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EDITORIAL WORDS

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Editor in Chief
Borneo J Pharm

Assalamu'alaikum Wr. Wb.

Alhamdulillahirabbil 'alamin. The next edition of **Borneo Journal of Pharmacy** (*Borneo J Pharm*), has been published at May 2022. This edition contains ten articles consisting of Pharmacology-Toxicology, Pharmacognosy-Phytochemistry, Pharmaceutical, Microbiology Pharmacy, Natural Product Development, and Clinical-Community Pharmacy. This edition includes writings from four countries including India, Indonesia, Nigeria, and Sri Lanka. The authors come from several institutions, including Universitas Pancasila, University of Jaffna, Universitas Muhammadiyah Prof. Dr. HAMKA, Universitas Setia Budi, Federal University Birnin Kebbi, Kano State Polytechnic, Universitas Muhammadiyah Palangkaraya, Prathima Institute of Medical Sciences, Bhaskar Pharmacy College, Ganapathy Degree College, Universitas Muhammadiyah Malang, Universitas Muhammadiyah Magelang, and Universitas Andalas.

Editorial boards are fully aware that there are still room for improvement in this edition, hence with all humility willing to accept constructive suggestions and feedback for improvements to the publication for the next editions. The editorial board would like to thank all editors and reviewers, and contributors of the scientific articles who have provided the repetoire in this issue. We hope that all parties, especially the contributors, could re-participate for the publication in the next edition on August 2022.

Wassalamu'alaikum Wr. Wb.

Palangka Raya, May 2022

Editor-in-Chief

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Author Guidelines

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1. Writing is the result of research or review of critical analysis study in the field but not limited to Pharmacology-Toxicology, Pharmacognosy-Phytochemistry, Pharmaceutical, Analytical Pharmacy-Medicinal Chemistry, Natural Product Development, Clinical-Community Pharmacy, Management Pharmacy, and other Pharmacy aspects which have never been published in other scientific publication media.
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   e. Methodology, Research method consists of 2 sub-chapters including materials and methods. If data analysis is specific, or the way of analysis is novel, then data analysis subchapter could be added. The sub-chapters are written without numbering or bullet.
   f. Results and Discussion, at least contain three subjects (1) description of results, (2) interpretation or explanation of results, and (3) comparative of results with previous studies results. If the subchapters very length could be made sub-sub-chapters with Arabic numbering. Description of the results can be in the form of tables and figures with a serial number (Table uses a sequence of Roman numerals and placed at the top, while figures using Arabic numeric sequence and placed in the below).
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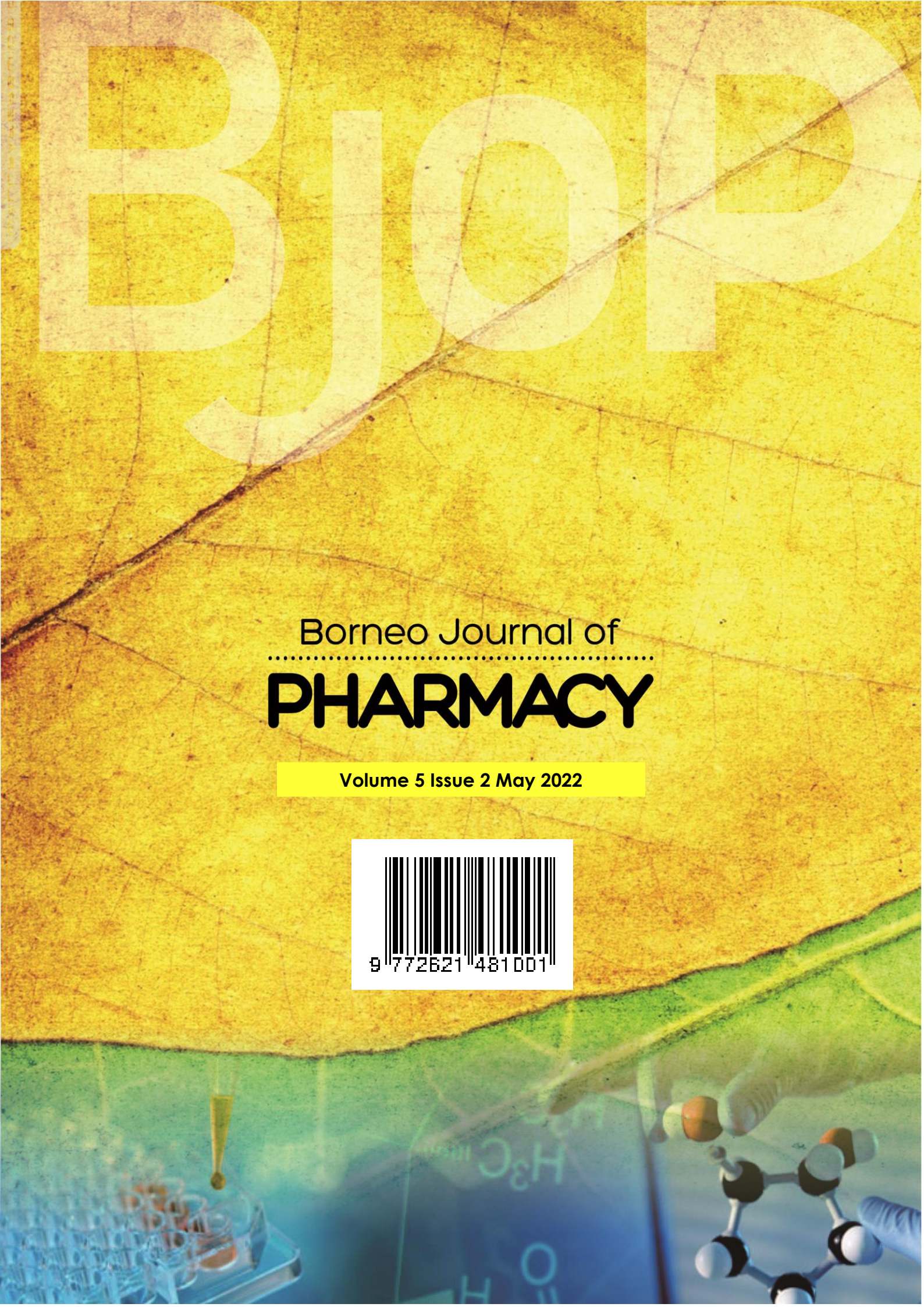
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Research Article

Anti-Inflammatory and Analgesic Activity of *Musa balbisiana* Peels *In Vivo*

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Musa balbisiana peels

Abstract

Musa balbisiana Peels (MBP) contains high levels of flavonoids, alkaloids, tannins, saponins, and triterpenoids. Flavonoids function to slow down the inflammatory process by inhibiting the arachidonic acid, forming prostaglandins, and releasing histamine. This study aimed to examine the anti-inflammatory and analgesic effects of MBP decoction. This study used the Winter method for anti-inflammatory assay by induction of carrageenan on the soles of rat's feet and Sigmund's method for analgesic assay with intraperitoneal induction of acetic acid in mice. Group I as a negative control, group II as a positive control with diclofenac sodium, group III as a low dose (200 mg/kg BW of MBP), group IV as a medium dose (400 mg/kg BW of MBP), and group V as a high dose (800 mg/kg BW of MBP decoction). The percentage of inhibition in the anti-inflammatory test in rats for groups II, III, IV, and V was 34.43%, 17.68%, 25.53%, and 25.4%, and the percentage of effectiveness for the anti-inflammatory test, respectively, was 51.35%, 74.15%, and 74.01%. The results of the percentage inhibition of the analgesic test in mice for groups II, III, IV, and V were 55.25%, 38.52%, 44.53%, and 49.31%, and the percentage of effectiveness for the analgesic test, respectively, followed by 69.71%, 80.59%, and 89.24%. Based on the results, it can be concluded that the decoction of the MBP has an anti-inflammatory and analgesic effect.

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INTRODUCTION

Inflammation is a complex biological response of vascular tissue to noxious stimuli such as pathogens, damaged body cells, or irritants[1]. Inflammation is triggered by releasing chemical mediators from damaged tissues and cell migration[2]. Pain, redness, swelling, and tissue and organ dysfunction are signs of inflammation[3]. This is a protective response made by the body against tissue damage caused by various stimuli[4]. In such cases, their defense reactions may cause progressive tissue injury, and anti-inflammatory or immunosuppressive drugs may be required to modulate the inflammatory process[5].

Pain is the most common symptom when inflammation occurs and can reduce the quality of life. Pain is an unpleasant sensory and emotional feeling associated with tissue damage[6]. Pain is often described as either noxious (noxious, protopathic) or harmless (non-noxious, epicritic), for example, light touch, warmth, or light pressure. Most people feel disturbed, uncomfortable, and tormented by the pain[7]. Many people cannot stand it and try to relieve pain by using painkillers or analgesics. Many different types of therapy have been developed to reduce pain caused by inflammation. In controlling inflammation and pain, drugs that can inhibit the disease are needed, so anti-inflammatory and analgesic drugs are needed, better known as anti-inflammatory and analgesic drugs (NSAIDs)[8]. The principal mechanism of action of NSAIDs, as analgesics, is the blockade of prostaglandin synthesis through cyclooxygenase inhibition (COX-1 and COX-2

enzymes) so that the production of PGI2 (prostacyclin) by COX-1 as a gastroprotective is also inhibited[9]. So if the therapy for pain and anti-inflammatory is carried out for a long time and requires high doses of drugs, that can be affected by side effects on the stomach[10].

Many herbal plants have been developed and have therapeutic effects for inflammation and pain, such as modern allopathic drugs with a single active substance with a single target pathway of action. Herbal medicines consist of various active molecules that work synergistically with various action targets[11]. The utilization of natural resources in the form of plants has long been used to cure diseases[12]. One of the plants used in medicine is *Musa balbisiana* (one type of banana). *Musa balbisiana* developing research, especially in terms of pharmacology and phytochemicals, is based on indications of medicinal plants that some people with empirically proven efficacy have used[13].

Musa balbisiana peels (MBP; **Figure 1**) have not been used optimally as traditional medicine, even though the fruit is widely used and consumed in the community. The use of this peel can undoubtedly reduce the organic waste of the MBP. The MBP contains high flavonoid content. Based on the results of the phytochemical screening conducted, it was known that the MBP contained flavonoids, alkaloids, tannins, saponins, and triterpenoids[14]. The GC-MS analysis showed that the major component of *M. balbisiana* extract was difluroisocyanotophosphine[15]. By inhibiting the arachidonic acid metabolic pathway, the formation of prostaglandins, and the release of histamine, flavonoids function as an anti-inflammatory or slow down the inflammatory process[16]. Flavonoids are anti-inflammatory agents because flavonoids in the body act to inhibit lipoxygenase enzymes, the role in leukotriene biosynthesis[17].

Figure 1. *Musa balbisiana* fruit

In addition to inhibiting the metabolism of arachidonic acid by reducing prostaglandins production, flavonoids also inhibit the secretion of lysosomal enzymes, which are inflammatory mediators. Inhibition of these inflammatory mediators can inhibit the proliferation of the inflammatory process[18]. Saponins are thought to interact with many membrane lipids. Membrane lipids such as phospholipids are precursors of prostaglandins and other inflammatory mediators. Saponins are thought to inhibit the increase in vascular permeability so that edema as a sign of inflammation does not occur[19]. Tannins have antioxidant activity, and antioxidants act as an anti-inflammatory in various ways, including inhibiting the production of O_2 oxidants by neutrophils, monocytes, and macrophages[20]. Based on these things, the authors are interested in further researching the anti-inflammatory effect that will be tested using the Winter method and the analgesic effect that will be tested using the Sigmund method of MBP decoction. The community has widely used this decoction method to manufacture traditional medicine because the extraction process is easy to do and does not require special tools. The anti-inflammatory and analgesic research carried out using MBP decoction aims to prove that the MBP has anti-inflammatory and analgesic effects.

MATERIALS AND METHODS

Materials

Musa Balbisiana peels fruit used in this study was obtained from Trirahayu Village, Negeri Katon District, Pesawaran Regency, Lampung Province, Indonesia. The experiment used Sprague Dawley (SD) male white rats aged 2-3 months with a bodyweight of 150-200 g as an anti-inflammatory test, and the Deutsche Denken Yoken strain male white mice aged 2-3 months old and weighed 25-30 g as an analgesic test, developed at the Non-Ruminant and Animal Hope Laboratory, Faculty of Animal Husbandry, IPB University. Other materials include diclofenac sodium, 0.5% CMC sodium, 1% carrageenan, 3% acetic acid, aquadest, feeding tube, stopwatch, and plethysmometer.

Methods

Plant determination

Plant samples identified as *M. balbisiana* were determined at Herbarium Bogoriense, Botany, Indonesian Institute of Sciences, Research Center for Biology, Cibinong Science Center, Bogor, Indonesia, with number 353/IPH.1.01/If.07/II/2020.

MBP decoction preparations

As much as 10 g of MBP powder was added with 200 mL of water, then it was heated until the volume was half of the initial volume with occasional stirring and then filtered through a filter heat sufficiently to obtain the desired volume of 100 mL.

Anti-inflammatory test

This research was carried out after obtaining ethical approval number 101/II/2021/KEPK from the Health Research Ethics Committee Universitas Pembangunan Nasional Veteran Jakarta. All actions were taken by minimizing pain and suffering in experimental animals[21]. An anti-inflammatory test was carried out using the Winter method to form edema on the paw of rats[22]. Before the experiment was carried out on rats, rats fasted for ± 18 hours while still being given water. The rats were weighed on the day of testing; 25 rats were taken at random and divided into five groups, respectively, with five rats each. Group I was a control (-) and given aquadest + 0.2 mL 1% carrageenan; Group II was the control (+) and was given 8.02 mg/200 g BW diclofenac sodium + 0.2 mL 1% carrageenan; while Groups III, IV, and V were the dose group that was given orally MBP decoction of 200; 400; and 800 mg/kg BW, respectively, and given 0.2 mL 1% carrageenan on the soles of the feet rat. Before being treated, measure the initial volume of the rat's paws by dipping the rat's paws into the plethysmometer. In the treatment of each anti-inflammatory test group, the rats were given the preparation of the test substance orally according to the dose of each treatment group. Thirty minutes later, the rat's paws were induced with 0.2 mL 1% carrageenan intraplantar continued to measure the volume of rat paw edema every hour for five hours. The calculations for area under the curve (AUC), percentage of anti-inflammatory (% antiinflammatory), and percentage of anti-inflammatory effectiveness (% effectiveness) occurring in the test group were presented in **Equations 1** to **3.**

$$\text{AUC} = \frac{(\text{Vn}-1+\text{Vn})(\text{tn}-\text{tn}-1)}{2} \qquad \dots [1]$$

$$\%\ \text{antiinflammatory} = 1 - \left(\frac{\text{the average value of AUC}}{\text{the average of AUC control}}\right)\text{x}100\% \qquad \dots [2]$$

$$\%\ \text{effectiveness} = 1 - \left(\frac{\text{t\% anti}-\text{inflammatory test}}{anti-inflammatory\ of\ diclofenac\ sodium}\right)x100\% \qquad \dots [3]$$

Vn : Volume of rat paw at hour/minute n
Vn-1 : Volume of rat paw at hour/minute (n-1)
tn : Hour n or minute n
tn-1 : Hour (n-1) or minute (n-1)

Analgesic test

The analgesic test was performed using the Sigmund method[23]. Before the experiment was conducted on mice, the mice fasted for ± 18 hours while still being given water. On the test day, the weight of the mice was weighed, and 25 mice were

taken at random and divided into five groups, with five mice each. Group I was a control (-) and was given aquadest + 0.2 mL/20 g BW 3% acetic acid; Group II was a control (+) and was given 81.16 mg/20 g BW diclofenac sodium + 0.2 mL/20 g BW 3% acetic acid; while Groups III, IV, and V were the dose group that was given orally MBP decoction of 200; 400; and 800 mg/kg BW, respectively, and given 0.2 mL/20 g BW 3% acetic acid. In the treatment of each analgesic test group, mice were given the test substance orally by the treatment dose of each group. Thirty minutes later, the mice were induced with 0.2 mL/20 g/BW 3% acetic acid intraperitoneally. Then the mice were placed in the cage; after the administration of acetic acid, the mice would give a writhing response which was indicated by moving a pair of front legs that were pulled forward and a pair of hind legs that were pulled back and rubbing their stomach against the bottom of the cage. The mice were observed, then the number of stretches shown by the mice was recorded every five minutes for an hour. The calculations for AUC were given in **Equation 4**, while % antiinflammatory and % effectiveness was calculated using **Equations 2** and **3**.

$$\mathrm{AUC} = ((\sum n - 1 + \sum n)(tn - tn - 1))/2 \quad \ldots [4]$$

∑n : Number of mice writhing in hours/minute n
∑n-1 : Number of mice writhing in hours/minute (n-1)
tn : Hour n or minute n
tn-1 : Hour (n-1) or minute (n-1)

Data analysis

The AUC values of data between each treatment group were analyzed using the SPSS® Statistical Analysis version 20. If the data on AUC values in all treatments had a normal and homogeneous distribution, the analysis would continue using one-way ANOVA (Analysis of Variance). If the results of the ANOVA test show that there is a statistically significant difference in each treatment, then the analysis will continue using the LSD (Least Significant Difference) test with a significance level of 5% (0.05) to determine whether there is a difference between each treatment. However, if the AUC value data has no requirements of a normal distribution and no homogeneity, the test continued with the Kruskal-Wallis method[24].

RESULTS AND DISCUSSION

Anti-inflammatory test

The data on the average volume of rat paw edema showed that the administration of the test substance reduced the volume of rat paw edema in the third hour after being induced by carrageenan. This shows that the ability of the test substance preparation can inhibit the increase in the volume of edema. The data is displayed in graphical form, as shown in **Figure 2**. Assessment of the effectiveness of anti-inflammatory drugs and looking at the increase and decrease in the volume of edema on the rat's paw can also be seen from the calculation of AUC. The greater the AUC value, the less effective an anti-inflammatory drug is. The results of the average AUC value in all treatment groups, the negative control AUC value was higher than all other test preparation groups. This indicates that carrageenan can induce the formation of edema on the soles of the rats' feet.

The AUC value of the test preparation and the positive control group was lower than the AUC value of the negative control group, indicating that the whole group of the test substance for the MBP decoction and the positive control had an anti-inflammatory effect. Based on the three decoction doses, it was found that the stew with a dose of 800 mg/kg BW was better at inhibiting the formation of edema in the soles of the rats' feet, as indicated by the lowest average AUC value compared to the other two groups (**Table I**). Based on the statistical test, it was found that there was a significant difference between the negative and the positive groups, and the three doses showed a decrease in edema volume compared to the negative group ($p<\alpha = 0.05$). There was a significant difference between the positive group and the dose group of MBP 200; 400; and 800 mg/kg BW, which indicated that the volume of edema in the positive group was smaller than that of the three-dose groups. There was no significant difference between the three-dose groups ($p<\alpha = 0.05$).

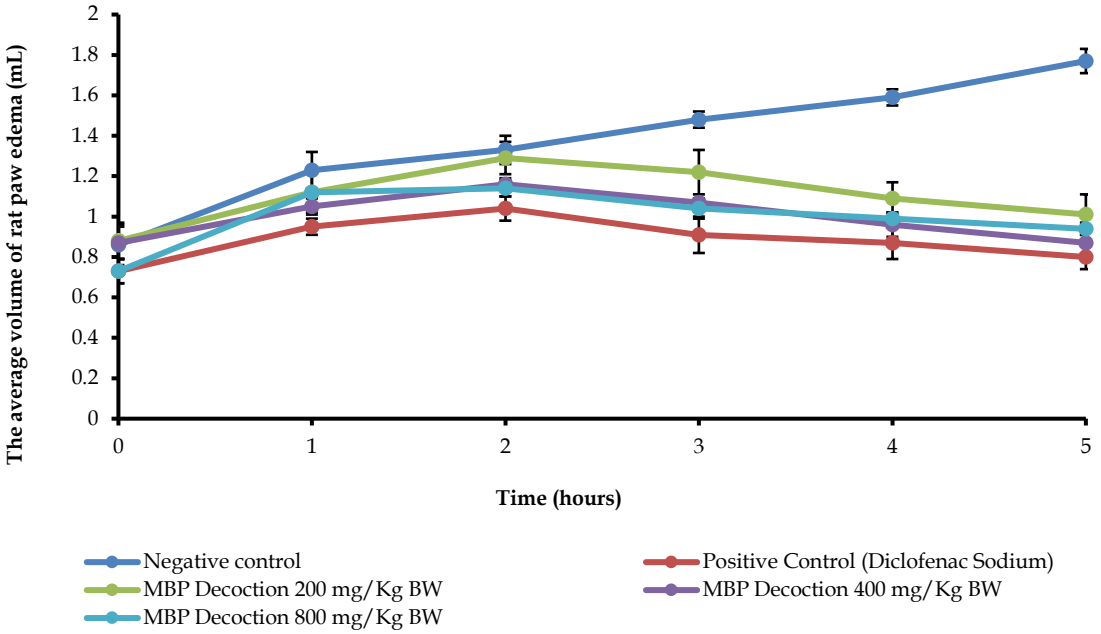

Figure 2. Correlation between time and the average volume of rat paw edema

Table I. The AUC values in the anti-inflammatory test

Groups	Value of AUC on rats (mL.hours)					Average ± SD
	1	2	3	4	5	
Negative control	6.74	7.31	6.98	7.06	6.31	6.88 ± 0.38
Positive control	4.62	4.09	4.50	4.53	4.83	4.51 ± 0.27
MBP 200 mg/Kg BW	5.44	6.00	6.25	4.75	5.86	5.66 ± 0.59
MBP 400 mg/Kg BW	5.30	5.17	4.93	4.86	5.36	5.12 ± 0.22
MBP 800 mg/Kg BW	5.02	5.18	4.93	5.03	5.40	5.13 ± 0.17

Analgesic test

Data on the average number of writhing in mice showed a decrease in the number of writhing in the 20th minute after being induced with acetic acid. This indicates the ability of the test substance to inhibit the increase in the number of writhing in mice. The data is displayed in graphical form, as shown in **Figure 3**. Assessment of the effectiveness of analgesic drugs and looking at the increase and decrease in the number of stretches in mice can also be seen from the AUC value. The smaller the AUC value, the greater the effectiveness of an analgesic drug. The results of the average AUC value can be seen that all doses have an analgesic effect because the negative control AUC value is higher than the positive control AUC value and other doses, but the 800 mg/kg BW has the lowest AUC value compared to the group other doses, as shown in **Table II**. There was a significant difference between the negative and positive groups and the three doses of MBP, which showed a decrease in the number of stretches in the positive and the three doses compared to the negative groups. There was a significant difference between the positive and three MBP dose groups, which showed that the number of stretches of the positive group was smaller than the three MBP dose groups. There was a significant difference between the MBP group at a dose of 200 mg/kg BW compared to the MBP group at a dose of 400 and 800 mg/kg BW, which showed the number of stretching of the MBP group at a dose of 200 mg/kg BW was more than that in the MBP group at a dose of 400 and 800 mg/kg BW. Moreover, there was a significant difference between the MBP group at a dose of 400 and 800 mg/kg BW, which showed that the MBP group at a dose of 400 mg/kg BW was more stretched than the 800 mg/kg BW group ($p<\alpha = 0.05$).

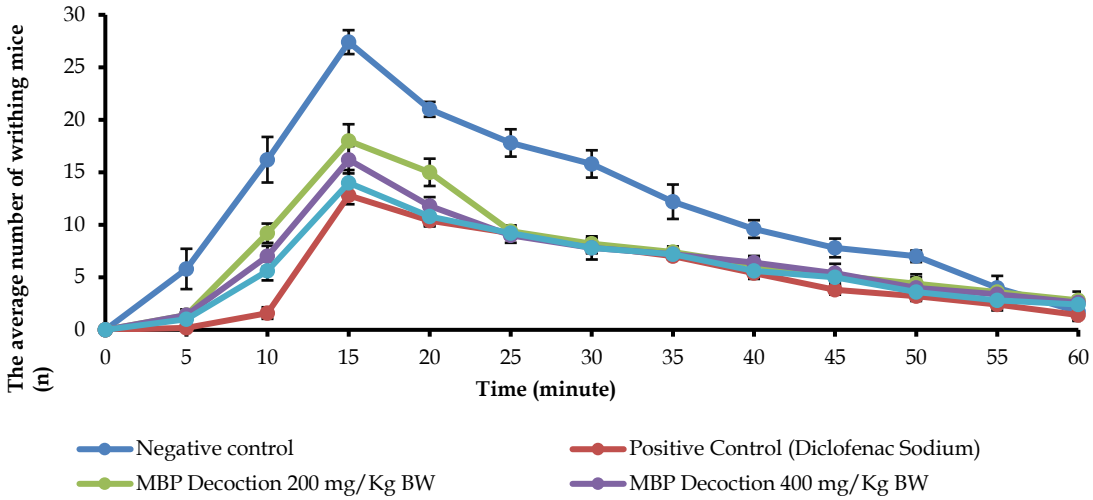

Figure 3. Correlation between time and the average number of writhing

Table II. The AUC values in the analgesic test

Groups	Value of AUC on mice (n)					Average ± SD
	1	2	3	4	5	
Negative control	732.5	735	657.5	710	722.5	711.5 ± 31.75
Positive control	330	322.5	332.5	312.5	320	323.5 ± 8.02
MBP 200 mg/Kg BW	430	450	432.5	475	435	444.5 ± 18,74
MBP 400 mg/Kg BW	392.5	395	390	420	407.5	401.0 ± 12.57
MBP 800 mg/Kg BW	372.5	355	372.5	372.5	360	366.5 ± 8.40

Percentage of inhibition of edema and writhing

The percentage of inhibition can be calculated from the average AUC data for the test and negative control groups, as shown in **Table III**. The positive control had better inhibition of edema and inhibition of the number of stretches than the test preparation group. It can also be seen that a decoction dose of 800 mg/kg BW (analgesic test) had the most significant inhibition of the amount of writhing compared to other decoction doses and a dose of 400 mg/kg BW (anti-inflammatory test).

Table III. % inhibition of edema and writhing of mice

Groups	% inhibition	
	% anti-inflammatory	% analgesic
Diclofenac sodium	34.43	55.25
MBP 200 mg/Kg BW	17.68	38.52
MBP 400 mg/Kg BW	25.53	44.53
MBP 800 mg/Kg BW	25.48	49.31

Percentage of anti-inflammatory and analgesic effectiveness

The percentage of effectiveness was calculated by comparing the average AUC of the test group with the average AUC of the positive control (diclofenac sodium), as shown in **Table IV**. The analgesic test preparation group at a dose of 800 mg/kg BW had better effectiveness as an analgesic compared to other doses, and the anti-inflammatory test preparation group at a dose of 400 mg/kg BW had a better anti-inflammatory effect than the analgesic test preparation group at a dose of 400 mg/kg BW with other doses.

Table IV. % anti-inflammatory and analgesic effectiveness

Groups	% effectiveness	
	% anti-inflammatory	% analgesic
MBP 200 mg/kg BW	51.35	69.71
MBP 400 mg/kg BW	74.15	80.59
MBP 800 mg/kg BW	74.01	89.24

The anti-inflammatory and analgesic research carried out using MBP decoction aims to prove that the MBP has anti-inflammatory and analgesic effects. In the MBP, some compounds are efficacious as anti-inflammatory and analgesic. The compounds contained in the peels of the *M. balbisiana* are flavonoids which are thought to have anti-inflammatory and analgesic activity[25]. The method used for the preparation of preparations is adjusted to the efficacious compounds, so it is hoped that these compounds are present in the preparations made. The decoction was chosen in this study because the stew is a method that is easy to apply, and the solvent is easy to obtain[26]. In the decoction, water is used as a solvent with high polarity. This high polarity will cause flavonoids to be attracted more during the extraction process[27]. The community has widely used this decoction method to manufacture traditional medicines because the extraction process is easy to do and does not require special tools. This method was chosen because flavonoid compounds are readily soluble in water; this property is influenced by the presence of OH groups in their structure which causes flavonoids to have polar properties and can dissolve in polar solvents. In addition, the infusion method, which is almost similar to decoct, succeeded in extracting total flavonoids as measured by UV-Visible spectrophotometry[28].

The doses of MBP decoction used for anti-inflammatory tests were 200, 400, and 800 mg/kg BW. In this study, the moderate and low doses of 400 and 200 mg/kg BW were based on the dose of ethanolic extract of the plant, one of which was the same species as the *M. balbisiana*, specifically *Musa acuminata*, which had been carried out by previous studies, in which these doses have been shown to provide anti-inflammatory and analgesic effects[29]. In this study, different test animals were used; the anti-inflammatory test was used by rats because the paws of rats were more prominent, so it was easier to measure and observe, while the analgesic test was used by mice because the mice were more sensitive to pain than rats and the reaction of mice to pain was easier to observe than mice[30]. Rats show more complex reactions than mice due to higher brain function. These reactions are, for example, sniffing, licking the soles of the feet, straightening the feet, or other unknown reactions[31].

In testing the anti-inflammatory effect using the Winter method, the parameter used is the decrease in the volume of edema in the soles of the mice (mL) compared to time (hours). This method was chosen because it is a commonly used anti-inflammatory test method, easy to perform, and can be measured quantitatively. Edema formation was carried out using carrageenan as a chemical induction of inflammation. Carrageenan was used because it did not cause injury or tissue damage to the rat's paws[32]. Carrageenan is more sensitive to anti-inflammatory drugs than other anti-irritants. In the phases of edema formation, there is the release of mediators that initiate the inflammatory process. The presence of edema formation phases also makes it easier to see the work of the anti-inflammatory substances that were tested more precisely, especially those that have a mechanism by inhibiting prostaglandin biosynthesis and COX formation. Edema that develops can last for six hours and gradually decrease over a day[33].

The instrument used in the anti-inflammatory test to measure the volume of edema in the soles of the rat's feet was a plethysmometer connected to a burette. The liquid used is mercury because mercury does not wet the rat's feet, and measurements are based on Archimedes' law; if an object is placed in a liquid, it will exert an upward force or pressure equal to the volume being pushed or moved. In the burette, methylene blue liquid was used to make it easier to read at the time of measurement, and the measurements were taken three times which were then averaged. When measuring the other rat's paw, the right paw does not kick the tool and interfere with the view when inserting the foot into the mercury[34].

Anti-inflammatory test studies were conducted on negative and positive controls and MBP decoction of 200, 400, and 800 mg/kg BW. Before testing from the 1st to the 5th hour (measurement of the volume of the rat's feet after carrageenan induction), a test was conducted at the 0th hour (before carrageenan induction). This was done to determine and ensure that the rat's feet were not swollen and in normal condition. This test was only carried out for five hours because the peak of edema that formed could not be observed. After all, the edema only lasted for six hours before slowly healing[35].

In the anti-inflammatory test, it was found that the average volume of edema in the negative control did not decrease the volume of edema in the rats' feet. In the negative control, the treatment given to rats only gave aquadest so that there was no inhibition of edema formation. Based on the average volume of edema in the positive control and MBP decoction, there was a decrease in edema on the soles of the rats. This happened because the positive control and MBP decoction had inhibitory activity on edema formation[36].

Anti-inflammatory activity can also be seen from statistical testing of negative control with the positive control, and MBP decoction for each dose showed a significant difference. The decrease in edema volume in the positive control and the three doses of MBP decoction occurred in the 3rd or 2nd hour after carrageenan induction. This shows that diclofenac sodium and MBP decoction inhibit edema formation because carrageenan induction can cause the release of inflammatory mediators (prostaglandins) three hours after carrageenan induction. This is also by the mechanism of action of diclofenac sodium as an anti-inflammatory which works by inhibiting COX and prostaglandin synthesis[37].

