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Research Article

Impact of Liposomes on the Effects of Quercetin-Rich Flavonoid Fraction Extracted from French Marigold (*Tagetes patula* L.) on the Jurkat Cells

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Abstract

Background: Natural polyphenols are widely used in traditional medicine for treating various diseases. Despite healthful properties, phenolic compounds ingestion in the form of food does not provide concentration (due to their low solubility in water, poor absorption, and fast metabolism) enough to achieve systemic therapeutic effects in the body. This problem has been solved by creating various composite pharmaceuticals from phenolic compounds using different methods to ensure the stabilization of polyphenols.

In the present study, we investigated the effects of quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) alone and incorporated into DPPA (1,2-palmitoyl phosphatidic acid) and DPPC (dipalmitoyl phosphatidylcholine) liposomes on the viability of the intact and incubated under oxidative stress conditions Jurkat cells.

Materials and Methods: Quercetin-rich flavonoid fraction was extracted from a French Marigold (*Tagetes patula* L.) by TLC, HPLC, and LC-MS methods. Extract alone and in complex with Dipalmitoyl phosphatidylcholine (DPPC) and 1,2- dipalmitoyl phosphatidic acid (DPPA) liposomes were added to the Jurkat cells culture at a rate of 2 mg/mL^{-1}. Cell viability was assayed by the MTT test based on evaluating cellular dehydrogenase activity.

Results: The spectrophotometric absorption spectrum of the quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) shows two peaks corresponding to benzoyl (254nm) and cinnamyl (375nm) aromatic rings.

In the complex of quercetin-rich flavonoid fraction with DPPC and DPPA liposomes, the spectrophotometric absorption peak at 254nm was not detected, while the absorption intensity of the peak at 375nm was sharply reduced. The quercetin-rich flavonoid fraction alone and in combination with DPPC liposome increased intact and incubated under low- and high-intensity oxidative stress conditions Jurkat cells' viability but did not revealed effect in combination with DPPA liposome.

Conclusion: The quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) formts stable complexes with DPPC and DPPA liposomes that gives possibility to storage of high content of phenolic compounds in lipid nanocapsules.

Negative (–P-COO–) surface charge of DPPA liposomes tends their repelling by negatively charged cellular membranes, whereas polar phosphatidylcholine head of DPPC liposomes which contains as negative (–P-COO–) also the positive (N^+) charged groups is attracted to the cellular membrane, leads the release of quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) within the cell cytoplasm and ensures an enhancement efficiency of the delivery process.

Keywords: Flavonoids; Metabolism; Emulsion

Introduction

Flavonoids are the most significant group of polyphenols. Natural polyphenols, found in plants, are widely used in traditional medicine for treating various diseases [1-4]. During the last decades, natural ingredients have attracted the attention of scientists [5,6]. Studies usually are focused on the identification of compounds with new effective pharmacological properties and the investigation of the molecular mechanisms of their activities. Despite healthful properties, phenolic compounds ingestion in the form of food does not provide concentration (due to their low solubility in water, poor absorption, and fast metabolism) enough to achieve systemic therapeutic effects in the body [7].

This problem has been solved by creating various composite pharmaceuticals from phenolic compounds using different methods (cyclodextrin coatings [8], simple emulsions, lipid nanoparticles [9], or liposomes [10,11]) to ensure the stabilization of polyphenols. The genus Tagetes (Asteraceae) is native to the Americas but some of its members (in particular *T. erecta* and *T. patula*) commonly known as marigolds were naturalized in India, North Africa, and Europe as early as in the16th century [12]. Marigold was introduced to Georgia from India, and its ground-dried petals became one of the most popular local spices [13,14] with health-beneficial properties. The healing properties of Tagetes species have been implemented by folk medicine for centuries [16]; particularly, they are used for preparing ethnobotanical remedies against rheumatism, stomach and intestinal problems, kidney and hepatic disorders, fever, and pneumonia. The efficacy of oral administration of *T. patula* florets extracts against acute and chronic inflammation was confirmed in experiments with animal models [15,17]. Both hydrophilic and lipophilic fractions from *T. patula* petals showed the highest radical-scavenging and immunomodulatory capacities among all Georgian spices tested [18] One can suppose that the combination of hydrophilic and lipophilic antioxidants in the marigold flowers can contribute to their health effects. In addition, was found that the major flavonoid of Tagetes, quercetin, has anti-cancer activity [19].

