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Variables Affecting the Preparation and Characterization of Axitinib as Oral Niosmoes

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Abstract

Axitinib is anticancer drug act on different solid tumours by inhibition protein kinase enzyme which is responsible for support the tumours with blood demand. Due to its low solubility, it has low bioavailability when given orally also it is affected by liver metabolism. Axitinib formulated as (niosomes) for the first time to study the effect of different variables on the in vitro behaviour of vesicles to increase drug dissolution, absorption ultimately enhance drug oral bioavailability. Niosomal dispersion formulas were formulated by diverse techniques (thin film hydration method, phase reverse evaporation method and sonication method) via multiple kinds of surface-active agent (tween and span), cholesterol and dicetyl phosphate then evaluated for visual appearance, efficiency of drug entrapment (EE%), size of the vesicles, poly dispersity index, zetapotential, viscosity, invitro drug release and study the vesicles morphology by field emission-scanning electron microscopy **FE-SEM**. Numerous factors effect on entrapment efficiency (EE%) were studied including surfactant sum and sort, impact of surfactant combination, effect of different proportions of cholesterol, effect of DCP in different amount, impact of preparation procedures and impact of sonication time. The results showed that thin film hydration method with sonication for four minutes was the best method for preparation of axitinib niosomes, the EE% between (50.97%-98.24%), the particle size was found within the range (64.5 nm - 530.79 nm), zeta potential which was between (-16.6 to -31 mV), polydispersity index (PDI) <1 and viscosity between (584 – 889.6) centipoise. The niosomal dispersion F1 which contained 1:1 weight ratio (span60: cholesterol) was found with high EE% (96.5±0.16%), smallest particle size (64.5±0.5 nm), highest drug release (100%) of drug released at the end of 4 hours and FE-SEM photograph showed that niosomes were spherical with no aggregation and had a smooth surface. The results showed that the type and amount of surfactants used, cholesterol ratio, stabilizer and the time of sonication had a substantial effect on EE%, the size of the vesicles as well as drug release; which were investigated so as to optimize the niosomal dispersion of anti-tumour drug reaching to an optimum formula that may improve drug bioavailability.

Key words: Axitinib, Anticancer, Entrapment efficiency, Invitro behaviour, Surfactants, Vesicles.

تاثير المتغيرات على تحضير وتقييم حويصلات النايوسوم الفموية المحملة بدواء الاكستنيب

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الخلاصة

يعتبر الاكسيتنيب من الادوية المضادة للسرطان الجيل الثاني (يستخدم في علاج أنواع مختلفة من الخلايا السرطانية) حيث يقوم بتثبيط انزيم البروتين كاينيز المسؤول عن بناء اوعية دموية جديدة التي تمد احتياجات الخلايا السرطانية المتنامية بشكل سريع. يظهر دواء الاكستنيب توافر حيوي فموي قليل لكونه قليل الذوبان بالإضافة الى تأثره بالأيض عن طريق الكبد. تم تحضير الاكستنيب على شكل صيغ حويصلات غير ايونية (النايوسوم) للمرة الأولى لدراسة تأثير متغيرات مختلفة على سلوك الحويصلات داخل المختبر لغرض تحسين ذوبانيته وامتصاصه وبالتالي تحسن التوافر الحيوي للدواء. تم تحضير صيغ النايوسوم المتشتت بطرق تحضير مختلفة باستخدام أنواع مختلفة من عوامل الفعالة السطحية (سبان وتوين)، كوليسترول و دايسيتل فوسفيت وتم دراسة المظهر المرئي، كفاءة احتباس الدواء، حجم الحويصلات، مؤشرتعدد التشتت، قيمة جهد زيتا، اللزوجة ، تحرر الدواء وأيضا . دراسة شكل الحويصلة باستخدام مجهر المسح الالكتروني. تم دراسة تاثيرالعديد من المتغيرات (تأثير نوع وكمية عوامل الفعالة السطحية، تاثيراستخدام مزيج من عوامل الفعالة السطحية، تاثيرنسب مختلفة من الكوليسترول، تاثيركميات مختلفة من دايسيتل فوسفيت، تأثير طريقة التحضير و تأثير وقت السونيكيشن) .

حيث أظهرت النتائج ان نسبة احتباس الدواء بين (٩٨,٢-٥٠,٩٧٪) وحجم الحويصلات بين (٥٣٠,٧٩ -٦٤,٥ نانوميتر) وجهد زيتا بين (-١٦,٥ الى -٣١ ملي فولت) , مؤشر تعدد التشتت كان أصغر من واحد واللزوجة بين (٨٨٩,٦-٥٨٤) سينتيويس. صيغة النايوسوم المتشتت (اف ١) التي تتكون من نسب وزن متساوية١:١ من (سبان ٦٠: كوليسترول) لها كفاءة احتباس للدواء عالية (٩٦,٥±٠,١٦) وحجم حويصلي الأصغر (٦٤,٥±٠,٥نانوميتر) وتحرر للدواء اسرع (تحرر ١٠٠ %من الدواء خلال اربع ساعات) وشكل الحويصلات تحت مجهر المسح الالكتروني كانت كروية ومتساوية وموزعة بشكل متجانس. أظهرت النتائج ان استخدام طريقة ترطيب الغشاء الرقيق مع مسبار السسونيكيشن لمدة اربع دقائق كانت هي الطريقة الأفضل في تحضير الاكسيتنيب نايوسوم وان نوع وكمية عوامل الفعالة السطحية المستخدمة ونسب الكوليسترول و الموازن ومدة السونيكيشن لها تأثير ملحوظ على نسبة احتباس الدواء، حجم الحويصلات وكذلك تحرر الدواء والتي تم دراستها من اجل تحسين صيغة النايوسوم المتشتت المضاد للسرطان بغية الوصول لصيغة ملائمة تحقق تحسنا في التوافر الحيوي للدواء.

