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Journal of Medicinal Plants Research

*Review*

# A review on the botanical aspects, phytochemical contents and pharmacological activities of *Warburgia ugandensis*

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***Warburgia ugandensis*** **Sprague (Family Canellacea) commonly known as Ugandan greenheart or pepper bark tree, is a highly valued medicinal plant in traditional medicine with a broad spectrum of antimicrobial activity whose parts especially the leaves and stem bark have for long been used in the treatment and management of many diseases and health conditions such as stomachache, cough, toothache, fever, malaria, oral thrush, measles and diarrhea in African communities where the plant occurs. This review focused on the phytochemical contents, medicinal uses and antimicrobial activities of *W. ugandensis* based published peer reviewed articles. This review established that the high therapeutic value of *W. ugandensis* is attributed to the abundance of drimane sesquiterpenes in its stem bark and leaves. These chemicals have also made the plant to have potent antibacterial and antifungal activities. However, more pre-clinical and clinical trials need to be done to further validate the traditional medicine applications of *W. ugandensis* for possible drug discovery. Due to its high demand, *W. ugandensis* has been over exploited and hence its population is in drastic decline. Consequently, there is need for development of advanced and more rapid propagation techniques to increase its population and distribution in its natural environment to meet the ever-increasing demand.**

**Key words:** Antimicrobial, Canellaceae, medicinal uses, phytochemicals, *Warburgia ugandensis.*

## INTRODUCTION

*Warburgia ugandensis* Sprague (Family Canellaceae) is a plant with immense medicinal values with restricted

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distribution in tropical Africa (Muller et al., 2015). It is commonly known as the “Uganda Green Heart Tree” or the “Pepper bark tree” with a unique characteristic bitter-peppery taste (Njire et al., 2014). Owing to its antimicrobial activities, *W. ugandensis* have been widely used throughout its distribution range to treat and manage myriad of diseases and disorders since time immemorial including cough, toothache, common cold, bronchial infections, parasitic infections, stomachache, fever, malaria, oral thrush, muscle pain, cystitis, constipation, weak joints, measles, and diarrhea among others (Lovett et al., 2006; Henke, 1994; Kiringe, 2006; Kokwaro, 2009; Wamalwa et al., 2006). Indeed, *W. ugandensis* is a highly valued plant species in African traditional health systems especially within the communities where it naturally grows (Were et al., 2015). In fact, due to the increasing demand for chemical diversity in screening programs, and seeking therapeutic drugs from natural products, interest particularly, in medicinal plants throughout the world has significantly grown (Were et al., 2015). This tree species has a high pharmaceutical value with potent antimicrobial activity (Olila et al., 2001).

Scientific studies showed that stem bark and leaves of *W. ugandensis* contain a number of essential phytochemicals such as drimane sesquiterpenes including ugandensidial, polygodial, warburganal, and isopolygodial (Were et al., 2015). Consequently, the medicinal efficacy of this plant could be due to the presence of some of these phytochemicals (Kuglerova et al., 2011). The stem bark of *W. ugandensis* accumulates higher amounts of the medicinally active phytochemical compounds explaining its preference for medicinal use compared to other plant parts (Grieb et al., 2011). Unfortunately, the stripping off of the tree bark injures or kills the tree (Botha et al., 2004). And with the increasing demand in the global market, the wild population of *W. ugandensis* has been greatly exploited raising concerns regarding conservation and the sustainability of utilization (WHO, 2002).

The nature of bioactive compounds in medicinal plants and their activities are influenced by genetic and environmental factors including geographical locations (Ullah et al., 2012; Soureshjan and Heidari, 2014; Arya et al., 2010; Muchugi et al., 2012). Abuto et al. (2016) observed that the antimicrobial activities varied among *W. ugandensis* populations across the Kenya rift valley due to possibly genetic diversity. This therefore, implies that the medicinal efficacy of the same species of plants from different populations may vary. The fruit is inedible, all plant parts have a hot peppery taste, leaves and seeds are used to add flavour to curries (Dharani, 2011). The wood of *W. ugandensis* is resistant to insect attack and very strong hardwood (Maundu and Tengnas, 2005). The wood produces fragrance that persists over 4 years of storage. The tree also produces resin that is used locally in Uganda as glue to fix tool handles (Orwa, 2009).

## Botany and distribution of *W. ugandensis*

*W. ugandensis* is an evergreen plant with a spreading rounded crown (Figure 1a). It grows to about 4.5 to 40 m in height and diameter of about 70 cm at breast height (Figure 1a) with a smooth or scaly, pale green or brown stem bark (Orwa, 2009) (Figure 1b); leaves are simple, alternate, stalked, glossy, short, with a presence of dotted glands (Figure 1d); flowers are solitary or in small 3-4 flowered cymes, axillary, regular and bisexual (Maundu and Tengnas, 2005). The fruit is a berry, at first green and ellipsoidal, later sub-spherical and turning purplish on maturation (Figure 1c); seeds are compressed and more or less cordate, yellow-brown in colour (Beentje et al., 1994). In fact, *W. ugandensis* is a hermaphroditic plant. Ecologically, *W. ugandensis* occurs in lowland rainforests, upland dry evergreen forests, secondary bush and grasslands and on termitaria in swamp forests (Maundu and Tengnas, 2005; Orwa, 2009). *W. ugandensis* and other related species are native to Northeast Tropical Africa: Ethiopia; East Tropical Africa: Uganda, Tanzania and Kenya; Central Africa: Democratic republic of Congo and South Tropical Africa: Malawi, South Africa and Swaziland (Orwa, 2009) (Figure 2).

## Traditional medicinal uses of *W. ugandensis*

*W. ugandensis* has gained a lot of popularity due to the high demand for the medicinal extracts from its bark, roots and leaves for use by traditional healers (Wamalwa et al., 2006; Olila et al., 2001). *W. ugandensis* is one of the priority species for herbal medicine in Kenya (Hamilton, 2008). Herbal medicine extracted from bark, roots, young twigs, leaves and fruits are used by the traditional healers (Maundu and Tengnas, 2005). *W. ugandensis* is actually considered an integral part of African traditional medicine by very many herbalists and traditional healers across the continent. All plant parts of *W. ugandensis* are used by herbalists and traditional healers to treat a wide range of diseases. Most herbalists/traditional healers use the bark of the herbal plant for various herbal formulations but the leaves and roots are as well highly medicinal. Various plant parts are used to cure or alleviate ailments such as stomachache, malaria, toothache, erectile dysfunction, measles, candidiasis, weak joints among others (Table 1). The mode of common use is by chewing and swallowing the juice from the bark of *W. ugandensis* (Kubo et al., 1976); powdered form is also known to be very effective in the treatment of the same diseases (Githinji et al., 2010). The skills and knowledge about the traditional herbal medicines are normally passed on orally from generation to generation (Sindiga, 1994). The knowledge passed on include what each herbal plant treats, and how the plant part is harvested, which plant part to use and the mode of its administration.

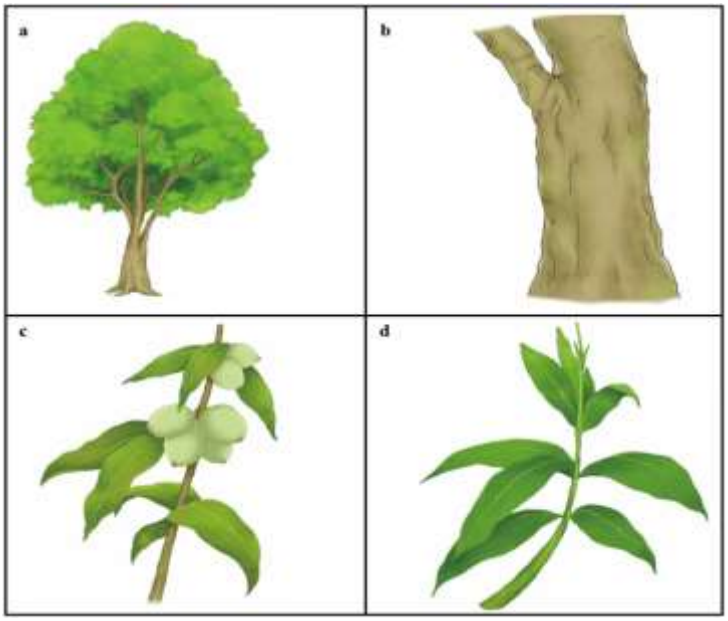


**Figure 1.** Morphology illustration of the main features of *W. ugandensis*: **a)** Whole plant of *W. ugandensis;* **b)** Stems of *W. ugandensis;* **c)** Fruits of *W. ugandensis;* **d)** Leaves of *W. ugandensis*. Source: Natural Chemotherapeutics Research Institute, Uganda.

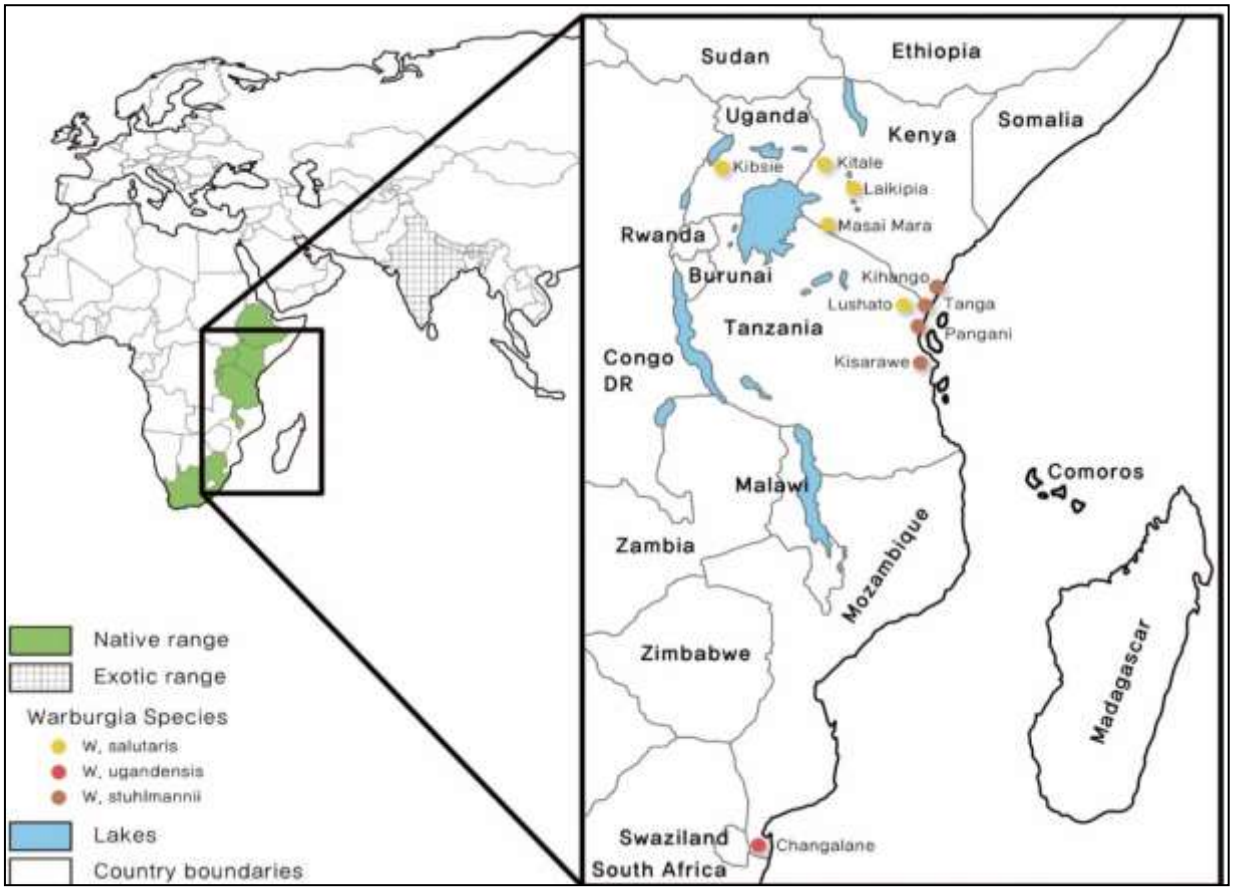


**Figure 2.** The distribution range of *W. ugandensis* and other related species (Muchugi et al., 2008).

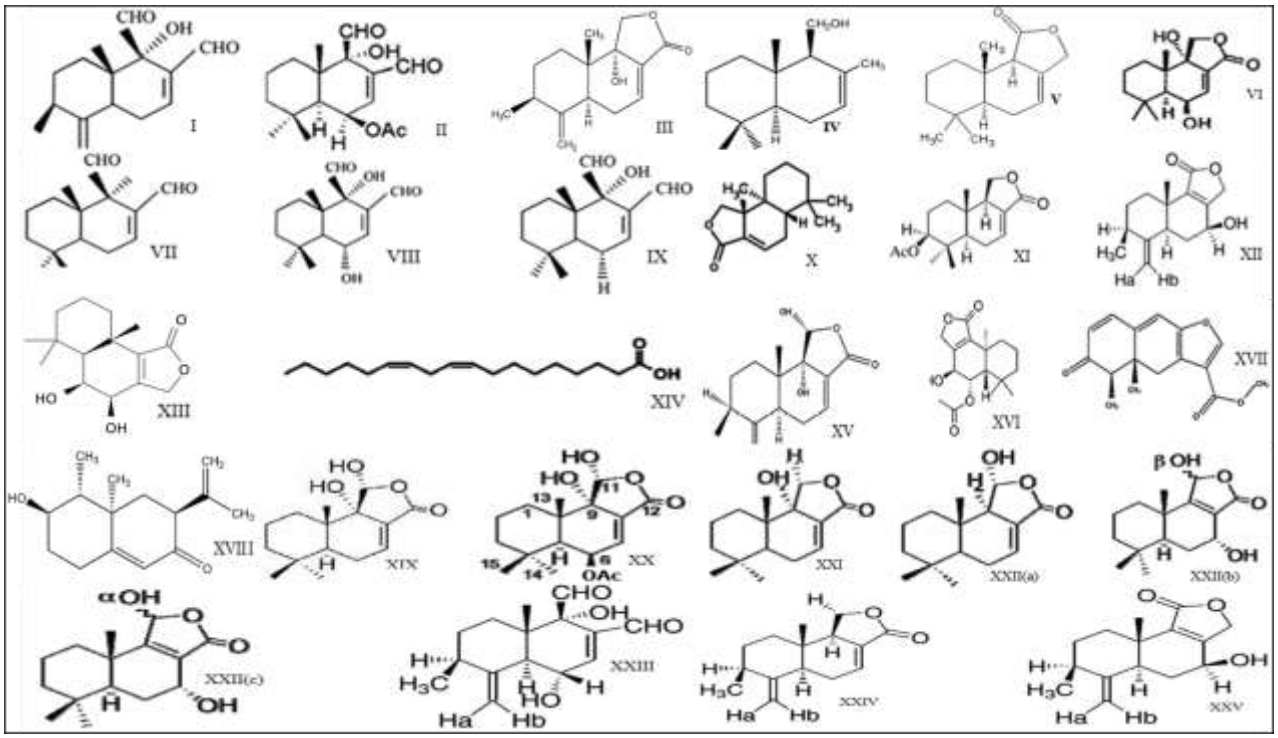

**Figure 3.** Molecular structures of compounds (I-XXV) in W. Ugandensis (Victor, 2013).

## Toxicity of *W. ugandensis*

Acute toxicity tests for extracts of *W. ugandensis* showed that the plant is not toxic at acute exposure to test organisms (Ahmad et al., 2017; Karani, 2013). Administration of single doses of varied concentrations of *W. ugandensis* to mice, no mortality was observed even at highest concentrations tested. *W. ugandensis* was classified as relatively harmless based on the scale of Loomis and Hayes (1976) with $LD_{50}$>5000 mg/kg body weight (Karani, 2013). In another study, *W. ugandensis* was showed to be non-toxic to *Drosophila melanogaster* at acute exposure but toxic at chronic exposure (Ahmad et al., 2017). *W. ugandensis* as well has been used as a medicinal plant whose bark extracts, leaves and young shoots have been used for ages with no adverse effects (Henke, 1994; Kokwaro, 1976; Mbuya et al., 1994). *W. ugandensis* stem bark extracts have been established to be non-toxic to BALB/c macrophages (Githinji et al., 2010). Consequently, it can be concluded that the use of *W. ugandensis* as a medicinal plant is indeed safe although further studies especially clinical studies on its safety still need to be conducted.

