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<td>M.D., Ph.D., Seoul National University Medical College, Seoul, Korea</td>
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Development and Validation of a Stability-Indicating RP-HPLC Method for the Simultaneous Determination of Sofosbuvir, Velpatasvir, and Voxilaprevir in Tablet Formulation

By Deepthi R & Gowri Sankar D

Abstract - Objective: The present study aimed to develop a stability-indicating reverse-phase high performance-liquid chromatography (RP-HPLC) method for the estimation of Sofosbuvir, Velpatasvir, and Voxilaprevir in tablet dosage form and validated in accordance with ICH guidelines.

Methods: The optimized conditions for the developed RP-HPLC method are Agilent C18 (250 mm×4.6mm, 5µ) column maintained at 30°C with a mobile phase consisting of Buffer(0.1%OPA) and Acetonitrile taken in the ratio 55:45%v/v on isocratic mode at flow rate 1.0ml/min. The sample was detected at 220 nm.

Results: The retention time of Sofosbuvir, Velpatasvir, and Voxilaprevir was found to be 2.17, 2.731 and 3.55 min respectively. The developed method was validated for accuracy, precision, specificity, ruggedness, robustness and solution stability.

Keywords: stability-indicating, method development, validation, RP-HPLC, sofosbuvir.

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Forced degradation studies were conducted by exposing the drug solution to various stress conditions such as acidic, basic, peroxide, neutral, photolytic and thermal conditions. The net degradation was found to be within the limits, indicating that the drug is stable in stressed conditions.

Conclusion: The developed method for the estimation of Sofosbuvir, Velpatasvir, and Voxilaprevir can be utilized for the routine analysis of the pharmaceutical dosage form.

Keywords: stability-indicating, method development, validation, RP-HPLC, sofosbuvir.

1. INTRODUCTION

Hepatitis C [1] is a liver infection which is caused by the Hepatitis C virus. The hepatitis C virus is a blood-borne virus and the most common modes of infection are through exposure to small quantities of infected blood. Globally, around 70 million people were suffering from Hepatitis C infection. Antiviral medicines like sofosbuvir, velpatasvir, and voxilaprevir, etc; can cure more than 95% of persons having Hepatitis C infection and reduce the causes of death.

Sofosbuvir [2] (Fig 1) is a nucleotide prodrug and a hepatitis C virus (HCV) NS5B polymerase inhibitor with potential HCV inhibiting activity. Used as an antiviral drug in the treatment of Hepatitis C virus. It is chemically (S)-Isopropyl 2-((S)-(((2R,3R,4R,5R)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)4-fluoro-3-hydroxy-4-methyl-tetrahydrofuran-2-yl) methoxy) (phenoxy) (phenylamino) propanoate.

Velpatasvir [3] (Fig 2) is a NS5A inhibitor which is used together with sofosbuvir to treat chronic Hepatitis C infection. Used as an antiviral drug in the treatment of Hepatitis C virus. Chemically it is methyl{(2S)-1-((2S,5S)-2-(9-((2S,4S)-1-((2R)-2-((methoxy carbonyl) amino)-2-phenylacetyl)-4-(methoxy methyl) pyrrolidin-2-yl)-1H-imidazol-4-yl)-1,11-dihydro[2] benzo-pyran[4',3':6,7]naptho[1,2-d][imidazol-2-yl]-5-methyl- pyrrolidin-1-yl)-3-methyl-1-oxobutan-2-yl} carbamate.

Figure 1: Structure of Sofosbuvir

Figure 2: Structure of Velpatasvir

Author α: Research Scholar, Department of Pharmaceutical Analysis, Andhra University, Visakhapatnam, Andhra Pradesh, India. e-mail: deepthi.pharma7@gmail.com
Voxilaprevir [4] (Fig 3) is a protease inhibitor and acts as a transporter of polypeptide. Used as an antiviral drug in the treatment of Hepatitis C virus, chemically it is \((1R,18R,20R,24S,27S,28S)N[(1R,2R)2\text{Difluoromethyl}]1\{\{1\text{methylcyclopropyl}sulfonyl\text{carbamoil}\}}\text{cyclopropyl}\}-28\text{-ethyl-13,13-difluoro-7-methoxy-24-(2-methyl-2-propanyl)-22,25-dioxo-21dioxide-4,11,23,26-tetra azapenta cyclo nonacosa-3(12),4,6,8,10-pentaene-27-carboxamide.}

**Figure 3: Structure of Voxilaprevir**

As per the literature survey [5-11], it is learned that very few HPLC methods have been reported for the determination of Sofosbuvir, Velpatavir, and Voxilaprevir individually and in combination by HPLC but there is no method for stability-indicating and simultaneous estimation of all the three drugs.

Therefore, there is a need to develop a rapid and reliable Stability-indicating HPLC method for the simultaneous determination of Sofosbuvir, Velpatavir, and Voxilaprevir in bulk and pharmaceutical dosage form.

**II. MATERIALS AND METHODS**

**a) Reagents and Chemicals**

Sofosbuvir, Velpatavir and Voxilaprevir working standards were procured from Spectrum Pharma Research Solutions, Hyderabad, as a gift sample. The VOSEVI tablets were supplied by the Medindia Pharma network. All the chemicals used were of AR grade purchased from Merck, Mumbai. All the solvents used were of HPLC grade purchased from Sigma-Aldrich, Mumbai.

**b) Chromatographic Conditions and Instruments**

WATERS HPLC 2695 SYSTEM equipped with quaternary pumps, Photo Diode Array detector, and an auto sampler integrated with Empower 2 software and Agilent C18 (250mm × 4.6mm, 5µ) column was used for the determination of Sofosbuvir, Velpatavir, and Voxilaprevir. The optimized conditions included 0.1% Orthophosphoric acid (OPA) and acetonitrile (55:45%v/v) as mobile phase run on an isocratic mode at flow rate 1.0ml/min. The column was maintained at 300C and detection was done at 220nm. Other equipment used in the method was Ultrasonic bath sonicator (BVK Enterprises) and weighing balance (Denver).

c) Preparation of Diluent

A mixture of water and acetonitrile in the ratio of 50:50%v/v was used as diluents.

d) Preparation of Mobile Phase

A mixture of 0.1% orthophosphoric acid and Acetonitrile in the ratio (55:45%v/v) was used as the mobile phase.

e) Preparation of Standard and Sample solutions

20mg, 5mg & 5mg of Sofosbuvir, Velpatavir and Voxilaprevir working Standards were transferred to 50ml of volumetric flasks separately, 3/4th of diluents (as mentioned) was added to all the three flasks and subjected for sonication for 10 minutes. The final volume was made up with diluents to obtain a final concentration of 400µg/ml of Sofosbuvir, 100µg/ml of Velpatavir, & 100µg/ml of Voxilaprevir respectively.

From the above stock solution, 1 ml was pipetted out into a 10ml volumetric flask and then the final volume was made with the same diluent. (40µg/ml of Sofosbuvir, 10µg/ml of Velpatavir and 10µg/ml of Voxilaprevir respectively)

10 Tablets (Vosevi) were weighed accurately and the average weight was calculated. An amount equivalent of 1 tablet was collected into a 50ml volumetric flask; 15ml of diluents was mixed and sonicated for around 30 minutes. It was then subjected to making the volume with diluents.

Filtered the solution and diluted 1ml of the above solution to 10ml with diluents. (40µg/ml of Sofosbuvir & 10µg/ml of Velpatavir & 10µg/ml of Voxilaprevir).

f) Method Validation

The developed method was validated in compliance with International Conference on Harmonization (ICH) guidelines [12, 13].

g) Specificity

The specificity of the method was determined by comparing the drug solution with the placebo solution and observed for the interference of placebo peak with drug peak.

h) Accuracy

The accuracy of the present method was determined by %recovery. The drug solution along with the sample was prepared in three concentration levels 50%, 100%, and 150%. Then the % recovery was calculated.

i) Precision

The precision of the method was estimated by injecting the six solutions of the standard into the HPLC system and the % relative standard deviation (%RSD) was calculated.
j) **Linearity**

The linearity of the method was developed by preparing series of dilutions ranging from 12.5µg/ml - 75µg/ml for Bictegravir, 50µg/ml-300µg/ml for Emtricitabine and 6.25µg/ml-37.5µg/ml for Tenofovir alafenamide respectively and injecting them into HPLC system.

k) **Ruggedness**

Ruggedness was determined by injecting the six solutions of the standard into HPLC for different days. The % RSD was calculated.

l) **Robustness**

Robustness of the method was determined by varying the optimized analytical conditions such as mobile phase composition by ±5%, flow rate by ±0.1ml/min and column temperature by ±5ºC.

m) **LOD and LOQ**

Calculation of limit of detection as well as Limit of quantification had been done by using standard equations: $\text{LOD} = 3.3 \times \sigma / S$, $\text{LOQ} = 10 \times \sigma / S$. Here $\sigma$ denotes for the standard deviation of intercepts of regression lines, $S$ denotes for slope.

n) **Solution stability**

Solution stability was estimated by analyzing the standard drug solution after storage for 24hrs under laboratory conditions.

o) **Forced degradation studies**

Forced degradation studies[14] were carried out for drug by exposing the drug solution to the various stress conditions such as acidic (2N Hydrochloric acid for 30min at 60ºC), basic (2N Sodium hydroxide for 30min at 60ºC), Oxidation (refluxing the drug solution with 20%H2O2), neutral (refluxing the drug in water for 6h at 60ºC), photolytic (exposing the drug solution to UV light by keeping the solution in UV chamber for 7 days or 200-watt hrs/m2 in photostability chamber), thermal (drug solution was placed in oven at 105ºC for 6hrs) conditions.

### III. Results

![Graph](image.jpg)

**Figure 4:** Optimized chromatogram of Sofosbuvir, Velpatasvir, and Voxilaprevir

**Table 1: System suitability and validation parameter results**

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<tr>
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<th>Voxilaprevir</th>
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<td>10175</td>
<td>126558</td>
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<tr>
<td>USP Tailing factor</td>
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<td>USP Resolution</td>
<td>4.5</td>
<td>-</td>
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<td>Precision (%RSD)</td>
<td>0.9</td>
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<td>Accuracy</td>
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<td>Linearity range (µg/ml)</td>
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<td>Correlation coefficient, $r^2$</td>
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<td>LOD (µg/ml)</td>
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<td>LOQ (µg/ml)</td>
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<td>Ruggedness (%RSD)</td>
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<td>Day2</td>
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<td>Robustness (%RSD)</td>
<td>1.2</td>
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<th>Voxilaprevir</th>
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<tr>
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<td>%Assay</td>
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<td>Thermal</td>
<td>98.22</td>
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%D- Percentage Degradation

Figure 5: Blank chromatogram

Figure 6: Standard chromatogram
Development and Validation of a Stability-Indicating RP-HPLC Method for the Simultaneous Determination of Sofosbuvir, Velpatasvir, and Voxilaprevir in Tablet Formulation

Figure 7: Sample chromatogram

Figure 8: Placebo chromatogram

Figure 9: Linearity plot of Sofosbuvir

Figure 10: Linearity plot of Velpatasvir
Development and Validation of a Stability-Indicating RP-HPLC Method for the Simultaneous Determination of Sofosbuvir, Velpatasvir, and Voxilaprevir in Tablet Formulation

**Figure 11:** Linearity plot of Voxilaprevir

![Linearity plot of Voxilaprevir](image)

The linearity equation is:

\[ y = 25052x + 1805. \]

\[ R^2 = 0.999 \]

**Figure 12:** HPLC Chromatogram of Acid Degraded sample

![HPLC Chromatogram of Acid Degraded sample](image)

**Figure 13:** HPLC Chromatogram of Alkali Degraded sample

![HPLC Chromatogram of Alkali Degraded sample](image)

**Figure 14:** HPLC Chromatogram of Peroxide Degraded sample

![HPLC Chromatogram of Peroxide Degraded sample](image)
IV. DISCUSSION

For the development of a new method for the simultaneous estimation of Sofosbuvir, Velpatasvir, and Voxilaprevir in bulk and pharmaceutical dosage form initially many mobile phases and many columns were tried to elute the drug peak with less tailing factor, more plate count and more resolution.

Waters HPLC Agilent C18 (250mm × 4.6mm, 5µ) column and Buffer: Acetonitrile (55:45%v/v) as mobile phase were selected based on peak parameters. The detection wavelength was found to be 220nm.

Prepared standard solution, sample solution, and blank solution were injected into the HPLC system and system suitability parameters were noted as summarized in Table 1 along with chromatograms as shown in fig. 4, 5, 6 and 7 respectively.

The developed method was found to obey Beer’s law in the concentration range of 10-60 µg/ml for Sofosbuvir, 2.5-15 µg/ml for velpatasvir and 2.5-15 µg/ml for Voxilaprevir with a correlation coefficient of 0.999 each respectively. A linear graph was plotted between concentration and peak area as shown in fig.
and results are summarized in Table 1.

The method was found to be accurate as the % recovery was 98.50%-101.50% for all the three drugs and was within the limits. The %RSD was found to be less than 1 for all the three drugs indicates that the method was precise. The method was found to be specific, as there is no interference of retention time of placebo peak with that of drug peak. The placebo chromatogram was shown as fig. 8.

