Original Article

Antibacterial Effect of Hypericum Perforatum and Calophyllum Inophyllum Against Some Bacteria Causing Infections in Cesarean and Episiotomy Wounds

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Abstract

Purpose: This study was carried out to examine the antibacterial effect of Hypericum perforatum essential oil and methanol extract and Calophyllum inophyllum seed oil against some bacteria that lead to recovery and infections in cesarean and episiotomy wounds.
Methods/Design: Bacteria isolated from bacterial cultures of cesarean and episiotomy incisions were identified using the conventional Vitek diagnostic method, automatized for validation, which is used in routine microbiology. Hypericum perforatum methanol extract, pure Calophyllum inophyllum seed oil, Hypericum perforatum essential oil prepared in Calophyllum inophyllum seed oil were used. The "agar disk diffusion" method, which is the most widely used and recommended method for antibacterial activity in natural plant extracts for the imaging of antibacterial activity, the microdilution method, also known as "Micro-well plate dilution" and using 96-well U-bottom microplates, were used. When two methods are evaluated in terms of sensitivity in the study, considering our disk diffusion test results, microorganisms obtained from the routine microbiology wound culture isolates have a high antibacterial effect due to C. inophyllum.
Results: The experiment was carried out with the microwell dilution method with regard to sensitivity, in which the first dilutions were initiated from ½ dilution, and a total of 8 dilutions were studied. The results of this experiment were particularly pleasant for C.inophyllum on Staphylococcus species, and the MIC values determined for 12 isolates from the corresponding extracts are presented.
Conclusion: As a result of the analyses, it was found out that Calophyllum inophyllum was more effective on bacteria than Hypericum perforatum.

Keywords: Bacteria, calophyllum inophyllum, cesarean, episiotomy, hypericum perforatum

Introduction

The increasing diversity of factors in infectious diseases and the rapid change of the drug susceptibility profiles of factors have guided science to find new antibacterial substances and develop R&D projects for "drug-resistant" isolates. This "drug-resistant" isolate increase in hospital-acquired infections has been much

higher. While many hospital-acquired infection cases and many hospital-acquired infection factors have been described, “postoperative incision infections” take an important place among these infection cases and they affect even the patient comfort and the success of the surgery significantly (Ahmed, 2012; Collee et al., 1996).

One of the puerperal infections is wound infections of the cesarean and episiotomy incisions. Episiotomy is not recommended in normal births nowadays, and it has been gradually decreasing in developed countries such as the USA, Belgium, and Sweden since 1983 while it is still routinely applied especially to primiparae in our country (Karacam, 2002). There is a limited number of studies on the rate of episiotomy in our country, and according to the results of these studies, it is stated that episiotomy is routinely applied in primigravidae, and according to the condition of the perineum in multigravidae (Karaçam & Erogu, 2003). Sayıner and Demirci (2007) stated in their study that episiotomy is applied to 96.7% of primiparous pregnant women and 51.8% of multiparous pregnant women, and its rate is 70.3% in all births (Sayıner & Demirci, 2007). According to Donmez et al., in Latin America, 70.0% of all births are performed by episiotomy and it is applied to 9 out of 10 primiparous women who have a vaginal birth, while episiotomy is performed in 94.2% of vaginal births in Brazil, more than 80.0% in Argentina and more than half of women having a vaginal birth in England (Donmez & Sevil, 2009).

There may be some complications after episiotomy. Among these, prolongation of the episiotomy recovery period, third- and fourth-degree perineal lacerations and infection are the most common complications. In the early period, hematoma, infection, cellulitis, abscess, pudendal nerve damage, pain, suture opening, increase in perineal laceration, edema, delayed defecation, and bleeding can be observed (Yanik, 2008). Long-term complications are rectal tone loss, rectal incontinence, rectovaginal fistula, scar tissue and painful sexual intercourse (Karaçam & Eroglu, 2003). According to Toker, when episiotomy care is not performed or if the wound site is not paid attention to, symptoms such as pain and rash appear in the episiotomy site, causing the mother to have a more difficult and stressful postnatal period. Furthermore, the mother's delayed defecation, urination and sexual desire caused by the discomfort in the episiotomy site lead to a lack of self-confidence and this affects and delays the baby-mother interaction (Toker, 2004).