## Phytochemistry of *W. ugandensis*

The high therapeutic values of *W. ugandensis* is attributed to the abundance of phytochemical compounds in it including drimane sesquiterpenes (Haraguchi, 1998; Frum et al., 2005; Frum and Viljoen, 2006; Jansen and De Groot, 1991; Kioy, 1990) and tannins and mannitol (Van Wyk and Gericke, 2000). Studies on this plant have indicated the presence of the following compounds (Figure 3) muzigadial (I), ugandensidial (II), muzigadiolide (III) (Mashimbye, 1999) drimenol (IV), drimenin (V) (Mohanlall and Odhav, 2009), pereniporin B (VI) and polygodial (VII) (Taniguchi and Kubo, 1993), mukaadial (VIII), warburganal (IX), cinnamolide (X), cinnamolide-3$\beta$-acetate (XI), deacetylugandensolide (XIII), 7α-hydroxy-8-drimen-11,12-olide (XII), linoleic acid (XIV), and 11α-hydroxy muzigadiolide (XV) in the stem bark (Kioy, 1990) and Ugandensolide (XVI), warburgin (XVII), warburgiadione (XVIII) (Mohanlall and Odhav, 2009). In addition, sesquiterpenes such as ugandenial A (XIX) (Xu et al., 2009), 9α-11α-dihydroxy,6ß-acetyl-cinnamolide (XX), 9α-hydroxycinnamolide (XXI) and dendocarbins A (XXII) (a), L (b) and M (c) were present (Wube et al., 2005). Coloratane sesquiterpenes 6α,9α-dihydroxy-4(13),7-coloratadien-11,12-dial (XXIII), 4(13),7-coloratadien-12,11-olide (XXIV), and 7b-hydroxy-4(13),8-coloratadien-11,12-olide (XXV) are present in stem bark of *W. ugandensis* (Wube et al., 2005) Monoterpenes (Kioy, 1990) and flavonol glycosides in the leaves (Manguro et al., 2003) (Figure 3).

## Antimicrobial activities

Many pharmacological studies have confirmed

**Table 1.** Traditional medicinal uses of *W. ugandensis.*

| Disease/Ailment | Plant part | Mode of preparation/administration | References |
|---|---|---|---|
| Cough | Bark | Dried chewed and swallowed. | (Kiringe, 2006; Kokwaro, 2009) |
| Constipation | Bark | Dried chewed and swallowed. | (Kokwaro, 2009) |
| Chest pains | Bark | Dried chewed and swallowed. | (Kiringe, 2006; Wamalwa et al., 2006) |
| Diarrhea | Bark, roots | Dried chewed and swallowed; fresh roots boiled and mixed with soup. | (Lovett et al., 2006) |
| Malaria | Bark, leaves, roots | Boiled in water and decoction drunk | (Lovett et al., 2006; Kokwaro, 2009; Wamalwa et al., 2006) |
| Fever | Bark | Dried chewed and swallowed. | (Kokwaro, 2009;Lovett et al., 2006) |
| Stomachache | Bark | Dried chewed and swallowed. | (Henke, 1994; Kiringe, 2006; Kokwaro, 2009) |
| Toothache | Bark | Dried chewed and swallowed, dried bark powder can be applied | (Kokwaro, 2009; Lovett et al., 2006) |
| Hernia | Bark | Dried chewed and swallowed. | (Lovett et al., 2006) |
| General body pains | Bark | Dried chewed and swallowed. | (Kokwaro, 2009; Lovett et al., 2006) |
| Fatigue | Bark | Dried chewed and swallowed. | (Lovett et al., 2006) |
| HIV-related opportunistic Infections | Bark | Dried chewed and swallowed. | (Mbwambo et al., 2009) |
| Loss of appetite | Bark | Dried chewed and swallowed. | (Kiringe, 2006) |
| Measles | Bark | Not specified | (Olila, 1993) |
| Muscle pains | Bark | Dried chewed and swallowed. | (Kokwaro, 2009) |
| Skin diseases | Leaves | Bath decoctions | (Wamalwa et al., 2006) |
| Common cold | Bark | Not specified | (Lovett et al., 2006) |
| Sinuses | Bark | Dried and ground to a snuff | |
| Throat infections | Bark | Bark decoction or bark mixed with animal fat | (Kiringe, 2006) |
| Tuberculosis | Bark | Not specified Ethiopia | (Wube et al., 2005) |
| Ulcers | Bark | Dried chewed and swallowed. | (Kiringe, 2006) |
| Weak joints | Bark | Weak joints | (Kokwaro, 2009) |
| Chest pain/Complaints | Bark | Dried chewed and swallowed Or Smoke from burning bark inhaled | (Kiringe, 2006; Wamalwa et al., 2006) |
| Yellow fever | Bark | Not specified | (Kuglerova et al., 2011) |
| Visceral leishmaniasis | Bark | Bark boiled in water/soup and taken orally | (Ngure et al., 2009) |
| Emetic | Bark, leaves | Hot decoction of bark or leaves | (Nanyingi, 2008) |
| Lung problems | Bark | Bark decoction | (Kiringe, 2006) |
| Snake bite | Bark | Not specified | (Olila, 1993) |
| Intestinal Worms | Bark | Not specified | (Lovett et al., 2006) |

antimicrobial activities of *W. ugandensis* extracts (Were et al., 2015; Kuglerova et al., 2011; Ahmed et al., 2013). The curative efficacy of extracts of *W. ugandensis* is attributed to the potent antifungal and antibacterial activities of the synergistic effects of the different bioactive phytochemical compounds in the plant (Olila et al., 2001; Haraguchi, 1998; Jansen and De Groot, 1991; Mashimbye, 1999; Taniguchi and Kubo, 1993). The pattern of antimicrobial inhibition of the various extracts of *W. ugandensis* varied with the solvents used for extraction and the part of the plant used as well as the regions where the samples were collected. It was observed that the DCM solvent extracts exhibited the highest

antimicrobial activity than the MeOH solvent extracts regardless of the plant part analyzed.

**Antibacterial and antimycobacterial activities**

*W. ugandensis* showed antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* in the agar well diffusion assay (Olila et al., 2001). This effect, however, was not demonstrable in the paper disc assay. Kuglerova et al. (2011) also showed that *W. ugandensis* stem bark exhibited antibacterial activity with a minimum inhibitory concentration (MIC) of 256 µg/ml against *S. aureus* and 512 µg/ml against *Enterococcus faecalis*. Similarly in Kitale and Rumuruti in Kenya, dichloromethane stem bark extracts of *W. ugandensis* exhibited inhibitory activity against *S. aureus* and *C. albicans* with mean zones of inhibitions both of 19.75 mm (Abuto et al., 2016). The preliminary bioassay results showed that *S. aureus* and *C. albicans* were the most susceptible microorganisms while *E. coli* was resistant to *W. ugandensis* extracts (Abuto et al., 2016). S*. aureus* was more sensitive than *E. coli* to *W. ugandensis* extracts (agar well assay). Since *S. aureus* is more often associated with secondary bacterial infections in measles than *E. coli* it may explain the value of the plant in measles therapy (Olila et al., 2001). The compound muzigadial has been found to be very active against *S. aureus* (Abuto et al., 2016). Antimycobacterial activity of *W. ugandensis* against *Mycobacterium aurum*, *M. fortuitum*, *M. phlei* and *M. smegmatis* were demonstrated using dichloromethane extract of the stem bark of *W. ugandensis* (Wube et al., 2005). Wube et al. (2005) further showed that the active constituents of *W. ugandensis* gave MIC values ranging from 4 to 128 mg/ml, compared to the antibiotic drugs ethambutol and isoniazid with MIC ranging from 0.5 to 8 mg/ml and 1 to 4 mg/ml respectively. Furthermore, muzigadial and muzigadiolide were found to have antimycobacterial activities against *M. fortuitum* (MIC of 16 µg/ml) and *M. phlei* (MIC of 64 µg/ml) respectively (Wube et al., 2005).

**Antifungal activity**

In a study conducted by Olila et al. (2001), they observed that the ethanolic extracts of the stem bark of *W. ugandensis* showed significant antifungal activity against *Candida albicans*. Similarly, warburganal which is one of the major phytochemicals contained in *W. ugandensis* exhibited a broad-spectrum antifungal activity against yeasts and filamentous fungi especially against *Saccharomyces cerevisiae*, *C. utilis,* and *Sclerotinia libertiana* (Kubo, 1995). Kuglerova et al. (2011) also observed that extracts from stem bark of *W. ugandensis* showed strong antifungal activity with a MIC of 256 µg/ml against *C. albicans* due to the presence of the muzigadial compound in the plant. Furthermore, *W. ugandensis* stem bark and leaves extracts were found to exhibit antifungal activity against *C. utilis* (Mbwambo et al., 2009; Ahmed et al., 2013; Taniguchi et al., 1983).

**Antiplasmodial activity**

*W. ugandensis* stem bark extract has showed potent activity against *Plasmodium knowlesi* and *P. Berghei* (Taniguchi et al., 1978). In fact, in an *in vitro* experiment, the methanol extracts from various parts of *W. ugandensis* exhibited antiplasmodial activity with an $IC_{50}$ value of less than 5 mg/ml against both chloroquine-sensitive and chloroquine resistant strains of *P. falciparum* (Taniguchi et al., 1978). This result sets a basis upon which future antimalarial drugs could be developed and typically explained the continuous use of the plant in traditional medicine for the treatment and management of malaria throughout its distribution range.

**Other activities**

Beside the activities discussed above, the presence of drimane sesquiterpenoids in *W. ugandensis* makes the plant a vital insect pest controlling agent (Olila et al., 2001; Frum et al., 2005; Xu et al., 2009; Nanyingi et al., 2010; Kubo et al., 1977). Similarly, warburganal and muzigadial showed strong inhibitory effects on the feeding of larvae of the monophagous *Spodoptera exempta* and the polyphagous *Spodoptera littoralis* at a concentration of 0.1 ppm in a regular leaf disk method (Nanyingi et al., 2010). *W. ugandensis* also exhibited antifeedant activity against *S. frugiperda*, *Heliothis armigera* and *H. virescens* (Kubo et al., 1977).

Drimane sesquiterpenoids in *W. ugandensis* are known to have cytotoxic activities that is, toxic effects at cellular level (Frum et al., 2005; Taniguchi and Kubo, 1993; Nanyingi et al., 2010; Meinwald et al., 1978). For example, ethanolic leaf extracts of *W. ugandensis* exhibited cytotoxic activity (95% CI), against brine shrimp larvae with reference to cyclophosphamide, a standard anticancer drug (Mbwambo et al., 2009). In an *in vitro* study, *W. ugandensis* bark exhibited potent cytotoxic activity on KB cell line with 99% and 64% inhibition at 10 and 1 mg/ml, respectively (Xu et al., 2009).

The hexane extract of *W. ugandensis* was strongly antileishmanial against *Leishmania major* and *Leishmania donovani* with $IC_{50}$ value of 9.95 and 8.65 respectively (Ngure et al., 2009). *W. ugandensis* demonstrated trypanocidal activity against *Trypanosoma brucei in vitro* (Olila et al., 2001). A cytotoxic sesquiterpene characterized as muzigadial was isolated from *W. ugandensis* and used to treat trypanosomiasis and other parasitic diseases in animals (Meinwald et al., 1978; Kioy, 1990). Rugutt et al. (2006) in Kenya, showed

*W. ugandensis* activity against soil pathogens namely *Fusarium oxysporum*, *Alternaria passiflorae* and *Aspergillus niger.* All these activities clearly justify the continued use of *W. ugandensis* in traditional medicine to treat and manage myriad of diseases and disorders.

## CONCLUSION

In all the communities where *W. ugandensis* occurs and particularly across Africa, it is medicinally highly valued and used by the local populations to treat and manage wide range of ailments and health conditions indicating that the species is a very vital source of ethnomedicine. Besides, there have not been records of its toxicity in populations where its leaves and bark have been used as vital sources of medicine. Several pharmacological studies have been carried out on *W. ugandensis* extracts leading to the identification and isolation of highly medicinal compounds backing up some of its documented traditional use in treatment of disease across Africa. There is no much documentation providing evidence of proprietary medicinal product development from *W. ugandensis* yet it has been proven through many studies of its potent therapeutic values. The need for clinical research on the extracts of *W. ugandensis* could be the research gap needed to make these products attractive for further pharmaceutical product development. Further research is therefore important on *W. ugandensis* to develop products with highly valued medicinal contents. In all or most of the areas, the local people use the raw extracts directly in the treatment and management of various ailments without precise standardized dosage across the discipline. Therefore, further preclinical and clinical studies ought to be done to standardize the use of this plant in treatment and management of a number of disease conditions. It is also important to note that the ever-increasing use of *W. ugandensis* in the treatment and management of various ailments has led to over exploitation hence decreasing its population drastically. Consequently, there is need for advanced and rapid propagation techniques to increase the population and distribution of *W. ugandensis*

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# Antibacterial and antioxidant potential of *Albizia anthelmintica* as a medicinal plant on pathogenic veterinary isolates

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***Albizia anthelmintica*** **is a medicinal plant belonging to the Fabaceae family. It is widely used by smallholder farmers and pastoralists to treat internal parasites in their livestock. This study aimed to determine the antibacterial and antioxidant potential of** ***A. anthelmintica*** **on pathogenic veterinary isolates. 100% hexane (He100), 100% chloroform (Ch100), 100% ethanol (E100), and 70% ethanol (E70) extracts of the roots and barks of** ***A. anthelmintica*** **were tested against four bacterial strains (*Escherichia coli, Clostridium perfringens, Salmonella enterica* serovar Typhimurium and *Proteus mirabilis*). Thin layer chromatography- 2, 2-diphenyl-1- picryl hydrazyl (TLC-DPPH) assay was used to examine antioxidant potential of extracts. Antimicrobial activity was determined using the disc diffusion method and minimum inhibiting concentrations (MICs) values were determined using the micro-titre broth-dilution method. At a concentration of 500 µg/ml, E70 roots extract showed the highest % DPPH inhibition of 66.9%. Among the bark extracts, the highest free radical scavenging activity was observed in E70 extracts with 58.9% DPPH inhibition. Phytochemical analysis of the plant extracts revealed the presence of compounds which are known to exhibit medicinal properties such as tannins, terpenoids, quinones, saponins and fatty acids phenols. E100 bark extracts contained most of these compounds except flavonoids. Only alkaloids were not detected in any of the roots or bark extracts. Ch100 bark extracts showed the highest antimicrobial activity and all bacterial isolates were resistant to the E100 root extracts. Ch100 root extracts showed the lowest minimum inhibition concentration of 0.625 mg/ml against *S. enterica* serovar Typhimurium. Findings of this study show that some of the root and bark extracts of *the A. anthelmintica* plant have both antimicrobial and antioxidant properties. These findings can possibly be relevant in the development of novel medication against veterinary pathogens. Furthermore, this study will guide similar studies.**

**Key words:** Antibacterial, antioxidant, *Albizia anthelmintica,* phytochemical, minimum inhibitory concentrations (MICs).

## INTRODUCTION

Medicinal plants have been used for many decades around the world to prevent or treat infectious and non-infectious diseases. As a result of the resistance of disease causing bacteria to conventional therapy, the use

of medicinal plants has been on an increase (Notka et al., 2004).

Around 80% of the world population relies on alternative medicines from plants with approximately 70,000 plants being utilised (Thomas, 2000). A variety of plant parts such as flowers, roots, stems and fruits have been found useful in drug development because of their medicinal properties (Prior, 2003).

Medicinal plants have great pharmacological importance because they possess bioactive molecules. These molecules are produced by metabolic pathways and genetic structures that are unique to each plant species. It has been proved that the environment can influence the amount of phytochemicals that can be found in each plant species (Thomas, 2000). Phyto-chemicals are therefore being used in the production of novel medicines as an alternative to synthetically produced drugs.

Pastoral farming remains very active in Botswana not only for the sustenance of families but also as a viable source of income through beef exports. Veterinary diseases remain a great threat to livestock production in Botswana. The diseases can adversely impact livestock health, lead to loss of income, lack of food security and cause productivity losses. There are different ways through which veterinary diseases can be managed. These include among others quarantining of diseased animals, breeding control, regulation of entry into farm lots and the development of better antibiotics, diagnostics tools, vaccines and vector control techniques (Sharma, 2016).

As Sharma (2016) explains, diseases cause poor livestock performance and medicines are too expensive and inaccessible to most smallholder farmers. Because of the cultural and ecological diversity of Botswana, there is substantial knowledge on the proper management and utilization of the different medicinal plants in the country to alleviate diseases that affect livestock. Therefore, without modern medicine, traditional medicinal plant extracts will continue to be an important component of smallholder farming in Botswana for the foreseeable future (Sharma, 2016).