الكلمات المفتاحية: اكسيتنب، مضاد سرطان، كفاءة الاحتباس، سلوك في المختبر،عوامل الفعالة السطحية، حويصلات.

Introduction

Angiogenesis or aberrant vascularization is considered as both a hallmark of solid tumours and a hallmark of tumour recurrence. Tumours establish blood supply early in their development upon growing beyond a few millimeters. Blood and lymphatic vessels are conduits for cancer cell transportation toward new sites, i.e. metastasis [(1)]. Angiogenesis inhibitors can be designed to block the formation of new blood vessels and the growth of tumours would thereby be halted but not eliminated[(2)]. Axitinib is an effective and selective inhibitor of vascular endothelial growth factor receptors 1, 2, and 3. And cut down the growth of the tumours by prevent angiogenesis [(3)]. This antiangiogenic agent act as a single agent against numerous solid tumours including metastatic renal cell carcinoma (mRCC), non-small cell lung cancer (NSCLC), thyroid cancer and melanoma and exhibited anticancer activity with tolerable safety profile[(4)].

Axitinib it is classified according to biopharmaceutical classification system as class II (low solubility and high permeability), it is a weak base, white to light-yellow powder, non-hygroscopic with a pKa (4.8), the partition coefficient (octanol/water) is 3.5, molecular weight is 386.47 Daltons, it is highly soluble at low pH but the solubility declines rapidly as the pH increases above 2[(5)]. Limitation of drug absorption due to poor solubility considered as a potential problem that can be encountered when delivering an active agent via the oral route [(6)].

To improve the solubility of poorly soluble drugs various techniques are available, such as traditional techniques as (micronization, amorphous forms, chemical modification of drug and inclusion complex etc)[(7)], as well as nanoparticles and nanocarrier technology as liposomes and niosomes [(8, 9)].

Niosomes are one of the auspicious drug carriers that consist of bilayer structure which created by self-assembly of surface-active agent and cholesterol in an aqueous medium. They have extended shelf life and display high stability also they are biodegradable, biocompatible and non-immunogenic [(9)] .

The niosomal approach assumed to improve the poor oral bioavailability drugs through passing the anatomical barrier of gastrointestinal tract [(10)], also the niosomes as nanoscale carrier, increased surface area which lead to enhance drug dissolution rate [(11)].

The aim of this work is to prepare niosomal dispersions containing anticancer drug (axitinib) and adjust through considering the factors influencing influencing the vesicle conduct reaching to the best formula that may enhance the drug oral bioavailability.

Materials and Method

Materials

Axitinib, span 20, span 40, span 60, span 80, tween 20, tween 60, tween 80, cholesterol and dicetyl phosphate (DCP) were purchased from china (sales tunchem company). Monobasic sodium phosphate, dibasic sodium phosphate and tribasic sodium phosphate were from India (alpha chemika). Organic solvents (methanol, chloroform) were obtained from merck (darmstadt, germany). Other chemicals with analytical grade.

Preparation of axitinib loaded niosomal dispersions

Thin film hydration technique was used to prepare niosomal dispersions using various types of surfactants (span 20,40,60,80 and tween 20,60,80), cholesterol as well as charge inducing agents [dicetyl phosphate (DCP)] as shown in Table 1. Each niosomal dispersion was prepared by dissolving the surfactant, cholesterol and DCP in chloroform and methanol in proportion 1:1 (v/v) in rounded bottom flask and the drug dissolved in 10 ml of methanol and added to first mixture. The organic solvent was evaporated using rotary evaporator rotated at 130 r.p.m at 60°C till a smooth dry lipid film was formed.

The film was then hydrated with 10 ml of phosphate buffer saline pH 7.4 at 60°C for 1hour and sonicated using ultrasound probe sonication applied for 4 minutes. The obtained dispersions were kept in a refrigerator at 4°C [(12)]. This method applied for the preparation of F1-F28, while formula F29 was prepared by sonication method where the surfactant, cholesterol, DCP and drug were dispersed in the aqueous phase. This dispersion was then bath sonicated for 10 min at 60°C and further ultrasonicated by probe sonicator for 4 minutes [(13)]. Formula (F30) was formulated by reverse phase evaporation process where a mixture of surface-active agent span 60, cholesterol and DCP

solubilized in 9 ml of a chloroform/methanol blend (2:1 v/v). By using a rotary evaporator at 60°C the organic solvents were gradually vaporized under reduced pressure till a thin dry film was obtained on the inner wall of the rotating flask. The film was redissolved in 12 ml chloroform and a solution containing 5 mg drug dissolved in 4 ml methanol with 6 ml phosphate buffered saline (pH 7.4). The mixture was sonicated in bath sonicator for 2 min, vortexed by hand and re-sonicated again for another 2 min in a bath sonicator. The obtained dispersion was rotary evaporated to interrupt the gel formed instantly then 10 ml phosphate buffered saline (pH 7.4) was added and rotary evaporation was persisted for an additional 15 minutes duration to confirm the elimination of remaining organic solvent. The niosomal dispersion was kept overnight at 4°C[14].

