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The preparation and Evaluation of Some Metronidazole Formulations as Gels and The study of Their Medicinal Activities Against Pathogenetic Microorganisms Causing Dermatitides

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Abstract

In the present study, a labor has been made to develop gel type of transdermal therapeutic system comprising different concentrations of metronidazole with hydrophilic polymeric combinations using solid dispersion method. Two main parts including chemical and biochemical were discussed in this study. In the chemical part, *In vitro* drug release and kinetics of drug release were studied for all the prepared pharmaceutical formulations in this study. The prepared formulations including G_3 and H_3 followed zero order, Korsmeyer-Peppas model and Fickian ($n<0.5$) transport mechanism (diffusion controlled release). In the biochemical part, the biochemical activities of all the prepared formulations (as antimicrobials) were studied (*in vitro*) in four types of microbes including *Staphylococcus aureus* (Gram-positive), *Staphylococcus epidermidis* (Gram-positive), *Candida albicans* (yeast) and *Trichophyton* sp. (mold) by using agar diffusion method. These formulations showed an increase in their biochemical activities against these isolates and these activities an associated with the increase in drug concentrations which were loaded with the selected polymers in this study and in addition to their possession of highest antimicrobial and biochemical activities to inhibit the growth of these microbial isolates as compared to pure drug and commercial gel at ($p<0.05$). Also, the prepared formulations including G_0, G_3, H_0 and H_3 were applied (*in vivo*) to the skin of male rabbits for studying subacute toxicity test (LD_{50}), skin (back and rectal) irritation tests and the created skin irritation treatment test. These formulations were not poisonous and non irritant, while H_3 formulation possessed of ability to treat the created skin irritation during (10-12) days.

Key words : Metronidazole Gels, *In Vitro* & *In Vivo* Studies.

Introduction

In recent years, the development of transdermal dosage form designed to have systemic effects has been attracting increasing attention, due to the several advantages that this administration route offers, such as a better control of blood levels, a reduced incidence of systemic toxicity, an absence of hepatic first-pass metabolism etc. Drug delivery via the skin is not a simple task. The outermost, and least permeable, layer of the skin, the stratum corneum (SC), is a formidable barrier both to water transport out of the body and to inward chemical permeation. In fact, the majority of drugs does not appear to penetrate the skin at a rate sufficiently high for therapeutic efficacy and only the most potent ones with appropriate physicochemical characteristics are valid candidates for transdermal delivery[(1)]. The most difficult aspect of the transdermal delivery system is to overcome the barrier of stratum corneum against foreign substances. It is well known that the penetration rate of drugs through the stratum corneum can be increased with appropriate vehicles and transdermal penetration enhancers, owing to their ability to increase the solubility of drug and/or enhancers in pharmaceutical formulations and to change the structure of lipophilic and/or keratinized domains in stratum corneum[(2)]. For skin care, and the topical treatment of dermatological diseases, a wide choice of vehicles ranging from solid to semisolids and liquid preparations, is available to clinicians and patients. Within the major groups of semisolid preparations, the use of transparent gels has expanded, both in cosmetics and pharmaceuticals[(3)]. Creams, gels, ointments and pastes are some of the topical semisolids in use for many decades. Out of various semisolids dosage forms, the gels are becoming more popular due to ease of application and better percutaneous absorption then other semisolid preparations. Effectiveness of topical applications mainly depends upon its rate and extent of drug release from the base[(4)]. Metronidazole was used as a model drug, which is an anti-microbial and anti-inflammatory agent with topical and systemic action that can be incorporated into several pharmaceutical forms[(5)]. It was used as a model drug in this study due to its very effectiveness for the therapy of mucous membrane diseases as vaginal infections[(6, 7)], skin irritation, inflammatory papules, pustules, and erythema of rosacea[(8-22)]. It is being used to treat anaerobic (B.fragilis) burn wound infections[(23)] and periodontal infections[(24)], when administered topically. As well as it has also proved to be very effective for the therapy of protozoal infections (trichomoniasis, , lambliasis (giardiasis), amoebiasis)[(25)], numerous anaerobic infections especially gram negative bacteria. It has been found to be mutagenic in bacterial assay[(26)] and murine spermatozoa[(27)] when administered orally. Many studies have been conducted concerning the efficacy of metronidazole as a local spermicide[(28)]. Another important rational of using metronidazole, is its unique, low molecular weight offering the greater permeation a benefit through vaginal epithelial membrane[(6, 7)]. Various dosage forms available in the market are solutions, suppositories, creams, ointments, gels, foams, sprays, tablets, capsules, films, etc[(29)]. Topical chemotherapeutic preparations meant for burns should not inhibit the reepithelization and cause injury to viable cells[(23)]. Not only the chemotherapeutic component but also the base used should not inhibit healing. In this study, the utility of gels were prepared from Carbopol 940 (C_{940}) and hydroxyl ethyl cellulose (HEC) for the controlled release of metronidazole, has been investigated.

Materials And Methods

Materials

Metronidazole was supplied by (NDI Co.- Iraq & Sigma–Aldrich Co. USA). Hydroxy ethyl cellulose (HEC) was supplied by (Aldrich Co., Germany). Carbopol940 (C_{940}) was supplied by (Sigma-Aldrich Co., Germany). Nutrient agar (NA) and Sabouraud's dextrose agar (SDA) were supplied by (Hi Media Lab., India). Muller–Hinton Agar (MHA) was supplied by (Titan Biotech Co., Rajasthan, India). All other chemicals were of reagent grade.

Methods

1. Chemical Part

1.1 The Separation and Purity of Metronidazole NDI®

Metronidazole NDI® was separated and was purified from commercial metronidazole tablets (NDI) which were available in the commercial local pharmacies by using two solvents including methanol and distilled water[30, 31]. After that, the material was left to dry at the room temperature.

1.2 Melting Point Determination of Metronidazole NDI®

To investigate from the purity of metronidazole NDI®, Tms of the pure metronidazole and metronidazole NDI® were determined by capillary method (open capillary tubes) after the dry process.

1.3 Preparation of Topical Gels

As a first step, C_{940} gel (G_0) and C_{940} & HEC gel (H_0) were prepared from two polymers, as shown in table (1), by solid dispersion method[32, 33] without metronidazole, as follows:

For preparing G_0, the required quantity of C_{940} was weighed accurately and was sprinkled slowly on the surface of distilled water for 2hrs. The polymer was continuously stirred by a magnetic stirrer till it was allowed soaking for 24hrs and get homogenous dispersion. With continuous stirring, other additives were added to naturalized gel as follows:

1) Dimethyl sulfoxide (DMSO) was added for acting as permeation enhancer.
2) Ethylene glycol (EG) was added for acting as a co-solvent.
3) Triethanol amine (TEA) was added (as ingredient latest) for acting as a crosslinked agent, as a swelling agent and for maintaining the pH of the gel (is an appropriate neutralizing agent)[34].

For preparing H_0, HEC was weighed accurately and was dissolved in determined amount of distilled water using a magnetic stirrer (solution 1). Also C_{940} was weighed accurately, was sprinkled slowly on surface in determined amount of distilled water for 2hrs using a magnetic stirrer till it was allowed soaking for 24hrs and get homogenous dispersion (solution 2). Solutions (1&2) were fully mixed and the additives were added. After that, the prepared gels (G_0&H_0) were weighed.

Table (1) Semisolid formulations of two gels

Ingredients	C_{940} gel (G_0)	(C_{940}+HEC) gel (H_0)
C_{940}	1.0%	1.0%
HEC	0.0	0.50%
TEA	0.50%	0.50%
EG	10%	10%
DMSO	0.5%	0.5%
Distilled water	100mL	100mL

As a second step, each 1g of gel (G_0&H_0) was loaded with determined amout of metronidazole[23], as follows:

After the addition of DMSO and EG in the previous part from the preparation of gel (G_0&H_0), the determined amount of metronidazole was weighed accurately. It was dissolved in methanol in another beaker except in the case of the biological activity assay. Buffer phosphate pH 7.0 was used as solvent to dissolve determined amout of metronidazole because of alcohols are used as antiseptics to kill microorganisms. It was then added to the mixture. Finally, TEA was added to the mixture for producing the various formulations metronidazole gels as shown in table(2). The prepared gels in the previous parts were packed in a wide mouth glass jar. The mouth was covered with screw capped plastic lid after covering aluminum foil and the gels were kept in a dark and cool place.

Table (2) Semisolid formulations of various metronidazole gels

Metronidazole (mg)/1g of gel (G_0& H_0)	Formulation code	
2.5	G_1	H_1
5.0	G_2	H_2
10.0	G_3	H_3
15.0	G_4	H_4
20.0	G_5	H_5
25.0	G_6	H_6
33.0	G_7	H_7

It is worth mentioning that all of the determined amounts of metronidazole in table (2) were dissolved in 1mL of the solvent (methanol or phosphate buffer pH 7.0).

