Colchicine as a Natural Toxicant
Stability-Indicating RP-HPLC Method

Highlights

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Colchicine as a Natural Toxicant and Methods of its Analysis

By Gorainova Daria Anatolievna

Abstract- Colchicine is an indispensable medical alkaloid of the Colchicaceae family. It is still used in the treatment of gout, recurrent illness, Behcet's disease and many other diseases. Despite the centuries-old experience of using this alkaloid by humans, fatal cases of poisoning are still encountered. Materials and methods. The authors analyzed specialized literature in databases and search engines Google Scholar, PubMed, Web of Science, Scopus, eLibrary on the toxicological, pharmacological, technological and clinical significance of colchicine. Results. Pharmacodynamics, clinical signs and pathological changes in case of colchicine poisoning are described based on the data of contemporary literature. In addition, the basic principles of the qualitative and quantitative determination of substances from biological material are illustrated.

Keywords: colchicine; Colchicum autumnale; toxicology.

GJMR-B Classification: NLMC Code: QV 600
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I. Introduction

The aim of our study is to determine the value of colchicine as a toxicant poison, consider its pathophysiological effects on the body, based on clinical cases, qualitative and quantitative determination and isolation from biologic material.

Relevance: Colchicine - is an alkaloid that is isolated from plants of the Colchicaceae - Colchicum autumnale and Gloriosasuperba. Colchicine has been used in medicine for the treatment of gout for more than 2000 years [1]. Colchicine is also used in the treatment of a wide range of diseases, in particular for the prevention of the development of amyloidosis in patients with Mediterranean familial fever (FMF) [2].

This herbal preparation is of interest from the point of clinical toxicology, because along with its unique healing properties, it has high toxicity, expressed in violation of almost all organs and systems of the body.

In addition, colchicine has a rather narrow therapeutic window, which can be difficult to determine for a particular patient [3, 4]. Accidental colchicine poisoning is periodically observed when C. autumnale or G. Superba are mistaken for Alliumursínnum and Ipomoeábatátas, respectively [5-8].

II. Materials and Methods

The toxicological, pharmacological, technological and clinical significance of colchicine was analyzed in specialized literature using electronic data-bases and search engines Google Scholar, PubMed, Web of Science, Scopus, eLibrary etc.

III. Results and Discussion

Colchicine is used for treatment of:
• acute gout and for the prevention of exacerbations, especially during the first few months of treatment with allopurinol or agents that promote the excretion of uric acid [10];
• recurrent illness (familial Mediterranean fever) [2, 11];
• primary AL amyloidosis;
• Behçet's syndrome;
• idiopathic thrombocytopenic purpura;
• pericarditis;
• primary biliary cirrhosis;
• gangrenous pyoderma, etc. [12]

There is no clear boundary between therapeutic, toxic and lethal doses of colchicine in both children and adults, but it is believed that the drug is relatively safe at a dose of up to 0.015 mg / kg, toxic - over 0.1 mg / kg, and can cause death in dose over 0.8 mg / kg. In addition, there need to be said that toxic effect with colchicine appears earlier than therapeutic [13].

The fatal cases of colchicine poisoning by the enteral route in a dose of 7 to 26 mg are described [14-16]. However, in 1966 a clinical case of 350 mg of orally taken colchicine was described and the patient survived [17]; although some authors doubt the accuracy of calculating the claimed dose [18].

The therapeutic use of colchicine in the treatment of recurrent illness according to current clinical guidelines is considered safe [19]; however, adverse reactions from the gastrointestinal system are often encountered in the treatment of acute attacks of gout, even taking into account clinical recommendations: in 80% of patients, before the onset of clinical improvement or at the same time, there are undesirable reactions from the gastrointestinal tract [20]. In addition, colchicine has a rather narrow therapeutic window, which can be difficult to determine for a particular patient [3, 4]. Accidental colchicine poisoning is periodically observed when C. autumnale or G. Superba are mistaken for Alliumursínnum and Ipomoeábatátas, respectively [5-8].
When analyzing cases of poisoning by plants, for example, Colchicum autumn, it should be taken into account that in addition to colchicine (Fig. 1) (C22H25NO6), the plant also contains colchamine (Fig. 2) (C21H25NO5). The amount of alkaloids in the seeds can reach 1% or more; less in tubers and flowers and least in leaves. In addition, colchicum contain a certain amount of saponin substances [2].