Table1. Composition of the prepared niosomal dispersions of axitinib

Formulation codes	Surfactant type	Axitinib (mg)	Surfactant: cholesterol ratio	Dicetyl phosphate (DCP) (mg)	Mixer type and time
F1	Span 60	5	50 mg :50mg	5	Probe sonication/4minutes
F2	Span 60	5	75mg :50mg	5	Probe sonication/4minutes
F3	Span 60	5	100 mg:50mg	5	Probe sonication/4minutes
F4	Span 40	5	50mg:50mg	5	Probe sonication/4minutes
F5	Span 40	5	75mg:50mg	5	Probe sonication/4minutes
F6	Span 40	5	100mg :50mg	5	Probe sonication/4minutes
F7	Span 20	5	50mg:50mg	5	Probe sonication/4minutes
F8	Span 20	5	75mg :50mg	5	Probe sonication/4minutes
F9	Span 20	5	100 mg:50 mg	5	Probe sonication/4minutes
F10	Span 80	5	50 mg:50 mg	5	Probe sonication/4minutes
F11	Span 80	5	75 mg:50 mg	5	Probe sonication/4minutes
F12	Span 80	5	100 mg:50 mg	5	Probe sonication/4minutes
F13	Tween 20	5	50 mg:50 mg	5	Probe sonication/4minutes
F14	Tween 20	5	75 mg:50 mg	5	Probe sonication/4minutes
F15	Tween 20	5	100 mg:50 mg	5	Probe sonication/4minutes
F16	Tween 60	5	50 mg:50 mg	5	Probe sonication/4minutes
F17	Tween 60	5	75 mg:50 mg	5	Probe sonication/4minutes
F18	Tween 60	5	100 mg:50 mg	5	Probe sonication/4minutes
F 19	Tween 80	5	50 mg:50 mg	5	Probe sonication/4minutes
F20	Tween 80	5	75 mg:50 mg	5	Probe sonication/4minutes
F21	Tween 80	5	100 mg:50 mg	5	Probe sonication/4minutes
F22	Span80+ tween80	5	50 mg:50 mg	5	Probe sonication/4minutes
F23	Span60+ tween60	5	50 mg:50 mg	5	Probe sonication/4minutes
F24	Span20+ tween20	5	50 mg:50 mg	5	Probe sonication/4minutes
F25	Span60	5	50 mg:25 mg	5	Probe sonication/4minutes
F26	Span60	5	50 mg:75 mg	5	Probe sonication/4minutes
F27	Span60	5	50 mg:50 mg	2.5	Probe sonication/4minutes
F28	Span60	5	50 mg:50 mg	10	Probe sonication/4minutes
F29*	Span60	5	50 mg:50 mg	5	Bath sonication 10 minutes then probe sonicated /4 minutes
F30**	Span 60	5	50 mg:50 mg	5	Bath sonication
F31	Span 60	5	50 mg:50 mg	5	Extensive vortex/ 10 minutes
F32	Span60	5	50 mg:50 mg	5	Probe sonication/6minutes
F33	Span60	5	50 mg:50 mg	5	Probe sonication /8minutes

*Prepared by sonication method ** prepared by reverse phase evaporation method

Entrapment efficiency (EE%) calculation

The entrapment efficiency of niosomal dispersion was determined using the centrifugation method, formulas (F1-F33) where each formula was placed in an eppendorf tube and then rotated at 10,000 rpm for 60 minutes at 4°C using a cooling centrifuge to get a pure supernatant [15]. By a UV spectrophotometer set at 260 nm, the free drug in the supernatant was determined[16]. The value of drug entrapment efficiency was calculated via the

equation below:

$$Entrapment\ Efficiency\ (\%) = [(dt - df)/dt] \times 100 \ldots\ldots\ldots (1)$$

Where: dt is the total concentration of drug and df is the concentration of free drug[17].

Variables impact on the entrapment efficiency of the prepared niosomal dispersions

Effect of surfactants type on EE%

Formula F1 (span60), F4 (span40), F7 (span20), F10 (span 80), F13 (tween 20), F16 (tween 60) and F19 (tween 80) were prepared using 1:1 surfactant : cholesterol weight ratio and 5mg of DCP in order to determine the effect of surfactants type on niosomal entrapment efficiency [18].

Effect of surfactant's amount on EE%

Formula F1- F21 were formulated to demonstrate the impact of different amounts of surfactants (span60, span40, span20, span80) keeping the same amount of cholesterol while varying surfactants ratio as (1:1, 1.5:1, 2:1 surfactant: cholesterol weight ratio) where F1-F3 was used to study the influence of various amount of span 60, F4-F6 to study the effect of different amount of span 40, F7-F9 to investigate the impact of various amount of span 20, F10-F12 to determine the impact of different amount of span 80, F13-F15 to determine the effect of different amount of tween 20 , F16-F18 to study the effect of different amount of tween 60 and F19-F21 to study the effect of different amount of tween 80 [19].

Effect of surfactant's combination on EE%

The impact of surfactant's combination on EE% was studied in formulas F22-F24 which were formulated to contain combination of equal amount (1:1) weight ratio of (span80/tween80) ,(span60/tween60) and (span20/tween 20) respectively with maintain the same surfactant: cholesterol weight ratio (1:1) and DCP [20].

Effect of different ratios of cholesterol on EE%

The effect of cholesterol ratios on EE% was studied in formulas F1, F25 and F26 which were prepared to contain various ratios of cholesterol with the equal amount and type of surfactant (span 60) as 1:1, 1:0.5 and 1:1.5 surfactant : cholesterol ratio respectively with maintain the same amount of drug and DCP [21].

Effect of different amount of DCP on EE%

Formula F27 and F28 were prepared containing 2.5 and 10mg of DCP in comparison to F1 (containing 5 mg of DCP), keeping the same weight ratio of span60: cholesterol (1:1) to study the effect of different amount of charge inducer on EE% [22].