The statistical results of the positive control with MBP decoction of 200, 400, and 800 mg/kg BW showed a significant difference, and this indicates that diclofenac sodium as a positive control has better anti-inflammatory activity than the three doses of MBP decoction. However, there was no significant difference in the three doses of MBP decoction. This means that increasing doses of MBP decoction did not affect the inhibitory activity of edema or anti-inflammatory activity. When viewed from the AUC value of the average volume of the soles of the rat's paw, a dose of 800 mg/kg BW gave a better anti-inflammatory effect than a dose of 200 and 400 mg/kg BW.

The method used for the analgesic test used in this study is the Sigmund method; this method uses chemical stimulation with glacial acetic acid. 3% glacial acetic acid, 0.2 mL/20 g BW, can induce mild pain in mice, as indicated by writhing. The choice of this method is because this method is a simple method, easy to do, and commonly used. This method is also more specific for drugs that are thought to have prostaglandin inhibitory activity. The pain caused by glacial acetic acid only lasts for an hour and then gradually subsides[23,38]. The parameter measured in this method is the number of stretches of mice compared to time (minutes). No different from the anti-inflammatory test in this test, the test animals were fasted ± 18 hours before being given treatment; this was done so that the stomach organ was empty and there was no food left so that the test preparation that was absorbed by the body optimally was not disturbed by the existing food. Symptoms seen in mice when they feel pain after administration of acetic acid are characterized by contraction of the abdominal wall so that the legs are pulled back, stretch, and the abdomen touches the base of the space it occupies; this symptom is called writhing. This method's administration of the preparation was carried out 30 minutes before induction of glacial acetic acid and then observed for 60 minutes every five minutes. This aims to see that the test preparation work provides a protective effect against the pain caused by the inducer[39].

In the analgesic test, it was found that the average number of writhing of mice in the negative control was more than the average number of stretches of the positive control and preparations of MBP decoction at doses of 200, 400, and 800 mg/kg BW. This happened because the negative control was only given aquadest so that there was no inhibition of prostaglandin synthesis. On the other hand, the average number of stretches decreased in the positive control and preparations of MBP decoction. This shows that diclofenac sodium and the three doses of MBP decoction have analgesic activity. In the statistical test, it can also be seen that there is a significant difference between the negative control and the positive control and the three doses of MBP decoction. It can also be stated that the activity inhibits the synthesis of prostaglandins. This happens because acetic acid induces pain by stimulating the release of free arachidonic acid from the phospholipid tissue resulting in the formation of COX and prostaglandins so that drugs that can reduce the number of mice writhing due to the induction of glacial acetic acid can inhibit prostaglandin synthesis[40]. In the statistical test, positive control with three doses of MBP decoction has a significant difference, and this indicates that the positive control has better analgesic activity than the three doses of MBP decoction. Then the increase in the dose of MBP decoction at a dose of 200, 400, and 800 mg/kg BW showed that there was a significant difference between the three doses, so it can be said that the increase in the dose gave an increase in the analgesic effect.

In this study, the decoction of MBP at a dose of 200, 400, and 800 mg/kg BW has been shown to have % anti-inflammatory activity with 17.685; 25.53%; and 25.48%, and % effectiveness with 51.35%; 74.15%; and 74.01%, respectively. Furthermore, MBP decoction with a dose of 200, 400, and 800 mg/kg BW has an analgesic effect, with the % inhibition of writhing in mice at 38.52%, 44.53%, and 49.31%, and % effectiveness respectively 69.71%, 80.59%, and 89.24%. This finding is similar to the study conducted by Yuei *et al*.[29] investigating banana peels' anti-inflammatory and analgesic activity, especially the potency of the popular Cavendish variety consumed. In their study, two different doses, 200 mg/kg and 400 mg/kg bark ethanol

extract, were administered to rats by oral administration. The hot plate test showed a good analgesic reaction for an extract dose of 400 mg/kg rats treated at 60 minutes, comparable to a positive control of diclofenac sodium. The anti-inflammatory test showed good inflammatory action at six hours, comparable to positive control. The greatest inhibition of inflammation was seen at six hours which was 63% in rats receiving an extract dose of 400 mg/kg. These findings suggest that Cavendish bark exhibits analgesic and anti-inflammatory activity. After the two tests were carried out in this research, it could be seen that the MBP decoction had anti-inflammatory and analgesic activity. The anti-inflammatory and analgesic activity of MBP decoction involves the presence of compounds in the MBP which are attracted when decoction, one of which is a flavonoid[41]. By inhibiting the arachidonic acid metabolic pathway, the formation of prostaglandins, and the release of histamine, flavonoids function as an anti-inflammatory or slow down the inflammatory process[42].

CONCLUSION

The decoction of the MBP at a dose of 200, 400, and 800 mg/kg BW could have an inhibitory effect on edema on the soles of the rat's feet induced by 1% carrageenan solution and exerted an inhibitory effect on the amount of writhing in mice induced by acetic acid.

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AUTHORS' CONTRIBUTION

Ni Made Dwi Sandhiutami: conceptualization, data curation, formal analysis, funding acquisition, methodology, project administration, resources, supervision, validation, and writing -review & editing. **Sondang Khairani**: data curation, formal analysis, methodology, supervision, validation. **Rika Sari Dewi**: formal analysis, methodology, supervision, validation. **Zainur Rahman Hakim**: supervision, validation, editing and writing -review & editing. **Anita Rahmi Pradani**: investigation, visualization, and writing - original draft.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest..

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Research Article

Comparative Analysis of Qualitative and Quantitative Phytochemical Evaluation of Selected Leaves of Medicinal Plants in Jaffna, Sri Lanka

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Abstract

The traditional system of medicine in Sri Lanka has shown much better improvement, has fewer side effects, and is less expensive than modern synthetic drugs in the treatment of many diseases. The objective of the present study was to comparatively evaluate the qualitative and quantitative analysis of phytochemical constituents of leaves of *Murraya koenigii* (L.) Spreng., *Tinospora cordifolia* (Wild) Hook.f., *Enicostemma axillare* (Lam) A. Raynal, and *Gymnema sylvestre* R. Br. were collected from Jaffna District. The shade-dried leaves were powdered and extracted with ethanol using the cold extraction technique. These ethanolic extracts were subjected to phytochemical analysis using recommended laboratory techniques. The one-way analysis of variance (ANOVA) and Tukey's multiple comparisons at probability value ($p < 0.05$) were used in the statistical analysis of the data. Phytochemical screening showed the presence of alkaloids, flavonoids, tannins, terpenoids, steroids, saponins, phenols, and glycosides. *Murraya koenigii* shows the highest phenol and alkaloid contents (1960.71±66.88 and 19.42±0.26). *Enicostemma axillare* shows the highest flavonoid and tannin contents (22.27±0.86 and 1.26±0.017). Therefore, *E. axillare* and *M. koenigii* can be used as nutraceuticals in traditional medicine.

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INTRODUCTION

Medicinal herbs have been known for ages as a rich source of medicinal agents for the prevention of diseases and ailments worldwide[1]. According to the World Health Organization, up to 80% of the world's population still relies on traditional treatments such as herbs for their primary health care[2]. In Sri Lanka, four traditional medicinal systems have been adopted: Ayurvedic medicine, Siddha, Unani, and Deshiya Chikitsa[3]. Plants and herbal medicines are primarily employed in the Ayurveda and Deshiya Chikitsa medicinal systems to treat many diseases[4]. Even though synthetic pharmaceuticals are readily available and very successful in curing numerous diseases in today's society, some people still prefer to use traditional folk medicines since they have fewer side effects[5].

More than 13,000 secondary metabolites have been isolated from the medicinal plants. The secondary metabolites serve as defense molecules or perform specialized functions in plants. These secondary metabolites possess medicinal properties, including antidiabetic and antioxidant activity[6,7]. Alkaloids, phenolics, terpenoids, flavonoids, saponins, xanthones, polysaccharides, and other compounds have been reported to have antidiabetic activity[8].

Plants have medical value because they contain chemical compounds that have a specific physiological effect on the human body. Alkaloids, flavonoids, tannins, and phenolic compounds are essential bioactive molecules found in plants[9]. *Murraya koenigii* (L.) Spreng., *Tinospora cordifolia* (Wild) Hook.f., *Enicostemma axillare* (Lam) A. Raynal, and *Gymnema sylvestre* R. Br. are commonly available in the Jaffna district and used to treat many diseases in traditional folk medicine. The leaves of these

plants are high in bioactive chemicals such as polyphenols, alkaloids, and flavonoids, which have a variety of bioactive properties, including antioxidant, anticancer, antibacterial, antidiabetic, and hepatoprotective properties[10-13].

Phytochemicals are naturally occurring compounds in various parts of the plants which can protect the liver by providing medicinal value or nutrients. These phytochemical compositions of the different medicinal plants mainly depend on the region where those are cultivated, the climate of that particular region, and the method and period of collection[14]. Therefore, the present study was to comparatively evaluate the qualitative and quantitative analysis of phytochemical constituents of leaves of above mentioned four medicinal plants.

MATERIALS AND METHODS

Collection of plant materials

The selected fresh leaves of four different medicinal plants (**Table I** and **Figures 1-4**) were collected from Jaffna District from September to October 2020. These plants were botanically authenticated in the National Herbarium Centre, Department of National Botanic Gardens, Peradeniya, Sri Lanka.

Table I. Medicinal plants used for the study

Plant Botanical name	Family	Common name		
		Sinhala	Tamil	English
Murraya koenigii (L.) Spreng.	Rutaceae	*Karapincha*	*Kariveppillai*	Curry leaf
Enicostemma axillare (Lam) A. Raynal	Gentianaceae	Nahi, Maja-Makka booti	*Vallaruku*	Indian white head
Gymnema sylvestre R. Br.	Apocynaceae	*Masbedda*	*Kurincha*	Cow plant
Tinospora cordifolia (Wild) Hook.f.	Menispermaceae	*Raskinda*	*Seenthil*	Moonseed

Figure 1. *Murraya koenigii*

Figure 2. *Tinospora cordifolia*

Figure 3. *Enicosrtemma axillare*

Figure 4. *Gymnema sylvestre*

Preparation of plant materials

The collected fresh leaves were washed with tap water for several times to remove the soil and dust particles, and those were air-dried systematically at room temperature for three weeks to avoid direct loss of phytoconstituents from sunlight. The shade-dried plant leaves were ground using the pulverizer and sieved up to 80 meshes. It was then homogenized to a fine powder and kept in air-tight containers separately for further analysis at room temperature (31±3°C).

Preparation of plant extracts

The leaf powder of each medicinal plant was extracted with ethanol using the cold extraction technique. A total of 50 g of powdered materials of each plant's leaves were separately weighed and placed in 500 ml of culture bottles. 150 mL of 100% absolute ethanol (1 : 3) was added to it and mixed well. The lid of each bottle was covered with parafilm. The solution was kept for five days with occasional shaking using a shaker at 150 rpm for 15 minutes every morning and evening. After that, those were filtered through Whatman No.1 filter paper. The part of filtered content was concentrated using a rotatory evaporator (Buchi), and another part was kept in the refrigerator at 4°C for further use. The analysis was done for three replicates of each medicinal plant leaf.

Qualitative analysis of phytochemicals[15-20]

The preliminary phytochemical screening of the ethanol extracts of each medicinal plant leaves powder was carried out using recommended laboratory procedures to detect the presence of different phytochemicals such as alkaloids, flavonoids, tannins, steroids, glycosides, phenols, terpenoids, saponins, coumarins, anthraquinones and quinines.

Phytochemical screening for flavonoids (alkaline reagent test)

Each 2 mL of filtered sample was mixed with a few drops of 20% NaOH. The formation of intense yellow color was detected. Then, a few drops of 70% diluted hydrochloric acid were added, and the yellow color disappeared. The formation and disappearance of the yellow color indicate the presence of flavonoids.

Phytochemical screening for phenols (ferric chloride test)

Each 2 mL of filtered sample was mixed with 2 mL of 5% aqueous $FeCl_3$.The formation of the blue color points out the occurrence of phenols.

Phytochemical screening for tannins (ferric chloride test)

Each 2 mL of filtered sample was added with 10% of alcoholic $FeCl_3$. The formation of the black/brownish blue directs the occurrence of tannins.

Phytochemical screening for alkaloids (Dragendroff''s test)

Each 2 mL of filtered sample was dissolved individually in dilute hydrochloric acid and filtered. The filtrate was treated with Dragendroff''s reagent (solution of potassium bismuth iodide). The formation of a red precipitate indicates the presence of alkaloids.

Phytochemical screening for terpenoids (chloroform test)

Each 2 mL of filtered sample was added with 0.5 mL chloroform with 0.5 mL of acetic anhydride and a few drops of concentrated sulfuric acid. The formation of reddish-brown precipitate directs the presence of terpenoids.

Phytochemical screening for anthraquinones

Each 2 mL of filtered sample was added with potassium hydroxide. The blood red colour shows the presence of anthraquinones.

Phytochemical screening for saponin (foam test/frothing test)

Each 2 mL of filtered sample was added with 4 mL of distilled water. It will be mixed well and shaken vigorously. If foam will be produced continues for ten minutes, it designates the presence of saponins.

Phytochemical screening for quinones

Each 1 mL of filtered sample was added with 1 mL of sodium hydroxide. The formation of blue, green, or red colors shows the presence of quinones.

Phytochemical screening for coumarins

Each 1 mL of 1% filtered sample was added with 3-4 drops of 1% KOH in absolute ethanol. The formation of yellow color directs the occurrence of coumarins.

Phytochemical screening for glycosides (Keller-Kiliani test)

Each 2 mL of filtered sample was added with 0.5 mL glacial acetic acid, three drops of 1% aqueous $FeCl_3$ solution, and 0.5 mL H_2SO_4 concentrated. A brown ring formed between the layers, which showed the entity of cardiac steroidal glycosides.

Phytochemical screening for steroids

It was carried out by Salkowski's test. About 2 mL of sample was mixed with 2 mL of chloroform. Then, 2 mL of concentrated H_2SO_4 was added to it. If steroids are present, the chloroform layer will appear red, and the acid layer will show greenish-yellow fluorescence.

Quantitative analysis of phytochemicals

Quantitative analysis for total phenolic content (Folin-Ciocalteu colorimetric method)

About 20 µL of each filter was added to the test tube using a micropipette. 1.58 µL was added to each above test tube. 100 µL of Folin-Ciocalteu reagent was added to each test tube. They were mixed well using a magnetic stirrer and allowed for eight minutes after stirring. 300 µL of a sodium carbonate solution was added to each stirred solution. They were heated in a water bath at 40°C for 30 minutes. They were permitted to cool. They were again stirred well. The Absorption of each

sample was measured using a spectrophotometer at 765 nm wavelengths. A curve chart for each solution was prepared by using absorbance and concentration. The three replicates were prepared for each sample. Using the standard curve, the total phenolic content was determined and expressed in mg gallic acid equivalent (mg GAE) per g of dry matter using the following linear equation based on the calibration curve (**Figure 5**)[21].

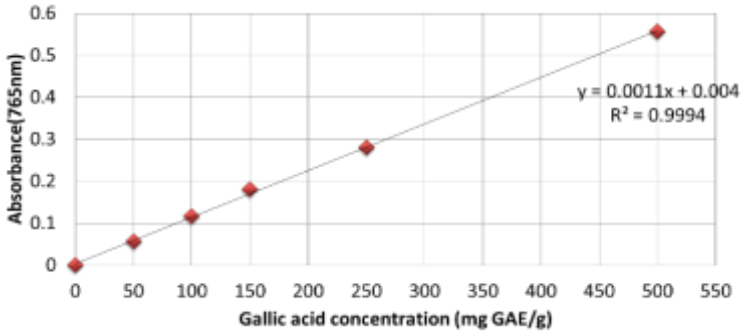

Figure 5. Standard curve for total phenolic content

Quantitative analysis for total flavonoid content (aluminum colorimetric method)

Each 0.25 mL filtered sample was added with 4.5 mL of distilled water. 0.3 mL of 5% $NaNO_2$ solution was added and allowed for 5 minutes. 0.3 mL of 10% of $AlCl_3$ was mixed and incubated for 5 minutes. 2 mL of 1N NaOH was added, and the entire volume was made to 10 mL with distilled water and mixed well. The absorbance of each sample was measured at 510 nm using a spectrophotometer. Blank was prepared using the above reagents and distilled water instead of sample. A curve chart for each solution was prepared by using absorbance and concentration. The three replicates were prepared for each sample. The flavonoid content was calculated as mg catechin equivalent (mg CAE) per gram of dry matter using the calibration curve and the following linear equation (**Figure 6**)[22].

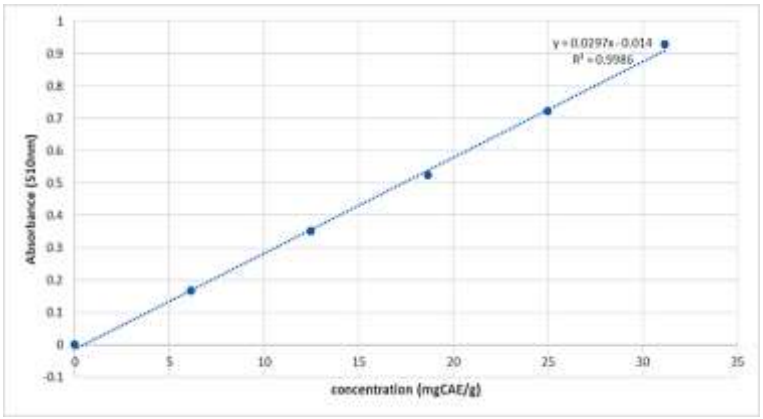

Figure 6. Standard curve for total flavonoid content

Quantitative analysis for total tannin content (Folin-Ciocalteu colorimetric method)

Each 0.5 mL of filtered sample was added with 3.75 mL of distilled water and 0.25 mL of Folin-Ciocalteu reagent, 0.5 mL of 35% sodium carbonate. The absorbance of each sample was measured at 725 nm using a spectrophotometer. The blank was prepared using the above reagents with distilled water instead of the sample. A curve chart (**Figure 7**) for each solution was prepared by using absorbance and concentration. The three replicates were prepared for each sample. The estimation of the total tannin content was carried out in three replicates. The tannin content of the samples was measured in mg/ml of tannic acid[23].

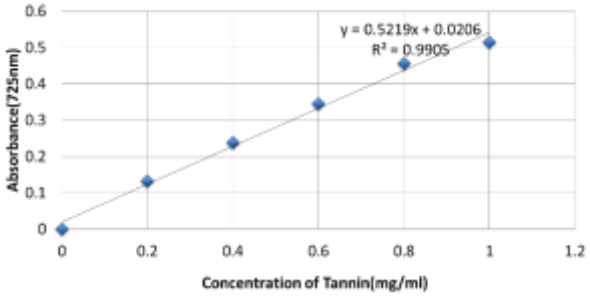

Figure 7. Standard curve for total tannin content

Quantitative analysis for total alkaloid content

About 5 g of the three samples of each powder material were balanced into a 250 mL beaker, and 200 mL of 20% of acetic acid was added and enclosed to stand for 4 hours. They were filtered, and the extract was concentrated using a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to each extract until the precipitous was completed. The whole solution was permitted to settle down, and the precipitate was collected by filtration through the accurately weighed filter paper. The filtrate is the alkaloid, which was dried in the oven for four hours and balanced. Total alkaloid content was measured as mg per g of air-dried material using the **Equation 1**[24].

$$\%\ Alkaloid\ content = \frac{w1-W2}{M} \times 100\% \quad \ldots [1]$$

W_1 : weight of the precipitate with the filter paper
W_2 : weight of the empty filter paper
M : weight of the sample

Statistical data analysis

The results were analyzed using one-way analysis of variance (ANOVA) and Tukey's multiple comparisons at probability value ($p \leq 0.05$) using the SAS statistical program (version 9.1.3). In each analysis, three replicates were maintained for each sample.

RESULTS AND DISCUSSION

Qualitative analysis of phytochemicals

The presence or absence of phytochemicals was evaluated using qualitative analysis of leaves from selected four medicinal plants. The results are provided in **Table II**. Saponins are found in all four plants, according to the study. Saponins contain a variety of functions, including the ability to precipitate and coagulate red blood cells, as well as the ability to bind cholesterol. It also shows foam formation in aqueous solutions and hemolytic action, and saponins have traditionally been employed as detergents and molluscicides. In addition to their industrial applications as foaming and surface-active agents, saponins have beneficial health effects against various diseases[25].

Table II. Preliminary phytochemical screening of ethanolic extracts of selected plant leaves

Phytochemicals	*M. koenigii*	*G. sylvestre*	*T. cordifolia*	*E. axillare*
Tannin (black colour)	+	+	+	+
Saponins (foam)	+	+	+	+
Flavonoid (yellow color)	-	+	+	+
Alkaloid (red precipitate)	+	+	+	+
Quinone (green or red color)	-	-	-	-
Anthraquinones (blood red color)	-	-	-	-
Glycoside (brown ring)	+	+	+	+
Terpenoids (reddish-brown precipitate)	+	-	-	+
Steroids (greenish yellow fluorescence)	+	+	+	+
Phenol (blue color)	+	+	+	+
Coumarins (yellow color)	+	+	+	+

"+" color change/precepitation observed & "-" color change/precepitation not observed

Plant steroids are vital for their cardiotonic properties and are employed in nutrition, herbal medicine, and cosmetics manufacturing. Steroids are used to stimulate bone marrow and promote growth. It promotes lean body mass and aids in preventing bone loss in older men[26]. As a result of this study, steroids were found in all four plants. Many studies have been carried out on the anti-hypoglycemic activity of terpenoids of herbal plant origin. *Murraya koenigii* and *E. axillare* show the presence of terpenoids. Flavonoids also show a wide variety of essential activities, including antihyperglycemic activity[27]. According to the study, flavonoids are present in *G. sylvestre, T. cordifolia*, and *E. axillare*.

Results show the presence of alkaloids in all selected plants. Thus, alkaloids can be concluded as one of the healers in medicinal plants, and many natural bio-resources studied may prove to be of significance in naturopathy and have properties that may be further investigated[28]. Many bioactive molecules in herbal plants may prove to be promising therapeutic tools. Previous research has proven that glycosides have a high potential in curing diabetes mellitus and many other diseases[29]. The glycosides are found in all four plants, according to the results of this study. Several natural phenolic compounds in medicinal plants provide anti-inflammatory, antioxidant, antimicrobial, and neuroprotective properties. Results show the presence of phenols in all selected plants.

According to the results, quinone & anthraquinones are absent in all selected plants. Coumarins are present in all selected plants. Coumarins have many biochemical and pharmacological properties which may be effective against diabetes and its complications, some of which are of potential therapeutic interest[30]. Tannins are thought to have various properties, including analgesic, anti-diabetic, and anti-inflammatory properties. Tannins have the potential to be an efficient kidney-relieving medication. Tannins are present in all selected plants, according to the results. These phytochemicals have a significant influence on hypoglycemic activity[31]. Therefore, they help to reduce diabetes. They contain antibacterial as well as antihyperglycemic properties. The use of natural chemical compounds from plants as antibacterial and antifungal agents is an intriguing technique for developing bioactive products and pharmaceuticals that could become practical therapeutic tools in the coming years[32].

Quantitative analysis of phytochemicals

The quantitative phytochemical analysis of different medicinal plant leaves is tabulated in **Table III**. Phenolic compounds are a broad and diversified class of chemicals that comprise a variety of secondary aromatic metabolites found in plants. It has been reported to have antioxidant, anti-diabetic, and antibacterial effects, among other biological activities[33]. Phenolic compounds have a wide range of pharmacological effects. Phenol's antioxidant activity mainly derives from its redox characteristics, hydrogen donors, and singlet oxygen quenchers[34]. Gallic acid has also been observed to play a synergistic role in drug-herb interactions, resulting in increased therapeutic benefit and fewer side effects. The results of this study show that the total phenolic content was significantly highest in *M. koenigii*, followed by *E. axillare*, *G. sylvestre*, and *T. cordifolia*. The antioxidant activity of ethanolic and water extracts of curry leaves is relatively high at all concentrations, but it increases as the sample concentration increases.

Table III. Quantitative analysis of phytochemicals of selected plant leaves

Plant species	Phenol (mgGAE/g)	Flavonoid (mgCAE/g)	Tannin (mg/ml)	Alkaloid (%)
M.koenigii	1960.71 ± 66.88	15.42 ± 3.50	1.223 ± 0.011	19.42 ± 0.26
E.littorale	856.84 ± 35.4	22.27 ± 0.86	1.26 ± 0.017	10.38 ± 0.31
G.sylvestre	616.92 ± 19.6	14.67 ± 1.35	1.23 ± 0.014	6.62 ± 0.25
T. cordifolia	325.61 ± 23.84	15.03 ± 1.42	1.24 ± 0.008	13.50 ± 0.33

Flavonoids are hydroxylated phenolic compounds that plants produce in response to microbial infection and have been discovered to have antibacterial properties in vitro against a wide range of pathogens. Flavonoids' antioxidative activities are attributable to various processes, including scavenging free radicals, chelation of metal ions like iron and copper, and inhibiting enzymes that generate free radicals[35]. Catechins have an anti-hyperglycemic effect, reducing blood sugar while also regulating insulin release. Catechins also have antiviral properties. The results of this study show that the total flavonoid content was highest in *E. axillare*, followed by *M. koenigii, G. sylvestre*, and *T. cordifolia*.

Tannins can inhibit the growth of many microorganisms such as fungi, yeasts, bacteria, and viruses. The results of this study show that the total tannin content was highest in E. *axillare*, followed by *M. koenigii, G. sylvestre*, and *T. cordifolia*. Tannins have antioxidant properties. They are cardio-protective, anti-inflammatory, anti-carcinogenic, and anti-mutagenic, among other things. Tannins increase glucose absorption while inhibiting adipogenesis, making them viable treatments for non-insulin-dependent diabetes mellitus (NIDDM)[36].

Plant cells are highly sophisticated chemical factories that produce secondary metabolites like alkaloids which possess significant biological properties. They exhibit good anti-microbial activity against a few bacterial pathogens causing common infections. Alkaloids are a vast and structurally diverse collection of chemicals that have been used as scaffolding for antibacterial medications like metronidazole and quinolones[37]. Alkaloids help to regulate hypoglycemic activity also[38]. The results of this study show that the total alkaloid content was highest in *M. koenigii*, followed by *G. sylvestre, T. cordifolia*, and *E. axillare*.

CONCLUSION

Medicinal plants and phytochemicals have much importance in the present scenario in developing countries where resources are limited. Regular uptake of herbal medicines containing these phytochemicals can benefit many health problems. The results of preliminary phytochemical screening using ethanolic extracts of *M. koenigii, G. sylvestre, T. cordifolia*, and *E. axillare* leaves are presented in this work. Leaves of *E. axillare* and *M. koenigii* are rich in critical specific phytochemicals and higher amounts of total phenolic and flavonoid contents than other plants. Therefore, *E. axillare* and *M. koenigii* can be used as multi-functional medicinal herbs in the traditional system of medicine and to prepare ready-to-use functional products and nutraceuticals.

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AUTHORS' CONTRIBUTION

Gowri Rajkumar: interpreted, conceived, design the analysis, supervised the experimental works and also the correction of the manuscript. **Panambara Arachchilage Harini Rangana Panambara**: performed the experiments and initially drafted the manuscript. **Vinotha Sanmugarajah**: contributed in the experimental works and assisted for manuscript writing.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Review Article

A Review of Anti-hyperglycemic Effects of Curry Leaf Tree (*Murraya koenigii*)

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Abstract

Diabetes mellitus is becoming a metabolic disease that is defined by the level of hyperglycemia. Nowadays, it has a serious threat to public healthiness in throughout the world. Constituents and extracts isolated from diverse natural resources, mainly plants, have constantly been a rich store for controlling and treating diabetes problems. Numerous researches are ongoing to identify the suitable traditional medical drugs, medicinal herbs, and resources for managing this condition. *Murraya koenigii* Spreng (family Rutaceae) is commonly known as a 'curry leaf tree' locally. It is widely scattered in India and Sri Lanka, and leaves are commonly used for cooking. And also mainly used for various health conditions such as diabetes, anemia, diarrhea, and others. The present review aimed to critically review the anti-hyperglycemic effect of the *M. koenigii* based on the review, *in vitro, in vivo*, and clinical studies. Based on this review, the *M. koenigii* possess flavonoids, phenols, saponins, alkaloids, tannins, and cardiac glycosides. It has shown a potential anti-hyperglycemic effect on induced diabetic rats. This review reported the potential of *M. koenigii* and its extract to be a high-value dietary product in terms of its anti-hyperglycemic effects and industrial profits. Therefore, the present review supports the researchers and readers/users to realize the importance of using *M. koenigii* in managing diabetes mellitus. Further, this review provides a valuable document for future scientific-related clinical trials in diabetic patients.

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INTRODUCTION

Diabetes is a metabolic condition primarily demarcated by the level of hyperglycemia, giving rise to a risk of microvascular damage. It is related to reduced life expectancy, significant morbidity due to specific diabetes interconnected microvascular problems, increased risk of macrovascular impediments, and reduced quality of life[1]. It is a global disease found in all nations of the world. In the last two to three decades, there has been an explosive upsurge in people with diabetes[2]. According to World Health Organization, about 422 million people in low-and middle-income countries have diabetes, and 1.6 million deaths occur due to diabetes each year[3]. Many herbal remedies are used in many countries to control and manage diabetes mellitus. Commonly, these medicinal herbs effectively manage the diabetic condition for a long time due to their various biological constituents, such as saponins, glycosides, polysaccharides, flavonoids, alkaloids, and terpenoids, which are possessed anti-diabetic activities[4]. Several *in vitro* and *in vivo* studies have been supported in recent years showing the potential effects of curry leaf tree or *Murraya koenigii* Spreng (family Rutaceae) therapies and improved blood glucose control to manage the diabetic condition. This tree is commonly known as sweet neem and is distributed throughout tropical zones and widely used for various health issues such as diabetes, diarrhea, anemia, ulcer, obesity, inflammation, and others, in the traditional medical system of Sri Lanka[5,6].

Therefore, the present review was to aim to do a critical review of the antihyperglycemic effect of *M. koenigii* based on reviews, *in vitro*, *in vivo*, and clinical studies from all available sources such as past and recent traditional textbooks, research articles, original research papers, websites, reputed scientific databases and other related documents during the year of 2021 at Jaffna District, Sri Lanka. This review article will be valuable to the documented indication of the antihyperglycemic special effects of *M. koenigii*.

CLASSIFICATION OF *MURRAYA KOENIGII*

Classification and common names of *M. koenigii* presented in **Table I**[7].

Table I. Classification of *M. koenigii*

Classification	Identity
Domain	Eukaryote
Kingdom	Plantae
Subkingdom	Viridiplantae
Infrakingdom	Streptophyta
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Rosanae
Order	Sapindales
Family	Rutaceae
Genus	Murraya J. Koenig ex L.
Species	*Murraya koenigii* (L.) Spreng.
Common Name	Karapincha
English Name	Curry Leaf Tree
Sanskrit Name	Girinimba, Krishnanimba
Tamil Name	Kariveppilai
Hindi Name	Currypatta

DISTRIBUTION OF *MURRAYA KOENIGII*

Murraya koenigii tree is a tropical to a subtropical tree distributed and cultivated throughout India, and it can be found in moist forests in Bhutan, Nepal, Pakistan, Sri Lanka, Thailand, Vietnam, and Laos. Its propagation is done by seeds[8].

MORPHOLOGY OF *MURRAYA KOENIGII*

Macroscopical features

It is a semi-deciduous aromatic shrub or small tree which is about 2.5 m up to 6 m in height and 15-40 cm in diameter with a short trunk, thin, smooth grey or brown bark (**Figure 1**). The leaves are imparipinnate, glabrous, and very intensely aromatic and about 30 cm long, each bearing 9-25 leaflets, short-stalked, alternate, and have a reticulate venation. Flowers are small, white funnel-shaped, fragrant, bisexual, calyx deeply five clefts, and pubescent. Petals five, free, whitish, glabrous, and with dotted glands. Fruits occur in close clusters, small, round to oblong in shape, glandular, thin pericarp enclosing one or two seeds having spinach green color[9,10].