Forced degradation studies results indicate that the drug was found to be stable in various stress conditions as net degradation was found to be within the limits. The chromatograms were shown in fig. 12-17 and results were summarized in Table 2.

V. Conclusion

A specific, precise, stability-indicating method was developed for the determination of Sofosbuvir, Velpatasvir, and Voxilaprevir in pure and tablet dosage form using RP-HPLC. The method was validated by using various validation parameters and the method was found to be linear, precise, accurate, specific and robust. From the degradation, studies conducted it is concluded that Sofosbuvir, Velpatasvir, and Voxilaprevir were stable at high concentrations of Acid, Base, Peroxide, Thermal, UV and Water stress study conditions. The run time was 5min which enables rapid quantitation of many samples in routine and quality control analysis of tablet formulations.

Acknowledgment

The authors are thankful to the pharma research solutions, Hyderabad for providing the Sofosbuvir, Velpatasvir, and Voxilaprevir as the gift samples and also for providing required facilities to carry out this research work.

Conflict of Interests

The authors claim that they have no conflict of interest. It has not meant to publish elsewhere. Moreover, it has not meant simultaneously presented for publication elsewhere.

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Impact of Pharmacist Interventions on Direct-Acting Antivirals Sustained Virologic Response and Drug-Drug Interactions

By Marcel Nogueira

Introduction- Infection by HCV is a growing global concern, given its effect on the mortality rate [1]. It is an important cause of cirrhosis, hepatocellular carcinoma, and liver transplant [2, 3]. In 2015, 71 million people were living with HCV. Several studies have shown that the number of new cases declined from the second half of the twentieth century. However, the incidence rate in 2015 was 23.7% (1.75 million new infections by HCV). This increase is related to different mechanisms of transmission. Besides the growing number of young injecting drug users in rural areas, there are reports of HCV transmission among men who have sex with men (MSM) infected with HIV. [4]. In 2016, the World Health Organization (WHO) showed overall goals for the elimination of HCV infection by 2030. This include a 90% reduction in new cases of chronic hepatitis C, a reduction of 65% of deaths, and treatment of 80% of eligible patients[5]. The old therapy in chronic hepatitis C has been a challenge because of the adverse events related to the use of oral ribavirin (RBV) and subcutaneous administration of peginterferon (PEG-IFN). This old therapy had low rates of SVR. In 2015, direct-acting antivirals (DAAs) were incorporated in Brazil. DAAs shows a better efficacy and safety profile, and has a better tolerability for patients [6].

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1. Introduction

Infection by HCV is a growing global concern, given its effect on the mortality rate [1]. It is an important cause of cirrhosis, hepatocellular carcinoma, and liver transplant [2, 3]. In 2015, 71 million people were living with HCV. Several studies have shown that the number of new cases declined from the second half of the twentieth century. However, the incidence rate in 2015 was 23.7% (1.75 million new infections by HCV). This increase is related to different mechanisms of transmission. Besides the growing number of young injecting drug users in rural areas, there are reports of HCV transmission among men who have sex with men (MSM) infected with HIV. [4]. In 2016, the World Health Organization (WHO) showed overall goals for the elimination of HCV infection by 2030. This include a 90% reduction in new cases of chronic hepatitis C, a reduction of 65% of deaths, and treatment of 80% of eligible patients[5]. The old therapy in chronic hepatitis C has been a challenge because of the adverse events related to the use of oral ribavirin (RBV) and subcutaneous administration of peginterferon (PEG-IFN). This old therapy had low rates of SVR. In 2015, direct-acting antivirals (DAAs) were incorporated in Brazil. DAAs shows a better efficacy and safety profile, and has a better tolerability for patients [6]. The Brazilian Ministry of Health has issued a protocol with the criteria for eligible patients and guidelines for the treatment of chronic hepatitis C. DAAs such as sofosbuvir (SOF), daclatasvir (DCV) and simeprevir (SMV) have been made available. In the second half of 2017, 3D (ombitasvir/paritaprevir/ritonavir + dasabuvir)(OBV/PTV/r +DSV) were included into this protocol [7]. Despite the aforementioned benefits over the old therapy, DAAs therapy presents a high risk of drug-drug interactions (DDIs)[7], [8]and there are some contraindications for all DAAs regimens [9]. The use of cytochrome P450 (CYP)/P-glycoprotein (P-GP) inducers (such as carbamazepine and phenytoin) are contraindicated, because of the risk of reduced concentrations of DAAs and high risk of virological failure [9]. Thus, it is essential to evaluate the continuous-use medication before starting treatment. DAAs have interactions with many drugs, especially in HCV-HIV co-infected patients in antiretroviral therapy [7]. CYP3A4 is the metabolic pathway for protease inhibitors such as SMV and NS5A inhibitor (DCV). These drugs can interact with enzyme inhibitors such as ketoconazole [10], [11], and inducers of CYP3A4, such as dipyrene and phenobarbital [12]. Similarly, daclatasvir (DCV) acts as a substrate and an inhibitor of P-glycoprotein (P-GP). Moreover, DCV is a weak inhibitor of organic anion transporters (OAT1B1/OATP1B3) and breast cancer resistance protein (BCRP) [11]. Sofosbuvir (SOF) is less involved in this, but it is as P-GP substrate and concomitant use of P-GP inducers should be avoided [10].

As a specialist in the management of pharmacotherapy, clinical pharmacist contributes to patient care by promoting the rational use of drugs and providing pharmacotherapy services [13]. A clinical pharmacist can identify cases of medication nonadherence, and provides support to hepatologists, optimizing patient care [14]. As well as encourages prevention measures, contributes to the reduction of HCV transmission, increases adherence to treatment and monitors adverse reactions [15]. Thereby, patient understands risks and benefits of pharmacotherapy, improving adherence and treatment outcome[16]. The involvement of the clinical pharmacist is beneficial for hepatology team because DDIs are a common event in the treatment of chronic hepatitis C. The identification and management of this is an intensive resource that requires adjustments to pharmacotherapy, in addition to continuous monitoring of patients. The assessment of DDIs in DAAs therapy and pharmacist interventions was recently published in the scientific literature [15], [17]. Nevertheless, in these studies, it was unclear whether the medical staff approves the pharmacist interventions.

II. Aim of the Study

Our primary objective was to evaluate the impact of pharmacist interventions related to DDIs on SVR. As secondary objectives: 1) to quantify DDIs identified by drug class and drug interaction potential; 2) to quantify pharmacist interventions recommended to medical staff and patient.
III. Ethics Approval

Research Ethics Committee (Plataforma Brasil - protocol number 81497617.1.0000.0068) approved this retrospective study conducted under the STROBE Initiative. Informed consent was not ethically required for this research.

IV. Method

a) Participants

We included patients with chronic hepatitis C, with DAAs prescription (SOF/DCV/SMV) with or without RBV or PEG-IFN, that received medication counseling by the Clinical Pharmacy of Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HCFMUSP). We excluded patients who died, who had DAAs therapy suspended or without the final hepatitis C virus RNA-polymerase chain reaction test (HCV RNA-PCR).

b) Setting

We assessed data tabulated in Microsoft Excel between December 2015 and June 2017, collected from patients of infectious disease, liver transplantation, and gastroenterology outpatient services of HCFMUSP, a public tertiary teaching hospital. Before starting DAAs therapy, all patients were referred for Clinical Pharmacy of HCFMUSP and received medication counseling. This service promotes the rational use of medicines, patient care, and recommends conducts for medical staff to optimize pharmacotherapy. Concomitant use of drugs was analyzed by the electronic prescription system or by manual prescriptions. All included patients have received medication counseling by Clinical Pharmacy as established by the following steps: 1) individual or group counseling supported by an information leaflet that addresses issues such as chronic hepatitis C, HCV transmission, prevention, medication, adherence and patient care during DAAs therapy; 2) DDI analysis on the HEP Drugs Interactions [8] and as necessary, pharmacist intervention addressed to medical staff, for management of DDIs; 3) Individualized guidance to facilitate medication administration times, according to routine of each patient; 4) tabulation of baseline characteristics, DDIs and pharmacist interventions on the database. By identifying DDIs, Clinical Pharmacy staff performed management of DDIs according to the clinical experience of each pharmacist and severity of interaction. Discussions were conducted with medical staff to solve this, in addition to sending letters when face-to-face contact was not possible. 5) DAAs dispensation. After these steps, all patients were referred for medical staff to authorize starting treatment. We performed the acceptance of pharmacist interventions accessing electronic medical records, new medical prescriptions, and by telephone follow-up.

Hence, we divided patients into three different groups: 1) Drug Interaction Avoided (DIA), those with pharmacist interventions approved, 2) Drug Interaction Persisted (DIP), those pharmacist interventions not accepted for any reason; 3) no drug interaction (NDI).

c) Variables

The primary endpoint was SVR, defined as an undetectable viral load, three months after completion of DAAs therapy [7]. Among the secondary endpoints are: 1) number of DDIs (identified by drug or drug class); 2) severity of each DDIs according to HEP Drug Interactions – weak interaction, potential interaction and do not coadminister [8]; 3) number and types of pharmacist interventions classified as alter administration time, alternative medication, discontinuation, dosing adjustment, laboratory monitoring tests and monitoring for side effects.

d) Data sources/measurement

For the primary outcome, we used logistic regression to compare SVR rates between DIA, DIP and NDI groups. The results were collected from electronic hospital records and recorded on the database. To minimize the risk of bias, three authors (MSN, NLL, and GDRS) performed double-checking of all collected data presented in this study.

e) Study sample size

No sample size calculation was done before the conduction of this study. We recruited all patients from December 2015 to June 2017, who met the inclusion criteria. A post-doc analysis was conducted with G*Power [18] to estimate the achieved power of the primary outcome (association between SVR and groups of intervention by logistic regression), considering \( \alpha = 5\% \) and observed effect size (OR), sample size and two-tailed regression model \( R^2 \).

f) Quantitative variables

The baseline characteristics include gender distribution, age, ethnic origin, DAAs regimen, treatment duration, HCV genotype, and presence of cirrhosis. We used frequency and percentage for categorical variables (total sample and for each group: DIA, DIP, NDI). We defined the continuous variables as mean and standard deviation.

g) Statistical methods

We compared the baseline characteristics between groups by the chi-square test for categorical variables and analysis of variance (ANOVA) for continuous variables. We used intention-to-treat (ITT) for missing data of SVR and obtained the odds ratio (OR) was obtained by a logistic regression model, including SVR as a dependent variable, groups (DIA, DIP and NDI) as independent variable and age, sex, genotype, and presence of cirrhosis as control variables. We
considered the level of statistical significance to be 5%, and performed all analyses using STATA 13 (Stata Corp, Texas, USA).

V. RESULTS

a) Participants

We included 1046 patients with chronic hepatitis C, with DAAs prescription. After Clinical Pharmacy guidance, patients were divided into groups DIA (n=273), DIP (n=26), and NDI (n=747). In total, we excluded 74 patients (74/1046, 7.7%). Of these patients, ten had suspended treatment by adverse events, and 64 did not present the final HCV RNA-PCR test. No information was found about the death of patients on the electronic medical records.

b) Descriptive data

Overall, there was a ratio of 48.9% men, 51.0% women and mean age of 58.0 ± 11.42. A statistically significant difference of mean age was found between DIA and NDI groups. The ethnic majority consisted of Caucasians in all groups. The most common DAAs regimen was SOF + DCV + RBV (48.4%) and the overall treatment duration was 12 weeks (84.7%). We observed a higher frequency of genotype 1b (37.0%), followed by 1A (32.9%). Cirrhotic corresponded to 49.5% of overall patients, with statistically significant difference among the three groups. The baseline characteristics are available in table 1.

c) Outcome data

Our data showed a total of 299 patients (28.5%) identified with DDIs. In this group of patients, 273 had pharmacist intervention approved by the medical staff (DIA), and 26 were not approved (DIP). The total number of DDIs was 464, and 286 (61.6%) were identified only with DCV. For DCV interactions, identified drugs were composed of calcium channel blockers such as amlodipine, diltiazem and verapamil (n=85, 29.7%), followed by levotheroxine (n=59, 20.6%) and statins (n=46, 16.0%) (Table 2). The clinical pharmacists performed one hundred thirty-four interventions, such as alteration administration time and 261 monitoring for side effects. Given the DDIs between DAAs and levotheroxine or warfarin, the medical staff accepted 54 interventions for laboratory monitoring tests (52.4%), and thirty-five alternative medication interventions (34.0%) because of contraindicated interactions between SOF, DCV, SMV, RBV, and drugs such as dipyrone (metamizole), anticonvulsants (phenobarbital, phenytoin, primidone, carbamazepine), amiodarone and dexamethasone. Paracetamol was recommended for medical staff to replace dipyrone. Drugs such as valproic acid, ethosuximide, lamotrigine and levitiracetam were recommended (after withdrawal) for patients with anticonvulsants prescription. Propafenone and prednisone were recommended to replace amiodarone and dexamethasone, respectively. Dosing adjustment (n=7, 6.7%) was requested for daclatasvir 90 mg and daclatasvir 30 mg (as a result of CYP3A4 inducer – efavirenz and CYP3A4 inhibitor such as ritonavir, respectively). Moreover, dosing adjustments for amlodipine (10 mg to 5 mg/day), atorvastatin and simvastatin (both to 20 mg/day) because of potential interaction with DCV, were requested. In seven cases (n=7, 6.79%), discontinuation of drugs such as dipyrone, dexamethasone, orlistat, and colestyramine, was suggested for patients with no treatment indication. In the DIP group, 16 interventions for laboratory monitoring tests (61.5%), eight for the alternative medication (30.7%) – given the use of contraindicated drugs such as dipyrone, anticonvulsants, and dexamethasone, and two for DCV dosing adjustment were not approved. The acceptance rate of pharmacist interventions was 79.8% (Table 3). Our team identified three hundred thirteen drugs (68%) as potential interaction, 103 (23%) weak interaction, and 43 (9%) as “do not coadminister” (Figure 1). All identified DDIs by group (DIA and DIP) are available in Appendix A and B.

d) Main results

Intention-to-treat analysis (ITT) revealed an overall SVR rate of 80.1% (n=838/1046). In the DIA and DIP groups, SVR rates were 86.1% (n=235/273) and 57.7% (n=15/26), respectively. In the NDI group, 78.7% of patients (n=588/747) achieved SVR. The logistic regression compared SVR rates among the three groups. The results demonstrate that the DIA group had a greater probability of SVR compared to the NDI group (OR: 1.51; 95% CI 1.00 - 2.28; p=0.048). The DIP group had lower probability of SVR compared to DIA group (OR: 0.26; 95% CI 0.10 - 0.62; p=0.003) and NDI group (OR: 0.39; 95% CI 0.17 - 0.90; p=0.029) (Table 4). The post-doc analysis resulted in an estimated achieved power of 99%, considering α=5%, effect size=1.51, sample size=1046, and R²=0.0361.

