

NANOENCAPSULATION AUGMENTS RELEASE EFFICACY AND GLUCOSE TOLERANCE OF 14-DEOXY, 11, 12-DIDEHYDRO ANDROGRAPHOLIDE LOADED POLYCAPROLACTONE NANOPARTICLES IN STREPTOZOTOCIN-NICOTINAMIDE INDUCED TYPE 2 DIABETES

NAGALAKSHMI K.^a, SUJATHA S.^{a*}

Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur 603203

Email: sujatha.sa@ktr.srmuniv.ac.in

Received: 25 Jul 2017, Revised and Accepted: 10 Oct 2017

ABSTRACT

Objective: This study was designed to study the release efficacy and glucose tolerance of 14-deoxy, 11, 12-didehydroandrographolide loaded polycaprolactone nanoparticles in streptozotocin-nicotinamide induced type 2 diabetes.

Methods: Biodegradable polymer based novel drug delivery systems had brought a considerable attention to improve therapeutic efficacy and bioavailability of various drugs. In this study, 14-deoxy-11, 12-didehydroandrographolide (sparingly water soluble) loaded polycaprolactone (nano-DDA) was synthesized using polyvinyl alcohol and tween20 as surfactants. MTT assay was performed to analyse the cytotoxicity of both the formulations on L6 myoblasts. Free DDA and nano-DDA were administered orally to the streptozotocin-nicotinamide induced experimental diabetic rats for 45d. Oral glucose tolerance test (OGTT) was carried out at the end of the study. After one week washout period, animals were administered with free and nano-DDA and release efficacy of DDA from polymer matrix and concentration of glucose were analysed.

Results: MTT assay revealed that nano-DDA prepared using tween-20 as a surfactant elicited cytotoxicity towards L6 myoblasts, whereas nano-DDA prepared using polyvinyl alcohol as a surfactant remained non-toxic till 10 μ M. OGTT studies revealed an initial increase of glucose at 30 min followed by a progressive decrease in the glucose level. In rat plasma, a gradual decrease in glucose level was observed up to 32h (139 mg/dl) for free DDA, whereas nano-DDA exhibited a major decrease in glucose concentration at 32h (115 mg/dl) which continued even after 48h (117 mg/dl).

Conclusion: A slow and sustained release of DDA from the polymer matrix substantiated that nanoencapsulation enhanced the oral bioavailability of DDA which resulted in decreasing the concentration of glucose which could be due to the pronounced antihyperglycemic activity of nano-DDA over free DDA.

Keywords: DDA, Nanoencapsulation, Release efficacy, Plasma glucose, Antihyperglycemic activity, Bioavailability

© 2017 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open-access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>)
DOI: <http://dx.doi.org/10.22159/ijap.2017v9i6.21607>

INTRODUCTION

Diabetes mellitus(DM) is a metabolic condition characterized by impaired glucose tolerance due to the dysfunction of pancreatic β cells or increased resistance to insulin action in the peripheral tissues (mainly skeletal muscle and adipose tissue) [1, 2]. The alarming rate of worldwide incidence of Diabetes Mellitus constitutes a global health issue. As reported by International Diabetes Federation (IDF) in 2015, about 415 million people were diagnosed with DM and is projected at 642 million by 2040 with an average increasing case of 3 people every second [3].

Among the two types of diabetes, Type II Diabetes Mellitus is the most commonly diagnosed form of diabetes found among 90-95% of the diabetic population [4], characterized by insulin resistance(IR), which is a hallmark of type 2 diabetes. Impaired insulin secretion and insulin resistance leads to the dysregulation of glucose homeostasis.

According to patient's perspective, oral administration of the drug is absolutely not painful and omits uncomfortable interventions. However, the oral bioavailability of bioactive compounds is limited by the protease enzymes in the gastrointestinal tract and hydrophobicity of the bioactive compounds [5]. In the food and pharmaceutical industries, the controlled release systems have emerged extensively to release the active substances Eg: drugs and nutrients [6]. The advancement in material science and nanoengineering are being prioritized for encapsulating therapeutic agents into a nanoformulation for targeted drug delivery [7, 8].