Microscopical features

Murraya koenigii shows the presence of unicellular trichomes with obliterated lumen, parenchymatous pith in the petiole, long pericyclic fiber in the midrib, large cruciferous stomata, and prismatic calcium oxalate crystals[11,12]. The root shows tetrarch to pentarchy stele, phelloderm fibers are absent, and concentric grains of parenchyma is present. Powder of *M. Koenigii* are green in color with no distinct odor or taste, unicellular, curved trichomes, two-layered palisade, a portion of secretory canals, well-developed pericyclic fibers, and a few crystals of calcium oxalate are the important categorizing

characters[13]. *Murraya koenigii* powder fluoresces brownish-black. When treated with 1 N methanolic sodium hydroxide, the powder illustrates yellowish-white color and, when mounted in nitrocellulose, produces chocolate fluorescence[14,15].

Figure 1. Different parts (**a**: leaves; **b**: flowers; **c**: seeds; **d**: fruits; **e**: powder; **f**: juice) of *M. koenigii*

GROWING SEASON OF *MURRAYA KOENIGII*

This plant has flowers and green leaves during the spring, summer, and rainfall. The leaves drop off during their' resting period in the winter. They like full sun and well-drained soil, which should be the dry side, and they need fertilizer in the month of summer[16,17]. The fruiting season is from the end of June to the end of August, and July is considered the peak fruiting season. This plant can grow to a tree up to 6 m tall in warm, humid climates, but it can also be grown up very successfully in a pot as a much smaller plant[18,19].

LITERATURE REVIEW STUDIES

The plant has main constituents such as caryophyllene, terpene, carvomenthone, menthol, menthone, citral, and linalyl acetate, which contribute to the flavor[20]. Leaves, seeds, flowers, and fruit of *M. koenigii*, contain constituents responsible for a variety of numerous biological processes[21]. A review study mentioned that traditionally *M. koenigii* leaves are used in Ayurveda medicine to manage diabetes, and mahanimbine is a carbazole alkaloid present in leaves, stem bark, and root of *M. koenigii* has a beneficial effect in the controlling the diabetes mellitus[22]. Another review related to the role of medicinal herbs in treating diabetes has reported that *M. koenigii* is included under the herbs of glucose-lowering effects[23]. Another review study informed that the *M. koenigii* acts as an anti-diabetic due to decreases oxidative stress by acting on Paraoxonase activity with Koenimbidine, Murrayacine, and Murrayazolinine chemical constituents[11]. Qais *et al.*[24] informed in their review article on anti-diabetic plants that the *M. koenigii* plants showed a significant hypoglycemic effect on carbohydrate metabolism using experimental rats. Based on the numerous *in vivo* studies, some reviews stated that *M. koenigii* leaves possess statistically significant anti-hyperglycemic effects in diabetic rats[25-30]. Numerous review-related studies mentioned that the leaves, fruit, and fruit juice have anti-diabetic properties[31-33].

In vitro phytochemical studies

Leaves of *M. koenigii* exposed tannins in the aqueous extract and quinones, coumarin, and sugar in both the alcoholic extracts[34]. Different fractions of ethanol leaves extract of *M. koenigii* indicated the occurrence of saponins, alkaloids, flavonoids, tannins, and cardiac glycosides[35-37]. Phytochemical screening exhibited the occurrence of carbohydrates, alkaloids, steroids, and flavonoids in the different extracts of the *M. koenigii* plant[38-46]. The study in India established that flavonoids, phenol, tannins, saponins, terpenoids, reducing sugar, and alkaloids are present in both urban and coastal areas of *M. koenigii*[47]. The hydro-distillate essential oil of *M. koenigii* leaves showed the most potent antioxidant activity within the concentration range[48]. The FRAP and DPPH assays confirmed that the highest total flavonoids and phenolic contents extracted from the curry leaf in Malaysia showed the highest antioxidant activity[49].

Phytochemicals such as flavonoids, monoterpenes, terpenoids, stilbenes, lignans, coumarins, alkaloids, and others, have been proposed as effective supplements for diabetes management and prevention of its long-term complications *in vitro* and *in vivo*[50]. Further, studies have proven that various phytochemical components of anti-diabetic herbs such as flavonoids, alkaloids, saponins, tannins, and terpenes were responsible for the anti-diabetic activities of the plants, and they mentioned that the flavonoids were observed to be the most popular anti-diabetic principle among the phytochemicals[51]. Based on the phytochemicals related to studies of *M. koenigii* also proven that it has flavonoids in its different extracts[52].

In vivo animal studies

Numerous comparative animal studies proved that the different extracts of the *M. koenigii* have shown anti-hyperglycemic effects in diabetic rats. Vinuthan *et al*.[53] found that the daily oral administration of aqueous (600 mg/kg BW) and methanol extracts (200 mg/kg BW) of *M. koenigii* for eight weeks exhibited a significant reduction ($p < 0.05$) in alloxan-induced diabetic rats when associated to control group. Another study found that the aqueous and methanol extracts of the *M. koenigii* have significantly declined the blood glucose level in streptozotocin-nicotinamide (STZ-NA) induced diabetes rats throughout 28 days of treatment[54]. An animal study found that the orally administered ethanol extract of *M. koenigii* at a dose of 200 mg/kg/BW/day for 30 days exhibited a significant reduction in blood glucose levels in STZ-induced diabetic rats[55]. Fauziah *et al*.[56] suggested that the treatment of ethanol leaves extract of *M. koenigii* at several doses (50% mL/10 g BW, 70% mL/10 g BW, and 90% mL/10 g BW) for 14 days of treatment significantly affected the decrease on blood sugar levels in alloxan-induced diabetic mice. Fraction (1 and 2) of ethanol leaf extract (400 mg/Kg BW) of *M. koenigii* showed significantly ($p > 0.05$) decreased blood sugar levels by 72% when compared to the group of alloxan-induced diabetic rats[35].

Lawal *et al*.[57] found that the orally administered at the various dose levels (at 100 mg/kg, 150 mg/kg, and 200 mg/kg BW), its aqueous leaf extract for seven days possessed hypoglycaemic activity in normal and alloxanized diabetic rats. Tembhurne and Sakarkar[58] mentioned that the user leaves could control body wait and maintain the glycemic levels in diabetic patients because those leaves suggested a potent hypoglycaemic activity in high-fat obese rats. Orally administrated, its aqueous leaf extract (200 mg/kg BW) showed anti-hyperglycemic activity greater than glibenclamide in STZ-induced diabetic rats for 28 days[59]. Al-Ani *et al*.[60] recommended that its aqueous leaf extract (200 mg/kg and 400 mg/kg) for a month exhibited significant ($p < 0.001$) enhancement in blood sugar levels against cellular oxidative damage in STZ-induced diabetic rats. El Amin *et al*.[61] recommended that the orally administered aqueous extracts of *M. koenigii* show a significant ($p \geq 0.05$) anti-hyperglycemic effect (range 55.6-64.6%) compared to the metformin (62.7%). Oral administration of *M. koenigii* aqueous extract (300 mg/kg, p.o) significantly reduced the blood glucose level in the diabetic group for 28 days in alloxanized diabetic rats[62].

Bhat *et al*.[63] recommended that its chloroform extract has significant inhibition (IC_{50} values of 1.96, 1.06, and 2.68 µg/mL) with porcine pancreatic α-amylase (56.40%) as well as murine pancreatic and intestinal glucosidases as compared with acarbose. A study identified that the orally administrated chloroform leaf extracts (250 and 500 mg/kg BW) for 30 days ensued in a significant reduction of blood sugar from 296.62±20.12 to 80.22±03.63 in alloxan-induced albino rats[64]. Ahmed *et al*.[65] found that its chloroform leaves extract has shown significant antidiabetic outcomes at doses of 250 and 500 mg/kg BW in alloxan-induced diabetic albino rats on intraperitoneal injection when matched to the control group. Another study

justified the combination of *M. koenigii* leaves extract (150 mg/kg; p.o.) and *V. vinifera* seeds extract (100 mg/kg; p.o.) have shown the potential antidiabetic effect after 21 days of treatment in alloxan-induced diabetic rat[66].

Its hydroalcoholic extract treatment exhibited a significant antidiabetic effect by restoring blood glucose and HbA1C level compared to the control group in STZ-induced rats[67]. A study demonstrated that feeding a diet containing various doses of curry leaves caused a maximal reduction in blood sugar in STZ-induced mild and moderate diabetic rats after seven days[68]. Another study confirmed that its ethanolic extract significantly reduced blood glucose levels at both doses, 200 and 400 mg/kg BW, in STZ-NA-induced rats[69]. The researchers observed that the curry leaf extract could decrease blood glucose levels from 387.0±15.6 mg/dL to 214.0±26.6 mg/dL after ten days in diabetic ob/ob mice[70]. A study indicated that its aqueous extract has a favorable effect in bringing down the severity of diabetes[6]. Sudha *et al*.[59] stated that its aqueous leaf extract was superior to glibenclamide in STZ-induced diabetic rats. Its leaves in tea bags have comparable activity with glibenclamide in reducing the blood glucose levels in alloxan-induced diabetes Wistar albino rats[71]. A single oral administration of variable dose levels of aqueous extract of *M. koenigii* showed a marked improvement in the sub, and mild diabetic rabbits in glucose tolerance test, and they suggested that it may be prescribed for controlling diabetes mellitus[6].

Human studies – Clinical trials

A clinical trial was conducted to assess the efficacy of *M. koenigii* leaves powder in reducing the blood glucose level of the 60 type II diabetic patients (30 participants in the experimental and 30 in the control group) in a rural area of Medavakkam, Chennai, that found that 10 g of *M. koenigii* leaves powder for 14 days along with their food showed a significant variance between the pre and post-prandial blood glucose level in the experimental group[72]. Another clinical trial found that the use of *M. koenigii* leaves juice (100 mL twice a day for seven days) has shown significantly ($p < 0.00003$) to reduce the blood sugar level among the 20 experimental groups of diabetic subjects when compared with 20 control group subjects who are above 40 years of age at selected hospitals, Puducherry[73]. Another clinical trial reported a significant difference after the administration of *M. koenigii* leaves powder in the average fasting and post-prandial blood glucose at a 5% significance level among the diabetic patients[74,75]. Several review studies recognized that *M. koenigii* has anti-diabetic potency due to carbazole alkaloid present in *M. koenigii*. **Table II** summarizes the results of different phytochemical, *in vitro*, and *in vivo* studies.

Table II. Summary of different studies of *M. koenigii*

Preparation / extract	Effects
***In vitro* studies**	
Aqueous extract & alcoholic extracts	Phytochemicals[34]
Ethanol extract	Phytochemicals[35-37]
Different extracts	Phytochemicals[38-48]
Hydro-distillate essential oil	Antioxidant activity[49]
Aqueous extract	Antioxidant activity[50]
***In vivo* studies**	
Aqueous and methanol extracts	Hypoglycaemic effect in alloxan-induced diabetic rats[53]
Methanol extract	Hypoglycaemic effect in STZ-NA induced diabetes rats[54]
Ethanol extract	Hypoglycaemic effect in STZ-induced diabetes rats[55,69]
	Hypoglycaemic effect in alloxan-induced diabetic mice[36,56]
Aqueous leaf extract	Hypoglycaemic effect in alloxan-induced diabetic rats[57,59,66]
	Hypoglycaemic effect in STZ-induced diabetes rats[59-62]
	Hypoglycaemic effect in diabetic rabbits[6]
Chloroform extract	Hypoglycaemic effect[63]
	Hypoglycaemic effect in alloxan-induced diabetic rats[64,65]
Hydroalcoholic extract	Hypoglycaemic effect in STZ-induced diabetes rats[67]
Diet containing various doses of curry leaves	Hypoglycaemic effect in STZ-induced diabetic rats[68]
Leaf extract	Hypoglycaemic effect in diabetic *ob/ob* mice[70]
leaves tea bag	Hypoglycaemic effect in alloxan-induced wistar albino rats[71]
Human studies – Clinical trials	
Curry leaves powder	Reduce blood glucose in type II diabetic patients[73]
Curry leaves juice	Reduce blood glucose in diabetic patients[72]

The phytochemical studies demonstrated that the *M. koenigii* possess the major constituents as phenols, saponins, alkaloids, flavonoids, tannins, and cardiac glycosides, which are secondary metabolites effective for antioxidant therapy. Therefore, *M. koenigii* can act as an anti-diabetic plant to decrease oxidative stress because oxidative stress also induces diabetes from free radicals. Based on the animal studies, numerous qualified animal studies were proved that the different extracts; aqueous, methanol, ethanol, and chloroform extracts of the *M. koenigii* in different doses and different periods have shown significant ($p \leq 0.05$) hypoglycemic effects in alloxan-induced as well as STZ-induced diabetic rats when compared with the control group and standard drug. Although human studies found that using *M. koenigii* leaves juice or powder has shown a significant decrease the blood glucose in diabetic patients, the study population, period of study and evaluation methods, and others are not enough to decide the conclusion. The longitudinal studies should be carried out to make a final decision.

CONCLUSION

This present review confers the 'anti-hyperglycemic effect of the *M. koenigii* as capable herbal material in managing diabetes mellitus due to its availability, efficacy, and clinical safety based on the literature review, *in vitro*, *in vivo*, and related clinical studies. This review reported the potential of curry leaf and its extract to be a high-value dietary product in terms of its anti-hyperglycemic effects and industrial profits. Therefore, the present review provides a valuable document for the researchers to do the future scientific-related clinical trials in diabetic patients.

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AUTHORS' CONTRIBUTION

Vinotha Sanmugarajah: collected data, conceived and design the analysis, writing of the paper. **Gowri Rajkumar**: contributed data, conceived and design the analysis.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Mini-Review

Pharmacognosy, Phytochemical, and Pharmacology of Wijaya Kusuma (*Epiphyllum oxypetalum* (DC.) Haw.) – An Update Review

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Wijaya Kusuma

Abstract

In Indonesia, *Epiphyllum oxypetalum* (DC.) Haw. is known as Wijaya Kusuma. The plant is grown for home decorating and used widely as medicine in some areas. This narrative review discusses the pharmacognosy, phytochemical, and pharmacology aspects of *E. oxypetalum*. The review is limited to original articles and abstracts available in Science Direct, PubMed, and Google Scholar. The keyword used to search the articles was "*Epiphyllum oxypetalum*". The plant contains proteins, amino acids, alkaloids, saponins, terpenoids, steroids, flavonoids, tannins, glycosides, and resins. The plant has pharmacological activities such as anti-inflammatory, antimicrobials, antidiabetic, and antioxidant properties. Researchers interested in developing *E. oxypetalum* as a medicinal plant might use this review as a reference.

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INTRODUCTION

Indonesia has a lot of biodiversity and plants that can be used as a source of traditional medicine. Plants used as a source of medicine have existed since ancient times, both hereditary and scientifically proven. Plants have been an integral part of pharmacotherapy throughout history. Medicinal plants have an essential role in the discovery of bioactive molecules[1]. One of the plants that have the potential to be developed as a medicinal plant is *Epiphyllum oxypetalum* (DC.) Haw. or Wijaya Kusuma (family: Cactaceae).

Epiphyllum oxypetalum is an ornamental with much history and is widely used to decorate homes. The plant has the potential to be employed as a medicine. *Epiphyllum oxypetalum* is a plant native to Southern Mexico, but it can also be found in North America and Southeast Asia. The plant is also known as night-blooming cereus because of its likeness to a lotus flower. In Indonesia, *E. oxypetalum* has a story that is particularly popular in Central Java. It is said, if someone sees the flowers blooming, then his wishes will come true and achieve success. Another popular myth in the culture is that the *E. oxypetalum* flower does not always bloom, depending on the planter. Many people plant this flower, hoping that it will bring them luck[2].

Epiphyllum oxypetalum may reach a height of 2-6 m, are ancient with a green hue, and have dark green leaves. The trunks and shoots of these plants can reach a diameter of 2 cm or more, are woody, and have many branches. The leaves on these plants are low sideways and lancet-shaped. Glossy green leaves on the upper surface and underside of sharp-pointed leaves, thinning, wavy, and serrated leaves, the top of narrow leaves in a linear fashion with the interest of 1.6 to 1.8 mm, nocturnal (bloom at night) funnel-shaped, and scented[3]. The growth of flowers is heavily influenced by light and wind[4]. *Epiphyllum oxypetalum* is growing in an area with no light, and only a tiny breeze does not blossom until it is fully mature. The temperature has a significant impact on the growth of plant germination[5].

Many *E. oxypetalum* research articles have been published, but there is no review of this plant's pharmacognosy, phytochemical, and pharmacological aspects. In an era with more scientific publications than ever, article reviews are an essential type of scientific writing. Article reviews were intended to highlight key aspects of contemporary research and compare them to prior studies on related subjects[6]. Based on this, we highlight the potential of this plant from pharmacognosy, phytochemical, and pharmacological aspects through a narrative review. This review is aimed to provide researchers with a summary of information on *E. oxypetalum's* potential as a medicinal plant.

PLANT CLASSIFICATION

Table I shows the classification of *E. oxypetalum*. Species information is essential for the identification stage of medicinal plants. This is useful to prevent errors in collecting and using plant samples[7].

Table I. Classification of *E. oxypetalum*[2]

Classification	Identity
Kingdom	Plantae
Subkingdom	Tracheobionta
Phylum	Magnoliophyta
Class	Magnoliopsida
Sub Class	Hamamelidae
Order	Caryophyllales
Family	Cactaceae
Genus	*Epiphyllum*
Species	*Epiphyllum oxypetalum*
Synonims	*Cereus oxypetalum, Epiphyllum purpusii, Phyllocactus oxypetalus, Phyllocactus purpursii, Cactus oxypetalus, Epiphyllum acuminatum, Phyllocactus acuminatus, Phyllocactus guyanensis, Phyllocactus grandis, Cereus latifrons* Pfeiffer, *Epiphyllum latifrons*
Local Name	Wijaya Kusuma (Indonesia); tan hua (China); bakawali (Melayu); queen of the night; orchid cactus; beauty under the moon (International); brahma kamala, nishagandhi (India); kadupul (Sinhala).

PHARMACOGNOSY

The identification of a plant is critical for the advancement of traditional medicine. Observations at the microscopic and macroscopic levels help to achieve this objective[8]. Devi *et al.*[3] reported that the transverse section of *E. oxypetalum* leaf from Bangalore district, Karnataka, India showed the presence of upper epidermis, paracytic stoma, cystolith crystal, mesophyll with midrib vascular tissue, mesophyll with the upper epidermis, needle-shaped crystals, starch grains, xylem vessels, phloem, sclerenchyma of bundle sheath, and pith tissue, xylem vessels, phloem layer, sclerenchyma patches of bundle sheath. Meanwhile, the powder leaves of *E. oxypetalum* microscopically show the presence of star-shaped calcium oxalate crystals, tetracytic stoma, anisocytic stoma, starch grains, and xylem vessel with spiral wall thickening.

The epidermis is the cell layer that protects the surface of leaves, flowers, fruits, seeds, stems, and roots. The epidermis protects tissues from external effects and is a regulator of gas exchange in the leaves. Stomata and trichomes are formed from the epidermis[9]. Anisocytic stomata are observed in *E. oxypetalum* leaves. Anisocytic stomata have three adjacent cells of different sizes around each guard cell[10]. Stomata are involved in gas exchange by controlling water loss during transpiration and absorbing CO_2 during photosynthesis. Because of the importance of stomata in the photosynthesis process, it will impact the generation of metabolites in plants[11]. Mesophyll tissue contains chloroplasts in cells[12].

Non-specific characteristics like a loss of drying and ash content impact the quality of plant material. Loss in drying and total ash value of *E. oxypetalum* dried leaves was 2 ± 0.10% and 4.6 ± 0.4%[3]. Ingale and Mansoori[13] reported that the loss on drying in the stem extract was 22.6158 g/100 g, and the leaves extract was 10.4658 g/100 g. Meanwhile, the ash content of the stem extract was 2.0625 g/100 g, and the leaves extract was 2.6024 g/100 g.

The loss on drying aims to provide a maximum range linked to the number of compounds lost during the drying process, whereas the ash content intends to offer an overview of the internal and external mineral content from the starting process to the generation of the extract[14]. The location where the plant samples were gathered, and the extraction solvent might

impact the concentration of chemicals in the plant. Environmental elements such as nutrition supplies, pH, growth location, humidity, and light are the key factors that influence the concentration of chemicals in a plant[15]. *Epiphyllum oxypetalum* was sampled from several sites across India for this study. As a result, environmental factors significantly impact the concentration of chemicals in plants, resulting in a wide range of results in each research. Microbial contamination is caused by excess moisture, whereas microbial decomposition is suppressed by low water content[3].

PHYTOCHEMICAL

Epiphyllum oxypetalum leaf powder has a carbohydrate content of 0.0237 ± 0.001 mg/0.5 mL[3], protein content was 14 mg/g, lipids content was 4.6 mg/g, and niacin content was 0.18 mg/g[16]. While, the levels of phenolics, flavonoids, tannins are 19.09 ± 0.08 g/0.6 mL, 8.728 ± 0.02 g/mL, and 31.32 ± 0.08%, respectively[3]. The leaves and flowers of *E. oxypetalum* have been extensively studied in the research, as well as the development of pharmaceuticals. Several studies on the chemical composition of this plant have been published. **Table II** summarizes the chemical composition of *E. oxypetalum*.

Plant chemicals are selectively soluble in suitable solvents. The extraction method used and the sample at various sites in each study can impact compound results. Maceration and soxhlet are two typical procedures researchers use to extract chemical components of *E. oxypetalum*. Maceration does not go through a heating process, so it is unlikely that the compounds contained are damaged[17]. The long maceration process allows the compound to be extracted completely. Soxhlet extraction is a highly effective hot extraction process. However, it should be noted that hot extraction can damage the samples' compounds for thermolabile compounds[18].

The choice of solvents considerably impacts the extraction efficiency of any traditional technique. The polarity of the substance to be studied is the most significant consideration when selecting a solvent. In choosing a solvent for bioactive component extraction, consider molecular affinity between the solvent and the solute, mass transfer, the use of a co-solvent, environmental safety, human toxicity, and financial feasibility[19].

Based on **Table II**, flowers and leaves of *E. oxypetalum* are reported to have metabolites that play a role in pharmacological activity. Alkaloids play a role in activities such as anticancer, antimalarial, and antihyperglycemic. Saponins play a role in antibacterial and antioxidant activities. Tannins play a role in antibacterial activity. Flavonoids have antioxidant properties[20]. Most of these compounds can be dissolved in either methanol, ethanol, or water[19].

Table II. Chemical composition of *E. oxypetalum*

Part of plant	Sample location	Extraction method and type of extract	Chemical component
Flowers	Hosur, Krishnagiri district, Tamil Nadu, India	Maceration, hexane extract	Alkaloids, saponins, terpenoids[20]
		Maceration, chloroform Extract	Proteins and amino acids, terpenoids[20]
		Maceration, ethanol extract	Alkaloids, terpenoids[20]
		Maceration, water extract	Steroids, flavonoids, tannins[20]
	Denpasar and Badung, Bali, Indonesia	Fractionation, petroleum ether fraction	Alkaloids, triterpenoids and saponins[21]
Leaves	Bangalore district, Karnataka, India	Soxhlet extraction, methanol Extract	Carbohydrates, proteins, tannins, phenols, alkaloids, flavonoids, sterols, saponins[3]
		Soxhlet extraction, water extract	Carbohydrates, proteins, tannins, alkaloids, sterols, saponins[3]
		Soxhlet extraction, petroleum ether extract	Carbohydrates, tannins, sterols, alkaloids[3]
		Soxhlet extraction, ethanol extract	Carbohydrates, proteins, tannins, phenols, alkaloids, sterols, saponins[3]
	Bangalore district, Karnataka, India	Soxhlet extraction, sequentially ethanol extract	Carbohydrates, proteins, tannins, phenols, alkaloids, saponins, glycosides, steroids, terpenoids, resins[16]
		Soxhlet extraction, sequentially acetone extract	Saponins, glycosides, proteins, steroids, terpenoids, phenols, resins, tannins[16]
		Soxhlet extraction, sequentially petroleum ether extract	Glycosides, proteins, steroids, terpenoids, resins[16]

Several chemical constituents of *E. oxypetalum* were identified using the gas chromatography-mass spectroscopy (GC-MS) method[20,22,23]. The summary can be seen in **Table III**. Hexadecanoic acid was detected in leaves and flowers. This compound

is reported to have potential as an antioxidant, flavor, pesticide, hemolytic, and other. Ethanol extract of *E. oxypetalum* leaves also contains flavonoids ([7- hydroxy-3(1,1-dimethyl prop-2enyl) coumarin) and fatty acids (oleic acid, nonadecanoic acid, and hexadecanoic acid)[20].

Table III. Chemical constituents identified in *E. oxypetalum*

Part of plant	Extraction method and type of extract	Chemical component
Flowers	Maceration; chloroform, ethanol, hexane and aqueous extracts	Hexadecanoic acid, ethyl ester Nonadecanoic acid Oleic acid 11-tridecen-1-ol 1-octadecyne Hexadecanal, Spiro[androdt-5-ene-17,1-cyclobutan]-2-1-3-hydroxy 1,6;3,4-dianhydro-2-deoxy-. beta.-DLyxo- hexopyranose di-n-decylsulfone, 7-hydroxy-3(1,1-dimethyl prop-2enyl) coumarin Pterin-6-carboxylic acid[20]
Leaves	Soxhlet extraction; ethanol extract	4-hydroxy-2-methylacetophenone Megastigmatrienone 4-((1E)-3-hydroxy-1-propenyl)-2 methoxyphenol n-hexadecanoic acid Octadecanoic acid Phytol Cholesta-22,24-dien-5-ol, 4,4-dimethyl Stigmasterol 22-stigmasten-3-one Heptacosane Nonadecane, 2- methyl- Spinasterone 4,22-stigmastadiene-3-one Tetracosane Hentriacontane Stigmast-4-en-3-one Testosterone cypionate[24]
	Cold percolation, methanol extract	10-octadeconoic acid, methyl ester; 1,2-benzenedicarboxylic acid, butyl octyl ester; 1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester; Cyclopropanebutanoic acid, 2-[(2-[(2 [(2pentylcyclopropyl)methyl]cyclopropyl)methyl]cyclopropyl)methyl]-, methyl ester; 17-(1,5-dimethylhexyl)-10,13-dimethyl-3 styrylhexadecahydrocyclopenta(a)phenanthren-2-one; Ergosteryl acetate; Ethanol, 2-(9-octadecenyloxy)-,(Z)-; Glycine,N-[(3a,5a,7a,12a)-24-oxo-3,7,12 tris [(trimethylsilyl) oxy]cholan-24-yl] -, methyl ester; (5a) pregnane -3,20 a-diol, 14a, 18a-[4-methyl-3-oxo- (1-oxa-4-azabutane-1,4-diyl)]-, diacetate; and Rhodopin[23]

Several structures of essential compounds in *E. oxypetalum* are presented in **Figure 1**. A molecular docking study reported that megastigmatrienone and testosterone cypionate found in *E. oxypetalum* leaves have the potential to be developed as an antiviral[22].

PHARMACOLOGY

The summary of pharmacological activity from *E. oxypetalum* is shown in **Table IV**. Because animal tests are expensive, time-demanding, and susceptible to ethical controversy, *in vitro* procedures are frequently preferred over *in vivo* assays[25]. According to **Table IV**, most *E. oxypetalum* research has been done *in vitro*. The leaves are more studied than the flowers. Flowers may be more challenging to obtain because not all plants planted can produce flowers.

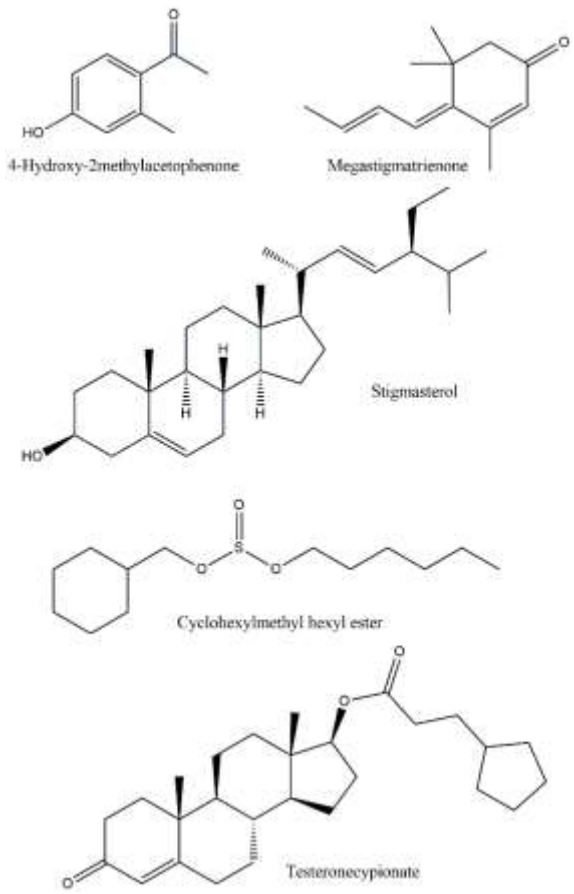

Figure 1. Several chemical structures identified in *E. oxypetalum*[20,22,23]

Alcohols, both ethanol and methanol, as polar solvents, are more often used in the test. This solvent can extract metabolites with a wider polarity, such as polyphenols, flavonoids, tannins, alkaloids, glycosides, terpenoids, and steroids[26]. The extraction methods that are widely used are maceration and soxhlet extraction. Both of these methods are commonly used in the extraction of medicinal plants[27]. However, currently, the use of non-conventional extraction techniques (such as ultrasonic-assisted extraction or microwave-assisted extraction) is auspicious to be applied in the development of this plant to produce effective, efficient extracts and environmentally friendly[28,29]. The plant metabolites play a role in the pharmacological effects of plants. Each pharmacological activity of the plant will be discussed at separate points.

Antioxidant

Antioxidants are chemical substances that, when consumed sufficiently, can prevent damage produced by the oxidation process[30]. In other words, the body needs a substance such as an antioxidant that helps protect against free radical attack[31]. The method that has been used to test antioxidant activity is the DPPH method and hydrogen peroxide scavengers which were tested on samples of methanol extract, ethanol extract, water extract, and petroleum ether fraction. DPPH•, on the other hand, is not a natural radical, but its reaction mechanism with antioxidants is close to that of peroxyl radicals ROO•[32]. When the DPPH solution is mixed with a substrate that can donate a hydrogen atom, it gives rise to a reduced form with a loss of purple color[33]. In this test, ascorbic acid, Trolox, gallic acid, butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) are often used as references[34].

The secondary metabolites, such as flavonoids and saponins, contribute to this activity. Saponins can reduce superoxide by forming hydroperoxide intermediates to prevent damage by free radicals. The antioxidant mechanism of steroids is by scavenging reactive species, such as superoxide and chelating metals (Fe^{2+} and Cu^{2+})[35]. While flavonoids are polyphenols that can donate hydrogen atoms to free radicals, the antioxidant activity of polyphenolic compounds can be generated from a neutralization reaction or at the termination of a chain reaction[36].

