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The effects of process variables on physicochemical properties and *in-vitro* cytotoxic activities of 5-fluorouracil nanoparticles against squamous cell carcinoma (SCC)

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ABSTRACT.

Background: A 2^3 factorial experimental design was used to study the effects of polymer type, Neem and Acacia (A), the nature of the organic phase (B) and the homogenization speed (C) on the particle sizes (S), the amount of drug entrapped (E) and the in-vitro release properties of the formulations ®.

Objective: This study was aimed at quantifying the effects of three production variables on the physicochemical and cytotoxic activities of 5-Fluorouracil nanoparticles.

Methods: 5-Fluorouracil polymeric nanoparticles (TD1-8) were prepared using emulsion evaporation technique. The particle sizes were estimated using the dynamic light scattering technique, in-vitro drug release studies were conducted using membrane dialysis bag, while the amount of drug entrapped was spectrophotometrically determined. The in-vitro cytotoxic activities of the formulations were estimated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

Results: The effects of A, B, C, AC, BC, and ABC on S, E and R were expressed as coefficients of the polynomial equations generated. The effects of A, B and C on E were positively correlated, but that of A and B on R and S were inversely proportional. The ranking for combined effects on the R was, AC ≤ BC≤ ABC, while that of E and S were, BC≤ ABC ≤AC and ABC≤ BC ≤AC respectively. All formulations show significantly different cytotoxic action against HSC-1 cells. Cell mortality of TD3, 6, 7and 8 were above 80% while that of TD1, 2, 4 and 5 range between 62.2-65.6 %. Interestingly 5FU polymeric nanoparticles with low particle sizes released higher percentage of the entrapped drug with corresponding higher cell mortality.

Conclusion: The two natural biodegradable polymers (Neem and Acacia) investigated were successfully used to synthesize 5 FU polymeric nanoparticles. The production parameters investigated in this study had significant effects on the physicochemical and cytotoxic properties of 5FU nanoparticles.

Key words: Biodegradable polymers, emulsion solvent evaporation technique, factorial design and 5-Fluorouracil.

Effets des variables du processus sur les propriétés physicochimiques et les activités cytotoxiques *in vitro* des 5-fluorouracil nanoparticules contre le carcinome épidermoïde (CE)

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RESUME

Contexte: Une conception expérimentale factorielle de 2^3 a été utilisée pour étudier les effets du type de polymère, Neem et Acacia (A), la nature de la phase organique (B) et la vitesse d'homogénéisation (C) sur la taille des particules (S), la quantité de médicament piégé (E) et les propriétés de libération *in vitro* des formulations ®.

Objectif: Cette étude vise à quantifier les effets de trois variables de production sur les activités physicochimiques et cytotoxiques des nanoparticules de 5-fluorouracile.

Méthodes: Des nanoparticules de polymère de 5-fluorouracile (TD1-8) ont été préparées à l'aide de la technique d'évaporation en émulsion. Les tailles de particules ont été estimées à l'aide de la technique de diffusion dynamique de la lumière, des études *in vitro* de libération de médicament ont été menées à l'aide d'un sac de dialyse membranaire, tandis que la quantité de médicament piégé a été déterminée par spectrophotométrie. Les activités cytotoxiques *in vitro* des formulations ont été estimées par dosage du bromure de 3-(4, 5-diméthylthiazole-2-yl) -2, 5-diphényltétrazolium (MTT).

Résultats: Les effets de A, B, C, AC, BC et ABC sur S, E et R ont été exprimés sous forme de coefficients des équations polynomiales générées. Les effets de A, B et C sur E étaient positivement corrélés, mais ceux de A et B sur R et S étaient inversement proportionnels. Le classement des effets combinés sur le R était, AC ≤ BC≤ ABC, tandis que celui de E et S était, BC≤ ABC ≤AC et ABC≤ BC ≤AC respectivement. Toutes les formulations présentent une action cytotoxique significativement différente contre les cellules HSC-1. La mortalité cellulaire des TD3, 6, 7 et 8 était supérieure à 80% tandis que celle des TD1, 2, 4 et 5 variait entre 62,2 et 65,6%. Fait intéressant, les nanoparticules de polymère 5FU avec de petites tailles de particules ont libéré un pourcentage plus élevé du médicament piégé avec une mortalité cellulaire correspondanteplus élevée.

