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Antibacterial and Phytochemical Activity of *Gliricidia sepium* against Poultry Pathogens

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Authors' contributions

This work was carried out in collaboration between all authors. Author AKJ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript and managed literature searches. Authors AKJ, OMK and OAG managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To examine antibacterial and phytochemical activity of *Gliricidia sepium* in the treatment of poultry pathogens.
Place and Duration of Study: the work was carried out in the Department of Microbiology Federal University of Technology Akure, Ondo State, between January 2013 to July 2014.
Methodology: The methanol, ethanol, aqueous, acetone and petroleum ether soluble crude and fractions extract of *Gliricidia sepium* leaf were examined for antibacterial activities against selected poultry pathogens, Phytochemical and possible sources of antioxidant were examined. The antibacterial activity of the crude and fractions were carried out against four poultry bacteria isolates using the agar well diffusion and paper disk methods respectively. For all the tests, the significance was determined at the level of $P< 0.05$.

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Results: In the antibacterial activity using agar well diffusion it was found that ethanol extract possessed highest zone of inhibition of 25.90±0.10 millimeters against *S. typhi* while the least zone of inhibition of 15.00±0.00 millimeters was recorded with petroleum ether against *S. flexneri.* The antioxidant DPPH test was performed with appreciable level of both ferric reducing antioxidant properties and free radical scavenging activities were of better expression in ethanolic extract than others. Likewise, ethanol extract has the highest concentration of phenol with a value of 7.57±0.00 mg/g and the highest flavonoid content was recorded in ethanol extract with a value of 3.94±0.02 mg/g.
Conclusion: The antibacterial activity showed by the extract of *G. sepium* was notable therefore the extract of this plant can be harnessed effectively in control of the growth of poultry pathogens.

Keywords: Antibacterial activity; poultry; phytochemical; Gliricidia; pathogens.

1. INTRODUCTION

Poultry is one of the fastest growing segments of the agricultural sector in Nigeria. Production of eggs and broilers has been rising at a rate of 8 to 10% per annum. Eggs and chicken meat are important rich sources of protein, vitamins and minerals. Poultry provides rich organic manure and is an important source of income and employment to millions of farmers and other persons engaged in allied activities in the poultry industry [1]. Many States in the world rely upon the poultry industry for a substantial portion of their agricultural income. The diseases of bacterial etiology present important factors in poultry production, therefore the sources of spreading the infection in poultry flocks and possible economic losses, which they induce, need to be investigated. For instance congestion of poultry at the poultry houses induces increased pathogenicity of some microbial agents, especially bacteria which could cause infection with high rate of morbidity and mortality [2].

Medicinal plants are gifts of nature to cure limitless number of diseases among human beings and animals. The abundance of plants on the earth's surfaces has led to an increasing interest in the investigation of different extracts obtained from traditional medicinal plants as potential sources of new antimicrobial agents [3]. This study was carried out with the objective to examine antibacterial and phytochemical activity of *Gliricidia sepium* in the treatment of poultry pathogens.

2. MATERIALS AND METHODS

2.1 Collection and Identification of the Plant Material

Gliricidia sepium plant was collected from forest in Ipinsa village of Ondo State, Nigeria according to the method of [4]. The identification and authentication of the plant material was carried out in the herbarium of the Department of Crop Soil and Pest Management Federal University of Technology Akure, Ondo State.

2.2 Preparation of the Plant Extract

Fresh leaves of *G. sepium* were separated from the stems and thereafter air-dried for two weeks at room temperature (25±2ºC). The dried leaves were pulverized by grinding machine (brand Retsch Gmbh 5657 HAAN, model type SM1, West Germany) into smooth powder. The powder was further sieved by 1.18 mm sieve. Different extracting solvents were used for the extraction which include both polar to non-polar: acetone, methanol, ethanol, petroleum ether and aqueous. For each of the solvent, 150 g were dissolved in 560 ml of solvents. The mixture was kept for 72 hours in a tightly sealed amber glass at room temperature, protected from sunlight and mixed several time by shaking. The paste was then filtered with muslin cloth. The filtrate was subjected to rotary evaporator under reduced pressure. These extracts were reconstituted with Dimethyl sulphoxide (DMSO,70%) to prepare different concentrations that ranged from 25 to 200 $mgml^{-1}$) and kept in refrigerator till used.

