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Optimization of Particle Size Distribution with Gaussian Analysis of Albumin Microcarriers Cross-linked by Natural Phenolic Compounds

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Highlights

- This paper focuses on the use of natural phenolic compounds as cross-linking agent.
- Microcarriers of Albumin protein were synthesized with these agents.
- Particle size optimization using Gausian analysis was achived.

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Keywords

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Abstract

The biodegradation of albumin into natural products and nontoxicity besides its antigenicity has many advantages in controlled drug delivery of therapeutic agents. A bifunctional covalent bonding agent, glutaraldehyde is extensively used for linking amine groups of albümin microparticles/microcarriers (AlbMC's). But its cytotoxicity and the rapid calcification of the glutaraldehyde-treated tissue limit the use of glutaraldehyde. Phenolic compound showed non-covalent and covalent chemical interactions with proteins. The objective of this research is to prepare three different natural phenolic compound cross-linked/stabilized AlbMC's and estimate the cross-linker concentration which is giving narrow size distributions since it is important to gain higher surface area. The influence of qallic acid (GA), tannic acid (TA) and quercetin concentrations on AlbMC's size was investigated by Gaussian function analysis of microcarriers determined after optical micrograph measurements. Gallic acid (GA) stabilized AlbMC's have 3.35 ± 0.71 µm average mean size distribution while it was 3.56 ± 0.71 µm for Quercetin and 3.71 ± 0.69 µm for TA stabilized microcarrier formations. Average mean particle size distribution of AlbMC's synthesized with synthetic cross-linker, glutaraldehyde was calculated as 5.12 ± 0.50 µm. All statistical analysis were evaluated by MATLAB program. New approach for albumin microcarrier synthesis by using phenolic compounds as a cross-linker can be proposed as an alternative microcarrier preparation system with narrow size distributions.

1. INTRODUCTION

Polymers from different origins such as natural, synthetic and modified natural are commonly used for the preparation of microcarriers. The natural polymeric carrier system, albumin microcarriers (AlbMCs) have been shown to be very suitable systems for drug targeting and drug delivery to the target tissues or cells by evading other tissue from undesirable effects in the field of biomedicine [1-5]. Their advantages are biodegradability, lack of toxicity, non antigenicity, stability from the physical and chemical aspect, and can be removed from the vascular system by phagocytosis [2-4]. Albumin also has numerous binding sites for external ligands like antibiotics [2-5] and the size of particles, degree of stabilization, and site of metabolism are the main factors influencing the extent of metabolism [6].

A bifunctional covalent bonding agent, glutaraldehyde (Figure 1) is extensively used for linking amine groups of AlbMCs. But its cytotoxicity like asthmatic symptoms, rhinitis, and skin irritation and the rapid calcification of the glutaraldehyde -treated tissue limit the use of glutaraldehyde [5, 7]. Recently, glutaraldehyde was described as having a cytotoxic effect on isolated hepatocytes [8].

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Similar phenolic and polyphenolic compounds besides flavonoids constitute one group of plant secondary metabolites. Condensed and hydrolysable tannins are known as natural polyphenols distributed in plants. Polymeric flavonoids or proanthocyanidins constitute the main structures of condensed tannins while hydrolysable tannins like gallotannins and ellagitannins can be hydrolyzed to yield phenolic acids and carbohydrate. Phenolic acids are also known as hydroxybenzoates and Gallic acid is the main phenolic acid obtained after hydrolysis [9,10]. Gallic acid (GA) (Figure 1) is found in various products and is known as natural and nonenzymatic antioxidant [11,12]. Tannic acid (1,2,3,4,6-pentagalloyl-Oglucose) (Figure 1) is one of the most studied hydrolyzable tannin [13,14]. The basic flavonoid structure consists of two phenyl groups joined by a three carbon bridge which is open or involved in a heterocyclic ring [15]**.** Quercetin (Figure 1) is one representative flavonoid with anti-oxidant, anti-inflammation, and anti-tumor properties. These effects were greatly the results of the inhibitory action demonstrated against protein/enzyme structures in various organisms [13,16-19].