Effect of preparation method on EE%

The impact of preparation method was studied in F29 and F30 prepared by different methods (sonication method and phase reverse evaporation method) respectively compared with thin film hydration method (used to prepare F1) in order to study the effect of preparation method on EE% [23].

Effect of type of mixing on EE%

Formula F31 used to demonstrate the impact of type of mixing (10minutes of extensive vortex) on EE% compared with F1 (probe sonication), knowing that both formulas were prepared by thin film hydration process [24].

Effect of sonication time on EE%

Formula F32 and F33 were prepared to investigate the impact of sonication time on EE%, where both prepared by thin film hydration method followed by probe sonication but F32 was probe sonicated for 6 minutes and F33 for 8 minutes in comparison with F1 (sonicated for 4 minutes) [25].

Characterization and evaluation of the prepared niosomal dispersions

Visual appearance

The prepared formulas (F1-F33) were investigated visually for any sign of precipitation by placing the formulas in transparent containers [26, 27].

Determination of vesicles size, surface charge, polydispersity index and viscosity.

The size of the vesicles, the charge (zeta potential) on the particle surface, size range of the vesicles (polydispersity index) were measured for the prepared niosomal dispersions formulas which showed highest percent of EE% (F1-F5 and F8 - F10) using 90Plus particle size analyzer. The device working principle depends on dynamic light scattering system (DLS) [27], which detected electrophoretic movement of charged particles under an applied electric field from doppler shift of scattered light. The light scattering was measured after 1 ml of each niosomal dispersion formula diluted with phosphate saline pH7.4 was injected in to a folded capillary zeta cell at room temperature [28, 29]. Viscosity was studied by Brookfield programmable DV-E III viscometer using spindle s63 with an optimum speed 50 r.p.m [30].

In vitro release study

The dialysis membrane method was used to study in vitro release study of niosomal dispersions (F1-F5 and F8- F10). Also, the drug release profile of selected formula compared with the drug release from marketed oral tablet.

The dialysis membrane (3500 MWCO) was quite cleaned and immersed in distilled water over night. After closing one end with a clip, 10 ml of each niosomal dispersion (containing 5mg drug) was poured into dialysis bag and the bag was closed with another closure clip. The Dialysis bag then immersed in a jar of dissolution apparatus (paddle type) containing 500 ml of 0.1 N HCl (pH 1.2) for 2 hours followed by transferring the dialysis membrane to a jar containing 500 ml of phosphate duffer saline pH (6.8) and 2%SLS for 4 hours. The

temperature was kept at 37 °C and rotated100 rpm. Aliquots (5mL) were taken and substituted with a freshly prepared medium in pre-determined time interval then drug content analysed using a UV/visible spectrophotometer at 260 nm [31, 32] .

Selection of the optimum niosomal dispersion formula

The choice of the optimum formula based on its visual observation, highest entrapment efficiency, smaller particle size, morphology and best drug release.

Study the morphology of niosomes

To study the shape and morphology of niosomes, field emission scanning electron microscopy was used (FE-SEM) [32] . A drop of optimized niosomal formulation was taken and placed on an aluminium stub with adhesive silver tape. Under vacuum, aluminium stubs were stored overnight [33].

Statistical analysis

Data are expressed as the mean ± standard deviation. Statistical analysis was conducted using SPSS software (Statistical Packages for Social Sciences- version 24). Unpaired *t*-test and one-way analysis of variance (ANOVA) were used to compare between two and multiple groups, respectively. $P < 0.05$ was considered significantly different.

Result and Discussion

Entrapment efficiency of the prepared niosomal dispersions

Thirty-three niosomal dispersion formulas were formulated to assess the efficacy of various contents and variables on the entrapment efficiency of axitinib niosomes. The entrapment efficiency for all the prepared niosomal formulas was calculated and the results were shown in Table 2.

Table 2. Entrapment efficiency of the prepared niosomal dispersion formulas

Formula code	EE% Mean ±SD	Formula code	EE% Mean ±SD
F1	96.55 ± 0.16	F16	79.91±0.54
F2	94.81±0.45	F17	73.55±0.66
F3	96.46±0.12	F18	75.89±0.57
F4	95.21± 0.95	F19	77.78±0.41
F5	98.24± 0.25	F20	70.96±0.62
F6	81.53±0.5	F21	71.88±0.68
F7	75.22±0.11	F22	60.8±0.53
F8	95.29±0.05	F23	60.85±0.614
F9	95.63±0.42	F24	58.9±0.34
F10	92.36± 0.21	F25	70.74±0.42
F11	86.6±0.41	F26	69.84±0.38
F12	86.63±0.24	F27	73.41±0.53
F13	80.77±0.68	F28	78.92±0.2
F14	81.63±0.24	F29	60.93±0.58
F15	87.52±0.52	F30	50.97±0.78
		F31	96.69±0.54
		F32	85.67±0.69
		F33	80.78±0.69

n=3 (experiments were done as triplicate)

***Effect of different variables on the entrapment efficiency of the prepared niosomal dispersion formulas*:**

Effect of surfactants type on EE%

Entrapment efficiency of vesicles was influenced by the type of surfactant used. The obtained data showed that the EE% of vesicles contained span 60 (F1) higher than span 40 (F4), span 20 (F7) and span 80 (F10). All these formulas contain the same weight ratio of surfactant: cholesterol (1:1) with the same amount of DCP and drug as shown in (Table 2), because span 60 which has a longer saturated alkyl chain (C18) compared to span 40 which has alkyl chain (C14) and span 20 has alkyl chain (C12) [34]. Span 80 has the same alkyl chain of span60 but the nature of the hydrophobic alkyl chain is unsaturated [35]. that lessen the encapsulation of drug as found in (F10), since the double bond made the chain bend, that's means the adjacent molecule cannot be tight when they form the membrane of vesicles [36] . Vesicles prepared with surfactants possess low transition temperature (Tc) values had low EE% such as(span 20 and span 80), while span 40 and span60 possess (hight Tc values) formed vesicles with high EE% [37, 38], because they are more likely to be in an ordered gel form thus lowering bilayer leaking [39, 40].

