

Original Article

EVALUATION OF ETHOSOMAL EPIGALLOCATECHIN-3-GALLATE GEL FORMULATIONS AS AN ANTIOXIDANT AND THERMOGENIC AGENT

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ABSTRACT

Objective: The aim of this study was to investigate *in vitro* and *in vivo* behavior of the prepared ethosomal formulations of green tea extract, which in turn would result in enhancing their therapeutic efficacy as antioxidants and thermogenic agents in obese mice.

Methods: Fat Wistar rats were acclimated for at least 7 d in environmentally controlled cages, then they were divided into five groups: 1st received only distilled water, 2nd received an oral dose of green tea extract, in 3rd group green tea extract loaded gel base was applied on the previously shaven dorsal side of rats and in the other 2 groups selected green tea extract loaded Ethosomal gel base were applied on the previously shaven dorsal side of rats. Total antioxidant capacity by ferric reducing ability of plasma (FRAP) method, catalase enzyme activity, malondialdehyde (MDA) and thiobarbituric acid reactive substance (TBARs) levels, and real-time qPCR of UCP2 and 3 were then determined.

Results: Formulation of green tea extract as ethosomal preparations exhibited a controlled release rate due to the reservoir action of ethosomes. The levels of TBARs and MDA were lower in groups supplemented with green tea extracts compared to control group and in groups (3-5) received it transdermal (P<0.0001 in all 3 groups) was lower than group received it orally with P = 0.0081. The messenger RNA levels of UCPs 2 and 3 in BAT were increased in those 3 groups.

Conclusion: The results support the efficiency of ethosomal gel in penetrating the lipid rich biological membrane. The *in vivo* study confirms the antioxidant and thermogenic behavior of transdermal applied green tea extract.

Keywords: Bioavailability, Lecithin, Green tea extract, Transdermal, Ethosome, Uncoupling proteins (UCPs) and Real-Time Polymerase Chain Reaction (RT-PCR)

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INTRODUCTION

Thousands of years ago (started in prehistoric times), natural products, such as plants, microorganisms, animals, and marine organisms, have been used to relieve and treat many diseases [1]. The replacements of normally used drugs with natural products, must of course, have presented a tremendous challenge to humans all overages. Green tea (*Camellia sinensis*) (fam. Theaceae) has been confirmed as safe agent with many benefits as one of the extremely important natural products [2]. Epigallocatechin gallate (EGCG) is the main effective component in green tea leaves extract and is responsible for many health beneficial effects. EGCG has a high antioxidant activity that can provide protection to the body from the risk of cardiovascular diseases, diabetes mellitus, cancer, and neurodegenerative disorders, obesity and exhibits anti-inflammatory effects [3, 4].

In alkaline media (like small intestine) EGCG is found to be of low absorption rate as less as 5% of EGCG reach the systemic circulation [5]. The majority amount of EGCG thereafter will be degraded by intestinal micro flora in the colon [6].

To overcome these problems, an alternative drug administration route such as transdermal drug delivery system is used, which can deliver drugs through the skin up to the systemic circulation [7]. This route is characterized by avoidance of gastrointestinal degradation; first pass effect, and drug fluctuations in the blood [8]. To provide optimal antioxidant and thermogenic effects, then the bioactive component of green tea (i.e., EGCG) should penetrate the skin. It is an issue, because of high polarity value of the hydrophilic molecule (log p=0.48), and a considerable molecular weight of (458.37 da) [9, 10].

To overcome this, formulation of green tea leaves extracts into a carrier-based lipid (i.e., ethosomes) is done to enhance its penetration through skin. Ethosomal carriers are systems of soft vesicles. They are mainly composed of (ethanol) and phospholipid

(lecithin) at relatively high concentrations, with water as a non-solvent. Interacting with lipid molecules (found in polar head group region), ethanol reduces the phase transition temperature (T_m) of the stratum corneum lipids, leading to an increase in fluidity. Moreover, this reaction results in an increase in membrane permeability. In addition, there is an interaction between ethosome itself and the stratum corneum barrier. Ethanol also gives the vesicles flexibility and softness [11]. Ethosomes can deliver hydrophilic and hydrophobic probes to a depth of 170 μm with greater intensity than the control systems [12]. However, high cost of phospholipids and their variable purity are problems of ethosomes.

Brown adipose tissue (BAT) is an important site for thermogenesis. Due to its high metabolic capability to maintain body temperature in cold conditions or to waste food energy, it is currently considered a novel therapeutic option for obesity management. BAT thermogenesis is mainly dependent on the action of uncoupling proteins (UCPs), which causes conversion of the driving force of adenosine triphosphate (ATP) synthesis into heat via uncoupling the process of oxidative phosphorylation in the mitochondria [13].

