination of the retained phenolic and flavonoid contents of extract along with the ME excipients [47]. Hence, from the results obtained, we can sequence the scavenging activity of hydrogen peroxide of various test samples as AA>GBME>EGB761.

NO scavenging activity

The percentage inhibition of NO free radical was plotted against various concentrations (10-100 µg/ml) of test samples (EGB761 and GBME) as represented in Fig. 3. GBME showed % inhibition of 93.1±1.72% at its highest concentration of 100 µg/ml, whereas the % inhibition for standard (AA) was found to be 97.43±1.23% and EGB761=88.2±1.6% on the same concentration. Furthermore, it can be concluded from the represented result data of dose-response curve that both of the test samples are concentration dependent. Hence, exhibiting the enhanced scavenging potential of GBME than EGB761 and ME without extract.

In vitro cytotoxicity studies: MTT assay

Cytotoxicity of mentioned test samples was tested on *Vero* cell line, using MTT assay. The results show (Fig. 4) that pure extract is more toxic (63.14±0.72% cell viability) than GBME (80.62±1.05% cell viability) at its highest concentrations (100 µg/ml). Results also showed a very low cytotoxic effect (95.2±0.42%) of the ME system (only ME with no extract loading) indicating the nontoxic nature of the excipients used for the ME system (Fig. 5). These results were expected as the excipients selected for the formulation (ME) were within the GRAS limits. However, the label claim for the GBME is 60 µg/ml, and till this range, no significant cellular toxicity was observed.

Stability testing of GBME and EGB761 extract

Stability of the test samples was checked after storing them at room temperature (37°C) for 12 months and testing the retention ability of their AO content left after storing them for mentioned time by the same methods: ABTS and DPPH assay. It was being observed that EGB761 had shown a significance loss of AO in comparison to GBME (85.8±0.49% vs. 68.7±1.41% AO activity) in case of ABTS assay. Similarly, through DPPH assay analysis also, EGB761 reflected a prominent loss and degradation of therapeutic phytochemicals with loss of AO activity of 65.2±1.06%

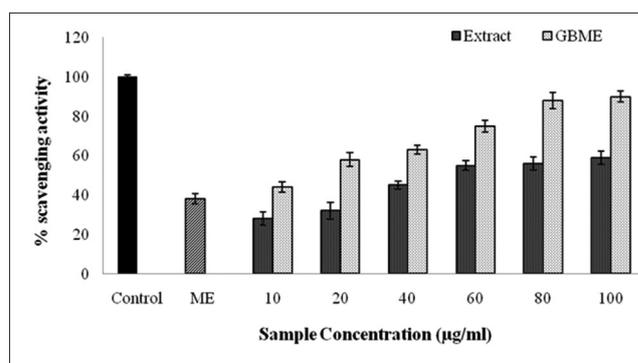


Fig. 2: Graph depicting H₂O₂ scavenging activity (%) of ME, EGB761, and GBME at different concentrations. ME: Microemulsion without EGB761, GBME: EGB761 loaded microemulsion, EGB761: Extract of *Gingko biloba*

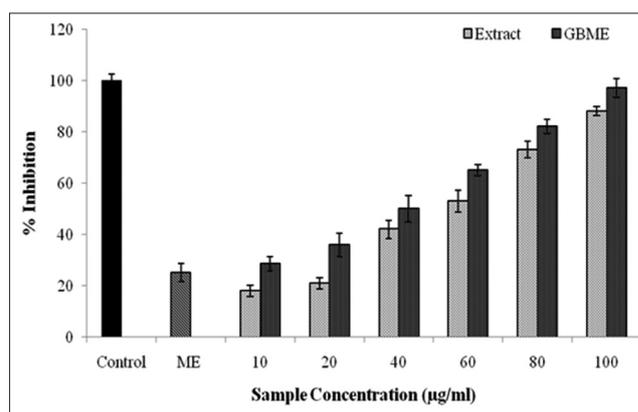


Fig. 3: Graph depicting nitric oxide scavenging activity (%) of ME, EGB761 and GBME at different concentrations. ME: Microemulsion without EGB761, GBME: EGB761 loaded microemulsion, EGB761: Extract of *Gingko biloba*

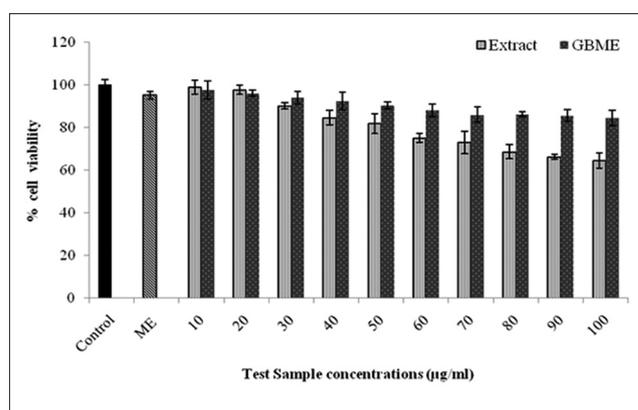


Fig. 4: In vitro cytotoxicity analysis of the optimized EGB761, ME and GBME at various concentrations (10-100 µg/ml) after 12 hrs of treatment by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay. ME: Microemulsion without extract, GBME: EGB761 loaded microemulsion, EGB761: Purified extract of *Gingko biloba*

in comparison to GBME (90.11±1.2%). As mentioned before the freshly prepared samples had higher AO of GBME (ABTS=89.2±0.78% and DPPH=94.6±0.04%) than EGB761 (ABTS=74.1±0.51%, DPPH=77.6±1.20%). Therefore, from this comparative data, we can conclude that ME system has enhanced the stability and shelf life of the

