# CZECH UNIVERSITY OF LIFE SCIENCE

## Faculty of Tropical AgriScience

### Department of Crop Science and Agroforestry



# *In vitro* growth-inhibitory effect of *Calophyllum inophyllum* ethanol leaf extract against diarrhoea-causing bacteria

Master's Thesis

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# Declaration

I hereby declare that this thesis entitled "*In vitro* growth-inhibitory effect of *Calophyllum inophyllum* ethanol leaf extract against diarrhoea-causing bacteria" is my own work and all the sources have been quoted and acknowledged by means of complete references. I also declare that this work has not been and is not being submitted for any other degree.

In Prague, 20th July 2017

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Bc. Tomáš Kudera

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# Abstract

The purpose of this study was to investigate the *in vitro* growth-inhibitory effect of *Calophyllum inophyllum*, a medicinal plant traditionally used in Samoan herbal medicine to cure gastrointestinal disorders, against the selection of diarrhoea-causing bacteria. In order to assess that, the minimum inhibitory concentration (MIC) of *C. inophyllum* ethanol leaf extract was determined against six diarrhoea-causing bacteria, namely *Clostridium difficile* infant, *Clostridium perfringens*, *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. The influence of the plant extract on bacterial growth kinetics was further evaluated by slightly modified broth microdilution method. The plant extract showed significant inhibitory activity against *C. perfringens* and *L. monocytogenes* (MIC = 128 μg/mL) followed by *C. difficile* (MIC = 512 μg/mL). Monitored growth curves also showed that the plant extract at 1/2 of MIC inhibits bacterial growth by distinct extension of the lag phase or suppression of the whole growth rate in *C. difficile* and *L. monocytogenes*, respectively. These results demonstrate significant anti-clostridial and anti-listerial activity of *C. inophyllum* ethanol extract that seems to be promising material for development of new antibacterial agents.



**Keywords:** *Calophyllum inophyllum*, Alexandrian laurel, intestinal infections, antibacterial activity, anti-clostridial, anti-listerial, plant extract

# Abstrakt

Účelem této práce bylo stanovit *in vitro* inhibiční aktivitu kalaby obvejčité (*Calophyllum inophyllum*), rostliny tradičně používané v samojské bylinné medicíně pro léčbu průjmových onemocnění, proti vybraným bakteriím způsobujícím střevní infekce doprovázené průjmem. Pro testování byl použit etanolový extrakt z listů, přičemž minimální inhibiční koncentrace (MIK) byly stanoveny pomocí bujónové mikrodiluční metody. Bakteriální střevní patogeny použité pro tento experiment byly *Clostridium difficile* infant, *Clostridium perfringens*, *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes* a *Salmonella enterica*. Vliv přítomnosti rostlinného extraktu na růstovou křivku bakterií byl dále stanoven pomocí modifikované bujónové mikrodiluční metody. Rostlinný extrakt prokázal výraznou *in vitro* inhibiční aktivitu proti *C. perfringens*, *L. monocytogenes* (MIK = 128 μg/mL) a *C. difficile* (MIK = 512 μg/mL). Sledováním vlivu extraktu na růstové křivky bakterií bylo dále prokázáno, že u *C. difficile* a *L. monocytogenes* dochází k významné inhibiční aktivitě i u koncentrací nižších než stanovených MIK, přesněji na úrovni 1/2 MIK. V případě *C. difficile* došlo k výraznému opoždění nástupu lag fáze, zatímco u *L. monocytogenes* ke značnému zmírnění a zpomalení jejího celkového průběhu. Výsledky této práce demonstrují významnou antiklostridiální a antilisteriální aktivitu etanolového extraktu z listů kalaby obvejčité, která se tak jeví jako slibný zdroj nových antibiotických látek používaných pro léčbu těchto infekčních průjmových onemocnění.



