Phytochemical Analysis, Antimicrobial Activity of *Euclea divinorum* (Magic guarri) leaves, tender stems, root bark extracts and Formulation of a Herbal-based Toothpaste for Dental caries Control

MBABAZI IMMACULATE

BSc (Ed)

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2021

DECLARATION PAGE

Declaration by the Candidate

This thesis is my original work and has not been presented for a degree in any other University. No part of this thesis proposal may be reproduced without the prior written permission of the author and/or Moi University.

Signature  Date……12th January 2021…….

Mbabazi Immaculate

(MSC/ACH/9/18)

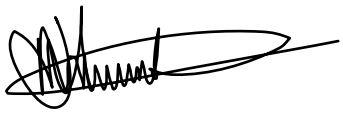
Declaration by Supervisors

This thesis has been submitted for examination with our approval as her University Supervisors.

Signature  Date……12th January 2021……

Dr. Phanice Wangila

University of Kabianga, Kericho, Kenya and Adjunct Senior Lecturer, Moi University, Eldoret, Kenya.

Signature  Date……12th January 2021……

Dr. Isaac O. K'owino

Masinde Muliro University of Science and Technology, Kakamega, Kenya and Adjunct Senior Lecturer, Moi University, Eldoret, Kenya.

DEDICATION

This work is dedicated to my beloved parents, Mr. Bamwesigye Johnson and Mrs. Lovence Bamwesigye and children; Ahabwe Keran and Amara Darin Kayla, and my husband Mr. Tumwekwase Dan who supported me both emotionally and financially throughout the course without forgetting the families of my lovely aunt Mr. and Mrs. Joseph Nduhuura and my uncle, Mr. Milton Nuwagaba.

ABSTRACT

Dental caries is a significant global health challenge as it affects people throughout their lifetime causing pain, discomfort, disfigurement and even deaths with approximately 3.5 billion people affected. The responsible oral pathogens have become resistant to the available drugs most of which have side effects like tooth discoloration, vomiting and change of oral micro-biota. The purpose of this study was to identify potential active ingredients and make a safe toothpaste from *Euclea divinorum* Hern locally known as *omuswa* (Luhya) which has been used in a long time to manage dental caries. The objectives of the study were to compare the phytochemicals, antimicrobial activity of organic extracts of *E. divinorum* leaves, tender stems and root bark against *Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 25923, *Candida albicans* and *Escherichia coli* ATCC 25922 and formulation of a herbal toothpaste from its most active extract. Plant parts of *E. divinorum* were collected from Elgeiyo Marakwet, Rift Valley located in the North Rift region of Kenya. Shade-dried samples were ground into powder and successively extracted with hexane, dichloromethane and ethanol. The methods used to characterize the extracts were classical phytochemical screening, Ultra Violet-Visible Spectrophotometry, Fourier Transform Infrared Spectroscopy and Gas chromatography/mass spectrometry while the antimicrobial activity was determined by agar disc diffusion method. Results showed the presence of alkaloids, phenols, saponins, flavonoids, steroids, cardiac glycosides, tannins, terpenes, and volatile oils in qualitative phytochemical screening. UV- Visible spectra of extracts of various parts had the absorption maxima in the region of 370 – 700 nm implying the presence of flavonoids and their derivatives plus conjugated systems of multiple bonds. FT-IR results showed O=C-O-C, =C-H, C-O, Ar-C=C, C-H, CN, –OH among others that revealed the presence of alcohols, phenols, alkanes, alkenes, alkynes, aldehydes, carboxylic acids, aromatics, nitro compounds and amines in the extracts. A total of 30 compounds were identified by GC-MS in the hexane and DCM extracts of *E. divinorum*. The major compounds were 3,4-Methylenedioxybenzylacetone, Eicosane, Tetratriacontane, Hexatriacontane, 9-Hexadecen-1-ol, 2-Hydroxy-2-methyl-8,8-diphenyl-octa-5,7-dien-3-one, 1,4-Naphthoquinone and Octacosanal, some of which have been reported to possess antimicrobial activity. The *E. divinorum* extracts (leaves, stems and root bark extracts) showed inhibitory activity against *Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. Ethanolic *E. divinorum* root bark extract had a MIC of 50 µg/ml for *S. aureus* and 25 µg/ml for *E. coli* and *C. albicans*. It follows that *E. divinorum* ethanolic root bark extract should be used for the formulation of herbal toothpaste. Different compounds with antimicrobial activity were identified in the extracts of *E. divinorum* hence the difference activity against the selected microbes observed. The formulated toothpaste may be used for the control of dental caries. However, its physical and chemical parameters should be investigated.

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LIST OF ACRONYMS

C. albicans	*Candida albicans*
E. coli	*Escherichia coli*
E. divinorum	*Euclea divinorum*
DMSO	Dimethyl sulphoxide
DCM	Dichloromethane
FT-IR	Fourier Transform Infrared
g	Grams
GC-MS	Gas Chromatography- Mass Spectrometer
GTF	Glucosyltransferase
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
MIC	Minimum Inhibitory Concentration
mL	Milliliter
MS	Mass spectroscopy
ppm	Parts per million
S. aureus	*Staphylococcus aureus*
S. mutans	*Streptococcus mutans*
SPE	Solid Phase Extraction
°C	Degrees Celsius
T. vulgaris	*Thymus vulgaris*
TLC	Thin Layer Chromatography
ZOI	Zone of inhibition

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Chapter 1 : INTRODUCTION

1.1. Background of the study

Bacteria exist freely in the mouth without causing any harm to the person's health provided their concentration does not increase beyond the minimum limit. However, beyond that limit, these bacteria are known to cause tooth infection and decay. *Streptococcus mutans* is considered as the main species that is responsible for tooth infection and decay (Kiani, Firoozian, & Moradkhani, 2017; Rostinawati, Aryani, & Is, 2018). These bacteria metabolize carbohydrates that remains after a meal causing fermentation of carbohydrates, a process that produces acids which results in tooth decay (Okpalugo, Ibrahim, & Inyang, 2009).

Proper hygiene is a necessity for healthy teeth and gums, and this can be achieved through effective removal of dental plaque. Alcohols and antibiotics such as penicillin, erythromycin, ampicillin and chlorhexidine have been reported to prevent dental caries in animals and humans but are not used because of their adverse side effects and the resistance of bacteria to them (Gomashe, Sharma, & Kasulkar, 2014; Ulusoy, 2019). Today, toothpastes and mouthwashes containing antimicrobial agents have been used worldwide for mouth hygiene to prevent dental caries such as plaque and gingivitis (Ion & Chetrus, 2013).

Fluorides as active ingredients in toothpastes play an important role in the reduction of dental caries and control of gingivitis and most of the toothpastes and mouthwashes on market today contain fluorides as anticaries. However, fluoridated toothpaste is not recommended for children under 6 years of age (Stovell, Newton, & Lynch, 2013) and many problems like pigmentation of the teeth, irritation, oral cancer, changes in the intestinal and oral flora and vomiting are associated with use or ingestion of these toothpastes (Ulusoy, 2019).

Recent studies are focused towards developing natural remedies with use of medicinal plants as an alternative for antibiotics and other synthetic antimicrobial agents because they are cheap, effective, readily available and have no adverse effects (Gomashe *et al.*, 2014). Thus, formulating toothpaste with herbs containing compounds with antimicrobial activities is helpful in control of dental caries (Davies, 2008).

Euclea divinorum is a tree or shrub which is deciduous or evergreen and grows at lower altitudes. The root decoction of the plant is used for treatment of cardiovascular disorders, diarrhea (Randrianarivony & Andriamihajarivo, 2016), gonorrhea, candida, syphilis and oral diseases (Chinsembu, 2016). The small stems and the barks of its roots have been used by the local people of Kenya and Uganda to clean and whiten their teeth. Its use to whiten the teeth is an added advantage over those plants that have been used in formulation of toothpaste.

Many compounds have been isolated from different plants that have been used to treat and clean teeth by people in the rural areas and have been found to have antimicrobial properties against oral pathogens (Palombo, 2011).

However, there are limited reports on the total phenolic content, total flavonoid content and antimicrobial activities of the leaves, tender stems and root barks of *E. divinorum* as well as characterization of these extracts.

1.2. Problem statement

There is increased resistance by oral pathogens to the currently available antibiotics (Bhattacharya, Ishnava, & Chauhan, 2016; Chauhan, 2010), increased prevalence of dental caries in developing and middle income countries (Braimoh, Umanah, & Ilochonwu, 2014), changes in oral and intestinal microbiota since most antibiotics have a broad spectrum antimicrobial activity (Marsh, 1992), and side effects caused by the anti-caries agents like vomiting, diarrhea, oral cancer, and tooth staining (Ahmad *et al.*, 2018; Park, You, Lee, Baek, & Hwang, 2003). Thus, there is need for alternative prevention and treatment options for oral diseases that are safe, effective, cheap and locally available.

Tooth brushing with toothpaste is mostly used as a preventive measure for dental caries. However, most of the toothpastes on the market today contain fluorides as the active ingredients against cariogenic organisms as well as a teeth whitening agent (Mangilal & Ravikumar, 2016) which when used in high amounts or swallowed causes dental and bone fluorosis (Manji & Fejerskovi, 1990), brain damage and neurological disorders (Takahashi & Nyvad, 2011).

Most of the toothpastes contain fluorides as the active ingredients which when used in high cause tooth discoloration (Stovell *et al.,* 2013). In the extra-cellular environment of maturing enamel, an excess of fluoride ions alters the rate at which enamel matrix proteins are enzymatically broken down and the rate at which the subsequent breakdown products are removed. Fluoride may also indirectly alter the action of protease via a decrease in the availability of free calcium ions in the mineralization environment. This results in the formation of enamel with less mineralization. This hypomineralized enamel has altered optical properties and appears opaque and lusterless relative to normal enamel.

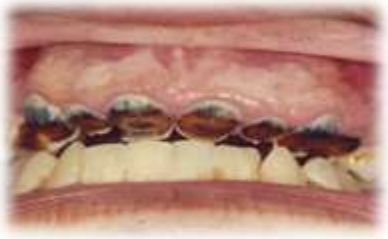


Figure 1.1: Demineralized teeth

All these issues in combination with the modern dynamics of development have resulted in consumer interest for natural additives as the active ingredients in toothpaste instead of the synthetic ingredients. Plant extracts have been found to contain active ingredients as antimicrobials against oral pathogens that cause dental caries (Verkaik *et al.,* 2011).

There is need to identify potentially safe active ingredients from leaves, tender stems and root bark extracts of *Euclea divinorum*. This research also attempted to make toothpaste from *Euclea divinorum* Hern which has been in use for a long time to manage dental caries (Al-Fatimi, 2019).

1.3. Significance of the study

This study was intended to bridge the gap between the available knowledge on the medicinal purposes of *E. divinorum* especially treatment of oral diseases and its antimicrobial activity against *Streptococcus mutans* and *Candida albicans* and the problems associated with the use of synthetic ingredients. It also formulated a herbal toothpaste with anticaries activity.

The use of natural products is required because they are eco-friendly (they impart less or no harm to the environment as well as living organisms as compared to synthetic products).

1.4. Justification of the study

An active ingredient with less or no side effect as compared to the synthetic ingredient and one which the oral pathogens are not resistant to is required in toothpaste formulation for both the young and the adults. Plants that have been used for the treatment of dental caries have been characterized qualitatively and quantitatively using different techniques.

Some plant extracts have been incorporated in toothpastes as the active ingredients to treat and prevent dental caries. *Euclea divinorum* is one of the medicinal plants used for the treatment of oral diseases and the root bark extract previously inhibited the growth of *S. mutans* (Geyid *et al.*, 2005). Although it has not been used in the formulation of toothpaste, it may even be a better option than other plants that have been used since the root barks whiten teeth. Thus, it could provide teeth whitening agents without using synthetic whiteners in the toothpaste formulated.

1.5. Objectives of the study

1.5.1. General objective

The main objective of the study was to analyze the phytochemicals and antimicrobial activities of the leaf, tender stems and root bark extracts of *E. divinorum* and formulate toothpaste for the control of dental caries.

1.5.2. Specific objectives

The specific objectives of this study were to.

i. Analyze the phytochemical constituents in the leaf, tender stem, and root bark extracts of *E. divinorum*.
ii. Determine the total flavonoid and total phenolic contents of the leaf, tender stem, and root bark extracts of *E. divinorum.*

iii. Determine antimicrobial activity of the leaf, tender stem, and root bark extracts of *E. divinorum* against *S. pyogenes*, *S. aureus*, *E. coli* and *C. albicans* microorganisms.

iv. Formulate a toothpaste from *E. divinorum* extracts and evaluate its antimicrobial activity against *S. pyogenes*, *S. aureus*, *E. coli* and *C. albicans* microorganisms.

1.6. Hypotheses (null hypotheses)

i. There are no differences in the phytochemical composition of *E. divinorum* leaf, tender stem and root bark extracts.

ii. There is no significant difference in the composition of total phenolic and flavonoid content in the extracts of *E. divinorum.*

iii. There is no significant difference in the antimicrobial activities of the leaf, tender stem and root bark extracts of *E. divinorum* against *S. pyogenes*, *S. aureus*, *E. coli* and *C. albicans* microorganisms.

iv. There is no significant difference between the antimicrobial activities of the formulated toothpaste and Colgate herbal against *S. pyogenes*, *S. aureus*, *E. coli* and *C. albicans* microorganisms.

Chapter 2 : LITERATURE REVIEW

2.1. Bacterial infections of the teeth and their management

2.1.1. Dental caries

Dental caries refers to the destruction of the tooth as a result of bacterial activities and causes discomfort and pain if left untreated (Gautam & Shrestha, 2018). It is the most common non-communicable diseases in the world and expensive to treat requiring 5% to 10% of the health care budget (WHO, 2017). Other than crushing food to facilitate digestion, the teeth also play an important role in beauty of the face as they contribute to the shape of human face. However, dental cavities and gingival diseases caused by poor hygiene are the most common problems that affect teeth (Ghelichli, 2014).

Streptococcus mutans synthesizes extracellular polysaccharides which can be up to 40% glucan. These are the polysaccharides which make up the bulk of plaque. The bacteria produce these polysaccharides from sucrose from the food remains by the use of glucosyltransferases enzymes (glucosyltransferase B (GTF B), GTF C and GTF D), which each synthesize their own glucans (Bowen & Koo, 2011).

Biofilm dental plaque is a milk-like thin layer around the teeth which contains more than 600 different microorganisms. *Streptococcus mutans* is the major bacterial species found in the mouth that causes dental caries (Kiani *et al.,* 2017). In the mouth, bacteria exist freely without causing harm. However, these bacteria become very dangerous to the teeth especially when their concentration exceeds a certain limit. They react with sugars from the food after a meal causing fermentation. Fermentation produces organic acids which erodes the enamel and eventually causes dental cavities in a process known as demineralization (Abou Neel *et al.*, 2016). The enamel is made up of hydroxyapatite which reacts with the acids produced from the process of fermentation resulting in dental cavities as illustrated in Equation 1 and Figure 2.1.

$$Ca_{10}(PO_4)_6(OH)2 + 8H^{+} \longrightarrow 10Ca^{2+} + 6(HPO_4)^{2-} + 2H_2O$$

Equation 1: Formation of tooth cavities.

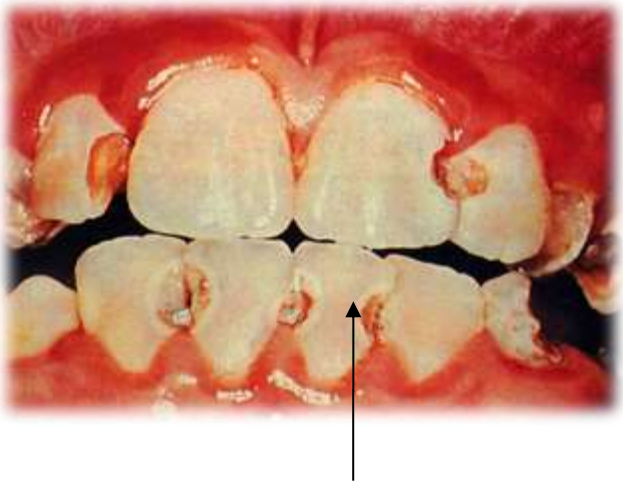


Figure 2.1: Dental cavities caused by acid erosion of the enamel

Bacterial activity at the tooth-gum interface leads to irritation of the gingival which leads to its inflammation. Gingival is a fibrous tissue covered by a mucosal membrane (Ghelichli, 2014). Various studies have proven that the prevalence of periodontal disease is high and dental plaque is associated with this prevalence. Clinical studies have also shown that the occurrence of gingivitis is favored by the presence of plaque (Gupta *et al.*, 2015).

2.1.2. Bacteria associated with dental caries

The oral cavity contains numerous microorganisms up to 700 different species consisting of both bacteria and fungi whose interactions result in dental caries and mucosal infections (Koo, Andes, & Krysan, 2018). Supragingival plaque consists of mainly Gram-positive bacteria that are responsible for dental caries while sub-gingival plaque is composed of Gram-negative species and are responsible for gingivitis and periodontal diseases (Schnack, 2019).

Research was carried out to find the oral biota in dental caries patients and the most prevalent microflora were found to be *Streptococcus mutans* (43 %), *E. coli* (26%), *Enterococcus* species (20%), *Pseudomonas aeruginosa* (7%) and *Staphylococcus aureus* (40%) (Rajaprabu, Siddharthan, Poongothai, Manikandan, & Hemalatha, 2018). *Staphylococcus aureus* was also the most prevalent with 46.4% in the oral cavity in supragingival plaque and saliva according to Ohara-Nemoto, Haraga, Kimura, and Nemoto (2008).

The interaction between *Candida albicans* and streptococcal biofilm is the most common cause of childhood caries although it is unable to colonize mineralized tooth enamel by itself. The presence of high amounts of sucrose sugars in the mouth increases the physical co-adhesion between *C. albicans* and streptococcal biofilms enhancing the microbial count, aciduricity and providing a substrate for exopolysaccharides α-glucans production by streptococcal glycosyltransferases (Gtfs) that enhance co-adhesion and bacterial–fungal tooth colonization (Koo *et al.,* 2018). *Streptococcus pyogenes* has been reported as the pathogen associated with the progression of periodontal diseases (Trapp & Scott, 2017).

2.1.3. Prevalence of dental caries

A technical report from the World Health Organization (WHO) indicated that dental caries is the most prevalent of all health conditions especially in middle income countries with half of the world's population being affected. In 2015, it was ranked the first global burden with 2.3 billion people having decayed permanent teeth (WHO, 2017) and the most common childhood disease, five times more than asthma (Schnack, 2019).

According to WHO statistics, 60-90% of school children and about 100% of adults have dental cavities (Ghelichli, 2014). Another report investigating dental cavities, gingivitis and the treatment needs among twelve year old indicated a prevalence of dental carries of 53% with a high number of girls affected than boys while that of gingivitis was found to be 77.7% (Owino, Masiga, & Macigo, 2010).

A comprehensive review on the global epidemiology of dental caries and severe periodontitis reported that the decay component is very high in low-income countries with the lowest prevalence among 12-year-olds in high-income countries and among people between 35 and 44 years old. Although the prevalence of dental caries has decreased over the past four decades, that of periodontitis is high with approximately 10% of the global population affected (Frencken *et al.,* 2017).

2.1.4. Prevention of dental caries

Different measures have been employed to prevent dental caries and research done to evaluate their effectiveness. In the recent years, numerous reports have been presented about control of dental plaque and gingivitis using different dentifrices (Davies, 2008). A study on the prevalence of dental caries, knowledge and attitude towards oral care in Chennai, Tamil Nadu was conducted on selected visually impaired individuals from

four schools. It was reported that 42% of the selected individuals had fair oral hygiene, 33% had good and 25% had poor oral hygiene. About 0.7% of the selected impaired individuals were reported to clean their teeth by rubbing salt onto the teeth with fingers, 8.2% rubbed toothpowder with fingers, 1.5% used neem stick while 89.6% used toothbrush and toothpaste. Among those that used toothpaste and toothbrush for their oral hygiene, 2% brushed more than twice a day, 29.5% twice a day whereas 68.5% brushed only once a day (John, Daniel, Paneerselvam, & Rajendran, 2017).

2.1.5. Dentifrices

Dental caries has been prevented using dentifrices. A dentifrice is a product used in maintaining oral hygiene for preventing tooth decay and for mouth freshness. Dentifrices are of different kinds and these include gels, toothpastes, toothpowders and mouthwashes. Toothpaste, gels and mouth washes are the most commonly used dentifrices which differ in their physical states due to different formulations as shown in Table 2.1

Table 2.1: Forms of dentifrices and their respective ingredients

Toothpaste	Mouthwash	Gel
Therapeutic agent	Therapeutic agent	Therapeutic agent
Abrasive	-	Less abrasive
Surfactants	Surfactants	Less surfactants
Humectants	Humectants	Humectants
Flavor	Flavor	Flavor
Flow modifier	-	Flow modifier
Preservatives	Preservatives	Preservatives
Color	Color	Color
Binders	-	Binders
-	Ethanol	-
Water	Water	Water

Source: Vranic *et al*. (2004).

A good dentifrice should have the following properties.

1. It should not irritate the mouth or be toxic.
2. It should have a good abrasive effect. It should be able to remove plaque and any other debris deposits from the teeth.
3. It should not impart any stain onto the teeth.
4. It should have a prolonged effect i.e. it should keep the mouth fresh and clean
5. It should be cheap and easily accessible (Mamatha & Kumar, 2017).

