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Original Article

Nanoencapsulation of Phthalate from *Melastomastrum Capitatum* (Fern.) in Chitosan-Nps as a Target Mediated Drug Delivery for Multi-Drug Resistant Pathogen

Cletus A. Ukwubile[1]*, Emmanuel O. Ikpefan[2], Otalu Otalu[3], Salihu Njidda[4], Alexander E. Angyu[5], Nnamdi D. Menkiti[6].

[1]Department of Pharmacognosy, Faculty of Pharmacy, University of Maiduguri, Maiduguri, Nigeria
[2]Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmacy Delta State University, Abraka, Nigeria
3 Department of Microbiology, Faculty of Natural Sciences, Kogi State University Anyigba, Nigeria
[4] Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmacy, University of Maiduguri, Nigeria
[5] Department of Biological Sciences, Faculty of Science, Taraba State University Jalingo, Jalingo, Nigeria
[6] Department of Chemistry, Faculty of Physical Science, Ahmadu Bello University, Zaria, Nigeria

*Corresponding Author E-mail: doccletus@yahoo.com & caukwubile@unimaid.edu.ng

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ABSTRACT

Background: Chitosan nanoparticle (chitosan-NPs) is a polymer obtained from the exoskeletons of crustaceans, and has been applied recently as a carrier for many drug agents. Multi-drug resistance has been the major set-back in the treatment of microbial infections globally.
Methods: Dibutyl phthalate (DBP) isolated from *Melastomastrum capitatum* leaves was encapsulated in chitosan-NPs and its antimicrobial activity was evaluated on selected multi-drug resistant pathogens. The isolated phthalate was characterized by FTIR, NMR and GC-MS. Chitosan-NPs encapsulated phthalate was prepared by ionic gelation of glutaraldehyde cross-linker. Antimicrobial activity of nano encapsulated drugs was carried by agar well diffusion at 0.5 µg/mL concentration. *In vivo* activity of nano encapsulated drugs were determined in thirty Swiss albino rats weighing 100-150g. Chitosan-NPs encapsulated treatment groups were administered at 0.5 µg/mL (i.p.) as compared with ciprofloxacin positive control group at 2.5 µg/mL.
Results: Chitosan-NPs encapsulated phthalate showed the strongest zones of inhibition against *VRE ATCC 29212, MRSA NCTC 13435, Candida albicans ATCC 19231*, and *Clostridiodes difficile NCTC14385*. Significant inhibition of bacterial growths was achieved by CSDBP encapsulated phthalate both *in vitro* and *in vivo* studies due to low concentrations in ALT, ALP, AST and creatinine, and high volume of WBC in rats. Non-Fickian drug release was observed by the formulations.

Conclusion: The study showed that chitosan-NPs mediated drug delivery exhibited strong antimicrobial activity with sustained release against multi-drug microbes in this study. This is promising, and can be employed as mediation for multi-drug resistant pathogens.

Keywords: Dibutyl phthalate, Chitosan-nanoparticles, *Melastomastrum capitatum*, Multi-drug resistant, Antimicrobial, Pathogens.

1. Introduction

Antimicrobial resistance (AMR) is described as an opposition by any microbe to antimicrobial drugs that are developed to inhibit their metabolic activities or kill them. AMR can develop in bacteria, fungi, endo-parasites (like flatworms and roundworms), as well as viruses. AMR, or drug resistance, develops when microbes can no longer respond to a drug treatment such as antibiotics, antifungal and antiviral that are used in treating them effectively. AMR can result in certain diseases and infections being difficult to control, hence, staying longer inside the body, pro-longed hospital stays in health centres, high economic and social costs of ailments, a higher risk of disease spreading to other people, and a greater chance of deaths due to diseases [1].

The World Health Organization (WHO) defines antimicrobial resistance as the ability of a microbe to resist the pharmacology of an antimicrobial agent that was once able to treat such infection caused by that pathogen. This specified resistance that is linked to pathogenic bacteria is broken down into two subtypes: Microbiological and clinical. Microbiologically-called resistance, is the most prevalent and emerges from mutant or acquired genes that enable the bacteria to withstand certain antibiotics-related metabolic processes. In both instances of acquired resistance, the bacteria may pass through conjugation, transduction, or transformation to the genetic catalyst for resistance, and this confers them with resistance [2].

A major concern is that AMR could result in a post-antibiotic era; a situation in which antibiotics would no longer be efficacious on bacteria. This means that in the 20th century, widespread disease or diseases and cuts and bruises that were simple to treat might become lethal [2]. Microbial resistance to drugs such as antimicrobial agents may be due to microbial behaviour, microbial mutation, selective pressure, gene transfer, human behaviour, and phenotypic change, inappropriate use of antimicrobial agents, wrong diagnosis, hospital use, and agricultural use of antimicrobial agents on farm animals [3-5]. Multi-drug resistance (MDR) has been reported in *Mycobacterium tuberculosis* (Mtb) infection, *Methicillin-resistant Staphylococcus aureus* (MRSA), *Neisseria gonorrhoea*, *Escherichia coli*, HIV, fungal agents such as *Candida albicans*, *Aspergillus*, and as well as *Plasmodium* responsible malaria [6].

Currently, some infections and diseases are unresponsive to current chemotherapy irrespective of route of administration [6]. There is therefore an urgent need to find suitable treatment alternatives toward overcoming these resistances developed by microbes to achieve the desired objectives. In certain situations, this implies using a combination of various drugs, a process termed multiple-drug therapy (MDT) to effect the treatment of such ailments or infections [7]. Recently, specific therapeutic measures have been designed to treat infection resulting from certain bacterium like *Clostridium difficile*. Some of these include using a

bacteriophage, use of monoclonal antibodies, and development of vaccines, faecal microbiota transplant, and use of probiotics [8-10]. Among the priorities of the WHO, combatting antibiotic resistance remains a huge concern. The World Health Assembly adopted a global action plan regarding antibiotic resistance by microbes [11]. The purpose of this initiative was to ensure that medicines for the prevention and treatment of infectious diseases are safe, effective and reliable.

Despite all these strategies in combatting antimicrobial resistances among MDR pathogens, there has not been much success recorded as these pathogens seem to continuously evolve new mechanisms to evade the effects of drug agents. Hence, researchers are seeking for new forms of therapeutic measures which are capable of overcoming resistance posed by these MDR pathogens in their hosts [12]. One of such alternatives is the use of a carrier in the form of chitosan that can pass through the body unnoticed by the host's immune system and deliver its contents at specific site of interest [13-15].

Chitosan nanoparticle is a linear amino polysaccharide consisting of a β-linkage of 1→4 N-acetyl glucosamine and glucosamine units. It is a colourless white amorphous, hard, inelastic and nitrogenous substance [16]. It has a variety of applications owing to its high biodegradability, non-toxicity, accessibility, mode of preparation as well as antimicrobial activities. Chitosan has essentially been applied as carriers of various anticancer drugs, antifungal, and other drugs. It is also used in bio-medical industries to deliver various drugs, in agriculture to target pure line of seeds through a process called hybridization, in oil industries to clear oil spillage, in environmental pollution control, in water treatment, in pipe manufacturing, among others [17, 18].

Dibutyl phthalate is an organic compound used often as a plasticizer because it has low toxicity. It has a chemical formula of $C_6H_4(CO_2C_4H_9)_2$, oily and colourless in nature, but commercial samples are usually yellow in colour. Further, it is a derivative of phthalate from phthalic acid. In human, they are mostly found in liver tissue and other bio-fluids, such as urine, saliva and blood. In plant, they are common in red raspberries. In biological systems, phthalic acid has been reported to possess antiviral, antibacterial, antimicrobial, and anticancer activities [19-21]. Its derivatives have been used as plasticizer in plastic industries as well as coating for pharmaceuticals [22].

