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*Full Length Research Paper*

# Ethnobotanical study of indigenous knowledge on medicinal plants used to treat diseases in selected districts of Amhara Regional State, Ethiopia

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This study was conducted in four districts of Amhara Region, Ethiopia. The aim of the study was to assess medicinal plants found in Menz gera, Siyadebirna Wayu, Antsokiya gemza and Bugna districts, Amhara region, Ethiopia. The ethnobotanical surveys were carried out from September 2017 to June 2018 using a semi-structured checklist consisting of questions or issues prepared in advance. Two separate questionnaires were prepared, one for the local healers and the other for the local community. A total of 376 (94 per district) informants were selected using purposive sampling technique and asked both open and close-ended questions. The interviews and observations were based on and around this checklist and some issues were raised promptly depending on responses of an informant. All plant species found in each district were recorded, collected, pressed and identified following Flora of Ethiopia and Eritrea. From the total 376 informants 238 were males and the rest 138 were females. A total of 77 medicinal plants were reported and distributed in 28 families. Family Lamiaceae was distributed in all four districts followed by 5 families which were distributed in three districts. Leaf 47 (56.63%) was the plant part widely used followed by stem 12 (14.46%), root 8 (9.64%) and seed 7 (8.43%), while the rest include fruit 3 (3.61%), bulb 3 (3.61%), flower 1 (1.20%). *Ocimum lamiifolium Hochst.* and *Citrus durantifolia* were the plants with the highest fidelity level values, each scoring 100%, followed by *Moringa stenopetala (Bak.f.) Cuf.* (96%). From the present study, the highest number of medicinal plants was observed in Antsokiya Gemza District followed by Siyaderina wayu, Bugna and Menz Gera, with 29, 24, 15 and 9 number, respectively. In these four districts the highest number of plants was used to treat wound followed by abdominal disease/pain. Pounding and grinding of the plant parts to make a powder were the most frequently used methods of traditional medicine preparation. Traditional medicine is the base for the modern medicine, but nowadays, the work is not respected by most people so it needs further attention especially in changing the attitude of the community towards traditional medicine.

**Key words:** Amhara Regional State, disease treated, medicinal plants, plant part.

## INTRODUCTION

Ethnobotanical studies are often significant in revealing locally important plant species especially for the discovery

of crude drugs. Right from its beginning, the documentation of traditional knowledge, especially on the

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medicinal uses of plants, has provided many important drugs of modern day (Cox and Balick, 1996; Flaster, 1996).

The medicinal plant of Ethiopia and the developing countries play major supplementary roles to the limited modern health care available. The development of useful and widely used drugs like Digoxin and Digitoxin, from *Digitalis* leaves; quinine from the cinchona bark; reserpine from *Rauwolfia serpentina*; morphine from *Papaver somniferum*; cocaine from *Erythroxylon coca* and the anti-cancer Vincristine and Vinblastine from *Carthartus troseus* of Madagascar and again anti-cancer compound, bruceatin, from the Ethiopian plant, *Brucea antidysenterica*, just to name a few are examples of the contributions of traditional pharmacopoeia (Desta, 1988).

The various literatures available show the significant role of medicinal plant in primary health care delivery in Ethiopia where 70% human and 90% livestock population depend on traditional medicine again similar to many developing countries particularly that of Sub-Saharan African countries. Those plants are part of the economic commodity for some members of the society which make their livelihood on their collection, trade and medicinal practices by practitioners or healers. It thus has a substantial potential to make contributions to the economic growth and alleviation of poverty in the country. Its proper management protects the environment and conserves biodiversity. The traditional health care is deep rooted in oral and written pharmacopoeias. Ethiopian plants have shown very effective contributions for some ailments of human and domestic animals. Such plants include *Phytolacca dodencadra* Aklilu (1965), many species of *Maytenus* studied by National Cancer Institute, USA (Kupchan et al., 1972) and many species that show anti-malarial (Nkunya, 1992).

Traditional medicine still remains the main resource for a large majority (80%) of the people in Ethiopia for treating health problems, and a traditional medical consultancy including the consumption of the medicinal plants has a much lower cost than the modern medical one (Abebe and Hagos, 1991; Asfaw et al., 1999; Addis et al., 2001). Out of the total flowering plants reported from the world, more than 50,000 are used for medicinal purposes (Govaerts, 2001; Schippmann et al., 2002).

In Ethiopia, about 800 species of plants are used in the traditional health care system to treat nearly 300 mental and physical disorders. The wide spread use of traditional medicine among both urban and rural population in Ethiopia could be attributed to cultural acceptability, efficacy in the country, socio-cultural background of the different ethnic groups as well as historical developments, which are related to migration, introduction of foreign culture and religion. Previous studies showed the existence of traditional medical pluralism in the country. In Ethiopia, either the knowledge from herbalists is passed secretly from one generation to the next

through words of mouths or their descendants inherit the medico-spiritual manuscripts (Pankhurst, 1965; Pankhurst, 1990; Slikkerveer, 1990; Abebe and Ayehu, 1993; Vecchiato, 1993). The study of Ethiopian medicinal plants has not been realized as fully as that of India or other traditional communities elsewhere (Iwu, 1993). In Ethiopia, though there has been some organized ethnomedicinal studies, there is limited development of therapeutic products and the indigenous knowledge on usage of medicinal plants as folk remedies are getting lost owing to migration from rural to urban areas, industrialization, rapid loss of natural habitats and changes in life style. In addition, there is a lack of ethnobotanical survey carried out in most parts of the country.

In view of these, documentation of the traditional uses of medicinal plants is an urgent matter and important to preserve the knowledge. Furthermore, most of the ethnomedicinal studies in northern part of Ethiopia are focused on 'Medihanit Awakie' (professional traditional practitioners) and the ancient medico-magical and/or medico-spiritual manuscripts and old Gee'z manuscripts (Abebe and Ayehu, 1993; Abbink, 1995; Pankhurst, 2001), and ignore the knowledge of ordinary people in the locality (Fassil, 2005). Thus, the purpose of this study is to assess the traditional uses of medicinal plants by the ordinary people in four different districts of Amhara region, Ethiopia and to provide baseline data for future pharmacological and phytochemical studies.

## MATERIALS AND METHODS

### Description of the study area

The study was conducted in four districts (Menz gera, Siyadebirna wayu, Antsokiya gemza and Bugna) of Amhara region, Ethiopia. From these the first three are located in North Shewa Zone and the last district is located in North wollo Zone. Menz gera is located at the eastern edge of the Ethiopian highlands in the Semien Shewa Zone. It is bordered on the south by Menz Lalo, on the southwest by Menz Keya, on the west by the Qechene River which separates it from the Debub Wollo Zone, on the north by Geshe Rabel, on the northeast by Antsokiyana Gemza, and on the east by Efratana Gidim.

The majority of the inhabitants practiced Ethiopian Orthodox Christianity, with 99.56% reporting that as their religion. Siyadebirna wayu is located in the Semien Shewa Zone; is bordered on the south by the Oromia Region, on the west by Ensaro, on the north by Moretna Jiru, and on the east by Basona Werana. The majority of the inhabitants practiced Ethiopian Orthodox Christianity, with 99.58% reporting that as their religion. Antsokiya gemza is the other part of the North Shewa Zone; is bordered on the south by Efratana Gidim, on the southwest by Menz Gera Midir, on the west by Geshe, and on the north and east by the Oromia Zone. The majority of the inhabitants were Orthodox Tewahedo, with 75.98% reporting that as their religion; while 23.15% were Muslim, and 0.82% Protestant. The last district is Bugna which is located in the northwest corner of the Semien Wollo Zone; is bordered on the south by Meket, on the west by the Debub Gondar Zone, on the north by the Wag Hemra Zone, and on the east by the Lasta woreda. The highest point in Bugna is Mount Abuna Yosef, on the

border with Gidan; other notable peaks include Mount Qachen. Rivers include the Tekezé, which has its source in this woreda. The majority of the population practiced Ethiopian Orthodox Christianity, with 98.28% professing this belief; while 1.59% of the population said they were Muslim (Figure 1).

North Shewa zone is one of 10 Zones in the Ethiopian Amhara Region. The highest point in the Zone is Mount Abuye Meda (4012 meters); other prominent peaks include Mount Megezez. North Shewa has a population density of 115.30. While 214,227 or 11.66% are urban inhabitants, a further 112 or 0.01% are pastoralists. The three largest ethnic groups reported in Semien Shewa were the Amhara (95.73%), the Oromo (2.14%), and the Argobba (1.71%); all other ethnic groups made up 0.42% of the population. Amharic is spoken as a first language by 96.97%, and 2.32% spoke Oromiffa; the remaining 0.71% spoke all other primary languages reported. North Wollo is the other zone in the Amhara Region of northern Ethiopia. Part of its southern border is defined by the Mille River. Its highest point is Mount Abuna Yosef. Most of this Zone is mountainous and characterized by steep slopes, which are unsuitable for agriculture and severely limits the cultivated area. A survey of the land in this zone shows that 24% is arable or cultivable, 4.6% pasture, 0.37% forest, 17.4% shrub land, 47.3% degraded or unusable, and the remaining 6.3% for all other uses. North Wollo has a population density of 123.25. While 155,273 or 10.35% are urban inhabitants, a further 2 persons are pastoralists. The largest ethnic group reported in North Wollo was the Amhara (99.38%); all other ethnic groups made up 0.62% of the population. Amharic is spoken as a first language by 99.28%; the remaining 0.72% spoke all other primary languages reported.

### Survey of medicinal plants

The ethnobotanical surveys were carried out from September 2017 to June 2018 using a semi-structured checklist consisting of questions or issues prepared in advance. There were two separate questionnaires, one for the traditional healers and the other for the local community.

The questionnaires were open-ended and close-ended. The interviews were based on and around this checklist and some issues were raised promptly depending on responses of an informant. First the questions for questionnaire and interview were written in Amharic then translated into English during paper writing. Prior to the administration of the questionnaire, conversations with the informants were held with the assistance of local farmers and help us in getting relevant information.

A total of 376 informants were questioned and interviewed; per each district 94 informants were involved. Out of about 336,092 inhabitants (168,739 females and 167,353 males) of the four districts (Wikipedia), these included a total of 238 males and 138 females; of which, fourteen were male local healers (three from Menzgera, three from Siyadebirna wayu, five from Antsokiya and three from Bugna district). The female and male informants' age ranges from 35 to 85 years and the mean age is 48 and 60 years respectively.

The informants were selected purposively and appointment was made prior to the visits. The informants such as teachers, agricultural professionals, health workers, local farmers and healers were asked to give their knowledge about the plants they use against a disease, plant parts harvested, method of preparation of the remedy and details of administration. Besides, observations were made on how they harvest the plant part, prepare the remedy and take the medicine. Specimens of the reported medicinal plants were collected during regular systematic walk in the fields and identified by botanists at the National Herbarium of Addis Ababa University following Flora of Ethiopia and Eritrea. Voucher specimens were deposited at the National Herbarium of Addis Ababa University, Ethiopia.

### Data analysis

All the data collected in the field were analyzed using qualitative and quantitative data analysis approach. For qualitative data, first the main themes were identified. Secondly, the main themes were assigned, then the responses were classified under the main themes. Finally, the themes and response were integrated into the text. On the other hand, the quantitative data were analyzed using Statistical Package for the Social Sciences software and presented in the form of table and graph.

## RESULTS AND DISCUSSION

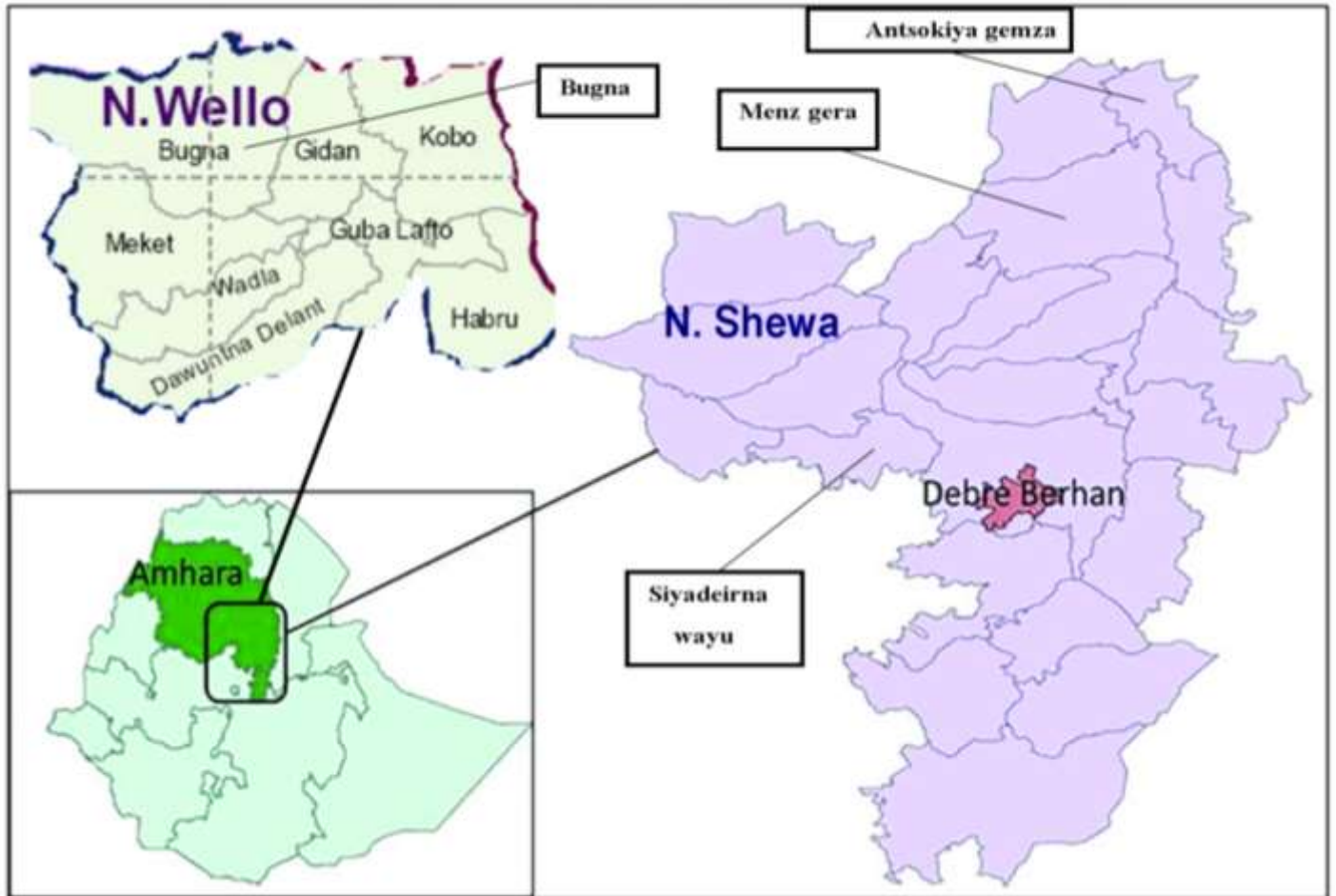
### Medicinal plant species diversity and distribution

A total of 77 medicinal plants were reported by the local community including the healers from the study area as being used for treatment of different disease in the area. These plants are distributed in 28 families. Family Lamiaceae was distributed in all four districts followed by 5 families which were distributed in three districts. Lamiaceae is among the most represented families in the flora of Ethiopia and Eritrea (Flora of Ethiopia and Eritrea, 1995; Flora of Ethiopia and Eritrea, 2004; Flora of Ethiopia and Eritrea, 2006). Other 12 and 10 families were distributed in two and one districts respectively (Figure 2). Other studies conducted in different parts of the country (Giday and Ameni, 2003; Tanto et al., 2003; Tadesse et al., 2005) also revealed the highest contribution of these families (Lamiaceae, Asteraceae and Fabaceae) to the Ethiopian medicinal flora.

### Knowledge of local healers on plant preparation

The local healers or practitioners used several methods of preparation of traditional medicines from plants. Pounding and grinding of the plant parts into powder were the most frequently used methods of traditional medicine preparation. According to Tilahun (2009), pounding and crushing are the most frequently employed methods to prepare remedies in single and multiple preparations. According to Rindos (1984), knowledge on plant use is the result of many years of human interaction and selection on the most desirable and successful plants present in the immediate environment at a given time. According to the practitioners, the powder as a result of grinding or pounding is a good strategy for preserving the plant materials that are not available both in dry and rainy seasons. It was also cited that, these are effective methods for the complete extraction of the active ingredients or potential content of the plant; they increase the curative power of the medicine or its efficacy, as both increases the healing power of the remedy through faster physiological reaction. The prepared medicinal plant is taken immediately or preserved for later use. The multiple prescriptions reported by the healers usually contain a range of





**Figure 1.** Map of the study area. Geographical location of Menz gera, Siyadebirna wayu, Antsokiya gemza and Bugna districts Amhara region, Ethiopia.

pharmacologically active compounds; in some cases, it is not known which ingredients are important for the therapeutic effect and some are used as adjuvants (Schulz et al., 2001). According to the local healers, the local communities including families of the practitioners do not want to learn and know about traditional medicine even they do not respect the healers; they see them as devil's followers. Even though, they still pass the traditional knowledge to their son. The traditional knowledge in the family or community is passed from male parent to his first-born son (Bishaw, 1990; Tesfu et al., 1995).

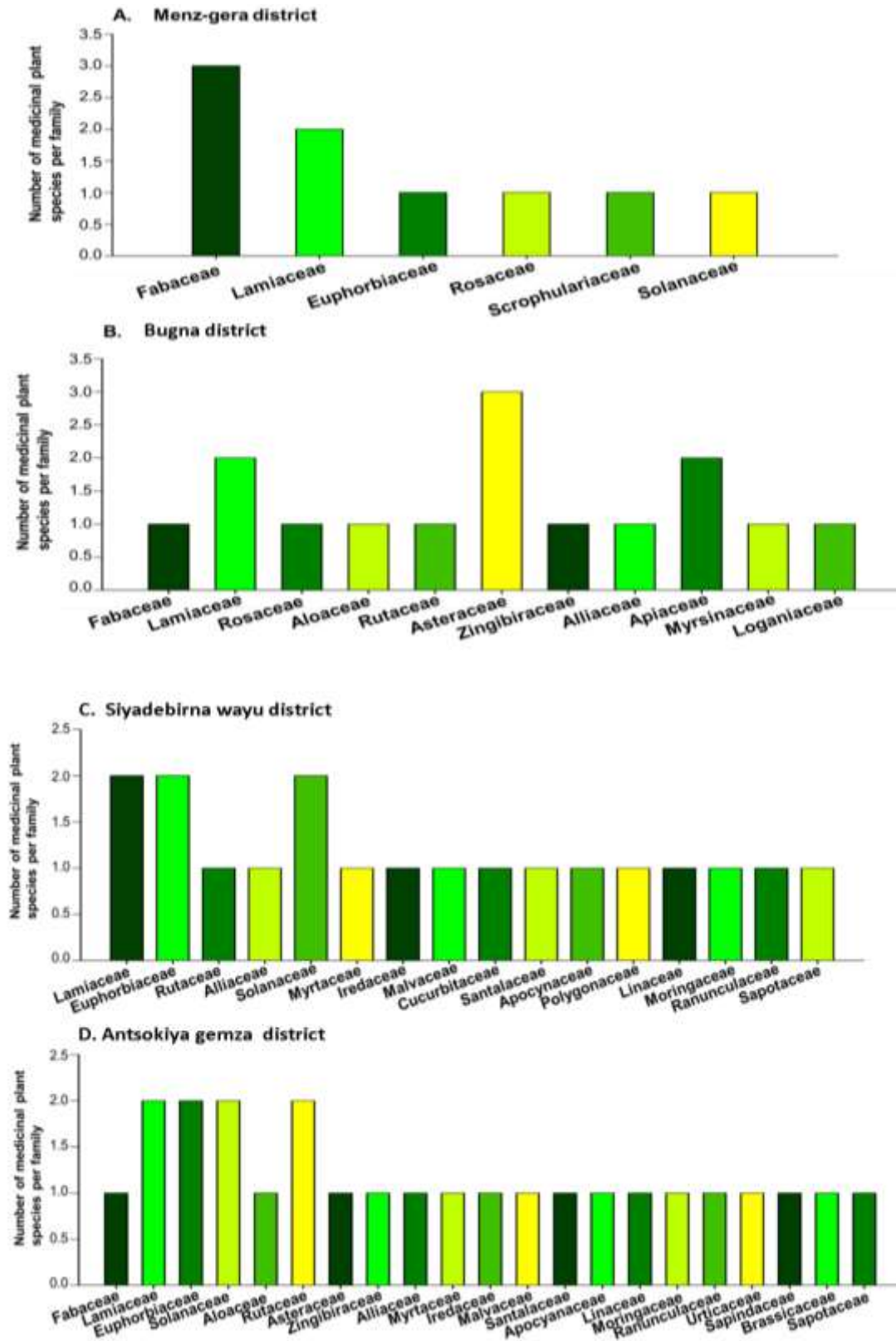
#### Plant part(s) used

Plant part(s) used for medicinal purposes indicated that leaf 47 (56.63%) was the plant part widely used followed by stem 12 (14.46%), root 8 (9.64%) and seed 7 (8.43%); while the rest include fruit 3 (3.61%), bulb 3 (3.61%), flower 1 (1.20%); the plant part for 2 (2.41%) species was

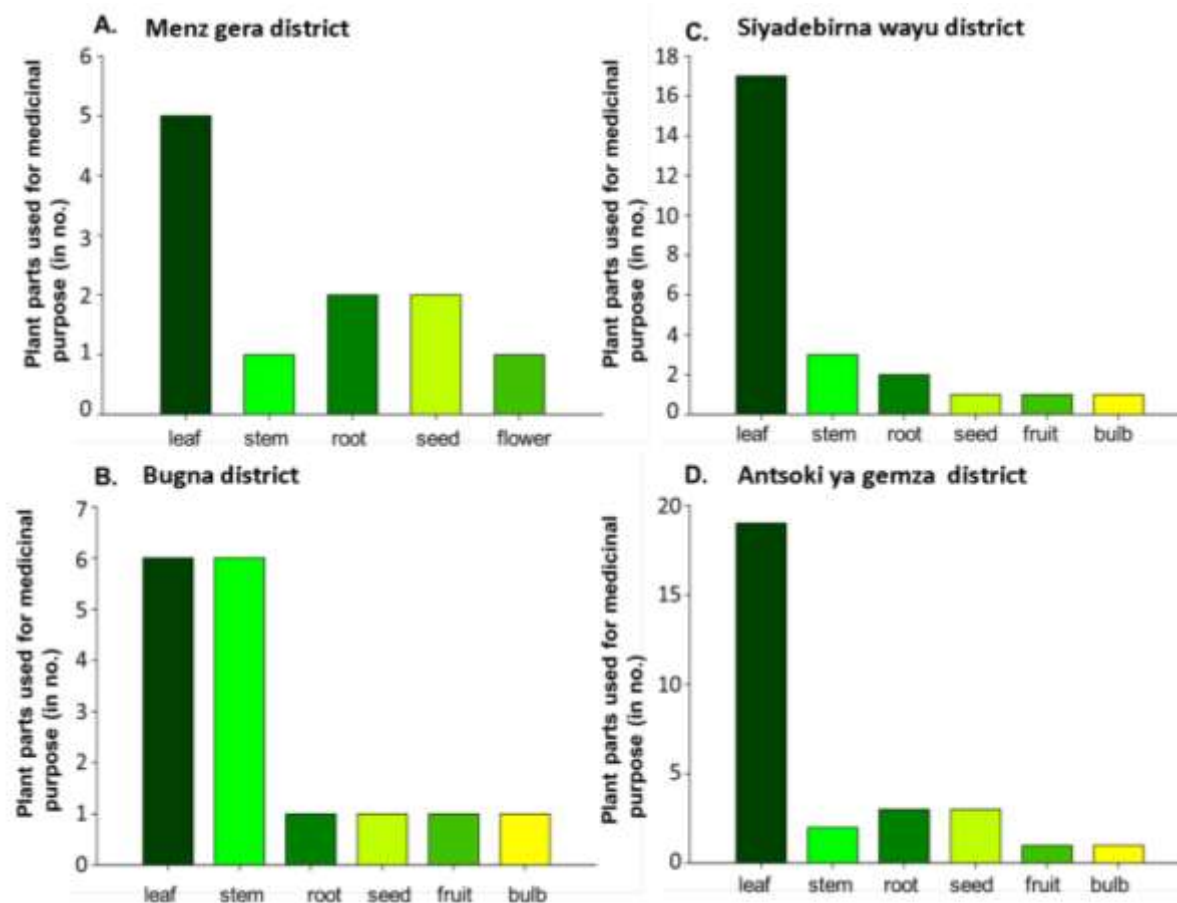
not specified. In all districts leaf is the widely used plant part (Figure 3). Previous reports in Ethiopia Flora of Ethiopia and Eritrea (1995), Flora of Ethiopia and Eritrea (2004), Flora of Ethiopia and Eritrea (2006) have also shown that leaves were the most commonly used to treat various health problems. Based on the family, 4 different parts of the plant under fabaceae family were used for medicinal purpose followed by 6 families from which 2 plant parts were used and from the remaining 18 families one plant part was used to treat different diseases (Table 1).