VI. DISCUSSION

Our study shows the impact of pharmacist interventions related to DDIs on the clinical outcome of DAAs therapy in 1046 patients. Although we emphasize that our findings reveal an overall SVR of 80.1%, we present a larger sample of patients comparing to others real-life studies of Cheinquer et al. (n=219) [19], Ferreira et al. (n=296) [20] and AI444040[21](n=211). Cheinquer demonstrated the effectiveness of DAAs (SOF/DCV/SMV), 3D therapy (OBV/PTV/r + DSV) and SOF/ledipasvir (LDV), with or without RBV, and showed a higher SVR rate (>90%). Ferreira aimed to evaluate the effectiveness of (SOF/DCV/SMV/LDV/PEG-IFN) with or without RBV, while the clinical study AI444040 assessed the effectiveness of SOF+DCV with or without RBV [21]. Both presented higher SVR rates
DDIs are also common in therapy with other DAAs. Maasoumy et al. demonstrated that 49% of patients were affected by DDIs with protease inhibitors (boceprevir and telaprevir), and management is required [6]. Other data suggest that the management of DDIs can be performed by laboratory monitoring tests, dosing adjustment, alternative medication, or discontinuation, when necessary [6, 15, 17, 22]. Langness et al.[15] observed DDIs frequency with DAAs such as SOF/LDV, 3D, SMV/SOF, and SOF/RBV. Commonly recommended interventions for the management of each interaction were discontinuation (for contraindicated drugs, supplements, and herbal products), as well as monitoring for side effects. In our retrospective cohort study, the risk of self-medication and the use of supplements or herbal products (such as St. John’s wort) were part of medication counseling for all patients. Therefore, we only consider the discontinuation intervention to those with DDIs in prescriptions. Besides Ottmann assess SVR, the author identified and quantified a total of 554 DDIs in 300 patients on DAAs therapy [17]. Ottmann’s study presented a greater focus on LDV/SOF and 3D. Only nine patients (3.0%) used SOF + DCV + RBV and of those, six had 11 DDIs identified (n=11/554 2.0%). Among the drug classes involved in DDIs, there are statins (n=87, 15.7%), calcium channel blockers (n= 63, 11.4%) and analgesics (n=48%, 8.6%). The most commons pharmacist interventions were dosing adjustment (29.6%), alternative medication (6.9%), and discontinuation (4.5%). Overall, 191 interventions were accepted (84.1%). We can compare our results of identified DDIs and the acceptance rate of pharmacist interventions. Our data present a higher frequency of laboratory monitoring tests (n=54, 52.4%) and alternative medication (n=35, 34%) approved interventions than dosing adjustment. This is explained by a higher proportion of patients using LDV/SOF or 3D scheme in Ottman’s study. These DAAs act as inhibitors of various transporters (OATP1B1/3 OATP2B1, P-gp, BCRP) and different metabolic pathways (CYP3A4/5, UGT1A1, CYP2D6) in addition to inducing CYP2C19 [22].

Our study has some limitations. We instructed patients to do not start DAAs therapy until receive medical authorization (after medication counseling by Clinical Pharmacy), but we cannot guarantee that all patients followed this conduct. Probably, some have started treatment after medication counseling and dispensation. We advised patients about DDIs and several pharmacist interventions were performed by sending letters to the external medical staff. Possibly, some of them did not handed it to the medical staff and therefore, were included in DIP group. The clinical pharmacy staff advised all patients about the risks of self-medication and herbal product consumption. We told to avoid dipyrone during DAA therapy, because of the risk of interaction [8, 12]. Dipyrone is one of the most consumed over-the-counter drugs in Brazil [23], and we must consider the hypothesis that not everyone followed these advices.

Because of methodological limitations of a retrospective cohort study, we did not classify cirrhotic patients according to the Child-Pugh score. Probably, this would make it possible to understand SVR rates showed in our findings.

VII. Conclusion

Although the overall rate of SVR was lower than other real-life studies, our results indicate that the DIA group had a significant probability of SVR compared to DIP and NDI groups. Furthermore, this in DAA therapy are common and the medical staff should not neglect it. Pharmacist interventions may contribute to the effectiveness of DAAs therapy and makes it possible to avoid treatment failures caused by DDIs.
Conflicts of interest: All authors have no conflict of interest to declare.

Financial Support: None to declare.

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Cellular Trafficking of Nanocarriers in Alveolar Macrophages for Effective Management of Pulmonary Tuberculosis

By Vipul A. Sansare, Deepa U. Warrier & Ujwala A. Shinde

Abstract- The aim of the present study was to design mannose anchored rifampicin nanostructured lipid carrier for active targeted drug delivery to alveolar macrophages. Targeting ligand, N-octadecylmannopyranosylamine was synthesized and characterized. Rifampicin loaded nanostructured lipid carriers were composed of stearic acid, oleic acid and targeting ligand and were prepared by melt homogenization ultrasonication. The N-octadecyl-mannopyranosylamine decorated rifampicin loaded nanostructured lipid carriers were further characterized for physical state of component, in-vitro release, in-vitro lung deposition, drug loading as well as drug antimicrobial activity on Bacillus subtilis strain. Moreover cytotoxicity and cell internalization ability were evaluated on alveolar macrophages RAW 264.7 cell lines by confocal laser scanning microscopy. The nanostructured lipid carriers exhibited good aerodynamic characteristics and sustained drug release profile with preserved antimicrobial activity. The studies on cell lines demonstrated non-cytotoxicity of nanocarriers.

Keywords: pulmonary tuberculosis, rifampicin, inhalable nanostructured lipid carriers, mannose conjugation, macrophages selective drug delivery.

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Abstract-The aim of the present study was to design mannose anchored rifampicin nanostructured lipid carrier for active targeted drug delivery to alveolar macrophages. Targeting ligand, N-octadecl-mannopyranosylamine was synthesized and characterized. Rifampicin loaded nanostructured lipid carriers were composed of stearic acid, oleic acid and targeting ligand and were prepared by melt homogenization ultrasonication. The N-octadecl-mannopyranosylamine decorated rifampicin loaded nanostructured lipid carriers were further characterized for physical state of component, in-vitro release, in-vitro lung deposition, drug loading as well as drug antimicrobial activity on Bacillus subtilis strain. Moreover cytotoxicity and cell internalization ability were evaluated on alveolar macrophages RAW 264.7 cell lines by confocal laser scanning microscopy. The nanostructured lipid carriers exhibited good aerodynamic characteristics and sustained drug release profile with preserved antimicrobial activity. The studies on cell lines demonstrated non-cytotoxicity of nanocarriers. The mannose anchored nanocarriers were found to internalize efficiently in cell cytoplasm than unconjugated nanocarriers. The prepared alveolar macrophages targeted rifampicin loaded nanostructured lipid carrier exhibited suitable features for inhaled therapy and could be considered as a promising avenue for tuberculosis therapy by means of a dry powder inhaler device.

Keywords: pulmonary tuberculosis, rifampicin, inhalable nanostructured lipid carriers, mannose conjugation, macrophages selective drug delivery.

1. Introduction

Tuberculosis (TB) has remained, unambiguously, a significant health care problem since long times, particularly in developing and under developed countries. The chemotherapy for the treatment of TB is extremely difficult due to the long treatment period and patient noncompliance, leading frequently to the emergence of multidrug resistant (MDR) strains. (Lienhardt, Vernon, and Raviglione 2010) The therapy becomes more complicated and compromised with the emergence of HIV/AIDS pandemic. (Control 2003) In this context, new and improved drug delivery strategies for existing drugs may play a crucial role in the TB management. (Costa et al. 2016) (Sung, Pulliam, and Edwards 2007) Targeted and sustained release chemo-therapy offers a great potential in tuberculosis treatment by achieving greater specificity of delivery and improved therapy. (Hwang et al. 2008) Because M. tuberculosis is known to infect alveolar macrophages (AMs) and affect the pathogenesis of tuberculosis, there have been renewed interests in targeting of anti-tuberculosis drugs to these cells. (Lawlor et al. 2011)

The etiological agent of TB is located in the AMs, more specifically inside the acidic compartments of the phagosomes and phagolysosomes. The infected macrophages have been reported to overexpress certain receptors, which can efficiently be targeted with appropriate drug delivery systems. (Cohen et al. 2018) AMs are pivotal regulators of immunological homeostasis and key effector cells in first line host defence. Intracellular location of bacteria protects them from host defence mechanisms and restricts penetration of antibiotics. (Alexandru-flaviu and Cornel 2014) It is, therefore, necessary to maximize drug uptake by AMs for efficient sterilization of microbial load. Once targeted, cells themselves could serve as a vehicle from where drug would be released from carrier system. However, inadequate specificity for macrophages and poor internalization potential of carriers constitute critical obstacles to success of such delivery systems. TB infection leads to AMs activation which over express mannose (MR-CD 206 and CD 163) (Ernst 1998) (Stahl and Ezekowitz 1998) receptors. Such activated macrophages can recognize and facilitate internalization of carriers bearing mannosylated carbohydrate molecules. (Jain et al. 2012) (Azad and Schlesinger 2011) Therefore an effort has been directed towards development of ligand-decorated carrier systems for cell-selective targeting. Such carriers are efficiently phagocytised by AMs and deliver high payload of drug. (Zaki and Tirelli 2010) Nanotechnology platforms are currently being explored for sustained delivery anti TB drugs to lungs. SLN has emerged as promising nanocarriers with superior physicochemical characteristics viz. small size, biocompatibility and deep-lung deposition ability. (Chakraborty et al. 2009) Dual effect of prolonged drug release and rapid drug transport could be achieved by means of SLNs. (Weber, Zimmer, and Pardeike 2013) Local delivery to lungs by inhalation has become one of the most attractive administration routes to target TB infection's cellular
reservoir, while reducing systemic adverse effects. (Pham, Fattal, and Tsapis 2015) (Hickey 2013) Lipids used in fabrication of SLNs are biodegradable, non-immunogenic; whose functionality can be easily modified and hence enhancing tendency of phagocytic uptake by macrophage cells. (Mehnert and Mader 2001) The benefits of inhalable lectin-targeted carriers could lead to greater patient compliance and curtail the emergence of drug-resistant M. tuberculosis strains.

Lipid-based particulate systems for TB inhaled therapy have been investigated less deeply though they were generally recognized as safe, non-swellable upon contact with the lung moisture and, consequently, able to retain the embedded drug before the target site. Among the lipid-based particulate systems, most of the studies have focused on liposomes (Chono et al. 2007); (Taylor et al. 2015); (Pandey, Sharma, and Khuller 2004); (Rojanarat et al. 2012); (Vyas et al. 2004); (Zaru et al. 2009)) whereas less attention has been paid to the solid lipid particles (Chuan, Li, and Yang 2013); (Pandey, Sharma, and Khuller 2005)) although their advantages over liposomes in terms of physical stability. Solid Lipid Microparticles (SLM), constituted by a solid lipid core stabilized by a surfactant at the surface, represents an advantageous approach to improve TB management. SLM exhibited several favourable properties as production without organic solvents, high drug loading levels and long-term stability. Furthermore, they could be considered proper to provide values of aerodynamic diameter essential for the particle deposition in the deep lung. (Maretti et al. 2014)

Various studies have shown that the mannose receptors have high affinity for carbohydrates, are specifically expressed by activated macrophages in tuberculosis infection. Therefore, drug loaded particulates conjugated to carbohydrate can ensure targeting and internalization of drug by lectin positive macrophages in pulmonary tuberculosis. (Jain et al. 2012), (Veira et al. 2018) After internalization of these particulates, release of the drug in sustained manner helps in achieving improved therapeutic benefits with lower doses. Lipid particulate carriers are one of the antimicrobial drug delivery platforms that have attracted much attention currently. Lipid particulates can provide a sustained release of the carried antimicrobial payloads, which can effectively eliminate the infectious microbes harbored at the lymphatic sites. Moreover, currently the pulmonary drug delivery is the preferred route of administration of aerosolized drugs in the treatment of pulmonary TB, delivering the drug directly to the site of infection through inhalation of an aerosolized delivery. On the basis of aforementioned key points the present project envisaged the development of novel lipid particulate formulation in which carbohydrate anchored lipid particulates loaded with rifampicin (RIF) for targeted and sustained delivery via pulmonary route with reduced systemic side toxicities and improved patient compliance.

