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TESIS DOCTORAL

New active natural phytocompounds for the treatment and prevention of infectious diarrhea

Nuevos fitocompuestos activos naturales para el tratamiento y prevención de diarreas infecciosas

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Reda Mohamed Mohamed Salem

Directores

Carmina Rodríguez Fernández
Narcisa Martínez Quiles
José Vicente Sinisterra Gago

Madrid, 2015

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NEW ACTIVE NATURAL PHYTOCOMPOUNDS FOR THE TREATMENT AND PREVENTION OF INFECTIOUS DIARRHEA

Thesis submitted in fulfilment of the requirements for PhD degree

Reda Mohamed Mohamed Salem

Supervisors

Carmina Rodríguez Fernández

Narcisa Martínez Quiles

José Vicente Sinisterra Gago

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Tesis presentada en cumplimiento de los requisitos para el grado de doctor

Reda Mohamed Mohamed Salem

Supervisores

Carmina Rodríguez Fernández

Narcisa Martínez Quiles

José Vicente Sinisterra Gago

Madrid, 2015

Dª. CONCEPCIÓN GIL GARCÍA,

DIRECTORA DEL DEPARTAMENTO DE MICROBIOLOGÍA II DE LA FACULTAD DE FARMACIA DE LA UNIVERSIDAD COMPLUTENSE DE MADRID,

CERTIFICA: Que Dª. **Reda Mohamed Mohamed Salem** ha realizado en el Departamento de Microbiología II de la Facultad de Farmacia de la Universidad Complutense de Madrid, bajo la dirección de los doctores **Carmina Rodríguez Fernández**, **Narcisa Martínez Quiles y José Vicente Sinisterra Gago,** el trabajo que presenta para optar al grado de Doctor con el título:

"*New active natural phytocompounds for the treatment and prevention of infectious diarrhea*"

Y para que así conste, firmo la presente certificación en Madrid, 2015

Fdo. Profa. Dra. Dª Concepción Gil García

Catedrática de Microbiología

Dra. CARMINA RODRÍGUEZ FERNÁNDEZ,

PROFESORA TITULAR EU, DEL DEPARTAMENTO DE MICROBIOLOGÍA II DE LA FACULTAD DE FARMACIA DE LA UNIVERSIDAD COMPLUTENSE DE MADRID,

CERTIFICA: Que Dª. **Reda Mohamed Mohamed Salem** ha realizado en el Departamento de Microbiología II de la Facultad de Farmacia de la Universidad Complutense de Madrid, bajo la dirección de los doctores **Carmina Rodríguez Fernández**, **Narcisa Martínez Quiles y José Vicente Sinisterra Gago,** el trabajo que presenta para optar al grado de Doctor con el título:

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Fdo. Profa. Dra. Dª Carmina Rodríguez Fernández

Dra. NARCISA MARTÍNEZ QUILES,

PROFESORA, DEL DEPARTAMENTO DE MICROBIOLOGÍA I DE LA FACULTAD DE MEDICINA, DE LA UNIVERSIDAD COMPLUTENSE DE MADRID,

CERTIFICA: Que Dª. **Reda Mohamed Mohamed Salem** ha realizado en el Departamento de Microbiología II de la Facultad de Farmacia de la Universidad Complutense de Madrid, bajo la dirección de los doctores **Carmina Rodríguez Fernández**, **Narcisa Martínez Quiles y José Vicente Sinisterra Gago,** el trabajo que presenta para optar al grado de Doctor con el título:

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Fdo. Profa. Dra. Dª Narcisa Martínez Quiles

Dr. JOSÉ VICENTE SINISTERRA GAGO,

PROFESOR EMÉRITO, DEL DEPARTAMENTO DE QUÍMICA ORGÁNICA Y FARMACÉUTICA, DE LA FACULTAD DE FARMACIA, DE LA UNIVERSIDAD COMPLUTENSE DE MADRID,

CERTIFICA: Que Dª. **Reda Mohamed Mohamed Salem** ha realizado en el Departamento de Microbiología II de la Facultad de Farmacia de la Universidad Complutense de Madrid, bajo la dirección de los doctores **Carmina Rodríguez Fernández**, **Narcisa Martínez Quiles y José Vicente Sinisterra Gago,** el trabajo que presenta para optar al grado de Doctor con el título:

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A mis padres

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Summary

Introduction

Enteric infections remain a significant public health problem. Worldwide, the infectious diarrhea is the second leading cause of childhood morbidity and mortality, and raise one of the most common human suffering with high morbidity and associated long term sequelae. Furthermore long term diarrhea is associated to cognitive and development deficiency in early childhood ages (under five years age). Infectious diarrheal disease is associated with poverty, and child mortality from it is higher in low-income than in high-income countries and in populations in crisis situations. In particular, enteric infections (diarrheal disorders) were responsible for an estimated 4.6 million deaths worldwide from around 1 billion episodes of diarrhea annually in children. Despite the widespread use of oral rehydration therapies (ORT) and an increased understanding of the pathogenesis of diarrhea, 1.9 million children less than five years of age still die from these illnesses every year according to recent WHO and UNICEF reports. Enteric infections outbreaks occur through the ingestion of contaminated food or water (fecal-oral route transmission) or by direct contact (person to person). The main clinical symptomatology of enteric infections can include either gastrointestinal, systemic affectations or both successively. The main symptom of *gastrointestinal affectation* is ***diarrhea***. Other frequent symptoms are fever, nausea, vomiting and abdominal pain, depending on microbial pathogen. *Systemic affectations* imply more serious syndromes including hemolytic uremic syndrome as the one produced by enterohaemorrhagic *E. coli* (EHEC), meningitis, malabsorption disorders, cardio-neurological alterations, arthritis and hepatitis. WHO defines diarrhea as the "*passage of loose or watery stools at least three times in a 24 h period*", the diarrhea occurs as a result of the failure or ineffectiveness of the digestive tract to perform its absorption function, by different mechanisms that contribute to loss of fluids and electrolytes following microbial infections. The etiological agents of enteric infections are bacteria, viruses, protozoa, fungi and helminthes.

In developing countries the most recurrence causative agents are bacteria. *Escherichia coli* is the predominant facultative anaerobe of the human colonic microbiota. *Escherichia coli* gradually emerged as a cause of diarrhea. Diarrheagenic *E. coli* (DEC) cause infection by a variety of complex mechanisms. These include adherence, production of toxigenic mediators, invasion of the intestinal mucosa, and transportation of bacterial proteins into the host cells. Diarrheagenic *E. coli* (DEC) is classified into six characterized categories: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adhering *E. coli* (DAEC). Enteropathogenic *E. coli* (EPEC) is the leading cause of morbidity and mortality of children under five years old in developing countries. Using a type-three secretion system the bacterial effector proteins are transferred to the host cell cytosol and affect multiple physiological functions, ultimately leading to diarrheal disease. EPEC attach to cells and form actin-rich structures called **pedestals**, which contain many proteins that play unknown functions during pedestal formation. Injected Tir binds to Intimin on the bacterial surface and promotes attaching of bacteria intimately and effacing lesion formation (loss of absorptive microvilli), leading to assembly of cytoskeleton intracellular actin on the cellular surface. Rearrangements of the actin cytoskeleton compose a pedestal-like structure where the bacterium tightly cups the cells, leading to degeneration of brush border microvilli and occurrence of diarrhea. **Oral Rehydration Therapy (ORT)** and nutritional support should be performed before any other therapeutic agent. **Antibiotics** are required for the treatment of bacterial gastroenteritis in those patients suffering from severe complications of gastroenteritis, including dissemination of the disease or sepsis. Generally, antimicrobial treatment tends to speed up the clinical resolution of diarrhea, prevents the progression of the disease and reduces the severity of associated symptoms as fever, vomiting and abdominal pain.

Antibiotics induce bacterial cell death based on the bacterial cell function that can be inhibited by the drug-target interaction. The mechanism includes interference with cell wall synthesis, interference with nucleic acid synthesis, inhibition of protein synthesis, metabolic pathway inhibition and alterations of cell membrane. Currently, there are many synthetic antibiotics for treatment of various diseases caused by microorganisms. Some of these microorganisms have become resistant to antibiotics, rendering them ineffective. The global emergence of bacterial resistance particularly *E. coli* to the antimicrobial agents has increased. The resistance to antimicrobials is due to several causes: genetic plasticity of the microorganisms, unnecessary and inappropriate use, including inadequate dosing, low quality drugs, and increased mobility of the world population. A great number of bacterial isolates are showing resistant to practically all available therapeutic agents. Therefore, the problem has been building up over decades, and today many common and life-threatening infections are becoming difficult or even impossible to treat, sometimes turning a common infection into a life-threatening one. So, there is a continuing need to search for new antibacterial agents to overcome antibiotic resistance. Medicinal plants remain an integrated part of cultures for diseases treatment since ancient times. For these reasons, focusing on alternative herbal medicine has been increased for discovering new therapeutic/antibacterial agents. Much more interest has been paid to extracts and biologically active compounds that were isolated from plant species. This interest in the use of phytocompounds was due to the emergence of side effects of synthetic compounds and the need to find new compounds, including new antibiotics to treat multiresistance bacteria. Therefore, plants can offer a new source of antibacterial agents. It is well known that phytochemical constituents and secondary metabolites are responsible for the activity of plants. The majority of these secondary metabolites work as a defense mechanism against predators and pathogens. The major groups of phytocompounds and bioactive constituents that work as antimicrobial agents are phenols, polyphenols, quinones, tannins, coumarines, flavonols, flavone, flavonoids, terpenoids, essential oils and alkaloids. There is an important direction that

can help us to overcome the problem of microbial resistance is to dedicate our efforts toward scientific research in order to discover new antimicrobial agents. In Europe, nearly 1500 species of medicinal and aromatic plants are widely used in Albania, Bulgaria, Croatia, France, Germany, Hungary, Poland, Spain, Turkey, and the United Kingdom. Many medicinal plants are used as antibacterial agents. It has been reported that *Allium sativum*, *Zingiber officinale*, Methanolic extract of *Tussilago farfara*, *Equisetum arvense*, *Sambucus nigra*, *Aesculus hippocastanum* and *Taraxacum officinale* possess antibacterial activity. Likewise, juices of blueberry, sour grape, Isabella grape, wild blackberry, essential oil of *Rosmarinus officinalis* and *Salvia officinalis* showed antibacterial activity. Moreover, essential oils obtained from *Origanum vulgare* showed effective antibacterial activity. Furthermore, methanol and aqueous extracts of the leaves of *Syzygium cumini* presented good antibacterial active agent. As well, ethanol extract and acetone extract of *Cinnamonum zeylanicum* and *Trachyspermum ammi* displayed good antibacterial activity. *H. sabdariffa* calyces extract has different biological potential activities. It can be used as an anti-obesity agent, treatment of atherosclerosis, diabetes, liver disease and metabolic syndrome. Furthermore, it can be used as antihypertensive and cardioprotective effects in type II diabetic patient with mild hypertension. Its aqueous and ethanol extracts can be used as food preservatives. It showed a dose dependent inhibitory effect against food spoilage of ground beef and apple juice by *S.* Typhimurium DT104, *E. coli* O157:H7, *L. monocytogenes*, *S. aureus* and *B. cereus*. Likewise, it inhibits the growth of MRSA, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii*. Due to the important problem of children deaths in the developing country, it is essential to investigate the use of new natural phytocompounds as antibacterial agents.

Objectives

Enteric infections, which are food and water borne diseases, are a serious public health problem worldwide. Microorganisms are the main cause of enteric infections; therefore their control may significantly reduce the food

and water-borne enteric infections outbreaks. The public is becoming increasingly conscious of problems with the over prescription and misuse of traditional antibiotics, and the failure of treatments due to the appearance of new bacterial strains antibiotic resistant. The use of plant extracts and natural phytochemicals with proved antimicrobial properties can be of great significance in the therapeutic use. In recent years, secondary metabolites as phenolic compounds, terpenoids, flavonoids and alkaloids have received a great deal of attention due to their diverse biological functions. Some natural substances have effective antimicrobial properties and they have been used as alternative treatments. Today, the use of plants as treatment of illness is accepted worldwide. In developing countries, the use of medicinal plants has significantly increased due to the low income of the population. Herbs and spices are generally considered to be safe and have been proved to be effective against certain ailments. Therefore we aimed to study the antimicrobial properties of selected medicinal plant extracts, as follows:

I. Evaluation of the *in vitro* antimicrobial activity of selected plant extracts, against Gram-negative and Gram-positive bacteria including some of the most frequent etiological agents of enteric infections and fungi.

II. Characterization of the antimicrobial activity of the natural extract with best activity and the most sensitive/interesting microorganism.

III. Determination of the *in vitro* cytotoxicity of the selected extract.

IV. Analysis of the effect against enteropathogenic *E. coli* (EPEC) of the selected extract during *in vitro* infection of cell cultures.

V. Chemical fractionation of the selected natural extract.

VI. Assessment of the antibacterial activity of the fractions.

Results

In the present study, the aqueous extracts of five plants (*Hibiscus sabdariffa*, *Cinnamomum zeylanicum*, *Origanum vulgare*,

Rosmarinus officinalis and *Thymus vulgaris*) were screened for their antimicrobial activity against 12 pathogenic bacteria (Gram-positive and Gram-negative), and 3 fungi (yeasts). The antimicrobial activity was assayed by using broth microdilution assay and disc diffusion test. *H. sabdariffa* extract showed the highest antibacterial activity against Gram-positive and Gram-negative, with MICs ranged between 4-10 mg/mL and MBCs ranged between 5-25 mg/mL. In disc diffusion test, *H. sabdariffa* extract produce an inhibition halo of 10.5 mm against EPEC. *H. sabdariffa* extract did not show fungicidal activity. Time killing studies showed bactericidal activity of *H. sabdariffa* extract (MBC 25 mg/mL) at 10 h against EPEC, with the endpoint at 23 h. This extract did not show cytotoxicity on macrophages but affected HeLa cells as previously reported.

The *H. sabdariffa* extract induces bacterial filamentation in EPEC. This morphological change reduces the ability of bacteria to adhere to epithelial cells *in vitro*. The *H. sabdariffa* extract reduces numbers of the pedestals formation induced by EPEC during the *in vitro* HeLa cells infection, in a time dependent manner. The *H. sabdariffa* extract inhibits pedestals formation in case of simultaneous treatment (EPEC infection and *H. sabdariffa* treatment simultaneously). This inhibition can be due to the induction of the filamentous morphology which would reduce the ability of the bacteria to adhere to HeLa cells and consequently no pedestals were formed. The pedestals cannot be formed in HeLa cells that were pre-treated with *H. sabdariffa* extract, before infection by EPEC. These results suggest that *H. sabdariffa* could be used both as potential preventive and treatment agent of gastroenteritis produced by EPEC.

The *H. sabdariffa* aqueous extract was fractionated using different organic solvent based on polarity. The acetone fraction (AC) was the

most active fraction with antibacterial activity, followed by further acetone extraction from ethanol extraction (AC-EtOH). They have antibacterial activity against tested bacteria at MIC 5 mg/mL and MBC at 5 mg/mL, except for EPEC with values of 8 mg/mL. The sub-fractions 2, 3 and 4 of acetone fraction had MIC at 5 mg/mL and MBC at 5 mg/mL for all tested bacteria.

This is the first study that demonstrates time kill kinetics of *H. sabdariffa* extract against EPEC, showing the effect of *H. sabdariffa* extract on pedestals formation induced by EPEC in HeLa cells and presents new active antibacterial fraction (acetone fraction). Furthermore, it opens door to investigate the molecular mechanism of *H. sabdariffa* extract as antibacterial agent and determining the active compounds of *H. sabdariffa* extract as antibacterial agent.

Conclusions

H. sabdariffa extract has antibacterial activity and could be used as antimicrobial agent for enteric infections, particularly produced by EPEC. The *H. sabdariffa* extract induces bacterial filamentation in EPEC and reduces the ability of bacteria to adhere to epithelial cells *in vitro*. This morphological change might be mediated through the interaction of active compounds with molecular targets from bacterial cell wall, leading to bacterial filamentation and death. *H. sabdariffa* extract could be used as potential protective agent against enteric infections particularly by EPEC. The antibacterial activity was present mainly in the 2, 3 and 4 acetone fractions. Therefore, the active compounds could be purified from these fractions in the future, in order to be used in pharmaceutical formulations for enteric infections.

Resumen

Introducción

Las enfermedades causadas por infecciones entéricas siguen siendo uno de los problemas más importantes de salud pública. La diarrea infecciosa es a nivel mundial la segunda causa de morbilidad y mortalidad infantil, con 4,6 millones de muertes de los aproximadamente un billón de episodios de diarrea al año reportados en niños. Los trastornos diarreicos conllevan además un gran sufrimiento dejando secuelas a largo plazo, entre las que se encuentran la deficiencia cognitiva y de desarrollo en los primeros cinco años de la infancia. Las enfermedades diarréicas infecciosas están altamente ligadas con la pobreza, y la mortalidad infantil es mayor en los países con menor producto interior bruto, especialmente en situaciones de crisis. A pesar del uso generalizado de las terapias de rehidratación oral (TRO) y de un mayor conocimiento de la patogénesis de la diarrea infecciosa, 1,9 millones de niños menores de cinco años siguen muriendo de estas enfermedades cada año según informes recientes de la OMS y UNICEF. Los brotes de infecciones intestinales se producen a través de la ingestión de alimentos o agua contaminados (transmisión vía fecal-oral) o por contacto directo (persona a persona). Los principales síntomas clínicos de las infecciones entéricas incluyen los síntomas gastrointestinales o las afecciones sistémicas, pudiendo darse sucesivamente. El principal síntoma de afectación gastrointestinal es la diarrea. Otros síntomas frecuentes son fiebre, náuseas, vómitos y dolor abdominal, en función de los patógenos microbianos. Las afecciones sistémicas implican síndromes más graves, incluyendo el síndrome urémico hemolítico (como el causado por la bacteria EHEC), meningitis, trastornos de malabsorción, alteraciones cardio-neurológicas, artritis y hepatitis. La OMS define la diarrea como “la deposición, tres o más veces al día (o con una frecuencia mayor que la normal para la persona) de heces “sueltas o líquidas”. La diarrea se produce como resultado de la insuficiencia o ineficacia del tracto digestivo para realizar su función de absorción, esto ocurre mediante diferentes mecanismos que contribuyen a la pérdida de líquidos y electrolitos tras la

infección microbiana. Los agentes etiológicos de las infecciones entéricas son bacterias, virus, protozoos, hongos y helmintos.

En los países en vías de desarrollo los agentes causantes de las infecciones entéricas más recurrentes son las bacterias. *Escherichia coli* es una bacteria anaerobia facultativa predominante en la microbiota intestinal del colon en los humanos que con el paso del tiempo se asoció a la aparición de diarreas. Las distintas cepas de *E. coli* diarreicas (DEC, de las siglas en inglés Diarrheagenic *E. coli*) infectan las células del hospedador mediante una variedad de mecanismos complejos, dentro de los cuales se incluyen la adhesión y/o la invasión de la mucosa intestinal, la producción de toxinas y la inyección de proteínas bacterianas en las células hospedadoras. Las bacterias *E. coli* diarreicas se clasifica en seis categorías: *E. coli* enteropatógena (EPEC), *E. coli* enterohemorrágica (EHEC), *E. coli* enterotoxigénica (ETEC), *E. coli* enteroinvasiva (EIEC), *E. coli* enteroagregativa (CEEA), y *E. coli* de adherencia difusa (DAEC). *E. coli* enteropatógena (EPEC) es la causa más importante de morbilidad y mortalidad en niños menores de cinco años en los países en vías de desarrollo. EPEC se adhiere a las células intestinales y mediante un sistema de secreción denominado tipo tres, inyecta las proteínas efectoras bacterianas que se transfieren al citosol de la célula hospedadora afectando a múltiples funciones fisiológicas que en última instancia conducen a la enfermedad diarreica. EPEC se adhiere a las células intestinales formando una lesión histopatológica caracterizada por la degeneración local de las microvellosidades de absorción, la adherencia íntima de la bacteria a la célula hospedadora y la formación de una estructura rica en filamentos de actina denominada **pedestal**. Los pedestales contienen además muchas otras proteínas celulares cuya función durante la formación de dicha estructura es desconocida. La proteína efectora Tir (de las siglas en inglés Translocated Intimin Receptor) es inyectada en la célula, se inserta en la membrana plasmática e interacciona con intimina, una proteína de la superficie bacteriana, promoviendo la fijación de las bacterias de manera íntima, y dando lugar al

ensamblaje de filamentos de actina en la superficie de la célula hospedadora. Esta reorganización del citoesquelto de actina de la célula da lugar a la formación del pedestal, donde la bacteria queda literalmente apoyada en la superficie celular de manera fija, lo que conduce a la degeneración de las microvellosidades del borde en cepillo y la ocurrencia de la diarrea. La **terapia de rehidratación oral** (TRO) y el suplemento nutricional se deben realizar antes de administrar ningún otro agente terapéutico. Los **antibióticos** se requieren para el tratamiento de la gastroenteritis bacteriana en aquellos pacientes que sufren complicaciones graves, como por ejemplo pacientes donde la enfermedad se ha expandido por el organismo provocando sepsis. Generalmente, el tratamiento antimicrobiano consigue prevenir la progresión de la enfermedad y reduce la gravedad de los síntomas clínicos asociados como fiebre, vómitos y dolor abdominal.

Los antibióticos inhiben la función celular bacteriana como resultado dela interacción fármaco-diana, provocando así la muerte celular bacteriana. Los distintos mecanismos mediante los cuales actúan los antibióticos incluyen la inhibición de la síntesis de la pared celular, la inhibición de la síntesis de ácidos nucleicos y de proteínas, la inhibición de rutas metabólicas y alteraciones de la membrana celular. Actualmente, hay muchos antibióticos sintéticos para el tratamiento de diversas enfermedades causadas por microorganismos. Algunos de estos microorganismos se han vuelto resistentes a los antibióticos, haciéndolos ineficaces. La aparición de la resistencia bacteriana los agentes antimicrobianos, particularmente de cepas de *E. coli*, se ha incrementado a nivel global. La resistencia a los antimicrobianos se debe a varias causas: plasticidad genética de los microorganismos, el uso innecesario e inapropiado de los antimicrobianos (incluyendo la dosificación inadecuada y los medicamentos de baja calidad), y el aumento de la movilidad de la población mundial. Un gran número de cepas bacterianas están mostrando ser resistentes a prácticamente todos los agentes terapéuticos disponibles. De este modo, el problema ha ido aumentando durante décadas, y hoy en

día muchas infecciones comunes se están convirtiendo en infecciones difíciles o incluso imposibles de tratar, y así una infección común potencialmente mortal puede convertirse en una amenaza para la vida de la persona. Por lo tanto, hay una continua necesidad de buscar nuevos agentes antibacterianos para superar la resistencia a los antibióticos. El uso de plantas medicinales para el tratamiento de las enfermedades es una práctica arraigada en las distintas culturas desde la antigüedad. Por ello, últimamente se han incrementado los tratamientos de medicina alternativa a base de hierbas para el descubrimiento de nuevos agentes terapéuticos y antibacterianos. Debido a la aparición de efectos secundarios frente a determinados compuestos sintéticos y a la necesidad de encontrar nuevos compuestos para tratar bacterias multiresistentes, incluyendo nuevos antibióticos, los extractos y compuestos biológicamente activos aislados a partir de distintas especies de plantas (fitocompuestos) están despertando un gran interés. Por lo tanto, las plantas se presentan como una nueva fuente de agentes antibacterianos. La mayoría de los constituyentes fitoquímicos y metabolitos secundarios responsables de la actividad de las plantas, funcionan como un mecanismo de defensa contra patógenos y depredadores. Los principales grupos de fitocompuestos y componentes bioactivos que funcionan como agentes antimicrobianos son: fenoles, polifenoles, quinonas, taninos, cumarinas, flavonoles, flavonas, flavonoides, terpenoides, aceites esenciales y alcaloides. De este modo, dirigir nuestros esfuerzos hacia la investigación científica para descubrir nuevos agentes antimicrobianos nos puede ayudar a superar el problema de la resistencia microbiana. Cerca de 1.500 especies de plantas medicinales y aromáticas son ampliamente utilizadas en distintos países Europeos como Albania, Bulgaria, Croacia, Francia, Alemania, Hungría, Polonia, España, Turquía y el Reino Unido. Muchas plantas medicinales poseen actividad antibacteriana, como por ejemplo *Allium sativum*, *Zingiber officinale*, el extracto metanólico de *Tussilago farfara*, *Equisetum arvense*, *Sambucus nigra*, *Aesculus hippocastanum* y *Taraxacum officinale*. Del mismo modo, los zumos de arándano, uva agria, uva “Isabella”, mora silvestre, y el aceite esencial obtenido de *Rosmarinus officinalis*, *Salvia*

officinalis y *Origanum vulgare* muestran actividad antibacteriana. Los extractos acuoso y metanólico de las hojas de *Syzygium cumini* presentan un agente activo antibacteriano. Además, el extracto de etanol y el extracto de acetona de *Cinnamonum zeylanicum* y *Trachyspermum ammi* muestran también actividad antibacteriana eficaz. El extracto de los cálices de *H. sabdariffa* presenta un amplio rango de actividades biológicas, pudiéndose utilizar como tratamiento contra la obesidad, la aterosclerosis, diabetes, enfermedades del hígado y otros síndromes metabólicos. Además, se puede utilizar como antihipertensivos y presenta efectos cardioprotectores en los pacientes diabéticostipo II con hipertensión leve. Por otro lado, sus extractos acuosos y etanólicos se pueden usar como conservantes de alimentos, habiéndose demostrado un efecto inhibidor dependiente de la dosis en el deterioro de los alimentos tales como carne picada y zumo de manzana debido a *S.* Typhimurium DT104, *E. coli* O157:H7, *L. monocytogenes*, *S. aureus* y *B. cereus*. Asimismo, inhibe el crecimiento de MRSA, *K. pneumoniae*, *P. aeruginosa* y *A. baumannii*. La elevada mortalidad en niños en países en vías de desarrollo como resultado de infecciones entéricas pone de manifiesto la importancia de investigar nuevos principios activos y fitocompuestos naturales para descubrir nuevos agentes antibacterianos y su potencial uso terapéutico.

Objetivos

Las enfermedades entéricas cuya causa principal son las infecciones por microrganismos transmitidos por agua o alimentos contaminados, son un grave problema de salud pública en todo el mundo. El control de los microorganismos puede reducir significativamente los brotes de infecciones entéricas debidos a la ingestión de comida o agua contaminada. La humanidad es cada vez más consciente del grave problema que constituye el fracaso de los tratamientos actuales, debido a la aparición de nuevas cepas bacterianas resistentes a múltiples antibióticos. El uso de extractos de plantas y fitoquímicos naturales con propiedades antimicrobianas probadas puede por tanto ser de gran importancia en el uso terapéutico. En los últimos años, metabolitos

secundarios como compuestos fenólicos, terpenoides, flavonoides y alcaloides han recibido una gran atención debido a sus diversas funciones biológicas. Algunas sustancias naturales tienen propiedades antimicrobianas eficaces y han sido utilizados como tratamientos alternativos, habiéndose aceptado en todo el mundo el uso de determinadas plantas para el tratamiento de ciertas enfermedades. En los países en vías de desarrollo, el uso de plantas medicinales ha aumentado significativamente debido a los bajos ingresos de la población. Se ha demostrado que ciertas hierbas y especias consideradas generalmente como seguras, son eficaces contra ciertas dolencias. Por todo ello, en esta tesis nos propusimos estudiar las propiedades antimicrobianas de una selección de extractos de plantas medicinales, estableciéndose los siguientes objetivos:

I. Evaluación de la actividad antimicrobiana *in vitro* de extractos de una selección de plantas sobre bacterias Gram-negativas y Gram-positivas causantes de infecciones entéricas y sobre hongos.

II. Caracterización de la actividad antimicrobiana del extracto natural con mayor actividad sobre el microorganismo más sensible o interesante de los estudiados.

III. Determinación de la citotoxicidad *in vitro* del extracto natural seleccionado.

IV. Análisis del efecto sobre *E. coli* enteropatógena (EPEC) del extracto natural seleccionado mediante la infección *in vitro* de una línea celular estable de células humanas.

V. Fraccionamiento químico del extracto natural seleccionado.

VI. Evaluación de la actividad antibacteriana de las distintas fracciones.

Resultados

En el presente estudio, fueron seleccionados los extractos acuosos de cinco plantas (*Hibiscus sabdariffa*, *Cinnamomum zeylanicum*, *Origanum vulgare*, *Rosmarinus officinalis* y *Thymus vulgaris*) por su actividad antimicrobiana frente a diversas cepas de bacterias patógenas (12 Gram-positivas y Gram-negativas en total), y 3 hongos (levaduras). La actividad antimicrobiana se analizó mediante dos ensayos microbiológicos, ensayo

de microdilución en caldo y prueba de difusión en disco. El extracto de *H. sabdariffa* mostró la mayor actividad antibacteriana sobre las bacterias Gram-positivas y Gram-negativas estudiadas, con CMI que oscilaron entre 4-10 mg/ml y CMB oscilaron entre 5-25 mg/ml. En la prueba de difusión en disco, el extracto de *H. sabdariffa* produjo un halo de inhibición de 10.5 mm contra EPEC. Sin embargo, el extracto *H. sabdariffa* no mostró actividad fungicida. Además, las curvas de letalidad mostraron actividad bactericida del extracto de *H. sabdariffa* (CMB 25 mg/mL) sobre EPEC a partir de las 10 horas y hasta las 23 horas. Este extracto no mostró citotoxicidad en macrófagos J774, pero presentó un effecto anti-proliferativo de células como se ha descrito previamente en células de carcinoma epitelial humano (células HeLa) en cultivo.

Se observó que el extracto de *H. sabdariffa* induce filamentación de la bacteria EPEC. Este cambio morfológico reduce la capacidad de las bacterias para adherirse a las células epiteliales cultivadas *in vitro* (células HeLa). Asimismo, se demostró que el extracto de *H. sabdariffa* reduce el número de pedestales formados por EPEC durante la infección de las células HeLa, de manera dependiente del tiempo de tratamiento. Además, en el caso de tratar simultáneamente las células con *H. sabdariffa* e infectarlas con EPEC se observó una inhibición en la formación de pedestales. Esta inhibición puede ser debida a la filamentación de EPEC en presencia del extracto, lo que reduciría la capacidad de las bacterias para adherirse a las células HeLa y en consecuencia la formación de pedestales. En este estudio se observó también que los pedestales no se formaron en las células HeLa que fueron tratadas con extracto de *H. sabdariffa* previamente a la infección por EPEC. Estos resultados sugieren que *H. sabdariffa* podría ser utilizado como potencial agente preventivo y como posible tratamiento de la gastroenteritis producida por EPEC.

El extracto acuoso de *H. sabdariffa* se fraccionó utilizando varios disolventes organicos de distinta polaridad. La fracción de acetona (AC) fue la fracción con mayor actividad antibacteriana, seguida por extracción

adicional de acetona de la extracción de etanol (AC-EtOH). Ambos presentaron actividad antibacteriana sobre todas las bacterias estudiadas con valores de CMI de 5 mg/mL y CMB a 5 mg/mL, a excepción de EPEC con valores de 8 mg/mL de CMB. Las sub-fracciones 2, 3 y 4 de la fracción de acetona tenían CMI en 5 mg/mL y CMB a 5 mg/mL para todas las bacterias probadas.

Este es el primer estudio que demuestra la cinética de las curvas de letalidad del extracto de *H. sabdariffa* sobre la bacteria EPEC. En este estudio se observa un claro efecto de dicho extracto sobre los pedestales formados tras la infección de EPEC en células HeLa y se descubre una nueva fracción activa con actividad antibacteriana (fracción de acetona). Nuestro trabajo abre así nuevas vías de estudio para investigar el mecanismo molecular del extracto de *H. sabdariffa* como agente antibacteriano y la determinación de los compuestos activos responsables de dicha actividad.

Conclusiones

El extracto de *H. sabdariffa* tiene actividad antibacteriana y podría ser utilizado como agente antimicrobiano en infecciones entéricas, particularmente producidas por EPEC. El extracto de *H. sabdariffa* induce la filamentación de la bacteria EPEC reduciendo la capacidad de las bacterias para adherirse a células epiteliales en cultivo. Este cambio morfológico podría estar mediada a través de la interacción de los compuestos activos de este extracto con dianas moleculares de la pared celular bacteriana, que conduce a la filamentación bacteriana y a la muerte de la bacteria. De este modo, el extracto de *H. sabdariffa* podría ser utilizado como potencial agente protector contra las infecciones entéricas provocadas particularmente por EPEC. Se corroboró que la actividad antibacteriana estaba presente principalmente en las fracciones 2, 3 y 4 de la fracción de acetona. Por lo tanto, los compuestos activos pueden ser purificados a partir de estas fracciones en el futuro, con el fin de ser

utilizado en formulaciones farmacéuticas para uso terapéutico de infecciones entéricas.

ABBREVIATIONS

Abbreviations

AC extract: Acetone soluble extract

AC from EtOH extract: Acetone soluble extract from ethanol soluble extract

AAF: aggregative adherence fimbriae

A/E: Attaching and effacing

AMR: Antimicrobial resistance

AKT: Serine/Threonine-specific protein kinase

BGA: Brilliant Green Agar

BFP: bundle forming pilus

CFU/ml: Colony Forming Unit/milliliter

CFA: Colonization factor antigen

cGMP: Cyclic guanosine monophosphate

CDC: Centers for Diseases Control and Prevention

DEC: Diarrheagenic *Escherichia coli*

DAEC: Diffusely adhering *Escherichia coli*

DRA: Down regulated in adenoma

EtOAc extract: Ethyl acetate soluble extract

EtOH extract: Ethanol soluble extract

Esp: *Escherichia coli* secretion protein

EU: European Union

Erk: Extracellular signal-regulated kinase

Ext: Extract

FasL: Fas ligand

Fra: Fraction

GAPPD: Global Action Plan for the Prevention and control of Pneumonia and Diarrhea

HUS: Haemolytic Uremic Syndrome

LB: Luria Bertani media

LT: Heat labile enterotoxin

LDL: Low density lipoprotein

MAPK: Mitogen activated protein kinase

MHA: Mueller Hinton Agar

MHB: Mueller Hinton Broth

MOI: Multiplicity of Infection

MIC: Minimum Inhibitory Concentration

MBC: Minimum Bactericidal Concentration

MRSA: Methicillin Resistant *Staphylococcus aureus*

NHE3: Sodium–hydrogen exchanger3

NOD: Niucleotide-binding oligomerization domain

NLRs: (NOD)-Like-receptors

ORT: Oral rehydration therapy

PI3K: Phosphoinositide 3-kinase

SS: Salmonella-Shigella agar

STEC: Shiga toxin-producing *Escherichia coli*

Stx: Shiga toxin

ST: Heat stable enterotoxin

SGLT1: Sodium–glucose cotransport 1

Tir: Translocated intimin receptor

TLC: Thin Layer Chromatography

TLRs: Toll-like receptors

TPC: Total phenolic compounds

TTSS: Type III secretion system

TATFAR : Transatlantic Taskforce on AMR

UPEC: Uropathogenic *Escherichia coli*

UTI: Urinary tract infection

UNICEF: United Nations Children's Fund

US: Untited States

VRBG: Violet-Red-Bile-Glucose

W/V: Weight/Volume

WHO: World Health Organization

XLD: Xylose Lysine Desoxycholate agar

YPD: Yeast Extract-Peptone-Dextrose medium

INTRODUCTION

Introduction

1. Gastrointestinal Microbiology and enteric infections

This is a fascinating time for gastrointestinal Microbiology. Enteric infections (whose etiological agents are bacteria, viruses, fungi and protozoa) remain a significant public health problem. Worldwide, the infectious diarrhea is the second leading cause of childhood morbidity and mortality, and raises one of the most common human suffering with high morbidity and associated long term sequelae. On the other hand, recent culture-independent metagenomic methods and the next-generation sequencing techniques along with bioinformatics developments have revealed an unprecedented microbial biodiversity in the human intestine and "our other genome", the human microbiome.

Advances in the past 10 years in the fields of gastrointestinal physiology, innate immunity and enteric bacterial virulence mechanisms highlight the multifactorial nature of infectious diarrhea. Moreover, the recognition of *Helicobacter pylori* and its role in gastric disease by Warren and Marshall earned a Nobel Prize in 2005 (Warren, 2005). The discovery of bacterial secretion systems and translocate effectors has unlocked numerous mechanisms of pathogenesis (Hodges and Hetch, 2012). The finding that eukaryotic cells perceive and respond to bacteria through the expression of Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-containing (NOD)-like-receptors (NLRs), is a major step toward understanding molecular interactions between the host and gut microbiota or microbial pathogens (Ishii *et al.*, 2008). The discovery of *quorum sensing* as a means of inter-cell communication between bacterial populations (Anand and Griffiths, 2003; Waters and Basler, 2005) and even with host (Holm and Vikström, 2014) cells has provided insight into pathogenic mechanisms. The multiple-sited impact of the commensal gut microbiota on whole host metabolism (Burcelin, *et al.*, 2011; Arumugam, *et al.*, 2011), as well as its role in human health (Clemente, *et al.*, 2012), and many diseases states, including inflammatory and irritable bowel syndromes,

obesity (Spor, *et al.*, 2011), even brain disorders (Borre, *et al.*, 2014), is now accepted and will undoubtedly lead to new therapeutic strategies. And the recently discovered *Human Microbiome* (Huttenhower, *et al.*, 2012) will allow a better understanding of the role of this complex intestinal community in human health and disease.

On the clinical side, for the time being, we are witnessing the emergence of more virulent strains of enteric pathogens as well as the failure of antimicrobial treatments owing to the appearance of new bacterial antibiotic resistances and antibiotic overuse. The use of active *natural phytocompounds* with proved *antimicrobial* and *antiquorumsensing* activities could be significant for the *treatment* and *prevention* of gastro-enteric infections. The benefits of *probiotics* and *nutraceuticals* in the treatment of medical disorders are being studied nowadays (Gerritsen, *et al.,* 2011; Rauch and Lynch, 2011; Rahal *et* al., 2012). Furthermore, we are becoming increasingly aware of the presence of gut pathogens through the widespread outbreaks of foodborne diseases and the relevance of water and food safety controls in human health.

2. Global burden of infectious diarrhea

First estimates of the global incidence of childhood mortality and morbidity became available in the early 1980s. The most important infectious causes of childhood morbidity and mortality are pneumonia and diarrhea (as it has been already mentioned) (Fig. 1). 81% of deaths associated with pneumonia and 72% of those associated with diarrhea occur in the first 2-5 years of life (Walker *et al.,* 2013), suggesting that an increased emphasis on prevention and treatment in neonates and young children is crucial. Both diseases are associated with poverty, and child mortality from them is higher in low-income than in high-income countries and in populations in crisis situations. In particular, enteric infections (diarrheal disorders) were responsible for an estimated 4.6 million deaths worldwide from around 1 billion episodes of diarrhea annually in children. Despite the widespread use of oral rehydration therapies (ORT) and an increased

understanding of the pathogenesis of diarrhea, 1.9 million children less than five years of age still die from these illnesses every year according to recent WHO and UNICEF reports (WGO, 2012).

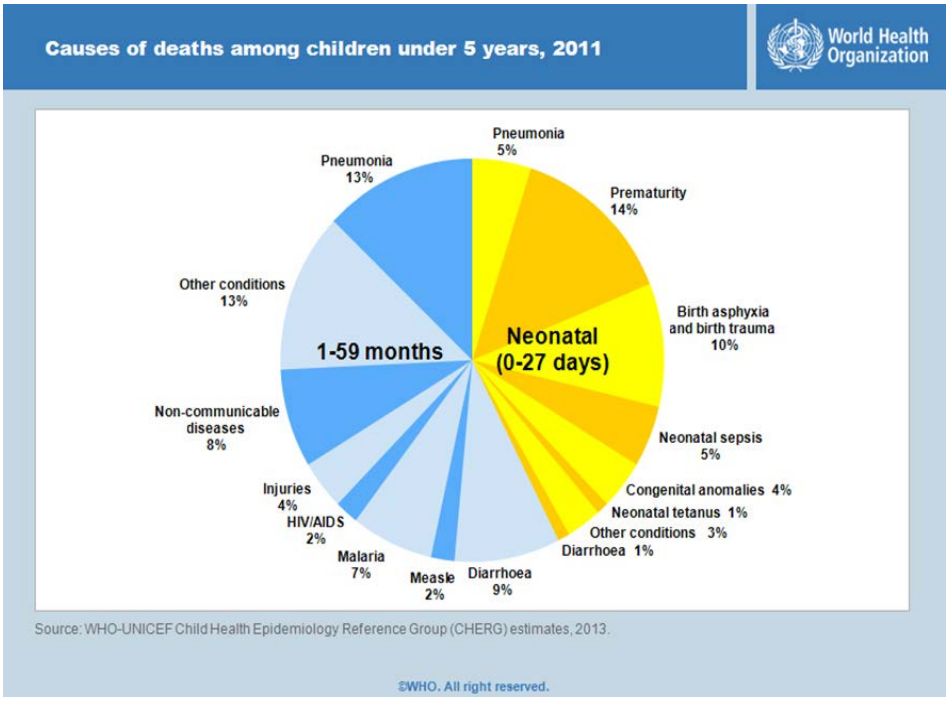

Figure 1. Report of the World Health Organization (WHO) 2011. Diagram of World Health Organization showing that diarrhea, the main symptom of enteric infections, represents the second leading cause of death among children less than five years worldwide in 2010. The figure represents 10 % of total death among children younger than five years of age.
Adapted from WHO-UNICEF Child Health Epidemiology Reference Group (CHERG) Estimates, 2013

In the developed world, deaths caused by diarrheal illness are rare, and the effects of these illnesses are often measured in financial terms (Thapar and Sanderson, 2004). It is usually a mild disease in most of European countries but it is still accompanied by large number of hospital admissions and not negligible number of deaths. Approximately 179 million cases of acute enteric infection occur in the United States each year (Wikswo and Hall, 2012). It is a major source of morbidity and hospitalization in children younger than five years, accounting for 200 000 hospital admissions with approximately 300 deaths and greater than 1.5 million visits to primary care

providers annually in US. These hospitalizations lead to $250 million in direct medical costs and $1 billion in indirect costs (Churgay and Aftab, 2012). It is estimated that 80% of children death in South Asia and Africa due to infectious diarrhea (WHO/UNICEF, 2009).

Enteric infection afflicts people of all ages around the world. Although etiological agents are not found in many cases, the nature of most acute diarrheal diseases is suggested by epidemiologic behaviour showing case clustering spread in families and occurrence among travellers.

Gastroenteritis is a clinically significant disease. Clinical studies in Fortaleza Brazil and other countries show clearly that patients with diarrhea persisting longer than 7-14 days demonstrate short fall in growth and cognitive development (Niehhaus, 2002; Nataro, 2013). The prevalence of infectious diarrhea is estimated at 1 to 1.5 episodes per person per year, and although most cases are self-limited, the severity of illness can vary markedly depending on characteristics of both the pathogen and the host. Management of each episode, therefore, varies based on cause, severity of disease, the host and the host's comorbid illnesses.

Prompt recognition, diagnosis and treatment of infectious diarrhea may have significant public health implications. The primary care action is crucial in early control of local outbreaks as well as in preventing secondary transmission, especially in health care workers, day care workers and food handlers. Stool testing for diagnosis of specific pathogens can abbreviate illness, reduce morbidity, decrease development of antimicrobial resistance through pathogen-directed treatment, and help identify and trace public health outbreaks. With the development of new tools of diagnosis essential information has been gained in our understanding of the etiology, pathogenesis, epidemiology and control of enteric infections. Therefore, global Cooperation is needed to limit the spectrum of the enteric infections.

3. Route of enteric infections transmission

Enteric infections outbreaks occur through the ingestion of contaminated food or water (fecal-oral route transmission) or by direct contact (person to person) (Bartlett, 1996).

4. Symptomatology of enteric infections

The anamnesis (clinical symptoms and clinical patient history) of enteric infections shows the main clinical symptomatology can include either gastrointestinal, systemic affectations or both successively (Table 1).

Table 1. Clinical symptoms of enteric infections

Gastrointestinal affectation	Systemic affectation
Diarrhea Nausea Vomiting Abdominal pain Fever	Hemolytic uremic syndrome (EHEC), meningitis, malabsorption disorders, cardio-neurological alterations, arthritis and hepatitis. Chronic sequelae

The main symptom of *gastrointestinal affectation* is ***diarrhea***. Other frequent symptoms are fever, nausea, vomiting and abdominal pain, depending on microbial pathogen (Table 1). *Systemic affectations* imply more serious syndromes including hemolytic uremic syndrome (as EHEC), meningitis, malabsorption disorders, cardio-neurological alterations, arthritis and hepatitis. Chronic sequeale are present in 2-3% of food borne and water borne enteric diseases.