As early as 3000-5000 BC when toothpaste and mouthwashes were not commercially available, Egyptians made a dental cream by mixing powdered ashes of oxen hooves with myrrh, burned egg shells, pumice, and water (Gupta *et al.,* 2015). They had no toothbrushes but rather they used chewed stems to apply the ash onto the teeth. Later on, toothpaste was formulated as a powerful weapon against tooth decay and fluoride toothpastes were found to be more effective in the reduction of dental cavities (Abhay & Dinnimath, 2015).

Proper use of toothbrush and toothpaste is the most effective way to control plaque. Different formulations of toothpastes have been put in place for the control of dental cavities where synthetic and natural ingredients have been used. Toothpastes formulated with synthetic components have active ingredients like fluorides, triclosan, chlorhexidine, preservatives, whiteners and surfactants which may cause irritation of oral tissue (Davies, 2008).

Toothpaste containing triclosan reduced dental plaque and gingivitis (Davies, 2008). However, reports have suggested that triclosan, an active ingredient in many kinds of

toothpastes, can combine with chlorine in tap water to form chloroform which the United States Environmental Protection Agency classifies as a possible human carcinogen. An animal study revealed that the chemical might modify hormones hence causing hormonal imbalance in humans if consumed through its use. Other reports proved bacteria could be able to develop resistance to triclosan in a way which can help them to resist antibiotics in the body (Vranic *et al.*, 2004).

Fluorides as active ingredients in toothpastes play an important role in the reduction of dental caries and control of gingivitis, but cause surface stains. Fluoridated toothpaste is not recommended for children under 6 years of age (Stovell *et al.*, 2013).

Chlorhexidine is used in control of surface decay and dental plaque due to its high antibacterial activity. However, it has many problems like resistance (Jothika, Vanajassun, & Someshwar, 2015), pigmentation of the teeth, tongue and mouth, change of taste, irritation, oral dryness, scaling of gingival and negative systemic effects in ingestion (Ghelichli, 2014). It also has a broad-spectrum antimicrobial activity (Ravi, Nirupad, Chippagiri, & Pandurangappa, 2017) thus it may also kill the useful microorganisms in the mouth. The efficacy of Chlorhexidine is due to the fact that it binds to the oral tissues and is released slowly into the oral cavity but bitter taste and alteration of tastes which last for about four hours after using toothpastes or oral rinses containing chlorhexidine are the side effects (Eden, 2008).

A randomized single blind *in vivo* study aimed at determining the effectiveness of probiotics, chlorhexidine, and fluoride mouthwashes against *S. mutans* was conducted. Patients were selected at random and divided into four groups where the first group was told to rinse their mouths with 10 mL of 0.2 % Chlorhexidine mouthwash, the second

group with 10 mL of 500 ppm of fluoride Colgate mouthwash, third group with probiotic mouthwash and the last group with distilled water after brushing with non-fluoridated toothpaste. There was a significant difference between the bacterial count in the control and antibacterial agents but there was no significant difference among the antibacterial agents. The bacterial count in the Chlorhexidine group was observed to have increased significantly from 1.8 CFU to 19.58 CFU possibly due to resistance (Jothika *et al.*, 2015).

2.2. Medicinal plants that have been used for oral care in rural communities

Different medicinal plants have been used in maintenance of proper oral hygiene most especially by the local people. The WHO encourages the use of chewing sticks from the roots or stems.

Table 2.2 shows some of the medicinal plants that have been used for oral care with their respective local names or common names among different communities, part of the plants used and the source of information.

Table 2.2: Plants used for oral care

Scientific name (Family)	Local name	Medicinal use(s)	Part(s) used	Reference(s)
Aloe vera (Liliaceae)	*Kiruma*	Toothache, ulcers	Leaves	Ngari *et al.* (2014)
Asparagus setaceus (Asparagaceae)	Not reported	Toothache	Leaves	Odongo *et al.* (2018)
*Distemonanthus benthamianus (*Fabaceae)	*Movingui*	Dental cleaning and chewing sticks	Stems	Bankole *et al.* (2012)
Diospyros lycoides (Ebenaceae)	Not reported	Toothache and chewing stick	Stem	Mwonjoria *et al.* (2018)
Euclea divinorum (Ebenaceae)	*Mukinyii Uswo*	Gum bleeding, toothache and chewing sticks	Roots	Ngari *et al.* (2014)
Fagara zanthoxyloides (Rutaceae)	*Ata*	Chewing sticks	Root and stem	Adefisoye *et al.* (2012)

Scientific name (Family)	Local name	Medicinal use(s)	Part(s) used	Reference(s)
Massularia acuminata (Rubiaceae)	*Pako Ijebu*	Teeth cleaning	stems	Bankole *et al.* (2012)
Maytenus putterlickioides (Celastraceae)	*Muthunthi*	Toothache	Leaves	Kaigong and Musila (2015)
Psidium guajava	Guava	Relieves tooth pains	Leaves	Mamatha and Kumar (2017)
Salvia officinali (Lamiaceae)	*Sage*	Swollen gums	Whole plant	Tyagi (2015)
Senna didymobotrya (Caesalpininceae)	*Mwenu*	Mouth ulcers	Leaves and roots	Ngari *et al*. (2014)
Solanum incanum (Solanaceae)	*Mutongu*	Toothache	Fruits and roots	Ngari *et al.* (2013)
Terminalia glaucescent	Not reported	Toothache	Stems	Kolapo *et al.* (2008)
Withania somnifera (Solanaceae)	*Murumbae*	Bad mouth odor	Leaves	Ngari *et al.* (2014)
Ximenia caffra (Olacaceae)	*Olemo*	Toothache and mouth infection	Bark and root	Geissler *et al.* (2002)

2.3. Plant metabolites with antimicrobial activities against oral pathogens

Medicinal plants have been used traditionally to treat various diseases in many parts of the world and today, their use is common in rural areas of most developing countries. Natural products in medicinal plants have been proven to have many biologically active compounds that have been identified as potential compounds for new drug development (Palombo, 2011). Numerous studies focusing on the use of medicinal plant extracts to control oral pathogens have been conducted.

A study was conducted on the antibacterial activity of natural herbal extracts on *S. mutans* and their potential as active compounds in toothpastes. Chlorhexidine and ciprofloxacin were used as standards and the methanolic extracts of mango twigs and eucalyptus twigs showed comparatively large zones of inhibition as the standards. Mango and eucalyptus twig methanolic extracts showed larger zone of inhibition (20.0

± 0.0, 23.5 ± 0.5, 16.0 ± 1.0, and 11.25 ± 0.25 in mm) compared to pudina leaf and garlic extracts that showed no inhibitory activity against the tested bacteria. When the mango and eucalyptus twig extracts were analyzed for their minimum inhibitory concentrations (MIC), the mango twig extract was found to have higher activity at lowest levels than the eucalyptus extracts. Thus, the mango and eucalyptus twig extracts have the potential to be added to toothpaste as antibacterial agents (Ravi *et al.*, 2017).

2.3.1. Phenolic compounds

The root bark methanolic extract of *Morus alba* was investigated for its antibacterial activity against oral pathogens and it was found to contain an active compound known as Kuwanon which showed the MIC of 8 µg/mL against *S. mutans* which was comparable to those obtained by (Park *et al.*, 2003). The methanol extract of *P. guajava* leaves yielded quercetin-3-O-α-L-arabinopyranoside which is a flavonoid and showed a MIC of 2-4 µg/mL against *S. mutans* (Prabu, Gnanamani, & Sadulla, 2006).

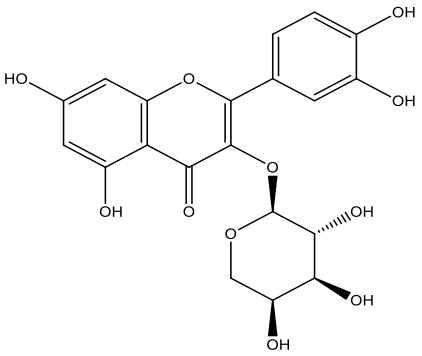
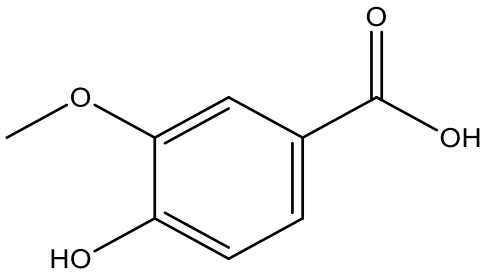


Figure 2.2: Structure of Quercetin-3-O-alpha-L-arabinopyranoside

Filali-Ansali *et al.* (2016) conducted a study on antioxidant and antimicrobial activities of chemical constituents from *Celtis australis*, vanillic acid, β-sitosterol and β-sitosterol-3-O-β-glucoside were found to be major constituents of methanol extract of leaves of *C. australis*. All compounds were tested for their antimicrobial activities and

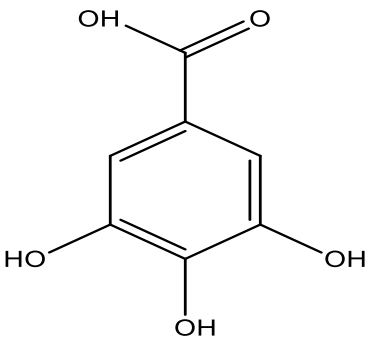
they showed significant activity against the selected bacteria strains. Vanillic acid had the strongest activity with MIC of 25 µg/mL against *B. cereus* (Filali-Ansari *et al.*, 2016).



vanillic acid

Figure 2.3: Structure of vanillic acid

The antibiofouling effect of polyphenols against *S. mutans* was investigated and most of the polyphenols used showed a significant reduction in biofilm formation. Gallic and tannic acids exhibited significant enzyme inhibition effects below their MICs and tannic acid had the greatest antibacterial activity against *S. mutans* with MIC of 0.4 mg/mL as compared to ascorbic acid, gallic acid, salicyclic acid, and quercetin which had MIC of 1.2-3.0 mg/mL (Taylor, Sendamangalam, Choi, Kim, & Seo, 2011; Veloz, Alvear, & Salazar, 2019).



Gallic acid

Figure 2.4: Structures of gallic acid

Extracts from citrus fruits contained active compound, Naringin that showed antimicrobial activity against growth of periodontal pathogens and other oral microorganisms with MIC of 125 mg/mL (Palombo, 2011).

Maldvin-3,5-diglycoside was identified as the active constituent of ethanol extract of *Alcea longipedicellata* flowers. When tested for its anticariogenic properties against oral pathogens had MIC of 160-200 µg/mL. The ethanol extract was isolated using silica gel column chromatography where the extract (2g) was loaded on the column and eluted using a continuous gradient of ethyl acetate. The isolates were subjected to FT-IR and carbon-13 NMR analysis for characterization (Babak *et al.*, 2007).

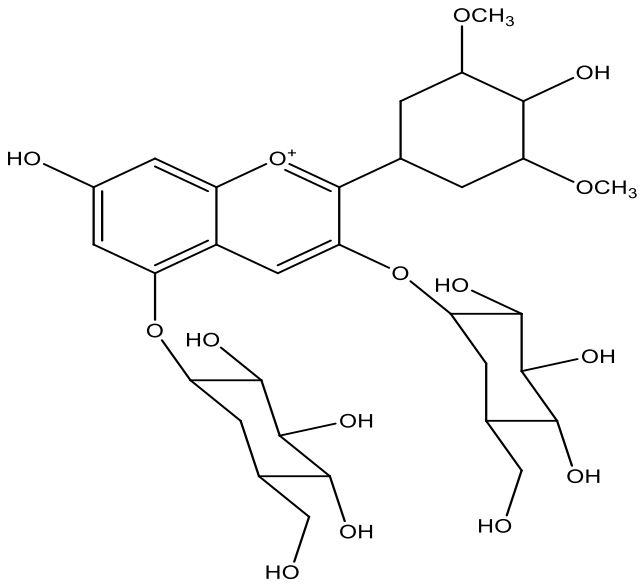


Figure 2.5: Structure of Maldvin-3,5-diglycoside

2.3.2. Fatty acids and their ester derivatives

Nalina and Rahima investigated the antibacterial effect of aqueous extracts of Piper betle leaves against *S. mutans* revealed that the extracts significantly reduced the acid producing properties of the bacterium. Chemical analysis with TLC and GC-MS showed hydroxychavicol, fatty acids (stearic and palmitic) and hydroxyl fatty acid

esters (stearic, palmitic and myristic) as the main constituents. The micrographs from Transmission Electron Microscopy showed that the extracts caused plasma membrane damage and nucleoid coagulation of *S. mutans* (Nalina & Rahim, 2007).

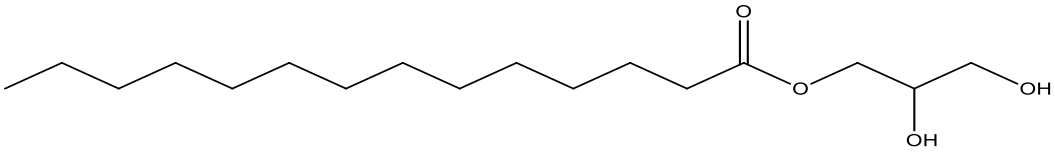


Figure 2.6: Structure of Myristic acid,2,3-bis(hydroxy)propyl ester

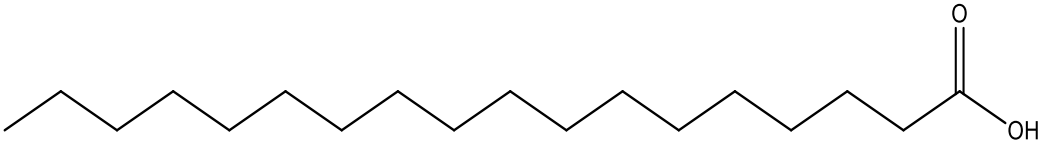


Figure 2.7: Structure of Stearic acid

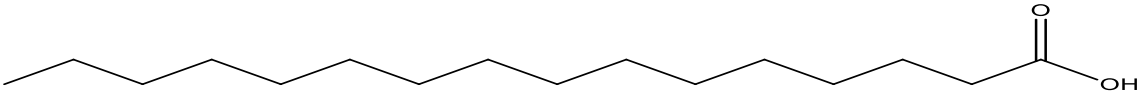


Figure 2.8: Structure of palmitic acid

2.3.3. Alkaloids

Hu *et al.,* conducted a study on *Coptidis rhizoma*'s ability to inhibit growth and proteases of oral pathogens was investigated and an alkaloid berberine was isolated which showed bactericidal activity with the greatest activity against *Porphyromonas gingivalis* (MIC of 20 µg/mL) and less activity against *Streptococcus* and *Lactobacillus* species (Hu, Takahashi, & Yamada, 2000).

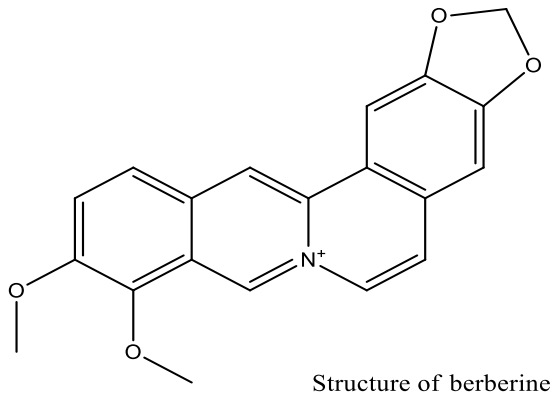


Figure 2.9: Structure of berberine, an alkaloid

2.3.4. Quinones and their derivatives

Quinones are group of plant and bacteria metabolites which often exhibit biological activities (Quintal-novelo & Rocio, 2013). Species in Ebenaceae family have been reported to have naphthoquinone derivatives (Babula, Adam, Havel, & Kizek, 2007). Previously, 7-methyl juglone, and other naphthoquinone derivatives were reported in *E. divinorum* root bark (Mebe, Cordell, & Pezzuto, 1998).

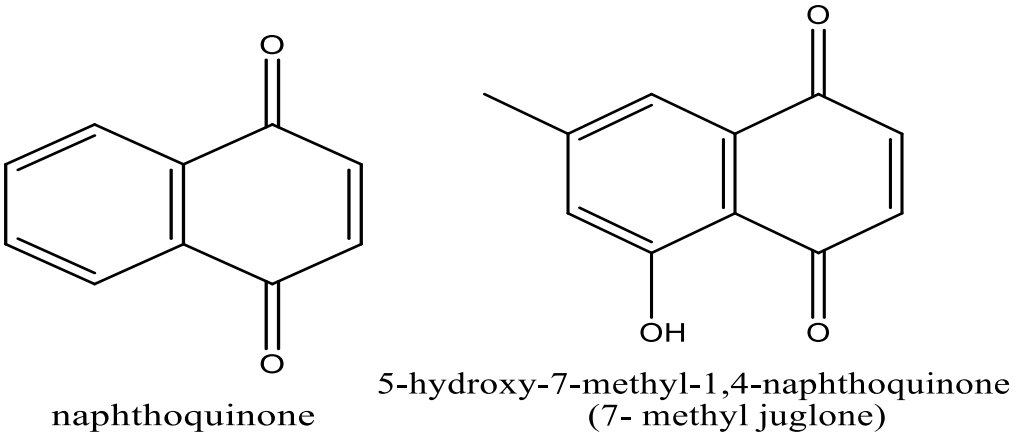


Figure 2.10: Some naphthoquinones identified in *E. divinorum* root bark

2.4. Toothpaste/mouthwash with natural ingredients (herbs)

Considering the reports about the side effects associated with the use of synthetic active ingredients against oral bacteria responsible for dental caries, many studies about

toothpaste formulation using the herbs as active ingredients for oral hygiene which range from antibacterial agents, detergents and whiteners have been conducted and such toothpastes have been found to be effective. The incorporation of plant extracts as active ingredient in oral care products provides an alternative cheap and safe means to people.

Toothpaste formulated with *Salvadora persica* and *Valeriana officinalis* which had been used to prevent and treat tooth diseases was found to be effective in control of growth of *S. mutans* (Ghelichli, 2014). Another study was conducted on formulating toothpaste with orange peel, banana peel and guava leaf extracts which was found to be helpful in reducing bacterial growth in the mouth (Sekar, Jasmin, & Ariffin, 2016).

A comparative study on multiple herbal (polyherbal) toothpaste and commercial toothpaste was conducted and the results showed that methanol extract of polyherbal formulations displayed significant antimicrobial activity against all selected human oral pathogens. Methanol extract exhibited broad spectrum of activity when compared with marketed brands of toothpastes and with standard Gentamicin. The polyherbal methanolic extract formulations showed to be more effective when compared with market (Sharma, Agarwal, Prakash, Pandey, & Singh, 2014). A study on formulating toothpaste using various forms neem twigs showed that the powder form contains tannins and fibers which were expected to be responsible for the tooth cleaning property and the antimicrobial activity (Patil, 2008).

An experiment on formulation and evaluation of herbal toothpaste and comparing it with marketed preparations (Colgate, Dabour Red, and Dantakanti) was conducted. An herbal toothpaste was formulated with plant extracts of neem leaves, guava leaves, and cinnamon barks among other ingredients which included camphor and honey. The

leaves were dried and crushed into fine powder before incorporating it in the toothpaste. The formulated toothpaste showed high antimicrobial activity against *S. aureus*. The formulation had comparable properties as the marketed ones (Deshmukh, Telrandhe, & Gunde, 2017).

Selihi *et al*. reported that *Thymus vulgaris* essential oils are mainly used in the food industry and in cosmetics as antioxidants and preservatives (Salehi, Rad, Contreras, & Carretero, 2018). These properties make *Thymus vulgaris* essential oil desirable in making toothpastes for control growth of mouth flora. Study on toothpaste formulation with *Thymus vulgaris* essential oil extract was conducted and when the toothpaste was evaluated for its chemical and physical parameters, the toothpaste was found suitable to be used to maintain oral hygiene (Kiani *et al.,* 2017).

Many plants have been used to treat dental carries and a lot of research has been conducted to evaluate antimicrobial activities of plant extracts against oral pathogens and their potential as active ingredients alternative to synthetic ingredients in toothpaste. Although *Euclea divinorum* has been used by the local people of Kenya to clean and whiten their teeth, there is limited information on the phytochemical composition of the plant extracts, and it has not been used to formulate toothpaste. The teeth whitening ability of this plant stimulated the interest to incorporate its extracts as active ingredient in formulating herbal toothpaste.

2.4.1. Taxonomy

Euclea divinorum, known as magic guarri in English and locally known as *nsikizi* (Orwa, Mutua, Kindt, Jamnadass, & Simons, 2009) by the Baganda of Uganda, *omuswa* by the Luhya, *olkinyie* by the Maasai (Onyango *et al.,* 2014) and *uswo* by the Kalenjin people of Kenya. It is classified as follows.

Kingdom	Plantae
Phylum	Spermatophyta
Class	Dicotyledonae
Order	Ebenales
Family	Ebenaceae
Genus:	*Euclea*
Species:	*E. divinorum*

2.4.2. Ethnobotany of *E. divinorum*

Euclea divinorum is a shrub or a tree about 6m tall often branching from the base or sometimes with a single stem and is ever green (Woldemedhin, Teshome, Shibeshi, & Sisay, 2017). The leaves are simple, lancoelate with entire or sometimes wavy margin and are alternately, sub-opposite or sometimes oppositely arranged onto the branches. Figure 2.11 shows the photograph of *E. divinorum* in its natural environment where it was collected.

