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Full Length Research Paper

Evaluation of anti-*Mycobacterium tuberculosis* activity of fractions from selected medicinal plants used traditionally for treating cough and respiratory disorders in South West of Nigeria

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Tuberculosis is a contagious airborne infection that mostly affects the lungs. The causative agent of tuberculosis in human is *Mycobacterium tuberculosis*. The emergence and dissemination of *M. tuberculosis* isolates that are resistant to multiple antimicrobial drugs represent a growing public health threat. Fractions from *Alafia barteri*, *Chasmanthera dependence*, *Chrysophyllum albidum*, *Emilia coccinea*, *Mezoneuron benthamianum*, *Phyllanthus muellerianus*, *Secamoni afzeli*, *Senna alata*, *Xylopia aethiopica* and *Acalypha fimbriata* were screened for activity against drug susceptible *M. tuberculosis* H_{37}Rv and the local isolates using proportion and nitrate reduction methods. The organisms used were *M. tuberculosis* H37Rv strain and the local isolates from TB patients. The standard antitubercular drugs used were isoniazid and rifampicin. No fractions from *A. barterii*, *C. dependens*, *E. coccinea*, *S. afzeli*, *S. alata* and *X. aethiopica* showed sensitivity against the *M. tuberculosis* strains. The hexane fraction of *C. albidum*, butanol fraction of *M. benthamianum*, ethyl acetate fraction of *P. muellerianus* and ethyl acetate fraction of *A. fimbriata* showed sensitivity with minimum inhibition concentration of 0.5 mg/ml. The ethylacetate and hexane fractions of *M. benthamianum* together with hexane fraction of *P. muellerianus* showed sensitivity with MIC value of 1.25 mg/ml. The highest MIC value of 2.5 mg/ml was obtained from hexane fraction of *A. fimbriata*. Thus, *C. albidum*, *M. benthamianum*, *P. muellerianus* and *A. fimbriata* possessed antimycobacterium tuberculosis activity and further research work would be required to assess possible antitubercular agents present in the four medicinal plants.

Key words: Tuberculosis, anti-mycobacterium, fractions, sensitivity and inhibition.

INTRODUCTION

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis*. It is transmitted from an in active tuberculosis patient by exposure to tubercle bacilli air-borne droplets from coughing or sneezing.

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Tuberculosis spreads easily in overcrowded settings and in conditions of malnutrition and poverty. In 1993, the World Health Organization (WHO) declared tuberculosis a global emergency because it killed more adults each year than any other infectious disease (Kandel et al., 2008).

Tuberculosis still constitutes a major health problem in Nigeria. According to the WHO, the estimated incidence of TB in Nigeria is 322 per 100,000 population with only 15% of the total burden of the disease in the country being notified in 2015 (Onyedum et al., 2017). Over 80 % of TB cases in Nigeria were still undetected while it claimed over 1.5 million lives annually in the country (Olokor, 2017).

Mycobacterium tuberculosis is successful in surviving the presence of toxic compounds because they produce effective permeability barriers comprising the outer membrane and the mycolate-containing cell wall on the surface (Liu et al., 2016). The ability of the organism to remain dormant or persistent within host cells for many years with the potential to be activated allows the bacterium to escape the immune system of the host (Meena and Rajini, 2010). Survival mechanisms of the bacterium include prevention of phago-lysosome fusion (Pieters, 2008), prevention of cell acidification (Queval et al., 2017) and protection against reactive nitrogen intermediates (RNI) (Rousseau et al., 2004).

A person infected with *M. tuberculosis* incurs 10% risk of developing active TB (WHO, 2007). Major risk factors for TB activation include HIV infection, recent contact with an infectious patient, initiation of an anti-tumor necrosis factor (TNF) treatment, receiving dialysis, receiving an organ or hematologic transplantation, silicosis, being in prison, being an immigrant from high TB burden countries, being a homeless person and being an illicit drug users (WHO, 2018). Alcohol consumption, particularly heavy consumption, is an important risk factor for tuberculosis (Lonroth et al., 2008; Rehm et al., 2009).

Adverse effects of antituberculosis drugs, drug interactions, high cost of drugs, shortage of drugs and a complex long time therapeutic regimen still make TB one of the major health challenges in the world (Arbex et al., 2010; Sotgiu et al., 2015). The emergence of Multi-Drug Resistant Tuberculosis (MDR-TB) and Extended-Drug Resistant Tuberculosis (XDR-TB) strains has threatened the efficacy of many existing antibiotics (Calligaro et al., 2014; Prasad et al., 2017).

Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. Natural products, either as pure compounds or as standardized plant extracts provide unlimited opportunities for new drug leads because of the unmatched chemical diversity (Mahalingam et al, 2011). Herbal drugs whether extract or decoction used against any pathogen will not cause the problem of drug resistance (Shashidhar et al., 2015).

Pure drugs or synthezised drugs are expensive and sometimes are not available in remote areas (Ammal and Bar, 2013). The search for new plant chemicals as antimicrobial agents becomes paramount because of an increase in antimicrobial resistance by pathogens and the emergence of new drug-resistant pathogens. Among the 11 currently used nature-derived TB drugs, seven of them were either isolated from microbes or semi-synthesized from microbial natural products (Liu et al., 2016) for example, streptomycin and kanamycin from *Streptomyces griseus* and capreomycin isolated from *S. capreolus* (Copp, 2003; Shu, 1998).

Rifampicin is a semi-synthetic drug that has been derived from Rifamycin, a product of *A. mediterranei* (Tribuddharat and Fennewald, 1999). Thus, plant kingdom can be looked at as an important source of new drugs for the treatment of TB because of its enormous chemical diversity (Gautam et al., 2007). The new drugs may not necessarily be new antibiotics but rather other drugs that prevent persistence within the host and leave the vegetative cells susceptible to treatment.

Anti-mycobacterium studies of some Nigeria medicinal plants demonstrate that they could be good sources of compounds with anti-mycobacterium activities worth of investigation (Mann et al., 2008; Ibekwe and Ameh, 2014). Some medicinal plants have been reported to possess anti-mycobacterium tuberculosis activity in Nigeria (Adeleye et al., 2008; Faleyimu et al., 2009). The selection of the ten medicinal plants is based on their usage by traditional practitioners in treating tuberculosis, cough or respiratory disorders.

The aim of this research work was to evaluate the anti-mycobacterium tuberculosis activity of the selected medicinal plants.

MATERIALS AND METHODS

Plant materials

The medicinal plants were obtained from Olokemeji Forest Reserve in Oyo state, Iberekodo market in Ogun state and Mushin market in Lagos state. The plants were identified by Mr T. K. Odewo of the Forestry Research Institute of Nigeria, (FRIN), Ibadan.

Preparation of extracts

80% ethanol solutions were added to the dried powdered samples of the plants. The mixtures were kept at room temperature for 72 h with gentle and intermittent shaking and thereafter filtered. Filtrates were dried at 42.5°C. Sequential extraction with hexane, ethyl acetate and butanol solvents were carried out. Table 1 shows the ten plants evaluated for anti-mycobacterium tuberculosis. Only *A. fimbriata* and *P. muellerianus* are of the same family, Euphorbiaceae, while others belong to different families. The Table 1 also shows the part of the plants used in the research work. The local name of the plant represents the name in Yoruba language. Table 2 shows the yield in both the 80% ethanol extraction and in the fractionation. No hexane fraction was obtained for *A. barteri*, *C. dependens*, *E. coccinea*, *S. afzelii, S. alata* and *X. aethiopica*. *A. fimbriata* was restricted to only hexane and ethylacetate partitioning.

Table 1. Plants for the anti-mycobacterium tuberculosis evaluation.

S/N	Name of plant	Family	Local name	Part of plant used
1	*Alafia barteri*	Apocynaceae	Agbari etu	Leaves
2	*Chasmanthera dependens*	Menispermaceae	Ato	Stem
3	*Chrysophyllum albidum*	Sapotaceae	Agbalumo	Cotyledon of seeds
4	*Emilia coccinea*	Compositae	Odundun'do	Leaves
5	*Mezoneuron benthamianum*	Leguminoseae	Jeninfinran	Leaves
6	*Phyllanthus muellerianus*	Euphorbiaceae	Egungun eja	Leaves
7	*Secamoni afzelii*	Asclepiadaceae	Ailu	Leaves
8	*Senna alata*	Caesalpiniaceae	Asunwon oyinbo	Leaves
9	*Xylopia aethiopica*	Annonaceae	Eru awonrika	Pods
10	*Acalypha fimbriata*	Euphorbiaceae	Jinwinni	Leaves

Table 2. Weight of extracts and fractions from the medicinal plants.

S/N	Medicinal plant	Weight of dried powdered sample (g)	Weight of ethanol extract (g)	Weight of butanol fraction (g)	Weight of ethylacetate fraction (g)	Weight of Hexane fraction (g)
1	*Alafia barteri*	50	0.95	0.57	0.28	-
2	*Chasmanthera dependens*	50	0.8	0.14	0.29	-
3	*Chrysophyllum albidum*	60	2.9	1.94	0.48	0.01
4	*Emilia coccinea*	50	0.8	0.13	0.25	-
5	*Mezoneuron benthamianum*	120	3.9	0.85	0.93	0.15
6	*Phyllanthus muellerianus*	50	1.2	0.16	0.34	0.01
7	*Secamoni afzelii*	40	1.7	0.05	0.13	-
8	*Senna alata*	34	1.2	0.16	0.25	-
9	*Xylopia aethiopica*	60	3.2	0.17	0.82	-
10	*Acalypha fimbriata*	60	1.2	-	0.21	0.05

Preparation of samples of roasted seeds of *C. albidum*

In line with the folklore usage of the seeds of *C. albidum* in treating tuberculosis infection, 100 sun dried seeds of the plant were put in a closed crucible and heated for 30 min at 50, 100 and 120°C separately. The seeds were removed from their shells after heating and were ground to powder for hexane extraction at room temperature. The yields were 0.13, 0.15 and 0.16 g of hexane extracts respectively. Various concentrations of the 50°C and the 120°C were subjected to anti-*M*. *tuberculosis* test as described above.