2.3 Test Organisms

The test organisms (poultry pathogens) used in this work were provided by the Department of microbiology, Federal Institute of Industrial Research Oshodi and was preserved under refrigerated condition until use. These test bacteria include *Escherichia coli* ATCC 8739, Staphylococcus *aureus* ATCC 25923*, Salmonella typhi* ATCC 6539 and *Shigella flexneri* ATCC 12022.

2.4 Standardization of Inoculum

The inocula were prepared from the stock cultures, which were maintained on nutrient agar slant at 4°C and sub-cultured onto nutrient broth using a sterilized wire loop. The density of suspension inoculated onto the media for susceptibility test was determined by comparison with 0.5 McFarland standard of Barium chloride solution [5].

2.5 Reconstitution of Extracts

The extracts were reconstituted and sterilized using Membrane filter (0.2 µm) before use. The required volume of extract was sterilized by using a syringe filter holder and the sterile extract was collected in the sterile bottle.

The recovery rate of extracts was calculated using the formula below.

% Recovery of Extract = WA/IW X100

Where IW = Initial weight of extracts, WA weight of extracts recovered after extraction.

2.6 Phytochemical Analysis of the Used Plant

2.6.1 Determination of total phenol

The total phenol content of the extract was determined by the method of Singleton et al. [6]. A known weight (0.2 g) of the extract was mixed with 2.5 ml of 10% Folin Ciocalteaus reagent and 2ml of 7.5% Sodium carbonate. The reaction mixture was subsequently incubated at 45°C for 40 mins, and the absorbance was measured at 700 nm in the spectrophotometer (Brand AJ, model 1C03, country England), gallic acid was used as standard phenol.

2.6.2 Determination of total flavonoid

The total flavonoid content of the extract was determined using a colorimetric assay developed by Jinsong et al. [7] by adding 0.2 g of the extract to 0.3 ml of 5% sodium nitrate solution ($NaNO_3$) at zero time. After 5min, 0.6 ml of 10% aluminum chloride solution ($AlCl_3$) solution was added and after 6 min, 2 ml of 1M sodium hydroxide solution was added to the mixture followed by the addition of 2.1 ml of distilled water. Absorbance was read at 510 nm against the reagent blank and flavonoid content was expressed as mg rutin equivalent.

2.6.3 Determination of ferric reducing property

To determine ferric reducing property, 0.25 g of the extract was mixed with 0.25 ml of 200 mM of Sodium phosphate buffer pH 6.6 and 0.25 ml of 1% potassium ferric chloride solution. The mixture was incubated at 50°C for 20min, thereafter 0.25 ml of 10% trichloricacetic acid (TCA) was also added and centrifuge at 2000 rpm for 10 min. Of the supernatant, 1ml was mixed with 1ml of distilled water and 0.2 ml of 20% Ferric chloride solution and the absorbance was measured at 700 nm [8].

2.6.4 Determination of free radical scavenging ability

The free radical scavenging ability of the extract was determined against DPPH (1, 1- diphenyl-2-picrylhydrazyl) using the method of Gyamfi et al. [8] by mixing 1g of the extract with 1ml of the 0.4 mM methanolic solution of the DPPH and the mixture was left in the dark for 30 min before measuring the absorbance at 516 nm.

2.6.5 Tannin determination

Extract of 0.2 g was weighed into a 50 ml sample bottle. 10 ml of 70% aqueous acetone was added and the solution was properly covered. The bottle was put in an ice bath shaker for 2 hours at 30°C .Each solution was then centrifuged and the supernatant stored in ice. 0.2 ml of each solution was pipetted into the test tube and 0.8 ml of distilled water was added. Standard tannic acid solutions were prepared from a 0.5 $mgml^{-1}$ of the stock and the solution made up to 1 ml with distilled water. 0.5 ml of Folin Ciocalteau reagent was added to both sample and standard followed by 2.5 ml of 20% Na_2CO_3 the solution were then vortexed and incubated for 40 minutes at room temperature. The absorbance was read at 725 nm against a reagent blank of the same solution and tannin concentration was calculated from a prepare standard tannic acid curve [9].