Figure 2.11: *Euclea divinorum* plant in its habitat

The plant is dioecious, usually grows in bushes, dry forest margins and open woodlands usually associated with Acacia species. It prefers calcareous valley clays and rocky grounds. It also grows on anthills and riverbanks in hot dry areas below 900 m. *E. divinorum* plants are distributed as native species in Botswana, Kenya, Namibia, South Africa, Swaziland, Tanzania, Uganda and Zimbabwe (Orwa *et al*., 2009).

2.4.3. General uses of *E. divinorum*

The fruits are edible, used in making beer, and are boiled to produce purple ink. In East Africa, the bark is used in preparation of fatty meat and milk soups, rich in phenolics thus used to produce ink that is used in dyeing baskets by the local people (Orwa *et al*., 2009). The bark is also used to produce high quality leather with a deep red colour. Roots of *E. divinorum* are chewed to colour the lips (Mwihaki *et al.*, 2012). In South Africa, the branches of this plant are hung above the entrance of huts and kraals to repel witches and other evil (Orwa *et al*., 2009).

2.4.4. Medicinal uses of *E. divinorum*

In Kenya, twigs are preferred to other parts of the plant as toothbrushes to clean the teeth. The root bark is traditionally used to treat toothache, headache, chest pain, ulcers (Magama, Pretorius, & Zietsman, 2003; Mwonjoria *et al.*, 2018), diarrhea, convulsions, cancer, skin diseases, and gonorrhea (Mebe *et al.*, 1998), fungal diseases, sores, wounds and abscesses (Mothana, Lindequist, Gruenert, & Bednarski, 2009). The leaves are used to treat noisy stomach, toothache and headache (Samie *et al.*, 2010).

2.4.5. Pharmacological activity of *E. divinorum* extracts

Mbanga, Ncube, and Magumura (2013) evaluated the antimicrobial activity of *Euclea undulata*, *Euclea divinorum* and *Diospyros lycioides* stem extracts on multi- drug resistant Streptococcus mutans. Thirty-one isolates of *S. mutans* were obtained from 47 carious teeth, tested for their sensitivity to eight antibiotics and the isolates that were

found to be resistant to at least four antibiotics were considered to be multi-drug resistant and were used during the study (Mbanga *et al.,* 2013). The author reported than the methanol, acetone and aqueous extracts of stems of *E. divinorum* exhibited inhibitory activities against four isolates of *S. mutans.* However, the acetone and aqueous extracts of *E. divinorum* exhibited higher antibacterial activity against four isolates than the methanol extract (Mbanga *et al.,* 2013).

The root extract of *E. divinorum* (dichloromethane: methanol mixture in the ratio of 1:1) inhibited the growth of all tested microorganisms and had a higher antibacterial activity than the activities of the stem and leaf extracts. It showed an inhibition of 9 ± 1 mm and 12 ± 1 mm against *S. mutans* and *S. sanguinis,* respectively. The stem and leaf extracts inhibited the growth of only *S. sanguinis* with zones of inhibition of 11 ± 2 mm and 10 ± 1 mm (Nyambe, 2014).

The root methanolic extract of *E. divinorum* exhibited antimicrobial activity to all tested strains of microbes (*S. aureus, Bacillus subtilis, Micrococuss flavus, E. coli, Pseudomonas aeruginosa* and *Candida maltosa*) and three multi-resistant strains of *Staphylococcus* strains (Mothana *et al.,* 2009).

The renoprotective effects of the crude extract (80% methanol) and solvent fractions (methanol aqueous) of *E. divinorum* leaves was evaluated against gentamicin-induced nephrotoxicity in rats. The other reported that pre-administration and co-administration of *E. divinorum* extracts along with gentamicin promotes in nephroprotection in rats. Acute toxicity testing results of the crude extract showed that it was the oral dose of 2000 mg/mL was safe and there was no change in behaviour seen in rats after 14 days of administration with extracts. The antioxidant activities of the extracts were evaluated and the maximum inhibition of DPPH at a concentration of 2000 µg/mL was found to

be 82.5%, 74.5% and 62.5% for methanol fraction, aqueous fraction and crude extract respectively (Feyissa *et al.*, 2013).

Mebe *et al.* (1998) isolated naphthoquinones, triterpenoids and flavonoids from chloroform extract of root barks and the isolated compounds were evaluated for their cytotoxicity against apanel cell lines. 3-β-(5-hydroxyferuloly)Lup-20-(30)-ene showed activity to only murine lymphatic leukemia and human breast cancer, 7-methyljuglone showed cytotoxicity to all tested cell lines.

The hexane extract was tested for its antifungal activity against *C. albicans* was reported to be inactive and methanol extract had weak activity. However, the antibacterial activity of the leaves, stem bark extracts showed some considerable activity against *S. aureus, E. coli* and other oral microbiome (Al-Fatimi, 2019).

2.4.6. Phytochemical studies conducted on the plant

Onyango *et al.* (2014) conducted a study of phytochemicals of medicinal plants used in milk and meat processing and preservation in Maasai Mara community in Kajiado district of Kenya. The authors reported the presence of tannins, saponins, alkaloids, steroids, terpenoids and reducing compounds in the methanol extract of root barks of *E. divinorum.* The water extract contained tannins, saponins, flavonoids, alkaloids, steroids, terpenoids, reducing compounds and flavones aglycones while the ether extract showed tannins, flavonoids, alkaloids, terpenoids, steroids and flavones aglycones (Onyango *et al.*, 2014).

Nyambe (2014) screened for the phytochemical constituents in the chewing sticks from *Diospyros lycioides* and *E. divinorum*. Anthraquinones, catenoids, saponins, tannins, polyphenols, and terpenoids were reported to be present in the dichloromethane:

methanol mixture extracts of all the parts of the plants (leaves, roots and the stems). Alkaloids were reported present in only the root extract (Nyambe, 2014).

Previous studies have been conducted to characterize the phytochemicals present in the root bark of *E. divinorum* which isolated naphthoquinones, triterpenoids and flavonoids. For example, 3-β-(5-hydroxyferuloly)Lup-20-(30)-ene, isodiospryrin, shinalene, catechin, lupeol, lupine,7-methyljuglone and betulin(3-5) were reported in the chloroform extract of *E. divinorum* root barks (Mebe *et al.,* 1998).

Isolation and structure elucidation of bioactive compounds in the leaves of *E. divinorum* led to the identification of two new compounds using UV-Vis, IR, ^{1}H, ^{13}C NMR, COSY, HMQC, HMBC and HREIMS (Mwihaki *et al.,* 2012). Euclenal A was identified as 8-hydroxy-3-methoxy-1-naphthaldehyde with a melting point of 207 °C to 210 °C while Euclenal B (4-hydroxy-3,8-dimethoxy-1-naphthaldehyde) had a melting point of 222 °C (Mwihaki *et al.,* 2012).

Similarly, flavonoids were characterized from aerial parts of *E. divinorum.* Extraction was performed in a Soxhlet apparatus for 12 hours using ethanol, and the resultant extract was partitioned between water and ethyl acetate. The ethyl acetate faction was then subjected to column chromatography using methanol: chloroform mixtures of increasing polarities. ^{1}H NMR, ^{13}C NMR, IR, MS and UV spectra provided information that was interpreted to give (2R:3R)-aromadendrin-3-O-β-L-arabinopyranoside, catechin, quercitrin and myricitrin (Dagne, Alemu, & Sterner, 1993).

2.4.7. Methods for analysis of phytochemicals

These include methods that have been employed to characterize compounds in oral medicinal plant extracts. Purification and separation of the plant extracts has been

employed by the use of chromatographic methods which include TLC and column chromatographic methods like HPLC (Altemimi, Lakhssassi, Baharlouei, Watson, & Lightfoot, 2017).

Structural elucidation of the bioactive compounds utilizes data from a wide range of spectroscopic techniques as well as classical methods. For example ultra violet visible (UV-Vis), infrared (IR), nuclear magnetic resonance (NMR) and mass spectroscopy. When the electromagnetic radiation is passed through an organic molecule, it absorbs some radiation which generates a spectrum as a measure of the amount absorbed.

2.4.7.1. Phytochemical screening

This is a classical method used to analyze plant metabolites present in the plant extract. Phytochemical screening assay is quick, cheap and simple to operate giving the researcher a quick answer on the classes of secondary metabolites present in extracts being investigated (Sasidharan, Chen, Saravanan, Sundram, & Latha, 2011).

2.4.7.2. Fourier Transform Infrared spectroscopy

An infrared radiation is passed through an organic molecule and it absorbs the radiation that causes vibrational changes in the molecule. This technique is quick, and it does not destroy the sample. Different bonds have different vibrational frequencies which is a characteristic frequency absorption band in the IR spectrum, and this enables identification of specific bonds (Altemimi *et al.,* 2017). FT-IR technique was used in identification of the functional groups in the leaf extracts of *Indigofera aspalathoides,* one of the plants used for the treatment of oral diseases and alkene, aromatic ring, alkynyl, carboxylic acid, and phenol groups were reported to be present (Kumar *et al.,* 2018). FT-IR characterization of chloroform extract of *E. divinorum* root bark showed the presence of conjugated esters (C=O), esters (C-O), alkenes ($C=CH_2$) and trans

alkenes (Mebe *et al.,* 1998). However, this technique has not been reported to have been used to characterize ethanol, dichloromethane and hexane extracts of *E. divinorum*.

2.4.7.3. Gas Chromatography/ Mass Spectrometry

GC-MS analysis is best suited for small molecular weight compounds (50-500 amu) that are thermally stable and volatile (Jorge, Mata, & António, 2016). The results from the NIST 2014 database do not give 100% assurance for the identification of any given compound in a sample. A high percentage similarity index (SI) could indicate that the match is most probably the identification of the molecule, and vice versa. However, GC-MS is a powerful tool that can assist in identifying possible compounds with high certainty, especially if a match exists in the National Institute of Science and Technology library (Jain, Soni, Jain, & Jeetendra, 2016).

Despite its wide application to characterize the composition of medicinal plant extracts, this technique has not been reported to have been used to characterize extracts of *E. divinorum*.

2.4.7.4. Ultraviolet-Visible Spectroscopy

This technique has been employed in characterization of medicinal plant extracts both qualitatively and quantitatively. Qualitative analysis involves conductive a scan from a range wavelength to obtain the wavelength maxima at with the extracts absorb light in the ultra violet and visible regions (Kalaichelvi & Dhivya, 2017). UV-Visible characterization of ethanol and chloroform extract of *E. divinorum* root bark leaves showed absorption peaks between 234 and 450 nm (Dagne *et al.,* 1993; Mebe *et al.,* 1998). However, this technique has not been reported to have been used to characterize ethanol, dichloromethane and hexane extracts of *E. divinorum*.

2.5. Summary of the Gap

A comparative study on antibacterial and antifungal activity of different parts of *E. divinorum* has not been reported, there is limited number of reports available on characterization and some techniques like GC-MS analysis have not been employed to characterize the extracts of *E. divinorum* even though its pharmacological studies and use in dental hygiene have been reported. Extracts of this plant have shown antibacterial activity against some oral pathogens thus need to further investigate the inhibitory effect of different extracts of parts of *E. divinorum* against selected microorganisms associated with dental caries and formulate a herbal based toothpaste for their control.

Chapter 3 : METHODOLOGY

3.1. Study design

This research was conducted both qualitatively (phytochemical screening of constituents in the extracts of leaves, tender stems and root barks) and quantitatively (the concentrations of phenolics and flavonoids plus antibacterial activity of both the extracts and the toothpaste formulated) using experimental design with purposive sampling.

3.2. Study area

The study area was Elgeiyo Marakwet rift valley from where samples (leaves, tender stems and root barks) were collected at coordinates 0°58’56.0”N35°35’16.5”E near St. Joseph’s Lawich Catholic.

3.3. Materials, Apparatus, Chemicals and Reagents

3.3.1. Apparatus and Materials

Apparatus and materials used in this study included deionized water, clean bags, ice box, sterile gloves, membrane filter, beakers, weighing balance, Whatman No.1 filter papers, petri dishes, conical flasks, test tubes, 500 µm sieve, oven, glass rod, measuring cylinders, sterile nutrient agar medium, Sterile discs, Eppendorf tubes, sample vials with lids, manila bags, water baths and a clock.

3.3.2. Chemicals and Reagents

The chemicals and reagents used in this work were; Gallic acid Analytical Reagent grade (AR), quercetin AR, ethanol AR, methanol AR, hydrochloric acid Laboratory grade (LG), sulphuric acid LG, sodium hydroxide Extra pure grade (EP), aluminium chloride EP, sodium nitrite EP, sodium carbonate EP, menthol crystals EP, sodium lauryl sulphate EP, sodium hydrogen carbonate EP, calcium phosphate EP, hydrated silica EP, calcium carbonate EPS, glycerol EP, ammonia sulphate EP, iron(iii) chloride EP, lead (II) acetate EP, chloroform LG, potassium iodide EP, acetic anhydride LG,

glacial acetic acid LG, magnesium turnings, dichloromethane (99.5 %), hexane (98.5 %) (Purchased from Pyrex East Africa Limited) Penicillin G AR and Dimethyl sulphoxide AR (purchased from Marty Enterprises Limited).

3.3.3. Equipment

The instruments used in this work were Rotary evaporator (Hahn Vapour Model no. HS-2005s, Korea), FT-IR (Shimadzu Fourier Transform Infrared spectrophotometer, Model FTS- 8000), Ultra Violet-Visible spectrophotometers (Beckham Coulter DU 720 – General Purpose UV/Vis spectrophotometer), an autoclave and Gas Chromatography Hyphenated to Mass Spectrometer equipment (Shimadzu QP 2010-SE GC-MS).

3.4. Sample collection

The study area was in Elgeiyo Marakwet rift valley located in the North rift region of Kenya where the samples used in this study were collected at coordinates 0°58’56.0”N and 35°35’16.5”E near St. Joseph’s Lawich Catholic.

Purposive and random sampling methods were used during the collection of samples from the field. The sampling was purposive because of prior knowledge about the availability of *E. divinorum* plants in the area. While in the field, the plants were selected at random.

Leaves were handpicked, tender stems were cut with a sterilized machete, and root barks were collected by digging them up from the ground with a sterilized hoe. The collected parts were kept separately in a clean and sterilized manila bag after which they were carried to the laboratory (Chemistry Laboratory at the Department of Biological Sciences) for sample preparation.

The plant was taken to the botanist (Mr. Tepeny at School of Sciences and Aerospace studies, Moi University, Kenya) for identification and classification. Fresh leaves, roots, flowers and stems were taken for identification.

3.5. Sample preparation

The leaves, tender stems and root barks of *E. divinorum* were washed under running tap water followed by distilled water and dried under a shade for four weeks due to humid conditions in the month of July. Respective samples were then warmed for 45 minutes in an oven at 30 °C to remove moisture before grinding separately to fine powder (APPENDIX I).

3.5.1. Extraction

Serial extraction method was used to obtain phytochemicals from the plant matrix, starting with hexane, dichloromethane and finally ethanol respectively from the matrix. Dry powdered leaves (200 g) were placed in a conical flask and macerated at room temperature for 48 hours in 98.5% hexane (800 mL) with occasional shaking. The samples were filtered through Whatman No. 1 filter papers under gravity. The filtrates were concentrated by evaporating hexane off using a rotary evaporator set at 45 °C while the residues labeled R1 were used in the subsequent extractions. The concentrated extracts were collected in labeled 50 mL sample vials, air dried at room temperature, weighed and masses of the extracts recorded (Felhi et al., 2017). The dry extracts were sealed and kept at 4 °C for further studies.

Residues (R1) were air dried, then macerated in 99.5 % dichloromethane (800 mL) at room temperature for 48 hours with occasional shaking. These were then filtered through Whatman No. 1 filter papers under gravity, and the second set of residues (labeled R2) were kept for further extraction. Filtrates obtained were concentrated by evaporating off the dichloromethane using a rotary evaporator with the temperature of

the water bath set at 35 °C and 22 revolutions per minute. The concentrated extracts were collected in a sample vial, air dried at room temperature, weighed and the respective weights recorded. The extracts were sealed in labeled sample vial tubes and kept at 4 °C for further studies.

Residues (R2) were air dried, then macerated in absolute ethanol (800 mL) at room temperature for 48 hours with occasional shaking. They were filtered and filtrates concentrated by rotary evaporation with the temperature of the water bath set at 65 °C and 22 rpm. The concentrated extracts were collected in a labeled sample vial, air dried at room temperature, weighed, weights recorded, sealed and kept at 4 °C for further studies.

Dry powders of tender stems and root barks were subjected to the same treatment as described in section 3.5.1 above to obtain respective hexane, dichloromethane and ethanol extracts which were labeled and kept for further analysis.

The extraction yield were obtained as the percentages of initial mass of the sample macerated using Equation 3 (Umaru, Badruddin, & Umaru, 2019).

$$Percentage\ yield\ (\%) = \frac{Mass\ of\ the\ extract\ obtained}{Mass\ of\ the\ sample\ macerated} \times 100 \qquad (3)$$

3.6. Phytochemical analysis

3.6.1. Phytochemical screening

Standard phytochemical screening procedures were carried out to test for the presence of alkaloids, flavonoids, cardiac glycosides, phenols, saponins, quinones, steroids, tannins, terpenes and volatile oils in all the extracts from leaves, tender stems and barks of roots of *E. divinorum* (Dey, Raihan, Sariful, Hamiduzzaman, & Monjur-Al-Hossain, 2012; Obwoge, Kinyua, Kariuki, & Magoma, 2014).

3.6.1.1. Alkaloids

To the extract (2 mL), hydrochloric acid (1%) was added and heated. Drops of the Wagner's reagent (1 mL) were then added to the resultant solution. Brown or reddish-brown precipitate indicated the presence of alkaloids (Sasidharan *et al.,* 2011).

3.6.1.2. Cardiac glycosides

Glacial acetic acid (1 mL) was added to the extract (2 mL) in a test tube followed by ferric chloride (3 drops) then concentrated sulphuric acid (1 mL). Presence of brown ring at the interface was used to indicate the presence of cardiac glycosides (Shabi, Kumari, & Chitarasu, 2014).

3.6.1.3. Flavonoids

Four drops of concentrated hydrochloric acid followed by magnesium turnings (0.5 g) were added to the extract (2 mL) in a test tube. A pink colour formation after 3 minutes was used to indicate the presence of flavonoids (Dey *et al.*, 2012; Shabi *et al.,* 2014).

3.6.1.4. Phenols

1% iron (III) chloride solution (1 mL) was added to the extract (2 mL) in a test tube. Blue or green color formation was an indication of presence of phenols (Prashant, Bimlesh, Mandeep, & Gurpreet, 2011).

3.6.1.5. Quinones

Concentrated sulphuric acid (1 mL) was added to the extract (1 mL) in a test tube. Formation of the red colour showed the presence of quinones (Shabi *et al.,* 2014).

3.6.1.6. Saponins

Distilled water (5 mL) was added to the extract (1 mL) in a test tube. The mixture was then shaken vigorously for two minutes. Appearance of foam lasting for 5 minutes confirmed the presence of saponins (Shabi *et al.,* 2014).

3.6.1.7. Steroids

Acetic anhydride (2 mL) was added to the extract (1 mL) in a test tube followed by concentrated sulphuric acid. A blue or green coloration indicated the presence of steroids (Obwoge *et al.*, 2014; Prashant *et al.*, 2011).

3.6.1.8. Tannins

In a test tube containing an extract (5 mL), a few drops of 1% solution of lead (II) acetate were added. Formation of a yellow or red precipitate indicated the presence of tannins (Rajiv, Deepa, Vanathi, & Vidhya, 2017a).

3.6.1.9. Terpenes

To the extract (2 mL), chloroform (5 mL), acetic anhydride (2 mL), concentrated HCl was added. Reddish brown colour at the interface was an indication of terpenes (Rajiv et al., 2017a).

3.6.1.10. Volatile oil

To the extract (1 mL), 90% ethanol was added, followed by 3 drops of ferric chloride. Formation of green colour indicated their presence (Shabi *et al.*, 2014).

3.6.2. Characterization of phytoconstituents in extracts of *E. divinorum*

3.6.2.1. Ultra Violet-Visible characterization

Dry powder of *E. divinorum* leaves (1.00 g) was placed in a conical flask macerated at room temperature for 48 hours in 98.5 % hexane (50 mL) with occasional shaking. The samples were filtered through Whatman No. 1 filter papers under gravity. The extract was centrifuged at 12,000 rpm for 10 minutes and again filtered. The sample was diluted 1:10 with hexane and then scanned at wavelength ranging from 210 nm to 800 nm using a UV/Vis spectrophotometer (Bashyam, Thekkumalai, & Sivanandham, 2015).

Leaves, tender stems, and root barks were extracted by sequential maceration with hexane, dichloromethane, and ethanol respectively and the same procedure as described in section 3.6.2.1 was adopted to obtain UV/Vis scan.