1.4 Standard Calibration Curve of Metronidazole

For the representation of standard calibration curve of metronidazole, the standard ($stock_1$) solution of metronidazole was prepared by weighing accurately 100mg of pure metronidazole dissolving in minimum quantity of methanol and diluting to 100.0mL with phosphate buffer pH7.0. 10.0mL of the $stock_1$ solution was taken and was diluted further to 100.0mL with phosphate buffer pH7.0 as $stock_2$ solution. 0.03, 0.06, 0.125, 0.25, 0.5, 1.0, 1.5, 2.0, 4.0 and 6.0mL were pipetted out of the $stock_2$ solution and were diluted to 10.0mL with phosphate buffer pH7.0 to obtain a concentrations range of 0.3 to 60.0µg/ml. The absorbance of all drug solutions was measured at 325nm using UV-spectrophotometer in triplicate readings (n=3) for avoiding any error case.

1.5 *In Vitro* Release (Dissolution) Studies

Dissolution testing is an essential requirement for the development and establishment of the release of drug model. In this study, skin permeation studies were performed using dialysis tube method[35]. 500mg of each prepared gel was placed in the synthetic dialysis bags and were immersed into 125mL phosphate buffer solution pH7.0 after it was fixed in sterilized beakers. Each beaker was accurately covered with glass watch and was fixed on a magnetic stirrer at 100rpm and 37±1^{o}C. 4mL aliquot of the dissolution fluid was withdrawn at regular time interval and was replaced with fresh quantity dissolution fluid. The samples were analyzed spectrophotometrically at 325nm to determine the dissolved drug concentration (drug content) using UV-spectrophotometer. All the experimental units were analyzed in triplicate (n=3).

1.6 *In Vitro* Release Kinetic Studies

In order to study the exact mechanism of drug release from the prepared formulations, the drug release data was analyzed according to zero order[36], first order[37], Higuchi square root[38] and Korsmeyer-Peppas model[39]. The criterion for selecting the most appropriate model was chosen on the basis of goodness of fit test.

2. Biochemical Part

2.1 *In Vitro* Study

2.1.1 Specimens Isolation and Collection

The total samples were collected from the patients who were consulted in the dept. of Dermatology, General Basrah Hospital. The collective samples were taken from skin (skin scraps and swabs from burn wounds) and mouth (swabs from oral mucous membranes) as follows:

1) Skin scraps: The patients with Tinea pedes, the surface was cleaned with 70% ethanol prior to the collection of the flakes of skin from the advancing border of the lesion by using two sterile glass slides.

2) Burn wounds swabs: The swab was taken from abscess of burn wound by sterile cotton swab stick.

3) Oral swabs: The patients with Candidiasis, the swab was taken from oral mucous membranes especially on the gingiva from the mouth by sterile cotton swab stick.

It is worth mentioning that skin diseases which include Tinea pedes, candidiasis and in addition to burn wounds were clinically diagnosed by dermatologist.

The collective samples have been taken to Bacteria and Fungi Laboratories (Lab) in Dept. of Biology, College of Science, University of Basrah, Iraq in order to be examined, diagnosed and isolated.

The collective samples from Tinea pedes and Candidiasis were planted on the sterile plate (peti dish's) surface of Sabouraud's dextrose agar (SDA) with 0.25g chloramphenicol and 0.5g cycloheximide. The former suppresses the growth of bacteria while the latter suppresses the contaminant fungi (that is, for isolating the fungi which can be a growth with this antibiotic existence from other fungi which can not be a growth with the same antibiotic existence). The collective samples from burn wounds were planted on the sterile plate surface of Nutrient agar (NA). Triplicate sterile plates for each sample were prepared and were incubated at 37 ℃ for 1-3days and at 37 ℃ for 3-7days for bacteria and fungi, respectively.

Various types from bacteria and fungi were defined after diagnosis. Four clinical microbial isolates which include *Staphylococcus aureus* (Gram-positive), *Staphylococcus epidermidis* (Gram-positive), *Candida albicans* (yeast) and *Trichophyton* sp. (mold) were selected for the study. Bacterial isolates which were isolated from burn wounds grew on screw capped vials slant surface of NA. While fungal isolates which were isolated from Tinea pedes and Candidiasis grew on sterile screw capped vials slant surface of SDA. The vials were incubated at 37 ℃ for 1-3 days and at 37 ℃ for 3-7 days for bacteria and fungi, respectively. After the growth appearance, the vials were maintained at 4℃ (as a stock$_1$ cultures for each clinical microbial isolates) in the refrigerator until the antimicrobial experiment assays.

2.1.2 Preparation of Media

NA and SDA were prepared for acting subcultures for each clinical microbial isolates, while Mueller hinton agar (MHA) was prepared for studying antimicrobial assay, as follows: 1) 2.8% NA media was prepared by dissolving 28g of NA in 1000mL sterile distilled water with gentle heat to dissolve the medium completely. 2) 6.5% SDA media was prepared by dissolving 65g of SDA with 0.25g chloramphenicol for suppressing the growth of bacteria in 1000mL sterile distilled water with gentle heat to dissolve the medium completely. 3) 3.8% MHA media was prepared by dissolving 38g of MHA in 1000mL sterile distilled water with gentle heat to dissolve the medium completely. All mixtures were placed in special vials, sterilized by autoclaving at 15 psi (121℃) for 15-20 min and were poured at 45℃ into each sterile Peti plate. These were allowed to solidify at the room temperature before use.

2.1.3 Inoculum' Preparation[40]

Before the preparation of microbial inocula, pure isolate for each clinical microbial species was subcultured (as $stock_2$ culture). $Stock_2$ cultures were prepared by transferring a loopful of colonies from the $stock_1$ cultures which were maintained at 4℃ on NA slants (bacteria) and SDA slants (fungi) for all clinical microbial species to plates of NA for bacteria and SDA with chloramphenicol for fungi. The plates were incubated at 37 ℃ for 1-3 days and at 37 ℃ for 3-7 days for bacteria and fungi, respectively. For the preparation of suspension (as inoculum) for each clinical microbial isolate, active cultures ($stock_2$) for each clinical microbial species were prepared by transferring a loopful of colonies from the $stock_2$ cultures for all clinical microbial species to sterile test tubes of 5mL of sterile saline (isotonic solution) (0.85%NaCl). These test tubes were incubated without agitation for 24hrs at 37 ℃ for bacteria and fungi. A suspension for each clinical microbial isolate was prepared and was adjusted to 1×10^6 colony forming units (cfu) mL^{-1} standardized by (0.5 Mac-Farland)[41] and using haemocytometer[42].

2.1.4 *InVitro* Antimicrobial Activity

The prepared semisolid formulations of gels which were loaded and not loaded with metronidazole in previous parts from the preparation, commercial gel (10mg of metronidazole loaded with 1g of gel) and in addition to the prepared liquid formulations of different concentrations of metronidazole, as shown in table(3), were used to test their antimicrobial activities.

Table (3) Liquid formulations of different concentrations of metronidazole

Metronidazole (mg)/1mL of solvent	0.0	2.5	5.0	10.0	15.0	20.0	25.0	33.0
Formulation code	S_0	S_1	S_2	S_3	S_4	S_5	S_6	S_7

2.1.5 Agar Diffusion Assay

The antimicrobial assay of all the formulations were evaluated by the zone of inhibition using the agar well diffusion assay[43], with slight modification according to the present experimental conditions. 0.2 mL of microbial inocula was placed on the surface of MHA media and then it was spread with L–shape glass rod. The plate was left for 10 min. Then individual plates were marked for the

organism being inoculated. Each plate was punched to make one well of 9.0mm diameter with the help of a sterile cork borer at the central site of the plates. The formulations of antimicrobial were placed in a central pore which included 0.5g for semisolid formulations and 0.5mL for liquid formulations.

A control experiment was set up using an equal amount of phosphate buffer pH 7.0 as control and sterile DMSO in central place of different antimicrobial formulations. These plates were incubated at 37 ℃ for 1-3 days and at 37 ℃ for 3-7 days for bacteria and fungi, respectively. The inhibition zones of each isolate were recorded in centimeter (cm) using ruler according to the growth of their controls. All formulations were tested in triplicates.

It is worth mentioning that the process of subculture for each clinical microbial isolates and the preparations of inocula for antimicrobial assay were happening in Biotechnology Lab, Dep. of Marine Biology. MSC, University of Basrah, Iraq.

2.1.6 Statistical Analysis

Data were presented as mean ± SD & standard error of the mean (SEM). The obtained data were subject to the analysis of variance (ANOVA) test to determine there LSD (Least Significant Difference) at probability level (p) < 0.05. Statistical analysis was performed with statistical software pakage of social sciences (SPSS) for windows (11.0 version)[44].