The pharmacological potential of bioactive compounds enormously depends on their solubility in water [9]. Unfortunately, the sparing solubility in water limits their bioavailability and clinical application. Hence site-specific or targeted drug delivery, reduction of systemic toxicity, improvement of physicochemical property, protection from biochemical degradation and improvement of environmental stability are the major challenges for designing an ideal delivery

system [2]. Recently, polymer-based drug delivery systems ventured many avenues for increasing efficacy and specific targeting of new, as well as already existing drugs. In this perspective, the biodegradable polymers are of prime interest due to their advantage of elimination of polymer metabolites out of the body by innate metabolic processes [10].

14-deoxy-11, 12-didehydroandrographolide (DDA) used in the present study is a diterpenoid of the annual herbaceous plant *Andrographis paniculata* (Family: Acanthaceae) which have been used for many ailments in India as well as in many Asian countries [11]. The limited availability of pharmacokinetic data of DDA resulted in the delay of further drug development and clinical uses. In our previous study, the enhanced bioavailability of nano-encapsulated DDA in experimental diabetic rats was reported [12]. In this present study, nano-DDA was formulated with two types of surfactants and their cell viability was analysed and the aim is to study the augmented glucose tolerance and release efficacy of the nanoencapsulated DDA (nano-DDA).

MATERIALS AND METHODS

Chemicals and reagents

Streptozotocin, Nicotinamide, Metformin, Polyvinyl alcohol and Tween 20 were procured from Himedia, Mumbai. Polycaprolactone was purchased from Sigma-Aldrich limited, Bangalore. 14-deoxy, 11, 12-didehydro andrographolide was purchased from Apeksha Research centre private limited, Indore, Madhya Pradesh, India.

Cell culture

Dulbecco's modified eagle's medium (DMEM) supplemented with 10% Fetal bovine serum (FBS) was used to culture L6 myoblasts (rat skeletal muscle cells) with streptomycin (75 μ g/ml), penicillin (120 units/ml), amphotericin B (3 μ g/ml) and gentamycin (160 μ g/ml) in 5% CO₂.

Formulation and characterization of nanoencapsulated DDA

Four formulations were attempted by varying the drug to polymer ratio, type of surfactant and sonication time. Polycaprolactone (PCL) was used as a biodegradable polymer. The nanoparticles containing PCL-DDA conjugate and free PCL were fabricated by solvent evaporation technique [13]. Briefly, known amounts of the DDA and PCL were added to 0.5 ml of dichloromethane, stirred to ensure that the materials were dissolved. The organic phase was introduced slowly into either 0.5% PVA or 1% Tween 20 solution resulting in an emulsion. Bandelin sonopuls sonicator (model UW2070, BANDELIN electronic GmbH and Co. KG, Berlin) was used to break down the resulting emulsion into nanodroplets. The solvent was evaporated with constant stirring using magnetic stirrer at 60 rpm for 4 h to form a colloidal suspension of nano-DDA. The nanoparticles were collected as a precipitate by centrifugation at 14000 rpm for 15 min. The pellet was air-dried and then stored at 4 °C. Empty PCL nanoparticles were prepared without the drug using the same methodology.

Assessment of cytotoxicity by MTT assay

The cytotoxic effect of nanoencapsulated drug prepared with polyvinyl alcohol (PVA) and tween 20 as surfactants was analysed by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay. The MTT assay is a colorimetric assay for evaluating cell viability. NAD(P)H-dependent cellular oxidoreductase enzyme reduces the yellow tetrazolium dye (MTT) to insoluble formazan crystals (purple colour). These enzymes are present largely in the cytosolic compartment of the cell. A solubilization solution was added to dissolve the insoluble product. The absorbance was read at 570 nm by a UV spectrophotometer [12-16].

$$\% \text{ Cell viability} = \frac{\text{O. D. of test}}{\text{O. D. of control}} \times 100$$

Measurement of release efficacy

After one week washout period, experimental animals were administered with free DDA (50 mg/kg b.w.) and nano-DDA (50 mg of nanoparticle-containing 9.4 mg DDA/kg b.w.)

The area under the curve (AUC) was calculated using the trapezoidal method. The release efficacy of DDA from polymer matrix formulation was extrapolated using the AUC. Release efficacy percentage was calculated using the following formula [17]:

$$\text{Release efficacy percentage} = \frac{\text{AUC}}{\text{AUC}_s} \times 100$$

Where AUC is the area under the curve of drug release chromatogram and AUC_s is the area under the curve of the standard sample.