3.6.2.2. FT-IR Characterization

The KBr pellets of samples were prepared by grinding 10 mg of hexane extract from the leaves, with 250 mg KBr (FT-IR grade). The 13 mm KBr pellets were prepared in a standard device under a pressure of 75 kN cm^{-2} for 3 min. The functional groups present in all the extracts were analyzed by a FT-IR spectrophotometer. The spectral resolution was set at 4 cm^{-1} and the scanning ranged from 400 to 4000 cm^{-1} (Nithyadevi & Sivakumar, 2015). The FT-IR spectra of all the extracts were obtained as described in the above procedure.

3.6.2.3. Gas Chromatography-Mass Spectrometry Analysis of Plant extracts

The hexane and Dichloromedthane extracts of the leaves, tender stems, and root barks were considered for GC-MS analysis. Samples were diluted in their respective solvents of extraction (1:10, m/v), ultrasonicated for 15 minutes, filtered separately through Whatman number one filter papers placed on a sanction filter funnel followed by 0.45 µm syringe filters and transferred into sample vials for GC-MS analysis.

A GC-MS Spectrophotometer coupled to an auto sampler was used for the analysis. Ultrapure Helium gas (99.99 %) was used as the carrier gas at a constant flow rate of 1 ml per minute. A BPX5 non polar column, 30 m long; 0.25 mm inner diameter; 0.25 µm film thickness, was used for separation. The GC was programmed as follows: the oven temperature was programmed from 50 ˚C (1 minute) to 250 °C (9 minutes) at a rate of 5 ˚C /min. Total run-time was 50 minutes. Only 1 µL of the sample was injected. Injection was done at 200 °C in split mode, with split ratio set to 10:1. The interface temperature was set at 250 °C. The EI ion source was set at 200 °C. Mass analysis was

done in full scan mode within a range of 50-600 atomic mass unit (a.m.u). Detected peaks were matched against the National Institute of Standards and Technology (NIST) 2014 mass spectrometry library for possible identification (Al-Owaisi, Al-hadiwi, & Khan, 2014; Alghamdi *et al.*, 2018).

3.7. Determination of total phenolic and total flavonoid contents

Total phenolic and total flavonoid contents were determined by Folin-Ciocalteau and aluminium chloride methods respectively in all the extracts as described in sections 3.7.1 and 3.7.2 respectively.

3.7.1. UV- Visible Spectrophotometric Analysis of Total Phenolic Content (TPC)

Phenolic content in the crude extracts was determined by the method reported by Johari and Khong (2019) with some modifications.

Crude hexane extract (0.1 g) from leaves was dissolved in hexane (25 mL). The resultant solution of this extract (0.5 mL) was taken into a vial in triplicate, mixed with 2.5 mL of Folin-Ciocalteau reagent followed by 6% of sodium carbonate (2.5 mL). The solutions were incubated in the dark at 25 °C for 30 minutes after which their absorbencies were measured at 760 nm using a DU 720 – General Purpose UV/Vis spectrophotometer.

To determine total phenolic content in the hexane, dichloromethane and ethanol extracts of the leaves, stem twigs and root barks, the respective dry extracts were dissolved in their respective solvents of extraction and the above procedure in 3.7.1 was repeated for each of the extracts. Gallic acid solution (500 ppm) was prepared as a stock solution from which solutions of 1, 10, 25, 75, 100 and 200 ppm were made. Each aliquot (500 µL) was taken into a vial and they were given the same treatment as the sample as described in section 3.7.1. A calibration curve was obtained from which the

TPC was determined as gallic acid equivalent in ppm (Johari & Khong, 2019). TPC was calculated as Gallic acid equivalent in ppm from the calibration curve using Equation 4.

$$\mathrm{TPC} = (\mathrm{c} \times \mathrm{DF} \times \mathrm{V}) \div \mathrm{M} \qquad (4)$$

Where *c* represents concentration of gallic acid from the calibration curve, DF represents the dilution factor, V represents volume of the extract used, and M represents mass of the extract.

3.7.2. UV- Visible Spectrophotometric Analysis of Total Flavonoid Content

Total flavonoid content (TFC) in the crude extracts were determined by the method reported by Pękal and Pyrzynska (2014) with some modifications.

Crude hexane extract (0.1 g) was dissolved hexane (25 mL). The resultant solution (1 mL) of this sample was put in a vial in triplicate and mixed with 5 % w/v of sodium nitrite solution (0.3 mL). After 5 minutes, 2 % w/v of aluminium chloride solution (0.5 mL), 1 M sodium hydroxide solution (2 mL) and finally distilled water (3.0 mL). The mixture was incubated at 25 °C for 20 minutes after which the absorbance of each was read at 510 nm.

The total flavonoid content in hexane, dichloromethane and ethanolic extracts of the leaves, tender stems and root barks was determined by first dissolving the extracts in their respective solvent of extraction and the above procedure was repeated for each of the extracts. Quercetin solution (500 ppm) was prepared from which solutions of 5, 10, 50, 75, and 100 ppm were made. 0.5 mL of each solution was taken into a vial and subjected to the same treatment as the samples as described in section 3.7.2. The

calibration curve was obtained from which the TFC was determined as quercetin equivalent in ppm (Pękal & Pyrzynska, 2014) using Equation 5.

$$TFC = (c \times DF \times V) \div M \qquad (5)$$

Where c represents concentration of quercetin from the calibration curve, DF represents dilution factor, V represents volume of the extract used and M represents mass of the extract.

3.8. Antibacterial activity of extracts

3.8.1. Antimicrobial screening assay

The antimicrobial activity of the extracts was assessed using disk diffusion method with surface plating to check the efficacy of the plant extract against four microorganisms namely, *Streptococcus pyogenes* ATCC 19615, S*taphylococcus aureus* ATCC 25923, *Candida albicans* and *Escherichia coli* ATCC 25922. The microbes were obtained from Egerton University Microbiology Laboratory, Nakuru, Kenya. The extracts were prepared by dissolving 0.05 g of the powders separately in 100 ml of 10 % dimethyl sulfoxide. The bacterial media were prepared according to the manufacturer's instructions.

Briefly, Mueller Hinton Agar (19 g)/ Subouraud Dextrose Agar (20 g) was dissolved in 500 ml of distilled water, autoclaved (at 121 °C, 1.5 bars) for 30 minutes and allowed to cool to 45 °C. The agar plates were prepared by pouring the prepared media onto sterilized plates and then allowed to solidify. The plates were seeded with respective microorganisms (0.1 ml) which were spread uniformly using a sterilized L-shaped rod. Three antimicrobial discs impregnated with 0.5 ml of different extracts placed aseptically on the agar surface. Discs soaked in 10 % dimethyl sulfoxide (DMSO) were used as the negative control and penicillin (10 µg/ml) was used as the positive control.

The plates were incubated at 37 °C for 24 hours (Farahmandfar, Esmaeilzadeh, Maryam, Shahrampour, & Bakhshandeh, 2019). Positive antimicrobial activities were established by the presence of measurable zones of inhibition.

3.8.2. Minimum inhibitory concentration

The method adopted here was described by Wetungu *et al.* (2014) with some modifications. The ethanol root bark extract was prepared by dissolving the dry solid extract in 1 % Dimethyl Sulphoxide (250 ml) to obtain solutions of 25, 50, 100, 200 and 400 µg/mL. The minimum inhibitory concentration (MIC) was determined by using Disk diffusion method adopting the same procedure in microbial screening assay (Wetungu *et al.*, 2014).

3.8.3. Formulation of toothpaste

Methylcellulose gum (2.00 g) was slowly added to distilled water (2.0 mL) and vigorously agitated until a smooth uniform mixture was obtained. Glycerol (4.0 mL) was added into the mixture and stirred thoroughly. Calcium carbonate (7.0 g), menthol crystals (0.50 g), hydrated silica (1.00 g), calcium phosphate (2.00 g), ethanol root bark extract (0.50 g), sodium hydrogen carbonate (2.00 g), and sodium lauryl sulphate (2.00 g) were added in the order one at a time while mixing with a stirring rod thoroughly (Grace, Darsika, Sowmya, Afker, & Shanmuganathan, 2015).

3.8.4. Antimicrobial activity of the formulated toothpaste

The formulated toothpastes were diluted with 250 ml of distilled water to make respective solutions of 400 µg/ml which were tested for their activity against *S. pyogenes, S. aureus, C. albicans* and *E. coli*.

Mueller Hinton Agar, Subouraud Dextrose Agar and agar plates were prepared as described for the extracts (section 3.8.1). The plates were seeded with microorganisms (0.1 ml) separately which were then spread uniformly using a sterilized L-shaped rod. Whatman discs (3) were separately soaked with toothpaste solutions (0.5 ml) were

placed onto surface of each agar plate containing the test microorganisms. Disks soaked in 10 % DMSO were used as the negative control and Colgate herbal fluoride toothpaste (Colgate Palmolive, China) as a positive control. The plates were later incubated at 37 °C for 18 hours.

3.9. Statistical analysis

All the data was reported as mean ± standard deviation of triplicate measurements. The data obtained was analyzed using both descriptive and inferential statistics. The percentage yield, total phenolic and total flavonoid contents were presented on respective bar graphs.

Total Phenolic Content, Total Flavonoid Content and antibacterial activities of extracts are continuous variables and since the same extracts (leaves, tender stems, root barks) considered are tested for the content in different solvents (hexane, dichloromethane and ethanol), one way repeated measures ANOVA was used for statistical data analysis after Mauchly's sphericity test was performed to check if the variances between all possible combinations are equal.

Chapter 4 : RESULTS AND DISCUSSION

4.1. Percentage yield

The extraction yields shown in Table 4.1 were expressed as the percentages of initial mass of the sample macerated (APPENDIX II).

Table 4.1: Organic extract yield of different parts of *E. divinorum* in %

Part used	Hexane	Dichloromethane	Ethanol
Leaves	1.13 ± 0.03	1.92 ± 0.17	2.51 ± 0.05
Tender stems	0.57 ± 0.04	1.74 ± 0.06	3.30 ± 0.26
Root bark	0.51 ± 0.01	2.35 ± 0.08	7.60 ± 0.20

Extraction using ethanol gave the highest yields in comparison to hexane and DCM with the root barks having the highest yield of 7.60 ± 0.2 % followed by tender stems (3.30 ± 0.26 %). For leaf extracts, a high yield was obtained using ethanol (2.51 ± 0.05 %) than using DCM (1.92 ± 0.17 %) and hexane (1.13 ± 0.03 %). This trend was also observed for the extracts of tender stems and root bark. However, these differences were not statistically different since the obtained p-value of 0.0722 was greater than 0.05; APPENDIX VIII). This result indicates that ethanol is a good solvent for extraction of the plant parts as compared to hexane and DCM probably indicating that most compounds in the parts of *E. divinorum* extracted could be polar, thus were able to dissolve in the more polar ethanol than other non-polar solvents used. Differences in solvent polarities used for extraction is known to play a key role in increasing the solubility of phytochemical compounds (Altemimi *et al.*, 2017). Further, differences in the structure of phytochemical compounds also determine their solubility in solvents of different polarities (Ingle *et al.*, 2017). Indeed, the three solvents used had different polarities arranged as hexane < DCM < ethanol. This change appears to be related not

only to the difference in the polarity of extracts of the components but also to the solvents used, which also plays a vital role in increasing the solubility of phytochemical compounds. Therefore, the results of the current study confirmed the effect of solvent extraction and the plant organ on the yield and consequently confirm the presence of both polar and non-polar compounds in the extracts.

4.2. Phytochemical Analysis Results

4.2.1. Phytochemical screening

Phytochemical screening tests carried out for different extracts of *E. divinorum* revealed the presence active phytoconstituents such as alkaloids, flavonoids, cardiac glycosides, phenols, saponins, quinones, steroids, tannins, terpenes and volatile oils in the extracts from leaves, tender stems and barks of roots of *E. divinorum* as shown in Table 4.2 and APPENDIX III.

Alkaloids were detected in only hexane extract of the root barks. Cardiac glycosides and steroids were detected in hexane extracts of the leaves, tender stems, and root barks and in dichloromethane extract of the tender stems. Flavonoids were present in ethanolic extract of the root barks only whereas phenols were detected in ethanol extracts of the leaves, tender stems and root barks only. Quinones were confirmed present in the hexane extracts of tender stems and root barks, dichloromethane extracts of leaves and tender stems, and ethanol extract of the root barks. Saponins and terpenes were present in only in the ethanol extracts of leaves, tender stems and root barks. However, tannins were detected in the hexane extract of the root barks and ethanol extracts of the leaves, tender stems and root barks. Volatile oils were detected in all the extracts with the exception of dichloromethane extract of the root bark of *E. divinorum.*

Table 4.2: Secondary metabolites identified in different extracts of *E. divinorum* parts.

Phytochemical	Solvent	Leaves	Tender stems	Root bark
Alkaloids	Dichloromethane	-	-	-
	Hexane	-	-	+++
	Ethanol	-	-	-
Cardiac glycosides	Dichloromethane	-	+++	-
	Hexane	++	+	+++
	Ethanol	-	-	-
Flavonoids	Dichloromethane	-	-	-
	Hexane	-	-	-
	Ethanol	-	-	+++
Phenols	Dichloromethane	-	-	-
	Hexane	-	-	-
	Ethanol	+	++	+++
Quinones	Dichloromethane	-	++	++
	Hexane	-	++	+++
	Ethanol	+	-	-
Saponins	Dichloromethane	-	-	-
	Hexane	-	-	-
	Ethanol	+++	+++	+++
Steroids	Dichloromethane	-	-	-
	Hexane	+++	+	+++
	Ethanol	-	-	-
Tannins	Dichloromethane	-	-	-
	Hexane	-	-	++
	Ethanol	++	++	+++
Terpenes	Dichloromethane	-	-	-
	Hexane	-	-	-
	Ethanol	+++	+++	+++
Volatile oils	Dichloromethane	+	+	-
	Hexane	+	++	+++
	Ethanol	+++	+++	+++

+++ represents very high, ++ indicates moderate, + indicates little/traces, and – indicates absent.

The phytochemical screening results of the present study agreed well with previous studies. Onyango *et al.* (2014) reported the presence of tannins, saponins, alkaloids, steroids, terpenoids and reducing compounds in the methanol and ether extracts of roots and powdered root barks of *E. divinorum* while its water extract contained tannins, saponins, flavonoids, alkaloids, steroids, terpenoids, reducing compounds and flavones aglycones. The ether extract showed the presence of tannins, flavonoids, alkaloids, terpenoids, steroids and flavones aglycones. Another study conducted reported the presence of anthraquinones, saponins, tannins, polyphenols, and terpenoids were reported to be present in the DCM: methanol extracts of leaves, roots and the stems of *E. divinorum*. However, alkaloids were present in only the root extract (Mwonjoria *et al.,* 2018). This is corroborated by the presence of alkaloids only in the hexane root bark extracts in the current study. The identified secondary metabolites could be responsible for the medicinal value of *E. divinorum*. For example, alkaloids are adenosine receptor antagonists, have analgesic, antitussive, stimulant, antispasmodic, antiprotozoal, antiarrhythmic, antipyretic, antimalarial, antitumor, vasodilating, aphrodisiac, antihypertensive and antibacterial activities (Ferrazzano *et al.,* 2011). Tannins have been reported to have antifungal, anti-inflammatory, antidiabetic, wound healing and antibacterial activities (Saxena, Saxena, Nema, Singh, & Gupta, 2013) while saponins possess hypocholesterolemic, antibacterial and anticancer agents. Terpenoids have analgesic, anticancer, antibacterial and anti-inflammatory properties.

4.2.2. UV-Visible spectra

Qualitative UV-Vis spectra of *E. divinorum* extracts were scanned at wavelength 210 to 800 nm to identify the compounds containing σ-bonds, π-bonds, and lone pair of electrons, chromophores and aromatic rings. The spectra and the peak values for the different extracts are shown in figures in Appendix III and APPENDIXV.

Leaf extracts recorded absorption at wavelengths of 410.0, 539.0, 699.0 nm (for hexane extract), 414.0, 453.0, 669.0 nm (for DCM extract) and 370.0, 436.0, 655.0 nm for ethanol extract. Tender stems recorded absorptions at 409.0, 674.0 nm (for hexane extract), 416.0, 452.0, 669.0 nm (for DCM extract) and 400.0, 665.0 for ethanol extract. Root bark recorded absorption at 369.0, 395.0, 446.0 nm (for hexane extract), 342.0, 420.0, 665.0 nm and 352.0, 439.0, 699.0 nm for ethanol extract.

These results are in agreement with Marimuthu *et al.* (2016) who also found similar findings as the absorption peaks fall in the same range. The presence of absorption peaks between 200 and 400 nm indicate the presence of unsaturated with sigma bonds and heteroatoms such as sulphur, oxygen and nitrogen. The absorption above 400 is due to extended system of multiple bonds that cause the molecules to absorb at a higher wavelength (Jain *et al.*, 2016). According to the UV-Vis spectra results of extracts of the leaves, tender stems and root barks, the presence of heteroatoms and unsaturated groups was indicated.

4.2.3. FT-IR results

Over the years, FT-IR spectroscopy has proven to be a valuable tool in the identification of the functional groups of compounds in plant extracts (Shabi *et al.*, 2014). The results are presented per the solvents used, hexane (Figures 4.2 to 4.4), ethanol (Figures 4.5 to 4.7) and DCM (Figures 4.8 to 4.10), respectively.

The results of FT-IR spectroscopic analysis of different extracts of *E. divinorum* revealed the existence of various phytochemical constituents. The FT-IR spectra were interpreted using the peak values as previously reported by other researchers (Kumar *et al.*, 2018). The spectrum of the standard is given in Figure 4.1

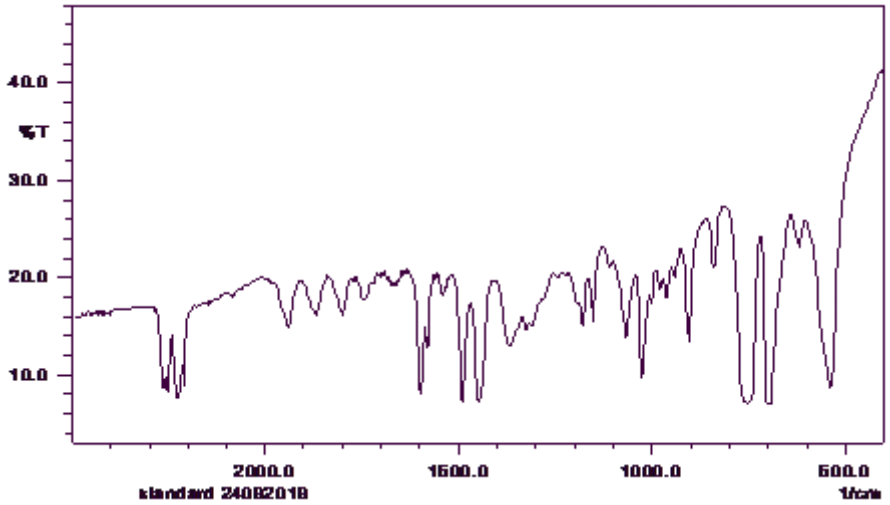


Figure 4.1: FT-IR spectrum of the standard (KBr)

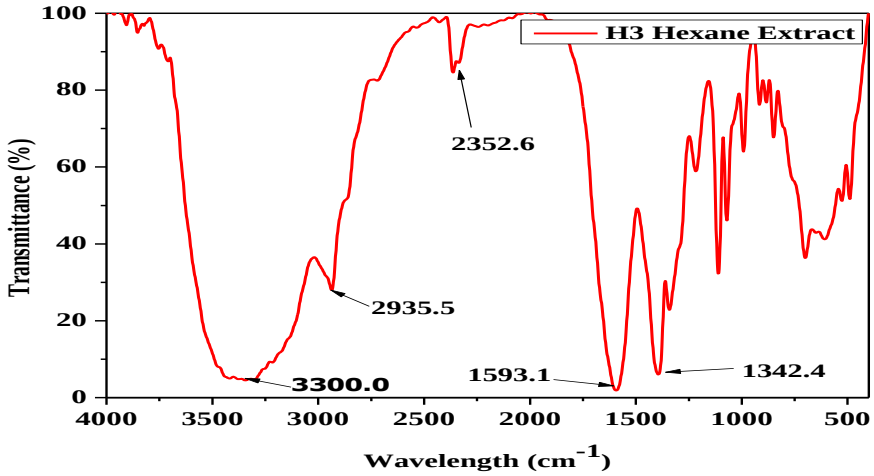


Figure 4.2: FT-IR spectrum of hexane root bark extract of *E. divinorum*

The intense absorption at 3300 cm^{-1} is due to stretching of -OH groups from phenolic compounds present in the extract (Jain *et al.*, 2016). The band at 2935.5 cm^{-1} is due to a C-H group asymmetric stretching which is -sp^3 hybridized carbon of alkanes. The sharp weak peak at 2353.6 cm^{-1} is due to absorption of the C≡N group. Absorption at 1593.1 cm^{-1} is due to stretching of Ar-C=C groups of aromatic alkenes. Absorption at 1394.1 cm^{-1} is due to Asymmetric in-plane bending of $–CH_3$. Absorption at 1340.4 cm^{-1} is due to Symmetric in-plane bending of $–CH_3$. The absorption at 1292 cm^{-1} is due to

O=C-O-C- stretch from the aromatic esters. The two absorption peaks at 1110.9 cm^{-1} and 1070.4 cm^{-1} are due to C-O stretching vibrations from secondary and primary alcohols respectively. The two weak bands at 991.3 and 914.2 cm^{-1} are due to =C-H bending vibrations of alkenes. The absorption band at 702 cm^{-1} is due to the bending vibrations of ≡C-H bond of alkyne.