2.2 *In Vivo* Study

2.2.1Animal Model

Sixty germany white male rabbits (young adult) weighing in range of (1.15-1.95 kg) were acclimatized for 50days before and during the study in Animal House of College of Science, University of Basrah, Iraq, under standard conditions at room temperature of (24±2℃), relative humidity (55±5%) and lighting (12hrs/day) with controlled care. The floor was spread with sawdust. Animals were fed with clover (trefoil), carrots, celery and lettuce, and they were supplied with water under strict hygienic conditions.

2.2.2 *In Vivo* Antimicrobials

The prepared formulations including G_0, G_3, H_0 and H_3 were topically applied to the animals for testing subacute toxicity, skin irritation and skin irritation treatment especially with H_3 formulation. These formulations were placed in big papers as carriers before the application. The diameter of these papers were in range (4.6-4.8cm) and they were described with impermeable for the materials and resistant to the temperature. These papers and the cotton wool were firmly secured in place with gauze and adhesive plaster to be occlusion.

2.2.3 Biochemical Application

The study contained three types of tests including subacute toxicity, skin irritation and skin irritation treatment tests. The determined area for the back of each animal was shaved with electrical clippers for removing hair till this area of the back became bare and smooth. The animals were left for one day before use.

2.2.3.1 Subacute Toxicity Test [45-48]

This test is sometimes also called LD_{50} (Median Lethal Dose or Lethal Dose 50%) dermal route. Twenty rabbits were used in this test. They were randomly divided into five groups containing four animals each as follows: The control group as group one was left untreated served. The prepared formulations including G_0, H_0, G_3 and H_3 were topically applied with doses (16g/kg) on the bare back of each animal to four groups as groups no. two, three, four and five, respectively. The animals were observed during three days. The data including the happened death or any other effects were recorded.

2.2.3.2 Skin Irritation Tests

2.2.3.2.1 Back Irritation Test **(14, 49-57)**

It is sometimes also referred to as the primary dermal irritation. In this test, twenty four rabbits were used. They were randomly divided into six groups containing four animals each as follows: The control group as group no. one was left untreated served. All prepared formulations including G_0, H_0, G_3 and H_3 were topically applied with doses 1g on $9cm^2$ subarea of the bare back of each animal to four groups. An aqueous solution of 1mL, containing 0.8% formalin soaked in $9cm^2$ cotton wool (standard irritant) was placed in the bare back of each animal as group no. six. The animals were observed for 24hrs for any sign of edema and erythrema.

2.2.3.2.2 Rectal Irritation Test **(58, 59)**

In this test, twenty rabbits were used. They were randomly divided into five groups containing four animals each as follows: The control group as group no. one was left untreated served. All prepared formulations including G_0, H_0, G_3 and H_3 were topically applied with doses 0.5g into the rectum of each animal to four groups. The animals were observed for 72hrs for any sign of sensitization (allergic reactions), photosensitization, edema and excess redness.

2.2.3.3 Skin Irritation Treatment Test **(8-23, 57)**

In this test, eight rabbits were used. They were randomly divided into two groups containing four animals each as follows: The control group as a first group was left untreated served. The second group contained animals which were irritant. The animals of the second group were treated with doses 1g of the formulation H_3 twice daily. This formulation was rubbed in lightly after applying it. The animals were observed for two weeks for any sign of the therapy and the cure.

Results and Discussion

1. Chemical Part

1.1 Melting Point Determination of Metronidazole NDI®

T_m of the metronidazole NDI® was found to be at 162.4ºC. This result was evident in that the metronidazole NDI® was pure through the correspondence of T_ms from the pure metronidazole and metronidazole NDI® to the large limit.

1.2 Standard Calibration Curve of Metronidazole **(61-64)**

After the scanning of $1x10^{-4}$ M of metronidazole solution (n=3) in UV region (200-400nm) by spectrophotometer, the maximum absorbance gave the λ_{max} for the drug at 325nm. So the standard calibration curve of metronidazole was developed at this wave length. The calibration curve was linear between (0.3-60µg/mL) concentration ranges. The manual standard calibration curve graph of metronidazole was determined in phosphate buffer of pH7.0 by plotting absorbance (y_1) against concentration (x) at 325nm by Microsoft Excel 2007, and it follows the Beer's law. Results were tabulated in table (5). The correlation coefficient (R^2) was found to be at 0.996. The slope (b) and intercept (a) were found to be at 0.045 and at 0.013, respectively according to the regressed line which was represented in the equation [1] and as shown in figure (1).

$$y_1=bx+a \quad \text{.....[1]}$$

where, y_1=practically calculated absorbance.

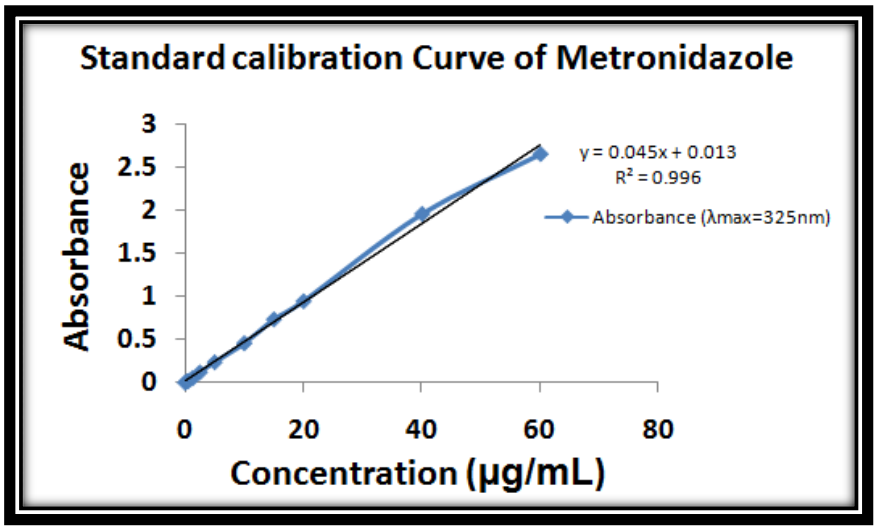

Fig (1) Standard calibration curve graph of metronidazole

In mathematics, the slope or gradient of a line described its steeper, incline. The slope is (in the simplest terms) the measurement of a line, and is defined as the ratio of the "rise" divided by the "run" between two points on a line, or in other words, the ratio of the altitude changes to the horizontal distance between any two points on the line. But in case of multiple points here is the method to calculate the slope and value of the intercept.

For the representation of standard regression curve graph, a least square method was used to calculate the slope (b) and intercept (a), as follows:

Step (1): Calculation of slope (b)

By the fomula:

$$b = \frac{n\sum xy - \sum x.\sum y}{n\sum x^2 - (\sum x)^2} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots [2]$$

where,

n = no. of observation (10)

Table (4) shows observation charts for the calculation of the slope (b) and intercept (a).

Table (4) observation charts for calculating the slope and intercept

S.NO	Concentrations (µg/mL)	Absorbance λ_{max}=325nm		
	x	y_1	x^2	xy_1
1.	0.3	0.016	0.09	0.0048
2.	0.6	0.023	0.36	0.0138
3.	1.25	0.052	1.5625	0.065
4.	2.5	0.121	6.25	0.3025
5.	5.0	0.238	25	1.19
6.	10.0	0.459	100	4.59
7.	15.0	0.737	225	11.055
8.	20.0	0.948	400	18.96
9.	40.0	1.964	1600	78.56
10.	60.0	2.660	3600	159.6
∑	154.65	7.218	5958.2625	274.34
n	10	10	10	10

The values in table (4) were put in equation [2] to calculate (b) value and which was equal to 0.0456.

Step (2): Calculation of intercept (a)

By the fomula:

$$a = \bar{y} - b\bar{x} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots[3]$$

where,

$\bar{y}$ = mean of y_1 (sum of observations / no. of observations)

$\bar{x}$ = mean of x (sum of observations / no. of observations)

b = slope

$\bar{x} = \Sigma X / 10 \longrightarrow \bar{x} = 15.465$

$\bar{y} = \Sigma y_1 / 10 \longrightarrow \bar{y} = 0.7218$

The values of $\bar{y}$ and $\bar{x}$ were put in equation [3] to calculate (a) value and which was equal to 0.0165.

Step (3): Calculation of regressed values

In the table (5), the calculation of regressed values of y_2 was followed according to the regressed line:

$$y_2 = bx + a \ldots\ldots\ldots\ldots\ldots\ldots[4]$$

where, y_2 = theoretically calculated absorbance (regressed y). Then the graph was plotted between the regressed values obtained from table (5).