**Klíčová slova:** *Calophyllum inophyllum*, kalaba obvejčitá, střevní infekce, antibakteriální aktivita, antiklostridiální, antilisteriální, rostlinný extrakt

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# List of Abreviations

ATCC - American Type Culture Collection

DSMZ - German Resource Centre for Biological Material

MHB - Mueller-Hinton broth

MIC - Minimum inhibitory concentration

WCB - Wilkins-Chalgren broth

# List of Appendices

# *In vitro* growth-inhibitory effect of *Calophyllum inophyllum* ethanol leaf extract against diarrhoea-causing bacteria

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# Contribution of authors

Tomáš Kudera was responsible for data collection, testing of *in vitro* growth-inhibitory of *C. inophyllum* ethanol leaf extract, data evaluation, and manuscript preparation. Johana Rondevaldová participated in plant sample preparation, antibacterial susceptibility testing and data evaluation. Rashmi Kant and Mohammed Umar were involved in the process of plant samples collection and identification. Eva Skřivanová was responsible for maintenance and culturing of anaerobic bacteria tested. Ladislav Kokoška as the main supervisor conceived and designed the study, and participated on manuscript preparation.

# 1. Introduction

Infectious diarrhoea still remains one of the main causes of global morbidity and mortality, especially in less developed countries for children aged under 5 years [1]. Bacterial agents associated with the most severe diarrhoeal episodes in these countries are *Escherichia coli*, *Shigella* spp., *Campylobacter jejuni*, *Salmonella* spp., and *Vibrio cholera* [2]. Other important diarrhoea-causing bacteria such as *Clostridium* spp., *Listeria monocytogenes* or *Enterococcus faecalis* are known to cause serious diseases even in the developed world [3]. Despite the high reduction of global mortality over the last few decades, especially due to the implementation of methods preventing fast dehydration of severely infected patients, diarrhoea remains one of the major human killers, and thus searching for further effective treatment practices is more than advisable [4].

Although the use of common antibiotics could play the major role in controlling diarrhoeal infections by reducing mortality among severely immunocompromised patients with invasive infections [5], by shortening the duration of an illness, or by decreasing secondary transmission of the pathogens [6]; their regular administration is in developing countries significantly restricted due to their low economical effectiveness [7], a high risk of seriousside-effects, and the growing resistance of several causal pathogens [8]. Finding a new, low-cost, easily available, and side-effect-free alternative to common antimicrobial therapy is therefore needed, such that the use of products derived from medicinal herbs such as extracts and their phytochemicals becomes the promising option [9]. A good example from previous studies is goldenseal (*Hydrastis canadensis* L.), the plant traditionally used to treat gastrointestinal infections from which an active antimicrobial compound berberine was later isolated, nowadays offered at the international market to alleviate diarrhoea [10].

In the territory of Pacific Islands, diarrhoea causes the third greatest burden of all present diseases [11]. In correspondence with this, the proportion of local medicinal plants traditionally used to treat diarrhoea is quite high, especially in the South Pacific [12]. Among these countries, relatively high ratio of such medicinal plants is used in traditional herbal medicine of Western Samoa, whereas many of them have not been laboratory tested for their antimicrobial activity yet [13]. Therefore, we performed

preliminary testing of ethanol extracts from 11 Samoan plant species, namely *Calophyllum inophyllum*, *Cordyline fruticosa*, *Inocarpus fagifer*, *Mussaenda raiateensis*, *Piper graeffei*, *Pometia pinnata*, *Premna serratifolia*, *Spondias dulcis*, *Syzygium malaccense*, *Thespesia populnea*, and *Trema cannabina* against diarrhoea-causing bacteria. Among these species, the extract of leaves of *C. inophyllum* produced results worth further investigation (T. Kudera and L. Kokoska, unpublished data).

*C. inophyllum* L. (*Calophyllaceae*), commonly known as Alexandrian laurel, is an evergreen medium to large tropical tree widely distributed along the coasts of Indian and Pacific Oceans especially in Melanesia and Polynesia [14]. Among its various medicinal uses throughout these regions [15], only in Western Samoa the leaf infusion is traditionally used to treat diarrhoea [13]. Despite the existence of previous studies reporting the antimicrobial activity of this part of the plant [16], a detailed research focused on its effect against diarrhoea-causing bacteria is still lacking. In the present study, we therefore examined *in vitro* growth-inhibitory effect of ethanol leaf extract of *C. inophyllum* against six diarrhoea-causing bacteria.

