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Phytochemical constituent, proximate analysis, antioxidant, antibacterial and wound healing properties of leaf extracts of *Chromolaena Odorata*

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ABSTRACT

This study investigated the phytochemical constituent, proximate analysis, antioxidant, antibacterial and wound healing properties of aqueous and ethanolic leaf extracts of Chromolaena odorata (L.). Results indicated the presence of saponins, phenols and tannins in aqueous and ethanolic leaf extracts. Proximate analysis indicated that the dried leaf contained ash (10.5%), crude fat (10.75%), fiber (14.9%), moisture (15%), crude protein (18.37%) and carbohydrate (30.48%). However, aqueous extract had high antioxidant and lipid peroxide inhibitory activity while ethanolic extract showed high phenolic and hydrogen peroxide inhibitory activity at 50, 100 and 250 µg/ml respectively. Furthermore, the antibacterial studies showed that the ethanolic extract of C. odorata inhibited the growth of S. aureus, S. typhi and E. coli at various degrees while S. aureus and S. typhi only were inhibited by the aqueous extracts. However, aqueous extract (coagulation: 15.18 ± 0.023 min; clotting 0.26 ± 0.014 min) showed significantly high ($p<0.05$) haemostatic activity than the ethanolic extract (Coagulation: 21.0 ± 0.696 min; Clotting 2.03 ± 0.035 min). The result of these studies indicates that leaf extracts of C. odorata possess antioxidant, antibacterial and haemostatic activities which explain its folkloric use in animal nutrition and human medicine.

Keywords: *Chromolaena odorata*, antioxidants, antibacterial and haemostatic.

INTRODUCTION

Chromolaena odorata (L.) R.M. King & H. Robinson formerly called *Eupatorium odoratum* (Siam weed), a herbaceous perennial that forms dense tangled bushes about 1.5 - 2.0 m in height

and has a characteristic aromatic smell [1]. In Nigeria it is referred to as 'obu inenawa' by the Igbos and 'ewe awolowo' by the Yorubas. It originally spread from southern Mexico to Argentina and Caribbean, but has been introduced into diverse ecological areas of tropical lands [2].*Chromolaena odorata* in tropical Africa has acquired a reputation as a medicinal herb for a variety of ailments including malaria, dysentery, toothache and fever [3]. Medicinally the plant decoction is taken as a remedy for cough and cold or in bath to treat skin diseases and its popularity as an effective therapy against diarrhoea, malaria fever, tooth ache, diabetes, skin diseases, dysentery and colitis has been severally documented [4]. In folk medicine, aqueous leaf extract of the plant is used as antiseptic for wound dressing [5]. Fresh juice from the leaf is also used as haemostatic to arrest bleeding from fresh cuts and to stop nose bleeding [1].

The medicinal values of plants have been claimed to lie in their phytochemical component including alkaloids, tannins, flavonoids and other phenolic compounds, which produce a definite physiological action on the human body [1]. Scientific reports had also shown that leaves of plants are major sources of antioxidants, antimicrobials and phytochemicals with medicinal values [6, 7]. Thus, in this present study the phytochemical constituents, proximate analysis, antioxidants, antimicrobials, clotting and coagulating properties of *C. odorata* had been investigated in a bid to scientifically prove the medicinal potentials of this obnoxious weed.

MATERIALS AND METHODS

Plant material

The leaf of *C. odorata was* collected from a farmland at Babcock University, Ilisan Remo, Ogun State, Nigeria and authenticated by Prof. E.B. Esan, a plant scientist and H.O.D Chemical and Environmental Sciences, School of Science and Technology, Babcock University, Ogun State, Nigeria.

Chemicals

DPPH (2,2 diphenyl-1- picrylhydrazyl hydrate), gallic acid and folin-ciocalteu's reagent were purchased from Sigma Aldrich, USA. All other chemicals and reagents used were of analytical grade.

Animals

Six Wister strain albino rats were obtained from the Preclinical Animal House, Department of Biochemistry, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan, Ibadan, Oyo State. Nigeria. The animals were given water and rat chow *ad libitum* for 14 d to allow for acclimatization before use.