Experimental animals and diet

Healthy adult male Wistar rats (150-200g) were used for the study. Rats were housed in polypropylene cages lined with husk in standard environmental conditions (temperature 25±2 °C with dark/light cycle 12/12h; 55±10% humidity). Priorly the experimental protocol was subjected to the Institutional Animal Ethical Committee and was approved by the same before beginning the experiment (53/IAEC/2011). Animals were fed with standard pellet diet and water ad libitum.

Experimental induction of diabetes

Streptozotocin (STZ) and nicotinamide (NIC) were dissolved in freshly prepared 0.1M citrate buffer (pH 4.5) and in normal physiological saline respectively and stored on ice prior to use. A single injection of Streptozotocin (45 mg/kg body weight), 15 min after the administration of nicotinamide (110 mg/kg body weight) was given intraperitoneally to induce Diabetes mellitus in overnight fasted rats. Hyperglycemia was confirmed by the raised blood glucose levels, determined on 3rd and then on 7th day after induction. The rats with blood glucose levels above 250 mg/dl were used for the study [18].

Experimental design and treatment of animals

Experimental design of free DDA

The rats were divided into seven groups comprising of five rats in each group. All rats except control and the diabetic group were administered with the drug (orally) for 45 d.

Group I-Control rats received Carboxymethylcellulose (0.5%)

Group II-Control rats administered orally with DDA (50 mg/kg body weight)

Group III-Diabetic rats

Group IV-Diabetic rats administered orally with DDA (10 mg/kg body weight)

Group V-Diabetic rats administered orally with DDA (25 mg/kg body weight)

Group VI-Diabetic rats administered orally with DDA (50 mg/kg body weight)

Group VII-Diabetic rats administered orally with Metformin (500 mg/kg body weight)

Experimental design for nano-DDA

Group I-Control rats received Carboxymethylcellulose

Group II-Control rats administered with nano-DDA (50 mg/kg b.w.)

Group III-Diabetic control rats (NIC+STZ induced)

Group IV-Diabetic rats administered with DDA (50 mg/kg b.w.)

Group V-Diabetic rats administered with nano-DDA (25 mg/kg b.w.)

Group VI-Diabetic rats administered with nano-DDA (50 mg/kg b.w.)

Group VII-Diabetic rats administered with Metformin (500 mg/kg b.w.)

Animals were administered with DDA for 45 d (1 ml/rat) in 0.5% carboxymethyl cellulose as a vehicle. DDA and Metformin were suspended in 0.5% CMC prepared in autoclaved distilled water prior to oral administration to experimental animals.

Oral glucose tolerance test (OGTT)

After treatment for 45 d with free and nano-DDA, glucose tolerance test was performed on overnight fasted rats [19]. Animals of all groups were administered with glucose (2g/kg) orally. Group IV, V, VI were treated orally with drug and group VII was administered with metformin (500 mg/kg), 30 min prior to oral glucose administration. The blood samples were withdrawn through retro-orbital plexus at 30, 60, 90 and 120 min after glucose administration.

Concentration of glucose

Blood samples were collected from retro orbital plexus of the rats at predetermined time intervals and plasma was separated immediately by centrifuging of heparinized blood at 3500 rpm for 10 min. The concentration of glucose in plasma samples was estimated using Robonik prietest GOD-POD glucose estimation kit, Robonik (India) Private limited, Mumbai, India.

Statistical analysis

Results were expressed as mean±SD. The data were analysed using Graphpad Prism 5.03 statistical software (Graphpad software Inc., La Jolla, CA). The statistical significance was evaluated by oneway analysis of variance (ANOVA) and Dunnet's multiple comparison tests was performed to determine significant differences between groups. The criteria for statistical significance were P<0.05.