#### Fidelity level index

Fidelity level (FL), as an estimation healing potential, is an important means to see for which ailment particular specie is more effective; accordingly, *O.lamiifolium Hochst.* and *C. durantifolia* were the plants having the highest level values, each scoring 100%, followed by *M. stenopetala (Bak.f.) Cuf.* (96%) (Table 2 and Figure 3).



**Figure 2.** The number of medicinal plants distribution in different districts per family. (A) Number of medicinal plants per family in Menz-gera district. (B) Number of medicinal plants per family in Bugna district. (C) Number of medicinal plants per family in Siyadebirna Wayu district. (D) Number of medicinal plants per family in Antsokiya-gemza district.



**Figure 3.** Plant parts used for medicinal purpose in four different districts. **(A)** Plant parts used for medicinal purpose in Menz-gera district. **(B)** Plant parts used for medicinal purpose in Bugna district. **(C)** Plant parts used for medicinal purpose in Siyadebirna Wayu district. **(D)** Plant parts used for medicinal purpose in Antsokiya-gemza district.

**Table 1.** The different families of the species with their plant parts used for medicinal purpose.

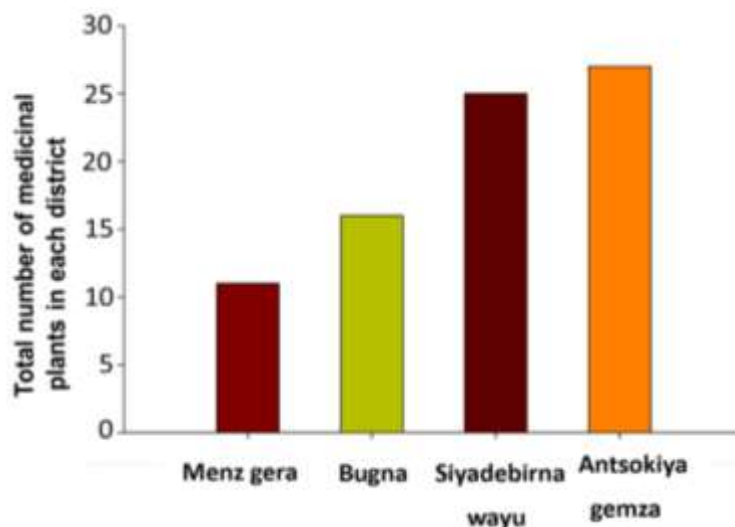
Family name	Plant part used for medicinal purpose						
	Leaf	Stem	Seed	Root	Flower	Fruit	Bulb
Fabaceae	✓	✓	✓		x	x	x
Lamiaceae	✓	✓	x	x	x	x	x
Euphorbiaceae	✓	✓	x	x	x	x	x
Rosaceae	x	x	✓	x	✓	x	x
Scrophulariaceae	✓	x	x	x	x	x	x
Solanaceae	x	x	x	✓	x	x	x
Aloaceae	✓	✓	x	x	x	x	x
Rutaceae	x	x	x	x	x	✓	x
Asteraceae	✓	✓	x	x	x	x	x
Zingiberaceae	x	x	x	✓	x	x	x
Alliaceae	x	x	x	x	x	x	✓
Apiaceae	✓	x	x	x	x	x	x
Myrsinaceae	x	x	x	x	x	✓	x
Myrtaceae	✓	x	x	x	x	x	x
Iredaceae	✓	x	x	x	x	x	x
Malvaceae	✓	✓	x	x	x	x	x

**Table 1.** Contd.

Cucurbitaceae	✓	x	x	x	x	x	x
Santalaceae	✓	x	x	x	x	x	x
Apocynaceae	✓	x	x	x	x	x	x
Polygonaceae	✓	x	x	x	x	x	x
Linaceae	x	x	✓	x	x	x	x
Moringaceae	✓	x	x	x	x	x	x
Ranunculaceae	✓	x	x	x	x	x	x
Urticaceae	✓	x	x	x	x	x	x
Sapindaceae	✓	x	x	x	x	x	x
Brassicaceae	x	x	x	x	x	x	x
Sapotaceae	✓	x	x	x	x	x	x

**Table 2.** Fidelity level (FL) value of medicinal plants.

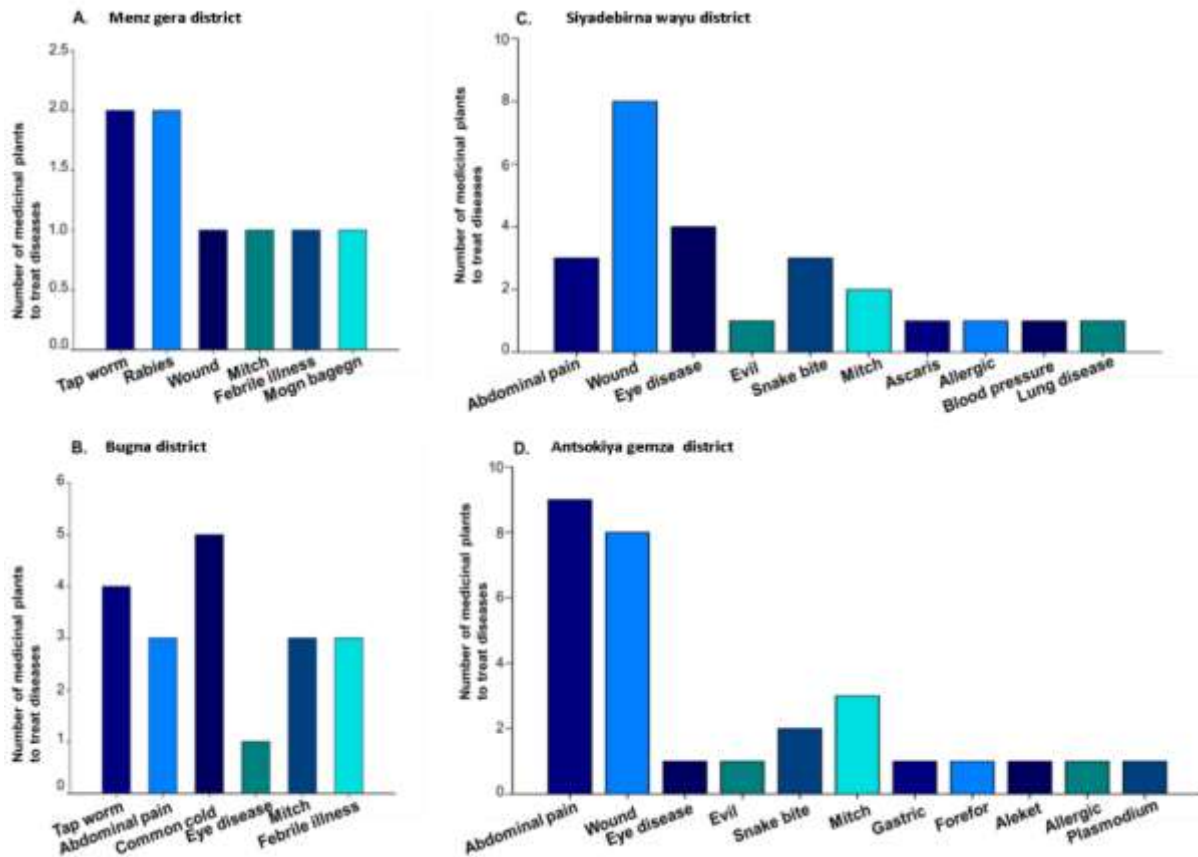
Species name	Local name	Use/s	Fidelity level (FL) (%)
<i>Ocimum lamifolium</i> Hochst.	Dama Kesse	Febrile illness	100
<i>Citrus durantifolia</i>	lomi	To clean mouth and kill microorganisms in the mouth	100
<i>Moringa stenopetala</i> (Bak.f.) Cuf.	Shiferaw	Blood pressure	96
<i>Hagenia abbyssinica</i>	Kosso	Abdominal disease / pain	93.75
<i>Embelia schimperi</i> Vatke	Inkoko	Tapeworm	92.85
<i>Linum usitatissimum</i> L.	Telba	Drying of abdomen	92.85
<i>Carissa spinarum</i> L.	Agam	Bite of snake	75

**Figure 4.** The total number of medicinal plants in each district.

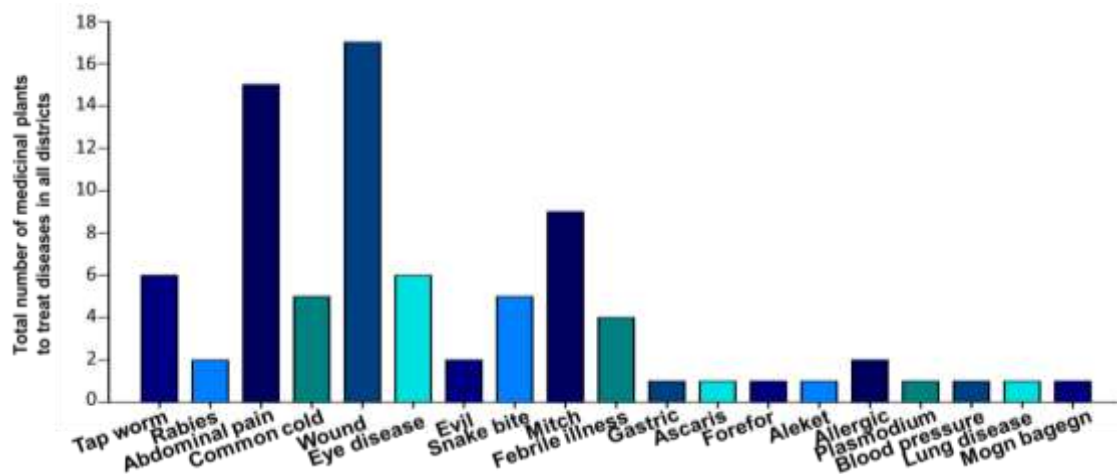
### Number and uses of medicinal plants

The number of medicinal plants was different from one district to the other. From the present study, the highest number of medicinal plants was observed in Antsokiya gemza district followed by Siyaderina wayu, Bugna and Menz gera, with 29, 24, 15 and 9 number, respectively (Figure 4). On the other hand, different medicinal plants

were used to treat different disease. In these four districts the highest number of plants was used to treat wound followed by abdominal disease/pain. According to MSWHO (2008), wounds are among the leading human disease. In Menz gera District, the highest number of plants was used to treat tapeworm and rabies followed by four different diseases with the same number of species. In Bugna, the highest numbers of plant were used to treat



**Figure 5.** The number of medicinal plants used to treat different disease. **(A)** Number of medicinal plants and its use in Menz-gera district. **(B)** Number of medicinal plants and its use in Bugna district. **(C)** Number of medicinal plants and its use in Siyadebirna Wayu district. **(D)** Number of medicinal plants and its use in Antsokiya-gemza district.



**Figure 6.** The total number of medicinal plants used to treat different disease in four districts.

common cold followed by tapeworm. In Siyadebirna wayu, the highest number of plants was used to treat wound followed by eye disease. In Ansokiya Gemza

District, the highest number of plants was used to treat abdominal disease/pain followed by wound (Figures 5 and 6).

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## CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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*Full Length Research Paper*

# Augmentation of docetaxel-induced tumor suppression in human ovarian cancer using selenium-enriched polysaccharides from *Pyracantha fortuneana* (Se-PFPs)

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*Pyracantha fortuneana* (Maxim.) contains many healthy ingredients, including selenium-enriched polysaccharides. Selenium-enriched polysaccharides from *P. fortuneana* (Se-PFPs) have anti-mutation and anti-oxidation activities, along with anti-tumor activity in ovarian cancer and breast cancer cells. Docetaxel (DTX) is widely applied in therapeutic treatment for ovarian cancer. However, its use in clinics is restricted due to its unfavorable side-effects. Thus there is a need to improve the therapeutic efficacy of Docetaxel (DTX). This study aimed to determine whether Se-PFPs and DTX can be used together to generate additive and/or synergistic effect in therapeutic treatment for ovarian cancer. *In vitro* and *in vivo* experiments were conducted to compare the anti-proliferative and apoptosis-inducing effects of the combined use of both drugs with that of each one used alone. Two ovarian carcinoma cell lines, SKOV3 and A2780 cells, were used and divided into four groups: 1) Saline control (vehicle); 2) Se-PFPs only; 3) DTX only; and 4) Se-PFPs+DTX. The apoptotic rate of group treated with a combination of Se-PFPs and DTX was found to be significantly higher than those of groups treated with either agent alone. This finding was confirmed by increased TdT-mediated dUTP nick end labeling (TUNEL)-positive rate, rate of apoptotic cells, enhanced cleavage of caspase-3 and PARP, and reduced cell viability and Ki-67 expression. The combined drugs did not significantly increase the toxicity HOSE cells, the human ovarian surface epithelial cells immortalized with hTERT. Similar results were obtained in the *in vivo* experiments by measuring tumor weight and volume. Thus, Se-PFPs can augment DTX tumor inhibitory effect. The combination of Se-PFPs and DTX can be a promising therapeutic strategy for ovarian cancer.

**Key words:** Selenium-enriched *Pyracantha fortuneana* (Maxim.) Li polysaccharides, docetaxel, tumor suppression, ovarian cancer.

## INTRODUCTION

Ovarian cancer is one of the most aggressive diseases characterized with widespread invasion and metastasis.

Its mortality is extremely high among gynecologic cancers. Docetaxel, a chemotherapy drug, has been

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applied to treat previously incurable ovarian cancer in recent years (Torre et al., 2018; Hou et al., 2018). However, more than 80% of patients with oophoroma at the stage III or IV succumb in the short term owing to relapse (Testa et al., 2018).

Compared with surgical treatment, natural plant extracts can have better effectiveness to fight disease and enhance life quality of patients (Al Sawah et al., 2015). *Pyracantha fortuneana* (Maxim.) is a shrub found in China, Vietnam, and Europe. Its fruits are of significant medicinal value. Selenium-enriched *P. fortuneana* Li polysaccharides (Se-PFPs), derived from *P. fortuneana* have been demonstrated to have an anti-mutation ability, is able protect immunologic function (Peng et al., 2016), and have a hepatoprotective effect (Yuan et al., 2015, 2010). Furthermore, it is also capable of inhibiting the growth of MDA-MB-231 breast cancer cells (Yuan et al., 2016). Moreover, Se-PFPs could inhibit the invasion and migration of ovarian cancer cells (Sun et al., 2016). Thus, treatment of ovarian cancer with Se-PFPs as an adjuvant in a short term deserves further exploration.

Docetaxel, derived from the Taxodaceae family, is one of the most studied taxanes of natural products (Kenmotsu and Tanigawara, 2015). It induces cell apoptosis through a mechanism involving suppressing microtubule dynamics (Azarenko et al., 2014) and arresting cell-cycle at the G2/M phase (Han et al., 2016). However, its clinical application is still limited due to its severe side-effects, such as nausea and fatigue (Sohail et al., 2018). Reduction dosage is an effective way to reduce side effects. Combination chemotherapy is brought into sharp focus on account of its lower toxicity and higher sensitivity. Researchers have already studied the effects of combination of taxanes with alkylating agents, topoisomerase inhibitors and anthracyclines (Joensuu et al., 2017). This study aimed to enhance the sensitivity of ovarian cancer cells to lower dosage of DTX by combining it together with Se-PFPs by analyzing the effects of the combination on induction of apoptosis and synergistic function.

## MATERIALS AND METHODS

### Reagents and cell lines

Selenium-enriched *P. fortuneana* fruiting bodies were obtained from Enshi mountain area in Hubei province, China. Se-PFPs were extracted from Se-enriched *P. fortuneana* as described by Yuan et al. (2015, 2010, 2016). Briefly, fruit were dried in an infrared dryer, and crushed into fine powder by a multifunctional disintegrator. The resulting products were refluxed and degreased twice with 5 L of petroleum ether at 75°C for 5 h each time. After cooling, the extract obtained was placed into hot water, concentrated under a vacuum at 55°C and precipitated with 4-fold volume of ethanol at 4°C overnight. The precipitate was dissolved in Sevag reagent to remove protein, followed by dialysis against distilled water for 48 h. After centrifugation at 2000×g for 15 min, the supernatant was pooled, condensed and lyophilized successively to obtain crude Se-PFPs.

Docetaxel purchased from Sigma-Aldrich (St. Louis, MO, USA)

was dissolved in dimethylsulfoxide (DMSO). Primary antibodies against cleaved caspase-3 and poly ADP ribose polymerase (PARP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SKOV3 and A2780 cells were initially obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37°C under 5% CO<sub>2</sub> in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin.

### MTT assay

Cell viability was determined as described by Yuan et al. (2012). Briefly, SKOV3 and A2780 cells were seeded at a density of 3000 cells per well in triplicate into 96-well plates, and exposed to 200 µg/mL Se-PFPs, 2 nM DTX, and 200 µg/mL Se-PFPs + 2 nM DTX for 24 and 48 h, respectively. Cells without exposure to these agents were taken as the negative control. Cell viability was determined using an MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) assay, and the absorbance was measured at 570 nm.

### Apoptosis assay

SKOV3 and A2780 cells were treated with 200 µg/ml Se-PFPs, 2 nM docetaxel (DTX) or 200 µg/mL Se-PFPs + 2 nM DTX for 48 h. Thereafter, the apoptosis was measured by two ways: part of the cells was measured by flow cytometry with Annexin V-fluorescein isothiocyanate (FITC) and 50 µg/mL propidium iodide (PI) staining, the remaining cells were fixed in 4% paraformaldehyde for 10 min and stained with 10 µg/mL Hoechst 33342 for 15 min, and apoptotic cells were counted under light microscopy.

### Western blot

The procedures of protein extraction from SKOV3 and A2780 cells or animal tissues and the protein concentration measurement were performed as described in our previous study (Yuan et al., 2015). The proteins (50 µg per well) were separated by SDS-PAGE (8 - 15%) and transferred onto polyvinylidene fluoride (PVDF) membrane. After being blocked with 5% defatted milk for 1 h, the membrane was incubated with the indicated primary antibodies overnight at 1:500 or 1:1000 dilution, and then incubated with corresponding secondary antibodies for 1 h. β-actin was used as the internal loading control. The membrane was developed and detected using enhanced Chemiluminescence Reagent (Pierce, Rockford, IL, USA) and exposed to X-ray film (Koda, Japan).

### Animal experiments

SKOV3 cells (4×10<sup>6</sup>) were subcutaneously injected into the flank of BALB/C female mice (30-day-old) following a protocol approved by the Ethics Committee Guide of China Three Gorges University. After ten days of injection, the mice were randomly assigned into four groups (10 mice in each group): 1) saline control (vehicle); 2) Se-PFPs only; 3) DTX only; and 4) Se-PFPs+DTX. The control group received saline intraperitoneal (i.p.) injections twice a week, as well as saline through oral gavage once a day. The mice treated with DTX only received DTX (5 mg/kg) injection i. p. twice a week, as well as saline through oral gavage once a day. The Se-PFPs+DTX treatment mice received Se-PFPs (250 mg/kg/day body weight) through DTX (5 mg/kg) i.p. injections twice a week and oral gavage once a day. The mice were weighed every other day, and the sizes of tumors were measured using a vernier caliper every three days. The mice were treated for 24 days. After the last



gavage, the mice in four groups were fasted for 16 h, weighted and euthanized through CO<sub>2</sub> inhalation. The tumors were then excised, weighted, and their volumes were calculated with the formula:  $\pi/6 \times \text{length} \times \text{width}$ . Specific organs, such as spleens, were excised and used for determining the activity and proportions of different immune cells. Blood was collected and used to determine the counts of white blood cells and measure the hepatic and renal function. The excised tumors were used to detect the proliferation and apoptosis rates.

#### Experimental design and calculation for CI

We performed the pretreatment and based on the results, the baseline data of the untreated control was used for normalization. We determined the dosage range for the possible coordination of drugs A and B and determined the fixed ratio of two drugs. At least three dosages and maximal six measures of drug A were selected. Given the fixed ratio of Se-PFPs: DTX = 1:2, then,

Se-PFPs (nM):	100	200	300	400	500
DTX (nM):	200	400	600	800	1,000
Combination:	A100+B200	A200+B400	A300+B600	A400+B800	A500+B1,000

The correspondingly matched dosages for drug DTX were calculated using software CalcuSyn. The experiments were repeated three times.

#### Immunohistochemical detection of Ki-67 and TUNEL assay for apoptotic cells

The partial tumor tissues were fixed in 4% formaldehyde and embedded in paraffin, and then cut into 4-micron sections. The commercial TM SP Kit (Zhongshan Corp., Beijing, China) was used to perform the immunohistochemical examination according to the manufacturer's instructions. The sections were exposed to 1:500 dilution of Ki-67 antibody (Santa Cruz Biotechnology) to evaluate the cell proliferation. The negative control was performed by using phosphate buffered saline; i.p., intraperitoneal (PBS) to replace Ki-67 antibody. For TUNEL assay, the other nearly frozen sections were used to detect the apoptotic cells using the TUNEL assay kit (Boehringer Mannheim, Indianapolis, IN, USA) following the manufacturer's instruction. Briefly, sections were treated with protease K at 37°C for 30 min, then incubated with a terminal deoxynucleotidyl transferase end labeling cocktail at 37°C for 2 h, and finally incubated with 50  $\mu$ L of FIFC in the dark for 1 h. The staining was scored as per Yuan et al. (2012).

#### Statistical analysis

For cell viability, Annexin V-FITC and morphologic analysis of apoptotic cells, values were presented as the means  $\pm$  standard deviation (SD) for at least three independent experiments done in triplicate. Statistical analyses were performed using one-way analysis of variance (ANOVA) or Student's t-test using Prism Graphpad 5 software.  $P < 0.05$  was set for statistical significance. Analysis of synergism was done using a commercial CalcuSyn software program (Biosoft, Ferguson, MO, USA). A synergistic, additive, or antagonistic interaction were defined as  $CI < 1$ ,  $CI = 1$ ,  $CI > 1$ , respectively.