The aim of this study was to optimize conditions for encapsulating green tea extract and examine the *in vitro* and *in vivo* efficacy of ethosomes on the rat skin when applied as topical formulation. Furthermore, the results should contribute to a better understanding of the antioxidant and thermogenic effects of green tea extract (EGCG) with advancing its bioavailability. In addition, messenger RNA (mRNA) expression of Uncoupler proteins types II and III genes related to thermogenesis and antioxidant effects were evaluated.

MATERIALS AND METHODS

Materials

We purchased Green tea leaves extract (*Camellia sinensis*) from (Andy Biotech Co. Ltd., China with standard potency 98.4 %).

Kindly, (Futures manufacture, Egypt) provided us with lecithin and (Sekem development foundation, Egypt) provided us with EGCG standard. A cellophane membrane (Molecular weight cut of 12000-14000) was purchased from United States (USA).

Methods

HPLC determination of EGCG in green tea extract

EGCG percentage was accurately measured spectrophotometrically by High performance liquid chromatography (HPLC) in green tea extract against EGCG standard [14] at 278 wavelength [15].

Preparation of ethosomes encapsulating green tea extract

Preparation of Ethosomes

Ethosomes were prepared by slight modification of cold method [11]. Initially Lecithin was taken and dissolved in ethanol by use of magnetic stirrer in completely closed flask at 30 °C. To this solution, 50 mg of the green tea extract dissolved in hot distilled water (30 °C) was added as fine stream by the use of syringe very slowly. The volume was made up

using distilled water (30 °C), thereafter whole system was whiskered for 15 min at 900 rpm. Further, it was sonicated for 5-15 min. Finally, the formulations were stored under refrigeration. We prepared 12 batches of ethosomes using varying concentrations of lecithin (1-3%) and ethanol (10-40%). (Table 1) The most two stabilized ethosomes with high entrapment efficiency and lower *Poly Dispersity Index* were selected for the preparation of two individual vesicle incorporated gel formulations and further selected *in vivo* study.

Characterization of ethosomes

Morphology and structure of ethosomes

For visualization of sample by Scanning Electron Microscopy (SEM), one drop of ethosomal system was mounted on a stub covered with a clean glass. The drop was spread out on the glass homogeneously. The ethosomal dispersion was spread on the glass slide using a glass rod. Formation of multilamellar vesicles was confirmed by examining the ethosomal suspension under an optical microscope with the magnification power of 100X (Olympus). Olympus camera was used for Photography.

Table 1: Composition of ethosomal vesicles

| Formula code | Lecithin % | Ethanol: water | Green tea extract (mg) |
|--------------|------------|----------------|------------------------|
| T1 | 1 | 10:90 | 50 |
| T2 | 1 | 20:80 | 50 |
| T3 | 1 | 30:70 | 50 |
| T4 | 1 | 40:60 | 50 |
| T5 | 2 | 10:90 | 50 |
| T6 | 2 | 20:80 | 50 |
| T7 | 2 | 30:70 | 50 |
| T8 | 2 | 40:60 | 50 |
| T9 | 3 | 10:90 | 50 |
| T10 | 3 | 20:80 | 50 |
| T11 | 3 | 30:70 | 50 |
| T12 | 3 | 40:60 | 50 |

12 batches of ethosomes were prepared using varying concentrations of lecithin (1-3%) and ethanol (10-40%).

Determination of entrapment efficiency (EE %)

The entrapment efficiencies of ethosomal preparations were determined by HPLC after dispersion in phosphate buffer. The ethosomal dispersion obtained was centrifuged at 10000 rpm. Then, supernatant was assayed for free drug [16].

Particle size and polydispersity index

Particle size and index polydispersity were measured using a particle size analyzer. The measurements were repeated. The polydispersity index was conducted to evaluate the particle distribution of ethosomes [17-19].

In vitro drug release studies

The *in vitro* permeation behaviour of green tea extract from ethosomal formulations was investigated using diffusion cell model. The diffusion cell was consisted of a cellophane membrane filled with ethosomal preparation, which was placed in a receptor compartment containing phosphate buffer agitated at 100 rpm at 37±0.5 °C. An aliquot of 2 ml sample was withdrawn at predetermined time intervals over 24 h and replaced immediately with an equal volume of fresh diffusion medium. The samples were filtered and analyzed using HPLC [20-23].

In vivo study

Grouping and dose administration

We used Fat Wistar rats (250±50 g) for our current study (supplied by the animal house in the faculty of medicine, Zagazig University). Ethical clearance for our study was obtained from the Institutional Review Board (IRB/08/2019), Zagazig University, Egypt. Rats were adapted for 7 d in environmentally controlled (23 °C±1 °C and equally half day dark/light cycle) cages, with free consumption of standard food and water and fasted overnight before the experiments. We divided them into 5 groups (ten rats per group):

- 1st GROUP (control): were given distilled water only
- 2nd GROUP (standard): were given (by oral feeding needles) oral dose of green tea extract.
- 3rd GROUP: we applied loaded gel base with green tea extract on the shaven dorsal side of rats.
- 4th and 5th GROUPS: we applied dose equivalent to 100 mg/kg body weight of selected green tea extract loaded Ethosomal gel base (T10 and T12) on shaven dorsal side of rats [24].