Table IV. Pharmacological activities of *E. oxypetalum*

Part of Plant	Type of extracts	Activities and methods	Results
Flowers	Methanol extract and petroleum ether fraction	Antioxidant, *in vitro*, diphenylpicrylhydrazine, DPPH method	The methanol extract and petroleum ether fraction (at 8000 ppm, 60 minute) was able to able to reduce DPPH free radicals by 70.00% and 155.1%, respectively[37].
	Methanol extract	Antihyperuricemia; *in vivo*	The extract (400 mg/Kg BW) can reduce 63.50% of uric acid on mice induced by *melinjo* and chicken liver juice raw[37].
Leaves	Ethanol extract and aqueous extract	Antioxidant, *in vitro*, DPPH and hydrogen peroxide scavenger methods	Both of extracts at 2000 and 500 µg/mL was able to inhibit DPPH radical and hydrogen peroxide scavenging[38].
	Alcohol extract and water extract	Anti-inflammatory, *in vitro*, human red blood cell membrane stabilisation and inhibition of protein denaturation method	Using *in vitro* techniques, the percentage inhibition of alcohol and aqueous extract was highest at 300 µg/mL, but in animal studies, the percentage inhibition of alcohol and aqueous extract was highest at 600 and 200 mg/Kg BW, respectively[39].
	Silver nano particles (AgNPs) synthesized from aqueous extract	Anti-bacteria, *in vitro*, tested using by Kirby-Bauer disc diffusion method	AgNP synthesized from aqueous extract of *E. oxypetalum* indicated the presence of anti-bacterial activity against *Propionibacterium acne, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*[40].
	Extract	Wound healing and kidney hispathological, *in vivo*	The combination of *Catharanthus roseus* and *E. oxypetalum* leaf extract at a concentration of 15% (topically) provided the best wound healing in guinea pigs compared to the administration of each extract alone. Histopathological parameter showed that both extracts were safe for the kidneys[41].
	Ethanol extract 96%	Anti-inflammatory, *in vivo*	In diabetic mice, topical treatment (ointment) of the extract of *E. oxypetalum* leaves accelerated wound healing time, with 20% *E. oxypetalum* extract displayed the highest effect[42].
	Petroleum ether, acetone, and ethanol extracts	Antimicrobe, *in vitro,* disc diffusion method	The maximum inhibition zone was indicated by acetone and petroleum ether extracts against *Escherichia coli* (14 mm); acetone extract against *Staphylococcus aureus* (14 mm); acetone and ethanol extracts against *Klebsiella pneumonia* (10 mm and 10 mm, respectively); and petroleum ether extract against *Bacillus subtilis* (12 mm). All extracts were tested at a concentration of 100 µg/mL. All extracts showed no activity against fungal pathogen[16].
	Methanol extract	Anti-inflammatory, *in vitro*, inhibition of albumin denaturation	The extract showed anti-inflammatory activity with a percent inhibition was 32%[37].
	Methanol extract	Anti diabetic, *in vitro*, α-amylase inhibitory assay	The extract showed the percent inhibition of α-amylase was 26%[37].
	Several active compounds (4-hydroxy-2-methylacetophenone, Stigmasterol, 6-octen-1-ol, 3,7-dimethyl, Megastigmatrienone, Cyclohexylmethyl hexyl ester, Testosterone cypionate	Antivirus, *in silico* study using molecular docking	Megastigmatrienone (5.02 kcal/mol) from *E. oxypetalum* leaves had higher binding interactions against *Treponema pallidum*, followed by megastigmatrienone (4.58 kcal/mol) with liver cirrhosis, and testosterone cypionate (7.084 kcal/mol) with Zika virus[22].

Antihyperuricemia

Determination of uric acid levels was determined by the enzymatic method using uric acid reagent FS-TBHBA (2,4,6-tribromo-3-hydroxybenzoic acid). The mechanism in this method is that the enzyme uricase oxidizes uric acid with the help of H_2O and O_2 into allantoin, CO_2, and H_2O_2. The H_2O_2 formed will react with 4-amino antipyrine and FS-TBHBA to form pink quinonimine; the peroxidase enzyme catalyzes the reaction[43]. Compounds that play a role in lowering uric acid levels are flavonoids. The flavonoid group of compounds inhibits the activity of xanthine oxidase and superoxidase, thereby reducing the formation of uric acid[44].

Anti-inflammatory

Epiphyllum oxypetalum has pharmacological activity as an anti-inflammatory. In a study by Dwita *et al.*[42], *E. oxypetalum* leaf extract contains secondary metabolites such as alkaloids, flavonoids, tannins, saponins, and steroids. Several studies have demonstrated the mechanism of flavonoids in wound healing by modulating the expression of cytokines and nitric oxide in the inflammatory phase.

Antidiabetic

The antidiabetic activity of methanol extract of *E. oxypetalum* leaf showed inhibition of α-amylase. α-amylase is helpful as a hypoglycemic agent to control hyperglycemia, especially in patients with type 2 diabetes mellitus. This enzyme delays carbohydrates and prolongs carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently reducing the postprandial rise in plasma glucose[45]. Secondary metabolite compounds such as phenolics, flavonoids, alkaloids, and steroids have antidiabetic activity[46].

Antibacterial

The chemical content of the *E. oxypetalum* leaf extract has the potential as an antibacterial, both against gram-negative and gram-positive bacteria, compared to antifungals[16]. When paired with antibiotics, nanoparticle technology using silver (silver nanoparticles, AgNPs) synthesized from an aqueous extract of *E. oxypetalum* is more effective. This formulation is both environmentally friendly and cost-effective and may be precious in biomedical applications[40].

AgNPs can be used effectively against many drug-resistant bacteria because their large surface area and small size make them easy to interact with substances and enhance their antibacterial efficacy. AgNPs can be a new generation of antimicrobial with broad-spectrum activity. Biological methods for the synthesis of nanoparticles have several advantages over chemical and physical methods because these methods do not involve chemical toxins, and sometimes reactions take place at very high temperatures. Using plants to synthesize nanoparticles can be an advantage over microorganisms because it eliminates the culture maintenance process[40].

The phenolic compounds contained in *E. oxypetalum* are one of the compounds suspected of having antibacterial activity[16]. Alkaloids are thought to have the ability as an antibacterial that can interfere with the peptidoglycan components of bacterial cells so that the cell wall layer is not formed completely. Terpenoids are other plant metabolites with antimicrobial, antifungal, antibacterial, and antiviral properties. The flavonoids act as an antibacterial agent by building complex molecules with proteins that damage the bacterial cell membrane's integrity. These substances can degrade cell walls and interfere with cell permeability. In addition to flavonoids, a type of polyphenolic compounds that have antibacterial action, particularly tannins[47].

Toxicity study

Safety is a major consideration in the development of medicinal plants. Eleven compounds found in methanol extract of *E. oxypetalum* leaves, through GC-MS analysis, were evaluated for their toxicity using QSAR – Toxicity Estimation Software Tool (TEST). Some of them were predicted to possess high to extreme toxicity against Daphnia *magna*, *Tetrahymena pyriformis*, and *Pimephales promelas*, such as oleic acid, eicosyl ester; hexadecanoic acid, 1-(hydroxymethyl)-1,2- ethanediyl ester; 17-pentatriacontene; cyclopropanebutanoic acid,2-[(2-[(2-[(2-pentylcyclopropyl)methyl]cyclopropyl)methyl]cyclopropyl)methyl]-,methyl ester; 17-(1,5-dimethylhexyl)-10,13-dimethyl-3-styrylhexadeca hydrocyclopenta(a)phenanthren-2-one; and ergosteryl acetate[23].

Among the chemicals found in *E. oxypetalum*, 0-octadecenoic acid, methyl ester and ethanol, 2-(9-octadecenyloxy)-, (Z) were harmless to development. Meanwhile, 1,2-benzenedicarboxylic acid, butyl octyl ester; 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester; cyclopropanebutanoic acid,2-[(2-[(2-[(2-pentylcyclopropyl)methyl]cyclopropyl)methyl]cyclopropyl)methyl]-,methyl ester; 17-(1,5-dimethylhexyl)-10,13-dimethyl-3-styrylhexadecahydrocyclopenta(a)phenanthren2-one; ergosteryl acetate; glycine,N-[(3a,5a,7a,12a)-24-oxo-3,7,12-tris[(trimethylsilyl)oxy]cholan-24-yl]-,methyl ester; and (5a)pregnane-3,20a-diol,14a,18a-[4-methyl-3-oxo-(1-oxa-4-

azabutane-1,4-diyl)]-,diacetate were poisonous. In oral rats, however, all compounds were predicted to have a low toxicity to nontoxic. Through *in silico* research, the usage of animals for toxicity estimates can be reduced[23]. This study was suspected that *E. oxypetalum* leaves are safe for humans and could be used to produce new medications in the future. Of course, before that, scientific evidence of *in vivo* toxicity from extracts and individual compounds of *E. oxypetalum* leaves in animal models is required.

FUTURE PROSPECTS

Based on the information provided, the *E. oxypetalum* plant in the future has the potential to be developed into a source of medicinal ingredients. So, it can be used as a raw material for natural medicine to treat various diseases. The flower and leaves of this plant contain chemical compounds in the form of primary and secondary metabolites. Steroid chemicals dominate the chemical substances detected in this plant, such as testosterone cypionate, stigmasterol, and others. No active chemical compounds against specific pharmacological actions have been isolated on this plant. These isolates will determine quality standards from the extracts or fractions production. In the future, it will also be essential to investigate the plant's roots to complete the information about the plant. The pharmacological activity of *E. oxypetalum* has the potential as an anti-inflammatory, a source of antioxidants, and antimicrobials. The chemical compounds discovered in these plants, particularly steroids, need to be researched further to see whether they may be used for additional therapeutic purposes, such as hormone treatment for fertility, contraception, or even aphrodisiacs.

CONCLUSION

Epiphyllum oxypetalum contains chemical compounds such as carbohydrates, proteins, amino acids, alkaloids, saponins, terpenoids, steroids, flavonoids, tannins, glycosides, and resins. This plant has pharmacological activity such as anti-inflammatory, antimicrobial, antidiabetic, and a source of antioxidants. *Epiphyllum oxypetalum* is a plant that is safe because it is not toxic. *Epiphyllum oxypetalum* has the potential to be investigated and developed further so that the plant's benefits can be shared with the rest of the community..

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AUTHORS' CONTRIBUTION

Chandra Adam Lesmana: conceptualization, investigation, data curation, resources, visualization, writing - original draft, and writing - review & editing. **Ni Putu Ermi Hikmawanti**: conceptualization, methodology, project administration, supervision, validation, visualization, writing - original draft, and writing - review & editing. **Agustin Yumita**: conceptualization, supervision, validation, writing - original draft.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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Research Article

Optimization of Quercetin Gel Formulation using Factorial Design Method and Antibacterial Test against *Propionibacterium acnes*

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Quercetin gel

Abstract

Quercetin is a flavonoid from a group of polyphenolic flavonoid compounds. Quercetin can be used as an alternative to acne treatment, predominantly triggered by *Propionibacterium acnes*. This study aimed to determine the effect and proportion of carbopol 940, propylene glycol, and glycerin on the physical quality of quercetin gel, the ability of the optimum formula in an antibacterial test, and its diffusion using Franz diffusion. This study uses the factorial design method for formula optimization. Optimization was carried out with the parameters of the physical quality of the gel tested, including viscosity, dispersibility, antibacterial, and Franz diffusion. The combination of carbopol 940, glycerin, and propylene glycol affected the physical quality test of quercetin gel, carbopol and glycerin significantly affected viscosity. In contrast, glycerin and propylene glycol significantly affected Franz's dispersion, antibacterial, and diffusion properties. The optimum proportion of the combination of carbopol 940, glycerin, and propylene glycol in the manufacture of quercetin gel using the factorial design method obtained a concentration of carbopol 940 of 0.5%, glycerin of 15%, and propylene glycol of 10%. The optimum formula ability in the antibacterial test was 22.20 mm, and the cumulative percent of quercetin penetrated was 97.91%.

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INTRODUCTION

Acne or acne vulgaris is an infection or inflammation that occurs in areas of the body that produce much oil, such as facial skin. The activation of excess oil causes acne, causing clogged pores or oil gland ducts on the facial skin and hair (pilosebaceous tract)[1]. A bacterial infection can also cause acne. The most dominant bacteria that can trigger the formation of acne is *Propionibacterium acnes*[2]. Acne treatment requires another alternative: natural compounds, such as quercetin. Quercetin is one of the flavonoids from a group of polyphenolic flavonoid compounds; quercetin is generally obtained as aglycones from several flavonoid glycosides[3]. Quercetin has a mechanism of action as an antibacterial that causes acne by inhibiting protein synthesis in bacteria and inhibiting the production of toxin metabolites in bacteria[4]. Based on the mechanism of action of quercetin, it can be used as an antibacterial treatment to facilitate its use and other considerations so that quercetin is prepared in advance.

Quercetin is a hydrophobic compound classified in the Biopharmaceutical Classification System (BCS) II, which means that quercetin has high permeability but low solubility[5]. The problem with quercetin in oral administration is that it has low bioavailability in the body, where its absorption is limited, its elimination is rapid, and it is extensively metabolized[6]. Problems that occur in quercetin gel preparations, the study's results[7] found that the largest inhibition zone was found in formula 1 with a concentration value of 0.05% w/w. In comparison, the minor inhibition zone was found in formula 3 with the highest concentration of 0.50% w/w; the higher the concentration, the greater the minimum inhibition zone obtained.

However, the results obtained in this study were contrary; the greater the concentration used, the smaller the minimum inhibition zone. This is because the phenol (-OH) group of quercetin will bind to HPMC to replace the –OH group of distilled water, resulting in decreased antibacterial inhibition[8]. So this study was further developed by using different gelling agents and different bacteria and optimizing the quercetin gel formula to obtain the optimum formula, which is expected to give better results. This study makes quercetin in gel preparations because it is to help treat acne by determining the optimum formula so that the quercetin gel preparation will be even more optimal in the composition of the formula concentration and antibacterial activity.

Transdermal dosage forms, one of which is a gel preparation. The gel is one of the dominant drug preparations used by the public[9]. Good pharmaceutical preparation must meet safety parameters but also have an optimum composition. A balanced composition between gelling agent and humectant will improve gel stability so that the gel can meet the parameters of pharmaceutical preparations that are good, effective, acceptable, and safe to use[10]. This study used carbopol as a gelling agent, propylene glycol, and glycerin as a humectant. The combination of humectants between propylene glycol and glycerin will maintain the stability of the preparation[11].

Testing the antibacterial activity of quercetin gel preparations uses the well diffusion method. The advantage of the well diffusion method is that it is easier to measure the area of the inhibition zone formed because the isolates are active not only on the top surface of the nutrient agar but also down to the bottom[12]. The well diffusion method is selected because it can accommodate a higher sample concentration than the disc method so that it diffuses faster in the media, which causes the inhibitory effect of bacteria to become stronger[13]. Based on this, it is known that quercetin compounds have problems in their bioavailability when made in oral preparations. Therefore, researchers are interested in making quercetin compounds in gel preparations, optimizing the quercetin gel formula using factorial design methods, and testing antibacterial activity against *P. acnes* bacteria. To obtain the optimum formula from the results of the physical quality test of the quercetin gel preparation and the antibacterial activity test using the well diffusion method.

MATERIALS AND METHODS

Tools and materials

The tools used in this study were analytical balance (AD-600i), micropipette, slide, microscope, hot plate (THERMO Scientific), centrifuge (HC6 Centrifuge), UV-Vis Spectrophotometry (T60), pH meter Atc, 30 viscometer NDJ-5S, incubator (ESCO Isotherm), autoclave, Laminar Air Flow (LAF), weights, and Franz cell diffusion (FDC-2C). The materials used in this study were quercetin, Carbopol 940, glycerin, propylene glycol, nipagin, nipasol, TEA, aqua PA, doxycycline antibiotics 30 m/disc, Nutrient Agar media, *P. acnes* ATCC 11827 bacteria, Mueller-Hinton Agar media, hydrogen peroxide, rabbit plasma, crystal violet, Lugol's, alcohol acetone, ethanol 96%, safranin, and phosphate buffer pH 7.4.

Sterilization of tools and materials

Equipment and materials used in the test were sterilized first. Tools such as test tubes, stirring rods, Erlenmeyer, Petri dishes, or test materials such as NA and MHA media aquadest were sterilized using an autoclave at 121°C for 15 minutes. Tools such as wire loops were sterilized by heating over a flame[14].

Media preparation

The manufacture of MHA media started by weighing 2.66 g of MHA and dissolved into an Erlenmeyer flask with distilled water until it reached a volume of 70 mL, then heated until homogeneous. The media was sterilized using an autoclave at 121°C for 15 minutes. The media was poured into a petri dish of 35 mL and allowed to solidify[15].

Bacterial suspension preparation

The suspension of the *P. acnes* ATCC 11827 test colonies obtained from the hospital and laboratory equipment chemical scientific supply-General Trade, North Jakarta, was rejuvenated by taking one dose of the colony from solid NA media into

a test tube containing 5 mL of physiological NaCl. The turbidity of the test colony suspension was standardized to the 0.5 McFarland standard (approximately 1.5 x 10^8 CFU/mL). The suspension should be used as the inoculum within 15 minutes[15].

Preparation of quercetin gel

The composition of the quercetin gel is presented in **Table I**. First, the weighing was carried out according to the formula, then grinding was carried out on the powder material taken. Carbopol swelling was carried out using distilled water at 37±2°C with constant grinding (mixture 1). Quercetin was dispersed into propylene glycol and then mixed until homogeneous (mixture 2). Nipagin and nipasol were dispersed into glycerin and mixed until homogeneous (mixture 3). Mixtures 2 and 3 were mixed and stirred using a stirrer (mixture 4). mixture 4 was added little by little into Mixture 1 until homogeneous by grinding. Distilled water was added until 100 mL. TEA was added drop by drop while grinding slowly until a homogeneous gel with good consistency was formed. The quercetin gel was evaluated, including organoleptic examination, homogeneity, viscosity, spreadability, pH, antibacterial, and Franz diffusion test[7].

Table I. Composition of Carbopol 940, glycerin and propylene glycol

Formula	Composition (%)		
	Carbopol 940	Glycerin	Propylene glycol
1	0,5	15	5
2	2	5	5
3	2	5	10
4	0,5	15	10
5	0,5	5	5
6	2	15	10
7	0,5	5	10
8	2	15	5

Physical quality check of quercetin gel

Organoleptic and homogeneity test

An organoleptic test was carried out by observing the quercetin gel preparation, including color, odor, and shape. The homogeneity test was carried out using a certain amount of preparations smeared on glass pieces. Observations were made on the preparations: the preparations had to show a homogeneous arrangement, and no coarse grains were seen; the homogeneity test was repeated three times[16].

Viscosity test

A viscosity test was carried out by adding 15 g of gel to the container. The test was carried out using a Viscometer NDJ-5S with rpm 30 and spindle No 4. The viscosity results were observed. The procedure was repeated three times. Good viscosity ranged between 3,000-50,000 cPs[17].

Spreadability test

The spreadability/dispersion test was carried out after 24 hours of manufacture. This test is carried out by taking 0.5 g of the gel preparation and placing it in the middle of a round glass that has been given a scale. Then covered with glass as the initial load and allowed to stand for a minute. After a minute of adding a load of 50 g, the diameter of the dispersion was measured. The load was then added 50 g and allowed to stand again for a minute, and the exact measurements were carried out as before for each addition of 50 g of load to 250 g, repeated three times[18].

pH test

The pH test was carried out using a pH meter by dissolving 1 g of the gel preparation in 10 mL of aquadest. The pH meter before use was calibrated using acetate buffer pH 4.0 and phosphate buffer pH 7.0. The calibrated pH meter was ready for use by dipping it into the gel. The pH value on the device was recorded, and repetitions were performed three times. The pH value of the gel preparation must be in the range of a neutral pH or suitable for the skin, between 4.5-6.5[19].

Antibacterial test

A suspension of 100 L of *P. acnes* bacteria was inoculated into six Petri dishes containing MHA media, then leveled on the media using L rods. Wells were made using a cork borer. 70 mg of gel was inserted into the wells that had been made; the holes were in the media with a diameter of 6 mm, and the size of the petri dish was 11 cm. One petri dish consisted of four wells for four formulas of the quercetin gel preparation; a total of six Petri dishes and two Petri dishes for positive control and negative control were then incubated for 24 hours at 37°C. The clear inhibition zone around the well was observed and measured using a caliper. The procedure was repeated three times[20].

Determination of the optimum formula for quercetin gel

The optimum formula was determined after testing the gel preparation's physical quality: viscosity, dispersibility, antibacterial, and *in vitro* penetration tests using Franz diffusion cells. The results of the physical quality of the gel preparation were then processed using expert design software using the factorial design method. The optimum formula selected was expected to have a desirability value close to 1. The optimum formula for the resulting quercetin gel was expected to have a suitable viscosity value of 3,000-50,000 cPs. The spreadability of a good gel preparation was 5-7 cm[11]. The antibacterial value in the strong category was in the range of values of 11-20 mm. *In vitro* penetration of Franz diffusion results reaches 40-90%[21].

RESULTS AND DISCUSSION

Organoleptic test

Organoleptic observations were seen from the gel preparation's shape, color, and odor, and the results obtained were recorded. The organoleptic results of eight quercetin gel formulas can be seen in **Figure 1** and **Table II**. Purpose organoleptic examination was carried out to determine the physical properties of the gel-based on the results of direct visual observation. Research conducted by Irianto *et al*.[22] related to organoleptic tests also observed the gel preparations made, including the gel preparation's shape, color, and odor. This test needs to be done because it relates to the convenience of use as a topical preparation. Observation of the homogeneity of the eight quercetin gel formulations gave good results, which looked homogeneous. Purgiyanti and Pratiwi[23] also reported that the gel homogeneity test was carried out to determine the mixing of each component in the gel manufacture.

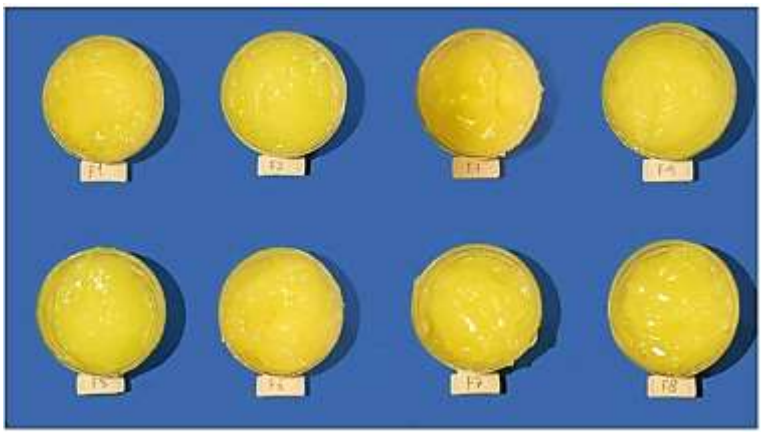

Figure 1. Quercetin gel preparation

pH test

The pH test in this study aims to determine whether the gel produced is acceptable for skin pH because it can cause skin irritation if not in accordance with skin pH. The results of the pH test for quercetin gel preparations can be seen in **Table III**. According to Gozali *et al*.[24], the skin's pH balance ranges from 4.5 to 6.5 because if the pH value is less than 4 and more than 7, it is feared that it can irritate the skin. In normal skin with balanced sebum secretion, the pH of facial skin is at 5.5. Meanwhile, oily skin tends to be more acidic, resulting in a lower pH, often owned by people with acne-prone and easily

irritated skin. The results of the pH test in this study were by the requirements if used on normal skin or acne-prone skin because the pH range obtained was still in the pH range of 4.5-6.5[11].

Table II. Organoleptic examination of quercetin gel

Formula	Shape	Color	Odor
1	Soft thick	Yellow	Typical
2	Soft thick	Yellow	Typical
3	Soft thick	Yellow	Typical
4	Soft thick	Yellow	Typical
5	Soft thick	Yellow	Typical
6	Soft thick	Yellow	Typical
7	Soft thick	Yellow	Typical
8	Soft thick	Yellow	Typical

Table III. pH examination of quercetin gel

Formula	pH±SD
1	5.6±0.05
2	5.5±0.20
3	5.8±0.05
4	5.6±0.10
5	5.6±0.15
6	5.7±0.15
7	5.6±0.15
8	5.6±0.10

Antibacterial test

The quercetin gel antibacterial activity test was carried out using the well diffusion method. The pitting method was chosen because this method can accommodate a higher gel concentration than the disc method. So that it diffuses more quickly in the media, which causes the inhibitory effect of bacteria to be stronger. The antibacterial activity test of quercetin gel can be seen in **Table IV**. The components that influence the antibacterial activity in this study are glycerin and propylene glycol. Glycerin has a preservative for antimicrobials if used in concentrations <20%; therefore, glycerin influences the results of antibacterial tests. Glycerin can also increase the active substance's permeability to increase the gel preparation's antibacterial inhibition[25]. According to Anastasia *et al*.[26], the addition of glycerin in the formula affects the activity of the inhibitory zone of the active substance, which can be seen from the comparison of the diameter of the inhibition zone in each formula. Zhang *et al*.[27] also stated that propylene glycol facilitates mixing between quercetin and gel base, so the higher the propylene glycol concentration is used, the antibacterial activity will increase. Propylene glycol has lipophilic properties, so it will help quercetin to penetrate the cell wall of bacteria. Propylene glycol has a synergistic effect helping the penetration of the active substance and works as an antimicrobial component.

Table IV. Antibacterial activity of quercetin gel

Formula	Inhibition zone±SD (mm)	Antibacterial activity category
1	18.88±0.06	Strong
2	15.47±0.44	Strong
3	15.79±0.72	Strong
4	18.68±0.05	Strong
5	17.39±0.02	Strong
6	16.62±0.02	Strong
7	17.66±0.02	Strong
8	16.42±0.27	Strong

Determination of the optimum formula for quercetin gel

Propionibacterium acnes grow optimally at a pH of 6-7[28]; the preparations obtained a pH of around 5. This causes the bacteria not to grow optimally because the pH is too acidic. This condition can increase the inhibitory power of the gel preparation, which is made more significant. In general, gel viscosity influences the inhibition of bacteria. This is because the high viscosity will inhibit the release of the active substance quercetin[29]. However, in this study, the large viscosity did not affect the release of quercetin because it was assisted by propylene glycol and glycerin. On the contrary, it would increase the

contact time of the gel with the skin, thereby increasing the antibacterial inhibition. The calculation results of the factorial design response to the value of viscosity, dispersion, antibacterial, and diffusion Franz can be seen in **Table V**. The equations presented arranged based on the factorial design chart for the response values of viscosity, spreadability, antibacterial, and Franz diffusion. Based on these data, it can be observed that the value of each parameter obtained varies with each formula. There are differences in these values. It was caused by Carbopol 940, glycerin, and propylene glycol levels.

Table V. Calculation of the factorial design response

Response	Factorial design equation
Viscosity	Y= +2191.50 (A) +0.6100 (B)- 66.480 (C)
Spreadability	Y= -0.3500 (A) +0.0175 (B)+0.04500 (C)
Antibacterial	Y= -1.385 (A) +0.107 (B) +0,029 (C)
Franz diffusion	Y= -15.17 (A) +0.880 (B)+0.549 (C)

In the equation generated from the viscosity response, it is known that the most influential factors in changing the viscosity value are Carbopol 940 and glycerin. The interaction between Carbopol 940 and glycerin components in each formula has a significant effect. The mechanism of gel formation by Carbopol 940 is by binding the solvent to the structure of the carbopol polymer so that crosslinks occur in the polymer, causing water to be trapped in it. When carbopol comes into contact with the water environment, anionic polyelectrolytes are formed, which then form a hydrogel structure[30]. When TEA is added, the negative charge repulsion along the polymer chain will increase, and the osmotic pressure inside the expanding polymer causes the formation of a three-dimensional structure. In other words, carbopol macromolecules combine to form flocks in solution. Increasing the polymer concentration in the aqueous environment will increase crosslinked polymer chains after adding the base agent. The higher the polymer concentration in the aqueous environment, the resulting gel network is denser and more compact so that the viscosity will increase[31]. According to the results obtained, glycerin affects viscosity. The mechanism of glycerin in increasing viscosity is by increasing the surface tension of the gel[32].

In the equation generated from the spreadability response, it is known that glycerin and propylene glycol are the most influential factors in changing the dispersion value. The interaction between the two components of glycerin and propylene glycol in each formula has a significant effect. The positive value of the equation is found in the components of glycerin and propylene glycol, which indicates that these two components affect the spreadability of the quercetin gel preparation. Semi-solid preparations will experience an increase in the number of dissolved particles caused by glycerin and propylene glycol. This will increase the interaction of the particles so that the movement of molecules in the preparation increases the spread of the gel[33]. If preparation has a high spreadability, the active substance will be evenly distributed and more effective in producing a therapeutic effect[34].

In the equation generated from the Franz diffusion response, it is known that glycerin and propylene glycol are the most influential factors in changing the dispersion value. Based on this equation, the value of the glycerin and propylene glycol components showed positive results, which indicated that the two components affected the percent penetration value of quercetin. Glycerin and propylene glycol cause dehydration of the skin membrane. This will increase the active substance's permeability from formulations with a water base[35].

The Design-Expert version 13 program will select a formula with the highest desirability so that the selected optimum formula will produce the desired physical properties of the gel. A good desirability value is close to 1. The Superimposed quercetin gel contour plot preparation is shown in **Figure 2**. Based on the contour plot in the figure, the optimum point is obtained, which is marked by a box labeled prediction accompanied by a given desirability value of 0.928.

The results of the physical properties test of the optimum quercetin gel formula were compared with the predicted values obtained from the Design-Expert program by statistical analysis using a one-sample t-test. The calculation is intended to determine the difference between the experimental value of the optimum gel formula and the predicted results from the program. These results indicate that the resulting formula has physical properties that match the predictions. All parameters of physical properties have a significance value greater than 0.05, so it can be concluded that the comparison of program prediction data with experimental values of the optimum quercetin gel formula is not significantly different. The optimum

formula composition obtained from the factorial design software shows that the proportion of the optimum formula concentration is carbopol 940 0.5%, glycerin 15%, and propylene glycol 10%. A comparison of the predicted value with the experimental value of the optimum formula can be seen in **Table VI**.

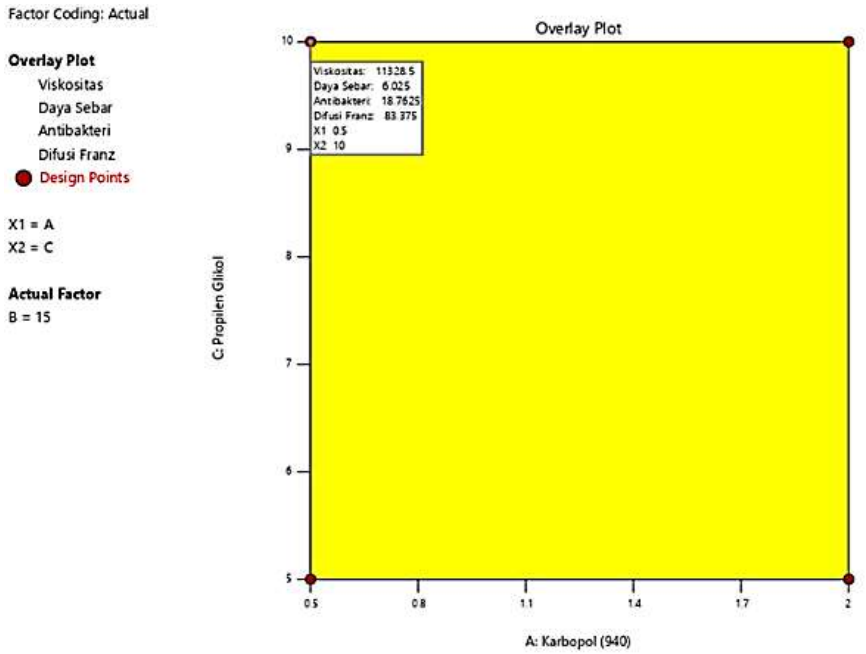

Figure 2. Superimposed quercetin gel contour plot. (Translation notes: *Viskositas*: viscosity; *Daya sebar*: spreadability; *Antibakteri*: antibacterial; *Difusi Franz*: Franz diffusion)

Table VI. Comparison of the predicted value with the experimental value of the optimum formula

Physical quality	Predictive value	Trial value	Sig.
Viscosity (cPs)	11,328	14,980	0.088*
Dispersibility (cm)	6.02	7.20	0.057*
Antibacterial/inhibition zone (mm)	18.76	22.20	0.053*
Franz diffusion (%)	83.37	97.91	0.051*

* Not significantly different

A one-sample t-test with a 95% confidence level was used to compare the predicted and experimental values. In the viscosity response, a value of >0.05 was obtained, so it was concluded that there was no significant difference between the predicted and experimental values. In the dispersion response, a value of >0.05 was obtained, concluding that there was no significant difference between the predicted and experimental values. In the antibacterial response, a value of >0.05 was obtained, concluding that there was no significant difference between the predicted and experimental values. In the Franz diffusion response, a value of >0.05 was obtained, concluding that there was no significant difference between the predicted and experimental values. It can be concluded that the results of the overall one sample t-test between the Franz diffusion predictions and the experimental values are not significantly different, indicating that the experimental values are close to the software predictions because the sign values of all tests are >0.05.