Test microorganisms

Klebsiella pneumonia, Staphylococcus aureus, Proteus vulgaris, Escherichia coli and *Pseudomonas aeruginosa* were obtained from Olabisi Onabanjo Teaching Hospital Sagamu, Ogun State. The microorganisms were sub cultured and the pure cultures re-sub cultured on nutrient agar slants and thereafter stored at 4^{o}C until required for study.

Extraction of plant material

Fresh leaf of *C. odorata* was air dried and then ground to fine powder. The pulverized samples (20 g) were soaked in 300 ml of 100% ethanol and 300 ml of distilled water for 72 h before extraction. The ethanolic and aqueous extracts were concentrated to dryness in a rotary evaporator and thereafter stored in refrigerator at 4°C until further use. However, for the antimicrobial activity, aqueous and ethanolic extracts were diluted separately with 30% dimethylsulphoxide (DMSO) to obtain 250 mg/ml, 200 mg/ml, 150 mg/ml, 100 mg/ml and 50 mg/ml concentrations stored at 15°C until required[8].

Antioxidant assay: Rapid Thin Layer Chromatography screening for antioxidant activity was carried out by spotting a concentrated ethanol and aqueous solutions of the extracts on silica gel plates. The chromatograms were developed in methanol : ethyl acetate (2 : 1) after which it was air-dried and sprayed with 0.2% $^{w}/_{v}$ DPPH spray in methanol. The chromatograms were visualized for the presence of yellow spots. The radical scavenging activity of leaf extracts was performed according to the DPPH spectrophotometric method of Mensor *et al*.[9]. One ml of 0.3 mM DPPH methanol solution was added to 2.5 ml solution of the extract or standard (100, 200 and 300µg/ml) and allowed to react at room temperature for 30 min. The absorbance of the resulting mixture was measured at 518 nm and converted to percentage antioxidant activity (AA %), using the formula:

$$AA\% = 100-\{(Abs\ sample - Abs\ blank) \times 100 / Abs\ control\}$$

Where Abs sample is the absorbance of sample and Abs control is the absorbance of control with the extract at 518 nm.

Blank=Methanol (1.0 ml) plus sample solution (2.0 ml), Negative control=DPPH solution (1.0 ml, 0.25mm) plus methanol (2.0 ml), ascorbic acid and Gallic acid were used as standards (Positive control).The scavenging reaction between (DPPH•) and an antioxidant (H-A) can be

written as: $(DPPH\bullet) + (H\text{-}A) \rightarrow DPPH\text{-}H + (A\bullet)$

Purple Yellow

Determination of total phenolic content: This was estimated as described by Singleton and Rossi[10] and modified by Gulcin *et al*.[11]. One ml aliquot of extracts or standard solution of gallic acid (10, 20, 30, 40 and50 mg/l) was added in a volumetric flask, containing 9 ml of water. One milliliter of folin-ciocalteu's reagent was added to the mixture and vortexed. After 5 min, 10 ml of 7% sodium carbonate was added to the mixture, and then incubated for 90 min at room temperature. After incubation the absorbance against the reagent blank was determined at 750 nm. A reagent blank was prepared using distilled water instead of plant extract. The amount of phenolic content in the extract was determined from the standard curve produced with varying concentrations (10, 20, 30, 40, 50 µg/ml) of gallic acid. The total phenolic content of the plant was then calculated as shown in the equation below and expressed as mg Gallic acid equivalent (GAE)/g fresh weight. All samples were analyzed in duplicates.

Equation…… C = c . m / V

Where:
C= total content of phenolic compound in gallic acid equivalent (GAE)/g
$C_=$ the concentration of gallic acid established from the calibration curve, µg/ml
V= volume of extract (ml)
m = weight of the crude plant extract (g).

Inhibition of lipid peroxidation: A modified Thiobarbituric Acid Reactive Substances (TBARS) assay was used to measure the lipid peroxide formed, using egg yolk homogenate as lipid-rich media [12]. Egg homogenate (0.5 ml, 10% v/v) was added to 0.1 ml of extract (1 mg/ml) and the volume made up to 1 ml with distilled water. Thereafter, 0.05 ml of $FeSO_4$ was added and the mixture incubated for 30 min. Next, 1.5 ml of acetic acid was added followed by 1.5 ml of TBA in SDS. The resulting mixture was vortexed and heated at 95°C for 60 min. After cooling, 5 ml of butan-1-ol was added and the mixture was centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm and converted to percentage inhibition using the formula:

(1 – E/C) x 100

Where C = absorbance of fully oxidized control, and
E = absorbance in the presence of extract.

