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Full Length Research Paper

Phytochemical analysis and *in vitro* antibacterial evaluation of leaf and bark extracts of *Alstonia boonei*

D. J. Ajose¹, O. F. Onifade^{2*} and I. I. Wemambu¹

¹Department of Biological Sciences, College of Natural and Applied Sciences, Bells University of Technology, Ota, Ogun State, Nigeria.

²Department of Food and Chemical Sciences, College of Natural and Applied Sciences, Bells University of Technology, Ota, Ogun State, Nigeria.

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The antibacterial effect of the leaf, bark and leaf and bark combined of *Alstonia boonei* on bacterial pathogens, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pneumonia*, *Proteus mirabilis*, *Klebsiella pneumonia*, *Shigella dysenteriae* and *Salmonella typhimurium* was determined using agar diffusion technique to investigate their efficacy as anti-bacterial agent. The phytochemical components of the leaf and bark were studied. The phytochemical screening of the ethanolic leaf extract indicated the presence of tannins, phlobatannins, alkaloids, and cardiac glycosides, reducing sugar, saponins, anthraquinones and steroids, while the bark contains alkaloids, flavonoids, saponins, phlobatannins, anthraquinones, steroids and reducing sugars. The extracts showed varying inhibitory effect against *P. aeruginosa* and *K. pneumonia*. *S. typhimurium* (13±0.27 mm at 100 mg/ml for the leaf extract) being the most susceptible at all concentrations, 12±0.21 and 12±0.62 mm at concentrations 100 and 50 mg/ml respectively for bark extract and similarly for the leaf + bark extract. The result of the study suggests that the extracts of the different component parts of the plant can be used for the treatment of infections caused by the test organisms.

Key words: *In vitro*, antibacterial, *Alstonia boonei*, extracts, agar diffusion.

INTRODUCTION

Medicinal plants are natural products which provide numerous essential services in the ecosystem (Firenzuoli and Gori, 2007). It is also taken internally or used to bath as a remedy for dizziness, and given after childbirth to aid the delivery of the placenta (Orwa et al., 2009). The leaves, pulped to a mash, are applied topically to reduce

oedema, and leaf sap is used to cleanse sores (Orwa et al., 2009).

Various ethnopharmacological studies that have been carried out on this plant products which showed that the extracts possess antimalarial, antipyretic, analgesic and anti-inflammatory properties (Ojewole, 1984, Olajide et

*Corresponding author. E-mail: onifadeolayinka@gmail.com, Tel.: +234(0)8036641466.

al., 2000; Bello et al., 2009; Onifade and Maganda, 2015) anthelmintic (Weshche et al., 1990), diuretic, spasmolytic and hypotensive properties (Kucera et al., 1972), immunostimulant property (Taiwo et al., 1998), antipsychotic and anxiolytic effect (Elisabetsky and Costa-Campos, 2006), reversible antifertility effect (Raji et al., 2005).

The stem bark is anti-venom for snake bites and also used in traditional medicine to treat painful urination, insomnia and chronic diarrhea (Asuzu and Anaga, 1991). Infusion of the root and stem bark is used as a remedy for asthma, while that from the stem bark and leaves is used to treat impotence (Opoku and Akoto, 2015). Therapeutically, the bark has been found to possess antimicrobial and antibiotic properties (Kam et al., 1997; Fakae, 2000; Hadi and Bremner, 2001). A decoction could be sweetened with pure honey and be taken up to 4 times daily as an effective painkiller for the following conditions: Painful menstruation (dysmenorrhoea), when associated with uterine fibroid or ovarian cysts in women; lower abdominal and pelvic congestion associated with gynaecological problems such as pelvic inflammatory diseases; and to relieve the painful urethritis common with gonococcus or other microbial infections in men (Adotey et al., 2012).

The cold infusion is also administered orally for the purpose of expelling round worms, threadworms (Abbiw, 1990), and other intestinal parasites in children. The bark decoction of *Alstonia boonei* is used with other preparations in the treatment of fractures or dislocation (Abbiw, 1990), jaundice for inducing breast milk and its latex is taken as a purgative (Adotey et al., 2012). *A. boonei* De Wild is regarded as one of few herbs with potential anti-HIV indicators (Adotey et al., 2012).

An array of chemical compounds has been isolated from *A. boonei*, which include alkaloids, tannins, iridoids, and triterpenoids (Akinmoladun et al., 2007). The alkaloids isolated from the plant include echitamine and other alkaloids, and the triterpenes b-amyrin, lupenol, and ursolic acid have all been isolated from leaves and stem bark (Adotey et al., 2012). Echitamine has anticancer activities (Adotey et al., 2012; Ashok et al., 2015), (Z)-9-Octadecenoic acid was found to be the most abundant volatile oil in the leaf and stem bark, while methyl (7 E)-7-octadecenoate was the most abundant in the root (Moronkola and Kunle, 2012).

In this study, the antibacterial activity of ethanol extracts of leaf, bark and equal combination of leaf and bark of *A. boonei* were analyzed against eight clinically pathogenic organisms using agar well diffusion method.

METHODOLOGY

Collection of samples

Fresh barks and leaves of *A. boonei* were obtained from the

botanical garden of the University of Lagos, Akoka (Lagos, Nigeria). Identification and authentication (LUH 6309) was done at the Herbarium, Faculty of Science, and Department of Botany of the University of Lagos. The leaves and stem were dried under shade for 2 weeks and ground into fine powder using a local grinding machine (the machine was pre-washed and dried before grinding to avoid contamination) at Oja market, Ogun State. The powder was stored in an air-tight vessel at room temperature.

Preparation of methanol extract

Extracts were prepared using the modified method of Opoku and Akoto (2015). 100 g of pulverized leaf and bark materials were soaked in 1000 ml of 70% ethanol separately and together (fifty grams each of leaf and bark) and left overnight. After 48 h, the mixtures were filtered with Whatman No. 1 filter paper and evaporated to dryness *in vacuo*. The crude extract was then stored at 4°C until further use.

Phytochemicals analysis

The phytochemical analysis was carried out according to Trease and Evans (2002). Chemical tests were carried out on the ethanolic extracts for the qualitative determination of phytochemical constituents.

Preparation of microorganism

The organisms used in this study were *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Shigella dysenteriae*, and *Salmonella typhimurium*. The strains were maintained at 37°C. Each bacterium was reactivated by inoculating onto sterile nutrient agar (Oxoid, CM003) plates and incubated appropriately. Mueller Hinton Agar (Oxoid, CM0337) medium was used as bacterial culture medium in the antibacterial assay.

Testing for antimicrobial activity

Antibacterial activity of ethanolic extracts of leaf and bark separately and leaf with bark together was tested using agar well diffusion method as described by Wemambu et al. (2018) with modification. 200 µl of bacteria suspension at 0.5 McFarland standards were aseptically introduced and spread using cotton swabs on surface of gelled sterile Muller Hilton agar plates. A well of about 8.0 mm diameter with sterile cork borer was aseptically punched on each agar plate. 100 µl of the reconstituted extracts at 100 mg/ml were introduced into the wells in the plates. A control well was made with 100 µl of the extracting solvent dimethylsulphoxide (DMSO) (undiluted). Plates were kept in laminar flow for 30 min for pre diffusion of extract to occur and then incubated at 37°C for 24 h. The diameter of the zone of inhibition around each well was measured.

RESULTS AND DISCUSSION

A. boonei has high quantities of alkaloids, tannins, saponins, steroids and flavonoids (which are the core antiplasmodial agents), these could be responsible for the antimalarial efficacy (Ogiangbe et al., 2010). From

Table 1. Phytochemical constituents of the extracts.

Constituent	Observation	
	Stem	Leave
Flavonoid	+	-
Tannin	+	+
Phlobatannin	-	+
Alkaloid	+	+
Cardiac glycosides	+	-
Steroid	+	+
Reducing sugar	+	+
Saponin	+	+
Anthraquinone	+	+

Table showing the presence/absence of phytochemicals in *A. boonei* stem bark and leaves; + = presence of compound; - = absence of compound.

Table 2. Antibacterial susceptibility pattern of test organisms to the crude extracts.

Isolate	Extract and concentration (mg/ml)											
	Leaf				Bark				Leaf + bark			
	100	50	25	12.5	100	50	25	12.5	100	50	25	12.5
<i>E. coli</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>S. aureus</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. aeru.</i>	12±0.43	12±0.87	-	-	-	-	-	-	-	-	-	-
<i>Strep. pneu.</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. mirabilis</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>K. pneu.</i>	13±0.17	11±0.56	-	-	-	-	-	-	-	-	-	-
<i>S. dysenteriae</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>S. typhi</i>	13±0.27	12±0.41	12±0.82	12±0.32	12±0.21	12±0.62	-	-	12±0.13	12±0.45	-	-

phytochemical screening, it was found that ethanolic extract of *A. boonei* stem bark contains alkaloids, flavonoids, saponins, phlobatannins, anthraquinones, steroids and reducing sugars. Tannins and cardiac glycoside were absent in the stem extract. Ethanolic extract of *A. boonei* leaves contains tannins, phlobatannins, alkaloids, cardiac glycosides, reducing sugar, saponins, anthraquinones and steroids. Flavonoids were absent (Onifade and Maganda, 2015), while steroidal compounds are known to behave like hormones, owing to their structural semblance. Tannins have also been found to be potentially anti-viral, anti-bacterial and anti-parasitic agents (Ene et al., 2008; Onifade and Maganda 2015). Tannins also hasten the healing of wounds and inflamed mucous membrane (Okwu and Okwu, 2004). Flavonoids are potent water-soluble antioxidants and free radical scavengers which prevent oxidative cell damage and have strong anticancer activity (Salah et al., 1995; Del-Rio et al., 1997; Okwu, 2004). Flavonoids also lower the risk

of heart diseases. Saponins are capable of neutralizing some enzymes in the intestine that can become harmful, building the immune system and promoting wound healing. Steroids and saponins have been found to be detrimental to several infectious protozoans (Delmas et al., 2000).

Three extracts (leaf, bark and leaf + bark) from the plant species, were screened for their potential antibacterial properties against *E. coli*, *S. aureus*, *P. aeruginosa*, *Streptococcus pneumoniae*, *P. mirabilis*, *K. pneumoniae*, *S. dysenteriae* and *S. typhimurium*. The extracts were prepared by sequentially extracting the plant material. The susceptibility pattern of the test organisms and phytochemical constituents of the extracts are represented in Tables 1 and 2. *E. coli*, *S. aureus*, *S. pneumoniae*, *P. mirabilis*, *S. dysenteriae* were all resistant to all extracts.

In this study, the antibacterial activity of ethanol extracts of leaf, bark and leaf + bark of *A. boonei* were analyzed against eight clinical isolates using agar well



Figure 1. *A. boonei*; leaves (left) and stem (right).

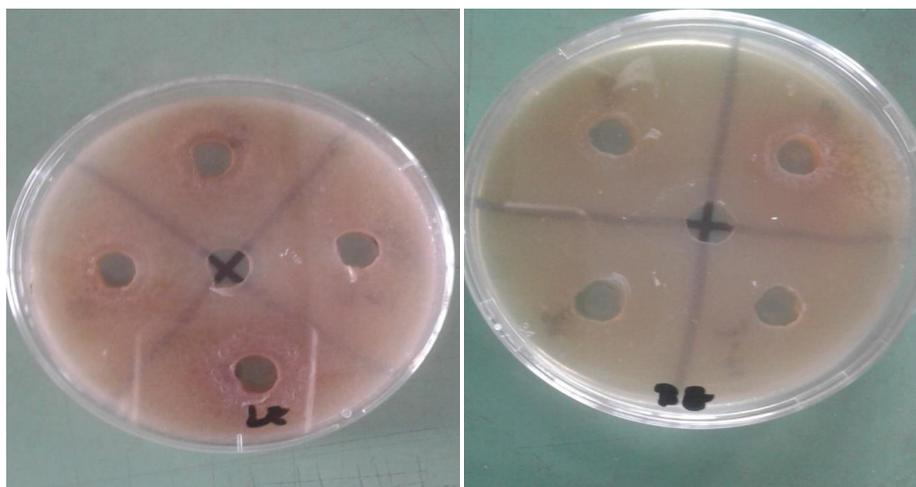


Figure 2. Plate showing the activity of crude leaf extract against *P. aeruginosa* (left) and bark extract (right) against *S. typhimurium* at concentrations 100, 50, 25 and 12.5 mg/ml. Negative control: DMSO (centre well).

diffusion method Figure 1. All test extracts showed varying degree of antibacterial activities at all tested concentrations which were compared with negative control DMSO that showed no activity with any of the extracts. *P. aeruginosa* was susceptible only to the leaf extract at 50 and 25 mg/ml and resistant to other extracts while *K. pneumoniae* was susceptible to leaf extract at concentrations 25 and 12.5 mg/ml and also resistant to other extracts. *S. typhimurium* was the most susceptible of all tested organisms Figure 2. . It had the highest zone of inhibition (13 ± 0.27) for leaf extract at 100mg/ml and lowest for other concentrations of the leaf extract and also at 100 and 50 mg/ml for both the bark extract and leaf + bark extract as presented in Table 2. The poor activity of the extracts could be as a result of the seventy per cent ethanol used for extraction as other researchers (Portillo et al., 2001; Alphonse et al., 2003; Koduru et al.,

2006; Aiyegoro et al., 2008; Ashafa et al., 2008; Igbiosa et al., 2009) have reported no activity with aqueous extracts against most bacterial strains. This could be due to the insolubility of the active compounds in water. Also, ethanol has a lower polarity as compared to methanol, and thus, may have not extracted all secondary metabolites from the samples used. It is also worthy to note that the resistant organisms could be highly resistant or multidrug resistant organisms and hence showing insensitivity to the extracts.

Conclusion

The result of this study shows that the tested organisms were more susceptible to the leaf extract than the bark the combined extracts. The combined effect of the leaf

and bark extract of the tested plant was additive.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Acquaintance of Botox: A cross-sectional survey among female students in Karachi, Pakistan

Shahlla Imam^{1*}, Wajiha Iffat², Sadia Shakeel², Ambreen Qamar³ Najaf Usman⁴, Nuzhat Sultana⁵ and Faisal Muhammad Khan¹

¹Institute of Pharmaceutical Sciences, Jinnah Sindh Medical University, Karachi – Sindh-Pakistan.