Animals were sacrificed after daily dosing for 7 d. A central longitudinal incision was made in the abdominal wall and blood samples were collected by cardiac puncture. Blood samples were centrifuged at 1500 × g for 20 min at 4 °C and the plasma was separated and stored at -20 °C until analyzed. The BAT was harvested, frozen immediately in liquid nitrogen, and stored at -70 °C. Liver from each animal was excised and its homogenate was prepared and centrifuged for 5 min at 4 °C. The supernatant obtained was further used for antioxidant assay [25]. Different specimens of the liver, kidney and brain were embedded in paraffin blocks, stained and observed for any changes under light microscope [26].

Assay of total antioxidant capacity (TAC)

We measured total antioxidant capacity by ferric reducing ability of plasma (FRAP) method, which is based on the capability of plasma to reduce Fe³⁺ to Fe²⁺ in the presence of TPTZ (2,4,6-tripyridyl-s-triazine) [27].

Determination of catalase activity

The activity of catalase (CAT) was assayed following (Pari and Latha) method [28].

Estimation of lipid peroxidation

We determined levels of malondialdehyde (MDA) and thiobarbituric acid reactive substance (TBARS) production by (Draper and Hadley) method [29].

Real-time qPCR

Total RNA was extracted from BAT using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The complementary DNA (cDNA) was prepared from 1µg RNA using Maxime RT PreMix (iNtRON). After cDNA synthesis, 2µl of first-strand cDNA was used for real

time PCR (RT-PCR) in 20µl reactions containing 10 µl of Universal SYBR Green PCR Master Mix (Qiagen, Chatsworth, CA, USA), 1µl of each primer and 4µl PCR grade water in thermal cycler (DTlite 4 DNA-TECHNOLOGY, Russia). The sequences of the sense and antisense primers used for amplification are shown in table 2.

Table 2: Primers used for the real-time quantitative polymerase chain reaction

| Gene | Sense primer (5'-3') | Antisense primer (5'-3') |
|----------------|----------------------|--------------------------|
| UCP-2 | GACCTATGACCTCATCAAGG | ATAGGTGACGAAACATCACCCAG |
| UCP-3 | ATGGACGCCTACAGAACCAT | TACGAACATCACCCAGTTCC |
| <i>β-actin</i> | AGAGCTACGAGCTGCCTGAC | AGCACTGTGTTGGCGTACAG |

The sequences of the sense and antisense primers for UCP-2, UCP-3 and *β-actin*

The $\Delta\Delta C_t$ method was used for measuring relative quantification [30]. We determined the $\Delta\Delta C_t$ value for each sample by calculation of the difference between the target gene Ct value and the *β-actin* Ct value as a reference gene. The normalized level of the target gene expression in each sample was calculated using the formula $2^{-\Delta\Delta C_t}$. Values were expressed as fold change relative to the control group.

Statistical analysis

All data are presented as mean±SD (standard deviation). We conducted Statistical analysis using (SPSS) (version 17; IBM Corporation, Armonk, NY, USA). $P < 0.05$ was considered as an

indication of a statistically significant difference.

RESULTS**HPLC determination of EGCG in green tea extract**

Amount of EGCG presented in green tea extract was measured at 278 wavelengths; this wavelength was advised by Huo *et al.* [31]. By optimizing HPLC conditions, the concentration of EGCG in the working green tea extract sample solution was determined (54.24 %) (fig. 1). To ensure the presence of suitable amount of EGCG within the extract with further dilutions, we constructed a calibration curve (fig. 2).

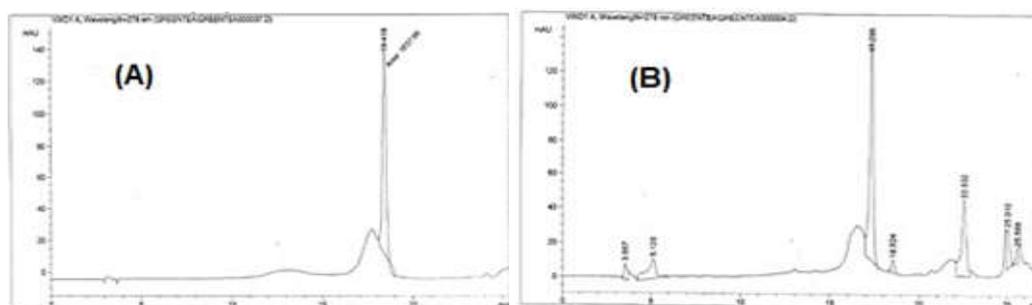


Fig. 1: HPLC profile of Green tea extracts (a) chromatogram of EGCG standard and (b) chromatogram of green tea extract, by optimizing HPLC conditions, the concentration of EGCG in the working green tea extract sample solution was determined (54.24 %)