The test organisms

The reference *M. tuberculosis* strain $H_{37}R_V$ labelled PT_{12} and the local isolates labelled PT_{10} were used. The local isolates were isolated from TB patients using standard methods. The organisms were sub-cultured in Middle Brook 7H9 broth supplemented with OADC at 37°C for 21-28 days and were confirmed acid fast gram positive bacillus using Ziehl Nelson stain.

Anti-*M. tuberculosis* test

The anti-*M. tuberculosis* test was done using proportion method. 5 ml of the filtered extract solutions (DMSO as solvent) was added to 15 ml of the homogenized egg LJ media to arrive at various concentrations ranging from 0.5 to 0.5 mg/ml. Each 20 ml medium was divided into 10 ml in universal containers. Standard drugs, isoniazid and rifampicin, at 0.2 and 0.4 µg/ml respectively, were added to LJ media accordingly. The media were slanted to form slopes. The LJ slopes without extracts and drugs were used as control. The slopes were insipissated (the slopes were thickened) at 85° C for 45 min, cooled and stored in a refrigerator at 4°C. Sterility and viability check were carried out before inoculation.

Inoculation of slopes with the bacteria

Bacterial dilutions 10^{-5} and 10^{-3} mg/ml were prepared for inoculation. 0.1 ml of the chosen bacterial dilutions was inoculated into all the labelled LJ slopes (Adeleye et al., 2008). The universal containers were loosely closed with caps to allow evaporation and were incubated at 37°C. The specimens were checked on the 7^{th}, 14^{th}, and 21^{st} days to ensure no contaminations. Readings were done on the 28^{th} day.

Nitrate reduction test

Nitrate reduction test was performed on all the slopes after 28 days. This involved addition of 2 ml Nitrate Substrate Broth, incubation at 37°C for 2 h, addition of 1 drop of 50% hydrochloric acid, 2 drops of AFB Nitrate Reagent A (sulfanilamide 0.2%), 2 drops of AFB

Table 3. Results of anti-mycobacterium tuberculosis test.

S/N	Medicinal plants	Fractions	Weight (mg/ml)	*Mycobacterium tuberculosis*	
				PT_{12}	PT_{10}
1	*Alafia barteri*	Butanol	25	R	R
		Ethylacetate	10	R	R
2	*Chasmanthera dependens*	Butanol	5	R	R
		Ethylacetate	10	R	R
3	*Chrysophyllum albidum*	Butanol	50	R	R
		Ethylacetate	10	R	R
		Hexane	0.5	S	S
4	*Emilia coccinea*	Butanol	5	R	R
		Ethylacetate	10	R	R
5	*Mezoneuron benthamianum*	Butanol	25	S	S
		Ethylacetate	25	S	S
		Hexane	5	S	S
6	*Phyllanthus muellerianus*	Butanol	5	R	R
		Ethylacetate	10	S	S
		Hexane	0.5	S	S
7	*Secamoni afzelii*	Butanol	5	R	R
		Ethylacetate	5	R	R
8	*Senna alata*	Butanol	5	R	R
		Ethylacetate	10	R	R
9	*Xylopia aethiopica*	Butanol	5	R	R
		Ethylacetate	25	R	R
10	*Acalypha fimbriata*	Ethylacetate	10	S	S
		Hexane	2.5	S	S
11	Isoniazid	-	0.2 µg	S	S
12	Rifampicin	-	0.4 µg	S	S

PT_{12}, The standard strain of $H_{37}Rv$; PT_{10}, the local isolate strain, R, resistance (no inhibition of bacteria growth), S, sensitive (bacteria growth was inhibited).

Nitrate Reagent B (naphthylethylenediamine dihydrochloride, 0.1 %) and a pinch of Nitrate Reagent C (Zinc dust). Colour change was examined for resistance while no colour changes were examined for sensitive.

Determination of minimum inhibition concentration (MIC)

Minimum inhibition concentration of the eight anti-*M. tuberculosis* fractions from the four medicinal plants was determined. The stock solution for each fraction contained 0.2 g of sample in 20 ml of DMSO. Various dilutions were made to arrive at 2.5, 1.25, 0.75, 0.5 and 0.25 mg/ml. The LJ media were duplicated to serve both the standard Mtb strain, PT_{12} and the local strain, PT_{10} to obtain 80 LJ slopes of samples. The anti-mycobacterium tuberculosis test procedure described above was used.

RESULTS AND DISCUSSION

Fractions from ethanolic extracts of the ten medicinal plants were evaluated for anti-mycobacterium tuberculosis activities. Table 3 shows the results of the anti-mycobacterium tuberculosis test. Butanol, ethylacetate and hexane fractions from *M. benthamianum*,

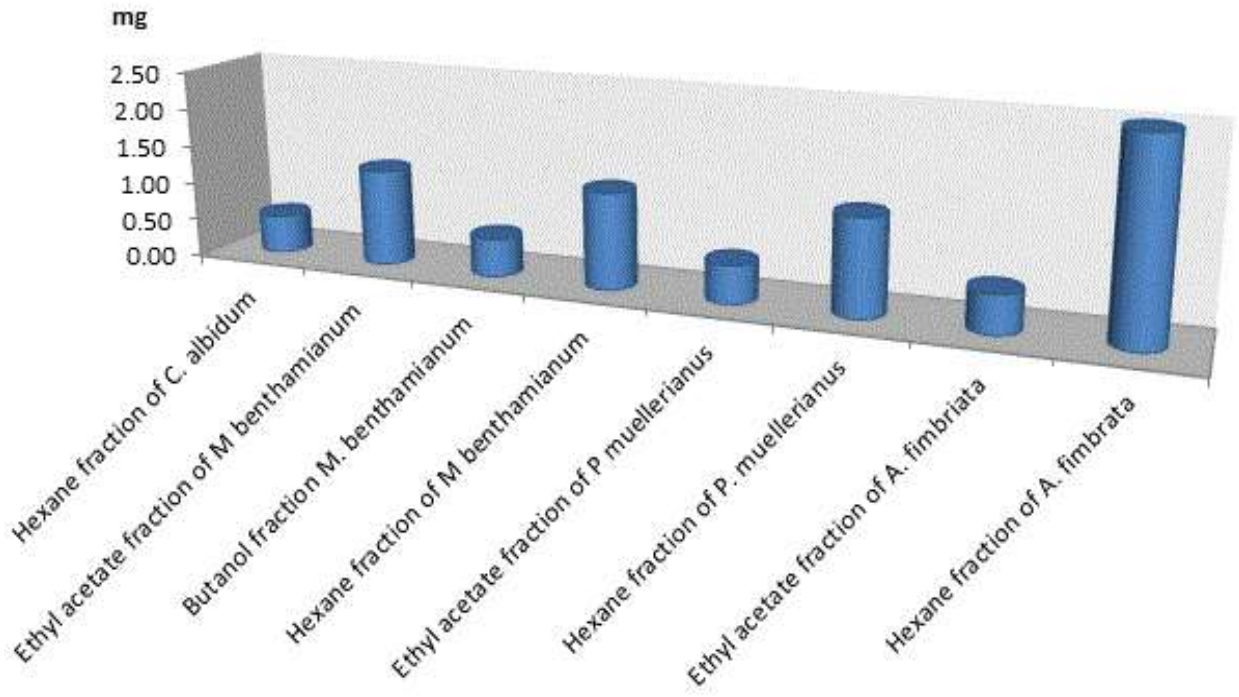

Figure 1. Minimum inhibition concentration of the anti-mycobacteruim fractions.

ethylacetate and hexane fractions from *P. muellerianus*, hexane fraction from *C. albidum* together with hexane and ethylacetate fractions from *A. fimbriata* inhibited the growth of the *M. tuberculosis* strains. No fractions from *A. barterii, C. dependens*, *E. coccinea*, *S. afzelii, S. alata* and *X. aethiopica* inhibited the growth of the bacteria. The medicinal plants were selected based on their antimicrobial activities and their traditional use in treating respiratory diseases.

The negative result obtained for *X. aethiopica* as shown in Table 3 is consistent with the earlier report by Adeleye et al. (2008) and Ogu (2011), of the ineffectiveness of the plant in inhibiting the growth of the *M. tuberculosis*. *X. aethiopica* had been reported to be antihypertensive (Gbadamosi and Kalejaye, 2017) and the association between hypertension and tuberculosis had been reported (Seegert et al., 2017). Thus, the use of *X. aethiopica* in combination therapy with other antitubercular medicinal plants by traditional practitioners of Southwestern Nigeria could have the advantage of limiting hypertension of the TB patients during the treatment period.

Figure 1 shows the results of the MIC. Hexane fraction of *C. albidum*, butanol fraction of *M. benthamianum*, ethylacetate fractions of both *P. muellerianus* and *A. fimbriata* had MIC value of 0.5 mg/ml. The ethylacetate and hexane fractions of *M. benthamianum* together with the hexane fraction of *P. muellerianus* showed minimum inhibition concentration of 1.25 mg/ml. The highest MIC value obtained was 2.5 mg/ml for hexane fraction of *A. fimbriata*. Extracts of *A. fimbriata* are used in the treatment of asthma and respiratory tract inflammation (Essiett and Okoko 2013). The anti-tuberculosis activity of an *Acalypha* specie, *Acalypha indica*, against multi-drug resistant *M. tuberculosis* isolates had been reported (Gupta et al., 2010).

Three fractions obtained from *M. benthamianum* inhibited the growth of *M. tuberculosis*. Gallic acid and its derivatives had been isolated from *M. benthamianum* (Tchinda et al., 2016). Gallic acid derivative isolated from another benthamianum species, *Disthemonanthus benthamianum*, had demonstrated antitubercular activity (Evina et al., 2017). Synthesized derivatives of gallic acid showed anti-tuberculosis activity (Ilango and Arunkumar, 2010).