In this study, dibutylphthalate, a member of the phthalate family was isolated from the leaves of *M. capitatum* and loaded into chitosan nanoparticle in order to determine its antimicrobial activities against selected multi-drug resistant (MDR) pathogens. The effect of crude extract of leaf and pure compound was compared with that of CSNPs-encapsulated phthalate as well. Therefore, the aim of this study was to determine the antimicrobial activity of nano encapsulated phthalate from *M. capitatum* leaf extract against multi-drug resistant pathogens.

2. Materials and Methods

2.1. Chemicals, reagents, apparatus and materials

In this present study, the following chemicals, reagents, apparatus and materials as well as others were used: n-hexane, ethyl acetate, n-butanol, methanol, chitosan powder 100G; No: CHTS 100G; Purity: 90+ % deacetylated; CAS No: 9012-76-4 (Chemsavers, USA), glutaraldehyde, 25% aq. soln; CAS:111-30-8 (Alfa Aesar® Great Britain), de-ionized water, acetone, glacial acetic acid, phosphate buffer saline solution (pH 7.4

), ASCEND 500 NMR; Probe Temp : 301K; Spin cell Temp : 306K (Brucker, USA), GC-MS; (Model 7890 A; Agilent Technologies, UK), alpha II FTIR (Brucker, USA), UV-vis spectrophotometer (Model B700; Shimadzu, China), Zetasizer (Model Nano ZS90 ;Malvern Panalytical Ltd, UK), nanospray drying apparatus (Model B-90 Shanghai Bilon instrument Co Ltd, China), thin layer chromatographic plates (Merck, Germany), 35 x 950 mm glass column, silica gel (60 F2S4 20 x 20 cm Merck, Germany), electronic blender (Model 5000 MH, Japan), rotary evaporator, haematological and Biochemical parameters testing kits (Roche, Germany), dialysis tubes (Benrocks Medicals Ltd, Nigeria), Mindray BC-2800 auto haematology analyzer (Guangzhou Medsinglong medical equipment Co., Ltd, China), haematocrit analyzer (Scimed Tech®, Boston, USA), among others. All chemicals, reagents and solvents were of analytical grades (Sigma Aldrich, USA).

2.2. Multi-drug resistant pathogens

Microorganisms that were notable for their resistant to antibiotics and other antimicrobial agents used in this study include*: Escherichia coli ATCC 35218*, *Vancomycin-* resistant *enterococcus (*VRE*) ATCC 29212*, *Candida albicans ATCC 19231*, *Neisseria gonorrhoea ATCC 49226*, *Clostridiodes difficile NCTC 14385*, *Klebsiella pnemoniae NCTC 13465*, and *Methicillin*-resistant *staphylococcus aureus (*MRSA*) NCTC 13435*.

2.3. Plant collection, authentication and preparation

Melastomastrum capitatum leaves were collected fresh from a forest in Taraba State, Nigeria, in wet season. The authentication was made at the herbarium unit Department of Botany, Ahmadu Bello University, Zaria, with a voucher of number 2761 deposited at the unit. Using an electronic blending machine (Made in Japan), the previously air-dried leaves (for two weeks) were crushed into fine powdered form, weighed, and kept in a clean sample bottle wrapped with aluminium foil to prevent direct sunlight [23].

2.4. Bio-guided fractionation of *M. capitatum* crude leaf extract

One thousand grams (1000 g) of powdered leaves was extracted in 2.5 Litres methanol (Sigma Aldrich, USA), by cold maceration technique for 48 hours, and concentrated *in vacuo* in a rotary evaporator. The dark jelly-like extracted obtained was partitioned successively in n-hexane (NH), ethyl acetate (EA), and n-butanol (NB) and bio-monitored by antimicrobial activity against selected microorganisms. All solvents used were of experimental grade with purity greater than 100% (Sigma Aldrich, USA).

2.4.1. Isolation of compound from most active fraction of *M. capitatum* leaf extract

Ethyl acetate fraction of the methanol leaf extract with the most antimicrobial activity was introduced into a column chromatography containing silica gel (60-120 mm particle size) stationary phase. The elution of the column was by gradient elution in increasing order of polarities of solvents (i.e. ethyl acetate: n-hexane, methanol: chloroform). Fractions were collected in 50 mL each bio-guided by antimicrobial activity. Fractions were equally monitored by TLC plates. Fractions with the same TLC profiles were combined to give fractions F1-F5 [24-26]. The most bioactive fraction by its antimicrobial activity (F2) was subjected further to silica gel column chromatography, and each bio-monitored by antimicrobial activity. The most bioactive F2 sub-fraction (F2.2; 58

mg weight) was checked for purity by obtaining a single spot on TLC plates and sharp peak from HPLC.

2.4.2. Structural elucidation of compound

2.4.2.1. High performance liquid chromatography (HPLC)

Agilent Technologies HPLC 1200 series with LC solution software, G1365B multi-wavelength detector, G1379B degasser, G1311A quaternary pump, G1329A/G1367B autosampler, G1330B thermal, and G1316A TC column was used to confirm the purity of the isolated compound. The flow rate of the apparatus was set at 1 mL/min [27].

2.4.2.2. Fourier transform infra-red (FT-IR) spectroscopy

The isolated compound was scanned for the presence of some functional groups in the range of 300-550 cm^{-1} within room temperature using Bruker alpha II FT-IR apparatus.

2.4.2.3. Nuclear magnetic resonance (NMR) Spectroscopy

NMR analysis of compound was performed on a Bruker NMR with 500 MHZ using deuterated chloroform (DCL_3) as standard solvent. The chemical shifts (δ) were expressed in parts per million (ppm) and the signal splitting patterns were also recorded as either singlets, doublets, triplets or quartets.

2.4.2.4. Gas chromatography- mass spectrometry (GC-MS)

GC analysis of compound was performed by an Agilent 7890A GC with a MS detector and column DB23 model number J&W 1222362 with internal diameter of 60 m × 250 µm × 0.25 µm (250 °C Max). The compound was dispensed into 1 mL sample vial and diluted with 1 mL ethyl acetate. The mixture was injected into GC-MS vials using a 0.45 µm needle. The injection was auto injection with volume of 1 µL, wash volume of 8 µL injection temperature of 250 °C and flow rate of l mL /min was employed with total flow of 6.4 mL and column flow of 0.57 mL/min. The pressure used was 16.0 kPa. The GC operating temperature condition was 50 °C for 2 min, increased at °C /min to 100 °C, held for 10 min and increased at 15 °C to 250 °C and then held for another 10 min. The starting m/z was 50 and ended with 500. The total runtime was 38 min [28].

2.5. Preparation of chitosan nanoparticle-encapsulated dibutyl phthalate

Chitosan encapsulated phthalate was prepared by spraying drying method. Briefly, 1 µg of isolated dibutyl phthalate (DBP) was dissolved in 10 mL deionized water. Similarly, 4 g of chitosan powder made from shrimp shells and crustaceans was accurately weighed on a Gemini-20 portal milligram scale balance into a clean 500 mL beaker, and dissolved in 100 mL deionized water containing 0.5 % v/v glacial acetic acid under magnetic stirring. Prepared solution of the dibutylphthalate was added to the beaker containing chitosan under constant stirring at 3000 rpm for 2 h. Glutaraldehyde solution was added in drop-wise at concentrations of 500, 1000, and 1500 µg/mL to each formulation (F1-F4). F1 was not cross-linked and used as control. The pH of the preparations was adjusted by adding 0.1M NaOH solution and stirred for 30 min. In order to enhance their targeting in *in vivo* study, 20 mg of folic acid was dissolved in 20 mL deionized water and 5 mL of the solution was added to the formulations, and stirred at 2000 rpm for 15 min. A nano-spray drying system [(version B-90), Shanghai Bilon instrument Co Ltd, China] was then used

to sprinkle the produced CSNP /DBP complexes for atomization. The spray dried chitosan nanoparticle loaded with phthalate (DBP) was weighed and stored in clean containers for further use.