Hydrogen peroxide scavenging activity: this was estimated using the modified method of Ruch *et al*.[13]. One ml of sample (50 - 250 µg/ml) was mixed with 0.1M phosphate buffer (pH 7.4) and 600 ml of a 43 mM solution of H_20_2 in the same buffer was added. The absorbance value at 230 nm of the reaction mixture was recorded at 0 min and then at every 10 min up to 40 min. For each concentration, a separate blank sample (devoid of hydrogen peroxide) was used for background subtraction. The percentage inhibition activity was calculated from

$$[(A_o-A_1)/Ao)]\times100$$

Where A_o= Absorbance of the control
$A_{1=}$ Absorbance of the extract/standards. All tests were done in triplicates

Phytochemical screening: Chemical tests were carried out on the aqueous and ethanolic extract for the qualitative determination of phytochemical constituents using standard procedures as described by Harborne [14] and Sofowora [15].

Proximate analysis: The chemical tests were carried out on the plant samples for the quantitative determination of physico-chemical constituents using standard procedures as described by Pearson [16].

Evaluation of antimicrobial activity: Agar diffusion method as described by Osadebe and Ukwueze[17] was adopted. Broth cultures of the test isolates (0.1 ml) containing $1x10^5$ cells/ml of organism was introduced into a sterile Petri dish and 15 ml molten nutrient agar added. The

content was thoroughly mixed and allowed to solidify. Three holes were made in the plates (about 5.0 mm diameter) using a sterile cork-borer and equal volume of leaf extracts were transferred into the holes using a Pasteur's pipette. Two Petri dishes containing a particular microorganism was used for each concentration of extract. The plates were allowed to stand for one hour for the pre-diffusion of the extracts to occur [18] and were incubated at 37°C for 24 h. At the end of incubation, the plates were collected and zones of inhibition that developed were measured. The average zones of inhibition were then calculated. The minimum inhibitory concentration (MIC) was calculated by plotting the logarithm of the concentration of extract against the square of zones of inhibition. The antilogarithm of the intercept on logarithm of concentration axis gave the MIC values [17, 18].

Wound healing assay (Haemostatic Activity): a modified method of Morton and Malone [19]. 1000 µl of Tween 80 solution and 1000 µl of the aqueous and ethanol extracts were added to different test tubes. 0.25 ml of the resulting solutions was orally administered to the different animals. At the end of administration, the animals' tails were inflicted using surgical blade and a few drops of the aqueous, ethanolic extracts and normal saline (control) were placed on the inflicted wounds on each animal. The time of clot was recorded. Subsequently the animals were subjected to cervical dislocation and 1 ml of blood was taken using a plastic hypodermic syringe and transferred into empty Ethylene Diamine Tetra Acetic acid (EDTA) bottles and 500 µl of each extracts and control (normal saline) were added and the time taken to coagulate the blood by each sample was recorded.

Statistical analysis: This was done with the aid of Windows Microsoft Excel and SPSS for windows; SPSS Inc., chicago, standard version 14.0 to determine differences between mean using analysis of variance (ANOVA). Data were reported as Mean ± Standard deviation.

RESULTS

Data from this study indicated the presence of terpenoids, tannns, saponins, phlobatannins, cardiac glycoside, anthraquinone, phenol and alkaloid in the extracts of *C. odorata* (Table.1). The proximate analysis showed carbohydrate was the highest (30.48%) while crude ash (10.50%) was the least (Table 2). Aqueous extract (42.56 ± 0.12%) had a significantly higher ($P<0.05$) percentage inhibition than ethanolic extract (3.29 ± 0.67 %). However, ethanolic extract (0.89 ± 0.849 mg GAE/g) had significantly ($p<0.05$) higher total phenolic content than the aqueous extract (0.24±0.071) of the same concentration. Rapid TLC screening for antioxidant activity was positive for all the plant leaves extracts. Furthermore, IC_{50} values for aqueous, ethanol, gallic acid and ascorbic acid were 22.50, 23.70, 15.90 and 17.0 µg/ml respectively (Table 3). The percentage scavenging ability of the extracts released by H_2O_2 (figure 1) showed that the aqueous extract of *C. odorata* had significant ($p<0.05$) higher activity at 100 and 250 µg/ml than ethanolic extract except at 50 µg/ml.