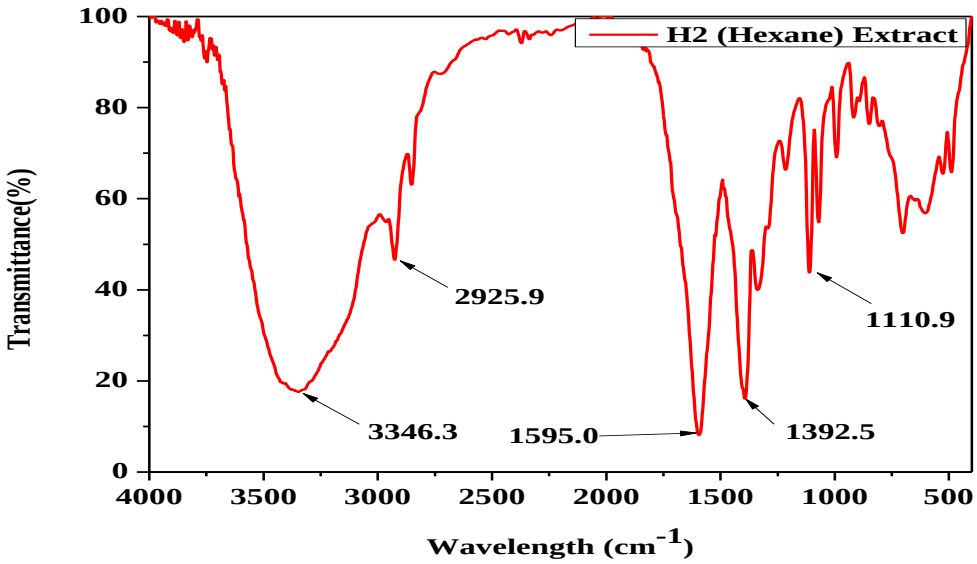


Figure 4.3: FT-IR spectrum of hexane extract of the tender stems

The intense absorption at 3346.3 cm^{-1} is due to stretching of -OH groups from phenolic compounds present in the extract i.e., hydrogen bonded phenols. The band at 2925.9 cm^{-1} is due to a C-H group asymmetric stretching which is SP^3 Hybridized carbon of alkanes. Absorption at 1595.0 cm^{-1} is due to stretching of Ar-C=C groups of aromatic alkenes. Absorption at 1392.5 cm^{-1} is due to Asymmetric in-plane bending of $–CH_3$. The two absorption peaks at 1110.9 cm^{-1} and 1070.1 is due to C-O stretching vibrations from secondary and primary alcohols respectively. The two weak bands at 991.3 and 910.2 cm^{-1} are due to =C-H bending vibrations of alkenes. The absorption band at 700 cm^{-1} is due to the stretching vibrations of -C-Br bond of aliphatic bromo-compounds (Gowri, Dharmalingam, Arockia, Prema, & Shobana, 2015).

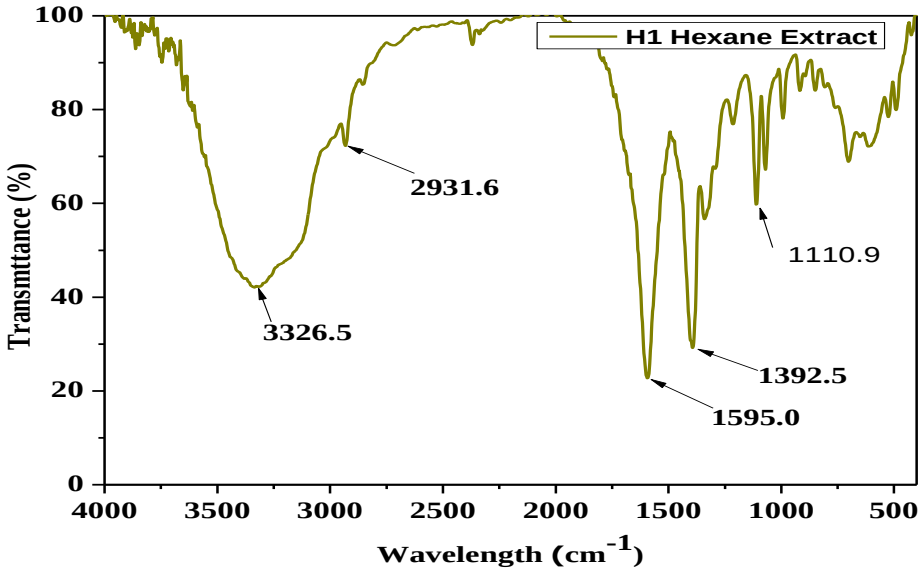


Figure 4.4: FT-IR spectrum of hexane leaf extract of *E. divinorum*

The intense absorption at 3326.5 cm^{-1} is due to stretching of -OH groups from phenolic compounds present in the extract (Jain *et al.*, 2016) or NH/OH stretching of amines and amides (Rajiv, Deepa, Vanathi, & Vidhya, 2017b). The band at 2931.6 cm^{-1} is due to a C-H group asymmetric stretching which is SP^3 Hybridized carbon of alkanes. The sharp weak peak at 2353.6 cm^{-1} is due to absorption of the C≡N group of nitrile compounds. Absorption at 1595.1 cm^{-1} is due to stretching of Ar-C=C groups of aromatic alkenes. Absorption at 1392.5 cm^{-1} is due to Asymmetric in-plane bending of $–CH_3$. The two absorption peaks at 1110.9 cm^{-1} and 1070.4 cm^{-1} is due to C-O stretching vibrations from secondary and primary alcohols respectively. The two weak bands at 991.3 and 914.2 cm^{-1} are due to =C-H bending vibrations of alkenes (Gowri *et al.*, 2015).

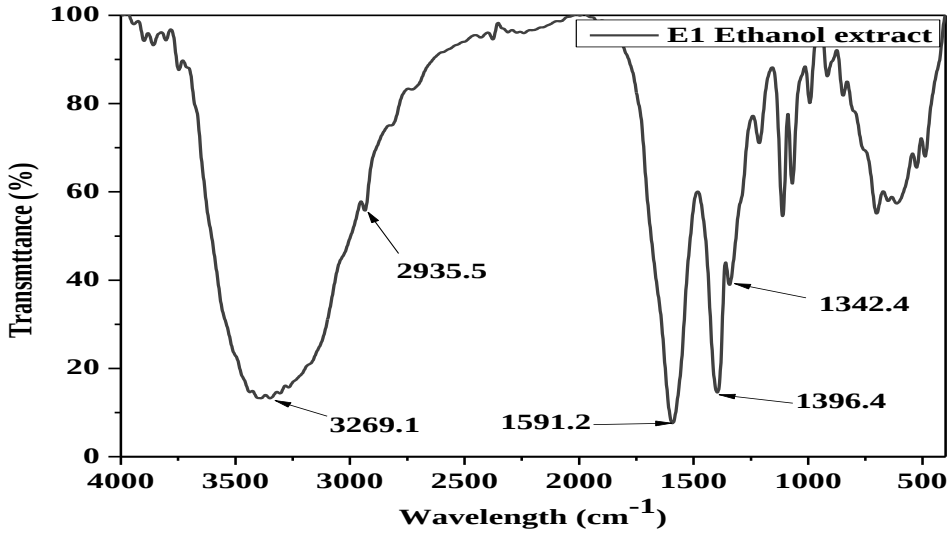


Figure 4.5: FT-IR spectrum of ethanol extract of the leaves of *E. divinorum*

The intense absorption at 3269.1 cm^{-1} is due to stretching of -OH groups from phenolic compounds present in the extract. The band at 2935.5 cm^{-1} is due to a C-H group asymmetric stretching which is SP^3 Hybridized carbon of alkanes. Absorption at 1591.2 cm^{-1} is due to stretching of Ar-C=C groups of aromatic alkenes. Absorption at 1396.4 cm^{-1} is due to Asymmetric in-plane bending of $–CH_3$. Absorption at 1342.4 cm^{-1} is due to Symmetric in-plane bending of $–CH_3$. The absorption at 1292.1 cm^{-1} is due to O=C-O-C- stretch from the aromatic esters. The two absorption peaks at 1110.9 cm^{-1} and 1070.4 is due to C-O stretching vibrations from secondary and primary alcohols respectively. The two weak bands at 991.3 and 914.2 cm^{-1} are due to =C-H bending vibrations of alkenes. The absorption band at 702 cm^{-1} is due to the bending vibrations of ≡C-H bond of alkyne.

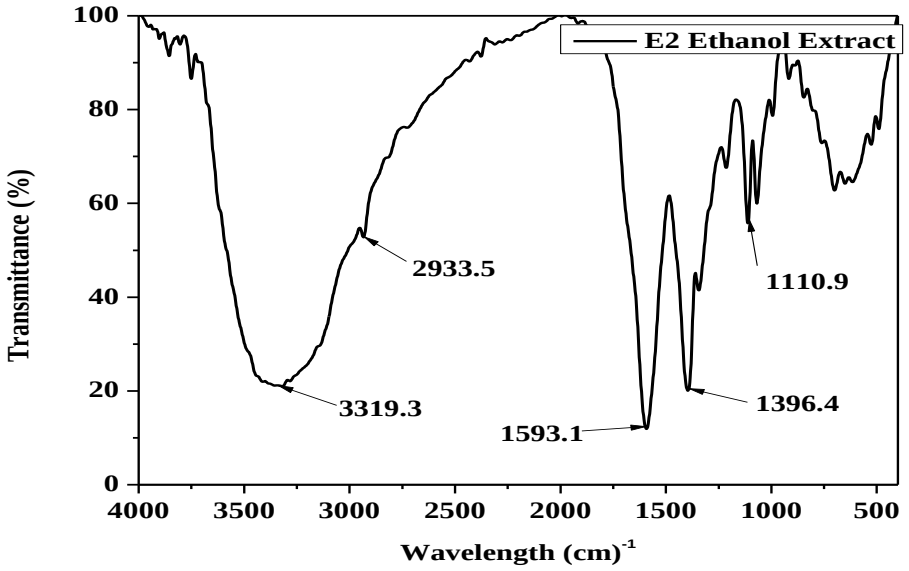


Figure 4.6: FT-IR spectrum of ethanol extract of the tender stems of *E. divinorum*

The intense absorption at 3319.3 cm^{-1} is due to stretching of -OH groups from phenolic compounds present in the extract. The band at 2933.1 cm^{-1} is due to a C-H group asymmetric stretching which is SP^3 Hybridized carbon of alkanes. Absorption at 1593.1 cm^{-1} is due to stretching of Ar-C=C groups of aromatic alkenes. Absorption at 1396.4 cm^{-1} is due to Asymmetric in-plane bending of $–CH_3$. Absorption at 1340.4 cm^{-1} is due to Symmetric in-plane bending of $–CH_3$. The absorption at 1292 cm^{-1} is due to O=C-O-C- stretch from the aromatic esters. The two absorption peaks at 1110.9 cm^{-1} and 1070.4 is due to C-O stretching vibrations from secondary and primary alcohols respectively. The two weak bands at 991.3 and 914.2 cm^{-1} are due to =C-H bending vibrations of alkenes. The absorption band at 700.1 cm^{-1} is due to the bending vibrations of ≡C-H bond of alkyne (Gowri *et al.,* 2015).

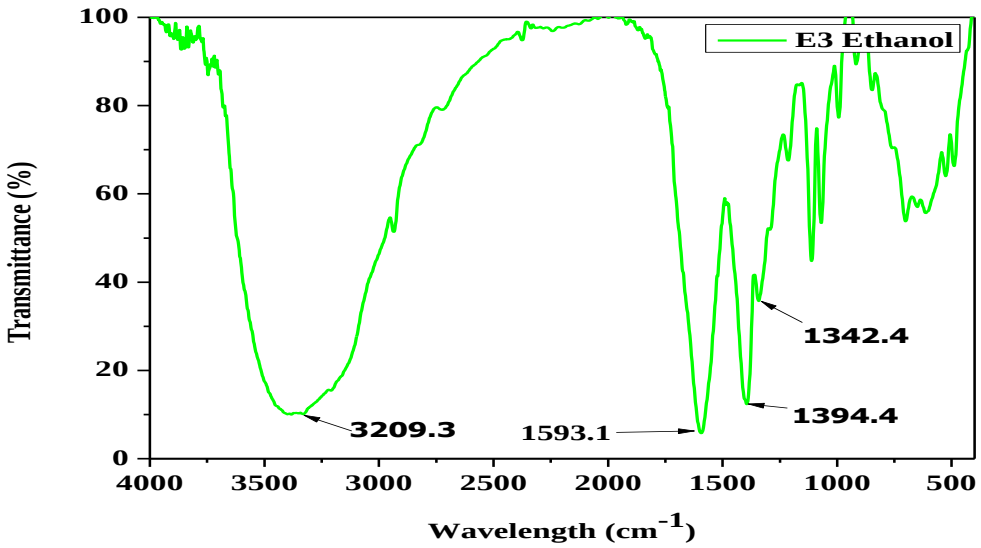


Figure 4.7: FT-IR spectrum of ethanol extract of the root barks of *E. divinorum*

The intense broad absorption at 3209.3 cm^{-1} is due to stretching of -OH groups from alcohols present in the extract (Murugan *et al.,* 2014). The band at 2941.3 cm^{-1} is due to a C-H group asymmetric stretching which is SP^3 Hybridized carbon of alkanes (Ashokkumar & Ramaswamy, 2014). Absorption at 1593.1 cm^{-1} is due to stretching of Ar-C=C groups of aromatic alkenes (Bayu, Nandiyanto, Oktiani, & Ragadhita, 2019). Absorption at 1394.4 cm^{-1} is due to asymmetric in-plane bending of $–CH_3$. Absorption at 1342.4 cm^{-1} is due to Symmetric in-plane bending of $–CH_3$. The two absorption peaks at 1100.1 cm^{-1} and 1059.4 is due to C-O stretching vibrations from secondary and primary alcohols respectively (Bayu *et al.,* 2019).

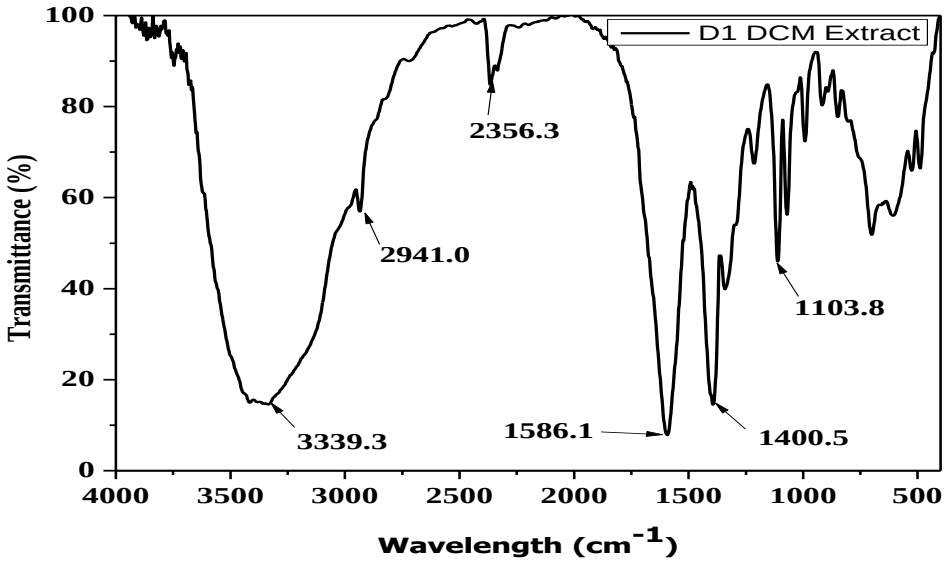


Figure 4.8: FT-IR spectrum of DCM extract of the leaves of *E. divinorum*

The intense absorption at 3339.3 cm^{-1} is due to stretching of -OH groups from phenolic compounds present in the extract (Ashokkumar & Ramaswamy, 2014). The band at 2941.0 cm^{-1} is due to a C-H group asymmetric stretching which is SP^3 Hybridized carbon of alkanes (Pachurekar & Ashwini, 2018). The sharp weak peak at 2356.3 cm^{-1} is due to absorption of the C≡N group. Absorption at 1586.1 cm^{-1} is due to stretching of Ar-C=C groups of aromatic alkenes. Absorption at 1400.1 cm^{-1} is due to Asymmetric in-plane bending of –OH bend (Bayu *et al.*, 2019). Absorption at 1340.4 cm^{-1} is due to Symmetric in-plane bending of $–CH_3$. The absorption at 1292.7 cm^{-1} is due to O=C-O-C- stretch from the aromatic esters. The two absorption peaks at 1103.8 cm^{-1} and 1099.4 is due to C-O stretching vibrations from secondary and primary alcohols respectively. The two weak bands at 991.3 and 914.2 cm^{-1} are due to =C-H bending vibrations of alkenes (Kalaichelvi & Dhivya, 2017; Papitha, Ravi, & Selvaraj, 2017).

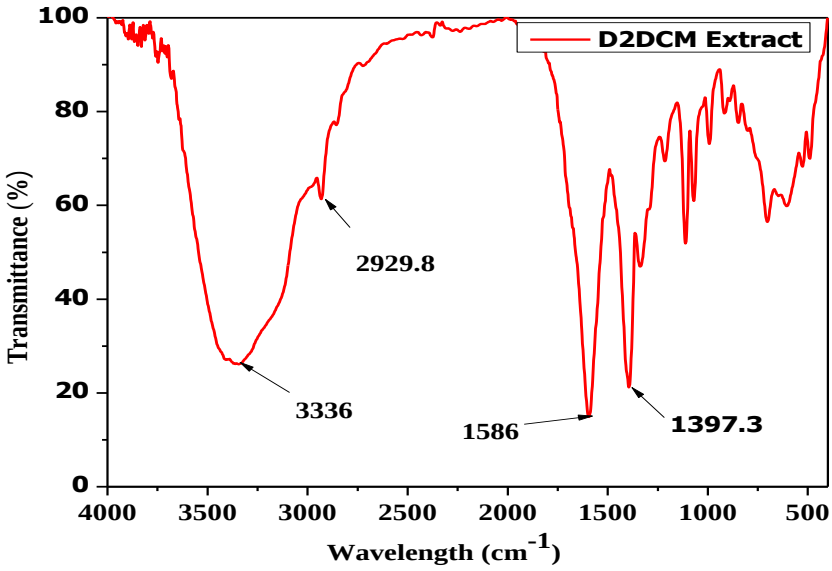


Figure 4.9: FT-IR spectrum of DCM extract of the tender stems of *E. divinorum*

The intense absorption at 3336 cm^{-1} is due to stretching of -OH groups from phenolic compounds present in the extract. The band at 2929.8 cm^{-1} is due to a C-H group asymmetric stretching which is SP^3 Hybridized carbon of alkanes. Absorption at 1586 cm^{-1} is due to stretching of Ar-C=C groups of aromatic alkenes. Absorption at 1397.3 cm^{-1} is due to Asymmetric in-plane bending of $–CH_3$. Absorption at 1339.4 cm^{-1} is due to Symmetric in-plane bending of $–CH_3$. The absorption at 1292 cm^{-1} is due to O=C-O-C- stretch from the aromatic esters. The two absorption peaks at 1110.9 cm^{-1} and 1070.4 is due to C-O stretching vibrations from secondary and primary alcohols respectively. The two weak bands at 991.3 and 914.2 cm^{-1} are due to =C-H bending vibrations of alkenes.

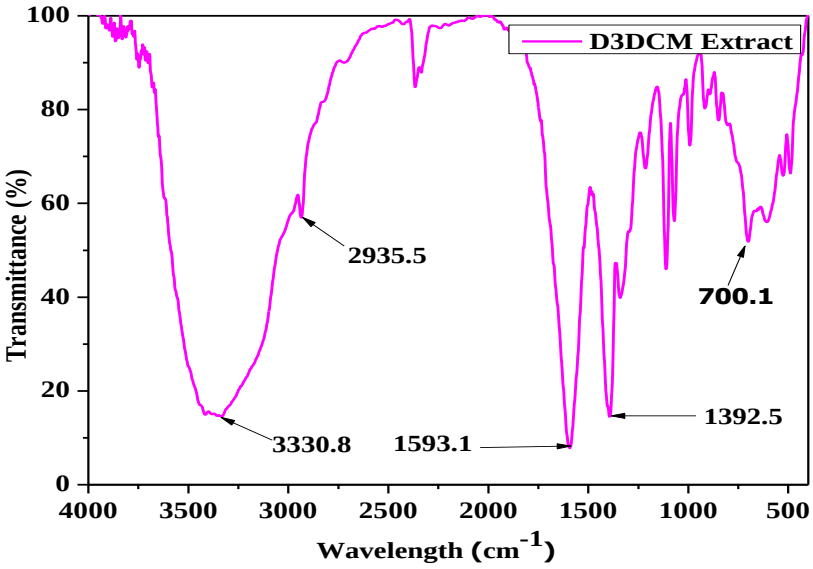


Figure 4.10: FT-IR spectrum of DCM extract of the root barks of *E. divinorum*

The intense absorption at 3330.8 cm^{-1} is due to stretching of -OH groups of polyhydroxy or phenolic compounds present in the extract (Gowri *et al.*, 2015). The band at 2935.5 cm^{-1} is due to a C-H group asymmetric stretching which is SP^3 Hybridized carbon of alkanes. The sharp weak peak at 2353.6 cm^{-1} is due to absorption of the C≡N group. Absorption at 1593.1 cm^{-1} is due to stretching of Ar-C=C groups of aromatic alkenes. Absorption at 1392.5 cm^{-1} is due to Asymmetric in-plane bending of $–CH_3$. Absorption at 1340.4 cm^{-1} is due to Symmetric in-plane bending of $–CH_3$. The absorption at 1292 cm^{-1} is due to O=C-O-C- stretch from the aromatic esters or $–NO_2$ groups. The two absorption peaks at 1110.9 cm^{-1} and 1070.4 is due to C-O stretching vibrations from secondary and primary alcohols respectively. The two weak bands at 991.3 and 914.2 cm^{-1} are due to =C-H bending vibrations of alkenes. The absorption band at 700.1 cm^{-1} is due to the bending vibrations of ≡C-H bond of alkyne.