CONCLUSION

The factorial design equations obtained from the response to the viscosity, dispersibility, antibacterial, and Franz diffusion tests can be concluded that the combination of carbopol 940, glycerin, and propylene glycol has a significant effect. The optimum formula composition from the predicted factorial design is carbopol 940 of 0.5%, glycerin 15%, and propylene glycol 10%. Moreover, the optimum formula ability of quercetin gel against antibacterial test has an inhibition zone value of 22.20 mm and penetration of the active substance quercetin with a cumulative percentage of penetrated quercetin in the gel

preparation of 97.91%. Comparison of the results of the predicted value and the experimental value of the physical quality test of the quercetin gel, it can be concluded that the overall results of the test using the one-sample t-test between the predicted value and the experimental value were not significantly different, because the sign values of all tests were >0.05.

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AUTHORS' CONTRIBUTION

M. Andi Chandra: research team leader, validation, and article writing. **Ilham Kuncahyo**: supervision, validation, methodology. **Ana Indrayati**: supervision, methodology.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The author declares there is no conflict of interest and equivalent.

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Review Article

Campylobacter Species, Microbiological Source Tracking and Risk Assessment of Bacterial pathogens

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Source attribution

Abstract

Campylobacter species continue to remain critical pathogens of public health interest. They are responsible for approximately 500 million cases of gastroenteritis per year worldwide. Infection occurs through the consumption of contaminated food and water. Microbial risk assessment and source tracking are crucial epidemiological strategies to monitor the outbreak of campylobacteriosis effectively. Various methods have been proposed for microbial source tracking and risk assessment, most of which rely on conventional microbiological techniques such as detecting fecal indicator organisms and other novel microbial source tracking methods, including library-dependent microbial source tracking and library-independent source tracking approaches. However, both the traditional and novel methods have their setbacks. For example, while the conventional techniques are associated with a poor correlation between indicator organism and pathogen presence, on the other hand, it is impractical to interpret qPCR-generated markers to establish the exact human health risks even though it can give information regarding the potential source and relative human risk. Therefore, this article provides up-to-date information on campylobacteriosis, various approaches for source attribution, and risk assessment of bacterial pathogens, including next-generation sequencing approaches such as shotgun metagenomics, which effectively answer the questions of potential pathogens are there and in what quantities.

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INTRODUCTION

Campylobacter spp cause campylobacteriosis, a chronic enteric infection. *Campylobacter* spp. are among the leading causes of gastroenteritis globally[1]. Importantly, World Health Organization (WHO) has identified *Campylobacter* species as one of the high-priority antimicrobial resistance. The evolution of antimicrobial resistance poses an additional threat to modern medical procedures, rendering current intervention measures geared towards curtailing the menace ineffective and increasing the mortality rate, causing treatment failure and infections—the spread of resistance genes through the environment[2]. Although the environment has been described as the reservoir of antibiotic-resistant bacteria which can be transmitted to humans, the environmental load of antibiotic-resistant *Campylobacter* is scarcely investigated[3]. Ingestion of contaminated water, as well as food, is the principal risk factor of campylobacteriosis[4].

There are various methods of source tracking and microbial risk assessment, most of which rely on conventional microbiological techniques. Detection of fecal indicator organisms such as *Escherichia coli* has been used as a traditional surface water pollution monitoring and risk assessment method[5]. However, this method is hampered by several limitations: poor correlation between indicator organism and pathogen presence and the inability of the method to indicate the source

of fecal pollution since indicator organisms are excreted by some warm-blooded animals, although source tracking is an essential tool for public health risk characterization and the subsequent implementation of remediation and control strategies[6-8]. In the last few years, novel microbial source tracking methods have emerged to mitigate these challenges. These include library-dependent microbial source tracking and library-independent source tracking. However, library-dependent microbial source tracking methods have several setbacks, such as poor interspecies sensitivity, specificity, and overall accuracy[9]. Interestingly, library-independent techniques such as quantitative PCR (qPCR) have allowed the accurate study of fecal pollutants in environmental samples, including water, by quantifying the host-specific microbial source by tracking gene markers[10]. In the library, independent techniques, *Bacteroidales*, as bacteria with a strict requirement for the absence of oxygen inhabiting the human and animal gut with a higher population relative to *E. coli*, are typically used as the target[11]. Host-specific *Bacteroidales* 16S rRNA gene markers have been developed for diverse hosts to segregate human and non-human fecal sources in the environment[12]. However, instead of targeting *Bacteroidales* 16S rRNA, a recent study reports that bird feces could be discriminated from other fecal sources by targeting bacterial taxonomic groups like species of *Helicobacter* with better results[13]. Again, these methods are not without limitations. For example, studies have reported that variations in geographical locations could seriously interfere with the performance and results of these microbial source tracking techniques[14,15]. Equally, it is impractical to interpret qPCR-generated molecular markers to establish the exact human health risks even though it can give information regarding the potential source and relative human risk[16]. Other techniques of microbial source tracing depend on the results of antibiotics resistance and carbon utilization assays[17]. In light of the limitations of these methods, it is, therefore, necessary to look inward to find alternative options that are robust in terms of sensitivity and specificity.

Recent advances in next-generation sequencing (NGS) approaches (**Figure 1**), such as shotgun metagenomic sequencing, have resulted in its widespread application in every aspect of microbiology, microbial source tracking inclusive[18]. Shotgun metagenomics can effectively answer the questions of what potential pathogens are there in a sample by identifying virulence and resistance genes and in what quantities[19]. When analyzed using an appropriate source tracking algorism, shotgun metagenomics data becomes a powerful tool for microbial source tracking and risk assessment[20].

Shotgun metagenomics is widely applied in environmental and clinical studies[21]. Metagenomics sequencing has been used to systematically study antibiotic genes associated with the human microbiome[22], study the links of the microbiome with inflammatory bowel diseases[23], and, importantly, track outbreaks of human pathogens[24]. Therefore, we set out to provide information on various source attribution methods and risk assessment of bacterial pathogens, highlighting the potential of next-generation sequencing in combination with machine learning technology.

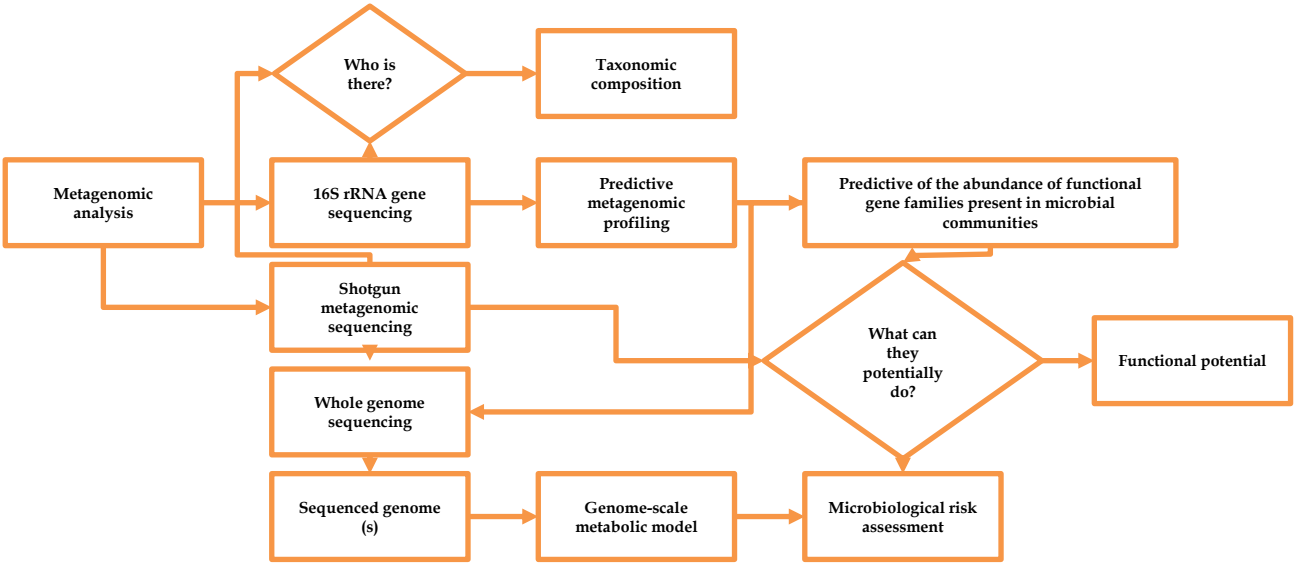

Figure 1. The links between metagenomics and microbial risk assessment[25]

MEDICALLY IMPORTANT *Campylobacter* spp., RESISTANCE GENES, AND RESERVOIRS

Campylobacter species, gram-negative, slender, spirally curled, and microaerophilic bacteria are essential etiologic agents of gastroenteritis in humans, responsible for approximately 500 million cases of gastroenteritis per year globally[1]. Veron and Chatelain[26] were the first to carry out a broad taxonomic study on the *Campylobacter* genus and classified them into four different species: *C. fetus, C. coli, C. jejuni*, and *C. sputorum* nearly five decades ago. Ever since, at least 36 species and 14 subspecies of *Campylobacter* have been described[27]. These include *C. upsaliensis, C. ureolyticus, C. helveticus, C. rectus, C. showae, C. gracilis, C. hominis, C. curvus, C. concisus, C. insulaenigrae, C. hyointestinalis*, and *C. lanienae*. Of all these, *C. jejuni* and *C. coli* are considered to be the leading cause of human campylobacteriosis[27,28]. Various extra-gastrointestinal conditions and autoimmune diseases, especially Guillain–Barre syndrome, have been mainly linked to *C. jejuni*[27]. However, pathogenicity in other species such as *C. lari, C. fetus, C. ureolyticus, C. upsaliensis, C. hyointestinalis*, and *C. concisus* has been documented[29,30]. Species of *C. fetus* have been isolated in septicemia patients and are frequently described as the etiologic agent of poor fertility and miscarriage in humans and animals[27]. It is therefore clear that the accurate tracking of these pathogens is crucial given their wide-ranging medical significance, especially as a number of them have been identified to harbor antibiotic resistance genes (**Table I**).

Table I. Medically important *Campylobacter* spp., resistance genes, and reservoirs

Campylobacter spp.	Resistance genes	Primary reservoir	References
C. jejuni	CmeDEF, *erm*(B), *aadE*, *sat4*, *aphA-3*, *tet*(O), *ant-like* A, *ant-like* B, *ant(6)-Ia*, *sat-1*, *sat-4*, *lnuC*, *ant(6)-Ib*, *aad9*, *aph(3)-IIIa*, *aph(2)-IIIa*, *hpt*, *apmA*, bla_{OXA-61}, *gyr*A and CmeABC	Dogs and cats	27,31-35
C. coli	*erm*(B), CmeABC, *aadE*, *sat4*, *aphA-3*, *tet*(O), bla_{OXA-61}, *cat*, *cfr*(C), *gyr*A, *ant-like* A, *ant-like* B, *ant(6)-Ia*, *sat-1*, *sat-4*, *lnuC*, *ant(6)-Ib*, *aad9*, *aph(3)-IIIa*, *aph(2)-IIIa*, *hpt*, *apmA* and *lnu*Cs	Dogs, cats, pigs and poultry	27,31,33-37
C. upsaliensis	*tet*(O) and *gyr*A	Dogs and cats	38,39
C. fetus subsp. *fetus*	*gyr*A, *tet*(44) and *ant(6)-Ib*	Cattle and sheep	40,41
C. rectus	*erm*(B)	Human oral cavity and dogs	42-44
C. hyointestinalis	*gyr*A	Cattle, pig and sheep	44,45

Campylobacter jejuni and *C. coli* exhibit intrinsic resistance to bacitracin, novobiocin, penicillin, rifampicin, trimethoprim, sulfamethoxazole, vancomycin, and most of the cephalosporins, whereas resistance to aminoglycosides, quinolones, macrolides, ketolides, amphenicols, and tetracyclines is usually acquired[46-48]. Although macrolides, such as azithromycin, and fluoroquinolone, such as ciprofloxacin, are the primary and secondary drugs of choice for the treatment of campylobacteriosis, resistance to these important antibiotics among species of *Campylobacter* with the potential to bring about more severe consequences, including prolonging hospitalization and higher risk of invasive infection or even death, have been reported[27]. This is of enormous concern, particularly when the global public health experts are struggling to contain the menace of antimicrobial resistance. What is more concerning, though, is that various mechanisms of resistance and, in some cases, a combination of more than one mechanism have been identified in these pathogens[49]. **Table II** summarizes the various mechanisms of resistance identified in *Campylobacter* spp.

MICROBIAL RISK ASSESSMENT

Quantitative microbial risk assessment modeling has been used to evaluate the risk of disease from waterborne pathogens since the 1980s. It is a type of modeling used to outline the human risk of exposure to disease-causing microbes from the environment through a dose-response model[50]. These models consist of several probability steps that rely on literature or primary data. Before the 2010 Haiti cholera epidemic, only a few studies, such as an analysis of the 1993 cryptosporidium outbreak in Milwaukee, Wisconsin, and an analysis of epidemic and endemic conditions caused by waterborne pathogens, applied mathematical modeling to study the transmission of the etiologic agents[51,52]. However, the Haiti cholera epidemic

shattered the country in 2010 and triggered a significant interest in applying infectious disease transmission modeling methods for waterborne microbial risk assessment; ever since significant progress has been made[53].

Table II. *Campylobacter* spp. mechanisms of resistance to various classes of antibiotics

Class of antibiotic	Mechanism of antibiotic resistance	References
Aminoglycosides such as gentamicin, amikacin, tobramycin, neomycin, and streptomycin.	a). Enzymatic modification and inactivation of antibiotics	[27,54]
Macrolides, lincosamides and ketolides. Examples include erythromycin, roxithromycin, azithromycin and clarithromycin.	a). Target mutation in 23S rRNA or/and ribosomal proteins L4 and L22 b). Modification of the ribosomal target by methylation through *erm*(B) c). Multidrug efflux pump (CmeABC) and altered membrane permeability	[55,56]
Quinolones such as levofloxacin (Levaquin), ciprofloxacin (Cipro), ciprofloxacin extended-release tablets, moxifloxacin (Avelox), ofloxacin, gemifloxacin (Factive) and delafloxacin (Baxdela)	a). Modification of DNA gyrase target (Thr86Ile) b). Multidrug efflux pump (CmeABC)	[27,57]
Tetracyclines such as tetracycline, doxycycline, minocycline and tigecycline	a). Protection of the ribosomal binding site by ribosomal protection proteins (RPPs) encoded by *tet*(O) b). Multidrug efflux pump (CmeABC)	[58,59]
β-Lactam antibiotics (penicillins and cephalosporins) such as carbenicillin, penicillin G, ticarcillin, ampicillin, nafcillin, cloxacillin, mezlocillin, oxacillin, and piperacillin.	a). Enzymatic inactivation of the antimicrobials by β-lactamase (OXA-61) b). Multidrug efflux pump (CmeABC)	[27,60]

Dose-response models are response curves produced by plotting the probability of a response outcome such as infection, illness, or death versus the known dose of the etiologic agent via an identified transmission route. Dose-response model is the main component of quantitative microbial risk assessment[61]. It is so crucial that a complete quantitative microbial risk assessment model is almost impossible to develop without it. Dose-response modeling can be regarded as a multidisciplinary area requiring substantial knowledge and skills in microbiology, pathology, mathematics, statistics, and computing[62]. In order to understand the procedure employed in the development and delivery of inoculum, as well as assess the employability of the data to the dose-response model, microbiology skills are necessary[63].

On the other hand, knowledge of pathology is required to assess the relevance and setbacks of identified exposure routes. To understand how to develop approaches to improve a model and write the required code to run such algorithms, computing and mathematics skills are needed. Furthermore, statistics knowledge is necessary for determining the confidence associated with employing the dose-response model across multiple hosts, pathogen strains, pathogen isolates, and routes of exposure[64]. In the design of a dose-response model, dosing experiments are typically carried out on animal models. Here a fixed concentration of pathogens is introduced to animals, and the resulting response is observed. The outcomes obtained are then incorporated into exponential or β-Poisson models, which will produce numerical constants that would calculate the probability of response outcome possible. Pathogen's concentration needed to trigger a response in ½ of the tested population would be regarded as either lethal dose-50 (LD_{50}) or infectious dose-50 (ID_{50})[61].

The dose-response models currently available in quantitative microbial risk assessment software packages are fixed, based on the pathogen(s) chosen or sole pathogen(s). The packages do not make it possible for researchers to choose a dose-response model or learn more about dose-response modeling in general, hindering users' ability to visualize and optimize the dose-response model[65]. For example, QMRASpot, a quantitative microbial risk assessment software developed by Kiwa Watercycle Research (KWR) which precisely models drinking water systems for the Dutch government, has its overall exposure pathway and dose-response models embedded, unchangeable, and cannot be independently visualized[66]. Similarly, The FDA-iRISK, an integrative comparative risk assessment system primarily designed for food-borne hazards, displays the dose-response model name and its functional forms. It also updates the dose-response model regularly using expert elicitation from dose-response experts, but the capability to choose, optimize or visualize the dose-response models is unavailable[67]. Noteworthy, dose-response models for many infectious bacteria, including antibiotic-resistant bacteria, are lacking, and whether dose-response between antibiotic-resistant and susceptible bacteria might vary remains unknown. Therefore, to bypass these limitations associated with dose-response models[61].

APPLICATION OF SHOTGUN METAGENOMICS AND MATHEMATICAL MODELS IN RISK ASSESSMENT

Application of high-throughput sequencing techniques such as shotgun metagenomics can allow genomic analyses and identification of genes present in genomes of all microbial communities and the protein in a sample without the need for prior culture in the laboratory[68]. Shotgun metagenomic sequencing is a type of sequencing that reads out the nucleotide bases of all microbial DNA present in a sample without targeting a particular genomic locus[69]. Here, microbial DNA is typically extracted and pruned into small chunks sequenced severally rather than targeting a specific genomic locus. This will produce DNA reads that align to distinct genomic locations for the various genomes present in the sample. This approach allows resistance and virulence genes to be identified, cloned, and functionally expressed[70]. In comparison to 16S rRNA gene amplicon sequencing, which only profiles targeted organisms or particular genes, shotgun metagenomics sequencing has been proven to provide results with enhanced resolution, better sensitivity, and more broad characterization of microbial communities in samples. This has led to its widespread application across the globe in various fields of scientific research[71].

Since its introduction almost two decades ago, metagenomics approaches have been applied to various studies, including characterizing endosymbiotic bacteria from the environment, identification of bacterial species capable of carrying out total ammonia nitrification, detecting of presence of antibiotic-resistant genes in bacteria from the gut, investigating human pathogen outbreak and study of diversity and function of microorganisms living in different types of water samples[72]. Specifically, shotgun metagenomics has been employed to characterize taxonomic and functional shifts in hot water microbiomes and established that unassembled short metagenomic reads were efficient for broadly screening for the potential presence and quantities of pathogens of interest in water[73]. Likewise, in a recent study, Chen *et al.*[74] carried outsource the identification of antibiotic resistance genes of an interconnected river-lake system using shotgun metagenomics and observed an abundance of assorted genes linked to sewage pollution from city effluents. Further, in a different study[75], a shotgun metagenomic study brings sand from freshwater beaches as a source of disease-causing bacteria. Hence, the exploitation of this approach in microbial risk assessment no doubt offers significant potential in discovering resistance and virulence genes among members of *Campylobacter* in the water system.

Targeted screening method using the 16S rRNA gene marker for bacteria and shotgun metagenomics approach, which allows for the broad-range simultaneous detection of all microorganisms using the complete genetic information in the sample, are the two classical approaches commonly employed to study the composition of metagenomics samples[76]. The 16S rRNA gene, found in the genetic material of every bacterium, has alternating and conserved regions. The conserved areas of the 16S rRNA gene allow for amplifying the nine variable regions using specific short single-strands of nucleic acid called primers. The amplification products are then processed for sequencing in a library construction process[77]. Typically, shotgun metagenomics constitutes six steps from study design to data validation. There is sample collection; processing and sequencing; pre-processing of the sequencing reads; profiling of taxonomic, functional, and genomic features; and data analysis[78]. Every stage of this multi-sequential requires careful preparation and excursion, especially since every step has several pitfalls that can affect the final result. To ensure the lysis reagent has access to the nucleic acid, adequate homogenization, and cell lysis before nucleic acid extraction must be achieved[79].

Phylogenetic analyses of pathogenic microbes using next-generation sequencing approaches like shotgun metagenomics are potent tools for tracking the origin of disease, examining the evolutionary relationships, and deciphering the transmission pathways[69]. Shotgun metagenomics is so robust that it can be employed in taxonomic characterization and understanding the relationships between microorganisms, their activities, and functionalities in a given environment. This way, interest can be in the presence of antibiotic resistance and virulence genes and their transcripts[80]. Using an appropriate bioinformatics analysis tool or microbial risk assessment model, data generated from shotgun metagenomics can be analyzed to investigate an outbreak, source attribution, and risk assessment, depending on the study's objectives. Therefore, the potential this kind of powerful approach holds cannot be overlooked[81].

APPLICATION OF WHOLE GENOME SEQUENCING AND METAGENOMICS IN OUTBREAK INVESTIGATION, SOURCE ATTRIBUTION AND RISK ASSESSMENT OF FOODBORNE PATHOGENS

Whole genome sequencing (WGS) and metagenomics are powerful tools in contemporary food safety studies because they make possible robust and timely detection, identification, and characterization of a wide range of foodborne pathogens. During an outbreak, a credible, rapid and powerful identification technique is invaluable in curtailing the etiologic agent's further spread and avoiding false source attribution[82]. Either culture-based or targeted techniques commonly identify foodborne pathogens. Targeted identification techniques such as PCR or ELISA, although rapid since they can be carried out without the need for prior culture, are not potent and therefore allow unrepresentative strains to go undetected. In addition, because of their low molecular level resolution, these techniques are incapable of establishing the link between an outbreak and detected pathogenic microorganisms[83].

In recent years, the development of novel source-tracking models has been rapidly triggered by a surge in the application of WGS in food safety and public health. Various models and machine learning algorithms have now replaced conventional risk assessment models. Bioinformatics data sharing tools make it particularly crucial as it allows efficient use of WGS and metagenomics in risk assessment, source tracking, and outbreak investigations, specifically at local, regional, national, and international levels[84]. Whole genome sequencing is a powerful molecular technique with a high ability to discriminate among isolates. Thus, it can be employed to establish the relationship between an outbreak and a specific pathogen. Although its laborious nature has limited its application to research settings rather than routine food screening, quite several researchers have successfully employed WGS for source tracking in retro-perspective studies of enterohemorrhagic *E. coli*[85], *Salmonella* Bareilly strain causing a foodborne outbreak[86], and protracted invasive listeriosis Outbreak in Germany[87].

Further, the use of WGS in outbreak investigation in the food industry by the United States Food and Drug Administration and the Centre for Disease Control is increasing. For the outbreak investigation, data generated from WGS studies are deposited to the GenomeTrakr, an open-access database. Currently, the GenomeTrakr database consists of laboratories in the US and worldwide, resulting in a significant data increase[88]. GenomeTrakr and similar databases employed in outbreak investigations are making it increasingly possible to decipher the links between sequence data from disease outbreaks on the one hand and food and environmental sources on the other. Similarly, the capacity of WGS to discriminate isolates based on their sources makes it possible to detect diffuse outbreaks by linking rare cases, which would ordinarily be regarded as sporadic cases lacking a common source. This will go a long way in mitigating disease outbreaks from their source[89].

Phylogenetic data can be employed in source attribution since source attribution aims to measure the corresponding significance of particular food sources and animal reservoirs for human cases of foodborne diseases. The genetic information could indicate possible relationships with specific hosts or reservoirs and therefore provide hints on a particular foodborne path's geographical distribution and transmission path[90]. In identifying transmission routes by determining the epidemiological links between reservoirs or sources of infections and supplanting the epidemiological data, WGS is an efficient technique. This approach has proven efficient for several foodborne pathogens such as *Salmonella*, replacing traditional source tracking methods, which are often insufficient and inaccurately attribute the source of contamination[91].

Metagenomics, as a technique that does not rely on a prior culture of samples, has the potential to contribute significantly to outbreak investigation, and risk assessment in food microbiology, particularly as it relates to the detection and characterization of non-culturable, fastidious microbes, the source attribution of risk related to virulence and resistance genes, as well as assessment of microbial risk in complex communities[82].

The application of metagenomics sequencing makes it possible for the synchronous detection and identification of the etiologic agent, antimicrobial resistance, and virulence genes, providing potential as a reliable technique for examining food and water quality[92]. The application of metagenomics in food safety to detect pathogenic microorganisms in foods is one major area that has received attention in recent years. In addition to detection and identification, analysis such as source attribution and risk quantification might be desired. The pathogenicity of some food pathogens, such as the *Bacillus cereus*,

which have very similar genomes, can be determined using virulence determinants encoded on their extrachromosomal DNA. The combination of data such as the presence of pathogens and specific virulence markers is necessary for risk assessment associated with these bacteria in contaminated food[82]. In order to detect foodborne pathogens using metagenomics, the application of shotgun sequencing has been recommended since it allows the detection and characterization of microorganisms from various forms of samples[93].

Using the metagenomics approach, detection of disease-causing bacteria involves taxonomic profiling of shotgun sequencing data using bioinformatics tools which could produce false results, especially at the species level. This could bring about the detection of less pathogenic or opportunistic pathogens rather than human pathogens, leading to underestimation or overestimation of the potential risk. It is, therefore, necessary to verify results[94]. Moreover, species-level identification is inadequate in assessing the potential risk of foodborne pathogens. Thus, it is necessary to determine virulence and resistance genes[95]. One other problem of taxonomic classification using metagenomics in risk assessment is that it detects hundreds of species of organisms, including those not of health significance, in a sample. Therefore, to detect species pertinent to risk assessment, it is indispensable to target pathogens, thence effortlessly removing trivial data for risk assessment. Doing this will no doubt minimize one of the major challenges of metagenomic studies, the difficulties associated with data analysis[96].

For risk assessment in food samples using metagenomic analysis, Grützke *et al*.[97] proposed a workflow in which the first identification of taxonomic units with kraken2 using the complete RefSeq database. Then from the list of species, human, animal, or plant pathogens are filtered, classified reads are extracted from the metagenomic dataset and verified with BLAST using the nucleotide database from the website of the National Center for Biotechnology Information (NCBI). Subspecies are resolved by determination of the closest available reference using Mash. Virulence factors are detected with SRST2 in combination with the Virulence Factor Database (VFDB). Metagenomics, especially when integrated into predictive models, has made a significant contribution to risk assessment investigations since it can answer questions related to risk assessment, such as what pathogens are found in food and how they interact as well as how environmental factors affect features of the foodborne pathogens such as virulence and resistance[82].

Despite its potential and numerous advantages, metagenomics sequencing has hurdles surrounding its applicability, efficiency, cost, and standardization. Shotgun sequencing, for example, is incapable of discriminating between viable and dead organisms. Interestingly, several wet-lab scientists and bioinformaticians are increasingly providing solutions to these challenges[98]. To assess the potential infection risk posed by *Campylobacter*, it is necessary to employ techniques that ascertain viability since the viability of pathogens is an essential parameter in food and water quality assessment[99]. Conventional culture-based techniques, which rely on the ability of viable microbes to take up nutrients and produce colonies in a culture medium, have been used for many years, but these methods are both arduous and time-consuming[100]. For example, *Campylobacter* spp. take a week or more to produce a positive detection result using the culture method. In addition, the sensitivity of culture methods is low since they are not always capable of detecting microbes in viable but nonculturable states, even though their detection is necessary to prevent disease outbreaks[101].

Various novel viability assays such as dye-based assays, phage-based assays, testing of cellular metabolism as well as the measurement of heat flow and ATP production have emerged in the last twenty years[102]. Viability PCR or vPCR has been widely employed, reviewed, and optimized as an efficient method for discriminating viable from inactivated cells. The underlying principle of vPCR is that it correlates viability with cell envelope permeability. Here, microorganisms in a sample are incubated with a dye such as a propidium monoazide (PMA). Following photo-activation, dye binds to exposed DNA and interferes with the amplification during PCR. Inactivated or dead cells with damaged membranes have their nucleic acids exposed to the dye. Once the dye-DNA complex is photo-activated, the amplification of non-viable cells is blocked[103]. On the contrary, viable cells having their cell membranes still intact exclude the dye, leading to strong quantitative PCR (qPCR) signals in the presence of the dye[104]. Viability PCR has been employed to study the viability of not just commonly studied bacteria but also fastidious bacteria, spore-forming bacteria, protozoans, fungi, and even viruses. This aggrandizes how efficient the technique is in distinguishing dead microbial cells from viable cells and how useful it can be in microbial risk assessment[105].

INFECTIOUS DISEASE TRANSMISSION AND QUANTITATIVE MICROBIAL RISK ASSESSMENT MODELING

Both infectious disease transmission and quantitative microbial risk assessment modeling have been employed to decipher the source and degree of infectious disease risk, the role of various routes of transmission as well as possible control strategies[106]. Infectious disease transmission modeling has been used for decades by infectious disease epidemiologists to carry out epidemiological studies. One such modeling framework is the susceptible–infectious–recovered, which models person-to-person contact and infection transmission in a given population and has been in use since the 1900s[107].

Infectious disease transmission models use mathematical equations to visualize the spread of pathogens within a population. They can be used to determine the direction and degree of disease outbreaks and generate information on factors that influence disease transmission and the impact of the containment strategy[108]. In infectious disease models, it is usually assumed that individuals infected with an infectious disease are capable of spreading the disease to other individuals in the population[109]. In order to understand this process of transmission, infectious disease transmission models use various variables representing the numbers of individuals of several different attributes associated with infection in a population. Typically, these attributes include susceptible, exposed, infected, and removed. In the infectious disease transmission modeling, whether an individual is regarded as infectious or otherwise must be considered[110]. In a population, those who are infectious are those who are infected and could potentially spread infectious agents to other individuals. In contrast, those who are not infected but can acquire the infection are regarded as susceptible individuals within the population[106].

On the other hand, individuals who associate with the infected individuals who might have been infected but are not yet infectious are regarded as exposed, and lastly, those who have recovered and are no longer infectious and are immune from re-infection are referred to as removed. Being removed may mean such an individual was killed by the infection or developed complete post-recovery immunity. The underlying point is that removed individuals are incapable of further transmitting the infection[111]. Mathematical models that rely on the susceptible, infectious and removed attributes are referred to as the Susceptible-Infectious-Removed (SIR) models. In SIR model, the flow of infection typically starts from susceptible to removal. Individuals usually start as susceptible, become infective at a given time, recover after a certain infectious period, and thence become removed. This way, the possibility of acquiring infection for a susceptible individual usually relies on the status of individuals in the SIR model, which is the leading principle for the classic non-linear dynamics within disease transmission. On the other hand, the timing of removal following infection (the infectious duration) typically does not depend on other individuals and their status[112].