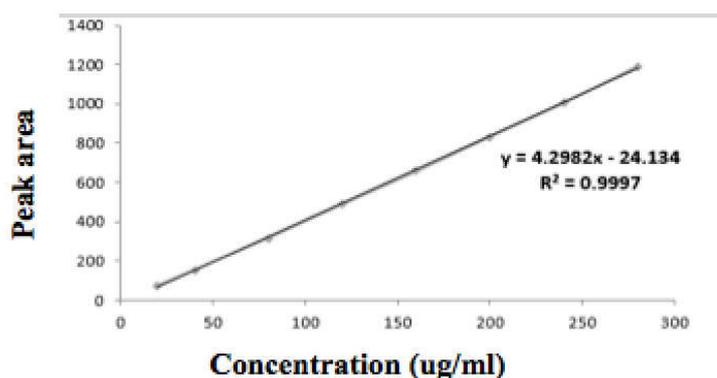


Fig. 2: Calibration curve of EGCG in green tea extract, to ensure the presence of a suitable amount of EGCG within the extract with further dilutions, we constructed a calibration curve

Preparation of ethosomes encapsulating green tea extract

Irrespective of whether transdermal absorption has a local effect or systemic action, the drug should pass through the *stratum corneum*. Because of the barrier effect of *stratum corneum*, the majority of

drugs cannot give their required therapeutic effect when applied topically. Recently, ethosomes are widely used for transdermal delivery because they not only easily pass through the skin but also increase the accumulation of drug in the skin. Dayan, N., and Toutiou, E., concluded that when compared with normal

liposometh, steady-state transdermal rate of *trihexyphenidyl hydrochloride* ethosomes composed of phospholipid, ethanol, and water increased by 87 fold [32]. In our current study, to enhance the transdermal penetration of green tea extract, ethosomal formulations were prepared and they were further incorporated into gels.

Ethosomal dispersal was enhanced with increasing amounts of lecithin concentration (1, 2, and 3%) and ethanol (10, 20, 30, and

40%). In all these dispersions, ethanol concentration with different percentages induced white milky suspensions without any significant differences under the macroscopic view. As one of the broad parameters for the evaluation of prepared ethosomes quality according to the basis of their particle size distribution, Poly Dispersity Index (PDI) was including with a value of 0-1 as reference. The preparations presenting PDI in this range were then evaluated based on their drug entrapment efficiency and vesicular size (table 3). PDI index range was (0.043-0.282).

Table 3: Entrapment efficiency, vesicle size and polydispersity index of the various ethosomal preparations

| F | Drug content % mean±SD | EE % mean±SD | Vesicle size (nm) mean±SD | Polydispersity index (PDI) mean±SD |
|-----|------------------------|--------------|---------------------------|------------------------------------|
| T1 | 63.77±2.49 | 60.93±2.34 | 184.22±2.33 | 0.067±0.003 |
| T2 | 71.08±1.37 | 64.51±0.98 | 134.82±0.72 | 0.049±0.001 |
| T3 | 73.18±0.94 | 69.21±1.33 | 112.65±1.56 | 0.038±0.002 |
| T4 | 76.4±0.81 | 71.39±1.52 | 100.23±1.3 | 0.043±0.003 |
| T5 | 79.29±2.01 | 73.94±2.27 | 222.32±0.98 | 0.210±0.019 |
| T6 | 83.4±0.11 | 78.82±0.42 | 200.81±1.44 | 0.088±0.008 |
| T7 | 85.46±1.61 | 79.9±2.3 | 169.2±2.74 | 0.067±0.005 |
| T8 | 87.14±0.67 | 81.59±1.68 | 145.72±1.07 | 0.058±0.002 |
| T9 | 89.78±0.3 | 85.63±1.03 | 314.04±1.32 | 0.279±0.013 |
| T10 | 90.9±1.22 | 87.91±2.69 | 309.68±0.78 | 0.121±0.011 |
| T11 | 91.93±0.52 | 88.5±1.94 | 299.5±0.99 | 0.282±0.024 |
| T12 | 93.32±1.54* | 90.11±0.81 | 287.06±1.01 | 0.117±0.029 |

Values are presented as mean±SD, PDI index range was (0.043-0.282), formulation T12* (93.32 %) showed maximized drug content with maximal ethanol content and increased lecithin concentration.

Shape and size of the vesicle are critical factors in transdermal drug delivery system therapeutic performance. In our current study, phase-contrast microscopy showed the surface morphology of ethosomes (fig. 3).