2.6.6 Determination of phytate

Phytate was determined according to the method of Wheeler and Ferrel [10] by soaking 2 g of sample in 100 ml of Hydrochlori acid solution for 3 hrs, filter through a No 1 Whatman filter paper and 25 ml of the filtrate was placed in inside a conical flask 5ml of 0.3% of ammonium thiocyanate solution as an indicator. In order to

give the solution its proper acidity, 53.5 ml of distilled water was added then titrated against 0.00566 g per milliliter of standard ferric chloride solution that contain about 0.00195 g of iron per milliliter until a brownish yellow colouration persist for 5 min.

2.6.7 Determination of oxalate

Oxalate determination was carried out by soaking 1 g of the sample in 75 ml of 1.5 NH_2SO_4 for 1 hr and filter through a No 1 Whatman filter paper. The filterate of 25 ml was insided a conical flask and then titrated hot (80-90°C) against 0.1 M $KMnO_4$ until a pink colour persist for 15 sec. [11].

2.7 Antibacterial Assay of the G. *sepium* Leaf Extracts

The antibacterial activity of extracts was determined by the agar well diffusion method as described earlier [12] with slight modification. After standardization of inocula, 0.1 ml of the 18 hr old broth cultures of the test organisms was aseptically placed into sterile Petri dishes and 15 ml of sterilized nutrient agar was poured aseptically on the it. The plates were swirled carefully for even distribution and allowed to gel. With the aid of sterile cork borer of 6 mm diameter, wells were bored on solidified agar medium. A concentration of 50 $mgml^{-1}$ of the extracts were prepared using 70% DMSO as the reconstituting solvent and filtered through 0.2 µm membrane filter. Each extract (0.5 ml) was then pipetted into the wells of appropriately labelled plates and holes. The plates were allowed to stand on the laboratory bench for 15 minutes to allow proper inflow of the extract into the medium before being incubated at 37°C for 24 hrs. The control was prepared by using 0.1 ml of the reconstituting solvent and incubated alongside with the extract. After incubation, zone of inhibition (diameter) was measured in millimeter The experiment was carried out in duplicates.

2.8 Antimicrobial Assay of the Column Fractions of Leaf Extracts

Antibacterial activity of the column fractions obtained after fractionation of extracts was determined by a slightly modified paper disc diffusion method [13]. The used bacterial isolates were grown in nutrient broth and sterile Petri dishes were seeded aseptically with 0.1 ml of the 18 hours old broth cultures of the test organisms and 20 ml of sterilized Mueller Hinton agar was poured aseptically on the seeded plates. The plates were swirled carefully for even distribution and allowed to gel. Standard size blank Whatman filter paper discs (6.00 mm in diameter) were put into bijou bottle, sterilized at 121°C for 15 minutes. The sterile paper discs were impregnated with 0.1 ml of extract dilutions reconstituted in minimum amount of extracting solvent at concentration of 50 $mgml^{-1}$ and were applied with the aid of sterile forceps at equal distances from each other on the seeded plates.

Filter paper discs dipped into sterile distilled water and allowed to dry were used as a control. The plates were then incubated at 37°C for 24 hrs. Antibacterial activity was determined inhibition zone around each paper disc.

2.9 Determination of Minimum Inhibitory Concentration (MIC) of Leaf Extracts

Minimum inhibitory concentrations (MICs) were determined by the agar diffusion technique as described earlier [14] with slight modifications. The MIC corresponds to the lowest concentration of the tested extracts, able to inhibit any visible microbial growth. Different concentrations of the extracts were prepared (25, 50, 100, 200 $mgml^{-1}$. Each concentration was used to impregnate paper disks. Then the disks were transferred into the Petri dishes containing the test organisms. The plates were incubated for 24 h at 37ºC. After incubation, the results in each plate were recorded. MIC of the extract was taken as the lowest concentration that showed no growth.

2.10 Statistical Analysis

All data obtained in this work were subjected to statistical analysis. Data are expressed as means ± SE (Standard Error). Significant differences between different treatment groups was tested using one –way analysis of variances (ANOVA) and significant results were compared with Duncan's multiple range tests using SPSS window 7 version 16 software. For all the tests, the significance was determined at the level of $P< .05$.

3. RESULTS

3.1 Percentage Recovery of Plant Extracts

Table 1 shows the percentage of each extract recovered after drying. It was noted that ethanol extract has the highest percentage of 13.81%

equivalent to 20.71 g of its initial weight followed by aqueous extract with 7.66% equivalent to 11.49 g of its initial weight.