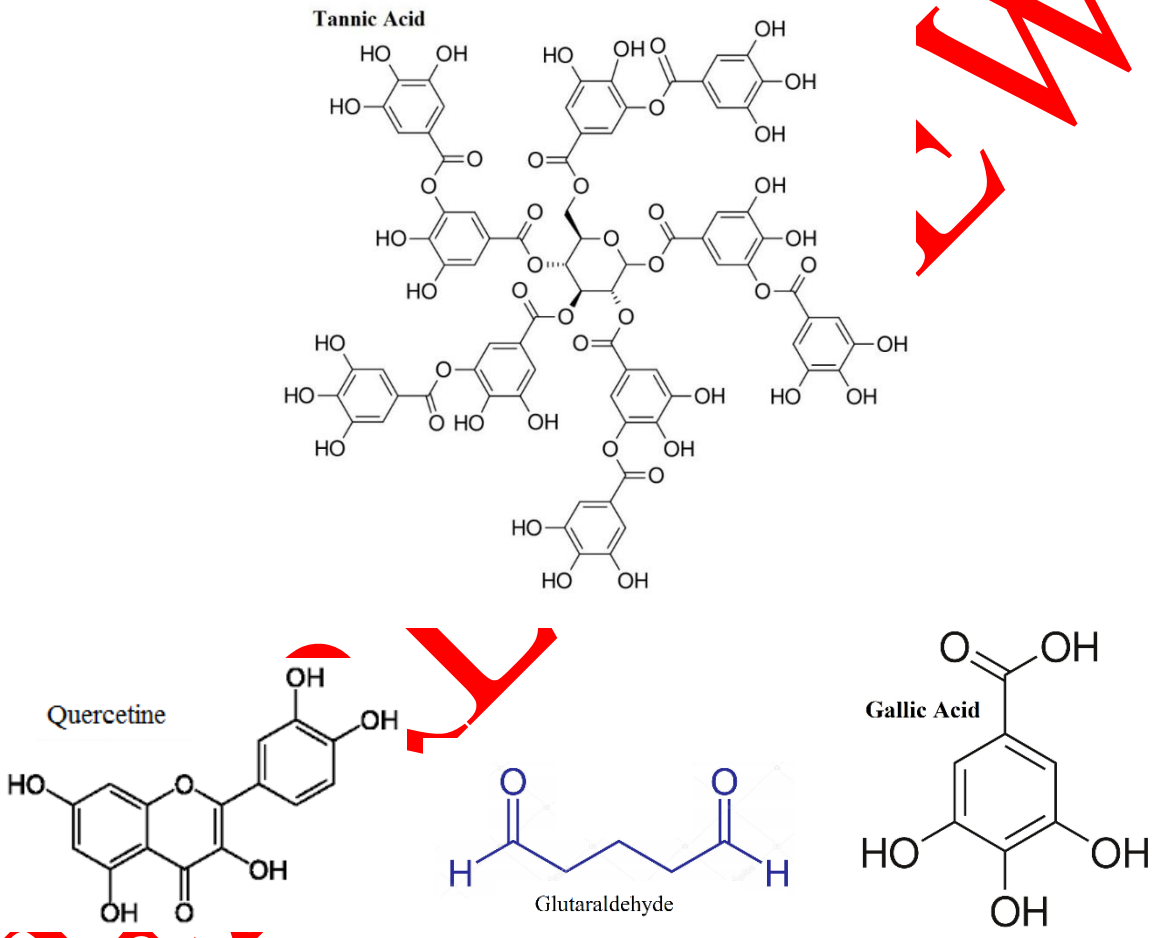


Figure 1. *Chemical structures of Tannic acid, Gallic acid, Quercetin, and Glutaraldehyde*

Polyphenols also characteristically possess a significant binding affinity to proteins, which can lead to the formation of soluble and insoluble protein–phenol complexes [20] or precipitate [21]. The chemical basis of the formation of these complexes are considered to be the result of hydrogen bonding, hydrophobic, ionic, and covalent depending on the protein and phenolic compound type [22-25]. It was reported that covalent bonds between phenolic compounds and proteins are more rigid and thermally stable than hydrogen bounding, ionic, and hydrophobic interactions [20, 25]. Cross-linking of gelatin was done with natural phenolic compounds caffeic acid (CA) and tannic acid (TA) above pH 9. The gelatin hydrogel's molecular mobility cross-linked with phenolic compounds significantly decreased compared with original gelatin gel [26]. The physical properties of gelatin hydrogels cross-linked with polyphenol extracted from *Fructus Chebulae* plant was found to be higher than genipin cross-linked gelatin hydrogels [27].

Gaussian distribution or Rosin-Rammler distribution is one of the specific representation of the polydisperse systems which quantifies the variety of sizes by the mean particle radius and the standart deviation of the radius divided by the mean radius [28]. The Generalized Gaussian (GG) distributions have found applications in many engineering studies and apllications. The main reason of this result comes from the flexible parametric form of its probability density function used to modeling many types of physical phenomena [29].

In the research, the natural phenolic compounds, GA, TA and Quercetin, were selected as cross-linker/stabilizer of the formation of albümin microparticles other than synthetic cross-linker glutaraldehyde due to their bounding capability with proteins or enzymes. So, we aimed to synthesize Albumin microcarriers in the presence of selected phenolic compounds (GA, TA and Quercetin) as stabilizer and/or cross-linking agent. Microcarrier formation was tested for different stabilizer concentrations and particle size diameters were measured under optic microscope. Gaussian size distribution was applied and phenolic compound concentrations, which give minimum size distributions were estimated.

2. MATERIAL METHOD

2.1. Materials

Bovine serum albumin, Gallic acid, Tannic acid, Quercetin, and diethyl ether were purchased from Sigma-Aldrich, Merch. Glutaraldehyde (25% in aqueous solution) was received from J.T.Baker. All other reagents were of analytical grade. Olive oil was gift from a local producer. Ultra-pure water was used in the preparation of all the solutions. All the solutions were prepared by using Ultrapure water (18.2 mΩ) obtained from a Millipore Elix 5UV Water Purification System (Merck, Czech Republic) and was used throughout.

2.2. Synthesis of Albumin Microcarriers

Albumin microcarriers were prepared by emulsion technique [30-33]. BSA solution of 50 mg/500 µl water was added dropwise to 50 ml olive oil containing beaker with stirring rate of 1000 rpm. The 0.5 mL solution of cross linker was dropped slowly to stabilize the formed protein droplets allowed to stir for 30 min. Then the oily phase was discarded after santrifugation for 10 min at 5000 rpm. The upper phase was removed and the remaining part was washed with diethyl ether three times to assure oil refinement. Cross-linker solution concentrations were selected to be equal or less than water solubility amounts [34-36]. Synthetic cross-linker (GAld) was also used to produce albumin microcarriers in order to assess microsphere sizes comparatively. The method mentioned above was also performed by the addition of the synthetic cross-linker solution of GAld (0.5 mL, 25% in aqueous solution).

2.3. Characterization Studies

Determination of particle sizes

AlbM's stabilized with Glu, GA, TA, and Quercetin were spreaded on a lamel and dried. The particle sizes of the microcarriers was determined by inverted microskope (MOTIC AE30). The diameters of the approximately 100 synthesized microcarriers were measured for each cross-linker concentration. Measurements were repeated at least three times and results were reported as mean ± standard deviation.

Particle size distributions

Particle size distributions of microcarriers cross-linked with GA, TA, Quercetin phenolic compounds and GAld were analyzed using MATLAB 7.02 program and statistical analysis was performed by Gaussian function to describe the normal distributions.

Statistical analysis

Experimental data were expressed as arithmetic means ± SD (n = 3 for all experiments), differences between groups were considered statistically significant when the probability (p) was less than 0.05.

3. THE RESEARCH FINDINGS AND DISCUSSION

3.1. Synthesis of Albumin Microcarriers (AlbMC's)

The selected natural phenolic compounds have different molecular weights and water solubility values. Molecular weights of GA, TA, and Quercetin were 170.12; 1701.20, 302.23 g/mol, respectively. Water solubility of GA was 11.9 g/L [34] while TA has the highest solubility with 1 g/0.35 mL (2850 g/L) [35]. Tannic acid concentrations were kept as gallic acid concentrations since it has more hydroxyl groups than gallic acid in order to avoid unreacted groups since albumin concentration will be kept constant. Quercetin has the minimum solubility between other phenolic compounds of 60 mg/L at 16°C water solubility [36]. The designed phenolic cross-linker concentrations dissolved in ultrapure water at neutral pH value were given in Table 1.