The ethylacetate and hexane fractions from *P. muellerianus* showed inhibition of the bacteria. Its butanol fraction was not sensitive as shown in Table 3. The leaves extract of *P. muellerianus* was reported to inhibit the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Doughari and Sunday, 2008). The leaves extract of *P. muellerianus* possessed anti-inflammatory property and the main constituent isolated from the plant, geraniin, had been shown to be anticarcinogenic, antihyperglycemic and antihypertensive (Boakye et al., 2016, Elendran et al., 2015). Only the hexane fraction of *C. albidum* inhibited the growth of *M. tuberculosis*. The traditional practitioners in Abeokuta, Ogun state part of

Table 4. Results of anti-mycobacterium tuberculosis test of the extracts of roasted seeds of *Chrysophyllum albidum*.

S/N	Samples tested	Concentrations	Mycobacterium tuberculosis	
			PT_{12}	PT_{10}
1	Hexane extract (50°C)	2 mg/ml	S	S
		1.5 mg/l	S	S
		0.4 mg/ml	S	S
2.	Hexane extract (120°C)	2 mg/ml	S	S
		1.5 mg/ml	S	S
		0.4 mg/ml	S	S
3	Rifampicin	0.4 µg/ml	S	S
4	Positive control	Agar inoculated only	R	R
5	Negative Control	Agar not inoculated	-	-

Nigeria, put roasted and powdered cotyledon of the seeds of *C. albidum* in honey for TB patients to lick for a period of one month. Table 4 shows the results of anti-*M. tuberculosis* test of the roasted seeds of *C. albidum*. There was no bacteria growth on the agar without inoculation (negative control) while there was growth on the agar inoculated (positive control). Both the hexane extracts of the 50 and 120°C roasted seeds showed sensitivity at 0.4 mg/ml. The fruit and the leaves extracts of *C. albidum* had been reported to possess high antimicrobial activity (George et al., 2018; Olasehinde et al., 2015).

Conclusion

There were no fractions from *A. barterii*, *C.dependens*, *E. coccinea*, *S. afzeli*, *S. alata* and *X. aethiopica* that showed sensitivity against the drug susceptible *M. tuberculosis* strains. Hexane fraction from *C. albidum*, butanol, ethylacetate and hexane fractions from *M. benthamianum*, ethylacetate and hexane fractions from *P. muellerianus* and *A. fimbriata* showed sensitivity against *M. tuberculosis* H_{37}RV and the local isolate from TB patients. The hexane extracts of the roasted seeds of *C. albidum* were also sensitive to the *M. tuberculosis* strains. The active fractions would be investigated for the presence of anti-mycobacterium tuberculosis agents.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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A comparative study of the histopathological modifications of adrenal gland in STZ-induced diabetic Wistar rats administered with selected herbal plants versus Glimepiride

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This study investigated the comparative study of four herbal extract versus Glimepiride on the histomorphological modification of adrenal gland in STZ induced diabetic rats with a view to understanding their antidiabetic properties. Forty- two healthy adult Wistar rats (*Rattus novergicus*) with an average weight of 153.4 g were randomly divided into seven groups (n=6). STZ (65 mg/kg) dissolved in citrate buffer was administered intraperitoneally to animals in groups B to G while animals in group A received equivalent volume of citrate buffer. Plant extracts (100 mg/kg) were administered daily (orally) to animals in groups C to F and glimepiride (anti-diabetic drug) to animals in group G for 14 days. After the expiration of the study, the animals were sacrificed and the adrenal gland was excised, fixed in 10% formol saline for histology and morphometric analysis. Result showed that body weights of diabetic rats significantly decreased when compared with control and other groups. Also, adrenal weight, and thickness of the cortex were significantly increased ($P<0.05$) in diabetic rats compared with control and other groups. Also, thickness in medulla of adrenal gland of group B was decreased significantly ($p < 0.05$) when compared with control and other groups. The histology and morphometric analysis revealed that the adrenal gland in the group treated with *Citrullus lanatus* seed shaft showed a better histoarchitectural outline of all the four plant extracts used. This study suggested that *C. lanatus* seed shaft could be a better alternative therapy in ameliorating diabetic-associated disorders of the adrenal gland.

Key words: Diabetes, *Psidium guajava, Veronia amygdalina, Ficus mucuso, Citrullus colocynthis,* adrenal gland.

INTRODUCTION

The extracts of medicinal plants have been established to ameliorate and protect different diseases which have been used by the majority of the world population for thousands of years. Herbal drugs are prescribed and used

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widely because of their effectiveness in curative, less side effects and not expensive.

Citrullus colocynthis (CC) (family of Cucurbitaceae) commonly known as colocynth or bitter apple, is one of the plants which has been used for anti-diabetic in traditional medicine (Shafaei et al., 2014). The name of this extract was derived from its bitter flavor which is similar to colocynthine (Shafaei et al., 2014). The colocynth which originated from tropical Asia and Africa is now widely distributed in other parts of the world (Azzi et al., 2015). *C. colocynthis* contained lycopene, ascorbic acid and citruline which are valued source of natural antioxidants. These mentioned functional ingredients act as protection against chronic health disorders like cancer insurgence and cardiovascular disorders (Shafaei et al., 2014). Lycopene is a lipophilic carotenoid stored in adipose tissues that reduces the obesity and hyperglycemic conditions (Madhava et al., 2011).

V. amygdalina (VA) is from Asteraceae family. It is a perennial plant with height between 1 and 6 m. It is soft wooded and a multipurpose with rapid regenerating shrub (Nwosu et al., 2013). All parts of the plant are useful in pharmacologically research and both the roots and leaves are useful in phyto-medicine for management of various diseases in humans and animals (Tugume et al., 2016). The leaves could also used traditionally to induce fertility in women (Adedapo et al., 2014). It possesses antioxidant benefits (Oyeyemi et al., 2017), enhances the immune system, decreased blood sugar when compared to untreated diabetic animals in a study conducted using streptozotocin-induced diabetic laboratory animals (Oyeyemi et al., 2017) and *in vitro* antihelmintic and antiparasitic properties (Ademola and Eloff, 2011). A number of researches have shown the anti-diabetic properties of VA (Oyeyemi et al., 2017). The *in vivo* anti-diabetic activity has also been demonstrated (Tugume et al., 2016).

Ficus mucuso (FM) with a common name called fig is a semi-deciduous spreading savannahs tree with greenish flower and a very tiny numerous seeds (Ahoua et al., 2012). Apes and indeed humans (Kamanzi, 2002) depends so much on *Ficus* as part of their diet because of the high nutritive value. The antioxidant status and beneficial effects of *Ficus* have been documented (Ahoua et al., 2012) as well as ameliorative role of its extracts on the biochemical profiles (Ayoka et al., 2014).

Guava (*Psidium guajava* L.) (PG) possess some phytochemicals which had been documented, such as phenolic compounds, carotenoids and vitamins, mainly ascorbic acid (C) and tocopherol (E), which are effective free-radical scavengers (Jiménez-Escrig et al., 2001; Tesfahun and Habtamu, 2017). Some of these substances are effective in the treatment of the diseases (Tesfahun and Habtamu, 2017). The regular consumption of significant amounts of fruits and vegetables of *P. guajava* has been promoted by specialists to prevent degenerative and chronic diseases due to its antioxidants property (Chiari-Andreo et al., 2017).

Diabetes is a group of metabolic diseases characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycaemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the adrenal gland eyes, kidneys, nerves, heart, and blood vessels (Ismail et al., 2016). Diabetes mellitus (DM) is a chronic disease leading to impairment of the functions of many systems, such as the cardiovascular, immune, and central nervous systems through hyperglycemia, polyuria, polydipsia, and natriuresis (Ismail et al., 2016).

Diabetes mellitus (DM) is primarily caused when there is dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis. Studies investigating the association between diabetes and adrenal gland morphology had documented that adrenal volume is increased in patients with diabetes (Carsin et al., 2016; Schneller et al., 2014).

This research is significant because it could help diabetics to know the comparative study of four herbal extract versus Glimepiride on the histomorphological modification of adrenal gland in STZ induced diabetic rats in the treatment of diabetes patients.

MATERIALS AND METHODS

Animal management

Forty- two healthy adult Wistar rats (*Rattus novergicus*) with an average weight of 153.4 g were procured from the animal house of College of Health Sciences, Obafemi Awolowo University, Ile - Ife, Osun State. The animals were kept under standard laboratory condition of good lighting, moderate temperature, and adequate ventilation in a hygienic environment. They were feed on standard rat chow of balance diet. The animals were placed under standard laboratory protocols as stipulated by the Institutional Animal Care and Use Committee.

Animal grouping and treatment

The animals were randomly divided into seven groups of 6 animals each: Group A, control normal rats administered with equivalent volume of citrate buffer by oral method; Group B, experimentally-induced diabetic rats with streptozotocin (65 mg/kg), administered intraperitoneally; Group C, induced diabetic rats with streptozotocin (65 mg/kg) treated with aqueous extract of VA leaves (100 mg/kg), dissolved in normal saline for 14 days administered orally; Group D, induced diabetic rats with streptozotocin (65 mg/kg) treated with aqueous extract of shaft of CC seeds (100 mg/kg), dissolved in normal saline for 14 days given orally; Group E, induced diabetic rats with streptozotocin (65 mg/kg) treated with aqueous extract of PG (100 mg/kg) orally, dissolved in normal saline for 14 days; Group F, induced diabetic rats (65 mg/ kg) treated with aqueous extract of FM (100 mg/kg) orally, dissolved in normal saline for 14 days; Group G, experimentally-induced diabetic rats with streptozotocin (65 mg/ kg) treated with a standard antidiabetic drug (2 mg/kg of glimepiride) dissolved in normal saline for 14 days administered orally.

Table 1. The effects of extracts on relative weight of adrenal gland.