The vesicles prepared by using tween 20 (F13) , tween60 (F16) and tween80 (F19) (all containing the same surfactant: cholesterol weight ratio 1:1 with same DCP amount) showed lower EE% compared with vesicles prepared by (span 60) F1, (span 40) F4, (span 20) F7 and (span80) F10, this is due to that the HLB value of surfactants has

crucial rule to vesicles formation so the surfactants with high HLB 14-17 such as (tween20,60,80) have high aqueous solubility and gave low entrapment efficiency [40]. The alkyl chain in tween 80 is unsaturated which leads to leaky bilayers in niosomes and low EE% [41]. Based on HLB values, the affinity for lipids was predicted to be in span more than tween as illustrated higher EE% with formulas containing span compared with those containing tween, this suggested that hydrophilic surfactants destabilized the lipid bilayers which lead to decrease EE% [42].

Effect of surfactants amount on EE%

To study the effect of surfactants amount, different niosomal dispersions were prepared with span 60 (F1, F2, F3), span40 (F4, F5, F6), span20 (F7, F8, F9), span80(F10, F11, F12), tween20 (F13, F14, F15), tween60 (F16, F17, F18) and tween 80 (F19, F20, F21) each surfactant was used in three different ratios of surfactant: cholesterol(1:1, 1.5:1 and 2:1) with the same amount of drug and DCP.

The results exhibited that increasing the amount of span 60 from 1(F1) to 2 (F3) weight ratio had no significant difference (P>0.05) in their EE%. which was high in both formulas. The high EE% was due the long saturated alkyl chain of span60 and highest phase transition temperature [43].

Span 40 and span 80 when increased from 1(F4 and F10) to 2 (F6 and F12) weight ratio lead to significant reduction (P≤0.05) in EE%, as the lipophilic drugs are intercalated almost completely within the lipid bilayer of the vesicles leading to a competition between the surfactant at high concentration and drug which led to exclusion of the drug from the bilayer so decreased the EE% [44]. In addition the existence of the double bond in the alkyl chain of span 80 leads to high membrane permeability, low density and low EE%[45]. While the EE% of formulas prepared by span 20 was increased upon increasing the surfactant amount from 1(F7) to 2 (F9) weight ratio, this might be related to increase the total lipid concentration so higher lipid concentration (amount of lipophilic surfactant) might have caused higher encapsulation volume and thus increase entrapment efficiency of lipophilic drug [46].

In all formulas containing tween (F13-F21) when the amount of surfactant increased there was no significant (P> 0.05) change in EE% in comparison to formulas containing span, as the hydrophilic surfactants (tween) destabilized the lipid bilayers and caused diffusion of the drug in the aqueous medium during preparation [47].

Effect of surfactants combination on EE%

Entrapment efficiency was reduced when used surfactants combination (span and tween) as shown in (F22-F24) in comparison to formulas containing one surfactant, because the combination of surfactants reduced their potential in solubilizing the lipophilic drug [49 , 50], and the same result was observed in carotene niosomes prepared by mixture of span 20/tween 20 [50]. The least encapsulation efficiency (58.9%) was observed with combination of span 20/tween 20 (F24) with respect to combination of span80/tween 80 (F22) and span 60/tween60 (F23) due to the higher HLB of the mixture (span20/tween20) which reduced its potential in solubilizing the lipophilic molecule also the liquid nature of these surfactants lead to more drug permeability throughout the bilayer and reduced the EE% [20].

Effect of different ratios of cholesterol on EE%

Entrapment efficiency of drug was observed to be increased with increasing the cholesterol weight ratio from 0.5 as in (F25, EE% equal 70%) to 1 in (F1, EE% equal 96%) while entrapment efficiency decreased on additional increase in cholesterol ratio to 1.5 as in (F26, EE% equal 69%) keeping the same type and amount of surfactant (span 60) and the same amount of drug and DCP. Upon increasing cholesterol ratio from 0.5 to 1, the hydrophobicity and stability of bilayers vesicles increased and leaking of drug decreased which may cause efficiently trapping the hydrophobic drug into bilayers as the vesicles formed [51]. But on higher amount of cholesterol (1.5) in F26, it may compete with the drug for packing space within the bilayer hence excluding the drug [52].

Effect of different amount of dicetyl phosphate (DCP) on EE%

The negatively charged molecules DCP applied in niosomes for increasing the steadiness of niosomes through electrostatic repulsion which prevent vesicles aggregation and coalescence [53]. Formula F1 had (EE% 96%) contains 5 mg of DCP, the phosphate groups of DCP aligned next to the polar heads of span 60 and the extended double hydrocarbon chains of DCP oriented parallel to the hydrocarbon chains of surfactants, impart more packing by filling any disorder through the bilayer membrane of niosomes [54]. When the DCP amount increased from 5mg (in F1) to 10 mg (in F28) contain the same type and ratio of surfactant (span60) : cholesterol (1:1) the EE% reduced from 96.55% to 78.92% since using high amount of stabilizer (DCP) may prevent the niosomes creation and reduced the EE% [55]. Same result observed with fluconazole liposomes where incorporation of dicetyl phosphate decreased the entrapment efficiency of the drug [56].

