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Quantitative effects of formulation process variables on antitumor activities of doxorubicin nanoparticles

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ABSTRACT

Drug therapy failure is still a recurring clinical phenomenon attributed to a host of factors among which are formulations shortcomings resulting from processing and manufacturing variables leading to different therapeutic effectiveness of pharmaceutical products of the same chemical entity but manufactured by different companies. This study was aimed at investigating the effects of process variable on therapeutics activities of doxorubicin nanoparticles using a 2^3 factorial experimental design to study the effects of three independent variables, the production technique (A), the homogenization speed (B) and the drying technique (C) on the therapeutic activity(antitumor) of doxorubicin nanoparticles. Eight (8) different formulations, (DX_{1-8}) of doxorubicin nanoparticles were prepared using both single and double emulsion evaporation techniques. The yield and the amount of drug entrapped were mathematically calculated. The release profiles of the formulations were determined using a type II USP dissolution apparatus and a UV spectrophotometer. Estimations of therapeutic effectiveness of the formulations were carried out through cell mortality assay using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) technique. Doxorubicin nanoparticles formulations were successfully prepared with yield ranging from 72.9-90.5% indicating high scale up possibility. The differences in antitumor activities observed were significant ($p \leq 0.05$), which may be as a result of marked differences in percentage drug entrapped and drug release profile of the formulations. The factors investigated in this study independently and jointly affected the antitumor activities of the doxorubicin formulations significantly and were ranked, A ≥ AC ≥ C ≥ ABC ≥ B. This study clearly linked therapeutic effectiveness (cytotoxicity/antitumor) of doxorubicin nanoparticles to the manufacturing variables. There is therefore the need for pharmaceutical manufacturers to optimize their manufacturing processes to prevent drug therapy failure.

Keywords: Breast Cancer, Doxorubicin, Factorial design and Drug therapy failure.

1 INTRODUCTION

Pharmaceutical processing exposes active pharmaceutical ingredients to range of physical treatments such as drying, granulation, emulsification etc. Batch to batch variation in quality of pharmaceutical formulations may due to different manufacturing process variables of which the types of equipment used, intrinsic nature of pharmaceutical aids, drying techniques and method of manufacturing are most prominent (KUMAR, *et al*., 2013).

The nature of the polymers used as drug delivery vehicle was reported to significantly altered the mechanical and the disintegrating properties of paracetamol tablets (AKIN-AJANI, *et al.*, 2005). Likewise the mode of incorporating the disintegrants and the drying techniques were shown to significantly affect the physicochemical properties of paracetamol and metronidazole tablet formulations (ODEKU & AKINWANDE, 2012; OYENIYI & ACHOR, 2014).

The release profile of the active agent from the formulation was equally shown to be dependent on the process and manufacturing variables (BEJUGAM, *et al.,* 2005). There is therefore the need to quantify the effects of the production variables on the therapeutic action of the pharmaceutical formulation with the aim of optimizing the drug manufacturing processes and guide against drug therapy failure (DTF) which is a clinical situation in which the therapeutic responses by the patients fail to meet up the standard responses even though the right pharmaceutical products were administered at the right dosage regimens (FIGUERAS, *et al*., 2003).

Many factors are reported to be responsible for drug therapy failure of which adulteration of pharmaceutical products, drug faking, patient's non-adherence, a drug's poor bioavailability or lack of efficacy, medication error, adverse reactions, drug resistant, tissue necrosis and formulation shortcomings are prominent among others (BARIWAL, *et al.,* 2019). DTF is a big concern in cancer chemotherapy, inherent resistant of the cancerous cells due to heterogeneity of most cancer types is considered as root cause of all drug therapy failure (ALFAROUK, *et. al.,* 2015; WANG, *et al.,* 2018).

Most factors enumerated above with the exception of formulation shortcomings had received adequate investigations and solutions proffered by way of recommendations. Formulation shortcomings are variation in drug effectiveness (cytotoxicity per dose administered) arising from product manufacturing variables that may have significant effects on the therapeutic usefulness of the formulated pharmaceutical products.