2.5.1. Characterization of chitosan-NP-encapsulated DBP

The formulated MC-loaded CSNPs were characterized in terms of particle size, surface morphology or shape, percentage drug entrapment, FTIR spectroscopy, swelling index, thermal analysis, and *in vitro* drug release kinetics. Protocols guiding each characterization were followed [31,32].

2.6. *In vitro* antimicrobial studies

2.6.1. Test multi-drug pathogens

Multi-drug organisms used were all standard cultures obtained on demand from the Veterinary Research Institute, Vom, Jos, Nigeria. These are *E. coli ATCC 35218*, *VRE ATCC 29212*, *Candida albicans ATCC 19231*, *Neisseria gonorrhoeae ATCC 49226*, *Clostridiodes difficile NCTC 14385*, *Klebsiella pneumonia NCTC 13465*, and *MRSA NCTC 13435.* The microbes were cultured in appropriate media prior to use.

2.6.2. Susceptibility testing of chitosan-NPs-dibutyl phthalate (CSDBP)

The antimicrobial activity was determined by the agar well diffusion method following standard protocols. Petri dishes containing Muller Hinton Agar (MHA) (Oxoid, UK) was used for bacteria and Potato Dextrose Agar (PDA) for *Candida albicans*. Wells were made into each culturing plates using a 5mm sterile cork borer according to each resistant organism. Wells were each filled with 0.5 µg/mL of chitosan encapsulated phthalate (CSDBP). Microbial suspensions were prepared each and 0.2 µg/mL of the microbial suspension was added to each well [33]. Two sets of control plates one containing standard antibiotic (ciprofloxacin 5 mg) and the other DBP were also prepared in the same way as stated above. Plates were then incubated for 2 h at 37 °C ± 2 °C for 24 hours. The plates were observed for the zone of clearance around the wells and measured. The experiments were performed in replicate [33, 34].

2.6.3. Minimum inhibitory concentrations (MIC) of chitosan- NPs-DBP

The MIC assay was performed in 96-well plates following the protocols of the National Committee for Clinical Laboratory Standards with modifications [35]. Stocks of the CSDBP were prepared at a final concentration of 1 µg/mL using deionized water. Prepared CSDBP solution was added to reach a 0.5 mg/mL concentration within a final volume of 200 µL. After this, serial dilutions of the CSDBP were performed by taking 100 µL from the previous solution to every next well, with 100 µL of culture media, and discarding the last 100 µL from the last dilution so the tested concentrations were 1.25–20 $\times 10^{-4}$ µg/mL. Then, 100 µL of the microbial suspensions were each added to each test well to achieve a final concentration of 10^5 cells/mL, and stirred at 200 rpm for 5 min, then, incubated at 37 °C. After 24 h of incubation, the MIC was determined as the concentration at which no significant growth was observed by the naked eye. All tests were performed in triplicate.

2.6.4. Minimum bactericidal concentration (MBC) of CSDBP

The MBC chitosan encapsulated phthalic acid was determined in order to know if the microbes were killed or only their growth was inhibited. In this assay, blood agar was prepared according to the

manufacturer's instructions, sterilized at 121 °C for 15 minutes. It was poured into sterile culturing plates, and left to solidify. The plates were labelled accordingly to correspond to the MIC wells. The contents of the MIC in the proceeding 96-well tubes in the serial dilution were sub cultured into the plates by dipping a sterile wire loop and streaking the surface of the agar in the culturing plates. The plates were then incubated at 37 ^{o}C for 24 hours after which they were checked for growth of colonies. The plate with the lowest concentration of CSDBP without growth was taken as the MBC [35].

2.7. *In vivo* antimicrobial study of chitosan-NPs- loaded phthalate (CSDBP)

2.7.1. Laboratory animals

Thirty Swiss albino rats of opposite sexes weighing between 100-150 grams for were purchased from the animal house of the Department of Pharmacology, University of Jos, Nigeria. Ethical approval for the use of these animals was granted by the Ethical Committee of the University of Jos with approval number of F17-00379.

Figure 1. One of the Swiss albino rats used for the study

2.7.2. Experimental design and in vivo antimicrobial study

A tape stripping infection model (or skin infection model) was performed according to the methods previously described by Escárcega-González *et al.* [37] with little modifications. Swiss albino rats were divided into seven groups of three rats per group. Rats in groups II to VII were anesthetized with pentazocine injection (Sosetiech®, USP) by intra-peritoneal injection of 2.5 mL of the anaesthesia while group I served as the control. After anaesthesia, the backs of the rats were shaved in an area of 5 cm^2 with a trimmer. Furthermore, an area of 2.5 cm^2 of the hairless skin of the rats was tape stripped five times in succession using an elastic adhesive bandage [37].

Skin damage was confirmed by reddish and glistening skin without bleeding. This procedure was carried out in all the rats. In order to induce infection in the rats, inoculum of bacteria was carried out by placing in the already created wound in tape stripping process, 100 µL of a 10^5 cells/mL bacterial culture in log phase (rapid growth phase) obtained from the MBC experiment.

The control group was with the wound on the skin without infection, and treated with 100 µL of saline solution every 24 h for 3 days in the damaged zone, while the infected groups were treated with 5 mg/mL ciprofloxacin (Apotex, USP) antibiotic for bacteria and nystatin for fungus and CSDBP every 24h for 3 days after infection. While treatment was ongoing, the rats were allowed to have free access to food and clean drinking water. At the end of the investigation, rats were sacrificed 24 h on day three of treatment and blood samples were immediately collected in EDTA bottles for liver function variable review. Thereafter, 100 mg of the wound was removed and centrifuged at 100 rpm for 5 min with 5 mL PBS solution with a 1:100 dilution factor used to carry out a pour plate technique to determine the

CFUs of living bacteria and results were compared with that of the control.

Liver function analysis: To determine the effect of CSDBP on liver functions of treated rats, blood and urine samples were taken and analysed using a haematology auto analyser (BC-3800, UK). Creatinine, WBC, alanine aminotransferase (ALT), alanine phosphatase (ALP) and aspartate aminotransferase (AST) were the biochemical parameters determined.

2.8. Statistical analysis

Data were expressed as mean±SE. Significant difference between means in groups were compared using one-way analysis of variance (one-way ANOVA) as analysed by SPSS statistical software version 22. The value of $p \leq 0.05$ was considered as statistically significant.

3. Results

3.1. Bio-guided fractionation of *M. capitatum* crude extracts

Fractionation of crude methanol extract with n-hexane (NH), ethyl acetate (EA), n-butanol(NB) and aqueous(AE) against selected multi-drug pathogens showed the ethyl acetate fraction II (F2) was the most active having the highest diameter zone of inhibition (19.4mm - 55.2 mm) after 24 h incubation, hence the strongest antimicrobial activity when compared to other fractions at the same concentrations (Table 1).