²Dow College of Pharmacy, Dow University of Health Sciences, Karachi – Sindh-Pakistan.

³Department of Physiology, Institute of Oral Health Sciences, Dow University of Health Sciences, Pakistan.

⁴Faculty of Pharmacy, Jinnah University for Women, Karachi Pakistan.

⁵Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, University of Karachi, Pakistan.

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The cosmetic treatment strategies have been considerably popular over the last few decades among the population with respect to age, gender, and ethnicity. These procedures are easily accessible safer, minimally invasive, and more precise. Botox therapy is one of the most common non-surgical cosmetic treatment. Thus, the current study was accomplished to assess the knowledge of female students towards Botox therapy for beautification as well as for therapeutic purposes. The study was conducted from June 2018 to December 2018 in different medical colleges and universities of Karachi-Pakistan. Overall, three hundred and eighty-six female students participated in the study. Descriptive statistics were used to reveal the students' demographic information. Pearson's chi-squared test was carried out to estimate the relationship between independent variables and responses. The response rate was 77.2%. The mean age of study participants was 26.44±3.33 years. The findings of the present study revealed that 58.3% of the participants were aware of Botox therapy and found it effective in cosmetology. The unexpected side effects, high procedure costs, and lack of cosmetologists were thought to be the main reasons that limit people from using Botox in Karachi-Pakistan.

Key words: Botox, cross sectional survey, knowledge, Karachi, Pakistan.

INTRODUCTION

Cosmetic treatments have been very popular around the world, among men and women. The increasing demand for cosmetic procedures in recent years could be attributed to higher disposable incomes, and media portrayal reduced the stigma of cosmetic procedures. The period underway emphasizes an accelerated development in cosmetics trade with accessibility of

marketed product for beautifying women. The majority of cosmetic procedures performed were nonsurgical, with laser hair removal, chemical peels, microdermabrasion and botulinum toxin type A injection (Lee and Lee, 2016). Botulinum toxin, also called, "miracle poison," is one of the most noxious natural substances known to mankind. It is produced by anaerobic fermentation of the bacterium

*Corresponding author. E-mail: shahlla.imam@jsmu.edu.pk.

Clostridium botulinum, an anaerobic, gram-positive, spore-forming rod commonly found on plants, like vegetables, fruits, and seafood. Numerals of different strains of *C. botulinum* have been recognized, including seven immunologically distinct serotypes (type A–G) (Archana, 2016; Dutta et al., 2016; Awan, 2017; Mya et al., 2019). Aesthetic use of botulinum toxin type A is one of the most widely studied formulation for cosmetic and therapeutic purposes (Sundaram et al., 2016; Satriyasa, 2019). The U.S. Food and Drug Administration has approved Botox (Botulinum toxin-A) treatment for alleviating eye-muscle disorders and improving the appearance of frown lines between the eyebrows (Al-Ghamdi et al., 2015; Batisti et al., 2017; Dayan et al., 2018). The literature findings suggested the efficacy of Botox in treatment of spasmodic dysphonia, essential voice tremor, headache, cervical dystonia spasmodic torticollis, masticatory myalgia, sialorrhoea, temporomandibular joint disorders, bruxism, blepharospasm (eyelid closure), hemifacial spasm and nasal allergy and allergic rhinitis. It also has remarkable effects in treating chronic pain, disorders of localized muscle spasms, migraine and neuropathic pain (Dutta, et al., 2016). Some adverse reactions reported to the FDA include respiratory problems, dysphagia, seizure, flulike syndrome, facial, muscle weakness, ptosis and bruising or swelling at the site of injection (Yiannakopoulou, 2015). The current study was accomplished to assess the knowledge, attitude of female students towards Botox therapy for beautification as well as for therapeutic purposes.

MATERIALS AND METHODS

A cross sectional study was conducted from June 2018 to December 2018 in different medical colleges and universities of Karachi including both private and public sector. The questionnaire was developed after an extensive literature survey on the use of Botox injections in cosmetology (Yiannakopoulou, 2015; Awan, 2017; Mya et al., 2019; Kattimani et al., 2019). The questionnaire was examined by professionals and researchers to ensure the validity of the questions content. The study population comprised female undergraduate Pharmacy, Science and Medical students aged 20–34 years whose knowledge towards the use of Botox injection was assessed through a 25-items questionnaire. The paper-based questionnaire was distributed to the participants who participated in the study. Overall, three hundred and eighty-six undergraduate and graduate female students participated in the study. Some basic information was given to the respondents and all information collected from the study was kept strictly confidential.

Data analysis

Statistical Package for the Social Sciences (SPSS, version 20; SPSS Inc., Chicago, IL, USA) software was used for data analysis. Descriptive statistics were used to reveal students' demographic information. Pearson's chi-squared non-parametric test was carried out to estimate the relationship between socio-demographic factors and miscellaneous questions included in the questionnaire. The

results were described in terms of frequencies, percentages, and means. *P* value < 0.05 was considered as significant.

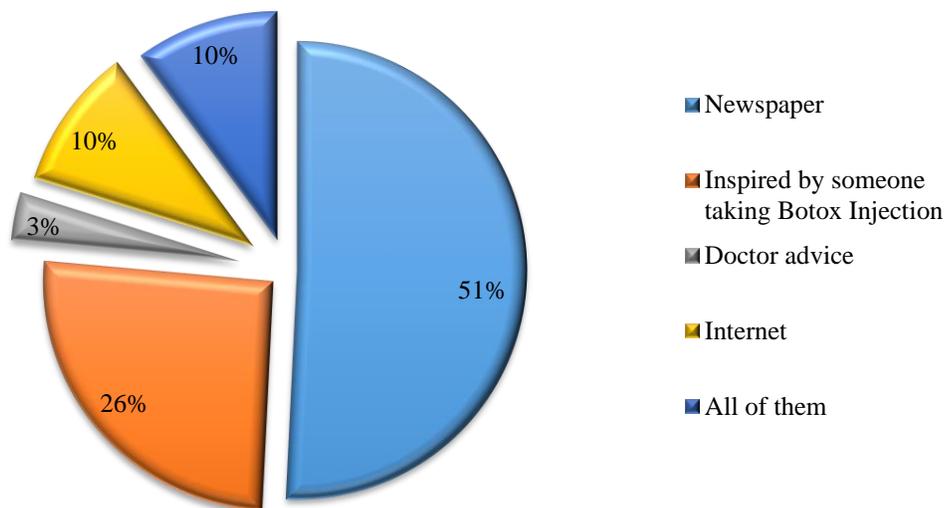
RESULTS

The questionnaire relied on self-reported responses from the participants. Out of the 500 survey forms, 386 participants successfully completed and returned the questionnaires, helping to achieve 77.2% response rate. Significant association ($p < 0.05$) was observed between the independent variable and responses of the respondents. The females who participated in the study were undergraduate and graduate medical (46.6%), pharmacy (33.4%) and science (19.9%) students. The mean age of study participants was 26.44 ± 3.33 years with minimum age being 20 years and maximum age 34 years. The demographic information of the respondents is presented in Table 1. Around 70% of the respondents opined that people should be conscious about beauty. Approximately 58.3% of the participants were aware of Botox injection and half of the population opined that the use of Botox in cosmetology is correct and 63.5% of participants believed that Botox is FDA approved. The majority of the respondent (64.0%) thought that Botox can be used without surgery and merely (32.4%) agreed that Botox can be used as an OTC drug. The majority of population (60.6%) believed that Botox injection may cause serious/unknown reactions and the participants (66.6%) sided against the use of Botox during pregnancy or breast feeding. The mass population (70.2%) agreed that Botox is an expensive therapy whereas 72.5% opined that female prefers Botox injections more as compared to male. The knowledge of students regarding Botox is illustrated in Table 1. As regards students' source of acquisition of information about Botox injection, more than half (50.8%) of the study population considered print and electronic media as major sources of knowledge. Figure 1 shows the other commonly prevailed source of knowledge about Botox therapy.

Nearly about 37.3% considered that Botox should be used above 40 years of age. Around 61.7% knew that Botox is used for skin treatment whereas 21.0% considered it to be used in overactive bladder or for any other medical purpose. The effect of Botox lasts for about 1 - 4 months (23.8%), 5 - 8 months (34.7%) and 9 - 12 months (41.5%) according to the opinion of the participants. Participants thought that time taken by Botox to produce its full effects is 3-7 days (43.3%), 1-2 days (23.6%) and more than 7 days (33.2%). Half population (53.6%) thought that Botox injection temporarily paralyzes the muscles whereas 26.4 and 19.9% thought that Botox injection partially and permanently paralyzes the muscles respectively. When respondents were asked about the expected price of Botox treatment, 13.0% opined above 15,000 rupees, 31.1% above 25,000 rupees whereas 56.0% thought more than 50,000 rupees. A mere 19.4% thought that Botox treatment should be

Table 1. Demographic characteristics of the study population (N=386).

Characteristics	N (%)
Age groups (years)	
20 – 24	139 (36)
25 -29	194 (50.3)
30 - 34	53 (13.7)
Educational Institute	
Public sector	150 (38.9)
Private sector	236 (61.1)
Educational Field	
Medical	180 (46.6)
Pharmacy	129 (33.4)
Science	77 (19.9)

**Figure 1.** Students' sources of information about Botox Injection.

given more awareness in our country as cosmetology. The restricted use of Botox according to students' perceptions were down to unexpected side effects (41.5%), expensive (28.5%), lack of awareness (21.5%) and lack of cosmetologists in Karachi, Pakistan (8.5%) as the main reasons that limit people from using Botox. Figure 2 depicts the respondents' opinion about health professionals who qualified to use Botox injection.

DISCUSSION

Botox injections, facial fillers, face-lifts, and eye-lid surgery, to name a few aesthetic facial procedures, have become a magic potion for several women (Mya et al.,

2019). Several women apply the procedures to amplify their physical beauty and self-worth, some observed the procedures as extremely risky, and still some disagreed that the procedures stemmed from the social deflation of later on life (Chang et al., 2016). The present study assessed the knowledge about cosmetic surgery and perceived satisfaction with Botox therapy among female students in Karachi, Pakistan. In the current study, half of the population accepted the cosmetic procedures because of the multiple aspects of facial appearance including youthfulness, and attractiveness. The vast majority (70%) of the respondents opined that people should be conscious about beauty and they would like to pay an amount on beauty or skin care for better looking. The participants had a sound knowledge of cosmetic

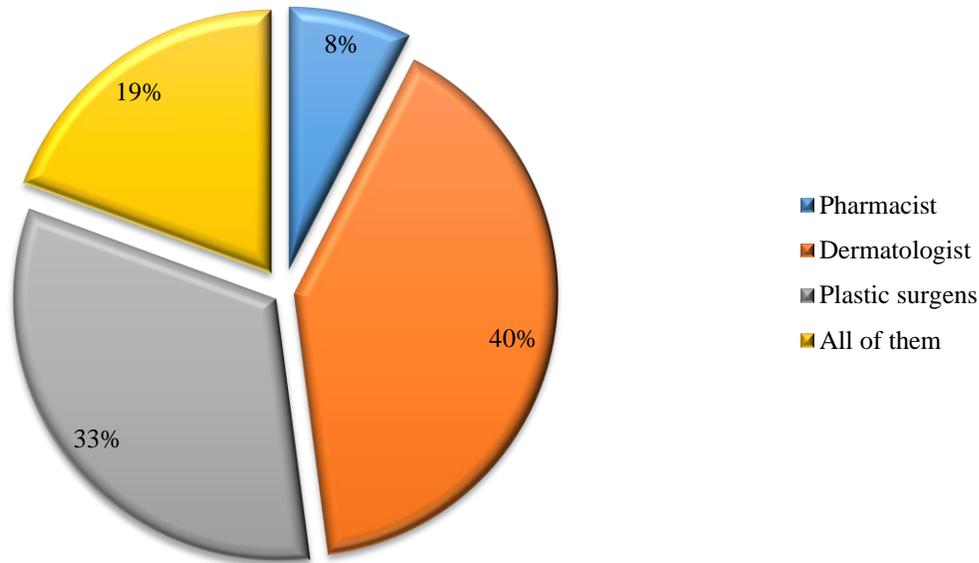


Figure 2. Students' opinion about the professionals qualified to use Botox injection.

Table 2. Knowledge of students with regards to use of Botox therapy (N=386).

Variable (Statement)	Responses N (%)		P-value
	Yes	No	
Should people be conscious about beauty?	269 (69.7)	117 (30.3)	0.007
Will you like to pay amount on your beauty or skin care?	252 (65.3)	134 (34.7)	0.028
Are you aware of Botox injection?	225 (58.3)	161 (41.7)	0.025
Do you think the use of Botox in cosmetology is correct?	194 (50.3)	192 (49.7)	0.053
Should people get complete information about Botox before therapy?	255 (66.1)	131 (33.9)	0.039
Do you think that Botox is FDA approved?	245 (63.5)	141 (36.5)	0.008
Do you think Botox can be used without surgery?	247 (64.0)	139 (36.0)	0.010
Do you think that Botox injection is a new drug therapy?	232 (60.1)	154 (39.9)	0.034
Do you agree that Botox can be used as an OTC drug?	125 (32.4)	261 (67.6)	0.862
If doctor advice you for Botox therapy will you prefer to use it?	161 (43.0)	220 (57.0)	0.261
Do you think Botox injection affects areas away from site of application?	237 (61.4)	149 (38.6)	0.107
Do you think Botox injection may cause serious / unknown reactions?	234 (60.6)	152 (39.4)	0.038
Do you think that Botox can be used during pregnancy or breast feeding?	129 (33.4)	257 (66.6)	0.334
Do you think Botox is an expensive therapy?	271 (70.2)	115 (29.8)	0.008

Statistical significance was analyzed with Pearson chi-square test. *P<0.05.

surgery and awareness regarding Botox injection. Although a large proportion of the respondents (60.6%) have awareness about the risks associated with cosmetic surgery, they believed that Botox injection may cause harmful/unwanted reactions. It has been reported that there are some issues regarding side effect and complication following the Botox injection (Satriyasa, 2019). The knowledge of students regarding Botox is illustrated in Table 2.