However upon reducing the amount of DCP from 5mg (in F1) to 2.5 (F27) contain the same type of surfactant span60 and same ratio of surfactant : cholesterol (1:1) the EE% reduced from 96.55% to 73.41% that's could be explained due to electrostatic charges induced chain tilt and subsequent changes in the lateral packing of the bilayers by the effect of charge inducing agents, the same result observed in flurbiprofen niosomes [57].

Effect of preparation method on EE%

Formula F29 and F30 were prepared by sonication and reverse phase evaporation method respectively and had EE% 60% and 50% compared with F1 which was prepared by thin film hydration (TFH) method and had EE% (96%) , because in F1, the probe tip sonicator used deliver high energy input to the prepared dispersion causing more reduction in particle size and increasing its surface area leading to higher EE [58]. Same result obtained in preparation of liposomes loaded with betel leaf (Piper betle L.) ethanolic extract prepared by thin film hydration [58].

Effect of type of mixing on EE%

To study the effect of type of mixing on EE%, F31 which its preparation involved 10 minutes of extensive vortex compared with F1 involved probe sonication for 4 minutes. The result showed the same high EE% in both formulas but there was a significant difference in vesicles particle size where vesicles size prepared by vortex mixing was (1190nm) indicating the lack of enough energy (using vortex mixer) lead to vesicles aggregation [59]. While the vesicles size prepared with probe sonication in F1 were small in size (64.5nm) due to high energy in put which reduced the particle size and disperse them in homogeneous manner [60].

Effect of sonication time on EE%

Formula F32 and F33 were formulated by thin film hydration process and probe sonicated for 6 and 8 minutes respectively in comparison to F1 which sonicated for 4 minutes, the EE% decreased from 96% (F1) to 85% in (F32) and 80% in (F33) because the sonication for a long time may cause a slightly temperature raise in niosomes dispersions (despite of putting the sample into ice water bath) because of high energy gaining through probe sonication process [61]. Also excessive sonication time could cause serious disruption of vesicles structures lead to great drug leakage from niosomes and low EE % [62].

Characterization of and evaluation the prepared niosomal dispersions

Visual observation

The niosomal preparations (F1-F33) appeared as a milky dispersion and formulas formulated by thin film hydration process where homogenous and no precipitate appeared when put it at room temperature overnight indicating their stability un like the formulas prepared by sonication and reverse phase evaporation method. Same results observed with niosomes of niflumic acid [63].

Determination of vesicles size, surface charge, polydispersity index and viscosity

The vesicles size, surface charge, poly dispersity index and viscosity were determined for formulas which had high EE% (F1, F2, F3, F4, F5, F8, F9, F10) and presented in table 3. The niosomal vesicles size was found within the range 64.5 nm to 530.7 nm (while F31was gave large particle size 1190nm so it was excluded.

The results showed that surfactants with high HLB value led to increase the vesicle size significantly ($P \leq 0.05$). Where niosomes containing different types of span: cholesterol ratio (1:1) as F1(containing span 60; HLB 4.7) has smallest vesicles size (64.5nm) than F4 (containing span 40; HLB 6.7) with vesicle size (108.42 nm) and F10 (containing span 80; HLB 8.6) had vesicle size (133.3nm) and F8 (containing span 20; HLB 8.6) had vesicle size (212.5 nm.), because when HLB values moving towards hydrophilic region (increased) the surface free energy of the vesicles was increased and water uptake of the surfactants increased leading to larger size of vesicles [64].

Increasing the amount of surfactants lead to decrease the particle size as shown in F8 and F9 (containing span 20 1.5:1 and 2:1 ratio respectively) and F4 and F5 (containing span 40 1.5:1 and 2:1 respectively), because increasing surfactant ratio lead to particle stabilization (enhanced interface stabilization) by forming a thin layer around the newly formed surface [65]. While in F1, F2 and F3 (containing span 60: cholesterol 1:1, 1.5:1 and 2:1 ratio respectively) the particle size increased with increased surfactant concentration due to the interface stabilization reached maximum extent, beyond which, the increase in surfactant concentration lead to increased viscosity of the medium reduces the net shear stress available for the droplet break down, leading to an increase in the particle size [66]. Same results observed with preparation of methotrexate nanohybrid (nanoparticles) for the possible treatment of osteosarcoma [67].

The result also showed that all the formulas had polydispersity index < 1. Low value of polydispersity index is considered to be required to uniform distribution and homogeneity of the particles within the preparation. Values close to zero indicated a homogenous dispersion [68].

Zeta potential which was between -16.6 to -31 mV, the negative value attributed to the presence of DCP common negative charge inducer additive that imparts a negative charge on the surface of niosomes so preventing vesicle aggregation and fusion while maintaining vesicle integrity and uniformity, consequently all formulas showed good stability [69].

The viscosity of niosomal dispersion increased with increase the surfactant amount as shown in F1 ,F2 and F3 (containing different ratio of span60), F4 and F5 (containing different ratio span 40) as well as F8 and F9 (containing different ratio span20) keeping the same amount of cholesterol [70]. Span 40 (Tc = 42°C) and Span 60 (Tc = 53°C) produced milky viscous niosomal dispersion because they have high transition temperatures and are solids at room temperature, so they act as gelators by themselves. While span80

and span 20 had lowest transition temperatures (16°C for span 20 and −12°C for span 80) and both are liquid at room temperature with lower viscous niosomal dispersion. The same results observed with formulation of pro-niosomal transdermal carrier systems for flurbiprofen [71].