3.2. Isolation of phthalate (DBP) from the most active fraction

The most active antimicrobial ethyl acetate (EA) fraction II (F2) further produced four sub-fractions (F2.1, F2.2, F2.3, and F2.4). Fractions F2-1-F2.4 re-chromatographed by gradient elution bio-monitored by antimicrobial activity showed that F2.2 sub-fraction was the most active of F2 fraction. F2.2 yielded a compound that was greenish and amorphous powder weighing 68 mg. TLC plate showed a single spot while HPLC apparatus indicated a sharp peak area and constant retention time with the standard

3.2.1. Structural elucidation of isolated *M. capitatum* phthalate

The compound isolated from *M. capitatum* was isolated as a white crystalline isolate with retention factor (R_f) of 0.83 (in ethyl acetate: n-hexane) while the melting point was observed to be −35 °C and its solubility (in water) was 13 mg/L (25 °C). The ^{1}H-NMR spectrum (500 MHz, $CDCl_3$, δ, ppm, J/ Hz) showed three downfield singlets at δ 7.25 (3H, J= 2.2,8.1 Hz) and a singlet upfield at δ 5.70 (2H, J= 2.2, 8.2) as well as a doublet at δ 5.30 (1H, J=2,16) signals for methine carbons (C1-5) at δ 55.18, 65.64, and 66.86 ppm, quaternary carbons (C4-C6) at δ 76.90, 77.05, and 77.20 ppm as well as aromatic groups (C7-C8) at δ 79.12 and 79.15 ppm [Fig. 2 and 4]. As confirmed by DEPT-135-NMR spectra, COSY-NMR of isolated phthalate showed correlations with 2H protons at 4.3 ppm, and 3H protons at 5.2 ppm. HSQC also showed linkages between these protons and carbons at 3.25, 3.49, 3.68, 3.88, 3.95, 4.14 and 4.30ppm, HMBC showed bond connections with protons at 3.21, 3.60, 4.20, 4.25, 4.40 and 5.21 ppm with 2H ,3H, 4H and 5H protons. The mass spectrum showed an attached methyl group which correlated with chemical shift δ 7.25 (5H, J= 8.2) (Figure 3, 5-7).

