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FORMULATION AND CHARACTERIZATION OF ACTIVATED CHARCOAL AND METRONIDAZOLE LAYERED TABLETS AND EVALUATION OF THE *IN VIVO* PERFORMANCE OF METRONIDAZOLE – ACTIVATED CHARCOAL FORMULATION IN SPRAGUE DAWLEY® RAT MODEL INFECTED WITH *ESCHERICHIA COLI* O157:H7

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Keywords:

Activated charcoal, Metronidazole, Layered tablets, *Escherichia coli* O157:H7, *in-vitro* release kinetics

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ABSTRACT: Background: Due to *Escherichia coli* O157:H7 bacteria's peculiar biochemical characteristics it is considered an emerging pathogen. The aim of this study is to design, develop and evaluate the efficacy of a single formulation combining adsorptive and anti infective properties in the treatment of diarrhoea caused by *Escherichia coli* O157:H7 in an animal model. **Method**: A bilayered tablet of metronidazole and activated charcoal (AC) formulated via direct compression was developed using hydrophilic mucoadhesive polymer xanthan gum in varying concentrations to ensure modified release of metronidazole in the first layer and activated charcoal with microcrystalline cellulose for instant disintegration in the second layer. *Escherichia coli* infected Sprague Dawley® rats was utilized to evaluate the efficacy of the formulation. **Results:** Swelling studies reflected an affinity for the polymer to swell in pH 6.8 where drug release and swelling was influenced by ionic strength of the medium owing to the conformational changes in the xanthan gum in this media with an increase in the zeta charge from -28.5 mV to -40.2 mV, drug release being predominantly by zero order. Treatment with activated charcoal and metronidazole reflected a negative result for identification of *Escherichia coli* O157:H7 by the third day of treatment in stool with symptoms cessation occurring by the second day of treatment. **Conclusion**: The synergistic potential of a nitro-imidazole and an adsorbent has been evaluated and established with treated groups showing cessation of symptoms within twenty four hours thus effective in the treatment of *Escherichia coli* O157:H7 associated diarrhea.

INTRODUCTION: Metronidazole a nitro imidazole derivative is a commonly used antibiotic in the treatment of anaerobic infections associated with diarrheal disease as well as clostridium difficile infections. [1]

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Diarrheal disease is estimated to have caused 1.1 million deaths in people aged five and older in Africa in 2011. [2] The treatment primarily targets three mechanisms, adsorption of causative organism, antibiotics act by attacking the cellular structure, as well as the inherent biological processes of the micro organisms and drugs affecting intestinal motility. [3] Prevalence rates of diarrhoea associated with *Escherichia coli* O157:H7 from across Nigeria range from 2.7% to 25%. [4]

Metronidazole is highly effective in treatment of diarrhoea associated with susceptible anaerobic organisms. Due to the fact that aerobic and anaerobic infections coexists in diarrheal disease, metronidazole thus is used as drug of choice in evaluating the adsorption of microorganism on the prepared activated charcoal as an adjuvant in the treatment of diarrhea. [1, 5]

Direct compression has been utilized as a simple and effective technique in the preparation of controlled release matrix systems incorporation the polymer with the drug substance. Depending on the modification of formulation parameters, various drug release rates and patterns can be obtained. The swelling mechanisms and kinetics of drug release from branched anionic polysaccharide like xanthan gum consisting of two β – D glucose units linked through the 1 and 4 positions functions as a release retarding barrier. [6]

Bhattacharya and Staniforth studied dosage form devices which incorporate polymeric muco-adhesives systems, floating drug delivery devices, bilayered tablets using xanthan gum. [7, 8] In the gastrointestinal tract where a disease occurs, residence time at various sites presents a drawback which precludes the optimization of the dosage form administered. Suksiripattanapong et al. elucidated its use in polymeric systems to ensure controlled release of the drug incorporated into these systems. As the fluid penetrates these matrixes, swelling of the polymer occurs and drug diffusion ensuing from the system at a pace associated with the polymer xanthan gum is experienced. [9]

Bilayered tablets are tablets with two mutually exclusive layers which are usually represented by two clearly different colors. These layers may consist of the same or different drugs with modulated release layer for immediate release while the other layer for a more sustained or extended release. [10]

This type of dosage form involves combination therapy which minimizes problems associated with poly-pharmacy and patient compliance. The bilayered tablet concept is equally useful in drugs which require synergy to elicit profound pharmacological action. [11, 12]

In this present study bilayered tablets of metronidazole and activated charcoal and metronidazole tablets formulated via direct compression using xanthan gum as hydrophilic polymer were designed and evaluated comparing the physicochemical characteristics, drug release profile and release kinetic of all the formulations.

Despite the advances in drug development, *Escherichia coli* O157:H7 associated diarrhoea currently has no specific treatment. The resultant heamolytic-uremic syndrome is a life-threatening condition which leads to death in 25% of patients who do not recover with fluid replacement.[13]

The objective of the study is to design, characterize and evaluate a new formulation regimen comprising an adsorbent and antibacterial as a bilayered tablet *in vitro*. The *in vivo* performance of the metronidazole – activated charcoal formulation will be evaluated in Sprague Dawley® rat model infected with *Escherichia coli* O157:H7 with metronidazole concentrations in plasma evaluated via Liquid chromatography tandem mass spectrometry (LC-MS/MS).

MATERIALS AND METHOD:

Materials: Metronidazole powder obtained from Nacalai Tesque Inc. Kyoto Japan was utilized for tablet formulation Lot number V2E8244; USP Grade Activated charcoal obtained from Friendemann Schmidt chemicals C3000-1-1000 120500-0215 Germany with BET surface area 2058m^2/g (AC1); and with BET surface area 2153m^2/g (AC), Xanthan gum (XA) obtained from R&M Essex UK Batch Number PHOU90811; Magnesium Stearate (MS) from Sigma Aldrich. Mucin from porcine stomach from Sigma Aldrich Bound sialic acid 0.5-1.5% Batch number #099K7011USA. *Escherichia coli* O157:H7 (Migula) Castellani and Chalmers ATCC® 43895 subculture.

All other reagents and chemicals used were of analytical and pharmaceutical grade.

Tablet formulations: Dry granulation accompanied by direct compression was utilized to formulate the bilayered tablets (**Table 1**), utilizing a 12 mm-diameter die on an infrared hydraulic press (Perkin- Elmer, Cambridge, UK) [14, 15, 16].

The mass of the initial layer in the tablets was varied to maintain a constant aspect ratio for each layer 1.4: 1 so the final ejected bilayered tablet height was 10 mm. [17, 18] The tablets were evaluated for hardness, Compaction and Bi-Layer Tablet Fracture Force using a E-Z 50 materials tester machine.

TABLE 1: COMPOSITION OF VARYING METRONIDAZOLE AND METRONIDAZOLE/ACTIVATED CHARCOAL BILAYERED TABLETS EXPRESSED IN % W/W

Ingredients	1.5% XA	3%XA/ MS	3% XA	3%XA/ AC	5% XA	5%XA/ AC	7.5%XA	7.5%XA /AC	10%XA	10% XA/AC
MCC (micro crystalline cellulose)	33.36	33.0	33.33	15.19	33.5	14.64	33.43	14.89	35.33	14.29
Xanthan gum (XA)	1.5	3.0	3	3	5	5	7.5	7.5	10	10
10% Pre-gelatinized starch	36.8	34.16	35.33	23.0	33.2	21.55	30.73	18.79	26.33	16.9
Metronidazole	26.67	26.67	26.67	28.57	26.67	28.57	26.67	28.57	26.67	28.57
Magnesium stearate (MS)	1.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Talc	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67
AC Layer				28.57		28.57		28.57		28.57
	100	100	100	100	100	100	100	100	100	100

XA= Xanthan gum, AC= Activated charcoal, MS= Magnesium stearate, MCC = micro crystalline cellulose.