Since self-perceived knowledge of cosmetic procedures could play a key role in influencing the choices of potential

cosmetic therapy, people should get complete information about Botox before therapy (Kwolek and Block, 2019). Even though Botox is a prescription medication injected into muscles for several cosmetic and medical purposes, it was noted that just over thirty (32.4%) agreed that Botox can be used as an OTC drug. The study population demonstrated that it could be used without surgery. The study indicated that females prefer to use Botox injections more as compared to male. It was also found that the influence of print and electronic media including television, internet, newspaper, and magazine reporting

of women's health and age-related illnesses forced women to use the non-surgical treatments (Figure 1). A reported study indicated that considerable interest in undergoing cosmetic surgery simply based on the information that they had obtained from television shows and magazines influenced the female attitudes towards cosmetic surgery (Ng et al., 2014). Although it was found that the majority of the respondents did not intend to undergo Botox procedures, less than 50% considered Botox should be used above 50 years of age. The finding of the present study also revealed that around 61.7% of participant agreed that Botox used for skin treatment is generally acceptable if indicated. The cosmetic procedures involved the face, including skin resurfacing and facial lesion. Several studies confirmed the effectiveness of Botox in the treatment of cosmetic and dermatological diseases (Satriyasa et al., 2019; Awan 2017).

Though Karachi-Pakistan has seen growing interest in the past few years in cosmetic procedures, the findings of the present study revealed that the participant's knowledge and attitude towards cosmetic surgery were reasonable while, receiving cosmetic surgery was not common in current study population. Nonetheless, there is a lack of sound information awareness and cost has slowed down the growth of cosmetic surgery as well as the risks associated with these procedures. There is limited data available on the number of cosmetic procedures performed on different age groups in Karachi-Pakistan. Figure 2 depicts the respondents' opinion that dermatologist and plastic surgeons were believed to be the most qualified professionals.

Conclusion

The present study proposes some insights into the attitudes and extent of knowledge on cosmetic and aesthetic procedures among students in Karachi, Pakistan. This study analyzed the students-reported outcomes following effective and aesthetic Botox treatment and concluded that dermatologists and cosmetologists should work to boost up the general awareness towards the spectrum of cosmetic and clinical applications of Botox. It would be a step towards personal care problems in Karachi-Pakistan and will ultimately improve the skin and overall health of society. Hence, future studies should be conducted to investigate the amount of information available to consumers regarding new cosmetic treatments that are emerging with technological advancements.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of the immunomodulatory properties and microbial bioburden of three commercial herbal mixtures sold in Awka, Anambra State Nigeria

Christopher E. Enemor^{1*}, Malachy Ugwu and Chukwuemeka Nworu²

¹Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria.

²Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria Nsuka, Nigeria.

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The consumption of herbal medicine and herbal medicinal products has been on the rise lately. This has led to an increase in research on herbal medicine and its different formulations to gain more knowledge in their constituents, therapeutic effects, its mechanism, and their undesired or toxic effects. The purpose of this study was to evaluate toxicity profile, phytochemical constituents, microbial quality and immunomodulatory properties of Goko cleanser®, Beta cleanser® and Weifa body defense mixtures®. Acute oral toxicity, phytochemical screening and the microbial quality was evaluated. The immunomodulatory activities of the herbal mixtures were studied using the Carbon Clearance Test, Cyclophosphamide Induced Neutropenia, Delayed-type Hypersensitivity Test and Humoral Antibody Assay with the Sheep Erythrocytes as antigen. The result indicated that the herbal mixtures showed no toxic effect on the test animals. The phytochemical analysis showed an adequate presence of immunomodulatory phytochemicals. The result also revealed that Beta cleanser® was contaminated with *S. aureus* and *E. coli*. The three test herbal mixtures, at doses tested, increased the phagocytic index by stimulating the reticuloendothelial cells and increasing their phagocytosis ability. The test herbal mixtures also showed significant protection against cyclophosphamide-induced neutropenia by increasing the depleted levels of leucocytes. The herbal mixtures aided the mobilization of macrophages and memory T cells as seen in the result of the Delayed-Type Hypersensitivity Test. The result of the humoral antibody test showed that the herbal mixtures exhibited a dose-dependent stimulatory effect on B cell maturation and differentiation into antibody-secreting plasma cells.

Key words: Immunomodulatory, Phytochemistry, Microbial Quality, Toxicity profile, Herbal Formulation.

INTRODUCTION

Herbal medicines are in great demand in the developed world for primary health care because of their efficacy,

safety and lesser side effects. They offer therapeutics in age-related disorders like memory loss, osteoporosis,

*Corresponding author. E-mail: enemoremmanuel@gmail.com.

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immune disorders, etc. for which no modern medicine is available (Tyler, 2000). Medicinal plants play significant roles in the prevention and treatment of various diseases. In nature, there are various medicinal plants which are used as immunomodulator agents (Singh et al., 2011). *Vernonia amygdalina* has been proven to strengthen the immune system through many cytokines regulation and increase in mean absolute CD4 count (Erasto et al., 2007; Momoh et al., 2012.) *Moringa oleifera* antibacterial activity, immunomodulatory activity (Anwar and Bhanger, 2003); *Morinda citrifolia* stimulates the release of several mediators from murine effector cells, including TNF- α , interleukin-1beta (IL-1 β), IL10, IL-12, interferon gamma (IFN- γ) and nitric oxide (Hirazumi and Furusawa, 1999). There appears to be an overwhelming increase in the public awareness and usage of herbal medical products in the treatments and or prevention of diseases in Nigeria. With this increased usage, the safety, efficacy and quality of these medicines have been an important concern for health authorities and health professionals (Okunola et al., 2007; Oreagba et al., 2011). Microbiological assessment of non-sterile products, such as herbal mixtures, is particularly pertinent in view of the fact that microbial contamination can reduce or even eliminate the therapeutic effect of drugs or cause drug-induced infections (Adesanya et al., 2007). Microbes presented in drugs not only make them hazardous from the infectious standpoint, but may also change the chemical, physical and organoleptic properties of the drugs or change the contents of active ingredients. Microorganisms and their toxic metabolites, which persist even after the death of the primary contaminants, can convert drugs to toxic products (Esimone et al., 2007). The presence of low level of pathogenic microorganisms, higher levels of opportunistic pathogens or bacterial toxic metabolites, which persist even after the death of the primary contaminants, can render the medicinal product ineffective (Ratajczak et al., 2014). In Nigeria, studies have shown that many patients rely on the use of herbal remedies in managing infectious diseases and immune boosting (Falodun and Imieje, 2013; Ekeanyawu, 2011). A questionnaire-based study by Oreagba et al. (2011) showed that 267 (66.8%) out of the 388 individuals recruited for the study had used herbal medicine at one point in their lives. These remedies however, have not been properly studied to confirm their label claims. This implies that these patients would be on drugs which may have no direct effect on both disease progression and quality of the patients' life. On the other hand, these herbal remedies contain bioactive constituents which may have either a positive or negative effect on therapeutic outcome. There is the need to confirm the label claims and safety profile of commercially available herbal medicines and mixtures. This study is geared towards the evaluation of the microbial bioburden and the immunologic claims of herbal mixtures popularly sold and

consumed in Anambra State, Nigeria.

MATERIALS AND METHODS

Test herbal mixtures

Goko cleanser®, Beta cleanser® and Weifa Body defense mixtures® (five bottle each) were purchased in Eke Awka market in Anambra state Nigeria. Table 1 shows the composition and other information about the products.

Equipment and instrument

These include incubator (Genlab UK), Autoclave (EQUITRON Medica, Instrument India), UV-Vis Spectrophotometer (JENWAY 6505, Bibby Scientific Ltd., UK).

Culture media and other reagents

Muller Highton Agar (Titan; Rajasthan India), Nutrient Broth (LabM; United Kingdom), Mannitol Salt Agar (HiMedia; Mumbai India), MacConkey Agar (Biotech United Kingdom), Salmonella Shigella Agar (Titan Biotech; Rajasthan India), Distilled Water, Normal Saline (Table 1).

Experimental animals used

Albino rats of both sexes, with weights range of 80-120 g, were used. They were housed under the standard condition of temperature (25 \pm 10°C) and relative humidity (60 \pm 10%) and fed with standard pellets diet and water. They were housed in the animal house in the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka. The animals were kept to acclimatize for about a week before they were randomly divided into the different experimental groups. The use of animals in this research was in accordance with the guidelines approved by the Animal Ethical Committee, Nnamdi Azikiwe University Awka Nigeria.

Microbial assay

Determination of the microbial load of the herbal sample was carried by the technique outlined by Oluyeye and Adelabu (2010). Exactly 10 ml of each sample (Goko herbal mixture®, Beta herbal mixture®, Weifa body defense mixture®) was aseptically transferred into a corresponding sterile tube containing 90 ml of sterile distilled water and ten-fold serial dilution was carried out into three containers (1/10, 1/100 and 1/1000 respectively). One milliliter of each dilution of the test herbal mixtures was mixed with 15 ml of sterile molten standard plate count agar (Muller Highton Agar) and 15 ml molten Sabouroud dextrose agar for bacteria and fungi respectively, and then poured into petri dishes. This was done in triplicate. The plates were allowed to set and incubated at 37°C and 24 h for bacterial counts and at 27°C for four days for fungal counts. Isolation and identification of potential pathogens in the herbal samples was also carried out using MacConkey agar, Manitol salt agar and Salmonella-Shigella agar. Potato dextrose agar was used to isolate fungi. For each herbal sample, 1.0 ml of the mixture was aseptically transferred onto each medium and spread on the surface with glass spreader. The plates were incubated at 37°C for 72 h. Pure cultures were obtained from the plates and stored

Table 1. Herbal mixtures with their composition.

Name of product	Composition	Producers	Batch Number	Date manufactured	Expiring Date	Nafdac Reg. number
Goko cleanse r®	<i>Vernonia amygdalina</i> -12% <i>Saccharum officinarum</i> -11.5% <i>Allium sativum</i> -13% <i>Cajanus cajan</i> -11.5% Caramel -1.5% <i>Zingiber officinale</i> -0.5% Treated Water-QS	Goko Herbs West Africa Ltd	0005	08/2014	07/2017	A7-0804L
Beta Cleanse r®	<i>Aloe vera</i> -25ml <i>M. oleifera</i> -20ml C. <i>aurantifolia</i> -15ml C. <i>officinarum</i> -20ml S. <i>offocarium</i> -15ml <i>Allium sativa</i> -5ml Treated water-100ml	SJOESYLY CONSERNS LTD	008	09/2014	08/2017	A7-0738L
WEIFA BODY DEFEN SE®	Roots and herbs Treated water <i>Dacryodes edulis</i> <i>Garcinia spp</i> <i>Allium sativum</i>	Benbela Tradomedical s Ltd	WBDA L/05	01/2014	01/2017	

onagar slants and kept in the refrigerator until used for biochemical identification. The limits presented in the European Pharmacopoeia (Microbial Quality of Pharmaceutical preparations; category 3B), for each test, was used to assess the result.

Phytochemical screening

The herbal mixtures were screened for the presence of various

phytochemical constituents using standard methods as earlier described (Trease and Evans, 2009; Akinjogunla et al., 2010).

Determination of acute toxicity

The acute oral toxicity study was conducted on each of the products as described by Lorke (1983). The study was conducted in two phases using a total of 39 rats. In the first phase, 27 rats were

divided into three groups of nine per group and the nine of each group was further divided three groups of three rats per group. Groups one to three were given 10, 100 and 1000 mg/kg body and 1000 mg/kg body weight of Beta herbal mixtures®, and the third group received 10, 100 and 1000 mg/kg body weight of Weifa body defense mixtures® respectively, to possibly establish the range of doses producing any toxic effect and the rats were watched for 24 h for mortality rate. The death pattern in the first phase determined the dose for the second phase. Depending on the death pattern, further specific doses (1600, 2900, 3600, and 5000 mg/kg) of Goko herbal mixture®, Beta herbal mixture® and Weifa body defense® were administered to three rats (one per dose) to further determine the LD₅₀ value. The herbal mixtures were serially diluted with sterile water and administered orally and the animals observed for 24 h. The LD₅₀ was calculated as the geometric mean of the maximum dose that did not result in lethality and the least toxic dose that produce death in the albino rats.

Processing of sheep red blood cells for use as an antigen

Blood samples were obtained from the jugular vein of a healthy sheep maintained in the animal house of Faculty of Pharmaceutical sciences, Nnamdi Azikiwe University Awka, Nigeria and into a 5 ml EDTA bottle. Then red blood cells were washed thrice with copious volume of sterile normal saline by centrifugation at 3000× g for 10 min. The final cell volume was adjusted to a concentration of 1×10^9 cells/ml and used for immunization and challenge.

Selection of doses

The doses for the study were selected based on the outcome of the oral toxicity studies done up to dose level of 5000 mg/kg body weight. On the account of no death, doses of 100, 200 and 400 mg/kg body weight was used.

Experimental protocols for determination of immunological parameters

The animals were numbered, weighed and divided into eleven different groups of six animals per group as follows:

Group 1: positive control to receive pellet and distilled water
 Group 2: negative control to receive 100 mg/kg body weight of Noni®
 Group 3: received 100 mg/kg body weight of Goko herbal mixtures®
 Group 4: received 200 mg/kg body weight of Goko herbal mixtures®
 Group 5: received 400 mg/kg body weight of Goko herbal mixtures®
 Group 6: Received 100 mg/kg body weight of Beta herbal mixtures®
 Group 7: received 200 mg/kg body weight of Beta herbal mixtures®
 Group 8: received 400 mg/kg body weight of Beta herbal mixtures®
 Group 9: received 100 mg/kg body weight of Weifa body defense®
 Group 10: received 200 mg/kg body weight of Weifa body defense®
 Group 11: received 400 mg/kg body weight of Weifa body defense®

Carbon clearance test

This test assesses the phagocytic activity of reticuloendothelial system (RES). It was conducted as described by Tripathi et al. (2012). Briefly, eleven groups of animals were used. Group 1 and 2

are positive (100 mg/kg body weight of Noni®) and negative control respectively; Group 3 to 5 received Goko herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively; Group 6 to 8 received Beta herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively while group 9 to 11 received Weifa body defense mixture® at increasing doses of 100, 200 and 400 mg/kg body weight respectively. The treatment was done daily for 10 days. Carbon ink suspension was injected via the tail vein to each rat 48 h after the tenth day treatment. Blood samples (25 µl) was withdrawn from the retro-orbital plexus under mild ether anesthesia at 0 and 15 min after injection of colloidal carbon ink and lysed in 0.1% sodium carbonate solution (3 ml). The optical density was measured spectrophotometrically at 660 nm. The phagocytic index was calculated using the following formula:

Where OD₁ and OD₂ are the optical densities at time t₁ and t₂, respectively (Barbuddhe et al., 1998).