3.2 Minimum Inhibitory Concentration of Crude Extracts on the Test Organisms

Table 2 shows the result of MIC of the extracts on the test organisms. The MIC value of the extract ranges from 25 to 200 $mgml^{-1}$. The MIC value of the aqueous extract was 200 $mgml^{-1}$ against test organisms. However only ethanol and methanol possessed an MIC of 25 $mgml^{-1}$ against *E. coli.*

3.3 Antibacterial Activities of *Gliricidia sepium* Crude Extracts

Table 3 shows the inhibition zone in millimeters for crude extracts; acetone, methanol, ethanol, aqueous and petroleum ether after 24 hours of incubation. All the extracts demonstrated good antibacterial activity against selected pathogens. The zone of inhibition ranged from 13.13±0.61 mm to 25.90±0.10 mm. It was discovered that ethanol exhibited the highest potency (25.90±0.10 mm) against *Salmonella typhi* and least activity was recorded with the ethanolic extract against *S. aureus.*

3.4 Antibacterial Activity of *G. sepium* Fraction

Table 4 shows the total ten fractions obtained from the leaves extracts. After 24 hours of incubation, zone of inhibition of the entire fraction showed inhibitory effect ranged from 1.05±0.03 to 21.00±0.06 mm. In the entire fractions, only fraction FC3 was resisted by the test organisms. Fractions FC1 exhibited a zone of inhibition ranged 7.90±0.06 to 11.00±0.00 mm against the test organisms. *Escherichia coli* and *S. typhi* exhibited resistance to FP1 fraction while only *S. typhi* showed resistance to FP2 fraction. For FM2 fraction all the test organisms except *S. aureus* showed resistance. For FM3 fraction only *S. aureus* was sensitive while for FM4 only *S. flexneri* was sensitive and *E. coli, S. aureus* and *S. typhi* were resistant. For FM5, both *E. coli* and *S. flexneri* were sensitive but *S. aureus* and *S. typhi* displayed resistance and for FM6 only *E. coli* was sensitive.

Table 1. Percentage recovery of plant extracts

	Initial weight (IW)(g) of the extract	Weight of extract after extraction (WA) (g)	(%) recovery = WA/IW × 100
Pet ether	150	4.5	3%
Aqueous	150	11.49	7.66%
Ethanol	150	20.71	13.81%
Methanol	150	6.5	4.33%
Acetone	150	9	6%

Table 2. Minimum inhibitory concentration (mg/ml)

Extracts	*S. aureus*	*S. flexneri*	*S. typhi*	*E. coli*
Pet ether	100	100	200	50
Aqueous	200	200	200	200
Ethanol	50	200	100	25
Methanol	50	200	100	25
Acetone	100	200	100	50

Table 3. Zone of Inhibition (mm) of crude extracts (agar well diffusion method)

Extracts	*Escherichia coli*	*Staphylococcus aureus*	*Shigella flexneri*	*Salmonella typhi*
Aqueous	$18.43±0.40^{Ab}$	$17.63±0.15^{Da}$	$19.33±0.28^{Bc}$	$20.00±0.00^{Dd}$
Ethanol	$23.43±0.40^{Db}$	$13.13±0.61^{Aa}$	$25.50±0.50^{Ec}$	$25.90±0.10^{Ec}$
Methanol	$20.00±0.00^{Bc}$	$14.50±0.50^{Ba}$	$16.00±0.00^{Ab}$	$16.00±0.00^{Ab}$
Acetone	$25.00±0.00^{Ec}$	$25.00±0.00^{Ec}$	$20.00±0.00^{Cb}$	$17.00±0.00^{Ba}$
Pet ether	$21.50±0.50^{Cc}$	$15.00±0.00^{Ca}$	$23.50±0.50^{Dd}$	$20.50±0.50^{Cb}$
Control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Data are presented as means ± S.E (Standard Error) (n=3). Mean with the same superscript letter (s) along the same row (lower case) or columns (upper case) are not significantly different (p≤ .05)

Table 4. Zone of inhibition (mm) of *G. sepium* fractions against selected pathogens (paper disk method)