Table 3. Niosomal vesicle size, zeta potential poly dispersity index (PDI) and viscosity of the prepared niosomal dispersion formulas

Formula code	Niosomal vesicle size (nm) mean ±SD	Zeta potential (mean ±SD)	PDI mean±SD	Viscosity (centipoise) mean ±SD
F1 span60 1:1	64.5±0.5	-21.66±0.57	0.218±0.01	752.3±2.5
F2 span60 1.5:1	198.63±3.17	-31±1	0.32±0.02	765.3±5
F3 span60 2:1	530.79±5.45	-26.66±1.52	0.3±0.03	807.3±6.4
F4 span40 1:1	108.42±2.1	-27.33±2.3	0.413±0.1	872±4.3
F5span40 1.5:1	92.1±0.73	-24±1	0.194±0.23	889.6±1.5
F8 span20 1.5:1	212.5±0.85	-25.66±0.57	0.387±0.13	655±5.5
F9span20 2:1	122.19±0.46	-21.33±0.57	0.37±0.14	715±4.5
F10 span801:1	133.3±0.52	-16.6±1.15	0.125±0.1	584±4.1

n=3 (experiments were done as triplicate)

In vitro release study

In vitro drug release of axitinib from formulas which had high EE% (F1, F2, F3, F4, F5, F8, F9, F10) was biphasic involving an early release phase (in 0.1N HCl) followed by continuous release in intestinal media, The results are presented in (Figure 1). The initial release phase may result from drug desorption from the outer surface of the niosomes and the second passive release phase might rely on gradual drug diffusion through the membrane[72], also, the drug is weak base, so it is more soluble in acidic medium (0.1N HCl). But the entire amount of loaded drug was not released in 0.1N HCl from the niosomes, while the drug released completely in acidic medium of stomach from marketed tablets (100% of drug released within 20 minutes) as shown in(Figure 2), this may be due to entrapment of the drug in the lipophilic region (bilayer membrane) of niosomes which protected it from the acidic medium (stomach) while in intestinal medium the drug released in continuous manner [16].

There were many variables affecting the drug release including surfactant type and amount, where in F1, F2, F3 containing span60 in different weight ratio showed drug release (100%, 53 % and 61.4 % respectively) at the end of 4 hrs. There was a significance decrease (P≤0.05) in the release profile as the ratio of span 60 increased in F2 and F3 in comparison to F1 containing 1:1 surfactant ratio, similar result observed with F8 and F9 containing span 20. The retardation in drug release as the amount of surfactant increased because the surfactant act as a depot for the release of the drug so when increased more drug entrapped and less leakage from noisome to dissolution media [16]. The same result observed in baclofen niosomes [73].

Formula F4, F5 containing span 40 showed release (65 % and 63.51 % respectively) with non-significant difference P>0.05 when the amount of span 40 increased from 1 (F4) to1.5 (F5) as span 40 has longer alkyl chain (C18) and high transition temperature (42°C) which lead to rigid bilayer and provided more stable vesicles and slow drug release [57], similar result obtained with metformin hydrochloride loaded niosomes [74].

Formula F10 containing span 80 (1:1) surfactant: cholesterol ratio showed slowest drug release because span 80 has mono unsaturated alkyl chain (presence of double bond) and lowest transition temperature (-12°c) [75]. Same result observed with simvastatin niosoms [36].

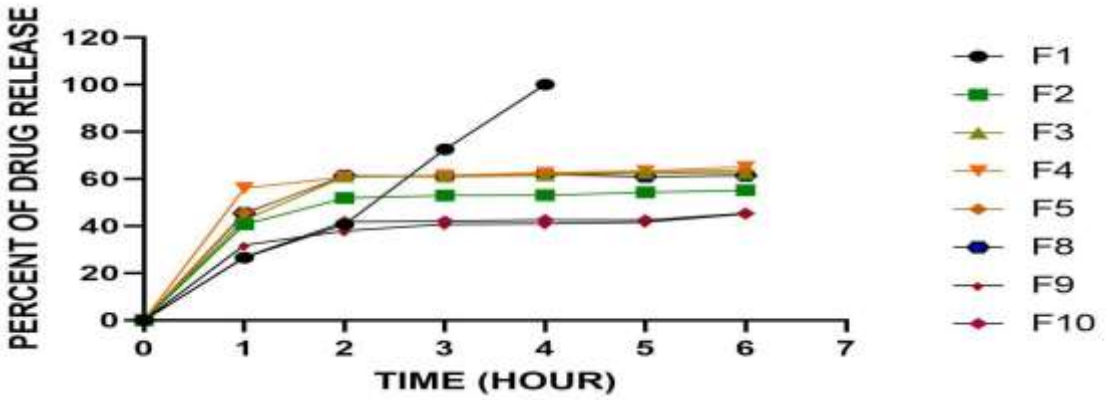

Figure 1. In vitro release profile of axitinib niosomal dispersion formulas (F1, F2, F3, F4, F5, F8, F9, F10) in simulated gastric medium (pH 1.2) for 2hr followed in BPS (pH 6.8) for 4 hr in simulated intestinal

medium at 37 °C.