The incidence rate of infection in the episiotomy site is 0.1%. Shy and Eschenbach divided episiotomy infections into four groups according to the depth of the wound. Simple episiotomy infection is limited to the involvement of skin and superficial fascia. Initially, it can be treated with broad-spectrum antibiotics that are also effective on streptococcus, staphylococcus, Enterobacteriaceae, and anaerobes also including B. fragilis. Infection may develop in two layers of superficial fascia. Necrotizing fasciitis is the infection of subcutaneous tissue extending to the deep fascia. The episiotomy wound may appear normal because the skin is not primarily involved. Despite minimal local findings, the patient's clinical outcome is poor. Treatment should include the combination of clindamycin, ampicillin, and gentamicin in addition to radical debridement. Myonecrosis is an extremely rare form of episiotomy infection caused by C. perfringens. Surgical resection and high dose penicillin treatment should be applied (Kadanali & Karagoz, 2012; Kawakita & Landy, 2017; Zuarez-Easton et al., 2017).

In addition to the medical treatments applied to prevent all these complications, non-pharmacologically various substances and methods are used. According to Duran et al., in our country, there is no standard practice in episiotomy care yet. Each hospital provides care according to its own procedures and uses various antiseptic solutions, epithelial and anesthetic pomades (Duran, Eroglu & Sandikçi, 2007). Toker reported that there is a limited number of studies on the effect of episiotomy care and antiseptic solutions on the healing process of episiotomy wounds (Toker, 2004).

Since ancient times, plants have taken an important place in improving human health. Hypericum perforatum, known as St John’s wort, is a perennial plant (Curtis & Levsten, 1990). H. perforatum has long been used both orally and topically for wound healing within alternative medicine of various countries (Ozturk, Korkmaz & Ozturk, 2007).

The pharmacological property of this plant contains antibacterial, anticancer, antiplatelet, anti-inflammatory, antiviral, UV protection activity, antioxidant activity, and wound healing activity. Traditionally, cleaning gums removed from the shell of the plant have been recorded for

use in the treatment of wounds and ulcers (Prabakaran & Britto, 2012).

In a study conducted, anti-microbial activities of methanol and n-hexane extract of C. inophyllum fruit peel against Staphylococcus aureus and Mycobacterium smegmatis were demonstrated. The methanol crude extract exhibited a higher inhibition zone in both S. aureus and M. smegmatis with 58.1% and 46.9%, respectively, while the n-hexane crude extract exhibited a slightly lower inhibition zone in these microbes with 53.8% and 37.5%, respectively (Ha et al., 2009).

In the 1930s, French researchers started to be interested in the wound healing properties, as well as the anti-neuralgic properties of C. inophyllum oil. The wound healing properties of C. inophyllum oil have traditionally been recognized for a long time. C. inophyllum oil accelerates the formation of granulation tissue and regeneration of epidermal cells of the skin (Guba, 1996).

The purpose of this study was to examine the antibacterial activity of H. perforatum and C. inophyllum oil extracts against some bacteria that cause recovery and infections in episiotomy wounds.

Materials and Methods

Plant material: The H. perforatum sample used in this study was collected from Erzurum, between Pazaryolu-Erzurum 100. km, in August 2018. The voucher specimens of the plant have been deposited in the Herbarium of Atatürk University, Faculty of Pharmacy, Erzurum, Turkey (AUEF 1354).

Extraction: 27 g of shade dried flowering tops of the plant were extracted with 200 ml methanol at 40 °C. For this solid–liquid extraction step heating mantle and reflux cooler were used. At the end of 3 hours the extract was filtered and the methanol evaporated under vacuum. The residue (methanol extract 4.99 g) was dissolved in water and frozen at -84 °C. Finally lyophilized at -50 °C.

Oil preparation: For oil preparation Tamanu oil (Calophyllum inophyllum Seed Oil) purchased from Art De Huile (Arın Derin Health Services Industry and Trade Company). The shade dried flowering tops of H. perforatum (2 g) macerated in Tamanu oil (6 ml) for 1 week at room temperature. Everyday the macerate was kept in thermostatic bath at 50 °C for 60 min. At the end of 1 week the macerate filtrated. Thus the H. perforatum maceration oil was ready for further experiments.