Colchamine blocks mitosis at the metaphase stage due to antimycotic action. Colchamine reduces blood pressure, inhibits lymphopoiesis and leukopoiesis, causes diarrhea and lowers the pain threshold. Accumulates in tissues. It is much less toxic than colchicine and is used in the treatment of malignant neoplasms. With exophytic and endophytic forms of skin cancer, colchamin ointment is used. It causes the decay of the tumor, but it should be applied carefully, avoiding contact with the mucous membranes. With inoperable cancer of the upper third of the stomach or esophagus, colchamine tablets with sarcolysin are prescribed.

Formulae of the active substances:

**Pharmacokinetics:** The maximum concentration of colchicine in blood plasma occurs approximately 2 hours after ingestion. Bioavailability is less than 50% (approximately 45%) [26]. It is believed that absorption of colchicine from the gastrointestinal tract is limited by the action of P-glycoprotein. Absorbed colchicine is found in high concentrations in the kidneys, liver and spleen. It undergoes oxidative demethylation in the liver with the participation of the cytochrome P450 isoenzyme CYP3A4. 2 main metabolites (metabolites of the 1st phase of biotransformation) are formed and 1 is a secondary (metabolite of the 2nd phase of biotransformation); the concentration of metabolites in plasma is negligible. Excretion of colchicine and its metabolites occurs through hepatobiliary secretion into the intestine, then with feces. Such a mechanism of excretion determines the effectiveness of the use of adsorbents even a day after poisoning [4]. With preserved renal function, their excretion of colchicine is only 10–20% of the total. The mean half-life is approximately 28 hours [27]. Enterohepatic circulation is detected 4-6 hours after oral administration. Most of the administered dose is excreted through the intestines.

a) **Interaction of colchicine with other drugs**

Colchicine is a substrate for P-glycoprotein and cytochrome P450 isoenzyme CYP3A4. If treatment with a P-glycoprotein inhibitor or CYP3A4 inhibitor is really necessary, then the dosage of colchicine needs to be adjusted if the patient's kidneys and liver function well, otherwise such combinations should be avoided [28]. Cases of myopathy and rhabdomyolysis have been reported in those who took statins, fibrates, cyclosporin (Ciclosporin) or digoxin (Digoxin) together with colchicine.

Thiazide diuretics can increase serum uric acid levels and interfere with colchicine activity.

b) **Toxicological significance**

Colchicine is a capillary poison; it causes severe circulatory disturbance, in particular severe hyperemia of the mucous membrane of the stomach and intestines, causing an increase in excitability and violent peristaltic movements of the intestine (cholera-like diarrhea). Colchicine also affects the kidneys, causing polyuria, albuminuria, hematuria, even anuria; paralyzes the central nervous system, causing death from respiratory paralysis. External clinical manifestations in case of colchicine poisoning do not occur immediately, but after a few hours, since they are apparently the result of the action of products of the conversion of colchicine in the body of a mammal (into dioxicolchicine) [20].

Colchicine binds to the intracellular protein tubulin, preventing the formation of microtubules, which inhibits normal mitotic cell division. In addition, colchicine slows down endo- and exocytosis, biomodification of proteins in the Golgi apparatus, stabilizes lysosome membranes, and generally changes the cell geometry [9]. The mechanism of action of the drug is associated with a decrease in the migration of leukocytes to the area of inflammation and the suppression of phagocytosis of microcrystals of uric acid salts. In addition, it blocks cell division in the anaphase and metaphase stages, prevents the degranulation of neutrophils and the development of amyloidosis, as it reduces the formation of amyloid fibrils. These mechanisms explain both the therapeutic use of colchicine and its toxicological effect. The active substance quickly stops an acute attack of gout.
effect of it is observed in the first 12 hours after the start of treatment in 75% of patients.

Since colchicine blocks mitosis, in case of poisoning with this substance, the greatest disorders develop in tissues with high mitotic activity: in the bone marrow, gastrointestinal tract, and hair follicles [21, 22].