Table 4 shows the results of the antimicrobial effects of the extract on the test isolates. As observed, the zones of inhibition decreased with decrease in the concentration of the extract. The extracts did not inhibit the growth of *P. aeruginosa*, *P. vulgaris*, and *K. pneumoniae* at any of the concentrations used. The highest zone of inhibition was exhibited on *S. aureus* with a zone diameter of 14 mm at a concentration of 250 mg/ml ethanolic extract while the lowest zone of

growth of inhibition was 1mm at 150 mg/ml concentration for ethanolic extract on *E. coli*. The ethanolic extract at 250 mg/ml, 200 mg/ml and 150 mg/ml only showed minimal inhibition on *E. coli* while no inhibition was recorded at other concentrations used. The lowest MIC was obtained with *S. aureus* with 0.1mg/ml while the highest was 0.8mg/ml for *E. coli* (Table 5).

Table 1: Phytochemical analysis of aqueous and ethanolic leaf extracts of *Chromolaena odorata.*

Phytochemicals	Aqueous	Ethanolic
Terpenoids	-	++
Tannin	+	++
Saponin	++	+
Phlobatannin	++	-
Cardiac glycoside	-	++
Flavonoids	-	-
Cardenolides	-	-
Anthraquinones	-	+
Phenol	++	+
Alkaloid	++	-
Volatile Oil	-	-

++ = *abundant* + = *trace* - = *absent*

Table 2: Proximate analysis result of the blended leaves of *Chromolaena odorata.*

Plant material	Moisture %	Fiber %	Fat %	Protein %	Ash %	Carbohydrate %
C. odorata	15	14.9	10.75	18.37	10.5	30.48

Table 3: Quantitative determination of total phenol, antioxidant and lipid peroxidative activity of *Chromolaena odorata*

Sample extract	Concentration (µg/ml)	Percentage antioxidant activity	Total Phenol (mg/g GAE)	Percentage inhibition of lipid peroxidation
Aqueous	100	75.61±0.070	0.24±0.707	42.56±0.12
	200	77.66± 0.212		
	300	78.62±0.059		
		22.50*		
Ethanolic	100	76.44±0.007	0.89±0.849	3.29±0.67
	200	77.39 ± 0.134		
	300	77.95±0.069		
		23.70*		
Gallic acid	100	81.55±0.25		
	200	82.00± 1.55		
	300	83.56±1.05		
		15.90*		
Ascorbic acid	100	82.10±0.44		
	200	81.58±0.04		
	300	82.32±2.05		
		17.00*		

**IC50 value in µg/ml (concentration of sample required for 50% inhibition of DPPH radical activity).*

Wound healing assay showed that the aqueous extract used the lowest time in clotting (0.26 ± 0.012 min.) as compared to ethanolic extract (2.03 ± 0.035 min.) and the control (6.88 ± 0.007 min.) which used the longest time in clotting. Also, in coagulation test the aqueous extract (15.18

± 0.023 min.) also used the lowest time in coagulation as compared to the ethanolic extract (21.78 ± 0.696 min.) and the control (162.28 ± 17.371 min.).