Conclusion: Les deux polymères biodégradables naturels (Neem et Acacia) étudiés ont été utilisés avec succès pour synthétiser 5 nanoparticules polymères FU. Les paramètres de production examinés dans cette étude ont eu des effets significatifs sur les propriétés physico-chimiques et cytotoxiques des nanoparticules de 5FU.

Mots-clés: Polymères biodégradables, technique d'évaporation des solvants en émulsion, conception factorielle et 5-Fluorouracil.

INTRODUCTION

Natural and biodegradable polymers are undoubtedly excipients of choice in polymeric nano drug delivery system. They are cost effective, non toxic, mostly inert and are readily available in nature.[1] Neem gum (NG) and acacia gum (AG) are both biodegradable polymers with multifunctional applications in drug delivery system. They are used as emulsifiers as well as binders in tablet manufacturing.[2, 3] Various biodegradable polymers are reportedly used as nano drug delivery vehicles, with improved therapeutic efficacy in cancer chemotherapy and they may be fabricated to offer both targeted drug delivery and sustained drug release.[4]

Squamous cell carcinoma (SCC) is a type of skin cancer which can manifest in any part of the skin and other organs in the body covered by squamous cell with ability to metastasize if not adequately treated.[5] Among the non-melanoma skin cancer, SCC presented the highest mortality rate even though, basal cell carcinoma (BCC) is the most common.[6] Risk factors associated with SCC involves exposure to radiation such UV light, viral infection such as human papiloma virus (HPV), smoking, ethnicity and dietary factors.[7] Though there is no tangible epidemiological data in Nigeria, due to un-coordinated and faulty recording system in most hospitals, the incidence of SCC in recent time is on the increase worldwide.[8] Surgery (followed by plastic reconstruction) and chemotherapy or combinations of both are the most effective treatment plans adopted by most clinicians as recommended by many international guidelines and conventions. Destructive radiotherapy and photodynamic therapy with 5-aminolevulinic acid (ALA) may be used in limited cases.[9, 10, 11]

Factorial experimental design can be deployed to eliminate trial and error method that characterizes pharmaceutical formulations and/or to optimize pharmaceutical formulations within the shortest possible time.[12, 13, 14]

In this study individual and combined effects of three process variables, nature of emulsifier (A), the type of organic phase (B) and the speed of homogenization (C) on the released properties of the nanoparticles (R), the entrapment efficiency of the formulation (E) and the particle size (S) was evaluated. These were evaluated statistically at low level (–) and high level (+). Eight set of experiments were designed and carried out. Each set was designed to investigate individual effects and combined or interacting effects of the independent variables on the selected dependent variables, (a 2^3 factorial design). Table1 provides an insight to the possible experimental combinations and polynomial equations obtained by subjecting independent variable values to multiple regression analysis Eq1.

$$Y = \beta_0 + \Sigma \beta_1 x_i + \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad Eq1$$

Where Y is the dependent variable, β_0 is the mean response of the experimental runs and β_1 is the coefficient of factor x

Table 1: Batch formulary for the nanoemulsion

Batches	Code	A	B	C (r/s)
TD_1	$A_LB_LC_L$	NG	CM	900
TD_2	$A_HB_HC_L$	AG	AM	900
TD_3	$A_HB_HC_H$	AG	AM	1200
TD_4	$A_LB_HC_L$	NG	AM	900
TD_5	$A_HB_LC_L$	AG	CM	900
TD_6	$A_HB_LC_H$	AG	CM	1200
TD_7	$A_LB_HC_H$	NG	AM	1200
TD_8	$A_LB_LC_H$	NG	CM	1200

METHODS

Materials

5-Fluorouracil, Dulbecco's Modified Eagle's Medium (DMEM) and tetrazolium dye, 3-(4,5-dimers dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich, (St Louis, MO, USA), Fetal bovine serum from Himedia India, while Acacia and Neem gum powders were prepared and standardized in the formulation laboratory in the Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University Sokoto (UDUS). All other reagents used were analytical grade. Milli-Q water was from Millipore Corp. (Billerica, MA, USA)