Breast cancer which is the abnormal and uncontrolled cell growth of the breast still remains the most invasive cancer in women and the second leading cause of all cancer related deaths in women. The triple-negative breast cancer (TNBC) growth and developments are not associated nor linked to presence of estrogen, progesterone receptors and human epidermal growth factor receptor 2 (HER2) proteins (NEVE, *et al.,* 2001; IQBAL, *et al.,* 2014). TNBC is generally more aggressive

(metastasizing quickly to other tissues in the body) and still retain the lowest 5 year survival rate of about 58% compared to 80% of non-triple negative breast cancer (NTNBC), (Gonçalves, *et al., 2018*; ANDERS, *et al., 2016*; PEROU, *et al.,* 2000).

Doxorubicin still feature prominently in cancer chemotherapy and the choice of MDA-MB-231 cell line for this investigation is base on the fact that, MDA-MB-231 is a triple-negative breast cancer cell line and a versatile tool for many cancer-related research applications, including observing drug effects, migration and invasion assays (PARK, *et al.,* 2018; JAIN, 2000).

This study is aimed at quantifying the effects of production technique (A), speed of homogenization (B) and the drying method (C) on the *in-vitro* cytotoxic activities, (T_R) of doxorubicin nanoparticles using a 2^3 factorial experimental design.

2 MATERIAL AND METHODS

2.1 MATERIALS

MDA-MB- 231 cell line from Bioarry, (U.S.A), Doxorubicin, 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 and gelatin from Himedia (India).All other reagents used are analytical grade. Milli-Q water was from (Millipore Corp., Billerica, MA, USA).

2.2 EXPERIMENTAL DESIGN

A 2^3 factorial experimental design was used to assess the influence of production technique (A), speed of homogenization (B) and the drying method (C) on the *in-vitro* cytotoxic activities of doxorubicin nanoparticles (T_R), the yield of the nanoparticles (Y) and the degree of drug entrapment by the polymers (EE). The three independent variables were evaluated at two statistical levels (high (H) or low (L) levels).

For A, double emulsion evaporation was considered as the high level while the simple emulsion evaporation technique was the low level. Nanoparticles dried using vacuum drying technique was the high level while those lyophilized were consider as the low level. The high and low levels of homogenization speed were 1500 and 1000 rpm respectively. The eight possible experimental combinations are; $A_LB_LC_L$, $A_LB_HC_L$, $A_LB_HC_H$, $A_LB_LC_H$, $A_HB_LC_L$, $A_HB_HC_L$, $A_HB_HC_H$ and $A_HB_LC_H$. Polynomial equations were obtained by subjecting independent variables to multiple regression analysis Eq1.

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$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 \quad \text{......... .... Eq1.}$$

2.3 FORMULATION OF POLYMERIC NANOPARTICLES

Doxorubicin nanoparticles were fabricated in batches according to table 1. A 0.1 % w/v solution of doxorubicin in diethyl ether was prepared and transferred into a 200 mL beaker set on variable speed magnetic stirrer, (PCE- MSR 150 GmbH, Germany). Similarly 0.2 % w/v aqueous gelatin suspension (to which 1mL of Sodium dodecyl sulphate was added) was equally prepared in a test tube and added drop wise into the organic solution of doxorubicin while the magnetic stirring was continue at the speed dictated by the batch formulary (table 1) to form the primary water in oil emulsion (W/O) (JAIN, 2000; McCALL, 2013).

The W/O emulsion was thereafter converted to a nano suspension by continuous stirring on the magnetic stirrer for about 4 hrs in order to remove the organic solvent. The nanoparticles were thereafter harvested by ultracentrifugation at 13,400 rpm for 30 minutes at 4°C. Batches to be dried by vacuum drier were conducted in a vacuum dryer (model LC 6210, Zhengzhou, China) operating at 20 ^{0}C while all other batches were freezed at – 40 ºC and lyophilized using a lyophilizer (FD-10-TP Labfreeze Instrument, Hunan China) (LEE, *et al.,* 2013; LEHMANN, *et al.,* 2011; GUNDLOORI, *et al.,* 2019).