Swelling Index of tablets: This was carried out to evaluate the effect of the polymer xanthan gum on the hydration characteristics of the tablets. [19] Both simulated gastric fluid (SGF) 0.1 N HCL, and simulated intestinal fluid (SIF), pH6.8 were utilized as dissolution medium. Varying concentrations of xanthan gum were utilized in the tablets as reflected in **Table 1**, and the effects of the diverse concentrations of this anionic polymer was reflected in the swelling studies as in the method described by Sujja-areevath *et al.* [19, 20]

The experiments were performed in triplicate. The percentage increase in weight due to absorbed liquid or water uptake was estimated at each time point from Equation 1:

% swelling index = (Wi – Wo) / Wo × 100 .....Equation 1

Initial weight of the tablet prior to immersion (Wo) and final weight of the tablet after immersion (Wi) was utilized to determine the swelling index. Measurement of swelling and erosion rates of metronidazole tablets was carried out, after immersion of tablets in the test medium, to relate the observed phenomena of drug release with the rate of polymer hydration. Weighed tablets (W_0) were placed in the closed plastic containers with the mesh underneath the tablets, rotating at 150 rpm using Environment Shaker–Incubator (model ES-20, Biosan, Latvia), [20, 21] with the dissolution medium of 0.1 N HCL (HCL, pH 1.2) or phosphate buffer (pH 6.8) at 37 ± 0.5°C. After 2, 5, 10, 20, 60, and 120 minutes, each container was removed from the incubator, the tablet with the mesh was withdrawn from the medium and blotted to remove excess water and then weighed (Wi) on an analytical balance [21] (model AG204, Mettler–Toledo, Greifensee, Switzerland).

Morphological examination of swollen metronidazole tablets: Morphological examination of the swollen tablets was carried out using a General imaging Co. digital camera 10.1 megapixel Model CI0033 USA equipped with GE Aspheric zoom lens 3 X 5.2–15.6 mm 1:3.5 - 6.4. Photo imaging was performed on each tablet formulation after hydrating in 0.1 N HCL or pH 6.8 phosphate buffer at varying time intervals.

Differential Scanning Calorimetric (DSC) studies: DSC studies for the pure metronidazole and the prepared tablets were analyzed in order to study the polymorphic changes in the drug as well as its interaction with the excipients. [22] Samples of pure drug and powdered tablets were weighed directly in pierced aluminium pans and scanned between 25°C to 190°C at a heating rate of 10ºC / min under static nitrogen gas at a pressure of 20N on aluminum 40 micro liter/ml and 50 ml / min flow rate . [22-25]

Mucoadhesion studies of metronidazole and metronidazole/activated charcoal tablets:

Texture analyzer method: The mucoadhesive force of the metronidazole tablet formulation was determined by measuring the force of detachment of the tablet from the intestinal mucosal of a pig using an Instron texture analyzer/ Force transducer Model 2519-104 Capacity 500N S/N 59649 using the method described by Teakuchi *et al.*[26-2]

Mucoadhesion study via mucin particle method: It was expected that the surface property of the mucin particles might be changed by the adhesion of the polymer i.e. xanthan if the polymer has a mucoadhesive property. The occurrence of such change was detected by measuring the zeta potential with a Zetamaster (Malvern Instruments, UK). [27-29]

Dissolution rate of metronidazole and metronidazole/activated charcoal tablets and Curve fitting and Kinetics of Drug release: Medium utilized was 900 mls simulated intestinal fluid (SIF pH 6.8). The dissolution of the metronidazole and metronidazole and activated charcoal layered tablets intended for sustained release containing varying concentrations of xanthan gum was utilized for this study. Distex dissolution System model Evolution 6300 with a programmable syringe pump dissolution auto sampler model Evolution 4300 and a biochrom Libra 312 UV spectrophotometer were utilized for the dissolution studies.

A single tablet is dropped into the designated hole into the dissolution medium SIF adjusted to pH6.8 900 ml contained in a 1000 ml flask. The flask is cylindrical with a hemispherical bottom. The flask is maintained at 37±0.5^{o}C by an automated constant temperature bath. The dissolution medium was heated up to 37^{o}C by the automated thermostat. Rotation of 75 revolutions per minute (rpm) was utilized; 5 ml of sample was withdrawn at 5mins, 10mins, 15 mins, 30mins, 1hr, 2hrs, 3hrs, 4 hr, 6hr, 8hr, 9hr, 12hr, 15hr, 18hr, and 24hr time intervals and filtered thrice. 5 ml of the dissolution medium maintained at 37^{o}C was added after each sample withdrawal. Each test was done with six replicates.

The amount of dissolved metronidazole was determined from UV absorbance at the wave length of maximum absorbance at 322 nm of filtered portion of the solution under test [12]. The release pattern was evaluated and was fit into varying kinetic models to further explain the release mechanism of the formulated tablets. The data was fit into Zero order release kinetics, First order release kinetics, Higuch's square root of time equation and the Korsemeyer Peppas release kinetics. [30]

Stability study: The bilayered metronidazole/ activated charcoal tablets were strip packed (Al–Al strip, 0.04mm) and subjected to accelerated stability testing detailed in the draft consensus guideline stability data package for registration in climatic zones III and IV as per ICH 2002 guidelines (40 °C ± 2 °C / 75% RH ± 5% RH). The samples were analysed periodically (0, 15, 30, 60, 90, and 180 days) and evaluated for the different physico-chemical parameters viz. appearance, weight variation, thickness, hardness, drug content which was evaluated via *in vitro* release studies. [31, 32]

Experimental animal model: One hundred and twenty (120) male Sprague Dawley® rats weighing 350–400g at the start of the experiment were allowed to acclimatize in their new environment for seven days prior to commencing the experiment. They were housed, each rat in an individual polypropylene and metal cages equipped to provide food and water. The animals were maintained under controlled conditions of temperature (20°C ± 2°C), relative humidity (45 ± 10%) with a 12 hour light and dark cycle, lights went off at 7pm. They had access to a standard 2016 diet i.e. Harlan Teklad Diet, Oxon, England and clean drinking water *ad libitum*. The animal care units at Melior Discovery Inc. 860 Springdale drive Exton PA 19341 USA and the College of Medicine University of Lagos, Lagos Nigeria, were utilized for this study after ethical approval was granted by the ethics committee of the College of Medicine University of Lagos, Lagos Nigeria.