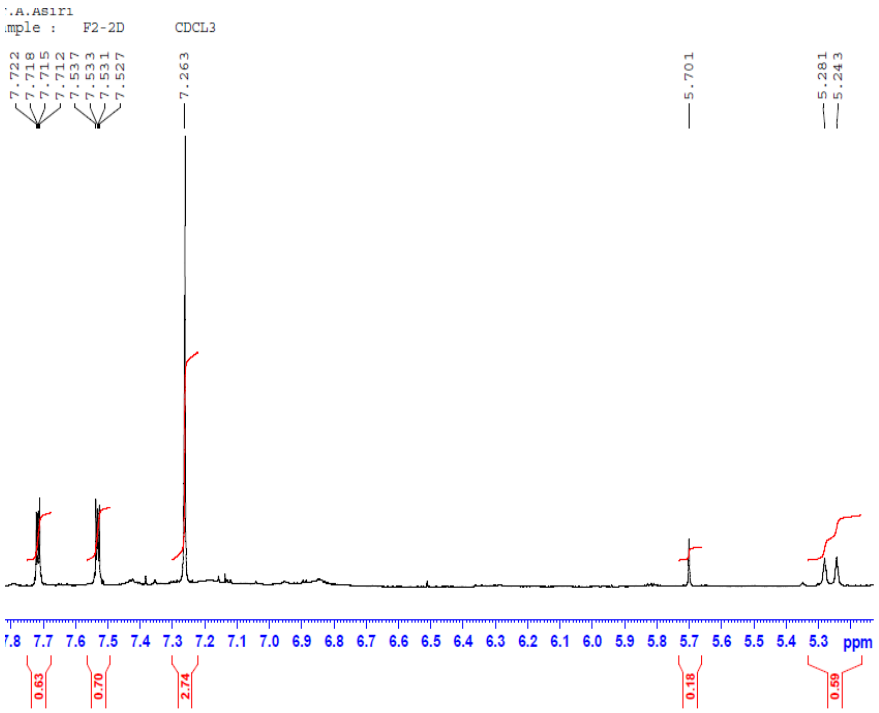

Figure 2. ^{1}H-NMR spectrum of dibutyl phthalate

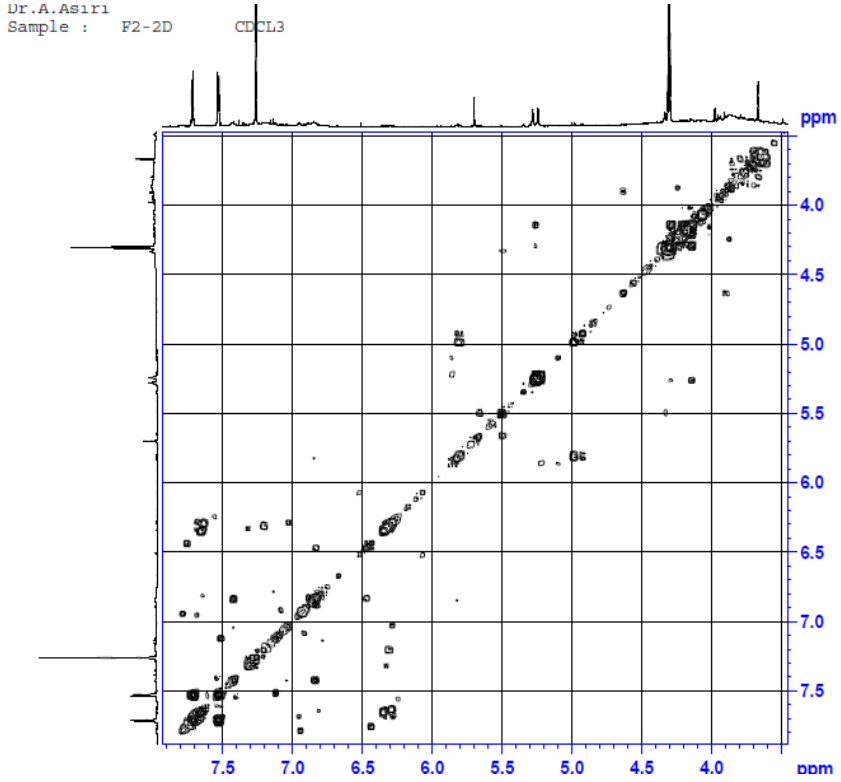

Figure 3. COSY-NMR spectrum of dibutyl phthalate

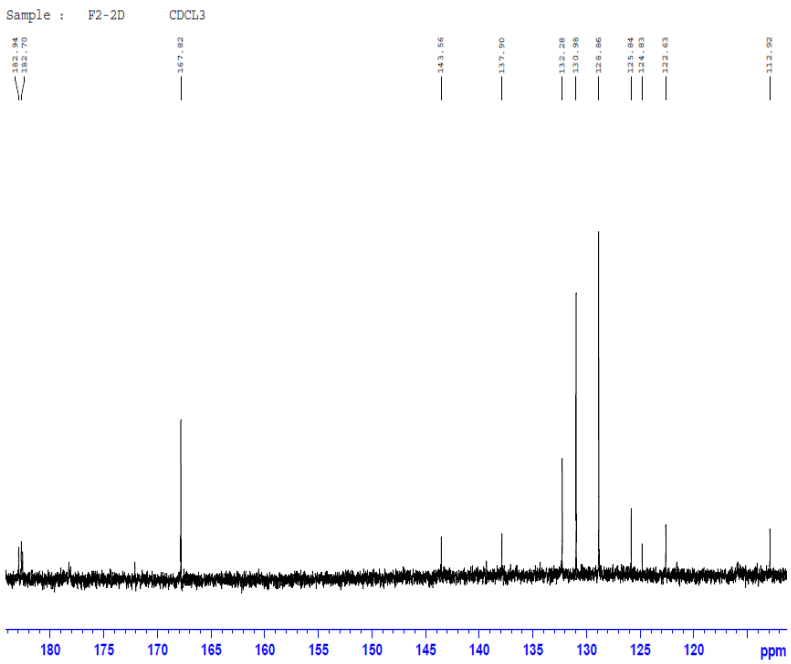

Figure 4. ^{13}C-NMR spectrum of isolated dibutyl phthalate

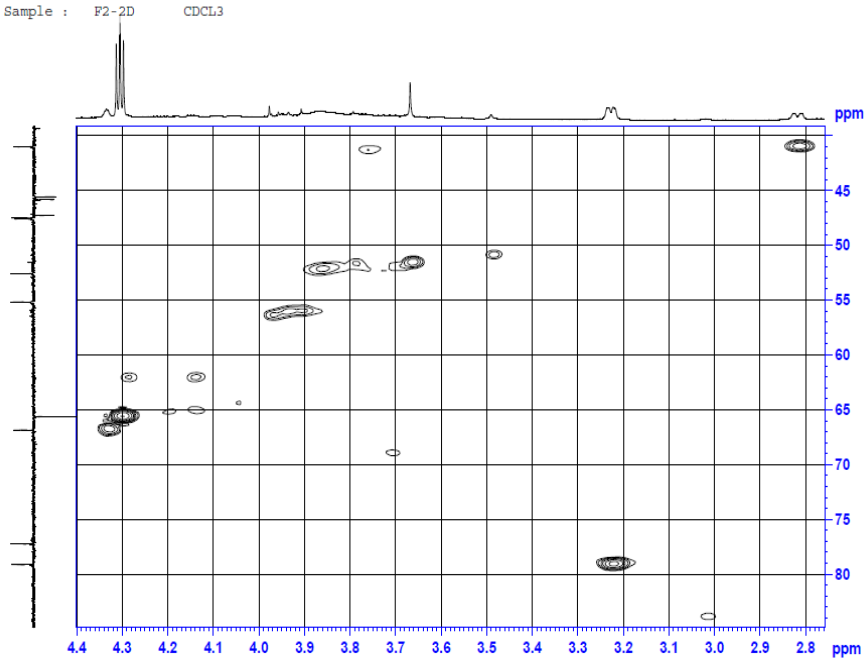

Figure 5. HSQC-NMR of isolated DBP

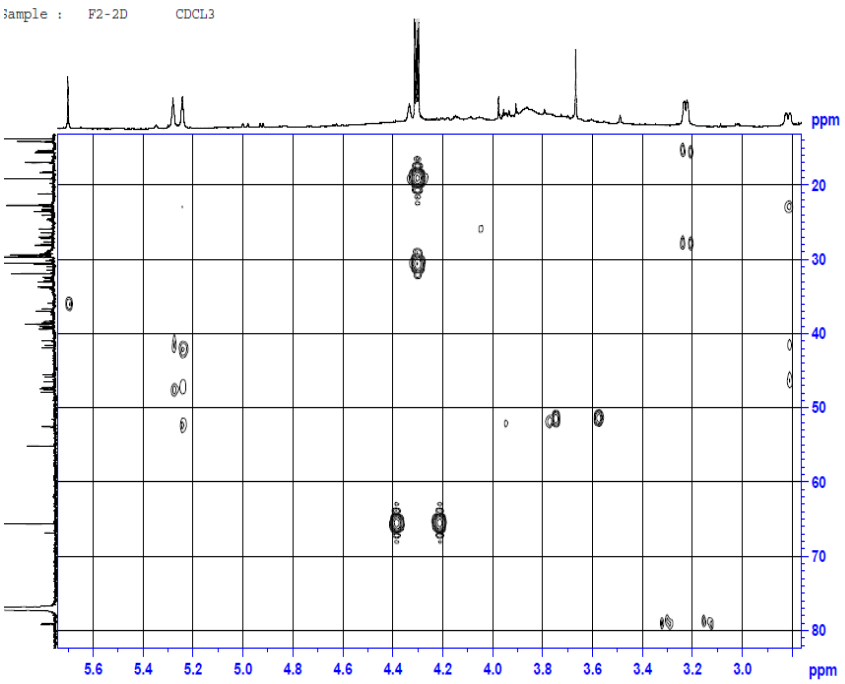

Figure 6. HMBC-NMR of isolated DBP

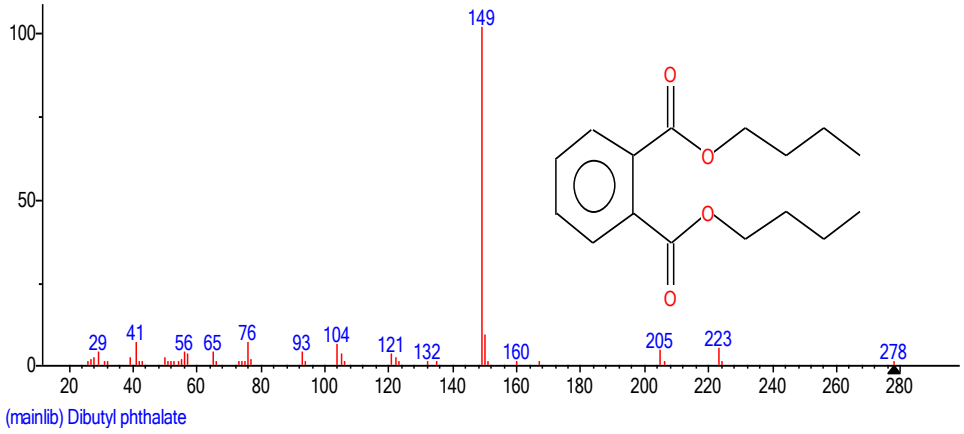

Figure 7. Mass spectra of dibutyl phthalate from *M. capitatum* leaves

3.3. Formulation and characterization of nano encapsulated DBP from *M. capitatum*

The shape of the formulated nanoparticles as revealed by the SEM (Phenom world) was circular (Figure 8), while the sizes as revealed by Malvern Zetasizer ranges from 204-248 nm, and zeta potential from 22.13±0.62v to 25.88±0.66v. FTIR analysis of formulations revealed the presence carbon atoms attached with oxygen as well as aromatic functional groups at 300-400 cm^{-1} wavelength. From the result, formulation code F6 had the highest % yield of 46.80, and % entrapment efficiency (% EE) of 57.60, while F1 had the lowest % EE of 30.00, whereas batch code F4 had the highest swelling index of 46.00%. The highest cumulative drug release in *in vitro* study was recorded in formulation batch code F6 with 98.41 µg, while the non-cross link code F1 had the lowest value of 28.20 µg in 8 hours (Table 1). Drug release mechanism as characterized with the Korsmeyer–Peppas model showed results of fitting with diffusional exponent (n = 0.6215) which confirmed a

non-Fickian or anomalous mechanism of drug release (figure 8). The release pattern in all the formulations were sustained over time (Figure 9).

