

EVALUATION OF ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF LEAVES, FRUIT AND BARK OF *KIGELIA AFRICANA*

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Abstract

In vitro antibacterial activity of extracts was tested against six bacterial strains viz. *Staphylococcus aureus*, *Proteus vulgaris*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Citrobacter amalonaticus* by agar-disc diffusion method. Ethanol and n-hexane were used as negative control and oxytetracycline was used as a positive control. Ethanolic and aqueous extracts of bark and leaves of *Kigelia africana* showed remarkable activity against various bacterial strains as compared to n-hexane. *S. aureus* and *E. coli* were proved as highly sensitive strains while *K. pneumoniae* was the resistant strain as the extracts formed no inhibition zone against it. The percentage of antioxidant activity of different parts of *Kigelia* was assessed by DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical assay. Quercetin was used as a standard antioxidant which showed 93.6% inhibition. *Kigelia* bark extract showed good antioxidant activity i.e., 67.33% inhibition, fruit extract possess moderate antioxidant activity i.e., 62.66% inhibition while leaves showed the poor antioxidant activity i.e., 59.66% DPPH inhibition respectively. Overall, the comparative analysis revealed that bark extract exhibited the most remarkable antibacterial as well as antioxidant activity as compared to leaves and fruit extracts.

Key words: *Kigelia africana*, Bacterial strains, Disc-diffusion method and DPPH-radical scavenging assay.

Introduction

Kigelia africana is a member of family Bignoniaceae popularly known as the cucumber or sausage tree because of the huge fruits (average 0.6m in length and 4 kg in weight), which hangs from long fibrous stalks (Cragg & Newman, 2001). The leaves are opposite or in whorls of three, 30-50 cm long, and flowers are bell shaped, orange to reddish and 10 cm wide. Individual flowers do not hang down but oriented horizontally (Joffe, 2003). Flowers are bisexual, very large, 2-4.5 cm long, widening and incurving up wards (Grace *et al.*, 2002). The sausage tree is fast growing and can mature in 4 to 5 years. It begins to flower from the age of 6 years. Mature fruits can be found on trees throughout the year (Jackson & Beckette, 2012).

Indigenous knowledge of herbal medicine is a big source of the modern knowledge (Kakar *et al.*, 2012). Throughout the world plants are used to treat various infectious diseases. They provide natural products that are used against infectious diseases (Walter *et al.*, 2011). The crude extracts of all family members of Biognoniaceae show antifungal activity, hepatoprotective, anti plasmodial, antioxidant, anticancer and antitumor activity etc (Choudhury *et al.*, 2011). The ethanolic extract of stem bark affect the muscle co-ordination and stimulant effect on the central nervous system (Owolabi *et al.*, 2008). Aqueous leaf extract show antidiarrheal activity as well. *Kigelia* also show the potential to increase the fertility (Azu *et al.*, 2010).

Due to the presence of active compounds in *K. africana* aqueous bark extract, it shows wound healing activity, particularly in relation to burn and bacterial infections (Sharma *et al.*, 2010; Alam & Singh, 2011). In West Africa, leaves are used for stomach and kidney

ailment, snakebite, and wounds, while stem and twigs are effective against wounds, snake bite, rheumatism as well as stomach and kidney ailments. Fruit is useful against constipation, gynecological disorders and hemorrhoids. It also acts as an anti-psoriasis and anti-eczema (Jackson & Beckette, 2012). Plant leaves comprises of essential nutrients which are comparable to other green leafy vegetables such as spinach (Glew *et al.*, 2010). The boiled fruits and roots are used to produce red and yellow dye respectively. Plant is used in sun block creams, after sun lotions and cosmetics etc. (Jackson *et al.*, 2000).

Extracts of *Kigelia* plant have strong molluscicidal activity against adult snail (DeSantos *et al.*, 2001) and anti-diabetic activity (Nyarko *et al.*, 2005) as well. Hence, *K. africana* is used in traditional medicine for many years. The objectives of present study were to determine the antibacterial and antioxidant potential of leaves, fruit and bark of this species and to use as an active drug in future.