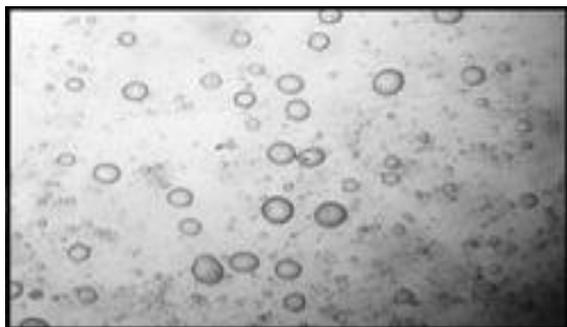


Fig. 3: Microscopical image of ethosomal dispersion

Phase contrast microscopy showed the surface morphology of ethosomes

Minimum and maximum vesicle sizes were observed to be 100.23 nm and 314.04 nm, respectively, depending on the concentration of ethanol and lecithin. The vesicles size was found to increase with lecithin concentration increase from (1-3%), while it was inversely related to concentration of alcohol (higher alcohol concentrations accompanied with lower vesicle size). This observation agreed with the Elsayed *et al.* findings [33] that higher concentration of ethanol allows a surface negative net charge to the vesicular systems by surface characteristics manipulation, this leads to a decrease in vesicles size. When we kept lecithin amount at 1% while increased ethanol concentration from 10% to 40%, a decrease in vesicles size from (184.22 to 100.23 nm) was observed. Likewise, vesicles size decreased from (222.32 to 145.72) nm and from (314.04 to 287.06 nm) with lecithin

concentration 2% and 3%, respectively and with the same increase in ethanol concentration (table 3).

Entrapment efficiency is used as a quality parameter, which has a direct effect on the delivery potential of the vesicular systems and to be determined in the formulated ethosomal vesicles, the drug content in each preparation was to be determined first. Drug content ranged from 63.77±2.49 % to 93.32±1.54 % that shows good capacity of formulation to hold the drug. Formulation T12 (93.32 %) showed maximized drug content with maximal ethanol content and increased lecithin concentration.

Ethanol and lecithin amounts and concentrations had a positive effect on the entrapment of the herbal extract inside lipid vesicles. Entrapment efficiency was showed to be 87.91 % with 20% ethanol and 3% lecithin formulation (T10), while increased to 88.5% and 90.11% with alcohol concentration increase to 30% and 40%, respectively, and lecithin concentration kept unchanged at 3% (table 3). This increase is probably due to accelerated green tea extract solubility in water present in the ethosomal core. Concentration of ethanol in the ethosome system should be maintained below 45%. In our study, when concentration exceeded 40%, drug leakage from the lipid bilayer started, leading to decreased entrapment percentage. Our data agreed with Paolino *et al.* findings and conclusion [34].

***In vitro* drug release studies through cellophane membrane**

The initial drug release should be fast enough to guarantee the maintenance of therapeutic drug levels *in vivo* in a timely way [35, 36].

As shown in (fig. 4), the release of all prepared ethosomal formulations after 24 h exceeded 50 %. Green tea extract formulated as ethosomal preparations showed a controlled release rate due to ethosomes' reservoir action. The impulse effect over the first six hours was clearly observed, with a following step of slower release, this finding might be linked with the feature of the bilayer or ethosomal structure [37-39]. The release rate then increased gradually with ethanol concentration increasing from 20 to 40 %. Moreover, a higher released amount was observed with increasing lecithin concentration which might be related to increased drug solubility [40] as well as increased lipid bilayer fluidity, which leads to drug release improvement [41, 42].

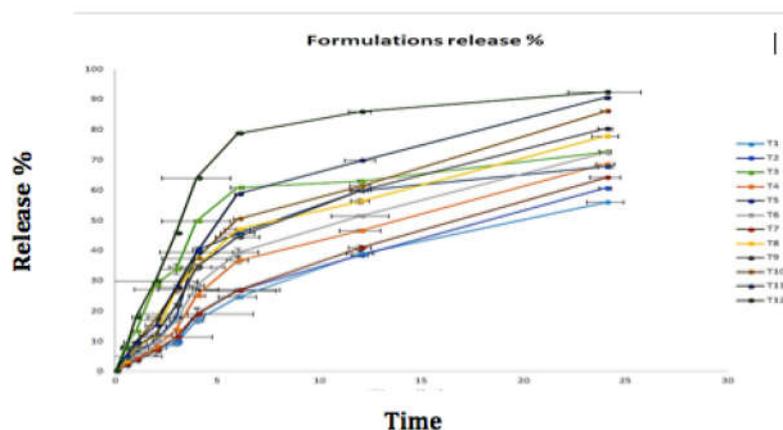


Fig. 4: Release profiles of all the prepared ethosomes (each value was an average of three measurements \pm SD)

The release of all prepared ethosomal formulations after 24 h exceeded 50 %. Green tea extract formulated as ethosomal preparations showed a controlled release rate due to ethosomes' reservoir action.