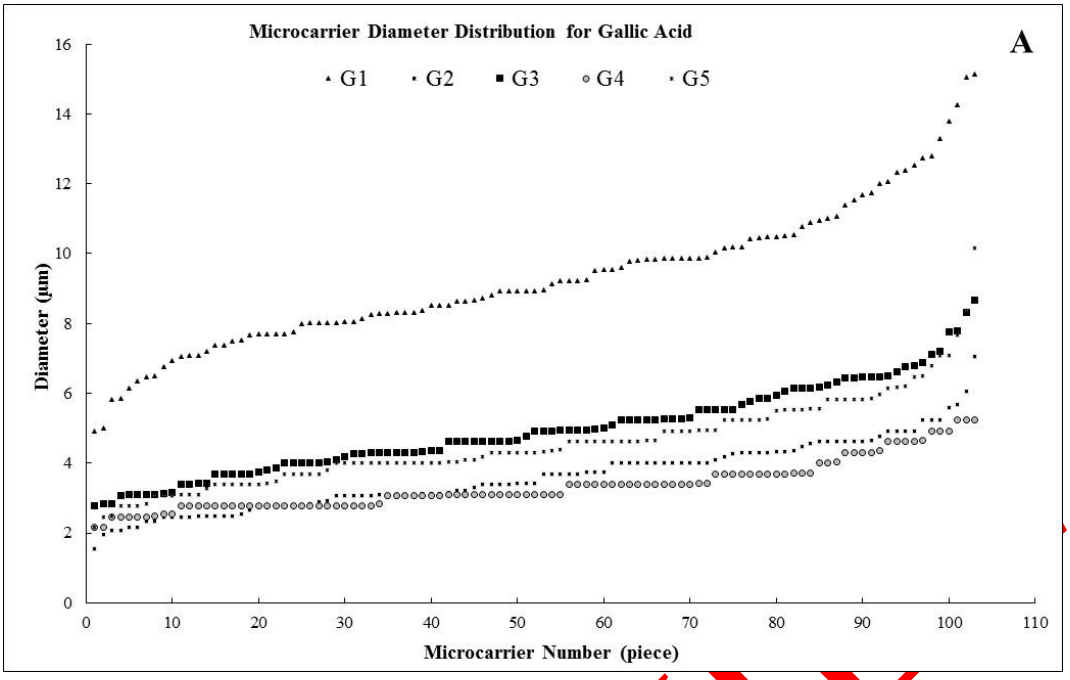
Table 1. *Selected natural cross-linker concentration amounts of Gallic acid, Tannic acid, and Quercetin in the synthesis of albumin microcarriers*

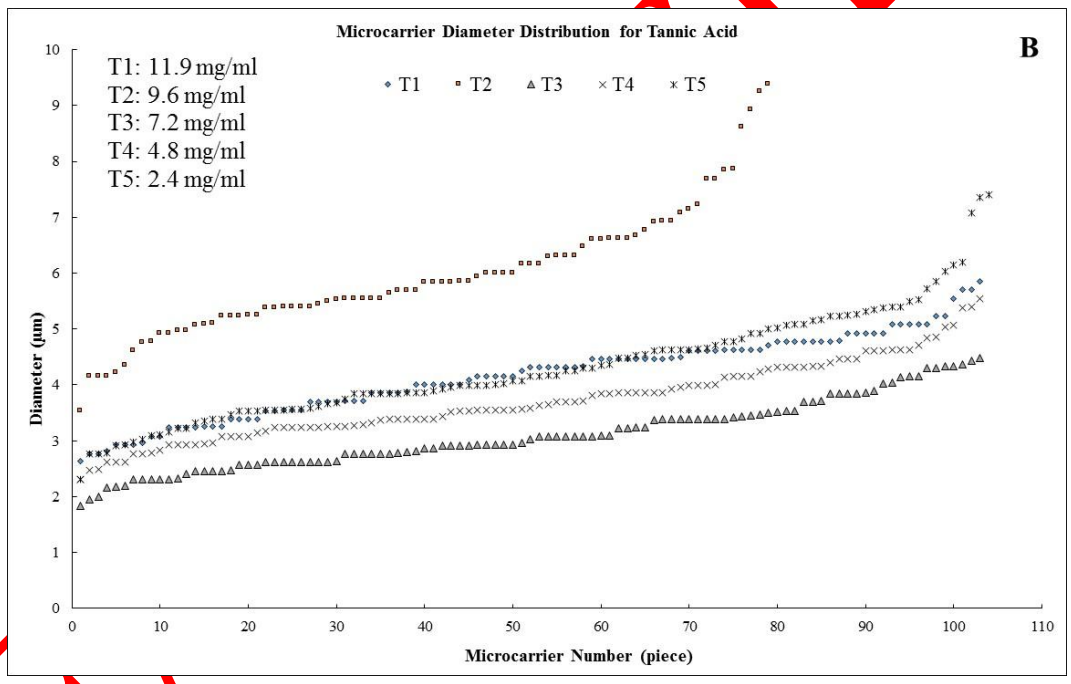
Natural crosslinkers	*C-1	*C-2	*C-3	*C-4	*C-5
Gallic acid (MW:170.12 g/mol w. s.: 11.9 g/L)	11.9 g/L 69.95 mM	9.6 g/L 56.43 mM	7.2 g/L 42.32 mM	4.8 g/L 28.22 mM	2.4 g/L 14.11mM
Tannic acid (MW: 1701.20 g/mol w. s.: 2850 g/L)	11.9 g/L 6.995 mM	9.6 g/L 5.643 mM	7.2 g/L 4.232 mM	4.8 g/L 2.822 mM	2.4 g/L 1.411 mM
Quersetin (MW: 302.23 g/mol w. s.: 60 mg/L)	0.06 mg/ml 0.199 mM	0.05 mg/ml 0.165 mM	0.04 mg/ml 0.132 mM	0.03 mg/ml 0.099 mM	0.02 mg/ml 0.066 mM

*C: Concentration; w.s.: water solubility

The formation of microstructures was achieved for each phenolic compound concentration and the average microcarrier diameter size of each concentration was found. Experiments were repeated at least three times and the average values were calculated. Size diameters of microcariers interacted with gallic acid, tannic acid and quercetin were plotted in Figure 2. The measured micro carrier diameters were approximately 100 pieces. The concentration of phenolic compound affected the formation of microcarrier and carrier diameter ranges changed for each concentration. AlbMC diameters of gallic acid cross-linker was between 2-4.5 µm for 9.6, 7.2, 4.8, and 2.4 mg/mL while 6-14 µm size diameters were measured for the highest concentration value, 11.9 mg/Ml (Figure 2A). Tannic acid aided microcarier formation has the same tendency and 2-5 µm particle sizes were estimated for 11.9, 7.2, 4.8, and 2.4 mg/mL TA. microcariers of 4-9.5 µm were found for 9.6 mg/mL TA (Figure 2B). The formation of microcariers in the presence of Quercetin was also given in Figure 2C. Cross-linker, quercetin concentrations for 0.03 and 0.06 mg/mL resulted with the higher AlbMC's diameters. Glutaraldehyde cross-linker leads to the conformation of microcarriers about 4-5.5 µm diameters.