#### Ethical considerations

The animal experiments were approved by the Ethics Committee

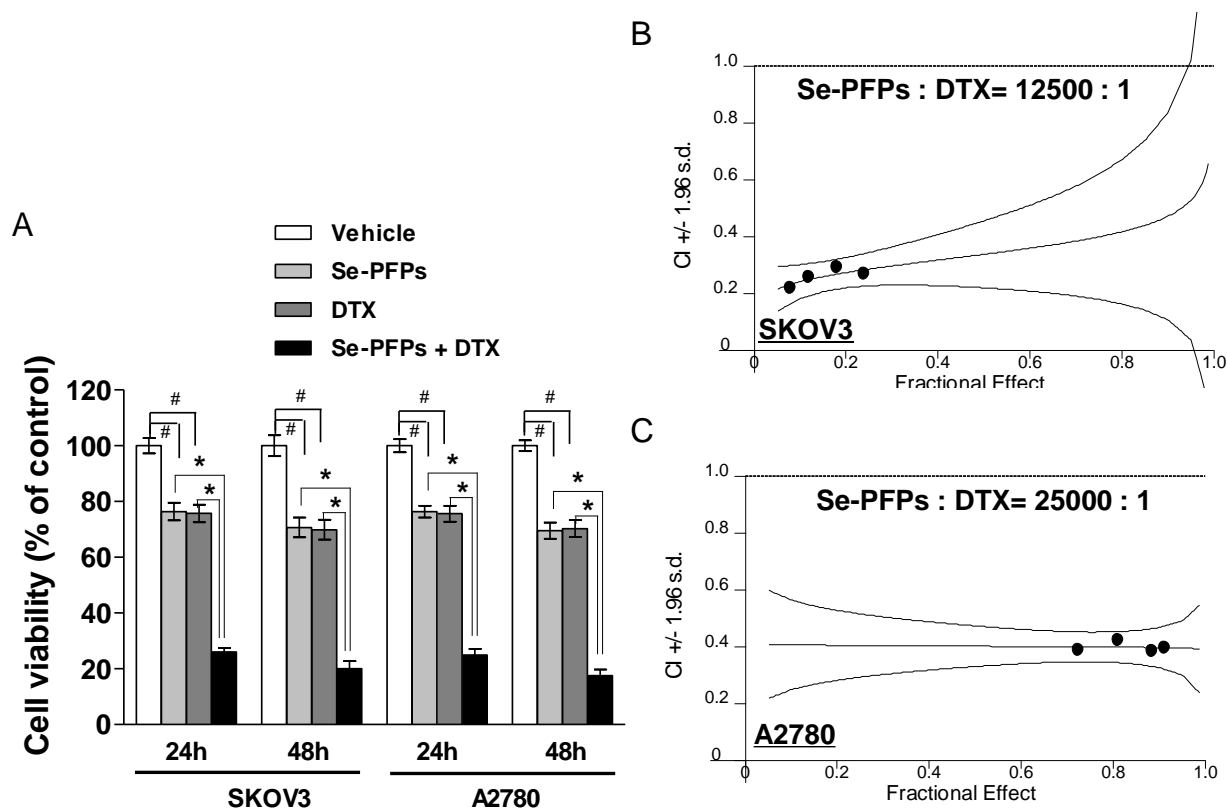
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## RESULTS AND DISCUSSION

### Se-PFPs and DTX interacted synergistically to inhibit cell proliferation and induce apoptosis in ovarian cancer cells

Figure-1A indicates that Se-PFPs or DTX alone significantly suppressed the proliferation of SKOV3 and A2780 cells at 24 and 48 h, respectively ( $P < 0.05$ ). However, a combination of Se-PFPs and DTX showed a significant and synergistically inhibitory effect on the proliferation of both SKOV3 and A2780 cells ( $P < 0.05$ , Figure 1A-C). To clarify whether reduced cell proliferation after Se-PFPs and DTX treatment was caused by induction of cell death, this study carried out cell apoptosis assays using multiple methods. Annexin V and propidium iodide (PI) staining showed that treatment of 200  $\mu$ g/ml Se-PFPs or 2 nM docetaxel individually for 48 h caused cell apoptosis by 14.9 and 15.8% in SKOV3 cells, and by 14.3% and 15.4% in A2780 cells, respectively. However, the treatment of cells with a combination of Se-PFPs and DTX resulted in a significant increase in the percentage of apoptotic cells to 67.5 and 68.8% in SKOV3 and A2780 cells, respectively ( $P < 0.01$ , Figure 2, panels A and B). It is noteworthy that, Se-PFPs and DTX did not show significantly synergistic effect on induction of apoptosis of HOSE cells, an immortalized non-tumorigenic human ovarian surface epithelial cell line (Figure 2 panel C). The synergistic apoptosis-inducing effect was further validated by elevated PARP cleavage in combined treatment of SKOV3 and A2780 cells with Se-PFPs and DTX (Figure 2B). We also discovered that combination index (CI) values of Se-PFPs and DTX were less than 1.0 for both SKOV3 and A2780 cells, indicating a highly synergistic interaction between Se-PFPs and DTX administration in inducing apoptosis in both SKOV3 and A2780 cells (Figure 2C-D).

*P. fortuneana* has been used as a traditional Chinese medicine for thousands of years because of its wide range of pharmacological activities. It has been demonstrated by previous researches that Se-enriched polysaccharides possess the antiproliferation and antitumor activities in multiple cancer lines (Mao et al., 2016), especially in breast cancer (He et al., 2013) and osteosarcoma (Wang et al., 2013). Polysaccharides induce apoptosis through many signaling pathways, such as mitogen activated protein kinase (MAPK) (Zhang et al., 2013) and NF- $\kappa$ B signaling pathway (Lee et al., 2014). It has been known that the NF- $\kappa$ B can control many cellular processes, including inhibition of apoptosis, induction of proliferation and cell invasion. Kinoshita et al. (2010) and Zhang et al. (2003) have shown that some drugs can lead to a high level of DTX-induced activation of caspase-3 in cancer cells through inhibiting NF- $\kappa$ B expressions. In addition, another kind of paclitaxel in the



**Figure 1.** Synergistic interaction of Se-PFPs with DTX to inhibit proliferation in ovarian cancer cells. (A) SKOV3 cells and A2780 cells were exposed to 200 µg/ml Se-PFPs, 2 nM DTX or 200 µg/ml Se-PFPs + 2 nM DTX for 24 and 48 h, respectively. Cell viability was measured using MTT method. The data are expressed as the ratio to the control cells. (B) SKOV3 and (C) A2780 cells were incubated with a range of Se-PFPs (200 to 500 µg/ml) and DTX (2 to 5 nM) alone and in combination at a fixed ratio (e.g. SKOV3, 12500:1; A2780, 25000:1) for 48 h. At the end of the exposure, the cell viability was measured as mentioned above. Fractional effect values were analyzed by CalcuSyn software. CI values <1.0 indicate a synergistic interaction. All the experiments were repeated thrice independently, and the data were expressed as mean ± SD. #*p* < 0.05; \**p* < 0.01, comparing with the indicated group.

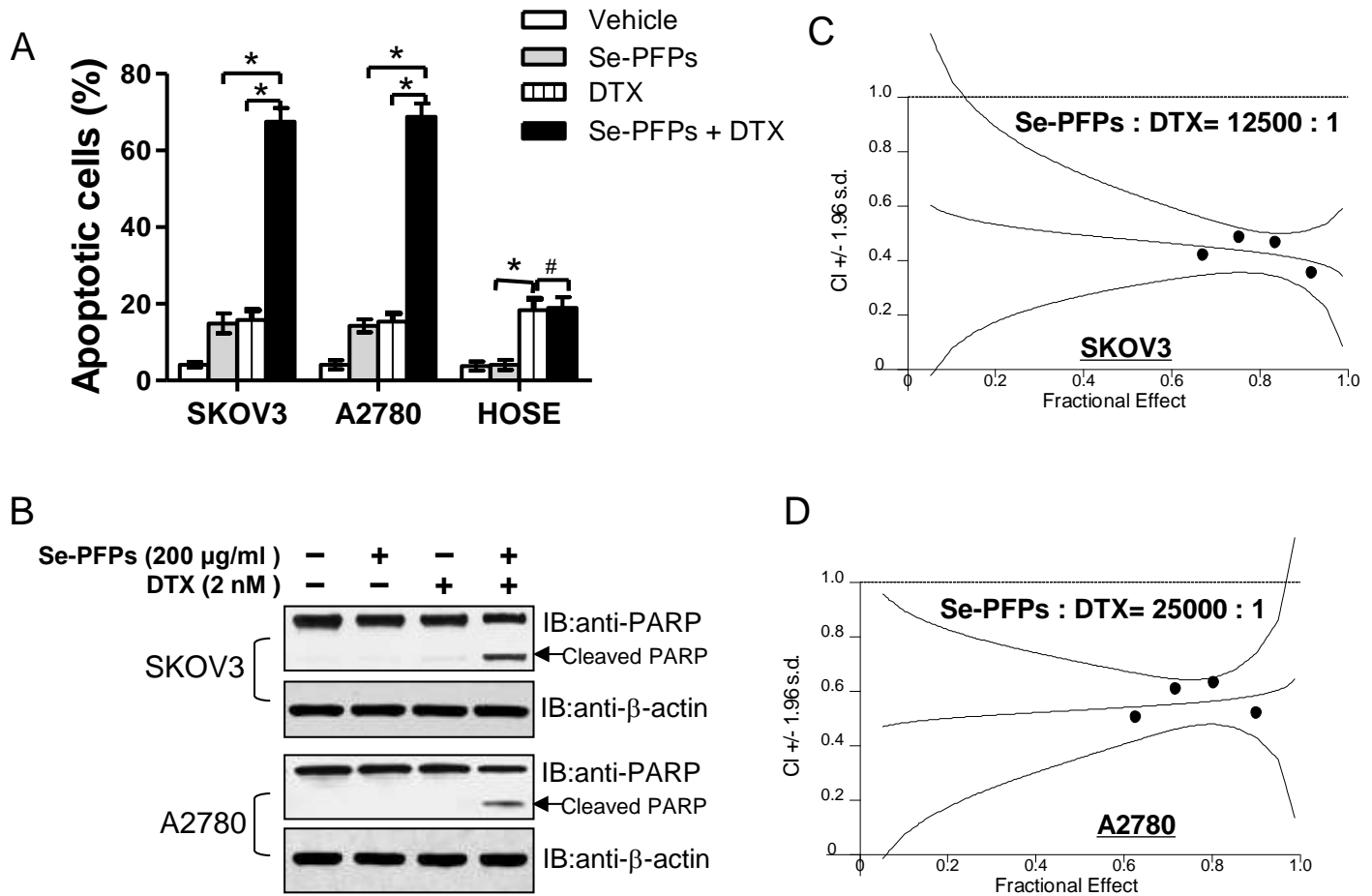
taxane family has been proved to have inhibitory effects on NF-κB family (Kinoshita et al., 2016; Zhang et al., 2003). Based on these observations, the increased caspase-3 activation in our study suggests that the combination therapy might also function through inhibition of the NF-κB activation. Thus, Se-PFPs and DTX may induce apoptosis of ovarian cancer cells through a common signaling pathway, which may be one of the cellular and molecular bases underlying their synergistic effect. However, further study is needed to confirm this hypothesis.

The combination therapy was initially used in treatment of lymphomas and leukemia (Andrew and Brown, 2017). Nowadays, it is widely used for treatment of malignant tumors. Without a single established criterion, it is difficult to clearly examine whether the combination is of antagonism or synergy. Some effective methods have been applied, such as the CI-isobologram (Liu et al., 2017) and the CI method. To assess the effects of Se-PFPs and DTX combination treatments, this study conducted a statistical analysis by determining CI values

in this research. The CI values have shown that the interaction between Se-PFPs and DTX has moderately synergistic effects against A2780 and SKOV3 cell lines. Similar results have been revealed by cell viability analysis, Annexin V and PI staining. Moreover, the similar synergistic effects demonstrated in our animal experiment suggest that the combined treatment has great potential in the real clinical application.

#### Se-PFPs enhanced DTX-induced inhibition on tumor growth and induction of apoptosis *in vivo*

To verify whether the *in vitro* findings in cultured cells could be repeated *in vivo*, nude mice xenografted model carrying SKOV3 cells were divided into four groups and treated with saline control; 5 mg/kg DTX (i.p. twice a week); 250 mg/kg Se-PFPs (oral gavage, once a day) or a combination of 5 mg/kg DTX + 250 mg/kg Se-PFPs, respectively. The effects of combination group were then compared with those of the other three groups. The mice



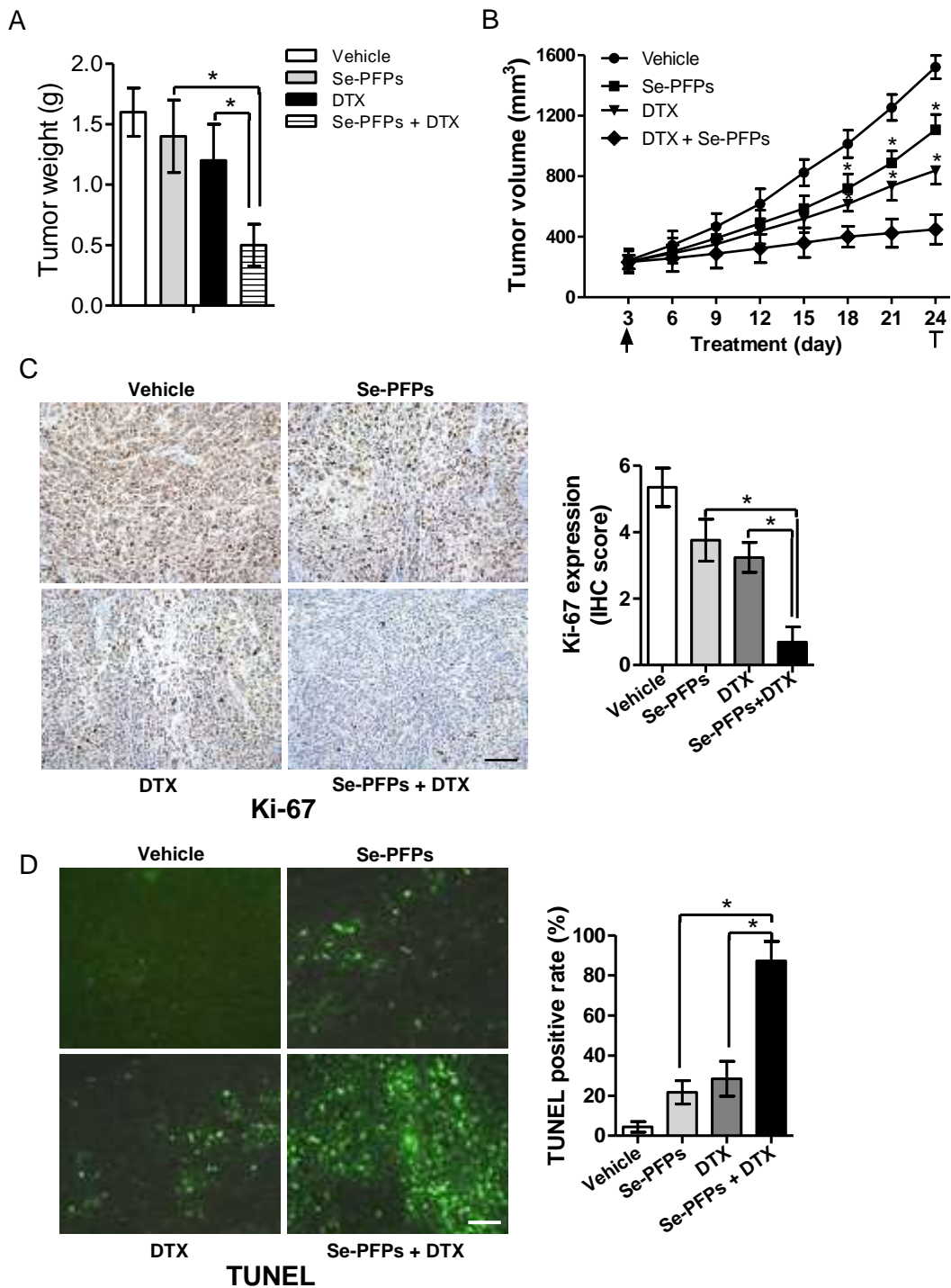
**Figure 2.** Co-administration of Se-PFPs and DTX induced apoptosis in ovarian cancer cells. (A) SKOV3, A2780 cells and (B) immortalized, non-tumorigenic HOSE cells were treated with 200 µg/ml Se-PFPs, 2 nM DTX or 200 µg/ml Se-PFPs + 2 nM DTX for 48 h, respectively. Apoptosis was determined by flow cytometry as described in this study. (C) SKOV3 and A2780 cells were exposed to 200 µg/ml Se-PFPs ± 2 nM DTX for 48 h, the cells were lysed and proteins separated by SDS-PAGE, and Western blot analysis was performed to monitor the degradation of PARP. (D) SKOV3 and A2780 cells were incubated with a range of Se-PFPs (200 to 500 µg/ml) and DTX (2 to 5 nM) alone and in combination at a fixed ratio (e.g. SKOV3, 12500:1; A2780, 25000:1) for 48 h. At the end of the exposure, apoptosis was measured by flow cytometry. Fractional effect values were analyzed by CalcuSyn software. CI values <1.0 indicate a synergistic interaction. All the experiments were repeated three times independently, and the data were expressed as mean ± SD. \* $p < 0.01$ , comparing with the indicated group, whereas # $p > 0.05$ , comparing with DTX group.

treated with either Se-PFPs or DTX exhibited reduced tumor volume to a certain extent while the mice treated with combination of Se-PFPs and DTX displayed decreased tumor weight and tumor volume after 18 days compared with those in groups of mice treated with vehicle control, DTX or Se-PFPs alone, respectively. At 24 days, the difference in the reduction ranges between the treatment with combined DTX or Se-PFPs and those treated with either DTX or Se-PFPs became much larger ( $P < 0.05$ , Figure 3A and B). However, there were no differences in body weight of mice among these groups (data not shown).

In order to evaluate the potential mechanisms by which Se-PFPs augment DTX-induced tumor-inhibition effect, the sections from the treated mice were stained antibody against Ki-67 (a marker for proliferation), or subjected to

TUNEL assay (for apoptotic cells). Figure 3C and D indicate that the immunohistochemical staining of Ki-67 and TUNEL assay revealed that proportion of Ki-67-positive cells was significantly decreased while the proportion of TUNEL positive cells was significantly increased in xenografted tumor tissues of mice treated with combination of DTX or Se-PFPs, as compared with those of mice treated with vehicle control, DTX or Se-PFPs alone, respectively ( $P < 0.05$ ). Consistent with our findings of *in vitro* experiments, these results obtained from the *in vivo* experiments support the conclusion that Se-PFPs can augment DTX-induced tumor inhibition in ovarian cancer.

This study clearly demonstrated that Se-PFPs could augment synergistically the anti-cancer function of DTX in ovarian cancer. However, the underlying molecular



**Figure 3.** Augmentation of DTX-induced tumor inhibition *in vivo* using Se-PFPs. The (A) weight and (B) volume of tumors from SKOV3-xenografted mice were shown. SKOV3 cells ( $4 \times 10^6$ ) were injected subcutaneously into mice. After ten days of injection, the mice were treated as described in this study. The tumor size was measured every 3 days. After 24 days of treatment, the mice were euthanized by CO<sub>2</sub> inhalation. The tumors were separated and weighed. C) IHC staining for Ki-67 (200x) and (D) TUNEL (40x). \* $p < 0.05$  (n=10), compared with the combined treatment group.

mechanisms are not clearly understood and still needs to be further elucidated. It has been reported that DTX can

induce suppression of peripheral blood mononuclear cells (PBMC) without decreasing expression levels of Th1-

derived cytokines. Whether or not the combination of DTX and Se-PFPs are involved in immunomodulation of different types of immune cells and in regulation of proinflammatory cytokines requires to be further investigated. Furthermore, based on the results of the experiments with HOSE, it can be concluded that the combination of DTX and Se-PFPs has relatively slight impact on normal ovarian cells (Figure 2C-D). However, it remains to be confirmed with other types of normal cells and to be determined whether inhibitory effects of their combination are tumor-specific; if it is proved to be the case, the combination therapy of DTX and Se-PFPs will be of therapeutic and clinical significance.

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## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# **Comparative analysis of capsaicin in twenty nine varieties of unexplored Capsicum and its antimicrobial activity against bacterial and fungal pathogens**

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In this study, the capsaicin content of chile pepper extracts from 29 unexplored varieties of *Capsicum* (twenty five varieties of *Capsicum chinense* and five of *Capsicum annuum*) was quantified and correlated with the antimicrobial potential against bacterial and fungal pathogens. The capsaicin content and bactericidal activity against numerous human pathogens (*Salmonella*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, *Candida albicans*) was compared to identify the most effective chile pepper varieties in the treatment of bacterial and fungal pathogens. The capsaicin content of the tested varieties varied from 29 to 42,633 ppm (139 - 682,135 SHU). On average, the fruits of *C. chinense* cultivars contained much higher concentrations of capsaicin than *C. annuum* cultivars. The undiluted chile peppers extracts with capsaicin concentrations greater than 25,000 SHU demonstrated bactericidal and antifungal effects. Overall, it was determined that *L. monocytogenes* and *S. aureus* were more susceptible to the antimicrobial effects of capsaicin than *Salmonella* and *E. coli* O157:H7, while *C. albicans* was markedly more susceptible than all bacterial species examined. The extract of the sixth most pungent cultivar, *C. chinense* Bhut Jolokia Red, showed the greatest antimicrobial potency of all screened peppers. The antimicrobial activity of pepper extracts was not directly correlated with increasing capsaicin concentrations, indicating that various *Capsicum* cultivars may possess distinct capsaicin derivatives. This is the first study which showed the relationship between capsaicin contents in different *Capsicum* varieties and their antimicrobial potential, and opens avenues in the study of capsaicin derivatives and their role in health and medicines.

**Key words:** Capsicum, pathogen, capsaicin, peppers, antimicrobial, bactericidal.

## **INTRODUCTION**

The emergence of antibiotic resistance has prompted scientists to explore medicinal plants, not only to ascertain claims of efficacy and safety, but also to develop phytochemical based new drugs as alternatives

to antibiotics. Plants of the genus *Capsicum*, colloquially referred to as chile peppers, have been widely grown for food and medicinal purposes for centuries. It is believed that the Aztec and Tarahumara peoples used chile

peppers as herbal remedies for coughs and bronchitis. Chile peppers have traditionally been used as antiseptics in African cultures, and in gastrointestinal and anti-inflammatory applications in India (Corson and Crews, 2007; Cichewicz and Thorpe, 1996). Capsaicin, the major bioactive molecule in *Capsicum*, has garnered medical attention due to its analgesic, anticancer, cardioprotective and antimicrobial properties (Omolo et al., 2014; Dorantes et al., 2008).

Additionally, capsaicin provides a unique characteristic and pungent taste to each variety of *Capsicum*. All plants of the *Capsicum* genus produce varied amount of capsaicin, however, only a few chile pepper varieties have been scientifically examined for their antimicrobial properties against bacterial and fungal pathogens. The antimicrobial activity of chile pepper extracts have been tested on a small selection of *Capsicum annum*, *Capsicum baccatum*, *Capsicum frutescens*, *Capsicum pubescens*, and *Capsicum chinense* cultivars, representing some of the milder members of the genus *Capsicum* (Cichewicz and Thorpe, 1996; Sanatombi and Sharma, 2008; Al Othman et al., 2011; Dorantes et al., 2000; Bacon et al., 2017). Only the Habanero variety of the most pungent *Capsicum* species, *C. chinense*, has been examined for bactericidal activity. Similarly, *C. chinense* varieties with extremely high capsaicin concentrations, such as the Trinidad Moruga Scorpion, Trinidad Douglah, Trinidad 7-Pot, Trinidad Scorpion, and Bhut Jolokia (“Ghost Pepper”) have never been tested for medicinal use (Omolo et al., 2014; Mills-Robertson et al., 2012).

The “hotness” of chile peppers has historically been expressed in Scoville Heat Units (SHUs), which was determined by a panel of human taste testers according to the Scoville organoleptic test. Once analyzed according to this method, chile peppers are sorted by their degree of pungency. There are five levels of pungency classification on the Scoville scale: non-pungent (0 – 700 SHU), mildly pungent (700 – 3,000 SHU), moderately pungent (3,000 – 25,000 SHU), highly pungent (25,000 – 80,000 SHU) and very highly pungent (>80,000 SHU) (Al Othman et al., 2011).

The organoleptic method is neither precise nor objective, and has led to the advancement of analytical methods to determine the capsaicin content of chile peppers. One such method is an antibody based capsaicin assay determines the amount of capsaicinoids present in peppers through comparisons to known standards in 96 micro-well plates and the use of a spectrophotometer.