Table 1 - Batch Formulary for production of doxorubicin nanoparticles

Batches	**Experimental Combination**	**Production Variables**
DX_1	$A_L B_L C_L$	• Simple emulsion • 900 rpm • Spray drying
DX_2	$A_L B_H C_L$	• Simple emulsion • 1,200 rpm • Spray drying
DX3	$A_L B_H C_H$	• Simple emulsion • 1,200 rpm • Freeze drying

DX4	$A_LB_LC_H$	• Simple emulsion • 900 rpm • Freeze drying
DX5	$A_HB_LC_L$	• Double emulsion • 900 rpm • Spraying drying
DX6	$A_HB_HC_L$	• Double emulsion • 1,200 rpm • Spraying drying
DX7	$A_HB_HC_H$	• Double emulsion • 1,200 rpm • Freeze drying
DX8	$A_HB_LC_H$	• Double emulsion • 900 rpm • Freeze drying

For the double emulsion evaporation technique the primary emulsion (W/O) was prepared as described above. The resulting emulsion was however re-dispersed in the aqueous phase and homogenized for about 25minutes to form the double emulsion (W/O/W) (IQBAL, *et al.,* 2015). The double emulsion was however converted to nanoparticles suspension by evaporation of the organic solvent through continuous magnetic stirring for 4 hours. Harvesting and drying of the nanoparticles were as described above.

2.4 DETERMINATION OF PERCENTAGE YIELD

The weight of the nanoparticles produced was determined and expressed as the percentage of the raw material used, (OYENIYI, et al.,2017a).

$$\text{Yield} = \frac{\text{weight of obtained nanoparticles}}{\text{weight of raw materials used}} \quad \text{.........Eq2}$$

2.5 SIZE DISTRIBUTION AND ZETA POTENTIAL

Size distribution and zeta potential measurement was performed using a Zetasizer nano ZS (Malvern Instruments Ltd., UK). Non-invasive backscatter optics technique was applied in all determination which was carried out on the formulation suspended in PBS 7.4

2.6 DETERMINATION OF ENTRAPMENT EFFICIENCY

Known quantity of the nanoparticles was suspended in about 1mL phosphate buffer 7.4 (PBS) and sonicated using a probe sonicator to liberate the drug from the polymeric shell. The resultant dispersion was passed through a membrane filter with pore sizes of about 0.22 µm. The amount of doxorubicin in the filtrate was determined spectrophotometrically (RAMA, *et al.,* 2014). The encapsulation efficiency (EE) of the formulation was calculated using equation 2:

$$EE = \frac{\text{Amout of drug in the Filtrate}}{\text{initial amount of drug added}} \quad \ldots\ldots\ldots\ldots\text{Eq.3}$$

2.7 *IN-VITRO* DRUG RELEASE STUDY

The release study was conducted using the USP dissolution test apparatus (type II). Known weight of the sample was introduced into the dissolution basket completely wrapped will dialysis membrane (0.01 µm pore size). This was then suspended in a 1L beaker containing 900mL of freshly prepared PBS 7.4 maintained at 37 ^{O}C. At predetermined time interval 5mL of the media was withdrawn and replaced with fresh PBS 7.4. The quantities of doxorubicin released from the nanoparticles formulation were determined UV-Vis spectrophotometer (Beckman Instruments, Fullerton, CA, USA) operating at 480nm and expressed as percentage cumulative drug release (United States Pharmacopeia, 2008).

2.8 CELL VIABILITY ASSAY

The cytotoxicity of each batch was evaluated through the MTT assay which estimates the amount of viable cells after treatment of known quantity of MDA-MB-231 cell line with the formulations. MDA-MB-231 cells were cultured in Dulbecco's modified eagle's medium (DMEM) without phenol red and supplemented with 10% fetal bovine serum. The cell culture medium was maintained at 37°C in a humidified incubator containing 5% CO_2 atmosphere.

The cultured MDA-MB-231 cells were sure to be trypsinized confluent cell and mono layers and free flowing when examined under microscope. The cells in the exponentially growing phase were used for cytotoxicity experiments. Specifically the cells were plated at a density of 5×103 cells/well (optimal seeding density) in 96 well plates and kept at 37°C in 5% CO_2 atmosphere in a CO_2 incubator (Model MCO-15AC; Sanyo Electric Biomedical Co. Ltd., Osaka, Japan).