RESULTS AND DISCUSSION

Cell viability assay

The capability of cells in reducing MTT delivers an indication of mitochondrial integrity and activity, which could be inferred as a measure of viability of cell [20]. The results of the cell viability study were presented in the form of percentage viability remains after treatment with PVA and Tween 20 formulations (fig. 1). The results revealed that the nano-DDA (Tween20) treated cells, exhibited a significant, (P<0.05) decrease in cell viability on the L6 myoblasts. The percentage viability of L6 cells after treatment with the aforementioned formulation was found to be 67% in 1nM. A major decline in the cell viability was noticed at 10µM (34%). But the cells treated with nano-DDA (PVA) did not display any change in cell

viability (98% viable on par with the control). In addition, nano-DDA (PVA) incubated L6 myoblasts did not elicit cytotoxicity to L6 myoblasts up to 10 μ M. This is suggestive of the compatibility of nano-DDA (PVA) with a living system. Hence nano-DDA with PVA as the surfactant has been used in further experimental procedures, omitting nano-DDA with tween20 as surfactant since it elicited a decline in cell viability. ChibuezeIhekwereme (2014) reported that calu-3 cells (bronchial adenocarcinoma of the airway) incubated with 0.2% Tween 20 for 60 min exhibited cytotoxicity with 57.1% \pm 8.1% viability of the cells [21]. Atefeh Hekmat *et al.* (2016)

stated that Docetaxel-Loaded Nanomicelles using Tween 20 as a surfactant resulted in a higher C26 cell death rate [22]. Vimala *et al.* (2011) reported that usually at the end of the preparation of nanoencapsulation, the surfactant used was removed by centrifuging the nanoparticles. But the surfactant which adsorbed on the surface of the nanoparticles could not be removed completely due to the formation of the interconnected network formed on the surface [23]. According to Sahoo *et al.* (2002) up to 13% of surfactant level is permissible which could influence the physical and biological properties of the nanoformulation [24].

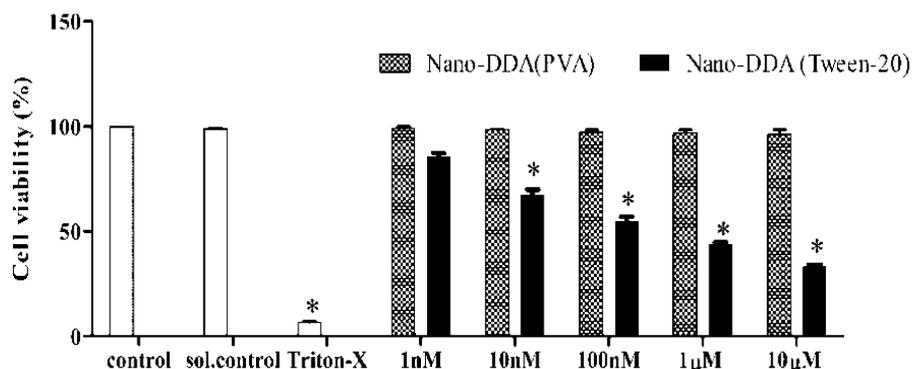


Fig. 1: Cell viability of nano-DDA (PVA) and nano-DDA (Tween-20) using MTT assay. The values are mean \pm SD, (*) p<0.05 as compared with control, sol. control-Solvent control

Release efficacy percentage (%)

The *in vitro* release of DDA loaded nanoparticles appeared to be two-stage processes. Initially there was a burst release of DDA from polymer matrix was observed. After that release of DDA was at constant rate. Generally, drug release from the polymer matrix depends on the diffusion of the drug, the erosion and the swelling of the polymer matrix and degradation of polymer [25]. The release efficacy of DDA from nanoparticulate matrix is tabulated (table 1). Release of DDA from nano DDA (DDA loaded PCL nanoparticles)

occurred in a controlled manner. When the nanoparticles were exposed to the aqueous environment, the initial burst release which was less than 20% that lasted up to 4h. Later, a gradual and sustained release of drug from the nanoparticulate matrix, which increased up to 74% in 16h followed by a fall in release efficacy, was observed. At 48 h about 4% of DDA release was observed. The slower and sustained release of DDA may possibly be endorsed the diffusion of the DDA loaded within the core of the nanoparticle matrix. You ling *et al.* (2008) reported that paclitaxel loaded PLGA nanoparticles exhibited a two-staged release mode from the nanoparticle [25].