Cyclophosphamide induced neutropenia studies

The albino rats were divided into 11 groups with each group having six animals each. Group 1 and 2 are positive (100 mg/kg body weight of Noni®) and negative control respectively; Group 3 to 5 received Goko herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively; Group 6 to 8 received Beta herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively while group 9 to 11 received Weifa body defense mixture® at increasing doses of 100, 200 and 400 mg/kg body weight respectively, daily for 10 days. On the 11th day, blood samples were withdrawn from the animals via retro orbital puncture into an EDTA container. A neutropenic dose of cyclophosphamide (30 mg/kg body weight) was administered on the 11th, 12th, and 13th days one hour after the administration of the treatment intra-peritoneally (i.p). Blood samples were withdrawn on the 14th day of the experiment by retro orbital puncture. Haematological parameters were studied (total white blood cell (WBC) counts and differential leucocyte count (DLC)) prior to and on the 3rd day after injection of cyclophosphamide. Data collected were expressed in mean and standard error of mean (S.E.M).

Delayed type hypersensitivity reaction

Animals were divided into eleven groups. Group 1 and 2 are positive (100 mg/kg body weight of Noni®) and negative control respectively. Group 3 to 5 received Goko herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively; Group 6 to 8 received Beta herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively while Group 9 to 11 received Weifa body defense mixture® at increasing doses of 100, 200 and 400 mg/kg body weight respectively for 5 days. On the fifth day, the animals were immunized with 0.1 ml of SRBCs suspension containing 1.0×10^9 cells/ml inter- peritonuosly (i.p). For 14 days, the animals in Group 3 to 11 were fed with their respective herbal mixtures and on the 19th day, they were sensitized again and then were fed with their respective herbal mixtures for another seven days. On the 8th day after immunization, the thickness of the right hind footpad was measured using a venier calliper. The animals were again challenged by the injection of 1.0×10^9 SRBCs footpad in the left leg. The thickness of the footpad was measured again after 24 h. The difference between the pre and post challenge footpad thickness was estimated and represents an index of the delayed

Table 2. Total Fungal and Bacteria population in the test herbal products.

Herbal product	Mean fungal count in CFU/ml \pm SEM	Inference	Mean aerobic bacteria Count in CFU/ml \pm SEM	Inference
Goko herbal cleanser®	$0.23 \times 10^2 \pm 0.083$	Pass	$3.2 \times 10^2 \pm 0.780$	Pass
Beta cleanser®	$0.82 \times 10^2 \pm 0.101$	Pass	$8.7 \times 10^2 \pm 0.891$	Pass
Weifa body defense®	$0.20 \times 10^1 \pm 0.022$	Pass	$1.1 \times 10^1 \pm 0.060$	Pass

type hypersensitivity (DTH) response. The DTH response was obtained from this formula (Corrier and DeLoach, 1990):

$$\frac{\text{Left foot pad challenged with antigen} - \text{right foot pad control}}{\text{Left foot pad challenged with antigen}} \times \frac{100}{1}$$

Humoral antibody determination

This was done using sheep erythrocyte agglutination test (SEAT) as described by Kumar et al. (1996) and Ray et al. (1991). Briefly, animals were divided into eleven groups, each having six rats. Group 1 and 2 are positive (100 mg/kg body weight of Noni®) and negative control respectively; Group 3 to 5 received Goko herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively; Group 6 to 8 received Beta herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively while Group 9 to 11 received Weifa body defense mixture® at increasing doses of 100, 200 and 400 mg/kg body weight respectively, daily for 10 days. All the animals were injected with 0.25 ml of 1×10^9 SRBC/ml on 6th, 8th, and 10th days to achieve maximum titer of antibody. On day 11 blood was collected and serum separated by centrifuging at 3000xg for 15 min. The serum was diluted serially with normal saline in separate test tubes. Serial dilutions that were made are 20, 40, 80 up to 1280th. To these serial dilutions, 50 μ l of SRBC was added and incubated at 37°C for 18 h. All the tubes were then subjected to physical examination visually for agglutination and compared with control. The highest dilution (lowest concentration of serum) giving hemagglutination was taken as the antibody titer for that group. The antibody titer was expressed in the graded manner, the minimum dilution being ranked as 1, and mean ranks of different groups was compared for statistical significance.

Statistical analysis

Results obtained were analysed using one-way analysis of variance (ANOVA) expressed as mean and standard error of mean to test for variations of the different parameters observed in the study. Test of significance was at $P < 0.05$. The Microsoft excel 2010 was used.

RESULTS

Microbial assay

The results show that the total fungal count ranges from 0.2×10^1 to 0.9×10^2 cfu/ml (Table 5). The result also shows that the total aerobic bacteria count is less than

0.9×10^3 cfu/ml with Weifa body defense been the least contaminated herbal product (Table 5). The result for the total *Escherichia coli* count in the herbal mixtures showed that Beta herbal cleanser®, after repeating the microbial quality assay, is contaminated with *E. coli* with the value of 0.3×10^1 CFU/ml (Table 6). The same herbal product (Beta cleanser®), from the result of the total *Staphylococcus aureus* count, has a *S. aureus* count of 0.8×10^1 CFU/ml and *Salmonella* spp. count of 0.4×10^1 CFU/ml. From the reference (Microbial Quality of Pharmaceutical preparations; category 3B; European Pharmacopoeia), the herbal preparations all passed the limit test for fungi and aerobic bacteria (not more than 10^2 CFU/ml and not more than 10^4 CFU/ml respectively). Beta cleanser® failed the limit test for *E. coli* and *S. aureus* (absence of *E. coli* and *S. aureus* according to the reference material) as these organisms were isolated from the herbal product after inoculating it in their selective media. On the surface of the Salmonella shigella agar, few black colonies of *Salmonella* spp. were observed but not much enough to conclude that Beta cleanser® also failed the limit test for the organism. Two biochemical tests were conducted for the isolated microorganisms. *S. aureus* was positive for catalase test, *E. coli* was positive for indole, and *Salmonella* spp was negative for both indole and catalase test (Tables 2 to 4).

Phytochemical screening of the herbal mixtures

The three herbal mixtures contain high level of tannin (Table 7). Weifa body defense mixture® also has excess of alkaloid and flavonoids, while Goko herbal mixture® has normal alkaloids and flavonoids. The herbal mixtures lacked reducing sugar in the exception of Weifa body defense mixture® which has traces of it.

Acute oral toxicity testing

The results of the first stage of oral acute toxicity test for the herbal mixtures are shown subsequently. From Tables 8 to 10 (for the first phase of the test), it was observed that after the administration of the specific doses of the herbal product, no animal died. The result of

Table 3. *E. coli*, *S. aureus* and *Salmonella* spp population in the test herbal products.

Herbal product	Mean <i>E. coli</i> Count in CFU/ml \pm SEM	Inference	Mean <i>S. aureus</i> count in CFU/ml \pm SEM	Inference	Mean <i>Salmonella</i> spp. count in CFU/ml \pm SEM	Inference
Goko herbal cleanser®	Nil	Pass	Nil	Pass	Nil	Pass
Beta cleanser®	$0.3 \times 10^1 \pm 0.006$	Fail	$0.8 \times 10^1 \pm 0.004$	Fail	$0.4 \times 10^1 \pm 0.020$	Pass
Weifa body defense®	Nil	Pass	Nil	Pass	Nil	Pass

Table 4. Phytochemical screening of the herbal mixtures.

Bioactive compound	Goko herbal mixture®	Beta herbal mixture®	Weifa body defense®
Reducing sugar	–	–	+
Saponins	++	++	++
Flavonoids	+	++	++
Proteins	+	+	+
Tanins	+++	+++	+++
Alkaloids	++	+	+++
KEY: -	= absence		
+	= trace		
++	= present		
+++	= excess		

Table 5. Acute oral toxicity test of Goko Herbal Mixture®.

First stage of acute toxicity			
Number of animals used	Dose administered (mg/kg)		Results
Group one			
3.0	10.0		No death
3.0	10.0		No death
3.0	10.0		No death
Group two			
3.0	100.0		No death
3.0	100.0		No death
3.0	100.0		No death
Group three			
3.0	1000.0		No death
3.0	1000.0		No death
3.0	1000.0		No death

the first phase of the test where no death was recorded led to the second phase of the test which was conducted with the animals given higher doses of the herbal products. The result of the second stage acute oral toxicity for the herbal mixtures is shown in Table 11. From Table 11, it was also observed that after the administration of the mixtures at higher doses, no death was recorded.

Carbon clearance test

The carbon clearance assay result of this work is shown in Figure 1. Goko cleanser® shows an enhanced carbon clearance activity in the test animal with the dose of 100 mg/kg been significant when compared to the control result. Beta cleanser®, at doses of 100 and 400 mg/kg, indicated a significant increase in the phagocytic index

Table 6. Acute oral toxicity test of Bata herbal mixtures®.

		First stage of acute toxicity	
Number of animals used		Dose administered (mg/kg)	Results
Group one			
	3.0	10.0	No death
	3.0	10.0	No death
	3.0	10.0	No death
Group two			
	3.0	100.0	No death
	3.0	100.0	No death
	3.0	100.0	No death
Group three			
	3.0	1000.0	No death
	3.0	1000.0	No death
	3.0	1000.0	No death

Table 7. Acute Oral Toxicity Test of Weifa Body Defense Mixtures®.

		First stage of acute toxicity	
Number of animals used		Dose administered (mg/kg)	Results
Group one			
	3.0	10.0	No death
	3.0	10.0	No death
	3.0	10.0	No death
Group two			
	3.0	100.0	No death
	3.0	100.0	No death
	3.0	100.0	No death
Group three			
	3.0	1000.0	No death
	3.0	1000.0	No death
	3.0	1000.0	No death

when compared to the negative control and to other two herbal mixtures. At doses of 100 and 200 mg/kg, Weifa body defense mixture® significantly enhanced the phagocytic index when compared to the control group and to Goko cleanser® and Beta cleanser® mixtures.

Cyclophosphamide induced immunosuppression

In this study, Goko cleanser® at dose of 400 mg/kg body weight, Beta cleanser® at doses of 100 and 200 mg/kg body weight and Weifa body defense mixture® at doses of 100 and 400 mg/kg body weight showed a significant percentage reduction of the inhibition effect of

cyclophosphamide on total white blood cell count in treated rats when compared to the control group. In the evaluation of the percentage reduction of the lymphocyte count, Goko cleanser® at dose of 100 mg/kg body weight showed a significant percentage reduction in lymphocyte count (50.08%) when compared to the control group (73.10%). Beta cleanser® and Weifa body defense mixture® at doses of 200 and 400 mg/kg body weight showed significant reduction in the percentage lymphocyte count when compared to the control group. Goko cleanser® at doses of 100 and 400 mg/kg, Beta cleanser® at doses of 100 and 200 mg/kg and Weifa body defense mixture® at dose of 200 mg/kg body weight all showed a significant percentage reduction in neutrophil

Table 8. Second stage of acute oral toxicity test.

Acute oral toxicity of Goko herbal mixture®		
Number of animals used	Doses administered (mg/kg)	Result
1.0	1600.0	No death
1.0	2900.0	No death
1.0	3600.0	No death
1.0	5000.0	No death
Acute oral toxicity of Beta herbal mixtures®		
Number of animals used	Doses administered (mg/kg)	Result
1.0	1600.0	No death
1.0	2900.0	No death
1.0	3600.0	No death
1.0	5000.0	No death
Acute oral toxicity of Weifa body defense mixture®		
Number of animals used	Doses administered (mg/kg)	Result
1.0	1600.0	No death
1.0	2900.0	No death
1.0	3600.0	No death
1.0	5000.0	No death

Table 9. Effect of Goko cleanser®, Beta cleanser® and Weifa body defense mixtures® on Phagocytic Index Values.

Treatment groups	Mean absorbance ± SEM		Phagocytic index ±SEM
	0 min	15 min	
Positive control	0.1066±0.00101	0.0098±0.00038	0.0691*
Negative control	0.0592±0.00055	0.0212±0.00079	0.0025
Goko 100 mg/ml cleanser®	0.0978±0.00083	0.0198±0.00085	0.0462*
Goko cleanser® 200 mg/ml	0.1020±0.00098	0.0101±0.00122	0.0669NS
Goko cleanser® 400 mg/ml	0.1064±0.00144	0.0095±0.00036	0.0699NS
Beta cleanser® 100 mg/ml	0.0732±0.00104	0.0292±0.00127	0.0266**
Beta cleanser® 200 mg/ml	0.0964±0.00151	0.0108±0.00210	0.0633NS
Beta cleanser® 400 mg/ml	0.0980±0.00087	0.0090±0.00066	0.0691*
Weifa defense® 100 mg/ml body	0.0988±0.00800	0.0213±0.00102	0.0444*
Weifa defense® 200 mg/ml body	0.1045±0.00137	0.0158±0.00221	0.0547*, **
Weifa defense® 400 mg/ml body	0.1184±0.00107	0.0102±0.00109	0.0720NS

Values are expressed in mean ± S.E.M., n=6

*= P<0.05 as compared with the control group

**=P<0.05 as compared with the three herbal mixtures

NS= P>0.05 not significant as compared to control (Negative) group.

count when compared to the control group (Table 12 and Figures 2 to 4).

Delayed type hypersensitivity reaction

Goko herbal cleanser®, Beta cleanser® and Weifa body

defense® mixtures produced some significant percentage increase in the paw volume of the immunized animals. From Figure 5, Goko cleanser® showed a significant effect in the increase in the paw volume of the immunized rats. Doses of 200 and 400 mg/kg body weight showed a significant percentage increase in the paw volume when compared to the control group. Beta cleanser® at dose of

Table 10. Effect of Goko cleanser®, Beta cleanser® and Weifa body defense mixtures® on the Cyclophosphamide induced leucopenia (Total WBC Counts).

