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In vitro stabilization and *in vivo* improvement of ocular pharmacokinetics of the multi-therapeutic agent baicalin: Delineating the most suitable vesicular systems

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ABSTRACT

Baicalin is a multi-purpose flavonoid used in the treatment of different ocular diseases. Owing to its poor stability in basic pH and its poor solubility, a suitable carrier system is needed to enhance its ocular therapeutic potential. Therefore, the objective of this work was to prepare and contrast different baicalin vesicular systems; namely liposomes, penetration enhancer vesicles PEVs and transfersomes. Results revealed that baicalin vesicles exhibited suitable particle size and zeta potential, high entrapment efficiency and controlled release. Depending on the vesicular composition, selected formulations were able to resist physical changes of particle size, zeta potential, entrapment efficiency and *in vitro* release after storage for 3 months, while retarding the degradation of baicalin. Selected vesicular formulations displayed equivalent or superior antioxidant potential compared to baicalin solution, with absolute superiority over ascorbic acid reference, while demonstrating sterilization endurance and safety on ocular tissues. Pharmacokinetic studies revealed that transfersomes displayed the fastest onset of action, while liposomes displayed the highest extent of absorption as concluded from the $T_{max,}$ C_{max}, and $AUC_{0-\infty}$ values with 4-5 folds increase in bioavailability compared to baicalin control solution. This delineates baicalin vesicular systems as a promising platform for treatment of ocular diseases such as inflammation, cataract and diabetic retinopathy.

Keywords: Baicalin, vesicles, stability, penetration enhancer, labrasol, pharmacokinetic studies.

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1. Introduction

Nutraceuticals are defined as compounds which are derived from natural sources, with proven beneficial effects in the prevention and treatment of diseases (**Hang et al., 2016**). When used *via* the ocular route, nutraceuticals were shown to be ~~of great value in~~ important for the treatment of eye-related diseases such as inflammation, dry eye, cataract and glaucoma (**Xiao et al., 2014; Abdelkader et al., 2015; Oh et al., 2015; Natesan et al., 2017**). However, despite the multi-therapeutic traits of nutraceuticals, their undesirable physicochemical properties ~~for ocular delivery~~ halter the exhibition of their full ocular biological activities (**Sahni, 2012**). Furthermore, topically applied ophthalmic therapeutic agents generally suffer very poor absorption in the eye due to the presence of various ~~eye~~ protective mechanisms such as blinking and lacrimation, in addition to the complex anatomy and physiology of the eye, which necessitates the design of new delivery carriers **(Sahoo et al., 2008; Araújo et al., 2009)**.

Drug carriers of optimum lipophilicity such as vesicular systems were shown to optimize drug permeation through the non-polar pathways of the eye, improve the ocular contact time, provide sustained release of drugs, reduce the required drug dose, allow good patient compliance, decrease adverse effects, in addition to being biocompatible with eye tissues **(Biju et al., 2006; Sultana et al., 2006)**. They were also shown to enhance the therapeutic index, stability and solubility of drugs **(Kamboj et al., 2013; Bseiso et al., 2015, 2016; Abdelgawad et al., 2017; Fadel et al., 2017)**. Of equal importance to the choice of delivery carrier is the smart choice of ocular vesicular additives ~~is another effective approach~~ to enhance the trans-corneal passage of drugs. Such additives include penetration enhancers such as labrasol **(Liu et al., 2009)** and bile salts such as sodium taurocholate and sodium glycocholate **(Dai et al., 2013)**.

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Baicalin; (7-glucuronic acid 5, 6 dihydroxyflavone) is a bioactive flavonoid extracted from the dried roots of Chinese herb *Scutellaria baicalensis Georgi*, with a large molecular weight of 446 g/mol, poor solubility, and instability in basic pH (**Liang et al., 2009; Wu et al., 2011**). Baicalin was known to exhibit various pharmacological activities in the eye, including anti-inflammatory, antioxidant and anti-angiogenic~~esis~~ effects. As a result, this flavonoid can prevent and treat ~~reduce the risk of~~ ocular diseases, including age-related macular degeneration (AMD) and diabetic retinopathy (DR) **(Spector and Garner, 1981; Kowluru, 2001; Wu et al., 2011; Xiao et al., 2014)**. Owing to its powerful antioxidant and anti-apoptotic properties, it was also shown to protect the retina against ischemia and oxidative stress **(Jung et al., 2008)**. Moreover, it was shown to inhibit aldose reductase (AR) enzyme activity in rat lens, which consequently reduces the risk of cataract formation **(Park et al., 2007)**. Baicalin also exerts anti-inflammatory effects in uveitis models induced by lipopolysaccharides (LPS) and prostaglandin E2 (PGE2) in rabbits **(Nagaki et al., 2001; Nagaki et al., 2003)**.

All of the previous studies strongly nominate baicalin as a promising nutraceutical for treatment of ocular diseases. However, since baicalin is unstable in basic solutions~~,~~ and ~~would~~ poorly permeates on its own into the eye tissues, it was essential to encapsulate it within a suitable delivery carrier such as vesicular systems. Therefore, the aim of this work was to select the most suitable vesicular composition for baicalin among liposomes, penetration enhancer vesicles PEVs, and transfersomes, ~~which would~~ to provide optimum stabilization and enhanced ocular pharmacokinetics. The vesicular systems were characterized for their particle size, zeta potential, entrapment efficiency, *in vitro* release, physical stability, morphology and antioxidant potential. Selected formulations were examined for their ocular safety and pharmacokinetic efficacy compared to baicalin conventional solution.

2. Materials and Methods

2.1. Materials

Soybean Phosphatidylcholine (Epikuron 200) was kindly provided by Cargill Texturizing solutions (Deutschland GmbH & Co. Hamburg, Germany). Labrasol® was kindly provided by Gattefosse' Co., France. Baicalin was purchased from Skin actives company (Gilbert, Arizona, United States). Sorbitol, cholesterol, sodium glycocholate, sodium taurocholate, span 60, phosphate citrate buffer tablets (pH 5.5), dialysis tubing cellulose membrane avg. flat width 33mm (1.3 in), ascorbic acid, formaldehyde and 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) were purchased from Sigma Chemical Co. (St. Louis, USA). Disodium hydrogen phosphate, potassium dihydrogen phosphate, methanol, sodium chloride and chloroform were purchased from El-Nasr Pharmaceutical Co. (Cairo, Egypt). Methanol, water, acetonitrile and glacial acetic acid ~~of~~ (HPLC grade) were purchased from Fisher Scientific Co., UK. Ortho-Phosphoric acid (analytical grade) was purchased from Honeywell Burdick & Jackson®, Germany. Ketamine hydrochloride 50 mg/ml was purchased from T3A pharma, (Giza, Egypt). Xylazine 20 mg/ml was purchased from Adwia pharmaceuticals Co., (Cairo, Egypt). Uranyl acetate was purchased from Allied signal, ~~(~~Germany~~)~~.

2.2. Preparation of different baicalin vesicular systems

Baicalin vesicles of different compositions were prepared using the thin film hydration technique **(Nasr et al., 2008a, Bsieso et al., 2015)**, as shown in **Table 1**. One hundred milligrams of the drug (1% w/v) in addition to different amounts of soybean phosphatidylcholine (PC) (Epikuron 200), penetration enhancer (labrasol), steroidal lipid (cholesterol), bile salts (sodium taurocholate and sodium glychocolate), or surfactants (span 60) were dissolved in 20 ml

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methanol. The organic solvent was evaporated by rotary evaporator (model IKA RV 10 basic, Germany) under reduced pressure at 40°C for PC based vesicles and 60°C for span 60 based vesicles (**Nasr et al., 2008a; El Zaafarany et al., 2010**), and rotated at 150 rpm. The dry lipid film was hydrated with 10 ml of phosphate-citrate buffer (pH 5.5) through portion-wise addition. The vesicular dispersions were mechanically rotated for 45 minutes after hydration, and kept in the refrigerator in the liquid form.