Definitions of ***diarrhea*** include increases in volume or fluidity of stools, changes in consistency, and increased frequency of defecation. The measurement of stool fluid content is impractical and assessment of stool frequency is preferred for diagnostic purposes. WHO defines diarrhea as the "*passage of loose or watery stools at least three times in a 24 h period*",

but emphasizes the importance of change in stool consistency and appearance rather than frequency, and the usefulness of parental insight in deciding whether children have diarrhoea or not (WHO, 1995). Blood in stool could indicate an acute diarrheal illnesses or dysentery, irrespective of frequency (Baqui, 1991; WHO, 1994). Diarrheal disorders can further be divided into **acute** and **chronic**, allowing some categorization of causes and associated management. **Acute diarrheas**, the most usual form of diarrheal illness, have an abrupt onset, resolve within 14 days, and are mostly caused by infections. **Chronic diarrheas** last for at least 14 days. **Persistent diarrheas** usually arise secondary to infections in the presence of complications such as malnutrition; whereas the remaining chronic diarrheas are mainly due to congenital defects of digestion and absorption. Persistent and chronic diarrheas often overlap.

The diarrhea occurs as a result of the failure or ineffectiveness of the digestive tract to perform its absorption function, by different mechanisms that contribute to loss of fluids and electrolytes following microbial infections. These events are linked to specific toxins and other virulence factors (Viswanathan *et al*., 2009).

4.1 Classification of diarrhea

Diarrhea can be classified according to different criteria (Vu HT, 2012):

A. Duration:

- **Acute diarrhea:** When the symptoms usually continue less than two weeks and can be cured after three or four weeks.
- **Chronic diarrhea:** When the diarrhea continue more than 4 weeks.

B. Physiopathology:

- **Osmotic:** It can be caused by ingestion of non-absorbable solutes or by disease states that interfere with normal solute absorption.
- **Secretory:** It results from a disturbance in the balance between absorption (primarily via villous epithelial cells) and secretion (primarily via crypt cells).

- **Inflammatory:** It occurs when there is a damage to mucosal lining or brush border which leads to loss of protein rich fluids and decrease ability to absorb these lost fluids.
- **Penetrating:** It includes the bacteria that penetrate to the submucosa
- **Steatorrhea:** It is characterized by the presence of excess of fat in feces. It includes any process that affects the digestion and absorption of fat as coeliac disease and pancreatic insufficiency.
- **Motility disturbance/functional:** Intestinal motility disturbance can cause increase of the intestinal passages and decrease the time mucosal contact for absorption.
- **Antibiotic–associated diarrhea:** It results from an imbalance in the intestinal microbiota caused by antibiotic therapy. Another consequence of antibiotic therapy leading to diarrhea is overgrowth of potentially pathogenic organisms such as *Clostridium difficile*.

C. Etiopathogenesis:

- **Non infectious diarrhea** can be caused by some diseases as irritable bowel syndrome, hyperthyroidism, diabetes, colorectal cancer, etc.; as well as chemical compounds.

- **Infectious diarrhea,** produced by enteric pathogens including bacteria, viruses, fungi and protozoa.

5. Etiological agents of enteric infections

As it has been already mentioned, the etiological agents of enteric infections are bacteria, viruses, protozoa, fungi and helminthes (Table 2). Medically, diarrhea is subdivided into non-inflammatory and inflammatory, mainly based on the presence of leucocytes in feces. However, considering a more recent view of inflammation, it should be taken into account that all pathogens are able to induce an inflammatory response in the intestine due to the recognition of their Pathogen-Associated Molecular Pattern (PAMPs) detected by Pattern Recognition Receptors (PRRs), found in epithelial and immune cells.

Table 2. Etiological agents of infectious diarrhea

(modified from Mensa *et al.*, 2014)

	Secretory ("non-inflamatory") diarrhea[1]	"Inflammatory" diarrhea[2]
	It induced by enterotoxins that act directly on the secretory mechanisms in feces. It does not contain leucocytes cells	It can be mediated by cytotoxines and invasiveness inducing destruction of mucosal cells leading to the inflammatory response
Bacteria	*Staphylococcus aureus* *Bacillus. cereus* *Clostridium perfringens* *Escherichia coli* (enterotoxigenic*, enteropathogenic, enteroaggregative* and diffusely adhereing) *Vibrio cholerae* *Aeromonas hydrophila* *Salmonella* spp. no Typhi* *Tropheryma whipplei*	*Campylobacter jejuni* *Escherichia coli* (enterohemorragic and invasive) *Shigella* spp* *Vibrio* spp *Yersinia enterocolitica* *Clostridium difficile* *Plesiomonas shigelloides** *Klebsiella oxytoca* *Listeria monocytogenes* *Mycobacterium spp* *Laribacter hongkongeniss* Enterocolitis neutopenic
Viruses	Rotavirus* Norovirus* Astrovirus Adenovirus Hepatitis A	Cytomegalovirus Herpes simplex virus (HHV1) Enteric Adenovirus (serotypes 40, 41) Sapovirus Enterovirus Pestivirus Picornavirus Torovirus
Protozoa	*Cryptosporidium parvum* *Cyclospora cayetanensis* *Giardia lamblia* *Cytoisospora belli*	*Entamoeba histolytica** *Schistosoma mansoni* *Balantidium coli* *Dientamoeba fragilis*
Fungi and helminthes	Microsporidia	*Trichinella spiralis* *Strongyloides stercoralis*

(*) Microorganisms more frequently involved in travel-diarrhea

(1) Watery diarrhea with copious stools without blood. Afebrile course, except in cases of viral infection. Absence of leukocytes on microscopic examination of the feces. Usually due to increased fluid secretion into the ***small intestine*** due to the effect of neurotoxins or enterotoxins, or mucosal surface invasion caused by virus or protozoa.

(2) Frequent and little bulkystools, often with fever. There may be *tenesmus* and diarrhea may contain blood or mucus and leukocytes. It is generally due to inflammation of the lining of the ***colon*** by microbial invasion or effect of cytotoxins.

5.1 *Escherichia coli*

Escherichia coli is the predominant facultative anaerobe of the human colonic microbiota. *E. coli* is the type species of the genus *Escherichia*, which contains mostly motile gram-negative bacilli within the family Enterobacteriaceae (Nataro, 1998). The genus *Escherichia*, originates from the german pediatrician Theodor Escherich, who discovered the bacteria in 1885. The species *coli*, means "from the colon", which is the microorganism's natural habitat. Most strains of *E. coli* live and grow harmlessly in the gastrointestinal tract (commensal microbiota)of animals and humans (Ingerson-Mahar, 2011). Infections due to pathogenic *E. coli* may be limited to the mucosal surfaces or can be spread throughout the human body. These pathogenic strains of *E. coli* are broadly categorized as either diarrheagenic *E. coli* or extraintestinal pathogenic *E. coli* (ExPEC) (Wiles, *et al*., 2008).

Three general clinical syndromes result from infection with inherently pathogenic *E. coli* strains (Nataro, 1998; Ausina and Moreno, 2006):

5.1.1 Enteric infection

5.1.2 Urinary tract infection

5.1.3 Sepsis / neonatal Meningitis

5.1.1 Enteric infection by *Escherichia coli*

Escherichia coli gradually emerged as a cause of diarrhea. Diarrheagenic *E. coli* (DEC) cause infection by a variety of complex mechanisms. These include adherence, production of toxigenic mediators, invasion of the intestinal mucosa, and transportation of bacterial proteins into the host cells (Khan and Steiner, 2002). Diarrheagenic *E. coli* (DEC) is classified into six characterized categories (Fig. 2): enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adhering *E. coli* (DAEC) (Nataro and Kaper, 1998). Each type has unique features in its interaction with eukaryotic cells to induce diarrhea.

A. Enteropathogenic *E. coli* (EPEC) adheres to small bowel enterocytes and destroys the normal microvillar architecture inducing the characteristic attaching and effacing (A/E) lesion and cytoskeletal changes including the accumulation of polymerized actin beneath the adherent bacteria. EPEC as infectious agent may cause persistent and chronic diarrhea in children lasting longer than 1 week (Nataro, 2006).

B. Enterohaemorrhagic *E. coli* (EHEC) also induces the attaching and effacing (A/E) lesion, but in the colon. The distinguishing feature of EHEC is the elaboration of Shiga toxin (Stx), systemic malabsorption that leads to potentially life-threatening complications. EHEC causes bloody diarrhea (haemorrhagic colitis), non-bloody diarrhea and haemolytic uremic syndrome (HUS).

C. Enterotoxigenic *E. coli* (ETEC) adheres and colonizes the surface of small bowel mucosa and induce watery diarrhea by the secretion of heat-labile (LT) and/or heat-stable (ST) enterotoxins.

D. Enteroaggregative *E. coli* (EAEC) The basic strategy of EAEC infection is the colonization in the intestinal mucosa predominantly that of the colon, followed by secretion of enterotoxins and cytotoxins. Studies on human intestinal explants indicate that EAEC induces mild, but significant, mucosal damage. These effects are most severe in colonic sections. Mild inflammatory changes are observed in animals. Models and evidences indicate that some EAEC strains may be capable of limited invasion of the mucosal surface. The most dramatic histopathological finding in infected animal models is the presence of a thick layer of auto-aggregating bacteria adhering loosely to the mucosal surface.

E. Enteroinvasive *E. coli* (EIEC) invades the colonic epithelial cells followed by lyses of the endocytic vacuole, then, the bacteria move through the cell by nucleating actin microfilaments. The bacteria

may move laterally through the epithelium by direct cell-to-cell spread or may exit and re-enter the baso-lateral plasma membrane.

F. Diffusely adhering *E. coli* (DAEC) is characterized by the development of finger like projection of epithelial cells, which wrap around the bacteria but without complete internalization. Then activates signal transduction cascades that are necessary to induce diarrhea (Kaper, Nataro *et al*., 2004). The complete pathogenesis of DAEC to induce diarrhea is not completely understood yet.

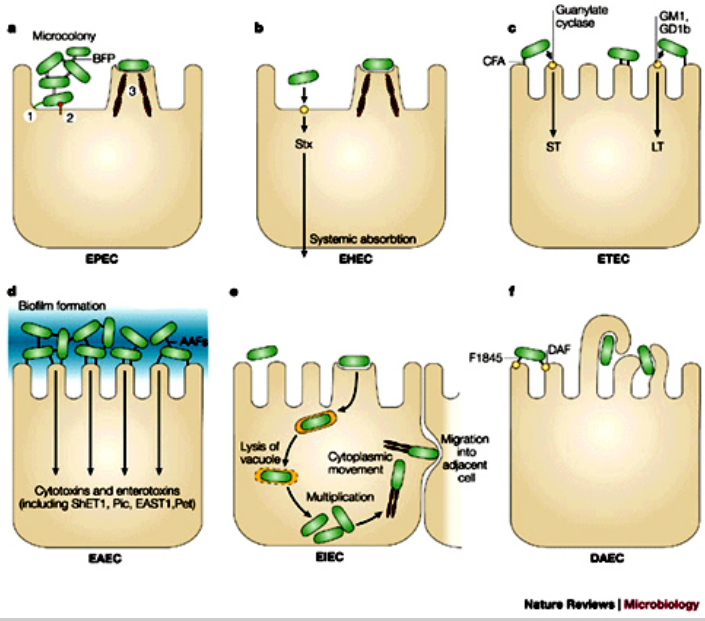

Figure 2. Categories of Diarrheagenic *Escherichia coli*.
Diarrheagenic *Escherichia coli* are classified in six categories. Each category has unique feature in its interaction with eukaryotic cells to induce diarrhea.
(a) Enteropathogenic *E. coli* (EPEC) (b) Enterohaemorrhagic *E. coli* (EHEC)
(c) Enterotoxigenic *E. coli* (ETEC) (d) Enteroaggregative *E. coli* (EAEC)
e) Enteroinvasive *E. coli* (EIEC) (f) Diffusely adhering *E. coli* (DAEC).

AAF: aggregative adherence fimbriae. BFP: bundle-forming pilus. CFA: colonization factor antigen. DAF: decay-accelerating factor. EAST1: enteroaggregative *E. coli* ST1. LT: heat-labile enterotoxin. ShET1: *Shigella* enterotoxin 1. ST: heat-stable enterotoxin.
Adapted from (Kaper, Nataro, *et al*., 2004)

5.1.2 Urinary tract infection

Urinary tract infections (UTIs) are very severe health problems worldwide. UTIs can be classified depending on the part of urinary tract. Lower urinary

tract infection is called cystitis and represents 95%, and the upper urinary tract infection is called acute pyelonephritis which is a complication that involves kidneys. 80 % of UTIs are caused by UPEC (Zalewska-Piatek, 2011).

5.1.3 Sepsis/neonatal Meningitis

Meningitis is defined as an inflammation of the membrane that surrounds the brain and the spinal cord. Meningitis symptoms are characterized by fever, neck stiffness, and mental status changes (Chavez-Bueno and McCracken, 2005). *Escherichia coli* K1 is the most prominent Gram-negative bacterium that causes meningitis in neonates with rates of fatality ranging from 5 to 30% of infected infants (Mittal, *et al*., 2011).

5.2 Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* (EPEC) infection is a major cause of infantile diarrhea in the developing world. Using a type-three secretion system the bacterial effector proteins are transferred to the host cell cytosol and affect multiple physiological functions, ultimately leading to diarrheal disease. EPEC attach to cells and form actin-rich structures called **pedestals**, which contain many proteins that play unknown functions during pedestal formation (Nieto-Pelegrin 2014). Injected Tir binds to Intimin on the bacterial surface and promotes attaching of bacteria intimately and effacing lesion formation (loss of absorptive microvilli), leading to assembly of cytoskeleton intracellular actin on the cellular surface. Rearrangements of the actin cytoskeleton compose a pedestal-like structure where bacterium tightly cups the cells, leading to degeneration of brush border microvilli (Figs. 4). Some translocated effectors that alter the transepithelial resistance include *E. coli* secreted protein F (EspF), Mitochonderial-associated protein (Map) and *E. coli* secreted protein G (EspG) (EPEC secreted protein G). EspF decreases both sodium-hydrogen exchanger 3 (NHE3) and sodium–glucose cotransport 1 (SGLT1) activity in addition to disruption of tight junctions. Map acts along with EspF to decrease SGLT1 activity and tight junction function. EspG1 and EspG2 decrease Cl^-/OH^-

exchange by down regulated in adenoma (DRA) exchanger and also impair tight junctions. The combined activities of these proteins and other effectors on ion transport alter the osmotic gradient leading to subsequent movement of water into the intestinal lumen causing diarrhea (Fig. 3) (Viswanathan, 2009).

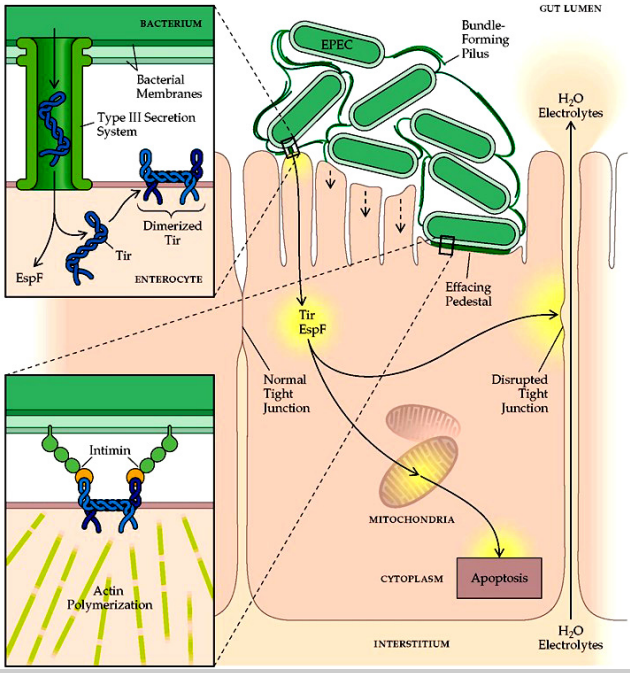

Figure 3. Schematic representation of the mechanism of action of enteropathogenic *Escherichia coli* infection. After initial adherence, EPEC causes the localized effacement of microvilli and intimately attaches to the host cell surface forming the characteristic attaching and effacement (A/E) lesion. EPEC secretes the virulence factor Tir (translocated intimin receptor) through a Type-III Secretion System (T3SS) into the cytoplasm of the target cell. Tir is then serves as the receptor for the bacterial surface protein intimin in the plasma membrane of the host cell. The binding induces a clustering in the membrane that activates the cellular machinery for actin polymerization to form the pedestal on which EPEC sits. EspF causes the loss of intestinal barrier function through disruption of tight junctions and induces host cell death by apoptosis.
Adapted from http://what-when-how.com/acp-medicine/infections-due-to-*Escherichia-coli*-and-other-enteric-gram-negative-bacilli-part-1/

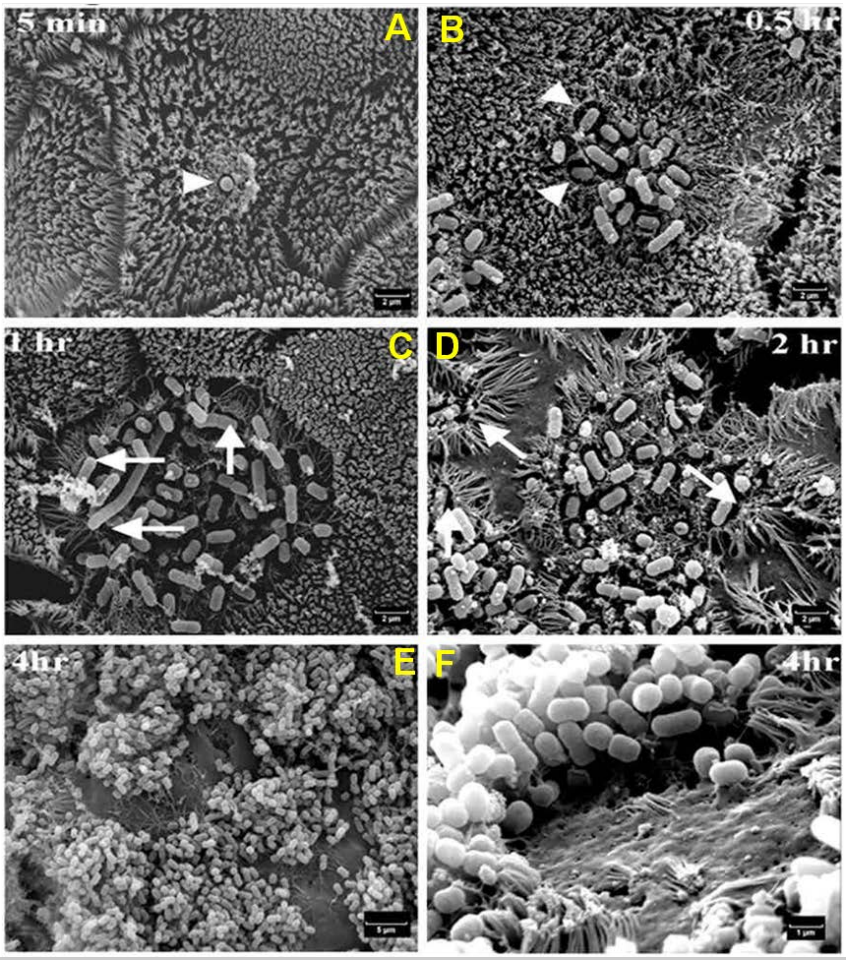

Figure 4. Kinetics of EPEC-induced microvilli damage. Scanning electron micrograph (SEM) showing **(A)** One attached bacterium to Caco 2 cells after 5 min infection. **(B)** Small microcolonies are formed after 30 min of EPEC infection. **(C)** Bacteria appearing to sink (arrowheads) into the brush border. **(D)** Importantly, loss of microvilli from around the infection site occurs at later times, with microvilli appearing to be pulled toward the bacteria (arrows). **(E and F)** Extensive loss of microvilli from around the infection site by 2 h with practically all microvilli lost by 4 h.
Adapted from (Dean *et al.*, 2006)

6. Outbreaks of enteric infections worldwide

In the period of 1950-54, enteric infections constituted the main cause of death among infants between 4 weeks and 1 year of age, in Venezuela. It represented the 52% of total death at this age; followed by respiratory tract

diseases that represented 22% of total death, then the infectious diseases that represented more than 6%.

Among infants under 5 years of age, in the same period, gastroenteritis was the major cause of death and represented 36% of total death followed by pneumonia which represented 15% of total death (Curiel and Ochoa, 1959).

Another example of the devastation that enteric infections can took place in the city of Surat (Gujarat State, India), between the 29 of October and the 3 of December in 1965, a total of 418 persons were hospitalized, of which 344 were children under two years of age. It was reported that 34 out of 81 examined specimens contained *E. coli* O_{86}:B_7 and one sample contained O_{126}:B_{16}. The route of transmission could not be determined (Pal, *et al*., 1969).

In 1963, the Newfoundland public health laboratories recorded an epidemic infantile gastroenteritis that led to 100 death cases of a total of 1071 affected children. 76% of this infantile enteric infection outbreak was due to enteropathogenic *E. coli* strain O_{111}:B_4 infection. This strain was isolated from aproximadtely 500 samples (Severs, *et al*., 1966)

During the period of 1995 to 2000 in European countries the enteric infections outbreaks were estimated by European surveillance systems (Table 3), including diagnostic reference laboratories, local public health staff, food inspectorates, physicians and systematic national surveillance. The gastroenteritis outbreaks were calculated in each country annually the outbreaks by national population. The data are shown in table 3.

Table 3. Outbreaks of enteric infections in European countries during 1995-2000 (Lopman, et al., 2003)

Country	Numbers of outbreaks / million in population
Denmark	37
England and Wales	1643
Finland	125
Italy	2
Slovenia	45
Spain	236
Sweden	856
Netherland	301

The most frequently reported etiological pathogens for those enteric infections outbreaks were viral agents.

During 2001-2002, the Centers for Disease Control and Prevention (CDC) reported 31 outbreaks of gastroenteritis that occured in 19 states of United State due to drinking polluted water by sewage (waterborne disease outbreak). These outbreaks caused 7 deaths, 51 hospitalizations from 1020 affected people. During the period from 30th of May 2004 to 8th of September 2004, was reported an outbreak of gastroenteritis between resident and visitors to South Bass Island, Ohio. Among 1450 total affected people, 21 people (1%) were hospitalized and no deaths. The symptoms reported by patients were diarrhea (83%), abdominal crambs (80%), nausea (77%), vomiting (50%), fever (45%) and bloody diarrhea (5%). The etiological agents of the outbreak were *Campylobacter jejuni*, norovirus, *Giardia intestinalis*, and *Salmonella* Typhimurium; that were identified in 16,

9, 3, and 1 people, respectively of a total of 1 450 persons (O'Reilly, *et al*. 2007).

Furthermore, it was reported by the American National Outbreaks Reporting System (NORS), the first illness onset of person-to-person contact of acute gastroenteritis during January 2009 up to December 2010 in United States (Fig. 5).

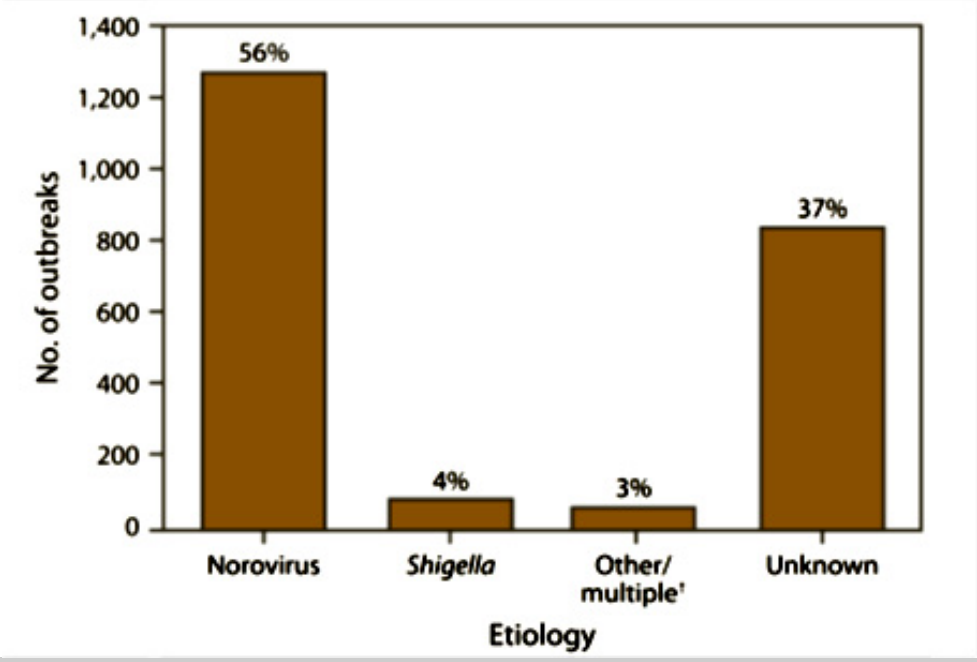

Figure 5. Numbers and percentages of outbreaks of acute gastroenteritis transmitted by person-to-person contact. National Outbreak Reporting System, United States, 2009–2010 reported that norovirus is the most common cause of gastroenteritis outbreaks while *Shigella* is the second common cause.

Norovirus outbreaks represented the highest number and percentage 1 270 (56%) etiology which were reported. *Shigella* was the second most commonly reported etiology, although it accounted for only 86 (4%) of all reported outbreaks. Other single-etiology outbreaks were suspected or confirmed to be caused by *Salmonella* (n=16), rotavirus (n=10), Shiga toxin-producing *E. coli* (STEC) (n=11), *Giardia lamblia* (n=5), *Cryptosporidium spp.* (n=9), *Clostridium difficile* (n=1) (Wikswo, 2012).

Furthermore, in Spain, it was determined the size and epidemiological characteristics of foodborne outbreaks due to Norovirus in Catalonia between October 2004 and October 2005. They observed that from 181 outbreaks reported during the study period, 72 were caused by *Salmonella* and 30 by norovirus (NoV); the incidence rates were 14.5 and 9.9 per 100 000 person-year, respectively (Martinez, *et al*., 2008).

One of the most recent and important outbreaks happened in Germany in 2011, where *E. coli* O_{104}:H_4 strain caused a large outbreak of hemolytic uremic syndrome (HUS) and bloody diarrhea. The isolated strain possesses new combination of pathogenic features that combines some of the virulence factors of both EHEC (Stx but not the type III secretion and Tir/Intimin system) and EAEC (especially the adherence mechanisms) (Scheutz, Nielsen *et al*., 2011). The outbreak affected 4 000 people including 900 with HUS, which resulted in 54 deaths (Karch, *et al*., 2012).

7. Treatments of enteric infections

Enteric infections remain a common health problem worldwide. **Oral Rehydration Therapy (ORT)** and nutritional support should be performed before any other therapeutic agent. The most suitable treatment for viral gastroenteritis and parasitic gastroenteritis is ORT which includes rehydration and maintenance of fluids with oral rehydration solutions beside the appropriate nutrition (King, *et al*., 2003; Santosham, *et al*., 2010). Before widespread use of oral rehydration solutions, the treatment of diarrhea was restricted to intravenous fluid replacement that required patient visits to a health care facility to access the appropriate equipment. The new methods for prevention, management and treatment of diarrhea, including an improved oral rehydration formulation, zinc supplementation and rotavirus vaccines make now the time to revitalise efforts to reduce the diarrhea mortality worldwide (Santosham, *et al*., 2010).

Antibiotics are required for the treatment of bacterial gastroenteritis in those patients suffering from severe complications of gastroenteritis, including dissemination of the disease or sepsis. Generally, antimicrobial

treatment tends to speed up the clinical resolution of diarrhea, prevents the progression of the disease and reduces the severity of associated symptoms as fever, vomiting and abdominal pain (Diniz-Santos, *et al*., 2006).

Antibiotics induce bacterial cell death based on the bacterial cell function that can be inhibited by the drug-target interaction. The mechanism includes interference with cell wall synthesis, interference with nucleic acid synthesis, inhibition of protein synthesis, metabolic pathway inhibition and alterations of cell membrane (Kohanski, 2010).

The use of **probiotics** has gained an important attention in recent years for their potential use as anti-gastroenteritis treatments. Probiotics are defined as live microorganisms that are administered in the adequate amounts to induce health benefits to the host. These benefits include: prevention of pathogen proliferation and function, stimulation of the host´s immune function and promotion of mucosal barrier integrity. However, their beneficial mechanisms of action are not completely known and further studies are needed (Britton and Versalovic, 2008).

7.1 Treatment of enteric infections produced by *Escherichia coli*

Diarrhea that is caused by enterotoxigenic *E. coli*, enteropathogenic *E. coli*, enteroaggregative *E. coli* and diffusely adhering *E. coli* can be treated by antibiotics to quickly relieve symptoms, such as 2nd or 3rd generation cephalosporin antibiotics, aztreonam beta-lactams associated with beta-lactamase inhibitors (clavulanic acid, sulbactam, tazobactam). But the more severe diarrhea that is caused by enteroinvasive *E. coli* and enterohaemorragic *E. coli* can be treated by Ciprofloxacine 500 mg/12h oral, 3-5 days or with other alternative treatment such as clotrimoxazol 160-800 mg/12h oral for 5 days, or azithromycin 500 mg/day for 3-5 days (Vu HT *et al*., 2012).

7.2 Problems of the use of antibiotics

Currently, there are many synthetic antibiotics for treatment of various diseases caused by microorganisms. Some of these microorganisms have become resistant to antibiotics, rendering them ineffective. The resistance to antimicrobials is due to several causes: genetic plasticity of the microorganisms, unnecessary and inappropriate use, including inadequate dosing, low quality drugs, and increased mobility of the world population (Bisi-Johnson, 2012). A great number of bacterial isolates are showing resistant to practically all available therapeutic agents. Therefore, the problem has been building up over decades, and today many common and life-threatening infections are becoming difficult or even impossible to treat, sometimes turning a common infection into a life-threatening one (WHO, 2012).

The CDC estimates that, more than two million people get sick every year by antibiotic-resistant infections in United States, with at least 23,000 deaths (Frieden, 2013). Pathogenic bacteria show an enormous array of resistence mechanisms against antibiotics. The mechanisms of microbial resistance include:

1- Decreased uptake of the antimicrobial: reducing entry of the antimicrobial agent by alteration of cell permeability; or expulsion of the antimicrobial from the cell, via general or specific efflux pumps.

2- Conversion of active drugs to inactive derivatives by enzyme(s) produced by resistant organisms.

2- Synthesis of alternative enzymes for the enzymes that the antimicrobial agents inhibit.

3- Modification and mutation of the antimicrobial targets.

4- Overproduction of the antimicrobial agent targets.

7.2.1 Antimicrobial resistance of *Escherichia coli*

It is reported that diarrheagenic *E. coli* (DAE) are important pathogens that have shown an increased resistance to many antibiotics. In 2008, at University of Uyo Teaching Hospital and University of Uyo Health Center in

Auna General Hospital, Akwa Ibom State, Nigeria, 75.4 % of isolated DAE showed high antibiotic resistance against tetracycline, 73.9 % were resistant to ampicillin, 68.1 % were resistant to gentamycin, and 46.4 % showed moderate resistance to chloramphenicol and 43.5 % were resistant to cephalothine (Akinjogunla, 2009).

A recent study showed that most of DAE isolated from fecal samples of children under five years of age, were resistant to ampicillin, erythromycin, nalidixic acid and cephalexin. The sensitivity pattern against azithromicin, cotrimoxazole and ciprofloxacin was moderately resistant, while that of mecillinam, ceftriaxone and gentamicin was quite satisfactory (Roy, *et al*., 2013).

Likewise, in USA *Escherichia coli* was isolated from human and food animal samples during 1950-2002 to determine the changes in antimicrobial drug resistance. A total of 1 729 *E. coli* isolates were tested for susceptibility to 15 antimicrobial drugs. *E. coli* isolates showed a significant increase of resistance for ampicillin, sulphonamide, tetracycline and gentamicin. It was concluded that multidrug resistance in *E. coli* increased from 7.2 % during 1950s to 63.6 % during 2000s (Tadesse, *et al*., 2012).

Moreover, another study identified the degree of *E. coli* resistance to amoxicillin/clavulanic acid, ceftriaxone, gentamicin, tetracycline, ciprofloxacin, and sulfamethoxazole/trimethoprim. *E. coli* strains (9 enterotoxigenic *E. coli* (ETEC) and 5 enteropathogenic *E. coli* (EPEC) were isolated from the cloacae of chickens that showed resistance to at least one of the commonly used antibacterial agents. They observed that, all 9 ETEC were resistant to tetracycline, 3 were resistant to sulfamethoxazole/trimethoprim and 1 to ciprofloxacin. All 5 EPEC were resistant to tetracycline, 4 were resistant to gentamicin, 4 were resistant to sulfamethoxazole/trimethoprim and 3 to ciprofloxacin. Therefore, this study confirm that *E. coli* has antibacterial resistance that can be a health hazard to human since chicken and eggs are very common foods (El-Rami, *et al*., 2012).

All mentioned studies prove that there is a continuing need to search for new antibacterial agents to overcome antibiotic resistance. Therefore we will try to shed light on the current efforts for saving life and to specifically overcome the bacterial resistance problem.

7.3 New approaches for saving lifes

Several national, regional and global initiatives are engaged in tackling Anti-microbial Resistance (AMR), and more specifically antibiotic resistance. In 1998, the World Health Assembly of the WHO urged member states to promote suitable directions to face antibiotic resistance. In 2000, WHO considered the rise of AMR a universal crisis, and in 2001 it started to release its first universal strategy for its containment. In 2009, a Transatlantic Taskforce on AMR (TATFAR) was organized to increase EU and US collaboration, and it has focused on improving the pipeline for new drugs. In the same year, the Swedish government promoted the issue during its EU presidency, particularly in relation to drug development.

In 2010, the European Commission launched a Joint Programme initiative on AMR to enhance a more cohesive approach to research and integrate relevant scientific fields to create a shared vision. This was followed by a 12-point EU Commission in 2011 ‘Action Plan against the Rising Threats from Antimicrobial Resistance. Similarly, 2011 saw a six-point policy package from WHO. The 2011 World Health Day was based on the premise of ‘no action today, no cure tomorrow’. In 2012, the first meeting of medical societies in India resulted in a roadmap to tackle resistance in India. Moreover in 2013, the United Kingdom published a roadmap. Furthermore, a number of other initiatives have been developed (Merrett, 2013). Briefly, there is an important direction that can help us to overcome the problem of microbial resistance is to dedicate our efforts toward scientific research in order to discover new antimicrobial agents.

The integrated Global Action Plan for the Prevention and control of Pneumonia and Diarrhea (GAPPD) proposed three sides of a framework to reduce the diarrheal morbidity and mortality (Fig. 6). The first side of

framework is to protect children by establishing good health practices from birth, including breastfeeding for six months, complementary feeding and vitamin A supplementation. The second side of the framework is to prevent diarrhea illness in children with vaccines, using safe drinking water and sanitation, washing hands with soap, reducing household air pollution. The third framework is to treat children who are ill with appropriate treatments, improved care seeking and referral, case management at the health facility and community level, supplies with low-osmolarity ORS, zinc, antibiotics and oxygen and continued feeding (WHO/UNICEF, 2013).

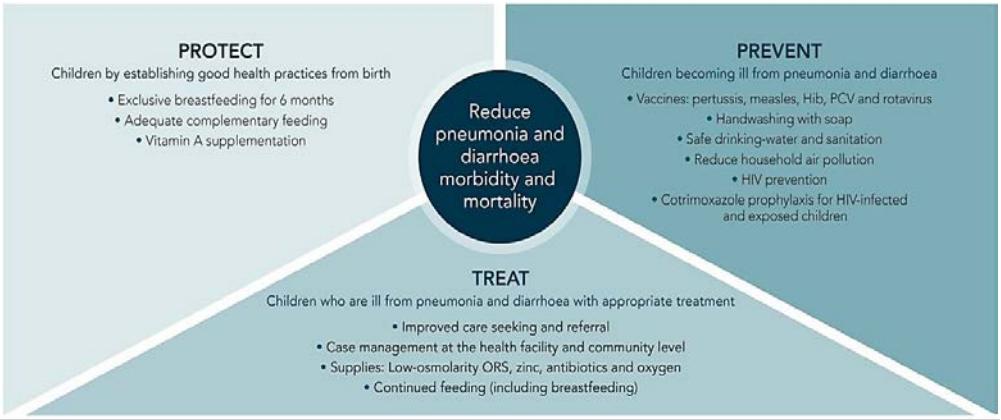

Figure 6. Protect, prevent and treat framework. The integrated Global Action Plan for the Prevention and control of Pneumonia and Diarrhea (GAPPD) proposed three sides of a framework to reduce the diarrheal morbidity and mortality. Adapted from (WHO/UNICEF, 2013)

7.4 Global use of medicinal plants

Medicinal plants have been employed to treat diseases since ancient times, and their use had remained an integral part of many cultures worldwide. From the total number of known plant species on Earth (estimated as 250 000–500 000), only a small fraction have been investigated for their antimicrobial activity and only 1–10% of plants are used. Among 109 new antibacterial drugs approved during the period 1981–2006, 69% originated from natural products, and 21% of antifungal drugs were natural derivatives or compounds simulating natural products (Savoia, 2012).

The WHO estimates that 80% of people living in developing countries utilize traditional medicine. Developed countries also use medicinal plants.

In Germany, 600-700 plant-based medicines are plentiful for prescription and they can be prescribed by 70% of German physician (Ehrlich, 2011).

In the last two decades in United States, there is a public dissatisfaction with the cost of medical prescriptions, which led to an interest in returning to use herbal medicine (Motaleb, *et al*., 2011). The use of medicinal plants is widespread in China, India, Japan, Pakistan, Sri Lanka and Thailand. In China approximately 40% of the total medicinal consumption is attributed to traditional medicines. In Japan, herbal medicinal preparations are more in demand than pharmaceutical preparations.

In Europe, nearly 1 500 species of medicinal and aromatic plants are widely used in Albania, Bulgaria, Croatia, France, Germany, Hungary, Poland, Spain, Turkey, and the United Kingdom (Hoareau,1999). In 19^{Th} century, medicinal plants were the main source to sustain health. In 1828, the German chemist Friedrich Wohler tried to synthesize the ammonium cyanate from silver cyanide and ammonium chloride, but accidently synthesized urea. Urea was the first synthetic organic compound, and 100 years following the Wohler`s discovery, the phytomedicine was forgotten due to the synthetic compound revolution. Then, in 1980s started a resurgence of interest in the use of plants as a source of bioactive phytocompounds.

Much more attention has been recently paid to extracts and biologically active compounds that were isolated from plant species. This interest in the use of phytocompounds was due to the emergence of side effects of synthetic compounds and the need to find new compounds, including new antibiotics to treat multiresistant bacteria. Therefore, plants can offer a new source of antibacterial agents (Ahmed, 2006).

It is well known that phytochemical constituents and secondary metabolites are responsible for the biological activity of plants. The majority of these secondary metabolites work as a mechanism of resistance against predators and pathogens (Zahin, *et al*., 2010). The major groups of phytocompounds and bioactive constituents that work as antimicrobial

agents are phenols, polyphenols, quinones, tannins, coumarines, flavonols, flavone, flavonoids, terpenoids, essential oils and alkaloids (Cowan, 1999).

7.5 Plants as anti-enteric infections agents

Many medicinal plants are used as antibacterial agents. It has been reported that *Allium sativum* (garlic) and *Zingiber officinale* (ginger) possess antibacterial activity against multi-drug resistant pathogens and they can be used for prevention of drug resistant microbial diseases. Their antibacterial activity were tested against five Gram-negative (*E. coli*, *Enterobacter* spp., *P. aeruginosa*, *Proteus* spp., *Klebsiella* spp.) and two Gram-positive (*S. aureus* and *Bacillus* spp.) multidrug resistant bacteria isolates (Karuppiah and Rajaram, 2012). *Allium sativum* offers a hope for developing alternative drugs and a good potential as antitubercular drug (Gupta, *et al*., 1999; Dini, *et al*., 2011).

Another study investigated the antibacterial activity of the aqueous garlic extract, apple vinegar and apple vinegar-garlic extract combination against 5 Gram-positive and 9 Gram-negative bacteria. It showed that aqueous extract of garlic and apple vinager-garlic extract combination possess wide spectrum activity against all tested bacterial isolates while the apple vinager possess slight antibacterial activity (Hindi, 2013).

Methanolic extracts of *Tussilago farfara*, *Equisetum arvense*, *Sambucus nigra*, *Aesculus hippocastanum* and *Taraxacum officinale* were investigated for antibacterial activity against *E. coli* strains resistant to ampicillin and chloramphenicol (Hleba, *et al*., 2013). It was studied, on one side, 10 *E. coli* strains isolated from milk from conventional breeding of cow, and on the other, 10 *E. coli* isolated from ecological breeding. All medicinal methanolic extracts possess antibacterial activity against 10 *E. coli* that were isolated from the conventional breeding of cow. In addition, the other 10 *E. coli* that were isolated from ecological breeding of mare (adult female of horse) were susceptible to *Tussilago farfara* medicinal plants (Hleba, *et al*., 2013).

Likewise, the antibacterial activity of commercial fruit juices as pineapple, blueberry, pink pear, sweet aji, corozo, starfruit, Santander's medlar, sour

grape, Isabella grape, and wild blackberry and the ethanolic extracts of bryophytes, two mosses (*Sphagnum magellanicum* and *Hypnum amabile*) and two liverworts (*Metzgeria decipiens* and *Trichocolea tomentosa*) was assessed against *B. subtilis*, *S. aureus*, *P. aeruginosa* and *E. coli* and compared to ampicillin and clindamycin antibiotics. This study showed that the juices of blueberry, sour grape, Isabella grape and wild blackberry and all ethanolic extracts of bryophytes were active against at least two of the evaluated bacteria with different percentage of inhibition (Rodríguez-Rodríguez, *et al*., 2012).

Essential oils of *Rosmarinus officinalis* and *Salvia officinalis* showed antibacterial activity against *Escherichia coli*, *Salmonella Enterica* ser.Typhi, *S. enterica* ser. Enteritidis, and *Shigella sonnei* (Bozin, *et al*. 2007). The ethanolic extract of *R. officinalis* was active against Methicillin- Resistant *S. aureus* (MRSA) (Jarrar, *et al*., 2010). Moreover, essential oils from *Origanum vulgare* were bactericidal against *S. aureus*, *B. subtlis*, *L. monocytogenes*, *C. perfringens*, *C. botulinum*, *E. coli*, *P. aeruginosa*, *S. enterica* ser. Typhimurium, *C. jejuni* and *V. cholerae*, with minimum inhibitory concentrations (MIC) ranging from 0.1 to 4 mg/mL (Derwish, *et al*., 2010).

Furthermore, the antimicrobial activity of crude methanol and aqueous extracts of the leaves of *Syzygium cumini* was evaluated against Gram-negative bacteria such as *S.* ser. Enteritidis, *S.* ser. Typhi *A*, *S.* ser. Paratyphi A, *S.* ser. Paratyphi B, *P. aeruginosa* and *E. coli;* and Gram-positive bacteria *B. subtilis*, and *S. aureus*. The results of this study showed that both extracts inhibited the tested Gram-negative and Gram-positive bacteria, but the methanol extract was more potent than the aqueous extract (Gowri and Vasantha, 2010). As well, ethanol extract of cinnamon bark (*Cinnamomum zeylanicum*) and ajowan fruits (*Trachyspermum ammi*) showed antibacterial activity against *Pseudomonas* spp., while acetone extract of spices displayed highest activity against *E. coli*. This study suggests that the ethanol extract of *C. zeylanicum* and *T. ammi* revealed a

significant scope to develop a novel broad spectrum of antibacterial herbal formulations (Usha, *et al*., 2012).

7.5.1 *Hibiscus sabdariffa*

Hibiscus sabdariffa belongs to the *Malvaceae* family. The genus *Hibiscus* includes from herbaceous annuals or perennials to small shrubs and small trees. The leaves are alternate, simple, ovate tolanceolate, often with serratedor lobedmargin. The flowersare large, conspicuous, trumpet-shaped, with five petals, white tones of roses, red, purple or yellow, orange, 4-15 cm in size. The fruit is a capsule containing several seeds in each locule. They are typical in subtropical and tropical areas around the world, with an origin centered mainly in South East Asia. It was introduced and cultivated since ancient times for use as ornamental, food and medicinal plant.

The world's largest producers are China and Thailand. The production is lower in Mexico, Egypt, Senegal, Tanzania, Mali and Jamaica, and the best quality in the world comes from Sudan, though sparingly. In the delta of the Gangesin India, where it is called *mesta*, is grown for its fiber plant stem, very tough. In Brazil it is grown organically in the area of Minas Geraisas Flor dos Hibiscus, where they have developed a variety of organic products. The plant grows as a home garden plant in the tropical countries, including Caribbean, México, Central America, India, Africa, Brazil, Australia, Hawaii, Florida and Phillipines. In Sudan, it is a major crop of export (Mahadevan, *et al*., 2009). In English, it is called Roselle, Sorrel, Red sorrel, Jamaica sorrel, Indian sorrel, Guinea sorrel, Sour-sour (Mahadevan, *et al*., 2009).