Methods

Drug/excipient interaction studies:

Fourier-transform infrared (FTIR) spectroscopy technique was used to study the possible drug /excipient interactions. Known quantities of 5FU, Neem and Acacia powders were mixed separately with analytical grade KBr (1:100 ratio) and thereafter compressed into pellets with hand held hydraulic press.The pellets were then scanned in an inert atmosphere over a wave number range of 4000–400 cm^{-1} in a Magna IR 750 series II FTIR instrument (Jasco, FTIR 4200, Japan). The procedure above was likewise repeated for a mixture of the drug and the excipients, (5 FU, Neem and Acacia).

Production of polymeric nano particles

Batch production of 5 FU polymeric nano particles were carried out as reported by Piñón-Segundo et al., (2012). This allow for the investigations into the effects of production variables on the physicochemical and released properties of 5 FU nan of or mulation produced by emulsion solvent evaporation technique (ESET). Mucilage of the gum as specified in the batch formulary (table 1) was prepared using Milli-Q water and set on a magnetic stirrer. The drug dissolved in the organic phase was thereafter added drop wise to the aqueous phase with continuous stirring. The homogenization seed was carried out using a high-speed homogenizer (IKA Laboratory Equipment, Model T10B Ultras-Turrax, Staufen, Germany) for an hour before raising the temperature to 50 ^{0}C while the homogenization was continued for another 30 minutes to evaporate the organic solvent. The nano particles were thereafter harvested by centrifugation at 45,000 rpm for 5 minutes. The obtained nano particles were properly washed and dried and kept in -40 ^{0}C freezer and lyophilized. The dried nano particles were then transferred to an airtight labelled container and stored in 4 ^{0}C refrigerator until when needed.[15]

Morphology of the formulated 5 FU polymeric nanoparticles

Transmission electron microscopy (TEM) was used to study the morphology of the formulations. Sample of lyophilized 5 FU nano particles were re-suspended in Milli-Q water and fixed on standard carbon coated copper grid (mesh). The mesh was then air dried for 5 hours before loading it into the TEM machine. The morphology of the nano particles was examined and images captured using a transmission electron microscope (TEM), (FEI type FP5018/40 Tecnai G2 Spirit Bio TWIN, USA).

Determination of particle size and zeta potential

A Zeta sizer, nano ZS with DTS software (Malvern Instruments Ltd., UK) was use to obtain the average particle size. The Nano ZS uses dynamic light scattering technique to estimate the average particle size at an angle of 1730 and temperature of about 25 0C. Prior to the measurements, all samples were diluted with Milli-Q water in disposable cells to have a suitable scattering intensity. The zeta potential determination was carried out in a disposable plain folded capillary zeta cells. The zeta potential was calculated from the electrophoretic mobility of nano particles under an electric field of 40 V/cm and application of Helmholtz–Smoluchowski equation.[16]

Determination of percentage drug entrapped

The success of the formulation depends on the ability and the amount of drug entrapped by the polymers. Known weight (1mg) of the formulation was re-suspended in 5mL of phosphate buffer solution (PBS pH 7.4) and sonicated with a probe sonicator operating at frequency of 20kHz for about 20 seconds, (150VT, Biologics, Inc USA) to liberate the entrapped drug. The resulting solution was filtered and amount of drug spectrophotometrically determined using a UV-Vis spectrophotometer, (Beckman 220 Instruments, Fullerton, CA, USA) operating at 266 nm.

***In-Vitro* drug release studies**

Estimations of the amount of drug released from the formulations were carried out using a BP dissolution apparatus. A known weight of the formulation was placed in a dialysis membrane bag (MWCO 20 kDa ; Spectrum Labs, Rancho Dominguez, CA). The two end of the dialysis membrane were tied and the bag was suspended with the aid of a thread into the dissolution media, (1000mL phosphate buffer solution pH 7.4 PBS). At regular interval 5mL of the dissolution media was withdrawn, filtered and

the amount of drug released was quantitatively determined using a UV-Vis spectrophotometer, (Beckman 220 Instruments, Fullerton, CA, USA) operated at 266 nm. Equal volume of fresh PBS was however added to replace the withdrawn sample.