Group	Absolute weight of adrenal glands (g)	Relative weight of adrenal glands (%)
A (Control)	0.06 ± 0.01[a]	0.06 ± 0.01[ab]
B (Diabetic)	0.04 ± 0.00[a]	0.03 ± 0.00[a]
C (Diabetic +*Veronia amygdalina*)	0.06 ± 0.01[a]	0.03 ± 0.00[a]
D (Diabetic + *Citrullus lanatus* seed shaft*)*	0.08 ± 0.01[a]	0.03 ± 0.00[a]
E (Diabetic + *Psidium guajava*)	0.05 ± 0.00[a]	0.03 ± 0.00[a]
F [Diabetic + *Fiscus mucuso* (SPP)]	0.20 ± 0.13[b]	0.09 ± 0.06[b]
G (Diabetic + Glimepiride)	0.05 ± 0.01[a]	0.02 ± 0.00[a]

Values are given as Mean ± SEM in each group. In the table ab differs significantly at p<0.05 while a and b does not differ significantly at p < 0.05 (using one way ANOVA with SNK).

Preparation of extracts

The plant leaves were procured from a local market in Ile-Ife metropolis in Osun state, Nigeria. The leaves were taken to the herbarium in the Department of Botany, Obafemi Awolowo University, Nigeria for identification. The leaves and shaft of the plants were air dried and powdered in a warring blender. The extraction process of the plant leaves of *V. amygdalina* (425 g), *P. guajava* (970 g), *F. mucuso* (370 g) and shaft of *C. colocynthis* (615 g) were prepared by dissolving it in 2.9 L, 3.19 L, 3.5 L and 2.2 L of distilled water respectively for 72 h with intermittent shaking. Thereafter, the solution was filtered using a filter paper. The filtrate was then concentrated *in vacuo* at 35°C using a rotator vacuum evaporator (Buchi Rotavapor, R110 Schweiz). The extracts were oven dried at 37°C, and the respective percentage yield (3.00, 2.65, 5.34 and 1.76 g) were stored until ready to use. The aliquot portion of each of the extracts were weighed and dissolved in normal saline for use on each day of the experiment.

Induction of diabetes

Diabetes mellitus was induced in groups B, C, D, E, F, and G by a single intraperitoneal injection of 65 mg/kg body weight of streptozotocin (Tocris Bioscience, UK) dissolved in 0.1 M sodium citrate buffer (pH 6.3) (Topal et al., 2013). Diabetes was confirmed in animals 48 h after induction, by determining fasting blood glucose level using a digital glucometer (Accu-chek® Advantage, Roche Diagnostic, Germany) consisting of a digital meter and the test strips using blood samples obtained from the tail vein of the rats. The animals were stabilized for 28 days before the commencement of extract and glimepiride administration. The fasting blood glucose was subsequently monitored throughout the experimental period. Animals in group A were given equal volume of citrate buffer used in dissolving streptozotocin intraperitoneally.

Method of administration of extracts

The animals were fed orally using orogastric tube. The animals were held with a glove with the left hand such that the neck region was held by the fingers to still the neck while being fed. Treatment was done at 07.00 h every day before the animals were fed over a period of two weeks (14 days).

Sacrifice and specimen collection

The animals were sacrificed by cervical dislocation 24 h after the expiration of research. The Adrenal glands were excised and weighed, following midline-abdominal incision.

Histological evaluation

The harvested adrenal glands were fixed in 10% formal saline for a minimum of 48 h and processed routinely for paraffin embedding. Serial sections were obtained at 5 µm from a rotary microtome (Bright B5040, Huntington England) and stained using routine haematoxylin and eosin method.

Photomicrography

Stained sections were viewed under a Leica DM750 microscope (Leica Microsystems, Heerbrugg, Switzerland) with digital camera attached (Leica ICC50) and digital photomicrographs were taken which were also imported onto the ImageJ version 1.48 (NIH, Bethesda, MD, USA) software for histomorphometric analysis of thickness of cortex and medulla in adrenal gland.

Statistical analysis

Data were expressed as mean ± SEM and analysed using One-way ANOVA, followed by Student Newman-Keuls (SNK) test for multiple comparisons. Significant difference was taken as p<0.05.

RESULTS

Effects of extracts on relative weight of adrenal gland

Body weights of diabetic rats significantly decreased (P<0.05) compared with other groups. Adrenal weight was significantly increased (P<0.05) in diabetic rats compared with other groups. Herbal plants extract-treated diabetic rats showed a significant increase in body weight (P<0.05) and a significant decrease in adrenal weight (P<0.05) in comparison with diabetic rats (Table 1).

Effects of extracts on histomorphometric thickness of cortex and medulla

There was significant decrease (P<0.05) in the thickness

Table 2. The effects of extracts on histomorphometric thickness of cortex and medulla.

Group	Adrenal cortex			
	Zona Glomerulosa (µm)	Zona Fasciculata (µm)	Zona Reticulosa (µm)	Adrenal Medulla (µm)
A (Control)	30.75±1.15	120.5 ± 2.34	43.61 ± 2.97	257.2 ± 31.69
B (Diabetic)	38.74 ± 0.63	131.5 ± 2.97	56.64 ± 1.93	91.97 ± 0.85
C (Diabetic + *Veronia amygdalina*)	32.39 ± 1.60	125.1 ± 2.90	48.02 ± 3.18	182.3 ± 10.07
D (Diabetic + *Citrullus lanatus* seed shaft)	31.13 ± 1.47	125.1 ± 2.36	44.92 ± 4.50	229.7 ± 42.28
E (Diabetic + *Psidium guajava*)	32.69 ± 2.27	127.1 ± 5.12	48.41 ± 2.85	138.9 ± 20.07
F [Diabetic + *Fiscus mucuso* (SPP)]	35.89 ± 1.50	127.4 ± 4.76	50.65 ± 1.57	151.7 ± 13.15
G (Diabetic + Glimepiride)	32.99 ± 2.52	128.2 ± 5.22	48.75 ± 2.58	155.7 ± 1.96

Values are given as Mean ± SEM (using one way ANOVA with SNK).

of the cortex of group B (Diabetic) compared with groups C, D, E, F and G and decreased significantly (P<0.05) in diameter of medulla in adrenal gland of diabetic rats when compared with the control and other groups. The herbal plants extract-treated diabetic rats showed a significant (P<0.05) increase in cortex and medulla total thickness when compared with diabetic (group B) (Table 2).

Histological findings

The adrenal glands of control rats revealed normal appearance and were seen surrounded by thin connective tissue capsule (Figure 1A). Sections of STZ diabetic rat's adrenal glands (Figures 1B) showed distortion in zona glomerulosa, zona fasciculate, dilated and congested sinusoids in the cortex. Normal zona glomerulosa and zona fasciculate were revealed in the extract administration groups (Figures 1C-1F) as compared to standard drug administration group (Figure 1G).

DISCUSSION

Experimental diabetes was induced by streptozotocin (STZ), led to the alteration of adrenal gland in wistar rats. This has been reported earlier that diabetes can be induced by means of chemical destruction or surgical removal of a part of the β-cell mass, feeding high-sugar diets, and drugs such as streptozotocin (STZ) and alloxan (Carsin-vu et al., 2016). In the present study, the effect of four different herbal plants on the adrenal gland in STZ-induced diabetes was carried out.

The histological result revealed that groups B treated with STZ had distortion of the histology of the adrenal gland tissues while group C to F improved hyperglycemia by four herbal plants which could be attributed to the fact that some components of herbal plants enhanced the insulin-stimulated glucose uptake of rat adipocytes (Obike et al., 2014).

In this new research, STZ-induced diabetes resulted in a significantly decreased in the rat's body weight and a significantly increased in adrenal gland weight mostly in group B. This is in accord with the findings of Mustata et al. (2005) and Ghada et al. (2015), who reported that diabetic rats were significantly reduced in body weight and significantly increased in adrenal gland weight. Reduction in body weight in group B might be due to the increased in muscle wasting and loss of tissue proteins (Shirwaikar et al., 2006). It could also be lack or deficiency of carbohydrate needed for the energy metabolism, which resulted in degradation of structural proteins (Pepato et al., 1996). Significant increased in the body weight of diabetic rats treated with the four different herbal plants compared with the diabetes group might be due to the blood glucose stabilization effect which is more effective for each other and prevents the loss of body weight differently.

The findings of this study shows that STZ-induced diabetes had significant increase in the total thickness of adrenal cortex and increased in the diameter of medullar, which may be explained on the basis of the increased in the thickness of zona fasciculate, zona glomerulosa and zona reticulosa in the cortex as well as increase in diameter of medullar in adrenal gland. Ghada et al. (2015) reported that STZ-induced diabetes causes a notable hypertrophy of the cells of the zona fasciculata, which may explain the increase in total thickness of the adrenal cortex and medullar in the present study.

Administration of the extracts improves the histoarchitecture of the Adrenal gland and by extension restores its functionality. The groups administered with *C.colocynthis* extract demonstrated a distinct regenerative capacity over the other three extract.