Fractions	*Escherichia coli*	*Staphylococcus aureus*	*Shigella flexneri*	*Salmonella typhi*
FP1	0.00 ± 0.00^{Aa}	7.90 ± 0.05^{Bb}	10.00 ± 0.00^{Bc}	0.00 ± 0.00^{Aa}
FP2	1.05 ± 0.03^{Bb}	10.93 ± 0.07^{Cc}	10.07 ± 0.00^{Bc}	0.00 ± 0.00^{Aa}
FCI	7.90 ± 0.06^{Ca}	13.90 ± 06^{Dc}	15.23 ± 0.72^{Cd}	11.00 ± 0.00^{Bb}
FC3	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}
FM1	21.00 ± 0.06^{Ec}	20.57 ± 0.36^{Fc}	15.03 ± 0.00^{Cb}	0.00 ± 0.00^{Aa}
FM2	0.00 ± 0.00^{Aa}	11.93 ± 0.18^{Db}	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}
FM3	0.00 ± 0.00^{Aa}	15.30 ± 0.15^{Eb}	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}
FM4	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}	15.04 ± 0.00^{Cb}	0.00 ± 0.00^{Aa}
FM5	8.90 ± 0.56^{Dc}	0.00 ± 0.00^{Aa}	10.10 ± 0.33^{Bc}	0.00 ± 0.00^{Aa}
FM6	8.90 ± 0.35^{Db}	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}
Control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Data are presented as means ± S.E (Standard Error) (n=3). Mean with the same superscript letter (s) along the same row (lower case) or columns (upper case) are not significantly different (p≤.05) Keys: FP = Fraction from Pet Ether, FC= Fraction from Chloroform, FM = Fraction from Methanol

3.5 Phytochemical Analysis

Fig. 1 shows that the concentration of total phenolic content in ethanol extract has highest of phenolic content with 2.86±0.07 mg/mg and the least phenolic content recorded with acetone extract as 1.90±0.02 mg/g. The same figure also revealed the concentration of total flavonoid content with the *Gliricidia sepium* ethanol extract possessing the highest values of 3.94±0.02 mg/g and the acetone extract has relative content of 0.91±0.02 mg/g. Fig. 2 showed percentage composition of DPPH or free reducing scavenging ability. The reagent 2, 2-diphenyl-1-picrylhydrazyl (DPPH) is a free radical and reduction of this chemical by probable antioxidants result in loss of absorbance. Thus, the degree of discolouration of the solution indicates the scavenging efficiency of the added substance. The results of free radical scavenging properties of the extracts expressed in percentage DPPH activities are shown in Fig. 2. All the plant extracts exhibited moderate to high antioxidant activities. From the extracts, highest antioxidant activity was observed in ethanol (81.54±0.03%) followed by methanol (71.46±0.00%), pet ether (61.00±0.03%) and the least activity was obtained from acetone (52.41±0.41%). The value obtained for free radicals antioxidant properties (FRAP) was recorded that ethanol extract displayed highest value of 34.95±0.08% and the acetone was found to possess relative value of 12.23±0.00%.

3.6 Anti-nutrient Content

Fig. 3 shows the composition of Anti-nutrient phytochemicals present in *Gliricidia sepium*. The phytate present was recorded with relatively high value in ethanol extract as 19.78±0.00 mg/g and the least value recorded in petroleum ether. Likewise oxalate, the highest value was recorded against ethanol extract with 2.54±0.00 mg/g while the least value was observed in petroleum ether. In the case of tannin the highest value was obtained in aqueous extract as 2.56±0.00 mg/g and the least observed in petroleum ether as 1.11±0.06 mg/g.

4. DISCUSSION

Differences were noticed in the antibacterial activities of the crude and fractions of the considered plants extracts. These attributes are linked to the differences in the chemical components of the plants extract such as tannins, alkaloids, phenols, flavonoids and saponins [4]. The results of antibacterial activities of both the crude and fraction extracts demonstrated high inhibitory potency against the tested pathogenic bacterial isolates. The ethanol, methanol, acetone, and petroleum ether extracts showed higher inhibitory activity than aqueous, this is connected to the inability of aqueous to display effective inhibition activity at low concentration based on its weak for the extraction of bioactive components from this plant majorly for antimicrobial purposes.