For comparative purposes, provesicles (with the same composition) (**Table 1**) were also prepared by the previously described method, with the difference that two grams of the carrier material sorbitol was included in the preparation, to obtain the vesicles in solid form **(Ahn et al., 1995, Aburahma and Abdelbary, 2012)**. Two grams of sorbitol were placed in the flask together with 100 mg of baicalin dissolved in 20 ml methanol in addition to different amounts of PC, labrasol, cholesterol, bile salts (sodium taurocholate and sodium glychocolate) and span 60 (Table 1). The organic solvent was then evaporated by rotary evaporator under reduced pressure at 40°C for PC based vesicles and at 60°C for span 60 based vesicles. The flask was then rotated at 150 rpm for 1 hour for complete drying. The dry powder was stored in the refrigerator as such, to be reconstituted when needed using phosphate-citrate buffer of pH 5.5.

2.3. Characterization of the prepared vesicular systems

2.3.1. Determination of the pH of the vesicular formulations

The pH values of the prepared baicalin formulations were determined using pH meter (pHs-3BW microprocessor pH/Mv/Temperature meter, Shanghai, China). The pH meter was first calibrated using pH 7 and pH 4 buffer solutions. For the pro-formulae, reconstitution was first done with phosphate citrate buffer pH 5.5, and then their pH values were measured.

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2.3.2. Determination of the particle size, polydispersity index and zeta potential

The freshly prepared liquid preparations and reconstituted dispersions of baicalin provesicles were analyzed for their particle size, polydispersity index and zeta potential using zetasizer (NanoZS 3600, Malvern Instruments, Worcestershire, UK), after appropriate dilution **(Bsieso et al., 2015; Fadel et al., 2017; Arafa and Ayoub, 2017; Barakat et al., 2017)**.

2.3.3. Determination of baicalin entrapment efficiency EE% in vesicular systems

The EE% of baicalin was assayed indirectly by measuring the concentration of free drug in the vesicular dispersion. The non-encapsulated "free" baicalin was separated by centrifugation (Power Spin LX centrifuge, UNICO, USA) at 4000 revolutions per minute ~~rpm at~~ and 25 °C for 60 minutes using viva spin tubes (PES membrane, MWCO 300 kDa, Sartorius, Stedium, Nieuwegein, Netherlands) **(Bal et al., 2010)**. EE% of the provesicles was determined after reconstitution of the powder with phosphate-citrate buffer pH 5.5. An aliquot of the filtrate was taken and diluted with methanol, and the amount of free drug was determined by HPLC method using C18 ODS column (Hypersil 250mm x 4.6 mm; 5um, Thermo scientific) and a mobile phase composed of methanol: water containing 0.2% acetic acid at a ratio of 94:6 (v/v), at a detection wavelength 279 nm **(Ashraf et al., 2016)**. The EE% was calculated according to the following equation **(Youshia et al., 2012)**:

$$EE\% = \frac{Wt - Wf}{Wt} \times 100 \qquad \text{(eq. 1)}$$

Where Wt is the total amount of drug and Wf is the amount of free drug analyzed in the filtrate.

2.3.4. *In vitro* drug release

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The *in vitro* release of baicalin from the vesicular systems was studied using dialysis tubing and a modified USP dissolution technique (Hanson, USA) **(Mouez et al., 2016)**. The dissolution medium used was 200 ml of freshly prepared phosphate buffer solution of pH 6.5 **(Liu et al., 2011)**, ~~chosen~~ to provide sink condition for the drug and avoid any significant instability during the release experiment resulting from its hydrolysis to the aglycone form catalyzed by pH above 6.6 **(Wu et al., 2011)**. The dialysis tubing previously soaked in the dissolution medium was fixed to one end of a special glass cylinder (open at both sides, and of diameter 2.5 cm), and allowed to touch the surface of the dissolution medium, while the other end was ~~The glass cylinders were~~ fixed ~~from one end~~ to the metallic shaft of the apparatus rotating at 75 rpm ~~while the other end was allowed to touch the surface of the dissolution medium~~ **(Nasr et al., 2008b)**.

Accurately measured volumes of the formulations (taken directly from the liquid vesicular formulations and after reconstitution of the pro-vesicles) equivalent to 10 mg baicalin were diluted with 1 ml buffer and transferred to the cylinders, which were allowed to touch the surface of the dissolution media maintained at 35 °C ± 0.5 **(Galassi et al., 2007)**. An aliquot of the dissolution medium was taken at different time intervals (0.25, 0.5, 1, 1.5, 2, 3, 4, 6 and 8 hours) with compensation of the withdrawn volume using buffer, and assayed using the HPLC method described in the section 2.3.3.

2.3.5. Stability studies

All baicalin liquid formulations ~~(~~and pro-vesicles in powdered form~~)~~ were stored in well closed vials in the refrigerator at 4±1°C for 90 days **(Bsieso et al., 2015; Bseiso et al., 2016)**. The pro-vesicles were reconstituted with phosphate citrate buffer pH 5.5 at the time of assessment. Re-evaluation of the samples was made regarding pH, particle size, zeta potential

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and EE%. Furthermore, selected formulations were tested for the change in the *in vitro* release pattern after three months storage as well. Finally, comparison of the chromatograms of stored baicalin vesicles with freshly prepared samples was performed to check the presence of baicalin degradation peaks.

2.3.6. Transmission electron microscopy (TEM) examination

Selected vesicular formulations were examined by TEM (model JEM 1010, Joel, Tokyo, Japan), to visualize the shape of the resulting vesicles, after negative staining using 1% uranyl acetate (**Mehanna et al., 2009; Ruozi et al., 2011; Said et al., 2017**).

2.3.7. DPPH free radical scavenging assay

The antioxidant effect of the selected baicalin formulations was determined by the DPPH assay. The assay is based on the fact that DPPH accepts hydrogen atoms from an antioxidant; with the antioxidant activity being proportional to the change of DPPH color (from purple to yellow) in test samples as monitored with UV spectrophotometer (Milton Roy, Spectronic 1201, United States). DPPH exhibits an obvious absorption maximum at 515 nm giving a characteristic purple color (**Woźniak et al., 2004; Hwang et al., 2005; MacDonald-Wicks et al., 2006; Moon and Shibamoto, 2009**).

Freshly prepared (0.004% w/v) methanolic solution of ~~2,2- diphenyl-1-picrylhydrazyl radical~~ DPPH was prepared and stored in the dark at 10 °C. Appropriate dilutions of baicalin solution and baicalin vesicular formulations were prepared to yield the following concentrations (32, 16, 8, 4, 2, 1 ug/ml). Ascorbic acid solution was used as reference standard antioxidant. An aliquot of five microliters of each solution was added to 200 ul of DPPH solution in 96 well plates, and absorbance values were recorded immediately to monitor the reduction in absorbance

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at 515 nm. Assessment was done for pro-vesicles after reconstitution. The IC_{50} values were computed and the calculation of percentage inhibition of DPPH radical (PI) was done according to the following equation **(Yen and Duh, 1994)**:

$$PI= \frac{AC-AT}{AC} \times 100 \qquad \text{(eq. 2)}$$

Where AC is the control absorbance and AT= sample absorbance

2.3.8. Studying the effect of sterilization by gamma irradiation

The selected baicalin formulations were sterilized prior to *in vivo* experiments by receiving a dose of gamma irradiation of 5 KGy (cobalt-60 gamma chamber 4000-A, Bhabba Atomic Research Center Bombay, India) **(Youshia et al., 2012)**. The particle size and EE% of the formulations were re-measured after sterilization by gamma irradiation as previously mentioned in sections 2.3.2 and 2.3.3 respectively.

2.3.9. Measurement of the osmolality of the vesicular formulations

The osmolality values for the selected formulations were measured using semi-micro osmometer K-7400, Knauer, Netherlands).

2.4. *In vivo* evaluation of the selected baicalin vesicular formulations

White albino rabbits weighing 2.0-2.5 Kg were involved in the *in vivo* studies. All animal procedures were approved by the Research Ethics Committee of the Faculty of Pharmacy, Ain Shams University, ~~with~~ acceptance number (REC-ASU 25).

2.4.1. Ocular irritation studies

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Ocular irritation studies were conducted according to the Draize technique **(Draize et al., 1944)**. Draize test takes into consideration the changes in the eye's anterior segment, such as the density and area of corneal opacification, severity of iritis and redness of conjunctiva. A high score indicates that the compound ~~will be irritating~~ is irritant to human eyes **(Wilhelmus, 2001)**.