After 12 hours of incubation, the medium in the wells was replaced with fresh medium treated with the PBS solution of the formulations (1µg/mL). After 48 hours, MTT dye solution was added to each well and the incubation was continued for another 4 hours. The medium in each well containing unbound MTT and death cells was removed by suction. The formazan crystals were solubilized with 100 µL dimethylsulfoxide, and the solution vigorously mixed to dissolve the reacted dye. The absorbance of each well was determined by reading the, optical density (OD) values at 595nm using DMSO as a blank. The amount of viable cells was thereafter determined using equation 4 (OYENIYI & BISWAJIT, 2017b).

Cell viability (%) = Mean OD/Control OD x 100% …………………… Eq. 4

2.9 STATISTICAL ANALYSIS

Data are presented as the average mean ± standard deviation. The significance of the difference between treatment groups was evaluated using unpaired Student's two-tailed t-test. $p \leq 0.05$ was considered statically significant.

3 RESULTS AND DISCUSSION

3.1 YIELD

The yield is an important criterion when making judgment about mass production of the nanoparticles (RAVAL, *et al.,* 2018). The yield of doxorubicin nanoparticles ranges from 72.9 to 90.5 %. This is high enough to justify scale up and mass production of doxorubicin nanoparticles by both techniques investigated in this study, table 2. Drying is one of the key unit operations in pharmaceutical manufacturing processes of which the method of drying and the equipment used had been shown to affect the quality and stability of pharmaceutical products (EMANI, *et al.,* 2018; IZUTSU, 2018; VASS, *et al.,* 2019).

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The drying methods (B) had a significant ($p \leq 0.05$), effect on the yields as batches of doxorubicin nanoparticles dried by freeze drying (DX1, 2, 5 and 6) gave a lower yield when compared to those dried using tray vacuum dryer (DX3, 4, 7, and 8). Freeze drying is multistage processes in which some particles of drug may be lost during final stage (sublimation) and this may be responsible of it comparative lower yield. The nature of polymer (A) and the speed of homogenization (C) equally had significant effects ($p \leq 0.05$) on the yield of the nanoparticles. The quantitative effects of A, B, C and the interacting effects (AC,BC, and ABC) on the yield of doxorubicin nanoparticles were ranked, AC =C ≥ BC= ABC ≥ A ≥ B and data generated (table2), were best fitted to a polynominal equation (Eq. 5).

$$Y_{(yield)} = 80.9 + A - 0.7B + 7.7\,C + 7.7\,AC - 5.4BC - 5.4ABC \ldots\ldots\ldots\text{Eq. 5}$$

Advocacy for the use of optimization equations expressing the contributing effects of each independent variable and their interacting effects before approving drug manufacturing processes is base on the fact that optimization equation saves time and minimizes wastage (AUDU-PETER & IBRAHIM, 2013). The drying technique (C) is observed to be the most influential factor and must be given adequate considerations for optimal yield (PATIL & BHOSKAR, 2014).

3.2 PARTICLE SIZE AND ZETA POTENTIAL

The particle sizes of all the formulations were in nano scale, ranging from 181 to 254nm. The particle size of nanoparticles is critical to the absorption, distribution and the release of the active drug molecules from the formulation, similarly cellular uptake of the nanoparticles may be facilitated by fabricating nanoparticles with smaller dimensions (HALEY & FRENKEL, 2008).

All the doxorubicin nanoparticle formulations investigated in this study were negatively charged. Equally charged nanoparticles were reported to be more stable as the nanoparticles will remain dispersed with the formulations (GUPTA*, et al.,* 1988).