Previous studies have reported some similar histopathological findings (Wu et al., 2004). The plant extracts used for the study, are common herbal plant used traditionally in the management of diabetes, amongst the South Wast, Nigeria. Three of these plants (*C. colocynthis, V. amygdalina* and *P. guajava*) have been

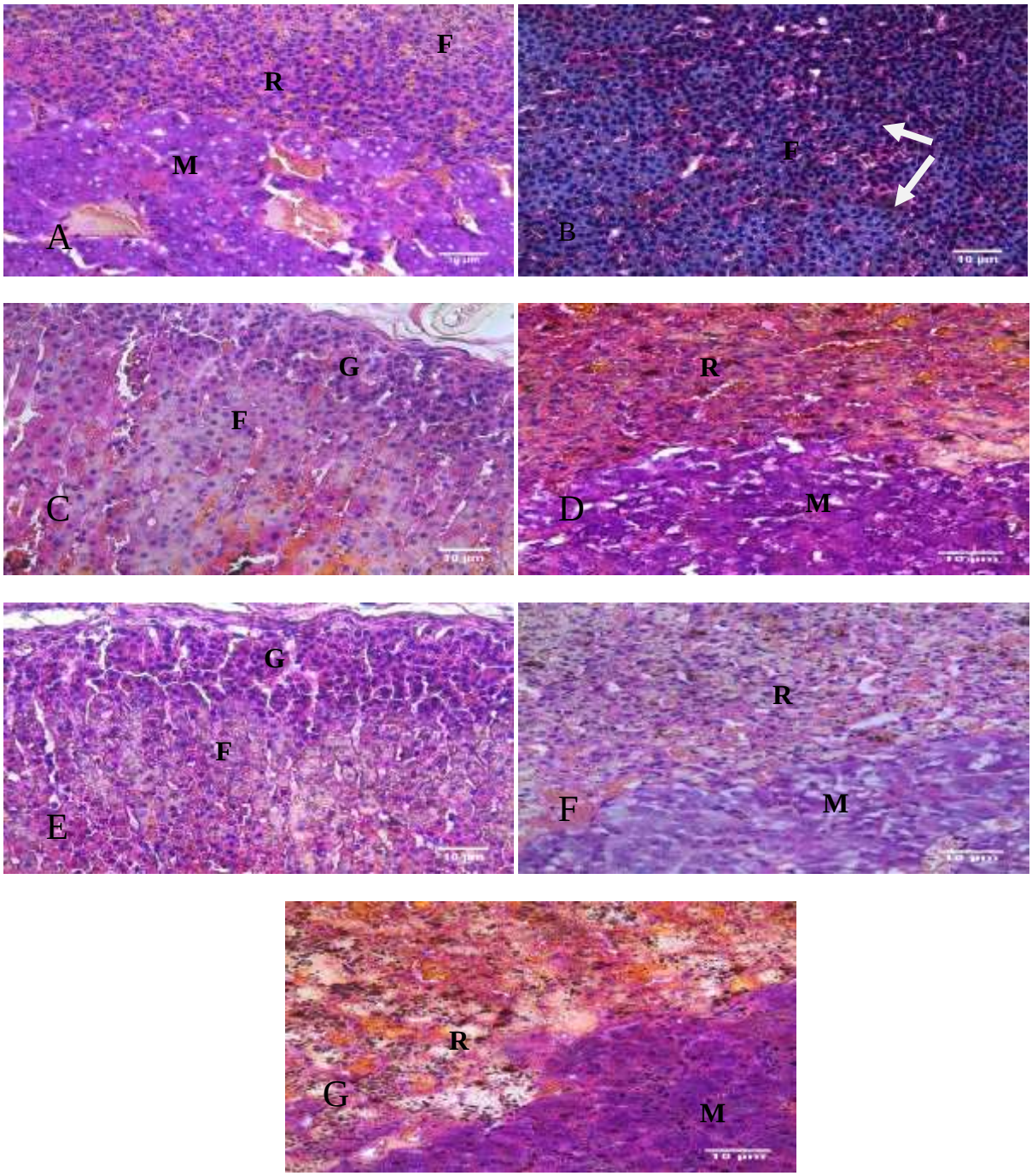

Figure 1. Photomicrographs of Adrenal Gland of Groups A, B, C, D, E, F, and G. (H&E x400). G, zona glomerulosa; F, zona fasciculate, R: zona reticulosa; M, medulla. The white arrow pointed to distorted in zona fasciculate. The extract treated groups (C-F) reveals Adrenal tissue regenerations which shows a remarkable reversible cellular injury as compared with group B.

reported to possess anti-diabetic properties (Akpaso et al., 2011). The four medicinal plants used in this study are well known for their antioxidant properties which are due to their high level content of flavonoids (komolafe et al., 2013).

Conclusion

Treatment of diabetic rats with four herbal plants markedly improves ultrastructure of the adrenal gland with their antioxidant properties which are due to their

high level content of flavonoids. Therefore this study suggests that *C. colocynthis* could be better in ameliorating diabetic-associated disorders of the adrenal gland.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Anti-oxidant and anti-microbial study of *Adiantum capillus veneris* and *Pteris quadriureta* L

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***Adiantum capillus veneris* (ACV) and *Pteris quadriureta* (PQ), two common ferns belonging to Pteridophyta family, has been used in traditional Ayurvedic and Unani medicine against numerous human ailments since ancient times. This study was designed to analyse the presence of various phytochemicals in the ACV and PQ leaves and their pharmacological activities. The methanol extract of ACV and PQ leaves was screened for the presence of various primary and secondary metabolites such as proteins, lipids, phenols, flavonoids, alkaloids, saponins, and tannins. Anti-oxidant, anti-bacterial, and anti-fungal activities were also analysed for methanolic extracts of ACV and PQ leaves using various methods. Various metabolites such alkaloids, phenols, flavonoids, saponins and tannins in the ACV and PQ leaves were found. Phenols and flavonoids were present in high concentration when compared with other metabolites. The results also showed that methanolic extracts of ACV and PQ leaves have anti-oxidant, anti-haemolytic, anti-bacterial, and anti-fungal activities. The pharmacological activities such as anti-oxidant, anti-haemolytic, anti-bacterial, and anti-fungal activities of ACV and PQ leaves might be due to the presence of phenols and flavonoids.**

Key words: *Adiantum capillus veneris*, *Pteris quadriureta,* anti-bacterial, anti-fungal, anti-oxidant, phytochemicals.

INTRODUCTION

Inverse correlations between antioxidant status and human diseases such as cancer, aging, neuro-degenerative disease and atherosclerosis have been reported (Halliwell, 1997; Fusco et al., 2007; Malliaraki et al., 2003; Rajendran et al., 2014). Many plant-derived non-nutritive compounds and dietary natural compounds present in food materials have been reported to possess antioxidant properties. Advantages of using phytochemicals include their abundance, less toxicity and low cost (Lee et al., 2017). Therefore, in recent years, the researchers are more interested to investigate the pharmacological behaviour of medicinal plants including antioxidant and antimicrobial properties.

Adiantum capillus veneris (ACV), a common fern belonging to Pteridophyta family, has been used in traditional Ayurvedic and Unani medicine against numerous human ailments since ancient times (Pandey and Rizvi, 2009; Pandey et al., 2013; Ahmed et al., 2012). ACV contains various secondary metabolites including triterpenes, flavonoids, phenylpropanoids, carotenoids, quercetin, rutin, shikimic acid, violaxanthin, and zeaxanthin (Ibraheim et al., 2011; Hussein et al., 2016; Vadi et al., 2017). ACV has been used as anti-fertility, anti-candidal, anti-viral, contraceptive, cough suppressant, blood cleanser, diaphoretic, diuretic, expectorant, hepatoprotective, menstrual stimulant and

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wound healer (Singh et al., 2008; Abbasi et al., 2010). *Pteris quadriureta* (PQ), another common fern from the same Pteridophyta family, is known for its anti-helmintic activity (Nayar, 1959). This plant is also used as a phytoremediation which removes toxic contaminants from soil and water. It removes heavy metals like arsenic and selenium (Singh and Upadhyay, 2014; Feng et al., 2015).

The present study was designed to investigate the antioxidant and antimicrobial activities of the methanolic extract of ACV and PQ leaves. These two plants have been analysed for the presence of various phytochemicals. Also, the antioxidant and anti-haemolytic activities of ACV and PQ extracts have been assessed. In addition, ACV and PQ extracts were also tested for their anti-bacterial and anti-fungal activities.

MATERIALS AND METHODS

Collection, identification and processing of plants

ACV and PQ plants were collected from Kodaikanal hills, Tamil Nadu on the 15 July, 2016 and identified by Regional Plant Resource Centre, Odisha Biodiversity Board (No. 2175). The leaves were washed thoroughly under running tap water and dried in hot air oven at 50 to 60°C for 3 to 4 h. The dried leaves were then powdered using the blender and stored at 4°C in air tight bottles.

Preparation of plant extract

Plant extraction was carried out using various solvents such as petroleum ether, chloroform, acetone, methanol and water with 20 g of powdered sample and 250 ml of respective solvent using a Soxhlet apparatus for 48 h. The extract was then filtered using Whatman No.1 filter paper and the filtrate was kept in a hot-air oven at 37°C to allow the solvents to evaporate and stored at 4°C.

Phytochemical analysis

Methanolic extracts of ACV and PQ leaves were screened for the presence of various bioactive compounds such as phenols, tannins, flavonoids, steroids, alkaloids, terpenoids, triterpenoids, phytosterols, glycosides, cardiac glycosides, anthraquinone glycosides, phlobatannins, quinine, coumarins, and saponins.

Quantification of chlorophyll

About 100 mg powdered sample was soaked in 10 ml of dimethyl sulfoxide (DMSO): acetone mixture (1:1) for overnight in the dark and absorbance was read at 663 and 645 nm. Total chlorophyll content was calculated using the following equations (Harborne, 1973):

Chlorophyll a (C_a) = (12.25 × OD at 663) - (2.79× OD at 645) × 10 / (1000×wt.)
Chlorophyll b (C_b) = (21.50 × OD at 645) - (5.10× OD at 663) × 10 / (1000×wt.)
Total Chlorophyll (C) = (7.15 × OD at 663) + (18.71× OD at 645) × 10 / (1000×wt.)

Estimation of protein

Protein estimation of the samples was done by using the extraction of dried, fresh, or frozen plant material in 0.1 sodium hydroxide (NaOH) for 30 min. 100 µl aliquots of centrifuged supernatant were analysed with 5 ml Bio-Rad Bradford dye reagent (Coomassie brilliant blue G-250) diluted 1:4 and containing 3 mg/ml soluble polyvinyl pyrollidone. Absorbance was recorded at 595 nm after 15 min against a NaOH blank and the samples were calibrated against a BSA standard in NaOH (Jones et al., 1989).

Quantification of lipids

About 10 g of dried powdered sample was taken for the lipid extraction using 150 ml of petroleum ether for 16 h at a solvent condensation rate of 2 to 3 drops/s according to American Association for Clinical Chemistry (AACC) method 30 to 25 with minor modifications of sample size and extraction time. The extract achieved was concentrated and evaporated at room temperature. Then, the weight of extract was taken which is the total lipid content and expressed as mg/g dry matter (Harborne, 1973).