Table (5) The calculation of regressed values of y_2

S. NO.	Slope * conc. (bx)	Intercept (a)	Regressed values (y_2=bx+a)
1.	0.3*0.0456(x_1)=0.01368	0.0165	0.01368+0.0165=0.318(y_1)
2.	0.6*0.0456(x_2)=0.02736	0.0165	0.02736+0.0165=0.0438(y_2
3.	1.25*0.0456(x_3)=0.057	0.0165	0.057+0.0165=0.0735(y_3)
4.	2.5 *0.0456(x_4)=0.114	0.0165	0.114+0.0165=0.1305(y_4)
5.	5.0*0.0456(x_5)=0.228	0.0165	0.228+0.0165=0.2445(y_5)
6.	10.0*0.0456(x_6)=0.456	0.0165	0.456+0.0165=0.4725(y_6)
7.	15.0*0.0456(x_7)=0.684	0.0165	0.684+0.0165=0.7005(y_7)
8.	20.0*0.0456(x_8)=0.912	0.0165	0.912+0.0165=0.9285(y_8)
9.	40.0*0.0456(x_9)=1.824	0.0165	1.824+0.0165=1.8405(y_9)
10.	60.0*0.0456(x_{10})=2.736	0.0165	2.736+0.0165=2.7525(y_{10})

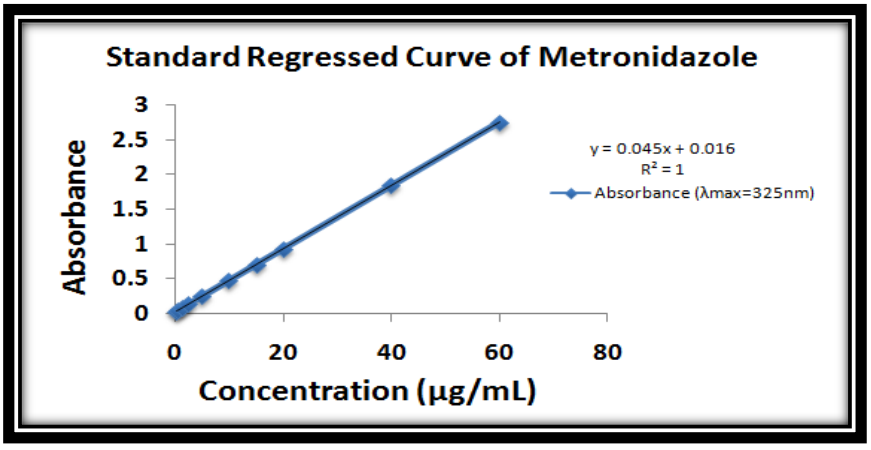

Fig (2) Standard regression curve graph

The absorbance (y_2) was found to be the line of regression and the curve was found to be the standard regressed curve. In figure (2), the value of R^2 (regression coefficient) was found to be equal to 1, and this means that the line is perfectly straight.

The above work is done because the values of slope and intercept was directly calculated from the software and the manual calculation found a tedious job. Also the improper way to handle the glassware and some minor negligence in plotting the graph can differ the results. Finally, the goal of the representation of the standard regression curve graph {figure (2)} was to obtain the regressed line {y_2=bx+a, equation[4]} which it was useful to calculate the metronidazole released (%) in *in vitro* release studies and *in vitro* metronidazole release kinetic studies.

1.3 *In Vitro* Release and Kinetics of Metronidazole

After the application of dialysis tube method[65] which was followed in *in vitro* metronidazole release study, the data were obtained and they were plotted between % cumulative drug release (y-axis) against the time (hrs) (x-axis) by Microsoft Excel 2007 as shown in figures (3, 4) to explain the behavior of metronidazole release (µg/mL) from the prepared formulations with an increasing time. Each figure (3, 4) included number of the prepared formulations with the same method which were different in metronidazole concentrations (mg/mL).

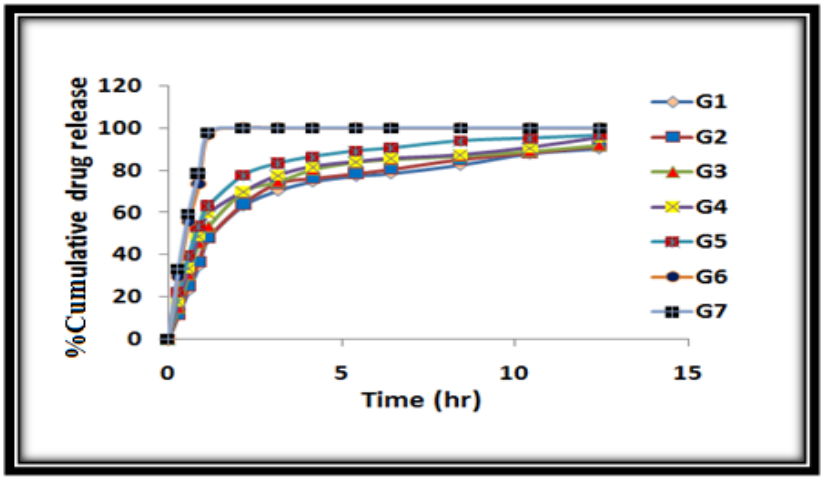

Fig (3) *In vitro* release profile metronidazole from gel G_1 to G_7

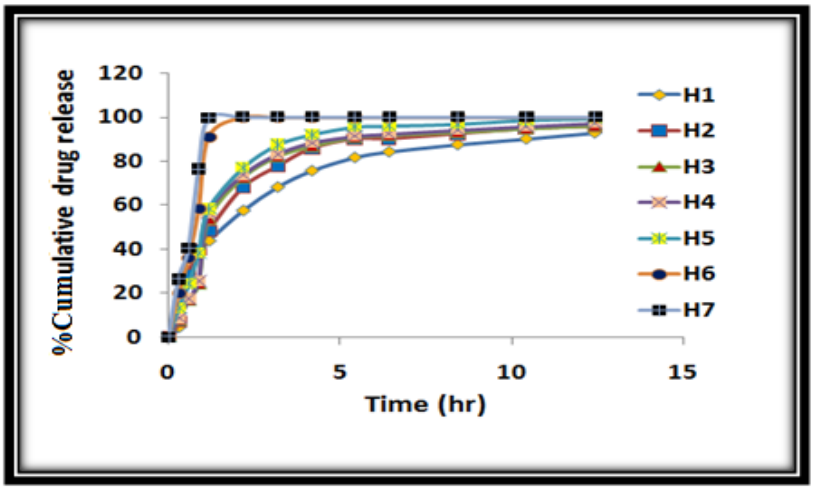

Fig (4) *In vitro* release profile metronidazole from gel H_1 to H_7

Generally, the figures (3, 4) showed that the metronidazole release (µg/mL) from each prepared formulation increased with increasing the time. Also in each figure (3, 4), the released metronidazole concentration (µg/mL) from the prepared formulation with a minimum concentration (mg/mL) of metronidazole at the determined time was lower as compared to the released metronidazole concentration (µg/mL) from the prepared formulation with a maximum concentration (mg/mL) of metronidazole at same the time. These results corresponded to the results obtained from the inhibition zones of *in vitro* biochemical studies of same the prepared formulations in the biochemical part. Figure (5) showed an initial burst release of G_3 and H_3 within first 15min. This observation was expected to inhibit the growth of the microorganisms assaied in *in vitro* biochemical study. The percentages of drug release from these formulations Vs. time were increasing according to the sequence $G_3 < H_3$.

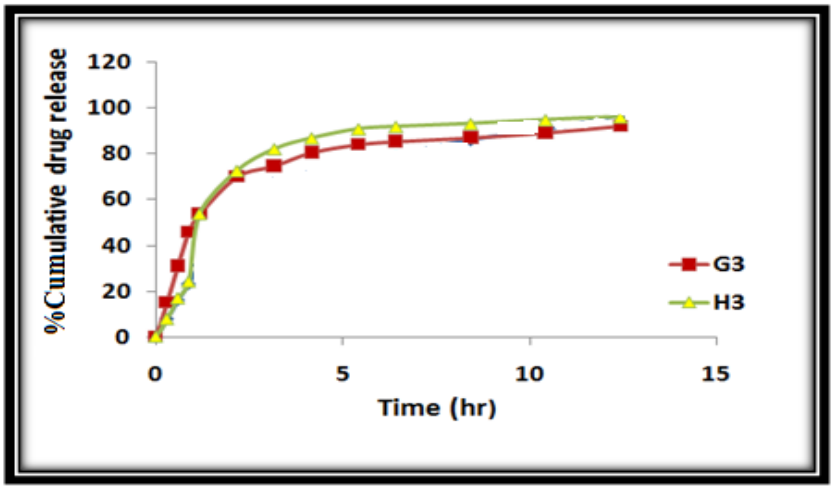

Fig (5) *In vitro* release profile metronidazole from various formulations G_3 and H_3

When the comparison between G_3 and H_3, the drug release was higher in H_3 than in G_3. At pH 7.0, C_{940} is present in the ionized state and, as a result polymeric network gets loosened comparatively, attributing to the higher drug release. An increase in the polymer (C_{940}) content was associated with a corresponding decrease in the drug release rate[66]. This could be due to the extensive swelling of the polymers with a created thick gel barrier for drug diffusion. The drug release was increased linearly with the addition 0.5% HEC to 1% C_{940} and causing more pores and channels in the formulation. HEC was considered hydrophilic polymer and it acts as resortable carriers[67]. Also the results which were obtained from the figure (5) about G_3 and H_3 confirmed that the viscosity of G_3 is higher as compared to H_3 through their agreement with other studies from the public researches in this connection and which showed that the viscosity of the gel base may play an important role in modifying the release of the drug into the receptor compartment when the diffusion through the gel matrix is a rate-determining step[68, 69], viz., they showed that the viscosity valuses of the 1% C_{940} gel and 1% HEC gel were found to be 33000cps and 22000cps, respectively. The investigation of these viscosity values revealed that the 1% C_{940} gel has a greater viscosity than the 1% HEC gel[65]. Consequently, there was a decreased release of metronidazole from G_3 compared to H_3.