# 2 Material and methods

## 2.1 Plant material

The leaves of *C. inophyllum* were collected from the trees growing in the coastal areas of thecapital city Apia (13°49'43.5"S 171°46'01.5"W), located at Upolu island of the Independent State of Samoa, in September 2015. The plant was authenticated by Tomáš Kudera and a voucher specimen (no. 2404KBFR0) has been deposited in the herbarium of the Department of Botany and Plant Physiology of the Faculty of Agrobiology, Food and Natural Resources of the Czech University of Life Sciences Prague (CZ).

## 2.2 Preparation of plant extracts

Dried sample of *C. inophyllum* leaves was homogenized using Grindomix mill (Retsch, Haan, DE) and 15 g of dry matter was extracted for 24 h in 450 ml 80% ethanol (Sigma-Aldrich, Prague, CZ) at room temperature using laboratory shaker (GFL, Burgwedel, DE). The extract was then filtered and concentrated using rotary evaporator (Büchi Labortechnik, Flawil, CH) in vacuo at 40°C. Dried residues were subsequently diluted in 100% dimethyl sulfoxide (Penta, Prague, CZ) to obtain stock solution of the final concentration 51.2 mg/mL and stored at -20°C until their use. The yield of dry residues was 16.6 %.

## 2.3 Microorganisms and media

The antibacterial activity was determined against six representatives of both Gram-positive/-negative and aerobic/anerobic diarrhoea-causing bacteria. Standard American Type Culture Collection (ATCC) strains, namely *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *L. monocytogenes* ATCC 7644, *S. enterica* ATCC 13076, were obtained from Oxoid (Basingstoke, UK). *C. perfringens* DSM 11778, was purchased from the German Resource Centre for Biological Material (DSMZ) (Braunschweig, DE). *C. difficile* infant KK4 was isolated from faecal samples of healthy infants aged from 1 to 6 months. Mueller-Hinton broth (MHB) (Oxoid, Basingstoke, UK) was used as a growth medium for all tested aerobic bacteria, whereas further supplementation by 1 % of glucose (Sigma-Aldrich, Prague, CZ) was done in case of *E. faecalis*. Both

anaerobs (clostridia) were in difference cultured in Wilkins-Chalgren broth (WCB) (Oxoid, Basingstoke, UK) enriched by 5 g/L soya peptone and 0.5 g/L cystein, grown under anaerobic condition.

## 2.4. Determination of minimum inhibitory concentrations (MIC)

For the effective assessment of in vitro antimicrobial activity, the specific broth microdilution method using 96-well microtiter plates was employed according to the guidelines of the Clinical and Laboratory Standards Institute [17], modified by Cos *et al* [18]. *C. inophyllum* extract was twofold diluted in MHB/WCB (100 μL) in a ranges of 1-512 μg/mL using automated pipetting platform Freedom EVO 100 (Tecan, Männedorf, CH) and a manual multichannel pipette (Eppendorf, Wesseling-Berzdorf, DE) for assessment of aerobic and anaerobic bacteria, respectively. All bacterial cultures were diluted to contain $1.5 \times 10^8$ CFU/mL and subsequently inoculated with the suspension in microtiter plate. Microplates were then incubated for 24 h at 37°C. The plates inoculated with clostridia were prepared and incubated in Whitley A35 Anaerobic Workstation (Don Whitley Scientific, West Yorkshire, UK). Bacterial growth was determined by the absorbance measurement by Cytation 3 Imaging Reader (BioTek, Vermont, USA) at 405 nm. The lowest extract concentration showing at least ≥80% reduction of microbial growth compared to the positive growth control was considered as MIC. Tetracycline (Sigma-Aldrich, Prague, CZ), previously dissolved in ethanol (Lach-Ner, Neratovice, CZ), was tested as positive antibiotic control in concentration range of 0.125-64 μg/mL for aerobic bacteria and *C. perfringens*, whereas for *C. difficile* the antibiotic was diluted in a range of 0.015625-8 μg/mL. All tests were performed as three independent experiments each carried out in triplicate, and the final results are presented as median/modal values.