The percentage recovery of *Gliricidia sepium* extract varied in the five solvents used. Comparatively, ethanol gave higher yield than water and others solvents. This observation is in accordance with [15] who worked on the leaves of three *Pistacia* species and reported that the recovery level of plant extracts is dependent and vary with the different extracting solvent. Also the high percentage recovery obtained in ethanol might be due to the organic nature of ethanol as well as being polar which allow them to actively

dissolve the chemical components of the leaves. The observation is also supported by [16] who reported that the most active components are generally insoluble, hence it is expected that low polarity organic solvents would yield more percentage recovery.

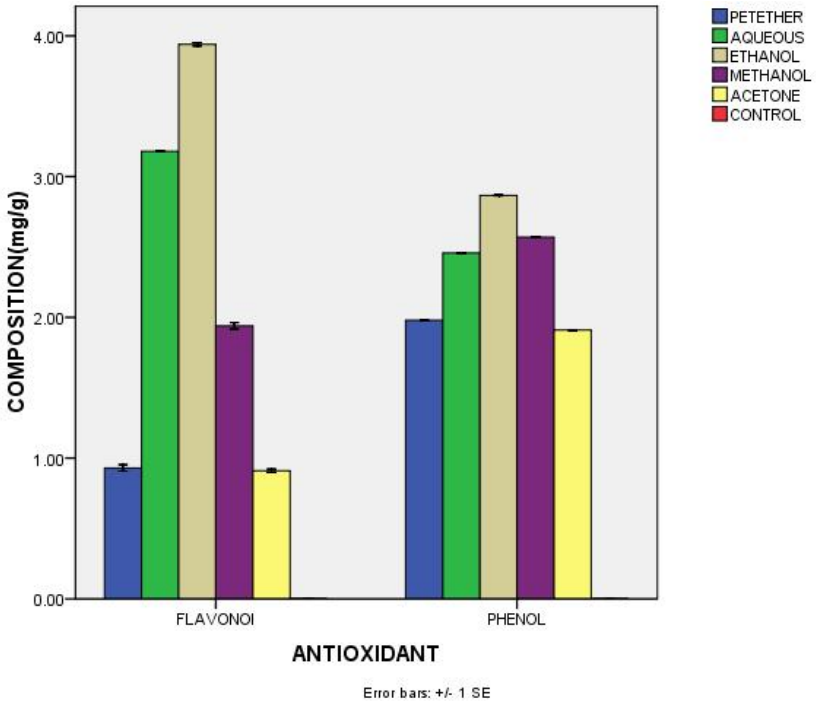

Fig. 1. Antioxidant composition (mg/g) in *Gliricidia sepium*

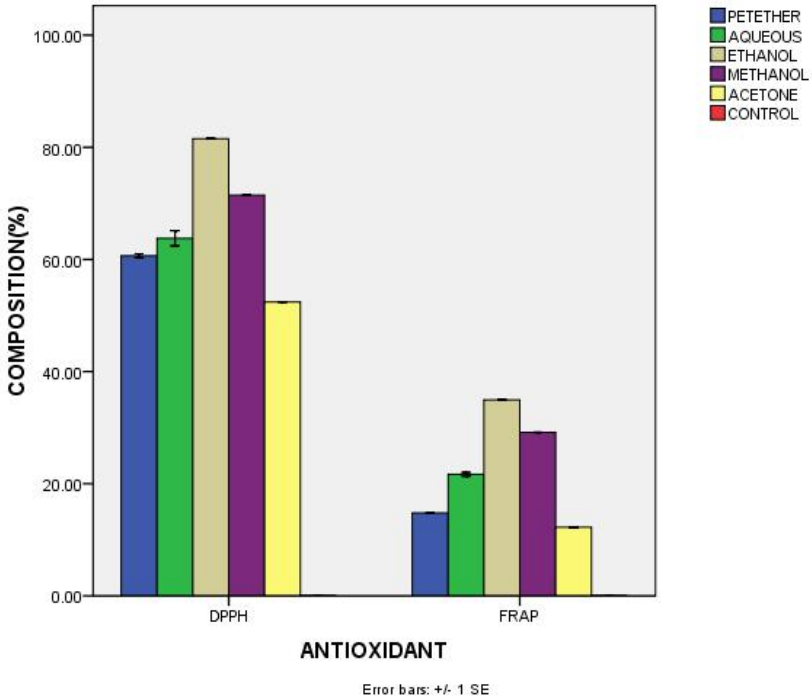

Fig. 2. Antioxidant composition (%) in *Gliricidia sepium*

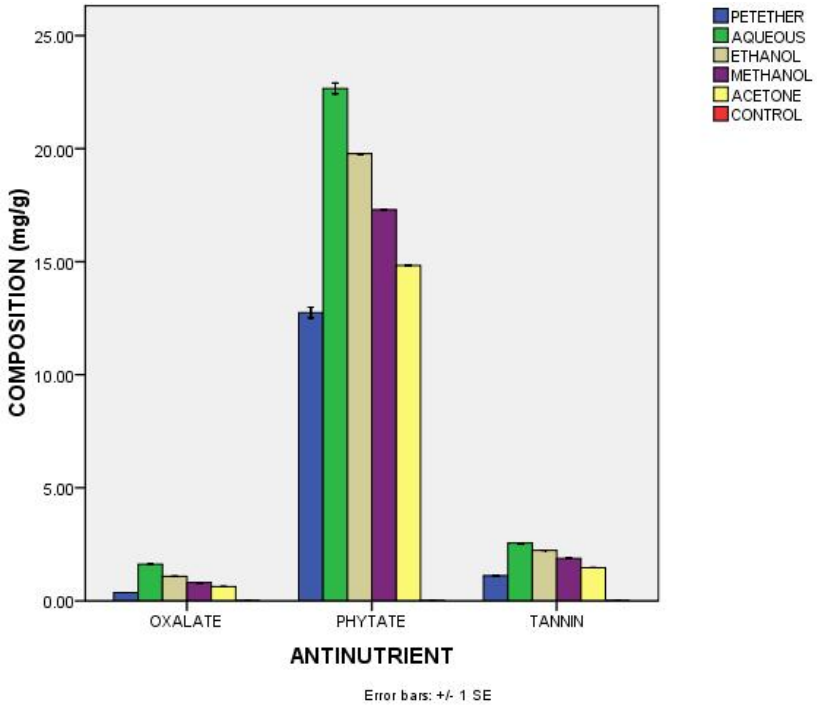

Fig. 3. Antinutrient composition (mg/g) in *Gliricidia sepium*

It was also observed that there is variation in the antimicrobial effect of the extract on the bacteria with the same Gram reaction. *E. coli, Shigella flexneri, and Salmonella typhi* which are Gram negative organisms, even though they produce different antimicrobial activity as indicated by their respective zones of inhibition. This observation is in line with [17] who worked on the adaptive resistance of some organisms and emphasized that the sensitivity to antimicrobial agents among microorganisms is not only dependent on cell but rather depend largely on other factors like the ability to form capsules, slime layer or spores.

The observed reduction in the effectiveness of the antimicrobial activity of the ethanolic column fractions reveals that *G. sepium* possess more than one active component which work in a synergistic manner. This agrees with [18] who reported that the activity of plant extracts can sometimes change after fractionation and a pure component eventually obtained may lack the activity of the original extract.