Alternatively, Liquid Chromatography (LC) and High-Performance Liquid Chromatography (HPLC) have been

used to determine capsaicin content in peppers (Perkins et al., 2002; Sanatombi and Sharma, 2008; Al Othman et al., 2011). These analytical methods are more time consuming and costly than antibody-based assays. The antibody based capsaicin assay was selected for this study as it is a high throughput, rapid method that has demonstrated reliability of capsaicin content determinations for whole fruits in numerous other studies (Perkins et al., 2002; Stewart et al., 2007; Rollyson et al., 2014; Kantar et al., 2016).

Monitoring cellular respiration is a common way to assess the viability of cell cultures. This practice uses biochemically active compounds that change color in the presence of electrons generated by cellular respiration. Resazurin is a deep blue, tetrazolium-based, non-toxic, oxidation-reduction dye that is reduced intracellularly to the pink compound, resorufin, by electron-generating enzymes involved in microbial respiration (Ahmed et al., 1994; O’Brien et al., 2000; Byth et al., 2001; Węsierska-Gądek et al., 2005; Fai and Grant, 2009).

The resazurin assay is non-destructive to the cell, and is useful for monitoring the viability of numerous microorganisms under environmental stressors (Byth et al., 2001; Twigg, 1945; Liu, 1989; Guerin et al., 2001; Mariscal et al., 2009). The assay is simple, sensitive, rapid, robust, reliable, relatively inexpensive, and serves to assess antibacterial properties of natural products such as plant compounds (Zhi-Jun et al., 1997; Gloeckner et al., 2001; Sarker et al., 2007; Vega-Avila and Pugsley, 2011; Rampersad, 2012).

In this study, the resazurin assay was used as an indicator of cell viability after exposure to chile pepper extracts, to test 29 previously untested chile pepper varieties for their antimicrobial properties. The 29 chile pepper extracts were tested against several pathogenic organisms: the Gram-positive bacteria *Listeria monocytogenes* and *Staphylococcus aureus*, Gram-negative bacteria *Escherichia coli* O157:H7 and *Salmonella* Typhimurium, and the pathogenic yeast *Candida albicans*.

Presently, there has been no report where the capsaicin contents of the fruits of several *Capsicum* cultivars have been correlated with antimicrobial activity against bacterial and fungal pathogens. Many of the *Capsicum* varieties regarded as the “hottest in the world” have never been examined for antimicrobial properties.

Therefore, the unique focus of the current study was to cultivate 29 varieties of *Capsicum*, including the hottest known varieties, together in a controlled environment and study the correlation of capsaicin contents with antimicrobial potential against bacterial and fungal pathogens. We identified a *Capsicum* variety with low

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capsaicin content and showed bactericidal activity, very similar to the variety with highest capsaicin content. These results of the current study provide new direction to explore derivatives of capsaicin or additional bioactive molecules with applications in the treatment of topical and digestive human diseases caused by bacterial and fungal pathogens.

## MATERIALS AND METHODS

The resazurin dye powder was obtained from Difco Inc. (Corpus Christi, TX). The Capsaicin Plate Kit (Cat. # 20-0027) was purchased from Beacon Analytical Systems Inc. (Saco, ME). The capsaicin powder (99.1% pure) was purchased from Chem-Impex Int'l Inc. (Wood Dale, IL) and stored at 4°C. SSB14B stainless steel beads (0.9-2.0 mm diameter) were purchased from Thomas Scientific (Swedesboro, NJ), for tissue homogenization using a Gold Bullet Blender (Next Advance, Inc., Averill Park, NY). BBLTM tryptic soy agar (TSA), Becto™ tryptic soy broth (TSB), yeast extract peptone dextrose (YPD) media and BBLTM brain heart infusion (BHI) broth were purchased from Becton, Dickinson and Company (Sparks, MD).

### Cultivation of pepper plants

Seeds of 29 pepper varieties were purchased from various seed companies across North America (Table 1) to explore their phenotypic diversity. Seed samples were procured from different geographic regions, and represent two different *Capsicum* species (*C. chinense* and *C. annuum*) (Table 1). Seed were sown 0.6 cm deep in Master Garden Premium potting mix (Premier Tech Horticulture, Ltd., Ontario, Canada) in a 32-cell tray (volume = 150 cm<sup>3</sup>). Trays were covered with a clear plastic lid (approx. 10 cm above media) and were placed in a greenhouse maintained at 23°C air temperature. When seeds germinated and cotyledons unfolded, the lid was removed and plants were grown in the same environment for an additional 9-14 weeks under natural daylight plus 25 μmol·m<sup>-2</sup>·s<sup>-1</sup> supplemental irradiance (0800-0200 HR; Sunblaze T5 fluorescent lights, Sunlight Supply, Inc., WA, USA; +1.62 mol m<sup>-2</sup> d<sup>-1</sup>). Plants fertilized with Pure Blend Pro Grow (3-2-4), Pure Blend Pro Bloom (2-3-5), and Cal-Mag Plus (all from Botanicare, AZ) for a ten-week period following the manufacturer's recommendations. All the chile pepper plants were transplanted in the University of Minnesota agricultural fields in June of 2015, developed by applying Sustane 4-6-4 Organic Plant Fertilizer (Sustane Natural Fertilizer, Inc., MN) fertilizers as recommended by Bosland et al (Paul et al., 2012). Fruits were harvested in September 2014 (Figure 1). All the ripened pepper fruits were harvested from three plants of each chile pepper cultivar variety and stored at -20°C.

### Microbiological methods

*L. monocytogenes* Scott A, *Salmonella* Typhimurium LT2, and *Escherichia coli* O157:H7 EDL933 bacterial cultures were obtained from the Food Safety Microbiology Laboratory (Department of Food Science and Nutrition), while the *S. aureus* 6538 and *C. albicans* ATCC 10231 cultures were obtained from the Hegeman Laboratory (Department of Horticultural Science), at the University of Minnesota – Twin Cities (St. Paul, MN).

*L. monocytogenes* was streak plated on BHI agar media, while the other bacterial strains were plated on TSA agar medium.

*Candida albicans* was grown in YPD agar medium. The plates were incubated overnight at 37°C for bacterial strains and at 30°C for *C. albicans*, and subsequently stored at 4°C. The test cultures were prepared by inoculating 5 ml of respective broth media and incubating overnight at 37°C for bacterial strains and at 30°C for *C. albicans* with shaking at 150 rpm.

### Preparation of chile pepper extracts

The 29 chile pepper varieties included 24 very highly pungent varieties (samples 1 - 24) and five non-pungent varieties (samples 25 - 29) (Figure 1). For each variety, one pepper fruit was randomly selected from three different plants of each variety and treated independently, providing a total of 87 chile pepper extracts. The samples were prepared by crude methanol extraction as previously described (Beacon Analytical Systems, 2011), and this method was selected as methanol extractions of chile peppers have been shown to exhibit the greatest antimicrobial activity (Bacon et al., 2017). Whole pepper fruits were briefly washed under running tap water and dried with paper towels. 5 g of pepper fruits were cut into small pieces of approximately 5 mm and transferred to a 50 ml conical tube containing eight stainless steel beads, and homogenized in a Bullet Blender tissue homogenizer for 12 min to make a paste. The resultant pepper paste was mixed with 12.5 ml of 100% methanol and the slurry was further homogenized for 12 min. The methanolic fraction without the cell debris was centrifuged for 23 min at 4,000 rpm. The resultant pepper extracts were filter-sterilized through a 0.2 μm Acrodisc filter (Pall Corporation, NY). The methanolic pepper extracts were diluted 1:2 using sterile deionized water and stored at 20°C until used for the assays. The capsaicin contents of all the 87 extracts were determined using the Capsaicin Plate Kit, as per the manufacturer's instructions (Beacon Analytical Systems, 2011).

For the resazurin based cell viability assays, 0.02% (w/v) resazurin dye was prepared in deionized water, filter-sterilized through a 0.2 μm Acrodisc syringe filter and stored at 4°C until use (Sittampalam et al., 2014). For each test, 50, 25, 12.5, and 6.25 μl of 1:2 diluted pepper extract were added into each well of a sterile 96 well microtiter plate and diluted with BHI for *L. monocytogenes*, or TSB for all other test microbes to obtain 1:4, 1:8, 1:16 and 1:32 dilutions respectively. The resulting volume in each test well was 100 μl.

The microbial inoculum was prepared from an overnight culture by adjusting the OD<sub>600</sub> to 0.08 or 0.13, representing approximately 5 × 10<sup>6</sup> or 5 × 10<sup>7</sup> CFU/ml yeast and bacteria cells respectively. 100 μl of the bacterial or yeast cell inoculum was pipetted into each test well. As a positive control, an equal amount of methanol was mixed with BHI broth for *L. monocytogenes* and TSB for all other microbes and was pipetted into the positive control well and diluted with sterile broth media to a volume of 100 μl. 100 μl of inoculum was then added for a total volume of 200 μl. 100 μl of the appropriate broth was pipetted into the growth control well, along with 100 μl of inoculum. An equivalent amount of the methanol in the samples used was pipetted into the negative control well. The methanol was diluted with the appropriate broth to a total of 200 μl. 20 μl of the 0.02% resazurin dye was added into each well. The plates were incubated for 2 h at 37°C, photographed, and the results were recorded based on change in color from deep blue to pink or purple. The changes in color were observed and scored as 1 (no change in resazurin color), 0.5 (changed to indigo/purple) and 0.0 (changed to pink). Averages < 0.5 are reported as 0. Averages < 1 but ≥ 0.5 are reported as 0.5. Averages ≥ 1 are reported as 1.

### Statistical analysis

A student's *t*-test analysis was used to determine the statistical



**Table 1.** Summary of Chile pepper cultivar and their source used in the current study.

Chile pepper cultivar	Species	Seed source
Carolina Reaper		Puckerbutt Seeds, Fort Mill, SC, USA
Scotch Bonnet		Reimer Seeds, Saint Leonard, MD, USA
7-pod Congo SR Gigantic		
Brainstrain Yellow		
Trinidad 7-pot Primo		Pepperlover.com
7-pot Brown		
Trinidad 7-pot Brainstrain Red		
Yellow Moruga		
Trinidad Scorpion		
7-pot		
Trinidad Douglah		Refining Fire Chiles, San Diego, CA, USA
Trinidad 7-pot	<i>C. chinense</i>	
Habanero Orange Blob		
Trinidad Scorpion Chocolate		
Tobago Scotch Yellow		Dr. Joe Delaney, St. Paul, MN, USA
Tobago Scotch Bonnet Red		
Brown Scotch Bonnet		
Trinidad Moruga Scorpion		Chile Pepper Institute, Las Cruces, NM, USA
Bhut Jolokia Red		
7-Pot Jonah		
Congo Trinidad		
Trinidad Large 7-pod Yellow		Hugo Feed Mill and Hardware, Hugo, MN, USA
Brown Trinidad Moruga Scorpion		
HHP Moruga		
Orange Blaze		
Red Ruffled Pimiento		
Red Majesty Sweet		Hugo Feed Mill and Hardware, Hugo, MN, USA
Tasty Paprika GL	<i>C. annuum</i>	
Romanian Rainbow		

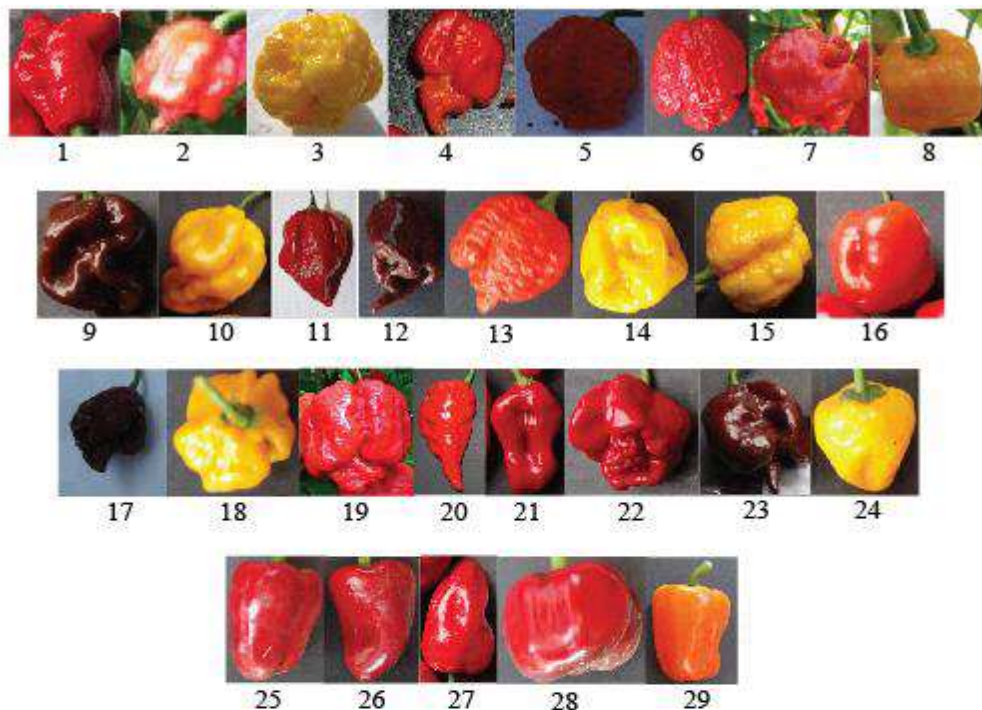
significance of the difference between samples, and between samples and standards.

## RESULTS

### Determination of capsaicin content

The 29 varieties of *Capsicum* used in this study were harvested from the farm field and are shown in Figure 1. The mature fruits of 15 *Capsicum* were red, 8 were yellow, whereas 6 were brown in color. The details of 29 varieties of *Capsicum* and their seed source are mentioned in Table 1. The extracts of chile peppers were subjected to quantitative estimation of capsaicin and its

correlation with antimicrobial activity. The extracts of 29 *Capsicum* varieties were subjected to quantitatively estimate the capsaicin content (ppm) as determined previously. The capsaicin content in 29 varieties varied from 29 to 42,633 ppm (Figure 2). In some extracts, the high standard deviation in capsaicin content is a reflection of variation in levels of capsaicin in three different fruits of the same plant, similar to variation reported in previous studies (Bacon et al., 2017). The capsaicin content (ppm) values were then multiplied by 16 to obtain Scoville Heat Unit (SHU) conversion values and summarized in Table 2. Based on SHU values, the twenty nine *Capsicum* varieties were classified into three broad categories. Nineteen *Capsicum* varieties were



- |                                      |                                    |
|--------------------------------------|------------------------------------|
| 1. 7 - Pod Congo SR Gigantic         | 16. Congo Trinidad                 |
| 2. Carolina Reaper                   | 17. Brown Scotch Bonnet            |
| 3. Brainstrain Yellow                | 18. 7 - Pot                        |
| 4. Trinidad Scorpion                 | 19. Trinidad Moruga Scorpion       |
| 5. 7 - Pot Jonah                     | 20. Bhut Jolokia Red               |
| 6. Trinidad Douglah                  | 21. Tobago Scotch Bonnet Red       |
| 7. Trinidad 7 - Pot Primo            | 22. HHP Moruga                     |
| 8. Habanero Orange Blob              | 23. Brown Trinidad Moruga Scorpion |
| 9. Trinidad Scorpion Chocolate       | 24. Scotch Bonnet                  |
| 10. Tobago Scotch Yellow             | 25. Red Majesty Sweet              |
| 11. Trinidad 7 - Pot                 | 26. Romanian Rainbow               |
| 12. 7 - Pot Brown                    | 27. Tasty Paprika GL               |
| 13. Trinidad 7 - Pot Brainstrain Red | 28. Red Ruffled Pimento            |
| 14. Trinidad Large 7 - Pod Yellow    | 29. Orange Blaze                   |
| 15. Yellow Moruga                    |                                    |

**Figure 1.** Fruits of chile peppers of 29 varieties. To prepare the chile pepper extracts, 3 fruits of chile peppers of each variety were randomly selected from each *Capsicum* plant.

classified as very highly pungent (>80,000 SHU), four as highly pungent (25,000-80,000 SHU) and six as non-pungent (0-700 SHU) as shown in Table 2. Trinidad 7 - Pot Brainstrain Red was the most very highly pungent variety with 682,136 SHU. Interestingly, all the five *C. annuum* varieties and one *C. chinense*, the Tobago Scotch Bonnet Red, were classified as non-pungent varieties. The six non-pungent varieties showed very trace amounts of capsaicin ranging from 139-470 SHU. Moreover, among non-pungent varieties, Tobago Scotch Bonnet Red showed more capsaicin content (470 SHU) as compared to all the five *C. annuum* varieties (139-241

SHU).

#### Evaluation of antimicrobial activity

The extracts of 29 varieties of *Capsicum* were tested for antimicrobial activity using a resazurin dye based assay using microwell plates. The capsaicin content in the extracts was correlated with antimicrobial activity. Different dilutions (1:4, 1:8, 1:16 and 1:32) were tested for antimicrobial activity against *L. monocytogenes*, *S. aureus*, *Salmonella*, *E. coli* O157:H7, and *C. albicans*.

**Table 2.** Summary of Capsaicin content of 29 chile pepper cultivars used in this study. Cultivars with similar capsaicin could be grouped together to make generalized conclusion.

Levels of pungency	Chile pepper cultivar	*SHUavg	*µg/mlavg
Very Highly pungent (> 80,000 SHU)	Trinidad 7 - Pot Brainstrain Red	682.135 ± 308.499	42.633 ± 13.031
	Trinidad Moruga Scorpion	649.544 ± 47.816	40.596 ± 2.989
	Trinidad 7 - Pot Primo	618.371 ± 254.201	38.648 ± 15.888
	Trinidad 7 - Pot	617.228 ± 182.863	38.577 ± 11.429
	HHP Moruga	556.929 ± 5.165	34.808 ± 323
	Bhut Jolokia Red	531.873 ± 100.398	33.242 ± 6.275
	7 - Pot Jonah	520.570 ± 26.809	32.536 ± 1.676
	Trinidad Douglah	474.156 ± 124.912	29.635 ± 7.807
	Trinidad Scorpion	452.326 ± 269.854	28.270 ± 16.866
	Brown Trinidad Moruga Scorpion	373.536 ± 65.883	23.346 ± 4.118
	7 - Pot	373.332 ± 36.784	23.333 ± 2.299
	7 - Pot Brown	310.395 ± 116.800	19.400 ± 7.300
	Trinidad Large 7 - Pod Yellow	181.834 ± 18.947	11.365 ± 1.184
	Yellow Moruga	171.877 ± 12.481	10.742 ± 1780
	Trinidad Scotch Yellow	159.662 ± 48.874	9.979 ± 3.055
	Trinidad Scorpion Chocolate	143.120 ± 6.044	8.945 ± 378
	Brown Scotch Bonnet	133.698 ± 74.470	8.356 ± 4.654
	Brainstrain Yellow	120.093 ± 68.353	7.506 ± 4.272
	7 - Pod Congo SR Gigantic	114.891 ± 30.554	7.181 ± 1.910
Highly pungent (25,000- 80,000 SHU)	Habanero Orange Blob	75.117 ± 16.779	4.695 ± 1.049
	Congo Trinidad	60.479 ± 10.484	3.780 ± 655
	Carolina Reaper	44.448 ± 25.544	2.778 ± 1.597
	Scotch Bonnet	27.789 ± 7.275	1.737 ± 455
Non-pungent (0-700 SHU)	Tobago Scotch Bonnet Red	470 ± 223	29 ± 14
	Red Majesty Sweet	241 ± 16	15 ± 1
	Romanian Rainbow	231 ± 10	14 ± 0.6
	Orange Blaze	202 ± 14	13 ± 0.9
	Red Ruffled Pimento	183 ± 6	11 ± 0.4
	Tasty Paprika GL	139 ± 5	9 ± 0.3

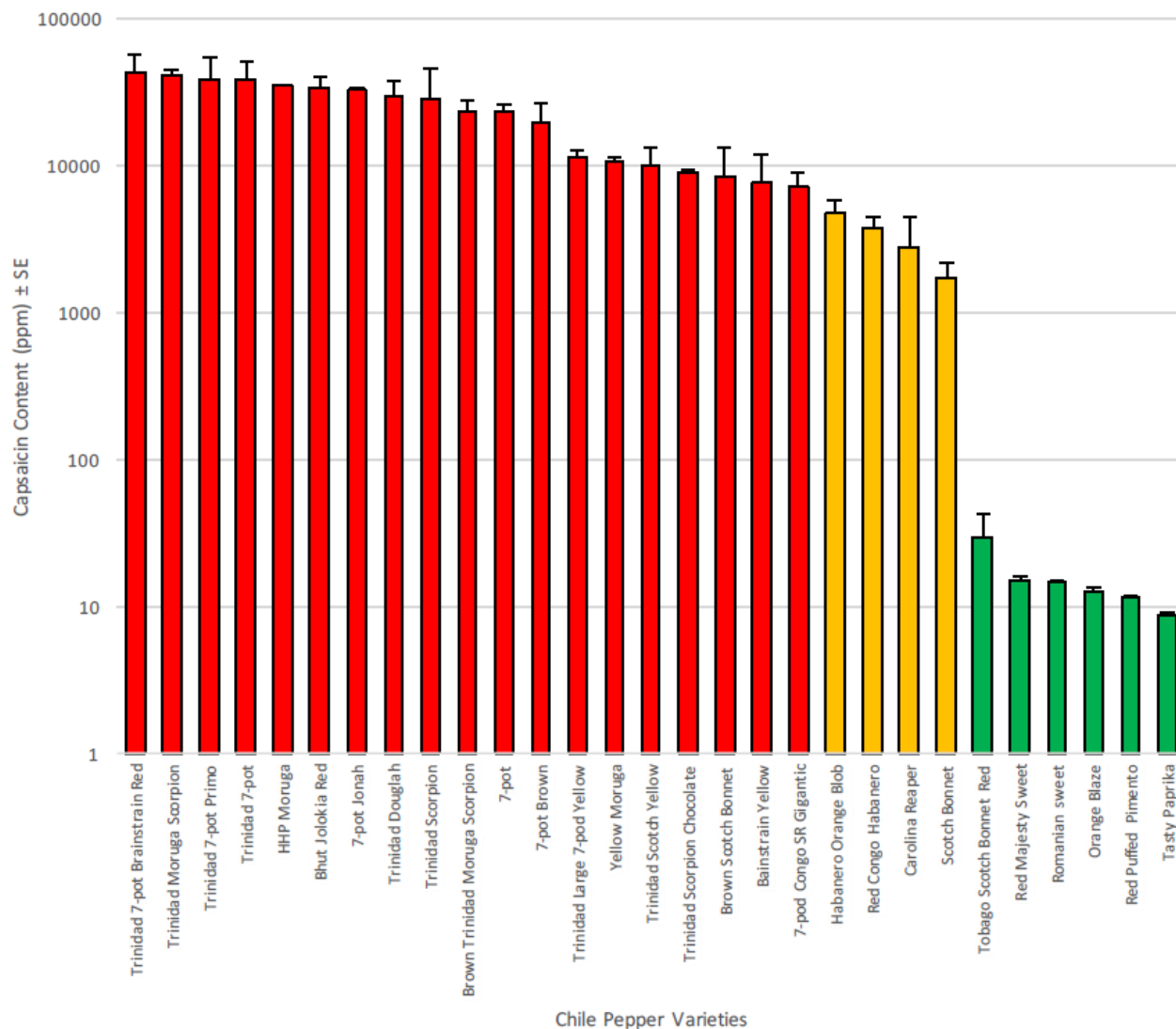
\*The data represent the average capsaicin content of triplicate samples (in SHU and µg/ml). The wide standard error margins (samples 1 – 24) are reflective of the wide range of capsaicin contents observed within the same chile pepper varieties.

After resazurin dye addition, color change was observed after 2 h. The results are presented in numerical scores based on the change in original dark blue color of resazurin. A dark blue, purple and pink colors represent 1.0 (bactericidal), 0.5 (partial bactericidal) and 0.0 (bacteriostatic) scores respectively. In addition, the negative control had a score of 1, while the positive and growth controls both had a score of 0.