Because of the great potential of medicinal plants in the manufacturing of antimicrobial drugs, this study screened the plant species *Albizia anthelmintica* for the antibacterial and antifungal activity of its extracts. *A. anthelmintica* belongs to the kingdom Plantae (Order Fabales; family Fabaceae; subfamily Mimosoideae) (Grade et al., 2008). *Albizia* species have high content of phenolic compounds such as triterpenoids and saponins.

In Africa, *Albizia* plant species are commonly used in the treatment of conditions such as diarrhea, coughs and rheumatism. Plants such as these have proved crucial to the identification of novel agents of therapy (Grade et al., 2008). The study aimed to determine the antibacterial potential of *A. anthelmintica* as a medicinal plant on pathogenic veterinary isolates. Additionally, its potential antioxidant properties were investigated.

## MATERIALS AND METHODS

### Sample collection and extract isolation

The sample site for this study was Sikwane village in Kgatleng district, Botswana. It is located at 55 km to the east of Gaborone. *A. antihelmintica* roots and bark samples were randomly collected and their identity confirmed at the University of Botswana Herbarium (Voucher No: 009). Four bacterial veterinary pathogenic strains identified as *Escherichia coli, Clostridium perfringens, Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *Proteus mirabilis* were collected from the Department of Biological Sciences, University of Botswana, Gaborone. The bacterial strains were previously isolated from diseases cattle and goats in Botswana. Cultures of these bacteria were maintained in Mueller-Hinton nutrient agar slants at 28°C. Roots and bark samples were washed, dried and blender ground into powder. The powder was then soaked in 100% hexane, 100% chloroform, 100% ethanol and 70% ethanol. The solvents were then rota evaporated to retain crude extract.

### Determination and measurement of antioxidant activity of extracts

Free radical scavenging activity of plant extracts was determined using the semi quantitative TLC- DPPH method. Briefly, 1 mg/1000 µl methanol solutions for each extract were prepared. To determine the scavenging capacity of extracts for the free radical 2, 2-diphenyl-1-picryl hydrazyl (DPPH), thin layer chromatography was used as described by Yeboah and Majinda (2004). Methanol was used as a control. The following equation was used to plot a bar graph of inhibition concentration at 500 µg/ml.

$$\%DPPH\ radical\ scavenging\ activity = 1 - [Asample \setminus Acontrol] \times 100$$

*$A_{sample}$ and $A_{control}$ are absorbances of extract samples and the control, respectively.

### Phytochemical screening and antimicrobial activity determination

Phytochemical screening was performed according to the methods described by Mazimba et al. (2006). Extracts were screened for the presence of the following: flavanoids, tannins, saponins, alkaloids, terpenoids, quinones and fatty acids phenols. Antimicrobial activities of *A. anthelmintica* extracts (100% hexane, 100% chloroform 100% ethanol and 70% ethanol) were determined using a modified Kirby-Bauer disc diffusion method (Bauer et al., 1966). Antimicrobial assays were performed in duplicates and discs impregnated with 100 µl Trimethoprim and 100 µl of distilled water were used as positive and negative controls, respectively. Results were interpreted as follows: Sensitive (S), Intermediary Resist

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(IR) and Resistant (R) which is in accordance with the standard measurement of inhibitory zones in millimetre (mm).

#### Minimum inhibition concentrations (MIC) determination

MIC values were determined using the micro-titre broth-dilution method. Muller Hinton broth was used as the primary medium for the tube dilution to determine the MIC for each microorganism as described in Wikler (2008). MIC values of *A. anthelmintica* extracts were determined using two-fold broth micro-dilution to prepare extract concentrations of 80 and 40 mg/ml. Trimethoprim and Muller-Hinton broth containing bacterial isolates were used as positive and negative controls, respectively.

#### Statistical analysis

The data of various analyses were expressed as mean ± standard deviation. All tests were carried out in triplicate to improve accuracy. Data was analysed using one way analysis of variance (ANOVA) followed by Dunnet's test. In the experiments, $P<0.05$ was taken to be significant.

## RESULTS

Plant extract concentrations (15.6 to 1000 µg/ml) in different solvents were observed on the TLC sheet (Figures 1 and 2). These concentrations showed moderate activities, indicated by the faint yellow colouration over the purple DPPH background in comparison with the bold colouration of the gallic acid standard. In the bark extracts, the highest activities were observed in the 100% chloroform extracts, 70% ethanol extracts and 100% ethanol extracts while the 100% hexane extracts showed little activity (Figure 1). Similar trends were observed in roots extracts (Figure 2).

Percentage inhibitions of bark and roots extracts were determined at 500 µg/ml concentrations (Figure 3). From the highest inhibition to the lowest inhibition, the observed inhibition percentages for the different extracts were as follows: 70% ethanol roots (66.9%); 70% ethanol barks (58.9%); ethanol 100% roots (49.2%); chloroform barks (49%); hexane bark (25.5%); ethanol 100% barks (14.6%); chloroform roots (7.3%) and hexane roots (4.9%). The standard (gallic acid) displayed 86.7% DPPH inhibition at a dose of 500 µg/ml

Phytochemical screening of all extracts was performed. Both 100% chloroform bark and roots extracts contained only one type of compound (terpenoids). Ethanol (100%) bark extracts contained all phenolic compounds except flavonoids (Table 1). Among all the compounds, terpenoids are the only ones present in all extracts. Chloroform barks showed the greatest antimicrobial activity of all the extracts. Barks of 100% ethanol extracts and 70% ethanol extracts showed the least antimicrobial activities (Table 2). All bacterial isolates were resistant to 100% ethanol roots extracts. Therefore, 100% ethanol roots displayed minimal antimicrobial activity.

MIC values determination showed a 0.625 mg/ml for chloroform roots against *S.* Typhimurium. The highest recorded MIC value was 5 mg/ml for both chloroform barks and hexane barks against *P. mirabilis* and *C. perfringens*, respectively (Table 3). 100% hexane barks recorded an MIC value of 2.5 mg/ml against *E. coli*.

## DISCUSSION

All the extracts of *A. anthelmintica* in this study showed antioxidant properties. Any substance that scavenges free radicals qualifies as an antioxidant. DPPH is a proton free radical. Scavenging of proton free radicals is a common mechanism of eliminating oxidants from biological systems (Krystyna and Anna, 2013). In this study, different *A. anthelmintica* extracts scavenged DPPH radicals to varying extents. Dasgupta and De (2007) has shown that plant extracts are mixtures of different scavenging compounds that operate synergistically to counter radical activity. The success of these extracts in suppressing DPPH radicals depends on their ability to donate a hydrogen or electron atom to react with DPPH radical. There were variations in the scavenging activities of the same extracts in different solvents. These variations could be due to the unequal distribution of the antioxidant molecules in the different parts of the plant (Krystyna and Anna, 2013).

Phytochemical analysis of the plant extracts showed that some of their constituents such as tannins, flavonoids, saponins and terpenoids may have medicinal potential. Audu et al. (2007) explain that phenolic compounds are amongst the largest and most abundant plant metabolites with a variety of biological properties such as antimicrobial, anti-apoptosis, anti-aging, anti-carcinogen and anti-inflammation. The study revealed the presence of fatty acids phenols in 4 of the 8 *A. anthelmintica* roots and bark extracts. Several studies, such as Himesh et al. (2011), have shown that most medicinal plants have phenolic compounds and natural antioxidants such as flavonoid, phenolic acids and tocopherols.

The present study revealed the presence of tannins in all ethanol roots extracts except 100% ethanol roots extracts. Zhao et al. (2010) has shown that tannins operate by binding to proline rich proteins to inhibit the synthesis of proteins. In this study, flavonoids were only detected in the 100% hexane bark extracts. Plants synthesize flavonoids to respond to infections by microbes and *in vitro,* they have been used effectively against different microorganisms (Parekh et al., 2006). Therefore, production of flavonoids is reactionary rather than routine which might explain their scarcity in most of the extract samples. The activity of flavonoids is dependent on their ability to form complexes with extracellular and soluble proteins as well as bacterial cell walls (Parekh et al., 2006).

Saponins were present in all ethanol extracts irrespective of the concentration of the solvent or the nature of the plant extract. They were however not present in any other solvent. Saponins do have the

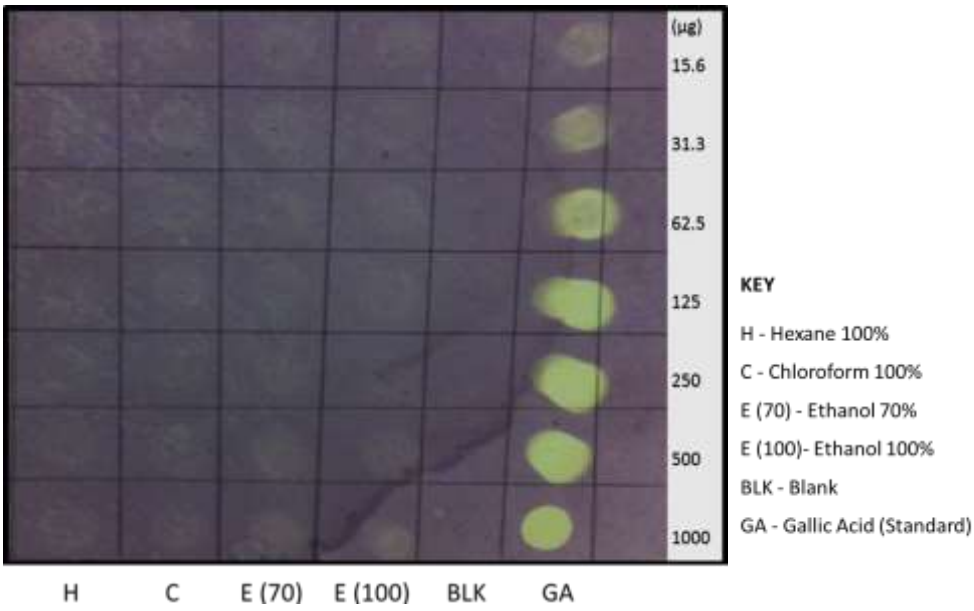


**Figure 1.** TLC-DPPH assay showing antioxidant activities of different concentrations of *Albizia anthelmintica* bark extracts spotted on a TLC sheet.

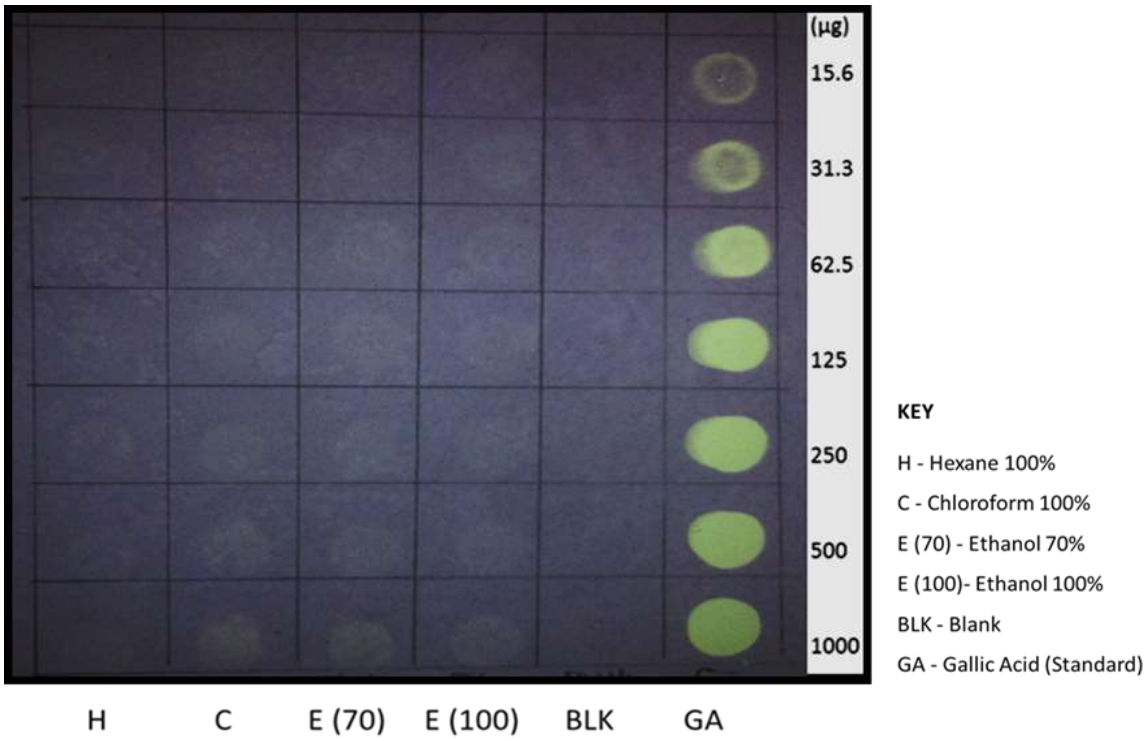


**Figure 2.** TLC-DPPH assay showing antioxidant activities of different concentrations of *Albizia anthelmintica* roots extracts spotted on a TLC sheet.

characteristic of foaming in aqueous solutions (Marin et al., 2001). In this study, terpenoids were the only compounds detected in all plant extracts. Croteau (1998) has shown that terpenoids have an important role as plant hormones and as a plant defence mechanism against microbial diseases and insect herbivores. This,

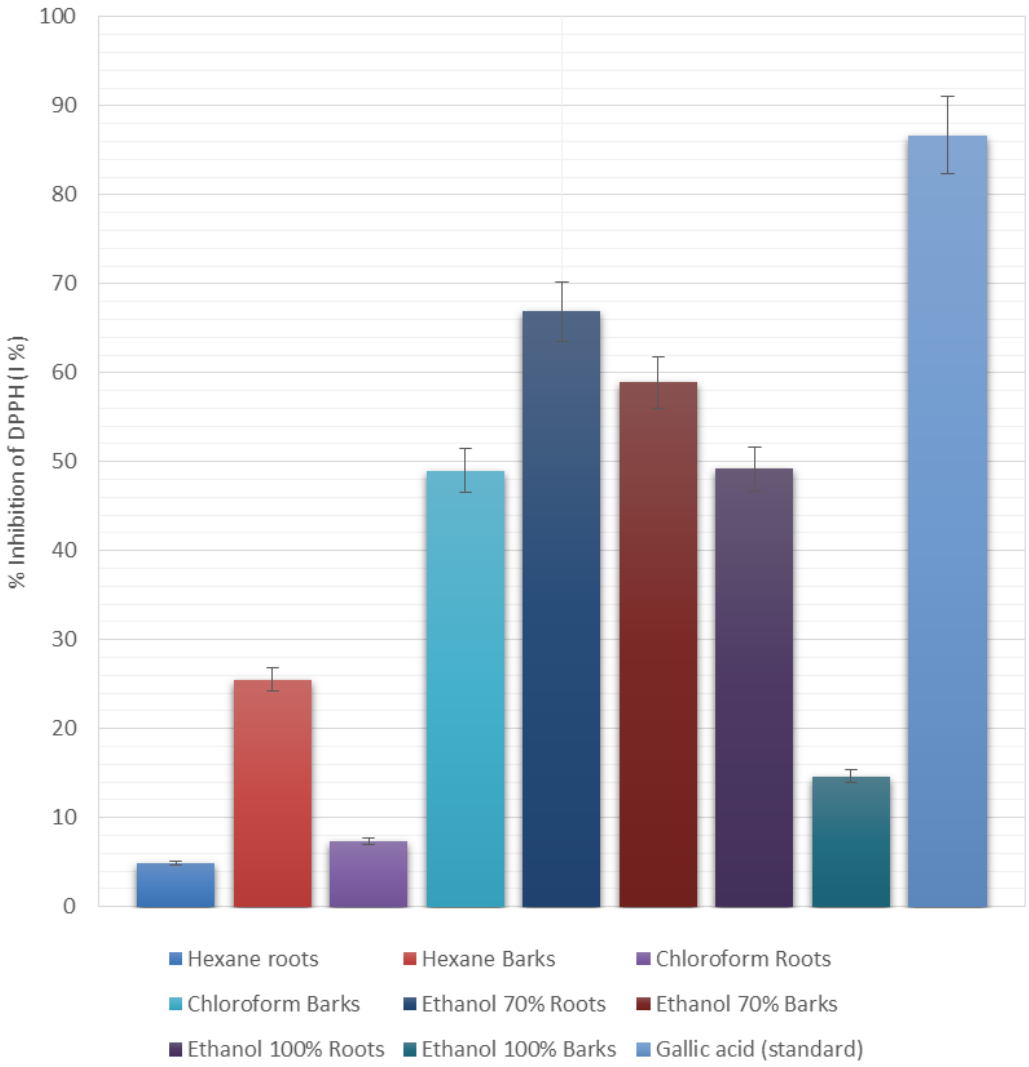


**Figure 3.** Percentage DPPH scavenging activity of 500µg/ml of different *Albizia anthelmintica* extracts.

**Table 1.** Phytochemical screening of *Albizia anthelmintica* extracts.

| Compound | 100% Hexane | | 100% Chloroform | | 70% Ethanol | | 100% Ethanol | |
|---|---|---|---|---|---|---|---|---|
| | Roots | Barks | Roots | Barks | Roots | Barks | Roots | Barks |
| Flavonoids | - | + | - | - | - | - | - | - |
| Tannins | - | - | - | - | + | + | - | + |
| Alkaloids | - | - | - | - | - | - | - | + |
| Terpenoids | + | + | + | + | + | + | + | + |
| Fatty acids phenol | + | - | - | - | + | + | - | + |
| Quinones | - | + | - | - | - | - | + | + |
| Saponins | - | - | - | - | + | + | + | + |

+: Present, -: absent.

therefore, means that they are indispensable to plant systems hence their ubiquitous presence across all extracts. Indeed, terpenoids have been attributed to a wide array of medicinal properties such as anti-

**Table 2.** Inhibitory zones of *Albizia anthelmintica* extracts on four different bacterial isolates.

| Test microorganisms | Plant extracts | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Hexane | | Chloroform | | 100% Ethanol | | 70% Ethanol | |
| | Roots | Barks | Roots | Barks | Roots | Barks | Roots | Barks |
| *Escherichia coli* | S | I | R | I | R | R | R | R |
| *Clostridium perfringens* | S | I | I | S | R | R | I | R |
| *Salmonella enterica* serovar Typhimurium | I | S | S | S | R | I | R | I |
| *Protius mirabillis* | R | I | I | S | R | R | I | R |

S: Sensitive, IR: Intermediary Resistant, R: Resistant.