II. Material and Methods

a) Material

Rifampicin was kindly gifted by Lupin Ltd, India. Soya lecithin S-100 was gifted by Lipoid, Germany. Stearic acid was purchased from Loba Chem. Ltd., India. Tween 20, D-mannose, mannitol, L-leucine and oleic acid were purchased from S.D. Fine-Chem Ltd. India. Stearylamine was purchased from TCI Chem. Pvt. Ltd. India. Nutrient agar and nutrient broth were purchased from Himedia, India. Nile red was purchased from Sigma Aldrich India. Alveolar macrophage cell line (RAW 264.7) was purchased from National center for cell science, India. All other regents and chemicals were purchased locally.

b) Synthesis and characterization of N-octadecylmannopyranosylamine

Synthesis was carried out by method as reported by Witoonsaridsilp W et al. (Witoonsaridsilp et al.2012) with slight modification. Briefly, a 5 mM of stearylamine was dissolved in 15 ml ethanol and heated up to 70°C, after which 5 mM of D-mannose was added with continuous stirring (200 rpm). This solution was stirred for 15 min till mannose was completely dissolved. The solution was cooled to 40°C and diluted with 35ml n-hexane. The reaction was monitored with thin layer chromatography. Hexane: ethyl acetate (8:2) was used as mobile phase and spots were detected in presence of UV light. The obtained crystals were collected at room temperature and characterized by FTIR, Mass and NMR spectroscopy.

c) Preparation of RIF NLCs

RIF NLCs were prepared by melt homogenization ultrasonication method. (Tran et al. 2014) In practice RIF (25 mg) was dissolved in stearic acid: oleic acid (500 mg) and melted at 70°C. 0.25 % w/v soya lecithin S-100 (25 mg) was transferred in melted lipid. Aqueous surfactant solution containing 10 ml of distilled water and 1.5% w/v of Tween 20 was injected using syringe (21 gauge) into molten lipid mass and stirred at 4000 rpm for 10 minutes at 70°C using overhead stirrer (Remi, India). The obtained pre emulsion was subjected to probe sonication (VCX500, Sonics and materials, U.S.A.) at 20% amplitude for 10 minutes and cooled to room temperature. For further particle size reduction, the NLC dispersion was subjected to homogenization using high pressure homogenizer (Stansted, UK) at 20,000 psi for 3 cycles. Mannose conjugated RIF NLCs prepared by NODM addition in molten lipid mass (10% w/w of total lipid). NLCs were labelled with nile red (0.001% w/v) for cellular uptake studies.
d) Particle size, zeta potential and entrapment efficiency determination

Particle size and zeta potential of RIF NLCs were determined with the aid of photon correlation spectroscopy (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK) at 25°C. Sample was appropriately diluted with distilled water to prevent inter-particle scattering. The percentage entrapment efficiency of RIF in the RIF loaded NLC dispersions was determined using the indirect method. The RIF loaded NLC dispersions were subjected to ultra-centrifugation at 80,000 rpm for 1h at 4°C using Optima Max XP ultracentrifuge (Beckman Coulter, U.S.A.) to separate the unentrapped drug. Supernatant was diluted and analyzed by UV spectrophotometry at $\lambda_{max}$ of 337 nm using methanol AR as blank for quantification of unentrapped or free RIF. Percentage entrapment efficiency was calculated by using equation 1.

$$\text{Percent entrapment efficiency} = \frac{(WL - WF) \times 100}{WL} \ldots (1)$$

e) Development of RIF loaded NLCs based DPI

Mannitol was used as carrier for pulmonary delivery of NLCs. NLC dispersion was mixed with mannitol at 1:2 total lipid to mannitol ratio. L-leucine (1%w/v) was added as anti-adherent and the resulting NLCs dispersion was spray dried using spray drier (Labultima-222, India). (Pilcer and Amighi 2010)

f) Particle size and morphology

Particle size of spray dried NLCs was investigated using Malvern Zetasizer Nano ZS at 25°C. The morphology of the spray dry dried NLCs was viewed by means of scanning electron microscopy (SEM) (Philips XL 30, Japan). The SEM samples were loaded on aluminium stub with carbon adhesive tape and gold was applied for electron conductivity. Samples were scanned at a voltage 10kV and the images were taken.

g) Assay of RIF in spray dry powder

Spray dried NLCs equivalent to 3mg of RIF (369.23mg) was dissolved in 100 ml of methanol AR and maintained in darkness for 24 h at room temperature; subsequently the solution was subjected to bath sonicator. The concentration of RIF was measured spectrophotometrically at 337 nm wavelength. (V-530, Jasco, Japan). Finally the percentage of RIF in spray dried NLCs were compared to initial value and the percentage of RIF entrapped in the spray dried NLCs was calculated. The value considered was average of three determinations.

h) In-vitro release study

RIF release from NLCs and RIF solution were examined on weighed samples using dialysis diffusion technique at 37±0.5°C and quantification was carried out by spectrophotometric method. Briefly, 3mg of RIF and equivalent of RIF NLCs were separately dispersed in 4 ml of simulated lung fluid (SLF) pH 7.4. The resulting dispersion was added to dialysis bag (MWCO 13000-14000 Da, Himedia, India) and was dialyzed separately against 150 ml of SLF (pH 7.4). At predetermined intervals, 5ml aliquots were withdrawn filtered through 0.45 µm membrane filter and RIF content was determined spectrophotometrically. The release medium was replenished with an equal volume of fresh SLF maintained at same temperature. Each experiment was performed in triplicate and the mean value of percent cumulative release and standard deviation at each time point were calculated.

i) In-vitro lung deposition of spray dried NLCs

Lung deposition of spray dried NLCs was assessed using Andersen Cascade impactor (ACI) (Cooply Scientific). NLCs equivalent to 0.3 mg of RIF was filled in each HPMC capsule size 3 (ACG, India). The flow rate was maintained to 60 ± 5 L/min. The spray dried NLCs in each capsule were aerosolized for 10 seconds using inhaler device (Lupihaler®). Methanol AR was used to rinse particles deposited on each stage and RIF content was determined spectrophotometrically. The mass median aerodynamic diameter (MMAD), % fine particle fraction (FPF), fine particle dose (FPD) were calculated from the percentage of RIF propelled from delivery device.

j) Differential scanning calorimetry (DSC) of RIF NLCs and components

Thermograms of RIF, stearic acid, stearic acid: oleic acid (8:2), mannitol, RIF NLCs and NODM conjugated RIF NLCs were recorded by DSC (Perkin-Elmer Pyris 1, USA) in order to investigate the effect of formulation process on the physical state of the components. The samples were heated from 30°C to 300°C at a heating rate of 10°C/min with nitrogen purging (20 mL/min) and endotherms were recorded.

k) Antimicrobial activity

In-vitro antibacterial activity study of RIF NLCs was performed using agar well diffusion technique to investigate whether RIF activity was maintained in the lipid particles. Nutrient broth and Bacillus subtilis (B.subtilis) ATCC 6633 were used as growth medium and microorganism strain respectively. The wells in agar plate were filled with 100µl of sterile RIF solution as reference. Each solution was serially diluted (two folds) to construct the calibration curve by relating inhibition zone diameter to RIF concentration of standard solution. Other well in agar plate was filled with RIF NLCs (10 ppm) dissolved in methanol. The plates were incubated at 37 ± 2°C and inhibition zone diameter was measured. The inhibition zone diameter produced by the RIF NLCs was plotted on the calibration curve to calculate concentration of RIF in RIF NLCs.
i) In-vitro cytotoxicity study

RAW 264.7 cell lines were seeded in 96 well plate at density 1*10^4 cells in 100 µl of Dulbecco’s modified Eagle’s Medium (DMEM) high glucose with 10 % Fasting blood sugar (FBS) medium. The plates were incubated in anaerobic condition with 5 % CO₂ at 37 °C for 48 h to obtained complete monolayer. Cells were then incubated with serial dilutions (1, 10, 50, 100 µM) of samples (RIF, unloaded NLCs, RIF NLCs, NODM conjugated RIF NLCs), medium as negative control and dimethyl sulphoxide as positive control for 37 °C, 5% CO₂ for 48h. After incubation times, the methyl thiazole tetrazolium test (MTT) was performed as per procedure described in (Mosmann 1983) The results were expressed as percentage of cell viability.

m) Cell internalization study

The samples (unconjugated NLCs and mannose conjugated NLCs) were suspended in phosphate buffer (PBS) and diluted using DMEM to a final NLC amount of 0.25mg/ml. RAW 264.7 cells were plated in 6-well plates (300,000 cells/well) and incubated for 12 h with the sample suspension at 37°C. After 12 h incubation, cells were washed with PBS and observed by confocal laser scanning microscopy (DMIRE2, Leica Microsystems GmbH, Wetzlar, Germany). (Maretti et al. 2014)

III. Result and Discussion

a) Synthesis and characterization of NODM

FTIR spectrum of D-Mannose, stearylamine and synthesized (NODM) were recorded and shown in Fig. 1. In spectrum of D-Mannose, broad peak at 3398 cm⁻¹ and intense peak at 2926 cm⁻¹ indicate the presence of –OH stretching and –CH₂ stretching vibrations. Vibrational signals at 1064 and 1638 cm⁻¹ indicate C=O stretching of either alcohol or aldehyde groups in mannose. In the spectrum of stearylamine, sharp lower intensity peak at 3331 cm⁻¹ indicate –NH₂ stretching of primary amine group of stearylamine. Vibrational signals at 2917 and 2849 cm⁻¹ indicate –CH₂ stretching of long alkyl chain. These two peaks were found to be more intense than that of mannose due to presence of long alkyl chain in stearylamine. Vibrational peaks at 1606 and 1471 cm⁻¹ indicate presence of –NH₂ and –CH₂ bending. Spectrum of NODM showed lower intensity peak at 3383 cm⁻¹. This is due to combination of –NH₂ stretching of stearylamine and –OH stretching of mannose. Peak at 1606 cm⁻¹ observed in stearylamine appear at lower intensity in NODM, indicating the conversion of primary amine (stearylamine) to secondary amine (NODM). Reduced intensity of peaks at 1606 cm⁻¹ and 3383 cm⁻¹ indicates the secondary amine linkage between mannose and stearylamine.

NMR and mass spectrum of synthesized NODM is shown in Fig. 2 and 3 respectively. In proton NMR spectrum, presence of lower intensity -NH proton signal at 2.7 ppm indicates the secondary amine linkage between mannose and stearylamine. In mass spectrum, M⁺ peak at 432.4 indicates the presence of desired compound, since the molecular weight of NODM was 431.

b) Preparation and evaluation of RIF NLCs

NLCs loaded with RIF were developed in perspective of a pulmonary therapy of tuberculosis with an objective to investigate the suitability of lipid carrier to target the RIF inside the alveolar macrophages. A major advantage of these lipid particles as drug carrier is related to the biocompatibility and preparation technique that avoids organic solvents. The particle size and zeta potential of prepared NODM conjugated NLCs were found to be 240.9 nm (PDI: 0.135) and -43.3 mV. High negative value of zeta potential indicates formation of stable NLC dispersion. It was found that RIF could be entrapped with relative high efficiencies in NLCs dispersion (52±0.88 %) due to careful selection of lipids and surfactants as well as partial lipophilic nature of RIF.

c) Development and characterization of RIF loaded NLCs based DPI

i. Physical properties of spray dried RIF NLCs

Particle size of spray dried RIF NLCs was found to be 409.5 nm with PDI 0.324 which indicates, NLCs are able to redisperse in water after dissolution of carrier. The NLCs dispersion showed particle size of 240.9 nm and after spray drying, particle size of NLCs was 409.5 nm; the increase in particle size after spray drying could be due to melting and aggregation of lipid matrix during spray drying.

SEM photomicrographs of RIF and RIF NLCs are shown in Fig. 4a and 4b. RIF is reported to be crystalline, rod shaped crystals This was evident from the SEM image of RIF which showed clear elongated crystals ranging from 10-20 µm in size. The spray dried NLCs were observed to be nearly spherical in shape and smooth surface which is suitable for lung delivery. Moreover, the entrapment of RIF did not modify the NLCs morphology.