Table 3: Summarization of compound loss (%) in flavonone group of GBME samples after the period of 12 months

S.No.	Name of phyto compounds	Amount present before storage	Amount present after storage (μg)	Calculated % loss
1.	Quercetin	0.54 ± 0.002	0.47 ± 0.026	12 ± 0.001
2.	Kaempferol	0.24 ± 0.011	0.21 ± 0.005	14 ± 0.002
3.	Isorhamnetine	0.12 ± 0.084	0.10 ± 0.061	16 ± 0.01

GBME: Extract of *Gingko biloba* loaded microemulsion

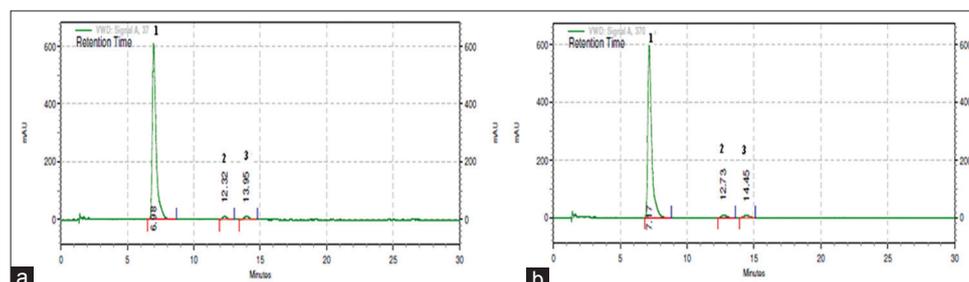


Fig. 5: (a) Representing high-performance liquid chromatography-ultraviolet (HPLC-UV) chromatogram of freshly prepared extract of *Gingko biloba* loaded microemulsion (GBME) samples, showing peaks of flavonone groups (quercetin, kaempferol, and isorhamnetine). The peak identified as: (1) quercetin, (2) kaempferol, and (3) isorhamnetine. (b) Representing HPLC-UV chromatogram of 12 months stored GBME samples, showing peaks of flavonone group (quercetin, kaempferol, and isorhamnetine). The peak identified as: (1) quercetin, (2) kaempferol, and (3) isorhamnetine

EGB761 extract significantly. Vicentini *et al.*, 2011, have also shown that the ME system increases stability. Their formulation had quercetin in w/o ME with span80 (surfactant), tween 80 (cosurfactant) and canola oil as an external phase. Evaluation of AO of the formulation after 12 months indicated that the functional groups were retained. In this study, also the quantity assessment results of flavonone groups (quercetin, kaempferol, and isorhamnetin) present reflected that the GBME formulation was quite stable with no significant alteration in its therapeutically essential groups of phytochemicals as HPLC analysis of freshly made GBME showed concentration of $0.54 \pm 0.002 \mu\text{g}$, $0.24 \pm 0.011 \mu\text{g}$, and $0.12 \pm 0.084 \mu\text{g}$ (Fig. 5a) for quercetin, kaempferol, and isorhamnetine, respectively. Whereas, the stored samples (12 months) showed the retention of $0.47 \pm 0.026 \mu\text{g}$ (quercetin), $0.21 \pm 0.005 \mu\text{g}$ (kaempferol), and $0.10 \pm 0.061 \mu\text{g}$ (isorhamnetin) of the analyzed compounds, suggesting very negligible loss of the groups (Fig. 5b). Therefore, it can be concluded from the above results that the encapsulation of EGB761 extract in ME system has also enhanced its shelf life with respect to its important therapeutic compounds intact (Table 3).

Statistical analysis

The obtained result data were expressed by calculating mean of all experiments done in triplicates \pm standard error along with calculation of data significance at $p < 0.01$ by one-way ANOVA (50).

CONCLUSION

This study was conducted to evaluate if the developed formulation of EGB761 (*G. biloba* extract) could retain its therapeutic index with enhanced shelf life as the most effective therapeutic phytochemicals of EGB761 extract, flavone glycosides (quercetin, kaempferol, and isorhamnetin) are reported to get degraded and metabolized in the gut, if taken orally in conventional form. *G. biloba* is a very important plant for treating nervous disorders, and there are many research groups working on to improve its therapeutic index and stability by various methods. We had developed a ME system earlier, and in this study, we have evaluated if we could achieve the aims as mentioned above. From this study, we can conclude that the AO and stability efficiency of EGB761 extract has enhanced after formulating it into ME system (GBME). Collectively, the experimental results suggested that the GBME formulation can effectively scavenge ROS and could provide effective protection against oxidative damage. The said ME were found to be equally efficient (in fact slightly more) in exhibiting their AO activity which might have been enhanced due to the already existing intrinsic

activity of the carrier system components (ME). Furthermore, the major constituents of the extract were also found to be almost constant after 12 months storage at room temperature. Besides this, the amount and ratios of excipients (oil, surfactants and cosurfactants) used for the formulation of ME are listed and are under the GRAS limits and that may be the reason that formulation was found to be quite safe with the toxicity studies. The stability and shelf life of the formulation was also augmented in comparison to the pure extract. Thus, the developed ME of EGB761 extract could serve as a potential and effective delivery system and further can be analyzed *in vitro* and *in vivo* for its therapeutic index and safety.

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