Materials and Methods

Antibacterial activity

Bacterial strains: Six bacterial strains were collected from bacteriology section of Bahawal Victoria Hospital and propagated in Biochemistry lab at University College of Veterinary and Animal Sciences, Islamia University, Bahawalpur. These strains include *Staphylococcus aureus*, *Proteus vulgaris*, *Citrobacter amalonaticus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The bacterial cultures were maintained on nutrient media and glycerol stocks were made by adding 150 μ L glycerol and 850 μ L cultures in the Eppendorf tubes.

Media preparation: The following media were used for the antibacterial studies:

a. Nutrient broth: Nutrient broth was prepared by dissolving 1.3 gm of nutrient broth in 100 mL distilled water. Sterilize in the autoclave at 121°C for 20 minutes.

b. Nutrient agar: Nutrient agar was made by dissolving 2.8 gm of nutrient agar in distilled water. Sterilize in the autoclave at 121°C for 20 minutes.

Extraction of plant materials: Following extracts were made to take antibacterial assay:

a. Ethanol extract: 10 gm of plant powder was added to 100 mL of absolute ethanol in a conical flask and was covered with aluminum foil to avoid evaporation of ethanol. After 42 hours, the extract was filtered with common Whatman filter paper.

b n-Hexane extract: 10 gm of plant powder was added to 100 mL of n-hexane in a conical flask and was covered with aluminum foil to avoid evaporation of ethanol. After 42 hours, the extract was filtered with common Whatman filter paper.

c Aqueous extract by freeze thaw method: Phosphate buffer saline (PBS) was made by dissolving sodium chloride (4 gm), potassium chloride (0.1 gm), potassium dihydrogen phosphate (0.1 gm), and disodium hydrogen phosphate (1.16 gm) in 200 mL distilled water. 10gm of plant powder was added in 100 mL PBS (phosphate buffer saline, pH=7.2) in a conical flask, mixed well and placed in freezer. After 24 hours, the extract was thawed. The process of freeze and thaw was repeated three times, centrifuged at 4000 rpm and filtered by using Whatman filter paper.

Cutting of discs from disc diffusion method: The discs were cut with the help of disc cutter from common whatman filter paper. The discs were dipped in respective plant extracts to test the antibacterial activity against cultured microbes. Negative controls were prepared using discs impregnated with 100µL of the solvents i.e., ethanol and n-hexane. Pre-soaked discs from commercially available antibiotic- oxytetracycline (positive control) were used as standards for comparison.

Antibacterial assay: Bacterial strains were taken from glycerol stocks and a loop full of culture was added to the sterilized 3 mL nutrient broth. The cultures were incubated at 37°C for 24 hours at constant shaking. Observe the turbidity in the test tubes. Take OD₆₀₀ of cultures and bring the OD₆₀₀ at 0.4. Take 1 mL of culture and spin at 12000 rpm aspirate the supernatant and left 100 µl of supernatant on pellet. Dissolve the pellet and apply on agar.

Determination of zone of inhibition: The antibacterial activity assay was performed by agar disc diffusion method. The molten nutrient agar was poured into sterilized petri dishes. When the media was solidified,

plates were inoculated with 100 µL of the respective organism by glass spreader and incubate at 37°C for 1 hour. The pre-soaked discs (6 mm in diameter) in different extracts were placed on the agar medium seeded with respective micro-organisms. Pre-soaked discs from Oxytetracycline were used as a positive control and discs impregnated with solvent i.e ethanol and n-hexane was used as negative control. The plates were then incubated at 37°C for 24 hours to allow maximum growth of micro-organisms. The antibacterial activity of test samples was determined by measuring the diameter of zone of inhibition expressed in millimeter. The transparently cleared zones show bactericidal activity while the cleared zones containing micro-colonies showed bacteriostatic activity (Bauer *et al.*, 1966; Colle & Marr, 1989).

Antioxidant activity

Preparation of samples: Leaves, bark and fruits were carefully washed with tap water and rinsed with distilled water. All plant materials were spread in a clean stainless tray and air-dried under shade at room temperature for 10-15 days. Dried leaves were crushed and ground into coarse powder with mortar and pestle and the shade-dried fruits and bark were powdered in the electric grinder. The powdered material was kept in nylon bags.