Albino rabbits were divided into four groups, of three rabbits each. The selected sterile formulations (V1, V9 and V15) or the baicalin control solution (in phosphate buffer pH 5.8) **(Wu et al., 2011)** were instilled in the right eye, with the left eye serving as normal control. A volume of 0.1 mL was instilled every 4 hours, four times daily for a period of one week **(Liu et al., 2011)**. The corneal condition was monitored after the following inspection time intervals from the initial dose: 4 h, 12 h, 24 h, 48 h, 72 h and after one week. The conjunctival congestion, swelling and discharge were evaluated, and given scores ranging from 0 to 3, 0 to 4 and 0 to 3, respectively. Iris hyperaemia was graded from 0 to 4. The ~~assessment criteria applied according to Draize technique~~ administered formulation was considered to be non-irritant from 0 to 3.9, slightly irritant from 4 to 8.9, moderately irritant from 9 to 12.9 and severely irritant from 13 to 16, upon comparing the mean total score values for the three treated eyes.

2.4.2. Histopathological assessment

All animal groups described in the previous section were sacrificed on day 7 by decapitation. The eyeballs were excised, washed with saline solution and immediately placed in a formalin solution 10% (v/v) for 24 hours, stained with hematoxylin and eosin (H&E) stain and then examined under the light microscope (Axiostar plus, Zeiss, New York, NY) for histopathological abnormalities **(Tayel et al., 2013)**.

2.4.3. *In vivo* pharmacokinetic study in rabbits

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2.4.3.1. Animals

The rabbits were housed in stainless steel cages and kept in light-controlled and air-conditioned chambers. ~~The~~ Feeding on standard laboratory diets and water *ad libitum* was ensured. Before starting the experiments, a medical checkup was done by a veterinarian to check their physical condition and to ensure the lack of obvious clinical abnormalities. The rabbits were kept under anesthesia during the experiment by injecting ketamine hydrochloride along with xylazine **(El-Laithy et al., 2011)**.

2.4.3.2. Sample collection and baicalin calibration curve in presence of aqueous humor

Drug-free aqueous humor was obtained from healthy rabbits using a small needle inserted across the cornea, just above the corneoscleral limbus in the anterior chamber of the eye. Samples were collected in vials, stored in freezer at −20ºC for later analysis and then thawed at room temperature for constructing a calibration curve **(Fresta et al., 1999; Chetoni et al., 2004)**. One hundred and fifty microliters of blank aqueous humor was added to 850 µl of baicalin methanolic solution, providing a final volume of 1 ml with final baicalin concentrations of (0.9, 1.5, 4.5, 6, 12 and 15 ug/ml). The mixture was then vortexed (IKA® VORTEX Genius 3, IKA®-Werke GmbH & Co. KG, Germany) for 1 min and centrifuged (model MC-14, A&E lab Co., UK) at 14,500 rpm for 10 min. The supernatant containing dissolved baicalin was filtered then injected to the HPLC column to be analyzed for construction of the calibration curve.

2.4.3.3. HPLC instrumentation

The quantitative determination of baicalin pharmacokinetic parameters in rabbits' eyes was done by HPLC using Agilent C18 column (250mm x 4.6 mm; 5um) with a mobile phase consisting of methanol: 0.2% phosphoric acid in water at a ratio of 47:53 **(Zhiyan et al., 2010)**, at a flow rate of 1 ml/min at 279 nm.

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2.4.3.4. Instillation of formulations and experimental setup

Forty eight healthy albino white rabbits were divided into four groups, of twelve rabbits per group, and three individual eyes were used for each time interval. The selected sterile vesicular formulations (V1, V9, V15) or the baicalin control solution (dissolved in phosphate buffer pH 5.8) were instilled (100 µl) in the conjunctival sac. Upon administration, the lower eye-lid was slightly pulled downward and away from the eye to allow the formulations or the control solution to ~~gather and~~ accumulate over the surface of the cornea in the lower cul-de-sac, and then the lower lid was lifted again to mix the prepared formulations with the lacrimal fluids of the eye **(Law et al., 2000)**. The rabbits were anaesthetized systemically using ketamine hydrochloride (intramuscular injection, 50 mg/ kg) in addition to xylazine as a muscle relaxant (intramuscular injection, 10 mg/kg) **(El-Laithy et al., 2011)**. The loading of the formulations was carefully achieved by two instillations (of 50 µl each) using a micropipette, while avoiding touching and damaging the eye **(Sun et al., 2007)**. At specific time intervals of 5, 15, 30, 60, 120, 240 and 360 minutes after drug instillation, aqueous humor samples were extracted from the anterior chamber of the eye by a small needle, and then collected and stored at -20ºC for further analysis ~~by~~ using HPLC.

For baicalin quantitative analysis, 150 µL of the collected aqueous humor from each eye was taken and completed to 1 ml by methanol. The samples were then mixed using a vortex for 1 minute and then centrifuged at 14,500 rpm for 10 minutes as previously mentioned in section 2.4.3.2. Twenty microliters of the supernatant were taken and injected into the HPLC and analyzed as described in section 2.4.3.3.

2.4.4. Pharmacokinetic analysis

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Baicalin pharmacokinetic parameters in aqueous humor were determined using Kinetica 5.0 software for PK/PD non-compartmental data analysis. The maximum drug concentration (C_{max}, ug/ml) and the time to achieve this peak (T_{max}, min.) were calculated. The areas under the concentration– time curves (AUC $_{0-\infty}$) were calculated by the linear trapezoidal rule. Results were expressed as mean ± standard deviation of the mean (SD) of three determinations.

2.5. Statistical analysis

All data were expressed as mean ± standard deviation (n=3). One way Anova and unpaired t-test was done using Graphpad® Instat software. A difference between means was reported significant if the P value was less than or equals to 0.05.

3. Results and Discussion

3.1. Preparation of baicalin vesicular systems

As shown in **Table 1**, formulations V1- V20 containing 100 mg baicalin (1% w/v) were prepared. Cholesterol was included in some formulations as a supplementary lipid component, because it was reported to improve the order and rigidity of the lipid bilayer (**Jain et al., 2014**). Appropriate amounts of soybean PC were used to form the lipid bilayer of the formed liposomes at a lipid: drug ratio (1.6:1) to (2:1) (**Ramana et al., 2010**). Regarding the pro-vesicles, a ratio of 1:10 lipid:sorbitol was employed for their preparation, as it was reported to be the most optimum ratio (**Ning et al., 2005**).

For niosomal formulations (V5-V8), span 60 was chosen as the bilayer forming agent. In formulae V9- V12, labrasol was used in the preparation of PEVs of baicalin, since it was reported to enhance the permeation of the drug into the corneal tissues of the eye when used as

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such, while being non-irritant to the eye at 0.5- 3% concentrations (**Liu et al., 2009; Huang et al., 2015**). Bile salts such as sodium taurocholate and sodium glycocholate were used for the preparation of the transfersomal formulations V13-V20. These bile salts were reported to enhance the ocular mucosal membrane permeability by breaking down the eye mucous and disrupting the cell junctions, therefore, they widen the tight junctions between the cells, leading to penetration of drugs through the paracellular and transcellular route (**Mahaling and Katti, 2016**). It was also reported that liposomes containing sodium deoxycholate caused toxicity or irritation to corneal epithelial cells and the cornea of rabbits, while liposomes containing sodium taurocholate and sodium glycocholate exhibited low toxicity and greatly improved the permeability in the eye (**Dai et al., 2013**), therefore, in this work, the later bile salts were used for the preparation of baicalin transfersomes.

Most baicalin vesicles were successfully prepared with no aggregation using the described thin film hydration technique (using methanol alone as a solvent owing to the insolubility of baicalin in chloroform). Only niosomal formulations V5 and V7 and proniosomal formulae V6 and V8 showed aggregates and flocculation immediately upon hydration of the thin film and reconstitution of the vesicles, which may be attributed to the inadequate hydration of the span 60 thin film created solely using methanol ~~by the buffer~~. Hence, these formulations were excluded from further characterization steps. The utilized pH 5.5 was chosen for hydration of the vesicles ~~was chosen for~~ to maintain~~ing~~ the stability of baicalin in the eye **(Xing et al., 2005)**, and it was proven to be non-irritant to the eye as well **(Lockington et al., 2012; Baranowski et al., 2014; Lim et al., 2014**).