The release (dissolution) of metronidazole from G_3 and H_3 was evaluated by different

kinetic models. The release patterns of these formulations were evaluated and calculated manually by Microsoft Excel 2007 using different equations including zero order, first order, Higuchi plot and korsmeyer-Peppas plot. These patterns were shown in figures (6-9).

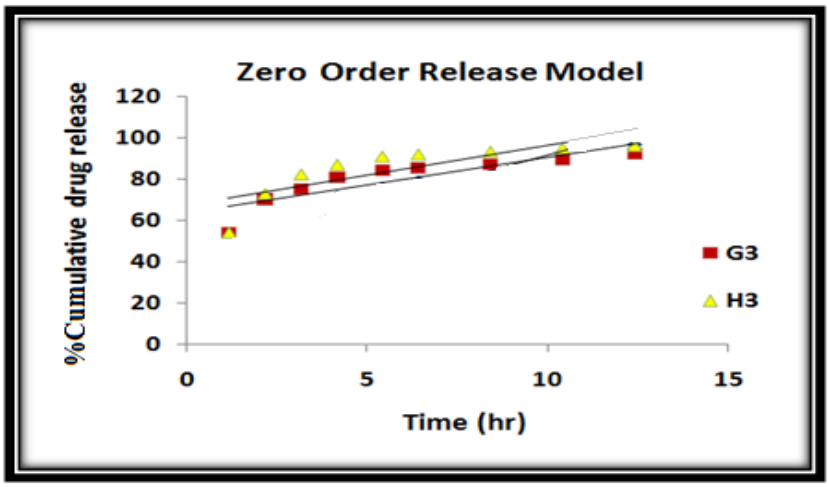

Fig (6) Zero order release plot of G_3 and H_3 formulations

The release data were analyzed according to these equations depicted in table (6). The interpretation of data was based on the value of the resulting regression coefficients. G_3 and H_3 followed zero order.

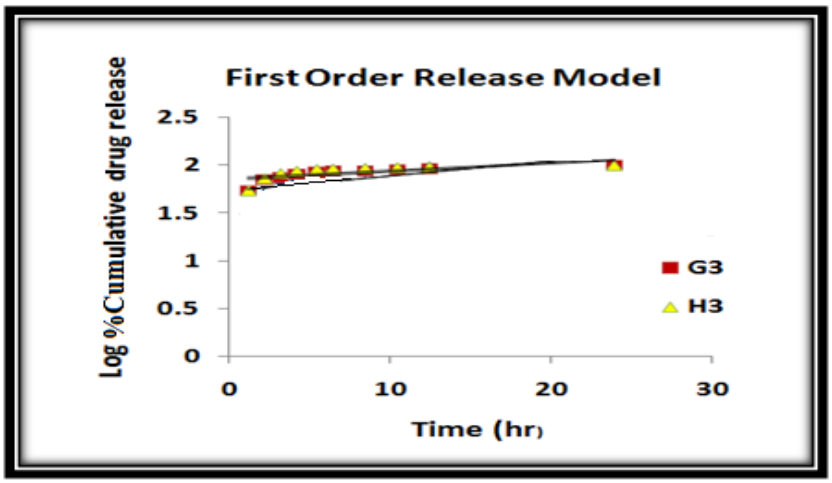

Fig (7) First order release plot of G_3 and H_3 formulations

To understand the mechanism of release of metronidazole from the formulations G_3 and H_3, the drug release data were fitted into Higuchi model and korsmeyer-Peppas model. The drug release data showed that the highest regression coefficient values were for korsmeyer-Peppas model.

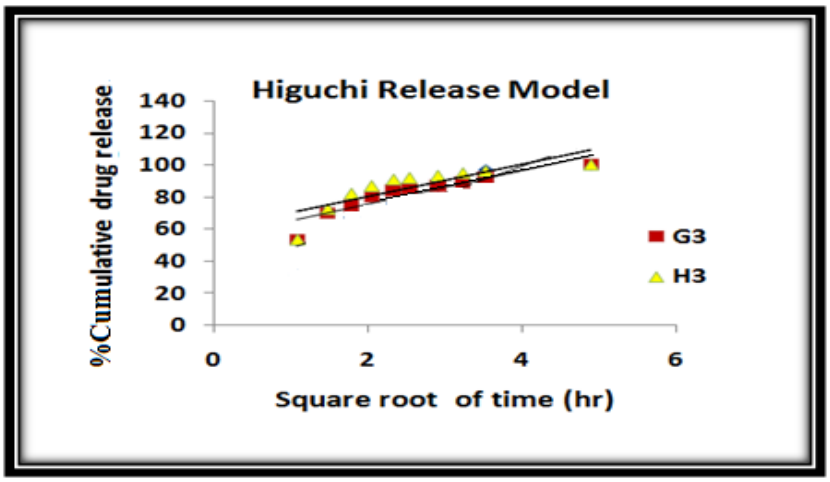

Fig (8) Higuchi plot of G_3 and H_3 formulations

Also the table (6) showed that all the formulations followed Fickian (n<0.5) transport mechanism (release mechanism), viz., diffusion controlled release .

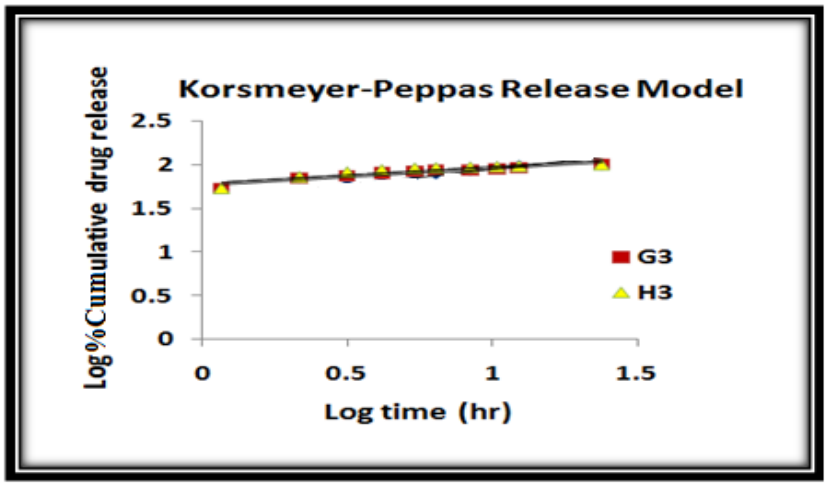

Fig (9) Korsmeyer-Peppas plot of G_3 and H_3 formulations

Table (6) Correlation coefficient and constants of different kinetic models

Formula code	Zero order		First order		Higuchi model		Korsmeyer-peppas model	
	R^2	K_0	R^2	K_1	R^2	K_H	R^2	n
G_3	0.741	2.693	0.576	0.08	0.834	10.61	0.907	0.188
H_3	0.646	2.898	0.421	0.007	0.676	10.19	0.806	0.188

When the observation of R^2 values was for zero order for all the formulations, the sequence with increasing R^2 values were shown as follows: $H_3<G_3$

Whenever R^2 values were high, the rates of drug release were slow. This explaination conformed with the explaination of figure (9).

3. Biochemical Part

4. 2.1 *In Vitro* Study

2.1.1 The Influences of S_1 to S_7 on the Microbial Isolation Growth

The values of inhibition zones (cm) were different from one isolate to another toward the different concentrations of metronidazole solutions (S_1 to S_7) (liquid formulations). Statistical analysis results indicated the existence of a significant

difference between these concentrations as shown in figure (10). The highest inhibition value was 4.52cm for *Staphylococcus aureus* isolate by S_7 and the lowest inhibition value was 1.94cm for *Staphylococcus epidermidis* isolate by S_3. The figures (11) showed *in vitro* biochemical activity of S_0 and S_3 against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Candida albicans* and *Trichophyton* sp. isolates . Also these results indicated the existence of a significant difference at $p<0.05$, viz., S_7 which was used against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Trichophyton* sp. and S_6 which was used against *Candida albicans* showed high significant difference as compared to remaining concentrations as shown in table (7).