In many patients with impaired renal function, the usual doses of colchicine can cause colchicine myoneuropathy, which often remains unrecognized. Skeletal muscle damage usually occurs to a greater extent than damage to peripheral nerve fibers, and is expressed in proximal muscle weakness and increased serum creatine kinase. The abolition of colchicine contributes to the remission of these symptoms within a few weeks, and the resolution of the neuropathic component is slower. Examination of the proximal muscles reveals a noticeable pathological spontaneous activity, but at first the condition itself is usually mistakenly diagnosed as polymyositis or uremic myopathy. An analysis of the sources confirms that impaired renal function is the primary risk factor for the development of colchicine myoneuropathy, and adjusting the dosage of colchicine is recommended as a priority measure [23, 24, 25].

There is evidence that colchicine myoneuropathy can occur in people with normal kidney function.

The occurrence of rhabdomyolysis was also described. Potentially fatal effects include neutropenia, thrombocytopenia, pancytopenia, acute renal failure, and congestive heart failure [7].

Clinical cases: Scientists from "Research Institute of Ambulance them. N.V. Sklifosovsky" have reported about 2 cases of colchicine poisoning. Patient M. (Pt 1), 48 years old was delivered 12 hours after eating several C. autumnalebulbs. Patient P. (Pt 2), 78 years old was admitted on the third day also after the erroneous eating of several C. autumnale bulbs. Both victims ate fried bulbs of the plant, confused with A. sativum. After 4-6 hours, both patients developed nausea, repeated vomiting, and loose stools. Upon admission, hemodynamic, respiratory, and blood counts (hemoglobin, red blood cells, formula) were within normal limits, and consciousness was not impaired. The patients’ condition progressively worsened due to inadequate breathing, which required a transfer to mechanical ventilation, as well as associated acute heart failure, which was regarded as cardiogenic shock. Treatment of Pt 1 included gastric lavage with the introduction of activated charcoal, forced diuresis, maintaining water-electrolyte balance, and mechanical ventilation. Pt 2 was performed hemodiafiltration for detoxification. The death of P-ta 1 occurred on the 5th, P-t 2 - on the 6th day from the moment of poisoning [29].

Clinical picture: The first signs of poisoning appear after 6-24 and even 48 hours. The first symptoms of the toxic effects of colchicine are usually: loose bowels, nausea and vomiting, abdominal pain. Their appearance should be a signal for the abolition of colchicine or a decrease in its dose. If this is not done or, conversely, the dose is increased, then diarrhea becomes profuse, gastrointestinal bleeding may occur, a rash may appear on the skin, and renal and liver failure develops.

The appearance of a reaction similar to toxic epidermal necrosis is described; within 10 days, alopecia, leukocytosis (recurrent type), stomatitis may appear. Death with acute overdose of colchicine can occur due to respiratory depression, cardiovascular collapse, sepsis (with the development of pancytopenia) [8].

Pathological changes: Autopsy data correspond to the clinical manifestations of poisoning. The mucous membrane of the stomach and intestines is severely inflamed and has a large number of hemorrhages. In severe cases, these changes can be hemorrhagic, especially in the large intestine, the mucosa of which is very swollen, vitreous, with a mass of small or continuous hemorrhages.

Intestinal contents are often bloody stained. Hemorrhages are present on the mucous and serous membranes of other organs. The kidneys and brain are strongly hyperemic and also have hemorrhages. The heart muscle is altered.

c) Chemo-toxicological analysis of colchicine

In modern chemo-toxicological analysis, liquid-liquid extraction is one of the main methods for the isolation of alkaloids and other toxic substances from biological material of a corpse, biological fluids (urine, blood), gastric lavage, and a number of other objects.

For each alkaloid, there is a pH range at which it is extracted with maximum amounts of water-immiscible organic solvents. The maximum degree of colchicine extraction is achieved in an acidic environment [24]. As an extractant, it is advisable to use ethyl alcohol, chloroform, dichloroethane, diethyl ether.