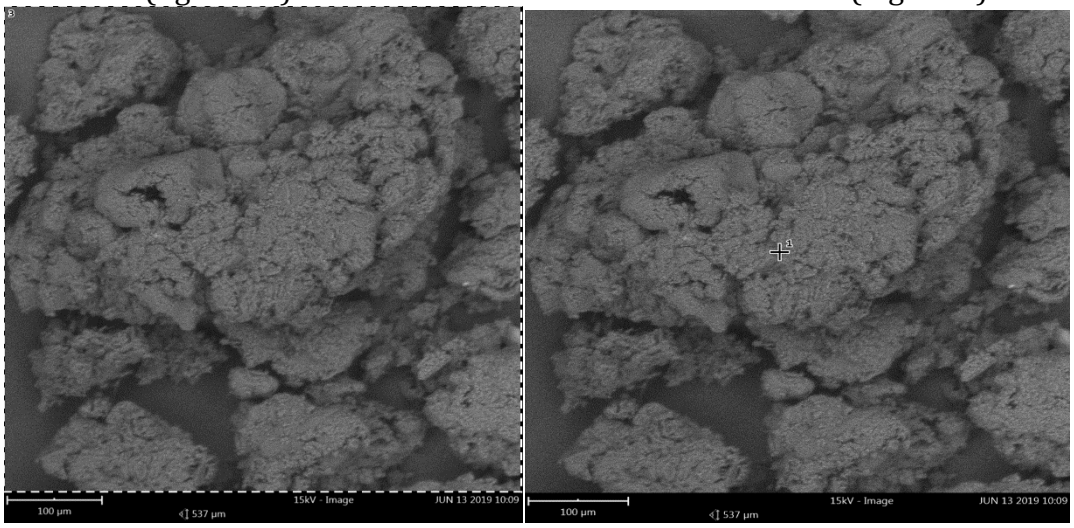

Figure 8. SEM images of F6 formulation FOV: 537 µm, Mode: 15kV - Image, Detector: BSD Full

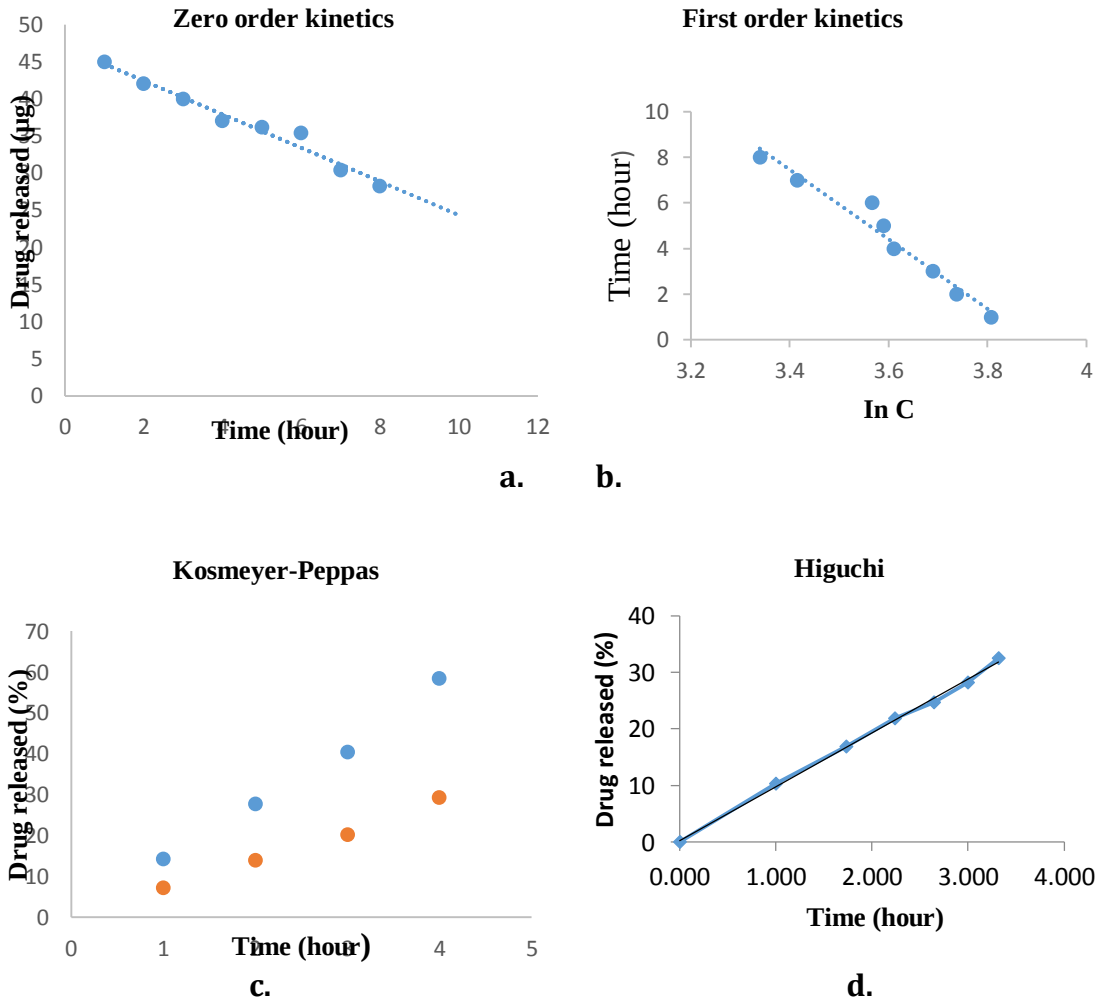

Figure 9. Drug kinetics of formulation batch code F6 (K = 0.517/h, n=0.6215)

Table 1. Physical characteristics of the formulation codes.

Batch code	F1	F2	F3	F4	F5	F6
Final weight (g)	2.22	2.24	2.28	2.16	2.32	2.34
Initial weight (g)	5	5	5	5	5	5
Percentage yield (w/w %)	44.40	44.80	45.60	43.20	46.40	46.80
Swelling index (%)	44.50	40.00	30.00	46.00	42.00	41.5
Entrapment efficiency (%)	30.00	37.60	39.80	51.20	51.60	57.6
In vitro drug release in 8h(µg)	28.20	68.80	76.00	82.40	40.01	98.41

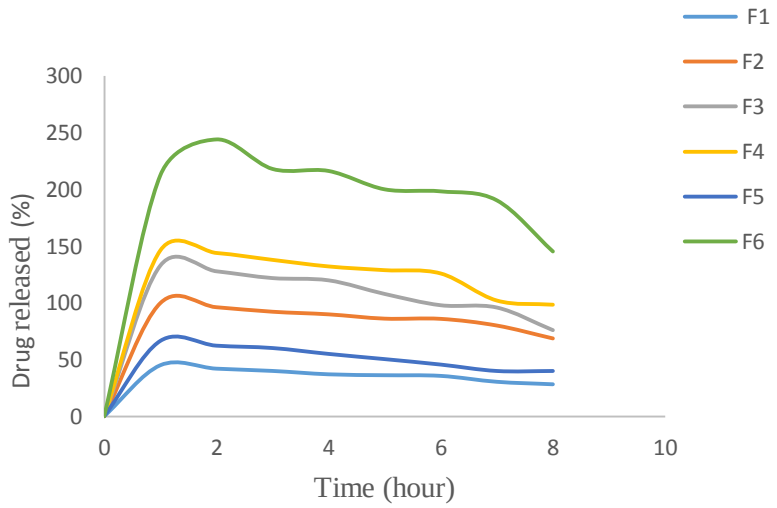

Figure 10. Dissolution profile of the matrix system at different concentrations of the cross linking and non-cross linking agents

3.4. *In vitro* antimicrobial study of chitosan-NPs encapsulated phthalate

In Table 2, chitosan-NPs encapsulated samples showed significant diameter zones of inhibitions against certain multi-drug resistant pathogens than others. CSPA showed the highest zone of inhibition against *VRE ATCC 29212* and *MRSA NCTC 13435,* while nano encapsulated standard antibiotic(ciprofloxacin) produced better diameter zones of inhibition against the microbes.