Table 1: Release efficacy of DDA from PCL polymer matrix

Time (h)	AUC (nano-DDA) (ng)	Release efficacy (%)
0.5	0	0
1	15.59 \pm 0.72	1.20 \pm 0.03
2	91.76 \pm 2.45	7.094 \pm .96
4	253.41 \pm 4.67	19.59 \pm .75
8	572.02 \pm 3.67	44.22 \pm 2.49
16	961.72 \pm 4.89	74.35 \pm 2.96
24	499.66 \pm 3.26	38.63 \pm 2.56
32	182.16 \pm 2.95	14.083 \pm 0.83
48	54.82 \pm 2.56	4.23 \pm 0.025

The values are expressed as mean \pm SD, n=3 consecutive experiments, AUC-area under curve

Oral glucose tolerance test

The effect of DDA on reducing the plasma glucose level in glucose loaded (2g/kg) rats was displayed in fig. 2. Initially, there was a sharp elevation in 30 min after glucose loading in all groups. DDA treated rats displayed a dose-dependent decline in the glucose level from 60 to 120 min similar to the control rats. However, the restoration from hyperglycemia was not observed even at 120 min in the diabetic rats, which differs significantly (P<0.05) with control rats, in which the glucose level gradually reverted back to the normal level after 30 min. The results from the observation clearly indicated that the increment in the blood glucose found in the oral glucose tolerance turned into

significantly greater in diabetic rats than the treatment groups. Zhaoxia Liu *et al.* (2013) reported that compared to diabetic control, DTH extract treated group showed significant hypoglycaemia and steady decline which exhibited a similar effect to metformin on OGTT [26]. Gerardo *et al.* (2000) reported that insulin-loaded PLGA formulation did not show any increase in blood glucose levels even at 800 mg/kg subcutaneous load of glucose to rats, which substantiated that the formulation is bioactive and useful in controlling elevated blood glucose levels [27]. Lei song *et al.* (2014), stated that the blood glucose was found to be effectively lowered in overnight fasted diabetic animals administered with nanoencapsulated insulin-chitosan complexes [28].

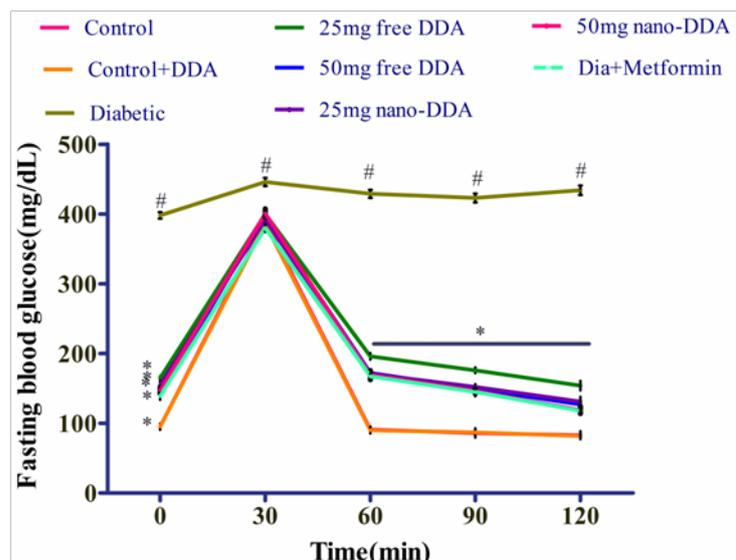


Fig. 2: Effect of free and nano-DDA on Oral glucose tolerance test in streptozotocin-nicotinamide-induced diabetic rats. Data represented as mean \pm SD, n=5.*p<0.05 as compared with diabetic group, #p<0.05 as compared with control, dia-diabetic

Concentration of glucose

The blood plasma profile of the rats after oral administration of free and nano-DDA was analysed. As displayed in table 2, the plasma of free DDA treated rats exhibited a dose-dependent decrease in glucose concentration. A major decrease in glucose level (115 mg/dl) was observed at 2h in which the T_{max} for free DDA as reported by Nagalakshmi *et al.* (2017) [12]. There was a gradual decrease in glucose level was observed which lasted up to 32h. At 48 h, an elevation in the glucose level was observed which could be due to the lesser concentration of DDA in plasma. On the other hand, the nano-DDA treated rats showed a dose dependant decrease in the

glucose concentration and a major decrease in the glucose level (102 mg/dl) at 16h, which was the T_{max} for nano-DDA as reported in our previous studies. In contrast to free DDA, nano-DDA maintained a declined glucose level of 117 mg/dl even at 48 h, which was about 189 mg/dl in free DDA treated rats at 48h. As reported by Zadeh *et al.* (2017), nanoencapsulation of insulin using PVA as a surfactant enhanced the absorption of insulin via the intranasal route of delivery [17]. Seema *et al.* (2017) reported that upon administration of repaglinide loaded microspheres to experimental diabetic rats, a glucose level of the formulation started to decrease significantly after 1 h which continued up to the eighth h indicating controlled release of drug from the formulations [29].

