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MESSAGE FROM EDITOR IN CHIEF

Dear Colleagues,

It is a pleasure to introduce the 2nd issue of EMU Journal of Pharmaceutical Sciences Volume 7, 2024. I would like to congratulate all participants, editorial board, and the journal secretarial for the hard work achieved out.

As a member of 'DergiPark' Akademik, an establishment under the Scientific and Technological Research Council of Türkiye (TÜBİTAK), EMU Journal of Pharmaceutical Sciences continues its journey on the welcoming, pure reviewing, and publication of scientific studies in diverse fields related to pharmaceutical sciences. The journal will continue to serve, promote, and disseminate scientific research information globally while giving scientists and researchers a higher platform to share their scientific word in the whole world. It is important to remind that the journal is free of submission or acceptance fee.

In the soon period, we are planning to launch the 3rd issue of the year.

Looking forward to your scientific contributions,

Best wishes,

Prof. Dr. H. Ozan Gülcan

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GUIDE FOR AUTHORS

EMU Journal of Pharmaceutical Sciences (EMUJPharmSci) covers the research on all aspects of Pharmacy presented as original articles, short reports and reviews.

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c) All authors' full names (*Font: Times New Roman Font Size: 11)*.
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State the objectives of the work and provide a brief background of the literature related with the topic. The novelty and the aim of the study should be clearly stated.

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a) Give a brief and clear description of the materials and methods used. Subtitles can be given as appropriate.
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- Descriptive titles should be given at the top of the tables and at the bottom of the figures.
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Comparison of In Vitro Ibuprofen Release Rates from HPMC and Carbopol 934® Gels

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Abstract

Topical drug delivery systems have become a critical area of research because of their ability to deliver active pharmaceutical ingredients directly to the target site, thereby reducing systemic exposure and associated side effects. Non-steroidal anti-inflammatory drugs like ibuprofen are frequently used in these systems for their strong pain-relieving and anti-inflammatory properties. In the present study, an HPMC gel formulation containing the same amount of ibuprofen (5% w/w) was prepared as an alternative to the market formulation, which is prepared with Carbopol 934® as the gelling agent. The aim was to compare the two formulations in terms of the release rate of the active substance. The study results demonstrated that the HPMC gel containing ibuprofen, formulated as an alternative to the market formulation, meets pharmaceutical criteria in terms of pH, viscosity, appearance, and active ingredient content (90-105%). Furthermore, the release rate of ibuprofen from gel was statistically significantly different compared to the market formulation prepared with Carbopol 934® ($p<0.05$). Based on the findings, it can be concluded that the prepared gel formulation may serve as an alternative to the market formulation containing the same amount of active ingredient. This is particularly desirable for enhancing the onset of anti-inflammatory and analgesic effects by increasing the release rate.

Keywords

Carbopol 934®, HPMC, ibuprofen, release rate, physicochemical controls.

Article History

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INTRODUCTION

Topical drug delivery systems have emerged as a pivotal area of research due to their capability to deliver active pharmaceutical ingredients directly to the site of action, thus minimizing systemic exposure and associated side effects. Non-steroidal anti-inflammatory drugs (NSADs) like ibuprofen are widely used for their potent analgesic and anti-inflammatory effects. The formulation of ibuprofen for topical application presents unique challenges, primarily revolving around enhancing its solubility, stability, and permeation through the skin (Oba et al., 2021; Hundscheid et al., 2023; Sung et al., 2020).

In the realm of topical gels, the choice of gelling agent plays a critical role in determining the drug release rate and overall efficacy of the formulation. Hydroxypropyl methylcellulose (HPMC) and Carbopol 934® are two widely used polymers in the pharmaceutical industry. HPMC, a semi-synthetic derivative of cellulose, is renowned for its biocompatibility, mucoadhesive properties, and ability to form clear, stable gels. It provides a controlled release profile, which is advantageous for maintaining therapeutic drug levels over an extended period (Montes et al., 2022; Hundscheid et al., 2023). Additionally, rheological properties of HPMC can be fine-tuned to optimize patient compliance and ease of application (Montes et al., 2022).

Carbopol 934® is a high molecular weight, cross-linked polyacrylic acid polymer. Its ability to form highly viscous gels even at low concentrations makes it a preferred choice for many topical formulations. Carbopol 934® gels are known for their excellent bioadhesion and clarity characteristics which are desirable traits for cosmetic and pharmaceutical applications. However, the release kinetics of ibuprofen from Carbopol 934® gels can be influenced by factors such as polymer concentration, pH, and the presence of electrolytes (Mahmoud et al., 2020).

This study seeks to investigate and compare the release rate of ibuprofen from an HPMC-based gel formulation to that of a commercially available Carbopol 934®-based gel. Understanding these dynamics is crucial, as they directly impact the therapeutic efficacy, onset of action, and patient adherence. While previous studies have explored the individual characteristics of HPMC and Carbopol 934® gels, comprehensive comparative analyses focusing on ibuprofen release are limited. By systematically evaluating the release profiles, we aimed to elucidate the potential advantages and limitations of using HPMC

versus Carbopol 934® as gelling agents in topical ibuprofen formulations. This investigation could a basis for future formulation strategies, ultimately enhancing the effectiveness of topical NSAID therapies and improving patient outcomes. Our findings may also contribute to the broader field of topical drug delivery, providing insights that could be applied to other active pharmaceutical ingredients and therapeutic areas.

MATERIALS AND METHODS

Materials

Ibuprofen, HPMC, and Carbopol 934® were obtained from Sigma-Aldrich (USA). Carbopol 934® ibuprofen (Carbopol-IBU) that was used as a reference was commercially available. All other chemicals and reagents used in this study were of analytical grade.

The equipments used in the study include: Electric balance: Mettler Toledo (USA), viscometer: Thermo Scientific Viscotester (USA), pH meter: Ohaus (USA), water bath: N-Biotech (Korea), magnetic stirrer: Velp Scientifca (USA) and UV/Visible spectrophotometer: Shimadzu UV-1800 (Japan).

Methods

Spectrophotometric analysis of ibuprofen

The stock solution was prepared by dissolving 50 mg of ibuprofen in 100 mL of 0.1 N NaOH solution. Using the prepared stock solution, the wavelength at which ibuprofen exhibits maximum absorbance was determined. The stock solution was then diluted to various concentrations, and the absorbance values of these dilutions were measured at the determined wavelength. The calibration equation was subsequently established based on these absorbance values (Kashyap et al., 2020; USP 32, 2009).

Preparation of HPMC-ibuprofen (HPMC-IBU) gel

The HPMC gel was prepared by dispersing the required amount of HPMC in distilled water under continuous stirring. The mixture was heated to 70°C and stirred until a homogeneous solution was obtained. After cooling to room temperature, ibuprofen was added and dispersed completely. The gel contained 5% (w/w) ibuprofen (Hasnain et al., 2020).

In vitro evaluation of ibuprofen gels

In vitro evaluation and characterization of ibuprofen gels were carried out via organoleptic controls, pH determination, viscosity measurement, and analysis of the percent ibuprofen content in the prepared gel formulations. For the analysis of

ibuprofen content in the gel formulations, 1 gr of the gel was mixed with 50 mL of pH 6.8 phosphate buffer. The mixture was stirred continuously using a magnetic stirrer for 12 hours, followed by ultrasonic bath treatment. The solution obtained was passed through a 0.45 µm membrane filter, and suitable dilutions were prepared to quantify the active ingredient using a UV spectrophotometric technique in triplicate (Pradal, 2020).

Organoleptic evaluations

The visual appearance and texture of the ibuprofen gel formulations were examined to determine their uniformity.

pH measurement

Ibuprofen gel formulations were dispersed in distilled water. A digital pH meter (Ohaus, USA) was utilized to measure their pH levels. The measurement was done in triplicate.

Viscosity

The viscosity of the gel formulations was assessed using a Thermo Scientific Viscotester (USA). The measurements were conducted in triplicate at room temperature, utilizing spindle No. 94 at a speed of 15.0 rpm (Boshrouyeh et al., 2023; Hasnain et al., 2020).

Assay

The concentration of ibuprofen in the gels was quantified using a UV spectrophotometric method. Initially, a standard solution of ibuprofen was prepared by thoroughly mixing 100 mg of ibuprofen in 1000 mL of a 6.8 pH buffer solution in a volumetric flask. The standard solution was then serially diluted to create a range of solutions with different concentrations of ibuprofen. The absorbance of these solutions was measured at 272 nm using UV spectrophotometry. Analytical parameters were determined using ANOVA (Mancini et al., 2021; USP 32, 2009).

In vitro ibuprofen release studies

The amount of ibuprofen in the hydrogel formulations was determined by accurately weighing and placing 2 gr hydrogel samples into dialysis bags made of xylene cellulose acetate. These bags were then immersed in a pH 6.8 phosphate buffer and maintained in a beaker with a water bath at 37 °C ± 0.5 °C, equipped with a heater and stirrer. The ibuprofen content of the samples was measured at 15, 30, 60, 90, 120, and 150 minutes using a UV spectrophotometric method. Each measurement was performed in triplicate (Theochari et al., 2021).

Statistical analysis

To optimize the ibuprofen gel formulations, ANOVA was employed. A p-value of less than 0.05 was considered as an indicative of a statistically significant difference. The results were presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Spectrophotometric analyses of ibuprofen

The results show that the absorbance value of ibuprofen at 272 nm was consistent with prior studies. The method validation parameters are shown in Table 1, and the calibration curve and equation are presented in Figure 1.

Table 1: Analytical method validation parameters for the assay of ibuprofen.