Preparation of extracts: The simple extraction procedure was adopted for these medicinal plants. 10 g each of dry powdered plant materials were soaked in 100 mL of ethanol at room temperature for 36 hours. Here, the extraction was done with solvent under shaking conditions. The respected extracts were then filtered through a whatmann filter paper, in order to obtain an aqueous extract and solvent was removed completely under reduced pressure. Filtered extract was collected in a conical flask. The ethanol extract was evaporated by rotary evaporator at 45°C to get the crude extracts for the determination of antioxidant activity (Sigaroodi *et al.*, 2008).

Evaluation of antioxidant activity: The percentage of antioxidant activity (AA%) of each extracts was assessed by DPPH free radical assay. The measurement of the DPPH radical scavenging activity was performed according to methodology described by Brand *et al.* (1995). The samples were mixed with the stable DPPH radical in an ethanol solution. The reaction mixture consisted of 5 µL of sample, 95 µL of DPPH radical solution of 0.5 mM in ethanol. DPPH was reduced when it reacts with an antioxidant compound. The color changes from deep violet to light yellow. The absorbance was noted at 517 nm after 100 min of reaction using ELISA. The mixture of ethanol and sample served as blank. The control solution was prepared by mixing ethanol and DPPH radical solution. The scavenging activity percentage (AA%) was determined according to Mensor *et al.* (2001).

$$AA\% = 100 - \frac{[(Abs_{sample} - Abs_{blank}) \times 100]}{Abs_{control}}$$

(Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and Abs_{control} is the absorbance of the control). Quercetin was used as a standard antioxidant.

Statistical analysis: Values were mean \pm SD (standard deviation) of three replicates. All experiments were performed atleast, three times (unless indicated otherwise) and were highly reproducible. Data collected was analyzed statistically by applying one-way ANOVA using Statistica software 12 and means were separated by least significant different test at $p < 0.05$ (Steel *et al.*, 1996).

Results

Results of antibacterial activity: The results revealed that the tested ethanolic, n-hexane and aqueous extract of *K. africana* possessed significant antibacterial activity against various bacterial strains.

Antibacterial assay of leaves of *K. africana*: The results showed that the ethanol formed maximum inhibition zone (i.e. 12 mm) against *S. aureus* and *E. coli* and average zone against *K. pneumonia* while it formed 3 mm zone of inhibition against *C. amalonaticus*. Moreover it was observed that *K. africana* formed minimum zones against *P. vulgaris* and *P. aeruginosa* that is 2 mm mean value. In case of n – hexane control, maximum zones (10 mm inhibition zone) were observed against *E. coli* and *S. aureus* whereas minimum zone of inhibition was observed against *K. pneumonia* (Fig. 1, Table 1).

Ethanol extract of *Kigelia* leaves showed maximum activity against *E. coli* (i.e. 22 mm mean value) and moderate activity was shown against *P. vulgaris* and *P. aeruginosa* (i.e. 5 mm mean value) respectively. While no inhibition zones were formed against two bacterial strains namely *K. pneumonia* and *C. amalonaticus* (0 mm mean value). The result showed that *E. coli* was highly sensitive and *K. pneumonia* and *C. amalonaticus* are the resistant strains. Aqueous extract showed maximum antibacterial activity against *C. amalonaticus* (7 mm mean value) and moderate values were observed against *S. aureus* (5 mm mean value), *P. aeruginosa* (4 mm mean value), *P. vulgaris* (4mm mean value). While the *K. pneumoniae* possessed least value (0 mm mean value). It means that *C. amalonaticus* is highly sensitive and *K. pneumonia* is resistant one (Fig. 1).

As standard (positive control), oxytetracycline showed significant zone of inhibition against *S. aureus* (20 mm mean value) and least significant (7 mm mean value) against *K. pneumoniae* while moderate values were observed against rest of bacterial strains. It revealed that *S. aureus* is highly sensitive and *K. pneumoniae* is resistant strain. Overall, the zones produced by *C.*

amalonaticus, *K. pneumonia*, *P. aeruginosa*, *P. vulgaris* and *E. coli* were found to be statistically highly significant while *S. aureus* was revealed as non-significant (Fig. 2).