The deep red sour drink is prepared from dry calyces and is called **roselle** (a name for the flower) or **rosella** (Australia), **karkadeh** in Egypt, Sudan, Italy and Russia, **agua de Jamaica** or **flor de Jamaica** in Latin America, **Arhul ka phool** in India, **Chai Kujarat** in Iraq, **Chai Torsh** in Iran, **gumamela** in the Philippines, **bissap**, **tsoborodo** or **wonjo** in West Africa, **sorrel** in Jamaica, Barbados and Trinidad and Tobago, **zobo** juice in Nigeria, **red sorrel** in the wider Caribbean, and other names in other

regions, including the U.S., where it is sometimes known as simply **Jamaica** (Omemu, *et al*., 2005)**.**

The aqueous brew has a tart, cranberry-like flavor, and sugar is often added to sweeten the beverage. It contains vitamin C and minerals and is used traditionally as a mild medicine. In west Sudan a white hibiscus flower is favored for its bitter taste and is customarily served to guests. Hibiscus brew contains 15-30% organic acids, including citric acid, malic acid, and tartaric acid. It also contains acidic polysaccharides and flavonoid glycosides, such as cyanidin and delphinidin, which give it its characteristic deep red colour (Ali, *et al*., 2005).

7.5.1.1 Medicinal uses of Hibiscus sabdariffa

H. sabdariffa calyces extract has different biological potential activities (Fig. 7). It can be used as an anti-obesity agent as was showed in an *in vivo* study using rats that were fed with basal diet and in parallel the same basal diet supplemented with *H. sabdariffa* extract. The rats were fed with basal diet supplemented with *H. sabdariffa* extract showed decrease in weight gain and in food consumption (Carvajal-Zarrabal, *et al.*, 2009).

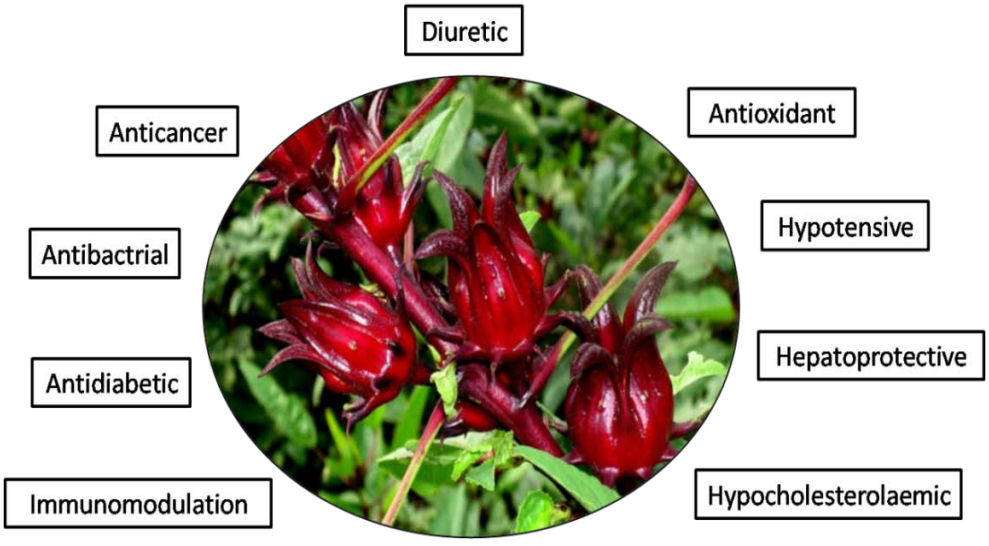

Figure 7. *Hibiscus sabdariffa* plant and its biological potential activities. (Modified from Da-Costa-Rocha 2014).

It has been shown that the aqueous extract of *H. sabdariffa* inhibits adipocyte differentiation through the modulation of the PI3-K/Akt and ERK

pathway that plays important roles during adipogenesis (Kim, *et al*., 2007). Also the aqueous and ethanol extracts can be used as food preservatives. In fact both extracts showed a dose dependent inhibitory effect against food spoilage of ground beef and apple juice by *S.* Typhimurium DT104, *E. coli* O157:H7, *L. monocytogenes*, *S. aureus* and *B. cereus* (Chao and Yin, 2009). Likewise, it inhibits the growth of MRSA, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii* (Liu, *et al*., 2005).

Another study reported that *H. sabdariffa* polyphenol extract reduced kidney mass in streptozotocin induced diabetic nephropathy in rats. Additionally it reduced triglyceride, total cholesterol and LDL serum content in streptozotocin treated rats (Lee, *et al*., 2009). Moreover it could be used as anticancer agent acting on the p53 signaling and p38 MAPK/FasL cascade pathway. Interestingly, it was shown to induce apoptosis in human gastric carcinoma cells (Lin, Huang, *et al*., 2005). Several *H. sabdariffa* extracts displayed different activities against atherosclerosis, diabetes, liver disease and other metabolic syndrome (Lin, Chen, *et al*., 2011). It has been suggested its use as protective agent against acetaminophen induced liver cell death mediated by oxidative stress (Liu, Wang, *et al*., 2010).

The red sour tea is antihypertensive and cardioprotective in type II diabetic patient with mild hypertension (Mozaffari-Khosravi, *et al*., 2009). Moreover, there is a report that supports using *H. sabdariffa* as an antihypertensive agent. Whereas crude methanolic extract of *H. sabdariffa* induced relaxation (vasodilator) of isolated aortas from spontaneously hypertensive rats. The relaxation effect acts via endothelium-dependent and independent vasodilator pathways through activation of endothelium-derived nitric oxide/cGMP-relaxant pathway (Ajay, *et al*., 2007).

H. sabdariffa ethanol and aqueous extracts are antipyretics in experimental animals (Reanmongkol and Itharat, 2007).

Due to important problem of children deaths in the developing countries, it is interesting to investigate the use of natural products as antibacterial agents.

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OBJECTIVES

OBJECTIVES

Objectives

Enteric infections, which are food and water borne diseases, are a serious public health problem worldwide. Microorganisms are the main cause of enteric infections; therefore their control may significantly reduce the food and water-borne enteric infections outbreaks. The public is becoming increasingly conscious of problems with the over prescription and misuse of traditional antibiotics, and the failure of treatments due to the appearance of new bacterial strains antibiotic resistant. The use of plant extracts and natural phytochemicals with proved antimicrobial properties can be of great significance in the therapeutic use. In recent years, secondary metabolites as phenolic compounds, terpenoids, flavonoids and alkaloids have received a great deal of attention due to their diverse biological functions. Some natural substances have effective antimicrobial properties and they have been used as alternative treatments. Today, the use of plants as treatment of illness is accepted worldwide. In developing countries, the use of medicinal plants has significantly increased due to the low income of the population. Herbs and spices are generally considered to be safe and have been proved to be effective against certain ailments. Therefore we aimed to study the antimicrobial properties of selected medicinal plant extracts, as follows:

I. Evaluation of the *in vitro* antimicrobial activity of selected plant extracts, against Gram-negative and Gram-positive bacteria including some of the most frequent etiological agents of enteric infections and fungi.

II. Characterization of the antimicrobial activity of the natural extract with best activity and the most sensitive/interesting microorganism.

III. Determination of the *in vitro* cytotoxicity of the selected extract.

IV. Analysis of the effect against enteropathogenic *E. coli* (EPEC) of the selected extract during *in vitro* infection of cell cultures.

V. Chemical fractionation of the selected natural extract.

VI. Assessment of the antibacterial activity of the fractions.

OBJECTIVES

MATERIALS AND METHODS

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Materials and Methods

1. Plant materials

Dried red calyces of *Hibiscus sabdariffa* var. ruber (red) were purchased from a local herbalist of Hurghada city (Egypt) which is imported from Sudan. Leaves and stem of *Rosmarinus officinalis,* leaves of *Origanum vulgare,* leaves of *Thymus vulgaris* and grounded bark of *Cinnamomum zeylanicum* or *Cinnamomum verum* (were obtained from a local herbal shop in Madrid, Spain. The botanical materials were identified in Dpt. of Plant Biology II, Faculty of Pharmacy, University Complutense of Madrid.

2. Preparation of the plant extracts

For *H. sabdariffa,* 10 g sample of the grounded dried red calyces were soaked in 100 mL of Milli Q sterile water for 24 h at RT. 10 g sample of the ground plants of *R. officinalis, O. vulgare, T. vulgaris* and *C. zeylanicum*, were soaked in 100 mL of Milli Q sterile water at 65 ºC for 30 min. Each aqueous plant extract was decanted and filtered using 0.22 µm sterile single use filters (Millipore Corporation, Bedford, MA, USA). The filtrates were lyophilized using a freeze-dryer (Freeze-drying works by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublimate directly from the solid phase to the gas phase) which it works at very low temperature (-40 ºC) and pressure. The lyophilized extracts were kept at 4 ºC, in the dark, until use.

3. Determination of total phenolic compounds (TPCs) content

Dried plants were prepared in sterile distilled water at 5-10% w/v as previously described. The total phenolic compounds contents were determined using the Folin-Ciocalteu method (Ainsworth and Gillespie, 2007) using gallic acid (Sigma) as standard. Folin-Ciocalteu reagent (VWR International Eurolab) is formed from a yellow color mixture of phosphotungstic acid, $H_3PW_{12}O_{40}$, and phosphomolybdic acid, $H_3PMo_{12}O_{40}$,

which, after oxidation of the phenols, is reduced to a mixture of blue oxides of tungsten, W_8O_{23}, and molybdenum, Mo_8O_{23}. The blue coloration produced has a maximum absorption in the region of 750 nm, and is proportional t o the total quantity of phenolic compounds originally present. The aqueous crude extract solution (10, 20 and 100µL) was oxidized with 500 µL of Folin-Ciocalteou reagent (VWR international Eurolab). Then the mixture was kept for a 2-5 min period. The reaction was neutralized by the addition of 400 µL of sodium carbonate Na_2CO_3 (75 g/L) (Panreac). The absorbance of the resulting blue color was measured at 750 nm after incubation in dark for 60 min at RT. The total phenolic content was expressed as mg gallic acid equivalents (GAE)/L (Sigma) (standard curve was prepared using concentration 2, 5 mg/L).

4. Bacterial strains and growth conditions

Stock cultures were maintained at 4 ºC on slopes of nutrient agar. Cultures for experiments were prepared by picking a colony from 24 h Mueller-Hinton agar (MHA) (Conda-Pronadisa) plate and it was re-suspended in Mueller-Hinton broth (MHB) medium (5 ml). Culture was grown aerobically for 20 h and at 37 ºC with shaking at 200 rpm. The cultures were diluted with fresh MHB to achieve absorbances at 600 nm; corresponding to 4×10^5 colony forming units (CFU/mL) based on 0.3 absorbance units (600 nm) to 2.5×10^8 CFU/mL. The strains of bacteria used in these studies were kindly provided by Dpt. of Microbiology II, UCM, unless otherwise clearly indicated. The **Gram-positive** tested bacteria are *Staphylococcus aureus* ATCC 29213, Methicillin Resistant *S. aureus* MRSA, *Enterococcus faecalis* ATCC 29212; *Listeria monocytogenes* ATCC 7644, *Listeria monocytogenes* isolated from meat (both *Listeria* were kindly provided by Dr. A. Zamora, Food Science and Food Hygiene Institut, Veterinary Military Centre of Defense, Madrid, Spain). The **Gram-negative** bacteria are *Klebsiella pneumoniae* KPBL, clinical isolate of *Pseudomonas aeruginosa and Burkholderia cenocepacia* J2315, *Escherichia coli* ATCC 25922, clinical isolate of Uropathogenic *E. coli* (UPEC); Enteropathogenic *E. coli*

(EPEC 0127:H6 strain E2348/69) (this strain was provided by Dr. Brett B. Finlay. University of British Columbia, Vancouver, Canada). *Salmonella enterica* serovar Typhimurium, *S. enterica* serovar Enteritidis (both *Salmonella* were kindly supplied by the National Center for Microbiology, Virology and Immunology Health.Carlos III Institut (Instituto de salud Carlos III), Madrid, Spain).

5. Fungal growth conditions

The fungal strains used in these studies were kindly provided by Dpt. of Microbiology II, UCM, unless otherwise clearly indicated. Fungal strains: *Candida albicans* SC5314 (ATCC MYA 2876), *Saccharomyces cerevisiae YPH499* (MA Ta ade2-101 trp-63 leu2-1 ura3-52 his3-Δ200 lys2-801), *S. cerevisiae BY4741* (MA Ta his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and *Yarrowia lipolytica* (P01a MATA leu2-270 ura3-302) (*Y. lipolytica* was provided by A. Domínguez, University of Salamanca, Spain). They were cultured on YPD agar plates. One colony of each strain was suspended in YPD broth medium (Yeast, Peptone, Dextrose) (Conda-Pronadisa) and incubated at 30 ºC for 20 h, and then they were diluted to achieve absorbances at 600 nm; corresponding to $4x10^6$ CFU/mL based on 1 absorbance unit to 2.5×10^7 CFU/mL for *C. albicans* and 1 absorbance unit at 600 nm to 1×10^7 CFU/mL for *S. cerevisiae YPH 499* and *S. cerevisiae BY4741* and *Y. lipolytica* for experimental conditions.

6. Antimicrobial activity

6.1 Microwell dilution assay

Minimal inhibitory concentration **(MIC)** and minimal bactericidal concentration **(MBC)** were determined through broth micro-well dilution assay (Fig. 8). The lyophilized plant extracts were dissolved in Mueller-Hinton broth (MHB) with serial dilution at specific concentrations w/v of each extract. 96-well microtiter plates were prepared by dispensing into each well 95 µl of each dilution of the lyophilized plant extract and 5 µL of inoculum (10^4 CFU; final concentration 10^5 CFU/mL) was added to each

tested dilution. The final volume in each well was 100 µL. Control wells were prepared with culture medium without inoculum, medium with bacterial inoculum and medium with plant extract without inoculum and plant extract alone, in same concentrations than used in the assay. Each concentration of the extract had a negative and positive control. The plate was incubated at 37 ºC on a shaker at 200 rpm for 16-18 h. Microbial growth in each concentration was determined by reading the respective absorbance at 600 nm using a standard microplate reader. Microbial growth was assessed by taking samples at 16-18 h and plating on MHA by using a replicator (drop assay) that were allow to grow for 24 h at 37 ºC.

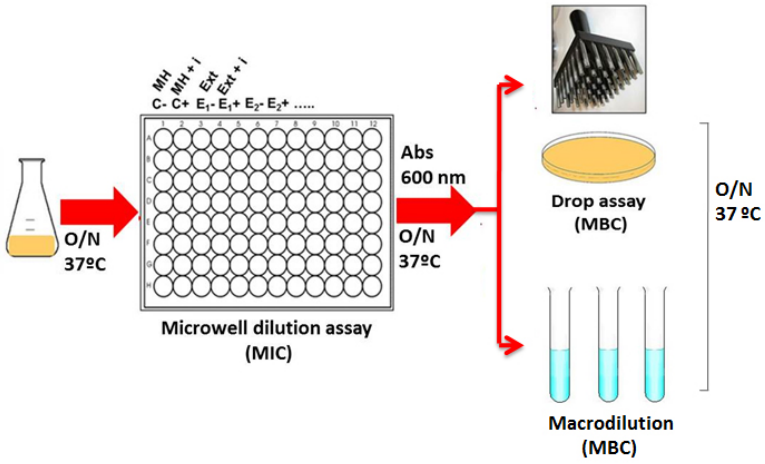


Figure 8. Microwell dilution assay.
The tested bacteria are incubated in MHB media at 37 ºC overnight. In a microwell plate, MH represent the negative control of inoculum (C-), MH+i represent the positive growth control of inoculum ($2x10^4$ CFU/well) (C+), Ext. represent the extract dissolved in MH without inoculum at specific concentration, Ext+i represent the extract dissolved in MH with inoculum ($2x10^4$ CFU/well) at the specific concentartion. Each microwell contains100 µl as final volume. The microwell plate is incubated at 37 ºC for 16-18 h. The microbial growth was determined by measuring the absorbance at 600 nm of each concentration to determine the minimal inhibitory concentration (MIC). Afterward, 1.5 µl of each microwell were droped onto Mueller-Hinton Agar (MHA) plate by using replicator to assess the minimal bactericidal concentration (MBC) and the rest of the microwell was mixed with 5 ml of broth MH media and incubated at 37 ºC with agitation for 16-18 h then the MBC was determined.

For assurance of the drop assay results, the rest of the microwell was mixed with 5 mL of MHB medium and incubated at 37 ºC with agitation for 16-18 h, then the MBC was determined by direct observation of presence or absence the turbidity. **MIC** is defined as the lowest concentration of the antimicrobial compound that inhibits the growth of microorganisms after

incubation. **MBC** is the lowest concentration of the compound that kills the bacteria.

6.2 Disc diffusion test

Antibiotic susceptibility by disc diffusion test was realized according to the Kirby-Bauer test (Cona, 2002). Two sterile discs (6 mm diameter) were soaked separately on the aqueous extract to obtain 35 and 70 mg/disc and then the discs were allowed to dry. These discs were placed on the surface of MHA plates, previously prepared with the selected bacteria. Each disc was pressed down to ensure complete contact with the agar surface and maintained at a distance no closer than 24 mm from each other (center to center). The plates contained EPEC and *E. coli* ATCC 25922 at a concentration of 10^8 CFU/mL (McFarland 0.5). The target bacteria EPEC and *E. coli* ATCC 25922 were added to melted MHA at 45 ºC in absence and presence of 0.5 % Tween 80 (Sigma) to facilitate the aqueous extract diffusion through the agar. This preparation was incubated at 37 ºC for a period of 24 h. Discs commercially prepared with an antibiotic (Ampicillin 10 µg/disc) (BD BBL) were used as a positive control. Antibacterial activity was determined as the lowest concentration of plant extract that produced an inhibition zone (halo) around a disc following the 24 h incubation. Antibacterial activity was evaluated from the diameter (mm) of the halo formed around the discs after 24 h incubation.

7. Time-kill curve studies

7.1 Time kill curves

Time-kill curve were done at 1% w/v (10 mg/mL) (MIC) and 2.5% w/v (25 mg/mL) (MBC) of plant extract. Overnight fresh culture bacteria were inoculated into MHB media at starting density of $4x10^6$ CFU/mL for both sample and control culture media. Cultures were incubated at 37 ºC for 24 h. Aliquots for the determination of viable counts were taken from test culture and growth control flasks at 0, 2, 4, 6, 8, 10, 12 and 24 h. Viable counts were determined by plating 100µl of known dilutions of the culture

samples on to MHA plates in duplicate. Cell count plates were incubated at 37 ºC for up to 48 h to allow bacterial growth. The number of colonies was multiplied by the assayed dilution. The growth curve was analysed by plotting log CFU/mL *versus* time and the bactericidal effect can be seen by a 3 log units decrease at the time specified (Cantón and Pemán, 1999).

7.2 Time-kill kinetics. Mathematical model for bactericidal activity

The killing kinetics of the bactericidal activity were analyzed by fitting the mean data at each time point to an exponential equation: $N_t = N_0 \text{ X } e^{-Kt}$, where N_t expresses the number of viable bacteria at time t, N_0 is the number of viable bacteria at the beginning of the experiment, K is the killing (or lethality) rate, and t is the incubation time. The exponential equation was transformed into a line by applying natural logarithms ($\log N_t = \log N_0 + Kt$). The K value was calculated, positive values of which indicate growth and negative values of which indicate killing. Thus, the time points of every killing curve were reduced to one value, K. The following parameters were derived from the killing equation: the mean times to achieve reductions in the proportions of viable cells of 50% (t_{50}=0.30103/K), 90% (t_{90} = 1/K), and 99% (t_{99} = 2/K) and the time to reach the bactericidal endpoint 99.9 % ($t_{99.9}$ = 3/K) for each concentration (Cantón, *et al*., 2004).

8. Microbial quality assays

To investigate the presence of ***Salmonella*** **spp.** 25 grams of plant calyces were transferred to a filter bag (Seward (Nessler), Stomacher lab system) under sterile conditions and 225 mL of buffered peptone was added. After 10 min of hydration at RT, the sample was homogenized for 1min in a homogenizer (Stomacher Lab Blender 400, Seward, Warthing, UK). Then the supernatant was transferred into a flask for incubation at 37 ºC for 24 h (pre-enrichment procedure).

To assess the presence of ***Salmonella*** **spp.,** after 24 h 1mL aliquots of the enrichment culture were added to two different types of broth culture media:

Selenite Brilliant Green broth media (Conda-Pronadisa) at 37 ºC and Rappaport-Vassiliadis broth media (Conda-Pronadisa) at 42 ºC for 24 h. Afterwards, a sample of the incubated selenite brilliant green broth media was laid over Salmonella-Shigella agar (SS) (Conda-Pronadisa) and Xylose Lysine Desoxycholate agar (XLD) (Conda-Pronadisa) for incubation at 37 ºC for 24 h. In parallel a sample of Rappaport-Vassiliadis liquid media (Conda-Pronadisa) was surface plated on the Brilliant Green Agar (BGA) (Conda-Pronadisa) for incubation at 37 ºC for 24 h.

To assess the presence of **aerobic mesophilic bacteria*, Enterobacteria, S. aureus***, ***B. cereus, E. coli*, *L. monocytogenes*** and **fungi**: 10 grams of plant calyces were transferred to a homogenizer bag under sterile conditions and 90 mL of buffered triptone were added.

For counting the **total viable aerobic** or **mesophilic bacteria,** the pour-plate method was used.1mL aliquots of 10^{-4}, 10^{-5} and 10^{-6} dilutions in peptone broth were mixed with 20 mL of Plate Count Agar (PCA) (Conda-Pronadisa) and plated by duplicates for each dilution. After incubation at 30 ºC for 24 h, plates were counted.

For detection of **enterobacteria** 1 mL aliquots of the homogenate (10^{-1}) were serially diluted up to 10^{-6}. 1mL of 10^{-1}, 10^{-2} and 10^{-3} were mixed with Violete-Red-Bile-Glucose (VRBG) (Conda-Pronadisa) in duplicate and were incubatedat 30 ºC for 24 h. For detection of ***E. coli*** 1mL of 10^{-1} and 10^{-2} dilutions were mixed with Coli-ID agar (bioMèrieux) in duplicate at 37 ºC for 24-48 h.

For detection of ***S. aureus***, the Most Probable Number (MPN) method was used.1mL 10^{-1}, 10^{-2} and 10^{-3} dilutions by triplicate each one were mixed with Chapman-Mannitol broth (Conda-Pronadisa) media by triplicate of each dilution for detection after incubation at 37 ºC for 24 h. To confirm the presence of ***S. aureus***, samples from incubated Chapman-Manitol broth media were plated on Baird-Barker agar (Difco) media and incubated at 37 ºC for 24 h.

To detect the presence of ***B. cereus***, a sample of 10^{-1} initial dilution was laid over Mossel agar (Conda-Pronadisa) and incubated at 30 ºC for 48 h.

To assess the presence or absence of ***L. monocytogenes***,: 10 g of the *H. sabdariffa* calyces were homogenized with Fraser broth media (bioMèrieux) and incubated at 30 ºC for 24-48 h, then a sample was laid over ALOA agar (bioMèrieux) and incubated at 37 ºC for 24 h. For **fungi detection**, duplicated samples of 10^{-1} and 10^{-2} dilutions were laid over Sabouraud agar (Conda-Pronadisa) to incubate for 2 to 4 days at 24 ºC.

9. Cell culture

HeLa, a human cervical epithelial cancer cell line was obtained from the American Type Culture Collection (ATCC). Cells were cultured in Iscove's Modified Dulbecco's Media (IMDM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Lonza BioWhittaker, Fisher Scientific) and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml, Invitrogen) at 37 ºC in a humidified atmosphere with 5% CO_2. Cell lines were routinely subcultured twice a week by detaching the monolayer with 0.25% trypsin - 0.03% ethylene diamine tetra acetic acid (EDTA) solution. The cell passage was recorded and cells were discarded at the 25^{th} passage. Frozen cell stocks were stored in liquid nitrogen using a freezing solution containing 20% of dimethyl sulfoxide (DMSO) as a cryoprotectant.

10. EPEC infection of HeLa cells and pedestal formation

For infections, HeLa cells were grown to 70-80 % confluence in tissue culture P100 plates in 5% CO_2 at 37 ºC. Cells were seeded at a density of 150,000 cells/well onto 6 well cell culture plates containing four heat-sterilized glass cover slips in each well and allowed to attach for 24 h. EPEC was grown overnight as previously mentioned.

For preactivation experiments, the bacteria was diluted 1:100 with Iscove´s modified Dulbecco`s media supplemented with 10 % fetal bovine serum without antibiotics in a P60 plate and were kept at 37 °C with 5% CO_2 for

2 h. After the incubation period, the absorbance at 600 nm was adjusted at 0.2. Then, 25 µl EPEC were added to each well of 6 well plates which contained HeLa cells and incubated for the indicated times at 37 °C in 5% CO_2. The Multiplicity of Infection (MOI) is defined as the number of bacteria added per cell at the beginning of the infection, and was calculated by considering that an absorbance at 600 nm of 1 is equivalent to 10^9 and that the number of HeLa cells per well counted the day of the infection. After the indicated time of infection, unattached bacteria were removed by washing cells three times with D-PBS, then the aqueous plant extract were added at the indicated concentrations and incubated for 1, 2 and 3 h, or cells were left uninfected as a control for the treatments.

11. Immunoflourescence experiments

Hela cells were fixed with formaline buffer solution (containing 4% w/v formaldehyde, Sigma) for 20 min at RT and then washed twice with D-PBS. The fixed cells were permeabilized with 0.05% Triton X-100 for 5 min. After washing with PBS three times, the cells were blocked with PBS containing 2% BSA for 10 min. The cells were stained with 1 µg / mL tetramethyl-rhodamine-isothiocyanate (TRITC)-phalloidin (Sigma) for 15 min at RT to visualize the actin cytoskeleton, and the pedestals. After three washes with PBS, the cells were stained with 4'-6-diamidino-2 phenylindole (DAPI, 300 nM) for 5 min at RT to visualize bacteria. After three washes with PBS the coverslips were allowed to dry at RT during 20 min and mounted using 5 µl of mounting medium (100 mM Tris-HCl pH 8.5, 10% Mowiol 4-88 (Calbiochem), 25% Glycerol with phenylnediamine (Sigma)). Micrographs were taken using a Nikon Eclipse TE 200-U fluorescence microscope equipped with a Hamamatsu camera. Images were processed with Adobe Photoshop. Quantification of pedestals was done by counting the number of pedestals of attached bacteria for a total of 40 infected cells in representative microscope fields.

12. Cell viability assays

12.1 Determination of cytotoxicity of the extract using the Rezasurin reduction test in J774 Macrophages

J774 murine macrophages were seeded ($7x10^4$ cells/well) in 96-well-flat-bottom microplates (Nunc) in 100 µL of RPMI 1640 medium (Sigma) supplemented with 10% of heat-inactivated fetal bovine serum (FBS). The plates were incubated at 37 C, 5% CO_2 to allow for cell attachment during 24 h. After the incubation time, the medium was replaced with 200 µL of the extract dissolved in RPMI medium at different concentrations, for another 24 h.

Rezasurin reduction test was used as an indicator for cell viability. Blank controls with the extracts dissolved in RPMI medium without cells and growth controls (untreated macrophages) were also included in each microplate. Afterwards, cytotoxicity was determined by addition of 1mM of the redox dye resazurin (20 µL/well) (resazurin sodium salt, Sigma) and measures were taken after 3 h of incubation by using a spectrofluorometer (λex 535 nm - λem 590 nm). Resazurin reduction test was used as an indicator of cell viability. Resazurin dye (blue and non-fluorescent) is reduced to resarufin (pink and highly fluorescent) by mitochondrial enzymes, which are carriers of diaphorase activities. These enzymes are probably responsible for the transference of electrons from $NADPH^+$ H^+ to resazurin, which is reduced to resorufin. The reduction of resazurin correlates with the number of alive macrophages.

12.2 Determination of cytotoxicity of the extracts on HeLa cells

HeLa cells were seeded at 150 000 cells per well of 6-well plates in IMDM medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics and incubated at 37 ºC in a humidified atmosphere with 5% CO_2 for 24 h. Then, the cells were treated with the aqueous plant extract of *H. sabdariffa* at different concentrations (5, 7 and 10 mg/mL) for 24 h;

afterwards, cells were washed and harvested in 10 mL of medium. Equal volumes (50 µL) of trypan blue and cell suspensions were mixed well and 5 µL were overlaid on a Neubauer chamber for cell counting under a light microscope. Live cells were counted per each well of the different concentrations and compared with the control of untreated cells. Trypan Blue is a blue acid dye that has two azo chromophores groups which cannot enter into the plasma membrane of live cells.

13. Fractionation of the aqueous extract of *Hibiscus sabdariffa* calyces

The lyophilized aqueous extract of *H. sabdariffa* (Fig. 9A) was fractionated using different organic solvents in order of increasing polarity: ethyl acetate, acetone and ethanol. First, lyophilized aqueous extract of *H. sabdariffa* calyces (40 g) were extracted with ethyl acetate (400 mL) at RT with magnetic stirring overnight (Fig. 9B). Then, the solvent (ethyl acetate) was filtered under vacuum (Fig. 9C), to obtain a supernatant and a residue. The supernatant was concentrated using a rotary evaporator (Rotavapor La BOROTA, 4000, Heidolph) (Fig. 10A) at 35 ºC, to obtain the **ethyl acetate soluble extract (0.170 g) (EtOAc extract)**. The residue obtained was dipped in acetone (400 mL) at RT with magnetic stirring overnight, to obtain a supernatant and a residue. The supernatant after filtration was concentrated using a rotary evaporator at 35 ºC, to obtain the **acetone soluble extract (1.1 g) (AC extract)** (Fig. 10B). Afterwards, the residue was immersed in 400 ml of ethanol at RT with magnetic stirring overnight. The supernatant was concentrated after filtration using a rotary evaporator at 35 ºC to obtaine the **ethanol soluble extract (20.6 g) (EtOH extract)**. This ethanol extract was immersed in 200 ml of acetone solvent. After filtration, the supernatant was concentrated using a rotary evaporator at 35 ºC to get the **acetone soluble extract from ethanol (AC-EtOH extract) (14.6 g).** All extracts were stored at 4 ºC till further analysis. The extraction process is showed in scheme 1.

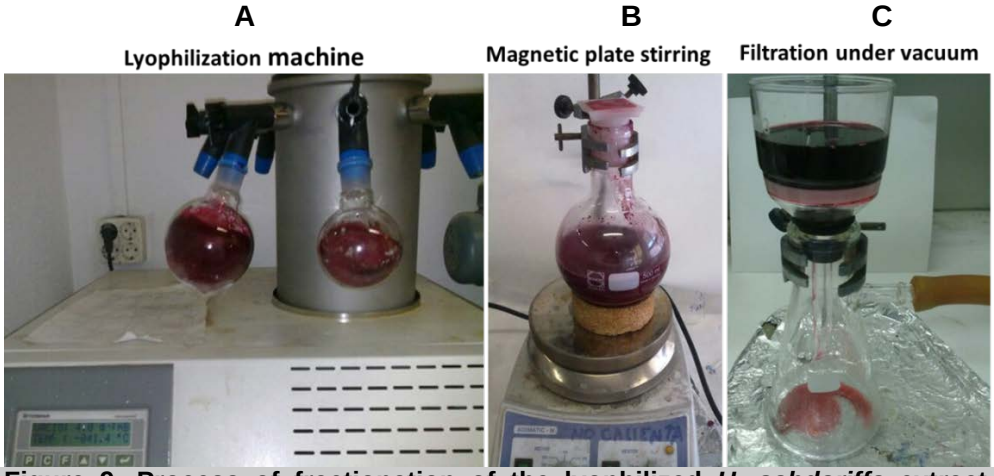


Figure 9. Process of fractionation of the lyophilized *H. sabdariffa* extract calyces. (A) Lyophilized *H. sabdariffa*calyces extract in the lyophilization machine. **(B)** the lyophilized H. sabdariffa calyces extract with the organic solvent on the magnetic stirring plate. **(C)** Filtration under vacuum to obtain a supernatant and a residue.

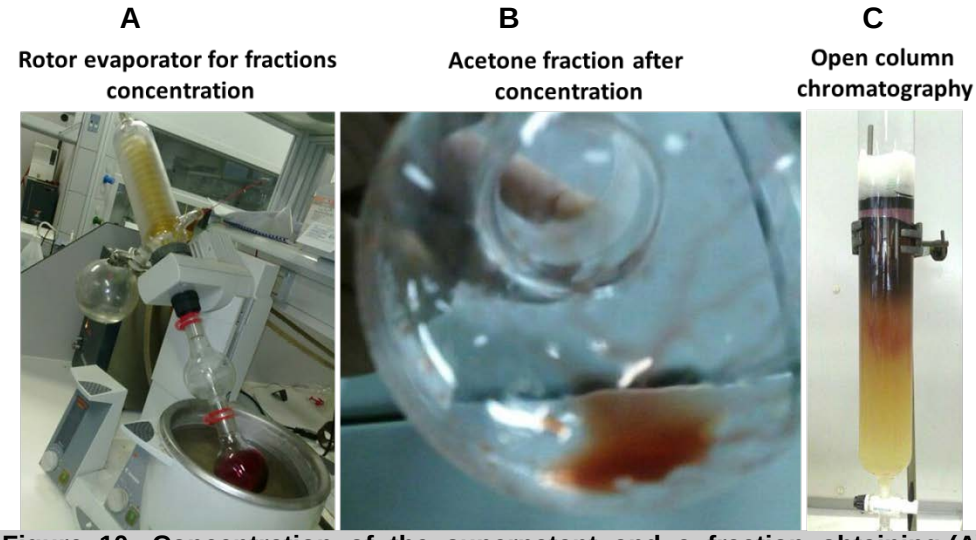
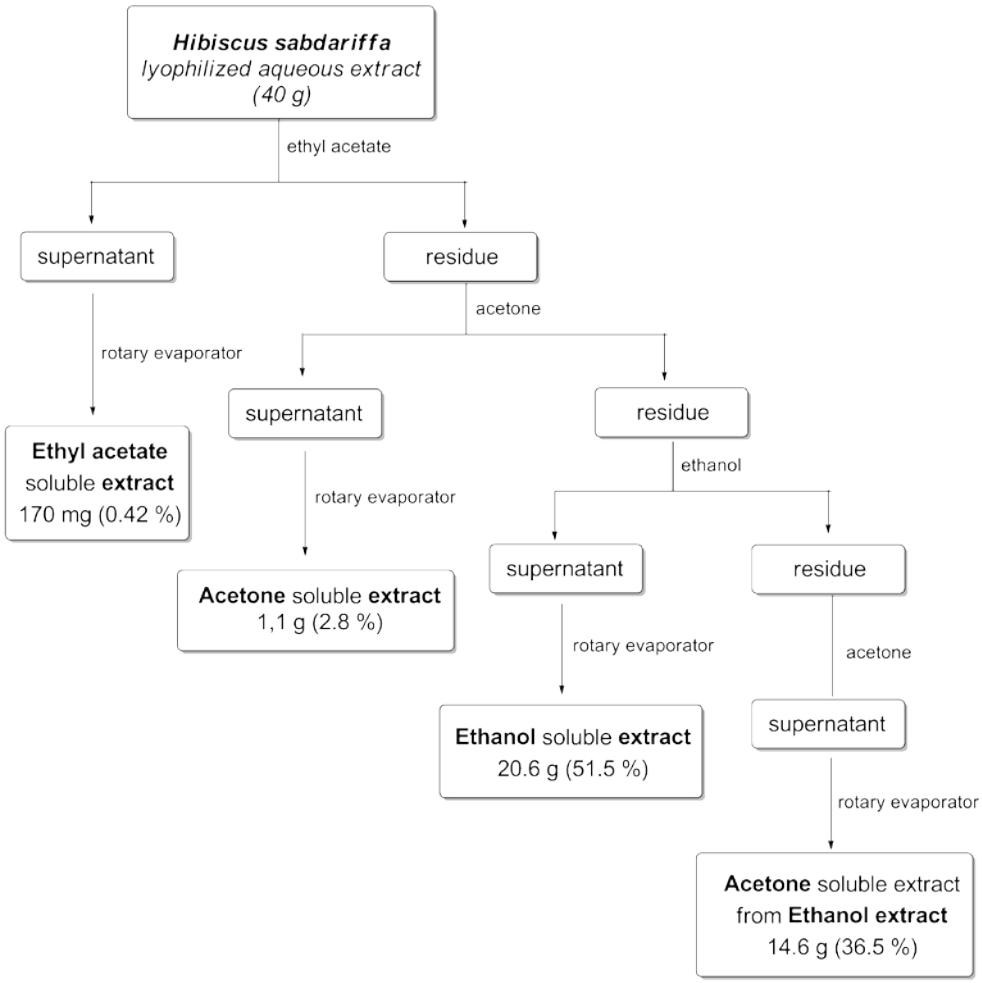


Figure 10. Concentration of the supernatant and a fraction obtaining.(A) Rotary evaporator machine to concentrate the supernatant obtained after filtration. **(B)** Acetone soluble fraction after concentration. **(C)** Silica gel column chromatography.



Scheme 1. Fractionation process of the aqueous extract of *H. sabdariffa* calyces using different organic solvent in order of increase the polarity.

Statistical Analysis were carried out using the two-tailed Student's *t*-test or Anova test and displayed graphically using GraphPad Prism software.

Graphs represent mean ± SD of three or two separated experiments. All experiments were repeated three times separately.

RESULTS

RESULTS

Results

1. Antibacterial activity of aqueous extracts

One of our objectives is to study the *in vitro* antibacterial activity of aqueous extracts of five medicinal plants *C. zeylanicum, O. vulgare*, *R. officinalis*, *T. vulgaris* and *H. sabdariffa* against Gram-negative bacteria Uropathogenic *E. coli* (UPEC), Enteropathogenic *E. coli* (EPEC E 2348/69), *E. coli* ATCC 25922, *P. aeruginosa, K. pneumoniae, S.* Typhimurium*, S.* Enteritidis and Gram-positive bacteria *S. aureus*, Methicillin Resistant *S. aureus* (MRSA), *E. faecalis*, *L. monocytogens* ATCC 7644 and *L. monocytogenes* isolated from meat, by broth micro dilution assay. This method allows the determination of the MICs and MBCs. This method involve the incorporation of known concentrations of antimicrobial agents into the broth media then checking for the presence or absence of organism growth, after a suitable incubation period, generally 16-18 h (MIC). And from each tested micro well, 1.5 µL was inoculated onto MHA plates then checking the presence or absence of the organism growth after the suitable incubation period (16-18 h) (MBC).

1.1 Antibacterial activity of *Cinnamomum zeylanicum* extract

The antibacterial activity of aqueous lyophilized extract of *C. zeylanicum* was diluted in MHB media at concentrations of 5, 10, 20, 35, 70 mg/mL. To determine the MICs and MBCs, the broth micro dilution assay was used. The results showed an inhibition of bacterial growth for both Gram-positive and Gram-negative bacteria. The MIC for *S. aureus*, *E. faecalis* and *L. monocytogenes* was 5 mg/mL, while for *S. aureus* (MRSA) was 10 mg/mL (Fig. 11B). Regarding Gram-negative bacteria, we found that the MIC for EPEC, *E. coli,* UPEC and *K. pneumoniae* was 20 mg/mL except *S.* ser. Typhimurium and *S.* ser. Enteritidis was 10 mg/mL (Fig. 11A) (Table 4).

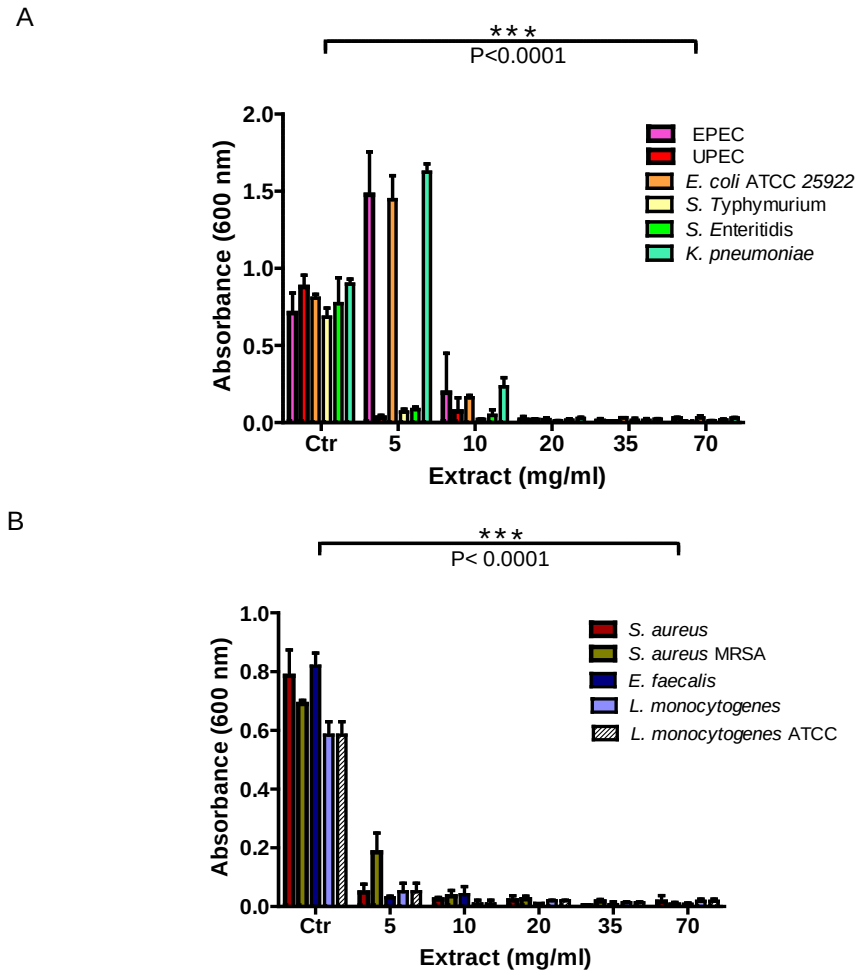


Figure 11. Antibacterial activity of aqueous extract of *C. zeylanicum* against Gram-negative and Gram-positive bacteria using drop assay. MIC of aqueous extract of *C. zeylanicum* against **(A)** Gram-negative bacteria and **(B)** Gram-positive bacteria by measuring the absorbance at 600 nm of bacterial growth after incubation at 37 ºC for 16-18 h. Graphs represent mean ± SD. Statistical analysis using two way ANOVA from two independent experiments ***, p<0.0001.

The MBC from extract of *C. zeylanicum* for Gram-positive bacteria *S. aureus*, Methicillin Resistant *S. aureus* (MRSA), *L. monocytogenes* and *L. monocytogenes* ATCC 7644 was 5 mg/mL. Regarding Gram-negative bacteria, it was founded that the MBC for enteropathogenic *E. coli*, Uropathogenic *E. coli*, *S.* ser. Typhimurium and *S.*ser.Enteritidis was 20 mg/mL, except for *E.coli* ATCC 25922 was 35 mg/mL and *K. pneumoni ae* was 70 mg/mL (Fig. 12) (Table 4).

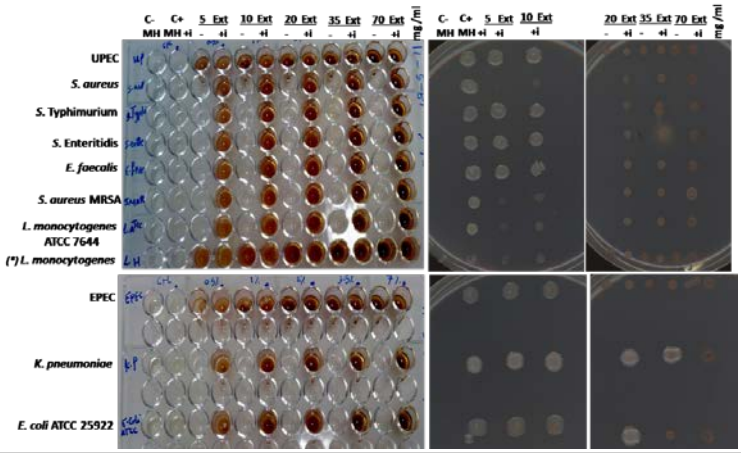


Figure 12. Antibacterial activity of aqueous extract of *C. zeylanicum* against Gram-negative and Gram-positive bacteria using drop assay.
MBC of aqueous extract of *C. zeylanicum* against Gram-positive and Gram-negative bacteria was assesed by inoculating 1.5 µL of each micro well into MHA medium.
Abbreviations: C= control, MH= Mueller Hinton, I= Inoculum, Ext= Extract, numbers as 5=5 mg/mL of the extract.
(*)*Listeria monocytogenes* was isolated from meat

Table 4. *Cinnamomum zeylanicum*

Bacteria	MIC					MBC				
	Extract (mg/mL)					Extract (mg/mL)				
	5	10	20	35	70	5	10	20	35	70
S. aureus	-	-	-	-	-	-	-	-	-	-
S. aureus MRSA	-	-	-	-	-	-	-	-	-	-
E. faecalis	-	-	-	-	-	+	+	-	-	-
L. monocytogenes	-	-	-	-	-	-	-	-	-	-
L. monocytogenes ATCC 7644	-	-	-	-	-	-	-	-	-	-
EPEC	+	+	-	-	-	+	+	-	-	-
E. coli ATCC 25922	+	+	-	-	-	+	+	+	-	-
UPEC	+	+	-	-	-	+	+	-	-	-
K. pneumoniae	+	+	-	-	-	+	+	+	+	-
*S. ser.*Typhimurium	+	-	-	-	-	+	+	-	-	-
S. ser. Enteritidis	+	-	-	-	-	+	+	-	-	-

(+) means that there is growth of bacteria (-) means that there is no growth of bacteria

1.2 Antibacterial activity of O*riganum vulgare* extract

The antibacterial activity of *O. vulgare* extract was assessed by using micro well dilution method (Fig. 8). The *O. vulgare* extract was diluted in MHB media at concentrations of 35, 70, 140 mg/mL. The results showed an inhibition for Gram-positive and Gram-negative bacteria. The MIC for Gram-positive bacteria *S. aureus*, Methicillin Resistant *S. aureus* (MRSA) was 35 mg/mL and for *E. faecalis* and *L. monocytogenes* was 35 mg/mL (Fig. 13B) (Table 5). The MIC for *S.* Typhimurium, *S.* Enteritidis was 70 mg/mL, and for UPEC was 140 mg/mL (Fig. 13A) (Table 5).