Parameters	Results
Linearity range (mM)	0.2-2.0
Slope (m)	0.3796
RSD (%)[1]	0.42
Determination of coefficient	0.03
LOD (µg/mL)[2]	0.0207
LOQ (µg/mL)[3]	0.09
RSD for accuracy	0.29

[1]RSD: Relative standard deviation
[2]LOD: Limit of detection
[3]LOQ: Limit of quantification

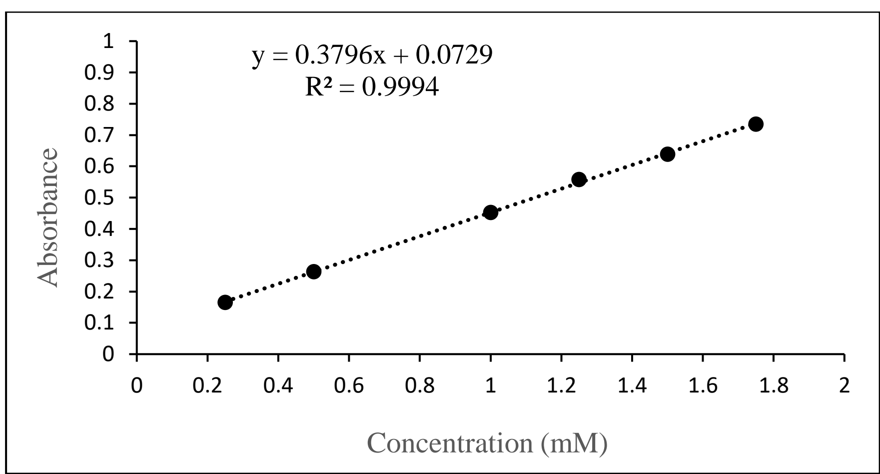

Figure 1: Calibration curve of ibuprofen.

In vitro evaluation of ibuprofen gels

Table 2 illustrates the physicochemical characteristics of the gels including pH, uniformity, viscosity, and percentage of ibuprofen content in the gel formulations.

According to the studies involving gels formulated with HPMC, the pH range of the gels is between 6.0 and 7.0 at room temperature. This range is associated with the neutral nature of HPMC and its compatibility with the pH values of other components in the gel. This characteristic suggests that the formulation is compatible with the skin and provides a suitable environment for the stability of active ingredients such as ibuprofen (Ardana et al., 2015; Nogami et al., 2021; Rahmani and Zulkarnain, 2023).

Due to the use of a medium molecular weight HPMC gel agent in the formulation, the viscosity of the prepared HPMC-IBU gel, considering its concentration, was as

expected within the medium-high range (1015±5.58 cPs). This viscosity value is suitable for a pharmaceutical gel, indicating that the formulation will form a thicker layer on the skin and will spread more slowly compared to water.

The ibuprofen content in the hydrogels was therapeutically adequate, with percentages ranging from 96.00 to 98.66% ± 1.32–2.77.

Table 2: The physicochemical characteristics of the formulated gel.

Formulations	pH±SD	Viscosity (cPs) ±SD (25°C)	Homogenity	Ibuprofen assay (%)±SD
HPMC-IBU gel	6.91±0.1	1015±5.58	+	96.14±1.32
Carbopol-IBU gel	6.87±0.5	1114±6.44	+	98.66±2.77

In vitro ibuprofen release studies

The therapeutic efficacy of gel formulations is closely linked to the release of ibuprofen. The release occurs through the three-dimensional network structure of the gels, either by passive diffusion or by erosion of the gel matrix due to degradation over time. Because ibuprofen is a small molecule, it can penetrate the stratum corneum, the outermost layer of the skin, primarily through passive diffusion. Additionally, the type and amount of gel agent used may affect the release of ibuprofen. As a result, the cumulative release profiles of ibuprofen were measured from the HPMC-IBU gels and the commercial Carbopol-IBU gel formulation. The release rate of ibuprofen from the new formulation prepared using HPMC was statistically significantly faster than that of Carbopol available on the market (p = 0.004). Release profiles from these gels that were tested in triplicate are shown in Figure 2.

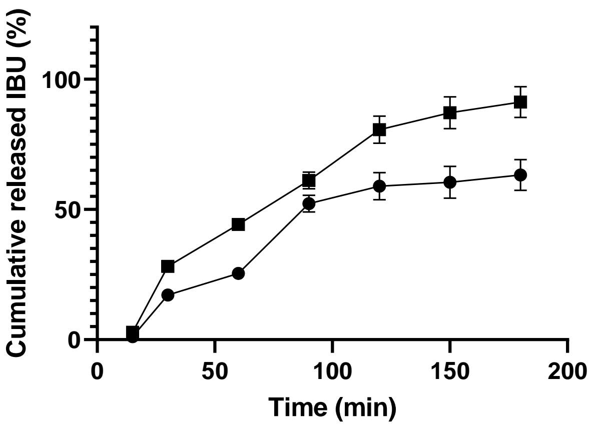
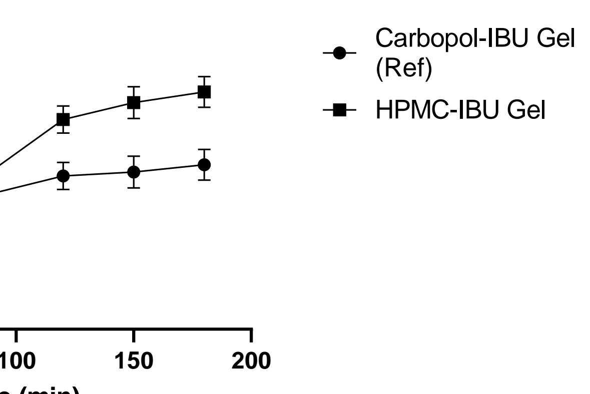

Figure 2: Release profiles of ibuprofen from gels.

CONCLUSION

In the present study, the aim was to prepare an ibuprofen gel formulation, frequently preferred for topical application in the pharmaceutical market due to its analgesic and anti-inflammatory properties, using HPMC as the gelling agent. The prepared gel formulation was compared with gels prepared with Carbopol, both in terms of physicochemical properties and the release rate of the active substance.

When the results were evaluated, it was determined that the physicochemical properties of the prepared HPMC gels were compatible with the references. The HPMC-IBU gel and the Carbopol 934®-IBU gel had comparable physicochemical properties, such as characteristic pH, viscosity, and appearance. The release rate of ibuprofen from the new formulation prepared using HPMC was statistically significantly faster than that from the Carbopol 934®. The reason for this difference is that the gel matrix formed with Carbopol 934® tends to have a higher viscosity, which can further slow the release rate of ibuprofen.

A gel containing HPMC can generally provide a more controlled and consistent release but does not form as viscous structure as Carbopol 934®. Therefore, the release rate of ibuprofen may be higher in the gel containing HPMC. HPMC forms a less viscous matrix that dissolves more rapidly, increasing the release rate of ibuprofen. On the other hand, Carbopol 934® may slow down the release of ibuprofen further by creating a more viscous structure. As a result, the prepared HPMC-IBU gel may be preferred over the market formulation, as it is desirable for the pain-relieving and anti-inflammatory effects to be observed in a short time after application.

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Determining the Assay of New Formulation Low Dosage Naltrexone HCl Capsules with a RP-HPLC Method

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Abstract

Naltrexone hydrochloride (NLX HCl) is a medication primarily used in the management of alcohol and opioid dependence. NLX HCl is an opioid antagonist that blocks the effect of opioids by binding to opioid receptors in the brain without activating them. This study aims to determine the assay amount of new formulation low dosage NLX HCl capsules by High-Performance Liquid Chromatography (HPLC). HPLC method is very precise, specific, simple, accurate and sensitive method for assay determination of new formulation NLX HCl (3.0 mg and 4.5 mg). The chromatographic separation was achieved using Eclipse XDB-12 C18 (150 mm x 4.6 mm, 5 µm particle size) column using UV detection at 280 nm. The mobile phase consisted of a variable mixture of Solution A and Solution B with gradient, run at a flow rate of 1.0 mL/min. According to our research, NLX HCl has a retention time of 10.5 minutes. The linearity range was established between 1.1-3.40 mg/mL.

Keywords

Assay, naltrexone hydrochloride, HPLC, formulation.

Article History

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INTRODUCTION

Naltrexone belongs to a class of drugs known as opioid antagonists. The chemical name of naltrexone hydrochloride (NLX HCl) is chemically (5α)-17-(cyclopropylmethyl)-4,5-epoxy-3,14-dihydroxymorphinan-6-one hydrochloride (Figure 1). NLX HCl is a medication approved by the Food and Drug Administration (FDA) to treat both alcohol use disorder (AUD) and opioid use disorder. Addiction is a well-known chronic brain condition (Younger et al., 2014). Opioids are a prevalent source of addiction and have been linked to a number of social and medical issues. Naltrexone is becoming a more widely used medication for treating opiate and alcohol addictions.

There are two types of NLX HCl: Full dose and low dose naltrexone (LDN). Full dose naltrexone is available by prescription in several countries in the form of 25 mg or 50 mg oral tablets. For up to 24 hours, a 50 mg tablet efficiently prevents the effects of heroin. LDN that is defined as a daily intake of 1 to 5 mg is a reversible competitive antagonist and acts momentarily inhibiting the brain's opioid receptors. Then, it increases the production of endorphins *via* a positive feedback mechanism. Clinical reports on LDN have indicated potential advantages for a number of illnesses, including multiple sclerosis (Agrawal, 2005), fibromyalgia (Patten et al., 2018), Crohn's disease (Jill et al., 2007), complex-regional pain syndrome (Chopra and Cooper, 2013), Hailey-Hailey disease (Campbell et al., 2018), and cancer (Lia et al., 2018).