As a control, pure capsaicin at 200 ppm (3,200 SHU) showed partial bactericidal effects on all the tested organisms. The minimum bactericidal concentration of pure capsaicin was approximately 250 ppm (4,000 SHU) for all microorganisms tested. The extracts with low capsaicin (<700 SHU), showed no effect on the cell viability of the microorganisms tested. For some extracts

even with high capsaicin content, showed partial bactericidal effect and it varied between *Capsicum* varieties as indicated in the Supplemental Table 1.

Among all the microorganisms, *C. albicans* was most sensitive to methanolic extracts of *Capsicum* and showed complete growth inhibition at 1:4 and 1:8 dilutions. Moreover, extracts of 20 *Capsicum* also showed partial growth inhibition even at 1:16 or up to 1:32 dilutions (Figure 3). At the 1:4 and 1:8 dilutions, the methanol concentrations in all the samples were high enough to kill the yeast cells as indicated by the positive control results. It was surprising to note that all the 29 extracts of *Capsicum* showed partial growth inhibition of both the Gram-negative bacteria (*S. Typhimurium* and *E. coli* O157:H7) even at 1:4 dilution. *S. aureus* and *L.*



**Figure 2.** Capsaicin content in 29 varieties.

*monocytogenes* showed highly varied responses to different extracts, which would confer a bactericidal effect on one of the organisms, but a bacteriostatic effect on the other.

Out of 19 very highly pungent *Capsicum* extracts, 14 showed bactericidal effect against *S. aureus* at 1:4 dilution. Extracts of the Bhut Jolokia Red cultivar showed partial bactericidal effect even at 1:16 dilution and extracts of the Trinidad Large 7 - Pod Yellow, Yellow Moruga, Trinidad Scorpion Chocolate, and Habanero Orange Blob showed partial bactericidal effect at 1:8 dilution. It is interesting to note that Habanero Orange Blob is highly pungent variety (75,117 SHU), but showed better antimicrobial activity against *S. aureus* and *L. monocytogenes* in contrast to Trinidad 7 - Pot Primo, which is classified as very highly pungent (618,371 SHU) variety and showed only partial bactericidal effect at 1:4

dilution. Similarly, Congo Trinidad with 60,479 SHU showed bactericidal effect, but is classified as highly pungent. All the six non pungent varieties showed no antimicrobial activity against any of the microorganisms tested.

Out of 19 very highly pungent *Capsicum* extracts, only 7 showed bactericidal effect against *L. monocytogenes* at 1:4 dilutions. Similar to *S. aureus*, extracts of Bhut Jolokia Red showed partial bactericidal effect even at 1:16 dilution, thus suggesting it is uniquely potent among other *Capsicum* varieties. Extracts of three very highly pungent *Capsicum* varieties, Trinidad Scorpion, Brown Trinidad Moruga Scorpion, and Trinidad Scorpion Chocolate, also showed partial bactericidal effect at 1:8 dilution. Similarly, Congo Trinidad with 60,479 SHU showed bactericidal effect at 1:4 dilutions, but classified as highly pungent. The highly pungent Carolina Reaper and Scotch Bonnet

**Table 3.** Antimicrobial scores for 29 cultivars of the chile peppers (*C. chinense* and *C. annuum*) tested against bacterial and fungal pathogens.

Chile pepper cultivar	Capsaicin <sub>avg</sub> (ppm)	<i>S. aureus</i> 6538	<i>S. Typhimurium</i> LT2	<i>E. coli</i> O157:H7 EDL933	<i>L. monocytogenes</i> Scott A	<i>C. albicans</i> ATCC 10231	Total score
Bhut Jolokia Red	33.242 ± 6.275	2.0	0.5	0.5	2.0	1	6.0
Trinidad Scorpion Chocolate	8.945 ± 378	1.5	0.5	0.5	1.5	1	5.0
Yellow Moruga	10.742 ± 1.780	1.5	0.5	0.5	1.0	0.5	4.0
Trinidad Large 7 - Pod Yellow	11.365 ± 1.184	1.5	0.5	0.5	1.0	0.5	4.0
Trinidad 7 - Pot	38.577 ± 11.429	1.0	0.5	0.5	1.0	1	4.0
Habanero Orange Blob	4.695 ± 1.049	1.5	0.5	0.5	1.5	0	4.0
Brown Trinidad Moruga Scorpion	23.346 ± 4.118	0.5	0.5	0.5	1.5	1	4.0
Trinidad Scorpion	28.270 ± 16.866	1.0	0.5	0.5	1.5	0	3.5
Trinidad Moruga Scorpion	40.596 ± 2.989	1.0	0.5	0.5	0.5	1	3.5
Trinidad Douglah	29.635 ± 7.807	1.0	0.5	0.5	0.5	1	3.5
Trinidad 7 - Pot Brainstrain Red	42.633 ± 13.031	1.0	0.5	0.5	0.5	1	3.5
HHP Moruga	34.808 ± 323	1.0	0.5	0.5	0.5	1	3.5
7 - Pot Jonah	32.536 ± 1.676	1.0	0.5	0.5	0.5	1	3.5
7 - Pot Brown	19.400 ± 7.300	1.0	0.5	0.5	0.5	1	3.5
7 - Pot	23.333 ± 2.299	1.0	0.5	0.5	0.5	1	3.5
Congo Trinidad	3.780 ± 655	1.0	0.5	0.5	1.0	0	3.0
Carolina Reaper	2.778 ± 1.597	0.5	0.5	0.5	0.5	0.5	2.5
7 - Pod Congo SR Gigantic	7.181 ± 1.910	1.0	0.5	0.5	0.5	0	2.5
Trinidad Scotch Yellow	9.979 ± 3.055	0.5	0.5	0.5	0.5	0	2.0
Trinidad 7 - Pot Primo	38.648 ± 15.888	0.5	0.5	0.5	0.5	0	2.0
Brown Scotch Bonnet	8.356 ± 4.654	0.5	0.5	0.5	0.5	0	2.0
Brainstrain Yellow	7.506 ± 4.272	0.5	0.5	0.5	0.5	0	2.0
Scotch Bonnet	1.737 ± 455	0.5	0.5	0.0	0.5	0	1.5
Tobago Scotch Bonnet Red	29 ± 14	0.0	0.0	0.0	0.0	0	0.0

varieties showed partial bactericidal effect at 1:4 dilution against *S. aureus*, and *L. monocytogenes*. All the six non pungent varieties showed no antimicrobial activity against any of the microorganisms tested.

In general, the Gram-positive bacteria, *S. aureus*, and *L. monocytogenes* were more susceptible to the bactericidal effects of capsaicin than the Gram-negative bacteria, *Salmonella* and *E. coli* (Figure 3). The screened yeast, *C. albicans*, was the most susceptible to capsaicin than both the Gram-positive and Gram-negative bacteria (Figure 3).

Table 3 summarizes the scores of all the results presented in the Supplementary data Table 1. The scores in the columns represent average data from three independent experiments as previously mentioned. The last column sums up these scores to determine the chile pepper sample with the highest antimicrobial score. Bhut Jolokia had the highest score of 6 and Tobago Scotch Bonnet Red showed lowest score of zero.

### Correlating capsaicin content with antimicrobial activity

Based on the student's *t*-test statistical analysis, it was

surprising that there is no significant difference between the scores from the chile peppers high in capsaicin ( $p > 0.05$ ). In contrast, there was a significant difference between the scores from the chile peppers low in capsaicin ( $p > 0.05$ ). However, the difference in scores was statistically significant among *Capsicum* varieties with high capsaicin and those with little to no capsaicin ( $p < 0.05$ ). The difference in scores was also statistically significant between the *Capsicum* extracts and the controls ( $p < 0.05$ ). In addition to this, that there was a moderate correlation between the capsaicin content and the antimicrobial scores listed in Table 3 and Figure 4.

### DISCUSSION

The Beacon Capsaicin Plate Kit assay is a polyclonal antibody assay specific for capsaicin determination of chile pepper fruits with reactivity to a limited number of closely related compounds (e.g. other capsaicinoids). Capsaicin is the most abundant capsaicinoid in chile peppers. The other capsaicinoids include dihydrocapsaicin, nordihydrocapsaicin, homodihydrocapsaicin and homocapsaicin. It has been established that milder

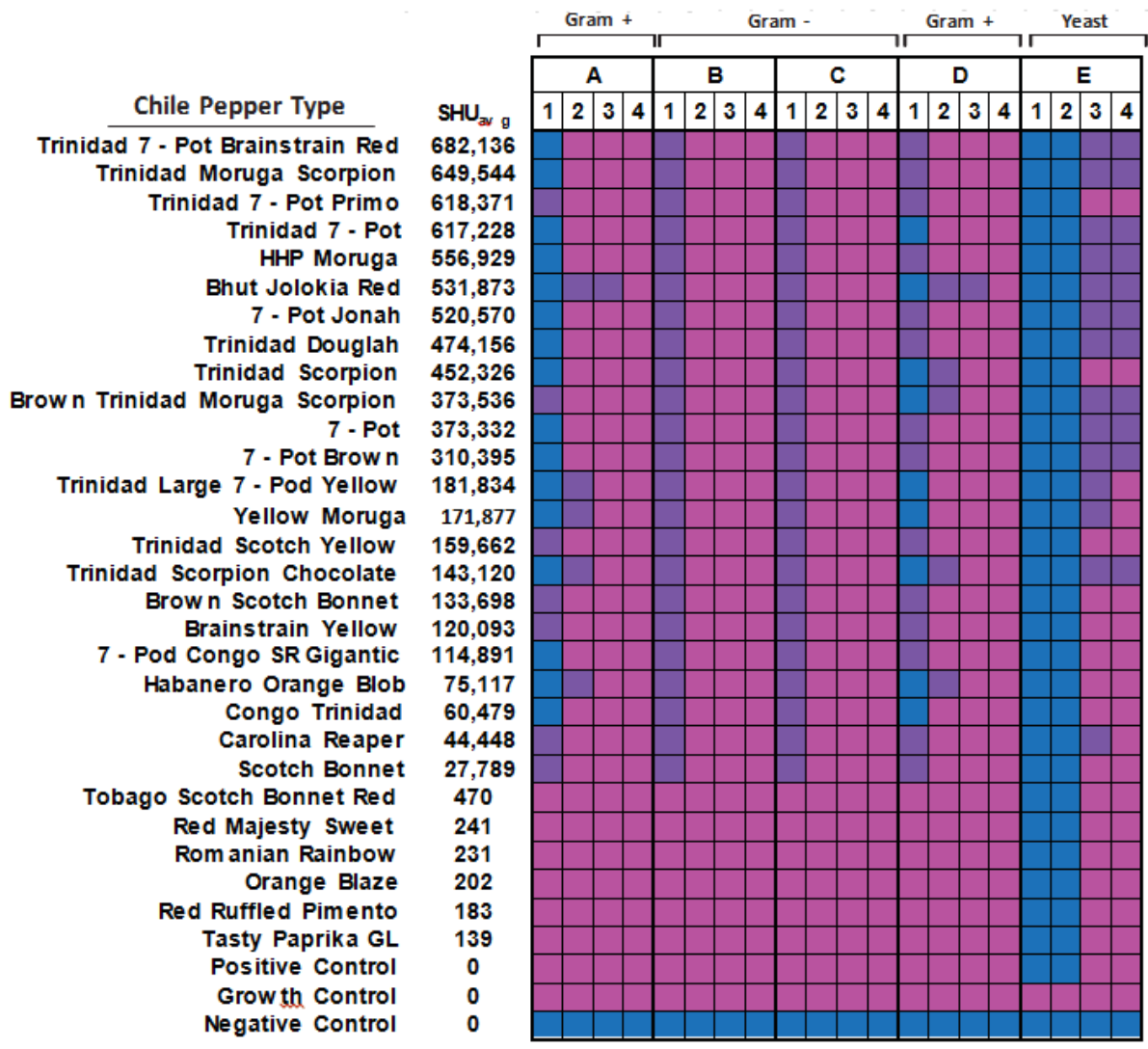


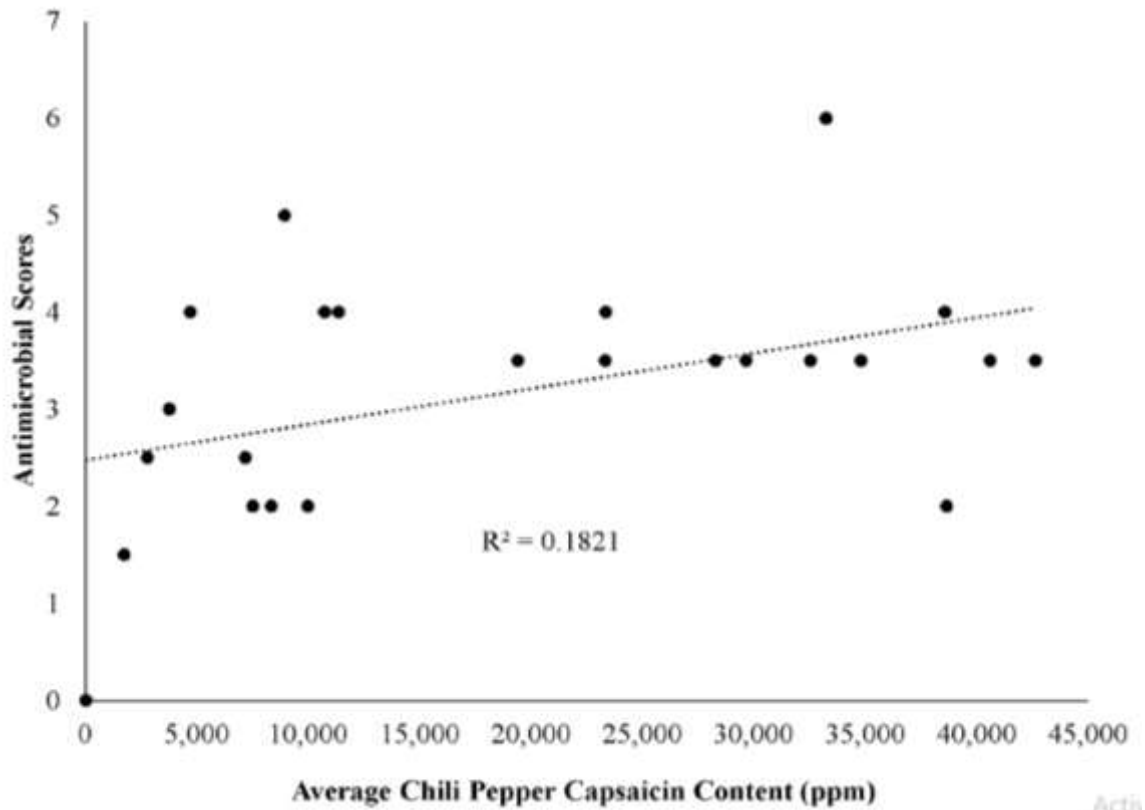
Figure 3. Extracts of 20 Capsicum.

varieties have capsaicinoid concentrations ranging from 0.003- 0.010% (w/w). Slightly hot varieties have capsaicinoids ranging from 0.01- 0.30% (w/w), while the hot varieties have a concentration range of 0.3- 1.0% (w/w) (Barbero et al., 2006). Based on this classification, the samples used herein from the *C. chinense* species were determined to be highly pungent, or very highly pungent. The samples from the *C. annuum* species were all non-pungent.

The amount of capsaicinoids in a chile pepper pod is dependent on the genetic makeup of the plant, the environment in which the plant is grown, the age of the fruit, and the position of the fruit on the plant. The heat level can vary considerably between plants of the same variety grown in a single field at the same time (Al Othman et al., 2011). When single plant heat levels of genetically identical chile pepper plants were compared

with the field average, it was found that individual plants could possess heat levels as much as 78% higher than the field average, indicating that plant to plant variation of an individual variety contributes to the chile pepper heat levels (Bacon et al., 2017). Similar variations were observed in the data obtained from the capsaicin assay in this study (Figure 2).

Capsaicinoid biosynthesis is unique to the genus *Capsicum* and results from the acylation of the aromatic compound, vanillylamine, with a branched-chain fatty acid. The presence of capsaicinoids is controlled by the *Pun1* locus, which encodes a putative acyltransferase. In its homozygous recessive state, *pun1/pun1*, capsaicinoids are not produced by the pepper plant (Rollyson et al., 2014). The trace amounts of capsaicin in the "sweet" chile peppers were most likely because ancestral chile peppers all produced capsaicin, and the



**Figure 4.** Antimicrobial scores for 29 cultivars of the chile peppers (*C. chinense* and *C. annuum*) tested against bacterial and fungal pathogens.

“sweet” chile varieties can revert to this ancestral trait.

The capsaicin data obtained in this study was used to determine whether there is a correlation between capsaicin content and bactericidal effects of the chile pepper extracts. While previous research suggests that capsaicin is the primary antimicrobial compound in chile peppers, data from this study implies that there might be other compounds or peptides with antimicrobial properties. Although Tobago Scotch Bonnet Red had very little capsaicin compared to the other samples of the *C. chinense* species, this cultivar showed some antimicrobial effects. This finding was unique because the other samples that had little capsaicin, but belonged to the *C. annuum* species, showed no antimicrobial properties. Since bactericidal activity of pure capsaicin was observed against all test organisms at >250 ppm (4,000 SHU), chile peppers lower than 4,000 SHU exhibiting partial or full bactericidal effect likely contain other antimicrobial compounds.

In this case, the Tobago Scotch Bonnet Red variety likely contains non-capsaicinoid antimicrobial compounds or peptides. Peptides isolated from the seeds of the habanero variety *C. chinense* have demonstrated antimicrobial effects against *S. aureus*, *E. coli* and other pathogenic organisms (Brito-Argaez et al., 2009). Such antimicrobial peptides may be responsible for the

inhibitory effects observed in the Tobago Scotch Bonnet Red cultivar. Additional studies to isolate and determine antimicrobial properties of these chemicals are underway.

Figure 3 summarizes the average results from the resazurin assay at the various dilution levels of chile pepper extracts against all organisms. Viable cells with active metabolism can reduce resazurin dye into the resorufin product, resulting in a color change in the test wells from dark blue to purple or pink. The quantity of resorufin produced is proportional to the number of viable cells (Promega Corporation, 2016). Therefore, a purple color in a test well implies fewer viable and consequently, fewer metabolically active cells.

*S. aureus* was tested against four different dilution levels of the pepper samples (1:4, 1:8, 1:16, and 1:32). At the 1:4 dilutions, 16 of the samples high in capsaicin showed total bactericidal effects, while 7 varieties showed partially bactericidal effects. When the samples were diluted 1:8, five of the samples high in capsaicin were partially bactericidal, while the rest showed no observable effects. At the 1:16 dilution, only Bhut Jolokia affected cellular viability of *S. aureus*. No bactericidal effects were observed for any of the samples at the 1:32 dilution.

None of the samples showed total bactericidal effects against *Salmonella* Typhimurium at the concentrations

tested. Instead, a partial bactericidal effect was observed (at the 1:4 dilution), indicated by a resazurin change in color to purple for 23 samples. A similar effect was noted for *E. coli* O157:H7 with a partial bactericidal effect observed for the same 23 samples.

Against *L. monocytogenes*, 9 samples had a bactericidal effect on the cells at the 1:4 dilution, while 14 samples were partially bactericidal. At the 1:8 dilution, 5 samples, Bhut Jolokia, Trinidad Scorpion, Brown Trinidad Moruga Scorpion, Trinidad Scorpion Chocolate and Habanero Orange Blob, exhibited partial bactericidal effects.

*C. albicans* showed greatest susceptibility to the samples with 14 samples showing a partial bactericidal effect at the 1:16 dilution. 11 samples diluted as much as 1:32 were still partially bactericidal to *C. albicans* cells. At the 1:4 and 1:8 dilutions, the methanol concentrations were high enough to kill the *C. albicans* cells, so the data for these two tests were included for completion only (Figure 3). Based on the findings shown in Table 3, it was concluded that *E. coli* O157:H7 and *Salmonella* Typhimurium were the least susceptible to the chile pepper extracts. This was expected, because both bacteria contain an additional outer cell membrane and are generally more resistant to antimicrobials (Ibrahim et al., 2010).

The susceptibility of the microbes tested to the chile pepper samples brings into question the mechanism by which capsaicin affects fungal cells. Kurita et al. (2002) suggest that capsaicin enters the cells and functions as a toxin, possibly to the membrane structure, and/or as an osmotic stressor. Work to understand investigate the possible mechanism is currently underway in our lab. Since hospital acquired skin infections due to multidrug resistant *Candida* spp. are on the rise worldwide (Borman et al., 2016), many of the chile peppers studied in this work may hold promising treatment remedies.

As previously mentioned, the ancient Aztec and Tarahumara cultures used chile peppers to treat hundreds of human diseases, and some historians believe the ingestion of chile peppers promoted a healthier gut and could treat some diarrheal diseases, possibly due to multicellular eukaryotic parasites. There have been studies demonstrating anti-parasitic properties of chile peppers against the ectoparasite *Ichthyophthirius multifiliis* to control disease in the aquaculture industry (Ling et al., 2012), but studies with human parasites have not yet been conducted. Future studies in these areas are warranted, since chile peppers may represent a relatively inexpensive way to treat many human diseases.

Table 3 shows the total overall scores of each pepper type for each microorganism tested in this study. Bhut Jolokia Red had the highest score, meaning it had the greatest effect on microbial viability on the organisms tested. Figure 4 illustrates the linear correlation ( $r$ ) between the antimicrobial scores and the capsaicin content of the *C. chinense* varieties. There was a moderate positive relationship between capsaicin

concentration and antimicrobial scores ( $r = 0.426$ ). Since there are over 30 classified chile pepper species, some may hold promise to treat human diseases, and studies in our lab are underway to investigate this further. Some question whether chile peppers are medically useful since they may irritate the patient. A recent study confirmed that human volunteers deemed capsaicinoid extract from the Bhut Jolokia chile pepper variety as an acceptable topical application for anti-arthritic and analgesic usage, thus overcoming an obstacle for future clinical applications of these important botanical sources (Sarwa et al., 2014, 2016). Since chile peppers are a widely accepted food item grown in every country in the world and known historically for broad-spectrum ethnopharmacological applications (Meghvansi et al., 2010), medical use of chile peppers, or isolated antimicrobial compounds, holds promise to treat many types of microbial infections and to numb pain caused by these associated diseases.

## Conclusions

In this unique study, 29 cultivars of chile peppers were grown under controlled environmental conditions and correlated the capsaicin content with antimicrobial properties against fungal and Gram-negative and Gram-positive human pathogens. Prior to this study, the hottest chile pepper to be tested for antimicrobial properties was a Habanero pepper (~100,000 SHU). The presented study examines many unexplored chile pepper varieties that have recently garnered attention due to their very highly pungent flavor. Included in the examined peppers are the hottest chile pepper varieties in the world (>1,000,000 SHU). Of the 29 cultivars tested, three very highly pungent cultivars (Trinidad 7 - Pot Brainstrain Red, Trinidad Moruga Scorpion, and HHP Moruga) with very high capsaicin content displayed bactericidal activity against *S. aureus* and *C. albicans*. Furthermore, the Bhut Jolokia (Ghost pepper) displayed bactericidal activity *S. aureus*, *L. monocytogenes*, and *C. albicans* even at 1:16 dilution of the extract, making it the most effective variety of *C. chinense* against the tested microbial panel. With the rise in multidrug resistant (MDR) "superbugs", specifically methicillin-resistant and vancomycin-resistant *St. aureus*, capsaicin and its derivatives from chile peppers should be further explored as antibiotic alternatives in the treatment of bacterial and fungal pathogens.

The results obtained in this study also highlight that capsaicin contents can vary widely between different cultivars of same *Capsicum* variety. Additionally, peppers with the highest capsaicin concentrations did not always correlate with the greatest antimicrobial activity, as the sixth most pungent variety, Bhut Jolokia Red, showed the greatest antimicrobial activity of all screen varieties.