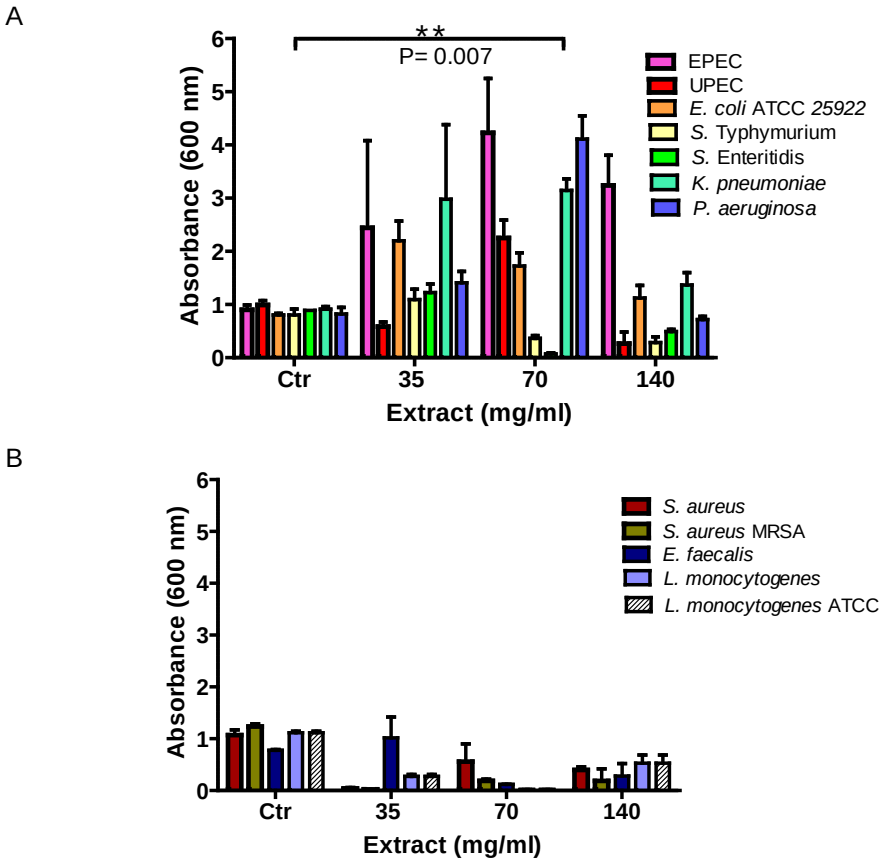


Figure 13. Antibacterial activity of *O vulgare* extract against Gram-negative and Gram-positive bacteria using drop assay. MIC of aqueous extract of *O. vulgare* against **(A)** Gram-negative bacteria and **(B)** Gram-positive bacteria by measuring the absorbance at 600 nm of bacterial growth after incubation at 37 ºC for 16-18 h. Graphs represent mean ± SD. Statistical analysis using two way ANOVA from two independent experiments **, p<0.007.

The MBC for Gram-positive bacteria *S. aureus*, Methicillin Resistant *S. aureus* (MRSA), and *L. monocytogenes* and *L. monocytogenes* ATCC 7644 was 35 mg/mL but for *E. faecalis* was 70 mg/mL. The MBC for *S.* ser. Typhimurium and *S.* ser. Enteritidis was 70 mg/mL and UPEC, *E. coli* ATCC 25922, EPEC and *K. pneumoniae* was 140 mg/mL. The aqueous extract showed no bactericidal activity against *P. aeruginosa* up to 140 mg/mL (Fig. 14) (Table 5).

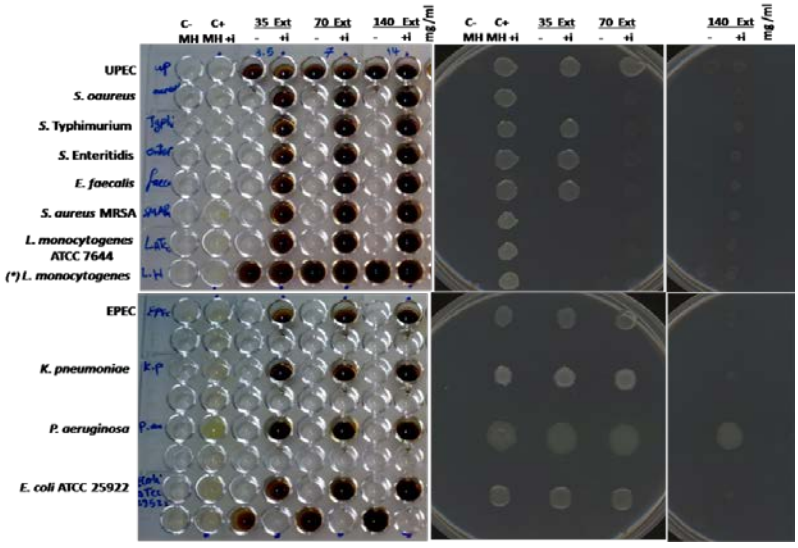


Figure 14. Antibacterial activity of *O vulgare* extract against Gram-negative and Gram-positive bacteria using drop assay.
MBC of aqueous extract of *O. vulgare* against Gram-positive and Gram-negative bacteria was assesed by inoculating 1.5 µL of each micro well into MHA medium. Abbreviations: C= control, MH= Müeller Hinton, i= Inoculum, Ext= Extract, numbers as 5= 5 mg/mL of the extract.
(*)*Listeria monocytogenes* was isolated from meat

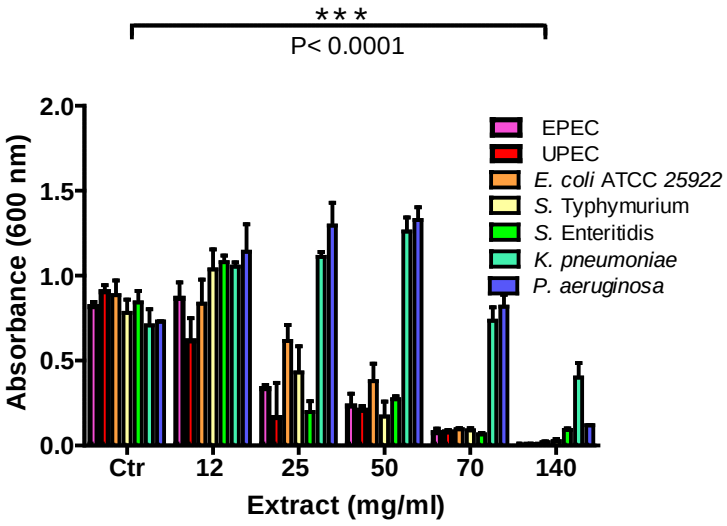
Table 5. *Origanum vulgare*						
	MIC			**MBC**		
Bacteria	Extract (mg/mL)			Extract (mg/mL)		
	35	70	140	35	70	140
S. aureus	_	_	_	_	_	_
S. aureus (MRSA)	_	_	_	_	_	_
E. faecalis	+	_	_	+	_	_
L. monocytogenes	_	_	_	_	_	_
L. monocytogenes ATCC 7644	_	_	_	_	_	_
EPEC	+	+	+	+	+	_
E. coli ATCC 29522	+	+	+	+	+	_
UPEC	+	+	_	+	+	_
K. pneumoniae	+	+	+	+	+	_
S. ser. Typhimurium	+	_	_	+	_	_
*S.*ser.Enteritidis	+	_	_	+	_	_
P. aeruginosa	+	+	+	+	+	+

(+) means that there is growth of bacteria (-) means that there is no growth of bacteria

1.3 Antibacterial activity of *Rosmarinus officinalis* extract

R. officinalis extract was diluted at concentartions 12, 25, 50, 70, 140 mg/mL. The extract showed an inhibition for Gram-positive and Gram-negative bacteria. The MIC for Gram-positive bacteria *S. aureus*, Methicillin Resistant *S. aureus* (MRSA) *and L. monocytogenes* was 12 mg/mL, and for *E. faecalis* was 25 mg/mL (Fig. 15B) (Table 6). Regarding Gram-negative bacteria, we found the MIC for UPEC, *E. coli* ATCC 25922, EPEC and *S.* ser. Typhimurium was 140 mg/mL (Fig. 15A) (Table 6). The *R. officinalis* extract showed MBC for Gram-positive bacteria *S. aureus*, Methicillin Resistant *S. aureus* MRSA*, L. monocytogenes* and *L. monocytogenes* ATCC 7644 at 12 mg/mL. The MBC for *E. faecalis* was 25 mg/mL. Regarding Gram-negative bacteria, we found the MBC for *S.* ser. Typhimurium, *S.* ser. Enteritidis and UPEC was 140 mg/mL and showed no bactericidal activity against *E. coli* ATCC 25922, EPEC*, K. pneumoniae* and *P. aeruginosa* up to 140 mg/mL (Fig. 16) (Table 6).

A



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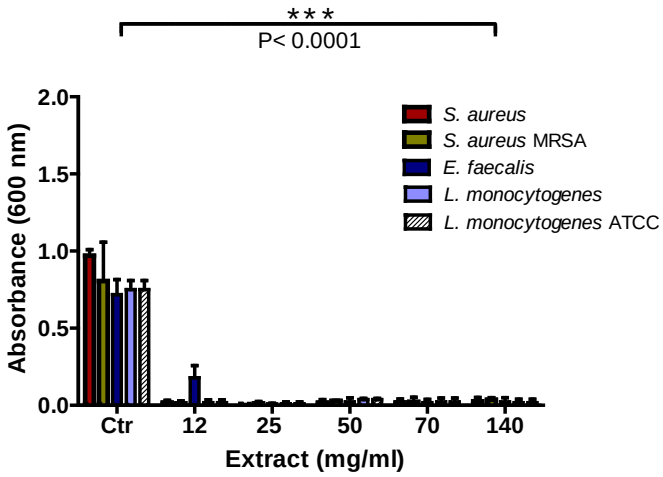


Figure 15. Antibacterial activity of aqueous extract of *R. officinalis* against Gram-negative and Gram-positive bacteria using drop assay. MIC of aqueous extract of *R. officinalis* against **(A)** Gram-negative bacteria and **(B)** Gram-positive bacteria by measuring the absorbance at 600 nm of bacterial growth after incubation at 37 ºC for 16-18 h. Graphs represent mean ± SD. Statistical analysis using two way ANOVA from two independent experiments ***, p<0.0001.

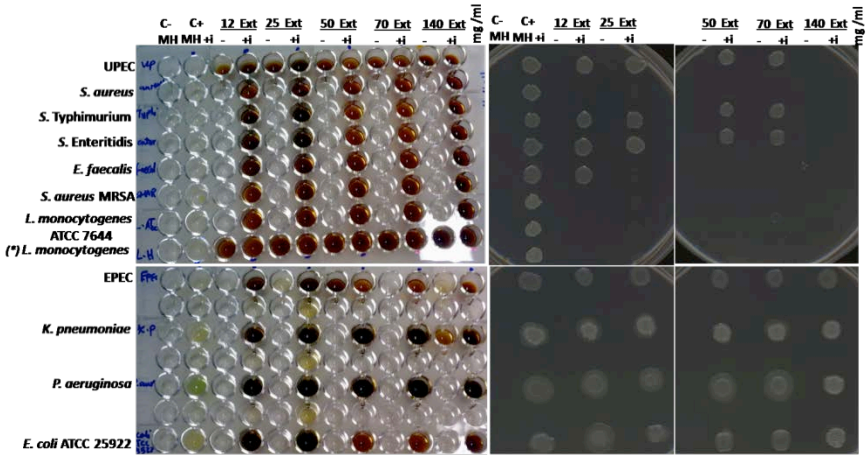


Figure 16. Antibacterial activity of aqueous extract of *R officinalis* against Gram-negative and Gram-positive bacteria using drop assay.
MBC of aqueous extract of *R. officinalis* against Gram-positive and Gram-negative bacteria by inoculating 1.5 µL of each micro well into MHA medium.
Abbreviations: C= control, MH= Mueller Hinton, i= Inoculum, Ext= Extract, numbers as 12 = 12 mg/mL of the extract.
(*)*Listeria monocytogenes* was isolated from meat

Table 6. *Rosmarinus officinalis*										
	MIC					**MBC**				
Bacteria	Extract (mg/mL)					Extract (mg/mL)				
	12	25	50	70	140	12	25	50	70	140
S. aureus	_	_	_	_	_	_	_	_	_	_
S. aureus (MRSA)	_	_	_	_	_	_	_	_	_	_
E. faecalis	+	_	_	_	_	+	_	_	_	_
L. monocytogenes	_	_	_	_	_	_	_	_	_	_
L. monocytogenes ATCC 7644	_	_	_	_	_	_	_	_	_	_
EPEC	+	+	+	+	_	+	+	+	+	+
E. coli ATCC 25922	+	+	+	+	_	+	+	+	+	+
UPEC	+	+	+	+	_	+	+	+	+	_
K. pneumoniae	+	+	+	+	+	+	+	+	+	+
*S.*ser. Typhimurium	+	+	+	+	_	+	+	+	+	_
S. ser. Enteritidis	+	+	+	+	_	+	+	+	+	_
P. aeruginosa	+	+	+	+	+	+	+	+	+	+

(+) means that there is growth of bacteria (-) means that there is no growth of bacteria

1.4 Antibacterial activity of *Thymus vulgaris* extract

T. vulgaris extract was diluted at concentrations of 35, 70, 140 mg/mL. *T. vulgaris* extract inhibited the bacterial growth for Gram-positive and Gram-negative bacteria. The MIC for *S. aureus*, Methicillin Resistant *S. aureus* (MRSA), *L. monocytogenes* and *E. faecalis* was 35 mg/mL (Fig. 17B) (Table 7). The MIC for UPEC, *E. coli* ATCC 25922, EPEC, *S.* ser. Typhimurium, *S.* ser. Enteritidis and *P. aeruginosa* was 35 mg/mL (Fig. 17A) (Table 7).

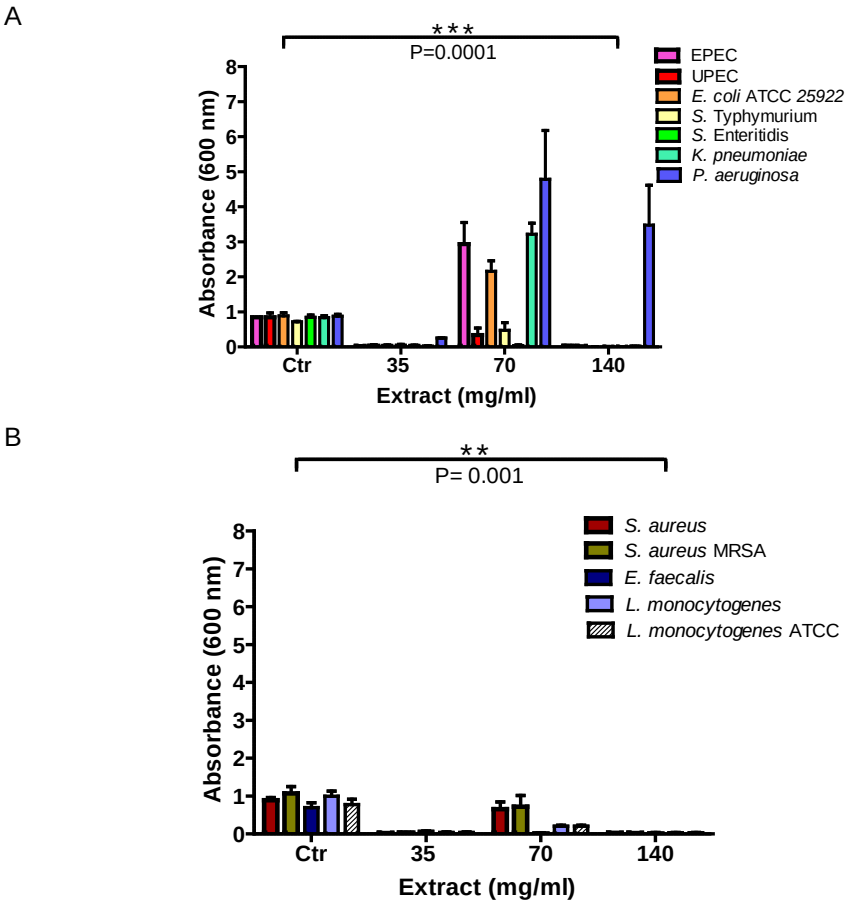


Figure 17. Antibacterial activity of aqueous extract of *T. vulgaris* against Gram-negative and Gram-positive bacteria using drop assay. MIC of aqueous extract of *T. vulgaris* against **(A)** Gram-negative bacteria and **(B)** Gram-positive bacteria by measuring the aborbance at 600 nm of bacterial growth after incubation at 37 ºC for 16-18 h. Graphs represent mean ± SD. Statistical analysis using two way ANOVA from two independent experiments ***, p=0.0001 and **, p<0.001.

The MBC for Gram-positive bacteria *S. aureus*, Methicillin Resistant *S. aureus* (MRSA), and *L. monocytogenes* and *L. monocytogenes* ATCC 7644 was 35 mg/mL but for *E. faecalis* was 70 mg/mL. The MBC for *S.* ser. Typhimurium and *S.* ser. Enteritidis was 70 mg/mL and UPEC, *E. coli* ATCC 25922, EPEC and *K. pneumoniae* was 140 mg/mL. The aqueous extract showed no bactericidal activity against *P. aeruginosa* up to 140 mg/mL (Fig. 18) (Table 7).

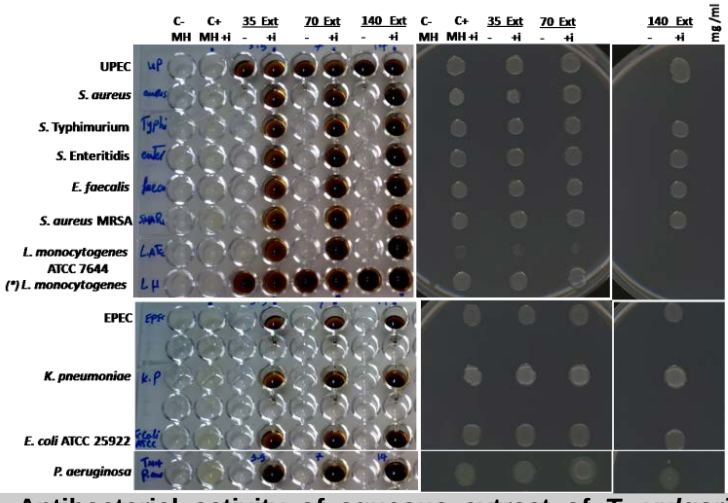


Figure 18. Antibacterial activity of aqueous extract of *T. vulgaris* against Gram-negative and Gram-positive bacteria using drop assay. MBC of aqueous extract of *T. vulgaris* against Gram-positive and Gram-negative bacteria by inoculating 1.5 µL of each micro well into MHA medium.
Abbreviations: C= control, MH= Müeller-Hinton, i= Inoculum, Ext= Extract, numbers as 35= 35 mg/mL of the extract.
(*)*Listeria monocytogenes* was isolated from meat

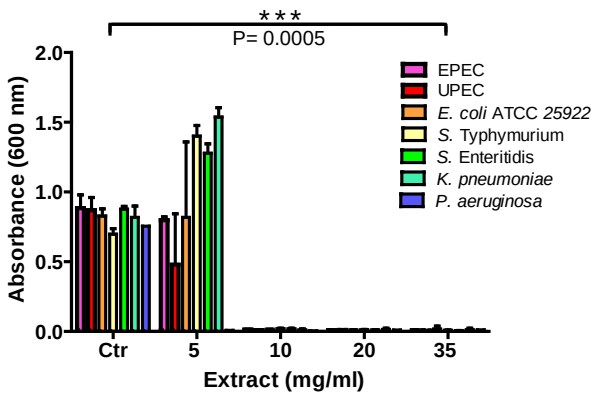
Table 7. *Thymus vulgaris*						
	MIC			**MBC**		
Bacteria	Extract (mg/mL)			Extract (mg/mL)		
	35	70	140	35	70	140
S. aureus	_	_	_	+	+	_
S. aureus (MRSA)	_	_	_	+	+	+
E. faecalis	_	_	_	+	+	+
L. monocytogenes	_	_	_	+	+	_
L. monocytogenes ATCC 7644	_	_	_	+	+	_
EPEC	_	_	_	+	+	+
E. coli ATCC 29522	_	_	_	+	+	+
UPEC	_	_	_	+	+	+
K. pneumoniae	_	_	_	+	+	+
S. ser. Typhimurium	_	_	_	+	+	+
S. ser. Enteritidis	_	_	_	+	+	+
P. aeruginosa	_	_	_	+	+	+

(+) means that there is growth of bacteria (-) means that there is no growth of bacteria

1.5 Antibacterial activity of *Hibiscus sabdariffa* extract

The *H. sabdariffa* extract showed significant inhibitory activity against Gram-positive and Gram-negative bacteria. The *H. sabdariffa* extract was diluted at concentartions of 5, 10, 20, 35 mg/mL. *H. sabdariffa* extract inhibited the bacterial growth of Gram-positive and Gram-negative bacteria. The MIC for Gram-positive bacteria *S. aureus* was 5 mg/mL, but for Methicillin Resistant *S. aureus* (MRSA) , *E. faecalis*, *L. monocytogens* ATCC 7622 and *L. monocytogenes* was 10 mg/mL (Fig. 19B) (Table 8). The MIC for UPEC, EPEC, *E.coli* ATCC 25922, *P. aeruginosa, K. pneumoniae, S.* Typhimurium*, S.* Enteritidis was 10 mg/mL (Fig. 19A) (Table 8) .

A



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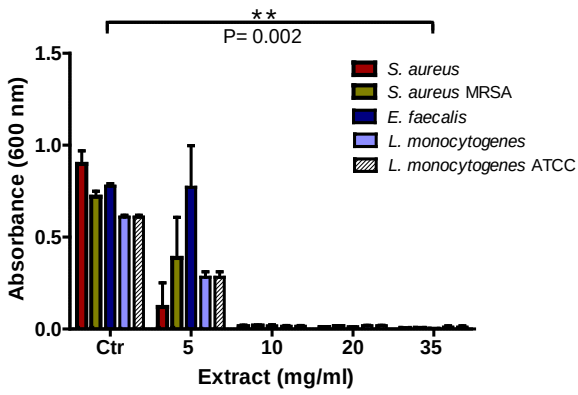


Figure 19. Determination of antibacterial activity of *H. sabdariffa* extract using drop assay: MIC of the aqueous extract against **(A)** Gram-negative bacteria and **(B)** Gram-positive bacteria by measuring the absorbance at 600 nm of the bacterial growth after incubation at 37 ºC for 16-18 h. Graphs represent mean ± SD. Statistical analysis using two way ANOVA from two independent experiments ***, p<0.0005 and**, p<0.002.

The MBC for Gram-positive bacteria *S. aureus*, *E. faecalis, L. monocytogens* ATCC 7644 and *L. monocytogenes* was 10 mg/mL, but for *S. aureus* (MRSA) was 20 mg/mL. The MBC for Gram-negative bacteria UPEC, *E.coli* ATCC 25922 and *P. aeruginosa* was 10 mg/mL, but for EPEC, *K. pneumoniae, S.* Typhimurium*, S.* Enteritidis was 20 mg/mL (Fig. 20C) (Table 8).

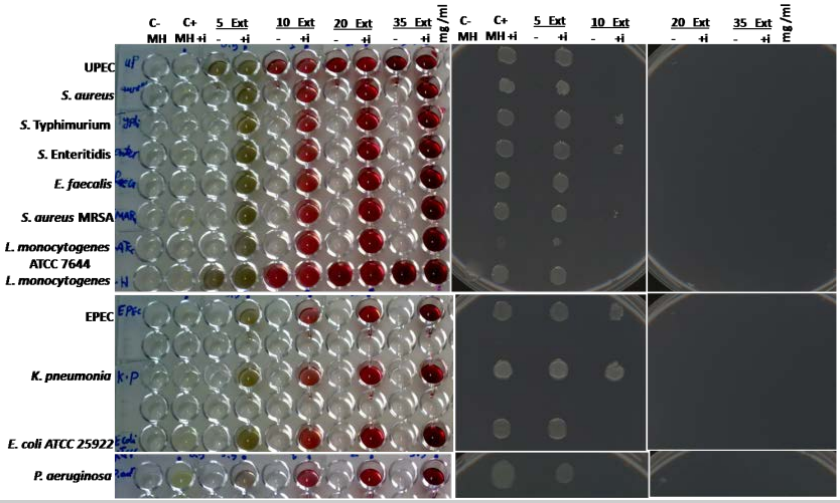


Figure 20. Determination of antibacterial activity of *H. sabdariffa* extract using drop assay: MBC of the aqueous plant extract on Gram-negative and Gram-positive bacteria by inoculating 1.5 µL of each micro well into Mueller-Hinton Agar (MHA) medium.
Abbreviations: C= control, MH= Müeller-Hinton, I= Inoculum, Ext= Extract, numbers as 5= 5 mg/mL of the extract.
(*)*Listeria monocytogenes* was isolated from meat

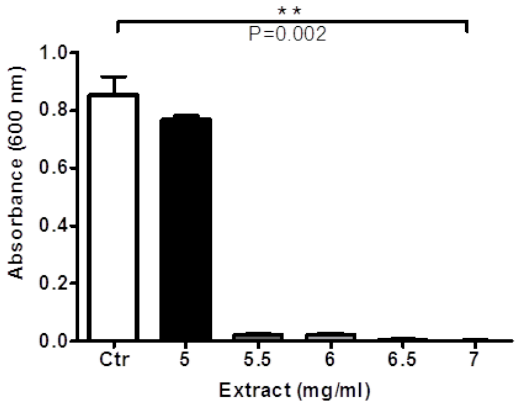
Table 8. *Hibiscus sabdariffa*

Bacteria	MIC				MBC			
	Extract (mg/mL)				Extract (mg/mL)			
	5	10	20	35	5	10	20	35
S. aureus	_	_	_	_	+	_	_	_
S. aureus MRSA	+	_	_	_	+	+	_	_
E. faecalis	+	_	_	_	+	_	_	_
L. monocytogenes	+	_	_	_	+	_	_	_
L. monocytogenes ATCC 7644	+	_	_	_	+	_	_	_
EPEC	+	_	_	_	+	+	_	_
E. coli ATCC 25922	+	_	_	_	+	_	_	_
UPEC	+	_	_	_	+	_	_	_
K. pneumoniae	+	_	_	_	+	+	_	_
S. ser. Typhimurium	+	_	_	_	+	+	_	_
S. ser. Enteritidis	+	_	_	_	+	+	_	_
P. aeruginosa	+	_	_	_	+	_	_	_

(+) means that there is growth of bacteria (-) means that there is no growth of bacteria

To determine the accurate MIC of *H. sabdariffa* against Gram positive and Gra m-negative bacteria, microdilution assay was used with low concentrations of *H. sabdariffa* extract at 5, 5.5, 6, 6.5 and 7 mg/mL against EPEC, *E. coli* ATCC 25922 (Gram-negative), *S. aureus* and *S. aureus* MRSA (Gram-positive). It was observed that the MIC for *S. aureus, S. aureus* MRSA and *E. coli* ATCC 25922 is 6 mg/mL, while for EPEC is 6.5 mg/mL (Fig. 21).

A



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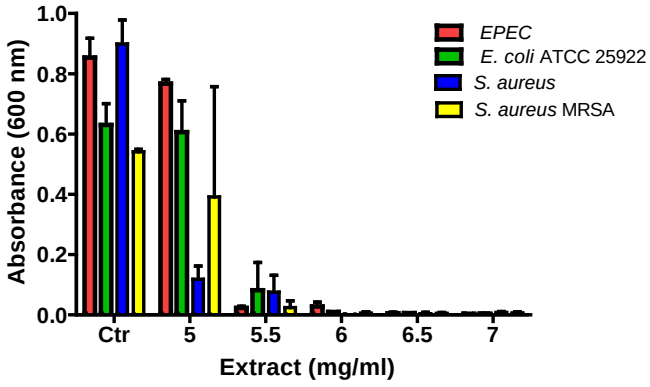

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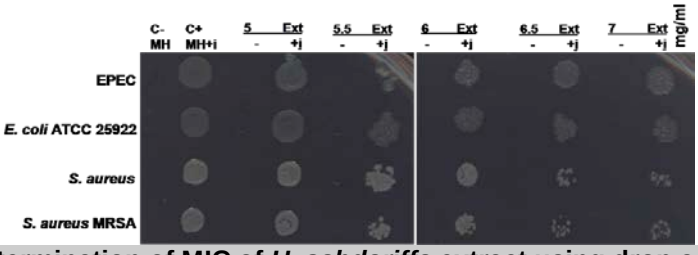


Figure 21. Determination of MIC of *H. sabdariffa* extract using drop assay. **(A)** MIC of *H. sabdariffa* against EPEC. **(B)** MIC of *H. sabdariffa* against two Gram-negative and two Gram-positive bacteria. **(C)** MBC of *H. sabdariffa* against Gram-negative and Gram-positive bacteria. Abbreviations: C= control, MH= Müeller Hinton, i= Inoculum, Ext= Extract, numbers as 5= 5 mg/mL of the extract.

The antibacterial activity of *H. sabdariffa* was determined dublicated in the same microwell plate using microdilution assay against EPEC, *E. coli* ATCC 25922 (Gram-negative), *S. aureus* and *S. aureus* MRSA (Gram-positive). The used concentrations are 5, 7, 10, 20 and 25 mg/mL. It was observed that the MIC is 7 mg/mL for EPEC, *E. coli* ATCC 25922, *S. aureus* and *S. aureus* MRSA in the two panels. But for the MBCs in the two panels were different, in the upper panel, the MBCs for EPEC, *E. coli* ATCC 25955, *S. aureus* and *S. aureus* MRSA are 25, 20, 10 and 20 mg/mL, respectively. In the lower panel, the MBCs for EPEC, *E. coli* ATCC 25922, *S. aureus* and *S. aureus* MRSA are 20, 20, 20 and 10 mg/mL, respectively (Fig. 22). This difference might due to the number of bacteria that was seeded by drop assay (replicator instrument).

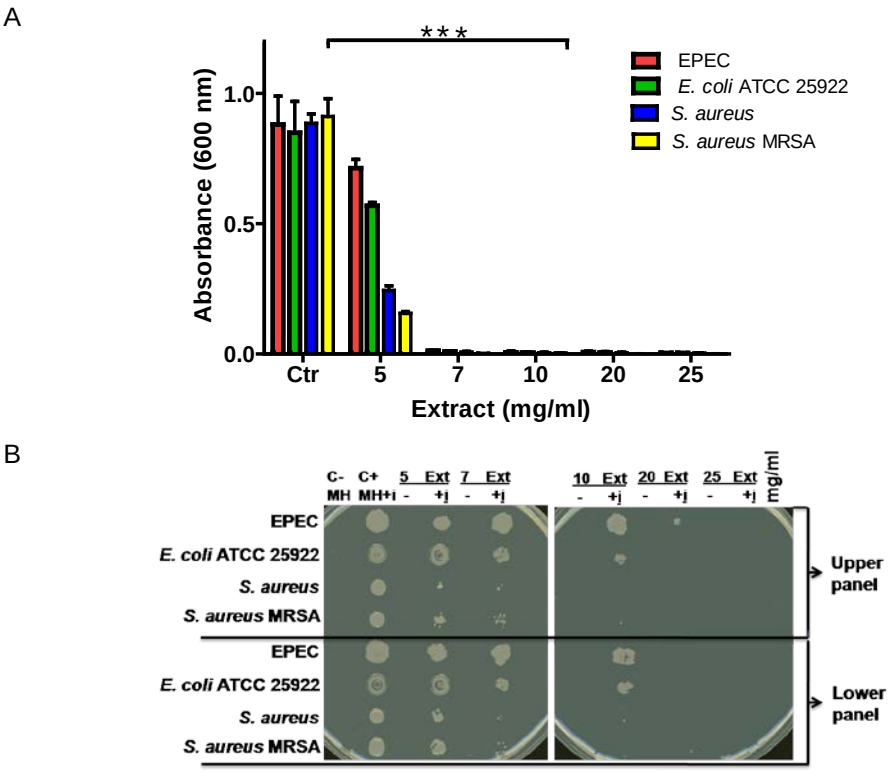


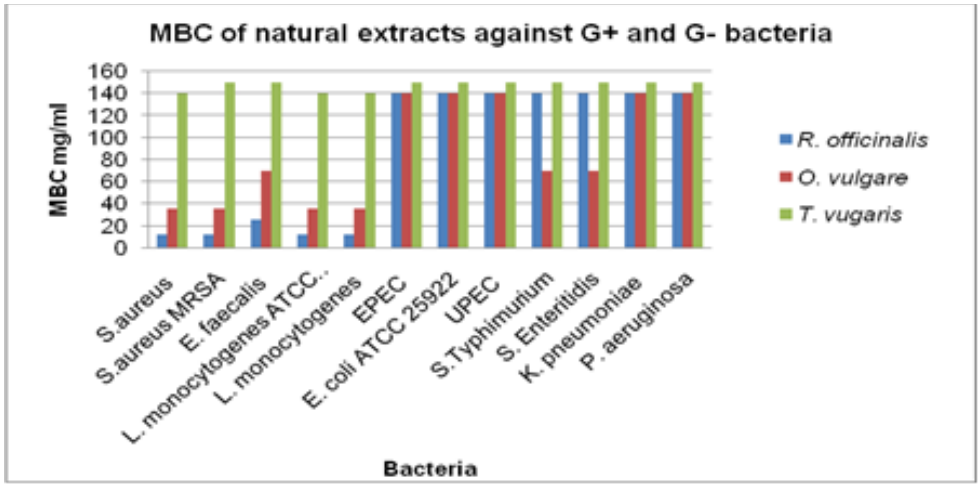
Figure 22. Determination of MIC and MBC of *H. sabdariffa* using drop assay. (A) MIC of *H. sabdariffa* against two Gram-negative and two Gram-positive bacteria. Graph represent mean ± SD. Statistical analysis using two way ANOVA from two independent experiments ***, p<0.0001. **(B)** MBC of *H. sabdariffa* extract against two Gram-negative and two Gram positive bacteria dublicated.
Abbreviations: C= control, MH= Müeller Hinton, i= Inoculum, Ext= Extract, numbers as 5= 5 mg/mL of the extract.

H. sabdariffa extract showed the best antibacterial activity. It presented the lowest MICs and MBCs of the screened medicinal plants in our study (Fig. 2 3 and 24) (Table. 9). In addition, it does not form precipitates and thus it does not interfere with the used methods. For those reasons it has been selected for further characterization studies.

Table 9. MIC and MBC of plant extracts

Bacteria	*C. zeylanicum*		*O. vulgare*		*R. officinalis*		*T. vulgaris*		*H. sabdariffa*	
	mg/mL		mg/mL		mg/mL		mg/mL		mg/mL	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus 29213	≤ 5	≤ 5	≤35	< 35	≤ 12	≤ 12	35	140	≤ 5	10
S. aureus MRSA	≤ 5	10	≤ 35	< 35	≤12	≤12	35	>140	10	10
E. faecalis	≤ 5	20	≤ 70	70	25	25	35	>140	10	10
L. monocytogenes	≤ 5	≤ 5	<35	< 35	≤ 12	≤ 12	35	140	10	10
L. monocytogenes ATCC 7644	≤ 5	≤5	<35	< 35	≤ 12	≤ 12	35	140	10	10
EPEC	20	20	> 140	140	140	>140	35	>140	10	25
E. coli ATCC 25922	20	35	>140	140	140	>140	35	>140	10	10
UPEC	20	20	≤140	140	140	140	35	>140	10	10
K. pneumoniae	20	70	>140	140	>140	>140	35	>140	10	10
S. ser. Typhimurium	10	20	70	70	140	140	35	>140	10	10
S. ser. Enteritidis	10	20	70	70	140	140	35	>140	10	10
P. aeruginosa	10	30	>140	>140	>140	>140	35	>140	10	10

A



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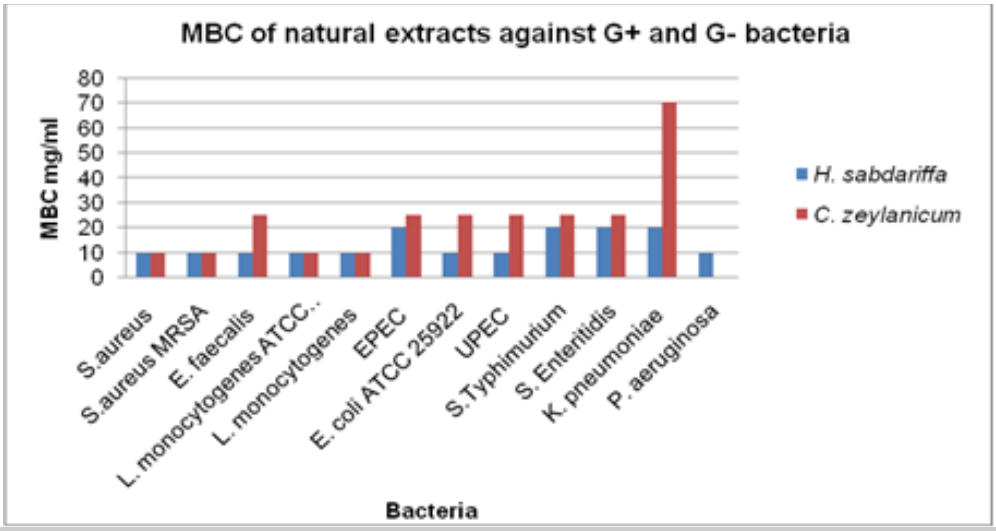


Figure 23. MBC of plant extracts against G-positive and G-negative bacteria. **(A)** MBC of *O. vulgare*, *R. officinalis* and *T. vulgaris* extracts against all tested Gram-negative and Gram-positive bacteria. **(B)** MBC of *H. sabdariffa*, *C. zeylanicum* against all tested Gram-negative and Gram-positive bacteria.

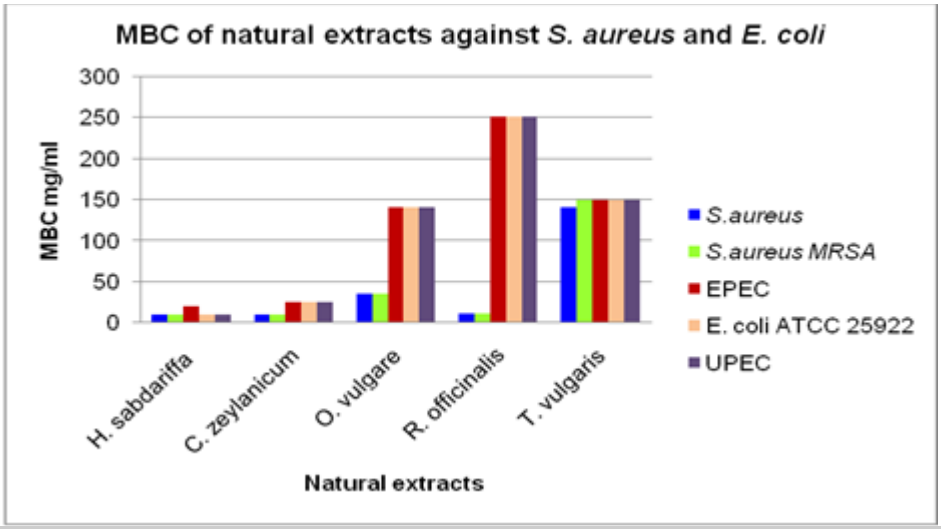


Figure 24. MBC of plants extract against Gram-positive and Gram-negative bacteria. MBC of *H. sabdariffa*, *C. zeylanicum*, *O. vulgare*, *R. officinalis* and *T. vulgaris* extracts against *S. aureus* and *E. coli*. *H. sabdariffa* showed the best antibacterial activity against both Gram-positive and Gram-negative bacteria. The sensitivity of Gram-positive bacteria to the natural extracts are higher than Gram-negative bacteria.

Table 9 and figures 23 and 24 showing that *H. sabdariffa* extract is the most potent antibacterial extract followed by *C. zeylanicum* extract, as they are effective against Gram-positive and Gram-negative. While the *R. officinalis*, *O. vulgare* and *T. vulgaris* extracts showed lower antibacterial activity than *H. sabdariffa* and *C. zeylanicum* against Gram-positive more than Gram-negative, which shows that Gram-positive bacteria are more sensitive to those natural extracts than Gram-negative bacteria.

1.5.1 Antibacterial activity of *Hibiscus sabdariffa* against EPEC using disc diffusion assay

The Kirby-Bauer agar diffusion test is the standardized method for determining antimicrobial susceptibility. The disc diffusion assay was used to assess the antibacterial activity of *H. sabdariffa* extract against EPEC (Fig. 25). Two paper discs (6 mm in diameter) (B and C) were impregnated with the *H. sabdariffa* extract (70 mg/disc) and one disc with 10 µg ampicillin commercially prepared. The impregnated discs were placed on an inoculated MHA plate. The *H. sabdariffa* extract showed zone of

inhibition represents 10.5 mm in MHA and compared with ampicillin antibiotic zone of inhibition which represents 11 mm.

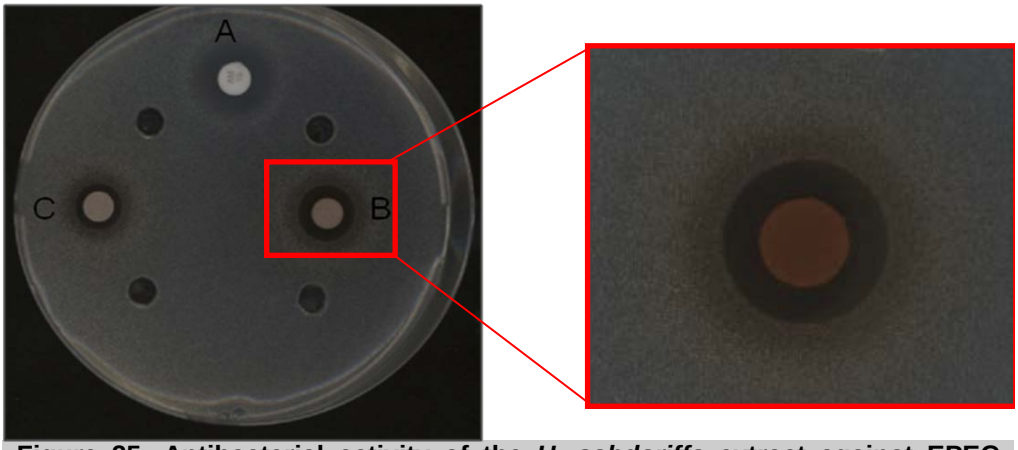


Figure 25. Antibacterial activity of the *H. sabdariffa* extract against EPEC growing on agar Müeller-Hinton by disc diffusion assay. **(A)** Ampicillin disc (10 µg/disc) showing zone of inhibition of EPEC bacterial growth (halo). **(B-C)** Aqueous extract discs (70 mg/disc) also showing zone of inhibition against EPEC bacterial growth (halo) after incubation at 37 ºC for 24 h.

1.5.2 Antibacterial activity of *Hibiscus sabdariffa* extract against *Burkholderia cenocepacia*

In addition the effect of *H. sabdariffa* extract was tested against *B. cenocepacia. B. cenocepacia* is a Gram-negative bacteria, which is an opportunistic pathogen and human infections are common in patients with cystic fibrosis and chronic granulomatous disease, and are often fatal. In cystic fibrosis, it can cause "cepacia syndrome" which is characterized by a rapidly progressive fever, uncontrolled broncho pneumonia, weight loss, and possibly death. The MIC and MBC of *H. sabdariffa* extract against *B. cenocepacia* represent 4 and 5 mg/mL, respectively (Fig. 26A and B).