Microcarrier diameters synthesized with the highest GA concentration were bigger than microcarriers synthesized with other GA concentration values. Lower GA amounts have closer particle diameters. The same tandency was observed in case of TA cross-linker. Particle diameters were increased for 9.6 g/L TA and the particle diameter changes were seemed to be narrowed at higher TA concentration of 11.9 g/L. The formations of microcarriers was also carried out by the addition of quercetin and the effect of concentration can be observed. Low quercetin concentrations showed higher differences in particle sizes. The synthetic cross-linker, GAld interacted with albumin protein leaded to the formation of highly uniform microparticle diameter range (Figure 2D).


Microcarrier Diameter Distribution for Gallic Acid
A
G1
G2
G3
G4
G5
Diameter (µm)
Microcarrier Number (piece)


Microcarrier Diameter Distribution for Tannic Acid
B
T1: 11.9 mg/ml
T2: 9.6 mg/ml
T3: 7.2 mg/ml
T4: 4.8 mg/ml
T5: 2.4 mg/ml
T1
T2
T3
T4
T5
Diameter (µm)
Microcarrier Number (piece)

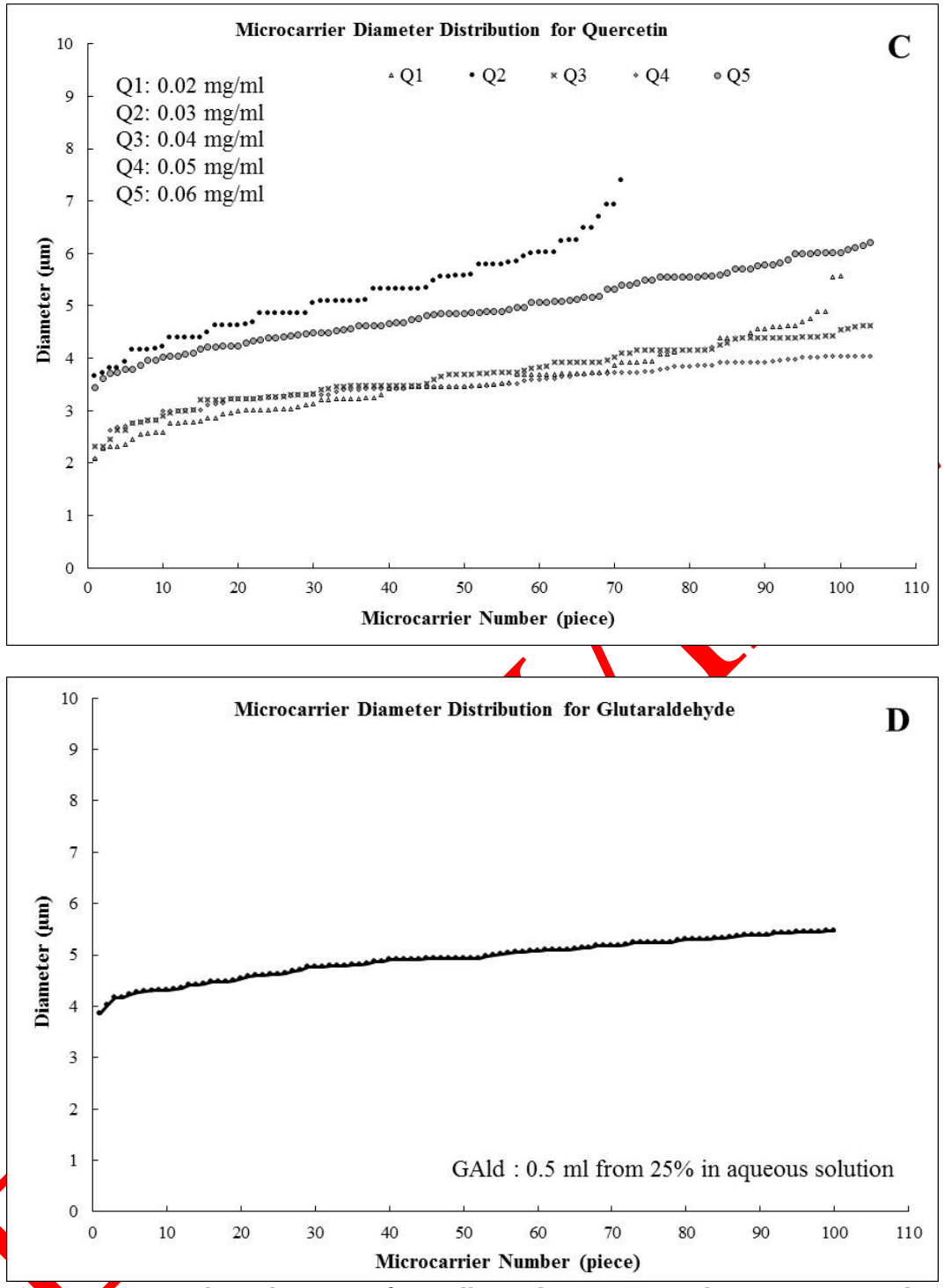


Figure 2. *Measured size diameters of A) Gallic acid, B) Tannic acid, C) Quercetin, and D) Glutaraldehyde stabilized AlbMC's*

The results indicate that cross-linker/stabilizer concentration cause differentiation in particles sizes. So, more thorough interpretation, Gaussian analysis has been done to predict better analysis of microcarrier size distributions and to determine the optimum concentrations of natural cross-linkers. The formed AlbMC's of each phenolic compound concentration was analyzed by MATLAB program and Gaussian distribution of each concentration value was plotted. The mean sizes of the microcarriers were given in Table 2.