Therefore, both T10 and T12 formulations were selected as the optimized green tea extract ethosomal formulations, that showed higher EE %, lower polydispersity index, as well as optimum release, consequently increasing penetrability and bioavailability [43].

In vivo study

Green tea extracts supplementation effect on ferric reducing ability of plasma (table 4), demonstrates the variation between the 1st group (control) and the others. FRAP level was noticed to be increased in groups supplemented with green tea extracts compared to control. It was higher in all transdermal receiving (3rd, 4th, and 5th) groups ($P < 0.0001$ in all 3 groups and 95% CI 0.7915 to 1.1485 in 4th group) than the 2nd group (orally receiving) with $P = 0.0035$ and 95% CI 0.1758 to 0.7642.

Table 4: Ferric reducing ability of plasma and changes in the activity of catalase and in TBARs and MDA production in different study groups

| Groups | FRAP (mg/ml) mean \pm SD | Catalase activity (Units/ml) mean \pm SD | TBARs (nmol/ml) mean \pm SD | MDA (μ mol/l) mean \pm SD |
|----------------|----------------------------|--|-------------------------------|----------------------------------|
| Control group | 1.13 \pm 0.19 | 2.4 \pm 0.29 | 5.04 \pm 0.78 | 3.5 \pm 0.21 |
| Standard group | 1.65 \pm 0.40 | 4.075 \pm 0.56 | 4.2 \pm 0.34 | 2.4 \pm 0.09 |
| Group III | 1.9 \pm 0.15 | 5.125 \pm 0.26 | 2.6 \pm 1.1 | 2.18 \pm 0.06 |
| Group IV | 2.1 \pm 0.19* | 5.075 \pm 0.23** | 3.01 \pm 0.34* | 2.21 \pm 0.043 |
| Group V | 2.1 \pm 0.29* | 5.425 \pm 0.46 | 3.2 \pm 1.01* | 2.14 \pm 0.05 |

Values are presented as mean \pm SD*: statistically significant compared to corresponding value of other groups ($P < 0.05$)

Additionally, Green tea extracts supplementation effect on the activity of catalase enzyme (table 4), demonstrates the variation between the 1st group (control) and the others. CAT enzyme activity levels were noticed to be influenced in groups supplemented with green tea extracts compared to control. It was better in all transdermal receiving (3rd, 4th, and 5th) groups ($P < 0.0001$ in all 3 groups and 95% CI 2.6387 to 3.3613 in group 5) than the 2nd group (orally receiving) with $P < 0.0001$ and 95% CI 1.5810 to 2.4190. In the current study, we confirmed that the activity of catalase enzyme is unregulated by catechins.

Also, Green tea extracts supplementation effect on production of thiobarbituric acid reactive substance (TBARs) and malondialdehyde (MDA) (table 4), demonstrates the variation between the 1st group (control) and the others. TBARs and MDA levels were noticed to be lower in groups supplemented with green tea extracts compared to

control group. Additionally, it was lower in all transdermal receiving (3rd, 4th, and 5th) groups ($P < 0.0001$ in all 3 groups and 95% CI-3.2959 to-1.5041 in-group 3) than the 2nd group (orally receiving) with $P = 0.0081$ and 95% CI-1.3653 to-0.2347.

On observation of plasma lipid profile changes (table 5), there was a decrease in cholesterol concentration by 12% in the 2nd group supplemented with green tea extracts orally compared to the 1st control group, and by 20%, 10% and 22% in transdermal receiving (3rd, 4th, and 5th) respectively. Regarding HDL level, no effect was observed in 2nd group (orally receiving) compared to control group. However, it increased in all transdermal receiving (3rd, 4th, and 5th) groups in relation to control group. The Triglycerides, LDL and VLDL levels were decreased in all green tea extract supplemented groups compared to control group.

Table 5: Changes in lipid profile in different study groups

| Groups | Cholesterol (mg/dl) mean \pm SD | HDL (mg/dl) mean \pm SD | Triglycerides (mg/dl) mean \pm SD | LDL (mg/dl) mean \pm SD | VLDL (mg/dl) mean \pm SD |
|----------------|-----------------------------------|---------------------------|-------------------------------------|---------------------------|----------------------------|
| Control group | 110 \pm 5 | 58 \pm 3 | 159 \pm 3 | 60 \pm 0.2 | 10 \pm 3 |
| Standard group | 91 \pm 3 | 58 \pm 3 | 126 \pm 3 | 50 \pm 0.9 | 12 \pm 5 |
| Group III | 89 \pm 2 | 64 \pm 3 | 102 \pm 5 | 43 \pm 0.9 | 13 \pm 3.2 |
| Group IV | 90 \pm 2.5 | 60 \pm 2 | 112 \pm 3 | 35 \pm 1 | 11 \pm 3 |
| Group V | 86 \pm 3 | 62 \pm 1.6 | 118 \pm 2.5 | 33 \pm 1.6 | 9 \pm 4 |

Values are presented as mean \pm SD: HDL levels were increased in all transdermal-receiving (3rd, 4th, and 5th) groups in relation to control group. The

Triglycerides, LDL and VLDL levels were decreased in all green tea extract supplemented groups compared to control group.