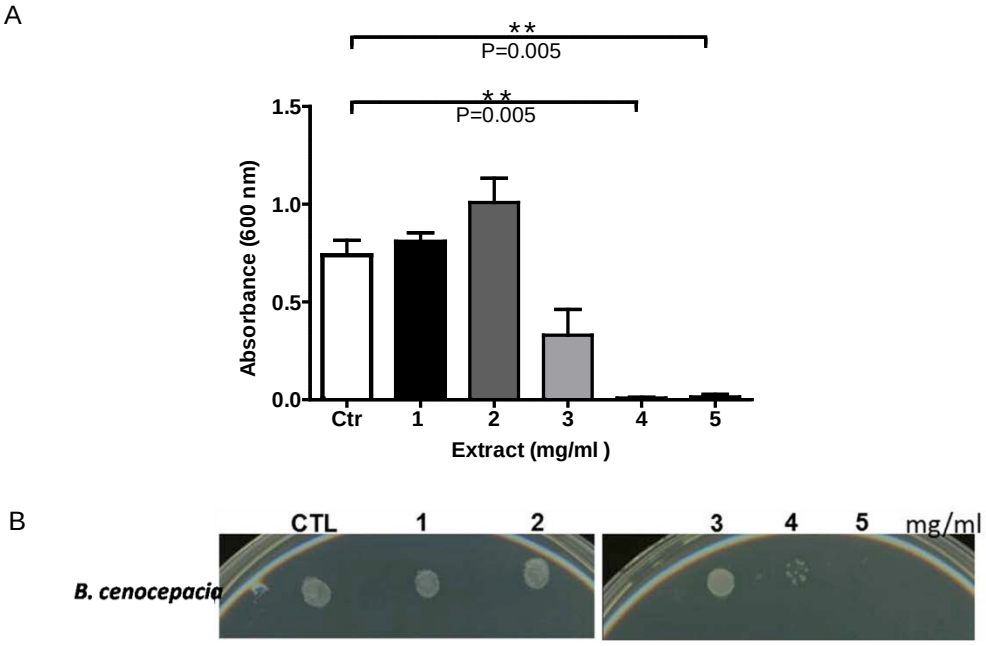
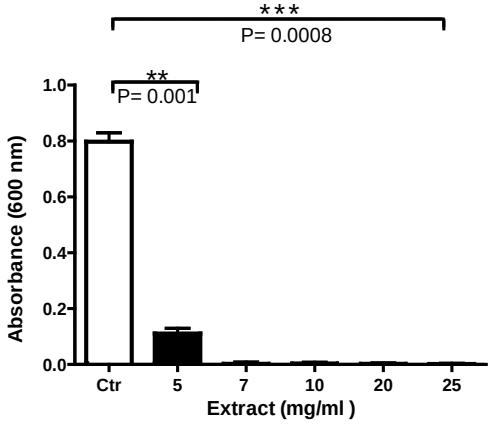


Figure 26. Determination of antibacterial activity of the *H. sabdariffa* extract against *B. cenocepacia* using drop assay. (A) MIC of the aqueous extract is determined by measuring the absorbance at 600nm of the bacterial growth after incubation at 37 ºC for 24 h. Graphic represent results from two independent experiments that were analyzed by Student´s *t-test* **, P=0.005. **(B)** MBC of the aqueous extract is determined by inoculating 1.5 µL aliquots of each microwell on MHA plates,at 37 ºC for 24 h.

1.5.3 MIC and MBC of *Hibiscus sabdariffa* extract against Enteropathogenic *E. coli* EPEC E 234/69

Because of EPEC is the main cause of the infantile diarrhea in developing countries, which is responsible for the children death, we determined the MIC of *H. sabdariffa* extract on EPEC using lower concentrations of 5, 7, 10, 20, 25 mg/mL of *H. sabdariffa* extract. The MIC against EPEC was 7 mg/mL (Fig. 27A), and MBC against EPEC was 25 mg/mL (Fig. 27B and C).

A



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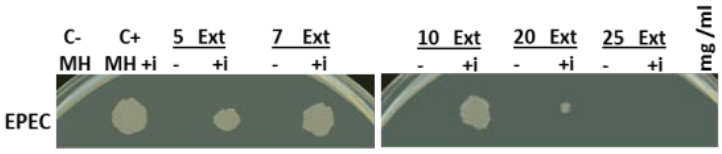

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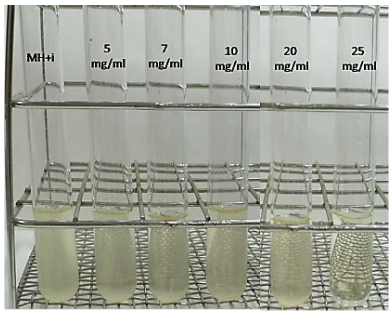


Figure 27. Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of *H. sabdariffa* extract on Enteropathogenic *Escherichia coli* (EPEC). (A) Graph showing the MIC of the aqueous plant extract on EPEC by measuring the absorbance of EPEC growth by using broth micro-well dilution assay. Graph represents results from two independent experiments that were analyzed by Student´s *t-test***, P= 0. 001; ***, P= 0.0008 for 5 mg/mL and the rest of concentrations, respectively. **(B)** Drop (spot) assay showing the MBC by plating 1.5 µL from each tested microwell of multiwell dilution plate onto MHA. **(C)** Macrodilution assay showing the MBC by adding the rest of microwell after drop assay to 5 ml of broth MH media and the growth of bacteria was checked after incubation for 16-18 h with agitation at 37 ºC.
Abbreviations: C= control, MH= Müeller Hinton, i= Inoculum, Ext= Extract, numbers as 5= 5 mg/mL of the extract.

1.5.3.1 Time-kill curves and time-kill kinetics

Lethality-time or time-kill curves provide important information on the dynamics of the microbicidal action of an antimicrobial agent and on

relationship between the concentration of the antimicrobial agent and its microbicidal activity. These time-kill studies are important parameters for the assessment of the bactericidal compounds efficacy.

Time kill curves were performed for EPEC using different concentrations of *H. sabdariffa* extract 10 mg/mL (1.4 x MIC) and 25 mg/mL (3.6 x MIC). The *bactericidal activity* is considered as the time needed to achieve a 3-log decrease in viability respect to the initial time of the used antibacterial concentration. This parameter is an acceptable index of bactericidal activity (Cantón and Pemán, 1999).

The results obtained in figure 28 shows that the 3-log decrease in cell viability occurred at 10 h. This is the time at which the extract concentration 25 mg/mL (MBC) is bactericidal.

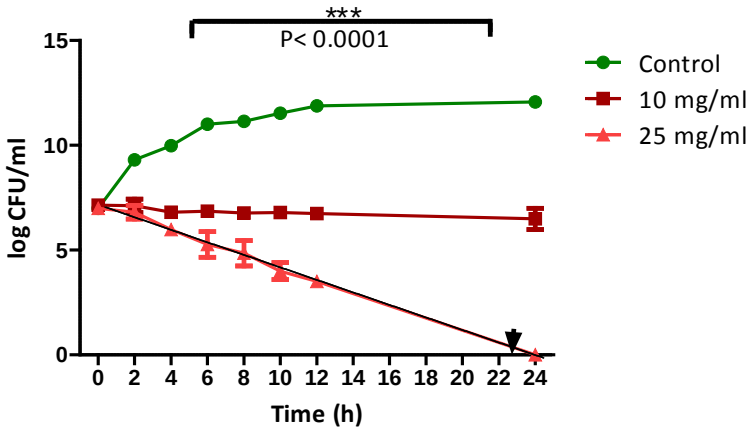


Figure 28. Time Kill kinetics of EPEC using *H. sabdariffa* extract. Time-killing curves representing the growth of EPEC *versus* time in absence (●: control) or presence of *H.sabdariffa* extract (■: 10 mg/mL; ▲: 25 mg/mL) in Müeller Hinton liquid media. Graphic represent results from two independent experiments that were analysed by two way ANOVA **, P<0.0001..

According to the calculation from figure equation, K values are: K_{25}=0.131h^{1}, K_{10}=0.018 h^{-1}. For 25 mg/mL (MBC), the following parameters are derived from the killing equation: the mean times to achieve reductions in the proportions of viable cells of 50% (t50)=2.3h, 90% (t90)=7.6h, and 99% (t99)=15.3 h, and the time to reach the bactericidal endpoint 99.9%

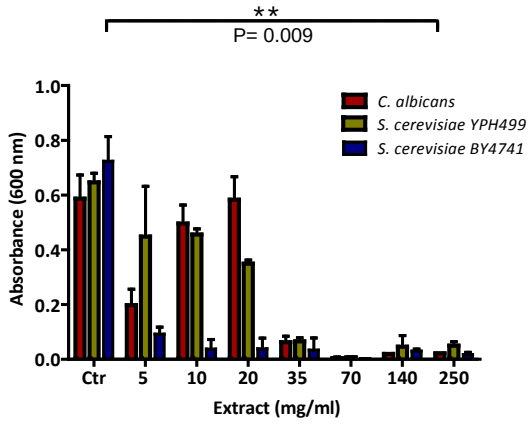
(t99.9)=23 h. For 10 mg/mL (MIC), the mean times to achieve reductions in the proportions of viable cells of 50% (t50)=16.5 h, 90% (t90)=55 h, and 99% (t99)=110 h and the time to reach the bactericidal endpoint 99.9% (t99.9)=165 h. These results suggest that the *H. sabdariffa* extract at MIC (10 mg/mL) the endpoint of bactericidal activity would be reached in 6 9 days. Whereas with the MBC (25 mg/mL) the endpoint is reached in 23 h. Nevertheless, it should be notice that we are working with a crude extract and not with a pure active principle.

2. Activity of *Hibiscus sabdariffa* extract on Fungi

In order to investigate the potential antifungal activity of the *H. sabdariffa* extract, two different fungi (yeasts) were used. The pathogenic yeast (fungi) *Candida albicans* and two strains of the non-pathogenic yeast *Saccharomyces cerevisiae*: *S. cerevisiae* YPH 499 and *S. cerevisiae* BY4741. The extract concentrations assayed were 5, 10, 20, 35, 70, 140 and 250 mg/mL by microwell and drop assays, as described in Material and Methods. The MICs were determined by measuring the absorbance of fungal growth at 600 nm in YPD liquid media (Fig. 29A).

The MBCs were determined by drop assay inoculating 1.5 µl aliquots of each microwell on YPD agar plates after incubation at 30 ºC for 24 h (Fig. 29B). We observed that the *H. sabdariffa* extract inhibits the growth of *S. cerevisiae* BY4741 at 10 mg/mL and *C. albicans*, *S. cerevisiae* YPH 499 at 70 mg/mL (Fig. 29A). The *H. sabdariffa* extract does not have fungicidal activity on *C. albicans* and *S. cerevisiae* BY4741 even up to 250 mg/mL. However at 140 mg/mL the extract has fungicidal activity against *S. cerevisiae* YPH 499 (Fig. 29B), but this concentration is too high to be considered as useful (or no toxic). These results imply that *H. sabdariffa* extract can not be used for treatment of fungal infections.

A



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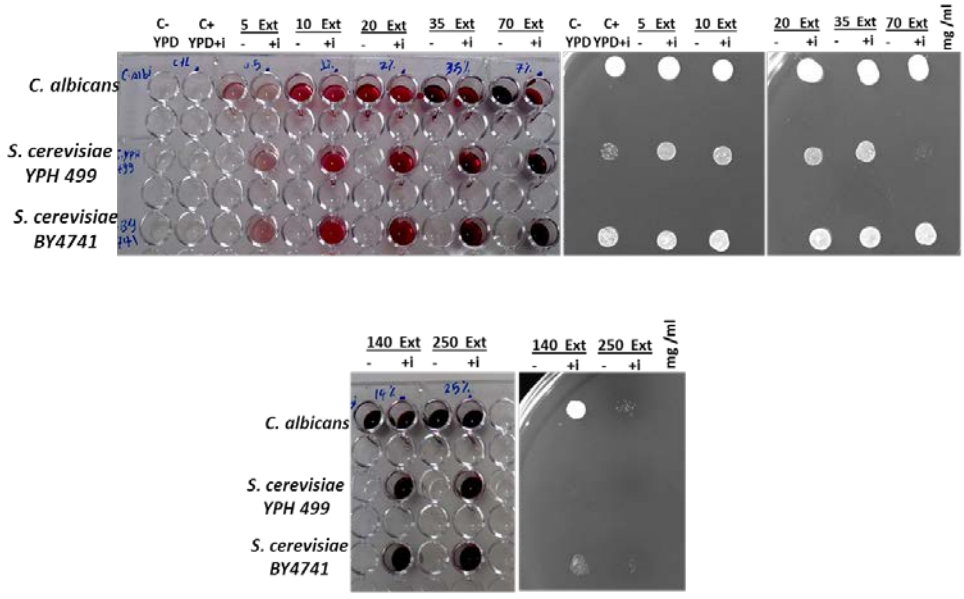

Figure 29. Effect of *H. sabdariffa* extract on Fungi. (A) Determination of the MIC of the *H. sabdariffa* extract against *C. albicans*, *S. cerevisiae* YPH499 and *S. cerevisiae* BY4741 using drop assay at 5, 10, 20, 35, 70, 140 and 250 mg/mL concentrations of the aqueous extract in Yeast Extract-Peptone-Dextrose (YPD) broth media at 30 ºC for 24 h. Graphs represent mean ± SD. Graph represent results from two independent experiments that were analyzed by two way ANOVA**, P= 0.009. **(B)** Determination of MBC and growth on YPD agar plates after incubation at 30 ºC for 24 h.

2.1 Effect of *H. sabdariffa* on the morphology of *Candida albicans*

Although it was not observed antifungal activity on the dimorphic yeast *C. albicans*, the potential effect on the morphology was investigated.

C. albicans undergoes the transition from blastospores which are round budding cells, to filamentous forms, which are elongated cells in response to high temperature (37 ºC) at neutral pH or in the presence of serum at 30 ºC (Kadosh and Johnson, 2005). It was observed at acidic pH of *H. sabdariffa* extract filamentous forms of *C. albicans* at different concentrations of the extract. We observed that the *H. sabdariffa* extract (pH= 4 at 5 mg/mL, pH=2.5 at 10 mg/mL and pH=2 at 20, 35 and 70 mg/mL) induces filamentous forms of *C. albicans* when they were incubated overnight in MHB medium at various concentrations, at 30 ºC (Fig. 30). The filamentous morphology was observed at 5, 10, 20, 35 mg/mL, although the cultures presented mixed morphologies (Fig. 30B, C, D and E). However, we could not detect filaments at higher concentrations of 70 mg/mL (Fig. 30F).

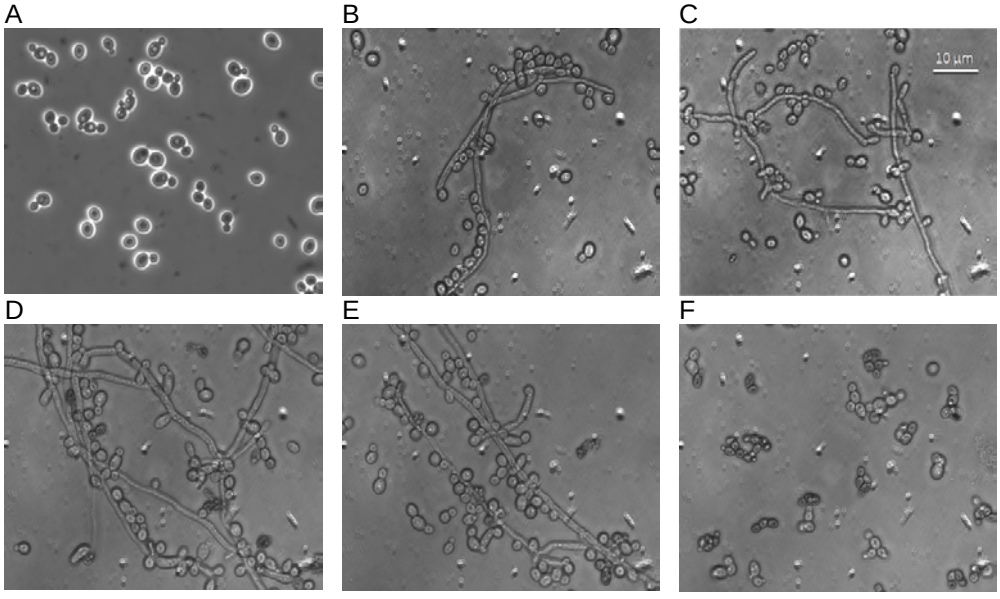

Figure 30. Effect of *H. sabdariffa* extract on *C. albicans* cells morphology. (A) *C. albicans* growth control without treatment of the aqueous extract**.** Treated *C. albicans* with the aqueous extract at **(B)** 5 mg/mL **(C)** 10 mg/mL **(D)** 20 mg/mL **(E)** 35 mg/mL **(F)** 70 mg/mL. All treated *C. albicans* transformed from blastospore to filament transition after incubation overnight (18 h) in Müeller-Hinton liquid medium at 30°C. Micrographs of phase contrast 400X. Scale bar is 10 µm.

2.2 Effect of *H. sabdariffa* on the morphology of *Yarrowia lipolytica*

In order to clarify if the observed effect of the extract on *C. albicans* cell morphology was a common effect to other dimorphic yeast, the *H. sabdariffa* extract was assayed with the non pathogenic yeast *Yarrowia lipolytica*.

In our study, we observed that in cultures of *Y. lipolytica* ($4x10^5$ CFU/mL) in YPD broth medium at 30 ºC, for 16-18h, the extract induces the mycelium form of *Y. lipolytica* in a dose response manner at concentration ranging from 5, 10, 20, 35 mg/mL (Fig. 31B, C, D and F). In this case, the culture cell morphology was more homogenous, and hypha were predominant. As previously observed with *C. albicans*, in *Y. lipolytica* the formation of mycelium was not produced at higher concentrations of 70 mg/mL of the aqueous extract (Fig. 31F).

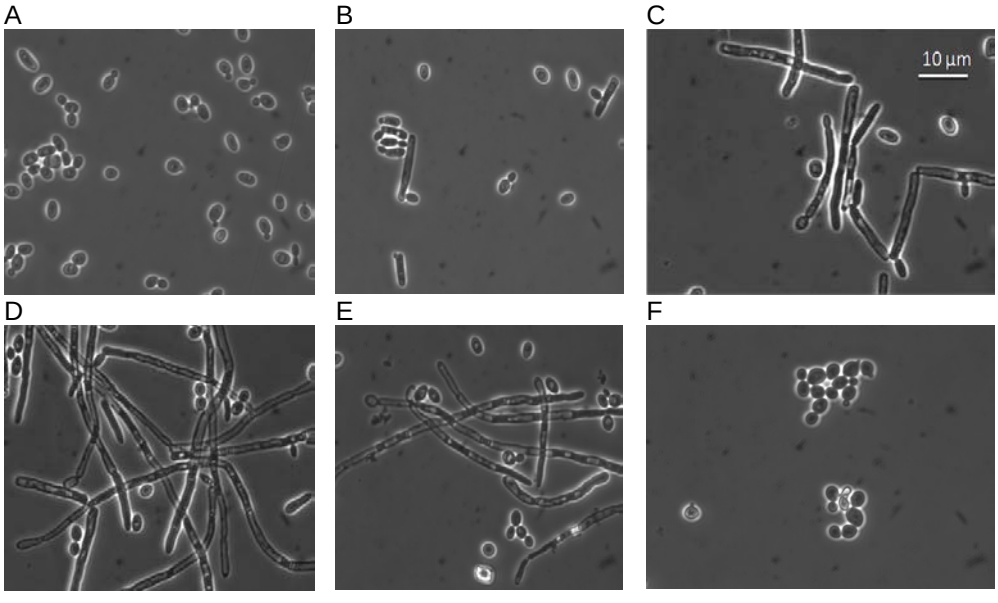

Figure 31. Effect of *H. sabdariffa* extract on *Y. lipolytica* cells morphology. (A) *Y. lipolytica* in absence of the aqueous extract (growth control).Treated *Y. lipolytica* with the aqueous extract at **(B)** 5 mg/mL **(C)** 10mg/mL **(D)** 20mg/mL **(E)** 35mg/mL **(F)** 70mg/mL in YPD liquid medium al 30 ^{o}C, 18h. Micrographs of phase contrast 400X. Scale bar represents 10 µm.

3. Microbial quality of the dried calyces of *Hibiscus sabdariffa*

The most potent antibacterial extract assayed was the aqueous extract of *H. sabdariffa*, both against Gram-negative and Gram-positive bacteria, especially against enteric pathogens, as showed previously. For this reason, the *H. sabdariffa* extract was selected for further studies and potential use as natural product to prevent or treat infectious diarrhea. Nevertheless, it is necessary to analyze the microbial quality of the plant material used before its potential oral use and further microbiological, molecular and chemical characterizations.

Medicinal herbs and plants can be considered either as ***pharmaceutical preparations*** or as ***food and beverages*** in terms of their microbial quality, in compliance with the European Pharmacopoeia (Ph. Eur.) and the Royal Spanish Pharmacopoeia (Ph.R.Sp.) on one hand; or in compliance with the Spanish Regulations on Food and Beverages (Codex Alimentarius) (Anderson and Calderón, 1999).

In addition other microorganisms have been investigated because their clinical and public health importance, such as *S. aureus*, *B. cereus and L. monocytogenes,* using standard microbiological methods. The results (Table 10) show that the calyces of *H. sabdariffa* that are used in our study did not have *Salmonella*, *E. coli*, *S. aureus*, *L. monocytogenes*, neither *B. cereus;* but it have Enterobacteria (18 CFU/10 g), and fungi ($5x10^2$ CFU /g). Although the dry calyces of *H. sabdariffa* have some Enterobacteria, and fungi, this batch can be used for human consumption because it complies with the microbial criteria for this type of products according to the European and Spanish regulations.

Table 10. Microbial quality of *H. sabdariffa* calyces	
Total viable aerobic count	$3x10^5$ CFU/g
Salmonella	absence /25 g
Enterobacteria	18 CFU/10 g
E. coli	absence / 10 g
S. aureus	MPN<3/g
B. cereus	$<10^2$ /g
L. monocytogenes	absence /10 g
Fungi	$5x10^2$ /g

4. Determination of total phenolic compounds content (TPC)

Phenolic compounds are a class of plant secondary metabolites. They are characterized by presence of one or more of six carbon rings and two or more phenolic hydroxyl group. For example, caffeic acid is a common representative of simple phenolic compounds that is present in tarragon and thyme, and is active against bacteria, viruses and fungi (Cowan, 1999; Karou, 2005). Polyphenols have direct antioxidant activity through inactivating harmful free radical such as lipid peroxides and by chelation of divalent metal ions, and have antioxidant activity indirectly through the induction of endogenous protective enzymes. Polyphenols content of aqueous plant extracts has been related to antibacterial activity due to their cytotoxic action on microorganisms, which include enzyme inhibition by the oxidized compounds (Cowan, 1999; Stevenson, 2007). Furthermore, it is well known that the phenolic compounds contribute to quality and nutritional value of plants in terms of modifying color, taste, aroma and flavor. Therefore we determined the TPCs content as described in Material and Methods (Point 3) of different aqueous plant extracts *Cinnamomun zeylanicum, Origanum vulgare*, *Rosmarinus officinalis*, *Thymus vulgaris*

and *Hibiscus sabdariffa*. The results obtained (Table 11) show that *O. vulgare and R. officinalis* extracts contain higher polyphenol compounds content (28 and 27.12 mg/g, respectively) than the rest of extracts, while *C. zeylanicum* and *T. vulgaris* have lower phenolic compounds content (9.9 and 8.9 mg/g, respectively) than the rest of extracts. *H. sabdariffa* contains 12.7 mg/g which is considered a moderate content of total phenolic compounds. Sirag *et al.,* (2014) determined the total phenolic compounds in ethanolic extract of *H. sabdariffa*. The phenolic content represents 41.07 mg/g. Their study determined phenolic compounds content higher than our value may be due to the extracts are different as our extract is aqueous extract and his extract is ethanolic (Sirag, *et al*., 2014).

Table 11. Total phenolic compounds content of the aqueous plant extracts

Plant extracts	Total phenolic compounds content (mg/g) (as mg gallic acid equivalents)
O. vulgare	28
R. officinalis	27.12
H. sabdariffa	12.7
C. zeylanicum	9.9
T. vulgaris	8.9

5. Cytotoxicity determination

5.1 Cytotoxicity of *Hibiscus sabdariffa* extract on Macrophages

In order to investigate the cytotoxicity effects of the *H. sabdariffa* extract, we measured cell viability as previously described (O'Brien, 2000), as mentioned in Materials and Methods. The J774 murine macrophage cell line was used to evaluate the cytotoxicity of the aqueous plant extracts by using the Rezasurin reduction test. It is considered that the cytotoxic

concentration is the one that reduces the cell viability of macrophages from 100% to 80-75%. In consequence, the cytotoxicity effect on viability of macrophages was assessed by determining the concentration of *H. sabdariffa* extract that reduced cell viability to 80-75 % compared to the cell viability of control untreated cells. The results which are presented in figure 32, indicate that the *H. sabdariffa* extract has no toxic effects on J774 murine macrophages cell line at 10, 50 and 100 µg/mL. Concentrations of the *H. sabdariffa* extract such as 1 mg/mL induce a pH change that was appreciable by the change in the color of culture medium. This change could contribute to macrophages death, and therefore we discard this concentration. All together these results indicate that the *H. sabdariffa* extract has not cytotoxic effects at concentrations of 10, 50 and 100 µg/mL.

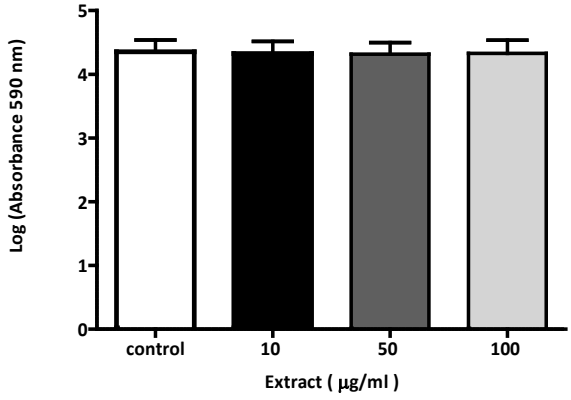

Figure 32. Effect of *H. sabdariffa* on cell viability. The *H. sabdariffa* extract did not show a significant decrease in cell viability of the J774 cell line (murine macrophages) at 10, 50 and 100 µg/mL concentrations. White bar represents the log of absorbance of resazurine in the presence of macrophages without the extract (blank). The black, dark grey and light grey bars represent the absorbance of resazurine in the presence of macrophages plus the aqueous plant extract at 10, 50 and 100 µg/mL, respectively. Results of three independent experiments analyzed by Student´s *t-test*

5.2 Cytotoxicity of *Hibiscus sabdariffa* extract on HeLa cells

Alteration of cell growth is another indicator of cytotoxicity. In other words, the capability of cells to divide is used as an index of toxicity. The

concentration of a given plant extract at which 50 percent of HeLa cells do not divide is called the **median inhibitory dose (ID_{50})** (Ekwall 1990). We measured the cytotoxicity effect of the *H. sabdariffa* extract on HeLa cells by using trypan blue exclusion assay. HeLa cells were plated at 150 000 cells per well of 6-well plate. After 24 h, cells were treated with the *H. sabdariffa* extract at 5, 7 and 10 mg/mL concentrations for 24 h. Then, the cells were harvested by trypsinization and counted under a microscope by adding trypan blue dye, which only enters dead cells. We observed that the *H. sabdariffa* extract did not show a significant decrease of growth of HeLa cells at 5 and 7 mg/mL concentrations. However, it showed a significant cell growth inhibition at 10 mg/mL. At such concentration HeLa cell growth was inhibited by approximately 50 % (Fig. 33).

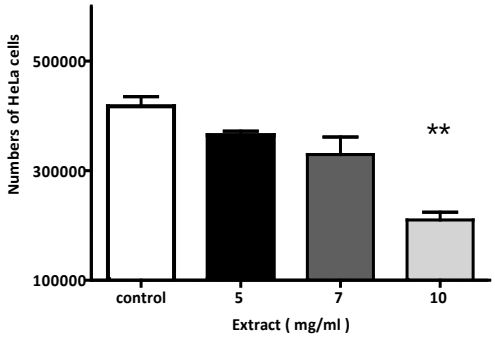

Figure 33. Cytotoxicity effect of *H. sabdariffa* extract on cell growth. White bar represents the numbers of HeLa cells without treatment (control). The black, dark grey and light grey bars represent the numbers of HeLa cells in the presence of *H. sabdariffa* extract at 5, 7 and 10 mg/mL, respectively. Results of two independent experiments are shown. Analysis was done by Student´s *t-test*

5.3 Cytotoxicity of *Cinnamomum zeylanicum* on Macrophages

Similarly, in order to investigate the cytotoxicity effects of the *C. zeylanicum* extract, we proceed as before (Point 12.1 of Material and Methods). The results which are presented in figure 34 showed that the cell viability of macrophages is decreased from 100 % to 82 % and to 81.5 % at concentrations of 50 and 100 µg/ml, respectively. These results indicate

that the *C. zeylanicum* extract is not toxic at 10, 50 and 100 µg/ml concentrations on J774 murine macrophages cell line, compared with the control, although it is close to the limit considered as cytotoxic, as previously mentioned.

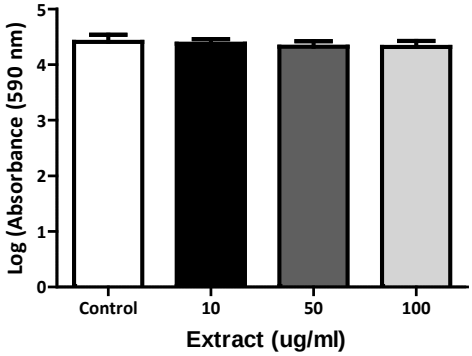

Figure 34. Effect of *C. zeylanicum* extract on cell viability. The aqueous plant extract of *C. zeylanicum* **does not affect** the cell viability of J774 cell line murine macrophages at 50 and 100 µg/mL. White bar represents the log of absorbance of resazurine in presence of macrophages without the extract (blank). The black, dark grey and light grey bars represent the absorbance of resazurine in presence of macrophages plus the aqueous plant extract at 10, 50 and 100 µg/mL, respectively. Results of three independent experiments analyzed by Student´s *t-test.*

6 Effect on pedestal formation induced by EPEC

6.1 Effects of the *Hibiscus sabdariffa* extract on pedestal formation induced by EPEC

Since the *H. sabdariffa* extract shows antibacterial activity (Fig. 19 and 20) and does not have significant toxic effects on cells at concentrations up to 100 µg/mL (Fig. 32), we aimed to investigate its effect on pedestal formation induced by enteropathogenic *Escherichia coli* (EPEC) in HeLa cells. To that purpose, HeLa cells were seeded at 150 000 cells/well in 6 well plates and incubated for 24 h. Then, cells were infected at a MOI of 10 with preactivated EPEC for 3 h. After removing the unattached bacteria by washing 3 times with D-PBS, the cells were treated with the *H. sabdariffa* extract for 1, 2 and 3 h. The number of pedestals of treated and untreated

cells were quantified in 40 HeLa cells stained with TRITC-phalloidin and DAPI, to label the actin pedestals and the bacteria respectively.

The results obtained are shown in Fig. 35, where it can be observed that cells treated with the aqueous plant extract show a significant inhibition of pedestal formation while untreated cells show increase numbers of pedestals along time (Fig. 35 and 36).

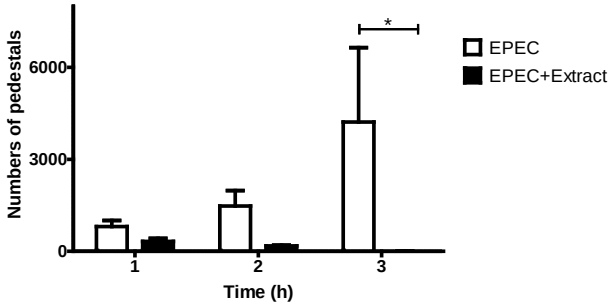

Figure 35. Quantitation of the number of pedestals after treatments with *H. sabdariffa* extract. HeLa cells were infected with EPEC for 1, 2 and 3 h. After washing off the unattached bacteria the number of pedestals formed was counted in cells treated with 10 mg/mL of aqueous extract of *H. sabdariffa* (black bars) and non-treated (white bars) cells. Graph represent results from two independent experiments that were analyzed by 2 way ANOVA test *, P=0.014.

These results indicate that the treatment with *H. sabdariffa* extract at 10 mg/mL of EPEC infected HeLa cells decreases the numbers of pedestals in a time dependent manner.

After demonstrating that the numbers of pedestals formed at 3 hours are decreased by treatments with the aqueous plant extract at 10 mg/mL concentration for 1, 2 and 3 h post-infection, we hypothesized that the aqueous plant extract could prevent pedestal formation. Two strategies were applied to test the hypothesis. Firstly, HeLa cells were treated with the aqueous plant extract for 3 h. After removing the aqueous plant extract, HeLa cells were washed 3 times before infection by EPEC for 3 additional hours (preinfection treatments, Fig. 37). Secondly, the aqueous plant extract and EPEC infection to HeLa cells were added simultaneously (simultaneous treatments, Fig. 38). In both strategies, we observed that the plant extract at 10 mg/mL concentration prevents pedestal formation.

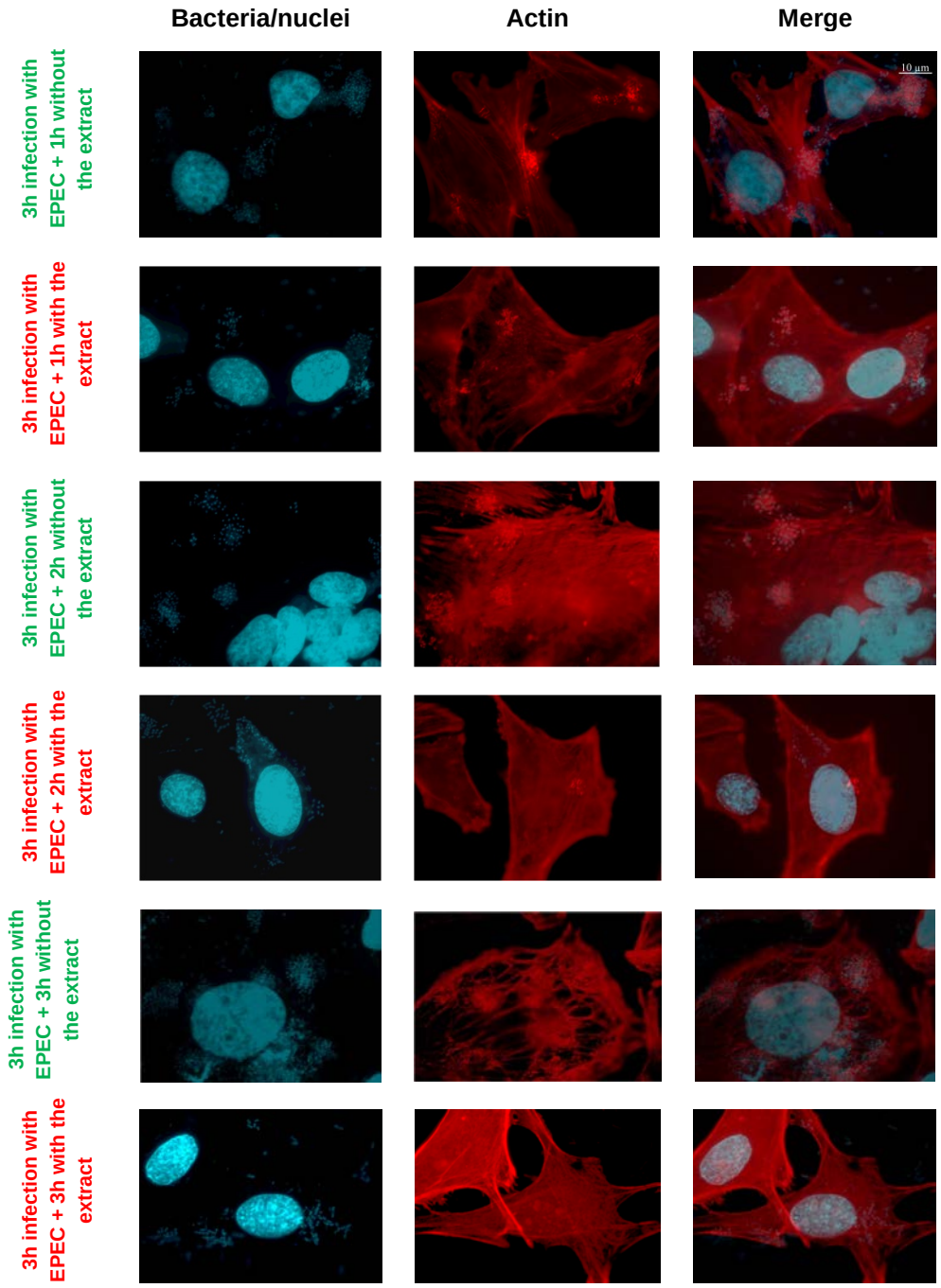

Figure 36. Effect of the treatment with the aqueous extract of *H. sabdariffa* on pedestal formation by EPEC. Visualization of pedestals after treatments. HeLa cells were infected with EPEC at a MOI of 10 for 3 hours. After washing off the unattached bacteria, the cells were treated with the aqueous plant extract of *H. sabdariffa* at 10 mg/mL or left untreated as a control for the indicated times. Then pedestals were visualized by fluorescence staining with TRITC-phalloidin (red pictures) to stain actin and DAPI to stain bacteria (blue pictures), respectively. Pictures were taken on an epifluorescence microscope at 1000X magnification. Images were merged with the Adobe Photoshop software. Scale bar represents 10 µm.

The first strategy is shown in Fig. 37 where it can be observed that pedestals are not formed by EPEC in HeLa cells, when the cells are previously treated with the aqueous extract at 10 mg/mL concentration, compared with untreated-infected cells for 3 h as a control of pedestal formation.

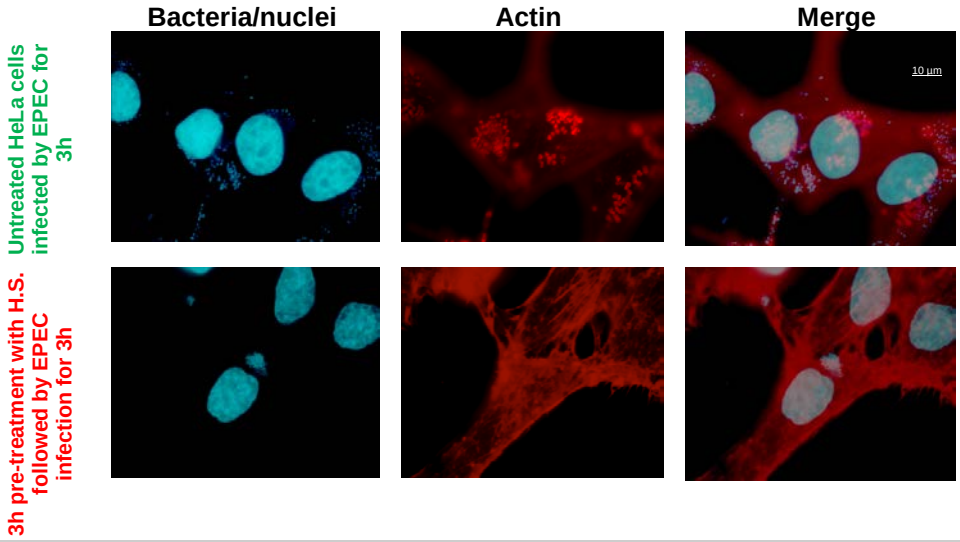

Figure 37. Effect of the treatment of the *H. sabdariffa* extract on pedestal formation by EPEC. HeLa cells were treated with the *H. sabdariffa* extract or left untreated, as a negative control, for 3 hours. After washing the cells, they were infected with EPEC at a MOI of 10 for 3 h. Then pedestals were visualized by fluorescence staining with TRITC-phalloidin (red pictures) to stain actin and DAPI to stain bacteria (blue pictures) respectively. Pictures were taken on an epifluorescence microscope at 1000X magnification. Images were merged with the Adobe Photoshop. Scale bar represents 10 µm.

The second strategy is shown in Fig. 38 where it can be observed that pedestals are not formed by EPEC in HeLa cells, when the *H. sabdariffa* extract at 10 mg/ml concentration and the bacteria (EPEC) were added to HeLa cells simultaneously and allow to proceed for 3 h. Untreated-infected HeLa cells are shown as a control of pedestal formation.

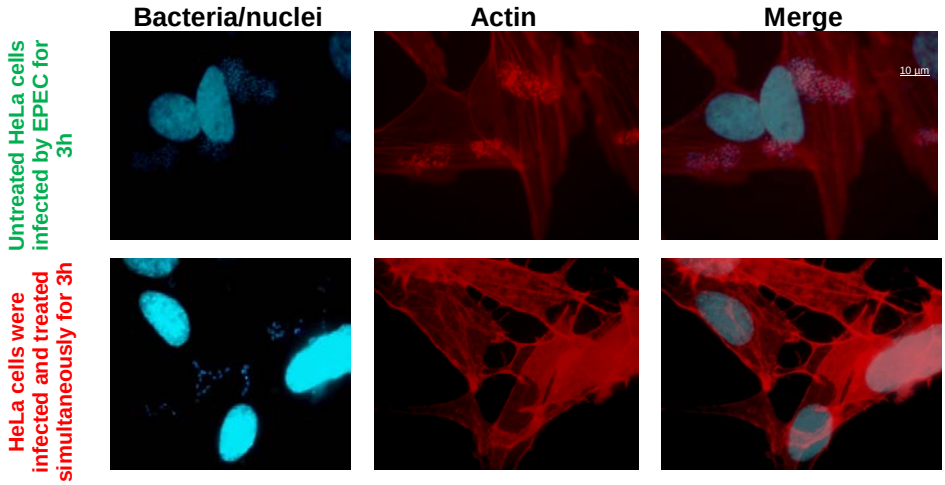

Figure 38. Effect of the treatment with the *H. sabdariffa* extract on pedestal formation by EPEC. The aqueous plant extract and EPEC were added to HeLa cells simultaeneously and infections were allow to proceed for 3 h. As a control, HeLa cells were infected by EPEC for 3 h without pretreatment. EPEC was added at a MOI of 10. Pedestals were visualized by flourescence staining with TRITC-phalloidin (red pictures) to stain actin and DAPI to stain bacteria (blue pictures) respectively. Picture were taken on an epifluorescence microscope at 1000X magnification. Images were merged with the Adobe Photoshop software. Scale bar represents 10 µm

6.2 Effect of *Cinnamomum zeylanicum* extract on pedestals induced by EPEC

After observing that the *C. zeylanicum* extract has antibacterial activity against Gram-negative and Gram-positive bacteria (Fig. 11 and 12), we decided to investigate its effect on pedestal formation induced by enteropathogenic *Escherichia coli* (EPEC) in HeLa cells. For that purpose, we proceed as before (Fig. 36). The results obtained are shown in Fig. 39, where it can be observed that cells treated with the aqueous plant extract of *C.* zeylanicum at 20 mg/mL show a significantly decreased number of pedestals compared to untreated cells (Fig. 39).

These results indicate that treatments of EPEC infected HeLa cells with the *C. zeylanicum* extract at 20 mg/mL decrease the numbers of pedestals in a time dependent manner.

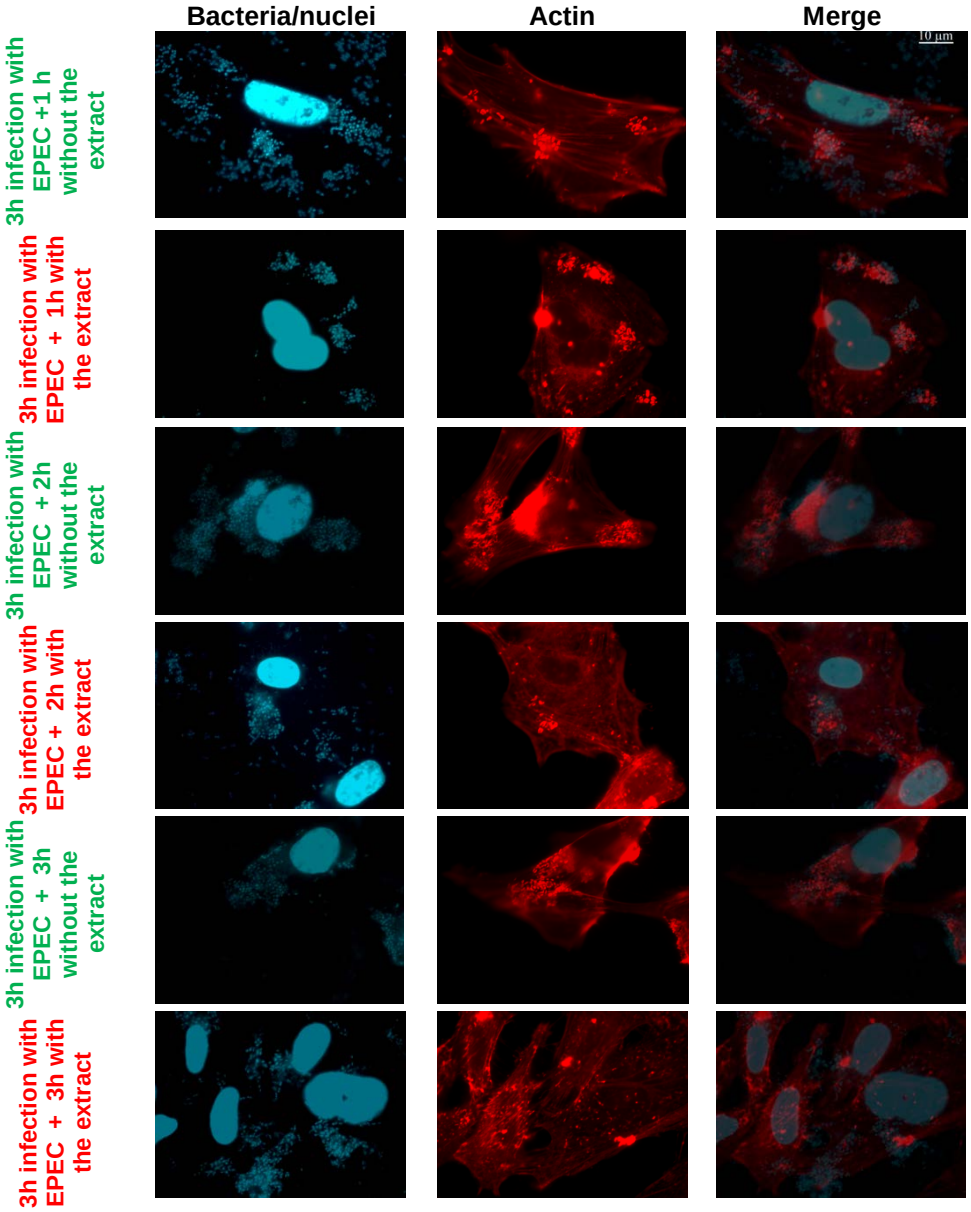

Figure 39. Effect of treatments with the *C. zeylanicum* extract on pedestal formation by EPEC. HeLa cells were infected with EPEC at a MOI of 10 for 3 hours. After washing-off the unattached bacteria, the cells were treated with the aqueous plant extract of *C. zeylanicum* at 20 mg/mL or left the cells untreated *as a control for the indicated times*. Then pedestals were visualized by fluorescence staining with TRITC-phalloidin (red pictures) to stain actin and DAPI to stain bacteria (blue pictures) respectively. Pictures were taken on an epifluorescence microscope at 1000X magnification. Images were merged with the Adobe Photoshop software. Scale bar represents 10 µm.