The physical state of NLCs were evaluated by DSC and compared with those of corresponding components (Fig. 5). RIF, stearic acid and mannitol displayed sharp endothermic peaks at 196.8°C, 63.2°C and 172.3°C due to their melting. (Ingh et al. 2010) (Alves et al. 2010) Furthermore, the endothermic peak for the mixture of stearic acid and oleic acid was shifted to 55.5°C and with lesser intensity than that for pure stearic acid. This indicates reduction in crystallinity of the lipid, which may be attributed to the formation of liquid lipid pockets within the solid lipid. The thermograms of RIF loaded NLCs displayed the presence of diminished and broad peaks of stearic acid and mannitol (Fig. 5b). (Ingh et al. 2010) This was due to a transition of crystalline to
amorphous state in the NLCs. The absence of sharp endothermic peaks related to the stearic acid in the DSC thermograms confirmed this hypothesis. This will possibly minimize the RIF expulsion form the lipid matrix facilitates the drug retention in the lipid matrix.

ii. In-vitro release study

In-vitro drug release study is a measurement of release of active pharmaceutical ingredient (API) from the formulation matrix, is important evaluation parameter for product development and quality control. In present study in-vitro release study of spray dried RIF NLCs was performed using dialysis tube diffusion technique by using dissolution apparatus. All studies were performed in triplicate and results are expressed in mean ± SD. Percent cumulative release of RIF from RIF solution and spray dried RIF NLCs is graphically represented in Fig. 6.

Spray dried RIF NLCs show sustained release profile up to 96 h whereas RIF solution showed 10 h release profile. This could be due to poor wettability of lipid nanocarriers particles and high lipid solubility of RIF. Similar result was reported by (Aboutaleb et al. 2012) (Mulla et al. 2017), where RIF release from RIF lipid microparticle showed sustained release profile in SLF pH 7.4 over a period of 100 h.

iii. In-vitro lung deposition of spray dried NLCs

ACI separates a sample into fractions based on inertia, which is a function of particle density, shape and velocity. Three important parameters determined from ACI study are MMAD, geometric standard deviation (GSD), FPF less than 5 μm. (Parumasivam et al. 2016) FPF indicates respirable fraction and it is a fraction of total inhaled drug that reaches the stage corresponding to cut off diameter of 5 μm. In- vitro lung deposition studies of spray dried RIF NLCs was performed using ACI. The amount of drug deposited on each stage was calculated and represented in Fig. 7.

Based on drug deposited on device, capsule and stages of ACI, Recovered Dose (RD), Emittted Dose (ED), FPD, FPF, MMAD and GSD were calculated. MMAD of spray dried formulation was 4.71, which is suitable for lung deposition. Approximately 34% of drug was deposited in the preseparator and induction port. This may be due to particle aggregation in presence of humidity. Higher emitted dose indicates good flow properties of powder.

d) Antimicrobial activity

The microbiological assay was performed on RIF NLCs dissolved in methanol: water mixture. Methanol was selected for complete extraction of RIF from NLCs without reducing biological activity. The B. subtilis ATCC 6633 strain was chosen because of its susceptibility to RIF. (Dey and Chatterji 2012) (Bemer-melchior, Bryskier, and Drugeon 2000) The zone of inhibition provided by dissolved NLCs was compared with those produced by standard RIF solution (Fig. 8).

NLCs produced inhibition zone diameter corresponding to the RIF loading value (5 ppm), thus providing evidence of antimicrobial activity preservation after entrapment of RIF in lipid matrix. The unloaded NLCs did not provide zone of inhibition indicating that lipid matrix did not interfere with the assay.

e) Cytotoxicity and internalization capacity by RAW 264.7 cell lines

Both cytotoxicity and capacity of NLCs to interact with alveolar macrophages were studied by means of macrophages RAW 264.7 cell lines. RIF loading level was considered for the selection of sample amount. To make NLCs fluorescent, Nile red was embedded into the lipid matrix. Nile red is lipid soluble dye and it is a vital stain for the detection of intracellular lipid material by confocal laser scanning microscopy. (Greenspan, Mayer, and Fowler 1985)

Concerning cytotoxicity of NLC samples at four different concentrations of RIF, MTT test results expressed as percent cell viability are shown in Fig 9. Rifampicin loaded NLCs exhibited a dose dependent cytotoxicity that increased with the concentration. However only negligible cytotoxicity with cell viability over 85% was found at 100 Mmol concentrations. These results are consistent with those observed for stearic acid based lipid carriers.

In order to clarify the location of NLCs in the cell, confocal microscopy was performed. The image obtained under filter set for red and blue fluorescence from the cells incubated with mannose conjugated NLCs (Fig. 10) was compared with that of non-conjugated NLCs. RAW 264.7 cells incubated with mannosylated NLCs revealed the presence of marked red fluorescent spots around the respective nuclei having size corresponding to that of nanoparticles indicating presence of NLCs inside the cell cytoplasm. Conversely, a negligible or slight fluorescent spots were observed from the cells incubated with unconjugated NLCs. Concerning mechanism of cell entry, receptor mediated endocytosis is established for efficient entry of mannosylated NLCs over unconjugated NLCs.

Due to lipophilic nature of RIF that is able to retain drug within NLCs matrix, as demonstrated by the in-vitro release study, it could be hypothesized that RIF remains embedded within the NLCs during the uptake process. Although release of RIF was negligible, there could be a possibility of leakage from NLCs, inside the macrophages to exert anti-tubercular activity, as observed both in-vivo and iv-vitro by other authors (Takenaga et al. 2008) owning to the intracellular biodegradation of the lipid matrix. Therefore, an anti-tubercular activity inside infected alveolar macrophages can be expected.

This preliminary phase of the study is not sufficient to predict the in-vivo effectiveness of the lipid based system in human TB treatment. The next step of
the study will consider effectiveness in infected cells and animals.

IV. Conclusion

Thus aim of the present study was to develop ligand conjugated RIF loaded nanostructured lipid carrier (NLCs) based dry powder for inhalation to provide AM targeting, reduce dose related side effects and formulate an acceptable dosage form. The major outcomes of this study was the successful synthesis of mannose conjugated lipid, entrapment of RIF within a lipid core and spray drying of optimized RIF NLCs dispersion. The RIF NLCs dispersion showed good quality control parameters (particle size below 300nm, PDI 0.135). The spray dried NLCs were found to be spherical, micron size particles thus showing suitability for pulmonary administration. The spray dried formulation showed efficient release of nanoparticles after dispersion in water. The formulation also showed antibacterial activity, in terms of zone of inhibition as compared to RIF solution. However, in-vivo organ distribution studies are necessary to confirm distribution of NLCs inside the alveolar macrophages. Thus, the developed RIF loaded NLCs based dry powder for pulmonary drug delivery may prove to be useful in the therapy of tuberculosis.

Conflict of interest

The authors declared that there are no conflicts of interest.

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**Fig. 3:** Mass spectrum of synthesized NODM

**Fig. 4:** SEM images of a) RIF b) Spray dried RIF NLCs

**Fig. 5a:** DSC thermograms of oleic acid, stearic acid and physical mixture stearic and oleic acid
Fig. 5b: DSC thermograms RIF, mannitol, spray dried RIF NLCs and mannosylated RIF NLCs

Fig. 6: *In-vitro* release profile of RIF suspension and RIF NLCs

Fig. 7: Comparative plot of % of drug deposited on each stage of ACI

Fig. 8: RIF antimicrobial activity from spray dried NLCs
Fig. 9: MTT test on RAW 264.7 cell line incubated with different concentrations of RIF, RIF NLCs and mannosylated RIF NLCs

Fig. 10: Confocal microscopy images of RAW 264.7 cells after nuclei staining and incubation with a) Mannosylated RIF NLCs b) Unconjugated RIF NLCs

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Hatchability Dry Cysts and Morphological Effects of Newly Hatching Nauplii of *Artemia Salina* (Linnaeus, 1758) after Exposed to Tributyltin Chloride

By Najla Mohamed Abushaala, Syaizwan Zahmir Zulkifli, Ahmad Ismail & Abduo Fattah Mohamed Elfituri

**Abstract**- In previous studies focused on a nauplii stage of Artemia sp as a model to acute toxicity tests to detection of antifouling as an active agent against fouling marine organisms as Tributyltin Chloride (TBTCl). This research aims to investigate the toxicities of (TBTCl) on hatching dry cysts and morphological changes on newly nauplii of Artemia salina. The range of TBTCl concentration was selected (5, 10, 15, 20, 25, 50, 75, 100, 150, 200 ng l⁻¹). The results showes TBTCl significantly reduced hatching percentages of *A. salina* cysts from the (5 to 200 ng l⁻¹). The 200 ng l⁻¹ TBTCl concentration showed no indication of hatching percentages among *A. salina* cysts. comparing with percentages in the control were 97%. The median effective concentration EC₅₀ of TBTCl was (46.48 ng l⁻¹). The survivors nauplii were used to study the effect TBTCl on morphological malformation as total length and body width of newly nauplii.

**Keywords**: *artemia cyst; acute-term mortality; ecotoxicology; hatching test; tributyltin chloride.*

**GJMR-B Classification**: NLMC Code: QV 55

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Hatchability Dry Cysts and Morphological Effects of Newly Hatching Nauplii of Artemia Salina (Linnaeus, 1758) after Exposed to Tributyltin Chloride

Najla Mohamed Abushaala, Syaizwan Zahmir Zulkifli, Ahmad Ismail & Abduo Fattah Mohamed Elfituri

Abstract- In previous studies focused on a nauplii stage of Artemia sp. as a model to acute toxicity tests to detection of antifouling as an active agent against fouling marine organisms as Tributyltin Chloride (TBTCl). This research aims to investigate the toxicities of (TBTCl) on hatching dry cysts and morphological changes on newly nauplii of Artemia salina. The range of TBTCl concentration was selected (5, 10, 15, 20, 25, 50, 75, 100, 150, 200 ngl⁻¹). The results showes TBTCl significantly reduced hatching percentages of A. salina cysts from the (5 to 200 ngl⁻¹). The 200 ngl⁻¹ TBTCl concentration showed no indication of hatching percentages among A. salina cysts. comparing with percentages in the control were 97%. The median effective concentration (EC₅₀) of TBTCl was (46.48 ngl⁻¹). The survivors nauplii were used to study the effect TBTCl on morphological malformation as total length and body width of newly nauplii. The higher rate of malformations of newly nauplii in 5 ngl⁻¹ TBTCl concentration was 32.00 ± 4.62. Because in this concentration is a chance to newly nauplii survival to a longer period in toxic solution, which gives clearly deformities. While the lower deformities (%) were1.00 ± 0.00 at 75 ngl⁻¹. Because the chance to survival newly nauplii is very weak and it was difficult to observed the deformities clearly. As for the other concentration of TBTCl the deformities (%) was between this means. Conclusion, finding indicated that when increasing TBTCl concentration affected the hatching rate and TBTCl can killa embryo of A. salina cysts in higher concentrations, while in low concentrations can effect on morphological changes (total length and body width) when exposure dry cysts to seawater contaminated with TBTCl.

Keywords: artemia cyst; acute-term mortality; ecotoxicology; hatching test; tributyltin chloride.

1. Introduction

Recently there are many research about the acute toxicity of Tributyltin Chloride (TBTCl) on marine organisms. In this research studying the acute toxicity tests on Artemia sp. in previous studies focused on a nauplii of Artemia sp. This research aims to investigate the toxicities of (TBTCl) of the hatching stage of dry cysts and morphological changes of newly hatching nauplii of Artemia salina. In most scientific research widely used Artemia sp. as a model marine organism for ecotoxicity test, due to it is large geographical distribution. Despite it is popularity, the use of Artemia sp. in toxicity check is subjected to a wide discussion, at the global level, more often than not due to a number of criticisms about low sensitivity and lack of protocol standardization. (George-Ares et al., 2003; Mayorga et al., 2010; Leis et al., 2014; Libralato, 2014 and Rotini et al., 2015). Biological influences of TBTCl on A. salina may additionally furnish clues for of the accumulation mechanisms in coastal ecosystems as nicely as of the mode of action of TBT in these organisms. A. salina and different Artemia species have been used in the literature for the screening of acute toxicities of booster biocides (Bartolomé and Sánchez-Fortún, 2005; Koutsasftis and Aoyama, 2008 and Rotini protocol et al., 2015). There are many advantages to use Artemia for example, adaptability to high temperature, adaptability to wide ranges of salinity, adaptability to varied nutrient resources, ease of culture, small body size and short life cycle (Nunes et al., 2006 and Koutsasftis and Aoyama, 2008). In addition, Artemia is low cost and can use it anywhere at any time.

Tributyltin chloriad is environmental hazards. The half of lives of tributyltin in the marine surroundings had been reported as nearly a number of days to weeks in water and from one to ten years in sediments (Huang et al., 2004 and Al-Rashdi, 2011). In the previous studies toxicities of booster biocides have been reported on embryos of some marine organisms such as freshwater mussels, zebra mussels, blue mussels, sea urchins, oysters, and sea squirts (Bellas et al., 2007; Fent, 1996 and Wang et al., 2012). High concentrations of BTs have been detected in lower trophic animals such as caprellids. It appears that TBT accumulates specially in caprellids in the marine ecosystem, irrespective of the trophic level in the food
Hatchability Dry Cysts and Morphological Effects of Newly Hatching Nauplii of *Artemia salina* (Linnaeus, 1758) after Exposed to Tributyltin Chloride

II. Materials and Methods

a) Hatching procedure and acute toxicity tests

The tributyltin chloride (TBTCl) used in toxicity tests was kindly provided by Sigma-Aldrich, USA (purity 96%). Stock solutions of TBTCl were prepared by diluting with artificial seawater up to 35% salinity. The range of concentration TBTCl was selected as (5, 10, 15, 20, 25, 50, 75, 100, 150, 200 ngl⁻¹). The experiments were performed in 50 ml test tube within tube racks that were submerged in water up to the midpoints of the tubes. Constant aeration, illumination (1000 Lux), and temperature (28 ± 1 °C) were maintained and the replicate number was three in the experiments each replicate 100 cysts of *A. salina* cysts. For each test group, added 40 ml from the test solution of different concentration TBTCl to test tubes, and then a 24 hours hatching period was initiated. After the hatching period, the number of newly hatched nauplii, viable hatched, cysts, and malformation newly hatched nauplii were counted. Hatching percentages (HPs) of cysts were determined by counting the number of completely hatched of nauplii. Hatching failure (found by subtracting the number of completely hatched nauplii from total group size). After that account hatchability (%), Deformity (%) and viable hatchability (%) by using the following formulae (Revathi and Munuswamy, 2010).