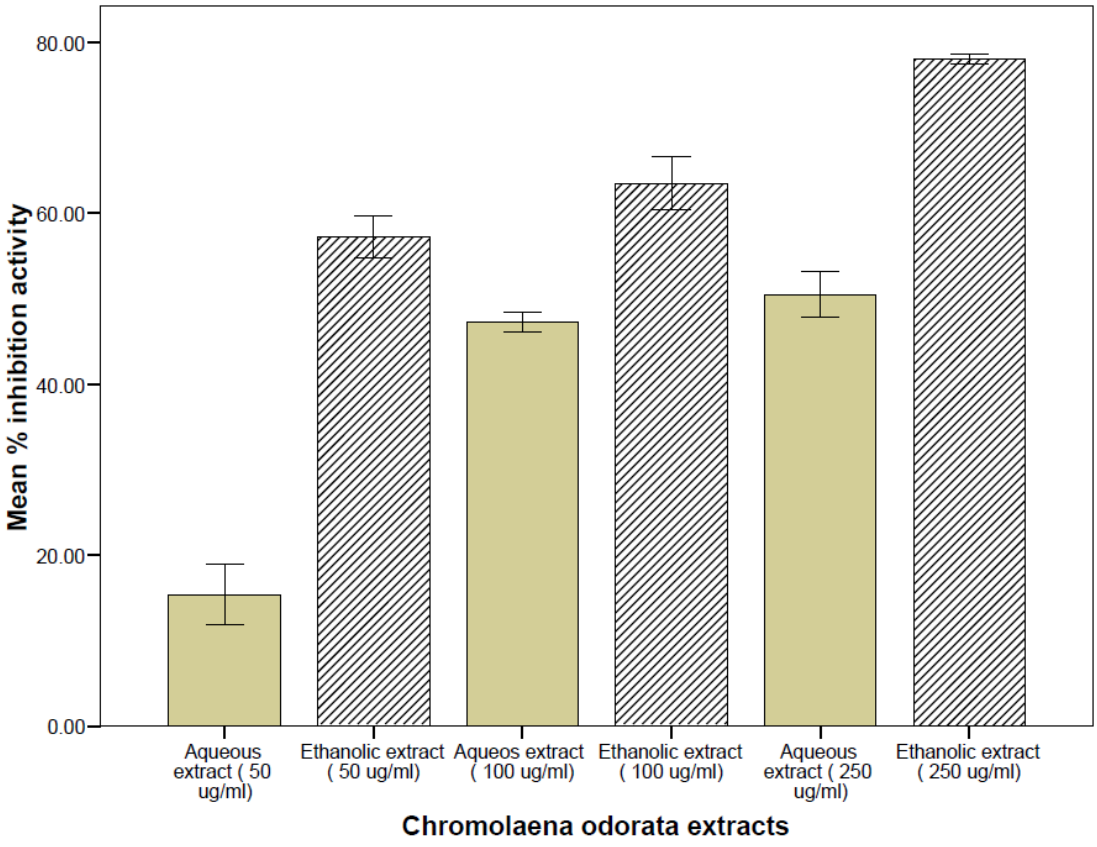

Fig 1: Percentage inhibition of hydrogen peroxide against 50, 100 and 250 µg/ml extracts of *Chromolaena odorata.*

Table 3: Zone of inhibitory activity of different concentrations of aqueous and ethanolic extracts of *Chromolaena odorata* against clinical bacteria.

Extract Concentration (mg/ml)		Zone of inhibition (mm)					
		E.coli	*S.aureus*	*P.aeruginosa*	*S.typhi*	*P.vulgaris*	*K. pnemoneae.*
250	Aqueous	NI	12	NI	9	NI	NI
	Ethanol	6	14	NI	10	NI	NI
200	Aqueous	NI	10	NI	7	NI	NI
	Ethanol	5	11	NI	8	NI	NI
150	Aqueous	NI	5	NI	6	NI	NI
	Ethanol	1	8	NI	6	NI	NI
100	Aqueous	NI	2	NI	4	NI	NI
	Ethanol	NI	5	NI	5	NI	NI
50	Aqueous	NI	1	NI	2	NI	NI
	Ethanol	NI	3	NI	3	NI	NI
Ethanol only	–	–	–	–	–	–	–

- = No activity *NI = No inhibition*

Table 4: Minimum Inhibitory Concentration (MIC) of aqueous and ethanolic extract of *Chromolaena odorata* for antibacterial activity

	MIC (mg/ml)					
Extracts	*E. coli*	*S. aureus*	*P. aeruginosa*	S. typhi	*P.vulgaris*	*Klebsiella*
Aqueous	Nil	0.2	Nil	0.3	Nil	Nil
Ethanol	0.8	0.1	Nil	0.2	Nil	Nil

Values are the average of at least three determination, The MIC for S. aureus and S.typhi are not significant (P < 0.05 significant).