7. Effect of *Hibiscus sabdariffa* extract on morphology of *E. coli* EPEC E 234/69 a

While we were doing EPEC infection experiments of HeLa cells in the experiments where EPEC and the extract were added simultaneously, we observed the preparations under a fluorescence microscope and noticed a filamentous morphology of EPEC after they were exposed to the *H. sabdariffa* extract at 10 mg/mL for 3 h compared to control (Fig. 40A).

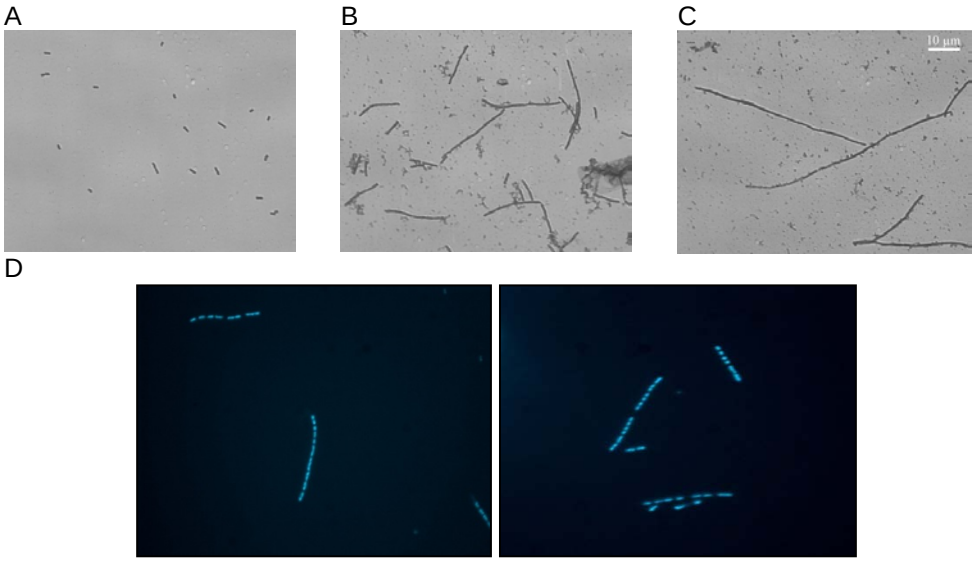

Figure 40. Effect of the *H. sabdariffa* extract on the morphology of EPEC. Simple staining with crystal violet of **(A)** Untreated EPEC (Control). **(B)** EPEC treated with the aqueous extarct of *H. Sabdariffa* at 10 mg/mL for 3 h. **(C)** EPEC treated with ceftazidime antibiotic at 0.5 µg/mL for 3 h. **(D)** EPEC stained with DAPI after exposure to the aqueous plant extract of *H. sabdariffa* for 3 h. Micrographs of phase contrast and epifluorescence. Scale bar represents 10 µm.

It was reported (Buijs, *et al*., 2007) that ceftazidime, a β-lactam antibiotic, induces a filamentous morphology of Gram-negative bacteria *E. coli* at sub-MIC or MIC concentrations. For this reason, using Gram´s and DAPI staining (Fig. 40D). We compared the effect of *H. sabdariffa* extract (10 mg/mL for 3 h) to the effect of ceftazidime (0.5 µg/mL for 3 h) on EPEC at 4 x 10^5 CFU/mL. Ceftazidime was used as a positive control of the induction of the filamentous morphology of EPEC (Fig. 40C). These results can be useful to investigate the mechanism of action of *H. sabdariffa* as an antibacterial agent.

8. Chemical fractionation of *Hibiscus sabdariffa* extract

8.1 Yield of fractions of *Hibiscus sabdariffa* extract calyces

10 g of *H. sabdariffa* is soaked overnight in 100 ml of mili Q water then is filtered, and subjected to lyophylisation. Thus from 100 mL of 10 % aqueous extract of *H. sabdariffa* calyces we obtained 5.4-5.6 g (55%). The yield of the other fractions was as follows: EtOAc fraction extract represented 0.42 %, the AC fractions extract represented 2.8 %, the EtOH represented 51.5 %, while the AC fraction from EtOH (initially originated from the EtOH extract) (AC-EtOH) extract represented 36.5 % (Scheme 1).

8.1.1 Antibacterial activity of fraction extracts

First, the different fractions were concentrated under vacuum using a rotary evaporator. The antibacterial activity was done using broth microdilution assay against two Gram-negative bacteria, EPEC and *E. coli* ATCC 25922. The growth of the bacteria in MHB media without extract was the bacterial growth control (Ctr) and the growth of bacteria in MHB media in presence of the extract was the experimental test (10 mg/mL) (Fig. 41).

Each extract (EtOAc, AC, EtOH, EtOH insoluble extract, AC-EtOH, and the AC insoluble extract from EtOH extract) was used at a concentration of 10 mg/mL against EPEC and *E. coli* ATCC 25922. We observed that the AC, EtOH and AC-EtOH extract showed an inhibitory (Fig. 41 B, C and H) and bactericidal (Fig. 41 E, F and K) concentrations against EPEC and *E. coli* ATCC 25922 at 10 mg/mL. While the EtOAc extract, EtOH insoluble extract and AC insoluble extract from EtOH extract showed no antibacterial activity (Fig. 41A, D, G, J, I and L).

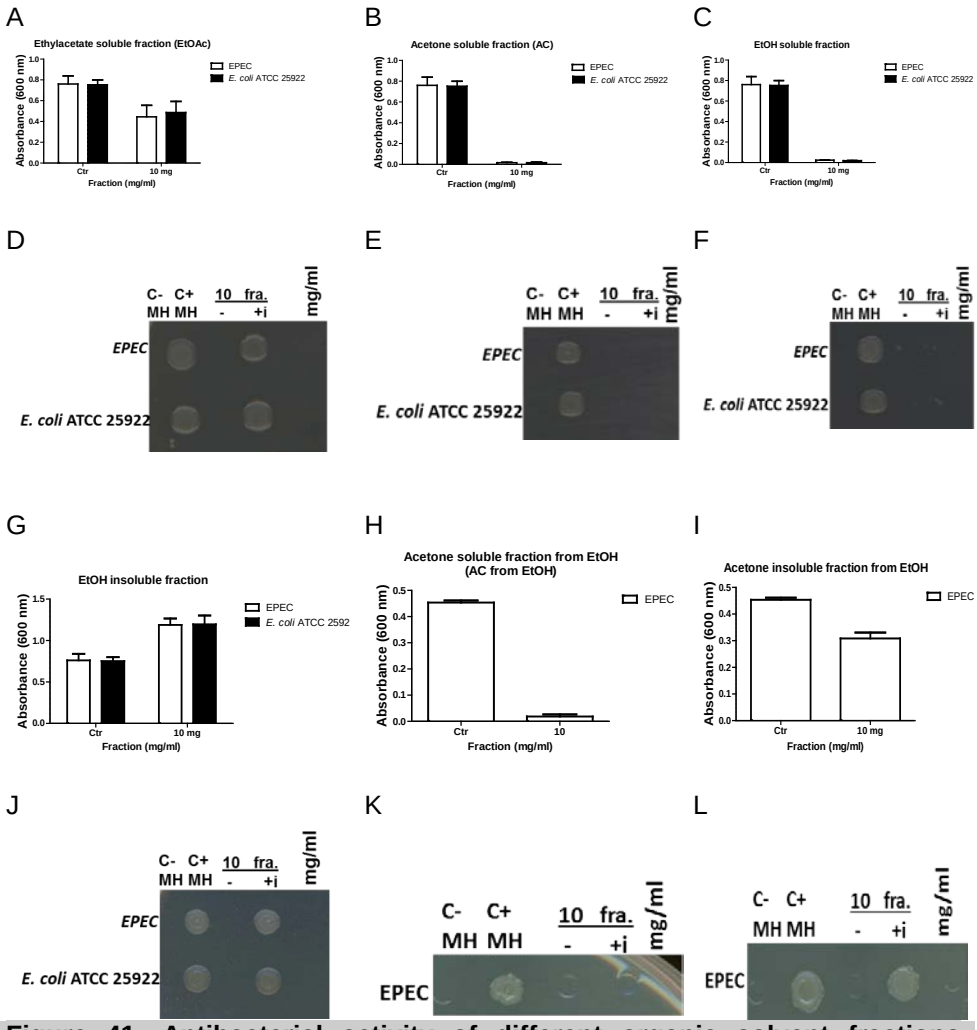

Figure 41. Antibacterial activity of different organic solvent fractions obtained from the initial aqueous extract of *H. sabdariffa* calyces. Minimal inhibitory concentrations of **(A)** Ethyl acetate soluble fraction. **(B)** Acetone soluble fraction. **(E)** Ethanol soluble fraction. **(F)** Ethanol insoluble fraction. (I) Acetone soluble fraction from ethanol. **(J)** Acetone insoluble fraction from ethanol. Minmimal bactericidal concentrations of **(C)** Ethyl acetate soluble fraction. **(D)** Acetone soluble fraction. **(G)** Ethanol soluble fraction. **(H)** Ethanol insoluble fraction. **(K)** Acetone soluble fraction from ethanol. **(L)** Acetone insoluble fraction from ethanol against Gram-negative bacteria by seeding 1.5 µL of each micro well into Mueller-Hinton Agar (MHA) medium.
Abbreviatons: MH= Mueller Hinton broth media, Ccontrol, i,inoculum, fra, Fraction, 10= 10 mg/mL

8.2 MIC and MBC of the active fraction extracts

We used the broth micro dilution assay to determine the MICs and MBCs of the AC and EtOH extracts at 2, 5, 8 and 10 mg/mL against two Gram-

positive bacteria *S. aureus* and Methicillin Resistant *S. aureus* (MRSA) and two Gram-negative bacteria enteropathogenic *E. coli* E 2348/69 (EPEC) and *E. coli* ATCC 25922.

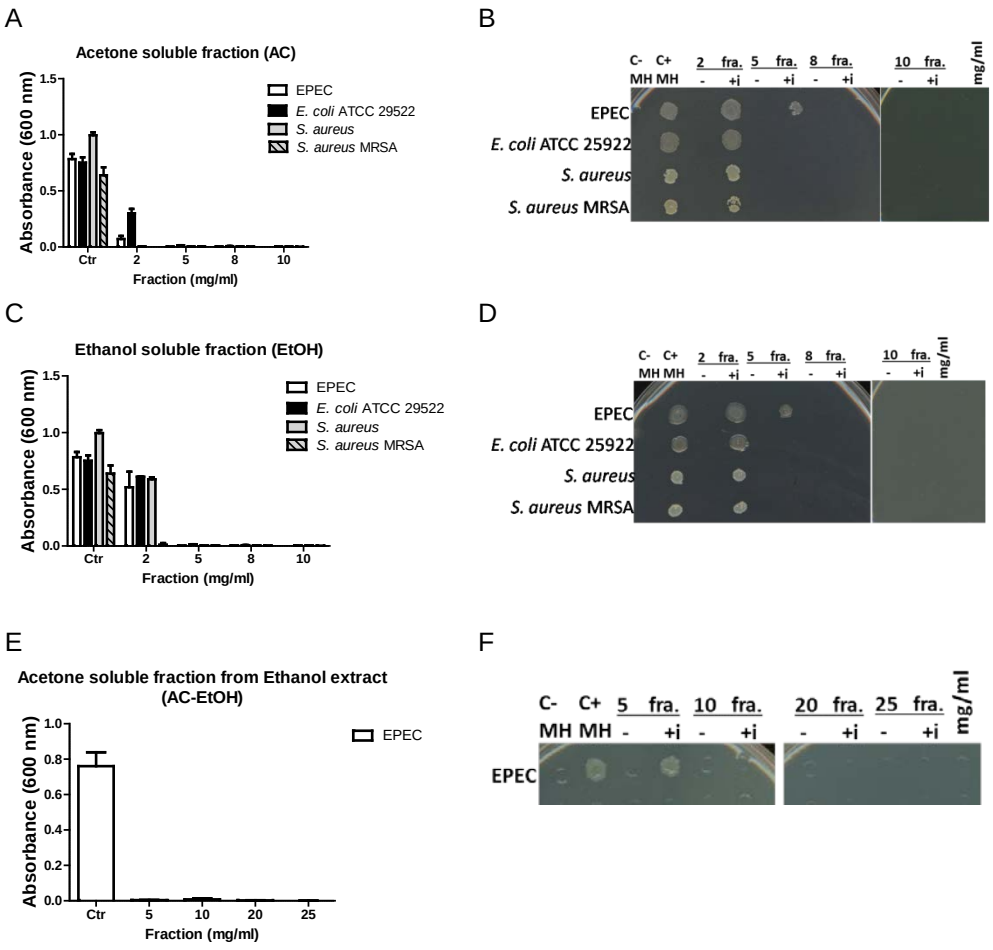

Figure 42. MIC and MBC of the active fractions by drop assay. MIC of (A) AC extract. **(C)** EtOH extract. **(E)** AC-EtOH extract. **MBC of (B)** AC extract. **(D)** EtOH extract. **(F)** AC-EtOH extract by seeding 1.5 µL of each micro well into MHA medium.
Abbreviations: MH= Mueller Hinton broth media, C = control, i= inoculum, fra.= Fraction, numbers as 5= 5 mg/mL

The MIC and MBC of AC-EtOH extract against EPEC were also determined using 5, 10, 20 and 25 mg/mL concentartions of the extract (Fig. 42).

We observed that the AC extract showed a MIC for *S. aureus* and MRSA at 2 mg/mL and against EPEC and *E. coli* ATCC 25922 at 5 mg/mL

(Fig. 42A), while the MBC against all of them was at 5 mg/mL except for EPEC that was 8 mg/mL (Fig. 42B) (Table 11).

Table 11. MIC and MBC of the active fractions

Bacteria	AC extract		EtOH extract		AC-EtOH	
	MIC	MBC	MIC	MBC	MIC	MBC
	Concentrations (mg/mL)					
EPEC	5	8	5	8	5	10
E. coli ATCC 25922	5	5	5	5		
S. aureus	2	5	5	5		
S. aureus (MRSA)	2	5	2	5		

EtOH fraction extract exhibited a MIC against MRSA at 2 mg/ml and for EPEC, *E. coli* ATCC 25922 and *S. aureus* at 5 mg/ml, while the MBC against all of them was at 5 mg/mL except for EPEC that was 8 mg/mL (Fig. 42C and D). The AC-EtOH extract showed a MIC against EPEC at 5 mg/mL (Fig. 42E) and a MBC at 10 mg/mL (Fig. 42F) (Table 11).

According to results presented in Figs. 41 and 42, we concluded that there are two active antibacterial extracts obtained from the initial aqueous extract of *H. sabdariffa* calyces: the AC extract and AC-EtOH extract. We decided to proceed to a further fractionation of them.

9. Fractionation of acetone soluble extract (AC extract)

1.1 grams of AC extract was fractionated using column chromatography (silica gel 60, 0.04-0.06 mm, 230-240 mesh), using as eluent a gradient mixture from dichloromethane (DCM)-methanol (MeOH) (20:1 v/v) to MeOH, increasing the polarity up to 100% of methanol. Silica gel weight was 156 g and a column with 4.7 cm of diameter and 30 cm of hight (Fig. 43A). As the solvent passed through the column and become in

contact with the sample (extract), it causes a separation of different compounds which were collected in galss tubes (Fig. 43A). The fractions collected were analyzed using Thing Layer Chromatography (TLC) (Fig. 43B and C). It was obtained six fractions (Table 12).

Table 12. The gradient mixture of eluent solvent used in the fractionation of AC extract.

Fraction	Eluent DCM:MeOH (v:v)	Tubes (15 mL / tube)	Weight (mg)
1	20 : 1	1-60	50
2	15 : 1	61-120	108
3	10 : 1	121-170	42
4	5 : 1	171-207	100
5	1 : 1	208-243	200
6	MeOH	244-280	576

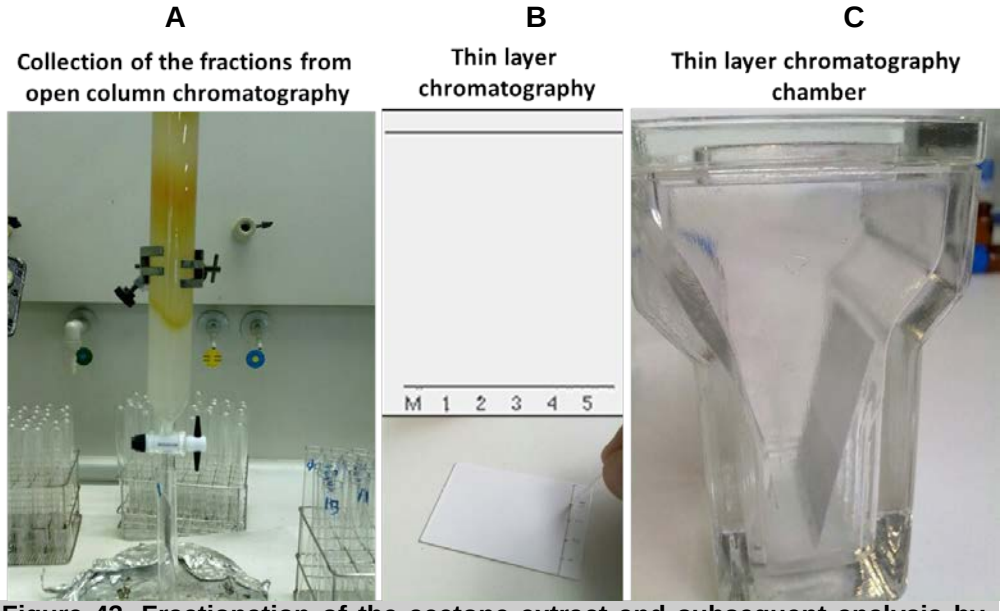

Figure 43. Fractionation of the acetone extract and subsequent analysis by TLC. (A) Collection of the fractions from silica gel column chromatography in tubes. **(B)** Spotting each tube on TLC. **(C)** TLC inside a special chamber with a specific mixture of eluent solvent to analyze the content of each tube.

9.1 Antibacterial activity of the fractions obtained from the fractionation of AC extract

We determined the minimal inhibitory and minimal bactericidal concentrations using the broth micro dilution assay (drop assay) of the six

acetone fractions at 5 and 10 mg/mL against two Gram-positive bacteria *S. aureus* and Methicillin Resistant *S. aureus* (MRSA) and two Gram-negative bacteria enteropathogenic *E. coli* E 2348/69 (EPEC) and *E. coli* ATCC 25922. We observed that the MICs of fraction 1 is 10 mg/mL (Fig. 44A) (Table 13), fraction 2, 3, 4 and 5 is 5 mg/mL and fraction 6 did not show an inhibition against any tested bacteria at the used concentrations (Fig 44C, 45A and C, 46A and C) (Table. 13). And the MBC of fractions 2, 3 and 4 is 5 mg/mL. MBC of fraction 5 is 10 mg/mL for EPEC, *E. coli* ATCC 29522 and *S. aureus* (MRSA). Fractions 1 and 6 showed no bactericidal activity against tested bacteria at the used concentrations (Fig. 44B and 46D) (Table 13). We concluded that the highly active fractions of AC extract are fractions 2, 3 and 4.

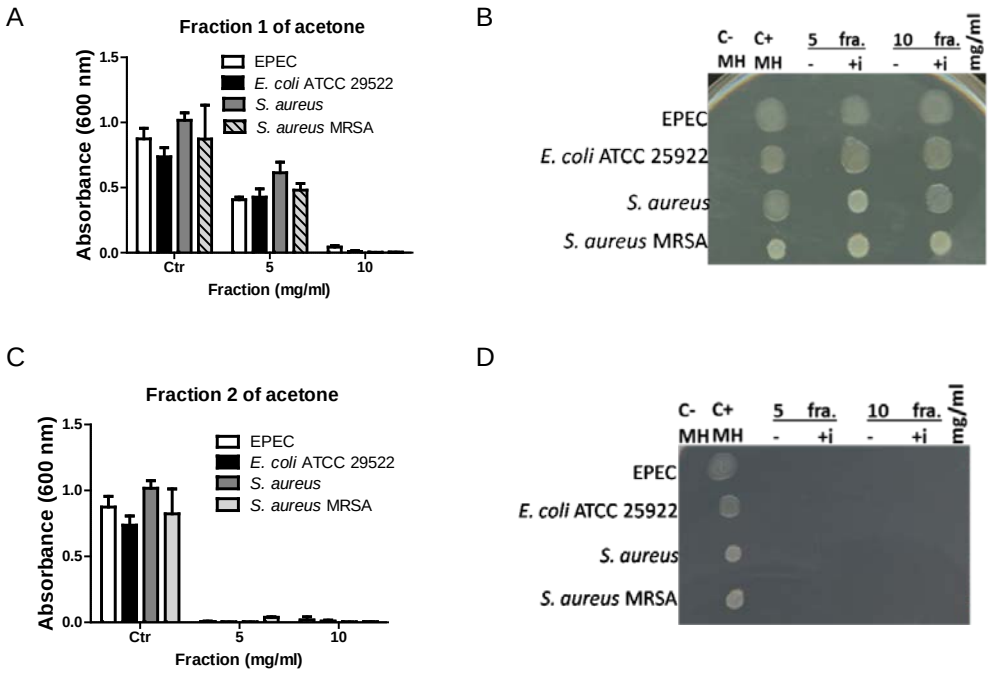

Figure 44. Antibacterial activity of fractions obtained from acetone soluble extract fractionation using drop assay. MIC of (A) Fraction 1**. (C)** Fraction 2**. MBC of (B)** Fraction 1. **(D)** Fraction 2 by seeding 1.5 µl of each micro well into Mueller-Hinton Agar (MHA) medium.
Abbreviations: MH= Müeller Hinton broth media, C = control, i= inoculum, fra.= Fraction, numbers as 5= 5 mg/mL.

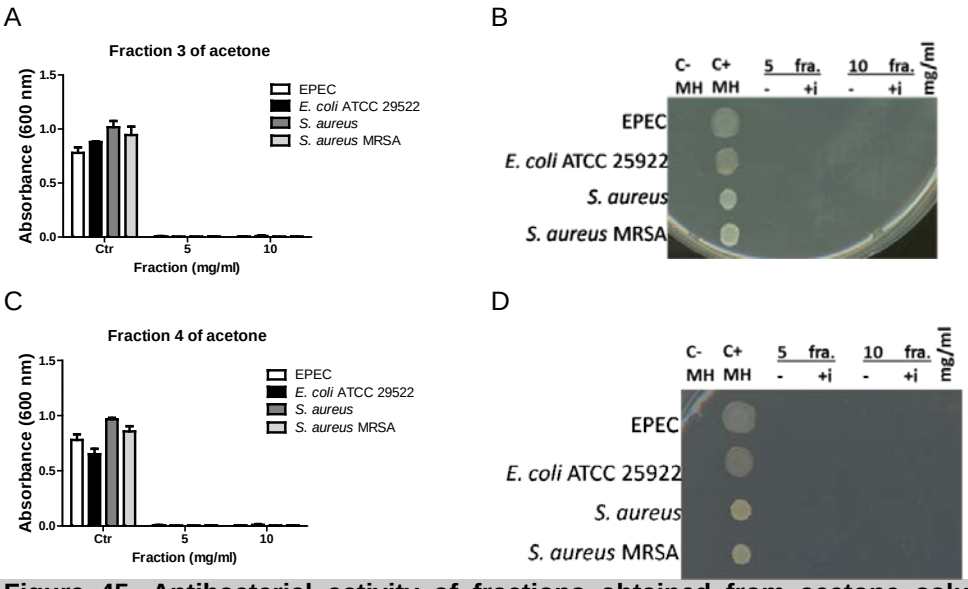

Figure 45. Antibacterial activity of fractions obtained from acetone soluble extract fractionation using drop assay. MIC of (A) Fraction 3**. (C)** Fraction 4**. MBC of (B)** Fraction 3. **(D)** Fraction 4 by seeding 1.5 µL of each micro well into Mueller-Hinton Agar (MHA) medium.
Abbreviations: MH= Müeller Hinton broth media, C = control, i= inoculum, fra.= Fraction, numbers as 5= 5 mg/mL.

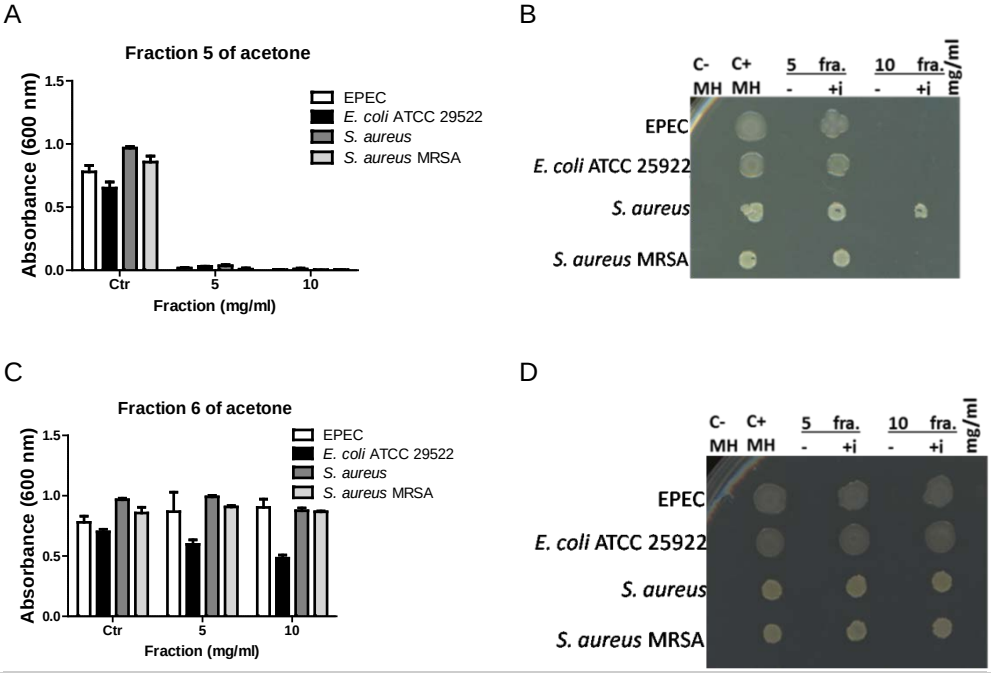

Figure 46. Antibacterial activity of fractions obtained from acetone soluble extract fractionation using drop assay. MIC of (A) Fraction 5**. (C)** Fraction 6**. MBC of (B)** Fraction 5.**(D)** Fraction 6 by seeding 1.5 µL of each micro well into Mueller-Hinton Agar (MHA) medium.
Abbreviations: MH= Müeller Hinton broth media, C = control, i= inoculum, fra.= Fraction, numbers as 5= 5 mg/mL.

Table 13. MIC and MBC of AC fractions.												
Bacteria	**Fraction 1**		**Fraction 2**		**Fraction 3**		**Fraction 4**		**Fraction 5**		**Fraction 6**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
	Concentrations (mg/mL)											
EPEC	10	>10	5	5	5	5	5	5	5	10	>10	>10
E. coli ATCC 25922	10	>10	5	5	5	5	5	5	5	10	>10	>10
S. aureus	10	>10	5	5	5	5	5	5	5	>10	>10	>10
S. aureus (MRSA)	10	>10	5	5	5	5	5	5	5	10	>10	>10

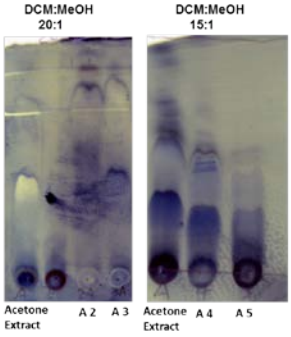

Figure 47. Thin layer chromatography stained with phosphomolybdic acid showing the acetone soluble extract and the antibacterial active fractions 2, 3, 4 and 5 obtained from acetone soluble extract using a gradient mixture of DCM:MeOH 20:1 and 15:1 as a mobile phase.

RESULTS

10. Fractionation of AC-EtOH extract

AC-EtOH extract (14 g) was fractionated by silica gel column chromatography. The eluent solvent was a gradient mixture of dichloromethane and methanol (DCM:MeOH, 10:1 to MeOH) increasing the polarity up to 100% of methanol. Silica gel weight was 445 g and was added to a column of 8 cm of diameter and 30 cm of hight. The fractions collected were analyzed on a TLC plate using the gradient mixture of DCM:MeOH (20:1 to MeOH) as the solvent system depends on the fraction polarity. 10 fractions were obtained (Table 14).

Table 14. The gradient mixture of eluent solvent used in the fractionation of acetone soluble extract from ethanol (AC-EtOH extract).			
Fraction	**Eluent DCM:MeOH (v:v)**	**Tubes (15 mL / tube)**	**Weight (mg)**
1	10 : 1	1-26	142
2	10 : 1	27 – 29	47
3	10 : 1	30 – 42	88.9
4	10 : 1	43 – 50	186
5	10 : 1	51 – 54	184
6	5 : 1	55 – 64	888
7	5 : 1	65 – 69	953.8
8	2 : 1	70 – 87	3700
9	1 : 1	88 – 100	1200
10	MeOH	100 – 130	3600

10.1 Antibacterial activity of fractions obtained from the fractionation of AC-EtOH extract.

Using broth microdilution assay the antibacterial activity of 10 fractions obtained from AC-EtOH extract was evaluated against EPEC by using 5, 10, 20, 25 mg/mL concentrations of each fraction. We observed that fractions 1, 7 and 10 have lower antibacterial activity than the rest of fractions.

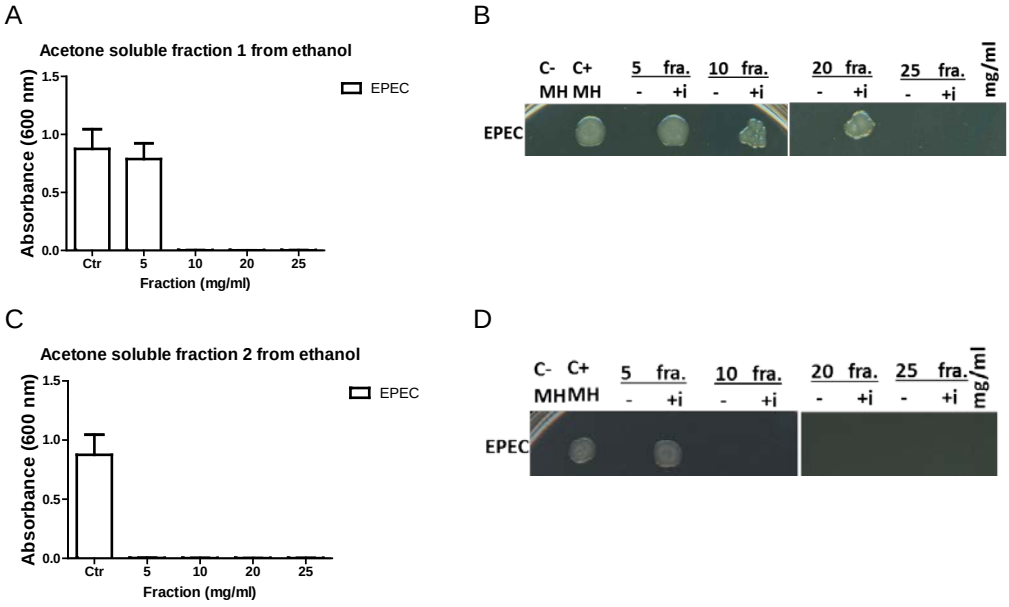

Figure 48. Antibacterial activity of fractions obtained from the AC-EtOH extract. MIC of (A) Fraction 1. **(C)** Fraction 2. **MBC of (B)** Fraction 1. **(D)** Fraction 2 by seeding 1.5 µL of each sample from a micro well into MHA plates.
Abbreviations: MH=Mueller Hinton, C = control, i= inoculum, fra.= Fraction

Fractions 1, 7 and 10 showed MIC at 10, 10 and 20 mg/mL, respectively (Fig. 48A, 51A and 52C). They showed MBC at 25, 10 and 20 mg/mL, respectively (Fig. 48B, 51B and 52D). While fractions 2, 3, 4, 5, 6, 8 and 9 showed MIC at 5 mg/mL (Fig. 48C, 49A, 49C, 50A, 50C, 51C and 52A) and exhibited MBC at 10 mg/mL (Fig. 48D, 49B, 49D, 50B, 50D, 51D, and 52B) (Table 15).

Table 15. MIC and MBC of AC from EtOH fractions

Bacteria	Fraction 1		Fraction 2		Fraction 3		Fraction 4		Fraction 5	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	Concentrations (mg/ml)									
EPEC	10	25	5	10	5	10	5	10	5	10
Bacteria	**Fraction 6**		**Fraction 7**		**Fraction 8**		**Fraction 9**		**Fraction 10**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
	Concentrations (mg/ml)									
EPEC	5	10	10	10	5	10	5	10	20	20

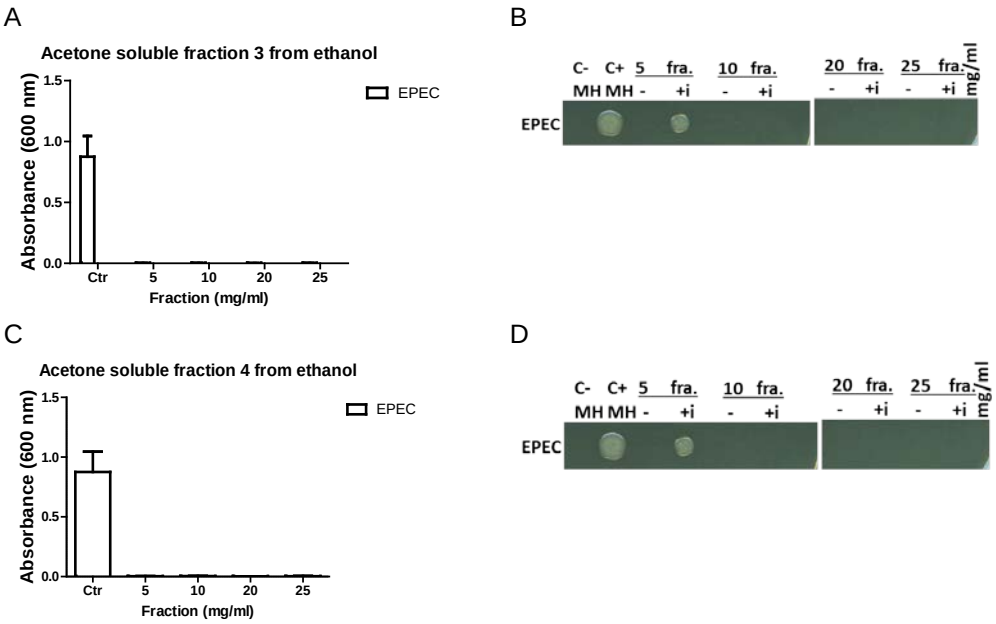

Figure 49. Antibacterial activity of fractions obtained from the AC-EtOH extract. MIC of (A) Fraction 3. **(C)** Fraction 4. **MBC of (B)** Fraction 3. **(D)** Fraction 4by seeding 1.5 µL of each micro well into MHA medium.
Abbreviations: MH= Mueller Hinton broth media, C = control, i= inoculum, fra.= Fraction, numbers as 5= 5 mg/mL.

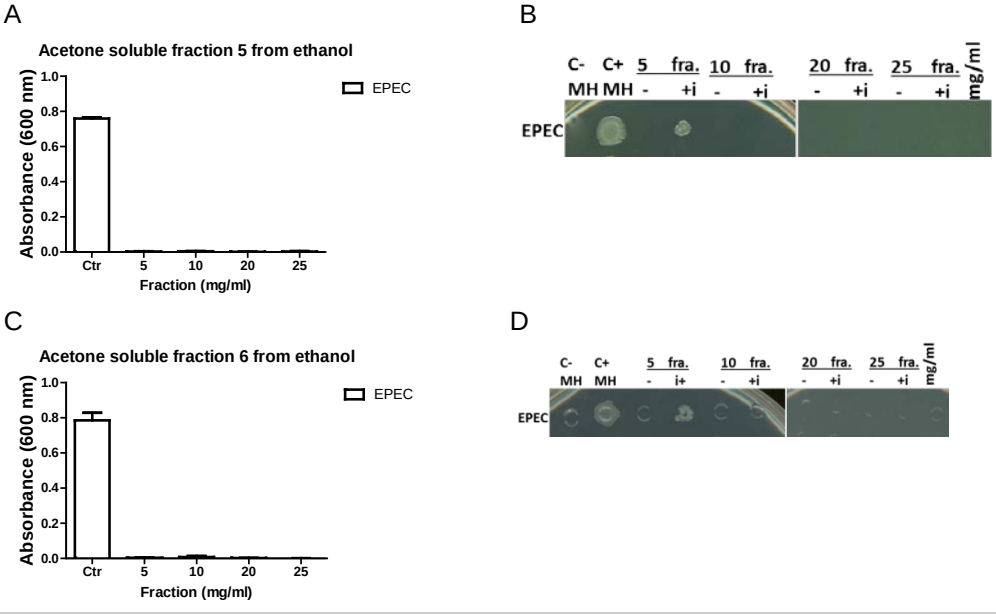

Figure 50. Antibacterial activity of fractions obtained from the AC-EtOH extract. MIC of (A) Fraction 5. **(C)** Fraction 6. **MBC of (B)** Fraction 5. **(D)** Fraction 6 by seeding 1.5 µL of each micro well into MHA medium.
Abbreviations: MH= Mueller Hinton broth media, C = control, i= inoculum, fra.= Fraction, numbers as 5= 5 mg/mL.

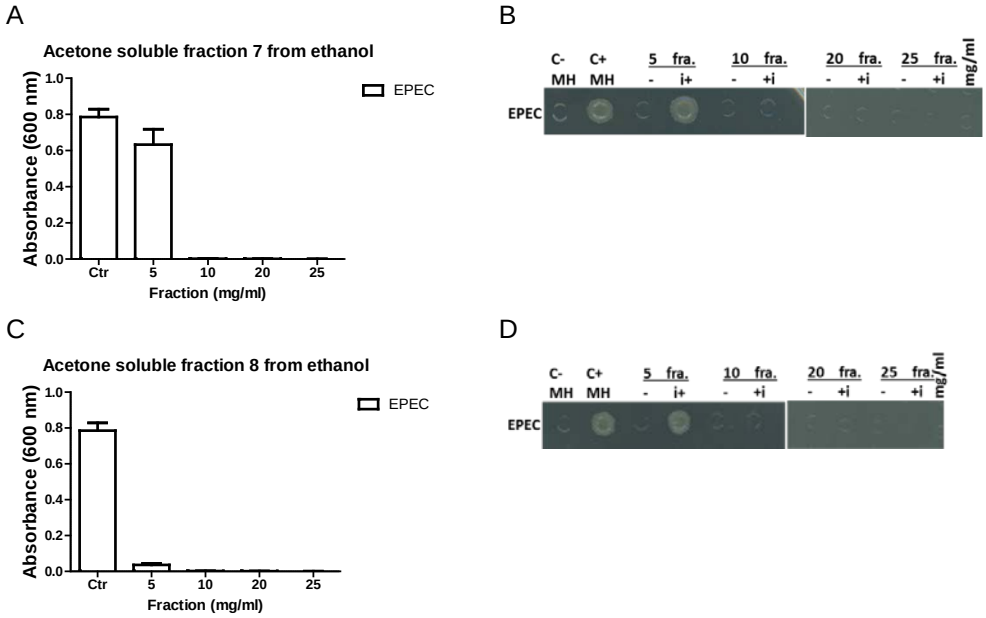

Figure 51. Antibacterial activity of fractions obtained from the AC-EtOH extract. MIC of (A) Fraction 7. **(C)** Fraction 8. **MBC of (B)** Fraction 7. **(D)** Fraction 8 by seeding 1.5 µL of each micro well into MHA medium.
Abbreviations: MH= Mueller Hinton broth media, C = control, i= inoculum, fra.= Fraction, numbers as 5= 5 mg/mL.

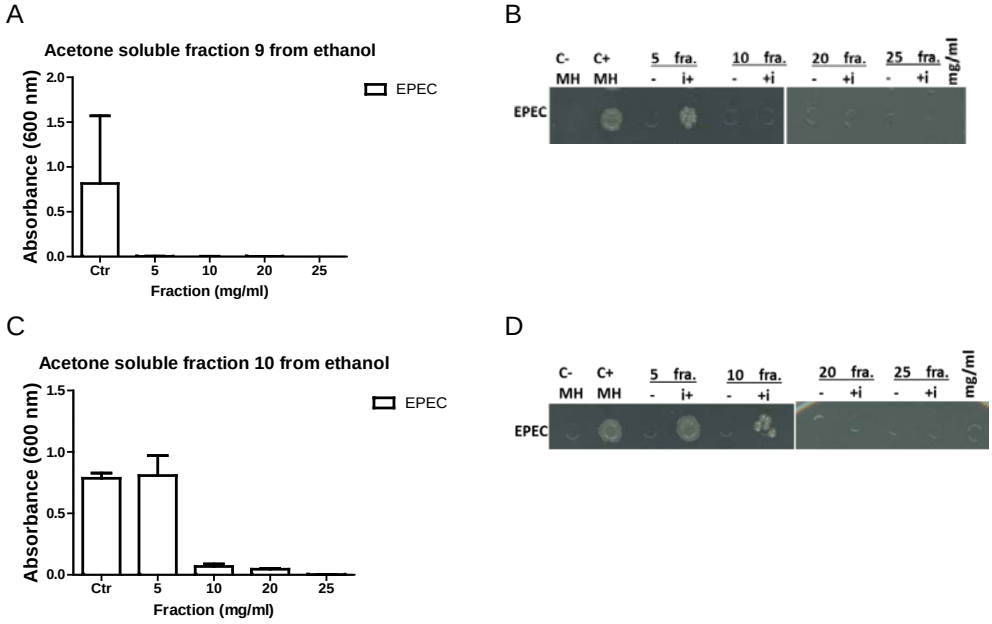

Figure 52. Antibacterial activity of fractions obtained from the AC-EtOH extract. MIC of (A) Fraction 9. **(C)** Fraction10. **MBC of (B)** Fraction 9. **(D)** Fraction 10 by seeding 1.5 µL of each micro well into MHA medium.
Abbreviations: MH= Mueller Hinton broth media, C = control, i= inoculum, fra.= Fraction, numbers as 5= 5 mg/mL.

DISCUSSION

DISCUSSION

Discussion

1. Antibacterial activity of aqueous plants extract

The interest in plants with antimicrobial properties has increased because of the current problems associated with the antibiotics and the appearance of bacterial resistance. There are needs for sustained efforts from government and pharmaceutical companies to develop new antimicrobial drugs to overcome bacterial resistance that cannot be faced by stewardship alone. The antibiotic resistance problem has been estimated to cost United States hospitals more than $ 20 billion annually and get patient to remain longer time in hospitals. Moreover, it was estimated that 2 million patients in the European Union get hospital acquired infections annually; leading to 175 000 patient death (Cooper and Shlaes, 2011). The antibiotic resistance is a global health problem that requires global action. There are many plant extracts with antimicrobial activity which have been recently reported (Rodríguez-Rodríguez, *et al*., 2012; Karuppiah and Rajaram, 2012; Hindi, 2013; Hleba, *et al*., 2013). Consequently we analysed the antibacterial activity of five selected plants. In the present study, aqueous extract of *H. sabdariffa, C. zeylanicum, R. officinalis*, *O. vulgare*, *T. vulgaris* were studied. We analysed their antibacterial activity against 12 pathogenic bacteria. Gram-negative bacteria: *E. coli* ATCC 25922, Enteropathogenic *E. coli* (EPEC E 2348/69), Uropathogenic *E. coli* (UPEC), *P. aeruginosa, K. pneumoniae, S.* ser. Typhimurium*, S.* ser. Enteritidis and against Gram-positive bacteria: *S. aureus*, Methicillin Resistant *S. aureus* (MRSA), *E. faecalis*, *L. monocytogens* ATCC 7622 and *L. monocytogenes* (strain isolated from meat).