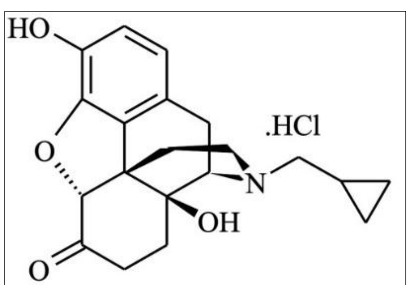


Figure 1: Structural formula of naltrexone hydrochloride.

High-Performance Liquid Chromatography (HPLC) is a sophisticated analytical technique that requires systematic procedure validation and refining for accurate compound separation, identification, and quantification. This study aims to determine the assay amount of new formulation low dosage NLX HCl capsules by HPLC. HPLC method is a very precise, specific, simple, accurate and sensitive method for assay determination of new formulation NLX HCl (3.0 mg and 4.5 mg). The chromatographic separation was achieved using Eclipse XDB-12 C18 (150

mm x 4.6 mm, 5 µm particle size) column using ultraviolet (UV) detection at 280 nm. The mobile phase consisted of a variable mixture of Solution A and Solution B with gradient, run at a flow rate of 1.0 mL/min.

MATERIALS AND METHODS

Equipment

The Agilent 1260 Infinity HPLC system was used for this study, which was outfitted with a solvent degasser, quaternary pump, auto sampler, column oven, and diode array detector.

Chemical and reagents

United States Pharmacopeia (USP) Naltrexone RS was obtained from USP Pharmacopeia. Sodium octanesulfonate, sodium acetate, glacial acetic acid and triethylamine were obtained from Sigma Aldrich and they were all reagent grade. HPLC grade methanol was purchased from local vendors. Water was obtained in-house using the Nanopure Diamond water system.

Chromatographic conditions

The assay analysis of new formulation NLX HCl (3.0 mg and 4.5 mg) capsules were done according to USP (USP 32, 2009).

The liquid chromatography is equipped with 280 nm detector and a 3.9 mm x 15 cm column that contains packing L1 and is programmed to provide at a flow rate of about 1 mL per minute a variable mixture of Solution A and Solution B. At the time the sample was injected into the equipment, the percentage of solution A was 100% over the next 35 minutes, the proportion of solution B was increased linearly to 100% and then over the next minute decreasing nearly to 100% of solution A.

Solution A: 1.08 g of sodium octanesulfonate and about 23.8 g of sodium acetate were dissolved in 800 mL of distilled water. Then, 1 mL triethylamine and 200 mL methanol were added into solution and mixed with magnetic stirrer machine. The pH of solution was adjusted to 6.5 ± 0.1 with glacial acetic acid, then filtered and degassed prior to use.

Solution B: 1.08 g of sodium octane sulfonate and about 23.8 g of sodium acetate was dissolved in 400 mL of distilled water, then 1 mL triethylamine and 600 mL methanol added into solution and mixed them with magnetic stirrer machine. The pH of solution was adjusted to 6.5 ± 0.1 with glacial acetic acid, then filter and degas prior to use.

Mobile phase: Use variable mixtures of Solution A and Solution B as directed for chromatographic system.

Procedure

Standard preparation: 22.5 mg of USP naltrexone RS was accurately weighted and added to 10 mL volumetric flask. 1.5 mL of methanol and 0.6 mL of 0.1 N hydrochloric acid was added and dissolved by swirling the flask and diluted with 0.1 M phosphoric acid to volume.

Sample preparation: Not fewer than 20 tablets were transferred into a tared container to determine the average tablet weight. The tablets were ground into a homogeneous mixture. An accurately weighed portion, equivalent to about 250 mg of NLX HCl was transferred to a 100-mL volumetric flask. About 80 mL of 0.1 M phosphoric acid was added and mixed or sonicated for at least 30 minutes. 0.1 M phosphoric acid was used to dilute to volume. The solution was mixed and filtered.

Procedure: Equal volumes (about 20 µL) of the standard preparation and the assay preparation were injected separately into the chromatograph. The chromatograms were recorded and the responses for all the peaks were measured. The quantity, in mg, of $C_{20}H_{23}NO_4{\cdot}HCl$ in the portion of NLX HCl was calculated using the formula:

$(377.86/341.41)100C\ (r_U / r_S)$

where, 377.86 and 341.41 are the molecular weights of NLX HCl and naltrexone, respectively; C is the concentration (mg/mL), of USP naltrexone RS in the standard preparation; and r_U and r_S are the peak responses of naltrexone obtained from the assay sample preparation and the standard preparation, respectively.

RESULTS AND DISCUSSION

Linearity

Linear calibration curves of the proposed method were obtained by diluting stock solutions with concentrations of 1.1, 1.68, 2.27, 2.83 and 3.40 mg/mL for NLX. Linearity was evaluated by fitting least-squares regression analysis. The analyses of calibration is shown in Figure 2.

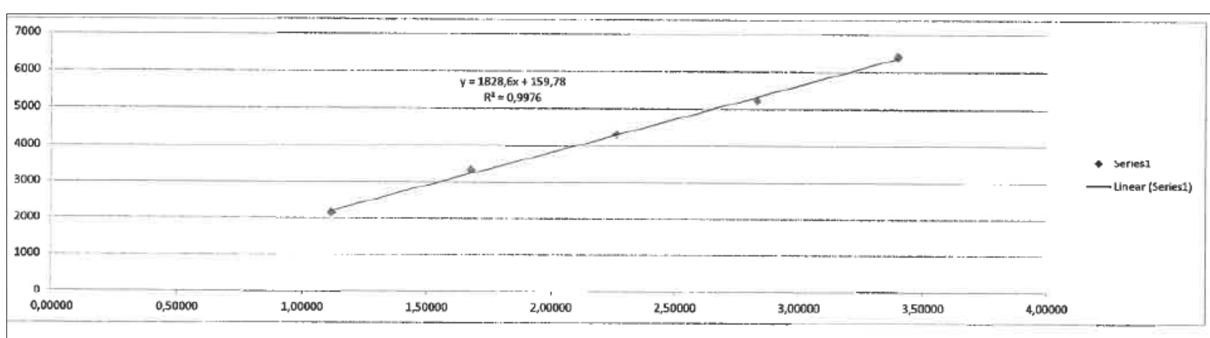


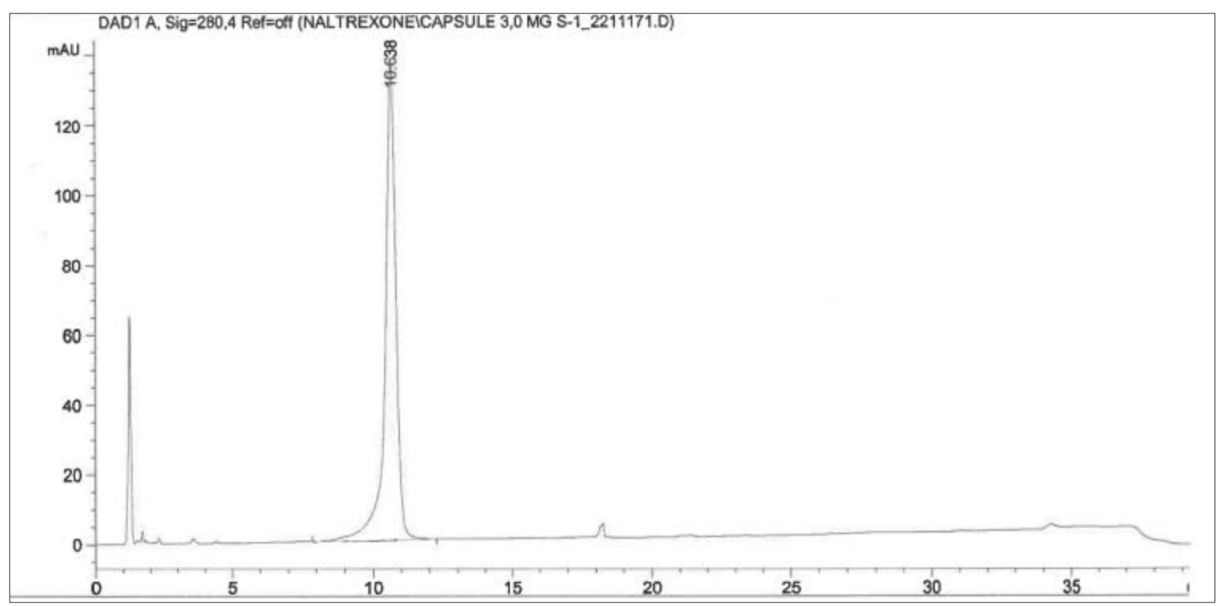
Figure 2: Calibration curve of NLX.

Assay analysis of NLX HCl 3.0 mg and 4.5 mg capsules

In a previous study, forced degradation studies of new formulation containing naltrexone were conducted (Yaghoubnezhadzanganeh and Burgaz, 2019). In this study, the assay analysis of new formulated NLX HCl 3.0 mg and 4.5 mg capsules (2 samples of each one) were conducted and results have been evaluated. The sample solutions have been prepared according to USP method (sample preparation) and measured by HPLC (Table 1). The chromatograms are shown in Figure 3.

Table 1: The assay results of NLX HCl 3.0 mg and 4.5 mg capsules.