Table 2 - Some Selected Properties of doxorubicin nanoparticles formulations

Batches	Combinations	Yield (%) ± SD	Particle size (nm) ± SD	ZP (mV)	CDR (%) ± SD	EE (%) ± SD	CV (%) ± SD
DX_1	$A_LB_LC_L$	72.9±0.02	254±0.03	-43.3	70.8±0.04	78.1 ±0.02	21.0 ±0.04
DX_2	$A_LB_HC_L$	73.1±0.02	198±0.04	-41.5	75.2±0.03	89.3±0.02	21.1±0.02
DX_3	$A_LB_HC_H$	84.2±0.01	195±0.01	-41.4	75.1±0.02	90.3±0.01	20.9 ±0.03
DX_4	$A_LB_LC_H$	89.3±0.02	232±0.02	-44.2	69.9±0.02	78.0±0.02	19.1± 0.02
DX_5	$A_HB_LC_L$	73.4±0.02	196±0.04	-43.4	86.6±0.03	92.5±0.01	12.1±0.02
DX_6	$A_HB_HC_L$	73.1±0.02	181±0.04	-38.0	97.1±0.04	98.5±0.03	10.1±0.02
DX_7	$A_HB_HC_H$	90.4±0.03	182±0.02	-38.2	95.6±0.01	98.6±0.02	10.8±0.02
DX_8	$A_HB_LC_H$	90.5±0.02	192±0.04	-40.0	86.8±0.02	92.6±0.02	11.3± 0.01

Keys: EE = % drug entrapped, CDR =% cumulative drug released, CV = % Viable Cells, ZP= Zeta potential

3.3 DRUG ENTRAPMENT

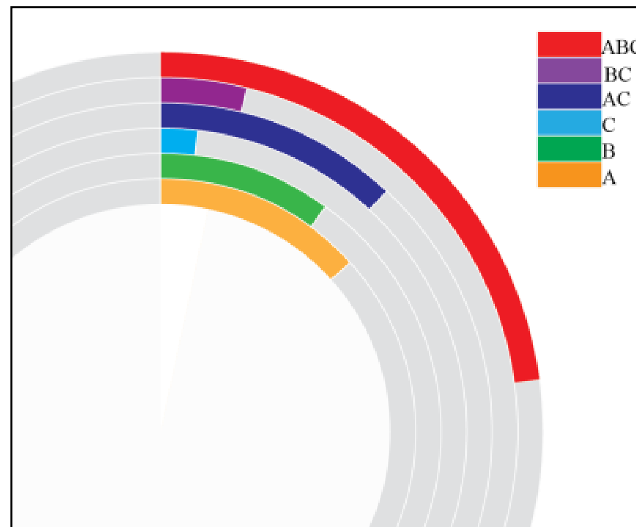
Different levels of entrapment were achieved for the eight formulation batches showing clearly the effect of these formulation factors on the amount of drug entrapped. The differences in entrapment efficiency values (EE) were significant ($p \leq 0.05$) indicating the need for judicious selection of manufacturing procedures. Even though EE of nanoparticles were reported to depend on the intrinsic properties and nature of the polymers employed in the manufacturing processes (LIECHTY & PEPPAS, 2012).

This study has clearly shown that other manufacturing variables such as the processing techniques play significant and unique roles for development of successful drug delivery system (NITTA & NUMATA, 2013). In this study the EE values were ranked $DX_4 \leq DX_1 \leq DX_2 \leq DX_3 \leq DX_5 \leq DX_8 \leq DX_6 \leq$ DX7. The effects of A, B, C and their interactions on EE were significant ($p \leq 0.05$) and the contributions of each of these on drug entrapped were expressed in equation 6.

$$Y_{EE} = 102.1 + 5.8A + 4.4B + 0.9C + 5.2AC + 4.0BC + 23ABC \text{..........Eq.6}$$

The ranking for the individual and the interacting effects was ABC ≥ A≥ AC≥ B≥ BC≥ C, fig 1. Generally the higher the EE values, the better the polymer usefulness as drug delivery vehicle (AGNIHOTRI, *et al.*, 2004).

Figure 1 - Effects of A, B, C, AC, BC, and ABC on EE



3.4 *IN-VITRO* DRUG RELEASE PROFILE

The release profile of a drug from the formulation is one of the key determinants of drug therapeutic responses of drug since the active drug must be released and transported to the receptor site before it can elicit its pharmacological action. Generally the release of the drug is influenced by both formulation and non formulating factors (ESIM, *et al.,* 2018; LINDNER & HOSSANN, 2010; ONNAINTY & GRANERO, 2019).