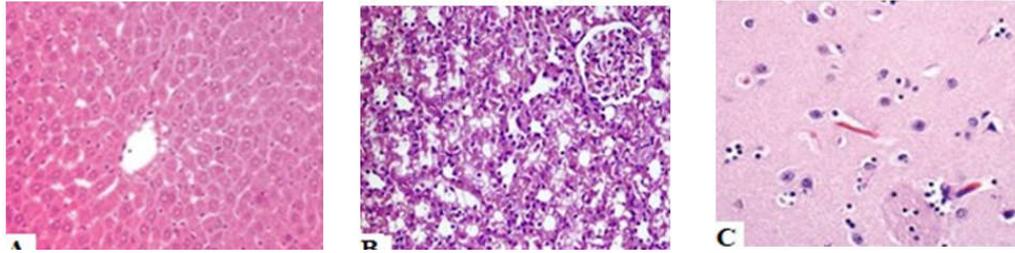


Fig. 5: Different histological specimens of experimental rats

Histopathological observation of tissues of various organs (brain, liver, and kidney) after administration of green tea extract (orally and transdermal) (fig. 5) showed no changes in the organs' tissues and cells of rats' specimens. Confirming that short time periods and such doses of oral and/or transdermal administration of green tea extract formulations doesn't cause any toxicity to brain, liver, nor kidney of the tested rats.

Fig. different histological specimens of experimental rats (H and E, $\times 400$) (a) liver, (b) kidney and (c) brain, show normal tissues with non-remarkable changes with control specimens.

To determine the relationship between EGCG supplementation and anti-oxidation or thermogenesis, the mRNA levels of UCP2 and UCP3

were measured in BAT. Transdermal administration of green tea extract formulations significantly increased the mRNA levels of UCP2 and UCP3 in groups 3-5 compared with those in the control group. Values for the 4th group are expressed as the fold change compared with those for the control group (mean \pm SD, n = 10). $p < 0.001$ vs. control group.

Aster Spathulifolius extract (when administered orally) causes increase in the mRNA synthesis of genes like *CPT1*, *UCP2*, and *UCP3* in the muscle of induced obese rats, which are involved in thermogenesis. We found that the expression of *UCP2* and *UCP3* in BAT were up-regulated significantly upon transdermal administration of green tea extract formulations (fig. 6, 7).

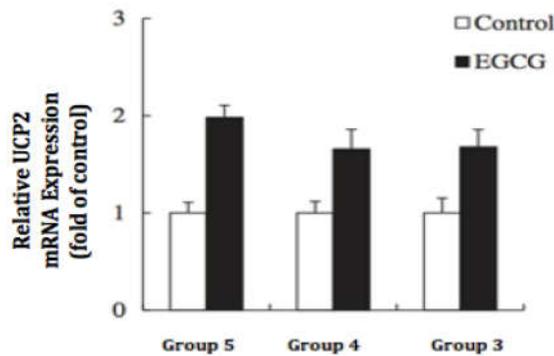


Fig. 6: Relative messenger RNA (mRNA) levels of UCP-2 gene, relative messenger RNA (mRNA) levels of UCP-2 gene in brown adipose tissue of rat in Epigallocatechin-3-gallate (EGCG) groups in relation to control groups

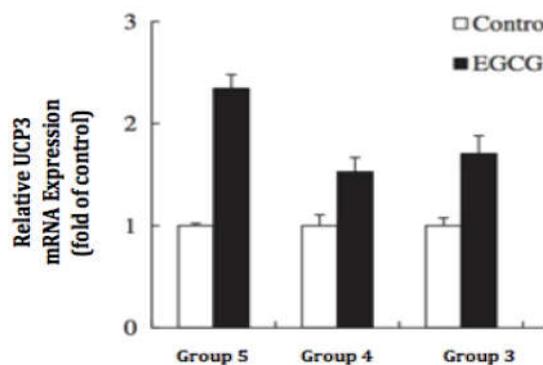


Fig. 7: Relative messenger RNA (mRNA) levels of UCP-3 gene, relative messenger RNA (mRNA) levels of UCP-3 gene in brown adipose tissue of rat in epigallocatechin-3-gallate (EGCG) groups in relation to control groups

DISCUSSION

In the present study, we found that *FRAP* was higher in all transdermal receiving (3rd, 4th, and 5th) groups ($P < 0.0001$ in all 3

groups and 95% CI 0.7915 to 1.1485 in 4th group) than the 2nd group (orally receiving) with $P = 0.0035$ and 95% CI 0.1758 to 0.7642. Forester and Lambert [44] and Chacko *et al.* [45] are in line with our findings.