Microcarrier mean particle size diameter was calculated as 9.28 ± 2.04 µm when GA concentration was 11.9 g/L (69.95 mM) and mean particle diameter ranged between 4.93 – 15.44 µm. GA concentration of

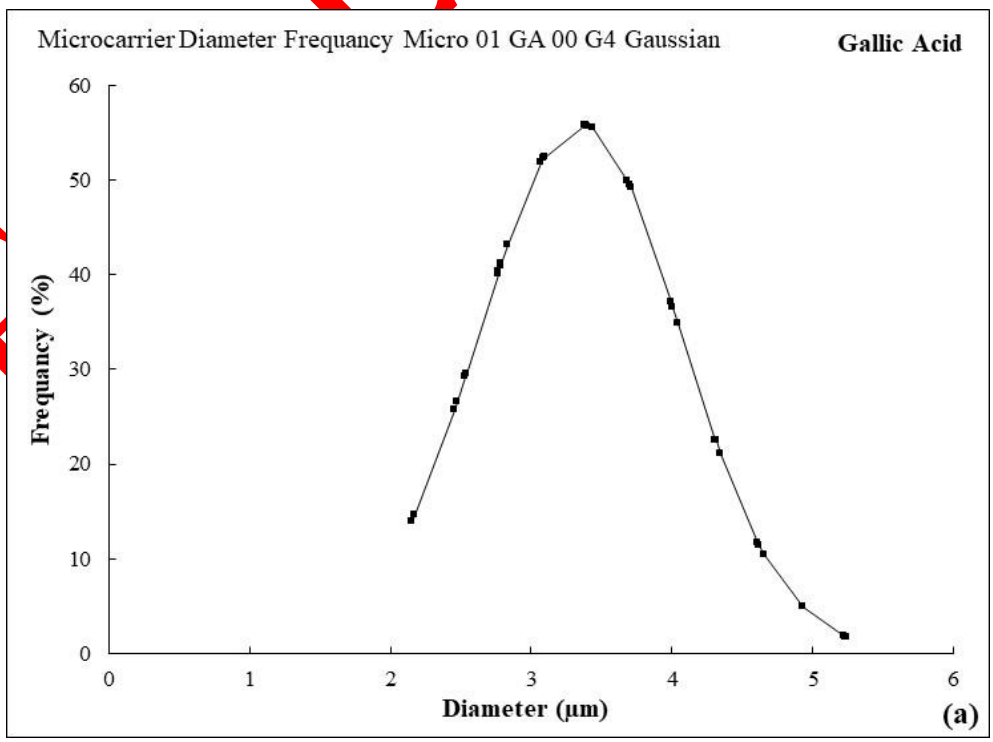
9.6 g/L (56.43 mM) resulted with 1.54 – 7.07 µm mean particle range and 3.62 ± 0.99 µm average mean particle size. Other GA concentration, 7.2 g/L (42.32 mM) leaded to 4.95 ± 1.27 µm average mean particle size with 2.77 µm minimum and 8.66 µm maximum mean diameters. Mean particle size range from 2.16 µm to 5.25 µm and average particle size diameter of 3.35 ± 0.71µm was found for GA concentration of 4.8 g/L (28.22 mM). The minimum GA concentration was 2.4 g/L (14.11 mM) and diameter of calculated average particle size was calculated as 4.57 ± 1.3 µm of 2.17-10.15 µm mean particle range. The results indicate that minimum size diameter range was obtained for 4.8 g/L (28.22 mM) GA concentration. Other phenolic compound, TA was used to form AlbMC's for each concentration. Particle sizes were investigated like GA size distributions and 3.71 ± 0.69 µm average particle size was calculated again for concentration of 4.8 g/L. (2.822 mM) Minimum mean particle size was 2.31 µm and maximum mean particle size of 5.55 µm was estimated. The reported results of TA have the minimum size distribution. GA and TA concentrations of 4.8 g/L or 28.22 mM GA and 2.822 mM TA concentrations can be selected as optimum values of phenolic acid and hydrolysable tannin compounds, respectively. Quercetin is the third phenolic compound with the lowest water solubility. The minimum particle size distribution was obtained in case of 0.2 g/L (0.066 mM) Quercetin concentration and average mean particle diameter of 3.56 ± 0.71 µm was calculated after Gaussian function analysis of the particles. Again, Quercetin concentration of 0.2 g/L (0.066 mM) can be selected as the optimum concentration value when this flavonoid structure will be used.

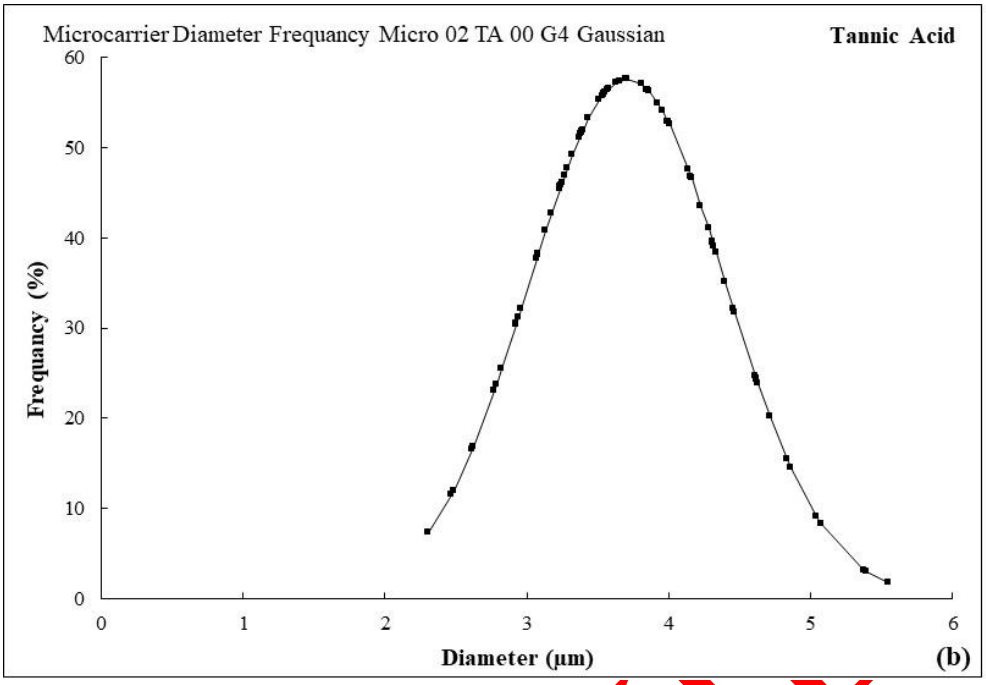
Table 2. *Maximum mean particle diameters calculated after Gaussian analysis of synthesized AlbMC's cross-linked with Gallic acid, Tannic acid, and Quercetin*