Table 2. Susceptibility testing of nanoencapsulated chitosan-NPs

Sample	Concentration(µg/mL)	Diameter zone of inhibition(mm)			
		E. coli	*VRE*	*C. albicans*	*N. gonorrhoeae*
CSDBP	0.5	36.50	58.40	41.13	39.11
DBP	0.5	22.10	32.11	26.00	18.20
MCE	0.5	11.50	28.13	12.22	6.13
CS-NPs	0.5	0	0	8.41	0
Ciprofloxacin	5	14.30	10.03	0	22.10
Nystatin	5	0	0	16.22	0
CS-Cipro	0.5	38.12	62.15	20.11	34.08

Sample	Concentration(µg/mL)	Diameter zone of inhibition (mm)		
		C. difficile	*K. pneumonia*	*MRSA*
CSDBP	0.5	38.20	36.50	46.30
DBP	0.5	28.50	30.10	38.04
MCE	0.5	16.40	12.03	10.20
CS-NPs	0.5	0	0	0
Ciprofloxacin	5	9.51	12.62	16.13
Nystatin	5	0	0	0
CS-Cipro	0.5	42.11	25.40	28.91

Pathogens: *E. coli ATCC 35218, VRE ATCC 29212, C. albicans ATCC 19231, N. gonorrhoeae ATCC49226, C. difficile NCTC 14385, K. pnemoniae NCTC 13465, MRSA NCTC 13435*. CSDBP (chitosan-NPs encapsulated phthalate; F6), DBP (dibutylphthalate), MCE (*Melastomastrum capitatum* crude leaf extract), CS-NPs (chitosan nanoparticles), CS-Cipro (chitosan-NPs encapsulated ciprofloxacin Tablet).

3.4.1. MIC and MBC studies

Chitosan-NPs encapsulated DBP showed the strongest MIC and MBC at all concentrations against multi-drug resistant pathogens (MDRPs) with values between 1.25-2.5 µg/mL were compared to other samples. Nano encapsulated standard antibiotic also displayed good MIC and MBC 2.5 µg/mL and 5.0 µg/mL against *VRE ATCC29212* and *MRSA NCTC 13435* (Table 3).

Table 3. MIC and MBC of samples against most potent pathogens

Sample	MDRPs	MIC (µg/mL)					MBC (µg/mL)				
		20	10	5	2.5	1.25	20	10	5	2.5	1.25
CSDBP	VRE	-	-	-	-	Sa	-	-	-	Sb	+++
	C. albicans	-	-	-	Sa	+	-	-	Sb	+	++
	C. difficile	-	-	-	Sa	+	-	-	Sb	+	++
	MRSA	-	-	-	Sa	+	-	-	Sb	+	++
DBP	VRE	-	Sa	++	++	+++	Sb	+	+	++	+++
	C. albicans	-	Sa	+	++	+++	Sb	+	++	++	+++
	C. difficile	-	-	-	Sa	++	-	-	Sb	+	++
	MRSA	-	-	-	Sa	+	-	-	Sb	+	++
MCE	VRE	-	Sa	+	++	+++	Sb	+	+	++	+++
	C. albicans	-	Sa	+	+	++	Sb	+	++	++	+++
	C. difficile	-	Sa	+	+	++	Sb	+	++	++	+++
	MRSA	-	Sa	+	+	++	Sb	+	++	++	+++
CS-Cip	VRE	-	-	-	Sa	+	-	-	Sb	+	++
	C. albicans	*	*	*	*	*	*	*	*	*	*
	C. difficile	-	-	Sa	+	++	-	Sb	+	++	+++
	MRSA	-	-	-	Sa	+	-	-	Sb	+	++

VRE ATCC 29212, C. albicans ATCC19231, C. difficile NCTC 14385, MRSA NCTC 13435. CSDBP (chitosan-NPs encapsulated phthalate; F6), DBP (dibutylphthalate), MCE (*Melastomastrum capitatum* crude leaf extract), CS-Cip (chitosan-NPs encapsulated ciprofloxacin tablet), Sa (MIC), Sb (MBC), * (not determined), - (no growth), + (light growth), ++ (moderate growth), +++ (dense growth).

3.5. In vivo study of chitosan-NPs encapsulated DBP against MRSA infected rats

From the result of biochemical analysis, CSDBP showed lower concentrations of most of the biochemical parameters in rats infected by *MRSA NCTC13435* except for WBC where significant increase in value was obtained. This result indicated that chitosan encapsulated phthalate improved the white blood cell maybe due to its specificity in delivery its content (Figure 11).

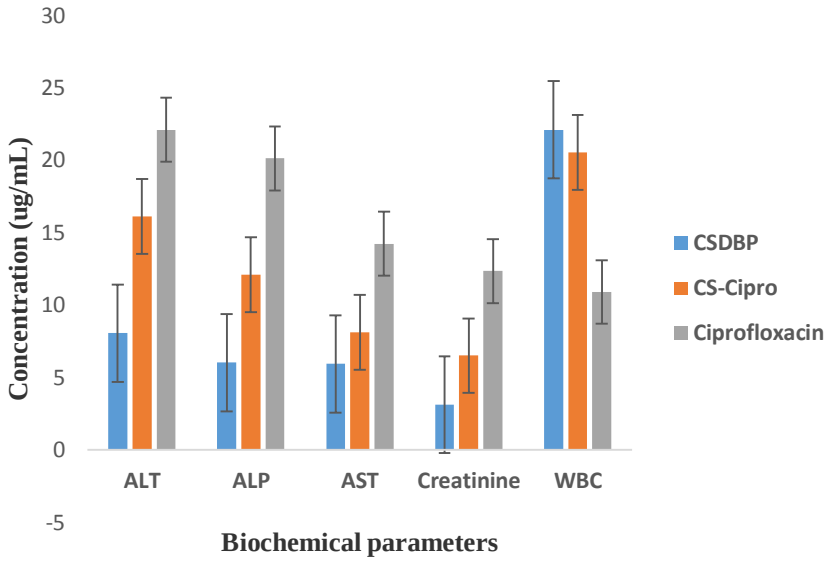

Figure 11. Biochemical parameters of CSDBP-treated *MRSA NCTC13435* infected rats

4. Discussion

It is believed that the emergence of multi-drug resistance is a direct consequence of the selective events imposed by the use of drugs such as antibiotics in clinical infections. This is probably true in terms of clinically relevant antibiotic resistance, involving a relatively high number of strains with high levels of resistance. Antibiotic medications have revolutionized the field of medicine, and has made possible our modern lifestyle of medication. However, because of the promotion and dissemination of antibiotic resistant bacteria, overuse of antibiotics undermines their effectiveness. Given the significance of antibiotics to modern medicine and the increasing apprehension about the threat of resistance, scientists are studying every aspect of area of antibiotic resistance.

Medicinal plants have been proven to be very effective in managing and treating of various microbial infections in health-care systems of many nations. They are used as antibacterial, anticancer, antiviral, analgesic, and anti-inflammatory agents. Treatment could be by decoctions, infusions, ointments or concoctions of crude extracts and by isolation of pure compounds from plant's parts.