The FT-IR spectra confirmed the presence of alcohols, phenols, alkanes, alkenes, alkynes, aldehydes, carboxylic acids, aromatics, nitro compounds and amines in different extracts of *E. divinorum*

Thus, the FT-IR spectra confirmed the presence of alcohols, phenols, alkanes, alkenes, alkynes, aldehydes, carboxylic acids, aromatics, nitro compounds and amines in the different extracts of *E. divinorum.* The various functional groups observed in different extracts reflected the biochemical profile of *E. divinorum* which could be responsible for its various medicinal properties.

4.2.4. GC-MS results

Various peaks were observed for the extracts in this study (APPENDIX X). The small peaks may be attributed to the compounds present in small quantities as well as disintegrated major compounds (Jain *et al.*, 2016).

A total of 30 compounds were identified in the hexane and DCM extracts of *E. divinorum*. The major compounds were 3,4-Methylenedioxybenzylacetone, Eicosane, Tetratriacontane, Hexatriacontane, 9-Hexadecen-1-ol, 2-Hydroxy-2-methyl-8,8-diphenyl-octa-5,7-dien-3-one, 1,4-Naphthoquinone and Octacosanal.

Hexane leaf extract contained 4 compounds, with Tetratriacontane (47.43 %) and 2-Hydroxy-2-methyl-8,8-diphenyl-octa-5,7-dien-3-one (13.53 %) being the major constituents (Table 4.2). Hexane extract of the tender stems recorded 12 compounds, one of which (Eicosane) was also identified in the hexane extracts of leaves and root bark. Only traces of Tetratriacontane were found in this extract. The major constituents were Eicosane (56.26 %) and Hexatriacontane (18.17 %). In the root bark extract, 8

compounds were identified. Hexatriacontane (70.91 %) and 1,4-Naphthoquinone (13.34 %) dominated (Table 4.3).

The DCM extract of the leaves showed presence of 9 compounds with Tetratriacontane (20.83 %), Eicosane (15.89 %) and Octacosanal (13.11 %) being the dominant components. On the other hand, DCM extract of the tender stems contained 5 phytoconstituents which included Eicosane (42.59 %) and Hexatriacontane (28.70 %) as the dominant components. The DCM extract of the root bark of *E. divinorum* produced a chromatogram with four prominent peaks which were identified to be 3,4-Methylenedioxybenzylacetone (76.01 %), 9-Hexadecen-1-ol (13.65 %), 2-Ethylhexyl acrylate (7.47 %) and 2,6,11-Trimethyldodecane (2.87 %) (Table 4.4).

Table 4.3: Compounds identified in hexane extracts of *E. divinorum* parts by GC/MS.

Sample	**Retention time (min)**	**Peak area (%)**	**Constituents**
Leaf extract	10.866	2.53	1-Methyl-2-Pyrrolidinone
	40.517	2.11	Eicosane
	44.488	**47.43**	Tetratriacontane
	45.991	**13.53**	2-Hydroxy-2-methyl-8,8-diphenyl-octa-5,7-dien-3-one
Tender stems extract	34.489	0.39	Palmitic acid
	34.889	1.12	Ethyl palmitate
	38.181	0.89	Ethyl-9,12-octadecadienoate
	38.327	0.62	Ethyl 9α-linolenate
	40.516	**56.26**	Eicosane
	41.753	0.66	4,8,12,16-Tetramethylheptadecan-4-olide
	42.336	1.97	Tetracosane
	43.939	0.83	9-Tricosene
	43.941	0.56	Heptacosanol
	46.609	**18.17**	Hexatriacontane
	46.910	Traces	Tetratriacontane
	47.142	4.82	Heneicosane
Root bark extract	27.772	**13.34**	1,4-Naphthoquinone
	29.549	0.73	7-Ethoxycoumarin
	30.873	3.71	Eicosane

Sample	Retention time (min)	Peak area (%)	Constituents
	31.367	**4.96**	Tetratetracontane
	35.395	0.78	4-Vinyl guaiacol
	40.765	**4.37**	Tetratriacontane
	41.428	0.94	Squalene
	46.920	**70.91**	Hexatriacontane

Peak areas in **bold** are for major constituents.

Table 4.4: Compounds identified in DCM extracts of E. divinorum parts by GC-MS

Sample	Retention time (min)	Peak area (%)	Constituents
Leaf extract	29.043	7.84	Octadecanal
	33.048	2.99	Cis, cis,cis-7,10,13-Hexadecatrienal
	34.895	1.38	Ethyl palmitate
	40.356	**20.83**	Tetratriacontane
	40.615	2.67	Tetradecyl acrylate
	41.415	5.86	Squalene
	44.488	**15.89**	Eicosane
	44.987	**13.11**	Octacosanal
	47.059	5.13	Heptacosanol
Tender stems extract	30.381	8.94	Tetratriacontane
	32.395	**28.70**	Hexatriacontane
	40.757	7.19	Tetratetracontane
	43.861	2.10	γ-Tocopherol
	46.618	**42.59**	Eicosane
Root bark extract	16.097	7.47	2-Ethylhexyl acrylate
	17.209	2.87	2,6,11-Trimethyldodecane
	31.286	**76.01**	3,4-Methylenedioxybenzylacetone
	40.608	**13.65**	9-Hexadecen-1-ol

Peak areas in **bold** are for major constituents.

Some of the compounds have been reported to have medicinal properties. For example, naphthoquinones such as 1,4-naphthoquinone have been reported to have antimicrobial activity (López *et al.,* 2014) and their presence in this species have been previously

reported (Mebe *et al.,* 1998). Various natural and synthetic naphthoquinone analogues are precursors in the synthesis of natural products and pharmaceuticals, which exhibit antibacterial, antifungal, antiviral, antitumor, trypanocidal, antimalarial, antileismanicidal, molluscicidal and insecticidal activities (Aminin & Polonik, 2020). Similarly, 1,4-naphthoquinone derivatives were reported to inhibit the growth of *C. albicans* and *S. aureus* (López *et al.,* 2014).

Squalene is another bioactive compound with several pharmacological activities including anticancer, antibacterial, antifungal, antioxidant and cardioprotective properties (Payum, 2016). Further, Palmitic acid and hydrocarbons such as Eicosane have antibacterial activity (Jayapriya & Shoba, 2015). Thus, the results of the current study support the antimicrobial activity of *E. divinorum* leaves, tender stems and root bark extracts against the selected microbes thus the use in formulation of toothpaste with root bark extract as the active ingredients.

4.3. Total Phenolic Content and Total Flavonoid Content results

4.3.1. Total Phenolic Content results

The total amount of phenolics contained in each of the extracts of *E. divinorum* parts was determined using the Folin-Ciocalteau method. Folin-Ciocalteau reagent consists of a mixture of sodium molybdate, sodium tungstate and other reagents which when added to the extracts, reacted with phenolic compounds to produce a blue complex solution which absorbed at 760 nm. That is to say, Mo(VI) species was reduced to Mo(V) species (Walker *et al.,* 2014).

A calibration curve shown in Figure 4.11 was prepared for UV-Vis Spectrophotometric quantitative analysis of total phenolic contents in all the solvent extracts of the different parts of *E. divinorum*. Linearity for gallic acid standard was

established from the range of 1 ppm to 100 ppm which was fitted on the straight line that gave the equation $y = 0.0022x + 0.0012$ and R^2 value of *0.9997*.

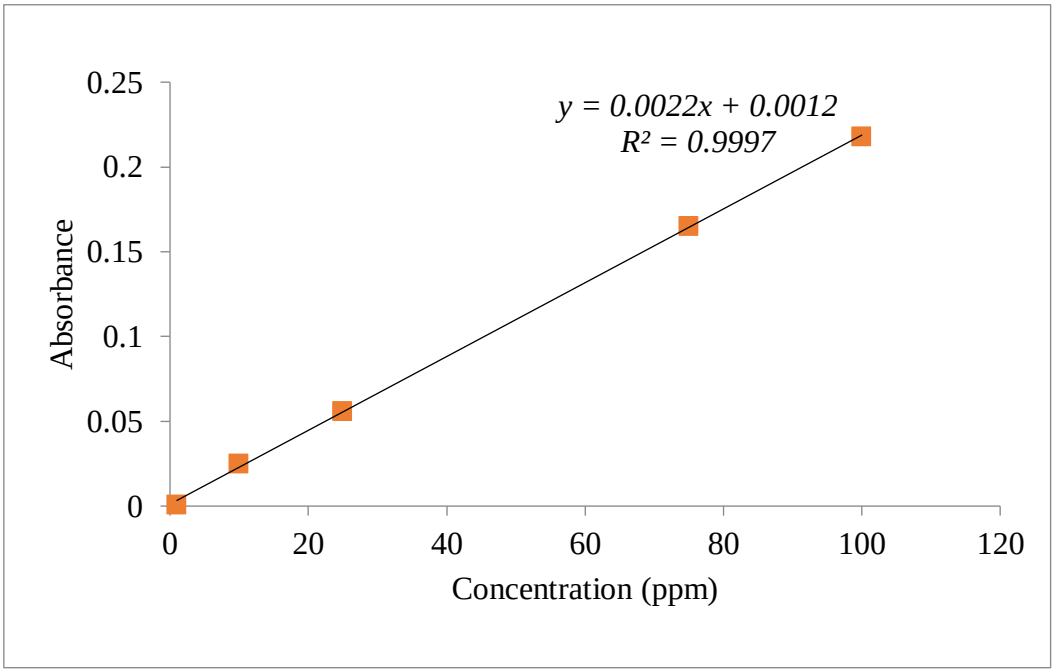


Figure 4.11: Calibration curve for Gallic acid standard

Table 4.5: TPC results in different solvent extracts in ppm

Part used	Hexane	Dichloromethane	Ethanol
Leaves	299.0 ± 2.6	1190.0 ± 12.0	1516.0 ± 17.1
Tender stems	231.0 ± 16.6	828.0 ± 11.4	2800.0 ± 5.7
Root barks	472.0 ± 6.3	1569.0 ± 5.3	3105.0 ± 3.3

All values are means ± standard deviations of triplicates, expressed as gallic acid equivalent in ppm.

The phenolic content is highest in ethanol extracts because ethanol being polar, it extracted more phenolic compounds which are also polar in nature with the root bark extract having the highest followed by tender stem and least in the leaf extract (Table 4.5, APPENDIX IVI).

Hexane being the most non polar, extracted the least phenolic compounds compared to those extracted by ethanol and DCM. The DCM tender stem extract contained the least amount phenolic compounds and the root bark contained the highest. Generally, the root bark extracts had the highest quantity of phenolic compounds compared to the rest of the parts of the plant extracted.

This could be due to the fact that phenolic compounds are polar thus were extracted by ethanol, a polar solvent. The results are comparable to those reported by Noreen *et al.* (2017) in which the TPC of different solvent extracts of *Coronopus didymus* was found to be highest in ethanol, followed by hexane and the least content found in DCM extract.

There were no significant differences ($p = 0.2161$) was greater than 0.05 in the TPC of the ethanol extracts of *E. divinorum* leaves, tender stems and root barks (APPENDIX VII). Similarly, there were no significant differences ($p = 0.9575$ which was also greater than 0.05) in the TPC of dichloromethane extracts of *E. divinorum* leaves, tender stems and root bark. No significant differences in mean TPC values of hexane extracts of *E. divinorum* leaves, tender stems and root bark was recorded ($p = 0.6476$, greater than 0.05). Low phenolic content in plant extracts indicate that the extract contains more flavonoid heterosides than aglycones (Ramos, Bezerra, Ferreira, Alberto, & Soares, 2017).

4.3.2. Total Flavonoid Content results

Aluminium chloride method which is based on the nitration of the aromatic ring of quercetin with its position 3 or 4 non-sterically hindered giving a yellow complex of aluminium formed that turns red after addition of sodium hydroxide (Pękal & Pyrzynska, 2014).

A calibration curve shown in Figure 4.12 was prepared for UV-Vis Spectrophotometric quantitative analysis of total flavonoid contents in all the solvent extracts of the parts of *E. divinorum*. Linearity for gallic acid standard was established from the range of 5 ppm to 100 ppm which was fitted on the straight line that gave the equation $y = 0.007x - 0.00201$ and R^2 value of 0.9999.

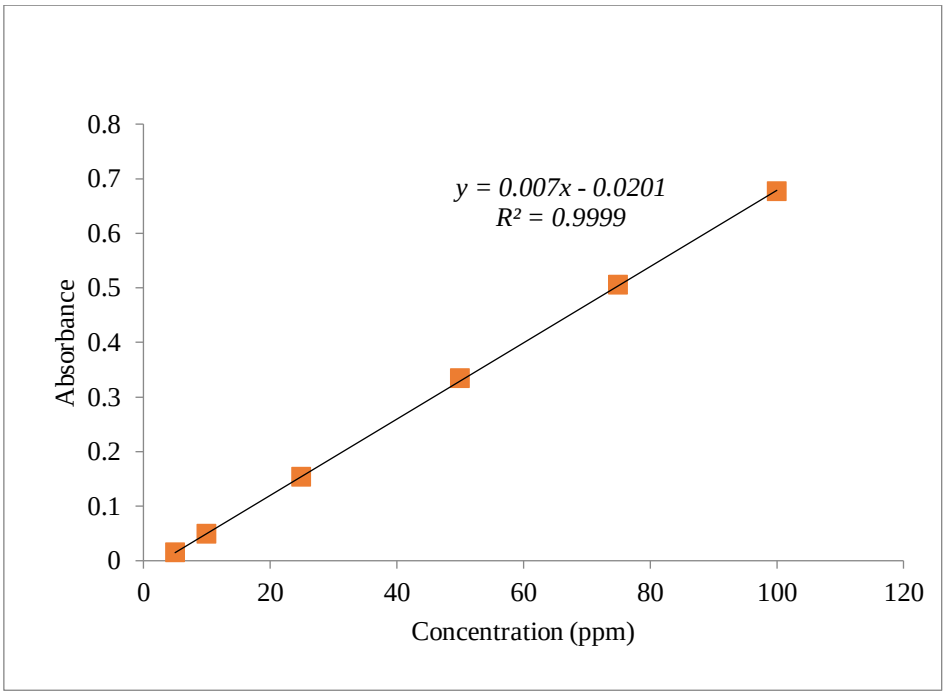


Figure 4.12: Calibration curve for quercetin standard

Table 4.6: TFC of the different solvent extracts of *E. divinorum* parts

Part extracted	Hexane	Dichloromethane	Ethanol
Leaves	84.3 ± 1.41	23.4 ± 0.40	63.1 ± 1.97
Tender stems	55.6 ± 1.33	27.6 ± 1.24	81.6 ± 2.88
Root bark	193.3 ± 1.09	96.1 ± 0.61	309.7 ± 11.9

All values are means ± standard deviations of triplicates in quercetin equivalent in ppm.

It was found that TFC were highest for ethanolic extracts compared to DCM and hexane extracts (Table 4.6, APPENDIX VI). In the root barks for instance, TFC were found to be 96.10 ± 0.61, 193.30 ± 1.09 and 309.70 ± 11.9 ppm for the DCM, hexane and ethanolic extracts respectively. However, the TFC in the leaves were lower for the ethanolic extract (63.10 ± 1.97 ppm) compared to the hexane extract (84.30 ± 1.41 ppm).

However, the statistical data analysis showed that there was no significant difference in TFC values in leaves, tender stems and root bark of *E. divinorum* ethanol extracts because the obtained statistical p-value of 0.0622 was greater than 0.05 level of significance. There was no significant difference in TFC levels in leaves, tender stems and root bark of *E. divinorum* dichloromethane extracts since the obtained statistical p-value of 0.2602 was greater than 0.05. There was no significant difference in TFC in leaves, tender stems and root bark of *E. divinorum* hexane extracts because the obtained p-value (0.2375) greater than 0.05.

This could have been because ethanol with the highest degree of polarity among the solvents, it is able to extract much of the flavonoids which are relatively polar in polar solvents (Prashant *et al.*, 2011).

A similar approach was done in comparing the total flavonoid contents in extracts of leaves, tender stems, and root barks of *E. divinorum.* It was found that the total flavonoid concentrations were recorded highly in all plant parts under ethanol extracts than under Dichloromethane and Hexane extracts. However, in leaves, TFC was recorded low under Ethanol extract (63.1 ppm) than under Hexane extract (84.3 ppm). This might be the case because ethanol having the highest degree of polarity as compared to hexane and DCM that are considered non polar, it is able to extract much of the flavonoids which are also polar (Okeke & Ezeabara, 2018).

Overall, it should be emphasized that the recovery of phytochemicals from plants are influenced by dielectric constant, chemical structure of organic solvents, and as well as chemical properties of plant phytochemicals (Felhi *et al.,* 2017b), explaining the variations observed in the phenolic and flavonoid contents obtained from the different parts of the plants using the three different solvents.

4.4. Antimicrobial activity results

4.4.1. Antimicrobial screening results

The results of antimicrobial assay showed that the crude extracts of *E. divinorum* displayed a notable inhibitory effect on the growth of tested microorganisms (Table 4.7). The activity was higher for ethanol extracts of leaves and root barks against most of the tested microorganisms. The results showed no inhibition activity of solvent used in dilution of the plant extracts (10 % DMSO) against studied bacteria and fungus. The positive control (penicillin) had comparable bioactivity against the studied microorganisms as the crude extracts. Crude ethanol root bark extracts of *E. divinorum* had a higher inhibitory effect on the growth of *S. pyogenes* and *E. coli* microorganisms than penicillin while the two showed a similar inhibitory effect against *C. albicans.*

Table 4.7: Antimicrobial activity of leaf, tender stem and root bark extracts of *E. divinorum*

Part	Extract	*S. pyogenes*	*S. aureus*	*E. coli*	*C. albicans*
Leaves	Hexane	5.67 ± 0.58	4.33 ± 0.58	0.00 ± 0.00	4.67 ± 0.58
	DCM	7.00 ± 1.00	1.00 ± 0.00	0.00 ± 0.00	2.33 ± 0.58
	Ethanol	21.00 ± 6.08	6.00 ± 0.00	0.00 ± 0.00	10.00 ±1.00
Stems	Hexane	5.33 ± 0.57	2.00 ± 0.00	00.0 ± 0.00	2.00 ± 0.00
	DCM	7.33 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	8.00 ±0.00
	Ethanol	7.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00	4.33 ± 0.58
Root bark	Hexane	8.00 ± 0.00	7.67 ± 0.58	5.67 ± 1.15	11.33± 0.57
	DCM	3.33 ± 0.58	4.00 ± 0.00	0.00 ± 0.00	6.67 ± 1.53
	Ethanol	25.67 ± 1.53	11.00 ± 3.61	10.33 ± 0.58	4.00 ± 0.00
	Penicillin	23.00 ± 2.65	17.67 ± 1.53	6.00 ± 1.00	4.00 ± 0.00
	1% DMSO	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Results are means of zone of inhibition diameter ± standard deviations (in mm) of triplicates.

More *et al.* (2008) investigated the ethanol extract of *E. divinorum* leaves and barks and tested for their inhibitory activity against oral pathogens. The authors reported that the extracts did not inhibit the growth of *C. albicans* while *S. mutans* was inhibited with a zone inhibition diameter of 6.0 mm and 2.4 mm at 2 mg and 3 mg of the plant extracts per disc (More *et al.*, 2008). Al-Fatimi investigated the antifungal activity of *E. divinorum* twigs and root barks under different solvents of extraction. The DCM extracts of *E. divinorum* roots showed the highest antifungal activity with inhibition diameter of 30 mm against *Trichophyton mentagrophytes.* It also exhibited comparable antifungal activity as ethyl acetate and methanol extracts of the root barks against the

tested fungal strains (Al-Fatimi, 2019). Aqueous extracts of *E. divinorum* twigs were reported to inhibit 50% of proteolytic activity of three strains of *Bacteroides gingivalis, Bacteriodes intermedius* and *Treponema denticol* at concentrations of 10 µg/ml up to 200 µg/ml (Homer, Manji, & Beighton, 1990).