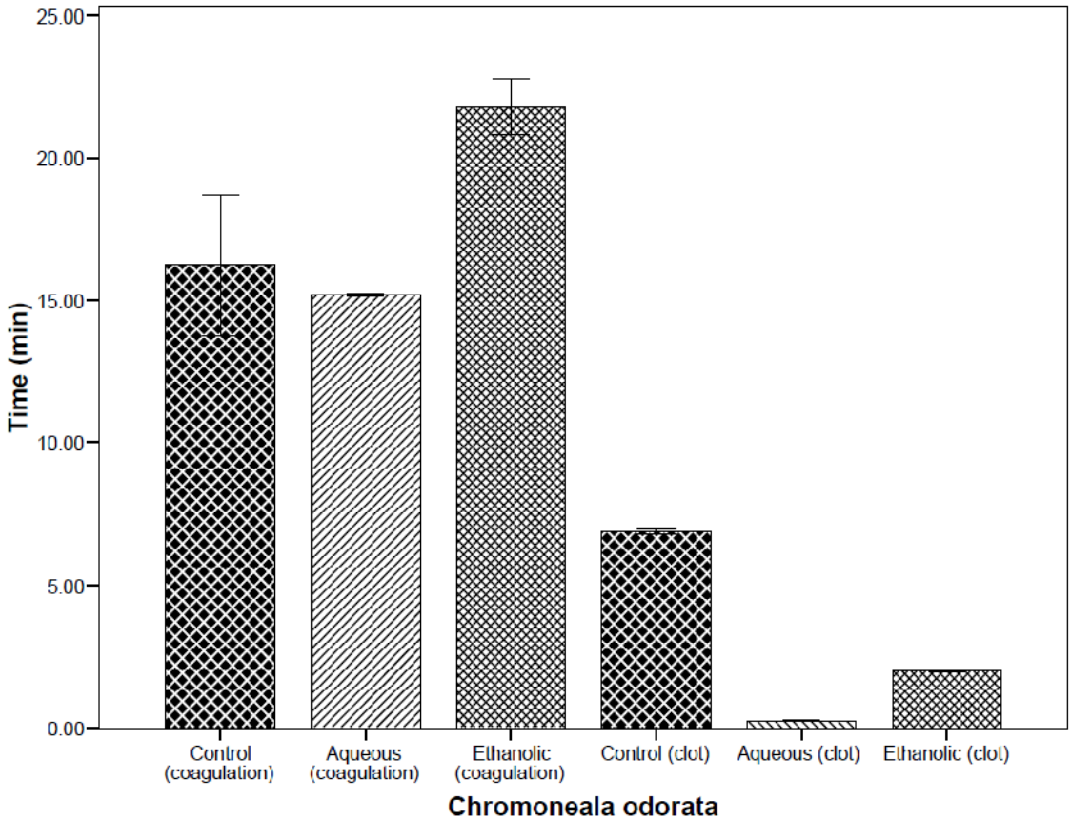

Fig 2: Mean coagulation and clotting time for aqueous and ethanolic extracts of *Chromolaena odorata.*

DISCUSSION

Recent studies on antioxidants and antibacterial properties of plant extracts could be attributed to the scientific evidences implicating plant phytochemicals and antioxidants in terminating or inhibiting deleterious chain reactions triggered by free radicals or reactive oxygen species [20, 21]. Radicals had been associated with redox related diseases including arthritis, diabetes, inflammation, cancer and genotoxicity [22, 23].

Phytochemical screening of *C. odorata* indicated the presence of saponins, phenols and tannins in aqueous and ethanolic leaf extracts. This suggests that *C. odorata* may possess hemolytic, anti-inflammatory, antioxidative, immunostimulant and antimicrobial activities [24]. Phlobatannins and alkaloids were present in the aqueous extract while cardiac glycosides,

anthraquinones and terpenoids were present in the ethanolic extract. The presence of phlobatannin indicates diuretic property and positive alkaloids suggest potentials of the extracts to help the white blood cells dispose harmful microorganisms and cell debris, and also improve cardiac conditions by reducing blood pressure, increasing circulation and inhibiting the accumulation of arteriosclerosis plaque and blood clots [25]. Pure cardiac glycosides administered as injections or tablets inhibit active transport of K^+ and Na^+ through membranes thereby strengthening heart muscle and the power of systolic concentration against congestive heart failure. The bitter taste of glycosides also prevents birds and insects from eating immature fruits and seeds and also abates the decay of damaged plant tissues. Anthraquinones are considered to be one of the most active agents in metastatic breast cancer [26].