All the procedures were in compliance with the American Psychological Association Guidelines for ethical conduct in the care and use of non human animals in research.[33]

Experimental Design 1: The rats were randomly divided into six groups of ten rats each. The treatment groups were classified from I to VI, [BET surface area of AC1 is 2058m^2/g (group V) and BET surface area of AC is 2153m^2/g (group VI)]. All the rats in each of the groups except group II were infected via oral administration of 1ml of 10^6 CFU of *Escherichia coli* O157:H7 and an incubation period of 2 days were allowed for symptoms of diarrhea specifically loose stool to manifest. All the drugs were administered orally by oral gavage, twice daily for a period of 5 days as shown in Table 2. Post infection with *Escherichia coli* O157:H7, stool was analyzed for the presence of the organism. All the stool samples from groups were collected every six hours and analyzed for the presence of the *Escherichia coli* O157:H7. [34-37]

TABLE 2: TREATMENT GROUPS FOR EXPERIMENTAL DESIGN 1

Group	Bacteria (*Escherichia coli* O157:H7)	Treatment
I	1ml of 10^6cfu/ml	Sterile water
II	1ml of Sterile water	Sterile water
III	1ml of 10^6cfu/ml	metronidazole alone 7.5mg/kg orally
IV	1ml of 10^6cfu/ml	5 mg/kg AC
V	1ml of 10^6cfu/ml	7.5 mg/k metronidazole and 5 mg/kg AC1 alone
VI	1ml of 10^6cfu/ml	7.5 mg/kg metronidazole and 5 mg/kg AC

AC1 = Activated charcoal with surface area 2058m^2/g
AC= Activated charcoal with surface area 2153m^2/g

Experimental Design 2: The rats were divided into three groups of ten rats each post infection with *Escherichia coli* O157:H7, Group A was administered metronidazole alone 7.5mg/kg orally and Group B was administered 7.5mg/kg metronidazole and 5 mg/kg AC1 as a single dose, Group C was administered 7.5mg/kg metronidazole and 5 mg/kg AC. (BET surface area of AC1 is 2058m^2/g and BET surface area of AC is 2153m^2/g) 0.5 ml of blood were collected via the tail vein of all the rats in each group at 0 hr, 1 hr, 3 hr, 6 hr and 8 hours after dosing and stored for centrifugation in heparinized collection tubes. The blood was immediately centrifuged at 250 rpm for 10 minutes at 4°C temperature, after which the supernatant plasma layer was separated and stored at −20 °C until analyzed. The plasma samples were analyzed for metronidazole concentrations using an LC- MS/MS methodology as described by Ilomuanya *et al.*[34]. All the rats were sacrificed 8 hours after dosing and the required organs were surgically removed via a lateral incision through the chest wall. Tissue samples from heart, lung, liver and stool samples were analyzed for metronidazole concentrations using the method developed by Uboh *et al.*[35] The samples were then extracted using liquid-liquid extraction (LLE) technique and assayed using LC- MS/MS.[34]

RESULTS:

Compression and Bi-Layer Tablet Fracture Force: The desired hardness for the bilayered tablets is solely dependent on the force of compaction of both the initial layer and the final bilayered tablets. [17] An increased force of compression was utilized to formulate the bilayered tablet compared to that required for the single tablets to ensure that they met BP specification on tablet hardness thus ensuring suitability for handling and storage as well as eliminating risk of increased friability and other inherent tableting defects (**Table 3**). With increasing final compaction force there was a progressive increase in the hardness of the bilayered tablet produced **(Fig. 1**).

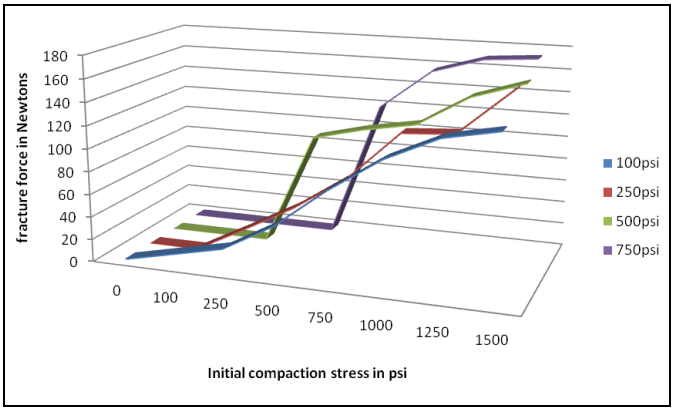

FIG. 1: EFFECT OF INITIAL COMPACTION STRESS ON THE FRACTURE FORCE OF THE ACTIVATED CHARCOAL / METRONIDAZOLE BILAYERED TABLET

An initial compaction stress of 100 psi, having a final compaction stress of 1500 psi gave an overall hardness of the tablet to be 140 Newton. However the initial compaction force of between 250 and 500 psi followed by a final compaction force of between 500 and 750 psi gave the desired hardness value for the bilayered tablets being an average of 110 N.

TABLE 3: EVALUATION OF DIFFERENT LAYERED TABLETS OF METRONIDAZOLE AND METRONIDAZOLE/ACTIVATED CHARCOAL TABLETS

Formulation	Hardness (Newton)	Weight variation (%)	Friability (%)	Diameter (mm)	Drug content(90-110%)
1.5%XA	110 ± 0.05	0.99 ± 0.03	0.30 ± 0.02	11.85 ± 0.01	99.73 ± 0.82
3%XA	112 ± 0.17	1.72 ± 0.03	0.35 ± 0.01	11.84 ± 0.01	101.0 ± 0.75
3%XA	118 ± 0.09	0.88 ± 0.1	0.28 ± 0.04	11.85 ± 0.01	99.83 ± 0.11
3%XA/AC	108 ± 0.11	1.07 ± 0.17	0.32 ± 0.08	11.85 ± 0.01	98.30 ± 0.02
5%XA	117 ± 0.08	1.1 ± 0.08	0.27 ± 0.02	11.85 ± 0.01	100.5 ± 0.33
5%XA/AC	110 ± 0.1	1.30 ± 0.01	0.29 ± 0.06	11.86 ± 0.03	98.34 ± 0.12
7.5%XA	115 ± 0.03	1.05 ± 0.08	0.28 ± 0.07	11.85 ± 0.02	100.30 ±0.22
7.5%XA/AC	108 ± 0.15	1.18 ± 0.06	0.30 ± 0.02	11.85 ± 0.02	98.30 ± 0.09
10% XA	114 ± 0.23	1.34 ± 0.43	0.35 ± 0.1	11.84 ± 0.01	99.79 ± 0.39
10%XA/AC	109 ± 0.34	1.99 ± 0.14	0.36 ± 0.09	11.85 ± 0.02	97.9 ± 0.26

All the values are expressed in ± standard deviation where n= 20
XA= Xanthan gum, AC= Activated charcoal

Swelling studies: Utilizing pH 6.8 for the swelling studies the duration of the experiment reflected a variation of percentage (%) swelling index from onset to 1260 minutes. An increased swelling index in neutral medium pH 6.8 was experienced compared with the acidic medium (**Fig. 2-3**).

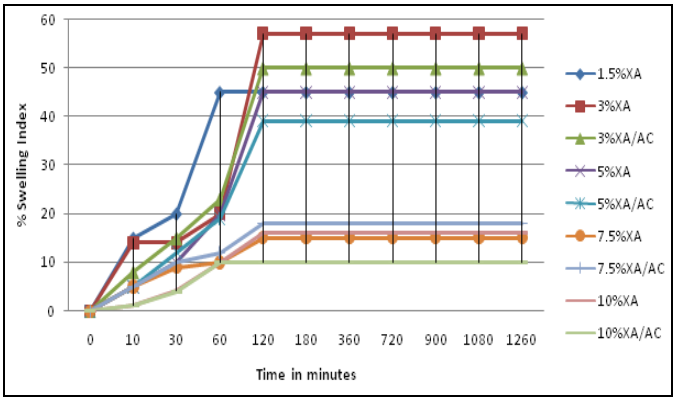

FIG. 2: SWELLING STUDIES OF METRONIDAZOLE AND METRONIDAZOLE/ ACTIVATED CHARCOAL LAYERED TABLETS AT pH 1.2 IN SIMULATED GASTRIC FLUID

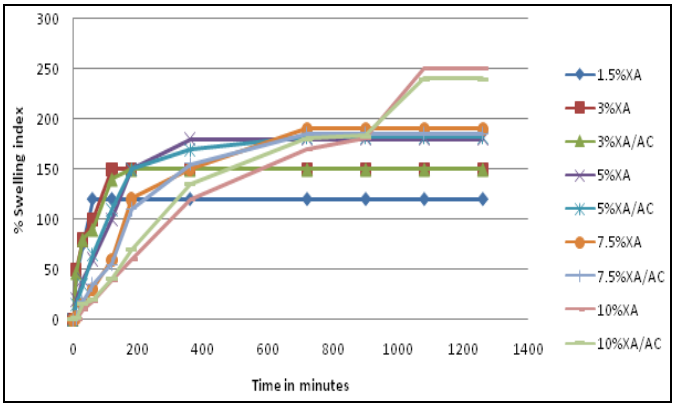

FIG. 3: SWELLING STUDIES OF METRONIDAZOLE AND METRONIDAZOLE/ ACTIVATED CHARCOAL LAYERED TABLETS AT pH 6.8 IN SIMULATED INTESTINAL FLUID.