Qualitative, quantitative analyses and chromatography are well described in special literature. As methods, high performance liquid chromatography is applicable. As detectors can be used: spectrometric, UV detector (a system of conjugated double bonds in a colchicine molecule) or a diode matrix. Chromatograph a solution of CO colchicine and CO colchamine, obtaining at least 3 chromatograms. The test solution and the colchicine CO solution are alternately chromatographed, obtaining at least 3 chromatograms. The calculation of the amount of alkaloids is carried out by the external standard method. [30, 31, 32].
IV. Conclusion

At excessive doses, colchicine can cause serious systemic toxicity. Acute colchicine poisoning is uncommon, but is associated with a high mortality rate. It is essential, therefore, that clinicians recognize and are familiar with colchicine poisoning symptoms, first aid and the basics of pathological changes and chemotoxicological analysis.

Acknowledgements

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References Références Referencias


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 x 4.6mm, 5µ) column maintained at 30ºC 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.

GJMR-B Classification: NLMC Code: QV 55

Strictly as per the compliance and regulations of:
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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-dioxo3,4-dihydropyrimidin1(2H)yl)4fluoro3hydroxy4methyltetrahydrofuran2yl)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-{2-[(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-oxobutane-2-yl} carbamate.

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 x 4.6mm, 5u) 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. LOD = 3.3×σ/S, LOQ =10×σ/S. Here σ 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**

![Optimized chromatogram of Sofosbuvir, Velpatasvir, and Voxilaprevir](https://via.placeholder.com/150)

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

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>USP Plate count</strong></td>
<td>Sofosbuvir</td>
</tr>
<tr>
<td></td>
<td>4088</td>
</tr>
<tr>
<td><strong>USP Tailing factor</strong></td>
<td>1.0</td>
</tr>
<tr>
<td><strong>USP Resolution</strong></td>
<td>4.5</td>
</tr>
<tr>
<td><strong>Precision (%RSD)</strong></td>
<td>0.9</td>
</tr>
<tr>
<td><strong>Accuracy</strong></td>
<td>98.50-101.50</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>Specific, No interference</td>
</tr>
<tr>
<td><strong>Linearity range (µg/ml)</strong></td>
<td>10-60</td>
</tr>
<tr>
<td><strong>Correlation coefficient, r²</strong></td>
<td>0.999</td>
</tr>
<tr>
<td><strong>LOD (µg/ml)</strong></td>
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</tr>
<tr>
<td><strong>LOQ (µg/ml)</strong></td>
<td>0.34</td>
</tr>
<tr>
<td><strong>Ruggedness (%RSD)</strong></td>
<td>Day1: 0.9</td>
</tr>
<tr>
<td></td>
<td>Day2: 0.9</td>
</tr>
<tr>
<td><strong>Robustness (%RSD)</strong></td>
<td>Flow rate: 1.2</td>
</tr>
</tbody>
</table>
Flow rate + 3.4 3.7 3.7
Column temperature – 2.8 2.7 2.9
Column temperature + 2.2 2.4 2.2
Mobile phase – 3.8 3.4 3.2
Mobile phase + 2.4 2.7 2.4
Solution stability (%RSD)
(0 hrs) 1.0 0.6 1.0
(24 hrs) 0.9 1.0 0.6

Table 2: Forced degradation studies result

<table>
<thead>
<tr>
<th>Stress condition</th>
<th>Sofosbuvir</th>
<th>Velpatasvir</th>
<th>Voxilaprevir</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Assay</td>
<td>%D</td>
<td>%Assay</td>
<td>%D</td>
</tr>
<tr>
<td>Acid</td>
<td>94.23</td>
<td>5.77</td>
<td>94.04</td>
</tr>
<tr>
<td>Base</td>
<td>95.35</td>
<td>4.65</td>
<td>95.10</td>
</tr>
<tr>
<td>Neutral</td>
<td>99.37</td>
<td>0.63</td>
<td>99.51</td>
</tr>
<tr>
<td>Peroxide</td>
<td>95.95</td>
<td>4.05</td>
<td>95.58</td>
</tr>
<tr>
<td>Photolytic</td>
<td>97.73</td>
<td>2.27</td>
<td>97.58</td>
</tr>
<tr>
<td>Thermal</td>
<td>98.22</td>
<td>1.75</td>
<td>96.43</td>
</tr>
</tbody>
</table>

%D- Percentage Degradation

Figure 5: Blank chromatogram

Figure 6: Standard chromatogram
Figure 7: Sample chromatogram

Figure 8: Placebo chromatogram

Figure 9: Linearity plot of Sofosbuvir

Figure 10: Linearity plot of Velpatasvir
**Figure 11:** Linearity plot of Voxelaprevir

![Linearity plot of Voxelaprevir](image)

\[ y = 25052x + 1805. \]

\[ R^2 = 0.999 \]

**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 11 respectively 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].