Quantification of saponins

To 50 mg of methanol extract, 100 ml of 20% ethanol was added and placed on a boiling water bath at 55°C with continuous stirring for 4 h. Then, the solution was diluted with 20 ml of diethyl ether and 5 ml of 5% sodium chloride and sent for centrifuge at 10000 rpm for 10 min. The obtained pellet was dried and saponins were estimated as percentage of the dried fraction (Harborne, 1973).

Quantification of alkaloids

Alkaloids were estimated by the method of Harborne with slight modifications (Harborne, 1973). Dried fraction (50 mg) of each fraction was mixed with 200 ml of 10% acetic acid in ethanol and the beaker was kept for incubation for 4 h. The mixture was concentrated up to one third of its total volume and then the ammonium hydroxide was added dropwise to precipitates the mixture. The precipitate was then washed with ammonium hydroxide and filtered. Alkaloids in the filtrate were calculated as percentage of the dried fraction.

Estimation of total phenol content

The total phenolic content was determined according to McDonald et al. (2001). To 1 ml of plant extract or standard, 5 ml of Folin Ciocalteau reagent and 4 ml of 7.5% sodium carbonate were added. The mixture was kept for 15 min under room temperature and eventually there was a formation of blue colour, read at 765 nm using UV/visible spectrophotometer. The total phenolic content was calculated against the calibration curve of gallic acid and the results were expressed as gallic acid equivalent (mg/g).

Estimation of total flavonoid content

The total flavonoid content was determined according to Chang et al. (2002). To 0.5 ml of plant extract or standard, 4.5 ml of methanol, 0.1 ml of 10% aluminium chloride and 0.1 ml of 1 M sodium acetate were added. Hence, the reaction mixture was kept at room temperature for 30 min and the absorbance was read at 415 nm using UV/visible spectrophotometer. The flavonoid content was calculated by calibration curve of quercetin.

Estimation of total tannin content

Total tannin content was determined by the method of Schanderl

(1970). To 1 ml of the plant extract or standard, 0.5 ml Folin-Ciocalteu phenol (FCP) reagent and 5 ml of 35% sodium carbonate was added and then the mixture was sent for incubation for 5 min at room temperature. Hence, there was a formation of the blue colour that occurred which was read at 640 nm using UV visible spectrophotometer. The tannin content was calculated by calibration curve of tannic acid and the results were expressed as gallic acid equivalent (mg/g).

Measurement of 1, 1- diphenyl-2-picrylhyorazyl (DPPH) radical scavenging activity

This assay of the methanolic extracts was performed by the scavenging activity of stable DPPH free radical by the method of Brand-Williams et al. (1995) with slight modifications. 1 ml of plant extracts of different concentrations including 50, 100, 150, 200 and 250 µg/ml were mixed with 0.1 mM DPPH solution in methanol. L-Ascorbic acid (1-100 µg/ml) was taken as standard with different concentrations and a blank was also used. Mixture of 1 ml methanol and 1 ml DPPH solution was used as control. The reaction mixture incubated for 30 min in dark and then the decrease in absorbance was measured at 517 nm using UV-Vis spectrophotometer. The reaction was carried out in triplicate manner. The inhibition % was calculated using the following formula:

Inhibition (%) = Ac - As / Ac × 100

where Ac is the absorbance of the control and As is the absorbance of the sample.

Measurement of total antioxidant assay (Phosphomolybdate assay)

This assay was carried out on the basis of the transformation of Mo^{6+} to Mo^{5+} to form phosphomolybdenum complex (Prior et al., 2005). In this assay, 300 µl of extract was incubated with a mixture of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate and the complete mixture incubated for 90 min. Hence, the absorbance was read at 695 nm and the results were expressed as AAE/100 mg dry weight of extract.

Measurement of $ABTS^{\cdot+}$ radical scavenging activity

The ability of antioxidant molecules to quench ABTS radical cation ($ABTSN^{+}$) was determined according to the method of Okamoto et al. (1992). A stable stock solution was prepared by adding 7 mM aqueous solution of ABTS with 2.45 mM potassium persulfate (final concentration) and then incubated the mixture to stand in the dark at room temperature for 16 h. 1 ml of $ABTSN^{+}$ stock solution was added to the 3 ml of sample solutions at various concentrations (2, 4, 6, 8, and 10 mg/ml). The contents were mixed properly and incubated at 3°C exactly for 30 min. Then, the absorbance was determined at 534 nm and the $ABTSN^{+}$ radical scavenging activity was calculated as follows:

$ABTS^{o+}$ scavenging effect (%) = Control abs_{534} – Sample abs_{534} / Control abs_{534} × 100%

Determination of anti-haemolytic activity

Anti-haemolytic activity was assessed by the spectrophotometric method of Yang et al. (2005) with slight modifications. From a normal healthy individual, 5 ml of blood was taken and centrifuged at 1500 rpm for 3 min (Institutional Human Ethics Committee No. 2189). Pellet of blood was washed three times with sterile phosphate buffer saline solution at pH 7.2. The pellet was re-suspended in normal 0.5% saline solution and 0.5 ml of the extract and various fractions (10, 50, 100, 200, 250 µg/ml in saline) were added in 0.5 ml of cell suspension. After incubation at 37°C for 30 min, the mixture was centrifuged at 1500 rpm for 10 min and absorbance was measured for the supernatant at 540 nm. For positive and negative controls, distilled water and phosphate buffer saline were used, respectively.

Estimation of superoxide radical scavenging assay

Superoxide radicals were generated by a modified method of Beauchamp and Fridovich (1971). The assay was based on the capacity of the sample to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin-light-nitroblue tetrazolium (NBT) system.

Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA, 0.1 mg NBT and various concentrations (50 to 250 µg) of sample extracts. Then the reaction mixture was incubated for 90 s. Immediately after incubation, the absorbance was measured at 590 nm. The mixture was covered with aluminium foil. The reaction mixture without extracts kept in dark served as blank. The percentage inhibition of superoxide anion generation was calculated as:

% Superoxide radical scavenging activity = (Control OD - Sample OD / Control OD) × 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC50) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Measurement of reducing power

The reducing power of the extracts was determined according to the method of Oyaizu (1986). The reaction mixture was made by adding 1 ml of extract with 2.5 ml of phosphate buffer and 2.5 ml of 1% potassium ferricyanide. The reaction mixture was incubated for 20 min at 50°C and 2.5 ml of 10% TCA was added and centrifuged. Hence, the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$, and the absorbance was read at 700 nm. The assay was carried out in triplicate, and the results are expressed as mean ± standard error (SE). Increase in absorbance of sample with concentrations indicates high reducing potential of the samples.

Control abs_{534} – Sample abs_{534} / Control abs_{534} × 100%

Assay of antimicrobial activities

Bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Salmonella enteric, Staphylococcus aureus,* and *Bacillus subtilis* and fungi such as *Trichophyton rubrum, Scedosporium apiospermum, Aspergillus fumigates, Aspergillus niger,* and *Aspergillus flavus* were collected and clinically isolated. Each bacterial strain was suspended in a nutrient broth and incubated for 18 h at 37°C. Nutrient agar (NA) and potato dextrose agar (PDA) were used for the study of anti-bacterial activity and anti-fungal activity, respectively. The nutrient broth cultured bacteria were spread over NA plate, whereas a 24 h cultured fungi was spread on PDA by using cotton swab. A 5 mm disc was dipped in each extract as well positive control solution such as ampicillin and itraconazole

Table 1. Percentage of yield extract of ACV and PQ leaves.

Solvent used	Yield (% w/w)	
	ACV	PQ
Petroleum ether	3.3	4.8
Chloroform	7.4	6.9
Acetone	8.5	8.2
Methanol	10.5	11.7
Water	6.1	6.6

(10 µg) for bacteria and fungi, respectively and placed on the swabbed agar plate. Each disc absorbs 15 µl of sample which is made up of 50 and 100 mg/ml concentration. The plates were then incubated at 37°C for 24 h for bacterial and 72 h for fungal pathogens. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone.

Statistical analysis

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

RESULTS

The percentage extraction yield of different extracts is shown in Table 1. The yield percentage of methanol extract of ACV and PQ was 10.5 and 11.7, respectively to that of dry powder. The yield percentage of methanol was higher than that of other solvents, and in the following order methanol>acetone>chloroform>water>petroleum ether. Since the yield percentage of methanol was higher than that of other solvents used, methanolic extracts of ACV and PQ leaves were used for further experiments. A large number of biologically active compounds were found in aqueous, methanol, acetone, diethyl ether and chloroform extracts of ACV and PQ. Several primary metabolites such as carbohydrates, proteins, and alkaloids, and secondary metabolites including coumarins, terpenoids, diterpenoids, flavonoids, phenols, tannins, saponins and steroids were found in the extracts of ACV and PQ (Table 2).

Next, various primary metabolites including proteins, chlorophyll, and lipids and secondary metabolites such as phenols, flavonoids, alkaloids, saponins, terpenoids, and tannins present in the methanolic extracts of ACV and PQ plants were quantified. Compared to PQ, ACV was found to have more amounts of primary and secondary metabolites. Methanolic extracts of ACV and PQ showed a higher concentration of phenols and flavonoids relative to other metabolites (Table 3).

The free radical scavenging activity of the methanol extracts of ACV and PQ leaves was determined by the DPPH method to evaluate the antioxidant activity of plant extracts. The extracts of each plant examined in the present study exhibited free radical scavenging activities and the highest activity was shown by ACV followed by PQ. At concentrations 10 to 200 µg/ml, the scavenging activities of ACV were 14.52 to 84.64%, while the scavenging activities of PQ were 8.71 to 71.78%. Percentage DPPH radical scavenging activities of both the extracts were dose dependent (Figure 1A). Further, ABTS radical cation scavenging activity of methanol extracts of ACV and PQ was analysed. The ABTS± scavenging activity of ACV was significantly higher than the PQ. At concentrations 10 to 200 µg/ml, the scavenging activities of ACV were 10.49 to 90.55%, while the scavenging activities of PQ were 2.36 to 68.74% (Figure 1B).