MTT assay for *in-vitro* cell viability studies

Trypsinized and monolayer confluent HSC-1 cell in the exponentially growing phase were used for this cytotoxicity experiment. The cells were cultured in DMEM media devoid of phenol red but supplemented with 10% fetal bovine serum (FBS). The cell culture medium was maintained at 37°C in a humidified incubator (Model MCO-15AC; Sanyo Electric Biomedical Co. Ltd., Osaka, Japan) containing 5% CO_2 atmosphere. The cells were plated in 96 well plate at density of 5×10^3 per well and were incubated for 12 hrs after which the media were carefully removed and replaced with reconstituted 5 FU nanoparticles formulation and re incubated for 48 hrs and the MTT dye solution were added to the wells and the incubation continued for another 4 hrs. At the end of the incubation, the medium in each well containing unbound MTT and death cells was removed by suction while formazan crystals were solubilized with 100 µLdimethylsulfoxide, and the solution was vigorously mixed to dissolve the reacted dye and was removed by suction. To estimate the amount of viable cells left, the well plates were introduced into the microplate reader (multimode plate reader, SpectraMax M5; Molecular Devices, CA, USA) and the absorbance of each well was read at 540 nm.

RESULTS

The FTIR spectra of pure Neem gum powder (NG), Acacia gum powder (AG), and 5FU and the mixture (5FU and polymers) were presented in figure 1, while the results of the transmission electron microscopy and the in-vitro cytotoxicity of the nanoparticles were as presented in figure 2 and 3 respectively