In the present study, we investigated the effects of quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) alone and incorporated into DPPA (1,2-palmitoyl phosphatidic acid) and DPPC (dipalmitoyl phosphatidylcholine) liposomes on the viability of the intact and incubated under oxidative stress conditions Jurkat cells.

Materials and Methods

Plant material

The flowers were collected from the plants of a local Georgian landrace of *T. patula* grown at an experimental plot near Tbilisi from seeds purchased from a commercial supplier. The collected flowers were air-dried in the shade at 25–30°C. The dried matter was stored in a closed glass container in a cool, dry place.

Extraction and purification of marigold constituents

The isolation was performed by sequential solvent extraction of *T. patula* flowers. A sample of 600 g of dried pulverized plant material was extracted with 1,2-dichloroethane in a Soxhlet apparatus for 48 h until color loss. The residue after the dichloroethane extraction was re-extracted with ethanol (solvent/plant matter ratio 1: 5) for the isolation of compounds of higher polarity. The solvents were evaporated under vacuum at 40°C and further separation of individual compounds from the dichloroethane extract was performed by column chromatography on a silica gel column with a chloroform-hexane solvent system. The elution of fractions from the column was started with hexane with a further increase of chloroform content in the system. A solvent mixture of 3% chloroform in hexane eluted compound 1 and compound 2 was eluted from the column with a 5% chloroform in hexane solution. In the ethanolic extract substances were separated on a silica gel column by elution with dichloroethane/methanol using thin-layer chromatography (TLC) for the characterization of the obtained fractions. The elution was started with dichloroethane with a subsequent stepwise increase of methanol content in the system. By elution with 2, 3, 5, 7, and 10% methanol in dichloroethane we received different fractions. Rechromatography of fractions Sephadex LH-20 and silica gel columolumn and further purification on a polyamide column with elution with aqueous ethanol we separated different compounds.

Thin layer chromatography (TLC)

The TLC separation was performed using silica gel plates from Merck (Germany). Separation of lipophilic compounds was performed in the solvent systems of dichloroethane-methanol (9: 1) and chloroform-methanol (9: 1). More polar compounds from ethanolic extracts were separated in the solvent systems of chloroform/methanol/water (26: 14: 3). The chromatograms

were inspected under UV light of 254 and 360 nm, before and after applying staining reagents for flavonoids detection. Flavonoids were detected as yellow spots revealed after heating the plates sprayed with a 1% ethanolic solution of aluminum chloride. Other compounds were detected by spraying 20% sulfuric acid solutions. After heating the sprayed plates to 100°C the compounds were revealed as spots of blue to green shades, depending on specific compounds.

High-pressure liquid chromatography (HPLC).

Separation was carried out on a Waters chromatograph (USA) using a Radia-Park C18 (8x10) chromatographic column, column temperature 350 C, flow rate 1.8 ml per minute. Sample quantity 20 µl, solvent system A-0.1% trifluoroacetic acid, B-60% acetonitrile-water (by volume). To 0.2 grams of dry spice powder add 40-degree alcohol, the ratio of material to solvent is 1/15, that is, 3 ml. For good extraction, the mixture was stirred and treated with ultrasound. Then left for 24 hours at room temperature. A portion of the obtained extracts was taken for analysis and filtered through a 0.22 millipore filter before HPLC analysis.