In another investigation (Mothana *et al.,* 2009), the root methanolic extract of *E. divinorum* exhibited antimicrobial activity against *S. aureus, Bacillus subtilis, Micrococuss flavus, E. coli, Pseudomonas aeruginosa* and *Candida maltosa* with zone of inhibition (ZOI) of 24, 12, 18, 11, 15 and 10 mm) and three multi-resistant *Staphylococcus* strains (*S. epidermidis* 847, *S. haemolyticus* 535 and *S. aureus* North German Epidemic Strain with ZOI of 24, 16 and 26 mm, respectively). Another study (Samie *et al.,* 2010) evaluated the antifungal activities of selected Venda medicinal plants against *C. albicans, Candida krusei* and *Cryptococcus neoformans* isolated from South African AIDS patients in which *E. divinorum* was one of the 76 medicinal plants tested. It was reported that hexane extract of *E. divinorum* leaves was more active against *Cryptococcus neoformans* with ZOI of 8 mm at 10 µl. No bioactivity was observed against the other tested fungi.

Ngari *et al.* (2013) reported that aqueous and DCM/methanol extracts of *E. divinorum* roots had no antimicrobial activity against *E. coli* while its DCM extract had ZOI of 10.30 ± 0.63 mm. DCM extracts had ZOI of 10.75 ± 1.60, 10.00 ± 0.41 and 00.00 ± 0.00 mm against *S. aureus, Bacillus subtilis* and *Lactobacillus acidophillus,* respectively. The DCM/methanol extracts had ZOI of 9.13 ± 0.72, 10.80 ± 0.25 and 6.70 ± 0.48 mm, respectively while the aqueous extracts had no bioactivity. In another study, crude root extracts of *E. divinorum* showed zone of inhibition of 9 mm and 13 mm for *S. mutans* and *S. sanguinis* respectively (Nyambe, 2014).

The antimicrobial activity of the different parts of *E. divinorum* could be due to the presence of antimicrobial compounds reported in this study (**Error! Reference source not found.**). Compounds identified include; squalene, tetradecyl acrylate, palmitic acid and ethyl ester in *E.* leaves and these compounds were reported to possess antimicrobial activity (Batovska, Todorova, Tsvetkova, & Najdenski, 2009). Furthermore, Ethyl-9-alpha-linolenate, Ethyl-9,12-Octadecadienoate, Ethyl palmitate and other fatty acids and their esters which a been reported to have antibacterial activities (Karimi, Lotfipour, Asnaashari, Asgharian, & Sarvari, 2019) were identified in our extracts of tender stems. Similarly, 7-Ethoxycoumarin, 1,4-Naphthoquinone derivative and squalene were identified and these have been reported to have antibacterial activity (López *et al.,* 2014; Lozano-Grande, Gorinstein, Espitia-Rangel, D´avila-Ortiz , & Mart´ınez-Ayala, 2018).

4.4.2. Minimum Inhibitory Concentration

The ethanolic extract of *E. divinorum* root bark was found to be the most active and its minimum inhibitory concentration (MIC) for the tested microorganisms was determined. It was found to have the highest activity against *S. pyogenes, S. aureus* and *E. coli* (Table 4.8).

Table 4.8: Zone of inhibition by different concentrations of ethanol root extract

Concentration (µg/mL)	*S. pyogenes*	*S. aureus*	*E. coli*	*C. albicans*
25	0.67 ± 0.58	0.00 ± 0.00	5.67 ± 2.08	2.00 ± 0.00
50	3.33 ± 0.58	3.00 ±0.00	12.00 ± 1.73	7.67 ± 1.53
100	4.00 ± 0.00	7.33 ± 2.31	14.00 ± 2.00	8.00 ± 1.73
200	6.00 ± 1.00	12.67 ± 1.53	13.67 ± 1.53	13.0 ± 1.73
400	25.0 ± 1.53	11.00 ± 3.61	10.33 ± 0.58	7.00 ± 1.00
DMSO (-ve control)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Zone of inhibition in mm

The MIC of the ethanolic extract of *E. divinorum* root bark against *S. pyogenes* was 25 µg/ml, 50 µg/ml for *S. aureus*, 25 µg/ml for *E. coli* and *C. albicans.* More *et al*. (2008) reported MIC ranging from 3.1 to 25.0 µg/ml for ethanolic extracts of *E. divinorum* root bark and leaves against a panel of Gram positive and Gram negative bacteria (*Actinomyces naeslundii*, *Actinomyces israelii*, *S. mutans*, *Actinobacillus actinomycetemcomitans* and *Porphyromonus gingivalis*). Nyambe (2014), reported MICs of 1250 and 2500 µg/ml for *E. divinorum* root fraction against *Streptococcus mutans* and *S. sanguinis*.

The minimum inhibitory concentration of root bark ethanol extract against *S. pyogenes* was 25 µg/mL, 50 µg/mL, for *S. aureus:* 25 µg/mL, for *E. coli* and 25 µg/mL for *C. albicans.* From the previous study, aqueous extracts of *E. divinorum* twigs were reported to inhibit 50% of proteolytic activity of three strains of bacteria: *Bacteroides gingivalis, Bacteriodes intermedius* and *Treponema denticol* at concentrations of 10 µg/mL up to 200 µg/mL (Homer *et al.*, 1990).

4.5. Antibacterial activity of the formulated toothpaste

Two toothpaste formulations were prepared where one (T2) was formulated with ethanol root bark extract of *E. divinorum* as the active ingredient against selected oral bacteria and fungus while the other formulation was prepared without the herbal extract (T1) to determine whether the toothpaste base alone can inhibit the growth of the selected microorganisms (Table 4.9). The results showed that toothpaste formulated with the ethanol extract of *E. divinorum* root barks was found to have higher activity against the tested oral microorganisms compared to Colgate herbal toothpaste that is formulated with fluoride as the active ingredient against selected microorganisms.

Colgate herbal toothpaste showed no activity against *E. coli* but had comparable activities against *S. aureus* and *C. albicans*. Toothpaste formulated without the herbal extracts showed no inhibition of *S. pyogenes, E. coli* and *C. albicans* since it had no active ingredients to inhibit the growth of organisms. Very little inhibition was observed against *S. aureus* possibly due to the ingredients used in toothpaste formulation.

Table 4.9: Zone of inhibition against selected oral pathogenic organisms by formulated toothpaste

Toothpaste	*S. pyogenes*	*S. aureus*	*E. coli*	*C. albicans*
T1	0.00 ± 0.00	1.33 ± 0.58	0.00 ± 0.00	0.00 ± 0.00
T2	22.67 ± 2.52	13.67 ± 0.58	7.67 ± 1.15	10.67 ± 1.15
Colgate herbal toothpaste	16.33 ± 2.08	14.67 ± 1.15	0.00 ± 0.00	7.00 ± 0.00

Zone of inhibition in mm.

These results are similar to those obtained by Sharma *et al.* (2014) where polyherbal toothpastes were formulated, and evaluated for their activity against S*treptococcus mutans, S. aureus* and *C. albicans.* The results from their study showed that the methanol extract of polyherbal formulation exhibited more activity against all the selected oral pathogens than the herbal marketed toothpaste used.

Wardhara *et al*. (2017) prepared a toothpaste with *Aloe vera* and Red Betel extracts revealed that the formulated toothpaste had comparable antibacterial activity against S. *mutans* (1.5 cm) to the market product with the ZOI of 1.9 cm whereas, no activity was observed when the toothpaste formulated without the herbal extract as the active ingredient (Wardhana, Warya, & Trisnawaty, 2017).

Chapter 5 : CONCLUSION AND RECOMMENDATION

5.1. Conclusion

Various secondary metabolites including flavonoids, alkaloids, tannins, phenols and saponins were found in extracts of *E. divinorum* leaves, tender stems and root barks. Their presence was also supported by the spectroscopic studies showing the characteristic peaks obtained in Infra-red, Ultraviolet and Visible regions. The presence of phenolics, alkenes, aldehydes, alcohols and aromatic compounds was also supported by the obtained FT-IR spectra. The GC/MS results confirmed the presence of compounds whose functional groups were detected during FT-IR and UV-Visible spectrometry. Root bark extracts contained the highest amount of phenolics and flavonoids. This study supports the use of the plant parts studied in traditional medicine. The ethanol extract of the root barks exhibited the highest activity against *Streptococcus pyogenes* and *Staphylococcus aureus*. The formulated herbal-based toothpaste was found to be as effective against selected microorganisms as Colgate herbal fluoride toothpaste.

5.2. Recommendations

The GC-MS results led to the identification of some potential therapeutic compounds that have been responsible reported to have antibacterial and antifungal activities thus there is need to isolate pure compounds and further elucidate their structure and determine the antimicrobial activity of pure isolates.

In vivo experiments need to be conducted on patients with dental caries to determine the efficacy of these extracts.

The formulated toothpaste should be evaluated for its physical and chemical characteristics to ascertain its safety before it can be used for the control of dental caries.

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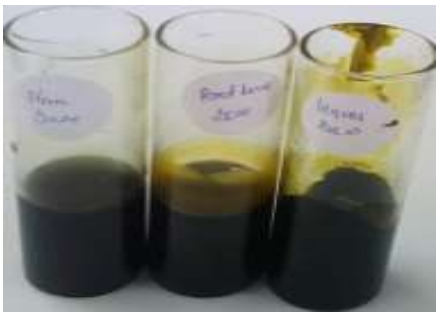
APPENDICES

APPENDIX I : Sample Preparation and extraction

Tender stems of *E. divinorum*

Root barks of *E. divinorum*

Dry ground powder of *E. divinorum* leaves, root barks and tender stems.



Hexane, DCM, and ethanol extracts of *E. divinorum* leaves, tender stems, and root bark.

APPENDIX II: Phytochemical Screening Results

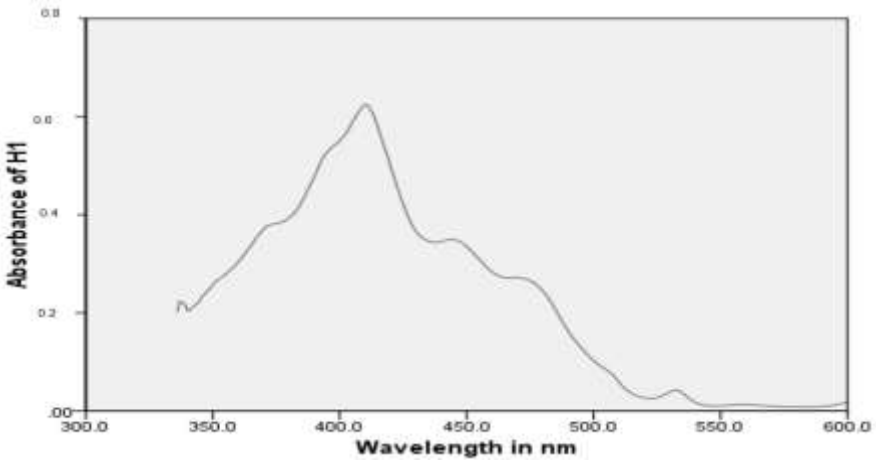
Test results for terpenes in ethanol extracts of tender stems, leaves and root barks respectively.

Test results for saponins in ethanol extracts of tender stems, root barks and leaves respectively.

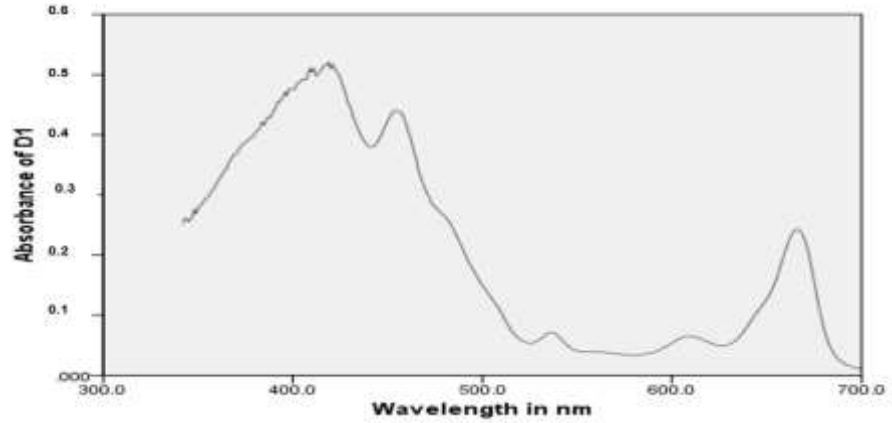
APPENDIX III: UV-Visible peak values of extracts of parts of *E. divinorum*

Plant part	Extract	Wavelength max (nm)	Absorption by the sample
Leaves	Hexane	410.0, 539.0, 699.0	0.690, 0.200, 0.140
	DCM	414.0, 453.0, 669.0	0.583, 0.435, 0.295
	Ethanol	370.0, 436.0, 655.0	0.617, 0.184, 0.13
Tender stems	Hexane	409.0, 524.0, 674.0	0.137, 0.008, 0.053
	DCM	416.0, 452.0, 669.0	0.343, 0.229, 0.085
	Ethanol	400.0, 524.0, 665.0	0.60, 0.008, 0.310
Root barks	Hexane	369.0, 395.0, 446.0	0.279, 0.297, 0.248
	DCM	342.0, 420.0, 665.0	0.686, 0.179, 0.95
	Ethanol	352.0, 439.0, 699.0	0.931, 0.761, 0.061

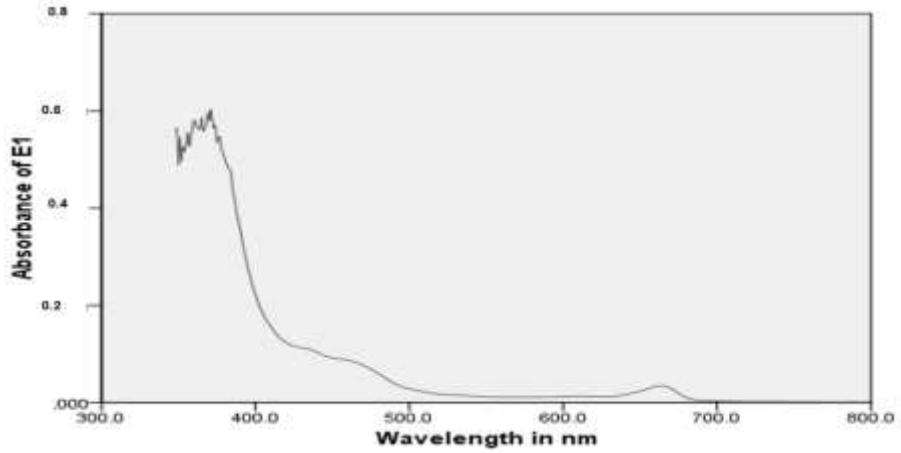
APPENDIX IV: UV – Visible Spectra for Extracts of *E. divinorum*



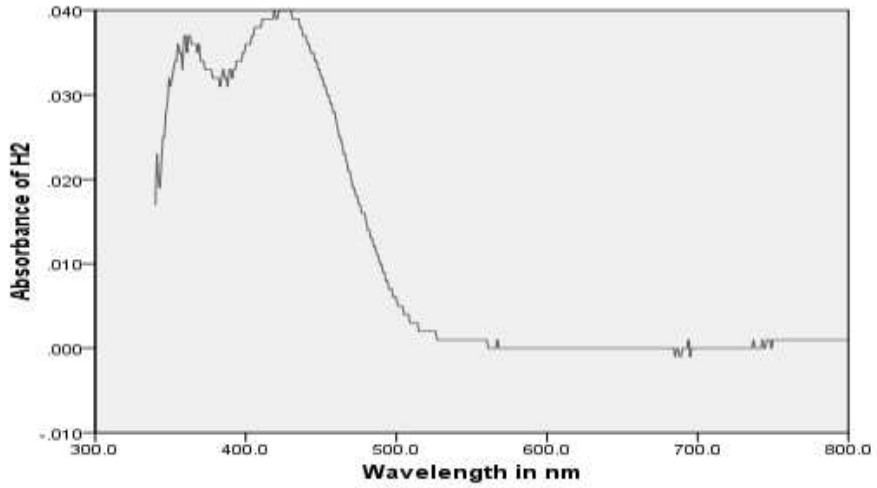
UV-Vis spectrum of hexane extract of the leaves of *E. divinorum*



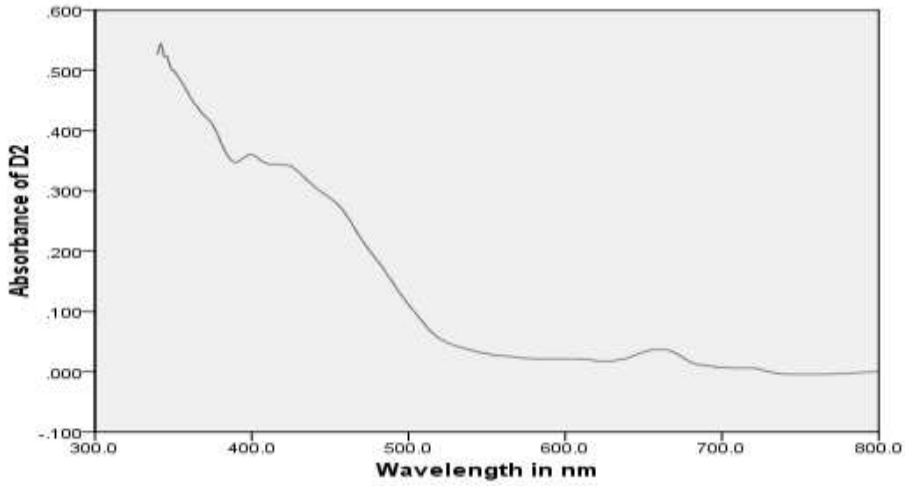
UV-Vis spectrum of DCM extract of the leaves of *E. divinorum*



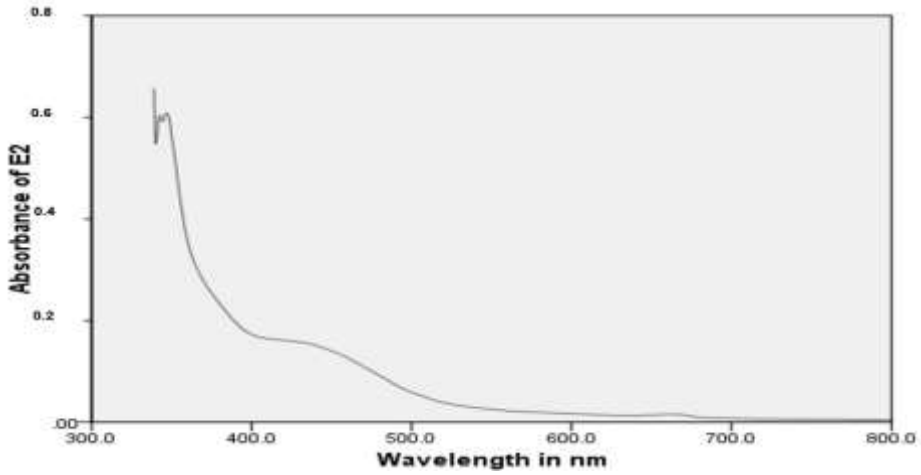
UV-Vis spectrum of ethanol extract of the leaves of *E. divinorum*



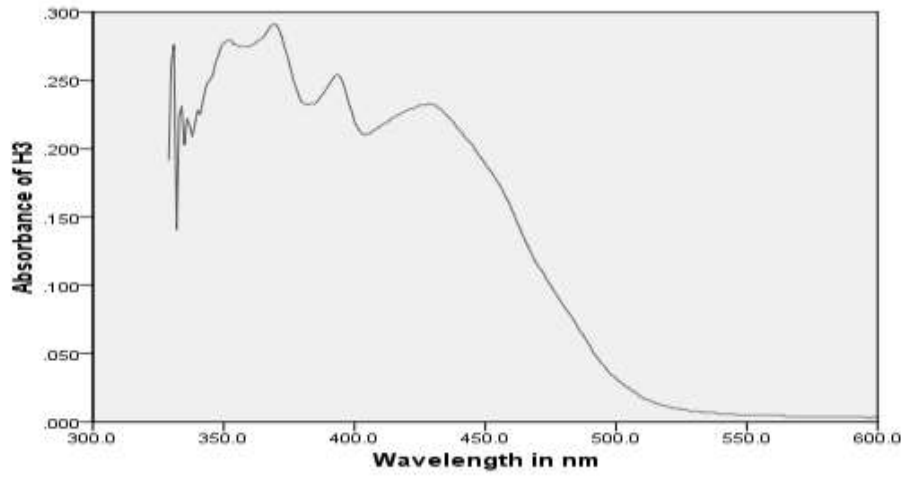
UV-Vis spectrum of hexane extract of tender stems



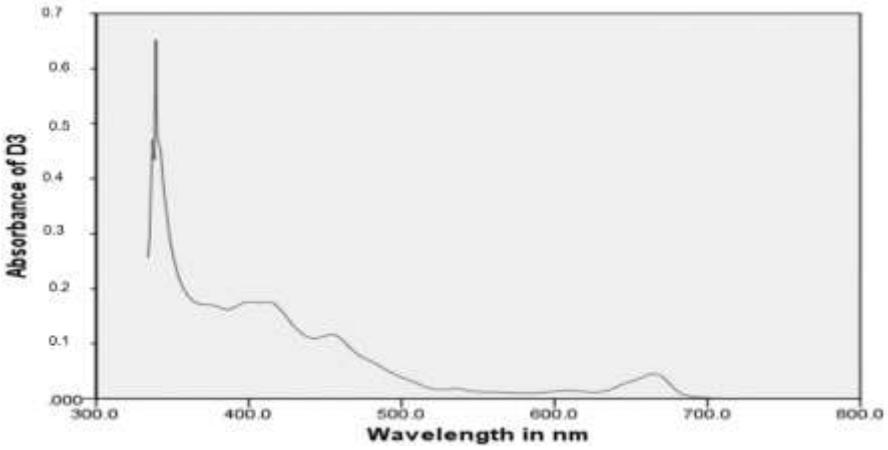
UV-Vis spectrum of DCM extract of the tender stems of *E. divinorum*