- Hatchability ($) = 100* (no. of hatched larvae) / (no. of total cyst in test)
- Deformity ($) = 100* (no. of deformed larvae) / (no. of hatched larvae)
- Viable hatchability ($) = 100* (no. of viable hatchability larvae in test) / (no. Total cyst in test).

b) Median effective concentration (EC₅₀)

The data on the hatchability inhibitor % of cysts was used in the estimation of 50% effective concentration (EC₅₀) in different concentration of TBTCl. The effective concentration EC₅₀ values were determined by using probit analysis in XL TEST-Pro (version 2014.5.03). And each end point was calculated by using the following formulae (Shimasaki et al., 2003).

c) Morphological abnormalities

The morphological abnormalities of *A. salina* newly hatched nauplii of completely hatched in each concentration of TBTCl toxicant and measurement the total length and width of the body (head width, abdominal width and tail width) have been measured (Alyuruk and Cavas, 2013).

III. Results and Discussion

Effects of Tributyltin Chloride on Cysts Hatchability in *Artemia salina*

1. Hatching Percentages (%)

Hatchability of the exposed *A. salina* cysts to different concentrations of TBTCl observed in this study is presented in *Figure 1*. The hatching percentages were shown to be affected by TBTCl concentrations. TBTCl significantly reduced hatching percentages of *A. salina* cysts at the various concentrations by using the following formulae (Revathi and Munuswamy, 2010). Hatchability (%) = 100* (no. of hatched larvae) / (no. of total cyst in test). The hatching percentages in the control were 97%, which is within the reported value of the manufacturer (minimum of 90% hatchability). Among these completely hatched cysts, 76% were active and 21% were viable hatched (completely hatched, but still not active). The remaining 3% cysts were found hatched after hatching period was prolonged for more than 24 to 48hr. From the results observed a significantly decrease hatching percentages from the 5 to 200 ngl⁻¹ and was TBTCl had varying effects on the hatching percentages of *A. salina* cysts (Figure 1). In 200 ngl⁻¹ showed complete hatching inhibition percentages of *A. salina* cysts. The cysts exposed to TBTCl within 24hr were unable to hatch even the hatching period was prolonged until 48hr. This result confirmed TBTCl can kill an embryo and inhibit hatchability of *A. salina* cysts.
A. salina cysts are a barrier to withstand external environmental condition. Disruption of these activities by certain concentration of TBTCl bioaccumulated into the cysts may cause death to the dormant embryo, and this finding is relative agreement with the results of this research have shown that the toxicity of TBTCl can impact the hatching process of A. salina cysts. This result is supported through Brix et al., (2006) studied estimated the median high-quality concentrations (EC₅₀) for metallic salts, suggesting that the hatching end point for A. franciscana is the most touchy examined to date for steel salts in saline environments and same in sensitivity with the most touchy tested to date for Cu. But in present finding A. salina cysts are more sensitive to TBTCl at lower concentration 5 ngL⁻¹ it was 25% cysts comparative with the control samples 3% cysts, and that mean TBTCl can inhibits hatching process and can kills dormant embryo in the low concentration and also when increasing the TBTCl concentration. Revathi and Munuswamy, (2010) investigated the effects of TBT on the embryonic development, and hatching success of eggs uncovered to TBT in the freshwater prawn brooder Macrobrachium rosenbergii, and observed TBT at 3.12 ppm, delayed the embryonic development and significantly reduced the hatchability of eggs as well. two on the different hand, the treated businesses showed impaired embryonic development with reduced body growth. Thus, TBT has appreciably retarded the embryonic improvement in the freshwater prawn M. rosenbergii. These studies clearly demonstrated the possible effects of toxicants particularly TBTCl on unhatched eggs or cysts of crustaceans, including A. salina was more sensitive to TBTCl at 5 ngL⁻¹, and the possible reason that TBT is more toxic to A. salina because the body size is small and it is life cycle is very short Figure 2 shows effect of different concentrations of TBTCl on the performances hatching of A. salina cysts percentages. Figure 2 (a) shows the completely hatching (%) that mean the newly hatching nauplii is active and healthy as shown in control. Several nauplii exposed to 10, 25 and 50 ngL⁻¹ were viable hatching (%) that mean the newly hatching nauplii completely hatching, but still not active and in the embryonic membrane (Figure 2 (b), (c) and (d)), while A. salina cysts exposed to 75 ngL⁻¹ TBTCl concentration was unable to completely break the cyst wall (Figure 2 (e)), while the A. salina cysts in the 100 ngL⁻¹ unable to hatching (Figure 2 (f)). This sequence of effects relatively showing the severity of TBTCl as its concentration increase in the aquatic environment.
2. Median Effective Concentration (EC\textsubscript{50}).

The median effective concentration EC\textsubscript{50} of TBTCI as shown in (Figure 3), at different concentration of TBTCI the A. salina nauplii completely hatching after 24hr was (EC\textsubscript{50} 46.48 ng\textsuperscript{l}/l), this is mean the TBTCI impacted the process of hatchability A. salina cysts and significantly reduced the hatchability cysts when increasing the concentration of TBTCI. Since there is a confined research on the inhibitory effects of the hatchability share cysts of A. salina it was once two examine EC\textsubscript{50} of TBTCI with one of a kind toxicants such as metals have been pronounced on the hatchability percentage of cysts for instance (Caldwell et al., 2003) studied A. salina, have been observed to inhibit hatching success of A. salina cysts in dose. A higher sensitivity was once discovered in the 24 and 72hr publicity EC\textsubscript{50} for 24hr was once 2.14 and 72hr was 0.023 µg ml\textsuperscript{-1}. This result is an settlement with (Brix et al., 2006) studied estimated the EC\textsubscript{50} of metallic salts are suggesting that the hatching endpoint for A. franciscana is the most sensitive examined to metals in marine environments. Meanwhile, Aly\textasciiacute{r} and Çavuş, (2013) mentioned their investigation related to the toxicities of diuron to the hatching stage of A. salina, their results showed that diuron should be a attainable hatching enzyme inhibitor and used to be substantially lowered the hatching proportion of A. salina cysts and prevented the hatching of cysts. Rotini et al., 2015 said in their learn about Artemia sp hatching assay is a touchy choice device to acute toxicity take a look at and the hatching test resulted extra touchy than acute mortality tests. The outcomes show the reliability and excessive sensitivity of this hatching assay on a short time and guide it is a useful application of first tier risk assessment techniques in the marine environment.
Figure 3: Median Effective Concentration 50% (EC$_{50}$ = 46.48 ngL$^{-1}$) between TBTCI Concentration and Hatchability Inhibition (%) of A. salina Cysts

3. Morphological Effects of A. salina Newly Hatching Nauplii after the Exposed the Cysts to Different Concentration of TBTCI

Analysis of the morphological deformities (%) of the means A. salina newly hatching nauplii after the exposed the cysts to different concentration of TBTCI as shown in Table 1. The higher rate of deformities of newly hatching nauplii at 5 ngL$^{-1}$ TBTCI concentration was 32.00 ± 4.62. Because in this concentration is a chance to newly nauplii stay a longer period survival, which gives greater opportunity to appear changes in shape and deformities. While the lower morphological deformities (%) were 1.00 ± 0.00 at concentration 75 ngL$^{-1}$. Because the chance to survival newly nauplii is very weak and continued growth and development to the body is slowly and it is difficult to note the deformities clearly. As for the other concentration of TBTCI the deformities (%) was between this means. That means the TBTCI different concentration can impact the morphological changes in newly hatching nauplii when exposure the dry cysts to artificial sea water contaminated with TBTCI.

Table 1: Morphological Deformities (%) of Means and SE of Newly Hatching Nauplii A. salina Affected by Different Concentration of TBTCI [N: number of cysts]

<table>
<thead>
<tr>
<th>TBTCI (ngL$^{-1}$)</th>
<th>N</th>
<th>Deformity (%)</th>
</tr>
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<tr>
<td>0</td>
<td>300</td>
<td>0.00 ± 0.00$^a$</td>
</tr>
<tr>
<td>5</td>
<td>300</td>
<td>32.00 ± 4.62$^c$</td>
</tr>
<tr>
<td>10</td>
<td>300</td>
<td>16.33 ± 3.76$^b$</td>
</tr>
<tr>
<td>15</td>
<td>300</td>
<td>9.00 ± 0.58$^{a,b}$</td>
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<tr>
<td>20</td>
<td>300</td>
<td>9.67 ± 1.46$^{a,b}$</td>
</tr>
<tr>
<td>25</td>
<td>300</td>
<td>6.33 ± 1.76$^{a,b}$</td>
</tr>
<tr>
<td>50</td>
<td>300</td>
<td>2.33 ± 0.33$^a$</td>
</tr>
<tr>
<td>75</td>
<td>300</td>
<td>1.00 ± 0.00$^a$</td>
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</tbody>
</table>

Because in this concentration is a chance to newly nauplii stay a longer period survival, which gives greater opportunity to appear changes in shape and deformities. While the lower morphological deformities (%) were 1.00 ± 0.00 at concentration 75 ngL$^{-1}$. Because the chance to survival newly nauplii is very weak and continued growth and development to the body is slowly and it is difficult to note the deformities clearly. As for the other concentration of TBTCI the deformities (%) was between this means. That means the TBTCI different concentration can impact the morphological changes in newly hatching nauplii when exposure the dry cysts to artificial sea water contaminated with TBTCI.
artificial sea water contaminated with TBTCI in the low concentrations.

The morphological deformities such as total length in the newly hatching nauplii as can be seen in the increase concentration of TBTCI, the total length of newly hatching nauplii A. salina will significantly decrease in general total length in newly hatching nauplii were represented in Table 2. This table is shown means of total length newly hatching nauplii shows the control group was (350.9 ± 49.6) µm, but the means for total lengths in different concentration of TBTCI 5, 10, 15, 20, 25, 50, 75 ngl⁻¹ were (284.6 ± 51.6), (266.8 ± 54.6), (282.2 ± 59.3), (294.9 ± 40.6), (288.8 ± 45.7), (274.8 ± 39.7) and (269.8 ± 54.6) µm, respectively. According to mean and standard error the lowest total length was (266.8 ± 54.6) µm at 10 ngl⁻¹ TBTCI concentration compared with the control group.

Table 2: Total Length of Newly Hatched Nauplii After 24hr Exposed to Different Concentration of TBTCI [N: number of nauplii]

<table>
<thead>
<tr>
<th>TBTCI (ngl⁻¹)</th>
<th>N</th>
<th>Mean ± SEM (µm)</th>
<th>Minimum (µm)</th>
<th>Maximum (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>35</td>
<td>350.9 ± 49.6</td>
<td>314.3</td>
<td>451.7</td>
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<td>35</td>
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<td>221.6</td>
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</tr>
<tr>
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<td>202.6</td>
<td>364.2</td>
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<tr>
<td>15</td>
<td>35</td>
<td>282.2 ± 59.3</td>
<td>212.5</td>
<td>398.9</td>
</tr>
<tr>
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<td>35</td>
<td>294.9 ± 40.6</td>
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<td>389.9</td>
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<tr>
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<td>216.5</td>
<td>352.1</td>
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<tr>
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<td>35</td>
<td>274.8 ± 39.7</td>
<td>244.8</td>
<td>393.3</td>
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<tr>
<td>75</td>
<td>35</td>
<td>269.8 ± 54.6</td>
<td>201.6</td>
<td>304.2</td>
</tr>
</tbody>
</table>

The morphological changes such as total length and width of body in the newly hatching nauplii as can be seen in Figures 4, 5, 6 and 7. In general the regression analysis (r) shown the high regression values and this demonstrates a strong inverse relationship between morphological measurements and increase TBTCI concentration. The head width is more affected compared to the total length r = 53 %. And the head is more caricatures and more sensitive to increasing of TBTCI r was 95%. While the abdomen and tail width of body shown moderately affected r = 89 % and decrease when the increasing concentration of TBTCI. In present study need to mention there are not enough studies about effects of TBTCI on the morphological changes in newly hatching nauplii so will be compare these findings with similar studies about nauplii exposed to different types of toxins. For example, Abushaala et al., (2015a) study effect of TBTCI on nauplii stage of A. salina and reported in their results the TBTCI had effect the morphology changes of nauplii A. salina. On the other hand, (Rao et al., 2007) studies toxicity of Organophosphates on morphology changes in nauplii A. salina and significant morphological alteration were noticed in nauplii. In under study Abushaala et al., (2015b) studied effect of Diorun on nauplii stage of A. salina their results shown the Diorun had effect the morphology changes in the nauplii stage of A. salina. Also Anderson, (2009) his find out about confirmed the impact of alcohol proportion on the improvement rate of A. salina nauplii.
Hatchability Dry Cysts and Morphological Effects of Newly Hatching Nauplii of *Artemia Salina* (Linnaeus, 1758) after Exposed to Tributyltin Chloride

**Figure 4:** Morphological of Total Length *A. salina* Nauplii after Exposed the Cysts to Different Concentration of TBTCI

**Figure 5:** Morphological of Head Width *A. salina* Nauplii after Exposed the Cysts to Different Concentration of TBTCI

**Figure 6:** Morphological of Abdomen Width *A. salina* Nauplii after Exposed the Cysts to Different Concentration of TBTCI
IV. Conclusion

In this study increased TBTCl concentration in solution could significantly decreased the hatchability percentage of A. salina cysts and prevented the hatching of larvae. And the EC50 value of TBTCl was once recognized as 46.48 ngl^-1 after 24hr exposure. Early nauplii of A. salina is sensitive to TBTCl contamination to reflect hatchability cysts and early life stage effects of toxicant. In addation, the TBTCl effect on morphological abnormality active newly nauplii A. salina. And significant morphological differences were observed in all nauplii exposed to the different concentration of TBTCl are used in this research. These results indicate that in this system TBTCl, it is proven environmentally toxic substances. In general, these results indicated that when increasing TBTCl concentration affected the total body length and the body width of A. salina newly hatched nauplii. In spite this result indicated that the system TBTCl is acutely toxic.