**Table 3.** Minimum Inhibition Concentrations (MIC) of plant extract against bacterial isolates.

| Plants extracts | MIC (mg/ml) | | | |
|---|---|---|---|---|
| | ***Clostridium perfringens*** | ***Escherichia coli*** | ***Salmonella enterica* serovar Typhimurium** | ***Proteus mirabillis*** |
| Hexane barks | 5 | 2.5 | - | - |
| Chloroform roots | - | - | 0.625 | - |
| Chloroform barks | - | - | - | 5 |

-: Absent.

carcinogenicity, anti-malaria, anti-ulcer, antimicrobial and diuretic activities (Aharoni et al., 2005). *A. anthelmintica* was previously associated with antibacterial activity because of monoterpenes that are present in essential oils made from this plant (Marin et al., 2001) which are effective against pathogenic veterinary isolates (Dasgupta and De, 2007).

In this study, 100% ethanol roots extract inhibited the growth of all bacterial isolates. *C. perfringens* is often involved in diseases in most domestic animals and some wildlife, including, poultry, sheep, goats, cattle, ostriches, dogs and cats (Niilo, 1993). Multidrug resistant *S.* Typhimurium and *E. coli* have been widely reported as causative agents of diarrhoea in cattle and small stock and still are a major cause of productivity and economic loss to cattle producers worldwide (Voetsch et al., 2004; Cho and Yoon, 2014).

*P. mirabilis* is one of the most common bacteria infecting the urinary tract in humans and dogs (Abe et al., 2017). *S.* Typhimurium showed intermediary inhibition and resistance to all ethanol extracts. MIC value of 0.625 mg/ml was observed on chloroform roots against *S.* Typhimurium. These MIC results were in agreement with the phytochemical screening results which indicated that chloroform roots contained only terpenoids.

In conclusion, findings of this study have shown that extracts of the *A. anthelmintica* plant species may be reliable sources of antioxidants and antimicrobials which can be used the development of novel drugs and the treatment of multi drug resistance veterinary pathogens.

Furthermore, this study provides valuable information that can not only guide future studies on medicinal plants but can also be an educational reference for students and scholars of Botswana.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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# *In vitro* antibacterial and antidiarraheic activity of root bark extract of *Anogeissus leiocarpa* (Combretaceae) during an experimental bacterial diarrhea induced by *Escherichia coli* extended-spectrum β-lactamases (ESBL) in albino Wistar rats

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**The present study was conducted to investigate *in vitro* and *in vivo* antibacterial activity of the root bark extracts of *Anogeissus leiocarpa* (DC) Guill. & Perr, in *Escherichia coli* extended-spectrum β-lactamases (ESBL)-induced diarrhea in rat. The antibacterial activity was performed *in vitro* by determining the inhibition zone using standard agar diffusion method as well as *in vivo* on *E. coli* infected Wistar rat model. Both minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were carried through microdilution method. Results obtained in this study indicated that ethanolic and acetatic extracts were only active on bacteria presenting an inhibition zone range from 8 to 16 mm. The MIC observed in agar slant tubes ranged from 6.25 to 50 mg/ml. The ethanolic fraction of *A. leiocarpa* (ETHA) showed the highest *in vitro* antibacterial activity against strains with MICs ranging from 6.25 to 12.5 mg/ml and MBCs ranging from 12.5 to 25 mg/ml. *In vivo*, after infection, diarrhea increased faeces frequency, weight and volume faeces and bacterial faeces load to a maximum on the 2nd day after infection ($P < 0.01$). ETHA normalized the appearance, weight, volume and water content of faeces. To all doses, like ciprofloxacin it reduced significantly ($P < 0.05$) the bacterial growth compared to control Lot (infected and untreated). The death rate in diarrheic control Lot was 50% by Day 14. No death was recorded in Lot treated with ciprofloxacin and Lot treated with ethanolic fraction at dose of 2222.22 mg/kg body weight (bw). This study supports the use of *A. leiocarpa* in the traditional treatment of bacterial infections and offer many perspectives in the search for new molecules against resistant microbial strains.**

**Key words:** *Anogeissus leiocapa*, antibacterial activity, *in vitro*, *in vivo*, therapeutic.

## INTRODUCTION

Diarrheal diseases, the third leading cause of death in the world, after respiratory diseases and AIDS (OMS, 2015) continue to be one of the leading causes of morbidity and mortality. Globally, they constitute the second leading cause of infant mortality in children less than five years (Bhutta et al., 2013), with about 2.5 billion diarrhea

episodes and 2.2 million deaths all years (Bahmani et al., 2015). Of the 2.2 million annual deaths, 37% of cases occur in sub-Saharan Africa (OMS, 2016). In Côte d'Ivoire, diarrhea represents 14% of the reasons for consultation in maternal and child health centers (Kouakou, 2012) and 15% of deaths of children less than 5 years (Liu et al., 2014). The main agents involved are viruses, protozoa and bacteria among which *Escherichia coli* is one of the most important etiologic agents (Asadi et al., 2010).

Given the magnitude of this situation, World Health Organization (WHO) has adopted a treatment based on oral rehydration (OR) and oral rehydration solution (OSR). However, the annual rates of use of OR and OSR, respectively of 0.39 and 1.02% are still very low (Birger et al., 2006). In addition to this program, antibiotics, although used, are becoming increasingly ineffective because of microbial resistance and toxicity problems. They are also still beyond the reach of population grants in developing countries because of their high cost. In this situation, it is necessary and urgent to offer to disadvantaged populations more accessible new therapeutic solutions taking into account their culture and their purchasing power.

To meet this challenge, exploring the rich Ivorian medicinal flora with nearly 800 species of medicinal plants (Aké-Assi, 1991) could offer alternative therapeutic solutions. It is in this context, that the present study focused on *Anogeissus leiocarpa* (DC) Guill. & Perr, a plant of the Ivorian medicinal flora. Phytochemical studies carried out on *A. leiocarpa* root barks revealed the presence of phenolic compounds (flavonoids, tannins, leucoanthocyanins and polyphenols), saponins and sterols (Moronkola and Kunle, 2014; Gbadamosi and Ogunsuyi, 2014). These compounds are known to have antimicrobial activity (Mann et al., 2014; Adamu et al., 2017).

This study focused on the *in vitro* and *in vivo* investigation of the antibacterial potential of Combretaceae, a selected medicinal plant of Côte d'Ivoire flora by using infected rats with *E. coli* extended-spectrum β-lactamases (ESBL).

## METHODOLOGY

### Plant material

The root barks of *A. leiocarpa* were used. These organs were harvested in January 2013 in Kouto (Bagoué region), a town located at 725 km north of Abidjan. This plant has been authenticated by Professor Aké-Assi of the National Floristic Center of Félix Houphouët-Boigny University and compared to the voucher specimen No. CNF 14798.

### Animals

Albinos white rats, male and female of Wistar strain aged 2 to 3 months and weighing between 180 to 200 g were used.

### Microorganisms

Nine bacterial strains involved in gastrointestinal disorders were used: *E. coli* CIP 7624 (ATCC 25922) (reference strain), eight clinical strains isolated from biological products: *E. coli* ESBL 13Y016 (isolated from urine), *Salmonella Typhi* 1586 (isolated from stool), *S. Typhi* 43PI16 (isolated from stool), *Pseudomonas aeruginosa* 131813 (isolated from stool), *Shigella dysenteriae* 1079PI/15 (isolated from stool), *Klebsiella oxytoca* (isolated from urine) and *Staphylococcus aureus* Met-R 1532C/10 (isolated from pus) and *Streptococcus* species. These strains come from the bio-bank of the Institut Pasteur of Côte d'Ivoire.

### Culture media and antimicrobial agents

The culture media used are: Müller-Hinton agar (Liofilchem®, Italia), Eosine blue agar of Methylene (Cultimed®, USA) and Mac Conkey agar (Cultimed®, USA).

### Preparation of the total aqueous extract and organic fractions of *A. leiocarpa*

Total aqueous extract was prepared according to Guede-Guina (1993) and ethyl acetate, dichloromethane and ethanol fractions according to Manga et al. (2013).

### Preparation of the bacterial inoculum

A volume of either 0.01, 0.1 or 1 ml of opalescent pre-culture broth was collected for *Pseudomonas*, enterobacteria and *Staphylococci*, respectively, and then diluted in a tube containing 10 ml of physiological saline to constitute dilution inoculum 10°.

### Preparation of concentration ranges

A concentration range of 500 to 7.81 mg/ml similar to that of Mann et al. (2014) was prepared by the double dilution method in 7 test tubes. These tubes were then sterilized by autoclaving at 121°C for 15 min (Bolou et al., 2011) and stored in a refrigerator at +4°C.

### Preparation of culture media

Culture media were prepared according to manufacturers' instructions (Liofilchem® and Cultimed®).

### Antibacterial sensitivity test

The agar well diffusion method was used (Irshad et al., 2012) for assessing the *in vitro* antibacterial activity of the prepared extract. Cefotaxime (CTX, 30 µg) for enterobacteria and gentamycin (GEN10 µg) for other bacteria served as positive controls. Only concentrations of extracts or fractions with an inhibition zone

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diameter (IZD) greater than 7 mm were reported in results

**Minimum inhibitory concentration (MIC)**

The incorporation of the plant extracts into Muller-Hinton (MH) agar was done using the double dilution method in agar slant tubes as described by Ouattara et al. (2013). Nine experimental tubes whose concentration varies to double dilution from 50 to 0.195 mg/ml and 2 control tubes, the growth control tube (TC) and the sterility control tube (TS) are prepared. The slope of the experimental tubes and that of the TC tube was seeded. The tubes were incubated at 37°C for 24 h. The MIC was the concentration of the first tube from which no microbial visible growth (Khaleel et al., 2016).

***Minimum bactericidal concentration (MBC)***

MBC is the lowest concentration of substance that leaves at most 0.01% of surviving germs. Using a loop calibrated at 2 µl, the contents of the tubes in which no haze was observed were seeded on MH (Box B) in parallel streaks 5 cm in length at the surface, starting with by the MIC tube. After 24 h incubation in an oven at 37°C, the numbers of colonies on the streaks of Box B with those of Box A were compared. In practice, the CMB corresponds to the concentration of the experimental tube whose number of colonies present on the streak is less than or equal to the number of colonies present on the streak of the dilution $10^{-4}$.

***In vivo* antibacterial activity**

***Experimental design***

Forty-two rats of both sexes previously deparasized through oral administration of 10 mg/kg body weight of tetracycline for 3 days (Ricicová et al., 2010) were used. They were divided into 7 Lots of six rats each. Rats from 6 Lots received, orally, 2 ml of the infective dose (Eman et al., 2008) evaluated at $2 \times 10^8$ CFU/ml with a sterile disposable needle-less syringe. The 7th batch is uninfected. Treatment started 48 h after the induction of diarrhea following the appearance of diarrheal faeces. For treatment, the infected rats received orally concentrations of the ethanolic fraction of *A. leocarpa* and ciprofloxacin (the reference antibiotic) according to the prescription of Venkatesan et al. (2005). Thus, daily and for 14 days, Lot 1 (uninfected and untreated) received 1 ml of distilled water; Lot 2 (infected and untreated) received 1 ml of distilled water; Lot 3 (infected and treated) received 2 ml of ETHA at dose of 69.44 mg/kg body weight corresponding to 1 × MIC; Lot 4 (infected and treated) received 2 ml at a dose of 271.87 mg/kg body weight (5 × CMI); Lot 4 (infected and treated) received 2 ml of ETHA at a dose of 631.3 mg/kg body weight (10 × CMI) ; Lot 6 (infected and treated) received 2 ml of ETHA at a dose of 2222.22 mg/kg body weight (40 × CMI) and Lot 7 (infected and treated) received 1 ml of ciprofloxacin at dose of 5 mg/kg body weight w.

***Enumeration of faecal E. coli***

Rats faeces were collected in sterile polyethylene sterile bags of 500 ml (LMR®, France) placed under the cage of the rats, twice daily for 2 weeks. One (1) g of faeces removed with a spatula was transferred to 9 ml of BMH, then vortexed for 10 s and incubated at 37°C for 3 to 4 h to obtain dilution inoculum10°. From this dilution 10°, dilutions ranging from $10^{-1}$ to $10^{-5}$ were prepared. This serial dilution were cultured (0.1 ml) in duplicate on Mac Conkey agar (two plates per dilution). After incubation at 37°C for 18 to 24 h, typical smooth pink color colonies were counted on two successive dilutions. Plates inoculated with a sample dilution that yields between 10 and 300 colonies per plate were read (ISO 7218, 2007). The *E. coli* faeces load (UFC per g of faeces) was calculated according to the formula proposed by ISO 7218 (2007):

$$\textit{E. coli} \text{ faeces load (UFC/g of faeces)} = \frac{\sum C}{V \times 1.1d}$$

where ΣC: sum of colonies counted on the two Petri dishes retained, V: volume of seeded inoculum on each Petri dish, and d: dilution corresponding to the first retained Petri dish, with the least diluted inoculum.

***Evaluation of the water content of faeces***

Fresh faeces was collected after 6 h and weighed individually to determine the wet weight (WW). Faecal samples were dried in a conventional oven at a temperature of 70°C. After 24 h, dried faeces were weighed again for determining their dry weight (DW) (Navarro et al., 2006). The difference between the WW and the DW helped to determine the water content of faeces (Navarro et al., 2006) expressed as a percentage, by the following relation:

$$\text{Water content (\%)} = \frac{WW - DW}{WW} \times 100$$

**Mortality rate and pathological manifestations**

The mortality rate of the rats in the different groups is calculated as the number of dead rats during the experiment compared to the total number of rats used in each Lot (Eman and Hoda, 2008). Faeces were checked continuously for color and consistency, the development of diarrhea in infected Lots, as well as any change in activity and behavior were recorded weekly throughout the experiment.

**Statistical analysis**

All data during this experience were expressed in three replicates. The results are expressed as mean ± standard error of means. They were determined by Dunnett's test using GraphPad Prism 7.0 Statistic Software and were considered significant at *p*-value less than 0.05.

**Ethical approval**

The experiments were conducted according to the ethical guidelines of Ethics Committee of Pasteur Institute of Côte d'Ivoire (Charter of Ethics of the Pasteur Institute, Text of September, 2012).