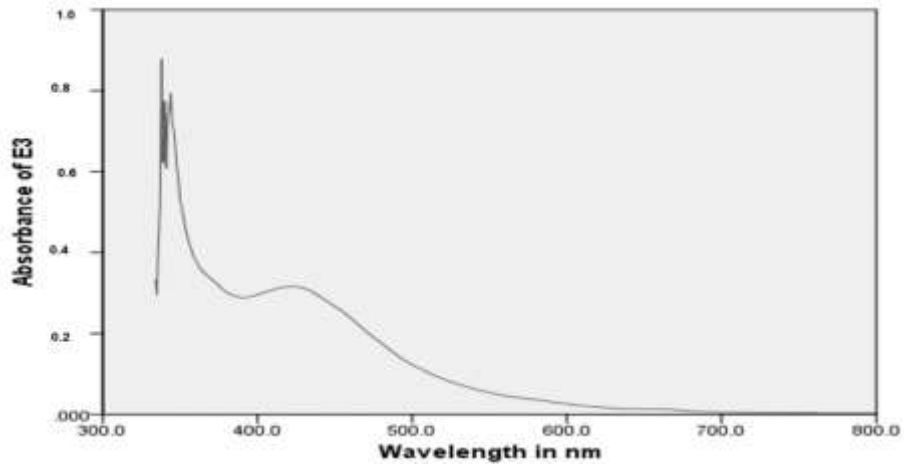
UV-Vis spectrum of ethanol extract of the tender stems of *E. divinorum*



UV-Vis spectrum of hexane extract of the root barks of *E. divinorum*

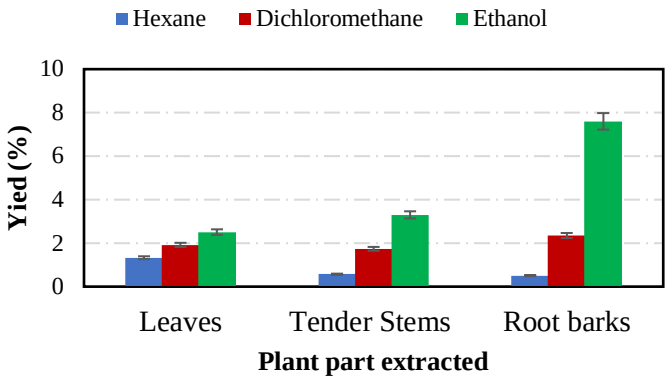


UV-Vis spectrum of DCM extract of the root barks of *E. divinorum*

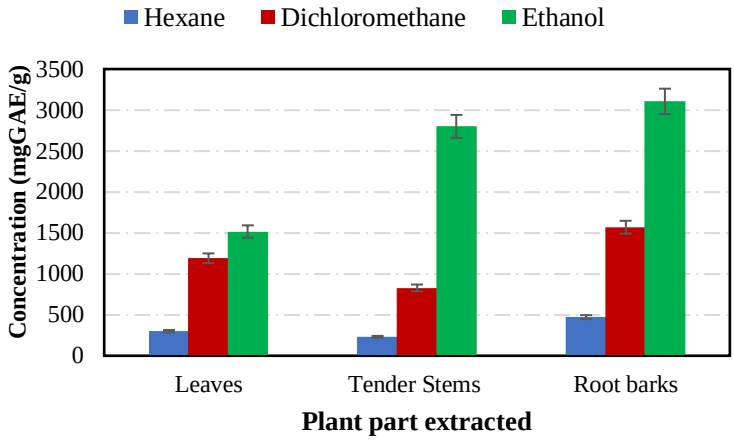


UV-Vis spectrum of ethanol extract of the root barks of *E. divinorum*

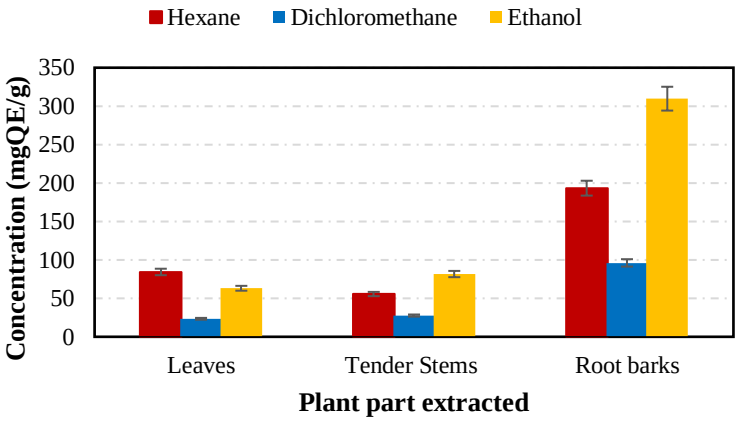
APPENDIX V: Percentage yield of the extracts



APPENDIX VI: Total Phenolic Content in extracts of *E. divinorum* parts



APPENDIX VII: Total Flavonoid Content in extracts of *E. divinorum* parts



APPENDIX VIII: Analysis of Variance for the yield of the extracts

SPSS output for percentage yield analysis

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	21.627	2	10.813	4.20269	0.07226	5.14325
Within Groups	15.438	6	2.573			
Total	37.064	8				

APPENDIX IX: Multivariate Analysis of Variance of TPC in ethanol extracts

```
. anova TPC subjectID ethanol, repeated(ethanol) bse(subjectID)

                          Number of obs =         9     R-squared     =  0.9999
                          Root MSE      = 8.85061     Adj R-squared =  0.9999

                   Source | Partial SS     df       MS           F     Prob > F
                ----------+-----------------------------------------------------
                    Model |  4266962.67      4  1066740.67   13617.97      0.0000
                          |
                subjectID |     4266602      2     2133301   27233.63      0.0000
                  ethanol |  360.666667      2  180.333333       2.30      0.2161
                          |
                 Residual |  313.333333      4  78.3333333
                ----------+-----------------------------------------------------
                    Total |     4267276      8    533409.5

Between-subjects error term:  subjectID
                     Levels:  3          (2 df)
          Lowest b.s.e. variable:  subjectID

Repeated variable: ethanol
                                        Huynh-Feldt epsilon        =  1.5453
                                        *Huynh-Feldt epsilon reset to 1.0000
                                        Greenhouse-Geisser epsilon =  0.6716
                                        Box's conservative epsilon =  0.5000

                                                  ------------ Prob > F ------------
                   Source |     df       F      Regular     H-F      G-G        Box
                ----------+-----------------------------------------------------
                  ethanol |      2      2.30     0.2161    0.2161   0.2487     0.2685
                 Residual |      4
                ----------+-----------------------------------------------------
```

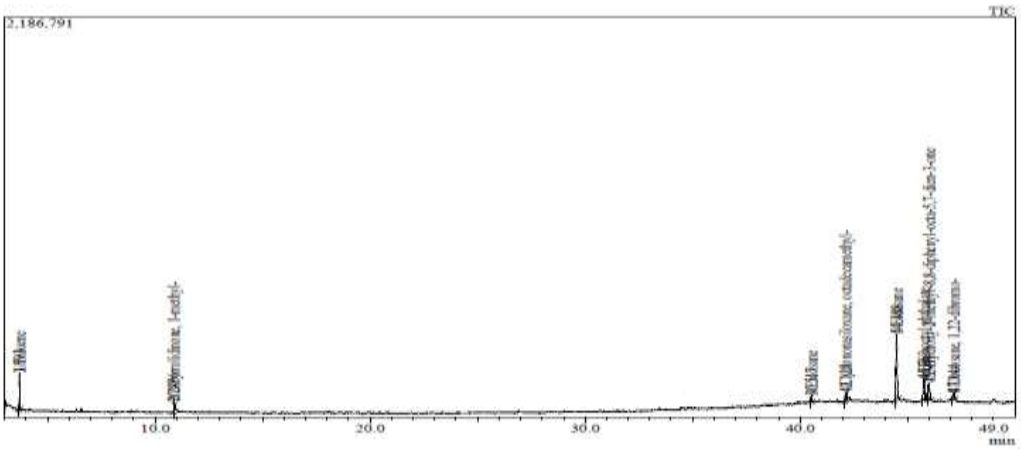
```
. anova TPC subjectID dmethane, repeated(dmethane) bse(subjectID)

                          Number of obs =       9     R-squared     =  0.9992
                          Root MSE      =  3.1798     Adj R-squared =  0.9984

                   Source | Partial SS    df       MS          F     Prob > F
                ----------+----------------------------------------------------
                    Model |  51534.4444     4  12883.6111    1274.20     0.0000
                          |
                subjectID |  51533.5556     2  25766.7778    2548.36     0.0000
                 dmethane |  .888888889     2  .444444444       0.04     0.9575
                          |
                 Residual |  40.4444444     4  10.1111111
                ----------+----------------------------------------------------
                    Total |  51574.8889     8  6446.86111

Between-subjects error term:  subjectID
                     Levels:  3          (2 df)
     Lowest b.s.e. variable:  subjectID

Repeated variable: dmethane
                                        Huynh-Feldt epsilon        =  0.6219
                                        Greenhouse-Geisser epsilon =  0.5287
                                        Box's conservative epsilon =  0.5000

                                                    ---------- Prob > F ----------
                   Source |  df       F    Regular     H-F      G-G      Box
                ----------+-----------------------------------------------------
                 dmethane |   2    0.04     0.9575    0.8929   0.8640   0.8534
                 Residual |   4
                ----------+-----------------------------------------------------
```

```
. anova TPC subjectID hexane, repeated(hexane) bse(subjectID)

                          Number of obs =         9     R-squared     =  0.9944
                          Root MSE      =   11.3944     Adj R-squared =  0.9889

                   Source | Partial SS    df       MS           F     Prob > F
                ----------+----------------------------------------------------
                    Model |  92838.6667     4  23209.6667     178.77     0.0001
                          |
                subjectID |  92712.6667     2  46356.3333     357.04     0.0000
                   hexane |        126     2          63       0.49     0.6476
                          |
                 Residual |  519.333333     4  129.833333
                ----------+----------------------------------------------------
                    Total |        93358     8    11669.75

Between-subjects error term:  subjectID
                     Levels:  3          (2 df)
      Lowest b.s.e. variable:  subjectID

Repeated variable: hexane
                                            Huynh-Feldt epsilon        =  0.5369
                                            Greenhouse-Geisser epsilon =  0.5091
                                            Box's conservative epsilon =  0.5000

                                                    ------------ Prob > F ------------
                   Source |  df       F    Regular     H-F      G-G        Box
                ----------+-----------------------------------------------------------
                   hexane |   2      0.49   0.6476    0.5668   0.5603     0.5581
                 Residual |   4
                ----------+-----------------------------------------------------------
```

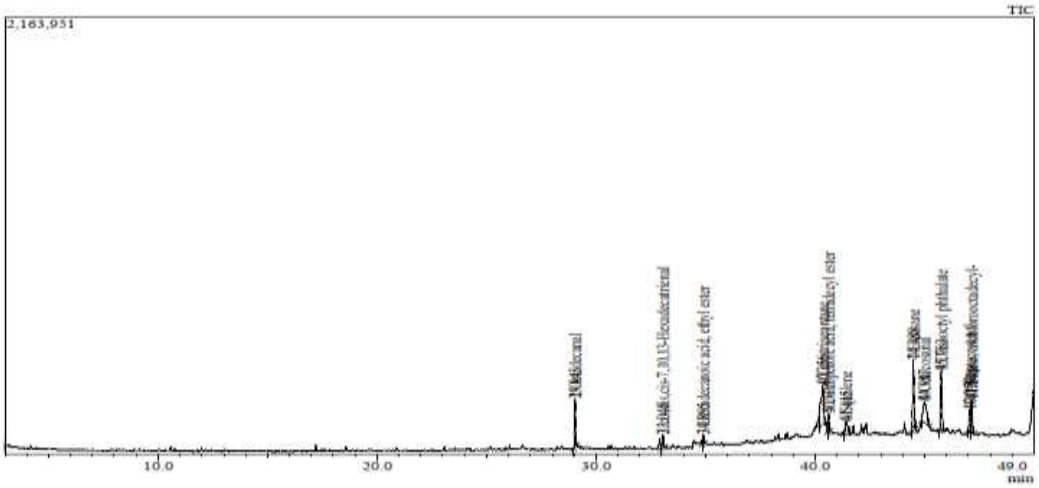
SPSS output for Total Flavonoid Content

```
. anova TFC subjectID ethanol, repeated(ethanol) bse(subjectID)

                          Number of obs =         9     R-squared     =  0.9999
                          Root MSE      =   1.29551     Adj R-squared =  0.9999

                   Source | Partial SS    df       MS           F     Prob > F
--------------------------+----------------------------------------------------
                    Model |  113203.627     4  28300.9067   16862.51     0.0000
                          |
                subjectID |   113183.42     2    56591.71   33718.99     0.0000
                  ethanol |  20.2066667     2  10.1033333       6.02     0.0622
                          |
                 Residual |  6.71333333     4  1.67833333
--------------------------+----------------------------------------------------
                    Total |   113210.34     8  14151.2925

Between-subjects error term:  subjectID
                     Levels:  3          (2 df)
       Lowest b.s.e. variable:  subjectID

Repeated variable: ethanol
                                        Huynh-Feldt epsilon        =  0.5831
                                        Greenhouse-Geisser epsilon =  0.5200
                                        Box's conservative epsilon =  0.5000

                                                    ---------- Prob > F ----------
                   Source |  df       F    Regular     H-F      G-G        Box
--------------------------+----------------------------------------------------
                  ethanol |   2    6.02     0.0622    0.1170   0.1294     0.1336
                 Residual |   4
--------------------------+----------------------------------------------------
```

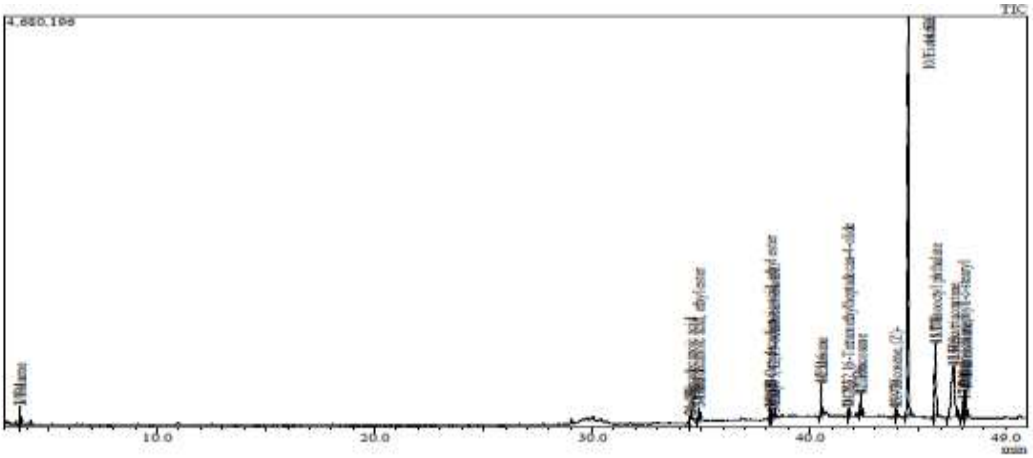
APPENDIX X: GC Chromatograms



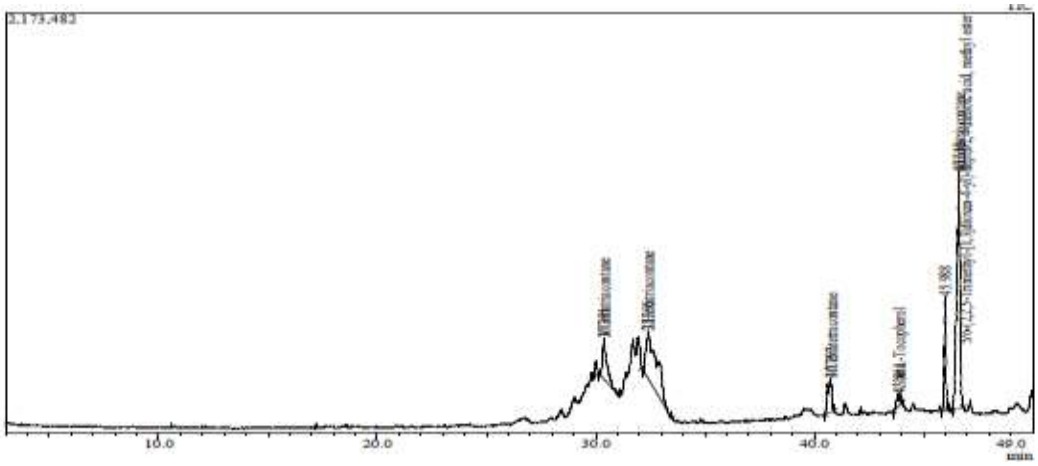
GC Chromatograph for hexane extract of the leaves



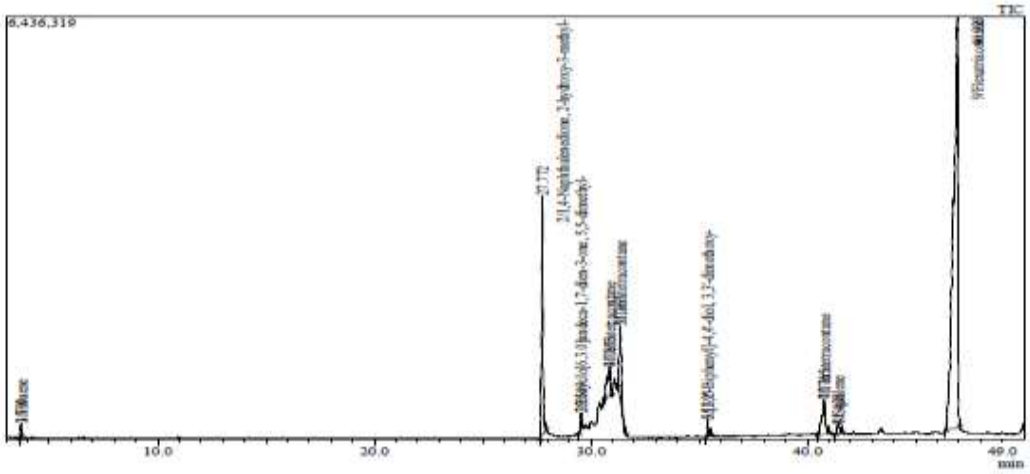
GC chromatogram for DCM extract of the leaves



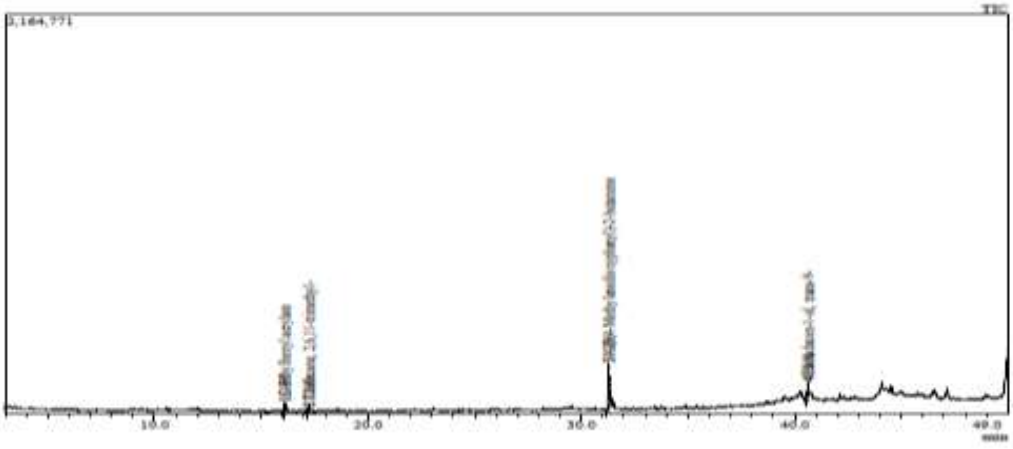
GC Chromatogram of hexane tender stems extract



GC Chromatograph for DCM tender stem extract

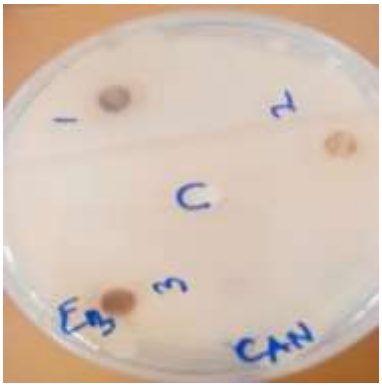


GC chromatogram for hexane root bark extract



GC chromatogram for DCM root bark extract

APPENDIX XI: Inhibition Zones of Microorganisms by the Extracts



Inhibition zones of *C. albicans* by ethanol and DCM extracts of the root barks respectively

Inhibition zones of *S. aureus* and *S. pyogenes* by ethanol and Hexane extracts of the root bark

Inhibition zones of *C. albicans* and *E. coli* by ethanol extract of the leaves and hexane extract

APPENDIX XII: Inhibition Zones by Formulated Toothpastes

No zone of inhibition for *S. pyogenes* and *S. aureus* by the formulated non-herbal toothpaste