Antibacterial assay of fruit of *K. africana*: The ethanolic extract of *K. africana* exhibited maximum antibacterial activity against *P. vulgaris* (6 mm mean value) while minimum zone of inhibition (2 mm mean value) was formed against *S. aureus* while rest of the bacterial strains namely *K. pneumoniae*, *P. aeruginosa*, *C. amalonaticus* and *E. coli* were found to be resistant strains as no zone of inhibition was produced against them (Fig. 3). Aqueous extract of *Kigelia* fruit showed maximum zone of inhibition against *P. vulgaris* (6 mm mean value) and *C. amalonaticus* (5mm mean value). It showed moderate inhibitory zone against *S. aureus* and *E. coli* (3 mm mean value). However, *K. pneumoniae* and *P. aeruginosa* were revealed as the resistant strains (Table 2).

In case of oxytetracycline, the largest zone of inhibition was observed against *E. coli* and *P. vulgaris* (23 mm mean value) and minimum against *C. amalonaticus* (5 mm mean value). Statistically, it has been proved that the zones produced by the fruit of *K. africana* against all bacterial strains were highly significant (Fig. 4).

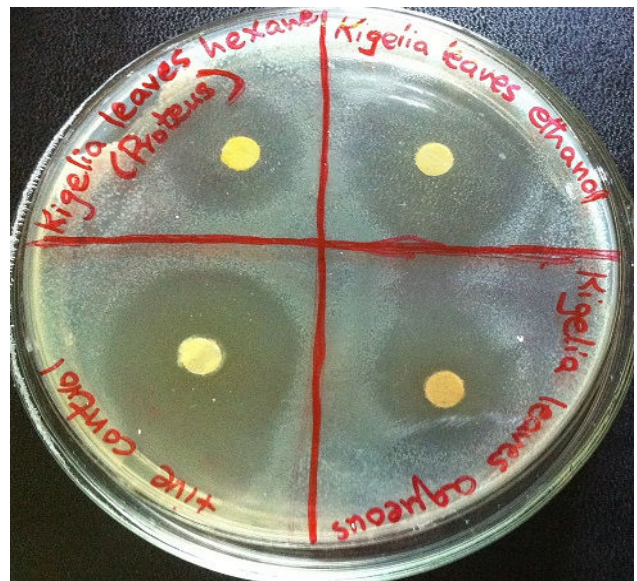
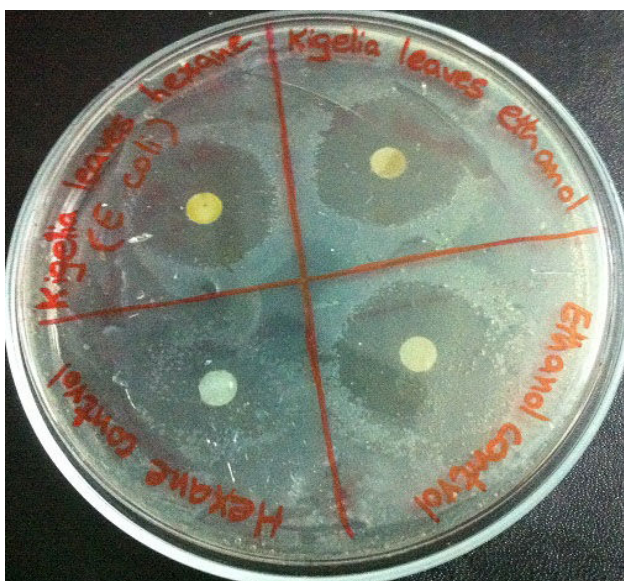
Antibacterial assay of bark of *K. africana*: Bark extract of *K. africana* possess good antibacterial activity against *E. coli* and minimum antibacterial activity against *K. pneumoniae* when tested with various extracts. Ethanolic extract of *Kigelia africana* bark showed maximum antibacterial activity against *E. coli* (10 mm mean value), moderate activity against *P. aeruginosa* and *P. vulgaris* (4 mm mean value) and least activity against *S. aureus* (1 mm mean value) while *K. pneumoniae* and *C. amalonaticus* were the resistant strains as no zone of inhibition was formed against them (Table 3). In case of n-hexane extract, *K. africana* bark extract produced resistance against all bacterial strains except *C. amalonaticus* (4 mm mean value) and *E. coli* (2 mm mean value) (Fig. 5).