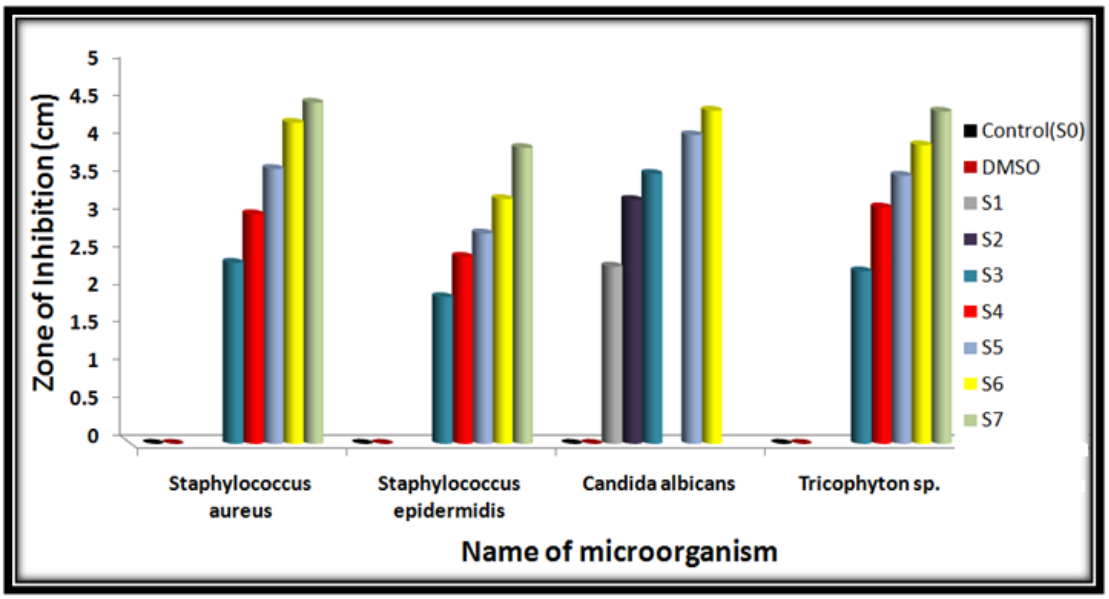

Fig (10) Histogram shows *in vitro* biochemical activities of S_0 as control, DMSO and different concentrations of metronidazole (S_1 to S_7) (liquid formulations)

Table (7) *In vitro* biochemical activities of different concentrations of metronidazole (S_1 to S_7) (liquid formulations)

Mean diameter of inhibition zone in cm (mean ± S.D)*				
Formula code	**Microorganisms**			
	Staphylococcus aureus	***Staphylococcus epidermidis***	***Candida albicans***	***Trichophyton* sp.**
S_0	NZ	NZ	NZ	NZ
S_1	-	-	2.35±0.131e	-
S_2	-	-	3.233±0.180d	-
S_3	2.4±0.396e	1.944±0.357e	3.579±0.097c	2.287±0.100e
S_4	3.044±0.406d	2.478±0.290d	-	3.133±0.192d
S_5	3.644±0.710c	2.789±0.378c	4.092±0.092b	3.554±0.184c
S_6	4.256±0.731b	3.244±0.421b	4.417±0.109a	3.958±0.176b
S_7	4.522±0.882a	3.922±0.633a	-	4.404±0.151a
DMSO	NZ	NZ	NZ	NZ

***: (n=3); a, b, c, d and e: The means which had the same letter indicated non existence significant differences between them and vice versa ($p<0.05$) for the same isolate; NZ: No Zone of inhibition**

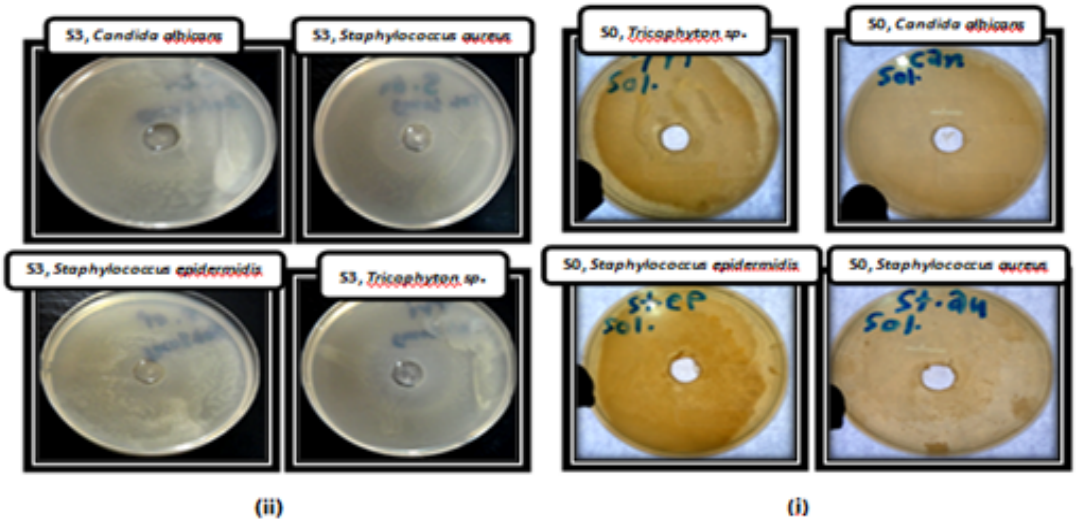

Fig (11) Photographs show *in vitro* biochemical activity of (i) S_0 as control and (ii) S_3 against the microbial isolates at 37℃ for 1 day and 3 days for bacteria and fungi, respectively

2.1.2 The Influences of G_0 to G_7 as Compared to Commercial Gel on the Microbial Isolation Growth

The values of inhibition zones (cm) were different from one isolate to another toward G_0, G_1, G_2, G_3, G_4, G_5, G_6 and G_7 (semisolid formulations) and they were compared with the values of inhibition zones of the commercial gel. Statistical analysis results indicated the existence of a significant difference between these formulations and as compared to commercial gel as shown in figure (12).The highest inhibition value was 6.51cm for *Candida albicans* isolate by G_6 and the lowest inhibition value was 2.22cm for *Staphylococcus epidermidis* isolate by G_0. The figures (13) showed *in vitro* biochemical activity of G_0 and G_3 against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Candida albicans* and *Trichophyton* sp. isolates. When the observation of figure (12), the inhibition values of the prepared formulations (G_0 to G_7) were superior as compared to the inhibition values of the commercial gel, viz., the prepared formulations (G_0 to G_7) are successful in the biochemical applications. Also these results indicated the existence of a significant difference at $p<0.05$, viz., G_7 which was used against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Trichophyton* sp. and G_6 which was used against *Candida albicans* that showed high significant difference as compared to other formulations as shown in table (8).

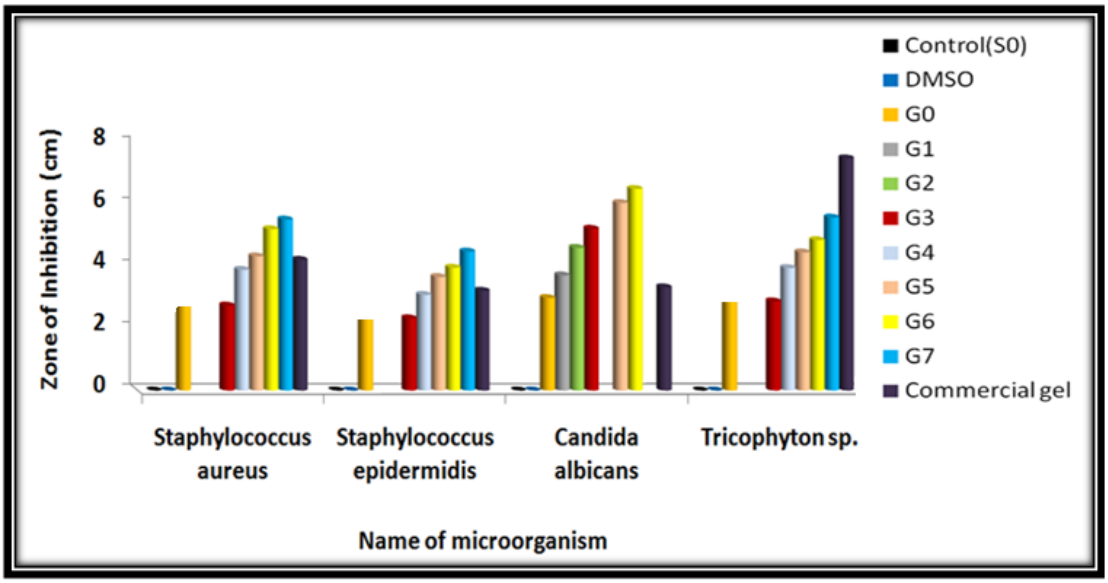

Fig (12) Histogram shows *in vitro* biochemical activities of S_0 as control, DMSO, the prepared formulations G_0 to G_7 and commercial gel (semisolid formulations)