Type of the solution	Concentration (mg/mL)	Average Area	Assay
Standard of naltrexone	2.30	3634	
Capsule containing 3 mg naltrexone	2.20	3558	% 93.03
Capsule containing 4.5 mg naltrexone	2.35	3859	% 93.97



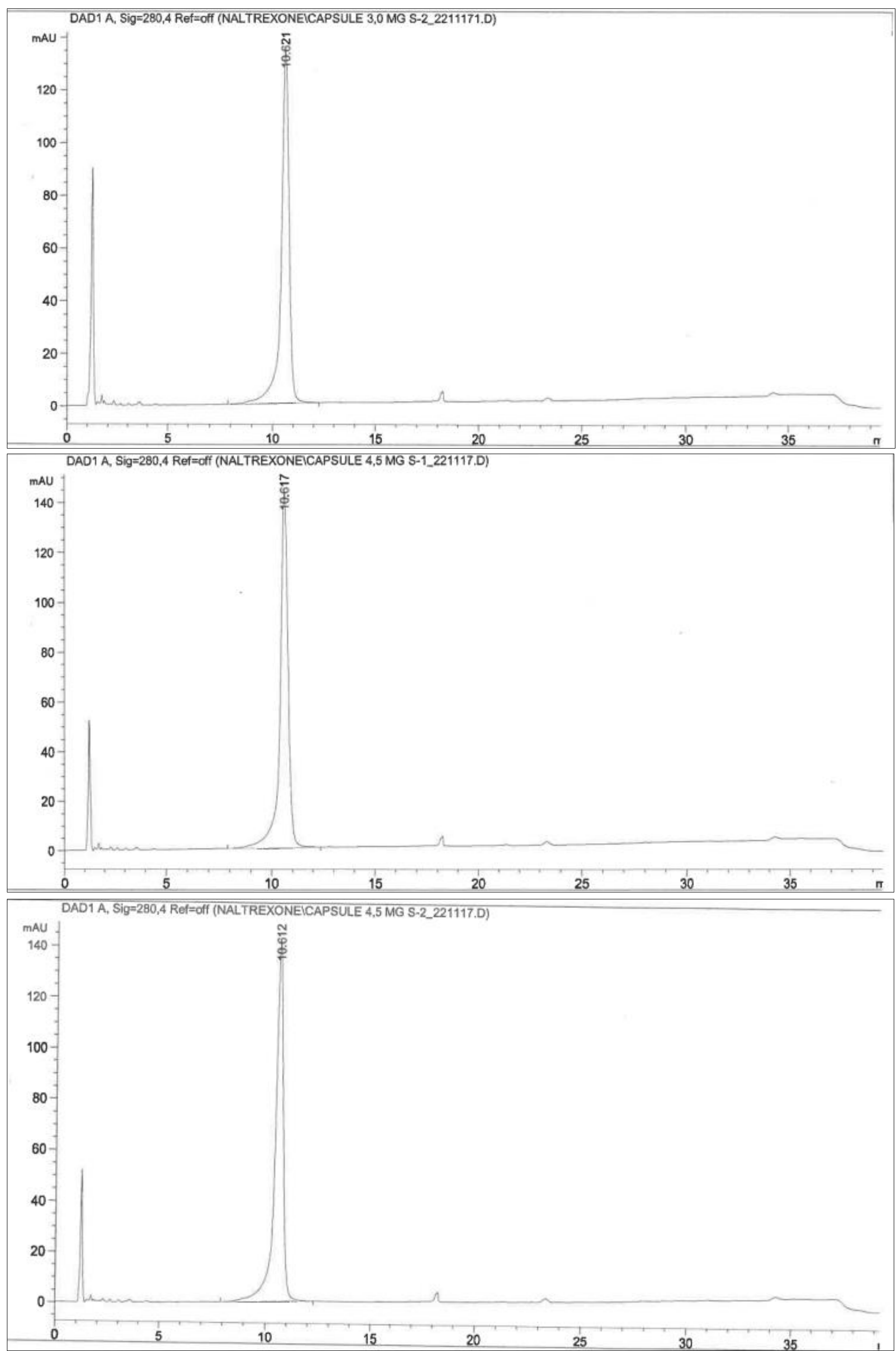


Figure 3: The chromatograms of NLX HCl 3.0 mg and 4.5 mg capsules.

CONCLUSION

The naltrexone hydrochloride quantification in pharmaceutical dosage form using the suggested RP-HPLC method is rapid, straightforward, linear, accurate, and precise. The quality-control of bulk medication and its preparations can therefore be carried out using the current RP-HPLC technique. The experiment was done successfully, the process was performed twice for each sample to get a great result. According to the USP, NLX HCl capsules contain not less than 90% and not more than 110% of the labeled amount of NLX HCl. The new formulated 3.0 mg and 4.5 mg NLX HCl capsules are in between the acceptance criteria.

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Antibacterial Activity of Naltrexone and Its Combination with Ciprofloxacin Against Gram Negative and Gram Positive Bacteria

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Abstract

Naltrexone, an opioid receptor antagonist, is commonly used in the treatment of alcoholism. Studies about the antibacterial activity of naltrexone are limited. In our study, we aimed to evaluate the antibacterial and synergistic activities of naltrexone against *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603. The results showed promising antibacterial activity against all tested pathogenic bacteria. Additionally, checkerboard assays revealed additive activity against *S. aureus* when combined with ciprofloxacin. Collectively, the data from our study suggest that naltrexone can further be used as a potential antibacterial source alone or in combination with other antibiotics in the treatment of bacterial infections.

Keywords

Antibacterial, checkerboard, ciprofloxacin, naltrexone.

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INTRODUCTION

Naltrexone is an opioid receptor antagonist that has been used in the treatment of alcohol and opioid dependence (Figure 1). It has been approved by Food and Drug Administration to be used in the therapy of alcohol and opioid use disorders (Lobmaier et al., 2011; Sudakin, 2016).

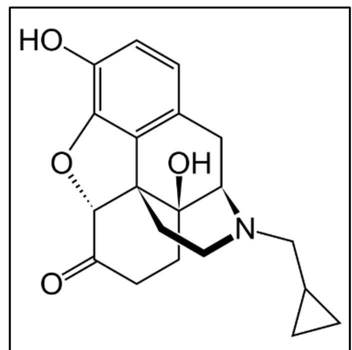

Figure 1: Structural formula of naltrexone.

Naltrexone is the most studied drug in the treatment for alcoholism (Lobmaier et al., 2011). It is commonly used for its ability to block the sedative effects of alcohols and other drugs, while also assisting in the reduction of the cravings towards addictive substances (Lobmaier et al., 2011; Sudakin, 2016). Low-dose naltrexone was reported to be a promising candidate for the off-label treatment of chronic pain due to its modulatory activity on glial cells although clinical trials are still warranted (Younger et al., 2014; Bolton MJ, et al., 2020). Another potential of naltrexone is its beneficial effect on inflammatory diseases such as Crohn's disease, multiple sclerosis, and fibromyalgia that is proposed to be via regulating the secretion of inflammatory cytokines including IL-6 and TNF-α and to provide therapeutic effects on cancers including B cell lymphoma and pancreatic cancer (Cant et al., 2017).

Due to reducing pro-inflammatory cytokine secretion, increasing the secretion of anti-inflammatory cytokines, modulating immune system via decreasing Th1 and Th17 cells, and because of immune-enhancer characteristics, naltrexone was proposed to promote the prevention and management of viral (such as COVID-19) and bacterial infections (El Shehaby et al., 2022). On the other hand, naltrexone was not studied for its direct antibacterial activity.

In this study, we aimed to demonstrate the antibacterial potency of naltrexone and its combination with ciprofloxacin, one of the most frequently prescribed commercial antibiotic against which the resistance has been increasing worldwide.

MATERIALS AND METHODS

Inoculum preparation

The antibacterial activity of naltrexone was investigated against *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 700603. The bacteria were subcultured on Mueller Hinton Agar (MHA). The media were incubated at 37 ºC. After 24 hours, 0.5 McFarland (1.5 x 10^8 cfu/mL) standard concentration of each bacterium was prepared within Mueller Hinton broth (MHB).

Minimum inhibitory concentration (MIC) determination

Antibacterial activity of naltrexone was demonstrated by broth microdilution method (Wikler, 2006). The final inoculum of the bacteria in the 96-well plates was 1 x 10^6 cfu/mL and the final concentrations of the sample ranged from 1 to 32 mg/mL. The highest concentration of naltrexone in MHB was used as the negative control and ciprofloxacin was utilized as the positive control. Incubation was conducted for 18 hours at 37 ºC. MIC was accepted as the minimum concentration of naltrexone that prevented the growth of each strain.

Minimum bactericidal concentration (MBC) determination

MBC was accepted as the lowest concentration of naltrexone that killed bacteria. Thus, 10 µL from each well (at the the concentration of MIC and higher) was inoculated on Mueller Hinton agar (MHA). The media were incubated for 18 hours at 37 ºC.

Interaction of naltrexone with ciprofloxacin

Checkerboard assay was used for the evaluation of the interaction of naltrexone with ciprofloxacin as previously described (Bellio et al., 2021). The final concentrations of the sample ranged from 0.5 to 32 mg/mL, whereas ciprofloxacin concentration ranged from 0.002 to 1 mg/L. Incubation was carried out for 18 hours at 37 ºC.

To determine the interaction of the naltrexone and ciprofloxacin in a combination, FIC index calculation (FIC Index = A / MICA + B / MICB) was used; where ‘A’ and ‘B’ are the MICs of each agent in combination within a single well plate; and MICA and MICB are the MICs of each agent individually. The interaction is accepted to be synergistic when FIC index was < 0.5; additive when 0.5-0.9; indifference when 1-4; and antagonistic when > 4.

Statistical analyses

All of the tests were done in triplicates. Statistical analyses were conducted by Students t-test.

RESULTS AND DISCUSSION

Antibacterial activities and combination tests

In order to assess the antibacterial activities of naltrexone, the microdilution method was used to detect MIC against *E. faecalis*, *S. aureus*, *E. coli*, and *K. pneumoniae*. MICs of naltrexone against the tested bacteria are shown in Table 1.

Table 1: MICs of naltrexone against Gram negative and Gram positive bacteria.