3.2. Characterization of the prepared vesicular systems

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3.2.1. Determination of the pH of the vesicular formulations

The pH of the prepared vesicular systems was around the pH 5.5, which was the pH of the buffer used in their preparation. This pH was suitable for maintaining the stability of baicalin in the eye **(Xing et al., 2005),** and was proved to be non-irritant to the eye as well **(Lockington et al., 2012; Baranowski et al., 2014; Lim et al., 2014)**.

3.2.2. Determination of the particle size and polydispersity index

The particle size values of the prepared baicalin vesicular systems ranged from (667-1341 nm) **(Table 2)**, except for formula V4 (proliposomes) and V20 (protransfersomes) which showed larger particle sizes of (2712 nm) and (2104 nm) respectively.

As could be inferred from the results, a general significant increase in particle size of liposomes and PEVs was displayed in the presence of cholesterol ($P<0.05$), which came in accordance with **Lee et al., 2005** and **Gallová et al., 2004** who attributed this to the general increase of lipid bilayer thickness upon addition of cholesterol. As for transfersomes, the change in particle size upon addition of cholesterol in the presence of sodium glycocholate and sodium taurocholate bile salts was non-significant as similarly observed by **Duangjit et al., 2013**, except for formula V20 where a significant and extreme increase in particle size was observed ($P<0.05$).

The preparation of baicalin vesicular systems in their pro-forms led to a general insignificant change in the particle size when compared to their liquid forms ($P>0.05$), suggesting that sorbitol preserved the particle size distribution pattern of vesicles without significant alteration. The change in bile salt type (sodium taurocholate/sodium glycocholate) didn't significantly affect the particle size of the transfersomal vesicles ($P>0.05$), except for

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V18/V20. This finding could be attributed to the similarity in their molecular weights, being 515 and 487 g/mol respectively.

The polydispersity index (PDI) values of all prepared vesicular systems were in the range of (0.47-0.85), except for formulae V3, V4, V12 and V20 which displayed higher polydispersity index values reaching 1 (data not shown), suggesting a non-homogenous and polydisperse population. This wide range of polydispersity is a normal finding for unextruded vesicles (**Zeisig et al., 1996; Nasr et al., 2008a**).

3.2.3. Determination of zeta potential of baicalin vesicles

The prepared formulations were all negatively charged, with charges ranging from (-14.4 to -31.5 mV) **(Table 2)**, which is indicative of suitable stability against vesicle aggregation and fusion. The negative charge of the vesicles could be ascribed to the presence of some fatty acids in the composition of the utilized soybean PC (Epikuron 200) ~~for vesicular preparation~~ (**Pinsolle et al., 2014**). PEVs had similar charge values to that of liposomes owing to the non-ionic nature of labrasol **(Bsieso et al., 2015)**. On the other hand, transfersomes displayed significantly higher negative charge values compared to liposomes and PEVs ($P<0.05$) owing to their content of either sodium taurocholate or sodium glycocholate which are anionic in nature **(D'Alagni et al., 1994; Dai et al., 2013; Mouez et al., 2016)**.

3.2.4. Determination of EE% of baicalin in vesicular systems

The EE% was directly measured for liquid dispersions, and for pro-vesicles after reconstitution **(Table 2)**. The EE% for baicalin in the vesicular systems ranged from (25.96-99%), in which the highest EE% was exhibited with liposomes, while lower EE% was achieved

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with transfersomes and PEVs. The high EE% values encountered with liposomes could be attributed to the lipophilicity of baicalin (log P = 1.27) **(Liang et al., 2009)**, leading to its subtle incorporation within lipid bilayers. Furthermore, the thin film hydration technique produces multilamellar vesicles, which are formed of multiple lamellae capable of incorporating and loading higher amount of baicalin ~~that~~ which has a hydrophobic nature **(Sharma and Sharma, 1997)**. ~~On the other hand~~ Regarding PEVs, labrasol® is known to be an amphipathic compound which was reported to cause destabilization of the phospholipid bilayer. The presence of labrasol has probably increased the solubilized portion of baicalin in the aqueous phase; ~~as a result it~~ causing an overall significant decrease in the EE% of baicalin within the vesicular bilayer ($P<0.05$) compared to liposomes **(Bsieso et al., 2015)**. Regarding the prepared transfersomes, they exhibited an optimum entrapment of baicalin ~~with~~ in the pro-vesicles reaching up to 99% with V18 and V20, but still the overall EE% values of transfersomes were less than those obtained with liposomal formulations. This could be ascribed to the presence of bile salts as edge activators, which were reported to increase the fluidity of the phospholipid bilayer and induce pore formation in the bilayers when they are in the liquid form (**Ahad et al., 2012**).

The presence of cholesterol in baicalin vesicular systems led to an overall increase in the EE% of baicalin, except with labrasol PEVs (V9/V11). The overall enhancement of baicalin EE% upon incorporation of cholesterol (CH) into liposomes and tranfersomes could be attributed to the fact that cholesterol increases the hydrophobicity of the phospholipid bilayer, thus, it increased the entrapment of baicalin within the vesicles **(Nasr et al., 2008a)**. Cholesterol was reported to be easily inserted into the phospholipid bilayer because of its structure and hydrophobic character **(Lee et al., 2005)**. In addition, it is known that cholesterol increases the microviscosity and rigidity of the lipid bilayer, and as a result it enhances its stability **(Moribe et**

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al., 1999, Chen et al., 2016) and decreases its permeability **(Du Plessis et al., 1996)**, leading to higher drug retention **(Joshi and Misra, 2001)**. On the other hand, in case of formulations (V9/V11), the decrease in EE% upon incorporation of cholesterol from 47.53% to 25.96% ($P<0.05$) may suggest some sort of competition between the hydrophobic cholesterol molecules and the amphipathic molecule of labrasol which has a solubilizing capacity for baicalin in the fluid vesicles, leading to an overall decrease in the labrasol content within the bilayers with consequent decrease in baicalin loading within the bilayer.

The provesicular formulations generally increased the EE% of baicalin, which could be attributed to the increased surface area caused by sorbitol leading to the entrapment of more baicalin inside the vesicles.

Regarding transfersomes prepared using sodium taurocholate, they displayed significantly higher EE% for baicalin in the liquid vesicles, which may be attributed to ~~its~~ the slightly lower HLB value of sodium taurocholate compared to sodium glycocholate (22.1 and 23.1) respectively, thus, imparting more hydrophobicity to the bilayer **(Kuneida and Sato, 1992)**. On the other hand, there was a non-significant effect of the bile salt type on the EE% in the pro-formulations ($P>0.05$).

3.2.5. *In vitro* drug release

The cumulative released percentages of baicalin ~~released~~ from different vesicular systems over a period of 8 hours are shown in **(Table 2),** and were found to range from 46.24 to 89.22%. Regarding liposomal and proliposomal formulations, the presence of cholesterol did not significantly affect the cumulative percent released of baicalin. This came in accordance to **Nasr et al., 2013**, who demonstrated that cholesterol presence in liposomes didn't exhibit a certain standard pattern regarding drug release. However, upon addition of cholesterol to labrasol PEVs

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and pro-PEVs formulations with more fluid membranes than those of liposomes, a significant decrease in the percent released of baicalin was observed. This ~~decrease~~ can be ascribed to the ability of cholesterol to modify and adjust membrane fluidity by limiting the movement of the relatively mobile hydrocarbon chains, which results in the reduction of the bilayer permeability and the efflux of the encapsulated drug, with concomitant prolonged drug retention (**Nagarsenker and Londhe, 2003**).

As can also be inferred from the results, the incorporation of cholesterol decreased the release of baicalin from transfersomal formulations, which could be interpreted similarly to PEVs based on the cholesterol stabilization ability on the fluid transfersomal and PEVs membranes. However, the non-significant effect of cholesterol on baicalin release from the pro-transfersomal counterparts might suggest a role for sorbitol in hindering the stabilizing effect of the cholesterol on the fluid transfersomal phospholipid membrane, as sorbitol was reported to have an effect on the phase transition temperature of phospholipids such as diplamitoyl phosphatidylcholine (**Saez et al., 1994**).