Treatment group	Total white blood cell count (WBC/mm ³)		
	Before	After	%Reduction
Positive control	12.02 ± 3.26	7.15 ± 2.13	41.50*
Negative control	16.72 ± 2.91	2.92 ± 1.72	82.63
Goko 100mg/ml cleanser®	18.04 ± 4.12	3.63 ± 0.96	80.05NS
Goko cleanser® 200 mg/ml	16.71 ± 4.44	3.90 ± 1.11	76.70**
Goko cleanser® 400 mg/ml	10.98 ± 3.02	4.41 ± 1.38	59.92*
Beta cleanser® 100 mg/ml	22.54 ± 4.81	4.48 ± 1.53	80.44*, **
Beta cleanser® 200 mg/ml	18.92 ± 4.03	3.95 ± 0.87	79.36*
Beta cleanser® 400 mg/ml	12.78 ± 3.88	5.65 ± 1.37	55.94NS
Weifa defense® 100 mg/ml body	15.43 ± 3.11	4.31 ± 1.13	72.19**
Weifa defense® 200mg/ml body	8.40 ± 2.94	3.47 ± 1.20	59.51*
Weifa body defense 400 mg/ml®	15.62 ± 2.72	9.04 ± 3.02	42.30*, **

Values are expressed in mean ± S.E.M., n=6

*= P<0.05 as compared with the control group

**=P<0.05 as compared with the three herbal mixtures

NS= P>0.05 not significant as compared to control (Negative) group.

Table 11. Effect of Goko cleanser®, Beta cleanser® and Weifa body defense® on Cyclophosphamide induced neutropenia (Lymphocyte counts).

Treatment group	Total lymphocyte count		
	Before	After	%Reduction
Positive control	32.82 ± 4.95	29.40 ± 6.04	10.42*
Negative control	41.62 ± 2.6	11.20 ± 2.98	73.10
Goko 100 mg/ml cleanser®	40.30 ± 4.69	16.11 ± 2.0	50.08*
Goko cleanser® 200 mg/ml	50.71 ± 1.89	33.52 ± 4.11	34.28**
Goko cleanser® 400 mg/ml	43.50 ± 5.21	37.21 ± 3.56	13.90**
Beta cleanser® 100 mg/ml	35.41 ± 3.10	12.23 ± 2.21	65.54NS
Beta cleanser® 200 mg/ml	31.92 ± 2.55	24.40 ± 3.65	23.51*
Beta cleanser® 400 mg/ml	42.21 ± 5.17	34.85 ± 1.69	17.55*
Weifa defense® 100 mg/ml body	44.60 ± 2.98	18.11 ± 2.89	59.42**
Weifa defense® 200 mg/ml body	38.76 ± 3.84	26.23 ± 2.91	32.40*
Weifa defense® 400 mg/ml body	46.04 ± 4.81	39.61 ± 4.02	13.98*, **

Values are expressed in mean ± S.E.M., n=6

*= P<0.05 as compared with the control group

**=P<0.05 as compared with the three herbal mixtures

NS= P>0.05 not significant as compared to control (Negative) group.

400 mg/kg body weight showed a significant percentage increase in paw volume of the immunized rats when compared to the control group, while at doses of 200 and 400 mg/kg there was a significant increase in the paw volume when the result is compared among the three test herbal mixtures. Weifa body defense mixture, at doses of 100, 200 and 400 mg/kg body weight showed significant percentage increase in paw volume of the immunized animal when the result was compared to control group

(Table 13).

Humoral antibody determination

To evaluate the effect of the test herbal mixtures on humoral response, its influence was tested on sheep erythrocyte specific humoral antibody titre in experimental animals. The antibody titre was interpreted as the highest

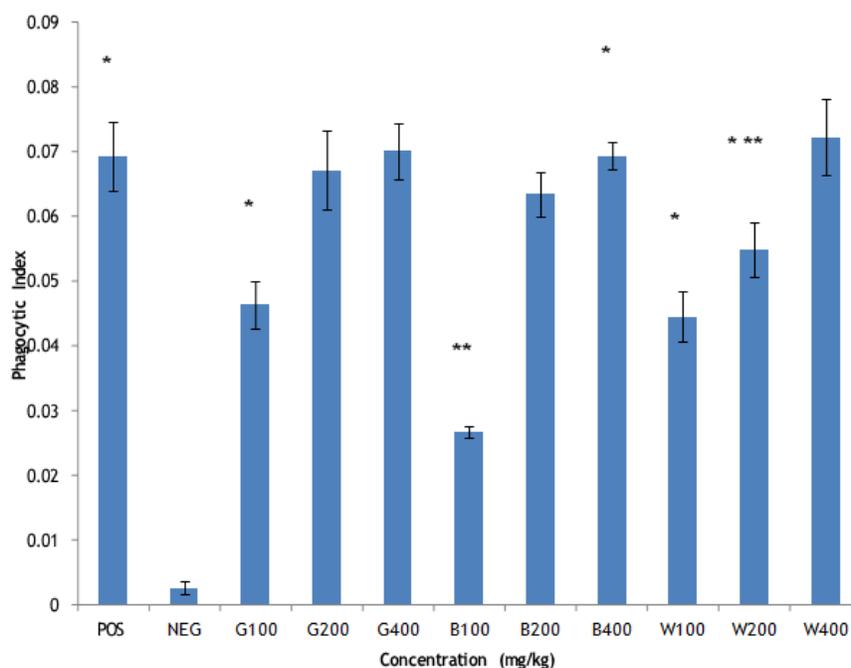


Figure 1. Phagocytic index of rats fed with Goko cleanser®, Beta cleanser® and Weifa body defense mixtures®.

Where G= Goko cleanser®, B= Beta cleanser® and W= Weifa body defense®.

*= P<0.05 as compared with the control group

**=P<0.05 as compared with the three herbal mixtures.

Table 12. Effect of Goko cleanser®, Beta cleanser® and Weifa body defense® on Cyclophosphamide induced neutropenia (Neutrophil counts).

Treatment group	Total neutrophil count		
	Before	After	%Reduction
Positive control	50.20 ± 6.90	38.40 ± 3.27	23.50*
Negative control	62.89 ± 3.21	19.02 ± 2.56	69.75
Goko 100 mg/ml cleanser®	56.21 ± 5.13	21.55 ± 2.18	61.66*
Goko cleanser® 200 mg/ml	61.00 ± 9.52	32.30 ± 3.39	47.05**
Goko cleanser® 400 mg/ml	45.34 ± 6.12	31.82 ± 7.40	29.81*
Beta cleanser® 100 mg/ml	52.65 ± 8.22	18.23 ± 2.12	65.37*
Beta cleanser® 200 mg/ml	38.45 ± 4.99	22.61 ± 4.11	41.23*
Beta cleanser® 400 mg/ml	47.44 ± 6.34	32.12 ± 4.78	32.30NS
Weifa defense® 100 mg/ml body	39.22 ± 2.82	17.32 ± 3.72	55.89**
Weifa defense® 200 mg/ml body	58.32 ± 6.91	39.81 ± 3.31	31.73*, **
Weifa defense® 400 mg/ml body	51.90 ± 3.83	40.61 ± 6.65	21.77NS

Values are expressed in mean ± S.E.M., n=6

*= P<0.05 as compared with the control group

**=P<0.05 as compared with the three herbal mixtures

NS= P>0.05 not significant as compared to control (Negative) group.

dilution that shows agglutination. In this study, Goko cleanser® at dose of 100 mg/kg body weight, Beta

cleanser® at 400 mg/kg and Weifa body defense® at doses of 100, 200 and 400 mg/kg body weight showed a

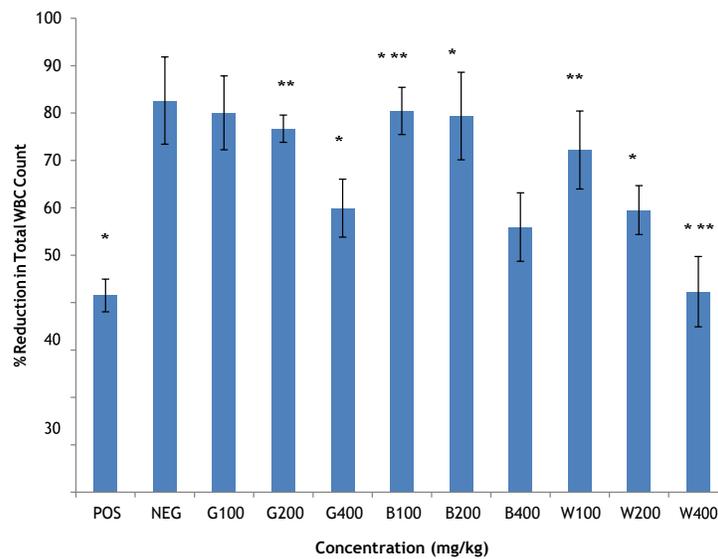


Figure 2. Cyclophosphamide induced leucopenia in rats fed with Goko cleanser®, Beta cleanser® and Weifa body defense mixtures® (Total WBC Counts).

Where G= Goko cleanser®, B= Beta cleanser® and W= Weifa body defense®.

*= P<0.05 as compared with the control group

**=P<0.05 as compared with the three herbal mixtures.

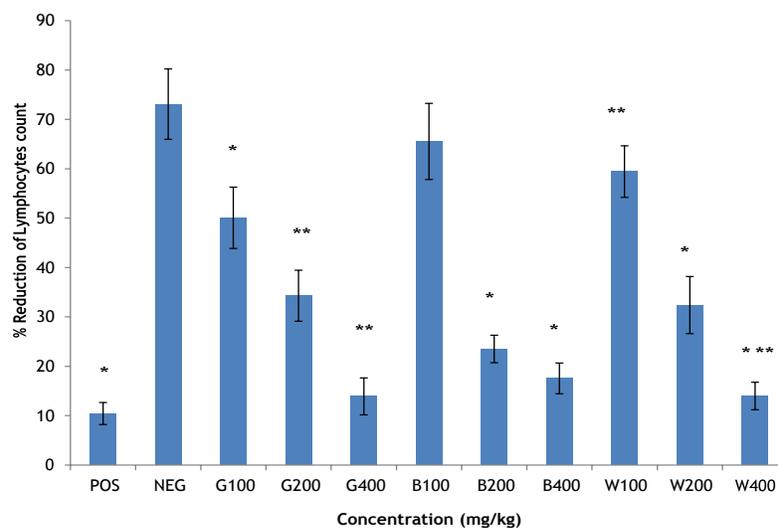


Figure 3. Cyclophosphamide induced neutropenia of rats fed with Goko cleanser®, Beta cleanser® and Weifa body defense mixtures® (Lymphocyte Counts).

Where G= Goko cleanser®, B= Beta cleanser® and W= Weifa body defense®.

*= P<0.05 as compared with the control group

**=P<0.05 as compared with the three herbal mixtures.

significant increase in augmenting antibody production. When the three herbal mixtures were compared using

statistical analysis (one way ANOVA), Goko cleanser® showed a significant effect at dose of 400 mg/kg body

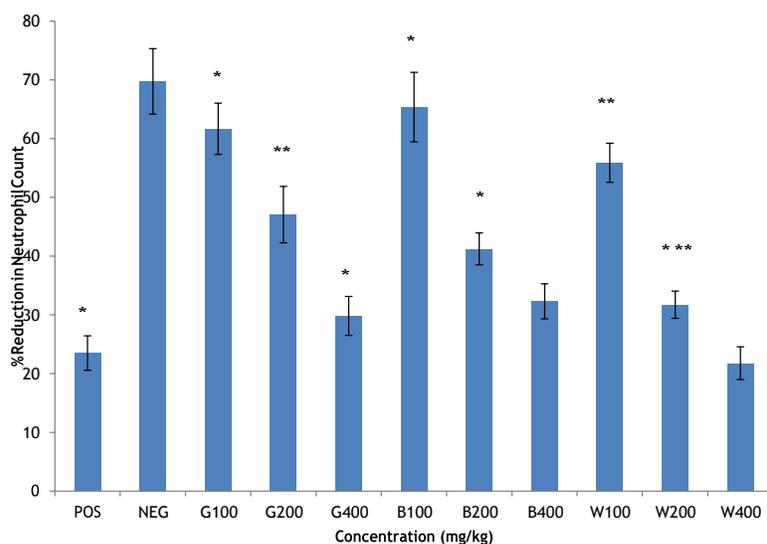


Figure 4. Cyclophosphamide induced neutropenia of rats fed with Goko cleanser®, Beta cleanser® and Weifa body defense mixtures® (Neutrophil Counts).

Where G= Goko cleanser®, B= Beta cleanser® and W= Weifa body defense®.

*= $P < 0.05$ as compared with the control group

**= $P < 0.05$ as compared with the three herbal mixtures.

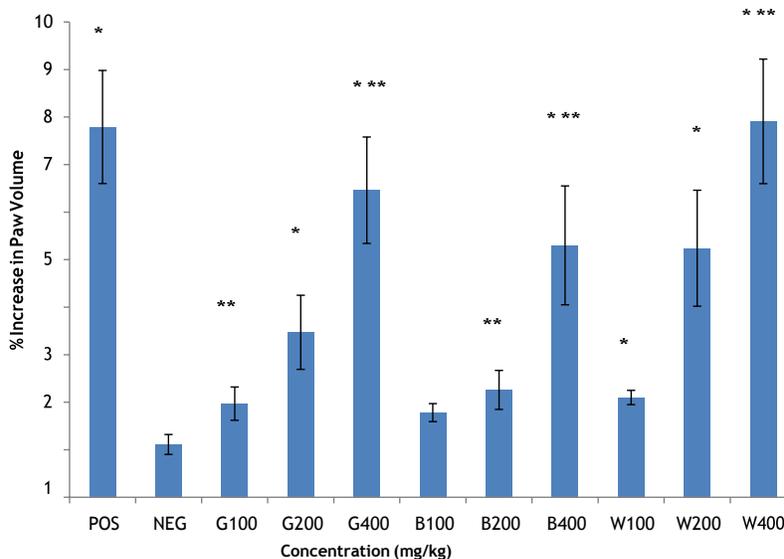


Figure 5. Delayed type hypersensitivity reaction of rats fed with Goko cleanser®, Beta cleanser® and Weifa body defense mixtures®.

Where G= Goko cleanser®, B= Beta cleanser® and W= Weifa body defense.

*= $P < 0.05$ as compared with the control group

**= $P < 0.05$ as compared with the three herbal mixtures.

weight, Beta cleanser® showed a significant effect at doses of 200 and 400 mg/kg body weight, while Weifa

body defense®, at dose of 400 mg/kg showed a significant effect as shown in Figure 6 and Table 14.