## 2.5 Bacterial growth kinetics analysis

For monitoring of bacterial growth kinetics, the protocol of broth microdilution method described above was used with following modifications. Plant extract was 2-fold diluted in MHB/WCB in ranges of 16-512 μg/mL, whereas tetracycline was prepared in ranges of 2-64, 0.015625-0.5 and 4-128 μg/mL for aerobic bacteria, *C. difficile* and *C. perfringens*, respectively. During 24 h of incubation, absorbance measurements were performed every hour and the regular orbital shaking conditions

were selected. The plates inoculated with anaerobes were prepared and incubated in Whitley A35 Anaerobic Workstation and manually withdrawn every hour for each absorbance measurement. To avoid oxygen contamination that could negatively affect growth of the anaerobic bacteria, for each measurement a special solitary plate was prepared (i.e., 25 identical plate copies for each experiment). All experiments were performed along with positive controls, neither with plant extract nor with tetracycline, to obtain reference growth curves. Results are presented as curves of bacterial growth calculated as the mean values of two independent experiments each performed in triplicate, demonstrated with apporopriate standard deviations.

# 3 Results

## 3.1 Minimum inhibitory concentration (MIC)

The growth-inhibitory effects of *C. inophyllum* ethanol leaf extract was determined *in vitro* for six representatives of both aerobic and anerobic diarrhoea-causing bacteria (Gram-positive and -negative) as MIC values. The data on susceptibility of all tested bacterial pathogens are summarized in **Table 1**. The results show that the most significant inhibitory effect was observed against *C. perfringens* and *L. monocytogenes* (MIC = 128 µg/mL), followed by moderate activity against *C. difficile* (MIC = 512 µg/mL). No inhibitory activity was observed against *E. faecalis*, *E. coli* and *S. enterica*. In general, Gram-positive strains were more susceptible to *C. inophyllum* ethanol leaf extract than Gram-negative ones. Tetracycline as the positive antibiotic control produced MICs in a range of 0.0625-16 µg/mL.

**Table 1:** *In vitro* growth-inhibitory effect of *C. inophyllum* ethanol leaf extract (CIE) on diarrhoea-causing bacteria

| **Bacterial strain** | **MIC (µg/mL)[a]** | |
|---|---|---|
| | **CIE** | **Tetracycline** |
| *Clostridium difficile* | 512 | 0.0625 |
| *Clostridium perfringens* | 128 | 16 |
| *Enterococcus faecalis* | >512 | 16 |
| *Escherichia coli* | >512 | 1 |
| *Listeria monocytogenes* | 128 | 0.25 |
| *Salmonella enterica* | >512 | 2 |

[a]MIC = Minimum inhibitory concentration (data are median or modal values of three independent experiments, each performed in triplicate)

## 3.2 Bacterial growth kinetics

The analysis of bacterial growth over 24 h in presence of different concentrations of *C. inophyllum* ethanol leaf extract compared to the standard growth and the positive antibiotic (tetracycline) controls was performed for all six diarrhoea-causing bacteria tested. The results, presented as growth curves of standard culture, MIC, and 1/2 of MIC, showed the concentration-dependent inhibitory effect of the tested plant extract on bacterial growth kinetics of *C. difficile* (**Figure 1**), *C. perfringens* (**Figure 2**), and *L. monocytogenes* (**Figure 3**).

Considering the growth of standard cultures, the results showed that all three bacteria exhibited slightly different growth kinetics. The exponential phase starts immediately at the beginning of the incubation of *C. perfringens*, whereas the lag phase of 5 and 3 h was needed in cases of *C. difficile* and *L. monocytogenes*, respectively. However, both clostridia then similarly exhibited a rapid exponential growth reaching further predominating stationary phase, which was in case of *L. monocytogenes* completely missing.