Isolation and suspension of bacteria: A total of 11 bacteria isolated from cesarean and episiotomy incisions sent from clinics, including 2 Pseudomonas aeruginosa, 2 Escherichia coli, 1 methicillin-resistant Staphylococcus aureus (MRSA), 1 methicillin-resistant Staphylococcus aureus (MSSA), 3 methicillin-resistant Coagulase-negative staphylococci (MRCNS), 1 methicillin-sensitive Coagulase-negative staphylococcus (MSCNS), 1 Enterococcus fecalis, and 1 Candida albicans isolate were used in the experiment. Bacteria were identified by the conventional Vitek diagnostic method, automatized for validation and used in routine microbiology. 0.5 Mc-farland $1x10^5$ CFU/ml main bacteria stock solutions were prepared from all of the isolates. All isolate stock solutions were diluted at 1/100 for subsequent use in experiments. The final isolate was used as the stock solution in the experiment without any waiting period (Dall'Agnol et al., 2003; Saroglou et al., 2007).

Preparation of main stock solutions of plant extracts: First, 200µg/ml main stock solution of dried methanol extract of "Hypericum perforatum" was prepared. In the experiment, Hypericum perforatum essential oil prepared in Calophyllum inophyllum seed oil, and pure Calophyllum inophyllum seed oil were used. For each of the oils, 0.10 mg/ml stock solutions with DMSO were prepared.

Preparation of the experimental setup: The "agar disk diffusion" method defined by Kirby-Bauer, which is the most widely used and recommended method for antibacterial activity in natural plant extracts for the imaging of antibacterial activity, and the microdilution method, also known as "Micro-well plate dilution" defined by Eloff and using 96-well U-bottom microplates, were used (Calvo et al., 2018). For the agar disk diffusion method, the Mueller Hinton Agar (1055.00, CONDA, PRANODISA) medium, which was placed into a medium plate with a thickness of 4 mm and diameter of 9 cm, was used. Before the study, 8 different concentrations from three separate extracts, gradually decreasing at the rate of ½ respectively, were prepared in sterile distilled water. After the first five dilutions, 6 mm blank paper disks prepared were saturated with 10 µl. Each disk was prepared in three for the repetition of the experiment. 50 µl of each of the 12 isolates

were placed into Mueller Hinton Agar media from the stock solutions prepared in advance, and their diffuse culture was applied to the whole surface by using sterile cotton swabs. After waiting for 15 min, disks containing 5 different concentrations of the active substances of three different extracts were arranged as 2.5 cm between them. All cultured plates were incubated in the incubator at 37 °C for 24h-48h. The inhibition zones around the disks were monitored for two days, and the existing zones were measured and recorded. As a common value, the averages of the experimental repetitions were taken as a single value.

For our microdilution study, the Brain Heart Broth (Merck 1.10493) medium was prepared first, and 50 µl was spread with an automatic pipette into all wells of the 96-well U-bottom plate. 50 µl from the three separate main stock extracts we prepared for the other experiment above was added, and dilution at the ½ ratio was achieved in the first well. Then, five separate decreasing proportions of active substance dilutions towards the right side were prepared for 3 separate extracts and 12 separate isolates for the three experiment repetitions. The final wells were left as the control wells. While no bacteria were placed in a well, no antibacterial agent was placed in another well. Then, 50 µl of the isolates prepared in 1/100 dilution and used in the other experiment were transferred to each of the five dilution wells. At this stage of our experiment, the "optical densities" of these plates were measured and recorded in terms of growth control in the ELISA reader device before the incubation. The plates were covered with slings against the risk of contamination and incubated with other cultures. Second measurements of the optical densities at the end of incubation and 0.1 µl from the wells was transferred.

Results

When two methods are evaluated in terms of sensitivity in the study, considering our disk diffusion test results, there is a high level of antibacterial activity of C. inophyllum especially observed in the studies and also on microorganisms obtained from the routine microbiology wound culture isolates (Uzunköy, 2005).Sensitivity was observed in disks, which did not exhibit significant differences between Staphylococcus species among these factors and even at very low concentrations. However, apart from Staphylococcus species, there was no effect on P.aeruginosa, E. fecalis, and C. albicans isolates, while sensitivity was observed in one of the E.coli isolates at high concentrations (Table 1).The experiment was carried out with the microwell dilution method with regard to sensitivity, in which the first dilutions were initiated from ½ dilution, and a total of 8 dilutions were studied. The results of this experiment were particularly pleasant for C.inophyllum on Staphylococcus species, and the MIC values determined for 12 isolates from the corresponding extracts are presented in the table below (Table 2).