Liquid Chromatography-Mass Spectrometry (LC-MS)

The samples were dissolved in HPLC-grade methanol and filtered through a Millex-HV Durapore (PVDF) membrane (0.22 µm) before being injected into the LC-MS instrument. Mass spectral analyses were carried out using the Ultraperformance LC-Quadruple Time of Flight (UPLC-QTOF) instrument (Waters Premier QTOF, Milford MA, USA), with the UPLC column connected online to a PDA detector and then to an MS detector equipped with an electrospray ion (ESI) source (used in ESI-positive mode). Separation was performed on a 2.1 × 50 mm i.d., 1.7 µm UPLC BEH C18 column (Waters Acquity). The chromatographic and MS parameters were as follows: the mobile phase consisted of 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B). The linear gradient program was as follows: 100% to 95% A over 0.1 min, 95% to 5% A over 9.7 min, held at 5% A over 3.2 min, and then returned to the initial conditions (95% A) in 4.2 min. The flow rate was 0.3 mL min−1 and the column was kept at 35°C. Masses of the eluted compounds were detected with a QTOF Premier MS instrument. The UPLC-MS runs were carried out at the following settings: capillary voltage of 2.8 kV, cone voltage of 30 eV, and collision energy of 5 eV. Argon was used as the collision gas. The range was 70 to 1,000 D. The MS system was calibrated using sodium formate and Leu-enkephalin was used as the lock mass. The MassLynx software version 4.1 (Waters) was used to control the instrument and calculate accurate masses. The quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) was identified using the molecular formulae calculated based on accurate mass and isotopic pattern information, and UV/visible spectra, in comparison with authentic standards of quercetin (Extrasynthese, Genay, France).

Cell culture and experimental design

The human T-cell leukemia lymphoblastoid Jurkat cells (DSMZ ACC 282) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen. The cells were grown in suspension culture at 37°C under 5% humidified CO_2 in bioactive medium RPMI 1640 (Gibco, Grand Island, NY, USA) containing inactivated embryonic bovine serum (Sigma, St Louis MO, USA), L-glutamine (4 mM), penicillin (100 U mL^{-1}), and streptomycin (100 U mL^{-1}). The experiments were carried out at cell densities of 0.3 to 0.6 × 10^6 cells mL^{-1}. To imitate the oxidative stress conditions, H_2O_2 (Sigma) was added to the Jurkat culture to reach the concentrations of 25 and 50 µM, corresponding to low and intermediate stress severity, respectively [20]. In the unstressed control treatment, water was added to the samples instead of H_2O_2. The quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) and in complex and without liposomes were added to the Jurkat cells culture at a rate of 2 mg/mL^{-1}.

Cell viability

Cell viability was assayed by the MTT test based on evaluating mitochondrial dehydrogenase activity [21]. Cell suspensions (2 × 10^6 cells mL^{-1}) were incubated with H_2O_2 and marigold preparations as described above. After the incubation period, the cells were harvested by centrifugation at 1500 g for 5 minutes, washed, and re-suspended in a fresh medium. The 8 mg mL^{-1} solution of 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) in buffer (140 mMNaCl, 5 mM HEPES, pH 7.4) was added to the cell suspension at a rate of 30 µL per 100 µL suspension and the mixture was incubated for 4 h at 37°C in a 5% CO_2 atmosphere. After this incubation, the supernatant was carefully removed and the colored formazan crystals produced from the MTT were dissolved in 100 µL of dimethyl sulfoxide

(DMSO). The absorption values of the solutions reflecting the cellular dehydrogenase activity were measured at 570 nm. The effects of various treatments on cellular activity and viability were expressed as percentages of their absorption values related to those of non-treated cells.

Preparation of DPPC and DPPA liposomes

For the preparation of complex liposomes containing quercetin, a ratio of 3:1 of quercetin and lipids was selected. Since quercitin is a molecule of hydrophobic nature, to the mixture of lipids molecules and quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) we first added a small amount of organic solvent, 1 μl of methanol, and then added 3 ml of distilled water heated to 60^0 C.