The growth of bacteria exposed to *C. inophyllum* extract and tetracycline at their MIC have confirmed the results obtained during the MIC endpoints determination. Therefore, the final absorbance values of bacterial growth were generally reduced by ≥80 % and no significant growth was observed during the whole 24 h of incubation period. In contrast, bacteria cultured in presence of plant extract and antibiotic at their 1/2 of MICs always exhibited the growth that was generally modified as follows: the lag phase was extended or/and the growth rate was suppressed resulting in a shallower curve giving a reduced final absorbance value. The longest delay of the onset of exponential phase caused by the presence of *C. inophyllum* ethanol leaf extract was observed in growth of *C. difficile* at concentration 256 μg/mL, where the lag phase was extended by approximately 14 h (Figure 1A).

Similarly large extension of the lag phase of *C. difficile* growth was also caused by tetracycline at concentration 0.03125 μg/mL (Figure 1B). Rather shorter delay of the onset of exponential growth (about 3 h) was caused by the tested plant extract at concentration 64 μg/mL in growth of *C. perfringens* (Figure 2C). In this case, the lag phase extension was considerably larger (about 10 h) when the bacteria were exposed to 8 μg/mL of tetracycline (Figure 2D). The growth of *L. monocytogenes* at 1/2 of MIC of

*C. inophyllum* ethanol leaf extract (64 μg/mL) was significantly suppressed and the maximum slope of the exponential phase was distinctly declined (Figure 3E). In case of tetracycline, the initial growth rate has also been reduced, however, after 14 h of incubation the exponential growth suddenly increased reaching the final absorbance value very close to the growth control (Figure 3F).

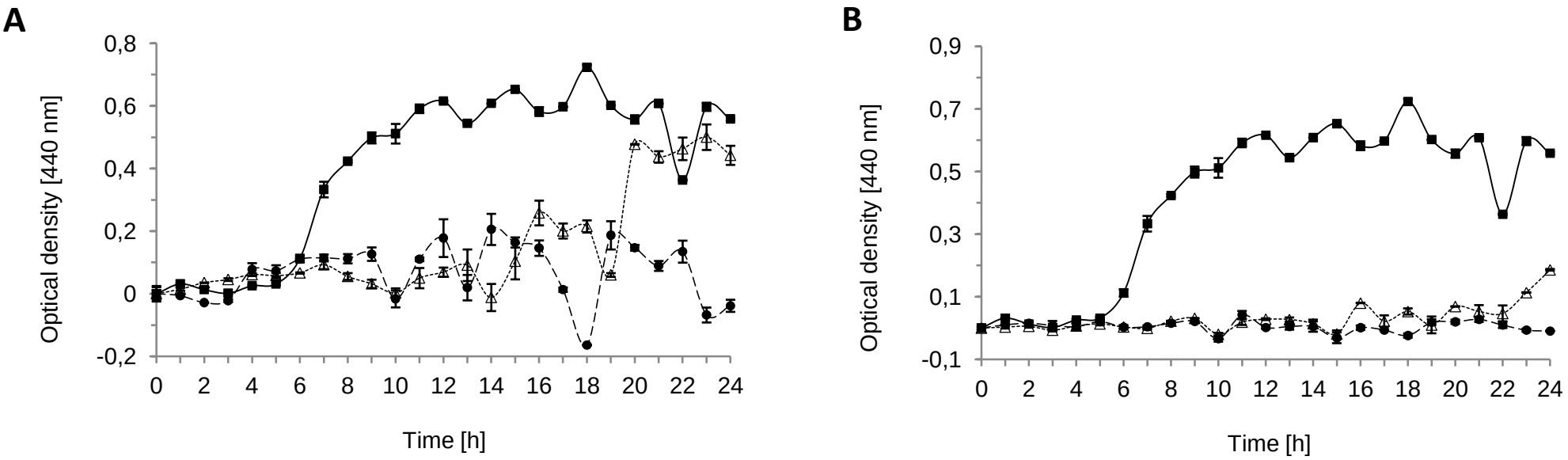


**Figure 1:** Growth kinetics of *C. difficile* over 24 h of incubation in presence of various concentrations of *C. inophyllum* ethanol leaf extract (A) and tetracycline (B), compared to their standard culture growth. Both graphs contain three growth curves: Growth control (■); growth at MIC (●) [512 (A), 0.0625 (B) µg/mL]; and the growth at 1/2 of MIC (Δ) [256 (A), 0.03125 (B) µg/mL].