CAT enzyme activity levels were noticed to be influenced in groups supplemented with green tea extracts compared to control. It was better in all transdermal receiving (3rd, 4th, and 5th) groups ($P < 0.0001$ in all 3 groups and 95% CI 2.6387 to 3.3613 in group 5) than the 2nd group (orally receiving) with $P < 0.0001$ and 95% CI 1.5810 to 2.4190. In the current study, we confirmed that the activity of the catalase enzyme is unregulated by catechins. Our results agreed with Li *et al.* [46] and Nelson *et al.* [47], However, opposed Pal *et al.* [48] and Chance *et al.* [49] findings, probably due to green tea extract use on induced injured animals.

Also, TBARs and MDA levels were noticed to be lower in groups supplemented with green tea extracts compared to control group. Additionally, it was lower in all transdermal receiving (3rd, 4th, and 5th) groups ($P < 0.0001$ in all 3 groups and 95% CI-3.2959 to -1.5041 in group 3) than the 2nd group (orally receiving) with $P = 0.0081$ and 95% CI-1.3653 to -0.2347. Our findings are in line with Mohamadin *et al.*, Awoniyi *et al.*, Messarah *et al.*, and Prabakar *et al.* [50-53] results.

On observation of plasma lipid profile changes, there was a decrease in cholesterol concentration by 12% in the 2nd group supplemented with green tea extracts orally compared to the 1st control group, and by 20%, 10% and 22% in transdermal receiving (3rd, 4th, and 5th) respectively. Regarding HDL level, no effect was observed in 2nd group (orally receiving) compared to control group.

However, increased in all transdermal-receiving (3rd, 4th, and 5th) groups in relation to control group. The Triglycerides, LDL and VLDL levels were decreased in all green tea extract supplemented groups compared to control group. Our results are consistent with the results of Basu *et al.*, Bornhoeft *et al.*, Cunha *et al.*, and Hassan *et al.* [54-57]. However, no effect of green tea supplementation on plasma lipid profile was the conclusion of Princen *et al.*, Erba *et al.* and Fukino *et al.* [58-60], which may be dose dependent effect.

Dulloo *et al.* [61] found an increase in the 24h energy expenditure by supplementation of green tea extract in humans. Subsequently, they reported an enhancement in the oxygen uptake rate for BAT by giving an ethanol extract of green tea, indicating the occurrence of BAT thermogenesis [62]. Green tea extract increases BAT thermogenesis via β -adrenoreceptor stimulation [63]. Green tea EGCG (via stimulating UCP2 expression in WAT) was observed to decrease diet-induced obesity [64]. Aster Spathulifolius extract (when administered orally) causes increase in the mRNA synthesis of genes like *CPT1*, *UCP2*, and *UCP3* in the muscle of induced obese rats, which are involved in thermogenesis [65]. We found that expression of *UCP2* and *UCP3* in BAT were up regulated significantly upon transdermal administration of green tea extract formulations.

CONCLUSION

Based on observations of present study, it can be concluded that a combination of 40% ethanol, 3% lecithin and 75% green tea extract (50 mg) can be used for the preparation of ethosomes with good entrapment efficiency. The *in vitro* release efficiency of ethosomal gel was found up to 92.49%, which support the potential of these carriers in penetrating the lipid rich biological membrane. The *in vivo* study confirms the antioxidant behavior of transdermal applied green tea extract. The findings suggest that EGCG may play important roles in regulating BAT thermogenesis and antioxidant effects for improving obesity. The present study revealed ethosomal gel as an efficient carrier for herbal extract.

AUTHORS CONTRIBUTIONS

Both authors designed for the study collected the data, analyzed, and wrote manuscripts. The first author was responsible for all the biochemical and molecular procedures. The second author performed all pharmaceutical experiments. All authors are responsible for financial support to complete the research.

ABBREVIATIONS

ATP-Adenosine triphosphate, BAT-Brown adipose tissue, CAT-Catalase activity, cDNA-Complementary DNA, CI-Confidence interval, EE-Entrapment efficiency, EGCG-Epigallocatechin gallate, FRAP-Ferric-reducing ability of plasma, HDL-High Density Lipoprotein, HPLC-High performance liquid chromatography, LDL-Low Density Lipoprotein, MDA-Malondialdehyde, PDI-Poly Dispersity Index, RNA-Ribonucleic acid, RT-PCR-Real Time Polymerase Chain Reaction, SD-Standard deviation, SEM-Scanning Electron Microscopy, TAC-Total antioxidant capacity, TBARs-Thiobarbituric acid reactive substance, Tm-Transition temperature, TPTZ-2,4,6-tripyridyl-s-triazine, UCPs-Uncoupling proteins, VLDL-Very Low-Density Lipoprotein

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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