AlbMC's cross-linked with	*C-1	*C-2	*C-3	*C-4	*C-5
Gallic acid	9.28 ± 2.04 µm	3.62 ± 0.99 µm	4.95 ± 1.27 µm	3.35 ± 0.71 µm	4.57 ± 1.30 µm
Tannic acid	4.16 ± 0.73 µm	6.01 ± 1.25 µm	3.10 ± 0.63 µm	3.71 ± 0.69 µm	4.31 ± 0.98 µm
Quarsetin	5.45 ± 1.14 µm	3.72 ± 0.48 µm	4.06 ± 0.73 µm	5.20 ± 0.83 µm	3.56 ± 0.71 µm

*C: cross-linker concentration; values represent the means of three independent replicates ± standard deviations, (p<0.05)

Synthetic cross-linker glutaraldehyde stabilized AlbMC's average mean particle diameter was estimated as 5.12 ± 0.50 µm of 3.86 µm minimum and 6.13 µm maximum mean particle sizes. Gaussian distributions of particles for phenolic compounds GA, TA, Quercetin, and synthetic cross-linker glutaraldehyde were given in Figures 3 and 4, respectively.





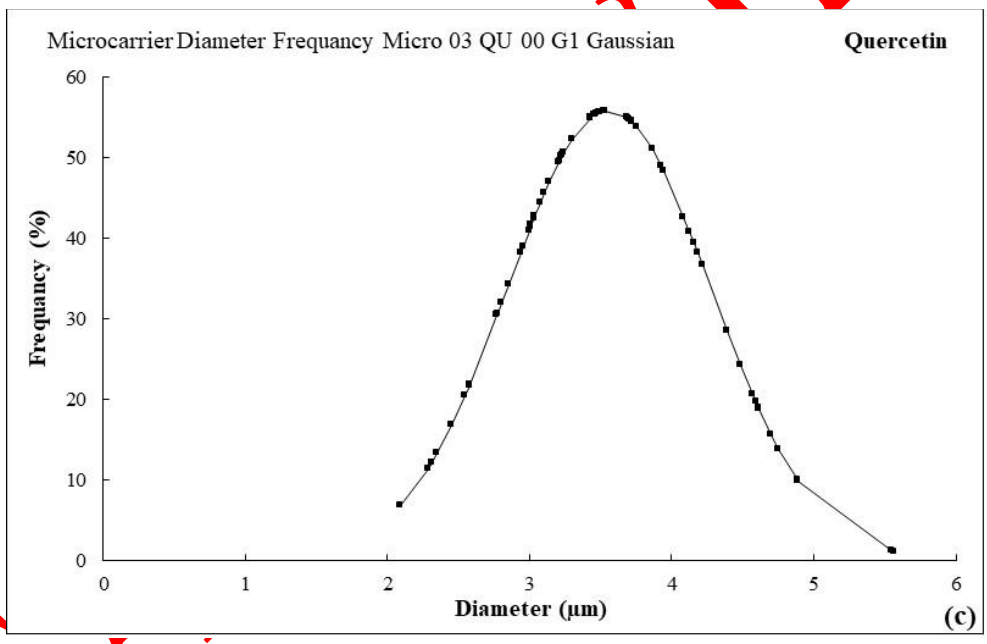


Figure 3. *Gaussian-like size diameters of AlbMC's: a) cross-linked white GA average particle diameter <d> =3.35 ± 0.71 µm, b) cross-linked white TA stabilized average particle diameter <d> =3.71 ± 0.69 µm, c) cross-linked white Quercetin stabilized average particle diameter <d> =3.56 ± 0.71 µm*