Aqueous bark extract of *K. africana* showed maximum zone of inhibition against *S. aureus* (15 mm mean value). 5 mm mean value of zone of inhibition was observed against *P. aeruginosa* and *P. vulgaris*. Least inhibition zone was formed against *C. amalonaticus* (3 mm mean value) while *K. pneumoniae* and *E. coli* were the resistant strains (Fig. 6). Oxytetracycline, used as positive control, showed largest zone of inhibition in case of *S. aureus* (25 mm mean value) and *E. coli* (20 mm mean value). However, least zone of inhibition was observed against *C. amalonaticus* (3 mm mean value). Overall, the studies revealed that the zones of inhibition produced by the bark extract of the respective plant against all bacterial strains were statistically highly significant.

Table 1. ANOVA for antibacterial assay from *K. africana* leaves and their inhibition zone in different plant extracts.

SV	df	<i>Staphylococcus aureus</i>	<i>Klebsiella pneumoniae</i>	<i>Pseudomonas aeruginosa</i>	<i>Proteus vulgaris</i>	<i>Citrobacter amalonaticus</i>	<i>Escherichia coli</i>
Replication	2	649.500 ^{ns}	0.000 ^{ns}	1.500 ^{ns}	0.667 ^{ns}	0.667 ^{ns}	1.500 ^{ns}
Extracts	5	916.800 ^{ns}	32.900 ^{**}	22.800 ^{**}	54.500 ^{**}	42.500 ^{**}	196.800 ^{**}
Error	10	648.700	0.800	0.700	1.067	0.667	0.700
Cv		169.80 %	28.25 %	20.92 %	7.47 %	19.60 %	8.37 %

ns = Non-significant, * = Significant, ** = Highly significant

Fig. 1.1. *S. aureus* (negative control).Fig. 1.3. *P. vulgaris* (leaves).Fig. 1.2. *E. coli* (negative control).Fig. 1.4. *C. amalonaticus* (leaves).Fig. 1. Antibacterial assay of leaves of *Kigelia africana*.Table 2. ANOVA for antibacterial assay from *K. africana* fruit and their inhibition zone in different plant extracts.

SV	df	<i>Staphylococcus aureus</i>	<i>Klebsiella pneumoniae</i>	<i>Pseudomonas aeruginosa</i>	<i>Proteus vulgaris</i>	<i>Citrobacter amalonaticus</i>	<i>Escherichia coli</i>
Replication	2	0.500 ^{ns}	0.167 ^{ns}	0.167 ^{ns}	0.677 ^{ns}	0.167 ^{ns}	0.000 ^{ns}
Extracts	5	173.300**	41.300**	30.500**	168.500**	21.600**	238.800**
Error	10	0.900	0.566	1.167	10.667	0.567	0.800
Cv		12.11%	23.77%	22.35%	10.16%	25.09%	11.18%

ns = Non-significant, * = Significant, ** = Highly significant

Table 3 ANOVA for antibacterial assay from *K. africana* bark and their inhibition zone in different plant extracts.

SV	df	<i>Staphylococcus aureus</i>	<i>Klebsiella pneumoniae</i>	<i>Pseudomonas aeruginosa</i>	<i>Proteus vulgaris</i>	<i>Citrobacter amalonaticus</i>	<i>Escherichia coli</i>
Replication	2	0.167 ^{ns}	0.167 ^{ns}	0.167 ^{ns}	1.167 ^{ns}	1.500 ^{ns}	0.167 ^{ns}
Extracts	5	260.100**	41.300**	24.400**	243.300**	11.300*	157.200**
Error	10	0.967	0.567	0.967	0.967	0.700	0.967
Cv		9.36%	23.77%	22.69%	5.31%	26.42%	10.92%

ns = Non-significant, * = Significant, ** = Highly significant

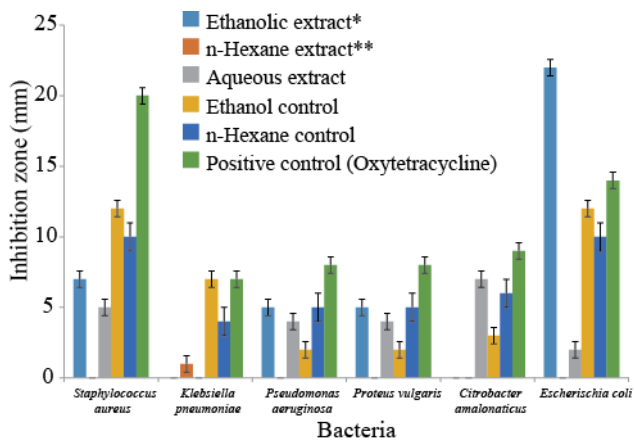


Fig. 2: Mean values of antibacterial assay of *Kigelia africana* leaves.
 * = activities mentioned are the zone of inhibition produced by ethanolic extract minus zone of inhibition produced by ethanol control
 ** = activities mentioned are the zone of inhibition produced by n-hexane extract minus zone of inhibition produced by n-hexane control