The data revealed no incident of dose dumping as all the formulations released the entrapped drug in prolong and sustained manners (Fig 2a &b). The factors investigated in this study individually and in combinations had remarkably significant ($p\leq$ 0.05) effects on the release profiles of the nanoformulations.

Figure 2 - Release profiles of doxorubicin nanoformulations, a and b.

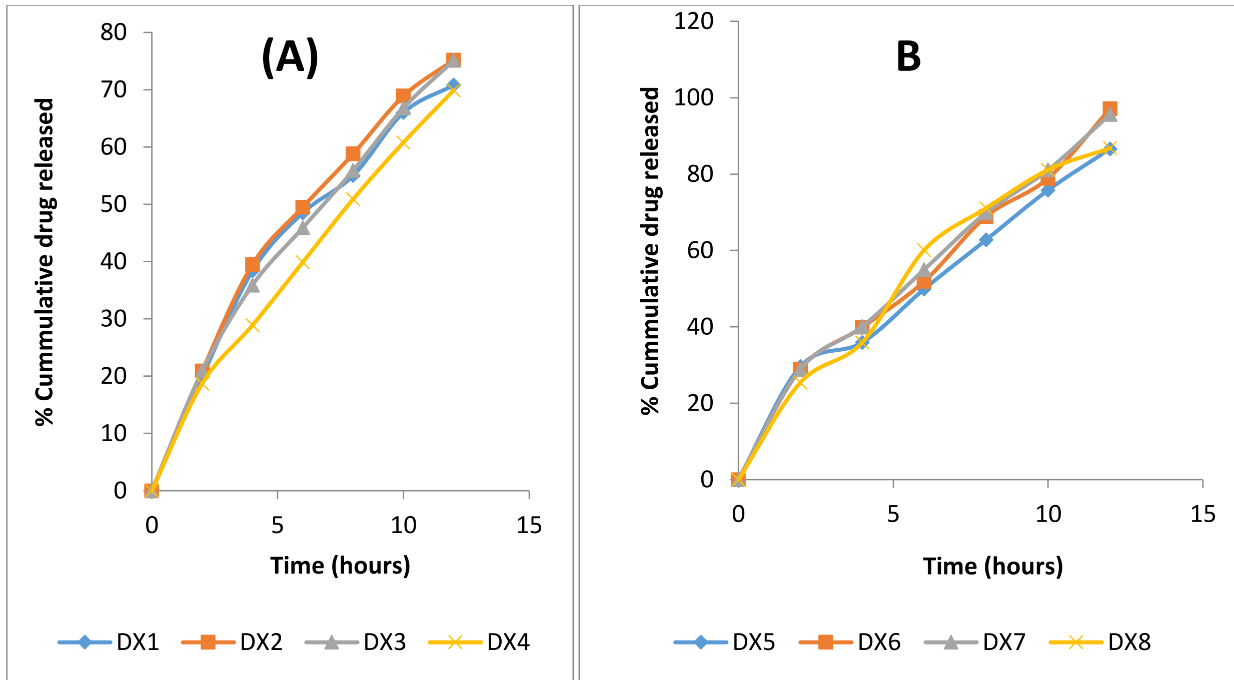
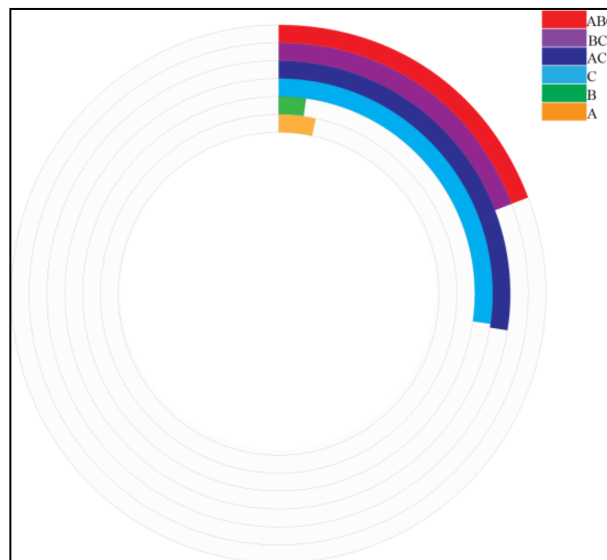