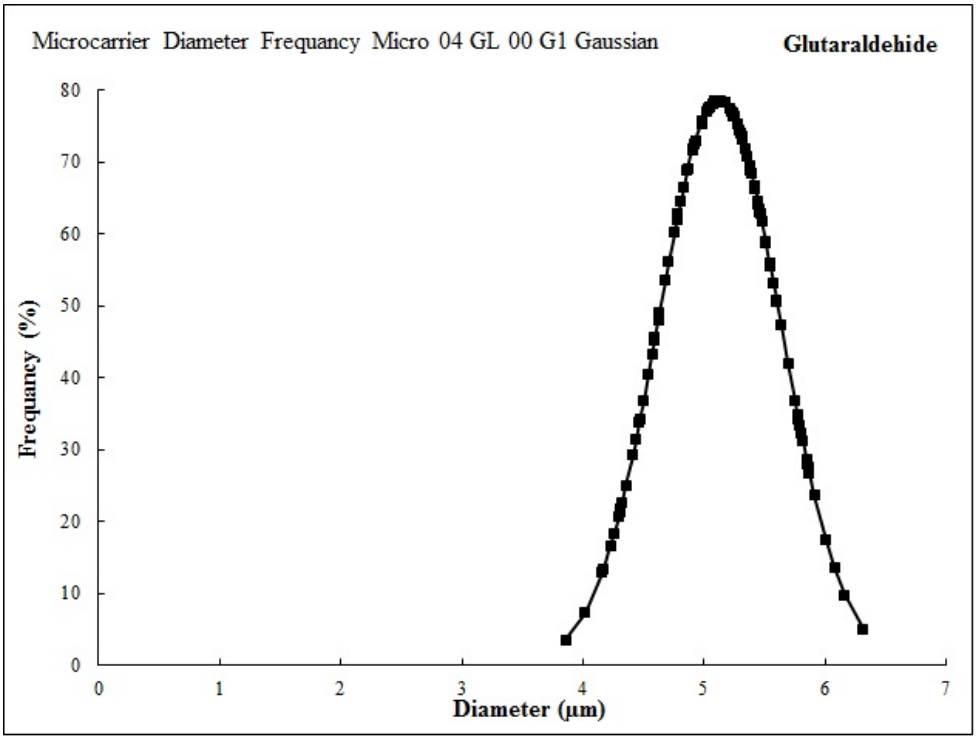
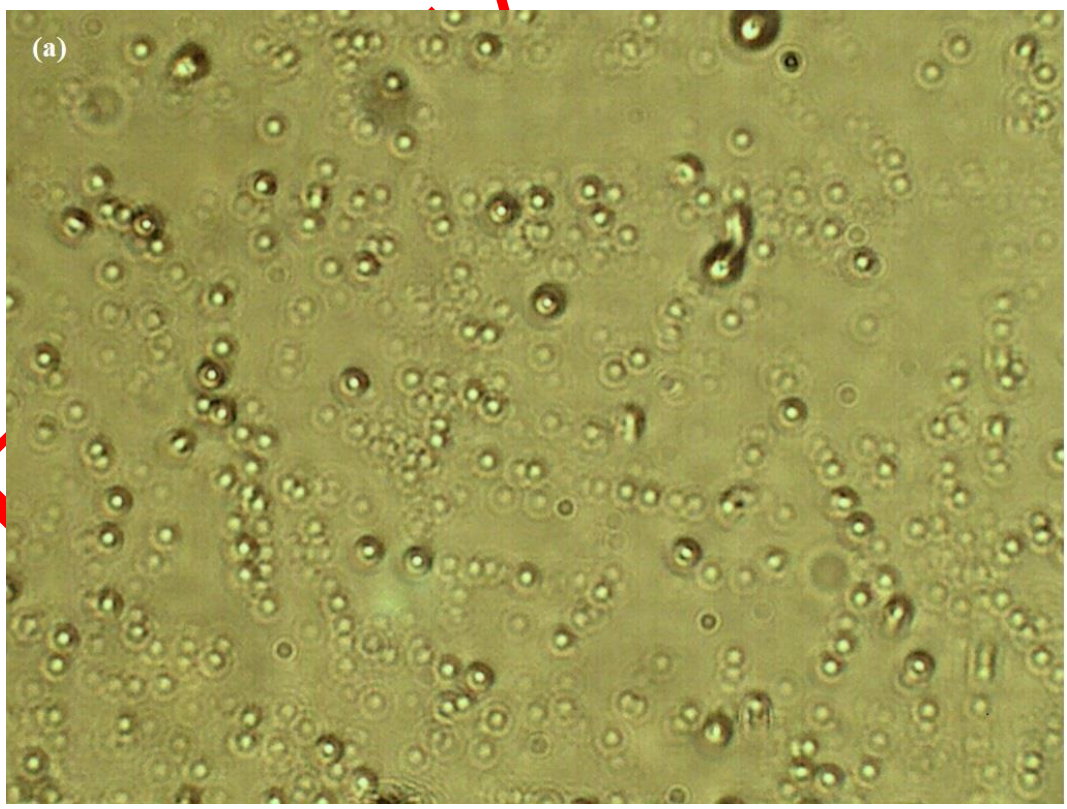
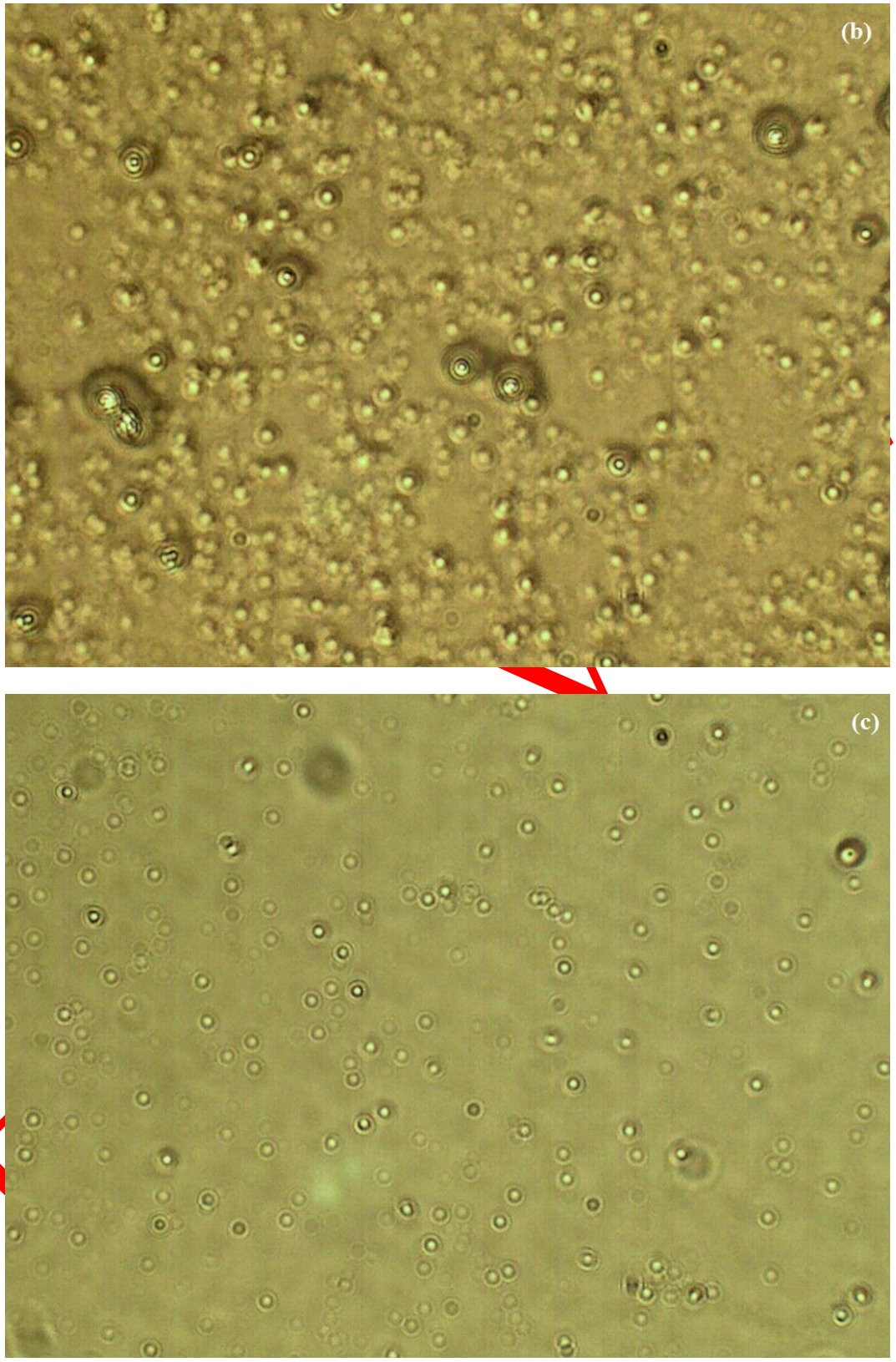


Figure 4. *Gaussian-like particle size distribution of glutaraldehyde cross-linked AlbMC's (average particle diameter, <d> =5.12 ± 0.50 µm)*

Optical micrographs obtained inverted microscope of microcarriers were shown in Figure 5.