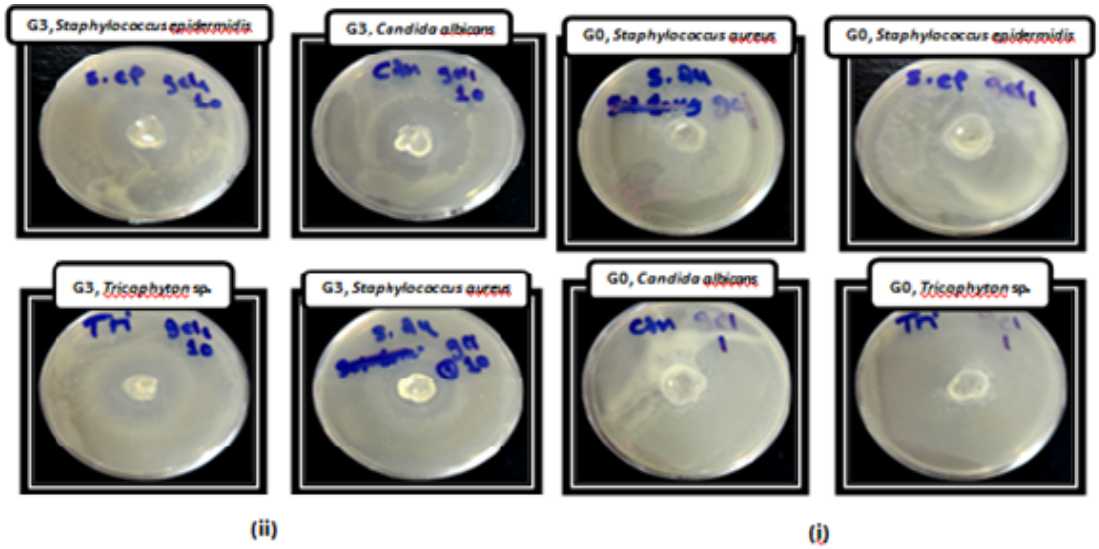

Fig (13) Photographs show *in vitro* biochemical activity of (i) G0 and (ii) G3 against the microbial isolates at 37℃ for 1 day and 3 days for bacteria and fungi, respectively

Table (8) ***In vitro*** **biochemical activities of different concentrations of metronidazole (G_0 to G_7) (semisolid formulations)**

Mean diameter of inhibition zone in cm (mean ± S.D)*				
Formula code	**Microorganisms**			
	Staphylococcus aureus	***Staphylococcus epidermidis***	***Candida albicans***	***Trichophyton* sp.**
G_0	2.537±0.066d	2.22±0.198f	2.987±0.033f	2.863±0.335f
G_1	-	-	3.721±0.802e	-
G_2	-	-	4.608±0.753d	-
G_3	2.744±0.492c	2.333±0.273e	5.225±0.396c	2.879±0.313e
G_4	3.9±0.724b	3.089±0.105d	-	3.958±0.261d
G_5	4.333±0.85b	3.667±0.193c	6.058±0.550b	4.462±0.152c
G_6	5.211±1.004a	3.967±0.295b	6.512±0.556a	4.858±0.251b
G_7	5.522±1.125a	4.489±0.372a	-	5.588±0.275a
Commercial gel	4.222±0.204	3.222±0.571	3.333±0.127	7.5±0.00
DMSO	NZ	NZ	NZ	NZ

***: (n=3); a, b, c, d, e and f: The means which had the same letter indicated non existence significant differences between them and vice versa ($p<0.05$) for the same isolate; NZ: No Zone of inhibition**

2.1.3 The Influences of H_0 to H_7 as Compared to Commercial Gel on the Microbial Isolation Growth

The values of inhibition zones (cm) were different from one isolate to another toward H_0, H_1, H_2, H_3, H_4, H_5,H_6 and H_7 (semisolid formulations) and they were compared to the values of inhibition zones of the commercial gel. Statistical analysis results indicated the existence of a significant difference between these formulations and as compared to commercial gel as shown in figure (14). The highest inhibition value was 6.64cm for *Candida albicans* isolate by H_6 and the lowest inhibition value was 3.11cm for *Trichophyton* sp. isolate by H_0. The figures (15) showed *in vitro* biochemical activity of H_0 and H_3 against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Candida albicans* and *Trichophyton* sp. isolates. When the observation of figure (14), the inhibition values of the prepared formulations (H_0 to H_7) were high superior as compared to the inhibition values of the commercial gel, viz., the prepared formulations (H_0 to H_7) are high success in the biochemical applications. Also these results indicated the existence of a significant difference at $p<0.05$, viz., H_7 which was used against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Trichophyton* sp. and H_6 which was used against *Candida albicans* that showed high significant difference as compared to other formulations as shown in table (9).

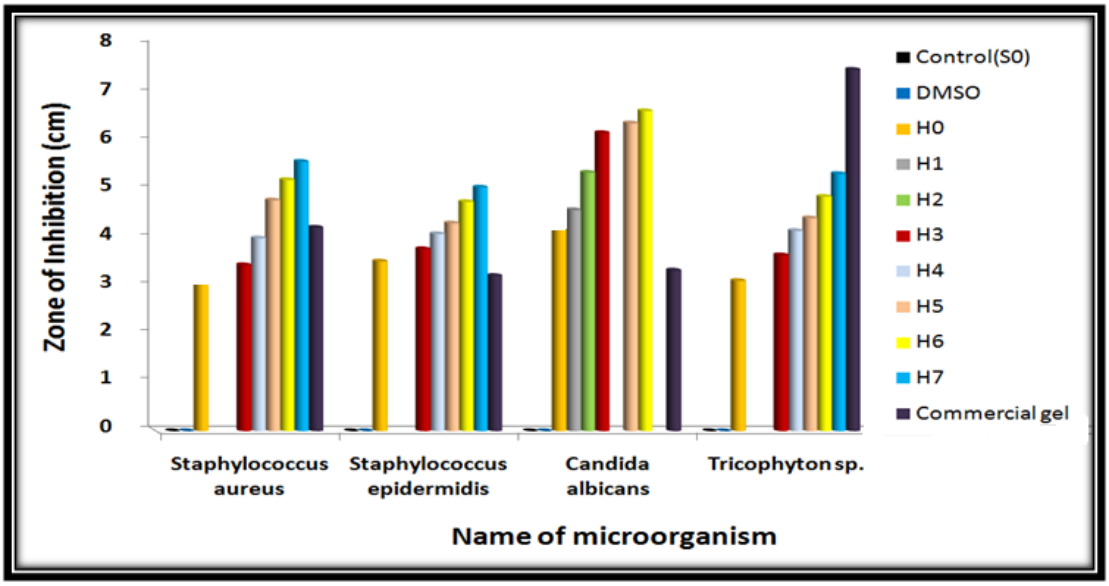

Fig (14) Histogram shows *in vitro* biochemical activities of S_0 as control DMSO, the prepared formulations H_0 to H_7 and commercial gel (semisolid formulations)

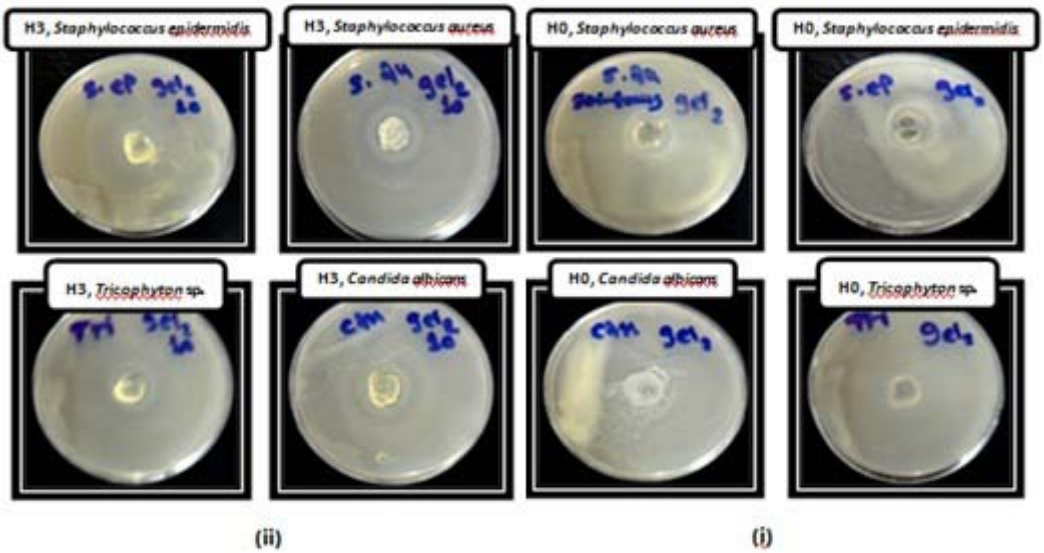

Fig (15) Photographs show *in vitro* biochemical activity of (i) H0 and (ii) H3 against microbial isolates at 37℃ for 1 day and 3 days for bacteria and fungi, respectively