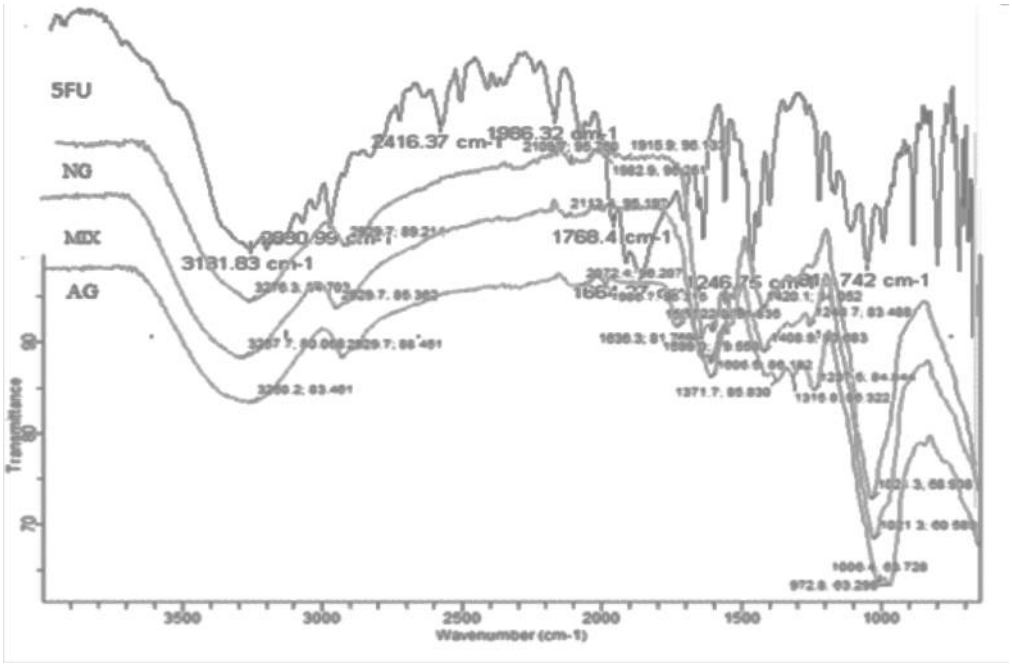


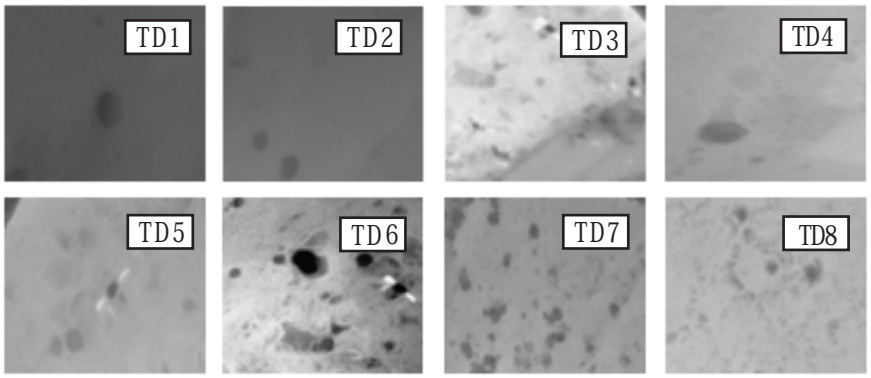
Figure 1: FTIR spectra of Neem, Acacia, 5FU and the mixture (MTX)

Values for the percentage drug released (R), the percentage drug entrapped (E), the average particle size (S), the yield and the zeta potential were presented in table 2.

Table 2: Selected properties of the polymeric nanoparticles of 5 FU

Properties	TD_1	TD_2	TD_3	TD_4	TD_5	TD_6	TD_7	TD_8
R (%)	66.6	68.2	86.4	75.4	75.6	89.6	`90.3	99.8
E (%)	88.2	88.4	58.8	82.4	88.2	56.8	58.1	57.8
S (nm)	118.0	118.0	60.0	98.0	99.0	58.0	58.5	62.0
Yield (%)	70.01	70.02	70.67	70.24	70.22	70.87	70.80	70.45
Zeta(mV)	13.04	13.04	13.01	13.03	13.03	13.01	13.01	13.01

Keys: R = cumulative drug released (%), E = % drug entrapped, S = mean nanoparticles sizes



Y_R= 83.9 -1.54A – 1.41B + 7.54C – 11.6AC – 10.6BC + 16.4ABC Eq2

Y_E = 68.6 + 2.0A + 0.84B + 6.8C + 13.6AC + 5.7BC +11.4ABC.....Eq3

Y_S=86.6 – 5.0A – 3B – 22C +110AC +66BC -330ABC.....Eq4

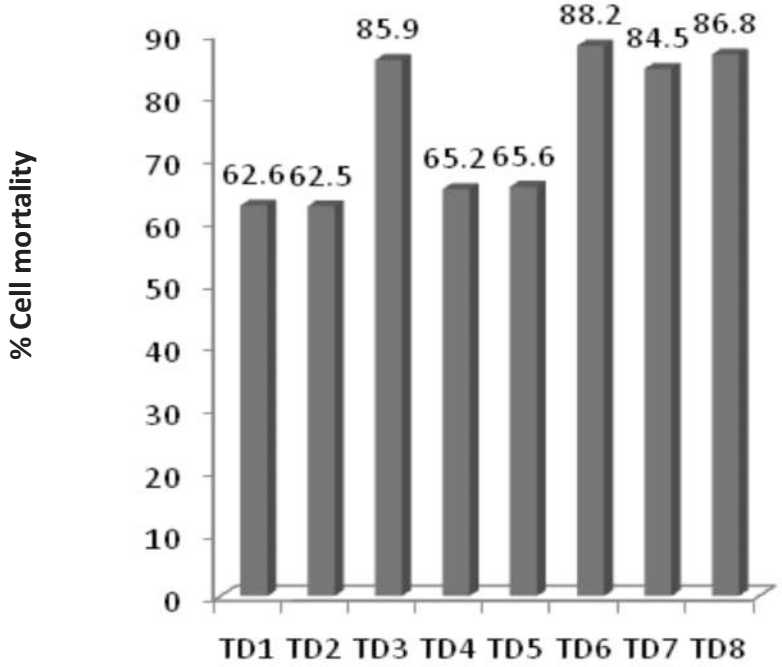


Figure 3: *In-vitro* cytotoxicity activities of 5 FU nanoparticles formulations

DISCUSSION

The NG, AG and 5FU spectra gave all the characteristic peaks, indicating that the materials are in the pure states. All the major peaks for the pure 5FU were equally observed in the mixture, indicating that there was no chemical interaction between the drugs and the excipients. The minor shifting in the peaks may be due to some beneficial physical interactions necessary for the formation of the nano particles.[17]