(b)
(c)

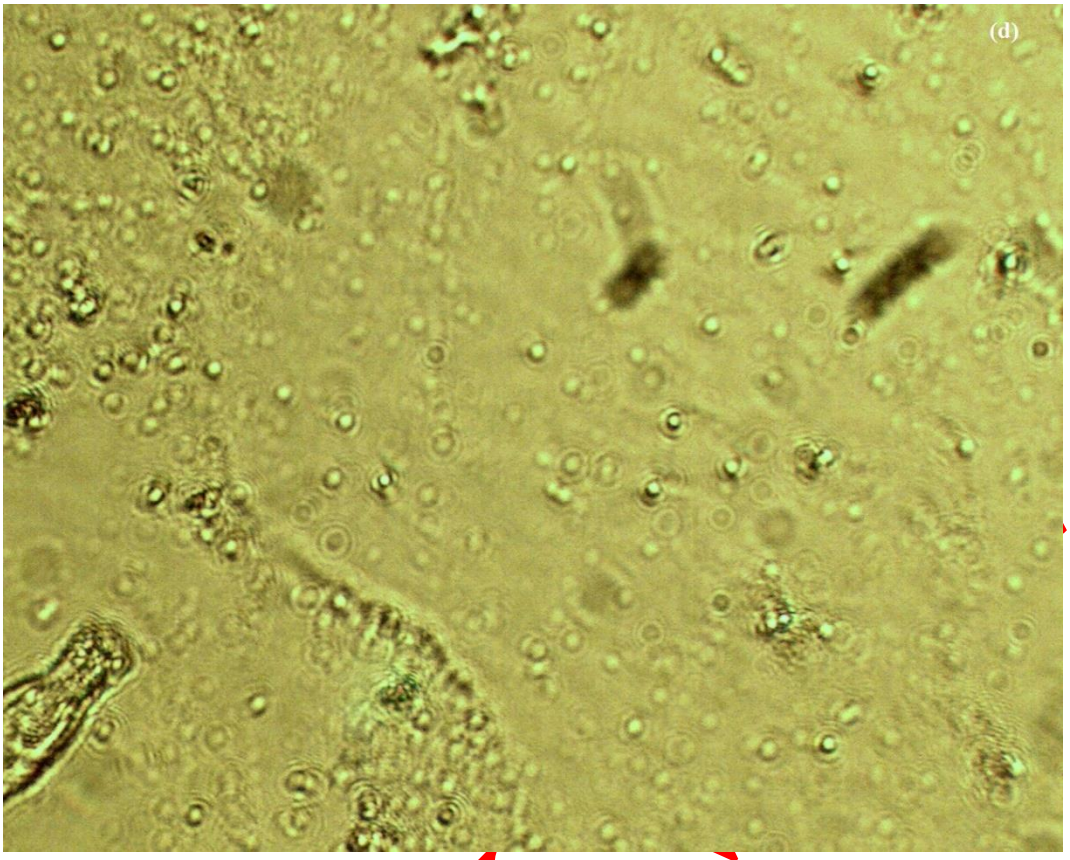


Figure 5. *Optical micrographs of AlbMC's stabilized whit; a) Glutaraldehyde; b) Gallic Acid, c) Tannic Acid; d) Quercetin*

Gelatin hydrogel was cross-linked with different concentrations genipin and polyphenols of a plant extract In order to decrease the poor mechanical properties. The gel strengths of hydrogels were tested and the highest gel strength obtained with polyphenols up to 360 µg polyphenols/g gelatin. On the other hand, the highest strength of samples cross-linked with genipin was 98.4 N/mm^2 with 300 mg genipin/g dry gelatin. The results indicate that hydrogen bonds formed by the cross-linking agent lead to increase in gel strength, but the mechanical properties of the gel will decrease when the concentration of the polyphenols exceeds a critical value which may result from precipitation [27]. The micrographs of AlbMC's having the minimum Gaussian size distributions confirm the stable microstructures. Molecular dynamic simulation and spectral techniques were used to investigate the interaction between gallic acid and lysozyme. It was found that the formation of a static complex and hydrogen bonding and hydrophobic interactions are the main driving forces obtained from the results of spectroscopy [37]. It can be said that the formation and size of microcarriers were affected by the molecular weight of phenolic compound: 3.35 µm (GA) < 3.56 µm (Quercetin) < 3.71 µm (TA). These effects are in agreement with a study reported previously for similar phenolic structures. Interaction of polyphenols like tannic acid, ellagic acid and gallic acid and BSA depends on molecular weights of phenolic compounds. Therefore, higher molecular weight of phenolic compound can be easily interact with protein molecule : tannic acid > ellagic acid > gallic acid. [38]. It was also reported that tannin-protein chemistry besides concentration, protein isoelectric point, pH, and ionic strenght of the solution is a factor in the formation of protein-tannin complex [20]. The concentrations of GA were selected between 69.95-14.11 mM, while ten times fewer than GA concentrations were applied for TA (6.995-1.411 mM. The least amounts of Quercetin, 0.199-0.066 mM, were used to form cross-linked albumin structures. In the literature, the phenolic compound concentrations was kept lower than 70 mM for natural cross-linkers of pentagalloyl glucose, ellagic acid and gallic acid for the investigation of interactions with bovine serum albumin. The analysis of the interactions of gallotannin (1,2,3,4,6-penta-**O**-galloyl-**β**-D-glucopyranose), and two simple phenolic compounds gallic acid and ellagic acid were performed by Circular Dichroism (CD) measurements. The structural changes at low concentrations (50 µM) cause slight changes in the secondary structure of the protein [39].

4. RESULTS

Microparticles based on the biodegradable polymer, bovine serum albumin (BSA), have been extensively investigated because of their excellent biocompatibility and biodegradability. In recent years, a continued interest in BSA microparticles has been carried out for the other types of crosslinking agents due cytotoxicity effects of synthetic compounds. We carried out experiment on protein microcarrier formation in the presence of three different phenolic compounds. It was found that phenolic compounds/cross-linkers interacted with protein structure depends on their molecular weights and concentrations. The molecular weights of natural compounds and concentrations of hydroxyl groups on phenolic structures dominates the formation of chemical interactions, which might also come from conformational changes of proteins. Particle size distributions, statistically analysed by Gaussion function gave important insignts on microstructure formation and cross-linker/stabilizer effect. Finally, the results of microcarrier formations and particle size distributions showed that natural phenolic compounds can be prefered as cross-linker or stabilizer, which can provide additional beneficial properties of natural phenolic compounds.

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CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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