The provesicular formulations didn't show a significant difference in the cumulative percent released of baicalin in formulations V1/V2, V13/V14, V15/V16 ($P>0.05$), while it significantly increased the cumulative percent release in the rest ($P<0.05$), which may be due to the increase in surface area caused by sorbitol which consequently increased the release of baicalin from the vesicles. **Ahn et al., 1995** reported an effect of sorbitol on the release of propranolol from proliposomes, however, this effect was non-significant.

Finally, transfersomes and protransfersomes formulated using sodium glycocholate exhibited significantly higher baicalin release compared to those formulated using sodium taurocholate ($P<0.05$), which could be ~~interpreted by~~ attributed to the lower HLB value of the

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latter; imparting more hydrophobicity to the membrane and retarding the release of baicalin. This correlated with the EE% results as well.

3.2.6. Effect of storage on the physical stability of baicalin vesicular systems

3.2.6.1. pH measurement

All vesicular formulations displayed non-significant changes in the pH values ($P>0.05$) upon storage, suggesting their stability.

3.2.6.2. Particle size and zeta potential

All vesicular formulations exhibited a non-significant change in particle size upon storage ($P>0.05$) except for some formulations, with a general non-significant change in zeta potential values (**supplementary material**). In terms of vesicular stability, transfersomes/protransfersomes and PEVs/proPEVs exhibited better storage stability than liposomes. The superiority of transfersomal formulations over other vesicular formulations in terms of physicochemical stability was also reported by **Mouez et al., 2016**.

In contrast to what was stated in the literature, some of the pro-vesicular formulations (V2 and V18) did not preserve the particle size of liposomes, and a significant increase in particle size of the vesicles was evident after three months storage ($P<0.05$).

3.2.6.3. Entrapment efficiency

Upon re-measuring ~~the~~ baicalin EE% after three months storage (**supplementary material**), all liposomal and pro-liposomal formulations showed slightly lower EE% ($P<0.05$), which may be linked to the change ~~of the~~ in particle size of the vesicular population after storage. Inclusion of cholesterol in the lipid bilayers of liposomes didn't significantly affect the change in

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baicalin EE% (P>0.05). The PEVs and pro-PEVs formulations displayed non-significantly different EE% results from the freshly prepared formulations (P>0.05), indicating their higher stability compared to liposomal formulations. Regarding transfersomes/pro-transfersomal formulations, it was evident that a statistically significant decrease in baicalin EE% occurred after three months storage (P<0.05), except for formula V20 in which no change occurred, and (V13, V19) in which a slight increase in EE% occurred. The change in EE% could be ascribed to the polydisperse nature of the prepared vesicles. Similarly to what was observed with particle size, pro-transfersomes didn't necessarily preserve the EE% of baicalin, as encountered with formulations V14 and V18.

Since most vesicular systems displayed similar storage properties regarding particle size and EE%, they further proceeded for re-testing their *in vitro* release properties upon storage, with only exclusion of some formulations. From the liposomal/proliposomal formulations, V1 was selected for further testing as it displayed the best particle size stability among its counterparts in addition to exhibiting the smallest particle size among them. From the PEVs/pro-PEVs formulations, all formulations were further tested for their *in vitro* release except for formula V11 which displayed doubling of its particle size upon storage and the smallest EE% value among its counterparts. ~~hence, it was excluded.~~ From the transfersomes/protransfersomal formulations, only V20 was excluded from the *in vitro* release study as it displayed the largest particle size among its counterparts.

3.2.6.4. *In vitro* release of baicalin

~~It was evident that~~ The release profiles were almost identical (data not shown) and the cumulative percent released of baicalin after 8 hours was non-significantly different for some

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formulations, while it was significantly different for others after storage for three months **(supplementary material)**. ~~(Table 2).~~

3.2.6.5. Monitoring degradation peaks of baicalin

Upon close inspection of the HPLC chromatograms of stored baicalin vesicular systems compared to freshly prepared ones, it was evident that degradation occurred with some formulations (V10,V13,V17,V18), as manifested by the appearance of degradation peaks of baicalin, while the other formulations displayed identical chromatograms to the freshly prepared samples **(Figure 1)**. Degradation of baicalin is pH and temperature-dependent, and was reported to be caused by the presence of 6,7 dihydroxyl groups in its benzene ring, yielding chalcone as the major degradation product in aqueous media **(Xing et al., 2005)**. As demonstrated in the current study, baicalin stability was found to be dependent on vesicular composition as well.

As indicated from the summarization of formulations stability results represented in **Table 3**, formulations V1, V9, V12, V15 and V16 demonstrated sufficient stabilization of baicalin with no degradation peaks upon 3 months storage, with overall desirable properties. Therefore, they were chosen for further morphological examination and testing of their antioxidant potential using the DPPH assay.

3.2.7. Transmission electron microscopy

Electron micrographs of formulations V1, V9, V12, V15 and V16 **(Figure 2)** displayed the shape and the core of well identified vesicles, showing ~~the~~ sealed ~~almost~~ spherical entities, similar to what was encountered with **Jain and Shastri, 2011** and **Bseiso et al., 2015**.

3.2.8. Antioxidant ~~assay~~ DPPH free radical scavenging assay

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As shown in **Figure 3** and **Table 4**, all vesicular formulations showed significant scavenging effect with the IC_{50} values ranging from 4.39 – 8.67 ug/ml. IC_{50} represents the concentration of baicalin causing 50% inhibition of the DPPH radical. Interestingly, the values of IC_{50} were significantly lower than ~~compared to~~ that of the ascorbic acid reference (14.2 ug/ml) (P<0.05), indicating that baicalin was stronger than ascorbic acid in its free radical scavenging activity. The hydroxyl group which is found at position C-6 of baicalin plays a role in its DPPH free radical scavenging activity **(Okawa et al., 2001)**. The antioxidant potential of baicalin is advantageous for cataract therapy, as antioxidants were reported to delay cataract formation and progression by reducing lipid peroxidation of the lens **(Zhao et al., 2000; Pollreisz and Schmidt-Erfurth, 2010; Thiagarajan and Manikandan, 2013)**.

Upon comparing the IC_{50} values of baicalin vesicular formulations to that of baicalin solution, it was evident that all formulations displayed either comparable or significantly lower IC_{50} values than the baicalin control solution (P<0.05) except for formulations V12 and V16, indicating the ability of most formulations to preserve and even potentiate the antioxidant ability of baicalin by encapsulation (**Tan et al., 2014; Nasr, 2016**). Components present in formulations V12 and V16 might have hindered the antioxidant ability, therefore, they were excluded, and formulations V1, V9 and V15 were further examined for their *in vivo* pharmacokinetic behavior. Worthy to note that the PEVs formula V9 and the transfersomal formula V15 displayed the highest antioxidant potential among their other vesicular counterparts, as indicated by their IC_{50} values.

3.2.9. Studying the effect of gamma radiation sterilization on the particle size and entrapment efficiency of selected vesicular formulations

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There was no-significant change in the particle size and EE% (P>0.05) of the formulations upon exposure to gamma radiation (data not shown), suggesting the suitability of this method for sterilization of baicalin vesicular formulations prior to their *in vivo* use.

3.2.10. Measurement of the osmolality of the vesicular formulations

The selected formulations (V1, V9, V15) were assessed for their osmolality, in both the freshly prepared and stored forms. Osmolality values were 320±4, 324±2.1, 313±2.4 mOsmol/kg for formulations V1, V9 and V15 respectively, which didn't significantly differ from the reported range for the lacrimal fluid (302-318 mOsmol/kg) (**Ramos Yacasi et al., 2017**), suggesting that the formulations were non-irritant to the ocular tissues. Upon storage of the formulations, no significant change in the osmolality values was observed (P>0.05), suggesting their stability.

3.3. *In vivo* evaluation of the selected baicalin vesicular formulations

3.3.1. Ocular irritation studies

Draize test results indicated that all baicalin vesicular systems as well as the baicalin control solution were non-irritant and of ~~with~~ good ocular tolerance. No ocular damages, hyperaemia or abnormalities were noted in the cornea, iris, or conjunctiva, which could be attributed to the biocompatible nature of the administered vesicles as well as the anti-inflammatory properties of baicalin **(Hsieh et al., 2007; Hou et al., 2012)**. This proves that the proposed vesicular systems were safe for opthalmic delivery, as also reported by **ElMeshad and Mohsen, 2016**.