Table 1. Disk diffusion results of H. perforatum and C.inophyllum

	Inhibition zones (mm)					
	H. perforatum essential oil in *C.inophyllum* seed oil					
Disk concentrations (mg/ml)/ isolates	0.1	0.05	0.025	0.0125	0.00625	0.003125
MRSA	15	14	13	12	10	8
MSSA	15	14	12	11	9	7
MRKNS-1	16	15	14	12	9	7
MRKNS-2	18	16	15	13	10	9
MRKNS-3	15	14	13	11	10	-
MSKNS	19	18	15	13	12	9
E.coli-1	-	-	-	-	-	-
E.coli-2	14	13	-	-	-	-

P.aeruginosa-1	-	-	-	-	-	-
P. aeruginosa-2	-	-	-	-	-	-
E. fecalis	-	-	-	-	-	-
C. albicans	-	-	-	-	-	-
			H. perforatum methanol extract			
Disk concentrations (µg/ml)/ isolates	200	100	50	25	12.5	6.25
MRSA	-	-	-	-	-	-
MSSA	7	-	-	-	-	-
MRKNS-1	7	-	-	-	-	-
MRKNS-2	9	-	-	-	-	-
MRKNS-3	-	-	-	-	-	-
MSKNS	-	-	-	-	-	-
E.coli-1	-	-	-	-	-	-
E.coli-2	-	-	-	-	-	-
P.aeruginosa-1	-	-	-	-	-	-
P. aeruginosa-2	-	-	-	-	-	-
E. fecalis	-	-	-	-	-	-
C. albicans	-	-	-	-	-	-
			C. inophyllum seed oil			
Disk concentrations (mg/ml)/ isolates	0.1	0.05	0.025	0.0125	0.00625	0.003125
MRSA	14	12	11	10	8	7
MSSA	17	14	13	9	9	7
MRKNS-1	16	11	10	9	8	-
MRKNS-2	17	11	10	9	9	8
MRKNS-3	14	12	11	10	8	8
MSKNS	17	16	14	13	9	8
E.coli-1	-	-	-	-	-	-
E.coli-2	13	12	-	-	-	-
P.aeruginosa-1	-	-	-	-	-	-
P. aeruginosa-2	-	-	-	-	-	-
E. fecalis	-	-	-	-	-	-
C. albicans	-	-	-	-	-	-

Table 2. MIC values of the microdilution test result of H. perforatum and C.inophyllum

MIC	*H. perforatum* essential oil in *C. inophyllum* seed oil	*H. perforatum* methanol extract	*C. inophyllum* seed oil
MRSA	0.0015625 µg/ml	-	0.003125 µg/ml
MSSA	0.0015625 µg/ml	200µg/ml	0.003125 µg/ml
MRKNS-1	0.003125 µg/ml	200µg/ml	0.00625 µg/ml
MRKNS-2	0.0015625 µg/ml	200µg/ml	0.0015625 µg/ml
MRKNS-3	0.00625 µg/ml	-	0.0015625 µg/ml
MSKNS	0.003125 µg/ml	-	0.0015625 µg/ml
E.coli-1	-	-	-
E.coli-2	-	-	-
P.aeruginosa-1	-	-	-
P. aeruginosa-2	-	-	-
E. fecalis	-	-	-
C. albicans	-	-	-

Discussion

Due to the increase in bacterial resistance to the existing antibiotics and the poor outcomes of the treatments, the need for different fields has increased (Chah et al., 2006). Some efforts are needed to find new antibiotics, to produce them locally and from cheap and renewable sources. Therefore, plants have started to be used in traditional medicine to treat a wide variety of human diseases (Sofowora, Ogunbodede & Onayade, 2013; Dawid-Pac, 2013). Calophyllum inophyllum and Hypericum perforatum are some of these plant species and have multiple uses (Barnes, Anderson & Phillipson, 2001; Léguillier et al., 2015). In the qualitative antimicrobial experimental study analysis, the classical agar disk dilution procedure was applied using Mueller Hinton Agar, and extracts and purified active substances obtained from C. inophyllum were tested against microorganisms, S. aureus (ATCC6538), V. angillarium (ATCC19264), E. coli (ATCC8739) and C. tropicalis (ATCC66029). The paper disks were impregnated with a 20 µl DMSO solution of each sample (1 mg/ml) and allowed to evaporate at room temperature. Oxacillin (20 mg of 1 mg/ml solution) was used as a positive control. The plates were incubated at 37 °C for 18 hours, and the diameter of the inhibition zone around the disk was measured at the end of the incubation period, and necessary recordings were made as a result of the measurement. As a result of the analysis, activity against S. aureus was observed, and nothing was observed against other microorganisms (Yimdjo et al., 2004).