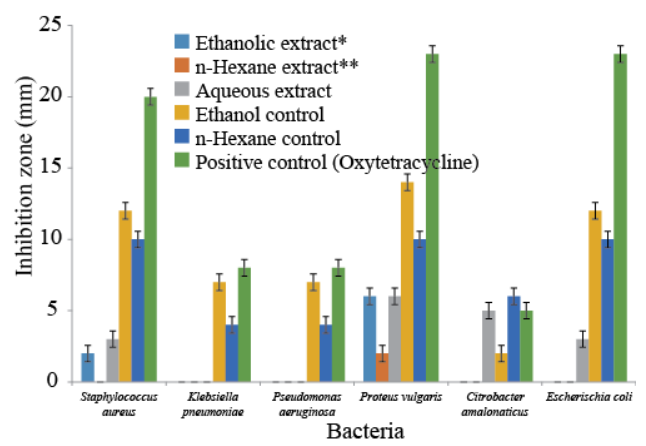


Fig. 4: Mean values of antibacterial assay of *Kigelia africana* fruit.
 * = activities mentioned are the zone of inhibition produced by ethanolic extract minus zone of inhibition produced by ethanol control
 ** = activities mentioned are the zone of inhibition produced by n-hexane extract minus zone of inhibition produced by n-hexane control

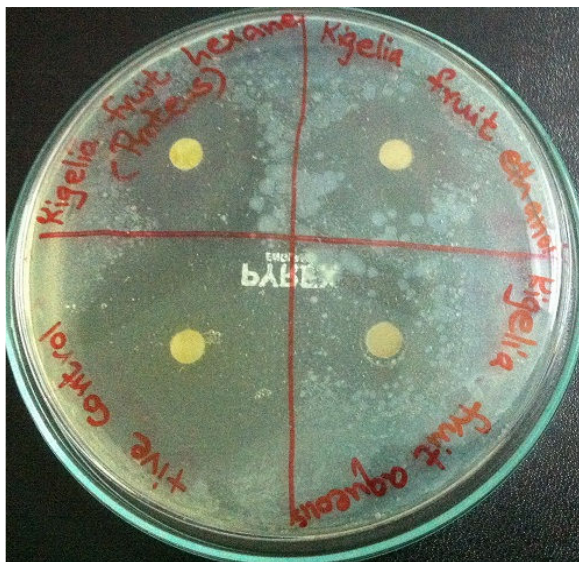


Fig. 3.1. *P. vulgaris*.

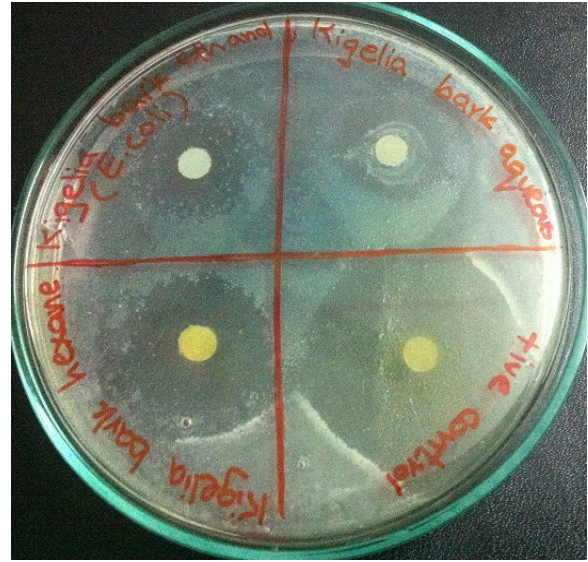


Fig. 5.1. *S. aureus*.