## RESULTS

### Antimicrobial activity of aqueous extract and organic fractions of *A. leiocarpa*

The effects of total aqueous extract and organic A. *leiocarpa* fractions obtained by agar well diffusion method on bacterial growth are shown in Table 1. The total aqueous extract and the dichloromethane fraction showed no activity on all the bacteria tested with inhibition zones

**Table 1.** Sensitivity of bacterial strains tested to aqueous extract and organic fractions of *Anogeissus leiocarpa*.

| Bacterial strains | Concentration of aqueous extract and organic fractions of *Anogeissus leiocarpa* (mg/ml) | | | | | | | Antibiotics (µg) | |
|---|---|---|---|---|---|---|---|---|---|
| | ETHA | | | EAA | | | EDMA ETAA | | |
| | $C_1$= 500 | $C_2$= 250 | $C_3$= 125 | $C_1$= 500 | $C_2$= 250 | $C_3$= 125 | $C_1$=500, $C_2$=250, $C_3$=125 | CTX (30) | GEN (10) |
| | Diameters of inhibition zone (mm) | | | | | | | | |
| *E. coli* ATCC 25922 | 13.42±1.22 | 10.19±0.50 | 8.46±0.32 | 11.40±0.40 | 9.61±0.88 | <7 | | 30 | ND |
| *E. coli*ESBL | 12.02±0.19 | 9.62±0.52 | ND | 10.71±0.56 | 8.14±0.11 | <7 | | 10 | ND |
| *P. aeruginosa* 131813 | 11.00±0.86 | 9.24±0.49 | 8.12±0.60 | 10.00±0.50 | 8.00±0.23 | <7 | | ND | 21 |
| *S. Typhi* 43PI16 | 15.33±0.88 | 14.00±0.58 | 10.67±0.68 | 14.01±0.84 | 11.30±0.25 | 9.46±0.77 | | 25 | 23 |
| *S. Typhi* 1586 | 14.67±1.33 | 12.33±0.33 | 9.08±0.86 | 15.07±0.61 | 12.37±0.33 | 10.05±0.14 | <7 | 28 | 30 |
| *S. dysenteriae* 1079PI15 | 12.71±0.28 | 10.22±0.33 | 8.03±0.50 | 11.33±0.58 | 9.09±0.47 | 8.43±0.46 | | ND | ND |
| *K. oxytoca* | 13.01±0.84 | 10.22±0.21 | 8.06±0.77 | 10.21±0.79 | 8.33±0.22 | <7 | | ND | ND |
| *S. aureus Meti-R* | 16.00±0.12 | 14.10±0.88 | 11.45±0.18 | 15.00±0.44 | 13.58±1.08 | 11.58±0.86 | | ND | ND |
| *Streptococcussp* | 16.64±0.56 | 13.05±0.83 | 9.14±0.23 | 14.17±0.20 | 12.48±0.31 | 9.69±0.27 | | ND | 15 |

The values were expressed as Mean±SEM. CTX: Cefotaxim; GEN: gentamycin; ESBL: extended spectrum beta-lactamase; Meti-R: meticillin resistant; ND: not determined. ETHA: ethanol fraction of *A. leiocarpa*, ETAA: total aqueous extract of *A. leiocarpa*; EDMA: dichloromethane fraction of *A. leiocarpa*; EAA: ethyl acetate fraction of *A. leiocarpa*.

diameters less than 8 mm.

Both ethanol (ETHA) and ethylacetate (EAA) fractions were active against all infectious bacteria tested with an IZD values ranging from 8 to 16 mm and 8 to 15 mm, respectively. Diameter of zone of inhibition decreased when reducing gradually the extract or fraction concentration excepted for EDMA and ETAA. On a general note ETHA exhibited higher sensitivity than EAA. Only *S. aureus* Meti-R and *Streptococcus* spp. displayed the highest sensitivity with an IZD up to 16.64 at 250 and 500 mg/ml, highest zone of inhibition observed with ETHA compared to EAA. With the exception of *E. coli* ESBL which is the main resistant strain at 125 mg/ml, the IZD of most bacteria tested with ETHA are larger than those of EAA. In addition, EAA at the concentration of 125 mg/ml revealed 3 resistant strains which are *E. coli* ATCC, *E. coli* ESBL and *P. aeruginosa* with an IZD below 8 mm. The inhibition zone of total aqueous extract and dichloromethane fraction at any concentration is below 8 mm. For standard drugs, extreme sensitivity was observed with an IZD that ranged from 10 to 30 mm.

## Antibacterial parameters of the total aqueous extract and organic fractions of *A. leiocarpa*

MIC, MBC and MBC/MIC ration values are shown in Table 2. The MICs values of all strains studied with both ETAA and EDMA extracts were above 50 mg/ml, thus their MBC and MBC/MIC ratio were not determined. The MICs of *A. leiocarpa* ranged from 6.25 to 12.5 mg/ml in ETHA fraction. For EAA fraction, it was 6.25 mg/ml for *S. Typhi* 43PI16 and *S. Typhi* 1586, but was up to 25 mg/ml for *E. Coli* ATCC, ESBL, and *P. aeruginosa* but it was up to 50 mg/ml for *Shigella dysenteriae* and *K. oxytoca*. The MBC/MIC ratio of EAA and ETHA fractions observed were strictly inferior to 4 corroborating the bactericidal effect against all bacteria tested.

## *In vivo* antibacterial activity

### *Infection rate, mortality in rats and faeces appearance*

The antibacterial activity *in vivo* was evaluated by increasing the concentration of ETHA shown in Table 3.

These results indicated antibacterial activity *in vivo* after 7 days of treatment especially from Lot 3 (ETHA 69.44 mg/kg body weight) to Lot 7 (ETHA 2222.22 mg/kg body weight) was correlated to a gradual decrease of mortality rate from 30 to 0%. However, apart from Lot 1 as control and untreated rats in Lot 2 which scored 40 and 10% mortality rate at 7 and 14 days, respectively, there was no mortality rate

**Table 2.** Antibacterial parameters of the total aqueous extract and organic fractions of *A. leiocarpa*.

| Extract | Antibacterial parameters (mg/L) | Gram negative bacteria | | | | | | | Gram positive bacteria | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ***E. coli* ATCC** | ***E. coli* ESBL** | ***S. Typhi* 43PI16** | ***S. Typhi* 1586** | ***P. aeruginosa* 131813** | ***S. dysenteriae* 1079PI15** | ***K. oxytoca*** | ***S. aureus* Meti-R** | ***Streptococcus* spp.** |
| EDMA | MIC | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 |
| | MBC | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 | >50 |
| | MBC/MIC | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| | Effect | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| EAA | MIC | 25 | 25 | 6.25 | 6.25 | 25 | 50 | 50 | 6.25 | 6.25 |
| | MBC | 25 | 50 | 6.25 | 6.25 | 50 | 50 | 50 | 6.25 | 12.5 |
| | MBC/MIC | 1 | 2 | 1 | 1 | 2 | 1 | 1 | 1 | 2 |
| | Effect | Bcid | Bcid | Bcid | Bcid | Bcid | Bcid | Bcid | Bcid | Bcid |
| ETHA | MIC | 12.5 | 12.5 | 6.25 | 6.25 | 12.5 | 12.5 | 12.5 | 3.12 | 3.12 |
| | MCB | 12.5 | 12.5 | 12.5 | 12.5 | 25 | 25 | 25 | 6.25 | 6.25 |
| | MBC/MIC | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| | Effect | Bcid | Bcid | Bcid | Bcid | Bcid | Bcid | Bcid | Bcid | Bcid |
| ETAA | MIC | > 50 | > 50 | > 50 | > 50 | > 50 | > 50 | > 50 | > 50 | > 50 |
| | MBC | > 50 | > 50 | > 50 | > 50 | > 50 | > 50 | > 50 | > 50 | > 50 |
| | MBC/MIC | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| | Effect | ND | ND | ND | ND | ND | ND | ND | ND | ND |

ND: Not determined; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; ETHA: ethanol fraction of *A. leiocarpa*; ETAA: total aqueous extract of *A. leiocarpa*; EDMA: dichloromethane fraction of *A. leiocarpa*; EAA: ethyl acetate fraction of *A. leiocarpa*.Bcid: bactericidal.

observable from Lots 3 to 7 (ciprofloxacin) recorded after 14 days (2 weeks) of treatment. Notably, Lot 6 with 2222.22 mg/kg body weight corresponding to 40 × MIC and the reference drug ciprofloxacin cleared the infection after 7 days of treatment.

### *Effect of ETHA on E. coli enumeration in rats diarrheal faeces*

The effect of ethanolic fraction on bacterial load in diarrheic faeces is as shown in Figure 1. It can be seen that after the 2 days of rats infection, the *E. coli* colony forming units per g of faeces (UFC) counted decrease from Lots 3 to 7 when the concentration of ETHA was increased progressively. Outstandingly, the UFC of Lot 6 is close to that of the Lot 7, the standard drug that drops rapidly to zero after 6 days of treatment but the UFC of Lot 2 remained higher than those with treatment during the 13 days of study.

### *Effect of ETHA on water content*

From the Figure 2 that shows the percent of facael water content, a significant decrease of water lost during the ETHA treatment was observed and it is strongly correlated to the diminishing feacal *E. coli* load in Figure 1. Only the rats treated with ciprofloxacin or cefotaxime showed no statistically significant difference ($p > 0.05$) in water content when compared with Lot 1 which represent not uninfected and untreated rats.

### *Effect of ETHA on the volume of diarrheal faeces*

To gain additional information on the antibacterial activity of ETHA, the effect of ETHA on the

**Table 3**. Infection and mortality rate during experiment.

| Lot | Before infection | | 24 h after infection | | 7 days after treatment | 14 days after treatment | Death rate (%) |
|---|---|---|---|---|---|---|---|
| | IR (%) | MR (%) | IR (%) | MR (%) | MR (%) | MR (%) | |
| Lot 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Lot 2 | 0 | 0 | 100 | 0 | 40 | 10 | 50 |
| Lot 3 | 0 | 0 | 100 | 0 | 30 | 0 | 30 |
| Lot 4 | 0 | 0 | 100 | 0 | 20 | 0 | 20 |
| Lot 5 | 0 | 0 | 100 | 0 | 10 | 0 | 10 |
| Lot 6 | 0 | 0 | 100 | 0 | 0 | 0 | 0 |
| Lot 7 | 0 | 0 | 100 | 0 | 0 | 0 | 0 |

MR: Mortality rate, IR: infection rate; Lot 1: no infected and no treated; Lot 2: infected and untreated; Lot 3: infected and treated with ETHA at dose of 69. 44 mg/kg bw corresponding to 1 × MIC; Lot 4: infected and treated with ETHA at dose of 271. 87 mg/kg bw corresponding to 5 × MIC; Lot 5: infected and treated with ETHA at dose of 631. 3 mg/kg bw corresponding to 10 × MIC; Lot 6: infected and treated with ETHA at dose of 2222.22 mg/kg bw corresponding to 40 × MIC; Lot 7: infected and treated with ciprofloxacin at dose of 5 mg/kg bw.

volume of diarrheic faeces investigation was perfomed on both volume and weight of the rats' faeces (Figures 3 and 4). There was a significant difference ($p < 0.01$) on both volume and weight of diarrheic faeces. Also, there was no significant ($p > 0.05$) decrease in infected and treated rats from 69.99 to 2222.22 mg/kg body weight when compared with uninfected and untreated rats. Notably, it is observed that the weight and volume estimated for the Lot 6 is almost similar to that of the drug control (Lot 7).

## DISCUSSION

The purpose of this study was to summarize the investigation of the antimicrobial properties of both the aqueous extract and organic fractions (ethyl acetate, dichloromethane and ethanol) of *A. leiocarpa* using antibacterial activity *in vitro* and *in vivo,* respectively.

This work made clear the advantages of the combination of these two experiments to obtain interesting information not only to gain insights into sensitivity or inhibitory measurement but also to obtain real time data from the direct effect of the fraction test on selected laboratory animal (the Wistar albino rats).

The results showed that microorganisms tested were susceptible to plant extracts and the highest inhibitory activity was observed for ethanol and ethyl acetate fractions which presented antibacterial activity against all bacteria examined.

These observations were consistent with previous study realized by Biyiti et al. (2004) who indicated that by a diffusion method, bacteria were sensible for IZD of 9 to 14 mm, very sensitive for IZD of 15 to 19 mm, highly sensitive for IZD larger than 20 mm whereas not sensible for IZD less than 8 mm, which is in accordance with the present results. Strains of *E. coli* ATCC 25922, *E. coli* ESBL, *P. aeruginosa*

131813, *S. Typhi* 43PI16, *S. Typhi* 1586, *S. dysenteriae* 1079PI15 and *K. oxytoca* were therefore sensitive to ethanolic fraction of *A. leiocarpa*. *S. aureus* Meti-R 1532C/10 and *Streptococcus* spp. were very sensitive to the ethanolic and ethyl acetate fractions of *A. leiocarpa* according to their inhibition diameters (14.57 ± 0.20 to 16.64 ± 0.56 mm). Similar results were obtained by Ichor et al. (2011) and Mann (2012), who obtained diameters of inhibition with strains of *S. Typhi*, *E. coli*, *Shigella* spp. and *P. aeruginosa*, ranging from 9 to 17 mm in the presence of the methanolic leaves extract of *A. leiocarpa*. In addition, Mann et al. (2008) showed that the ethanolic leaves extract of *A. leiocarpa* inhibit the *in vitro* growth of strains of *Pseudomonas* MDR, *S. aureus* Meti-R and *E. coli*.

MICs determined using the broth dilution method were correlated to those obtained by the diffusion method. The MIC value gave a measure of the antibacterial performance of antibiotics but it appeared that the MICs values of plant extracts and essential oils were not standardized. Also, it was important to note that there were no consensus on the inhibition concentration for natural products, hence the consistency of the antimicrobial activity results are based on the growth inhibition zones observed and the ratio of MBC/MIC estimated. The results of this study showed that, ethanolic fraction (ETHA) was the most active fraction which displayed the lowest MICs values, ranging from 3.12 to 12.5 mg/ml, while the ethyl acetate fractions (EAA) gave the

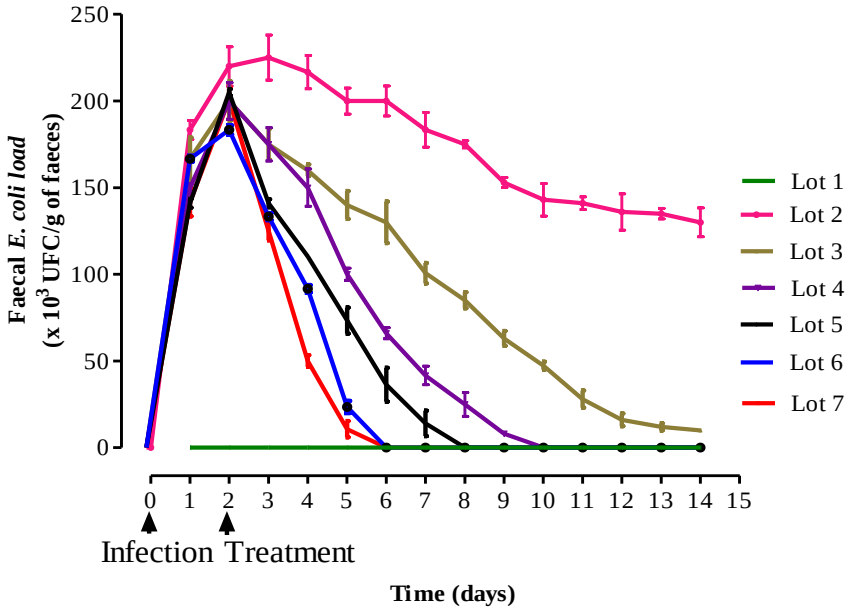


**Figure 1.** Evolution of faecal *Escherichia coli* ESBL load during experiment.

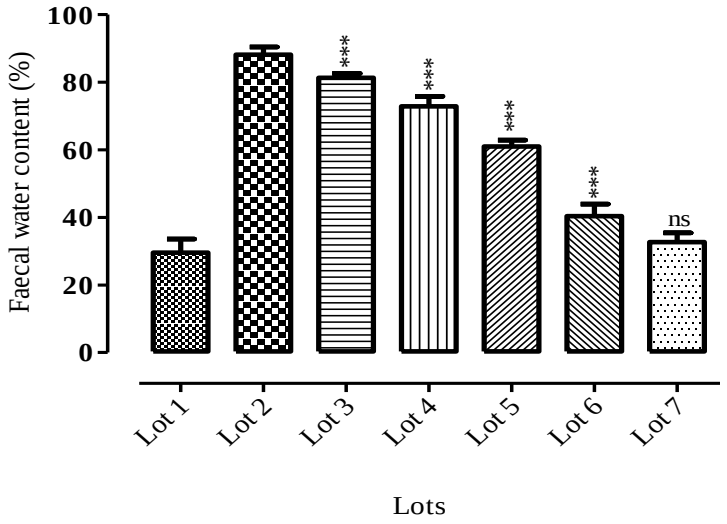


**Figure 2.** Variation of faecal water content during experiment. The values were expressed as Mean±SEM (n = 6 rats/Lot). ***p<0.001: significant difference compared to the infected and untreated Lot, ns: no significant when compared with the uninfected and untreated Lot at p<0.05. Lot 1: No infected and no treated, Lot 2: infected and untreated, Lot 3: infected and treated with ETHA at dose of 69,44 mg/kg bw corresponding to 1 × MIC, Lot 4: infected and treated with ETHA at dose of 271,87 mg/kg bw corresponding to 5 × MIC, Lot 5: infected and treated with ETHA at dose of 631,3 mg/kg bw corresponding to 10 × MIC, Lot 6: infected and treated with ETHA at dose of 2222.22 mg/kg bw corresponding to 40 × MIC, Lot 7: infected and treated with ciprofloxacin at dose of 5 mg/kg bw.