Furthermore, proximate analysis showed that *C. odorata* could serve as a good source of energy, flavor, minerals, protein and dietary fibre thus contributing to the palatability and supplementation of feed in animal nutrition [27, 28]. This may account for the use of plant in livestock production. However the relatively high moisture content of *C. odorata* content indicates a possible reduction in plant shelf life [29].

Results from various antioxidant assays (*in vitro*) in this investigation showed that the plant extracts possess antioxidant activity. Plant phenolics are major group of compounds acting as primary antioxidants or free radical scavengers [30]. The ethanolic extract of *C. odorata* showed higher phenolic than aqueous extract. A qualitative rapid thin layer chromatography screening for antioxidant activity of aqueous and ethanolic extracts were positive, as the colour of the DPPH spray changed from deep violet to yellowish spots indicating both extracts possess free radical scavenging activity, and may be considered good sources of antioxidants. Also, further study showed that the free radical scavenging power of the extracts of *C. odorata* increased in a concentration dependent manner as evident on the increased reduction of the stable DPPH radical [26]. Although, the scavenging power of both extracts were significantly low compared to standard gallic acid and ascorbic acid. Aqueous extract showed higher radical scavenging activity than the ethanolic extract as evident from the fifty percent inhibitory concentration values (IC_{50}) [26]. Inhibition of lipid peroxide formation showed that the aqueous extract indicated higher inhibitory activity than ethanolic extract. Formation of lipid peroxide has been shown to result from the cumulative effect of reactive oxygen species, disrupting the assembly of the membrane causing changes in fluidity and permeability, alterations in ion transport and inhibition of metabolic processes[31]. This result indicates that extracts have the ability to inhibit lipid peroxidation in biological system. The hydrogen peroxide scavenging test showed that ethanolic extract exhibited high hydrogen peroxide inhibitory activity than aqueous extract at the various concentrations tested. The ability of plant extract to scavenge H_2O_2 could also reflect its ability to inhibit the formation of hydroxyl radical *in vivo* and in a way protect the cell from oxidative damage [7].

Results from the antibacterial studies showed that the ethanolic extract of *C. odorata* inhibited growth of *S. aureus, S. typhi* and *E. coli* at various degrees while *S. aureus* and *S. typhi* only were inhibited by the aqueous extracts. The solvent used as control had no inhibitory effect on the test isolates indicating the efficiency of the plant extract. This may be due to the presence of phytochemicals such as phylobatanine, saponine, flavonoids, glycosides, tannins and alkaloids in the leaf extracts [32]. The low MIC exhibited by the extract on *S. aureus* and *S. typhi* is of great

significance in the health care delivery system since it could be used as alternative to orthodox antibiotics in the treatment of microbial infections especially as these bacteria frequently develop resistance to known antibiotics [33]. The Gram negative bacteria (*S. aureus*) were found to be more susceptible to the plant extract than the Gram positive bacteria. This could be due to the morphological differences in the cell wall of Gram positive bacteria, which is less complex and lacks the natural sieve effect against large molecules due to the small pores in their cell envelope [34]. This suggests that *C. odorata* antimicrobial mode of action might be due to the presence of flavonoids and tannins that binds to bacterial cell wall and inhibits its biosynthesis.

Plant extracts arrested bleeding from fresh wounds by reducing clotting and whole blood coagulation time which are important indices of haemostatic activity [6]. Studies on the haemostatic activity indicated that the aqueous extract showed a better haemostatic ability than the ethanolic extract. Haemostasis involves the spontaneous arrest of bleeding from damaged blood vessels which is important for initiation of tissue repair processes and prevention of tissue death through haemorrhage [6]. The reduction of coagulation time of whole blood by the leaf extracts is an indication that the extracts may also interfere with the blood coagulation pathways. The presence of saponins and tannins in the plant leaf is believed to be responsible for its haemostatic activity, thereby, supporting the traditional use of the plant leaf in wound healing [5]. In conclusion, this study indicates that leaf extracts of *C. odorata* possess bioactive metabolites, free radical scavenging, antibacterial and haemostatic activity which explains their use in animal nutrition and human medicine.

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