Agents		Gram positive bacteria		Gram negative bacteria	
		S. aureus **ATCC 25923**	***E. faecalis*** **ATCC 29212**	***E. coli*** **ATCC 25922**	***K. pneumoniae*** **ATCC 700603**
Sample (mg/mL)	Naltrexone	16 ± 0	16 ± 0	4 ± 0	2 ± 0
Control (mg/L)	Ciprofloxacin	0. 25 ± 0	1 ± 0.083	0.008 ± 0	0.25 ± 0.021

Data represented as the standard error of mean (±S.E.M).

Naltrexone showed promising antibacterial activity against all tested Gram positive and Gram negative bacteria with 16 mg/mL against *S. aureus* and *E. faecalis*, whereas 4 mg/mL against *E. coli*, and 2 mg/mL against *K. pneumoniae*. Furthermore, MBC assays revealed bactericidal effect against *S. aureus*, *E. coli*, and *K. pneumoniae* at 32 mg/mL, 16 mg/mL, and 16 mg/mL, respectively. On the other hand, no bactericidal effect was observed for *E. faecalis* even at the highest tested concentration (32 mg/mL).

Table 2: FIC index of the combinations against Gram positive and Gram negative bacteria.

Samples	Optimal Combination		FIC Index	
	Ciprofloxacin (mg/L)	**Naltrexone (mg/mL)**	**< 0.5**	**> 0.5**
S. aureus	0.125	4		0.625 (A)
E. faecalis	0.06	16		1.12 (I)
E. coli	0.002	4		1.25 (I)
K. pneumoniae	0.06	2		1.48 (I)

A: Additive. I: Indifference.

FIC index assay was used to obtain the interaction of naltrexone and ciprofloxacin against the tested bacteria. When binary combinations of naltrexone and ciprofloxacin were used against the tested bacteria, no antagonistic activity was observed as shown in Table 2. Furthermore, additive interaction was observed when 4 mg/mL of naltrexone and 0.125 mg/L ciprofloxacin was combined against *S. aureus*. The results revealed indifference effect for all other bacterial strains tested.

Naltrexone, especially low-dose naltrexone, is proposed to have antibacterial activity in addition to antifungal, antiviral, anti-helminthic, and immunomodulatory characteristics (Marangalo et al., 2024). Majority of the studies about antimicrobial effect of naltrexone focused on the immunomodulatory effects of the drug. Naltrexone was reported to be promising for management of acute endotoxic shock and viral infections such as COVID-19

because of reducing the secretion of proinflammatory cytokines such as tumor necrosis factor-α and regulating T-helper lymphocyte differentiation (El Shehaby et al., 2022; Greeneltch et al., 2004). Naltrexone was also shown to potentiate anti-HIV-1 activity of antiretroviral drugs in vitro (Gekker et al., 2001). However, studies investigating antibacterial activity of naltrexone in vitro are scarce.

CONCLUSION

Naltrexone, an opioid receptor antagonist, is commonly used in the treatment of alcoholism. The antibacterial results obtained with naltrexone revealed promising antibacterial activity against all tested Gram positive and Gram negative bacteria. Additionally, additive interaction was observed against *S. aureus* when optimal concentrations of naltrexone and ciprofloxacin was used. Collectively, our data suggest that naltrexone can be utilized as a potential source against Gram positive and Gram negative bacteria alone and/or within combinations with other antibiotics.

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Enhanced Antibacterial Efficacy of *Cymbopogon winterianus* Jowitt in Combination with Carvacrol

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Abstract

The continuous increase of antibiotic resistance and the lag in development of novel antibiotics are two important challenges in treatment of infectious diseases. Essential oils (EOs) and their combinations are promising alternatives for treatment of bacterial infections. *Cymbopogon winterianus* Jowitt EO, recognized by the European Medicines Agency for its sleep-enhancing and anxiety-relieving properties, also possesses sedative qualities, as noted in the German Commission E monograph, and aids in treating sleep disorders. The aim of the study was to investigate antibacterial activities of the *C.winterianus* EO and carvacrol combination. Commercial *C. winterianus* EO and carvacrol were tested for antibacterial activity against the American Type Culture Collection quality control strains *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Klebsiella pneumoniae* using the broth microdilution method. Ciprofloxacin served as the standard antimicrobial agent. Checkerboard assay was used to evaluate the combined effects of *C. winterianus* EO and carvacrol.

The results showed that *C. winterianus* EO exhibited moderate antibacterial activity against both Gram positive and Gram negative bacteria. The interaction between *C. winterianus* EO and carvacrol was additive against all tested bacteria. The minimum inhibitory concentration of carvacrol decreased by 2- to 8-fold when the carvacrol combined with *C. winterianus* EO, and carvacrol also enhanced the antibacterial activity of EO by 2- to 16-fold.

Keywords

Antibacterial, carvacrol, combination, *Cymbopogon winterianus,* essential oil, synergy.

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INTRODUCTION

Cymbopogon winterianus Jowitt is a member of the Poaceae family and is grown in South and Central America. The main constituents of *C. winterianus* essential oil (EO) which is known as Java citronella oil are geraniol, citronellol, and citronellal. These compounds are widely utilized in soap, fragrance, cosmetic, and flavoring industries worldwide (Wany et al., 2013). Citronella oil has diuretic, febrifuge, antimicrobial, and antispasmodic properties (Simic et al., 2008). There are many products that contain the oil which are used as biopesticides (Munda and Lal, 2020). In trade, the EO is divided into two categories: Java type citronella oil, which is derived from *C. winterianus* (superior type), and Ceylon type citronella oil, which is derived from *Cymbopogon nardus* (inferior type). Both oils that were medicinally used in the ancient and modern eras consist of different secondary metabolites such as terpenoids and alkaloids (Wany et al., 2013). Tropical or subtropical parts of Asia, Africa, and America are known as plant cultivation areas. Taiwan, Guatemala, Honduras, Malaya, Brazil, Ceylon, India, Equador, Madagascar, Mexico, and the West Indies remain as the largest producers for Java citronella oil (Shasany et al., 2000).

Carvacrol ($C_{10}H_{14}O$), known as 2-methyl-5-[1-methylethyl] phenol, is a phenolic monoterpenoid and a derivative of cymene that naturally is found in EOs (Imran et al., 2022). Commercial carvacrol is produced using biotechnological and chemical processes. Carvacrol is a lipophilic substance with a density of 0.976 g/ml at ambient temperature (25 °C); it is soluble in ethanol, acetone, and diethyl ether but insoluble in water (Yadav et al., 2009). Numerous biological activities of carvacrol including antiviral (Sánchez et al., 2015), antibacterial, antifungal (Nostro et al., 2004), antioxidant (Milos et al., 2012), and anticarcinogenic (Ozkan and Erdogan, 2011) effects. Although agar dilution, well or disk diffusion methods are used for the detection of antibacterial activities of natural products, broth microdilution technique remains as the golden standard method.

Combination studies are increasingly attracting attention in the scientific area. When drugs are tested in combinations, they can exhibit varying pharmacodynamic interactions such as synergy, additivity, antagonism, or indifference. Checkerboard assay in a 96-well microplate provides an effective assessment of the combined effects of natural products (Berenbaum, 1978; Doern et al., 2010). In the checkerboard test, two samples are tested in double serial dilutions, and the concentration of each drug is tested

individually and together. Therefore, it is possible to ascertain the impact of each individual medication, but also most importantly, the impact created by their combination (Berenbaum, 1978). Checkerboard analysis is utilized to measure synergy and determine how the combined effects of samples compared to their individual effects on potency.

In this study, it was aimed to investigate the antibacterial activity of *C. winterianus* and its interaction with carvacrol.

MATERIALS AND METHODS

American Type Culture Collection (ATCC) strains of *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 700603 were included in the study. All bacteria were deposited at the Faculty of Pharmacy, Eastern Mediterranean University, at – 85 °C and were refreshed prior to the assays.

Commercial *C. winterianus* EO was kindly supplied by Doallin Ltd., Istanbul. Ciprofloxacin and carvacrol were obtained from Sigma Aldrich (Germany). The properties of *C. winterianus* EO are given in Table 1.

Table 1: The properties of *C. winterianus* essential oil (Technical Data from Doalinn- Orlife Global Ltd.).

Organoleptic Properties	
Appearance	Clear mobile liquid
Color	Pale yellow to brownish
Odor	Citronellal-like, lemon, grassy
Physico-Chemical Properties	
Density at 20 ºC	0.880-0.895
Refractive index at 20 ºC	1.4650-1.4750
Flash point	94
Rotatory power	-5 / 1
Main Components (>0.5% by GCMS)	
Sulcatone	0.727
Citronellal	26.059
Linalool	0.908
Neoisopulegol	0.747
Caryophyllene	3.006
Citronellyl acetate	1.480
Neral	8.215
Geranial	10.907
Geranyl acetate	1.304
Delta cadinene	0.788
Citronellol	15.294
Geraniol	26.335

Antibacterial Activities and Checkerboard Assays

Broth microdilution method was used to investigate antibacterial activities of *C. winterianus* EO and carvacrol as suggested by Clinical and Laboratory Standards Institute (CLSI) with minor modifications (CLSI, 2023).

Stock solutions at the concentration of 1024 mg/mL were prepared using dimethyl sulfoxide (DMSO) (Sigma Aldrich, Germany). These solutions were then diluted using Mueller Hinton broth (MHB), adding Tween 80 when necessary. Two-fold serial dilutions of the compound and the EO were prepared in 96-well microplates. Each well was filled with 100 µL of the dilution and 10 µL of each bacterium. The final concentrations of the DMSO in the well was ≤3%. Each bacterium were included in the wells at the final concertation of 5 x 10^5 cfu/mL. Additionally, wells containing 3% DMSO with the bacterial inoculum and those with the compound or EO alone served as positive and negative controls, respectively. Ciprofloxacin was included as the reference antibacterial agent.