Acknowledgements

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Assessment of Antibacterial Activity and Minimal Inhibitory Concentration of Leaf Extract of Morinda Citrifolia Against Enterococcus Faecalis- An Invitro Study

By Venkata Teja Kavalipurapu, Apoorva Vasundhara Kaligotla & Gummuluri Sriram

Abstract- **Background:** When root canal infections are concerned, the causative factor is primarily the microbial inhabitation, which leads to the spread of infection. The literature evidence supports the aspect that frequent usage of antibiotics induces resistance and threatens the effectiveness of the treatment. So, the current issue that is faced globally is the development of resistant microbial species. To combat this interest, towards the usage of newer herbal horizons is increased.

**Materials and Methods:** The antibacterial effect of Ethanolic Leaf Extract of Morinda Citrifolia was investigated against Enterococcus Faecalis (E. Faecalis). Agar well diffusion and broth dilution methods were used to determine the Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

**Results:** The MIC of Ethanolic Extract of Morinda Citrifolia extract was found to be 250 µg/ml, and the MBC was 400 µg/ml

**Keywords:** bacteria, biofilm, endodontics, morinda citrifolia leaves, root canal.

GJMR-B Classification: **NLMC Code:** QV 744

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Conclusion: Ethanolic leaf extract showed better antimicrobial activity on E. Faecalis.

Keywords: bacteria, biofilm, endodontics, morinda citrifolia leaves, root canal.

I. INTRODUCTION

Endodontics is a field where complex root canal anatomy, dealt in a simplified way. Most of the treatment and therapeutic success attributed to the complete removal of tissues and contents from root canal space, including the dentinal and microbial debris (1),(2). When root canal infections are concerned, the causative factor primarily is microbial inhabitation, leading to the spread of infection into periapical space, causing apical periodontitis(3). It's a known fact that root canal infections cannot be attributed to a single species or a single microbe. The pathogenic resistant microbial colony is attributed to the formation of a complex biofilm(4),(5). So, the major hurdle in therapeutic endodontics is the complete removal of bacterial biofilm(5).

So, from the previous discussion, it’s understood that microbial inhabitancies are primary causation factors for root canal infections. Thus, therapeutic options and advancements tend to concentrate more on this aspect. Although the advancing front is towards the discovery of antimicrobial agents in eradicating the bacterial biofilm, it’s more neglected that the threat of antimicrobial resistance is also increasing. So, the current issue that is faced globally is the development of resistant microbial species(6). The literature evidence supports the aspect that, frequent usage of antibiotics induces resistance and threatens the effectiveness of the treatment (7),(8).

The evolving trend in new therapeutics is concentrating on a wide range of herbal products and natural plant extracts. The evidence also supports that these products exhibit a broad range of antimicrobial properties and therapeutic benefit(9),(10). So, many researchers and clinicians have shifted their interest in exploring the natural plant extracts and assessing their therapeutic effects. Although there is a wide range of natural products and plant extracts widely used for various medicinal purposes, the well-documented usage and benefits in endodontics are seen majorly with Morinda Citrifolia(11).

A study by Murray et al.,(12) stated that, the antimicrobial efficacy of Morinda Citrifolia juice was similar to 6% sodium hypochlorite, which is considered as a gold standard root canal disinfectant in endodontics. The currently available evidence is strongly in favor of sodium hypochlorite as the main endodontic irrigant(13).

The concentration of hypochlorite used and the contact time of hypochlorite with root canal walls deals with its effectiveness in reduction of microbes (14), especially E. Faecalis, which is known to be a resistant species responsible for the reinfections(15). But, the sad truth is that the choice of irrigant concentration and usage cannot be standardized in the clinical scenario as it is possible in an experimental scenario. So, in a clinical scenario, various studies compiled and showed evidence of developing a resistant E. Faecalis in failed primary root canal treatments(16),(17). Hypochlorite at lower concentrations might lead to the development of resistant strains (14).

Author ρ: e-mail: ramchinna05@gmail.com
So, this was the primary concern for the authors of the present study. We state that “Herbal products although might not replace primary root canal irrigant, but might be an adjunctive therapeutic option and a lot of research and therapeutic potential, still lack and to be translated in a clinical scenario”. So, our research majorly concentrates on assessing various plant extracts. Our previous research has focused on assessing the Ethanolic Fruit Extract of Morinda and proved to be beneficial against E. Faecalis(9). So, the present study formulated was to assess a step ahead to evaluate the beneficial antimicrobial effect of Ethanolic Leaf Extract of Morinda Citrifolia. The null hypothesis stated was, there was no significant antibacterial effect of ethanolic leaf extract of Morinda Citrifolia against E. Faecalis on using agar well diffusion and broth dilution methods.

II. MATERIALS AND METHODS

The study was approved by the Institutional Ethical Committee. Before the start of the research, the sun-dried powder of Morinda Citrifolia was collected. The extract preparation was similar to our previous study(9), where we used the dried fruit and seeds powder against E.Faecalis. In this present study, we concentrated mainly on the effect of dried leaf powder. The dried leaf powder was initially subjected to 1000ml of ethanol using Soxhlet extractor for 72, not exceeding the boiling point of the solvent. After which, the extract was filtered and vacuum dried at 45 degree Celsius. The obtained extract was refrigerated until use.

The strains used for testing were gram-positive E. Faecalis bacterial strains ATCC 29212. The bacterial strains were cultured in Luria broth agar (Himedia, Mumbai) and incubated at 37-degree celsius for 24 hours and maintained on nutrient agar slants at 4-degree celsius. The sterile spreader was used for the inoculation of these organisms across the media. The microorganisms were inoculated into molten Luria broth agar and poured into Petri dishes and solidified. Wells of uniform diameter were then prepared on the solidified agar. The discs were then impregnated with experimental test solution at different concentrations of 40, 60, 80, and 100µl, respectively. 10mg/ml of tetracycline was used as a positive control, and the solvent without plant extract was used as a negative control. Plates were incubated for 24 hours at 37-degree celsius, and the development of the inhibitory zone around the wells was measured in diameter and recorded.

MBC value was determined by subculturing the test dilution on a freshly prepared nutrient agar media. The plates were incubated further for 18-42 hours at 37-degree celsius. The highest dilution yielded no visible turbidity on nutrient agar and taken as MBC.

Determination of Minimum Inhibitory Concentration:

The MIC of the test solutions was determined using broth dilution methods using CLSI 2012 standard protocol (18).

The cultures were then incubated and subsequently, serially diluted to reach a density of 2×10^4 cells per ml. Cell counting was done using a hemocytometer. Luria broth (Himedia, Mumbai) was prepared and sterilized at 121-degree celsius, 15lbs for 15 minutes.

Two milliliters of Luria broth was dispensed into the tubes, and 100µL of cell culture was inoculated in it. Different concentrations of ethanolic leaf extracts of 7.5, 15.625, 31.25, 62.5, 125, 250, 500, and 1000µg respectively were added into the tubes. Positive and negative controls were similar to agar well diffusion.

Growth control was run parallel with every experiment. All the experimental tubes were incubated for 48 hours in anaerobic jars. After completion of the incubation period, the optical density was measured at 600nm. Each experiment was carried out in a triplicate set. The lowest possible concentration before the color change was considered as MIC. The percentage of bacterial inhibition was computed by an equation, as mentioned in our previous study(9).

III. STATISTICAL ANALYSIS

Data were analyzed using SPSS version 11. Multiple comparisons were made using one way ANOVA followed by LSD test for post hoc analysis. Statistical significance was considered for p<0.05.

IV. RESULTS

Table 1 depicts the MBC, and Table 2 illustrates the MIC values. Each value represented as a mean ± standard deviation. When results were compared, the statistically significant difference was noted as compared to the negative control.

V. DISCUSSION

From the results of the present study, it can be concluded that the ethanolic extract of morinda citrifolia leaf also seemed to have an efficient antibacterial activity on assessing using agar well diffusion and broth dilution methods. None of the previous literature on this specific aspect can be compared, as much of the literature is concentrated on the pathogenic medical microorganisms. When the results of the present study were compared with our previous study results, they showed inferior values on both MIC and MBC evaluation. So, it can be assessed that fruit extract seemed to have better antimicrobial properties as compared to leaf extract on ethanolic extraction.
Previous literature has reported the potent antimicrobial activity in the organic solvent as compared to water as an extracting compound, which indicated that active compounds resulting in antibacterial activity are more soluble in organic solvents (19). When the antimicrobial property of the herbal or natural plant products is evaluated critically, the enhanced effect cannot be attributed to a single compound or agent. It's a combined action of various bioactive compounds such as alkaloids, flavonoids, and other significant compounds promoting its activity (20).

Previous literature on morinda citrifolia has shown enormous evidence based data on its antimicrobial, antifungal, antioxidant, and anticancer properties (21), (22), (23). As discussed previously, the combined action of bioactive compounds in the plant extract of morinda citrifolia, which led to its enhanced antibacterial activity. The null hypothesis of the present study rejected, and results proved that leaf extract of morinda citrifolia possessed antimicrobial property on assessment.

When a limitation of the present study is considered, it's a first invitro study simulated to assess a single pathogen. But, when root canal infections are considered, it’s usually polymicrobial inhabitants (24). So, the effectiveness of the agent tested by this in-vitro study is not possible. So, better future studies should concentrate on combined agents on multiple endodontic pathogens to prove their effectiveness. In our perspective, there is still a long way for the natural plant extracts to be considered effective in clinical endodontics.

VI. Conclusion

Ethanolic leaf extract of morinda citrifolia seemed to possess antimicrobial properties against E. Faecalis and can be considered as an antimicrobial agent to treat root canal infections.

References Références Referencias


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1. Authors must go through the complete author guideline and understand and agree to Global Journals' ethics and code of conduct, along with author responsibilities.
2. Authors must accept the privacy policy, terms, and conditions of Global Journals.
3. Ensure corresponding author’s email address and postal address are accurate and reachable.
4. Manuscript to be submitted must include keywords, an abstract, a paper title, co-author(s’) names and details (email address, name, phone number, and institution), figures and illustrations in vector format including appropriate captions, tables, including titles and footnotes, a conclusion, results, acknowledgments and references.
5. Authors should submit paper in a ZIP archive if any supplementary files are required along with the paper.
6. Proper permissions must be acquired for the use of any copyrighted material.
7. Manuscript submitted must not have been submitted or published elsewhere and all authors must be aware of the submission.

Declaration of Conflicts of Interest

It is required for authors to declare all financial, institutional, and personal relationships with other individuals and organizations that could influence (bias) their research.

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Plagiarized content will not be considered for publication. We reserve the right to inform authors’ institutions about plagiarism detected either before or after publication. If plagiarism is identified, we will follow COPE guidelines:

Authors are solely responsible for all the plagiarism that is found. The author must not fabricate, falsify or plagiarize existing research data. The following, if copied, will be considered plagiarism:

- Words (language)
- Ideas
- Findings
- Writings
- Diagrams
- Graphs
- Illustrations
- Lectures

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1. Substantial contributions to the conception and acquisition of data, analysis, and interpretation of findings.
2. Drafting the paper and revising it critically regarding important academic content.
3. Final approval of the version of the paper to be published.

Changes in Authorship

The corresponding author should mention the name and complete details of all co-authors during submission and in manuscript. We support addition, rearrangement, manipulation, and deletions in authors list till the early view publication of the journal. We expect that corresponding author will notify all co-authors of submission. We follow COPE guidelines for changes in authorship.

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Unless specified in the notification, the Editorial Board’s decision on publication of the paper is final and cannot be appealed before making the major change in the manuscript.

Acknowledgments

Contributors to the research other than authors credited should be mentioned in Acknowledgments. The source of funding for the research can be included. Suppliers of resources may be mentioned along with their addresses.