In another study, a significant number of known and new xanthones were isolated from Calophyllum species of Sri Lanka in the last 5 years. The antimicrobial activity of Calophyllum xanthones was examined with a specific reference to methicillin-resistant Staphylococcus aureus (MRSA). These studies were carried out using the agar plate method. Calozeloxanthone, a xanthoxin isolated from C. moonii and C. lankensis, exhibited the highest activity against methicillin-resistant S. aureus (MRSA) strains at a concentration of 8.3 microg/ml. The calozeyloxanthone MIC value for S. aureus (MSSA and MRSA) ranges from 4.1 to 8.1 mg/ml. Therefore, calozeyloxanthone is intended to be used as an antimicrobial agent in the treatment of infections with S. aureus, including methicillin-sensitive S. aureus (MRSA) (Dharmaratne, Wijesinghe & Thevanasem, 1999).

In a study using the dilution method to test the antibacterial activity of Calophyllum inophyllum oil and to determine the minimal inhibitory concentration (MIC), 20 aerobic gram bacterial

species, 20 aerobic gram+ bacterial species, multidrug-resistant staphylococcus aureus and 2 anaerobic gram+ bacterial species (e.g. Propionibacterium acnes and Propionibacterium granulosum) were used (Mahboubi et al., 2014; Leguillier et al., 2015). As a result, Calophyllum inophyllum exhibited two distinct antibacterial effects; one against Gram+ bacteria with the direct inhibition of mitotic growth and the other one against Gram- bacteria due to further release of β-defensin 2 peptide by macrophages. According to these results, by using cell and bacterial cultures, it is observed that they confirmed the pharmacological effects of Calophyllum inophyllum in terms of wound healing and being an antimicrobial agent (Léguillier et al., 2015).

In the in vitro study, β-defensin 2 exhibited a dependent microbicidal effect against Gram-bacteria (Pseudomonas aeruginosa, Escherichia coli) and fungi (Candida albicans) at the concentrations of 1 to 3 μM (Liu et al., 2002). These data suggest that a small increase in β-defensin 2 release may be sufficient to improve host defense against cutaneous pathogens.

In another experiment, the oilogramme procedure was used to test the capacity of Calophyllum inophyllum oil to directly inhibit the growth of bacteria, to address the antibacterial properties of Calophyllum inophyllum oil and to determine the MIC values with herbal oils. This new technique based on oil/water emulsion does not require the use of organic solvents such as ethanol or DMSO, known to have cytotoxic effects in living cells for the MIC detection (Eldin & Sarhan, 2014; Lahlou, 2004). The results stated that it is more active against Gram+ bacteria. It can be said that Gram-bacteria have less permeable cell walls to antimicrobial compounds (Vlietinck et al., 1995). Upon examining the studies on Calophyllum inophyllum, it is observed that there are similar results with our study and it is effective at almost every concentration.

Considering the studies on Hypericum perforatum; in the study of Barnes et al., it was reported that hyperforin has antibacterial activity against Staphylococcus aureus (Barnes, Anderson & Phillipson, 2001). The components of Hypericum perforatum include hyperforin, hypericin, and hyperoside, isoquercitrin and epicatechin from the flavonoid group.

The antibacterial activity of hyperforin against multidrug-resistant S. aureus and Gram-positive bacteria, including Streptococcus pyogenes and Corynebacterium diphtheriae, was reported (Schempp et al., 1999b). Hyperforin did not show any growth inhibitory effect against Gram-negative bacteria such as Enterococcus faecalis, Escherichia coli and Pseudomonas aeruginosa or against Candida albicans (Schempp et al., 1999b). However, it was emphasized that the antibacterial effects of hyperforin were observed only at high concentrations (Voss, & Verweij, 1999). Upon examining the studies on Hypericum perforatum, it is observed that there are similar results with our study and the results are at high concentrations.

Conclusions: This study may be regarded as a source to develop new drugs to combat most of the drug-resistant bacteria, especially against postoperative infections. As a result of the analyses, it was found out that Calophyllum inophyllum was more effective on bacteria than Hypericum perforatum. Further studies, including standard bacterial isolates, should be definitely carried out at a more advanced level and on a larger scale.

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