Table 13. Effects of Goko cleanser®, Beta cleanser®, and Weifa body defense mixtures® on the delayed type hypersensitivity reaction in rats.

Treatment groups	DTH response % increase
Positive control	7.79 ± 0.041*
Negative control	1.11 ± 0.013
Goko cleanser® 100 mg/ml	1.97 ± 0.016**
Goko cleanser® 200 mg/ml	3.47 ± 0.032*
Goko cleanser® 400 mg/ml	6.46 ± 0.029*, **
Beta cleanser® 100 mg/ml	1.78 ± 0.020NS
Beta cleanser® 200 mg/ml	2.26 ± 0.025**
Beta cleanser® 400 mg/ml	5.30 ± 0.027*, **
Weifa body defense® 100 mg/ml	2.10 ± 0.017*
Weifa body defense® 200 mg/ml	5.24 ± 0.031*
Weifa body defense® 400 mg/ml	7.91 ± 0.037*, **

Values are expressed in mean ± S.E.M., n=6

*= P<0.05 as compared with the control group

**=P<0.05 as compared with the three herbal mixtures

NS= P>0.05 not significant as compared to control (Negative) group.

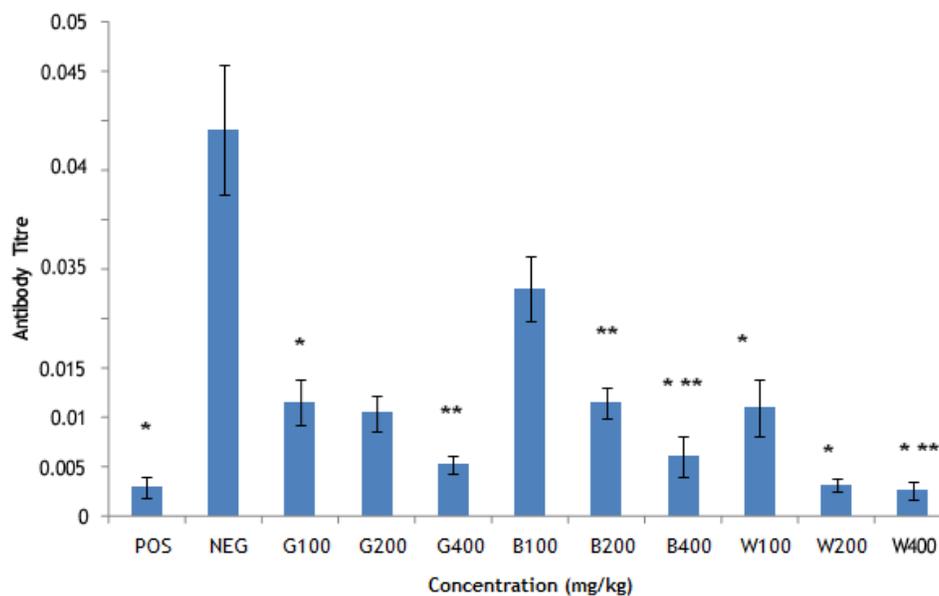


Figure 6. Humoral antibody determination in immunised rats fed with Goko cleanser®, Beta cleanser® and Weifa body defense mixtures®.

Where G= Goko cleanser®, B= Beta cleanser® and W= Weifa body defense.

*= P<0.05 as compared with the control group

**=P<0.05 as compared with the three herbal mixtures.

DISCUSSION

The presence of microbial contaminant in non-sterile pharmaceutical products can reduce or even inactivate the therapeutic activity of the product and has potential to adversely affect patients taking the medicines (Nakajima

et al., 2005). Some infectious disease outbreaks have been associated with the use of heavily contaminated raw materials of natural origin. Since the microbial quality of the herbal medicinal products were influenced by the environments and quality of the raw materials used during formulation, the manufacturers should ensure that

Table 14. Effect of Goko cleanser®, Beta cleanser® and Weifa body defense mixtures® on Humoral Antibody Determination Test.

Treatment	Mean Antibody Titre value±S.E.M.
Positive control	0.0029 ± 0.0011*
Negative control	0.0441 ± 0.0065
Goko cleanser® 100 mg/ml	0.0115 ± 0.0023*
Goko cleanser® 200 mg/ml	0.0104 ± 0.0018NS
Goko cleanser® 400 mg/ml	0.0052 ± 0.0009**
Beta cleanser® 100 mg/ml	0.0229 ± 0.0033NS
Beta cleanser®200 mg/ml	0.0114 ± 0.0015**
Beta cleanser® 400 mg/ml	0.0060 ± 0.0021* **
Weifa body defense® 100 mg/ml	0.0109 ± 0.0028*
Weifa body defense® 200 mg/ml	0.0031 ± 0.0007*
Weifa body defense® 400 mg/ml	0.0026 ± 0.0009* **

The antibody titre was interpreted as the highest dilution that shows agglutination Values are expressed in mean ± S.E.M., n=6.

*= P<0.05 as compared with the control group

**=P<0.05 as compared with the three herbal mixtures

NS= P>0.05 not significant as compared to control (Negative) group.

the microbial load is brought to a minimal safety level in the raw materials, finished dosage forms, and the packaging components, to maintain appropriate quality, safety, and efficacy of the products. Studies conducted on numerous herbal products sold and consumed in south east Nigeria showed that they were contaminated with bacteria and fungal isolates (Ujam et al., 2013). Govender et al. (2006) also reported contamination of herbal products with *Bacillus* spp., *Enterobacteriaceae* spp., *Salmonella* spp., *S. aureus*, *Penicillium* spp and *Aspergillus* spp. Moreover, elevated levels of bacterial and fungal contaminants, such as *Penicillium* spp., *Aspergillus* spp and *Fusarium* spp, have been observed in herbs and spices (Kneifel et al., 2002). In this study, *Staphylococcus aureus* and *E. coli* were isolated from Beta cleanser mixtures® and these contaminations can alter the physical, chemical and, to some extent, the pharmacological activity of the herbal product, and hence is said to be detrimental to consumers.

Studies have shown that different alkaloid extracted from numerous medicinal plants possesses a lot pharmacological activity including immunomodulatory activity (Manu and Kuttan, 2009). Kolodziej and Kiderlen (2005) attributed the immune modulatory effect of tanins extracted from different medicinal plant to their ability to cause macrophage activation. Pods of *Acacia concinna* (Leguminosae) contain several saponins which studies have shown to possess immunological adjuvant property (Ratiya et al., 2006). The result of this study was in concordance with the research work of other investigators on immunomodulatory effects of phytochemicals and suggests the origin of the immunological activity test of the herbal mixtures. At 5000 mg/kg body weight, Goko

herbal cleanser®, Beta cleanser® and Weifa body defense mixtures® were safe and non-lethal as revealed by the study, hence the oral acute toxicity of these herbal mixtures is greater than 5000mg/kg body weight.

Reticuloendothelial systems are class of cells that occur in widely separated parts of the human body and that have in common the property of phagocytosis, whereby the cells engulf and destroy bacteria, viruses, and other foreign substances and ingest worn-out or abnormal body cells. German pathologist Karl Albert Ludwig Aschoff introduced the term reticuloendothelial system in 1924, collating the cells based on their phagocytic activity. The carbon clearance assay was used to evaluate the effect on reticuloendothelial cell mediated phagocytosis (Jayathirtha and Mishra, 2004). When ink containing colloidal carbon is injected intravenously, the macrophages engulf the carbon particles of the ink. Rate of clearance of (carbon particles) ink from blood is known as phagocytic index. When colloidal ink containing carbon particles are injected directly into the systemic circulation, the rate of clearance of carbon from the blood by macrophage is governed by an exponential equation (Gokhale et al., 2003). Several researches have proven that some medicinal plants have the potential of stimulating the reticuloendothelial cells and increase their phagocytosis ability. Study on the immunomodulatory property of methanolic extract of *Swietenia mahagoni* seeds shows that the extract stimulated the reticuloendothelial system and hence increased the phagocytic index significantly (Hajra et al., 2012; Yadev et al., 2011) administered extracts of *Quisqualis indica* to albino rats and the extract appeared to enhance the phagocytic function by exhibiting a clearance rate of

carbon from the blood stream of the animal by the cells of the reticulo-endothelium system. Ethanolic extract of *Trigonella Foenum-Graeceum* were administered to albino mice and the result indicted an enhanced phagocytic function when compared to the control (Smriti et al., 2012). Methanolic Extract of *Swietenia mahagoni* seeds also enhanced phagocytic function on test animals (Subhadip et al., 2012). From the results obtained, it can be concluded that the test herbal mixtures possessed immunostimulatory property.

Cyclophosphamide is a chemotherapeutic agent used in many experimental protocols such as induced myelo-suppression in experimental animals. It is an alkylating agent of the nitrogen mustard type (Takimoto and Calvo, 2005). An alkylating agent adds an alkyl group to DNA. It attaches the alkyl group to the guanine base of DNA, at the number 7 nitrogen atom of the imidazole ring. This interferes with DNA replication by forming intrastrand and interstrand DNA crosslinks (Giraud, 2010). This was said to be the mechanism behind its myelo- suppression. White blood cells (WBCs), also called leukocytes or leucocytes, are the cells of the immune system that are involved in protecting the body against both infectious disease and foreign invaders. There are five main types: neutrophils, eosinophils, basophils, lymphocytes, and monocytes. Neutrophils are the most abundant white blood cell, constituting 60-70% of the circulating leukocytes (Alberts et al., 2002). They are important components in the surveillance and protection systems for a broad spectrum of host defenses. They play the main role as an effectors or killer cell for many types of antigenic challenges especially for infections. The primary functions of the neutrophils in host resistance are the migration towards the challenge, which is called 'chemotaxis' and the intracellular killing of microorganisms by the formation of oxygen radicals (Badway and Karnovski, 1980). Lymphocytes include natural killer cells (NK cells) (which function in cell-mediated, cytotoxic innate immunity), T cells (for cell-mediated, cytotoxic adaptive immunity), and B cells (for humoral, antibody-driven adaptive immunity). They are the main type of cell found in lymph, which prompted the name lymphocyte. Prevention of neutropenia induced by cyclophosphamide is suggested to be through the induction of the maturation and activation of macrophages which secretes substances such as colony stimulating factor and interleukin 1 (Heppner and Calabresi, 1976). At a standard dose of 30 mg/kg body weight, cyclophosphamide have been shown to decreases total white blood cell, neutrophil and lymphocyte counts in all groups of the experimental animals. Different studies have demonstrated that some medicinal plant can increase blood parameters of experimental animals administered with cyclophosphamide (Stalin and Sampath, 2013) showed that aqueous extract of *Leucas aspera* used to treat immune-suppressed mice gave a result that indicated an increase

in neutrophil and total leucocyte counts when compared to cyclophosphamide treated groups. Methanol extracts of fruits of *Solanum xanthocarpum* showed pronounced immunoprotective activity by increasing the depleted levels of total WBC count and RBC, percentage Hb, and percentage neutrophils adhesion in mice treated with cyclophosphamide at a dose of 30 mg/kg body weight (Rokeya et al., 2011; Gupta et al., 2010), in their study, demonstrated the immunomodulatory property of ethanolic (50%) extract of *Moringa oleifera* leaves on immune-suppressed rats. Eze et al. (2013), while studying the immunologic effects of *M. oleifera* methanolic leaf extract in chickens infected with Newcastle disease virus discovered that the extract stimulated the production of white blood cells. The result showed a dose dependent increase in total WBC and percentage neutrophil counts by an action on both the cellular and humoral immunity when the result is compared to the control group, Hence, the test herbal products are said to be immunostimulatory in action.

Delayed type hypersensitivity (DTH) reaction as the reaction takes two to three days to develop. It is not antibody mediated but a type of cell-mediated response. CD4+ helper T cells recognize antigen in a complex with MHC II major histocompatibility complex on the surface of antigen-presenting cells. These can be macrophages that secrete IL-12, which stimulates the proliferation of further CD4+ Th1 cells. CD4+ T cells secrete IL-2 and interferon gamma, inducing the further release of other Th1 cytokines, thus mediating the immune response. Activated CD8+ T cells destroy target cells on contact, whereas activated macrophages produce hydrolytic enzymes and, on presentation with certain intracellular pathogens, transform into multinucleated giant cells. The DTH response directly correlated with T-lymphocytes especially T-DTH-lymphocytes, therefore increased the effect on cell mediated immunity. When antigens are challenged T-cells, sensitized T-lymphocytes to convert lymphoblasts and secrete lymphokines, attracting more scavenger cells such as macrophages and basophils and induction becomes apparent within 24-72 h in test animals such as rats (Poulter et al., 1982). There are two different types of reactions capable of causing tissue injury in this way. The first, known as delayed type hypersensitivity, (DTH for short) is mediated by CD4+ helper T cells (Th-1 and Th-17 cells). The second, known as cell mediated cytotoxicity, is mediated by CD8+ T cells. The increased response indicates that ethanol extract of *Spilanthes acmella* leaves has a stimulating effect on B-lymphocytes and macrophages killing activity through NO release by stimulating T cell for the hypersensitivity reaction (Yadev et al. 2011). Studies by (Stalin and Sampath, 2013 ; Lu et al. (2007) indicated an increase in DTH reaction in mice in response to T cell dependent antigen; this revealed the stimulatory effect of aqueous extract of *L. aspera* and of *Actinidia*

macrosperma on T cells. In this research, sheep red blood cells (SRBC) are used as the antigen to induce delayed type hypersensitivity reaction in rats and this was used to evaluate the immunomodulatory effect of the herbal mixtures. In DTH reaction, T cells initiate the reaction which leads to activation and accumulation of macrophages, induce vasodilation, increase vascular permeability and as an end result, produces inflammation. This ultimately leads to the increase in the foot pad volume of the immunized animals (Dashputre and Niakwade, 2010). The result stated above shows that the three test herbal mixtures have immunostimulatory property.

Humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation to antibody secreting plasma cells (Ose and Muenster, 1968). Antibody functions as the effector of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells. Study by Hajra et al. (2012) showed that *Swietenia mahagoni* seeds extract significantly increased circulating antibody titre. This he suggested is as a result of an enhanced responsiveness of macrophages, T and B lymphocyte subsets involved in antibody synthesis. The result showed that high values of hemagglutinating antibody titre obtained in the case of methanolic extract of *Swietenia mahagoni* seeds indicated that immunostimulation was achieved through humoral immunity. Studies on the ethanolic extract of *Trigonella Foenum-Graeceum* leaves at a dose of 200 mg/kg body weight showed a significant agglutination and hence Antibody titre value when compared to the control group. Ethanol extract of *Spilanthus acmella* leaves, at a dose of 250mg/kg body weight showed an augmentation of the humoral response as evidenced by an enhancement of antibody responsiveness to sheep red blood cell antigen in rats as consequence of both pre and post-immunization drug treatment and this indicates the enhanced responsiveness of macrophages and B-lymphocyte subsets involved in antibody synthesis (Yadev et al., 2011). The result obtained shows that the test herbal mixtures have immunostimulatory property.

Conclusion

In conclusion, the three test herbal mixtures passed the microbial bioburden limit assay except for Beta cleanser® that failed the *S. aureus* and *E. coli* limit test, the presence of these microorganisms poses a danger to human upon consumption. The herbal mixtures contain some major plant phytochemicals such as flavonoids, saponins, tannins and alkaloids with established immunostimulatory/immunomodulatory activity. The toxicity profile test showed that the herbal mixtures were

relatively safe and post no acute toxic event upon consumption. This study has recognized the immunostimulatory properties of Goko cleanser®, Beta cleanser® and Weifa body defense mixtures® according to the outcome of the immunological assays done. Comparatively, Weifa body defense mixture exhibited the best immune potentiating activity than the other two herbal mixtures.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Cytotoxic effects on MCF-7 breast cancer cell lines, phenol and flavonoid contents, high performance liquid chromatography (HPLC) analysis and antioxidant activity of *Maerua pseudopetalosa* (Gilg and Bened) De Wolf fractions

Manal A Ibrahim^{1*} and Manal M Ramadan²

¹Department of Botany, Faculty of Science and Technology, Omdurman Islamic University, Sudan.

²Chemistry of Flavor and Aroma Department, Food industry and Nutrition Research Division, National Research Centre, Giza, Egypt.

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***Maerua pseudopetalosa* (Gilg and Bened.) De Wolf tubers which are used traditionally as antitumor agent in Sudan were subjected to separation by column chromatography technique. Eight fractions were obtained for the ethyl acetate extract and twelve for the ethanolic extract. The ethanolic fractions F₈, F₉, F₁₁ and F₁₂, with high bioactivity were subjected to further investigations. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used for assessment of the cytotoxicity. Remarkable cytotoxicity against Michigan Cancer Foundation-7 (MCF-7) was shown, for the first time. Actually, the results revealed that F₁₂ is a very promising one with remarkable activity against MCF-7 cell lines (43.51 µg/g at 72 h), high antioxidant activity (91.3% by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 97% by ABTS) and flavonoid and phenolic contents (11.75 and 20.72 mg/g). Six compounds were detected in F₉ (syrngic 11.65 µg/g, sinapic 8 µg/g) and F₁₂ (gallic 88.12 µg/g, caffeic 11.1 µg/g, sinapic 38.67 µg/g) which were not recorded in any previous work in the available literature by using high performance liquid chromatography (HPLC).**

Key words: Michigan Cancer Foundation-7 (MCF-7) breast cancer, *Maerua pseudopetalosa*, phenol and flavonoid contents, high performance liquid chromatography (HPLC) analysis, antioxidant activity.

INTRODUCTION

Sudan is rich in countless flora due to the diverse climatic and soil conditions in the different ecological regions.

Traditional medicines play a very important role in the health care system in Sudan and a high percentage of

*Corresponding author. E-mail: manalabdalla071@gmail.com. Tel: +249123176069.

the nomads still rely on local traditional healers. As in many other countries, biodiversity in Sudan is still maintained in some parts, which are known for the presence of so many species that are characterized for their undisputed cure for a large number of diseases (Ibrahim and EL Nure, 2016).

The use of plants in medicine is not limited or restricted to any region of the world. It is an old practice in various parts of the globe for both preventive and curative purposes. Dependence on herbs as medicine in the treatment of diseases is an adopted practice by a large proportion of the rural population because of its availability and affordability (Sani et al., 2009).

Phenolic compounds are commonly found in both edible and nonedible plants, and they have been reported to have multiple biological effects, including antioxidant activity. Free radicals have been implicated in the development of a number of disorders, including cancer, neuro-degeneration and inflammation (Halliwell, 2006a, b). The presence of antioxidants such as phenolics, flavonoids, tannins and proanthocyanidins in plants may provide protection against a number of diseases; for example, ingestion of natural antioxidants has been inversely associated with morbidity and mortality from degenerative disorders (Gülcin, 2012). Medicinal plants are therefore being investigated for their antioxidant properties, and the demand for natural antioxidants and food preservatives is increasing (Peschel et al., 2006). The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufacturers, and consumers as the trend of the future is moving toward functional food with specific health effects (Lo'ligier, 1991). Flavonoids and other phenolics have been suggested to play a preventive role in the development of cancer and heart disease. Ingestion of alcohol-free red wine or a phenolic compound mixture extracted from red wine has been shown to improve the antioxidant status of plasma in humans (Serafini et al., 1998; Carbonneau et al., 1998).

The plant, *Maerua pseudopetalosa* (Gilg and Bened.) De Wolf (family Cappariaceae), is a Sudanese medicinal plant which is traditionally used as remedy for cough and as cure for tumors. In the Nuba Mountains and the Republic of the South Sudan, the fruits are eaten but only during famine times and after careful preparations to remove the toxic substances (Henry, 1948).

Thirty three compounds were detected in tuber fractions by using GC/MS analysis; fraction 12 showed more cytotoxic effect, this was attributed to the presence of a proline derivative (proline-N-methyl-butyl ester). This compound might be considered as the cause of the high toxicity of the fraction; since free proline was used as an inhibitor of breast cancer development. Surprisingly, *M. pseudopetalosa* tubers were used in the folkloric

medicine in Sudan by the natives of the South Blue Nile State for the treatment of breast cancer growth without any knowledge of its chemical constituents (Ibrahim and EL Nure, 2015).

The plant kingdom represents an enormous reservoir of biologically active molecules and so far, only small fractions of plants with medicinal activity have been assayed. Nearly 50% of drugs used in medicine are of plant origin. There is therefore much current research devoted to the phytochemical investigation of higher plants that have ethnobotanical information associated with them. The phytochemicals (secondary metabolites) isolated are then screened for different types of biological activity (Harborne, 1998).

In addition, there will be need for the permanent search and development of new natural drugs. This need also arises from the advantage of certain natural products in controlling some diseases that chemicals fail to do. For example, the annonaceous acetogenins, extracted from pawpaw tree (*Asimina triloba* Dunal) were found to be the best effective against selected tumor type, e.g., squamatin is selective against the human prostate carcinoma cell line (PC-3) and a series of 9-carbonyl compounds work best against the human pancreatic tumor cell line (PaCa-2) (Ahammadshahib et al., 1993).

Breast cancer is one of the leading causes of death among women in the world. At the present, using of natural compounds such as medicinal plants in cancer therapy has aroused general because of its minimal side effect, safety and efficiency (Ribereau et al., 1997; Taixiang et al., 2005).

Approximately, one-third of the women with breast cancer developed metastases and ultimately died of the disease. MCF-7 is an estrogen receptor-positive human cancer cell line, derived from a patient with metastatic breast cancer (Parkin et al., 2001). Growth of MCF-7 cells is inhibited by tumor necrosis factor (TNF alpha). Many plants claimed to induce apoptosis in MCF-7 cells such as *Antrodia camphorate* (Levenson and Jordan, 1997) and *Gmelina asiatica*. The goal of screening medicinal plants is to search for excellent anticancer agent suitable for human malignancies, therefore, the aim of this study was to screen tuber fractions with respect to their total phenolic and flavonoid content, HPLC analysis, antioxidant activity in order to find new potential sources of natural anticancer drugs. The study is a part of a larger survey with other functional properties of this plant such as their antimicrobial, secondary metabolites, toxicity and GC/MS analysis.

MATERIALS AND METHODS

Plant

The plant under investigation (*M. pseudopetalosa*) was collected from the South of Sudan. The plant was authenticated at the

Department of Botany by Prof. Hatil H. Alkamali, Omdurman Islamic University.

Preparation of crude plant extracts

The plant material was air dried, ground into a coarse powder form and the dried ground tubers (1 kg) of the *M. pseudopetalosa* were soaked for 3 days in 1500 ml ethyl acetate and ethanol consecutively. Ethyl acetate extract gave 7.1 g while the ethanol extract gave 10.9 g; both with dark brown residue and were subjected to silica gel (230 - 400 mesh) column chromatography separation using stepwise gradient elution of n-hexane to chloroform, and chloroform to ethyl acetate and finally washing with pure methanol. Then, 100 ml portions were collected, concentrated and combined according to their similarity in spectrometric and thin layer chromatography (TLC) separation behaviors using suitable solvent systems. Ethyl acetate gave eight fractions while ethanol gave twelve fractions. Four fractions with bioactive effects were subjected to further investigations.

Total phenolic and flavonoid contents

The total phenolic content of the extract was determined by the Folin-Ciocalteu method (Kaur and Kapoor, 2002). Briefly, 200 L of crude extract (1 mg/mL) was made up to 3 mL with distilled water, mixed thoroughly with 0.5 mL of Folin-Ciocalteu reagent for 3 min, followed by the addition of 2 mL of 20% (w/v) sodium carbonate. The mixture was allowed to stand for a further 60 min in the dark and absorbance was measured at 650 nm. The total phenolic content was calculated from the calibration curve and the results were expressed as mg of gallic acid equivalent per g dry weight.

The total flavonoid content of crude extract was determined by the aluminium chloride colorimetric method (Chang et al., 2002). In brief, 50 L of crude extract (1 mg/mL ethanol) was made up to 1 mL with methanol, mixed with 4 mL of distilled water and then 0.3 mL of 5% NaNO₂ solution; 0.3 mL of 10% AlCl₃ solution was added after 5 min of incubation, and the mixture was allowed to stand for 6 min. Then, 2 mL of 1 mol/L NaOH solution was added, and the final volume of the mixture was brought to 10 mL with double-distilled water. The mixture was allowed to stand for 15 min, and absorbance was measured at 510 nm. The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg rutin equivalent per g dry weight.

Antioxidant properties

1,1-Diphenyl-2-picryl-hydrazyl assay

The antioxidant activity of the extract was determined by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay, as described earlier with some modifications (Villaño et al., 2007). Briefly, 200 L of each extract (100-500 g/mL) was mixed with 3.8 mL DPPH solution and incubated in the dark at room temperature for 1 h. The absorbance of the mixture was then measured at 517 nm. Ascorbic acid was used as a positive control. The ability of the sample to scavenge DPPH radical was determined from DPPH scavenging effect = (Control OD - Sample OD / Control OD) × 100.

ABTS⁺ free radical-scavenging activity

The determination of ABTS⁺ radical scavenging activity was carried out as reported by Dorman and Hiltunen (2004). Briefly, the

ABTS⁺ radical was generated by the reaction of 7 mM ABTS aqueous solution with K₂S₂O₈ (2.45 mM) in the dark for 16 h adjusting the absorbance at 734 nm to 0.700 at room temperature. The samples (10 µL) were added to 1490 µL ABTS⁺, absorbance at 734 nm was read immediately (A0) and after 6 min (A1). Several concentrations were measured, and the percentage inhibition [(A0-A1/A0) × 100] was plotted against the phenol content and IC₅₀ was determined (concentration of total phenol able to scavenge 50% of ABTS⁺ free radical).

Chromatography

HPLC instrument employed in the study was Make Waters Analytical system (USA) with alliance 2690 pump, automatic injector, UV-dual lambda observance detector and empower-2 software. The stationary phase used is C18 column. Calibration of the system was done by accurately weighing 0.01 g of standard solution (Merck, Germany) dissolved in 100 ml of HPLC grade water. 20 µl of different concentrations made from the standard stock solutions and samples were injected through a C18 Column. The mobile phase consisting of water: methanol (70:30 v/v) was degassed before use. Detection of stalk solution was done at 273 nm and flow rate was maintained at 1 ml/min. All the chemicals used are HPLC grade (99.8% pure). The methanol was obtained from Merck and Tri fluoro acetic acid from Finar. De-ionized water was obtained from Milli-Q (millipore, USA). The samples were run for minutes. All chromatographic data were recorded and processed using Autochro-300 software.

Anticancer activity

Cell cultures and treatments

Human breast cancer cell line (MCF-7) was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% MEM non-essential amino acid solution, and 1% penicillin streptomycin solution (10,000 units of penicillin and 10 mg of streptomycin in 0.9% NaCl) in a humidified atmosphere of 5% CO₂, 95% air at 35°C. The passage number range for cell lines was maintained between 20 and 25. The cells were cultured in 75 cm² cell culture flasks. For experimental purposes, cells were cultured in 96 well plates (0.2 ml of cell solution/well). The optimum cell concentration as determined by the growth profile of the cell line was 2×10⁵ cells/ml (Cells were allowed to attach for 24 h before treatment with tested extracts). The stock solution was filtered with Minisart Filters (0.22 µm). Working 2 fold serially diluted test materials were prepared. Cell monolayers were washed with PBS and the addition serially diluted materials were dispensed to the pre-cultured plates for determination of test materials toxicity (Romero et al., 2003).

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

The MTT assay is based on the protocol described for the first time by Mossmann (1983). The assay was optimized for the cell lines used in the experiments. Briefly, for the purposes of the experiments at the end of the incubation time, cells were incubated for 4 h with 0.8 mg/ml of MTT, dissolved in serum free medium. Washing with phosphate buffer saline (PBS 1 ml) was followed by the addition of DMSO (1 ml), gentle shaking for 10 min so that complete dissolution was achieved. Aliquots (200 µl) of the resulting

Table 1. Total phenol and flavonoid contents.

Sample	Total flavonoid	Total phenol
Fraction ₈	7.18 ± 0.32 ^b	9.99 ± 1.2 ^c
Fraction ₉	0.24 ± 0.01 ^d	9.51 ± 1.2 ^d
Fraction ₁₁	0.49 ± 0.01 ^c	11.18 ± 1.3 ^b
Fraction ₁₂	11.75 ± 0.66 ^a	20.72 ± 1.9 ^a

Values are means of three replications ± SD; Means with the different letter in each column are significantly different ($p < 0.05$).