3.3.2. Histopathological assessment of treated ocular tissues

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No histopathological changes were visible in the investigated ocular tissues (cornea, iris, retina or sclera) of the selected formulations (V1, V9 and V15) as shown in **(Figure 4)** when compared to the normal control. They showed no negative effects on these tissues such as edema, bleeding or presence of inflammatory cells. This supported the findings of Draize test in delineating the safety of the proposed vesicular systems.

3.3.3. Pharmacokinetic study

The pharmacokinetic parameters of baicalin liposomes (V1), labrasol PEVs (V9) and transfersomes containing sodium taurocholate (V15) were compared to those of baicalin control solution (**Table 5**). The mean aqueous humor drug concentration–time profiles after ocular administration of V1, V9 and V15 as well as baicalin control solution are illustrated in **(Figure 5)**. Regarding the rate of absorption, results revealed that the transfersomes (V15) showed the shortest t_{max} (15 min) similar to baicalin control solution (15 min), followed by the liposomes (30 min) then the penetration enhancer vesicles with labrasol (60 min). This came in accordance with **Dai et al., 2013**, who reported that liposomes containing bile salts were able to achieve a fast penetration across the cornea towards ~~to~~ the stroma owing to the flexibility imparted to the vesicles by the presence of bile salts, and their reported transient opening of the tight junctions. The non-significant difference in t_{max} between vesicular formulations and ocular control solution of the same drug was also encountered with **Sun et al., 2006**. On the other hand, the interesting observation that the liposomal formulation V1 and the PEVs formula V9 exhibited a higher t_{max} compared to the control solution and the transfersomal formulation V15 might suggest some sort of affinity of the aforementioned formulations to the outer surface of the cornea, leading to delayed appearance of the drug in the aqueous humor. The delay in ocular t_{max} of formulations

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compared to control is a common finding, provided that it is a sustained delivery system (**Wu et al., 2011**). Hence, it can be concluded that transfersomes were the fastest to be absorbed among the vesicular formulations.

Concerning the extent of absorption, results revealed the superiority of the baicalin liposomal formulation compared to the ocular baicalin control solution and the other vesicular formulations as revealed by the combined C_{max} and AUC values. Regarding the C_{max} values, they were 4.0736 ± 0.241 µg/ml, 2.313± 0.111 µg/ml for ocular liposomes (V1) and baicalin PEVs (V9) respectively, while it was 1.503± 0.0491 and 2.228 ± 0.128 µg/ml for baicalin transfersomes (V15) and the control solution respectively. On the other hand, the $AUC_{0-\infty}$ values were non-significantly different among vesicular formulations ($P>0.05$), but were significantly higher than the control solution ($P<0.05$), corresponding to a 5.4 folds, 4.4 folds and 4.6 fold increase in bioavailability with V9, V1 and V15 respectively. This could be attributed to their phospholipid content which enhances the permeation of poorly soluble drugs into the corneal surface and improves the residence time **(Patel et al., 2013)**. Upon close inspection of the concentration-time profiles in **Figure 5**, it can be noticed that formulations V15 and V9 increased the residence time of baicalin and delayed its elimination, followed by V1. This was manifested by the longer time it took for baicalin to disappear from the aqueous humor with these formulations respectively. This came in accordance with **Taha et al., 2014**, and could be attributed to enhanced residence of the vesicular formulations within the eye interior, coupled with a sustained release potential from them. Findings of the pharmacokinetic study indicate that vesicular systems were able to enhance the ocular bioavailability of baicalin, with transfersomes being the fastest and liposomes being the most bioavailable.

4. Conclusion

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In this work, different vesicular systems were prepared for baicalin, in order to delineate the most suitable system from the physicochemical and pharmacological point of view. Results revealed that all vesicular categories (liposomes, PEVs, transfersomes) were ~~proven~~ promising in terms of baicalin *in vitro* stabilization, sterilization endurance, safety, and pharmacokinetic superiority. This opens the opportunity for delivery of other drugs *via* the proposed systems with application in several ocular diseases. Futuristic studies will involve detailed elucidation of degradation mechanisms of baicalin using LC-MS, as well as conduction of pharmacodynamic studies on animal models of cataract and diabetic neuropathy.

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Declaration of interest

The authors report no conflicts of interest

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Figure legends

Figure (1): (a) Typical HPLC chromatogram of non-degraded formulations after storage for 3 months (b) HPLC chromatogram after storage for 3 months showing degradation peaks.

Figure (2): Negative stain electron micrographs of baicalin vesicles (a) liposomal formula V1 at 50000X (b) PEVs formula V9 at 8000X (c) pro PEVs formula V12 after reconstitution at 12000X (d) transfersomal formula V15 at 12000X (e) pro transfersomal formula V16 after reconstitution at 12000X.

Figure (3): DPPH free radical scavenging effects of baicalin vesicular formulations (V1, V9, V12, V15, V16) compared to baicalin solution control and ascorbic acid reference.

Figure (4): Micrographs of rabbit ocular tissues [cornea, iris, retina, sclera and choroid of the eye] (a) normal control, (b) treated with baicalin liposomes formula (V1), (c) treated with baicalin PEVs formula (V9), (d) treated with baicalin transfersomes formula (V15), respectively.

Figure (5): Mean baicalin aqueous humor concentration-time profiles in aqueous humor following administration of formulae V1, V9, V15 as well as baicalin control solution in rabbits' eyes.

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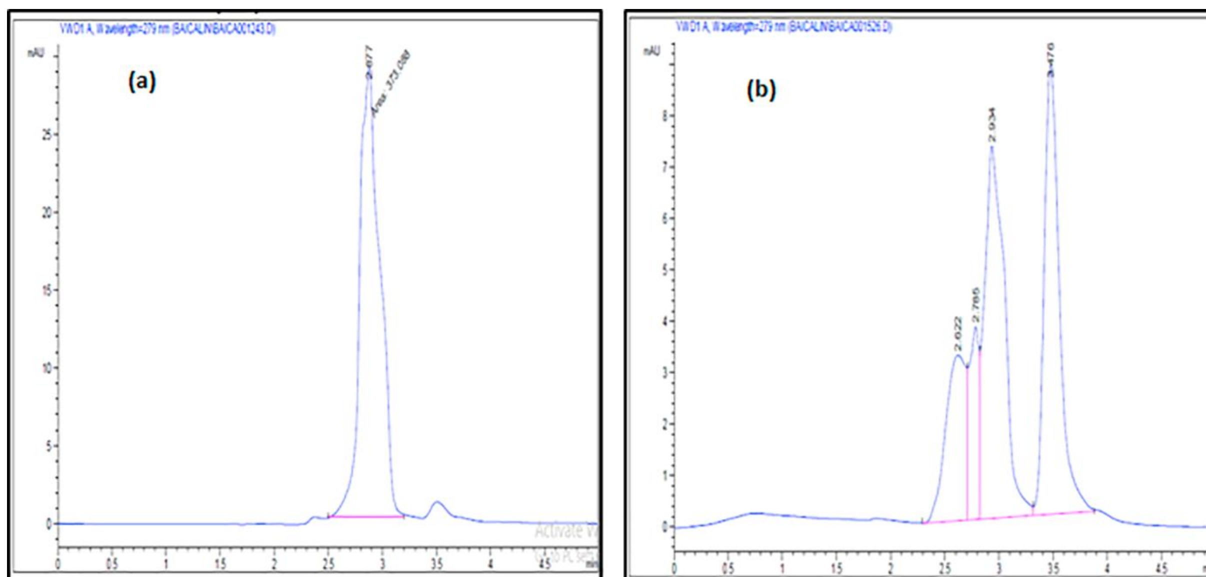