Formulation 3% XA to 5% XA did not swell to a great extent as 7.5% XA and 10% XA due to less hydration of the polymer layer. At the 30 minutes time point in the pH 6.8 % swelling index was 75% and 80% for 7.5% XA and 10% XA compared to 35% and 18% experienced in 5% XA and 3% XA. However over time, the values for the higher concentrations of XA increased % swelling index to 250% for 10% XA (**Fig. 4A**) at the 1080 minutes time point compared to %swelling index of 120% for 3% XA.

An increased concentration of xanthan gum in the tablets translated to the increased swelling observed. Swelling was examined for both single and layered tablets. The metronidazole layer of the XA/AC tablet was utilized for the studies and the results obtained were statistically similar to the values obtained from the plain tablets as seen in **Fig. 4B.**

There was a visual difference in the formation of a gel around each tablet (**Fig. 4A**). The gel strength significantly varied with the concentration of xanthan gum in the formulation of the tablet. Concentrations of 5% XA, 7.5% XA and 10% XA formulated tablets on hydration showed an enclosure in a gel layer leaving the inner core with varying degrees of hydration depending on the concentration of XA they contained (**Fig. 4 A-D**). This ensured that metronidazole is released in a controlled manner via zero order kinetics.

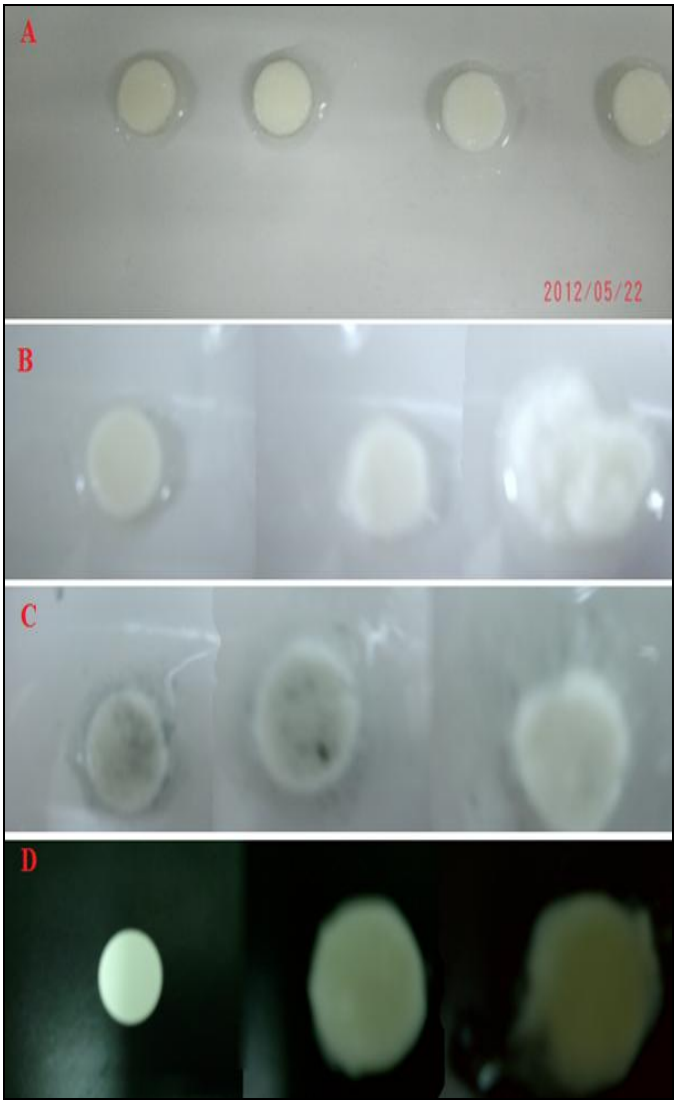

FIG. 4: MORPHOLOGICAL EXAMINATION OF SWOLLEN METRONIDAZOLE TABLETS SHOWING SWELLING OF 10% XA, 7.5% XA, 5% XA, 3% XA TABLETS (LEFT TO RIGHT) RESPECTIVELY AFTER EXPOSURE TO SIF FOR 10 MINUTES (A); SWELLING OF 5% XA TABLET AFTER 10 MINUTES, 60 MINUTES AND 180 MINUTES (B); SWELLING OF 5% XA/AC AFTER 10 MINUTES, 60 MINUTES AND 180 MINUTES (C); SWELLING OF 10% XA AFTER 30 MINUTES, 720 MINUTES AND 1260 MINUTES (D).

Differential Scanning Calorimetric (DSC) studies: There were no significant shifts in the endothermic peaks however there were changes in the enthalpy of fusions which is to be expected. The pure sample had a lower enthalpy of fusion-233.42 J/g than the test sample containing the granule formulation -108.21J/g, as shown in **Fig. 5**. The DSC findings of the matrix tablet powder, no major thermal event corresponding to chemical interaction was observed.

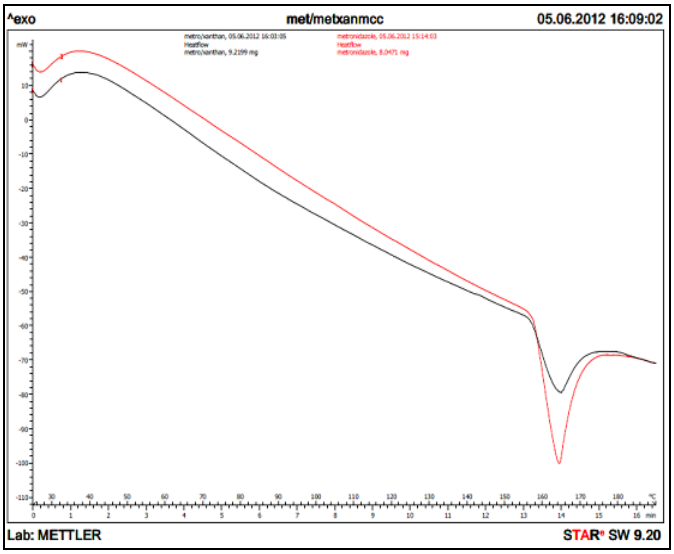

FIG. 5: COMPARATIVE DIFFERENTIAL SCANNING CALORIMETRIC THERMOGRAM SHOWING PURE METRONIDAZOLE AND TABLET SAMPLE CONTAINING XANTHAN GUM

Mucoadhesion Studies: An increase in xanthan gum concentration in the tablets caused a corresponding increase in mucoadhesion. The overall adhesion of the tablet was directly dependent on the formation of a gel layer on the surface of the tablet and this facilitated the adhesion to the pig's mucosal surface. It is therefore pertinent to say that a more tenacious gel is formed at concentrations of xanthan gum XA 10% and XA 7.5% and this can also be compared to the results obtained in the swelling studies earlier examined see **Fig. 4**. The formulation XA 10% had the highest value of 11.01± 1.07 N/cm^2 compared to 1.05 ± 0.43 N/cm^2 for XA 1.5% as shown below in **Table 4.**