highest MICs that ranged from 6.25 to 25 mg/ml apart from *S. dysenteria* and *K. oxytoca* but no antibacterial activity were recorded with ETAA and EDMA. These results were similar to those of Timothy et al. (2015) and

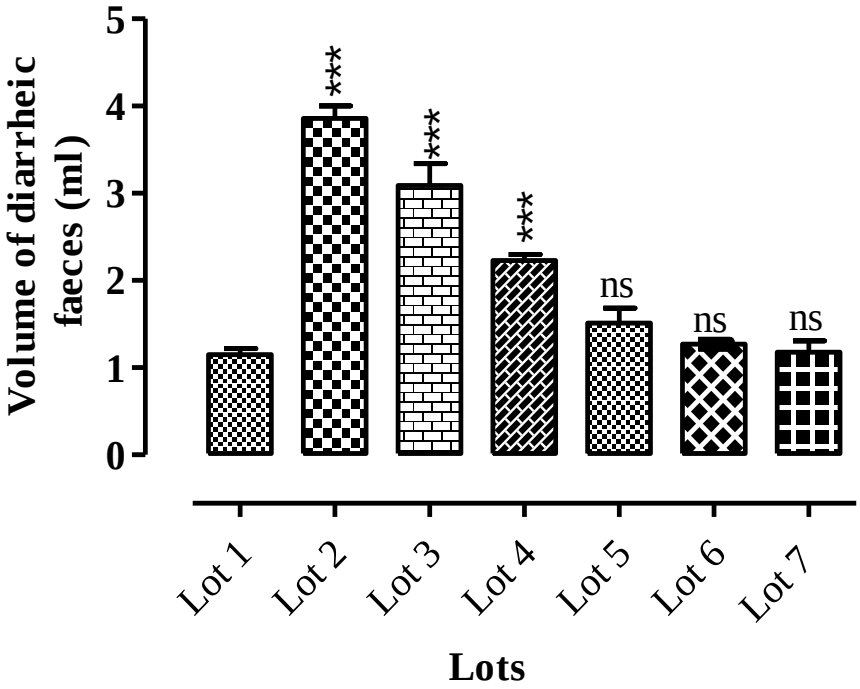


**Figure 3.** Volume of faeces of rats treated and untreated during experiment. Values are means±SEM. Each Lot includes 6 animals (n=6/Lot). ***p<0.001: significant difference compared to the uninfected and untreated Lot, ns: no significant at p<0.05 when compared with the uninfected and untreated Lot (Lot1). Lot 1: no infected and no treated, Lot 2: infected and untreated, Lot 3: infected and treated with ETHA at dose of 69.44 mg/kg bw corresponding to 1 × MIC, Lot 4: infected and treated with ETHA at dose of 271.87 mg/kg bw corresponding to 5 × MIC, Lot 5: infected and treated with ETHA at dose of 631.3 mg/kg bw corresponding to 10 × MIC, Lot 6: infected and treated with ETHA at dose of 2222.22 mg/kg bw corresponding to 40 × MIC, Lot 7: infected and treated with ciprofloxacin at dose of 5 mg/kg bw.

Ali et al. (2017) with MICs ranging from 6.3 to 44.6 mg/ml and 5 to 20 mg/ml, respectively with ethanolic and ethyl acetatefraction extract of *A. leiocarpa* barks. Although antibacterial activity was detected in both EAA and ETHA fractions against almost tested bacteria strains, the MBC values (minimum bactericidal concentration) also showed a similar pattern of activity from that of MIC. The extract action was bactericidal when the ratio of the MBC/MIC is <4 and bacterioatatic for MBC/MIC >4 (Berché et al., 1991).

Based on results scored, the EAA and ETHA extracts on sensitive bacteria were especially bactericidal against both Gram positive and Gram negative bacteria. This result could be explained by the fact that most antimicrobial active components were less polar compounds that were not water soluble and so the organic solvent extracts showed a more potent activity. Contrary to the use of dichloromethane which was an organic solvent, no bacterial activity has also been observed. That was in accordance with previous work of Mabiki et al. (2013) that showed for the most of part of terpenes/terpenoids compounds in dichloromethane extract. It was known that because of this chemical diversity, terpenes/terpenoids have great industrial uses as flavors, fragrances (Schwab et al., 2008), high grade lubricants, biofuels, agricultural chemicals and in the near future will play a more significant role in medicines (Niehaus et al., 2011).

On the whole, Gram-positive bacteria (*S. aureus* Meti-R 1532C/10 and *Streptococcus* spp.) were more sensitive than Gram-negative bacteria. Bari et al. (2010) corroborated the high resistance of some Gram-negative bacteria compared to Gram-positive bacteria. In fact, Gram-negative staining bacteria had efflux pumps that prevented the intracellular accumulation of antibacterial agents (Demetrio et al., 2015). It was noted that ethanolic fraction of *A. leiocarpa* demonstrated *in vitro* the most active antibacterial activity during this study. In order to confirm this interesting observation, this *in vivo* therapeutic activity was evaluated using an experimental bacterial diarrhea induced by the *E. coli* ESBL, a strain resistant to β-lactams.

Diarrhea could be measured by several parameters such as the water content, weight, volume, bacterial load

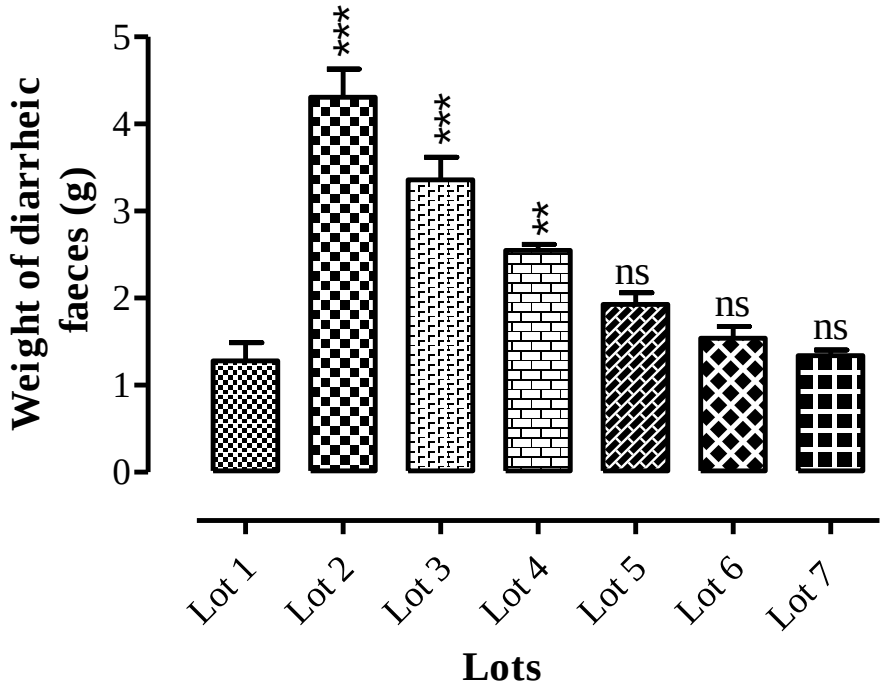


**Figure 4.** Weight of faeces of rats treated and untreated during experiment. Values are means±SEM. Each Lot includes 6 animals (n=6/Lot). ***p<0.001 and **p<0.01: significant difference compared to the uninfected and untreated Lot, ns: no significant at p<0.05 when compared with the uninfected and untreated Lot (Lot1). Lot 1: no infected and no treated, Lot 2: infected and untreated, Lot 3: infected and treated with ETHA at dose of 69.44 mg/kg bw corresponding to 1 × MIC, Lot 4: infected and treated with ETHA at dose of 271.87 mg/kg bw corresponding to 5 × MIC, Lot 5: infected and treated with ETHA at dose of 631.3 mg/kg bw corresponding to 10 × MIC, Lot 6: infected and treated with ETHA at dose of 2222.22 mg/kg bw corresponding to 40 × MIC, Lot 7: infected and treated with ciprofloxacin at dose of 5 mg/kg bw.

in the diarrhea feces and death of the specimen. For this instance, an infective dose of *E. coli* of $2 \times 10^8$ CFU/ml similar to that used by Mushtaq et al. (2005) was used to induce experimental *E. coli* diarrhea in rats. This dose brings about a colonization of the gastrointestinal tract in 24 h, and was responsible for dehydration, loss of appetite, appearance of demolded, and soft, liquid or semi-liquid light brown faeces (Forrester, 2002).

Mortality rate of 50% was observed in the untreated and infected Lot but no death was noted for both Lots treated with Ciprofloxacin and the ETHA fraction of 631.3 and 2222.22 mg/kg body weight, respectively. In fact, a significant reduction ($p < 0.001$ and $p < 0.01$) of rats' death was observed from 30 to 0% when increasing the dose of ETHA and a decrease of the rats weight and volume of diarrheal faeces indicating a progressive eradication of the *E. coli* inducted infection compared to infected and untreated Lots. These results illustrated the therapeutic action of the ETHA fraction.

These observation was corroborated by an important decreasing of faecal *E. coli* ESBL load during the experiment that may be ascribed to the healing power of the extract. After the treatment, the bacterial load of the infected and treated Lots decreased significantly ($p < 0.001$) as during the treatment and dose-dependent manner compared to the untreated infected Lot. Similar results were obtained by Niehaus et al. (2011) and Ibrahim and Sarhan (2015) when enumerating *E. coli* from diarrheal rat faeces. These results highlighted the therapeutic potentialities of the ethanolic fraction of *A. leiocapa.*

The antibacterial effect of *A. leiocarpa* extracts could be attributed to the bioactive compounds present in the extracts such as phenolic compounds (flavonoids, tannins, leucoanthocyanins and polyphenols), and saponins and sterols (Moronkola and Kunle, 2014). It has been suggested that bioactive compounds act through two main mechanisms of action. The first is related to their hydrophobic property, which facilitates their adhesion to the surface of bacteria, causing their instability (Jongbloed et al., 2007). The second was the inactivation of different bacterial molecules such as enzymes or receptors, following the binding of bioactive compounds (Pandey and Kumar, 2013). Some of these compounds

could lead to cell membrane perturbations and exert a β-lactam action on the transpeptidation of the cell wall. Other compounds could interact with the lipid bilayers in cell membranes, leading to the separation of the two membranes, thus leading to cellular swelling and cell death (Tshingani et al., 2017).

The reduction in the bacterial burden of faeces in infected and untreated rats may be explained by the protective action of their immune system against the pathogens (Lunga et al., 2014). The *in vitro* and *in vivo* analyses of antibacterial activity revealed the antibacterial and therapeutic potential of *A. leiocarpa*.

## Conclusion

This study showed that root barks of *A. leiocarpa* extracts possessed antibacterial activity by inhibiting *in vitro* bacterial growth. Its therapeutic activity was carried out against *E. coli* in experimentally infected rats by the significant reduction of defecation frequency, water content of faeces, weight and volume of diarrheic faeces, and *E. coli* load of faeces. The present results demonstrated that *A. leiocarpa* should be used in the traditional therapeutic arsenal against resistant infectious germs.

## Abbreviations

**ETHA,** Ethanol fraction of *Anogeissus leiocarpa*; **ETAA,** total aqueous extract of *Anogeissus leiocarpa*; **EDMA,** dichloromethane fraction of *Anogeissus leiocarpa*; **EAA,** ethyl acetate fraction of *Anogeissus leiocarpa.*

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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# Antioxidant and antiproliferative activities of ethanolic extracts of *Elateriospermum tapos* Blume (Euphorbiaceae)

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**Phytochemicals possessing free radical scavenging and antiproliferative activities play an important role in cancer chemoprevention. In this study, the ethanolic extracts prepared from fresh and dried leaves, bark, and seed skin of Pra (*Elateriospermum tapos* Blume.) were tested for their antioxidant activity using a ferric reducing ability of plasma (FRAP) assay. The antiproliferative activities against four human cancer cell lines, breast (MCF-7), colon (HT-29, HCT116) and cervical (HeLa) cancer cells, were examined using the MTT assay. Fresh leaf extract exhibited the greatest antioxidant activity by giving the greatest FRAP value, followed by bark, seed skin and dried leaf extracts, respectively, which were strongly correlated to their flavonoid content. Dried leaf extract with the highest total phenolic content exhibited the greatest antiproliferative activity against all cancer cell lines tested. The growth of cervical cancer cell line (HeLa) was the most sensitive to all plant part extracts tested with $IC_{50}$ values of 5.44 ± 0.57 µg/ml (fresh leaf), 5.41 ± 0.37 µg/ml (dried leaf), 6.39 ± 1.16 µg/ml (bark) and 7.03 ± 0.07 µg/ml (seed skin) at 72 h exposure. The non-cancer cell line (Vero) was more resistant to all plant part extracts when compared with the cancer cell lines. The ethanolic extracts of all *E. tapos* plant parts are promising for further purification and drug development.**

**Key words:** Antioxidant, antiproliferation, chemoprevention, *Elateriospermum tapos*, ethanolic extract.

## INTRODUCTION

The leakage of free radicals during respiration is an unavoidable consequence and may account for cell or tissue injury. The reactive oxygen species (ROS) are involved in both initiation and promotion stages of multistage carcinogenesis via damaging DNA, proteins and lipids (Klaunig and Wang, 2018; Perchellet, 1995). Antioxidants are generally known as the free radical scavengers, acting as the inhibitors at both initiation and promotion stages of carcinogenesis and protecting cells against oxidative damage. Chemoprevention involves the

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administration of chemical agents to block initiation and promotion processes of cancer development. The cancer prevention program of US National Cancer Institute using animal model or epidemiological studies includes (1) identification and characterization of chemopreventive agents, (2) establishment of efficacy and toxicity testing of candidate compounds in animal model systems, and (3) establishment of human intervention trials of promising chemopreventive agents (Boone et al., 1990; Golemis et al., 2018). The study at cellular level is definitely required prior to animal studies, although it has not literally mentioned.

Nowadays, cancer is the leading cause of the death of human especially breast, colon and cervical cancers (National Cancer Institute, 2009). In the United States, 1,735,350 new cancer cases and 609,640 cancer deaths are expected to occur in 2018 (Siegel et al., 2017). In Thailand, five types of cancers: breast, cervix, colorectal, liver and lung cancers are accounted for approximately 60% of the cancer burden (Virani et al., 2017). A tremendous number of cancer patients in the world face problem with high cost of chemotherapy especially in developing countries bringing about an inevitable option for development of herbal medicine. A number of research indicated that there are many types of plants and different parts of some edible plant extracts that contained high level of phenolic compounds and had significant anticancer activities (Ismail et al., 2012; Ramasamy et al., 2011; Sanseera et al., 2016; Senawong et al., 2014). Besides anticancer properties, these plants also possess antioxidant properties, preventing oxidative damage and tumorigenesis. Different prepared extracts of some plants and their parts have been reported to contain wide range of flavonoids, steroids, phenolics and saponins which may exert varied pharmacological activities like anticancer, antidiarrheal, antimicrobial and antioxidant (Gandhi and Mehta, 2013). Many research found different kinds of plants, vegetable, fruits that are able to cure cancer or prevent cancer, however the search for new anticancer plants is still needed in order to find more effective results and less toxic.