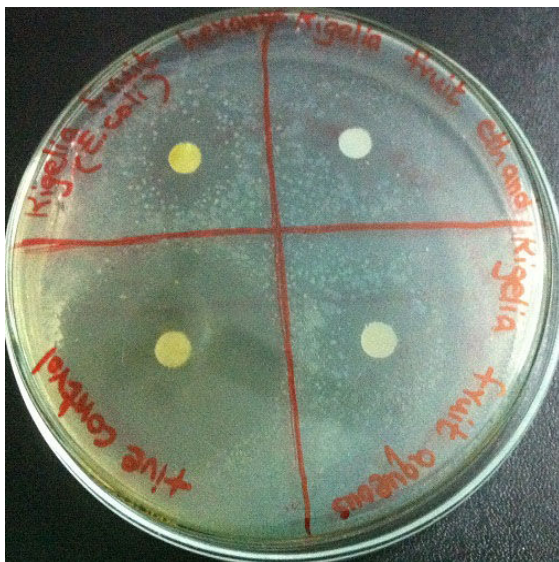


Fig. 3.2. *E. coli*.

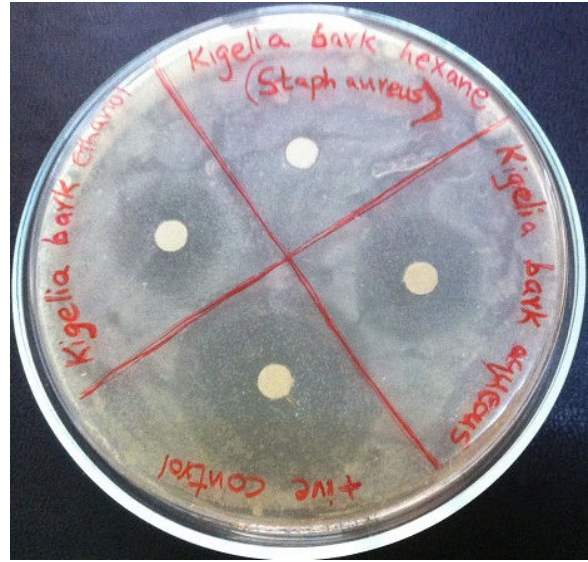


Fig. 5.2. *E. coli*.

Fig. 3. Antibacterial assay of *Kigelia africana* fruit.

Fig. 5. Antibacterial assay of *Kigelia africana* bark.

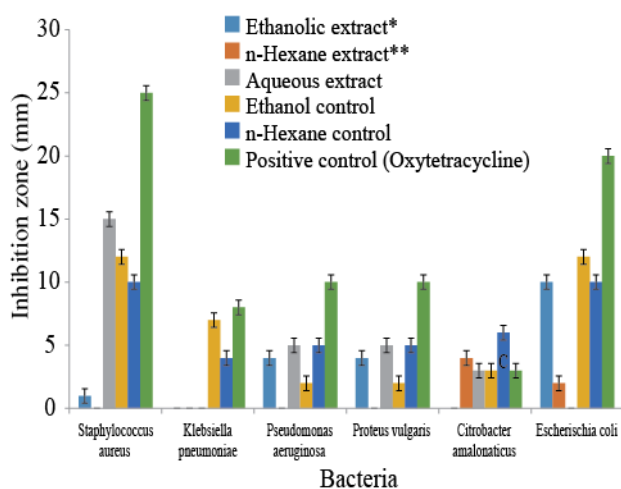


Fig. 6. Mean values of antibacterial assay of *Kigelia africana* bark.
 * = activities mentioned are the zone of inhibition produced by ethanolic extract minus zone of inhibition produced by ethanol control
 ** = activities mentioned are the zone of inhibition produced by n-hexane extract minus zone of inhibition produced by n-hexane control