In the current study, characterization of chitosan-NPs was based on the size, morphology, surface charge, swelling index, drug entrapment efficiency (%EE), and *in vitro* release employing sophisticated technology like Fourier transformed infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and Zetasizer (Table 1). From the study, encapsulated drug was well entrapped in chitosan-NPs with round-shaped morphology as confirmed by the

SEM (Figure 8), with size range of 204-248 nm, and zeta potential 22.13±0.62v to 25.88±0.66v. Similarly, there is a significant difference between crossed-linked formulations F2, F3, F4, F5 and F6 in the amount of drug released in *in vitro* studies within 8 h. The study further showed that cross-linked F6 formulation batch code has the highest cumulative drug release of 98.41 as compared with F1 batch code. This is because characteristics like the distribution of size, particle size diameter and charge affect the physical stability and the distribution of the *in vivo* distribution of the nanoparticles [16]. Also, features like physical stability and re-dispersibility of the polymer dispersion as well as the *in vivo* performance are affected by the surface charge of the nanoparticles.

The size of formulated nanoparticles in this study is very important because size reduction of targeted formulation and designing its pathways for suitable drug delivery system is a more basic and efficient approach that forms the basis of nanotechnology delivery system [17]. It is also important to mention that size reduction gives rise to different types of nanostructures that show unique physicochemical and biological properties in biological system. Because of smaller sizes of these formulations (F1-F6), CSDBP is readily available for biological tissues in *in vivo* experiment in this current study. Larger size of above 600nm is reported as not being suitable for drug delivery [18]. This is because it is advisable to use smaller range nanoparticle as nanotechnology offers drug in the nanometre size range which enhances the performance in a variety of dosage forms. This is the case of this present study where F6 batch code has a size of 204nm and highest drug entrapment efficiency as well as cumulative drug release. In addition, the release or dissolution pattern of F6 formulation sustained for the period of the experiment 8h. This release was confirmed by Kosmeyer-Peppas drug kinetic equation as non-Fickian in nature (Figure 9 a-d, 10).

The isolation of bioactive compound for antibacterial activities from the leaves of *Melastomastrum capitatum* showed that a phthalate family (figure 2-7) was accountable for the recorded biological activity. Characterization of the compound using various techniques such as FTIR, NMR, and GC-MS revealed the various functional groups likened with that of dibutylphthalate (DBP), a phthalate and derivative of phthalic acid. Phthalate family such as dibutylphthalate (DBP) has been shown to possess antimicrobial activities against selected organism like *Pseudomonas aeruginosa*. This finding corroborated with the results obtained from other studies (Table 2) where the compound was potent against all selected multi-drug resistant pathogens (MDRP). From the study, crude extract of *Melastomastrum capitatum* leaf (MCE) was more potent on *Vancomycin- resistant enterococcus* (VRE) with 28 mm zone of inhibition than any other pathogens that are multi-drug resistant (Table 2).

The presence of many compounds in the crude extract may be responsible for this activity, since various compounds act therapeutically in unique ways [31,37]. Similarly, highest zones of inhibition were shown by chitosan-NPs encapsulated phthalate (CSPDBP) with 58.40 mm for VRE and 46.30 mm for MRSA, while the isolated phthalate had diameter zones of 32.11 mm for VRE and 38.04 mm for MRSA. Nanoencapsulation of a standard antibiotic ciprofloxacin produced better diameter zone of inhibition (DZI) against *VRE* with 62.15 mm DZI, *MRSA* with 28.91mm DZI, and *C. difficile* with 42.11 mm DZI, while nystatin showed a DZI of 16.22 mm against *Candida albican* in *in vitro* study. Furthermore, chitosan-NPs encapsulated

phthalate showed a maximum MIC of 2.5 µg/mL against all MDRP when compared with the standard antibiotic (table 3). It has been reported that delivering therapeutic compounds to the desirable site is a major problem in treatment of many diseases due to the host's immune system. To overcome the body barrier, formulating nanoparticles with folic acid will help in specifying their targeting [17,18, 32], hence, the reason for using folic acid in this present study.

It was reported that conventional utilization of drugs is characterized by poor bio-distribution, limited effectiveness, undesirable side effects, and lack of selectivity [30,34]. Strategies like controlling drug delivery can potentially overcome these limitations by transporting drug to the place of action, hence another reason why folic acid was added in the formulations. This is because, from the study chitosan-NPs encapsulated DBP showed decrease in the values of liver enzymes (ALP, AST, ALT, and creatinine) and increased WBC concentration. This showed the ability of chitosan-NPs encapsulated phthalate to successfully kill multi-drug resistant *MRSA 13435* in infected rats (Figure 11). This is possible because CSDBP drug delivery system provides protection against rapid degradation or clearance of drug from the endothelial cells, and it also enhances drug concentration in target tissues. To achieve this, lower doses (0.5µg/mL) of folate chitosan-NPs loaded dibutyl phthalate (DBP) was used in the present study.

Finally, the study showed that nano encapsulated phthalate (DBP) possessed strong antimicrobial activities against multi-drug resistant pathogens such *VRE*, *MRSA*, *Candida albicans*, and *Clostridiodes difficile* when used at lower concentration in both *in vitro* and *in vivo* studies. This method of using chitosan nanoparticles as carrier for antimicrobial agents isolated from medicinal plant against multi-drug agent is novel and very promising, and should be sustained in order to overcome threats posed by multi-drug resistant pathogens in human health-care system.

5. Conclusion

From this study, chitosan-NPs encapsulated dibutylphthalate (DBP) showed the most potent antimicrobial activities against multi-drug resistant pathogens. Chitosan-NPs have shown immense capabilities in delivering drugs and isolated antimicrobial compound from a medicinal plant *M. capitatum* than the time when the drugs were used without chitosan-NPs a carrier. This was possible due to its biodegradability, smaller sizes, sustained release pattern, higher *in vitro* release and round shape of microsphere as well as low toxicity displayed in rats. This approach of drug delivery is safe, and it is recommended as a therapeutic measure for multi-drug resistant pathogens.

Abbreviations

NPs: nano particles; FT-IR: Fourier transform infra-red; GC-MS; gas chromatography-mass spectroscopy; NMR: nuclear magnetic resonance; HSQC: heteronuclear single quantum coherence, HMBC: heteronuclear multiple bond correlation, DEPT: distortionless enhancement by polarization transfer, COSY: Correlated spectroscopy, HPLC: high performance liquid chromatography, TLC: thin layer chromatography; AST: aspartate amino transaminase, ALP: alanine phosphatase, ALT: alanine amino transaminase; SEM: scanning electron microscope; MRSA: methicillin-resistant *Staphylococcus aureus*; VRE: vancomycin-resistant enterococcus; AMR: antimicrobial resistance; CS: chitosan; MCE: *Melastomastrum capitatum* extract; ATCC: American type culture collection;

NCTC: National type collection; MDR: multi-drug resistance; DBP: dibutyl phthalate, DZI: diameter zone of inhibition; MIC: minimum inhibition concentration; MBC: minimum bactericidal concentration; EDTA: ethylene diamine tetra acetic acid.

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Conflict of interests

We have no competing interests.

Consent for publications

All authors have read and approved the final manuscript for publication.

Availability of data and materials

Authors declare that all data are embedded in the manuscript.

Authors' contributions

C.A.U., E.O.I., O.O. and N.D.M., designed the study, carry out experiments, interpret the spectra, drafted the initial manuscript, and analyzed the data; S.N., performed the kinetic studies and interpret the data; while A.E.A., supervised the study, performed the statistical analysis and revised the manuscript before submission. All authors read the revised manuscript and approved it for final submission.

Ethics approval and consent to participate

The laboratory animals used in this study was approved for use by the research ethical committee of the University of Jos, Nigeria with approval code F17-00379.

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