A simple SIR model can be expanded to include additional attributes germane to the transmission dynamics of a particular disease of interest. The attribute 'exposed' is often included, resulting in a corresponding model referred to as Susceptible-Exposed-Infectious-Recovered (SEIR) model. Equally, addition or alteration of attributes transition is possible[113]. For example, individuals may lose their acquired post-recovery immunity over time, resulting in changing their status from the removed to the susceptible, thereby yielding the Susceptible-Infectious-Recovered-Susceptible (SIRS) model[114]. Similarly, the removed state can entirely be left out of the model if the infectious agent under study does not trigger the production of any form of post-recovery immunity, yielding Susceptible-Infectious-Susceptible (SIS) model[115].

One disadvantage of the basic SIR model is that it cannot discriminate whether an infected individual develops symptoms, even though this could be an essential transmission factor[109]. For instance, individuals infected with airborne respiratory pathogens are more likely to spread the infection if they develop frequent coughing and sneezing symptoms. Similarly, it is necessary to account for asymptomatic individuals for diseases in which asymptomatic infection (carriage) is the fundamental transmission driver, such as meningococcal or pneumococcal disease. Irrespective of the model specification, individuals are primarily assigned to a group based on specific health attributes, which could change from time to time. In the last ten years, modelers have faced increasing hurdles, the most important of which is the growing availability of genomic and other 'omics' data generated for diagnostic and surveillance, which has reformed the field of risk assessment[116].

However, recent advances in computer algorithms and machine learning technology offer researchers an efficient alternative that overcomes these challenges[117].

WGS, MACHINE LEARNING AND MICROBIAL RISK ASSESSMENT

Establishing the links between WGS or metagenomics data sets and specific risk indicators is especially important. However, the complex nature of genomic data concerning the number of microbial isolates remains a significant challenge, especially in applying conventional statistical tools[82]. Most microbial risk assessment models cannot discriminate strains in terms of their differences in resistance and virulence. Interestingly, machine learning technology and other novel models currently deployed in microbial risk assessment can analyze large data sets while accurately predicting the risk/in a population[118]. Machine learning algorithms are developed and employed for risk assessment. Over time, these algorithms are improved for better performance. These technologies can identify a combination of factors that allows the prediction of risk outcomes, thereby making risk assessment from big data sets more sensitive and reliable. Additionally, conventional risk assessment models usually use intermediate genetic interactions[119].

On the other hand, machine learning algorithms consider personal effects, which rely on interactions between environmental and genetic factors. Machine learning algorithms allow simultaneous prediction and interpretation using big data sets. Consequently, it is possible to unveil a particular phenotype and predict the presence of the protein from a sequence. With machine learning methods, it is also possible to carry out a microbial risk assessment with the flexibility to certain genetic acquired variations, which could favor the timely identification of strains with novel resistance or virulence determinants[118]. The applications of machine learning technology in genomics and as a placement for the classical genome-wide association studies have proliferated in recent years. Far-reaching disease indicators have been studied through their application to gene expression data, where computer algorithms learn to discriminate between various disease phenotypes. Other successful applications of machine learning algorithms in health and disease include a better understanding of the relationship between patient genotypes, gene-expression-related phenotypes, and patient outcomes in cancer research, as well as the discovery of regions in bacterial genomes code for antibiotic resistance. The application of machine learning algorithms in risk assessment using WGS data has been described. WGS data becomes a powerful tool for microbial source tracking and risk assessment when analyzed using an appropriate source tracking algorithm.

CONCLUSION

In conclusion, the evidence reviewed here provides valuable information on the various medically necessary *Campylobacter* spp, their mechanism of resistance, important reservoirs, and most importantly, how advanced molecular techniques are deployed in microbial risk assessment and source tracking. In particular, the limitations of conventional methods, which include time-consumption, poor sensitivity and specificity on the one hand, and the superiority of WGS and machine learning technology, which include high reliability and robustness, on the other hand, have been explored. The application of machine learning and NGS technologies offer massive potential since they can be deployed in combination to track sources of outbreaks and predict risks. If timely deployed, they could help tackle outbreaks from their sources, thereby minimizing casualties and other impacts. Noteworthy, these technologies, despite their numerous advantages, their deployment in resource-limited settings is constrained by factors such as lack of expertise and the cost.

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AUTHORS' CONTRIBUTION

Bashar Haruna Gulumbe: conceptualization, drafting of the manuscript, revision of the manuscript, and approval of the final draft. **Abbas Yusuf Bazata**: conceptualization, drafting of the manuscript, revision of the manuscript, and approval of the final draft. **Musbahu Abdullahi Bagwai**: drafting of the manuscript, revision of the manuscript, and approval of the final draft.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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Research Article

Formulation of Anti Acne Loose Powder of Bawang Dayak (*Eleutherine bulbosa* (Mill.) Urb.) Ethanol Extract

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Abstract

Bawang dayak (*Eleutherine bulbosa* (Mill.) Urb is one of the notable Iridaceae family, originating from Central Kalimantan, Indonesia. Previous studies have reported that *E. bulbosa* ethanol extract and its cream preparation have antibacterial properties that can inhibit the growth of acne-causing bacteria and cause no significant skin adverse reaction. This study aimed to make a loose powder preparation from *E. bulbosa* ethanol extract and determine its physical evaluation and antibacterial activity. Loose powder formulation was made with various concentrations of *E. bulbosa* ethanol extract, F0 (0%), F1 (5%), F2 (10%), and F3 (15%). Loose powder evaluates for organoleptic, homogeneity, and antibacterial activity by the disc diffusion method. The results show that *E. bulbosa* ethanol extract can produce a loose powder formulation. The color of the formula is rather yellow (F0), brown-ash (F1), and light brown (F2 and F3), which has a typical mint odor, smooth texture, and homogeneous. All formulations inhibited the growth of *Propionibacterium acnes, Staphylococcus epidermidis,* and *Staphylococcus aureus*. This present study showed the potential of Formula 3 (F3) as an anti-acne loose powder due to its organoleptic properties, homogeneity, and antibacterial activity, which has the largest inhibition zone diameter of 17.6 ± 3.1 mm.

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INTRODUCTION

Acne (acne vulgaris) is a skin condition of the sebaceous glands that is characterized by the development of sebaceous papules, cystic acne, inflammatory lesions, and involvement of the follicular canal and sebum production by *Propionibacterium acnes*, *Staphylococcus epidermidis*, and *Staphylococcus aureus*[1]. *Propionibacterium acnes* was involved in developing inflammatory acne by activating complements and metabolizing sebaceous triglycerides into fatty acids that irritate the follicular wall and surrounding dermis[2]. *Staphylococcus epidermidis* usually involves in superficial infections within the sebaceous unit[3]. Meanwhile, *S. aureus* growth could cause acne lesions[4]. *Propionibacterium acnes*, *S. epidermidis*, and *S. aureus* can be the target sites of anti-acne drugs[5].

The use of antibiotics to treat acne is usually done to reduce the bacterial population. However, overuse of antibiotics can lead to antibiotic resistance. Therefore, it is necessary to explore local medicinal plants to develop anti-acne drugs[6]. Bawang Dayak or *Eleutherine bulbosa* (Mill.) Urb.) is one of the notable Iridaceae family, originating from Central Kalimantan, Indonesia. This plant is also widely cultivated in Southeast Asia. The bulb part has been used traditionally among the Dayak community as folk medicine to treat several diseases[7,8]. *Eleutherine bulbosa* was known to have antibacterial properties against acne-causing bacteria, as reported in our previous studies[9-12]. Our other previous studies[13,14] also reported that cream of *E. bulbosa* ethanol extract could inhibit the growth of *P. acnes*, and it does not cause significant skin adverse reactions[15,16].

However, it is necessary to make a series of anti-acne preparations to increase the effectiveness of using *E. bulbosa* as an anti-acne. Topical products can be directly applied to the affected area, thus decreasing systemic absorption and increasing the exposure of the pilosebaceous units to the acne treatment[17]. One of the preparations for topical application is a loose powder. Loose powder is the original type of face powder that can easily absorb on the skin and free the face from oil[18]. Therefore, this study aims to make innovative loose powder preparations from *E. bulbosa* ethanol extract and to determine its physical evaluation and antibacterial activity. Formulating loose powder of *E. bulbosa* extract is needed as an alternative treatment for acne. So, in the end, it can be produced anti-acne product series from *E. bulbosa* ethanol extract.

MATERIALS AND METHODS

Materials

The materials used were *E. bulbosa* bulbs, peppermint oil, ZnO, menthol, corn starch, sterile talcum, blank antimicrobial susceptibility disc, strains of *P. acnes* ATCC 11827, *S. epidermidis* ATCC 12228, *S. aureus* ATCC 25923, Mueller-Hinton agar, 96% ethanol, NaCl, distilled water, branded loose powder (Wardah acnederm face powder). The main instruments used include an analytical scale, oven, blender, autoclave, incubator, rotary evaporator, hot plate, laminar airflow, and caliper.

Methods

Collection of plant

Fresh bulbs of *E. bulbosa* were collected from Sei Gohong Village, Bukit Batu Sub-District, Palangka Raya, Central Kalimantan, Indonesia. The plant was authenticated by Dr. Joeni Setijo Rahajoe from the Indonesian Institute of Sciences, Research Center for Biology, with specimen voucher 2119.

Preparation of plant extract

The plant materials were prepared by cutting the bulbs and drying them in the sun no later than 10 AM. The dried plant material is ground with a blender. The powdered plant materials were extracted by percolator using 96% ethanol. Then, a rotary evaporator was used to concentrate all extracts[14].

Formulation preparation

The formulation components used are listed in **Table I**. The components include ZnO, menthol, corn starch, sterile talcum, and peppermint oil. The loose powder formulation of *E. bulbosa* ethanol extract was made with three concentrations, 5%, 10%, and 15%. *Eleutherine bulbosa* ethanol extract was weighed and dissolved in ethanol, then some corn starch and sterile talcum were added and grounded until homogeneous. Meanwhile, menthol was dissolved with a bit of ethanol, then some corn starch, sterile talcum, and ZnO were added and grounded until homogeneous. The mixture of *E. bulbosa* ethanol extract was put into a mixture of menthol and ZnO, added peppermint oil, and grounded until homogeneous. The negative control formulation (F0) was prepared in the same procedure without adding *E. bulbosa* ethanol extract. The homogeneous formulation of loose powder was sieved through a 100-mesh sifter and packed[19].

Table I. Formulation of loose powder of *E. bulbosa* ethanol extract

Material	Amount (mg)			
	Negative control or Formula 0 (F0)	Formula 1 (F1)	Formula 2 (F2)	Formula 3 (F3)
Eleutherine bulbosa ethanol extract	0	500	1000	1500
Peppermint oil	10 drops	10 drops	10 drops	10 drops
ZnO	300	300	300	300
Menthol	100	100	100	100
Corn starch	4000	4000	4000	4000
Sterile talcum ad	10000	10000	10000	10000

Physical evaluation of loose powder

There were two evaluations of physical properties: organoleptic and homogeneity tests[20]:

1. Organoleptic test: Loose powder preparations that have been made were observed in color, odor, and texture.
2. Homogeneity test: The homogeneity test was done by visually observing the mixed color uniformity of the extract and powder base. It was carried out by spreading the powder sample on a white paper.

Antibacterial activity test

A loose powder formulation was tested to determine an antibacterial activity against *P. acnes*, *S. epidermidis*, and *S. aureus* using a disc-diffusion technique with three variations of concentration of 5%, 10%, and 15%. The 0.5 McFarland standard was prepared, and 10 mL was put into sterile tubes. The bacterial suspension was made by diluting the bacterial colonies in sterile physiological saline and adjusting the turbidity to 1-2x10^8 CFU/mL. A sterile cotton swab was dipped in a standardized bacterial suspension and used for uniform inoculation onto Mueller-Hinton agar plates. Then, all the discs were immersed in the solution of loose powder sample placed on the plates. A branded loose powder was used as a control. Discs immersed in a solution of branded loose powder were also placed on the plates. These plates were then incubated for 24 hours at 37°C[19]. The diameter of the inhibition zone was measured in mm using a caliper. The study was repeated three times for each loose powder formulation and control[21].

RESULTS AND DISCUSSION

Physical evaluation of loose powder

Organoleptic test

An organoleptic test was carried out to see the physical appearance of the powder preparations by observing the color, odor, and texture. The result of the organoleptic test showed that F0 had a rather yellow color, F1 had a brown ash color, while F2 and F3 had a light brown color (**Table II**). The color difference is due to differences in *E. bulbosa* ethanol extract concentration in the formulations. All formulations had a typical mint odor and smooth texture based on the odor and texture. Typical mint odor due to the addition of menthol and peppermint oil to the formulation to cover up the pungent odor of *E. bulbosa*. The loose powder formulations of *E. bulbosa* ethanol extract can be seen in **Figure 1**.

Table II. Observations of organoleptic loose powder formulations

Formulation (% concentration of extract)	Texture	Color	Odor
F0 (0 %)	Smooth	Rather yellow	Typical mint
F1 (5 %)	Smooth	Brown ash	Typical mint
F2 (10%)	Smooth	Light brown	Typical mint
F3 (15%)	Smooth	Light brown	Typical mint

Homogeneity test

This study showed that all formulation was homogeneous. The homogeneity test of the loose powder aims to see whether all the content is combined perfectly. Homogeneity is one of the requirements for the preparations of loose powder[22]. The loose powder is said to be homogeneous if all the ingredients that make up the powder are well mixed and there are no palpable ingredients.

Antibacterial activity test

The antibacterial activity was tested in triplicate against three acne-causing bacteria: *P. acnes*, *S. epidermidis*, and *S. aureus*. Based on the zone of inhibition, it could be classified into four categories: weak (<5 mm), moderate (5-10 mm), strong (10-20 mm), and very strong (>20 mm)[21,23]. Meanwhile, based on the antibacterial activities of extracts can be classified into three levels: weak activity (inhibition zone lower than 12 mm), moderate activity (inhibition zone between 12 and 20 mm), and strong activity (inhibition zone higher than 20 mm)[24].

Figure 1. The loose powder formulations: F0 (**a**), F1 (**b**), F2 (**c**) and F3 (**d**)

The results showed that two loose powder formulations of *E. bulbosa* ethanol extract (F1 and F2) had a weak inhibitory response against *S. aureus*, while F3 showed moderate inhibitory power. F1 and F2 had a moderate inhibitory power against *S. epidermidis*. However, F3 had a strong inhibitory response against *S. epidermidis* with an inhibition zone of 10.8 ± 0.8 mm. Meanwhile, based on the classification of antibacterial activities of extract[24], the three formulations (F1, F2, F3) had a weak activity against *S. epidermidis* and *S. aureus*, with the inhibition zones in the range of 2.9 ± 1.4 to 10.8 ± 0.8 mm. Furthermore, the antibacterial activity of the three formulations can be described as strong against *P. acnes*. The highest zone of inhibition produced by F3 was 17.6 ± 3.1 mm (**Table III** and **Figure 2**). This can occur due to differences in *E. bulbosa* ethanol extract concentration in each formulation. The higher the *E. bulbosa* ethanol extract concentration in the formulation, the higher the inhibition zone produced[21].

The ability to produce the clear zone was presumably dependent on the secondary metabolites possessed by the test sample[25]. This finding was due to flavonoids, alkaloids, saponins, and tannins in *E. bulbosa* ethanol extract[11], which could be responsible for the antibacterial properties observed. Eleutherol A, a flavonoid from *E. bulbosa*, inhibits cell wall synthesis in bacteria[26]. Alkaloids have an antibacterial ability and generally work through efflux pump inhibition activity. Most of the alkaloids are found to be bactericidal rather than bacteriostatic[27,28]. Saponins can cause bacterial cell contents' leakage through cell wall degradation followed by disruption of the cytoplasmic membrane and membrane proteins[29]. Tannins were known to have antibacterial properties against Gram-negative and Gram-positive human pathogens[30,31].

A previous study[32] reported that an anti-acne loose powder of ethanol extract of *Piper betle* leaves had antibacterial activity against one acne-causing bacteria, *S. aureus*. The inhibition zones of loose powder formulation of F1 (0%), F2 (5%), F3 (10%) and F4 (15%) were 1.05 mm, 5 mm, 6.11 mm, and 6.31 mm. The inhibition zones produced in this study were greater on a concentration of 15% of *E. bulbosa* ethanol extract in loose powder formulation (F3) against *S. aureus*, which is 7.9 ± 1.5 mm.

Table III. The inhibition zone of loose powder formulation of *E. bulbosa* ethanol extract and control

Formulation (% concentration of extract)	Zone of inhibition (mm) (mean ± SD; n=3)		
	P. acnes	*S. epidermidis*	*S. aureus*
F0 (0%)	6.0 ± 2.7	7.1 ± 0.4	2.1 ± 0.5
F1 (5%)	12.8 ± 0.1	6.6 ± 1.6	2.9 ± 1.4
F2 (10%)	16.1 ± 1.6	9.1 ± 0.5	4.1 ± 1.2
F3 (15%)	17.6 ± 3.1	10.8 ± 0.8	7.9 ± 1.5
Control	1.1 ± 0.2	1.8 ± 0.5	1.6 ± 0.7

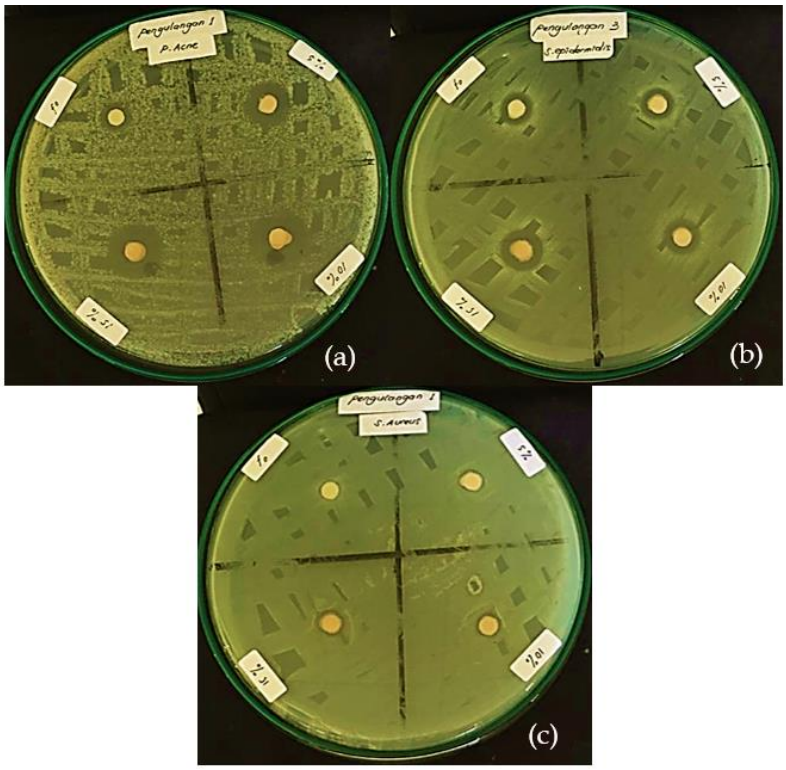

Figure 2. The antibacterial activity of loose powder formulation of *E. bulbosa* ethanol extract against *P. acnes* (**a**), *S. epidermidis* (**b**) and *S. aureus* (**c**)

Negative control (F0) also showed the inhibition zones against three bacterial tested. It can be caused by the presence of zinc oxide. Zinc oxide is known for its antioxidant properties and has been shown to help prevent UV damage. It is used for several dermatological conditions, including infections (warts, leishmaniasis), dermatitis (acne vulgaris, rosacea), pigmentary disorders (melasma), and neoplasias (basal cell carcinoma), and due to its non-toxicity, biocompatibility and antibacterial activity[33].

This study used a branded loose powder (Wardah acnederm face powder) as a control. It contains mica, corn (*Zea mays*) starch, kaolin, silica, zinc stearate, aqua, phenoxyethanol, dimethicone, salicylic acid, ethylhexylglycerin, hydrogen dimethicone, methicone, allantoin, *Epilobium angustifolium* flower/leaf/stem extract, fragrance, aluminum hydroxide, butylene glycol, sodium metabisulfite, *Glycine soja* (soybean) protein, tocopherol. Salicylic acid, *Epilobium angustifolium* flower/leaf/stem extract, and soybean protein are commonly used for acne treatment and have antibacterial activity[34-36].

The antibacterial activity of control was categorized as weak, with the inhibition zones against *P. acnes*, *S. epidermidis*, and *S. aureus* being less than 2 mm. When compared, the inhibition zones resulting from the three formulations of loose powder of *E. bulbosa* ethanol extract were more significant than the inhibition zones of the positive control. Therefore, it can be concluded that the loose powder formulation of *E. bulbosa* ethanol extract has better antibacterial activity against three bacteria that can cause acne.

CONCLUSION

Eleutherine bulbosa ethanol extract can be processed into a loose powder formulation. The color of the formula is rather yellow (F0), brown-ash (F1), and light brown (F2 and F3). Moreover, it has a typical mint odor, smooth texture, and is homogeneous. The highest zone of inhibition produced by F3 (15%) against P. acnes was 17.6 ± 3.1 mm. This present study showed the potential of formulation as anti-acne, but further research is needed to do irritation tests in rabbits and on human skin so it can be developed as an anti-acne loose powder product.

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AUTHORS' CONTRIBUTION

Susi Novaryatiin: conceptualization, funding acquisition, methodology, visualization, writing-original draft, writing-review & editing. **Nursheilla Rizky Amalia**: formal analysis, investigation, project administration, resources. **Syahrida Dian Ardhany**: conceptualization, funding acquisition, methodology, supervision, validation.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Review Article

The Current Perspectives in Clinical Research: Computer-Assisted Drug Designing, Ethics, and Good Clinical Practice

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Molecular superimposition
Vaccines

Abstract

In the era of emerging microbial and non-communicable diseases and re-emerging microbial infections, the medical fraternity and the public are plagued by under-preparedness. It is evident by the severity of the Coronavirus disease (COVID-19) pandemic that novel microbial diseases are a challenge and are challenging to control. This is mainly attributed to the lack of complete knowledge of the novel microbe's biology and pathogenesis and the unavailability of therapeutic drugs and vaccines to treat and control the disease. Clinical research is the only answer utilizing which can handle most of these circumstances. In this review, we highlight the importance of computer-assisted drug designing (CADD) and the aspects of molecular docking, molecular superimposition, 3D-pharmacophore technology, ethics, and good clinical practice (GCP) for the development of therapeutic drugs, devices, and vaccines.

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INTRODUCTION

The world is still fighting the Coronavirus Disease (COVID-19) pandemic, which practically challenged humankind on every front. It has become necessary for pharmaceutical companies to constantly develop new drugs targeting prevalent diseases and emerging and re-emerging ones[1-3]. Drug designing can be broadly classified into two categories: structure-based and ligand-based drug designing. The structure-based method considers the structures of both target and the ligand. At the same time, the ligand-based approach utilizes only the structure and target of the ligands[4,5]. Once the designing method is finalized, the new drug undergoes four phases before entering the consumer usage market. The drug development involves four phases, including phase 1, 2, 3, and 4[6].

Phase 1, also termed the drug development phase, evaluates humans' drug dosage and toxicity. A minimal amount of the drug is given to healthy and physiologically sound male volunteers. In this phase, the dosage with the first sign of toxicity is noted[7]. Phase 2 is considered a pre-clinical phase where the trial drug is assessed for its efficacy against a specific disease. In this phase, a small amount of the new drug is given to the patient volunteers, who are followed up on a timely basis. This phase decides the optimum dosage for patient use[8]. Phase 3 is called the clinical development phase, and wherein many patients are recruited to evaluate and confirm the results obtained in the previous two phases. The drug is compared with

the current treatments or uses a placebo, and its efficiency is identified. The complete data on the efficacy and safety of the drug is collected and placed before the international and national regulatory agencies like the Food and Drugs Administration (FDA), the United States of America (USA), and the Central Drugs Standard Control Organization (CDSCO), India for final approval of marketing[9]. Phase 4 involves post-marketing studies, which are also called pharmacovigilance. During this phase, the long-term safety and efficacy of the drug are assessed in a larger population group[10].

Various techniques to discover drugs have evolved from finding a natural substance to treat diseases and using computer-assisted drug designing (CADD) for manufacturing the drugs (**Figure 1**). The latest addition to this array of technology is molecular docking and artificial intelligence[11,12]. The molecular docking process consists of two main stages: ligand conformation and positioning of the ligand within the target sites[13]. In the current review, we comprehensively discuss the nuances of clinical research, which include CADD, discovery, molecular docking, molecular superimposition, 3D pharmacophore technology, ethics, and good clinical practice (GCP).

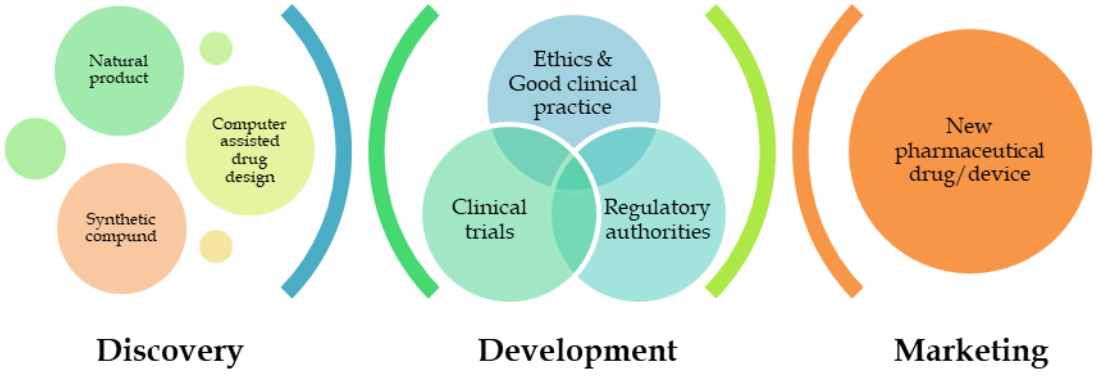

Figure 1. The process of new pharmaceutical drug/device discovery, development, and marketing

THE NUANCES OF DRUG DESIGN AND DISCOVERY

Drug design and discovery involve a complex process. Given the improved scientific and technological advances, the drug discovery process has shifted from the traditional processes to the more synthetic approaches. Drug design has transformed from when the drugs were discovered from the purification and alteration of a known natural substance to the novel technique of producing the drugs from chemicals. Improved knowledge of the disease, from physiological to molecular and atomic levels, and the availability of advanced technologies have significantly influenced the drug design and the discovery process[14]. The drug design and discovery process can be depicted in stages that include identifying the problem/disease, finalizing the compound, and conducting the phase-wise trials (phase 0, phase 1, phase 2, phase 3, and phase 4). After clearly understanding the process involved in drug design and discovery, we move towards developing and manufacturing the drug. An increased understanding of the disease/problem and the genetic basis of the disease enables the identification of the target protein that cures the disease[15] (**Figure 2**).

Since several diseases like Alzheimer's, Parkinson's, and malignancies have different contributing factors, identification of those factors and finding/discovering the modulating compounds using molecular and computer-assisted approaches are considered multidimensional approaches to drug discovery[16]. Although technological advancement proves to be a boon to drug design and discovery, there will still be issues identifying the appropriate drug target for a particular disease and the rational approaches to its discovery[17,18]. The essential components of drug design and discovery include the identification of a problem/disease/target. The case here could be when a satisfactory treatment is unavailable, or there is not yet any therapeutic drug available to treat. Once a target is identified, a search for any natural substance with known therapeutic value is searched and further analyzed for the hit compound, which is further purified and evaluated through clinical trials[19].

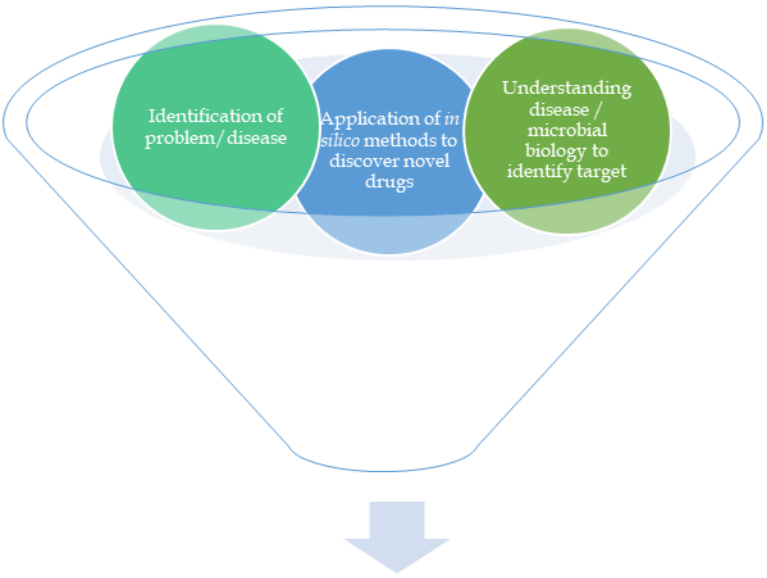

Figure 2. The process of drug design and discovery

The hit molecule is purified using medicinal chemistry studies. The pre-clinical studies are performed to assess the biological activities and toxicological characteristics of the cell cultures and experimental animals before being evaluated in humans in different phases of clinical research. The compound can be synthesized synthetically from chemicals or by modifying a known compound[20]. The CADD, also called the '*in silico* method,' has been instrumental in studying and analyzing the compound in recent times. Even with the increased technological advances, the process of drug design and discovery is a lengthy (time-consuming), costly, complex, and highly unpredictable process[21]. Several avenues require therapeutic interventions to prevent and cure various diseases that include and are not limited to tumors, microbial infectious diseases like malaria, infectious diseases by antibiotic-resistant microorganisms, and other non-infectious conditions, as noted from previous reports[22-24].

Recently, some resolute researchers have discovered a novel Computational Analysis of Novel Drug Opportunities (CANDO), used to fight Ebola using the repurposed therapeutic CANDO Platform[25]. The automatic computer software is currently available to virtually screen and identifies the naturally available products with medicinal value as potential drug candidates[14]. The available technologies have been instrumental in analyzing naturally available bioactive compounds for their anticancer properties, as noted in a recent research report. In this study, the plants of Origanum species were found to produce bioactive oils (carvacrol) that have potential anti-tumor properties, as observed from the experimental animal studies[26]. The availability and accessibility of advanced computational methods in drug design and discovery have increased the productivity and success of developing newer drugs. It has helped screen several naturally available and known natural compounds for their therapeutic value[27]. Recently a public-private partnership (PPP) has been initiated to collaborate and produce newer anti-tubercular drugs in a project called more medicines for tuberculosis (MM4TB)[28].

MOLECULAR DOCKING: A CLUE TO DRUG DESIGN AND DISCOVERY

After identifying the target and finding the desired compound/hit, the most critical drug design and discovery process is to validate the compounds' complementarity with the molecular docking technique. Molecular docking studies enable researchers to find the best confirmation between the protein target and the ligand[13]. Molecular docking identifies the configuration where the protein-ligand complex shows maximum interaction with the least energy. It also finds different protein targets and inhibitors of the target proteins and designs appropriate molecules or ligands to bind to them. This

process is influenced by several factors, including intramolecular (bond length, bond angle) and intermolecular forces (electrostatic, van der Waals forces, and others). The docking type includes protein-protein, protein-ligand, lock-key, and fitting and flexible docking[29,30]. Molecular docking is a computational methodology where the target protein and ligand interactions are carefully studied regarding their best sites of attachments/interactions. The molecular docking studies use computer programs to analyze various ligand-protein binding confirmations and rank these confirmations, which forms an essential aspect of pharmaceutical research[31]. The discovery of whole human genome sequencing has improved the understanding of various disease processes and has been instrumental in identifying better drug targets and binding sites. Molecular docking also helps study the small molecule binding affinities to the target protein and the biochemical processes involved in the ligand-protein bindings[32].