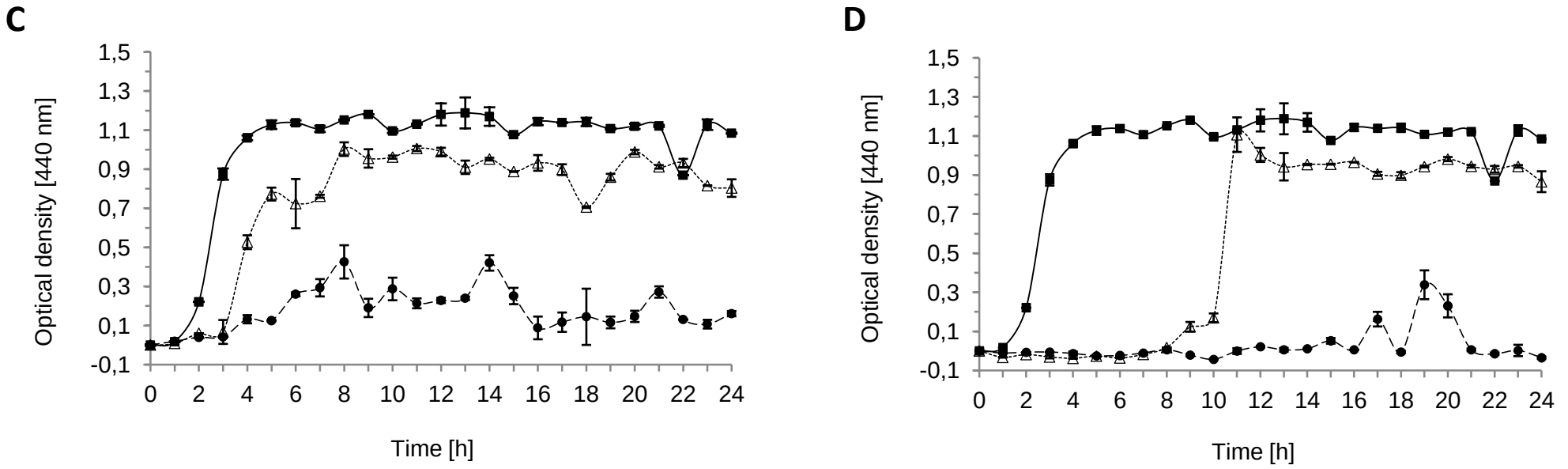


**Figure 2:** Growth kinetics of *C. perfringens* over 24 h of incubation in presence of various concentrations of *C. inophyllum* ethanol leaf extract (C) and tetracycline (D), compared to their standard culture growth. Both graphs contain three growth curves: Growth control (■); growth at MIC (●) [128 (C), 16 (D) µg/mL]; and the growth at 1/2 of MIC (Δ) [64 (C), 8 (D) µg/mL].