TABLE 4: EFFECT OF XANTHAN GUM CONCENTRATION IN METRONIDAZOLE TABLETS ON MUCOADHESION

Formulation	Mucoadhesion(Newton/cm^2)
1.5%XA	1.05± 0.43
3%XA	2.78 ± 1.10
3% XA containing MS	2.99 ±1.01
3%XA/AC	3.87± 0.32
5%XA	5.77 ± 1.32
5%XA/AC	5.07 ± 0.23
7.5%XA	6.99 ± 0.78
7.5%XA/AC	7.97± 1.12
10% XA	11.01 ± 1.07
10%XA/AC	11.07 ± 1.11

The results are expressed as Mean ± SD and n = 6
XA= Xanthan gum, AC= Activated charcoal, MS= Magnesium stearate

The mucin particle suspension involved utilizing varying concentrations of xanthan gum. This brought about a change in the zeta potential of the mucin particles. Zeta potential is the electric potential in the interfacial double layer at the location of the slipping plane against a point in the bulk fluid away from the interface. The resultant zeta potential moved to a higher negative value because of the negative charge of the polymer xanthan gum which is anionic as shown in **Fig. 6A**. These results are similar to those obtained by Takeuchi *et al.* [26, 27] hence the feasibility of the mucin particle method for evaluating the mucoadhesive property of xanthan gum as a polymer. In the presence of excipients and mucin alone there was no significant change in the zeta potential (**Fig. 6B**). The mucoadhesive property can be said as a direct result of incorporation of the xanthan gum.

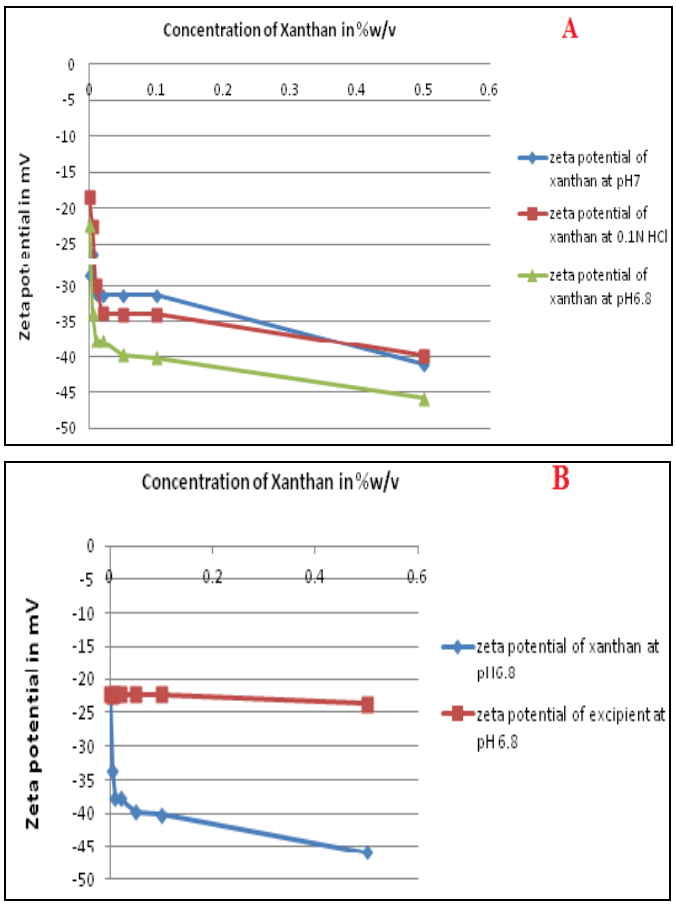

FIG. 6: ZETA POTENTIAL OF MUCIN PARTICLES IN XANTHAN GUM AT pH 6.8, pH 7.0, 0.1 N HCL(A) AND ZETA POTENTIAL OF MUCIN PARTICLES IN XANTHAN GUM AT pH 6.8 IN COMPARISON WITH ZETA POTENTIAL OF MUCIN PARTICLES IN THE TABLET EXCIPIENTS (B)

The two methods used in evaluating the mucoadhesive property of xanthan gum clearly show that as the concentration of xanthan gum in the formulation or in the particulate system increases, there also is a reflective increase in the mucoadhesive property whether it's measured via zeta potential or in N/cm^2. The mucoadhesive value is greatly affected by the environment, at pH 6.8 maximum adhesive property of xanthan is observed.

Drug release kinetics Studies: Due to the presence of xanthan gum in the formulation, a shear reversible property was observed in the tablet when exposed to aqueous media [16, 19] due to its inherent helical structural property. With increasing concentrations of xanthan gum contained in the tablet (plain or bilayered) a corresponding increase in viscosity around the circumference of the tablet due to the reordering of its conformation into a random coil.

In the formulation containing 5% XA, at 540 minutes 99.8% of metronidazole was completely released, 94.05% was obtained for the 5% XA/AC formulation as shown in **Fig. 7**. There was a formation of a gel layer round the surface of the tablet lending to its sustained release behavior. However the release characteristic was best fitted to the Higuchi model as in **Table 5**.

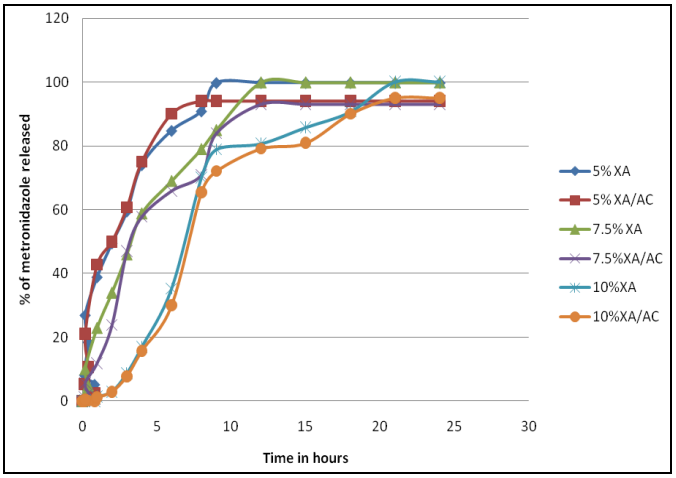

FIG. 7: RELEASE OF METRONIDAZOLE TABLETS AND METRONIDAZOLE ACTIVATED CHARCOAL BILAYERED TABLETS CONTAINING 5% XA, 7.5% XA, 10% XA AND 5% XA/AC, 7.5% XA/AC, 10% XA/AC

XA=xanthan gum; AC= activated charcoal

TABLE 5: KINETIC RELEASE PARAMETERS OBTAINED FROM FITTING METRONIDAZOLE DISSOLUTION DATA TO VARIOUS KINETIC MODELS.