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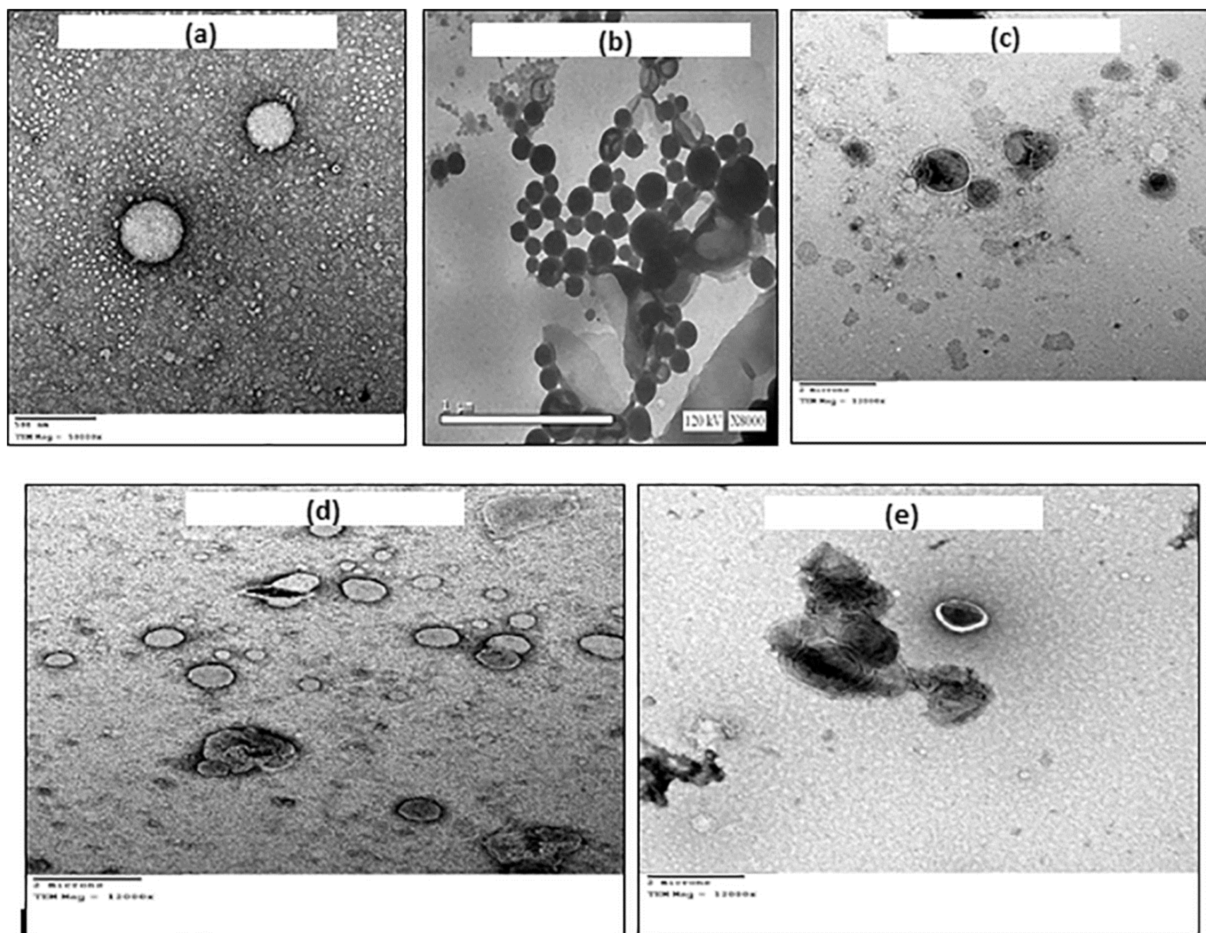

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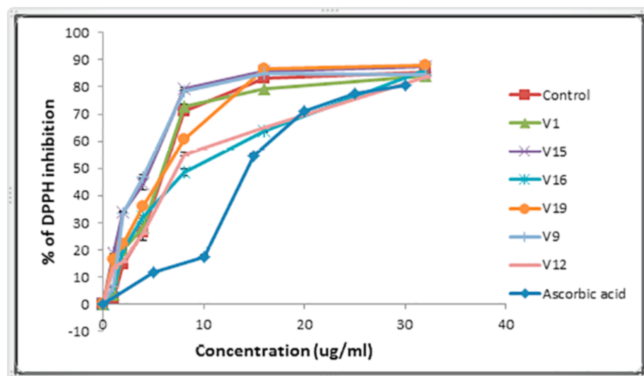

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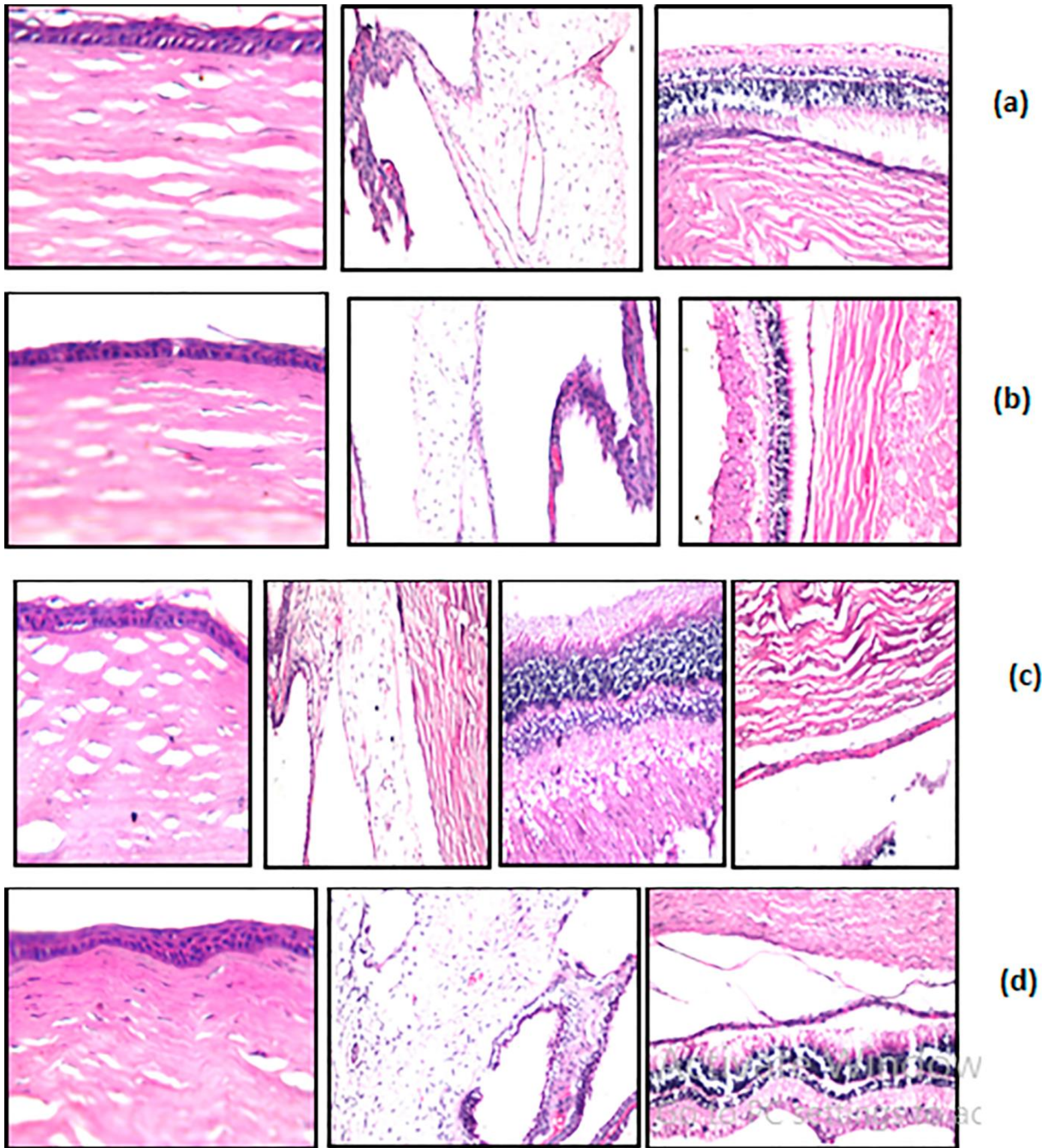
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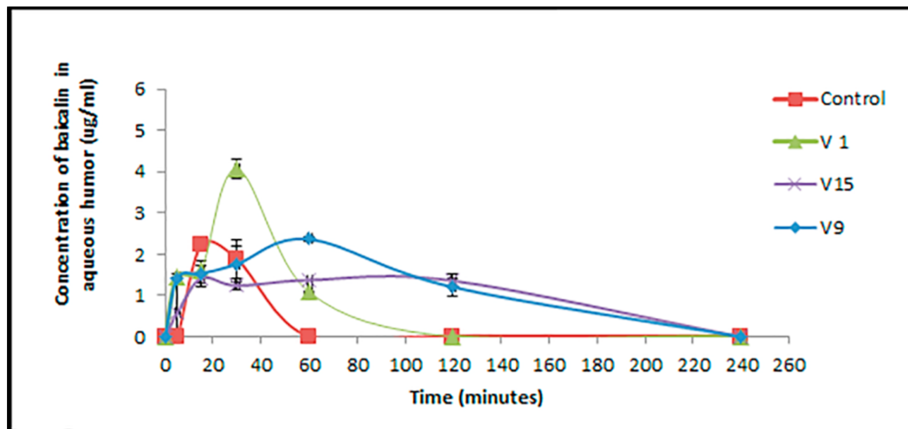