The microplates were incubated at 37 °C under an aerobic environment for 16 to 20 hours. The minimum inhibitory concentrations (MICs) of the compound and EO were identified as the lowest concentrations that visually inhibited bacterial growth. MIC was further confirmed by addition of 10 µL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) at the concentration of 5 mg/mL.

The antimicrobial activity of binary combinations of *C. winterianus* EO and carvacrol was assessed using checkerboard assay following CLSI guidelines with slight modifications (CLSI, 2023). The compounds and EOs were prepared at concentrations eight times greater than their MICs. Two-fold decreasing serial dilutions of the compound were combined with two-fold increasing serial dilutions of the EO, each at 50 µL. The first rows and columns of the plate included only either the compound or the EO. Finally, 10 µL of bacterial suspension at the concentration of 5 x 10^6 cfu/mL was added to each well.

Fractional inhibitory concentration index (FICI) was calculated by adding the FICs of the compound and the EO. The FIC for the compound was determined as the ratio of its MIC in combination to its MIC when tested alone; while the FIC for the EO was the ratio of its MIC in combination to its MIC alone

.

FICI was calculated using the formula below:

$$\text{FICI} = \frac{\text{MIC } carvacrol \text{ (in the combination)}}{\text{MIC } carvacrol \text{ (alone)}} + \frac{\text{MIC } EO \text{ (in the combination)}}{\text{MIC } EO \text{ (alone)}}$$

The interaction between carvacrol and the EO was classified as; synergistic when FICI ≤ 0.5, additive when 0.5 < FICI < 1, indifferent when 1 ≤ FICI ≤ 4, and antagonistic when FICI > 4 (Berenbaum, 1978). All of the experiments were performed in triplicates.

RESULTS

Antibacterial Activities of *C. winterianus* EO and Carvacrol

The MICs of the EO of *C. winterianus* against *E. coli*, *S. aureus*, and *E. faecalis* were 8 mg/ml whereas the MIC was 4 mg/ml against *K. pneumoniae*. The MIC of carvacrol was 0.25 mg/ml against all of the strains tested. *C. winterianus* EO and carvacrol had lower antibacterial activity than ciprofloxacin (Table 2).

Table 2: MICs (mg/mL) of carvacrol and *C. winterianus* essential oil.

Species	*C. winterianus* (mg/ml)	Carvacrol (mg/ml)	Ciprofloxacin (mg/L)
E. coli	8	0.25	0.008
K. pneumoniae	4	0.25	0.015
S. aureus	8	0.25	0.125
E. faecalis	8	0.25	0.5

Antibacterial Interactions between *C. winterianus* EO and Carvacrol

In the present study, the interaction between *C. winterianus* and carvacrol was found to be additive against the Gram positive and Gram negative bacteria tested. Synergistic, indifference and antagonistic interactions were not detected.

Combinations tested against the bacteria and the FICI results of the best combinations are given in Table 3.

Table 3: FICI of *C. winterianus* essential oil and carvacrol combinations.

Species	Concentrations for the combination (mg/mL)		FICI (Interaction)*
	C. winterianus	Carvacrol	>0.5
E. coli	1	0.125	0.625 (A)
K. pneumoniae	0.25	0.125	0.562 (A)
S. aureus	2	0.125	0.75 (A)
E. faecalis	4	0.03	0.62 (A)

*A: Additive.

When *C. winterianus* oil and carvacrol were individually tested against the bacteria, it was observed that they were effective at relatively high concentrations, contrary to their combinations. The activity of EO against *K. pneumoniae, E. coli, S. aureus*, and *E. faecalis* was increased with carvacrol addition by 16-, 8-, 4-, and 2-fold, respectively. In addition, *C. winterianus* increased the efficacy of carvacrol 2-fold against *E. coli, K. pneumoniae, S. aureus* and 8-fold against *E. faecalis* (Table 3).

DISCUSSION

In a previous study, EO of *C. winterianus* showed antibacterial activity against *S. aureus*, *Staphylococcus epidermidis*, *Streptococcus mutans*, *Salmonella* Typhimurium, and *C. albicans* (Verma et al., 2020). In another study, *C. winterianus* EO was reported to reveal moderate antibacterial activity against *Bacillus cereus*, *Micrococcus luteus*, and *S. aureus* but less activity against Gram negative bacteria. The MIC of *C. winterianus* against *S. aureus* was 2 µL/mL. MICs against Gram negative bacteria including *E. coli*, *Proteus mirabilis*, and *Salmonella* Enteriditis were 4-6 µL/mL (Simic et al., 2008). The activity of EO of *C. winterianus* against fifteen strains of *C. albicans* was determined by MIC, minimum fungicidal concentration (MFC) and time-kill methods. The oil showed antifungal activity against *C. albicans*. MIC of the oil ranged from 78 to 625 µg/mL, whereas MFC was in between 312 and 1250 µg/mL (Oliveira et al., 2011). It was also noticed that geraniol, one of the main components of *C. winterianus* EO, presented many pharmacological properties including antibacterial and antifungal activity (Lira et al., 2020). Combination studies using geraniol and different EOs have been carried out. The most commonly used technique in these studies was checkerboard assay. Combinations of geraniol and several standard antibiotics such as norfloxacin and chloramphenicol were found to be synergistic for majority of the strains tested. Antagonism was reported with tetracycline (Lira et al., 2020). However, the combination studies are limited. Collectively, findings of the present study were promising against *S. aureus*, *E. faecalis*, *E. coli* and *K. pneumoniae*. Combinations of both EO and carvacrol additively increased the antibacterial activity of one another.

Consequently, the additive effect of EO and carvacrol appears promising, potentially offering new strategies against the tested

bacteria. Nevertheless, additional *in vivo* studies are necessary to explore the effectiveness of combinations of EOs with antibiotics as well as to assess different formulations.

CONCLUSION

As a result, the combined use of EOs and carvacrol is promising. Since the oral use of EOs is limited, studies on externally used products containing these combinations can be planned in the future.

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Chronotoxicity Studies in Pharmaceutical Science: A Comprehensive Review

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Abstract

Chronotoxicity evaluates the time-dependent toxicity of xenobiotics together with an individual's circadian rhythm. Suprachiasmatic nuclei located in the hypothalamus regulate circadian rhythms in individuals. Circadian rhythms are important for human health, metabolic processes, inflammation, and various cancers. This comprehensive review aims to provide an overview of the literature on chronotoxicity, circadian pharmacokinetics, and chronoefficiency. Our literature search was conducted using databases including "Web of Science," "PubMed," and "Science Direct." We used the keywords "circadian rhythm dysregulation," "chronotoxicity of therapeutics," "chronotoxicity," and "time-dependent toxicity" for our literature search. Chronopharmacokinetics studies pharmacokinetic changes related to dosage time. Light plays an important role in circadian rhythm by stimulating ganglion cells. The stimulus is transferred to the suprachiasmatic nuclei and other parts of the brain that regulate the circadian rhythm. Evaluation of the risks and benefits of various therapeutic options requires detailed knowledge of the complex mechanisms that regulate circadian rhythms.

Keywords

Chronotoxicity, chronopharmacokinetic, chronotherapeutics, chronoefficacy, circadian rhythms.

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INTRODUCTION

Chronopharmacology is a scientific field that studies time-dependent physiological responses to drugs. In particular, it examines the influence of endogenous biological rhythms on the outcome of medical treatment. It has been proposed that chronotherapeutic methods can improve the efficacy of many drugs. For instance, chronotherapy is a successful treatment approach for various types of cancers. Chronotherapy improves the tolerability and antitumor efficacy of anticancer drugs in both experimental animals and patients with cancer. Since 2010, toxicologists and pharmacologists have increasingly focused on circadian rhythms, focusing on understanding their consequences (Ayyar et al., 2021).

Chronotoxicology is a subfield of chronopharmacology that focuses on the undesired and adverse effects of xenobiotics on living organisms in relation to their circadian rhythms. Circadian rhythm, known as the internal biological clock, is the cycle of physical, mental, and behavioral changes within organisms that naturally occurs in every 24 hours in response to the environment. Circadian rhythms are thought to be regulated and maintained by the “central clock” or “master clock” which is located in the suprachiasmatic nuclei (SCN) of hypothalamus (Koopman et al., 1989). The circadian timing system plays a crucial role in numerous biological processes and parameters, as depicted in Figure 1.

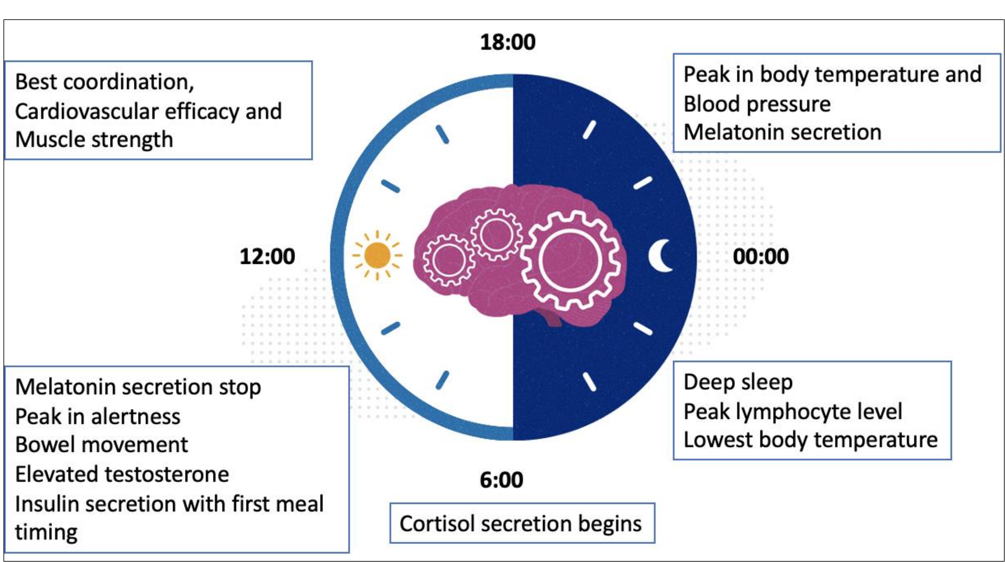

Figure 1: Biological process underlying circadian rhythm.