Table 2: Glucose concentration (mg/dl) at various time points after oral administration of nano-DDA (PVA) formulation to diabetic rats

Time(h)	free DDA	nano-DDA
0.5	143 \pm 2.4	125 \pm 2.78
1	137 \pm 1.5	122 \pm 3.67
2	115 \pm 1.37	118 \pm 2.89
4	121 \pm 1.37	112 \pm 2.65
8	126 \pm 2.65	109 \pm 2.82
16	129 \pm 2.33	102 \pm 3.98
24	131 \pm 2.9	109 \pm 2.95
32	139 \pm 2.48	115 \pm 3.1
48	189 \pm 3	117 \pm 2.7

Data represented as mean \pm SD, n=5, PVA-polyvinyl alcohol

CONCLUSION

The oral administration of nanoencapsulated DDA exhibited an enhanced oral bioavailability of DDA than free DDA, by reducing the first-pass metabolism of DDA. However, further studies using clinical trials may confirm the results obtained in this study. Henceforth, the nano-DDA can be considered as a potential therapeutic candidate in the antidiabetic drug. This work opens a door for discovery of novel potential antihyperglycemic agents.

FUNDING SOURCE

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

ACKNOWLEDGEMENT

The authors gratefully acknowledge Dr. D. Alwin, Central Animal House, SRM Medical college hospital and Research centre, SRM University, Kattankulathur-603203 for technical support in an animal study.

CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest.

REFERENCES

- Samadder A, Das S, Das J, Khuda-Bukhsh AR. Relative efficacies of insulin and poly (lactic-co-glycolic) acid encapsulated nano-insulin in modulating certain significant biomarkers in arsenic intoxicated L6 cells. *Colloids Surf B* 2013;109:10–9.
- Vijayan M, Jose R, Jose S, Abraham S, Joy J. Study on quality of life assessment in diabetic retinopathy among patients with type 2 diabetic patients. *Asian J Pharm Clin Res* 2017;10:116–9.
- IDF diabetes atlas-Home n.d. Available from: <http://www.diabetesatlas.org>. [Last accessed on 13 Jun 2017].
- Kumar R, Patel D, Prasad S, Sairam K, Hemalatha S. Antidiabetic activity of alcoholic root extract of *Caesalpinia digyna* in streptozotocin-nicotinamide-induced diabetic rats. *Asian Pac J Trop Biomed* 2012;2:S934–40.