Declaration of funding sources

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Preparing your Manuscript

Authors can submit papers and articles in an acceptable file format: MS Word (doc, docx), LaTeX (.tex, .zip or .rar including all of your files), Adobe PDF (.pdf), rich text format (.rtf), simple text document (.txt), Open Document Text (.odt), and Apple Pages (.pages). Our professional layout editors will format the entire paper according to our official guidelines. This is one of the highlights of publishing with Global Journals—authors should not be concerned about the formatting of their paper. Global Journals accepts articles and manuscripts in every major language, be it Spanish, Chinese, Japanese, Portuguese, Russian, French, German, Dutch, Italian, Greek, or any other national language, but the title, subtitle, and abstract should be in English. This will facilitate indexing and the pre-peer review process.

The following is the official style and template developed for publication of a research paper. Authors are not required to follow this style during the submission of the paper. It is just for reference purposes.
**Manuscript Style Instruction (Optional)**

- Microsoft Word Document Setting Instructions.
- Font type of all text should be Swiss721 Lt BT.
- Page size: 8.27” x 11””, left margin: 0.65, right margin: 0.65, bottom margin: 0.75.
- Paper title should be in one column of font size 24.
- Author name in font size of 11 in one column.
- Abstract: font size 9 with the word “Abstract” in bold italics.
- Main text: font size 10 with two justified columns.
- Two columns with equal column width of 3.38 and spacing of 0.2.
- First character must be three lines drop-capped.
- The paragraph before spacing of 1 pt and after of 0 pt.
- Line spacing of 1 pt.
- Large images must be in one column.
- The names of first main headings (Heading 1) must be in Roman font, capital letters, and font size of 10.
- The names of second main headings (Heading 2) must not include numbers and must be in italics with a font size of 10.

**Structure and Format of Manuscript**

The recommended size of an original research paper is under 15,000 words and review papers under 7,000 words. Research articles should be less than 10,000 words. Research papers are usually longer than review papers. Review papers are reports of significant research (typically less than 7,000 words, including tables, figures, and references)

A research paper must include:

a) A title which should be relevant to the theme of the paper.
b) A summary, known as an abstract (less than 150 words), containing the major results and conclusions.
c) Up to 10 keywords that precisely identify the paper’s subject, purpose, and focus.
d) An introduction, giving fundamental background objectives.
e) Resources and techniques with sufficient complete experimental details (wherever possible by reference) to permit repetition, sources of information must be given, and numerical methods must be specified by reference.
f) Results which should be presented concisely by well-designed tables and figures.
g) Suitable statistical data should also be given.
h) All data must have been gathered with attention to numerical detail in the planning stage.

Design has been recognized to be essential to experiments for a considerable time, and the editor has decided that any paper that appears not to have adequate numerical treatments of the data will be returned unrefereed.

i) Discussion should cover implications and consequences and not just recapitulate the results; conclusions should also be summarized.
j) There should be brief acknowledgments.
k) There ought to be references in the conventional format. Global Journals recommends APA format.

Authors should carefully consider the preparation of papers to ensure that they communicate effectively. Papers are much more likely to be accepted if they are carefully designed and laid out, contain few or no errors, are summarizing, and follow instructions. They will also be published with much fewer delays than those that require much technical and editorial correction.

The Editorial Board reserves the right to make literary corrections and suggestions to improve brevity.
**Format Structure**

It is necessary that authors take care in submitting a manuscript that is written in simple language and adheres to published guidelines.

All manuscripts submitted to Global Journals should include:

**Title**

The title page must carry an informative title that reflects the content, a running title (less than 45 characters together with spaces), names of the authors and co-authors, and the place(s) where the work was carried out.

**Author details**

The full postal address of any related author(s) must be specified.

**Abstract**

The abstract is the foundation of the research paper. It should be clear and concise and must contain the objective of the paper and inferences drawn. It is advised to not include big mathematical equations or complicated jargon.

Many researchers searching for information online will use search engines such as Google, Yahoo or others. By optimizing your paper for search engines, you will amplify the chance of someone finding it. In turn, this will make it more likely to be viewed and cited in further works. Global Journals has compiled these guidelines to facilitate you to maximize the web-friendliness of the most public part of your paper.

**Keywords**

A major lynchpin of research work for the writing of research papers is the keyword search, which one will employ to find both library and internet resources. Up to eleven keywords or very brief phrases have to be given to help data retrieval, mining, and indexing.

One must be persistent and creative in using keywords. An effective keyword search requires a strategy: planning of a list of possible keywords and phrases to try.

Choice of the main keywords is the first tool of writing a research paper. Research paper writing is an art. Keyword search should be as strategic as possible.

One should start brainstorming lists of potential keywords before even beginning searching. Think about the most important concepts related to research work. Ask, “What words would a source have to include to be truly valuable in a research paper?” Then consider synonyms for the important words.

It may take the discovery of only one important paper to steer in the right keyword direction because, in most databases, the keywords under which a research paper is abstracted are listed with the paper.

**Numerical Methods**

Numerical methods used should be transparent and, where appropriate, supported by references.

**Abbreviations**

Authors must list all the abbreviations used in the paper at the end of the paper or in a separate table before using them.

**Formulas and equations**

Authors are advised to submit any mathematical equation using either MathJax, KaTeX, or LaTeX, or in a very high-quality image.

**Tables, Figures, and Figure Legends**

Tables: Tables should be cautiously designed, uncrowned, and include only essential data. Each must have an Arabic number, e.g., Table 4, a self-explanatory caption, and be on a separate sheet. Authors must submit tables in an editable format and not as images. References to these tables (if any) must be mentioned accurately.
Figures

Figures are supposed to be submitted as separate files. Always include a citation in the text for each figure using Arabic numbers, e.g., Fig. 4. Artwork must be submitted online in vector electronic form or by emailing it.

Preparation of Electronic Figures for Publication

Although low-quality images are sufficient for review purposes, print publication requires high-quality images to prevent the final product being blurred or fuzzy. Submit (possibly by e-mail) EPS (line art) or TIFF (halftone/photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Avoid using pixel-oriented software. Scans (TIFF only) should have a resolution of at least 350 dpi (halftone) or 700 to 1100 dpi (line drawings). Please give the data for figures in black and white or submit a Color Work Agreement form. EPS files must be saved with fonts embedded (and with a TIFF preview, if possible).

For scanned images, the scanning resolution at final image size ought to be as follows to ensure good reproduction: line art: >650 dpi; halftones (including gel photographs): >350 dpi; figures containing both halftone and line images: >650 dpi.

Color charges: Authors are advised to pay the full cost for the reproduction of their color artwork. Hence, please note that if there is color artwork in your manuscript when it is accepted for publication, we would require you to complete and return a Color Work Agreement form before your paper can be published. Also, you can email your editor to remove the color fee after acceptance of the paper.

Tips for Writing a Good Quality Medical Research Paper

1. Choosing the topic: In most cases, the topic is selected by the interests of the author, but it can also be suggested by the guides. You can have several topics, and then judge which you are most comfortable with. This may be done by asking several questions of yourself, like "Will I be able to carry out a search in this area? Will I find all necessary resources to accomplish the search? Will I be able to find all information in this field area?" If the answer to this type of question is "yes," then you ought to choose that topic. In most cases, you may have to conduct surveys and visit several places. Also, you might have to do a lot of work to find all the rises and falls of the various data on that subject. Sometimes, detailed information plays a vital role, instead of short information. Evaluators are human: The first thing to remember is that evaluators are also human beings. They are not only meant for rejecting a paper. They are here to evaluate your paper. So present your best aspect.

2. Think like evaluators: If you are in confusion or getting demotivated because your paper may not be accepted by the evaluators, then think, and try to evaluate your paper like an evaluator. Try to understand what an evaluator wants in your research paper, and you will automatically have your answer. Make blueprints of paper: The outline is the plan or framework that will help you to arrange your thoughts. It will make your paper logical. But remember that all points of your outline must be related to the topic you have chosen.

3. Ask your guides: If you are having any difficulty with your research, then do not hesitate to share your difficulty with your guide (if you have one). They will surely help you out and resolve your doubts. If you can't clarify what exactly you require for your work, then ask your supervisor to help you with an alternative. He or she might also provide you with a list of essential readings.

4. Use of computer is recommended: As you are doing research in the field of medical research then this point is quite obvious. Use right software: Always use good quality software packages. If you are not capable of judging good software, then you can lose the quality of your paper unknowingly. There are various programs available to help you which you can get through the internet.

5. Use the internet for help: An excellent start for your paper is using Google. It is a wondrous search engine, where you can have your doubts resolved. You may also read some answers for the frequent question of how to write your research paper or find a model research paper. You can download books from the internet. If you have all the required books, place importance on reading, selecting, and analyzing the specified information. Then sketch out your research paper. Use big pictures: You may use encyclopedias like Wikipedia to get pictures with the best resolution. At Global Journals, you should strictly follow here.
6. Bookmarks are useful: When you read any book or magazine, you generally use bookmarks, right? It is a good habit which helps to not lose your continuity. You should always use bookmarks while searching on the internet also, which will make your search easier.

7. Revise what you wrote: When you write anything, always read it, summarize it, and then finalize it.

8. Make every effort: Make every effort to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in the introduction—what is the need for a particular research paper. Polish your work with good writing skills and always give an evaluator what he wants. Make backups: When you are going to do any important thing like making a research paper, you should always have backup copies of it either on your computer or on paper. This protects you from losing any portion of your important data.

9. Produce good diagrams of your own: Always try to include good charts or diagrams in your paper to improve quality. Using several unnecessary diagrams will degrade the quality of your paper by creating a hodgepodge. So always try to include diagrams which were made by you to improve the readability of your paper. Use of direct quotes: When you do research relevant to literature, history, or current affairs, then use of quotes becomes essential, but if the study is relevant to science, use of quotes is not preferable.

10. Use proper verb tense: Use proper verb tenses in your paper. Use past tense to present those events that have happened. Use present tense to indicate events that are going on. Use future tense to indicate events that will happen in the future. Use of wrong tenses will confuse the evaluator. Avoid sentences that are incomplete.

11. Pick a good study spot: Always try to pick a spot for your research which is quiet. Not every spot is good for studying.

12. Know what you know: Always try to know what you know by making objectives, otherwise you will be confused and unable to achieve your target.

13. Use good grammar: Always use good grammar and words that will have a positive impact on the evaluator; use of good vocabulary does not mean using tough words which the evaluator has to find in a dictionary. Do not fragment sentences. Eliminate one-word sentences. Do not ever use a big word when a smaller one would suffice.

Verbs have to be in agreement with their subjects. In a research paper, do not start sentences with conjunctions or finish them with prepositions. When writing formally, it is advisable to never split an infinitive because someone will (wrongly) complain. Avoid clichés like a disease. Always shun irritating alliteration. Use language which is simple and straightforward. Put together a neat summary.

14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.
20. **Think technically:** Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. **Adding unnecessary information:** Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. **Report concluded results:** Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. **Upon conclusion:** Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

**INFORMAL GUIDELINES OF RESEARCH PAPER WRITING**

**Key points to remember:**

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

**Final points:**

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

*The introduction:* This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

*The discussion section:*

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

**General style:**

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

**To make a paper clear:** Adhere to recommended page limits.
**Mistakes to avoid:**

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

**Title page:**

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

**Abstract:** This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

**Reason for writing the article**—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

**Approach:**

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

**Introduction:**

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

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XVII
The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

*Materials may be reported in part of a section or else they may be recognized along with your measures.*

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.
Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."
Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

**Approach:**

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

**The Administration Rules**

Administration Rules to Be Strictly Followed before Submitting Your Research Paper to Global Journals Inc.

*Please read the following rules and regulations carefully before submitting your research paper to Global Journals Inc. to avoid rejection.*

**Segment draft and final research paper:** You have to strictly follow the template of a research paper, failing which your paper may get rejected. You are expected to write each part of the paper wholly on your own. The peer reviewers need to identify your own perspective of the concepts in your own terms. Please do not extract straight from any other source, and do not rephrase someone else's analysis. Do not allow anyone else to proofread your manuscript.

**Written material:** You may discuss this with your guides and key sources. Do not copy anyone else's paper, even if this is only imitation, otherwise it will be rejected on the grounds of plagiarism, which is illegal. Various methods to avoid plagiarism are strictly applied by us to every paper, and, if found guilty, you may be blacklisted, which could affect your career adversely. To guard yourself and others from possible illegal use, please do not permit anyone to use or even read your paper and file.
Please note that following table is only a Grading of "Paper Compilation" and not on "Performed/Stated Research" whose grading solely depends on Individual Assigned Peer Reviewer and Editorial Board Member. These can be available only on request and after decision of Paper. This report will be the property of Global Journals.

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<td>Unclear summary and no specific data, Incorrect form Above 200 words</td>
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<td>No specific data with ambiguous information Above 250 words</td>
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<td>Introduction</td>
<td>Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited</td>
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<td>Methods and Procedures</td>
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<td>Result</td>
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<tr>
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<td>Complete and embarrassed text, difficult to comprehend</td>
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<td>Irregular format with wrong facts and figures</td>
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<td>Discussion</td>
<td>Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited</td>
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<td>Conclusion is not cited, unorganized, difficult to comprehend</td>
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