Therefore, we propose the notion that different capsaicinoids have different antimicrobial properties. This



opens the avenue for synthetic biology to improve the activity of capsaicin to treat multiple diseases.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# Hepato-protective potentials of *Sterculia setigera* stem-bark extract on acetaminophen induced hepato toxicity in Wistar albino rats

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The study was set to investigate the potency of stem bark extract of *Sterculia setigera* as a hepato-protective agent against acute administration (overdose) of acetaminophen in experimental animals. Experimental animals were grouped into six treatments with each group containing five rats. Group 1 was the placebo, Group II was the standard treatment orally administered acetaminophen at a dosage of 250 mg/kgbw and thereafter treated with the standard drug silymarin at 100 g/kgbw after 6 h, to Group III (negative treatment) was orally administered acetaminophen only, at a dosage of 250 mg/kgbw without follow up treatment with standard drug (silymarin). Groups IV, V and VI were orally administered 70% methanol stem bark extract at a dosage of 200, 400 and 600 mg/Kgbw six hours after being orally administered with the hepatotoxic acetaminophen. The trial treatment was carried out for a period of three weeks. The inadequacy of herbs used in curing of liver diseases and other dysfunctions caused by allopathic drugs is enough reason to focus on systematic scientific research to evaluate some species of plants that are traditionally claimed to possess hepato-protective activities.

**Key words:** *Sterculia setigera*, stem-bark, extract, Wistar albino rats.

## INTRODUCTION

Liver is the largest organ in the body, contributing about 2% of the body weight in the average human. It is connected with most of the physiological processes which include growth, immunity, nutrition, energy

metabolism, reproduction, synthesis and secretion of bile, albumin, prothrombin and the reduction of the compliments which are the major effectors of the hormonal branch of the immune system (Dey and Saha,

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2013). Hepatotoxicity is the inflammation of the liver general associated with various drugs used in the modern medicine, different chemicals, toxins and viruses (Ravikumar et al., 2005; Stierum et al., 2005).

Hepatic problems along with heart problems are the major causes of death across the world. Roger and Pamplona (2001) and EllahiBukish et al. (2014) reported that, two million people die annually from liver related disorders with 60,000 occurring from hepatitis B alone. The WHO fact sheets (2005) also reveals that, more than 170 million people have long term liver infections with hepatitis C virus (Bartholomew et al., 2014).

Numerous medicinal plants and their formulations are being used for disorders in ethnomedical practices and in traditional system of medicine in different parts of the world. This situation arose from the fact that, conventional drugs used in the treatment of liver diseases are often unavailable, inaccessible and unaffordable particularly to the rural poor that suffer most, the burden of the disease (Anurag et al., 2013).

For instance, cirrhosis was the twelfth leading cause of death in the United States in 2007 and represented a large economic burden with the national cost of treatment ranging from \$14 million and above, in addition to the \$2 billion as an indirect cost due to loss of work productivity and reduction in the health-related quality of life (HRQOL). Depending on the disease aetiology, this has been on the progressive rise and is expected to significantly increase to \$2.5million and \$10.6 million respectively by the year 2028 (Teschke et al., 2012; Christopher and Taosheng 2017). Unwanted side effects experienced with most of the orthodox drugs are another major reason for a search of an alternative source of drugs against liver diseases.

The plant *Sterculia setigera* Del, of the family: Sterculiaceae is a savannah, widespread in the savannah areas of the tropical Africa. It is mostly found in the open savannah woodlands often characterised by stony hills. It is widespread in Nigeria and called by different tribes with different names. For instance, it is called Kukuuki in Hausa, Kokongiga in Nupe, Bo' boli in Fulfulde, Sugubo in Kanuri, Ose-awere/Esofunfun/Etula in Yoruba, Ompla/Upula in Idoma, Upuru in Igede, Ufia in Igala and Kaume-ndul/Kumenduur in Tiv languages (Igoli, et al., 2005).

This plant has wide application as a traditional remedy to various ailments in Nigeria. For instance, the Yorubas use its burnt stem bark in the preparation of black soap against dermatosis (Adjanahun et al., 1991), the Igedes also employ the stem bark decoction to treat diarrhoea (Almagoul et al., 1985). Several tribes in the North central Nigeria use the mercerated bark from the plant for the treatment of dysentery (Igoli et al., 2003). This work therefore, set to evaluate the potency of this plant (*Sterculia setigera*) as a hepato-protective agent that will be more affordable, accessible and readily available to the rural poor in Nigeria and the rest of sub-Saharan Africa.

## MATERIALS AND METHODS

### Plant material

The ethno-botanical survey was carried out in the surrounding villages namely, Old/New Awuru, Koro, Popo, Kere, Lubaruru and Dogongari villages around New-Bussa in Borgu local government area of Niger State. The main aim was to ascertain from the local people (particularly the elderly ones), the plant species commonly utilised in the traditional management of liver diseases. Part(s) utilised, method of preparation and period of harvest were also enquired from the interviewees. The identity of the plant was confirmed by Mr Musa Idris in the Department of Forestry, Federal College of Wildlife Management, New Bussa, Nigeria. The plant was deposited at the Forestry Research Institute Herbarium with an assigned voucher number FIH/Garba/NBS/1467.

### Preparation of the extract

The crude extract was prepared based on the method described by Garba et al. (2015). Briefly, fifty gram of the dried sample was pulverised to powdered form and cold extracted in 400 ml of 70% v/v (methanol/water mixture). Extraction lasted for 48 h. The extract was filtered using muslin cloth and the solvent was removed and recovered using rotary evaporator. The extract was then transferred into a sterile universal bottle and stored at 4°C until required for use. The yield of the extract was 5.46 g/50 g or 10.92% of the whole sample extracted.

### Phytochemical analysis

The phytochemical analysis of the extract from stem bark of *S. setigera* was carried out based on coloration and precipitation test as described by Trease and Evans (2002) and Sofowara (1982).

#### Test for alkaloids

0.5 g of extract was diluted into 10 ml with acid alcohol, boiled and filtered. To 5 ml of the filtrate was added 2 ml of dilute ammonia. 5 ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggen dorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggen dorff's reagent) was regarded as positive for the presence of alkaloids.

#### Test for phenols

1 ml of crude extract and Iron (III) chloride were mixed for 2 min. Formation of a deep bluish green colouration of the mixture indicate the presence of phenols.

#### Test for tannins

0.5 g of the extract was boiled with 10 ml of distilled water in a test tube and then filtered. A few drops of 10% of ferric chloride was added and observed for brownish green or a blue-black coloration.

#### Test for terpenoids (Salkowski test)

To 0.5 g of the extract was added 2 ml of chloroform. Concentrated

H<sub>2</sub>SO<sub>4</sub> (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

#### **Test for cardiac glycosides**

1 g of the extracts was treated with 2 ml of glacial acetic acid, a drop of 10% FeCl<sub>3</sub> and 1 ml of concentrated H<sub>2</sub>SO<sub>4</sub>. The appearance of brown coloration indicates the glycosides.

#### **Test for flavonoids**

5 ml of dilute ammonia was added to the aqueous portion of the extract followed by concentrated sulphuric acid (1 ml). A yellow coloration that disappears on standing indicates the presence of flavonoids.

#### **Test for saponins**

To 0.5 g of extract was dissolved in 5 ml of distilled water in test tube. The solution was shaken and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken after which it was observed for the formation of an emulsion.

#### **Test for anthraquinones**

1 ml of the plant crude extract was mixed with 1 ml of chloroform, then 10% NH<sub>3</sub> solution was added to the mixture. A brick red precipitate indicate the presence of anthraquinones.

#### **Test for phlobatannins**

0.2 g of the crude extract was mixed with 5 ml of 1% HCl in a test-tube and heated for 2 min. A red precipitate indicates the presence of phlobatannins.

#### **Test for steroids**

Five drops of concentrated H<sub>2</sub>SO<sub>4</sub> was added to 0.2 g of the extract. A reddish brown colour indicates the presence of steroids.

### **Experimental animals**

Thirty experimental animals (Wister rats) were purchased from animal house of the Federal University of Technology, Minna, Niger State. The rats were housed in a rat Pen(s) measuring 3 m × 2 m × 2.5 m. The floor surface was overlaid with sawdust which was changed at three days intervals to prevent mould growth. They were properly fed with rat's pellets and water *ad libitum*. They were allowed twelve days to get properly acclimatised with our laboratory conditions. The handling of the animals in the course of experimental work was done strictly based on the Canadian Council on Animal Care guidelines (CCAC, 1999).

### **Experimental design**

Thirty experimental animals (Wister rats) were grouped into six of five rats each (n=5).  
Group I was the placebo  
Group II was the standard treatment orally administered acetaminophen at a dosage of 250 mg/kgbw and thereafter treated

with the standard drug silymarin at 100 g/kgbw after six hours. Group III (negative treatment) was orally administered acetaminophen only, at a dosage of 250 mg/kgbw without follow up treatment with standard drug (silymarin). Groups IV, V and VI were orally administered 70% methanol stem bark extract at a dosage of 200, 400 and 600 mg/Kgbw six hours post oral administration with the hepatotoxic acetaminophen. The trial of induced toxicity and follow-up treatments with both standard drug and the extract was carried out for a period of three weeks.

### **Blood collection and measurement of parameters**

On the 22nd day, the experimental animals were sacrificed and the blood sample was collected in a heparinised and non-heparinised sample bottles for haematological and serum biochemical analysis respectively. The haematological parameters were determined using the automated haemato-analyser Sysmex kx21, (product of Sysmex corporation, Japan).

### **Calculation of absolute values**

The different absolute values such as, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated from values of RBC, PCV and Hb as follows: MCV (millimicron) = PCV% × 10 / RBC count (x million per mm<sup>3</sup>); MCH (picogram) = Hb g/dl × 10 / RBC count (x million per mm<sup>3</sup>) and MCHC (picogram) = Hb g/dl × 100 / PCV %

### **Determination of biochemical parameters**

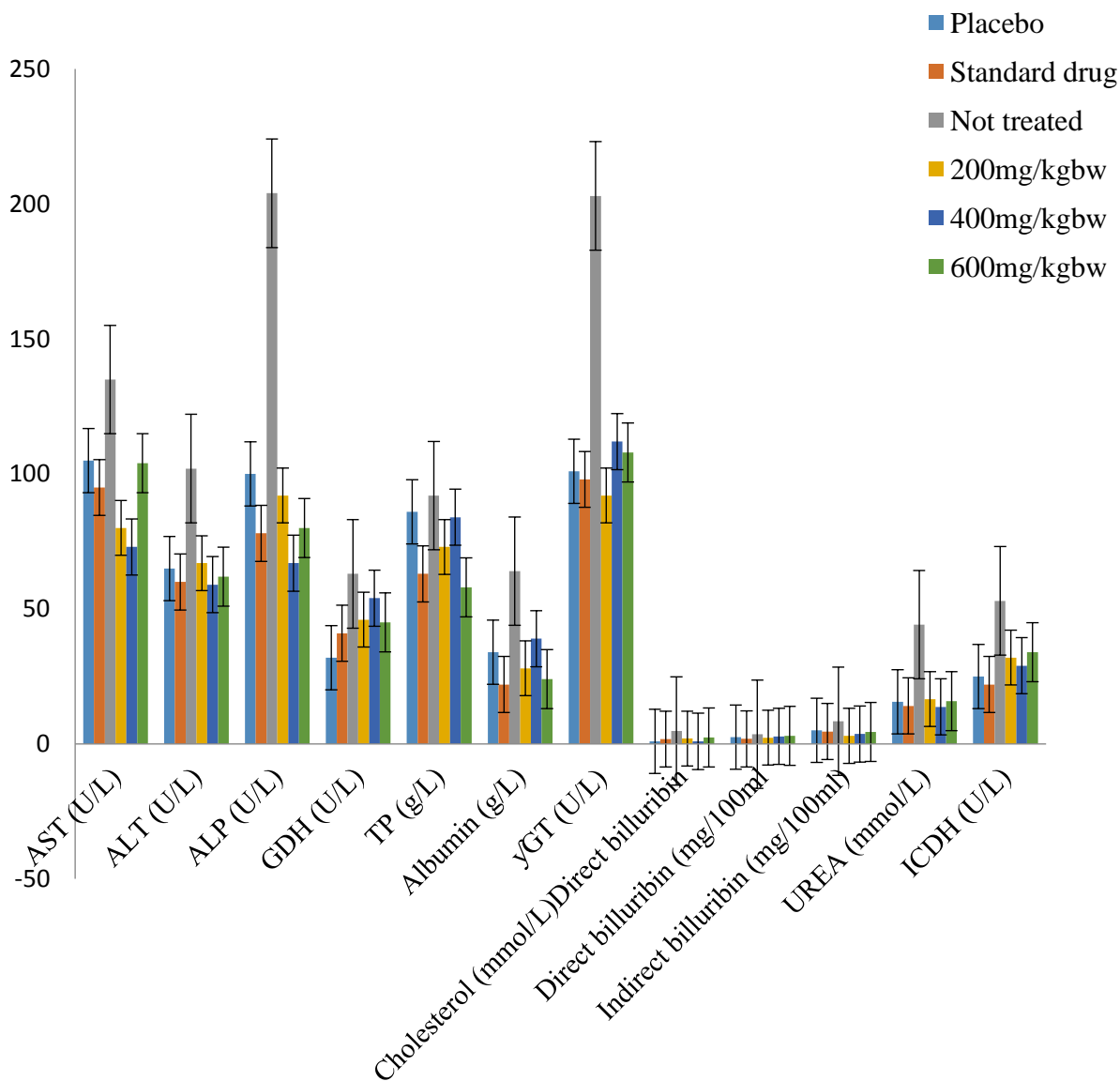
The biochemical analyses were determined for Alkaline phosphatase (ALP) based on methods of Tietz (1995) (Gornall et al., 1949), Aspartate transaminase (AST), Alanine transaminase (ALT), Gamma glutamyl transferase (γGT), and Isocitrate dehydrogenases (ICDH) as described by Reitman and Frankel (1957). While the serum total protein concentration was estimated by Biuret method as described by Gornall et al. (1949).

### **Statistical analysis**

The data are presented as mean ± S.E.M. All the data were analysed by one-way ANOVA and differences between the means were assessed with Duncan Multiple comparison test. Differences were considered significant at p ≤ 0.05. All analyses were carried out using Statistical Package for Social Science (SPSS) version 2.0 (USA).

## **RESULTS AND DISCUSSION**

From the result obtained in Figure 1, it is clear that the extract substantially protects the liver from the oxidative damage that usually characterise the continuous administration of acetaminophen. This becomes vivid from the ALT concentrations in all the other treatments (Placebo inclusive) that appear not to be significantly different (p ≤ 0.05) from one another, but with quite significant difference (p ≤ 0.05) from the values obtained from the negative control. Of interest is the fact that, the extracts from this plant appeared to be nearly as effective



**Figure 1.** Biochemical parameters of rats administered different concentrations of *Sterculia setigera* stem bark extract. AST = Aspartate transaminase, ALT = Alanine transaminase, ALP = Alkaline phosphatase, GDH = Glucose dehydrogenase, TP = Total protein, γGT = Gamma glutamyl transferase, ICDH = Isocitrate dehydrogenase.

as the standard drug (silymarinR) in maintaining the integrity of the liver cells, since high values of ALT is an indication of inflammation of the hepatocytes (Masto et al., 2018).

Conversely, while there is no significant difference ( $p \leq 0.05$ ) between the AST concentration in the standard treatment, 200 and 400 mg/Kgbw, there appeared to be significant difference between these groups and the placebo, negative control and 600mg/kgbw and the former groups showed no significant difference ( $p \leq 0.05$ ) in their AST concentrations. Myocardial injury/damage, might be the possible cause for the rise in the AST level in the former groups/treatments (Gao et al., 2015). The

ALP level is known to increase due to increased bone deposition, liver damage, hyperthyroidism, biliary tract disease, intestinal damage, hyperadrenocorticism, corticosteroid administration, barbiturate administration, and generalized tissue damage (including neoplasia) (Rangboo et al., 2016).

The levels falling within the normal range (in rats) in the standard group and the groups administered various concentrations (200, 400 and 600 mg/kgbw) is an indication of the extracts at these concentrations possessing similar protective and homeostatic role as the standard drug (silymarinR), while high value in the placebo group might not be unconnected to the raised

**Table 1.** Effect of 70% methanol stem bark extract of *S. setigera* on haematological parameters in acetaminophen induced hepatotoxic rats.

Treatment	HB(g/dl)	PCV (%)	MCV (mmicron)	MCH (pg)	MCHC (g/L)	RBC x10 <sup>6</sup> /mm <sup>3</sup>	PLC (x10 <sup>3</sup> /mm <sup>3</sup> )	TWBC (x10 <sup>3</sup> /mm <sup>3</sup> )	NEU (%)	LEU (%)	MON (%)
Placebo	22.5±2.34 <sup>c</sup>	30.0±4.3 <sup>b</sup>	56.0±3.34 <sup>a</sup>	30±2.22 <sup>a</sup>	75±1.33 <sup>a</sup>	5.0±5.32 <sup>c</sup>	1050±32.45 <sup>a</sup>	32.0±3.23 <sup>a</sup>	24.0±1.34	50.0±3.23 <sup>a</sup>	26.0±2.32
Positive control(Std drug)	25.9±2.35 <sup>c</sup>	32.0±4.21 <sup>b</sup>	63.0±5.55 <sup>c</sup>	32.0±2.12 <sup>a</sup>	80.9±1.23 <sup>d</sup>	8.0±4.23	2298±21.14 <sup>d</sup>	53.7±2.32	0.6±1.23 <sup>a</sup>	81.0±5.11 <sup>d</sup>	13.0±2.11 <sup>a</sup>
Negative control	13.5±2.57 <sup>a</sup>	20.0±3.56 <sup>a</sup>	73.0±4.21 <sup>d</sup>	41.0±2.63 <sup>b</sup>	67.5±1.14 <sup>b</sup>	3.2±3.22 <sup>a</sup>	1287±22.08 <sup>c</sup>	74.0±1.11 <sup>d</sup>	0.9±1.22 <sup>b</sup>	74.0±7.23	17.0±2.23
200mg/kgbw	19.4±2.45 <sup>b</sup>	20.0±1.32 <sup>a</sup>	46.0±4.22 <sup>a</sup>	50.0±2.67 <sup>c</sup>	97.0±1.56 <sup>c</sup>	4.0±3.32 <sup>b</sup>	1132±22.89 <sup>b</sup>	62.0±3.32 <sup>c</sup>	10.0±1.23 <sup>b</sup>	72.0±4.33 <sup>c</sup>	18.0±2.32 <sup>b</sup>
400mg/kgbw	17.9±2.45 <sup>b</sup>	20.0±1.32 <sup>a</sup>	43.0±3.23 <sup>a</sup>	46.0±2.13 <sup>b</sup>	89.5±0.32 <sup>bc</sup>	4.0±4.11 <sup>b</sup>	1023±21.13 <sup>a</sup>	50.0±2.22 <sup>b</sup>	11.0±1.45	72.0±5.23 <sup>c</sup>	17.0±2.12 <sup>b</sup>
600mg/kgbw	19.8±2.34 <sup>b</sup>	20.0±1.34 <sup>a</sup>	66.0±5.76 <sup>c</sup>	67.0±2.65 <sup>d</sup>	99.0±1.23 <sup>c</sup>	4.5±4.33 <sup>b</sup>	1118±31.22 <sup>b</sup>	62.1±1.14 <sup>c</sup>	12.0±0.12 <sup>c</sup>	66.0±3.45 <sup>b</sup>	22.0±2.32 <sup>c</sup>

Values are mean ±SEM of 3 determinations. The values along the row with different superscripts are significantly different ( $p \leq 0.05$ ).

**Table 2.** Qualitative phytochemical screening of *S. setigera*.

Plant sample	Tannins	Flavonoids	Alkaloids	Phenol	Saponin	glycoside
<i>Sterculia setigera</i>	+	+	+	+	+	+

levels of circulating steroids and biliary disease that may be inherent in some of the animals within the group (Tang et al., 2017) (Figure 1).

Despite its competing efficacy with the standard drug, it suffice to state that, the significant difference ( $p \leq 0.05$ ) observed in the GDH concentration between the groups treated with the extract and the standard groups confirms that, liver necrosis which is one of the side effects of orthodox drugs against liver diseases (Lemasters, 1999) is more pronounced in the extract compared to the standard drug and this will require further fractional purification to eventually isolate the notorious compound responsible for the observed necrosis. Other parameters such as  $\gamma$ GT, Albumin, Cholesterol, Direct and indirect billuribin were all found not to be significantly different ( $p \leq 0.05$ ) from the positive control but significantly different ( $p \leq 0.05$ ) from the negative

control and this is a clear attestation to the fact that their levels increases due to fatty meals, hepatic or biliary diseases (Ashtari et al., 2015).

Moreover, one unique pharmacological properties that could be envisaged to be possessed by *S. setigera* extract is its non-polycytonaemic effect when compared with the standard drug as shown in Table 1. The RBC, Hb and PCV values were significantly higher ( $p \leq 0.05$ ) in the group administered the standard drug when compared with the groups administered the extract. However, it remains to be established if the cause is relative or absolute. While the lower values obtained in the negative group might be attributed to the anaemia that usually characterised liver necrosis (Franchini et al., 2016) (Table 1). However, administration of both the standard drug and the *S. setigera* extract were observed to stimulate the immune mechanism of

the tested animals which might be extrapolated to mean the presence of some unusual compounds in both the standard drug and the extract since the placebo group were observed to have relatively lower values (Table 1). Additionally, higher Platelet counts observed in the group administered standard drug and the negative group further corroborates the earlier observation.

Qualitative analysis of the extract shows the presence of Flavonoids and phenols among other phytochemicals (Table 2). One of the flavones Naringin has been shown to play a significant role as a hepatoprotective agent (Lee et al., 2004). It was observed by Jayaraman and Namasivayam (2011) that, the Naringin is converted in monogastrics to Naringenin which was found to be a potent hepatoprotective agent. The possible presence of such compound in the flavonoids found to be contained in such plant might

attribute to the observed effect.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# Phytochemical analysis, antioxidant, antibacterial and combinational effects of medicinal plants used by Bapedi traditional healers to prepare herbal mixtures

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The aim of this study is to evaluate the biological activities of some plant species used in the preparation of herbal mixtures and determine the combinational effects. The plant materials were collected from traders who use them to prepare herbal mixtures claimed to be blood purifiers, pain-relievers, anti-diarrheal and aphrodisiac medicines. An aqueous decoction of each plant species was prepared. Thin layer chromatography (TLC) was used to establish the phytochemical profiles of the decoctions. Standard chemical tests were performed to screen for the presence of phyto-constituent groups. Total polyphenol content of the decoctions was also determined. The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay and ferric reducing power were used to assess the antioxidant activities of the extracts. The micro-broth dilution assay was used to determine antimicrobial activity. Fractional inhibitory index was used to determine the consequence of mixing different decoctions together. The phytochemical fingerprints of the decoctions showed that water mainly extracted polar compounds from the plants. Terpenoids, tannins and steroids were detected in all the decoctions. The decoctions of *Delonix elata*, *Veltheimia capensis*, *Sarcostemma viminalis*, and "Tšhikwana" had the lowest antioxidant activity. The twigs of *Kirkia wilmsii* exhibited the greatest antioxidant activity ( $EC_{50}$  15.71  $\mu$ g/mL) and further showed significant broad-spectrum antibacterial activity against diarrheagenic pathogens. The twigs of *K. wilmsii* had the greatest amount of total phenolics followed by *Hypoxis hemerocallidea* (corm) and *Monsonia angustifolia*. Majority of the aqueous extract combinations yielded indifferent and additive interactions. Although synergistic effects are sought after when mixing different plant extracts, this study demonstrated the complexities associated with combinations of aqueous extracts and the importance of appropriate knowledge on which species of plants should be mixed to enhance their therapeutic properties.

**Key words:** Phytochemical screening, antioxidant, antimicrobial, combinational effects.

## INTRODUCTION

Since the earliest of times, plants have been broadly used in various traditional healthcare systems to treat numerous diseases and to promote human health. In addition, they are vital to the development of

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pharmaceutical drugs because half of the drugs in the world are plant derivatives (van Wyk et al., 2013).