Figure 3 - Effects of A, B, C, AC, BC, and ABC on CDR



The optimized quantitative contributions of A, B, C, AC, BC, and ABC on percentage cumulative drug released CDR were as expressed in eq.7 and fig.3 which shows that the production technique had the most significant effect on CDR, this may be due to that fact that the double emulsion evaporation technique was able to entrapped higher amount of the active drug during the manufacturing processes.

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$$Y_{CDR} = 82.1 + 9.4A + 3.6B - 0.3C - 2.8AC - BC - 10.2\ ABC \ldots\ldots\ldots\ldots Eq.7$$

The ranking for the individual effects and the interacting effects of the independent variables on CDR was ABC ≥ A≥ B≥ AC≥ BC≥ C (fig 3) indicating that the speed of homogenization (C) had the most remarkable significant effect on CDR.

3.5 CYTOTOXICITY OF THE FORMULATIONS

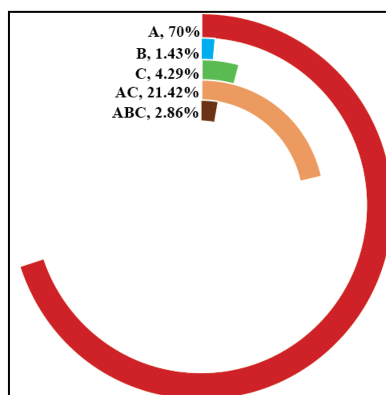
The antitumor activity of each formulation was presented as the percentage number of viable tumor cells at the end of MTT assay. Each of the formulation gave different values of cytotoxicity and the quantitative effects of the production variables on cell cytotoxicity were significant ($p \leq 0.05$), and the data obtained were used to generate polynomial equation (eq.8)

$$Y_{VC} = 15.8 - 4.9A - 0.1B - 0.3C + 1.5AC + 0.2ABC \ldots\ldots\ldots\ldots Eq.8$$

Figure 3, shows the percentage contributions of each independent production variables and the interacting effect on the antitumor activity of the produced nanoparticles. Changing the production technique from simple to double emulsion evaporation improved the antitumor actions formulations. This may be due to the fact that the batches produced by double emulsion evaporation technique (DEET) incorporated and released higher percentage of doxorubicin as shown in table 2.

There interaction between speed of homogenization of the emulsion and the drying technique (BC) was zero, suggesting that BC had no effect on antitumor actions of the formulations, Eq. 8. The ranking of individual and combined effects on cytotoxicity of the formulations was, A ≥AC ≥C ≥ABC ≥B, (fig. 4).

Figure 4 - Spiral chart showing the contributions of A, B, C, AC and ABC on antitumor activities of doxorubicin nanoparticles



Pharmaceutical manufacturers are not under any obligation to fully disclose their production technique which invariably remain trade or technological secrete but unfortunately this is key determinant of the therapeutic activities of pharmaceutical products. Investigating and quantifying the effects of production variables on therapeutic action of drugs is wholesome and may serve as excellent guide for future manufacturing activities.

This may also be a wakeup call on regulative authorities to mandate pharmaceutical companies to demonstrate the effects of their adopted production techniques on the expected therapeutic activities of their products before final approval is given for registration, manufacturing and sale of pharmaceutical products.

4 CONCLUSION

Over the years researcher had attempted to optimized production processes without directly focusing on the therapeutic activities of the drugs. This study had clearly link cytotoxicity of doxorubicin nanoparticles to the production technique (A), the speed of homogenization (B), drying technique (C) and the combinations of these independent variables investigated in this study. It is therefore critical for the regulatory authorities to make additional regulations which will require pharmaceutical manufacturers to optimize the manufacturing process in relation to therapeutic activities of drug in addition to pharmaceutical equivalent evaluations.

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