*Elateriospermum tapos* Blume, locally known in Thai as Pra, Kra, or Perah, is a plant in the family Euphorbiaceae, distributed across the national highland rain forest or the high humidity mountain located in the southern part of Thailand especially in the Bantad Mountain Range in Trang and Nakornsrithammarat provinces (Chayamarit and van Welzen, 2005). This plant is also abundant in lowland forests throughout Malaysia especially in northern parts of Peninsular Malaysia (Ling et al., 2006).

The well-known medicinal plant *Suregada multiflora* Baill. of the same family (Euphorbiaceae) has been recorded for its anticancer activity (Itharat et al., 2004) and has been used in Thai traditional medicine (Chayamarit and van Welzen, 2005), however, there is no recorded data for the use of *E. tapos* in cancer treatment. The white and sticky latex exuding from its bark, leaves and fruit stalks, is used for the treatment of cracked sole of the foot (Chai et al., 1989). Our recent data from a screen program searching for natural products with anticancer properties indicated that *E. tapos* plant parts exhibited a promising antiproliferative activity against several human cancer cell lines (Tisadoldilok and Senawong, 2017). In this study, the ethanolic extracts prepared from fresh and dried leaves, bark, and seed skin of Pra were further investigated for their antioxidant activity using ABTS and ferric reducing ability of plasma (FRAP) assays comparing with the previous results from (2,2-diphenyl-1-picryl-hydrazyl-hydrate) (DPPH) assay. The anticancer activities of the extracts characterized by the half maximal inhibitory concentration ($IC_{50}$) values against four human cancer cell lines, breast (MCF-7), colon (HT29, HCT116) and cervical (HeLa) cancer cell lines, were evaluated using MTT assay.

## MATERIALS AND METHODS

### Plant materials

Fresh and dried leaves, bark, and seed skin of Pra (*E. tapos*) were collected from Nakornsrithamaraj Province, Thailand, during August - October, 2015. Taxonomic identification was approved by the Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok, Thailand. A voucher specimen (voucher number ST15001) is deposited at Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand.

### Preparation of plant extracts

The plant parts used in this study included fresh and dried leaves, seed skin and bark. The leaves, seed skin and bark of Pra (*E. tapos*) were cleaned with running tap water to remove any external material. The leaves of Pra were divided into fresh and dried samples. To prepare the dried samples, the plant parts were dried in a hot air oven at 40 to 50°C for 24 h. The fresh plant parts were cut into small pieces, whereas the dried plant samples were ground. Both fresh and dried samples of the plant parts (500 g) were soaked in 95% ethanol (2,000 ml) for seven days at room temperature. The supernatant was collected and filtered through a filter paper. The solid residue was repeatedly extracted 3 times with ethanol. The filtrates from each extraction were combined and the solvent was evaporated under reduced pressure using a rotary evaporator to yield the crude ethanolic extract of each plant part. All the crude extracts were weighed and kept in a refrigerator until they were used.

### Determination of total flavonoid content

Total flavonoid content of the plant part ethanolic extracts was measured using a colorimetric method as previously described (Chuenchom et al., 2016). The flavonoid content was expressed as mg Rutin equivalent per gram of the extract.

### FRAP assay

The antioxidant activities based on the ferric reducing ability of *E.*

**Table 1.** Antioxidant activities of ethanolic extracts of Pra (*E. tapos*).

| Plant parts | Antioxidant activities | | [a]Total Phenolics (mg GAE/1 g extract) | Total flavonoids (mg of Rutin/1 g extract) |
|---|---|---|---|---|
| | [a]DPPH $IC_{50}$ (µg/ml) | FRAP (mmol $FeSO_4$ equivalents/1 g extract) | | |
| Fresh leaf | 0.10±0.00 | 130.18±5.31 | 161.16±6.81 | 236.64±6.99 |
| Dried leaf | 20.00±0.01 | 22.49±2.12 | 198.77±6.26 | 89.58±11.06 |
| Seed skin | 21.00±0.01 | 66.13±2.16 | 69.02±1.66 | 97.42±2.11 |
| Bark | 9.00±0.01 | 58.02±2.49 | 121.86±4.36 | 151.00±2.69 |
| Trolox | 3.10±0.01 | 244.78±1.40 | | |

[a]Previous published results (Tisadoldilok and Senawong, 2017)
Values are given as mean ± S.D. of triplicate experiments.
Trolox was used as the reference standard.

*tapos* ethanol crude extracts were analyzed according to the method of Benzie and Strain (1996) with some modifications. A reagent was prepared fresh by mixing 10 ml of 300 mM acetate buffer with 1 ml of 10 mM 2, 4, 6-Tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM of hydrochloric acid (HCl) and 1 ml of 20 mM $FeCl_3.6H_2O$. The freshly prepared FRAP reagent was pre-warmed at 37°C for 5 min. Thereafter, a blank reading was taken at 595 nm using a plate reader. Subsequently, 3 µl of sample, standard or positive control (each dissolved in 10% DMSO) and 9 µl of water was added to 90 µl of the FRAP reagent. Absorbance readings were measured instantly upon addition of the FRAP reagent and again at 4 min after the start of the reaction. The change in absorbance in the 4 min reaction was calculated by comparison to the absorbance changes of $FeSO_4.7H_2O$ against a standard curve (100-1,000 µM) tested in parallel. Results were expressed as millimoles (mmol) $FeSO_4$ equivalents per gram of the extract. All experiments were carried out in triplicate.

#### Cell culture

Human cervical adenocarcinoma (HeLa) and colorectal adenocarcinoma (HT29) cell lines were obtained from Dr. P. Picha (National Cancer Institute, Bangkok, Thailand). Human colorectal carcinoma (HCT116) and breast adenocarcinoma (MCF-7) cell lines were kindly provided by Dr. O. Tetsu (University of California, San Francisco, U.S.A.). The non-cancer Vero cells were kindly provided by Dr. S. Barusrux (Khon Kaen University, Khon Kaen, Thailand). All cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) (Gibco-BRL) at 37°C in a humidified atmosphere with 5% $CO_2$.

#### Antiproliferative activity assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess antiproliferative activity of the plant extracts against human cancer cell lines. Briefly, cells were seeded in a 96-well plate ($10^4$ cells/well) and incubated for 24 h. Different concentrations (0.5-100 µg/ml) of the plant ethanolic extracts were added to the cells and incubated for 24, 48 and 72 h. Vehicle control groups were added with 0.5% dimethyl sulfoxide (DMSO). After exposure of the cells to the plant ethanolic extracts, the medium was removed, and the cells were incubated with MTT (Sigma Chemical Co., St Louis, MO) (0.5 mg/ml in PBS) for 2 h. DMSO was used to dissolve the formazan dye, which was detected by a microplate reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.) at 550 nm. A reference wavelength used in this study was at 655 nm to subtract optical density caused by dead cells and cell debris. The absorbance of formazan dye was proportional to the number of viable cells. The percentage of viable cells was calculated using the following equation:

% Cell viability = [Sample ($A_{550}$-$A_{655}$)/Control ($A_{550}$-$A_{655}$)] × 100

The half maximal inhibitory concentration ($IC_{50}$) values from each experiment were estimated by plotting x-y and fitting the data with a straight line (linear regression). The average of $IC_{50}$ values was calculated from three independent experiments.

#### Statistical analysis

Data from three independent experiments were expressed as mean ± standard deviation (SD). Statistical analyses were carried out using the statistical program SPSS version 17.0 for windows (SPSS Corporation, Chicago, IL). The criterion for statistical significance was set at $p<0.05$.

## RESULTS AND DISCUSSION

### Antioxidant activities of plant part ethanolic extracts of Pra (*E. tapos*)

Our previous results indicated that the ethanolic extracts of *E. tapos* plant parts exhibited antioxidant activity by the DPPH radical scavenging assay (Tisadoldilok and Senawong, 2017). In this study, the antioxidant capacity of the plant part ethanolic extracts were determined by a FRAP assay, expressed as mg $FeSO_4$/g sample, and compared the results with the previous DPPH results. According to DPPH and FRAP assays, fresh leaf extract, compared with those of other *E. tapos* plant part extracts, exhibited the greatest antioxidant capacity by giving the least $IC_{50}$ value for DPPH assay and giving the greatest value for FRAP assay (Table 1). Fresh leaf and bark extracts appeared to exhibit antioxidant capacity more potent than dried leaf and seed skin extracts when compared the results from DPPH and ABTS assays. Fresh leaf extract showed approximately 31 times greater antioxidant activity than that of Trolox from DPPH assay.

**Table 2.** Antiproliferative activity of ethanolic extracts of plant parts of *E. tapos* represented by $IC_{50}$ values after exposures to non-cancer and cancer cell lines for 24, 48 and 72 h.

| Parts used of *E. tapos* | $IC_{50}$ values (means ± SD; µg/ml) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Vero cells* | | | HeLa cells | | | HCT116 cells | | | HT29 cells | | | MCF-7 cells | | |
| | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| Fresh leaf | 63.28±2.57 | 42.99±4.08 | 14.69±2.54 | 17.63±2.37 | 7.42±0.78 | 5.44±0.57 | 14.22±0.66 | 9.21±0.20 | 5.69±0.39 | 76.72±8.16 | 10.90±0.26 | 4.80±0.17 | >100 | 87.98±2.78 | 12.28±0.35 |
| Dried leaf | 46.49±7.95 | 17.35±0.47 | 13.08±2.07 | 10.40±0.65 | 6.67±0.68 | 5.41±0.37 | 11.43±0.97 | 9.55±0.17 | 8.74±1.02 | 26.22±1.36 | 15.31±1.14 | 6.68±0.21 | 20.06±2.18 | 17.91±0.89 | 13.60±0.49 |
| Bark | 92.03±3.12 | 73.65±2.02 | 45.66±1.04 | 45.81±2.23 | 9.22±2.60 | 6.39±1.16 | 61.64±3.66 | 25.97±1.15 | 23.42±2.42 | 91.81±1.76 | 22.17±1.03 | 16.57±0.58 | 93.24±3.28 | 45.35±2.82 | 24.19±0.69 |
| Seed skin | 99.10±2.49 | 92.07±2.61 | 83.15±4.25 | 13.26±0.07 | 8.20±0.05 | 7.03±0.07 | 25.19±5.31 | 12.78±0.46 | 11.57±0.46 | 70.14±0.90 | 36.45±0.73 | 21.80±0.19 | 87.94±1.67 | 50.59±0.33 | 49.32±0.87 |

*Non-cancer cell line.

Whereas in a FRAP assay, fresh leaf extract exhibited antioxidant activity values approximately 1.9 times less than those of Trolox. The FRAP assay was demonstrated to have similar predictive power as the DPPH assay on *E. tapos* plant part antioxidant activity. In addition, total flavonoids of the ethanolic plant part extracts correlated well with their antioxidant activities. The difference in antioxidant activities of the extracts from fresh and dried leaves might probably be due to the extraction procedure or drying process. Some active volatile compounds may have been destroyed or evaporated during the processing of samples so that the antioxidant activities of dried and fresh leaf extracts are different (Table 1). This observation is consistent with other studies (Alabri et al., 2014; Vaidya et al., 2014). The strong correlation between total flavonoids and the antioxidant capacity of *E. tapos* plant parts indicates that flavonoids of the plant parts may be responsible for antioxidant activity. Phenolic compounds including flavonoids may act as free radical scavengers by acting as hydrogen or electron donors, singlet oxygen quenchers and metal chelators (Riachi and De Maria, 2015).

## Antiproliferative activities of ethanolic extracts of plant parts of Pra (*E. tapos*)

Our previous study demonstrated that ethanolic extracts of *E. tapos* plant parts exhibited antiproliferative activities against HeLa, HCT116, HT29 and MCF-7 cells at screening concentration of 100 µg/ml (Tisadoldilok and Senawong, 2017). In this study, antiproliferative activities of ethanolic extracts of the plant parts against four cancer cell lines (HeLa, HT29, HCT116 and MCF7) and a non-cancer cell line (Vero) were further investigated by MTT assay to obtain the $IC_{50}$ values. According to dose-response curves (Figures 1 to 4), all plant part ethanolic extracts inhibited proliferation of all cancer cell lines tested in a dose- and time-dependent manner. Cellular sensitivities determined as the $IC_{50}$ values against each cell line are summarized in Table 2 in accordance with the dose-response curves in Figures 1 to 4.

Cellular sensitivities differed depending on types of cells and extracts. In dried leaves, no further enzymatic or metabolic alteration of natural compounds would become possible further, whereas in fresh leaves there remains a possibility of formation of new compounds as secondary metabolites in responses to light and other factors. Although there is no significant difference in total phenolic contents between the extracts from dried and fresh leaves of moringa, the dried leaves significantly promoted the crude extract with higher total flavonoid content (Vongsak et al., 2013). In this study, fresh and dried leaf extracts of *E. tapos* were studied comparatively. Fresh leaf extract of *E. tapos* exhibited most effective growth inhibition against cervical cancer (HeLa) and colon cancer (HCT116) cell lines for 24 and 48 h exposures, and most effective inhibition against colon cancer (HT29) cell line for 72 h exposure (Figure 1 and Table 2). Dried leaf extract was more effective than fresh leaf extract on growth inhibition in all cancer cell lines tested for 24 h exposure (Figures 1, 2 and Table 2). However, no significant difference on growth inhibition in all cancer cell lines was observed between fresh and dried leaf extracts for 72 h exposure (Figures 1, 2 and Table 2).

Bark extract effectively inhibited the growth of HeLa, HCT116 and HT29 cells for 48 and 72 h exposures with less toxicity on the non-cancer Vero cells (Figure 3). The breast cancer MCF-7 cell line was more resistant than other cancer cell lines, but more sensitive to bark extract than the non-cancer cell line (Figure 3 and Table 2). Seed skin extract was more potent than Bark extract on growth inhibition of HeLa and HCT116 cells at exposure times of 24 and 48 h (Figures 3, 4 and Table 2). Among all plant part extracts used in this study, seed skin extract showed the least toxicity against the non-cancer Vero cells (Figure 4 and Table 2).

Based on the criteria of the American

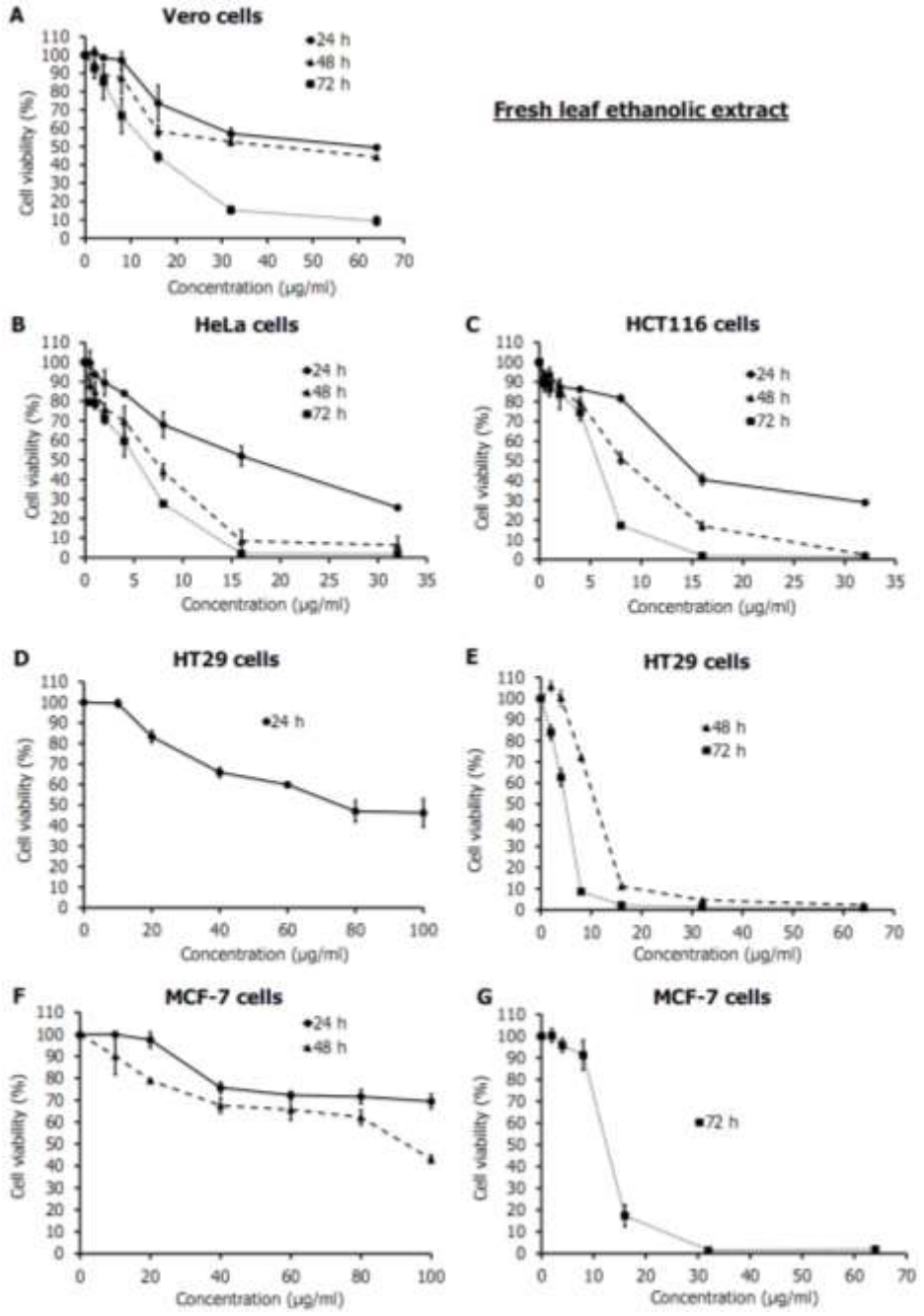


**Figure 1.** Effect of fresh leaf ethanolic extract of *E. tapos* on proliferation of Vero (A), HeLa (B), HCT116 (C), HT29 (D, E) and MCF-7 (F, G) cells, treated for 24, 48 and 72 h. The cell viability was calculated by comparison with the control, 0.5% DMSO treated. The results were shown as mean ± S.D. (n = 6).