Phytochemicals such as phenolic compounds, alkaloids, diterpenoids, steroids, alkaloids and other complex compounds are secreted by plants as a defence mechanism against infections, predators and/or to also survive abiotic factors in the environment. Phytochemicals have been shown to have antioxidant, anticarcinogenic, antimicrobial, antiallergic, antimutagenic, and anti-inflammatory properties (Alesiani et al., 2010). It is, therefore, not entirely startling that traditionally, many medicinal plants were and continue to be used for treatment of diverse ailments (Madikizela et al., 2017).

The utilisation of plants for their therapeutic properties has been a commonplace practice in South Africa. This practice was motivated and accelerated by the abundance of diverse medicinal plant species which are spread across the country. As such, each geographically distal cultural group in the country may have different pharmacopeia for the treatment of similar ailments (Madikizela et al., 2017).

Through experience accumulated over the long use of medicinal plants and the increasing occurrence of health ailments in communities, traditional healers generally no longer prepare herbal remedies using a single plant species. Instead, they combine different plants species in an effort to increase efficacy and the healing potential of the therapies.

Due to the increased demand in plant-based products, the plant trade industry provides job opportunities and thus a much-needed source of income. This benefit brings about an intense competition that may motivate adulteration or the distribution of products of a substandard quality coupled with unsubstantiated claims (Ndhkala and van Staden, 2012).

The Limpopo Province has a high distribution of Bapedi traditional health therapists and they are distributed mainly in the Capricorn, Sekhukhune and Waterberg districts which collectively include more than 17 municipalities in which they are operational (Semenya and Potgieters, 2015). The traditional healers situated at the Capricorn district municipality sell the concoctions use plants such as *Hypoxis hemerocallidea*, *Drimia elata*, *Sarcostemma viminalis* and *Kirkia wilmsii* to prepare aqueous herbal mixtures prescribed for immune boosting, diarrhoea and relief of pain. The plants used by these traders have been reported to have numerous biological activities.

Eloff et al. (2010) demonstrated the antimicrobial activity of *K. wilmsii* by using hexane, acetone, methanol and dichloromethane extracts of its leaves against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporium canis* and *Sporothrix schenckii*. Katerere and Eloff (2008) reported significant antimicrobial activity of an ethyl acetate extract of the leaf and corm of *H.*

*hemerocallidea* against *S. aureus* and *E. faecalis*. The bulb *D. elata* has been reported to be used for treatment of asthma and pain (Semenya and Potgieters, 2015).

In the Limpopo Province it is mixed with several plants for the treatment of sexually transmitted infections (Semenya et al., 2013).

Although, the named medicinal plants were all used to prepare aqueous herbal mixtures, Matotoka and Masoko, (2018) reported only moderate antibacterial activity of the herbal mixtures against diarrhoeagenic microorganisms. In addition, the antioxidant activity of the herbal mixtures fell short to be comparable to ascorbic acid.

Needless to say, one has to then consider the consequence of plant combinations on the intended therapeutic activity of herbal mixtures. Several studies have reported that phytochemicals from different plant species can interact, and the outcomes of these interactions may be synergistic, antagonistic and/or non-interactive (Orchard and van Vuuren, 2017; van Vuuren and Viljoen, 2011). While the herbal mixtures sold at Ga Maja are prepared to improve biological activity, the underlying interactions of the different plants species is poorly understood. It was therefore, the focus on this study to assess the biological activities of the medicinal plants used by the traders to prepare the herbal mixtures and determine their combinational effects.

## MATERIALS AND METHODS

### Plant collection

The plants were collected in late summer from traditional healers in Lebokwagomo, Limpopo Province. The plant species in this study were selected for analysis because they have been claimed by the traders and traditional healers to be the ingredients that are used in the preparation of their commercialised herbal mixtures. The plant species were deposited at University of Limpopo's Larry Leach herbarium where voucher specimens were obtained. Table 1 shows the list of the collected plant species. During drying, the plant material was stored at room temperature away from sunlight. Large pieces such as the corms, twigs and stems were cut into smaller pieces to increase the surface area of the parts to allow for a quicker drying period. A commercial blender was used to grind the dried material into fine powders. During the course of this study, the powdered plant material was stored in air-tight glass containers and kept away from sunlight to prevent possible photo-oxidation reactions.

### Extraction

The herbal mixtures are normally prepared by the traders by boiling all the plant material together in a pot that is heated over wood fire. The boiling time ranges from 5 to 10 min. Therefore, to gain access to similar phytochemicals as the traders, separate water decoctions of each plant material was prepared. The decoctions were prepared by heating 10 mL of distilled water in a glass beaker for 5 min. Once the water began to boil, 1 g of the ground plant material was added to the water. The plant material was stirred thoroughly with a glass rod throughout the boiling process. After extraction, the extracts were filtered through Whatman No. 1 filter paper into pre-

**Table 1.** Collected plant species used by traditional healers to prepare herbal mixtures.

Plant material	Family	Voucher number	Vernacular name	Ethnopharmacological application
<i>Kirkia wilmsii</i> (leaves)	-	SS 94	<i>Legaba/Modumela</i>	
<i>Kirkia wilmsii</i> (corn)	<i>Kirkiaceae</i>	SS 94	<i>Legaba/Modumela</i>	Hypertension (Semenya and Potgieters, 2015)
<i>Kirkia wilmsii</i> (twigs)	-	SS 94	<i>Legaba/Modumela</i>	
<i>Hypoxis hemerocallidea</i> (corn)	<i>Hypoxidaceae</i>	SS 115	<i>Monna maledu</i>	Testicular tumours, impotency, cardiac diseases (Drewes et al., 2008).
<i>Monsonia angustifolia</i> (leaves)	<i>Geraniaceae</i>	121393	<i>Tee ya thaba</i>	Aphrodisiac (Fouche et al., 2015)
<i>Drimia elata</i> (corn)	<i>Hyacinthaceae</i>	S 18	<i>Sekanama</i>	Treatment of human immune-deficiency virus infections (Semenya et al., 2013)
<i>Sarcostemma viminale</i>	<i>Asclepiadaceae</i>	121404	<i>Moema</i>	Wound healing and treatment of ulcers (Semenya et al., 2013)
<i>Vahlia capensis</i>	-	121394	<i>Makgonatšohle</i>	-
" <i>Tšhikwana/Moroto wa tšhwene</i> " (Powder)*	-	-	-	-

*Tšhikwana/Moroto wa tšhwene* is a powdered plant mixture that is prepared by the traditional healers with claims that it has aphrodisiac and blood purifying properties. Among other unspecified plants species, the plant mixtures were said to be prepared by mixing *D. elata*, *S. viminale*, *V. capensis*. The traditional healers were unable to provide us with the full list of plant species for preparation because they indicated they secrecy of the recipe is essential for them to maintain a good relationship with their ancestral gods.

weighed glass vials and freeze-dried.

## Phytochemical analysis

### *Thin layer chromatography fingerprint profiles*

The method used to establish the phytochemical fingerprint profiles of the extracts was that of Kotze and Eloff (2002), detailed by Elisha et al. (2017).

The dried plant extracts were reconstituted with distilled water to a concentration of 10 mg/mL. 10 µL of each of extract was loaded onto thin layer chromatography (TLC) plates (Merck, Silica gel F<sub>254</sub>). The TLC plates were developed using solvents (Merk, technical grade) which differed in polarities that ranged from non-polar to polar (Table 2).

Once the mobile phase had reached the solvent-front, the chromatograms were removed from the TLC chambers. The mobile phases were prepared as detailed in Table 2. Chromatograms were visualised under ultraviolet (UV) light 254 nm. The TLC plates were sprayed with vanillin spray reagents (0.1 g vanillin [Sigma], 28 ml methanol, 1 ml sulphuric acid [Sigma]) and heated at 110°C for optimal colour development.

## Phyto-constituents

### *Total phenolics*

The total phenolic content of the plant decoctions was determined by using the Folin-Ciocalteu reagent method (Tambe and Bhambar, 2014), with minor modifications. The extracts (10 mg/mL) were diluted with 490 µL of distilled water to make up a final volume of 500 µL. This was followed by the addition of 0.25 mL of Folin-Ciocalteu reagent (Sigma) in each test tube. Sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) (Sigma) (1.25 mL) was added and the mixtures were incubated in the dark at room temperature for 30 min. An ultraviolet/visible (UV/VIS) spectrophotometer was used to determine the absorbance of the mixtures at 550 nm. A blank and the standard curves were prepared in a similar manner, except that the plant extracts were replaced by distilled water for the blank. Tannic acid (Sigma) was used as the standard for this procedure; whereby varying concentrations of tannic acid (1.25 - 0.08 mg/mL) were prepared. The results obtained from the linear regression formula of the tannic acid standard curve were expressed as milligram tannic acid equivalence/gram of extract (mg of TAE/g extract). The experiment was conducted in triplicates and independently repeated three times.

## *Total flavonoids*

The total flavonoid content was determined using the aluminium chloride method (Tambe and Bhambar, 2014). Briefly, 100 µL of 10 mg/mL of the decoctions was added to 4.9 mL of distilled water in a clean test tube. To this reaction mixture, 300 µL of 5% sodium nitrite (NaNO<sub>2</sub>) (Rochelle) dissolved in distilled water was added and the mixture was left at room temperature for 5 min. This was followed by the addition of 300 µL of 10% aluminium chloride (AlCl<sub>3</sub>) (Rochelle) (dissolved in distilled water). The reaction was allowed to stand for 5 min at room temperature. After the elapsed time, 2 mL of sodium hydroxide (NaOH) (Rochelle) was added to the solution. The mixture in the test tube was then made up to 10 mL with distilled water. Quercetin (Sigma) was used as a standard; whereby different concentrations (500 - 31.5 µg/mL) were prepared. The absorbance of the experimental samples and the standard were determined using a UV/VIS spectrophotometer at a wavelength of 510 nm. The blank was prepared in the same manner as the experimental samples with 100 µL of distilled water added instead of the extracts. The total flavonoid content of the samples was expressed as milligram quercetin equivalence/ gram of extract (mg QE/g extract).

**Table 2.** Phyto-constituents present in the decoctions of plant species used to prepare the herbal concoctions.

Phyto-constituent	Plant species											
	KwT	KwL	KwC	SV	HH	VC	MA	DE	PM1	PM2	PM3	PM4
Terpenoids	+	+	+	+	+	+	+	+	+	-	-	-
Alkaloids	-	-	-	-	-	+	-	-	-	+	-	-
Saponins	+	-	+	-	+	+	-	+	+	+	+	+
Flavonoids	+	+	+	+	-	+	-	-	+	+	-	-
Phlbatannins	-	-	-	-	-	-	-	-	-	-	-	-
Cardiac glycosides	+	+	+	-	+	+	+	+	+	-	+	+
Tannins	+	+	+	+	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	+	+	+	+	+	+	+	+

(+): Present, (-): absent, Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm); SV: *S. viminale*; HH: *H. hemerocallidea*; VC: *V. capensis*; MA: *M. angustifolia*; DE: *D. elata*; PM (1, 2, 3, 4): Different *Tshikwana* powders.

### Total tannins

The Folin-Ciocalteu method (Tambe and Bhambar, 2014) was used to determine the tannin content in the concoctions. Briefly, 100  $\mu\text{L}$  of 10 mg/mL of the concoctions and aqueous plant extracts was added to a clean test tube containing 7.5 mL of distilled water. The Folin-Ciocalteu reagent (Sigma) (0.5 mL) was added to the mixture and vortexed. Ten millilitres of a 35% solution of sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) was added to the mixture. The mixture in the tube was transferred to a 10 mL volumetric flask and the volume of the mixture was made up to 10 mL with distilled water. The mixture was shaken and kept at room temperature for 30 min in the dark. Gallic acid (Sigma) was used as a standard and reference standard solutions (1.0 - 0.625 mg/mL) were prepared. The absorbance for the solutions was measured against a blank that was prepared in the same manner as the test solutions. A UV/VIS spectrophotometer was used to measure the absorbance at 725 nm. Tannin content was expressed as milligram gallic acid equivalence/ gram of extract (mg GAE/g extract). The experiment was conducted in triplicates and independently repeated three times.

### Free radical scavenging activity assay

#### Thin layer chromatography-DPPH fingerprint profiles

Thin layer chromatography (TLC) coupled with 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma) was used to screen for possible antioxidant compounds in the plant decoctions. Deby and Margotteaux (1970)'s procedure was performed as detailed by Adamu et al. (2014). The chromatograms were prepared and developed in an identical manner to phytochemical fingerprinting. DPPH solution (0.2% w/v) was prepared by dissolving 0.2 g of the DPPH free radical in 100 mL of methanol. This solution was sprayed onto the air-dried chromatograms. The presence of antioxidant activity was indicated by the development of yellow bands against a purple background.

#### Quantitative free radical scavenging activity

The free radical scavenging activity of the extracts was determined by using the DPPH method (Chigayo et al., 2016), with modifications. Briefly, different concentrations of the extracts (250 - 15.63  $\mu\text{g/mL}$ ) were prepared to a volume of 1 mL of the solution. L-ascorbic acid (Sigma) was used as standard by preparing the same concentration range as the extracts. To these solutions, 2 mL of 0.2

mmol/L DPPH solution dissolved in methanol was added and vortexed thoroughly. The solutions were left to stand in the dark for 30 min at room temperature. The control solution was prepared by adding 2 mL of 0.2 mmol/L DPPH to 1 mL of distilled water. After the elapsed time, the solutions were analysed with a UV/VIS spectrophotometer set at a wavelength of 517 nm. The experiment was run in duplicate and repeated three times. Free radical scavenging activity of the extracts was expressed as percentage inhibition of DPPH from the control solution. The percentage was calculated as follows:

$$\%Inhibition = \frac{Ac - As}{Ac} \times 100$$

where Ac is the absorbance of the control solution and As is the absorbance of the extracts.

#### Ferric reducing power

The antioxidant activity of the decoctions was further evaluated using the ferric reducing power method (Vijayalakshmi and Ruckmani, 2016; Ahmed et al., 2012). Extract stock solutions and L-ascorbic acid (Sigma; 1.25 mg/mL) were serially diluted to obtain five different concentrations of the samples (625 - 39  $\mu\text{g/mL}$ ). The different concentrations of the solutions (2.5 mL) were mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (Rochelle) (1% w/v in distilled water), respectively, in a test tube. Thorough vortexing was done after addition of solutions. The mixtures were incubated at 50°C for 20 min. 2 mL of trichloroacetic acid (Rochelle) (10% w/v in distilled water) was added to the test tubes after incubation. The mixtures were centrifuged at 3000 rpm for 10 min and 5 mL of the resulting supernatant was transferred to a clean test tube. To this solution, 5 mL of distilled water and 1 mL ferric chloride (0.1% w/v in distilled water) were added consecutively with thorough vortexing after each addition. A UV/VIS spectrophotometer was used to read the absorbance of solutions at 700 nm wavelength. The blank for this procedure was prepared by the same procedure as the tests samples whereby in place of the extracts, an equal volume of distilled water was added. L-Ascorbic acid was used as a standard. The experiments were performed in duplicates and repeated three times.

#### Micro-dilution assay

The antimicrobial activity of the plant decoctions was evaluated by

**Table 3.** Total phenolic, flavonoid, tannin content and half maximal effective free radical scavenging activity (EC<sub>50</sub>) of the plant decoctions.

Plant species	Phenolics (mg TAE/g extract)*	Flavonoids (mg QE/g extract)*	Tannins (mg GAE/g extract)*	EC <sub>50</sub> * (µg/mL)
<i>K. wilmsii</i> (leaves)	61.51 ± 0.07	1.68 ± 0.12	16.62 ± 0.28	18.50 ± 0.242
<i>V. capensis</i>	53.65 ± 0.08	0.46 ± 0.01	47.02 ± 2.57	203.47 ± 4.743
<i>S. viminale</i>	52.84 ± 0.05	0.01 ± 0.04	3.85 ± 0.16	207.25 ± 4.780
<i>K. wilmsii</i> (Twigs)	88.84 ± 0.11	4.90 ± 0.09	22.76 ± 0.22	15.71 ± 0.245
<i>H. hemerocallidea</i>	65.69 ± 0.01	4.57 ± 0.21	8.08 ± 0.31	112.19 ± 0.783
<i>k. wilmsii</i> (Corn)	63.06 ± 0.08	1.45 ± 0.10	6.85 ± 0.09	34.45 ± 0.316
<i>D. elata</i>	53.29 ± 0.06	1.19 ± 1.36	6.97 ± 0.87	329.04 ± 2.116
<i>M. angustifolia</i>	66.84 ± 0.06	4.22 ± 0.05	8.21 ± 0.13	40.87 ± 0.030
<i>Tshikwana 1</i>	50.18 ± 0.11	9.44 ± 0.49	16.75 ± 0.16	109.67 ± 0.146
<i>Tshikwana 2</i>	50.83 ± 0.06	1.23 ± 0.07	8.28 ± 0.09	535.91 ± 0.371
<i>Tshikwana 3</i>	63.16 ± 0.15	3.70 ± 0.05	8.46 ± 0.04	85.85 ± 0.142
<i>Tshikwana 4</i>	52.5 ± 0.19	1.94 ± 0.20	-	237.53 ± 0.214

(-): No detectable concentration; mg TAE/g: milligram tannic acid equivalence per gram of plant extract; mg QE/g: milligram quercetin equivalence/ gram of plant extract; milligram gallic acid equivalence/ gram of plant extract. \*Values are mean of triplicates ± standard deviation (SD) (n=3)

determining the minimal inhibitory concentration against: *E. coli*, *P. aeruginosa*, *S. aureus*, *E. faecalis* and *C. albicans*. These species are common causative agents of infectious diarrhoea (Ahmed et al., 2012). The broth micro-dilution assay by Eloff (1998) and the modified version by Masoko and Eloff (2005) to suit fungal growth requirements were used. Sterile distilled water (100 µL) was added to each well of a round bottom 96 well microtitre plate. The extracts were concentrated to 10 mg/mL using distilled water and 100 µL of the solutions were separately serially diluted to 50% with the distilled water in the wells of the 96 well microtitre plates. Each culture (100 µL) was separately added to each well. The antibiotic Amphotericin B (Sigma) was used as a positive control and sterile distilled water as the negative control. The microtitre plates were covered with laboratory plastic wrap and incubated for 24 h at 37 °C for bacteria and at 25°C for the yeast. After incubation, *p*-iodonitrotetrazolium chloride (INT; Sigma) dissolved in sterile distilled water was used to detect microbial growth. The metabolically active microorganisms reduce the tetrazolium salt to a purple formazan. A volume of 40 µL of INT (0.2 mg/mL) was added to each well of the microtitre plates and further incubated for 30 min (bacteria) and 2 to 3 h (yeast). Minimum inhibitory concentration (MIC) values of the extracts were recorded as the concentrations of the lowest clear wells of each extract that was able to inhibit growth. The assay was repeated three times in duplicate.

### Synergistic, additive and antagonistic interactions

The effects of the combination of structurally different bioactive phytochemicals from the various plant decoctions were studied. The same *in vitro* methods as described earlier were used for assessing the combinational effects of the plant decoctions on antioxidant and antibacterial activity respectively. The ratio used for each mixture was a 1:1 of the extracts.

The fractional inhibitory concentration (FIC) of each plant extract was calculated in order to determine types of chemical interactions that occur when they are mixed (van Vuuren and Viljoen, 2011). However, this method was modified to optimise the analysis of the extract combinations towards antioxidant activity. For antioxidant activity, the FIC value for each extract in a combination was calculated by dividing the EC<sub>50</sub> value of the combination by the EC<sub>50</sub>

value of each plant decoction placed in the combination (equation a). For antibacterial activity, the MIC was used instead of the EC<sub>50</sub> values (equation b). The fraction inhibitory index ( $\sum FIC$ ) was then calculated by adding the two FIC values of the plant extracts in a combination (equation c).

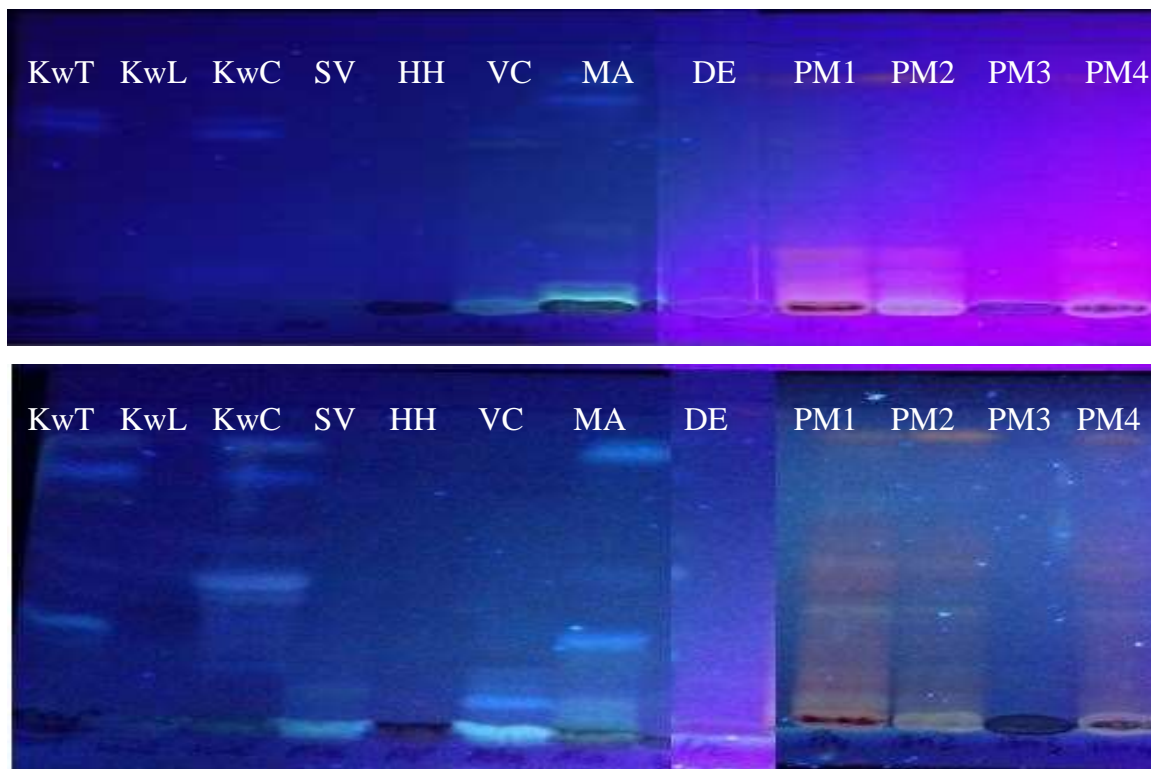
The interpretations of the fractional inhibitory index to explain the effect of the mixture of the decoctions are detailed in Table 3. Briefly,  $\sum FIC$  values ≤ 0.5 demonstrate synergistic interactions. For additive effects of the combinations,  $\sum FIC$  are > 0.5 to 1.00. The interpretation for indifference was  $\sum FIC$  that are > 1.00 - ≤ 4.00. Antagonistic interactions were interpreted as  $\sum FIC$  that is >4.00.

$$FIC = \frac{EC_{50}(\text{Combination A,B})}{EC_{50}} \quad (1)$$

$$FIC = \frac{MIC(\text{Combination A,B})}{MIC A} \quad (2)$$

$$FIC \text{ index} = \sum FIC = FICA + FICB \quad (3)$$

where EC<sub>50</sub> is the concentration of the extract that was about to inhibit 50% of the DPPH. MIC is the minimum inhibitory concentration of an extract towards microbial growth. FICA is the FIC for the first extract in the combination and FICB is of the second extract. Additive interactions occur when the therapeutic effect is of the sum of the individual components. The synergistic effects of a combination refer to when the therapeutic effect of the combination is greater than that of the individual constituents. Antagonistic interactions mainly refer to chemical interactions which result in the reduction or loss of a biological activity compared to the individual Phyto-constituents (Wang et al., 2011). Indifferent interactions indicate the non-interactive reaction of a combination that results in neither an additive nor antagonistic effect (van Vuuren and Viljoen, 2011).