Table 2. Antioxidant activity by DPPH assay.

Sample	Concentrations (%)				
	20	40	60	80	100
8	13.1	22.7	34.6	51.4	70.8
9	13.9	26.0	39.9	60.2	74.1
11	19.8	38.9	68.2	77.7	82.7
12	27.4	41.9	73.6	88.2	91.3

solutions were transferred in 96-well plates and absorbance was recorded at 560 nm using the microplate spectrophotometer system (Spectra max190-Molecular Devices). Results were analyzed with the Soft max pro software (version 2.2.1) and are presented as percentage of the control value. The relation between surviving fraction and extract concentration is plotted to get the survival curve for cell line after the specified time. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated (Mossmann, 1983).

RESULTS AND DISCUSSION

Total phenolic and flavonoids contents

Phenolic compounds are one of the major chemical classes of plants' secondary metabolites. They play an important role in the defense of plants against pathogens, diseases, parasites, and predators (Bhattacharyya et al., 2014). Moreover, they involve in a number of physiological mechanisms such as antioxidant activity. They also play an important role in stabilizing lipid peroxidation (Wei and Shioh, 2001). The total phenolic contents in the examined tuber fraction extracts using the Folin-Ciocalteu reagent is expressed in term of gallic acid equivalent. The values obtained for the concentration of total phenols are expressed as mg of GA/g of extract (Table 1). The highest concentration of phenols was measured for fraction 12 (20.72±1.9 mg/m) and this may increase anticancer activity of this fraction since flavonoids and phenolic compounds have been suggested to play a preventive role in the development of cancer and heart disease. Also fraction 11 represented concentration of 11.18±1.3 mg/ml, while fractions 8 and 9 exhibited low concentrations. On the other hand, the

values of flavonoids content represented high concentration with fraction 12 (11.75 ± 0.660), while fraction 8 showed 7.18 ± 0.32 mg/g. The lowest flavonoid content was measured in fractions 11 and 9.

Antioxidant activity

Reactive oxygen species, such as single oxygen, superoxide ion, hydroxyl ion and hydrogen peroxide, are highly reactive toxic molecules, which are generated normally in cells during metabolism. They cause severe oxidative damage to proteins, lipids, enzymes and DNA by covalent binding and lipid peroxidation, with subsequent tissue injury. Natural antioxidant agents have attracted much interest because of their ability to scavenge free radicals (Saeed et al., 2012).

Test for antioxidant activity represented strong activity in fraction 12 at a concentration of 100 mg/ml when we used DPPH and ABTS (91.3 and 97%) (Tables 2 and 3). Also F₈, F₉ and F₁₁ showed high antioxidant activity with DPPH method at a high concentration (100 mg/ml), the values were 70.8, 74.1 and 82%, respectively.

On the other hand, ABTS method exhibited high values at a concentration of 100 mg/ml, the fractions F₁₁, F₉, F₈ have activity equal to 88.9, 90.3 and 80.2%, respectively.

High performance liquid chromatography (HPLC) analysis

HPLC analysis can be used for classification of herbs based upon secondary metabolites. Extract yield at optimum condition was then analyzed by HPLC for

Table 3. Antioxidant activity by ABTS assay.

Sample	Concentrations (%)				
	20	40	60	80	100
8	41.6	58.4	70.1	75.3	80.2
9	51.8	66.0	73.4	82.8	90.3
11	50.1	56.8	69.3	75.4	88.9
12	56.9	68.6	80.1	88.3	97.0

Table 4. Retention time, peak area and concentration of compounds present in F₁₂ as revealed by HPLC analysis.

Compound	Retention time	Peak area	Concentration (mg/ml)
Gallic	5.7	237.432	88.121
Caffic	21.4	78.225	11.106
Sinapic	23.8	295.678	38.674

Table 5. Retention time, peak area and concentration of compounds present in F₉ as revealed by HPLC analysis.

Compound	Retention time	Peak area	Concentration (mg/ml)
Syrngic	23	51.408	11.65
Sinapic	33.8	78.225	8.00

quantifying bioactive compound.

The HPLC analysis of tuber fractions showed some interesting results (Table 4). Fraction 12, which proved to be the highest cytotoxic fraction, has got 3 compounds as revealed by the HPLC analysis. One of the compounds present in fraction 12 is a gallic acid which belongs to phenolic compounds. This compound might be considered as the cause of the high anticancer activity of the fraction; since gallic acid potent high antioxidant activity is linked to anticancer agent as reported by Lo^o-liger (1991).

Fraction 12 represented high concentration of gallic acid with a value of 88.121 µg/ml. Gallic acid (3,4,5 trihydroxybenzoic acid) is a phenolic compound present in most plants. This metabolite is known to exhibit a range of bioactivities including antioxidant, antimicrobial, anti-inflammatory, and anticancer (Felipe and Salgado 2016). However, this molecule attracts the interest of researchers mainly for its antioxidant capacity (Kim, 2007). Other pharmacological activities described in the literature are anticancer (Chia et al., 2010). Also, sinapic acid (3,5 dimethoxy-4-hydroxycinnamic acid) which belongs to the class of phenolic acid, exhibited 38.674 µg/ml. It has been tested and reported against various pathological conditions such as cancer inflammation,

diabetes and oxidative stress (Kikuzaki et al., 2002). The less concentration is shown by caffeic acid (3,4 dihydroxycinnamic acid 11.106 µg/ml). Caffeic acid is present in several medications of popular use, mainly based on propolis; moreover, it is acting as a carcinogenic inhibitor (Greenwald, 2004). On the other hand, fraction 9 (Table 5) reflected less concentration of syrngic and sinapic with values of 11.65 and 8 µg/ml, respectively.

Anticancer activity

Tuber fractions were evaluated *in-vitro* for their anticancer activity against MCF-7 cell lines using MTT assay. 5-fluorouracil is one of the most commonly used drugs to treat cancer (positive control) and the plant extracts were used at different concentrations for 24, 48 and 72 h. The MTT assay is a sensitive, quantitative and reliable colorimetric assay that measure cell viability. The assay is based on the capacity of the cellular mitochondrial dehydrogenase enzyme in living cells to reduce the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue/purple formazan product which is

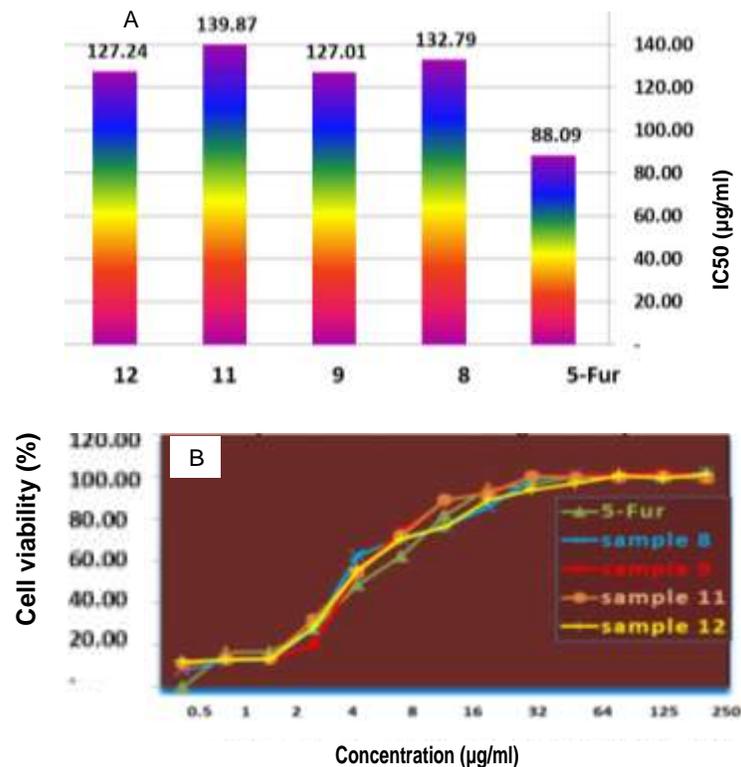


Figure 1. (A) Evaluation of IC₅₀ after 24 h of test compounds to human breast cancer cell line (MCF-7). (B) Evaluation of viability % of human breast cancer cell line (MCF-7) post treatment with test materials for 24 h compared with 5-fluorouracil using TMM assay.

insoluble in water. The amount of formazan produced is directly proportional to the cell number in a range of cells lines (Riss et al., 2016; Gerlier and Thomasset, 1986). Also, it is accurate because of its ability to describe the relationship between the amount of active cells with absorbance obtained from measuring its 50% inhibition concentration value (IC₅₀) (Behera et al., 2003). The lesser the IC₅₀ value, the higher the potential of the tested extract to inhibit cell proliferation. The principle of the MTT assay is to measure the activity of mitochondrial dehydrogenase in converting MTT into formazan. The concentration of formazan, which has a blue color, can be determined with a visible spectrophotometer and it has positive correlation with the number of living cells because the reduction event only exists when the mitochondrial reductase is produced (mitochondria is still active and this indicates that the cell is alive) (Chapdelaine, 2001).

As a matter of fact, the results revealed that F12 (43.15 µg/ml) is a very promising one with remarkable IC₅₀ against MCF-7 breast cancer cell when compared with 5-fluorouracil (51.22 µg/ml) used as standard drug at 72 h (Figure 3A). The other fractions F11, F9 and F8 showed IC₅₀ equal to 66.98, 48.11 and 60.45 µg/ml, respectively

(Figure 3A). As the cell viability decreases the inhibition increase and that led to more potent drug, this is clearly shown in Figures 1, 2 and 3B.

Phytochemicals isolated from herbs have emerged as a new and promising source of anticancer remedies, or as adjuvants for chemotherapeutic drugs, to enhance their efficacy and decrease side effects (De Vita et al., 2000).

Gallic acid is a possible cause of the anticancer activity observed for fractions (F12); since Felipe and Salgado (2016) reported that the gallic acid was known to display some anticancer and antioxidant activity. Moreover, the presence of gallic acid is restricted to this fraction. This compound might be considered as the cause of the high toxicity of the fraction; since gallic acid was found to inhibit the growth of breast cancer cell MCF-7 as reported by Wang et al. (2014). He suggested it as a possible application in breast cancer therapy. However, Zheng et al. (2001) also referred to the potent antioxidant of the cinapic acid which may provide another explanation to increase antioxidant and anticancer effects of this fraction.

Furthermore, the presence of caffeic acid in fraction 12 might also be taken as another proof for the increased anticancer activity of the fraction compared to fraction 9

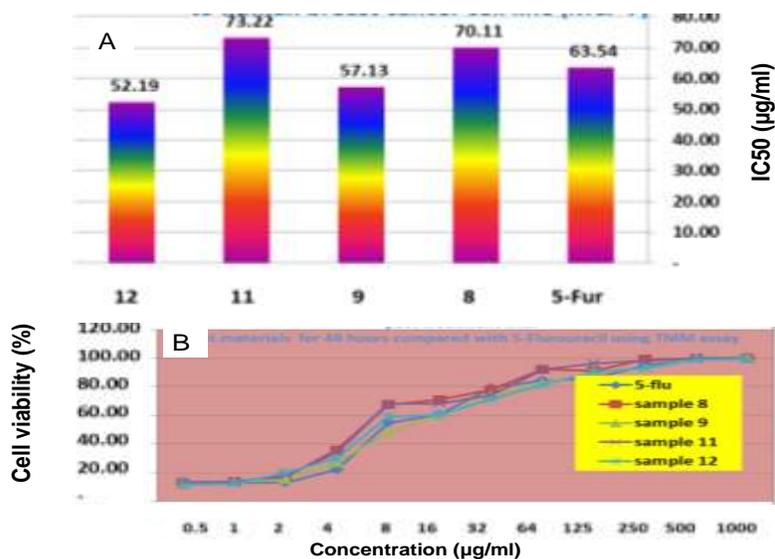


Figure 2. (A) Evaluation of IC50 after 48 h of test compounds to human breast cancer cell line (MCF-7). (B) Evaluation of viability % of human breast cancer cell line (MCF-7) post treatment with test materials for 48 h compared with 5-fluoracil using TMM assay.

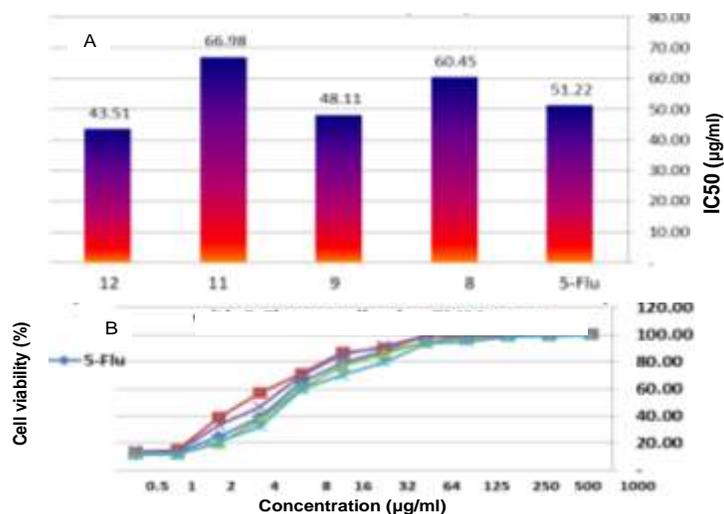


Figure 3. (A) Evaluation of IC50 after 72 h of test compounds to human breast cancer cell line (MCF-7). (B) Evaluation of viability % of human breast cancer cell line (MCF-7) post treatment with test materials for 72 h compared with 5-fluoracil using TMM assay.

which lacks this acid in spite of its high IC₅₀ value (48.11 µg/ml).

Surprisingly, *M. pseudopetalosa* tubers were used in the folkloric medicine of the natives of the South Blue Nile State in Sudan for the treatment of breast cancer growth without any knowledge of their chemical constituents (Ibrahim and EL Nure, 2015).

Conclusion

This study indicated that the tuber fractions contained high amounts of phenolic compounds and exhibited strong antioxidant activities. Gallic acid, cinapic acid, and caffeic acid are concentrated largely in fraction 12 in comparison with the other fractions and also represented

anticancer effect more than 5-fluorouracil which is used as anticancer chemo therapeutic drug. Hence, the plant tubers may be used as a new and promising source of breast cancer remedies, or as adjuvants for chemotherapeutic drugs to decrease side effects.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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