The crude extracts of the plants used were visibly active on the tested bacteria isolates due to the combinative therapeutic action of the various bioactive compounds contained in the plants. This was expected because the various inhibitory components present were not separated into single entity for directional effect on certain bacteria as does by synthesized antibiotics [19].

In this study, the phytochemical analysis showed that *G. sepium* possessed free radicals which have been implicated in many disease conditions. Herbal drugs containing free radical scavengers are gaining importance in treating such diseases. Many plants extract exhibit efficient antioxidant properties due to their phytoconstituents, including the phenolics. The total phenolic contents in plant extracts of the species *G. Sepium* depends on the type of extract, mainly the polarity of solvent used in extraction. High solubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction [20]. *Gliricidia sepium* has good amount of flavonoid. Flavonoid in animal diet reduces the risk of cancer [21]. Amongst phytochemical present is tannin which constitute one of the important secondary metabolites, have antimicrobial activity and are present widely in different groups of plants. [22] confirmed that tannins suppress methanogenesis directly through their anti-methanogenic and indirectly through antiprotozoal activities. Also, tannin is antioxidants and can improve resistance

to heat stress [23]. The presence of phytate and oxalate are known to show medicinal activity as well as exhibits physiological activity [24].

5. CONCLUSION

The extract of *G. sepium* demonstrated good antibacterial activity against the poultry bacteria pathogens therefore the plant can be a good source of drug. This potential drug source can be harnessed to produce antibacterial agents that can be used in poultry.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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