5. Erel G, Kotmakçı M, Akbaba H, Sozer Karadağlı S, Kantarcı AG. Nanoencapsulated chitosan nanoparticles in the emulsion-based oral delivery system: *in vitro* and *in vivo* evaluation of insulin loaded formulation. *J Drug Delivery Sci Technol* 2016;36:161-7.
6. Dhana Lekshmi UM, Poovi G, Neelakanta Reddy P. *In vitro* observation of repaglinide engineered polymeric nanoparticles. *Dig J Nanomater Biostructures* 2012;7:1-18.
7. Mahapatro A, Singh DK. Biodegradable nanoparticles are an excellent vehicle for site-directed *in vivo* delivery of drugs and vaccines. *J Nanobiotechnol* 2011;9:55.
8. Soonthornsit N, Hemstapat W, Pitaksutheepong C, Pitaksuteepong T. Enhancement of stability, release and *in vitro* digestibility of mulberry stem extract using microemulsions. *Int J Pharm Pharm Sci* 2017;9:103-9.
9. Nizar Awish Jassem NAR. Formulation and *in vitro* evaluation of Azilasartan medoxomil nanosuspension. *Int J Pharm Pharm Sci* 2017;9:110-9.
10. Dash TK, Konkimalla VB. Poly-ε-caprolactone based formulations for drug delivery and tissue engineering: a review. *J Controlled Release* 2012;158:15-33.
11. Ooi JP, Kuroyanagi M, Sulaiman SF, Muhammad TST, Tan ML. Andrographolide and 14-deoxy-11, 12-didehydroandrographolide inhibit cytochrome P450s in HepG2 hepatoma cells. *Life Sci* 2011;88:447-54.
12. Nagalakshmi K, S Sujatha, D Alwin. DDA loaded PCL nanoparticles enhances the oral bioavailability of DDA in diabetes-induced experimental rats. *Int J Pharm Pharm Sci* 2017;9:198.
13. Al-marzook FA, Omran R. Cytotoxic activity of alkaloid extracts of different plants against breast cancer cell line. *Asian J Pharm Clin Res* 2017;10:168-71.
14. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol Annu Rev* 2005;11:127-52.
15. Fröde TS, Medeiros YS. Animal models to test drugs with potential antidiabetic activity. *J Ethnopharmacol* 2008;115:173-83.
16. Sarvesh Sharma, Vimal Kumar. *In vitro* cytotoxicity effect on MCF-7 cell line of co-encapsulated artesunate and curcumin liposome. *Int J Pharm Pharm Sci* 2017;9:123-8.
17. Pari L, Saravanan G. Antidiabetic effect of cogent db, a herbal drug in alloxan-induced diabetes mellitus. *Comp Biochem Physiol-C Toxicol Pharmacol* 2002;131:19-25.
18. Kamaraj N, Rajaguru PY, Issac P kumar, Sundaresan S. Fabrication, characterization, *in vitro* drug release and glucose uptake activity of 14-deoxy, 11, 12-didehydroandrographolide loaded polycaprolactone nanoparticles. *Asian J Pharm Sci* 2017;12:353-62.
19. Nasiri Zadeh S, Rajabnezhad S, Zandkarimi M, Dahmardeh S, Mir L, Ali Darbandi M, *et al.* Mucoadhesive microspheres of chitosan and polyvinyl alcohol as a carrier for intranasal delivery of insulin: *in vitro* and *in vivo* studies. *MOJ Bioequiv Availab* 2017;3:1-8.
20. Jain NK, Jain SK. Development and *in vitro* characterization of galactosylated low molecular weight chitosan nanoparticles bearing doxorubicin. *AAPS PharmSciTech* 2010;11:686-97.
21. Ihekwereme C, Esimone C, Shao D, Agu RU. Preliminary studies on validation of calu-3 cell line irritation and toxicity. *Pharmaceutics* 2014;6:268-80.
22. Atefeh Hekmat, Hossein Attar, Ali Akbar Seyf Kordi, Maryam Iman, JmR aafari. New oral formulation and *in vitro* evaluation of docetaxel-loaded nanomicelles. *Molecules* 2016;21:17-9.
23. Vimala K, Yallapu MM, Varaprasad K, Reddy NN, Ravindra S, Naidu NS, *et al.* Fabrication of curcumin encapsulated chitosan-PVA silver nanocomposite films for improved antimicrobial activity. *J Biomater Nanobiotechnol* 2011;2:55-64.
24. Sahoo SK, Panyam J, Prabha S, Labhasetwar V. Residual polyvinyl alcohol associated with poly (D,L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. *J Controlled Release* 2002;82:105-14.
25. Ling Y, Huang Y. Preparation and release efficiency of poly (lactic-co-glycolic) acid nanoparticles for drug-loaded paclitaxel. 7th asian-pacific conf. Med Biol Eng Berlin Heidelberg: Springer Berlin Heidelberg; 2008. p. 514-7.
26. Liu Z, Wan L, Yue Y, Xiao Z, Zhang Y, Wang Y, *et al.* Hypoglycemic activity and antioxidative stress of extracts and corymbiferin from *swertia bimaculata* *in vitro* and *in vivo*. *Evid Based Complement Altern Med* 2013:1-12. <http://dx.doi.org/10.1155/2013/125416>
27. Carino GP, Jacob JS, Mathiowitz E. Nanosphere based oral insulin delivery. *J Controlled Release* 2000;65:261-9.
28. Song L, Zhi Z, Pickup JC. Nanolayer encapsulation of insulin-chitosan complexes improves the efficiency of oral insulin delivery. *Int J Nanomed* 2014;9:2127-36.
29. Seema K, Megha S, Abhisek P. Ethylcellulose floating microspheres of antidiabetic agent: *in vitro* and *in vivo* evaluation. *Int J Appl Pharm* 2017;9:44-9.