GJMR-B Classification: NLMC Code: QW 4

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

Marcel Nogueira

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 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 dipryrone 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 (BRCP) [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 hepatitis 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.

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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 after 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 α=5% and observed effect size (OR), sample size and two-tailed regression model R².

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 amiodipine, diltiazem and verapamil (n=85, 29.7%), followed by levothyroxine (n=59, 20.6%) and statins (n =46, 16.0%) (Table 2). The clinical pharmacists performed one hundred thirty-four interventions, such as ordering additional 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 levetiracetam 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 amiodipine (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 AI444040assessed the effectiveness of SOF+DCV with or without RBV [21]. Both presented higher SVR rates.
Ottman’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 common 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.

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 Ottman assess SVR, the author identified and quantified a total of 554 DDIs in 300 patients on DAAs therapy [17].
Conflicts of interest: All authors have no conflict of interest to declare.

Financial Support: None to declare.

References Références Referencias


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.

GJMR-B Classification: NLMC Code: QV 55
Cellular Trafficking of Nanocarriers in Alveolar Macrophages for Effective Management of Pulmonary Tuberculosis

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-octadecyl-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-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 drug release with preserved antimicrobial activity. 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) (Emst 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-swelling 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 favorable 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, maninitol, 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 35 ml 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 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 probe sonication (VCX500, Sonics and materials, U.S.A.) at 20% amplitude for 10 minutes and cooled to room temperature. 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 1 h 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.

Percent entrapment efficiency $= (WL - WF) \times 100 ÷ WL.......... (1)$

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 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 3 mg of RIF (369.23 mg) 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 dry 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, 3 mg 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, 5 ml 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 the 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 (AGC, 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 (Lupiharler®). 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 100 μl 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, stearic amine 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 stearic amine, 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), Emitted 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 streaic 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 in-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. 1:** FTIR spectrum of D-mannose, strearylamine and NODM

**Fig. 2:** Proton NMR spectrum of synthesized NODM
**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 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 TBTCI 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 ng l⁻¹). The results showed TBTCl significantly reduced 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. The higher rate of malformations of newly nauplii in 5 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. The higher rate of malformations of newly nauplii in 5 ng l⁻¹ TBTCl concentration was 32.00 ± 4.62. Because in this concentration is a chance to survival newly nauplii survival to a longer period in toxic solution, which gives clearly deformities. While the lower deformities (%) were1.00 ± 0.00 at 75 ng l⁻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; Koutsashtis and Aoyama, 2008 and Rotini 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 Koutsashtis and Aoyama, 2008). In addiction, 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 frome 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
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, and malformed newly hatched nauplii were counted. Hatching percentages (HPs) of cysts were determined by counting the number of completely hatched 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 a 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 hatchability 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 a 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 TBTCI 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 TBTCI 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 (EC50s) 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 TBTCI at lower concentration 5 ng/l it was 25% cysts comparative with the control samples 3% cysts, and that mean TBTCI can inhibits hatching process and can kills dormant embryo in the low concentration and also when increasing the TBTCI 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 TBTCI on unhatched eggs or cysts of crustaceans, including A. salina was more sensitive to TBTCI at 5 ng/l, 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 TBTCI 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 ng/l 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 ng/l TBTCI concentration was unable to completely break the cyst wall (Figure 2 (f)), while the A. salina cysts in the 100 ng/l unable to hatching (Figure 2 (f)). This sequence of effects relatively showing the severity of TBTCI as its concentration increase in the aquatic environment.
2. Median Effective Concentration (EC$_{50}$).