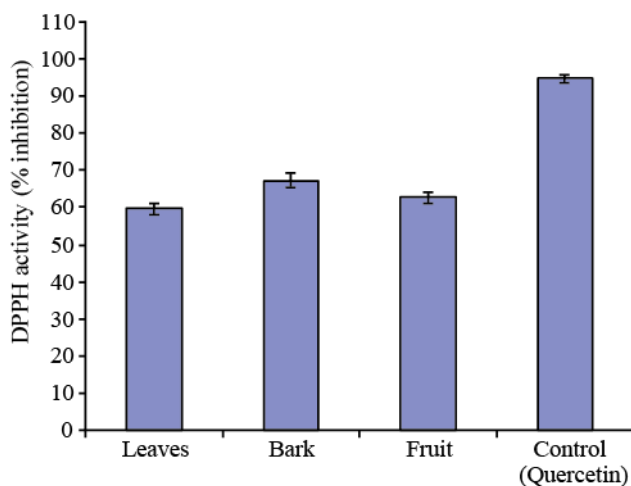


Fig. 7. Antioxidant activity of different parts of *Kigelia africana* ± SD.

In a nutshell, different parts of *K. africana* possessed remarkable antibacterial activity against *E. coli* and *S. aureus* strains and least activity against *K. pneumoniae*. It was clear that among six bacterial strains, *S. aureus* and *E. coli* were highly sensitive strains in both leaves and bark extract while *P. vulgaris* was found to be sensitive in case of fruit extract. However, *K. pneumoniae* was proved as resistant strain in all extracts. Oxytetracycline, which was used as a positive control, showed promising results in all bacterial strains. Moreover, ethanolic extract and aqueous extract showed significant antibacterial activity as compared to n-hexane extracts respectively.

Results of antioxidant activity: The percentage of antioxidant activity of different parts of *K. africana* was assessed by DPPH free radical assay. Quercetin was used as a standard antioxidant which showed 94% inhibition. Results revealed that the bark of *Kigelia* possesses good antioxidant activity (67.33%), fruit extract showed moderate antioxidant activity (62.66%) and leaves showed the poor antioxidant activity (59.66%) respectively. In a nutshell, all the selected parts of species showed remarkable DPPH radical scavenging activity in varying concentrations as shown in Fig. 7.

Discussion

Antimicrobial compounds from plants represent a potentially novel source of antimicrobial substances since they act against bacteria via mechanisms that are different from those of currently used antibiotics and may thus have a clinical value in the treatment of antibiotic resistant antimicrobial strain (Eloff, 1998; Naveed *et al.*, 2013). Our results revealed that the ethanolic and aqueous extract of various parts of *K. africana* possess good antibacterial activity as compared to n-hexane extracts respectively (Figs. 1-6). Hence, extracts of *K. africana* may be used as the alternative source for treating several infectious disease caused by various pathogens (Dyary *et al.*, 2014; Hamad *et al.*, 2014).

Oxygen is an element obligatory for life where living systems have evolved to survive in the presence of molecular oxygen, which has double-edged properties, being essential for life; it can also aggravate the damage within the cell by oxidative events (Shinde *et al.*, 2012). Oxidative stress results when the balance between the productions of ROS exceeds the antioxidant capability of the target cell (Ahmad *et al.*, 2009). The antioxidant defense system in most living cells is composed of two components: antioxidant enzymes (endogenous antioxidants), and small molecule antioxidants (exogenous antioxidant) (Mugwerua & Rusling, 2006).

Diet plays a vital role in the production of the antioxidant defense system by providing essential nutrient antioxidants such as vitamin C, vitamin E, and β -carotene. Plants are considered as one of the most important and interesting subjects that should be explored for the discovery and development of newer and safer drug candidates (Hamid *et al.*, 2011; Xiao CW *et al.*, 2014). The scavenging of the stable DPPH radical is widely used to assess antioxidant activity in a short time compared with other methods. This assay allows comparison of the reactivity of powerful antioxidants such as BHT and ascorbic acid with those present in extracts against DPPH radical (Manzoor *et al.*, 2013). Results revealed that the bark of *K. africana* had good antioxidant activity while the leaves of plant show poor antioxidant activity (Fig. 7).

The herbal remedies have become more popular in the treatment of minor ailments (Bakht *et al.*, 2013; Pieri *et al.*, 2014). *Kigelia* is one of the most important sources of new bio-active compounds but there is need for more such approaches to find out more effective chemical compounds. This plant has great potential to be developed as drug by pharmaceutical industries. It is suggested that this plant could be used as an additive in the food industry providing good protection against oxidative damage. However, further studies are needed to isolate the exact active components which are responsible for antibacterial and antioxidant activities.

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