	Zero order model		1st order model		Higuchi model		Korsemeyer Peppas model			Hixon-crowell model	
	R^2	Ko	R^2	K_1	R^2	K_H	R^2	n		R^2	K_{HC}
1.5%XA	0.9997	0.1011	-	-	-	-	0.9992	1.0	non fickian/ zero order	-	-
3%XA	0.9995	0.6798	-	-	-	-	0.9994	0.9999	non fickian/ zero order	-	-
3%XA	0.9991	0.1411	-	-	-	-	0.9993	0.9999	non fickian/ zero order	-	-
3%XA/AC	0.9973	0.5523	-	-	-	-	0.9994	0.9999	non fickian/ zero order	-	-
5%XA	0.9368	0.1159	0.7934	0.0034	0.9982	4.3885	0.9392	0.6771	anomalous	0.704	-0.2648
5%XA/AC	0.9456	0.8761	0.9860	-0.0024	0.9795	4.8186	0.9392	0.6771	anomalous	0.660	-0.2964
7.5%XA	0.9833	0.3422	0.7075	0.0031	0.9969	4.1269	0.8141	0.3850	-	0.659	-0.3506
7.5% XA/AC	0.9892	0.8794	0.9725	-0.0015	0.9797	4.0450	0.8140	0.3799	-	0.723	0.3844
10%XA	0.9901	0.5831	0.9725	0.0015	0.9198	3.3040	0.9912	1.6843	Super case II transport	0.772	0.1911
10% XA/AC	0.9911	0.6890	0.9343	-0.0009	0.9194	3.4510	0.9912	1.6840	Super case II transport	0.782	0.1889

K……...Kinetic rate constant characteristic of the drug/polymer system
R^2 ……Linear regression analysis of data used characterizes the mechanism of release
n……Diffusion exponent used to characterize mechanism of release

This could be explained under the assumption that the gel layer formed some sort of insoluble matrix which may facilitate Fickian diffusion. A higher XA concentration in the tablet formulation i.e. 7.5% XA and 10% XA showed a reduced initial drug release of about 34% and 5% at 120 minutes respectively. A more consistent gel could be visually seen around the tablets at different time intervals (as in the swelling studies) due to increased hydration and water sorption accompanied by a zero order release mechanism (**Fig. 4A**). This led to increasing the drugs diffusion path way and thus modulates the drug release over time irrespective of the initial concentration of drug in the tablet. An increase in the concentration of the polymer XA in the formulation significantly affected the overall time of release of metronidazole from the tablet thus influencing their release profiles as well.

The zero order kinetic model effectively explains the release of metronidazole from most of the formulations i.e. 1.5% XA and 3% XA, 3% XA/AC, 3% XA+MS. The release of metronidazole is independent of the initial concentration of drug within the tablet and thus uniform release is facilitated over time (**Fig. 7).** The dissolution values were fitted into the Korsemeyer Peppas kinetic model and as reflected in **Table 5** can account for the release in 3% XA and 3% XA/AC via Fickian diffusion and zero order as earlier explained. Case II relaxation which refers to the erosion of the polymeric chain can be best used to explain the release kinetics of metronidazole from the formulations 10% XA and 10% XA/AC. This is due to the change in the thickness and porosity of the gel structure. A complex hydrated layer through which the drug gradually diffuses out is formed and this layer ensured prolonged release of the drug via zero order over a period of 24 hours. Thus the mechanism or kinetics of release observed usually encompasses more than one mechanism as shown in **Table 5**. In some cases drug release occurred due to combination of macromolecular relaxation and a somewhat Fickian diffusion. The diffusion exponent in 5% XA and 5% XA/AC was between 0.5 and 1.0 thus lending credence to an anomalous mechanism despite having exponent values of 0.94 and 0.95 respectively for zero order release.

Stability studies: According to ICH guidelines Q1E Step4 [38] six month accelerated stability study (40 ± 2 °C/75±5% RH) for the optimized formulations showed negligible change over time for the parameters like appearance, weight variation, thickness, hardness, and drug content. [31, 38] The results showed that humidity had a marked influence on metronidazole stability, with degradation accelerated at high temperature.

The effects of light and temperature on metronidazole stability were only significant at the 90th day (**Fig. 8**). The most adverse condition leading to the rapid degradation of metronidazole was 40°C/ 75% RH. Under these conditions, the combined effects of temperature and relative humidity appeared to be synergistic. The presence of higher than 85% of metronidazole as drug content was obtained in the stability study over the period of analysis, thus reflecting that leeching of metronidazole by the activated charcoal layer was negligible. Shelf life was obtained by linear extrapolation of zero-order kinetics, was found to be 2.14 years for XA/AC 7.5%, 2.99 years for XA 7.5% and 2.63 years for XA/AC 5%, 2.81 years for XA 5%.

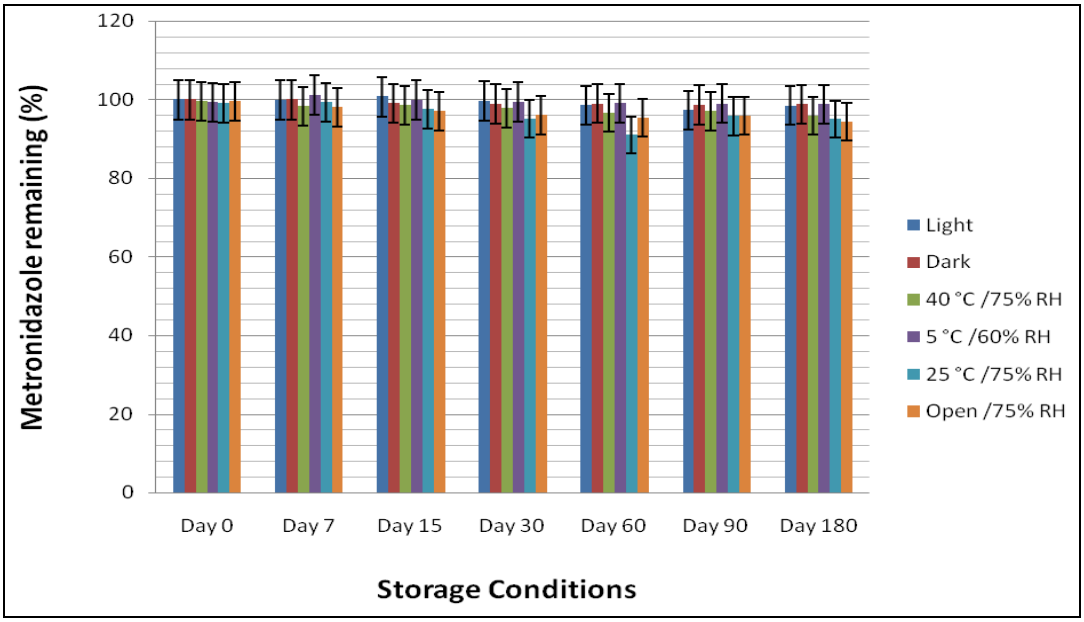

FIG. 8: THE STABILITY OF METRONIDAZOLE IN THE METRONIDAZOLE-ACTIVATED CHARCOAL (XA/AC 5%) FORMULATION. DATA SHOW THE MEAN PERCENTAGE METRONIDAZOLE CONCENTRATION AND STANDARD DEVIATION.

Estimation of Metronidazole in biological samples: For the LC- MS/MS method, a linear correlation with $R^2 = 0.9999$ was obtained, between the metronidazole/IS (Internal standard D_9 Clenbuterol) peak areas ratios and metronidazole concentrations over the range of 50 to 500 ng/ml. The limit of detection and quantification were found to be 50ng/ml and 75ng/ml, respectively.

Experimental Animal Model: The effects of individual treatments on each group were evaluated as shown in **Table 6** where deaths were recorded in groups I, III and IV. The control group and the group treated with metronidazole alone had a survival rate of less than 60% at the end of the treatment cycle. On day 2 of administration of metronidazole and activated charcoal a reduced amount of wet stool was observed over a period of four hours.