Table (9) ***In vitro*** **biochemical activities of different concentrations of metronidazole (H_0 to H_7) (semisolid formulations)**

Mean diameter of inhibition zone in cm (mean ± S.D)*				
Formula code	**Microorganisms**			
	Staphylococcus aureus	***Staphylococcus epidermidis***	***Candida albicans***	***Trichophyton* sp.**
H_0	2.935±0.807f	3.522±0.494f	4.312±0.367f	3.117±0.143f
H_1	-	-	4.587±0.885e	-
H_2	-	-	5.363±0.679d	-
H_3	3.444±0.255e	3.778±0.597e	6.187±0.675c	3.65±0.251e
H_4	4.0±0.1d	4.089±0.629d	-	4.154±0.278d
H_5	4.789±0.451c	4.311±0.760c	6.388±0.586b	4.417±0.246c
H_6	5.211±0.627b	4.756±0.800b	6.642±0.479a	4.863±0.335b
H_7	5.589±0.401a	5.056±0.829a	-	5.337±0.253a
Commercial gel	4.222±0.204	3.222±0.571	3.333±0.127	7.5±0.00
DMSO	NZ	NZ	NZ	NZ

***: (n=3); a, b, c, d, e and f: The means which had the same letter indicated non existence significant differences between them and vice versa ($p<0.05$) for the same isolate; NZ: No Zone of inhibition**

Metronidazole was selected as the anti-inflammatory and the antimicrobial agent for this study because it has been shown to be highly effective toward dermatitides and microbes[70]. After drug application, metronidazole readily permeates mammalian cells as well as anaerobic and aerobic microbial cell membranes by diffusion, to achieve a steady state intracellular concentration. When the nitro group of the compound is reduced by nitroreductase (ferrodoxin-like electron transport protein), a concentration gradient is created and more drug enters the cells. The reduction of metronidazole leads to the release of decomposition products with toxic properties (nitro, nitroso, nitroso-free radicals and hydroxylamine derivatives)[71]. It is believed that these intermediates interfere with deoxyribonucleic acid (DNA) synthesis. Once in the cell, metronidazole binds to the DNA strands and disrupts the helical structure of the molecule. DNA strands breakage and inhibition of nucleic acid synthesis occurs, which ultimately leads to cell death. This process results in a rapid killing of the microorganisms especially anaerobes. The hydroxymetabolite of metronidazole also exhibits antimicrobial activity. This suggests that there may be synergism between the parent drug and its metabolite, which may account for the greater than expected clinical efficacy of the drug in treating microbial infections[72]. It is worth mentioning that metronidazole is primarily metabolized in the liver and its metabolites are excreted in the feces[73].

HEC and C_{940} were considered as bio (muco) adhesive pharmaceutical polymers[74, 75], viz.,their bioadhesive property was high (+ + +). Whilst, the additives were used in very little amounts and they were considered unactive materials toward microbes, as follows: EG was used as cosolvent to increase the solubility of the drug in the vehicle (polymer). DMSO was used as permeation enhancer and it was considered as a control plate in several public researches. TEA was used as a crosslinked agent, to maintain the pH of gel (is an appropriate neutralizing agent)[34] and as a swelling agent closely enabling packing and the tight binding between the long and side chains.

2.2 *In Vivo* Study

2.2.1 Subacute Toxicity Study

The results of subacute toxicity test (LD_{50} dermal route) were not shown any state of death or any other effects in all groups. That is, the behavior of these animals was normal when they were compared with the control group to a period of three days. These results proved that the prepared formulations which were topically applied to the bare back of animals were not toxic according to the scheme of toxicity classification. This scheme represented that the material is considered non-toxic when this material did not cause a state of death in animals with dose (>15g/kg)[76] . In addition, these results were in accord with the apparent results of the medical administrations of topical metronidazole as gel, cream and lotion. these medical administrations proved that the minimal absorption of metronidazole and consequently its insignificant plasma concentration which happened after topical administration[13, 49, 50, 77-79]. The hydrogels (C_{940} and HEC) which were used in the preparation of various formulations as gels, they were considered biodegradable materials and their analyitical results were non-toxic according to the noval researches in this connection[80-83].

2.2.2 Skin Irritation Studies

2.2.2.1 Back Irritation Study

All the formulations were homogenous in the texture and showed no skin irritation (safety) for all groups of animals as compared to the control group upon the application for 24hrs except group no. eight which was found to be irritant because it was treated with 0.8% formalin as standard irritant also upon the application for 24hrs.

2.2.2.2 Rectal Irritation Study

All the formulations were found to be non-irritant to rectums of animals.

2.2.3 Skin Irritation Treatment Study

Skin irritation state was considered one of indications and clinical appearances which was treated with topical metronidazole[13, 29, 49, 50, 76-79]. The prepared formulation H_3 was worthy to cause relative improvement on a high level from skin irritation. The treatment continued till skin irritation became disapparent and healing completely at approximate period in range of (10-12) days. This result was in accord with the recent public researches in this connection.

Conclusions

In vitro drug release study of all the prepared formulations confirmed that the drug release was increased linearly with increasing the time and in the same type of the prepared formulations, the released drug from the prepared formulation with minimum concentration of drug at determined time was lower as compared to the released drug from the prepared formulation with maximum concentration of drug at same the time. *In vitro* drug release kinetics study of the prepared formulations (G_3 and H_3) confirmed that the drug release was increased according to the sequence $G_3<H_3$ and these formulations followed zero order, Korsmeyer-Peppas model and Fickian ($n<0.5$) transport mechanism (diffusion controlled release). With increasing drug concentration (mg/mL) which was loaded with the used polymers in the preparation of the formulations, R^2 values will decrease and drug release will increase. This will cause to make these prepared formulations are unuseful and are not obedient to controlled and slow drug release systems. The prepared formulations including G_0 and H_0 have a good inhibition toward the selected microorganisms according to describe their polymers with biochemical activities, while the prepared formulations including G_1 to G_7 and H_1 to H_7 have high inhibition

toward the same selected microorganisms as compared to pure drug and commercial gel at $p<0.05$. *In vivo* study confirmed that the prepared formulations including G_0, G_3, H_0 and H_3 were not poisonous and non irritant and H_3 formulation possessed of ability to treat the created skin irritation during (10-12) days.

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تحضير بعض صيغ المترونيديزول كهلاميات وتقييمها ودراسة فعالياتها الدوائية ضد الاحياء المجهرية المرضية المسببة للألتهابات الجلدية

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الخلاصة

في الدراسة الحالية, جرى العمل لتطوير أنواع من الهلاميات لنظام علاجي عبر الجلد مؤلفا من تراكيزمختلفة من المترونيديزول مع أتحادات بوليمرية آلفه (محبه) للماء بأستخدام طريقة التشتت الصلب. لقد تضمنت هذه الدراسة جانبين رئيسيين تمثلا بالجانب الكيميائي والجانب الكيموحياتي. ففي الجانب الكيميائي تبين بأن الصيغ المحضرة G_3 و H_3 أتبعت المرتبة الصفرية, نموذج (Korsmeyer–Peppas), وميكانيكية الانتشار المسيطر ((n<0.5) Fickian transport) . أما في الجانب الكيموحياتي فقد تمت دراسة الفعاليات الكيموحياتية (خارج الجسم الحي) لكل الصيغ المحضرة (كمضادات الجرثومية) وذلك لأربع عزلات جرثومية أشتملت *Staphylococcus aureus* (الموجبة لصبغة كرام), *Staphylococcus epidermidis* (الموجبة لصبغة كرام) , *Candida albicans* (خميرة), و *Trichophyton* sp. (عفان) بطريقة الأنتشارالمتبعة بالحفر. وقد تبين بأن إزدياد الفعاليات الكيموحياتية لهذه الصيغ تجاه تلك العزلات قد ترافق مع الزيادة في تراكيز الدواء المحمل مع البوليمرات المختارة في تلك الدراسة, وبالاضافة إلى إمتلاك تلك الصيغ لفعاليات كيموحياتية وضد جرثومية عالية لتثبيط نمو العزلات الجرثومية وذلك بالمقارنة مع الدواء لوحده ومع الهلام التجاري عند مستوى إحتمالية أقل من 0,05(p<0.05). كما تم تطبيق الصيغ المحضرة G_0 , G_3 , H_0 و H_3 على جلد أرانب ذكور لدراسة السمية ((subacute toxicity (LD_{50}), إختباري إثارة (تهيج) الجلد, وإختبار معالجة إثارة (تهيج) الجلد المستحدث. وقد تبين بأن تلك الصيغ لم تكن سامة أو مثيرة (مهيجة) للجلد. أما بالنسبة للصيغة الدوائية المحضرة H_3 فقد إمتلكت القدرة على معالجة إثارة (تهيج) الجلد المستحدث خلال فترة زمنية تراوحت (10–12) أيام.

كلمات مفتاحية: هلاميات المترونيديزول, دراسات خارج وداخل جسم الكائن الحي.