A mixture of quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) (3 mg) and lipids (1 mg) (DPPC and DPPA from Lipoid, Newark, New Jersey) was dissolved in 3 mL distilled water at 50 °C followed by intensive shaking until a homogeneous liposomal suspension without aggregates of quercetin molecules was obtained. Since liposomes encapsulate about 50% of GTCs, we added 0.2 μg of GTC–liposome complexes to 100 mL of the incubated cell suspension. Quercetin solubility is high in lipids and alcohol, while this compound is quite water-insoluble [22], the quercetin molecule can bind to hydrophobic parts of the liposomes and can also be incorporated into the internal volume (lumen) of the liposomes. Quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) was placed inside liposomes in complexes prepared by the method described by Mdzinarashvili., *et al.* [23]. To equalize the liposome diameters (200 nm), the suspension of complex liposomes was finally extruded through a nanoporous membrane.

Statistics

The trials were performed in five replications. The statistical analysis of the obtained results, including the calculation of means and standard deviations, was conducted using the IBM SPSS Statistics program. The statistical significance of the differences between the treatment results versus the non-treated control was analyzed by pairwise comparison using the Student's -test at values of ≤0.001, ≤0.01, and ≤0.05 designated as, and, respectively.

Results and Discussion

Composition of *T. patula* Extracts

Several flavonoids, belonging to the group of flavonols (quercetin, quercetagetin, patuletin, Lutein, glucosylated (quercetin-3-glucoside, quercetagetin-7-glucoside) and di-glucoside (quercetagetin-3,7-diglucoside) derivatives of quercetin and quercetagetin) were separated and identified in the marigold extracts by TLC, HPLC and LC-MS methods. Such composition of flavonoids is typical of *T. patula* [15,24] The quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) was used for further trials with Jurkat cells (Figure 1).

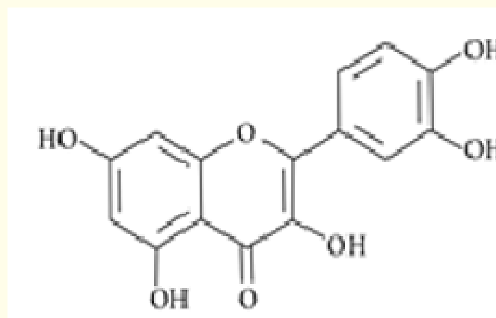

Figure 1: The quercetin molecule (a - benzoyl, b – cinnamyl).

Spectrophotometric absorption spectrum of pure quercetin quercetin in MDPPA and MDPPC liposomes

The absorption spectrum of the quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) shows two peaks at 254 nm and 375 nm, which belong to different conjugated aromatic rings: the first absorption peak at 254 nm corresponds to benzoyl, and the second absorption peak at 375 nm corresponds to cinnamyl [25] (Figure 1, 2).

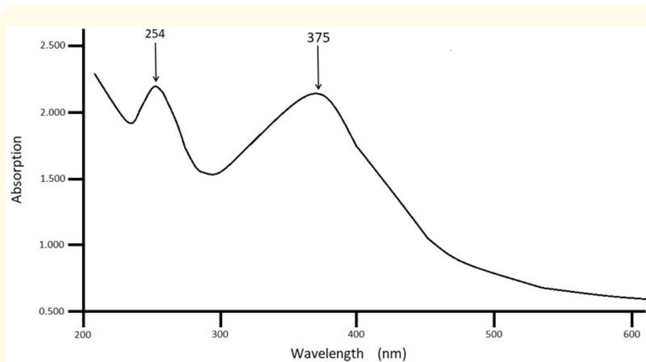

Figure 2: Spectrophotometric absorption spectrum of quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.).

In the complex of quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) with DPPC and DPPA liposomes, the spectrophotometric absorption peak at 254 nm wavelength, characteristic for the benzoyl group of quercetin, was not detected, while the absorption intensity of the peak at 375 nm wavelength, characteristic of the cinnamyl group, was sharply reduced (Figures 3, 4).