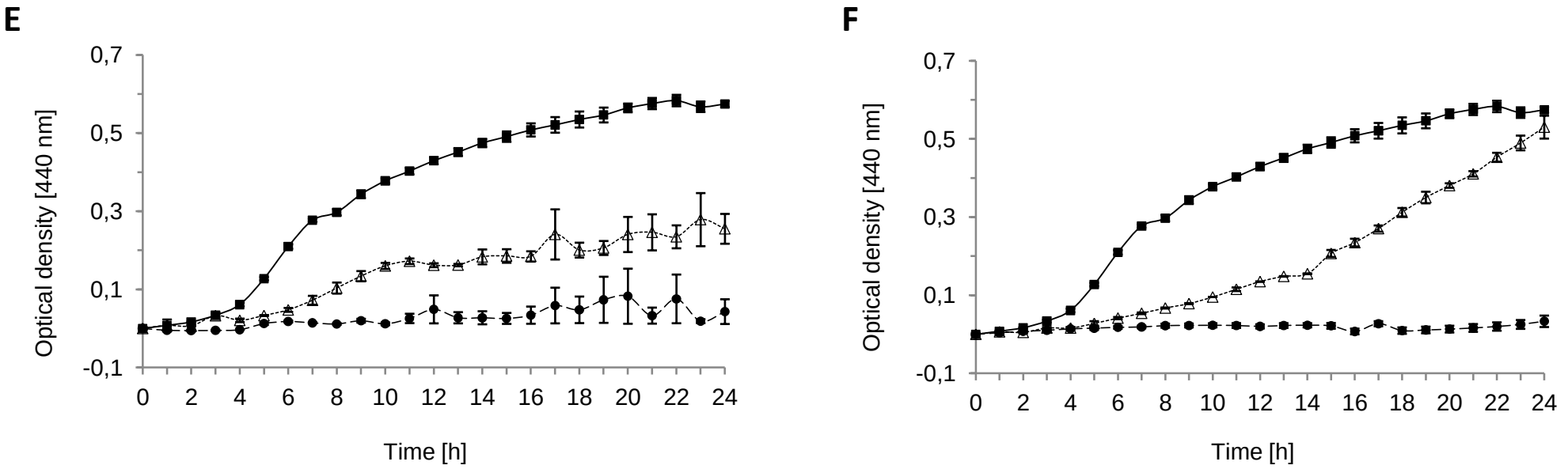


**Figure 3:** Growth kinetics of *L. monocytogenes* over 24 h of incubation in presence of various concentrations of *C. inophyllum* ethanol leaf extract (E) and tetracycline (F), compared to their standard culture growth. Both graphs contain three growth curves: Growth control (■); growth at MIC (●) [128 (E), 0.25 (F) µg/mL]; and the growth at 1/2 of MIC (Δ) [64 (E), 0.125 (F) µg/mL].

# 4 Discussion

The results of this study showed significant *in vitro* growth-inhibitory effect of *C. inophyllum* extract against three Gram-positive diarrhoea-causing bacteria, namely *C. difficile*, *C. perfringens*, and *L. monocytogenes*. In correspondence with our findings, Ali *et al* [16] described a potential activity of buthanol, chloroform and ethanol leaf extracts of *C. inophyllum* against Gram-positive bacteria. Despite the existence of above mentioned study, there are no reports on antibacterial effect of the extract on specific growth-kinetic curves of the tested pathogens. In addition, this is the first report on its anti-clostridial and anti-listerial effect.

As far as the chemical constituents responsible for antibacterial action of the extract are concerned, various classes of secondary metabolites such as coumarins, flavonoids, triterpenes, and xanthones have previously been identified as the main bioactive constituents of the plant [19]. For example, Yimdjo *et al* [20] described antibacterial effect of some calophyllic acid, phenylcoumarine, and xanthone derivatives isolated from root bark and seed against *S. aureus*, whereas calophyllolide was a compound with the highest activity. Since *C. inophyllum* leaves are known as a rich source of the same constituents [21], it is possible to suppose that they could contribute to their anti-clostridial and anti-listerial action observed in this study. Another group of compounds present in relatively high amounts in *C. inophyllum* leaves are friedelin-type triterpenoids, such as canophyllol, canophyllic acid, and friedelin [22]. Ali *et al* [16] described their significant inhibitory effect against Gram-positive (*Corynebacterium diptheriae, Staphylococcus aureus*) and Gram-negative (*Klebsiella pneumoniae, S. typhi, Proteus mirabillis*) bacteria. In another study, Noundou *et al* [23] reported a high inhibitory effect of friedelin against *Bacillus cereus*, *E. faecalis*, and *E. coli* with MICs values in a range of 16-63 μg/mL. Nevertheless, the studies evaluating anti-clostridial and anti-listerial effect of all above mentioned compounds are missing.