The importance of circadian rhythm in maintaining both systemic and tissue-level homeostasis, is well recognized. Disruption of the rhythm has direct consequence for human health, disorders, and diseases. In-depth studies on the daily patterns of cells

and tissues have uncovered significant effects of the circadian rhythm on how medicines and xenobiotics are processed and their consequences on the body (Ayyar et. al., 2021). For instance, metabolic syndrome, inflammation, and cancer are caused by disruptions in the circadian rhythm. Plants represent a crucial natural reservoir of diverse underexplored bioactive compounds. Therefore, the investigation of plant metabolites and their biological effects remains a focal point of scientific interest. The ultimate goal is to discover bioactive natural compounds and to advance the development of alternative, green, and sustainable technologies that can reduce or eliminate the reliance on hazardous substances in everyday life (Clairambault, 2007). In addition, circadian rhythm may change the severity of some diseases as listed below:

- Myocardial infarction and stroke incidence are high in the early morning due to a rapid rise in blood pressure (Mohd Azmi et al., 2021).
- Individuals with asthma are generally more susceptible to nocturnal exacerbations, primarily owing to the accumulation of inflammatory cells in the airways overnight (Mason et al., 2020).
- Rheumatic arthritis patients experience joint stiffness and pain in the morning or after a period of inactivity (Jacob et al., 2020).
- Patients with osteoarthritis experience pain in the afternoon, which could be due to decreased secretion of cortisol, an anti-inflammatory hormone, at night (Koyanagi, 2021).

Extensive investigations in mice and rats have been conducted to elucidate the complexities of circadian rhythm effects. Researchers have discovered notable differences in the regular physiological functions and harmful effects of drugs by subjecting laboratory animals to alternating periods of light and darkness lasting 12 hour each (Emmer et al., 2018). Within the 24 hour cycle, various biological processes such as cholesterol production and glucose homeostasis (Mason et al., 2020), oxidative stress levels (Budkowska et al., 2022), and cortisol equilibrium (Mohd Azmi et al., 2021), undergo variations. Recognizing that changes in circadian rhythms might affect how pharmaceuticals work in a person's body highlights the importance of considering individual variations in weekly, seasonal, and yearly circadian rhythms when assessing the balance between the risks and benefits of a therapeutic approach.

MATERIALS AND METHODS

A comprehensive and thorough search was performed on the Web of Science, PubMed, and ScienceDirect platforms to identify relevant papers. The search scope included studies that were published between 2014 and 2024, with a thorough examination of the chosen literature. To ensure the retrieval of publications closely linked to the research emphasis, a carefully selected set of keywords, such as “circadian rhythm dysregulation”, “chronotoxicity of therapeutics”, “chronotoxicity”, and “dosage time-dependent toxicity”, were used. Only publications written in English and available as full text were included in study, guaranteeing a meticulous and thorough examination of the pertinent scientific literature. Articles discussing chronotoxicity studies in cancer and cardiovascular diseases were targeted for this research.

Pharmacokinetic and Pharmacodynamic Base Chronotoxicity

Pharmacokinetic that deals with disposition process and fate of the drug is generally divided into four key components: Absorption, distribution, metabolism, and extraction (ADME). ADME describes what the body does to the drug. The four steps determine drug and metabolite concentrations in target tissues and organs, thereby effecting drug efficacy and toxicity (Okyar et al., 2024).

Pharmacokinetic behavior is influenced by various physiological factors including blood flow, gastric motility, enzyme activity, and renal function. Dosing time is also a variable that affects pharmacokinetics, that is referred to dosing time-dependent pharmacokinetics or chronopharmacokinetics (Koyanagi, 2021). Light plays a significant role in regulating the human circadian cycle via effectively synchronizing our internal clocks. When light reaches the eye, it predominantly stimulates the retinal ganglion cells which convert photon energy into an electrical signal. This vital information is subsequently transmitted directly to the suprachiasmatic nucleus neuron (SCN) and the other particular brain regions essential for controlling circadian elements such as sleep, alertness, and mood (Clairambault, 2007). The series of reactions entails the production of humoral, metabolic, and neurological signals, which act as messengers to synchronize the numerous internal clocks in the body. This synchronization results in a cohesive rhythmic arrangement of several cellular activities (Albrecht, 2020). SCN generates autonomous circadian rhythms through two interconnected molecular feedback loops in

circadian locomotor output cycles kaput (CLOCK) gene expression. These loops oscillate over about 24 hours to regulate their own expression. The positive loop is regulated by transcription factors, specifically brain and muscle ARNT-like protein 1 (BMAL1) and CLOCK. As a result of this activation, the period and cryptochrome genes (*per1*, *per2*, *cry1*, and *cry2*) are transcribed. Afterward, PER and CRY proteins gather in the cytoplasm, combining to create the PER/CRY heterodimer. The heterodimer then moves to the nucleus, where it reduces its own production by blocking Clock/Bmal1-mediated transcription. In addition, this complex transcriptional network has a far-reaching impact on the regulation of multiple genes found in different tissues, referred to as clock-controlled genes (CCGs), which are crucial for cellular physiology and metabolism (Korenčič et al., 2014). The dynamic characteristics of drug absorption and pharmacokinetics have been widely studied in animals and humans, and involve many medications (Ohdo, 2010).

Cytochrome (CYP)-mediated metabolic reactions can be either detoxification or bioactivation, where this metabolic pathway plays an essential role in determining drug chronotoxicity. Acetaminophen (APAP)-induced hepatotoxicity is primarily caused by the formation of the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI), which is mediated by CYP2E1 (Bielefeld et al., 2018). Studies revealed that the hepatotoxicity of APAP exhibits a strong circadian rhythm, with more severe toxicity occurring in the evening but milder toxicity in the morning in mice. This variation is attributed to the diurnal expression of CYP2E1, which is high in the nighttime and low in the daytime. Nutrient and drug transporters in the gastrointestinal tract exhibit a circadian rhythm, as documented by the circadian differences in the absorption of nonsteroidal anti-inflammatory drugs (NSAIDs) such as diclofenac, indomethacin, and ketoprofen, which exhibit higher absorption after administration of the drug in the morning. The increased rate of absorption may be explained by daily variations in the gastric transit time and intestinal blood flow (Fernandez et al., 2011).

Propranolol, a β-blocker used to treat hypertension, exhibits a greater efficacy when administered in the morning. The lipophilic nature of propranolol may contribute to its rapid absorption in the morning, which is thought to be caused by daily changes in gastrointestinal physiological factors such as gastric pH and gastric emptying rate. Intestinal Abcc2, an ATP-binding cassette efflux transporter, is associated with circadian changes. The

absorption of chemotherapeutic agents, such as methotrexate (MTX), is typically higher when Abcc2 expression is low but lower when Abcc2 is high. Due to lower intestinal Abcc2 expression in dark phase, tissue accumulation and toxicity of MTX are higher than those in light phase. Numerous unwanted side effects such as hepatotoxicity and nephrotoxicity have been reported in patients treated with MTX (Jacob et al., 2020).

Limited research has been conducted on diurnal fluctuations in the dispersion of drugs in various tissues from the overall blood circulation. Circadian shifts can affect the transportation of small-molecule medications by causing diurnal changes in cardiac output and blood flow rates to organs, including the brain, liver, skin, and muscles (Bicker et al. 2020). Additionally, ion channels, transporters, and efflux pumps display diurnal fluctuations in tissue expression, possibly affecting the transport and removal of drug substrates. Protein binding in the plasma and tissue plays a crucial role in drug distribution (Ayyar et. al., 2021). Albumin and other plasma proteins show daily fluctuations in their presence in the bloodstream. These variations can modify the proportion of unbound drugs in the plasma, thereby affecting their distribution. The levels of lidocaine in the plasma exhibit a 2-fold variation based on the number of hours in a day, potentially because of the time-dependent changes in albumin concentrations influenced by the circadian rhythm (Bruguerolle et al., 1983).

The circadian timing system is essential for optimizing metabolism and energy expenditure, with fasting-feeding cycles and rest-activity rhythms serving as the significant timing signals. The system also significantly affects drug metabolism, namely circadian pharmacokinetics and pharmacodynamics, resulting in alterations in therapeutic effectiveness and toxicity (Erkekoglu and Baydar 2012; Levi et al., 2007). The liver is the primary organ responsible for drug biotransformation in the body. Factors that influence drug metabolism include the rate of blood flow in the liver, the activity of certain enzymes, and the binding of drugs to plasma proteins (Fernandez et al., 2011). Circadian rhythms are evident in the gene expression and activity of many enzymes that play a role in drug metabolism. Core clock genes regulate the transcription of several enzymes, and circadian fluctuations have been detected in almost all variables that affect hepatic metabolism (Sukumaran et al., 2010). Recent advancements in molecular biology techniques have revealed both the direct and indirect processes by which core clock genes regulate the transcription of circadian drug-metabolizing enzymes (Dong et al., 2020). Bmal1 and Clock induce circadian

rhythmicity in the expression of CYP2a4/5, Ugt1a1, Fmo5, and Sult1a1 by directly activating their transcription via binding to E-box elements in the promoter regions of these genes. Bmal1/Clock also indirectly controls the activity of drug-metabolizing enzymes, Bmal1, and the daily expression of CYP3a11 through Dbp and Hnf4α (Lin et al., 2019).