Of all the newer *in silico* techniques available for drug discovery, molecular docking is considered a key concept for successful drug discovery using structure-based drug design (SBDD)[33]. Identification of newer molecular entities/blockbuster drugs is a tedious and costly affair that the newer molecular docking technology can overcome[34]. Using molecular docking, the novel binding site for the drug (HIV-1 integrase) for combating human immunodeficiency virus (HIV) infection was discovered[35]. In recent times, the molecular docking mechanism has been used to study the molecular and quantum mechanics of the proteins, using these studies to discover newer antimicrobial therapeutic agents and assess the role of larger protein-protein complex interactions in developing drugs[36]. In the SBDD, the ligand/protein binding capacity with the receptor is analyzed for the strengths of the bond, stability, and affinities using various scoring parameters[37]. There are now ligand libraries available, and it is effortless to virtually screen the ligand compatibility with a protein or a receptor target[38]. The molecular docking technique enables high throughput screening of multiple ligands and their complementarity with the potential receptors (**Figure 3**)[39].

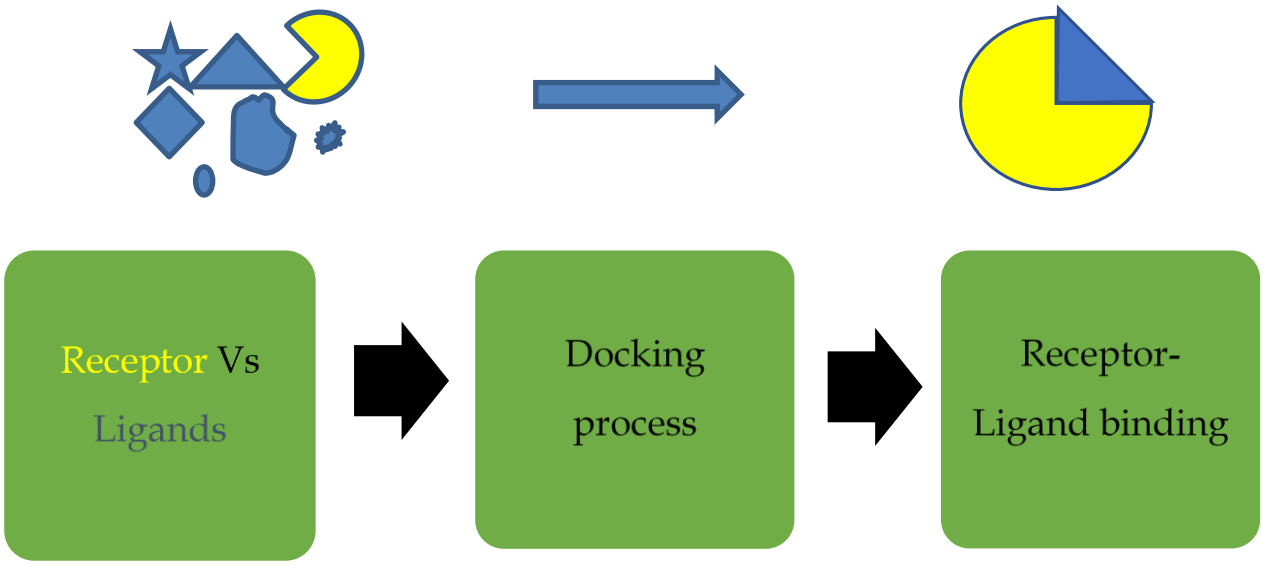

Figure 3. Molecular docking process to assess multiple ligands and their complementarity with the receptors

SYNTHESIS OF PHARMACOPHORE ELEMENTS USING CADD

The most significant part of a drug design and discovery is the synthesis of three-dimensional ligands, also called 3D-pharmacophore elements. The CADD enables the development of pharmacophore elements and to study of the spatial arrangements and the electrochemical properties of the ligands[40]. The 3D-pharmacophore ligands help understand the ligand's binding abilities to the protein/enzyme. The pharmacophores are the carrier molecules/ligands which help bind the drug/protein. Pharmacophores are the molecules that bind to the target proteins and bring about the needed biological

response (treatment). The activities of the pharmacophore elements depend on the hydrogen bond donor/acceptor, positively/negatively charged, aromatic/aliphatic rings or moieties, and hydrophobicity[41,42].

X-ray crystallographic studies are used to study the molecular structure/confirmations of the ligand (spatial arrangements and electrochemical properties) and the receptor. High-affinity ligands are more suitable for attachment to the receptors and show no steric repulsions with receptors. The pharmacophore technology assists in studying the ligand's binding sites (high affinity/low affinity), modifying the binding site/molecular structures to improve the binding capabilities of the ligand with the receptor/protein (**Figure 4**)[43]. Pharmacophore-based ligand synthesis methods will help identify the suitable biological target, as noted from a recent study that found hepatocyte growth factor receptor (c-Met) as a suitable target for new compounds[44]. The quantitative structure-activity relationship (QSAR) and three-dimensional ligand-based pharmacophore models are frequently used to identify the target binding sites on the ligand, as noted from the research studies on Alzheimer's disease[45].

In CADD, synthesizing pharmacophore elements is crucial for designing and discovering a new drug. Recent research elaborated on using a pharmacophore model to synthesize new quinolone derivatives for their antioxidant activities[46]. Pharmacophore modeling was used to synthesize a ligand-based pharmacophore model to synthesize the serotonin receptor antagonist, which has a therapeutic application in managing various clinical conditions, including anxiety and others[47]. Because CXCR2 is an essential receptor in the development and metastasis of cancerous conditions, the ligand-based pharmacophore model was prepared using the computational method (virtual screening) to synthesize the CXCR2 antagonists[48].

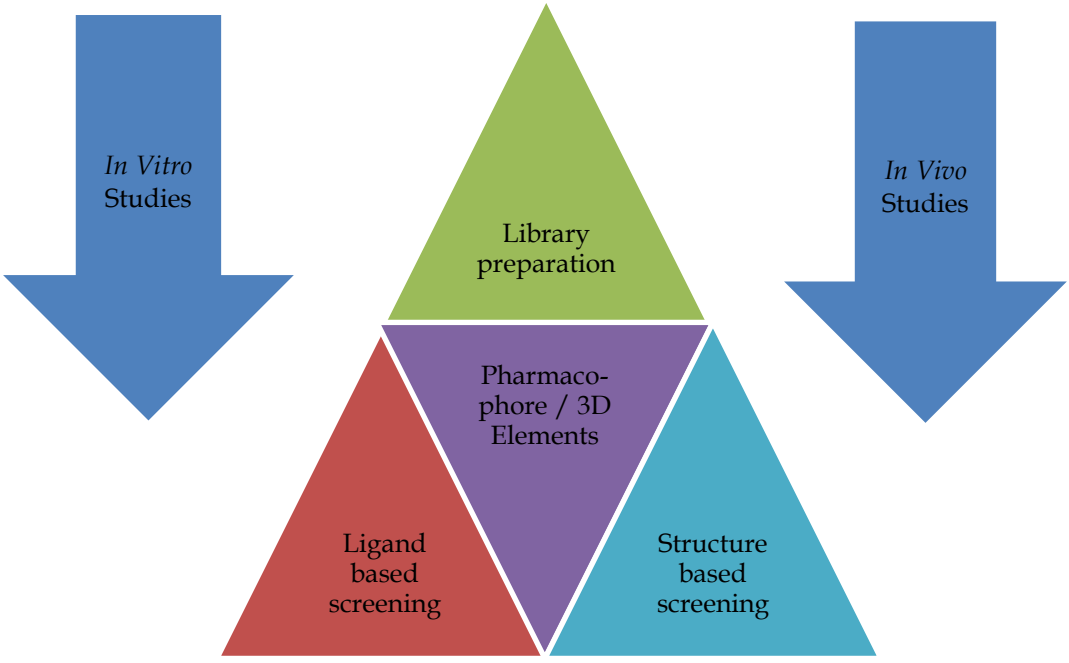

Figure 4. Pharmacophore based ligand screening

THERMODYNAMICS OF DRUG DESIGN AND DISCOVERY

The success of the drug design and the discovery depends on the thermodynamics of the ligand-receptor complexes. This concept discusses the conformational modes of the ligand and its multiple binding sites to the protein/receptor. It also elaborates on the two important mechanisms of assessing the binding affinities of the ligands to the protein/receptor molecules: free energy perturbation (FEP) and thermodynamic integration (TI)[49]. The nature of the receptors includes those without the endogenous ligand (enzymes, ion channels, proteins, and nucleic acids) and those with the endogenous

regulatory ligands (hormones, auto acids, neurotransmitters, growth factors, and cytokines). By using the CADD, the conformational properties of the ligand-receptor/protein complex may be studied/understood using the quantum chemical methods that include the Schrödinger equation. In this equation, the molecule is considered a collection of positively charged nuclei and negatively charged electrons moving under the influence of Coulombic potentials[50].

The ligand and the receptor interactions will decide the complex's stability and the drug delivery potential. The protein-ligand of the ligand-receptor interactions depends on the complexes' enthalpy and entropy[51]. The bioactive conformational energies of the ligand-receptor/protein complexes assume greater significance because the higher the affinity, the greater the complexes' stability. The affinities depend on the free energy difference (ΔG) between the bound ligand-protein complex and the unbound protein and the ligand[52]. The water affinities and the hydrophobicity associated with the stable ligand-receptor complexes depend on the protein's polar, non-polar, and topographical complex concavities, as noted in a previous study[53].

Drug design and discovery is a complex process involving several versatile research areas. The ligand-binding ability of the receptor (drug-target complex) is checked using thermodynamic studies, and those ligands which are faulty can be eliminated, and those with improved binding capacity can be selected for further research. The thermodynamic studies include the assessment of the free energies (ΔG) of the ligands, their enthalpies (ΔH), and the entropies (ΔS)[54]. Thermodynamics is the study of the heat change that occurs when two molecules interact. It is used to identify inhibitors and antagonists to minimize antimicrobial drug resistance due to mutations, reduce side-effects caused by non-specific attachments, and water solubility to increase bioactivity, as noted from the available research findings[55]. The lead optimization studies apply thermodynamics considering three essential aspects that include the presence of appropriate enthalpy in the hydrogen bonds, there is favorable entropy in hydrophobic interactions, and conformational changes that are entropically unfavorable[56].

MOLECULAR SUPERIMPOSITION AND MOLECULAR MECHANICS INVOLVED IN DRUG DESIGN

Among the most significant advantages of CADD, the technique of molecular superimposition assumes great significance. Understanding the process of molecular superimposition and molecular mechanisms involved in drug design and discovery is essential. After preparing a 3D-pharmacophore element, the molecular superimposition helps to compare different molecules for their conformational properties and ability to bind or fit into the model. The molecular superimposition may be done using either the atoms/fragments or the molecules. Molecular superimposition can be rigid or flexible[57]. The computer method QUASIMODI is used to perform superimposition and the Patterson-density-based similarity index, and the electron-density derived similarity is applied to optimize the confirmations. The FLEXS, FLASHFLOOD, SUPERFLEX-SIM, and the FLASH methods are applied to perform a flexible alignment. The semiflexible approach can be applied using the computer program, the SUPERPOSE, and the CATALYST[58]. However, molecular superimposition ensures that various atoms and molecules are checked for their confirmations, and binding abilities, the stability of a 3D-pharmacophore element also depends on the molecular mechanics of the molecule that is assessed. The molecules are a combination of atoms, and the stability of the complex depends on the bond lengths, bond angles, torsional angles, and the non-bonded distances between atoms of the molecule[59].

Clathrin is a protein present on the cell membranes of eukaryotes with various functionalities that include the uptake of bacteria, membrane-bound proteins, and others. A recent study reported using a flexible docking mechanism to identify the confirmations on the clathrin for its binding ability to the Bolinaquinone to inhibit its activities[53]. Most synthetic drugs are synthesized by using organic molecules containing carbon atoms. Therefore, medicinal chemists play an active role in drug design and discovery. Molecular mechanics involve synthesis, alteration, and representation of 3D structures of the molecules. Molecular mechanics include applying computational technologies to study the molecular and biological properties of various protein/receptors/targets using theoretical and experimental data. The molecular mechanics involve

X-ray crystallographic studies to understand the 3D conformation of the molecule and the ability of the molecule to bind to the target/receptor. Molecular mechanics are inexpensive and easy to manage and are used to reproduce molecular confirmations matching and adjusting the bond lengths, bond angles, and torsion angles to equilibrium values to the one it has been designed to bind/attach[60]. The QSAR study is a technique that quantifies the anatomical and biological properties of the molecules/ligands/proteins. The physicochemical properties include hydrophobicity, structural, ion-ion interactions, and steric effects. A recent study attempted to combine the molecular docking technique with the QSAR method to find the binding sites on the transforming growth factor-β (TGF-β) necessary to stop invasion and tumor metastasis[61].

3D-PHARMACOPHORE ELEMENTS IN DRUG DESIGN AND DISCOVERY

The 3D-pharmacophore and the typical feature of the pharmacophore include hydrogen-bond donors and acceptors, positively and negatively charged ions, and hydrophobicity. The pharmacophore elements form the basis/core of medicinal chemistry. The pharmacophores are synthesized by using the active molecules in such a way that they retain the biological activity, and a slight change in the configuration of the molecules may influence the biological activities. The pharmacophore technology is to synthesize the ligand and receptor antagonists, as noted in the case of dopamine antagonist receptors and the serotonin (5-hydroxy tryptophan) receptors. The 3D-pharmacophore elements are prepared using the atoms and the molecules bound by various bonds/forces like the hydrogen bonds, electrostatic forces, and the van der Waals forces. Also, the pharmacophore elements may contain the heteroatoms such as oxygen, nitrogen, and polar functional groups such as carboxylic acids, amides, and hydroxy groups[62].

There are two types of pharmacophore elements, structure-based (X-ray) and ligand-based (derived from active compounds) pharmacophore elements[63]. Since not all protein structures have been elucidated, the ligand-based pharmacophore synthesis is most opted by the researchers. The software used in the molecular modeling pharmacophores includes the MOE and Phase[64]. Pharmacophore technology is essential in drug design when the structural data on a target receptor is unavailable. The pharmacophore method is used to perform lead discovery, lead optimization, and to assess the similarity and variations in the structural confirmations of the ligand and the receptor[65]. According to the international union of pure and applied chemistry (IUPAC), the pharmacophore is defined as the interactions of molecular structures to their molecular target by the steric and electric features and defining a specific biological property. The pharmacophore technique uses molecular interaction to define a ligand's binding ability to the receptor, including features such as hydrogen bond donors, hydrogen bond acceptors, positive and negative charged ion groups, and hydrophobic regions[66].

HUMAN PARTICIPANTS IN CLINICAL TRIALS

Clinical research is usually undertaken to solve a current medical/public health problem. The problem in most instances would be the patients suffering from various diseases that include both infectious (microbial infections) and non-infectious conditions. The solution looked for is to find a treatment for a disease that has neither a therapeutic intervention available nor a vaccine present, and when the current treatment is plagued with complications/severe adverse effects. Although the pharmaceutical substances are designed based on CADD and other *in silico* methodologies, they are tested on healthy and diseased people to assess their safety and efficacy before being approved by the regulatory authorities for prescription purposes. The regulatory bodies stress the need for human subjects' protection, informed consent, and support for the families of trial participants during clinical trials[67,68]. It was recommended to provide aids and tools consisting of detailed information about the trial to potential volunteers and facilitate better decision-making[69,70]. The regulatory agencies in France have enforced 'Jardé law,' an improved clinical trial directive that enhances the protection of the rights of trial participants[71]. Clinical trials involve special population groups like the children, pregnant women, and elderly aged, among other vulnerable groups, which may potentially pose ethical and legal obligations (**Figure 5**)[72-76].

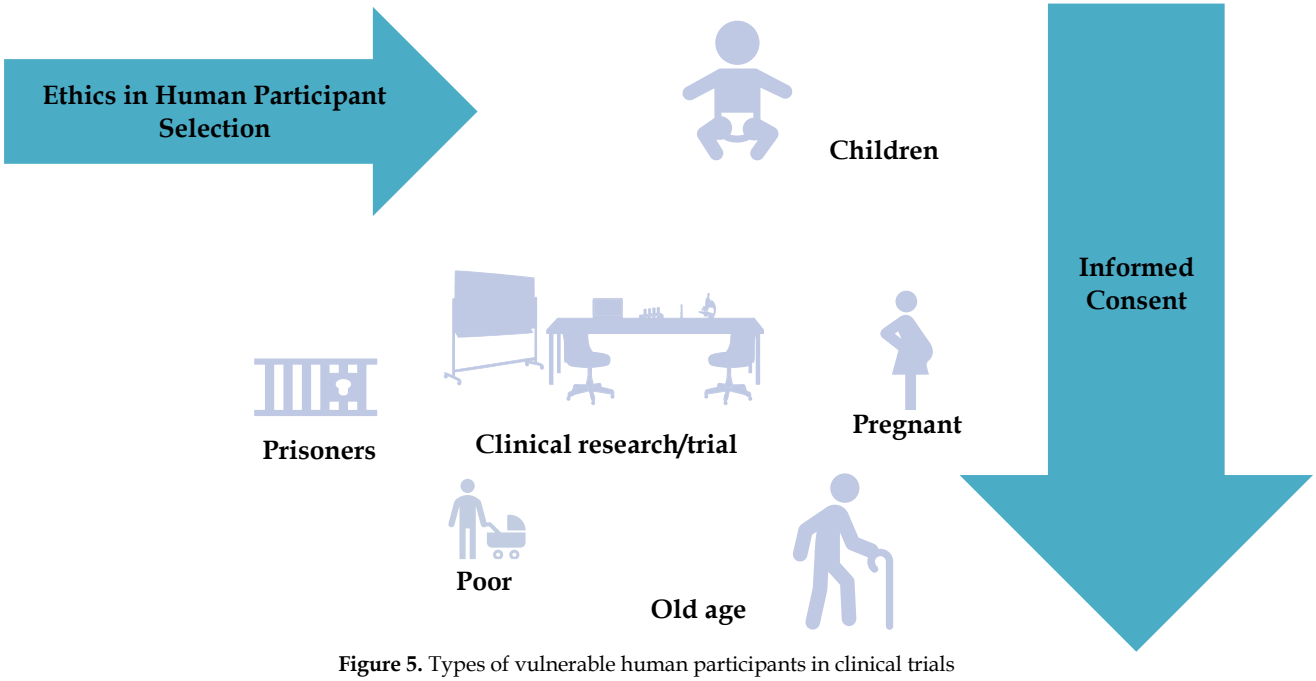

Figure 5. Types of vulnerable human participants in clinical trials

ETHICS IN CLINICAL RESEARCH: PROTECTION OF HUMAN RIGHTS

Although clinical research is conducted for the welfare of the people, the ethical concerns of the subjects participating in the research must be satisfied. This concept delineates the policies that protect the human rights of the subjects taking part in the clinical research. The institutional review boards (IRBs) play vital roles and responsibilities in regulating the conduction of clinical research. The IRBs emphasize the functional regulations and ethical considerations during cooperation and collaborating research that should strictly be followed by the institute that engages (also the institute which do not engage in human subject research) in research involving the human subject. Clinical research involving human subjects is critical in protecting human rights concerning the humans participating in the research. In most instances, it applies to the risk and burdens that a human subject participating in clinical research is exposed to during clinical research. The first such regulation regarding ethics in clinical research involving human subjects was the Nuremberg code, followed by the 1964 Helsinki declaration by the World Health Organization (WHO). It was later followed by the Belmont report by the United States of America[77].

With the international guidelines as a parameter, national guidelines for protecting human rights were implemented by the respective countries, including India's Indian Council for Medical Research's (ICMR) Ethical Guidelines for Biomedical Research on Human Subjects in the later years[78]. The ethical code of conduct during clinical research involving human subjects has gained significance due to the infamous human experiments during World War I and II. Also, the Tuskegee Syphilis human research that led to unethical practices involving a particular group of humans, including the prisoners and mentally ill people, was instrumental in framing ethical code in clinical research involving humans[79]. Clinical research becomes ethical by satisfying seven requirements that include research to enhance further understanding of a disease/condition, scientific methodology used while conducting the research, including appropriate participants after following scientific procedures, and favorable risk-benefit ratios. An independent review board approves the study protocol, informed consent is taken without any influence, and respect for privacy protects the well-being[80].

Since clinical research is conducted for a good social cause and the improvement of human health, such research must satisfy ethical concerns and justify the research concerning its social value requirement (SVR). The SVR is justified in all cases of clinical research, which satisfy eight ethical concerns that include safeguarding the rights of participants who cannot give consent, respect for autonomy, investigator integrity (not exposing subjects to undue risks), deceiving participants

(promising undue advantages/incentives), not exploiting the participants, stewardship of public resources (spending for a social cause), imparting public trust (benefiting public), compensating any deviations for the above rules (make sure the competent adults are recruited for research on the non-social cause, only expose to no more than moderate risks, compensate the undue risk with benefits, preserving privacy, and not use public funds)[81].

ETHICS IN CLINICAL RESEARCH: INFORMED CONSENT

Informed consent is the process of obtaining the approval and voluntary acceptance by the individual participating in the clinical research. Also, the informed consent acts as a bridge between the investigators and the participants as to how the research work is conducted (research flow), the interventions, the risks involved to the benefits, and the necessary precautions in case of any adverse events. It would sum up the process of gaining confidence and satisfaction to the effect that all ethical concerns are/will be appropriately addressed. In clinical research or any other research, informed consent assumes tremendous significance from an ethics perspective if humans are involved. The institutional review boards play a key role in ensuring the contents/elements of the informed consent form and the process of obtaining it from the participants. Clinical researchers must understand the process and elements of informed consent while obtaining the consent from the specially-abled group participating in the research following the council for the international organization of medical sciences regulations (CIOMS). In emergency circumstances, the informed consent and all other issues related to the informed consent become an exception[82].

Informed consent should not only be considered a formality but a legal compulsion/obligation, as observed by a previous report from India[83]. The challenges of the informed consent obtaining process were elaborated in previous research that noted that the informed consent process might be influenced by religious sentiments, patient perceptions, specially-abled groups/vulnerable populations (children, pregnant women), and the general local, social, and cultural characteristics of the population[84]. Informed consent has many elements, including the fact that the participants are fully aware of the research work, the potential risks, and other aspects of human rights. Informed consent in clinical research should address the elements like when informed consent is required and how the consent is obtained from the participants of a clinical research study. The most significant aspect of informed consent is autonomy (deciding to participate in clinical research and discontinuing at any time). Informed consent is practiced by imparting certain functionalities in the conduction of clinical research that includes protecting privacy, and autonomy, respecting participant values, protecting and promoting the welfare of study participants, preserving trust, satisfying all regulatory requirements, and overall research integrity[85].

GOOD CLINICAL PRACTICE: THE PROCESS OF INVESTIGATIONAL NEW DRUG APPLICATION

Clinical research is conducted following the good clinical practice guidelines laid down by the international conference of harmonization (ICH). These are universally followed throughout the world during the conduction of clinical research involving human participants. All the stakeholders in clinical research, including the principal/investigators and the sponsor, have specified roles and responsibilities. Once a new compound is discovered, an investigational new drug (IND) application must be submitted for the conduction of clinical research. The process of an IND application is generally as per the national and international guidelines/ authorities like the food and drugs administration (FDA), US. The FDA plays a significant role in the process of IND application in case of drugs related to life-threatening illnesses and in the management of imports and exports of the drugs concerning IND[86].

Clinical research is, in most instances, undertaken to identify a new drug. Such a process involves the identification of a problem/disease, identifying a potential molecule/drug, and evaluating the drug through different phases of clinical research. During this process, the first step towards clinical research requires the approval of the IND by appropriate regulatory authorities like the FDA. The INDs are the candidates who have been pre-tested, are found to be

pharmacologically active, and do not pose any risk to humans. The IND is evaluated for its potential toxicity by animal testing even before using it on humans. Only after passing phase 0 the IND proceeds further for an application for its approval through different phases of clinical research where it is evaluated on humans[20]. Depending on its uses, the IND are of various types that include the investigator IND (he/herself initiates the drug trial), the emergency use IND (for treating emergencies by the investigator), and the treatment IND (an experimental drug showing promise is tried as a treatment in cases of serious illness). Also, the IND can be of two types, the commercial and the research IND (**Figure 6**)[87].

The most significant part of clinical research is the implementation of good clinical practice (GCP) guidelines. Once the lead compound is identified and optimized, the next step toward drug discovery is the application for a new drug testing (investigational new drug application-INDA). The potential drug is approved for animal testing (pre-clinical phase to assess for safety and toxicity) and later in humans (clinical research phase 1-4)[88]. While the clinical research is being conducted, the GCP guidelines must be followed at various stages. The GCP guidelines state that regulatory authorities must satisfactorily evaluate the clinical trials like the FDA. The FDA must evaluate each phase of a clinical trial. The GCP guidelines ensure that the clinical trials are approved by the regulatory authorities (IRB), ensuring the trial processes, designing the case report form (CRF), analyzing research planning, and assessing the study reports at regular intervals after completion of the study[89].

Figure 6. Types of Investigational new drug (IND)

GOOD CLINICAL PRACTICE: REGULATORY AUTHORITIES AND CLINICAL TRIAL PROTOCOLS

Among many other procedural processes involved in clinical research, clearance from the regulatory authorities like the FDA is a must before starting clinical research. These authorities monitor the activities before, during, and after the conduction of clinical research. Historically, the GCP guidelines are formulated by the meetings after the Nuremberg code, the declaration of Helsinki, the Belmont Report, recommendations by the respective countries (USA-FDA), and the WHO guidelines. In every step of clinical research, the role played by the regulatory authorities assumes great significance. The

application for an IND is a systematic process that includes three sets of forms, the FDA form 1571 (study protocol), the form 1572, which gives the information about the investigator and the site of investigation, and the FDA form 3674, which contains the clinical trial registration at the respective national agencies[90].

The sponsor and investigators are responsible for updating the modifications/amendments in the study protocol both to the institutional review boards and to the FDA. Also, they are entitled to notify any information amendments (increase/decrease in drug exposure), safety reports (reporting adverse events), and annual reports detailing the status of the study. Protection of human rights, the safety of the participating subjects, and the reliability of the data being generated imply the quality of the clinical research. A rigorous review of the study protocol by the respective institutional review boards and stringent informed consent practices will demonstrate the high scientific standards of a clinical trial study. Continuous monitoring of the trial and regular audits will ensure the quality of a trial[91].

Most clinical trials evaluate the efficacy, safety, and adverse events associated with medical products, including drugs. The clinical trial involves a large group of qualified medical professionals, including the principal investigator, co-investigators, clinical research associates, and the sponsors who fund the trial (**Figure 7**). The clinical trial must follow a protocol (background and purpose of study, trial design, infrastructure required, procedural details, and statistical methods to analyze results), standard operating procedures (SOP), study manuals, and other guidelines, including a well-structured plan of action document. All the deviations in the protocol must be so as not to harm the study participants, and any harm must be addressed and informed to the regulatory authorities[92]. To avoid bias in reporting results, rejection of the results by the sponsor or the regulatory authorities must be appropriately addressed. Although the governments are liberal and encouraging concerning permission for clinical research activities (research and drug manufacturing), unless the GCP guidelines are adhered to, no clinical research will result in positive results[93-95].

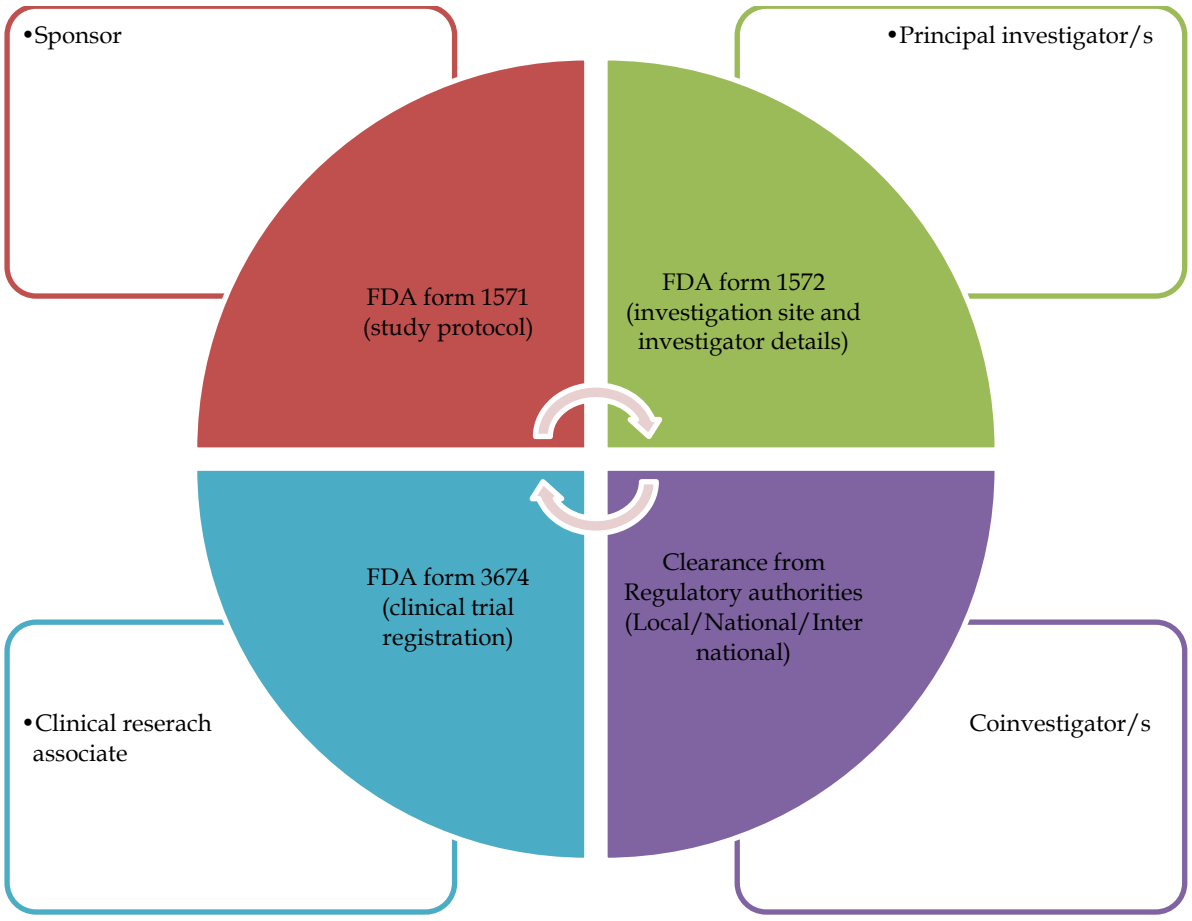

Figure 7. Essential elements of a clinical trial

CONCLUSION

Clinical research is an emerging area with great scope for research. Currently, clinical research aims to find faster solutions to modern, existing, and emerging diseases, including the novel Severe Acute Respiratory Syndrome CoV-2 (SARS-CoV-2) responsible for Coronavirus Disease-19 (COVID-19). The non-availability of a vaccine for HIV, Dengue virus, and no proper treatment for several other microbial infections and tumors, among other life-threatening illnesses, are responsible for the increased focus on clinical research. Several microbial infectious diseases need better antimicrobial therapeutics due to increased antibiotic resistance. Tuberculosis is plagued by multidrug resistance, and therefore, the control of the spread of infection has become a challenge. The *in silico* methodologies discussed in this review may be applied to virtually screen/identify drug candidates and minimize the cost and time taken to develop new drugs. An improved understanding of molecular modeling techniques and *in silico* methods are instrumental in studying the potential drug candidates' pharmacokinetic and pharmacodynamic properties. Adhering to the GCP guidelines on ethics for protecting human/participant's rights and acquiring informed consent from all the participants as prescribed by the regulatory agencies are prerequisites for conducting a successful clinical research/trial.

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AUTHORS' CONTRIBUTION

All authors contribute equally.

DATA AVAILABILITY

Not applicable.

CONFLICT OF INTEREST

There are no conflicts of interest.