Whereas *C. perfringens* and *L. monocytogenes* are known as typical foodborne pathogens causing diarrhoea, *C. difficile* infections are generally linked with antibiotic-associated diarrhoea observed in hospitalised patients [3]. Besides the option of using *C. inophyllum* leaves to treat diarrhoea by inhibition of gut pathogenic bacteria, its application as food preservative could be another way of its utilisation as it has already been hypothesised in previous studies. For example, Odedina *et al* [24] described a high

anti-listerial potential of *Rhodomyrtus tomentosa* ethanol leaf extract for further new food preservative development. Despite the fact that *C. inophyllum* ethanol leaf extract shows a promising antibacterial activity, its safety profile is crucial aspect for practical future application. As the folk medicinal preparations from *C. inophyllum* leaves are particularly applied externally (healing of skin and eye disorders [12]**)**, there is significant evidence of their oral administration [13, 15]. Although Carratu *et al* [25] classified *C. inophyllum* as not admitted in food supplements due to the presence of resins, there are actually no existing toxicological studies on the oral toxicity of this plant part. Therefore, a pharmacological and toxicological evaluation of the plant is needed before considering further ways of its application in treatment of clostridiosis and listeriosis.

# 5 Conclusion

The findings of this study show the significant *in vitro* growth-inhibitory effect of *C. inophyllum* ethanol leaf extract against diarrhoea-causing bacteria *C. difficile, C. perfringens* and *L. monocytogenes*. Bacterial growth kinetic data demonstrate that the plant extract markedly inhibits the growth of the pathogens by significant extension of the lag phase or suppression of the whole growth rate as observed for *C. difficile* and *L. monocytogenes*, respectively. These results can be therefore helpful in further research targeting the development of new anti-clostridial and anti-listerial agents derived from the active antibacterial constituents isolated from *C. inophyllum* leaves. However, further pharmacological and toxicological evaluation is needed before it can achieve clinical application.

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# Appendix 1: Letter of Acceptance of a Manuscript

TJPR Tropical Journal of Pharmaceutical Research
Official Open Access Journal of Pharmacotherapy Group
Faculty of Pharmacy, University of Benin, Benin City, Nigeria
http://www.tjpr.org

11-Jul-2017

**Manuscript no:** 20158110
**Our ref:** 2017071115

**Prof L Kokoska**
KamÃ½ckÃ¡ 129, 165 21 - Suchdol
Prague, Czech Republic

Dear Prof Kokoska,

**Letter of Acceptance of a Manuscript Submitted to Trop J Pharm Res**
**Re: In vitro growth-inhibitory effect of Calophyllum inophyllum leaf ethanol extract against diarrhoea-causing bacteria**

I am pleased to inform you that your manuscript with the above title has now been accepted for publication in Trop J Pharm Res and is slated for publication in vol 16(9), September 2017.

The galley proof will be sent to you in due course and you will be required to respond within 48 hours.

Congratulations and thank you for your interest in Trop J Pharm Res.

**Professor Patrick O Erah**
Editor, Trop J Pharm Res
*Email*: editor-reg@tjpr.org *Tel*: (+234) 0805 526 3622

# Appendix 2: List of author's publications

## Scientific papers

**Kudera T.**, Rondevaldová J., Kant R., Umar M., Skřivanová E., Kokoška L. *In vitro* growth-inhibitory effect of *Calophyllum inophyllum* ethanol leaf extract against diarrhoea causing bacteria. *Tropical Journal of Pharmaceutical Research*, 2017, in press.

## Symposium abstracts

Šmíd F., **Kudera T.**, Rondevaldová J., Kokoška L. *In vitro* growth-inhibitory effect of plant-derived compounds against diarrhea causing bacteria.The Australian Society for Microbiology, Annual Scientific Meeting, July 6-9, 2014, Melbourne, Australia (no. 432),