Drug excretion, whether in its original form or as metabolites, is the ultimate stage in the process of drug elimination. The kidney is the main organ responsible for the disposal of majority of medicines. However, many pharmaceuticals are also excreted through the bile and eliminated in the feces (Ayyar et al., 2021). Circadian rhythms are observed in all three processes involved in kidney excretion, namely glomerular filtration, tubular secretion, and tubular reabsorption. Research indicates that the glomerular filtration rate (GFR) exhibits a diurnal fluctuation, reaching its highest point during daylight hours and decreasing throughout the nighttime in humans (Koopman et al., 1989). The diurnal variation in renal clearance, mainly for medicines eliminated via glomerular filtration with minimal protein binding, depends on GFR variability and plasma protein binding fluctuations. The fluctuation in plasma protein binding over 24 hours can also impact the daily variations in renal excretion (Sukumaran et al., 2010). The kidneys have an inherent circadian timing system that regulates certain genes responsible for managing secretion/absorbtion of sodium ions, water balance, and the transport of nutrients and xenobiotics. Certain transporters in the proximal tubules of the kidney exhibit circadian cycle. The passive reabsorption of a drug is influenced by its lipophilicity, pKa, urine pH, and flow rate; all of which exhibit diurnal variations (Zuber et al., 2009).

Chronotoxicity of Therapeutic Drugs

Within the field of cancer, the use of a portable programmable pump is considered as a revolutionary method to improve the efficacy of cytotoxic medications delivered through systemic routes (Mak et al., 2020). This technique selectively delivers chemotherapy during the circadian rhythm, when healthy tissues are most sensitive, to minimize the dosage during periods of increased vulnerability.

An example of such an improved technique is the precise timing of the distribution of 5-fluorouracil (5-FU), a commonly used medication in the adjuvant, neoadjuvant, and metastatic therapy of many types of cancers, such as breast, esophageal, and colorectal cancers (Maeda et al. 2018) Interestingly, the injection of 5-FU specifically targets cancer cells but other cells in the body are also affected (Mohd Azmi et al., 2021). 5-FU causes

hematotoxicity depending on the time of administration. Administration of 5-FU at a dose of 300 mg/kg resulted in a significant and statistically measurable reduction in the average number of white blood cells (WBC) circulating in the bloodstream within a 24 hour timeframe, leading leukopenia. Mice treated with 5-FU at zeitgeber time (ZT) 5 and 21 showed maximum and minimum leukopenia, respectively (Bouali et al., 2023). Treatment with 5-FU near the daily awakening period leads to the least damage to the bone marrow and intestine. It enhances key genes' expression and enzymatic activities in the pyrimidine metabolic pathway, resulting in the greatest antitumor effect and the best survival. Moreover, for the optimal timing of effective cancer therapy, it is crucial to know the time of day when tumors reproducibly express proliferative targets relevant to DNA synthesis or cell division. Several chemotherapeutic drugs are toxic to both cancerous and healthy cells in the process of cell division. Furthermore, these drugs often exhibit cytotoxic effects at specific cell cycle stages. For example, drugs such as 5-FU and irinotecan are more effective against cells that are in the S phase (DNA synthesis) of cell division. The number of cells in the S and G2/M phases increased by approximately 50% during the second half of the dark period in the bone marrow tissue of mice, whereas G0/G1 cells were predominant during the light period. The optimal time for administering 5-FU, irinotecan, docetaxel, and gemcitabine in mice is during the early light period, when BCL2 expression (antiapoptotic) is high but proapoptotic BAX expression, is low. However, some anticancer drugs such as oxaliplatin which produces DNA cross links do not have phase specificity (Okyar et al., 2024).

Dihydropyrimidine dehydrogenase (DPYD) is a rate-limiting enzyme that detoxifies 5-FU. Several studies have revealed that the transcription and activity of DPYD reach its peak level during the midnight hours, which could impact the drug's tolerability when administered at these times (Cardinali et al., 2023).

Cisplatin is a highly effective chemotherapeutic agent that has ototoxicity as a notable side effect. Cisplatin has been shown to correlate with circadian timing, since it exhibits the highest efficacy as an antitumor agent and the lowest toxicity to the kidneys when administered during the active (dark) phase of the light-dark cycle in rodents. These findings suggest that administration of cisplatin during the active phase reduces the likelihood of ototoxicity in Fischer rats. This finding aligns with the reduced kidney toxicity observed in animals exposed to cisplatin during nighttime. The substantial differences in threshold shifts

between light and dark exposures suggest that the timing of exposure has a notable influence on susceptibility to cisplatin-induced hearing loss (Ma et al., 2023).

Oxaliplatin is one the therapeutics currently used for the treatment of colorectal cancer (Bruguerolle et al., 1983). It is widely used in combination with 5-FU and irinotecan. The combination therapy can cause hematological toxicity depending on the time of administration (Dulong et al., 2022). Preclinical studies on the chronotoxicity of oxaliplatin carried out on male mice, revealed diurnal patterns of hematological and intestinal toxicities. The most pronounced consequences were detected around ZT7, corresponding to the start of light in a 12-hour light/12-hour darkness cycle when the mice were exposed to these alternating cycles (Boughattas et al., 1994).

The human cardiovascular system exhibits disting diurnal patterns over 24-hour cycles, including variations in the heart rate, blood pressure, circulating catecholamines, blood coagulation indicators, vascular endothelial function, and autonomic nervous system activity. Significantly, various cardiovascular systems undergo changes in the morning. Notably, these changes in the morning rhythm are linked to the development of Cardiovascular Disease (CVD) (Buurma et al., 2019).

Platelets are key role in arterial thrombosis, and aspirin prevents it by suppressing platelet function, with patients prescribed a maintenance dose of 75-325 mg daily. Despite lifestyle modifications and drug-based interventions, 10-33% of these patients experience relapse of cardiovascular events within 5 years, with the exact causes unknown but likely multifaceted (Buurma et al., 2019). Aspirin was administered orally. To decrease the fraction of uncontrolled platelets during the morning hours to 5%, one can align with the circadian cycle and consume aspirin before going to bed (Bonten et al., 2014; Krasińska et al., 2021).

Clopidogrel, a frequently given antiplatelet medication for stroke and heart attack, is metabolized by many CYP450 enzymes, including carboxylesterase 1 (CES1). A recent study revealed notable difficulties in achieving successful treatment results with clopidogrel, mainly due to drug resistance caused by differences in the expression of enzymes responsible for metabolizing the medication. Significantly, multiple instances of hepatotoxicity have been documented. Research suggests that clopidogrel has a strong diurnal pattern in its efficacy in wild-type mice, with a more powerful antiplatelet effect during the active phase (ZT 22) than during the resting period (ZT10). This is consistent with clinical observations showing improved effectiveness when clopidogrel is administered to humans in the morning.

Deletion of CLOCK increased the toxicity of clopidogrel and disturbed its daily cycle. In addition, the liver toxicity of clopidogrel is linked to daily variation in the expression of CES1D, which contributes to the complexity of its negative effects (Ma et al., 2023). Therefore, chronopharmacology and chronotoxicology are two important phenomena that significantly affect the fate of medicine (Kaur et al., 2013). Figure 2 provides an overview of various drugs that may be influenced by the phenomena.

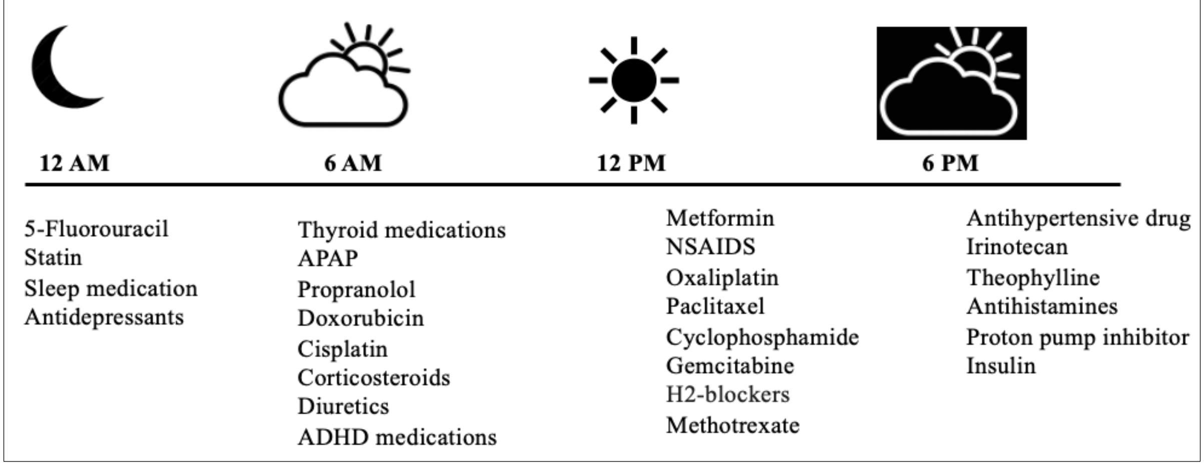

Figure 2: Certain medications whose efficacy and toxicity profiles are influenced at different times of the day.

CONCLUSION

The pharmacokinetic and pharmacodynamic characteristics of drugs show variations related to genetic predisposition, gender, and age of the individuals. The timing of drug administration has important effects on the efficacy and toxicity profiles of the drugs. Studying the regulation and dysregulation of chronotoxicity will help in the development of chronoefficient therapeutic options. Further research and the application of these findings could lead to more effective and safer pharmacotherapy regimens.

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