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Graphical abstract

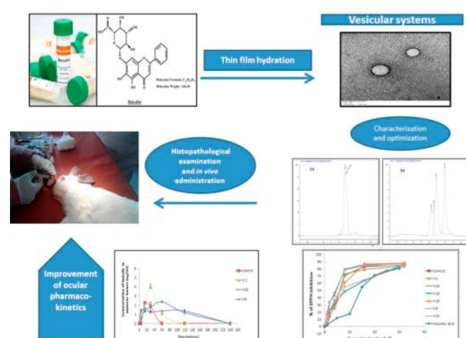

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Table (1): Composition of the prepared baicalin vesicles

Formula code*	Type of vesicular system	Bilayer forming material		Additives	
		Type	Amount (mg)	Name	Amount
V1	Liposomes	Soybean PC	200	-	-
V2	Proliposomes		200	Sorbitol	2 gm
V3	Liposomes		160	Cholesterol	40 mg
V4	Proliposomes		160	Sorbitol Cholesterol	2 gm 40 mg
V5	Niosomes	Span 60	200	-	-
V6	Proniosomes		200	Sorbitol	2 gm
V7	Niosomes		160	Cholesterol	40 mg
V8	Proniosomes		160	Sorbitol Cholesterol	2 gm 40 mg
V9	PEVs	Soybean PC	200	Labrasol	300 ul
V10	Pro-PEVs		200	Labrasol Sorbitol	300 ul 2 gm
V11	PEVs		160	Labrasol Cholesterol	300 ul 40 mg
V12	Pro-PEVs		160	Labrasol Cholesterol Sorbitol	300 ul 40 mg 2 gm
V13	Transfersomes		170	Na glycocholate	30 mg
V14	Pro-Transfersomes		170	Na glycocholate Sorbitol	30 mg 2 gm
V15	Transfersomes		170	Na taurocholate	30 mg
V16	Pro-Transfersomes		170	Na taurocholate Sorbitol	30 mg 2 gm
V17	Transfersomes		160	Na glycocholate Cholesterol	30 mg 40 mg
V18	Pro-Transfersomes		160	Na glycocholate Cholesterol Sorbitol	30 mg 40 mg 2 gm
V19	Transfersomes		160	Na taurocholate Cholesterol	30 mg 40 mg
V20	Pro-Transfersomes		160	Na taurocholate Cholesterol Sorbitol	30 mg 40 mg 2 gm

***All formulae contained 100 mg baicalin. The odd numbered formulae were hydrated with 10 ml phosphate-citrate buffer (pH 5.5) yielding a vesicular dispersion ~~with~~ of 1% w/v baicalin loading, while the even numbered formulae were the pro-forms of the respective aforementioned formulae prepared using sorbitol as a solid carrier.**

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Table 2: Characterization of baicalin vesicular formulations in terms of particle size, zeta potential, entrapment efficiency and cumulative percent released after 8 hours (n=3).

Formula Code	Vesicular System	Particle size (nm) Mean ± S.D.	Mean Zeta Potential± S.D. (mV)	Entrapment efficiency (EE%) Mean± S.D.	Cumulative percent released after 8 hours Mean± S.D.
V1	Liposomes	667.9± 157	-16.8±1.86	94.09± 0.202	67.76 ± 7.31
V2	Pro liposomes	730.3±90.44	-17.8±0.99	98.92± 0.010	74.44 ± 11.29
V3	Liposomes	946±111.1	-16.3±0.98	95.30± 0.166	63.65 ± 3.72
V4	Pro Liposomes	2712±161	-14.4±4.1	98.45± 0.025	82.86 ± 1.58
V9	PEVs	674±43.27	-15.5±0.35	47.53± 0.738	65.81 ± 1.77
V10	Pro PEVs	688.24±26	-17.3±1.34	48.99± 0.88	89.22 ± 2.68
V11	PEVs	1256±42	-17.2±1.56	25.96± 2.22	46.24 ± 5.43
V12	Pro PEVs	1341±90	-22±2.47	78.14± 0.096	77.73 ± 5.18
V13	Transfersomes	978±114	-30.5±0.38	41.52± 0.768	83.10 ± 2.09
V14	Pro transfersomes	959±85	-24.8±0.85	97.8±0.1	79.13 ± 0.43
V15	Transfersomes	976±219	-31.5±2.11	54.07± 0.243	59.20± 4.21
V16	Pro transfersomes	961±51	-31±0.7	97.86± 0.074	64.52 ± 3.23
V17	Transfersomes	944.7±105	-27.4±1.77	48.76 ± 0.46	63.80 ± 3.45
V18	Pro-transfersomes	849±71	-23.7±1.48	99±0.1	81.00 ± 3.47
V19	Transfersomes	960.1±31.06	-27.8±1.81	77.65± 0.511	50.89± 1.49
V20	Pro transfersomes	2104±463	-24.4±1.13	99± 0.012	66.58 ± 2.20

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Table 3: Summary of stability/instability incidents encountered with the different baicalin vesicular formulations after 3 months storage

Formula code	Instability parameter	Other exclusion criteria	Status
V1	N.A.*		
V2	Doubling of particle size	-	Excluded
V3	Doubling of particle size	-	Excluded
V4	N.A.	The largest particle size among liposomal counterparts	Excluded
V9	N.A.	-	
V10	- Appearance of baicalin degradation peaks - Significant decrease in cumulative percent released	-	Excluded
V11	Doubling of particle size	-	Excluded
V12	N.A.	-	
V13	- Appearance of baicalin degradation peaks - Significant decrease in cumulative percent released	-	Excluded
V14	Significant decrease in EE%	-	Excluded
V15	N.A.	-	
V16	N.A.	-	
V17	- Appearance of baicalin degradation peaks - Significant decrease in cumulative percent released - Significant decrease in EE%	-	Excluded
V18	- Appearance of baicalin degradation peaks - Significant decrease in cumulative percent released - Significant decrease in EE%	-	Excluded
V19	- Significant decrease in cumulative percent released	-	Excluded
V20	N.A.	The largest particle size among transfersomal counterparts	Excluded

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- **N.A.* : Not applicable owing to the stability of the formula with respect to change in particle size, zeta potential, EE%, cumulative percent released and baicalin degradation after refrigeration storage for 3 months.**
- **The shaded formulations proceeded to the next characterization steps**

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Table (4): Inhibitory activity of the selected baicalin formulations on DPPH free radical as represented by IC_{50} values (n=3).

Formula Code	**IC_{50} (ug/ml) Mean± S.D.**
Baicalin control solution	6.11 ± 0.43
V1	5.9 ± 0.3
V9	4.39 ± 0.51
V12	7.28 ± 0.46
V15	4.67 ± 0.31
V16	8.67 ± 0.41
Ascorbic acid (Reference standard)	14.2 ± 0.4

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Table 5: Comparative pharmacokinetic parameters of baicalin following ocular administration of the selected formulations; liposomes, PEVs, transfersomes (V1, V9 and V15) and baicalin control solution in rabbits (n=3).

Pharmacokinetic parameter	Baicalin control solution	V1	V9	V15
C_{max} (µg/ml)	2.228± 0.128	4.073± 0.241	2.313± 0.111	1.503±0.0491
T_{max} (Min)	15	30	60	15
$AUC_{0-\infty}$ (µg h/ml)	0.544± 0.327	2.379± 0.050	2.945± 1.032	2.528± 0.151

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