There are different bioassays that can be used to evaluate the antibacterial activity of different plants extracts. In our study, we used both disc diffusion test and microwell dilution assay. Disc diffusion test is not appropriate for non-polar samples, because they do not diffuse well through the agar. This method does not distinguish between bacteriostatic and bactericidal effects. Microwell dilution assay (Fig. 8) was used to evaluate the MIC of the

selected plants aqueous extracts. In order to determine the MBC, is necessary to verify if there is cell growth after removing the antimicrobial agent. In general, in the scientific literature the drop assay is the most used method; but this method alone has some limitations in terms of sensitivity. In our working group (Dpt. of Microbiology II, UCM) we have obtained lower MBC values when using drop assay alone than the ones obtained with another complementary method such as macrodilution. This methodological strategy was set-up earlier by B. Conde (Dpt. Microbiology II, UCM) working with natural compounds (Conde *et al.,* 2013; Mohamed, Conde *et al*., 2013).

For this reason, the MBC of *H. sabdariffa* extract against EPEC, was determined by both drop and macrodilution assays. For drop assays 1.5 µL aliquots from each microwell were inoculated on MHA plates. For macrodilution assay (Fig. 8), the remaining volume from each microwell was diluted in 5 mL of liquid media, incubated at 37 ºC with agitation and checking the bacterial growth by turbidity.

1.1 Antibacterial activity of aqueous plants extract by agar diffusion assay

Agar diffusion assay was used to evaluate the antibacterial activity of the aqueous extracts of the five selected plants. The aqueous extract of *H. sabdariffa* was the only aqueous extract that showed a zone of inhibition (halo) of 10.5 mm in Müeller-Hinton agar (MHA) inoculated with EPEC. The ampicillin control halo was 11 mm (Fig. 18). This result suggests that *H. sabdariffa* extract could have a potential inhibitory effect over EPEC that we further investigate.

The agar diffusion assay was done with all the selected aqueous plants extract. The MHA was prepared with and without addition of Tween 80 (0.5%) to facilitate the diffusion of the aqueous extracts through the agar. Furthermore, we used Hexane organic solvent to dissolve the lyophilized aqueous extract (after lyophilization) of the plants for the purpose of facilitating the diffusion process through the agar.

We observed that any of the aqueous extracts of *C. zeylanicum, O. vulgare*, *R. officinalis* and *T. vulgaris*, or lyophilized aqueous extracts dissolved in Hexane solvent induce zone of inhibition through MHA (data not shown). These results indicate that the *H. sabdariffa* extract is very polar, whereas it diffused into the Mueller Hinton agar easily; on the contrary, the lyophilized *H. sabdariffa* extract did not dissolve in hexane organic solvent. The others aqueous plants extract did not show zone of inhibition (halo), probably because they contain less polar compounds than *H. sabdariffa* and agar diffusion assay is not appropriate for non-polar samples.

1.2 Antibacterial activity of aqueous plants extract by drop assay (Broth micro dilution assay)

1.2.1 *Hibiscus sabdariffa* extract

***H. sabdariffa* extract** displayed **the best** antibacterial activity: all tested Gram-positive and Gram-negative bacteria were inhibited (MIC) at concentrations ranging between 5 and 10 mg/mL of the aqueous extract, and were killed (MBC) at concentrations ranging between 10 and 20 mg/mL of the aqueous extract (Fig. 19 and 20) (Table 8).

Fullerton *et al* (2011) checked the antimicrobial activity of phenolic extract of *H. sabdariffa* calyces using disc diffusion method against *Escherichia coli* O157:H7 isolated from food, veterinary and clinical samples. The results of that study showed that sorrel was more effective at 10%, while at 5%, and 2.5% showed lower activity, it induced a bacterial growth inhibition zones of 12.66 mm, 10.75 mm and 8.9 mm, respectively. These results indicated that sorrel is effective at the different concentrations that were charged on the paper disc (Fullerton, Khatiwada *et al*. 2011).

In addition, Elsayed and Elshafei (2011) evaluated the antimicrobial activity of aqueous extract of *H. sabdariffa* under different extraction conditions (cold, hot and autoclaved) using agar well diffusion method and different types of media against *Bacillus mycoides*, *Escherichia coli* and *Candida albicans* (Elshafei 2011). The results of this study revealed that the

H. sabdariffa extract has antimicrobial activity against the tested microorganisms with variable degrees depending on the extraction conditions, assay medium and the test strain under study. In addition, this study reveals that *H. sabdariffa* extracts contain stable and heat resistant components.

Both of the previous studies determined the inhibitory effect of the extract against the tested microorganisms using agar diffusion method. They did not evaluate the bactericidal or fungicidal effect of the plant extracts. Our study is more comprehensive because we evaluated the inhibitory effect, the bactericidal and fungicidal effects against tested microorganisms. These two studies are consistent with our study suggesting the possible use of the *H. sabdariffa* extract as antimicrobial agent.

Other study by Ali *et al* (2011) evaluated different activities of the ethanolic extract of *H. sabdariffa* at 250 and 500 mg/kg body weight in young Swiss-albino mice, including anti-inflammatory activity using xylene-induced ear oedema and antidiarrheal activity induced by castor oil ingestion. They suggested using calyces of *H. sabdariffa* in traditional medicine as anti-inflammatory and antidiarrheal agents (Ali, Ashraf *et al*. 2011). Although this study analyzed the antidiarrheal activity of the ethanolic extract it did not analyze the effect of the ethanolic extract of *H. sabdariffa* against microorganisms directly, as we have done.

Taken together our results until this point, we think that is reasonable to conclude that the aqueous extract of *H. sabdariffa* can be potentially used as an antibacterial agent, and it will be a promise for treatment of gastroenteritis.

Afterward, it was interesting to evaluate the antibacterial activity of *H. sabdariffa* against *Burkholderia cenocepacia,* Gram-negative bacteria that are important human pathogen. It is currently considered as one of the main causes of infections of hospitalized patients, especially for sickle cell disease patients, oncology and burn patients and chronic lung disease patients, particularly those suffering from cystic fibrosis that have

a weakened immune systems. *B. cenocepacia* are often resistant to most common antibiotics (Torbeck, *et al*. 2011). We observed that the *H. sabdariffa* extract significantly inhibited the growth of *B. cenocepacia* with a MIC of 4 mg/mL and killed it with a MBC of 5 mg/ml (Fig. 26). These results indicated that the *H. sabdariffa* extract possess a potent antibacterial activity against *B. cenocepacia* and shows promise for his use for treatment of hospitalized patient infections produced by *B. cenocepacia*.

1.2.2 *Cinnamomum zeylanicum* extract

C. zeylanicum extract exhibited an antibacterial activity **lower than** the antibacterial activity that was showed by ***H. sabdariffa*** against the tested Gram-positive and Gram-negative. ***C. zeylanicum* aquous extract** showed MIC ranging between 5 - 20 mg/mL, and showed MBC at 5 - 70 mg/mL (Fig. 11 and 12) (Table 4). Maidment *et al*. 2006 using disc diffusion assay showed the antimicrobial activity of aqueous, alcoholic extracts and essential oil of ***C. zeylanicum*** against *Escherichia coli* B and *Staphylococcus aureus* and the yeast *Saccharomyces cerevisiae*. They found that alcoholic extract had greater activity than aqueous extract and essential oil had greater activity than both aqueous and alcoholic extracts. These results are consistent with our study, as the ***C. zeylanicum* extract** showed antimicrobial activity (Maidment, 2006).

Bayoub, *et al*., verified the antibacterial activity of ethanol extract of 13 medicinal plants. *C. zeylanicum*, *T. vulgaris* and *R. officinalis* were among these 13 plants. The MIC of the ethanol extract was determined against *L. monocytogenes* using microdilution method. The MICs of *C. zeylanicum*, *T. vulgaris* and *R. officinalis* extracts were 0.4, 1.56 and 5.25 mg/mL, respectively. Furthermore, they used agar well diffusion method inoculating *L. monocytogenes* ATCC 19117, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *E. faecalis*, *K. pneumoniae*, *E. cloacae*, and *A. baumannii* strains. The diameter of inhibition zone of the ethanol extracts tested against *L. monocytogenes*. The ethanol extract of *C. zeylanicum*, *T. vulgaris* and *R. officinalis* showed 22, 19 and 16 mm of inhibition zone. No inhibition

zone was detected of *T. vulgaris* and *R. officinalis* extract against *K. pneumoniae. C. zeylanicum* and *R. officinalis* showed no inhibition zone against *E. faecalis* (Bayoub *et al*., 2010).These results consistent with our results as C. zeylanicum water extract showed better activity than both water extract of *R. officinalis* and *T. vulgaris.* The antibacterial activity of ***C. zeylanicum*** **extract** probably can be attributed to its essential oil content (Baratta *et al.,* 1998; Senhaji *et al.,* 2007; Rana *et al.,*2011). Generally essential oils antibacterial activity is complex, but Ranasinghe *et al.* (2013) reported that the antibacterial activity of essential oils arise from their hydrophobic nature that lead to disruption of bacterial cell wall and consequently leakage of bacterial components (Ranasinghe, *et al*., 2013).

1.2.3 *Origanum vulgare* extract

O. vulgare extract presented **moderate** antibacterial activity against the Gram-positive and Gram-negative bacteria tested, as it exhibited MIC ranging between 35 - 140 mg/mL and MBC between 35 – 140 mg/mL (Fig. 13 and 14) (Table 5). We observed that the MIC for Gram-positive bacteria *S.aureus*, Methicillin Resistant *S. aureus* (MRSA) was 35 mg/mL and for *E. faecalis* and *L. monocytogenes* was 70 mg/mL (Fig. 13B). Regarding Gram-negative bacteria, we found the MIC for *S.* Typhimurium, *S.* Enteritidis was 70 mg/mL, and for UPEC was 140 mg/mL (Fig. 13A) (Table 5). The aqueous extract of *O. vulgare* showed minimal bactericidal concentration (MBC) for Gram-positive bacteria *S. aureus*, Methicillin Resistant *S. aureus* (MRSA), and *L.monocytogenes* and *L. monocytogenes* ATCC 7644 was 35 mg/mL but for *E. faecalis* was 70 mg/mL. Regarding Gram-negative bacteria The MBC for *S.* Typhimurium and *S.* Enteritidis was 70 mg/mL and UPEC, *E. coli* ATCC 25922, EPEC and *K. pneumoniae* was 140 mg/mL, but it did not show bactericidal activity against *P. aeruginosa* at the used concentrations (Fig. 14) (Table 5).

Ashraf *et al* (2011) evaluated the antibacterial activity of chlorophorm, methanol and aqueous extract of *O. vulgare* by agar well diffusion assay. The study reported that the chlorophorm and methanol extracts has better

antibacterial and antifungal activity than aqueous extract (Ashraf 2011). This study is consistent with our study as both studies showed that the aqueous extract did not inhibit *P. aeruginosa*. And their study showed that the aqueous extract did not inhibite *S. typhi*, *E. coli* ATCC 25922 and *S. aureus,* while our aqueous extract inhibits those bacteria probably because of higher concentrations used in our study. They used various concentrations of the dried plant extracts including 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL and 62.5 µg/mL. Although the chlorophorm and methanol extracts of *O. vulgare* in Ashraf study showed better antibacterial activity than the aqueous extract of *O. vulgare*, we used the aqueous extract to compare its antibacterial activity with the aqueous extract of the other selected plants in our study.

Abdul sattar *et al.* (2012) studied the antimicrobial activity of water extract of *T. vulgaris*, *O. vulgare* and *R. officinalis* at 100, 200, 300, and 400 mg/mL concentrations using agar well diffusion method. The antimicrobial activity was evaluated against *Streptococcus mutans, Lactobacillus acidophilus* bacteria that cause dental caries and *Candida albicans* (Abdul sattar 2012). They reported that the water extract of these plants possess antimicrobial activity: the best one is *T. vulgaris* followed by *O. vulgare* and then *R. officinalis*. This study is consistent with our results in that the aqueous extracts of the three selected plants possess antibacterial activity. Although in our study *O. vulgare* had the greatest antibacterial activity compared to *R. officinalis* and *T. vulgaris*. However we should keep in mind that the microorganisms analysed in both studies were different.

1.2.4 *Rosmarinus officinalis* extract

R. officinalis extract **render *a* lower** antibacterial activity than ***O. vulgare*** against both the Gram-positive and Gram-negative bacteria tested. It displayed MIC ranged between 12 and 140 mg/mL, and MBC ranged between 12 and 140 mg/mL, but it did not show bactericidal activity against *E. coli* ATCC 25922, EPEC*, K. pneumoniae* and *P. aeruginosa* (Fig. 15 and 16) (Table 6).

Several researchers have reported the antimicrobial activity of ***R. officinalis*** essential oil (Bozin 2007; Okoh et al. 2010; Zaouali *et al*. 2010). Thus Moreno *et al.* (2006) reported that the antibacterial activity of ***R. officinalis*** is due to their polyphenol content, more specifically to carnosic acid $C_{20}H_{28}O_4$ and rosmarinic acid $C_{18}H_{18}O_{18}$ (Moreno, 2006).

Abu-Shanab, 2004 investigated the antibacterial activity of hot water, methanol and ethanol extracts of five plants. The dried extracts of *Syzygium aromaticum*, *Cinnamomum cassia*, *Salvia officinalis*, *Thymus vulgaris* and *Rosmarinus officinalis* against *S. aureus*, *Bacillus subtilis* ATCC 6633 multi-drug resistant *P. aeruginosa* and enterohemorrhagic *E. coli* O157 EHEC. Agar diffusion method and microdilution method were used. The concentrations used for agar diffusion method was 100 mg/mL and for micro dilution method were from 0.781 to 6.25 mg/mL to determine the MIC of each plant extract. They observed that the water extract of all plants screened by disc diffusion method showed an inhibitory effect ranges between 10-13 mm against *S. aureus* MRSA and *B. subtilis* but no effect was detected against *P. aeruginosa* and *E. coli* (EHEC). Furthermore this water extracts did not inhibite any of the tested microorganisms at the concentrations used by micro dilution method. Ethanol extract of *R. officinalis* inhibited the growth of *S. aureus* MRSA and *B. subtilis* at 1.5 and 3.125 mg/mL, respectively, and *T. vulgaris* inhibited them at 3.125 and 6.25 mg/mL, respectively. No inhibition effect detected for the tested Gram-negative. This study is consistent with our study, as their results indicated that the Gram-positive bacteria are more sensitive to the crude extracts of all the species studied than Gram-negative bacteria (Abu-Shanab, 2004).

1.2.5 *Thymus vulgaris* extract

T. vulgaris extract reported **the lowest** antibacterial activity of the selected plants. It showed MIC at 35 mg/mL for the Gram-positive and Gram-negative bacteria tested (Fig. 17A and B), and MBC for three Gram-positive bacteria *S. aureus*, *L. monocytogenes* and *L. monocytogenes* ATCC 7622 at 140 mg/mL. The aqueous extract of ***T. vulgaris*** showed no bactericidal

activity against all tested Gram-negative bacteria, UPEC, E. *coli* ATCC 25922, EPEC, *K. pneumoniae*, *P. aeruginosa,* *S.* Typhymurium, *S.* Enteritidis, and two Gram-positive bacteria Methicillin Resistant *S. aureus* (MRSA) and *E. faecalis* (Fig.18) (Table 7). The bacteria were inhibited completely at 35 mg/mL, and at higher concentration of 70 mg/mL it seems that they grow and no inhibition which is not logic. We interpret that it is due to the precipitate was formed of the extract in each well contain it and interfere with OD reading.

Kon and Rai (2012) demonstrated the antibacterial activity of essential oil of ***T. vulgaris*** using disc diffusion method against *S. aureus* and *E. coli*. *S. aureus* showed higher susceptibility to essential oil than *E. coli* (Kon, 2012). These results are consistent with our results in the inhibition of both *S. aureus and E. coli* growth. However we did not find bactericidal activity against *E. coli*.

Nzeako *et al*. (2006) evaluated the water extract of ***T. vulgaris*** using well agar diffusion technique against *S. aureus, P. aeruginosa, E. coli*, *S. pyogenes*, *Corynebacterium species, Salmonella species*, *Bacteroides fragilis* and *Candida albicans.* This study showed that the aqueous extract of *T. vulgaris* possess antibacterial activity only against *S. aureus* at 25% v/v (Nzeako 2006). They evaluated the essential oil of thyme against the previous microorganisms. The essential oil of *T. vulgaris* showed inhibition against all tested microoorganisms *S. aureus, P. aeruginosa, E. coli*, *S. pyogenes*, *Corynebacterium species, Salmonella species*, *Bacteroides fragilis* and *Candida albicans* at 0.39, 1.56, 0.78, 0.78, 0.39, 0.39, 1.56, and 0.10 % v/v. These results are consistent with our results, as the aqueous extract showed no bactericidal activity for *E. coli* and *S. species*. But our study showed an inhibition for all tested microorganisms. This inhibition results is conflicting with Nzeako *et al*. (2006) results might be due to the method used for evaluation of the antibacterial activity. In their study was used only agar diffusion assay while in our study we used both agar diffusion and drop assays.

Based on the previous results, we concluded that the Gram-positive bacteria are more sensitive to plants extract than Gram-negative bacteria (Fig. 23 and 24). Consistent with our study, Mohamed Bokaeian *et al*, (2014) showed that Gram-positive *S. aureus* is more sensitive to *H. sabdariffa* extract than Gram-negative *E. coli*, as the highest MIC was 20 mg/mL against two *E. coli* and the least MIC was 1.25 mg/mL against three *S. aureus* (Bokaeian, 2014). Furthermore, Shan *et al*. (2007) study showed that Gram-positive bacteria (*S. aureus*, *L. monocytogenes* and *B. cereus*) were generally more sensitive to 46 extracts from dietary spices and medicinal herbs than Gram-negative bacteria (*E. coli* and *S. anatum*) (Shan, 2007).

In general, comparing our results with the ones in the literature was quite difficult due to the heterogeneity of some factors that affect the experimental conditions such as the used CFU/mL, method used for extraction of the plant extract, the solvent used in the extraction method, the used extract is lyophilized (in case of aqueous extracts) or not, and the assay used for MIC and MBC determination. Additionally, the same plant from different countries shows different results due to the difference between the environmental conditions that lead to the difference in the active constituent's content.

2. Total phenolic compounds content of the plant aqueous extracts

Generally, the antibacterial activity of plants extract is attributed to the presence of several secondary metabolites such as alkaloids, flavonoids, phenols and polyphenols, flavones, flavonols, terpenoids (Cowan, 1999) that can act individually or synergistically.

Subsequently to the evaluation of the antibacterial activity of the selected aqueous plants extract, it was interesting to determine the polyphenol content of the selected plants as a parameter of plants quality and because it is documented that polyphenols have antibacterial activity (Cowan, 1999;

Karou, 2005). Therefore, we hypothesized that both parameters, polyphenol content and antibacterial activity could correlate.

Total polyphenol content was determined using Folin-Ciocaltaeu method (Sharma, *et al*. 2011). As shown in table 11, *O. vulgare* and *R. officinalis* possessed the highest crude extract content of phenolic compounds with values of 28 and 27.12 mg/g, respectively. *H. sabdariffa* is considered to have a moderate crude extract content of polyphenols with a value of 12.7 mg/g. While *T. vulgaris* presented the lowest crude extract content of polyphenols with a value of 8.9 mg/g. *C. zeylanicum* polyphenol content is slightly higher than that of *T. vulgaris* with a value of 9.9 mg/g (Table 11). These results reveal that the polyphenol content is not correlated with the antibacterial activity. Thus the aqueous plant extract with the highest polyphenol content did not present the highest antibacterial activity. In the present study *O. vulgare* presented the highest polyphenol content but it does not presented the highest antibacterial activity. As mentioned, *H. sabdariffa* presents moderate content of polyphenol but it possesses the highest antibacterial activity.

Sirag *et al.* (2014) determined the total phenolic compounds in ethanolic extract of *H. sabdariffa*. The phenolic content represents 41.07 mg/g. Their study determined phenolic compound content higher than our plant extract content may be due to the extracts are different as our extract is aqueous extract and their extract is ethanolic (Sirag 2014) .

To summarize this part, the order of the plants extract in terms of polyphenol content is *O. vulgare* > *R. officinalis* > *H. sabdariffa* > *C. zeylanicum* > *T. vulgaris*. The order of the plants extract in terms of antibacterial activity is *H. sabdariffa* > *C. zeylanicum* > *O. vulgare* > *R. officinalis* > *T. vulgaris*. Stagos, *et al*. (2012) reported the same conclusion using 24 extracts from Greek domestic *Lamieceae* species: he found that the polyphenol content is not correlated with antibacterial and antioxidant activities (Stagos, Portesis *et al*., 2012). Mahboubi *et al*. (2014) study consistent with our results, they showed that the antibacterial activity

of five plants extract (*Thymus vulgaris*, *Thymus caramanicus*, *Zataria multiflora*, *Ziziphora clinopodioides* and *Ziziphora tenuior*) is not correlate with phenolic compounds contents (Mahboubi, *et al*., 2014). In contrast, the study of Shan *et al*., (2007) showed that the antibacterial activity of 46 extracts from dietary spices and medicinal herbs was closely associated with their phenolic constituents (Shan, 2007). These results reveal that the phenolic compounds in our aqueous plants extract are not the major compounds responsible for the antibacterial activity. Therefore it is possible that other compounds (Cowan, 1999) are responsible for the antibacterial activity found acting individually or alternatively, they can synergize.

After screening the antibacterial activity of the five aqueous plants extract, we decided to select *H. sabdariffa* aqueous extract as it showed the best antibacterial activity to complete the aims of our study. Therefore, we next determined the microbial quality of the dried calyces of *H. sabdariffa*.

3. Microbial quality of *Hibiscus sabdariffa* calyces

Naturally, plants have high level of microorganisms including bacteria and fungi. Microbial contamination of dried plants could be caused by inappropriate methods of cultivation, collection, harvesting, unsuitable transportation, prolonged drying and storage, inadequate hygiene of producers and climatic conditions. Microbial contamination can chemically transform some plants constituents to other metabolites not initially found in the herbal plants which may reduce or inactivate their therapeutic activity. Herbal plants which are used without boiling water have different limits of suitability regarding the presence of specific microorganisms than herbal plants used with boiling water (Table 16). Medicinal herbs and plants can be considered either as *pharmaceutical preparations* or as *food and beverages* in terms of their microbial quality, in compliance with the European Pharmacopoeia (Ph. Eur.) and the Royal Spanish Pharmacopoeia (Ph. R. Sp.) (Real Farmacopea Española, R. F. E.) on one hand; or in compliance with the Spanish Regulations on Food and Beverages (Codex Alimentarius). According to the Ph. Eur. and Ph. R. Sp.

medicinal plants are included in category 4 which states that herbal remedies consisting solely of one or more vegetable drugs (whole, reduced or powdered).

When considered as *food and beverages*, most of medicinal plants are included into the group of *stimulating food and derivatives*, Codex Alimentarius Chapter XXV, under the epigraph of "plant species for use in feeding infusions". This title groups the vegetal species, or their parts, used in feeding for their physiological action, because of the characteristic flavor and taste (Real Decreto 3176/1983 of November 16; B.O.E 28-12-83) (Table 17).

Table 16. Microbial criteria for pharmaceutical preparation (European Pharmacopoeia)

	Herbal remedies to which boiling water is added before use	Herbal remedies to which boiling water is not added before use
Total viable aerobic count	Not more than 10^7 /g or mLof aerobic bacteria Not more than 10^5 /g or mL of aerobic fungi	Not more than 10^5 /g or mL of aerobic bacteria Not more than10^4 /g or mL of fungi
E. coli	10^2 /g or mL	Absence in 1g or 1mL
Salmonella		Absence in 10 g or 10 mL
Enterobacteria		Not more than 10^3 /g or mL

Table 17. Microbial criteria for stimulating foods and derivatives. Microbial Regulation R.D. 1534/1483 of April 27; B.O.E. 27-5-83[(1)].

Total viable aerobic count	Maximum 1x10^6 CFU/g
Salmonella-Shigella	Absence /25 g
E. coli	1x10^4 CFU/g
B. cereus	1x10^2 CFU/g
Fungi	Maximum 1x10^4 CFU/g

[(1)] Microbial Regulation for tea and other plant infusions. Spanish Regulations on Food and Beverages.

The microbial contamination of herbal plants may reduce or inactivate their therapeutic activity and some plants sensitive constituents could be transformed chemically to poisonous compounds by contaminating microorganisms (Ahmed, 2006). The evaluation of the microbial quality was done by using the standard colony-count method which is commonly used for detecting and determining the number of microorganisms in plant materials to evaluate the food safety and commercial quality. The present study show that the calyces of *H. sabdariffa* that are used in our study did not present, *E. coli*, *S. aureus*, *L. monocytogenes*, but it has Enterobacteria (18 CFU/10 g), *B. cereus* ($<10^2$ CFU/10 g) and fungi ($5x10^2$ CFU/g). Although the calyces of *H. sabdariffa* have been contaminated with some of Enterobacteria, *B. cereus* and fungi, it can be used for human consumption due to its acceptable microbiological criteria that is consistent with the microbial criteria shown in table 16 and 17. According to the the European microbial criteria that is mentioned in table 16 and 17. Therefore, the results (Table 10) show that there are no faecal contaminations and suggested adequate measures during cultivation, collection, harvesting, drying, packaging and an adequate hygiene of producers. The implication of these results is that the dried *H. sabdariffa* calyces are appropriate for human consumption and can be used as a medicinal herbal plant.

In 2009, Adebayo-tayo *et al*., isolated several microorganisms from the dried *H. sabdariffa* calyces collected from different markets in Uyo (Eastern Nigeria). They isolated *S. aureus, B. subtilis, Bacillus* spp*. E. faecalis, Micrococcus* spp. and *Klebsiella* spp. The associated fungi found were *Aspergillus flavus, A. terreus, A. glaucus, Penicillium citrinum, Fusarium oxysporum, Rhizopus* spp*.* and *Mucor* spp. *A. glaucus* had the highest frequency of occurrence among the isolated fungi (Adebayo-tayo, 2009). This study proved that the majority of the collected samples of dried *H. sabdariffa* calyces are below the acceptable limit for human consumption which conflicting with our study results.

Another study of Omemu *et al*. (2005) in Nigeria, reported that the microorganisms isolated from the dried calyx and the juices included the

fungi, *Aspergillus niger, Aspergillus flavus, Rhizopus oligosporus, Penicillium citrinum, Mucor* spp., *Saccharomyces cerevisiae,* and *Candida krusei,* while *Bacillus subtilis*, *Pseudomonas.* spp., *S. aureus, Streptococcus faecalis, Escherichia coli, Proteus mirabilis, Serratia* spp., *Lactobacillus brevis* and *Lactobacillus fermentum* represented the bacterial isolates (Omemu, 2006).

These two studies are contradicting our study probably due to the different origin of dried *H. sabdariffa* calyces. Our dried calyces were purchased from local herbalist of Egypt, accordingly soil, climatic conditions, cultivation, harvesting, drying, packaging and hygiene of producers could be different than in Nigeria.

Afterwards, because of the good antibacterial activity presented by *H. sabdariffa* aqueous extract, it was relevant to evaluate the cytotoxicity in *in vitro*. So that, in order to evaluate the cytotoxicity of the aqueous extract of *H. sabdariffa*, the cell viability of both macrophages and HeLa cell lines was measured after their treatment with the aqueous extract as an index of cytotoxicity.

4. Evaluation of the cytotoxicity

4.1 Cytotoxicity of *Hibiscus sabdariffa* on macrophages

We used the J774 macrophage cell line to assess the cytotoxicity of *H. sabdariffa* as previously described (O'Brien, 2000). The cytotoxicity was assessed by determining the concentration that decreases the cell viability of macrophages to 80-75 %. *H. sabdariffa* extract was used at 10, 50 and 100 µg/mL. It was observed that the *H. sabdariffa* extract has not cytotoxic effects at the used concentrations (Fig. 32). We avoided using higher concentrations with macrophages due to the pH change that was appreciable by the change in the color of culture medium (that was corroborated by measuring the pH (pH= 4 at 1 mg/mL) that could lead to macrophages death. Therefore the *H. sabdariffa* extract can be used with safety and efficacy as antibacterial agent for human consumption.

4.2 Cytotoxicity of *Hibiscus sabdariffa* extract on HeLa cells

The cytotoxicity of *H. sabdariffa* was also evaluated using infection experiemnts of HeLa cells because it is a frequently used infection model. First at all, we evaluated the cytotoxicity of the *H. sabdariffa* extract at different concentrations. We observed (Fig. 33) that the aqueous extract of *H. sabdariffa* at 5 and 7 mg/mL did not show any significant effect on the growth of HeLa cells. However, it shows a significant cell growth inhibition at 10 mg/mL. At such concentration HeLa cell growth was inhibited by approximately 50 %. These results at 5, 7 and 10 mg/mL concentrations were confirmed by using 4 different initiall cell densities ($1x10^5$, $2x10^5$, $3x10^5$ and $4x10^5$ cells/well) of HeLa cells.

Khaghani *et al*. (2011) evaluated the cytotoxicity of *H. sabdariffa* extract from calyces. They used two types of cell lines, namely human breast adenocarcinoma MCF-7 (a breast-cancer cell line) and normal human fetal foreskin fibroblast HFFF. The concentrations of the aqueous extract that were used ranged between 0.05 and 0.5 mg/mL (Khaghani, 2011). They observed that the *H. sabdariffa* extract significantly decreased by 45% the number of MCF-7 cells at 0.5 mg/mL after 72 h exposure. On the contrary the *H. sabdariffa* extract was not cytotoxic for the HFFF cell line after incubation of 24-72 h at all tested concentrations. These results reveal that the *H. sabdariffa* extract has a selective cytotoxicity. These results are consistent with our findings: we observed a significant decrease of HeLa cell numbers to approximately 50%, after 24 h incubation with the aqueous extract at 10 mg/ml. This cytotoxic effect on cancer cell lines is probably due to an anti-proliferative effect. Comparable results were obtained by Akim *et al*. (2011) showing that *H. sabdariffa* extract has antiproliferative effects on different cancer cell lines as Caov-3, MCF-7, MDA-MB-231 and HeLa cells (Akim, 2011) which further supports our results.

Furthermore, Onyenkewe *et al*. (1999) and Ali *et al*. (2005) in an *in vivo* study using rats, reported that the LD_{50} of *H. sabdariffa* calyces extract was

above 5000 mg/kg, suggesting that the extract is substantially non-toxic (Onyenekwe, *et al*. 1999; Ali, *et al*. 2005). From all these studies we concluded that the *H. sabdariffa* has potential safety for human consumption as an antibacterial agent.

4.3 Cytotoxicity of *Cinnamomum zeylanicum* on macrophages

Cytotoxicity of *C. zeylanicum* extract was assessed using the J774 macrophage cell line as previously described (O'Brien, 2000). The cytotoxicity was assessed by determining the concentration that decreases the cell viability of macrophages to 80-75 %. *C. zeylanicum* extract was used at 10, 50 and 100 µg/ml. The results (Fig. 34) indicate that the *C. zeylanicum* extract is not toxic at 10, 50 and 100 µg/ml concentrations on J774 murine macrophages cell line, compared with the control, although it is close to the limit considered as cytotoxic, as previously mentioned.

Singh *et al.* (2009) analyze the cytotoxic effect of an aqueous cinnamon extract from the bark of *C. zeylanicum* compared with that of commercially available cinnamaldehyde and was performed on various human and mouse cell lines as well as in primary cells. The aqueous cinnamon extract proved to be more cytotoxic to cancerous cells at concentrations just above 0.16 mg/mL (containing 1.28 µM cinnamaldehyde) around which the commercial cinnamaldehyde (1.6 µM) had no cytotoxic effect. At a critical concentration of 1.28 mg/mL (containing 10.24 µM cinnamaldehyde), aqueous cinnamon extract treatment resulted in 35-85% growth inhibition of the majority of the cancerous cells, whereas at a similar concentration (10 µM) commercial cinnamaldehyde treatment resulted in 30% growth inhibition of only SK-N-MC cells with no effect on other cell lines (Singh, *et al*. 2009). These results suggest that aqueous cinnamon extract had a significant inhibitory effect on the majority of cancer cells and thus may suggest to be a chemotherapeutic agent and proved that lower concentrations than 0.16 mg/mL is not inducing cytotoxic effect to cancer cells.

Recently, because of recurrence outbreaks caused by *E. coli* worldwide, particularly the significant outbreak that occurred in Germany 2011 due to *E. coli* O104:H4 strain that possesses a new mixture of pathogenic features from both EHEC (Stx but not the type III secretion and Tir/intimin system) and EAEC (specially the adherence mechanisms). Enteropathogenic *E. coli* (EPEC) 2348/69 was selected instead of EHEC because our laboratory safety level authorization prevent using EHEC as it is a level 3 microorganism. So that, we analysed the antibacterial activity of the *H. sabdariffa* extract against EPEC using 5, 7, 10, 20, 25 mg/mL concentrations. We observed that EPEC was inhibited (MIC) at 7 mg/mL and was killed (MBC) at 25 mg/mL (Fig. 27). These results imply that the *H. sabdariffa* extract as a potential antibacterial activity against EPEC and might be used for treatment of gastroenteritis induced by EPEC.

5. Effect on pedestal formation induced by EPEC

5.1 Effect of *Hibiscus sabdariffa* extract on pedestal formation

EPEC adheres to intestinal cells, promotes characteristic A/E lesions and the formation of disease-associated actin-enriched structures called pedestals. A widespread *in vitro* model of infection is the use of HeLa cells. To investigate the effects of *H. sabdariffa* extract on pedestal formation, HeLa cells were treated for 3h with the aqueous extract **after** infection by EPEC for 3h; at this time point pedestals should have been readily detectable.

We observed that the *H. sabdariffa* extract blocked pedestal formation while the infected untreated HeLa cells displayed an increase in the number of pedestals along time (Fig. 35 and 36). These results indicate that the *H. sabdariffa* extract can inhibit pedestal formation induced by EPEC even if the bacteria are already attached to the cells.

After observing that the *H. sabdariffa* extract decreases the number of pedestals, we hypothesized that the aqueous extract may prevent pedestal formation. To comprehend that purpose, two strategies were used

1- Treating HeLa cell for 3h with the aqueous extract **before** infection by EPEC (**preventive treatment**).

2- Treating HeLa cells for 3h with the aqueous extract during EPEC infection (**simultaneous treatment)**.

With the first strategy, we observed that pedestals were not formed which prove that the aqueous extract can prevent their formation (Fig. 37). With the second approach, we also observed that pedestals were not formed (Fig. 38).

Importantly, while performing the infection experiments, we observed that *H. sabdariffa* extract induced a filamentous morphology of the bacteria. We interpret that the extract was inhibiting the final steps of the bacterial division and decided to further investigate the important finding, discussed in detail below.

Our study represents the first of its class to address the effect of *H. sabdariffa* extract on pedestal formation by EPEC. Furthermore, we propose a previously unreported mode of action as antibacterial agent against EPEC: inhibition of cell division. We concluded that the aqueous extract of *H. sabdariffa* could have a potential use for treatment and prevention of gastroenteritis produced by EPEC. Future studies will aim to analyse the effect *in vivo*, using *Citrobacter rodentium* infection of mice.

6. Probable mechanism of action of *Hibiscus sabdariffa* as antibacterial agent

Surprisingly, using fluorescence microscopy while performing the HeLa cell infection experiments, we observed that treatments of EPEC with the *H. sabdariffa* extract induced a filamentous morphology; in which cells continue to elongate but do not separate (probably implying no septa formation).

Next Gram´s and DAPI staining was performed to compare this phenotype with the one produced by ceftazidime, an antibiotic known to induce filamentous morphology of Gram-negative bacteria. Thus, EPEC was treated with ceftazidime at MIC 0.5 µg/mL for 3h, and in parallel, we use the *H. sabdariffa* aquous extract at 10 mg/mL for 3h. We compared both conditions to untreated EPEC as a negative control (Fig. 40). We observed that the filamentous morphology of EPEC that is induced by *H. sabdariffa* is similar to the one induced by ceftazidime. However, at higher concentrations of aqueous extract of *H. sabdariffa*, such as 20 mg/mL (bactericidal concentration), the filamentous morphology was not observed.

Buijs *et al*. in 2007 reported that ceftazidime and cefotaxime induce a filamentous morphology in bacteria. Ceftazidime is a β-lactamic antibiotic with affinity for penicillin-binding protein (PBP-3) and PBP-1. At low concentrations, ceftazidime inhibits PBP-3 leading to filament formation, but at higher concentrations inhibits PBP-1 resulting in rapid bacterial lysis. In addition, it was reported that the capacity to produce filaments correlates with the MIC (Buijs, 2007). Therefore, probably for that reason we could not observe the filamentous morphology of treated EPEC with *H. sabdariffa* at higher concentrations such as 20 or 25 mg/mL.

It has been reported that this morphological change (filament induction) reduces the ability of bacteria to adhere to epithelial cells (Zhanel, 1992). This would explain why there was a significant inhibition of pedestal formation by EPEC in all conditions tested. We can conclude that the probable mechanism of action of our aqueous extract as antibacterial agent is similar to the one of ceftazidime. Collectively, *H. sabdariffa* extract antibacterial activity could be due to the interference of the active compounds with bacterial cell wall synthesis, inducing the filamentous morphology at lower concentrations and bacterial death, at higher concentrations.

After proving that the *H. sabdariffa* extract is a very potent antibacterial agent against Gram-negative and Gram-positive bacteria that can

potentially be used for treatment and prevention of gastroenteritis induced by EPEC and establishing its safety for human consumption, it was interesting to study the rates of bacterial killing. Therefore we performed time kill analysis.

7. Time kill studies

Time kill curve is an accepted index of bactericidal activity which is used for assessment of the efficacy of bactericidal agents. The rates of bacterial killing after exposure to the antibacterial agents should achieve a 3-log decrease in viability. The concentrations used in time kill study were 10 (1.4 x MIC) and 25 (3.5 x MIC) mg/mL of the *H. sabdariffa* extract against EPEC at $4x10^6$ CFU/mL. The cell counts were determined at 0, 2, 4, 6, 8, 10, 12, and 24 h of drug exposure compared to the cell counts of untreated EPEC as a control for growth. 10 mg/mL is the maximum concentration used for inhibition of pedestal formation without affecting immunostaining of HeLa cells, moreover its induction to filamentous morphology of EPEC was compared to ceftazidime antibiotic. For these reasons, it was decided using 10 mg/mL in this time kill study instead of 7 mg/mL. From Fig. 28 the bactericidal activity of ***H. sabdariffa*** extract started at 10 h. As the 3-log decrease in cell viability is occurred at 25 mg/mL (MBC) (Fig. 28). At 10 mg/mL is an inhibitory concentration, as no decrease in cell viability observed during 24 h. According to the calculation from figure equation, K values are: K_{25} = 0.131 h^{-1}, K_{10} = 0.018 h^{-1}. For 25 mg/mL (MBC), the following parameters are derived from the killing equation: the mean times to achieve reductions in the proportions of viable cells of 50% (t50) = 2.3h, 90% (t90) = 7.6 h, and 99% (t99) = 15.3 h, and the time to reach the bactericidal endpoint 99.9% (t99.9) = 23 h. For 10 mg/mL (MIC), the mean times to achieve reductions in the proportions of viable cells of 50% (t50) = 16.5 h, 90% (t90) = 55 h, and 99% (t99) = 110 h and the time to reach the bactericidal endpoint 99.9% (t99.9) = 165 h. These results suggest that the *H. sabdariffa* extract at MIC (10 mg/mL) the endpoint of bactericidal activity would be reached in 6.9 days. Whereas with the MBC (25 mg/mL) the endpoint is reached in

23 h. Nevertheless, it should be notice that we are working with a crude extract and not with a pure active principle. These results can interpret the wide range between the MIC (7mg/mL) and the MBC (25 mg/mL) against EPEC is due to the time required for the bactericidal activity of *H. sabdariffa* is concentration dependent. This is the first study showing the bactericidal activity rate of *H. sabdariffa* extract against EPEC. It can be concluded that *H. sabdariffa* extract is a potential antibacterial agent specifically against EPEC.

8. Effect of *Hibiscus sabdariffa* extract on Fungi

After proving that the *H. sabdariffa* extract has a potent antibacterial activity against Gram-negative and Gram-positive bacteria, it was pertinent to investigate its antifungal activity. We tested the extract activity against *C. albicans*, and two strains of *S. cerevisiae, YPH 499* and *BY4741.* Drop assay was performed using the aqueous extract at 5, 10, 20, 35, 70, 140 and 250 mg/mL concentrations. It was observed that the *H. sabdariffa* extract inhibits the growth of *S. cerevisiae BY4741* at 10 mg/mL. However the MIC for *C. albicans* and *S. cerevisiae YPH 499* was 70 mg/mL (Fig. 29A).

Thereafter, it was found that the *H. Sabdariffa extract* has no fungicidal activity on *Candida albicans* and *Saccharomyces cerevisiae BY4741,* even at concentrations up to 250 mg/mL. However at 140 mg/mL concentration has fungicidal activity on *Saccharomyces cerevisiae YPH 499* (Fig. 29B). Consistent with our results, the study by Elsayed and Elshafei (2011) recently reported that the *H. sabdariffa* extract is able to inhibit the growth of *Bacillus mycoides*, *Escherichia coli* and *Candida albicans* in the ranges of 0.32 ± 0.13 - 1.50 ± 0.05 mg/mL using agar well diffusion method (Elshafei, 2011). However Olaleye (2007) reported that the methanolic extract of *H. sabdariffa* is not able to inhibit *Candida albicans* up to 80 mg/mL (maximum concentration used in this study) using agar diffusion method.

Fitzgeralda *et al*. (2003) suggested that the *H. sabdariffa* extract can be used as a preservative agent for food (Fitzgeralda, 2003). Therefore, the *H. sabdariffa* extract could not be used for treatment of fungal diseases, due to its inability to induce fungicidal activity.

8.1 Dimorphism in fungi

The word dimorphism (Greek *dis* = two; *morphe* = form), is used in mycology to describe the property that is possessed by some fungi to adapt two different vegetative morphologies, depending on the growth conditions. However, the denotative value of this term, in our discipline, not reduced to duality of yeast-hypha phenotype, because under the heading dimorphic fungi includes a number of organizations with more than two interconvertible morphologies, distinct, coexisting in certain circumstances and some authors suggest the term pleomorphic or polymorphic (Rodríguez, 1987)**.**

The phenomenon of dimorphism is the capacity displayed by different fungi to grow in two forms: yeast and hypha (mycelium). The conditions that induce the dimorphic transition of yeast to hypha or *vice versa* are very diverse, including changes in temperature, pH, the gaseous atmosphere of growth, or the presence of specific compounds in the culture media (Coelho, 2010). Several fungal species display a morphological change called dimorphism and it is associated to pathogenesis. Dimorphism can be defined as the capacity of fungi to grow in two distinct morphological forms as yeast form or filamentous form. This phenomenon is induced as a response to the environmental conditions as pH, temperature, nutritional status, gaseous atmosphere of growth (CO_2 content) or presence of specific compounds in the culture media. This ability of switching among these morphologies (yeast and mycelium forms) is required for virulence although both forms have been identified during infection (Ruìz-Herrera, 2012).

8.2 Effect of *Hibiscus sabdariffa* extract on *Candida albicans* morphology

In *C. albicans*, the dimorphic transition can be induced by a range of environmental conditions and molecules including pH, temperature, nutritional factors, serum (Jacobsen, 2012) or quorum sensing (Albuquerque, 2012). At low pH, < 6, *C. albicans* cells predominantly grow in the yeast form, while at a high pH, > 7, hyphal growth is induced (Soll, 1986). In contrast, in presence of *H. sabdariffa* extract (at low pH, ≤ 4) *C. albicans* undergoes the transition to hyphae (Fig. 30). In order to obtain more conclusive results and to understand the regulation of this phenomenon at the molecular level, further studies are needed.

There are several virulence factors of *C. albicans* contributing to its pathogenicity. These properties include adhesiveness to host cells, secretion of degradative enzymes, and the ability to undergo a reversible morphological transition from the blastospore to filamentous forms (Ruìz Herrera, 2012). Therefore our results indicate that the induction of a filamentous morphology at low concentrations of 5, 10, 20, 35 mg/mL could be attributed to the increase of its virulence. At higher concentrations 70 mg/mL (MIC) we could not observed the phenotype due to the growth inhibitory effect of the aqueous extract against *C. albicans* (Fig. 30). Alternatively, it is possible that *H. sabdariffa* contain an active constituent able to induce filamentation of *C. albicans*. Further studies are required to prove any of these two hypothesises.