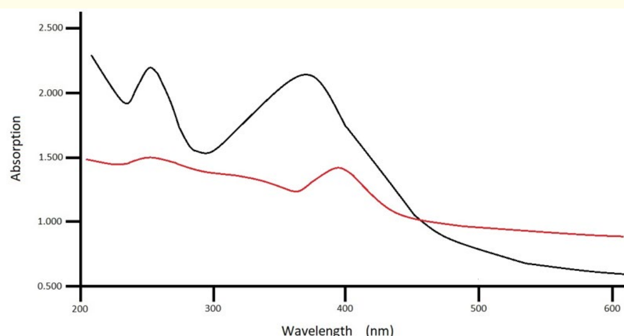

Figure 3: Spectrophotometric absorption spectrum of quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) (a) and DPPC liposome-encapsulated quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) (b).

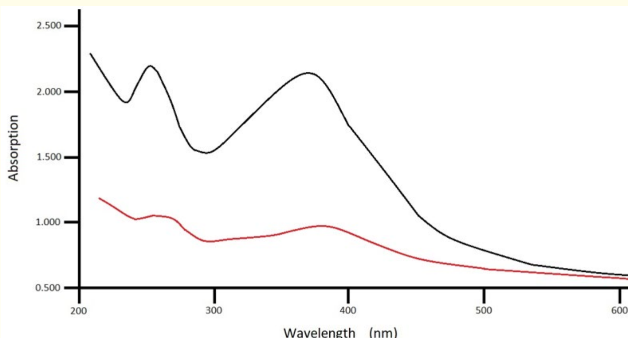

Figure 4: Spectrophotometric absorption spectrum of quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) (a) and DPPA liposome-encapsulated quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) (b).

Effects of quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) on Jurkat Cells Viability

Adding a quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) to intact Jurkat culture caused a certain increase in cell viability above the control level (by 20%), which may be related to the enhancement of mitochondrial dehydrogenase activity and cell proliferation level (Figure 5).

Oxidative stress induced by exogenous H_2O_2 in a dose-dependent manner reduced the viability of Jurkat cells (Figure 5). The quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) had enough high protective effects against H_2O_2-induced damage of the Jurkat cells (at low and intensive oxidative stress conditions). The superiority of quercetin over other phenolic compounds in protecting Jurkat cells against H_2O_2-induced death was earlier shown by Zhang., *et al*. [26].

Figure 5 shows that DPPC and DPPA liposomes alone did not affect intact and incubated under oxidative stress Jurkat cells viability.

The quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) in combination with DPPC liposome increased the viability of intact Jurkat cells by 20% and did not change in combination with DPPA liposome.

The viability of Jurkat cells incubated at both low- and high-intensity oxidative stress conditions under the influence of quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) in combination with DPPC liposome increased by 25%, and 18%, respectively, but did not change when incubated with DPPA liposome-encapsulated quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) (Figure 5).

Discussion

Based on the analysis of spectrophotometric data, we obtained information on the structure of the complex of quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) with DPPC/DPPA liposomal nanoparticles.

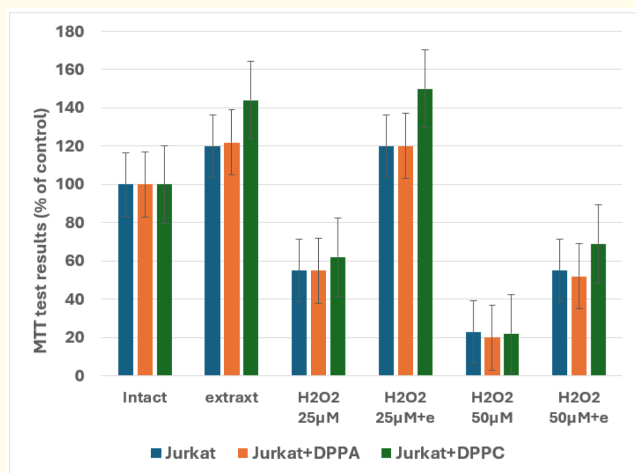

Figure 5: Effects of quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) on the viability of the Jurkat cells (% of the non-treated control). Error bars represent standard deviations of five replications. Values marked with asterisks were significantly different from the non-treated control according to Student's -test at values of ≤0.001, ≤0.01, and ≤0.05 designated as ***, **, and *, respectively.