TABLE 6: EVALUATION OF THE CHANGES IN THE STOOL CONSISTENCY AFTER TREATMENT IN TREATED AND CONTROL GROUPS OF THE EXPERIMENTAL ANIMAL MODELS

Group	Serological identification of *Escherichia coli* O157:H7in the stool (+) / number of loose Stool in 6 hours							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 10	Day 14	Number of animals still alive
I	+/6	+/7	+/8	+/7	+/7	+/6	+	5
II	-	-	-	-	-	-	-	10
III	+/7	+/4	+/6	+/5	+/6	+/7	+	4
IV	+/8	+/4	+/3	+/3	+/1	+	+	9
V	+/7	+/2	+/1	+	+	-	-	10
VI	+/3	+/1	-	-	-	-	-	10

Marked reduction in the number of wet stools was observed over the first 48 hours of the study in groups treated with metronidazole and activated charcoal i.e. groups V & VI. Presence or absence of the bacteria in stool sample was utilized as treatment end points for this study. Group V reflected a negative result for identification of *Escherichia coli* O157:H7 on the fifth day of treatment and this was retained till the tenth day of the study as reflected in **Table 7**. In comparison, group VI reflected a negative result for serological identification of *Escherichia coli* O157:H7 on the third day of treatment and this was retained till the tenth day of the study, thus showing the superiority of metronidazole and activated charcoal (AC) having a higher BET surface area (than AC1) in the adsorption of *Escherichia coli* O157:H7. Groups I, III and IV all through the duration of the study showed positive results for the presence of *Escherichia coli* O157:H7.

TABLE 7: AVERAGE COUNT OF *ESCHERICHIA COLI* O157:H7 IN THE STOOL EXPRESSED AS cfu/g OF STOOL.

Group	Average Bacterial (*Escherichia coli* O157:H7) count in the stool expressed as cfu/g of stool.														
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Number of animals still alive
I	3×10^5	4.7×10^6	4×10^5	7×10^4	2.4×10^5	1.2×10^5	1.5×10^6	6.8×10^5	6.1×10^2	2.4×10^2	5.9×10^2	5.7×10^2	10^2	190	5
II	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10
III	3.1×10^6	7×10^5	7.8×10^5	6.7×10^5	4.5×10^5	6.7×10^5	7×10^5	8.3×10^4	3×10^4	$2.3\ 10^4$	3×10^3	2.5×10^3	1.1×10^2	108	4
IV	2.7×10^5	5.7×10^5	7×10^4	6.8×10^3	8×10^2	6.7×10^2	4.4×10^2	4.3×10^2	3.1×10^2	3.1×10^2	120	102	93	29	9
V	2×10^6	4.7×10^3	10^2	340	380	149	-	1	-	-	-	-	-	-	10
VI	2.9×10^6	10^2	-	-	-							-		-	10

DISCUSSION: The anti-diarrheal role of metronidazole and activated charcoal combination was evaluated for their *in vivo* performance in Sprague Dawley® rats models infected with *Escherichia coli* O157:H7. The rat's defecation pattern prior to infection allowed daily sampling. The adult rats defecated 39 ± 3 fecal pellets per day. The majority of the pellets were defecated during the light cycle of the experiment. Fecal mass expressed as dry weight obtained as 6.88 ± 0.2g/day was obtained prior to infection with *Escherichia coli* O157:H7. After oral administration of *Escherichia coli* O157:H7, 40% of the animals exhibited diarrhoeal symptoms of loose stool which was confirmed using SMAC agar and *Escherichia coli* O157:H7 antisera. Only animals that had established diarrhoea were used for this study.

Tissue and plasma concentrations of metronidazole in groups treated with either metronidazole alone or metronidazole and activated charcoal did not show significant variability using experimental design 2. This reflected that adsorption of metronidazole in the presence of activated charcoal occurred but not to such an extent as to affect absorption of the antibacterial into the tissue and plasma (**Fig. 9**), thus ensuring that optimal plasma concentration of this nitro-imidazole is available systemically for activity against susceptible organisms.

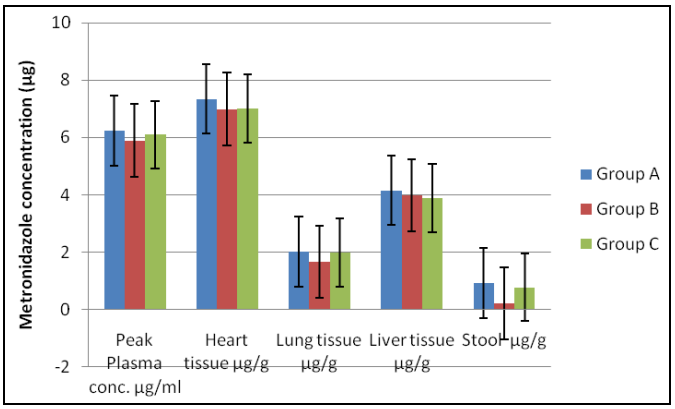

FIG. 9: CONCENTRATION OF METRONIDAZOLE AT PEAK PLASMA CONCENTRATION IN DIFFERENT TISSUES IN SPRAGUE DAWLEY® RATS.

Peak plasma concentration was obtained at three hours for the rats administered with metronidazole alone at 6.2 µg/ml while metronidazole/AC showed a peak plasma concentration at three hours of 5.89 µg/ml. Similar results have been obtained by Sagan et al. and Silva *et al.*, [39- 41] where peak plasma concentrations of 5.6 to 7.5 µg/ml have been reported to occur between 2 to 3 hours after oral administration of metronidazole (10mg/kg). These results are in agreement with *in vitro* data obtained via pharmacodynamic modeling [8] and adsorption modeling studies in our studies where effective concentrations of metronidazole in the presence of activated charcoal did not vary significantly from the systems which were developed to simulate metronidazole dissolution alone.

There was no significant difference between the two results analyzed; the amount of drug released from both the single tablet and the layered tablet was similar. Metronidazole concentrations in plasma (**Fig. 10**) and stool samples (**Fig. 11**) were detected using previously described and validated LC-MS/MS via liquid-liquid extraction process at 2.94 minutes retention time.