Antioxidant potential of the methanol extract of ACV and PQ was further estimated using potassium ferric cyanide reduction method. The presence of reductants (antioxidants) in the plant extract causes the reduction of Fe^{3+}/Ferric cyanide complex to Fe^{2+} form. Therefore, the Fe^{2+} complex can be monitored by measuring the formation of Perl's

Prussian blue at 700 nm. It was observed that the reducing power of ACV and PQ was increased from 19.08 to 81.41% and 9.13 to 75.31%, respectively at concentrations 10 to 200 µg/ml. This may be due to the presence of secondary metabolites in the extract (Figure 1C). Further, ACV (42.24% at 50 µg/ml concentration) also showed potent superoxide activity as compared to PQ (32.68% at 50 µg/ml concentration) (Figure 1D). The phosphomolybdate assay was used to determine the total antioxidant capacity of samples. In this assay, Mo^{6+} is reduced to Mo^{5+} by antioxidant potential of the extract. The antioxidant capacity of methanolic extract of ACV was more than that of PQ. The percentage of activities of ACV and PQ were 53.16±.3 and 40.55±1.2, respectively (Table 4).

Then, the anti-haemolytic activity of methanolic extracts of ACV and PQ leaves using a biological test based on free radical-induced erythrocytes lysis in human blood was analysed. Lipid oxidation of human blood erythrocyte membrane mediated by H_2O_2 induces membrane damage and subsequently haemolysis. The results showed that ACV exhibited a maximum anti-haemolytic

Table 2. Preliminary phytochemical screening of ACV and PQ leaves.

Plant constituent	Extracts									
	Aqueous		Methanol		Acetone		Petroleum Ether		Chloroform	
	ACV	PQ	ACV	PQ	ACV	PQ	ACV	PQ	ACV	PQ
Alkaloids	+	+	+	+	+	+	+	+	+	+
Anthraquinone glycosides	+	-	-	-	-	-	-	-	-	-
Carbohydrate	+	+	+	+	+	+	+	+	+	+
Cardiac glycosides	+	+	+	+	+	+	-	-	-	-
Coumarins	+	+	+	+	+	+	+	+	+	+
Diterpinoids	+	+	+	+	+	+	+	-	+	-
Flavonoids	+	+	+	+	+	+	+	+	+	+
Phenols	+	+	+	+	+	+	+	+	+	+
Phlobatannins	-	-	-	-	-	-	-	-	-	-
Phytosterols	+	+	+	+	-	+	-	-	+	+
Protein	+	+	+	+	+	+	+	+	+	+
Quinones	+	-	-	-	-	-	-	-	-	-
Reducing sugar	-	-	-	-	-	-	-	-	-	-
Saponins	+	+	+	+	+	-	+	-	+	+
Steroids	+	+	+	+	+	-	+	+	+	+
Tannins	+	+	+	+	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+	+	-	+	-

The presence of phytochemical is indicated by '+' and absence is indicated by '-'sign.

Table 3. Quantitative phytochemical screening of methanol extracts of ACV and PQ leaves.

Metabolites		Weight (mg/g dw)	
		ACV	PQ
Primary metabolites	Chlorophyll	1.96±004	1.20±006
	Protein	1.23±02	1.19±01
	Lipids	14.71±12	12.62±15
Secondary metabolites	Tannin	96.17	67.23
	Terpenoids	9.09	17.92
	Alkaloid	1.02	2.21
	Phenols	21.17	10.83
	Flavonoids	35.33	17.69
	Saponins	11.05	6.17

Values are expressed as the mean ± SD (n = 3)

activity followed by PQ. The percentage of activities of ACV and PQ were 79.07±1.05 and 70.78±7, respectively (Table 4). Moreover, the RBC haemolysis is a more sensitive system for evaluating the antioxidant properties of the phytochemicals. The anti-haemolytic activity of ACV and PQ may be due to the presence of phenols and flavonoids in the extracts.

Tables 5 and 6 show the anti-bacterial and anti-fungal activities of methanol extracts of ACV and PQ leaves. Two concentrations (50 and 100 mg/ml) of extracts were tested against five different bacteria including *B. subtilis*, *E. coli*, *P. aeruginosa*, *S. enteric*, and *S. aureus* and five different fungi including *A. niger, A. fumigates, A. flavus, T. rubrum,* and *S. apiospermum*. Zone of inhibition for the following was measured in mm. It has been observed that there was a significant increase in the zone of inhibition, on increasing the concentration of extracts (Figures 2 and 3).

DISCUSSION

Medicinal plants are very much in demand because of their biological properties and bioactive compounds

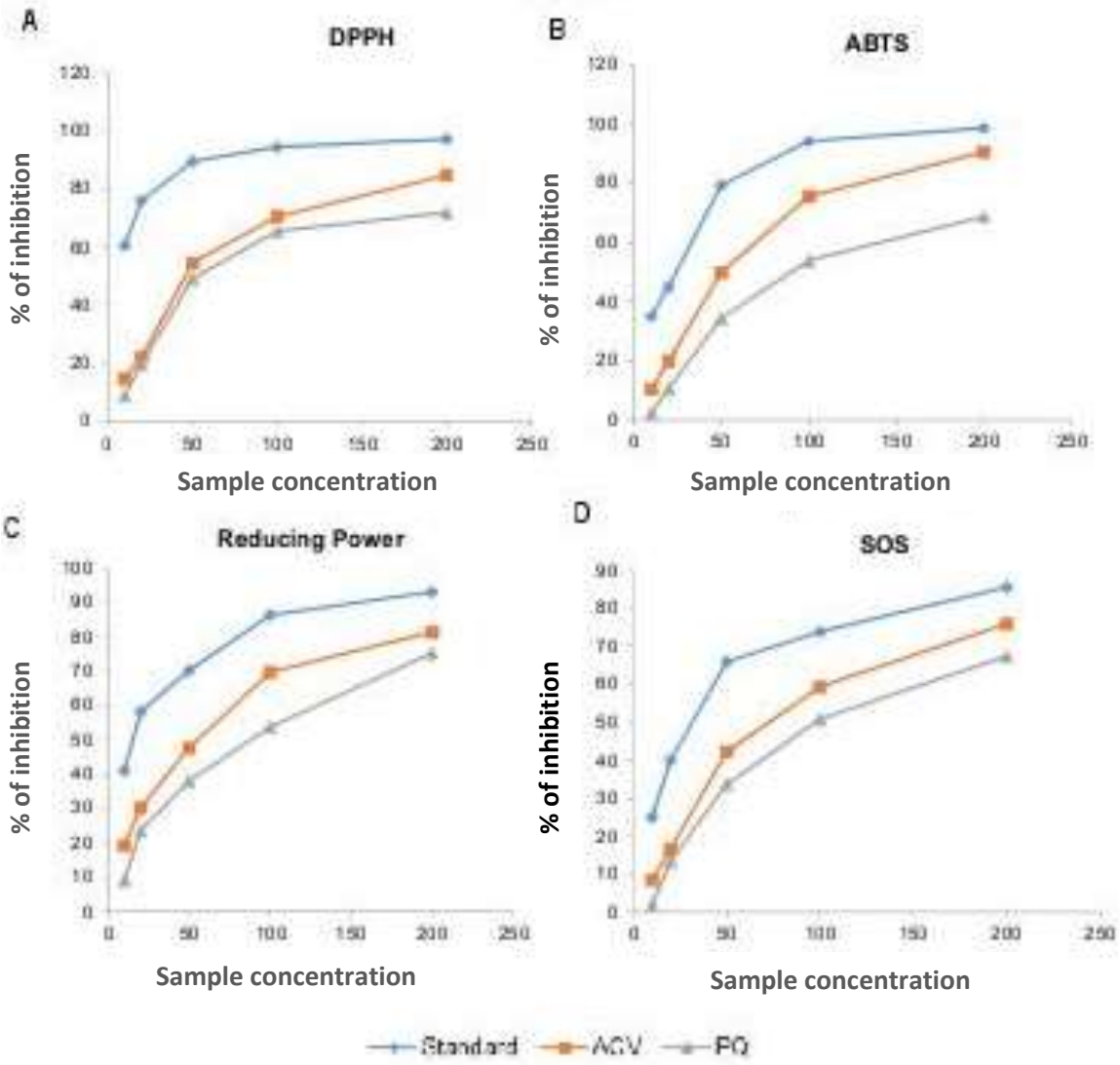

Figure 1. Anti-oxidant activity of methanol extracts of ACV and PQ leaves by DPPH, ABTS, Reducing power and SOS. A. DPPH free radical scavenging activity of methanol extract of ACV and PQ. Values are means of triplicate determinations (n = 3) ± standard deviation. B. ABTS radical scavenging activity of methanol extract of ACV and PQ. Values are means of triplicate determinations (n = 3) ± standard deviation. C. Reducing power of methanol extracts of ACV and PQ. Values are means of triplicate determinations (n=3) ± standard deviation. D. SOS radical scavenging activity of methanol extract of ACV and PQ. Values are means of triplicate determinations (n = 3) ± standard deviation. Ascorbic acid was used as standard.

which are well known to act against various diseases (Misra, 2013; Atanasov et al., 2015; Pandey and Rizvi, 2009). In the present study, it has been shown that methanolic extracts of ACV and PQ leaves possess anti-oxidant, anti-bacterial, anti-fungal, and anti-haemolytic activities.

Phytochemical analysis gives the basic information about the bioactive components present in the plant extract (Hosseinzadeh et al., 2015). In the present study, the qualitative and quantitative analysis of methanol extracts of ACV and PQ leaves showed the presence of various secondary metabolites such as alkaloids, anthraquinones, cardiac glycosides, phenols, flavonoids, saponins, tannins and terpenoids. Several researchers reported that secondary metabolites including alkaloids, phenols and flavonoids, contribute to the biological activities of the plant (Dipankar et al., 2011; Oliveira et al., 2014). Quantitative analysis revealed that the extracts contained a high concentration of flavonoids, phenols and tannins. It is well known that phenols and flavonoids possess various biological activities such as anti-viral, anti-inflammatory, anti-cancer, anti-haemolytic and anti-oxidative potential (Beg et al., 2011; Bertrand Sagnia et al., 2014; Ameni et al., 2015). The anti-oxidant and anti-

Table 4. Anti-oxidant and anti-hemolytic activities of methanol extracts of ACV and PQ leaves.