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Short Communication

Trends of Influenza's Symptoms Drug Search Terms in Indonesian-Language using Google Trends in the Covid-19 Pandemic

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Abstract

Covid-19 has spread globally and causes severe acute respiratory syndrome. The symptoms of covid-19 have similarities with influenza, such as cough, fever, runny nose, and sore throat. Therefore, the internet sources tend to have an increasing search related to influenza symptoms drugs. This study aims to assess the search trend of influenza symptoms drugs using google trend analysis in Indonesia. We explore Google trend analysis using search terms in the Indonesian language related to influenza symptoms drugs from December 6th, 2020 to November 30th, 2021. The positive confirmed cases were obtained from the Indonesian government website https://covid19.go.id/. Our results demonstrated the increasing search terms related to influenza drug symptoms during July and August. The highest term search was "*obat batuk*". The positive covid-19 confirmed cases in Indonesia increased during July and August. During the peak of the covid-19 outbreak in Indonesia in July-August 2021, there was an increase in Google Trends searching related to influenza's drug symptoms.

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INTRODUCTION

The covid-19 pandemic has affected more than 200 countries worldwide, including Indonesia. The first cases of covid-19 in Indonesia were detected on March 2nd, 2020, in Jakarta, the capital city of Indonesia. Up to April 19th, 2022, according to WHO (https://covid19.who.int/), there were 503,131,834 total cumulative cases of Covid-19 around the world, and there were 6,200,571 cumulative deaths, with 11,324,243,310 vaccines were administered around the world. At the end of March 2020, Indonesia's case fatality rate (CFR) reaches 8.9%. During covid-19, to prevent the spread of covid-19, the Indonesian government implied stay-at-home notification[1].

Covid-19 causes similar symptoms to the influenza virus, such as cough, fever, rainy nose, sore throat, and fatigue. In some cases, covid-19 can complicate differential diagnosis. Since influenza and covid-19 showed similar symptoms of respiratory disease, influenza pandemics seem to be a model of covid-19 pandemics. Moreover, covid-19 symptoms can cause weakness, taste disorder, and myalgia[2,3]. The patients who present those symptoms should be promptly checked for SARS-CoV-2 infection. The diagnostic testing for covid-19 includes molecular testing, serology testing, and other laboratory assessment. The pharmacology treatments of covid 19 include antiviral drugs, anti-inflammatory drugs, low molecular weight heparins, plasma, and hyperimmune immunoglobulins[4].

Anxiety, stress, depressive symptoms, and post-trauma growth (PTG) are other effects of the Covid-19 pandemic. This condition is influenced people's health-seeking behavior. A study in Lahore, Pakistan, found a change in people's health-seeking behavior. During the Covid-19 pandemic, the trend of self-medication was increasing, and the number of people visiting hospitals decreased[5].

In Indonesia, a study in four provinces (East Java, Central Java, Riau, and South-East Celebes) also found that during the Covid-19 pandemic, people tended to do self-medication compared to visit health center services or hospitals[6]. A qualitative study in Makassar, Indonesia, also found the increasing trends of self-medication because people were afraid to visit the hospital. They also thought medicine from drug stores could cure their diseases, such as influenza symptoms[7]. In Indonesia, self-medication was used for influenza symptoms (such as fever, headache, and cough), diarrhea, acute pain, and indigestion[8]. During the Covid-19 pandemic, Indonesia's top three diseases cured by self-medication were fever, flu, and cough. People get information related to the medication from family, friends, or the internet[9].

Nowadays, the internet is a popular source of health information. One internet that can be used for searching health care information is Google Trends. Since 2004, Google Trends has explored web behavior topics or terms. In Google Trends, users can use up to five topics or terms, and the result will be displayed as a set of time series. Google Trends have become a powerful tool to demonstrate epidemiologic surveillance. It is believed to be reliable for surveys related to RSV worldwide[10]. Previous research on covid-19 has been published using google trends[11-13]. However, the use of Google Trends to assess the drug-related terms in Indonesia is limited. Therefore, this study aimed to investigate influenza symptoms' trends in drug searching in Indonesia. Moreover, the positive confirmed cases during covid-19 in Indonesia were searched to obtain the peak of the covid-19 outbreak.

MATERIALS AND METHODS

Google Trend Searching

Google Trends were used to search influenza symptoms drug terms in Indonesian between December 6th, 2020, and November 30th, 2021. The exploration data was shown by a graphic that indicated relative search volume (RSV). The terms related influenza's drugs in Indonesian language were "*obat flu*", "*obat batuk*", "*obat pilek*", "*obat demam*", and "*obat sakit tenggorokan*". "*Obat flu*" means "influenza medications," while "*obat batuk*," "*obat pilek*," "*obat demam*," and "*obat sakit tenggorokan*" means "cough medications," "runny nose medications," "fever medications," and "sore throat medications," respectively. The terms were searched in comparison of those five terms/ keywords and individual search of those five terms/ keywords.

Positive Covid-19 Confirmed Cases in Indonesia

To obtain the positive Covid-19 confirmed cases in Indonesia, the Indonesian government website (https://covid19.go.id/) and WHO website (https://covid19.who.int/) were accessed. Both websites provided information related to the Covid-19 cases in Indonesia, including positive Covid-19, confirmed cases, recovered Covid-19 cases, and mortality cases of Covid-19 in Indonesia.

RESULTS AND DISCUSSION

Figure 1 shows Indonesia's positive confirmed cases of covid-19 from December 2020 to November 2021. This figure indicated increasing covid-19 positive cases in Indonesia during July and August. Previous research showed that the case fatality rate (CFR) of covid-19 reached 8.9% at the end of March 2020. To minimize and prevent the covid-19 spread in Indonesia, the central government applied the regulation of a social distancing project called *Pembatasan Sosial Berskala Besar* (PSBB) by April 2020. The implementation of PSBB depends on the local governments because the central government gave the authority of implementation to the local governments. However, before the local governments implemented the PSBB, they had to wait for approval from the Indonesian Ministry of Health. Thus, the implementation of PSBB, such as time and policy, is diverse in each area of Indonesia[14,15].

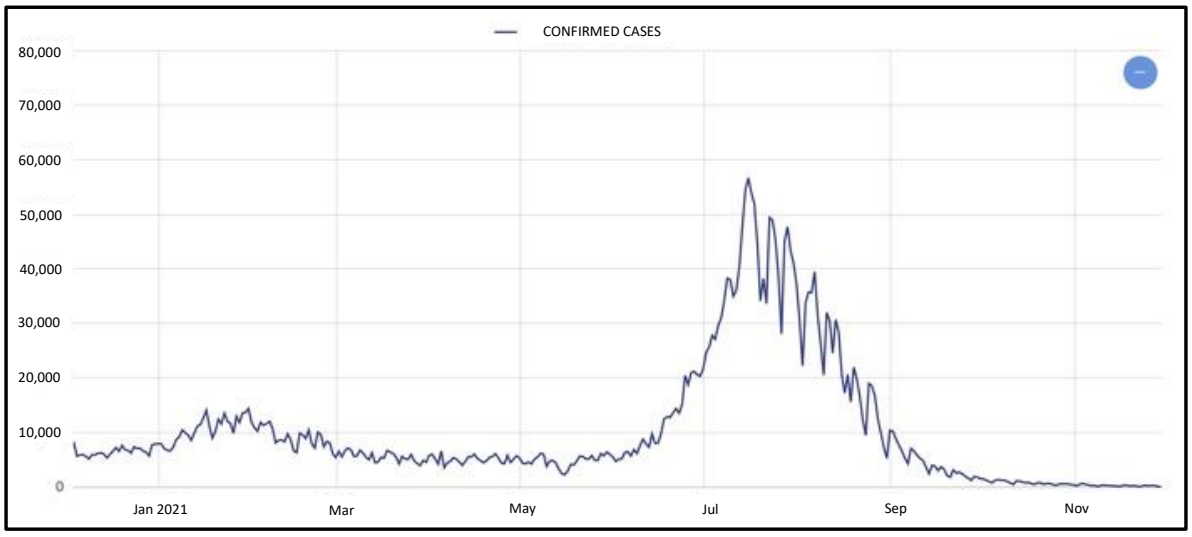

Figure 1. Positive confirmed cases of Covid-19 in Indonesia from December 2020 to November 2021

Using the search terms related to drug-related influenza symptoms, this study reported searching trends during the Covid-19 outbreak in Indonesia, from December 6th, 2020, to November 30th, 2021. The RSV of search terms "*obat flu*", "*obat batuk*", "*obat pilek*", "*obat demam*", dan "*obat sakit tenggorokan* in comparison were displayed in **Figure 2**. This figure illustrated the increasing trends in all five search terms during July and August. The main symptoms of Covid-19 are fever, cough, fatigue, slight dyspnea, sore throat, headache, conjunctivitis, and gastrointestinal issues. The highest RSV was found on the term "*obat batuk*". This result aligns with previous studies that cough is one common symptom of Covid-19. Cough is one of the usual symptoms of Covid-19, which can persist for weeks or months after the infection[16]. Another longitudinal study in Geneva found that cough and loss of taste or smell were common symptoms in the early phase[17]. A systematic review also found that cough was a common symptom of Covid-19 in 25 studies. Based on the studies in this systematic review, among 1000 people, around 655 people would get a cough. Of 655 people who got a cough, 142 people would have Covid-19. Of the rest of the 345 who did not get a cough, around 68 people would get Covid-19[18]. However, the result of the current study is different from the result of Lai *et al*. in 2020, which found the most common symptom of Covid-19 was fever, followed by a cough[19].

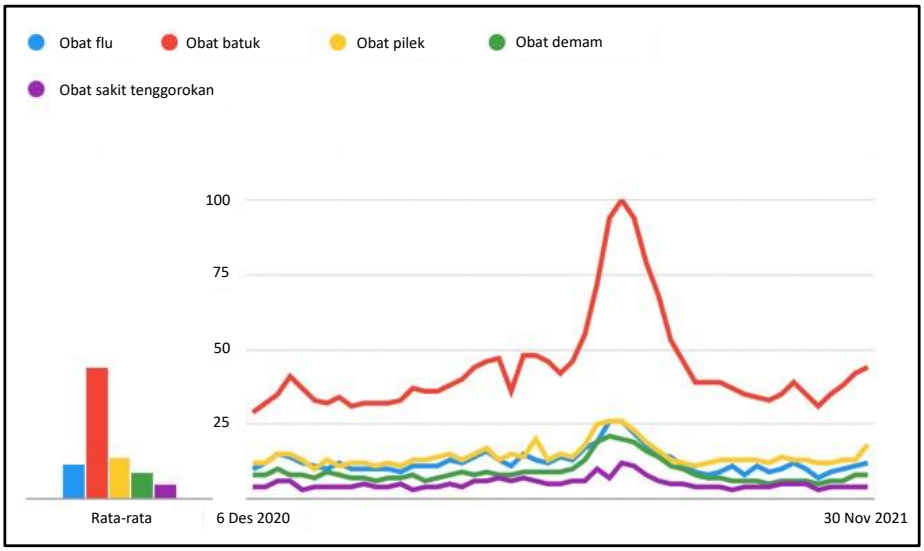

Figure 2. Google Trends Search Terms of "*Obat flu*", "*Obat batuk*", "*Obat pilek*", "*Obat demam*", and "*Obat sakit tenggorokan*" from December 6th, 2020 to November 30th, 2021

Next, to determine each term's individual trend, the keyword using Google Trends was conducted. Figure 3 demonstrates the individual figure of each term. All the individual figures showed an increasing trend between July and August 2021. Terms of "*obat flu*", "*obat batuk*", "*obat pilek*", "*obat demam*", and "*obat sakit tenggorokan*" were found to have the similar trend. The frame time of increasing individual trend search of "*obat flu*", "*obat batuk*", "*obat pilek*", "*obat demam*", and "*obat sakit tenggorokan*" is similar with the increasing of covid-19 positive cases in Indonesia. This condition can be related to anxiety and stress. During the Covid-19 pandemic, Indonesian people's prevalence of mental health problems, such as stress, anxiety, and depression, increased. Information from the mass media could be one of the reasons. A study in China found that the use of media mass was associated with negative affect, anxiety, and stress[20]. In Indonesia, the use of media mass is also positively associated with anxiety. The content of information from media mass is also related to people's mental health. Stressful content was more associated with negative affect, stress, and anxiety. On the other hand, positive content, such as heroic acts and disease prevention, was associated with positive affect and less depression[21]. Between July and August 2021, more negative information, such as positive cases and death news, was in the mass media. This stressful content could lead to anxiety in the community.

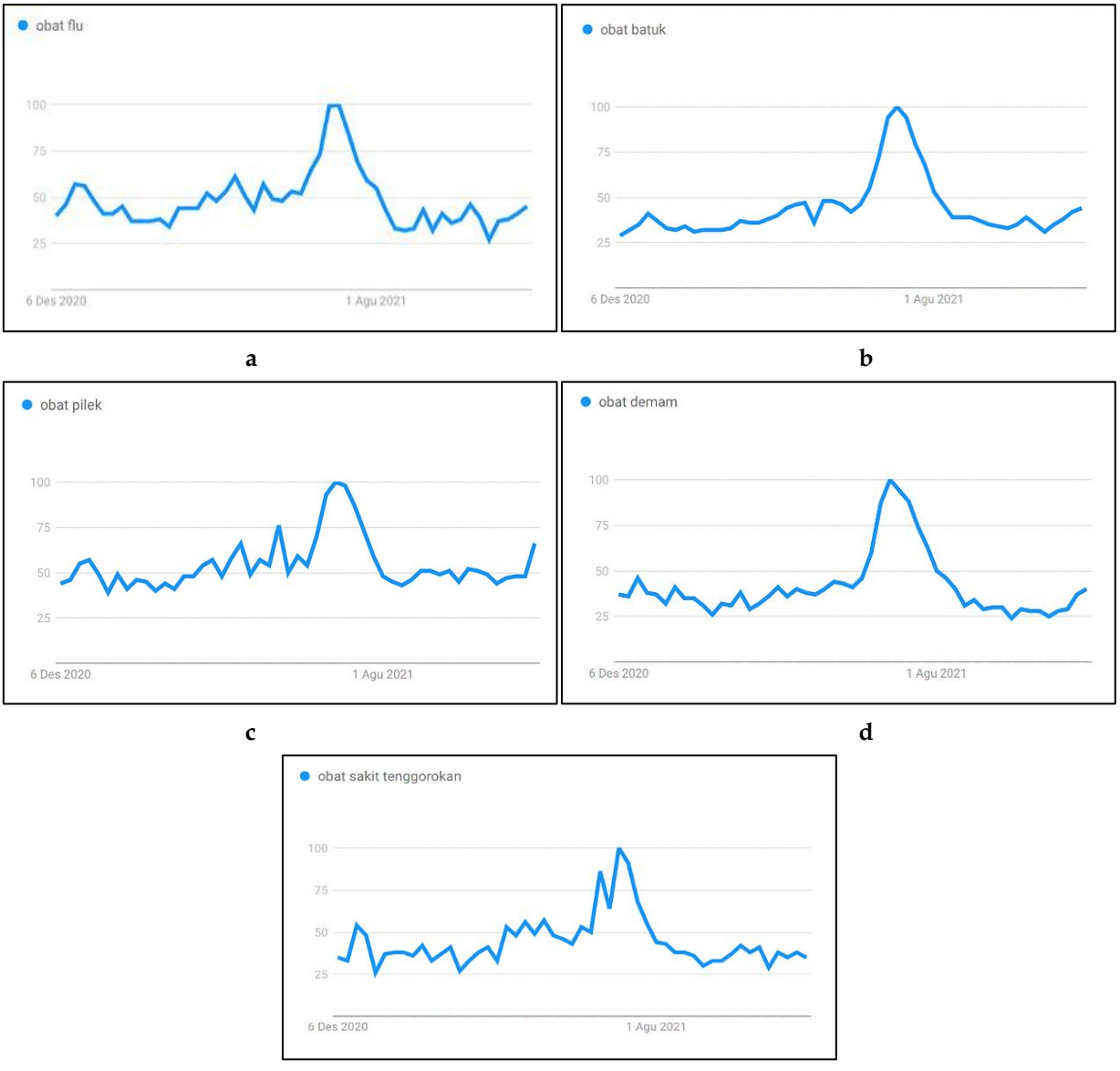

Figure 3. Google Trends Search for Individual Term of "*Obat flu*" **(a)**, "*Obat batuk*" **(b)**, "*Obat pilek*" **(c)**, "*Obat demam*" **(d)**, and "*Obat sakit tenggorokan*" **(e)** from December 6th, 2020 to November 30th, 2021

The trend shown by combination terms or individual terms related to influenza's symptoms drugs showed a similar pattern with positive Covid-19 confirmed cases trends from the government website. The increasing Google Trend search and positive Covid-19 confirmed cases peaked in July and August 2021. This result may be related to the increase in anxiety. Anindyajati *et al*.[22] found that one in five people in Indonesia got anxiety during the pandemic of Covid-19. One of the riskiest groups is people who are suspected cases of Covid-19. However, the anxiety could make people think twice before they go to the hospital during the Covid-19 pandemic. A study in Indonesia found that during the Covid-19 pandemic, people tended to self-medicate compared than visiting health center services or hospitals[6]. Other studies found that during the Covid-19 pandemic, fever, flu, and cough were the top three diseases cured by self-medication. The medicine information usually comes from family, friends, or the internet. A study found that during the second wave of the outbreak of Covid-19 (during June 2021), people self-medicate based on information they got through social media, such as WhatsApp[23]. In the future, Google Trends analysis related to the Covid-19 drugs, such as antiviruses and vitamins, need to be investigated.

CONCLUSION

During the covid-19 outbreak peak in Indonesia, reported in July and August, there were increasing Google search terms related to influenza's symptoms drugs. The highest Google search terms were observed on "Obat batuk" terms. Further research related to the correlation of positive covid-19 confirmed cases with Google search terms related to the drug using statistics analysis needs to be explored.

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AUTHORS' CONTRIBUTION

Nailis Syifa': data collection, formal analysis. **Nurul Purborini**: data collection, formal analysis.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Effect of Drug Information Service on Clinical Outcome of Patients with Type 2 Diabetes Mellitus in Padang, Indonesia

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Abstract

Type 2 diabetes mellitus (T2DM) has been a health burden worldwide, including Indonesia. However, T2DM therapy needs a long and complex process, which patients often do not favor, thus making them not take medications as instructed and negatively affecting the clinical outcomes. This study aimed to understand the effect of Drug Information Service provision on the clinical outcomes of T2DM patients. This quasi-experimental study was conducted using one group pre-post-test design. The fasting blood glucose levels as the clinical outcome were measured before and after the intervention. A drug information service was provided through direct explanation to the patients. Sociodemographic data were analyzed descriptively. The difference in fasting blood glucose before and after the intervention was assessed using Wilcoxon signed-rank test. Forty patients participated in this study. Most participants are female (N=34; 85%) and receive two-drugs combination therapy of metformin and sulfonylureas (N=32; 77.5%). Although there is a decrease in mean fasting blood glucose level after intervention (174.92±59.561 vs. 184.20±49.768), there is no significant difference between fasting blood glucose levels pre-intervention and post-intervention ($p > 0.05$). It is concluded that despite the noticeable decline in blood glucose level after drug information service, its effect on blood glucose control is insignificant.

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INTRODUCTION

Diabetes mellitus (DM) has been a health burden worldwide, including Indonesia. Type 2 diabetes mellitus (T2DM) is the most common type of DM characterized by insulin resistance[1]. In 2019, it was estimated that 463 million (9.3%) adults worldwide suffered from DM. This number is predicted to increase to 578 million in 2030. It is also estimated that 4.2 million people lost their lives due to DM and its complications. Meanwhile, over 700,000 people over 15 in Indonesia suffer from DM, while more than 13,000 originated from West Sumatra[2,3].

Persistent hyperglycemic conditions in uncontrolled DM can cause acute or chronic complications. Among acute complications were diabetic ketoacidosis and diabetic coma. Meanwhile, the chronic complications of DM are nephropathy, neuropathy, and cerebrovascular disease[4].

Blood glucose control is essential to prevent those complications, observed through several parameters such as fasting blood glucose, postprandial blood glucose, and glycated hemoglobin[5]. Blood glucose control is influenced by several factors, such as demographic and clinical characteristics. However, it was also well understood that patients who take antidiabetic medications as instructed are likely to have lower glycated hemoglobin levels and better control of DM-related

comorbidities[6]. Nevertheless, T2DM therapy needs a long and complex process, often not favored by patients[7]. Several studies also suggested that many patients have low adherence to T2DM medication regimens[8-10].

Several interventions can improve patients' understanding and behavior related to their medications, for example, educational video and smartphone-based education[11]. However, in Indonesia, patients may not always have adequate access to technology. Thus, there is a need to develop a simple approach to implement in Indonesia's primary health care setting[12].

Drug information service is one of the pharmaceutical services that can improve clinical outcomes in T2DM. Drug information service is a part of pharmaceutical care delivered in public health centers and other settings like hospitals or pharmacies[13]. Drug information service is defined as a service by pharmacists to provide accurate, precise, and up-to-date information to doctors, pharmacists, other health professionals, and patients. This service includes providing and disseminating information to consumers, both actively and passively, answering questions from patients and health professionals, and creating media of information such as leaflets, drug labels, posters, and newsletters[14].

Few studies have examined the impact of drug information on patient outcomes. A review by Rutter *et al*.[15] from 20 studies concluded that drug information service affects patient outcomes positively. Previous studies and a review article also shows that pharmaceutical care intervention, which includes providing medication information to patients with T2DM, had a positive impact on clinical outcome[16-18]. A study in France shows that tailored information about the disease, diet, and drug treatment improved patients' HbA1c levels[19]. However, studies that reported the effect of drug information services on clinical outcomes in T2DM patients are relatively rare. Thus, we conduct a quasi-experimental study to understand the effect of drug information service provision on the clinical outcome of T2DM patients at Andalas Public Health Center in Padang, Indonesia.

MATERIALS AND METHODS

Materials

This study was conducted at Andalas Public Health Center in Padang, West Sumatra, Indonesia, from August to October 2021. The tools used in this study were data collection forms, stationery, and laptops. Meanwhile, the materials used were drug information sheets and patients' data compiled by the public health center.

Methods

Study design

This quasi-experimental study was conducted using one group pre-post-test design. All participants in this study were given a drug information service. The fasting blood glucose level was measured before and after the intervention. The staff at the public health center performed the blood glucose level measurement.

Population and sampling

The population of this study was the patients with T2DM who were registered in a Chronic Disease Management Program (*Program Pengelolaan Penyakit Kronis*/PROLANIS) at Andalas Public Health Center in Padang, West Sumatra, Indonesia. The sample was chosen according to the inclusion criteria and exclusion criteria. The inclusion criteria were adult T2DM patients ≥18 years who received oral antidiabetic medication and consented to participate. The exclusion criteria were the patients who dropped out from the study or were referred to other healthcare facilities.

Intervention

A drug information service was provided through direct explanation to the patients. The drug information in this study consisted of the medication indication, instruction on medication use, and the side effects of each medication. A drug information guide (https://doi.org/10.5281/zenodo.6496273) was developed for oral antidiabetic agents that were commonly used in the public health center. Patients were also reminded to take their medications as instructed and to return 30 days later for a follow-up period.

Data analysis

Sociodemographic data were analyzed descriptively. The data distribution of fasting blood glucose was analyzed using the Shapiro-Wilk test. The association between patients' gender, age group, and the number of medications with fasting blood glucose levels were measured using the independent t-test method. Meanwhile, the difference between types of comorbidities with blood glucose levels was measured by one-way ANOVA. A Pearson correlation test was also performed to analyze the correlation between the duration of DM and patients' blood glucose levels. The difference in fasting blood glucose before and after the intervention was assessed using Wilcoxon signed-rank test because the data on blood glucose levels after intervention were not normally distributed.

Ethical approval

This study had obtained ethical approval from the Research Ethics Committee, Faculty of Medicine Universitas Andalas, and registered under No. 391/UN.16.2/KEP-FK/2021.

RESULTS AND DISCUSSION

From August to October 2021, 73 patients were recruited for this study. Six patients did not attend the follow-up, four moved to other healthcare facilities for control or medical treatment, while 23 did not attend the healthcare center on time (30 days after the previous visit). Thus, only the data from 40 patients were included for further analysis. Most participants were female (N=34; 85%) and did not work, either homemakers or pensionary (N=35; 90%), as seen in **Table I**. **Table II** shows that most patients also had T2DM for 1 to 5 years (N=36; 90%). Besides, most patients (N=29, 72.5%) also had comorbidities, mostly hypertension (N=17, 42.5%), although other comorbidities such as hypercholesterolemia were also found.

Table I. Sociodemographic characteristics of participants

Characteristics	Number of subjects (N=40)	Percentage (%)
Gender		
Male	6	15
Female	34	85
Age (years)		
18-59	20	50
≥60	20	50
Last education		
Elementary school	15	37.5
Junior high school	9	22.5
Senior high school	12	30
Diploma/bachelor degree	5	10
Occupation		
Worked	5	10
Not worked	35	90

Table II. Clinical characteristics of participants

Characteristics	Number of subjects (N=40)	Percentage (%)
Duration of T2DM		
< 1 year	4	10
1-5 years	36	90
Number of comorbidities		
0	11	27.5
1	20	50
2-3	9	22.5
Type of comorbidities		
Hypertension	17	42.5
Hypercholesterolemia	3	7.5
Hypertension + hypercholesterolemia	6	15
Other	3	7.5

Generally, most participants received the combination of two oral antidiabetic drugs from biguanide (metformin) and sulfonylurea class of therapy (N=29; 72.5%), as shown in **Table III**. The most common sulfonylurea drugs administered to the patients were glimepiride, used by 31 participants (77.5%). According to the T2DM management guideline in Indonesia, the first-line medication for T2DM is metformin due to its good effectiveness, low hypoglycemia risk, neutral effect on body weight, improved cardiovascular outcome, and low cost. Meanwhile, sulfonylurea monotherapy could cause side effects such as hypoglycemia and body weight gain[2,20]. Combined antidiabetic therapy is recommended when the glycemic target is not reached. Hence, the high percentage of combination therapy in this study implies that the patients may need more than one antidiabetic medication to achieve the glycemic target[21].

Table III. Participants' medication profile

Characteristics	Number of subjects (N=40)	Percentage (%)
Number of medication		
1 oral antidiabetic agent	8	20
2 oral antidiabetic agents	32	80
Type of antidiabetics		
Metformin	5	12.5
Glimepiride	2	5
Gliquidone	1	2.5
Metformin+glimepiride	29	72.5
Metformin+glibenclamide	2	5
Glimepiride+gliquidone	1	2.5

Indonesian National Formulary has a set of criteria that manage the administration and restrictions of the different antidiabetic drug classes. Metformin and specific sulfonylurea agents (glibenclamide, glimepiride, and glipizide) can be administered in primary health care facilities[22]. The availability of these drugs on the national formulary may explain why participants received these drug classes for antidiabetics. Although gliquidone is not listed as the medication for patients in primary health care, it can be administered for the back-referral program[23]. A back-referral program is a health service that provides treatments and medications based on the recommendation of a specialist physician for patients with chronic diseases in primary health care[24].

Due to the restrictions at the time of the study and the high cases of COVID-19 in the area, we could obtain fasting blood glucose data as the clinical outcome. Besides HbA1c, fasting blood glucose is also one of the monitoring parameters useful in T2DM patients[25,26]. Compared to HbA1c, fasting blood glucose is a direct, widely accepted, and inexpensive measure[27]. For patients taking oral antidiabetics, blood glucose monitoring also can be considered to assess changes in blood glucose control, monitor the effect of foods on postprandial blood glucose, and changes in blood glucose levels during illness[28].

Before the intervention, participants' fasting blood glucose levels ranged from 95-295 mg/dL. An analysis of the difference in blood glucose levels across different comorbidities and medications was also conducted (**Table IV**) to check for any significant differences. However, no characteristics were associated with patients' blood glucose levels before intervention ($p > 0.05$). It showed that the pre-intervention blood glucose levels were not different among participants of different gender, ages, duration of T2DM, type of comorbidities, and a number of medications. In other words, this means that patients had no difference in baseline blood glucose levels. In contrast, other studies reported otherwise. A study in China suggested that older age and fewer than 12 years of education were associated with poor glycemic control[29]. Meanwhile, another study in Ethiopia found that comorbidities, disease duration (more than seven years), and combination therapy that included insulin were predictors of poor glycemic control in patients with T2DM[30].

Thirty days after the intervention, patients' fasting blood glucose levels ranged from 113-364 mg/dL (**Table V**). This data showed that not all participants successfully achieved the target of blood glucose control. Guidelines released by the American Diabetes Association and the Indonesian Association of Endocrinology (*Perhimpunan Endokrinologi Indonesia*/PERKENI) recommend that adults with diabetes achieve pre-prandial capillary plasma glucose of 80-130 mg/dL[31].

Table IV. Relationship between participants' characteristics with fasting blood glucose levels (pre-intervention)

Characteristics	p-value
Gender	0.430
Age	0.670
Duration of T2DM	0.075
Type of comorbidities	0.208
Number of medication	0.665

Table V. Comparison of fasting blood glucose levels (pre-intervention and post-drug information service intervention)

Variable	Pre-intervention		Post-intervention	
	N	%	N	%
Blood glucose level (mg/dL)				
<130	5	12.5	10	25
130-199	18	45	21	52.5
≥200	17	42.5	9	22.5
Mean±SD	184.20±49.768		174.92±59.561	
Median	182.5		158	
*p**	0.096			

*Mann Whitney U Test, significance level is indicated by p <0.05

Despite an increase in both minimum and maximum levels of fasting blood glucose post-intervention, patients' fasting blood glucose levels under 130 mg/dL increased from 12.5% to 25% (**Table V**). The mean and median of this parameter also decreased slightly. However, there was no significant difference between fasting blood glucose levels pre-intervention and post-intervention (p >0.05). Although post-interventional blood glucose was not significantly different from the baseline level, the slight decrease in the mean and median in this study might be worth exploring further. This finding differs from previous studies that documented pharmacists-led interventions could improve patients' blood glucose control.

In a study in Pakistan[32], the intervention involved pictorial charts and verbal communication related to diabetes management. The patients were followed up one month after the baseline. Meanwhile, in a study in Nigeria[33], the intervention was given in two consecutive face-to-face interviews and educational sessions, with a three-month follow-up period. Other studies in Indonesia suggested that educational videos, patient counseling, and drug information provided by pharmacists could improve patients' HbA1c[34,35]. However, another study in Indonesia also did not find a significant effect of drug information service on blood glucose levels, despite lower blood glucose levels observed in the intervention group[36]. These studies suggested the advantages of using a multimodal educational method for patients, not only relying on direct explanation to significantly affect patients' blood glucose control. Moreover, glycemic control was also influenced by multiple factors which are not always related to medications, such as dietary control[37], and other physical-related factors, such as BMI and central obesity[38]. This study was conducted when the COVID-19 cases were still high in Indonesia, which made more intensive and comprehensive educational provision to patients impossible. Besides, the fasting blood glucose monitoring needs to be accompanied by other glucose monitoring parameters such as HbA1c, as it reflects blood glucose control in the longer term. The sample of this study is also relatively small, which may not be representative of T2DM patients who received care at primary health care facilities in Indonesia. Further studies involving more patients and control groups are needed to examine drug information's effect in a more robust study design.

CONCLUSION

It is concluded that the provision of drug information results in lower blood glucose levels of T2DM patients at Andalas Public Health Center, Padang, Indonesia, even though the effect is not statistically significant.

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AUTHORS' CONTRIBUTION

Lailaturrahmi: conceptualization, methodology, formal analysis, writing – original draft, writing – review & editing, project administration, funding acquisition. **Fuji Araswati**: investigation, formal analysis, writing – original draft. **Armenia**: conceptualization, methodology, supervision. **Rahmi Yosmar**: conceptualization, methodology, writing – review & editing, supervision, funding acquisition.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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