The median effective concentration EC$_{50}$ of TBTCI as shown in (Figure 3), at different concentration of TBTCI the $A. salina$ nauplii completely hatching after 24hr was (EC$_{50}$ 46.48 ng/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$_{50}$ 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$_{50}$ for 24hr was once 2.14 and 72hr was 0.023 mg/ml. This result is an achievement with (Brix et al., 2006) studied estimated the EC$_{50}$ of metallic salts are suggesting that the hatching endpoint for $A. franciscana$ is the most sensitive examined to metals in marine environments. Meanwhile, Alyuğurk 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 exact 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\textsubscript{50} = 46.48 ng L\textsuperscript{-1}) between TBTCI Concentration and Hatchability Inhibition (%) of \textit{A. salina} Cysts

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

Analysis of the morphological deformities (%) of the means \textit{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 ng L\textsuperscript{-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 ng L\textsuperscript{-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 \textit{A. salina} Affected by Different Concentration of TBTCI [N: number of cysts]

<table>
<thead>
<tr>
<th>TBTCI (ng L\textsuperscript{-1})</th>
<th>N</th>
<th>Deformity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>300</td>
<td>0.00 ± 0.00\textsuperscript{a}</td>
</tr>
<tr>
<td>5</td>
<td>300</td>
<td>32.00 ± 4.62\textsuperscript{c}</td>
</tr>
<tr>
<td>10</td>
<td>300</td>
<td>16.33 ± 3.76\textsuperscript{b}</td>
</tr>
<tr>
<td>15</td>
<td>300</td>
<td>9.00 ± 0.58\textsuperscript{a,b}</td>
</tr>
<tr>
<td>20</td>
<td>300</td>
<td>9.67 ± 1.46\textsuperscript{a,b}</td>
</tr>
<tr>
<td>25</td>
<td>300</td>
<td>6.33 ± 1.76\textsuperscript{a,b}</td>
</tr>
<tr>
<td>50</td>
<td>300</td>
<td>2.33 ± 0.33\textsuperscript{a}</td>
</tr>
<tr>
<td>75</td>
<td>300</td>
<td>1.00 ± 0.00\textsuperscript{a}</td>
</tr>
</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 ng L\textsuperscript{-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 ng/l 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 ng/l 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 (ng/l)</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 ± 49.6d</td>
<td>314.3</td>
<td>451.7</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>284.6 ± 51.6c</td>
<td>221.6</td>
<td>377.8</td>
</tr>
<tr>
<td>10</td>
<td>35</td>
<td>266.8 ± 54.6abc</td>
<td>202.6</td>
<td>364.2</td>
</tr>
<tr>
<td>15</td>
<td>35</td>
<td>282.2 ± 59.3ab</td>
<td>212.5</td>
<td>398.9</td>
</tr>
<tr>
<td>20</td>
<td>35</td>
<td>294.9 ± 40.6c</td>
<td>223.8</td>
<td>389.9</td>
</tr>
<tr>
<td>25</td>
<td>35</td>
<td>288.8 ± 45.7bc</td>
<td>216.5</td>
<td>352.1</td>
</tr>
<tr>
<td>50</td>
<td>35</td>
<td>274.8 ± 39.7abc</td>
<td>244.8</td>
<td>393.3</td>
</tr>
<tr>
<td>75</td>
<td>35</td>
<td>269.8 ± 54.6a</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 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.
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 EC_{50} value of TBTCl was once recognized as 46.48 ng/L\(^{-1}\) 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 addtion, 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.

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References


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).

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

GJMR-B Classification: NLMC Code: QV 744
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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).
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 dried leaf powder was initially subjected to 100ml 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.

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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 2×104 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 inhabitations (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


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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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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.
**Criterion for Grading a Research Paper (Compilation)**

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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<tr>
<td><strong>Abstract</strong></td>
<td>Clear and concise with appropriate content, Correct format. 200 words or below</td>
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<td></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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<tr>
<td><strong>Introduction</strong></td>
<td>Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads</td>
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<td><strong>Methods and Procedures</strong></td>
<td>Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake</td>
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<tr>
<td><strong>Result</strong></td>
<td>Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited</td>
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