Plant	Phosphomolybdenum assay	Anti-hemolytic activity
	% of activity	
ACV	53.16±.3	79.07±1.05
PQ	40.55 ±1.2	70.78±.7

Values are means of triplicate determinations (n=3) ± standard deviation.

Table 5. Anti-bacterial activity of methanol extracts of ACV and PQ leaves.

Bacteria	Antibiotic (Zone of Inhibition in mm)	ACV (Zone of Inhibition in mm)		PQ (Zone of Inhibition in mm)	
		50 mg/ml	100 mg/ml	50 mg/ml	100 mg/ml
Bacillus subtilis	29±1.14	26±1.33	35±2.05*	26±1.31	37±2.49*
Escherichia coli	24±1.20	26±1.28	36±1.64*	22±0.44	32±1.15*
Pseudomonas aeruginosa	22±0.95	22±0.59	28±1.09*	22±0.87	25±0.61*
Salmonella enteric	27±2.01	25±1.38	31±2.17*	25±1.02	41±1.92*
Staphylococcus aureus	30±0.84	32±2.05	41±2.86*	26±0.47	34±1.43*

Values are means of triplicate determinations (n=3) ± standard deviation. *($p<0.05$) Significantly different from antibiotic.

Table 6. Anti-fungal activity of methanol extracts of ACV and PQ leaves

Fungi	Antibiotic (Zone of Inhibition in mm)	ACV (Zone of Inhibition in mm)		PQ (Zone of Inhibition in mm)	
		50 mg/ml	100 mg/ml	50 mg/ml	100 mg/ml
Aspergillus niger	24±0.55	21±0.83	27±1.44	22±0.62	28±1.27
Aspergillus fumigates	42±3.27	28±2.41	31±0.91*	24±1.09	35±1.90*
Aspergillus flavus	27±1.11	28±1.03	33±2.19*	24±0.70	29±0.97
Trichophyton rubrum	39±2.10	29±2.35	31±1.37*	27±1.59	36±2.35
Scedosporium apiospermum	29±0.82	27±1.07	40±2.92*	25±0.73	43±1.77*

Values are means of triplicate determinations (n=3) ± standard deviation. *($p<0.05$) Significantly different from antibiotic.

microbial activity observed in the present study may be due to the presence of phenols and flavonoids in ACV and PQ extracts.

As the scavenging of DPPH radical depends on electron transfer/donating ability, the radical scavenging activity of extracts could be related to the presence of phenols, thus contributing to their electron transfer/ hydrogen donating ability (Bab and Malik, 2015; Diemdo et al., 2014; Saha and Verma, 2016). Both ACV and PQ showed a less percentage of inhibition for DPPH radical scavenging activity as compared to well-known antioxidant ascorbic acid. However, methanol extracts of ACV leaves exhibited a higher antioxidant capacity than PQ. Similarly, Hamid et al. (2017) reported that *Adiantum venustum* extracts exerted DPPH radical scavenging activity. ACV and PQ methanolic extract also showed effective scavenging activity of superoxide and ABTS radical. It has been reported that phenols and flavonoids have anti-radical and anti-oxidant activities (Agarwal, 2011; Saxena et al., 2012). It also has been studied by Sowndhararajan et al. (2013) that tannins are more capable to reduce free radicals (ABTS_+) due to their molecular weight, the number of aromatic rings and nature of hydroxyl group's substitution than the specific functional groups.

The presence of phenolic compounds in the extracts causes the reduction of Fe^{3+}/Ferric cyanide complex to ferrous form. Similar observation between the polyphenolic constituents in terms of dose dependent and reducing power activity have been reported for several plant extracts including ferns (Lai et al., 2009). Superoxide radical can lead to the formation of hazardous hydroxyl radicals as well as singlet oxygen which results in oxidative stress and DNA damage (Lobo et al., 2010; Khanna et al., 2014; Rahal et al., 2014). In the present study, ACV and PQ showed significant superoxide scavenging activity and the scavenging potential may

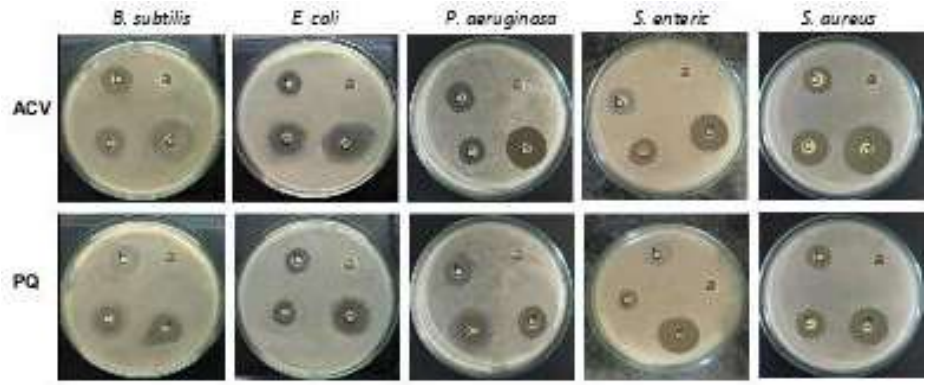

Figure 2. Anti-bacterial activity of methanol extracts of ACV and PQ leaves. a. Control, b. Positive control, c. 50 mg/ml, d. 100 mg/ml.

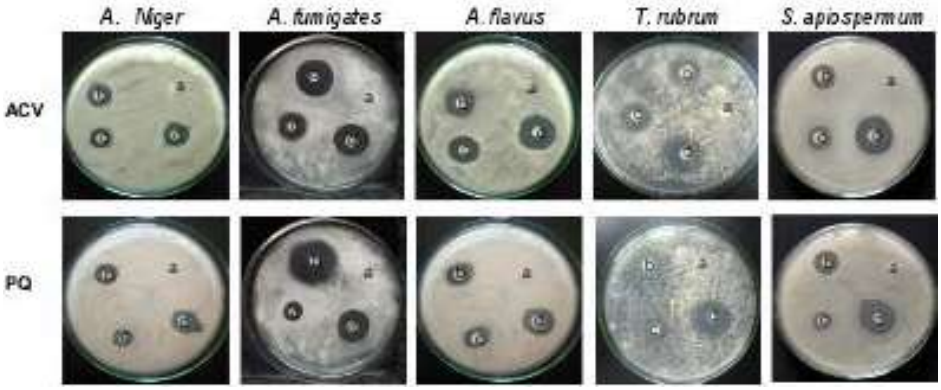

Figure 3. Anti-fungal activity of methanol extracts of ACV and PQ leaves. a. Control, b. Positive control, c. 50 mg/ml, d. 100 mg/ml.

be due to the presence of bioactive phytoconstituents such as phenols and flavonoids. Similarly, Kaur et al. (2017) reported that fern extract showed significant superoxide radical scavenging activity. Recent studies proved that phenolic compounds reduce the Mo^{6+} into Mo^{5+} leading to the formation of a green phosphomolybdate complex. The phosphomolybdate has the hydrogen and electron donating ability that helps to detect the antioxidants such as ascorbic acid, α-tocopherol, and some phenolic, cysteine, and aromatic amines (Malliaraki et al., 2003; Prior et al., 2005). The methanolic extracts of ACV and PQ showed significant total antioxidant capacity which may be due to the presence of phenols.

Lipid peroxidation can injure every molecule of the biological system and can break the DNA strands which lead to mutation and cancer (Barrera, 2012; Zhong and Yin, 2015). Due to the heavy accumulation of poly-unsaturated fatty acids and haemoglobin, the erythrocytes can be damaged severely such that it can lead to oxidative damage resulting in haemolysis (Asgary et al., 2005; Pandey and Rizvi, 2010). The compounds present in ACV and PQ extracts are capable of anti-haemolytic and anti-lipid peroxidation activities, which is evident from inhibition of erythrocyte lysis with increasing concentration of extracts. In line with the present findings, Kaur et al. (2017) reported that fern extract showed significant anti-haemolytic activity. The methanol extracts of ACV and PQ were more effective in inhibiting microbial growth and this may be due to the presence of sterols and secondary metabolites. Similarly, Ishaq et al. (2014) reported that fern extract shows significant antimicrobial activity against various strains of bacteria and fungi.

The present investigation suggests that bioactive compounds from ACV and PQ leaves possess potential anti-oxidant and anti-microbial activities. However, isolation and preparation of phytochemicals from ACV and PQ and assessment of their impact on various health improvements/control of free radical mediated diseases through *in vitro* and *in vivo* studies are needed. Such identified potential and natural constituents could be exploited as cost effective food/feed additives for human

and animal health.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

ABTS, 2,2'-Azino-bis(3)-ethylbenzothiazoline-6-sulphonic acid; **ACV,** *Adiantum capillus veneris*; **DPPH,** 1,1-diphenyl-2-picrylhydrazyl; **NA,** nutrient agar; **NBT,** nitro-blue tetrazolium; **PDA,** potato dextrose agar; **PQ,** *Pteris quadriureta*; **UV,** ultraviolet.

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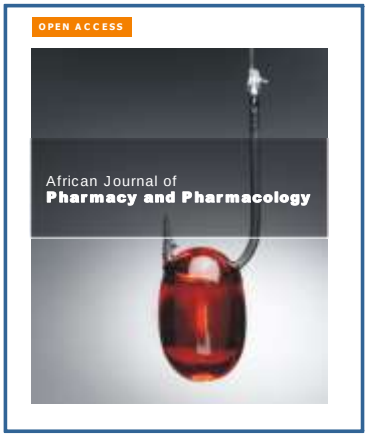

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