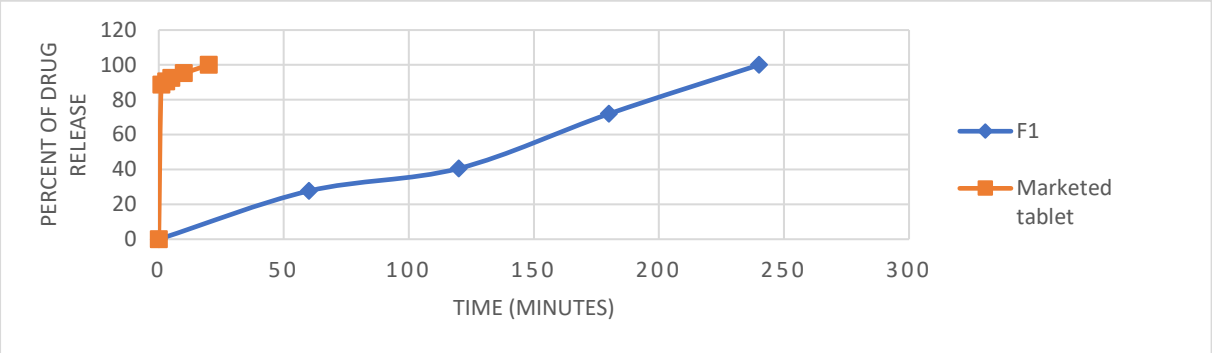

Figure 2. in vitro drug release profile of axitinib niosomes (F1) and marketed tablet.

Selection of the optimum niosomal dispersion formula

Formula (F1) containing surfactant: cholesterol ratio 1:1 was selected as optimum formula due to high EE% (96.5%), smallest particle size (64.5nm), highest drug release (release 100% in 4 hours).

Study the morphology of optimum niosomal dispersion

The FE-SEM photograph of niosomes F1 (Figure 3) showed that niosomes were spherical in shape without aggregation, with smooth surface and their diameter within nanoscale [76]. Similar morphology obtained with study of candesartan cilexetil niosomes [77].

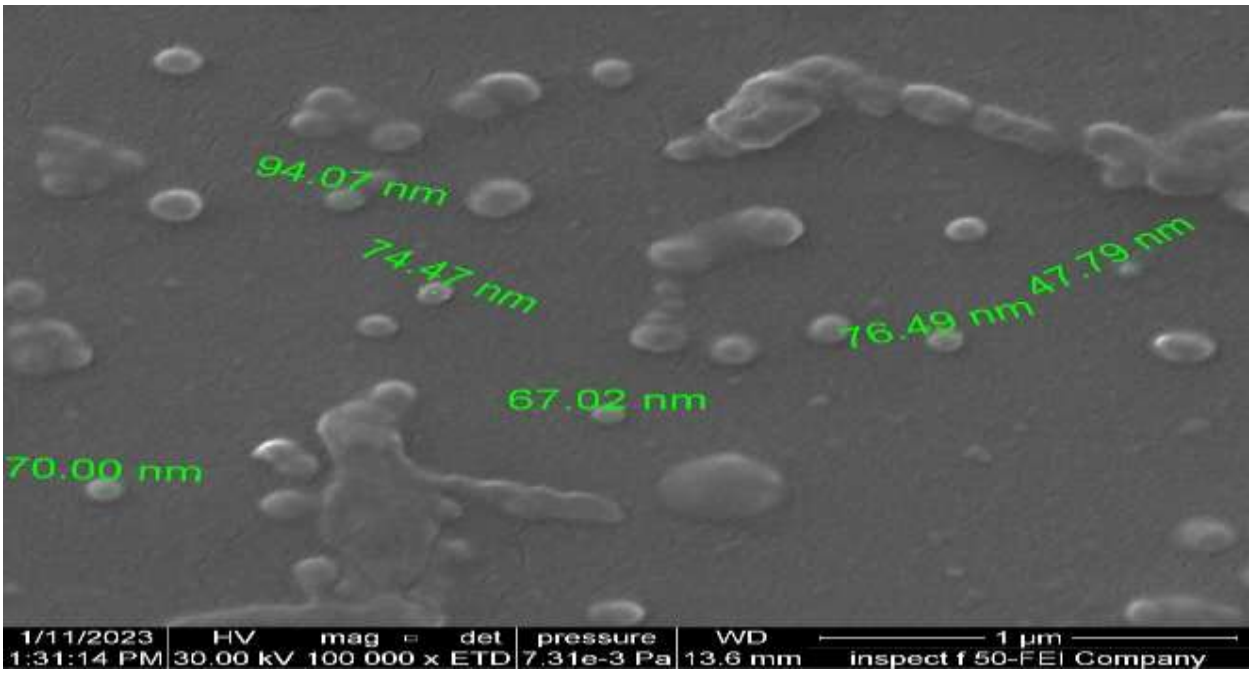

Figure 3. Morphology of F1 by field emission scanning electron microscopy (FESEM).

Conclusion

Axitinib niosomes prepared successfully using thin film hydration method with sonication for four minutes. Formula (F1) containing span60: cholesterol (1:1) weight ratio and 5mg of stabilizer showed high EE%, small particle size and highest drug release comparison to other formulas, so these variables (type and amount of surfactant, cholesterol ratio, amount of stabilizer and sonication time) exhibited remarkable effect on the in vitro behaviour of niosomal dispersion of (F1) which is expected to improve the oral bioavailability of drug through crossing the anatomical barrier of gastrointestinal tract via transcytosis of M cells at the intestinal lymphatic tissues, also by entrapment of the drug in nano scale vesicles (niosomes) lead to increased surface area so enhanced the drug dissolution, absorption ultimately improved the drug oral bioavailability.

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Conflicts of Interest

The authors would like to confirm that there are no conflicts of interest with this publication.

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Ethics Statements

The study did not need ethical approval from an ethics committee.

Author Contribution

The authors contributed to the manuscript equally.

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