**Figure 1.** Chromatograms showing a variety of 245 nm UV light fluorescent compounds present in the various plant decoctions. The extracts were eluted with non-polar (BEA) to polar (EMW) mobile systems. Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm); SV: *S. viminale*; HH: *H. hemerocallidea*; VC: *V. capensis*; MA: *M. angustifolia*; DE: *D. elata*; PM (1, 2, 3, 4): Different *Tshikwana* powders.

## RESULTS AND DISCUSSION

### Phytochemical analysis

The TLC separated phytochemicals were visualised under ultraviolet (UV) light of 254 nm wavelength. Under this spectrum, electrons in compounds undergo transition from a ground state to an excited state. When the electrons are de-excited to the ground state, they release energy in the form of an electromagnetic radiation wavelength of a lower energy than the UV light that range within the visible spectrum. The latter results in structurally diverse compounds to fluoresce and is visualised in different colours.

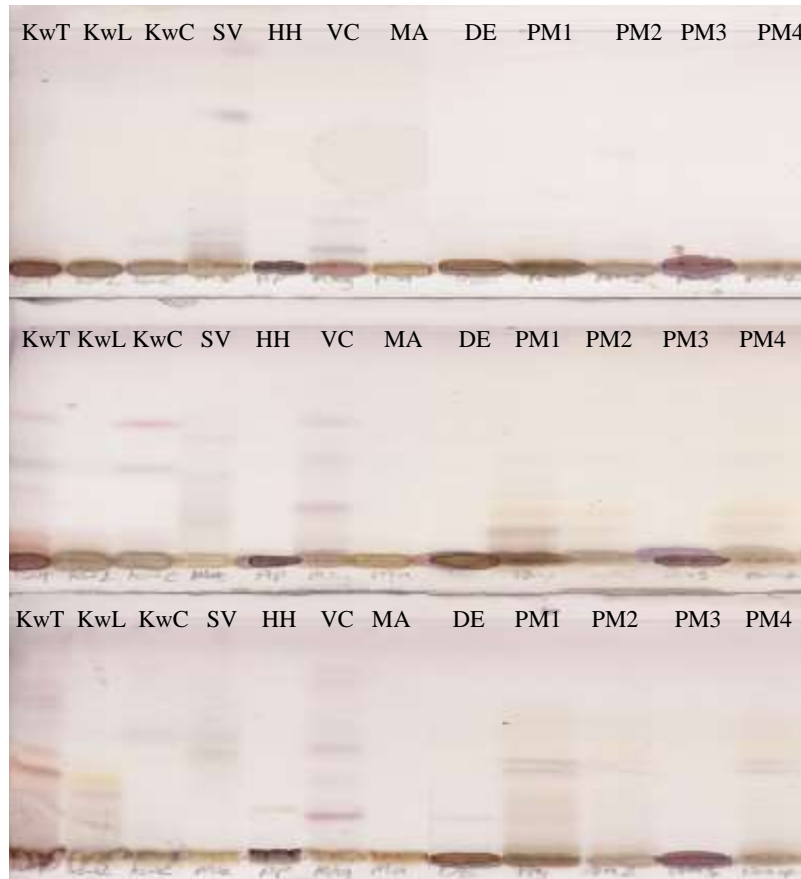
The number of coloured bands on a chromatogram reflects the number of fluorescent compounds present in the decoctions. The decoctions of the plants contained most polar compounds because many of them were visible in the chromatogram developed with the polar mobile phase (EMW) followed by the CEF and BEA chromatograms respectively (Figure 1). This observed trend has demonstrated that water extracted mainly polar compounds. This is because water has exhibited extreme polarity and as such it was expected to extract majority of polar compounds and discriminate against non-polar

compounds. Barba et al. (2016) have also reported the same principle.

The fluorescence indicated that the compounds consisted of conjugated double bonds and/or extended pi ( $\pi$ ) electron configurations (Ahmed et al., 2014). Polyphenols have been reported to have lengthy conjugated aromatic systems in their chemical structures (Dai and Mumper, 2010). Therefore, the various fluorescing compounds in Figure 1 may be due to the presence of polyphenolic compounds or their analogues.

The vanillin-sulphuric acid spray was used to establish the phytochemical profiles of compounds that were otherwise unable for fluoresce. The profile of these non-fluorescent compounds also showed that majority of them were polar (Figure 2). Major chemical classes that were detected were terpenoids (purple or bluish-purple bands), flavonoids (pinkish, yellow or orange) and proanthocyanidins (pink) (Ahmed et al., 2014; Tagana et al., 2011; Dai and Mumper, 2010).

Key phyto-constituents were qualitatively screened from the plant decoctions. The presence of terpenoids, tannins and steroids were common in all the extracts (Table 2). Terpenoids/terpenes were reported to have antibacterial and antioxidant activity (Zengin and Baysal, 2014). Flavonoids and tannins were documented to



**Figure 2.** Chromatograms of plant decoctions showing various coloured compounds that reacted with vanillin-sulphuric acid reagent. Different colours developed after heating the sprayed chromatograms at 110°C. Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm); SV: *S. viminalis*; HH: *H. hemerocallidea*; VC: *V. capensis*; MA: *M. angustifolia*; DE: *D. elata*; PM (1, 2, 3, 4): Different Tšhikwana powders.

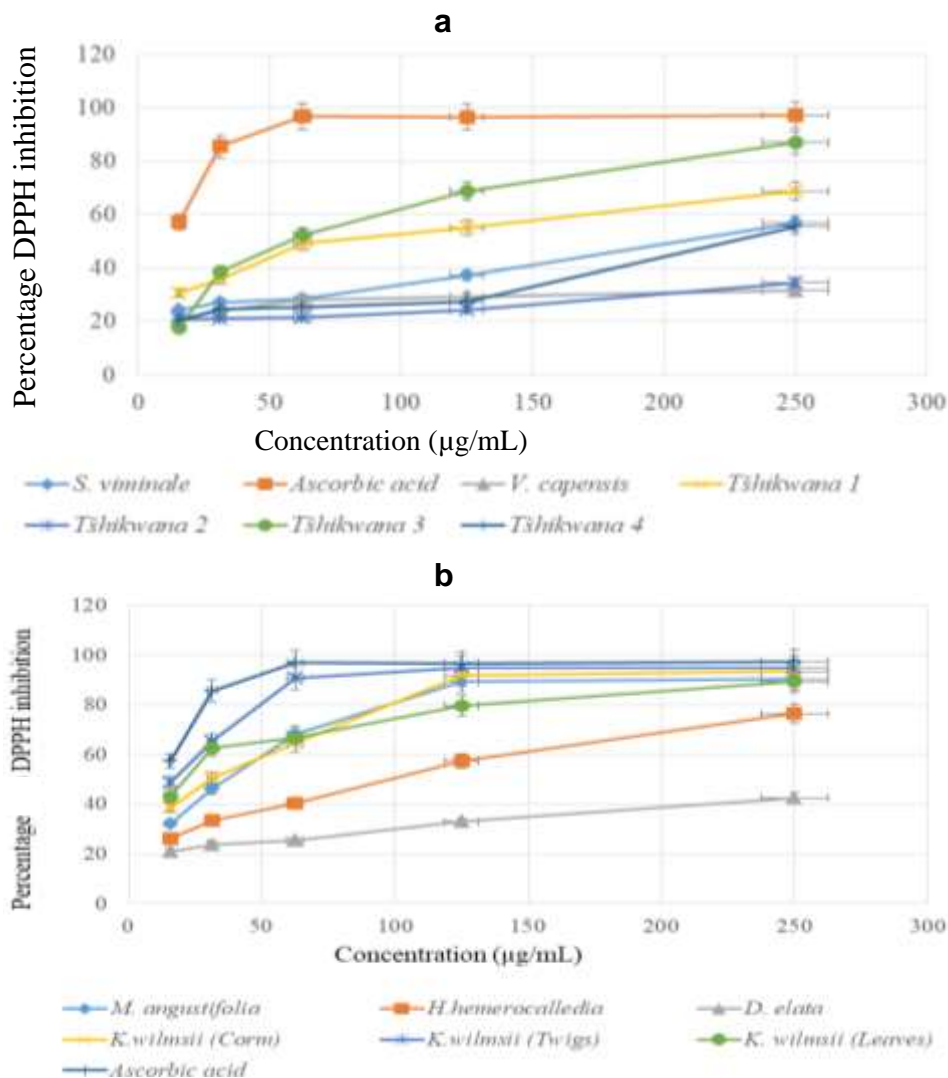
possess antioxidant, anti-allergic, anti-inflammatory, antimicrobial and anticancer properties (Valifard et al., 2014; Balasundram et al., 2006). The biological activities associated with saponins include anti-inflammatory, antimicrobial and cytotoxic effects (Sarikurkcu and Tepe, 2015). The presence of the different phyto-constituents in the plant extracts demonstrated an increased probability for these structurally diverse compounds to chemically interact and affect biological activity by either increasing or decreasing it.

The twigs of *K. wilmsii* (88.84 mg TAE/g extract) had the greatest amount of total phenolics followed by *Monsonia angustifolia* (66.84 mg TAE/g extract) and *H. hemerocallidea* (65 mg TAE/g extract). The flavonoids were highest in “Tšhikwana” 1 (PM1) (9.44 mg QE/g extract), followed by the twigs of *K. wilmsii* (4.90 mg QE/g extract) and *H. hemerocallidea* (4.57 mg QE/g extract). Tannin content was greatest in *V. capensis* (47.02 mg GAE/g extract) followed by the twigs of *K. wilmsii* (22.76 mg GAE/g extract) (Table 3). The high concentrations of

these polyphenolic constituents may be basis for the biological activity of the plant species.

Higher extraction temperatures were reported to enhance solubility and mass transfer rate of phytochemicals into a solvent. In addition, under such settings, the solvent is able to penetrate the cell membrane and matrices which improves the rate of extraction (Dai and Mumper, 2010). Henceforth, the choice by the traders to use boiling water as an extractant enables them to obtain considerable concentrations of bioactive compounds (phenolics, flavonoids and tannins) across the different parts of the various plant species especially the hard pieces such as the twigs and corms. Phenolics consist of one or more polar hydroxyl functional groups (Dai and Mumper, 2010), thus their high concentrations in the water decoctions was a reasonable outcome.

Studies on phenolics have led to the discovery of the biological and physiological properties, which include anti-microbial, antioxidant, anti-inflammatory, cardio-



**Figure 3.** (a) Percentage free radical (DPPH) inhibition by plants and the “*Tshikwana*” mixtures. L-ascorbic acid was used as a standard to which the activity of the extracts was compared. (b) DPPH inhibition of plant species with higher scavenging activity.

protective and vasodilatory activities (Goszcz et al., 2017; Shanmugapriya et al., 2013). The presence of these polyphenolic phyto-constituents in the plant decoctions displays a host of potential beneficial ways they can be used to improve health.

### Free radical scavenging activity

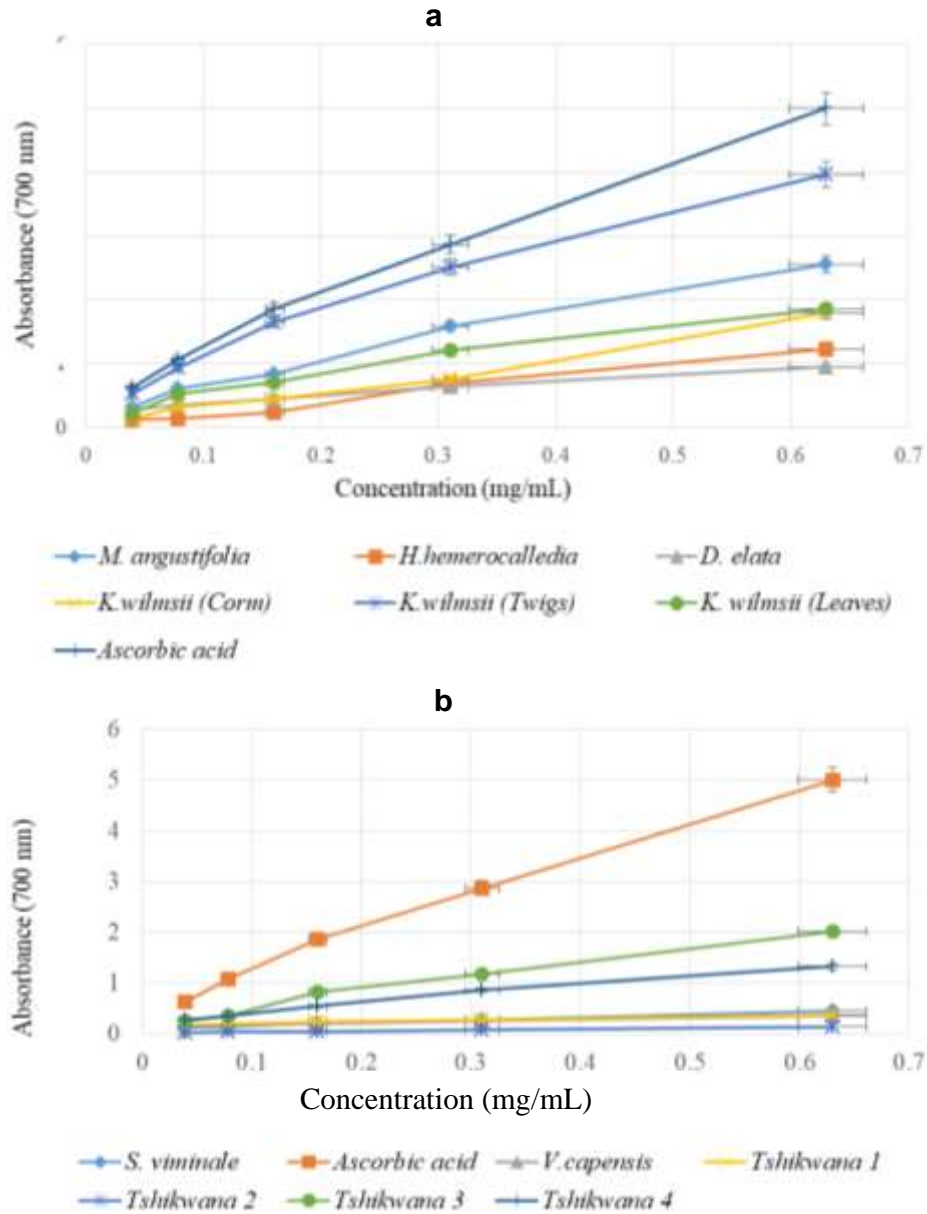
The antioxidant activity of the decoctions was compared with that of the standard, L-ascorbic acid (Figure 3a, b). The decoctions of *D. elata*, *V. capensis*, *S. viminale*, “*Tshikwana*” powders had the lowest antioxidant activity (Figure 3a). However, the twigs of *K. wilmsii* exhibited the greatest activity among the extracts and was comparable to that of L-ascorbic acid. This high activity was also

observed in the extracts from the leaves of *M. angustifolia* (Figure 3b).

The half maximal effective concentration ( $EC_{50}$ ) to inhibit DPPH was determined to further elucidate the effectiveness of the free radical scavenging activity of the extracts. Low  $EC_{50}$  values indicate that a small amount of the extract is required to inhibit half the amount of total the free radical in a solution. Higher  $EC_{50}$  values indicate the converse. For example, only 15.71  $\mu\text{g/mL}$  of *K. wilmsii* twig extract was required to inhibit 50% of DPPH in a solution whilst, a greater concentration of “*Tshikwana*” 2 (535.91  $\mu\text{g/mL}$ ) and *D. elata* (329.04  $\mu\text{g/mL}$ ) was required (Table 3). Of the plants, *K. wilmsii* displayed noteworthy antioxidant activity.

The species of *Kirkia* has been associated with high levels of fatty acids, flavonols, isocoumarin, lignans,





**Figure 4.** (a) The ferric reducing power of the plant decoctions compared with L-ascorbic acid. (b) The ferric reducing power of plants extracts at different concentrations expressed as absorbance at 700 nm wavelength.

neolignans, carotenoids, phenols and tannins which haven isolated from different parts such as the leaves, bark of the stem and roots (Maroyi, 2016). These compounds may be responsible for the high antioxidant activity of the twigs extract of the plant.

### Ferric reducing power

The ferric reducing power of the extracts was compared to that of L-ascorbic acid. As shown in Figure 4a, b,

greater absorbance was indicative of higher antioxidant activity. Moreover, the reducing power of the samples showed a concentration-dependent relationship. The twigs of *K. wilmsii* demonstrated the highest activity amongst the plant extracts followed by *M. angustifolia* (Figure 4a).

The ferric reducing power together with the free radical scavenging activity demonstrated that the aqueous extracts have more than one mode of antioxidant activity, that is, they can donate both protons and electrons. Furthermore, they showed that the different modes of

**Table 4.** Minimum inhibitory concentrations (MIC) of various plant decoctions against diarrheagenic bacteria.

Microorganism	MIC (mg/mL)												
	KWT	KWL	KWC	MA	SV	VC	HH	DE	PM1	PM2	PM3	PM4	Amp (µg/mL)
<i>E. coli</i>	0.16	0.31	1.25	0.04	5	5	0.63	> 5	2.5	2.5	1.25	1.25	0.08
<i>P. aeruginosa</i>	0.63	1.25	1.25	1.25	2.5	5	2.5	5	2.5	2.5	2.5	0.63	0.16
<i>E. faecalis</i>	0.63	1.25	2.5	0.63	2.5	> 5	0.63	> 5	1.25	2.5	2.5	2.5	0.13
<i>S. aureus</i>	0.63	2.5	2.5	1.25	5	5	1.25	5	2.5	2.5	0.63	1.25	0.16
Average	0.51	1.33	1.88	0.79	3.75	> 5	2.5	> 5	2.19	2.5	1.72	1.41	0.13

Kw (T, L, C): *K. wilmsii* (twigs, leaves, corn), SV: *S. viminalis*, HH: *H. hemerocallidea*, VC: *V. capensis*, MA: *M. angustifolia*, Del: *D. elata*, PM (1, 2, 3, 4): Different "Tshikwana" powder.

activity can vary in strength and efficacy. For example, an extract can have excellent free radical scavenging activity but low reducing power and vice versa. The combined effect of these different modes of action may increase the efficacy of the extracts to reduce oxidative-stress related disorders.

Some of the plant materials used, particularly "Tshikwana" powders, *S. viminalis*, and *D. elata* had the lowest reducing powers (Figure 4b) and free radical scavenging activity (Figure 3a). Therefore, these plant species may be omitted when preparing herbal mixtures. This would reduce the rate of harvest of these species and contribute to their conservation.

### Antimicrobial micro-dilution assay

The traders claimed that the plant species used to prepare the herbal mixtures had anti-diarrheal activity, hence, their inclusion in the recipe. To validate this ethnopharmacological use, four different strains of common diarrhoeagenic bacteria (*E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis*) and a yeast isolate (*C. albicans*) were selected to determine the antimicrobial activity of the plant decoctions.

The micro-dilution assay was used to determine the lowest effective concentration of the crude plant decoctions able to inhibit the growth of pathogenic microorganisms. This lowest concentration of the extract responsible for inhibitory effects was taken as the MIC with units of milligram per millilitre (mg/mL).

MIC values equal or less than 1.0 mg/mL were considered as noteworthy/significant antimicrobial activity (Orchard and van Vuuren, 2017). *S. viminalis*, *V. capensis*, *M. angustifolia*, *D. elata* and "Tshikwana" powders generally showed weak antibacterial activity. Significant activity against the four bacterial strains was observed with the decoction of the twigs of *K. wilmsii* (KWT) followed by the leaves of *M. angustifolia* (MA) with average MICs of 0.51 and 0.79 mg/mL, respectively across the Gram positive and negative strains (Table 4).

It was also remarkable that the Gram-negative bacteria (*E. coli* and *P. aeruginosa*) were generally more

susceptible to the plant decoctions. The antimicrobial activity of the extracts against the Gram-negative bacterial strains may be attributed to their abilities to inactivate microbial adhesion mechanisms, enzymes (peptidyl transferase) and cell membrane transport proteins (Ahmed et al., 2012). The plant species that have significant antibacterial activity against the diarrhoeagenic pathogens demonstrated their potential to be used to treat infectious diseases such as diarrhoea as indicated by the traders.

Further studies are required to isolate and characterise the antibacterial compounds in the extracts to investigate in-depth the mechanism of action that would lead to pharmacological effects. Active compounds isolated from plant resources may be used for further toxicological studies and pharmaceutical development (Gado et al., 2018).

### Combinational effects

A large number of combinations resulted in indifferent outcomes (Table 5). Despite this, some combinations which include *K. wilmsii* and *M. angustifolia* extracts demonstrated significant activity (MIC < 1 mg/mL). The only synergistic interaction detected was from the combination of *M. angustifolia* and *H. hemerocallidea* against *P. aeruginosa* (MIC of 0.63 mg/mL). The combinations that consisted of *V. capensis*, *S. viminalis* and *D. elata* generally resulted in antagonistic interactions. From Table 4, it was shown that these three species of plants had weak antibacterial activities against the diarrhoeagenic pathogens. Therefore, the presence of these plants in the combinations decreases the antimicrobial activity because they may be diluting active compounds in the solution.

There were also no synergistic effects of the combinations towards improving activity of the individual extracts. Most of the interactions were antagonistic and indifferent. Even so, the combinations exhibited significant growth inhibitory potential against *C. albicans* because the MIC values were lower than 1 mg/mL (Table 5). The combinational effects of the different plant

**Table 5.** Combinational effects of the plant decoctions towards antimicrobial activity.

Combination	<i>E. faecalis</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>C. albicans</i>
	FIC index ( $\sum FIC$ )				
KWT + KWL	0.75	2,5	6	3	4
KWT + KWC	1.25	2,5	6	4,5	3
KWT + MA	2	3	6	20	3
KWT + HH	2	3	5	5	2.25
KWL + KWC	0.75	2	2	2,5	3
KWL + MA	1.5	3	4	9	0,75
KWL + HH	3	1,5	4,5	3	18
KWC + MA	0.63	4	2	8.25	2
KWC + HH	2.5	3	3	3	10
MA + HH	2	4	0.38	17	1.25
SV + VC	1.5	1	1.5	1	4.97
SV + DE	1.5	1	1.5	1	4.97
SV +KWT	5	2.25	2.5	4.14	9.79
SV + KWL	3	1.5	3	4.28	9.79
SV + KWC	2	1.5	3	2.5	12.03
SV + MA	2.5	2.5	3	16.16	3.03
SV + HH	5	2.5	2	4.47	5.97
VC + DE	1	1	1	1	2
VC + KWT	4.5	2.25	4.5	8.06	8.31
VC + KWL	2.5	1.5	2.5	8.56	16.63
VC + KWC	1.5	1.5	2.5	2.5	9.06
VC + MA	4.5	2.5	2.5	32.3	9.06
VC + HH	4.5	2.5	1.5	4.5	3
DE + KWT	4.5	4.5	4.5	8.06	8.31
DE + KWL	2.5	1.5	2.5	4.28	16.63
DE + KWC	1.5	1.5	2.5	2.5	9.06
DE + MA	4.5	2.5	2.5	16.28	9.06
DE +HH	4.5	2.5	1.5	4.47	3

Kw (T, L, C): *K. wilmsii* (twigs, leaves, corm), SV: *S. viminale*, HH: *H. hemerocallidea*, VC: *V. capensis*, MA: *M. angustifolia*, DE: *D. elata*, PM (1, 2, 3, 4): Different "Tshikwana" powders,  $\sum FIC$ : Sum of fractional inhibitory concentrations,  $\sum FIC \leq 0.5$ : Synergistic,  $\sum FIC > 0.5 - 1.00$ : Additive,  $\sum FIC > 1.00 - \leq 4.00$ : Indifferent,  $\sum FIC > 4.00$ : Antagonistic.

species suggested that the decoctions of the plants would be more efficacious when used singularly than in combinations.

## Conclusion

The noteworthy biological activities exhibited by some of the plant species validate their potential for use for therapeutic purposes. Although synergistic effects are sought after when mixing different plant species, this study demonstrated the complexities that are associated with mixing plant decoctions. Moreover, adequate knowledge is required to know which species of plants should be mixed to enhance a therapeutic response because diverse chemical groups are prone to interact

and may alter the strength of biological activity.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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