As it follows from the research results, in the absorption spectrum of the extract of marigold fraction flavonoids rich in quercetin, two peaks at 254 nm and 375 nm wavelengths were observed. These peaks belong to the conjugated aromatic rings of benzoyl (254 nm) and cinnamyl (375 nm) in the quercetin molecule [25] (Figure 2).

DPPC and DPPA liposomes have similar structural organizations at a basic level - both lipid molecules create standard bilayer liposomes with "hydrophobic" interaction stabilizing lipid tails and electrostatic interaction between the hydrophilic lipid heads.

In the spectrophotometric absorption spectrum of the quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) encapsulated into the DPPC and DPPA liposomes, the peak characteristic for the benzoyl group (254 nm) was not detected (Figures 3, 4), which should be related to the connection of the hydrophobic benzoyl group to the hydrophobic tails of the liposomes.

In the spectrophotometric absorption spectrum of the complex of quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) with DPPC and DPPA liposomes, the intensity of the peak at 375 nm characteristic for the cinnamyl group decreased dramatically (Figures 3, 4). The cinnamyl group is characterized by its hydrophilic nature, which causes its location on the surface of the lipid bilayer of DPPC and DPPA liposomes; it partially sinks into the layer of the lipid heads, which leads to a decrease in the absorption intensity of the peak at 375 nm. The obtained result confirms the formation of stable complexes of quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) with DPPC and DPPA liposomes.

There are significant differences between the DPPC and DPPA liposomes. The DPPA liposomes with negative (–P-COO–) surface charge in a neutral aqueous environment will tend to be repelled by negatively charged (ζ-potential) cell membranes, whereas polar phosphatidylcholine head of DPPC liposomes contains both a negative (–P-COO–) and a positive (N+) charged groups [27,28]; consequently, the DPPC liposomes are attracted to the negatively charged (ζ-potential) cell membranes.

The results of the study showed that pure DPPC and DPPA liposomes did not affect the viability of intact Jurkat cells incubated under different intensity oxidative stress conditions.

The quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) in combination with DPPC liposome increased the viability of intact Jurkat cells by 20%. During the incubation[n of Jurkat cells under both low- and high-intensity oxidative stress conditions with the quercetin-rich flavonoid fraction extract incorporated into the DPPC liposome, Jurkat cells' viability increased by 25%, and 18%, respectively. The effectiveness of quercetin-rich flavonoid fraction extract within the DPPC liposomes may be, firstly, related to the storage of high content of phenolic compounds in lipid nanocapsules, secondly, the interaction of the liposomes with the cell membrane presumably can lead to the release of quercetin-rich flavonoid fraction extract within the cell cytoplasm, which ensures an enhancement efficiency of the delivery process. Consequently, DPPC liposomes increased the antioxidant effect of the quercetin flavonoid fraction on the intact and incubated under oxidative stress conditions Jurkat cells, it can modulate its bioactivity and therapeutic effects.

Conclusion

The quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) formts stable complexes with DPPC and DPPA liposomes that gives possibility to storage of high content of phenolic compounds in lipid nanocapsules.

Negative (–P-COO–) surface charge of DPPA liposomes tends their repelling by negatively charged cellular membranes, whereas polar phosphatidylcholine head of DPPC liposomes which contains as negative (–P-COO-) also the positive (N^+) charged groups is attracted to the cellular membrane, leads the release of quercetin-rich flavonoid fraction extracted from French Marigold (*Tagetes patula* L.) within the cell cytoplasm and ensures an enhancement efficiency of the delivery process.

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