In this study, the two biodegradable polymer investigated were successfully utilized in production of polymeric nano particles of 5FU, with yield that varies from batch to batch, (70.01-70.87 %). The differences in batch to batch yield were statistically insignificant p(0.05), and were high enough for mass production of the nano particles. The nano particles were all spherically shaped with average particle size ranging from 50 – 118 nm, (figure 2 and table 2). Formulations produced with high homogenization speed (TD3, 6, 7 and 8) had particle sizes below 90nm, while those (TD1, 2, 4 and 5) produced with low speed had particle sizes above 90 nm. Increasing the speed of homogenization resulted in significant reduction in the dimension of the nano particles, ($p \leq 0.05$). Iacovella and Glotzer 2012 reported that the method and the speed of homogenization greatly affect the particle sizes of nano sphere.[18] The ability of a polymer to entrap drug and serve as a drug delivery vehicle is an intrinsic property unique to each polymer which may affect its physicochemical properties and by extension its pharmaceutical usefulness.[19,20]

The % drug entrapped (E) ranged from 56.8- 88.4. The batches produced with low homogenization speed (TD1, 2, 4 and 5) all entrapped 5 FU well above 80% while those with high homogenization speed TD3, 6, 7 and 8 have E values blow 60%. Higher E value is most desirable in nano particles production in order to minimize wastage. All the formulations show significant difference in their in-vitro drug release profile (R). However batches produce with high homogenization speed had R values above 80% while those with low speed had values below 80%. These observed differences were statistical significant at $p \leq 0.05$ and the speed of homogenization (C) appears to have the greatest influence when compared to the polymer nature (A) and type of organic phase (B) and this is supported by the polynomial equations 2,3 and 4 derived from the data obtained.

The ranking for the individual effects (A, B and C) on S, E and R follow the same pattern $C \geq A \geq B$. The interacting effects of AC, BC, and ABC on R, E and S were all significant ($p \leq 0.005$) and were provided by the polynomial equation 2,3,and 4. ABC had a positive influence on E and R but an inverse proportion on S. BC and AC on the other hand had positive effects on S and E but an inverse proportion effect on R. The ranking for the interacting effects on S and E were $AC \geq BC \geq ABC$ and $AC \geq ABC \geq BC$ respectively, while the ranking for R was $ABC \geq AC \geq BC$. While the polymer type (A) and the homogenization speed (C) mostly influenced the nano particles sizes and the percentage drug entrapped, the release properties of the nano particles were greatly influenced by all the factors ABC. Careful selections of these variables is therefore of great importance in the production of polymeric nano particles by emulsion solvent evaporation methods. The cytotoxic activities of the formulations were given in Figure 3. There were significant differences, ($p \leq 0.05$) in cytotoxic activities of the formulations. Interestingly, TD3, 6, 7 and 8 which had smaller particle sizes and higher percentage of drug release resulted in more deaths of HSC-1 cells as compared to TD1, 2, 4 and 5. Drug formulations with smaller particle sizes had shown improved therapeutic activities because the smaller particle sizes present increased surface area which aid faster drug dissolution and absorption since drug particle were able to pass through physiological barrier more easily. [21]We were unable to carry out an in-vivo study on the optimized formulations due to lack of fund.

CONCLUSION

This study adequately investigates the use of natural biodegradable polymers as nano drug delivery vehicles and the results obtained show that both polymers are suitable for the production of polymeric nano particles of 5 FU and the three factors investigated, individually and jointly affect both the physicochemical properties and cytotoxic activity of 5FU nano particles.

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