8.3 Effect of *Hibiscus sabdariffa* extract on *Yarrowia lipolytica* morphology

Y. lipolytica is non-pathogenic dimorphic yeast. It has the capacity to metabolize hydrocarbons and lipids, to accumulate large amount of organic acids and to secrete heterologous proteins. It represents a very good model for studying fungal dimorphism. It has been described that the transition yeast to hypha in *Y. lipolytica* is induced as a response to neutral pH or

high temperature or presence of serum (Rodríguez and Domíngez, 1984) or in presence of N-acetylglucosamine as the only carbon source, as only induction factor (Rodríguez and Domínguez, 1990). Interestingly, we observed hypha formation of *Y. lipolytica*, although the pH of our *H. sabdariffa* extract was acidic (low pH < 4) at 30 ºC (Fig. 31). These conditions are non-filament inducing conditions. But at higher concentration 70 mg/mL of the aqueous extract those filaments were not observed. These results are analogous to the observed with *C. albicans*.

9. Fractionation of the *Hibiscus sabdariffa* extract

The lyophilized *H. sabdariffa* extract was fractionated using different organic solvents. The fractionation process was carried out by using organic solvents according to their differential polarity. Due to the fact that the lyophilized *H. sabdariffa* extract is a very polar extract, we started the fractionation with the less polar organic solvent and continue with the higher polar organic solvents.

Hexane was the first solvent used in our fractionation process and as expected we did not obtain an extract from the lyophilized *H. sabdariffa* extract. Then **ethyl acetate**, **acetone** and **ethanol** solvents were used. EtOAc extract presented the lowest yield (0.42 %) while EtOH extract presented the highest yield (51.5 %). Acetone extract presented law yield (2.8 %) and AC-EtOH extract presented moderate yield (36.5%) (Scheme 1). This implies that the compounds that *H. sabdariffa* calyces contain are highly polar.

The antibacterial activity of EtOAc, AC, EtOH extracts and the residue was evaluated against EPEC and *E. coli* ATCC 29522, AC-EtOH extract was evaluated against EPEC only, using micro dilution assay at 10 mg/mL as preliminary test. We observed that the active extracts are AC, EtOH and AC-EtOH extracts, as they showed antibacterial activity against EPEC and *E. coli* ATCC 29522 at 10 mg/mL and AC-EtOH extract showed antibacterial activity against EPEC at 10 mg/mL (Fig. 41).

We determined the MIC and MBC of the active extracts (AC and EtOH) against EPEC, *E. coli* ATCC 29522, *S. aureus* and Methicillin Resistant *S. aureus* (MRSA) at 2, 5, 8 and 10 mg/mL. But AC-EtOH extract used against EPEC only at 5, 10, 20 and 25 mg/mL. We observed that AC and EtOH extracts showed MIC against EPEC, *E. coli* ATCC 29522, *S. aureus* and Methicillin Resistant *S. aureus* (MRSA) at 5 mg/mL, and MBC at 5 mg/mL for *E. coli* ATCC 29522, *S. aureus* and Methicillin Resistant *S. aureus* (MRSA) and 8 mg/mL for EPEC (Fig. 42) (Table 11). AC-EtOH extract showed MIC and MBC against EPEC at 5 and 10 mg/mL, respectively.

AC-EtOH extract originates after thin layer chromatography (TLC) analysis to fractionate AC and EtOH extracts, we observed that EtOH extract contains bands similar to the AC extract bands. So that, we decided to treat EtOH extract with acetone solvent to obtain AC extract from EtOH extract (AC-EtOH). The antibacterial activity of both AC-EtOH extract and the residue of EtOH extract was evaluated against EPEC. We observed that the AC-EtOH extract has MIC at 5 mg/mL and MBC at 10 mg/mL, while the residue did not show antibacterial activity at 10 mg/ml (Fig. 41). All together we concluded that the antibacterial active constituents present in the AC extract as well as AC-EtOH extract.

We continue the isolation process, therefore, AC extract was fractionated using column chromatography with silica gel as stationary phase and using gradient eluent of DCM:MeOH 20:1 increasing the polarity gradually up to MeOH 100% as mobile phase (Table 12). Six fractions of AC extract were obtained (Table 12). Their antibacterial activity was evaluated against two Gram-negative bacteria EPEC, *E. coli* ATCC 29522, and two Gram-positive bacteria *S. aureus* and Methicillin Resistant *S. aureus* (MRSA) at 5 and 10 mg/mL concentrations. We observed that fractions 2, 3 and 4 showed the strongest antibacterial activity, as they exhibited MIC at 5 mg/mL and MBC at 5 mg/mL for all tested bacteria (Fig. 44 C, D, and 45A, B, C and D) (Table 13). Fraction number 5 showed fainter antibacterial activity than fractions 2, 3 and 4 (Fig. 46 A and B). Fractions 1 and 6 showed no

bactericidal activity against tested bacteria at the used concentrations (Fig. 44B and 46D) (Table 13).

Similarly the AC-EtOH extract was fractionated using column chromatography with silica gel as stationary phase and using gradient eluent solvent of DCM:MeOH 10:1 increasing the polarity gradually up to 100% MeOH as mobile phase (Table 14). 10 fractions were obtained from AC-EtOH extract (Table 14). Their antibacterial activity was evaluated against EPEC only, because it is our research interest and to save fractions quantities for further fractionation, at 5, 10, 20 and 25 mg/mL. We observed that the fractions 2, 3, 4, 5, 6, 8, 9 showed the best antibacterial activity, as they exhibited MIC against EPEC at 5 mg/mL and MBC at 10 mg/mL (Fig. 48C, D, 49A, B, C, D, 50A, B, C, D, 51C, D and 52A, B) (Table 15). But fractions 1, 7 and 10 displayed lower antibacterial activity than the rest of 10 fractions (Fig. 48A, B 51A, B and 52C, D) (Table 15). These results reveal that specifically fractions 2, 3 and 4 of AC extract displayed stronger antibacterial activity than the fractions of AC-EtOH extract which probably implies that the active constituents are more concentrated in AC extract than in the AC-EtOH extracts if we hypothesized that both extracts contain the same active constituents.

TLC result of both extracts (AC and AC-EtOH extract) was observed under UV light to conclude that both extracts have similar bands on TLC using DCM:MeOH 10:1 as mobile phase (Fig. 53) implicating that both extracts could contain similar active compounds.

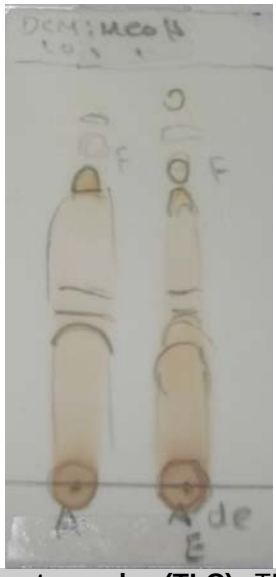

Figure 53. Thin layer chromatography (TLC). TLC was marked showing the running pattern of compounds through TLC of AC extract (A) and AC-EtOH extract (A de E) using solvent mixture of DCM:MeOH 10:1 as a mobile phase.

All together, we concluded that the stronger antibacterial activity of some fractions versus others is probably due to the presence of higher concentrations of active antibacterial compounds. Alternatively, they may contain specific compounds that act synergistically. Therefore, if these synergistic compounds are separated they loss their antibacterial activity. This could explaine the lack of antibacterial activity obtained in the last fractionation performed to get fractions A, B and C, although their parental fraction showed antibacterial activity (data not shown).

The antibacterial activity of *H. sabdariffa* has been attributed to numerous compounds (secondary metabolites) found in the calyces. There are different secondary metabolites of calyces that have been identified as protocatechuic acid, phenolic acids, organic acids, saponins and alkaloids (Liu, Tsao et al. 2005; Olaleye 2007).

Morales-Cabrera and others, (2013) examined the antimicrobial activity of five varieties of *H. sabdariffa* against *S.* Typhimurium *and S. Choleraesuis*. Aqueous, methanol and the **ethanol extract** were tested and it was determined that ethanol extracts were the most effective against

Salmonella (Morales-Cabrera 2013). However, the **methanol extract** of *H. sabdariffa* showed antibacterial activity against *E. coli* O157:H7 (EHEC) that was isolated from food, veterinary and clinical samples (Fullerton 2011). In a different study, the **ethanolic extract** showed better antibacterial activity than the aqueous extract of *H. sabdariffa* against *E. coli*, *S. aureus*, *P. aeruginosa* and *S. mutants* (Al-Hashimi, 2012).

In conclusion, the majority of currently published reports that ethanolic or methanolic extracts of *H. sabdariffa* calyces showed higher antibacterial activity than the aqueous extract. The present study is the first study reporting that the **acetone soluble extract** showed better antibacterial activity against tested bacteria than the aqueous extract. Our result could be of use for pharmaceutical formula preparation for treatment of enteric infections specifically induced by EPEC. Therefore, we conclude that the crude aqueous extract or the fractions of *H. sabdariffa* can be used for treatment of gastroenteritis even more considering that this plant is already used as a tea.

10. Perspectives

Future studies will aim to:

To study the potential molecular target of *H. sabdariffa* in the bacteria cell wall to induce bacterial filamentation.

Determination of the active compounds of *H. sabdariffa* as antibacterial agents. This in progress with prof. Angel Rumbero (Dpt. of organic chemistry, Fac. of Science, UAM).

Determination of the *in vivo* antibacterial effect of the new natural extract in animal model (mice) infected with *Citrobacter rodentium* (etiologic agent equivalent to human infection with EPEC) by:

(a) Determining the bacterial count in the stool of infected and infected/treated groups (collect stool at days 7 and 14, end of the experiment) and determine bacterial count.

(b) Determining the amount of water in the stool.

(c) Determining the inflammatory store.

(d) Staining for selected protein (tight junction and inflammation).

To determine the effectiveness of *H. sabdariffa* extract in protecting barrier function of intestinal monolayers. The *in vitro* experimental study can be achieved by using a specific intestinal cell line (Caco 2) grown in transwells. These will be used to assess the new plant extract's ability to protect intestinal barrier function (measuring transepithelial resistance) in monolayers infected with EPEC. Moreover, staining these monolayers for tight junction proteins (occluding, ZO1) will exhibit the efficiency of new plant extract in protection of tight junction structure.

We will determine the efficiency of *H. sabdariffa* extract in protecting host intestinal cells with EPEC-induced inflammatory response. In the designed experimental groups, we will assess if the extract attenuates the inflammatory signaling pathway (NF-kB) and cytokine expression (IL-8) induced by EPEC infection.

A collaboration with Dr. S. Savkovic (NorthShore University Healthsystem, Evanston, Illinois, USA) have been already stablished in order to address these aspects.

CONCLUSIONS

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Conclusions

The antibacterial activity of five natural plant extracts were assayed against Gram-positive (*Staphylococcus aureus*, Methicillin Resistant *S. aureus* (MRSA), *Enterococcus faecalis*, *Listeria monocytogens* ATCC 7644 and *L. monocytogenes* isolated from meat); and Gram-negative bacteria (Uropathogenic *Escherichia coli*, Enteropathogenic *E. coli* (EPEC E 2348/69), *E. coli* ATCC 25922, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella* Typhimurium and *S*. Enteritidis).

1. The antibacterial *Hibiscus sabdariffa* extract has antibacterial activity against all tested Gram-positive and Gram-negative bacteria. *Cinnamomum zeylanicum* extract has antibacterial activity against all tested Gram-positive and Gram-negative bacteria. *Origanum vulgare* extract showed antibacterial activity against Gram-positive and Gram-negative bacteria except *P. aeruginosa. Rosmarinus officinalis* is only active against Gram positive bacteria. Also *Thymus vulgaris* is only active against Gram positive bacteria.

The most active extracts are *H. sabdariffa* and *C. zeylanicum.* In general, Gram-positive bacteria are more sensitive than Gram negative bacteria. The sensitivity ranges are similar for both extracts, except *Klebsiella pneumonia*, that is the less sensitive with *C. zeylanicum*.

2. The bactericidal activity of *H. sabdariffa* is dose and time dependent, with a bactericidal endpoint t99.9 of 23h, for EPEC.

3. *H. sabdariffa* calyces can be a source of antibacterial compounds against Gram-negative and Gram-positive bacteria. But not for antifungal compounds due to the lack of fungicidal activity against *Candida albicans*.

4. *H. sabdariffa* extract has an antiproliferative effect over HeLa cells, in agreement with previous results obtained with other transformed cell lines.

5. *H. sabdariffa* total extract inhibits and prevents pedestal formation in treated Hela cells.

6. The bactericidal action over EPEC and the inhibitory activity of *H. sabdariffa* extract over pedestal formation might be, at least in part, due to the induction of bacterial filamentation, which can be mediated through the interaction of some active constituents with a molecular target, inducing incomplete cell wall synthesis.

7. The most active fractions obtained from *H. sabdariffa* extract are the fractions 2, 3 and 4 obtained from the “acetone extract”. Those fractions can be used for further isolation of active compounds as antibacterial agents, or for obtaining pharmaceutical preparations for the treatment of enteric infections, specifically those produced by EPEC.

REFERENCES

REFERENCES

References

Abdul sattar BA, Hassan AM, and Hassan AS. (2012). In Vitro Antimicrobial Activity of *Thymus Vulgaris*, *Origanum Vulgare* and *Rosmarinus Officinalis* Against Dental Caries Pathogens. Ibn Al-Haitham Journal for Pure and Applied Science. 25: 1-7.

Abu-Shanab B, Adwan G, Abu-Safiya D, *et al.* (2004). Antibacterial activities of some plant extracts utilized in popular medicine in Palestine. Turkish Journal of Biology. 28: 99-102

Adebayo-tayo BC, and Samuel UA. (2009). Microbial quality and proximate composition of dried *Hibiscus sabdariffa* calyxes in Uyo, Eastern Nigeria. Malaysian Journal of Microbiology. 5: 13-18.

Ahmed I, Aquil F, and Owais M. (2006). Modern Phytomedicine: Turning medicinal plants into drugs. Wiley-VCH Verlag GmbH and Co. KGaA, Weinheim. 1-369

Ahmed B, Shakoori FR, Ali SS, *et al*. (2009). Antimicrobial Resistance Pattern and Plasmid Analysis of *Escherichia coli* from Patients Suffering from Acute Diarrhoea in Azad Kashmir, Pakistan. Zoological Pakistan Journal. 41: 371-380.

Ainsworth EA, and Gillespie KM. (2007). Estimati on of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. Nature protocols. 2: 875-877.

Ajay M, Chai HJ, Mustafa AM, *et al.* (2007). Mechanisms of the anti-hypertensive effect of *Hibiscus sabdariffa* L. calyces. Journal of Ethnopharmacology. 109: 388-393.

Akim AM, Ling LC, Rahmat A. *et al*. (2011) Antioxidant and anti-proliferative activities of Roselle juice on Caov-3, MCF-7, MDA-MB-231 and HeLa cancer cell lines. African Journal of Pharmacy and Pharmacology. 5: 957-965.

Akinjogunla OJ, Eghafona NO, and Ekoi OH. (2009). Diarrheagenic *Escherichia coli* (DEC): prevalence among in and ambulatory patients and susceptibility to antimicrobial chemotherapeutic agents. Journal of Bacteriology Research. 1: 34-38.

Albuquerque P, and Casadevall. (2012). Quorum sensing in fungi_ a review. Medical Micology. 50: 337-345.

Al-Hashimi AG. (2012). Antioxidant and antibacterial activities of *Hibiscus sabdariffa* extracts. African Journal of Food Science. 6: 506-512.

Ali BH, Al Wabel NA, and Blunden G. (2005). Phytochemical, pharmacological and toxicological aspects of *Hibiscus sabdariffa* L. a review. Phytotherapy Research. 19: 369-375.

Ali MK, Ashraf A, Biswas NN. (2011). "Antinociceptive, anti-inflammatory and antidiarrheal activities of ethanolic calyx extract of *Hibiscus sabdariffa* Linn. (Malvaceae) in mice. Journal of Chinese Integrative Medicine. 9: 626-631.

Anand SK and Griffiths MW. (2003). Quorum sensing and expression of virulence in *Escherichia coli* O157:H7. International Journal of Food Microbiology. 85: 1-9.

Anderson MDRP, and Calderón V. (1999). Microbiología alimentaria: metodología analítica para alimentos y bebidas. Ediciones Diaz de Santos. 1-441

Arumugam M, Raes J, and Pelletier E. (2011). Enterotypes of the human gut microbiome. Nature. 473: 174-180.

Ashraf Z, Muhammad A, Tareq AH, *et al*. (2011). In vitro antibacterial and antifungal activity of methanol, chloroform and aqueous extracts of *Origanum vulgare* and their comparative analysis. International Journal of Organic Chemistry. 1: 257-261.

Ausina RV and Moreno GS. (2006). Tratado SEIMC de enfermedades infecciosas y microbiologia clinica. Ed. Medica Panamericana S. A.

Baqui AH, Black RE, Yunus M, *et al*. (1991). Methodological issues in diarrhoeal diseases epidemiology: definition of diarrhoeal episodes. International Journal Epidemiology. 20: 1057-1063.

Baratta MT, Deans SG, and Ruberto G. (1998). Antimicrobial and antioxidant properties of some commercial essential oils. Flavour Fragrance Journal. 13: 235-244.

Bartlett CL. (1996). An overview of emerging footborne and waterborne diseases. East Mediterranean Health Journal. 2: 51-60.

Bayoub K, Baibai T, Mountassif D, *et al*. (2010). Antibacterial activities of the crude ethanol extracts of medicinal plants against *Listeria monocytogenes* and some other pathogenic strains. African Journal of Biotechnology. 9: 4251-4258.

Bisi-Johnson MA., and Obi CL. (2012). *Escherichia coli* and *Salmonella* Species: Molecular Landscape and Therapeutic Considerations: a Review. Journal of Medical Science Advances. 1: 1-16.

Bokaeian M, Sheikh M, Shahi Z, *et al*. (2014). Antimicrobial activity of *Hibiscus sabdariffa* extract against human pathogen. International Journal of Advanced Biological and Biomedical Research. 2: 433-439.

Borre YE, O'Keeffe GW, and Clarke G. (2014). Microbiota and neurodevelopmental windows: implications for brain disorders. Trends in Molecular Medicine. 20: 509-518.

Bozin B, Mimicka-Duck N, Samojlik I, *et al*. (2007). Antimicrobial and antioxidant properties of rosemary and sage (*Rosmarinus officinalis* L. and *Salvia officinalis* L., *Lamiaceae*) essential oils. Journal of Agricultural and Food Chemistry. 55: 7879-7885.

Britton RA, and Versalovic J. (2008). Probiotics and gastrointestinal infections. Interdisciplinary Perspectives on Infectious Diseases. 2008: 1-10.

Buijs J, Dofferhoff AS, Mouton JW, *et al*. (2007). Continuous administration of PBP-2- and PBP-3-specific beta-lactams causes higher cytokine responses in murine *Pseudomonas aeruginosa* and *Escherichia coli* sepsis. The Journal of Antimicrobial Chemotherapy. 59: 926-933.

Burcelin R, Serino M, Chabo C, *et al*. (2011). Gut microbiota and diabetes: from pathogenesis to therapeutic perspective. Acta Diabetologica. 48: 257-273.

Byarugaba DK. (2010). Mechanisms of antimicrobial resistance. Antimicrobial resistance in developing countries. Springer Ed. Chapter 2: 15-29.

Cantón E, and Pemán J. (1999). Curvas de letalidad en antifúngicos. Revista Iberoamericana de Micología. 16: 82-85.

Cantón E, Pemàn J, Espinel-Ingroff A, *et al*. (2004). Patterns of Amphotericin B Killing Kinetics against Seven Candida Species. Antimicrobial Agents and Chemotherapy. 48: 2477-2482.

Carvajal-Zarrabal O, Hayward-Jones PM, Pedroza-Hernandez MF, *et al.* (2009). Effect of *Hibiscus sabdariffa* L. dried calyx ethanol extract

on fat absorption-excretion, and body weight implication in rats. Journal of Biomedicine Research International. 2009: 1-5.

Chao CY and Yin MC. (2009). Antibacterial effects of roselle calyx extracts and protocatechuic acid in ground beef and apple juice. Foodborne Pathogens and Disease. 6: 201-206.

Chavez-Bueno S and McCracken GH. (2005). Bacterial meningitis in children. Pediatric Clinics of North America. 52: 795-810.

Churgay CA, and Aftab Z. (2012). Gastroenteritis in Children: Part I. Diagnosis. American Academy of Family Physicians. 85: 1059-1062

Clemente JC, Ursell LK, and Parfrey LW. (2012). The impact of the gut microbiota on human health: an integrative view. Cell. 148: 1258-1270.

Coelho MAZ, Amaral PFF, and Belo I. (2010). *Yarrowia lipolytica*: an industrial workhorse. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. 3: 930-944.

Cona T. (2002). Condiciones para un buen estudio de susceptibilidad mediante test de difusión en agar. Revista Chilena de Infectología. 19: 77-81.

Conde B, Mohamed R, Franco ME, *et al*. (2013). Actividad antimicrobiana de un nuevo extracto vegetal procedente de Venezuela. XXIV Congreso de Microbiologia SEM 2013.

Cooper MA and Shlaes D. (2011). Fix the antibiotics pipeline. Nature. 472: 32-32.

Cowan MM. (1999). Plant products as antimicrobial agents. Clinical Microbiology Reviews.12: 564-582.

Curiel D, and Ochoa E. (1959). Statistics of gastroenteritis mortality in Venezuela. Bulletin of the World Health Organization. 21: 353-361.

Da-Costa-Rocha I, Bonnlaender B, Sievers H, *et al*. (2014). *Hibiscus sabdariffa* L. - A phytochemical and pharmacological review. Food Chemistry. 165: 424-443.

Dean P, Maresca M, Schüller S, *et al*. (2006). Potent diarrheagenic mechanism mediated by the cooperative action of three enteropathogenic *Escherichia coli*-injected effector proteins. Proceedings of the National Academy of Sciences of the United States of America. 103: 1876-1881.

Derwish E, Benziane Z, Manar A, *et al*. (2010). Phytochemical analysis and in vitro antibacterial activity of the essential oil of *Origanum vulgare* from Morocco. American- Eurasian Journal of Scientific Research. 5: 120-129.

Dini C, Fabbri A, and Geraci A. (2011). The potential role of garlic (*Allium sativum*) against the multi-drug resistant tuberculosis pandemic: a review. Annali dell'Istituto Superiore di Sanità. 47: 465-473.

Diniz-Santos DR, Silva LR, and Silva N. (2006). Antibiotics for the empirical treatment of acute infectious diarrhea in children. Brazilian Journal of Infectious Diseases. 10: 217-227.

Ehrlich SD. (2011) Herbal medicine: A review. University of Maryland Medical Center.

Ekwall B, Silano V, Zucco F, *et al*. (1990). Toxicity tests with mammalian cell cultures. John Wiley & Son Ltd. Chapter 7: 75-99.

El-Rami FE, Sleiman FT, and Abdelnoor AM. (2012). Identification of virulence genes among antibacterial-resistant *Escherichia coli* isolated from poultry. Advanced Studies in Biology. 4: 385-396.

Elsayed MA, and Elshafei AM. (2011). Antimicrobial activity of roselle (*Hibiscus sabdariffa*) calyces and clove (*Syzygium aromaticum*) buds-water extracts using different extraction conditions. Advances in Food Sciences. 33: 141-145.

Fitzgeralda DJ, Stratford M, and Narbad A. (2003). Analysis of the inhibition of food spoilage yeasts by vanillin. International Journal of Food Microbiology. 86: 113-122.

Friden. CDC (US). (2013). Antibiotic resistance threats in the United States, 2013. Centers for Disease Control and Prevention, US Department of Health and Human Services. 1-114.

Fullerton M, Khatiwada J, Johnson JU, *et al.* (2011). Determination of antimicrobial activity of sorrel (*Hibiscus sabdariffa*) on *Escherichia coli* O157:H7 isolated from food, veterinary, and clinical samples. Journal of Medicinal Food. 14: 950-956.

Gerritsen J, Smidt H, and Rijkers GT. (2011). Intestinal microbiota in human health and disease: the impact of probiotics. Genes & Nutrition. 6: 209-240.

Gowri SS, and Vasantha K. (2010). Phytochemical screening and antibacterial activity of *Syzygium cumini* L. (*Myrtaceae*) leaves extracts. International Journal of PharmTech Research. 2: 1569-1573.

Gupta RL, Jain S, Talwar V, *et al*. (1999). Antituber-cular activity of garlic (*Allium sativum*) extract on combination with conventional antitubercular drugs in tubercular lymphadenitis. Indian Journal of Clinical Biochemistry. 14:12-18.

Gyawali CP, Henderson KE, and Thomas MF. (2012). Gastroentrología. Manual Washington de Especialidades Clinica: Washington

university in St. Louis. School of Medicine. 3ª Ed Wolters Kluver/ Lippincott Williams & Wilkins. 1-350.

Hindi NKK. (2013). *In vitro* antibacterial activity of aquatic garlic extract, apple vinegar and apple vinegar - garlic extract combination. American Journal of Phytomedicine and Clinical Therapeutics. 1: 42-51.

Hleba L, Petrová J, Rovná K, *et al*. (2013). Antibacterial activity of some wild medical plants extract to antibiotic resistant *Escherichia coli*. Journal of Microbiology, Biotechnology and Food Sciences. 2: 1215-1224.

Hoareau L, and DaSilva EJ. (1999). Medicinal plants: a re-emerging health aid. Electronic Journal of Biotechnology. 2: 56-70.

Hodges K, and Hecht G. (2012). Interspecies communication in the gut, from bacterial delivery to host-cell response. The Journal of Physiology. 590: 433-439

Holm A, and Vikström E. (2014). Quorum sensing communication between bacteria and human cells: signals, targets, and functions. Frontiers in Plant Science. 5: 1-7.

Huttenhower C, Gevers D, *et al* (2012). Structure, function and diversity of the healthy human microbiome. The Human Microbiome Project Consortium. Nature. 486: 207-214.

Ingerson-Mahar M, and Reid A. (2011). *E. coli* : God, Bad, and Deadly. American Academy of Microbiology Report. 1-16.

Ishii KJ, Koyama S, Nakagawa A, *et al*. (2008). Host innate immune receptors and beyond: making sense of microbial infections. Cell Host Microbe. 3: 352-363.

Jacobsen ID, Wilson D, Wachtler B, *et al.* (2012). *Candida albicans* dimorphism as a therapeutic target. Expert Reviews. 10: 85-93.

Jarrar N, Abu-Hijleh A, and Adwan K. (2010). Antibacterial activity of *Rosmarinus officinalis* L. alone and in combination with cefuroxime against methicillin-resistant *Staphylococcus aureus*. Asian Pacific Journal of Tropical Medicine. 3: 121-123.

Kadosh D, and Johnson AD. (2005). Induction of the *Candida albicans* filamentous growth program by relief of transcriptional repression: A genome-wide analysis. Molecular Biology of the Cell. 16: 2903-2912.

Kaper JB, Nataro JP, and Mobley HLT. (2004). Pathogenic *Escherichia coli*. Nature Reviews Microbiology. 2: 123-140.

Karch H, Denamur E, Dobrindt U, *et al.* (2012). The enemy within us: Lessons from the 2011 European *Escherichia coli* O104:H4 outbreak. EMBO Molecular Medicine. 4: 841-848.

Karou D, Dicko MH, Simpore J, *et al.* (2005). Antioxidant and antibacterial activities of polyphenols from ethnomedicinal plants of Burkina Faso. African Journal of Biotechnology. 4: 823-828.

Karuppiah P, and Rajaram S. (2012). Antibacterial effect of *Allium sativum* cloves and *Zingiber officinale* rhizomes against multiple-drug resistant clinical pathogens. Asian Pacific Journal of Tropical Biomedicine. 2: 597-601.

Khaghani S, Razi F, Yajloo M, *et al*. (2011). Selective cytotoxicity and apoptogenic activity of *Hibiscus sabdariffa* aqueous extract against MCF-7 human breast cancer cell line. Journal of Cancer Therapy. 2: 394-400.

Khan MA, and Steiner TS. (2002). Mechanisms of Emerging Diarrheagenic *Escherichia coli* Infection Current Infectious Disease Reports. 4: 112-117.

Kim JK, So H, Youn MJ, *et al.* (2007). *Hibiscus sabdariffa* L. water extract inhibits the adipocyte differentiation through the PI3-K and MAPK pathway.Journal of Ethnopharmacology. 114: 260-267.

King CK, Glass R, Bresee JS, *et al.* (2003). Managing acute gastroenteritis among children: oral rehydration, maintenance, and nutritional therapy. Morbidity and Mortality Weekly Report (MMWR) Recommendations and Reports. 52: 1-16.

Kohanski MA, Dwyer DJ, and Collins JJ. (2010). How antibiotics kill bacteria: from targets to networks. Nature Reviews Microbiology. 8: 423-435.

Kon K, and Rai M. (2012). Antibacterial activity of *Thymus vulgaris* essential oil alone and in combination with other essential oils. Neusantara Bioscience. 4: 50-56.

Lee WC, Wang CJ, Chen YH, *et al.* (2009). Polyphenol extracts from *Hibiscus sabdariffa* Linnaeus attenuate nephropathy in experimental type 1 diabetes. Journal of Agricultural and Food Chemistry. 57: 2206-2210.

Lin HH, Chen JH, and Wang CJ. (2011). Chemopreventive properties and molecular mechanisms of the bioactive compounds in *Hibiscus sabdariffa* Linne. Current Medicinal Chemistry. 18: 1245-1254.

Lin HH, Huang HP, Chen JH, *et al. (*2005). Hibiscus polyphenol-rich extract induces apoptosis in human gastric carcinoma cells via p53 phosphorylation and p38 MAPK/FasL cascade pathway. Molecular Carcinogenesis. 43: 86-99.

Liu KS, Tsao SM, and Yin MC. (2005). In vitro antibacterial activity of roselle calyx and protocatechuic acid. Phytotherapy Research. 19: 942-945.

Liu LC, Wang CJ, Lee CC, *et al.* (2010). Aqueous extract of *Hibiscus sabdariffa* L. decelerates acetaminophen-induced acute liver damage by reducing cell death and oxidative stress in mouse experimental models. Journal of the Science of Food and Agriculture. 90: 329-337.

Lopman BA, Reacher MH, Duijnhoven YV, *et al.* (2003). Viral gastroenteritis outbreaks in Europe, 1995-2000. Emerging Infectious Diseases Journal. 9: 90-96.

Mahadevan N, Shivali KP, and Kamboj P. (2009). *Hibiscus sabdariffa* Linn.- An overview. Natural Product Radiance. 8: 77-83.

Mahboubi A, Kamalinejad M, Ayatollahi A, *et al.* (2014). Total phenolic content and antibacterial activity of five plants of Labiatae against four foodborne and some other bacteria. Iranian Journal of Pharmaceutical Research. 13: 559-566.

Maidment C, Dyson A, Haysom I. (2006). A study into the antimicrobial effects of cloves (*Syzgium aromaticum*) and cinnamon (*Cinnamomum zeylanicum*) using disc diffusion assay. Nutrition and Food Sciences. 36: 225-230.

Mandell and Bennett´s. (2000). Principles and practice of infectious diseases. Churchill Livingstone Ed. (Fifth edition). 1: 1-1534.

Martinez A, Dominguez A, Torner N, *et al*. (2008). Epidemiology of foodborne norovirus outbreaks in Catalonia, Spain. BMC Infectious Diseases. 8: 1-7.

Meades GJR, Henken RL, Waldrop GL, *et al.* (2010). Constituents of cinnamon inhibit bacterial acetyl CoA carboxylase. Planta Medical Journal. 76: 1570-1575.

Mensa J, Gatell JM, García-Sánchez JE, *et al*. (2014). Guía de Terapéutica Antimicrobiana. Editorial Antares.

Merrett GLB. (2013). Tackling Antibiotic Resistance for Greater Global Health Security. Center on Global Health Security. Chatman House. 1-16.

Mittal R, Krishnan S, Gonzales-Gomez I, *et al.* (2011). Deciphering the roles of outer membrane protein A extracellular loops in the pathogenesis of *Escherichia coli* K1 meningitis. Journal of Biological Chemistry. 286: 2183-2193.

Mohamed R, Conde B, Nieto-Pelegrìn E, *et al*. (2013). Un nuevo fitoextracto natural como potencial antidiarreico frente a *Escherichia coli* enteropatógena. XXIV Congreso de Microbiología SEM 2013.

Morales-Cabrera M, Hernández-Morales J, Leyva-Rúelas G, *et al*. (2013). Influence of variety and extraction solvent on antibacterial activity of roselle (*Hibiscus sabdariffa* L.) calyxes. Journal of Medicinal Plants Research. *7*: 2319-2322.

Motaleb MA, Hossain MK, Sobhan I, *et al*. (2011). Selected Medicinal Plants of Chittagong Hill Tracts. International Union for Conservation of Nature. Bangladesh.1-128

Moreno S, Scheyer T, Romano CS, *et al*. (2006). Antioxidant and antimicrobial activities of rose mary extracts linked to their polyphenol composition. Free Radical Research. 40: 223-231.

Mozaffari-Khosravi H, Jalali-Khanabadi BA, Noori-Shadkam M, *et al.* (2009). The effects of sour tea (*Hibiscus sabdariffa*) on hypertension

in patients with type II diabetes. Journal of Human Hypertension. 23: 48-54.

Nataro JP. (2006). Atypical enteropathogenic *Escherichia coli*: typical pathogens?. Emerging Infectious Diseases. 12: 696-696

Nataro JP, and Kaper JB. (1998). Diarrheagenic *Escherichia coli*. Clinical Microbiology Reviews. 11: 142-201.

Nataro JP. (2013). Diarrhea among children in developing countries. Advances in Experimental Medicine and Biology. 764: 73-80.

Niehhaus MD, Moore SR, and Patrick PD. (2002). Early childhood diarrhea is associated with diminished cognitive function 4 to 7 years later in children in a northeast Brazilian Shanty town. The American Society of Tropical Medicine and Hygiene. 66: 590-593.

Nieto-Pelegrin E, Meiler E, Martinez-Quiles N, *et al*. (2014). Crk Adaptors negatively regulate actin polymerization in pedestals formed by enteropathogenic *Escherichia coli* (EPEC) by binding to Tir effector. PLOS Pathogenes. 10: 1-16.

Nzeako BC, Al-Kharousi ZSN, and Al-Mahrooqui Z. (2006). Antimicrobial activities of Clove and Thyme extracts. Sultan Qaboos University Medical Journal. 6: 33-39.

O'Brien J, Wilson I, Orton T, *et al*. (2000). Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. European Journal of Biochemistry. 267: 5421-5426.

Okoh OO, Sadimenko AP, and Afolayan AJ. (2010). Comparative evaluation of the antibacterial activities of the essential oils of *Rosmarinus officinalis* L. obtained by hydrodistillation and solvent free microwave extraction methods. Food Chemistry. 120: 308-312.

Olaleye MT. (2007). Cytotoxicity and antibacterial activity of Methanolic extract of *Hibiscus sabdariffa*. Journal of Medicinal Plants Research. 1: 9-13.

Oldfield EC, and Wallace MR. (2001). The role of antibiotics in the treatment of infectious diarrhea. Gastroenterology Clinics of North America. 30: 817-836.

Omemu AM, Edema MO, Atayese AO, *et al*. (2006). A survey of the microflora of *Hibiscus sabdariffa* (Roselle) and the resulting “Zobo” juice. African Journal of Biotechnology. 5: 254-259.

Onyenekwe PC, Ajani EO, Ameh DA, *et al.* (1999). Antihypertensive effect of roselle (*Hibiscus sabdariffa*) calyces infusion in spontaneously hypertensive rats and a comparison of its toxicity with that in Wistar rats. Cell Biochemistry Function. 17: 199-206.

O'Reilly CE, Bowen AB, Perez NE, *et al.* (2007). A waterborne outbreak of gastroenteritis with multiple etiologies among resort island visitors and residents: Ohio, 2004. Clinical Infectious Diseases. 44: 506-512.

Pal SC, Rao CK, Kereselidze T, *et al.* (1969). An extensive community outbreak of enteropathogenic *Escherichia coli* O86: B7 gastroenteritis. Bulletin of the World Health Organization. 41: 851-858.

Rahal EA, Kazzi N, Nassar FJ, *et al*. (2012). *Escherichia coli* O157:H7—Clinical aspects and novel treatment approaches. Frontiers in Cellular and Infection Microbiology. 2: 1-7.

Rana IS, Singh A, and Gwal R. (2011). In vitro study of antibacterial activity of aromatic and medicinal plants essential oils with special reference to cinnamon oil. International Journal of Pharmacy & Pharmaceutical Sciences. 3: 376-380.

Ranasinghe P, Pigera S, Premakumara GAS, *et al.* (2013). Medicinal properties of 'true' cinnamon (*Cinnamomum zeylanicum*): a systematic review. BMC Complementary and Alternative Medicine. 13: 1-10.

Rauch M, and Lynch, SV. (2012). The potential for probiotic manipulation of the gastrointestinal microbiome. Current Opinion in Biotechnology. 23: 192-201.

Reanmongkol W, and Itharat A. (2007). Antipyretic activity of the extracts of *Hibiscus sabdariffa* calyces L. in experimental animals. Songklanakarin Journal of Science and Technology. 29: 29-38.

Rodríguez C, Lopéz MC and Domínguez A. (1990). Macromolecular synthesis during the yeast-mycelium transition in *Yarrowia lipolytica*. Experimental Micology. 14: 1-12.

Rodríguez C and Domíngez A. (1984). The growth characteristics of *Saccharomycopsis lipolytica*: morphology and induction of mycelium formation. Canadian Journal of Microbiology. 30: 605-612.

Rodríguez C. (1987). Dimorfismo en *Yarrowia lipolytica*: caracterización fenotípica, síntesis de macromoléculas y movilización de sustancias de reserva durante la transición levadura-hifa. Universidad de Salamanca. Tesis doctoral.

Rodriguez-Rodriguez JC, Samudio-Echeverry IJP, and Sequeda-Castañeda LG. (2012). Evaluation of the antibacterial activity of four ethanolic extracts of bryophytes and ten fruit juices of commercial interest in Colombia against four pathogenic bacteria. Acta Horticulturae. 964: 251-258.

Roy S, Shamsuzzaman SM, and Mamun KZ. (2013). Antimicrobial resistance pattern of diarrheagenic *Escherichia coli* isolated from

acute diarrhea patients. International Journal of Pharmaceutical Science Invention. 2: 43-46.

Ruìz-Herrera J. (2012). Dimorphic fungi: Their importance as models for differentiation and fungal pathogenesis. Bentham Science Publishers. 1-196.

Santosham M, Chandran A, Fitzwater S, *et al.* (2010). Progress and barriers for the control of diarrheal disease. Lancet. 376: 63-67.

Savoia D. (2012). Plant-derived antimicrobial compounds: alternatives to antibiotics. Future Microbiology. 7: 979-990.

Scheutz F, Nielsen EM, Frimodt-Moller J, *et al.* (2011). Characteristics of the enteroaggregative Shiga toxin/verotoxin-producing *Escherichia coli* O104:H4 strain causing the outbreak of haemolytic uraemic syndrome in Germany, May to June 2011. Eurosurveillance. 16: 1-6.

Senhaji O, Faid M, Kalalou I, *et al*. (2007). Inactivation of *Escherichia coli* O157:H7 by essential oil from *Cinnamomum zeylanicum*. The Brazilian Journal of Infectious Diseases. 11: 234-236.

Severs D, Fardy P, Acres S, *et al.* (1966). Epidemic gastroenteritis in Newfoundland during 1963 associated with *E. coli* 0111:4. Canadian Medical Association Journal. 94: 373-378.

Shan B, Cai YZ, and Corke H, *et al*. (2007). The *in vitro* antibacterial activity of dietary spice and medicinal herb extracts. International Journal of Food Microbiology. 117: 112-119.

Sharma GN, Sati N, and Sanadya J, *et al*. (2011). Phytochemical screening and estimation of total phenolic content in *Aegle marmelos* Seeds. International Journal of Pharmaceutical and Clinical Research. 3: 27-29.

Singh R, Koppikar SJ, Paul P, *et al.* (2009). Comparative analysis of cytotoxic effect of aqueous cinnamon extract from *Cinnamomum zeylanicum* bark with commercial cinnamaldehyde on various cell lines. Pharmaceutical Biology. 47: 1174-1179.

Sirag N, Elhadi MM, Algaili AM, *et al*. (2014). Determination of total phenolic content and antioxidant activity of Roselle (*Hibiscus sabdariffa* L.) Calyx ethanolic extract. Standard Research Journal of Pharmacy and Pharmacology. 1: 34-39.

Soll DR. (1986). The regulation of cellular differentiation in the dimorphic yeast *Candida albicans*. Bioessays. 5: 5-11.

Spor A, Koren O, and Ley R. (2011). Unravelling the effects of the environment and host genotype on the gut microbiome. Nature Reviews Microbiology. 9: 279-290.

Stevenson DE and Hurst RD. (2007). Polyphenolic phytochemicals – just antioxidants or much more?. Cellular and Molecular Life Sciences. 64: 2900-2916.

Stagos D, Portesis N, Spanou C, *et al.* (2012). Correlation of total polyphenolic content with antioxidant and antibacterial activity of 24 extracts from Greek domestic *Lamiaceae* species. Food and Chemical Toxicology. 50: 4115-4124.

Tadesse DA, Zhao S, Tong E, *et al.* (2012). Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950-2002. Emerging Infectious Diseases Journal. 18: 741-749.

Thapar N, and Sanderson IR. (2004). Diarrhoea in children: an interface between developing and developed countries. The Lancet. 363: 641-653.

Torbeck L, Raccasi D, Guilfoyle DE, *et al.* (2011). *Burkholderia cepacia*: This Decision Is Overdue. PDA Journal of Pharmaceutical Science and Technology. 65: 535-543.

Usha M, Ragini S, and Naqvi SMA. (2012). Antibacterial activity of acetone and ethanol extracts of Cinnamon (*Cinnamomum zeylanicum*) and Ajowan (*Trachyspermum ammi*) on four Food Spoilage Bacteria. International Research Journal of Biological Sciences. 1: 7-11.

Valgas C, Machado de Souza SM, Smânia EFA, *et al*. (2007). Screening methods to determine antibacterial activity of natural products. Brazilian Journal of Microbiology. 38: 369-380.

Viswanathan VK, Hodges K, and Hecht G. (2009). Enteric infection meets intestinal function: how bacterial pathogens cause diarrhoea. Nature Reviews Microbiology. 7: 110-119.

Vu, HT. (2012). Diarrhea. In: Washington Manual of gastroenterology Subspecialty Consult. Gyawali, CP. Ed.Wolters Kluwer/ Lippincott Williams and Wilkins. 3rd edition.

Walker CLF, Rudan I, and Liu L. (2013). Global burden of childhood pneumonia and diarrhoea.The Lancet. 381: 1405-1416.

Warren JR. (2005). *Helicobacter* – the ease and difficulty of a new discovery. Nobel Lecture. 292-305.

Waters CM., and Bassler BL. (2005). Quorum Sensing: Cell-to-Cell Communication in Bacteria. Annual Review of Cell and Developmental Biology. 21: 319-346.

WGO. (2012). Acute diarrhea in adults and children: a global perspective. World Gastroentrology Organization. 1-24.

WHO. (1995). The treatment of diarrhoea: a manual for physicians and other senior health workers, WHO/CDR/95·3. Geneva: World Health Organization. 1-50

WHO. (1994). The management of bloody diarrhoea in young children: WHO/CDD/94·49. Geneva: World Health Organization. 1-20

WHO/UNICEF. (2013). Ending Preventable Child Deaths from Pneumonia and Diarrhea by 2025. World Health Organization. 1-64.

WHO/UNICEF. (2009). Diarrhea: Why children are still dying and what can be done. World Health Organization. 1-68.

WHO. (2012). The evolving threat of antimicrobial resistance Options for action. World Health Organization. 1-125.

Wikswo ME, and Hall AJ. (2012). Outbreaks of acute gastroenteritis transmitted by person-to-person contact-United States, 2009-2010. MMWR Surveillence Summary. 61: 1-12.

Wiles TJ, Kulesus RR, and Mulvey MA. (2008). Origins and virulence mechanisms of uropathogenic *Escherichia coli*. Experimental and Molecular Pathology Journal. 85: 11-19.

Zahin M, Khan MSA, and Ahmad I. (2010). Ethnomedicinal plants derived antibacterials and their prospects. Ethnomedicine: A Source of Complementary Therapeutics. 6: 149-178.

Zalewska-Piatek PMZ. (2011). Urinary tract infections of *Escherichia coli* strains of chaperone-usher system. Polish Journal of Microbiology. 60: 279-285.

Zaouali Y, Bouzaine T, and Boussaid M. (2010). Essential oils composition in two *Rosmarinus officinalis* L. varieties and incidence for antimicrobial and antioxidant activities. Food and Chemical Toxicology. 48: 3144-3152.

Zhanel GG, Hoban DJ, and Harding GKM. (1992). Subinhbitory antimicrobial concentrations: A review of *in vitro* and *in vivo* data. Canadian Journal of Infectious Diseases. 3: 193-201.