National Cancer Institute, the crude extract promising for further purification should have $IC_{50}$ values lower than 30 µg/ml (Suffness and Pezzuto, 1990). In this study, $IC_{50}$ values of ethanolic extracts from fresh leaves, dried leaves, and seed skin against HeLa and HCT116 cells at all exposure times (24, 24 and 72 h) are less than 30 µg/ml, indicating that ethanolic extracts from these plant parts are promising for further purification and drug development. The taraxerane triterpene, 2,3-seco-taraxer-14-ene-2,3,28-trioic acid 2,3-dimethyl ester, was previously isolated from the leaves of *E. tapos* and exhibited cytotoxic activity against small cell lung carcinoma cell line (NCI-H187) (Pattamadilok and Suttisri, 2008). Although antioxidant activity of the plant

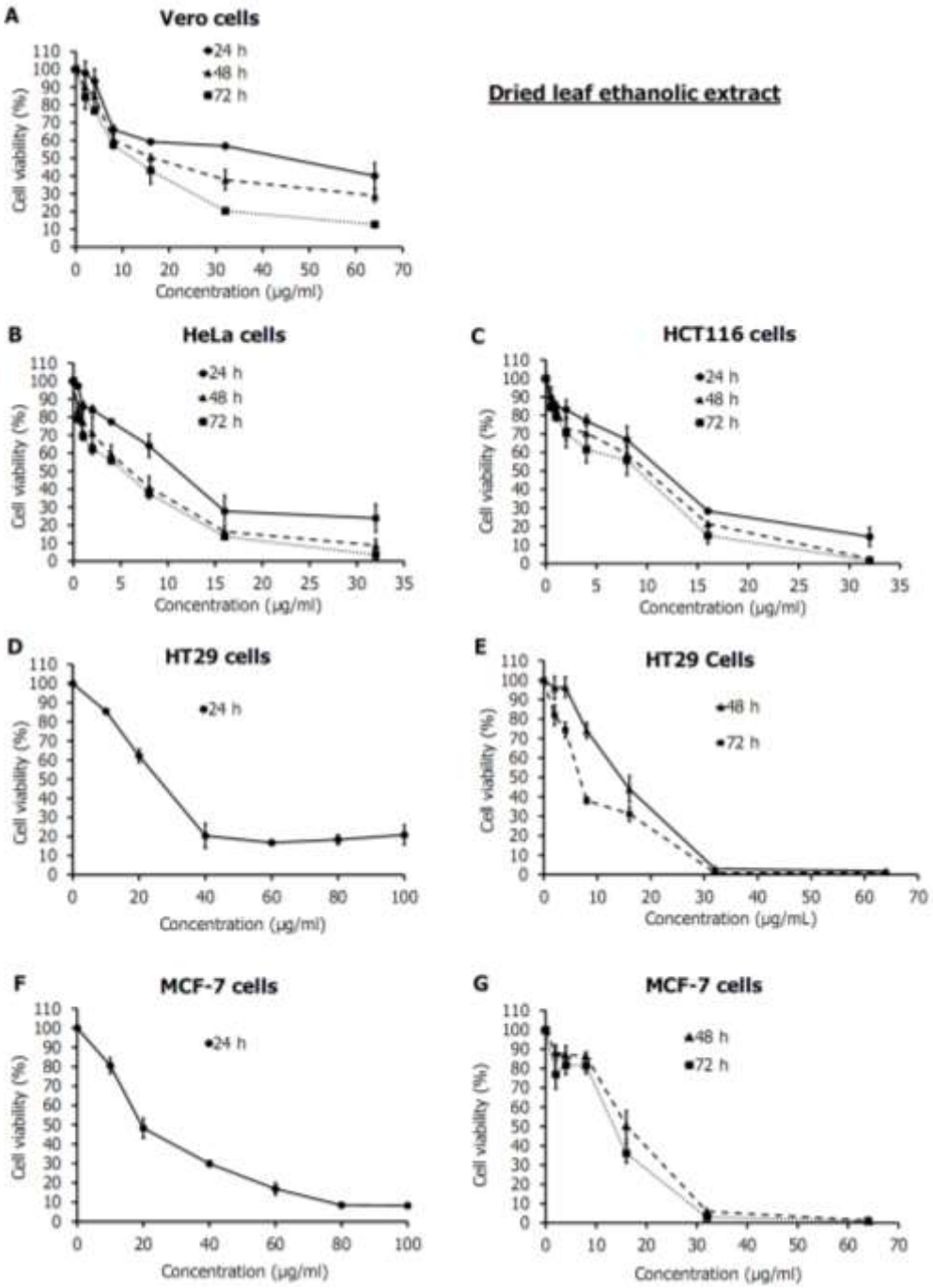


**Figure 2.** Effect of dried leaf ethanolic extract of *E. tapos* on proliferation of Vero (A), HeLa (B), HCT116 (C), HT29 (D, E) and MCF-7 (F, G) cells, treated for 24, 48 and 72 h. The cell viability was calculated by comparison with the control, 0.5% DMSO treated. The results were shown as mean ± S.D. (n = 6).

part extracts was strongly correlated with their flavonoid content, antiproliferative activity was not correlated with their flavonoid content (Tables 1 and 2), suggesting that type of flavonoids may have a greater impact on their antiproliferative activity. However, antiproliferative activity was correlated well with their total phenolic content.

## Conclusion

Comparing to the antioxidant values of the other plant parts of *E. tapos*, fresh leaf showed the greatest antioxidant activity by giving the least $IC_{50}$ value for DPPH assay and the greatest FRAP value. The growth of

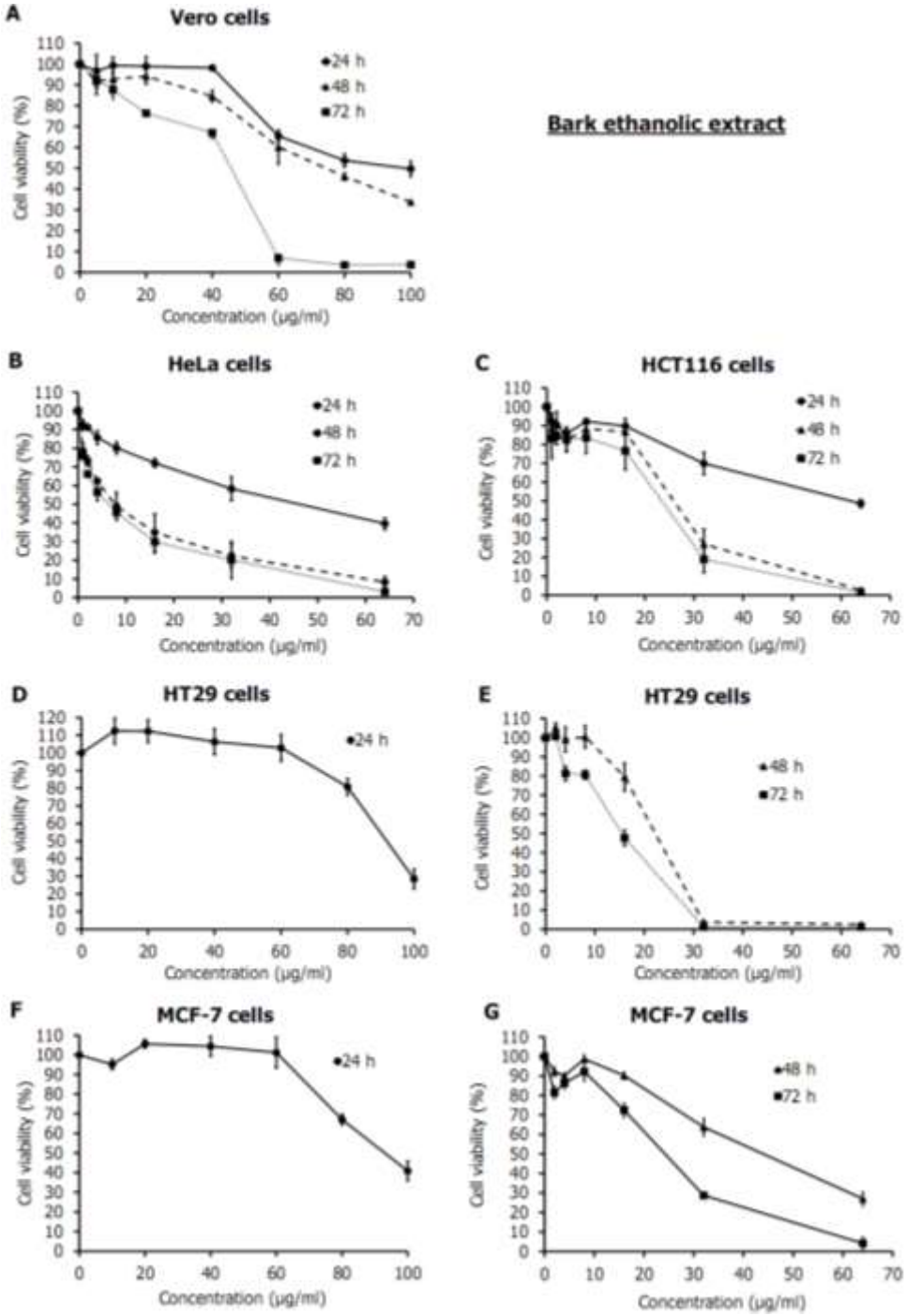


**Figure 3.** Effect of bark ethanolic extract of *E. tapos* on proliferation of Vero (A), HeLa (B), HCT116 (C), HT29 (D, E) and MCF-7 (F, G) cells, treated for 24, 48 and 72 h. The cell viability was calculated by comparison with the control, 0.5% DMSO treated. The results were shown as mean ± S.D. (n = 6).

cervical cancer cell line (HeLa cells) was most sensitive to all plant part extracts in comparison to other cancer cell lines studied, especially to dried leaf extract. The viability of the non-cancer cell line (Vero cells) was also progressively decreased with increasing concentrations of the extracts, but more resistant to the extracts when compared with the cancer cell lines. The ethanolic extracts from these plant parts are promising for further purification and drug development as their $IC_{50}$ values against cancer cell lines are less than 30 µg/ml. The data

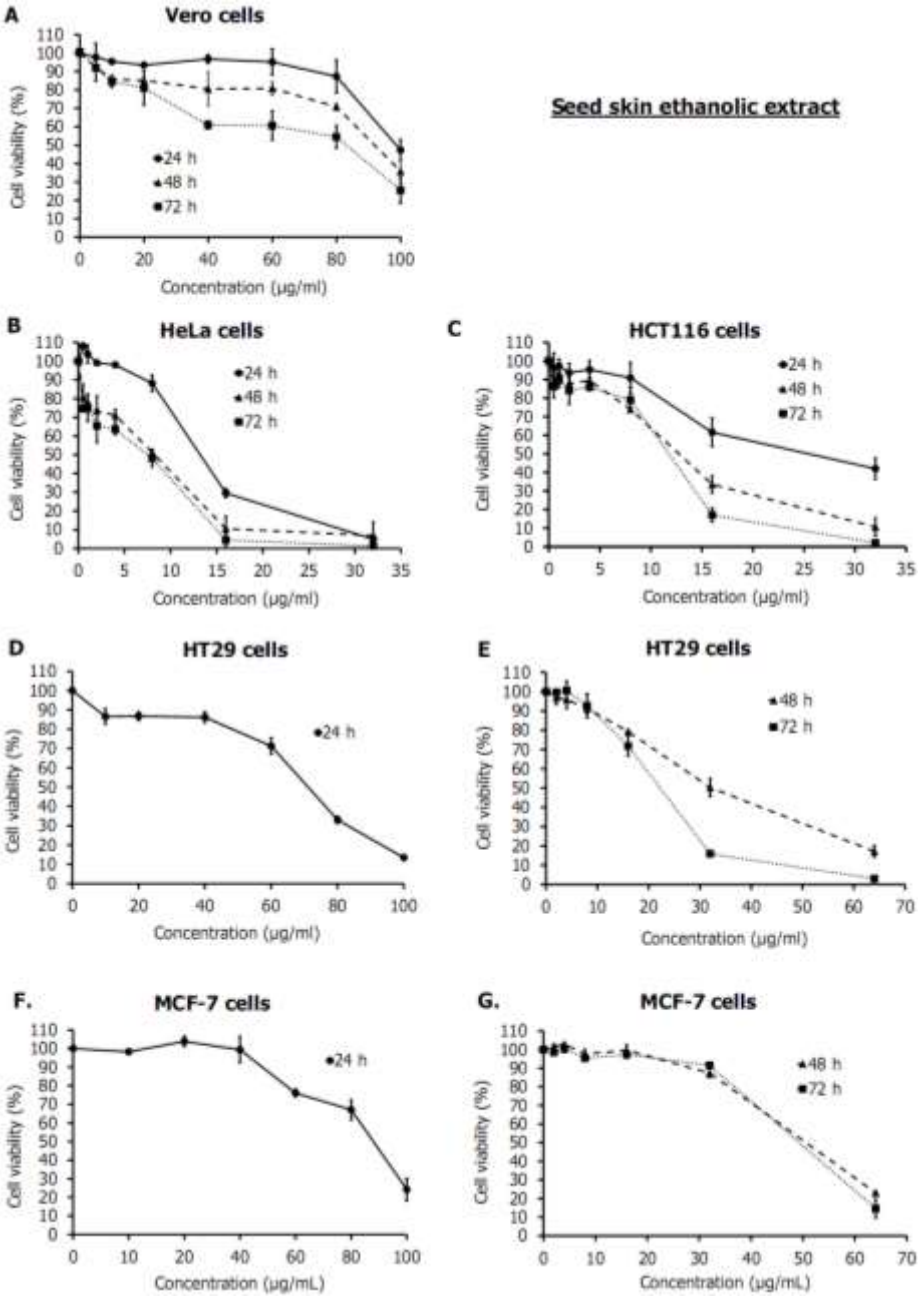


**Figure 4.** Effect of seed skin ethanolic extract of *E. tapos* on proliferation of Vero (A), HeLa (B), HCT116 (C), HT29 (D, E) and MCF-7 (F, G) cells, treated for 24, 48 and 72 h. The cell viability was calculated by comparison with the control, 0.5% DMSO treated. The results were shown as mean ± S.D. (n = 6).

finding from this research will be used for further studies on development of the herbal cancer medicine in the future. In addition, the reported antineoplastic properties of these plant parts would promote the cultivation and preservation programs of this plant in local area.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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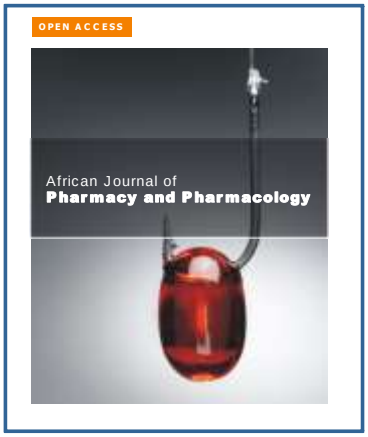








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