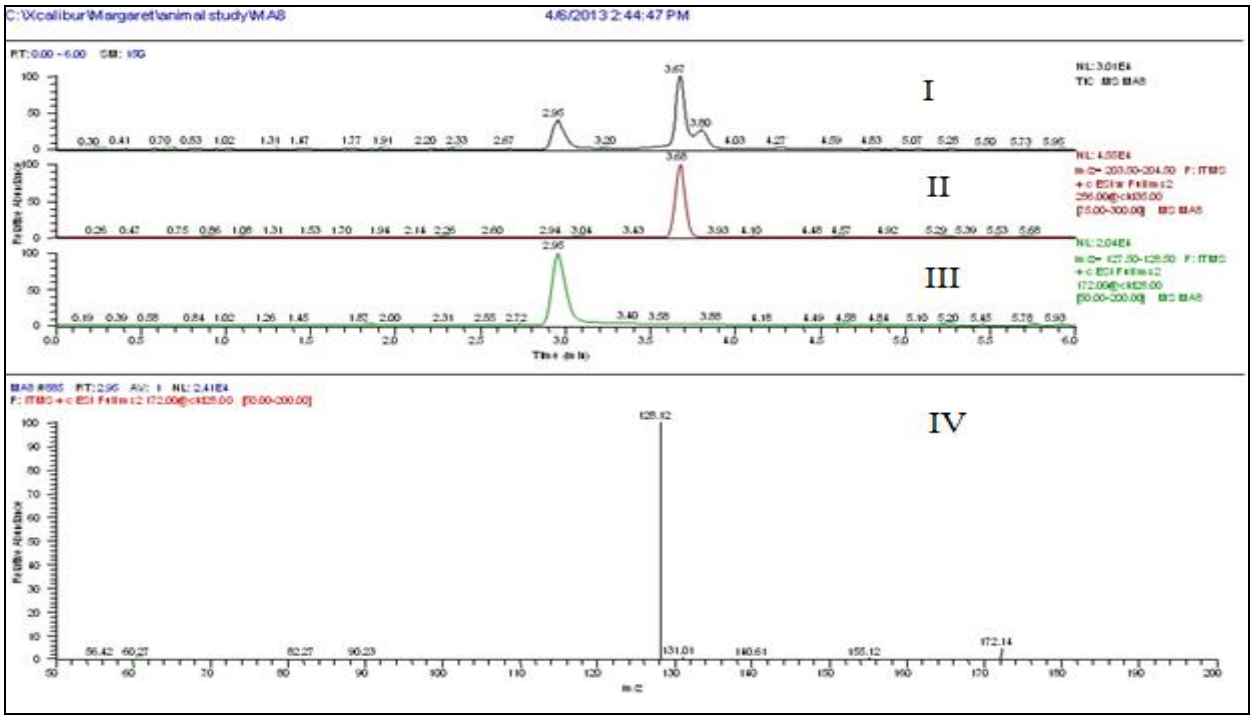

FIG. 10: LC MS/MS CHROMATOGRAM OF METRONIDAZOLE IN PLASMA SHOWING (I) TOTAL ION CURRENT TIC OF METRONIDAZOLE PLUS IS WITH RETENTION TIME OF 3.66MINUTES, (II) INTERNAL STANDARD (IS) WITH RETENTION TIME OF 3.66 MINUTES (III) METRONIDAZOLE WITH RETENTION TIME OF 2.94 MINUTES AND (IV) PRODUCT ION SPECTRUM FOR METRONIDAZOLE *(m/z* 172→128) AFTER ADMINISTRATION OF METRONIDAZOLE AND ACTIVATED CHARCOAL.

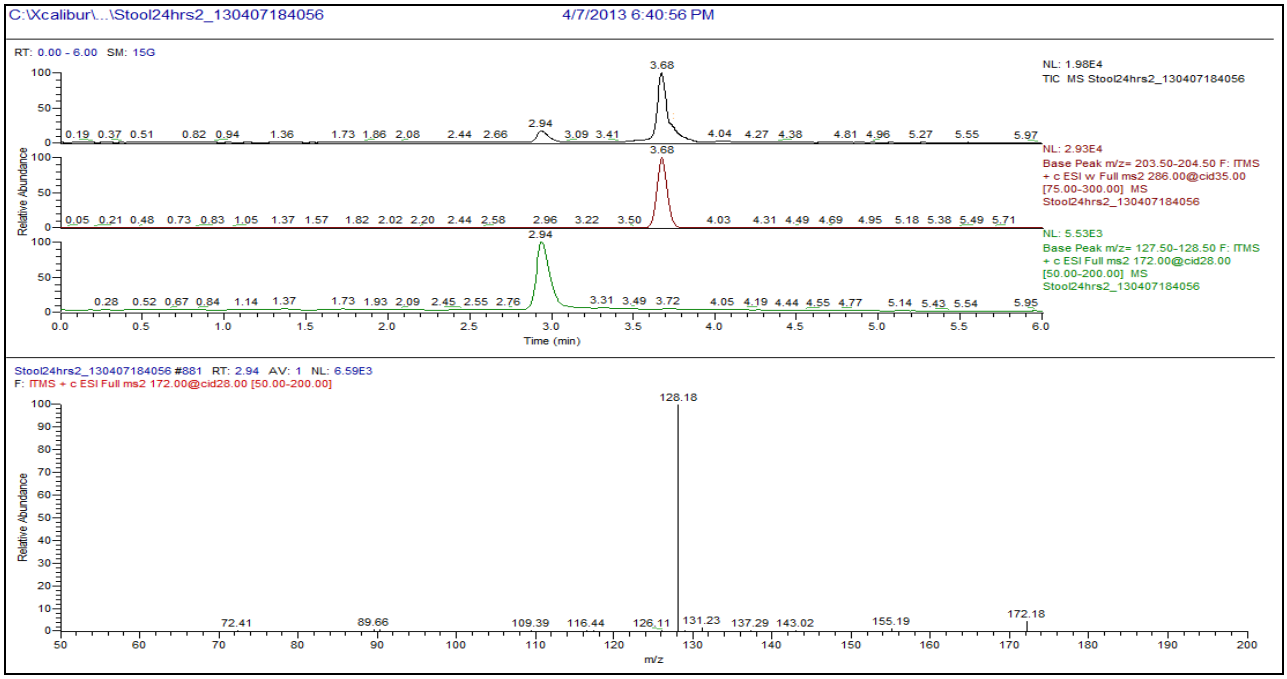

FIG. 11: LC MS/MS CHROMATOGRAM OF METRONIDAZOLE IN STOOL SHOWING (I) TOTAL ION CURRENT TIC OF METRONIDAZOLE PLUS IS WITH RETENTION TIME OF 3.66MINUTES, (II) INTERNAL STANDARD (IS) WITH RETENTION TIME OF 3.66 MINUTES (III) METRONIDAZOLE WITH RETENTION TIME OF 2.94 MINUTES AND (IV)PRODUCT ION SPECTRUM FOR METRONIDAZOLE *(m/z* 172→128)

There was no significant difference in the treatment outcome for the groups I and III. This result is in consonance with Hermsen *et al.*, that metronidazole has no activity against *Escherichia coli*. [8, 42] At the end of the animal study apart from the negative control group, only the groups which had the adsorbent as well as the antibacterial recorded absence of *Escherichia coli* O157:H7 in the stool at the end of the treatment period. *Escherichia coli* O157:H7 associated diarrhoea currently has no specific treatment. The standard guidelines for treatment include fluid replacement and blood pressure support to prevent death from dehydration, 60% of victims recover without treatment in five to 10 days. There is no evidence that antibiotics improve the course of disease, and treatment with antibiotics may precipitate kidney complications. [43-45] Haemolytic-uremic syndrome is a life-threatening condition which leads to death occurs in 25% of patients who do not recover; this condition leads to death in at least 5% of patients closely monitored in intensive care units. [44]

Certain novel treatment strategies, such as the use of anti-induction strategies to prevent toxin production and the use of anti-Shiga toxin antibodies [44] have also been proposed but are still at experimental stages. Studies within Nigeria have shown that the degree of contamination of food varying from vegetables to meat products is very high ranging from 15% to as high as 53%. More alarming is the fact that sampling from cooked sources also reflect rate of contamination with *Escherichia coli* O157:H7 as high as 25%. [45]

Thus the usefulness of the proposed combination of antimicrobial and adsorbent in the treatment of diarrhoea associated with *Escherichia coli* O157:H7 has been elucidated. Its activity clearly shown in the *Escherichia coli* O157:H7 Sprague Dawley® rat Diarrhoeal model, and is superior to available forms of management which do not lead to immediate clearance of the bacteria from the intestinal tract.

CONCLUSION: Direct compression of hydrophilic polymer and drug release modifier xanthan gum directly influences the release characteristics of metronidazole from single or bilayered tablet formulation with activated charcoal. The synergistic potential of a nitro-imidazole and an adsorbent has been evaluated and established with treated groups showing cessation of symptoms within twenty four hours thus effective in the treatment of *Escherichia coli* O157:H7 associated diarrhea. This information will pave way for development of this drug combination by the pharmaceutical industry for use in treatment of mild to moderate hemorrhagic diarrhea.

CONFLICT OF INTEREST: The researchers declare no conflict of interest

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